Study on the Reaction of Monofunctional Fluorescent Reagents in Organic Solutions by Fluorometry

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Rections of some fluorescent reagents with substrates, used widely in biochemistry for quantitative analysis of some functional groups in aqueous media, have been studied in organic solutions with a view that they may be used also in the area of synthetic polymer science. Reactions studied by fluorometry were (i) 5-dimethylamino-1-naphthalensulfonyl chloride with butylamine, (ii) 2-naphthalenesulfonyl chloride with butylamine, and (iii) *N*-(1-naphthyl)maleimide with 1-pentanethiol. In polar solvents all the fluorescent probes studied are too labile and converted spontaneously into fluorescent compounds. In less polar solvents, however, such side reactions do not take place and the reaction of fluorescent reagents with substrates proceeds quantitatively with a simple second-order kinetics. The second-order rate constants for the three reactions are of the order of 10^{-3} to 10^{-1} M^{-1} s^{-1} in the range of about 30 to 50 °C, and they increase with the solvent polarity.

Fluorescent probes have been utilized widely in biochemistry either for analysing quantitatively functional groups in proteins or for probing the environment around the functional groups. In the former case, a component to be analyzed is converted by the reaction with a proper probe into a fluorescent derivative, and its quantity is determined from the emission intensity. In the later case, the details of the spectrum, lifetime, quantum yield, and depolarization provide information about the functional group and its surroundings. Two sorts of fluorescent probes have been used mainly.[1] The one is called fluorescent thiol reagent and comprises certain nonfluorescent *N*-substituted maleimides. They react with thiol compounds in aqueous solution to give fluorescent adducts.[2] The other is called fluorescent amine reagent and comprises nonfluorescent sulfonyl chlorides such as 5-dimethylamino-1-naphthalenesulfonyl chloride,[3] and nonfluorescent "fluorescamine."[4] They react almost quantitatively with primary amines in aqueous solution to give fluorescent products. Since these fluorescent reagents themselves are not soluble in water, it is a common practice to dissolve them first in an organic solvent such as acetone or dioxane then mix the solution with an aqueous solution of substrate.

A useful extension of the fluorescent probes is to make the probes react with substrates in organic solvents which are commonly used in studies on organic reactions or synthetic polymers. If it has become feasible to use the fluorescent probes in organic solutions in a similar manner to in aqueous solution, the fluorescent probes will be utilized more widely in organic chemistry or in polymer science. Only little has been reported, however, on the reaction kinetics of the fluorescent reagents, especially on the reaction in nonaqueous solvent.[5-7]

To advance the utilization of the fluorescent probes more widely, we investigated the reaction of monofunctional fluorescent amine reagents or sulfonyl chlorides with butylamine as well as of monofunctional thiol reagent with 1-pentanethiol in various solvents.

Experimental

Materials. *N*-(1-Naphthyl)maleimide (NMI) was prepared using a modified method of Tsou *et al.*[8] from 1-naphthylamine and maleic anhydride with acetic anhydride as dehydrating agent and sodium acetate and triethylamine as catalysts (51% yield). *N*-Butyl-5-dimethylamino-1-naphthalenesulfonamide (DNSBA) was prepared in a similar manner to the preparation of *N*-ethyl-5-dimethylamino-1-naphthalenesulfonamide.[8] A chloroform solution of 5-dimethylamino-1-naphthalensulfonyl chloride (dansyl chloride; DNSCl) (15 ml; 0.19 mol dm^{-3} (M†)) was mixed with a chloroform solution of butylamine (*n*-$BuNH_2$) (15 ml; 1.86 M) at room temperature. After 6 h the solution was washed with 0.1 M aqueous HCl solution and distilled water, dried over Na_2SO_4 overnight then the solvent was removed. The yellow residue was recrystallized from diethyl ether to give yellow-orange crystals which melted at 95—97 °C (40% yield). The structure was ascertained by NMR and IR. NMR ($CDCl_3$): δ 2.9 (N–C$\underline{H}_3$, -SO_2NH–C$\underline{H}_2$–), δ 5.3 (–SO_2N$\underline{H}$–), *etc.* IR: 3300 cm^{-1} (NH), 1320 and 1150 cm^{-1} (SO_2), *etc.* The purity was confirmed by TLC.

