Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 1, pp. 61–69, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

Chloroisothiocyanatoquinolines as fluorogenic derivatizing agents for primary and secondary amines

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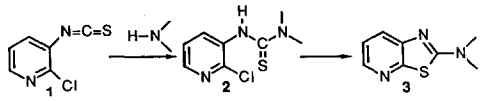
Abstract: Two new prechromatographic LC fluorogenic derivatizing agents, 2- and 4-chloro-3-isothiocyanatoquinoline (**4** and **5**) have been synthesized and shown to react smoothly with primary and secondary amines to produce fluorescent thiazoloquinolines. Compounds **4** and **5** hydrolyse in aqueous base and the rates of this reaction compared with the rates of derivatizations with amino acids indicate that the hydrolysis reaction interferes with derivatization when UV detection is used. The kinetics of derivatization with ordinary amines indicate that this reaction is quite facile, although less nucleophilic amines, e.g. aniline, react slowly. The pK_as for first protonations indicate that derivatives of **4** would be unprotonated and those of **5** would be protonated with typical RP-LC mobile phases. The Stokes shift for protonated derivatives of **5** is nearly 200 nm. The excess of unreacted derivatizing reagent interferes with UV detection of some analytes; but when fluorescence detection is used this excess produces only a small negative peak. With fluorescence detection the sensitivity of this method is about 0.8 μM at *S/N* of 2, and the response of peak height to concentration is linear over at least two decades of concentration.

Keywords: *Chemical derivatization; 2- and 4-chloro-3-isothiocyanatoquinoline; fluorescence; LC; primary and secondary amines.*

Introduction

In their search for new LC amine derivatizing agents Stobaugh and co-workers have shown that 2-amino-3-isothiocyanatoquinoline (L.L. McChesney, S.C. Bernstein, S.V. Kakodkar and J.F. Stobaugh, in preparation) (**1**) reacts with primary and secondary amines to give the expected thioureas (**2**), which cyclize to thiazolopyridines (**3**) either spontaneously or upon acidification depending on the system. This work suggests that chloroisothiocyanatoquinolines might similarly derivatize amines to give products that are more useful for fluorescence detection.



2- and 4-chloro-3-isothiocyanatoquinoline (**4**) and (**5**) were synthesized. These compounds easily formed derivatives with primary and secondary amines in acetonitrile (ACN). The rates of these reactions were measured. Compounds **4** and **5** also derivatize amino acids in aqueous base, and these rates were measured, but under these conditions the derivatizing reagents hydrolyse leading to a variety of products which tend to interfere with analyte detection using the 254 nm UV detector, and so the rates of hydrolysis were also measured. The fluorescence of the derivatives depends on their states of protonation. The pK_as for these protonations were determined spectroscopically, and the excitation and emission spectra for the protonated and neutral forms were obtained. The derivatizing procedure for ordinary amines is simple, and the derivatives separate easily on a C-8 column leading to clean peaks from the fluorescence detector, with only minimum interference from the large excess of derivatizing reagent. The derivatization of *n*-butylamine with **5** gave good HPLC linearity over a hundred-fold range of concentrations.

Experimental

Equipment

Melting points were taken on a hot-stage microscope and are uncorrected. UV spectra were recorded with a Coleman Hitachi Model 124 Double Beam Spectrophotometer, IR

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spectra on nujol mulls with a Perkin–Elmer 1420 Ratio Recording Infrared Spectrophotometer, fluorescence spectra with a SLM/Aminco DMX-1000 Spectrofluorometer, and NMR spectra with a Varian T-60 Spectrometer. Mass Spectra were obtained from The University of Kansas, courtesy of Prof. John F. Stobaugh. The chromatograph used was a Beckman Model 330 Isocratic Liquid Chromatograph with a Model 110A Pump, 20 μl injection loop, 254 nm Analytical Optical Unit. The fluorescence detector was a Fluoro-Tec Filter Fluorometer (St John Associates, Inc., Beltsville, MD, USA), with a mercury vapour UV lamp, a 245–390 nm band-pass excitation filter, and a 500 nm cut-off emission filter. All recrystallizations were accompanied by charcoal treatment. The amines used in the syntheses of **6** and **7** were distilled and stored over KOH. Elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN, USA).

