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Practical syntheses of dyes for difference gel electrophoresis

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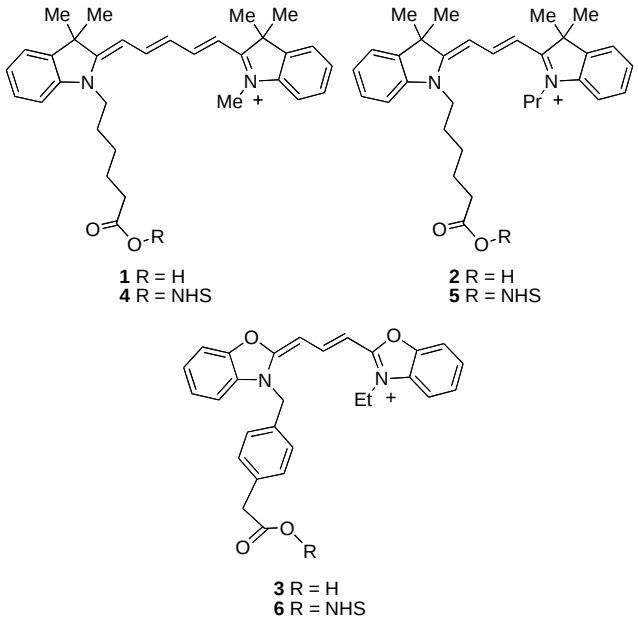
Abstract—The three dyes (two indocyanine and one benzoxazolium) useful in difference gel electrophoresis—methyl Cy5 **1**, propyl Cy3 **2**, and the benzoxazolium dye Cy2 **3**—and their NHS esters have been prepared by efficient routes in good overall yield from inexpensive precursors.

1. Introduction

The use of fluorescent dyes in biology is ever-increasing and serves as the basis for many advancements in science;[1] for example, the sequencing of the human genome. Fluorescent two-dimensional difference gel electrophoresis is a relatively new technique for the multiplex quantitative analysis of the component proteins of related, but different, protein samples.[2] This technique has been quite useful in solving problems related to comparing different protein samples by two-dimensional gel electrophoresis. The application of this technique requires a supply of fluorescent dyes with different absorption characteristics activated for attachment to the proteins of interest. Usually, this signifies a group of several indocyanine dyes, although recently other dyes have also been used, in particular, the three dyes methyl Cy5 **1**, propyl Cy3 **2**, and the benzoxazolium dye Cy2 **3** (Scheme 1).[3] The dyes are generally converted into their *N*-hydroxysuccinimide esters **4**–**6** for attachment to the proteins. Although these dyes are well-known and even commercially available,[4] there is no detailed experimental procedure for their efficient synthesis. We report here detailed procedures for the synthesis of these three difference gel electrophoresis (DIGE) dyes from relatively inexpensive precursors.



Scheme 1. Dyes methyl Cy5, propyl Cy3, and Cy2.

2. Results and discussion

For the synthesis of the methyl Cy5 dye **1** and its NHS ester **4** (Scheme 2), the three-carbon spacer **7** had to be prepared. Condensation of commercially available malondialdehyde bis(dimethyl acetal) with aniline under acidic conditions afforded the anilino anilinium salt **7** in 85% yield.[5] The reaction of **7** with the commercially available 1,3,3-trimethyl-2-methyleneindoline **8** in refluxing acetic acid afforded the anilinobutadienyl salt **9** in 66% yield.[6] The second component of the dye, the indolium salt **11**, was prepared by alkylation of commercially available 2,3,3-trimethyl-3*H*-indole **10** with 6-bromohexanoic acid in dichlorobenzene at 110 °C for 12 h to give 67% yield of **11**.[7] Finally, the reaction of