1-Pentanethiol (*n*-AmSH), 2-naphthalenesulfonyl chloride (NDSCl), DNSCl, and *n*-$BuNH_2$, all from Tokyo Kasei, were used without further purification. All the solvents (from Wako Pure Chemical Ind.) were of nonfluorescent "Dotite Luminasol" or "Dotite Spectrosol" grade except pyridine (Reagent grade) and used as received.

Apparatus. The NMR spectra were obtained on a 100-MHz JEOL MH-100 spectrometer. Infrared spectra were measured by using the KBr pellet technique with the aid of a JASCO IR-G spectrometer.

Kinetic studies by fluorometry were performed with a JASCO FP-550 spectrofluorometer using a 1 cm × 1 cm quartz cell with a cap without degassing. Fluorescence was measured using excitation waveloength at 290 nm for NMI, 280 nm for NDSCl, and 340 nm for DNSCl and observed in the range of 325—345 nm for NMI, 330—355 nm for NDSCI, and 480—505 nm for DNSCl, respectively. Fluorescence data are reported without spectral correction, except fluorescence spectra in the wavelength from 400 to 550 nm which were corrected by using the standard solution of quinine sulfate in 0.05 M H_2SO_4.

Results and Discussion

Reaction of 5-Dimethylamino-1-naphthalenesulfonyl Chloride with Butylamine. In the course of investigation of the reaction of 5-dimethylamino-1-naphthalenesulfon-

† 1 M = 1 mol dm^{-3}.

yl chloride (DNSCl) with butylamine (n-$BuNH_2$) in organic solvents, we found that the stability of DNSCl has to be taken into account. DNSCl is unstable at room temperature and even without amines it is converted by an unknown reaction to fluorescent products in polar solvents such as methanol (slow), acetonitrile, pyridine, DMF (rapid), and dimethyl sulfoxide (rapid). No further detailed study of the reaction was carried out in these solvents. One of the probable mechanisms of the solvolysis, however, may be analogous to that proposed for pyridine.[9]

$$RSO_2Cl + C_5H_5N \longrightarrow RSO_2N^+C_5H_5Cl^- \quad (1)$$

The reaction product in Eq. 1 is presumably fluorescent, since DNSCl is nonfluorescent probably because of the intramolecular heavy-atom effect of chlorine.[10]

On the other hand, DNSCl is stable in less polar solvents such as THF, chloroform, and ethyl acetate. So the reaction rate were measured in these solvents. The reaction mechanism is most reasonably formulated as a bimolecular nucleophilic substitution on sulfur, similar to that for the reaction of benzenesulfonyl chloride with aniline.[11]

$$RSO_2Cl + H_2NR' \longrightarrow RSO_2NHR' + HCl \quad (2)$$

In organic solvents, hydrogen chloride formed reacts immediately with amine.[12]

$$HCl + H_2NR' \longrightarrow H_3N^+R'Cl^- \quad (3)$$

Then the overall reaction is expressed as

$$RSO_2Cl + 2H_2NR' \longrightarrow RSO_2NHR' + H_3N^+R'Cl^-. \quad (4)$$

N-Butyl-5-dimethylamino-1-naphthalenesulfonamide (DNSBA), the reaction product of DNSCl with n-$BuNH_2$, was prepared and purified separately to examine its fluorescence prior to kinetic measurement. Figure 1 shows its absorption and fluorescence spectra in ethyl acetate. Although the absorption maxima are invariant with solvent polarity, the fluorescence maxima (λ_{max}) shift gradually to longer wavelength with increasing solvent polarity as is known for dansylamine.[13] The λ_{max} of DNSBA are 467, 487, 497, 499, 502, and 518 nm in cyclohexane, benzene, tetrahydrofuran, ethyl acetate, chloroform, and N,N-dimethylformamide, respectively. The plot of the energy of fluorescence maxima *vs.* solvent polarity parameter