Synthesis

*2-Chloro-3-isothiocyanatoquinoline (**4**).* All glassware was oven-dried, and the reaction was run under an atmosphere of dry N_2. A solution of 850 mg (4.76 mmol) 3-amino-2-chloroquinoline [1, 2] and 2.20 ml (2.15 g, 27.2 mmol) pyridine (distilled and stored over KOH) in 75 ml THF (passed through alumina, distilled, and stored over 3A molecular sieves) was, over the course of 1 h, dropped into a stirred solution of 1.00 ml (1.51 g, 13.1 mmol) thiophosgene (Aldrich) in 35 ml THF. Stirring was continued for another hour and the solvent was stripped. The residue was taken up in CH_2Cl_2, washed twice with cold water and once with saturated NaCl–H_2O, dried over $MgSO_4$, reduced to *ca* 10 ml, and chromatographed on 40 g silica gel (Fisher, 100–200 mesh), eluting with CH_2Cl_2. The residue from the first fraction was recrystallized from ACN. IR ν 2030 cm^{-1} (N=C=S); MS *m/e* 220, 222, M^+, ^{35}Cl, ^{37}Cl.

*4-Chloro-3-isothiocyanatoquinoline (**5**).* This was made by an identical procedure starting with 3-amino-4-chloroquinoline [3]. IR ν 2010 cm^{-1} (N=C=S); MS *m/e* 220, 222, M^+, ^{35}Cl, ^{37}Cl.

*2-n-Butylaminothiazolo(5,4-b)quinoline (**6a**).* A warm solution of 86.0 mg (390 μmol) **4** in 3.0 ml ACN was treated with 39.0 μl (28.9 mg, 394 μmol) *n*-butylamine. After a few minutes the product, which had precipitated, was collected by filtration and recrystallized from EtOH–H_2O. IR ν 3150 (N—H), 1615, 1560, 1205, 740 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.6–2.0 (m, 7 H, —$CH_2CH_2CH_3$), 3.57 (t, 2 H, N–CH_2), 6.16 (s, broad, 1 H, N—H), 7.2–8.2 (m, 4 H, aromatic), 8.08 (s, quinoline-4-H).

*2-Di-n-propylaminothiazolo(5,4-b)quinoline (**6b**).* A warm solution of 80.4 mg (364 μmol) **4** in 2.8 ml ACN was treated with 51.0 μl (37.6 mg, 372 μmol) di-*n*-propylamine. The mixture was allowed to stand for *ca* 15 min, treated with 2 ml 10% NaOH–H_2O, and stored in the freezer for 2 days during which time the product crystallized, and was then collected and recrystallized from EtOH–H_2O. IR ν 1610, 1600, 1535, 1335, 740 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.98 (t, 6 H, CH_3), 1.68 (sextet, 4 H, —CH_2—), 3.54 (t, 2 H, N—CH_2), 7.2–8.1 (m, 5 H, aromatic), 8.08 (s, quinoline-4-H).

*2-Anilinothiazolo(5,4-b)quinoline (**6c**).* Treatment of a warm solution of 83.1 mg (376 μmol) **4** in 2.80 ml ACN with 33.0 μl (35.5 mg, 362 μmol) aniline gave a mixture from which the solvent was stripped. The residue was triturated with 2 ml 10% aq. NaOH, washed with cold water, and recrystallized from 95% EtOH. IR ν 1630, 1595, 1570, 740 cm^{-1}.

*2-n-Butylaminothiazolo(4,5-c)quinoline (**7a**).* A warm solution of 86.0 mg (390 μmol) **5** in 3.2 ml ACN was treated with 39.0 μl (28.9 mg, 395 μmol) *n*-butylamine. The hydrochloride of the product crystallized at room temperature, and after 2 h this solid was collected by filtration, dissolved in water, and the neutral product was precipitated with 10% NaOH–H_2O. This product was recrystallized from EtOH–H_2O. IR ν 3180 (N—H), 1590, 1325, 760 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.93 (t, 3 H, CH_3), 1.2–2.0 (m, 4 H, —CH_2CH_2—), 3.4 (t, 2 H, N–CH_2), 6.42 (s, broad, 1 H, N—H), 7.1–8.2 (m, 4 H, aromatic), 8.93 (s, 1 H, quinoline-2-H).

