A Highly Selective Hydrogen-Deuterium Exchange
in Indolium Heptamethine Cyanines

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## Dedicated to the memory of Dr. Roland K. Robins

Chemical shift assignments in ^{1}H nmr spectra of four tricarbocyanines have been made. Studies by ^{1}H nmr have shown a rapid acid-catalyzed proton exchange at positions 1',7' and a much slower exchange at positions 3',5' of the heptamethine chain. Base catalysis results in a slow exchange at positions 1',7' only.

Indolium cyanine dyes are of immense interest as near-infrared absorbing and fluorescing chromophores [1]. In general, these dyes show greater chemical stability and photostability than similar cyanines derived from other heterocyclic systems [2]. The heptamethine derivatives, such as **1-4** (Figure 1), are of particular interest because
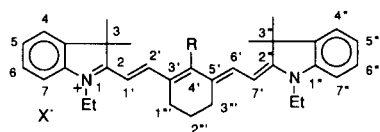
1: R = Cl, X = I
2: R = OMe, X = I
3: R = H, X = ClO$_4$
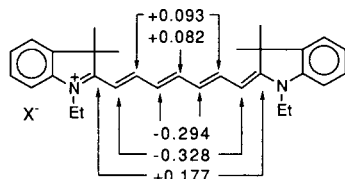
4a: X = I
4b: X = ClO$_4$

Figure 1. Structures of cyanine dyes **1-4**, a numbering system (shown for **1-3**), and point charges at selected carbon atoms of **4**, as obtained by the ampac method. Note that the classical resonance structure of **4** poorly represents the electron density distrution suggested by the ampac calculation. See foonote [17] for additional information.

they absorb in the region of 700-850 nm that matches closely the wavelength of 785 nm of the commercially available GaAlAs diode laser [3]. A number of laser applications of the dyes in the areas of quantum electronics, optoelectronics [3] biology, and bioanalytical chemistry [4,5] have been suggested recently. Research is underway in our laboratories to develop an analytical method for

ultratrace determination of proteins, including HIV-1-specific proteins, in crude clinical isolates by using cyanine dyes as fluorescent labels for the biomolecules [6,7]. The approach takes advantage of the inherently low interference in the near-infrared region, so it may not be necessary to prepurify the biological mixtures. Our systematic studies involve the development of chemistries suitable for the labeling [6,8] and work directed toward a better understanding of chemical and spectral properties of cyanine dyes in solution [7,9]. Literature data on the latter subject is extremely limited [1,10,11]. In particular, few cyanines have been characterized by complete chemical shift assignments in their ^{1}H nmr spectra [10-13]. In part this may be due to difficulties in obtaining pure samples of highly polar dyes [14].

In this paper we report chemical shift assignments for all protons of cyanine dyes **1-3, 4b**. The ^{1}H nmr spectroscopy was also used to study the effects of acid and base on the dyes.

The shift assignments for N1(1'')CH$_2$ and C3(3'')(CH$_3$)$_2$ in all dyes, and for C3'(5')CH$_2$ in **1-3** were straightforward (Table 1). Subsequent nOe experiments resulted in identification of signals of the corresponding adjacent protons, and gave the all-*trans* conformation, as shown in Figure 1, in all cases studied. This geometry was further supported by decoupling experiments, which gave coupling constants $J \geq 12$ Hz for the adjacent protons of the heptamethine chain. Molecular modeling results also consistently suggested the all-trans geometry [15].

As can be seen from Table 1 the methine resonances are spread over a wide range of chemical shifts, with the highest δ values observed for protons at the carbon atoms C2' and C6'. The protons at C1' and C7' nearest to the ring nitrogen atoms exhibit the lowest chemical shifts [10,11,16]. In order to understand this rather unusual phenomenon the point charges at the carbon atoms of the chromophore of **4b** were calculated (Figure 1). It was

Table 1

^{1}H NMR Spectral Data of Dyes 1-4 (deuteriochloroform, δ)

