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Fluorescein Isothiocyanates: Improved Synthesis and Purity

Spectral Studies[1]

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Modification of the methods of synthesis by direct reaction of the isomeric aminofluoresceins with a limited excess of thiophosgene in the presence of calcium carbonate has yielded the 5- and 6-isothiocyanatofluoresceins of high purity. The ir and nmr spectra of these isomers are described.

Fluorescein isothiocyanate has been widely used as a fluorescent labeling agent (1) since its introduction in 1958 (2). This reagent was accepted without complete establishment of structure or standards of purity.

The method of synthesis of the isothiocyanate was modified from the procedure (3) for the isocyanate which employed the isomeric 5- and 6-nitrofluoresceins as key intermediates. While these isomers had been readily separated on the basis of differential solubility of their acetates in acetic anhydride (3), it was later that structures were assigned to these acetates by Corey and Churchill (4).

Lack of purity of the fluorescein isothiocyanates remains a problem in their optimal use in protein labeling (5–7). Physical and chemical methods for examining this purity have been reviewed and applied to commercially available samples (8). More recently, Cherry *et al.* (9) have examined the purity of commercially available fluorescein isothiocyanates based upon their bovine serum albumin labeling efficiency. They recommend 70% minimal purity for immunofluorescein applications.

It is the purpose of this paper to describe a modification of synthesis of the fluorescein isothiocyanate isomers which yields products of improved purity.

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METHODS

Spectra. Nuclear magnetic resonance spectra were obtained with a Varian A-60A spectrometer in acetone with tetramethylsilane as the internal standard. Infrared spectra were obtained in dimethylsulfoxide in silver chloride cells and as solid state spectra in potassium bromide and as Nujol or Flurolube mulls with a Perkin–Elmer model 337 spectrophotometer.

5-Isothiocyanatofluorescein. A suspension of 4.9 g (0.0141 mole) 5-aminofluorescein (10) and 5 g (0.05 mole) finely pulverized calcium carbonate in 150 ml of acetone was vigorously stirred while 1.2 ml (0.0161 mole) thiophosgene was added all at once. Stirring was continued 1 hr at room temperature and then 2 hr under reflux. The warm mixture was filtered to separate the calcium salts, and these salts were extracted and washed with boiling acetone. The combined acetone solutions were concentrated to about 60 ml, and petroleum ether (60–70°) was added until permanent turbidity resulted. After the mixture had stood, impurities precipitated in the form of a viscous oil which was separated by filtration. More petroleum ether was added, and cooling gave 2.9 g (52.7% yield) of orange-yellow crystals which slowly decomposed above 160°C. After two recrystallizations from acetone–petroleum ether, an orange-yellow product was obtained which gave single spots upon silica gel tlc in two systems [System I: ethyl acetate:pyridine:acetic acid (50:1:1). System II: dimethylformamide:chloroform:28% ammonium hydroxide (10:5:4)]. Infrared (dimethylsulfoxide) 2110 (NCS), 1760 cm^{-1} (C=O); nmr (acetone-d_6) δ 6.60–6.78 (6H, m with most pronounced peaks at 6.68 and 6.75, xanthene −H), 7.35 (1H, d, J = 8 Hz, C_7 − H), 7.78 (1H, d of d, J = 8, J = 2 Hz, C_6 − H), 7.93 (1H, d, J = 2 Hz, C_4 − H), 8.85 (broad s, acidic −H).

Anal. Calcd for $C_{21}H_{11}NO_5S$: C, 64.78; H, 2.85; N, 3.56; S, 8.23. Found: C, 64.85; H, 2.96; N, 3.54; S, 8.27.

