

Synthesis and Biological Evaluations of 2,3-Diarylthiophene Analogues of Combretastatin A-4

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Supplementary Information

Experimental

Chemistry

General

Unless otherwise noted, all materials were obtained from commercially available sources and were used without purification. The reaction process was monitored by TLC with silica gel plates (thickness 250 μm , Indicator F-254) under UV light. The products were purified by column chromatography (60 $\text{\AA}$, 200-300 mesh, Qingdao Ocean Chemicals) with the designated solvents. The melting points were measured using a hot-stage microscope (X-4, Beijing Taike Ltd.) and are uncorrected. ESI-MS and EI-MS were measured on an Agilent 1100-sl and an Agilent 6890N mass spectrometer, respectively. High-resolution mass spectra (HRMS) were obtained using a Bruker Daltonics micrOTOF-Q mass spectrometer equipped with electrospray ionisation (ESI). ^1H and ^{13}C NMR spectra were obtained in CDCl_3 solution using Bruker AVANCE 400 (^1H , 300 MHz) and Bruker AVANCE 600 (^{13}C , 150 MHz) spectrometers, respectively, with TMS (tetramethylsilane) as the internal reference. The chemical shifts were reported in ppm downfield from tetramethylsilane and proton-proton coupling constants (J) in Hz. For ^1H NMR: CDCl_3 7.26 ppm; for ^{13}C NMR: $^{13}\text{CDCl}_3$ 77.0 ppm.

General synthetic procedures for deoxybenzoin 12a-e

A solution of 3,4,5-trimethoxybenzaldehyde (0.02 mol) and *p*-toluenesulphonyl hydrazide (0.02 mol) in anhydrous alcohol (150 mL) was stirred at room temperature for 40 min. The resulting solution was protected from light, and sodium hydroxide (0.02 mol) and the appropriate benzaldehyde (0.01 mol) were added. Then the reaction mixture was heated at 55 °C for 48 h. The solvent was then removed under reduced pressure and extracted with ethyl acetate (100 mL $\times$ 3). The combined organic layer was dried over anhydrous Na₂SO₄, filtered and concentrated to yield the crude product. Flash chromatography (silica gel, hexanes/ethyl acetate) was used to purify the deoxybenzoin products.

1-[4-methoxy-3-(phenylmethoxy)phenyl]-2-(3,4,5-trimethoxyphenyl)-ethanone (compound 12e): (4.3 g, 50.9%); Yellow Solid; mp 74-76 °C; MS (EI) *m/z*: 422 (M⁺, 16%), 241 (89), 181 (32), 91 (100); δ_H (300 MHz; CDCl₃) 3.82 (9 H, s), 3.95 (3 H, s), 4.13 (2 H, s), 5.18 (2 H, s), 6.45 (2 H, s), 6.91 (1 H, d, *J* 8.4), 7.31-7.46 (5 H, m), 7.62 (1 H, d, *J* 2.1), 7.66 (1 H, dd, *J* 2.1, *J* 8.4).

Synthesis of α -hydroxyphosphonate 14

A solution of 3,4,5-trimethoxybenzaldehyde (0.1 mol), dimethyl phosphate (0.12 mol), and sodium methoxide (0.015 mol) in anhydrous methanol (20 mL) was vigorously stirred for 1 h at 50 °C. The solvent was then removed under reduced pressure and extracted with ethyl acetate (100 mL $\times$ 3). The combined organic layer was washed with brine, dried over anhydrous Na₂SO₄, filtered and concentrated to yield the crude product, which was used without further purification.

General synthetic procedures for deoxybenzoin 17a-c

In a round-bottomed flask maintained under a nitrogen atmosphere, appropriate amounts of α -hydroxyphosphonate **14** (10 mmol) and 3,4-dihydro-2H-pyran (18 mmol) as well as 1.5 mol% *p*-TsOH·H₂O were dissolved in dry toluene (30 mL). The resulting mixture was heated to 55 °C for 2 h, and dry THF (5 mL) was then added to the mixture. Upon cooling to -45 °C, 10.5 mmol *n*-butyllithium (15% in hexane, 1.6 mol/L) was added dropwise to the mixture, and the contents of the flask were stirred for 1 h under a nitrogen atmosphere. Then, the solvent for the appropriate benzaldehyde present at 10 mmol in anhydrous THF was added dropwise, and the solution was stirred for another 4 h. Upon solvent removal by rotary evaporation, the residue was dissolved in a mixture of 20 mL MeOH and HCl aq. (3 mL, 12 M) and stirred at room temperature for 1 h. Next, the flask was stored at -10 °C to allow the deoxybenzoin to precipitate as a solid. The pure product

was a white solid and could be obtained from this crude mixture by recrystallisation from methanol (10 mL).

2-(4-methoxy)-1-(3,4,5-trimethoxyphenyl)-ethanone (compound 17a): (2.3 g, 72.3%); White Solid; mp 85-86 °C; δ_H (300 MHz; CDCl₃) 3.79 (3 H, s), 3.89 (6 H, s), 3.90 (3 H, s), 4.19 (2 H, s), 6.87 (2 H, d, *J* 9.0), 7.17 (2 H, d, *J* 9.0), 7.26 (2 H, s).

2-(4-methoxy-3-nitrophenyl)-1-(3,4,5-trimethoxyphenyl)-ethanone (compound 17b): (2.7 g, 75.0%); White Solid; mp 81-83 °C; MS (EI) *m/z*: 361 (M⁺, 32%), 181 (100); δ_H (300 MHz; CDCl₃) 3.92 (6 H, s), 3.93 (3 H, s), 3.96 (3 H, s), 4.26 (2 H, s), 7.07 (1 H, d, *J* 9.0), 7.25 (2 H, s), 7.45 (1H, dd, *J* 2.4, *J* 9.0), 7.78 (1 H, d, *J* 2.4).

2-[4-methoxy-3-(phenylmethoxy)phenyl]-1-(3,4,5-trimethoxyphenyl)-ethanone (compound 17c): (3.2 g, 74.8%); White Solid; mp 74-76 °C; δ_H (300 MHz; CDCl₃) 3.85 (3 H, s), 3.86 (6 H, s), 3.90 (3 H, s), 4.13 (2 H, s), 5.11 (2 H, s), 6.81 (1 H, d, *J* 8.4), 6.82 (1 H, s), 6.85 (1 H, d, *J* 8.4), 7.22 (2 H, s), 7.28-7.35 (5 H, m).

General synthetic procedures for (*E*)- and (*Z*)-3-chloro-2,3-diarylacrylaldehydes 13a-e and 18a-c

The deoxybenzoin (3.2 mmol) was dissolved in an ice-cooled solution of tetrahydrofuran (5 mL) and anhydrous dimethylformamide (15 mL), and phosphorus oxychloride (32 mmol) was added over a period of 30 min. After 24 h at room temperature, the slurry was poured into water (50 mL) and stirred at 0 °C for 1 h. The solvent was then removed under reduced pressure and extracted with ethyl acetate (100 mL × 3). The combined organic layer was washed with brine, dried over anhydrous Na₂SO₄, filtered and concentrated to yield the crude product, which was used without further purification.

General synthetic procedures for esters 3a-e and 6a-c

A 1 M solution of sodium alkoxide, freshly prepared from 2 mmol of sodium, and 20 mL alcohol were added to 3-chloro-2,3-diarylacrylaldehyde (1 mmol), which was in 5 mL of alcohol solvent. Ethyl 2-mercaptoacetate (1.05 mmol) was then added, and the resulting mixture was stirred at 0 °C for 4 h. The mixture was stirred at room temperature for another 12 h. The solvent was then removed under reduced pressure and extracted with ethyl acetate (100 mL × 3). The combined organic layer was washed with brine, dried over anhydrous Na₂SO₄, filtered and concentrated to yield the crude product. Flash chromatography (silica gel, hexanes/ethyl acetate)

was used to purify the product ester.

5-(4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid ethyl ester (compound 3a): (0.22 g, 50.6%); White Solid; mp 54-56 °C; MS (EI) m/z : 428 (M^+ , 100%), 413 (46), 97 (36), 71 (49), 57 (83), 43 (91); δ_H (300 MHz; $CDCl_3$) 1.40 (3 H, t, J 7.0), 3.71 (6 H, s), 3.87 (3 H, s), 3.95 (3 H, s), 4.38 (2 H, q, J 7.0), 6.50 (2 H, s), 7.00 (2 H, d, J 8.8), 7.39 (2 H, d, J 8.8), 7.80 (1 H, s).

5-(3-bromophenyl)-4-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid ethyl ester (compound 3b): (0.20 g, 42.5%); Yellow Solid; mp 55-57 °C; MS (ESI) m/z : 477.0 ($M+H^+$); δ_H (300 MHz; $CDCl_3$) 1.40 (3 H, t, J 7.0), 3.70 (6 H, s), 3.86 (3 H, s), 4.40 (2 H, q, J 7.0), 6.44 (2 H, s), 7.18 (1 H, m), 7.24 (1 H, m), 7.44 (1 H, m), 7.53 (1 H, m), 7.84 (1 H, s).

5-(4-methoxy-3-nitrophenyl)-4-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid ethyl ester (compound 3c): (0.20 g, 43.1%); Yellow Solid; mp 112-114 °C; MS (EI) m/z : 473 (M^+ , 100%), 458 (39), 195 (64); δ_H (300 MHz; $CDCl_3$) 1.41 (3 H, t, J 7.0), 3.73 (6 H, s), 3.87 (3 H, s), 3.97 (3 H, s), 4.39 (2 H, q, J 7.0), 6.44 (2 H, s), 7.01 (1 H, d, J 8.8), 7.44 (1 H, dd, J 2.0, J 8.8), 7.82 (1 H, s), 7.90 (1 H, d, J 2.0).

5-(2-naphthalenyl)-4-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid ethyl ester (compound 3d): (0.23 g, 52.4%); White Solid; mp 135-137 °C; MS (ESI) m/z : 449.1 ($M+H^+$); δ_H (300 MHz; $CDCl_3$) 1.42 (3H, t, J 7.0), 3.59 (6 H, s), 3.86 (3H, s), 4.41 (2 H, q, J 7.0), 6.49 (2 H, s), 7.34 (1 H, dd, J 1.7, J 8.6), 7.50 (2 H, m), 7.73 (1 H, d, J 8.6), 7.79 (2 H, m), 7.89 (1 H, s), 7.93 (1 H, d, J 1.7); δ_C (150 MHz; $CDCl_3$) 13.4, 50.9 (2 $\underline{OCH}_3$), 59.9, 60.3, 105.3 (2 $\underline{CH}$), 125.6, 125.7, 125.9, 126.7, 127.0, 127.1, 127.4, 129.9, 132.2, 134.7, 137.8, 152.1(2C), 161.2.

5-[4-methoxy-3-(phenylmethoxy)phenyl]-4-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid ethyl ester (compound 3e): (0.24 g, 45.6%); White Solid; mp 48-50 °C; MS (EI) m/z : 534 (M^+ , 19%), 91(100); δ_H (300 MHz; $CDCl_3$) 1.40 (3 H, t, J 7.0), 3.70 (6 H, s), 3.86 (3 H, s), 3.89 (3 H, s), 4.38 (2 H, q, J 7.0), 4.93 (2 H, s), 6.45 (2 H, s), 6.83 (1 H, d, J 8.3), 6.84 (1 H, s), 6.96 (1 H, dd, J 2.0, J 8.3), 7.28-7.31 (5 H, m), 7.79 (1 H, s).

4-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid ethyl ester (compound 6a): (0.19 g, 45.3%); Yellow Solid; mp 61-63 °C; MS (ESI) m/z : 429.1 ($M+H^+$); δ_H (300 MHz; $CDCl_3$) 1.40 (3 H, t, J 7.0), 3.67 (6 H, s), 3.81 (3 H, s), 3.86 (3 H, s), 4.38 (2 H, q, J 7.0), 6.52 (2 H, s), 6.85 (2 H, d, J 8.8), 7.21 (2 H, d, J 8.8), 7.78 (1 H, s).

4-(4-methoxy-3-nitrophenyl)-5-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid ethyl ester (compound 6b): (0.23 g, 47.6%); Yellow Solid; mp 57-59 °C; MS (ESI) m/z : 474.1 ($M+H^+$), 496.1 ($M+Na^+$); δ_H (300 MHz; $CDCl_3$) 1.41 (3 H, t, J 7.0), 3.72 (6 H, s), 3.87 (3 H, s), 3.96 (3 H, s), 4.39 (2 H, q, J 7.0), 6.50 (2 H, s), 7.01 (1 H, d, J 8.4), 7.40 (1 H, dd, J 1.8, J 8.4), 7.81 (1 H, s), 7.85 (1 H, d, J 1.8).

4-[4-methoxy-3-(phenylmethoxy)phenyl]-5-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid ethyl ester (compound 6c): (0.26 g, 48.4%); Yellow Solid; mp 55-57 °C; MS (ESI) m/z : 557.2 ($M+Na^+$); δ_H (300 MHz; $CDCl_3$) 1.39 (3 H, t, J 7.0), 3.67 (6 H, s), 3.85 (3 H, s), 3.93 (6 H, s), 4.38 (2 H, q, J 7.0), 4.98 (2 H, s), 6.50 (2 H, s), 6.82 (1 H, d, J 1.8), 6.84 (1 H, d, J 8.4), 6.87 (1 H, dd, J 1.8, J 8.4), 7.31-7.38 (5 H, m), 7.74 (1 H, s).

General synthetic procedures for acids 4a-e and 7a-c

The ester (0.5 mmol) was stirred with sodium hydroxide solution (1 M, 5 mL) and an equal volume of ethanol at 80°C for 2 h. The cooled solution was acidified with 5% hydrochloric acid, and the subsequent slurry was stirred for an additional hour. The solvent was then removed under reduced pressure and extracted with ethyl acetate (100 mL $\times$ 3). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4 , filtered and concentrated to yield the crude product. Flash chromatography (silica gel, hexanes/ethyl acetate) was used to purify the acid product.

5-(4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid (compound 4a): (0.18 g, 98.0%); White Solid; mp 129-131 °C; MS (EI) m/z : 400 (M^+ , 100%), 385 (52), 135 (21); δ_H (300 MHz; $CDCl_3$) 3.63 (6 H, s), 3.77 (3 H, s), 3.83 (3 H, s), 6.42 (2 H, s), 6.77 (2 H, s), 7.18 (2 H, s), 7.85 (1 H, s).

5-(3-bromophenyl)-4-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid (compound 4b): (0.17 g, 97.1%); Yellow Solid; mp 149-151 °C; MS (ESI) m/z : 449.0 ($M-H^+$); δ_H (300 MHz; $CDCl_3$) 3.70 (6 H, s), 3.87 (3 H, s), 6.44 (2 H, s), 7.19 (1 H, m), 7.24 (1 H, m), 7.46 (1 H, m), 7.54 (1 H, m), 7.93 (1 H, s).

5-(4-methoxy-3-nitrophenyl)-4-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid (compound 4c): (0.21 g, 95.2%); Yellow Solid; mp 215-217 °C; MS (ESI) m/z : 444.0 ($M-H^+$), 889.2 ($2M-H^+$); δ_H (300 MHz; $CDCl_3$) 3.74 (6 H, s), 3.90 (3 H, s), 3.99 (3 H, s), 6.53 (2 H, s), 7.04 (1 H, d, J 8.6), 7.43 (1 H, dd, J 2.0, J 8.6), 7.81 (1 H, d, J 2.0), 7.92 (1 H, s).

5-(2-naphthalenyl)-4-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid (compound 4d):

(0.20 g, 96.5%); Yellow Solid; mp 185-187 °C; MS (ESI) m/z : 421.1 ($M+H^+$); δ_H (300 MHz; $CDCl_3$) 3.58 (6 H, s), 3.85 (3 H, s), 6.49 (2 H, s), 7.32 (1 H, d, J 8.1), 7.49 (2 H, m), 7.71 (1 H, d, J 8.1), 7.79 (2 H, m), 7.92 (1 H, s), 7.98 (1 H, s); δ_C (150 MHz; $CDCl_3$) 56.0 ($2OCH_3$), 61.0, 106.3 ($2CH$), 126.7, 126.8, 127.7, 128.1, 128.2, 128.5, 130.7, 130.7, 133.0, 133.2, 137.6, 139.2, 153.2($2C$), 162.2.

5-[4-methoxy-3-(phenylmethoxy)phenyl]-4-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid (compound 4e): (0.24 g, 96.0%); Yellow Solid; mp 145-147 °C; MS (ESI) m/z : 529.2 ($M+Na^+$); δ_H (300 MHz; $CDCl_3$) 3.71 (6 H, s), 3.87 (3 H, s), 3.90 (3 H, s), 4.94 (2 H, s), 6.46 (2 H, s), 6.84 (1 H, d, J 8.4), 6.86 (1 H, d, J 1.8), 6.98 (1 H, dd, J 1.8, J 8.2), 7.35 (5 H, m), 7.88 (1 H, s).

4-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid (compound 7a):

(0.19 g, 96.0%); Yellow Solid; mp 138-140 °C; MS (ESI) m/z : 401.1 ($M+H^+$), 823.2 ($2M+Na^+$); δ_H (300 MHz; $CDCl_3$) 3.67 (6 H, s), 3.80 (3 H, s), 3.87 (3 H, s), 6.53 (2 H, s), 6.85 (2 H, d, J 8.4), 7.21 (2 H, d, J 8.4), 7.86 (1 H, s); δ_C (150 MHz; $CDCl_3$) 55.3, 56.0($2OCH_3$), 61.0, 106.4 ($2CH$), 114.0($2CH$), 127.9, 128.6, 130.3($2CH$), 137.6, 138.3, 138.8, 146.4, 153.1($2C$), 159.1, 167.5.

4-(4-methoxy-3-nitrophenyl)-5-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid (compound 7b):

(0.21 g, 95.3%); Yellow Solid; mp 211-213 °C; MS (ESI) m/z : 446.0 ($M+H^+$); δ_H (300 MHz; $CDCl_3$) 3.76 (6 H, s), 3.90 (3 H, s), 3.99 (3 H, s), 6.47 (2 H, s), 7.04 (1 H, d, J 8.6), 7.46 (1 H, dd, J 2.0, J 8.6), 7.92 (1 H, s), 7.93 (1 H, d, J 2.0).

4-[4-methoxy-3-(phenylmethoxy)phenyl]-5-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid (compound 7c):

(0.25 g, 97.4%); Yellow Solid; mp 128-130 °C; MS (ESI) m/z : 507.1 ($M+H^+$), 529.1 ($M+Na^+$); δ_H (300 MHz; $CDCl_3$) 3.71 (6 H, s), 3.87 (3 H, s), 3.90 (3 H, s), 4.94 (2 H, s), 6.46 (2 H, s), 6.85 (1 H, dd, J 1.8, J 8.2), 6.96 (1 H, d, J 8.4), 6.98 (1 H, d, J 1.8), 7.31 (5 H, m), 7.86 (1 H, s).

General synthetic procedures for 2,3-diarylthiophenes 5a-e and 8a-c

The acid (0.1 mmol), quinoline (10 mL), and copper (I) oxide (0.25 mmol) were heated at 145 °C for 4 h under nitrogen. Upon cooling, the mixture was filtered, and the residue was washed with CH_2Cl_2 . The combined filtrate and washings were extracted with a solution of 5% hydrochloric acid, 10% sodium carbonate and water. The combined organic layer was dried over anhydrous

Na₂SO₄, filtered and concentrated to yield the crude product. Flash chromatography (silica gel, hexanes/ethyl acetate) was used to purify the product.

2-(4-methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)-thiophene (compound 5a): (31 mg, 85.7%); White Solid; mp 75-77 °C; MS (EI) *m/z*: 356 (M⁺, 100%), 341 (63), 162 (23); δ_H (300 MHz; CDCl₃) 3.68 (6 H, s), 3.80 (3 H, s), 3.86 (3 H, s), 6.49 (2 H, s), 6.83 (2 H, d, *J* 5.2), 7.14 (1 H, d, *J* 5.2), 7.27 (3 H, m).

2-(3-bromophenyl)-3-(3,4,5-trimethoxyphenyl)-thiophene (compound 5b): (36 mg, 88.4%); Yellow Solid; mp 64-66 °C; MS (EI) *m/z*: 406 (M⁺, 64%), 404 (64), 391 (34), 389 (34), 360 (100), 345 (66), 326 (44), 311 (31), 195 (46), 147 (60), 98 (94); δ_H (300 MHz; CDCl₃) 3.69 (6 H, s), 3.86 (3 H, s), 6.47 (2 H, s), 7.16 (1 H, d, *J* 5.2), 7.21 (2 H, m), 7.34 (1 H, d, *J* 5.2), 7.38 (1 H, m), 7.52 (1 H, m).

2-(4-methoxy-3-nitrophenyl)-3-(3,4,5-trimethoxyphenyl)-thiophene (compound 5c): (32 mg, 80.7%); Yellow Solid; mp 123-125 °C; MS (ESI) *m/z*: 402.1 (M+H⁺); δ_H (300 MHz; CDCl₃) 3.72 (9 H, s), 3.87 (3 H, s), 6.47 (2 H, s), 6.95 (1 H, d, *J* 8.8), 7.14 (1 H, d, *J* 5.2), 7.34 (1 H, d, *J* 5.2), 7.38 (1 H, dd, *J* 2.4, *J* 8.8), 7.84 (1 H, d, *J* 2.4); δ_C (150 MHz; CDCl₃) 56.1 (2OCH₃), 61.0, 65.6, 106.4 (2CH), 114.4, 124.6, 125.9, 126.8, 130.4, 131.4, 134.6, 135.4, 138.6, 138.9, 151.4, 153.3 (2C), 153.4.

2-(2-naphthalenyl)-3-(3,4,5-trimethoxyphenyl)-thiophene (compound 5d): (34 mg, 89.2%); Yellow Solid; mp 116-118 °C; MS (EI) *m/z*: 376 (M⁺, 100%), 361 (67), 123 (71); δ_H (300 MHz; CDCl₃) 3.59 (6 H, s), 3.85 (3 H, s), 6.52 (2 H, s), 7.21 (1 H, d, *J* 5.2), 7.35 (1 H, dd, *J* 1.9, *J* 8.6), 7.36 (1 H, d, *J* 5.2), 7.46 (2 H, m), 7.70 (1 H, d, *J* 8.6), 7.78 (2 H, m), 7.90 (1 H, d, *J* 1.9); δ_C (150 MHz; CDCl₃) 55.9 (2OCH₃), 60.9, 106.4 (2CH), 124.4, 126.2, 126.3, 127.5, 127.6, 127.8, 128.0, 128.1, 130.2, 131.9, 131.9, 132.5, 133.4, 138.2, 138.5, 153.1 (2C).

2-[4-methoxy-3-(phenylmethoxy)phenyl]-3-(3,4,5-trimethoxyphenyl)-thiophene (compound 5e): (39 mg, 84.6%); Yellow Solid; mp 89-91 °C; MS (EI) *m/z*: 462 (M⁺, 99%), 308 (25), 91 (100); δ_H (300 MHz; CDCl₃) 3.69 (6 H, s), 3.85 (3 H, s), 3.88 (3 H, s), 4.93 (2 H, s), 6.47 (2 H, s), 6.82 (1 H, d, *J* 8.3), 6.84 (1 H, d, *J* 2.1), 6.93 (1 H, dd, *J* 2.1, *J* 8.3), 7.11 (1 H, d, *J* 5.2), 7.25 (1 H, d, *J* 5.2), 7.27-7.32 (5 H, m).

3-(4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)-thiophene (compound 8a): (30 mg, 84.6%); Yellow Solid; mp 81-83 °C; MS (EI) *m/z*: 356.1 (M⁺); δ_H (300 MHz; CDCl₃) 3.67 (6 H, s), 3.79 (3

H, s), 3.85 (3 H, s), 6.52 (2 H, s), 6.90 (2 H, m), 7.11 (1 H, d, J 5.4), 7.23 (2 H, m), 7.27 (1 H, d, J 5.4); δ_C (150 MHz; $CDCl_3$) 54.3, 54.9 ($2OCH_3$), 59.9, 105.4 ($2CH$), 112.8 ($2CH$), 122.6, 128.1, 128.9, 129.3 ($2CH$), 129.4, 136.4, 136.7, 136.8, 152.0 (2C), 157.6.

3-(4-methoxy-3-nitrophenyl)-2-(3,4,5-trimethoxyphenyl)-thiophene (compound 8b): (35 mg, 86.8%); Yellow Solid; mp 95-97 °C; MS (EI) m/z : 401.1 (M^+); δ_H (300 MHz; $CDCl_3$) 3.72 (6 H, s), 3.87 (3 H, s), 3.95 (3 H, s), 6.50 (2 H, s), 6.70 (1 H, d, J 8.4), 7.15 (1 H, d, J 5.4), 7.34 (1 H, d, J 5.4), 7.40 (1 H, dd, J 2.4, J 8.4), 7.88 (1 H, d, J 2.4 Hz); δ_C (150 MHz; $CDCl_3$) 56.1 ($2OCH_3$), 56.6, 61.0, 106.7 ($2CH$), 113.3, 124.5, 125.8, 129.0, 129.1, 129.6, 134.8, 134.9, 138.0, 139.5, 139.6, 151.7, 153.3 (2C).

3-[4-methoxy-3-(phenylmethoxy)phenyl]-2-(3,4,5-trimethoxyphenyl)-thiophene (compound 8c): (41 mg, 87.9%); Yellow Solid; mp 74-76 °C; MS (ESI) m/z : 485.2 ($M+Na^+$); δ_H (300 MHz; $CDCl_3$) 3.67 (6 H, s), 3.85 (3 H, s), 3.88 (3 H, s), 4.96 (2 H, s), 6.50 (2 H, s), 6.84 (1 H, dd, J 2.4, J 8.4), 6.89 (1 H, d, J 8.4 Hz), 6.90 (1 H, d, J 2.4), 7.06 (1 H, d, J 5.4), 7.25 (1 H, d, J 5.4), 7.31 (5 H, m).

General synthetic procedures for 3f, 4f, 5f, 6d, 7d and 8d

$TiCl_4$ (0.07 mmol) was added dropwise to each solution of the compounds **3e**, **4e**, **5e**, **6c**, **7c**, and **8c** (0.06 mmol) in CH_2Cl_2 (15 mL) at room temperature. The reaction was monitored by TLC and quenched by treatment with MeOH. The solvent was removed, and the residue was extracted with CH_2Cl_2 (100 mL $\times$ 3). The combined organic layer was dried over anhydrous Na_2SO_4 , filtered and concentrated to yield the crude product. Flash chromatography (silica gel, hexanes/ethyl acetate) was used to purify the products, **3f**, **4f**, **5f**, **6d**, **7d**, and **8d**.

5-(3-hydroxy-4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid ethyl ester (compound 3f): (25 mg, 93.9%); Yellow Solid; mp 61-63 °C; MS (EI) m/z : 444 (M^+ , 100%), 429 (50), 171 (26), 139 (25), 45 (48); δ_H (300 MHz; $CDCl_3$) 1.39 (3 H, t, J 7.0), 3.70 (6 H, s), 3.86 (3 H, s), 3.88 (3 H, s), 4.37 (2 H, q, J 7.0), 6.47 (2 H, s), 6.77 (1 H, d, J 8.4), 6.82 (1 H, dd, J 2.0, J 8.4), 6.94 (1 H, d, J 2.0), 7.80 (1 H, s); δ_C (150 MHz; $CDCl_3$) 14.4, 56.0, 56.1 ($2OCH_3$), 60.9, 61.2, 106.3 ($2CH$), 110.6, 115.5, 121.5, 126.6, 130.9, 131.1, 135.7, 137.4, 138.2, 145.3, 145.5, 146.9, 153.1 (2C), 162.3.

5-(3-hydroxy-4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid (compound 4f): (24 mg, 94.3%); Yellow Solid; mp 187-189 °C; MS (ESI) m/z : 417.1 ($M+H^+$); δ_H

(300 MHz; CDCl₃) 3.70 (6 H, s), 3.87 (3 H, s), 3.89 (3 H, s), 6.49 (2 H, s), 6.78 (1 H, d, *J* 8.4), 6.84 (1 H, dd, *J* 2.0, *J* 8.4), 6.96 (1 H, d, *J* 2.0), 7.89 (1 H, s); δ_C (150 MHz; CDCl₃) 56.0, 56.1 (2OCH₃), 61.0, 106.3 (2CH), 110.6, 115.5, 121.5, 126.4, 129.6, 130.8, 137.3, 137.5, 138.6, 145.6, 147.0, 147.1, 153.2(2C), 167.0.

2-methoxy-5-[3-(3,4,5-trimethoxyphenyl)-2-thienyl]-phenol (compound 5f): (21 mg, 92.0%); Yellow Solid; mp 51-53 °C; MS (EI) *m/z*: 372 (M⁺, 99%), 357 (38), 271 (45), 149 (72), 84 (100); δ_H (300 MHz; CDCl₃) 3.67 (6 H, s), 3.86 (3 H, s), 3.88 (3 H, s), 6.51 (2 H, s), 6.75 (1 H, d, *J* 8.3), 6.80 (1 H, dd, *J* 1.9, *J* 8.3), 6.96 (1 H, d, *J* 1.9), 7.13 (1 H, d, *J* 5.2), 7.27 (1 H, d, *J* 5.2); δ_C (150 MHz; CDCl₃) 56.0 (3OCH₃), 61.0, 106.3 (2CH), 110.5, 115.7, 121.5, 123.6, 127.6, 129.9, 132.1, 137.0, 137.5, 138.4, 145.4, 146.1, 153.0 (2C); HRMS (ESI) Calcd for C₂₀H₂₁O₅S [M + H]⁺ 373.1104, found *m/z* 373.1110.

4-(3-hydroxy-4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid ethyl ester (compound 6d): (25 mg, 93.5%); Yellow Solid; mp 65-67 °C; MS (ESI) *m/z*: 445.1 (M+H⁺); δ_H (300 MHz; CDCl₃) 1.40 (3 H, t, *J* 7.0), 3.69 (6 H, s), 3.86 (3 H, s), 3.89 (3 H, s), 4.38 (2 H, q, *J* 7.0), 5.66 (1 H, br s, OH), 6.54 (2 H, s), 6.75 (1 H, dd, *J* 1.8, *J* 8.4), 6.79 (1 H, d, *J* 8.4), 6.90 (1 H, d, *J* 1.8), 7.77 (1 H, s); δ_C (150 MHz; CDCl₃) 13.4, 55.0 (2OCH₃), 55.1, 59.9, 60.2, 105.4 (2CH), 109.6, 114.3, 120.0, 127.8, 128.1, 129.9, 135.1, 137.2, 137.3, 143.8, 144.5, 145.0, 152.1 (2C), 161.3.

4-(3-hydroxy-4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-2-thiophenecarboxylic acid (compound 7d): (23 mg, 91.4%); Yellow Solid; mp 201-203 °C; MS (ESI) *m/z*: 417.1 (M+H⁺), 855.2 (2M+Na⁺); δ_H (300 MHz; CDCl₃) 3.69 (6 H, s), 3.87 (3 H, s), 3.90 (3 H, s), 6.56 (2 H, s), 6.76 (1 H, dd, *J* 1.8, *J* 8.4), 6.80 (1 H, d, *J* 8.4), 6.91 (1 H, d, *J* 1.8), 7.86 (1 H, s); δ_C (150 MHz; CDCl₃) 56.0(3OCH₃), 61.0, 106.5 (2CH), 110.6, 115.3, 121.1, 128.5, 128.8, 129.6, 137.8, 138.4, 138.8, 145.6, 146.1, 146.8, 153.2(2C), 167.2.

2-methoxy-5-[2-(3,4,5-trimethoxyphenyl)-3-thienyl]-phenol (compound 8d): (21 mg, 95.4%); Yellow Solid; mp 91-93 °C; MS (EI) *m/z*: 372.1 (M⁺); δ_H (300 MHz; CDCl₃) 3.69 (6 H, s), 3.85 (3 H, s), 3.87 (3 H, s), 5.63 (1 H, br s, OH), 6.53 (2 H, s), 6.76 (2 H, m), 6.94 (1 H, d, *J* 1.4), 7.10 (1 H, d, *J* 4.8), 7.26 (1 H, d, *J* 4.8); δ_C (150 MHz; CDCl₃) 56.0(2OCH₃), 56.1, 60.9, 106.5 (2CH), 110.5, 115.4, 121.2, 123.6, 129.8, 130.0, 130.5, 137.4, 137.7, 138.0, 145.4, 145.6, 153.0(2C). HRMS (ESI) Calcd for C₂₀H₂₁O₅S [M + H]⁺ 373.1104, found *m/z* 373.1105.

Biological evaluation

Cell culture

The human fibrosarcoma cell line HT-1080, human gastric adenocarcinoma cell line SGC-7901 and human mouth epidermal carcinoma cell line KB were cultured in RPMI-1640 medium containing 10% FBS, 100 U/mL streptomycin and 100 U/mL penicillin at 37 °C in a humidified atmosphere containing 5% CO₂. All cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA).

In vitro anti-proliferative activity assay

The *in vitro* anti-proliferative activities of CA-4 (**1a**) and all of the target compounds were determined by MTT (Sigma) assay. Briefly, cells were seeded into 96-well plates at a density of $1-3 \times 10^4$ /well, depending on the growth rate of the cell line. Twenty-four hours later, triplicate wells were treated with media and the compounds being tested. After 72 h of incubation at 37 °C in 5% CO₂, the drug-containing medium was removed and replaced with 100 µL of fresh medium containing 5 mg/mL MTT solution. After 4 h of incubation, the medium with MTT was removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to each well. The plates were gently agitated until the purple formazan crystals were dissolved, and the OD₄₉₀ values were determined using a microplate reader (MK3, Thermo, Germany). The data were calculated and plotted as the percent viability compared to the control. The 50% inhibitory concentration (IC₅₀) was defined as the drug concentration that resulted in an absorbance of 50% of that of the untreated wells in the MTT assay.

Inhibition of tubulin polymerisation

The effects of compounds **8d**, **5f** and CA-4 (**1a**) on the polymerisation of tubulin were determined using a fluorescence-based tubulin polymerisation assay kit (Cytoskeleton-Cat.#BK011P) according to the manufacturer's protocol. Tubulin was resuspended in ice-cold G-PEM buffer (80 mM PIPES, 2 mM MgCl₂, 0.5 mM EGTA, 1 mM GTP, 20% (v/v) glycerol) and added to wells of a 96-well plate containing the designated concentrations of the drug or vehicle. The samples were mixed well, and tubulin assembly was monitored (emission wavelength of 420 nm; excitation wavelength of 360 nm) at 1 min intervals for 90 min at 37 °C using a plate reader (FASCalibur, BD Biosciences, USA). The IC₅₀ values were calculated after 20 min using SPSS software.

Immunofluorescence staining

Immunostaining was performed to detect the microtubule-associated tubulin protein after exposure to CA-4 (**1a**) and the investigated compound **8d**. SGC-7901 cells were seeded in a 24-well plate at 1×10^4 cells per well and grown for 24 h. The cells were treated with the vehicle or twice the IC₅₀ concentration of CA-4 (**1a**) or **8d** for 12 h. The control and treated cells were fixed with 4% formaldehyde in PBS for 30 min at -20 °C, washed twice with PBS and permeabilised with 0.1% (v/v) Triton X-100 in PBS for 5 min. The cells were then blocked with 5% bovine serum albumin (BSA) in PBS for 10 min. The primary α -tubulin antibody (Santa Cruz, CA) was diluted (1:100) with 2% BSA in PBS, and the plates were incubated overnight at 4 °C. The cells were washed with PBS to remove unbound primary antibody, and the cells were then incubated with FITC-conjugated antimouse secondary antibody diluted (1:1000) with 2% BSA in PBS for 3 h at 37 °C. The cells were washed with PBS to remove unbound secondary antibody, and the nuclei were stained with 4,6-diamino-2-phenolindol dihydrochloride (DAPI). Then, immunofluorescence was detected using a fluorescence microscope (Olympus, Tokyo, Japan).

Molecular modelling

The molecular modelling studies were performed with Accelrys Discovery Studio 3.0. The crystal structure of the tubulin DAMA-colchicine complex (PDB: 1SA0) was retrieved from the RCSB Protein Data Bank (<http://www.rcsb.org/pdb>). In the docking process, the protein protocol was prepared via several operations, including the standardisation of atom names, insertion of missing atoms in residues and removal of alternate conformations, insertion of missing loop regions based on SEQRES data, optimisation of short- and medium-sized loop regions with the Looper Algorithm, minimisation of the remaining loop regions, calculation of pK, and protonation of the structure. The receptor model was then typed with the CHARMM force field, and a binding sphere with a radius of 9.0 Å was defined with the original ligand (DAMA-colchicine) as the binding site. The CA-4 (**1a**) and **8d** compounds were drawn with Chemdraw and fully minimised using the CHARMM force field. Finally, CA-4 (**1a**) and **8d** compounds were docked into the binding site using the CDocker protocol with the default settings.

The target compounds

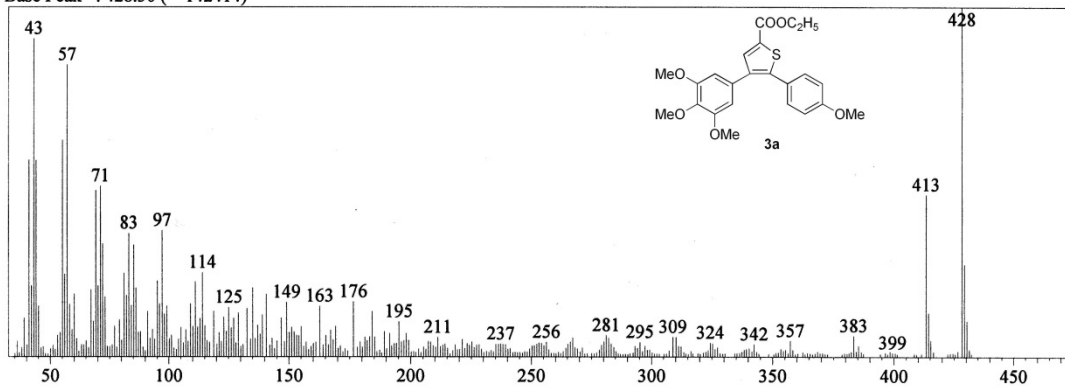
3a:

MS

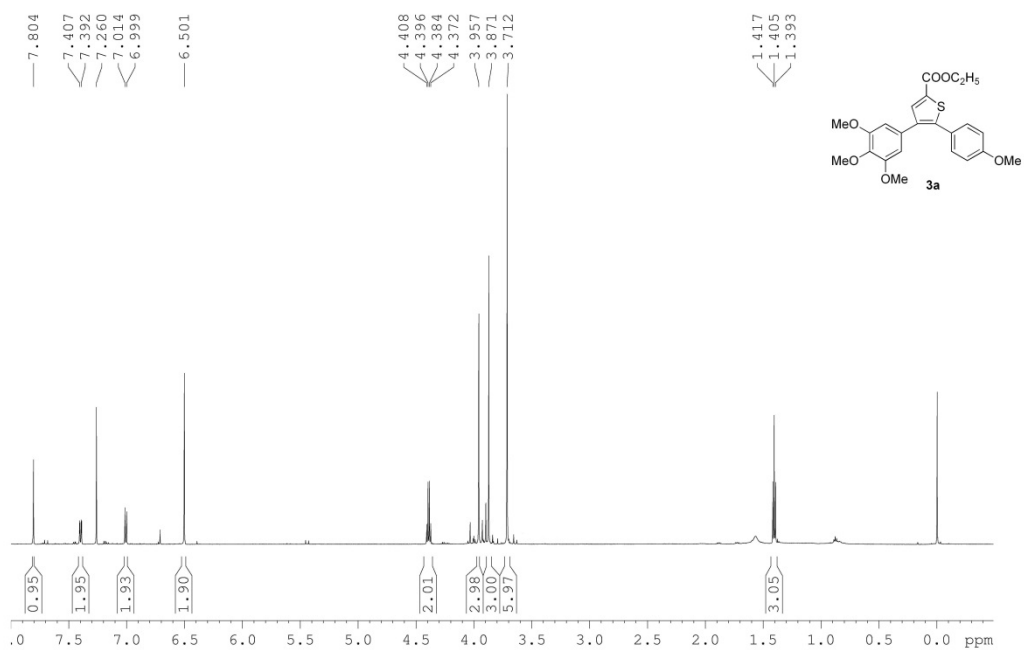
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Mass Peak # : 353 Ret. Time : 2.383

Base Peak : 428.30 (142414)

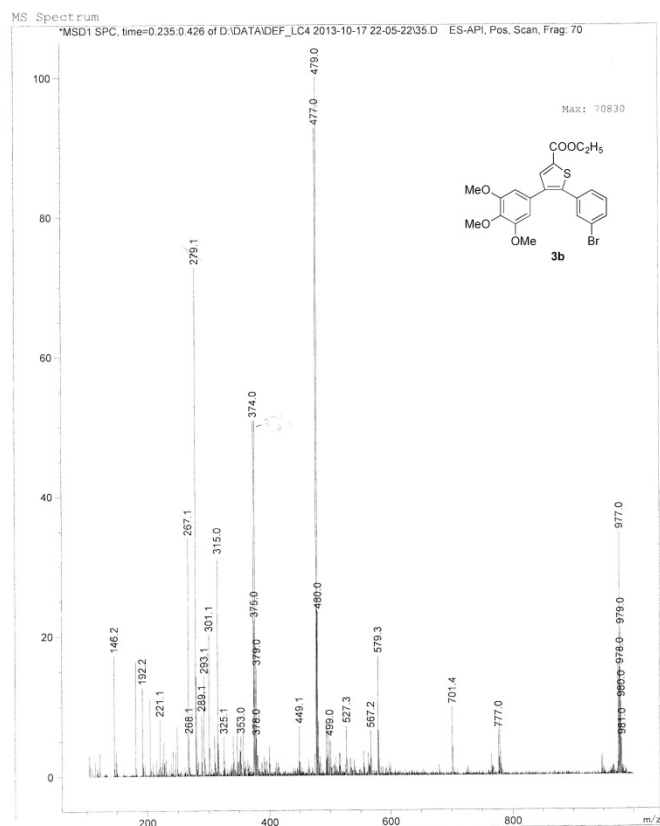


¹H NMR

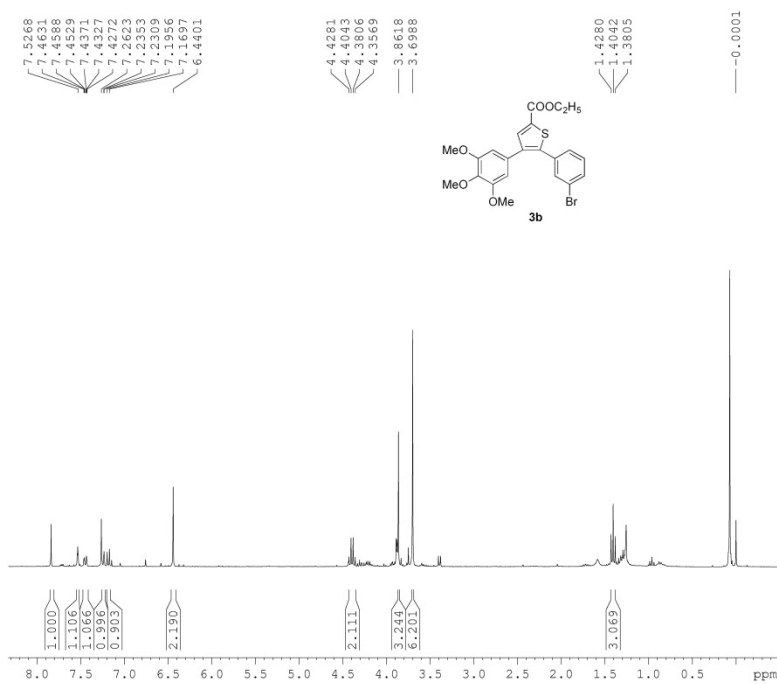


3b:

MS



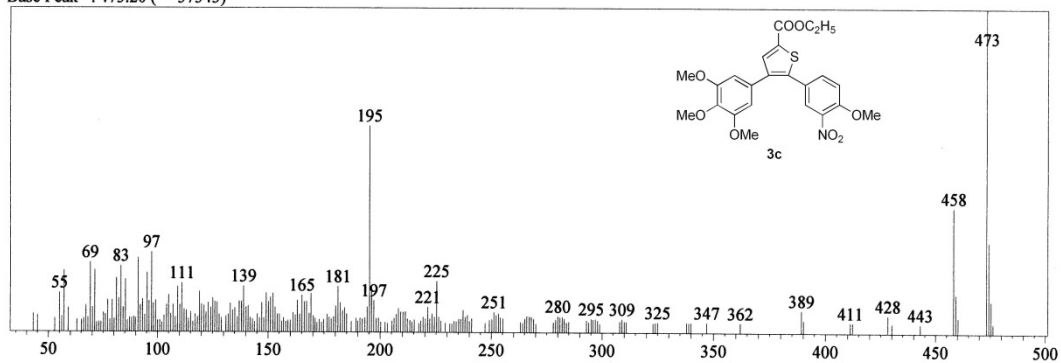
¹H NMR



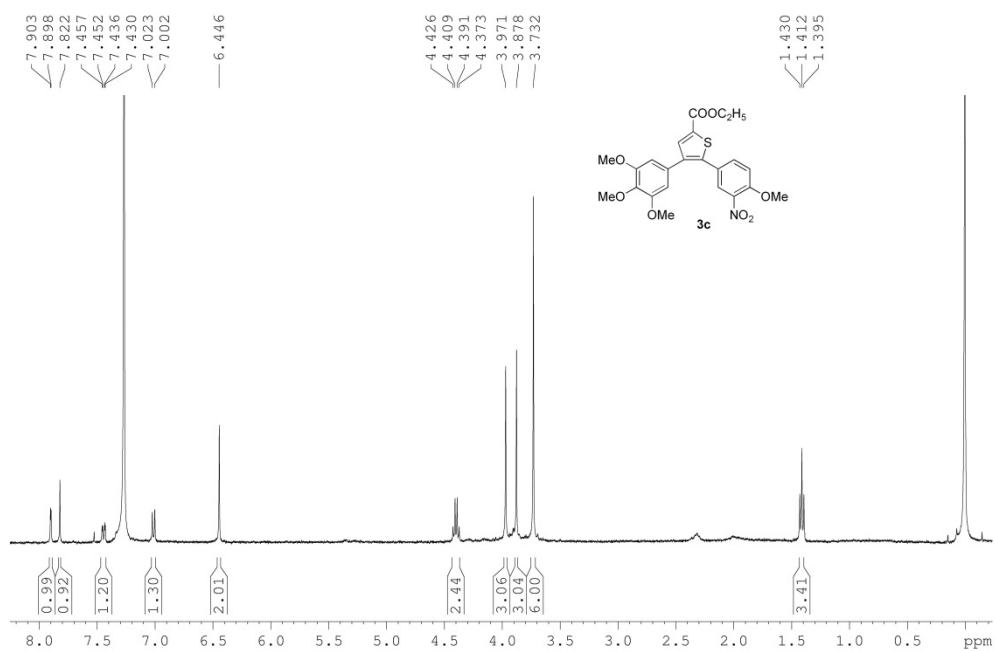
3c:

MS

Scan # : 361
Mass Peak # : 234 Ret. Time : 3.200
Base Peak : 473.20 (37343)



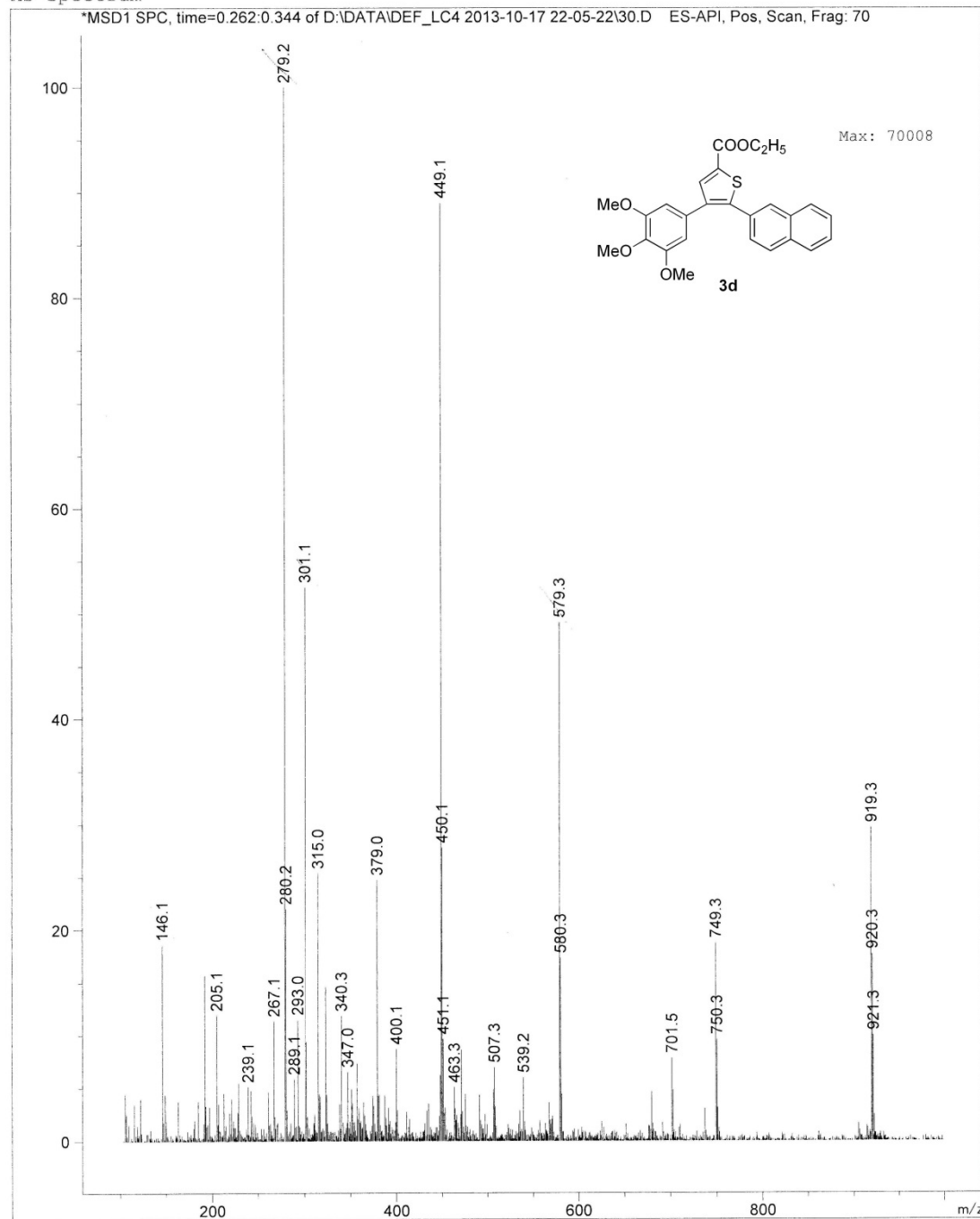
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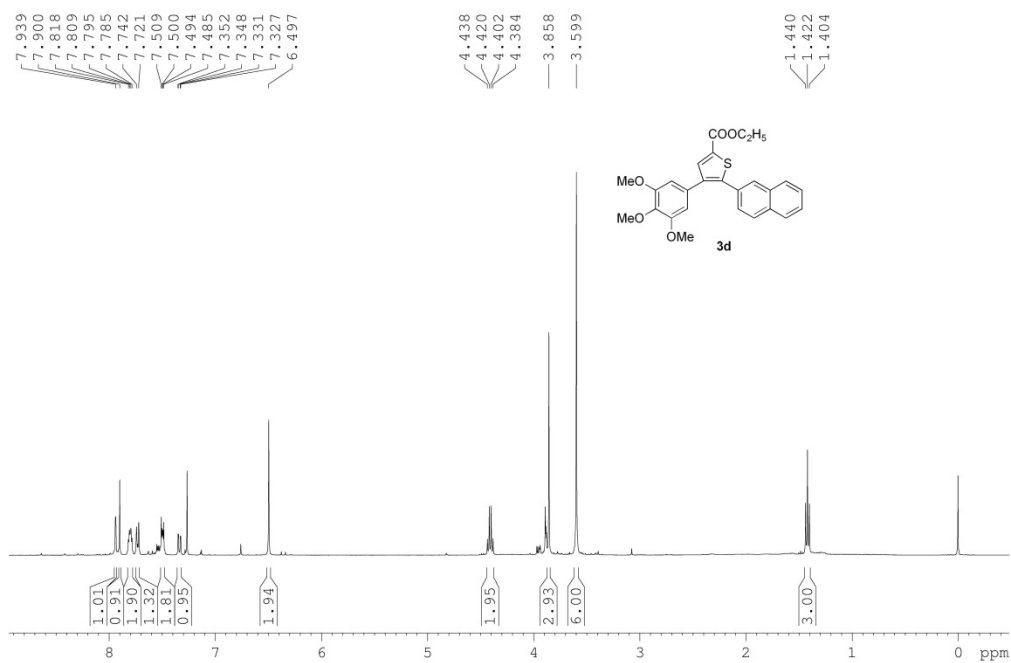
3d:

MS

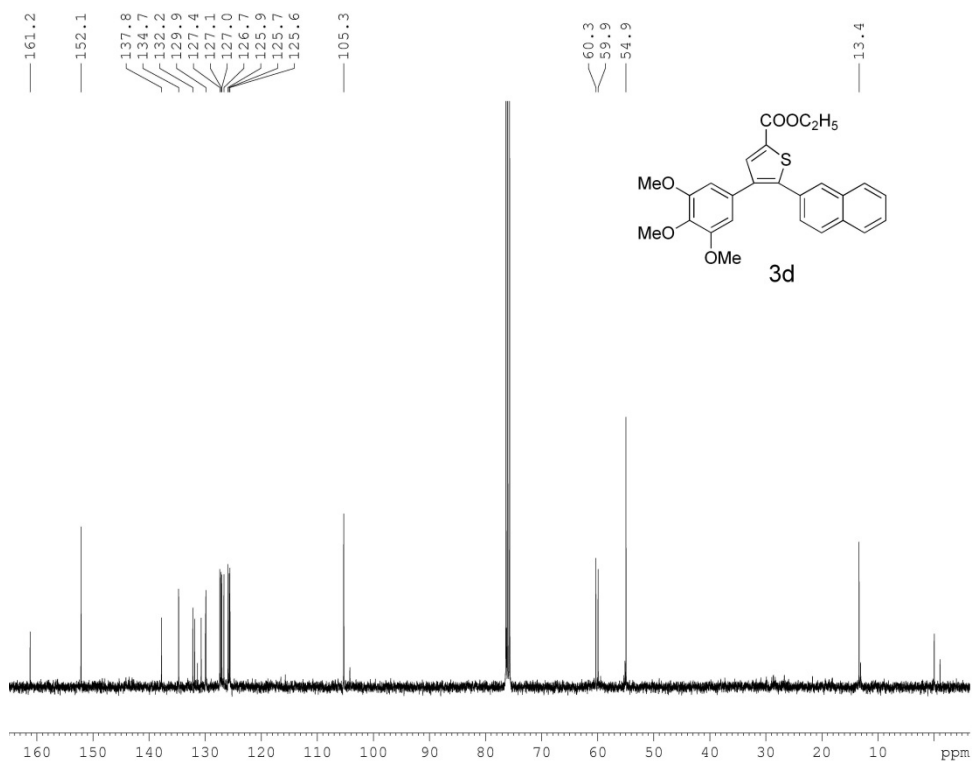
MS Spectrum



¹H NMR

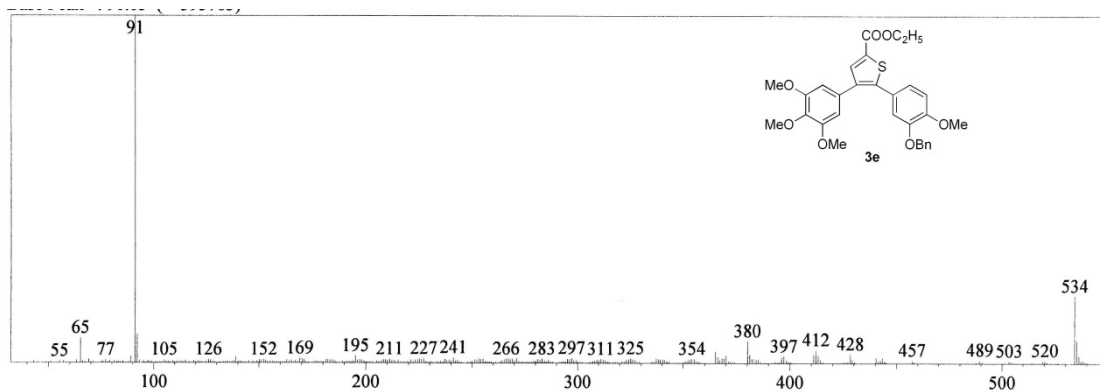


¹³C NMR

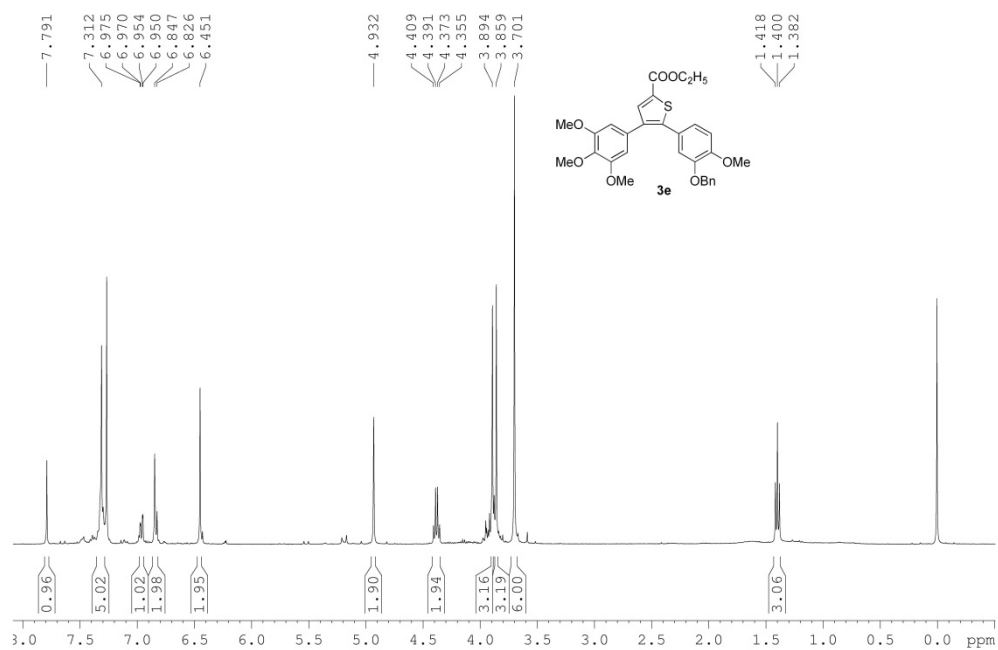


3e:

MS

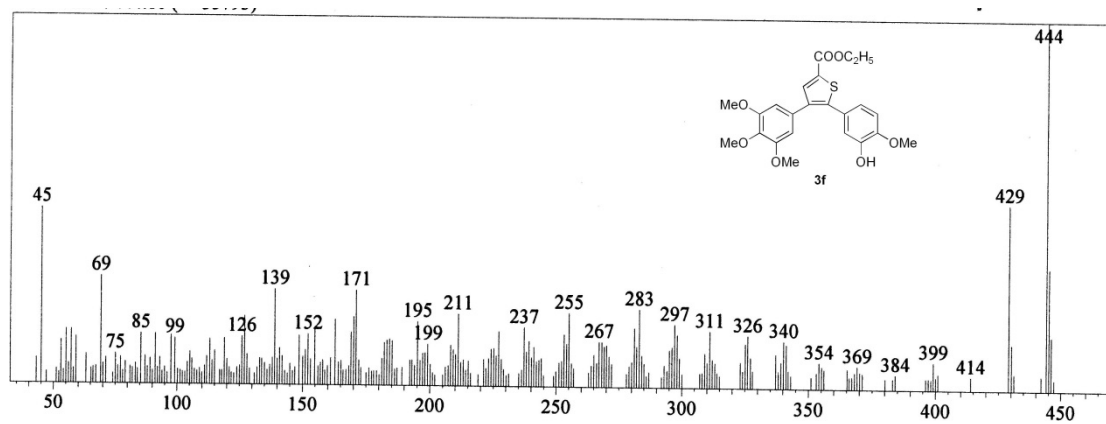


¹H NMR

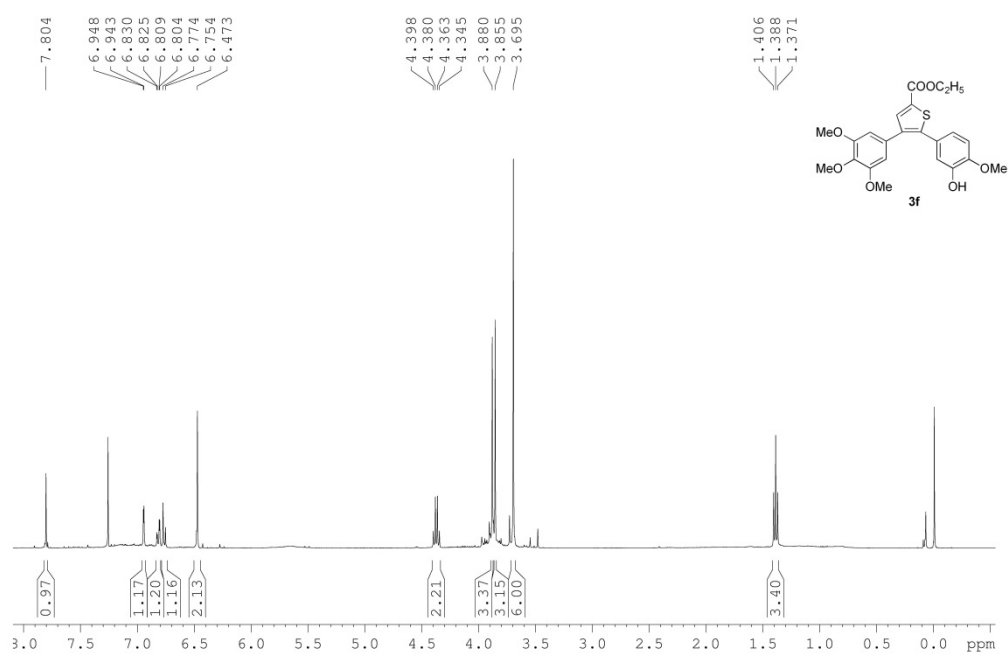


3f:

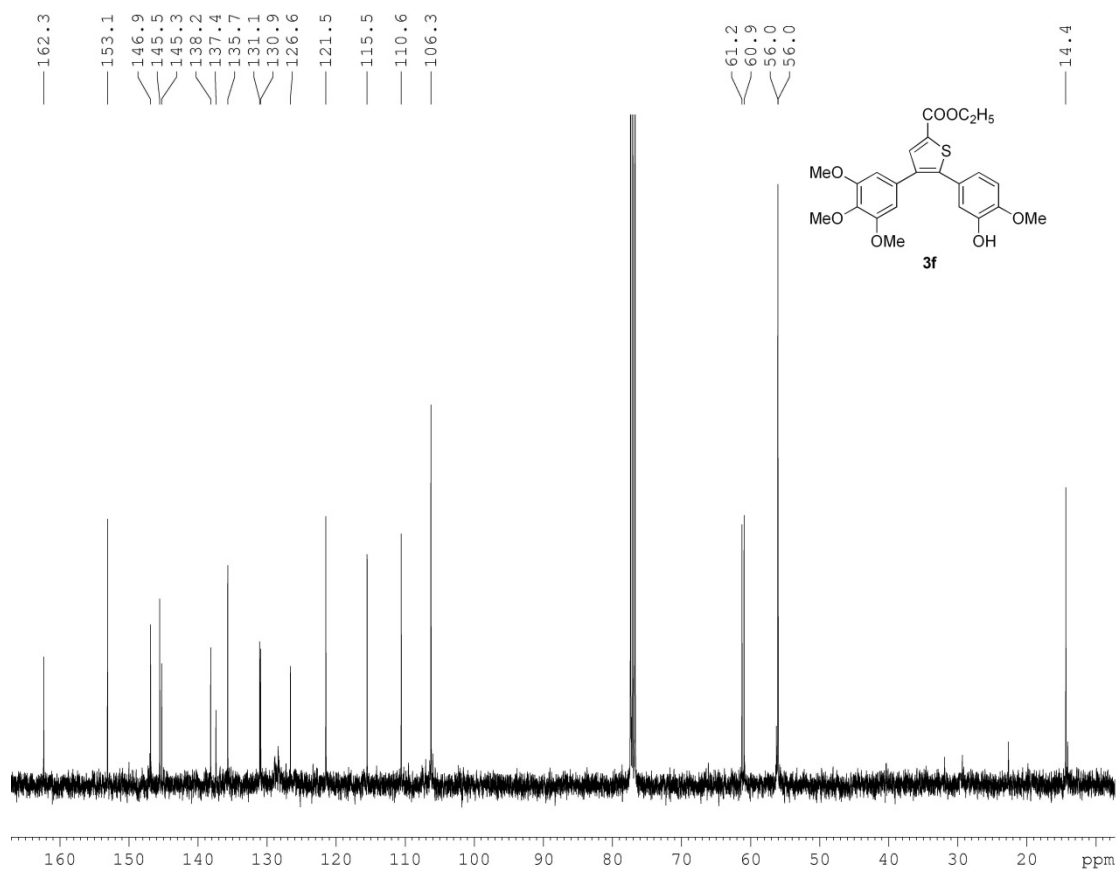
MS



^1H NMR

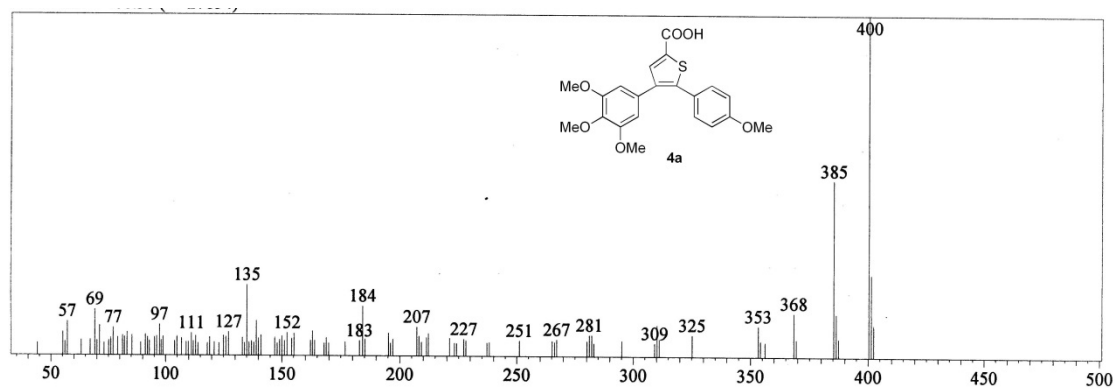


¹³C NMR

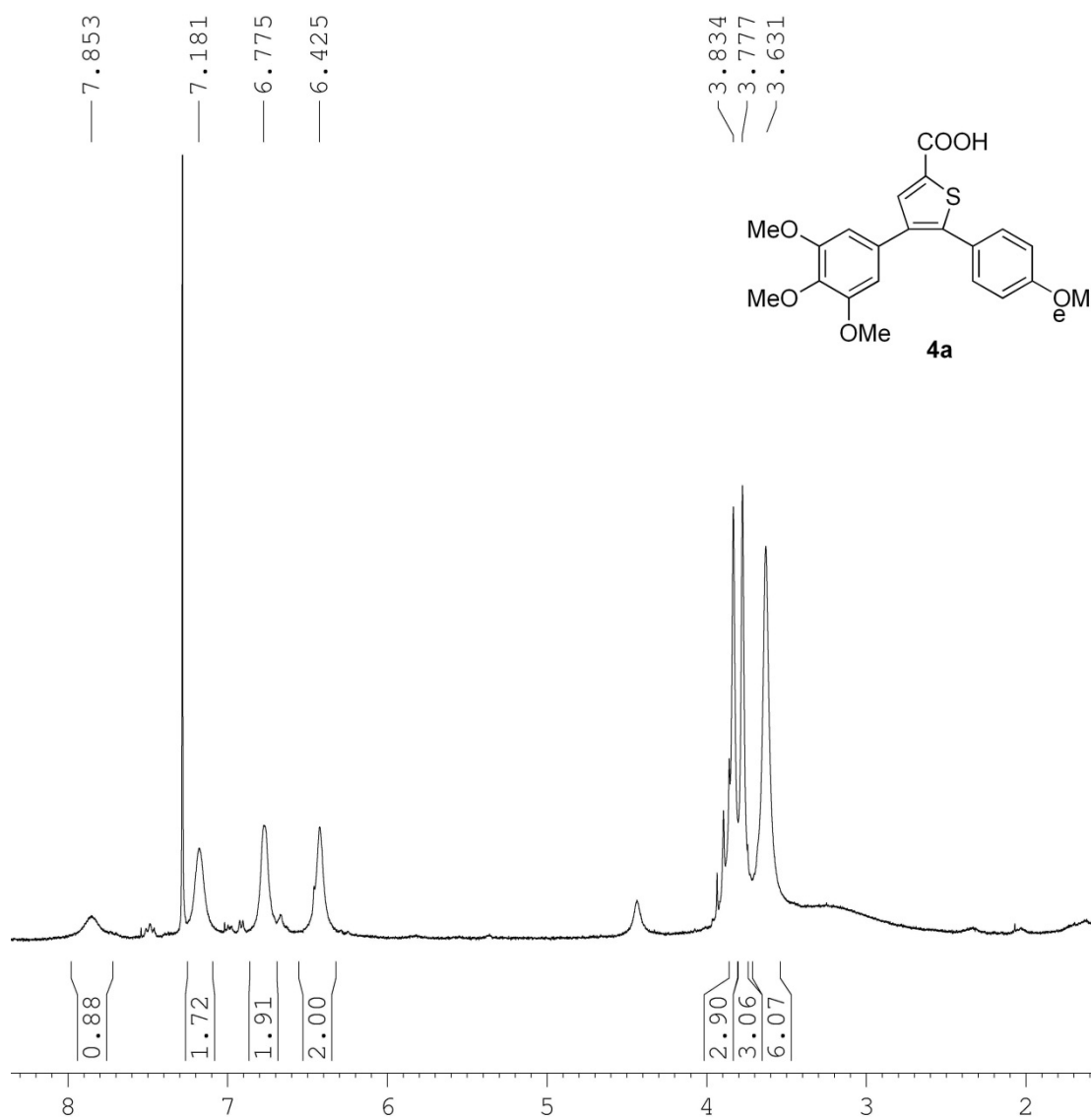


4a:

MS

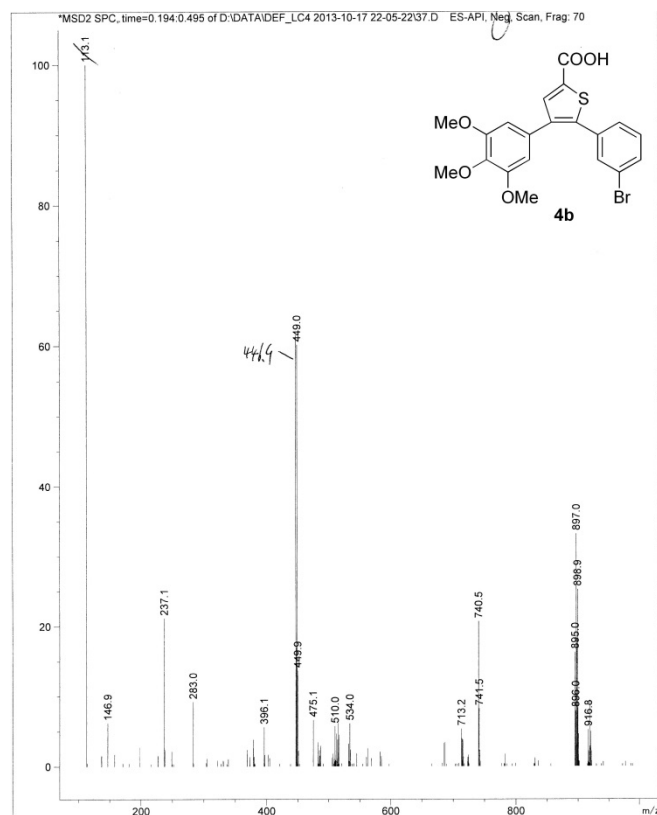


¹H NMR

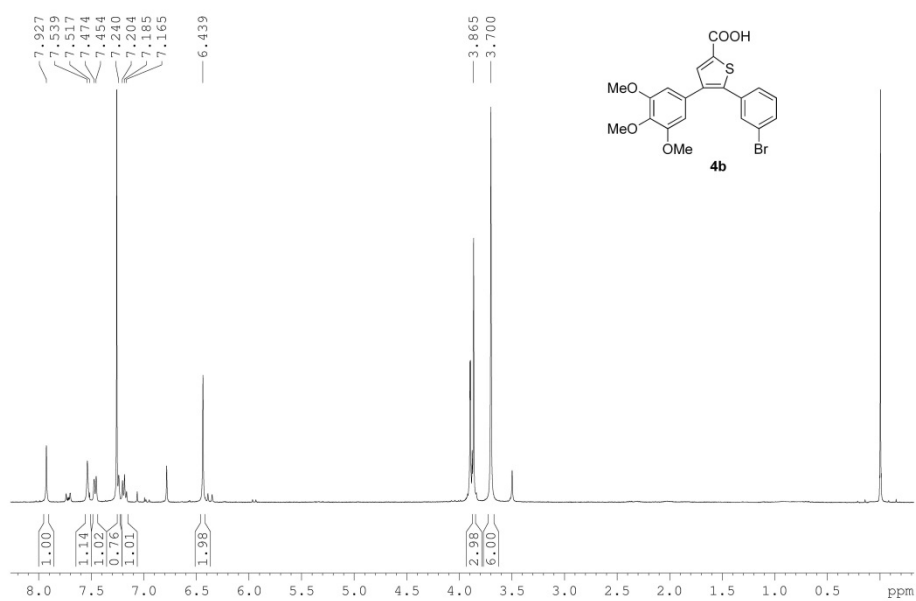


4b:

MS

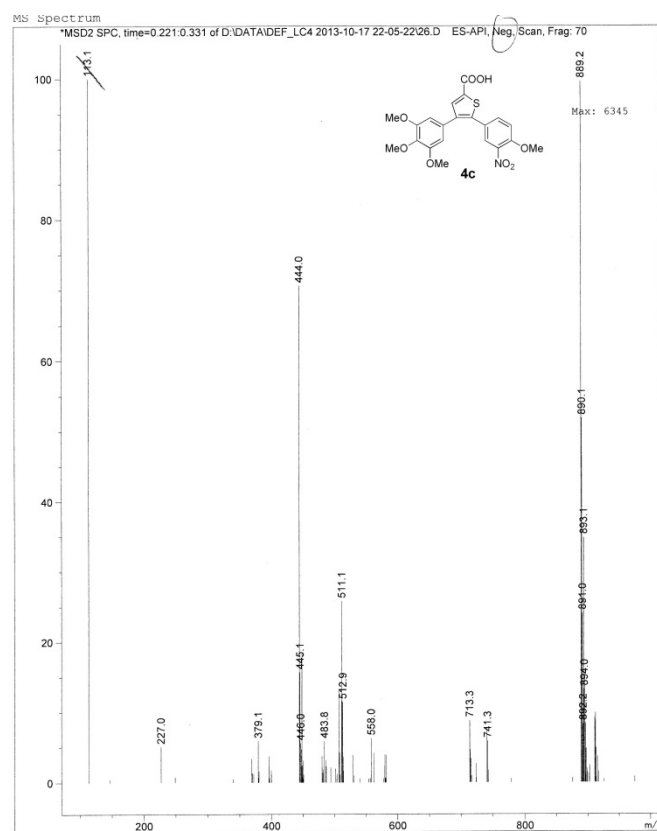


¹H NMR

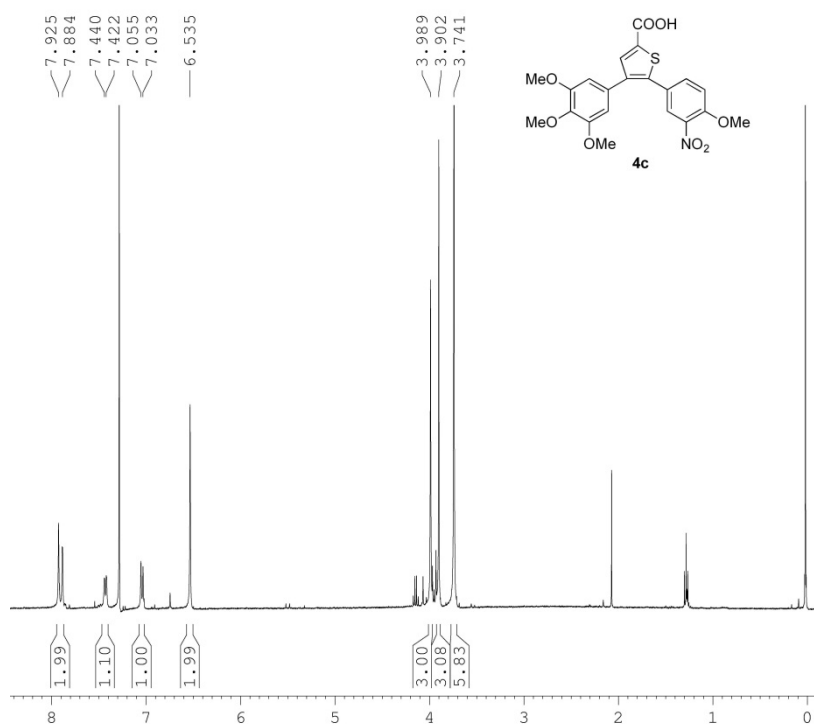


4c:

MS

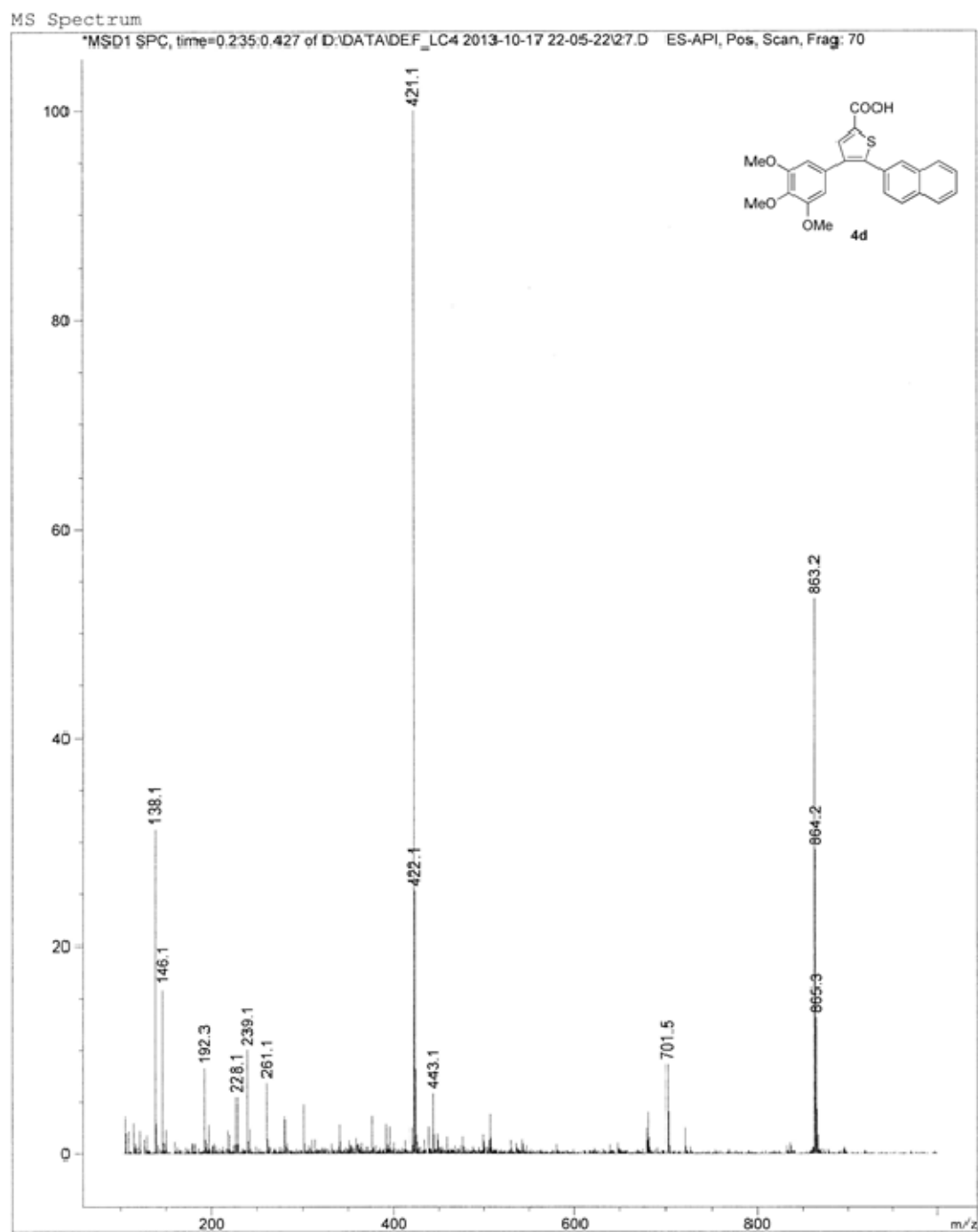


¹H NMR

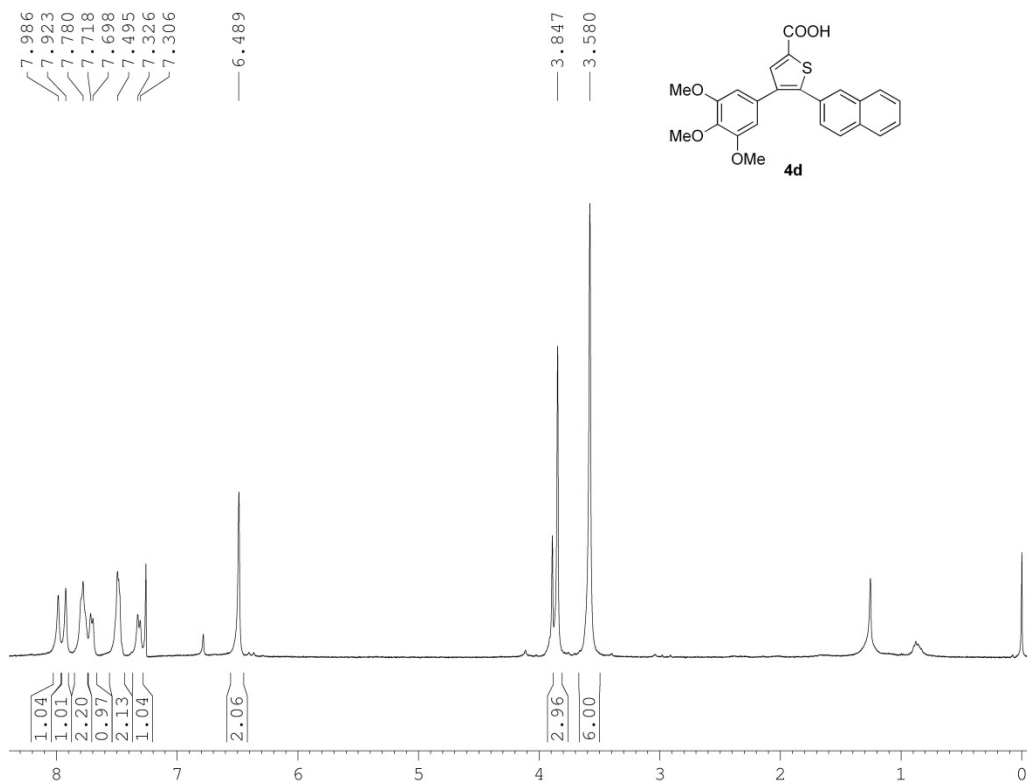


4d:

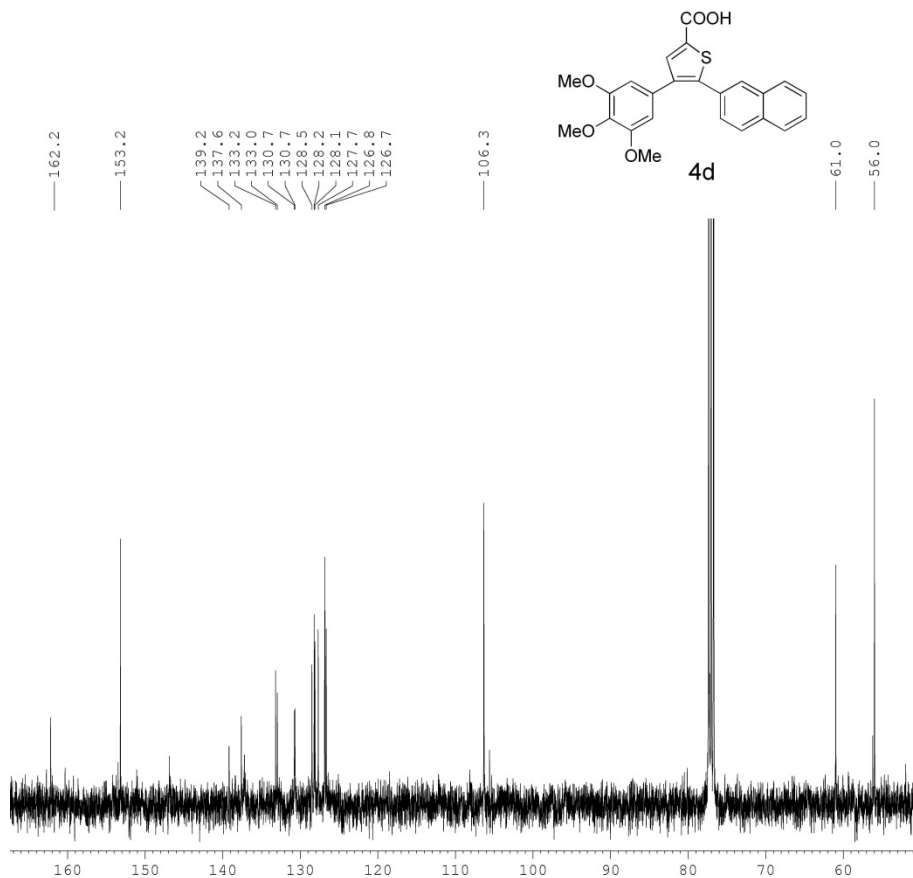
MS



^1H NMR

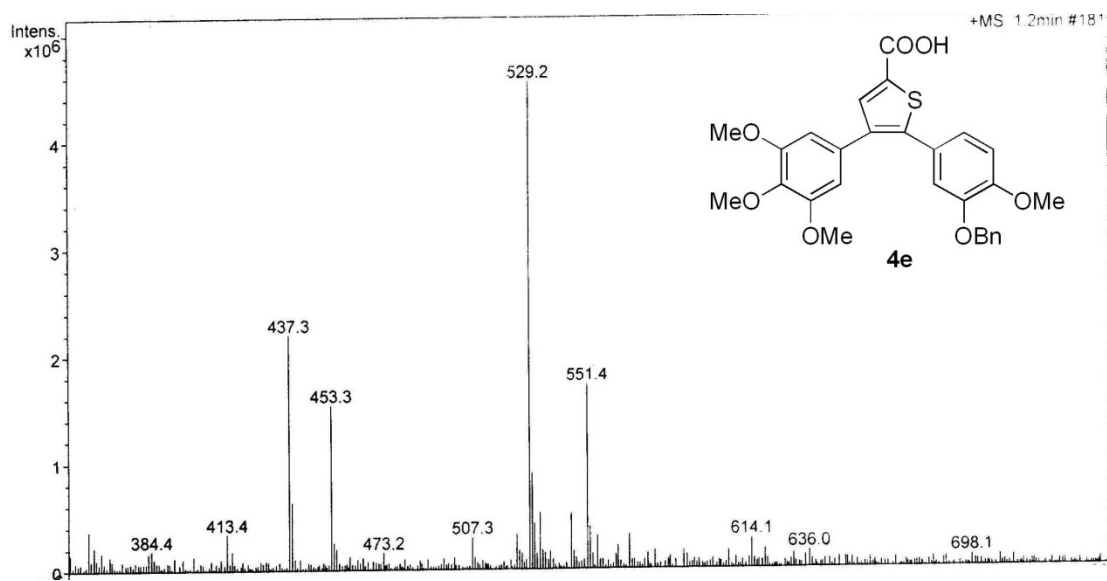


^{13}C NMR

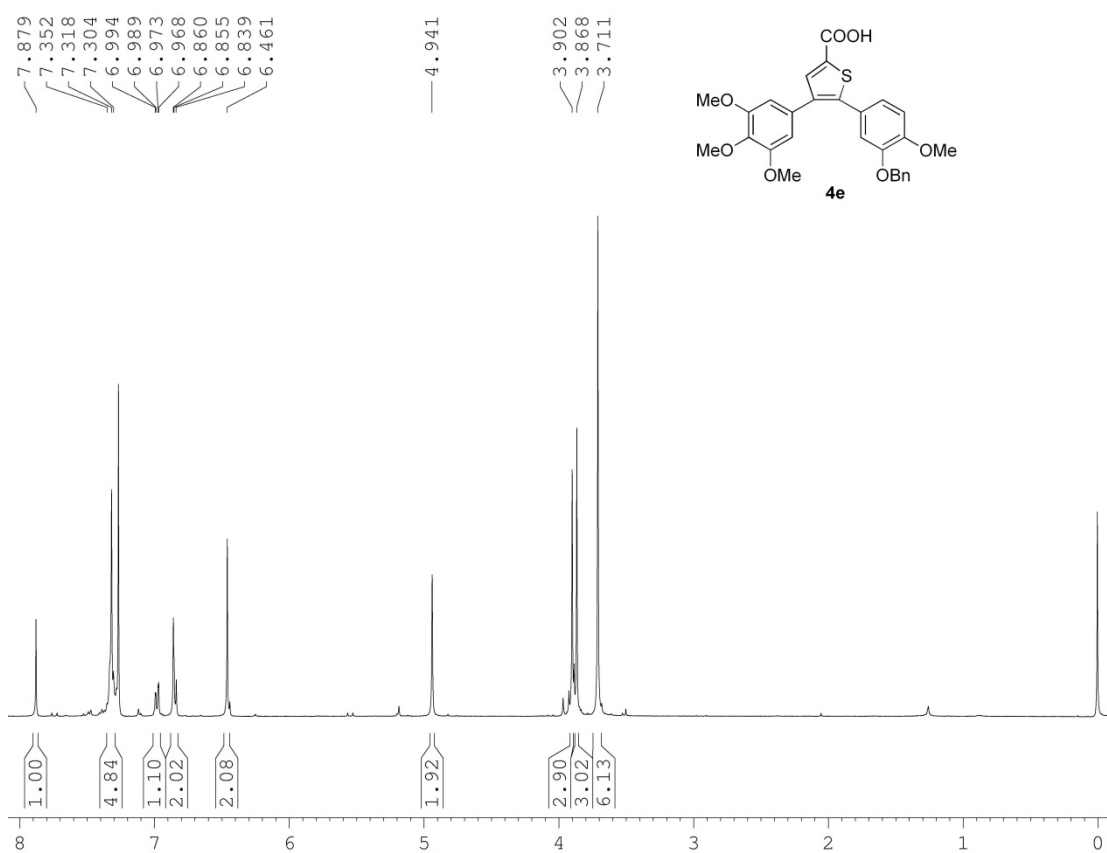


4e:

MS



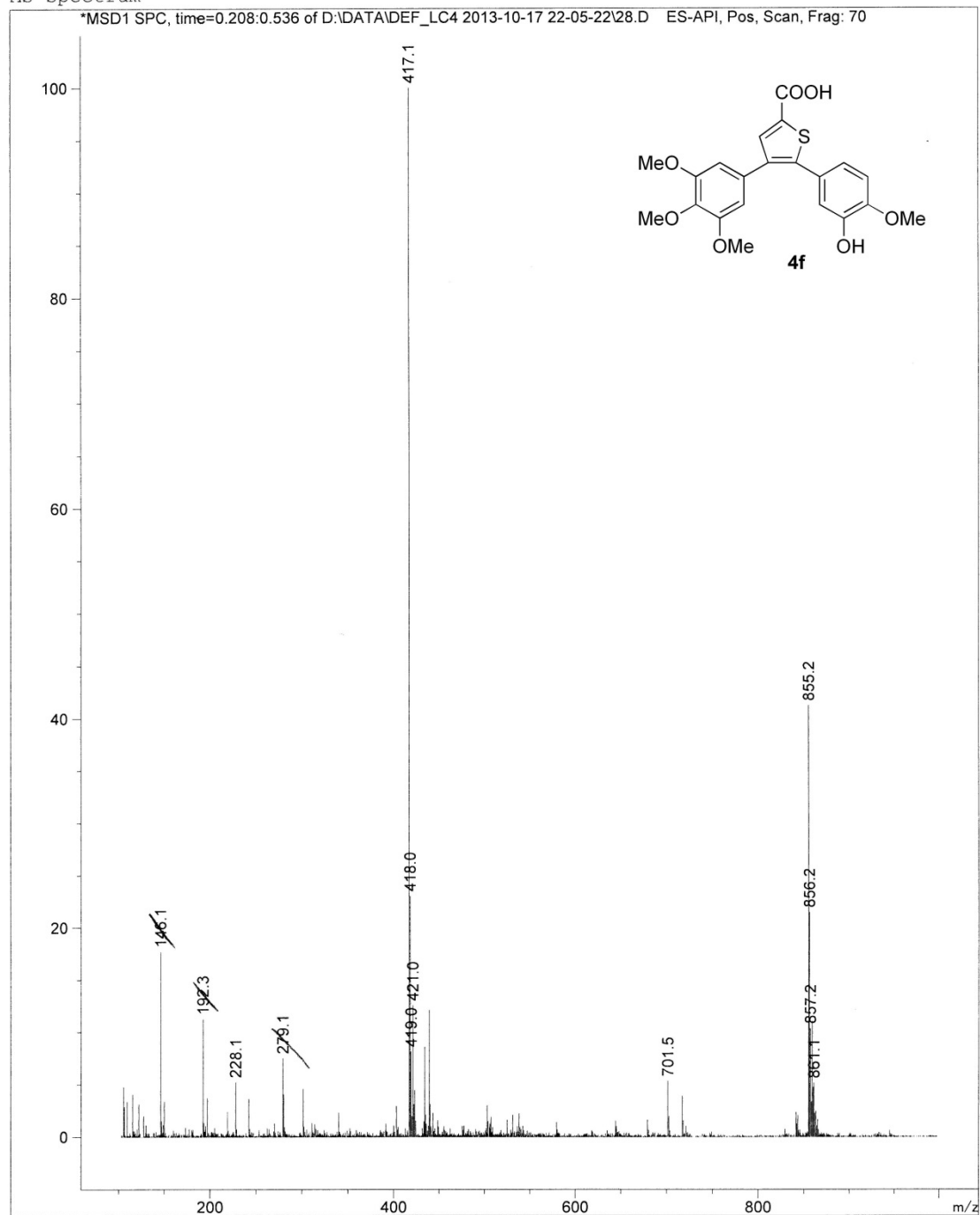
¹H NMR



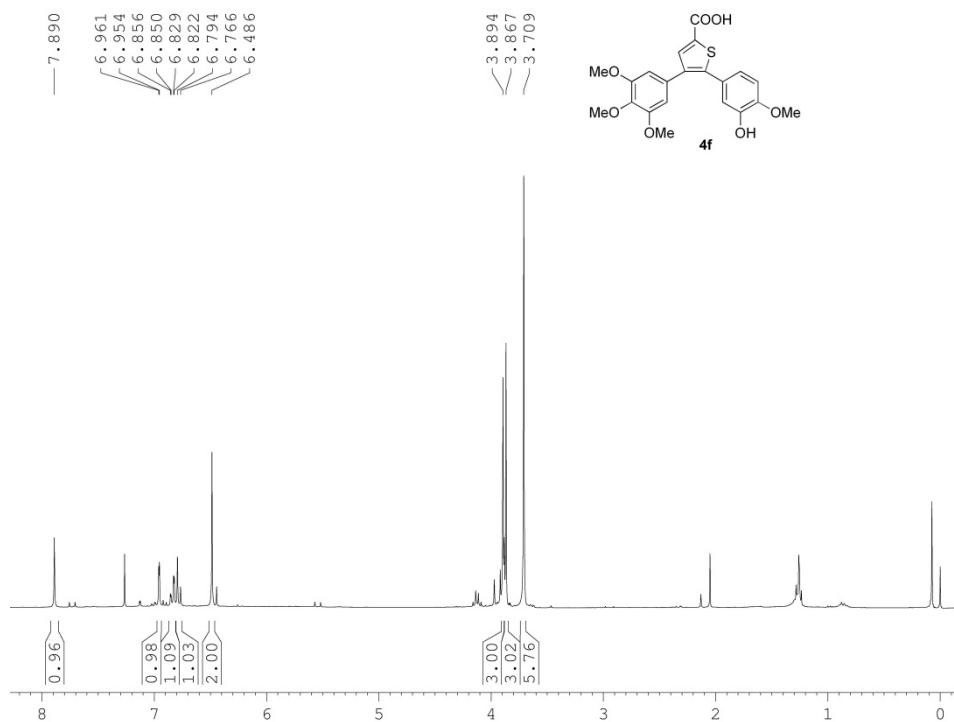
4f:

MS

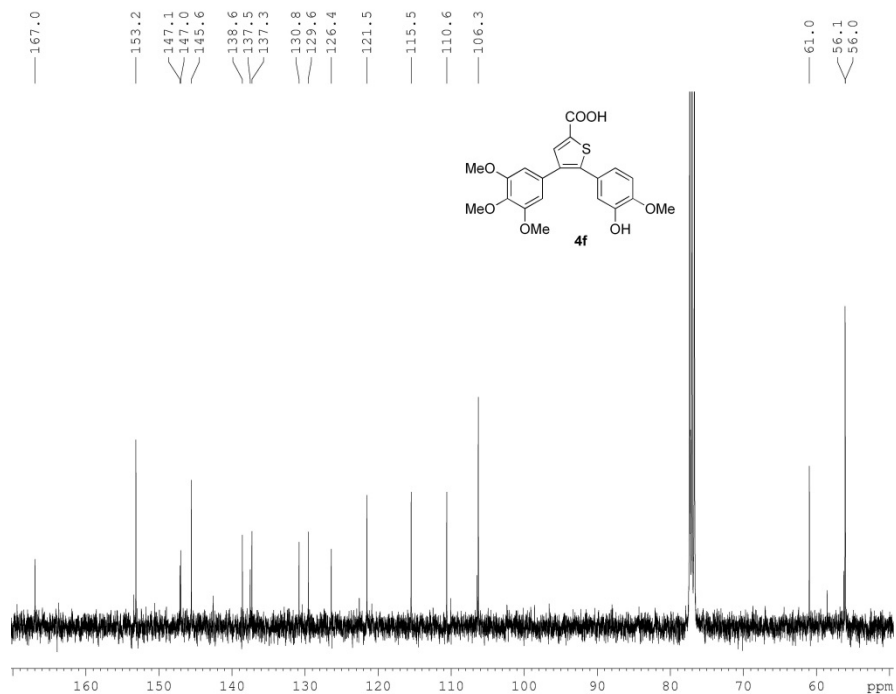
MS Spectrum



^1H NMR



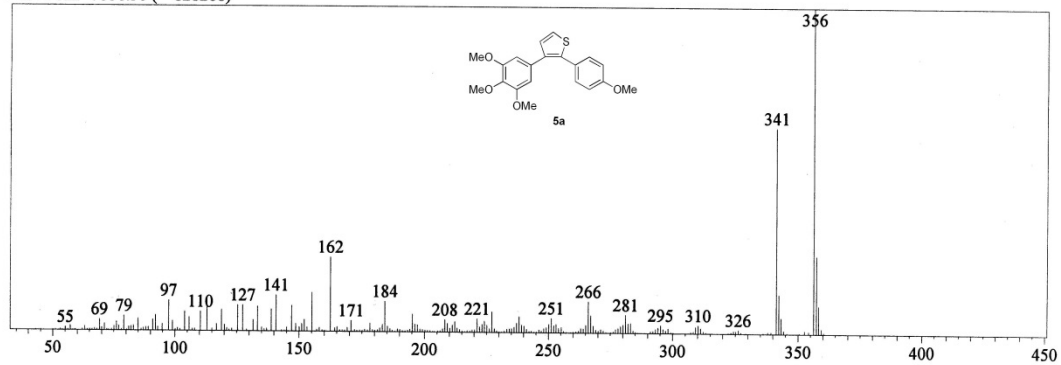
^{13}C NMR



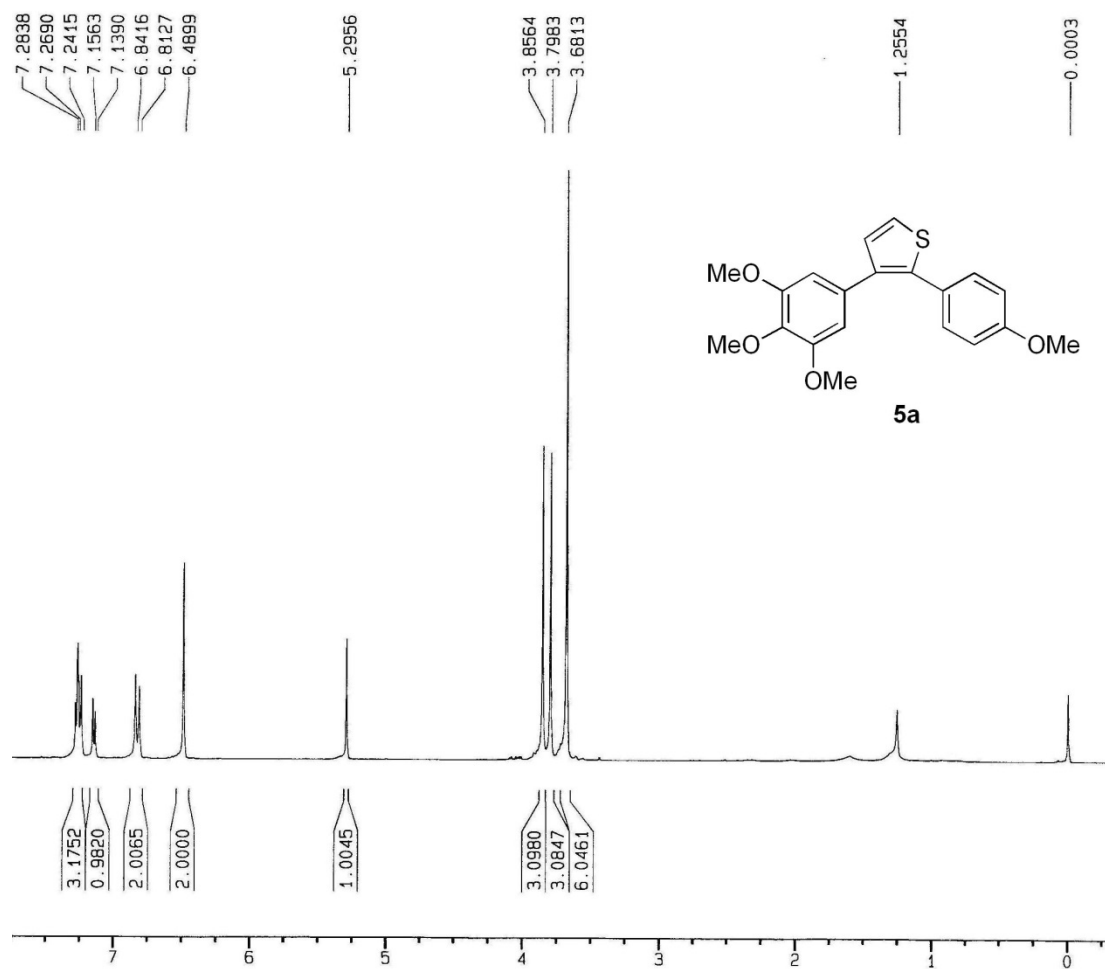
5a:

MS

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Base Peak : 356.30 (828268)



¹H NMR



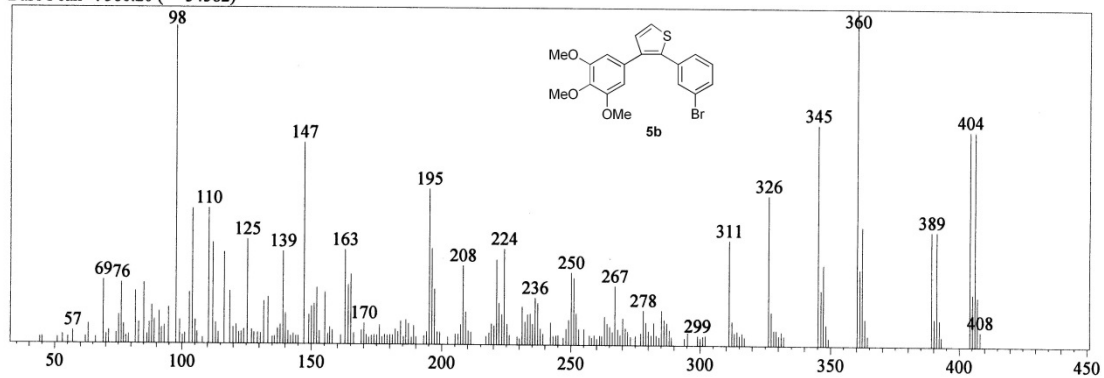
5b:

MS

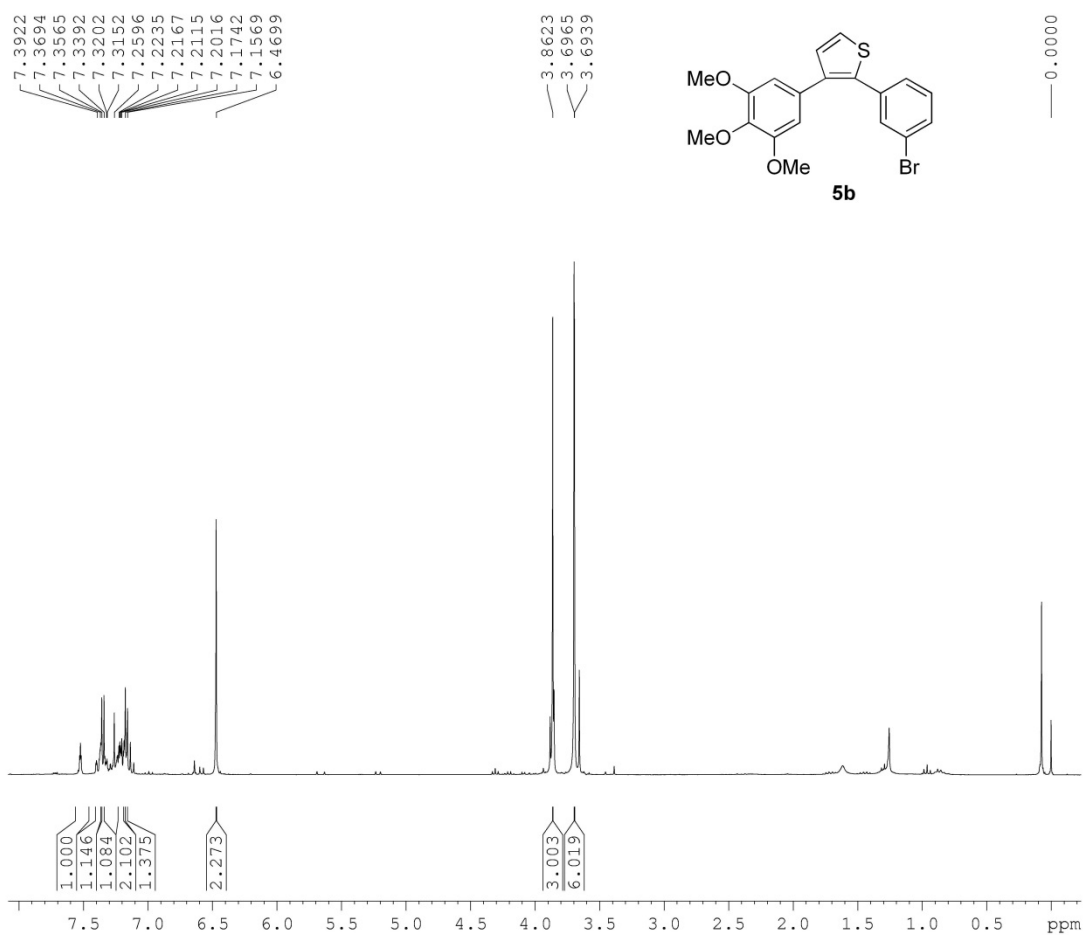
Scan # : 233

Mass Peak # : 223 Ret. Time : 2.133

Base Peak : 360.20 (54582)



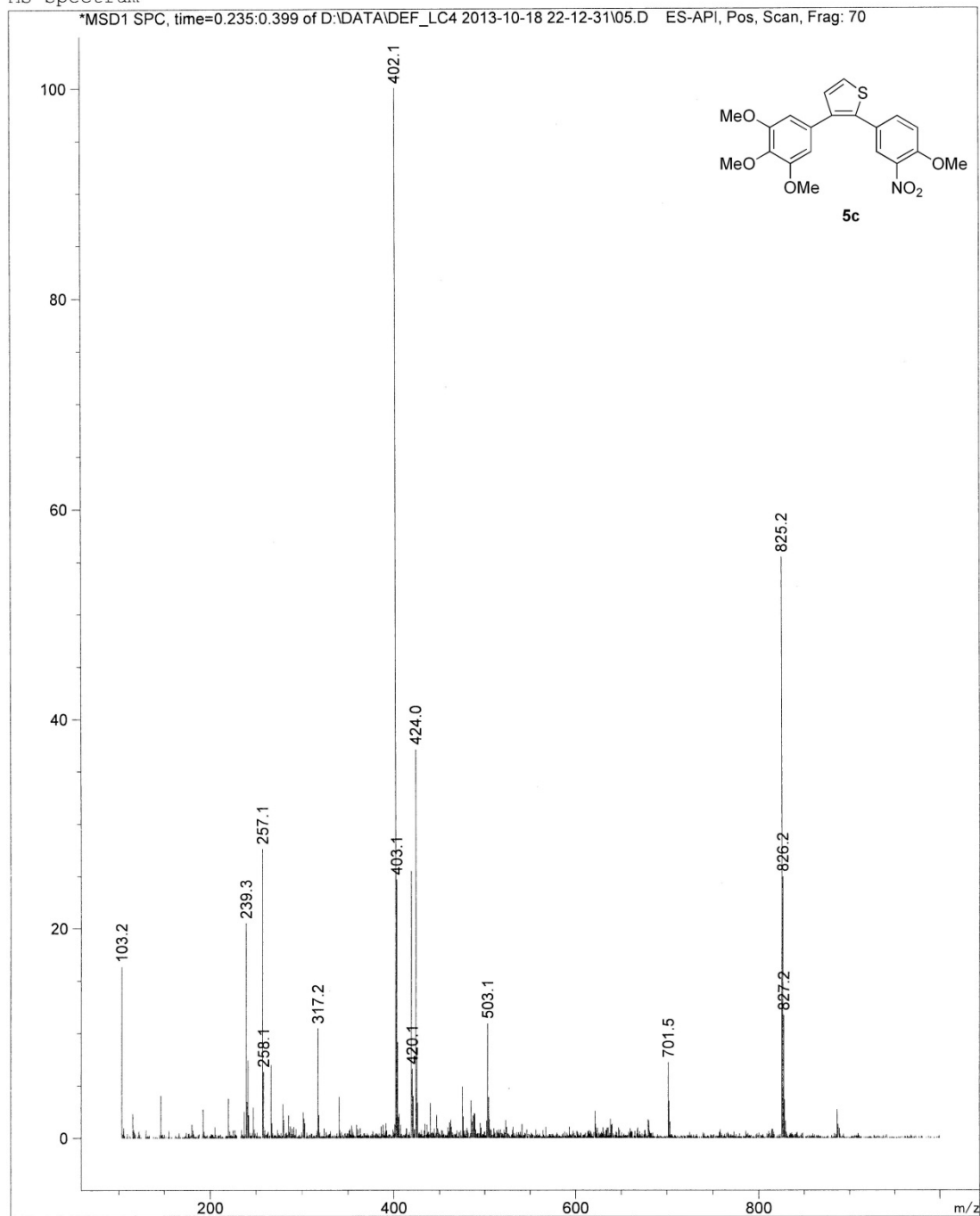
^1H NMR



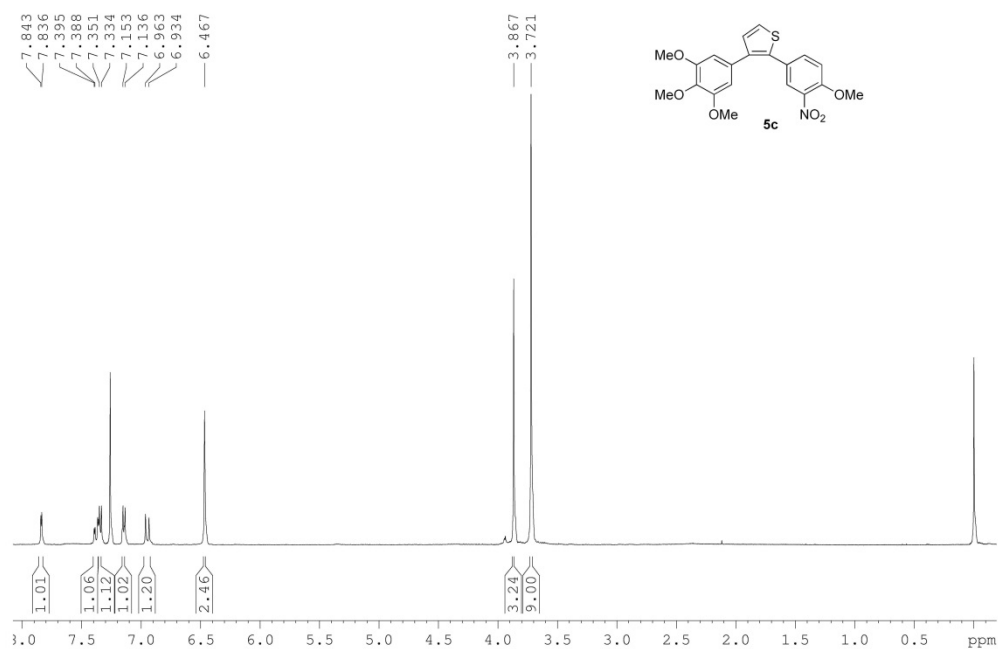
5c:

MS

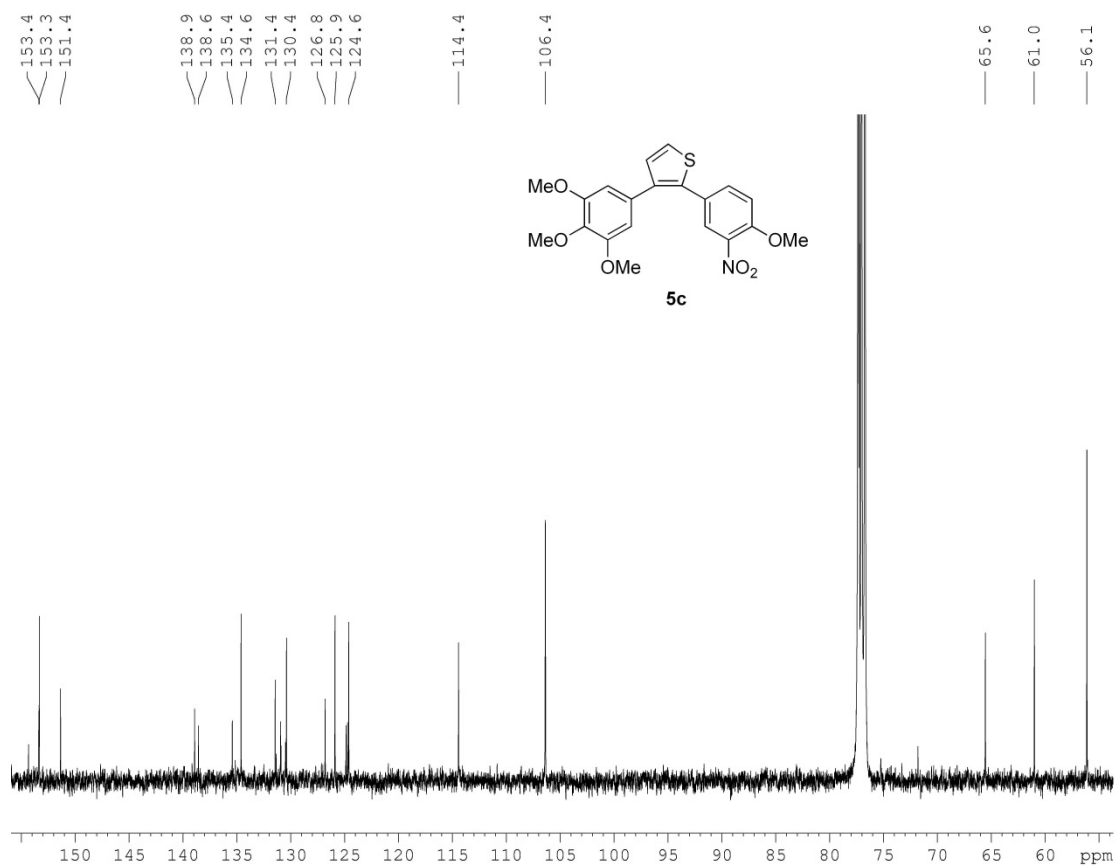
MS Spectrum



^1H NMR

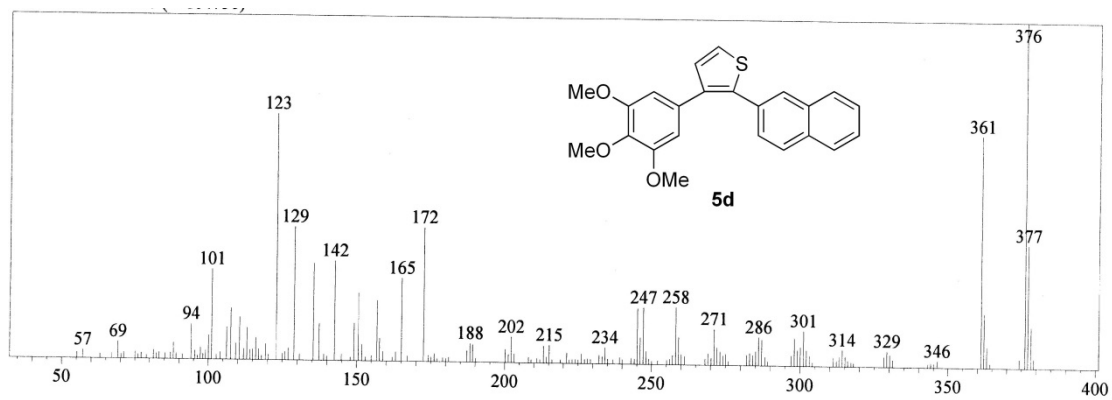


^{13}C NMR

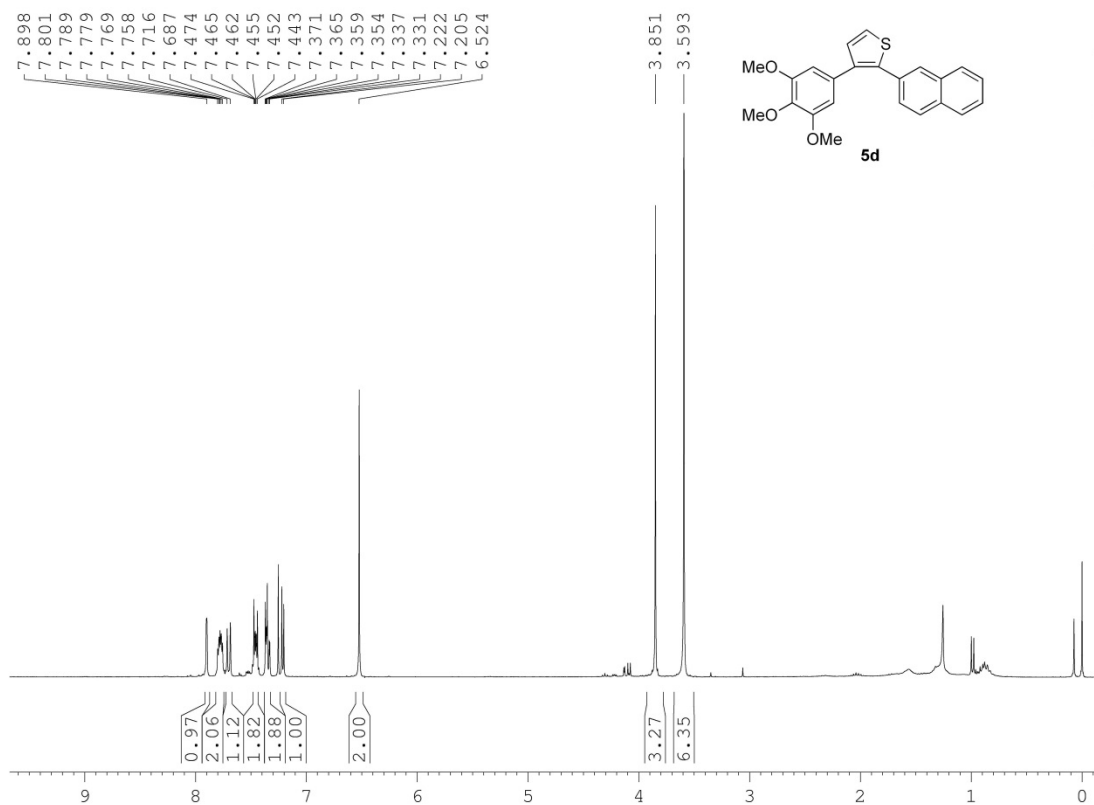


5d:

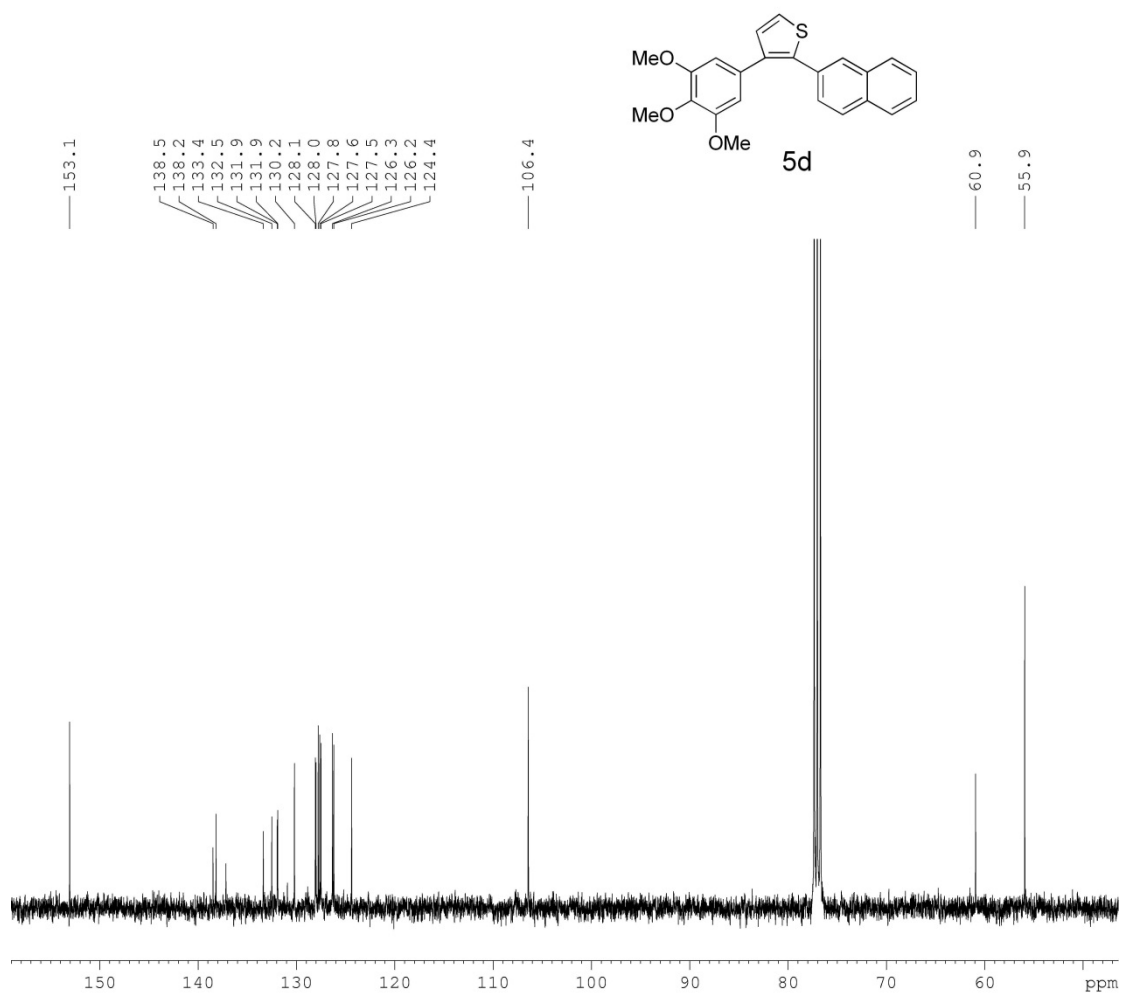
MS



¹H NMR

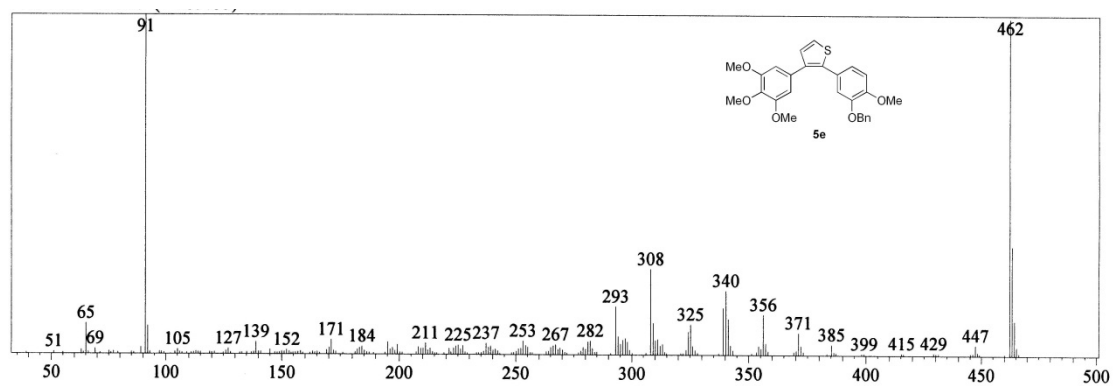


^{13}C NMR

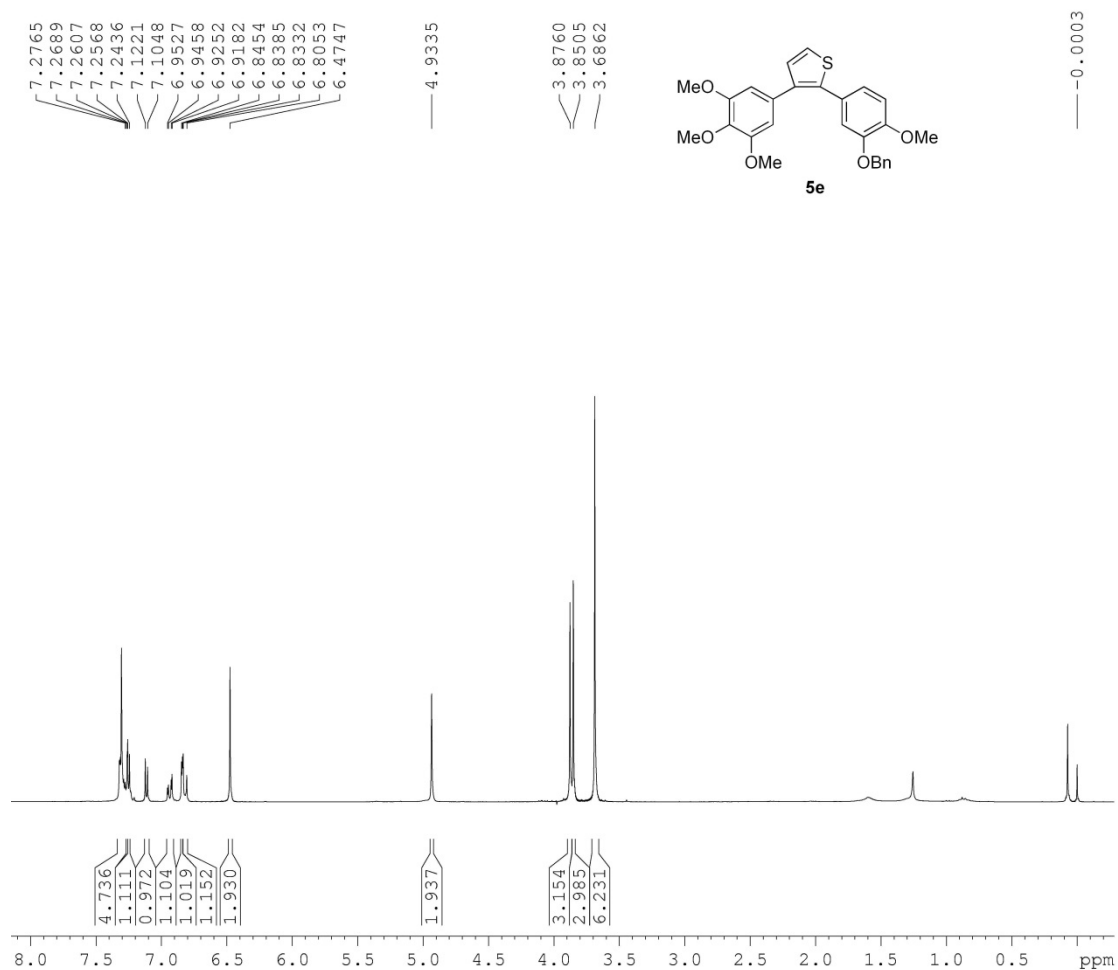


5e:

MS

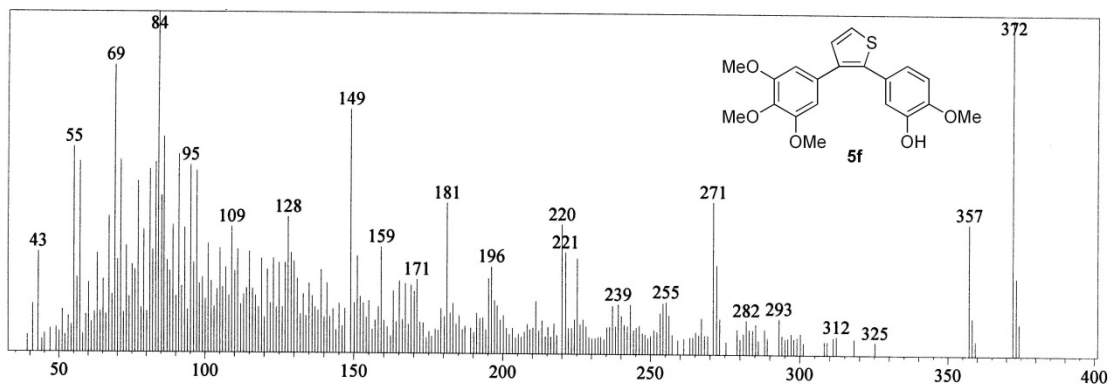


^1H NMR



5f:

MS



HRMS

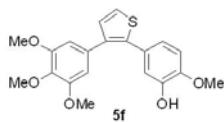
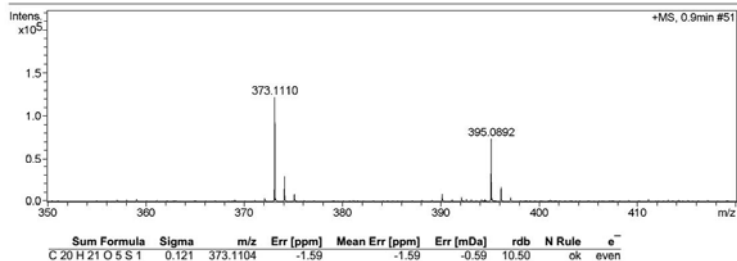
Mass Spectrum Molecular Formula Report

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Method	Idj_bga_jh.m	Instrument / Ser#	micrOTOF-Q 125
Sample Name	SJZQZ080320		
Comment			

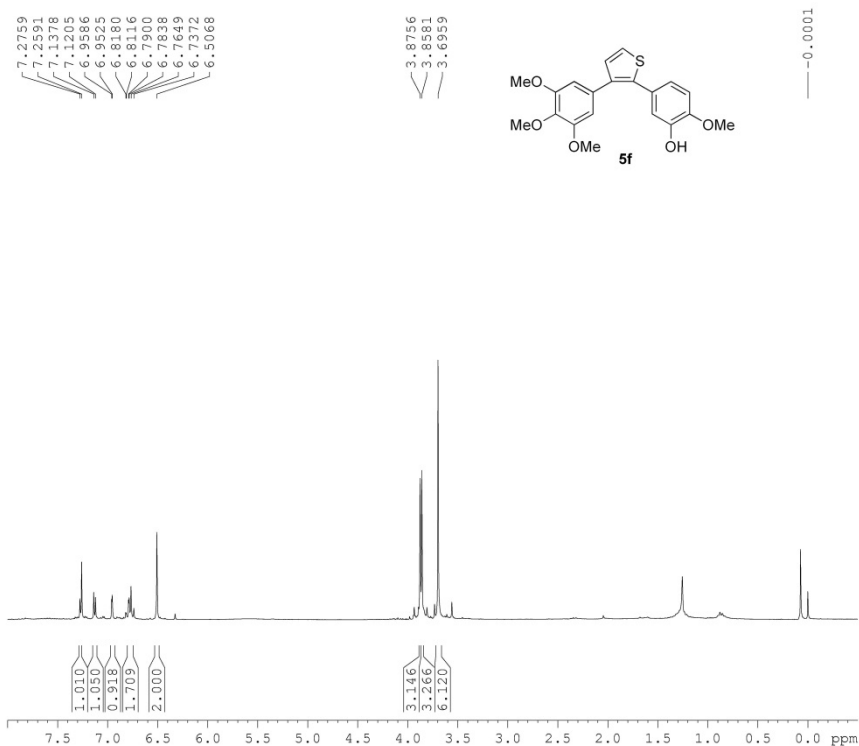
Acquisition Parameter			
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Scan End	1000 m/z	Set Collision Cell RF	250.0 Vpp
		Set Nebulizer	0.3 Bar
		Set Dry Heater	180 °C
		Set Dry Gas	4.0 l/min
		Set Divert Valve	Source

Generate Molecular Formula Parameter

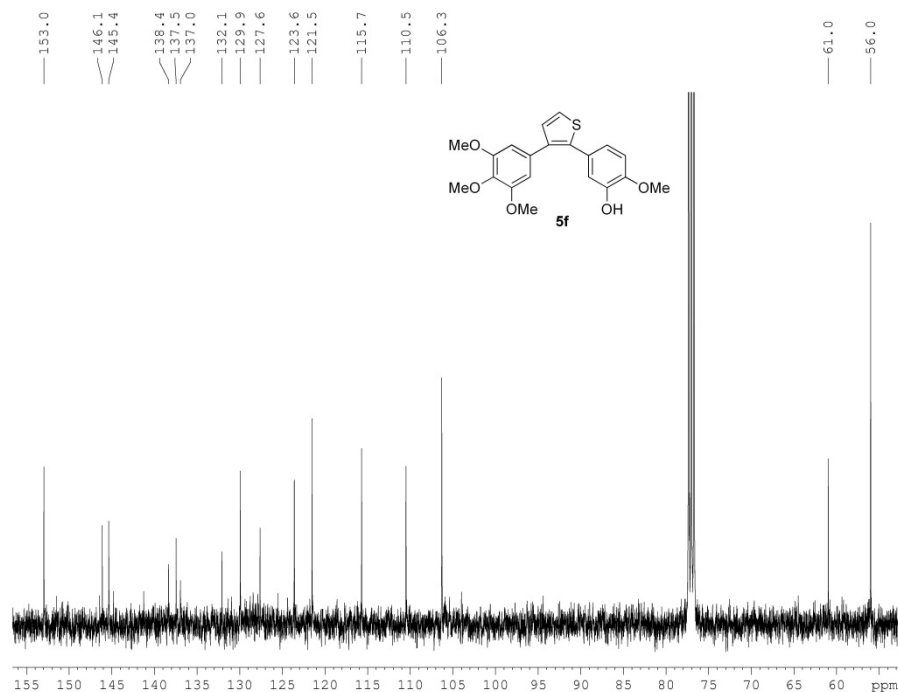
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Formula, max.			
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^1H NMR

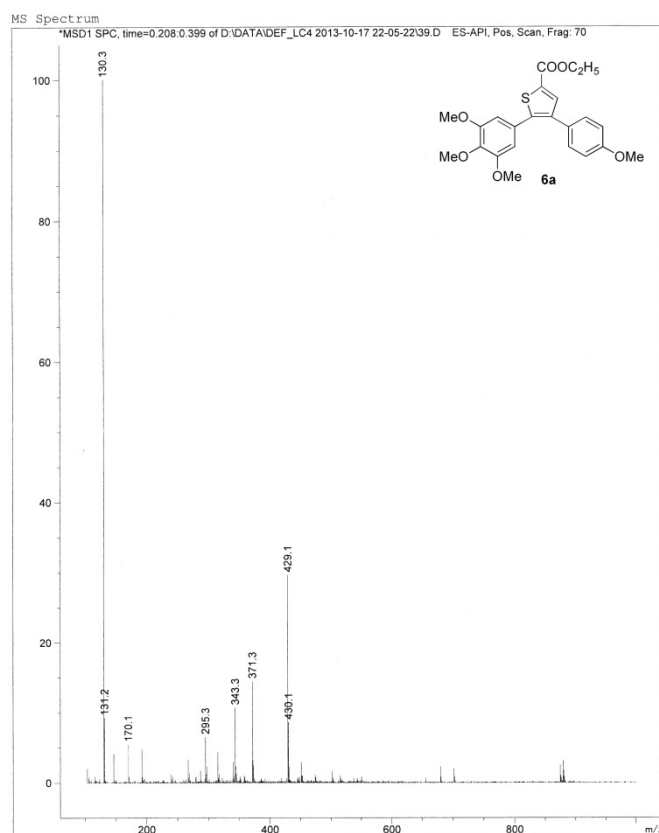


^{13}C NMR

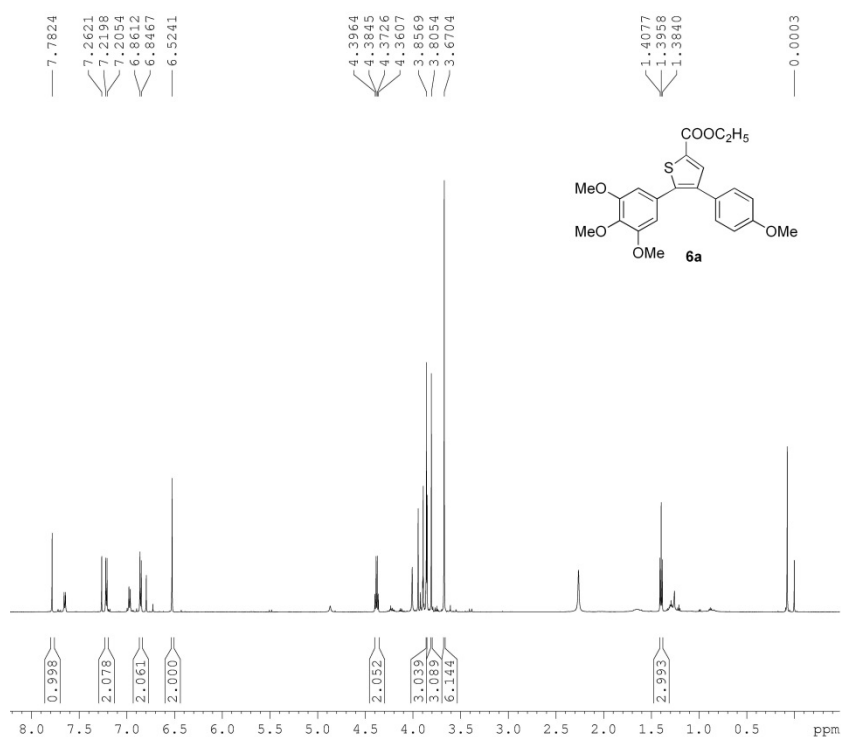


6a:

MS

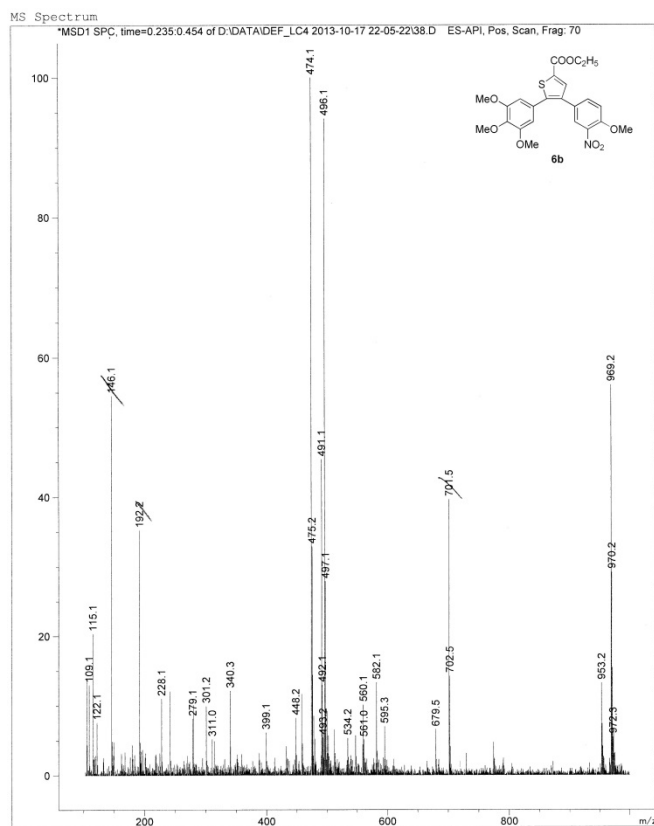


¹H NMR

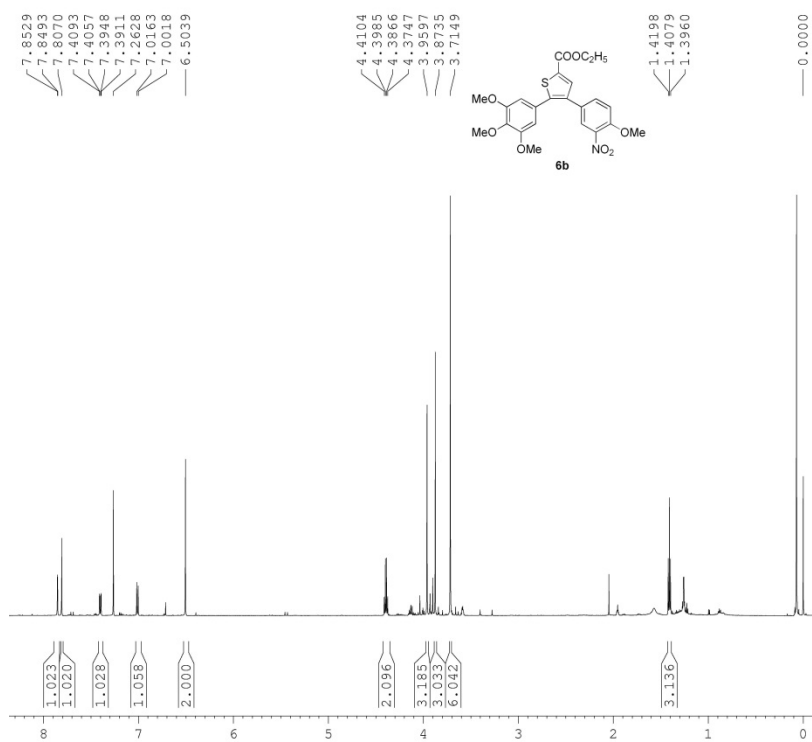


6b:

MS

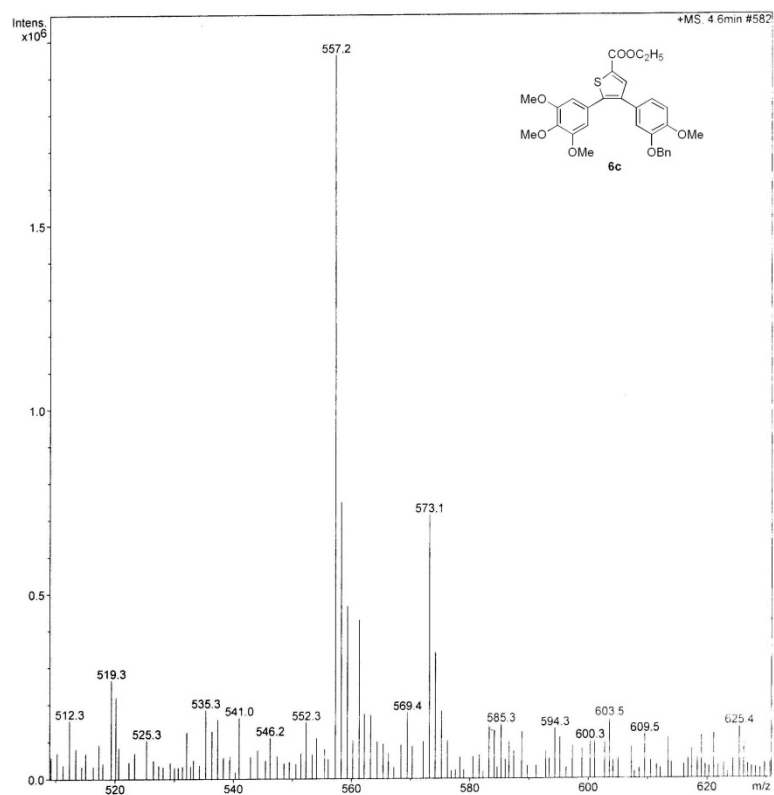


¹H NMR

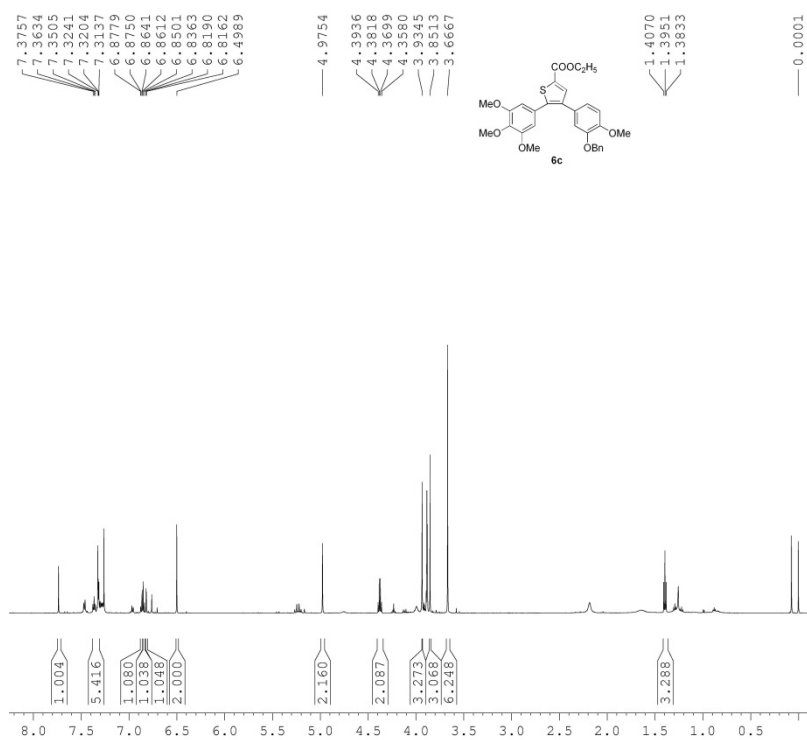


6c:

MS



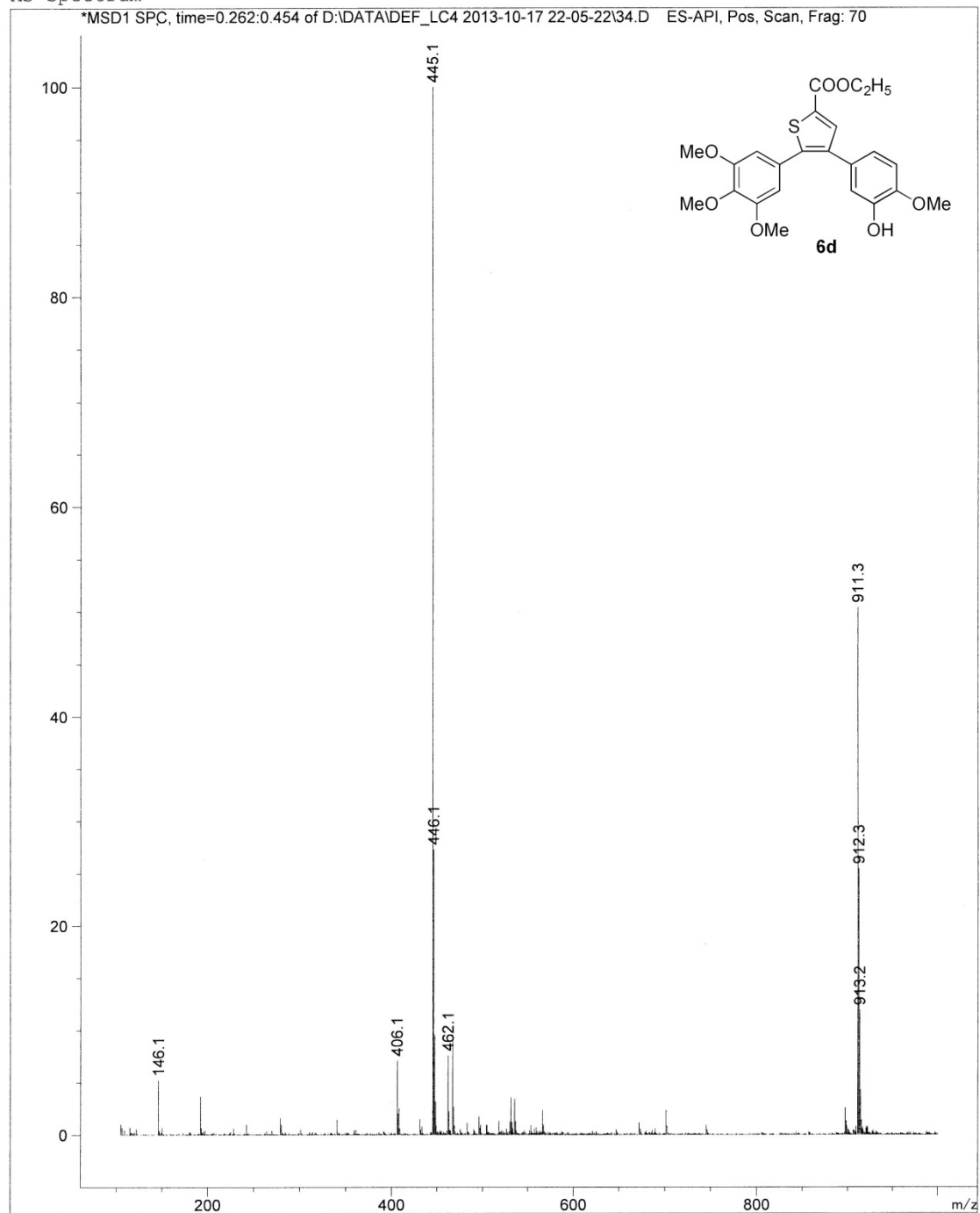
^1H NMR



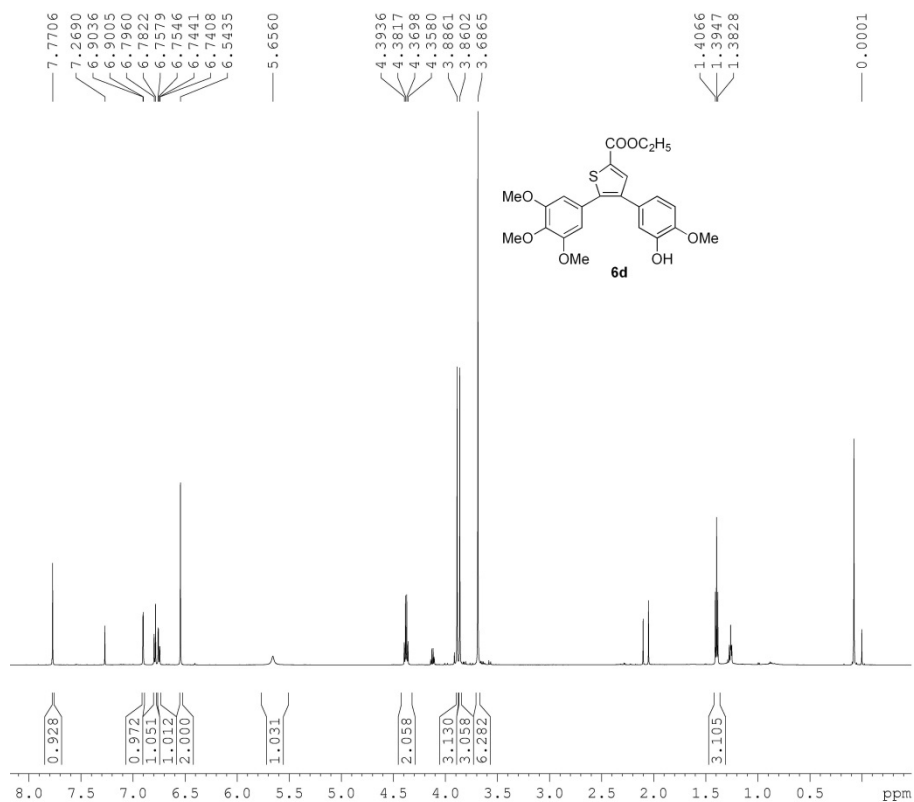
6d:

MS

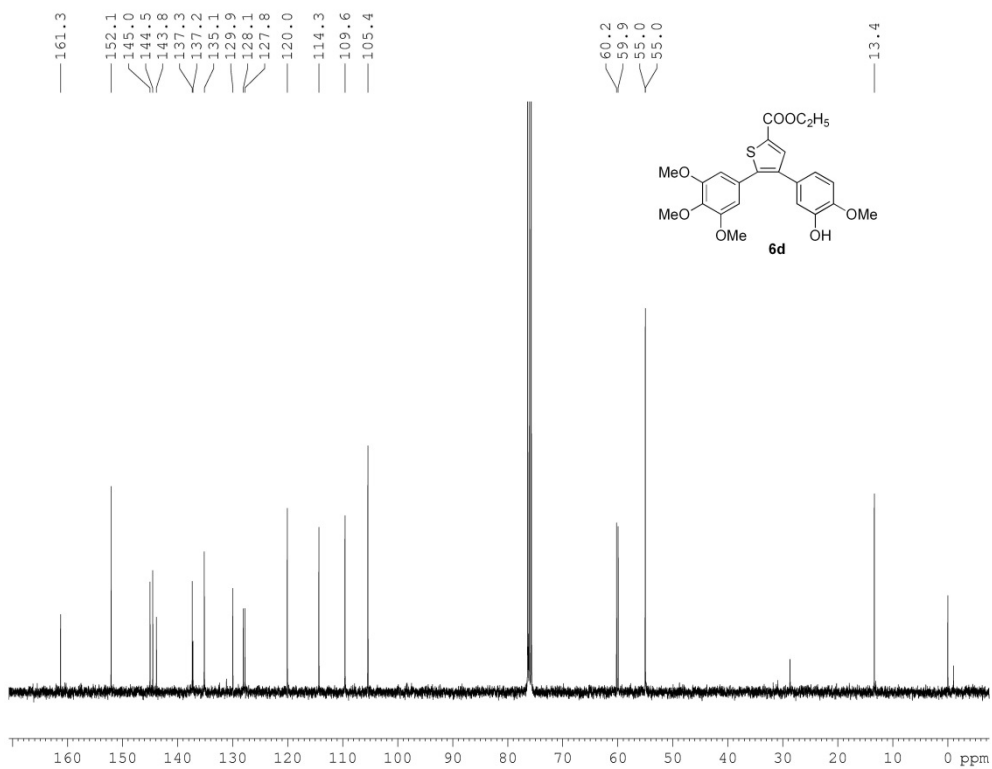
MS Spectrum



¹H NMR



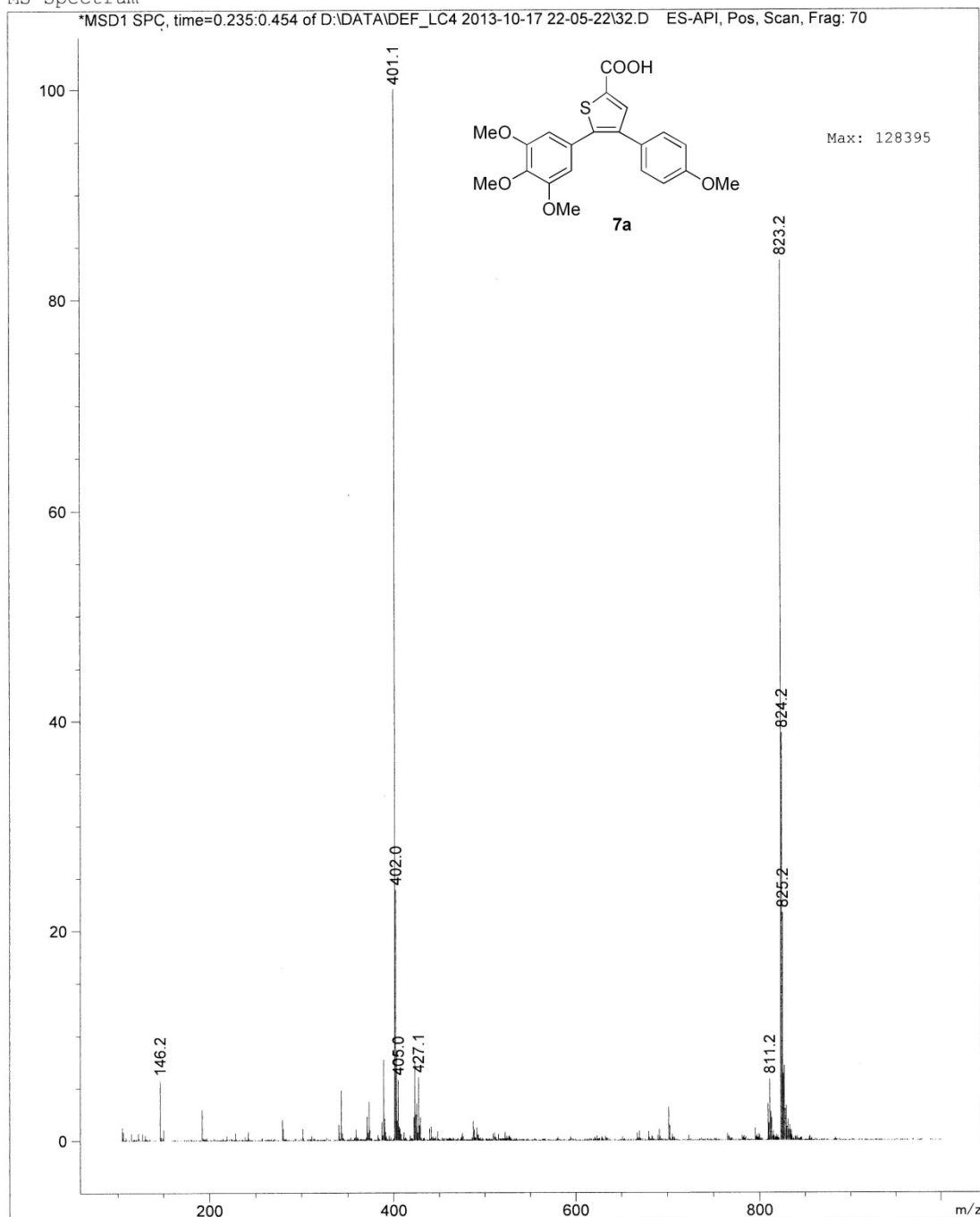
¹³C NMR



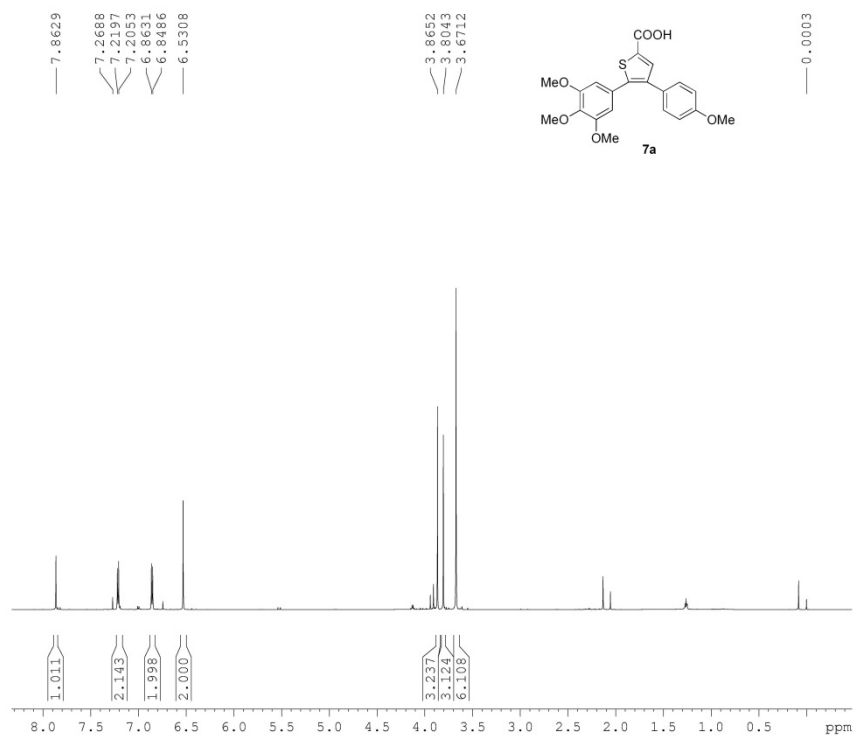
7a:

MS

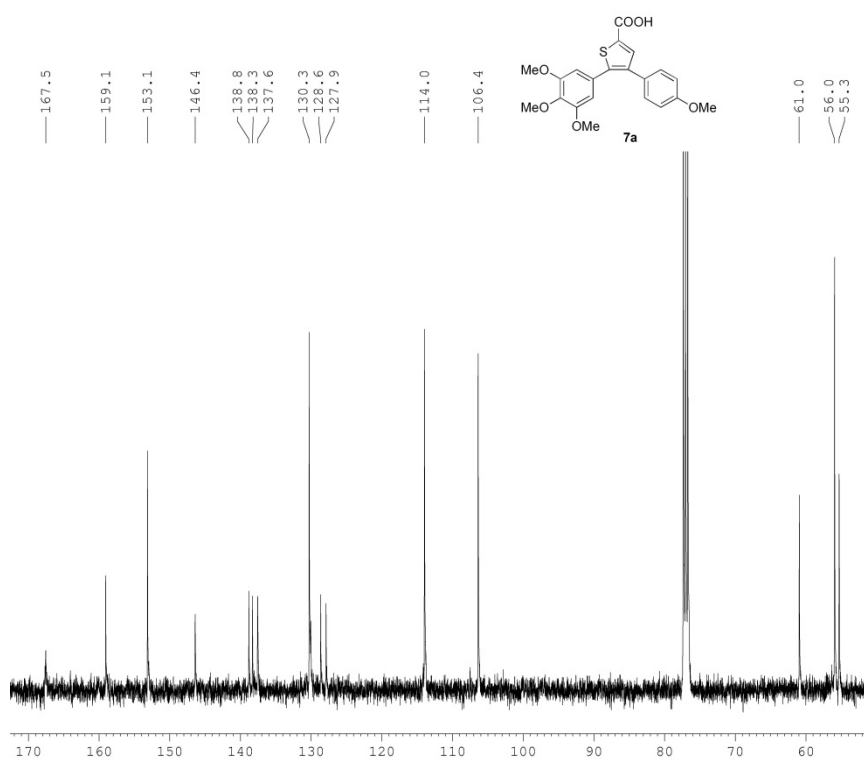
MS Spectrum



^1H NMR

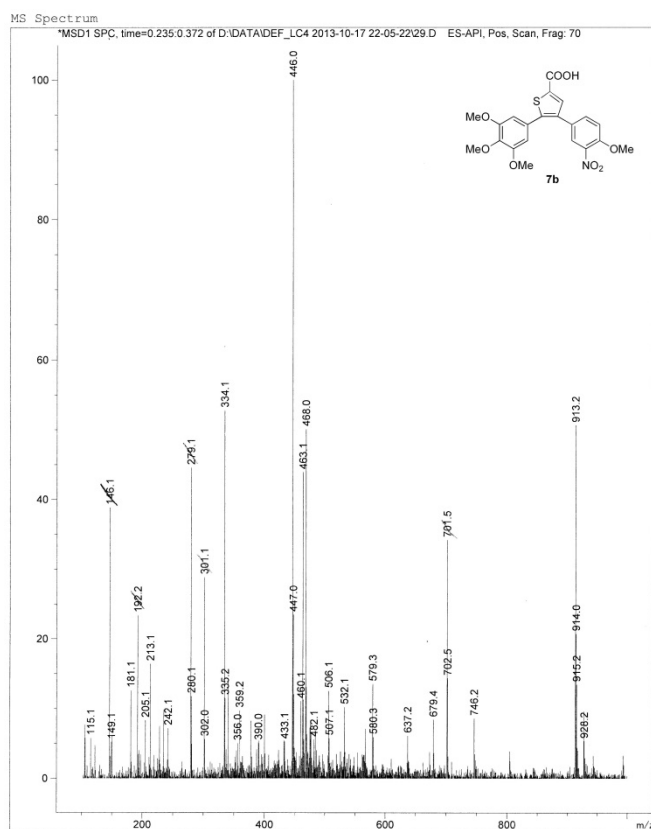


^{13}C NMR

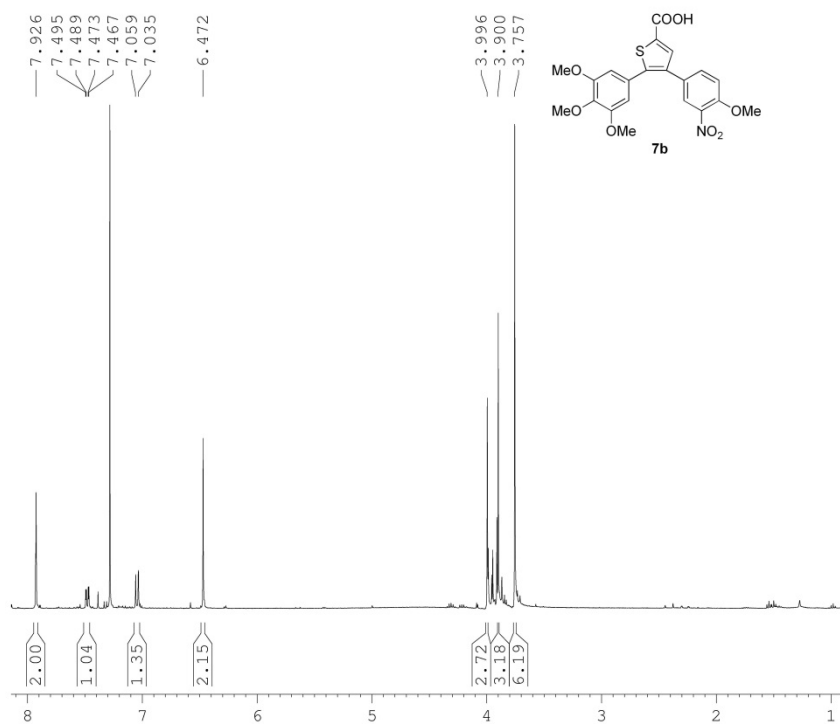


7b:

MS

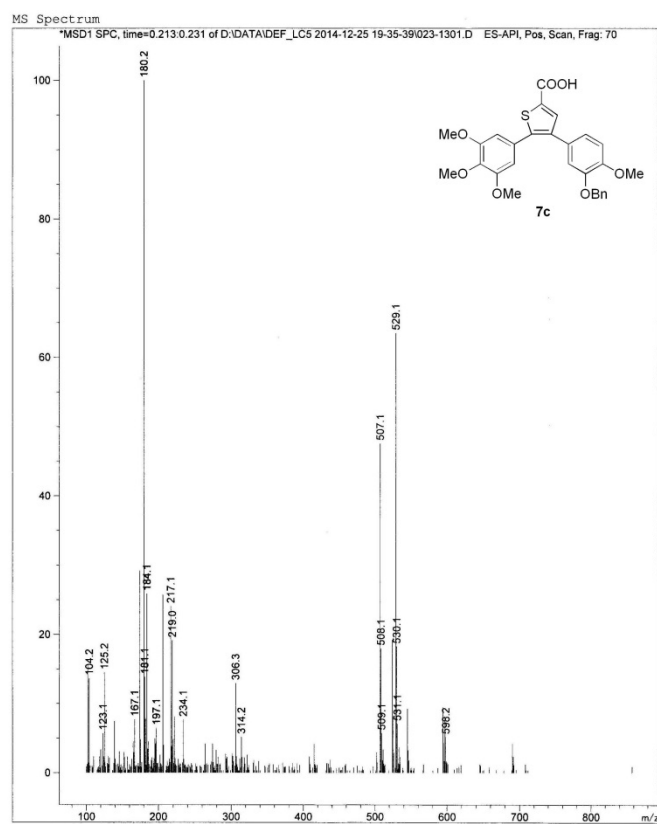


^1H NMR

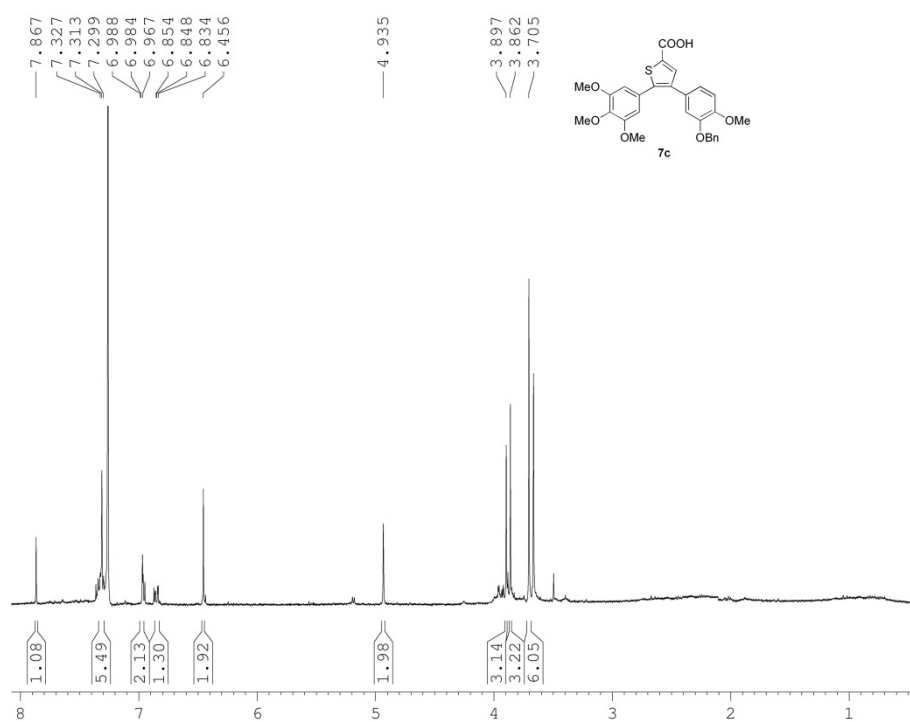


7c:

MS



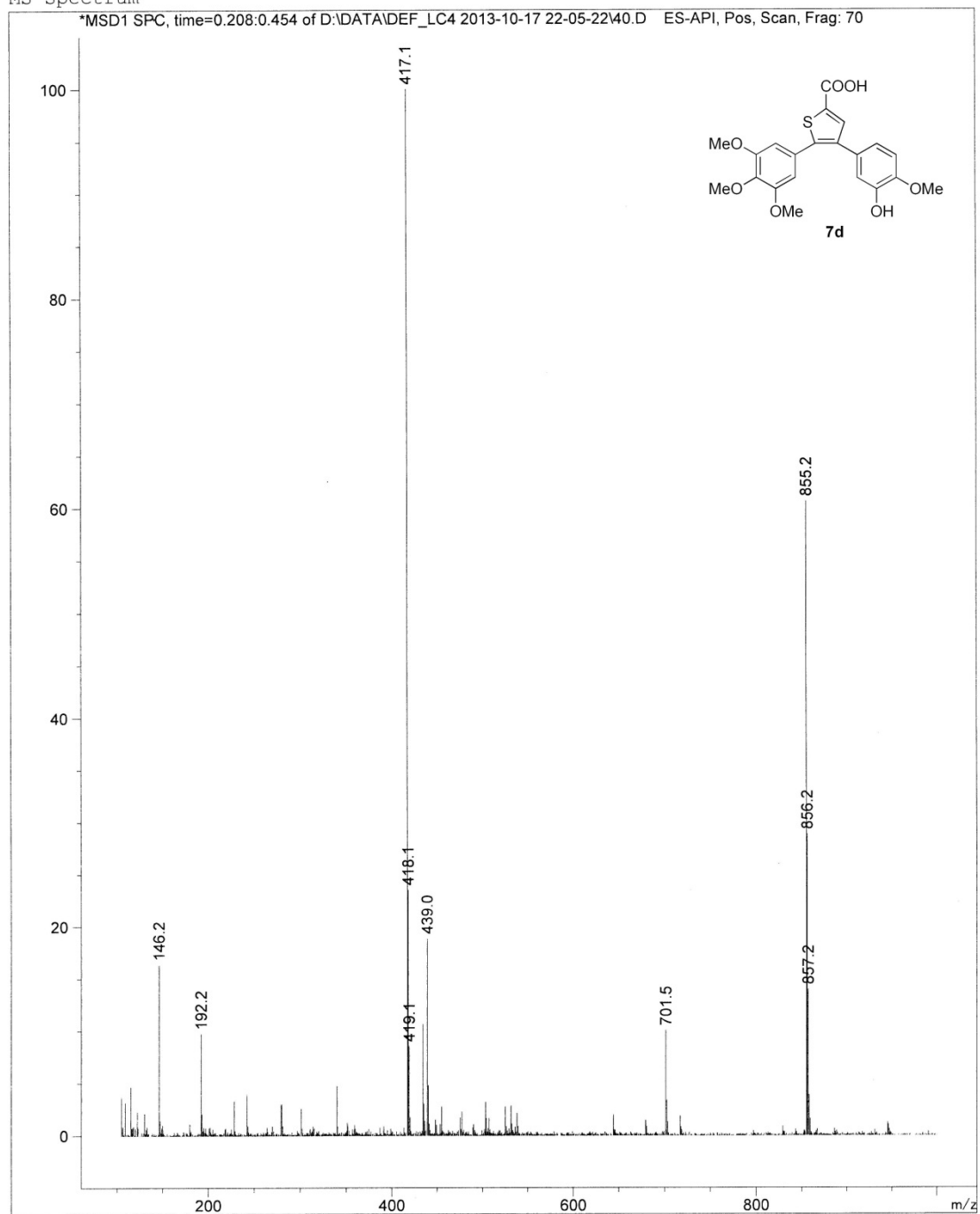
¹H NMR



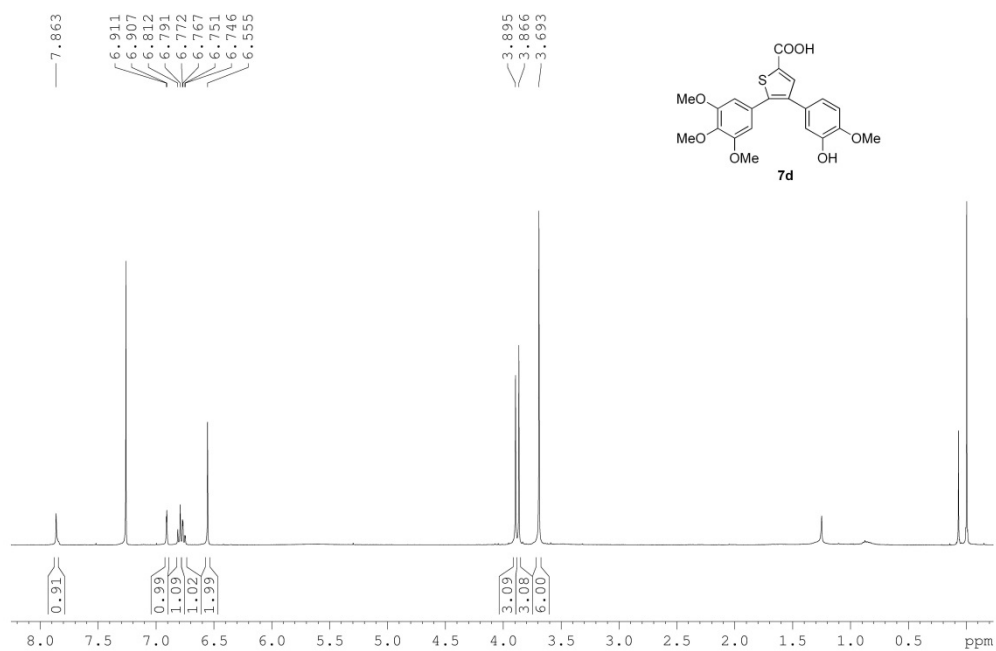
7d:

MS

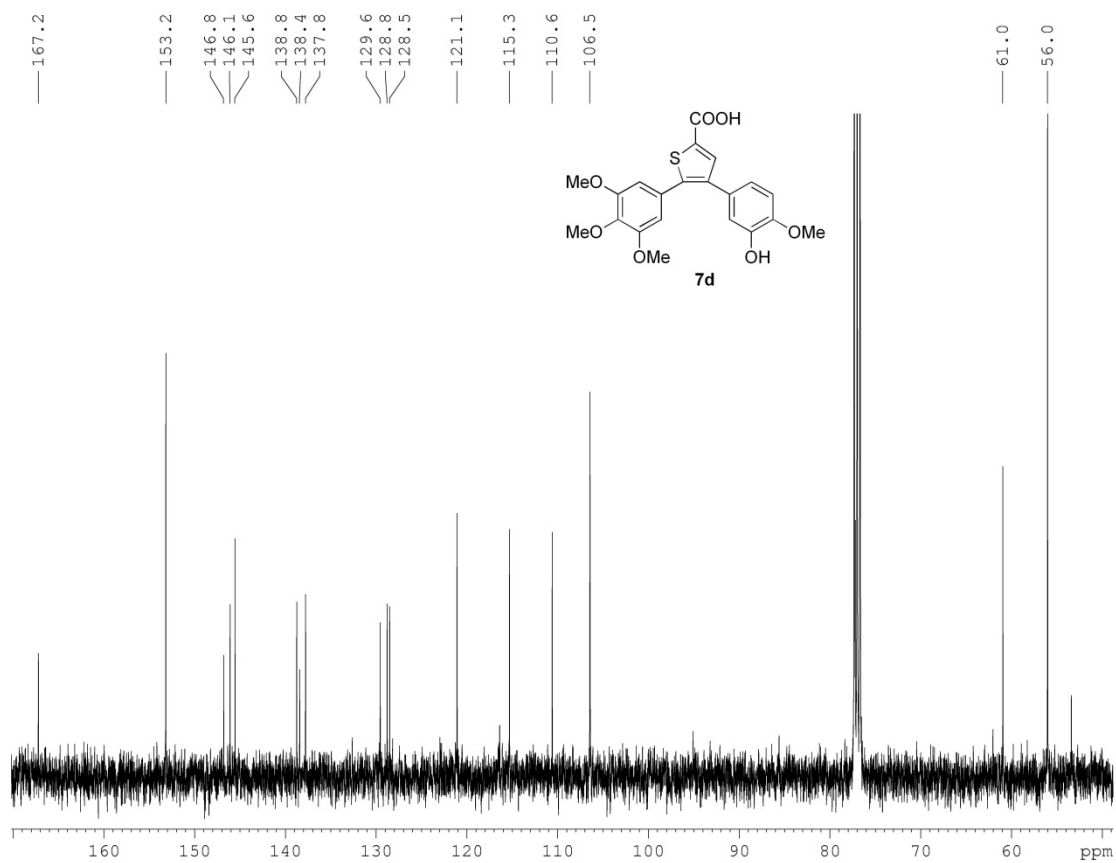
MS Spectrum



^1H NMR

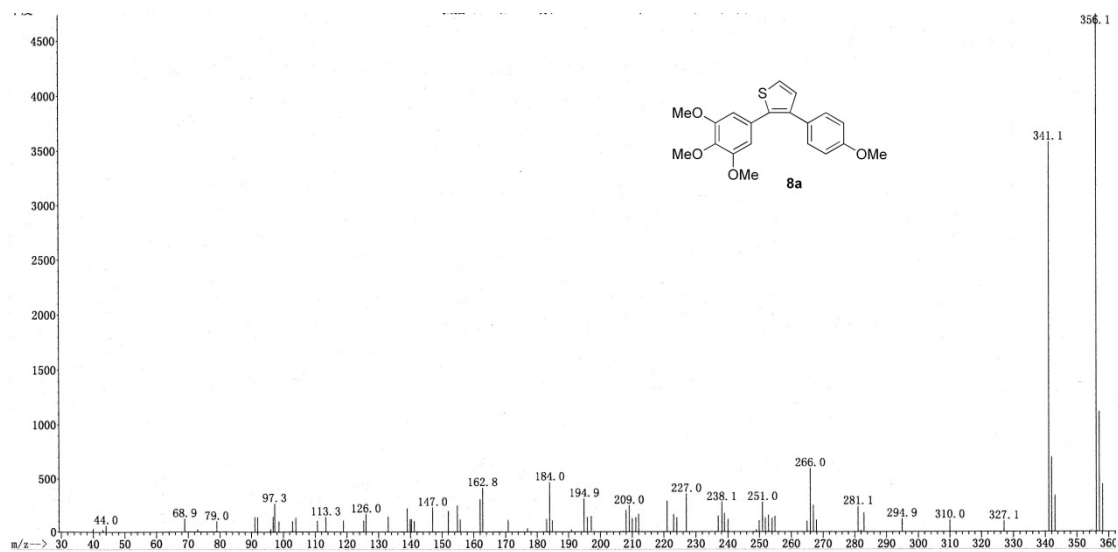


^{13}C NMR

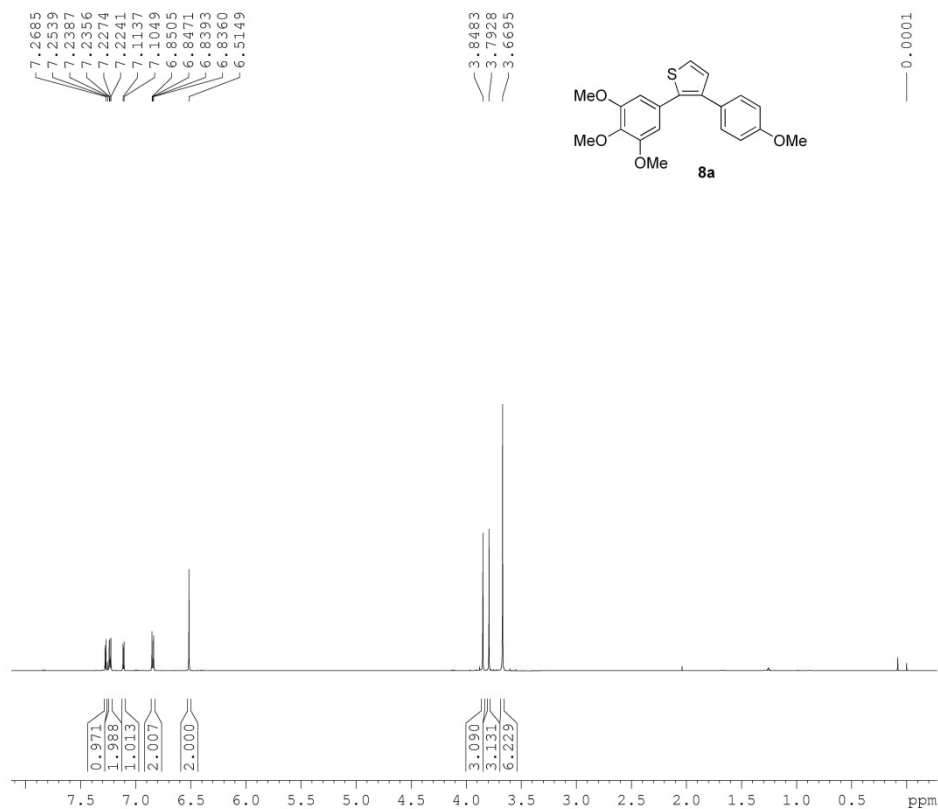


8a:

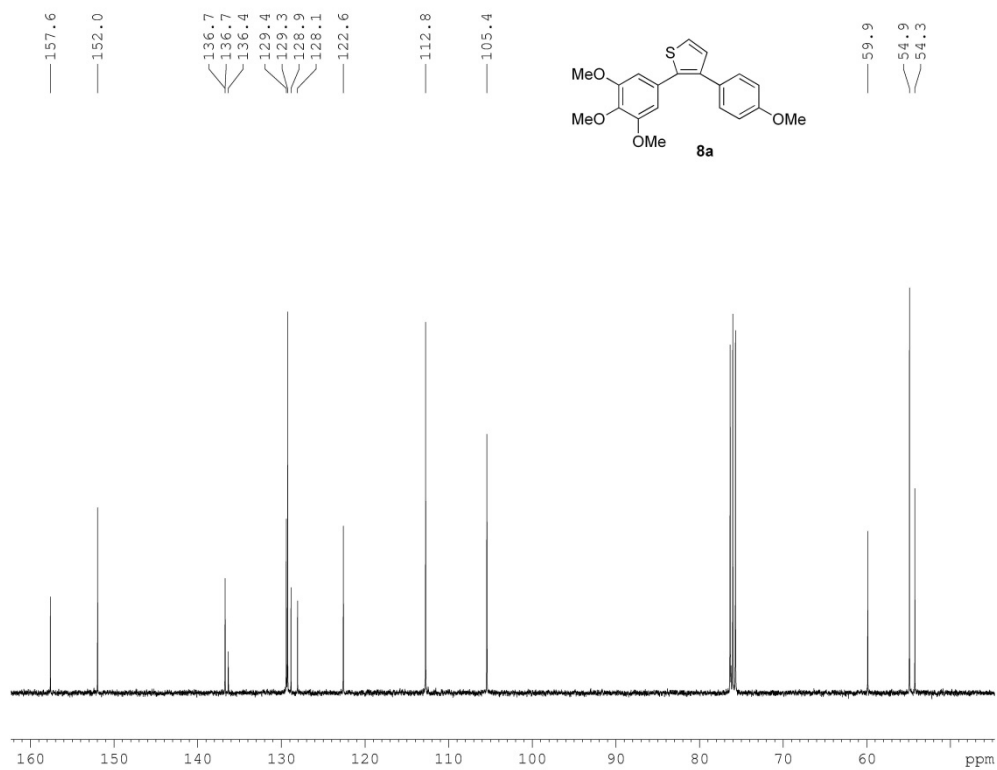
MS



^1H NMR

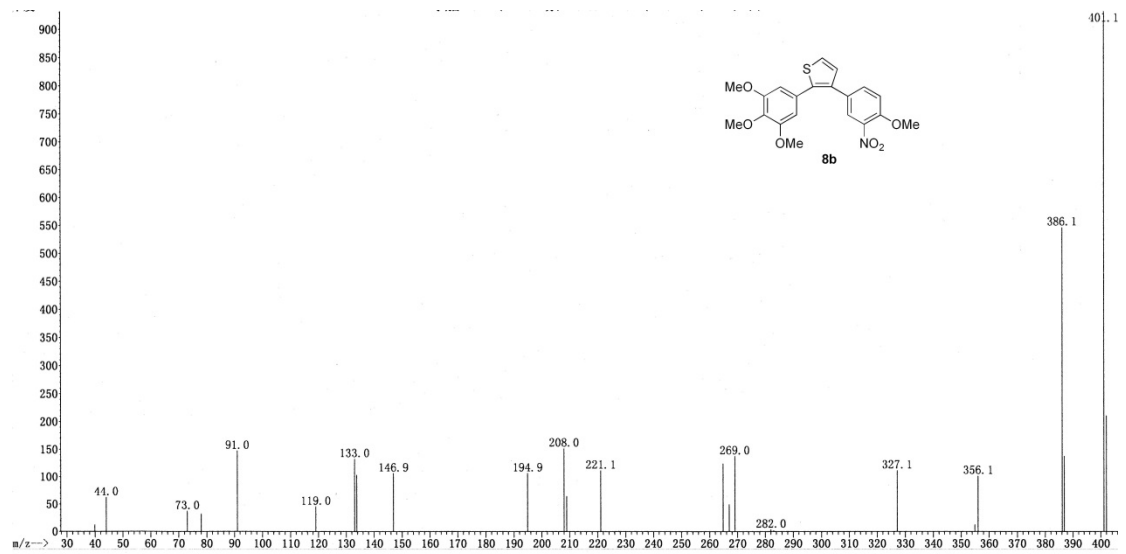


^{13}C NMR

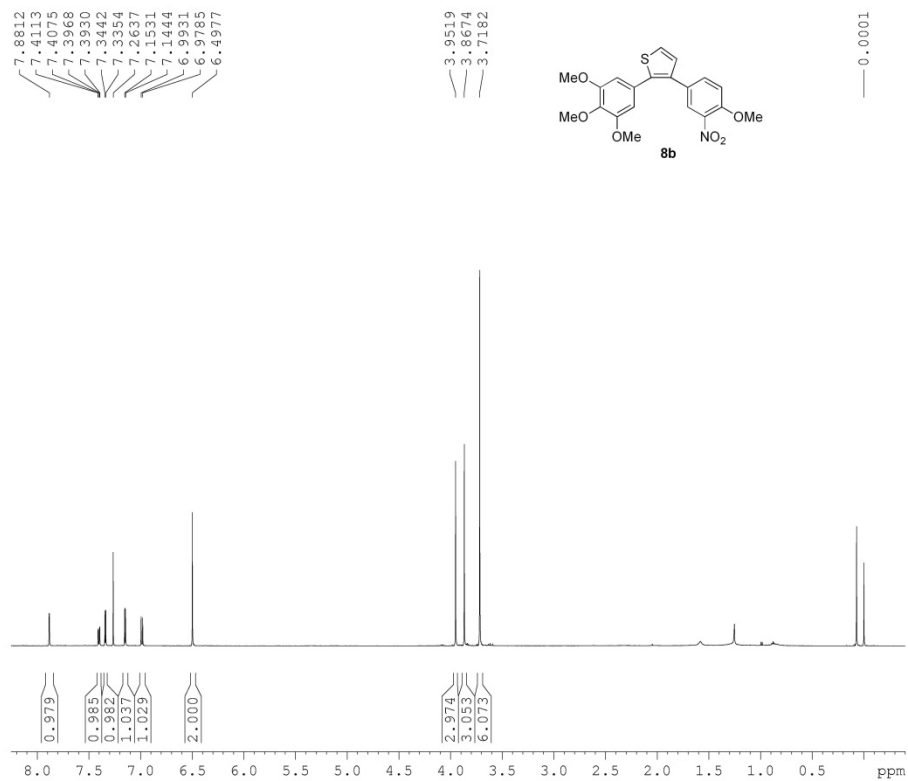


8b:

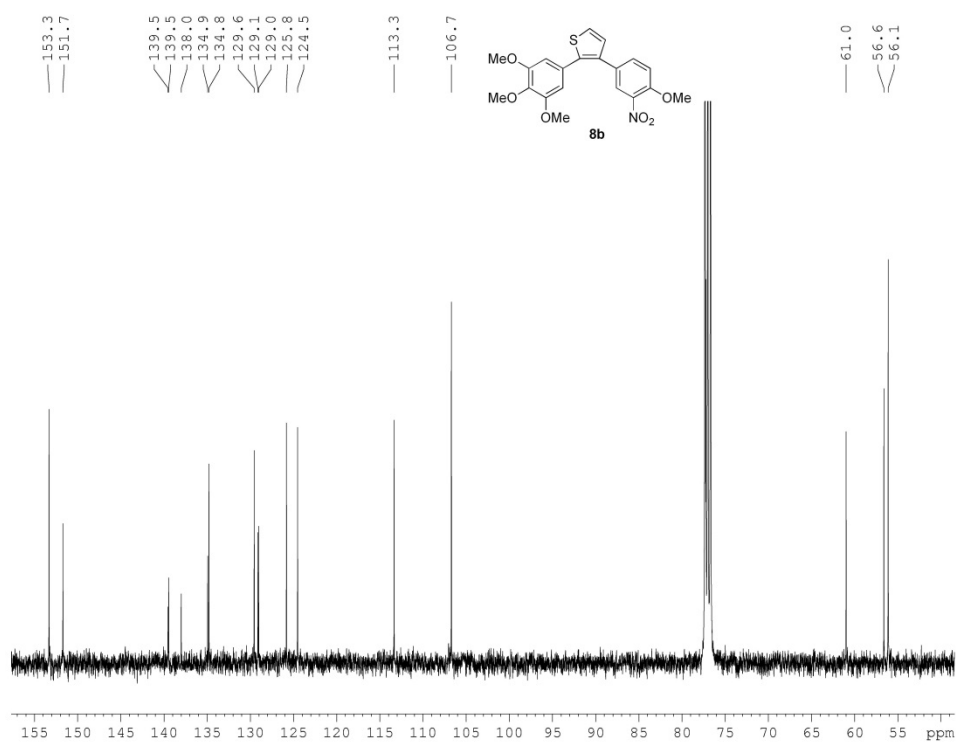
MS



¹H NMR

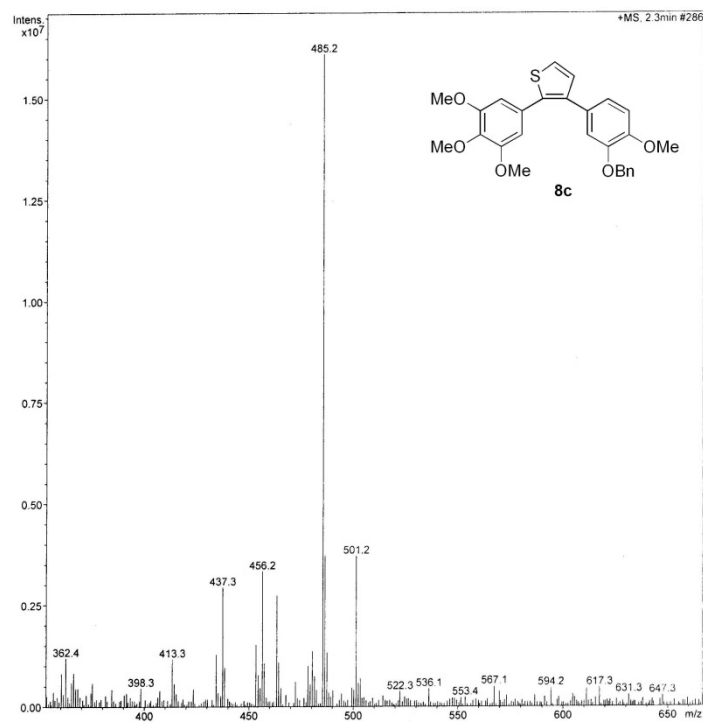


¹³C NMR

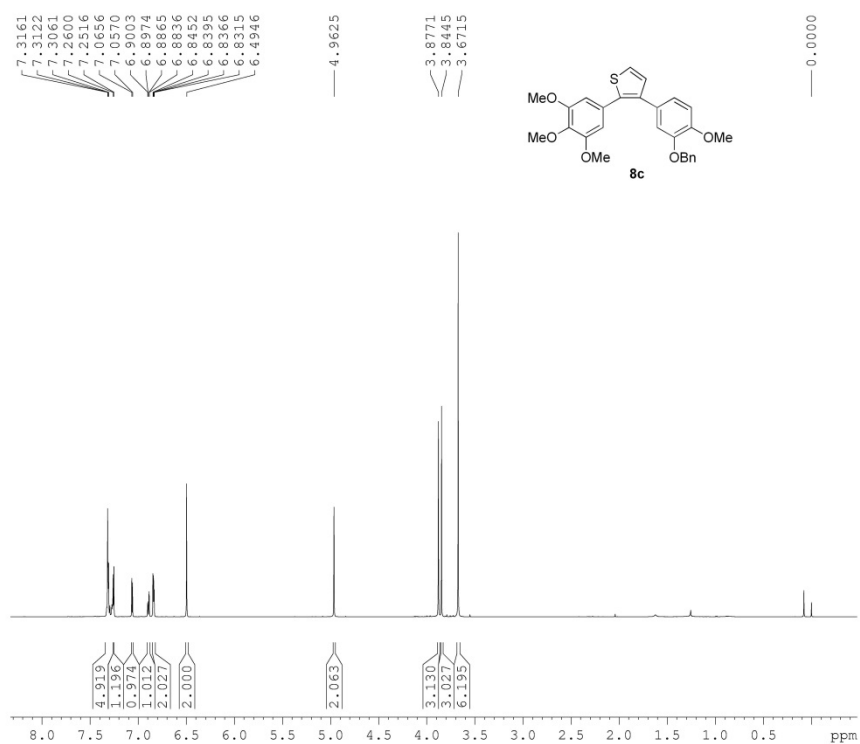


8c:

MS

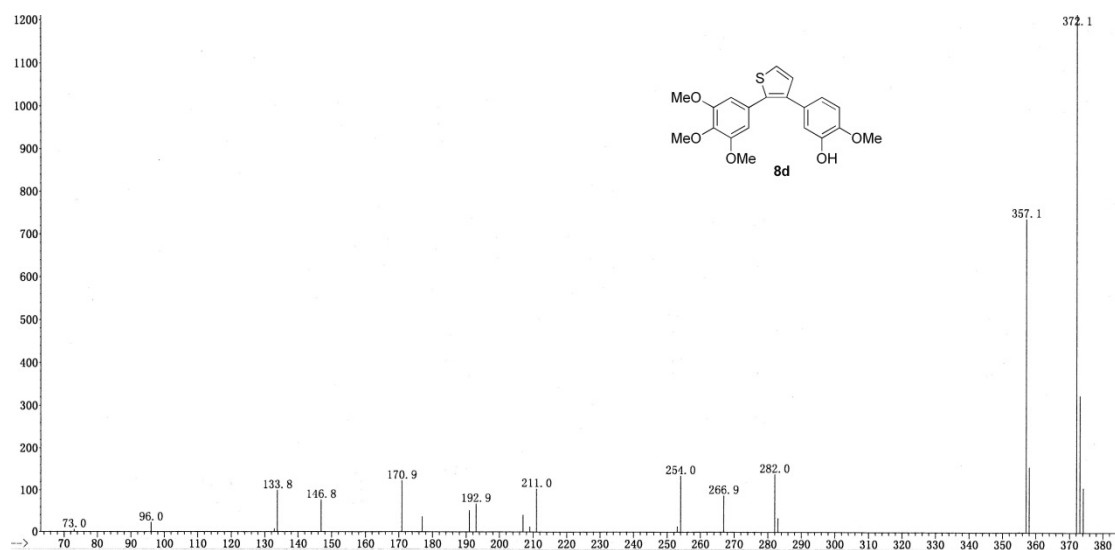


¹H NMR

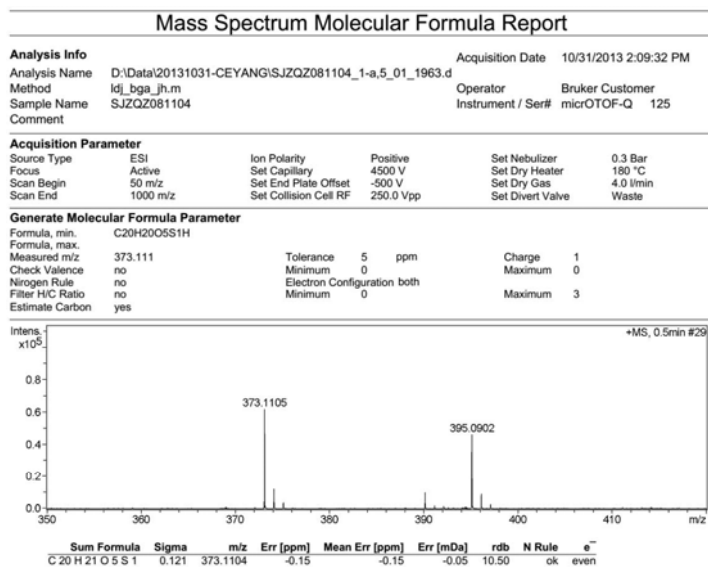


8d:

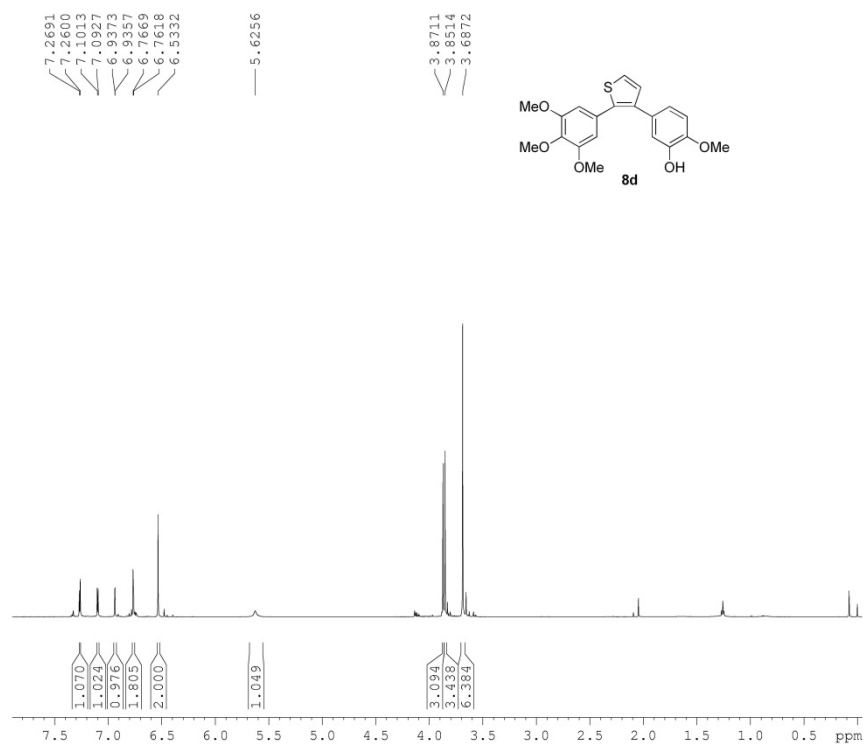
MS



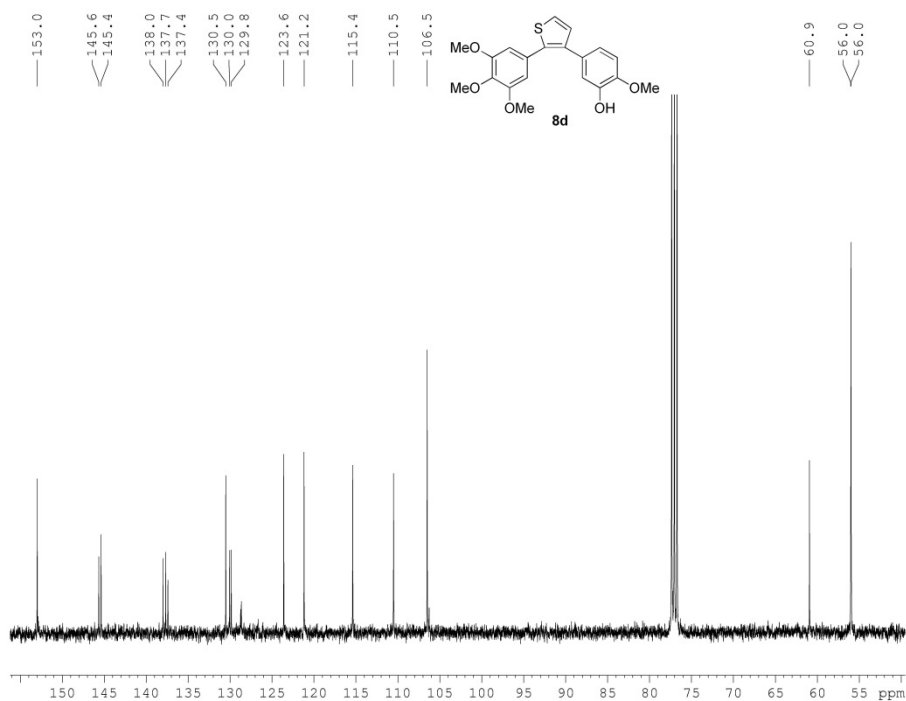
HRMS



¹H NMR



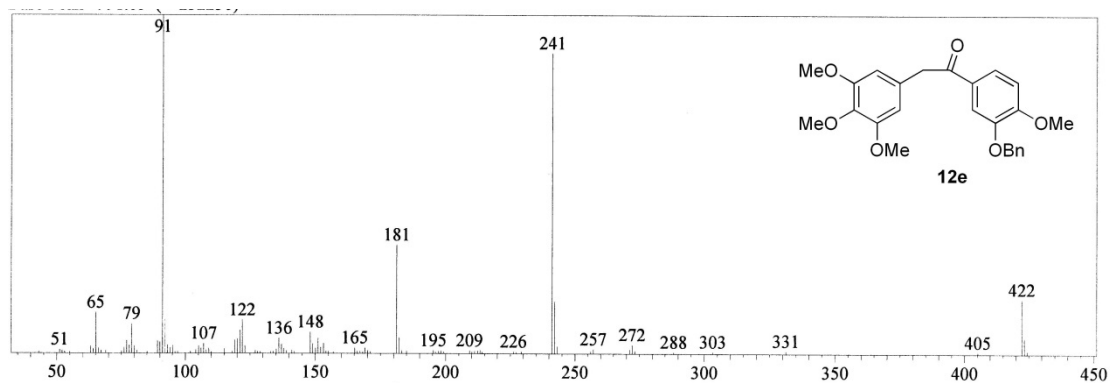
¹³C NMR



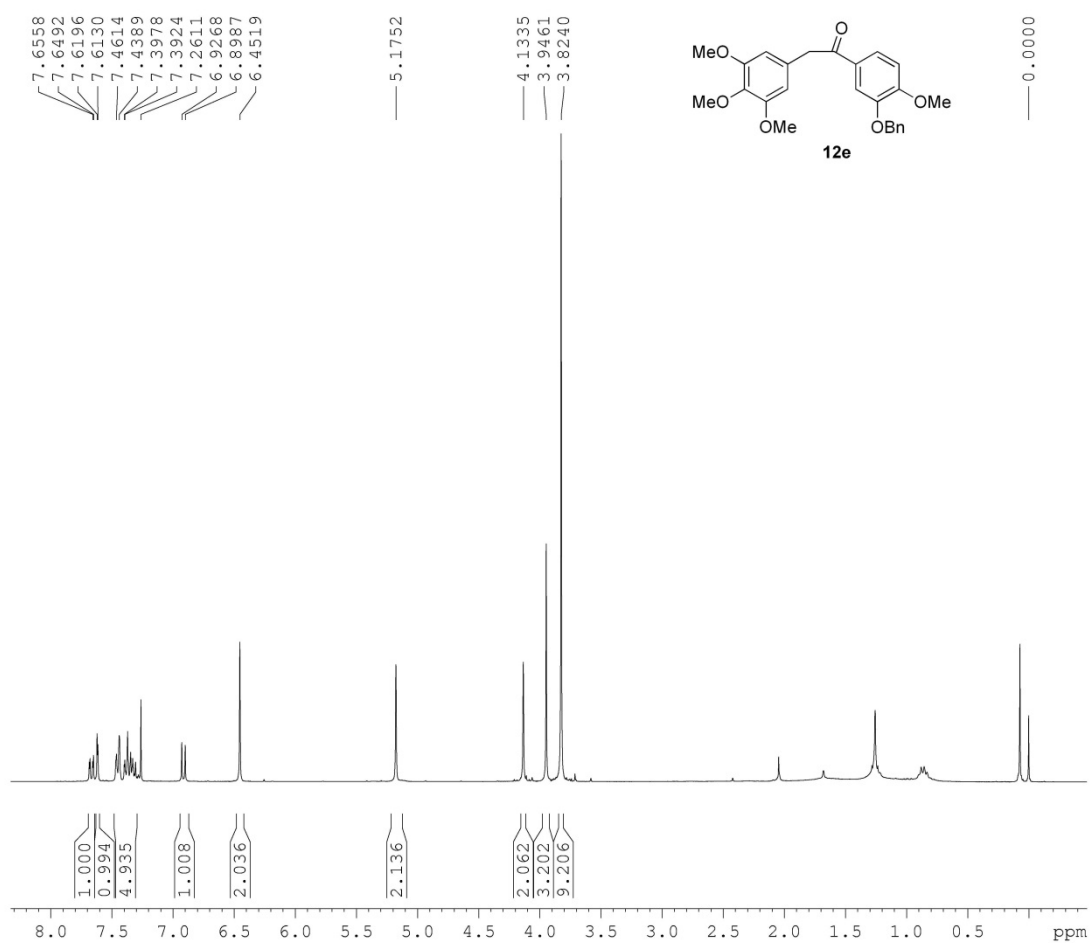
The intermediates

12e:

MS

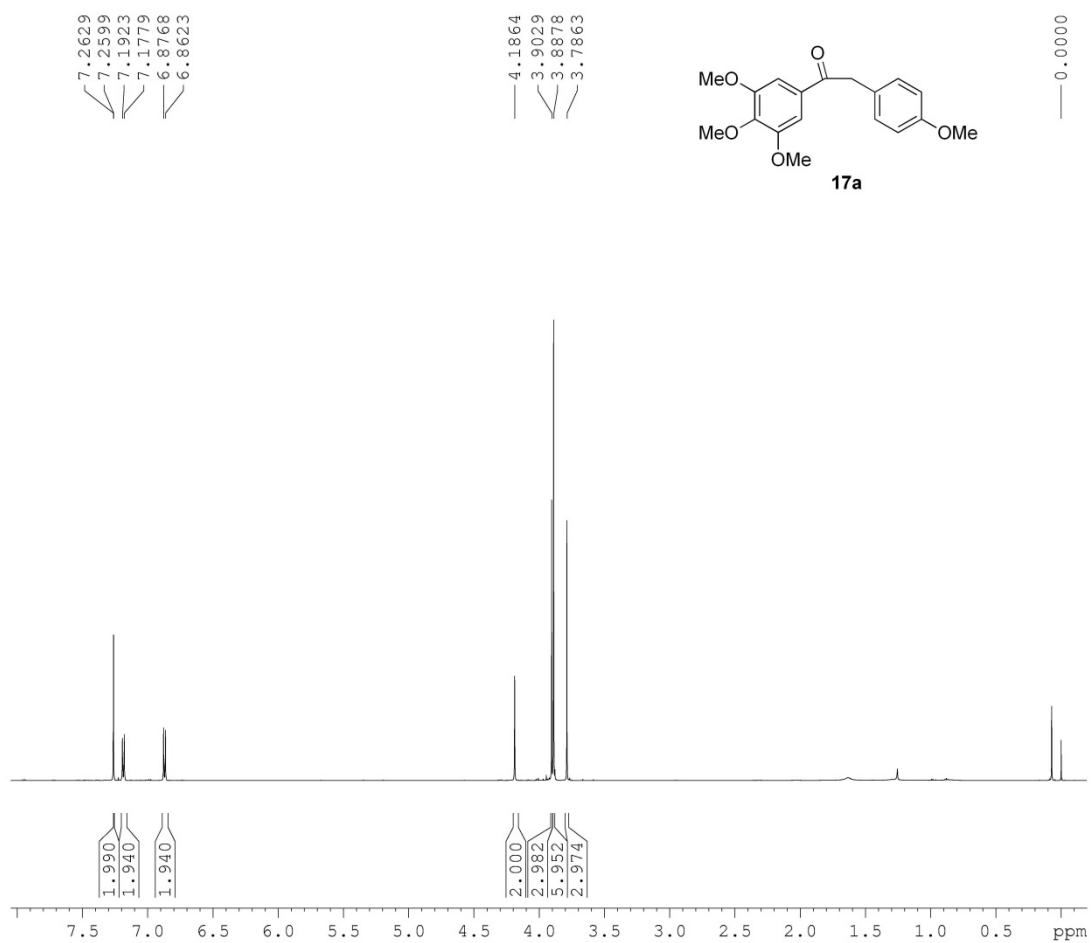


¹H NMR



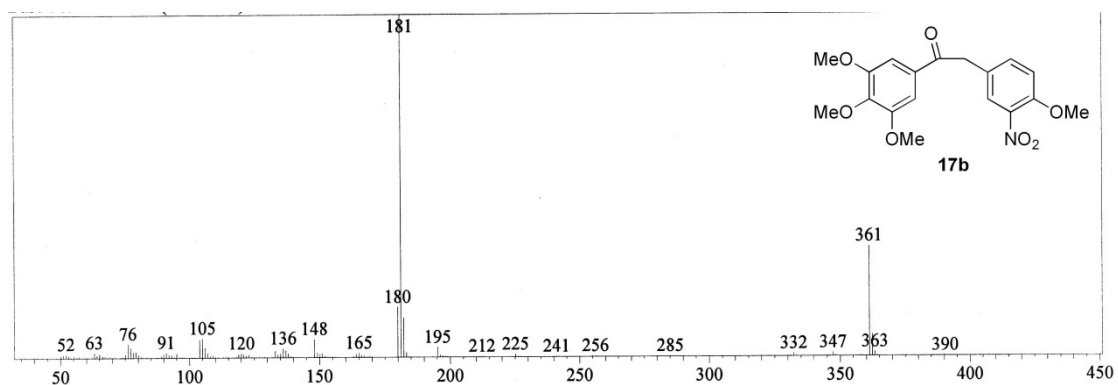
17a:

¹H NMR

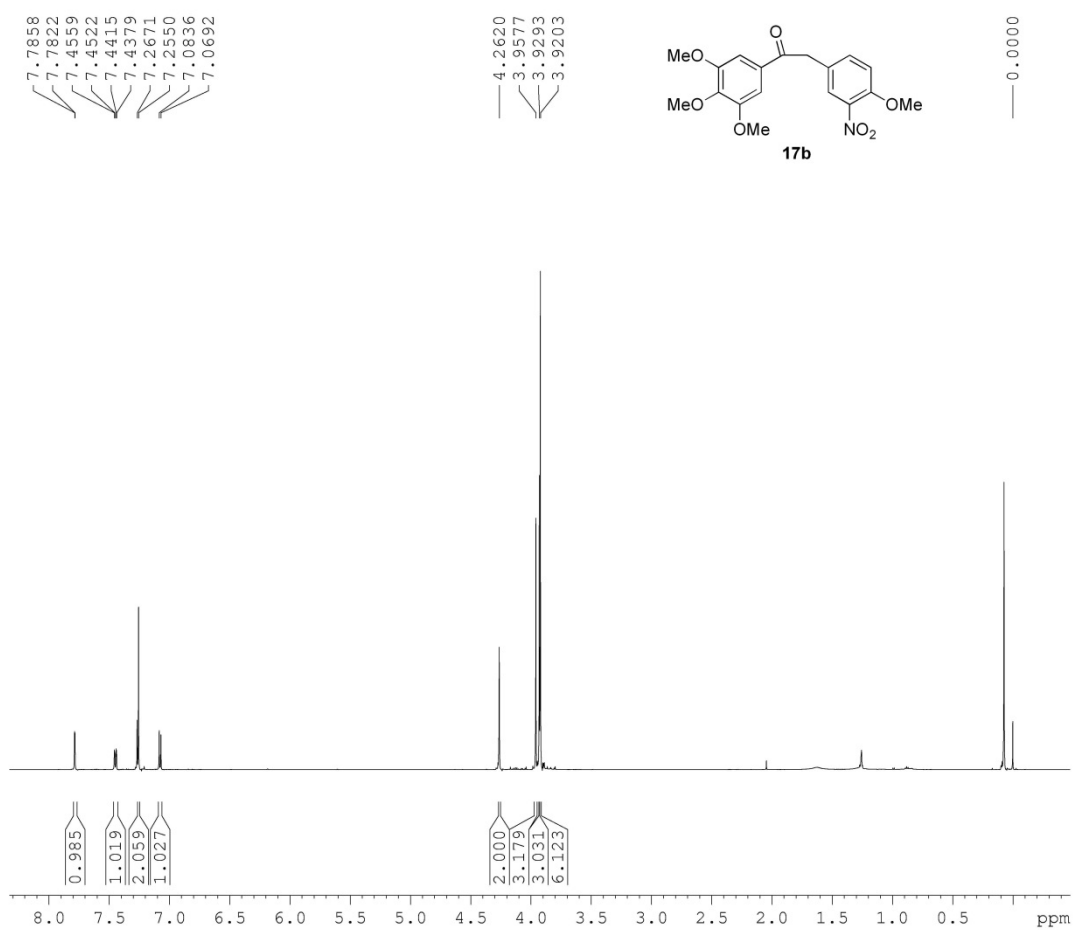


17b:

MS



¹H NMR



17c:

¹H NMR

