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Preliminary Communication

Synthesis of functional near infrared pyrrolopyrrole cyanine dyes for optical and photoacoustic imaging

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Abstract

Fluorescence lifetime and photoacoustic imaging (PAI) provide attractive strategies for *in vivo* molecular imaging in the near infrared (NIR) region. Pyrrolopyrrole cyanine (PPCy) dyes are promising molecules for these applications. In this study, we synthesized a chloro-functionalized non-fluorescent dye with high absorption coefficient suitable for PAI. Upon complexation with boron, the dye becomes highly fluorescent with lifetime of 3.6 ns, which is longer than most cyanine dyes commonly used for NIR optical imaging. The potential to use the non-fluorescent or fluorescent analogs of PPCy for fluorescence or photoacoustic imaging provides a versatile approach to image molecular processes and physiological events by these methods.

Keywords: fluorescence lifetime; molecular probes; photoacoustic imaging agent; spectroscopy.

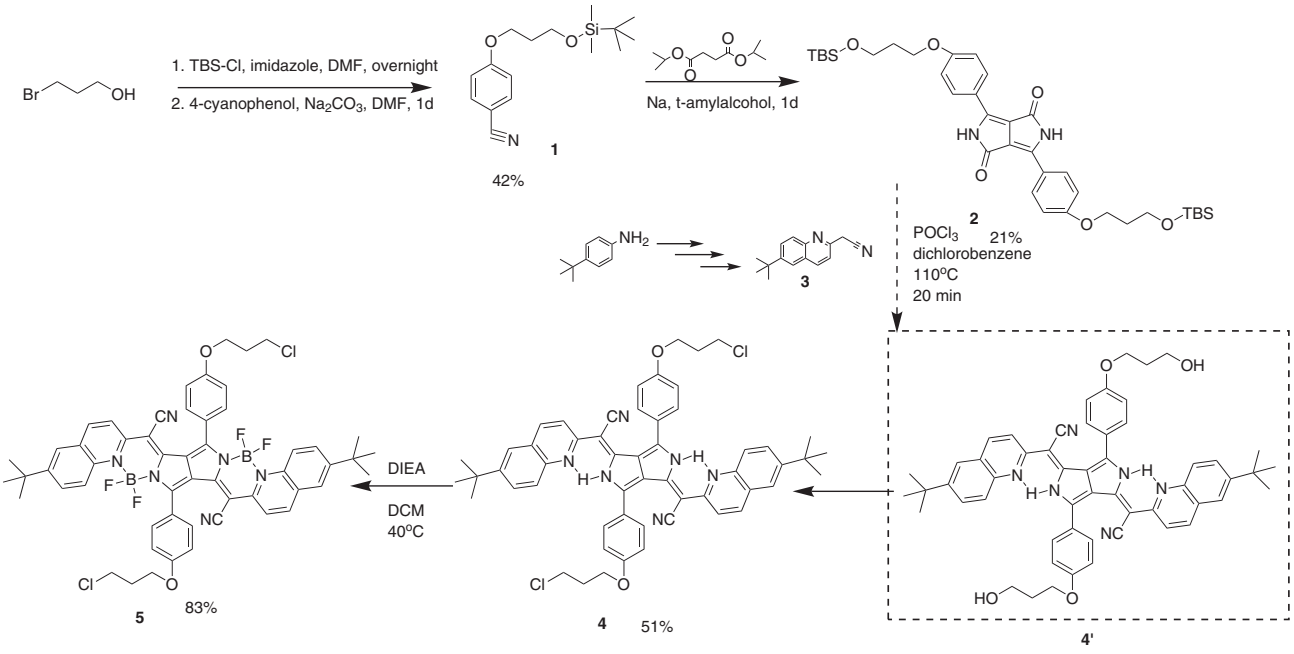
Introduction

Owing to relatively deep tissue penetration and low tissue autofluorescence in the near infrared (NIR) region between 700 and 900 nm, NIR fluorescent dyes are attractive in modern optical imaging of biological targets *in vivo* (Akers et al., 2008). The most common NIR dyes are the polymethine cyanine dye family, comprising benzoxazole, benzothiazole, indolyl, 2-quinoline, and 4-quinoline subclasses (Frangioni, 2003). These dyes have adjustable optical properties and high extinction coefficients, often >150 000 M/cm (Licha, 2002). In addition, many cyanine dyes can be easily conjugated to targeting moieties, imparting molecular specificity or selectivity to targeted cells or tissues (Achilefu, 2004; Bai et al., 2008; Ye et al., 2008).

