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Suitability of DTAF as a fluorescent labelling reagent for direct analysis of primary and secondary amines — spectral and chemical reactivity considerations

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Abstract: DTAF has been used successfully to prepare fluorescent labelled reagents for fluorescence polarization immunoassays. Its applicability as a derivatization reagent for direct fluorescence analysis of primary and secondary amines was evaluated. DTAF was shown to have spectral properties that closely resemble those of fluorescein and that are apparently insensitive to the presence of the triazine nucleus. Spectrally determined pK_a values also closely resemble those of fluorescein and other amino aryl-*s*-triazines. DTAF is prone to hydrolytic degradation with the rate of reaction increasing with increasing pH, until a pH value is reached at which ionization of the amine bridging the two aromatic nuclei occurs; at this pH the rate reaches a plateau value. Both primary and secondary amines react efficiently with DTAF, and the reactivity increases with the increasing basicity of the amine reactants. The reaction is pH-dependent, proceeding most efficiently at pH values at which both the "bridging amine" of DTAF and the amine substrate are unionized. Methyl substituted secondary amines were consistently more reactive than the corresponding primary amine, but further imposition of steric bulk about the amine nitrogen significantly reduced the reactivity of amines toward DTAF. In cases where such steric bulk is minimal, DTAF appears to be a suitable fluorescent labelling reagent for direct analytical applications.

Keywords: *Chemical derivatization, secondary amines, HPLC, fluorescence, DTAF.*

Chlorinated *s*-triazines undergo facile nucleophilic substitution reactions with amines forming amino triazines with loss of chloride ion [1–3]. Modification of the chloro-*s*-triazine system by incorporation of a fluorescent moiety on a carbon of the triazine nucleus provides a convenient means for introducing a fluorescent reporter group onto amines [4]. Such derivatization may have widespread applicability in the analysis of susceptible amines. To date, however, this reaction has been exploited only in preparing fluorescent-tagged reagents for fluorescence polarization immunoassays [5, 6]. For such applications, the fluoresceinyl substituted-chloro-triazine, DTAF, has been used. Although valuable for indirect analytical methods, fluorescent chloro triazines may also

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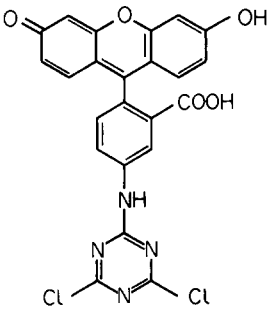


DTAF

have applicability as reagents for direct analysis of amines. After the derivatization reaction the product can be isolated chromatographically and detected by monitoring the fluorescence intensity of the column eluate. A number of fluorescent reagents for "pre-column" derivatization of amines have been described. Most of these reagents are suitable for derivatization of primary amines, but fail to form stable fluorescent products with secondary amines, or lack specificity and react indiscriminately with nucleophiles. Such reagents generally cannot be used in aqueous solution, since they are hydrolytically degraded, and this greatly limits their applicability.

Studies with simple chlorinated triazines suggest that compounds like DTAF may also react with secondary amines and display optical properties that would make them suitable as analytical reagents. This paper describes spectral and chemical reactivity characteristics of DTAF, which allow its suitability as an analytical reagent to be assessed. In particular, absorption and emission spectral properties of DTAF, its lability in aqueous media and its reactivity toward primary and secondary aliphatic amines are described. Such information will provide the basis for assessing the feasibility of broadening the applications of DTAF to include its use as an analytical reagent for pre-column derivatization of amines.

Materials and Methods

Apparatus

The chromatographic system consisted of an Altex pump (Model 110A), a Rheodyne fixed-volume loop injector (Model 7125) equipped with a 50 μl loop and a Schoffel FS-970 fluorescence detector equipped with a Corning 7-51 primary filter and a 500 nm cut-off secondary filter. Columns of ODS-Hypersil (Shandon; 150 × 4.6 mm i.d., 5 μm particles) were utilized in these studies. Chromatograms were recorded on a Houston Instrument Omniscribe recorder, and intensities were measured in terms of peak height.

The pH-stat system consisted of a Brinkman Metrohm Impulsomat (Model 614), pH meter (Model 632), Dosimat (Model 655), and a stirrer (Model E649). The experiments were performed in either a 15- or 25-ml titration vessel attached to a Haake D2L circulating temperature controller.

Ultraviolet-visible spectra were generated on a Perkin–Elmer spectrophotometer (Model 555). Fluorescence scans were obtained on a Perkin–Elmer fluorescence spectrofluorometer (Model 650-40) interfaced to the Perkin–Elmer Data Station (Model 3600).

DTAF reagent synthesis

DTAF was prepared according to the method of Barskii [4] using dry acetone as the reaction solvent. Spectral data and elemental analysis were identical with previously reported values [4].

DTAF degradation kinetics

The rate of hydrolysis of DTAF was determined at 30°C in a pH-stat system. 25 μl of an aqueous solution containing sufficient sodium perchlorate to maintain a total ionic strength of 0.01 was pipetted into a jacketed titration vessel. DTAF (250 μl of a 200 μg ml^{-1} solution in acetone) was added and the pH was adjusted with sodium hydroxide solution to the desired value. Samples were periodically withdrawn and analyzed by HPLC using a mobile phase of 22% (v/v) acetonitrile in ammonium acetate (0.01 M) at a flow rate of 1.0 ml min^{-1}.

The rate constants for the loss of DTAF at pH values 7.0 to 9.5 were determined from the initial rate of formation of the degradation product, and at pH values 10.0 to 12.0 from the first-order loss of DTAF.

Determination of the pseudofirst-order rate constants for the reaction of DTAF with various amines at pH 9.0

Potassium perchlorate solution (0.01 M; 10 ml) was pipetted into a water jacketed titration vessel and equilibrated to 30°C. The desired volume of DTAF stock suspension (1 mg ml^{-1} in acetone) was then added with enough acetone to bring the final volume to 12 ml. The pH was rapidly adjusted to pH 9 with sodium hydroxide solution (the DTAF was entirely in solution at this point) and 100 μl of amine solution (3.88×10^{-4}M) was added. Samples were periodically withdrawn and analyzed by HPLC using the mobile phases given in Table 1. In all cases, kinetic analysis was based on monitoring the formation of product. In some cases, where the half-life of formation of the product was

Table 1
Second-order rate constants for various amines reacted with DTAF

Compound	pK_a*	HPLC Mobile Phase† ACN:NH_4OAc	k_2 (M^{-1} min^{-1})‡
Benzylamine (BA)	9.33	32:68	214
N-Methylbenzylamine (NMBA)	9.54	35:65	3600
N-Ethylbenzylamine (NEBA)	9.64	38:62	233
N-Isopropylbenzylamine (NiPBA)	9.58	39:61	50
Butylamine (BuA)	10.06	25:75	251
N-Methylbutylamine (NMBuA)	11.01	33:67	12700
N-Ethylbutylamine (NEBuA)	11.10	38:62	2680
Dibutylamine (DiBUA)	11.36	42:58	9560
Proline	10.64	28:72	1490
Desipramine	10.09	60:40	15600

*All pK_a values were corrected to an ionic strength of 0.01, using the Debye-Huckel equation, and to 30°C by methods outlined by Perrin *et al.* [21]. The pK_a values listed are from Perrin *et al.* [22] except for desipramine which is froim Kenttamaa and Elfving [23].

†Products were analyzed on an ODS Hypersil column (5 μm particle size; 4.6 × 150 mm) using a mobile phase of acetonitrile (ACN):0.1 M ammonium acetate (NH_4OAc) solution expressed in (v/v) %. Flow rate = 1 ml min^{-1}. Changes in organic modifier concentration were necessitated by the broad range of product hydrophobicities. In all cases the mobile phase was adjusted to give a capacity ratio between 6 and 10.

‡Rate constants were divided by the fraction of the free amine present at pH 9.

less than the analysis time, several kinetic runs were carried out at a given pH in order to obtain a kinetic profile. The pseudofirst-order rate constants were obtained by computer fit using non-linear least squares curve-fitting.

Effect of pH on the rate of reaction of DTAF with N-*methylbenzylamine and* N-*methylbutylamine*

The pseudofirst-order rate constants for the reaction of DTAF with *N*-methylbenzylamine were determined at pH 7–11. Potassium phosphate buffers (0.01 M) were used in the pH range 7–9, while sodium carbonate buffers (0.01 M) were used in the range pH 9.5–11. 2 ml of buffer were pipetted into a polyethylene scintillation vial (Fisher Scientific). The buffer was equilibrated to 30°C and then 100 μl of a 1 mg ml^{-1} suspension of DTAF in acetone was added. The amine (20 μl of a 3.88×10^{-4}M solution in acetonitrile) was then added. Samples were periodically withdrawn and analyzed by HPLC using the conditions given in Table 1.

Results and Discussion

The absorption and fluorescence spectra of DTAF were examined in aqueous solution in the visible spectral range. Absorption properties were determined as a function of pH (Fig. 1). As pH increases, the absorbance at 435 nm, the wavelength of maximum absorption (λ_{max}) in acidic solution decreases, with a concomitant and disproportionate increase in absorptivity at 490 nm (λ_{max} at neutral and alkaline pH). A plot of absorbance at 490 nm versus pH (Fig. 2) allows the graphical determination of a single pK_a of 6.2 [7]. This pK_a can be assigned to the ionization of the phenolic function of the fluorescein moiety, based on previously reported pK_a values for fluorescein [8] (pK_a 6.50) and aminofluorescein [9] (pK_a 6.42). In the visible region, the spectral properties of DTAF are attributable to the fluorescein moiety and are not significantly influenced by the triazine nucleus and thus spectral characteristics are not expected to be significantly affected by displacement reactions involving a chlorine on the triazine ring.

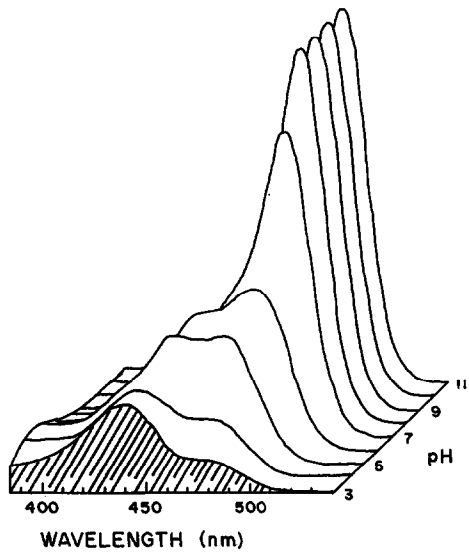


Figure 1
Visible absorption spectra of DTAF as a function of pH at 30°C.

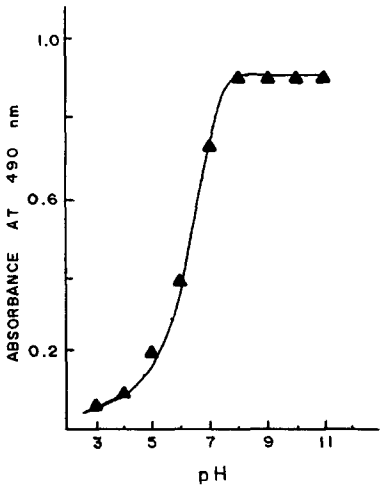


Figure 2
Plot of absorbance of DTAF at 490 nm versus pH. The pK_a was determined to be 6.18 at 30°C.

Fluorescence scans in which the excitation wavelength was varied while the emission wavelength was held constant at 700 nm indicated an excitation maximum at 490 nm. The emission maximum was determined to be 516 nm from scans in which the excitation wavelength was held constant at 450 nm and the emission spectrum was scanned from 470 to 700 nm. When fluorescence spectra were determined as a function of pH (Fig. 3), an increase in fluorescence intensity was observed with increasing pH to approximately 9; at higher pH, fluorescence decreased. A plot of fluorescence intensity at 516 nm versus pH (Fig. 4) is bell-shaped and reveals inflection points (signifying pK_a values) of 6.5 and 10.7. The pK_a of 10.7 can be assigned to deprotonation of the amine bridging the triazine and fluorescein ring systems, based on previously reported [10] pK_a values of sulphoarylamino dichlorotriazines (pK_a 10.5–10.7). Fluorescence spectra of DTAF closely resemble those of fluorescein and are also not significantly affected by the triazine ring or substituents on it.

Kinetics of DTAF hydrolytic degradation

The degradation of DTAF was studied at 30°C over the pH range 7–12. The pH was maintained with a pH-stat to avoid buffer effects. In the pH range 7–9.5, degradation was sufficiently slow to warrant calculation of rate constants from the initial rate of formation of degradation product. At higher pH, rate constants were determined from the first-order loss of DTAF. A pH-rate profile for DTAF degradation at 30°C and constant ionic strength was derived from these measurements (Fig. 5). Degradation rates increased with increasing pH, with a break in the curve observed at about pH 10.8. Slowing of degradation in this pH region is attributed to deprotonation of the amine bridging the fluorescein and triazine rings. Horrobin [10] showed that the degradation of sulphoaryl amino dichlorotriazines similarly slow appreciably as the pH approaches the pK_a of the bridging amine, despite the concomitant increase in hydroxide ion concentration present. Ackermann and Dussy [11] studying the solvolysis of arylamino dichlorodiazines and triazines in ethanol, observed a similar decrease in reactivity at pH values around the pK_a. Other workers [12–14] have observed rate suppression of hydrolysis of dihalogenated *s*-triazines due to electron releasing groups. Deprotonation of the bridging amine increases the electron density within the triazine ring, reducing

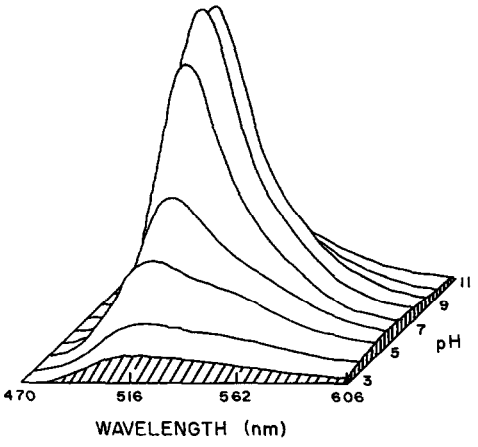


Figure 3
Fluorescence emission spectra of DTAF as a function of pH at 30°C. The excitation wavelength was 450 nm.

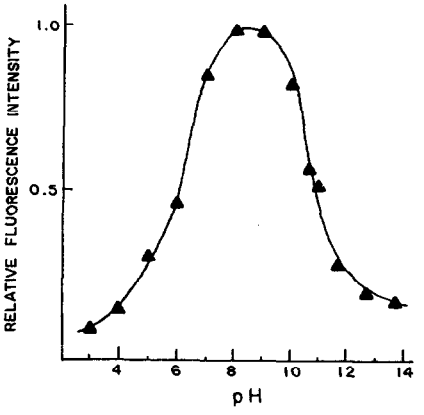


Figure 4
Plot of fluorescence intensity of DTAF at 516 nm as a function of pH. The excitation wavelength was 450 nm.

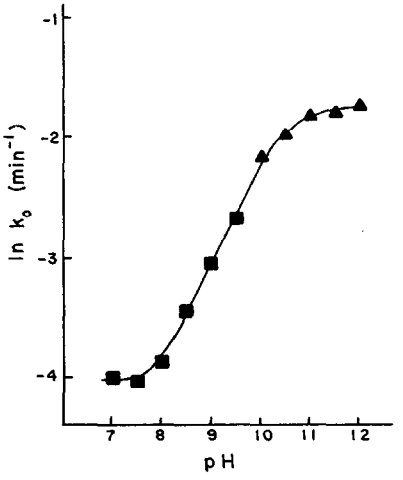
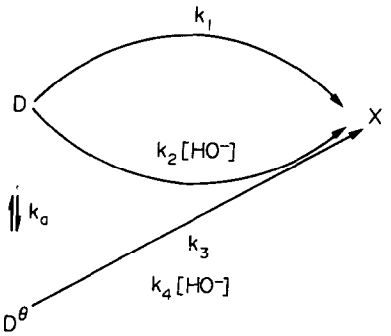


Figure 5
Plot of the pH-rate profile for the degradation of DTAF at 30°C and constant ionic strength of 0.01 M.

susceptibility to nucleophilic attack by water or hydroxide. Horrobin [10] has shown that at pH values substantially above the pK_a, hydrolysis of the less reactive deprotonated species again becomes significant, due to the high concentrations of hydroxide ions. However, characterization of DTAF degradation was not carried out in a pH region where reactivity of the deprotonated amine is significant.

Thus, the degradation of DTAF can be described by Scheme 1, where D and D^- are DTAF and its conjugate base (generated by deprotonation of the bridging amine), respectively, K_a is the ionization constant for that deprotonation and X represents the collective degradation products.



Scheme 1
Degradation of DTAF(D) in water.

Based on the above discussion, k_3 and $k_4[HO^-]$ are very much smaller than k_1 and $k_2[HO^-]$ in the pH range studied and can be neglected for this kinetic analysis. The kinetic expression describing this behaviour is represented by Equations 1 and 2,

$$k_{obs} = k_1 + k_2 f_u [HO^-] \quad (1)$$

$$k_{obs} = k_1 + \frac{k_2 K_w}{[H^+] + K_a} \quad (2)$$

where f_u is the fraction of DTAF in the unionized state (D) and K_w is the autoprotolysis constant for water. Similar rate expressions have been generated for the hydrolysis of 2,4-di(2-chloroethoxy)-*s*-triazine [15] and sulphoaryl amino dichlorotriazines [10].

The solid line in Fig. 5 represents a Simplex fit of the data to Equation 2. The rate constants for DTAF degradation determined from the fit of the data are $k_1 = 7.31 \times 10^{-5}$ min^{-1} and $k_2 = 56.1$ M^{-1} min^{-1}; this analysis afforded a pK_a for the bridging amine of 10.37 which is similar to the pK_a determined experimentally from fluorescence measurements (10.7). The magnitude of these rate constants is within the same range reported for the hydrolysis of other substituted chloro-*s*-triazines [10–15].

Kinetics of the reaction of DTAF with various amines

The rate of reaction of DTAF with various amines was determined at pH 9 (pH maintained with a pH stat) at a constant ionic strength of 0.01 M. Pseudofirst-order rate constants were determined by HPLC by measurement of formation of amine product, rather than disappearance of substrate, to remove from the kinetic analysis the effect of the amine as a general base catalyst for the hydrolysis of DTAF. Reactions were followed to at least 75% completion. The validity of the analytical method was

established by analyzing a series of samples of *N*-methylbenzylamine at known concentrations varying between 20 and 200 ng ml^{-1}. Samples were prepared in 0.01 M carbonate buffer (pH 9) and, following derivatization, were analyzed by HPLC with fluorescence detection. Over this concentration range the fluorescence response was linearly related to solute concentration (regression line: $y = 1.06x + 1.11$; $r^2 = 0.999$), demonstrating that the reagent forms a fluorescent derivative with *N*-methylbenzylamine.

The pseudofirst-order constants were measured as a function of DTAF concentration for each member of the series of amines being studied. Table 1 is a summary of the second-order rate constants determined from the slopes of plots of k_{obs} versus DTAF concentration. Secondary amines in which the *N*-substituent is methyl were consistently more reactive than the corresponding primary amines, but reactivity was apparently reduced by the introduction of further steric bulk around the amine nitrogen. There was a general trend toward increased reactivity with increasing amine basicity.

A Brønsted plot of the data (Fig. 6) reveals no direct correlation, however, between the second-order rate constant and the pK_a of the amine. This is not unexpected when the steric and electronic variability within the series of amines selected for study are considered. Similar trends (but not Brønsted correlations) were observed between the reactivity of amino acids toward 2,4-dichloro-6-(*o*-chloroanilino)-5-triazine and the pK_a of the amino function of the amino acid [16].

The reactivity of DTAF toward *N*-methylbenzylamine was also studied as a function of pH. The loss of DTAF is described in Scheme 2 and by Equations 3–5. Again, under the conditions of the reaction, D^- is assumed not to react with amine (f_u^{amine} is the fraction of reactant amine present in unionized form). Thus,

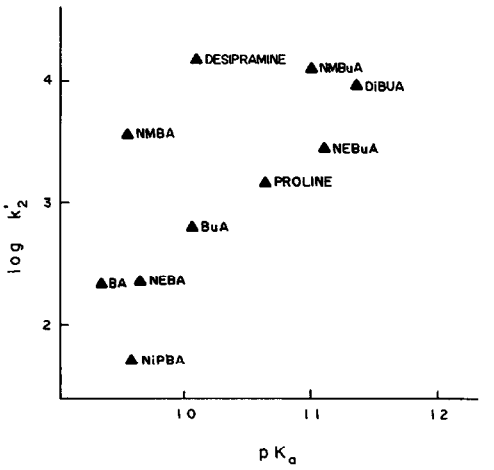
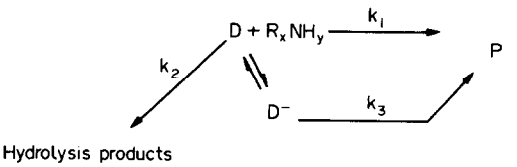


Figure 6
Plot of the second order rate constants for the reaction of DTAF with various amines versus the pK_a of the amine. See Table 1 for abbreviations.



Scheme 2
Reaction of DTAF(D) with amines to form fluorescent product (P).

$$\frac{dP}{dt} = k_1 [D] [R_xNH_y] + k_3 [D^-] \quad (3)$$

$$-\frac{dD}{dt} = (k_1 [R_xNH_y] + k_2) [D] \quad (4)$$

$$k_{obs} = k_1 + k_2 [HO^-] f_u^D f_u^{R_x NH_y} \quad (5)$$

Concentration versus time profiles were fitted to Equations 3 and 4 by non-linear least squares analysis to generate the second-order rate constant, k_1. Data were corrected for the loss of DTAF due to its degradation during the time course of the reaction. A pH-rate profile was generated (Fig. 7) from rate constants determined at constant buffer concentration, but not extrapolated to zero buffer concentration. The solid line represents the best fit to Equation 5. The pK_a for the bridging amine and the two rate constants were floated to achieve the best fit of the data; this results in calculated rate constants of $k_1 = 26.6\ M^{-1}\ min^{-1}$ and $k_2 = 3570\ M^{-2}\ min^{-1}$ and a kinetically determined value of 10.35 for the pK_a of the bridging amine (a value which agrees well with the pK_a determined by alternate means). These results and those of others [10, 17, 18] suggest that the reaction of DTAF with amines occurs most efficiently at pH values at which the reacting amine and the bridging amine of DTAF are unionized.

The effect of ionic strength on the reaction of DTAF with *N*-methylbenzylamine was studied in systems maintained at pH 9.5 (with a pH stat) in which ionic strength (μ) was varied from 1×10^{-5} to 1×10^{-2}M. Over this 1000-fold concentration range, there was no change in the rate constants for the reaction, i.e. at $\mu = 1 \times 10^{-5}$M, $k = 2.3 \times 10^{-2}$ min^{-1} and at $\mu = 1 \times 10^{-2}$M, $k = 2.2 \times 10^{-2}\ min^{-1}$.

The effect of amine concentration on the rate of reaction of DTAF with *N*-methylbenzylamine was studied at a constant pH of 9.5 (maintained with a pH stat; ionic strength 0.01). The amine concentration was varied 10-fold, while the DTAF concentration was held constant and in a minimum of 100-fold excess. Second-order rate constants for the displacement reaction increased linearily with amine concentration (Fig. 8), suggesting that the amine also plays a significant catalytic role in the

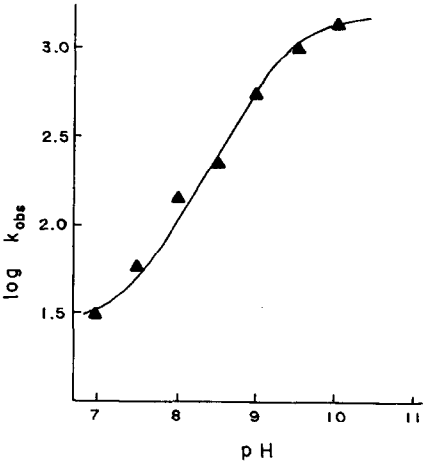


Figure 7
Plot of the ln (second order rate constant) for the reaction of DTAF with *N*-methyl benzylamine as a function of pH.

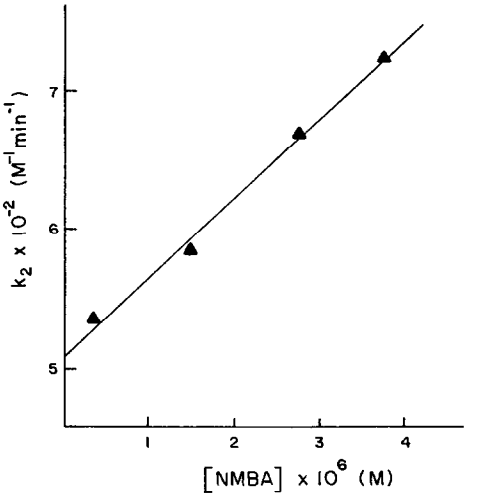


Figure 8
Plot of the second order rate constant for the reaction of DTAF with *N*-methylbenzylamine (NMBA) versus the concentration of NMBA at pH 9.5 and 30°C.

displacement reaction. This observation is consistent with the description by other authors [18–20] of the general base catalysis provided by amines in similar chemical systems. A possible mechanism is one in which the amine assists in the abstraction of a proton from the reacting amine in the transition state.

Conclusion

These results suggest that DTAF reacts efficiently with primary and secondary amines in aqueous solution. The reaction is carried out at approximately pH 9 to optimize reaction kinetics and product luminescence intensity. However, at this pH, hydrolytic degradation of DTAF also occurs. The efficient derivatization of secondary amines with DTAF suggests it to be a potentially useful reagent for the direct analysis of secondary amines in situations where the added sensitivity offered by a fluorescent label is desired.

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