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An efficient approach to the synthesis of water-soluble cyanine dyes using poly(ethylene glycol) as a soluble support

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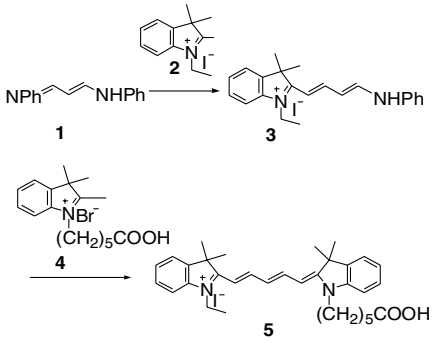
Abstract—An efficient synthesis approach to unsymmetrical water-soluble cyanine dyes has been established. Loading and activation of sulfoindolenium to poly(ethylene glycol) (PEG) have been achieved via a simple strategy. Cyanine dyes are released by the attack of heterocyclic carbon nucleophile and the cleavage of PEG-bound hemicyanine. The efficient approach delivers cyanine dyes in high purity without the nontrivial chromatographic separation.

Unsymmetrical water-soluble cyanine dyes (Cy3 and Cy5), originally described by Waggoner and co-workers,[1,2] have been proven valuable in numerous applications involving conjugation with proteins. However, these dyes were prepared by traditional solution-phase chemistry and must be purified by chromatography, the tedious process that resulted in a high cost of using these dyes. Traditional solution synthesis of unsymmetrical cyanine dyes is illustrated in Scheme 1. The reaction of nucleophilic heterocycle such as **2** with a polyene chain precursor such as amidine **1** gives a hemicyanine intermediate **3**. The hemicyanine is susceptible to react with a second molecule of heterocycle such as **4** to form unsymmetrical dye **5**, meanwhile it can also react with **2** to form the symmetrical dye, which often requires nontrivial chromatographic separation.[3–5] Therefore purification of the unsymmetrical dye is a particular problem in traditional solution synthesis.



Scheme 1. Solution synthesis of a cyanine dye.

Solid-phase synthesis is a powerful approach to the synthesis of important heterocyclic compounds[6–9] because of the ease of workup and purification.[10–13] From earlier research, it is known that solid-supported substrate attacked by heterocyclic nucleophile is an effective way for the synthesis of dyes. Balasubramanian's group improved the solid-phase synthesis of cyanine dyes employing sulfonyl chloride resin and polystyrene as loading materials.[14,15] But they also pointed out that the sulfonated heterocycles did not react well in the loading reaction, which led to only one sulfo-group attached to the cyanine dyes. Therefore, cyanine dyes synthesized by solid-phase method suffered poor solubility for biomolecular labelling.[14–18]

In recent years, soluble polymer-supported synthesis has been studied intensely.[19] In fact, the advantages of both homogeneous reaction (high reactivity) and solid-phase synthesis methods (easy isolation and purification of products) coexist in this technique. Our own interest grew out of a desire to develop a soluble polymer-supported synthesis strategy to synthesize unsymmetric cyanine dyes with high solubility. The structure of

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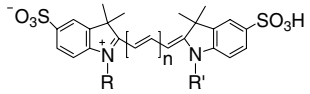


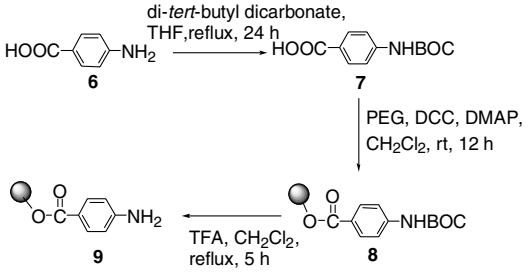
Figure 1. Structure of sulfoindocyanine dye. $R = CH_2CH_3$, $R' = (CH_2)_5COOH$, $n = 1, 2$.

sulfoindocyanine dyes synthesized in our work is shown in Figure 1. They consist of two nitrogen-containing heterocycles linked to each other by an odd-number polyene chain. Structural diversity is possible by varying the length of polyene chain, the nitrogen substituents or the heterocycle themselves. The sulfoindocyanine dyes have $-SO_3^-$ and $-SO_3H$ groups at 5 and 5′ positions, respectively, so as to increase their water solubility. The R′ substituent is carboxyl alkyl group, which can be activated to form succinimidyl ester for easy conjugation with biomolecule.