Unfortunately, conventional NIR cyanine dyes suffer from some limitations. First, some of these dyes decompose over time in ambient conditions and tend to aggregate in solutions. Second, the fluorescence quantum yields of many indocyanine dyes are typically <15% in aqueous environment and are readily photobleached under high laser beams. Finally, most available NIR cyanine dyes have similar fluorescence lifetimes (FLTs) around 1 ns, confining the scope of lifetime imaging applications with these NIR fluorophores to narrow lifetime range.

Consequently, there is a need to develop novel NIR dyes to improve signal intensity, sensitivity, photostability, and diverse FLT ranges for fluorescence imaging. Compared to conventional fluorescence imaging based on fluorescence intensity measurements, FLT imaging is less affected by dye concentration, photobleaching, light scattering, excitation intensity, and sample turbidity (O'Leary et al., 1996; Cerussi et al., 1997; Kuwana and Sevick-Muraca, 2002; Ntziachristos and Weissleder, 2002; Lichtman and Conchello, 2005). Development of NIR dyes with long FLTs is challenged by the energy gap law (Englman and Jortner, 1970; Caspar et al., 1982), causing a decrease in quantum yield and FLT at these long NIR wavelengths. Another attractive area of research with NIR dyes is photoacoustic imaging, which provides strong contrast, high resolution and deep tissue penetration by combining optical with ultrasound technologies (Wang, 2008). Imaging capability and accuracy of photoacoustic imaging is enhanced by using exogenous contrast agents. Because photoacoustic signals originate from heat after light absorption, contrast agents with high absorption coefficients and low fluorescence quantum yields are optimal for this application. Unfortunately, few NIR dyes possess these features.

Recently, a new class of NIR absorbing and fluorescent diketopyrrolopyrrole dyes has been developed (Fischer et al., 2007, 2009). These dyes exhibit high quantum yields (>0.50), low photobleaching (2% absorption drop after 60 min illumination compared to 93% absorption drop for indocyanine green) and long FLTs (2.5–3.8 ns) (Berezin et al., 2009). These properties are suitable for fluorescence imaging, whereas the non-fluorescent analogs are useful for photoacoustic imaging. However, the application of this class of dye in biomedical research is greatly limited owing to poor hydrophilicity and lack of functionality for subsequent conjugation with biomolecules such as peptides and proteins. The importance of functionalized pyrrolopyrrole cyanine (PPCy) for conjugation with peptides and proteins was recently reported (Fischer et al., 2010).



Scheme 1 Synthesis of PPCy.

Results and discussion

Here, we report the synthesis of functional PPCy dyes. The synthetic pathway for the PPCy dyes is shown in Scheme 1. The hydroxyl group in 3-bromopropanol was first protected with *t*-butyldimethylsilyl, and subsequent alkylation reaction with 4-cyanophenol yielded compound **1** with 42% yield. Next, diketopyrrolopyrrole **2** was synthesized by Stobbe-like condensation of diisopropyl succinate and **1** in a mixture of sodium *t*-amyl alcoholate and *t*-amyl alcohol. Quinoline acetonitrile **3** was prepared as previously reported (Fischer et al., 2009). Finally, the desired compound **4′** was obtained by heating a mixture of **2** and **3** in dichlorobenzene at 110°C in the presence of $POCl_3$. High reaction yield (52%) was achieved. Compound **2** first forms a monophosphorylated intermediate with $POCl_3$, followed by forming condensation product with **3** (Iqbal et al., 1988; Fischer et al., 2009). The yield of the reaction was largely affected by the solubility of both condensation partners. The quinoline acetonitrile **3** is soluble in many organic solvents, whereas **2** is only partially soluble in limited solvents. By changing the solvent from toluene to dichlorobenzene, the condensation reaction yield improved from 19% to 52%.

