Synthesis of Water-Soluble Near-Infrared Cyanine Dyes Functionalized with [(Succinimido)oxy]carbonyl Group

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Two heptamethine cyanine dyes suitable for labeling of biomolecules at a primary amino group with a near-infrared chromophore/fluorophore ($\lambda_{max}/(\lambda_{em}$ = 800/830 nm and 837/864 nm) have been synthesized from readily available starting materials. Despite the high molecular complexity of intermediate and final products, all these compounds have been obtained in an analytically pure form by using crystallization only.

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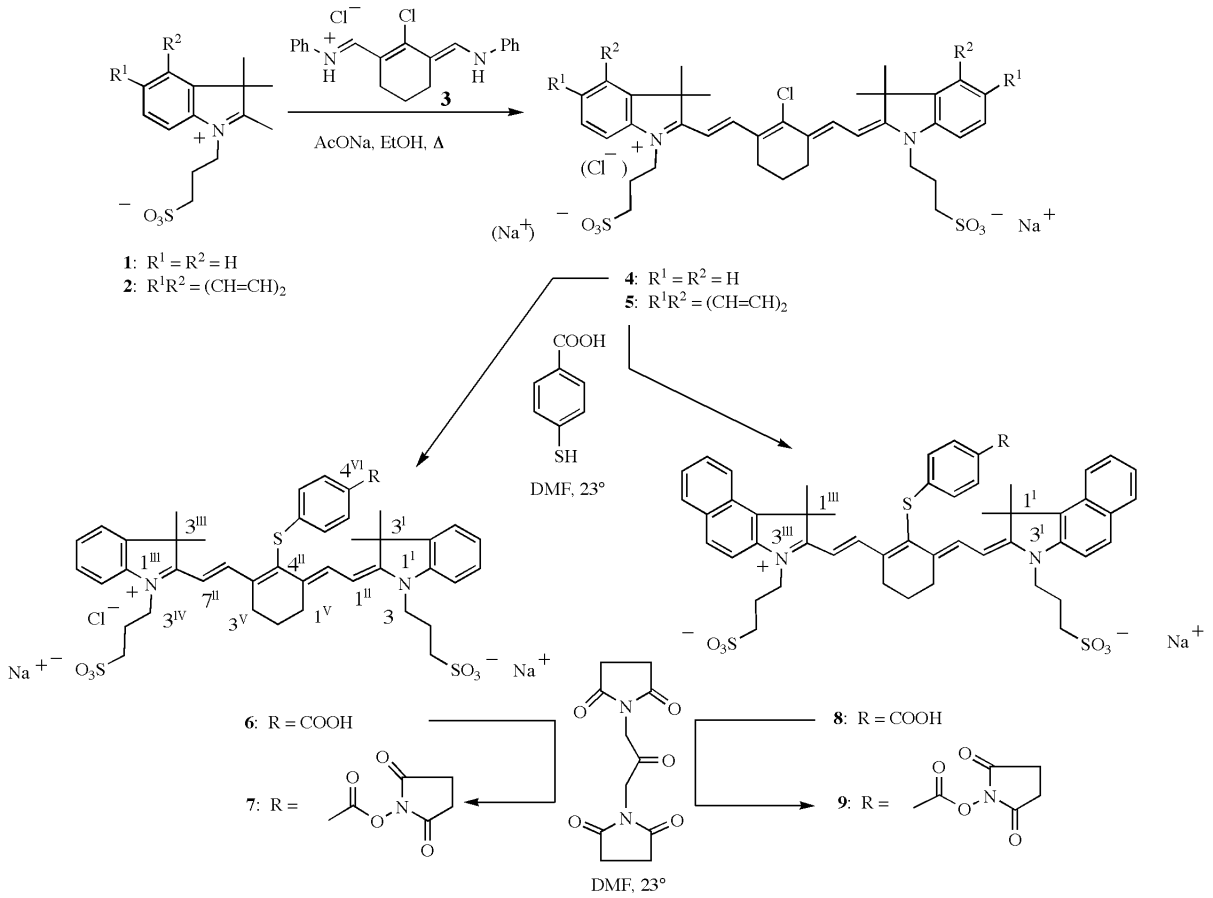
Cyanine dyes have emerged recently as an effective fluorescent label for biomolecules, owing to their large values of molar extinction coefficients and relatively large Stokes' shifts [1]. The labeling with a near-infrared cyanine is especially advantageous thanks to inherently low interference from biological media in the near-infrared region. Several functional groups have been developed to covalently attach a dye probe to a biomolecule. For example, iodoacetamido, maleimido, 2-pyridyldithio or methanesulfonylthio function on a dye molecule undergoes a selective reaction with thiol of a protein [2,3]. On the other hand, dye functionalized with an isothiocyanato group or a carboxylic acid ester derived from 4-sulfo-2,3,5,6-tetrafluorophenol or *N*-hydroxysuccinimide can selectively be attached to a primary amino group of a protein [4-13]. Another application of the amino-reactive near-infrared dyes is the synthesis of near-infrared labeled DNA oligomers for use in DNA sequencing [14-16]. Unfortunately, the preparation of dye intermediates or final reagents for the applications discussed above usually requires a tedious chromatographic purification. Functionalized cyanine des are normally purified by

