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Fluorescent Properties and Resonance Energy Transfer of 3,4-Bis(2,4-difluorophenyl)-maleimide

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Supporting Information

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I. Synthetic procedures used to generate the FRET peptide 10

The FRET peptide was generated by a solid-phase synthesis approach, followed by the subsequent coupling of the donor (fluorophore) and the acceptor (quencher) molecules. The fluorophore **1c** was coupled to the N-terminus and the DABCYL component (quencher) was linked to the amino group at the Lysine residue at the C-terminus. The peptide was generated in 25 µmol by using peptide synthesizer (Symphony, Peptide Technology Inc.). Fmoc-protective group was installed at the N-terminus with an Alloc protective group at the lysine residue on the C-terminus. In the first step the peptide resin was washed with DCM (3 x 5 mL x 30 s) and allowed to expand for 10 minutes in DCM under Ar atmosphere. Subsequent Fmoc - removal was performed by reacting the peptide resin with 20 % piperidine in DMF under Ar atmosphere for 20 min, followed by wash with DMF (3 x 30 s) and DCM (3 x 30 s).[1] Upon removal of Fmoc, while still in solution under Ar atmosphere, the peptide resin was reacted with the fluorophore **1c** (40.7 mg, 4 eq), HCTU (41 mg, 4 eq), and DIPEA (17.4 µl, 4 eq). The reaction continued for 120 min under Ar atmosphere and the system was stirred occasionally. After the completion of the coupling reaction, the peptide resin was again washed with DMF (3 x 30 s) and then DCM (3 x 30 s). Subsequent Alloc removal was performed by allowing the peptide resin to react with $PhSiH_3$ (24 eq, 81µL) in DCM (1 mL) and solution of $Pd(PPh_3)_4$ (3 mg, 0.10 eq) in DCM (3 mL) with Ar passing through and mechanical stir for about 15 min.[2] The resin was washed twice with DCM (8 x 30 s) and Kaiser test was performed to confirm the presence of free amine as a result of the successful removal of the Alloc group. Subsequently, the pre-activated quencher, 4-(4-dimethylamino-phenylazo)-benzoic acid 2,5-dioxo-2,5-dihydro-pyrrol-1-yl ester (35 mg, 4 eq) in DCM (3 mL) was reacted for 120 min with the resin peptide. The peptide resin was washed with DCM (8 x 30 s) and that process was repeated once more. The peptide was cleaved from the resin with 95 % THF, 2.5% H_2O, 2.5% triisopropylsilane and subsequently washed with DCM (5 x 30 s). The peptide was precipitated from cold diethyl ether and subsequently segregated from the solvent by centrifugation. The isolated peptide was dissolved in acetonitrile : water (30 : 70) and it was isolated as red powder after lyophilization. The mass of the final peptide was confirmed by MALDI–TOF, calculated 1704.71, and f1und 1727.39 (M + Na).

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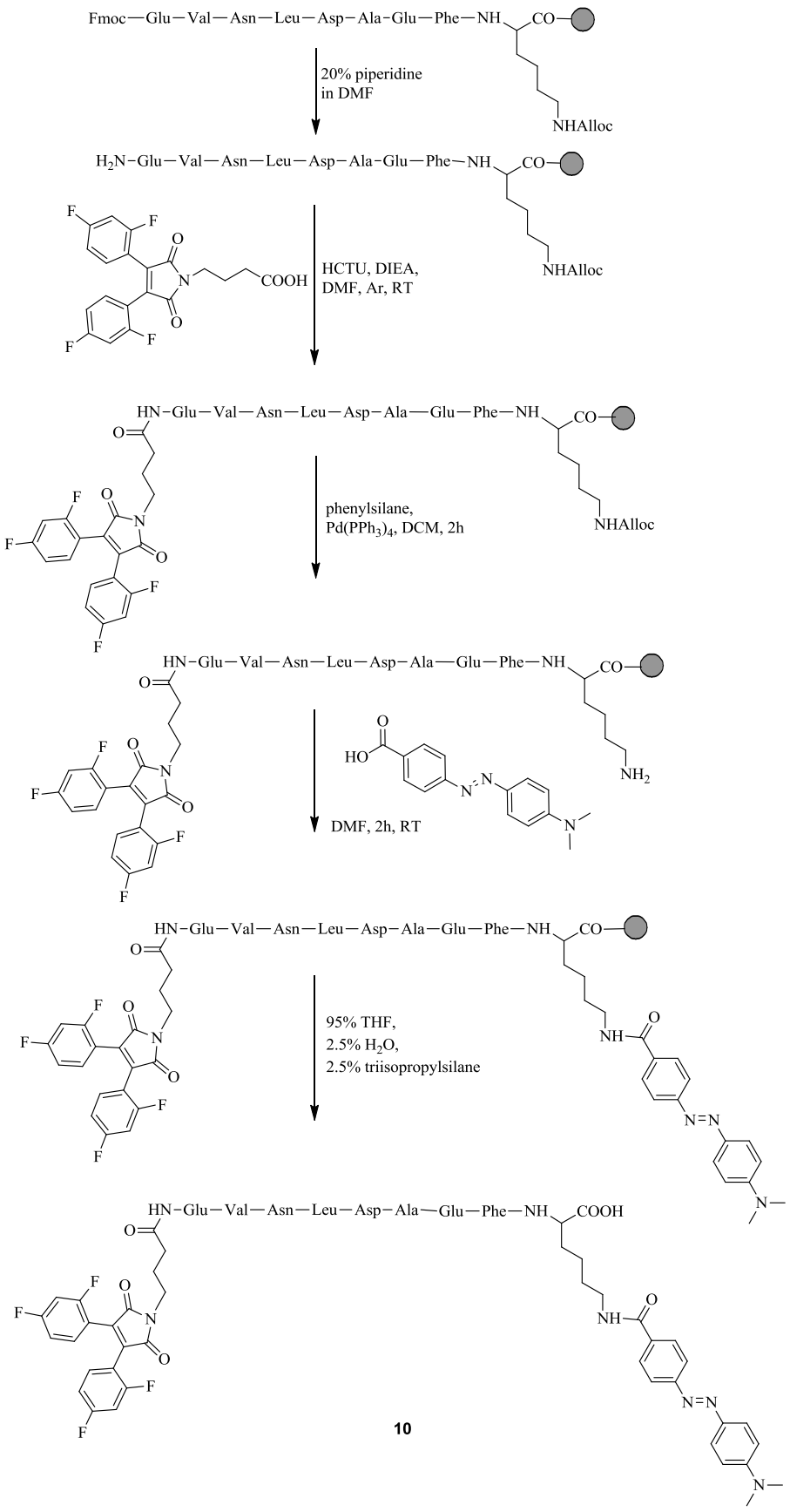

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Figure S1. *Synthetic approach to generate the FRET peptide with attached donor and acceptor moiety.*

II. Spectroscopic analysis and characterizations of 1, 1a, 1b and 1c

2.1 Quantum yields and extinction coefficients in different solvents

Extinction coefficient $\varepsilon_{(340)}$ (M^{-1} cm^{-1})	**1**	**1a**	**1b**	**1c**
hexanes	20,580	22,980	17,400	15,800
toluene	17,099	15,430	15, 400	15,320
DCM	48,400	28,320	24,150	23,110
CH_3OH	20,536	17,620	16,950	16,720
CH_3CN	13,490	18,560	11,200	12,530

Table 1. *Extinction coefficients of **1**, **1a**, **1b**, and **1c** (λ_{max} = 340 nm).*

Quantum yield (Φ_{fl})	**1**	**1a**	**1b**	**1c**
DCM	0.61	0.24	0.26	0.29

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Table 2. *Quantum yield of **1**, **1a**, **1b**, and **1c** measured in DCM against perylene as a reference (λ_{ex} = 340 nm, $\Phi_{fl}^{perylene}$=0.72).*[3]

2.2 Solvatochromism

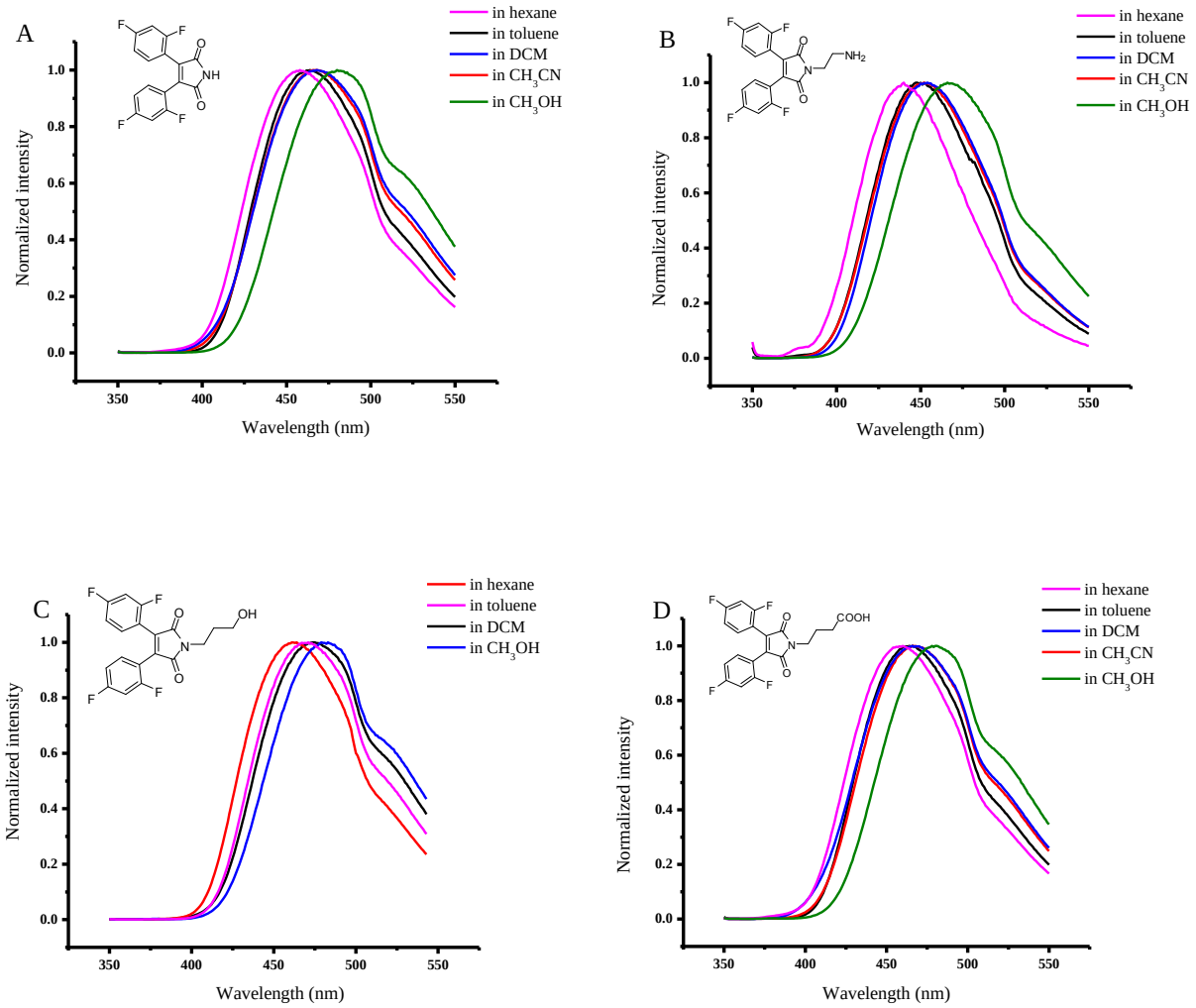

Figure S2. *Emission spectra of **1**, **1a**, **1b**, and **1c** recorded in various solvents (λ_{ex} = 340 nm), **A**. emission spectra of **1**, **B**. emission spectra of **1a**, **C**. emission spectra of **1b**, **D**. emission spectra of **1c**.*

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2.3 pH dependence of the emission.

