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Supporting Information

69451 Weinheim, Germany

Highly Fluorescent and Water-Soluble Diketopyrrolopyrrole Dyes for Bioconjugation**

Elodie Heyer, Pauline Lory, Jérôme Leprince, Mathieu Moreau, Anthony Romieu, Massimo Guardigli, Aldo Roda, and Raymond Ziessel**

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Supporting Information

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Abbreviations

The following abbreviations are used throughout the text of the ESI file: AcOH, acetic acid; ATR, attenuated total reflectance; BSA, bovine serum albumin; CV, cresyl violet; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; DPP; diketopyrrolopyrrole; ESI, electrospray ionization; EtOH, ethanol; EtOAc, ethyl acetate; FT-IR, Fourier transformed infrared spectroscopy; HRMS, high-resolution mass spectrometry; LRMS, low-resolution mass spectrometry/spectrum; MALDI-TOF, matrix-assisted laser desorption ionization - time-of-flight; NHS, *N*-hydroxysuccinimidyl; NMP, *N*-methylpyrrolidone; PBS, phosphate buffered saline; PyBrOP, bromotripyrrolidinophosphonium hexafluorophosphate; R6G, rhodamine 6G; RP-HPLC, reversed-phase high performance liquid chromatography; rpm, round per minute; rt, room temperature; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoresis; TEA, triethylamine; TEAB; triethylammonium bicarbonate; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography; TSTU, *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate.

General

Unless otherwise noted, all other commercially available reagents and solvents were used without further purification. TLC were carried out on Merck DC Kieselgel 60 F-254 aluminum sheets. The spots were directly visualized or through illumination with UV lamp (λ = 254/365 nm) and/or staining with a phosphomolybdic acid solution (4.8% wt. in EtOH). Column chromatography purifications were performed on silica gel (technical grade, pore size 30 Å, granulometry 63-200 μm was preferred for DPP derivatives to reduce non-specific adsorption of these dyes over the stationary phase) from Sigma-Aldrich. All chemicals were used as received from commercial sources without further purification unless otherwise stated. DIEA and NMP (peptide synthesis grade) were purchased from Iris Biotech GmbH. The HPLC-gradient grade acetonitrile (CH_3CN) was obtained from Carlo Erba. Phosphate buffered saline (PBS, 100 mM phosphate + 150 mM NaCl, pH 7.3), phosphate buffer (KH_2PO_4/K_2HPO_4, 66.7 mM, pH 7.05 or 7.70) and aq. mobile-phases for HPLC were prepared using water purified with a PURELAB Ultra system from ELGA (purified to 18.2 MΩ.cm). Triethylammonium bicarbonate (TEAB, 1.0 M) buffer was prepared from distilled TEA and CO_2 gas. 2-Aminoethane-1,1-disulfonic acid and its tributylammonium salt were prepared according to literature procedures[1]. This product is now commercially available from Strem Chemicals Inc. (Newburyport, MA, USA).

Instruments and methods

Centrifugation steps were performed with an Hettich Universal 320 instrument. ^{1}H- and ^{13}C-NMR spectra of compounds **3-9** were recorded either on a Bruker Avance 300 or on a Bruker Avance 500 spectrometer. Chemical shifts are expressed in parts per million (ppm) from the

[1]A. Romieu, D. Tavernier-Lohr, S. Pellet-Rostaing, M. Lemaire, P.-Y. Renard, *Tetrahedron Lett.* **2010**, *51*, 3304-3308.

residual non-deuterated solvent signal[2]. *J* values are expressed in Hz. Infrared (IR) spectra were recorded either with a Perkin-Elmer FT-IR spectrometer equipped with an ATR diamond apparatus on thin solid layer or with an universal ATR sampling accessory on a Bruker Alpha FT-IR spectrometer. Both analytical and semi-preparative HPLC were performed on a Thermo-Dionex Ultimate 3000 instrument equipped with a RS Variable Detector (four distinct wavelengths). For analyses, a flow-through back-pressure regulator was used. Ion-exchange chromatography (for desalting DPP-acid derivatives purified with TEAB as aq. mobile phase) was performed with an Econo-Pac® disposable chromatography column (Bio-Rad, #732-1010) filled with an aq. solution of Dowex® 50WX8 (Fluka, granulometry 595-841 μm ~ 5-10 g for 10 mg of dye, 14 × 70 mm bed), regenerated using aq. 10% HCl solution and equilibrated with deionized water. Low-resolution mass spectra (LRMS) were obtained with either a Bruker Amazon LS (ion trap) or a Thermo Finnigan LCQ Advantage MAX (ion trap) apparatus equipped with an electrospray ionization (ESI) source. High-resolution mass spectra (HRMS) were recorded on a Thermo LTQ Orbitrap XL apparatus equipped with an electrospray ionization (ESI) source. The purified BSA-DPP conjugates were characterized by MALDI-TOF mass spectrometry either on an Applied Biosystems Voyager DE PRO mass spectrometer or on a Bruker Ultraflex II LRF 2000 mass spectrometer (linear detector mode, sinapinic acid as a matrix, positive mode). UV-visible spectra was obtained either on a Varian Cary 50 scan spectrophotometer by using a rectangular quartz cell (Hellma, 100-QS, 45 × 12.5 × 12.5 mm, pathlength 10 mm, chamber volume: 3.5 mL) or on a Shimadzu UV-3600 dual-beam grating spectrophotometer with a 1 cm quartz cell. The molar absorption coefficients of DPP dyes were obtained as the slope of regression line A = f(concentration) created from absorbance measurements of three independant solutions (concentration in the range 10^{-5} to 10^{-6} M^{-1}). Fluorescence spectroscopic studies (emission/excitation spectra) were performed either with an HORIBA Jobin Yvon Fluorolog spectrophotometer (software DataMax) with a standard fluorometer cell (Labbox, LB Q, 10 mm) or on an HORIBA Jobin Yvon FluoroMax 4P spectrofluorimeter. Emission spectra were recorded under the same conditions after excitation at the corresponding wavelength (shutter: auto open, excitation and emission slit = 10 nm). All fluorescence spectra were corrected. Luminescence lifetimes were measured on a FL 920 Edimburgh Instruments spectrofluorimeter equipped with a R928 photomultiplier and a PicoQuant PDL 800-D pulsed diode connected to a GwInstect GFG-8015G delay generator. Emission wavelengths were selected by a monochromator. Lifetimes were deconvoluted with FS-900 software using a light-scattering solution (LUDOX) for instrument response.

Fluorescence quantum yields were measured at 25 °C by a relative method using cresyl violet (CV, Φ_F = 50% in EtOH), fluorescein (Φ_F = 90% in 0.1 N NaOH) or rhodamine 6G (R6G, Φ_F = 76% in H_2O) as a standard[3]. The following equation was used to determine the relative fluorescence quantum yield:

$$\Phi_F(x) = (A_S/A_X)(F_X/F_S)(n_X/n_S)^2\Phi_F(s)$$

where A is the absorbance (in the range of 0.01-0.1 A.U.), F is the area under the emission curve, n is the refractive index of the solvents (at 25 °C) used in measurements, and the subscripts s and x represent standard and unknown, respectively. The following refractive index value is used: 1.333 for H_2O, 1.477 for DMSO, 1.362 for EtOH, 1.337 for PBS, aq. 2.5% SDS and 0.1 N NaOH and 1.404 for THF.

[2]G. R. Fulmer, A. J. M. Miller, N. H. Sherden, H. E. Gottlieb, A. Nudelman, B. M. Stoltz, J. E. Bercaw, K. I. Goldberg, *Organometallics* **2010**, *29*, 2176-2179.
[3]J. Olmsted, III, *J. Phys. Chem.* **1979**, *83*, 2581-2584.

High-performance liquid chromatography separations

Two chromatographic systems were used for the analytical experiments and the purification steps respectively: System A: RP-HPLC (Thermo Electron Hypersil GOLD C_{18} column, 5 µm, 4.6 × 100 mm) with CH_3CN and aq. TEAB (50 mM, pH 7.5) as eluents [0% CH_3CN (5 min), followed by a linear gradient from 0% to 80% CH_3CN (40 min)] at a flow rate of 1.0 mL/min. Quadruple UV-vis detection was achieved at 220, 260, 495 and 530 nm. System B: semi-preparative RP-HPLC (SiliCycle SiliaChrom C_{18} column, 10 µm, 20 × 250 mm) with CH_3CN and aq. TEAB (50 mM, pH 7.5) as eluents [0% CH_3CN (5 min), followed by a gradient of 0% to 10% CH_3CN (5 min), then 10% to 50% CH_3CN (55 min)] at a flow rate of 20.0 mL/min. Quadruple UV-vis detection was achieved at 220, 260, 480 and 530 nm for DPP-thienyl derivatives and 220, 260, 445 and 495 nm for DPP-phenyl derivatives.

Synthesized compounds

*Di- and tetrasulfonated DPP dyes **7-9** were found to be soluble in D_2O but bad quality spectra were obtained (i.e., broad and poor-resolved peaks). Thus, all NMR spectra were recorded in [D_6]DMSO.*

3,6-Diphenyl-2,5-dihydropyrrolo[*3,4-c*]pyrrole-1,4-(2H,5H)-dione, DPP-phenyl (1)[4]. In a flame-dried Schlenk round-bottom flask, sodium metal (3.9 g, 169.6 mmol, 6.2 equiv.) was added to 2-methylbutan-2-ol (75 mL). The reaction medium was vigorously stirred and heated at 110 °C until complete consumption of Na^0, before adding 2-benzonitrile (8.5 mL, 83.0 mmol, 3.0 equiv.) in one portion. A solution of diethyl succinate (4.6 mL, 27.5 mmol, 1 equiv.) in 2-methylbutan-2-ol (18.0 mL) was added dropwise and the reaction medium was further stirred at 110 °C for 48 h. Thereafter, quenching of the reaction was performed by adding at 90 °C a solution of AcOH (18 mL) in CH_3OH (100 mL). The bright red suspension was centrifugated and washed several times with hot CH_3OH, until getting a colorless filtrate. The crude product was dried under vacuum to give a red powder (2.05 g, yield 26%) and used without further purification. IR (neat): ν = 3135, 3050, 2977, 2842, 2729, 1642, 1610, 1455, 1424, 1203, 1144, 813, 741, 631, 674, 662, 455 cm^{-1}. NMR data are identical to those reported by Shaabani *et al.*, and Takahashi and Mizuguchi[5].

3,6-Dithiophen-2-yl-2,5-dihydropyrrolo[*3,4-c*]pyrrole-1,4-(2H,5H)-dione, DPP-thienyl (2)[6]. In a flame-dried Schlenk round-bottom flask, sodium metal (3.2 g, 138.3 mmol, 5.0 equiv.) was added to 2-methylbutan-2-ol (75 mL), the resulting mixture was stirred at 110 °C until complete consumption of Na^0. The temperature was then decreased to 90 °C and 2-thiophenecarbonitrile (8.0 mL, 85.8 mmol, 3.0 equiv.) was added in one portion, affording a brown medium. A solution of diethyl succinate (4.8 mL, 28.7 mmol, 1.0 equiv) in 2-methylbutan-2-ol (18 mL) was added dropwise and the solution was stirred at 110 °C overnight. Thereafter, a solution of glacial AcOH (20 mL) in CH_3OH (100 mL) was added to the medium, resulting in the precipitation of a deep purple solid, collected by centrifugation. Several washes with hot CH_3OH were carried out until getting a colorless filtrate. The crude product was dried under vacuum to give a purple powder (7.9 g, yield 92%) and used without further purification. IR (neat): ν = 2988, 2797, 1639, 1600, 1430, 1413, 1183, 1129, 852, 778, 699, 538 cm^{-1}. NMR data are identical to those reported by Zou *et al.*[7]

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Di-*tert*-butyl DPP-phenyl *N,N*'-diacetate (3). DPP-phenyl **1** (105 mg, 0.36 mmol, 1 equiv.) was suspended in NMP (5 mL). Anhydrous K_2CO_3 (527 mg, 3.81 mmol, 10.6 equiv.) was added and the resulting mixture was heated at 120-125 °C (oil bath temperature) for 15 min. Thereafter, *tert*-butyl bromoacetate (0.509 mL, 3.47 mmol, 9.6 equiv.) in solution in NMP (2.5 mL) was added dropwise and the resulting mixture was heated at the same temperature for further 2 h. The reaction was checked for completion by TLC (CH_2Cl_2 100%) and the mixture was cooled to rt. Thereafter, this mixture was thrown into deionized water (25 mL) and the insoluble solid was recovered through centrifugation (3000 rpm, 15 min). The aq. phase was extracted with CH_2Cl_2 (3 × 40 mL) and the combined organic layers were used to dissolve the solid. The resulting organic phase was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The resulting residue was subjected to two column chromatography purifications. First purification: silica gel Sigma-Aldrich, 30 g, heptane 100%, then step gradient of CH_2Cl_2 in heptane from 0% to 100% to recover the desired product partially contaminated with NMP and *tert*-butylbromoacetate. Second purification: silica gel Merck Geduran® Si 60 (granulometry 63-200 μm), 20 g, step gradient of EtOAc in CH_2Cl_2 from 0% to 5%. Compound **3** was obtained as a bright yellow crystalline powder (94 mg, yield 51%). R_f 0.71 (CH_2Cl_2/EtOAc 9 : 1, v/v); IR (neat): ν = 2977, 2930, 1737, 1681, 1615, 1416, 1364, 1225, 1145, 1118, 1083, 985, 839, 797, 744, 703, 686 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ = 1.37 (s, 18 H, 2 × tBu), 4.41 (s, 4 H, 2 × N-C$\underline{H_2}$-CO_2tBu), 7.50 (m, 6 H, H-phenyl), 7.77 (m, 4 H, H-phenyl) ppm; ^{13}C NMR ($CDCl_3$, 126 MHz): δ = 28.0 (6 C), 44.3 (2 C), 82.7 (2 C), 109.8 (2 C), 128.0 (2 C), 128.8 (4 C), 129.1 (4 C), 131.4 (2 C), 148.4 (2 C), 162.4 (2 C), 167.6 (2 C) ppm; LRMS (ESI+): *m/z* 517.00 $[M + H]^+$ (100) and 1032.33 $[2M + H]^+$ (75), calcd for $C_{30}H_{32}N_2O_6$ 516.23; HRMS (ESI+): *m/z* 539.21574 $[M + Na]^+$ (100) and 1055.44253 $[2M + Na]^+$ (15), calcd for $C_{30}H_{32}N_2NaO_6^+$ 539.21526.

Di-*tert*-butyl DPP-thienyl *N,N*'-diacetate (4). DPP-thienyl **2** (100 mg, 0.34 mmol, 1 equiv.) was suspended in NMP (5 mL). Anhydrous K_2CO_3 (506 mg, 3.66 mmol, 11 equiv.) was added and the resulting mixture was heated at 120-125 °C (oil bath temperature) for 15 min. Thereafter, *tert*-butyl bromoacetate (0.489 mL, 3.40 mmol, 10 equiv.) in solution in NMP (2.5 mL) was added dropwise and the resulting mixture was heated at the same temperature for further 90 min. The reaction was checked for completion by TLC (CH_2Cl_2 100%) and the mixture was cooled to rt. Thereafter, this mixture was thrown into deionized water (25 mL) and the insoluble solid was recovered through centrifugation (3000 rpm, 15 min). The solid was washed with deionized water (25 mL), dissolved in CH_2Cl_2 (50 mL) and the resulting organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The resulting residue was purified by column chromatography (silica gel, 35 g, heptane 100%, then step gradient of CH_2Cl_2 in heptane 0% to 100% to remove *tert*-butylbromoacetate and finally elution with CH_2Cl_2/EtOAc 8 : 2, v/v to recover the desired product). Compound **4** was obtained as a dark reddish purple solid (112 mg, yield 64%). R_f 0.62 (CH_2Cl_2/EtOAc 95: 5, v/v); IR (neat): ν = 2976, 2926, 2853, 1739, 1650, 1566, 1422, 1409, 1366, 1224, 1151, 1122, 1027, 858, 744, 716 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ = 1.42 (s, 18 H, 2 × tBu), 4.79 (s, 4 H, 2 × N-C$\underline{H_2}$-CO_2tBu), 7.26 (dd, *J* 5.0, *J* 4.0, 2 H, H-thiophene), 7.61 (dd, *J* 5.2, *J* 1.0, 2 H, H-thiophene), 8.74 (dd, *J* 4.0, *J* 1.0, 2 H, H-thiophene) ppm; □□□□□□□□($CDCl_3$, 126 MHz): δ = 28.1 (6 C), 44.4 (2 C), 83.0 (2 C), 107.8 (2 C), 128.9 (2 C), 129.9 (2 C), 130.8 (2 C), 135.1 (2 C), 140.1 (2 C), 161.2 (2 C), 167.2 (2 C) ppm; HRMS (ESI+): *m/z* 551.12858 $[M + Na]^+$ (100) and 1079.26801 (38), calcd for $C_{26}H_{28}N_2O_6S_2Na^+$ 551.12810.

DPP-phenyl *N,N*'-diacetic acid (5). Di-*tert*-butyl ester **3** (78 mg, 0.15 mmol, 1 equiv.) was dissolved in CH_2Cl_2 (2.5 mL). TFA (1.39 mL, 18.05 mmol, 120 equiv.) was added and the

resulting reaction mixture was stirred for 4 h. The reaction was checked for completion by TLC (CH_2Cl_2/EtOAc 9 : 1, v/v) and the mixture was evporated under reduced pressure. The residual TFA was coevaporated with toluene. The resulting solid residue was triturated and washed with cyclohexane (2 × 30 mL) and finally dried under vacuum to give compound **5** as an orange solid (58 mg, yield 95%). R_f ~ 0.0 (CH_2Cl_2/EtOAc 9 : 1, v/v); IR (neat): ν = 3053, 2980, 2933, 1720, 1677, 1653, 1607, 1592, 1445, 1405, 1388, 1243, 1159, 1128, 1087, 1076, 789, 725, 687 cm^{-1}; ^1H NMR ([D_6]DMSO, 300 MHz): δ = 4.46 (s, 4 H, 2 × N-C<u>*H*</u>$_2$-CO_2H), 7.60 (m, 6 H, H-phenyl), 7.80 (m, 4 H, H-phenyl) ppm; ^{13}C NMR ([D_6]DMSO, 126 MHz): δ = 43.1 (2 C), 108.5 (2 C), 127.3 (2 C), 128.5 (4 C), 129.0 (4 C), 131.5 (2 C), 147.9 (2 C), 161.4 (2 C), 169.8 (2 C) ppm; HPLC (system A): t_R = 15.3 min (purity 95%); LRMS (ESI-): *m/z* 403.13 $[M - H]^-$ (100) and 806.80 $[2M - H]^-$ (70), calcd for $C_{22}H_{16}N_2O_6$ 404.10; HRMS (ESI-): *m/z* 201.04369 $[M - 2H]^{2-}$ (100) and 403.09398 $[M - H]^-$ (30), calcd for $C_{22}H_{15}N_2O_6^-$ 403.09356.

DPP-thienyl *N,N'*-diacetic acid (6). Di-*tert*-butyl ester **4** (100 mg, 0.19 mmol, 1 equiv.) was dissolved in CH_2Cl_2 (3 mL) and the resulting solution was cooled to 4 °C. TFA (0.9 mL, 11.7 mmol, 61.5 equiv.) was added and the resulting reaction mixture was stirred for 2 h. The reaction was checked for completion by TLC (CH_2Cl_2/EtOAc 9 : 1, v/v) and the mixture was evporated under reduced pressure. The residual TFA was coevaporated with toluene. The resulting solid residue was triturated and washed with cyclohexane (2 × 15 mL) and finally dried under vacuum to give compound **6** as a dark reddish purple solid (55 mg, yield 69%). R_f ~ 0.0 (CH_2Cl_2/EtOAc 9 : 1, v/v); IR (neat): ν = 3076, 2973, 2908, 1715, 1659, 1571, 1424, 1407, 1391, 1225, 1121, 1031, 854, 736, 728, 708, 697, 666 cm^{-1}; ^1H NMR ([D_6]DMSO, 300 MHz): δ = 4.78 (s, 4 H, 2 × N-C<u>*H*</u>$_2$-CO_2H), 7.41 (dd, *J* 4.8, *J* 3.9, 2 H, H-thiophene), 8.09 (dd, *J* 5.0, *J* 0.9, 2 H, H-thiophene), 8.66 (dd, *J* 3.8, *J* 0.9, 2 H, H-thiophene) ppm; ^{13}C NMR ([D_6]DMSO, 126 MHz): δ = 43.9 (2 C), 106.3 (2 C), 128.7 (2 C), 129.5 (2 C), 132.8 (2 C), 134.1 (2 C), 139.7 (2 C), 160.3 (2 C), 169.8 (2 C) ppm; HPLC (system A): t_R = 15.3 min (purity 99%); HRMS (ESI-): *m/z* 415.00462 $[M - H]^-$ (40) and 371.01497 (100, fragmentation peak corresponding to the loss of a single carboxyl group), calcd for $C_{18}H_{11}N_2O_6S_2^-$ 415.00530.

Disulfonated DPP-phenyl (7). DPP-phenyl dicarboxylic acid **5** (10.13 mg, 25.1 μmol, 1 equiv.) **5** was dissolved in NMP (250 μL) and DIEA (8.7 μL, 50.2 μmol, 2 equiv.) was added. Thereafter, 125 μL of a 0.24 M solution of PyBrOP in NMP (30.1 μmol, 1.2 equiv.) was added and the resulting reaction mixture was periodically vortexed for 15 min. Then, this crude acyl bromide DPP derivative was added dropwise to a pre-cooled (4 °C) 0.30 M solution of 2-aminoethane-1,1-disulfonic acid (tributylammonium salt) in NMP (417 μL, 125 μmol, 5 equiv.) containing 2.5 equiv. of DIEA, over a period of 15 min. The resulting reaction mixture was stirred at rt for 3 h. The progress of the amidification reaction was monitored by RP-HPLC (system A). A further amount of PyBrOP (0.3 equiv) was added and the mixture was stirred for further 1 h. Thereafter, the mixture was diluted with aq. TEAB (6 mL) and purified by RP-HPLC (system B). The product-containing fractions were lyophilized to give the TEA salt of compound **7**. Desalting by ion-exchange chromatography over Dowex H^+ resin (followed by lyophilisation) afforded water-soluble DPP dye **7** as a dark reddish purple amorphous powder (5.1 mg, yield 35%). ^1H NMR ([D_6]DMSO, 500 MHz): δ = 3.46 (t, *J* 6.0, 1 H, N-CH_2-C<u>*H*</u>$(SO_3H)_2$), 3.63 (t, *J* 6.0, N-C<u>*H*</u>$_2$-$CH(SO_3H)_2$), 4.28 (s, 2 H), 4.45 (s, 2 H), 7.57 (m, 6 H, H-phenyl), 7.81 (m, 4 H, H-phenyl), 7.86 (t, *J* 3.6, 1 H, N<u>*H*</u>) ppm; ^{13}C NMR ([D_6]DMSO, 126 MHz): δ = 38.8 (partially masked by DMSO), 43.1, 44.7, 74.6, 108.2, 108.8, 127.3, 127.4, 128.5 (2 C), 128.8 (2 C), 128.9 (2 C), 129.0 (2 C), 131.4, 131.5, 147.4,

148.6, 161.4, 161.5, 166.2, 169.8 ppm; HPLC (system A): t_R = 14.7 min (purity 94%); LRMS (ESI-): *m/z* 590.00 $[M - H]^-$ (100); LRMS (ESI+): *m/z* 894.73 $[M + H + 3TEA]^+$ (100), calcd for $C_{24}H_{21}N_3O_{11}S_2$ 591.06; HRMS (ESI-): *m/z* 590.05339 $[M - H]^-$ (100), calcd for $C_{24}H_{20}N_3O_{11}S_2^-$ 590.05447.

Disulfonated DPP-thienyl (8). DPP-thienyl dicarboxylic acid **6** (10.14 mg, 25.1 μmol, 1 equiv.) was suspended in NMP (250 μL) and DIEA (8.7 μL, 50.2 μmol, 2 equiv.) was added. Partial solubilization of fluorophore was obtained through periodic vortexing. Thereafter, 125 μL of a 0.22 M solution of PyBrOP in NMP (24.1 μmol, 1 equiv.) was added and the resulting reaction mixture was periodically vortexed for 15 min. Then, this crude acyl bromide DPP derivative was added dropwise to a pre-cooled (4 °C) 0.30 M solution of 2-aminoethane-1,1-disulfonic acid (tributylammonium salt) in NMP (400 μL, 120 μmol, 4.8 equiv.) containing 2.5 equiv. of DIEA, over a period of 30 min. The resulting reaction mixture was stirred at rt for 1 h. The progress of the amidification reaction was monitored by RP-HPLC (system A). A extra equiv. of PyBrOP was added and the mixture was stirred for further 2 h 30 min. Thereafter, the mixture was diluted with aq. TEAB and purified by RP-HPLC (system B). The product-containing fractions were lyophilized to give the TEA salt of compound **8**. Desalting by ion-exchange chromatography over Dowex H^+ resin (followed by lyophilization) afforded water-soluble DPP dye **8** as a dark reddish purple amorphous powder (6.5 mg, yield 43%). ^{1}H NMR ([D_6]DMSO, 500 MHz): δ = 3.47 (t, *J* 5.0, 1H, N-CH_2-C*H*($SO_3H)_2$), 3.68 (t, *J* 5.0, 2 H, N-C*H*$_2$-$CH(SO_3H)_2$), 4.66 (s, 2 H), 4.79 (s, 2 H), 7.37 (dd, *J* 3.5, *J* 4.9, 1 H, H-thiophene), 7.40 (dd, *J* 4.0, *J* 4.8, 1 H, H-thiophene), 8.01 (t, *J* 5.0, 1 H, N*H*), 8.06 (m, 2 H, H-thiophene), 8.59 (dd, *J* 1.0, *J* 4.0, 1 H, H-thiophene), 8.66 (dd, *J* 1.5, *J* 3.8, 1 H, H-thiophene) ppm; ^{13}C NMR ([D_6]DMSO, 126 MHz): δ = 38.8 (partially masked by DMSO), 43.4, 44.7, 74.8, 106.1, 106.6, 128.7, 128.8, 129.3, 129.4, 132.8, 133.1, 134.1 (2 C), 139.1, 140.3, 160.1, 160.4, 165.8, 169.6 ppm; HPLC (system A): t_R = 14.8 min (purity 99% at 495 nm and 95% at 445 nm); LRMS (ESI-): *m/z* 601.87 $[M - H]^-$ (100), calcd for $C_{20}H_{17}N_3O_{11}S_4$ 602.97; HRMS (ESI-): *m/z* 601.96713 $[M - H]^-$ (100), calcd for $C_{20}H_{16}N_3O_{11}S_4^-$ 601.96676. A trace amount of tetrasulfonated derivative **10** (< 1 mg) was also recovered during the RP-HPLC purification. LRMS (ESI-): *m/z* 788.87 $[M - H]^-$ (5) and 889.60 $[M - H + TEA]^-$ (100), calcd for $C_{22}H_{24}N_4O_{16}S_6$ 789.94.

DPP-phenyl (9). A significant amount of tetrasulfonated derivative **9** was also recovered during the RP-HPLC purification, and also subjected to ion-exchange chromatography over Dowex H^+ followed by lyophilization. A highly hygroscopic dark reddish purple amorphous powder was obtained (2.7 mg, yield 14%). ^{1}H NMR ([D_6]DMSO, 500 MHz): δ = 3.49 (t, *J* 5.0, 2 H, 2 × N-CH_2-C*H*($SO_3H)_2$), 3.64 (t, *J* 5.0, 4 H, 2 × N-C*H*$_2$-$CH(SO_3H)_2$), 4.27 (s, 4 H, 2 × N-C*H*$_2$-C(O)N-), 7.56 (m, 6 H, H-phenyl), 7.83 (m, 4 H, H-phenyl), 7.86 (t, *J* 5.0, 2 H, 2 × N*H*) ppm; ^{13}C NMR ([D_6]DMSO, 126 MHz): δ = 38.8 (2 C), 44.7 (2 C), 74.6 (2 C), 108.4 (2 C), 127.3 (2 C), 128.8 (4 C), 128.9 (4 C), 131.3 (2 C), 148.1 (2 C), 161.5 (2 C), 166.2 (2 C) ppm; HPLC (system A): t_R = 13.9 min (purity 99%); LRMS (ESI-): *m/z* 776.87 $[M - H]^-$ (20) and 877.67 $[M - H + TEA]^-$ (100), calcd for $C_{26}H_{26}N_4O_{16}S_4$ 778.02; HRMS (ESI-): *m/z* 388.00372 $[M - 2H]^{2-}$ (100), calcd for $((C_{26}H_{24}N_4O_{16}S_4)/2)^{2-}$ 388.00405.

Preparation of BSA-DPP conjugates

General procedure for the conversion into N-hydroxysuccinimidyl (NHS) ester: DPP dye carboxylic acid (1.7 μmole, 1 equiv., weighed in a 0.5 mL "eppendorf"-type microtube) was disoslved in DMSO (concentration 18.5 mM). 3 equiv. of DIEA (2.5 μL of a 2.0 M solution in NMP) were added and the resulting mixture was periodically vortexed for 5 min (all

compounds were perfectly soluble except DPP-thienyl *N,N'*-diacetic acid **6**). Then, 1.1 equiv. of TSTU (10 μL of a 180 mM solution in DMSO) was added and the resulting reaction mixture was protected from light (using aluminum foil) and periodically vortexed for 1 h. The reaction was checked for completion by ESI-MS. The resulting NHS ester was used in the next labeling step without purification.

*NHS ester **5-NHS***: LRMS (ESI+): *m/z* 502.08 $[M + H]^+$, 524.08 $[M + Na]^+$ and 562.16 $[M + Na + K - H]^+$, calcd for $C_{26}H_{19}N_3O_8$ 501.12.

*NHS ester **6-NHS***: LRMS (ESI-): *m/z* 511.48 $[M - H]^-$; LRMS (ESI+): *m/z* 535.67 $[M + Na]^+$, calcd for $C_{22}H_{15}N_3O_8S_2$ 513.03.

*NHS ester **7-NHS***: LRMS (ESI-): *m/z* 687.00 $[M - H]^-$, calcd for $C_{28}H_{24}N_4O_{13}S_2$ 688.08.

*NHS ester **8-NHS***: LRMS (ESI-): *m/z* 348.44 $[M - 2H]^{2-}$, 698.49 $[M - H]^-$ and 720.45 $[M + Na - 2H]^-$, calcd for $C_{24}H_{20}N_4O_{13}S_4$ 699.99.

Fluorescent labeling of BSA: The solution of NHS ester (see above, 15- or 30-fold excess according to protein) was added to a solution of BSA (500 μL, 1.8 mg/mL, 13.5 nmol) in phosphate buffer (pH 7.05 or 7.70). The resulting mixture was protected from light and periodically vortexed. The reaction was left at 4 °C overnight. Thereafter, this mixture was again periodically vortexed at rt for 4 h. After dilution with 1 mL of phosphate buffer, the solution was transferred to an ultra centrifugal filter device (Amicon Ultra 2 mL, Ultracel cut-off 30 kDa from Merck Millipore, ref. UFC203024) and centriguated at 4000 rpm for 15 min. For each fluorescent BSA conjugate, ca. 50 μL of solution were recovered. Confirmation of conjugation to the protein was achieved by MALDI-TOF mass spectrometry and gel electrophoresis using conditions described below.

Gel analysis of conjugates: As a quality control for each conjugate, SDS-PAGE were performed using a ECL™ Gel Box system (GE Healthcare). Each conjugate was separated by SDS-PAGE with a 4–20% gradient ECL Gel (GE healthcare) under non-reducing conditions. After electrophoresis at 160 V for 1 h, the gel was imaged with a MultiDoc-It™ Imaging System (UVP) using the 365 nm channel. The gel was finally stained with Coomassie Brilliant Blue G-250 (Fisher Scientific) to determine the molecular weight of conjugate.

The dye-to-protein ratio (F/P) of these conjugates was also determined spectrophotometrically by measuring their absorbance at 280 nm and at the λ_{max} of DPP-dye (460, 534, 455 and 529 nm for **BSA-5**, **BSA-6**, **BSA-7** and **BSA-8** respectively) and inserting the measured values into the following equation :

$$F/P = A_{max}\, P_{\varepsilon 280} / (A_{280}\, F_{\varepsilon max} - A_{max}\, F_{\varepsilon 280})$$

Where A_{280} is the absorbance of the protein at 280 nm, $P_{\varepsilon 280}$ is the extinction coefficient of the BSA protein at 280 nm (43 824 M^{-1} cm^{-1}), A_{max} is the absorbance of the DPP label as its absorption maximum, $F_{\varepsilon max}$ is the extinction coefficient of the fluorophore at the absorption maximum, and $F_{\varepsilon 280}$ is the extinction coefficient of the fluorophore at 280 nm (15 240 M^{-1} cm^{-1} for **5**, 16 270 M^{-1} cm^{-1} for **6**, 9 570 M^{-1} cm^{-1} for **7** and 13 095 M^{-1} cm^{-1} for **8**).

Figure S1. ^{1}H NMR spectrum of compound **3** recorded in $CDCl_3$ at 500 MHz.

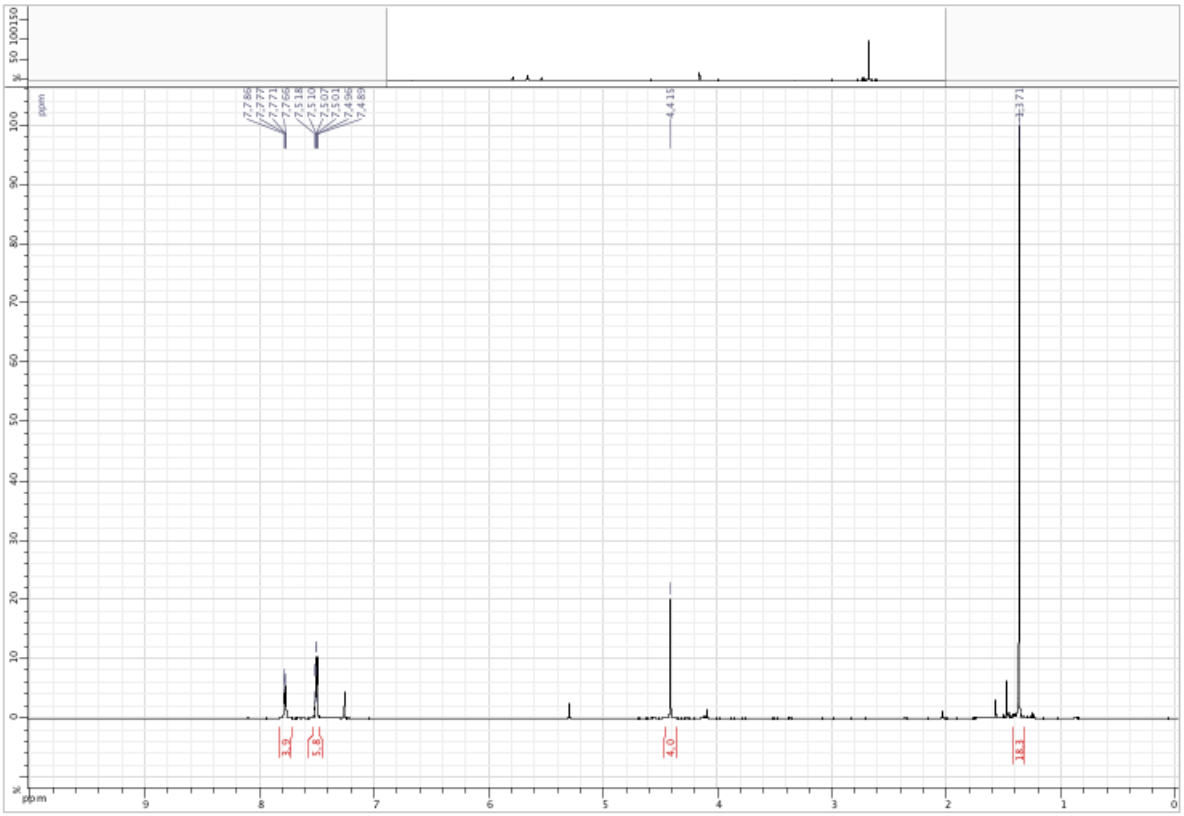


Figure S2. ^{13}C NMR spectrum of compound **3** recorded in $CDCl_3$ at 126 MHz.

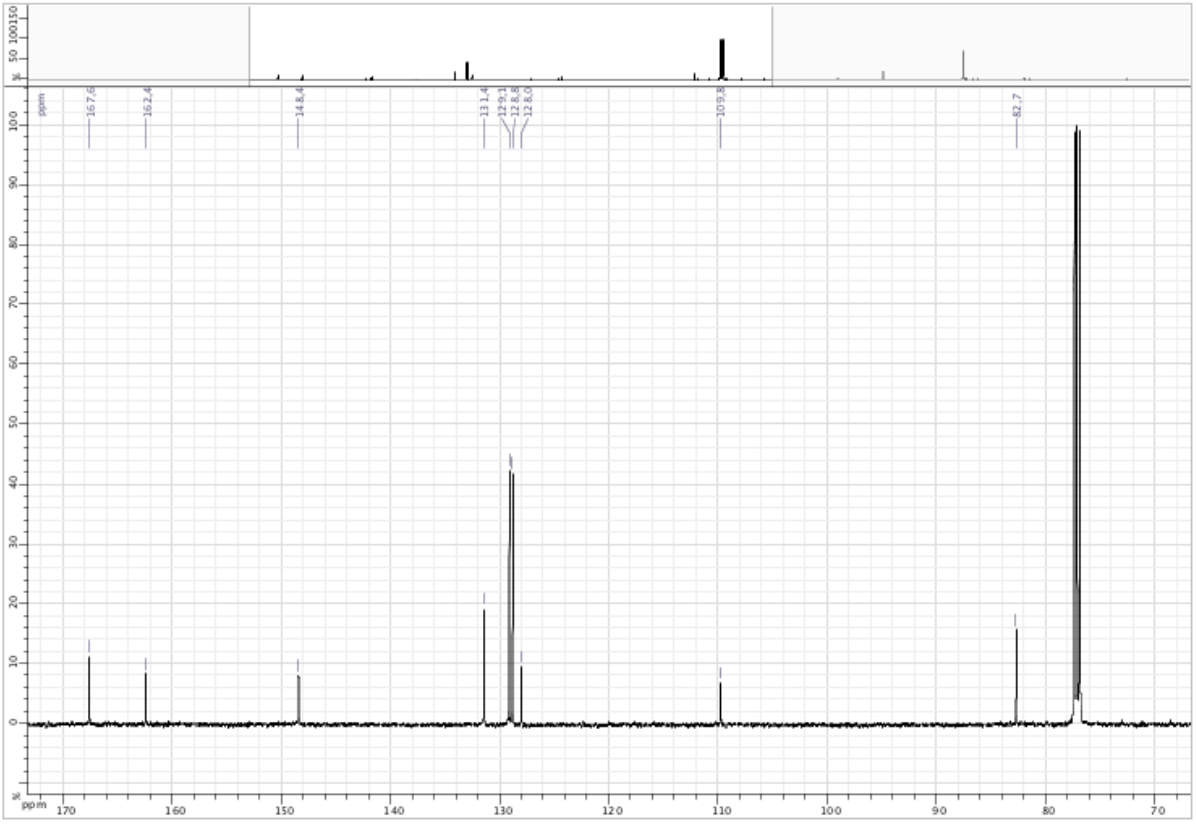


Figure S3. ESI+ mass spectrum (low resolution) of compound **3**.

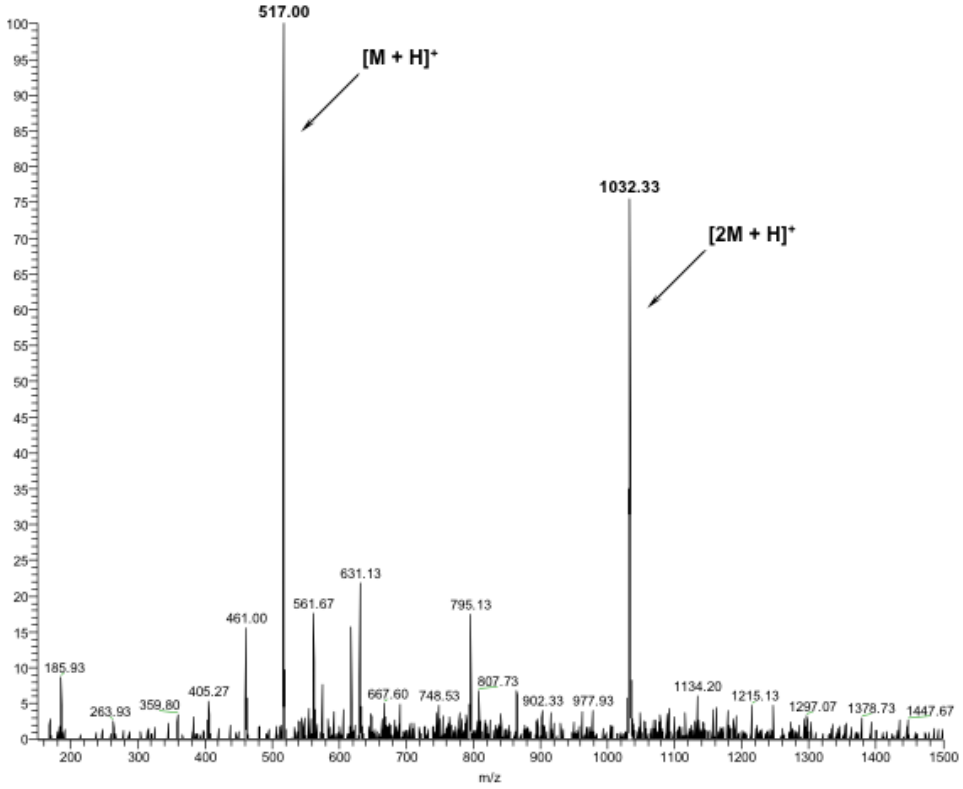
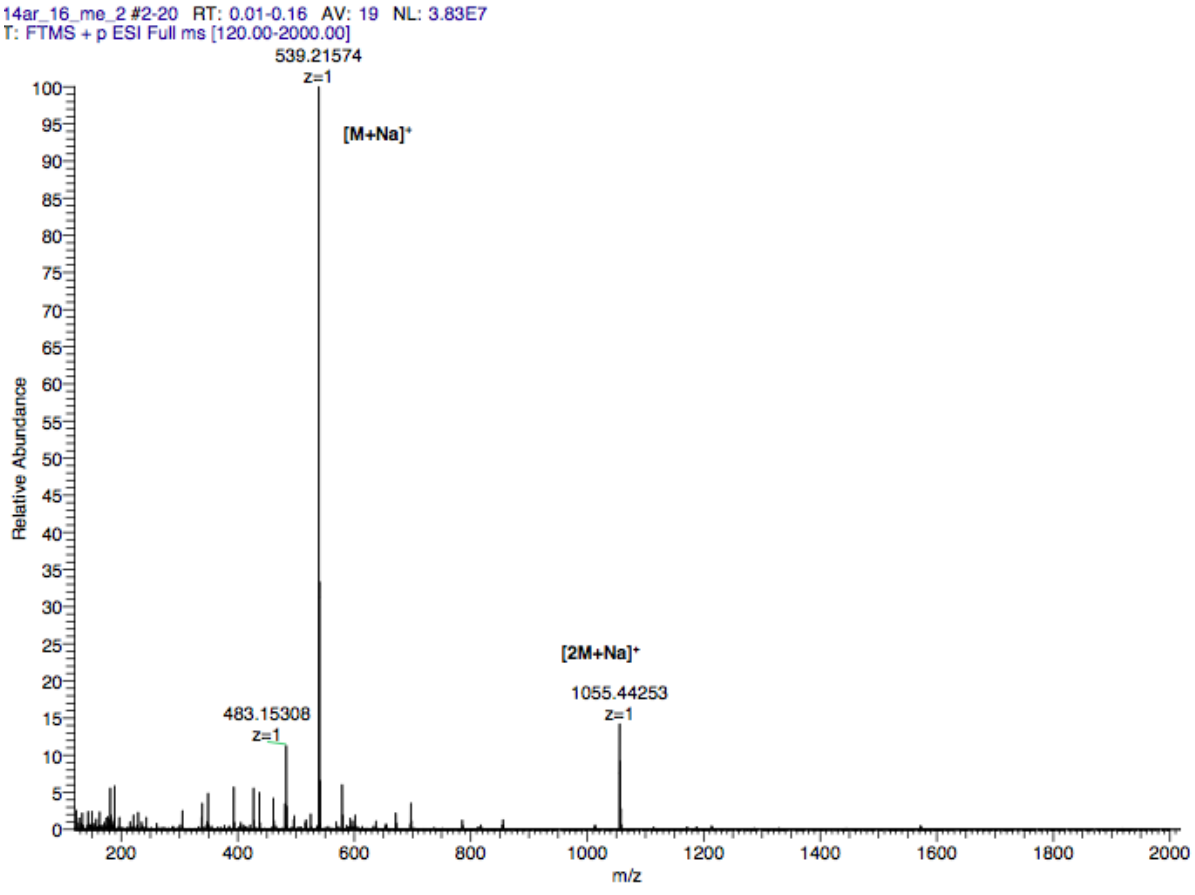


Figure S4. ESI+ mass spectrum (high resolution) of compound **3.**



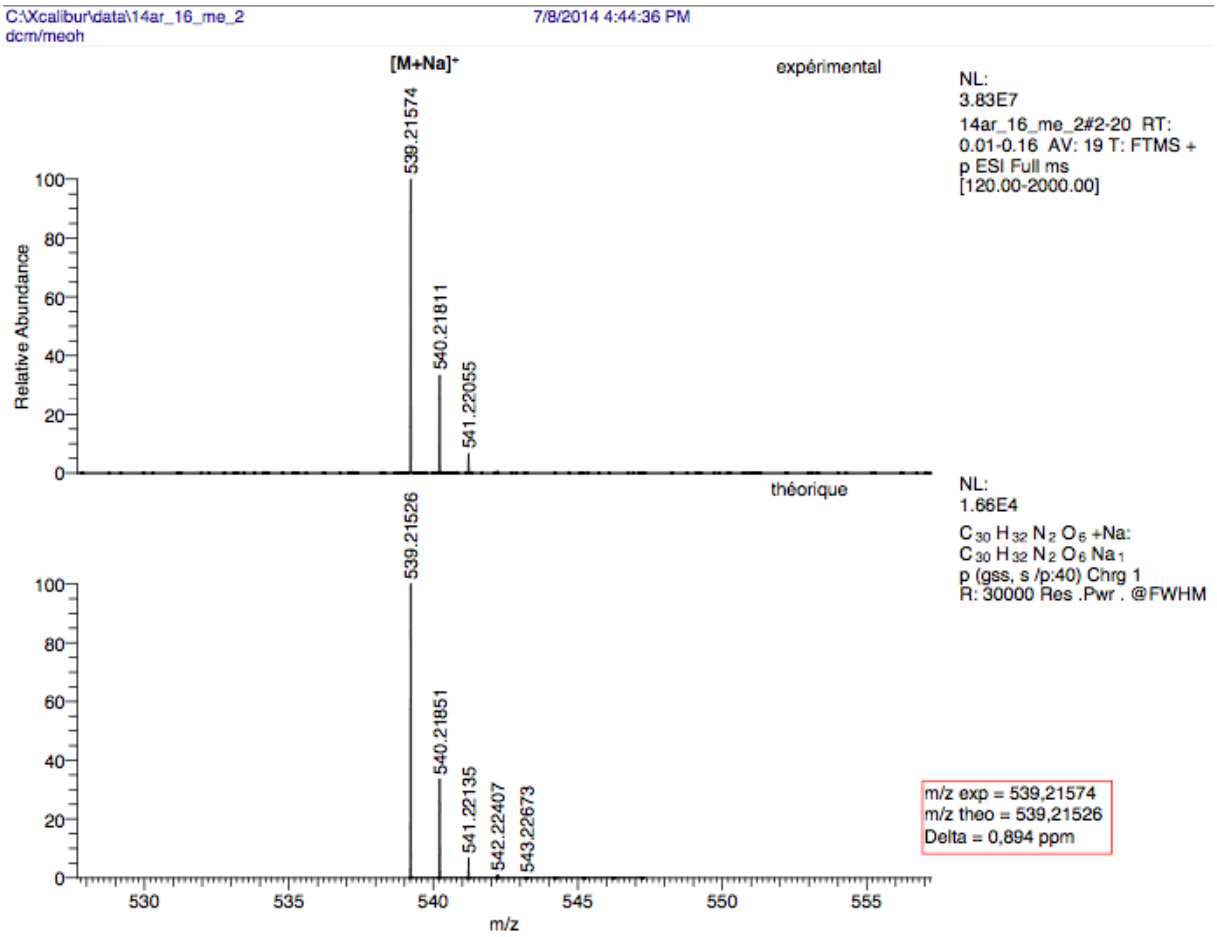


Figure S5. ^{1}H NMR spectrum of compound **4** recorded in $CDCl_3$ at 500 MHz.

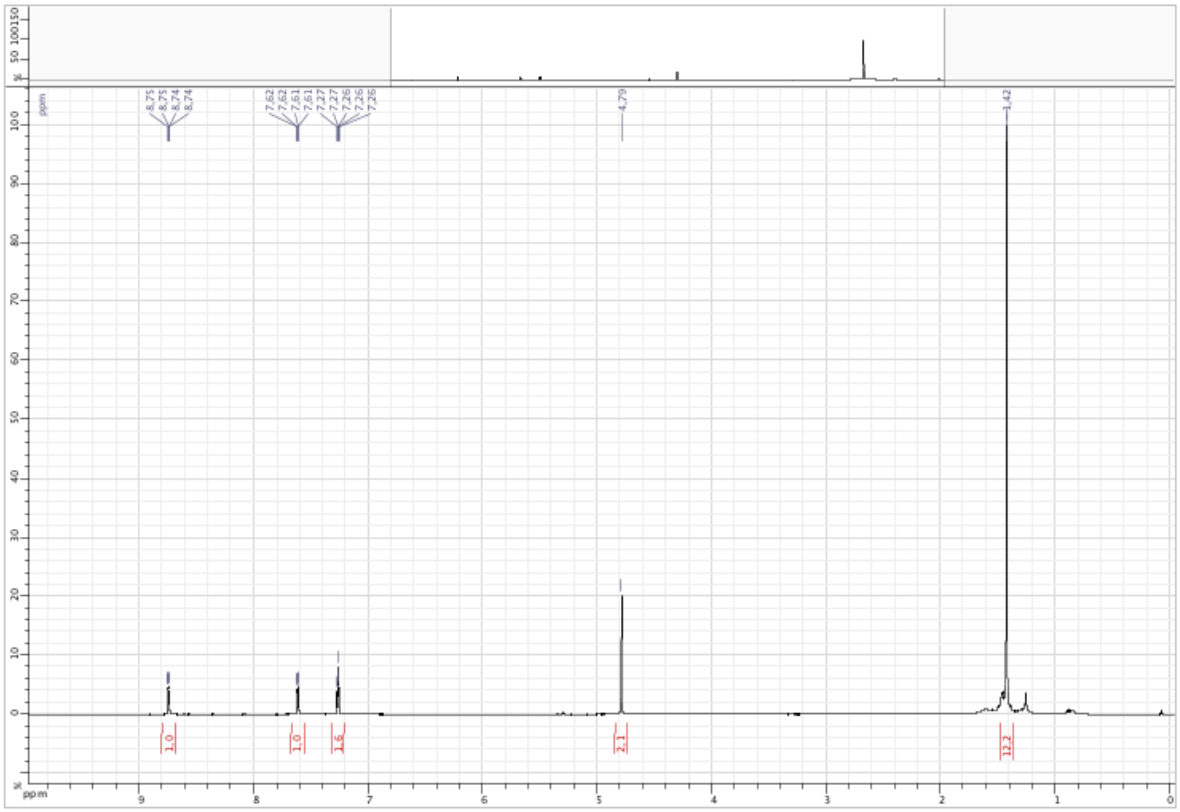


Figure S6. ^{13}C NMR spectrum of compound 4 recorded in $CDCl_3$ at 126 MHz.

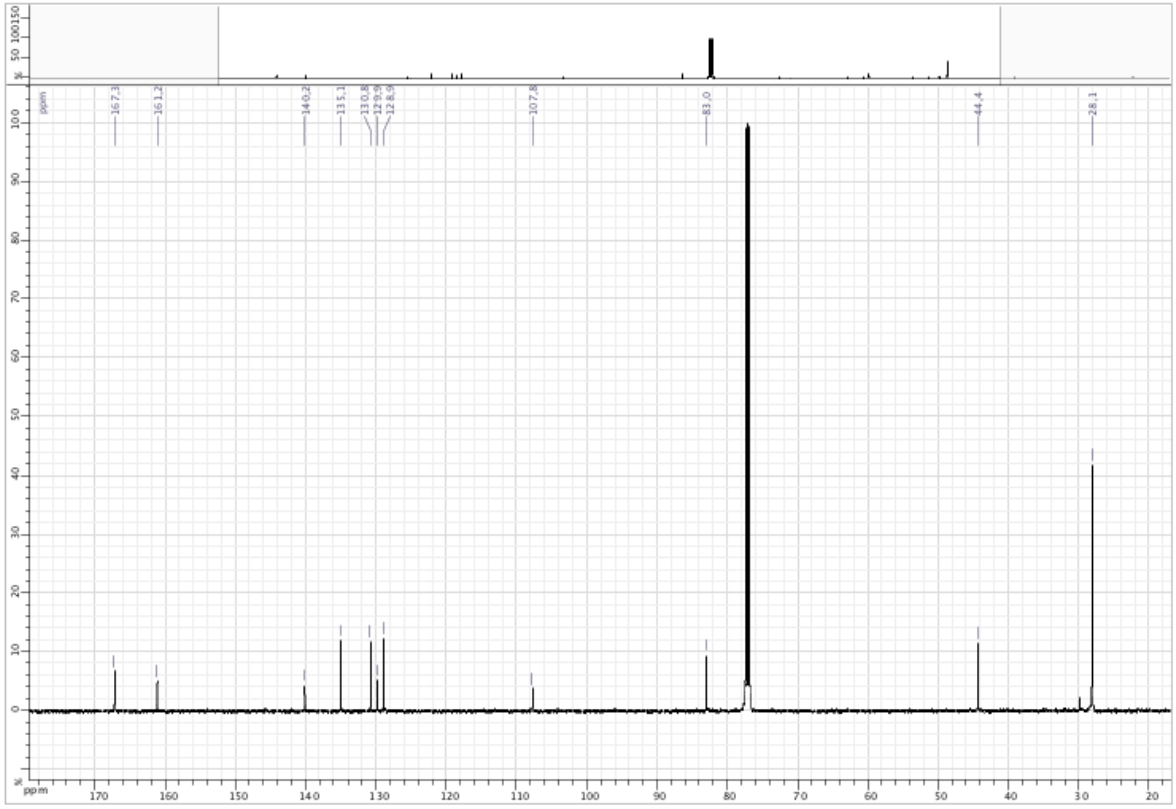
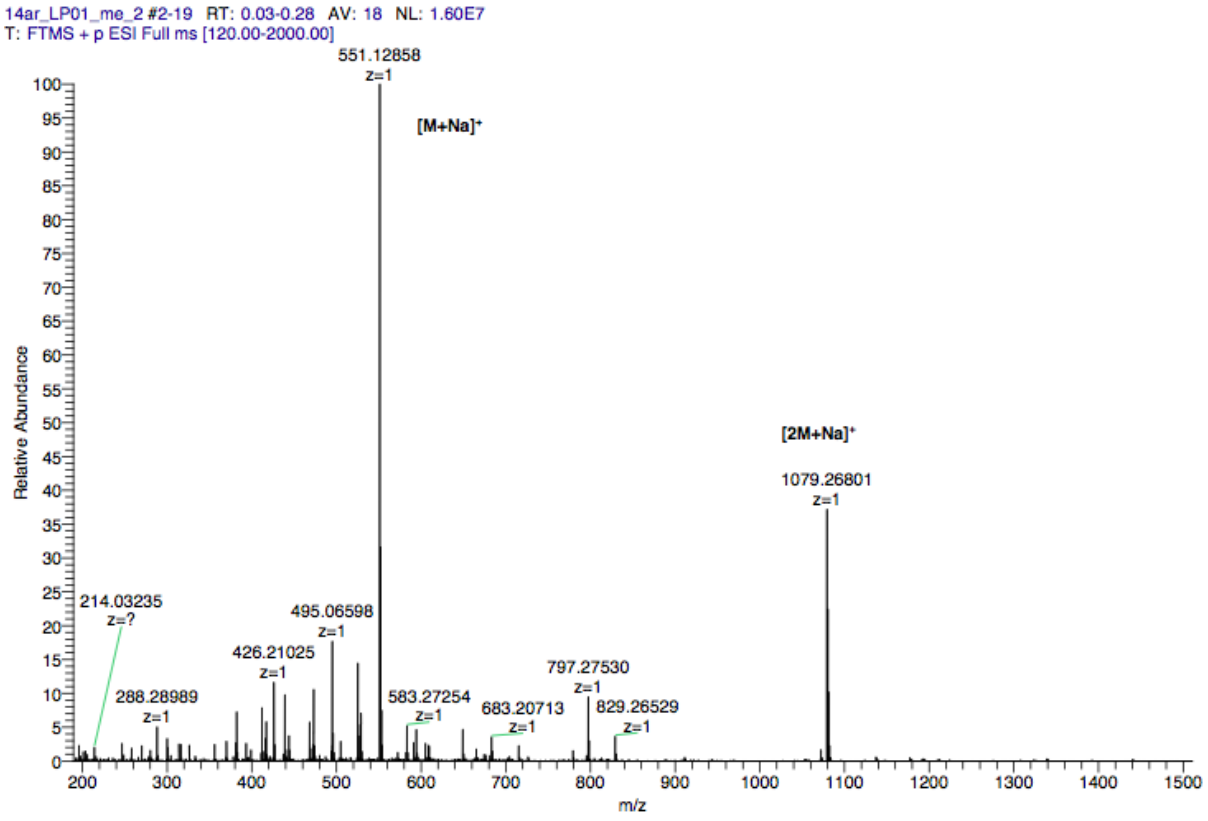


Figure S7. ESI+ mass spectrum (high resolution) of compound **4.**



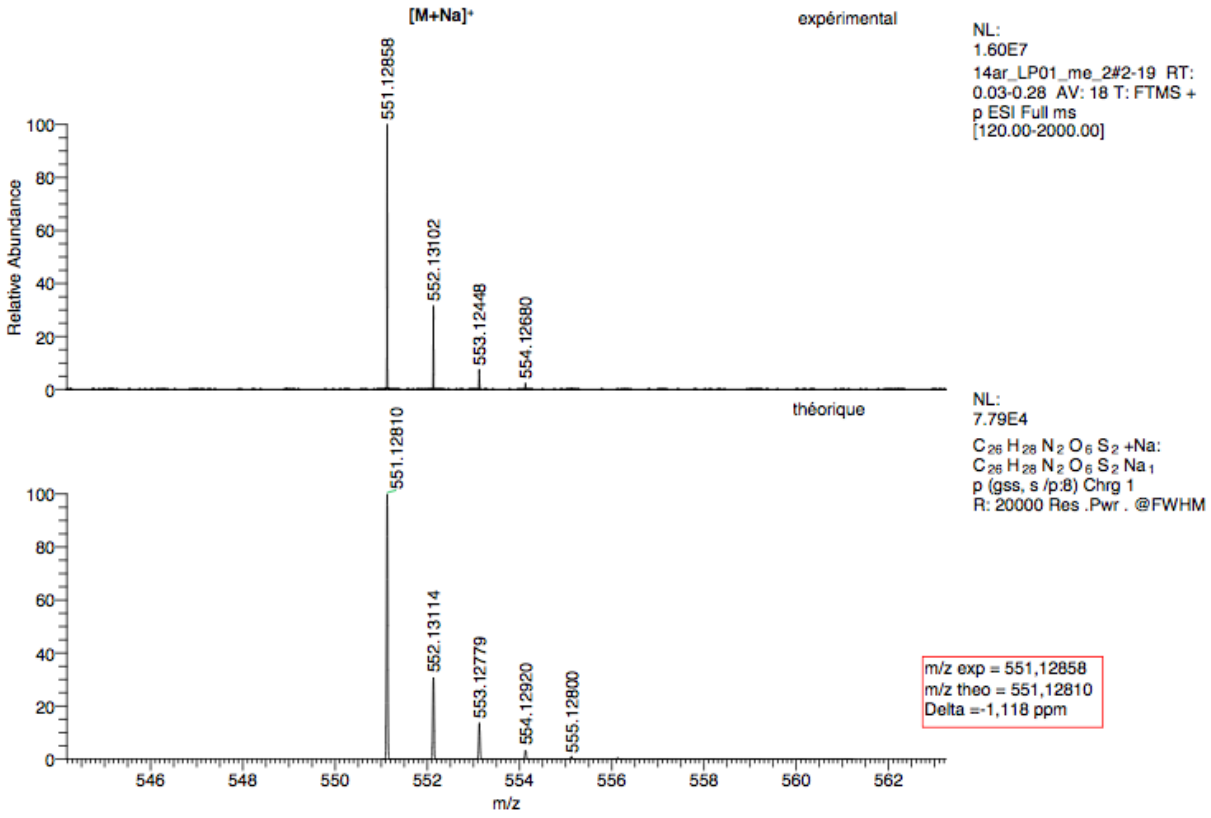


Figure S8. ^{1}H NMR spectrum of compound 5 recorded in [D_6]DMSO at 500 MHz.

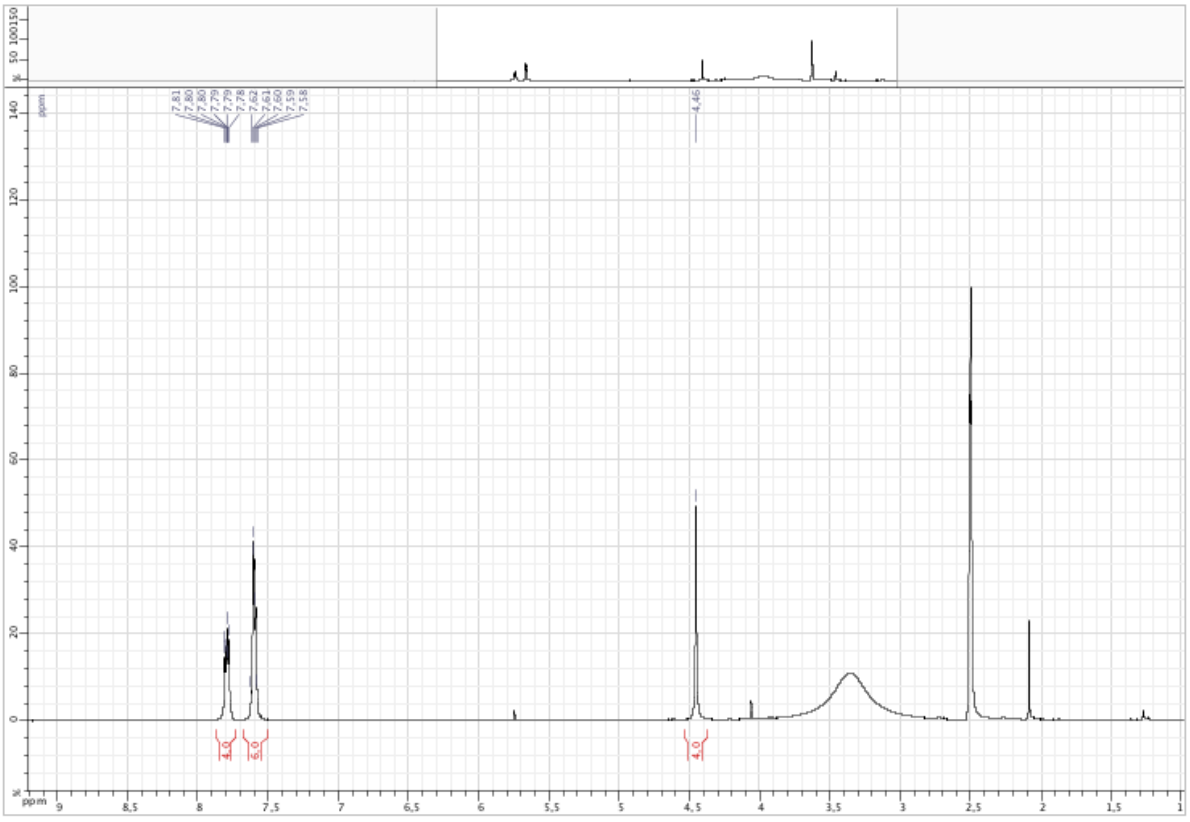


Figure S9. ^{13}C NMR spectrum of compound **5** recorded in [D_6]DMSO at 126 MHz.

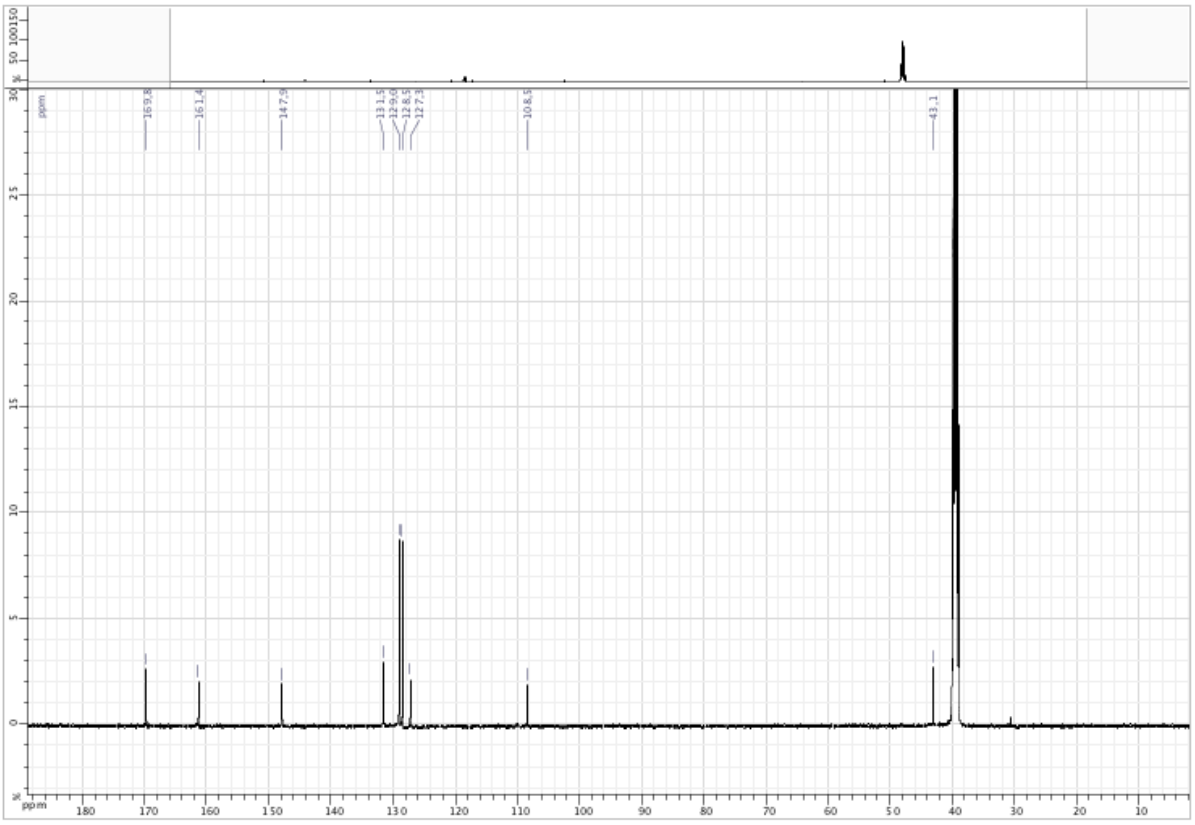


Figure S10. ESI- mass spectrum (low resolution) of compound **5.**

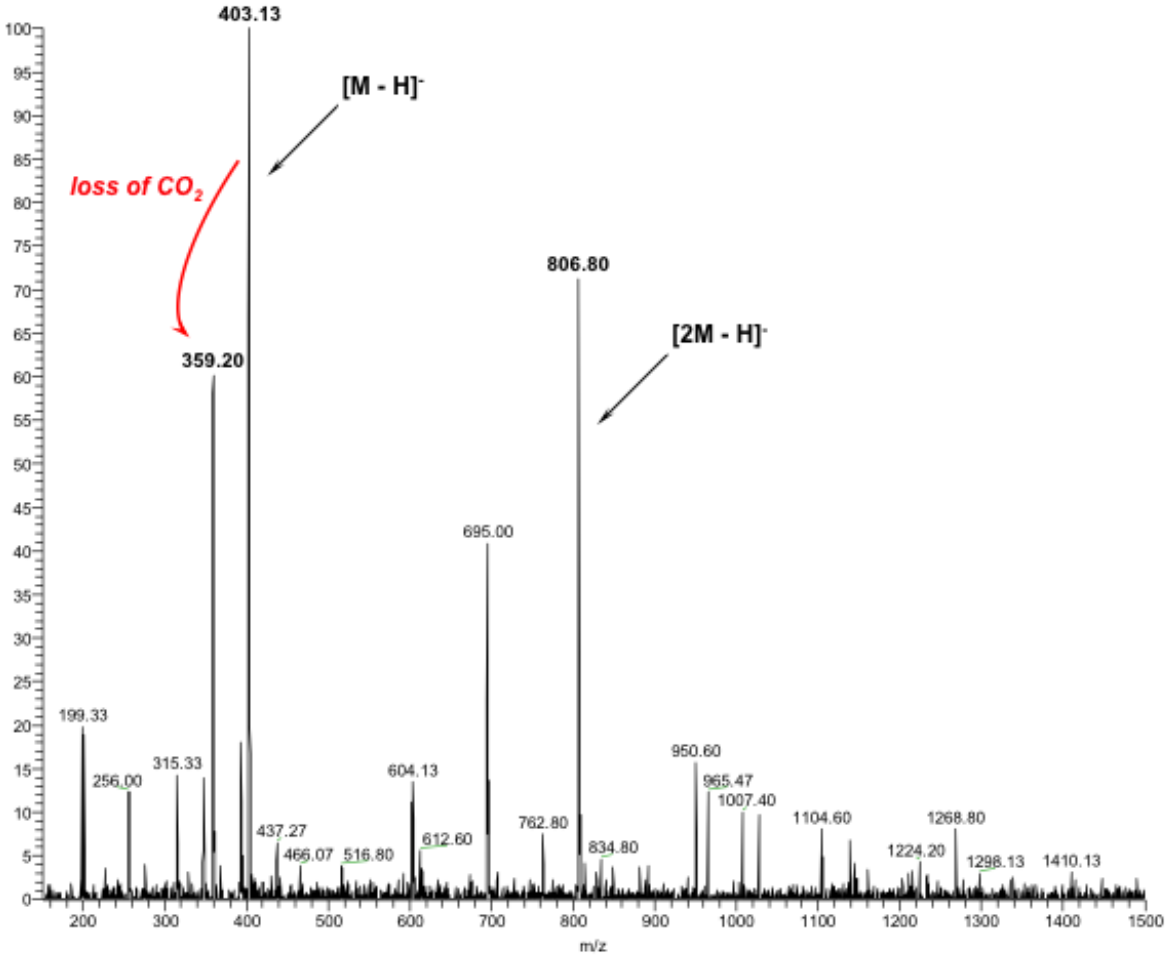


Figure S11. ESI- mass spectrum (high resolution) of compound **5.**

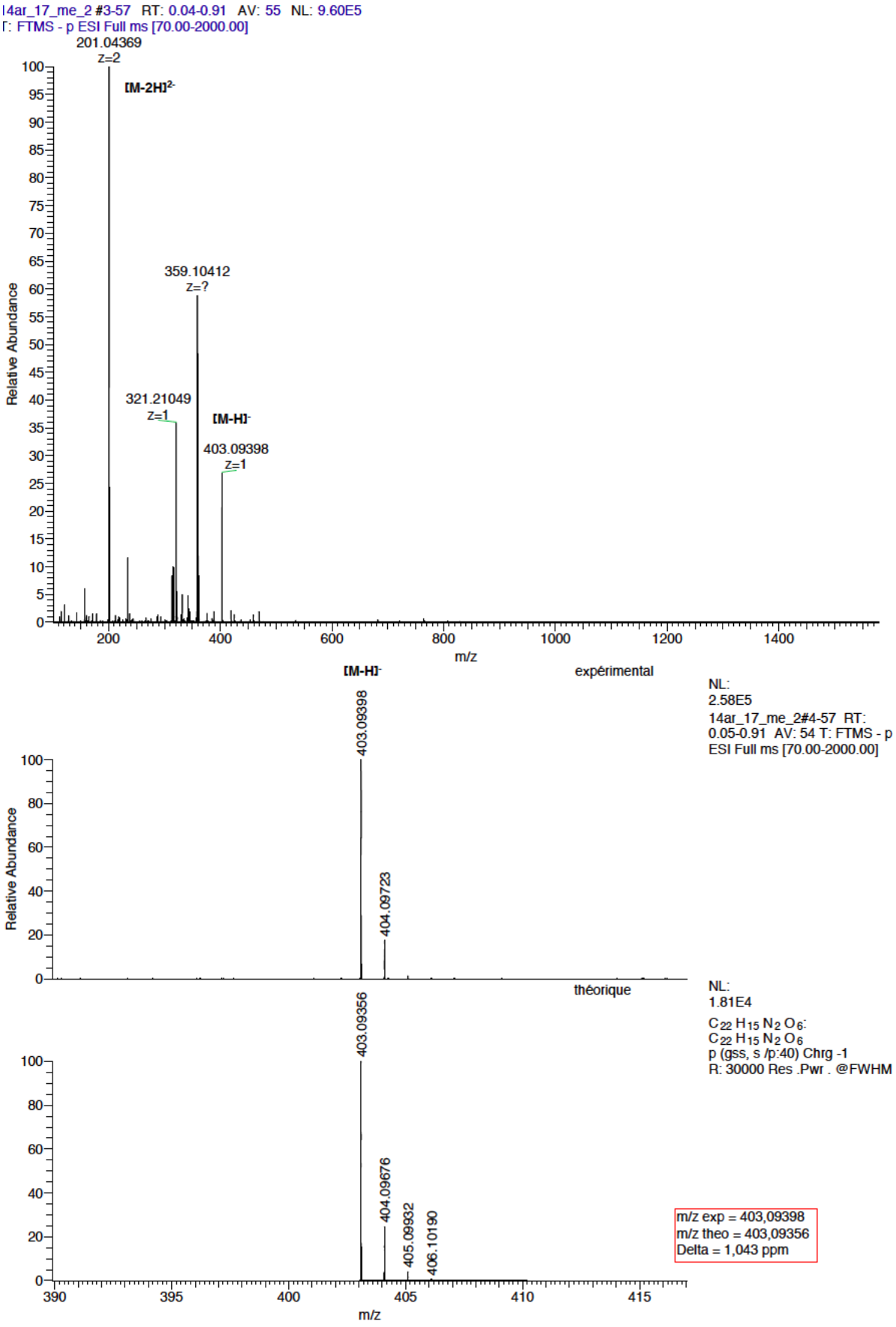
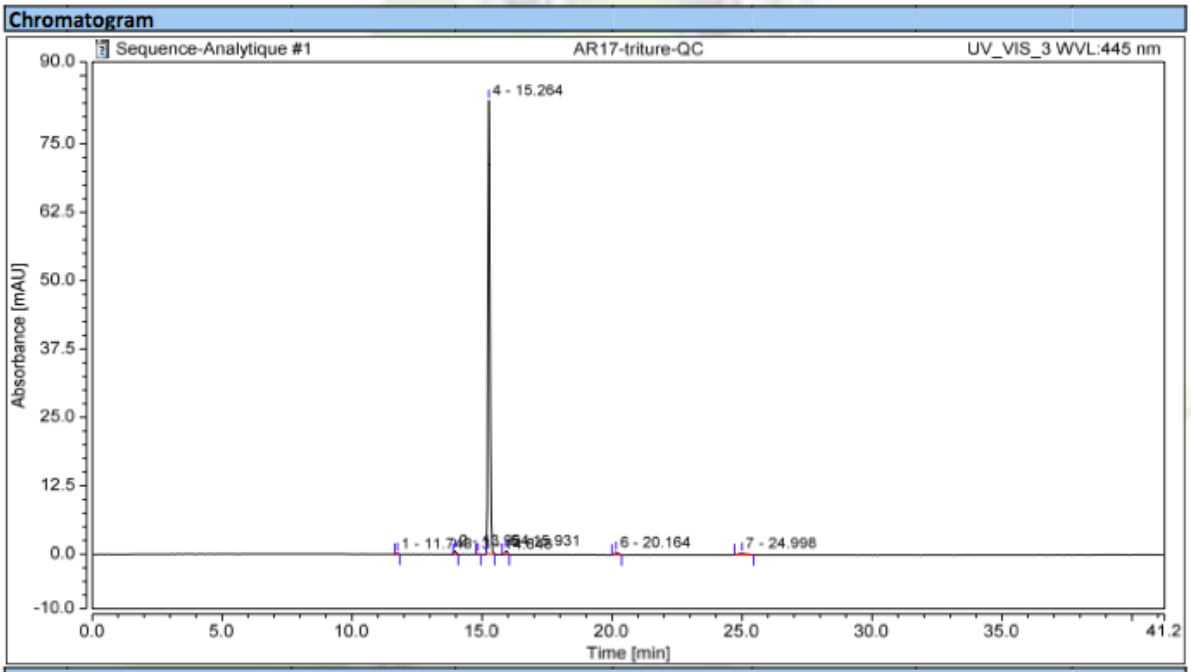

14ar_17_me_2 #3-57 RT: 0.04-0.91 AV: 55 NL: 9.60E5
T: FTMS - p ESI Full ms [70.00-2000.00]
201.04369
z=2
[M-2H]2-
359.10412
z=?
321.21049
z=1
[M-H]-
403.09398
z=1
Relative Abundance
m/z
[M-H]-
expérimental
403.09398
404.09723
théorique
403.09356
404.09676
405.09932
406.10190
NL:
2.58E5
14ar_17_me_2#4-57 RT:
0.05-0.91 AV: 54 T: FTMS - p
ESI Full ms [70.00-2000.00]
NL:
1.81E4
C22 H15 N2 O6:
C22 H15 N2 O6
p (gss, s /p:40) Chrg -1
R: 30000 Res .Pwr . @FWHM
m/z exp = 403,09398
m/z theo = 403,09356
Delta = 1,043 ppm

Figure S12. RP-HPLC elution profile (system A) of compound **5** (visible detection at 445 nm).



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		11.748	0.013	0.146	0.16	0.17	n.a.
2		13.964	0.067	0.791	0.84	0.93	n.a.
3		14.848	0.013	0.162	0.17	0.19	n.a.
4		15.264	7.662	82.912	95.67	96.92	n.a.
5		15.931	0.073	0.762	0.92	0.89	n.a.
6		20.164	0.083	0.487	1.04	0.57	n.a.
7		24.998	0.097	0.285	1.21	0.33	n.a.
Total:			**8.008**	**85.545**	**100.00**	**100.00**	

Figure S13. ^{1}H NMR spectrum of compound **6** recorded in [D_6]DMSO at 300 MHz.

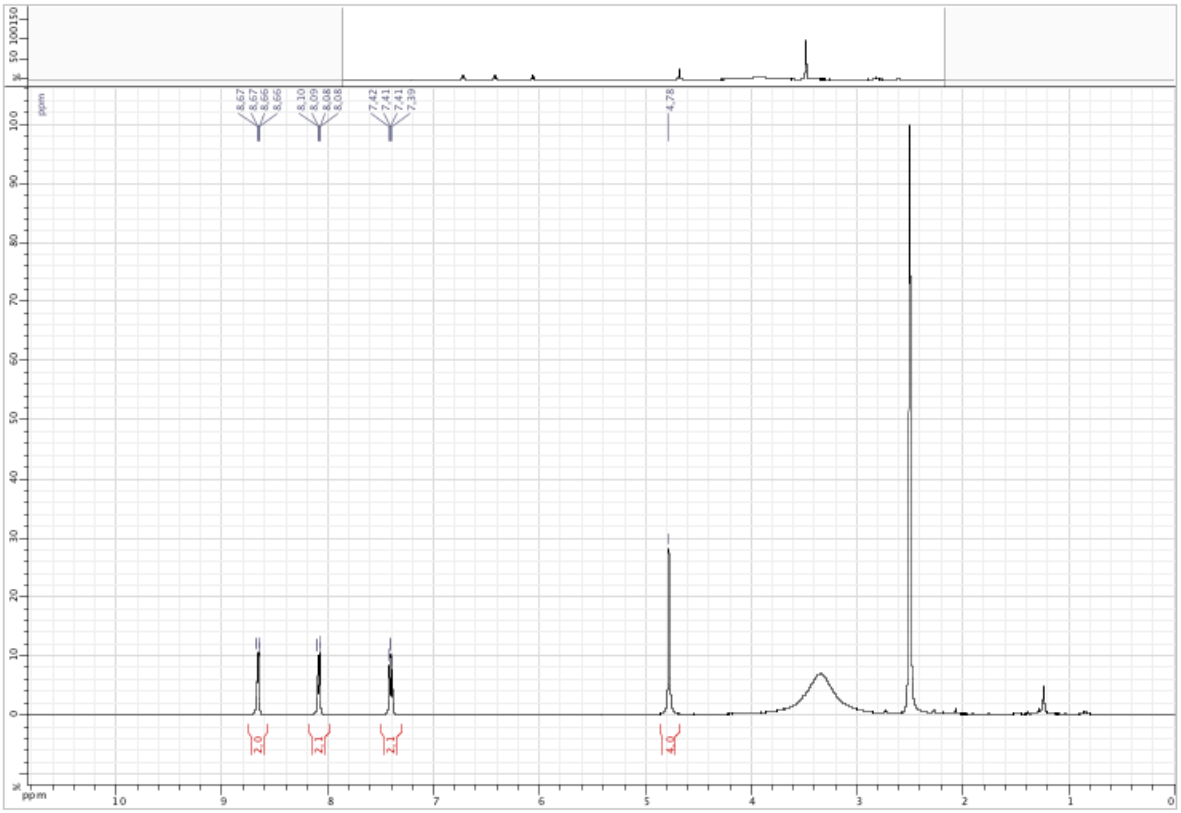


Figure S14. ^{13}C NMR spectrum of compound **6** recorded in [D_6]DMSO at 126 MHz.

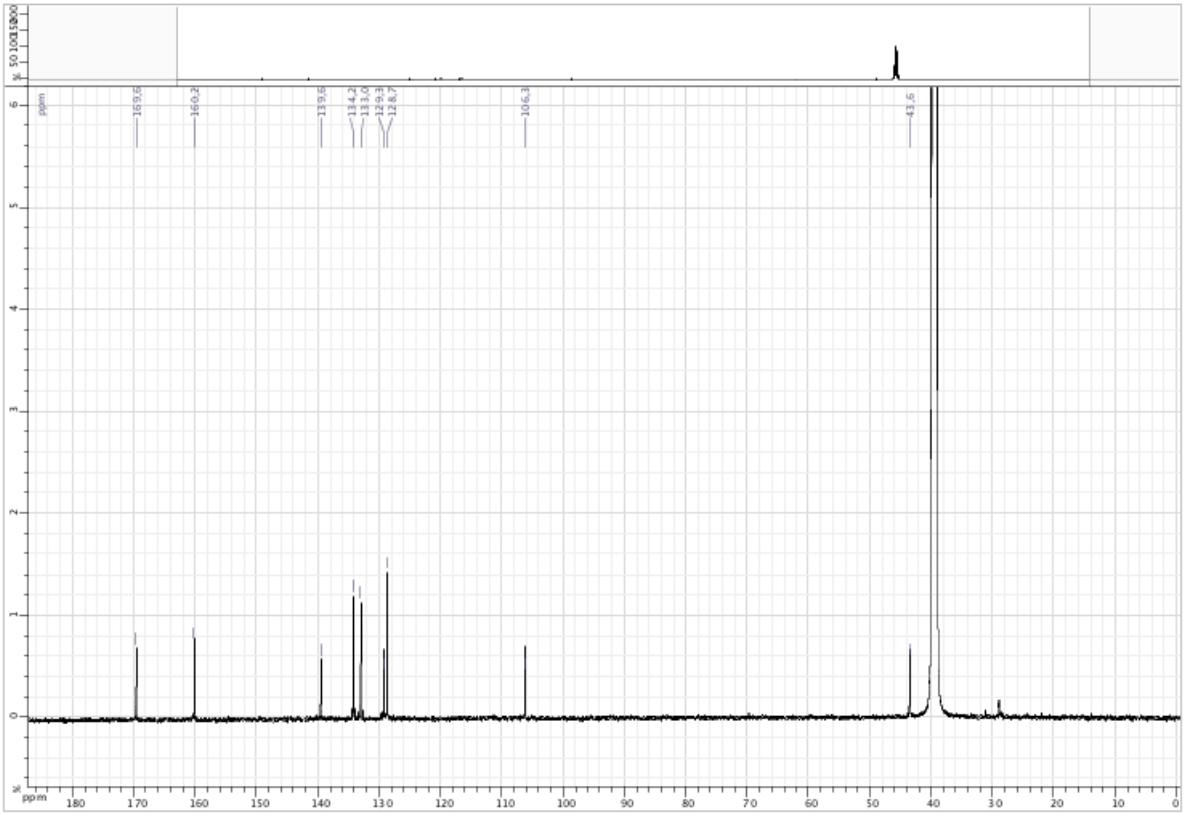


Figure S15. ESI- mass spectrum (high resolution) of compound **6**.

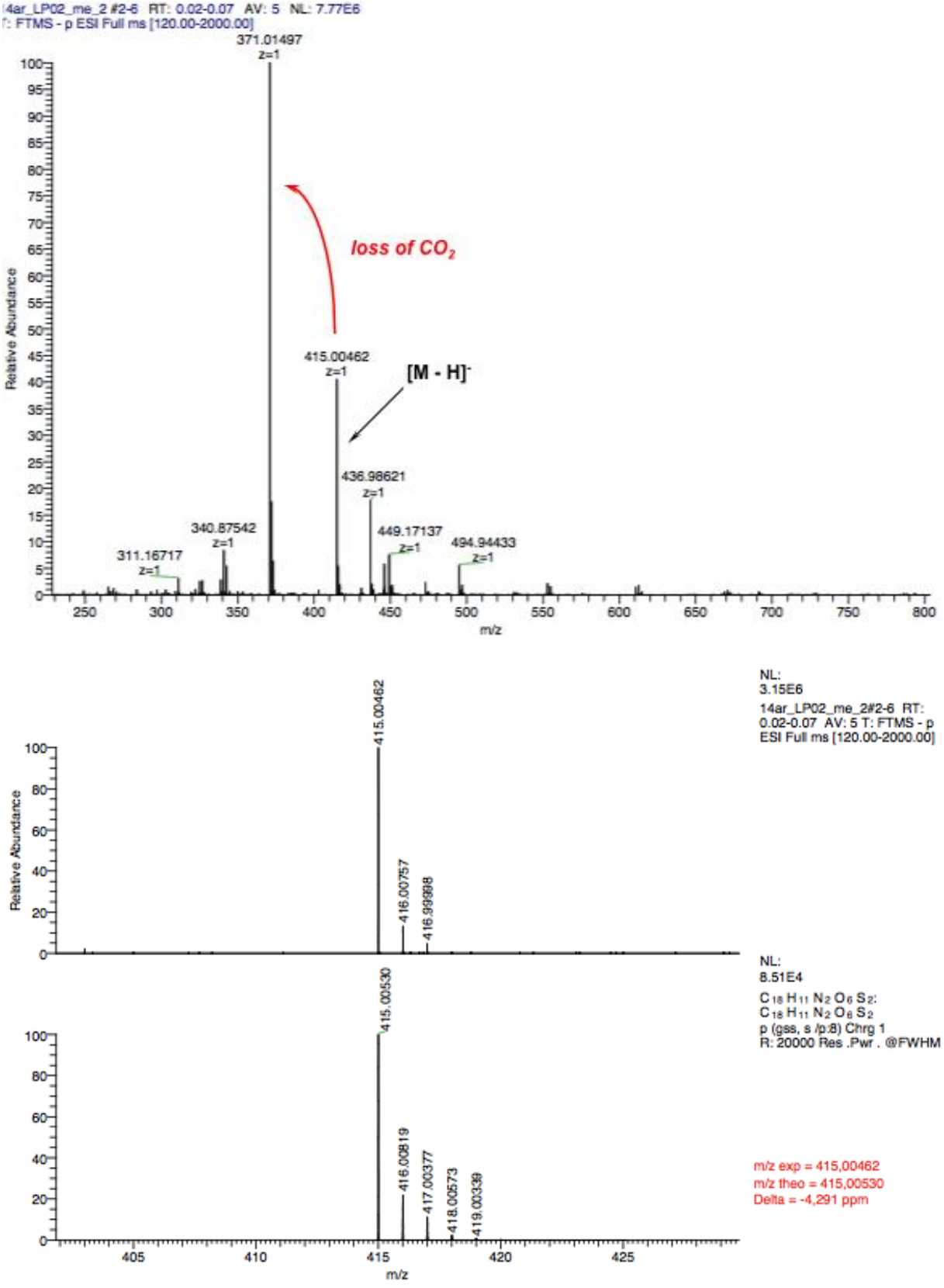
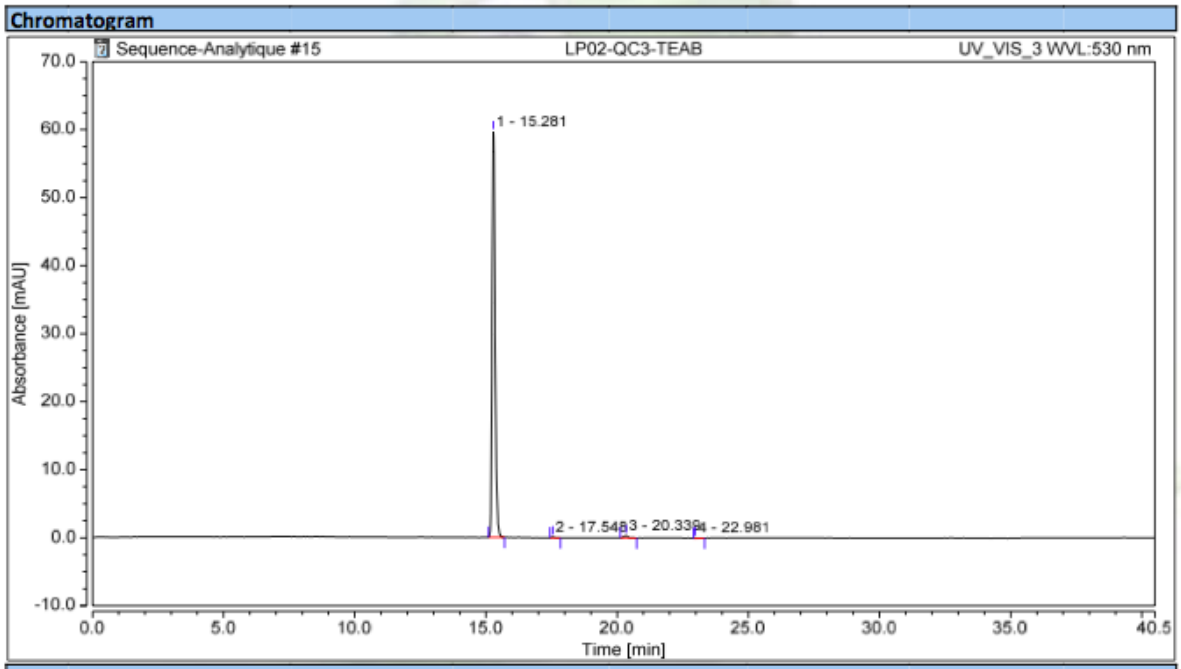


Figure S16. RP-HPLC elution profile (system A) of compound **6** (visible detection at 530 nm).



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		15.281	7.769	59.638	98.90	99.16	n.a.
2		17.548	0.014	0.103	0.18	0.17	n.a.
3		20.339	0.061	0.351	0.78	0.58	n.a.
4		22.981	0.011	0.049	0.14	0.08	n.a.
Total:			**7.856**	**60.141**	**100.00**	**100.00**	

Figure S17. ^{1}H NMR spectrum of disulfonated DPP-phenyl **7** recorded in [D_6]DMSO at 500 MHz.

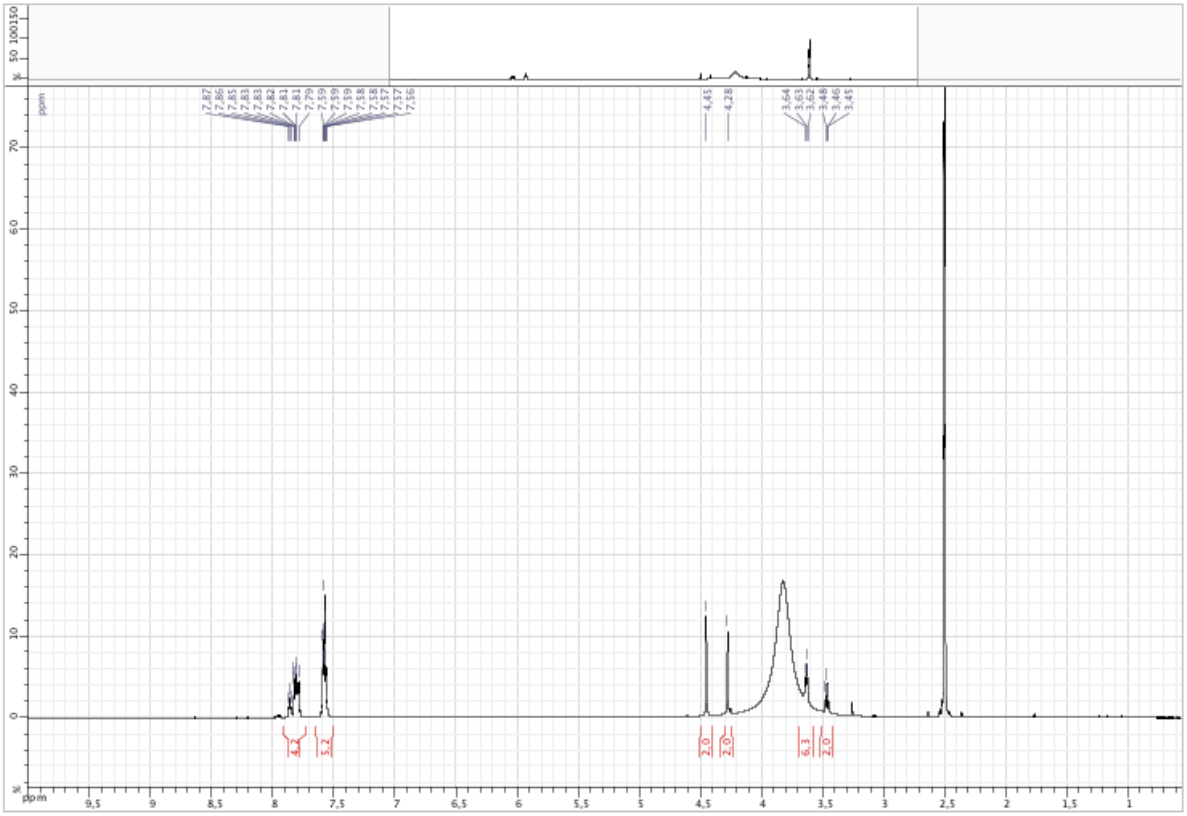


Figure S18. ^{13}C NMR spectrum of disulfonated DPP-phenyl **7** recorded in [D_6]DMSO at 126 MHz.

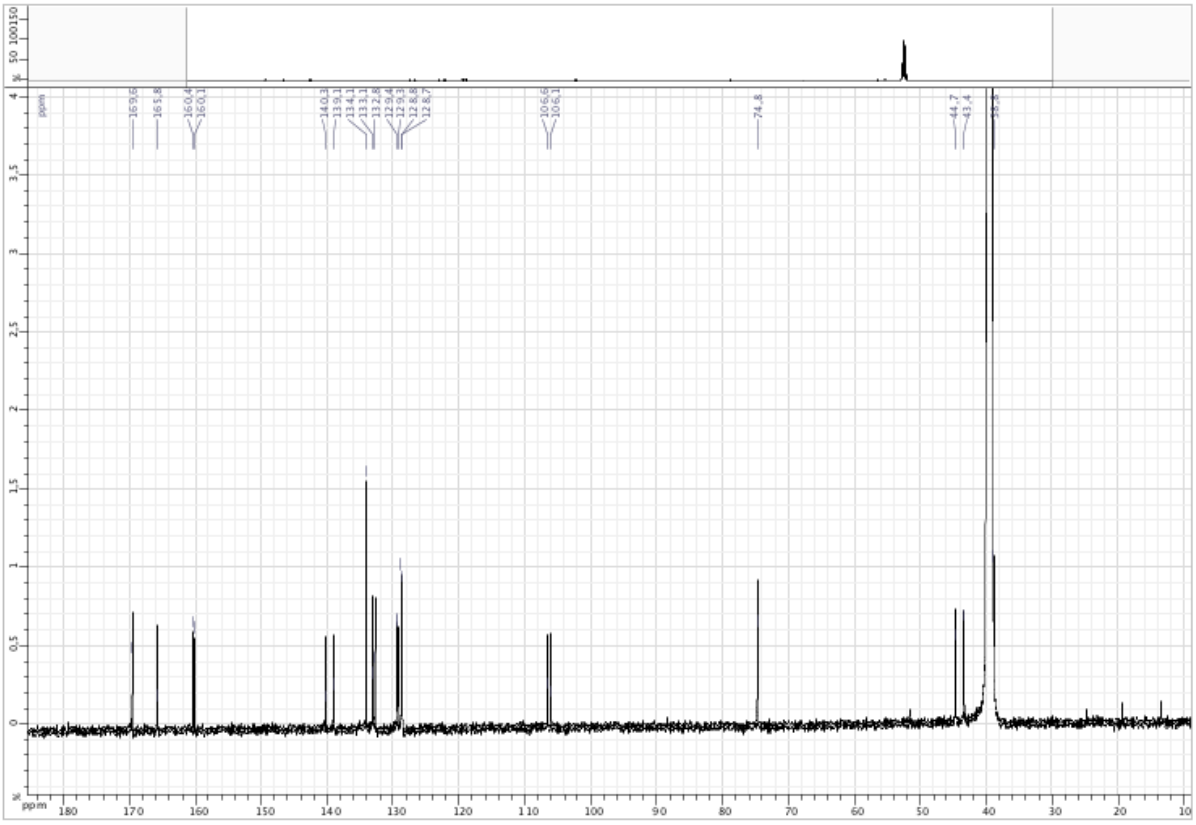


Figure S19. ESI- mass spectrum (low resolution) of disulfonated DPP-phenyl **7**.

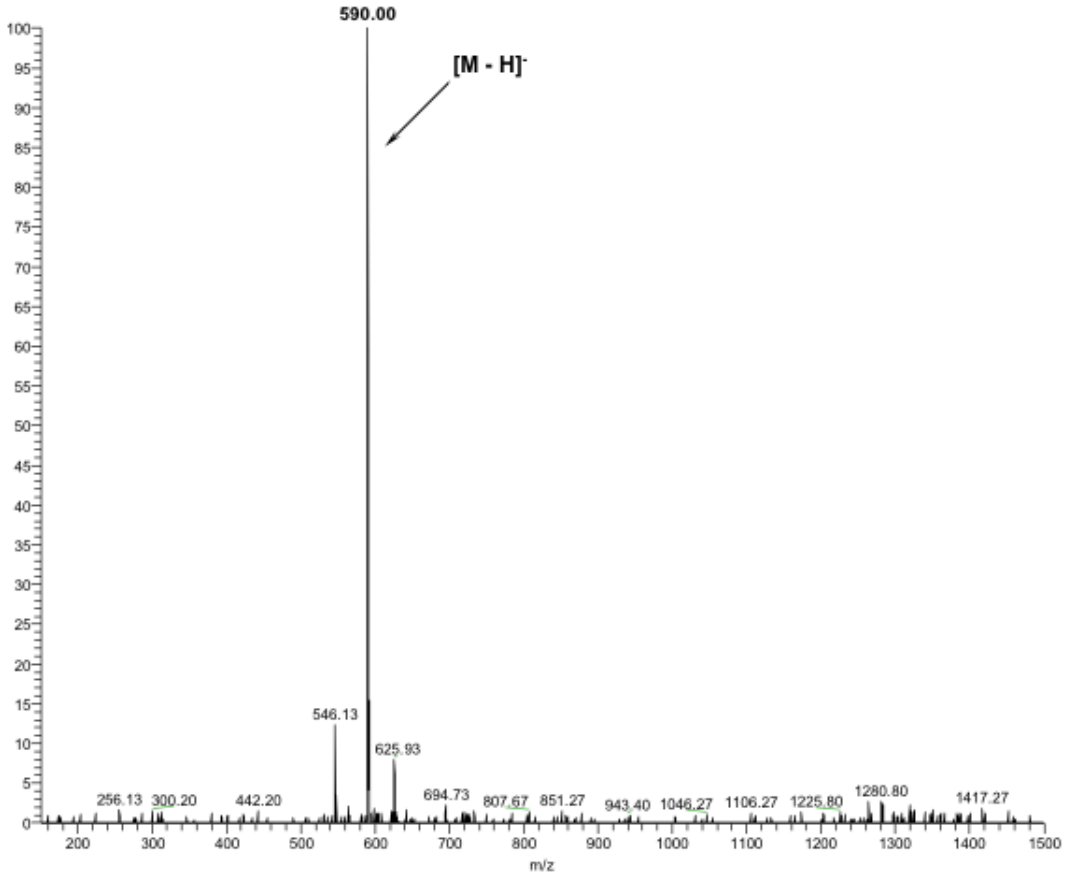


Figure S20. ESI+ mass spectrum (low resolution) of disulfonated DPP-phenyl **7.**

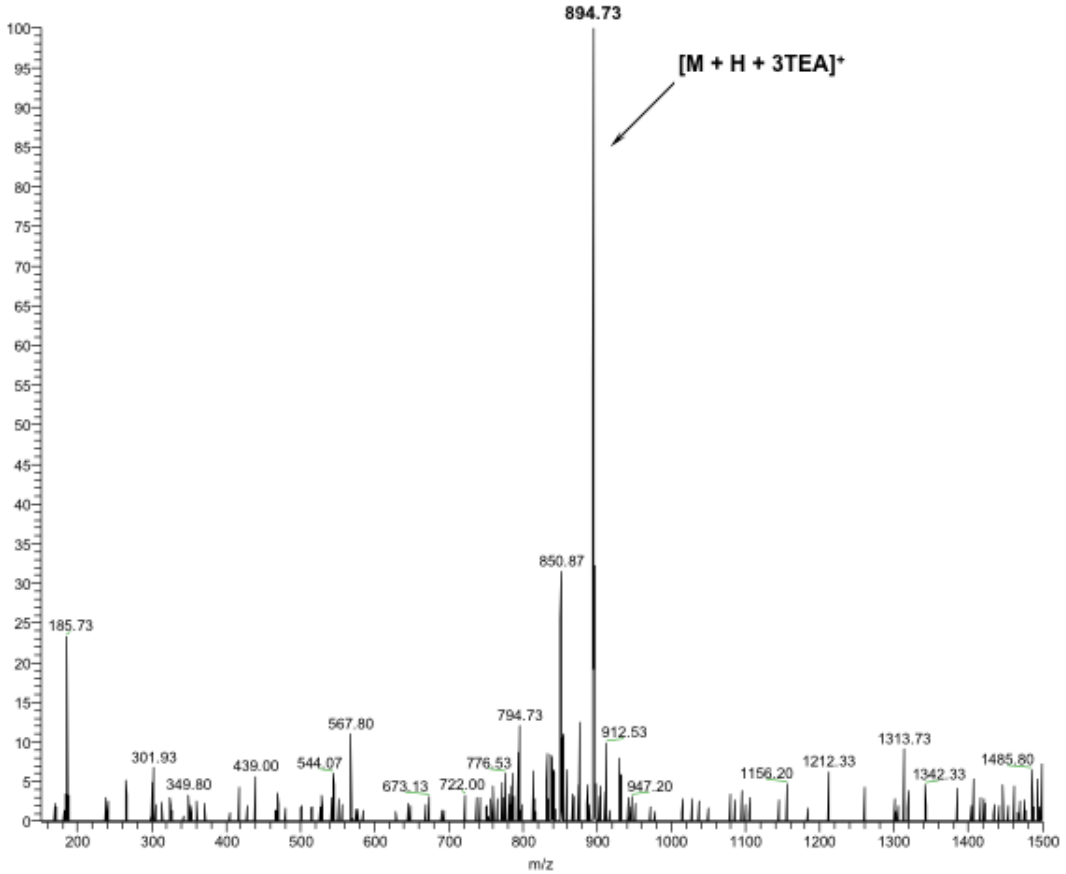


Figure S21. ESI- mass spectrum (high resolution) of disulfonated DPP-phenyl **7.**

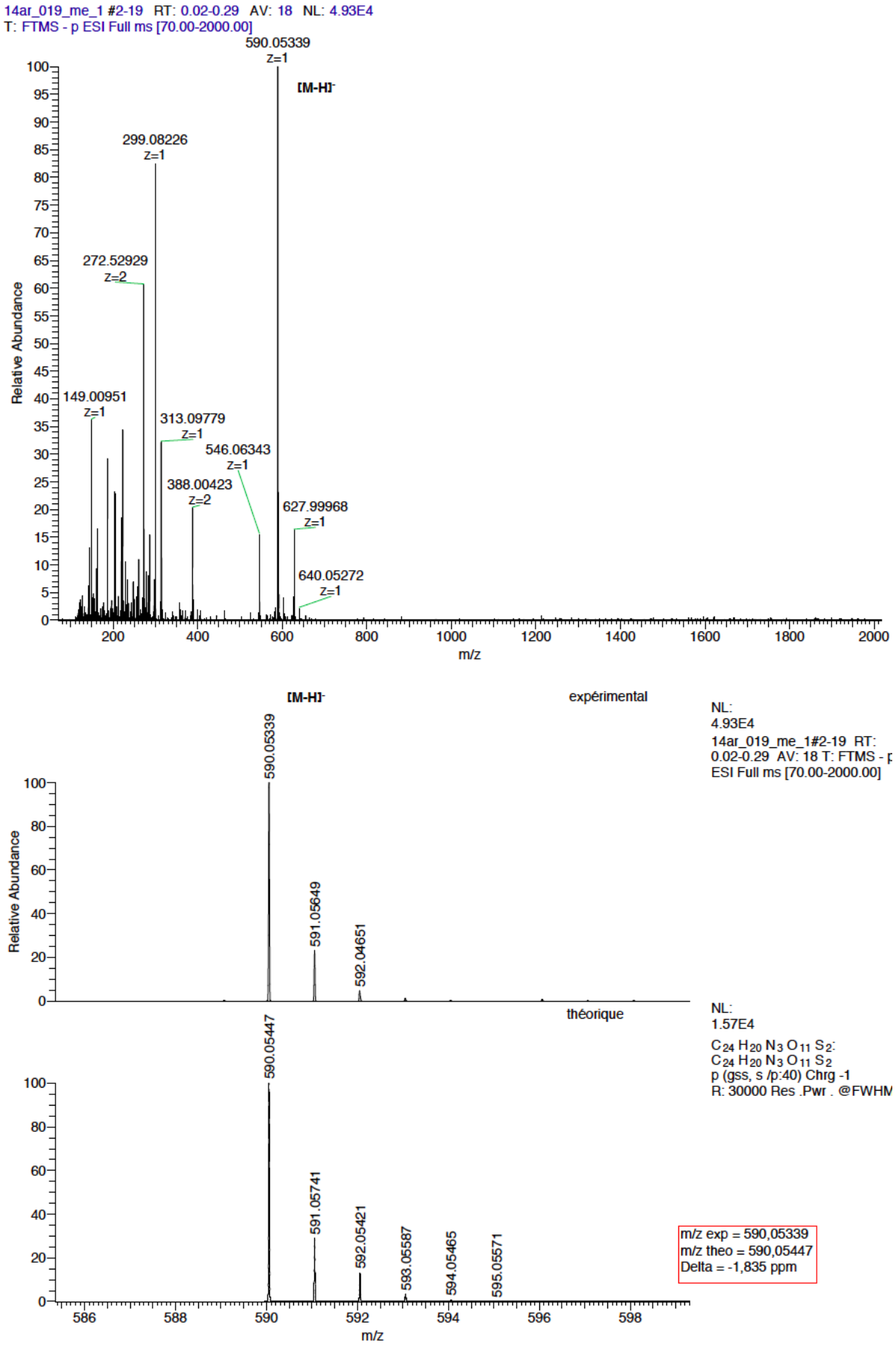
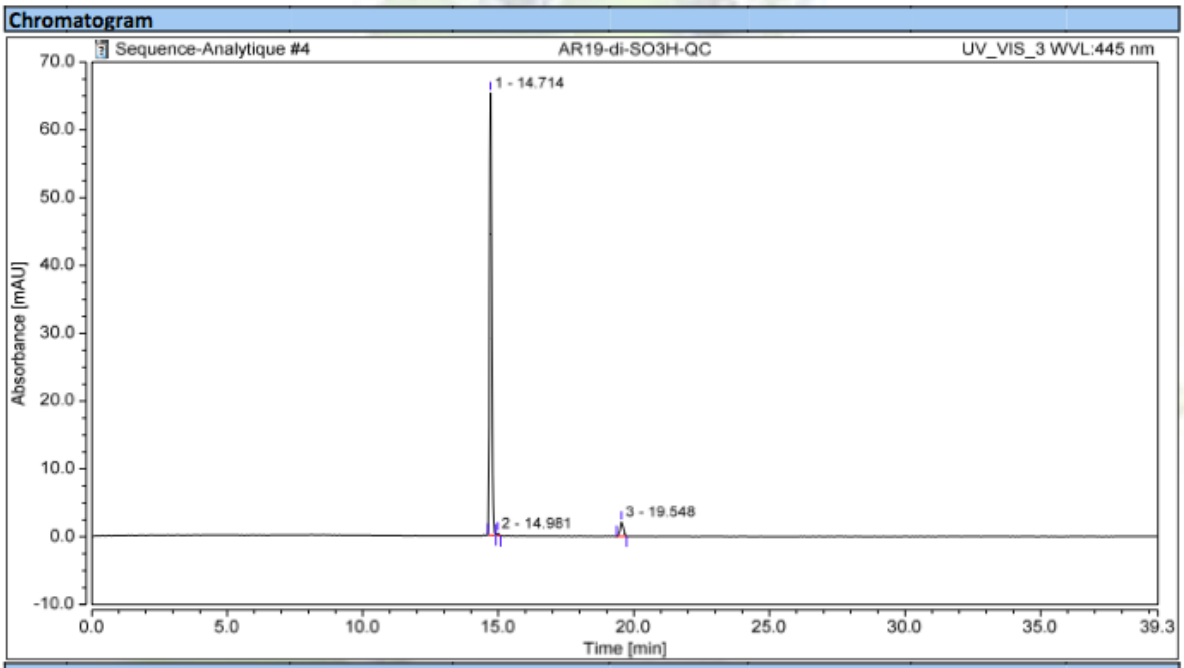


Figure S22. RP-HPLC elution profile (system A) of disulfonated DPP-phenyl 7 (visible detection at 445 nm).



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		14.714	5.778	65.279	94.58	96.29	n.a.
2		14.981	0.035	0.405	0.57	0.60	n.a.
3		19.548	0.296	2.112	4.85	3.11	n.a.
Total:			**6.109**	**67.796**	**100.00**	**100.00**	

Figure S23. ^{1}H NMR spectrum of disulfonated DPP-thienyl **8** recorded in [D_6]DMSO at 500 MHz.

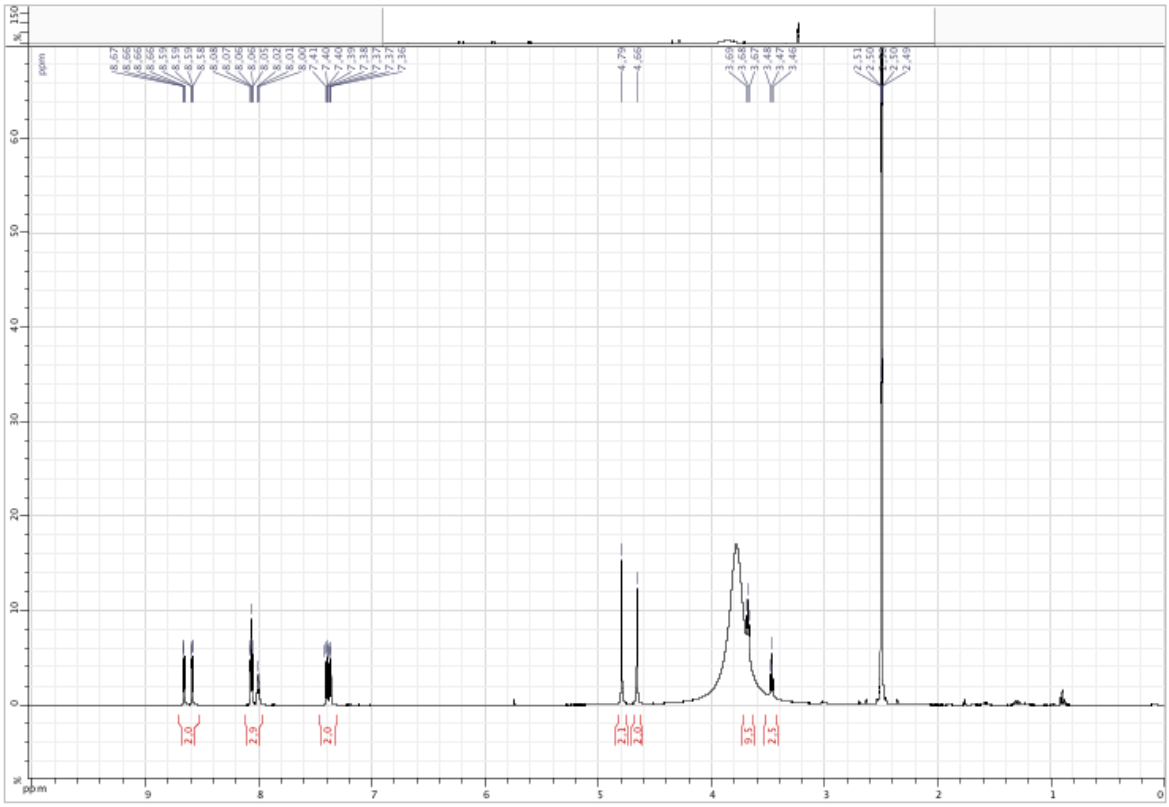


Figure S24. ^{13}C NMR spectrum of disulfonated DPP-thienyl **8** recorded in [D_6]DMSO at 126 MHz.

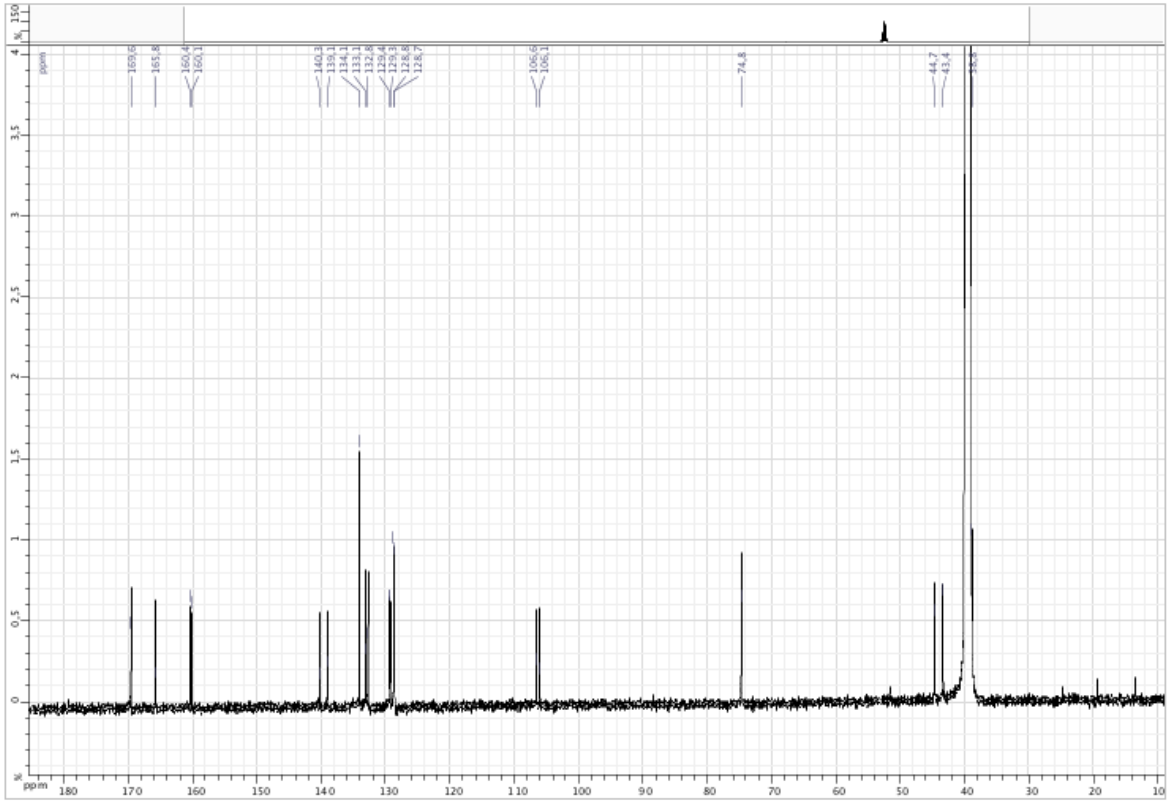


Figure S25. ESI- mass spectrum (low resolution) of disulfonated DPP-thienyl **8.**

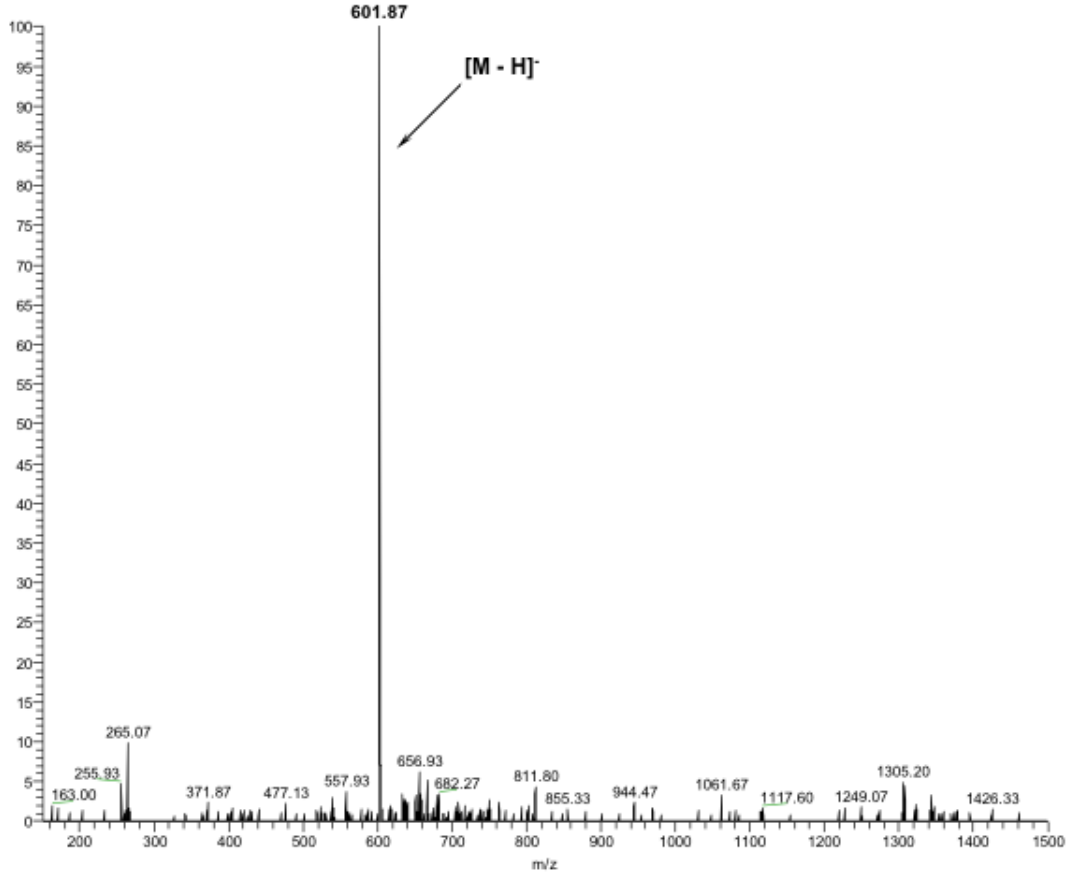
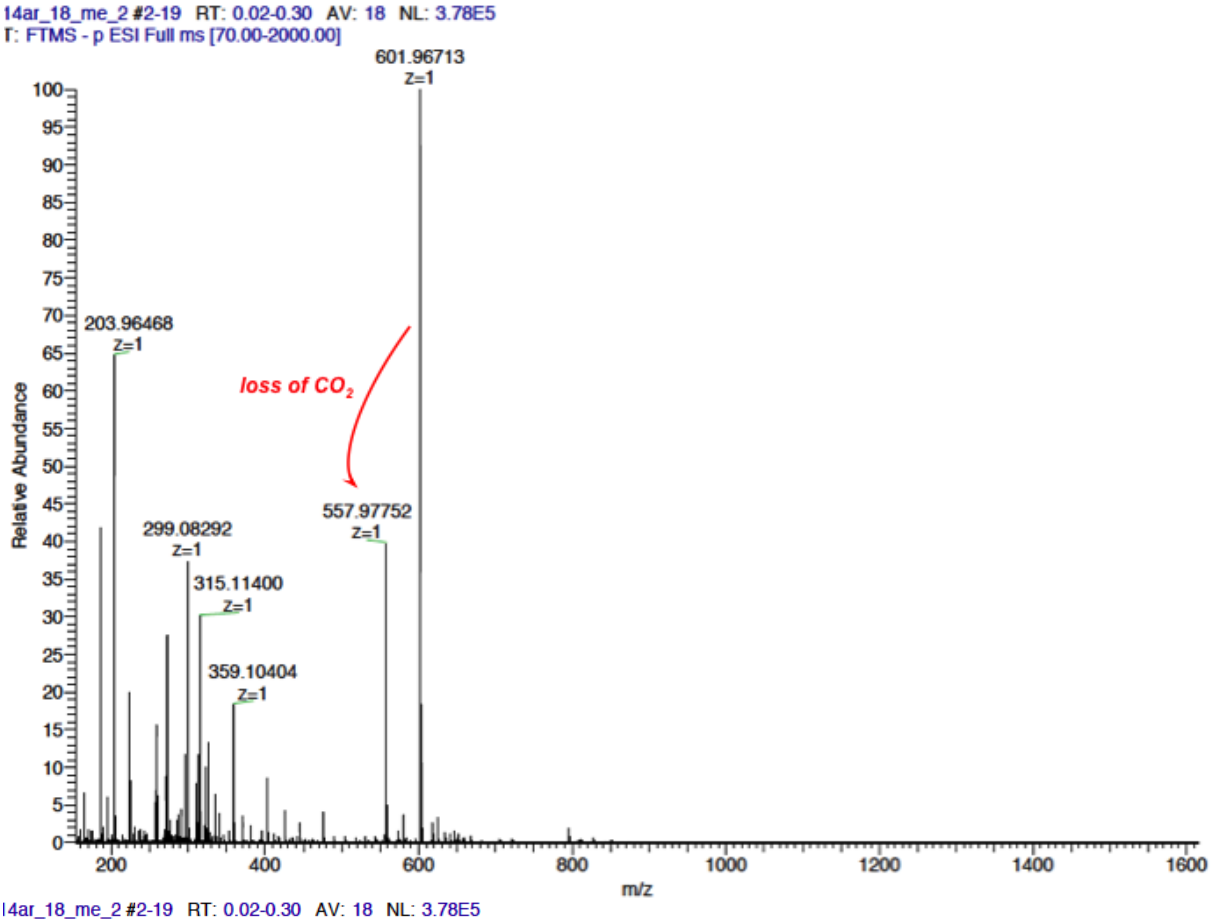


Figure S26. ESI- mass spectrum (high resolution) of disulfonated DPP-thienyl **8.**



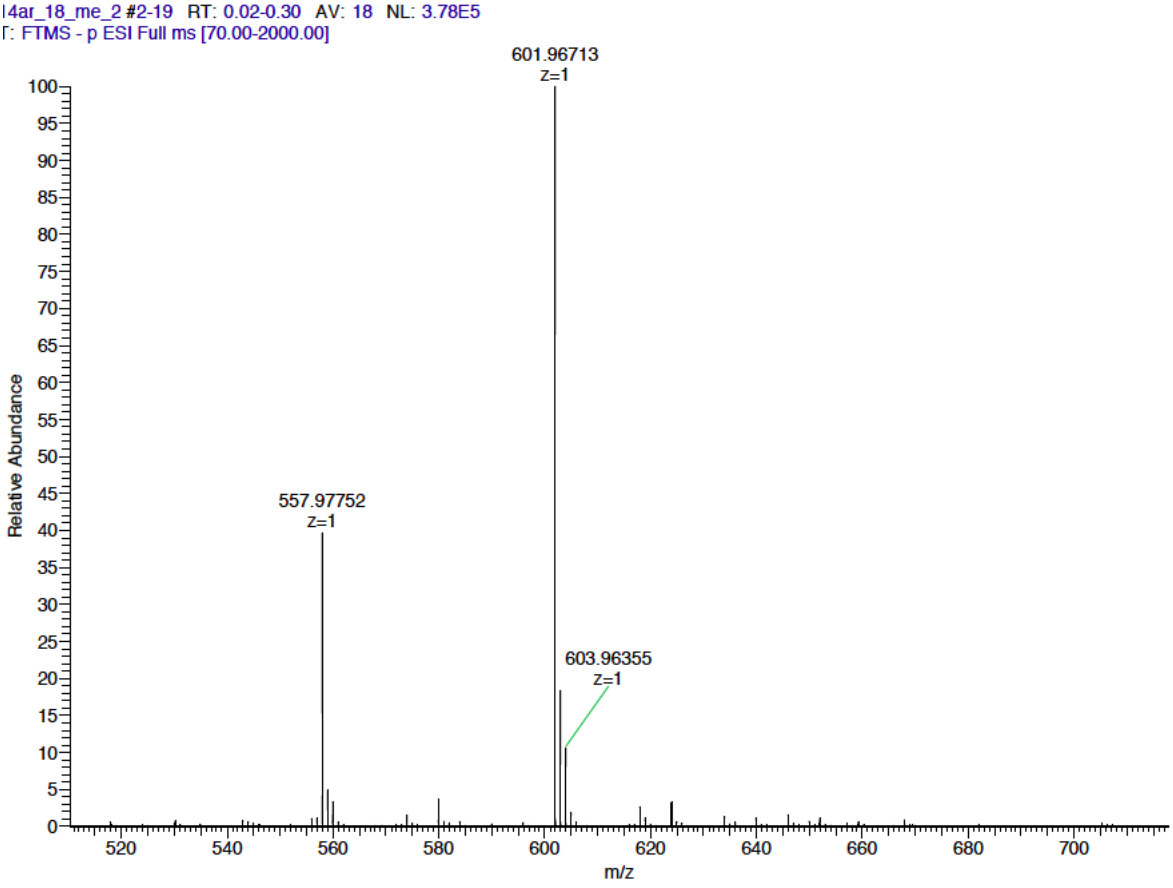
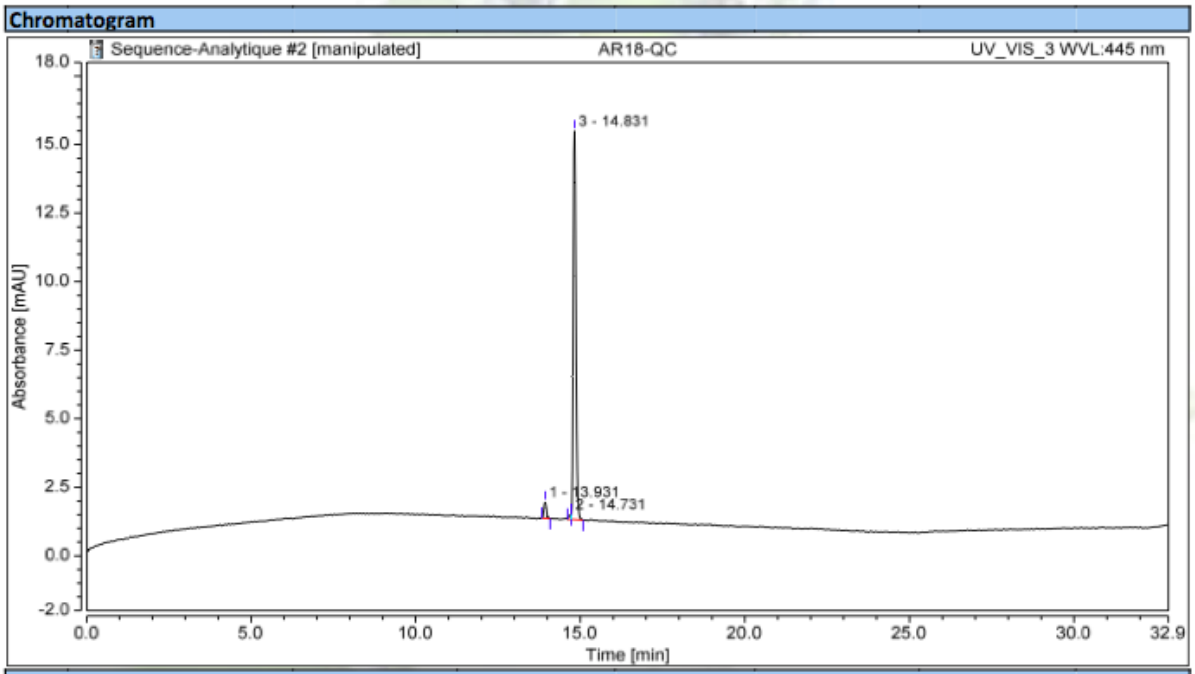


Figure S27. RP-HPLC elution profile (system A) of disulfonated DPP-thienyl **8** (visible detection at 445 nm).



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		13.931	0.051	0.593	3.70	3.96	n.a.
2		14.731	0.013	0.177	0.96	1.18	n.a.
3		14.831	1.317	14.178	95.34	94.85	n.a.
Total:			**1.381**	**14.947**	**100.00**	**100.00**	

Figure S28. ^{1}H NMR spectrum of tetrasulfonated DPP-phenyl **9** recorded in [D_6]DMSO at 500 MHz.

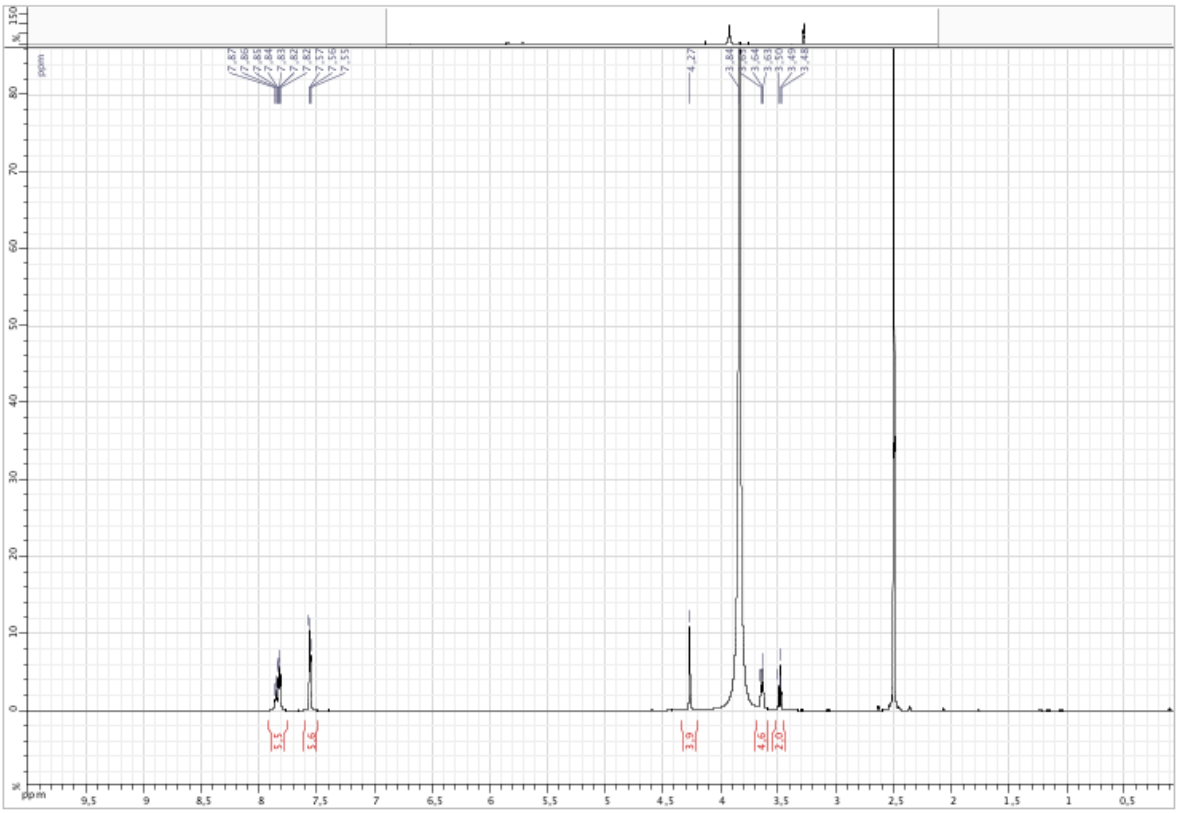


Figure S29. ^{13}C NMR spectrum of tetrasulfonated DPP-phenyl **9** recorded in [D_6]DMSO at 126 MHz.

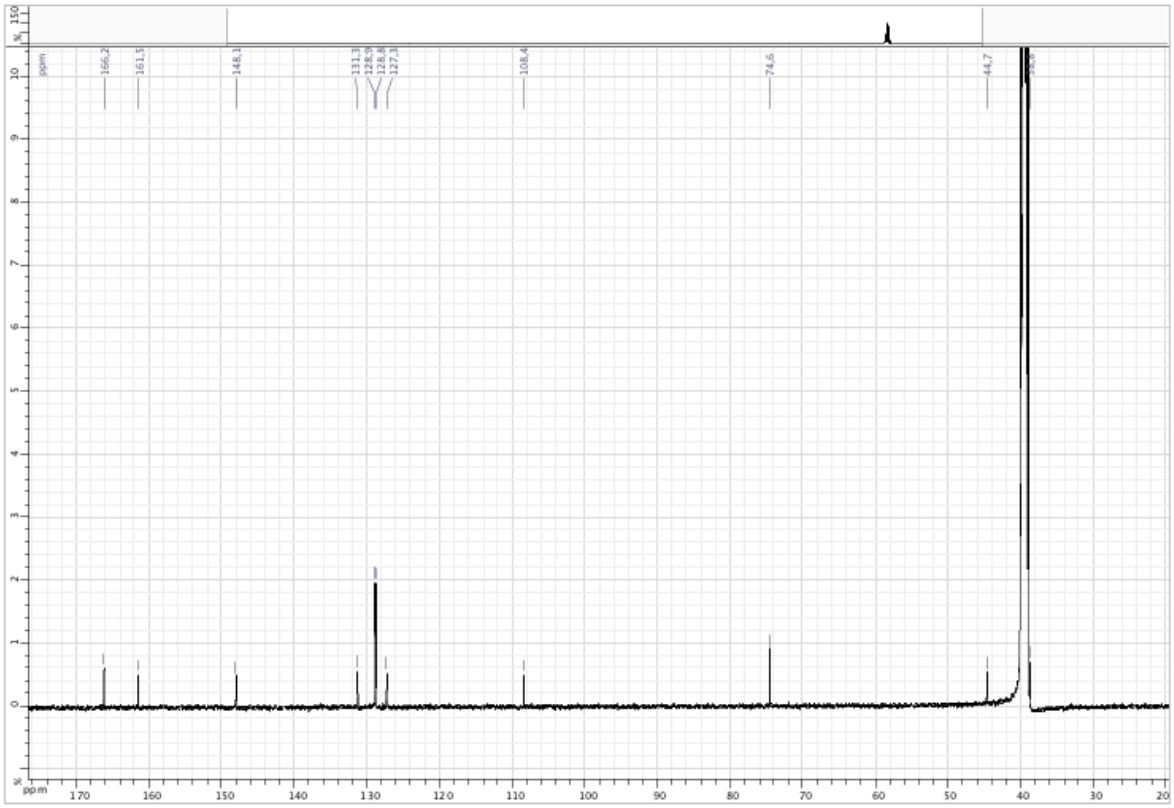


Figure S30. ESI- mass spectrum (low resolution) of tetrasulfonated DPP-phenyl **9**.

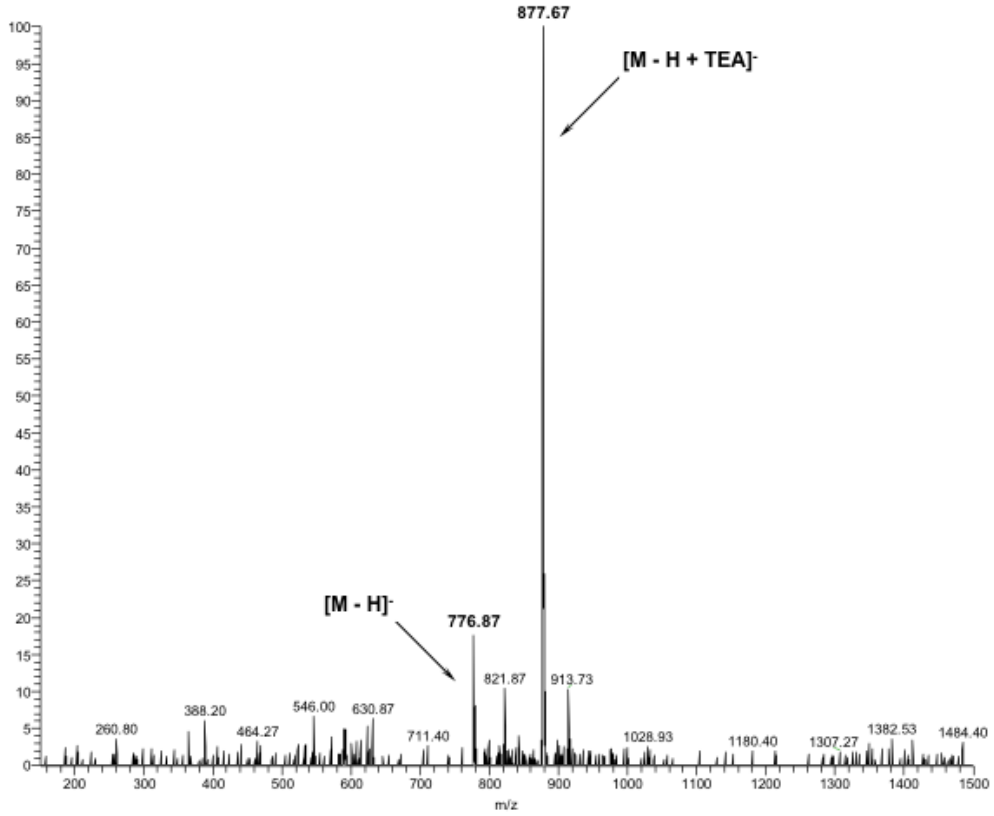
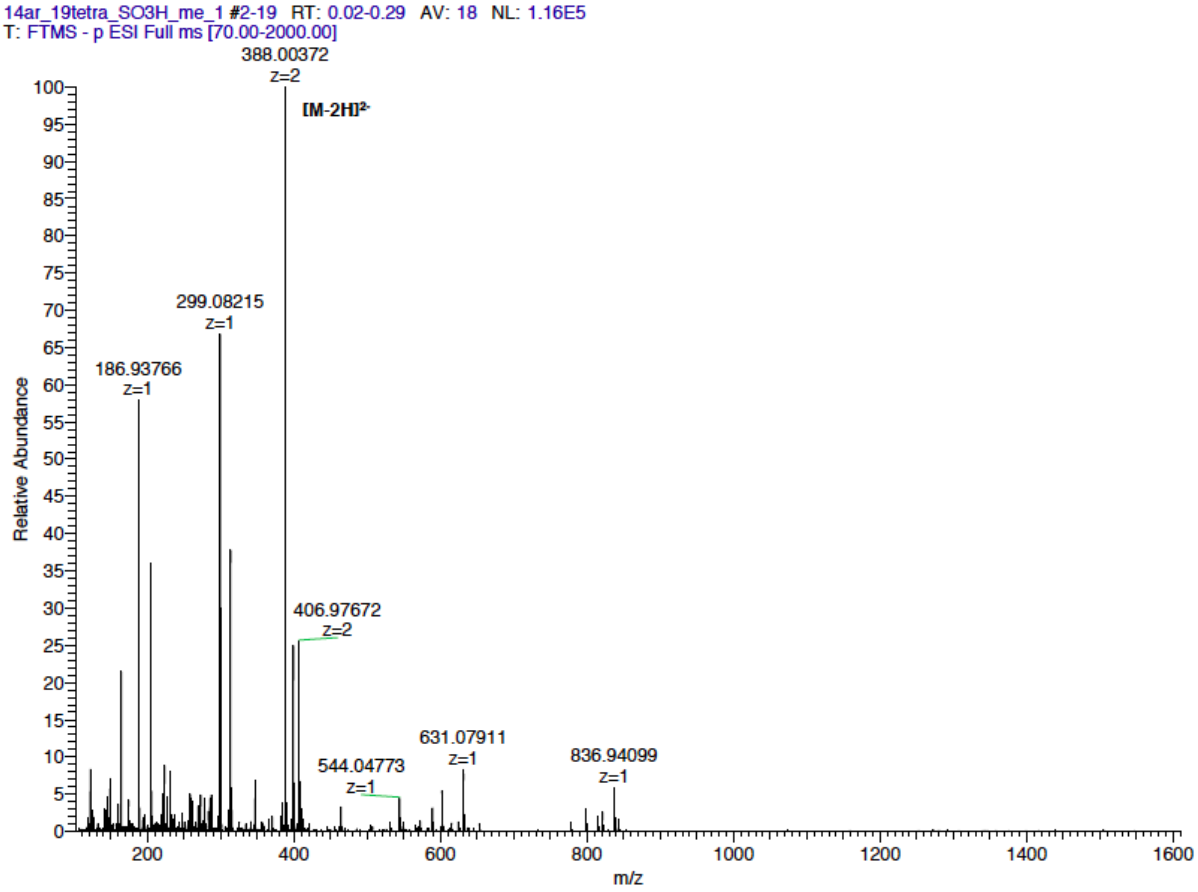


Figure S31. ESI- mass spectrum (high resolution) of tetrasulfonated DPP-phenyl 9



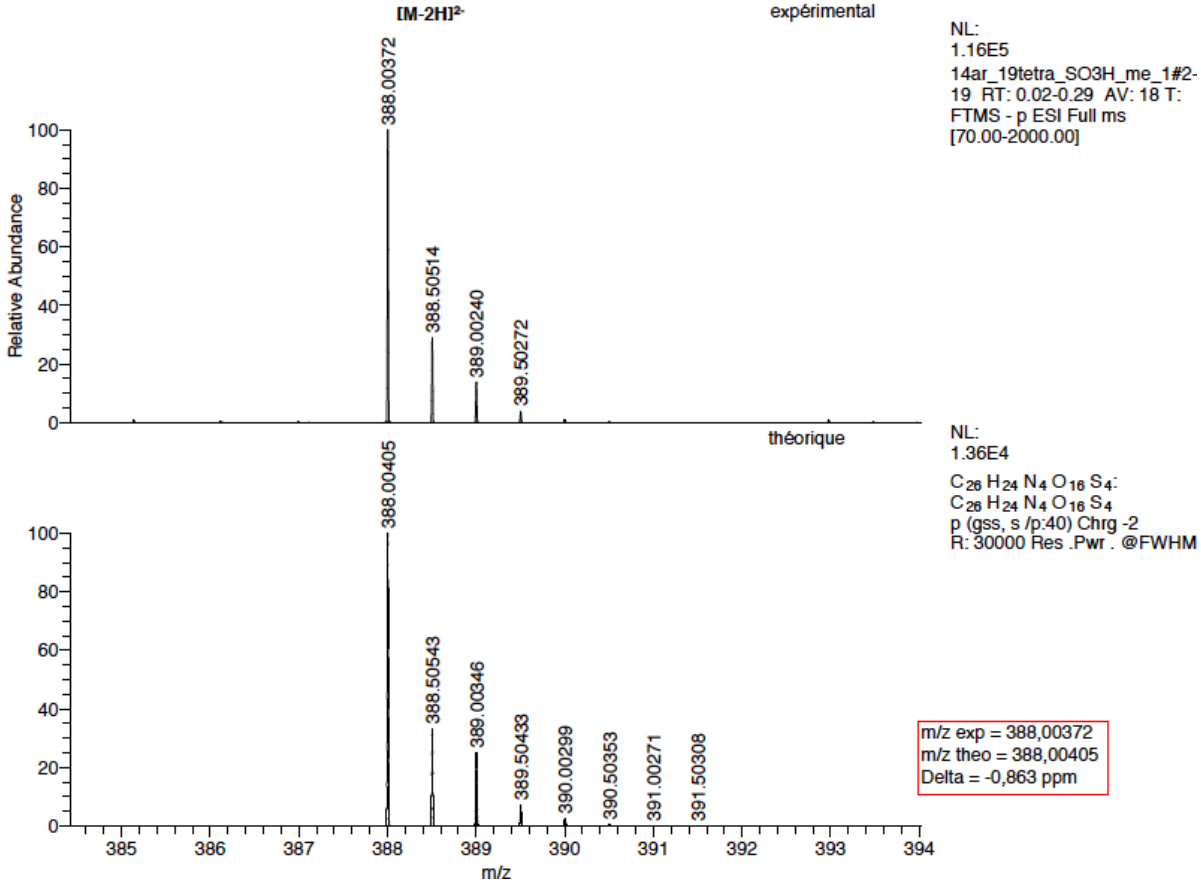
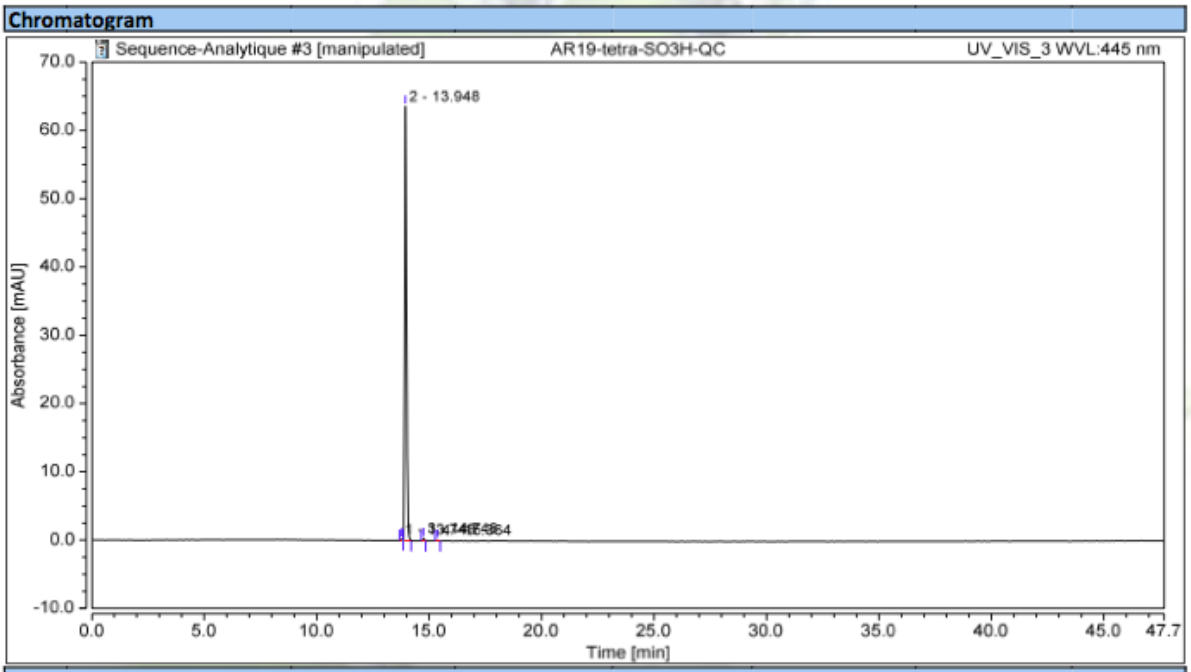

[M-2H]²⁻
expérimental
NL:
1.16E5
14ar_19tetra_SO3H_me_1#2-19 RT: 0.02-0.29 AV: 18 T: FTMS - p ESI Full ms [70.00-2000.00]
388.00372
388.50514
389.00240
389.50272
Relative Abundance
théorique
NL:
1.36E4
C26 H24 N4 O16 S4:
C26 H24 N4 O16 S4
p (gss, s /p:40) Chrg -2
R: 30000 Res .Pwr . @FWHM
388.00405
388.50543
389.00346
389.50433
390.00299
390.50353
391.00271
391.50308
m/z exp = 388,00372
m/z theo = 388,00405
Delta = -0,863 ppm
m/z

Figure S32. RP-HPLC elution profile (system A) of tetrasulfonated DPP-phenyl **9** (visible detection at 445 nm).



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		13.748	0.022	0.201	0.38	0.31	n.a.
2		13.948	5.802	63.617	99.00	99.00	n.a.
3		14.748	0.028	0.336	0.47	0.52	n.a.
4		15.364	0.008	0.106	0.14	0.16	n.a.
Total:			**5.861**	**64.259**	**100.00**	**100.00**	

Figure S33. ESI- mass spectrum (low resolution) of tetrasulfonated DPP-thienyl **10.**

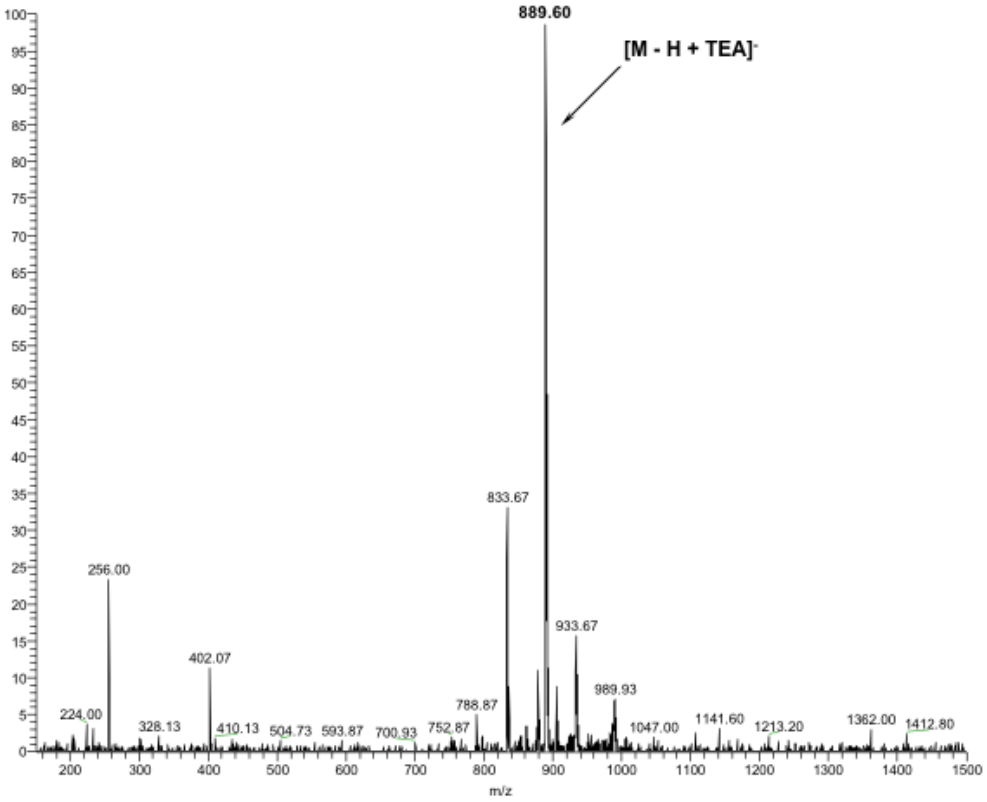


Figure S34. ESI+ mass spectrum (low resolution) of the crude NHS ester **5-NHS**.

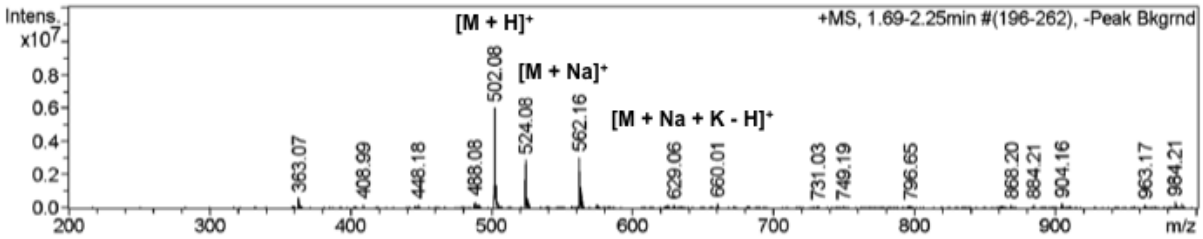


Figure S35. ESI+ (top) and ESI- (bottom) mass spectra (low resolution) of the crude NHS ester **6-NHS**.

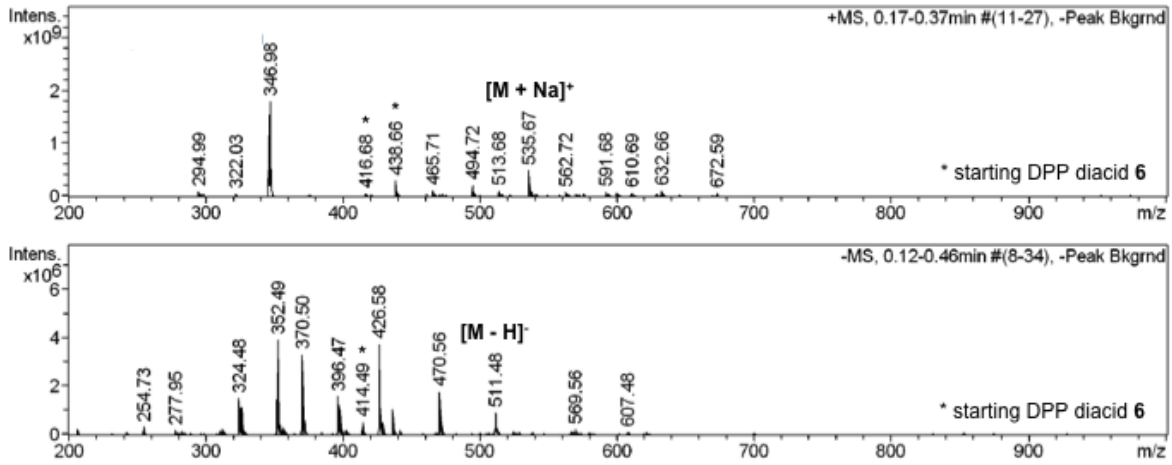


Figure S36. MALDI-TOF mass spectra of BSA (top) and fluorescent conjugates **BSA-6** (bottom left) and **BSA-8** (bottom right) (labelling conditions: 15 equiv. of DPP dye in phosphate buffer at pH 7.0) recorded in the positive mode (matrix: sinapinic acid).

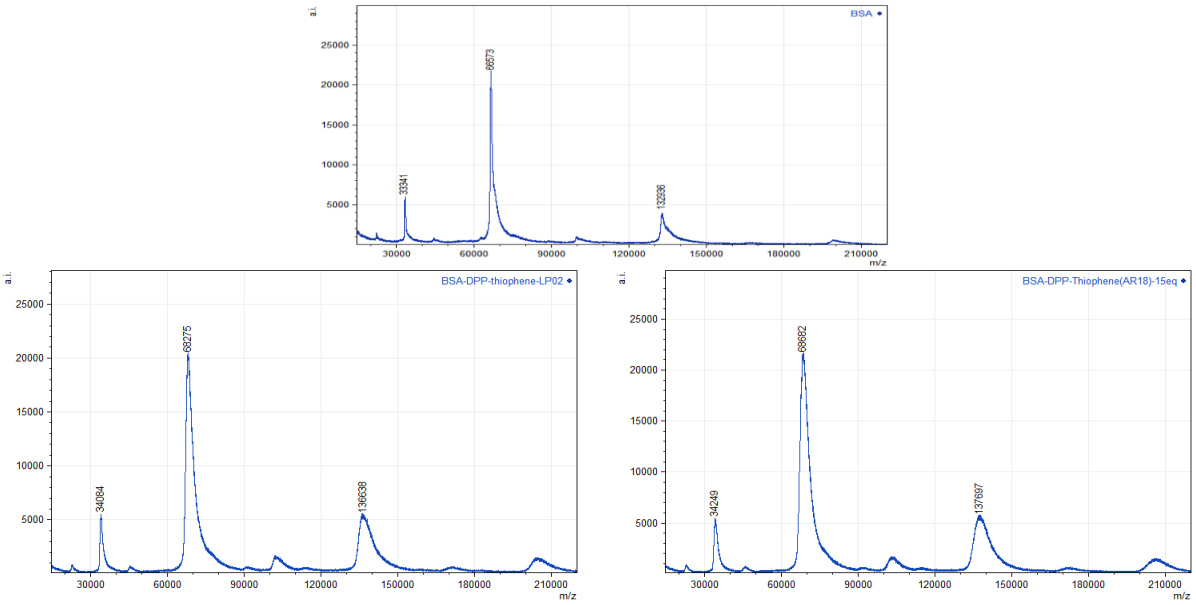


Figure S37. Gel images of conjugates: (left) fluorescence scan (upon illumination at 365 nm), (right) Coomassie stain.

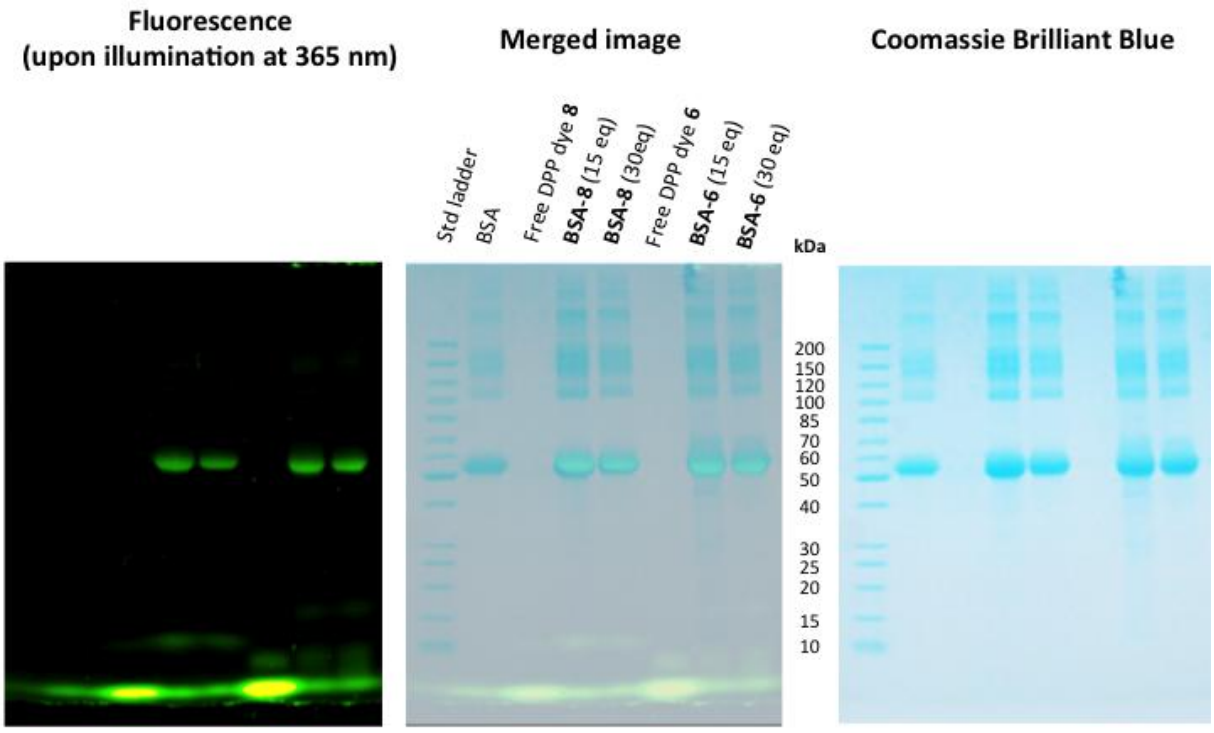
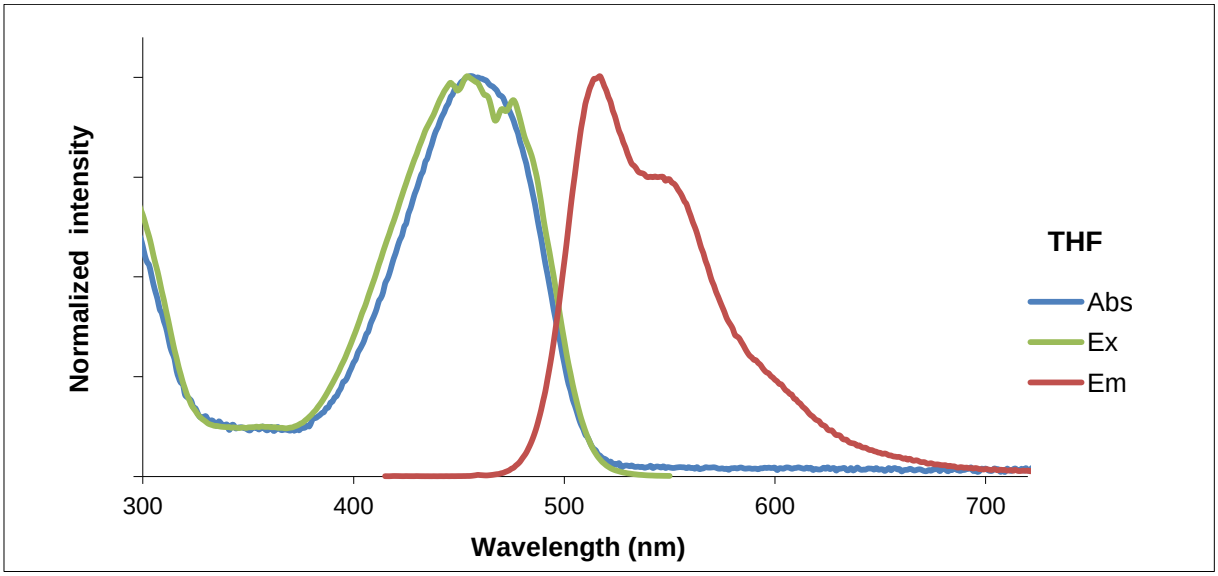


Figure S38. Normalized absorption, corrected emission (λ_{exc} = 400 nm) and excitation (λ_{em} = 580 nm) spectra of compound **3** in THF (top) and DMSO (bottom) at 25 °C.



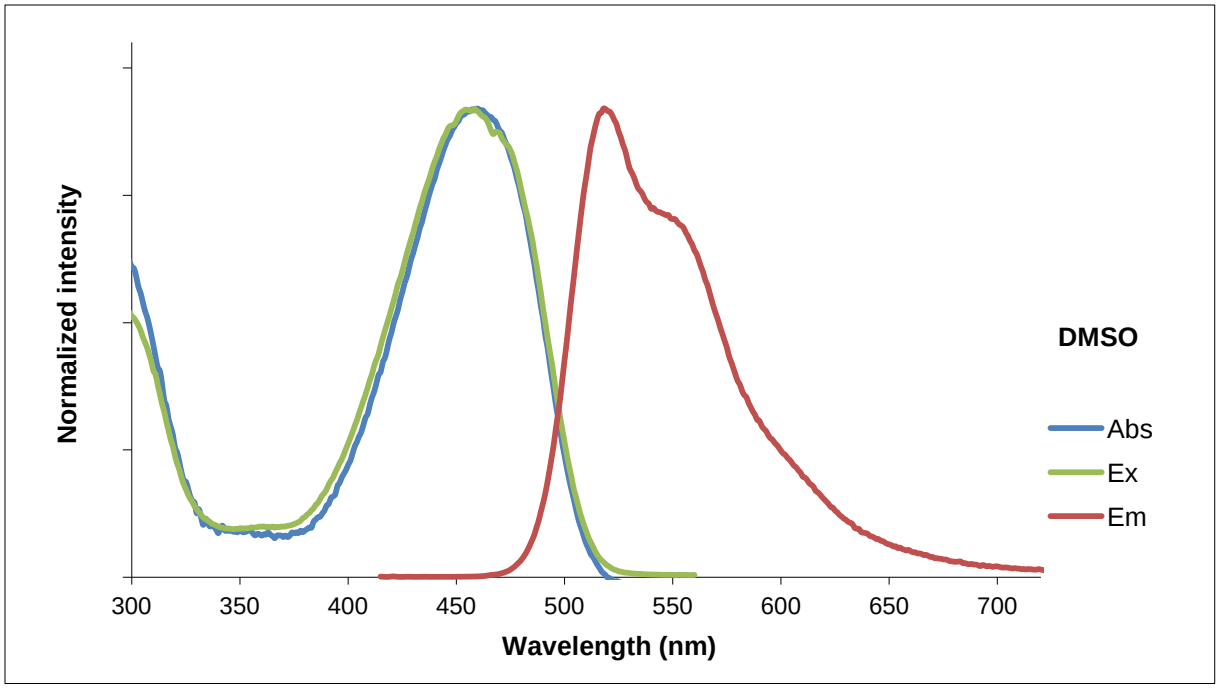
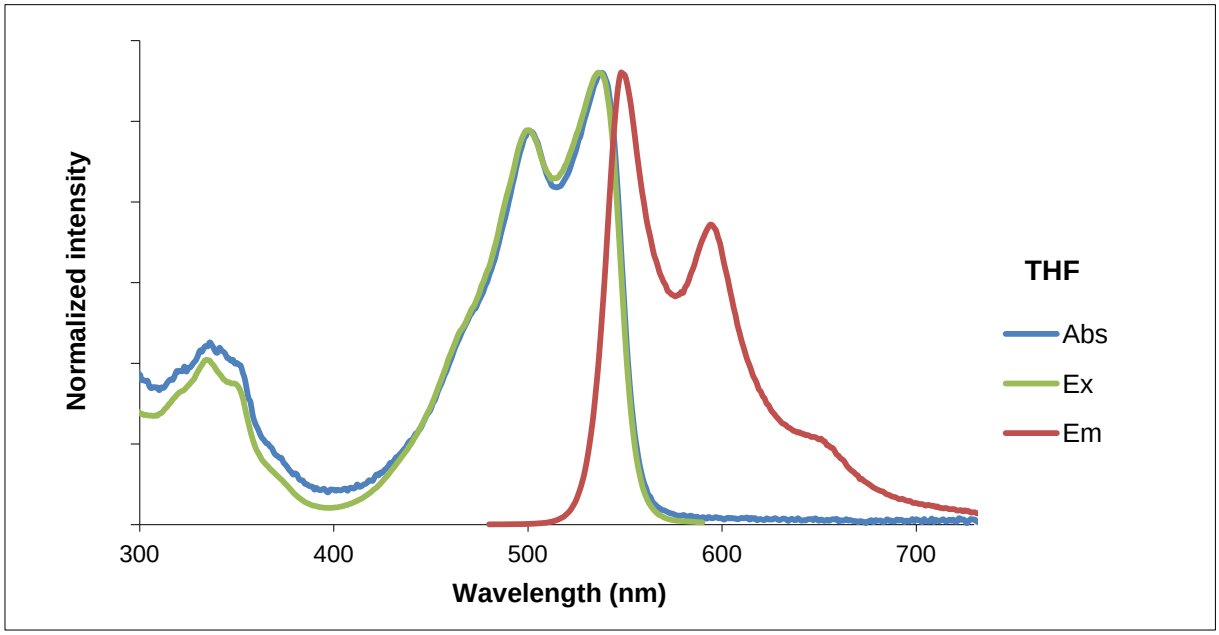


Figure S39. Normalized absorption, corrected emission (λ_{exc} = 460 nm) and excitation (λ_{em} = 620 nm) spectra of compound 4 in THF (top) and DMSO (bottom) at 25 °C**.**



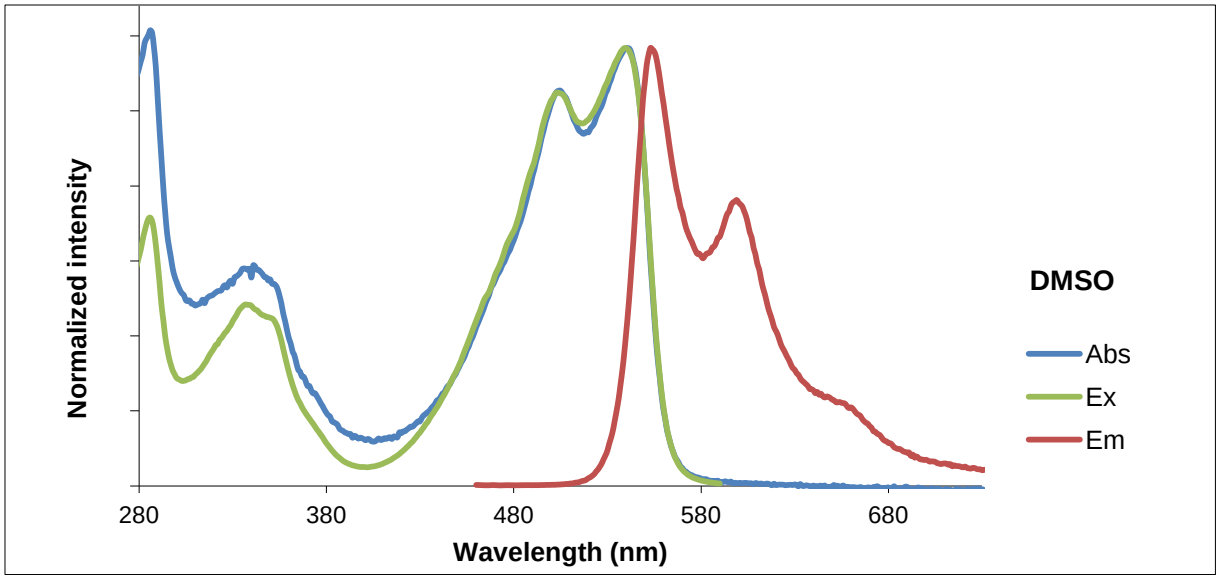


Figure S40. Normalized absorption, corrected emission (λ_{exc} = 400 nm) and excitation (λ_{em} = 610 nm) spectra of compound **5** in PBS at 25 °C.

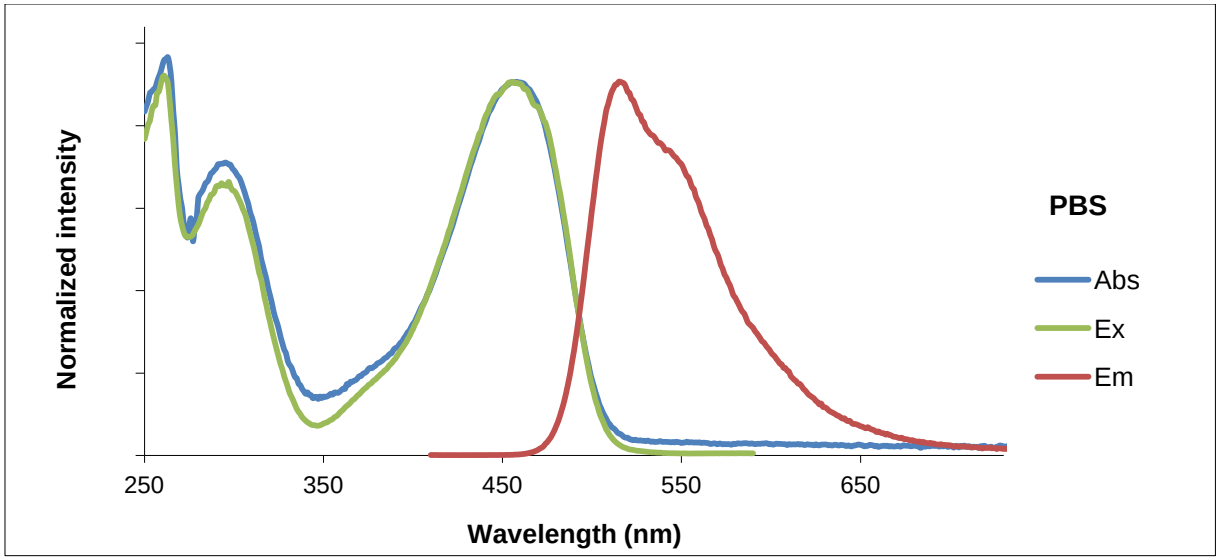


Figure S41. Normalized absorption, corrected emission (λ_{exc} = 450 nm) and excitation (λ_{em} = 600 nm) spectra of compound **6** in PBS at 25 °C.

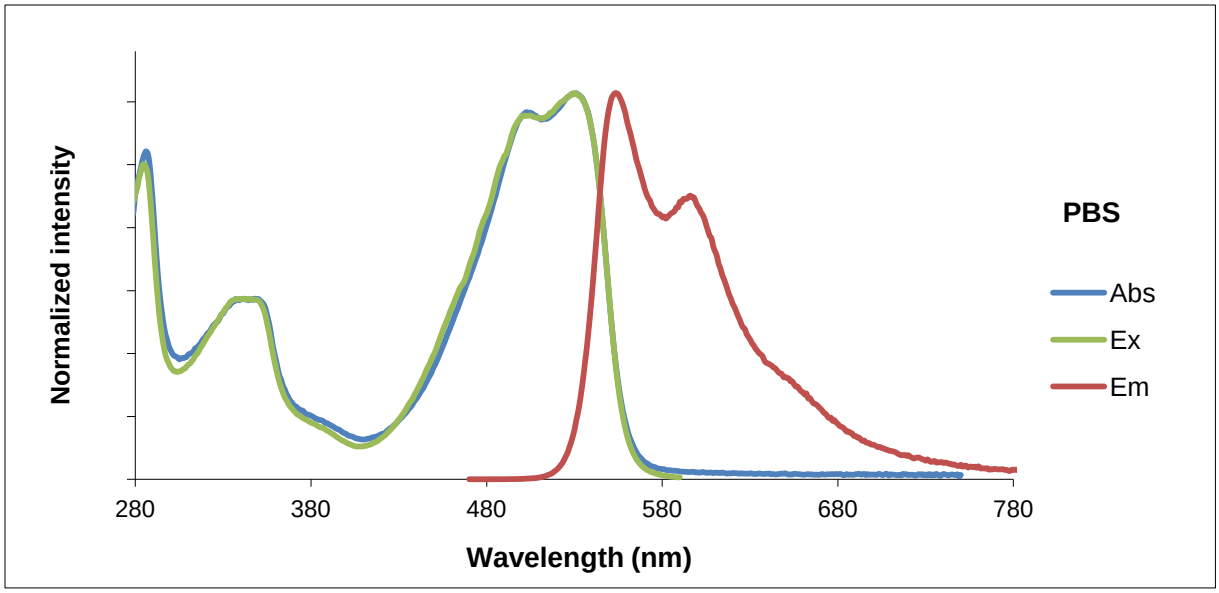


Figure S42. Normalized absorption, corrected emission (λ_{exc} = 450 nm) and excitation (λ_{em} = 550 nm) spectra of DPP-phenyl di-SO_3H **7** in H_2O + 2.5% SDS at 25 °C.

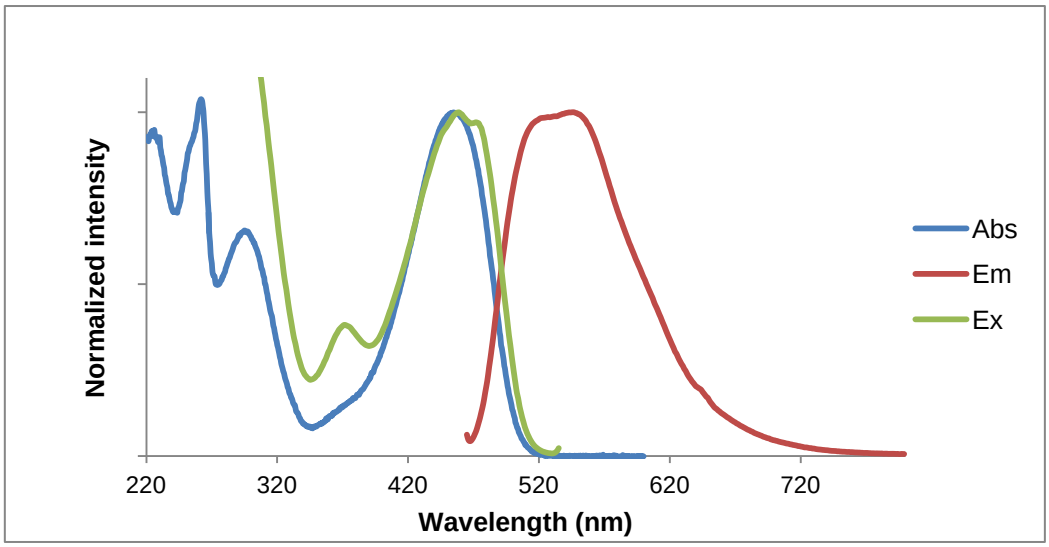


Figure S43. Normalized absorption, corrected emission (λ_{exc} = 450 nm) and excitation (λ_{em} = 590 nm) spectra of DPP-thienyl di-SO_3H 8 in H_2O + 2.5% SDS at 25 °C.

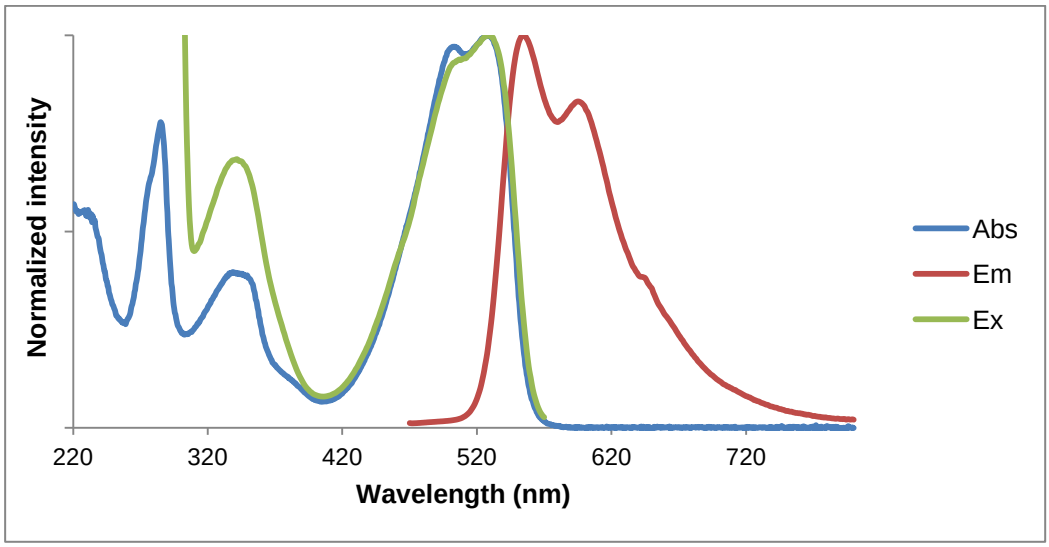


Figure S44. Normalized absorption, corrected emission (λ_{exc} = 450 nm) and excitation (λ_{em} = 550 nm) spectra of DPP-phenyl tetra-SO_3H **9** in PBS at 25 °C.

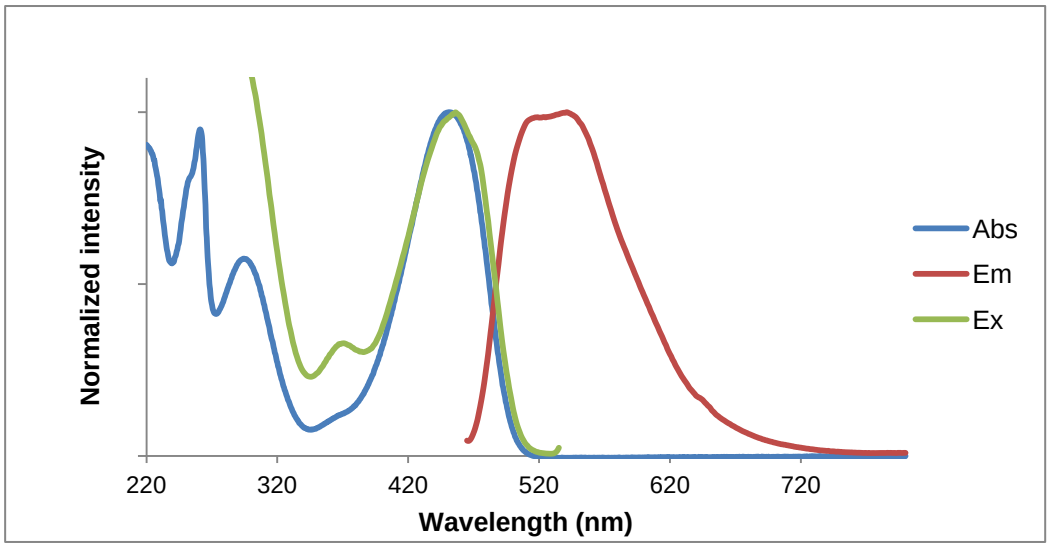


Figure S45. Normalized absorption, corrected emission (λ_{exc} = 450 nm) and excitation (λ_{em} = 550 nm) spectra of DPP-phenyl tetra-SO_3H **9** in H_2O + 2.5% SDS at 25 °C.

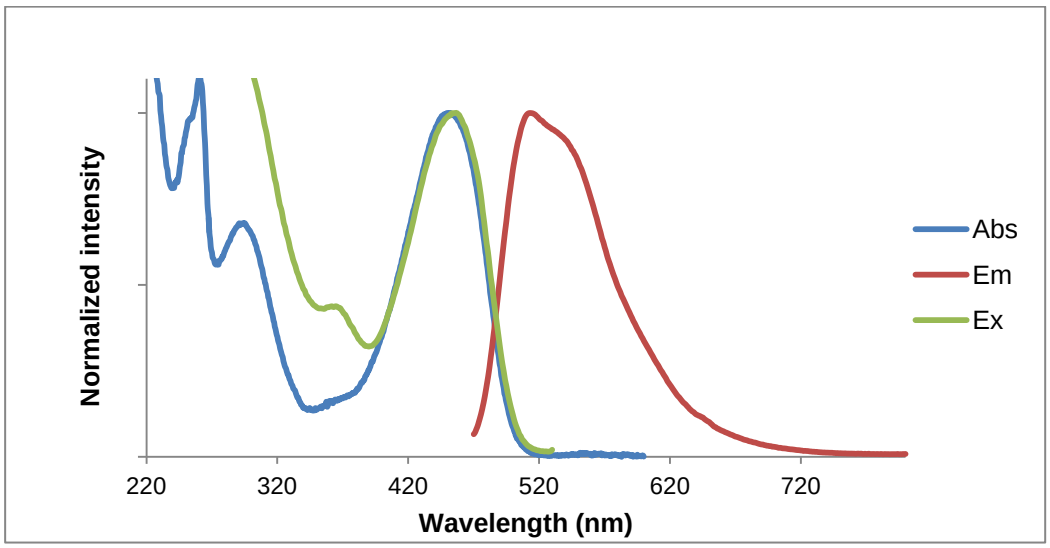


Figure S46. Normalized absorption, corrected emission (λ_{exc} = 450 nm) and excitation (λ_{em} = 590 nm) spectra of DPP-thienyl tetra-SO_3H 10 in PBS at 25 °C.

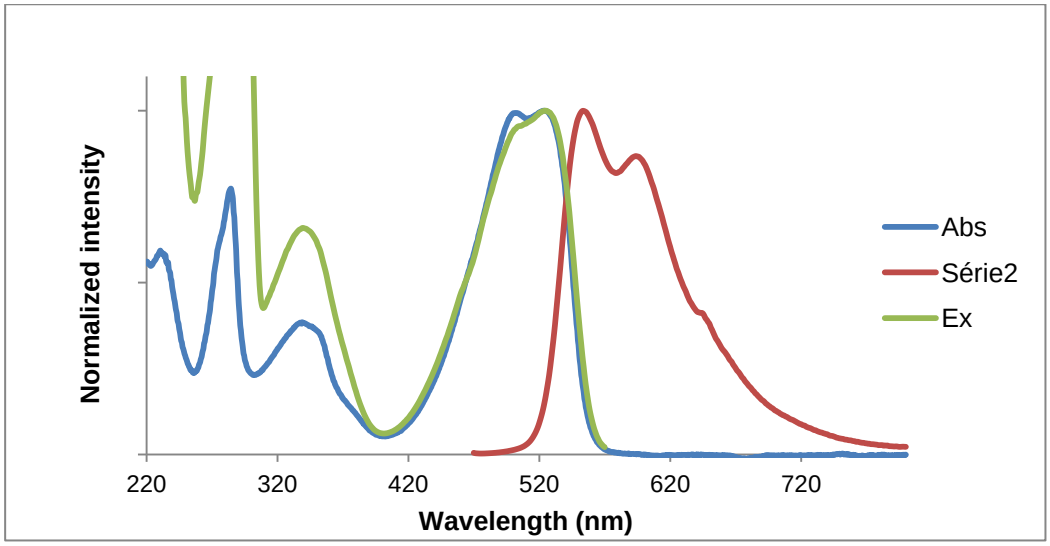


Figure S47. Normalized absorption, corrected emission (λ_{exc} = 450 nm) and excitation (λ_{em} = 590 nm) spectra of DPP-thienyl tetra-SO_3H **10** in H_2O + 2.5% SDS at 25 °C.

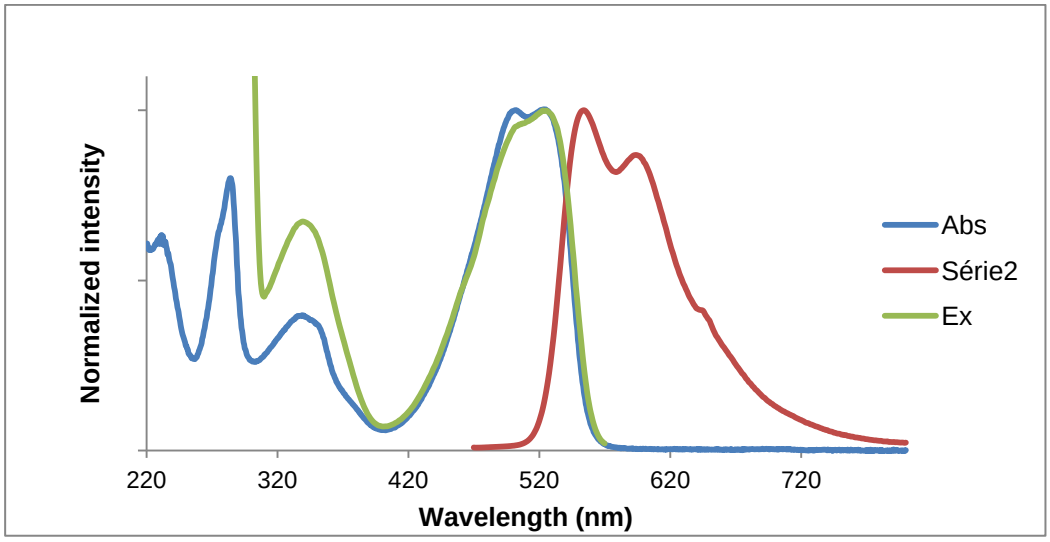


Figure S48. Normalized absorption, corrected emission (λ_{exc} = 450 nm) and excitation (λ_{em} = 590 nm) spectra of fluorescent bioconjugate **BSA-5** in PBS at 25 °C (labeling conditions: 15 equiv. of 5-NHS, pH 7.0).

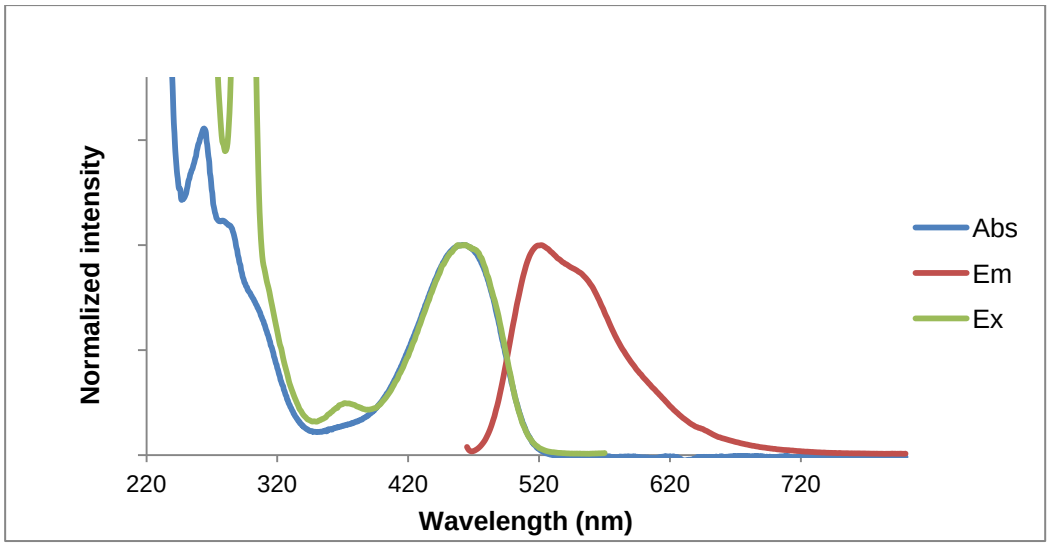


Figure S49. Normalized absorption, corrected emission (λ_{exc} = 450 nm) and excitation (λ_{em} = 590 nm) spectra of fluorescent bioconjugate **BSA-6** in PBS at 25 °C (labeling conditions: 15 equiv. of 6-NHS, pH 7.0).

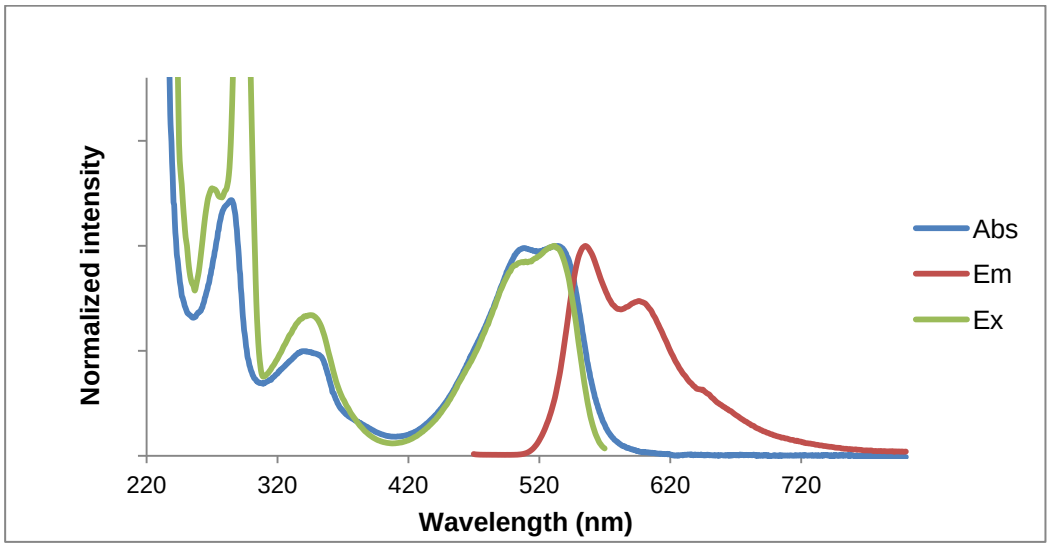
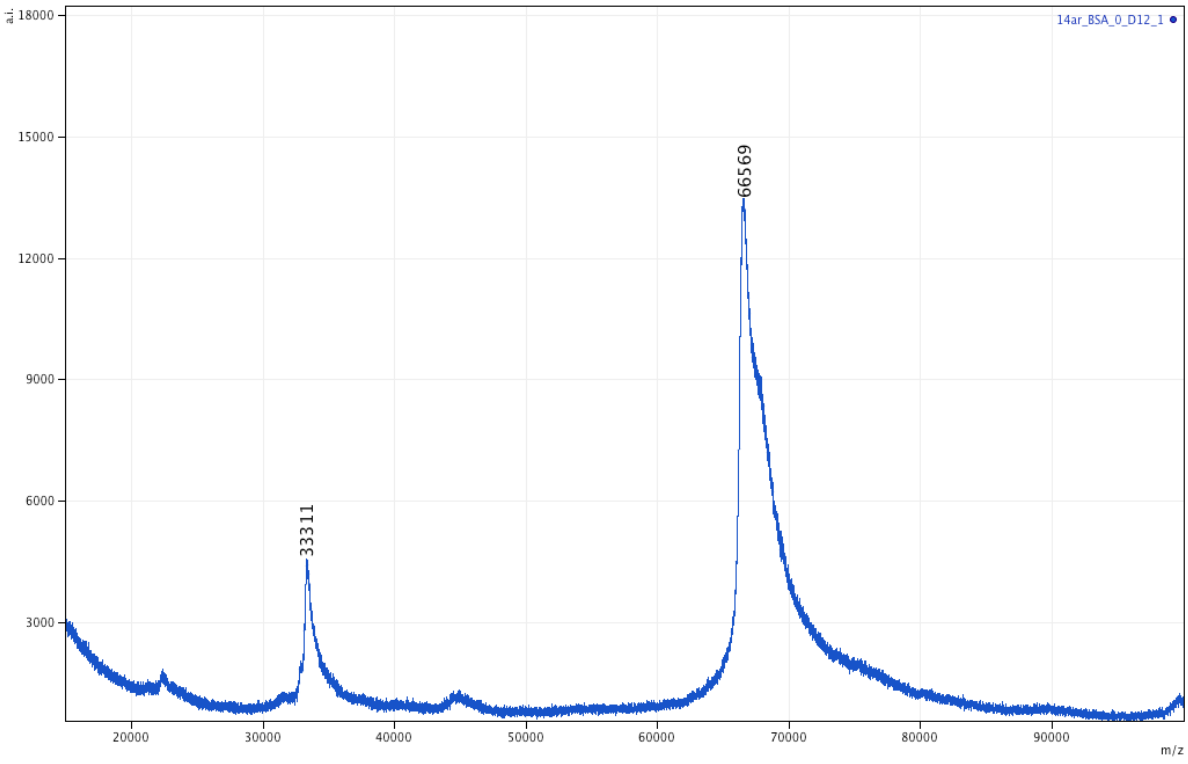


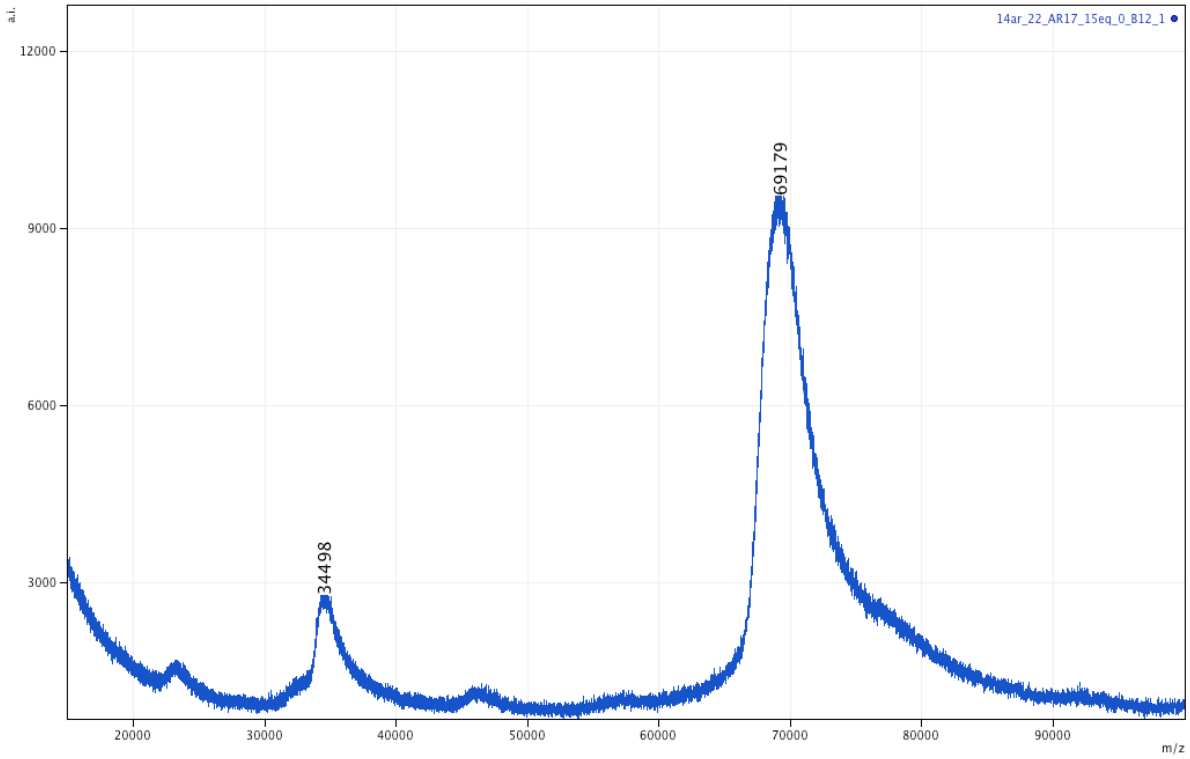
Figure S50. MALDI-TOF mass spectra of fluorescent bioconjugates **BSA-5** and **BSA-7** (linear detector mode, matrix: sinapinic acid, positive mode).

The two main peaks observed in all mass spectra are assigned to $[M + 2H]^{2+}$ and $[M + H]^{+}$.

a) BSA

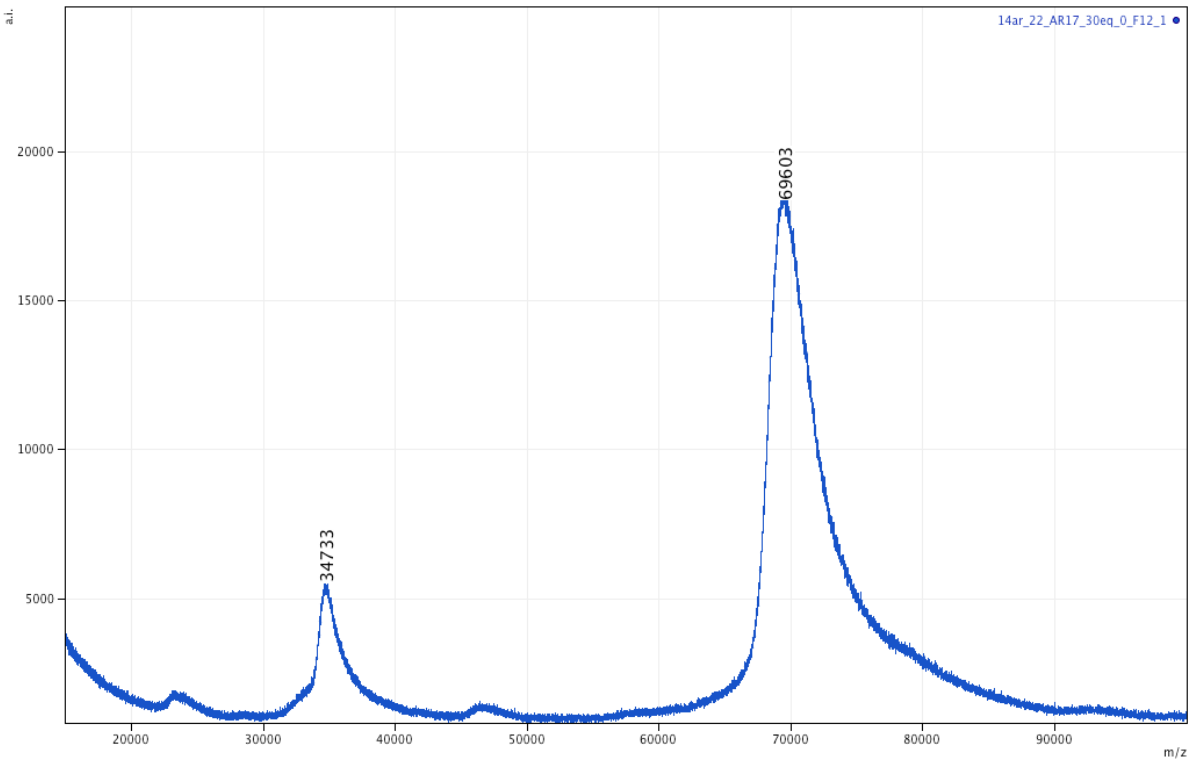


*b) **BSA-5**, labeling conditions: 15 equiv. of **5-NHS**, pH 7.0*



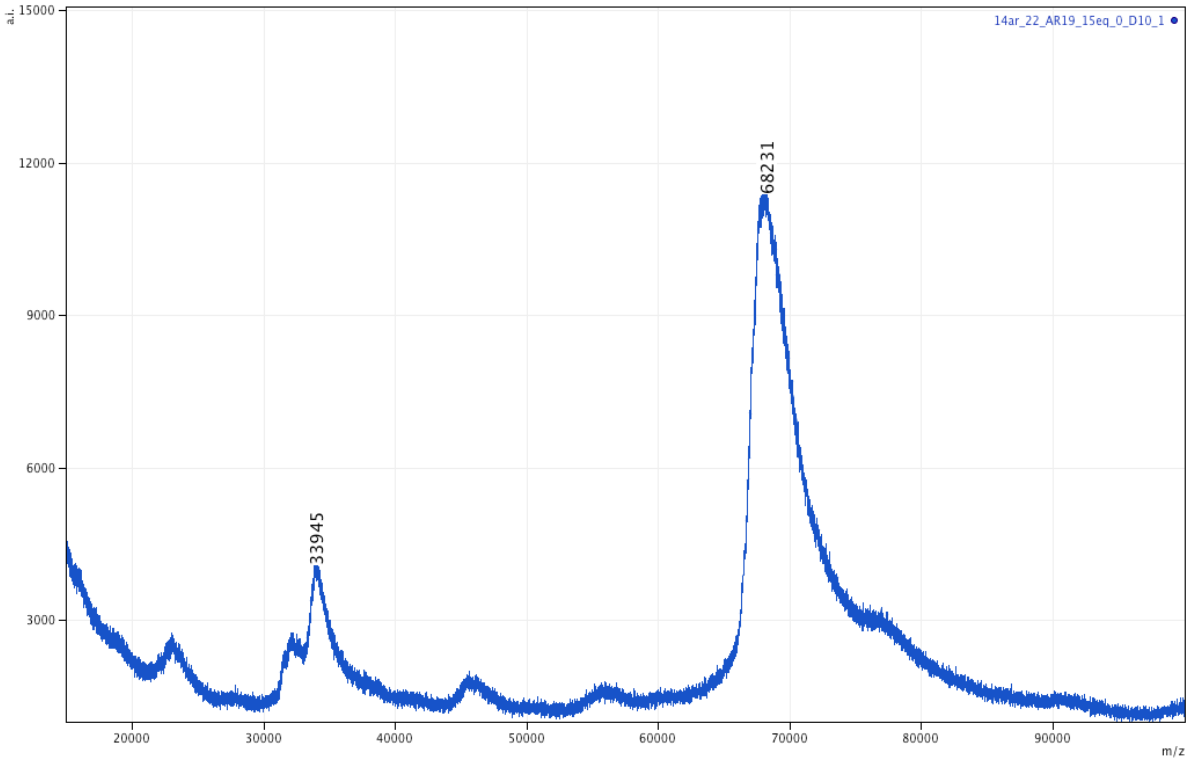
F/P (calcd. from the mass difference between BSA and labeled-BSA) = 6.8

*c) **BSA-5**, labeling conditions: 30 equiv. of **5-NHS**, pH 7.0*



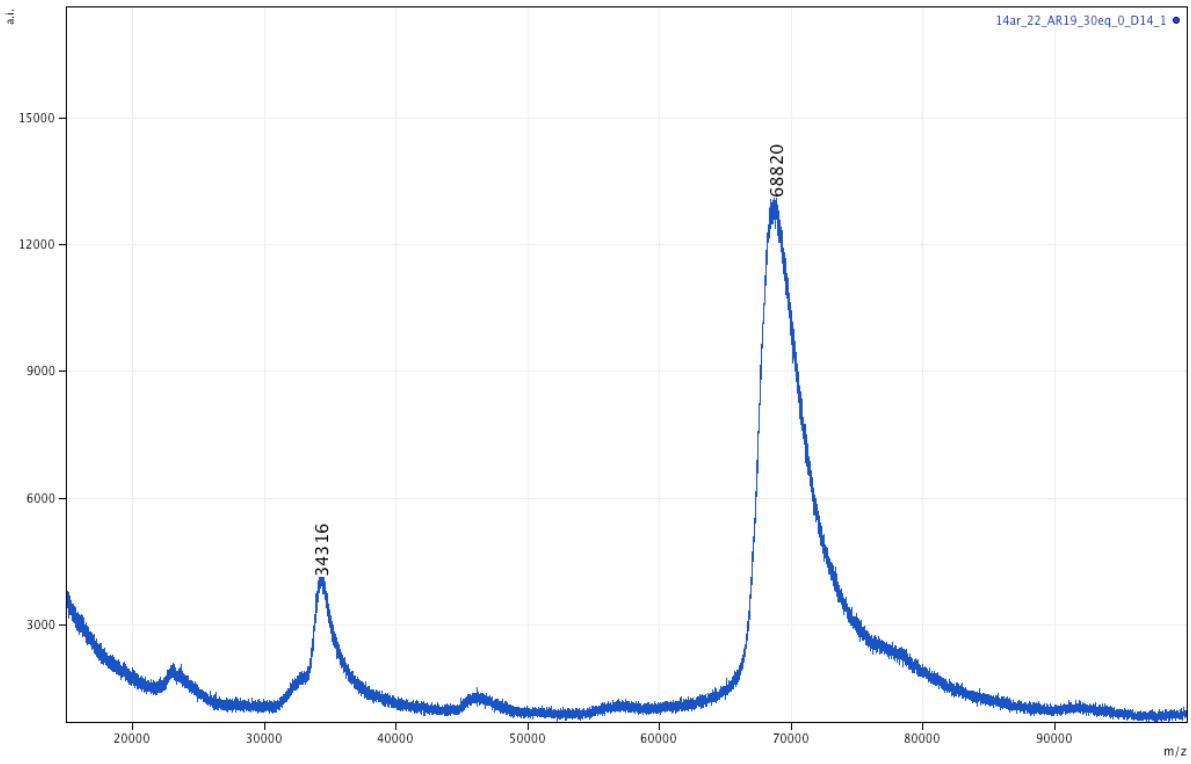
F/P (calcd. from the mass difference between BSA and labeled-BSA) = 7.9

d) ***BSA-7****, labeling conditions: 15 equiv. of* ***7-NHS****, pH 7.0*



F/P (calcd. from the mass difference between BSA and labeled-BSA) = 2.9

e) ***BSA-7****, labeling conditions: 30 equiv. of* ***7-NHS****, pH 7.0*



F/P (calcd. from the mass difference between BSA and labeled-BSA) = 3.9

Figure S51. Fluorescence spectra evolution during the photobleaching experiments for disulfonated DPP-thienyl dye 8 (at a concentration of 0.42 μM) in PBS (pH 7.2) and at rt.

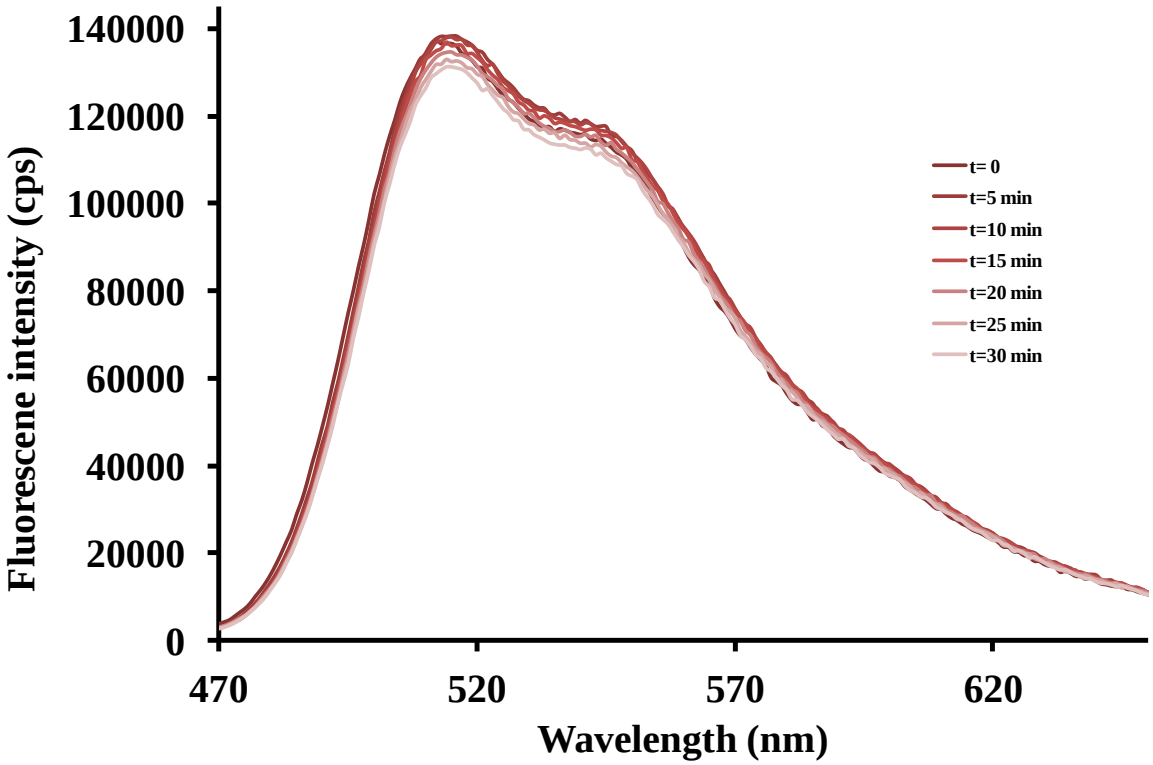


Figure S52. Fluorescence spectra evolution during the photobleaching experiments for fluorescein (at a concentration of 0.2 μM) in PBS (pH 7.2) and at rt.

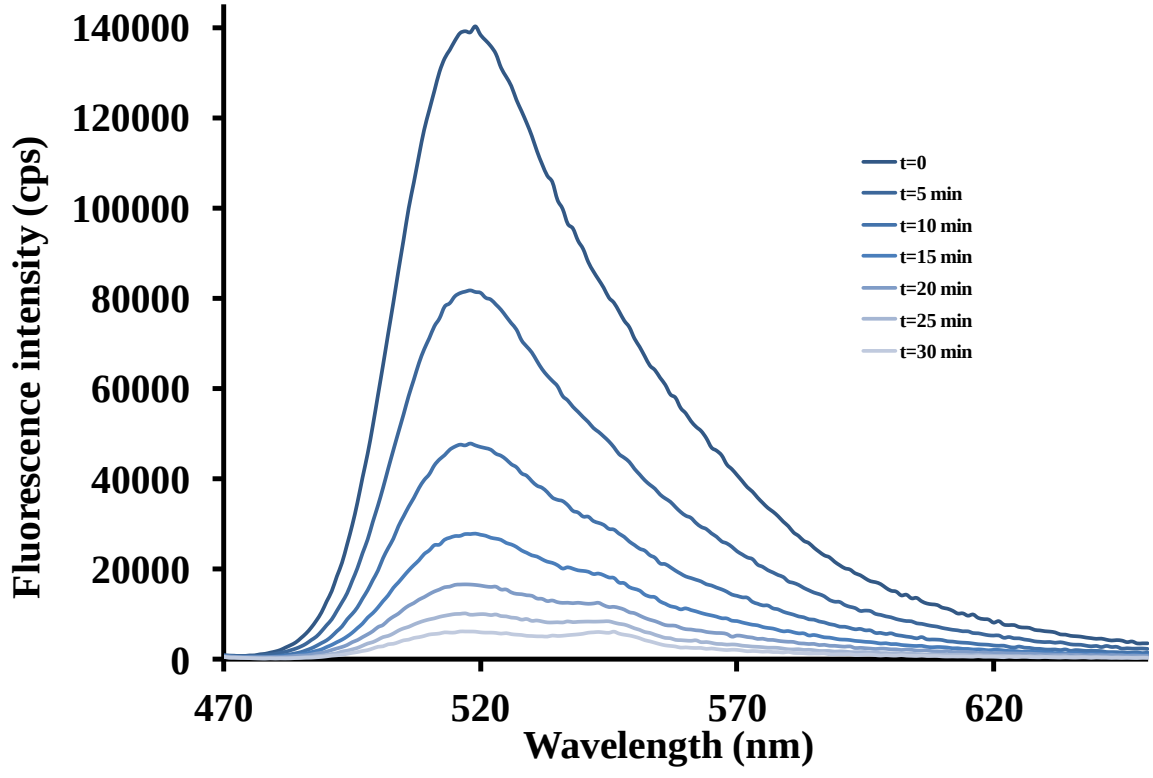


Figure S53. Photobleaching experiments for disulfonated DPP-thienyl dye **8** (**X**, concentration: 0.42 μM), dye 7 (●, concentration 0.40 μM), and for fluorescein (▲, concentration: 0.2 μM) in PBS (pH 7.2) and at rt.