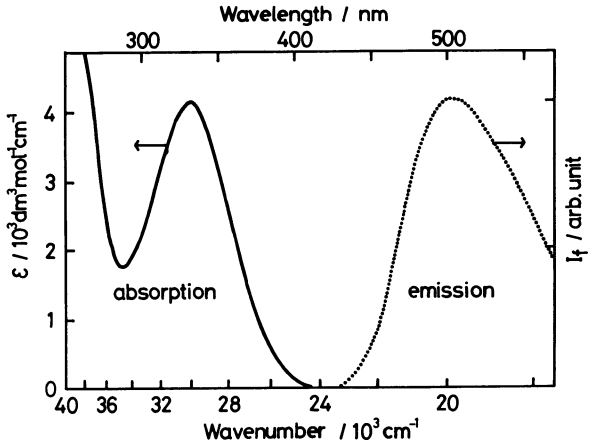


Fig. 1. Absorption and fluorescence spectra of DNSBA in EtAc.

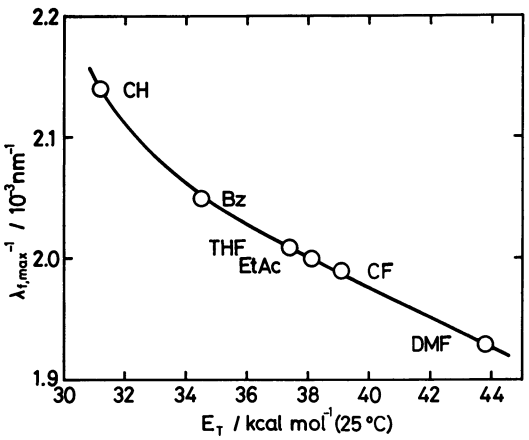


Fig. 2. Relationship between the energy of fluorescence maxima of DNSBA and the solvent-polarity parameter E_T in nonaqueous solents.

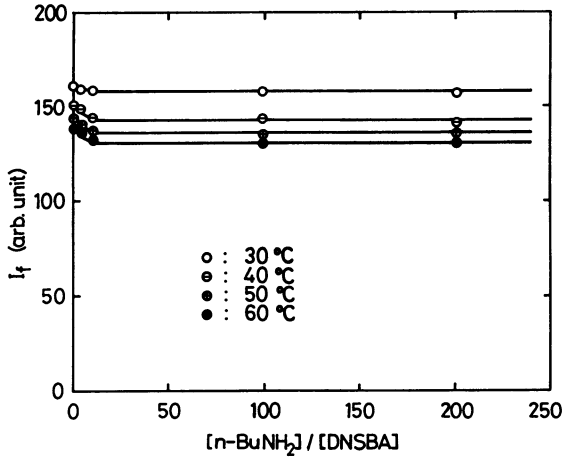


Fig. 3. The effect of addition of n-$BuNH_2$ on I_f of DNSBA in EtAc at several temperatures.

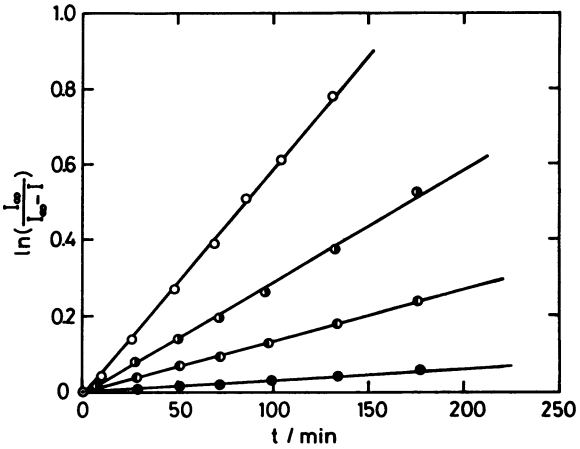


Fig. 4. Pseudo first-order plots of the reaction of DNSCl with n-$BuNH_2$ in THF at 50 °C: [DNSCl]=8.34×10^{-6} M; [n-$BuNH_2$]/[DNSCl]=40 (●), 200 (◐), 400 (◑), and 800 (○).