*2-Di-n-propylaminothiazolo(4,5-c)quinoline (**7b**).* A warm solution of 80.0 mg (363 μmol) **5** in 3.0 ml ACN was treated with 51.0 μl (37.7 mg, 372 μmol) di-*n*-propylamine. After allowing the reaction mixture to

stand at room temperature for about an hour the solvent was removed and the residue was stirred with 2 ml 10% NaOH–H_2O and cooled. The product, which was induced to crystallize, was obtained by filtration, washed with a little water, dried, and recrystallized from 30–60° pet. ether. IR ν 1665, 1650, 1640, 1315, 745 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.98 (t, 6 H, CH_3), 170 (sextet, 4 H, —CH_2—), 3.53 (t, 4 H, N—CH_2), 7.1–8.2 (m, 4 H, aromatic), 9.18 (s, 1 H, quinoline-2-H).

2-Anilinothiazolo(4,5-c)quinoline (7c). Compound **5** (120 mg, 543 μmol) in 2.80 ml ACN and 49.5 μl (53.3 mg, 543 μmol) were reacted and worked up by the same procedure used for **6c**. The product was recrystallized from EtOH–H_2O. IR ν 1630, 1570, 1560, 740 cm^{-1}.

Kinetics

Hydrolyses. Reaction medium (5.05 ml) was brought to 40.3°C and, as the timer was started, treated with 100 μl of a stock solution (104.4 mg of derivatizing agent **4** or **5** in 5.00 ml ACN). Aliquots were removed regularly and subjected to LC analysis for as long as the starting material peak did not interfere with the peak for the amine product of hydrolysis which was used for the kinetic analysis. The reaction medium in each case was a 0.050 M buffer (pH, buffer: 8.0, phosphate; 9.0, borate; 10.0, carbonate) made in 1:1 (v/v) H_2O–ACN. For each derivative the flow rate was 1.8 ml min^{-1}. The mobile phases were 70:30 and 60:40 (v/v) 0.050 M, pH 3.0 phosphate buffer–ACN, for **4** and **5**, respectively, and a 4.6 × 150 mm, 5 μm Hypersil ODS column was used. The amine peak heights as a function of time were analysed by a non-linear fitting program for first-order kinetics [4] written in Z-Basic for the Zenith 100 and modified for the Macintosh.

Derivatizations. The following stock solutions were used: both **4** and **5**, 81.7 mM in ACN; *n*-butylamine, di-*n*-propylamine, and analine, 1.31_5, 1.31_3, and 1.31_7 mM, respectively, in ACN; alanine and proline, 75.8 and 7.64 mM, respectively, in H_2O. The three ordinary amines were each reacted in the same way. A solution of 100 μl of the amine stock solution and 1700 μl of ACN was equilibrated at 40.3°C and, as the timer was started, treated with 200 μl of the derivatizing agent stock solution. Aliquots were removed and subjected to LC analysis: mobile phase, phosphate buffer (0.050 M, pH 3.0)–acetonitrile (30:70, v/v) (except for the reaction of **4** with di-*n*-propylamine in which the pH of the phosphate buffer was 2.5, and the percentages were 60:40); flow rate, 2.0 ml min^{-1}; column, 150 × 4.6 mm Spherisorb C-8, 5 μm particles (1 cm guard column); UV detection. The product peak height was followed as a function of time, and the data was analysed as described in the previous paragraph. Interference from the large peak due to excess **4** in its reaction with di-*n*-propylamine made it impossible to obtain more than one peak per run with a height significantly less than the height at t_∞. In this case the rate constant was obtained from a number of runs under identical conditions by using $k = (1/t_f)\ln[1/(1-f)]$, where f is the ratio of the peak during the run to the t_∞ peak, and t_f is the time at which that peak appears. The results from these several runs were averaged.

The kinetics of derivatizations of **5** with alanine and proline were carried out in 5.00 ml of aqueous-ACN buffers previously described for the kinetics of hydrolysis, to which 5 μl of amino acid stock solution and 100 μl of **5** stock solution were added. This last ingredient was added after the rest of the solution had equilibrated at 40.3°C, and as the clock was started. The rate constant for the alanine reaction was obtained by the growth in product (the thiourea analogous to **2**) peak height as a function of time (using the same data analysis as previously described), while the rate constant for the proline reaction was obtained using the t_f method described in the previous paragraph. The LC conditions were the same as above except that the mobile phase varied as to relative amounts of 0.050 M, pH 3.0 phosphate buffer and ACN:ALA at pH 10.0, 70:30; ALA at pH 9.0, 85:15; and PRO both pH 10.0 and 9.0, 90:10.