Protons	Dye [a]			
	1	2	3	4b [b]
NCH$_2$CH$_3$	4.28 (q, 4H)	4.19 (q, 4H)	4.08 (q, 4H)	4.12 (q, 4H)
NCH$_2$CH$_3$	1.46 (t, 6H)	1.45 (t, 6H)	1.43 (t, 6H)	1.42 (t, 6H)
C(CH$_3$)$_2$	1.72 (s, 12H)	1.70 (s, 12H)	1.72 (s, 12H)	1.69 (s, 12H)
4-H and 4"-H	7.38 (d, 2H)	7.38 (d, 2H)	7.34 (d, 2H)	7.35 (d, 2H)
5-H and 5"-H	7.26 (t, 2H)	7.22 (t, 2H)	7.20 (t, 2H)	7.21 (t, 2H)
6-H and 6"-H	7.41 (t, 2H)	7.41 (t, 2H)	7.36 (t, 2H)	7.37 (t, 2H)
7-H and 7"-H	7.18 (d, 2H)	7.12 (d, 2H)	7.07 (d, 2H)	7.09 (d, 2H)
1'-H and 7'-H	6.25 (d, 2H)	6.08 (d, 2H)	6.01 (d, 2H)	6.25 (d, 2H)
2'-H and 6'-H	8.35 (d, 2H)	8.08 (d, 2H)	7.75 (d, 2H)	7.84 (m, 2H)
3-H and 5'-H	--	--	--	6.66 (m, 2H)
4'-H or 4'-R	--	3.99 (s, 3H)	7.57 (s, 1H)	7.70 (m, 1H)
1'''-H and 3'''-H	2.77 (t, 4H)	2.64 (t, 4H)	2.52 (t, 4H)	--
2'''-H	1.99 (m, 2H)	1.94 (m, 2H)	1.95 (m, 2H)	--

[a] Coupling constants are 7.2 ±0.4 Hz for vicinal protons of the ethyl groups, 7.6±0.4 Hz for adjacent aromatic protons, and 13.2±0.4 Hz between 1'(7')-H and 2-(6')-H in all dyes 1-3 and 4b. The vicinal protons of the trimethylene bridge in 1-3 a are coupled with 6.5±0.4 Hz. For dye 4b the coupling constants between 2'(6')-H and 3'(5')-H and between 3'(5')-H and 4'-H are 12.0±0.4 Hz. [b] Impure dye 4a showed similar chemical shifts.

hoped that the observed chemical shifts could be correlated with the calculated charges [17]. Indeed, the computational results suggest that the protons at C1' and C7' should be strongly shielded in comparison to other protons, as observed. Moreover, a closer analysis of the data for 4b in Figure 1 and Table 1 reveals a strong correlation between the calculated point charges at all carbon atoms of the heptamethine chain and the experimental δ values for protons at these carbon atoms. The order of decreasing electron densities at the carbon atoms and the order of increasing chemical shifts for the protons are 1'(7'), 3'(5'), 4', 2'(6').
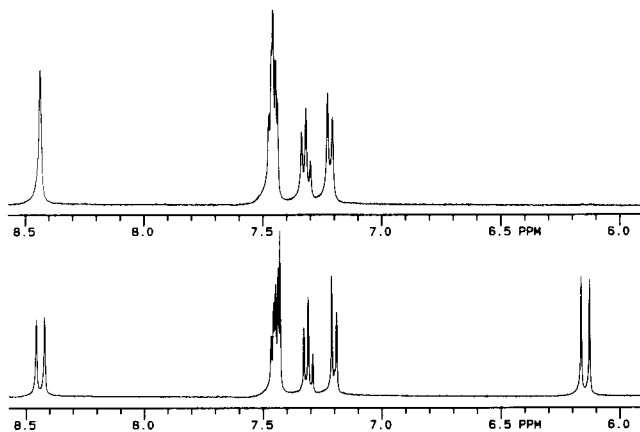

Figure 2. The methine and aromatic regions of the ^{1}H nmr spectra of 1 (10 mM) taken in a mixture of deuteriochloroform and methanol-d$_4$ (10:1) in the absence (bottom spectrum) and presence of 1.5 mM deuterium chloride (top spectrum). Note a singlet for 2'(6')-H and disappearance of a doublet for 1'(7')-H after the addition of acid. A signal for chloroform can be seen at δ 7.45.

