A NOVEL NEAR-INFRARED CYANINE DYE FOR BIOANALYTICAL APPLICATIONS

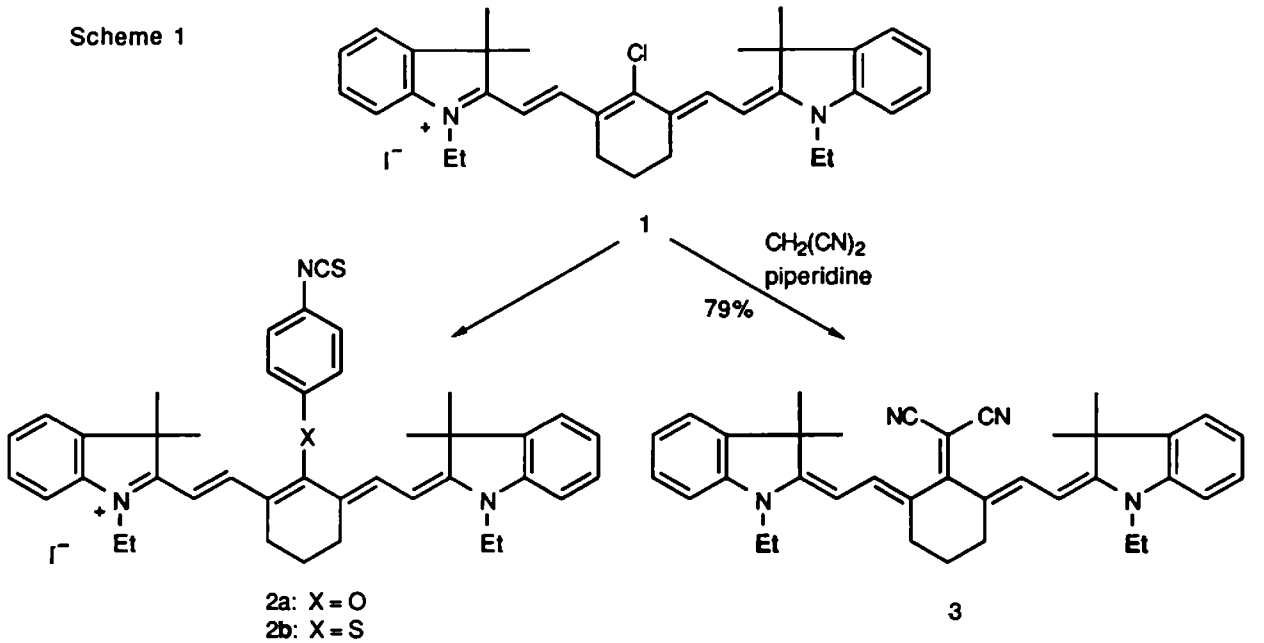
Malgorzata Lipowska, Gabor Patonay, and Lucjan Strekowski*
Department of Chemistry, Georgia State University, Atlanta, Georgia 30303, USA

Abstract : Synthesis of a stable heptamethine cyanine dye **10** substituted with an isothiocyanato function for labeling with a near-infrared chromophore of biomolecules at amino groups is presented.

Although the first near-infrared (NIR) absorbing dye was synthesized at the beginning of this century, the literature on the NIR dyes is quite limited (**1**). Recent years, however, have seen a renewed interest in the organic dye chemistry owing to a wide availability of inexpensive diode lasers which emit in the NIR region. NIR dyes have already found important applications as media for optical recording and thermal writing display, sensitizers for infrared photography, drugs in photodynamic therapy, and fluorescent probes in bioanalytical chemistry, to name a few. Cyanine dyes, in particular, have emerged recently as an effective fluorescent label for biomolecules, owing to their large values of molar extinction coefficients of up to 300000 $M^{-1}cm^{-1}$ and relatively large Stokes' shifts of up to 50 nm (**2**,3). For example, in conjunction with a GaAlAs diode laser emitting at 785 nm, dyes **2** (Scheme 1) synthesized by us (4,5) have been used with some success as fluorescent labels for nucleotides in a novel approach to DNA sequencing (6) and for determination of proteins of ultra low levels of concentration (4,7,8). Because of the lack of background interference from a biological medium in the NIR region, a detection limit as low as 10^{-14} M has been found for certain biomolecules (4,6). We strongly favor the isothiocyanate functionality over other known reactive groups for covalent labeling of macromolecules with an NIR chromophore. The SCN group is relatively stable under a wide range of pH conditions, yet it reacts rapidly and selectively with a primary amine on a biomolecule to form a stable thiourea linkage between the chromophore and the biomolecule. Of some concern is the low hydrolytic stability of the central ether and sulfide bridges in **2** and their bioconjugates, which results in partial loss of the chromophore during an analytical procedure (6-8). A novel, greatly improved dye label **10** (Scheme 2) is described in this paper.

