Microwave-assisted synthesis of thiophene fluorophores, labeling and multilabeling of monoclonal antibodies and long lasting staining of fixed cells

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SUPPORTING INFORMATION

I. General and Materials and Synthesis

Ia. General. Microwave experiment were carried out in a Milestone Microsynth Labstation operating at 2450 MHz monitored by a proprietary control unit. The oven was equipped with magnetic stirring, pressure and temperature sensors. Reactions were performed in a glass vessel (capacity 10 mL) sealed with a septum. The microwave method was power controlled (100 W maximum power input) and the samples were irradiated with the appropriate power to achieve the temperature of 80 °C. The typical process profile (temperature/pressure/energy vs time) for aqueous microwave-assisted Suzuki-type preparation of TF-STP esters is reported in Figure 1S.

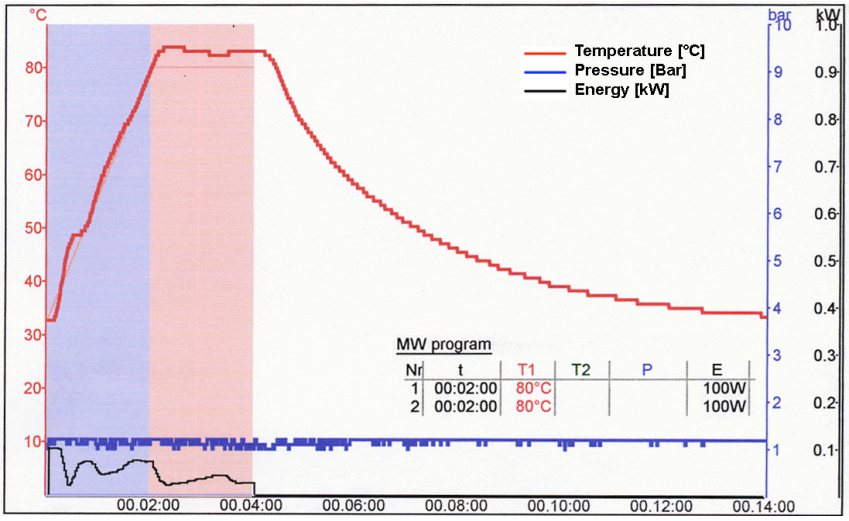


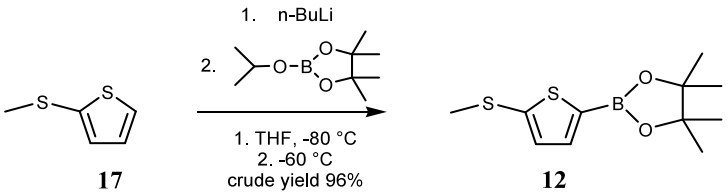
Fig. 1S. Typical process profile (temperature/pressure/energy vs time) for aqueous microwave-assisted Suzuki-type preparation of TF-STP esters.

All ^{1}H NMR, ^{13}C NMR and ^{19}F NMR spectra were recorded at room temperature on a Varian Mercury-400 spectrometer equipped with a 5-mm probe. Chemical shifts were calibrated using the internal DMSO-d_6, $CDCl_3$ or acetone-d_6 resonance which were referenced to TMS. For ^{19}F NMR spectra 0.5% fluorobenzene was added as an internal standard. The fluorobenzene was referenced to $CFCl_3$.[1] Mass spectra were collected on a ion trap Finningan Mat GCQ spectrometer operating in electron impact (EI) ionization mode. Each sample was introduced to the ion source region of GCQ *via* a direct exposure probe (DEP). Melting points were determined on Kofler bank apparatus and are uncorrected. UV-Vis spectra were recorded using a Perkin Elmer Lambda 20 spectrometer. Photoluminescence spectra were collected on a Perkin Elmer LS50B spectrofluorometer. The measurements were performed using an excitation wavelength corresponding to the maximum absorption lamda. The photoluminescence quantum yields were measured with reference to quinine sulfate as the standard and carried out as described in reference 15d (section Absorption and Photoluminescence measurements).

Ib. Materials and Synthesis. Unless otherwise noted, all operations were carried out under a dry, oxygen-free nitrogen atmosphere. All organic solvents were dried over 4-Å molecular sieves before use. THF was purified by refluxing and distilling with $LiAlH_4$, then was allowed to stand for 1h with sodium sheets before use. TLC was carried out with 0.2 mm thick of silica gel 60 F_{254} (Merck). Visualization was accomplished by UV light or phosphomolybdic acid solution. Preparative coloumn chromatographies were performed on glass coloumns of different sizes hand packed with silica gel 60 (particle sizes 0.040-0.063 mm, Merck) or florisil (100-200 mesh, Aldrich). Petroleum ether refers to the fraction of bp 40-70 °C.

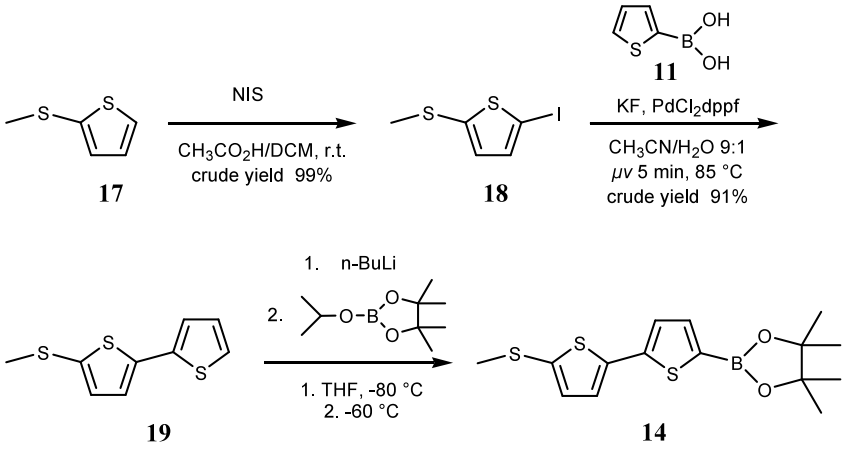
5-Bromo-2-thiophenecarboxylic acid (**1**), 2-thienylboronic acid (**11**), 2,2'-bithiophene-5-boronic acid pinacol ester (**13**), 2,2':5',2"-terthiophene-5-boronic acid pinacol ester (**15**), 2-(methylthio)thiophene (**17**), dichlorobis(triphenylphosphine)palladium(II) ($PdCl_2$dppp), *N*-iodosuccinimide, 2,3,5,6-tetrafluorophenol, 1,3-dicyclohexylcarbodiimide, sodium bicarbonate were purchased from Sigma-Aldrich Co; 1,1′-bis(diphenylphosphino)ferracene palladium(II)chloride dichloromethane complex ($PdCl_2$dppf), *n*-butyllithium 2.5 M solution in hexane, potassium fluoride were obtained from Acros Organics; 2-isopropoxy-4,4,5,5-tetramethyl-1,3,2-dioxaborolane from Alfa Aesar GmbH & Co KG.. The synthesis of compounds **3b-9b**[2] and **10b**[3] has already been reported.

Scheme S1. Synthesis of compound **12**.



4,4,5,5-Tetramethyl-2-[5-(methylthio)thiophen-2-yl]-1,3,2-dioxaborolane (12). To a stirred solution of 17 (1 mmol) in dry THF (4 mL) at –80° C was slowly added a 2.5 M solution of *n*-BuLi in hexane (1.2 mmol). The mixture was allowed to react at this temperature for 1 h, then at room temperature for an additional hour. After cooling at –60 °C, 2-isopropoxy-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (2.2 mmol) was added by a syringe. The resulting mixture was allowed to warm to room temperature and stirred overnight before quenching with water. The product was extracted with ether (2x25 mL). The combined organic layers were dried over anhydrous sodium sulphate and the solvent was removed under reduced pressure to provide the title compound which was utilized with no further purification. Yield 96%. Dark violet oil; EI-MS *m/z* 256 (M^+); ^{1}H NMR ($CDCl_3$, TMS/ppm) δ 7.47 (d, ^3J=3.6 Hz, 1H), 7.03 (d, ^3J=3.6 Hz, 1H), 2.53 (s, 3H), 1.32 (s, 12H).

Scheme S2. Synthesis of compound **14**.



2-Iodo-5-(methylthio)thiophene (18)

Under exclusion of light *N*-iodosuccinimide (1.1 mmol) was added stepwise to a solution of **17** (1 mmol) in acetic acid/methylene chloride 1:1 (20 mL). The mixture was left to stir overnight then quenched with ice. After separation of layers, the aqueous phase was extracted with dichloromethane (2x10 mL). The resulting organic layers were washed with potassium hydroxide (2x10 mL, 30% aqueous), and dried over anhydrous sodium sulphate. Evaporation of the solvent under reduced pressure gave the crude product which did not need further purification. Yield 99%. Clear yellow oil; EI-MS *m/z* 256 (M^+); ^{1}H NMR ($CDCl_3$, TMS/ppm) δ 7.09 (d, ^3J=4.0 Hz, 1H), 6.76 (d, ^3J=4.0 Hz, 1H), 2.46 (s, 3H); ^{13}C NMR ($CDCl_3$, TMS/ppm) δ 142.5, 137.3, 132.9, 74.2, 22.4.

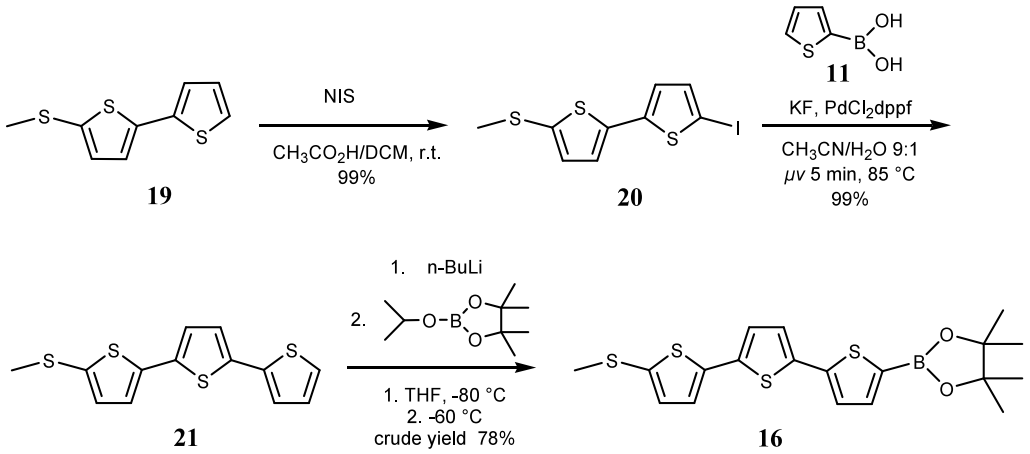
5-(Methylthio)-2,2'-bithiophene (19)

A mixture of **18** (1 mmol), 2-thienylboronic acid (**11**) (3 mmol), PdCl2dppf (0.5 mmol), KF (4 mmol) in CH_3CN/water 9:1 (3 mL) was irradiated with microwaves at 85 °C for 5 min. The reaction mixture was brought to room temperature and the solvent was evaporated under reduced pressure. The title compound was isolated by flash chromatography on silica gel with increasing amounts of EtOAc in petroleum ether as eluent. Yield 91%. Colorless oil; EI-MS *m/z* 212 (M^+); ^{1}H NMR ($CDCl_3$, TMS/ppm) δ 7.21 (dd, ^3J=4.8 Hz, ^4J=1.2 Hz, 1H), 7.15 (dd, ^3J=3.6 Hz, ^4J=1.2 Hz, 1H), 7.03 (d, ^3J=3.6 Hz, 1H), 7.02 (dd, ^3J=4.8 Hz, ^3J=3.6 Hz, 1H), 7.00 (d, ^3J=3.6 Hz, 1H), 2.52 (s, 3H); ^{13}C NMR ($CDCl_3$, TMS/ppm) δ 139.4, 136.9, 136.1, 131.6, 127.7, 124.4, 123.7, 123.6, 22.0.

4,4,5,5-Tetramethyl-2-[5'-(methylthio)-2,2'-bithiophen-5-yl]-1,3,2-dioxaborolane (14)

Prepared following procedures described for **12**. ^{1}H NMR of the crude product showed a mixture of **14**/staring material **19** in the ratio 70:30. After unsuccessful attempts to purify **14** (because of instability), the crude was utilized as obtained for the last synthetic step. Yellow-brown oil; ^{1}H NMR ($CDCl_3$, TMS/ppm) δ 7.51 (d, ^{3}J=3.6 Hz, 1H), 7.18 (d, ^{3}J=3.6 Hz, 1H), 7.07 (d, ^{3}J=3.6 Hz, 1H), 6.97 (d, ^{3}J=3.6 Hz, 1H), 2.51 (s, 3H), 1.35 (s, 12H).

Scheme S3. Synthesis of compound **16**.



19 **20** **21** **16**

5-Iodo-5'-(methylthio)-2,2'-bithiophene (20)

Prepared following the same method given for **18**. Yield 99%. Pale yellow solid; mp 57-59 °C; EI-MS *m/z* 337 (M^{+}); ^{1}H NMR ($CDCl_3$, TMS/ppm) δ 7.13 (d, ^{3}J=3.6Hz, 1H), 6.95 (d, ^{3}J=4.0Hz, 1H), 6.93 (d, ^{3}J=3.6Hz, 1H), 6.78 (d, ^{3}J=4.0Hz, 1H), 2.50 (s, 3H); ^{13}C NMR ($CDCl_3$, TMS/ppm) δ 142.8, 138.0, 137.6, 137.0, 131.5, 124.9, 124.2, 77.1, 21.9.