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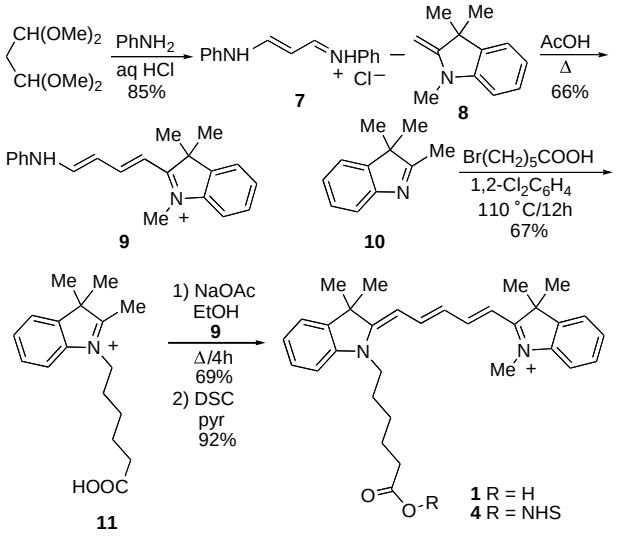
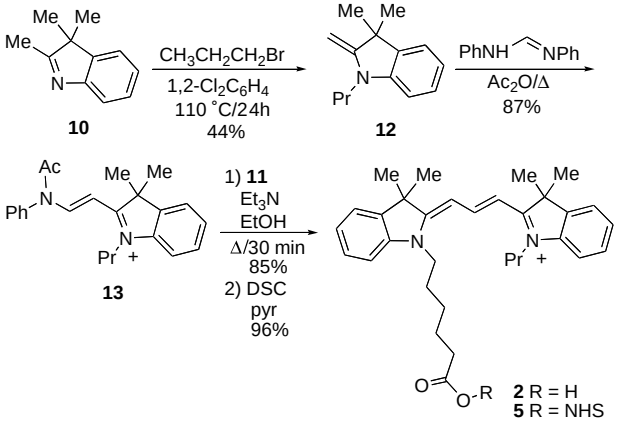


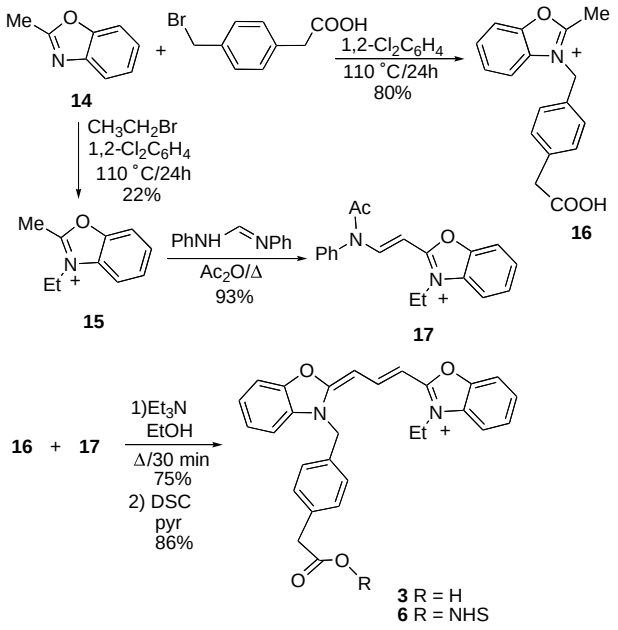
Scheme 2. Synthesis of dye methyl Cy5 and its NHS ester.

the activated indolium salt **9** with the methylindolium salt **11** in ethanol in the presence of sodium acetate afforded the desired dye **1** in 69% yield as a blue powder. The dye was easily converted into the *N*-hydroxysuccinimide ester (NHS) by treatment with commercially available *N*,*N*′-disuccinimidyl carbonate (DSC) in the presence of pyridine to give the activated dye **4** in 92% yield. All spectroscopic data, especially high field NMR and mass spectrometry, were in agreement with the structures assigned.[8]

The synthesis of the propyl Cy3 dye **2** and its NHS ester **5** (Scheme 3) began with the alkylation of the trimethylindole **10** with propyl bromide to give the 1-propyl-2-methyleneindoline **12**[9] in an unoptimized yield of 44%. Condensation with commercially available *N*,*N*′-diphenylformamidine in the presence of excess acetic anhydride as solvent afforded the acetanilidylvinyl indolium salt **13** in 87% yield. This compound was then reacted with the same methylindolium salt **11** in ethanol in the presence of triethylamine to give the desired dye **2** in 85% yield as a red powder.[10] The dye was easily converted into the *N*-hydroxysuccinimide ester (NHS) by treatment



Scheme 3. Synthesis of dye propyl Cy3 and its NHS ester.