Recently we have synthesized dye **1** (Scheme 1) and shown that the chlorine atom at the central position is easily substituted by the reaction with various heteroatom-centered nucleophiles (4). Dyes **2** among others (4,5,9) have been prepared by using this chemistry. Accordingly, our initial attempts to improve hydrolytic stability of the dye labels were to synthesize analogs of **2** without a heteroatom at the central position of the chromophore. Reactions of carbon nucleophiles containing a function that could be elaborated into the SCN group were studied. Compound **1** was inert toward cyanide anion and nucleophiles derived from diethyl malonate and ethyl acetoacetate under a variety of experimental conditions. Decomposition of **1** was observed under elevated temperatures. Surprisingly, the treatment of **1** with malononitrile and piperidine in ethanol at 23°C for 30 min followed by chromatography on silica gel with chloroform as an eluent

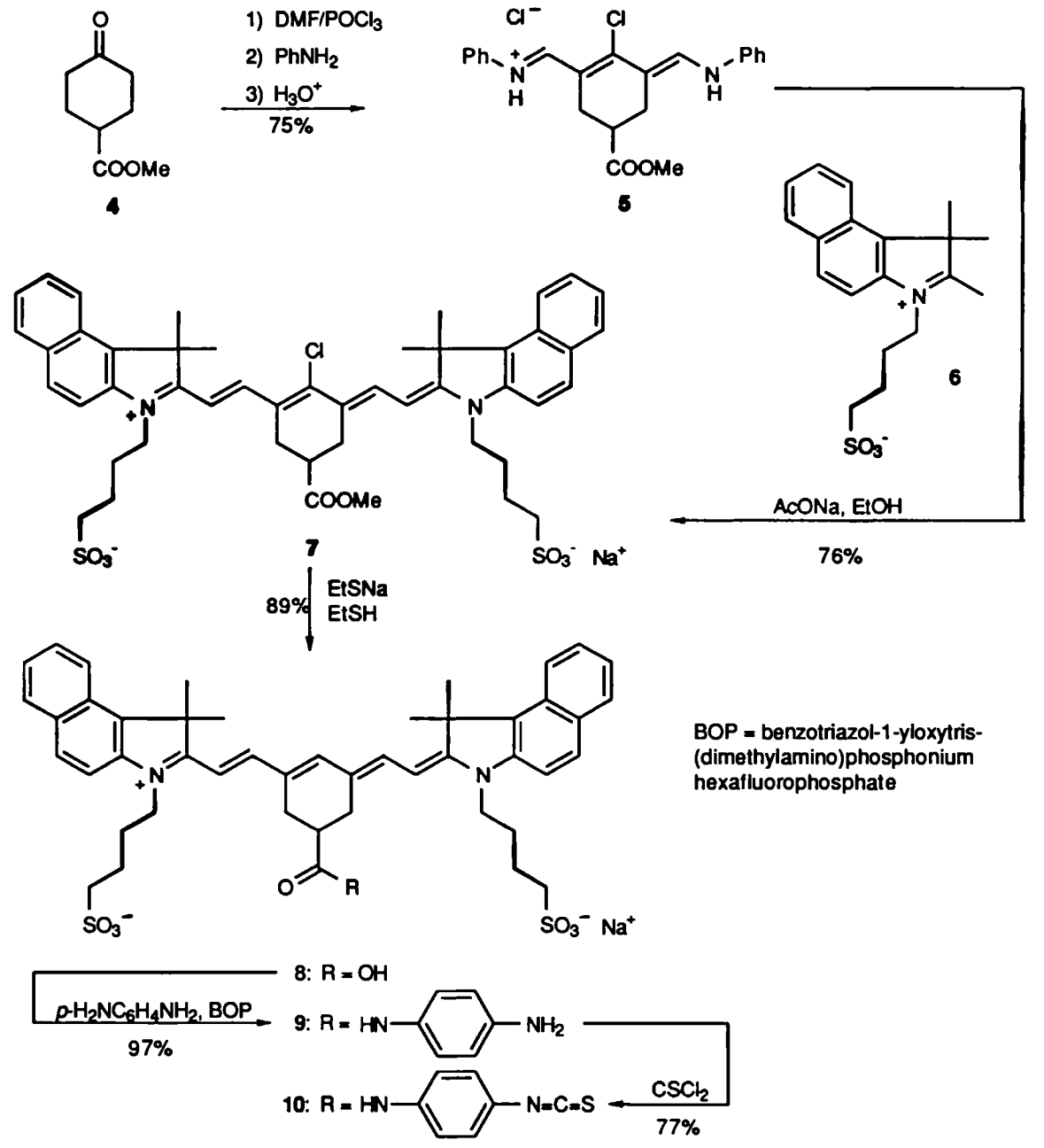
Scheme 1



gave product 3 in a 79% yield [IR (neat) 2188 cm^{-1}; VIS-NIR λ_{max}^{MeOH} 646 nm (ε 29600); FAB-MS *m/z* 541 (MH^+, 100%); HR-FAB-MS calcd for $C_{37}H_{41}N_4$ *m/z* 541.3331, found 541.3329]. Unfortunately, the cyano groups of **3** could not be hydrolyzed, and the discussed approach had to be abandoned.

The successful strategy involved a total synthesis of a functionalized dye starting with a keto ester **4** (10), the carboxylate function of which was retained through several-step transformations (Scheme 2). The treatment of **4** with a Vilsmeier-Haack reagent (11) followed by the reaction of the resultant dialdehyde intermediate with aniline gave the expected iminium derivative **5**. Quenching of the mixture resulted in partial hydrolysis of the methoxycarbonyl function of **5**, but the ester **5** and the corresponding carboxylic acid were easily separated by fractional crystallization from MeOH/Et_2O. Condensation of **5** with an indolium inner salt **6**, obtained by alkylation of 2,3,3-trimethyl-3*H*-benz[*e*]indole with 1,4-butane sultone (12), furnished dye **7** in a 76% yield after flash chromatography ($CHCl_3$/MeOH) and crystallization from MeOH/Et_2O [VIS-NIR λ_{max}^{MeOH} 817 nm (ε 194300); FAB-MS *m/z* 883 (M^+-Na, 100%); HR-FAB-MS calcd for $C_{48}H_{52}ClN_2O_8S_2$ *m/z* 883.2853, found 883.2848]. The treatment of **7** with the reagent system EtSNa/EtSH (4) resulted in the desired dechlorination and also caused demethylation of the ester function to give a carboxy derivative **8** in a one-pot transformation. This facile preparation of **8** is even more simplified by using a crude mixture of ester **5** with its corresponding carboxylic acid for the condensation with **6** followed by treatment of the resultant mixture of ester **7** and the corresponding carboxylic acid with EtSNa/EtSH. Dye **8** was purified by ultrafiltration through a Molecular/Por membrane type C with a molecular weight cut off of 500 daltons. This remarkable and highly efficient filtration process is actually dialysis under pressure. The solution of a crude dye (up to 100 mg) in H_2O/MeOH (9:1) is concentrated under a static pressure of nitrogen, with low molecular weight impurities, both organic and inorganic, being removed with the solvent through the membrane. The ultrafiltration of crude **8** with several cycles of concentration, dilution with a fresh solvent, and concentration, followed by removal of the solvent on a rotary evaporator at 30°C, and then crystallization of the residue from MeOH/Et_2O gave dye **8** of exceptional purity [VIS-NIR λ_{max}^{MeOH} 788 nm (ε 150000); FAB-MS *m/z* 835 (M^+-Na, 100%);