In order to obtain peaks in a relatively clear region of the LC for the reaction of **4** with proline, the reaction had to be quenched with acid. This stopped hydrolysis and also caused the initial product, the thiourea analogous to **2** to cyclize to the thiazoloquinoline similar to **6**. The amino acid stock solution was diluted by a factor of 10 and 10 μl of this solution was added to 1.00 ml of the aqueous-ACN buffer. After equilibrating the mixture at 40.3°C, 20 μl of **4** stock solution was added as the clock was started. At measured intervals 200 μl aliquots

were removed and quenched in 20 μl conc. HCl, resulting in a pH of <1. Each of these solutions was kept at 40°C for at least 10 min, and then subjected to HPLC analysis as described above using an 85:15 (v/v) mobile phase.

Spectroscopy

UV spectra. Stock solutions contained *ca* 14 mg of each derivative in 25.00 ml 95% EtOH. One ml of the stock solution was diluted to 100.0 ml with solutions of known pH. These were prepared using H_2O–EtOH (3:2, v:v). For pH 0.3 and 0.5 this solvent was brought to the appropriate pH with conc. HCl or H_2SO_4. Other pHs were obtained with 0.050 M buffers: 2.0, 2.5, 7.0, phosphate; 3.5, 6.0, succinate; 4.0, succinate or acetate; 5.0, acetate. The absorbances were obtained by subtracting the baseline values (solvent vs solvent) from the measured values (the average of two spectra). Spectra on solutions of fixed total derivative concentration were run until a basic (unprotonated) spectrum, an acidic (protonated), and a transitional spectrum were found. From the best of these spectra, according to the coincidence of the isosbestic points, two wavelengths were chosen where the differences were relatively large and the slopes were relatively small. The pK_a was obtained at each wavelength, and the two were averaged. $pK_a = pH + \log[(A_b - A_t)/(A_t - A_a)]$, where pH is the pH of the transitional solvent, and A_a, A_b, and A_t are the absorbances in the acidic, basic, and transitional solutions, respectively.

Fluorescence spectra. A stock solution of about 14 mg in 25.00 ml 95% EtOH was prepared for each derivative. A 1.00 ml aliquot of the stock solution was brought to 100.0 ml with 0.050 M phosphate buffer EtOH–H_2O (60:40, v/v), for pHs 2.48 and 7.40; or with EtOH–H_2O (60:40, v/v) brought to pH 0.39 with conc. H_2SO_4. The excitation and emission maxima were found for each of these solutions and the emission spectrum was scanned while exciting at that maximum wavelength. Similarly the excitation spectrum was scanned while monitoring at the emission maximum wavelength. In all measurements the emission intensity of the sample was compared with the emission of a rhodamine B instrumental standard to correct for variable lamp output. One measurement was done on each compound at each pH.

Derivatization with **5**

UV detection. Stock solutions in ACN of *n*-butylamine, di-*n*-propylamine, and aniline were made up in concentrations of 1.315, 1.313, and 1.317 mM, respectively. Aliquots of these stock solutions of 100 μl each were added to 1500 μl ACN. The resulting solution was allowed to equilibrate at 40.3°C and treated with 200 μl of the 81.7_5 μM solution of **5**. Five hours after the addition of the derivatizing agent the mixture was subjected to HPLC analysis. A 150 × 4.6 mm Spherisorb C-8, 5 μ particle column was used (1 cm guard column) at a flow rate of 2.0 ml min^{-1}, with a mobile phase of 70:30 (v/v) 0.050 M, pH 3.0 phosphate buffer–ACN. The 254 nm UV detector was used.

Fluorescence detection. This derivatization was run as described in the previous paragraph with the following exceptions. The solution of amines contained 50, 5, and 250 μl of the *n*-butylamine, di-*n*-propylamine, and aniline, respectively, stock solutions; and the previously described fluorescence detector was used.