The apparent charge density distribution, as obtained from the experimental and computational studies, has an important ramification for the properties of cyanine dyes towards acids and bases (vide infra). When treated with a catalytic amount of deuterium chloride in methanol-d$_4$ or a deuteriochloroform-methanol-d$_4$ mixture, all dyes 1-4 undergo a rapid hydrogen-deuterium exchange at the equivalent positions 1' and 7'. This phenomenon is conveniently studied by ^{1}H nmr, and the spectra of 1 obtained with a 10 mM concentration of the dye in the absence and presence of the acid are are given in Figure 2 for illustration. At the concentration of deuterium chloride of 1.5 mM the rate of this selective exchange is too rapid to be measured at the time scale of the nmr experiments. With 4b under the same conditions no appreciable exchange of other protons was observed even after 2 hours. Increasing the concentration of deuterium chloride to 15 mM under otherwise identical conditions resulted in a slow hydrogen-deuterium exchange at the positions 3' and 5' with the estimated half-time of 30 minutes, and the exchange was essentially complete after several hours at 23°. The exchange of protons at the positions 2', 4' and 6' was not observed by nmr, even after the strongly acidic solution of 4b had been allowed to stand at 23° for 24 hours.

All these reactions are fully reversible. For example, the addition of methanol to the acidic solution of the tetradeuterio derivative 4-1'D3'D5'D7'D resulted in a rapid exchange of hydrogen for deuterium at positions 1' and 7' and a much slower exchange at the positions 3', 5', as observed by ^{1}H nmr. Also, an attempted isolation of 1-1'D7'D, which included aqueous workup, resulted in a quantitative recovery of 1.
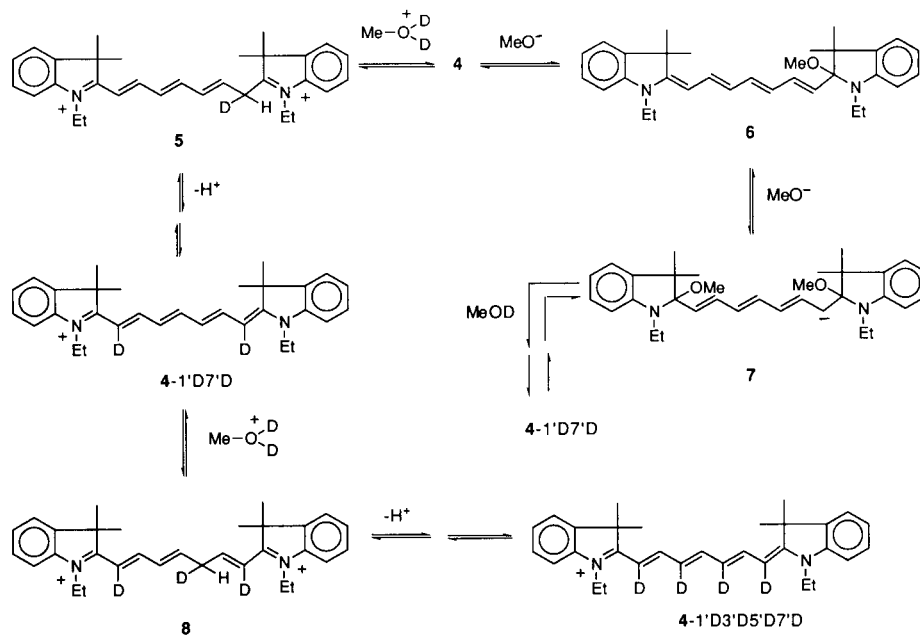
In contrast to the acid catalysis the exchange at the positions 1′,7′ is slow in the presence of base. The half-time of 1 hour was estimated by ^{1}H nmr for the reaction of **3** (10 mM) in methanol-d$_4$ in the presence of sodium methoxide (25 mM) at 23°. Also in contrast to the acid catalysis the selective hydrogen-deuterium exchange at the positions 1′,7′ was the only result of the methoxide ion-mediated reaction of **4b**. Interestingly, immediately after the base addition the green color of the dye solutions faded, and the aromatic and vinyl regions in the ^{1}H nmr spectra shifted upfield with a loss of resolution. The green color of the solutions and ^{1}H nmr spectra characteristic for tricarbocyanines were obtained again after a five-fold dilution of the samples with deuteriochloroform or methanol-d$_4$.