(Dimroth's E_T) for DNSBA in these solvents shows a satisfactory correlation as for dansylamine[13] (Fig. 2).

The proportionality between I_f and concentration of DNSBA holds for the concentration range [DNSBA]≦

1×10^{-5} mol dm^{-3} in all solvents used. The linearity does not hold at higher concentration of DNSBA. The temperature dependence of fluorescence intensity I_f of DNSBA in ethyl acetate is shown in Fig. 3, together with the effect of addition of n-$BuNH_2$ on I_f of the solution. The fluorescence intensity increases with a decrease in temperature and this may be a result of a decrease in the internal quenching of fluorescence.[14] The fluorescence intensity decreases by a few percent by the addition of n-$BuNH_2$ up to about ten fold excess to DNSBA in molality, but remains constant by further addition of n-$BuNH_2$. In any event, the effect of addition of n-$BuNH_2$ is not large and only a slight correction due to the effect is needed in the following kinetic studies.

Figure 4 shows the pseudo first-order plots of the reaction of DNSCl with excess n-$BuNH_2$ in THF at 50 °C. The second-order rate constants for the reaction in THF at 50 °C, calculated from the observed pseudo first-order rate constants and the concentration of n-$BuNH_2$ used, are entirely constant for widely different n-$BuNH_2$ concentrations as shown in Fig. 5. The second-order rate constants of the reaction in ethyl acetate (EtOAc) are also constant for different n-$BuNH_2$ concentrations. These facts show that the reaction is a simple bimolecular one in these conditions. The final fluorescence intensity of the reaction mixture was in fair agreement with that of the corresponding DNSBA solution as shown in Table 1. This confirms that the reaction proceeds quantitatively in these conditions.

Table 2 lists the second-order rate constants (k_2) of the reaction in various solvents mainly at 60 °C for the concentration of DNSCl ranging from 1×10^{-6} to 1×10^{-5} M. The correlation of k_2 to dielectric constant ε or $(\varepsilon-1)/(2\varepsilon-1)$ in various solvents is quite poor. On the contrary, the second-order rate constants are roughly proportional to the E_T values. This tendency may support the argument that the reaction of DNSCl with n-$BuNH_2$ is S_N2 type, because it is well known that the rate constant of a S_N2 reaction with solvated transition state becomes larger as the solvent becomes more dipolar aprotic in character.[15] The solvent effect of the present reaction may be different from that of the reaction of 2-thiophenesulfonyl chloride with anilines for which a linear correlation is found for the rate constants and the dielectric constants and a S_AN mechanism is proposed, bond-breaking being rate-determining step in aprotic solvents.[16]

Table 1. Final fluorescence intensity of the reaction mixture of DNSCl with n-$BuNH_2$ in EtAc

[DNSCl]/10^{-5} M	[n-$BuNH_2$]/10^{-4} M	Temp/°C	I_f	$I_{f,model}$	$I_f/I_{f,model}$
1.15	2.85	40	134	133	1.01
1.15	2.85	50	130	126	1.03
1.15	2.85	60	116	122	0.95
0.98	2.48	70	95	99	0.96

Table 2. Second-order rate constants of the reaction of DNSCl with n-$BuNH_2$ in nonaqueous solvents

Solvents	ε	E_T / kcal mol^{-1}	Temperature / °C	k_2 / M^{-1} s^{-1}
Dichloromethane	8.9	41.1	30	2.2×10^{-1}
Chloroform	4.7	39.1	50	3.7×10^{-2}
Ethyl acetate	6.0	38.1	60	2.0×10^{-1}
1,2-Diethoxyethane	5.1	—	60	1.8×10^{-2}
Tetrahydrofuran	7.4	37.4	50	1.7×10^{-2}
1,4-Dioxane	2.2	36.0	60	4.3×10^{-2}
Benzene	2.3	34.5	50	1.6×10^{-3}
Toluene	2.4	33.9	60	1.1×10^{-3}
Cyclohexane	2.0	31.2	60	$\ll1\times10^{-3}$

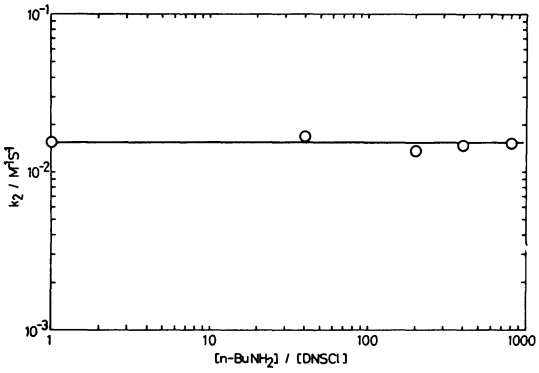


Fig. 5. Constancy of the second-order rate constants k_2 of the reaction of DNSCl with n-$BuNH_2$ in THF at 50 °C at different n-$BuNH_2$ concentration.

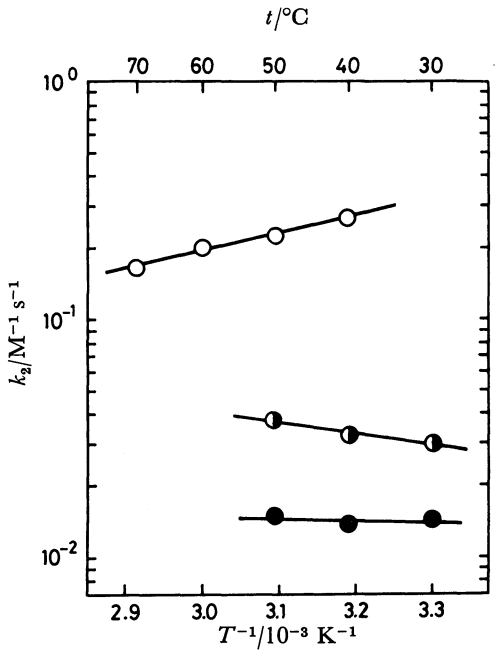


Fig. 6. Arrhenius plots of the second-order rate constants k_2 of the reaction of DNSCl with n-$BuNH_2$ in EtAc (○), chloroform (◑), and THF (●).

Arrhenius plots of the rate constants in ethyl acetate, chloroform, and tetrahydrofuran are shown in Fig. 6. The linear plot for the reaction in chloroform is normal but gives rather small activation energy (ΔE=2.1 kcal/mol). On the contrary, the reaction rate decreases with increasing temperature in ethyl acetate, and almost constant in tetrahydrofuran. In the case of the reaction of 2,4-dinitrobenzenesulfonyl chloride with n-$BuNH_2$ in

benzene, the amine is reported to act as a catalyst and the reaction is not of first-order with respect to amine.[12] In both ethyl acetate and tetrahydrofuran, however, the reaction of DNSCl with n-$BuNH_2$ is of first-order with respect to the amine as shown in Fig. 5. The anomalous temperature dependence of the reaction in these solvents may be explained as follows. In some S_N2 reactions, the degree of solvation of the reactants and the transition state are known to vary with solvent nature,[17] and the change in solvation is specified by the corresponding equilibrium constant which depends on the solvent nature as well as temperature. Accordingly, the cause of the anomaly may be ascribed to the temperature dependence of the equilibrium constant for the solvation. In this case, however, the reaction order is not affected and the reaction remains to be a simple bimolecular one.