Calibration of **5** *with* n-*butylamine*

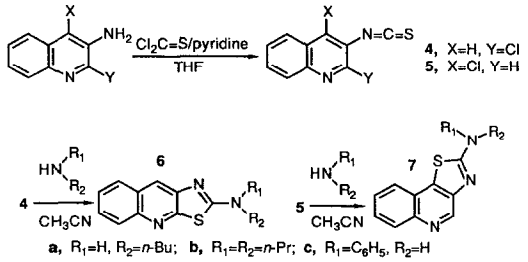
Ten amine concentrations were used to give final derivative concentrations ranging from 8.50 to 850 μM. Each concentration was run in triplicate. Each determination was run at 40.3°C, and contained 50 μl of 81.3 mM **5** in ACN so that the final solution (500 μl) contained an initial concentration of 8.13 mM **5**. Each reaction was allowed to proceed for a minimum of 20 min before being subjected to HPLC analysis as described in the preceding paragraph. The mean of the three peak heights for each concentration was calculated, as was the relative standard deviation. In two cases one of the three peaks could not be used because of interference from another peak in the chromatogram. In these two cases the range between the mean and the actual data points was used in place of σ. One concentration was discarded because of large differences among the three peak heights. The average relative standard deviation for the nine remaining points was 3% of the peak height.

Table 1
Analytical data for all new compounds

Compound	M.P. (°C)	Yield (%)	Formula	Calculated					Found				
				C	H	N	S	Cl	C	H	N	S	Cl
4	119.5–121	80	$C_{10}H_5ClN_2S$	54.43	2.28	12.69	14.53	16.07	54.32	2.28	12.57	14.80	16.22
5	87–88	79	$C_{10}H_5ClN_2S$	54.43	2.28	12.69	14.53	16.07	54.09	2.22	12.60	14.94	16.25
6a	171–173	60	$C_{14}H_{15}N_3S$	65.34	5.87	16.33	12.46		65.12	5.93	16.34	12.46	
6b	55.5–57.5	77	$C_{16}H_{19}N_3S$	67.33	6.71	14.72	11.23		67.08	6.75	14.55	11.30	
6c	254–255.5	61	$C_{16}H_{11}N_3S$	69.29	4.00	15.15	11.56		68.58	4.03	14.97	10.90	
7a	151.5–153	78	$C_{14}H_{15}N_3S$	65.34	5.87	16.33	12.46		65.12	5.93	16.30	12.92	
7b	69–71	69	$C_{16}H_{19}N_3S$	67.33	6.71	14.72	11.23		66.97	6.67	14.55	11.08	
7c	270.5–271.5	51	$C_{16}H_{11}N_3S$	69.29	4.00	15.15	11.56		68.60	4.03	15.07	10.82	

Results and Discussion

Compounds **4** and **5** were made by adding the known corresponding amines [1–3] in the presence of pyridine to thiophosgene in THF at room temperature. Compound **4** reacts readily with *n*-butylamine, di-*n*-propylamine, and aniline in ACN to give 2-*n*-butylamino-, 2-di-*n*-propylamino-, and 2-anilinothiazole(5,4-b)-quinoline, **6a**, **b** and **c**, respectively. Similarly, **5** yields 2-*n*-butylamino-, 2-di-*n*-propylamino-, and 2-anilinothiazolo(4,5-c)quinoline, **7a**, **b** and **c**. Analytical data for all new compounds are given in Table 1.



Compound **1** has been shown to derivatize a variety of amines in neutral organic solvents, however some amines, e.g. amino acids require aqueous base in order to insure that the unprotonated amino group is present (L.L. McChesney, S.C. Bernstein, S.V. Kakodkar and J.F. Stobaugh, in preparation). However, the isothiocyanato group hydrolyses in aqueous base and may give products which interfere with LC detection of the derivatives. The immediate hydrolysis product is the amine which may then react with starting material to give the thiourea and then possibly the thiazole. The rates of hydrolysis of **4** and **5** were measured as a function of pH by following the appearance of the amine peak in the HPLC. The results are shown in Table 2. These results indicate that the rates of hydrolysis are not particularly sensitive to the heterocyclic attachment of the isothiocyanato group.

Table 2
Pseudo-first-order rate constants ($k/10^{-3}$ s^{-1}) at 40°C for hydrolysis of derivatizing agents **1**, **4** and **5**

	pH			
Compound	7	8	9	10
1*	0.0075		0.03	0.8
4		<0.06	0.1	0.5
5			0.07	0.4

*From L.L. McChesney, S.C. Bernstein, S.V. Kakodkar and J.F. Stobaugh (in preparation).

