ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

Kinetically-controlled mechanism-based isolation of metabolic serine hydrolases in active form from complex proteomes: butyrylcholinesterase as a case study.

Hui Liu, Huimin Zhou, Huaqiao Du, Qiaoling Xiao and Marco Pistolozzi*
School of Biology & Biological Engineering, South China University of Technology, Higher Education Mega Center, Guangzhou, People's Republic of China
*E-mail: marco_pistolozzi@scut.edu.cn

Contents

S1. Chemicals, reagents and solvents.
S2. General methods for the chemical synthesis.
S3. Synthesis of the solid-supported probes.
S3.1 Resin activation with p-nitrophenyl chloroformate (**5**) (Strategies 1 & 2).
S3.2 Synthesis of sepharose-6Ahx-TTD-TBT (**6a**) (Strategy 1).
S3.3 Synthesis of sepharose-6Ahx (**7**) (Strategy 2).
S4. Determination of the amount of active carbonate groups on the resin (Strategies 1 & 2)
S5. Measurement of hBChE activity
S6. Sample preparation for the proteomic analysis
S7. Scheme S1
S8. ^{1}H -NMR, ^{13}C-NMR and HRMS spectra.

S1. Chemicals, reagents and solvents

6-aminohexanoic acid (6Ahx), Boc-6-aminohexanoic acid (Boc-6Ahx), boric acid, N,N'-carbonyldiimidazole (CDI), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), dioxane, hexanoic acid (Hx), iodoacetamide (IAA), potassium ferricyanide, sodium salicylate (SAL) , tetramethylammonium chloride (4MAC) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Aladdin (Shanghai, PRC).

Ammonium bicarbonate, ammonium persulfate (AP), bovine serum albumin (BSA), S-butyrylcholine iodide (BTCh), chloroform-d ($CDCl_3$), 4-(dimethylamino)pyridine (DMAP), 5-5'-dithiobis(2nitrobenzoic acid) (DTNB), formic acid (HPLC grade), glycerol, *rac*-ibuprofen sodium (IBU), methanol-d_4 (CD_3OD), p-nitrophenyl chloroformate (4NPCF), Sepharose CL-4B, N,N,N′,N′-tetramethylethylenediamine (TEMED) and 4,7,10-trioxa-1,13-tridecanediamine (TTD) were purchased from Sigma-Aldrich (Shanghai, PRC).

Acetonitrile (ACN), ammonium acetate, dichloromethane (DCM), ethanol (EtOH), isopropanol (IPA) and methanol (MeOH) were all HPLC grade and were purchased from Fisher Scientific (Loughborough, UK).

Acetone, acetic acid (HAc), hydrochloric acid (HCl), formaldehyde, potassium carbonate, sodium acetate (NaOAc), sodium chloride (NaCl), sodium citrate dihydrate, sodium hydroxide (NaOH), sodium phosphate monobasic dihydrate ($NaH_2PO_4 \cdot 2H_2O$), sodium phosphate dibasic dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$), sodium sulfate and sodium thiosulfate were purchased from Damao Chemical Reagent Factory (Tianjin, PRC)

Ammonia solution (NH_4OH, 25%), copper sulfate, ethanolamine, maleic acid and sodium tetraborate pentahydrate were purchased from Kelong Chemical Reagent Factory (Chengdu, PRC)

Rac-bambuterol hydrochloride (BMB) and *rac*-terbutaline hemisulfate (TBT) were kindly provided by Dongguan Keyphar (Dongguan, PRC).

Trifluoroacetic acid (TFA, LCMS grade) was purchased from Anaqua Chemicals Supply (Shanghai, PRC). Bromophenol blue, glycine, 30% polyacrylamide (37.5:1) and tris basic, were from Sangon Biotech (Shanghai, PRC). Leucine enkephalin was purchased from Waters (Shanghai, PRC), silver nitrate from Kemiou (Tianjin, PRC), and trypsin (sequencing grade modified, porcine) from Promega (Beijing, PRC).

S2. General methods for the chemical synthesis

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Ascend (400 MHz for ^{1}H, 101 MHz for ^{13}C) spectrometer in the indicated solvents.

ESI-TOF MS spectra were measured on a Thermo Fisher LTQ OrbitrapXL. The reactions were monitored by thin layer chromatography (carried out on Huanghai TLC HSGF254, 0.2mm, using DCM/MeOH 90/10 containing NH_4OH 0.25% w/v as mobile phase and UV light and 0.3% ethanolic ninhydrin containing 1% HAc for detection) and HPLC (see below)

Preparative normal phase chromatography separations were performed using silica gel 60 N (spherical, 200-300 mesh) (Qingdao ocean Chemical Co., LTD). Semi-preparative reverse phase chromatography separations were performed using Copure C18 SPE cartridges (2 g) fitted on a SPE vacuum apparatus. Analytical reverse phase chromatography was carried out using a Shimadzu LC-20A HPLC system equipped with a Waters XBridge BEH C18 (130Å, 5 μm, 100 mm x 4.6 mm i.d.), a guard column XBridge BEH C18 Sentry Guard Cartridge (130Å, 5 μm, 20 mm x 4.6 mm i.d.) and a SPD-M20A PDA detector. Mobile phases were based on ammonium acetate (50 mM, pH 4.6) (mobile phase A) and MeOH (mobile phase B). Separations were carried out using isocratic elution (A/B 50/50), unless otherwise noted.
Moisture-sensitive reactions (i.e., the resin activation reaction with 4NPCF and the synthesis of compound **1**) were carried out using oven-dried glassware and organic solvents with reduced water content. Anhydrous ACN and IPA were prepared by storing the commercial solvents over a 1-1.5 cm layer of molecular sieves (ACN, 3Å, IPA, 4Å) for at least 24 h. The water content of acetone was reduced by storing the commercial solvent over a 1-1.5 cm layer of calcium sulfate (4-8 h with occasional shaking), followed by filtration and storage over a 1.5-3 cm layer of molecular sieves (4Å). The molecular sieves were activated by microwave heating (Panasonic NN-GM333W, 400W, 1 min × 5 times). The solvents stored over molecular sieves were filtered before use.

S3. Synthesis of the solid-supported probes.

S3.1 Resin activation with p-nitrophenyl chloroformate (5) (Strategies 1 and 2)

Sepharose CL-4B (~10 g wet weight) was sequentially washed on a sintered glass funnel with 100 mL of each of the following ice-cold solutions: 30/70(v/v) acetone/water; 70/30(v/v) acetone/water; 100% acetone; anhydrous acetone; anhydrous ACN. The resin was suction-dried, then 6.2 g were suspended in a solution of p-nitrophenyl chloroformate (4NPCF, 371.5 mg, 1.54 μmol) in 5 mL of ice-cold anhydrous ACN. A suspension of 4-(dimethylamino)pyridine (DMAP, 697.8 mg, 5.76 μmol) in 5 mL of ice-cold ACN was added dropwise to the reaction mixture over 10 min, while gently stirring with a glass rod. The rod was washed with 2 mL of ice-cold anhydrous ACN, then the reaction vessel was capped, placed on a rocking table and tumbled end-over-end for 50 min at 4°C. The resulting p-nitrophenyl carbonate-sepharose CL-4B (**5**) was then sequentially washed with the following ice-cold solutions (100 mL each): 100% acetone; 5% HAc in dioxane; MeOH; anhydrous IPA. The resin was then stored in IPA at 4°C. The amount of active p-nitrophenyl carbonate groups immobilized on the resin was quantified by measuring the amount of p-nitrophenol released in alkaline conditions (see paragraph S4).

S3.2 Synthesis of sepharose-6Ahx-TTD-TBT (6a) (Strategy 1)

An aliquot of p-nitrophenyl carbonate-sepharose CL-4B (**5**) was washed on a sintered glass funnel with 100 mL of ice-cold water, then 5.0 g of suction-dried resin were weighted and added to a solution of compound **4** (65.4 mg) in 12 mL of ice-cold boric acid/sodium tetraborate buffer (0.2 M, pH 8.5). The reaction vessel was then capped, placed on a rocking table and tumbled end-over-end for 48 h at 4°C. The resulting

derivatized resin (**6**) was washed on a sintered glass funnel with 100 mL of ice-cold distilled water, then an aliquot (~80 mg) was sampled to measure the amount of residual carbonate groups (see paragraph S4). The rest of the gel was incubated with ethanolamine-HCl (0.1 M, pH 8.5) for 36 h to remove the residual carbonate groups. The resin was then sequentially washed on a sintered glass funnel with the following ice-cold solutions (100 mL each): ethanolamine-HCl 0.1 M pH 8.5, distilled water, NaOAc buffer (0.1 M, pH 4) containing 0.5 M NaCl, distilled water, NaH_2PO_4 0.1M/EtOH 80/20. The resin was stored in NaH_2PO_4 0.1M/EtOH 80:20 at 4°C. The amount of compound **4** immobilized on the resin was quantified by HPLC by measuring the amount of terbutaline released in alkaline conditions (see paragraph 3.4.2, main text).

S3.3 Synthesis of sepharose-6Ahx (7) (Strategy 2)

An aliquot of p-nitrophenyl carbonate-sepharose CL-4B (**5**) was washed on a sintered glass funnel with 100 mL of ice-cold water, then 6 g of suction-dried resin were weighted and added to a solution of 6Ahx (400 mg) in 10 mL of ice-cold sodium phosphate buffer (0.1 M pH 7.5). The reaction vessel was then capped, placed on a rocking table and tumbled end-over-end for 24 h at room temperature. The resulting derivatized resin (**7**) was washed on a sintered glass funnel with 100 mL of ice-cold distilled water, then an aliquot (~80 mg) was sampled to measure the amount of residual carbonate groups (see paragraph S4). The rest of the gel was incubated with ethanolamine-HCl (0.1 M, pH 7.5) for 24 h to remove the residual carbonate groups. The resin (**7**) was then sequentially washed on a sintered glass funnel with the following ice-cold solutions (100 mL each): ethanolamine-HCl 0.1 M pH 7.5, distilled water, NaOAc buffer (0.1 M, pH 4) containing 0.5 M NaCl, water, NaH_2PO_4 0.1 M/EtOH 80:20. The resin (**7**) was stored in NaH_2PO_4 0.1 M/EtOH 80:20 at 4°C. The amount of 6Ahx immobilized on the resin was estimated on the basis of the amount of residual carbonate groups present on the resin at the end of the reaction (see paragraph S4).

S4. Determination of the amount of active carbonate groups on the resin (Strategies 1 and 2)

About 80 mg of the stored gel were washed on a 500 µL spin filter with ice-cold distilled water (500 µL ×10). Aliquots of the washed gel (20 mg ×3) were incubated with 200 µL of NaOH 1 M on spin filters for 15 min at room temperature. The filtrate obtained after a brief spin (10 sec) using a table centrifuge was collected. Then the gel was further washed with NaOH 1M (100 µL ×3) and the eluates obtained after a brief spin (10 sec) using a table centrifuge were collected and pooled together with the first filtrate.

The absorbance at 400 nm of the resulting solution, opportunely diluted with NaOH 1 M, was measured by using a microplate reader. The concentration of carbonate groups was calculated using ε_{401nm} = 18,380 $M^{-1}cm^{-1}$.

The amount of active groups immobilized on the resin used for strategy 1 and strategy 2 were 41 µmol/g and 31 µmol/g of wet (spin filtered) resin, respectively.

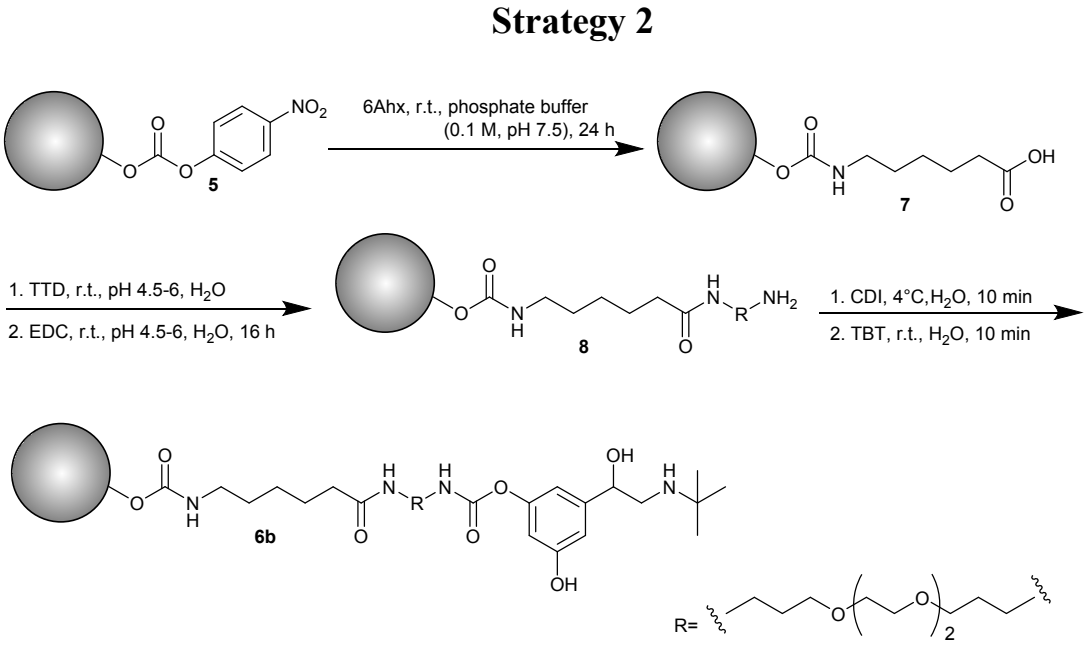
S5. Measurement of hBChE activity

The stock solutions of substrate (S-butyrylcholine iodide, BTCh) were freshly prepared in water. The stock solutions of 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB) were freshly prepared in sodium phosphate buffer (100 mM, pH 7). An aliquot of substrate working solution (25 µL) containing BTCh and DTNB in assay buffer was added to 225 µL of human plasma or purified hBChE opportunely diluted in assay buffer in a well of a 96-well plate. The enzyme activity was determined by monitoring the increase of concentration of yellow product at 412 nm over 2-5 min at 37°C or 25°C. The assay buffer used was sodium phosphate (100 mM, pH 8, at 37°C) or (100 mM, pH 7, at 37°C) or (50 mM, pH 8, 25°C) for the characterization of the kinetic of interaction between compound 3 and hBChE, the routine determination of the isolated hBChE yields and the determination of the specific activity, respectively. The final concentrations of BTCh in the same three instances were 1 mM, 1 mM and 0.4 mM and the concentrations of DTNB were 0.34 mM, 0.3 mM, and 0.3 mM, respectively. A blank sample containing assay buffer instead of a hBChE preparation was measured to account for the spontaneous conversion of the substrate into product, and the values obtained were subtracted from the measured activities.

S6. Sample preparation for the proteomic analysis

The aliquots of the ultrafiltrates obtained with resin 6a (strategy 1) showed in Fig. 7, lanes 4 and 5 (main text, 10 µL and 12 µL, respectively), were placed in two ultrafiltration units (Amicon® Ultra-0.5 mL, 30 KDa MWCO). After the addition of 400 µL of urea 8M in NH_4HCO_3 50 mM, the units were briefly shaken by gentle inversion then were briefly spun with a table centrifuge (~600 ×g, 5 s), and 2.5 µL of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) 1 M in water were added. The samples were vortexed then were briefly spun using a table centrifuge (~600 ×g, 5 s), and incubated under shaking (250 rpm) for 30 min at room temperature. The samples were then centrifuged (14,000 ×g, 20 min, 4°C). The filtrates were discarded and 400 µL of iodoacetamide (IAA) 50 mM in NH_4HCO_3 50 mM were added to each filter. The samples were vortexed then were briefly spun using a table centrifuge (~600 ×g, 5 s), and were incubated under shaking (250 rpm) for 30 min at room temperature, in the dark. The samples were centrifuged (14,000 ×g, 20 min, 4°C) then 400 µL of NH_4HCO_3 50 mM were added. This centrifugation-dilution step was repeated twice more then the sample was centrifuged a final time and 100 µL of NH_4HCO_3 50 mM were added, followed by 5 µL of trypsin 200 pg/µL in HAc 50 mM. The samples were vortexed then were briefly spun using a table centrifuge (~600 ×g, 5 s), then were incubated on a thermomixer (250 rpm) at 37°C, overnight (~16 h). After incubation, the samples were centrifuged (14,000 ×g, 20 min, 4°C) and 10 µL of 10% formic acid, v/v, in water were added to the filtrates. The filtrates were then dried using a centrifugal vacuum system (2.5 h, 45°C). One solution was reconstituted in 25 µL of 0.1% formic acid in water/ACN 95/5 and vortexed for 1 min. This solution was withdrawn with a micropipette and used to reconstitute the other sample. The resulting solution was analyzed by UPLC-HDMS^E .

S7. Scheme S1



Scheme S1. Synthesis of sepharose-6Ahx-TTD-TBT 6b (strategy 2). 6Ahx, 6-aminohexanoic acid; TTD, 4,7,10-trioxa-1,13-tridecanediamine; EDC, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride; CDI, N,N'-carbonyldiimidazole; TBT, *rac*-terbutaline hemisulfate

S8. ^{1}H-NMR, ^{13}C-NMR and HRMS spectra.

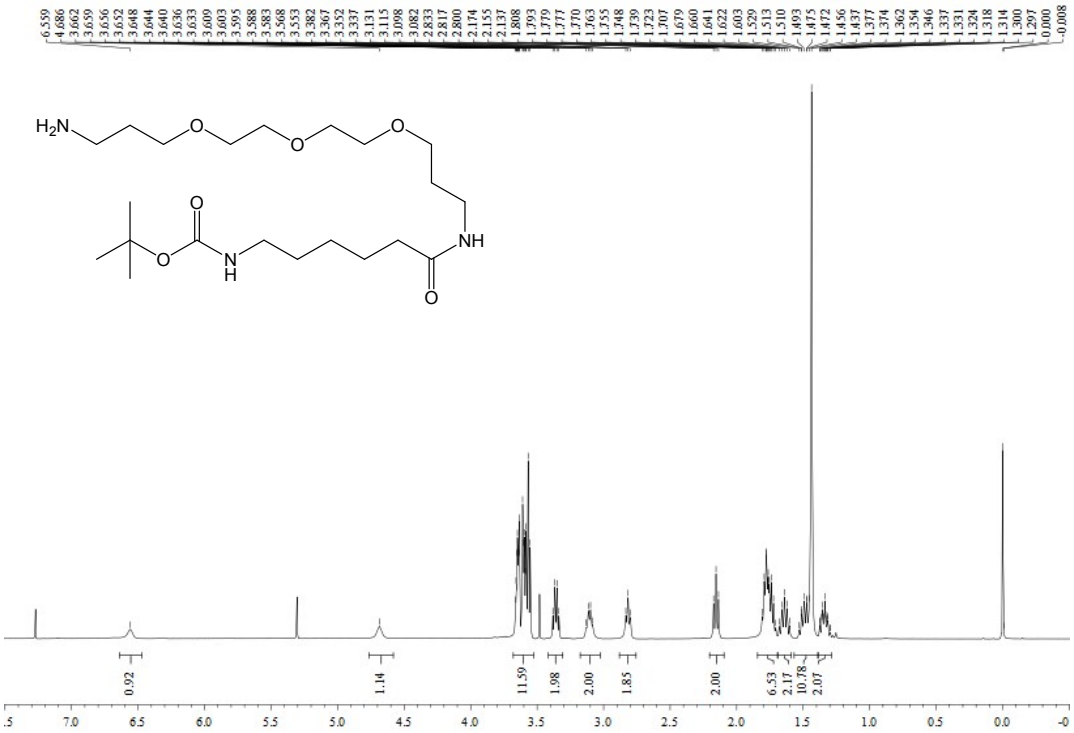


Figure S1. ^{1}H-NMR spectrum of compound **1** (Boc-6Ahx-TTD) ($CDCl_3$)

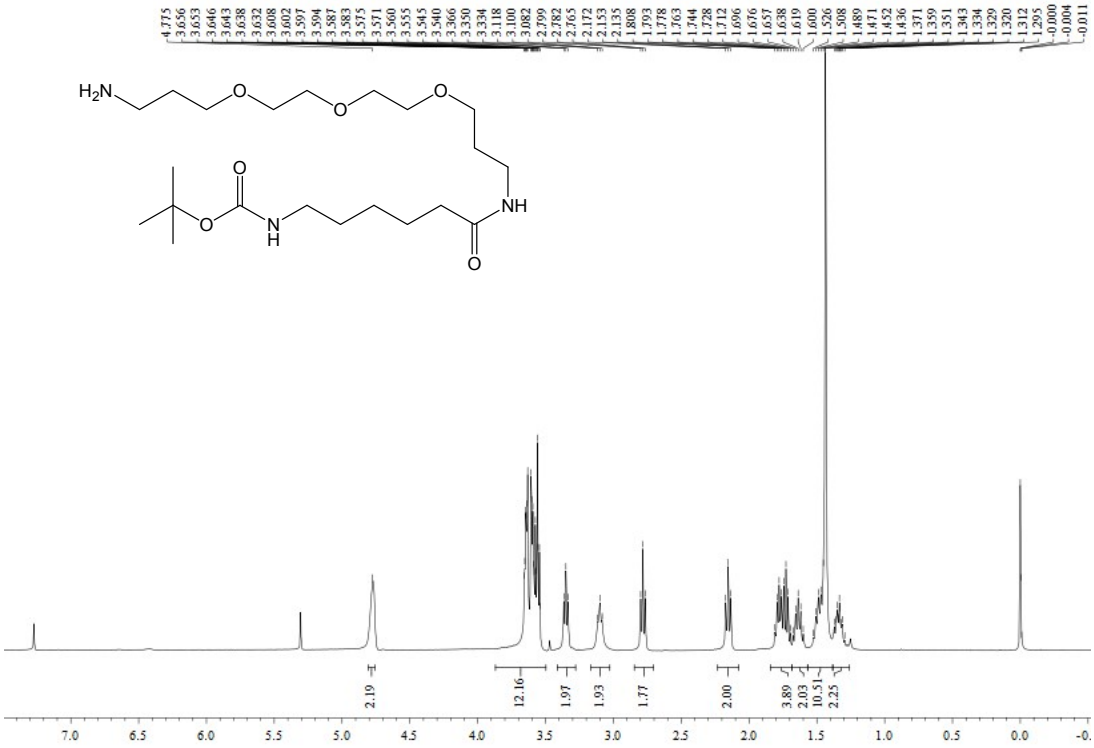


Figure S2. ^{1}H-NMR spectrum of compound **1** (Boc-6Ahx-TTD) ($CDCl_3$+D_2O). The same sample of figure S1 after addition of one drop of D_2O.

Figure S3. ^{13}C-NMR spectrum of compound **1** (Boc-6Ahx-TTD) ($CDCl_3$)

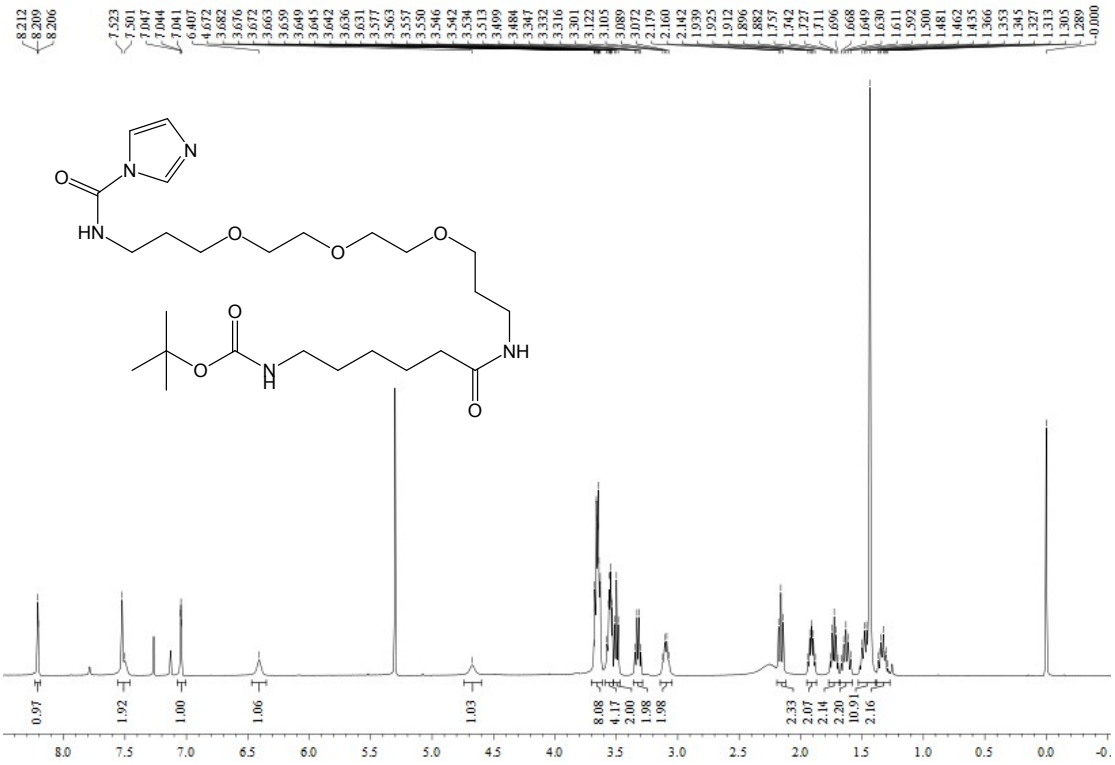


Figure S4. ^{1}H-NMR spectrum of compound **2** (Boc-6Ahx-TTD-imidazole) ($CDCl_3$)

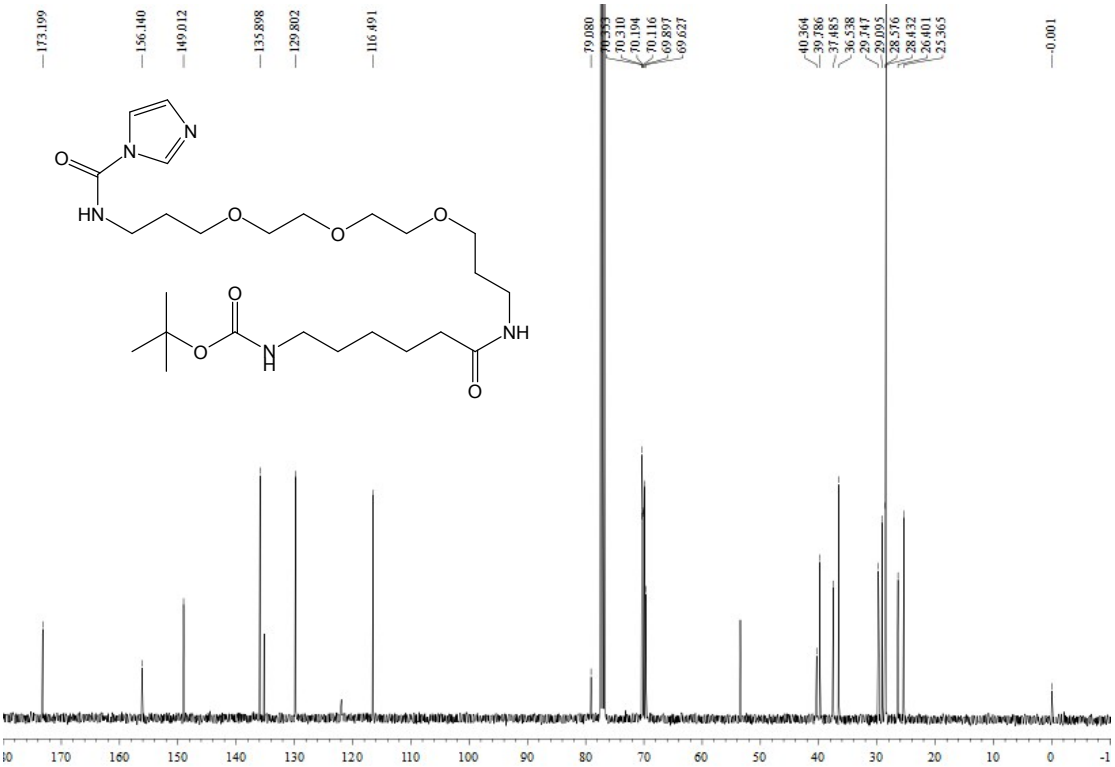


Figure S5. ^{13}C-NMR spectrum of compound **2** (Boc-6Ahx-TTD-imidazole) ($CDCl_3$)

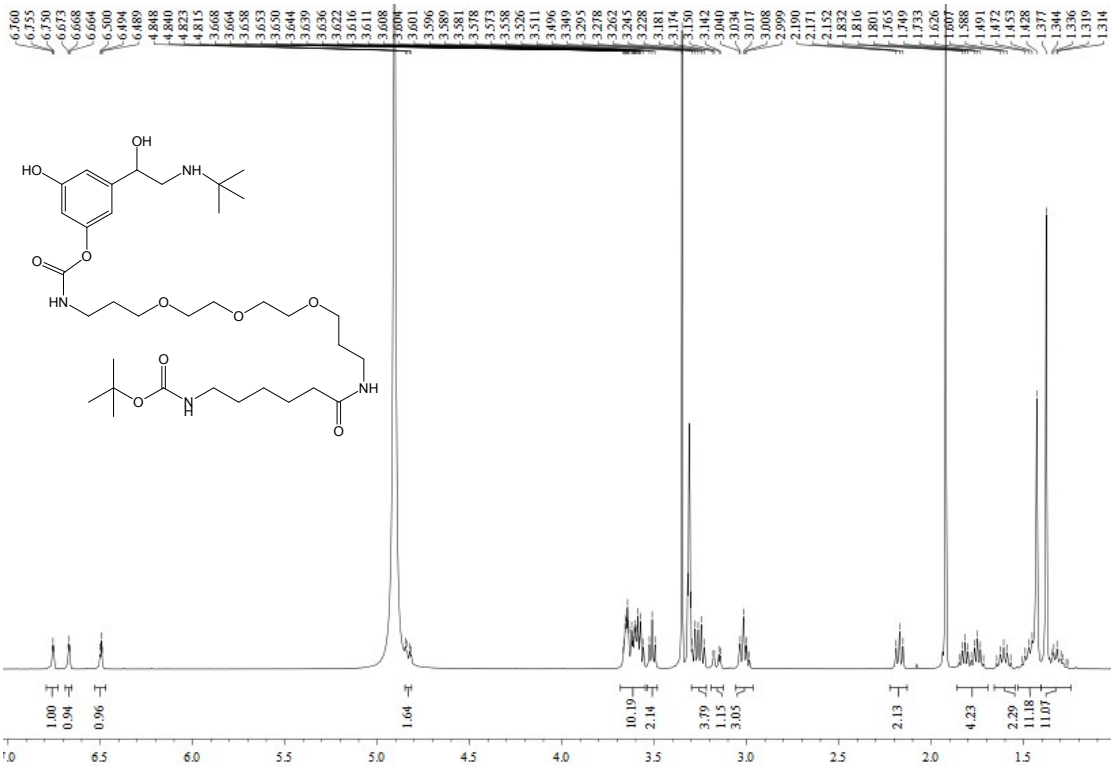


Figure S6. ^{1}H-NMR spectrum of compound **3** (Boc-6Ahx-TTD-TBT) (CD_3OD)

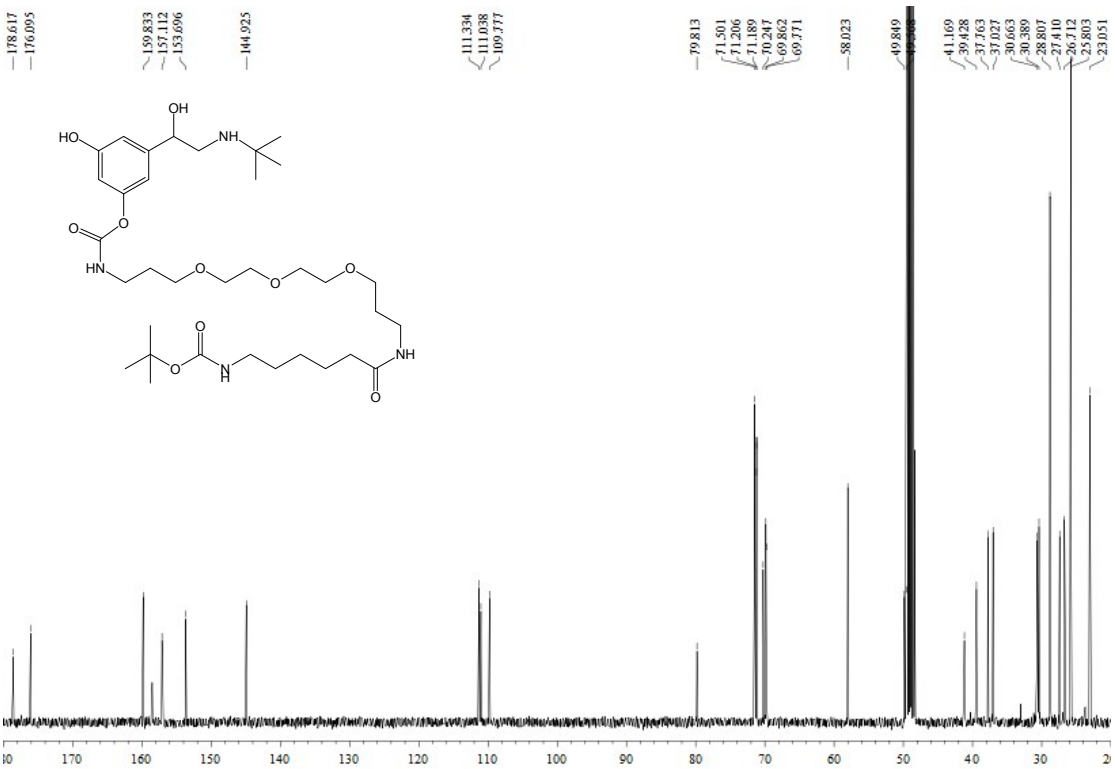


Figure S7. ^{13}C-NMR spectrum of compound **3** (Boc-6Ahx-TTD-TBT) (CD_3OD)

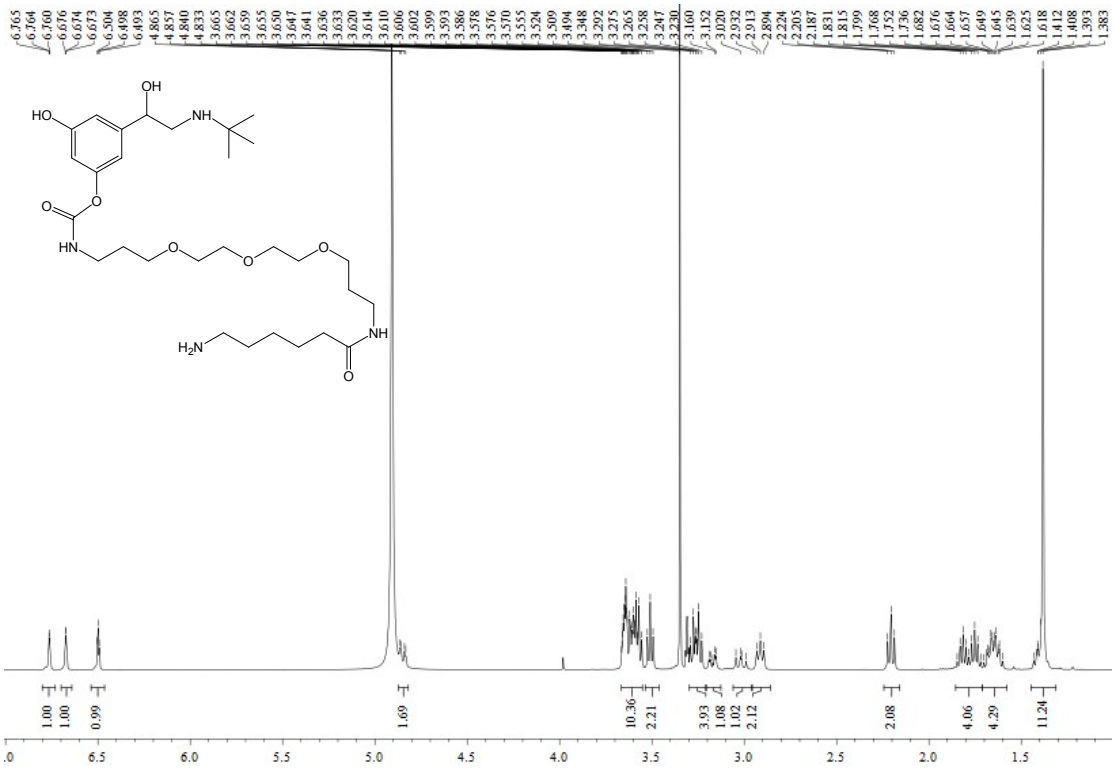


Figure S8. ^{1}H-NMR spectrum of compound **4** (6Ahx-TTD-TBT) (CD_3OD)

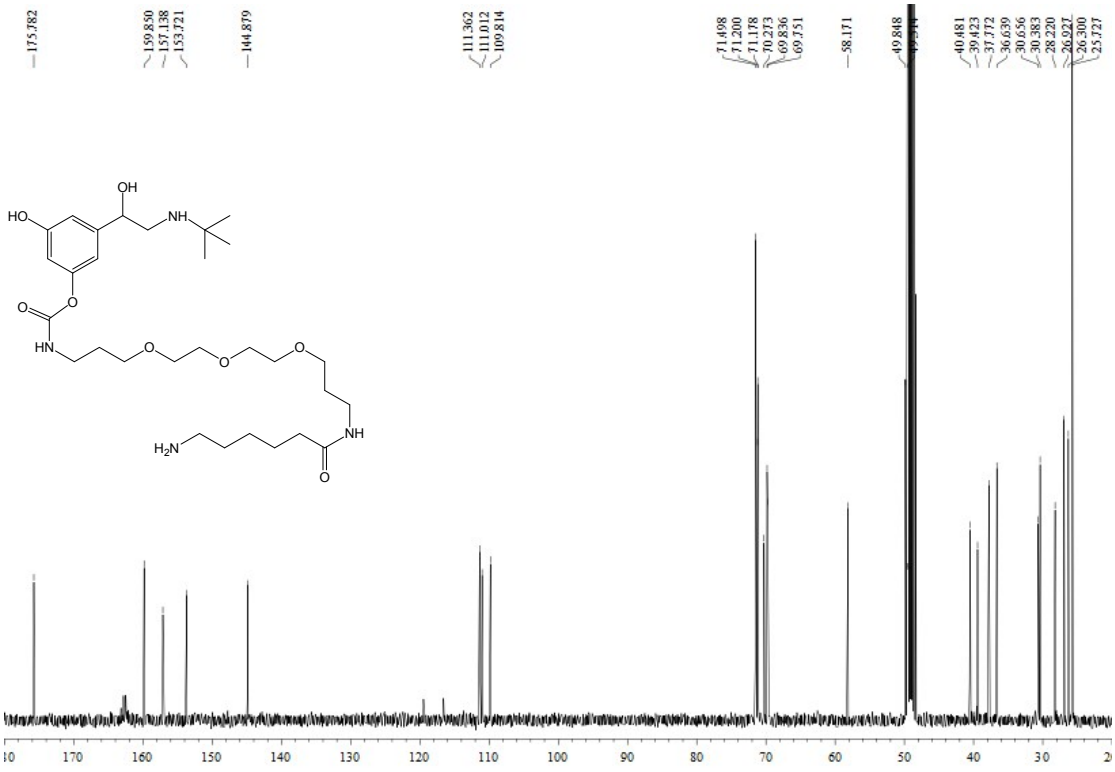


Figure S9. ^{13}C-NMR spectrum of compound **4** (6Ahx-TTD-TBT) (CD_3OD)

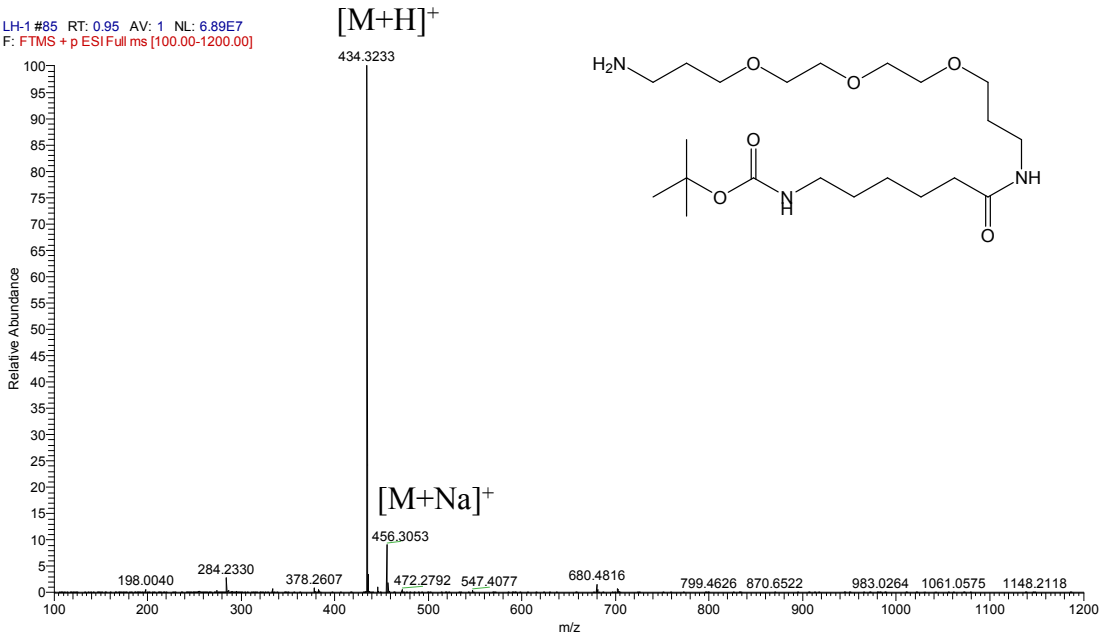


Figure S10. HRMS spectrum of compound **1** (Boc-6Ahx-TTD)

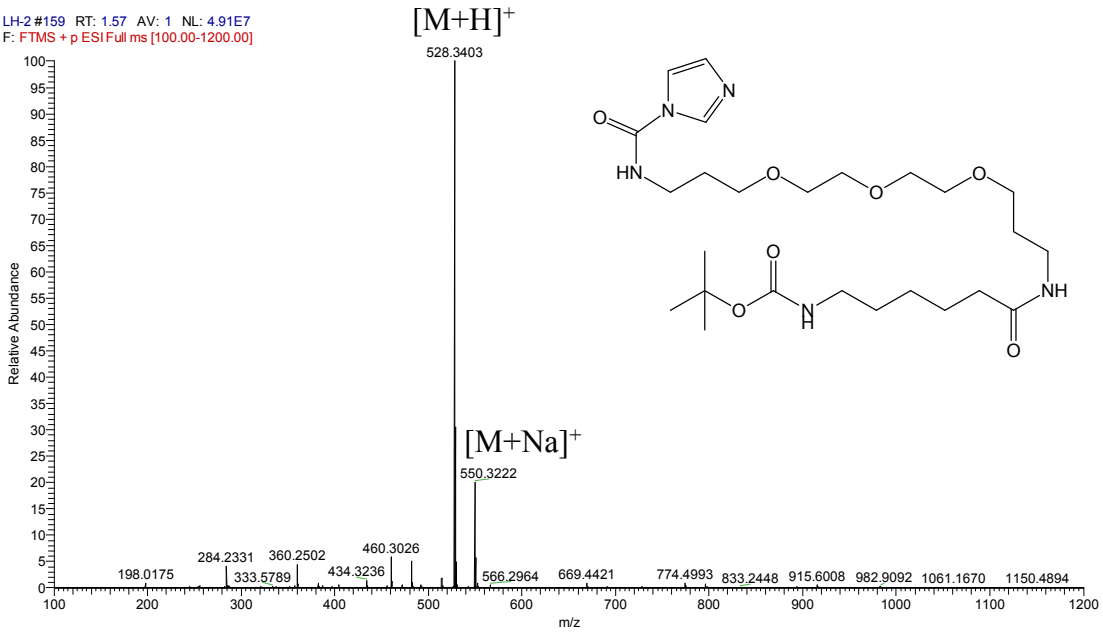


Figure S11. HRMS spectrum of compound **2** (Boc-6Ahx-TTD-imidazole)

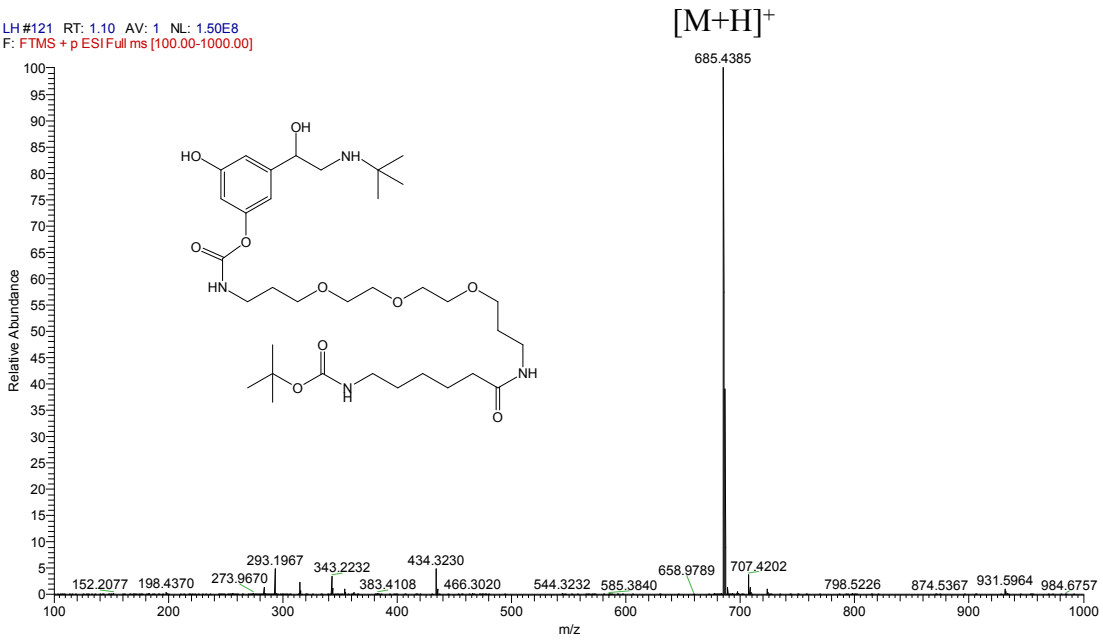


Figure S12. HRMS spectrum of compound **3** (Boc-6Ahx-TTD-TBT)

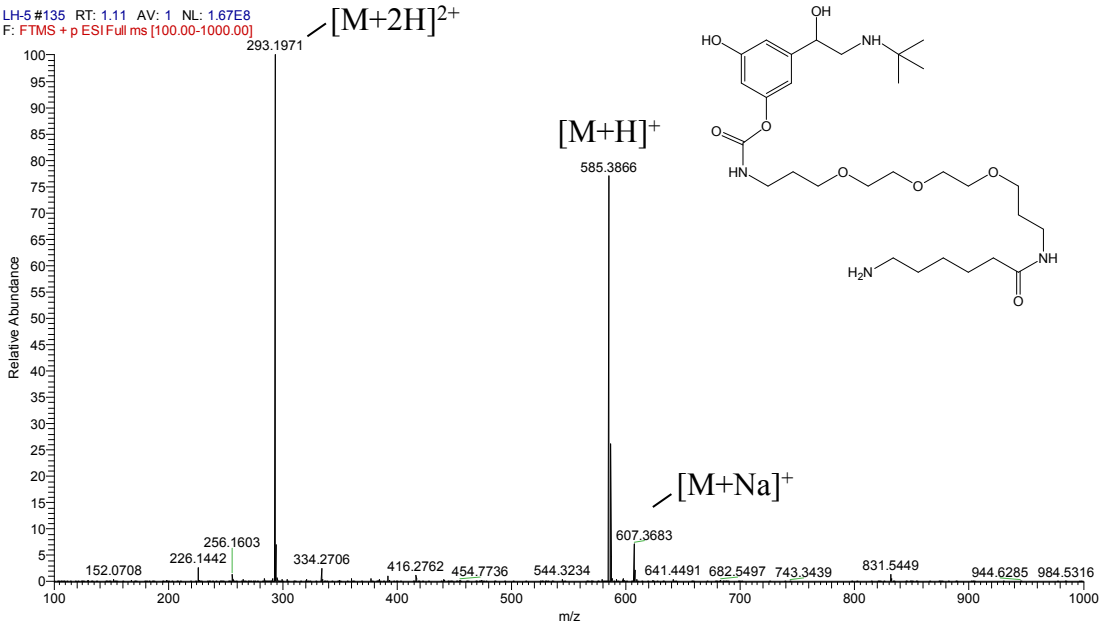


Figure S13. HRMS spectrum of compound **4** (6Ahx-TTD-TBT)