Scheme



reverse-phase hplc because their polarity is additionally increased by the presence of hydrophilic groups for an increased solubility in water.

This report pertains to a facile preparation of two near-infrared cyanine dyes functionalized with *N*-hydroxysuccinimide ester (**7** and **9** in Scheme). Good aqueous solubility of **7**, **9** is achieved by the presence of sulfonatopropyl groups in the molecules. Importantly, no chromatography was used throughout the synthetic routes, and simple crystallizations, in most cases single crystallizations directly from reaction mixtures, were adequate to obtain intermediate and final products in an analytically pure form.

Synthesis of chloro-substituted cyanine **4** by the reaction of indolium inner salt **1** with the iminium reagent **3** has been reported previously [11]. The analogous benzo-fused dye **5** was prepared in this work in a similar way from **2** and **3**. In an attempt to simplify purification of **4**, **5**, instead of hplc separation as described for **4** [11], a crude product was crystallized from ether. Indeed, the ^{1}H nmr spectrum of **4** or **5** obtained by this simple procedure showed the absence of any organic impurity. On the other hand, in spite of a careful filtration of the ether solution before crystallization, the elemental analysis of the crystallized dye **5** showed the presence of sodium chloride in a ratio of dye/NaCl of 1:1. Dye **4** as a known compound was not subjected to elemental analysis. Structures of disulfo-substituted cyanines, such as **5** in Scheme, normally are drawn with a sodium sulfonate moiety [8,11]. The other sulfo group has been thought to be ionized also and the resultant anionic sulfonate to be part of an inner salt by interacting with the cationic chromophore. At least for **5**, the presence of an equimolar amount of sodium chloride in ether solution and co-crystallization of dye with sodium chloride can be interpreted in terms of a dye structure in which two anionic sulfonate groups are neutralized by two sodium counter cations, and the cationic cyanine chromophore interacts with external chloride anion.

We have shown previously that the chlorine atom at the central meso position of heptamethine cyanines, such as in **4** and **5**, is efficiently replaced by nucleophiles that are good single electron donors. The reaction involves an $S_{RN}1$ mechanism and, accordingly, proceeds smoothly in solvents that support a single-electron-transfer process, such as *N*,*N*-dimethylformamide [5]. The treatment of **4** and **5** with 4-mercaptobenzoic acid in *N*,*N*-dimethylformamide furnished the respective carboxylic acid derivatives **6** and **8** in high yield. The isolation procedure was further simplified by dropwise dilution of a crude reaction mixture with ethanol/ether, which caused slow crystallization of **6** or **8**. Elemental analysis results showed that compound **6** forms an equimolar complex with sodium chloride while dye **8** has a classical structure with inner salt and sodium sulfonate moieties, as shown in Scheme.

The desired dye reagents **7** and **9** were obtained by esterification of the respective carboxylic acids **6** and **8** with disuccinimido carbonate. Again, it was highly rewarding to find out that these complex molecular entities could be isolated in a pure analytical form by direct precipitation from a crude reaction mixture. As with **6**, its ester derivative **7** co-crystallizes with an equimolar amount of sodium chloride. As with **8**, its ester **9** does not contain sodium chloride. These results show that crystallization of sodium chloride with sulfoalkyl-substituted cyanines is not a general phenomenon but depends on the individual structure of a dye.

The high polarity of esters **7** and **9** make them soluble in water, which is a desirable feature for labeling of water soluble molecules at a primary amino group. The high polarity is also responsible for their crystallization with water. The solid samples of **7** and **9** are also hydroscopic. As a result, solid samples of **7** and **9** slowly hydrolyze, even when stored in a dessicator over phosphorus pentoxide, reverting to the respective acids **6** and **8**. Nevertheless, in a series of preliminary experiments we encountered no problems with the synthesis of amides from simple primary amines and with labeling of proteins by using reagents **7** and **9** that had been stored for not longer than two days. Detailed results of the labeling studies will be published in due course.

Fluorescence analysis of complex biological systems in the near-infrared region is a much more sensitive technique than that based on absorption. When detecting near-infrared fluorescence, it is important to consider the separation of the absorption and emission, as a large Stokes' shift results in a decrease of the Rayleigh scatter [1]. The synthesized dye labels exhibit large Stokes' shifts, 30 nm for **6**, **7** and 27 nm for **8**, **9**; hence, a relatively small amount of scatter can be observed. Importantly, these dyes can efficiently be excited by using several commercial laser diodes.

EXPERIMENTAL

Upon heating, all dyes reported in this paper undergo partial decomposition at >170°, and then melting is observed at >200°. The ^{1}H nmr spectra were taken at 400 MHz in dimethyl sulfoxide-d_6 solution at 30°. Coupling constants smaller than 1 Hz are not reported. Near-infrared absorption (λ_{max}) and fluorescence (λ_{em}) were taken in methanol.

Synthesis of Dyes **4** and **5**.

The preparation of inner salt **1** by the reaction of 2,3,3-trimethyl-3*H*-indole with 1,3-propane sultone and condensation of **1** with *N*-[5-anilino-3-chloro-2,4-(propane-1,3-diyl)-2,4-pentadien-1-ylidene]anilinium chloride (**3**) to give dye **4** has been reported [11]. Similar procedures were used for the synthesis of inner salt **2** by alkylation of 2,3,3-trimethyl-3*H*-benz[*e*]indole with 1,3-propane sultone and the synthesis of dye **5** from **2**. Inner

salts **1** and **2** were crystallized by diluting a solution in methanol with ether. Dyes **4** and **5** were crystallized from ether.

3-(1',1',2' -Trimethyl-1' *H*-3 ' -benz[*e*]indolio)propanesulfonate (**2**).

This compound was obtained in a 95% yield after crystallization; ^{1}H nmr: δ 1.76 (s, 6H), 2.23 (quint, J = 7 Hz, 2H), 2.67 (t, J = 7 Hz, 2H), 2.94 (s, 3H), 4.78 (t, J = 7 Hz, 2H), 7.72 (t, J = 8 Hz, 1H), 7.78 (t, J = 8 Hz, 1H), 8.21 (d, J = 8 Hz, 1H), 8.23 (d, J = 9 Hz, 1H), 8.28 (d, J = 9 Hz, 1H), 8.35 (d, J = 8 Hz, 1H).

Anal. Calcd. for $C_{18}H_{21}NO_3S$: C, 65.23; H, 6.39; N, 4.23. Found: C, 64.87; H, 6.78; N, 4.14.

Sodium 3-[2'-[4"-Chloro-7"-[1'",1'"-dimethyl-3'"-(3^{IV}-sulfonatopropyl)-1'"*H*-benz[*e*]indol-3'"-ium-2'"-yl]-3",5"-(propane-1^V,3^V-diyl)-2",4",6"-heptatrien-1"-ylidene]-1',1'-dimethyl-1',2'-dihydro-3'*H*-benz[*e*]indol-3'-yl]propanesulfonate Complexed with Sodium Chloride (**5**).

This compound was obtained in a 95% yield after crystallization; ^{1}H nmr: δ 1.88 (m, 2H), 1.95 (s, 12H), 2.09 (m, 4H), 2.63 (m, 4H), 2.78 (m, 4H), 4.50 (m, 4H), 6.55 (d, J = 14 Hz, 2H), 7.50 (t, J = 8 Hz, 2H), 7.64 (t, J = 8 Hz, 2H), 7.85 (d, J = 9 Hz, 2H), 8.04 (d, J = 8 Hz, 2H), 8.07 (d, J = 9 Hz, 2H), 8.27 (d, J = 8 Hz, 2H), 8.36 (d, J = 14 Hz, 2H); nir: λ_{max} = 820 nm (ε = 300000 $M^{-1}cm^{-1}$).