The fluorescence of the reaction product of DNSCl with n-$BuNH_2$ can be excited at 350 nm or longer wavelength. Then DNSCl will be useful for a general fluorescent probe for studies in the field of polymer science and organic chemistry, because even the presence of chromophores such as phenyl groups and indole rings does not interfere the fluorescence measurement. This is the reason why we studied the reaction of DNSCl with n-$BuNH_2$ most thoroughly in various organic solvents as shown above. The situation is different for fluorescent probes such as 2-naphthalenesulfonyl chloride and N-(1-naphthyl)maleimide which are excited in the range of 270—280 nm. But these reagents are also useful in some cases as fluorescent probes to amino and mercapto groups. So the reaction of 2-naphthalenesulfonyl chloride with n-$BuNH_2$ and that of N-(1-naphthyl)maleimide with 1-pentanethiol were studied briefly in various organic solvents as shown below.

Reaction of 2-Naphthalenesulfonyl Chloride with Butylamine. 2-Naphthalenesulfonyl chloride (NDSCl) is also unstable at room temperature and converted by an unknown reaction to fluorescent products in such polar solvents as already listed in the preceding chapter. On the other hand, like DNSCl, NDSCl is stable in less polar solvents such as tetrahydrofuran and chloroform. So the reaction rate were measured in tetrahydrofuran (THF) and chloroform. The mechanism for the reaction of NDSCl with n-$BuNH_2$ may be similar to that of DNSCl with n-$BuNH_2$ as shown in Eq. 2 to 4. The first-order plots of the reaction in such solvents give straight lines, indicating that the reaction is first-order with respect to NDSCl and is a simple bimolecular one in these conditions. Second-order rate constants of the reaction were calculated from the observed pseudo first-order rate constants obtained for the reaction with n-$BuNH_2$ in large excess to NDSCl. The second-order rate constants of the reaction thus obtained are 4.6×10^{-2} $M^{-1}\,s^{-1}$ and 5.3×10^{-1} $M^{-1}\,s^{-1}$ in THF at 30 °C and in chloroform at 40 °C, respectively.

The emission spectrum of the product of the reaction of NDSCl with n-$BuNH_2$ in THF is shown in Fig. 7, together with that of naphthalene in THF. Figure 7 indicates that the emission spectrum is not much affected by the substitution of sulfamide group at β-position of the naphthalene ring.

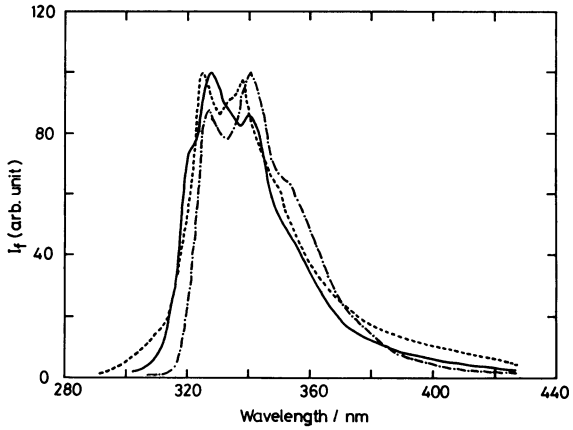
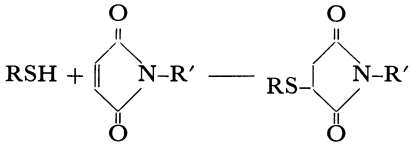


Fig. 7. Fluorescence spectra of naphthalene (——), the reaction product of NSCl with n-$BuNH_2$ (-·-·), and that of NMI with n-AmSH (------) in THF.

Reaction of N-(1-Naphthyl)maleimide with 1-Pentanethiol. Thiols and thiolate anions are known to add almost quantitatively to some nonfluorescent maleimides to give fluorescent adducts in aqueous solution.[6,18] So



(5)

the reaction is widely used for quantitative analysis for mercapto groups in proteins and amino acids such as cysteine.