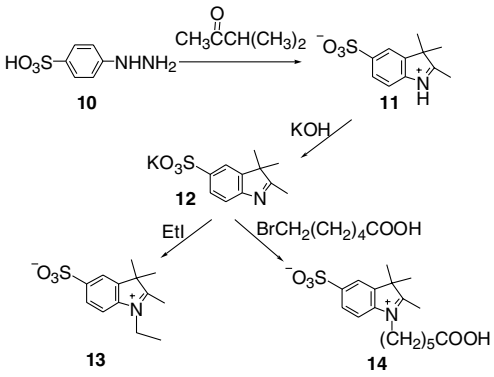
In order to realize the soluble polymer-supported synthesis, the preparation of aniline scaffold **9** was required (see Scheme 2). We selected poly(ethylene glycol) (PEG, MW2000) as a soluble support. The choice of using PEG was mainly motivated by its good solubility in dichloromethane (DCM) and its poor solubility in diethyl ether, which made the reaction homogeneous and the separation heterogeneous. Furthermore, the low cost and recyclability of PEG and its derivatives made them attractive candidates as the support for the synthesis of cyanine dyes.

The synthesis of PEG-bound aniline **9** began with 4-aminobenzoic acid **6** and was accomplished in three steps. The amino group of **6** was protected with di-*tert*-butyl dicarbonate to afford **7**,[20] which was coupled with PEG in the presence of DCC leading to the formation of PEG-bound BOC–aniline **8**.[21] Intermediate **8** was characterized by FT-IR and ^{1}H NMR. The loading level of the BOC–aniline group was 0.19–0.32 mmol/g determined by ^{1}H NMR integral method[22] using ethanol as an internal standard. It is stable to storage at ambient conditions. Subsequent cleavage of the BOC group under classical condition (TFA/CH_2Cl_2) afforded **9**, quantitatively. The removal of BOC group was confirmed by the complete disappearance of the methyl signal in the ^{1}H NMR spectrum. The loading yield of aniline group on PEG was also estimated by ^{1}H NMR integration method.

In addition to the scaffold of aniline **9**, another two important intermediates, 1-ethyl-2,3,3-trimethylindolenium-5-sulfonate **13** and 1-(ε-carboxypentynyl)-2,3,3-trimethylindolenium-5-sulfonate **14**, were prepared for the synthesis of cyanine dyes. Indolenium-5-sulfonate **11** was prepared from *p*-hydrazinobenzenesulfonic acid **10** and 3-methyl-2-butanone by conventional Fisher indole synthesis (Scheme 3).[23] The reaction of **11** and KOH gave 2,3,3-trimethylindolenium-5-sulfonic potassium salt **12**. And then **13** was obtained by refluxing **12** in large excess of ethyl iodide. The indolium salt **14** was prepared by the reaction of **12** with 6-bromohexanoic acid in dichlorobenzene at 110 °C for 48 h in 81% yield.