5-(Methylthio)-2,2':5',2''-terthiophene (21)

Prepared in the same manner as described before for **19**. Yield 99%. Clear yellow solid; mp 86-89 °C; EI-MS *m/z* 294 (M^{+}); ^{1}H NMR (Acetone-d_6, TMS/ppm) δ 7.45 (dd, ^{3}J=5.2Hz, ^{4}J=1.2Hz, 1H), 7.31 (dd, ^{3}J=3.6Hz, ^{4}J=1.2Hz, 1H), 7.22 (d, ^{3}J=4.0Hz, 1H), 7.19 (d, ^{3}J=3.6Hz, 1H), 7.18 (d, ^{3}J=3.6Hz, 1H), 7.10 (dd, ^{3}J= 5.2Hz, ^{3}J=3.6Hz, 1H), 7.07 (d, ^{3}J=4.0Hz, 1H), 2.54 (s, 3H); ^{13}C NMR (Acetone-d_6, TMS/ppm) δ 139.1, 137.0, 136.4, 136.3, 135.7, 131.7, 127.8, 124.5, 124.3, 124.2, 123.7, 123.6, 22.0.

4,4,5,5-Tetramethyl-2-[5'-(methylthio)-2,2':5',2''-terthiophene-5-yl]-1,3,2-dioxaborolane" (16)

Prepared according to the procedures given for **12**. Yield 78%. Microcrystalline yellow-green solid; mp 137-139 °C; EI-MS *m/z* 420 (M^+); ^{1}H NMR ($CDCl_3$, TMS/ppm) δ 7.52 d, ^3J=3.6Hz, 1H), 7.22 (d, ^3J=3.6Hz, 1H), 7.12 (d, ^3J=4.0Hz, 1H), 7.02 (d, ^3J=4.0Hz, 1H), 7.00 (d, ^3J=4.0Hz, 1H), 6.97 (d, ^3J=4.0Hz, 1H), 2.51 (s, 3H), 1.35 (s, 12H); ^{13}C NMR ($CDCl_3$, TMS/ppm) δ 143.6, 139.0, 137.9, 136.7, 136.4, 136.2, 131.7, 125.0, 124.9, 124.4, 123.9, 24.7, 22.0.

5-Bromothiophene-2-carboxylic acid 4-sulfo-2,3,5,6-tetrafluorophenyl ester, sodium salt (2)

To a solution of commercial **1** (103 mg, 0.5 mmol) and 4-sulfotetrafluorophenol, sodium salt,[4] (152 mg, 0.5 mmol) in 1 mL DMF and 15 mL acetone was added 1,3-dicyclohexylcarbodiimide (113 mg, 0.55 mmol) and the mixture was stirred at room temperature for 20 h. The resulting precipitate was removed by filtration and the filtrate was concentrated under reduced pressure to give a crude product. The product was purified by column chromatography on silica gel with 30% DCM in acetone as eluent to give 200 mg (81%) of the title compound as a white solid; one spot on TLC (R_f = 0.22, 30% DCM in acetone); ^{1}H NMR (DMSO-d_6, TMS/ppm) δ 8.03 (d, ^3J=4.0 Hz, 1H), 7.54 (d, ^3J=4.0 Hz, 1H); ^{13}C NMR (DMSO-d_6, TMS/ppm) δ 157.1, 139.0, 133.8, 130.2, 124.6.

General procedure for the synthesis of TF-STP esters.

A mixture of the opportune thienylboronic ester (**11-16**) (0.15 mmol), compound **2** (0.05 mmol), $PdCl_2$dppp (0.0025 mmol), $NaHCO_3$ (0.05 mmol) in DMF/water 2:1 (3 mL) was irradiated with microwaves at 80 °C for 2 min. The reaction mixture was brought to room temperature and the solvent was evaporated under reduced pressure. All TF-STP esters (**3a-8a**) were isolated by flash chromatography on florisil with increasing amounts of acetone in DCM as eluent.

2,2'-Bithiophenyl-5-carboxylic acid 4-sulfo-2,3,5,6-tetrafluorophenyl ester, sodium salt (3a)

Yield 80%. Amorphous white solid; mp>250°C; EI-MS *m/z* 358 (M^+ -SO_3Na); absorption maximum, 353 nm (ε 23200 $cm^{-1}M^{-1}$), emission maximum, 430 nm in DMSO; ^{1}H NMR (DMSO-d_6, TMS/ppm) δ 8.12 (d, ^3J=4.0Hz, 1H), 7.72 (dd, ^3J=5.2Hz, ^4J=1.2Hz, 1H), 7.64 (dd, ^3J=3.6Hz, ^4J=1.2Hz, 1H), 7.54 (d, ^3J=4.0Hz, 1H), 7.18 (dd, ^3J=5.2Hz, ^3J=3.6Hz, 1H); ^{13}C NMR (DMSO-d_6, TMS/ppm) δ 157.9, 147.7, 139.4, 135.2, 129.7, 129.4, 128.1, 126.2, 126.1.

5'-(Methylthio)-2,2'-bithiophenyl-5-carboxylic acid 4-sulfo-2,3,5,6-tetrafluorophenyl ester, sodium salt (4a)

Yield 72%. Yellow-green solid; mp>250°C; EI-MS *m/z* 404 (M^+ -SO_3Na); absorption maximum, 376 nm (ε 19900 $cm^{-1}M^{-1}$), emission maximum, 503 nm in DMSO; ^1H NMR (DMSO-d_6, TMS/ppm) δ 8.11 (d, ^3J=4.0Hz, 1H), 7.54 (d, ^3J=4.0Hz, 1H), 7.49 (d, ^3J=4.0Hz, 1H), 7.14 (d, ^3J=4.0Hz, 1H), 2.573 (3H, s); ^{13}C NMR (DMSO-d_6, TMS/ppm) δ 158.1, 147.2, 141.5, 139.6, 135.8, 131.2, 128.8, 126.3, 126.2, 21.05.

2,2':5',2''-Terthiophene-5-carboxylic acid 4-sulfo-2,3,5,6-tetrafluorophenyl ester, sodium salt (5a)

Yield 84%. Yellow solid; mp>250°C; EI-MS *m/z* 440 (M^+ -SO_3Na); absorption maximum, 400 nm (ε 24100 $cm^{-1}M^{-1}$), emission maximum, 508 nm in DMSO; ^1H NMR (DMSO-d_6, TMS/ppm) δ 8.14 (d, ^3J=4.0Hz, 1H), 7.63 (d, ^3J=4.0Hz, 1H), 7.59 (dd, ^3J=5.2Hz, ^4J=1.2Hz, 1H), 7.57 (d, ^3J=4.0Hz, 1H), 7.43 (dd, ^3J=3.6Hz, ^4J=1.2Hz, 1H), 7.38 (d, ^3J=4.0Hz, 1H), 7.13 (dd, ^3J=5.2Hz, ^3J=3.6Hz, 1H); ^{13}C NMR (DMSO-d_6, TMS/ppm) δ 157.9, 147.1, 139.4, 139.2, 136.1, 133,7, 129.3, 129.2, 127.4, 126.2, 126.16, 126.11, 126.0.

5''-(Methylthio)-2,2':5',2''-terthiophene-5-carboxylic acid 4-sulfo-2,3,5,6-tetrafluorophenyl ester, sodium salt (6a)

Yield 76%. Ocher yellow solid, mp>250°C; EI-MS *m/z* 486 (M^+ -SO_3Na); absorption maximum, 410 nm (ε 21100 $cm^{-1}M^{-1}$), emission maximum, 580 nm in DMSO; ^1H NMR (DMSO-d_6, TMS/ppm) δ 8.13 (d, ^3J=4.0Hz, 1H), 7.62 (d, ^3J=4.0Hz, 1H), 7.56 (d, ^3J=4.0Hz, 1H), 7.34 (d, ^3J=4.0Hz, 1H), 7.32 (d, ^3J=3.6Hz, 1H), 7.10 (d, ^3J=3.6Hz, 1H), 2.54 (3H, s); ^{13}C NMR (DMSO-d_6, TMS/ppm) δ 157.9, 147.0, 139.4, 138.5, 138.4, 137.0, 133,8, 131.6, 129.2, 126.33, 126.30, 126.2, 126.1, 21.25.

2,2':5',2'':5'',2'''-Quaterthiophene-5-carboxylic acid 4-sulfo-2,3,5,6-tetrafluorophenyl ester, sodium salt (7a)

Yield 82%. Orange solid, mp>250°C; EI-MS *m/z* 522 (M^+ -SO_3Na); absorption maximum, 429 nm (ε 31900 $cm^{-1}M^{-1}$), emission maximum, 555 nm in DMSO; ^1H NMR (DMSO-d_6, TMS/ppm) δ 8.14 (d, ^3J=4.0Hz, 1H), 7.64 (d, ^3J=4.0Hz, 1H), 7.57 (d, ^3J=4.0Hz, 1H), 7.55 (dd, ^3J=5.2Hz, ^4J=1.2Hz, 1H), 7.42 (d, ^3J=4.0Hz, 1H), 7.40 (d, ^3J=4.0Hz, 1H), 7.37 (dd, ^3J=3.6Hz, ^4J=1.2Hz), 7.31 (d, ^3J=4.0Hz, 1H), 7.10 (1H, dd, ^3J=5.2Hz, ^3J=3.6Hz); ^{13}C NMR (DMSO-d_6, TMS/ppm) δ 158.1,

147.2, 139.6, 138.8, 137.5, 136,6, 134.9, 134.1, 129.53, 129.50, 127.2, 127.1, 126.6, 126.5, 126.1, 125.6.

5'''-(Methylthio)-2,2':5',2'':5'',2'''-quaterthiophene-5-carboxylic acid 4-sulfo-2,3,5,6-tetrafluorophenyl ester, sodium salt (8a)

Yield 80%. Amorphous red solid, mp>250°C; EI-MS *m/z* 568 (M^+ -SO_3Na); absorption maximum, 434 nm (ε 34300 $cm^{-1}M^{-1}$) in DMSO, emission maximum, 625 nm in DMSO/H_2O 90:10; ^{1}H NMR (DMSO-d_6, TMS/ppm) δ 8.14 (d, ^3J=4.0Hz, 1H), 7.65 (d, ^3J=4.0Hz, 1H), 7.58 (d, ^3J=4.0Hz, 1H), 7.42 (d, ^3J=3.6Hz, 1H), 7.40 (d, ^3J=3.6Hz, 1H), 7.28 (d, ^3J=4.0Hz, 1H), 7.26 (d, ^3J=4.0Hz, 1H), 7.09 (d, ^3J=3.6Hz, 1H), 2.53 (3H, s); ^{13}C NMR (DMSO-d_6/D_2O 90:10, TMS/ppm) δ 157.9, 146.9, 139.2, 138.5, 137.7, 137.6, 136.7, 135.0, 134.1, 131.9, 131.8, 129.2, 126.9, 126.4, 126.3, 125.9, 125.7, 21.5.

II. NMR Spectra

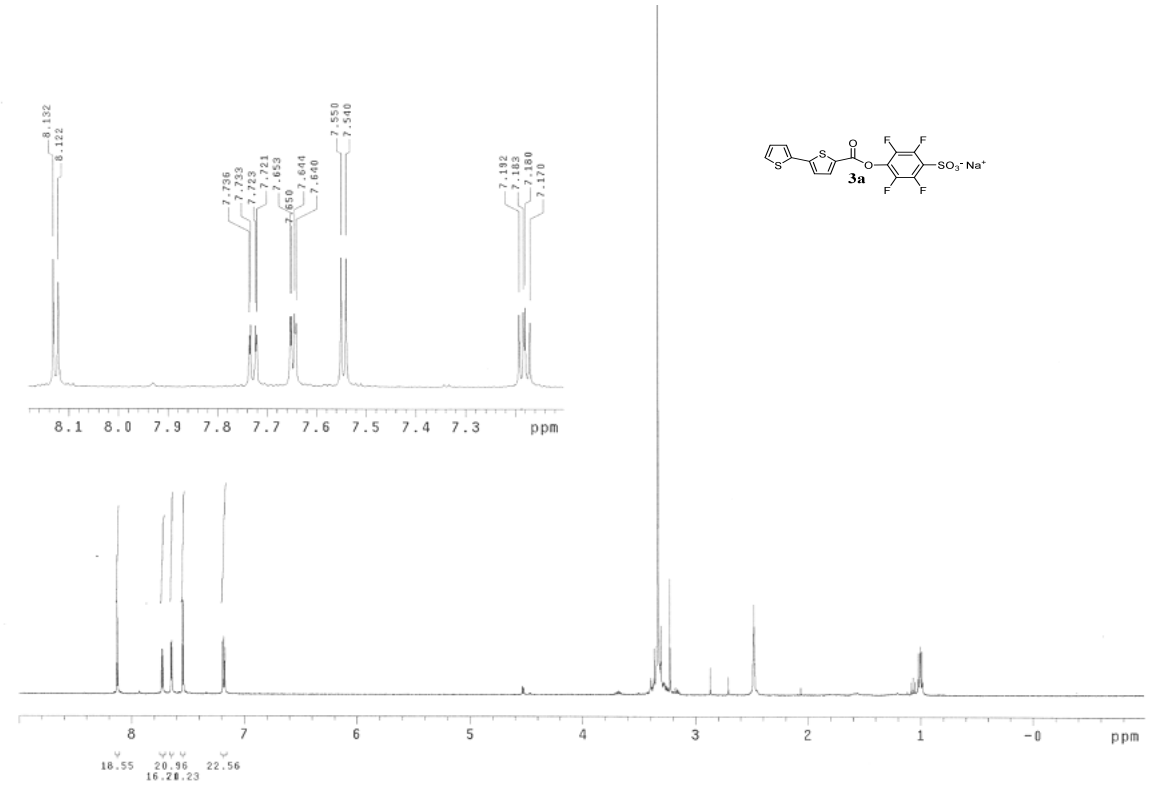


Figure 2S. [1]H NMR spectrum of **3a** in DMSO-d_6.

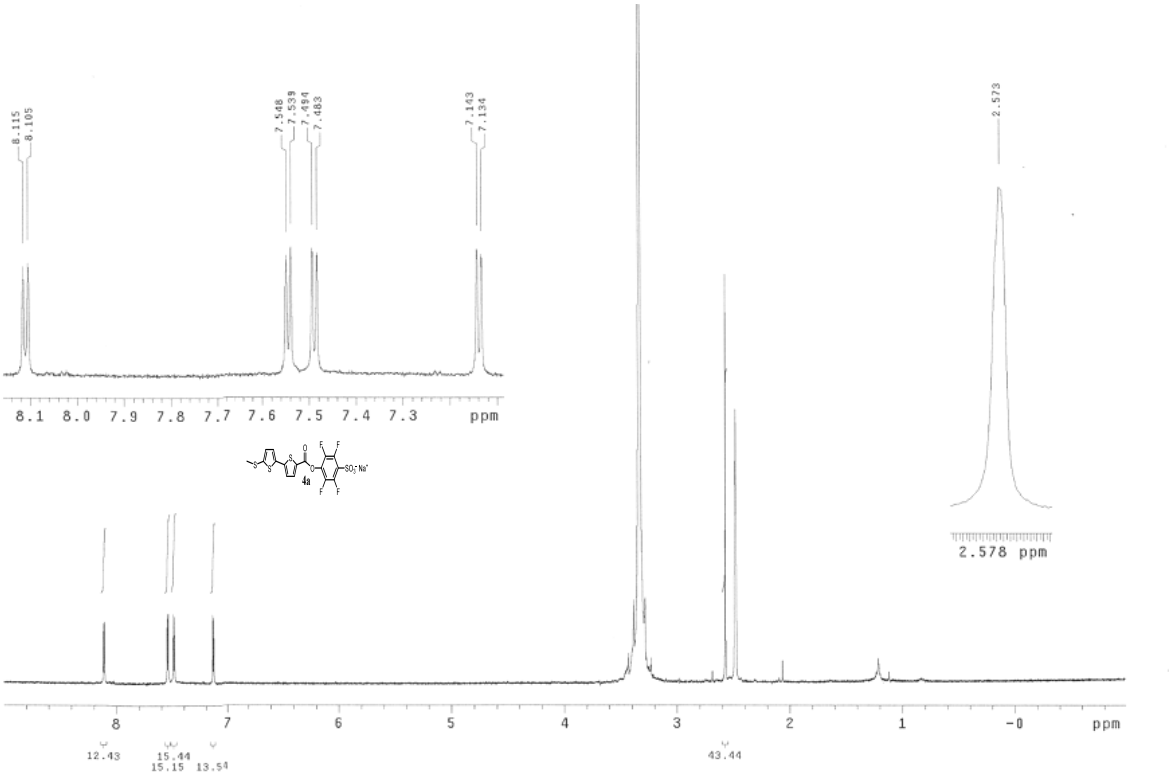


Figure 3S. [1]H NMR spectrum of **4a** in DMSO-d_6.

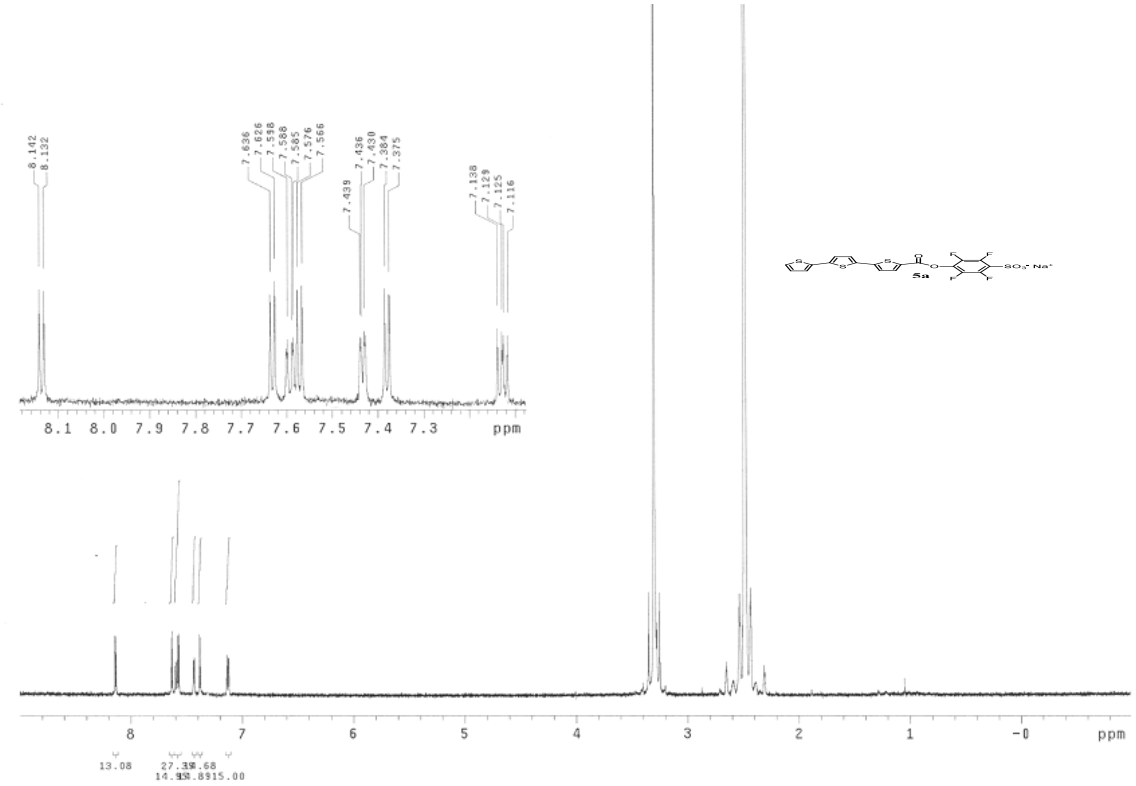


Figure 4S. [1]H NMR spectrum of **5a** in DMSO-d_6.

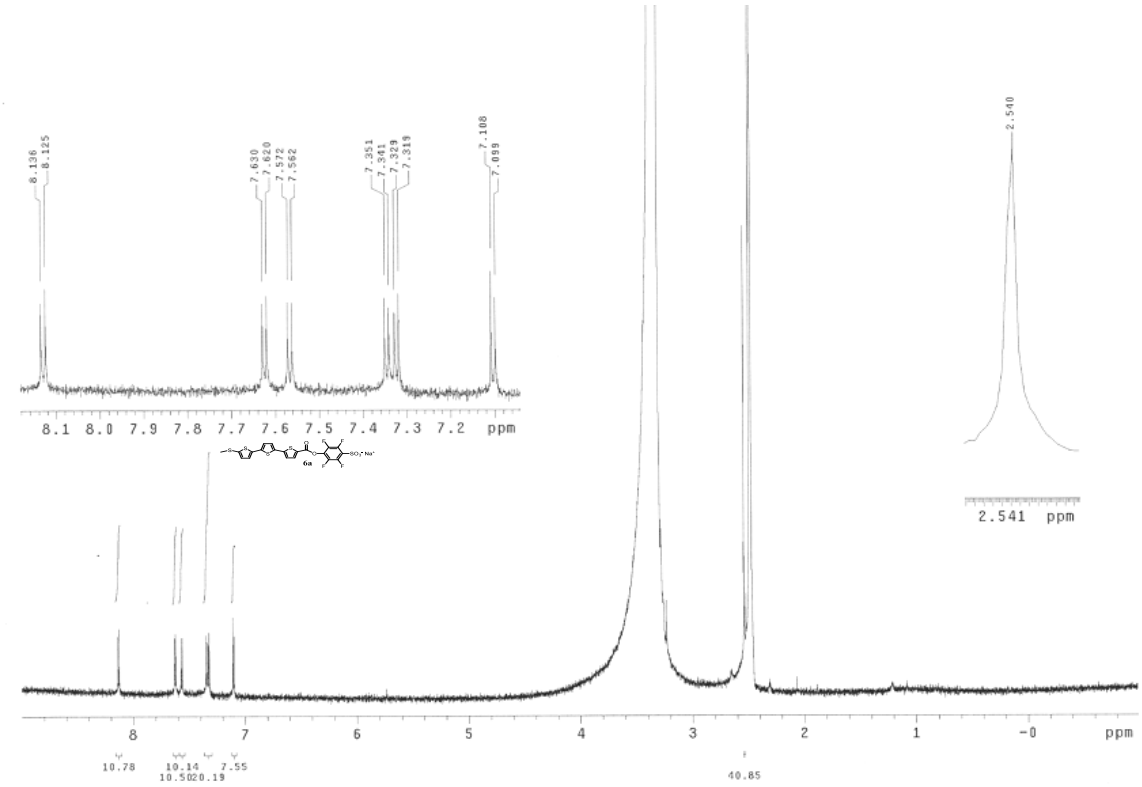


Figure 5S. [1]H NMR spectrum of **6a** in DMSO-d_6.

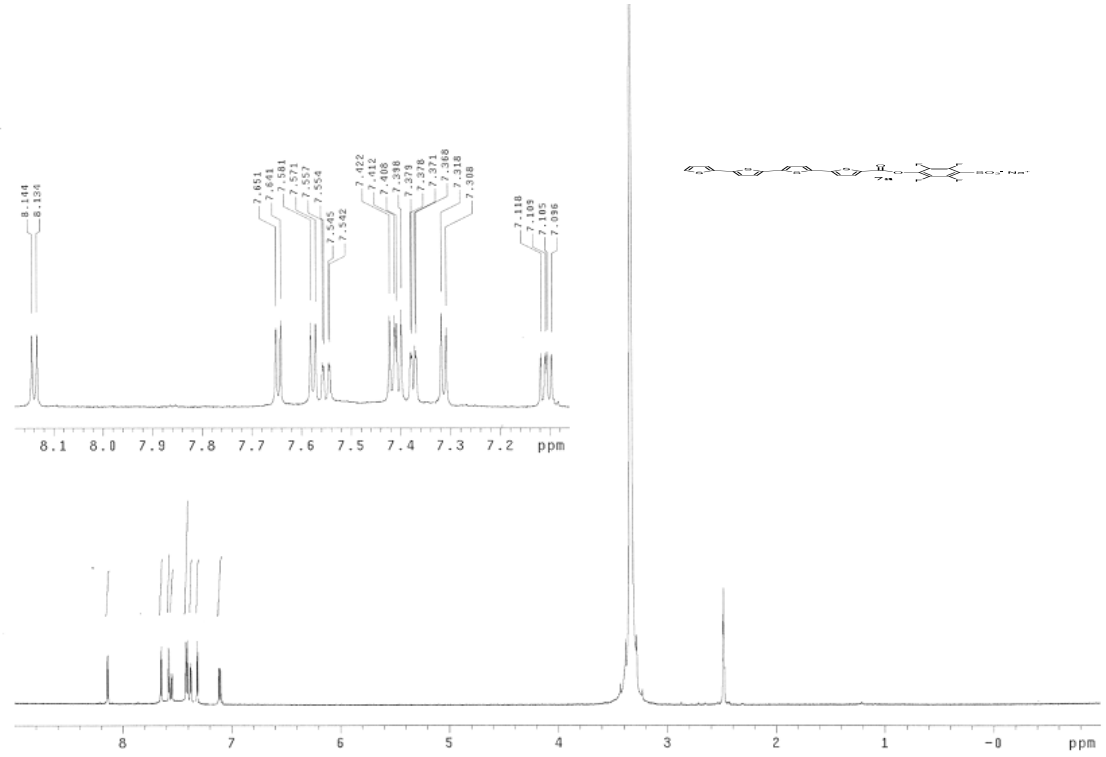


Figure 6S. [1]H NMR spectrum of **7a** in DMSO-d_6.

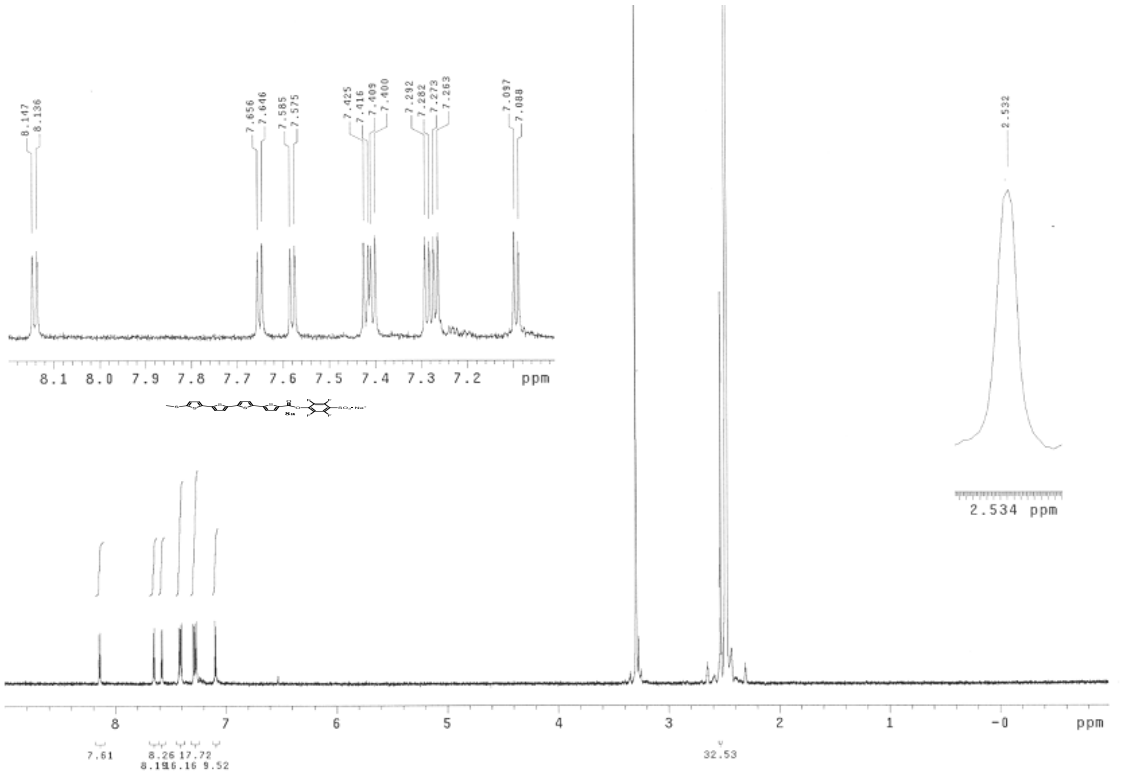


Figure 7S. [1]H NMR spectrum of **8a** in DMSO-d_6.

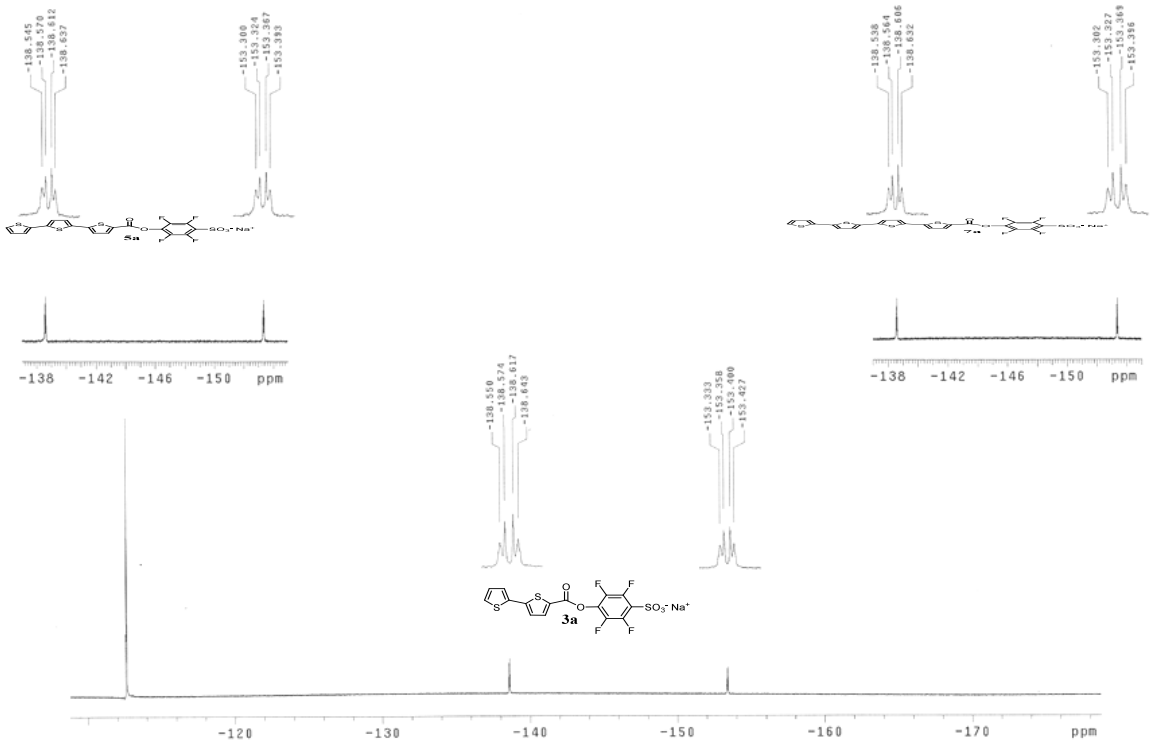


Figure 8S. ^{19}F NMR spectrum of **3a**, **5a** and **7a** in DMSO-d_6.

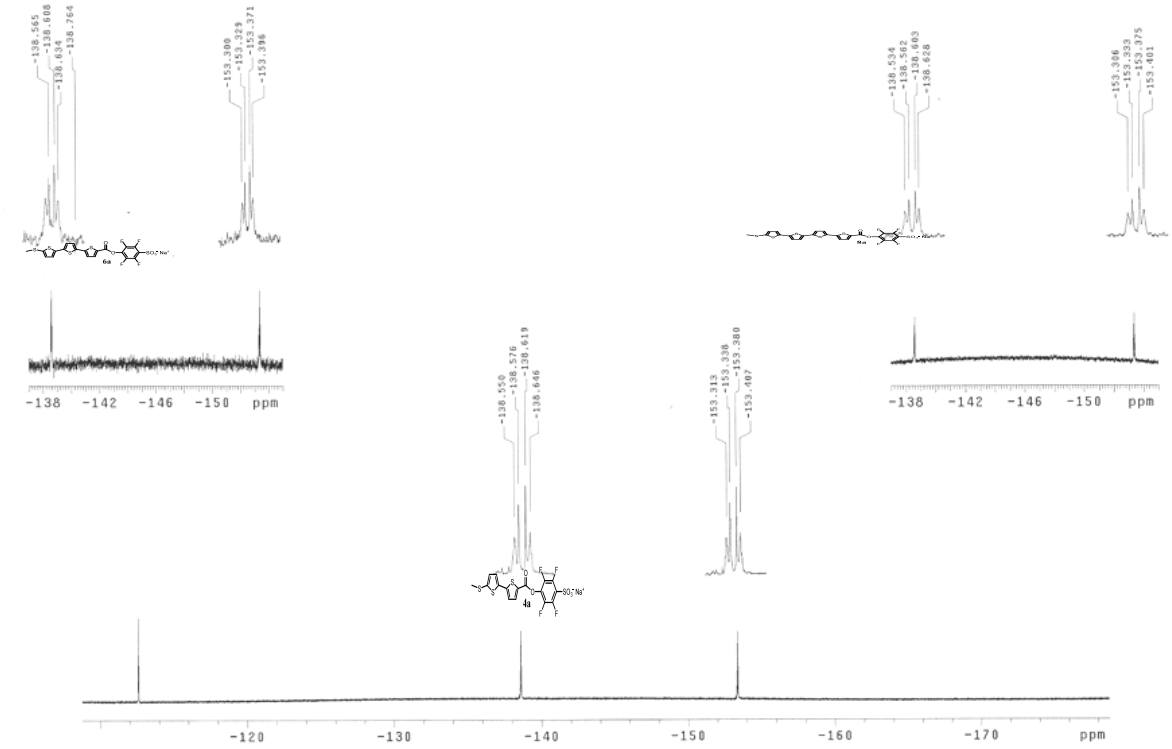


Figure 9S. ^{19}F NMR spectrum of **4a**, **6a** and **8a** in DMSO-d_6.

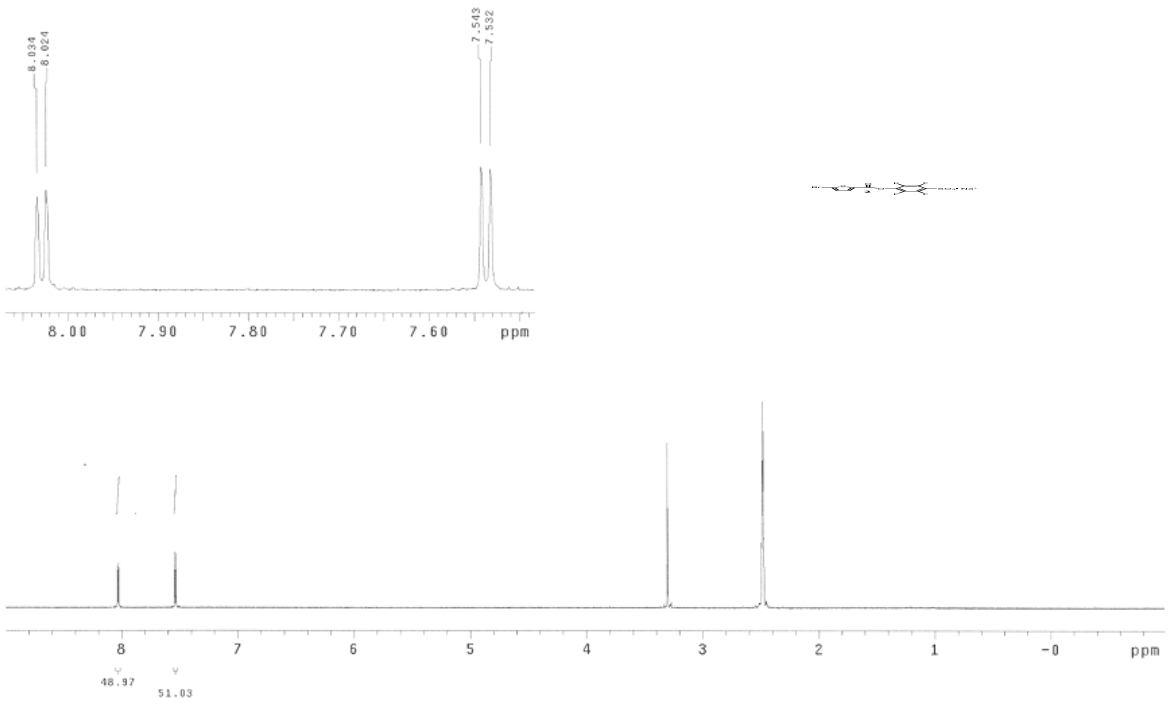


Figure 10S. ^{1}H NMR spectrum of **2** in DMSO-d_6.

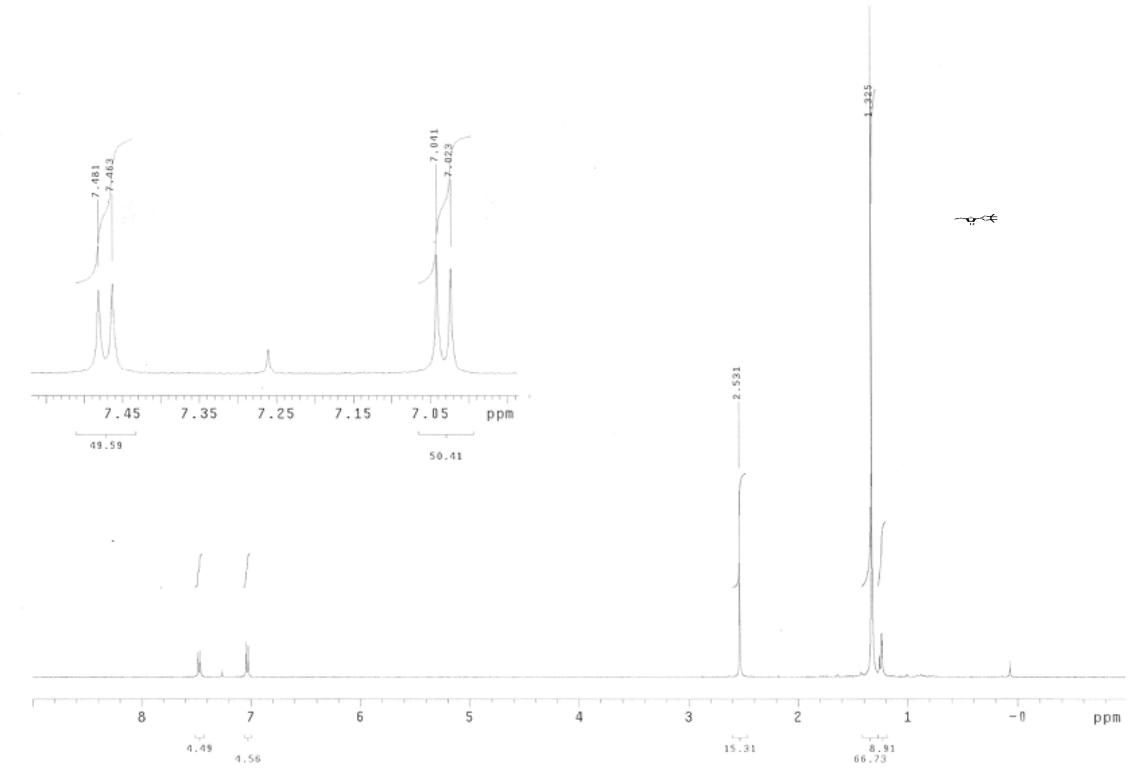


Figure 11S. ^{1}H NMR spectrum of **12** in $CDCl_3$.

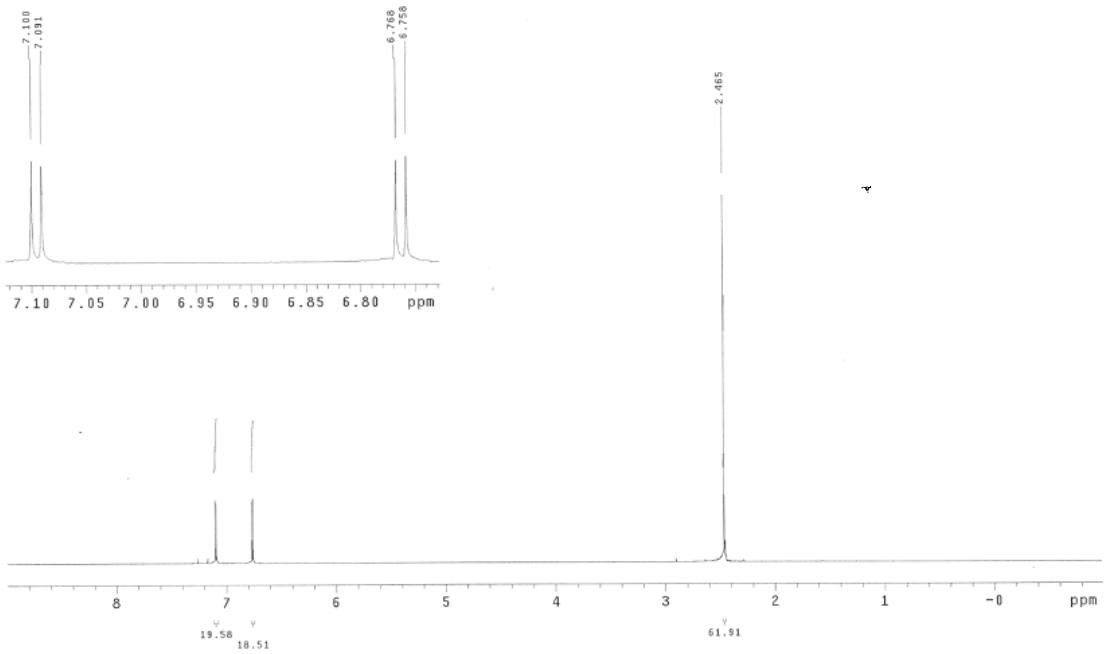


Figure 12S. ^{1}H NMR spectrum of **18** in $CDCl_3$.

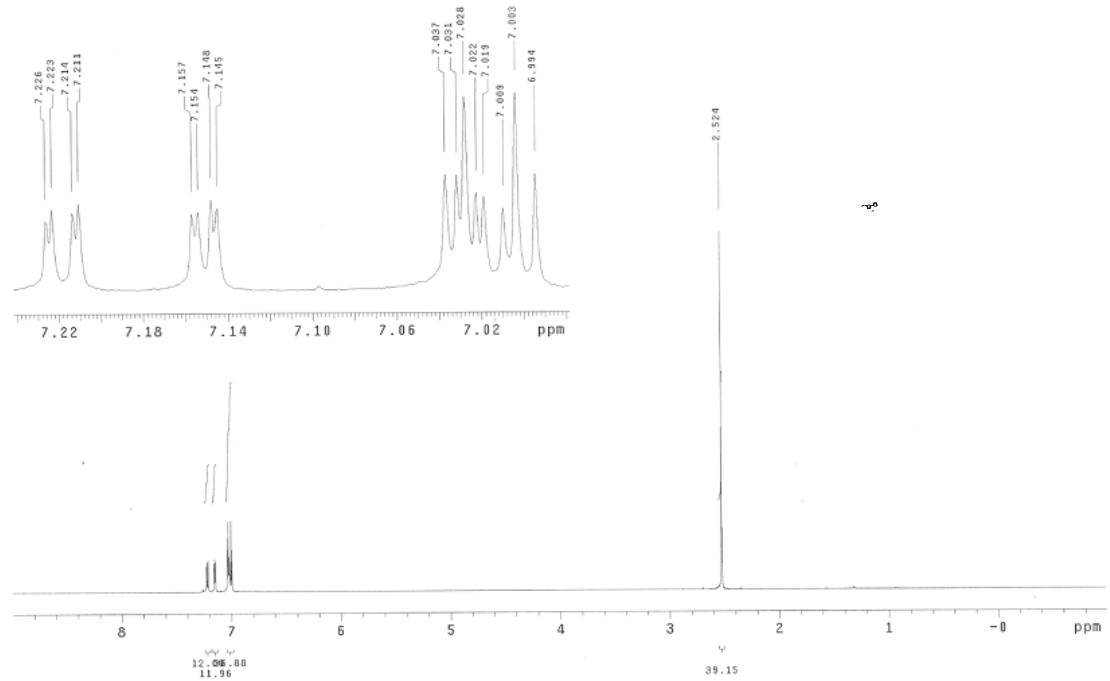


Figure 13S. ^{1}H NMR spectrum of **19** in $CDCl_3$.

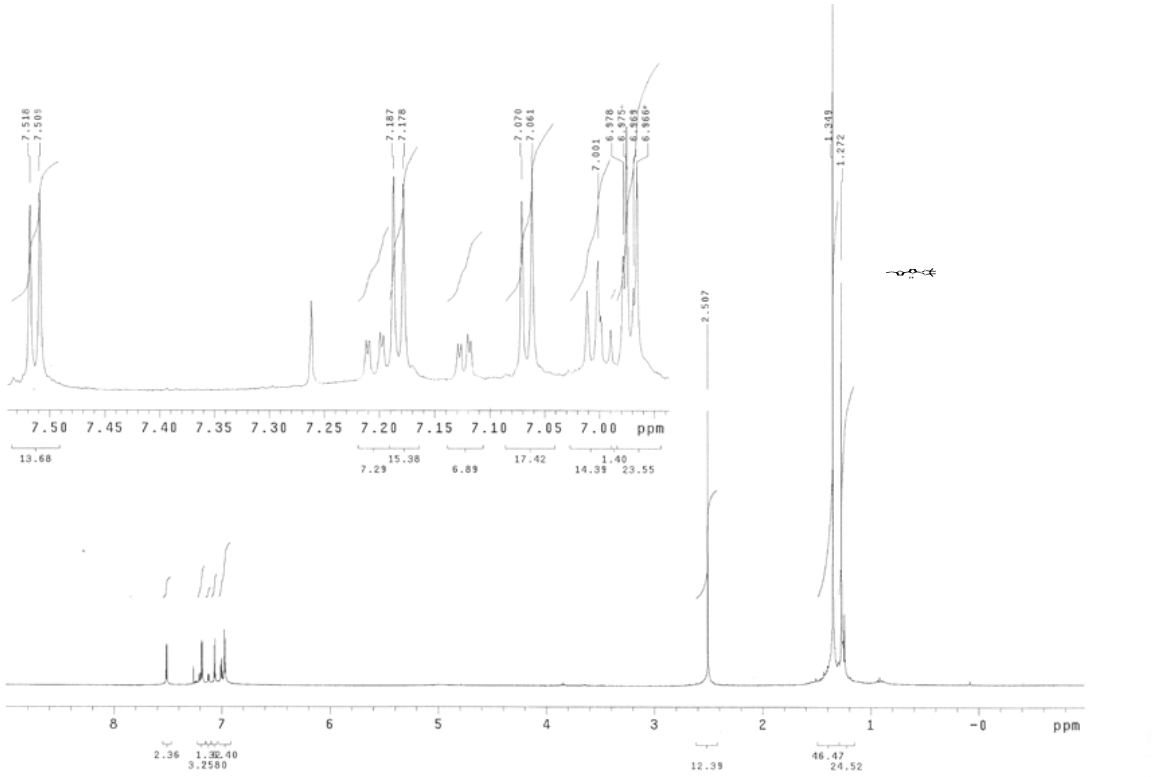


Figure 14S. ^{1}H NMR spectrum of **14** in $CDCl_3$.

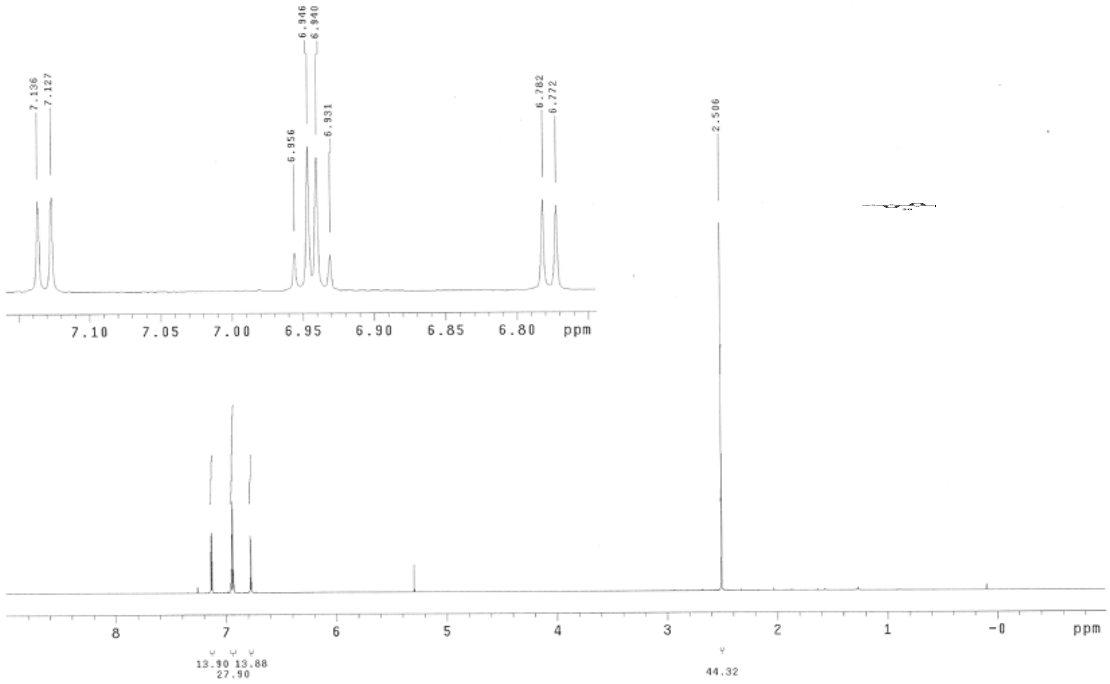


Figure 15S. ^{1}H NMR spectrum of **20** in $CDCl_3$.

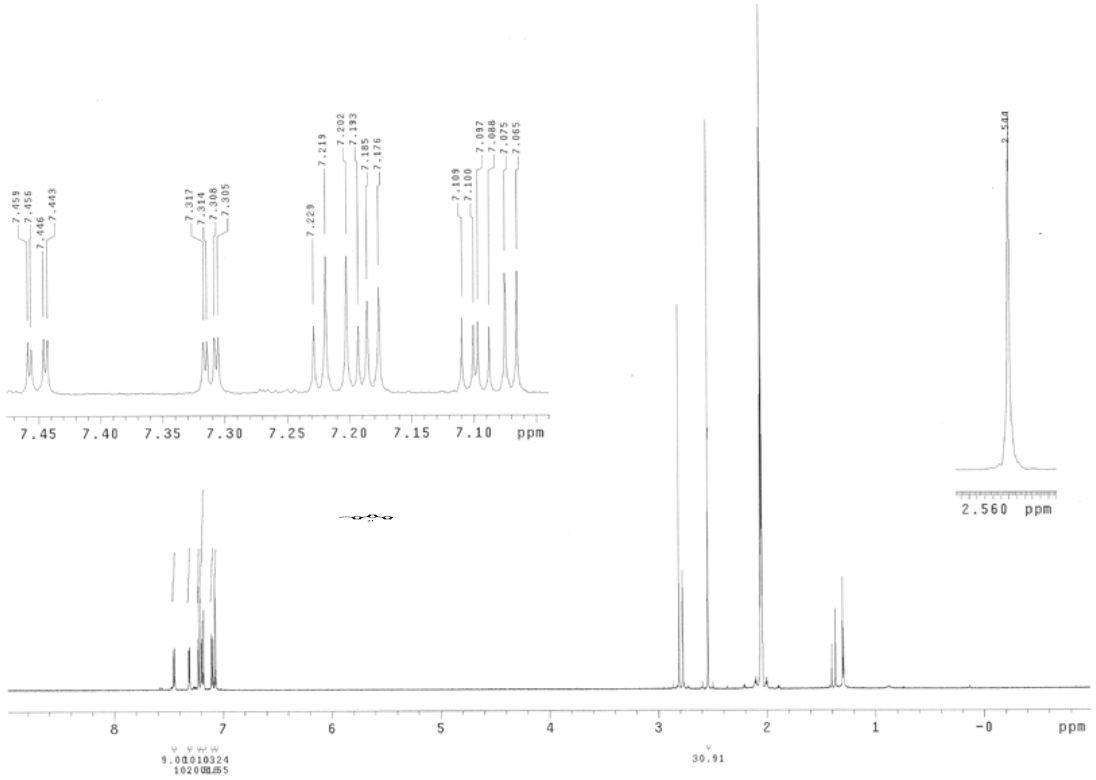


Figure 16S. ^{1}H NMR spectrum of **21** in aceton-d_6.

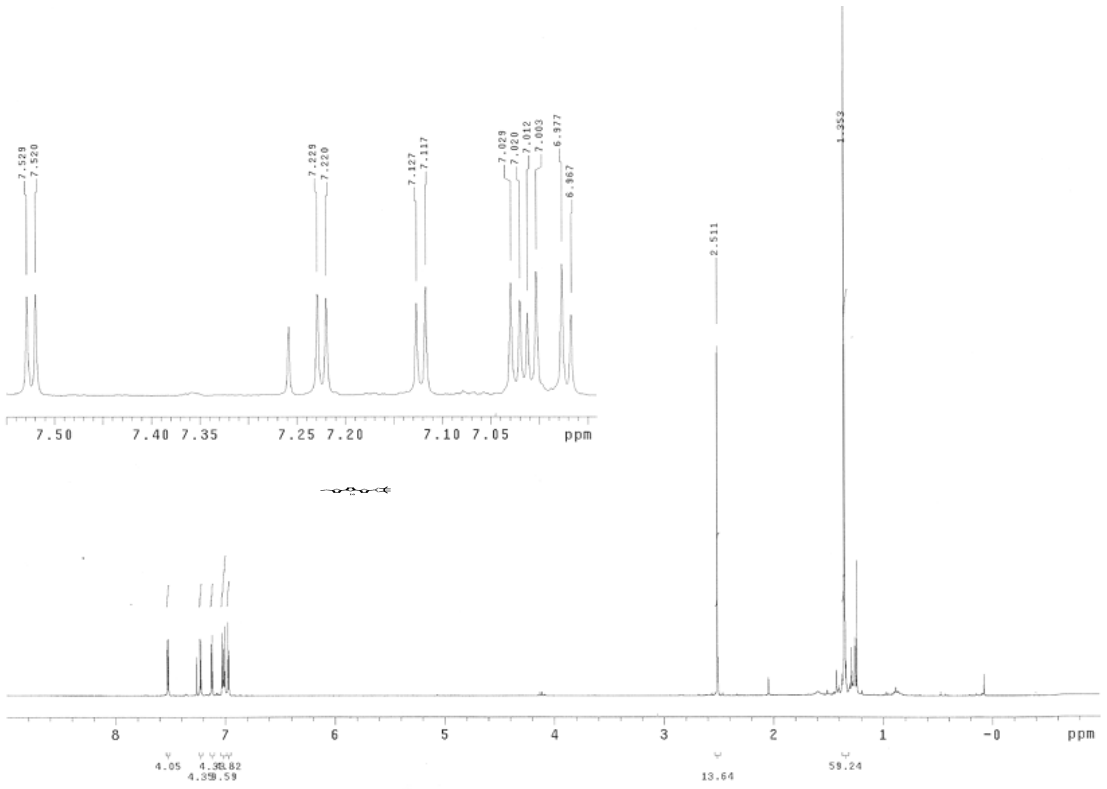


Figure 17S. ^{1}H NMR spectrum of **16** in $CDCl_3$.

III. Mass Spectra (note that all mass spectra show the molecular peak minus 103, corresponding to the weight of the SO_3Na fragment)

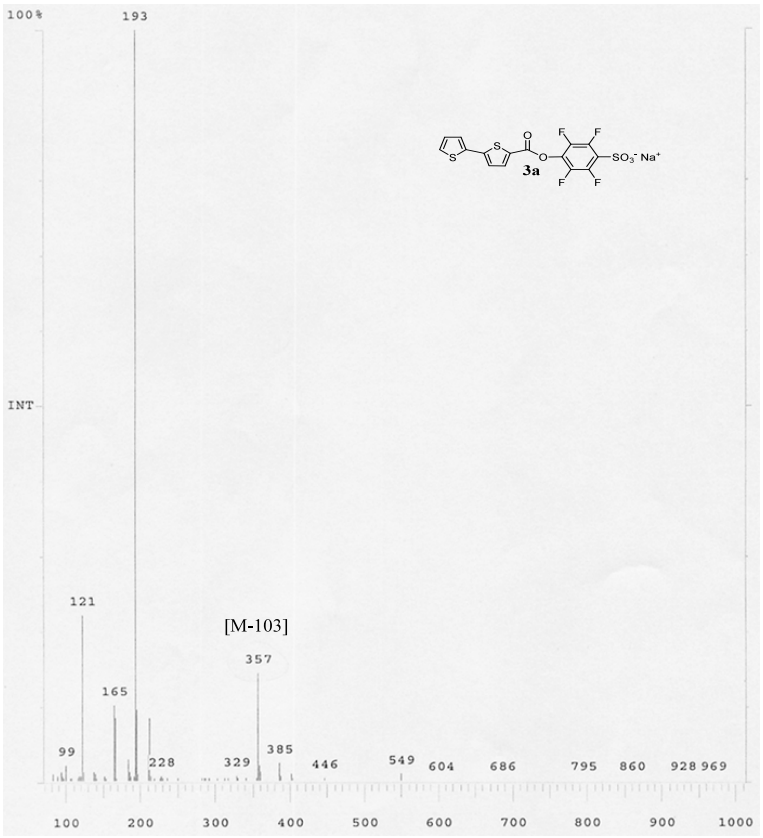


Figure 18S. EI mass spectrum of **3a**.

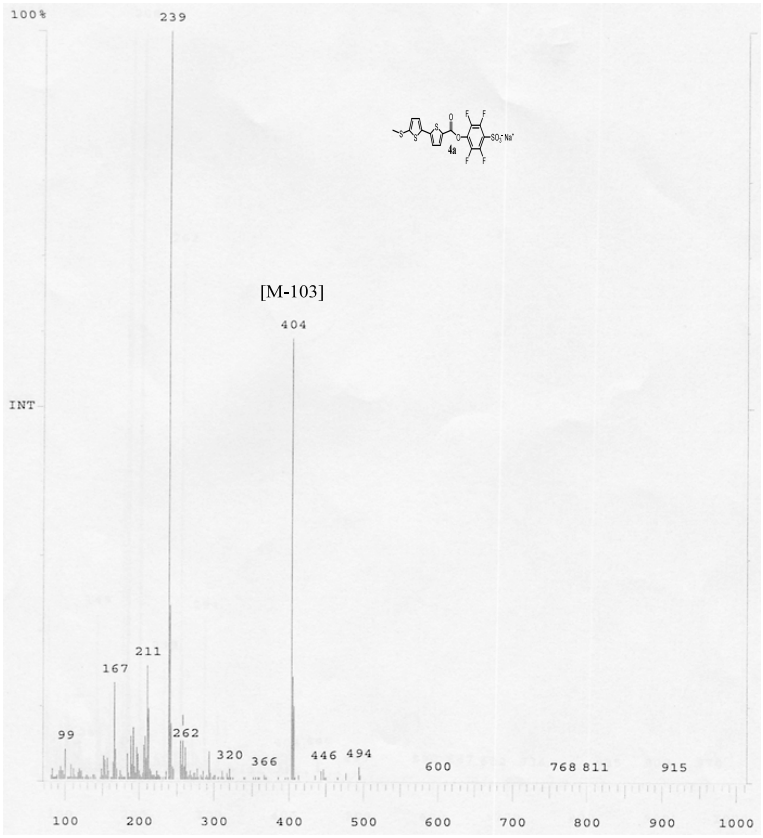


Figure 19S. EI mass spectrum of **4a**.

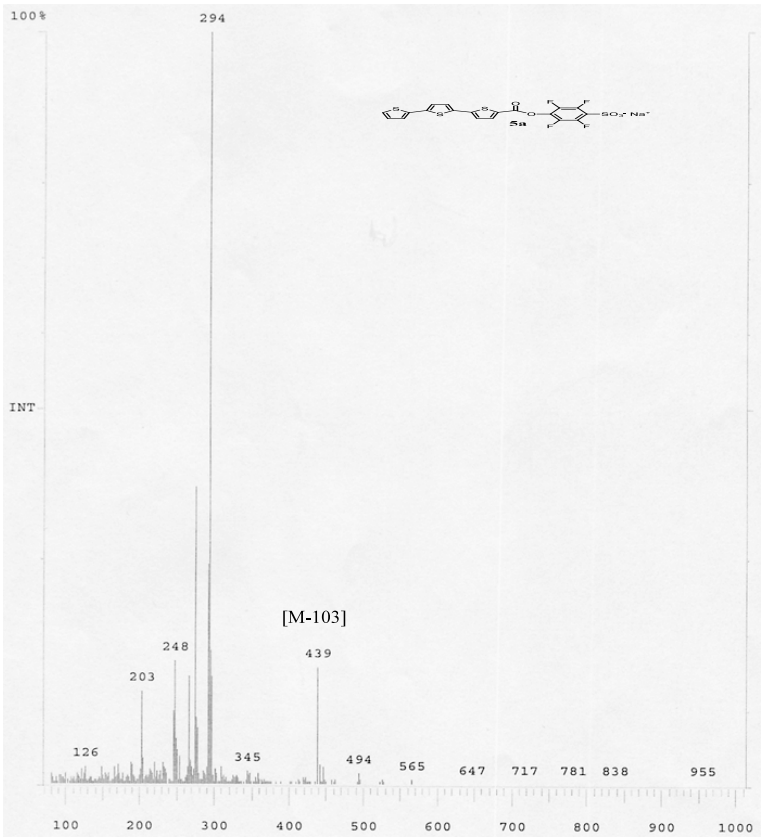


Figure 20S. EI mass spectrum of **5a**.

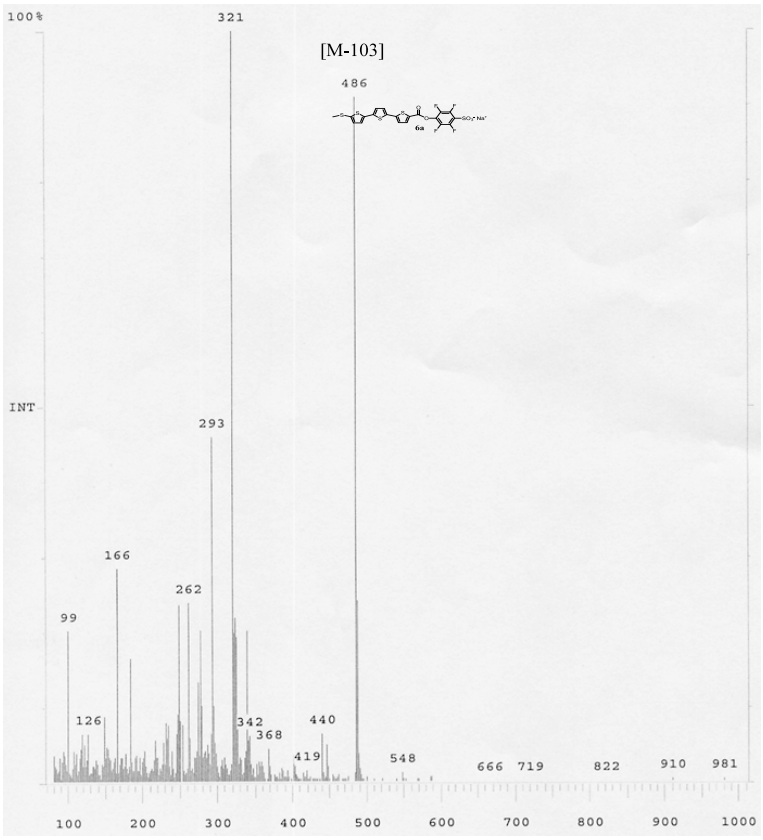


Figure 21S. EI mass spectrum of **6a**.

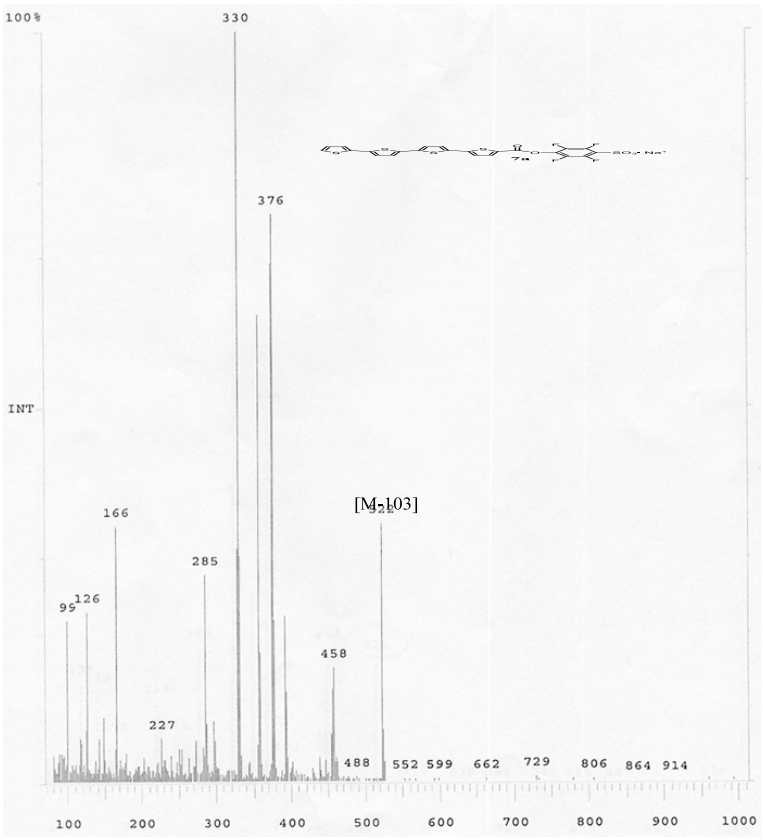


Figure 22S. EI mass spectrum of **7a**.

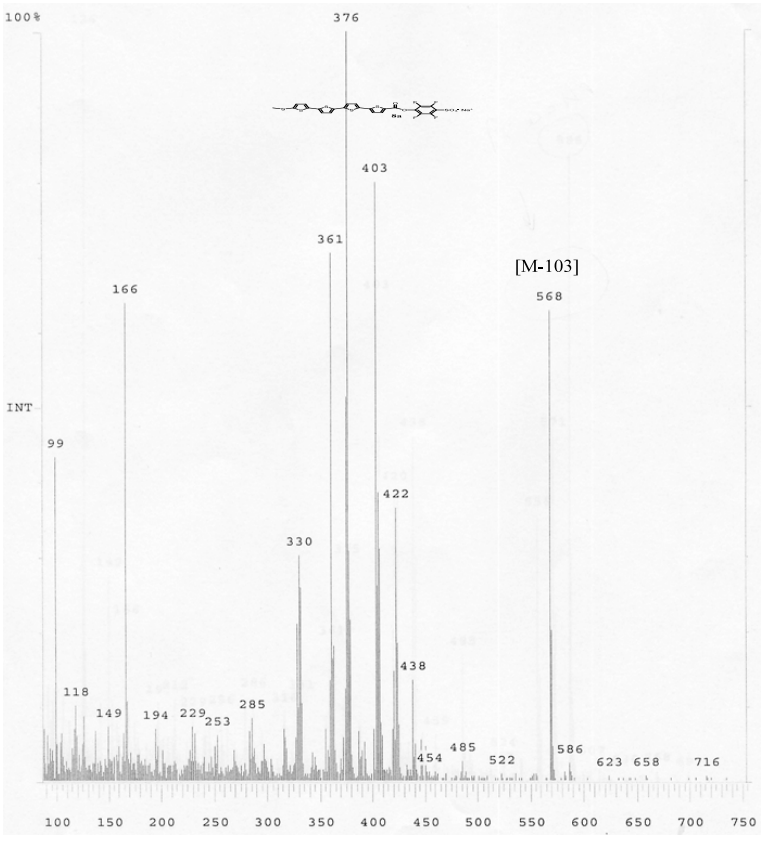
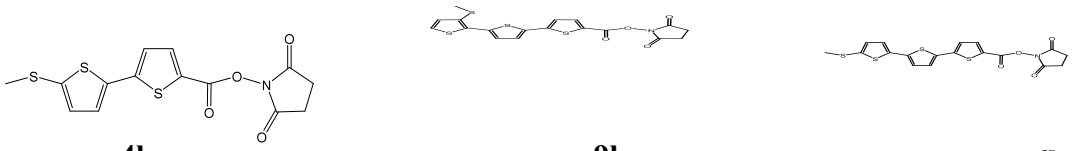


Figure 23S. EI mass spectrum of **8a**.

IV. Optical properties

IVa. *Absorption and photoluminescence spectra of fluorophores* 4b, 9b, 6b *as a function of the solvent*



4b **9b** **6b**

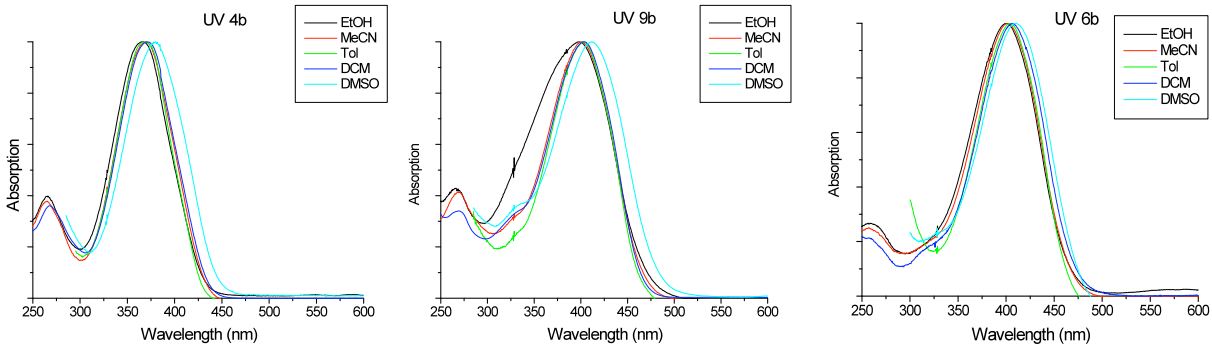


Figure 24S. Absorption spectra of TFs **4b, 9b, 6b** in ethanol (EtOH), acetonitrile (MeCN), toluene (Tol), dichloromethane (DCM) and dimethylsulfoxide (DMSO).

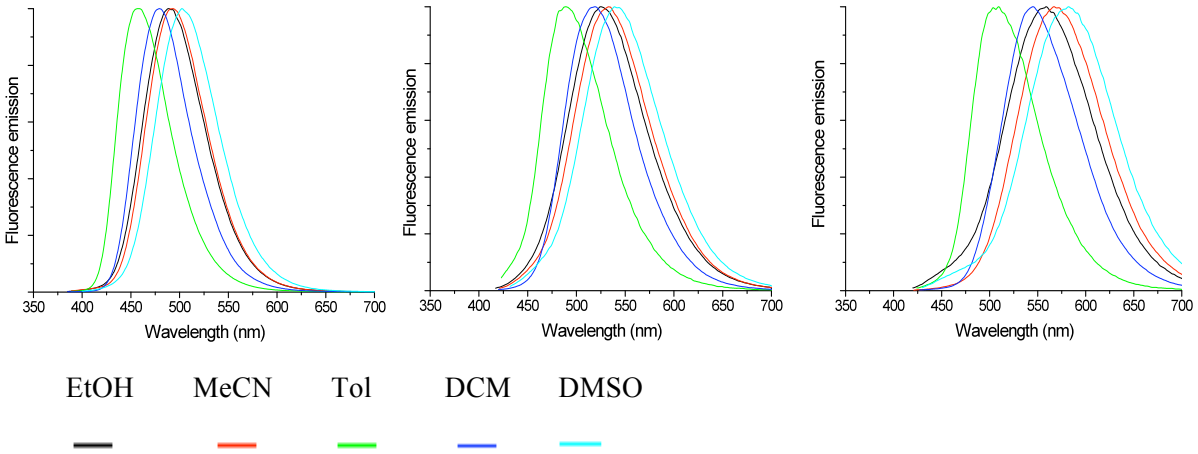


Figure 25S. Fluorescence spectra of TFs **4b, 9b, 6b** in different solvents.

Table 1S. Absorption (λ_{max}, nm) and photoluminescence (λ_{PL}, nm) wavelengths of TFs **4b,9b,6b** as a function of the dielectric constant (ε) of the solvent.

		4b		9b		6b	
Solvent	ε	λ_{max}	λ_{PL}	λ_{max}	λ_{PL}	λ_{max}	λ_{PL}
Tol	2.4	369	458	403	489	404	509
DCM	9.1	370	479	403	518	407	545
EtOH	25.0	365	489	397	525	400	559
MeCN	37.5	369	493	401	533	401	567
DMSO	48.9	380	502	412	539	410	582

IVb. *Photobleaching under irradiation with 100 W mercury lamp: comparison of TF* **6a** *with fluorescein*

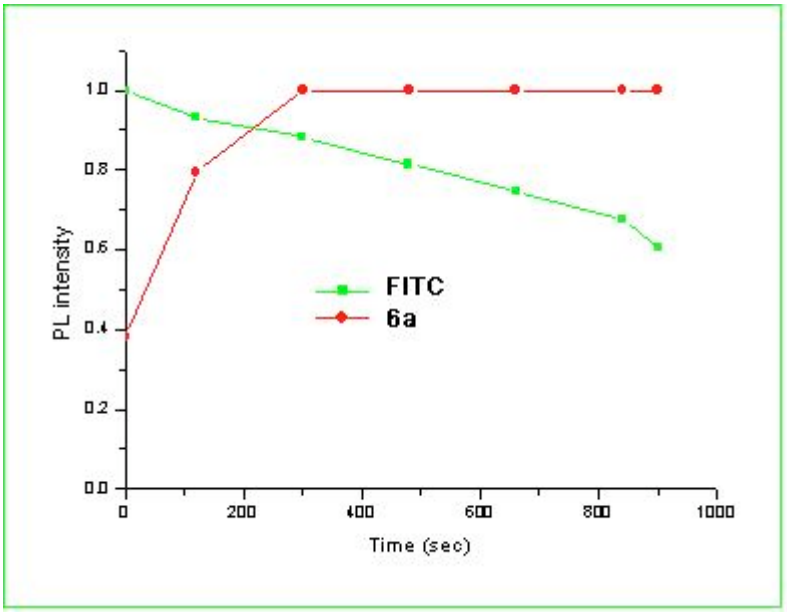


Figure 26S. Comparison of the photobleaching of **6a** and fluorescein as a function of time under irradiation with a 100 W mercury lamp Newport.

The solutions - 10^{-4} M in PBS Tween 0.5% pH 7.4 - were mantained under lamp excitation for a given time, then they were analysed with a Perkin Elmer Lambda 20 spectrometer and Perkin Elmer LS50 spectrofluorometer. Each point corresponds to fluorescence intensity for a fixed value of absorption (0.2) obtained by irradiation at the maximum absorption wavelength of the fluorophore.

IVc. *Quantum yield (□) measurements*

The concentration of air-equilibrated sample solutions was adjusted to obtain absorption values < 0.1 at the excitation wavelengths. Luminescence quantum efficiencies (□) were evaluated by comparing the intensities (*I*) of wavelength integrated corrected spectra with reference to quinine sulfate ($□_r$ = 0.546 in air-equilibrated 1N H_2SO_4)[1] and $[Ru(bpy)_3]Cl_2$ ($□_r$ = 0.028 in air-equilibrated

water), by using the following equation[5-7]: $\Phi = [(A \times n^2 / I) / (A \times n^2 / I)_r] \times \Phi_r$ where A and n are the absorbance values at the employed excitation wavelength, and the refractive index of the solvent, respectively.

V. Theoretical calculations

Amos-Burrows equation:[8]

$$E_{solv.}^{PL} = E_{vac.}^{PL} + \frac{0.623}{a^3}\left(2\left(\vec{\mu}_g \cdot \vec{\mu}_e - \|\vec{\mu}_e\|^2\right)(f-g)\right) + \left(\|\vec{\mu}_g\|^2 - \|\vec{\mu}_e\|^2\right)g$$

where $E_{vac..}^{PL}$ is the emission energy in vacuum, $f = (\varepsilon - 1)/(\varepsilon + 2)$, $g = (n^2 - 1)/(n^2 + 2)$, ε is the static dielectric constant, n is the refractive index at optical frequencies and a is related to the molecular volume.[9] In the above formula E is eV, a in Å and the dipoles in Debye.

The a value was fixed to 6.5 Å for TFs **9b** and **6b** and reduced by 15% for TF **4b** according to the reduced computed molecular volume.

All theoretical calculations were performed using the TURBOMOLE 5.9 program package.[10]

VI. Labeling of monoclonal antibodies (MoAbs)

VIa. ***Mono-labeling of anti-CD38 and anti-CD4 MOAbs***

<u>Comparative conjugation of TFs-STP and TFs-NHS with anti-CD38 monoclonal antibody</u>

Anti-CD38 antibody was exchanged over a column and dissolved in alkaline carbonate buffer pH 9.3 containing 0.5% tween-20. Then the solution was concentrated by microcon membrane with 50 kD cut off, to obtain a final protein concentration in the range of 2.5-3.0 mg/mL.

The TFs-STP and TFs-NHS were dissolved in anhydrous DMSO, immedialtely before use at a concentration comprised between 10mg/mL and 4 mg/mL, according to the intrinsic solubility of the fluorophores. Each fluorophore solution was added to the solution of the antibody in the amount needed to reach protein:fluorophore 1:15 molar ratio. After stirring for 1 min, the mixtures were incubated at room temperature for 30 min. The separation of the different conjugates from unreacted fluorophores was realized through a desalting column equilibrated with PBS pH 7.4 (storage buffer), using Sephadex G50 as medium. The optical characterization was performed using a Perkin Elmer Lambda 20 spectrometer and photoluminescence spectra were collected on a Perkin

Elmer LS50 spectrofluorometer. After UV measurements the bioconjugate was stabilized with a solution of phosphate buffer saline containing 0.5% (w/v) of bovine serum albumine (BSA) and 0.1% (w/v) of sodium azide. The determination of F/P and protein concentration was done by measuring the absorbance at 280 nm, at the maximum absorption wavelength of the fluorophore and by correcting for a factor corresponding to the absorption band around 280 nm present in the spectrum of the fluorophore.

Preparation of Anti-CD4-6b conjugate

Anti-CD4 antibody was exchanged over a column and dissolved in alkaline carbonate buffer pH 9.3 containing 0.5% tween-20 and the solution concentrated by microcon membrane with 50 kD cut off to obtain a final protein concentration of 3.0 mg/mL. Fluorophore **6b** was dissolved in anhydrous DMSO immediately before use at a concentration of 10mg/mL. The fluorophore solution was added to the solution of the antibody in the amount needed to reach protein:fluorophore 1:15 molar ratio. After stirring for 1 min, the mixture was incubated at room temperature for 120 min. The separation of the conjugate from unreacted fluorophore was realized through a desalting column equilibrated with PBS pH 7.4 (storage buffer), using Sephadex G50 as medium. The optical characterization was performed using a Perkin Elmer Lambda 20 spectrometer and photoluminescence spectra were collected on a Perkin Elmer LS50 spectrofluorometer. After UV measurements the bioconjugate was stabilized with a solution of phosphate buffer saline containing 0.5% (w/v) of bovine serum albumine (BSA) and 0.1% (w/v) of sodium azide. The determination of F/P and protein concentration was done by measuring the absorbance at 280 nm, at the maximum absorption wavelength of the fluorophore and by correcting for a factor corresponding to the absorption band around 280 nm present in the spectrum of the free fluorophore. Absorption and photoluminescence spectra of the conjugate are shown in Figure 20S.

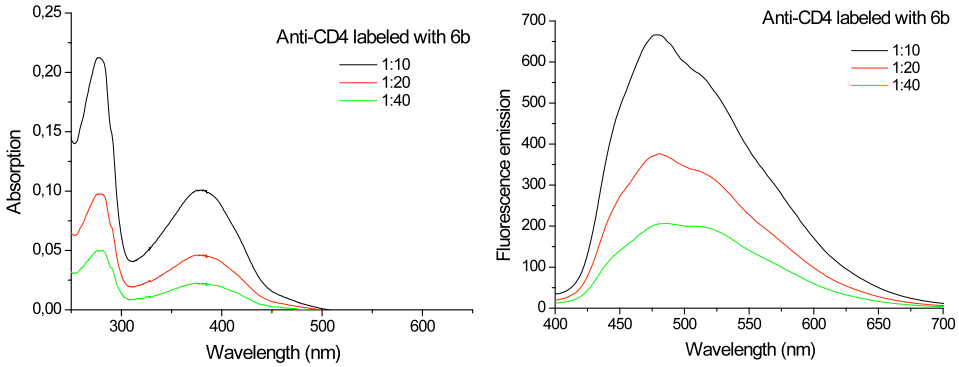


Figure 27S. Absorption and fluorescence spectra of anti-CD4 MoAb labeled with **6b**.

VIb. ***Multiple-labeling of anti-CD38 and anti-CD4 and white-emitting MoABs***

Procedure for the preparation of white emitting anti-CD38 using **3a** and **8a**

MoAb 3 mg/mL anti-CD38 **3a** 1:5, **8a** 1:30

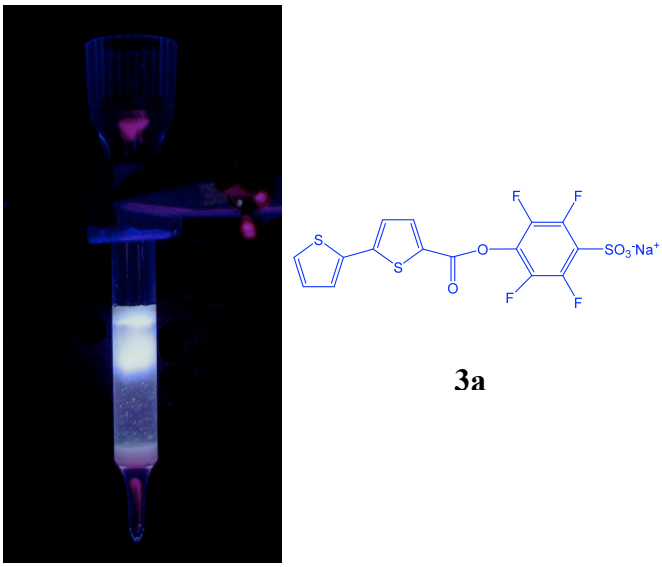
Anti-CD38 antibody was exchanged over a column and dissolved in alkaline carbonate buffer pH 9.3 containing 0.5% tween-20 and the solution concentrated by microcon membrane with 50 kD cut off to obtain a final protein concentration of 3 mg/mL. Fluorophores **3a** and **8a** were dissolved in anhydrous DMSO, immedialtely before use, the former at 10 mg/mL and the latter at 5 mg/mL. The fluorophore solutions were added simultaneously to the solution of the antibody in the amount needed to reach protein:fluorophore 1:5 molar ratio, for **3a**, and 1:30 molar ratio for **8a**. After stirring for 1 min, the mixture was incubated at room temperature for 30 min. The separation of the conjugate from unreacted fluorophores was realized through a desalting column equilibrated with PBS pH 7.4 (storage buffer), using Sephadex G50 as medium.

The optical characterization was performed using a Perkin Elmer Lambda 20 spectrometer and photoluminescence spectra were collected on a Perkin Elmer LS50 spectrofluorometer. After UV measurements the bioconjugate was stabilized with a solution of phosphate buffer saline containing 0.5% (w/v) of bovine serum albumine (BSA) and 0.1% (w/v) of sodium azide. The determination of F/P and protein concentration was done by measuring the absorbance at 280 nm, 350 nm, 420 nm and by correcting for a factor corresponding to the absorption band around 280 nm present in the spectrum of **3a** and **8a**. An additional correction factor is necessary for the determination of **3a** F/P because of the overlapping of **8a** absorption band in the **3a** absorption region.

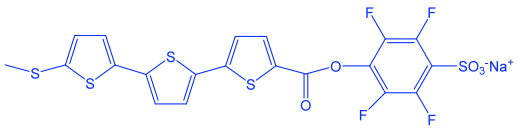
3 mg/mL anti-CD38 **3a** 1:5, **6a** 1:40

Anti-CD38 antibody was exchanged over a column and dissolved in alkaline carbonate buffer pH 9.3 containing 0.5% tween-20. Then the solution was concentrated by microcon membrane with 50 kD cut off to obtain a final protein concentration of 3 mg/mL. Fluorophores **3a** and **6a** were dissolved in anhydrous DMSO, immedialtely before use, at 10 mg/mL, the fluorophore solutions were added simultaneously to the solution of the antibody in the amount needed to reach protein:fluorophore 1:5 molar ratio, for **3a**, and 1:40 molar ratio for **6a**. After stirring for 1 min, the mixture was incubated at room temperature for 30 min. The separation of the conjugate from unreacted fluorophores was realized through a desalting column equilibrated with PBS pH 7.4 (storage buffer), using Sephadex G50 as medium. The optical characterization was performed using a Perkin Elmer Lambda 20 spectrometer and photoluminescence spectra were collected on a Perkin Elmer LS50 spectrofluorometer. After UV measurements the bioconjugate was stabilized with a solution of phosphate buffer saline containing 0.5% (w/v) of bovine serum albumine (BSA) and 0.1% (w/v) of sodium azide. The determination of F/P and protein concentration was done by measuring the absorbance at 280 nm, 350 nm, 405 nm and by correcting for a factor corresponding

to the absorption band around 280 nm present in the spectrum of **3a** and **6a**. An additional correction factor is necessary for the determination of **3a** F/P because of the weak overlapping of **6a** absorption band in the **3a** absorption region.

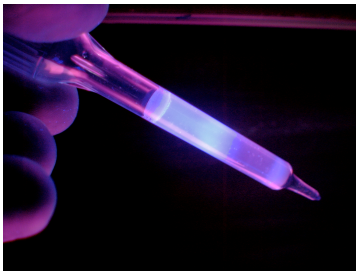


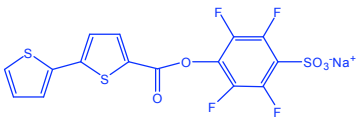
3a



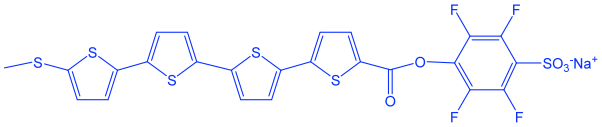
6a

Figure 28S. Photograph of the white emitting anti-CD38 MoAb obtained by multiple-labeling with **3a** and **6a** obtained by exciting with a 15 W UV lamp at λ_{exc}=364 nm. Spectra in the article.

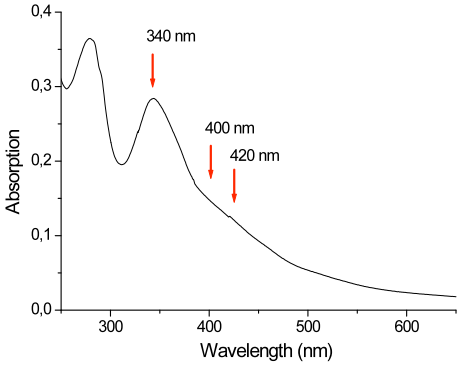




3a



8a



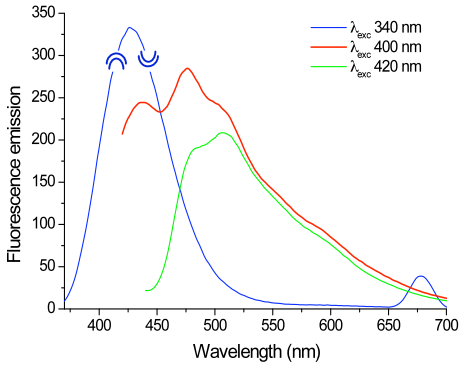
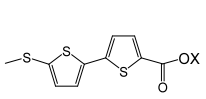
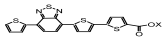


Figure 29S. Photograph and absorption and emission spectra of the white emitting anti-CD38 MoAb obtained by multiple-labeling with **3a** and **8a**. The photograph was obtained by exciting with a 15 W UV lamp at λ_{exc}=364 nm.

<u>Procedure for the preparation of white emitting anti-CD38 using **4b** and **10b**</u>



4b



10b

X = 

3 mg/mL anti-CD38 **4b** 1:30, **10b** 1:30

Anti-CD38 antibody was exchanged over a column and dissolved in alkaline carbonate buffer pH 9.3 containing 0.5% tween-20. Then the solution was concentrated by microcon membrane with 50 kD cut off, to obtain a final protein concentration of 3 mg/mL. Fluorophores **4b** and **10b** were dissolved in anhydrous DMSO, immedialtely before use, the former at 10 mg/mL and the latter at 4 mg/mL. The fluorophore solutions were added simultaneously to the solution of the antibody in the amount needed to reach protein:fluorophore 1:30 molar ratio. After stirring for 1 min, the mixture was incubated at room temperature for 30 min. The separation of the conjugate from unreacted fluorophores was realized through a desalting column equilibrated with PBS pH 7.4 (storage buffer), using Sephadex G50 as medium. The optical characterization was performed using a Perkin Elmer Lambda 20 spectrometer and photoluminescence spectra were collected on a Perkin Elmer LS50 spectrofluorometer, after UV measurements the bioconjugate was stabilized with a solution of phosphate buffer saline containing 0.5% (w/v) of bovine serum albumine (BSA) and 0.1% (w/v) of sodium azide. The determination of F/P and protein concentration was done by measuring the absorbance at 280 nm, 350 nm, 480 nm and by correcting for a factor corresponding to the absorption band around 280 nm present in the spectrum of **4b** and **10b**.

VII. Immunostaining

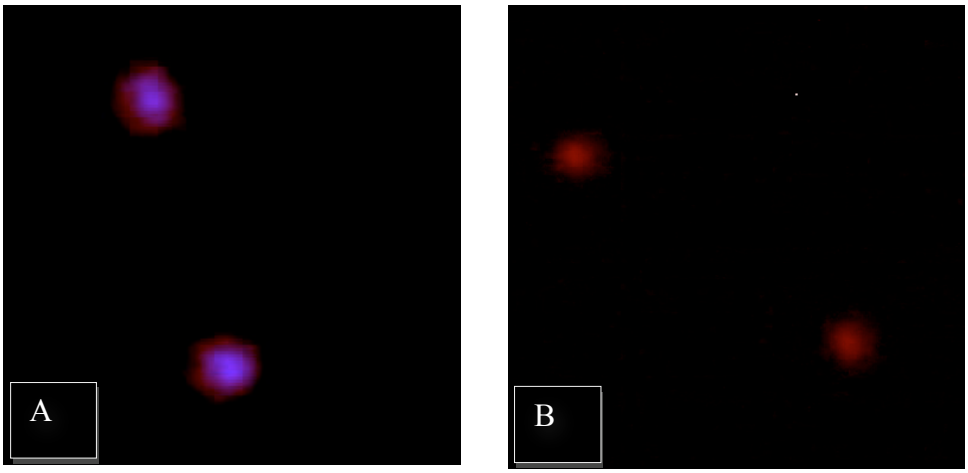


Figure 30S. A) Fluorescence microscopy images of MOLT 4 cell line stained using the anti-CD4 monoclonal antibody labeled with fluorophore **6b** and examined 1 month after preparation and counterstaining with DAPI. B) Same sample examined 3 months after preparation. Note the absence of blue fluorescence due to quenching of DAPI.
Images A and B have been obtained with the same sample as that of Figure 6 in the article. The sample slide was kept in air into a clean room at 18-22°C without any light protection.

Va. *Procedure for immunostaining*

Materials
DAPI was purchased from Invitrogen Corporation

Solutions used
-TBS Tween: 0.05M Tris Base / 0.9%NaCl / 0.05% Tween20 pH=7.6
-PEM Buffer: 80mM Pipes/5mM EGTA/2mMMgCl
-Blocking Solution: 1% BSA / 0.02% NaN3 in TBS Tween
-4% Formaldeide in PEM Buffer
-0.1M Ammonium Chloride in PEM Buffer
-6uM DAPI in TBS-Tween

Cells used
MOLT-4 cell line

Cell staining protocol
1: BLOCKING
-Centrifugation of cells suspension (5min; 1.200rpm; Room Temperature)
-Resuspension of pellet in Blocking Solution
-Incubation (60min; RT)
-Centrifugation of cells suspension (5min; 1.200rpm; Room Temperature)
-Resuspension of pellet in remaining supernatant and suspension divided into three tubes

2: IMMUNOSTAINING

-Used the scheme showed below:

-100ul Cells	+	Negative Control 1
-100ul Cells	+	10ul Bl. Buffer (Negative Control 2)
-100ul Cells	+	10ul CD4-AMZ12 (diluted 1:30 in Bl. Buffer)

-Incubation (180min; RT) and each tube diluted into 1mL of Bl.Buffer

3: WASHING
-Centrifugation of tubes (5min; 1.200rpm; RT)
-Resuspension of tubes in TBS-Tw
-Centrifugation of tubes (5min; 1.200rpm; RT)
-Resuspension of tubes in PEM Buffer

4: FIXATION WITH FORMALDHEYDE
-Centrifugation of tubes (5min; 1.200rpm; RT)
-Resuspension of tubes in 4% Formaldeide in PEM Buffer
-Incubation (20min; RT;in the dark)
-Centrifugation of tubes (5min; 1.200rpm; RT)
-Resuspension of tubes in PEM Buffer

5: QUENCHING OF AUTO-FLUORESCENCE
-Centrifugation of tubes (5min; 1.200rpm; RT)
-Resuspension of tubes in 0.1M Ammonium Chloride in PEM Buffer
-Centrifugation of tubes (5min; 1.200rpm; RT)
-Resuspension of tubes in 0.1M Ammonium Chloride in PEM Buffer
-Centrifugation of tubes (5min; 1.200rpm; RT)
-Resuspension of tubes in TBS-Tween

6: COUNTERSTAINNG WITH DAPI
-Centrifugation of tubes (5min; 1.200rpm; RT)
-Resuspension of tubes in 6uM DAPI in TBS-Tween
-Centrifugation of tubes (5min; 1.200rpm; RT)
-Pellet of each tubes in remaining supernatant

7: MOUNTING THE COVERSLIP
-Added 3ul of each tube on coverslide previously cleaned with Etanol 99%

VIII. References

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