In the case of the reaction of N-(1-naphthyl)maleimide (NMI) with 1-pentanethiol (n-AmSH) in organic solvents, we also found that the stability of NMI has to be taken into account. The maleimide ring is known to be readily hydrolyzed to form the corresponding maleamic acid derivative in alkaline aqueous solution.[19] In addition, highly electrophilic maleimide double bond is easily susceptible to anionic polymerization by appropriate initiators in strongly polar media such as N,N-dimethylformamide (DMF).[20] In fact, NMI was not stable at room temperature in DMF and fluorescence developed even without thiols. Figure 8 shows the change in fluorescence intensity with time of NMI in

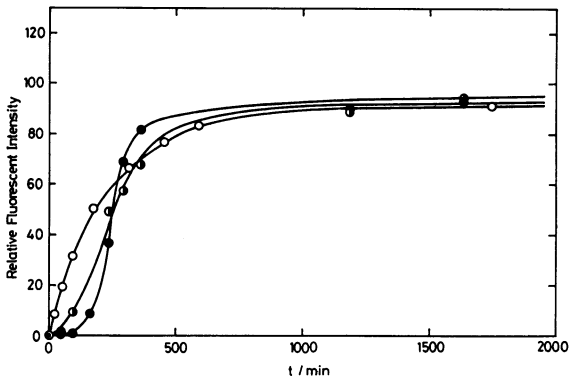


Fig. 8. Variation of the fluorescence intensity of NMI in DMF at 50 °C with time at volume fraction of acetic acid 0% (○), 9% (◑), and 33% (●).

DMF with various concentration of an acetic acid added to supress anionic polymerization. Although acetic acid seems to act as an inhibitor to any side reaction in the begining, its addition induces an autocatalytic reaction at a later stage. On the other hand, the first-order plots of the spontaneous deactivation reaction of NMI in DMF without acetic acid give straight lines. The first-order rate constant of the reaction is 6.5×10^{-5} s^{-1} at 50 °C and the value remains unaltered even when the equimolar amount of *n*-AmSH is added to the NMI solution. Although we did not study the detailed mechanism of the development of fluorescence of NMI in DMF, it is evident that the side reaction of NMI in DMF make impossible the kinetic study of the reaction of NMI with *n*-AmSH. Such a side reaction of maleimide ring is negligible for synthesis from bismaleimide compound and bisthiol in DMF, because the concentration of substrate is sufficiently high (10^{-1} to 10^{-2} M).[20] It should be noted, however, that the side reaction can not be ignored in the reaction of NMI as a fluorescent probe in DMF in which the concentration of substrate is limited (10^{-4} to 10^{-5} M).

NMI is stable in less polar solvents such as ethyl methyl ketone, benzene, and THF, and the fluorescence development of NMI in such less polar solvents is negligible for a few days at 50 °C. The reaction rate of NMI with *n*-AmSH in THF was followed by fluorometry. Since THF is miscible with water in all proportions, the effect of added H_2O is also studied. The pseudo first-order plots of the reaction of NMI with *n*-AmSH in THF at 50 °C with and without a small amount of H_2O give straight lines and the reaction proceeds quantitatively. The second-order rate constants of the reaction are evaluated as 4.3×10^{-2} M^{-1} s^{-1} in pure THF, 8.5×10^{-2} M^{-1} s^{-1} in THF with 0.3% H_2O, and 1.5×10^{-1} M^{-1} s^{-1} in THF with 3% H_2O. So the effect of the presence of H_2O is not very large.

The room temperature emission spectrum of the product of the reaction in THF is also shown in Fig. 7. The emission spectrum is not affected much by the substitution of thiol adduct of maleimide ring at α-position of naphthalene ring similarly to the case of NDSCl.

In conclusion, several reactions of some fluorescent reagents with substrates were confirmed to be simple second-order reactions as well as to proceed quantitatively in less polar solvents. Reaction rate of the fluorescent reagents studied here may be more rapid in polar aprotic solvents as is general for S_N2 type reactions. In polar solvents, however, all the fluorescent probes studied are too labile to study and converted spontaneously into fluorescent compounds. Application of the fluorescent probe to studies on organic reactions or synthetic polymers will be published elsewhere.[21]

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