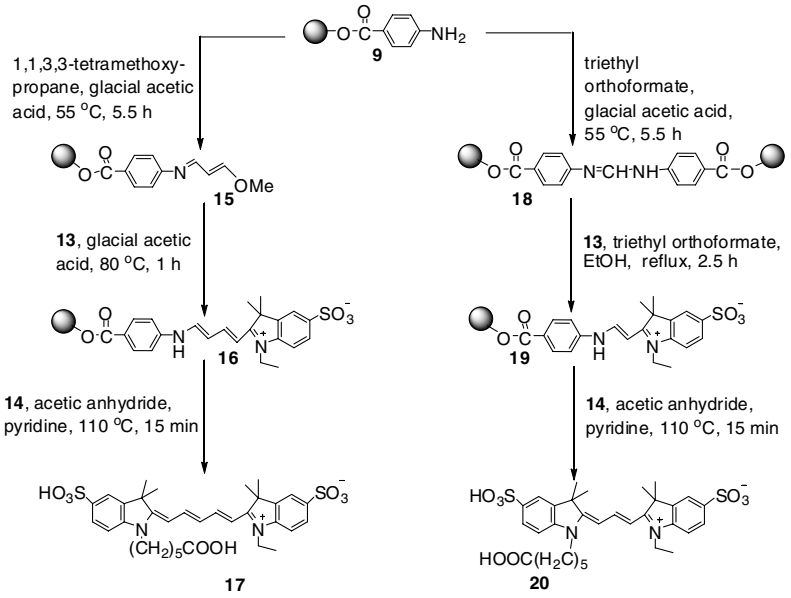


Scheme 2. Preparation of supported aniline on PEG.



Scheme 3. Synthesis of sulfonated heterocycles.

Our strategy for the polymer-supported synthesis of unsymmetrical water-soluble cyanine dyes is described in Scheme 4. The reaction of PEG-bound aniline **9** with 1,1,3,3-tetramethoxypropane or triethyl orthoformate in glacial acetic acid gave product PEG-bound-4-(3-methoxyallylideneamino)benzonic acid ester **15**[24] or PEG-bound formamidine **18**,[25] respectively. The structural difference between **15** and **18** was confirmed by ^{1}H NMR spectra. In the ^{1}H NMR spectrum of **15**, the signals at δ 8.06 (m, 3H), 7.85 (d, 1H), 7.11 (d, 2H), 6.65 (dd, 1H) indicated that a phenyl and three methine groups exist in molecule **15**. However, three main peaks at δ 8.53 (1H), 8.12 (4H) and 7.26 (4H) in the ^{1}H NMR spectrum of **18** showed that **18** has a symmetric structure in which hydrogen atom connected with nitrogen atom can move to another nitrogen atom by tautomerization. Subsequent reaction of **15** with heterocyclic carbon nucleophile **13** resulted in the formation of PEG-bound tetramethine hemicyanine dye **16**.[26] However, it was not successful in preparing similar PEG-bound dimethine hemicyanine **19** using the same conditions. A variety of solvents, temperatures, and reaction time for **19** were investigated. It was found that the reaction in ethanol with reflux gave by far the best result.[27] In fact, **16** or **19** could also react with **13** to give the byproducts (symmetrical dyes). However, the byproducts could be minimized by using equivalent **13** and appropriate reaction time. And the small quantity of symmetrical dyes could be precipitated with ethyl acetate, while the PEG-bound hemicyanine could be dissolved completely in it at 25 °C. The removal of the byproducts was confirmed by thin-layer chromatography (TLC).[28] Although a small quantity of unreacted **15** or **18** existed in the product, which did not affect the following reaction and the



Scheme 4. Polymer-supported synthesis of trimethine and pentamethine cyanine dyes.

purification of final products. The important step was to form unsymmetrical cyanine dye **17** or **20**. In this step, immobilized activated PEG-bound tetramethine hemicyanine **16** or PEG-bound dimethine hemicyanine **19** reacted with a substoichiometric quantity of the second heterocycle nuleophile **14** in a mixed solution of acetic anhydride and pyridine, so that all **14** is consumed and the unreacted hemicyanine would remain on the polymer. At the same time, the possibility of forming the symmetrical dyes would be eliminated. Finally, **17** or **20** was ably released by the attack of heterocycle **14** and the cleavage of immobilized hemicyanine **16** or **19**. When the reaction finished, the reaction mixture was cooled and the blue or red gummy product was precipitated with ethyl acetate. Because a small number of PEG-bound materials were precipitated at the same time, the gummy product was washed with DCM, which dissolved all PEG-bound materials. After being washed two or three times with DCM, pure product **17**[29] and **20**[30] were obtained in 23.7% and 21.6% yields for the final reaction, respectively, which were extremely higher than that of similar compounds synthesized by traditional solution method.[1,3] And furthermore the purity of these products were greater than 98% determined by HPLC.

