Supplementary Material for

Probing the Sequence of Conformationally-Induced Polarity Changes in the Molecular Chaperonin GroEL with Fluorescence Spectroscopy

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Nile Red maleimide, with the thiol-reactive maleimide unit spaced from the fluorescent Nile Red chromophore by a hexamethylene chain, was prepared from a Nile Red precursor bearing a phenolic hydroxyl group at the 2-position, 9-diethylamino-2-hydroxy-5H-benzo[a]phenoxazin-5-one (Ref. 1). The overall synthesis involves

preparation of the phenol-functionalized chromophore and a protected maleimide moiety followed by connection of the two parts via the C_6 spacer and finally deprotection to create the free maleimide.

Synthesis of the Nile Red phenol involves first nitrosation of 3-diethylaminophenol to give 5-diethylamino-2-nitrosophenol that in turn is oxidatively condensed with 1,6-dihydroxynaphthalene. The protected maleimide moiety was synthesized from the Diels-Alder adduct of maleic anhydride and furan (Ref. 2). Attachment of the protected maleimide to the dye may be done by formation of the ether last (Route "a") or the imide bond last (Route "b"). The Route "a" was primarily followed and Route "b" has no distinct advantages and was performed to confirm the structure of the common Diels-Alder protected intermediate. The retro Diels-Alder reaction producing the free maleimide was performed at 140ºC and driven to completion by removal of furan.

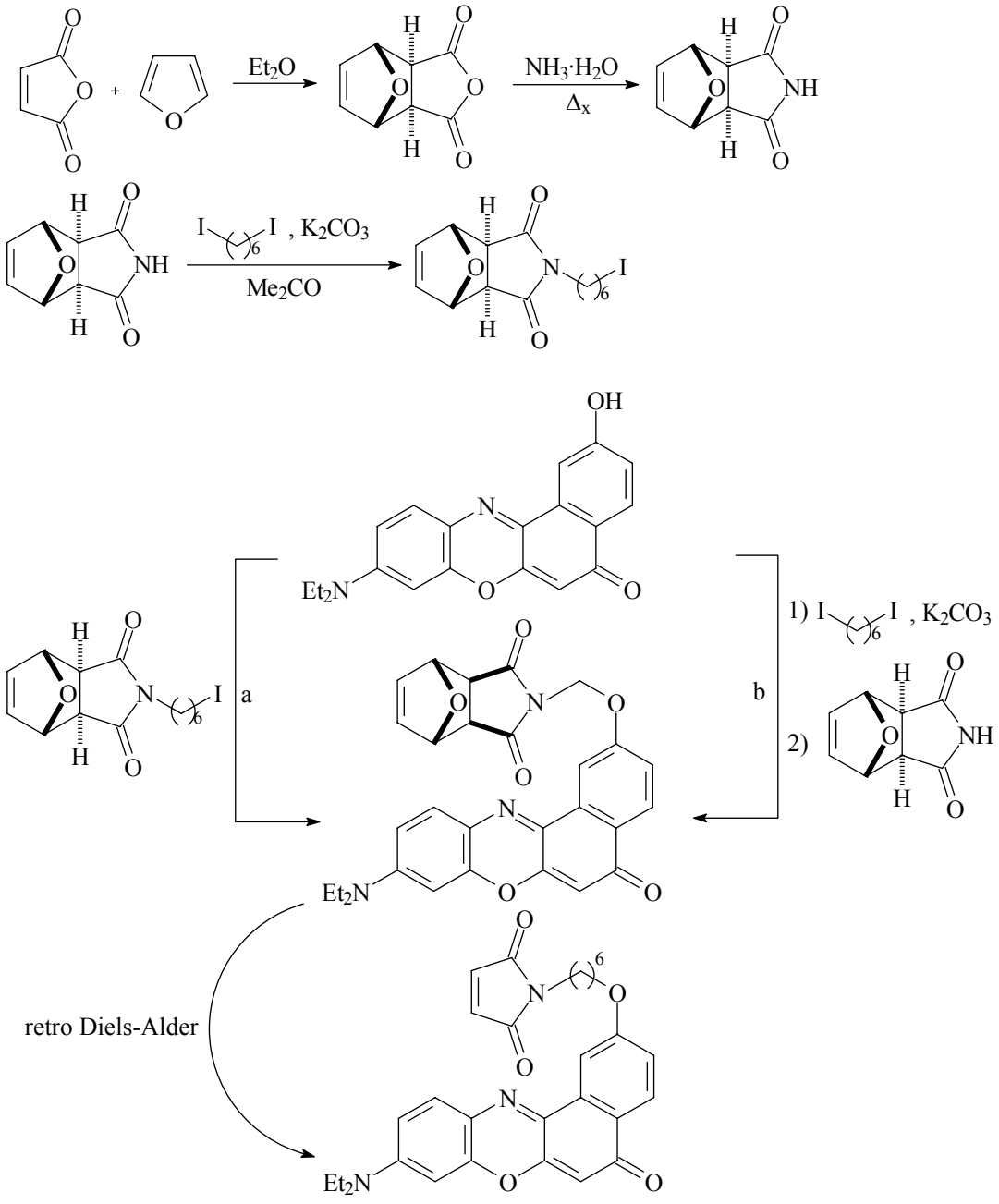

Figure S1. Nile Red maleimide synthesis. The top scheme shows the synthesis of the furan maleimide adduct and its monoalkylation with 1,6-diiodohexane. The bottom scheme shows two routes that differ only in the order of the steps to link the dye and maleimide precursor and final deprotection of the common product to the free maleimide.

9-Diethylamino-2-(6-iodohexyloxy)-5H-benzo[a]phenoxazin-5-one.

A 50 ml pear-shaped flask was charged with a stirring bar, Nile Red Phenol (9-diethylamino-2-hydroxy-5H-benzo[a]phenoxazin-5-one, 66 mg, 0.2 mmol), 1,6-diiodohexane (134 mg, 0.4 mmol), potassium hydroxide (55 mg, 0.4 mmol), and anhydrous dimethylacetamide (10 ml). The reaction mixture was stirred at 90°C for one hour (most of the starting material was consumed during first several minutes, as monitored by TLC with eluent of 1:1 hexanes and ethyl acetate). The solvent was evaporated and the residue was chromatographed on silica gel with a 1:1 mixture of hexanes and ethyl acetate to give 74 mg (79%) of the ruby red product which was used directly in next step. ^{1}H NMR ($CDCl_3$), δ 8.16 (d, 1H, J=9Hz); 7.97 (d, 1H, J=2.4Hz); 7.56 (d, 1H, J=9Hz); 7.13 (dd, 1H, J_1=9Hz, J_2=2.4Hz); 6.68 (dd, 1H, J_1=9Hz, J_2=2.4Hz); 6.43 (d, 1H, J=2.4Hz); 6.37 (s, 1H); 4.15 (t, 2H, J=6.6Hz); 3.47 (q, 4H, J=7.2Hz); 3.19 (t, 2H, J=6.6Hz); 1.84 (m, 2H); 1.54 (m, 2H); 1.42 (m, 2H); 1.25 (t, 6H, J=7.2Hz). ^{13}C NMR ($CDCl_3$), δ 182.2, 162.0, 152.1, 151.3, 147.2, 139.5, 139.4, 134.2, 131.5, 127.8, 125.8, 125.2, 118.5, 110.7, 106.7, 104.8, 96.7, 68.4, 45.6, 33.6, 29.9 (double intensity), 29.6, 12.8, 7.00.

(4-(6-iodohexyl)-10-oxa-4-azatricyclo[5.2.1.$0^{2,6}$]dec-8-ene-3,5-dione)

A 100 ml round-bottom flask equipped with a stirring bar, reflux condenser and nitrogen bubbler was charged with the furan-maleimide Diels Alder adduct (1.65 g, 0.01 mol), 1,6-diiodohexane (3.7 g, 0.011 mol), potassium carbonate (1.4 g, 0.11 mol) and acetone (35 ml). The reaction mixture was stirred at 40°C for 12 hours and the course of the

reaction was monitored by TLC (Rf = 0.47,1:1 hexane and ethyl acetate). After this time the reaction mixture was cooled, the solvent was evaporated and the residue was chromatographed with a gradient of hexane and ethyl acetate (3:1 to 1:1 mixture). Recrystallization from ethyl acetate yielded 1.0 g (26.6%) of the product as a white crystalline material, m.p. 71.5°C (DSC, 10°/min). ^{1}H NMR ($CDCl_3$), δ 6.52 (s, 1H); 5.25 (s, 1H); 3.46 (t, 2H, J=6.6Hz); 3.17 (t, 2H, J=6.6Hz); 2.84 (s, 2H); 1.8 (m, 2H); 1.56 (m, 2H); 1.45-1.26 (m, 4H). ^{13}C NMR ($CDCl_3$), δ 176.4, 136.7, 81.1, 47.6, 38.9, 33.4, 30.1, 27.5, 25.7, 7.1. Analysis calculated for $C_{14}H_{20}NO_3I$: C, 44.84; H, 4.83; N, 3.78. Found, C, 44.82; H, 4.84; N, 3.73. IR (cm^{-1}): 3022, 2998, 2937, 2896, 2854, 1693, 1404, 1373, 1349, 1286, 1249, 1199, 1172, 1147, 1094, 1026, 919, 875, 804, 721, 663, 593.

Furan protected Nile Red maleimide, 4-[6-(9-Diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)-hexyl]-10-oxa-4-aza-tricyclo[5.2.1.0^{2,6}]dec-8-ene-3,5-dione.

Route “a”

A 125 ml round-bottom flask fitted with a stirring bar and nitrogen inlet was charged with Nile Red phenol (9-Diethylamino-2-hydroxy-5H-benzo[a]phenoxazin-5-one, 370 mg, 1.1 mmol), 2-(6-iodohexyl)-3a,4,7,7a-tetrahydro-4,7-epoxy-1H-isoindole-1,3(2H)-dione (500 mg, 1.33 mmol), potassium carbonate (400 mg, 2.9 mmol), and dry dimethylformamide (3 ml). The reaction mixture was stirred at 65°C for 12 hours, cooled and applied directly to a silica gel column and then chromatographed with a mixture of hexane and ethyl acetate (1:1). After evaporation of the solvent, the residue was washed with distilled hexane (10 ml × 2), and dried in vacuum to give 418 mg (65%) of the

product as a red powder. ^{1}H NMR ($CDCl_3$), δ: 8.18 (d, 1H, J=9Hz); 8.01 (d, 1H, J=2.4Hz); 7.57 (d, 1H, J=9Hz); 7.14 (dd, 1H, J_1=9Hz, J_2=2.4Hz); 6.63 (dd, 1H, J_1=9Hz, J_2=2.4Hz); 6.50 (d, 1H, J=0.9Hz); 6.42 (s, 1H), 6.27 (d, 1H, J=0.9Hz); 5.26 (s, 2H), 4.14 (t, 2H, J=6.6Hz); 3.53-3.41 (m, 6H, overlap of N_1–H (q) and N_2–H (t)); 2.88 (s, 2H); 1.85 (m, 2H); 1.75-1.52 (m, 4H); 1.25 (t, 6H, J=6.6Hz). ^{13}C NMR ($CDCl_3$), δ: 183.3, 176.3, 161.8, 152.1, 150.7, 146.6, 140.1, 136.5 (double intensity), 134.1, 131.1, 127.7, 125.6, 124.7, 118.3, 109.5, 106.7, 105.3, 96.3, 80.9, 68.2, 49.9, 47.4, 38.9, 25.6, 26.4, 27.5, 29.0, 12.7.

Route “b”

Synthesis route “b” is the same as route “a” except that 9-Diethylamino-2-(6-iodohexyloxy)-5H-benzo[a]phenoxazin-5-one (74 mg, 0.156 mmol) and furan-maleimide adduct (33 mg, 0.2 mmol) were used as reactants. The product was obtained as a red powder, 55 mg (61%).

Nile Red maleimide, 1-[6-(9-Diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)-hexyl]-pyrrole-2,5-dione.

A one liter pear-shaped flask fitted with a stirring bar and a 40 cm Vigreaux column was charged with the Diels-Alder protected Nile Red maleimide (4-[6-(9-Diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)-hexyl]-10-oxa-4-aza-tricyclo[5.2.1.02,6]dec-8-ene-3,5-dione (4.0 g, 6.87 mmol), dichloromethane (100 ml) and toluene (250 ml). The mixture was gradually heated up to 140°C with a heating mantle and then gently refluxed for 12 hours permitting the removal of dichloromethane and furan as it was formed. After this time the solvent was evaporated, the residue dissolved in dichloromethane and

chromatographed on silica gel with 1:1 mixture of hexane and ethyl acetate. A total of 2.26 g (64%) of dark red product, which could be converted to a crystalline form by very slow evaporation of dichloromethane solution. M.p. 167°C (DSC, 10°C/min), ^{1}H NMR ($CDCl_3$), δ: 8.20 (d, 1H, J=9Hz); 8.01 (d, 1H, J=2.4Hz); 7.58 (d, 1H, J=9Hz); 7.16 (dd, 1H, J_1=9Hz, J_2=2.4Hz); 6.69 (s, 2H); 6.65 (dd, 1H, J_1=9Hz, J_2=2.4Hz); 6.42 (d, 1H, J=0.9Hz); 6.28 (s, 1H); 4.15 (t, 2H, J=6.6Hz); 3.55 (t, 2H, J=9Hz); 3.45 (q, 4H, J=9Hz); 1.85 (m, 2H); 1.68-1.56 (m, 4H); 1.44 (m, 2H); 1.25 (t, 6H, J=6.6Hz). ^{13}C NMR ($CDCl_3$), δ: 183.3, 170.9, 161.8, 152.1, 150.7, 146.9, 140.2, 134.1 (double intensity), 131.1, 127.7, 125.6, 124.7, 118.3, 109.5, 106.7, 105.3, 96.4, 68.2, 45.1, 37.8, 29.1, 28.5, 26.5, 25.7, 12.7. IR (cm^{-1}): 3003, 2943, 2868, 1736, 1621, 1601, 1579, 1406, 1350, 1319, 1114, 1083, 898, 862, 829, 799, 694. UV-Vis (CH_2Cl_2) λ_{max} 536 nm (ε_{max} = 41260); (CH_3OH) λ_{max} 552 nm (ε_{max} = 36170). Analysis calculated for $C_{30}H_{31}N_3O_5$: C, 69.26; H, 6.20; N, 8.10. Found, C, 70.16; H, 6.08; N, 8.18.

References

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