Electronic supplementary information (ESI) for

Endomorphin-1 analogs containing α-methyl-β-amino acid exhibit potent analgesic activity after peripheral administration

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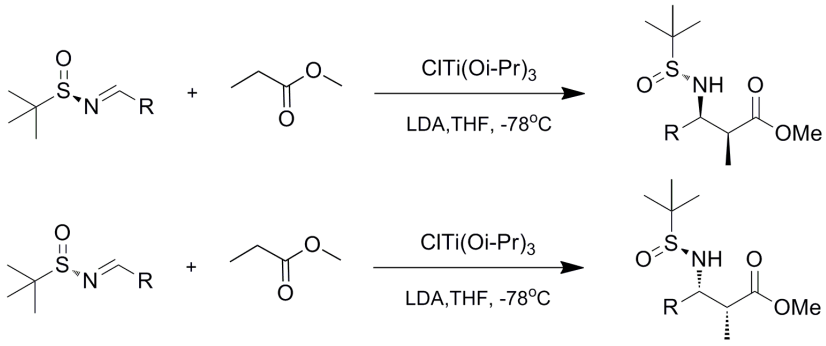
Table of Contents:

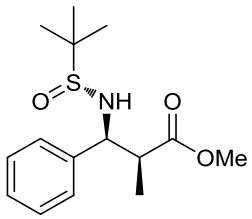
1. General remarks

All standard amino acids were commercially available. Mass spectra were measured with a Maxis 4G ESI-TOF analyzer (Bruker, U.S.A). Melting points were determined on a micro-melting point apparatus (AII-E, China). Analytical RP HPLC was carried out with a Waters Delta 600 instrument equipped with a Waters Deltapak C18 column (4.6 mm × 250 mm, 5 μm); absorbance was monitored at λ = 220 nm. The solvents for analytical HPLC were as follows: A, 0.1% TFA in acetonitrile; B, 0.1% TFA in water. The column was eluted at a flow rate of 1 mL/min with a linear gradient of A:B = 10:90 to A:B = 90:10 for 30 min, and a gradient of A:B=90:10 to A:B=10:90 for 5 min. The retention time is reported as t_R (min).

2. General procedure for synthesis of unnatural amino acids

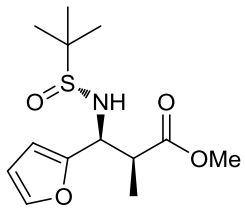


General procedure for the enolate addition to *N*-sulfinyl imine. A solution of *i*-Pr_2NH (1.25 equiv, 6.25 mmol) in THF was cooled to 0℃, and then *n*-Butyllithium (1.3equiv, 6.5 mmol) was added dropwise via syringe and the solution was stirred for 15 min. The solution was then cooled to -78℃ and methyl acetate (1.2 equiv, 6 mmol) was added via syringe and the reaction solution was stirred for 0.5h. To this solution, $ClTi(Oi\text{-}Pr)_3$ (2.6equiv, 13 mmol) dissolved in THF was dropwise at -78℃ and stirred for 45 min. A solution of sulfinyl imine (1 equiv, 5 mmol) in THF was added dropwise at -78℃ via syringe and the mixture was stirred for 5h. After that, NH_4Cl (sat.) was added dropwise at -78℃ and allowed to warm to room tempterature. Upon completion, the reaction was extracted with EtOAc and dried over Na_2SO_4. After concentration of the solvents, the residue was purified on a silica gel column to give the corresponding product.



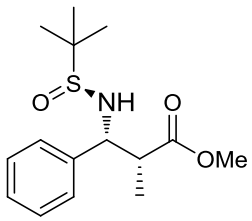
(*2S, 3S*)-methyl 3-((*R*)-1,1-dimethylethylsulfinamido)-2-methyl-3-phenylpropanoate (1)

Colorless oil; **^{1}H NMR** (300 MHz, $CDCl_3$): δ 9.83 (brs, 1H), 7.28-7.19 (m, 5H), 5.24 (d, 1H, *J* = 1.8), 4.86 (s, 1H), 2.83-2.75 (m, 1H), 1.24 (s, 9H), 1.07 (d, 3H, *J* = 7.2) ppm; **^{13}C NMR** (300 MHz, $CDCl_3$): δ 177.0, 138.7, 128.3, 128.2, 127.9, 58.8, 56.5, 45.4, 22.8, 10.7 ppm; ESI-TOF MS: m/z [$M+H^+$] calcd: 284, found: 284.1; yield 83%; dr = 87:13; $[\alpha]^{20}_{D}$ = -79 (c=1, $CHCl_3$).



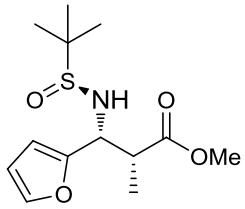
(*2S,3S*)-methyl 3-((*R*)-1,1-dimethylethylsulfinamido)-3-(furan-2-yl)-2-methylpropanoate (2)

Colorless oil; **^{1}H NMR** (300 MHz, $CDCl_3$): δ 10.37 (brs, 1H), 7.39-7.36 (m, 1H), 6.34-6.28 (m, 2H), 5.26 (d, 1H, *J* = 5.1), 4.87 (t, 1H, *J* = 3.6), 3.08-3.00 (m, 1H), 1.26 (m, 9H), 1.22 (d, 3H, *J* = 11.1) ppm; **^{13}C NMR** (300 MHz, $CDCl_3$): δ 175.6, 151.1, 141.2, 109.2, 107.6, 55.7, 54.2, 42.7, 21.7, 11.2 ppm; ESI-TOF MS: m/z [$M+H^+$] calcd: 274, found: 274.1; yield 80%; dr = 92:8; $[\alpha]^{20}_{D}$ = -63 (c=1,$CHCl_3$).



(*2R,3R*)-methyl 3-((*S*)-1,1-dimethylethylsulfinamido)-2-methyl-3-phenylpropanoate (3)

Colorless oil; **^{1}H NMR** (300 MHz, $CDCl_3$): δ 10.45 (brs,1H), 7.28-7.19 (m,5H), 5.22 (d, 1H, *J* = 2.1), 4.85 (t, 1H, *J* = 2.7), 2.83-2.75 (m,1H), 1.24 (s, 9H), 1.07 (d, 3H, *J* = 7.2) ppm; **^{13}CNMR** (300 MHz, $CDCl_3$): δ 176.0, 137.7, 127.3, 127.2, 126.8, 57.9, 55.5, 44.4, 21.8, 9.7 ppm; ESI-TOF MS: m/z [$M+H^+$] calcd: 284, found: 284.1; yield 83%; dr = 90:10; $[\alpha]^{20}_{D}$ = 79 (c=1,$CHCl_3$).