A simple isolation and purification procedure for the products was achieved, thanks to the loading of PEG, thus eliminating time-consuming chromatographic separation and the use of costly equipment. The products have high purities analyzed by HPLC and TLC. These dyes are well suited to protein labeling, microscopy imaging of living cells, fluorescence detection, etc., and their succinmidyl ester derivatives for protein labelling can be readily made using published syntheses.[2]

In summary, we have developed an efficient approach to the synthesis of unsymmetric water-soluble cyanine dyes from inexpensive precursors in a few steps. This method affords products in high purity without complicated chromatographic separation, and it is suitable to synthesize unsymmetrical water-soluble cyanine dyes bearing sulfo-groups. Further utilization of this procedure in the synthesis of other types of cyanine dyes, including heptamethine cyanine dyes and other hydrophilic heterocyclic compounds, is in progress in our laboratory.

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Supplementary data

Full experimental details for **7**–**14**, details for resins loading and spectroscopic data for all compounds. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2007.06.060.

References and notes

1. Mujumdar, S. R.; Mujumdar, R. B.; Grant, C. M.; Waggoner, A. S. *Bioconjugate Chem.* **1996**, *7*, 356–362.
2. Mujumdar, R. B.; Ernst, L. A.; Mujumdar, S. R.; Lewis, C. J.; Waggoner, A. S. *Bioconjugate Chem.* **1993**, *4*, 105–111.
3. Lin, Y.; Weissleder, R.; Tung, C.-H. *Bioconjugate Chem.* **2002**, *13*, 605–610.
4. Kim, J. S.; Kodagahlly, R.; Strekowski, L.; Potanoy, G. *Talanta* **2005**, *67*, 947–954.
5. Toutchkine, A.; Nalbant, P.; Hahn, K. M. *Bioconjugate Chem.* **2002**, *13*, 387–391.