Possible mechanisms that account for the observed different reactivities of the dyes in the presence of acid and base are illustrated for the reactions of a dye system **4**. We propose that the selective hydrogen-deuterium exchange at positions 1′,7′ in the presence of acid is kinetically controlled, and a dication **5** is the primary intermediate product [11,18]. This hypothesis is consistent with the order of point charges at the heptamethine chain, with the positions 1′,7′ being the most susceptible for deuteration/protonation. As can be seen from Figure 1, an electrophilic attack by acid should also take place at positions 3′,5′, albeit with a lower efficiency, as observed. A possible thermodynamic control of the selectivity in acid-catalyzed reactions was also considered. The thermodynamic control, however, appears to be less likely because the calculated heats of formation of the postulated dications **5** and **8** are similar [19].

The base catalysis apparently involves an initial addition reaction of methoxide ion at the position 2(2″) of the chromophore to give intermediate product **6**. A subsequent addition reaction with **6** would result in the formation of an anionic species **7**, a postulated precursor to the deuterio derivative **4**-1′(7′)D. The same sequence of reactions with **4**-1′(7′)D would produce a dideuterio derivative **4**-1′D7′D, the observed product. This mechanistic pathway is consistent with a kinetic control of a nucleophile addition at the most electrophilic site of the chromophore (Figure 1) which, at the same time, produces a thermodynamically stable system **6**. The instant decolorization of **4** upon treatment with sodium methoxide, as already mentioned, may be attributed to the formation of the adduct **6**. The facility of this addition reaction also explains the experimental finding that methylene hydrogens of the N-ethyl substituent do not undergo a hydrogen-deuterium exchange in the presence of methoxide ion.

In summary, we have shown highly selective proton exchange reactions of heptamethine cyanines. Both the acid and base catalyzed reactions can be explained in terms of the concept of point charges at the chromophore. The results may have important ramifications for selective synthetic modifications of the cyanine chromophore in nucleophile- and electrophile-mediated transformations [6,8].

EXPERIMENTAL

methylindolin-2''-ylidene)-4'-methoxy-3',5'-(propane-1''',3'''-diyl)-1',3',5'-heptatrien-1'-yl]-1-ethyl-3,3-dimethyl-3H-indolium iodide (**2**) [8], 2-[7'-(1''-ethyl-3'',3''-dimethylindolin-2''-ylidene)-3',5'-(pro-pane-1''',3'''-diyl)-1',3',5'-heptatrien-1'-yl]-1-ethyl-3,3-dimethyl-3H-indolium perchlorate (**3**) [6], and 2-[7'-(1''-ethyl-3'',3''-dimethylin-dolin-2''-ylidene)-1',3',5'-heptatrien-1'-yl]-1-ethyl-3,3-dimethyl-3H-indolium iodide (**4a**) [10,13] have been reported. With the exception of compound **4a**, all dyes **1-3** were obtained in analytically pure forms.

2-[7'-(1''-Ethyl-3'',3''-dimethylindolin-2''-ylidene)-1',3',5'-hepta-trien-1'-yl]-1-ethyl-3,3-dimethyl-3H-indolium Perchlorate (4b).

A solution of crude iodide **4a** (56 mg, < 0.1 mmole) in ethanol (10 ml) was treated with a solution of perchloric acid (0.15 mmole) in aqueous ethanol (5 ml). After cooling (0°) the resultant precipitate of **4b** was collected, washed with ether, and then crystallized three times from a mixture of methanol and ether. Upon heating, compound **4b** undergoes partial decomposition at > 150°, and then melting is observed at > 200°.

Anal. Calcd. for $C_{31}H_{37}N_2 \cdot ClO_4$: C, 69.32; H, 6.94; N, 5.22. Found: C, 68.98; H, 6.81; N, 5.05.

¹H NMR Studies.

All spectra were recorded at 23° on a Varian VXR-400 instrument operating at 400 MHz. Tetramethylsilane was used as an internal reference. Chemical shift assignments for all protons of cyanine dyes **1-3**, **4b** (10 mM in deuteriochloroform or methanol-d_4, 0.8 ml) were obtained with the help of nOe and decoupling experiments. The nOe difference spectra were taken in interleave mode with a 90° pulse width, relaxation delay $\geq 5T_1$ (typically 20-30 s). For each peak the irradiation power was optimized to deliver only enough power for saturation. These studies were assisted by molecular modeling of the most stable conformation of the dyes and ampac calculations of charges on the individual atoms of the dyes.

After the ¹H nmr spectrum in deuteriochloroform had been taken, the sample in the nmr tube was treated with methanol-d_4 (0.05 ml) containing a varying amount of deuterium chloride, and the spectrum was recorded again immediately after the acidification and then after standing. The period of time necessary to accumulate each spectrum was approximately 10 minutes. The spectra were also taken for a solution of the dye **1-3**, **4b** (10 mM) and sodium methoxide-d_3 (25 mM) in methanol-d_4, and then after fivefold dilution of the sample with methanol-d_4 or deuteriochloroform. Stock solutions of the base were prepared from methanol-d_4 and metallic sodium under a nitrogen atmosphere.

Molecular Modeling and Ampac Calculations.

The computations were run on a Silicon Graphics Iris and a Silicon Graphics 4d/380s. Preliminary molecular modeling of the dye cations was conducted in a pcmodel program with the mmx force field (a modified mm2 force field). The subsequent, full geometry optimization was carried out with the program ampac 2.1 (QCPE Program 506, Indiana University) and by using the am1 Hamiltonian. The procedure was stopped when the gradient was below 0.2 cal·mol⁻¹·Å⁻¹. The reported values of the point charges on individual atoms and the heat of formation correspond to the optimized geometries of the dye cations.

Acknowledgements.

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[14] In reference [6] we have mentioned briefly a convenient method of purification of cyanine dyes by a standard silica gel chromatography with a mixture of chloroform and methanol (up to 50% of methanol) as an eluent. Workup of the chromatography fractions consists of concentration and then treatment with chloroform to precipitate silica gel eluted with methanol. Cyanine dyes are soluble in chloroform. Additional purification can be achieved by crystallization of a perchlorate derivative as described for **4b** in this paper.

[15] Molecular modeling of **4** gave essentially a planar geometry including terminal aromatic moieties. The individual torsional angles of the chromophore system of **4** are less than 2°. With **1-3** the central part of the chromophore is deviated from coplanarity by about 8°.

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[17] The point charge on the individual atom in a molecule is defined as a difference between the positive core charge (*e.g.* +4 for a carbon atom) and the electron density on the atom. A total charge of +1 of the cation **4** was obtained after summing up the point charges at all atoms included in the molecular formula $C_{31}H_{37}N_2$ of this cation. The ampac calculations gave a positive charge less than unity for the sum of point charges at the two nitrogen atoms, the nine carbon atoms of the linker chain, and the seven methine hydrogen atoms of the cation **4**. This computational result is in agreement with the well known experimental fact that the terminal aromatic moieties of a cyanine cation are an integral part of the optical chromophore. It should be noted, however, that the point charges calculated by the ampac method are a representation of charge distribution across the molecule, rather than absolute values.

[18] The ¹H nmr spectrum of **1** in trifluoroacetic acid-d is consistent with a stable dication in which two deuterium atoms are present at the position 7' and one deuterium atom is at the position 1' (numbering system of Figure 1): δ 1.62 (t, 6H, CH₂*CH₃*, J = 7.6 Hz), 1.67 (s, 6H, C(CH₃)₂), 1.77 (s, 6H, C(CH₃)₂), 2.05 (p, 2H, 2'''-H, J = 6.5 Hz), 2.77 (t, 2H, 1'''-H, J = 6.5 Hz), 2.82 (t, 2H, 3'''-H, J = 6.5 Hz), 4.57 (q, 4H, *CH₂*CH₃, J = 7.6 Hz), 6.46 (s, 1H, 6'-H), 7.60-7.72 (m, 8H, aromatic), 8.79 (s, 1H, 2'-H). The yellow solution of **1** in trifluoroacetic acid had λ max = 420 nm and lacked a near infrared absorption band. Dilution of this sample with chloroform resulted in appearance of a green color, and the electronic spectrum was fully consistent with the presence of a heptamethine cyanine chromophore.

[19] The heats of formation calculated by the ampac method for dications derived from **4** by protonation at the position 1'(7') and the position 3'(5') are 514 kcal/mole and 512 kcal/mole, respectively.