6-Isothiocyanatofluorescein. This compound was synthesized from 6-aminofluorescein (10) by the same procedure used for the 5-isothiocyanate isomer. Recrystallization from acetone–petroleum ether was repeated until a single spot was obtained by tlc in Systems I and II. Infrared (dimethylsulfoxide) 2110 (NCS), 1760 cm^{-1} (C=O); nmr (acetone-d_6) δ 6.67–6.78 (6H, m with most pronounced peaks at 6.70 and 6.75, xanthene − H), 7.30 (1H, d, J = 2 Hz, C_7 − H), 7.63 (1H, d of d, J = 8, J = 2 Hz, C_5 − H), 8.05 (1H, d, J = 8 Hz, C_4 − H), 8.92 (broad s, acidic −H).

Anal. Found: C, 64.82; H, 2.94; N, 3.42; S, 8.27.

RESULTS AND DISCUSSION

Our first approach to improve the purity of fluorescein isothiocyanate involved an attempted purification of 5-aminofluorescein diacetate, since this compound, in spite of a wide melting range (145–160°C), was used as an intermediate without further purification in the earlier procedure (2). It is interesting that repeated recrystallization from methanol–water of the product of the reduction of 5-nitrofluorescein diacetate actually leads to a decrease in purity.

McKinney, Spillane, and Pearce (8) have reported that hydrogen chloride formed during reaction of aminofluorescein with thiophosgene results in the formation of a hydrochloride of fluorescein isothiocyanate. According to these authors, the extent of hydrochloride formation is not consistent, but, in all cases, the hydrogen chloride is extremely difficult to remove, and it is an important factor influencing purity and stability of the intermediate. It was, therefore, concluded in the present investigation that the usual use of a large excess of thiophosgene was to be avoided and that calcium carbonate should be added to the reaction mixture to prevent an accumulation of hydrogen chloride.

When 5-aminofluorescein diacetate was reacted with thiophosgene in this manner, 5-isothiocyanatofluorescein diacetate was isolated with elemental analysis values within reasonable agreement with theoretical values. Since the loss of excess hydrogen chloride also prevented the simultaneous cleavage of the ester groups which occurs in the original procedure (2), a separate hydrolysis was required. The undesirability of using mineral acids, owing to salt formation, resulted in the evaluation of systems involving aqueous sodium bicarbonate and dry ammonia–acetone. Both systems not only produced rapid cleavage of the acetate groups, but they also resulted in reaction with the isothiocyanato group.

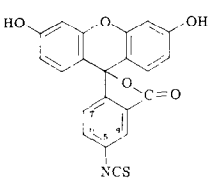
In order to avoid the necessity of acetate hydrolysis, aminofluorescein instead of aminofluorescein diacetate was used in the present procedure to obtain pure fluorescein isothiocyanate. The improved procedure of McKinney, Spillane, and Pearce (10), which employs a mixture of sodium hydrogen sulfide and sodium sulfide, was used in preference to the original Raney nickel reduction of the 5- and 6-nitrofluorescein isomers employed by Coons and Kaplan (3).

Each aminofluorescein isomer was separately reacted with thiophosgene in acetone in the presence of calcium carbonate. After these reactions were completed, calcium salts were separated by filtration, and since the calcium salts contained considerable product, they were extracted repeatedly with hot acetone. The fluorescein isothiocyanate isomers were

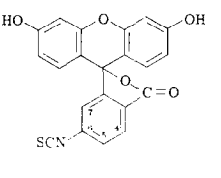
recovered from combined filtrates and extracts by evaporation of the acetone. Purification of residues was carried out by recrystallization from acetone–petroleum ether. The initial addition of petroleum either precipitated impurities in the form of a viscous oil, while further addition of petroleum ether produced yellow-orange crystals. Repeated recrystallizations from acetone–petroleum ether of each of the 5- and 6-isothiocyanatofluorescein isomers produced products of high purity. Single spots were observed upon silica gel tlc in two systems, and elemental analyses consistent with the theoretical values were obtained.

Nuclear magnetic resonance spectra were obtained from saturated solutions in acetone-d_6. While there was increased solubility in dimethysulfoxide-d_6, such solutions lack stability and were sensitive to the addition of traces of dry HCl.

In agreement with the previously published assignments for the nitrofluorescein acetates (4), the NMR spectrum of isomer I is consistent with the 5-isothiocyanato compound (A), while isomer II corresponds to the 6-isothiocyanato compound (B). These assignments for isomers I and II are also in agreement with the isomer assignments reached by Kramer, Klapper, and Miller (11), but our assignments do not agree with their NMR spectra for 5-isothiocyanatofluorescein. It would appear that these



A (isomer I)



B (isomer II)

authors have inadvertently reported the spectra of isomer II for that of isomer I.

The ir spectra of these isomers, and especially isomer I, are unusually sensitive to the media in which they are obtained. Both isomers exhibit absorption at 2110 cm^{-1} for the isothiocyanato group and at 1760 cm^{-1} for the carbonyl group when determined in dimethylsulfoxide (12). The solid-state ir spectra for the 5-isothiocyanatofluorescein isomer indicates the existence of both a yellow and red form of the compound analogous to those described for fluorescein per se (13). The yellow form has isothiocyanato absorption at 2030 cm^{-1}, but it exhibited no carbonyl bond when the compound was reduced in particle size in a mortar already moistened with mulling agent (Nujol or Flurolube). However, when ground in a dry mortar before the addition of mulling agent, the compound reddened in color and the resulting spectra had broad isothio-

cyanato absorption at 2030 cm $^{-1}$ with a shoulder at 2070 cm $^{-1}$, as well as carbonyl absorption at 1740 cm^{-1} with a shoulder at 1760 cm $^{-1}$. In KBr, the isothiocyanato band was at 2050 cm $^{-1}$ and a carbonyl band at 1730 cm^{-1}. 6-Isothiocyanatofluorescein was more uniform in its solid-state spectra, with the major isothiocyanato band at 2040 cm $^{-1}$ and carbonyl absorption at 1740 cm^{-1}.

REFERENCES

1. Nairn, R. C. (1964) Fluorescent Protein Tracing, 2nd ed., Williams and Wilkins Co., Baltimore, MD.
2. Burckhalter, J. H., and Seiwald, R. J. (1960) U. S. Patent 2,937, 186; Riggs, J. L., Seiwald, R. J., Burckhalter, J. H., Downs, C. M., and Metcalf, T. G. (1958) *Amer. J. Pathol.* **34**, 1081.
3. Coon, A. H., and Kaplan, M. H. (1950) *J. Exp. Med.* **91**, 1.
4. Corey, H. S., and Churchill, F. C., II (1966) *Nature (London)* **212**, 1040.
5. Goldstein, G., Slizys, I. S., and Chase, M. W. (1961) *J. Exp. Med.* **114**, 89.
6. Pittman, B., Hebert, G. A., Cherry, W. B., and Taylor, G. C. (1967) *J. Immunol.* **98**, 1196.
7. Hebert, G. A., Pittman, B., and Cherry, W. B. (1967) *J. Immunol.* **98**, 1204.
8. McKinney, R. M., Spillane, J. T., and Pearce, G. W. (1964) *Anal. Biochem.* **7**, 74.
9. Cherry, W. B., McKinney, R. M., Emmel, V. M., Spillane, J. T., Hebert, G. A., and Pittman, B. (1969) *Stain Technol.* **44**, 179.
10. McKinney, R. M., Spillane, J. T., and Pearce, G. W. (1962) *J. Org. Chem.* **27**, 3986.
11. Kramer, D., Klapper, H., and Miller, F. (1968) *Spectr. Lett.* **1**, 23.
12. McKinney, R. M., Churchill, F. C., II, Spillane, J. T., and Pearce, G. W. (1969) *Anal. Biochem.* **29**, 526.
13. Sklyar, Y. E., and Mikhailov, G. I. (1966) *J. Org. Chem. USSR* **2**, 894.