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A practical approach for the preparation of monofunctional azulenyl squaraine dye

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Abstract—The synthesis of monofunctional azulenyl squaraine dye **NIRQ$_{700}$** is described. The essential azulene intermediate **3**, 1-(methoxycarbonyl)-2-methylazulene, was achieved via [8+2] cycloaddition between lactone **2**, 2*H*-3-methoxycarbonyl-cyclohepta[*b*]furan-2-one, and the in situ generated vinyl ethers under high temperature and pressure conditions. Methylation on the cycloheptatriene ring of 2-methyl azulene **6** via Meisenheimer-type intermediate following Schrott's method formed the carboxylic acid intermediate **9**, 3-(2-methyl-azulen-4-yl)-propionic acid. Condensation of **9** with squaric acid provided the title compound **NIRQ$_{700}$** at moderate yields. The non-fluorescent squaraine dye **NIRQ$_{700}$** absorbed in a 600–700 nm range and potentially can be used to quench a number of available NIR fluorochromes in order to extend the spectrum of biological quenching assays.

Fluorescence resonance-energy transfer has long been used to study various biological events in vitro, such as protease kinetics[1] or nucleic acid hybridization.[2] To impart high signal changes in protease assays, a fluorescent donor and a non-fluorogenic chromophore are often covalently attached to the ends of a specific enzyme substrate. Resonance-energy transfer from the excited state of a fluorophore to a non-fluorogenic chromophore results in quenching of a fluorescence signal. Upon proteolytic cleavage of the substrate by enzymes, a fluorescent dye and a quencher are separated from each other, resulting in fluorescence.[1]

Fluorochromes typically used for the above assays fluoresce in the visible range ($\lambda = 400$–600 nm), so that the fluorescence signal can be conveniently visualized by spectrophotometers or by fluorescence microscopes. However, light in this range is not ideal for many in vitro and in vivo applications, because of autofluorescence in the visible spectrum, and because of strong absorption of photons by tissue and blood. Recently various near-infrared (NIR, $\lambda = 700$–900 nm) probes have shown great promise for in vivo imaging of various target molecules or biological events, such as receptors,[3–5] tumor associated proteases,[6–8] osteolastic activity[9] and thrombin.[10]

We have recently reported the application of a near-infrared fluorescence quencher, **NIRQ$_{750}$** (Fig. 1) for caspase activity detection. This probe was synthesized by dimerizing of commercially available guaiazulene with squaric acid.[11] During the course of work we found that the electronic donating isopropyl moiety on guaiazulene ring contributes to the bathochromic shift of the dye in the NIR region ($\lambda = 750$ nm). In a continued work we would like to look for more applications of azulenyl squaraine dyes as quenchers for commercial NIR fluorochromes in the 650–700 nm range. It is anticipated that substituting the guaiazulene half dye of **NIRQ$_{750}$** by an azulene will hypsochromically shift the absorbance wavelength by about 40–50 nm ($\Delta\lambda$).

The synthesis of **NIRQ$_{700}$** was started with a known procedure[12] by reacting the activated tropolone **1** with

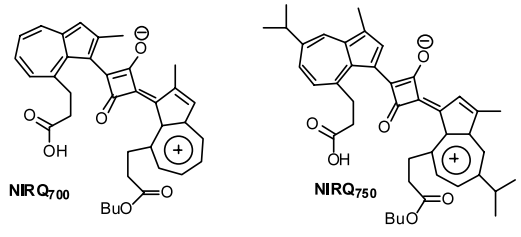

Figure 1. Molecular structure of the conjugatable NIR quenching, non-fluorescent azulenyl squaraine dyes **NIRQ$_{700}$** and **NIRQ$_{750}$**.

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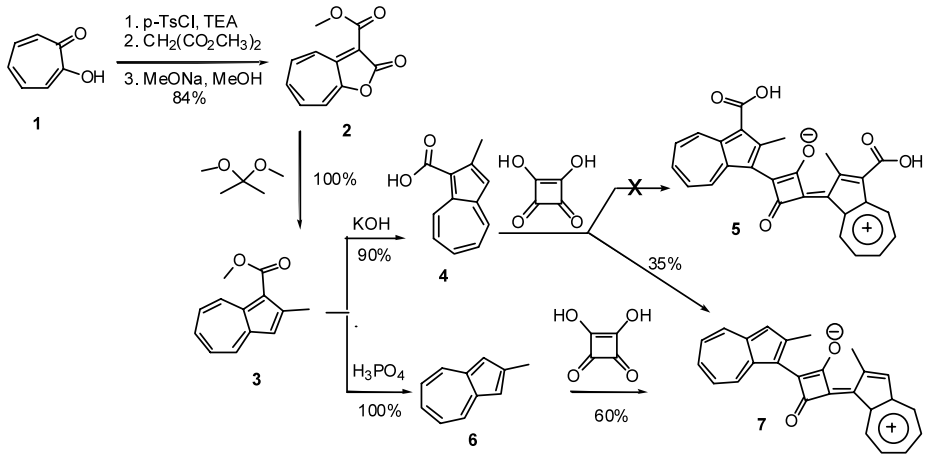


Scheme 1. Preparation of azulene derivatives and attempted synthesis of a azulenyl squaraine dye.

dimethyl malonate in the presence of sodium methoxide to obtain lactone **2** (Scheme 1). The crude product was purified by recrystallization using ethanol and dichloromethane to provide 84% yield. An azulene ring was prepared via [8+2] cycloaddition of lactone **2** with vinyl ether—a product of thermolysis of acetals.[13,14] This reaction was temperature and solvent dependent. In the absence of solvent, only a black tar-like decomposition material was recovered. The expected brownish-red liquid product **3** was obtained by heating **2** with 2,2-dimethoxypropane in anhydrous toluene at 200°C under pressure.[14]

The methyl ester of **3** was removed by saponification using potassium hydroxide and the product was purified by recrystallization with methanol and chloroform to yield the pink solid acid **4**. Subsequent condensation was carried out by refluxing compound **4** with squaric acid; unfortunately, instead of obtaining the expected azulenyl squaraine product **5** with the intact free carboxylic acid, an unexpected product **7** was observed. The absorption of **7** (green color) found about 180 nm higher than compound **4** in acetonitrile clearly demonstrating that the condensation process was successful (Fig. 2). However, the evidence of this decarboxylation was provided in the ^{1}H NMR and mass spectrometry. We thought that the 2-methyl group on the azulene five-membered ring may serve as an electron-donating group that foster the decarboxylation process. We then treating compound **3** in acidic condition using anhydrous phosphoric acid to support the hypothesis. The decarboxylation was smoothly completed in 5 min at 100°C and the brownish red solution of compound **3** turned to purple color of compound **6** quantitatively.

In order to regain a functional group for future conjugation, an insertion of a carboxylic moiety after condensation was attempted. The 2-methyl azulene **6** was first condensed with squaric acid to yield **7**. But, the subsequent *Friedel–Crafts alkylation* of introducing the 4-carboxylic group into the aromatic ring by succinic anhydride was not successful. In a separate reaction, we were able to introduce an alkyl chain into the aromatic ring of compound **6** via *Friedel–Crafts alkylation* using similar conditions (data not shown). For compound **7**, perhaps the electron density in the five-membered ring of azulene now delocalized to the squaryl ring making it inactive for alkylation.

An alternative approach of preparing monofunctional squaraine azulene analog was started from methylation on the seven-membered ring of compound **6** with methyllithium at room temperature in diethyl ether (Scheme 2). The mixture was refluxed overnight to form the azulenate ions of the Meisenheimer-type intermediate[15,16] as the color changed from deep blue to a pale yellow suspension. Addition of methanol at −70°C provided a colorless solution which subsequently dehydrogenated with *p*-chloranil gave a dark blue oil 2,4-dimethyl azulene **8** at 52% efficiency.[17,20] The absorbance maximum of 2,4-dimethyl azulene **8** was similar to that of intermediate **4**. A first trial of deprotonation of the methyl group with *t*-BuOK in THF under conditions described by Song et al. was not successful.[18] Using Schrott's method,[19] treating 2,4-dimethyl azulene **8** with *n*-BuLi at −40°C in the presence of diisopropyl amine, the nucleophilic substitution was carried out by adding bromoacetic acid dropwise to the reaction mixture at −40°C, at the end of the

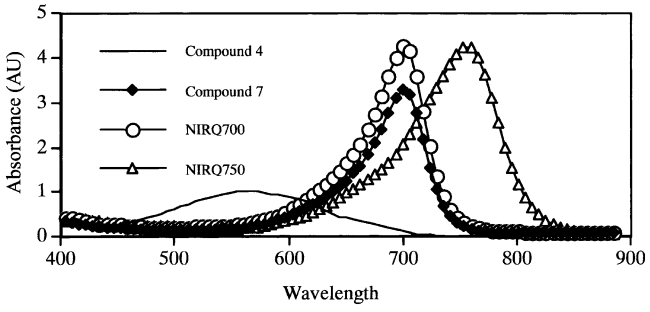
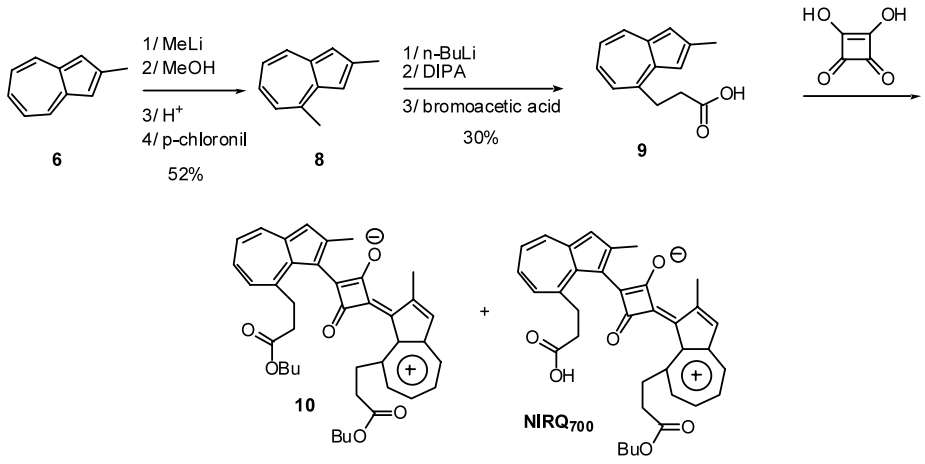


Figure 2. Absorption spectra of azulene derivatives in acetonitrile.



Scheme 2. Synthesis of azulenyl squaraine dye, **NIRQ$_{700}$**.

reaction the suspension was acidified with 2 M HCl and the carboxylic acid product **9**[20] was extracted by diethyl ether. Condensation between **9** and squaric acid under similar conditions to those described earlier[11] provided the mono- and di-ester, **NIRQ$_{700}$**[20] and **10** at 26 and 38% yield, respectively. The prepared **NIRQ$_{700}$** has an absorption maximum at $\lambda_{max}=700$ nm and a broad *absorbance* between $\lambda=600$ and 750 nm (Fig. 2).

In summary, the synthesis reported above describes a novel approach of preparing a monofunctional azulenyl squaraine dye **NIRQ$_{700}$**. Similar to the previously reported **NIRQ$_{750}$**, the newly synthesized compound **NIRQ$_{700}$** has no fluorescence, and is expected to be an efficient FRET quencher for 600–750 nm fluorochromes.[13] It is noteworthy that the absorption could be tuned conveniently by replacing the substitution group on the azulene rings.

Acknowledgements

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20. Data and procedure for representative products:
2. $R_f=0.48$ (9.5:0.2 CH_2Cl_2/MeOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.95 (s, 3H), 7.34 (ddd, $J=3.3$, 3.8, 3.3 Hz, 1H), 7.50 (t, $J=4.1$ Hz, 2H), 7.64 (m, 1H), 8.86 (d, $J=11.3$ Hz, 1H); ^{13}C NMR (200 MHz, $CDCl_3$) δ 51.6, 96.3, 119.2, 130.6, 134.0, 136.1, 139.6, 154.6, 158.6, 163.8, 165.1; FAB-MS: calcd $(M+H)^+$ ($C_{11}H_9O_4$) 205.18, found 205.11; elemental anal. calcd for $C_{11}H_8O_4$: C, 64.71; H, 3.95. Found: C, 64.26; H, 3.89; UV–vis (MeCN) $\lambda_{max}=$ 400 nm.
3. $R_f=0.63$ (CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.83 (s, 3H), 3.98 (s, 3H), 7.13 (s, 1H), 7.39 (t, $J=11.1$ Hz, 1H), 7.50 (t, $J=11.1$ Hz, 1H), 7.69 (t, $J=11.1$ Hz, 1H), 8.28 (d, $J=10.6$ Hz, 1H), 9.48 (d, $J=10.6$ Hz, 1H); ^{13}C NMR (200 MHz, $CDCl_3$) δ 18.1, 50.8, 86.2, 115.1,

120.2, 126.8, 127.7, 135.8, 137.2, 142.1, 143.2, 154.1, 166.6; MALDI-TOF MS: calcd $(M+H)^+$ ($C_{13}H_{13}O_2$) 201.23, found 201.22; UV–vis (MeCN) $\lambda_{max}=524$ nm. **4**. $R_f=0.35$ (CH_2Cl_2); ^1H NMR (200 MHz, THF-d_8) δ 2.82 (s, 3H), 7.13 (s, 1H), 7.38 (t, $J=9.46$ Hz, 1H), 7.43 (t, $J=2.44$ Hz, 1H), 7.69 (t, $J=10.1$ Hz, 1H), 8.30 (d, $J=9.76$ Hz, 1H), 9.59 (d, $J=10.1$ Hz, 1H); ^{13}C NMR (50 MHz, THF-d_8) δ 18.3, 116.6, 120.9, 127.4, 128.0, 136.5, 136.9, 137.9, 143.2, 144.2, 155.0, 167.4; MALDI-TOF MS: calcd $(M+H)^+$ ($C_{12}H_{11}O_2$) 187.22, found 187.19; UV–vis (MeCN) $\lambda_{max}=522$ nm.

6. $R_f=0.8$ (9.5:0.5 hexane/Et_2O); ^1H NMR (400 MHz, $CDCl_3$) δ 2.67 (s, 3H), 7.14 (t, $J=9.8$ Hz, 2H), 7.18 (s, 2H), 7.47 (t, $J=9.6$ Hz, 1H), 8.16 (d, $J=9.4$ Hz, 2H); ^{13}C NMR (200 MHz, $CDCl_3$) δ 16.7, 118.3, 123.0, 134.1, 135.3, 140.7, 150.3; HRMS (CI) calcd M^+ ($C_{11}H_{10}$) 142.2000, found 142.0781; UV–vis (MeCN) $\lambda_{max}=560$ nm.

7. $R_f=0.7$ (9.5:0.5 CH_2Cl_2/acetone); ^1H NMR (200 MHz, $CDCl_3$) δ 3.20 (s, 6H), 7.28 (s, 2H), 7.48 (m, 2H), 7.70 (m, 4H), 8.14 (d, $J=9.5$ Hz, 2H), 10.48 (m, 2H); ^{13}C NMR (50 MHz, 1:1 $CDCl_3$:MeOD) δ 19.0, 125.2, 128.1, 132.3, 132.8, 137.2, 140.2, 141.7, 147.4, 151.2, 156.5, 183.2, 185.3; MALDI-TOF MS: calcd $(M+H)^+$ ($C_{26}H_{19}O_2$) 363.43, found 363.36; UV–vis (MeCN) $\lambda_{max}=$ 700 nm, ε *(CH_2Cl_2, cm^{-1} M^{-1})= 120,000.*

8. A 1.4 M solution of MeLi (3.20 mL, 4.39 mmol) was added to a flame-dried flask containing a solution of 2-methyl azulene **6** (520 mg, 3.66 mmol) in anhydrous ether at room temperature. After refluxing overnight, the resulting suspension was added with MeOH (2 mL) at −70°C then with 2N HCl (10 mL) at room temperature. The organic layer was separated, and the aqueous solution was extracted with ether (3×20 mL). The combined organic solution was dried over $MgSO_4$, and evaporated under vacuum. The brown residue was redissolved in benzene (10 mL), then *p*-chloranil (899 mg, 3.66 mmol) was added portionally at room temperature. During this time, the solution changed gradually from brown to purple. After 48 h, the reaction was quenched with 1N NaOH (20 mL). The organic layer was washed with water, dried over $MgSO_4$, filtered, and concentrated to a purple oil. Chromatography with hexane afforded 300 mg (52%) of a dark purple oil: $R_f=0.4$ (hexane); ^1H NMR (200 MHz, $CDCl_3$) δ 2.65 (s, 3H), 2.85 (s, 3H), 7.02–7.15 (m, 3H), 7.20 (d, $J=6.4$ Hz, 1H), 7.42 (t, $J=9.8$ Hz, 1H), 8.17 (d, $J=9.5$ Hz, 1H); ^{13}C NMR (200 MHz, $CDCl_3$) δ 16.6, 24.3, 116.5, 118.7, 122.0, 126.5, 134.5, 138.1, 140.7, 144.1, 148.4; MALDI-TOF MS calcd. $(M+H)^+$ ($C_{12}H_{13}$) 157.23, found 157.42; UV–vis (MeCN) $\lambda_{max}=548$ nm.

9. A 2.5 M solution of *n*-BuLi (846 μL, 2.12 mmol) was added dropwise to a solution of 2,4-dimethyl azulene **8** (220 mg, 1.41 mmol) and diisopropyl amine (316 μL, 2.26 mmol) in ether (10 mL) at −40°C then reaction mixture was warmed up to 0°C for 30 min. A solution of bromoacetic acid (195.90 mg, 1.41 mmol) in ether (1 mL) was added dropwise to the reaction mixture after cooling the flask to −40°C. The reaction was stirred overnight as the temperature slowly raised up to room temperature. The reaction mixture was poured onto ice-cold water (30 mL) and the unreacted starting material **8** was extracted with ether (2×10 mL). The aqueous phase was acidified by 2 M HCl (20ml), and extracted with ether (20 ml). The organic solution was collected, dried over $MgSO_4$, filtered, and concentrated to a wet purple residue. Chromatography with CH_2Cl_2 afforded 91 mg (30%) of a dark purple oil: $R_f=0.1$ (CH_2Cl_2); MALDI-TOF MS calcd. $(M+H)^+$ ($C_{14}H_{15}O_2$) 215.26, found 215.30.

NIRQ$_{700}$. 4-Azulenepropanoic acid, 2-methyl **9** (120 mg, 0.56 mmol) and squaric acid (31.90 mg, 0.28 mmol) were refluxed for 5 h in a solvent mixture containing toluene (20 mL) and *n*-BuOH (20 mL), accompanied by water removal using Dean–Stark apparatus. The solvents were removed by rotavapor then the residue was redissolved in chloroform. Chromatography with 9:1 CH_2Cl_2/acetone afforded an oily green material (41 mg, 26%). $R_f=0.34$ (9.5:0.5 $CHCl_3$/acetone); ^1H NMR (200 MHz, $CDCl_3$, CD_3OD) δ 0.93 (t, $J=7.3$ Hz, 3H), 1.33 (m, $J=7.9$ Hz, 2H), 1.61 (q, $J=6.4$ Hz, 2H), 2.84 (t, $J=7.0$ Hz, 4H), 3.14 (s, 6H), 3.50 (t, $J=7.9$ Hz, 4H), 4.11 (t, $J=6.7$ Hz, 2H), 7.44 (s, 2H), 7.77 (m, 6H), 10.29 (d, d, $J=4.27$, 4.27, 2H); ^{13}C NMR (50 MHz, $CDCl_3$, CD_3OD) δ 13.7, 19.2, 29.8, 30.8, 34.1, 35.2, 65.2, 125.4, 131.0, 134.8, 139.0, 141.4, 146.8, 148.3, 149.4, 149.9, 155.5, 173.1, 175.1, 182.7, 185.8; ES-HRMS calcd $(M+H)^+$ ($C_{36}H_{35}O_6$) 563.2433, found 563.2431; UV–vis (MeCN) $\lambda_{max}=700$ nm, ε *(CH_2Cl_2, cm^{-1} M^{-1})= 84,000.*