Scheme 4. Synthesis of the benzoxazolium dye Cy2 and its NHS ester.

with *N*,*N*′-disuccinimidyl carbonate (DSC) in the presence of pyridine to give the activated dye **5** in 96% yield. Again, all the pertinent spectroscopic data, especially high-field NMR and mass spectrometry, were in agreement with the structures assigned.[8]

Finally, the synthesis of the Cy2 dye, the benzoxazolium dye **3**, and its NHS ester **6** (Scheme 4) required completely different coupling components. Thus, the commercially available 2-methylbenzoxazole **14** was alkylated with two different alkyl halides: alkylation with ethyl bromide gave the ethyl salt **15**[11] in 22% yield (this compound is also commercially available), while alkylation with the commercially available 4-bromomethylphenylacetic acid gave an 80% yield of the salt **16**.[12] Condensation of the ethyl salt with diphenylformamidine in the presence of excess acetic anhydride as solvent afforded the acetanilidylvinyl indolium salt **17** in 93% yield.[13] This compound was then reacted with the other benzoxazolium salt **16** in ethanol in the presence of triethylamine to give the desired dye **3** in 75% yield as a deep yellow powder. The dye was easily converted into the *N*-hydroxysuccinimide ester (NHS) by treatment with *N*,*N*′-disuccinimidyl carbonate (DSC) in the presence of pyridine to give the activated dye **6** in 86% yield. The spectroscopic data were in agreement with the structures assigned.[8]

3. Conclusion

We have thus developed simple practical methods for the synthesis of the three dyes—methyl Cy5 **1**, propyl Cy3 **2**, and Cy2 **3**—from inexpensive precursors in only a few steps and reasonable overall yields. The synthesis of each dye requires only 4 steps from commercially available materials and generally the yields are quite high, especially for the coupling of the components.

The utility of these dyes for DIGE is currently under investigation and will be reported shortly.[14]

4. Experimental

4.1. General information

All reactions were carried out under an atmosphere of nitrogen and all commercial reagents were used directly. ^{1}H and ^{13}C NMR NMR data were obtained on a Bruker 400 MHz spectrometer. ^{1}H NMR and ^{13}C NMR data are reported in parts per million (δ) downfield from tetramethylsilane. The following abbreviations are used: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). Infrared spectra were recorded on a Thermo Nicolet Avatar 370 FT infrared spectrophotometer as a liquid film or as a thin crystalline film. All IR data are reported in wavenumbers (cm^{-1}). Thin-layer chromatography (TLC) was performed using Merck silica gel 60 F254 0.2 mm alumina-backed plates. Visualization was accomplished using ultraviolet light or one of the following stains: anisaldehyde, phosphomolybdic acid, and potassium permanganate. Flash chromatography was carried out using ICN Biomedicals silica gel 60 (230–400 mesh).

4.1.1. *N*-((1*E*)-3-(Phenylimino)prop-1-enyl)benzenamine hydrochloride 7. A solution of distilled water (140 mL), HCl (10 mL), and aniline (7.4 mL, 0.08 mol) was added dropwise to a solution of distilled water (171 mL), HCl (8.5 mL), and malondialdehyde bis(dimethyl acetal) (10.5 mL, 0.06 mol) with stirring at 50 °C. The precipitate was isolated by filtration to give malondialdehyde dianil hydrochloride **7** (12 g, 85%) as an orange powder. [5] IR (neat): 3425, 1642, 1620, 1580, 1492, 1343, 1273, 1194, 749, 683 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.72 (2H, d, J = 13.2 Hz), 8.92 (2H, t, J = 12.4 Hz), 7.40 (8H, m), 7.20 (2H, m), 6.50 (1H, t, J = 11.5 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 158.89, 139.18, 130.33, 126.31, 117.86, 99.09.