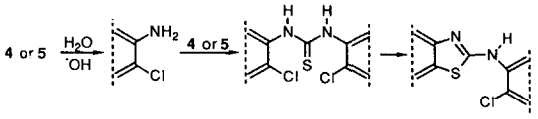


Table 3 lists the rates of reaction of a variety of amines with **4** and **5**. These experiments, as well as the kinetics of hydrolysis, were conducted using the LC with 254 nm UV detection. In the cases of the amino acids, because of interference from hydrolysis products, especially at higher pHs, this data was difficult to obtain. In addition, since the derivative is hydrolysing its concentration is changing, and the rate constants are not true pseudo-first-order constants. This data is thus less reliable than that for the reactions in ACN. These problems made it impossible to get any reliable figures for the reaction of **4** with alanine at any pH, in spite of trying a

Table 3
Pseudo-first-order rate constants ($k/10^{-3}$ s^{-1}) at 40°C for derivatizations with reagents **4** and **5***

		Solvent		
			Aqueous, pH	
Derivatizing reagent	Amine	ACN	9.0	10.0
4	*n*-$BuNH_2$	0.06		
4	*n*Pr_2NH	0.06		
4	PRO		2.0	6.0
5	*n*-$BuNH_2$	0.7		
5	*n*-Pr_2NH	5.0		
5	$\varnothing NH_2$	0.04		
5	ALA		0.4	0.6
5	PRO		3.0	4.0

*The data are normalized to a 1 mM concentration of derivatizing agent.

variety of mobile phases. In fact, of the two amino acids examined, only the secondary compound, proline, reacts at a rate sufficiently faster than hydrolysis to permit reasonably clean quantitation. In general, the secondary amines react faster than the primary aliphatic amines which react faster than the primary aromatic amine, under the same conditions, results which are to be expected. The rates of reaction of **4** and **5** with similar compounds under similar conditions are comparable in all cases except the reaction with di-*n*-propylamine in ACN. The reason for this exception is not understood. The data may be used to calculate the time required for 99% reaction and this knowledge is useful for planning prechromatographic derivatization procedures. For the ACN reactions, which in the present study were run with a derivatizing agent concentration of about 8 mM, these times range from 2 min for di-*n*-propylamine and **5**, to under 20 min for the other aliphatic amines with **4** or **5**, to 4.3 h for aniline with **5**. The kinetic results suggest that with UV detection, **4** and **5** are best used with amines that may be derivatized in non-hydrolysing media (not aqueous base), and that weakly nucleophilic amines, e.g. aniline, require long derivatization times.

Most of the derivatives could be seen to fluoresce under the hand-held UV lamp, but this phenomenon was dependent on the pH of the solvent. There are two possible sites for protonation of these compounds, the endocyclic quinoline nitrogen, and the amidine system encompassing the thiazolo and exocyclic nitrogen atoms. Stobaugh has shown that in **3** the former protonates first (L.L.McChesney, S.C. Bernstein, S.V. Kakodkar and J.F. Stobaugh, in preparation). The spectroscopic pK_as for the derivatives are: **6a**, 2.8; **6b**, 2.0; **6c**, 2.4; **7a**, 3.8; **7b**, 5.0; and **7c**, 5.8. One indicator of the accuracy of these results is the nature of the overlapping spectra. As can be

