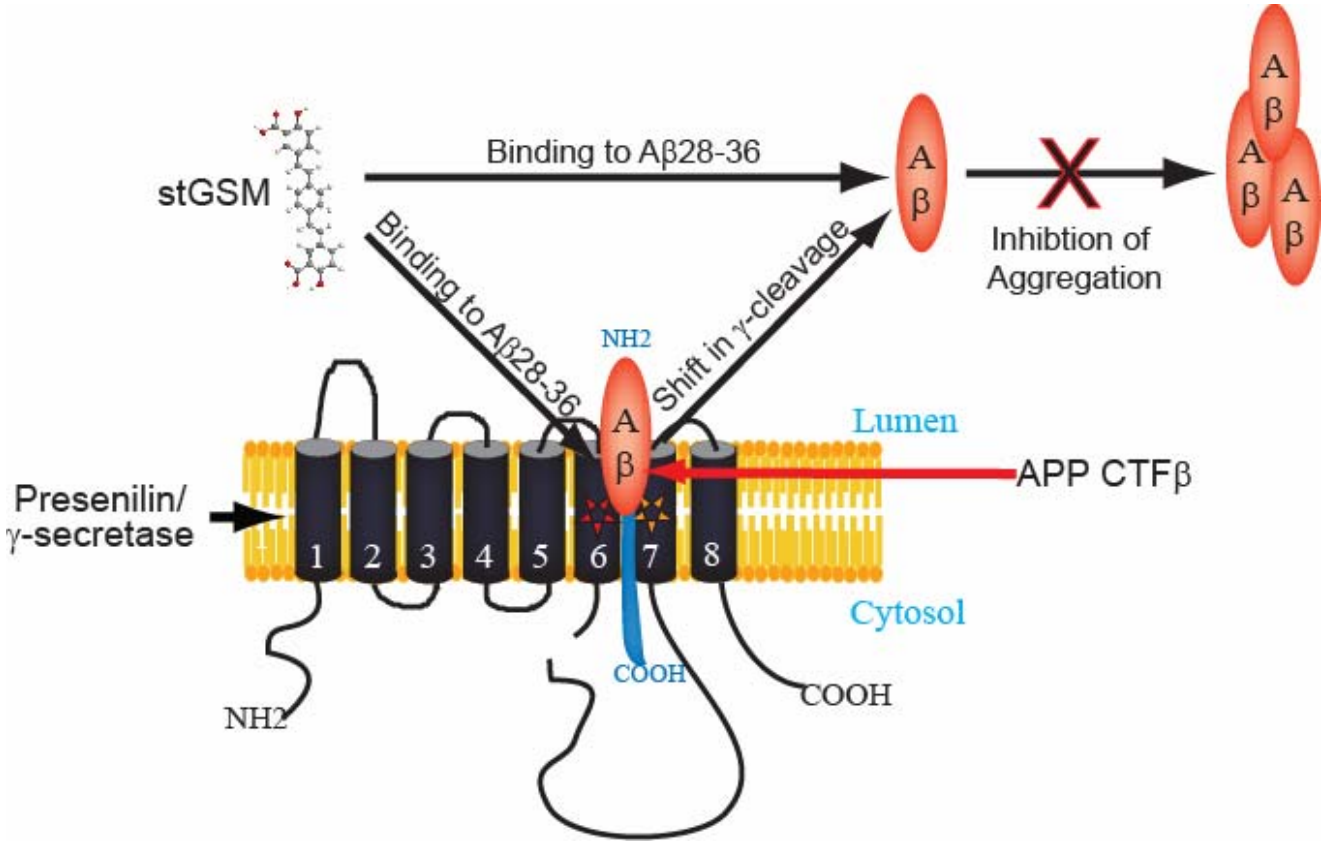
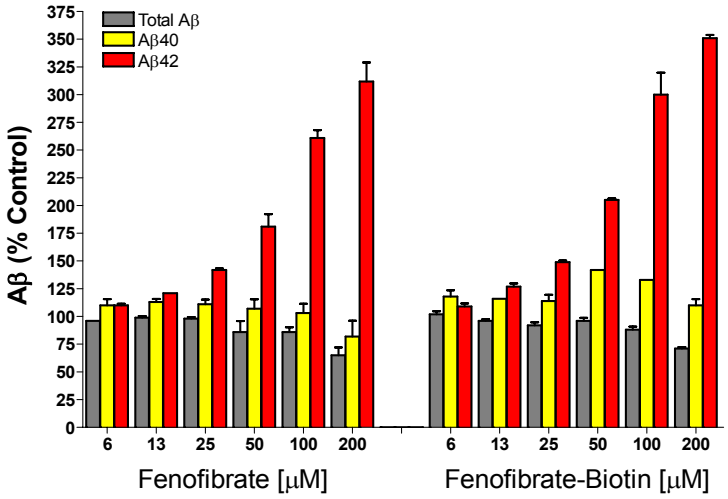
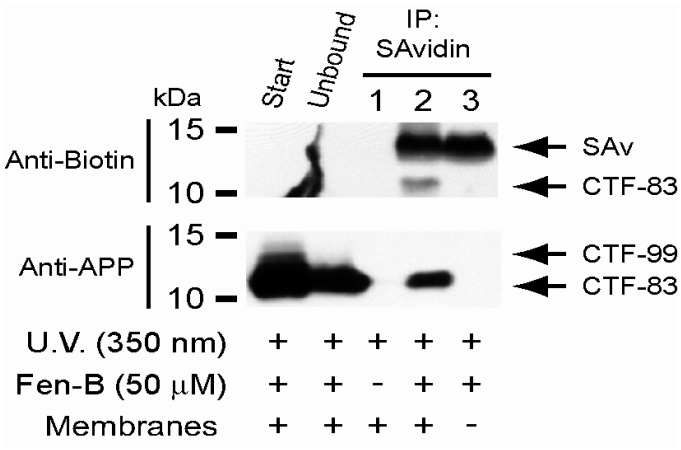


Supplementary Figure 1 . Effects of substrate targeting γ-Secretase Modulators (stGSMs). A simplified model of the γ-secretase complex and its interaction with APP-CTF substrate is shown. The γ-secretase complex is composed of presenilin N-terminal and C-terminal fragments which encode the active site aspartates in transmembrane domains 6 and 7 (indicated by the stars). Nicastrin, Aph-1 and Pen-2 that are integral but presumably non-catalytic components of γ-secretase complex are not shown. Using GSM photoprobes we discovered that these compounds do not label the catalytic or structural components of γ-secretase but interact directly with the substrate (APP-CTF) at residues Aβ28-36. The binding of stGSMs to APP-CTF could lead to changes in the cleavage site by shifting the position of the substrate in the membrane bilayer relative to the active site of γ-secretase or by inducing conformational changes of the enzyme complex and APP. Because stGSMs bind a site present both in substrate and in Aβ itself, stGSMs can inhibit Aβ aggregation. Therefore Aβ42 lowering stGSMs may have three mechanistically linked actions that could provide synergistic benefit for the treatment or prevention of AD. First they decrease production of the pathogenic Aβ42 peptide. Second they can directly inhibit Aβ aggregation. Third, by increasing levels of shorter Aβ peptides they may indirectly decrease Aβ aggregation.

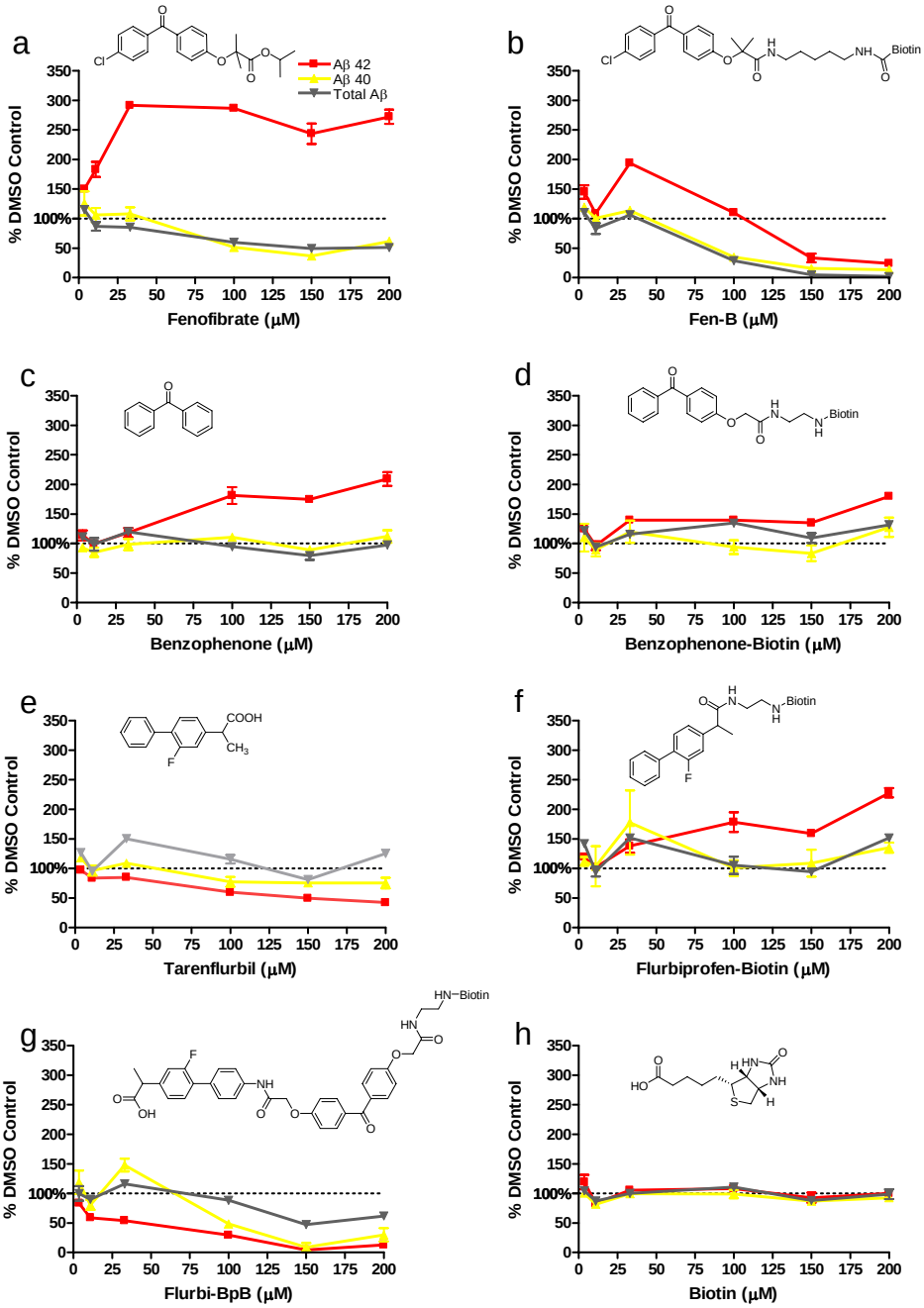
a



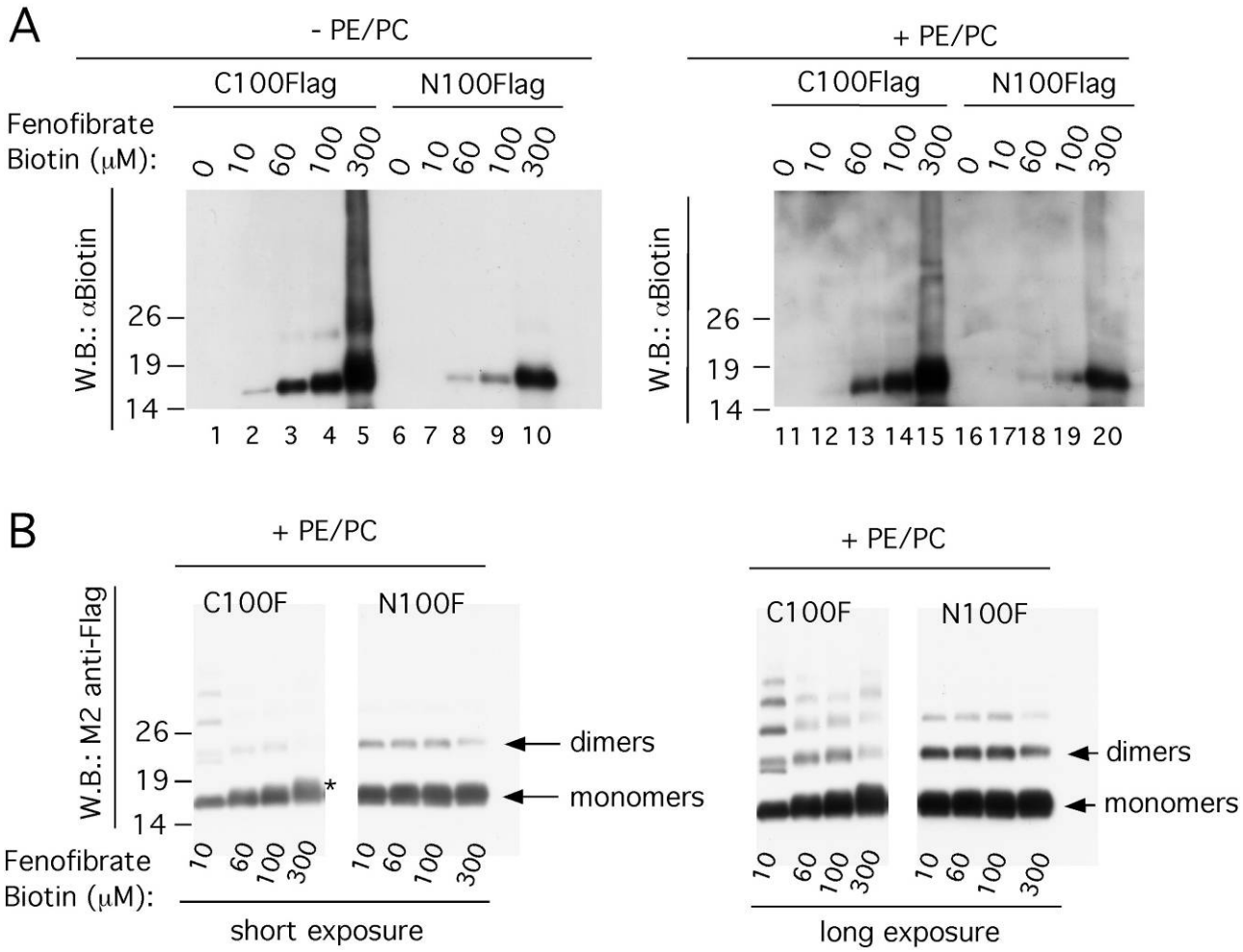
b



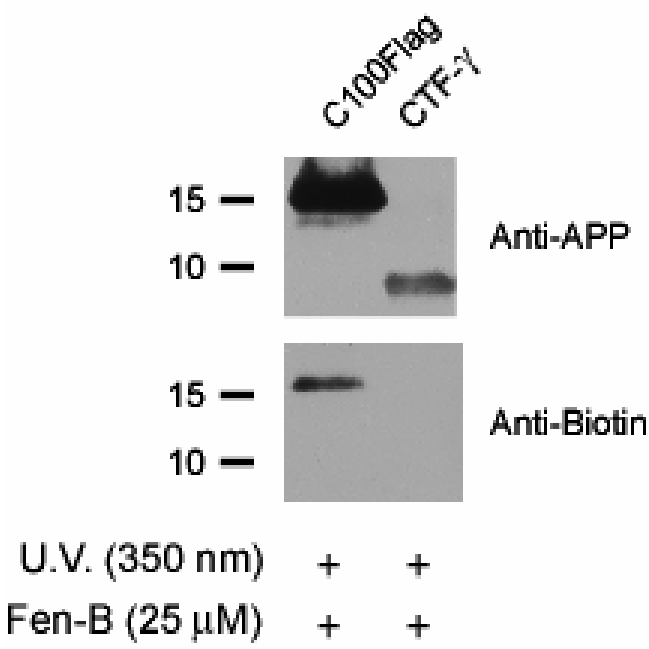
Supplementary Figure 2. Fenofibrate-Biotin (Fen-B) is an Aβ42 raising GSM that labels cell-derived APP-CTF. a) The effects of fenofibrate and fenofibrate-biotin (Fen-B) on Aβ production were investigated in a cell-free in vitro γ-secretase assay performed as described in the methods. The parent compound and photoprobe derivative both raise Aβ42 with similar potencies. Percent control Aβ species ± s.e.m., n=2. **b**) Fen-B labels APP-CTFs from cells lysates. CHAPSO solubilised membrane fractions from H4 APP-CTF-105-AP cells were irradiated (350nm) in the presence of Fen-B (50μM) for 30 mins. Biotinylated material was precipitated with streptavidin overnight (SAv). Beads were washed (3X) and then incubated with XT sample buffer, heated to 95°C, run on 12% Criterion XT gels, and transferred to nitrocellulose membranes. Blots were probed for biotin (Bethyl) and APP (CT20). Excess drug (Fen-B) and unlableled APP-CTF is detected in the flow-through from the beads (unbound lane). Biotin bound to streptavdin (SAv) is detected by the anti-biotin antibody. APP-CTF-83 is pulled down in lane 2 and is reactive with anti-biotin and anti-APP (CT20).



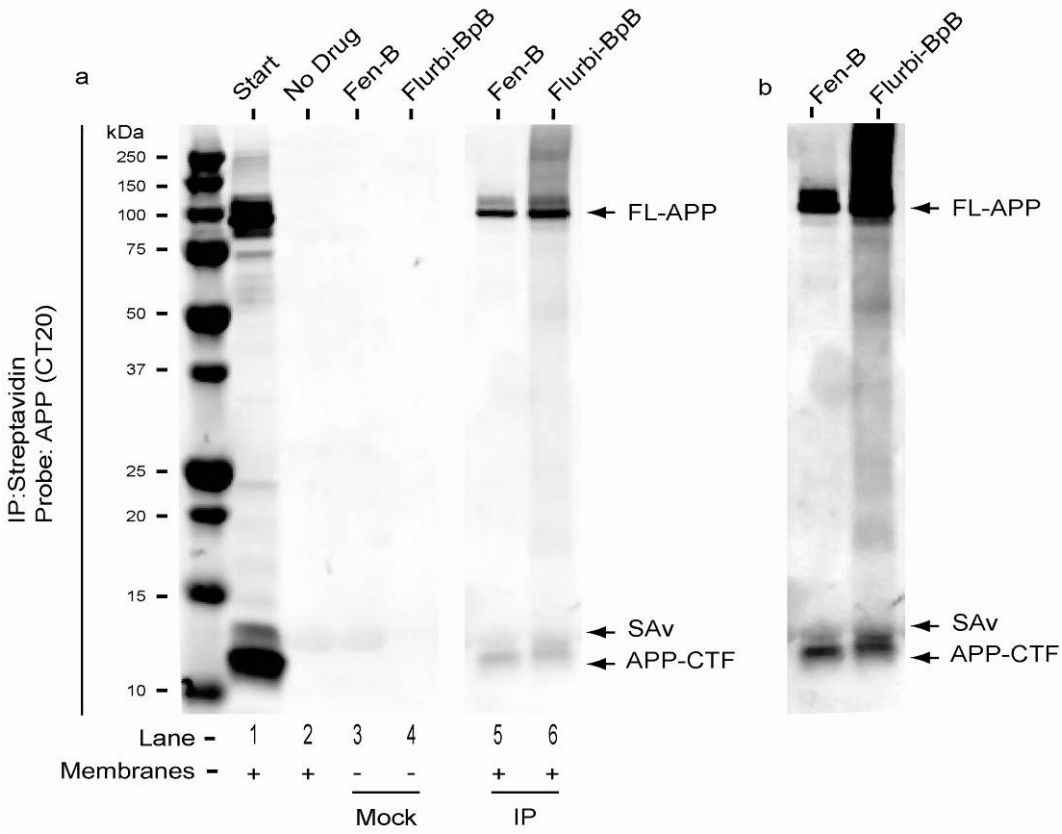
Supplementary Figure 3 . GSM Photoprobes and related compounds display differential ability to alter Aβ42. Parent GSMs and biotin tagged photoactivatable GSMs were evaluated for their effects on Aβ secreted from H4 Bri-C99 (CTF-β). Cells were treated with compounds for 6 hours and Aβ measured by ELISA. **a**) 33 μM fenofibrate raises Aβ42 300% with no changes in total or Aβ40 levels. Toxicity, based on cell morphology and XTT assay, was noted at higher concentrations (150, 200μM). **b**) Fenofibrate-Biotin raised Aβ42 (33μM, ~200% increase) but is toxic at higher doses. UV exposure increases toxicity of **b** and **g**. **c** and **d**) Both benzophenone and benzophenone-biotin increased Aβ42 in cells. **e**) Tarenflurbil lowered Aβ42 without decreasing total Aβ levels. **f**) Coupling of a biotin tag to flurbiprofen through the carboxylate creates a flurbiprofen biotin conjugate that raised Aβ42. **g**) Addition of a benozphenone-biotin tag (**d**) to the adjacent phenyl ring of Flurbipofen produces Flurbi-BpB that lowered Aβ42. **h**) Biotin alone had no effect on Aβ levels. These later results show that the benzophenone group of BpB is responsible for its increase in Aβ42 levels. Changes in Aβ (percent control) ± s.e.m., n=3.



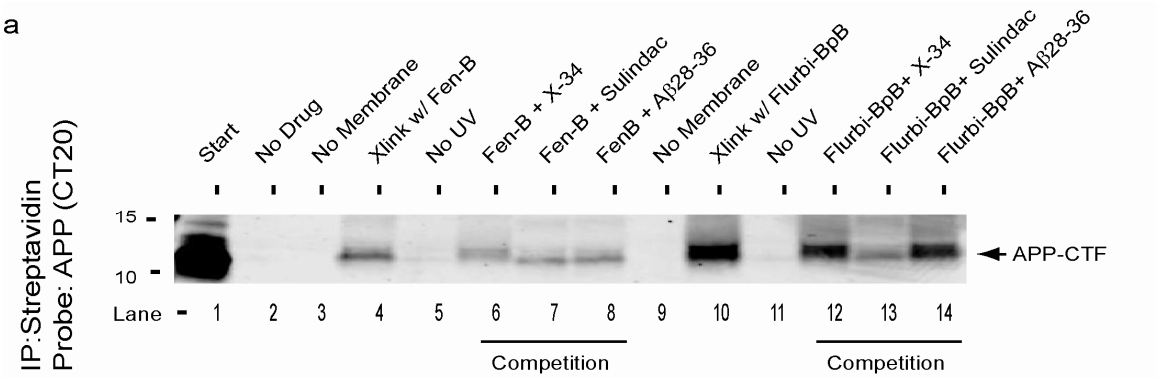
Supplementary Figure 4. Presence of γ-secretase does not prevent Fen-B labeling of C100F. Recombinant γ-secretase substrates (1μM) containing a Flag tag and based on the sequence of APP (C100F) and Notch (N100F) were crosslinked for 30 minutes in the presence of increasing concentrations of Fenofibrate-Biotin (Fen-B) and purified γ-secretase from γ-30 cells. **a**) Fen-B labeled C100F as demonstrated by a dose-dependent increase in a biotin reactive band that migrates at the same molecular weight as monomeric C100F (as seen in panel b). N100F is also labeled but only at higher concentrations of Fen-B (100, 300 μM). Addition of γ-secretase and phosphatidylcholine/ phosphatidylethanolamine (+PC/PE) does not prevent labeling. **b**) Increasing concentrations of Fen-B produce a gel shift in C100F monomers and higher order species. This effect is only noted in N100F at the highest concentration.



Supplementary Figure 5 . Initial Mapping of Fen-B labeling of C100Flag. The labeling of Fen-B to APP C100Flag and CTF-γ (CTF-50, rpeptide) was compared to determine the primary binding site. Peptides (5μM) were incubated in buffer (50 mM HEPES, pH 7.4) with Fen-B (25 μM) and crosslinked for 30 mins. Samples were separated on 12 % Bis-Tris Criterion XT gels (Bio-Rad) and probed for APP (antibody CT20) and Biotin (Bethyl, rabbit). Fen-B labeled C100F with no evidence for binding to CTF-γ; thus, the binding site likely residues in the N-terminal region (Aβ sequence) as demonstrated in Fig. 2.



Supplementary Figure 6. GSM Photoprobes label Full length APP and APP-CTFs in cell membrane fractions. a) Crude (microsomal) membrane fractions from H4-APP cells were isolated via nitrogen cavitation, sodium carbonate treatment, and centrifugation as described in the methods section. Membranes were resuspended in PBS using a glass-teflon homogenizer and pre-cleared of biotinylated material with streptavidin-plus ultralink beads (Pierce) at 4°C for 1hour, and then beads were pelleted at 20xg for 10mins. The membrane fraction (supernatant; Start. Lane 1) was split into different sample groups and crosslinked with the appropriate GSM photoprobe (Fen-B or Flurbi-BpB, both at 50μM) for 30 mins (350nm). Membranes were collected (100,00xg, 30mins) and washed with PBS via suspension/centrifugation 3 times to remove excess drug. Membrane pellets were solubilzed in RIPA buffer with protease inhibitor and centrifuged to remove insoluble material. Supernatants were incubated with streptavidin-ultralink beads (100ul) overnight to capture biotinylated material; beads were washed with RIPA three times, and eluted with XT sample buffer @ 95°C. Samples were separated on 12% Bis-Tris Criterion XT gels, transferred to PVDF (0.2μm), blocked in 0.5% casein, and probed for APP (CT20; 1:1000). Cell membranes irradiated in the presence of Fen-B (lane 5) or Flurbi-BpB (lane 6) and precipitated with streptavidin (IP) show labeling of both full-length APP (FL-APP; ~100kDa) and APP CTF (~12kDa). APP pulldown is not observed in control samples: membranes crosslinked in the absence of photoprobe drugs (lane 2) or photoprobes alone with beads (lanes 3, 4; Mock). Competition with GSMs (fenofibrate, sulindac sulfide) prevents labeling of FL-APP and APP CTF by Fen-B and Flurbi-BpB (data not shown). Minor non-specific reactivity with eluted streptavidin (SAv) is observed in all sample lanes (2-6) incubated with SAv beads. **b)** A longer exposure of lanes 5 and 6 from a) is shown to allow better visualization of APP CTF pulldown in the Fen-B and Flurbi-BpB crosslinked samples.

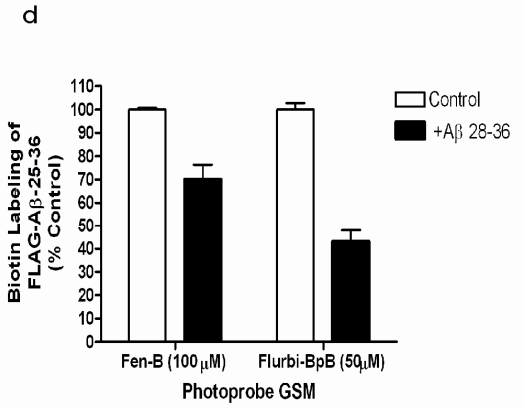


b

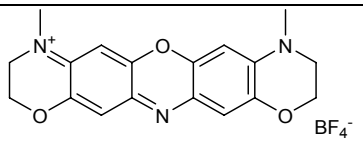
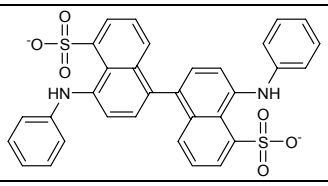
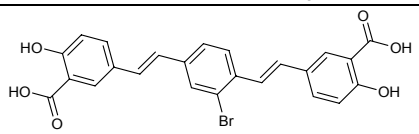
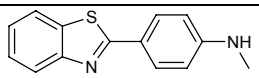
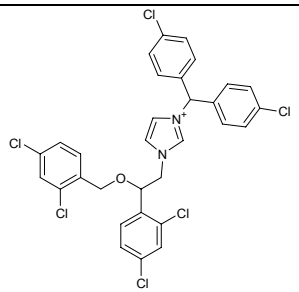
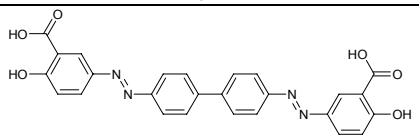
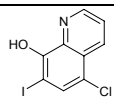
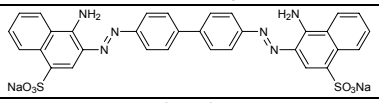
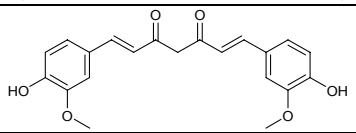
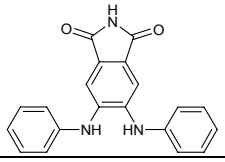
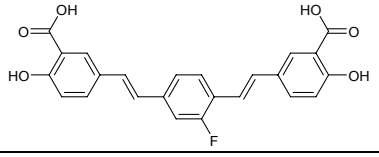
Sample	Integrated Intensity of APP-CTF (counts/mm^2)	APP-CTF Pulldown (% Control)
Fen-B (Control)	3.72	100
Fen-B + X-34	1.62	44
Fen-B + Sulindac	1.66	45
Fen-B + Aβ28-36	2.2	59

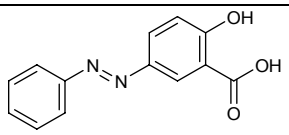
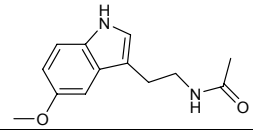
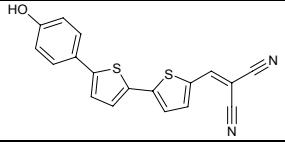
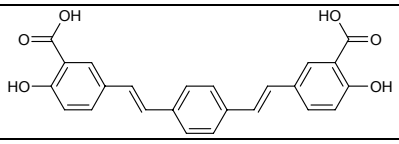
c

Sample	Integrated Intensity of APP-CTF (counts/mm^2)	APP-CTF Pulldown (% Control)
Flurbi-BpB (Control)	4.46	100
Flurbi-BpB + X-34	3.04	68
Flurbi-BpB + Sulindac	1.17	26
Flurbi-BpB + Aβ28-36	2.68	60



Supplementary Figure 7. Photolabeling of APP-CTF and Aβ25-36 by Fen-B and Flurbi-BpB is competed by GSMs and Aβ28-36. a) Membranes from H4-APP-CTF-C105-AP cells were isolated and purified, as described in supplementary figure 6. The pre-cleared membrane fraction (Start, Lane 1) was split into different sample groups and crosslinked with the appropriate GSM photoprobe (Fen-B or Flurbi-BpB; 25μM) +/- competitors (X-34, sulindac sulfide, Aβ28-36; 200μM). Samples were precipitated with streptavidin beads (IP) and analyzed via western for APP-CTF (CT20; 1:1000). Fen-B (lane 4) or Flurbi-BpB (lane 10) alone pull down APP-CTF (~12kDa). APP pulldown is not observed in control samples: membranes crosslinked without photoprobes drugs (lane 2) or photoprobes alone with beads (lanes 5, 11). Competition with GSMs (X-34 and sulindac sulfide) and the putative GSM binding region (Aβ 28-36) decreases labeling of APP- CTF by Fen-B and Flurbi-BpB. In **b)** and **c)** the degree of competition for APP-CTF pulldown is quantified. The amount of APP-CTF band in an individual experimental sample was measured (integrated intensity per mm^2) using the Odyssey infrared imaging system as described by the manufacturer (Li-Cor). The degree of APP-CTF crosslinking and pulldown by the GSM photoprobe alone (Fen-B or Flurbi-BpB; control) is compared to APP-CTF recovered after crosslinking in the presence of competing compounds (percent control). APP holoprotein shows similar labeling and competition profiles (not shown). **d)** Synthetic Aβ28-36 (100μM) competes for labeling of FLAG-Aβ-25-36 (10μM) by Fen-B or Flurbi-BpB. Biotin labelling was measured by M2 Flag ELISA with streptavidin-HRP for detection. Percent control ± s.e.m., n=2.

	Compound Name	Chemical Structure	Evidence for GSM Activity	Literature References
1	AOI-987		None	[1]
2	Bis-ANS		↓	[2-4]
3	BSB		↓	[5, 6]
4	BTA-1		None	[7, 8]
5	Calmidazolium chloride		↑	[9]
6	Chrysamine G		↓	[10, 11]
7	Clioquinol		None	[12]
8	Congo red		↓	[13, 14]
9	Curcumin		None	[15]
10	DAPH (4,5-Dianilinophthalimide)		↑	[16]
11	FSB		↓	[17]

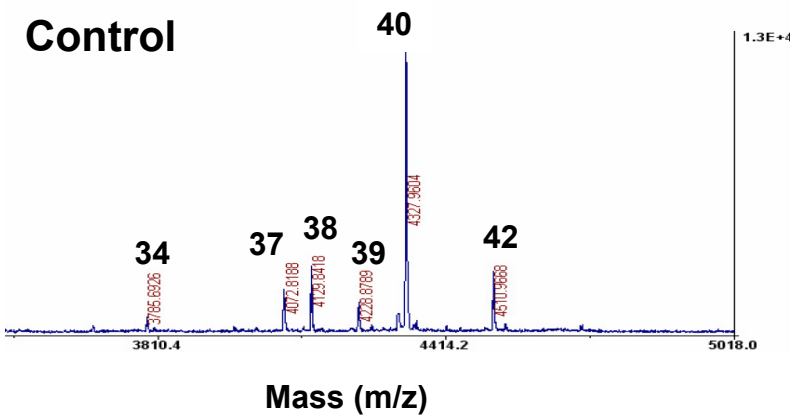
12	Half Chrysamine G		↓	18
13	Melatonin		None	19, 20
14	NIAD 4 amyloid dye		None	21
15	X-34 amyloid dye		↓	22, 23

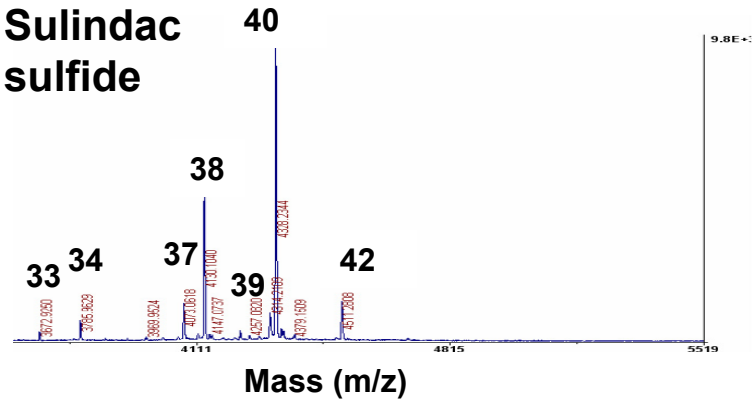
Supplementary Figure 8. γ-secretase modulatory (GSM) activity of compounds that bind Aβ or Aβ amyloid. A literature search was performed to identify compounds reported to bind to the Aβ peptide directly or amyloid. These candidates were initially tested in cell-based assays or cell-free in-vitro γ-secretase assays at two doses (10 and 100μM). Samples were analyzed via ELISA for effects on Aβ42, 40 and total levels and GSM activity of compounds are summarized as Aβ42 raising (↑), Aβ42 lowering (↓) or none detectable. The observed trend of GSM activity of compounds was consistent over 3 experiments with samples run in duplicate or triplicate.

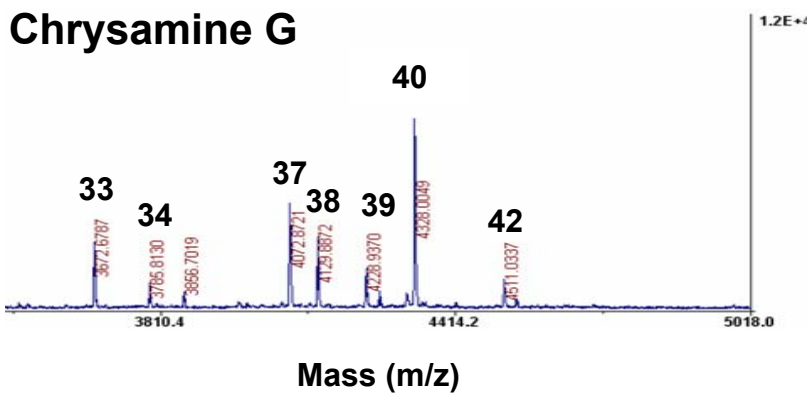
Supplementary references for Fig. 8.

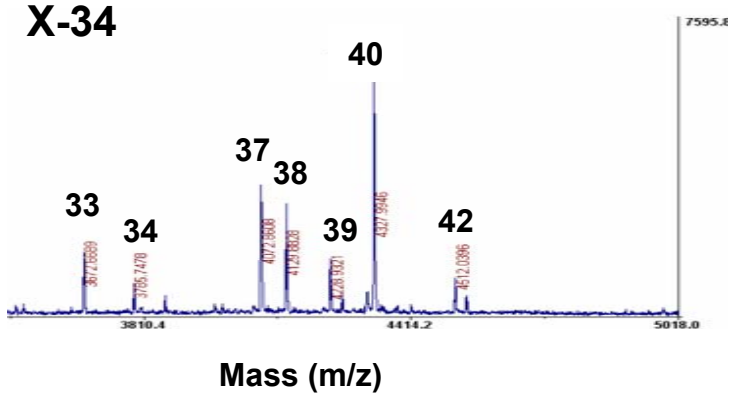
1. Hintersteiner, M. et al. In vivo detection of amyloid-[beta] deposits by near-infrared imaging using an oxazine-derivative probe. Nat Biotech 23, 577-583 (2005).
2. LeVine, I. I. I. H. 4,4'-Dianilino-1,1'-binaphthyl-5,5'-disulfonate: report on non-[beta]-sheet conformers of Alzheimer's peptide [beta](1-40). Archives of Biochemistry and Biophysics 404, 106-115 (2002).
3. Chen, Y.-R. & Glabe, C. G. Distinct Early Folding and Aggregation Properties of Alzheimer Amyloid-beta Peptides Abeta40 and Abeta42. J. Biol. Chem. 281, 24414-24422 (2006).
4. Ferrao-Gonzales, A. D. et al. Controlling {beta}-Amyloid Oligomerization by the Use of Naphthalene Sulfonates: trapping low molecular weight oligomeric species. J. Biol. Chem. 280, 34747-34754 (2005).
5. Skovronsky, D. M. et al. In vivo detection of amyloid plaques in a mouse model of Alzheimer's disease. PNAS 97, 7609-7614 (2000).
6. Schmidt, M. L. et al. The Fluorescent Congo Red Derivative, (Trans, Trans)-1-Bromo-2,5-Bis-(3-Hydroxycarbonyl-4-Hydroxy)Styrylbenzene (BSB), Labels Diverse {beta}-Pleated Sheet Structures in Postmortem Human Neurodegenerative Disease Brains. Am J Pathol 159, 937-943 (2001).

7. Mathis, C. A. et al. A lipophilic thioflavin-T derivative for positron emission tomography (PET) imaging of amyloid in brain. Bioorganic & Medicinal Chemistry Letters 12, 295-298 (2002).
8. Klunk, W. E. et al. The Binding of 2-(4'-Methylaminophenyl)Benzothiazole to Postmortem Brain Homogenates Is Dominated by the Amyloid Component. J. Neurosci. 23, 2086-2092 (2003).
9. Williams, A. D. et al. Structural properties of A{beta} protofibrils stabilized by a small molecule. PNAS 102, 7115-7120 (2005).
10. Klunk, W. E., Debnath, M. L. & Pettegrew, J. W. Chrysamine-G binding to Alzheimer and control brain: Autopsy study of a new amyloid probe. Neurobiology of Aging 16, 541-548 (1995).
11. Klunk, W. E., Debnath, M. L., