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Synthesis and Evaluation of Polyhydroxylated Near-Infrared Carbocyanine Molecular Probes

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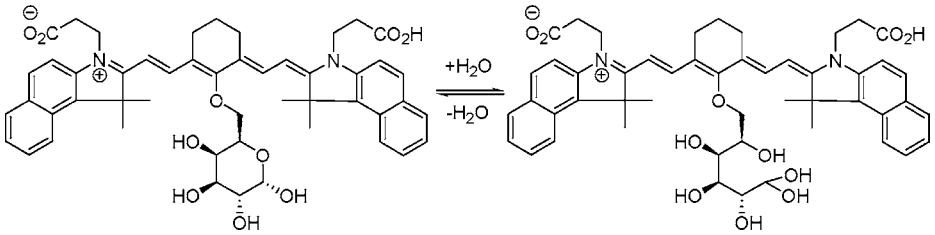
ABSTRACT



A new near-infrared (NIR) fluorescent molecular probe derived from indocarbocyanine dye and galactose was prepared, and the procedure was optimized. The presence of a nonionic polyhydroxyl moiety between hydrophobic groups enhances solubility and possibly minimizes aggregation in aqueous solutions. The structural framework of this molecule provides multivalent sites for labeling diverse molecules.

The resurgence of interest in detecting and imaging various diseases and analytes by optical methods has led to the development of methods for the synthesis and isolation of fluorescent probes in good yields and high purity.[1−4] Particularly, the use of carbocyanine (e.g., indocyanine green, ICG, Figure 1) and xanthene (e.g., fluorescein disodium salt) derivatives in humans shows that these fluorescent compounds are biocompatible, even at high concentrations of injected doses.[5] Because tissue autofluorescence and light absorption are low in the NIR wavelengths (750−900 nm), NIR light can travel several centimeters in heterogeneous systems such as cells and tissue phantoms. For this reason, molecular probes such as ICG and its reactive derivatives are useful for analyzing tissuelike heterogeneous samples by optical methods.[6,7]

The basic structural framework of many carbocyanine compounds consists of a linear polymethine unit that is flanked by symmetrical or nonsymmetrical aromatic groups.

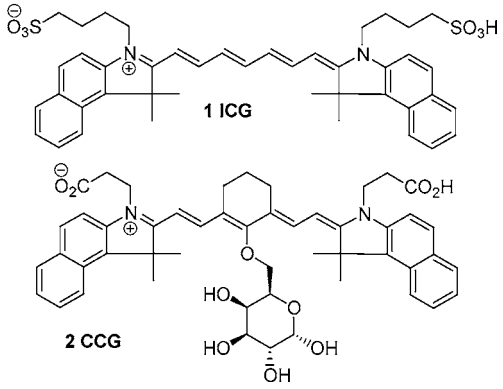


Figure 1. Structure of ICG and CCG.

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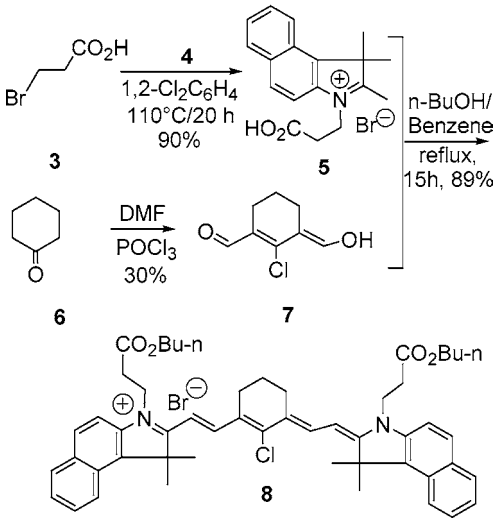
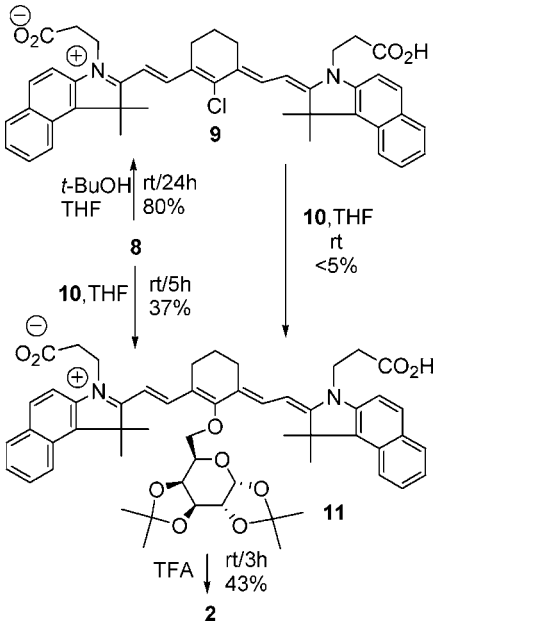
Scheme 1



Scheme 2



Predicated on previous findings that ICG derivatives that possess a conformationally constrained heptamethine moiety enhance fluorescence quantum yield and photochemical and photophysical stabilities of this class of compounds,[8,9] we designed and synthesized a novel NIR carbocyanine fluorescent probe containing galactose. The monosaccharide is incorporated in the central hydrophobic core of the chromophore system and anchored by ether linkage to the conformationally constrained cyclohexenyl group (CCG, **2**, Figure 1). Interestingly, the structural framework of the cyclohexenyl cypate-galactose **2** possesses multiple sites for homogeneous or heterogeneous labeling of biomolecules, drugs, and analytes with a single fluorescent molecule. Additionally, the nonionic polyhydroxyl group of galactose provides a mild hydrophilic environment for labeling molecules that are sensitive to the presence of strong anions such as the sulfonates used to improve water solubility in most carbocyanine molecular probes. CCG is also attractive because of the possibility of reducing aggregation of the probe in aqueous solutions.

The methods used to synthesize compound **2** are shown in Schemes 1 and 2. Reaction of 3-bromopropionic acid **3** with 2,3,3-trimethylbenzoindolenine **4** in 1,2-dichlorobenzene for 20 h at 120 °C gave the benzoindole carboxylic acid derivative **5** in 90% yield (Scheme 1). The intermediate **5** can be produced as needlelike crystals directly from the reaction mixture by controlled heating of the mixture between 100 and 105 °C in a cylindrical glass vessel. Synthesis of the second intermediate, 2-chloro-1-formyl-3-(hydroxymethylene)cyclohex-1-ene **7**, was performed by following a literature method.[10] We isolated **7** by H_2O/DCM extraction. The DCM phase was percolated through a $MgSO_4$ column, and the filtrate was concentrated on a rotary evaporator. Treatment of the residue with pentane gave the chloroaldehyde **7** as yellow crystalline solid. The chloraldehyde is not stable at room temperature and must be handled with care. For prolonged storage, **7** was converted to its stable aniline Schiff base.

In attempts to prepare chloro cylcohexenyl cypate (CCC, **9**, Scheme 2) from **7** and **5** using a literature method,[11] we obtained mixtures of **8**, **9**, and the mono-*n*-butyl ester. The ratios of these compounds varied under different reaction times and temperatures. For example, the proportion of CCC **9** in the mixture increased at lower temperatures and shorter reaction times relative to the conditions used to prepare **8**. The use of *n*-BuOH as a reaction solvent was beneficial in subsequent reactions under strongly basic conditions because it gave *n*-butyl esters of the carboxylic acid groups that served as a transient protecting group. Further optimization of the reaction conditions led selectively to the synthesis of **8** in excellent yield without base or acid catalysis. Evaluation of the reactivity of **7** and its aniline Schiff base derivatives (dianiline and dianiline monohydrochloride) showed that freshly prepared **7** gave excellent yield of the desired compounds compared with the aniline and aniline hydrochloride derivatives.

Etherification of **8** with galactose was accomplished by using 1,2,3,4-di-*O*-isopropylidene-D-galactopyranose (**10**) in freshly distilled THF. The solution was cooled to −78 °C, and a stoichiometric amount of *n*-BuLi was added to generate the reactive lithium alkoxide of D-galactopyranose. After

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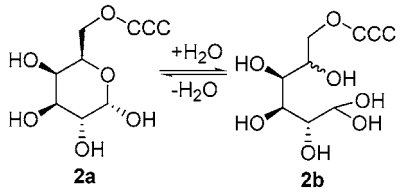
Scheme 3



adding **8** in solid form, the reaction mixture was allowed to warm to room temperature. Interestingly, the alkaline reaction condition also removed the *n*-butyl ester to give isopropylidene-protected **11** in good yields (30−50%).[12] We observed that the best workup conditions for isolating **11** were obtained by (i) neutralizing the crude mixture, with HBr, (ii) evaporating the solvent, and (iii) washing the solid residue with water. Deprotection of the isopropylidene group of **11** with TFA at room temperature for 3 h gave the expected compound **2** in 43% yield as a green solid. The product was purified by reverse-column chromatography.

CCC **9** is an important NIR fluorescent compound that can be used to prepare dendritic arrays of dye-labeled molecules because of the availability of multiple reactive sites. We found that *n*-butyl-protected **8** can be effectively deprotected with THF/*t*-BuONa to give **9** in high yield (80%). Rapid precipitation of **9** from THF facilitates the reaction, and isolation of **9** and prevents its degradation in the basic reaction mixture (Scheme 2). However, attempts to prepare **11** from **9** gave a very low yield of the expected compound (<5%), even in the presence of excess lithium galactopyranose salt.

Although we were able to identify the cyclic analogue by MS, both **11** and **2** exist predominantly in the hydrated linear gem diol form (Scheme 3).

Shown in Figure 2 are the absorption and fluorescence spectra of **2**, which is representative of the spectral properties of the new molecular probes prepared. All the compounds have similar absorption and fluorescence emission maxima in 20% aqueous DMSO. This indicates that direct substitution of the chloro group with an alkoxyl group at the central hydrophobic chromophore system had negligible effects on the spectral properties of the compounds. The small Stokes shift of <15 nm observed is typical of many NIR cyanine fluorescent probes. However, the absorption spectrum is broad, enabling excitation of the molecule at any wavelength between 650 and 810 nm. For example, exciting the probe at 750 nm and monitoring the fluorescence emission at 825 nm would minimize the challenge of rejecting stray excitation light by the emission filter.

Cyanine dyes are known to aggregate in aqueous solutions because of the structural arrangement of their hydrophobic core.[13,14] The aggregates affect dye solubility and optical properties. A common aggregation mode in cyanine dyes is *J*-aggregates, which are polymerlike stackings of molecules

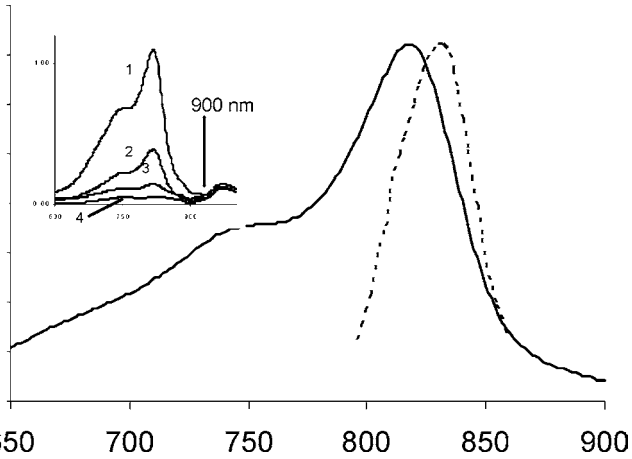


Figure 2. Absorption (solid line) and fluorescence (dashed line) spectra of **2** in 20% aqueous DMSO solution. *y*-Axis: Normalized absorption and fluorescence intensity in arbitrary unit. *x*-Axis: wavelength in nm. Inset: Absorption spectra of **2** at different concentrations (**1**, 3.0×10^{-6} M; **2**, 7.5×10^{-7} M; **3**, 1.9×10^{-7} M; **4**, 4.7×10^{-8} M). Peaks >900 nm are nearly unchanged.

in aqueous solvents. Spectroscopically, the *J*-aggregates are manifested by a new sharp absorption band at longer wavelengths with respect to the major long-wavelength absorption of monomers.[15] The small bumps at 972 nm observed in the absorption spectrum of **2** (Figure 2, insert) at different concentrations probably correspond to *J*-aggregates. This is similar to what was observed in the absorption spectrum of benzimidazolocarbocyanine.[15] The peak does not show noticeable increases in intensity at higher concentrations, and it is not as sharp as the *J*-aggregates band of benzimidazolocarbocyanine. We compared the aggregation pattern of **2** with the hydrophobic **8**. The results show that **8**, which is more susceptible to forming aggregates than **2**, has a small secondary absorption peak at 896 nm, possibly arising from *J*-aggregates. At the same molar concentration (3.0 μM), this peak is nearly 5 times more intense than that of compound **2** at 972 nm. Thus, the new molecular design may have reduced aggregate formation in aqueous medium.

Our preliminary results of the in vivo distribution of **2** in normal nude mice showed that **2** rapidly clears from blood and is predominately excreted by the hepatobiliary system within 5 min postinjection. The fast clearance from blood reduces background fluorescence, thereby enabling rapid visualization of target tissue.

In conclusion, we have synthesized a new conformationally constrained NIR fluorescent probe with enhanced water solubility and biocompatibility. The presence of a structurally constrained cyclic substructure, a nonionic polyhydroxyl moiety, and multiple reactive functional groups makes **2** a robust molecular probe for a variety of chemical and

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biological applications. We are currently exploring the use of this compound to study diseased tissue by optical methods.

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Supporting Information Available: Experimental procedures for all compounds, characterization for key compounds, and description and results of biodistribution studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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