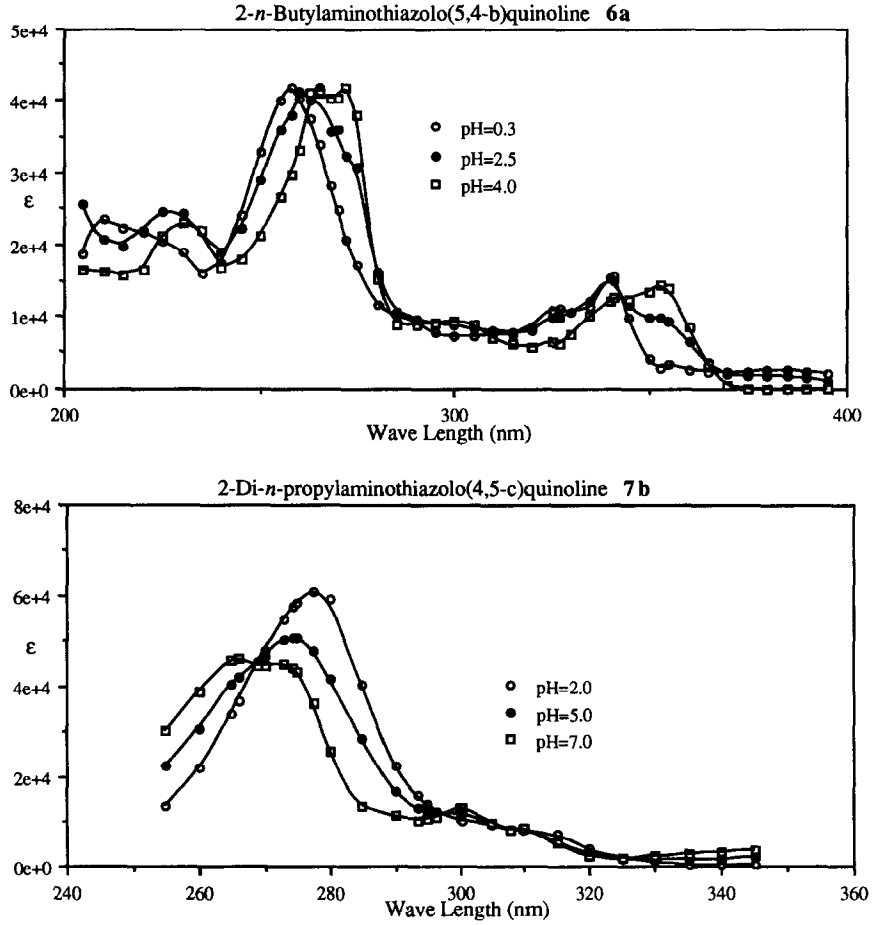


Figure 1
Representative UV spectra for pK_a determinations.

seen from Fig. 1 the isosbestic points in the spectra for **7b** are better defined than those for **6a**. In general the isosbestic points for the spectra in which the acidic medium is about pH 2 are better than those for which that pH is about 0.5. This may be due to interference from the doubly protonated species in the more acidic solvent. In any event the pK_as of **7** are significantly higher than those of **6**. Typical mobile phases for these HPLC analyses contain buffer at about pH 3 with about 30% ACN. A fluroescence detector will therefore respond to the protonated form of **7** and the unprotonated form of **6**.

The excitation and emission maxima, and the relative fluorescence intensities for **6a** and **b** and **7a** and **b**, in both protonated and unprotonated forms are given in Table 4. The protonated form of both di-*n*-propyl derivatives are the most fluorescent compounds in the table. The excitation maxima for **6** are about 50 nm longer than those for **7**, and protonation of each derivative leads to a large increase in its Stokes shift. The former property is useful in avoiding interference from possible adventitious impurities which, if they do fluoresce tend to have lower excitation maxima. The latter property is useful in avoiding stray light interference from a possible intense and perhaps broad excitation source. The pK_as of **7** suggest that these compounds will demonstrate this large Stokes shift in a typical mobile phase of pH 3.

The derivatization procedure is useful for the analysis of amines by UV and fluorescence detection. However, the derivatizing agents themselves absorb in the UV and therefore interfere with peaks from certain analyses. On the other hand, **5** does not give a positive peak with the present fluorescence detector, rather it quenches baseline fluorescence and gives a small negative peak. This is shown in Fig. 2 which shows the results of both UV and fluorescence detection. In both, the peaks for *n*-butylamine and aniline are clean. However, the di-*n*-propylamine peak is completely obliterated by the peak for excess **5** with UV detection. With fluorescence detection this last

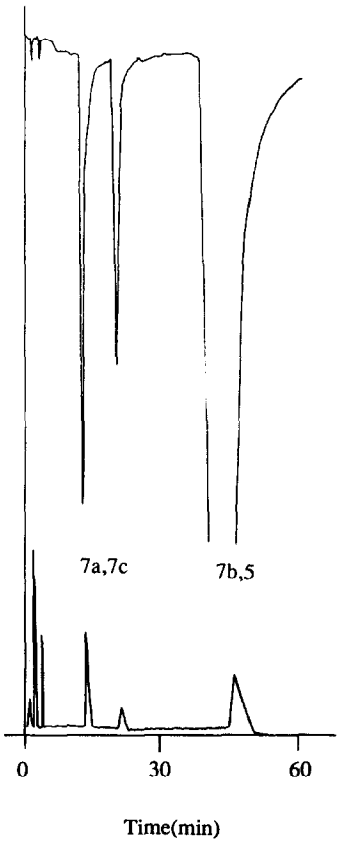


Figure 2
HPLC of derivations with reagent **5**, UV and fluorescence detection (upper and lower chromatograms, respectively; the superposition of two separate experiments). For details see Experimental section.