Scheme 2



HR-FAB-MS calcd for $C_{47}H_{51}N_2O_8S_2$ *m/z* 835.3087, found 835.3108]. An amidation reaction of carboxylic acid 8 with *p*-phenylenediamine in the presence of BOP was followed by transformation of the amino group in the resultant crude product 9 into an isothiocyanate function (4,5) to give the final functionalized dye **10**. Again, the purification of 10 was achieved by ultrafiltration and then crystallization as described above. The analytically pure dye **10** has the following characteristics: VIS-NIR λ_{max}^{MeOH} 785 nm (ε 152000); λ_{em}^{MeOH} 811 nm; ^{1}H NMR (400 MHz, DMSO-d_6) ∂ 1.70-1.87 (m, 8 H), 1.94 (s, 12 H), 2.50-3.08 (m, obscured by solvent absorption), 4.31 (m, 4 H), 6.33 (d, *J* = 14.4 Hz, 2 H), 7.42 (d, *J* = 8.8 Hz, 2 H), 7.49 (t, *J* = 7.2 Hz, 2 H), 7.65 (t, *J* = 7.2 Hz, 2 H), 7.74 (d, *J* = 9.0 Hz, 2 H), 7.77 (s, 1 H), 7.83 (d, *J* = 9.0 Hz, 2 H), 7.86 (d, *J* = 14.4 Hz, 2 H), 8.04 (m, 4 H), 8.24 (d, *J* = 8.8 Hz, 2 H), 10.53 (br s, 1 H); FAB-MS *m/z* 969 (M^+-Na + 2 H, 100%); HR-

FAB-MS calcd for $C_{54}H_{57}N_2O_7S_3$ *m/z* 969.3389, found 969.3385. Compound **10** was allowed to react with an aqueous solution of ethylamine to give the corresponding thiourea derivative quantitatively.

The synthetic chemistry and applications of NIR cyanine dyes have been plagued by instability and low solubility of these organic salts (1). It is not uncommon to observe partial decomposition of a dye during an attempted purification. In this paper we describe a facile synthesis and efficient purification of a stable dye **10**. Compound **10** contains sulfonatobutyl groups for increased solubility in water. The superior chemical and photochemical stability of **10** in comparison to that of a vast majority of other heptamethine cyanine dyes is due to the presence of a ring structure in the central portion of the chromophore. It has been noted previously (1) and confirmed in our studies that enhancement of the rigidity of the polymethine chain is very effective in improving the stability of cyanine dyes. We wish also to comment on the design of **10** as a symmetric structure. First, the symmetry factor greatly facilitates the final purification of the dye by crystallization. Second, it is known that cationic dyes display solvent effects in nucleophilic solvents, but the spectral shift of the λ_{max} values is generally low for symmetric dyes (1). It should be noted that the λ_{max}^{MeOH} of 785 nm of **10** perfectly matches the wavelength of 785 nm of the commercial GaAlAs diode laser.

Acknowledgements. This work was supported by grants from the National Institutes of Health (AI28903) and the National Science Foundation (CHE8920456).

References

(1) For a recent review, see: J. Fabian, H. Nakazumi and M. Matsuoka, Chem. Rev. *92*, 1197 (1992)

(2) L. A. Ernst, R. K. Gupta, R. B. Mujumdar and A. S. Waggoner, Cytometry *10*, 3 (1988)

(3) R. B. Mujumdar, L. A. Ernst, S. R. Mujumdar and A. S. Waggoner, Cytometry *10*, 11 (1989)

(4) L. Strekowski, M. Lipowska and G. Patonay, J. Org. Chem. *57*, 4578 (1992)

(5) M. Lipowska, G. Patonay and L. Strekowski, Synth. Commun. *23*, 3087 (1993)

(6) D. B. Shealy, M. Lipowska, J. Lipowski, N. Narayanan, S. Sutter, L. Strekowski and G. Patonay, Anal. Chem. *67*, 247 (1995)

(7) R. J. Williams, N. Narayanan, G. A. Casay, M. Lipowska, L. Strekowski and G. Patonay, Anal. Chem. *66*, 3102 (1994)

(8) R. J. Williams, M. Lipowska, G. Patonay and L. Strekowski, Anal. Chem. *65*, 601 (1993)

(9) L. Strekowski, M. Lipowska and G. Patonay, Synth. Commun. *22*, 2593 (1992).

(10) E. Hardegger, P. A. Plattner and F. Blank, Helv. Chim. Acta *27*, 793 (1944)

(11) S. M. Makin, L. I. Boiko and O. A. Shavrygina, Zh. Org. Khim. *13*, 1189 (1977)

(12) D. W. Heseltine and L. G. S. Brooker, US Patent 2,895,955 (1959); Chem. Abstr. *54*, 121 (1960)

Received February 17, 1995