4.1.2. 2-(4-Phenylamino-1*E*,3*E*-butadien-1-yl)-1,3,3-trimethylindolium chloride 9. A mixture of malondialdehyde dianil hydrochloride **7** (1 g, 4.49 mmol) and 1,3,3-trimethyl-2-methyleneindoline **8** (0.93 mL, 4.49 mmol) in glacial acetic acid (10 mL) was refluxed for 4 h. The solution was cooled to room temperature, the acetic acid was removed under reduced pressure, and the residue was purified by flash chromatography on silica gel (dichloromethane/hexane/methanol = 5:1:1) to give the product **9** as a red powder (0.9 g, 66%).[6] ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.18 (1H, d,J = 9.8 Hz), 7.52 (1H, t, J = 13.3 Hz), 6.74–7.36 (9H, m), 6.48 (1H, dd, J = 14.1, 9.9 Hz), 5.55 (1H, d, J = 12.5 Hz), 3.24 (3H, s), 1.55 (6H, s). ^{13}C NMR (100 MHz, DMSO-d_6): δ 129.41, 129.17, 128.04, 125.24, 121.71, 121.36, 120.41, 118.18, 107.27, 46.68, 28.38.

4.1.3. 1-(5-Carboxypentyl)-2,3,3-trimethyl-3*H*-indolium bromide 11. A mixture of 2,3,3-trimethyl-3*H*-indole **10** (0.2 g, 1.25 mmol) and 6-bromohexanoic acid (0.36 g, 1.80 mmol) in 1,2-dichlorobenzene was heated at 110 °C for 12 h. The solution was cooled to room temperature, and the residue obtained was filtered and washed with a mixture of acetonitrile/diethyl ether (1/1). The solid obtained was dried under vacuum to give the product **11** as a light red powder (2.3 g, 67%).[7] IR (neat): 3405, 2936, 1724, 1624, 1460, 1392, 1168, 767 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.93–7.97 (1H, m), 7.79–7.87 (1H, m), 7.56–7.64 (2H, m), 4.43 (2H, t, J = 7.7 Hz), 2.82 (3H, s), 2.19 (2H, t, J = 7.2 Hz), 1.81 (2H, m), 1.52 (2H, m), 1.50 (6H, s), 1.35 (2H, m). ^{13}C NMR (100 MHz, DMSO d_6): δ 196.98, 174.77, 142.33, 141.51, 129.85, 129.40, 123.99, 115.97, 54.62, 47.90, 33.83, 27.41, 25.87, 24.48, 22.47, 14.51.

4.1.4. 2-[5-[1-(5-Carboxypentyl)-1,3-dihydro-3,3-dimethyl-2*H*-indol-2-ylidene]-1,3-pentadien-yl]-1,3,3-trimethyl-3*H*-indolium, Methyl Cy5, 1. A solution of the anil **9** (0.20 g, 0.65 mmol), the acid 11 (0.18 g, 0.65 mmol), and anhydrous sodium acetate (0.11 g, 0.79 mmol) in absolute ethanol (50 mL) under nitrogen was refluxed for 4 h. The solid was purified by flash chromatography on silica gel (dichloromethane/methanol = 5:1) to give the methyl Cy5 dye **1** (0.22 g, 69%) as a blue powder.[4] IR (neat): 3406, 2925, 1716, 1575, 1470, 1425, 1371, 1335, 1217, 1146, 1016, 1040, 923, 796, 750, 708 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.30 (2H, t, J = 13.1 Hz), 7.58 (2H, d, J = 7.4 Hz), 7.34 (4H, m), 7.21 (2H, m), 6.53 (1H, t, J = 12.3 Hz), 6.25 (2H, dd, J = 13.8, 13.9 Hz), 4.06 (2H, t, J = 7.2 Hz), 3.56 (3H, s), 2.15 (2H, t, J = 7.2 Hz), 1.69 (2H, m), 1.64 (12H, s), 1.52 (2H, m), 1.32 (2H, m). ^{13}C NMR (100 MHz DMSO-d_6): δ 175.03, 173.69, 172.95, 154.47, 143.21, 142.47, 141.54, 141.47, 128.80, 125.83, 125.16, 125.05, 122.89, 122.76, 111.49, 103.77, 103.51, 55.39, 49.30, 43.67, 34.16, 31.56, 27.61, 27.44, 27.15, 26.12, 24.73.

4.1.5. 2-[5-(1,3-Dihydro-1,3,3-trimethyl-2*H*-indol-2-ylidene)-1,3-pentadienyl]-1-[6-[(2,5-dioxo-1-pyrrolidi-nyl)oxy]-6-oxohexyl]-3,3-dimethyl-3*H*-indolium, Methyl Cy5-NHS ester, 4. Anhydrous pyridine (0.1 mL) and *N*,*N*′-disuccinimidyl carbonate (DSC, 23.6 mg, 0.09 mmol) were added to a stirred solution of the acid **1** (0.36 g, 0.06 mmol) in dry DMF (2 mL) under nitrogen. The reaction mixture was stirred at 60 °C for 1.5 h. After evaporation of the solvent, the deep blue residue was purified by column chromatography on silica gel (dichloromethane/hexane/methanol = 5:1:1) to give the pure NHS ester of the dye **4** (0.33 g, 92%) as a blue powder.[3,4b] IR (neat): 2926, 1733, 1496, 1481, 1456, 1372, 1336, 1217, 1182, 1149, 1097, 1040, 1016, 924, 796, 756, 708, 668 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.30 (2H, t, J = 13.1 Hz), 7.58 (2H, d, J = 7.4 Hz), 7.36 (4H, m), 7.20 (2H, m), 6.53 (1H, t, J = 12.4 Hz), 6.25 (2H, t, J = 14.2 Hz), 4.05 (2H, t, J = 7.2 Hz), 3.56 (3H, s), 2.77 (4H, s), 2.64 (2H, t, J = 7.2 Hz), 1.68 (2H, m), 1.65 (2H, m), 1.64 (12H, s), 1.44 (2H, m). ^{13}C NMR (100 MHz, DMSO-d_6): δ 173.74, 173.26, 172.93, 170.71, 169.35, 154.49, 151.10, 143.21, 142.44, 141.54, 141.47, 129.11, 128.81, 125.83, 125.19, 125.05, 122.87, 122.76, 111.50, 103.44, 49.31, 49.03, 43.63, 31.54,

30.44, 27.61, 27.43, 26.84, 25.90, 25.66, 24.37. MS (EI): *m*/*z* (%) = 580 (100).

4.1.6. 3,3-Dimethyl-2-methylene-1-propylindoline 12. A mixture of 2,3,3-trimethyl-3*H*-indole **10** (0.2 g, 0.01 mol) and 1-bromopropane (2.28 mL, 0.02 mol) in 1,2-dichlorobenzene was heated at 110 °C for 24 h. The solution was cooled to room temperature, and the residue obtained was filtered and washed with a mixture of acetonitrile/diethyl ether (1/1). The solid obtained was dried under vacuum to give the 3,3-dimethyl-2-methylene-1-propylindoline **12** as a light red powder (0.11 g, 44%).[8] IR (neat): 2966, 2925, 1617, 1601, 1474, 1454, 1356, 1290, 1119, 931, 767 cm^{-1}. ^1H NMR (400 MHz, D_2O): δ 7.66 (1H, m), 7.60 (1H, m), 7.45–7.50 (2H, m), 4.65 (2H, s), 4.30 (2H, t, J = 7.4 Hz), 1.86 (2H, m), 1.42 (6H, s), 0.87 (3H, t, J = 7.4 Hz). ^{13}C NMR (100 MHz, D_2O): δ 141.77, 140.92, 129.75, 128.99, 123.26, 115.15, 54.33, 49.20, 21.72, 20.85, 10.18.

4.1.7. 2-(2-Phenylacetamido-*E*-1-ethenyl)-3,3-dimethyl-1-propylindolium salt 13. A mixture of *N*,*N*′-diphenylformamidine (0.35 g, 1.78 mmol) and 3,3-dimethyl-2-methylene-1-propylindoline **12** (0.30 g, 1.48 mmol) in acetic anhydride (10 mL) was refluxed for 30 min. The solution was cooled to room temperature, the solvent was removed under reduced pressure, and the residue was purified by flash chromatography on silica gel (dichloromethane/hexane/methanol = 5:1:1) to give the salt **13** as a light yellow powder (0.45 g, 87%). IR (neat): 2965, 2926, 1680, 1638, 1603, 1580, 1553, 1492, 1369, 1311, 1200, 1130, 996, 757 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 9.10 (1H, d, J = 14.2 Hz), 7.05–7.70 (9H, m), 5.34 (1H, d, J = 14.2 Hz), 4.06 (2H, t, J = 7.1 Hz), 2.05 (3H, s), 1.70 (6H, s), 1.60 (2H, m), 0.67 (3H, t, J = 7.4 Hz). ^{13}C NMR (100 MHz $CDCl_3$): δ 162.80, 154.24, 142.85, 139.56, 129.48, 128.41, 125.42, 123.32, 121.96, 119.42, 109.13, 94.35, 47.84, 45.19, 29.26, 27.86, 20.15, 11.45.

4.1.8. 2-[3-[1-(5-Carboxypentyl)-1,3-dihydro-3,3-dimethyl-2*H*-indol-2-ylidene]-1-propenyl]-3,3-dimethyl-1-propyl-3*H*-indolium, Propyl Cy3, 2. A mixture of the salt **11** (0.08 g, 0.28 mmol), enamide **13** (0.10 g, 0.28 mmol), and dry triethylamine (0.1 mL) in absolute ethanol (10 mL) was refluxed for 30 min. The solvent was removed under reduced pressure and the crude residue was purified by flash chromatography on silica gel (dichloromethane/hexane/methanol = 5:1:1) to give the dye propyl Cy3 **2** as a deep red powder (0.12 g, 85%).[9] IR (neat): 3407, 2969, 2934, 2876, 2734, 2673, 1557, 1453, 1427, 1242, 1192, 1130, 1030, 930, 754 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 8.31 (1H, dd, J = 13.4, 13.5 Hz), 7.61 (2H, d, J = 7.4 Hz), 7.36–7.47 (4H, m), 7.26 (2H, t, J = 7.1 Hz), 6.54 (1H, d, J = 13.5 Hz), 6.53 (1H, d, J = 13.4 Hz), 4.08 (2H, t, J = 7.2 Hz), 3.43 (2H, t, J = 7.2 Hz), 2.14 (2H, t, J = 7.2 Hz), 1.75 (2H, m), 1.66 (12H, s), 1.52 (2H, m), 1.40 (2H, m), 1.19 (2H, m), 0.84 (3H, t, J = 7.4 Hz). ^{13}C NMR (100 MHz DMSO-d_6): δ 174.20, 161.01, 146.45, 143.11, 141.37, 137.32, 129.11, 127.92, 126.13, 122.98, 122.21, 118.45, 105.73, 102.72, 74.11, 49.36, 44.06, 43.30, 30.26, 27.94, 27.90, 26.26, 20.89, 19.44, 11.79, 11.46.

4.1.9. 2-[3-(1,3-Dihydro-3,3-dimethyl-1-propyl-2*H*-indol-2-ylidene)-1-propenyl]-1-[6-[(2,5-di-oxo-1-pyrro-lidinyl)oxy]-6-oxohexyl]-3,3-dimethyl-3*H*-indolium, Propyl Cy3-NHS ester, 5. Anhydrous pyridine (0.1 mL) and *N*,*N*′-disuccinimidyl carbonate (DSC, 21 mg, 0.08 mmol) were added to a stirred solution of the dye **2** (27 mg, 0.05 mmol) in dry DMF (2 mL) under nitrogen. The reaction mixture was stirred at 60 °C for 1.5 h. After evaporation of the solvent, the deep red residue was purified by column chromatography on silica gel (dichloromethane/hexane/methanol = 5:1:1) to give the pure propyl Cy3-NHS ester **5** (31 mg, 96%) as a red powder.[3,4b] IR (neat): 2924, 2853, 1737, 1555, 1456, 1428, 1371, 1248, 1196, 1158, 1116, 1019, 930, 796, 579, 680 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 8.32 (1H, dd, J = 13.4, 13.4 Hz), 7.61 (2H, d, J = 7.6 Hz), 7.38–7.47 (4H, m), 7.26 (2H, t, J = 7.4 Hz), 6.48 (2H, d, J = 13.5 Hz), 4.09 (4H, m), 2.74 (4H, s), 2.66 (2H, t, J = 7.2 Hz), 1.74 (2H, m), 1.69 (2H, m), 1.66 (12H, s), 1.48 (2H, m), 1.19 (2H, m), 0.95 (3H, t, J = 7.4 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 174.20, 173.40, 169.17, 168.61, 151.50, 142.30, 140.52, 129.11, 128.86, 125.04, 122.03, 111.14, 110.96, 105.17, 48.90, 46.40, 44.20, 30.70, 29.71, 28.20, 27.21, 25.82, 25.62, 24.53, 21.17, 11.30. MS (EI): *m*/*z* (%) = 582 (100), 485 (22).

4.1.10. *N*-Ethyl-2-methylbenzoxazolium bromide, 15. A mixture of 2-methylbenzoxazole **14** (2.24 g, 0.01 mol) and bromoethane (2.5 mL, 0.03 mol) in 1,2-dichlorobenzene was heated at 110 °C for 24 h. The solution was cooled to room temperature, and the residue obtained was filtered and washed with diethyl ether. The solid obtained was dried under vacuum to give the salt **15** as a white powder (0.60 g, 22%).[10] This compound is also commercially available from various suppliers. IR (neat): 3084, 3047, 2974, 2929, 2859, 2729, 1593, 1462, 1388, 1188, 1147, 1025, 759 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 8.09–8.23 (2H, m), 7.71–7.80 (2H, m), 4.60 (2H, q, J = 7.3 Hz), 3.02 (3H, s), 1.43 (2H, t, J = 7.3 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.03, 147.91, 129.73, 129.04, 128.13, 115.00, 113.42, 42.38, 13.84, 13.40.

4.1.11. *N*-[(4-Carboxymethyl)phenylmethyl]-2-methylbenzoxazolium bromide, 16. A mixture of 2-methylbenzoxazole **14** (2.24 g, 0.01 mol) and 4-(bromomethyl)phenylacetic acid (3.22 g, 0.01 mol) in 1,2-dichlorobenzene was heated at 110 °C for 12 h. The solution was cooled to room temperature, and the residue obtained was filtered and washed with acetonitrile. The solid obtained was dried under vacuum to give the salt **16** as a light yellow powder (3.20 g, 80%).[11] IR (neat): 3014, 1733, 1579, 1456, 1360, 1226, 1164, 753 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 7.13 (4H, s), 7.01 (1H, br t, J = 7.8 Hz), 6.95 (1H, br d, J = 8.1 Hz), 6.85 (1H, br d, J = 7.8 Hz), 6.68 (1H, br t, J = 7.6 Hz), 5.20 (1H, d, J = 15.0 Hz), 4.19 (1H, d, J = 15.0 Hz), 3.48 (2H, s), 3.17 (3H, s). ^{13}C NMR (100 MHz DMSO-d_6): δ 173.18, 170.49, 153.38, 136.75, 133.98, 130.15, 130.04, 129.54, 129.46, 128.38, 119.71, 117.15, 50.79, 40.84, 22.21.

4.1.12. 2-(2-Phenylacetamido-*E*-1-ethenyl)-*N*-ethylbenzoxazolium salt, 17. A mixture of *N*,*N*′-diphenylformamidine (0.38 g, 1.98 mmol) and the salt **15** (0.26 g, 1.65 mmol) in acetic anhydride (10 mL) was refluxed for 30 min. The solution was cooled to room temperature, the solvent was removed under reduced pressure, and the residue was purified by flash chromatography on silica gel (dichloromethane/hexane/methanol = 5:1:1) to give the salt **17** as a light yellow powder (0.41 g, 93%).[12] IR (neat): 3084, 3064, 2978, 1719, 1646, 1613, 1589, 1491, 1466, 1413, 1372, 1319, 1252, 1151, 1004, 755, 702 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 9.19 (1H, d, J = 13.8 Hz), 7.26–7.83 (9H, m), 5.33 (1H, d, J = 13.8 Hz), 4.44 (2H, q, J = 7.4 Hz), 2.02 (3H, s), 1.31 (3H, t, J = 7.4 Hz).^{13}C NMR (100 MHz, $CDCl_3$): δ 173.25, 169.92, 162.86, 147.68, 146.88, 138.12, 131.30, 130.86, 129.62, 128.57, 127.85, 113.93, 112.21, 87.14, 42.36, 23.45, 13.79.

4.1.13. 2-[3-[3-[4-[2-carboxyethyl]phenylmethyl]-2(3*H*)-benzoxazolylidene]-1-propenyl]-3-ethylbenzoxazolium, Cy2, 3. A mixture of the enamide **17** (0.10 g, 0.32 mmol), the salt **16** (0.92 g, 0.32 mmol), and dry triethylamine (0.1 mL) in absolute ethanol (10 mL) was refluxed for 30 min. The solvent was removed under reduced pressure and the crude residue was purified by flash chromatography on silica gel (dichloromethane/hexane/methanol = 5:1:1) to give the dye **3** as a deep yellow powder (0.11 g, 75%). IR (neat): 3408, 2927, 1710, 1609, 1565, 1508, 1461, 1394, 1347, 1280, 1201, 1154, 1116, 1083, 978, 906, 748 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 8.38 (1H, dd, J = 13.2, 13.3 Hz), 7.16–7.48 (12H, m), 5.90 (1H, d, J = 13.3 Hz), 5.88 (1H, d, J = 13.2 Hz), 5.22 (2H, s), 4.12 (2H, br q, J = 7.2 Hz), 3.51 (2H, s), 1.30 (3H, t, J = 7.2 Hz). ^{13}C NMR (100 MHz $CDCl_3$): δ 174.31, 166.37, 162.25, 161.74, 148.00, 146.88, 146.73, 131.23, 130.93, 130.58, 130.38, 127.17, 126.33, 126.23, 125.59, 125.38, 111.01, 110.86, 110.76, 110.53, 85.95, 85.26, 47.41, 45.52, 39.60, 12.98.

4.1.14. 2-[3-[3-[[4-[2-[(2,5-Dioxo-1-pyrrolidinyl)oxy]-2-oxoethyl]phenyl]methyl]-2(3*H*)-benzo-xazolylidene]-1-propenyl]-3-ethyl-benzoxazolium, Cy2-NHS ester, 6. Anhydrous pyridine (0.1 mL) and *N*,*N*′-disuccinimidyl carbonate (DSC, 21 mg, 0.08 mmol) were added to a stirred solution of the dye **3** (25 mg, 0.05 mmol) in dry DMF (2 mL) under nitrogen. The reaction mixture was stirred at 60 °C for 1.5 h. After evaporation of the solvent, the deep yellow residue was purified by column chromatography on silica gel (dichloromethane/hexane/methanol = 5:1:1) to give the pure Cy2-NHS ester **6** (26 mg, 86%) as an orange powder. IR (neat): 2924, 2851, 1736, 1565, 1507, 1461, 1395, 1348, 1280, 1201, 1115, 1082, 747 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 8.40 (1H, t, J = 13.2 Hz), 7.22–7.49 (12H, m), 6.52 (1H, d, J = 13.2 Hz), 6.40 (1H, d, J = 13.2 Hz), 5.49 (2H, s), 4.31 (2H, br q, J = 7.1 Hz), 3.85 (2H, s), 2.78 (4H, s), 1.46 (3H, t, J = 7.1 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.99, 166.51, 162.18, 161.73, 146.95, 146.82, 132.87, 132.02, 131.37, 130.82, 130.13, 128.36, 126.09, 125.28, 125.08, 110.98, 110.90, 110.76, 110.66, 87.15, 86.49, 37.19, 25.60, 13.34. MS (EI): m/z (%) = 550 (100), 453 (6), 304 (6).

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