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SUPPORTING INFORMATION

Detoxification of amyloid β fibrils by curcumin derivatives and their verification in *Drosophila* Alzheimer's model

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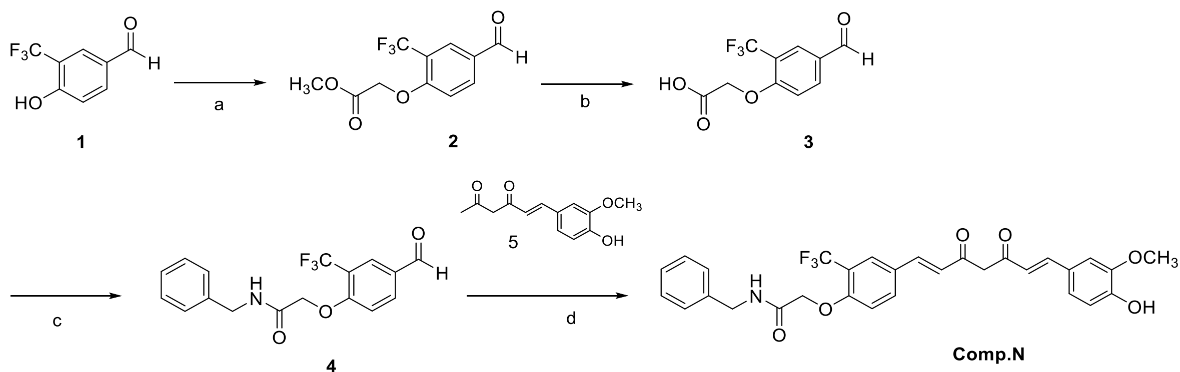

Scheme S1. Synthesis of compound **N**. Reaction condition: (a) acetone, methyl bromoacetate, K_2CO_3, 80 ºC, 94%; (b) NaOH 1 M, 100 ºC, 41%; (c) benzylamine, DMF, HOBt, DCC, DMAP, RT 24 h, 58%; (d) DMF, $B(OH)_3$, morpholine, MW 150 ºC, 15 min, 47%.

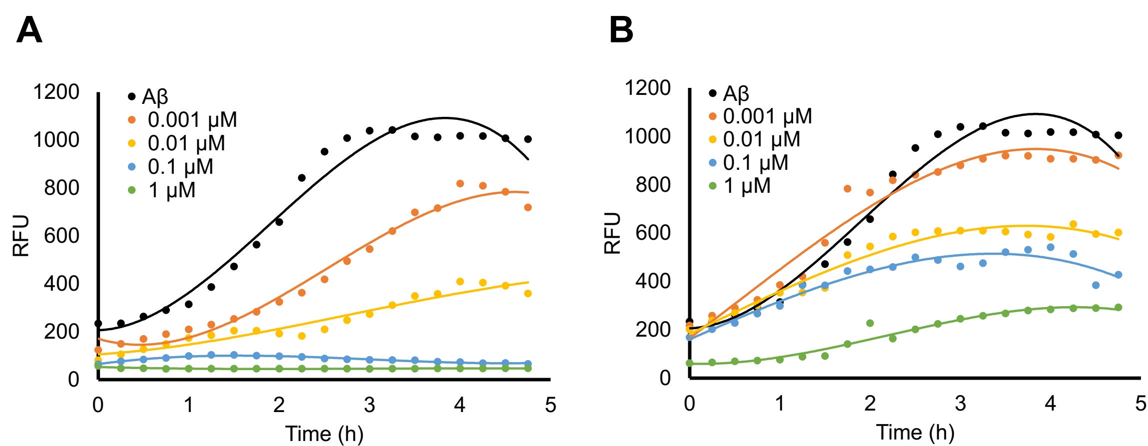

Figure S1. The aggregation kinetics of Aβ in the presence of comp. **B** (A) or comp. **N** (B) estimated by ThT assay. The Aβ (20 μM) was incubated with curcumin derivatives (0, 0.001, 0.01, 0.1, 1 μM) in PBS at pH 7.4 at 37 ºC.

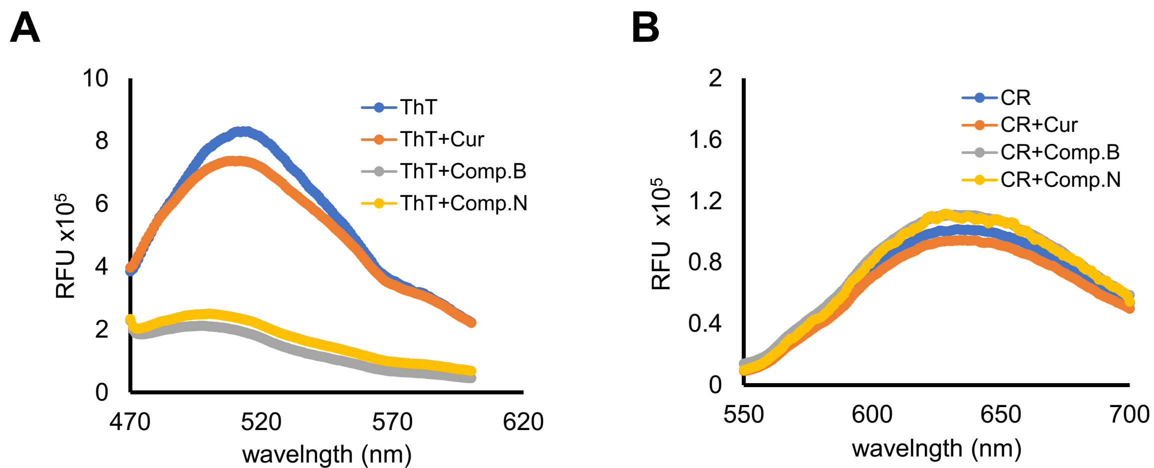

Figure S2. Quenching study of curcumin, compound **B**, and compound **N** in the presence of ThT (A) and CR (B).

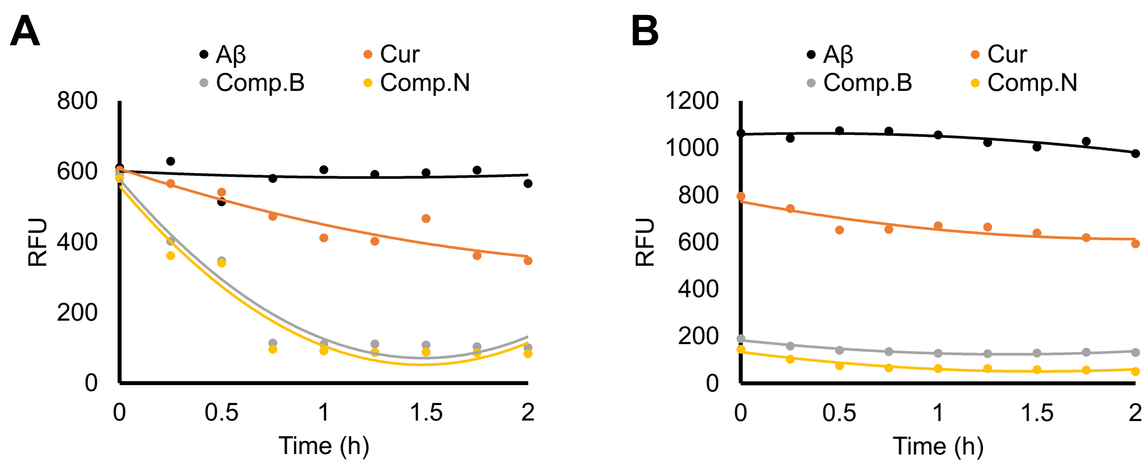

Figure S3. The disaggregation kinetics of Aβ fibril (10 μM) in the presence of curcumin derivatives (1 μM) monitored by CR assay (A) and ThT assay (B) in PBS at pH 7.4 at 37 °C.

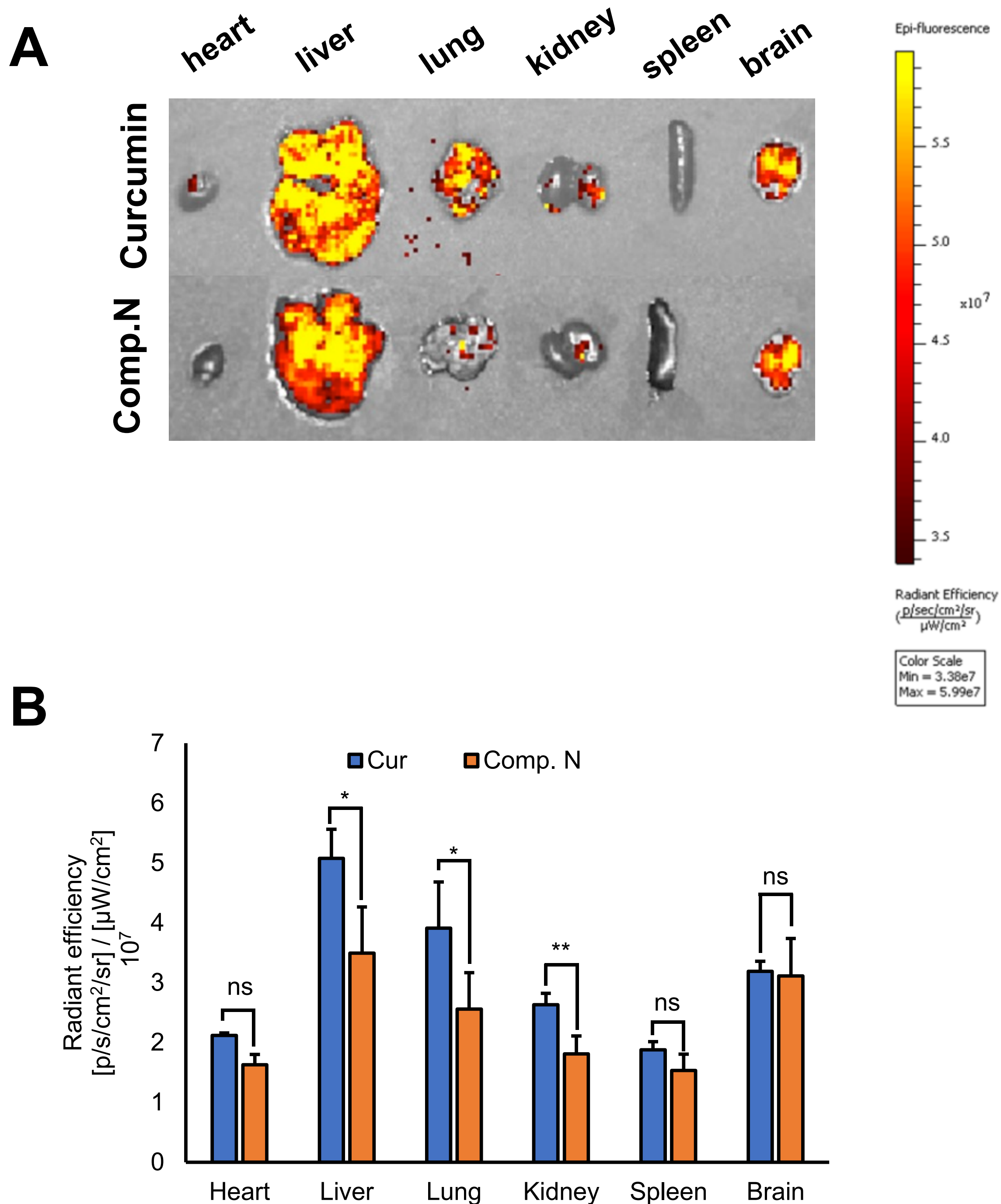

Figure S4. (A) *Ex vivo* fluorescence images of the heart, liver, lung, kidney, spleen, and brain of mice after 1 h injection. (B) Quantitative analysis of average radiant efficiency in each organ using IVIS imaging program. Data were expressed in mean ± SEM; *p <0.01, **<0.05 by two-sided Student's *t*-test, ns = not significant. Three mice were used for each imaging experiment.

Experimental Procedure

All the solvents used were in analytical standard grade. The NMR spectra were measured on a Bruker biospin AVANCE II (400 MHz for ^{1}H and 100 MHz for ^{13}C) or a Bruker biospin AVANCE III (500 MHz for ^{1}H, 125 MHz for ^{13}C, and 470 MHz for ^{19}F). Chemical shifts (δ) was reported in ppm relative to internal tetramethylsilane. The HRMS data were recorded on Bruker ESI-TOF-MS micrOTOF II instrument with sodium formate as the calibration standard. The negative stain transmission electron microscopy (TEM) images were measured by using TEM H-8100 (Hitachi) operated at 200 kV. Vanilin, potassium carbonate, morpholine, and boric acid were purchased from Wako Chemical (Japan). Acetylacetone, benzylamine, 4-dimetylaminopyridine, methyl bromoacetate, and hydroxybenzotriazole were purchased from Tokyo Chemical Industry (Japan). Microwave for synthesis was conducted on Biotage® Initiator+ instrument. Column chromatography was performed on silica gel Chromatorex (Japan). Purity analysis was determined by HPLC analysis using Inertsil ODS-3 5 µm (4.6 × 75 mm; GL Science) with a linear gradient of 0.1% formic acid in water/0.1% formic acid in MeCN detected by UV lamp for 30 min. Amyloid β ($A\beta_{42}$) peptide was purchased from Peptide Institute (Japan). Thioflavin t and congo red for fluorescence detection of $A\beta_{42}$ was purchased from Wako Chemical (Japan) and Sigma (USA), respectively.

General Procedure to Synthesis Compound 2

4-hydroxy 3-trifluoromethyl benzaldehyde (1 mmol) and potassium carbonate (3 mmol) were dissolved in DMF 10 mL. Methyl bromoacetate (1.1 mmol) was added then the mixture was refluxed for 2 h at 80 °C in oil bath. After filtration to remove the salt, the organic solvent was removed then the mixture was purified by column chromatography (silica gel, hexane:ethyl acetate 2:1).

Methyl 2-(4-formyl-2-(trifluoromethyl)phenoxy)acetate (2). Yield: 94%. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 3.82 ($-OCH_3$, s, 3H), 4.01 ($-OCH_3$, s, 3H), 4.76 ($-CH_2$, s, 2H), 6.88 (=CH, *J*=8.8, d, 1H), 8.05 (=CH, *J*=8.4, d, 1H), 8.18 (=CH, s, 1H), 9.96 (-CHO, s, 1H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 52.50, 65.46, 68.01, 112.86, 120.18, 121.39, 129.35, 134.95, 160.15, 167.76, 189.64. HRMS-ESI (m/z): calcd for $C_{11}H_{10}F_3O_4^+$ 263.0526; found 263.0535 $[M+H]^+$.

General Procedure to Synthesis Compound 3

Compound **2** (0.26 mmol) was dissolved in 0.5 mL methanol, then 2 mL of NaOH 1 M was added. The mixture was stirred for 30 min at 100 °C. The reaction was quenched by the addition of HCl 0.1 M then was extracted using ethyl acetate. The desired compound was purified by column chromatography (silica gel, hexane:ethyl acetate 2:1).

2-(4-formyl-2-(trifluoromethyl)phenoxy)acetic acid (3). Yield: 41%. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.08 (=CH, *J*=8.6, d, 1H), 8.09 (=CH, *J*=8.7, d, 1H), 8.21 (=CH, d, 1H), 9.99 (-CH, s, 1H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 64.91, 102.02, 112.68, 122.27, 125.09, 131.19, 131.59, 156.05, 170.16. HRMS-ESI (*m/z*): calcd for $C_{10}H_6F_3O_4^-$ 247.0224; found 247.0217 $[M-H]^-$.

General Procedure to Synthesis Compound 4

Compound **3** (0.5 mmol) was dissolve in DMF. To that solution Dicyclohexylcarbodiimide (1.5 mmol), hydroxybenzotriazole (0.5 mmol), N,N dimethyl amino pyridine (0.05 mmol) was added.

Benzylamine (0.6 mmol) added followed by stirring for 24 h. The white precipitate obtained was filtered and evaporated. The desired compound was purified by column chromatography (silica gel, hexane:ethyl acetate 2:1).

N-benzyl-2-(4-formyl-2-(trifluoromethyl)phenoxy)acetamide (4). Yield: 58%. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 4.67 ($-CH_2$, d, 2H), 4.82 ($-CH_2$, s, 2H), 5.41 (-NH, s, 1H), 7.12 (=CH, d, 2H), 7.24 (=CH, d, 1H), 7.31 (=CH, d, 2H), 7.45 (=CH, d, 1H), 8.20 (=CH, d, 1H), 8.26 (=CH, s, 1H), 10.01 (-CHO, s, 1H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 43.54, 67.96, 113.49, 127.77, 128.05, 129.13, 129.35, 130.52, 135.97, 137.53, 166.35, 189.61. HRMS-ESI (*m/z*): calcd for $C_{17}H_{14}F_3O_3Na^+$ 360.0818; found 360.0814 $[M+Na]^+$.

General Procedure to Synthesis Compound 5. Acetylacetone (10 mmol) and boric acid (10 mmol) were suspended in DMF. Vanillin (2 mmol) was added then followed by morpholine (2 mmol). The mixture was irradiated in microwave at 100 ºC for 10 min. The reaction mixture was quenched by HCl 0.1 N and extracted using ethyl acetate. The organic phase was dried using $MgSO_4$. The crude product was purified by column chromatography (silica gel, hexane:ethyl acetate 4:1) to obtain the desired compound.

(4-hydroxy-3-methoxyphenyl) hex-5-ene-2,4-dione (5). Yield: 65%. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 2.20 ($-CH_3$, s, 3H), 3.98 ($-OCH_3$, s, 3H), 5.68 (=CH, s, 1H), 5.87 (=CH, s, 1H), 6.37 (=CH, *J*=15.8, d, 1H), 6.97 (=CH, *J*=8.2, d, 1H), 7.06 (=CH, *J*=1.8 Hz, d, 1H), 7.13 (=CH, *J*=8.2, 1.8 Hz, dd, 1H), 7.58 (=CH, *J*=15.8 Hz, d, 1H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 26.59, 55.86, 100.69, 111.39, 115.88, 119.87, 123.09, 126.55, 140.47, 148.19, 149.41, 178.48, 196.85. LRMS-ESI (*m/z*): calcd for $C_{13}H_{14}O_4$ 234.09; found 235.13 $[M+H]^+$

General Procedure to Synthesis Compound N

Compound **5** (0.26 mmol) and boric acid (0.26 mmol) was suspended in DMF. Compound **4** (0.26 mmol) was added followed by morpholine (0.1 mmol). The mixture was irradiated by microwave at 150 ºC for 10 min. The HCl 0.1 N was added to quench the reaction. Crude product was extracted by ethyl acetate then dried using $MgSO_4$. The desired compound was purified by column chromatography (silica gel, hexane:ethyl acetate 2:1).

N-benzyl-2-(4-((1E,6E)-7-(4-hydroxy-3-methoxyphenyl)-3,5-dioxohepta-1,6-dien-1-yl)-2-(trifluoromethyl)phenoxy)acetamide (N). Yield: 47%. ^{1}H-NMR (400 MHz, CD_2Cl_2): δ (ppm) 3.97 ($-CH_3$, s, 3H), 4.56 ($-CH_2$, d, 2H), 4.69 ($-CH_2$, s, 2H), 5.89 ($-CH_2$, s, 2H), 6.57 (=CH, d, 1H), 6.64 (=CH, d, 2H), 6.92 (=CH, d, 2H), 7.09 (=CH, d, 1H), 7.14 (=CH, d, 1H), 7.17 (=CH, d, 1H), 7.19 (=CH, d, 1H) 7.3-7.4 (=CH, d, 5H), 7.62 (=CH, d, 1H), 7.65 (=CH, d, 2H), 7.75 (=CH, d, 1H), 7.85 (=CH, s, 1H), 8.01 (-NH, s, 1H). ^{13}C-NMR (125 MHz, DMSO-d_6) 42.46, 56.19, 68.02, 101.89, 111.89, 115.01, 116.15, 118.24 ($J_{c\text{-}f}$= 30, q), 121.55, 123.88, 124.36 ($J_{c\text{-}f}$= 270.8, q), 124.39, 126.69, 127.37 ($J_{c\text{-}f}$= 4.2, q), 127.71, 128.33, 128.79, 134.15, 138.36, 139.39, 141.97, 148.47, 150.01, 157.41, 167.16, 182.04, 185.15. ^{19}F-NMR (475 MHz, DMSO-d_6): δ (ppm) 60.68. HRMS-ESI: *m/z* calcd for $C_{30}H_{25}F_3NO_6^-$ 552.1639; found 552.1630 $[M-H]^-$. HPLC Purity 98.1%, retention time 6.20 min.

Preparation of Aβ monomer and fibril

Procedure on solubilizing the Aβ was conducted according the known literature by slightly modification[1]. Isoform of Aβ used in this study is the Aβ with 42-amino acid residue. To prepare the Aβ monomer stock, in amount 0.5 mg of the lyophilized Aβ (Peptide Institute) was dissolved in NaOH 2 mM by gently mixing without vortexing to obtain 500 μM as final concentration. The solution was centrifuged at 13,200 rpm, 4°C, and 10 min. Aβ fibril was prepared by incubating Aβ at concentration 20 μM in PBS pH 7.4 for 24 h at 37 °C.

Thioflavin T Assay

The Thiofalvin T (ThT) stock at concentration 2 mM was freshly prepared in tris glycine 10 mM pH 8.5. To observe the aggregation kinetics of Aβ, ThT was diluted by PBS pH 7.4 to reach final concentration of 40 μM. The Aβ was added to get final concentration of 20 μM then incubated at 37 °C for stimulate the aggregation process which monitored based on the fluorescence intensity measured at Ex/Em 430/480 nm using microplate reader (Tecan Infinite F200, Tecan, Switzerland). This assay was conducted in 384-well plate full black non-binding with frequent 15s of linear shaking before measurement. For the determination of inhibitory activity of aggregation process, curcumin derivatives (0, 0.001, 0.01, 0.1, 1 μM) was added and incubated along with Aβ following the previous reported.[2] To monitor the disaggregation kinetics, the Aβ fibril (20 μM) was incubated with ThT (40) and curcumin derivatives (1 μM), then the fluorescence intensity was measured at Ex/Em 430/480 nm. All samples were measured in triplicate.

Congo Red Assay

Congo Red (CR) stock at concentration 2 mM was freshly prepared in tris glycine 10 mM pH 8.5, then diluted in PBS at pH 7.4 to reach final concentration 40 μM. Curcumin derivatives was added at final concentration 10 μM followed by Aβ fibril (20 μM) then was incubated at 37 °C. After 24 h, the mixture was transferred in 384-well plate black bottom non-binding. For wavelength scanning, the fluorescence intensity was measured at an excitation of 490 nm and emission range from 510 to 700 nm while for the disaggregation kinetics the fluorescence intensity was measured at Ex/Em 490/619 nm. Fluorescence intensity was measured using microplate reader (SpectraMax iD5, Molecular Device, USA). All samples were measured in triplicate.

Quenching Study

The ThT or CR stock at concentration 2 mM was diluted into 40 μM in PBS at pH 7.4. Curcumin derivatives (1 μM) was added in the mixture then fluorescence spectra were recorded with excitation of 430 nm and emission range from 450 to 500 nm for ThT and excitation of 490 nm and emission range from 510 to 700 nm for CR. Fluorescence intensity was measured using microplate reader (SpectraMax iD5, Molecular Device, USA).

Negative Stain TEM

Elastic carbon grids (ELS-C10, STEM, Japan) was hydrolysed by ion coater (IB-2, Eiko, Japan) with 3 mA of plasma current for 40 s before applying sample solution. Briefly, 5 μL of Aβ solution with

or without curcumin derivatives was applied to a hydrophilic grid and incubated for 1 min at RT. After gently dried with filter paper, the grid sample was washed with Milli-Q water and dried again with filter paper two times. Finally, the grid was incubated with 5 µL of 1% Nano-W negative staining solution (NY, USA) for 1 min followed by complete drying using filter paper. The negative stained sample was observed using TEM H-8100 (Hitachi) operated at 200 kV.

Cell Culture

The N2a cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum. Briefly, N2a cells (5 x 10^3 cells/well) was grown on 96-well plate overnight at 37 ºC CO_2 5%. For the evaluation of protective effect was conducted by treating the N2a cells with monomeric Aβ (20 µM) and Aβ fibril (10 µM) in the absence or presence of curcumin derivatives 1 µM.

MTT Assay

An MTT reduction assay was conducted as described previously[3]. Briefly, MTT powder was dissolved in PBS pH 7.4 to obtain 5 mg/mL concentration stock, then was diluted into 0.5 mg/mL in DMEM medium. After removal of medium on 96-well plate containing-treated cell, each well was added by 100 µL MTT 0.5 mg/mL and incubated at 37 ºC CO_2 5%. After 3 h incubation, the medium was removed and 100 µL DMSO was added following the absorbance measurement at wavelength of 550 nm using microplate reader (Tecan Infinite F200, Tecan, Switzerland). Calculation of % cells viability was measured by dividing the absorbance of untreated cells with the absorbance of curcumin-treated cells after absorbance correction.

Immunofluorescence Staining

N2a (1 x 10^4 cells/well) was cultured on cover glass in 6-well plate overnight at 37 ºC CO_2 5%. Aβ fibril was prepared by incubating Aβ at concentration 20 µM in PBS pH 7.4 for 24 h at 37 ºC. Cells were treated by Aβ fibril (10 µM) and curcumin derivatives (1 µM) and incubated for 24 h. After washing with PBS, cells were fixed by 4% paraformaldehyde for 20 min. Cells were washed again by PBS before permeabilized by 0.1% Triton X-100 in PBS for 10 min. After additional washing by PBS, cells were blocked by 2% BSA in PBS for 30 min. After further washing by PBS, it was followed by incubation with primary anti-Aβ fibril (OC) monoclonal antibody conjugated with fluorescence dye ATTO594 (1:100 in 2% BSA, StressMarq; SPC-507D) for 1 h. Non-specifically bound primary antibody was washed by PBS before the incubation with primary anti-β-actin monoclonal antibody (1:300 in 2% BSA, santa cruz; sc-47778) for 1 h followed by 1 h incubation with secondary antibody anti-mouse IgG (1:300 in 2% BSA, santa cruz: sc-2010). Cells were washed again by PBS and the nucleus was stained by Hoechst 33342 (dojindo; 346-07951). All images were recorded by a Zeiss LSM 780 confocal microscope.

Brain Permeability Study

Curcumin and Compound **N** was dissolved in DMSO followed by the addition of kolliphor then stirred for 30 min. The mixture was added by PBS until the concentration of curcumin, DMSO, and

kolliphor reached 1 mM, 1%, and 15%, respectively. After stirring for 15 min, the mixture was sonicated for 15 min to homogenize the emulsion. The emulsion was sterilized by filtration and stored in room temperature until used. The Slc:ddY mice was injected intravenously from tail with emulsion. After 1 h, mice were sacrificed and collected its heart, liver, lung, kidney, spleen, and brain. After gently washing the organs with saline, the fluorescence profile was measured by IVIS imaging with Ex/Em 430/520 for curcumin and 430/540 for compound **N**. All protocols for *in vivo* study were approved by the Institutional Animal Care and Use Committee of Tokyo Institute of Technology.

Fly Strains

Flies were maintained at 25°C on standard fly food. Male flies were used in the experiment. *Elav-Gal* (#458) was obtained from the Bloomington *Drosophila* Stock Center (BDSC, Bloomington, IN, USA). *UAS-Aβ1-42#1* and *UAS-Aβ1-42#3* were kindly provided by Dr. Tsuda.[3]

Locomotor behavior

The climbing assay was based on a method previously described.[4] In brief, flies were reared at 25 °C, and adult flies one to five days old were collected and placed in a vial containing a compound. The compounds were 25 µM of curcumin, compound **B**, or compound **N** in Formula 4-24 Blue *Drosophila* Medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). After one day or two weeks at 25 °C, locomotor behavior was measured by climbing assay. 10 flies were distributed in an empty vial and allowed to adapt for 5 min at room temperature. The flies were tapped down to the bottom. Then flies that climbed above 4 cm in 10 s were counted and the percentage was calculated. This ratio was defined as the climbing index in this study. After resting for 1 min, the above procedure was performed 5 times per vial and the average was obtained. Statistical analyses were performed using Prism 9 (GraphPad Software, San Diego, CA, USA). Data were analysed using multiple comparison ANOVA with the Kruskal-Wallis test and Dunn's multiple comparisons between groups. The null hypothesis was rejected at a 0.05 level of significance.

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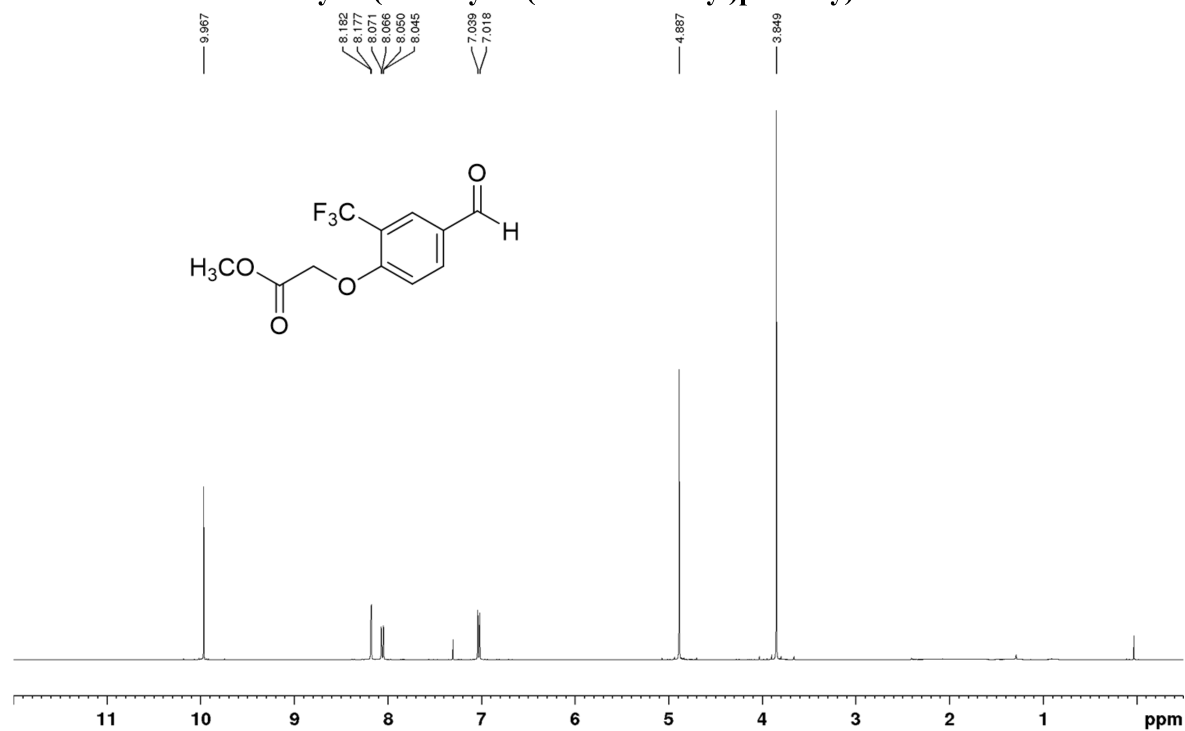
^{1}H-NMR Profile of methyl 2-(4-formyl-2-(trifluoromethyl)phenoxy)acetate



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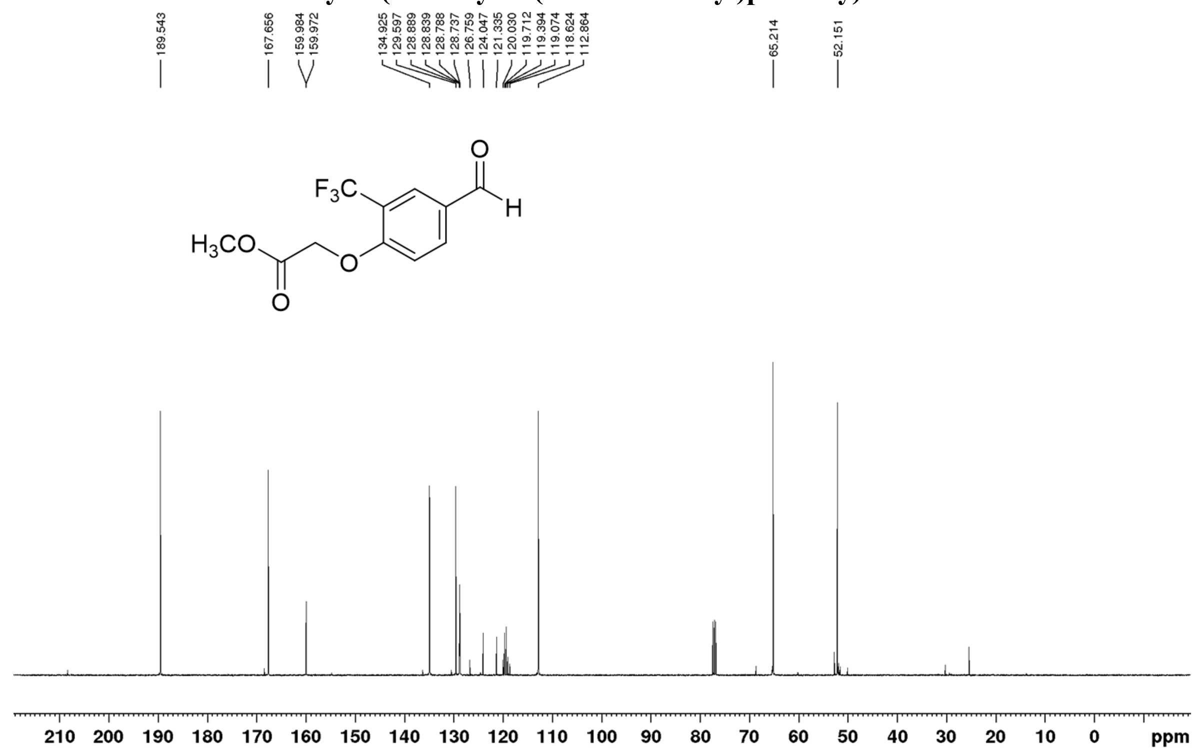

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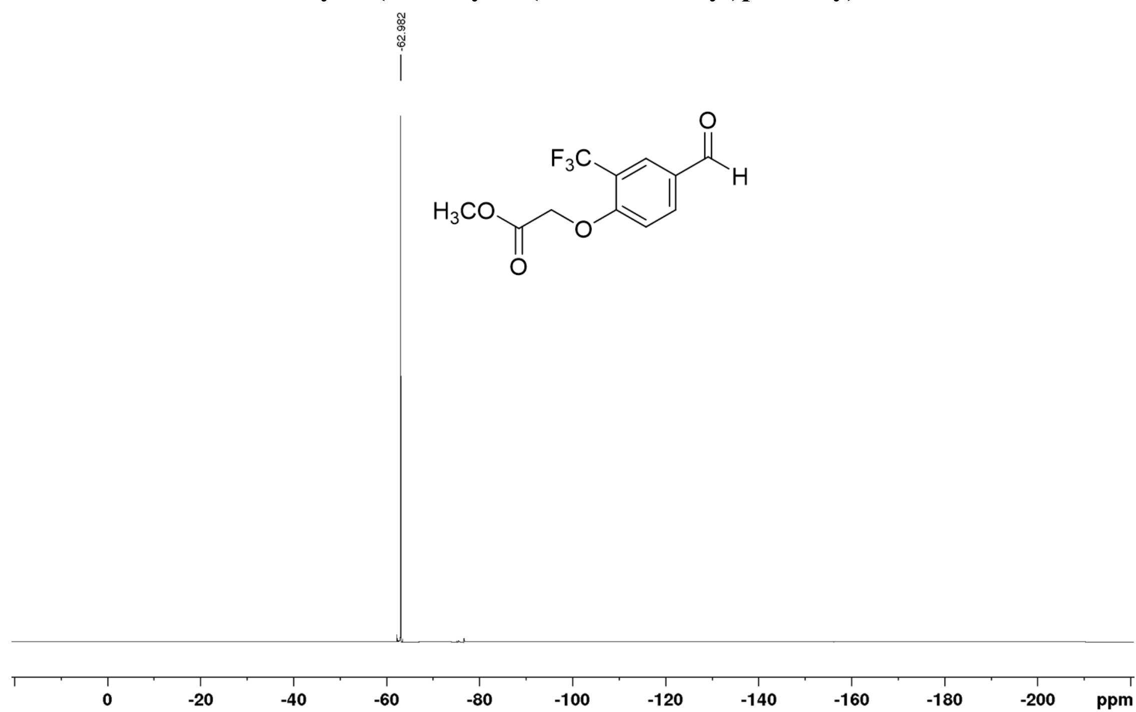

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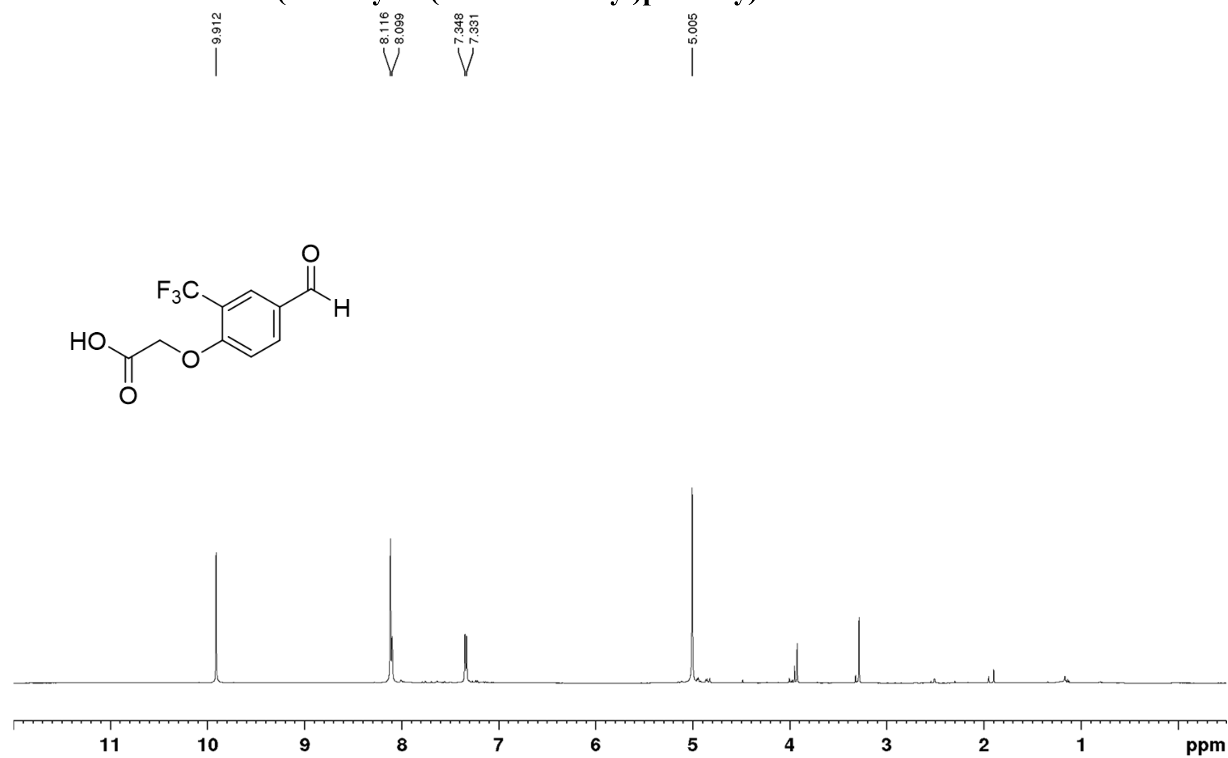

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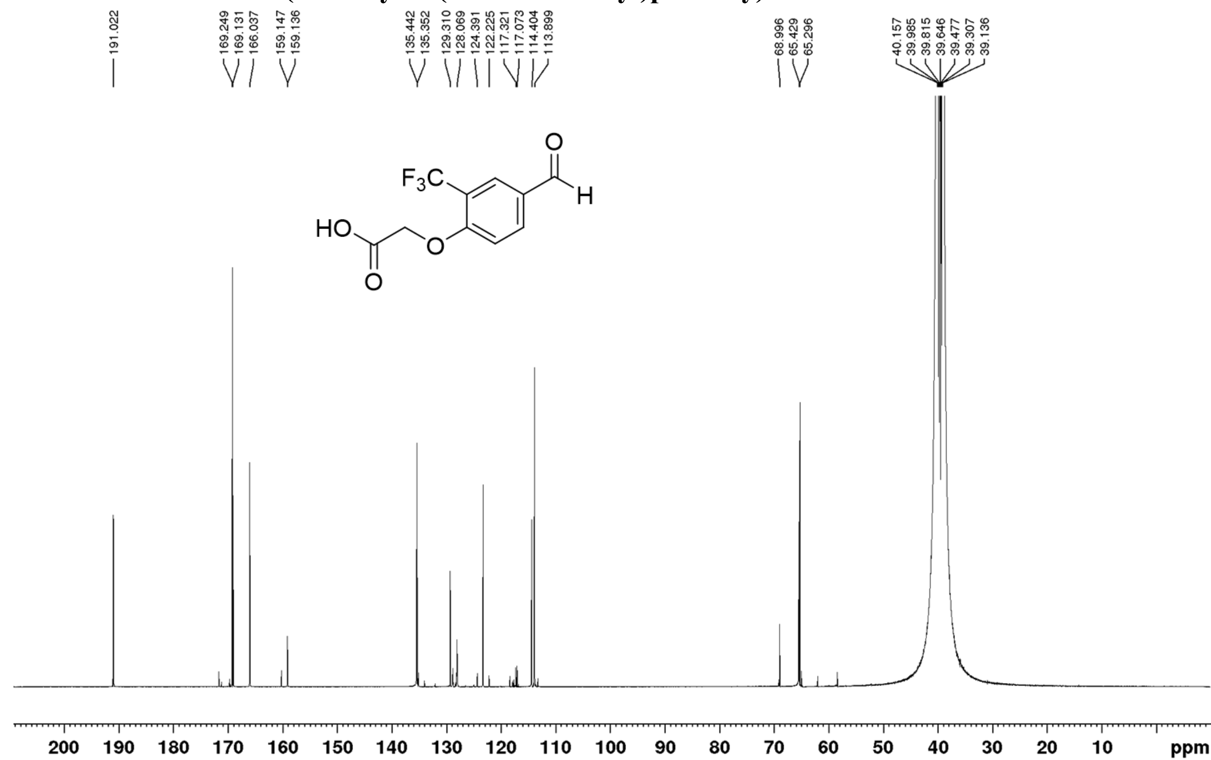

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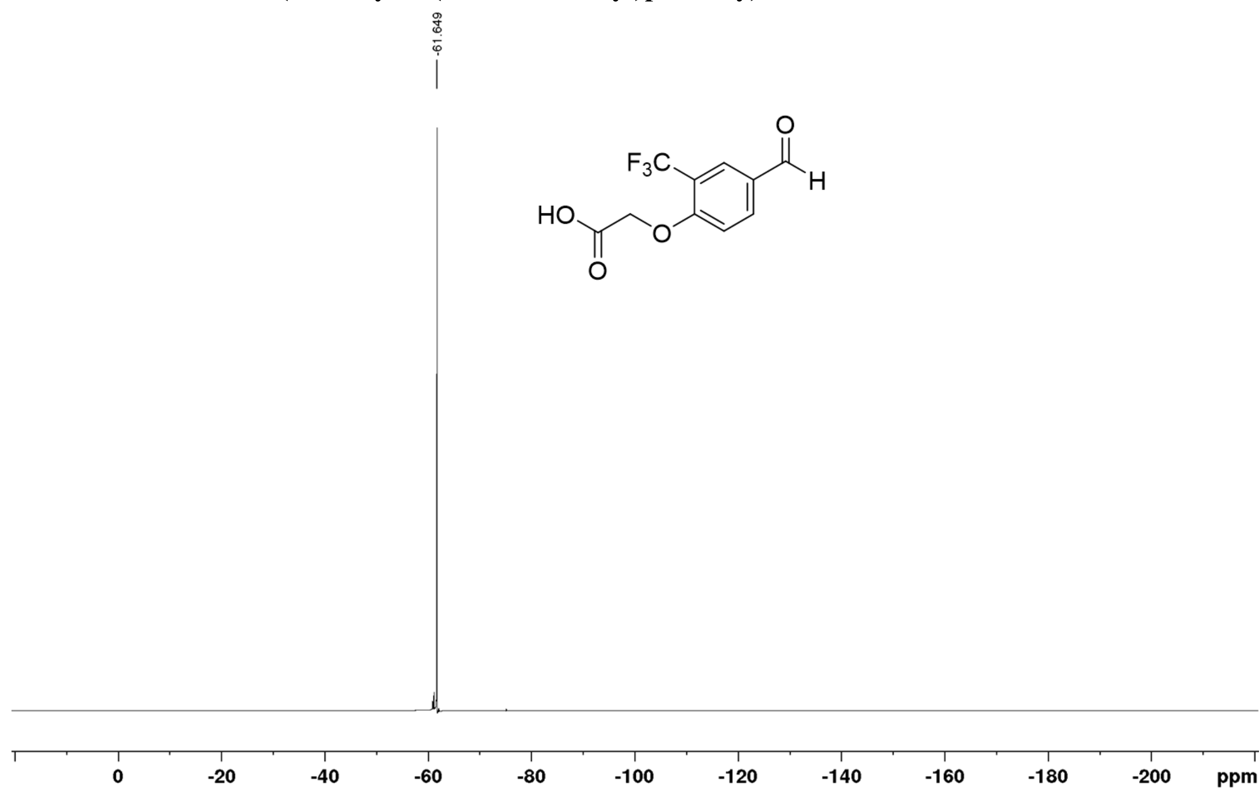
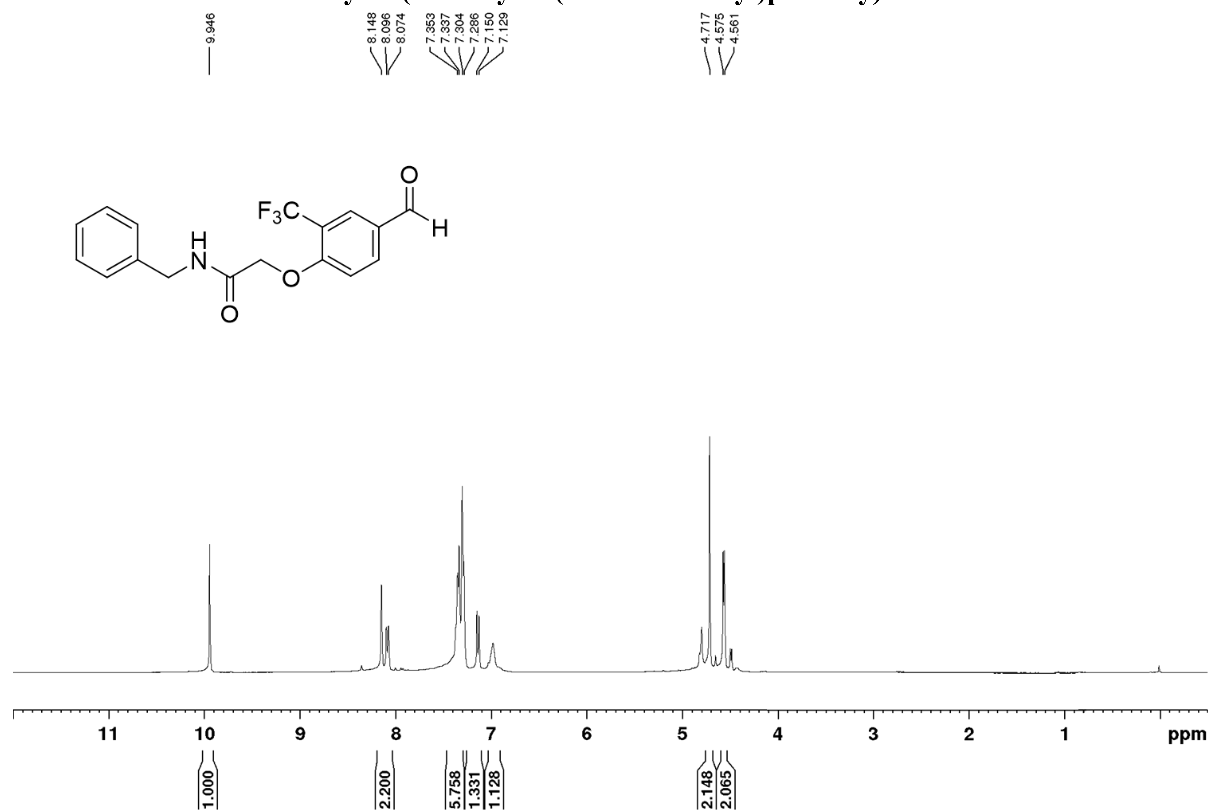


^{1}H-NMR Profile of N-benzyl-2-(4-formyl-2-(trifluoromethyl)phenoxy)acetamide



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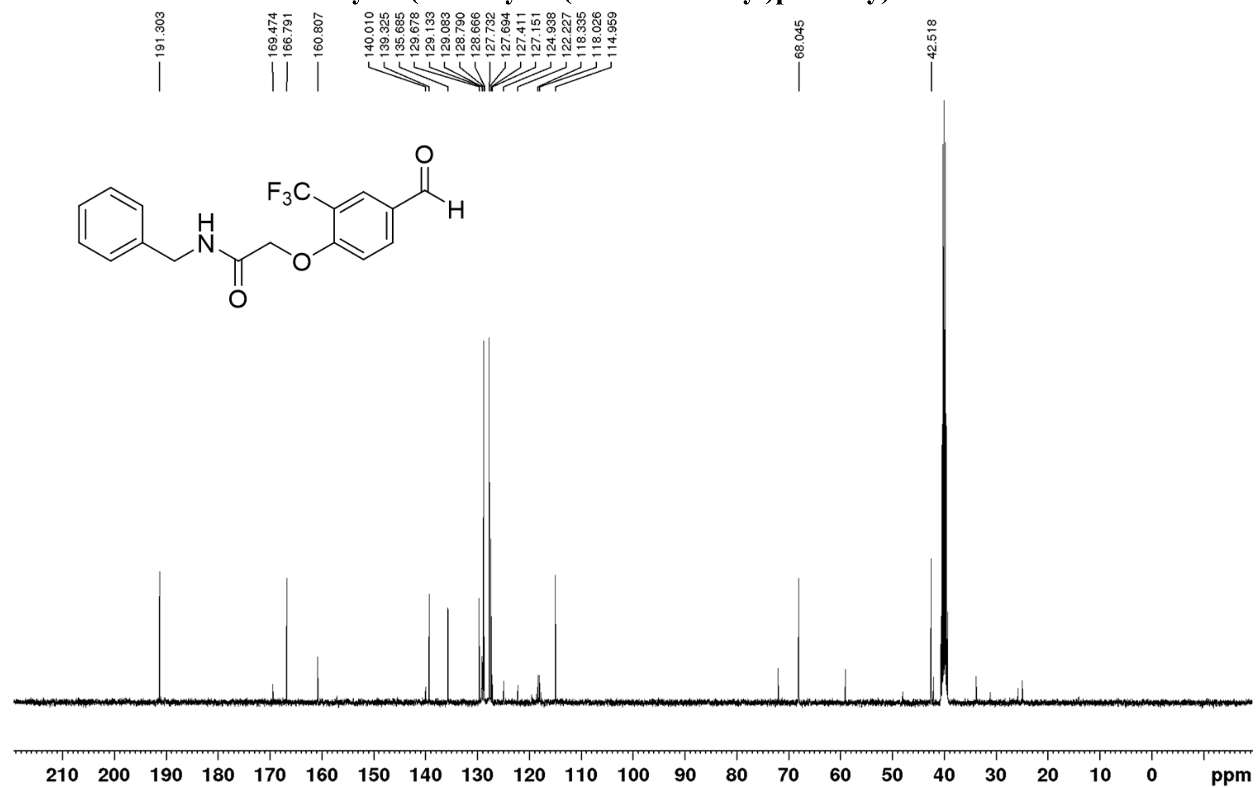

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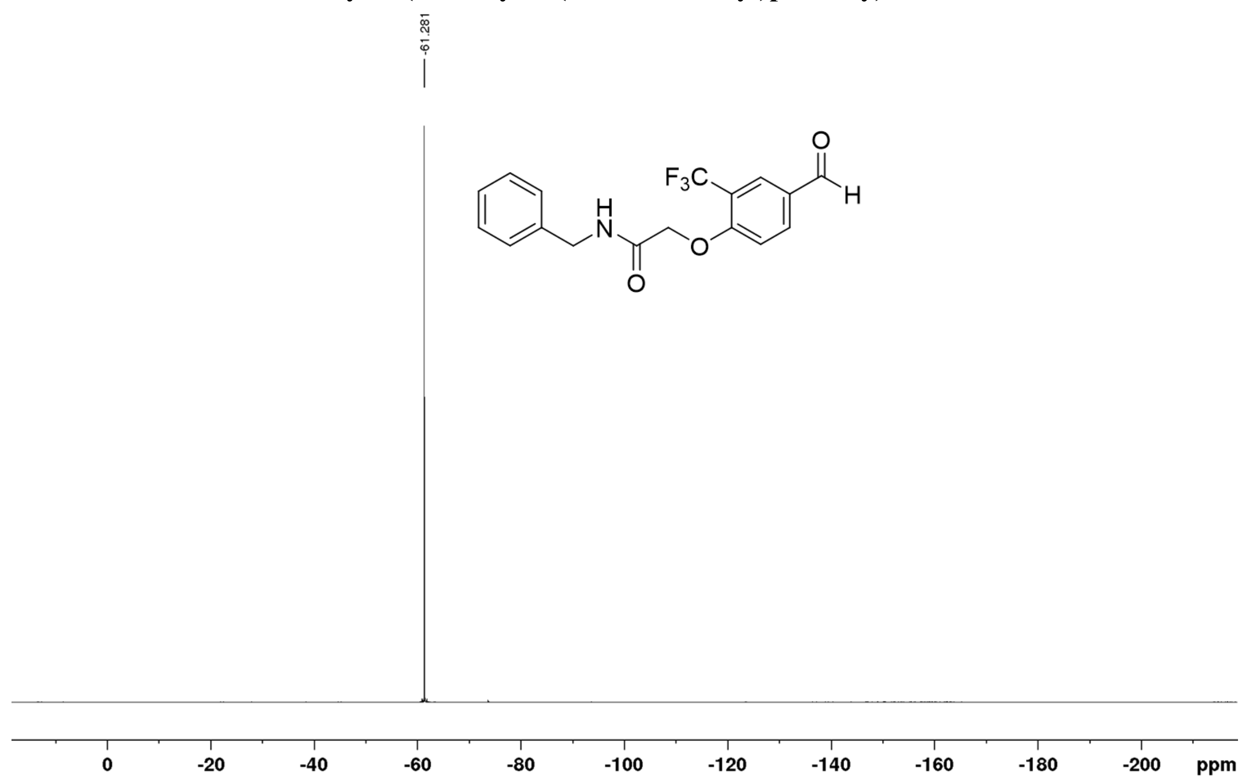
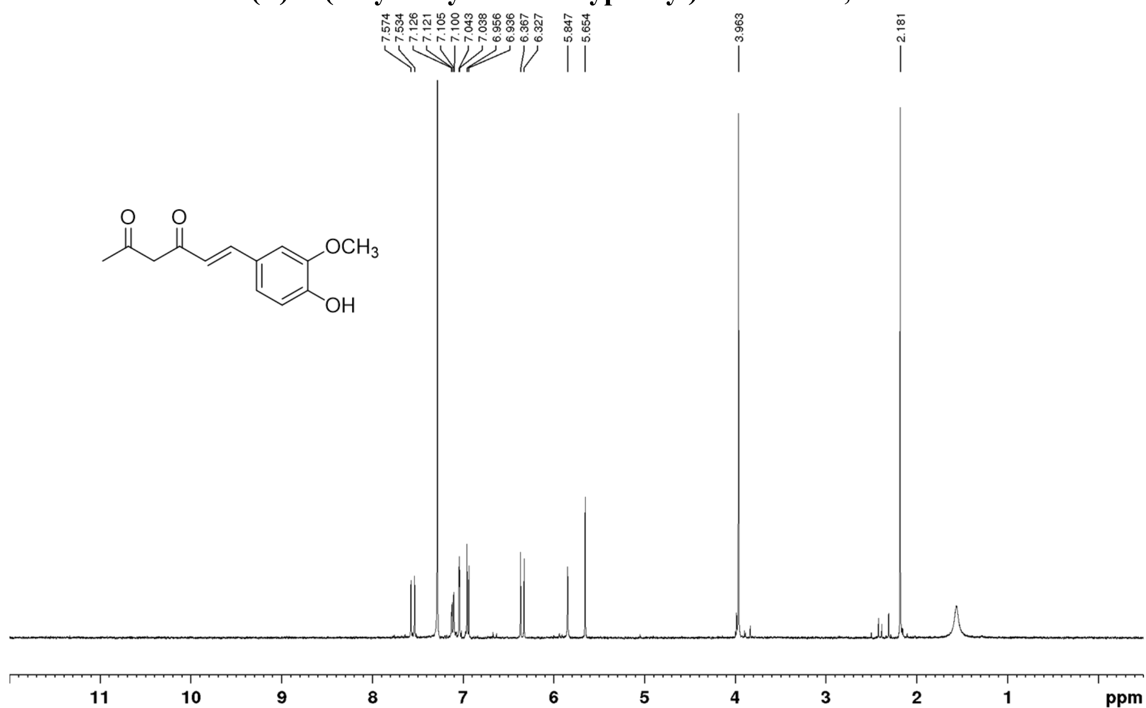


^{1}H-NMR Profile of (E)-6-(4-hydroxy-3-methoxyphenyl)hex-5-ene-2,4-dione



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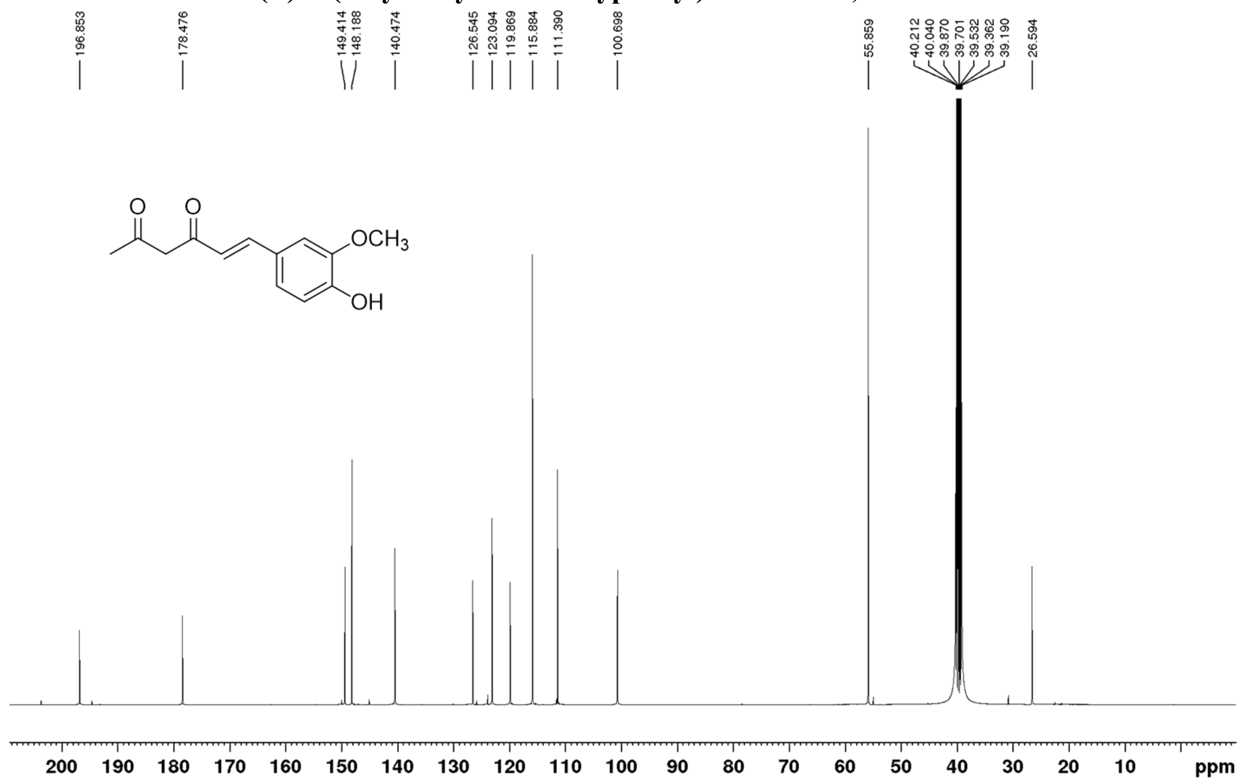
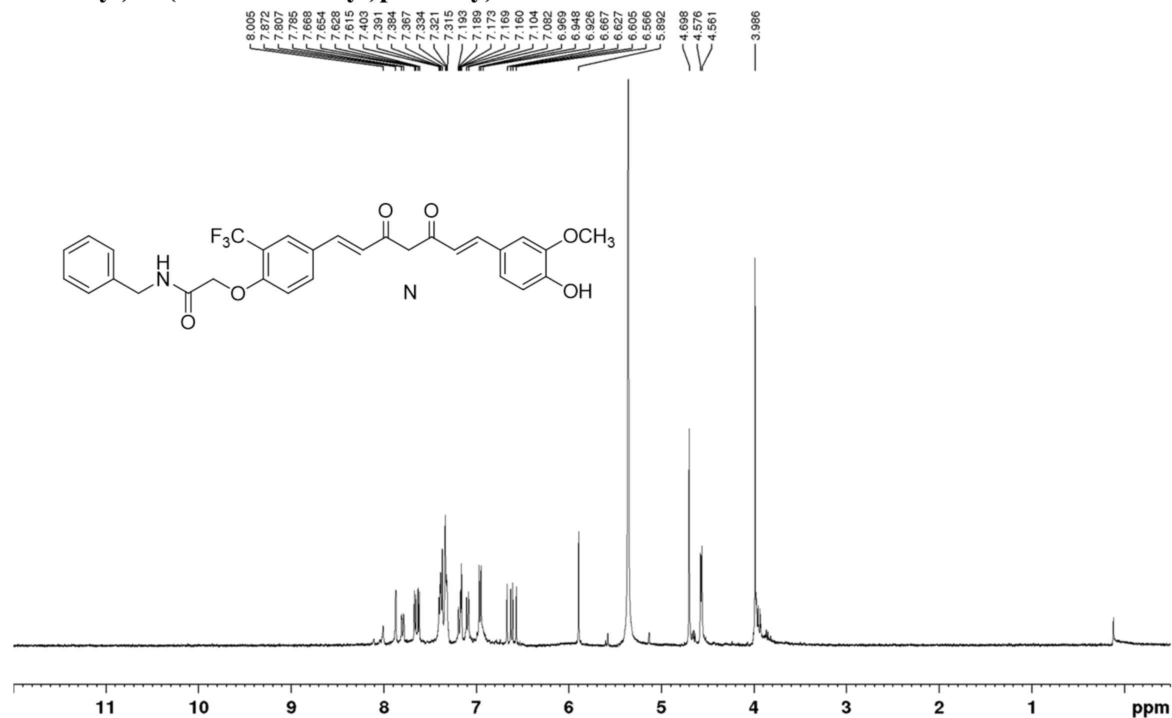
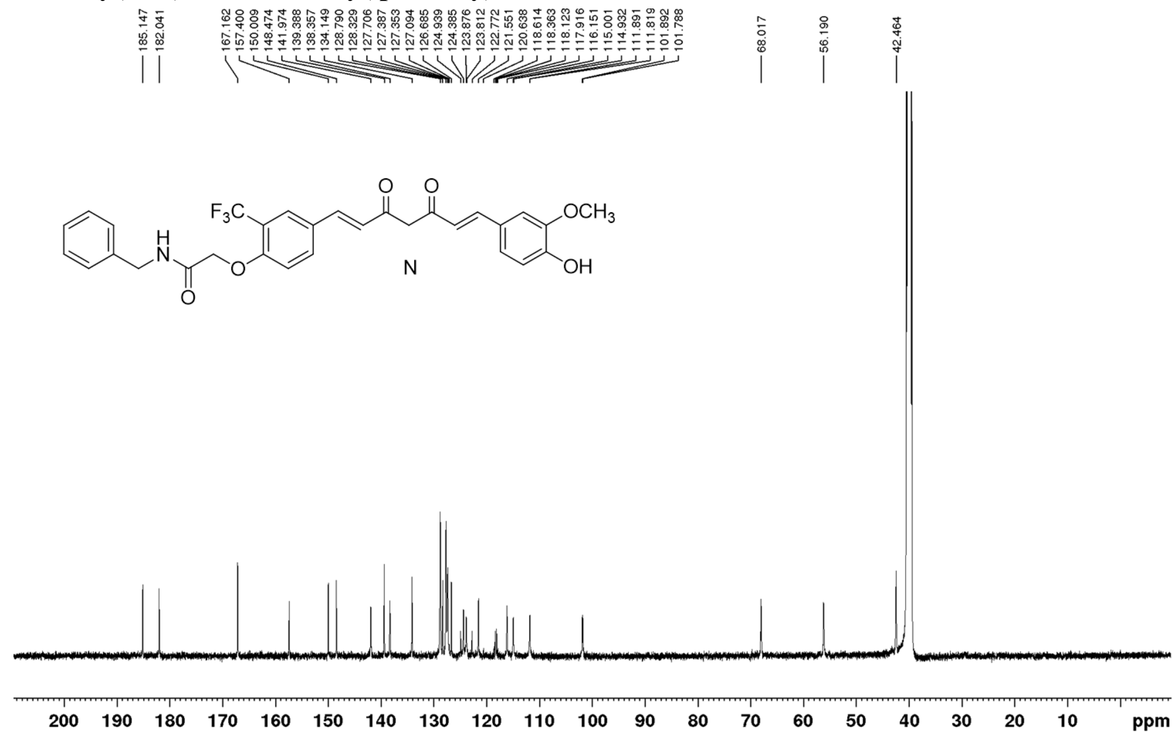


^{1}H-NMR Profile of N-benzyl-2-(4-((1E,6E)-7-(4-hydroxy-3-methoxyphenyl)-3,5-dioxohepta-1,6-dien-1-yl)-2-(trifluoromethyl)phenoxy)acetamide



^{13}C-NMR Profile of N-benzyl-2-(4-((1E,6E)-7-(4-hydroxy-3-methoxyphenyl)-3,5-dioxohepta-1,6-dien-1-yl)-2-(trifluoromethyl)phenoxy)acetamide



^{19}F-NMR Profile of N-benzyl-2-(4-((1E,6E)-7-(4-hydroxy-3-methoxyphenyl)-3,5-dioxohepta-1,6-dien-1-yl)-2-(trifluoromethyl)phenoxy)acetamide

HPLC Profile of Compound **N**