6. Thompson, L. A.; Ellman, J. A. *Chem. Rev.* **1996**, *96*, 555–600.
7. Krchňák, V.; Holladay, M. W. *Chem. Rev.* **2002**, *102*, 61–91.
8. Balkenhohl, F.; Bussche-Hunnefeld, C. V. D.; Lansky, A.; Zechel, C. *Angew. Chem., Int. Ed.* **1996**, *35*, 2288–2337.
9. El-Fayyoumy, S.; Mansour, W.; Todd, M. H. *Tetrahedron Lett.* **2006**, *47*, 1287–1290.
10. Seneci, P. *Solid-Phase Synthesis and Combinatorial Technologies*; John Wileys & Sons: New York, 2000.
11. Liu, Z.; Ruan, X.; Huang, X. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2505–2507.
12. Kilburn, J. P.; Lau, J.; Jones, R. C. F. *Tetrahedron* **2002**, *58*, 1739–1743.
13. Li, W.; Chen, Y.; Lam, Y. *Tetrahedron Lett.* **2004**, *45*, 6545–6547.
14. Mason, S. J.; Balasubramanian, S. *Org. Lett.* **2002**, *4*, 4261–4264.
15. Mason, S. J.; Hake, J. L.; Nairne, J.; Cummins, W. J.; Balasubramanian, S. *J. Org. Chem.* **2005**, *70*, 2939–2949.
16. Jung, M. E.; Kin, W.-J. *Bioorg. Med. Chem.* **2006**, *14*, 92–97.
17. Isacsson, J.; Westman, G. . *Tetrahedron Lett.* **2001**, *42*, 3207–3210.
18. Merrington, J.; James, M.; Bradley, M. *Chem. Commun.* **2002**, *2*, 140–141.
19. Gravert, D. J.; Janda, K. D. *Chem. Rev.* **1997**, *97*, 489–509.
20. Vigroux, A.; Bergon, M.; Zedde, C. *J. Med. Chem.* **1995**, *38*, 3983–3994.
21. Huh, K. M.; Bae, Y. H. *Polymer* **1999**, *40*, 6147–6155.
22. Talebpour, Z.; Haghgoo, S.; Shamsipur, M. *Anal. Chim. Acta* **2004**, *506*, 97–104.
23. Illy, H.; Funderburk, L. *J. Org. Chem.* **1968**, *33*, 4283–4285.
24. Preparation of PEG-bound-4-(3-methoxyallylideneamino)benzoic acid ester (**15**): A mixture of **9** (2.02 g, 0.90 mmol), 1,1,3,3-tetramethoxypropane (3.7 ml, 22.2 mmol) and glacial acetic acid (9 ml) was stirred and heated at 55 °C for 5.5 h. And then 30 ml cold diethyl ether was added to the solution with vigorous stirring to produce a dark yellow precipitate, which was filtered and collected. The precipitate was dissolved in dichloromethane (DCM) and precipitated with diethyl ether for further purification. Yield 1.56 g (73.0%). IR (KBr): 3435, 2885, 1704, 1639, 1601, 1465, 1114, 1060 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 8.10–8.02 (m, 3H), 7.85 (d, 1H, $J = 8.3$ Hz), 7.11 (d, 2H, $J = 8.2$ Hz), 6.65 (dd, 1H, $J = 2.7$, 8.3 Hz), 4.46–3.33 (m, PEG).
25. Preparation of PEG-bound formamidine (**18**): A mixture of **9** (3.02 g, 1.35 mmol), triethyl orthoformate (10 ml, 60 mmol) and glacial acetic acid (27 ml) was stirred and heated at 55 °C for 5.5 h. And then 50 ml cold diethyl ether was added to the solution with vigorous stirring to produce a yellow precipitate, which was filtered and collected. The precipitate was dissolved in DCM and precipitated with diethyl ether for further purification. Yield 2.43 g (80.1%). IR (KBr): 3430, 2881, 1701, 1638, 1604, 1534, 1111 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 8.53 (s, 1H), 8.12 (d, 4H, $J = 8.1$ Hz), 7.26 (d, 4H, $J = 8.1$ Hz), 4.50–3.39 (m, PEG).
26. Preparation of PEG-bound tetramethine hemicyanine (**16**): A mixture of **15** (0.810 g, 0.34 mmol), **13** (0.090 g, 0.34 mmol) and glacial acetic acid (7.5 ml) was stirred and heated at 80 °C under nitrogen atmosphere for 1 h. And then 30 ml cold diethyl ether was added to the solution with vigorous stirring to produce a brown precipitate, which was filtered and collected. The precipitate was dissolved in methanol and precipitated again with diethyl ether for further purification. At this stage, a little of symmetrical dye was existed in the product. And then the brown precipitate was dissolved in ethyl acetate. The PEG-bound tetramethine hemicyanine could be dissolved completely in ethyl acetate at 25 °C, while the symmetrical dye could not be dissolved in it. After the symmetrical dye was filtered, the ethyl acetate layer was concentrated under reduced pressure and then 30 ml cold diethyl ether was added to the solution with vigorous stirring. And then **16** was filtered and collected. Yield 0.812 g (83.9%). IR (KBr): 3440, 2885, 1704, 1627, 1599, 1467, 1173, 1113, 1061, 1027 cm^{-1}.
27. Preparation of PEG-bound dimethine hemicyanine (**19**): A mixture of **18** (2.35 g, 1.05 mmol), **13** (0.28 g, 1.05 mmol), triethyl orthoformate (0.34 ml, 2.08 mmol) and ethanol (5 ml) was stirred and heated to reflux under nitrogen atmosphere for 2.5 h. And then 50 ml cold diethyl ether was added to the mixture with vigorous stirring to produce a yellow precipitate, which was filtered and collected. The precipitate was dissolved in methanol and precipitated again with diethyl ether for further purification. At this stage, a little of symmetrical dye was existed in the product.
And then the yellow precipitate was dissolved in ethyl acetate. The PEG-bound dimethine hemicyanine could be dissolved completely in ethyl acetate at 25 °C, while the symmetrical dye could not be dissolved in it. After the symmetrical dye was filtered, the ethyl acetate layer was concentrated under reduced pressure and then 30 ml cold diethyl ether was added to the solution with vigorous stirring. And then **19** was filtered and collected. Yield 1.83 g (69.3%). IR (KBr): 3435, 2883, 1697, 1633, 1604, 1528, 1175, 1114 cm^{-1}.
28. Thin layer chromatography (TLC) was performed on silica gel plates (GF_{254}), which was developed with a mixture of *n*-butanol/acetic acid/water (v/v/v: 4/1/2).
29. Cleavage of PEG-bound hemicyanine and formation of Cy5 (**17**): A mixture of PEG-bound tetramethine hemicyanine **16** (0.501 g, 0.18 mmol), 1-(ε-carboxypentynyl)-2,3,3-trimethylindolenium-5-sulfonate **14** (0.033 g, 0.09 mmol), acetic anhydride (Ac_2O) (2 ml) and pyridine (1 ml) was stirred and heated at 110 °C under nitrogen atmosphere for 15 min. The mixture was cooled and the blue dye was precipitated with ethyl acetate. The gummy product **17** was washed with DCM until the blue free powder was obtained. Yield 0.014 g (23.7%). ^1H NMR (300 MHz, D_2O): δ 7.86–7.70 (m, 6H, 4-H, 4′-H, 6-H, 6′-H, β, β′ protons of the bridge), 7.27–7.25 (m, 2H, 7-H, 7′-H), 6.38 (dd, 1H, γ proton of the bridge, $J = 12.6$, 12.5 Hz), 6.11–6.00 (m, 2H, α, α′ proton of the bridge), 4.00–3.95 (m, 4H, α-, α′-CH_2), 2.18 (t, 2H, –CH_2COOH, $J = 7.0$ Hz), 1.72–1.27 (m, 21H, 3CH_2 groups, 1CH_3 and 2 $(CH_3)_2$ groups). ^{13}C NMR (300 MHz, D_2O): δ 183.1, 173.9, 173.7, 154.3, 144.2, 143.7, 141.9, 141.8, 139.4, 139.3, 126.6, 119.8, 111.1, 110.9, 104.0, 103.8, 49.1, 49.0, 44.1, 39.4, 37.1, 26.8, 26.7, 26.1, 25.6, 11.7. MALDI-TOF-MS: *m*/*z* for $C_{33}H_{40}S_2O_8N_2$ $[M]^+$ calcd 656.81. Found: 656.86. $[M+Na]^+$ calcd 679.80. Found: 679.90. HPLC (methanol–water) $t_R = 1.98$ min, purity >98%.
30. Cleavage of PEG-bound hemicyanine and formation of Cy3 (**20**): A mixture of PEG-bound dimethine hemicyanine **19** (0.592 g, 0.24 mmol), 1-(ε-carboxypentynyl)-2,3,3-trimethylindoleninium-5-sulfonate **14** (0.038 g, 0.11 mmol), acetic anhydride (Ac_2O) (2 ml) and pyridine (1 ml) was stirred and heated at 110 °C under nitrogen atmosphere for 15 min. The mixture was cooled and the red dye was precipitated with ethyl acetate. The gummy product **20** was washed with DCM until the red free

powder was obtained. Yield 0.015 g (21.6%). ^{1}H NMR (300 MHz, D_2O): δ 8.31 (t, 1H, β proton of the bridge, $J = 13.9$ Hz), 7.73–7.64 (m, 4H, 4-H, 4′-H, 6-H, 6′-H), 7.18–7.15 (m, 2H, 7-H, 7′-H), 6.22–6.17 (m, 2H, α, α′ proton of the bridge), 3.94–3.89 (m, 4H, α-, α′-CH_2), 2.03 (t, 2H, –CH_2COOH, $J = 7.1$ Hz), 1.64–1.15 (m, 21H, 3CH_2 groups, 1CH_3 and 2$(CH_3)_2$ groups). ^{13}C NMR (300 MHz, D_2O): δ 183.5, 175.8, 175.5, 151.9, 144.1, 143.7, 141.7, 141.5, 139.7, 139.60, 126.8, 119.8, 111.5, 111.3, 103.4, 49.3, 44.3, 39.6, 37.3, 27.1, 27.0, 26.6, 26.1, 25.6, 11.9. MALDI-TOF-MS: m/z for $C_{31}H_{38}S_2O_8N_2$ $[M+Na]^+$ calcd 653.20. Found: 653.09. HPLC (methanol) $t_R = 2.19$ min, purity >98%.