Anal. Calcd. for $C_{44}H_{46}ClN_2NaO_6S_2$•NaCl•5$H_2O$: C, 54.48; H, 5.82; Cl, 7.31; N, 2.89. Found: C, 54.63; H, 5.61; Cl, 7.60; N, 3.03.

Synthesis of Dyes **6** and **8**.

A solution of dye **4** or **5** (0.2 mmol) and 4-mercaptobenzoic acid (93 mg, 0.6 mmol) in *N*,*N*-dimethylformamide (10 mL) under a nitrogen atmosphere was allowed to stand for 24 hours at 23°. The mixture was stirred and treated dropwise with ethanol/ether (1:20, 20 mL). The resultant precipitate was collected by filtration, washed with ether and dried at 60°/1 mmHg.

Sodium 3-[2'-[4''-[4^{VI}-(Carboxyphenyl)thio]-7''-[3''',3'''-dimethyl-1'''-(3$^{I\ V}$-sulfonatopropyl)-1'''*H*-indol-1'''-ium-2'''-yl]-3",5"-(propane-1^V,3^V-diyl)-2",4",6"-heptatrien-1"-ylidene]-3',3'-dimethylindolin-1'-yl]propanesulfonate Complexed with Sodium Chloride (**6**).

This dye was obtained in a 66% yield; ^{1}H nmr: δ 1.39 (s, 12H), 1.93 (t, J = 6 Hz, 2H), 2.01 (quint, J = 7 Hz, 4H), 2.58 (t, J = 7 Hz, 4H), 2.82 (t, J = 6 Hz, 4H), 4.35 (t, J = 7 Hz, 4H), 6.55 (d, J = 14 Hz, 2H), 7.22 (t, J = 8 Hz, 2H), 7.38 (m, 4H), 7.49 (d, J = 8 Hz, 2H), 7.52 (d, J = 8 Hz, 2H), 7.87 (d, J = 8 Hz, 2H), 8.54 (d, J = 14 Hz, 2H); nir: λ_{max} = 800 nm (ε = 300000 $M^{-1}cm^{-1}$), λ_{em} = 830 nm.

Anal. Calcd. for $C_{43}H_{47}ClN_2NaO_8S_3$•NaCl•5$H_2O$: C, 52.30; H, 5.82; N, 2.84. Found: C, 52.47; H, 5.56; N, 2.82.

Sodium 3-[2'-[4''-[4^{VI}-(Carboxyphenyl)thio]-7''-[1''',1'''-dimethyl-3'''-(3^{IV}-sulfonatopropyl)-1'''*H*-benz[*e*]indol-3'''-ium-2'''-yl]-3",5"-(propane-1^V,3^V-diyl)-2",4",6"-heptatrien-1'-ylidene]-1',1'-dimethyl-1',2'-dihydro-3'*H*-benz[*e*]indol-3'-yl]propanesulfonate (**8**).

This dye was obtained in a 90% yield; ^{1}H nmr: δ 1.70 (s, 12H), 1.97 (t, J = 6 Hz, 2H), 2.09 (quint, J = 7 Hz, 4H), 2.65 (t, J = 7 Hz, 4H), 2.87 (t, J = 6 Hz, 4H), 4.49 (t, J = 7 Hz, 4H), 6.60 (d, J = 14 Hz, 2H), 7.47 (t, J = 8 Hz, 2H), 7.49 (d, J = 8 Hz, 2H), 7.61 (t, J = 8 Hz, 2H), 7.82 (d, J = 9 Hz, 2H), 7.92 (d, J = 8 Hz, 2H), 8.03 (d, J = 8 Hz, 2H), 8.05 (d, J = 9 Hz, 2H), 8.20 (d, J = 8 Hz, 2H), 8.69 (d, J = 14 Hz, 2H); nir: λ_{max} = 837 nm (ε = 360000 $M^{-1}cm^{-1}$), λ_{em} = 864 nm.

Anal. Calcd. for $C_{51}H_{51}N_2NaO_8S_3$: C, 65.22; H, 5.47; N, 2.98. Found: C, 65.37; H, 5.74; N, 3.34.

Succinimidyl Esters **7** and **9**.

A solution of dye **5** or **7** (0.1 mmol) and disuccinimido carbonate (31 mg, 0.12 mmol) in anhydrous *N*,*N*-dimethylformamide (5 mL) under a nitrogen atomosphere was allowed to stand at 23° for 24 hours. Then the solution was stirred and treated dropwise for 30 minutes with ether (25 mL). The resultant crystalline precipitate of **7** or **9** was filtered, washed with ether, and dried at 30°/0.5 mmHg for 2 hours.

Sodium 3-[2'-[4"-[[4^V'-[[(2^V",5^V"-dioxopyrrolidin-1^V"-yl)oxy]carbonyl]phenyl]thio]-7"-[3'",3'"-dimethyl-1'"-(3'^{V}-sulfonatopropyl)-1'"*H*-indol-1'"-ium-2'"-yl]-3",5"-(propane-1^V,3^V-diyl)-2",4",6"-heptatrien-1''-ylidene]-3',3'-dimethylindolin-1'-yl]propanesulfonate Complexed with Sodium Chloride (**7**).

This dye was obtained in a 90% yield; ^{1}H nmr: δ 1.40 (s, 12H), 1.94 (t, J = 6 Hz, 2H), 2.02 (quint, J = 7 Hz, 4H), 2.58 (m, 8H), 2.82 (t, J = 6 Hz, 4H), 4.35 (t, J = 7 Hz, 4H), 6.55 (d, J = 14 Hz, 2H), 7.22 (t, J = 8 Hz, 2H), 7.38 (m, 4H), 7.50 (d, J = 8 Hz, 2H), 7.52 (d, J = 8 Hz, 2H), 7.87 (d, J = 8 Hz, 2H), 8.55 (d, J = 14 Hz, 2H); nir: λ_{max} = 800 nm (ε = 300000 $M^{-1}cm^{-1}$), λ_{em} = 830 nm.

Anal. Calcd. for $C_{47}H_{50}N_3NaO_{10}S_3$•NaCl•6$H_2O$: C, 51.20; H, 5.67; Cl, 3.22; N, 3.81. Found: C, 50.87; H, 5.44; Cl, 3.63; N, 3.49.

Sodium 3-[2'-[4''-[[4^{VI}-[[(2^V'',5^V''-Dioxopyrrolidin-1^{VII}-yl)oxy]carbonyl]phenyl]thio]-7''-[1'''',1''''-dimethyl-3''''-(3'^{V}-sulfonatopropyl)-1''''*H*-benz[*e*]indol-3''''-ium-2''''-yl]-3",5"-(propane-1^V,3^V-diyl)-2",4",6"-heptatrien-1'-ylidene]-1',1'-dimethyl-1',2'-dihydro-3'*H*-benz[*e*]indol-3'-yl]propanesulfonate (**9**).

This dye was obtained in an 88% yield; ^{1}H nmr: δ 1.71 (s, 12H), 1.97 (t, J = 6 Hz, 2H), 2.09 (quint, J = 7 Hz, 4H), 2.64 (t, J = 7 Hz, 4H), 2.87 (t, J = 6 Hz, 4H), 2.89 (s, 4H), 4.48 (t, J = 7 Hz, 4H), 6.59 (d, J = 14 Hz, 2H), 7.47 (t, J = 8 Hz, 2H), 7.49 (d, J = 8 Hz, 2H), 7.61 (t, J = 8 Hz, 2H), 7.82 (d, J = 9 Hz, 2H), 7.92 (d, J = 8 Hz, 2H), 8.02 (d, J = 8 Hz, 2H), 8.04 (d, J = 9 Hz, 2H), 8.19 (d, J = 8 Hz, 2H), 8.69 (d, J = 14 Hz, 2H); nir: λ_{max} = 837 nm (ε = 360000 $M^{-1}cm^{-1}$), λ_{em} = 864 nm.

Anal. Calcd. for $C_{55}H_{54}N_3NaO_{10}S_3$•1.5$H_2O$: C, 62.13; H, 5.40; N, 3.95. Found: C, 62.00; H, 5.43; N, 3.58.

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