(*2R,3R*)-methyl 3-((*S*)-1,1-dimethylethylsulfinamido)-3-(furan-2-yl)-2-methylpropanoate (4)

Colorless oil; **^{1}H NMR** (300 MHz,$CDCl_3$): δ 10.21 (brs, 1H), 7.32-7.29 (m, 1H), 6.26-6.21 (m, 2H), 5.18 (d, 2H, J = 5.1), 4.80 (t, 1H, J = 4.8), 2.99-2.95 (m, 1H), 1.19 (s, 9H), 1.14 (d, 3H, J = 8.1) ppm; **^{13}C NMR** (300 MHz,$CDCl_3$): δ 175.7, 151.2, 141.2, 109.2, 107.6, 55.7, 54.2, 42.7, 21.7, 11.2 ppm; ESI-TOF MS: m/z [$M+H^+$] calcd: 274, found: 274.1; yield 80%; dr = 91:9; $[\alpha]^{20}_{D}$ = 61 (c=1,$CHCl_3$).

3. Peptide synthesis

General procedure for the synthesis of Boc-Tyr/Dmt-Pro-OH. A solution of Boc-Tyr/Dmt-OH (10 mmol), DCC (12 mmol), HOSu (12 mmol) in distilled THF (50 mL) was stirred at 0 ℃, H-Pro-OH (1.2 mmol) was dissolved in sat. aq. $NaHCO_3$ adjusted the pH value to 9-10, and then the reaction mixture containing Boc-Tyr/Dmt-OSu and H-Pro-ONa was stirred at 0 ℃ for 30 min and at room temperature overnight. When the reaction completed, it was extracted with THF. The extract was washed successively with 5% citrate acid in twice, and brine, dried over Na_2SO_4, and evaporated at reduced pressure. The residue was purified on a silica gel column (EA:MeOH = 10:1). Yield: 85–90%.

General procedure for the synthesis of Boc-Trp-Xaa-NH_2 (Xaa = (*S,S*)-AMBP, (*R,R*)-AMBP, (*S,S*)-AMBF, (*R,R*)-AMBF). A solution of Boc-Trp/Phe-OH (10 mmol), EDCI (16 mmol), HOBt (14.5 mmol), and DIEA (40 mmol) in distilled DCM (50 mL) was stirred at 0 ℃, then the H-Xaa-NH_2 (1.2 mmol) was dissolved in distilled DCM (10 mL) added slowly into the mixture stirred at 0 ℃ for 30 min, and at room temperature overnight. When the reaction completed, it was extracted with DCM. The extract was washed successively with 5% citrate acid, saturated $NaHCO_3$ in twice, and brine, dried over Na_2SO_4, and evaporated at reduced pressure. The

residue was purified on a silica gel column (DCM:MeOH = 15:1) with a yield range of 82–88 %.

General procedure for the synthesis of H-Tyr/Dmt-Pro-Trp-Xaa-NH_2 (Xaa = (*S,S*)-AMBP, (*R,R*)-AMBP, (*S,S*)-AMBF, (*R,R*)-AMBF). A solution of N-terminal fragments Boc-Tyr/Dmt-Pro-OH (1.0 mmol), EDCI (1.6 mmol), HOBt (1.45 mmol), and DIEA(4.0 mmol) in distilled DCM (10 mL) was stirred at 0 ℃. Deprotection of the Boc groups of C-terminal fragments (1.2 mmol) was performed with HCl/EA (1:4, v/v). Then, the reaction mixture containing Boc-Tyr/Dmt-Pro-OBt and deprotected C-terminal fragments H-Trp-Xaa-NH_2 was stirred in distilled DCM at 0 ℃ for 30 min, and at room temperature overnight. When the reaction completed, it was extracted with DCM. The extract was washed successively with 5% citrate acid, saturated $NaHCO_3$ in twice, and brine, dried over Na_2SO_4, and evaporated at reduced pressure. The crude protected tetrapeptides residue was purified on a silica gel column (EA:MeOH = 15:1) with a yield range of 70–82%.

The Boc-group deprotection was performed by treatment with HCl/EA (1:4, v/v) at room temperature for 2 h. After extracted with DCM:MeOH = 10:1, the solvent was evaporated at reduced pressure. The residue was purified by semi-preparative RP-HPLC, and characterized by TLC, TOF-MS, mp, and by RP-HPLC revealed that all the peptides were the desired peptides with >95% purity.

4. Materials and methods

4.1 Animals (Animal Center of Medical College of Lanzhou University, Gansu, People's Republic of China) were housed in a temperature-controlled environment (22 ± 1 ℃) under standard 12 h light/dark conditions and received food and water ad libitum. All animals were cared for, and experiments were carried out in accordance with the principles and guidelines of the American Council on Animal Care. All the protocols in this study were approved by the Ethics Committee of Lanzhou University (permit number: SYXK Gan 2009–0005), China.

4.2 Radioligand Binding Assay. In the experiments designed to define peptide specificity for μ-, δ- and κ-opioid receptors, HEK293 cells stably expressing MOP, DOP, or KOP (2.5–3.5×106 cells/tube) were incubated with 1.7 nM [^{3}H]DAMGO, 1.0 nM [^{3}H]DPDPE or 2.0 nM [^{3}H]U69,593 and 10^{-10}–10^{-4} M unlabeled ligands for

each experiments. The reaction was performed in 25 ℃ for 1 h in freshly prepared binding buffer (25 mM Hepes, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.4% bovine serum albumin [BSA], and 2.5 mM ethylenediaminetetraacetic acid [EDTA], pH 7.4). The reaction was stopped by rapid vacuum filtration through GF/C filters (Whatman, Maidstone, U.K.) using a cell harvester. The filters were washed twice with 6 mL ice-cold buffer and then dried for 1 h at 80 ℃. The radioactivity was measured by liquid scintillation counting (liquid scintillation counter, PerkinElmer). The affinity constants (*Ki*) were calculated according to Cheng and Prusoff with GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). The dissociation constant (K_d^{μ} = 0.6 nM, K_d^{δ} = 2.8 nM, K_d^{κ} = 2.9 nM) and the number of binding sites (B_{max}) were calculated by iterative curve-fitting analysis using at least seven concentrations of [^{3}H]DAMGO, [^{3}H]DPDPE or [^{3}H]U69,593 in a range of 0.085–8.5 nM, 0.10–5.05 nM, or 0.10–5.00 nM. Nonspecific binding was assessed in the presence of 10 μM naloxone, 10 μM naltrexone, or 10 μM nor-BNI.

4.3 Measurements of cAMP Accumulation. In vitro cAMP assays were performed as described in our previous reports. HEK293 cells which stably expressing the μ-opioid receptor were seeded in 24-well microtiter plates in the day before the assay. Before beginning the assay, cells in serum-free medium containing 1 mM IBMX, vehicle, or various concentrations (10^{-10}–10^{-4} M) of compounds and 50 μM forskolin for 0.5 h. After incubation, cells were lysed, and cAMP levels were assessed by a competition PKA binding assay. The concentrations of cAMP in lysates were calculated using the standard curve that was constructed using cAMP standard. Scintillation fluid was added, and radioactivity was quantified in a scintillation counter (liquid scintillation counter, PerkinElmer). Analysis of the data was performed using the Graph-Pad Prism software (version 5.0, San Diego, CA).

4.4 Metabolic Stability. A final protein concentration of 2.3 mg/mL in 50 mM Tris buffer, pH 7.4, was used for all incubations. RP-HPLC analysis determined the stability of peptides. Approximately 10 μL of peptide (10^{-2} M) stock solution was digested with 190 μL of rat brain homogenate at 37 °C in a final volume of 200 μL for incubation. 20 μL of the aliquots were withdrawn from the mixture at 0, 10, 30, 60, 120, 240, 480 min, and 90 μL acetonitrile was added immediately for precipitated proteins, placing the tube on ice for 5 min, added 90 μL of 0.5% acetic acid at the required time to prevent further enzymatic breakdown. The aliquots were centrifuged

at 13,000 g for 15 min at 4 °C. The obtained supernatants were filtered with filters and 50 μL of the filtrate was analyzed by RP-HPLC on a Waters Delta Pak C18 column (4.6 mm × 250 mm, Milford, MA), using the solvent system of 0.1% TFA in acetonitrile (A) and 0.1% TFA in water (B) with a linear gradient of A:B = 10:90 to A:B = 90:10 for 30 min and A:B = 90:10 to A:B = 10:90 for 5 min, the column was eluted at a flow rate of 1 mL/min. The degradation rate constants (k) were determined by least-squares linear regression analysis of logarithmic tetrapeptide peak area [($\ln(A_t/A_0)$] vs. time courses, with at least seven time points. The rate constants obtained were used to establish the degradation half-lives ($t_{1/2}$) as In2/k.

4.5 Tail-flick test. Male Kunming mice weighing 18−22 g were employed, various doses of analogs were injected intracerebroventricular (i.c.v.) or intravenous (i.v.), and the warm water tail-flick responses were measured at different times. Nociception was evoked by immersing the mouse tail in warm water (50 ± 0.2 ^{o}C) and measuring the latency to withdrawal. For the study involving the opioid antagonist, animals were pretreated with naloxone and naloxone methiodide before i.v. challenge with analogs. For each mouse, the tail-flick latency was recorded before treatment, and those with a latency of approximately 3−5 sec were selected. The latency to tail-flick was defined as the test latency, the corresponding cut-off time was set at 10 sec in order to minimize tissue damage. The antinociceptive response was expressed as the percent maximum possible effect (%MPE), calculated by the following equation: %MPE = 100 × (test latency – control latency) / (10 – control latency).

4.6 Formalin test. Male Kunming mice weighing 22−25 g were employed. The animals were acclimatized in a transparent acrylic observation chamber with a mirror positioned below the floor for a period of 15 min. Mice were pre-treated (i.v. injection) with test analogs 5 min before subcutaneous (s.c.) injection of 5% formalin solution (20 μL) into the right hind paw, and mice were immediately placed back in the observation chamber, and the time spent licking, shaking, and biting the injected paw at 0−5 min (first-phase) and 15−30 min (second-phase) was measured with a stopwatch. The nociceptive response was expressed as the second (sec), and 0.9 % saline was used as control.

4.7 Fecal pellet output. Male Kunming mice weighing 25−30 g were employed fasted 2 hour. Compounds were i.v. injected 5 min later and mouse was separately placed on

a metal grid-bottom cage, 2 hours as the cut-off time and the total number of fecal pellet produced was recorded. All the fecal material was collected and desiccated overnight in an oven at 50 ^{o}C and to obtain the dry weight.

4.8 Rotarod test. Male Kunming mice weighing 18−22 g were employed. An automated apparatus was used to evaluate the ability of mice to maintain balance for up to 5 min on a rod rotating at a constant speed of 16 rpm. Prior to starting data collection, mice were trained for 2–3 days and were given three trials per day in order to achieve maximal performance in the rotarod test. The mice were frequently monitored by the researchers. Mice were given three trials with 2-min breaks between trials. After i.v. injection test analogs, the latency to fall from the rotating drum was measured in seconds.

5. References

[1] Y. Wang, Y. Xing, X. Liu, H. Ji, M. Kai, Z. Chen, J. Yu, D. Zhao, H. Ren, R. Wang, A new class of highly potent and selective endomorphin-1 analogues containing alpha-methylene-beta-aminopropanoic acids (map), *J Med Chem* 55 (2012) 6224-36.

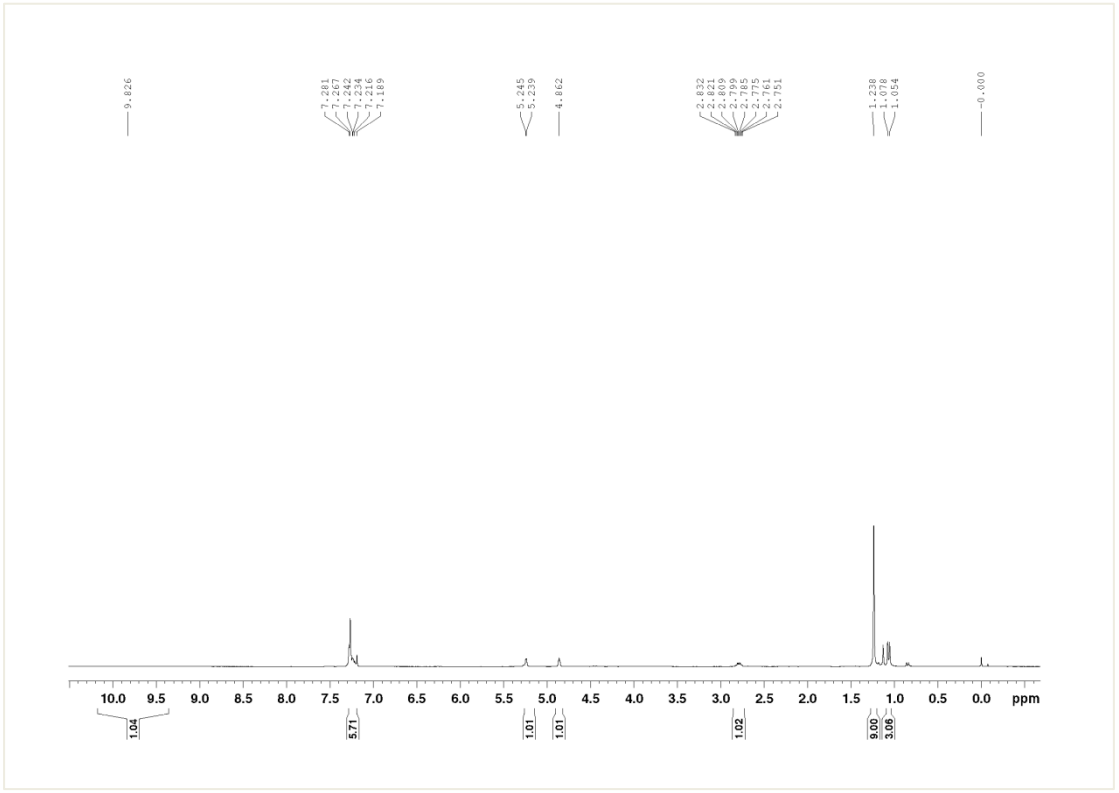
[2] T.P. Tang, J.A. Ellman, The tert-Butanesulfinyl Group: An Ideal Chiral Directing Group and Boc-Surrogate for the Asymmetric Synthesis and Applications of beta-Amino Acids, *J Org Chem* 64 (1999) 12-13.

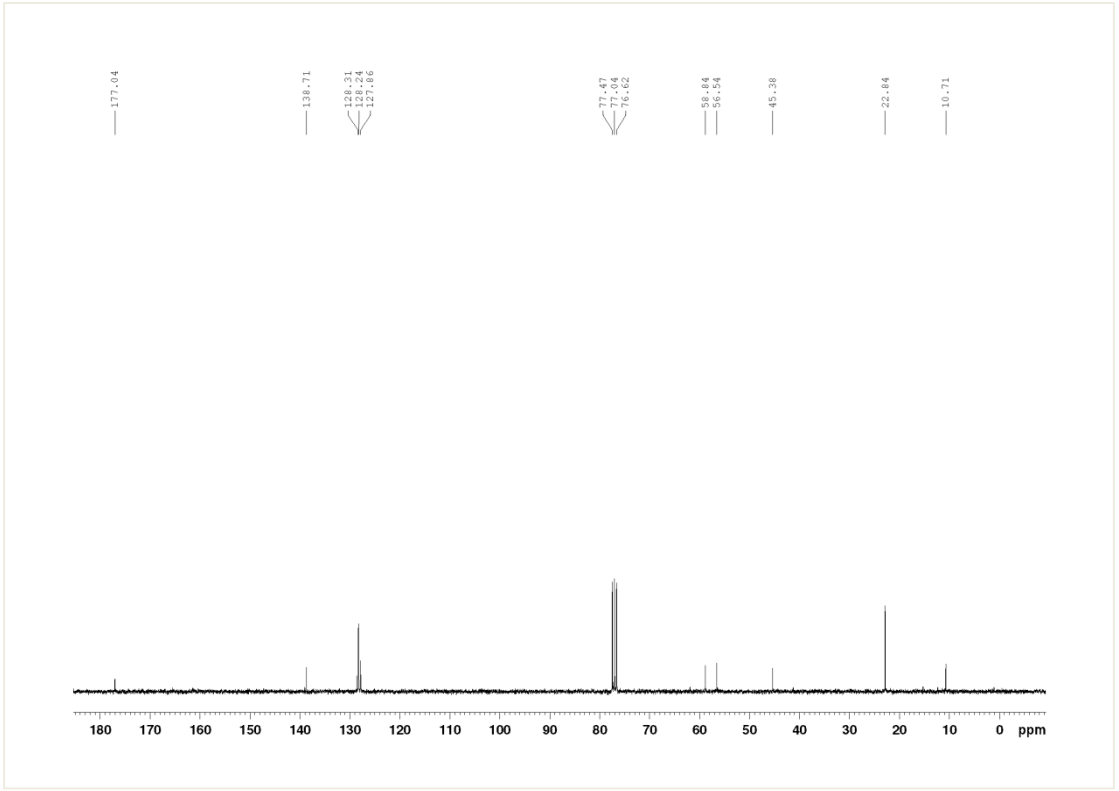
[3] T.P. Tang, J.A. Ellman, Asymmetric synthesis of beta-amino acid derivatives incorporating a broad range of substitution patterns by enolate additions to tert-butanesulfinyl imines, *J Org Chem* 67 (2002) 7819-32.

[4] Y. Wang, X. Liu, D. Wang, J. Yang, L. Zhao, J. Yu, R. Wang, Endomorphin-1 analogues (MELs) penetrate the blood-brain barrier and exhibit good analgesic effects with minimal side effects, Neuropharmacology 97 (2015) 312-21.

6. Copies of NMR and ESI TOF-MS spectra

(*2S, 3S*)-methyl 3-((*R*)-1,1-dimethylethylsulfinamido)-2-methyl-3-phenylpropanoate (1)





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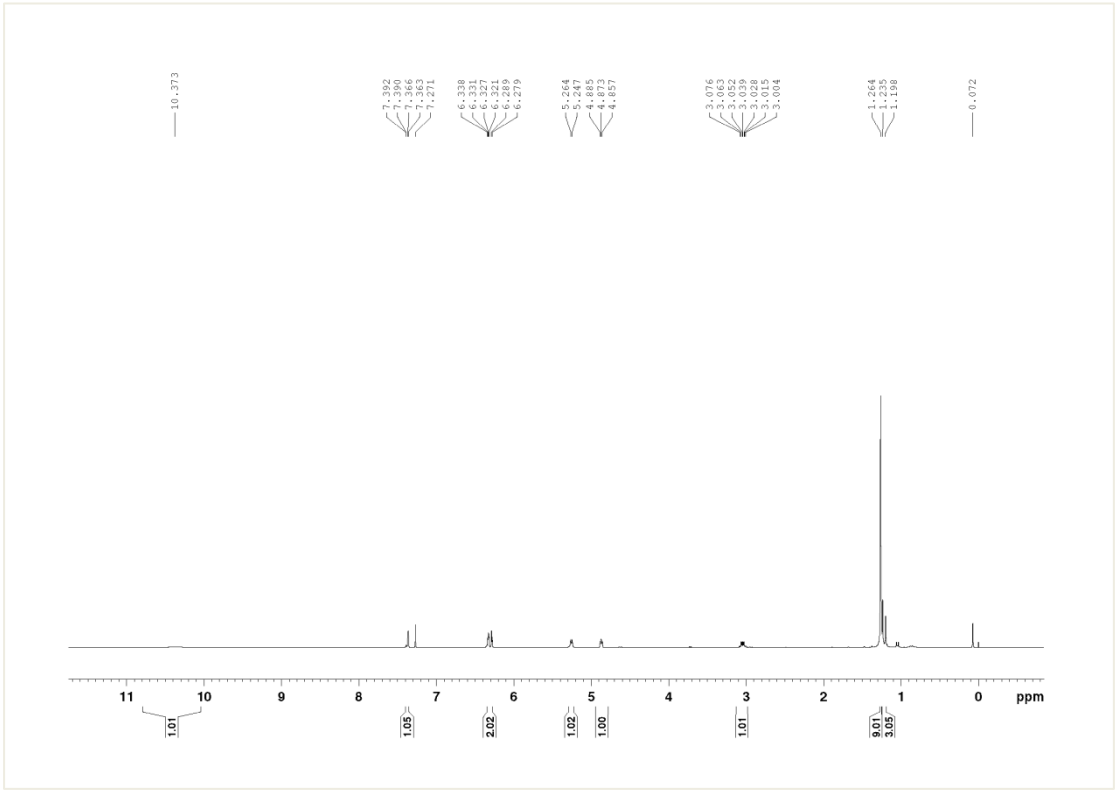

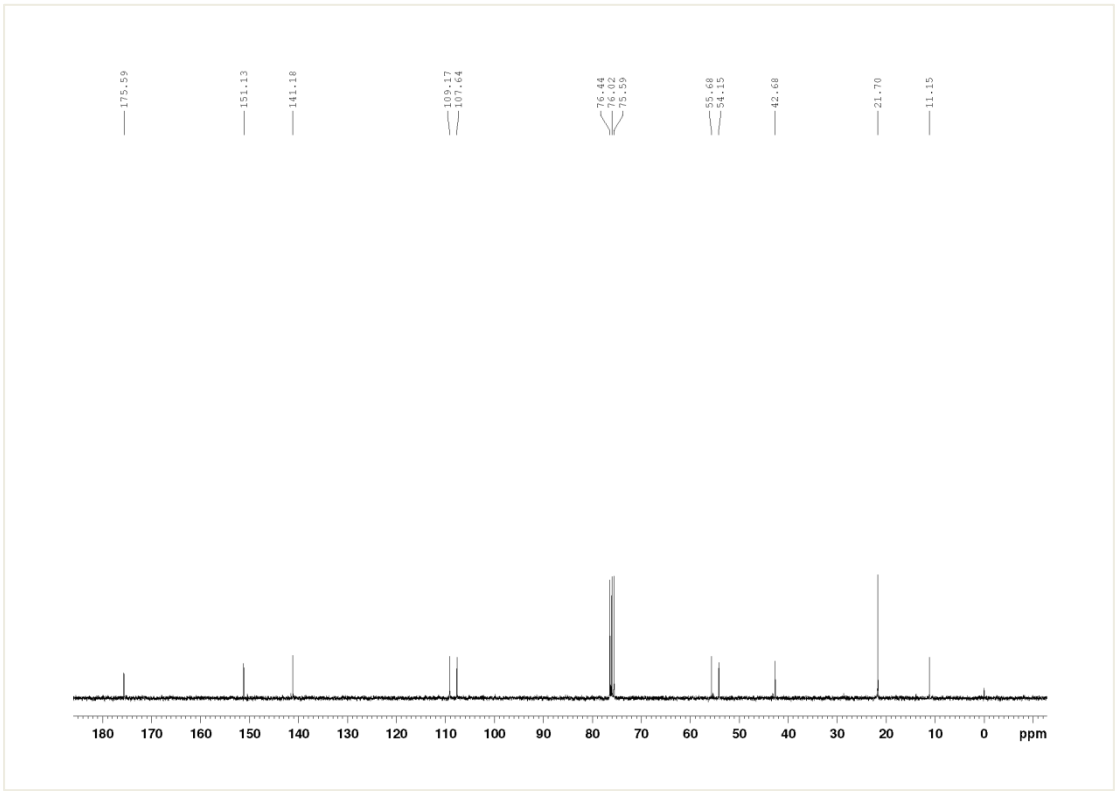

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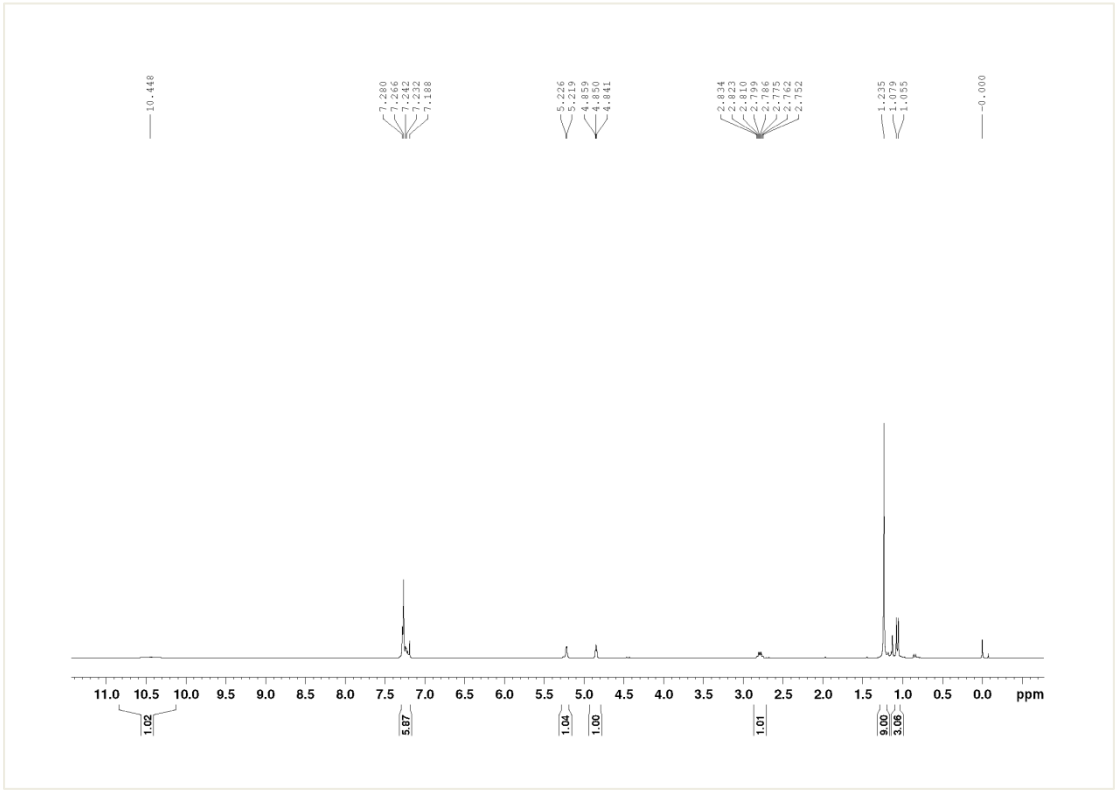

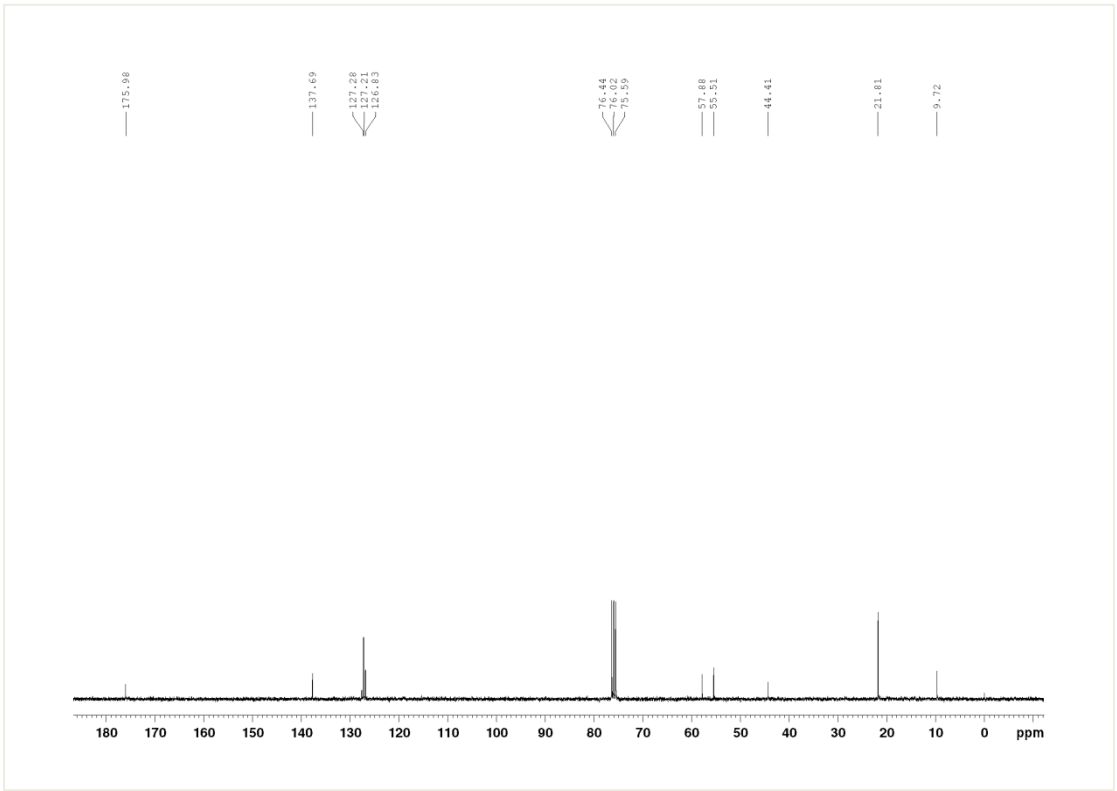

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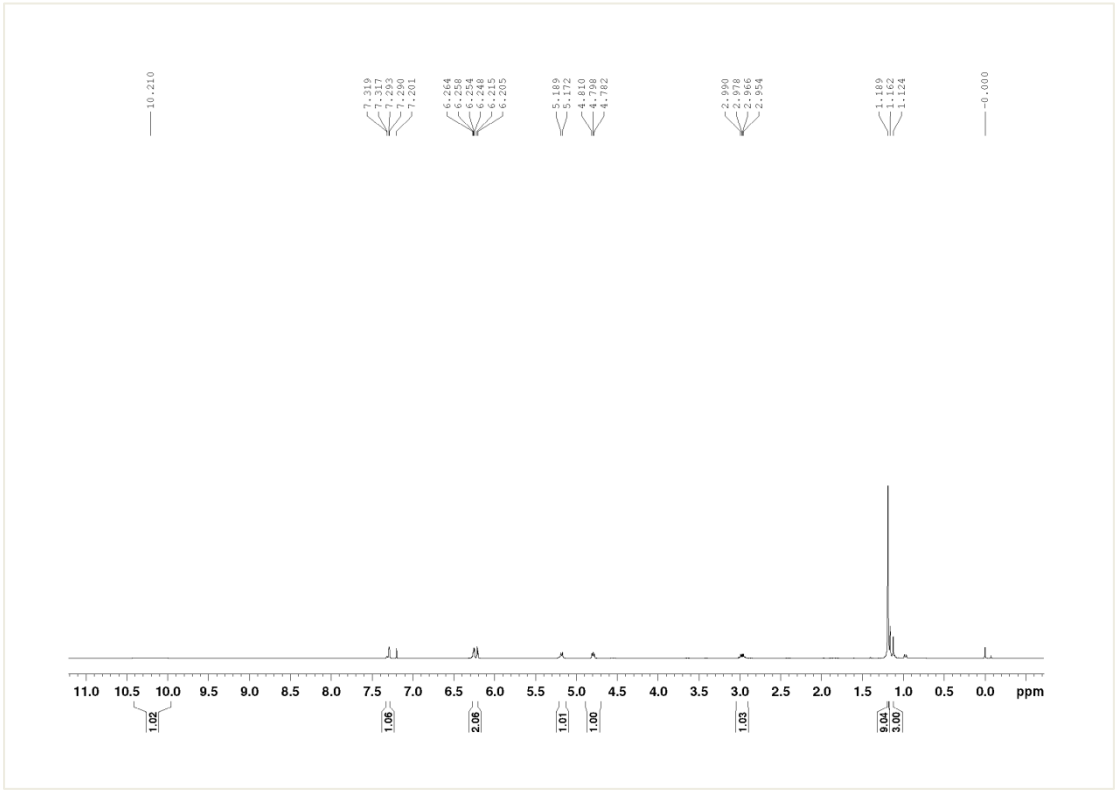
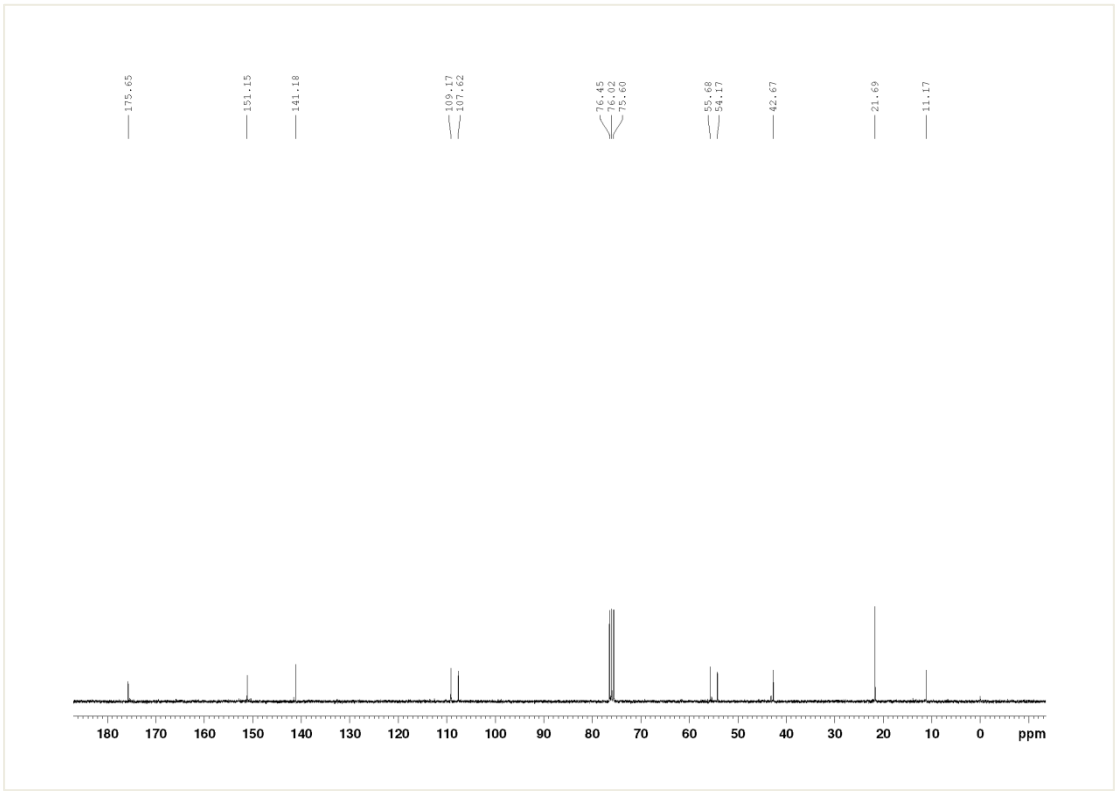
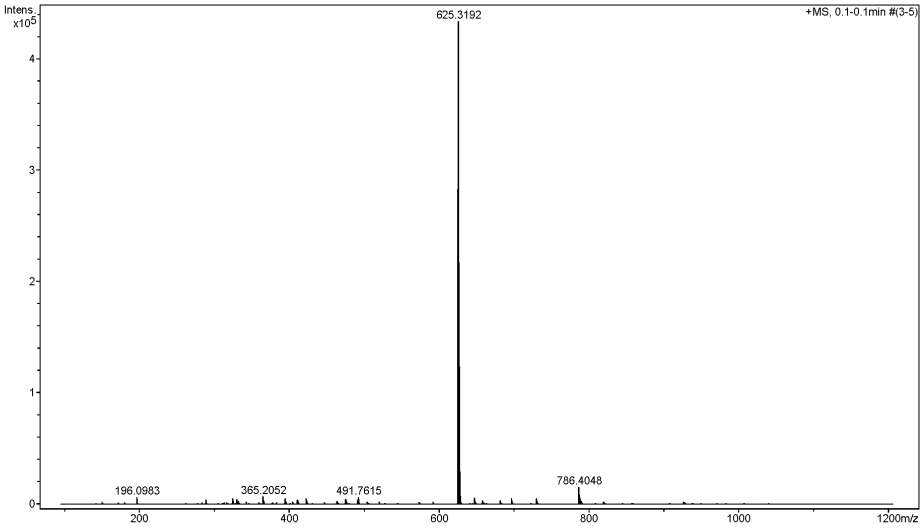




Analog 1:



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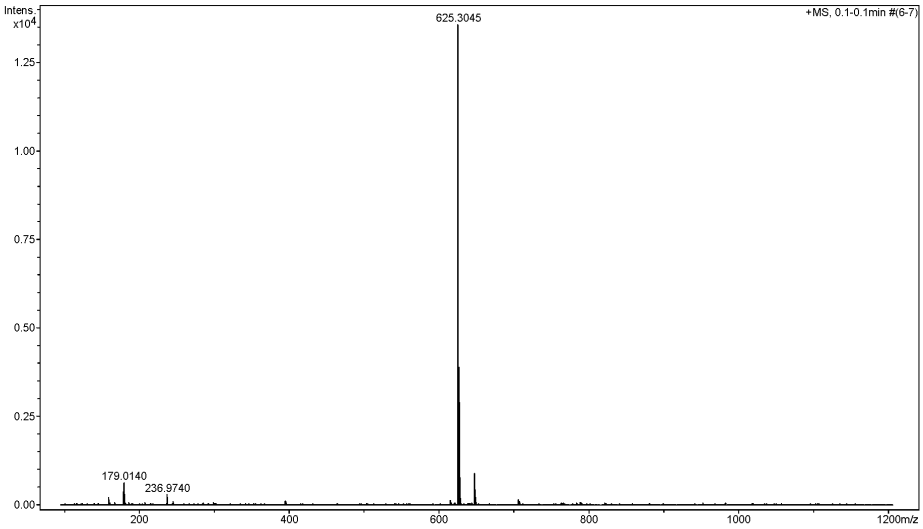

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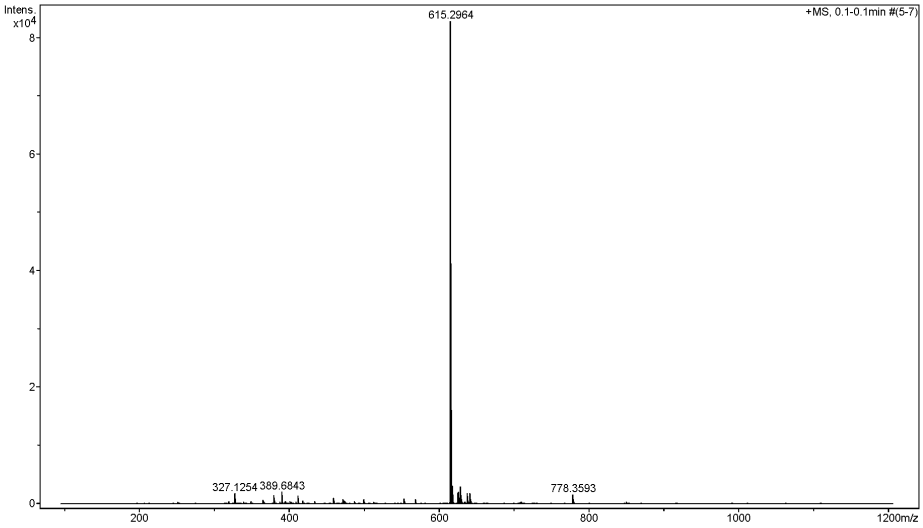

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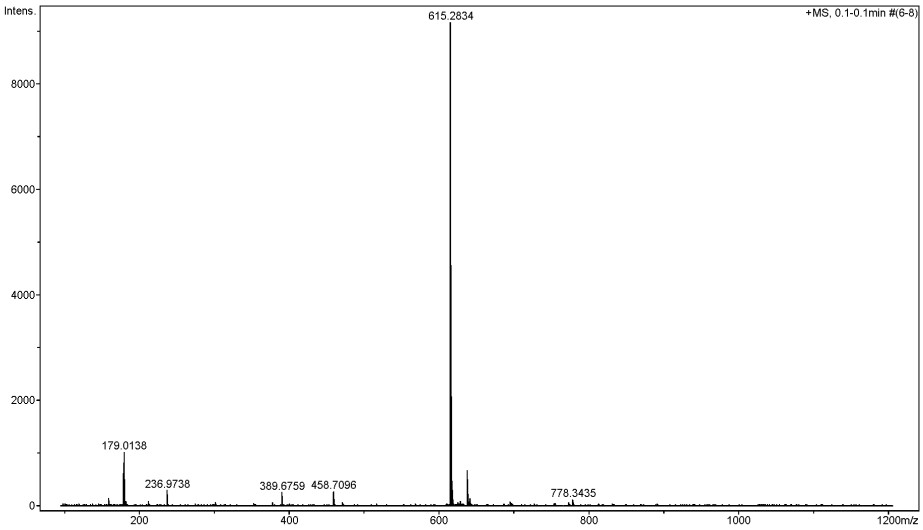

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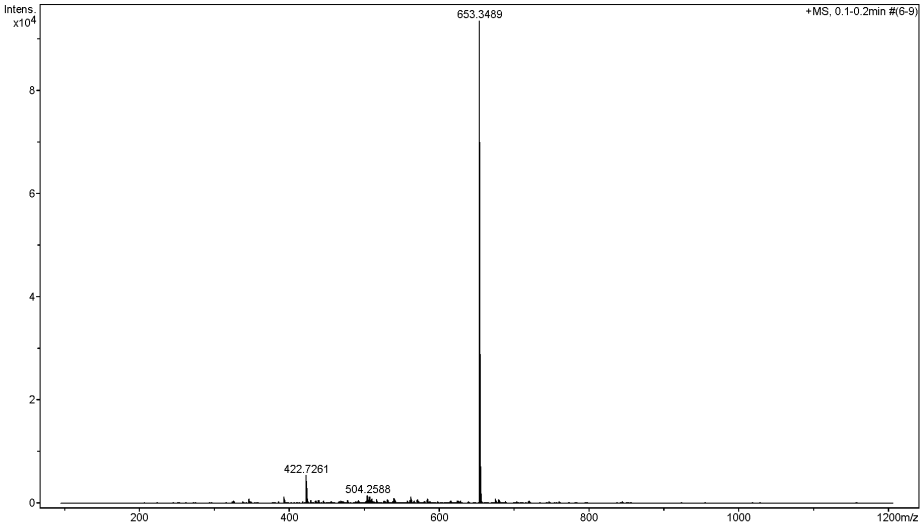

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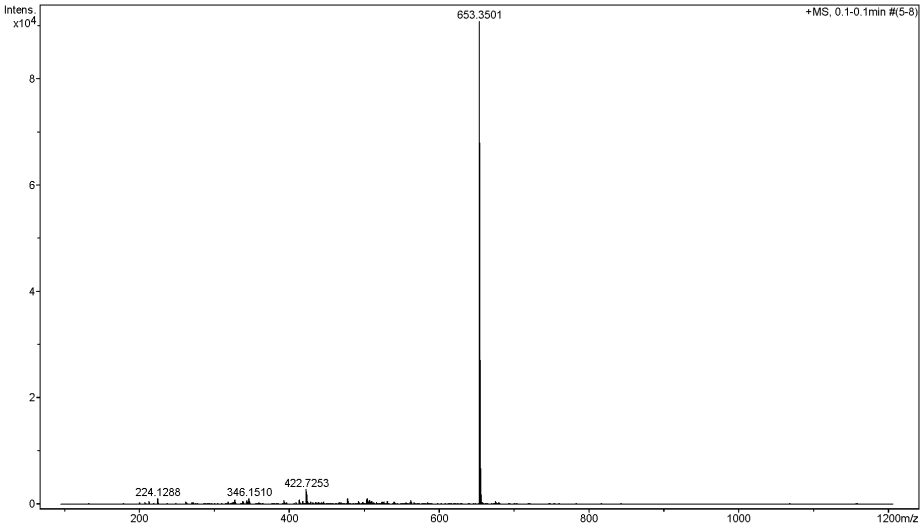

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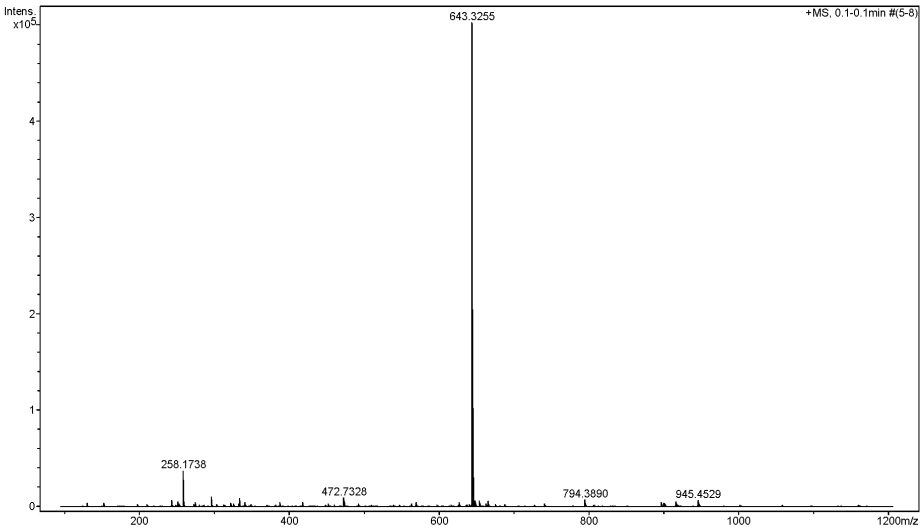

Analog 8: