Diarylcarbonates are a new class of deubiquitinating enzyme inhibitors

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Supporting Materials

Biochemistry Experimental Section

Materials. Bortezomib was from LC laboratories (Woburn, MA). G5 isopeptidase inhibitor 1 (50-230-7928) was from Calbiochem (Philidelphia, PA). His_6USP9x, ubiquitin aldehyde, HA-ubiquitin vinylsulfone, ubiquitin vinylsulfone, NSC 632839 hydrochloride was from Boston Biochem (Cambridge, MA). Cy5-ubiquitin-VME was from UbiQ. Antibodies: anti-K48-linked ubiquitin, clone APU2; anti-K63-linked ubiquitin, clone APU3, were from Millipore (Billerica, MA); H-552; anti-Mdm2, SC-13161 were from Santa Cruz (Santa Cruz, TX); anti-PARP, 9542; anti-Abl, 2862; β–tubulin, 2156 were from Cell Signaling Technologies (Beverley, MA). Anti-actin was clone AC-40, A3853 and anti-GAPDH was clone G9295. Anti-HA Clone 3F10 was from Roche (Indianapolis, IN). HRP conjugated secondary antibodies were from AbCam (Cambridge, MA). Compounds were synthesized as described in the Supporting Materials.

FACS analysis. FACS was carried out on a Beckman FACS-Calibur. For HEK 293T and K562, cells were resuspended by repeated pipetting/agitation of the incubation media, followed by dilution into PBS. For Cos-1, MCF-7, and CHO cells, media was removed and trypsin was added. Harvested cells were placed in FACS buffer (0.5% FBS in PBS with 3 μg/ml propidium iodide) 30 s prior to analysis. All data were analyzed using FlowJo V10, from TreeStar (Ashland, OR). Approximately 2500 cells were sorted per replicate. Cells were sorted by propidium iodide dye exclusion to give a “viable population”. GFP positive cells within this group were identified relative to untransfected controls. Then the geometric mean of the whole GFP positive population within the viable population was calculated. Typical transfection efficiencies for ^{G76V}ubiquitin-GFP were 60-70% for both Cos-1 and HEK 293T and 25-40% for CHO cells, based on GFP positive cells.

Lysate assays. Biochemistry experiments were performed as previously described [1, 2]. Cells overexpressing HA-ubiquitin were prepared as above. Pellets were typically stored at -80 °C until required, at which time they were thawed on ice. Cell lysis was performed in lysis buffer using a Dounce homogenizer. Crude lysate was centrifuged at 17000 g for 10 min at 4 °C, after which time the concentration of the lysate was adjusted to 1 mg/ml (Bradford assay with an IgG standard). The lysate was aliquoted into PCR strip tubes (typical volumes 75-50 μL) and compound in DMSO was added to this to give a final concentration of DMSO of 1%. Tubes were briefly centrifuged, overlaid with Chill-out wax (50 μL) and placed in a PCR machine at 37 °C with heated lid set to 37 °C. Aliquots (9 μL) were removed at the stated times and immediately quenched in (2X final concentration) reducing (dithiothreitol) loading buffer and frozen (-19°C) till required. Western blot analysis was carried out using standard methods. Samples were resolved by SDS-PAGE, transferred to PVDF [(0.45 μm) (Towbin buffer, tank apparatus, 90 V, 1 h, then overnight at 30 V, 4 °C)] then blocked in 15% milk in TBS-T HS (100 mM Tris HCl, pH 7.6, 500 mM NaCl, 0.5% Tween-20) for at least 2 h at RT. Afterward, membrane was washed in TBS-T HS then probed with anti HA-HRP (1:18000) for 1.33 h at RT. Membrane was washed 3 times in TBS-T HS (15 min) then once in TBS (15 min) and exposed to ECL II and visualized using blue biofilm. The dynamic range of the assay at the 2 h time point was approximately 5 for HEK 293T and 2.5 for Cos-1 cell lysates, which showed the same trend as observed for HEK 293T cells. When required, membranes were stripped in 100 mM glycine pH 4, 500 mM NaCl, 1% SDS, 5 mM BME, at 55 °C for 19 min, then analyzed.

Ubiquitin-VS activity profiling. Experiments were performed as previously described [1, 2]. Lysates (1.0 - 1.5 mg/ml) were treated with inhibitor (or 1% DMSO control) for 19-60 min at 37 °C (see text). HA-Ub-VS (0.7-1.5 μM) or Cy5-ubiquitin-VME (250 nM) was added and incubated for 19-25 min. Samples were quenched in 2X (final concentration) reducing Laemmli buffer. For

recovery experiments, a concentrated lysate (6 mg/ml) was treated with compound **C14** (250 μM) and incubated for 40 min. Afterward, the lysate was diluted to 0.6 mg/ml (final concentration of inhibitor 25 μM) in lysis buffer, then HA-Ub-VS was added (note, under these **final** conditions HA-Ub-VS outcompetes the inhibitor). Aliquots were quenched in 2x (final concentration) reducing Laemmli buffer.

Cell experiments were carried out as above with some modifications. For Cos-1 and MCF-7 cells, after trypsinization, the cell/trypsin mixture was diluted with complete media and centrifuged, then washed three times with PBS. Cell pellets were lysed on an ice/salt bath and lysate was centrifuged for 5 min.

Enzyme assays. Kinetic assays were performed on a Biotek plate reader at 25 ^{o}C by monitoring release of either: AMC by change in fluorescence (excitation wavelength 360 nm, emission wavelength 460 nm) or 2-naphthol by change in fluorescence (excitation wavelength 280 nm, emission wavelength 429 nm). For ficin and papain, enzyme was preincubated for 30 min with inhibitor prior to addition of substrate (Z-Arg-AMC). The final concentration of DMSO in all assays was 2%. Ficin and papain (8 μg/mL) were assayed in 100 mM potassium phosphate, pH 6.8, 0.4 mM β-mercaptoethanol with the substrate Z-Arg-AMC (300 μM; papain: K_m = 240 μM, k_{cat} = 0.07 s^{-1}).

SENP assays. The compounds were initially tested for the ability to inhibit the processing of SUMO2-modified RanGap1 by the catalytic domain of SENP6. The recombinant catalytic domain of SENP6 (200 nM) was preincubated with different concentrations of the inhibitor for 30 min at room temperature before adding the substrate Rangap1-SUMO2 (3 μM), the reaction was carried out at 37 ^{o} C for 10 min. The reaction was stopped adding SDS loading buffer and gel loaded for western blot analysis. Both SENP6 and Rangap1-SUMO2 contain a His tag and

are easily detected with an anti-His antibody. The reaction buffer used is: 50 mM Tris, pH 8, 20 mM NaCl, 5 mM DTT. The compounds were also assayed for the ability to inhibit the labeling of endogenous SENPs with the activity-based probe SUMO2-vinlysulfone in cell extracts [3]. HeLa cells were resuspended in a lysis buffer containing 50 μM inhibitor (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% TX100, 10 μM (each): leupeptin, MG132, 3,4-DCI and E64), and incubated for 30 min before adding the probe. As control, lysis was performed in the presence of NEM that efficiently prevented labeling of endogenous SENP6 and SENP1. The compounds did not affect the labeling profile of SENP6.

Progress curves for USP9x and USP7CD inhibition. To His_6-USP9x (0.7 nM) in degassed 50 mM HEPES, pH 7.7, 150 mM NaCl, 0.75 mM BME, was added Ub-AMC (300 nM; K_M= 340±100 nM, independently determined) and inhibitor (final concentration of DMSO = 1%). To His_6USP7CD (41 nM) in degassed 50 mM in degassed HEPES pH 7.7, 150 mM NaCl, 0.75 mM BME, was added UbAMC (814 nM; K_M=1.3±0.3 μM) The reaction was monitored by following the release of AMC. The concentration of AMC was determined from standard curves (note that the inhibitor quenches AMC fluorescence, so standard curves were determined for each inhibitor concentration). USP9x was unstable under these conditions, so a term was included to account for nonspecific inactivation. The progress was fitted to the following mechanism, using Dynafit.

$$E + S <==> ES \text{ (rapid equilibrium, } K_m = 340 \text{ nM)}$$

$$ES \rightarrow E + P \ (0.35\ s^{-1})$$

$$E \rightarrow \text{dead} \quad (0.0023 \pm 0.0001\ s^{-1})$$

$$E + I \rightarrow EI \ (k_{on})$$

Cysteine reactivity profiling. Hela cell lysates are treated with DMSO or the **C22** (50 μM) for 22 min followed by IAA-alkyne probe for 20 min. Click chemistry and peptide analysis was performed as described previously [4, 5].

Chemistry Experimental Section

Synthesis of compounds

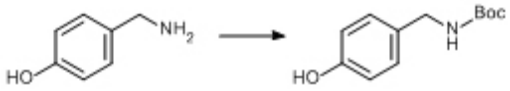
All reactions were carried out under an atmosphere of dry nitrogen supplied by a balloon. All solvents and amine bases were either distilled before use or bought dry over molecular sieves. All aqueous solutions were saturated unless otherwise stated.

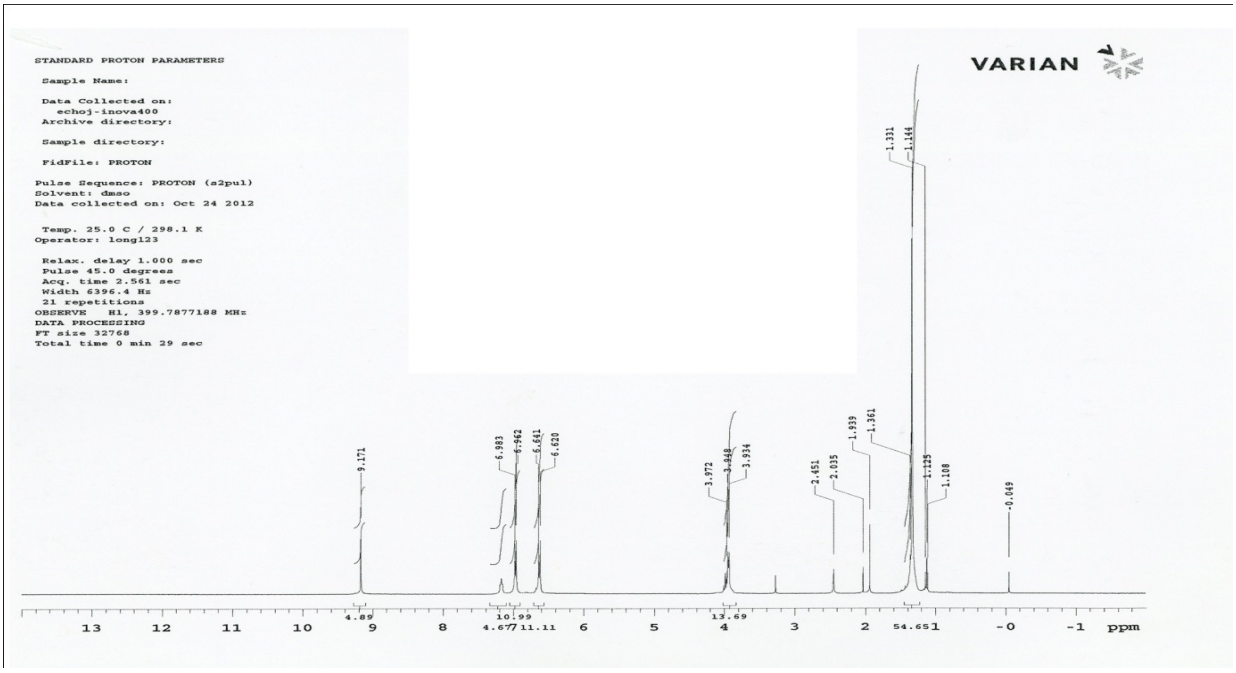
General procedure 1. To a round bottom flask was added molecular sieves and a stir bar and the flask was heated to dryness over a Bunsen flame, then cooled over vacuum for 20 min. The amine was added to the flask, then a stopper was added and the flask flushed with dry nitrogen. DMF/pyridine (1:1) was then added and the flask cooled to 4^{o}C. The chloroformate was then added drop-wise (neat if liquid or as a solution in DMF, if solid). After 1 h the reaction was warmed to rt and run overnight. After this time $NaHCO_3$ and EtOAc was added and the reaction stirred for 5 min. The aqueous phase was extracted, then the organic phase was washed 3 times with 10% $CuSO_4$, three times with water, then once with brine. Organic phase was then dried with magnesium sulfate, filtered and concentrated.

General procedure 2. The Boc protected compound was added to a dry flask, then $HCl.Et_2O$ was added. Mixture was stirred overnight after which time a solid formed. The solid was filtered, washed 3 times with Et_2O then concentrated in vacuo.

General procedure 3. A carboxylic acid, alcohol, EDCI and triethyl amine were stirred overnight in DMF. After this time reaction was diluted with EtOAc, and water was added. This mixture was stirred for 5 min, then phases were separated and the organic phase was washed three times with water, and once with brine. The organic phase was dried with magnesium sulfate, filtered and concentrated.

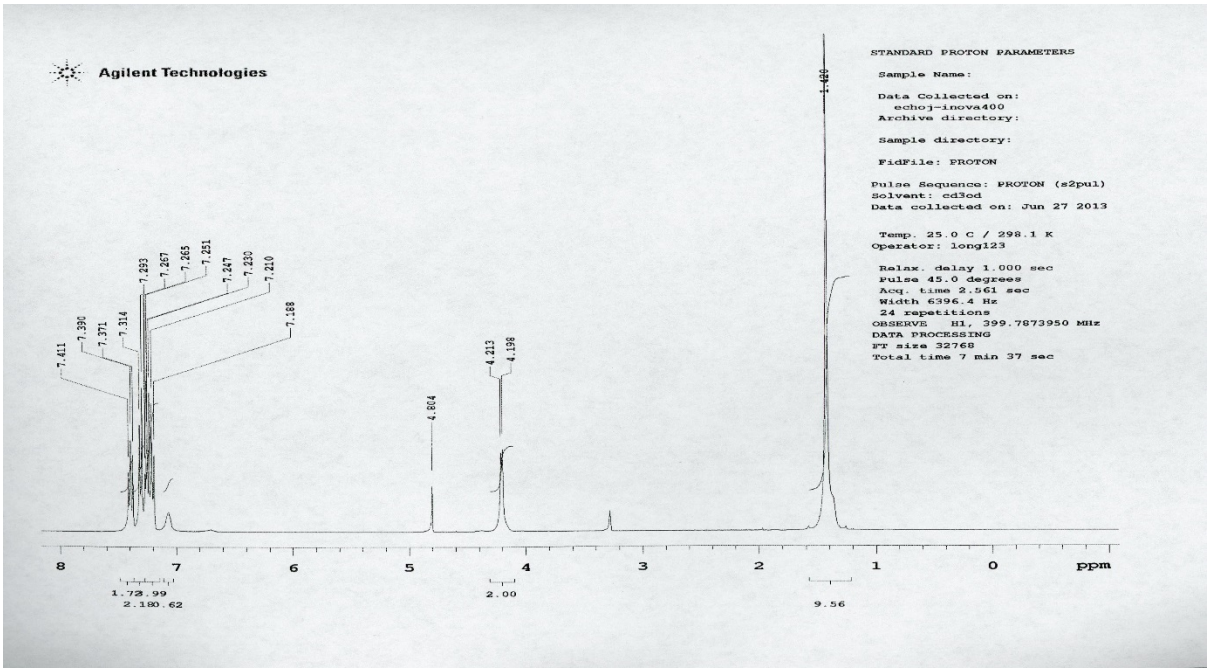
Synthesis of *tert*-butyl 4-hydoxybenzylcarbamate

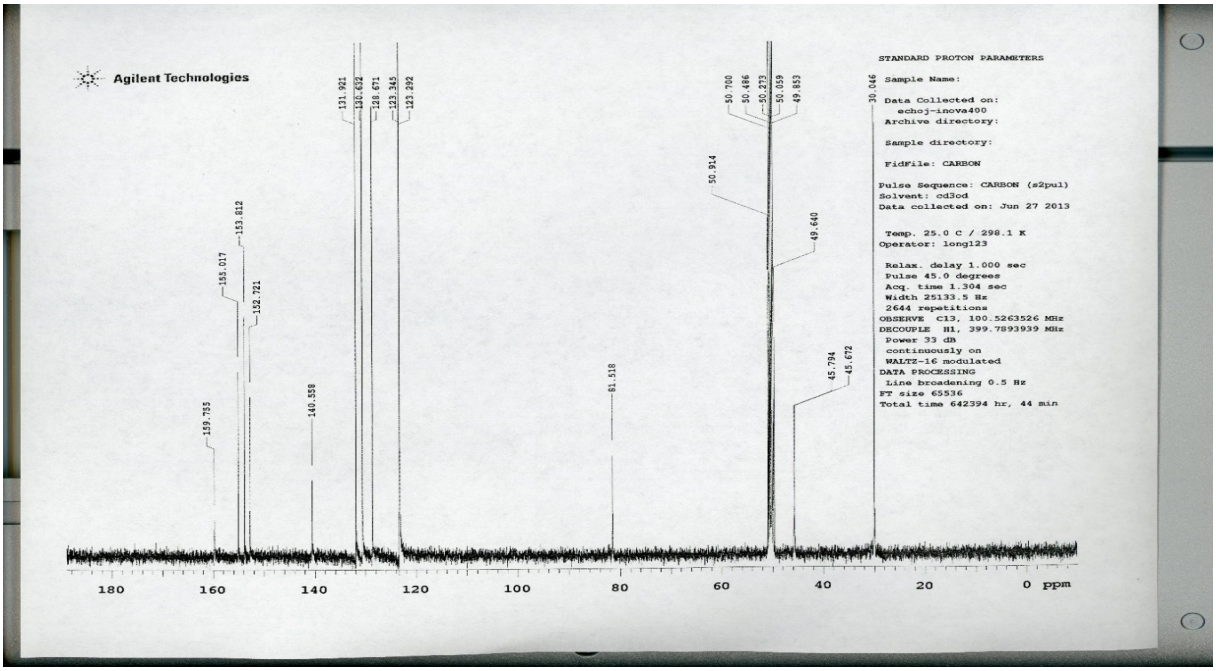




To 4-hyroxybenzylamine (5 g, 40 mmol) in DMF/pyridine (20 mL 5:1) was added Boc_2O XS at 4 ^oC and the reaction was stirred overnight at RT. At this point approx 0.5 ml 10 M NaOH was added to the reaction and stirring was continued. After 30 min 250 mL water and 200 mL EtOAc were added and the phases separated. The organic layer was washed sequentially with 10% copper sulfate (2 times), sodium bicarbonate and brine. Organic phase was then dried over magnesium sulfate, filtered and concentrated *in vacuo*. Chromatography on silica gel (elution 60-70% EtOAc in hexane) yielded the purified product as a white solid. δH (400 MHz, CD_3SOCD_3) 1.144 (9H, s); 3.936 (2H, d, J=5.6Hz); 6.630 (2H, d, J=8.4Hz); 6.951 (2H, d, J=8.4Hz); 7.081 (1H, br s); 9.171 (1H, s).

Synthesis of *N*-Boc 4-(aminomethyl)phenyl (phenyl) carbonate C3

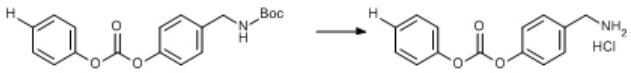


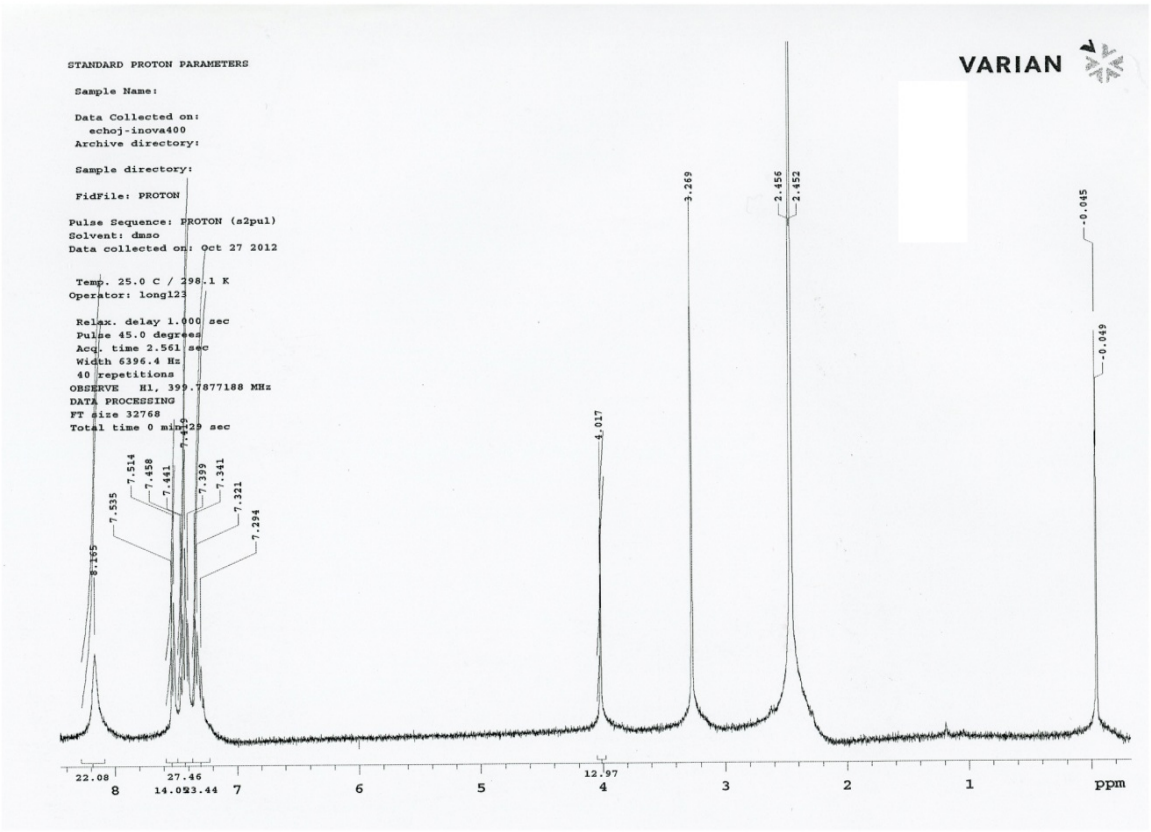


Following *General Procedure 1*, *tert*-butyl 4-hydoxybenzylcarbamate (500 mg, 2.2 mmol) was reacted with phenyl chloroformate (0.617 mg, 3.96 mmol) in DMF / pyridine (20 mL). Chromatography on silica gel (gradient from 5 % EtOAc in hexanes to 40 % EtOAc in hexanes) gave the target compound as a white solid (326 mg, 50%). δH (400 MHz, CD_3SOCD_3) 1.420 (9H,

s); 4.206 (2H, d, J=6.0Hz); 7.188-7.314 (6H, m); 7.6 (2H, t, J=7.6Hz). δC (100 MHz, CD_3SOCD_3) 30.046; 45.672; 81.518; 123.292; 123.345; 124.077; 128.617; 130.632; 131.921; 140.558; 152.721; 153.812; 155.017; 159.755.

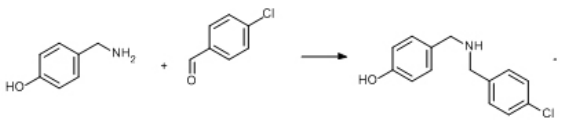
Synthesis of 4-(aminomethyl)phenyl (phenyl) carbonate hydrochloride C4

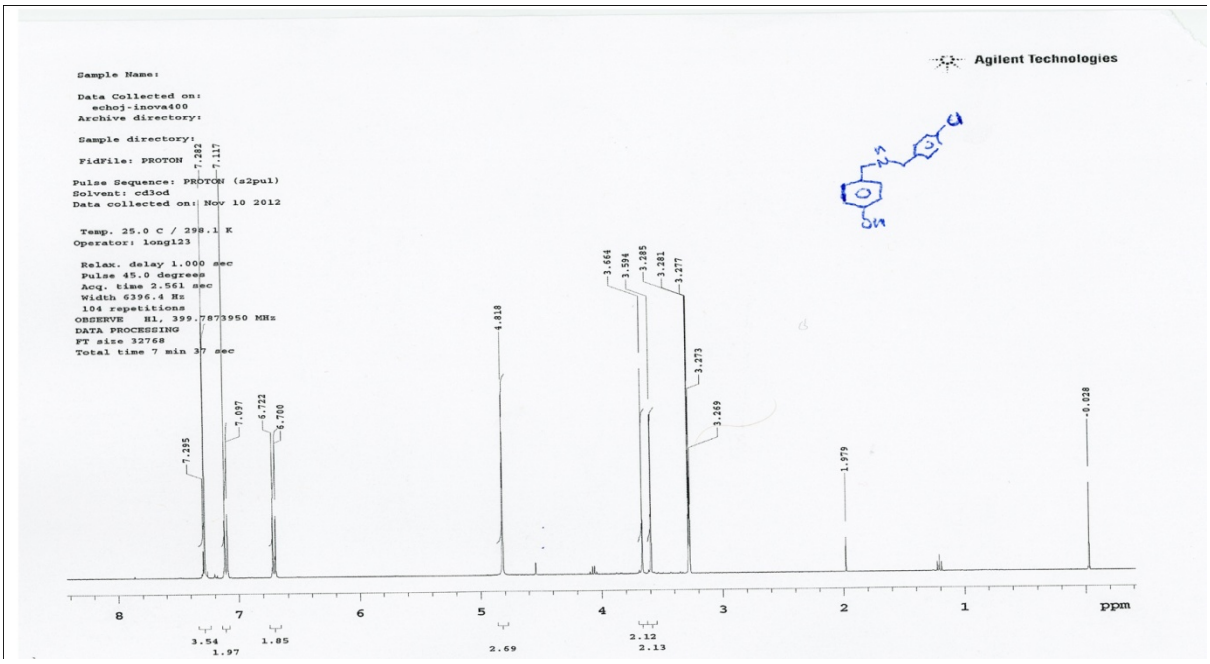




Following *General Procedure 2*, **C3** (90 mg, 0.26 mmol) was dissolved in 2 M HCl in Et_2O (20 mL) and stirred overnight at RT. The title compound was obtained as a white solid (36 mg, 50 %). δH (400 MHz, CD_3SOCD_3) 4.017 (2H, s); 7.294-7.341 (3H, m); 7.524 (2H, d, J=8.4Hz); 8.165 (3H, s). ESI^- (242 $M\text{-}H^+$ 100 %)

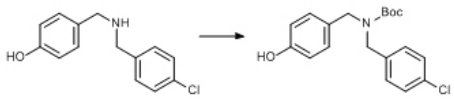
Synthesis of 4-chlorobenzyl 4-hydroxybenzylamine

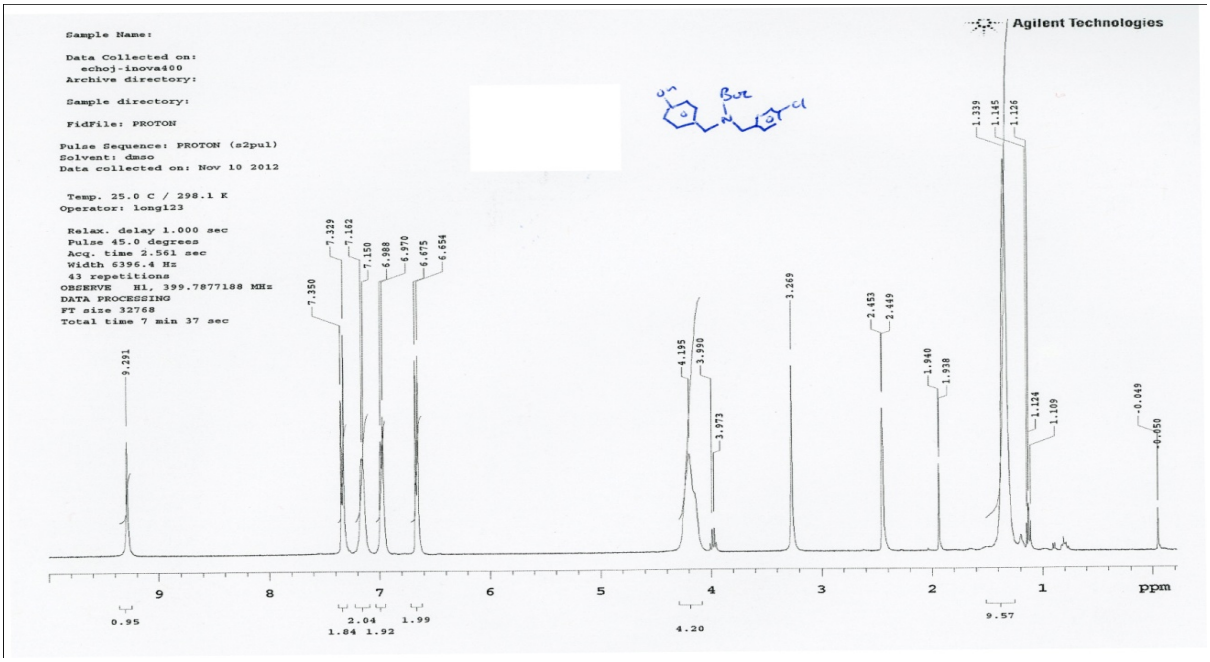




4-Chlorobenzaldehyde and 4-hydroxybenzylamine were mixed 1:1 in methanol (4 mL) and the reaction was stirred for 1 h at room temp followed by a further 1 h at reflux. After cooling to 4 ^{o}C and dilution into 20 mL total methanol, sodium borohydride (3 equivalents) was added portion-wise over 1 h. The reaction was allowed to stir for a further h, after which time 200 mL EtOAc was added and 250 mL water. Phases were separated and the organic layer was washed 3 times with sodium bicarbonate and then with brihone. Organic layer was dried with magnesium sulfate, filtered and concentrated to give the crude amine which was used without further purification. δH (400 mHz, CD_3OD) 3.594 (2H, s); 3.664 (2H, s); 7.711 (2H, d, J=8.8Hz); 7.107 (2H, d, J=8.8Hz); 7.282-7.295 (4H, m).

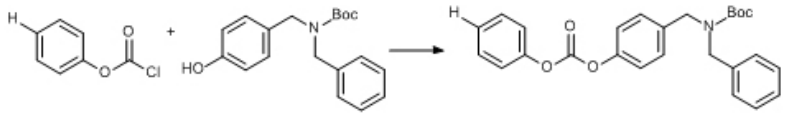
Synthesis of *N*-Boc 4-chlorobenzyl 4-hydroxybenzylamine

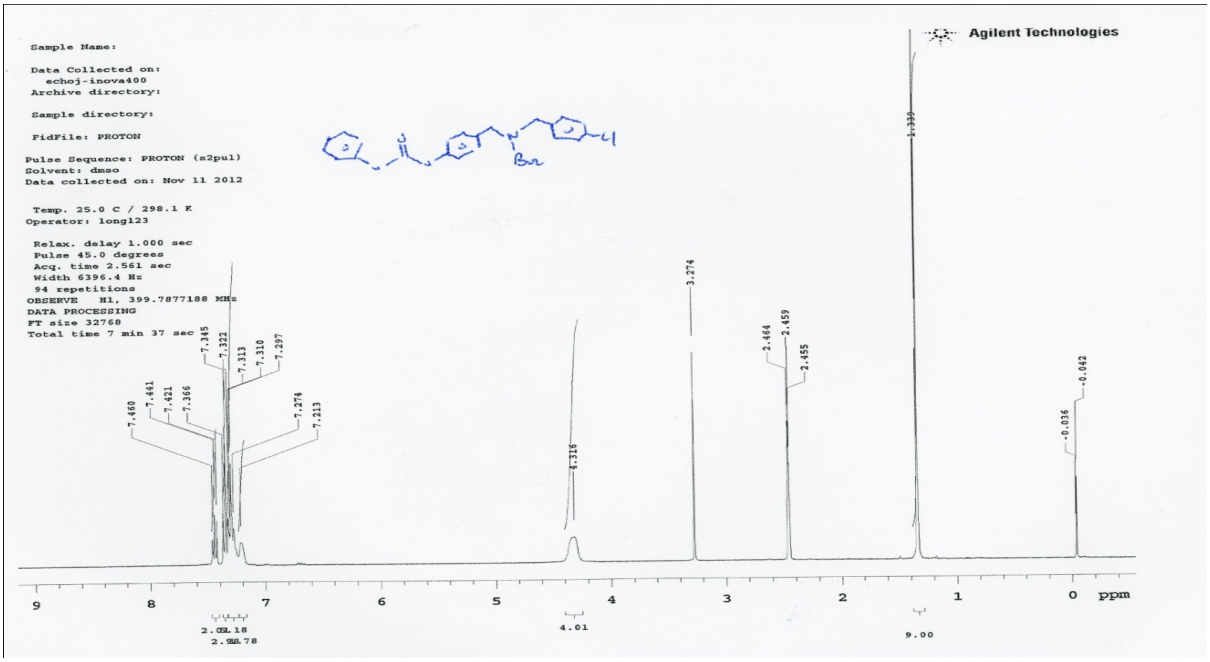




Amine was dissolved in DMF/pyridine (20 mL 5:1) was added Boc_2O XS at 4 ^oC and the reaction was stirred overnight at RT. At this point approx 0.5 ml 10 M NaOH was added to the reaction and stirring was continued. After 30 min 250 mL water and 200 mL EtOAc were added and the phases separated. The organic layer was washed sequentially with 10 % copper sulfate (2 times), sodium bicarbonate and brine. Organic phase was then dried over magnesium sulfate, filtered and concentrated in vacuo. Chromatography on silica gel (elution 30-40% EtOAc in hexane) yielded the purified product as a white solid. (note: peaks are broad due to rotameric equilibria about the N-Boc bond). δH (400 mHz, CD3SOCD3) 1.399; 4.195 (4H, br s); 6.664 (2H, d, J=8.4Hz); 6.979 (2H, d, J=7.8Hz); 7.156 (2H, m); 7.282-7.340 (2H, d, J=8.4Hz).

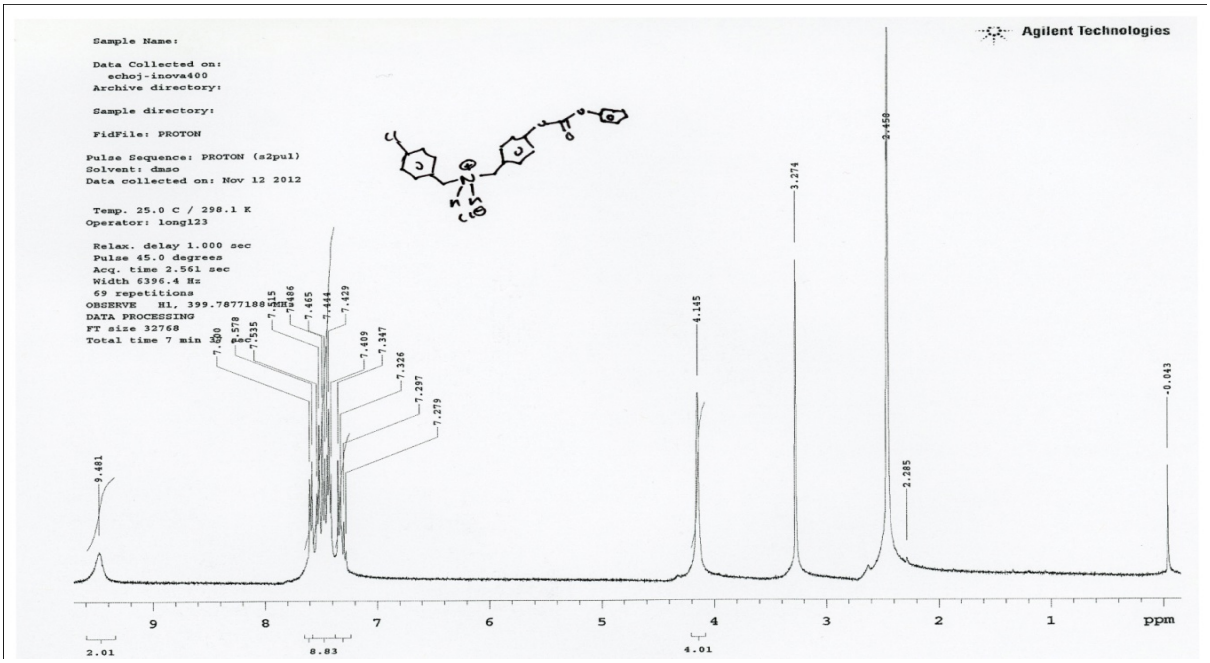
Synthesis of 4-(((4-chlorobenzyl) N-Boc amino)methyl)phenyl phenyl carbonate

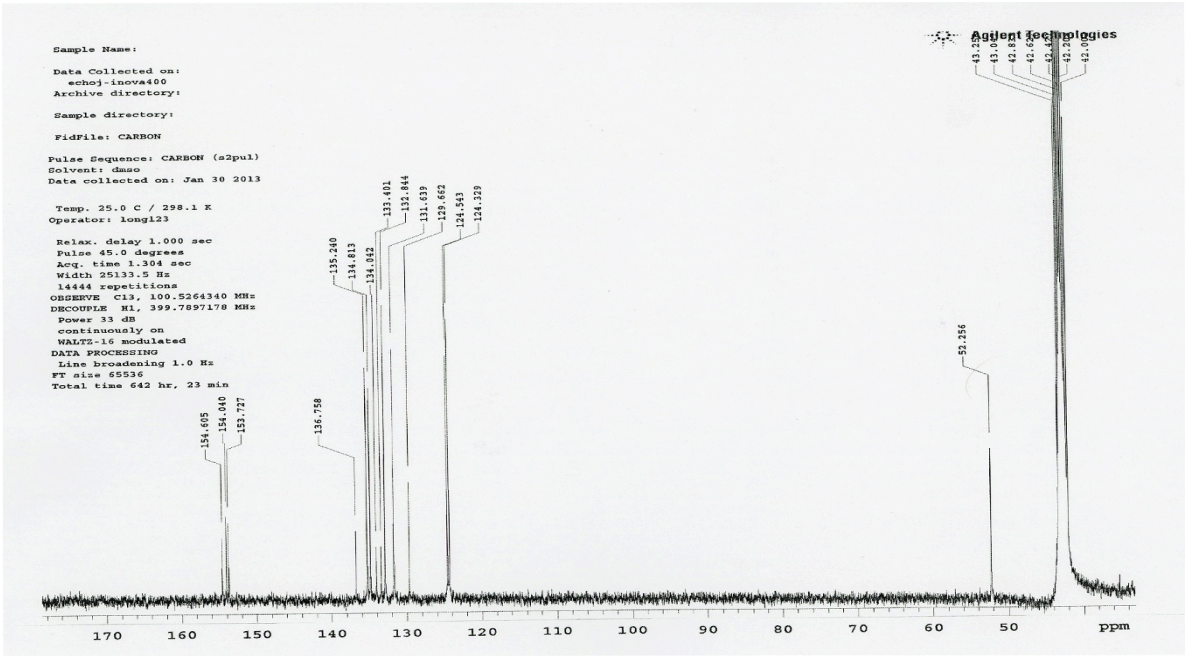


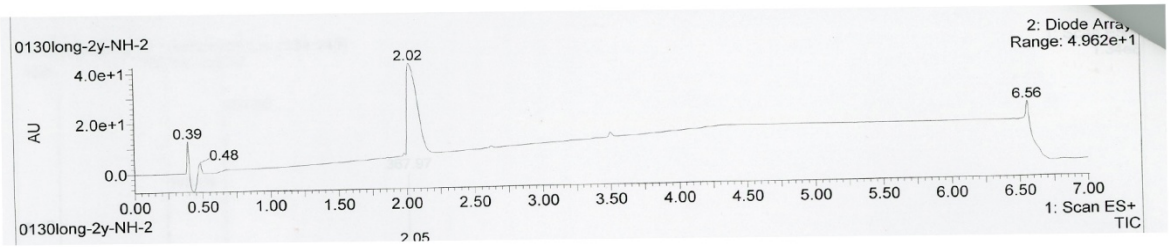


Following *General Procedure 1*, the title compound was prepared in 50% yield. δH (400 mHz, CD3SOCD3) 1.339 (9H, s); 4.316 (4H, br s); 7.274-7.366 (1H, br s); 6.979 (11H, m); 7.441 (2H, t, J=8.0Hz).

Synthesis of 4-(((4-chlorobenzyl)amino)methyl)phenyl phenyl carbonate hydrochloride C5

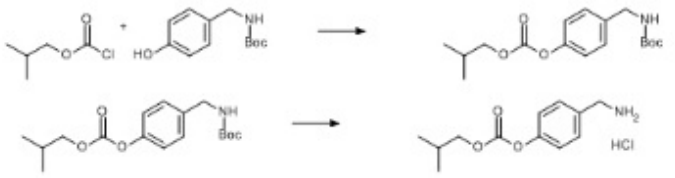


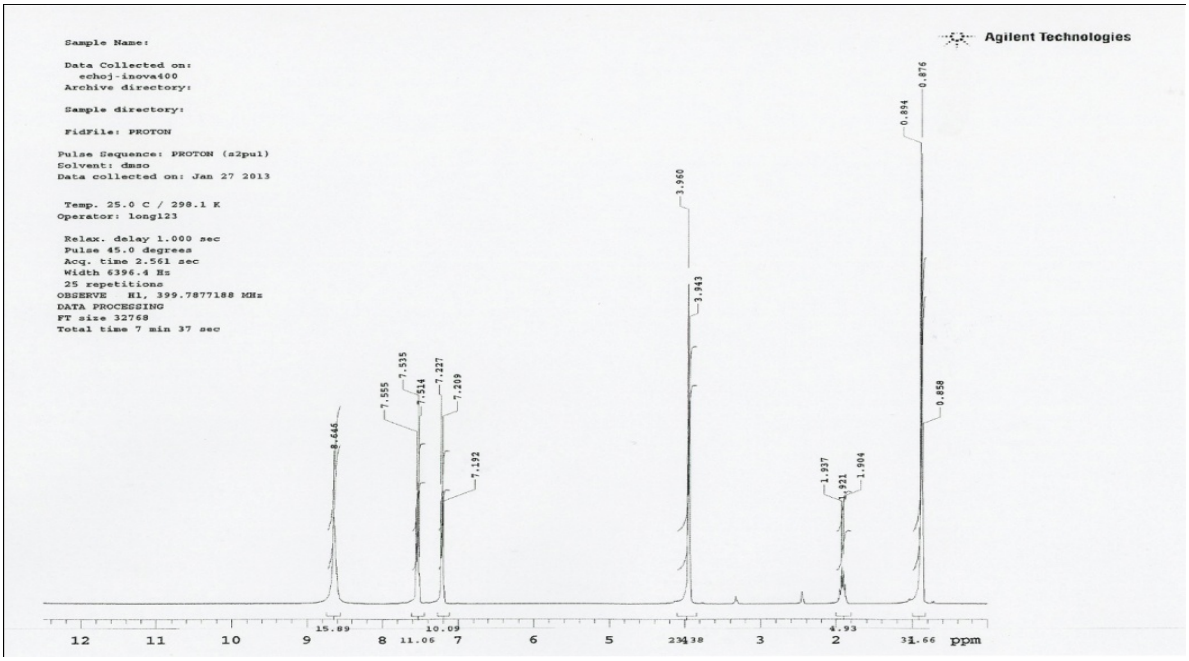


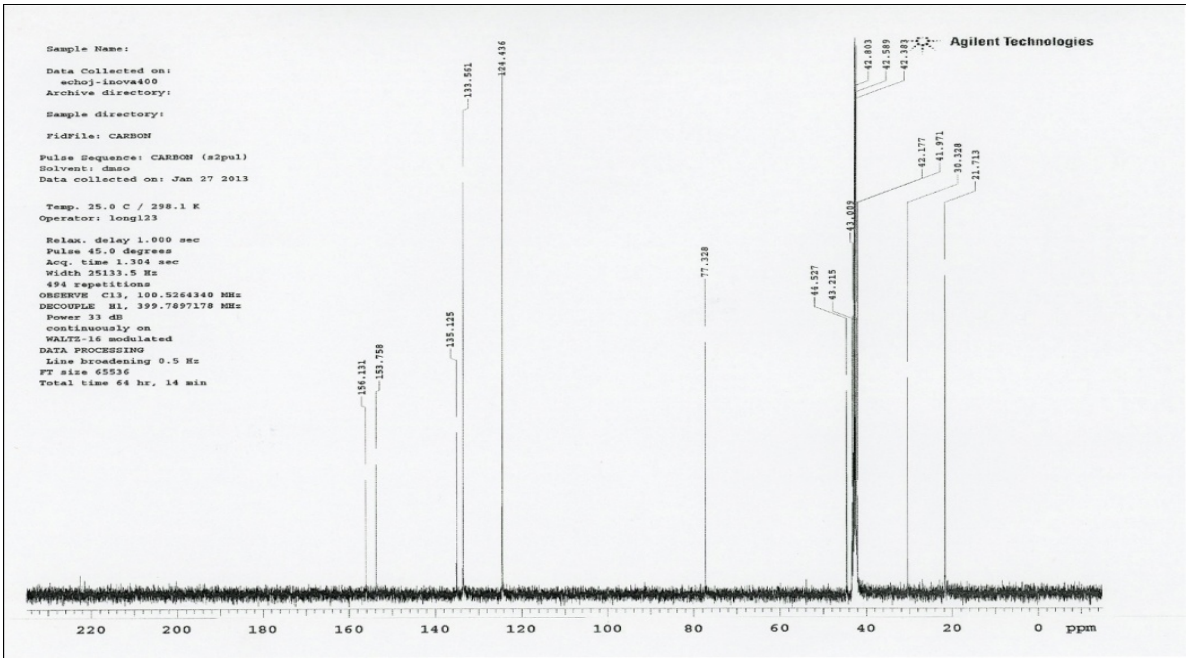


Following *General procedure 2*, The Boc protected precursor (100 mg, 0.22 mmol) was dissolved in HCl/Et_2O (20 mL) and stirred overnight at RT. The title compound was obtained as a white solid (30 mg, 35 %). δH (400 mHz, CD3SOCD3) 4.145 (4H, br s); 7.279-7.560 (13H, m); 9.481 (2H, br s). δC (100 mHz, CD3SOCD3) 52.256; 124.329; 124.543; 129.662; 131.639; 132.844; 133.410; 134.042; 134.813; 135.240; 136.758; 153.727; 154.040; 154.605. m/z (ESI^+) 368 (100% MH^+).

Synthesis of 4-(aminomethyl)phenyl *iso*-butyl carbonate hydrochloride C6

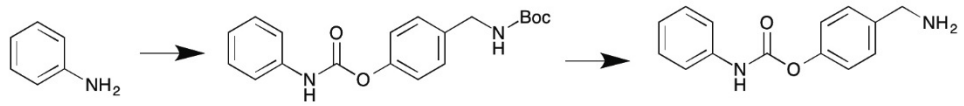


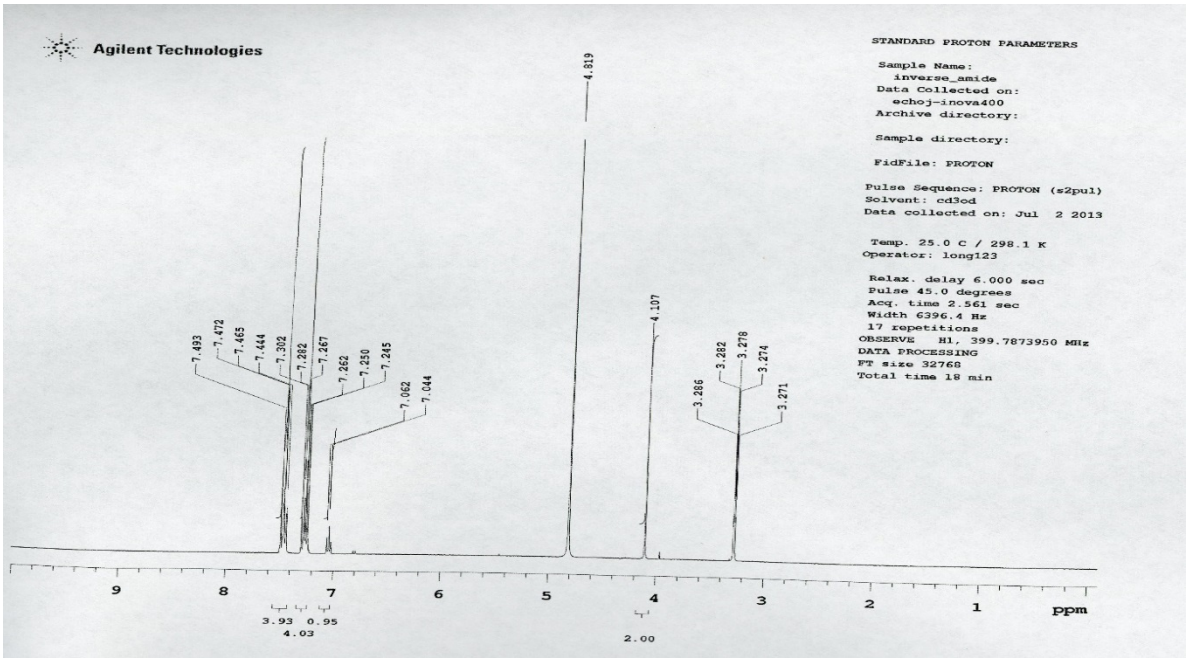


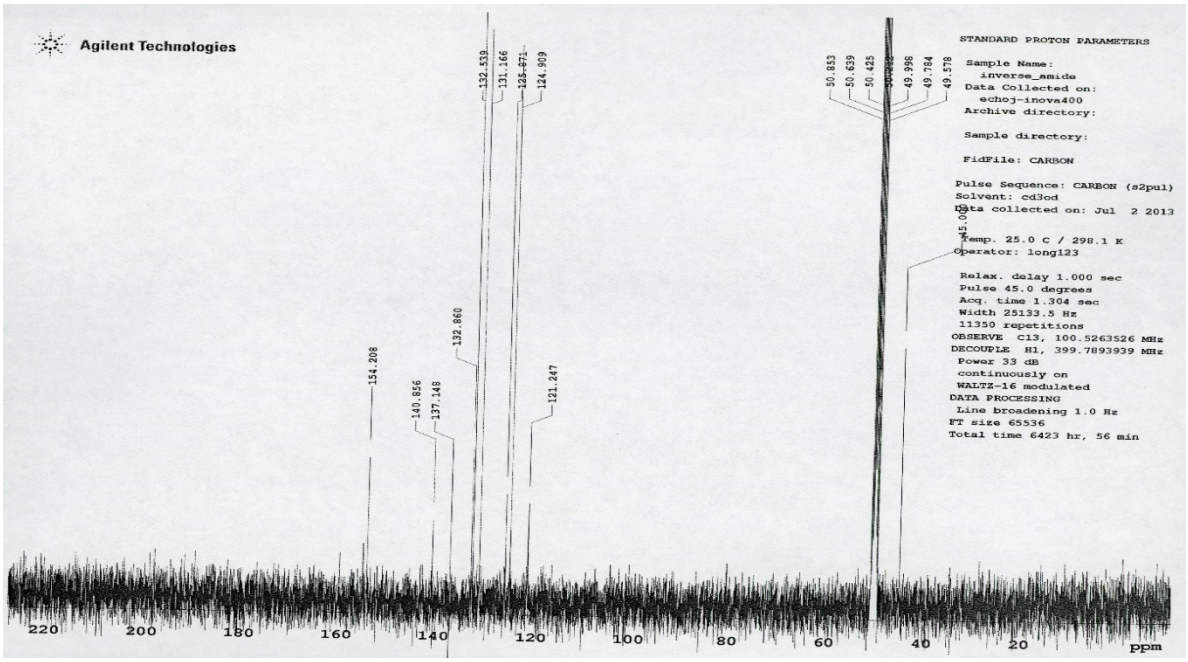


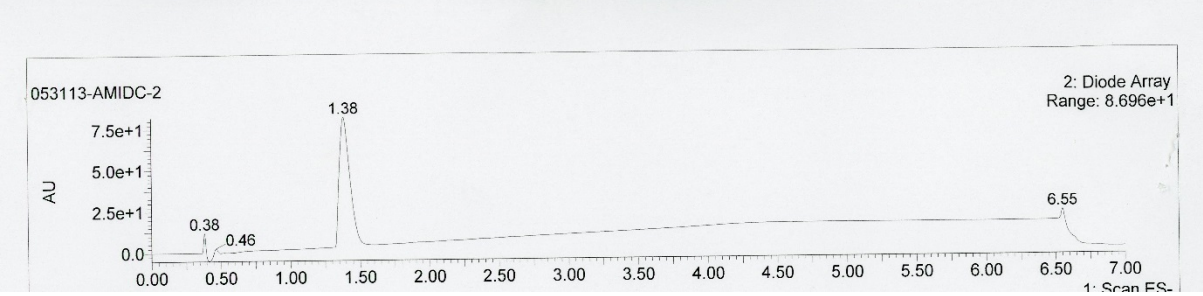
According to *General Procedure 1*, *iso*-butyl chloroformate was reacted with *tert*-butyl 4-hydoxybenzylcarbamate in DMF:pyridine (20 mL). Purification by chromatography on silica gel yielded **the Boc product**. Then according to *General Procedure 2*, **the Boc** product was treated with HCl/Et_2O to yield the title compound. δH (400 mHz, CD_3SOCD_3) 0.876 (6H, d, J=6.5Hz); 1.921 (1H, m); 3.95 (4H, m); 7.209 (2H, d, J=7.6Hz); 7.533 (2H, d, J=7.6Hz), 8.645 (3H, s). δC (100 mHz, CD_3SOCD_3) 21.813; 30.328; 44.527; 77.328; 124.436; 133.125; 135.125; 153.758; 156.156. m/z (ESI^+) 224 (100% MH^+).

Synthesis of Amide C7



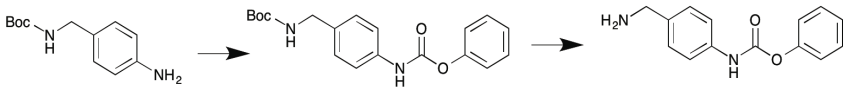


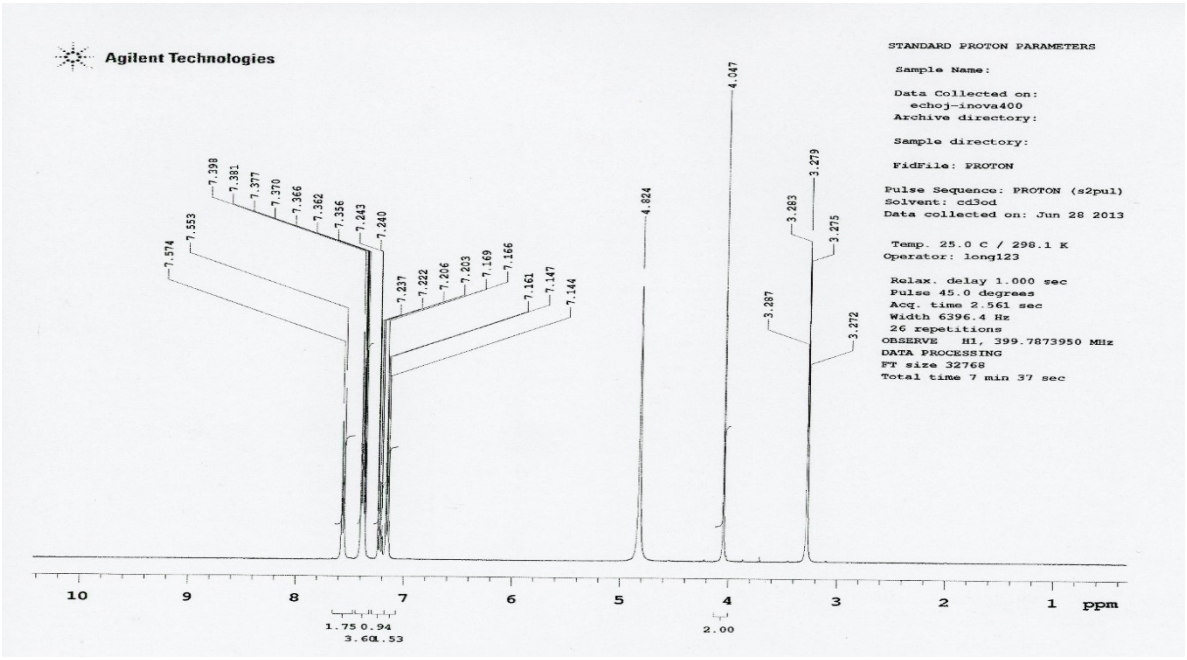


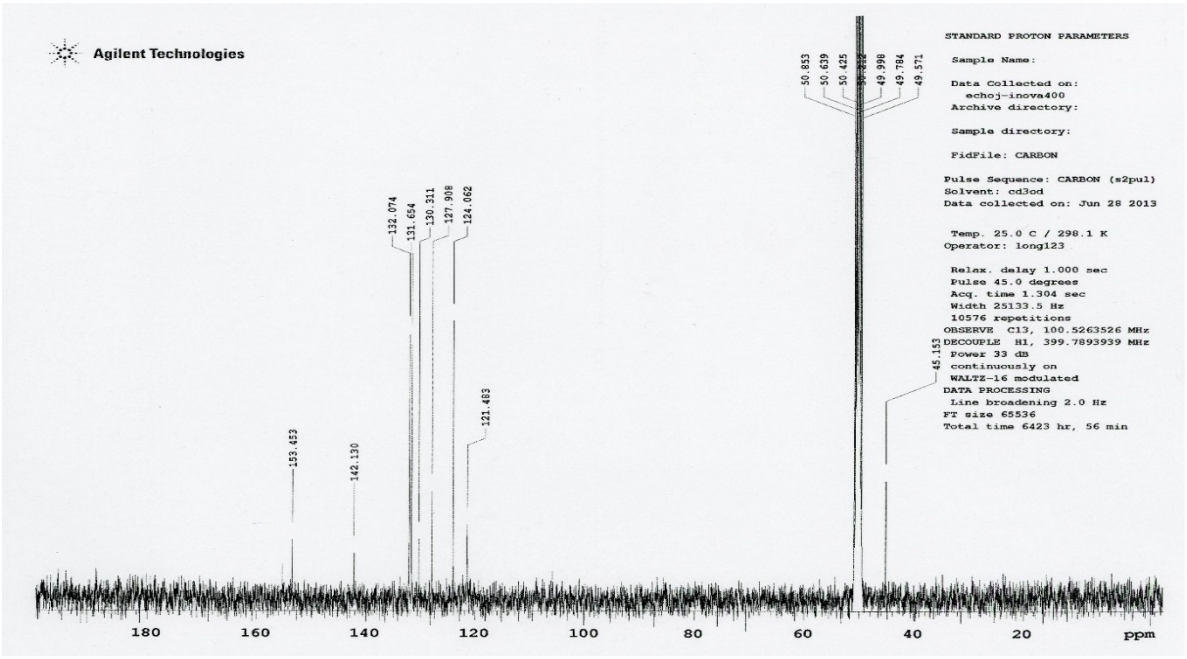


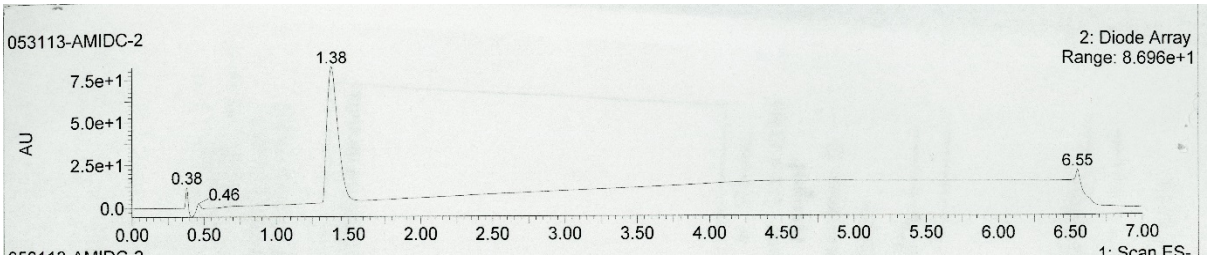
Phenyl *iso*cyanate (261 mg, 2.2 mmol) was reacted with *tert*-butyl 4-hydoxybenzylcarbamate (500 mg, 2.2 mmol) in pyridine (20 mL) at 4 ^{o}C for 1 h prior to warming to rt. The product of this reaction was treated according to *General procedure 2*, with HCl/Et_2O. This gave the titled product as a white solid (100 mg, 30%). δH (400 mHz, CD_3SOCD_3) 4.107 (2H, s); 7.044 (1H, t, J=7.2Hz); 7.245-7.302 (4H, m), 7.444-7.493 (4H, m). δC (100 mHz, CD_3SOCD_3) 45.000; 30.328; 121.247; 124.909; 125.871; 131.166; 132.539; 132.860; 137.148; 140.856; 154.208. m/z (ESI^+) 243.

Synthesis of Amide C8



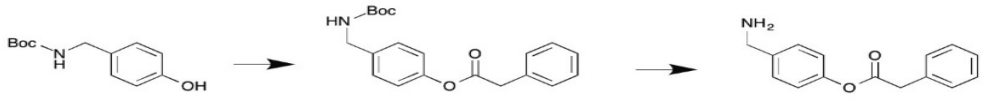


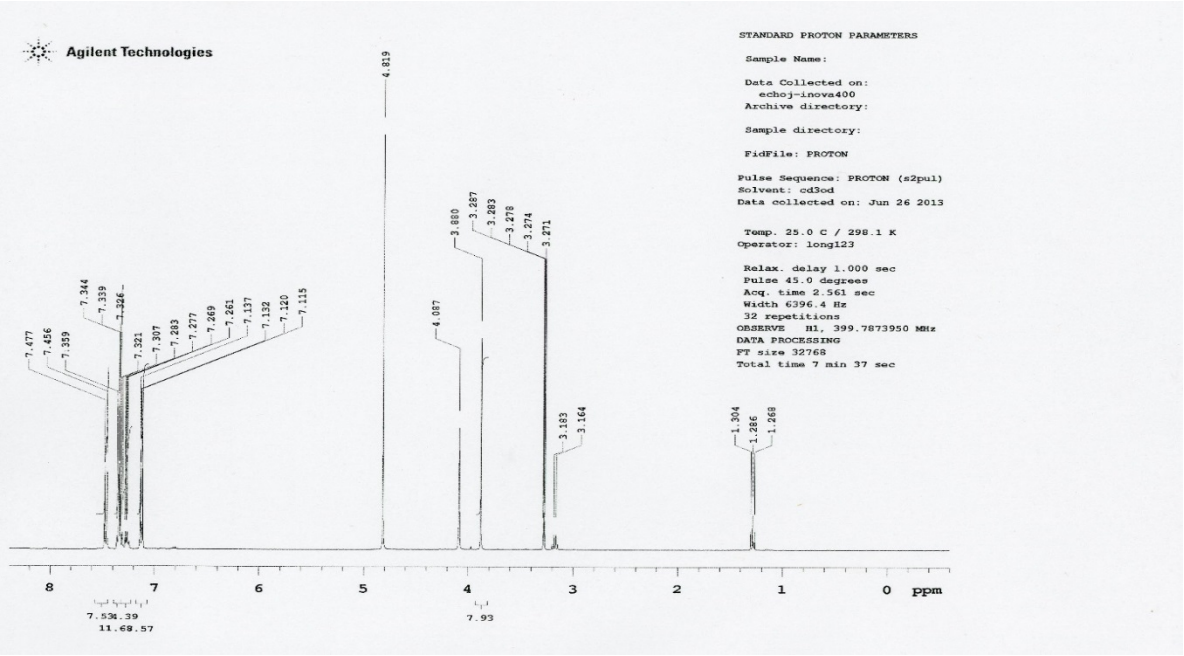


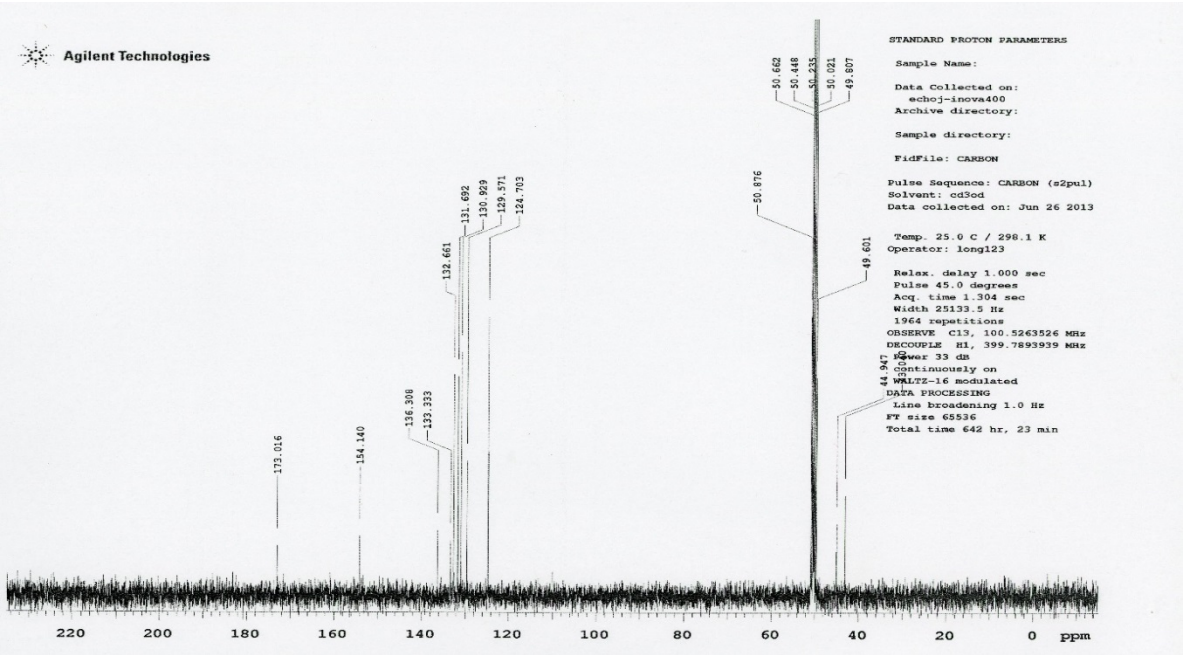


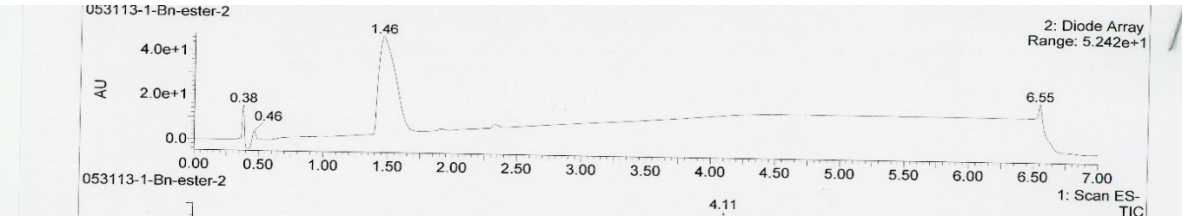
Following *General Procedure 1*, *tert*-butyl 4-aminobenzylcarbamate (500 mg, 2.2 mmol) was treated with phenyl chloroformate (342 mg, 2.2 mmol). Silica chromatography yielded the Boc compound (301 mg, 40%) as a white solid. Following *General procedure 2*, the Boc compound (300 mg, 0.9 mmol) yielded the title compound (148 mg, 70%). δH (400 mHz, CD_3SOCD_3) 4.047 (2H, s); 7.147-7.614 (1H, m); 7.166-7.243 (4H, m), 7.63 (2H, d, J=8.3Hz). δC (100 mHz, CD_3SOCD_3) 45.153; 30.328; 121.483; 124.062; 127.908; 130.311; 131.654; 132.074; 142.130; 153.453. m/z (ESI^+) 243.

Synthesis of 4-(aminomethyl)phenyl phenylacetate hydrochloride C9



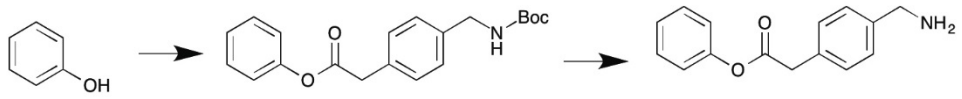


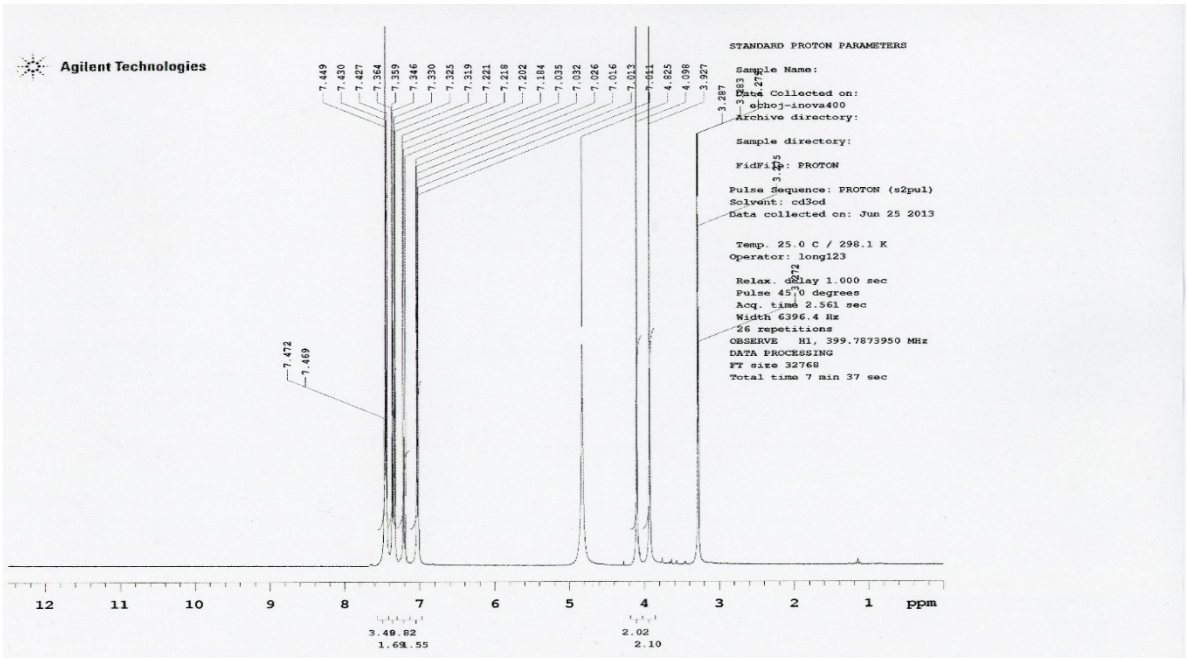


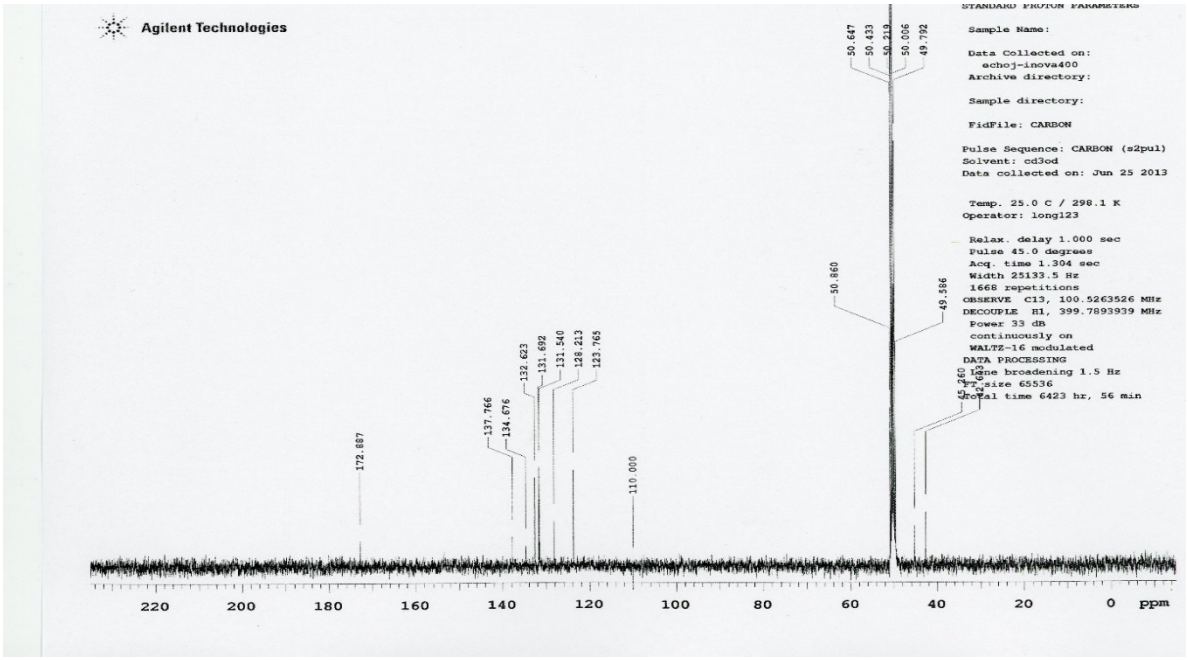


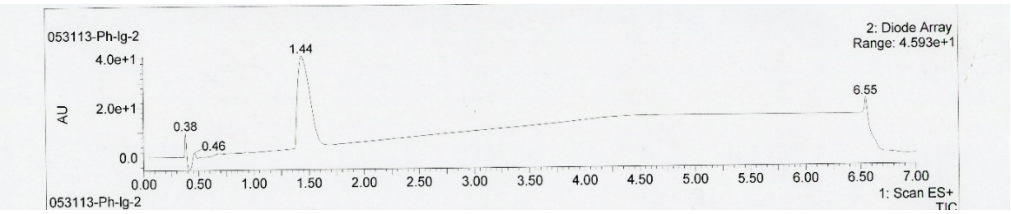
Following *General procedure 3*, phenylacetic acid (1.0 g, 7 mmol) was reacted with *tert*-butyl 4-hydoxybenzylcarbamate (1.5 g, 7.0 mmol), EDCI (2.1g, 14.0 mmol) and triethyl amine (0.9 g, 9.0 mmol) in DMF (20 mL). Then following *General procedure 2*, the product (500 mg, 1.4 mmol) was treated with Et_2O.HCl and stirred overnight. The solid was filtered and washed three times with Et_2O to give the pure compound as a white solid (212 mg, 60%). δH (400 mHz, CD_3SOCD_3) 3.880 (2H, s); 4.087 (2H, s); 7.120 (2H, d, J=8.2); 7.261 (1H, m); 7.326 (2H, m); 7.466 (2H, d, J=8.4). δC (100 mHz, CD_3SOCD_3) 43.040; 44.947; 124.703; 129.571; 130.929; 131.692; 132.661; 133.333; 136.308; 154.140; 173.016. m/z (ESI^+) 242.

Synthesis of Phenyl [4-(aminomethyl)phenyl]acetate hydrochloride C10



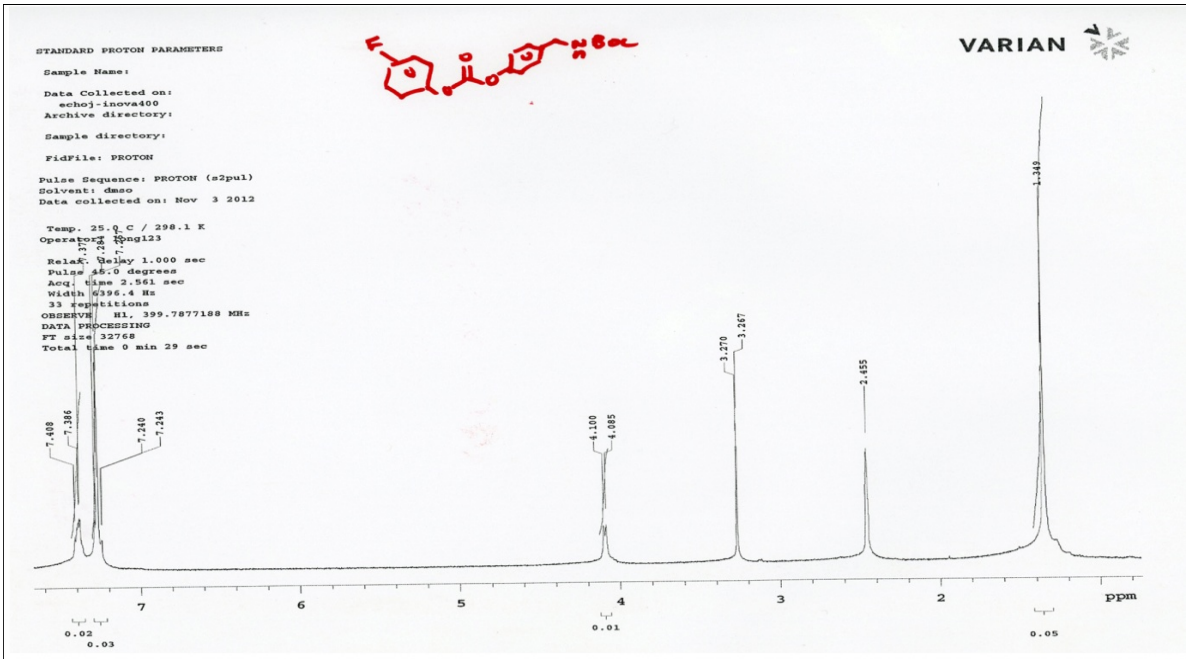


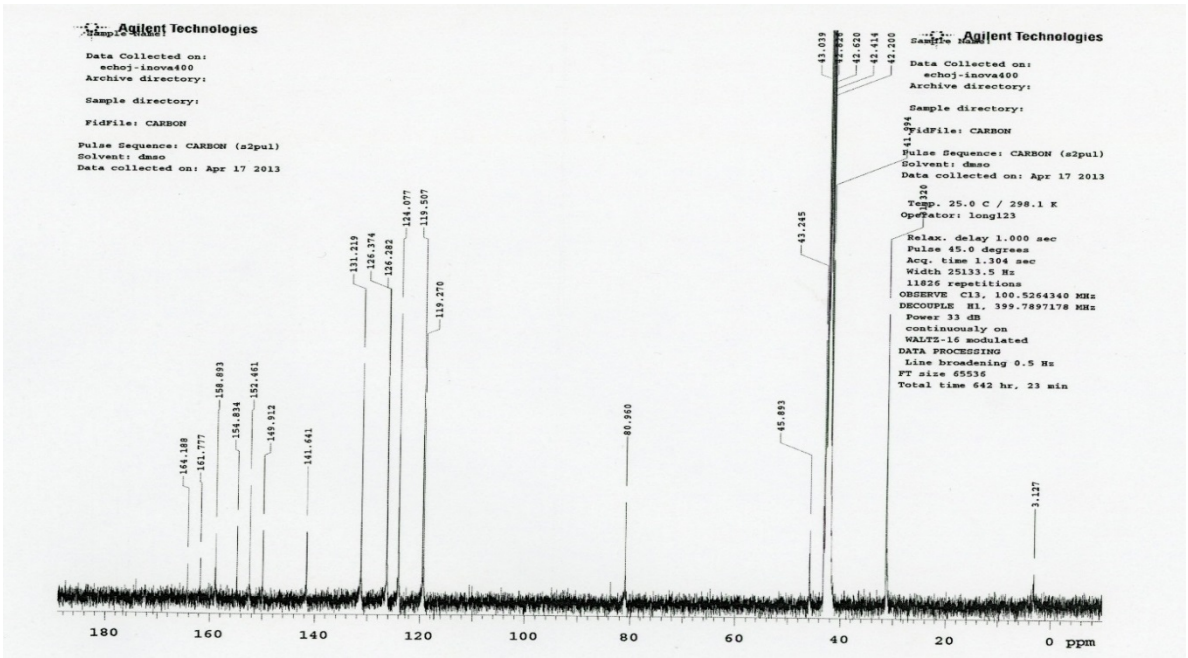




Following *General procedure 3*, *N*-Boc [(4-aminomethyl)phenyl]acetic acid (1.0 g, 3.7 mmol) was reacted with phenol (0.4 g, 3.7 mmol), EDCI (1.0 g, 7.4 mmol) and triethyl amine (0.4 g, 3.7 mmol) in DMF (10 mL). Then following *General procedure 2*, the product (400 mg, 1.1 mmol) was treated with Et_2O.HCl and stirred overnight. The solid was filtered and washed three times with Et_2O to give the pure compound as a white solid (222 mg, 80%). δH (400 mHz, CD_3SOCD_3) 3.927 (2H, s); 4.098 (2H, s); 7.120 (2H, d, J=8.2); 7.261 (1H, m); 7.326 (2H, m); 7.466 (2H, d, J=8.4). δC (100 mHz, CD_3SOCD_3) 42.643; 45.260; 110.000; 123.761; 128.213; 131.540; 131.692; 132.623.; 134.676; 137.766; 172.887. *m/z* = $C_{15}H_{15}NO_2$ 242 (MH^+)

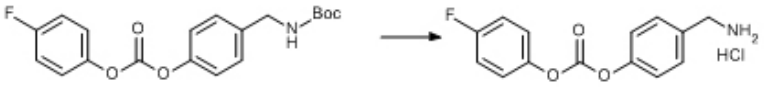
Synthesis of N-Boc 4-(aminomethyl)phenyl (4-fluorophenyl) carbonate

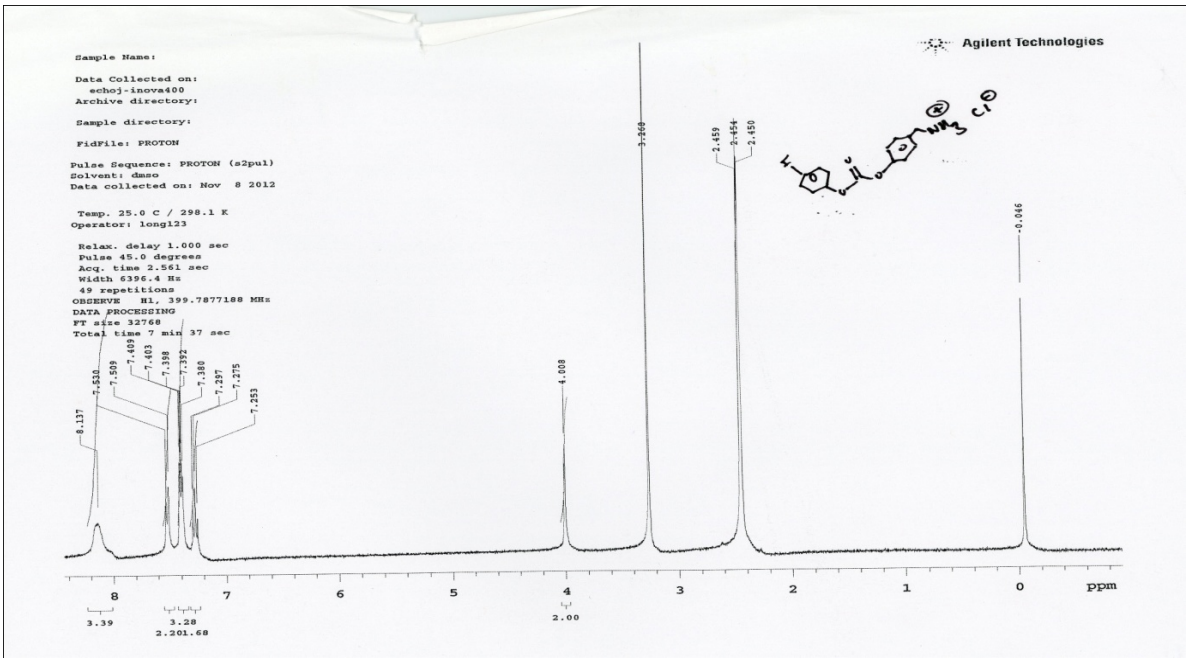


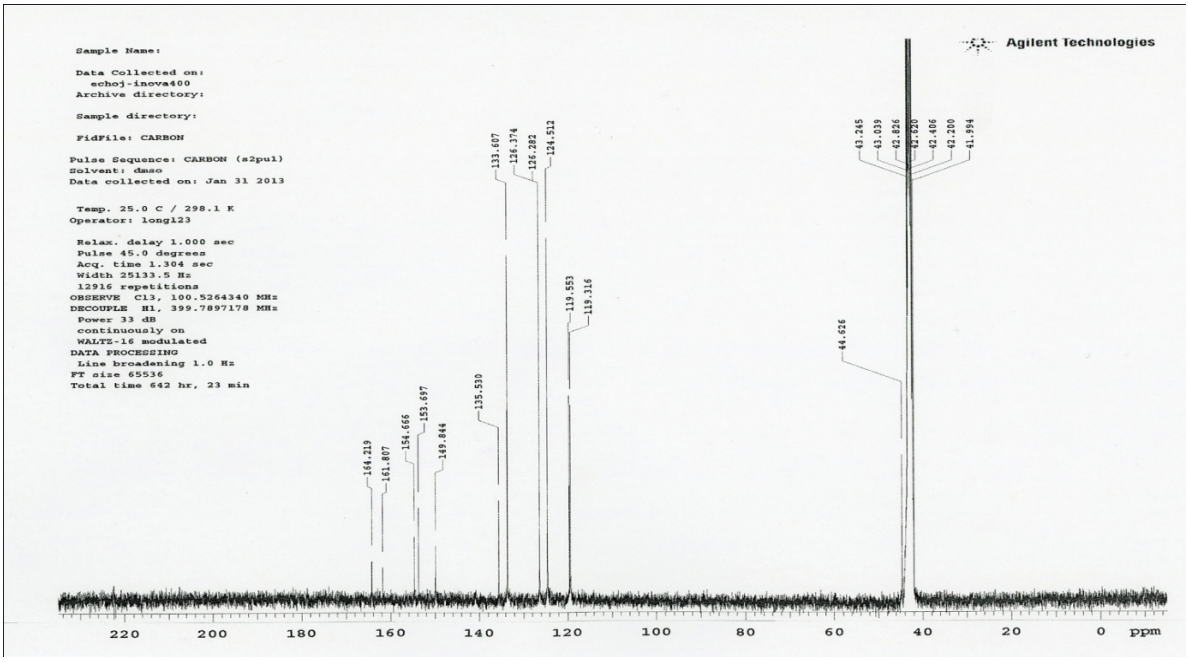


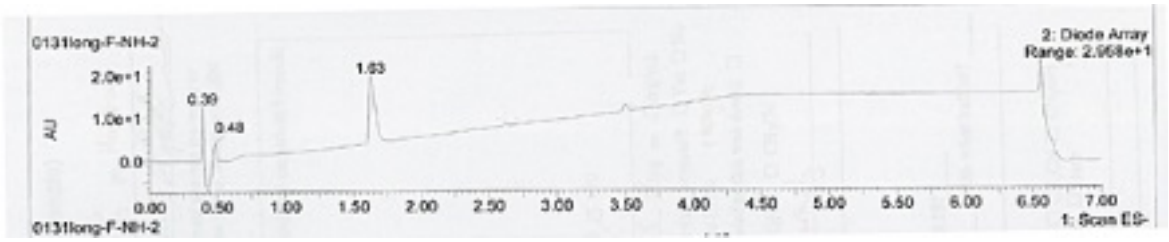
Following *General Procedure 1*, *tert*-butyl 4-hydoxybenzylcarbamate (500 mg, 2.2 mmol) was reacted with p-fluorophenyl chloroformate (0.689 mg, 3.96 mmol) in DMF / pyridine (20 mL). Chromatography on silica gel (gradient from 5% EtOAc in hexanes to 40% EtOAc in hexanes) gave the target compound as a white solid (326 mg, 50%). δH (400 MHz, CD_3SOCD_3) 1.349 (9H, s); 4.092 (2H, d, J=6.0Hz); 7.243-7.284 (4H, m); 7.377-7.408 (4H, m). δC (100 MHz, CD_3SOCD_3) 10.320; 45.893; 80.960; 119.270 + 119.507; 124.077; 126.282 + 126.374; 131.219; 141.641; 149.912; 152.461; 154.834; 158.893; 161.777; 164.188.

Synthesis of 4-(aminomethyl)phenyl (4-fluorophenyl) carbonate hydrochloride C11



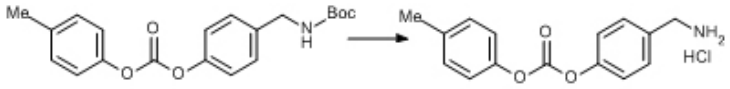


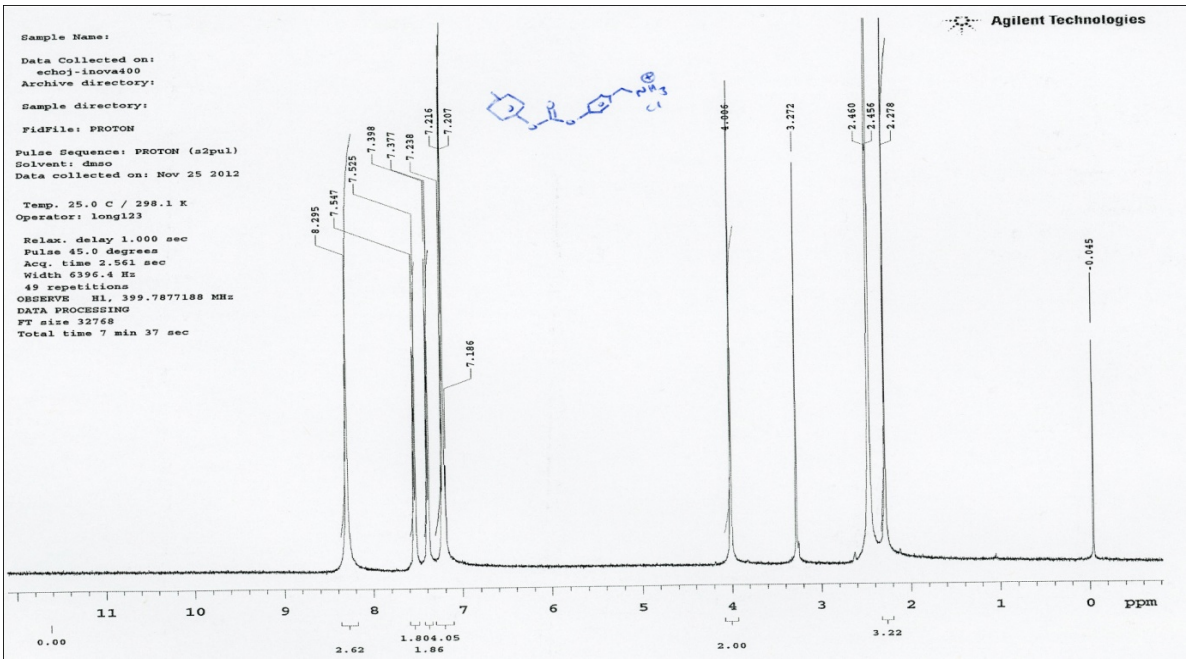


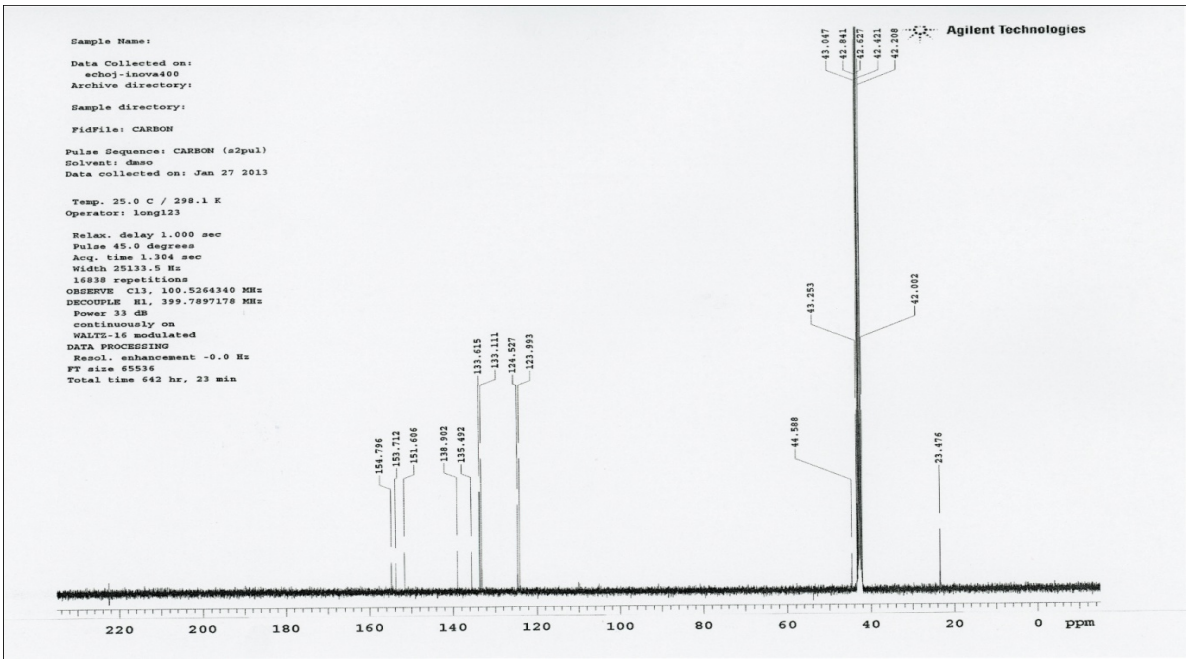


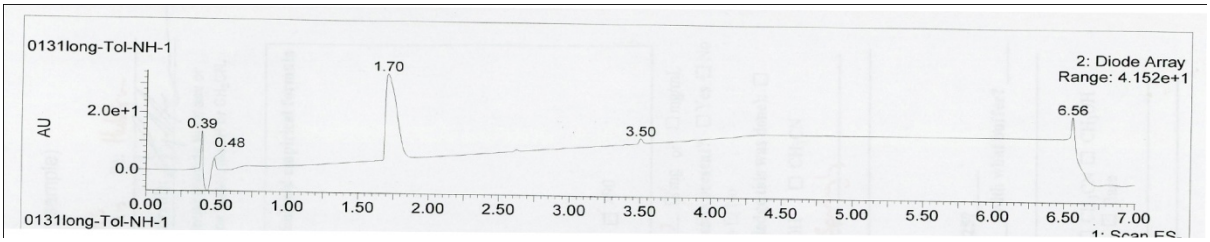
Following *General Procedure 2*, **Boc compound** (100 mg, 0.28 mmol) was dissolved in HCl/Et_2O (20 mL) and stirred overnight at RT. The title compound was obtained as a white solid (50 mg, 60 %). δH (400 MHz, CD_3SOCD_3) 4.008 (2H, s); 7.243-7.284 (4H, m); 7.261 (2H, d, J=8.8Hz); 7.380-7.409 (4H, m); 7.519 (2H, d, J=8.4Hz); 8.137 (3H, br s). δC (100 MHz, CD_3SOCD_3) 44.626; 119.316; 119.553; 124.512; 126.282; 126.384; 135.530; 149.844; 153.647; 154.666; 161.807; 164.219. m/z (ESI^+) 262 (100% MH^+).

Synthesis of 4-(aminomethyl)phenyl (4-methylphenyl) carbonate hydrochloride C12



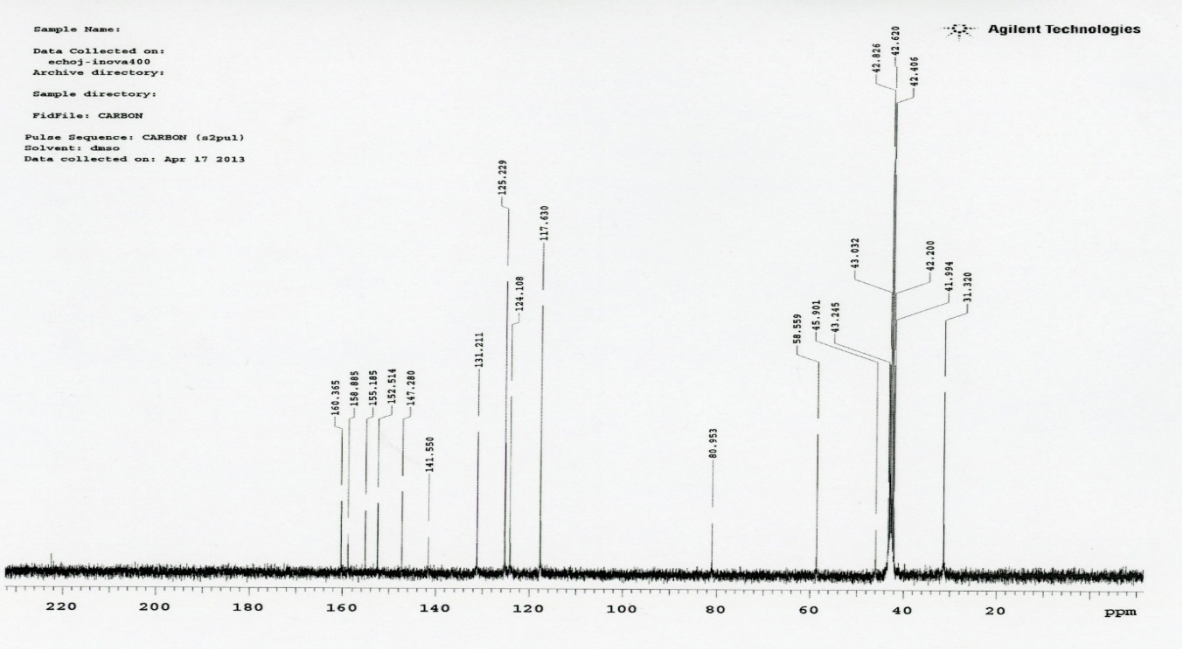






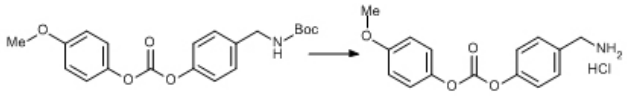
Following *General procedure 2*, **Boc compound** (100 mg, 0.28 mmol) was dissolved in HCl/Et_2O (20 mL) and stirred overnight at RT. The title compound was obtained as a white solid (24 mg, 30 %). δH (400 mHz, CD_3SOCD_3) 2.278 (3H, s); 4.006 (2H, s); 7.186-7.216 (4H, m); 7.387 (2H, d, J=8.4Hz); 7.535 (2H, d, J=8.5Hz); 8.295 (3H, s). δC (100 mHz, CD_3SOCD_3) 23.476; 44.588; 123.993; 124.527; 133.111; 133.615; 135.492; 138.902; 151.606; 153.712; 154.796. m/z (ESI^+) 258 (100% MH^+).

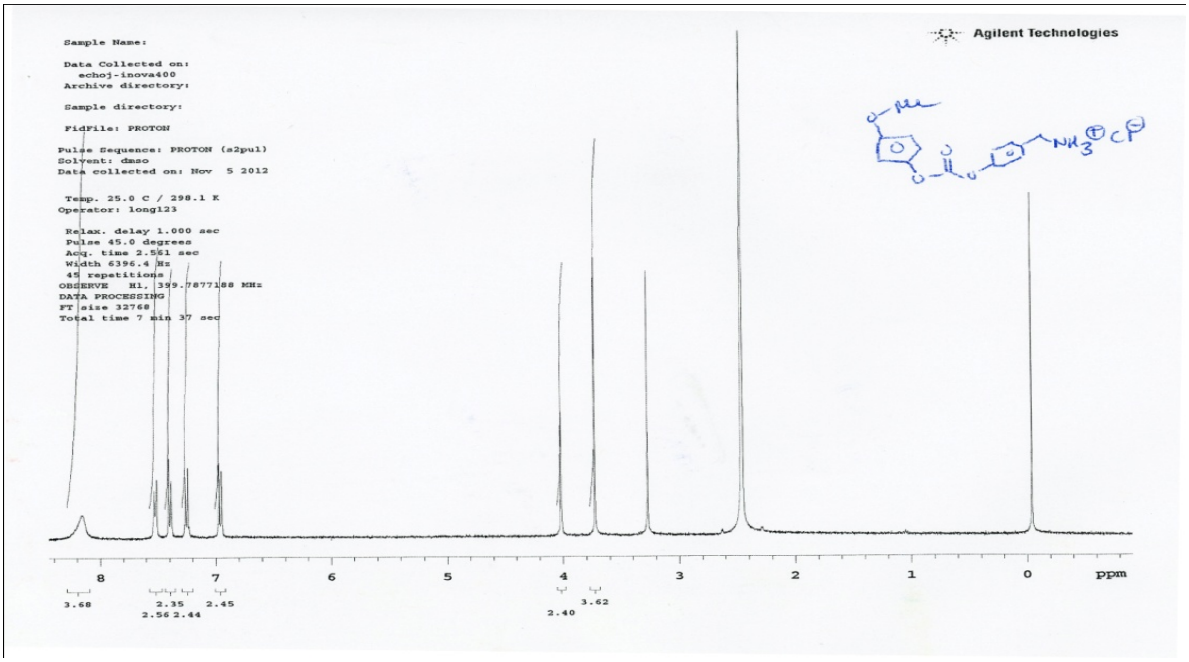
Synthesis of N-Boc 4-(aminomethyl)phenyl (4-methoxyphenyl) carbonate

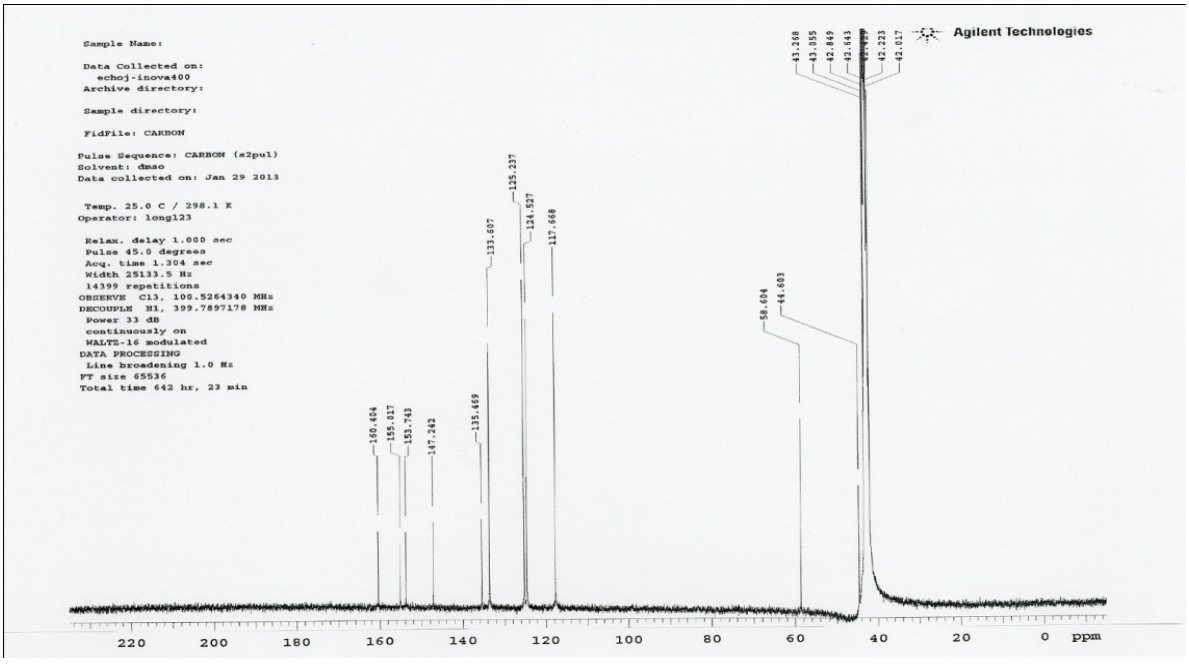


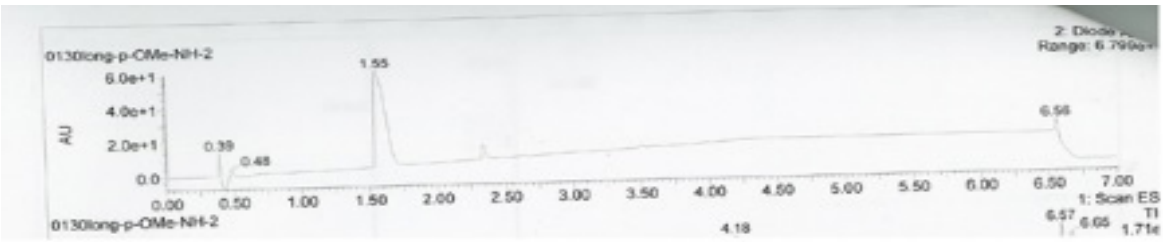
Following *General Procedure 1*, *tert*-butyl 4-hydoxybenzylcarbamate (500 mg, 2.2 mmol) was reacted with 4-methoxyohenyl chloroformate (1400 mg, 3.96 mmol) in DMF / pyridine (20 mL). Chromatography on silica gel (gradient from 5% EtOAc in hexanes to 40% EtOAc in hexanes) gave the target compound as a white solid (509 mg, 55 %). δH (400 mHz, CD_3SOCD_3) 1.355 (9H, s); 3.724 (3H, s); 4.106 (2H, d, J=9.6Hz); 6.912 (1H, d, J=9.2Hz); 7.233-7.264 (4H, m); 7.35 (1H, m). δC (100 mHz, CD_3SOCD_3) 31.320; 43.245; 45.901; 58.559; 80.953; 117.630, 124.108; 125.229; 131.211; 141.556; 147.280; 152.514; 155.185; 158.885; 160.365.

Synthesis of 4-(aminomethyl)phenyl (4-methoxyphenyl) carbonate C13



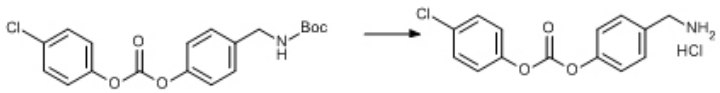


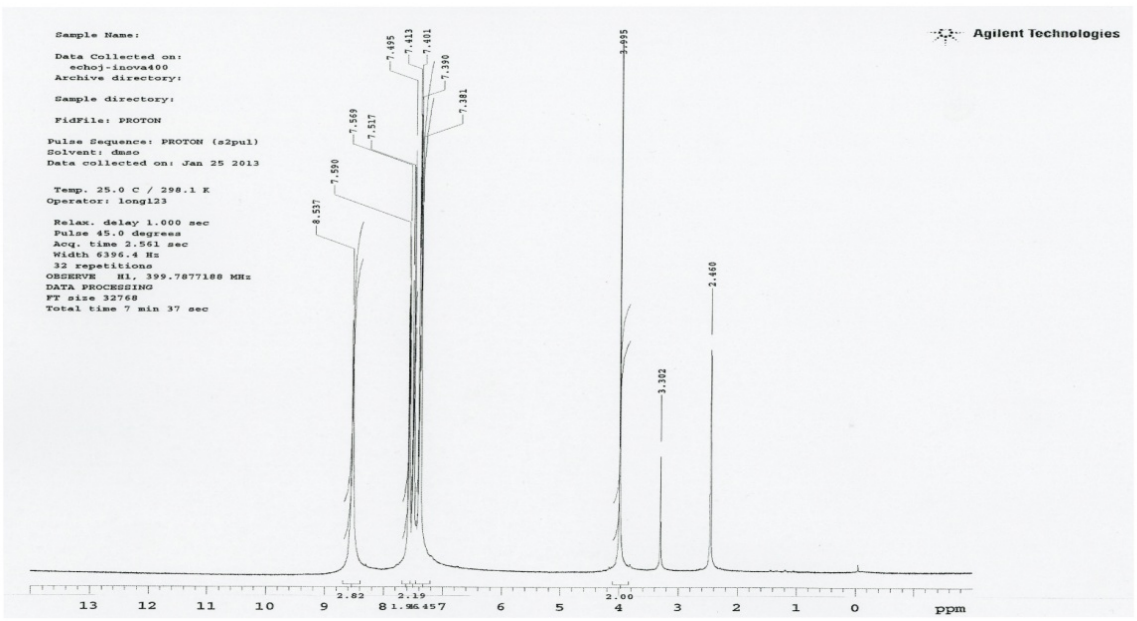


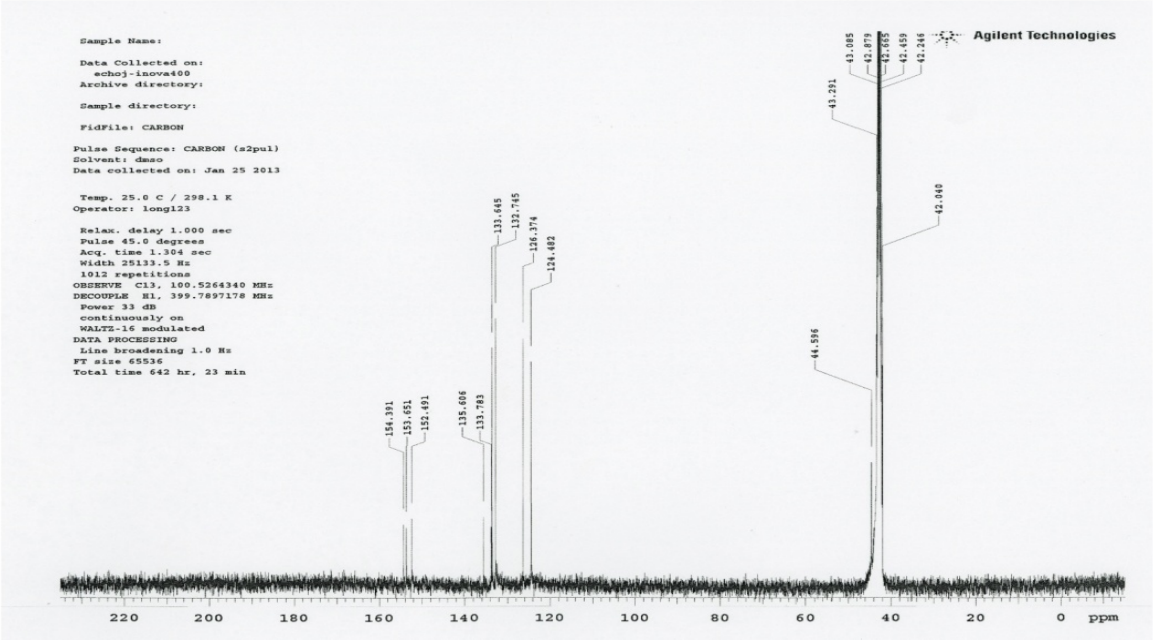


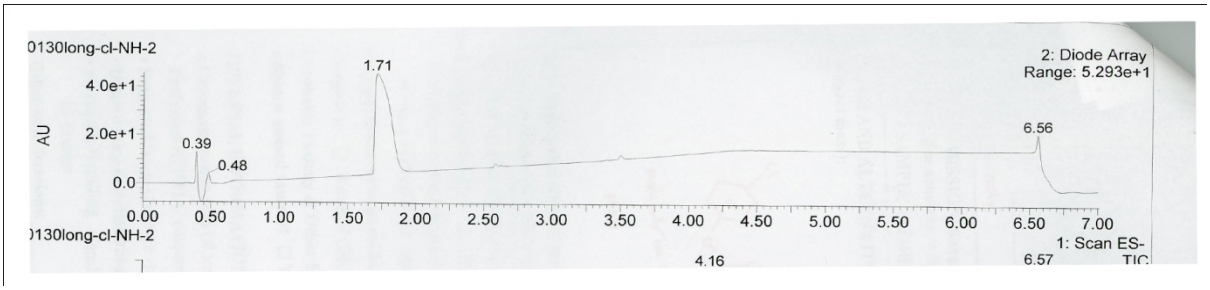
Following *General procedure 2*, **Boc compound** (160 mg, 0.43 mmol) was dissolved in HCl/Et_2O (30 mL) and stirred overnight at RT. The title compound was obtained as a white solid (60 mg, 45 %). δH (400 mHz, CD_3SOCD_3) 3.751 (3H, s); 4.052 (2H, s); 6.964 (2H, d, J=8.2Hz); 7.252 (2H, d, J=8.2Hz); 7.402 (2H, d, J=8.5Hz); 7.522 (2H, d, J=8.5Hz); 8.215 (3H, s). δC (100 mHz, CD_3SOCD_3) 160.404; 155.017; 153.743; 147.242; 135.469; 133.607; 125.237; 124.527; 117.668; 58.604; 44.603.m/z (ESI^+) 274 (100% MH^+).

Synthesis of 4-(aminomethyl)phenyl (4-chlorophenyl) carbonate hydrochloride C14



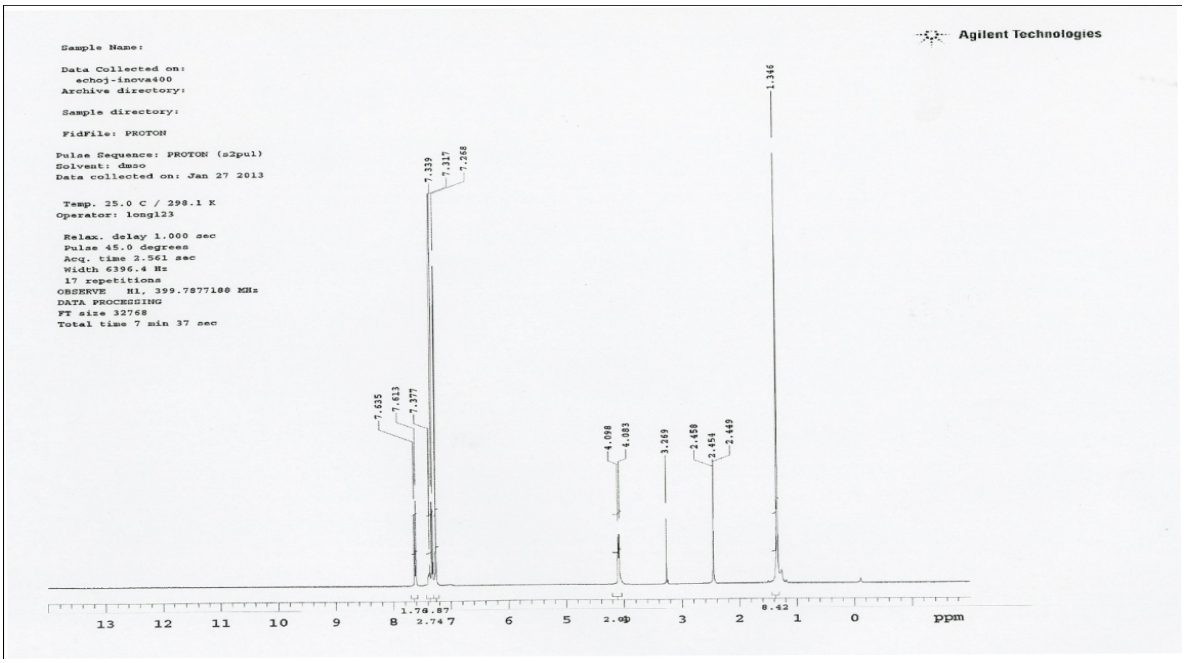


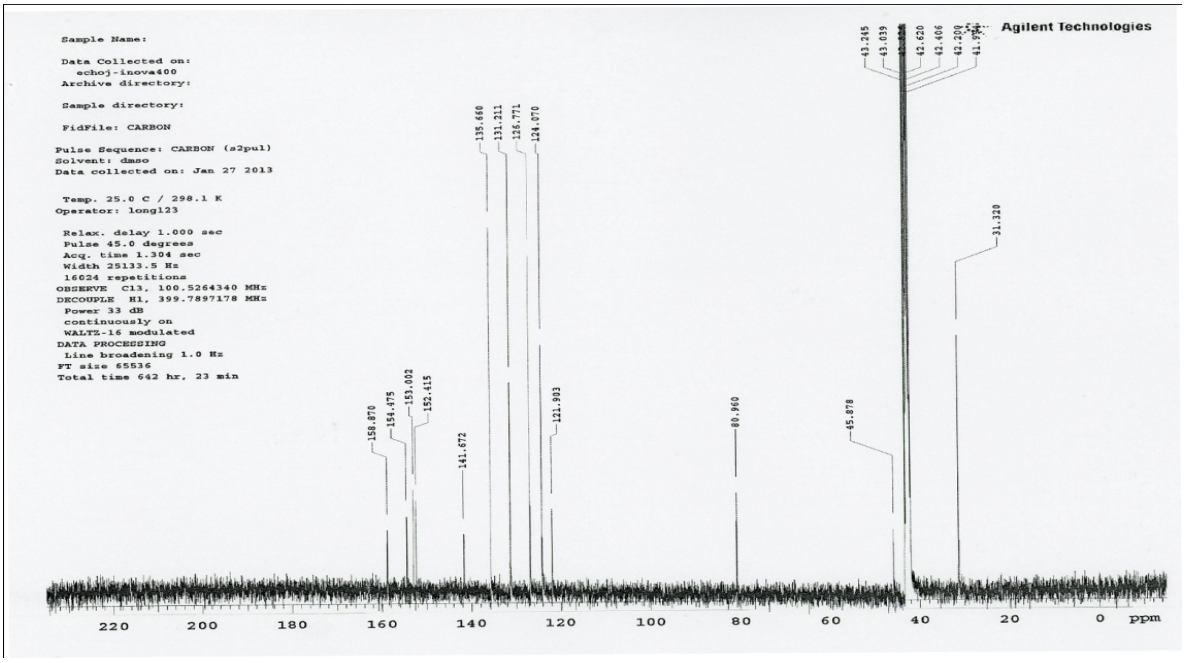


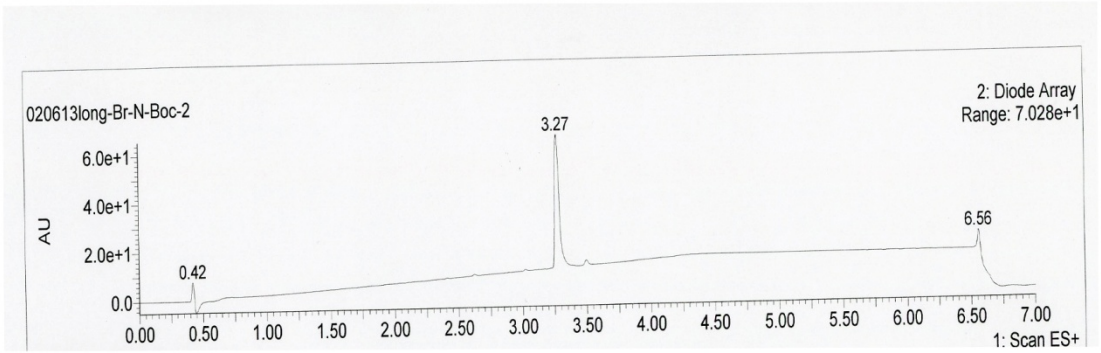


Following *General procedure 2*, **C23** (110 mg, 0.29 mmol) was dissolved in HCl/Et_2O (20 mL) and stirred overnight at RT. The title compound was obtained as a white solid (54 mg, 60 %). δH (400 mHz, CD_3SOCD_3) 3.995 (2H, s); 7.381-7.413 (4H, m); 7.506 (2H, d, J=8.8Hz); 7.580 (2H, d, J=8.8Hz), 8.537 (3H, s). δC (100 mHz, CD_3SOCD_3) 44.596; 124.482; 126.374; 132.745; 133.645; 133.783; 135.606; 152.491; 153.651; 154.391. m/z (ESI^+) 279 (100% MH^+).

Synthesis of N-Boc 4-(aminomethyl)phenyl (4-bromophenyl) carbonate

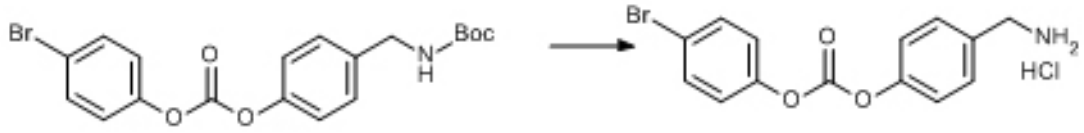


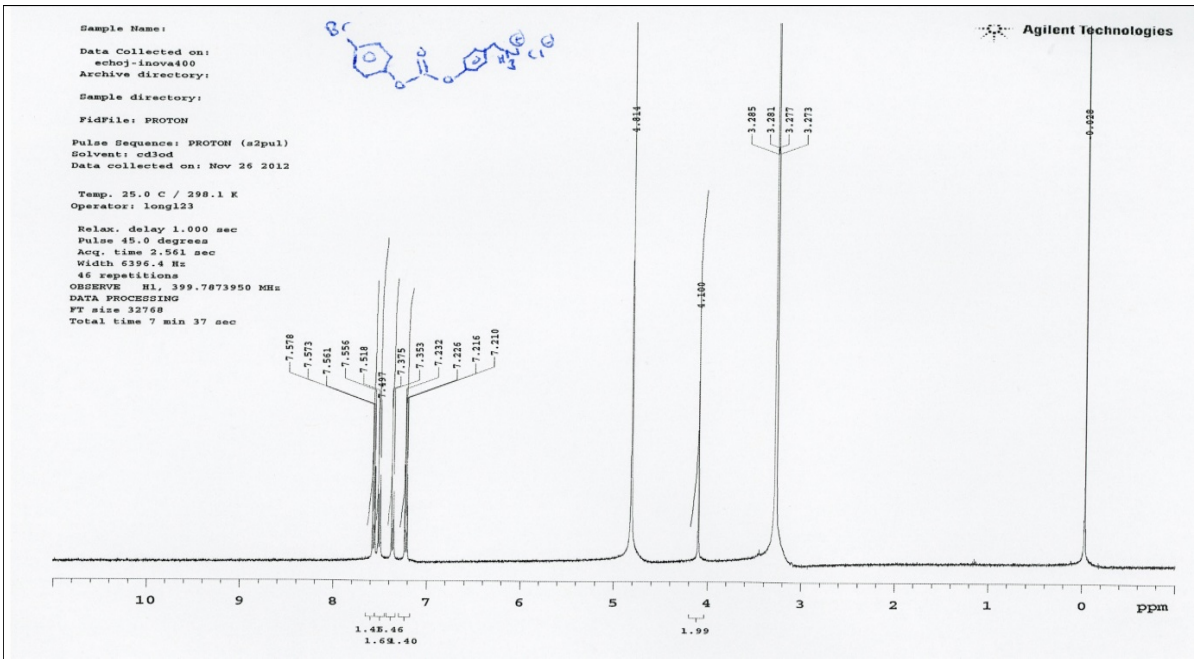


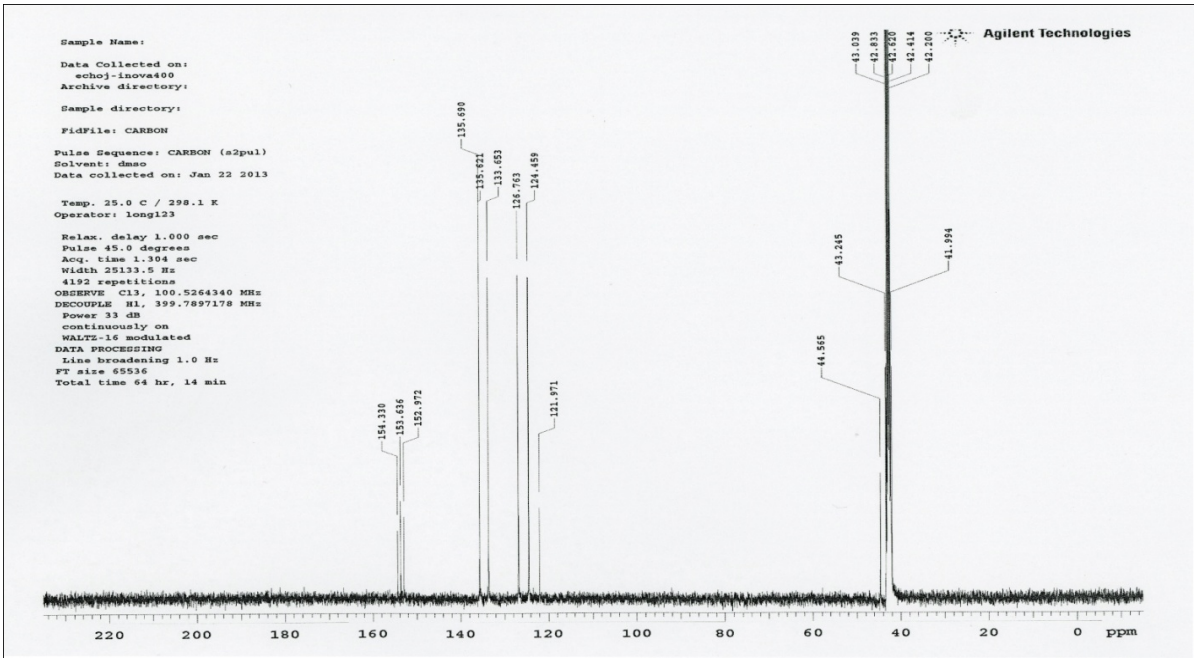


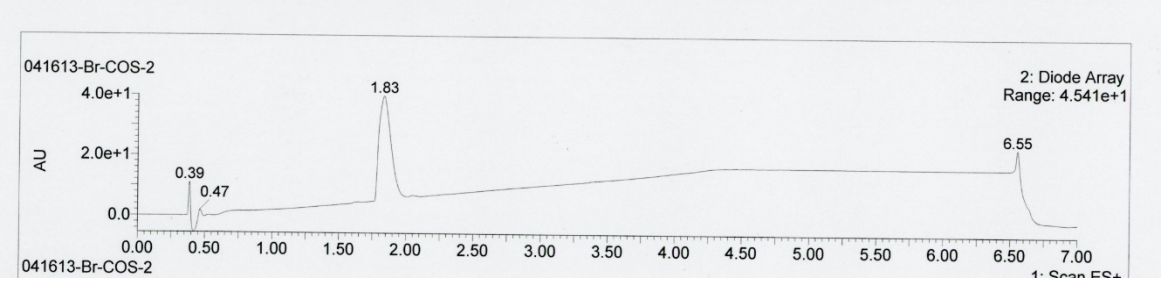
Following *General Procedure 1*, *tert*-butyl 4-hydoxybenzylcarbamate (500 mg, 2.2 mmol) was reacted with p-bromophenyl chloroformate (0.931 mg, 3.96 mmol) in DMF / pyridine (20 mL). Chromatography on silica gel (gradient from 5 % EtOAc in hexanes to 40 % EtOAc in hexanes) gave the target compound as a white solid (509 mg, 55 %). δH (400 MHz, CD_3SOCD_3) 1.346 (9H, s); 4.091 (2H, d, J=6.0Hz); 7.268-7.332 (4H, m); 7.367 (2H, d, J=8.8); 7.377 (1H, m); 7.624 (2H, d, J=8.8). δC (100 MHz, CD_3SOCD_3) 31.320; 45.878; 80.960; 121.903; 124.070; 126.771; 131.211; 135.660; 141.672; 152.415; 153.002; 154.475; 158.870. m/z (ESI^+) 440 (100% $M+NH_4^+$).

Synthesis of 4-(aminomethyl)phenyl (4-bromophenyl) carbonate hydrochloride C15



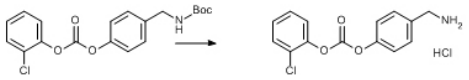


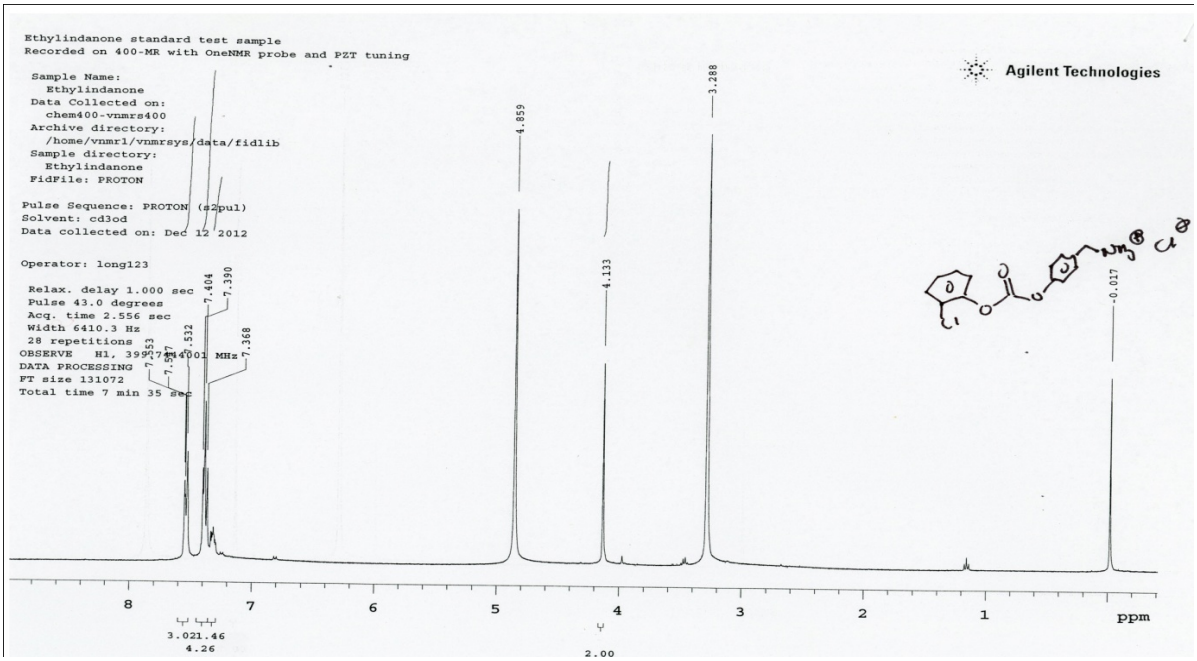


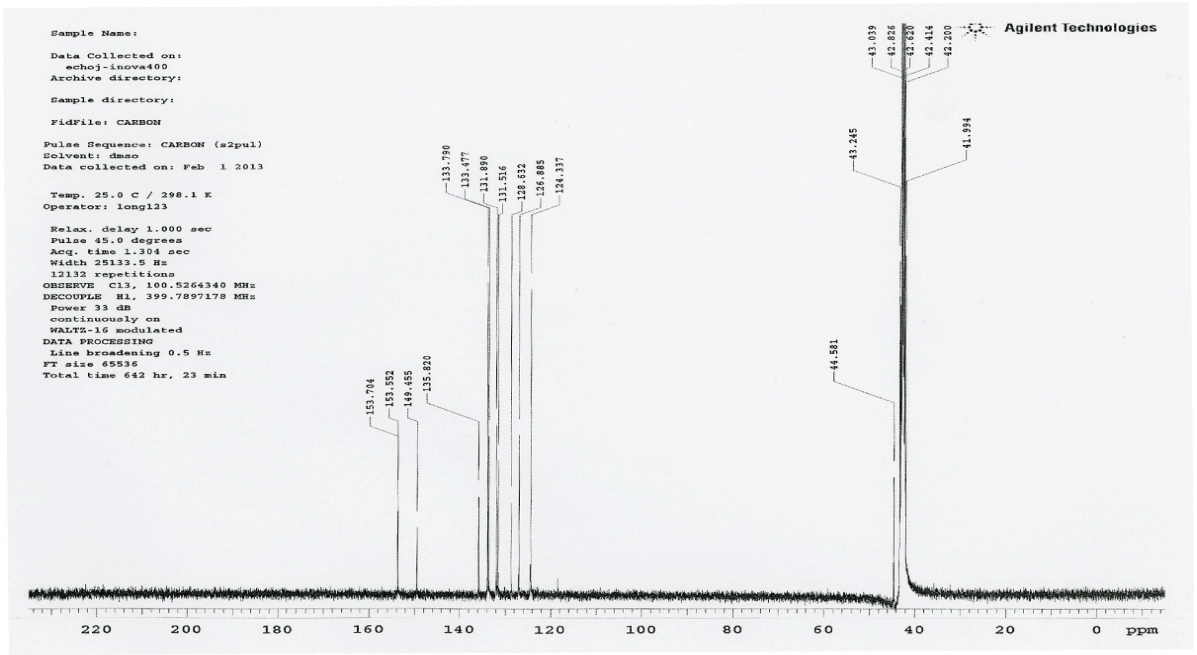


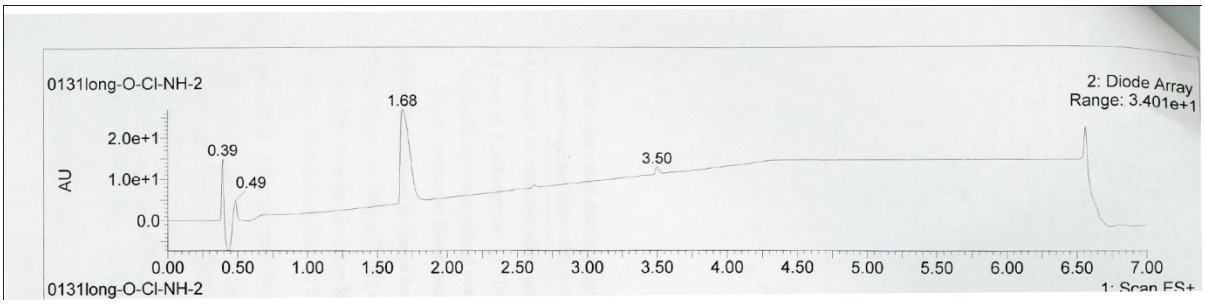
Following *General procedure 2*, **Boc compound** (150 mg, 0.36 mmol) was dissolved in HCl/Et_2O (20 mL) and stirred overnight at RT. The title compound was obtained as a white solid (51 mg, 40 %). δH (400 mHz, CD_3OD) 4.100 (2H, s); 7.216 (2H, d, J=4.8Hz); 7.364 (2H, d, J=8.8Hz); 7.555 (2H, d, J=8.8Hz); 7.573 (2H, d, J=4.8Hz). δC (100 mHz, CD_3SOCD_3) 44.565; 121.971; 124.459; 126.763; 123.653; 135.621; 135.690; 152.972; 153.636; 154.330. m/z (ESI^+) 322.

Synthesis of 4-(aminomethyl)phenyl (2-chlorophenyl) carbonate hydrochloride C16



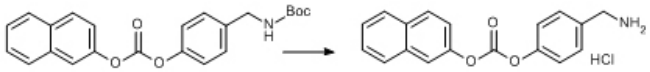


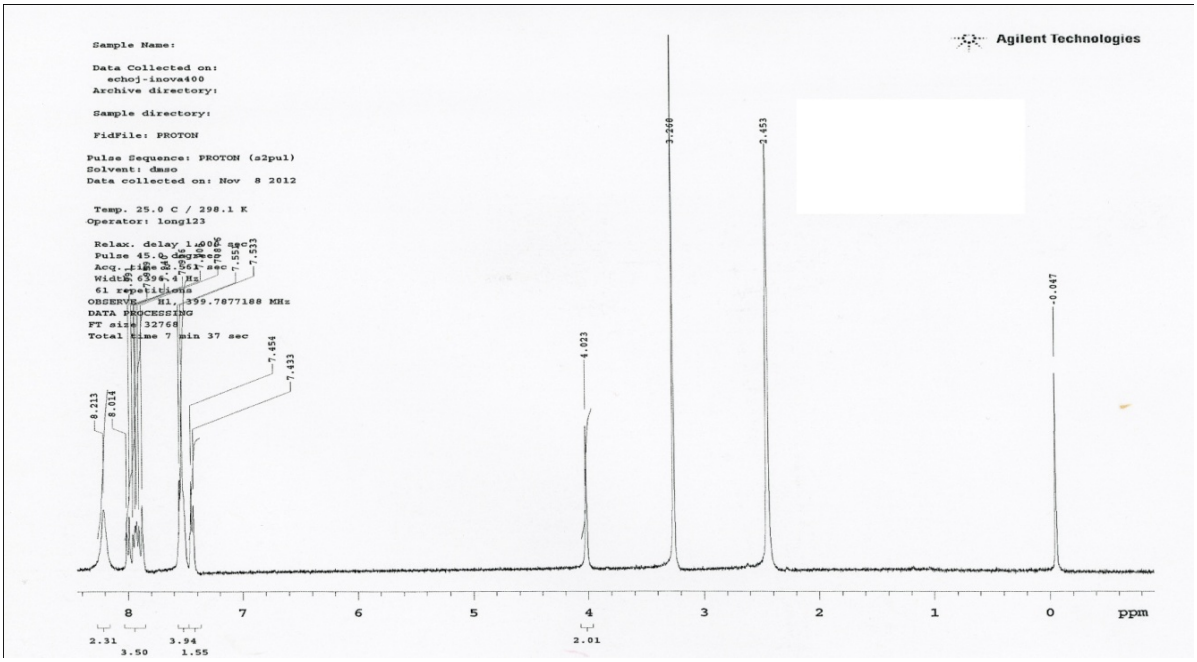


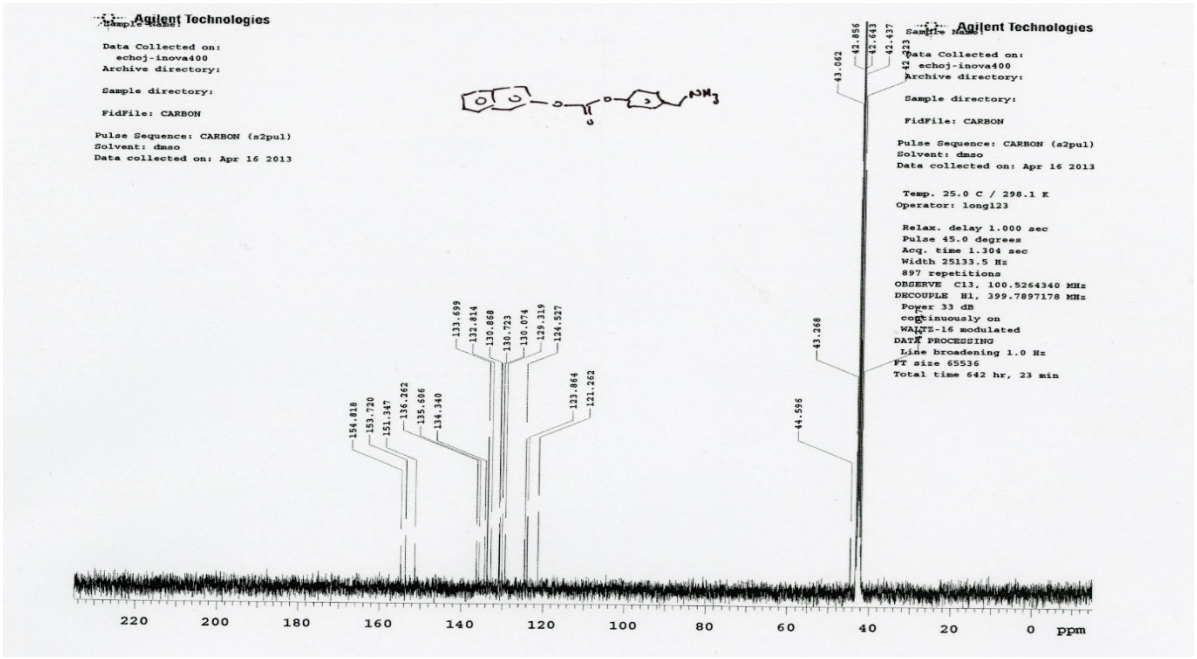


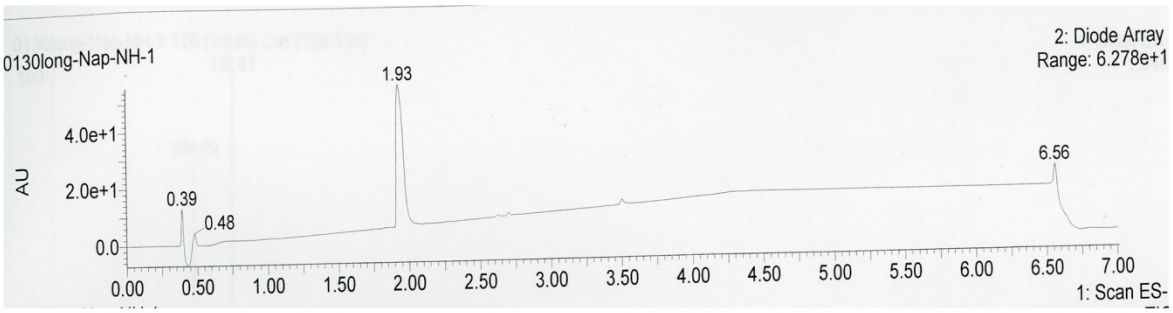
Following *General procedure 2*, the **Boc compound** (55 mg, 0.15 mmol) was dissolved in HCl/Et_2O (10 mL) and stirred overnight at RT. The title compound was obtained as a white solid (20 mg, 44 %). δH (400 mHz, CD_3OD) 4.133 (2H, s); 7.216 (2H, d, J=4.8Hz); 7.364 (2H, d, J=8.8Hz); 7.573 (2H, d, J=4.8Hz). δC (100 mHz, CD_3SOCD_3) 44.581; 124.337; 126.885; 128.632; 131.516; 131.890; 133.477; 133.790; 135.820; 149.455; 153.552; 153.704. m/z (ESI^-) 276 (100% $M\text{-}H^+$).

Synthesis of 4-(aminomethyl)phenyl (2-naphthyl) carbonate hydrochloride C17



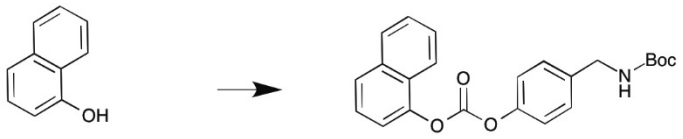


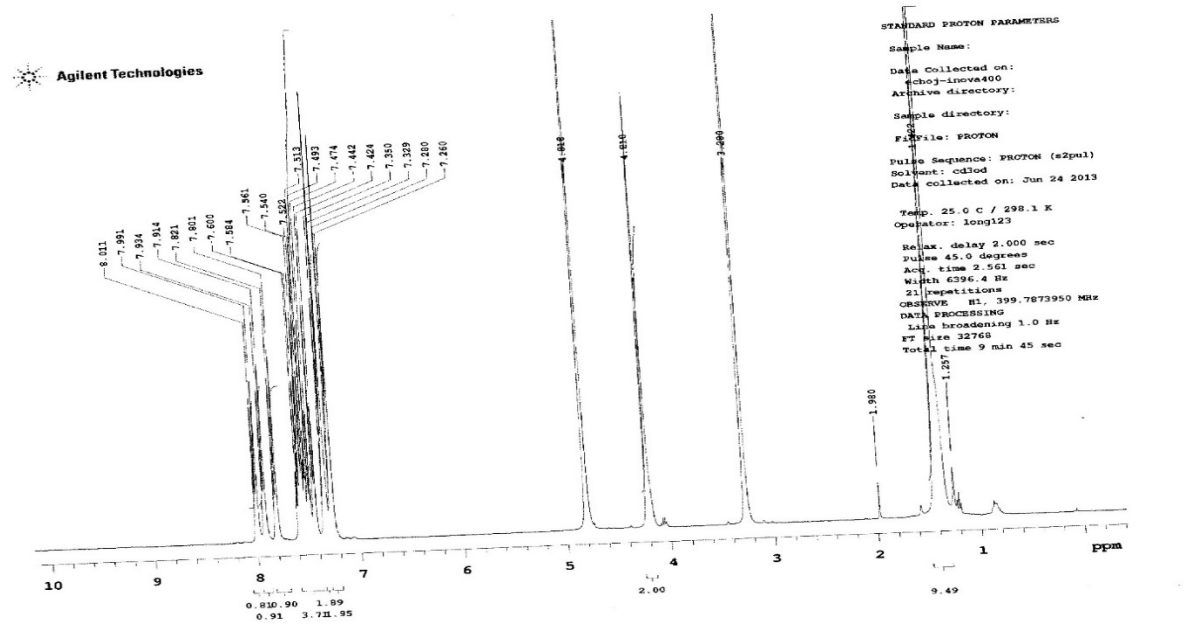


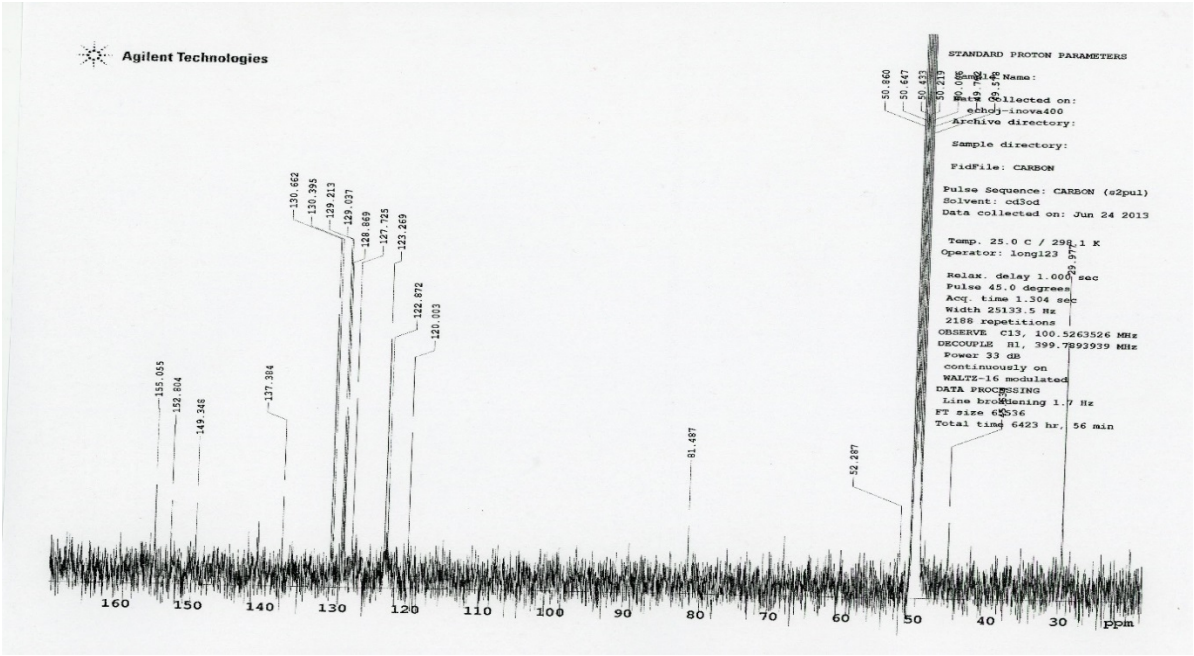


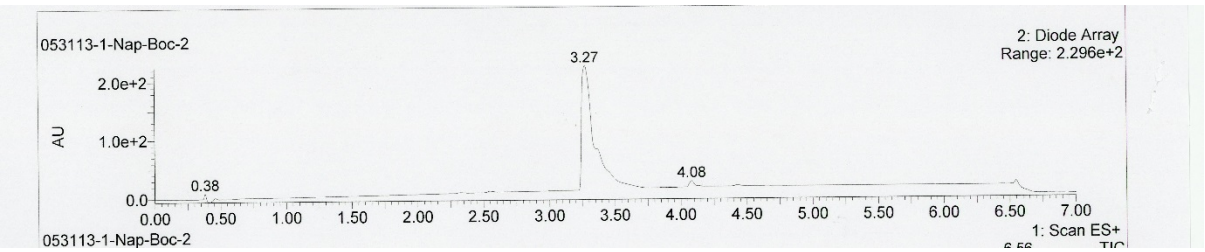
Following *General procedure 2*, **C19** (200 mg, 0.51 mmol) was dissolved in HCl/Et2O (40 mL) and stirred overnight at RT. The title compound was obtained as a white solid (67 mg, 40 %). δH (400 mHz, CD_3SOCD_3) 4.023 (2H, s); 7.444 (2H, d, J=8.4Hz); 7.533-7.551 (5H, m); 7.865-8.014 (4H, m); 8.213 (3H, s). δC (100 mHz, CD_3SOCD_3) 44.596; 121.262; 123.864; 124.527; 129.319; 130.074; 130.723; 130.868; 132.814; 133.699; 134.340; 135.606; 136.262; 151.347; 153.720; 154.818. m/z (ESI^+) 294 (100% MH^+).

Synthesis of *N*-Boc 4-(aminomethyl)phenyl (1-naphthyl) carbonate



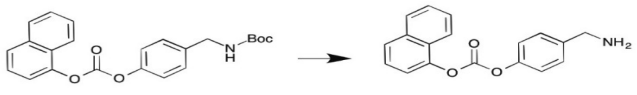


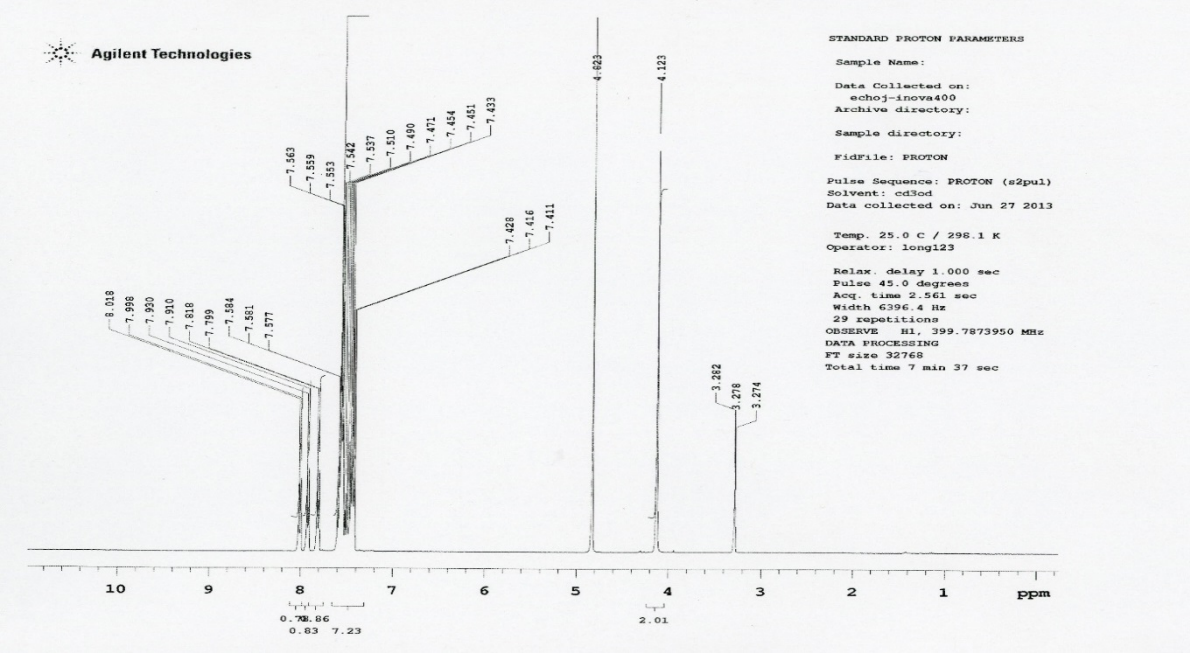


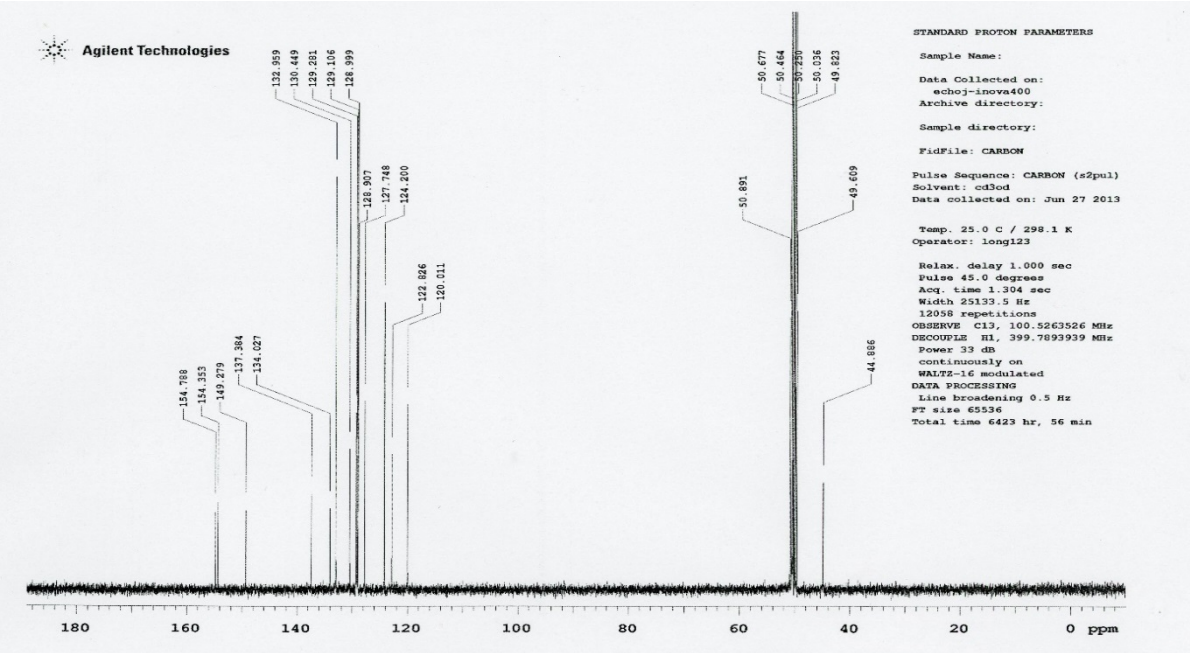


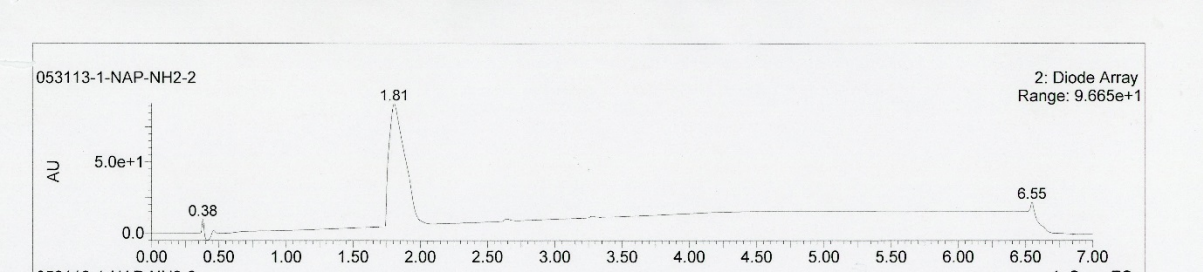
Following *General Procedure 1*, *tert*-butyl 4-hydoxybenzylcarbamate (500 mg, 2.2 mmol) was reacted with 1-naphthyl chloroformate (0.803 mg, 3.96 mmol) in DMF / pyridine (20 mL). Chromatography on silica gel (gradient from 5 % EtOAc in hexanes to 40% EtOAc in hexanes) gave the target compound as a white solid (750 mg, 80 %). δH (400 mHz, CD3SOCD3) 1.348 (9H, s); 4.210 (2H, d, J=6.0Hz); 7.260-7.561 (8H, m 7.871 (1H, d, J=8.4); 7.924 (1H, d, J=8.3); 8.001 (1H, d, J=8.3).

Synthesis of *N*-Boc 4-(aminomethyl)phenyl (1-naphthyl) carbonate hydrochloride C18



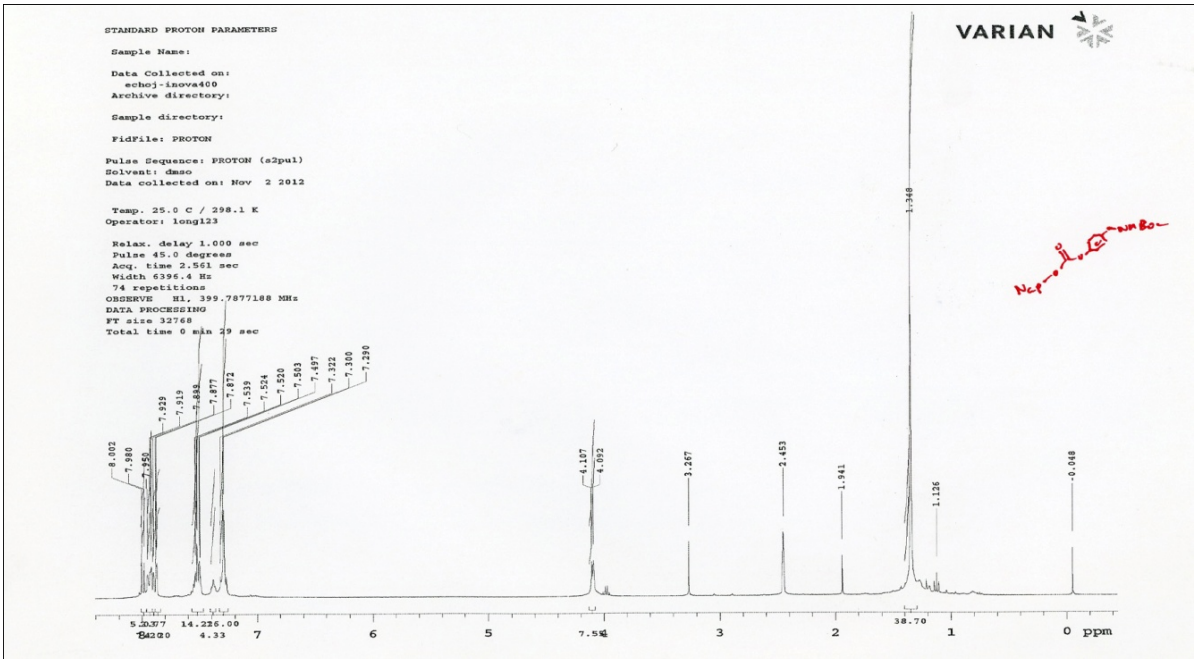


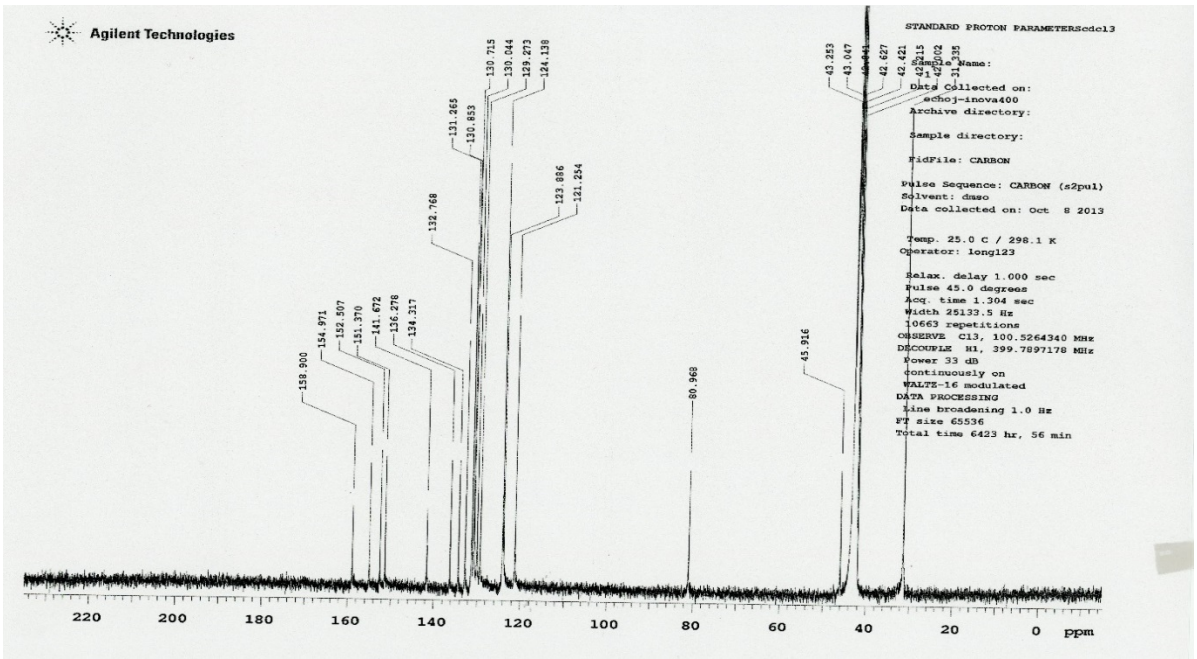




Following *General procedure 2*, **Boc compound** (400 mg, 1.02 mmol) was dissolved in HCl/Et_2O (80 mL) and stirred overnight at RT. The title compound was obtained as a white solid (67 mg, 20 %). δH (400 mHz, CD_3SOCD_3) 4.123 (2H, s); 7.411-7.563 (8H, m); 7.808 (1H, d, J=7.4); 7.920 (1H, d, J=8.0); 8.008 (1H, d, J=8.0). δC (100 mHz, CD_3SOCD_3) 44.886; 120.011; 123.826; 124.200; 127.748; 128.907; 128.999; 129.106; 129.281; 130.449; 132.959; 134.027; 137.076; 149.279; 154.788. m/z (ESI^+) 294 (100% MH^+)

Synthesis of *N*-Boc 4-(aminomethyl)phenyl (2-naphthyl) carbonate C19



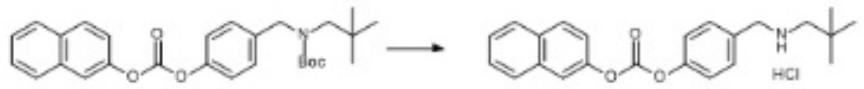


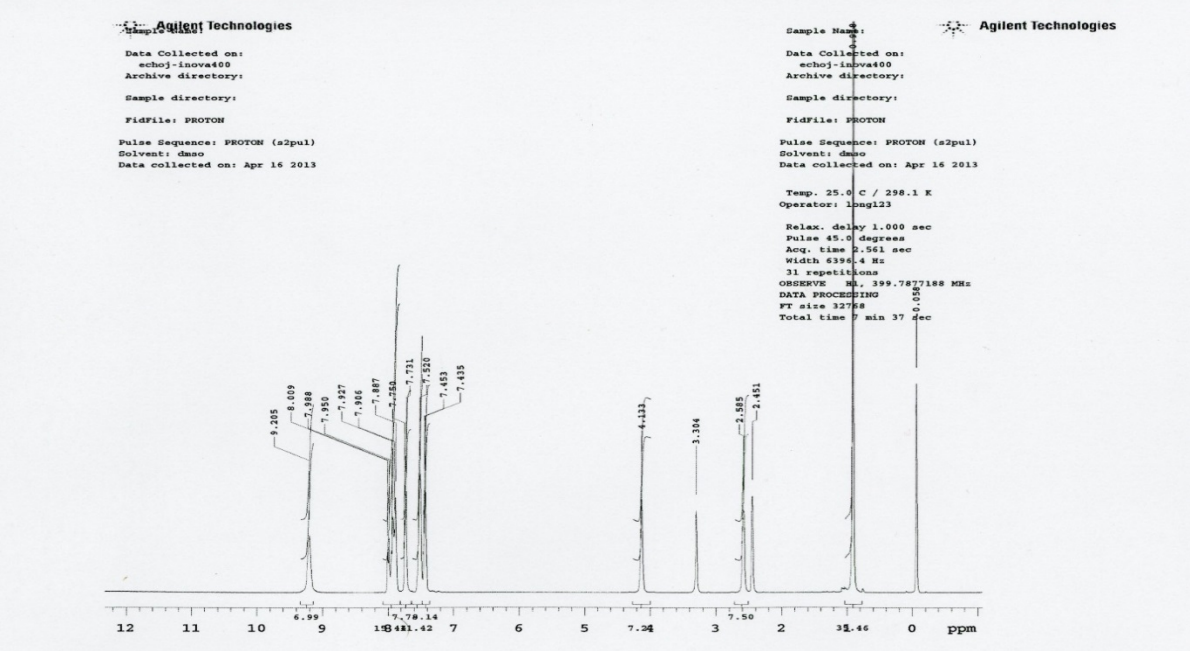
Following *General Procedure 1*, *tert*-butyl 4-hydoxybenzylcarbamate (500 mg, 2.2 mmol) was reacted with 2-naphthyl chloroformate (0.803 mg, 3.96 mmol) in DMF / pyridine (20 mL).

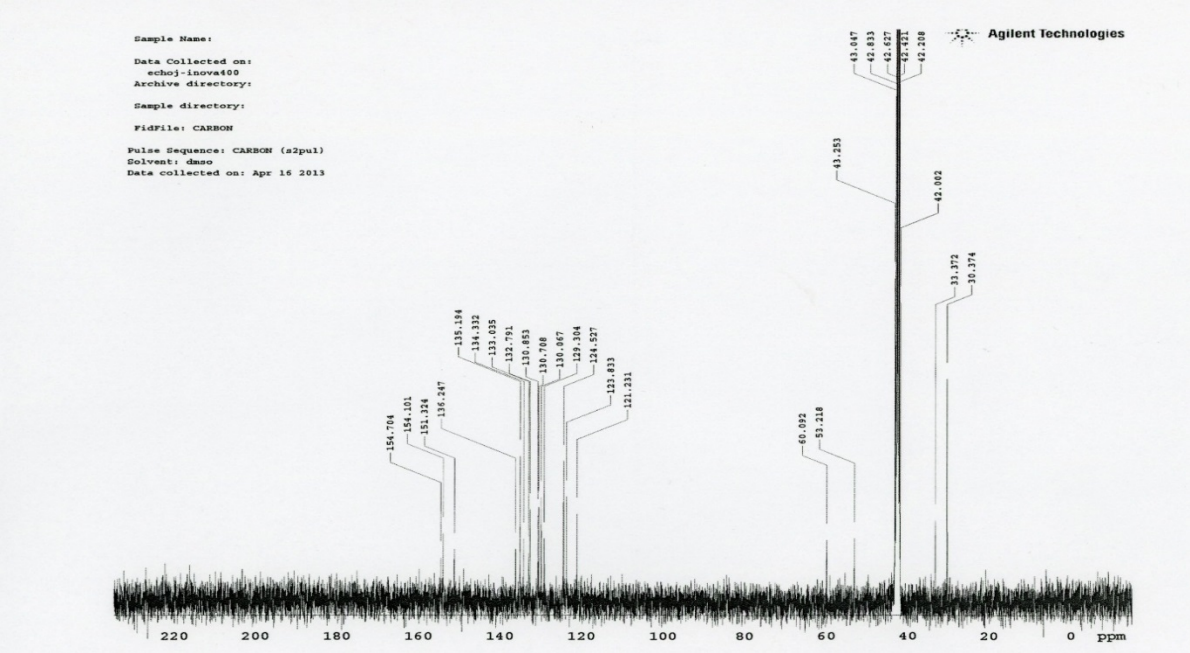
Chromatography on silica gel (gradient from 5 % EtOAc in hexanes to 40 % EtOAc in hexanes) gave the target compound as a white solid (509 mg, 55 %). δH (400 mHz, CD_3SOCD_3) 1.348 (9H, s); 4.099 (2H, d, J=6.0Hz); 7.290-7.322 (4H, m); 7.35 (1H, m); 7.497-7.539 (4H, m); 7.875 (1H, d, J=2.0Hz); 7.925 (1H, dd, J=8.2, 4.0Hz); 7.990 (1H, d, J=8.8Hz). δC (100 mHz, CD_3SOCD_3) 31.335; 45.916; 80.968; 121.254; 123.886; 124.138; 129.273; 130.044; 130.175; 130.853; 131.265; 132.768; 134.317; 136.278; 141.672; 151.370; 152.507; 154.971; 158.900.

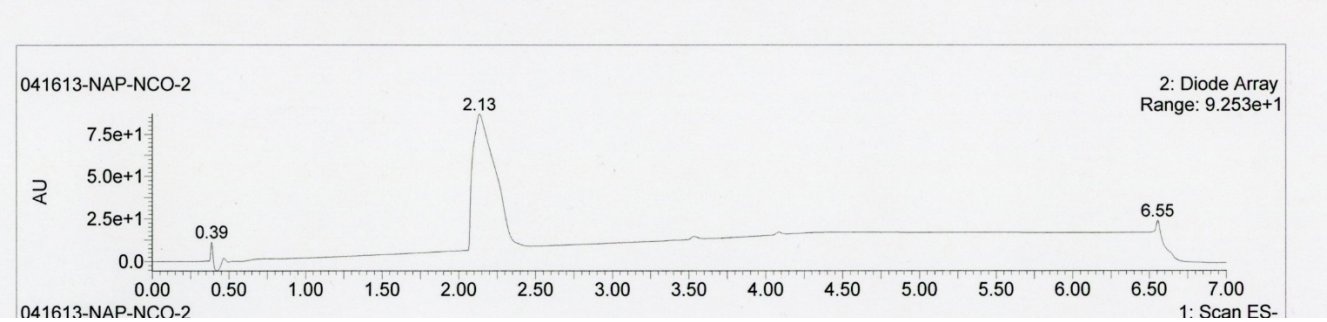
Synthesis of 4-(*N-neo*-pentylaminomethyl)phenyl (2-naphthyl) carbonate hydrochloride

C20



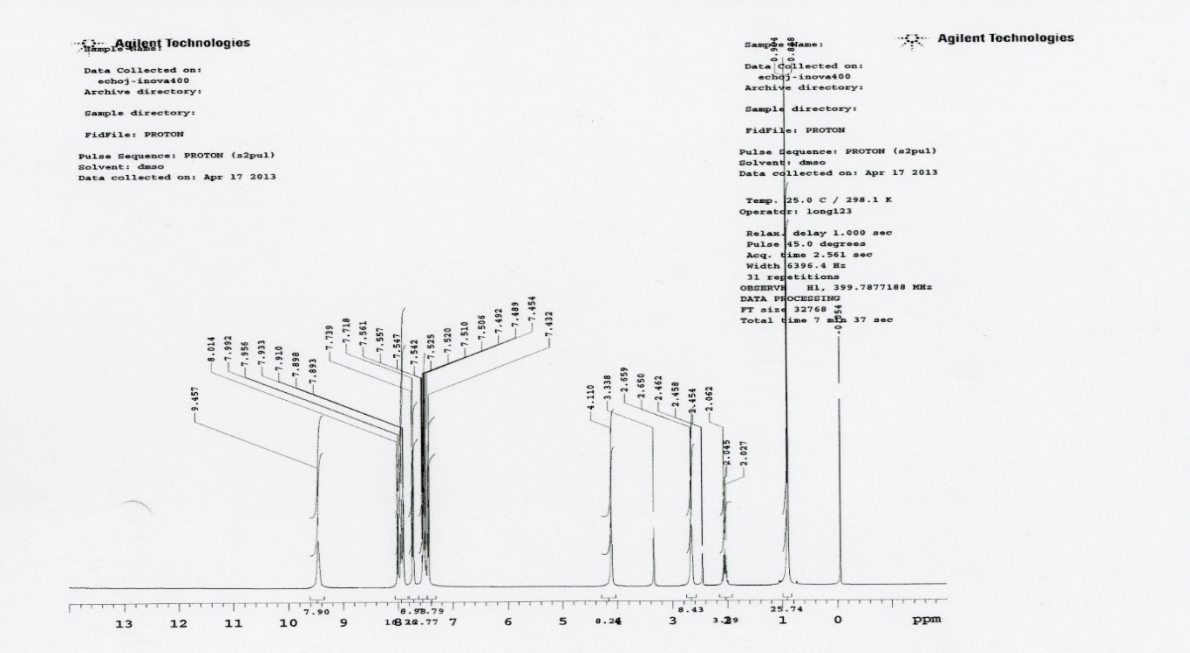


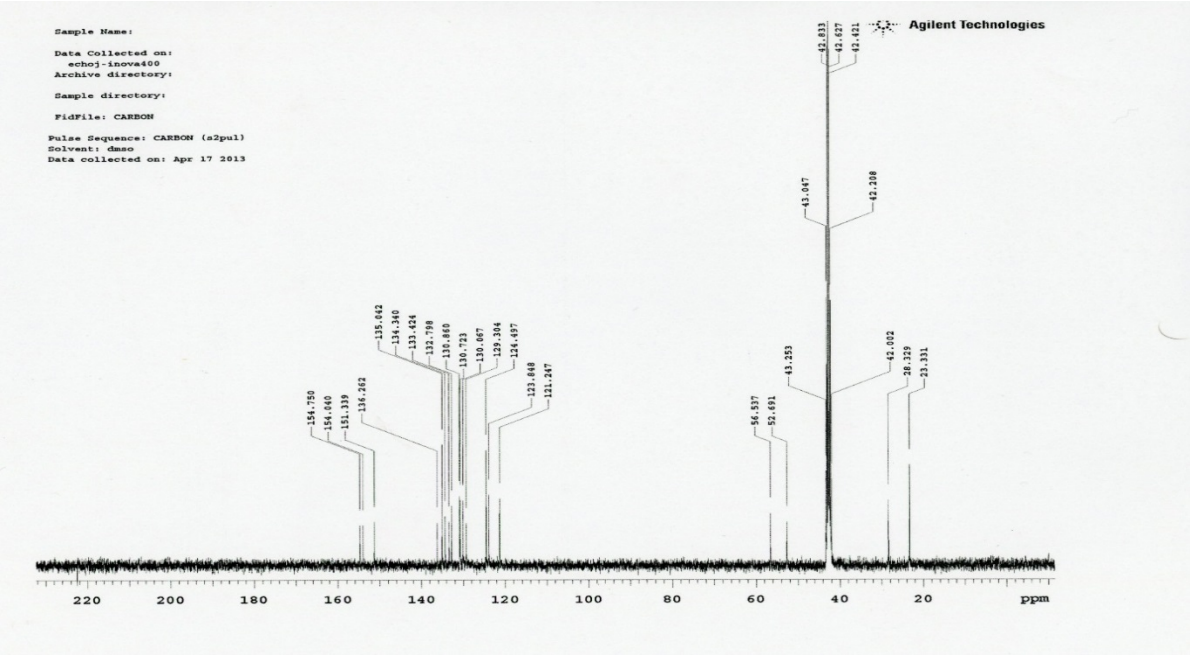


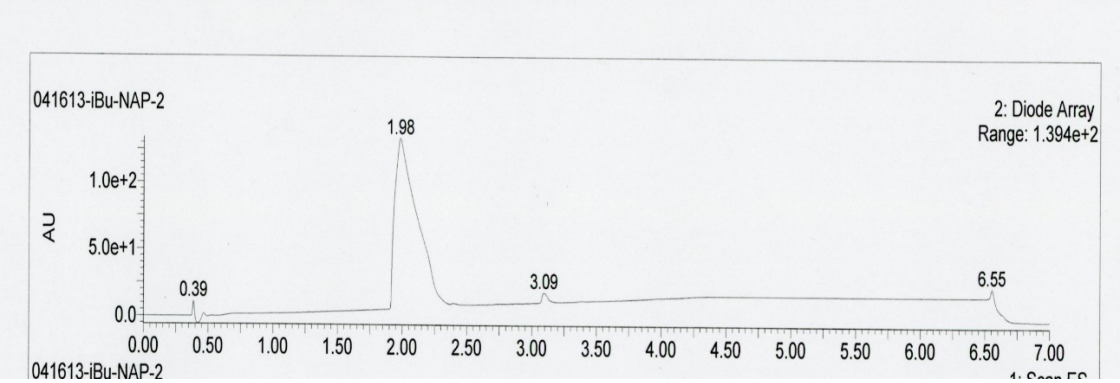


Following *General Procedure 2*, the corresponding Boc protected compound was dissolved in HCl/Et_2O (20 mL) and stirred overnight at RT. The title compound was obtained as a white solid. (400 MHz, CD_3SOCD_3) 0.918 (9H, s); 2.585 (2H, s); 4.133 (2H, s); 7.444 (2H, d, J=7.6Hz); 7.520 (3H, m), 7.740 2, d, J=7.6Hz); 7.887-8.009 (4H, m); 9.205 (3H, br s). (100 MHz, CD_3SOCD_3) 30.374; 33.372; 53.218; 60.092; 121.231; 123.833; 124.527; 129.304; 130.067; 130.708; 130.853; 132.791; 133.035; 134.332; 135.194; 151.324; 154.101; 154.704. m/z (ESI^+) 364.

Synthesis of 4-(*N*-iso-butylaminomethyl)phenyl (2-naphthyl) carbonate hydrochloride C21

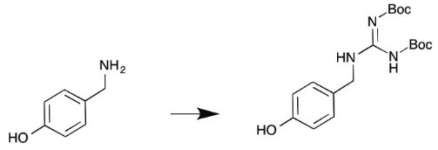


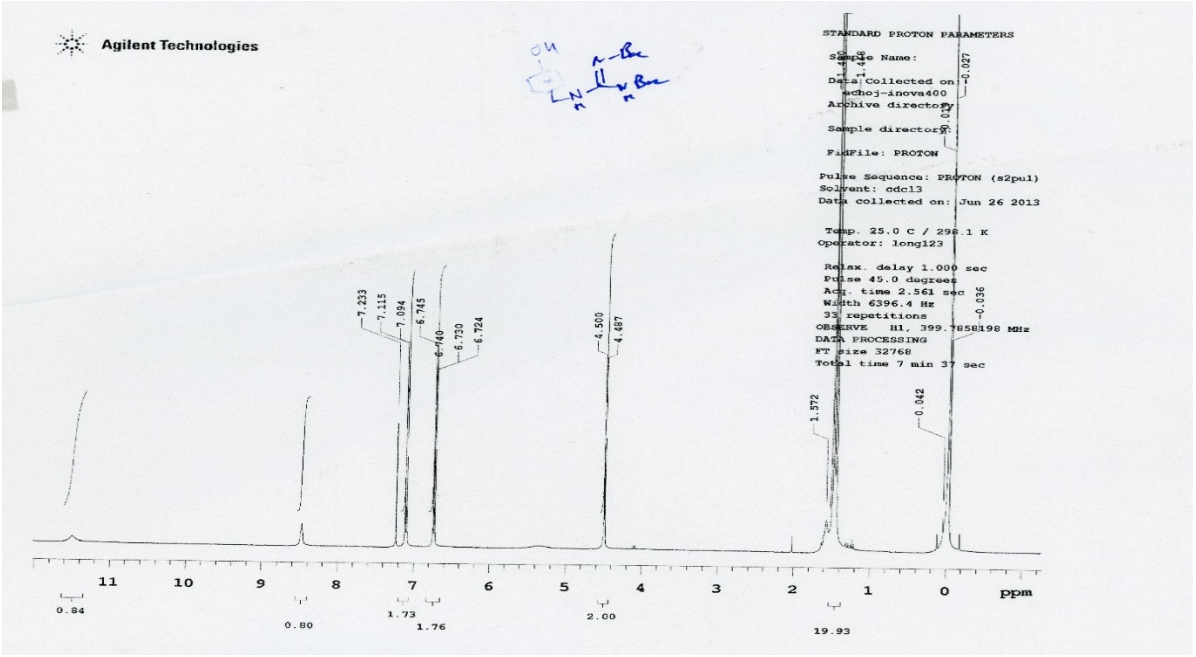




Following *General Procedure 2*, the corresponding Boc protected compound was dissolved in HCl/Et_2O (20 mL) and stirred overnight at RT. The title compound was obtained as a white solid. (400 MHz, CD_3SOCD_3) 0.886 (6H, d, J=5.2); 2.045 (1H, m); 2.650 (2H, m); 4.110 (2H, s); 7.443 (2H, d, J=8.8Hz); 7.489-7.561 (3H, m); 7.728 (2H, d, J=8.7Hz); 7.893-8.014 (4H, m); 9.457 (3H, br s). (100 MHz, CD_3SOCD_3) 23.331; 28.329; 52.691; 56.537; 121.247; 123.848; 124.497; 129.304; 130.723; 130.860; 132.798; 133.424; 134.340; 135.042; 136.262; 151.339; 154.040; 154.750. m/z (ESI^+) 350.

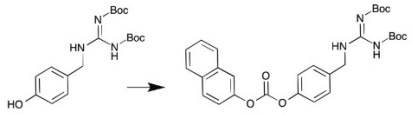
Synthesis of N,N-diBoc-4-(guanidinomethyl)phenol

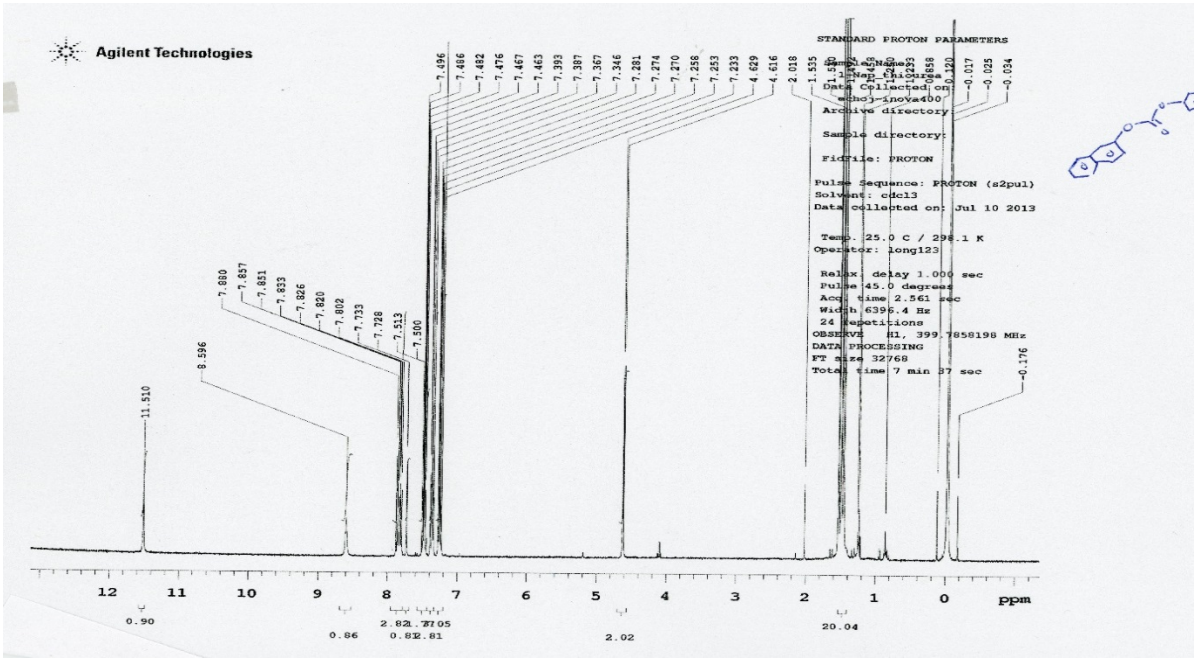




Following *General Procedure 1*, To a stirred solution of 4-hydroxybenzylamine (5 g, 40 mmol) 1,3-bis-(tert-butoxycarbonyl)-2-methyl-2-thiopseudourea (12 g, 4.4 mmol) in 10% DMF in CH_2Cl_2 438 mg (2.58 mmol) of silver nitrate was added (7.4 g, 4.4 mmol). A white precipitate indicative of silver methyl sulfide formed after 1 h. [i] The mixture was stirred overnight at room temperature. Purification by column chromatography on silica gel (gradient of hexane to ethyl acetate) gave the title compound (10.0 g, 70%). δH (400 mHz, CDCl3) 1.448 (9H, s); 1.480 (9H, s); 4.494 (2H, d, J=5.5Hz); 6.734 (2H, d, J=8.4Hz); 7.105 (2H, d, J=8.4Hz); 8.202 (1H, s); 11.250 (1H, s).

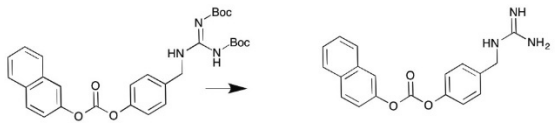
Synthesis of N,N-diBoc-4-(guanidinomethyl)phenyl naphthalene-2-yl carbonate

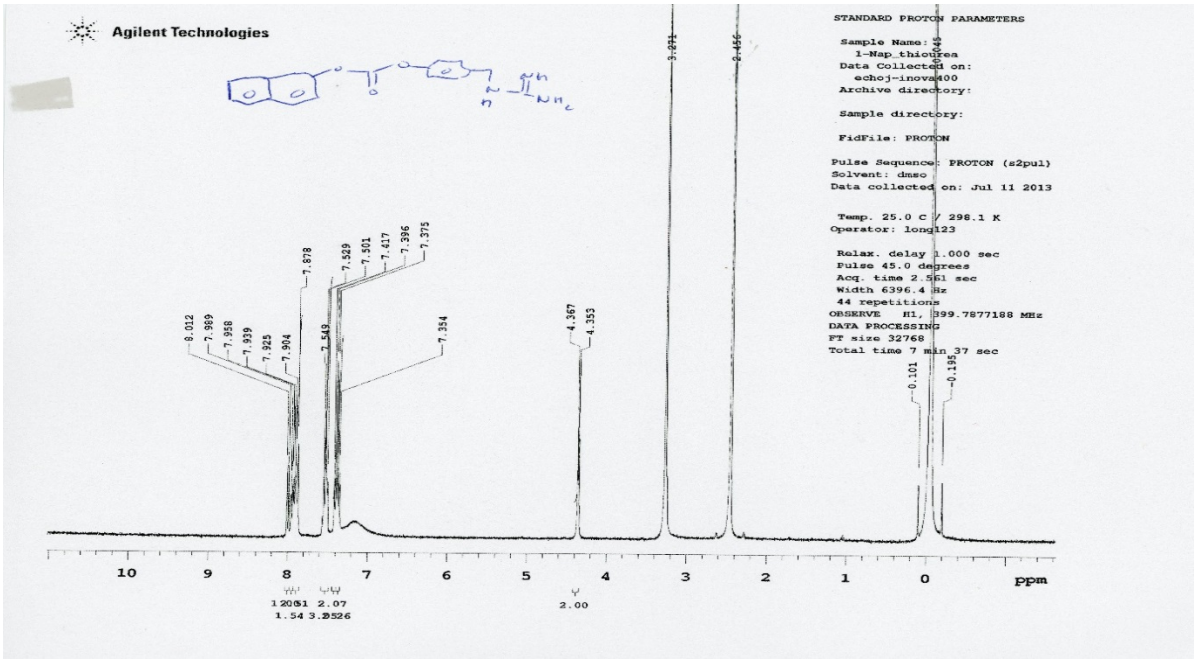


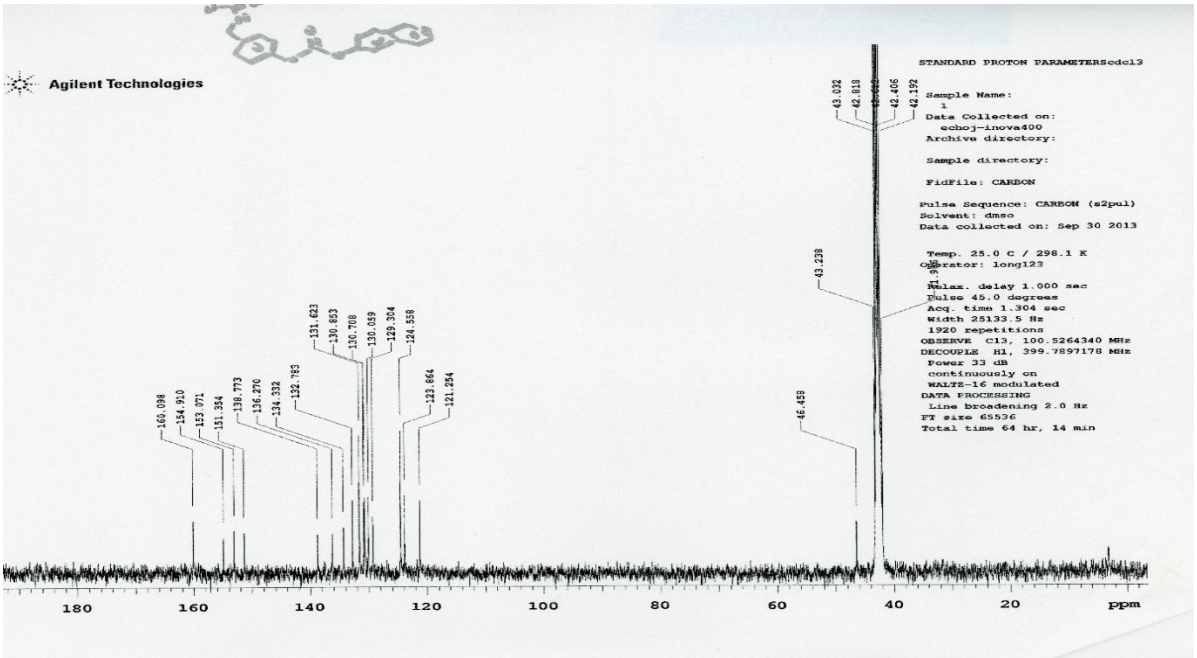


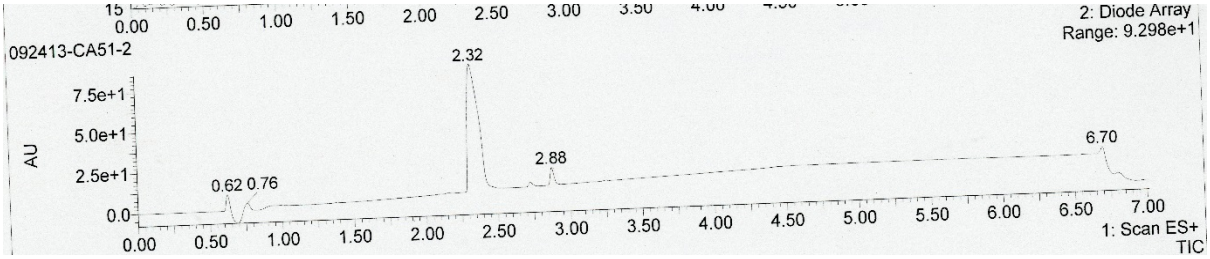
Following *General Procedure 1*, **diBoc compound** (500 mg, 1.34 mmol) was reacted with 2-naphthyl chloroformate (230 mg, 1.34 mmol) in DMF / pyridine (20 mL). Chromatography on silica gel (gradient from 5% EtOAc in hexanes to 40% EtOAc in hexanes) gave the target compound as a white solid (356 mg, 50%). δH (400 mHz, CD_3SOCD_3) δH (400 mHz, $CDCl_3$) 1.458 (9H, s); 1.492 (9H, s); 4.623 (2H, d, J=5.5Hz); 7.233-7.281 (3H, m); 7.346-7.395 (3H, m); 7.731 (1H, d, J=2.0Hz); 7.728-7.880 (3H, m); 8.596 (1H, s); 11.510 (1H, s).

Synthesis of 4-(guanidinomethyl)phenyl naphthalene-2-yl carbonate hydrochloride C22



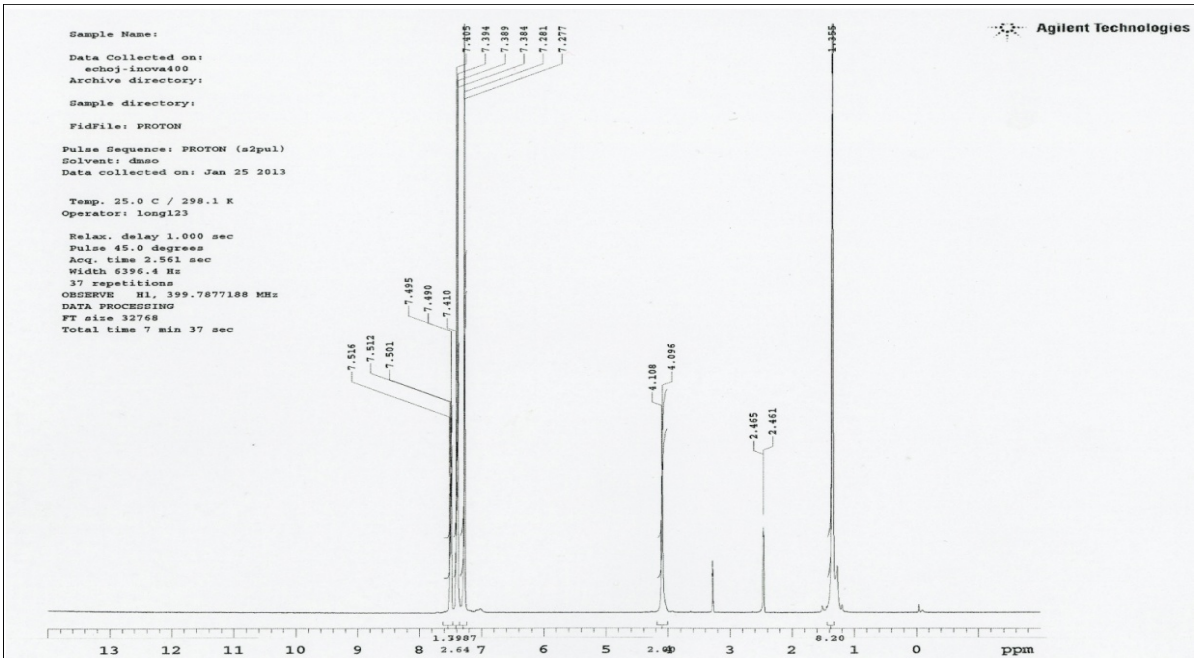


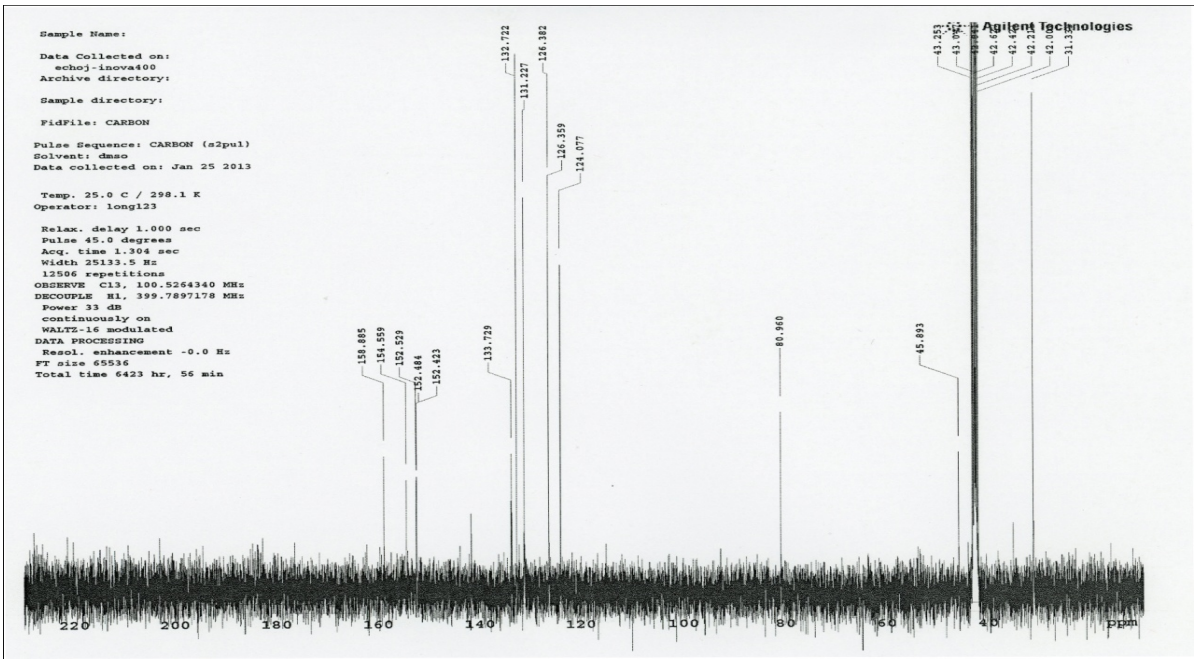


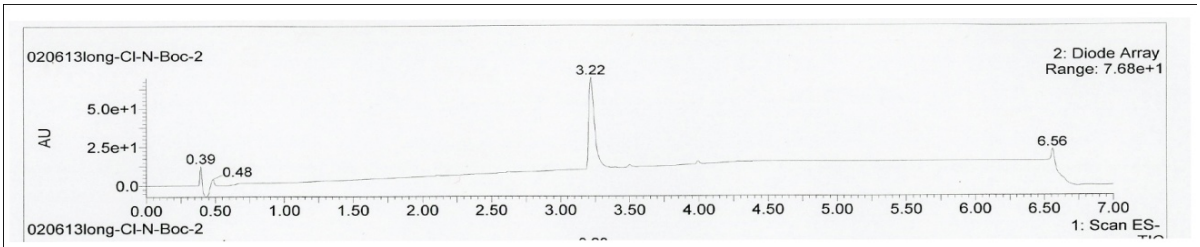


Following a modification of *General Procedure 1*, *The Boc compound* (300 mg, 0.56 mmol) was reacted with HCl.Et_2O at 50 ^oC for 4 h. The resulting precipitate was filtered, washed 3 times with Et_2O and used without further purification (75mg, 40%). δH (400 mHz, CD_3SOCD_3) 4.360 (2H, d, J=5.6Hz); 7.022 (2H, br s); (6H, m); 7.490 (2H, d, J=6.8Hz); 7.507 (1H, d, J=6.8Hz). δC (100 mHz, CD_3SOCD_3) 46.458; 121.254; 123.864; 124.558; 130.059; 130.708; 130.853; 131.623; 132.783; 134.332; 136.270; 138.773; 151.354; 153.071; 154.910; 160.098. m/z ($ESI^{+)}$ 336 (100% MH^+).

Synthesis of *N*-Boc 4-(aminomethyl)phenyl (4-chlorophenyl) carbonate C23







Following *General Procedure 1*, *tert*-butyl 4-hydoxybenzylcarbamate (500 mg, 2.2 mmol) was reacted with p-chlorophenyl chloroformate (0.931 mg, 3.96 mmol) in DMF / pyridine (20 mL). Chromatography on silica gel (gradient from 5% EtOAc in hexanes to 40% EtOAc in hexanes) gave the target compound as a white solid (509 mg, 55 %). δH (400 mHz, CD_3SOCD_3) 1.355 (9H, s); 4.102 (2H, d, J=4.8Hz); 7.277-7.405 (4H, m); 7.490 (2H, d, J=6.8Hz); 7.507 (1H, d, J=6.8Hz). δC (100 mHz, CD_3SOCD_3) 31.332; 45.893; 124.077; 126.359; 131.227; 132.722; 133.729; 141.021; 152.423; 154.559; 158.885. m/z (ESI^+) 378 (100% MH^+)

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3. Hemelaar J, Borodovsky A, Kessler BM, et al. Specific and covalent targeting of conjugating and deconjugating enzymes of ubiquitin-like proteins. *Mol Cell Biol.* 2004;24(1): 84-95.
4. Qian Y, Martell J, Pace NJ, Ballard TE, Johnson DS, Weerapana E. An Isotopically Tagged Azobenzene-Based Cleavable Linker for Quantitative Proteomics. *Chembiochem.* 2013;14(12): 1410-1414.
5. Weerapana E, Speers AE, Cravatt BF. Tandem orthogonal proteolysis-activity-based protein profiling (TOP-ABPP)--a general method for mapping sites of probe modification in proteomes. *Nat Protoc.* 2007;2(6): 1414-1425.