Upon purification, compound **4′** was characterized by ^{1}H NMR. All peaks seemed to correlate with the target structure. Further derivatization with CDI or chloroformate, however, failed to produce desired products. Considering that hydroxyl group and chloride have similar chemical shifts effect for protons bound to the neighboring carbon, we postulated that $POCl_3$ converted the hydroxyl group to chloride. Subsequent ^{13}C NMR (Figure 1, peak at 41.7 ppm indicates chloride) and FT-IR (data not shown) analysis verified the existence of chloride, instead of hydroxyl group, on the molecule (compound **4**).

After the structure of compound **4** was verified, we studied the spectroscopic properties of the dye. This PPCy molecule exhibits major and minor absorption peaks at 730 nm and 663 nm, respectively (Figure 2), with extinction coefficient of 58 000 м/cm in dimethyl sulfoxide (DMSO). No significant fluorescence was observed, as the flexible heteroaromatic end groups lead to efficient radiationless depopulation of the excited S_1 state (Fischer et al., 2009). Therefore, compound **4** is suitable as a contrast agent for photoacoustic imaging. However, incorporation of BF_2 into **4** yields compound **5**. This reaction rigidified the molecule and red-shifted the absorption (Figure 2) with good absorption coefficient (ε=111 000 м/cm). The resulting fluorescence quantum yield was high (81% in DMSO) and the characteristic long FLT of 3.6 ns was retained in compound **5**. These properties are excellent for FLT imaging studies.

In summary, we have reported the synthesis of PPCy and BF_2·PPCy dyes as functional NIR dyes with attractive optical properties. Compound **4** has strong absorption, and the boron complexed compound **5** has strong fluorescence in the NIR region. The use of $POCl_3$ provided a method for one-pot transformation of the hydroxyl to reactive chloride function. Derivatization of the chloride to other functional groups, such as carboxyl and amino, and subsequent bioconjugation of the PPCy to targeting moieties for biomedical imaging are currently in process. With a reactive chloride group, good absorption coefficient, long FLT and/or high fluorescence quantum yield, these dyes appear to be good candidates for molecular imaging applications.

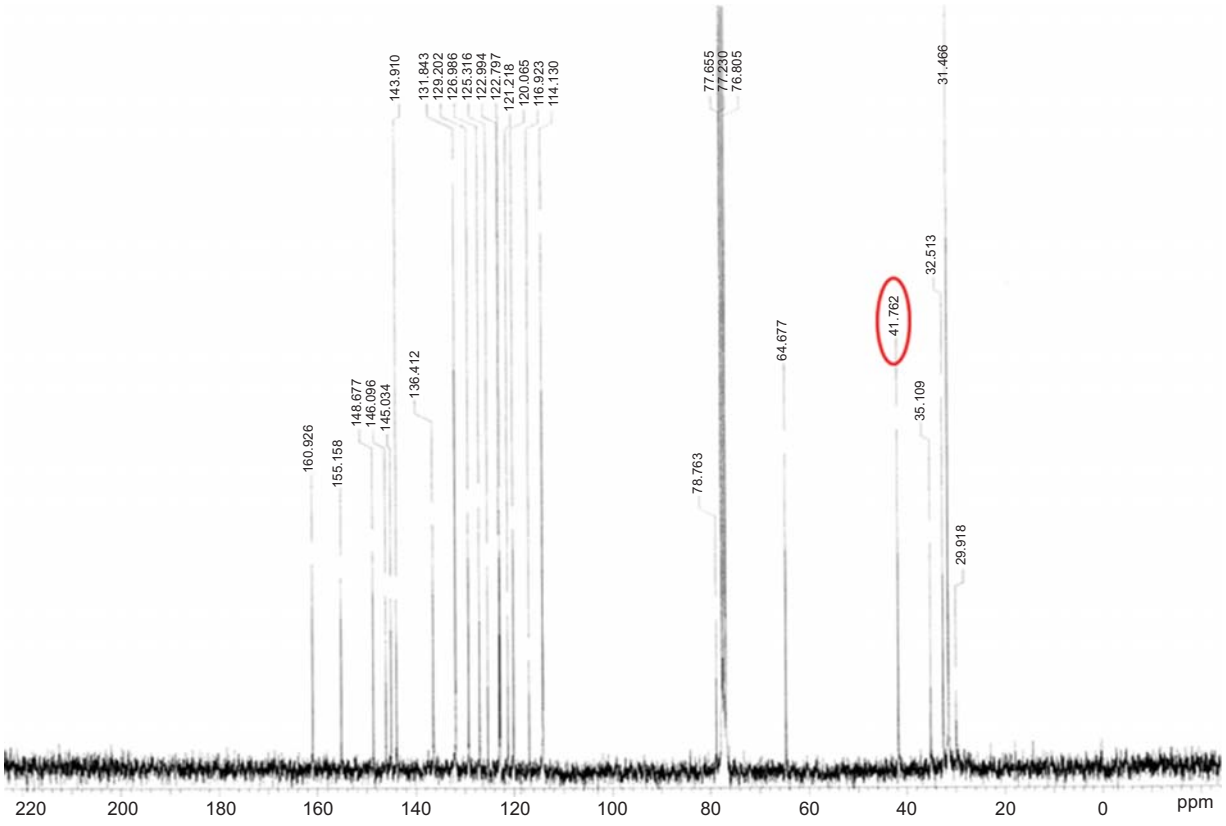


Figure 1 ^{13}C NMR spectrum of compound **4**.

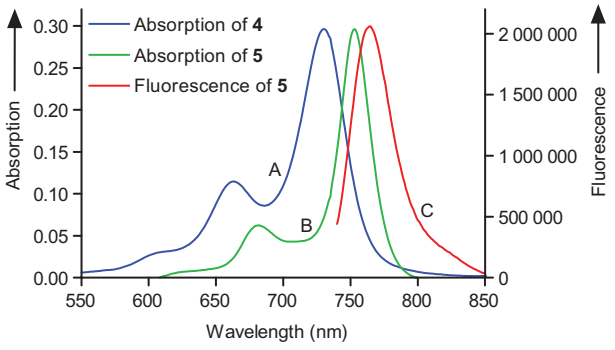


Figure 2 Absorption and fluorescence of PPCy and BF_2·PPCy in dichloromethane at room temperature.
(A) Absorption of **4** (blue), (B) absorption of **5** (green), and (C) fluorescence of **5**, excited at 730 nm.

Experimental details

Compound **3** was synthesized from 4-*t*-butyl aniline in three steps as reported previously (Fischer et al., 2009).

Synthesis of 4-(3-((*t*-butyldimethylsilyl)oxy)propoxy) benzonitrile (1)

To a solution of 3-bromopropanol (100 mmol) in anhydrous DMF (100 ml) was added imidazole (110 mmol) at 0°C. After 5 min, *t*-butyl dimethylsilyl chloride (110 mmol) was added and the resulting mixture was allowed to warm to room temperature. After stirring overnight, the reaction mixture was partitioned between saturated sodium bicarbonate solution (100 ml) and ethyl ether (200 ml). The separated ether layer was then washed with 1 M sulfuric acid solution (80 ml), followed by brine (80 ml). Next, the organic solution was dried over sodium sulfate and concentrated by rotary evaporation.

In another flask, a mixture of 4-cyanophenol (80 mmol) and sodium carbonate (100 mmol) in anhydrous DMF (100 ml) was stirred at 70°C under argon for 10 min. The freshly synthesized 3-bromopropoxy-*t*-butyl-dimethylsilane was added and the mixture was stirred at room temperature for 24 h. The reaction mixture was quenched with 1 M sulfuric acid and extracted with ether (3×150 ml). The extracted ether layers were combined, washed with brine and dried over sodium sulfate. After removal of the solvent under reduced pressure, the desired compound **1** (10 g, 43%) was eluted with 20% ethyl acetate in hexane by flash column chromatography. ^{1}H NMR 300 MHz ($CDCl_3$): δ 7.57 (d, *J*=9.3 Hz, 2H), 6.94 (d, *J*=9.3 Hz, 2H), 4.11 (t, *J*=6.3 Hz, 2H), 3.79 (t, *J*=6.3 Hz, 2H), 1.99 (m, 2H), 0.87 (s, 9H), 0.03 (s, 6H).

Synthesis of 3,6-bis(4-(3-((*t*-butyldimethylsilyl)oxy) propoxy)phenyl)pyrrolo[3,4-*c*]pyrrole-1,4 (2*H*,5*H*)-dione (2)

Sodium (0.65 mol) in *t*-amyl alcohol (30 ml) was heated at 100°C under argon atmosphere overnight. After all the metal sodium had reacted, compound **1** (30 mmol) was added in one portion. The resulting mixture was stirred at 100°C under argon atmosphere and a solution of diisopropyl succinate (15 mmol) in *t*-amyl alcohol (7 ml)

was added through a syringe pump over 20 h. The reaction mixture was slowly added to a stirring mixture of 50 ml methanol and 12 ml acetic acid. The red precipitate was filtered and washed with methanol (3×15 ml). After drying, compound **2** was isolated as a red solid (5 g, 49%). ^{1}H NMR 300 MHz ($CDCl_3$): δ 11.15 (s, 2H), 8.45 (d, *J*=8.7 Hz, 4H), 7.12 (d, *J*=8.7 Hz, 4H), 4.11 (t, *J*=6.0 Hz, 4H), 3.76 (t, *J*=6.0 Hz, 4H), 1.92 (m, 4H), 0.86 (s, 16H), 0.03 (s, 12H). MS (MALDI with α-cyano-4-hydroxycinnamic acid as matrix)$^+$ $[M+H]^+$ calcd. 665.3, found 665.1.

Synthesis of PPCy (4)

$POCl_3$ (0.8 mmol) was added to a mixture of **2** (0.1 mmol) and **3** (0.25 mmol) in anhydrous dichlorobenzene (3 ml) at 110°C. The reaction mixture was stirred under argon atmosphere for 30 min before being partitioned between $NaHCO_3$ solution and chloroform. The combined organic layers (3×30 ml) were combined and dried over Na_2SO_4. The solvent was then removed by rotary evaporation and crude product was purified on silica gel column starting with dichloromethane to 2% methanol in dichloromethane as eluent. Compound **4** was isolated as a green solid (44 mg, 52%). ^{1}H NMR 300 MHz ($CDCl_3$): δ 7.98 (d, *J*=8.7 Hz, 2H), 7.81–7.77 (m, 8H), 7.68 (s, 1H), 7.65 (s, 3H), 7.18 (d, *J*=8.7 Hz, 2H), 4.29 (t, *J*=5.7 Hz, 4H), 3.83 (t, *J*=6.3 Hz, 4H), 2.33 (m, 4H), 1.44 (s, 18H). MS (MALDI with α-cyano-4-hydroxycinnamic acid as matrix)$^+$ $[M+H]^+$; calcd. 885.3, found 885.3.

Synthesis of BF_2·PPCy (5)

A mixture of **4** (0.01 mmol) and DIEA (0.2 mmol) was heated to 40°C in dichloromethane (2 ml) in a sealed vial. After 5 min, $BF_3{\cdot}Et_2O$ (0.4 mmol) was added and the resulting mixture was heated at 40°C for another 2 h. The reaction mixture was washed with water and dried over Na_2SO_4. After the solvent was removed by rotary evaporation, the crude product was purified by column chromatography using chloroform as eluent. BF_2·PPCy was isolated as a green solid (7.8 mg, 83%). ^{1}H NMR 300 MHz ($CDCl_3$): δ 8.45 (d, *J*=9.9 Hz, 2H), 8.12 (d, *J*=9.0 Hz, 2H), 7.78–7.68 (m, 8H), 7.64 (d, *J*=2.4 Hz, 2H), 7.10 (d, *J*=8.7 Hz, 4H), 4.26 (t, *J*=5.7 Hz, 4H), 3.81(t, *J*=6.3 Hz, 4H), 2.31 (m, 4H), 1.36 (s, 18H).

Acknowledgments

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