Procedure used for a titration of 2,6-lutidine to 5 uM solution of 1 in CH_3CN

The pH dependence test was carried out in 1 cm path length quartz cuvette. A 10 mM stock solution of the fluorophore **1** in CH_3CN was prepared and 1 uL of 10 mM stock solution was added to 2 mL CH_3CN in a glass cuvette. The 5 uM final concentration of **1** in 2 mL CH_3CN in the cuvette was kept consistent through the entire titration experiment. A 8 M stock solution of the base, 2,6-lutidine was prepared by mixing 15 ml of 2,6-lutidine (MW = 107.15g/mol; density (d) = 0.92 g/cm^3) with 10 mL of CH_3CN. The base, 2,6-lutidine was gradually titrated to the solution of the fluorophore in CH_3CN in small increments: 320 eq = 1.6 mM 2,6-lutidine (0.5 uL of 8 M stock solution); 841 eq = 4.2 mM 2,6-lutidine (1 uL of 8 M stock solution); 1684 eq = 8.4 mM 2,6-lutidine (2 uL of 8 M stock solution); 2560 = 12.8 mM 2,6-lutidine (3 uL of 8 M stock solution); 4205 eq = 21 mM 2,6-lutidine (5 uL of 8 M stock solution).

Reversibility of the base-induced fluorescence quenching demonstrated through the neutralization of the basified solution of **1** with acid (TFA).

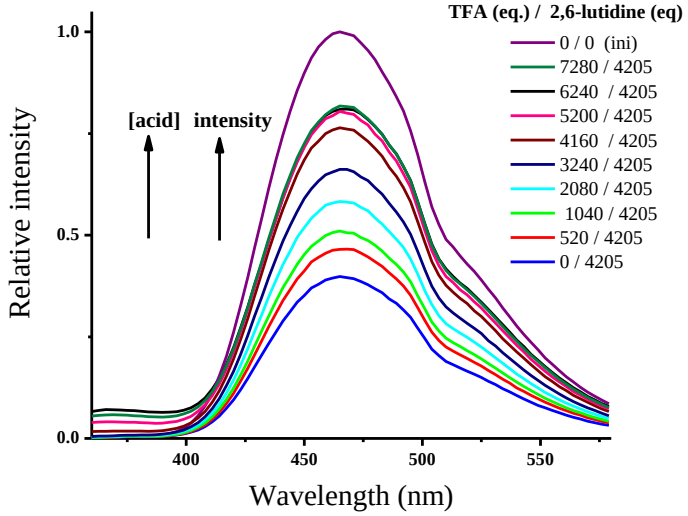

***Figure S3**. Emission spectra of **1** (λ_{ex} = 340 nm) in CH_3CN recorded through titration of acid, TFA (0 eq – 7280 eq) to the basified solution of **1** (10 uM of **1** in CH_3CN; 4205 eq of 2,6-lutidine). "Ini" marked curve refers to initial solution before addition of acid or base.*

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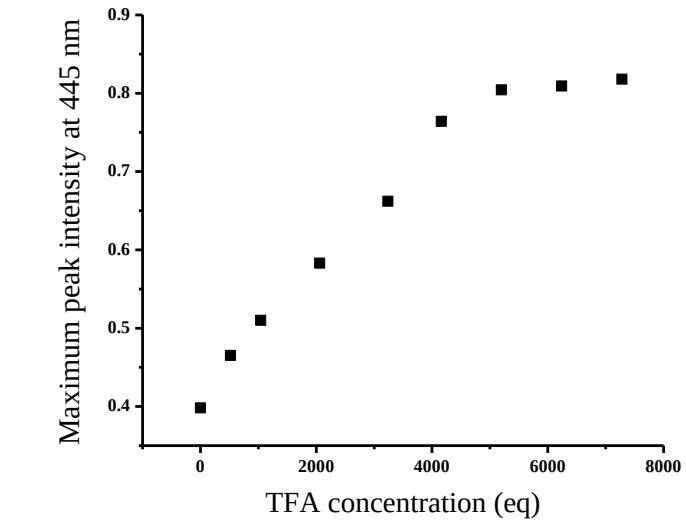

Figure S4. *Graphic representation of the correlation between the maximum emission intensity at 445 nm (λ_{ex} = 340 nm) and the corresponding acid concentration, TFA (0 eq – 7280 eq used to neutralize the basified solution of* ***1*** *(10 μM of* ***1*** *in CH_3CN; 4205 eq of 2,6-lutidine).*

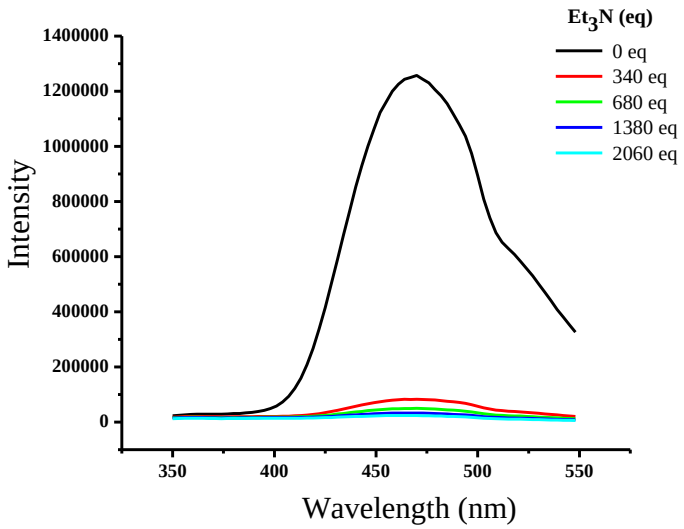

Figure S5. *Emission spectra of* ***1*** *(10 μM in CH_3CN, λ_{ex} = 340 nm) titrated with Et_3N (0 eq -2060 eq).*

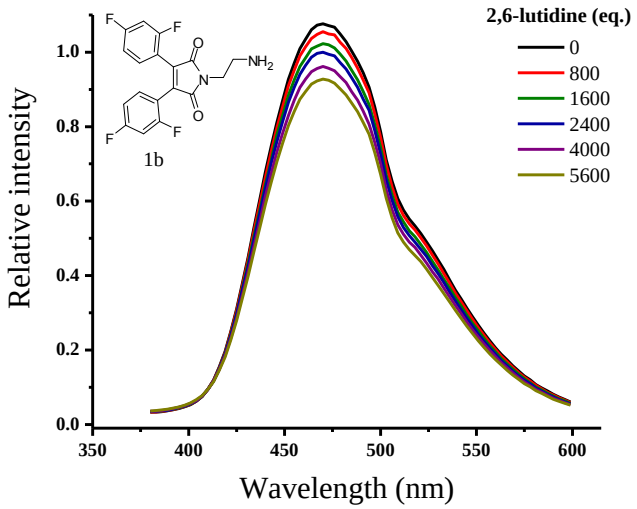

Figure S7*. Emission spectra of* ***1b*** *(5 μM in CH_3CN, λ_{ex} = 340 nm)) obtained from the titration of 2,6-lutidine (0 eq -5600 eq) demonstrating the smaller fluorescence quenching effect due to the functionalization at the N-position.*

III. Spectroscopic analysis and characterization of the FRET peptide system:

3.1 Spectral overlap

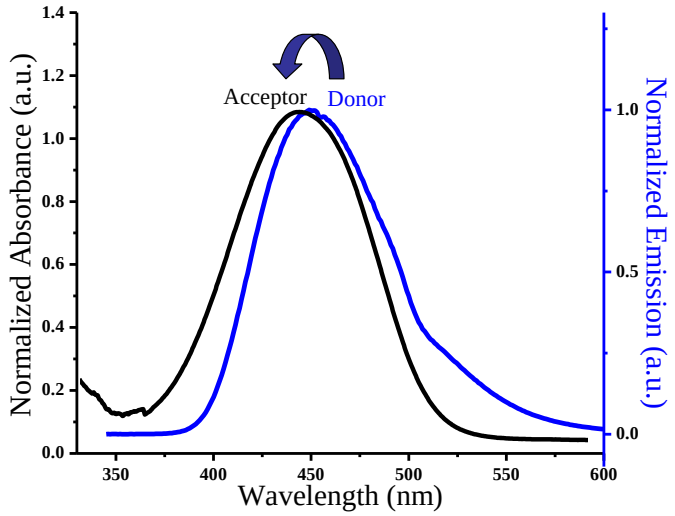

Figure S8. *Spectral overlap of the emission profile of* ***1c*** *(donor, blue line) and the absorption profile of the dark quencher (acceptor, black line), Dabcyl.*

3.2 Absorption and emission spectra of **9,** the covalently attached donor (**1b**) and quencher (Dabcyl)

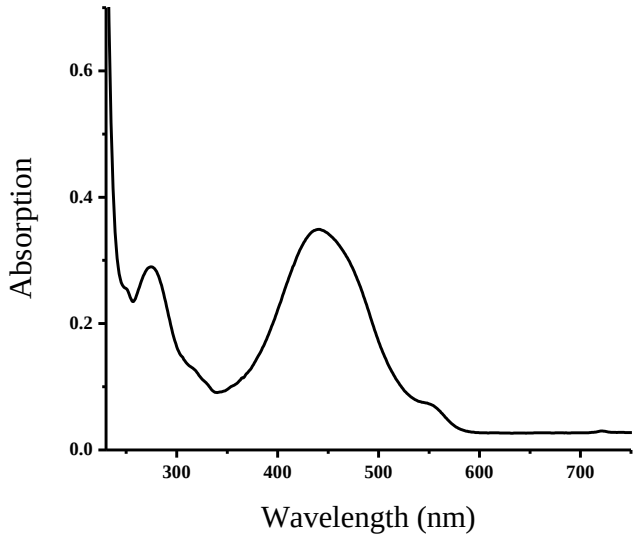

Figure S9. *Absorption spectrum of the covalently linked donor (**1c**) and the acceptor (Dabcyl) (λ_{exc} = 340 nm) (10 μM in DCM, used as a preliminary control to determine the proper quencher selection.*

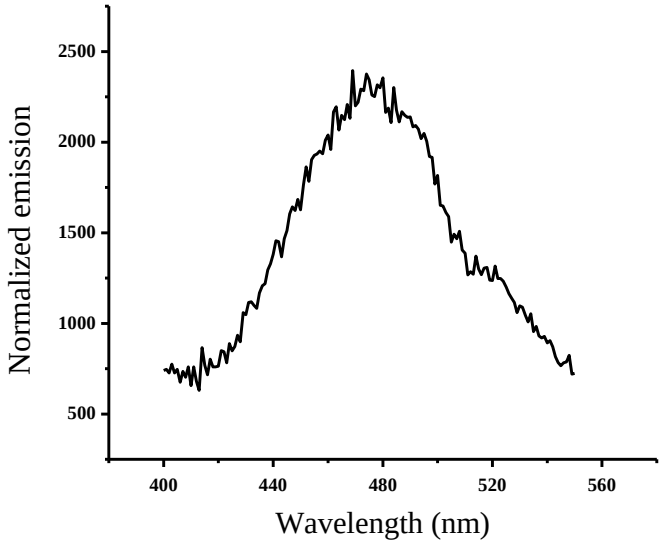

Figure S10*. Normalized emission spectra the covalently linked donor (**1c**) and the acceptor (Dabcyl) (λ_{exc} = 340 nm, 10 μM in DCM), used as a preliminary control to determine the proper quencher selection.*

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IV. Fluorimetric titration experiment used to rule out diffusional quenching between the donor (1c) and the dark quencher (Dabcyl), free in solution.

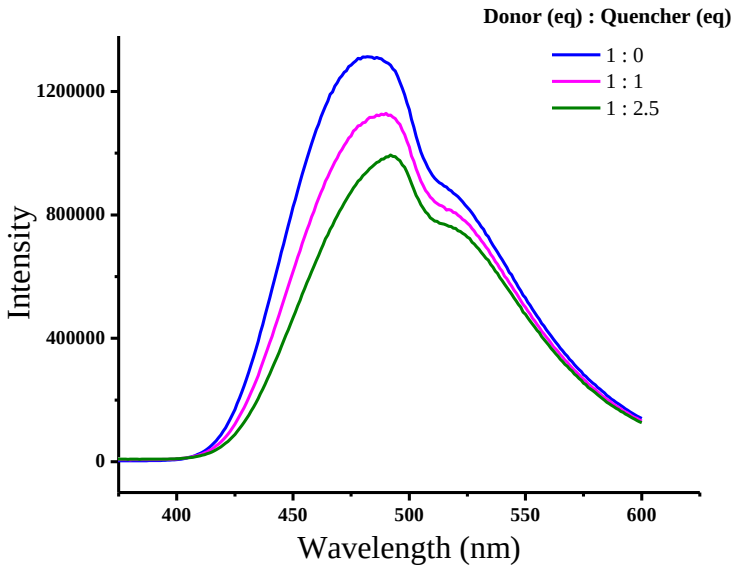

Figure S11. *Emission spectra recorded in a titration experiment in which the quencher molecule, Dabcyl, (0 eq – 2.5 eq in DCM) was titrated to the acceptor* ***1c*** *(10 μM in DCM, λ_{ex} = 340 nm).*

V. Conditions for the incubations of the FRET peptide with β-secretease and Lineweaver-Burk plot.

5.1 Incubation conditions of the FRET peptide with β-secretease

The sequence of this β-secretase FRET peptide is derived from the β-secretase cleavage site of the "Swedish" APP mutation and has been used previously in the AnaSpec Senso Lyte® 520 β-secretase Assay Kit. The incubation of the FRET substrate with β-secretase leads to the cleavage of the Leu-Asp bond and regaining of the fluorescence from the liberated fragment, containing the chromophore. All incubations were performed in 384-well micro plate at room temperature, for 1 hour and each well contained β-secretase, sodium acetate buffer (pH 4.5), and substrate in DMSO in total volume of 30 µL. Once the FRET off state was initiated due to the enzymatic cleavage, the fluorescent signal in relative fluorescent units (RFU) was continuously monitored with spectrofluorimeter upon λ_{exc} / λ_{em} = 340 nm / 460 nm. The progression of the enzymatic reaction was also monitored in a concentration dependent manner with the following substrate concentrations: 2.93 µM, 2.7 µM, 2.4 µM, 2.13 µM, 1.86 µM, 1.6 µM, 1.33 µM, 1.06 µM, 0.66 µM, 0.33 µM, 0.017 µM. The experiment was performed in three

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independent trials, carried out under the same incubation conditions which revealed that the enzymatic reaction followed Michaelis–Menten kinetics

5.2 Lineweaver-Burk plot of the enzymatic reaction

Linweaver-Burk plot[4] was used to calculate two important characteristics of the rate of the enzyme-mediated reaction, V_{max} and K_m (V_{max} = 27 μM /min and K_m = 0.24 μM).

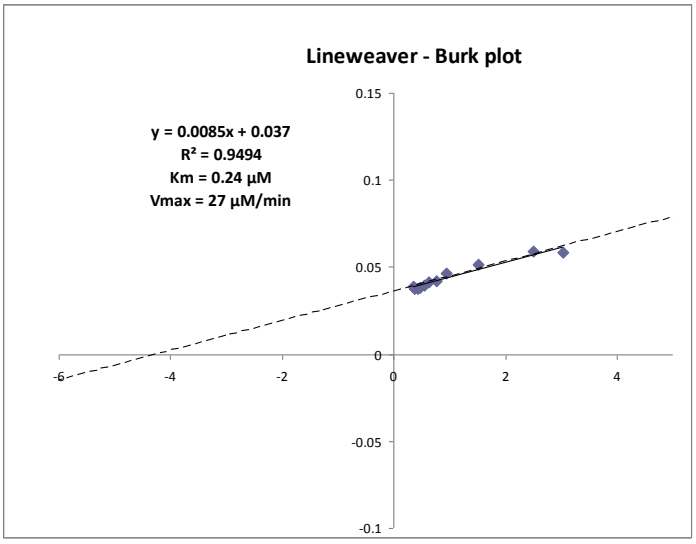

Figure S12. *Lineweaver-Burk plot of the enzymatic reaction.*

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VI. Spectroscopic analysis and characterization of the FRET system with bright acceptor.

6.1 Absorption of the acceptor (TPP)

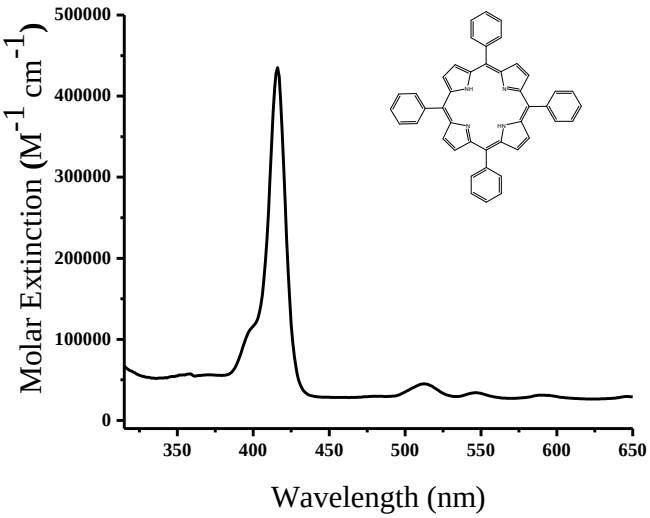

Figure S13. *Absorption of the acceptor (TPP).*

6.2 Emission spectra of the acceptor (TPP) alone in solution excited at 270 nm and at 420 nm

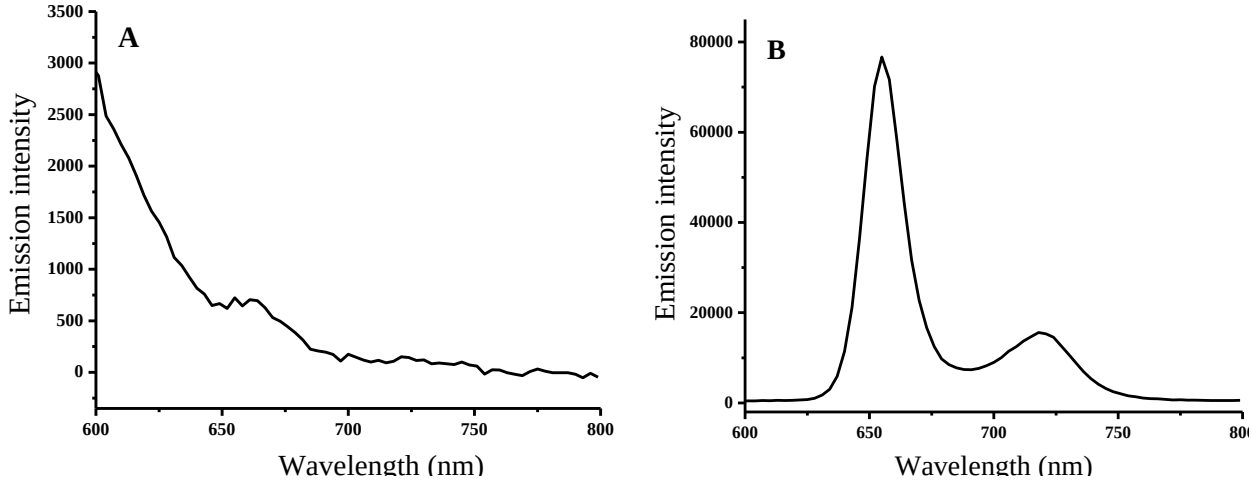

Figure S14. *Emission spectra of the acceptor (**TPP**) alone in toluene **A**. excited at λ_{exc} = 270 nm and **B**. excited at λ_{exc}= 420 nm.*

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6.3 FRET titration experiment between the donor (**1a**) and the bright acceptor (**TPP**).

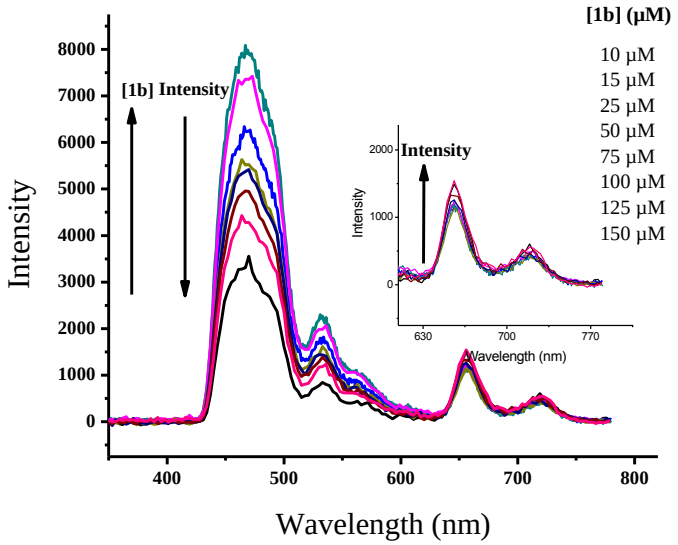

Figure S15*. Emission spectra obtained by titrating* ***1a*** *(10 – 150 μM) to* ***TPP*** *(25 μM) in toluene (*λ_{exc} *= 270 nm), demonstrating the FRET diffusion mechanism between the donor and acceptor molecule. The intensity of the donor emission decreased and the intensity of the acceptor emission increased while the concentration of the donor was gradually increased from 10 to 150 μM (constant concentration of the acceptor).*

6.4 Representation of the resonance energy ratio change (RRC).

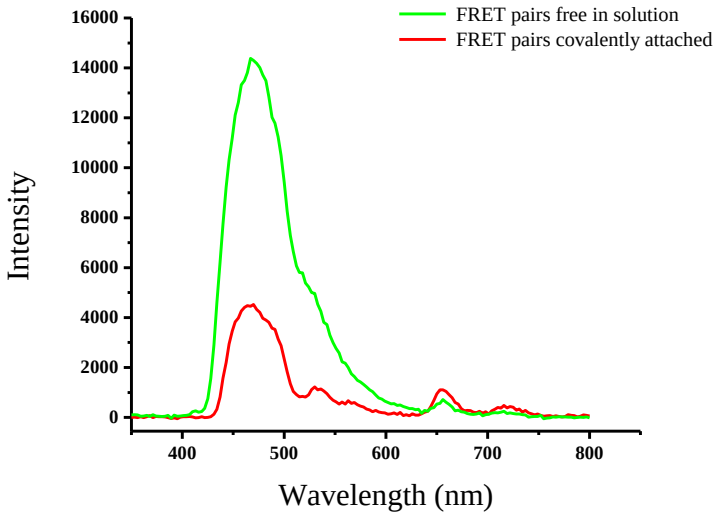

Figure S16. *Emission spectra of* ***A****. FRET pairs free in solution (toluene) and* ***B****. FRET pairs covalently attached at the same concentration (5μM in toluene).*

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VII. Calculation of the Förster-Radius (R_0)

The Förster radius (R_0) was calculated according to the following equation:[5]

$$R_0 = \left\{ \frac{9000(\ln 10)K^2 Q_d J}{128\pi^5 n^4 N_{av}} \right\}^{1/6} = 9.78 \times 10^3 \left\{ Q_d K^2 n^{-4} J \right\}^{1/6} \text{Å}$$

where K_2 is the orientation factor and is assumed to be 2/3 for randomLy oriented molecules, Q_d is the quantum yield of the donor molecule (**1c**), n is the index of refraction, N_A is the Avogadro's number, and J is the spectral overlap integral between donor and acceptor, represented as:

$$J_{DA} = \frac{\int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int F_D(\lambda) d\lambda}$$

where $F_D(\lambda)$ is the fluorescence spectrum of the donor, and ε_A is the extinction spectrum of the acceptor.[6]

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(6). Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*, 3 ed.; Springer: New York, 2006.

VIII. ^{1}H and ^{13}C

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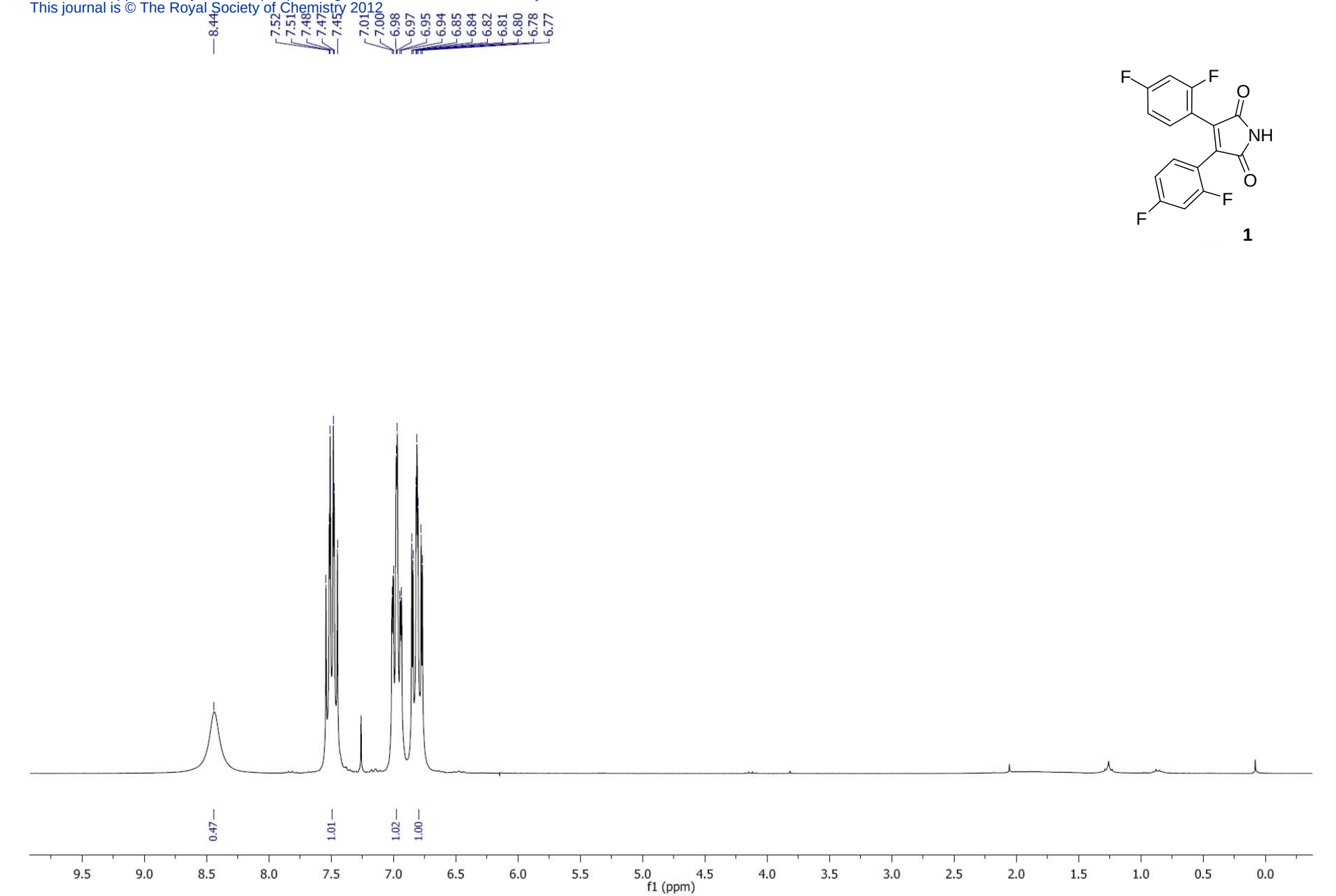

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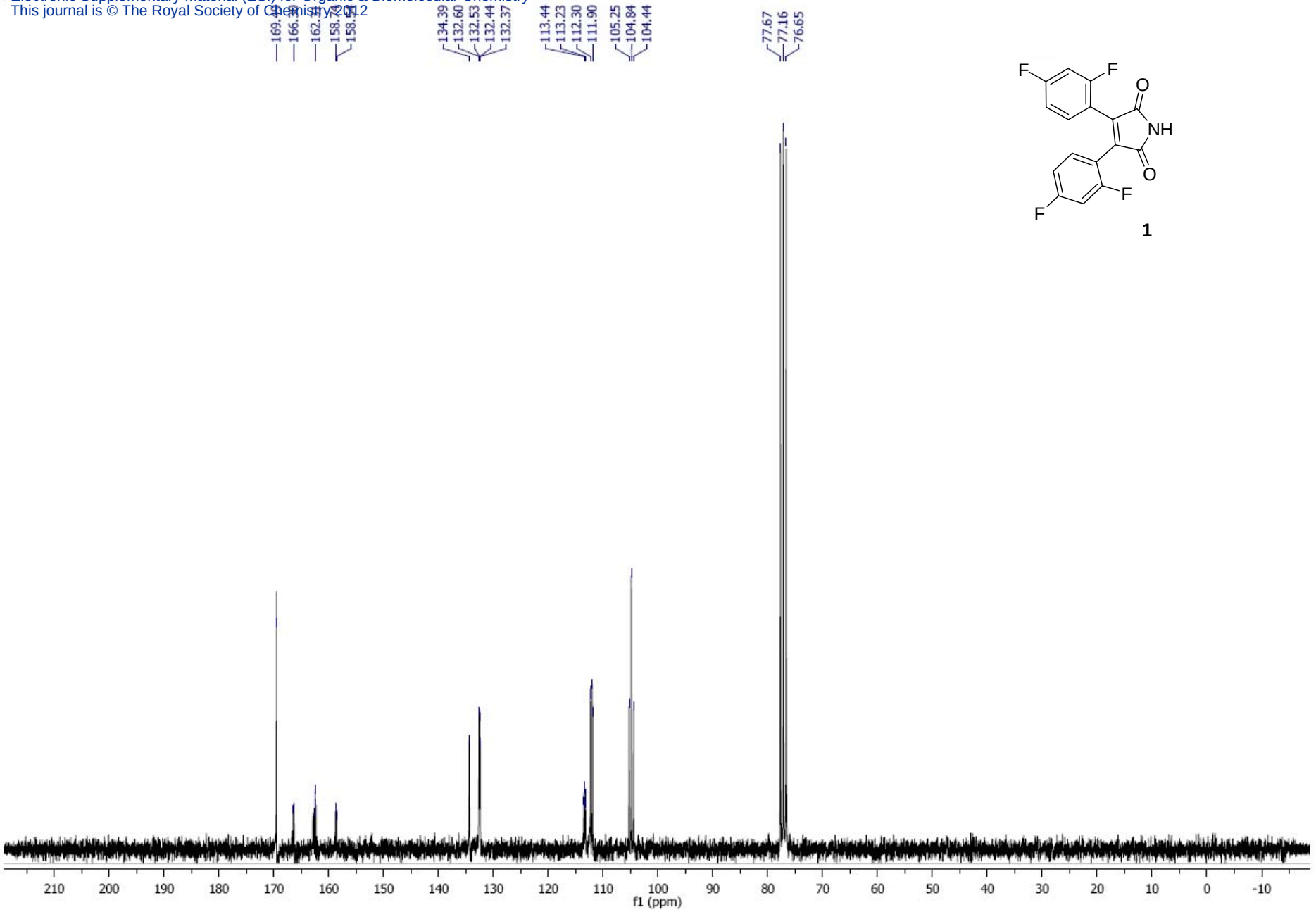

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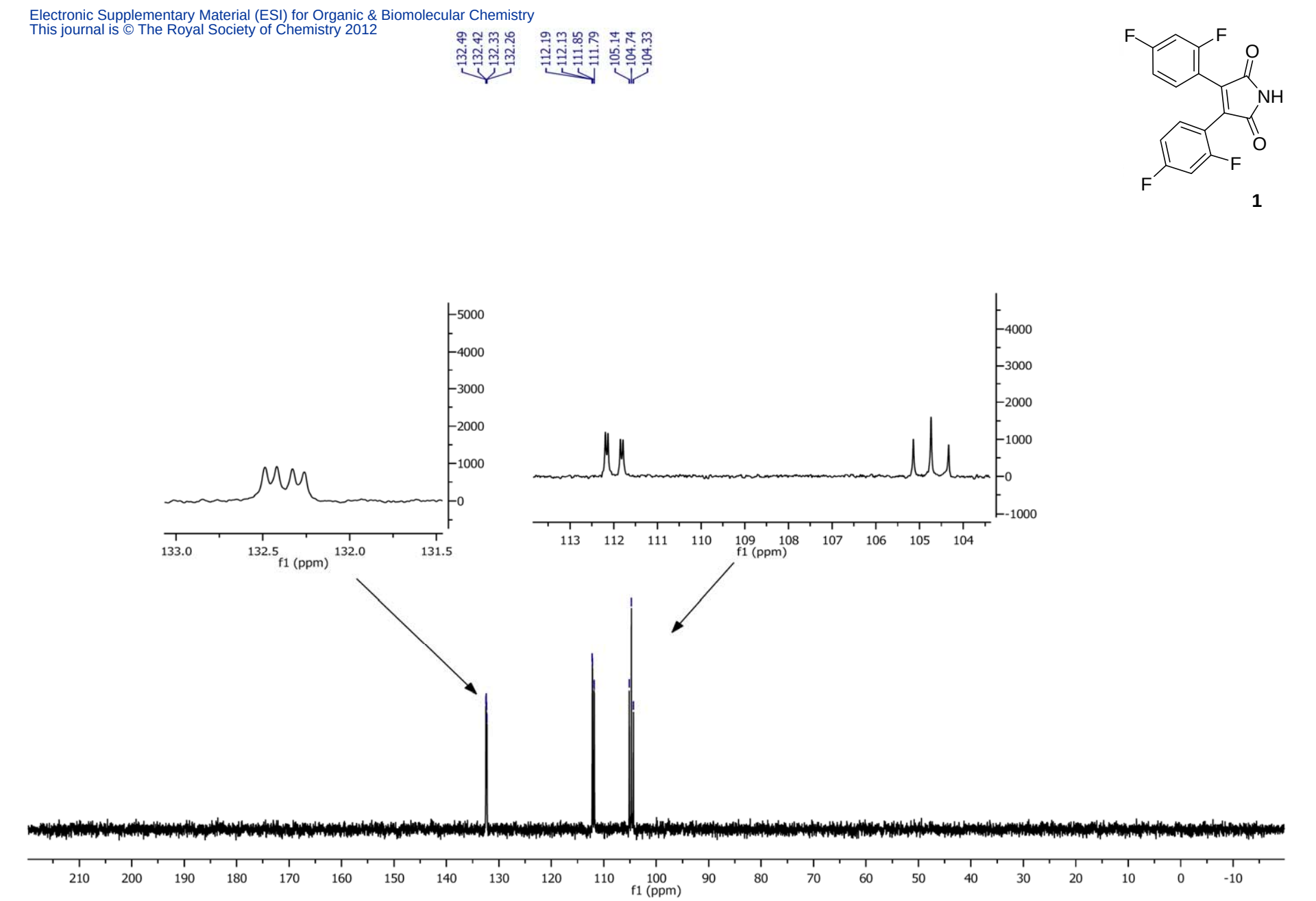

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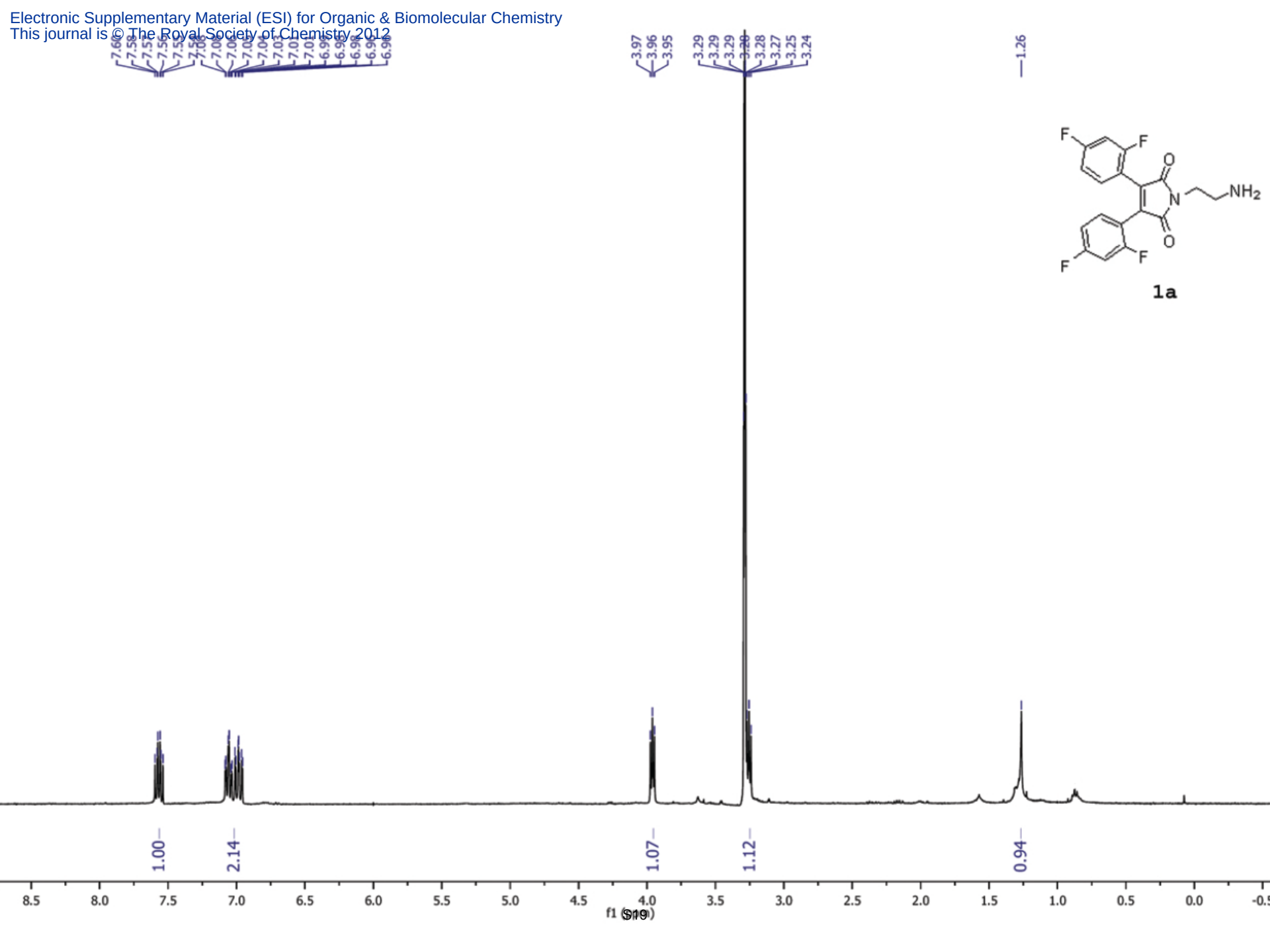

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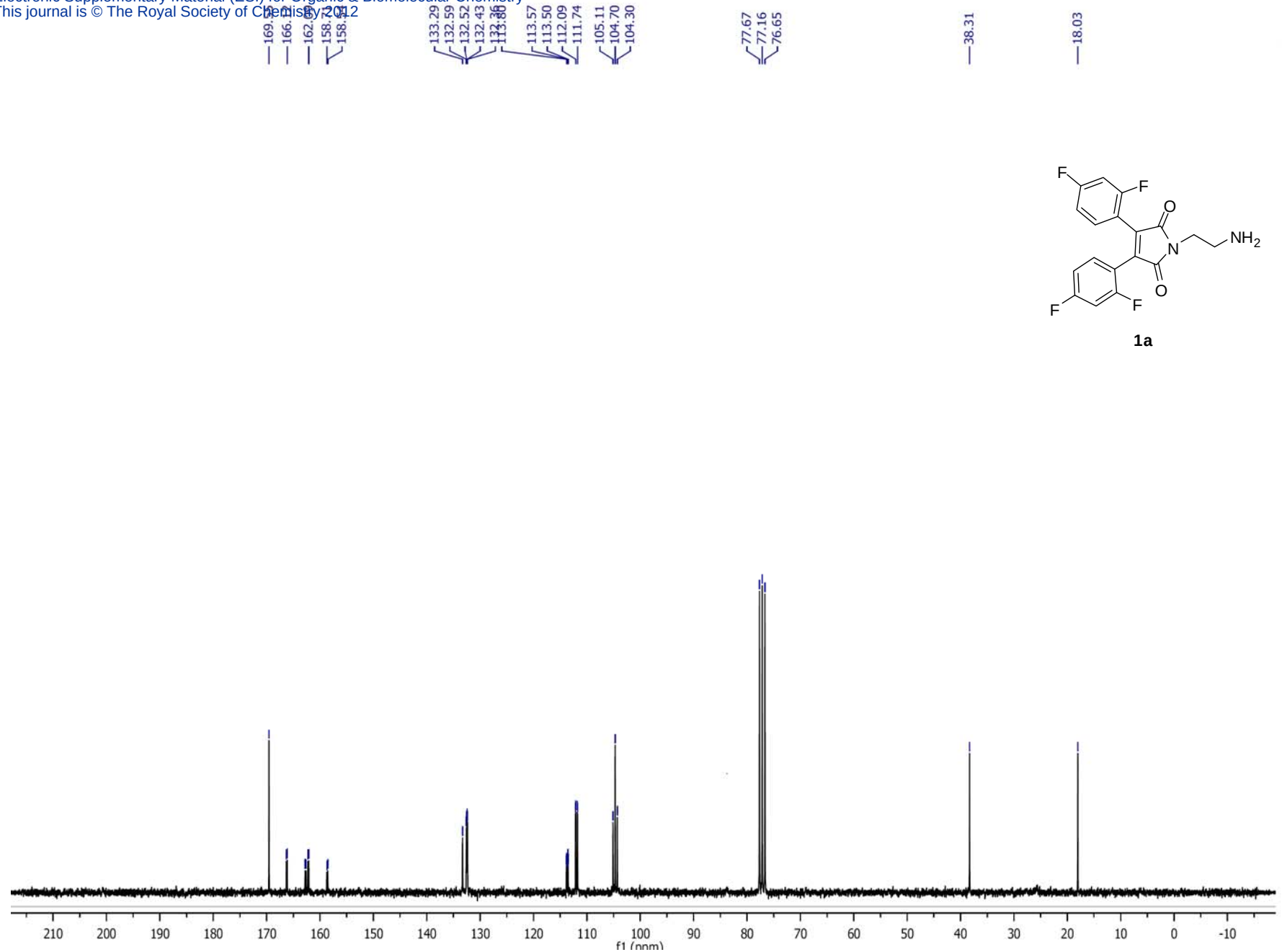

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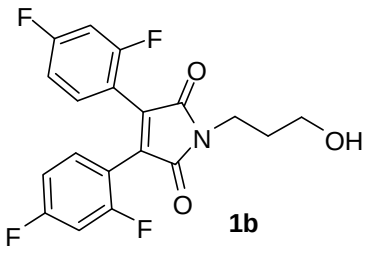

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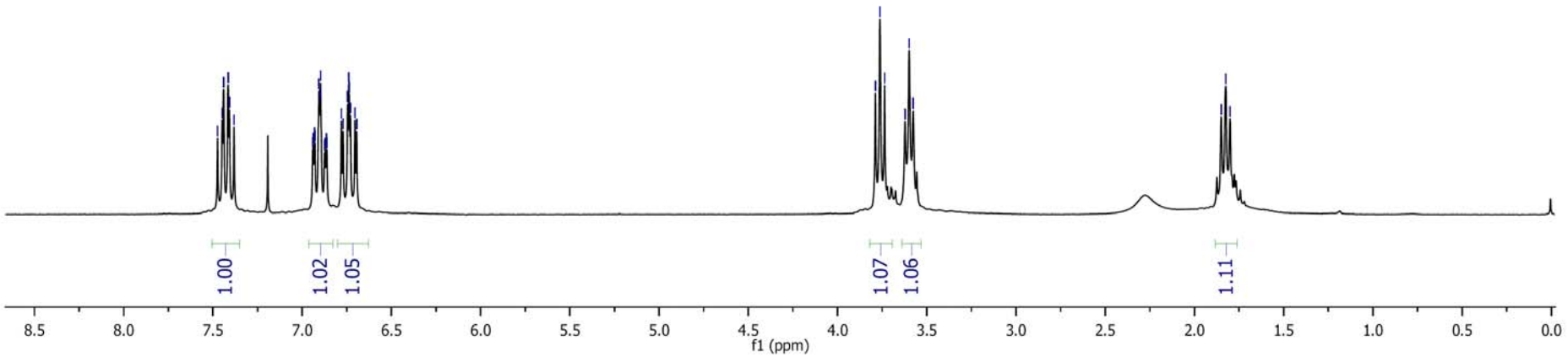

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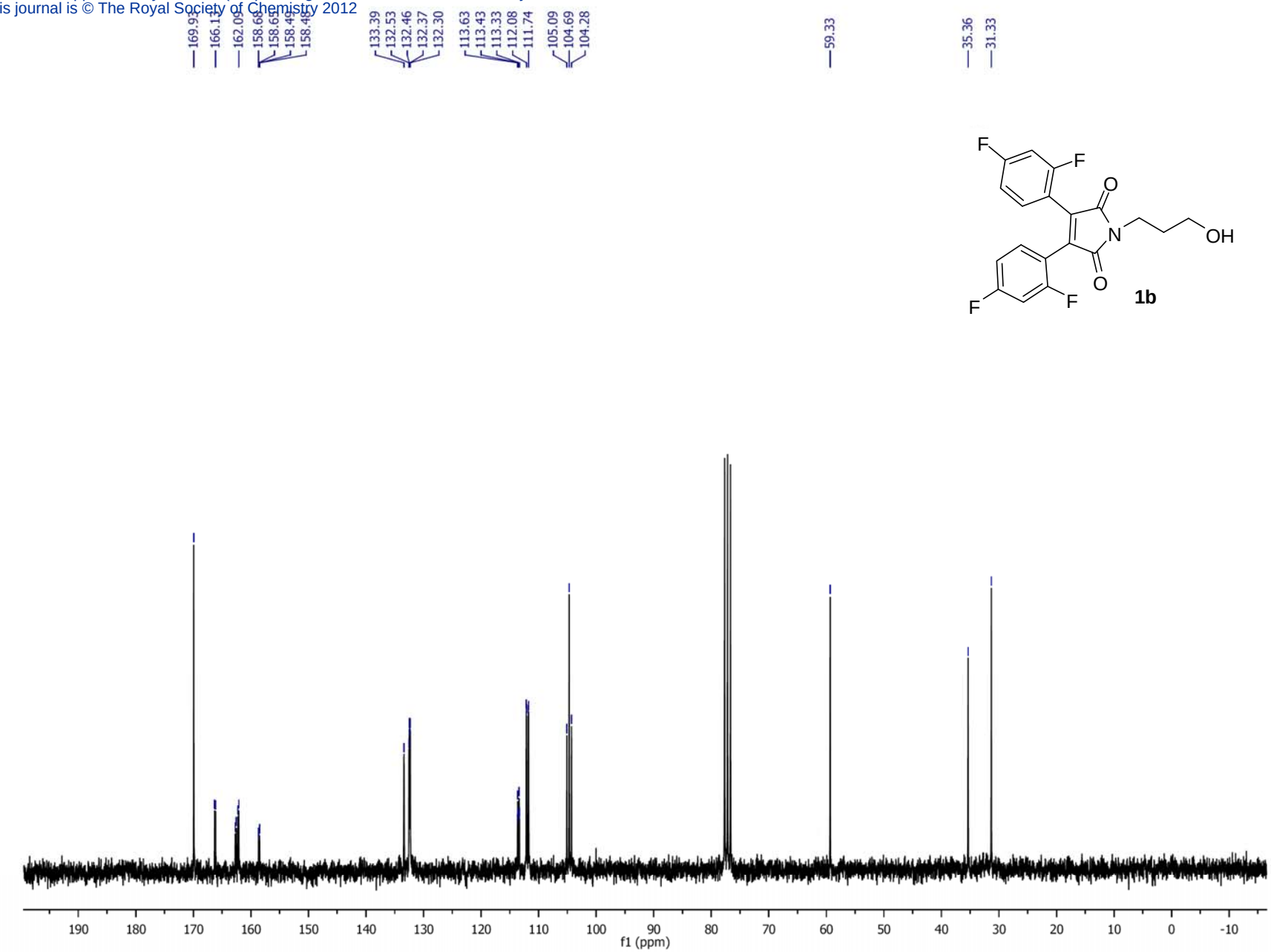

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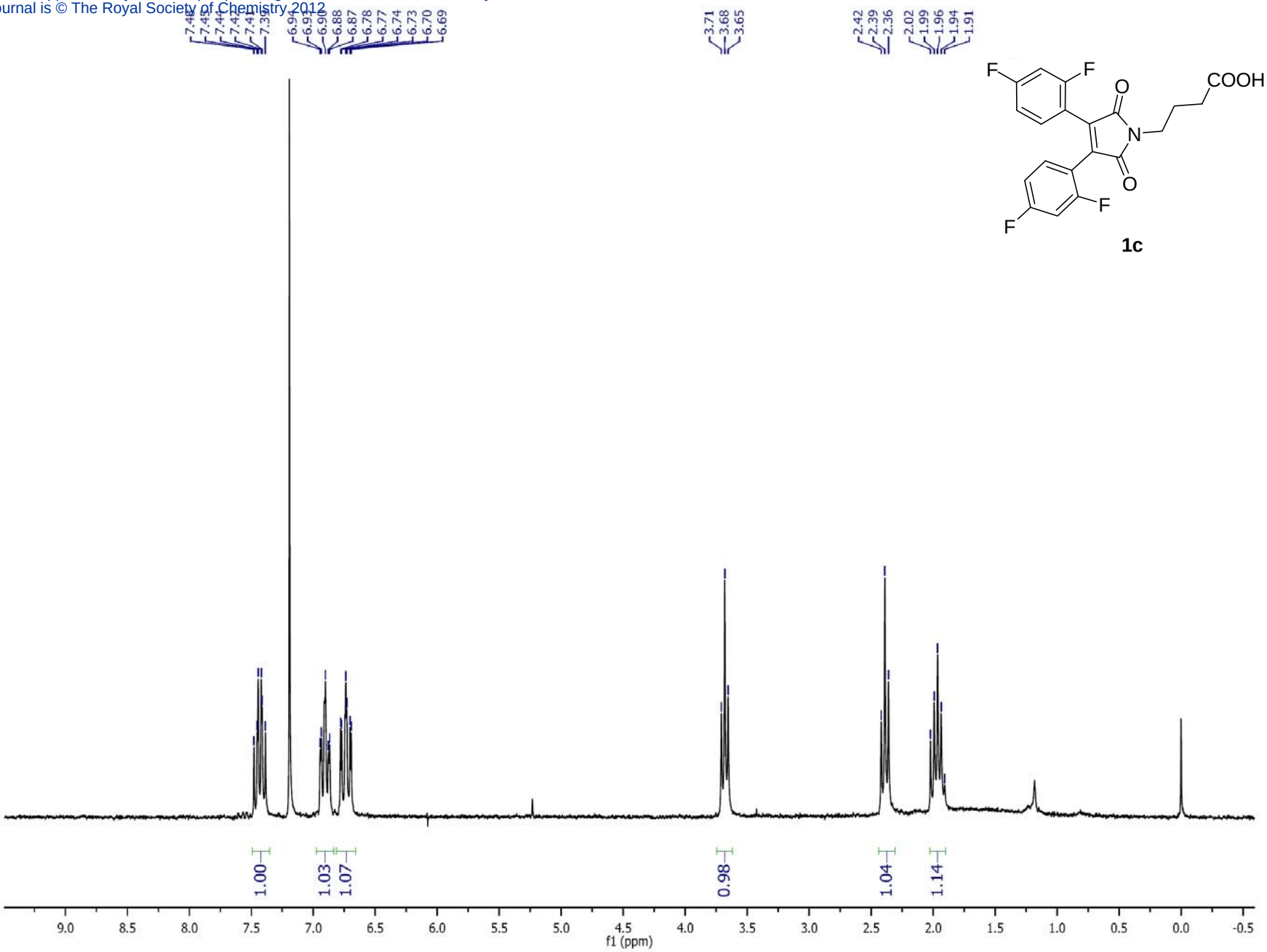

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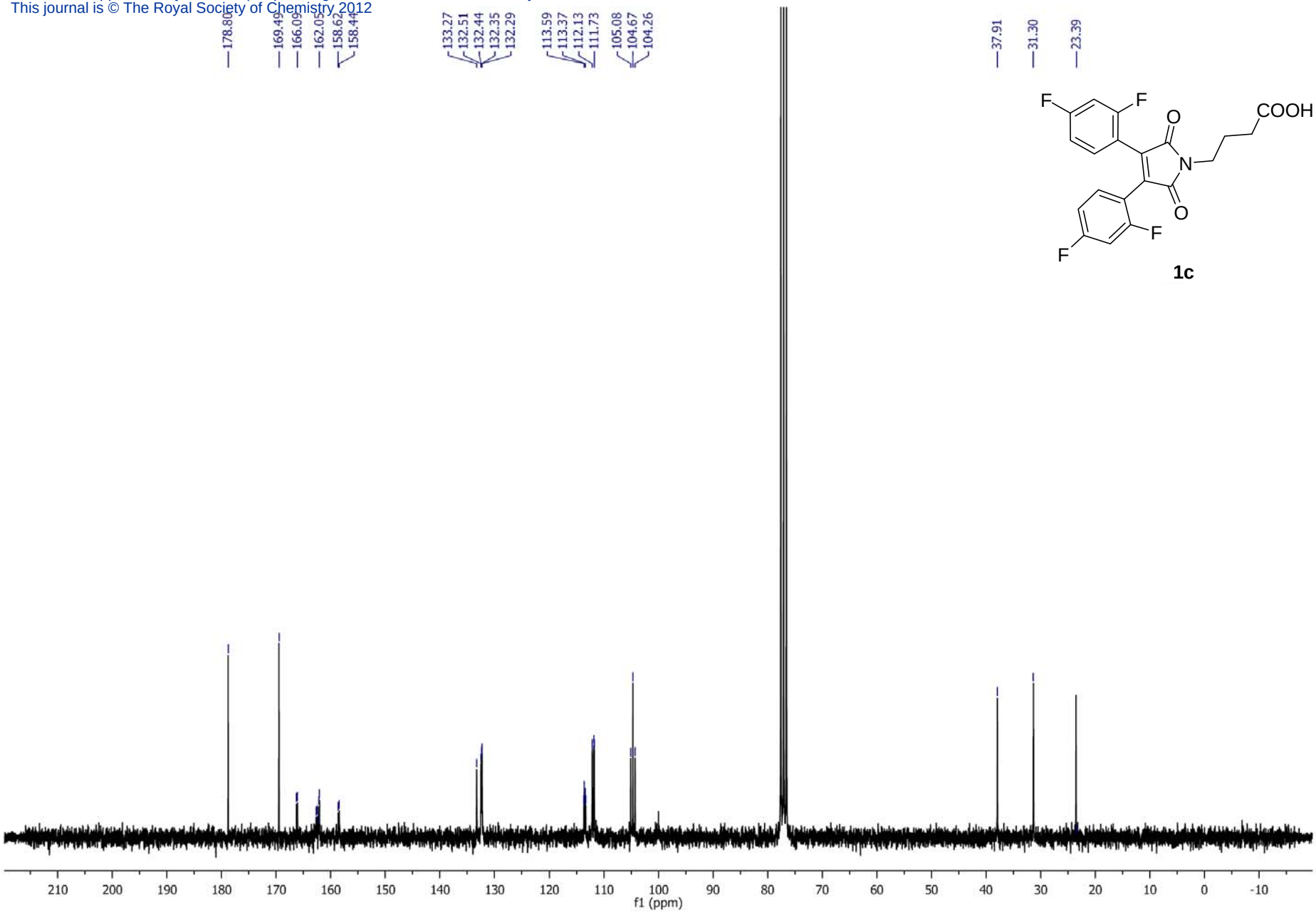

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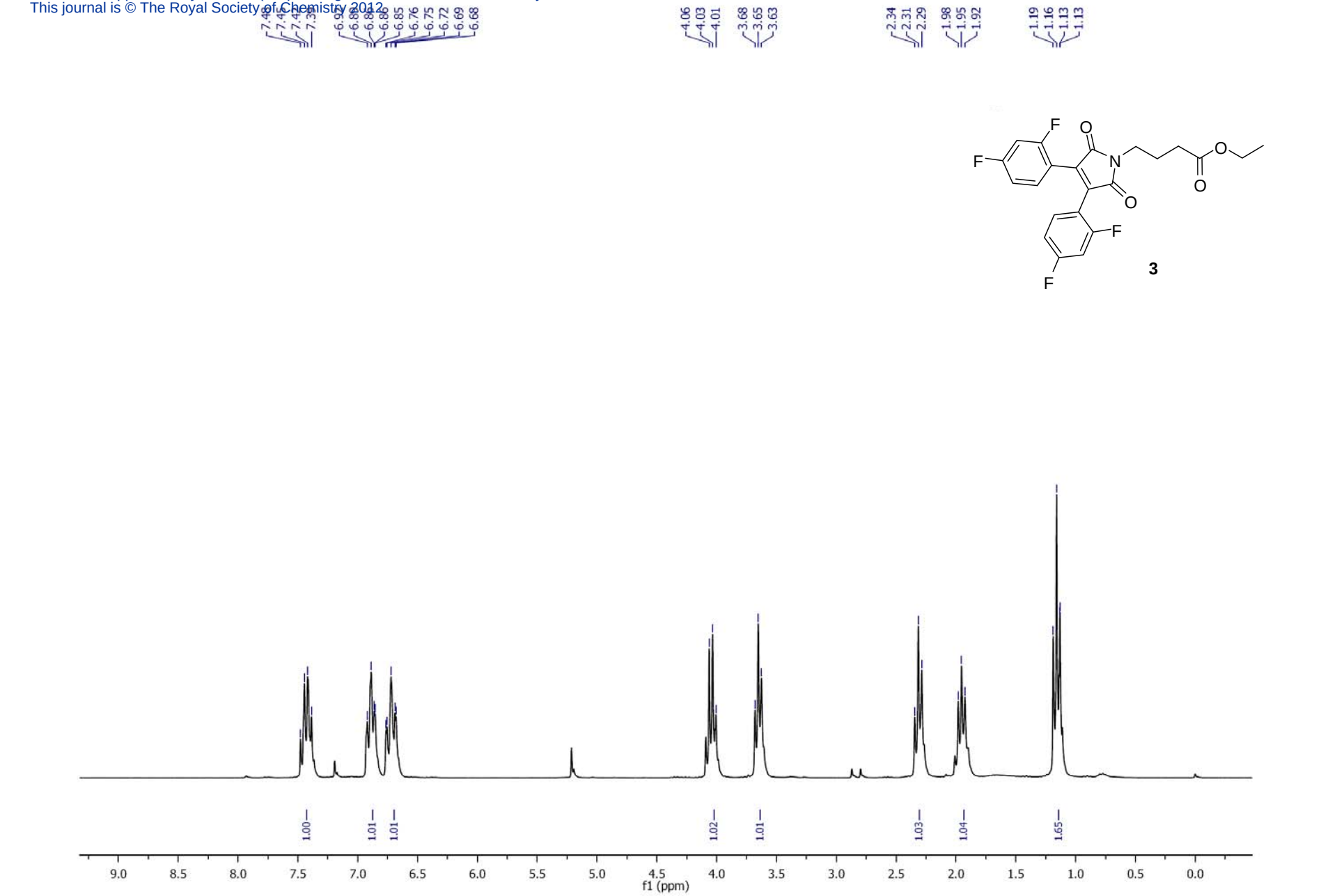

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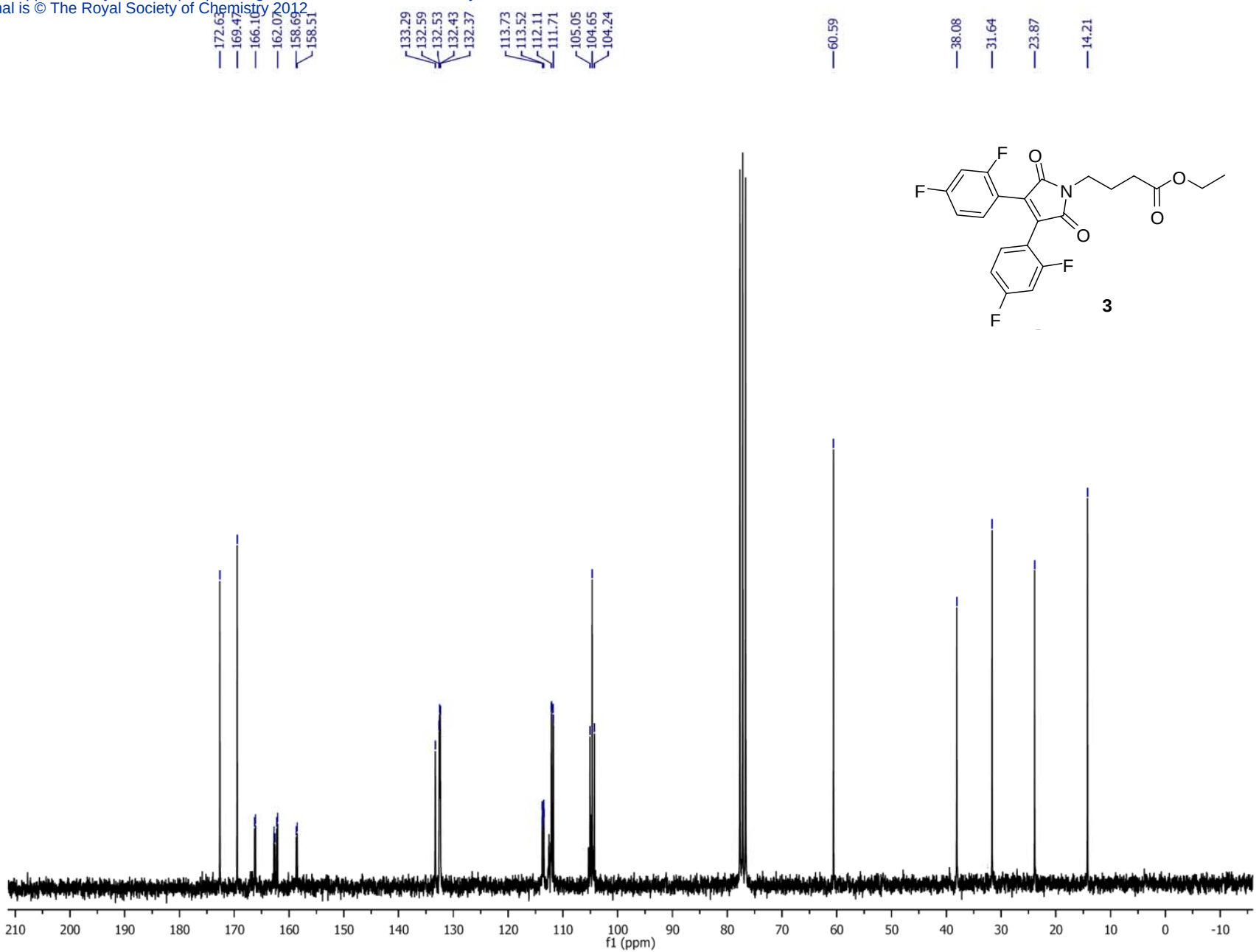

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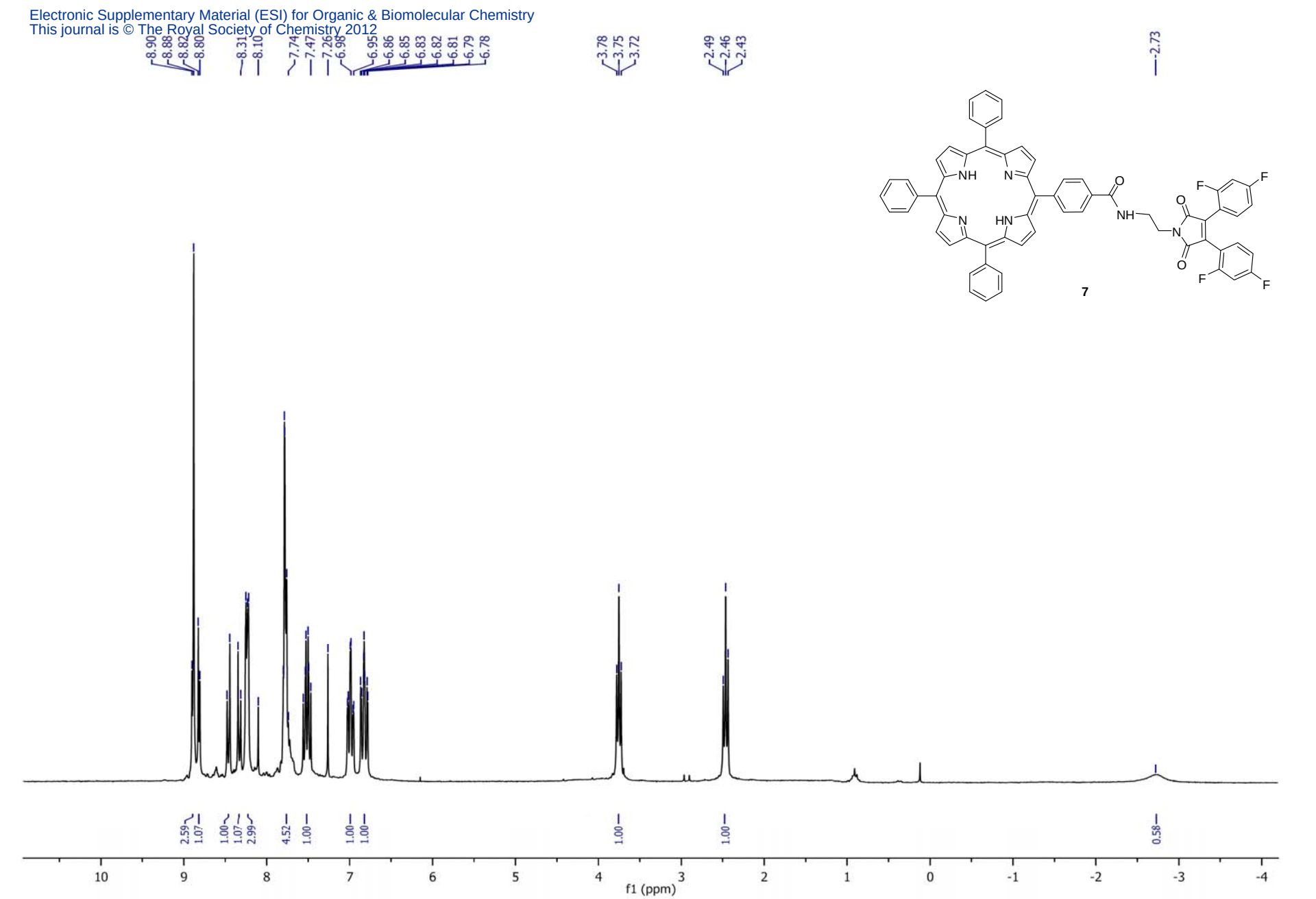

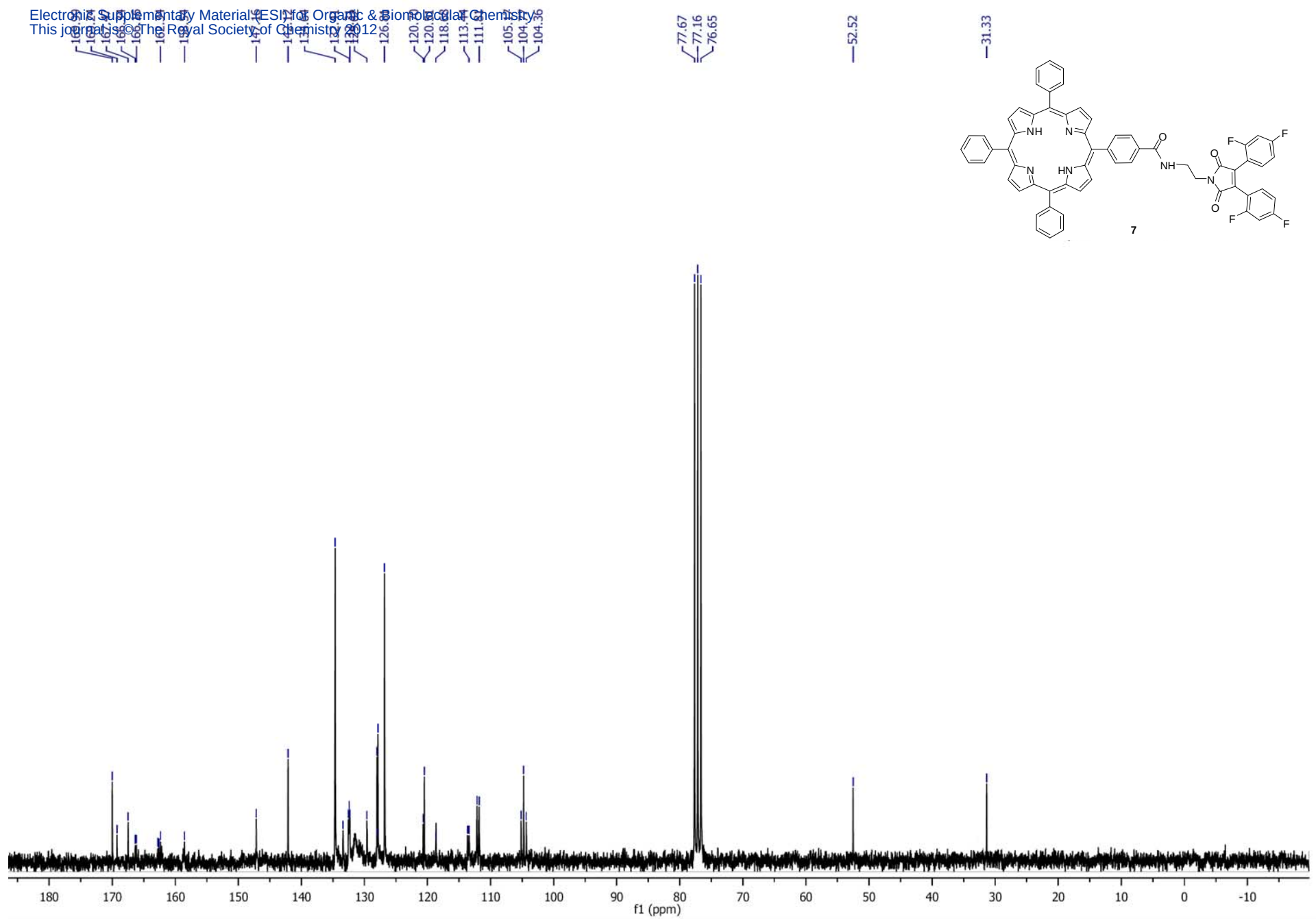
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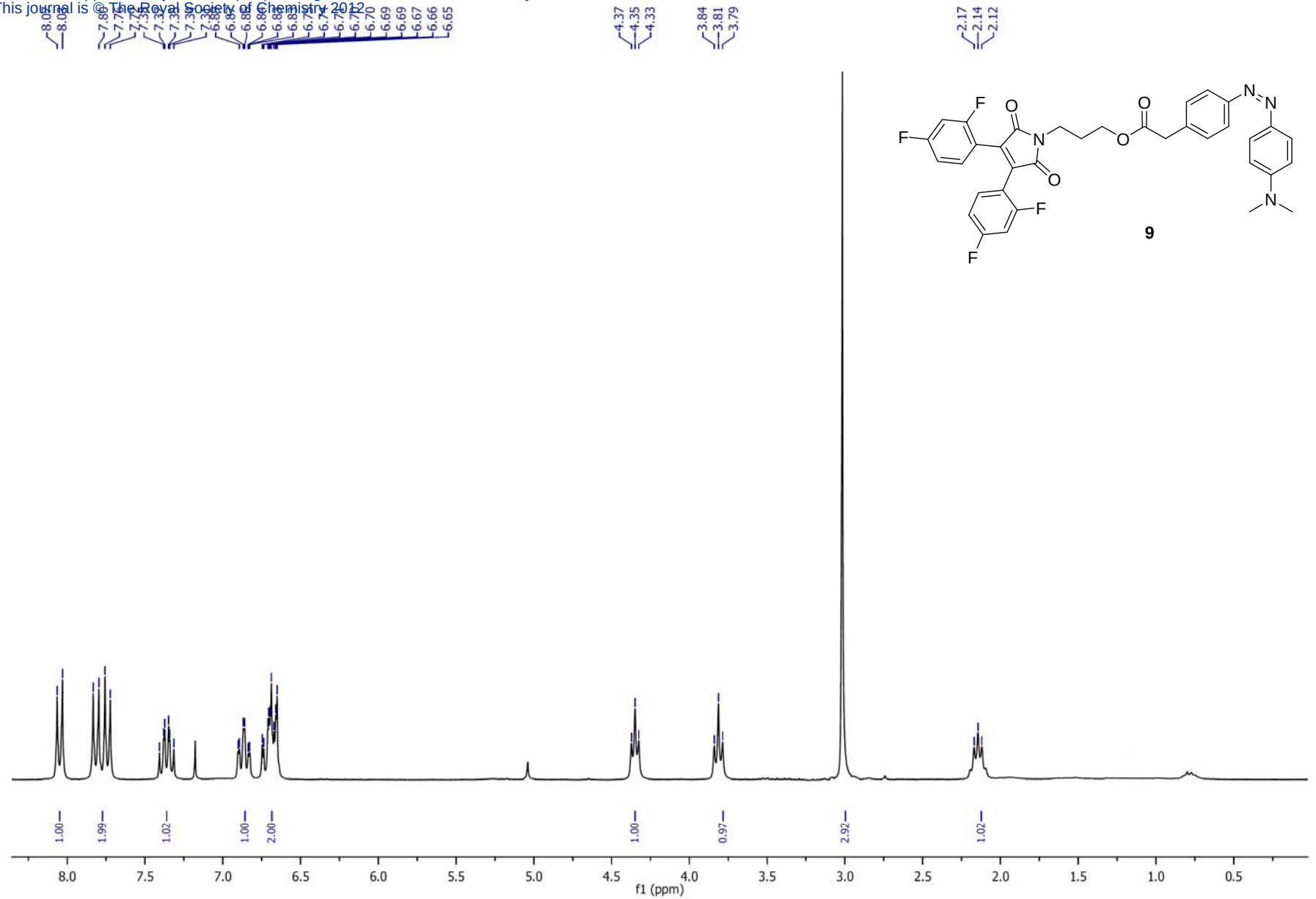

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