Table 4
Relative fluorescence intensities of derivatives

Compound	pH	Slit width (nm)*	λ (nm) ex	λ (nm) em	λ (nm) Δ	Relative fluorescence intensities
6a	0.39	1	340	443	143	29.8
6a	7.40	1	354	388	34	60.5
6b	0.39	1	343	461	118	79.3
6b	7.40	1	357	393	36	67.2
7a	2.48	4*	300	468	168	*
7a	7.40	1	299	397	98	16.2
7b	2.48	1	289	487	198	100
7b	7.40	2*	300	399	99	*

*The width of the four slits on the two monochromators. In the two cases where the slits had to be opened beyond 1 nm to measure the wavelengths the relative fluorescence has no meaning.

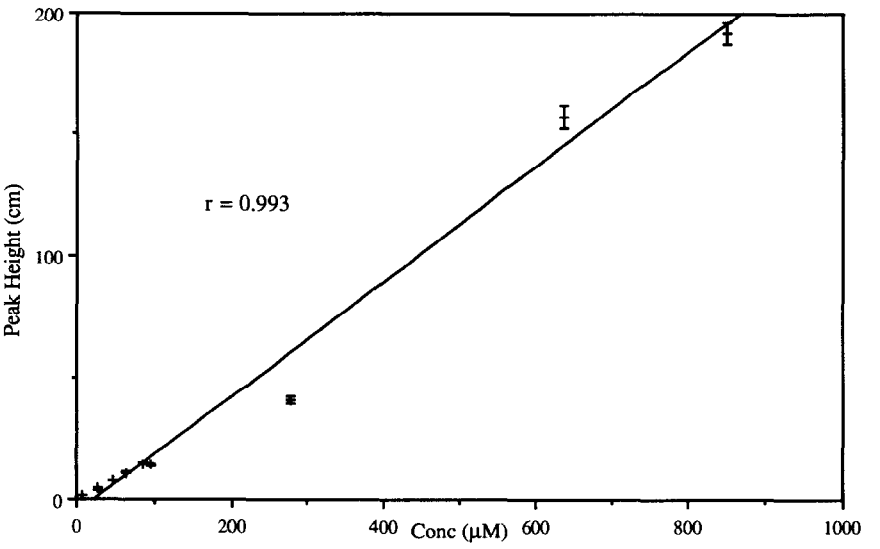


Figure 3
Calibration of reagent **5** with *n*-butylamine.

peak shows quite clearly except for a slight asymmetry due to the superposition of the negative peak for **5**. This negative peak was verified in a separate chromatogram not shown here. Note that Fig. 2 is the result of two independent chromatograms. The reproducibility of retention times is apparent from this figure, and is also supported by preliminary experiments with each of the individual derivatives. The cause of the peaks that come off shortly after the dead volume in the fluorescence chromatogram is not known. It is not due to microscopic air bubbles at the injection front since other experiments have shown that the pattern is reproducible.

Figure 3, the peak height vs concentration curve, shows good linearity with a correlation coefficient of 0.993. The sensitivity at *S/N* of 2 was about 0.8 μM, or a total of about 1.6 pmol. It is likely that the sensitivity might be improved by using a fluorescence detector with two monochromators, or by using a laser source to induce fluorescence.

Conclusions

Two new derivatizing agents have been added to the repertory of tools available for the fluorogenic derivatization of primary and secondary amines. Compounds **4** and **5** are most effective for amines which are unprotonated in organic or non-basic aqueous media. They react in less than an hour with aliphatic amines, but suffer the disadvantage of requiring long reaction times with less nucleophilic amines such as aniline. These derivatizing agents are best used with fluorescence detection where their interference with analyte peaks is small. The sensitivity obtained with **5** in this study, with the filter fluorescence detector suggests that significantly better results might be obtained with a more advanced detector.

Acknowledgements — We are grateful to Prof. John F. Stobaugh of The University of Kansas, who conceived the fundamental chemistry involved in these new derivatizing agents, for his help and encouragement with this project and for supplying the mass spectra, and to him and Prof. Christopher M. Riley for the loan of the fluorescence detector. We are also grateful to Prof. Robert Grubbs of Wright State University for the use of the spectrofluorometer. Acknowledgement is made to the Donors of the Petroleum Research Fund, administered by the American Chemical Society for support of this research.

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[*Received for review 22 October 1991; revised manuscript received 12 August 1992*]