Polycyclic Aromatic Isothiocyanate Compounds as Fluorescent Labeling Reagents

J. E. SINSHEIMER ˣ, V. JAGODIĆ, LJ. POLAK, D. D. HONG, and J. H. BURCKHALTER

Abstract □ Polycyclic aromatic isothiocyanates were synthesized in an attempt to produce new fluorescent agents for protein labeling and for use in microanalytical techniques. The relative fluorescence of these reagents, the required intermediates, and the derivatives were established. Of the compounds evaluated, the 9-acridine derivatives were the most promising.

Keyphrases □ Fluorescent labeling agents—synthesis of polycyclic aromatic isothiocyanates, relative fluorescence □ Isothiocyanates, polycyclic aromatic—synthesized as fluorescent labeling agents, relative fluorescence

In 1950, Coon and Kaplan (1) introduced fluorescein isocyanate for the fluorescent labeling of proteins. The subsequent synthesis of fluorescein and rhodamine isothiocyanates (2, 3) greatly advanced this technique because of major improvements in reagent stability and purity. These latter compounds are widely used for protein labeling, especially in antibody–antigen studies (4).

There is continued interest in these laboratories concerning the synthesis of improved fluorescent agents of greater stability and of contrasting color as compared to fluorescein isothiocyanate. The synthesis and evaluation of stilbene isothiocyanates were described previously (5). The present study describes the synthesis and evaluation of isothiocyanate derivatives of polycyclic aromatic compounds for potential use as protein labeling reagents and in fluorescent microanalysis of amines (6).

EXPERIMENTAL[1]

2-Isothiocyanato-7-methoxyfluorene (I)—A mixture of 0.9 g (0.0043 mole) of 2-amino-7-methoxyfluorene, 0.22 g (0.0022 mole) of calcium carbonate, and 200 ml of benzene was refluxed, and 0.4 ml (0.005 mole) of thiophosgene in 50 ml of benzene was added slowly from a separator. The mixture was refluxed for 3 hr and filtered by suction in the hood. Air evaporation of the filtrate afforded 0.95 g (88%) of a pink-gray precipitate. The compound was recrystallized from acetone, mp 128–129°; IR (KBr): 2950 (CH_3), 2100 (broad, NCS), 1610 and 1470 (aromatic), and 1240 (aromatic ether) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 328 (log ϵ 4.41) and 210 (4.52) nm; NMR ($CDCl_3$): δ 3.66 (s, 2, CH_2), 3.82 (s, 3, OCH_3), and 6.82–7.64 (m, 6, aromatic H).

Anal.—Calc. for $C_{15}H_{11}NOS$: C, 71.11; H, 4.39; N, 5.53; S, 12.66. Found: C, 71.12; H, 4.37; N, 5.45; S, 12.65.

[1] Melting points were in open capillary tubes with a Mel-Temp electric block and are corrected. Spectra were recorded on Perkin-Elmer model 337 IR, Beckman DK-2A UV, Aminco-Bowman 4-8106 fluorescence, and Varian A-60-A NMR spectrometers. Tetramethylsilane was used as the internal reference in NMR measurements. Microanalyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich.

1-Isothiocyanato-9-fluorenone (II)—A mixture of 1 g (0.005 mole) of 1-amino-9-fluorenone, 0.26 g (0.0026 mole) of calcium carbonate, 0.4 ml (0.005 mole) of thiophosgene, and 300 ml of benzene was treated in a similar manner as in the preparation of I. The solid was recrystallized from acetone to give 0.46 g (38%) of a yellow powder, mp 135–136.5°; IR (KBr): 2100 (broad, NCS), 1680 (C=O), and 1580 and 1560 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 340 (log ϵ 3.73), 284 (sh, 4.35), 268 (sh, 4.49), 252 (4.55), and 216 (4.64) nm; NMR ($CDCl_3$): δ 6.96–7.99 (m, aromatic H).

Anal.—Calc. for $C_{14}H_7NOS$: C, 70.86; H, 2.98; N, 5.90; S, 13.51. Found: C, 70.91; H, 3.06; N, 5.08; S, 13.61.

***N*-Benzyl-*N'*-(9-oxo-1-fluorenyl)thiourea (III)**—A mixture of 0.15 g (0.0006 mole) of II, 0.08 g (0.0007 mole) of benzylamine, and 30 ml of benzene was refluxed for 1.5 hr. Air evaporation of the solution yielded 0.1 g (45%) of a reddish-orange granular precipitate. It was recrystallized from ethanol–water, mp 184–185°; IR (KBr): 3270 and 3170 (secondary NH), 1650 (C=O), and 1570 and 1520 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 280 (sh, log ϵ 4.11), 260 (sh, 4.41), and 251 (4.46) nm; NMR [$(CD_3)_2SO$]: δ 4.78 (d, 2, J = 6 Hz, $CH_2C_6H_5$, 7.20–7.75 [m, 13 (12 aromatic H, 1 exchangeable NH)], and 9.55 (broad s, 1, NH).

Anal.—Calc. for $C_{21}H_{16}N_2OS$: C, 73.22; H, 4.69; N, 8.13. Found: C, 73.27; H, 4.79; N, 8.08.

***N*-Carboxymethyl-*N'*-(9-oxo-1-fluorenyl)thiourea (IV)**—A solution of 0.15 g (0.0006 mole) of II in 30 ml of acetone and 0.048 g (0.0006 mole) of glycine in 10 ml of 0.1 *M* $NaHCO_3$ was refluxed for 1.5 hr. The solution was cooled and a white precipitate was removed by filtration. The filtrate was adjusted to pH 4.0 with 10% HCl and was extracted with ether until the aqueous portion was almost colorless. The ether solution (dried over sodium sulfate) was evaporated, and the residue was recrystallized from acetone–water to give 0.12 g (60%) of an orange precipitate, mp 203.5–205° dec.; IR (KBr): 3300 (secondary NH), 1705 (COOH), 1645 (C=O), and 1580 and 1510 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 280 (sh, log ϵ 4.27) and 253 (4.63) nm; NMR [$(CD_3)_2SO$]: δ 4.28 (d, J = 5 Hz, 2, CH_2), 7.22–8.50 (m, 7, aromatic H), 9.24 (s, 1, NH), and 10.02 (s, 1, COOH).

Anal.—Calc. for $C_{16}H_{12}N_2O_3S$: C, 61.52; H, 3.88; N, 8.97. Found: C, 61.72; H, 3.92; N, 9.00.

4-Isothiocyanato-9-fluorenone (V)—Compound V was prepared from 4-amino-9-fluorenone in a similar manner as for II. A yield of 1.02 g (85%) was obtained from recrystallization from acetone–water, mp 140–141°; IR (KBr): 2100 (broad, NCS), 1690 (C=O), and 1570 and 1560 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 286 (sh, log ϵ 4.33), 271 (4.51), 248 (4.54), and 217 (4.87) nm; NMR ($CDCl_3$): δ 7.30–8.00 (m, aromatic H).

Anal.—Calc. for $C_{14}H_7NOS$: C, 70.86; H, 2.98; N, 5.90; S, 13.51. Found: C, 70.81; H, 2.97; N, 5.89; S, 13.36.

1-Isothiocyanato-4-methyl-9-fluorene (VI)—A mixture of 1 g (0.0048 mole) of 1-amino-4-methyl-9-fluorenone (7), 0.24 g (0.0024 mole) of calcium carbonate, 0.4 ml (0.005 mole) of thiophosgene, and 300 ml of toluene (dried over calcium hydride) was refluxed for 4 hr under the conditions used for preparing I. The filtrate was air evaporated in the hood, and the precipitate was recrystallized from acetone–water, affording 0.91 g (65%) of VI, mp 128–129°; IR (KBr): 2050 (broad, NCS), 1670 (C=O), and 1580, 1560, and 1540 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 284 (sh, log ϵ 4.06), 253 (4.39), and 217 (4.46) nm; NMR ($CDCl_3$): δ 2.49 (s, 3, CH_3) and 6.75–7.70 (m, 6, aromatic H).

Anal.—Calc. for $C_{15}H_9NOS$: C, 71.68; H, 3.62; N, 5.57; S, 12.76. Found: C, 71.58; H, 3.63; N, 5.48; S, 12.64.

***N*-Benzyl-*N'*-(4-methyl-9-oxo-1-fluorenyl)thiourea (VII)**—A mixture of 0.15 g (0.0006 mole) of VI, 0.08 g (0.0007 mole) of benzylamine, and 30 ml of benzene was refluxed for 1.5 hr. Air evaporation of the solution yielded 0.124 g (58%) of VII, which was recrystallized from acetone–water, mp 178–179°; IR (KBr): 3300 (secondary NH), 3020 (aliphatic CH), 1670 (C=O), and 1620 and 1550 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 282 (sh, log ϵ 3.92) and 254 (plateau, 4.40) nm; NMR [$(CD_3)_2SO$]: δ 4.80 (d, J = 6 Hz, 2, $CH_2C_6H_5$), 7.21–8.44 (m, 11, aromatic H), 9.47 (t, 1, NH), and 10.03 (broad s, 1, NH).

Anal.—Calc. for $C_{22}H_{18}N_2OS$: C, 73.71; H, 5.07; N, 7.82. Found: C, 73.66; H, 5.06; N, 7.82.

2-Isothiocyanato-9-acridone (VIII)—A mixture of 0.18 g (0.00086 mole) of 2-amino-9-acridone (8), 0.05 g (0.0005 mole) of calcium carbonate, 1 ml (0.013 mole) of thiophosgene, and 200 ml of toluene (dried over calcium hydride) was refluxed for 3 hr. The toluene was air evaporated in the hood. Then the residue, recrystallized from tetrahydrofuran, afforded 0.12 g (54%) of yellow-green plates, mp 338–340° dec.; IR (KBr): 3300 (NH), 2120 (broad, NCS), 1630 (C=O), and 1590, 1570, and 1520 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 327 (log ϵ 4.23), 314 (4.11), 293 (4.77), 247 (4.55), and 222 (4.35) nm.

Anal.—Calc. for $C_{14}H_8N_2OS$: C, 66.64; H, 3.20: N, 11.11. Found: C, 66.70; H, 3.45; N, 10.77.

***N*-(1-Butyl)-*N'*-(9-oxo-2-acridanyl)thiourea (IX)**—A mixture of 0.5 g (0.002 mole) of VIII and 1.5 g (0.02 mole) of *n*-butylamine in 300 ml of tetrahydrofuran was refluxed for 0.5 hr. The solvent was evaporated to dryness, and the residue was recrystallized from absolute alcohol to give 0.54 g (78%) of a yellow fluffy powder, mp 255° dec.; IR (mineral oil): 3050–3260 (broad, NH), 1635 (C=O), and 1600 and 1550 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 285 (log ϵ 4.61), 2.57 (4.68), and 213 (4.49) nm; NMR [$(CD_3)_2SO$]: δ 0.98 (t, J = 7 Hz, 3, CH_3), 1.25–2.02 (m, 4, CH_2), 3.33–3.85 (m, 2, CH_2), 7.17–8.38 [m, 8 (7 aromatic H and 1 exchangeable H), NH], 9.61 (broad s, 1, NH), and 12.12 (broad s, 1, NH).

Anal.—Calc. for $C_{18}H_{19}N_3OS$: C, 66.42; H, 5.90; N, 12.91. Found: C, 66.31; H, 5.96; N, 12.68.

***N*-Phenyl-*N'*-(9-acridinyl)thiourea (X)**—A solution of 0.5 g (0.0022 mole) of 9-isothiocyanatoacridine (9) and 0.25 g (0.0027 mole) of aniline in 100 ml of absolute alcohol was refluxed for 1 hr. The precipitate was filtered and washed with cold alcohol. Recrystallization from absolute alcohol afforded 0.63 g (90%) of product, mp 178–180°; IR (KBr): 3270 (secondary NH), 1630, 1560, and 1530 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 268 (log ϵ 4.61) and 241 (4.65) nm; NMR [$(CD_3)_2SO$]: δ 6.98–8.58 (m, aromatic H).

Anal.—Calc. for $C_{20}H_{15}N_3S$: C, 72.91; H, 4.60; N, 12.76. Found: C, 72.93; H, 4.68; N, 12.81.

***N*-Carboxymethyl-*N'*-(9-acridinyl)thiourea (XI)**—A modified literature procedure (10) was followed. A solution of 1.9 g (0.008 mole) of 9-isothiocyanatoacridine (9) in 100 ml of pyridine and 0.48 g (0.0064 mole) of glycine in basic water (20 ml of water adjusted to pH 9 with 1 *N* NaOH) was stirred and heated at 60° (water bath). The solution was maintained at pH 8–9 by adding 1 *N* NaOH dropwise until base consumption ceased (about 10 ml). Heating was continued for 0.5 hr longer. The excess isothiocyanatoacridine was extracted with benzene and the benzene extracts were discarded. The benzene content of the aqueous layer was reduced by air evaporation, and an equivalent of hydrochloric acid was added to precipitate crude XI. The precipitate was collected, dried, and recrystallized from methanol to give 1.3 g (55%) of product, mp 182–183°; IR (KBr): 3300 (secondary NH), 1720 (C=O), and 1630, 1590, and 1525 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 284 (sh, log ϵ 3.88), 253 (4.65), and 217 (4.30) nm; NMR [$(CD_3)_2SO$]: δ 4.28 (s, 2, CH_2) and 7.01–8.68 (m, 8, aromatic H).

Anal.—Calc. for $C_{16}H_{13}N_3O_2S$: C, 61.71; H, 4.22; N, 13.50. Found: C, 61.53; H, 4.24; N, 13.34.

***N,N*-Diethyl-*N'*-(9-acridinyl)thiourea (XII)**—A mixture of 0.14 g (0.0019 mole) of diethylamine and 0.5 g (0.0022 mole) of 9-isothiocyanatoacridine (9) in 100 ml of absolute alcohol was heated in a 60° water bath for 1 hr with occasional stirring. The solution was concentrated by air evaporation. Filtration and recrystallization of the precipitate from absolute alcohol afforded 0.51 g (76%) of a silky yellow precipitate, mp 184.5–186°; IR (KBr): 3080, 2980, and 2940 (aliphatic CH) and 1620, 1580, and 1520 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 285 (sh, log ϵ 4.24), 242 (4.67), and 220 (sh, 4.59) nm; NMR [$(CD_3)_2SO$]: δ 1.24 (m, 6, CH_3), 3.72 (q, 4, CH_2), 6.99–8.20 (m, 8, aromatic H), and 11.4 (s, 1, NH).

Anal.—Calc. for $C_{18}H_{19}N_3S$: C, 69.86; H, 6.20; N, 13.58. Found: C, 69.74; H, 6.18; N, 13.55.

***N*-(9-Acridinyl)thiourethane (XIII)**—A solution of 0.5 g (0.0022 mole) of 9-isothiocyanatoacridine (9) in 50 ml of absolute alcohol was treated with 10 ml of 10% HCl (aqueous). The solution was left at room temperature overnight, and a yellowish-orange precipitate was obtained. Recrystallization from acetone afforded 0.3 g (50%) of product as the hydrochloride salt, mp 138–140°; IR (KBr): 2800 (broad, HCl), 1590 and 1550 (aromatic), and 1220 (C=S) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 252 (log ϵ 5.17) nm; NMR [$(CD_3)_2SO$]: δ 1.40 (t, J = 7 Hz, 3, CH_3), 4.62 (q, J = 7 Hz, 2, CH_2), and 7.56–9.58 (m, 8, aromatic H).

Anal.—Calc. for $C_{16}H_{14}N_2OS \cdot HCl$: C, 60.27; H, 4.75; N, 8.79. Found: C, 60.54; H, 4.77; N, 8.70.

1-(9-Acridinyl)-4-benzyl-2-thiohydantoin (XIV)—The intermediate *N*-[α-benzyl(carboxymethyl)]-*N'*-(9-acridinyl)thiourea was prepared from 2 g (0.0085 mole) of 9-isothiocyanatoacridine (9), 0.7 g (0.0042 mole) of phenylalanine, and 10 ml of 1 *N* NaOH in a similar manner as for XI. A mixture of crude thiourea compound and 15 ml of acetic acid, saturated with hydrogen chloride gas, was stirred at room temperature for 6 hr in a stoppered erlenmeyer flask. The mixture was evaporated to dryness *in vacuo;* the residue, recrystallized from methanol, afforded 0.45 g (13%) of product, mp 176–178°; IR (KBr): 3320 (secondary NH), 1730 (C=O), and 1640, 1590, and 1530 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 253 (log ϵ 4.80) nm; NMR [$(CD_3)_2SO$]: δ 3.14 (d, J = 6 Hz, 2, CH_2), 5.20 (d, J = 6 Hz, 1, CH), 6.84–8.36 (m, 13, aromatic H), and 9.16 (s, 1, NH).

Anal.—Calc. for $C_{23}H_{17}N_3OS \cdot H_2O$: C, 68.80; H, 4.78; N, 10.47. Found: C, 68.55; H, 4.72; N, 10.44.

2-Thiono-3-(9-acridinyl) - 4-oxo-1,3-diazabicyclo[3.3.0]octane (XV)—A solution of 2.5 g (0.022 mole) of proline in 35 ml of Na_2HPO_4–H_3PO_4 buffer (pH 9.0) was treated with 1 g (0.0042 mole) of 9-isothiocyanatoacridine (9) in 35 ml of dioxane. The reaction mixture was stirred at room temperature for 10 hr and filtered. The filtrate was extracted with 10 100-ml portions of ethyl acetate (organic extracts were discarded), and the aqueous phase was made acidic with acetic acid until precipitation ceased (about 15 ml). Then the precipitate was filtered and dried in a vacuum desiccator to give the crude thiourea derivative. A mixture of the crude material and 10 ml of anhydrous acetic acid saturated with hydrogen chloride gas was stirred at room temperature for 10 hr in a stoppered erlenmeyer flask. The excess acid was evacuated *in vacuo,* and the remaining viscous material was recrystallized from methanol to give 0.21 g (15%) of product, mp 191–193°; IR (KBr): 2970 (aliphatic CH), 1720 (C=O), and 1630 and 1570 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 256 (log ϵ 4.77) and 223 (sh, 4.48) nm; NMR [$(CD_3)_2SO$]: δ 1.40–2.12 (m, 6, CH_2), 3.75 (m, 1, CH), and 7.54–8.71 (m, 8, aromatic H).

Anal.—Calc. for $C_{19}H_{15}N_3OS \cdot 0.5H_2O$: C, 66.64; H, 4.72; N, 12.27. Found: C, 66.40; H, 4.97; N, 12.45.

4-Isothiocyanatobenzophenone (XVI)—A suspension of 10 g (0.042 mole) of 4-aminobenzophenone hydrochloride in 150 ml of chloroform was stirred with 50 ml of aqueous sodium hydroxide (6.12 g, 0.053 mole) solution until 4-aminobenzophenone dissolved in chloroform. The mixture was cooled to 0°, and 6.14 g (0.053 mole) of thiophosgene was added at once. Stirring was continued for 3 hr at 0° and then for 1.5 hr under reflux. The chloroform layer was removed, dried, and evaporated to dryness *in vacuo.* The crude product was kept in a vacuum desiccator over phosphorus pentoxide and potassium hydroxide and then was recrystallized from petroleum ether (bp 60–75°) (charcoal). The yield was 8.6 g (82.5%) of light-yellow crystals, mp 81–82°; IR (mineral oil): 2100 (NCS) and 1640 (C=O) cm^{-1}; NMR ($CDCl_3$): δ 7.22–7.89 (m, aromatic H).

Anal.—Calc. for $C_{14}H_9NOS$: C, 70.27; H, 3.79; N, 5.85. Found: C, 70.49; H, 3.93; N, 6.15.

***N*-Benzyl-*N'*-(4-benzophenone)thiourea (XVII)**—A solution of 2 g (0.0084 mole) of 4-isothiocyanatobenzophenone and 4.5 g (0.042 mole) of benzylamine in 10 ml of alcohol was refluxed for 2 hr. Excess ethanol was removed, and crystallization of the product was initiated by adding a few drops of water and cooling. The yield was 2.3 g (79%), mp 125–127°. Recrystallization from chloroform–

petroleum ether (bp 60–75°) gave 2.1 g, mp 127–128°; IR (mineral oil): 3400 and 3030–3200 (NH), 1640 (C=O), and 1190 (C=S) cm^{-1}; NMR ($CDCl_3$): δ 4.88 (d, J = 5 Hz, 2, CH_2), 6.87 (m, 1, NH), 7.32–7.83 (m, 14, aromatic H), and 8.76 (broad s, 1, NH).

Anal.—Calc. for $C_{21}H_{18}N_2OS$: C, 72.81; H, 5.23; N, 8.08. Found: C, 72.61; H, 5.21; N, 7.86.

2-Anilino-5-nitrobenzyl Chloride (XVIII)—A solution of 10 g (0.039 mole) of 2-anilino-5-nitrobenzoic acid (11) and 8 g (0.038 mole) of phosphorus pentachloride in 160 ml of benzene was refluxed on a water bath for 1 hr. The solution was treated with charcoal and filtered while still warm. Petroleum ether (bp 30–45°) was added to the filtrate. The product crystallized as yellow crystals, yielding 8.7 g (80.5%). Two recrystallizations from benzene–petroleum ether afforded an analytical sample, mp 157–158°; IR (mineral oil): 3300 (NH), 1710 (C=O), and 1620, 1600, and 1580 (aromatic) cm^{-1}; NMR ($CDCl_3$): δ 6.99–9.22 (m, 8, aromatic H) and 9.73 (broad s, 1, NH).

Anal.—Calc. for $C_{13}H_9ClN_2O_3$: C, 56.44; H, 3.28; Cl, 12.81; N, 10.12. Found: C, 56.56; H, 3.31: Cl, 12.74; N, 10.09.

2-Nitro-9-[2-(diethylamino)ethylamino]acridine Hydrochloride (XIX)—A literature procedure (12) was modified and applied. To a solution of 5 g (0.018 mole) of XVIII in 100 ml of warm benzene, 2.1 g (0.018 mole) of *N,N*-diethylethylenediamine was added and the solution was refluxed for 30 min. Then 6 ml (0.065 mole) of phosphorus oxychloride was introduced and refluxing was continued on an oil bath for 2 hr. Cooling caused the crude product (mp 220°) to separate from solution. It was collected on a filter, washed with benzene, and recrystallized from ethanol (400 ml) to give 6.2 g (92%) of yellow needles, mp 223–224°; IR (mineral oil): 3260 (NH), 1640 and 1580 (aromatic), and 1530 and 1330 (NO_2) cm^{-1}; NMR [$(CD_3)_2SO$]: δ 1.30 (t, J = 7 Hz, 6, CH_3), 3.05–3.80 (m, 8, CH_2), 7.13–8.72 (m, 7, aromatic H), 9.40 (broad s, 1, HCl), and 10.58 (broad s, 1, NH).

Anal.—Calc. for $C_{19}H_{22}N_4O_2 \cdot HCl$: C, 60.88; H, 6.18; Cl, 9.46; N, 14.94. Found: C, 60.94; H, 6.19; Cl, 9.47; N, 14.94.

2-Amino-9-[2-(diethylamino)ethylamino]acridine Monohydrate (XX)—A mixture of 4.1 g (0.011 mole) of XIX, 8.8 g (0.039 mole) of stannous chloride dihydrate, and 10 ml of concentrated hydrochloric acid was refluxed on an oil bath for 1 hr. The solution was made alkaline by the addition of 100 ml of 5 *M* sodium hydroxide and kept warm until the stannous hydroxide dissolved. A product separated as a yellow oil, which solidified after cooling. The solid was collected on a filter and washed with sodium hydroxide solution and then with water. The yield was 3.1 g (86.4%), mp 127°. Recrystallization from benzene (charcoal)–petroleum ether (bp 30–45°) gave 2.4 g (67%) of silvery-white crystals, mp 127–128°. A second recrystallization gave an analytical sample, mp 127–129°; IR (mineral oil): 3360, 3300, 3240, and 3180 (NH) and 1640, 1600, and 1580 (aromatic) cm^{-1}; NMR ($CDCl_3$): δ 0.98 (t, J = 7 Hz, 6, CH_3), 2.32–2.68 (m, 6, CH_2), 3.33 (t, J = 6 Hz, 2, CH_2), 3.53 (s, 2, NH_2), 6.55–7.34 [m, 9 (7 aromatic H and 2 exchangeable H), H_2O], and 8.00 (broad s, 1, NH).

Anal.—Calc. for $C_{19}H_{24}N_2 \cdot H_2O$: C, 69.91; H, 8.03; H_2O, 5.52; N, 17.16. Found: C, 69.98; H, 7.95; H_2O, 5.36; N, 17.23.

2-Isothiocyanato - 9-[2-(diethylamino)ethylamino]acridine Hydrochloride (XXI)—To a solution of 3.1 g (0.0095 mole) of XX in 50 ml of acetone, 1.15 g (0.01 mole) of thiophosgene was added and the solution was refluxed for 1.5 hr. The light-brown solution was filtered, and petroleum ether (bp 30–45°) was added to the filtrate to obtain 3.35 g (83%) of light-yellow product, mp 164–168° dec. Recrystallization from chloroform (charcoal)–ether gave 3 g (74.5%) of white product, mp 167–168°; IR (mineral oil): 3280 (NH), 2070 (NCS), and 1650 and 1580 (aromatic) cm^{-1}; NMR ($CDCl_3$): δ 1.45 (t, J = 7 Hz, 6, CH_3), 3.10–3.43 (m, 6, CH_2), 3.90 (m, 2, CH_2), 7.02–7.90 [m, 9 (7 aromatic H and 2 exchangeable H), H_2O], 8.99 (broad s, 1, HCl), and 9.76 (broad s, 1, NH).

Anal.—Calc. for $C_{20}H_{22}N_4S \cdot HCl \cdot 1.5H_2O$: C, 58.03; H, 6.33; Cl, 8.56; H_2O, 6.53; N, 13.53; S, 7.75. Found: C, 58.11; H, 6.32; Cl, 8.69; H_2O, 6.41; N, 13.56; S, 7.69.

***N* - Benzyl - *N'* - [9-[2-(diethylamino)ethylamino]-2-acridinyl]thiourea (XXII)**—To a solution of 0.42 g (0.001 mole) of XXI in 4 ml of ethanol, 0.21 g (0.002 mole) of benzylamine in 2 ml of alcohol was added and the mixture was refluxed in a water bath for about 10 min. The mixture was cooled and a few drops of ether were added. The yield was 0.38 g (82.5%) of white crystals, mp 172°. Recrystallization from alcohol gave a melting point of 172–173°; IR (mineral oil): 3290 and 3140 (NH), 1650, 1600, and 1580 (aromatic), and 1075 (C=S) cm^{-1}; NMR [$(CD_3)_2SO$]: δ 1.00 (t, J = 7 Hz, 6, CH_3), 2.39–2.72 (m, 6, CH_2), 3.24–3.47 (m, 2, CH_2), 4.88 (d, J = 6 Hz, 2, $CH_2C_6H_5$), 7.00–7.77 (m, 12, aromatic H), 8.33 (m, 3, NH and H_2O), 9.56 (s, 1, NH), and 9.60 (s, 1, NH).

Anal.—Calc. for $C_{27}H_{31}N_5S \cdot H_2O$: C, 68.18; H, 6.99; H_2O, 3.79; N, 14.72; S, 6.74. Found: C, 68.26; H, 6.93; H_2O, 3.75; N, 14.69; S, 6.78.

9-(4-Nitrobenzyl)acridine (XXIII)—This compound was prepared from 9-benzylacridine according to Hunters and Shaw (13), mp 217–218° [lit. (13) mp 195–198°]; IR (mineral oil): 1540 (NO_2) and 1600 (aromatic) cm^{-1}; NMR ($CDCl_3$): δ 5.07 (s, 2, CH_2) and 7.5–8.2 (m, 12, aromatic H).

Anal.—Calc. for $C_{20}H_{14}N_2O_2$: C, 76.42; H, 4.49; N, 8.91. Found: C, 76.22; H, 4.37; N, 8.96.

9-(4-Aminobenzyl)acridine (XXIV)—A solution of 1.7 g (0.0057 mole) of XXIII in 200 ml of 95% alcohol and 4.51 g (0.02 mole) of stannous chloride in 10 ml of concentrated hydrochloric acid was refluxed for 1 hr on a water bath. The solvent was removed *in vacuo*, and the residue was neutralized with 10% sodium hydroxide. The precipitate was collected on a filter, washed with water, and recrystallized from alcohol. The yield was 1.26 g (80%), mp 231–233°; IR (mineral oil): 3430 (NH) cm^{-1}; NMR ($CDCl_3$): δ 3.25 (s, 2, NH_2), 4.65 (s, 2, CH_2), and 6.2–8.1 (m, 12, aromatic H).

Anal.—Calc. for $C_{20}H_{16}N_2$: C, 84.48; H, 5.67; N, 9.85. Found: C, 84.31; H, 5.74; N, 9.90.

9-(4-Isothiocyanatobenzyl)acridine (XXV)—In 150 ml of acetone was dissolved 1 g (0.0034 mole) of XXIV, and 0.77 ml (0.01 mole) of thiophosgene was added at once. The solution was refluxed for 1 hr. The light-yellow solid was collected and washed with acetone, yielding 1 g (77%), mp 217–220° dec. One gram (0.0027 mole) of hydrochloride was dissolved in chloroform and converted to the free base by shaking with 10% ammonia. The chloroform was evaporated and the residue was recrystallized from cyclohexane to afford 0.7 g (79% yield) of product, mp 156–157°; IR (mineral oil): 3300 (NH) and 2120 (broad, NCS) cm^{-1}; NMR ($CDCl_3$): δ 4.81 (s, 2, CH_2) and 6.82–8.1 (m, 12, aromatic H).

Anal.—Calc. for $C_{21}H_{14}N_2S$: C, 77.28; H, 4.32; N, 8.58. Found: C, 77.23; H, 4.34; N, 8.53.

9-(4-Nitro-α-methylbenzyl)acridine (XXVI)—A solution of 2.5 g (0.0088 mole) of 9-(α-methylbenzyl)acridine (14) in 10 ml of 10 *N* nitric acid was heated to 60° for 10 min. The mixture was cooled and the solid was collected to yield 3 g of the nitrate of starting material, mp 165–168°. The product was dissolved in concentrated sulfuric acid (20 ml), which resulted in a rise in temperature to 50°. The solution was cooled, poured over ice, and made alkaline with concentrated ammonia. The resultant solid was collected, dissolved in alcohol (70 ml), decolorized with activated charcoal, and precipitated by addition of water (30 ml). The yield was 1.5 g (52%) of product, which was recrystallized from alcohol, mp 171–172°; IR (mineral oil): 1580 (NO_2) cm^{-1}; NMR [$(CD_3)_2SO$]: δ 2.02 (d, J = 7 Hz, CH_3), 6.33 (q, J = 7 Hz, 1, CH), and 7.41–8.33 (m, 12, aromatic H).

Anal.—Calc. for $C_{21}H_{16}N_2O_2$: C, 76.81; H, 4.91; N, 8.53. Found: C, 76.66; H, 4.97; N, 8.64.

9-(4-Amino-α-methylbenzyl)acridine (XXVII)—A solution of 1.69 g (0.005 mole) of XXVI in 250 ml of alcohol and 1.5 g (0.02 mole) of stannous chloride in 10 ml of concentrated hydrochloric acid was refluxed for 45 min. The solvent was removed *in vacuo* and the residue was neutralized with 10% sodium hydroxide. The precipitate was collected on a filter, washed with water, and recrystallized from 75% alcohol. The yield was 1.1 g (75%) of XXVII, mp 190–191°; IR (mineral oil): 3425, 3310, and 3180 (NH) and 1630, 1590, and 1520 (aromatic) cm^{-1}; NMR [$(CD_3)_2SO$]: δ 1.96 (d, J = 7 Hz, 3, CH_3), 3.49 (s, 2, NH_2), 5.67 (q, J = 7 Hz, 1, CH), and 6.55–8.31 (m, 12, aromatic H).

Anal.—Calc. for $C_{21}H_{18}N_2$: C, 84.53; H, 6.08; N, 9.39. Found: 84.37; H, 6.12; N, 9.42.

9-(4-Isothiocyanato-α-methylbenzyl)acridine (XXVIII)—To a solution of 0.75 g (0.0027 mole) of XXVII in 7 ml of acetone, 0.77 ml (0.01 mole) of thiophosgene was added at once. The solution was refluxed for 1 hr. The yellow solid was collected on a filter and washed with dry acetone to yield 0.9 g of solid. The solid was dissolved in chloroform and converted to the free base with 10% ammonia. The yield was 1 g (70%) of product, mp 110–114°; IR (mineral oil): 2120 (broad, NCS) cm^{-1}; NMR ($CDCl_3$): δ 2.02 (d, J

Table I—Fluorescence Properties

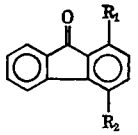
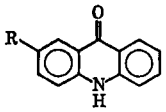
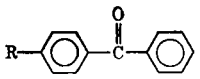
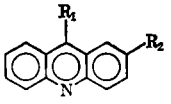
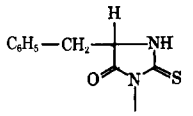
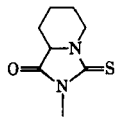
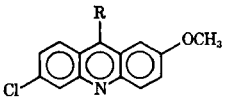
Compound	Compound Number or Source	pH[a]	Activation/ Fluorescence Wavelengths, nm	Relative Fluorescence[b]
Fluorenones				
R_1 / R_2				
NH_2 / H	—[c]	6.8	460/525	0.14
NCS / H	II	3.0	450/525	0.0044
$NHCSNHCH_2C_6H_5$ / H	III	11.0[d]	440/535	0.0096
$NHCSNHCH_2$-COOH / H	IV	10.0[d]	425/520	0.069
H / NCS	V	5.9	300/545	0.00026
NH_2 / CH_3	Ref. 7	5.9	470/535	0.23
NCS / CH_3	VI	5.9	360/535	0.0014
$NHCSNHCH_2C_6H_5$ / CH_3	VII	5.9	480/540	0.00029
Acridones				
R				
H	—[c]	9.0	400/425, 440	4.33
NH_2	Ref. 8	8.0	240/530	0.67
NCS	VIII	12.5	400/470, 480	0.49
$NHCSNHC_4H_9$	IX	9.0	400/470	0.039
Benzophenones				
R				
NH_2	—[c]	5.0	260/420	0.0021
NCS	XVI	7.0	257/420	0.0010
$NHCSNHCH_2C_6H_5$	XVII	2.0	302/405	0.0020
Acridines				
R_1 / R_2				
NH_2 / H	—[c]	5.9	270/440, 460	2.64
Cl / H	—[e]	3.0	270/440	0.088
NCS / H	Ref. 9	1.0	410/440, 460	0.67
$NHCSNHC_4H_9$ / H	Ref. 9	1.0	410/440, 460	0.013
$NHCSNHC_6H_5$ / H	X	1.0	400/440, 460	0.30
$NHCSNHCH_2$-COOH / H	XI	3.0	400/460	0.084
$NHCSN(C_2H_5)_2$ / H	XII	1.0	270/470	0.0069
$NHCSOC_2H_5$ / H	XIII	3.0	400/440	0.025
 / H	XIV	3.0	260/460	0.058
 / H	XV	12.5	400/490	0.046
$NHCH_2CH_2N(C_2H_5)_2$ / NCS	XXI	12.5	370/485	0.0033
$NHCH_2CH_2N(C_2H_5)_2$ / NHCSNH-$CHC_6H_5CH_2$	XXII	12.5	330/500	0.0016
$CH_2—C_6H_4—NH_2$ / H	XXIV	3.0	350/480	0.086
$CH_2—C_6H_4—NCS$ / H	XXV	7.0	360/430	0.20
$CH(CH_3)—C_6H_4—NH_2$ / H	XXVII	7.1	360/470	0.041
$CH(CH_3)—C_6H_4—NCS$ / H	XXVIII	8.0	365/430	0.17

Table I—(*Continued*)

Compound	Compound Number or Source	pH[a]	Activation/ Fluorescence Wavelengths, nm	Relative Fluorescence[b]
2-Methoxy-6-chloroacridines				
R				
$NHCH_2CH_2$—C_6H_4—NH_2	XXX	2.0	440/500	0.27
$NHCH_2CH_2$—C_6H_4—NCS	XXXI	11.0	270/490	0.03
$NHCH_2CH_2CH_2$—C_6H_4—NCS	XXXIV	11.0	430/500	0.40

[a] Compounds were measured in absolute alcohol–aqueous solutions (1:1) except where noted. Values given for pH are those of the aqueous solutions at maximal fluorescence. [b] Relative fluorescence is relative to a 1.60×10^{-6} *M* solution of fluorescein isothiocyanate at pH 10. [c] Aldrich Chemical Co. [d] Compounds measured in dioxane–aqueous solutions. [e] Eastman Organic Chemicals.

= 7 Hz, 3, CH_3), 5.71 (q, *J* = 7 Hz, 1, CH), and 7.18–8.38 (m, 12, aromatic H).

Anal.—Calc. for $C_{22}H_{16}N_2S$: C, 77.63; H, 4.74; N, 8.23. Found: C, 77.58; H, 4.84; N, 8.23.

2-Methoxy-6-chloro-9-[2-(4-nitrophenyl)ethylamino]acridine (XXIX)—One gram (0.0029 mole) of 2-methoxy-6-chloro-9-phenoxyacridine was dissolved in 4 g of phenol at 80°. Then 0.78 g (0.0042 mole) of 2-(4-nitrophenyl)ethylamine hydrochloride was added to the solution, which was heated at 140° for 7 hr. The mixture was cooled and poured into ether (10 ml), and the hydrochloride of XXIX was collected. The yield was 1.5 g, mp 255–258°. The base was liberated with cold 5% sodium hydroxide and recrystallized from methanol to give 1 g (82%) of yellow crystalline XXIX, mp 150–151°; IR[2]: 3300 (NH) and 1320 (NO_2) cm^{-1}; NMR [$(CD_3)_2SO$]: δ 3.18 (t, *J* = 7 Hz, 2, CH_2), 3.94 (s, 3, OCH_3), 4.10 (t, *J* = 7 Hz, 2, CH_2), and 7.21–8.38 [m, 11 (10 aromatic H and 1 exchangeable H), NH].

Anal.—Calc. for $C_{22}H_{18}ClN_3O_3$: C, 64.78; H, 4.45; N, 10.30. Found: C, 64.65; H, 4.51; N, 10.29.

2-Methoxy-6-chloro-9-[2-(4-aminophenyl)ethylamino]acridine (XXX)—A solution of 4.07 g (0.01 mole) of XXIX in 1 ml of methanol and 0.79 g (0.033 mole) of stannous chloride in 10 ml of hydrochloric acid was refluxed for 1.5 hr. The solvent was removed *in vacuo*, and the residue was neutralized with 5% sodium hydroxide to give 3 g (76%) of XXX. The product was recrystallized from 80% alcohol as yellow crystals, mp 130–131°; IR[2]: 3320 (NH_2) and 1600 (aromatic) cm^{-1}; NMR ($CDCl_3$): δ 2.70 (t, 2, CH_2), 3.58 (t, 2, CH_2), 3.63 (s, 3, OCH_3), 4.5 (broad s, 1, NH), and 6.16–7.62 (m, 10, aromatic H).

Anal.—Calc. for $C_{22}H_{20}ClN_3O$: C, 69.92; H, 5.33; N, 11.12. Found: C, 69.70; H, 5.43; N, 11.11.

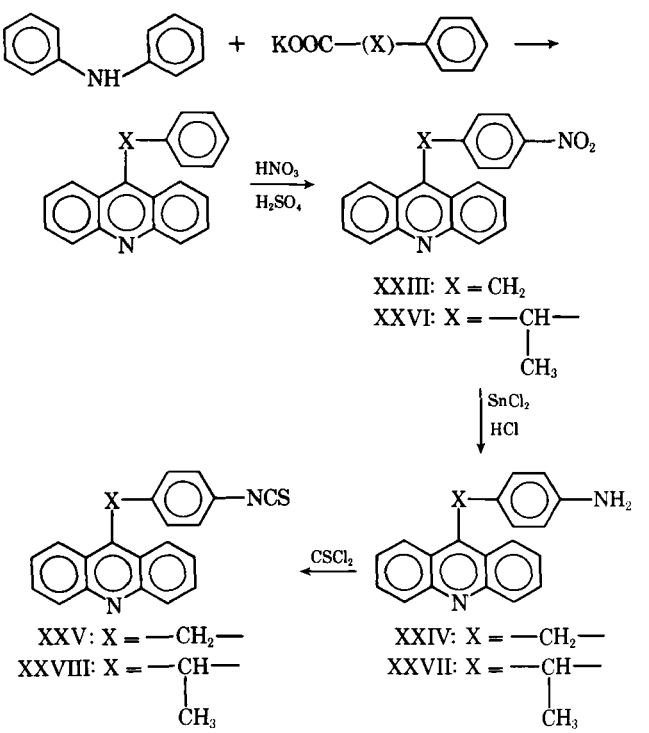
2 - Methoxy - 6 - chloro-9-[2-(4-isothiocyanatophenyl)ethylamino]acridine Hydrochloride (XXXI)—In 20 ml of acetone, 0.4 g (0.001 mole) of XXX was dissolved and 0.77 ml (0.01 mole) of thiophosgene was added at once. The solution was refluxed for 1.5 hr and the light-yellow precipitate was collected to yield 0.45 g (94%) of XXXI-HCl. It was recrystallized from methanol, mp 184–185°; IR[2]: 2120 (broad, NCS) cm^{-1}; NMR ($CDCl_3$): δ 3.15 (t, *J* = 7 Hz, 2, CH_2), 3.95 (s, 3, OCH_3), 4.2 (t, 2, CH_2), and 7.05–8.2 (m, 10, aromatic H).

Anal.—Calc. for $C_{23}H_{19}Cl_2N_3OS$: C, 60.54; H, 4.20; N, 9.21. Found: C, 60.31; H, 4.21; N, 9.20.

2 - Methoxy - 6 - chloro-9-[3-(4-nitrophenyl)propylamino]acridine (XXXII)—A quantity of 2.18 g (0.0065 mole) of 2-methoxy-6-chloro-9-phenoxyacridine (15) was dissolved in 9 g of phenol at 80°. Then 1.5 g (0.0065 mole) of 3-(4-nitrophenyl)propylamine hydrochloride (16) was added and heating was continued at 130° for 3 hr. The mixture was cooled and poured into ether (30 ml), and a solid was collected to give 3.2 g of XXXII-HCl, mp 229–231°. The free base was liberated with cold 10% sodium hydroxide. It was recrystallized from methanol to afford 2.5 g (91%) of product, mp 122–123°; IR[2]: 1330 (NO_2) cm^{-1}; NMR ($CDCl_3$): δ 2 (m, 2, CH_2), 2.7 (t, *J* = 7 Hz, 2, CH_2), 3.56 (t, *J* = 7 Hz, 2, CH_2), 3.78 (s, 3, OCH_3), and 6.90–8.0 (m, 10, aromatic H).

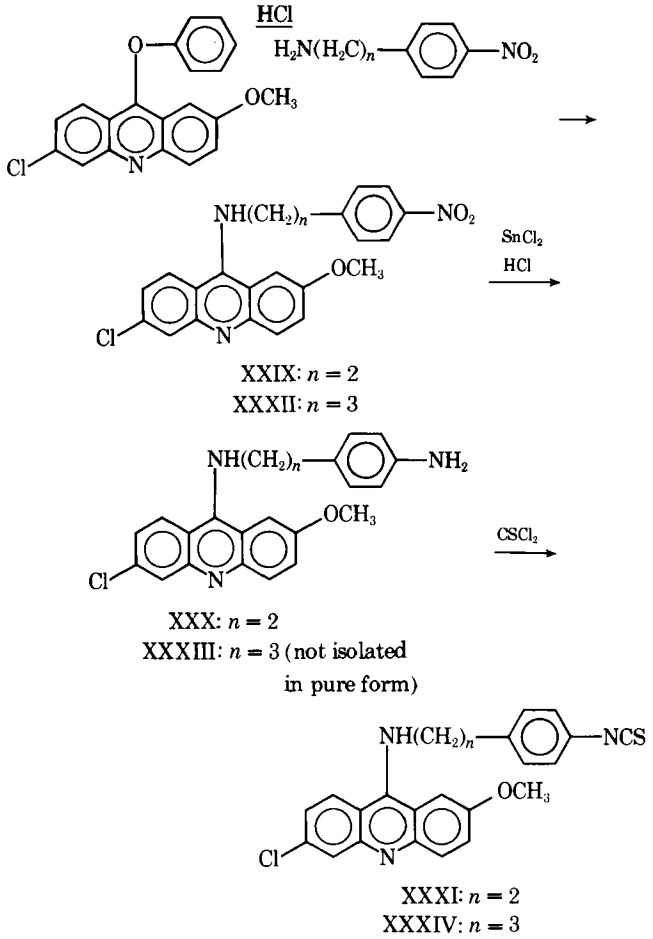
Anal.—Calc. for $C_{23}H_{20}ClN_3O_3$: C, 65.78; H, 4.78; N, 9.96. Found: C, 65.72; H, 4.79; N, 10.03.

2-Methoxy - 6-chloro-9-[3-(4-isothiocyanatophenyl)propylamino]acridine (XXXIV)—A solution of 4.2 g (0.010 mole) of nitro compound XXXII in 800 ml of methanol and 7.44 g (0.033 mole) of stannous chloride in 10 ml of concentrated hydrochloric acid was refluxed for 1.5 hr. It was refrigerated to yield 4 g of solid (87%), mp 275–278°. Conversion to the free base with 5% ammonia and recrystallization from 75% alcohol gave XXXIII, mp 90–92°. This solid was used without further purification as an acetone solution (1 g/50 ml), to which 1 ml of thiophosgene was added at once. The solution was refluxed for 1 hr, and the light-yellow solid was collected and washed with acetone to give 1 g of XXXIV-HCl, mp 230–235° dec. One gram of XXXIV-HCl was dispersed in chloroform and converted to the free base with aqueous 10% ammonia. The chloroform was evaporated and the residue was recrystallized from cyclohexane to afford 0.7 g (47%) of product as yellow needles, mp 132–133°; IR (mineral oil): 2120 (broad, NCS) cm^{-1};



Scheme I—Type A Compounds

[2] Fluorolube.



Scheme II—Type B Compounds

NMR ($CDCl_3$): δ 2.05 (m, 2, CH_2), 2.70 (t, J = 7 Hz, 2, CH_2), 3.65 (t, J = 7 Hz, 2, CH_2), and 7.0–8.05 (m, 10, aromatic H).

Anal.—Calc. for $C_{24}H_{20}ClN_3OS$: C, 66.42; H, 4.69; N, 9.68. Found: C, 66.23; H, 4.61; N, 9.71.

RESULTS AND DISCUSSION

The synthesis of polycyclic aromatic isothiocyanates appeared to be an attractive approach in the search for new microanalytical and protein labeling reagents. As rigid conjugated unsaturated structures, they presented the potential, after UV activation, for high fluorescence with minimal loss of energy by alternative pathways.

An initial survey of compounds was based upon the availability of suitable intermediates for the synthesis of isothiocyanate derivatives by reaction of the corresponding amines with thiophosgene, as previously described for the preparation of fluorescein isothiocyanate (17). Compounds I, II, V, and XVI were prepared in this manner from commercially available amines. Also, VI was prepared in the same manner from its corresponding amine synthesized in the laboratories for other purposes (7), and VIII was prepared from the amine described in the literature (8). In addition, 9-isothiocyanatoacridine was synthesized by reaction of the 9-chloro compound with silver thiocyanate as previously reported (9).

Previous studies with stilbene isothiocyanate (5) had indicated that the thiourea compounds formed by reaction of the isothiocyanates with amines, such as benzylamine, served as more suitable models for relative fluorescence studies than the reagents *per se*. In a similar manner, the *N*-benzyl and other thiourea derivatives were prepared from the aforementioned isothiocyanates (I, II, V, VI, VIII, and XVI) for comparison of fluorescence. The fluorescence characteristics of the compounds of interest are summarized in Table I. The fluorenes were not included because they lacked sufficient fluorescence for reliable measurement. Since 9-isocyanatoacridine (9) was among the more promising of the reagents synthesized, a further series of its derivatives (X–XV) was prepared and their relative fluorescence is also reported in Table I. It became evident that even with this more promising reagent, the derivatives of interest exhibited a considerable decrease in fluorescence.

Compound XXI was desired to examine the effect of isothiocyanato at the 2-position and to obtain increased water solubility as the amine hydrochloride salt. Compound XXI was prepared by the condensation of XVIII with *N,N*-diethylethylenediamine in the presence of phosphorus oxychloride to form XIX. Reduction with stannous chloride and hydrochloric acid yielded the 2-amino compound (XX), which was reacted with thiophosgene to give XXI as the hydrochloride salt. However, as indicated in Table I, this compound and its *N*-benzylthiourea (XXII) are not intensely fluorescent.

A study was initiated to remove the aromatic isothiocyanate moiety from conjugation with the acridine nucleus so as to retain, and not effect, the high fluorescence of the acridines. Examples of the following two types of isothiocyanates (A and B) were synthesized as indicated.

Type A compounds (XXIV, XV, XVII, and XVIII) (Scheme I) exhibited a moderate intensity of fluorescence (Table I). Type B compounds (XXX, XXXI, and XXXIV) (Scheme II) also showed moderate intensity. However, they were prone to cleave at the 9-position to yield 2-methoxy-6-chloro-9-acridone. This proved to be an important limitation in their synthesis and subsequent use.

Of the polycyclic compounds studied, the 9-acridine derivatives were the most promising reagents. Work has been most extensive with 9-isothiocyanatoacridine. However, its derivatives (X–XV) generally have a low intensity of fluorescence and use (6) depends upon a unique photochemical cyclization (9).

REFERENCES

(1) A. H. Coon and M. H. Kaplan, *J. Exp. Med.*, **91**, 1(1950).

(2) J. H. Burckhalter and R. J. Seiwald, U.S. pat. 2,937,186 (1960).

(3) J. L. Riggs, R. J. Seiwald, J. H. Burckhalter, C. M. Downs, and T. G. Metcalf, *Amer. J. Pathol.*, **34**, 1081(1958).

(4) "Fluorescent Protein Tracing," 3rd ed., R. C. Nairn, Ed., Livingstone, Ltd., Edinburgh, Scotland, 1969.

(5) J. E. Sinsheimer, J. T. Stewart, and J. H. Burckhalter, *J. Pharm. Sci.*, **57**, 1938(1968).

(6) A. DeLeenheer, J. E. Sinsheimer, and J. H. Burckhalter, *ibid.*, **62**, 1370(1973).

(7) E. E. Kilbourn, Ph.D. thesis, University of Michigan, Ann Arbor, Mich., 1968.

(8) A. Albert, "The Acridines," 2nd ed., Arnold, London, England, 1966, p. 50.

(9) A. DeLeenheer, J. E. Sinsheimer, and J. H. Burckhalter, *J. Pharm. Sci.*, **61**, 273(1972).

(10) M. Brenner, A. Niederwieser, and G. Pataki, in "Thin-Layer Chromatography," E. Stahl, Ed., Springer-Verlag, Berlin, Germany, 1965, p. 428.

(11) A. A. Goldberg and W. Kelly, *J. Chem. Soc.*, **1946**, 102.

(12) N. S. Drozdov, *Zh. Obshch. Khim.*, **8**, 937(1938).

(13) E. H. Hunters and E. N. Shaw, *J. Org. Chem.*, **13**, 674(1948).

(14) N. G. Bu-Hoi and J. Lecocq, *Rec. Trav. Chim.*, **64**, 250(1945).

(15) I. P. C. Guha and S. P. Mukherjee, *J. Indian Inst. Sci.*, **28A**, 63(1946).

(16) C. K. Ingold and I. S. Wilson, *J. Chem. Soc.*, **1927**, 813.

(17) J. E. Sinsheimer, V. Jagodić, and J. H. Burckhalter, *Anal. Biochem.*, **57**, 227(1974).

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[x] To whom inquiries should be directed.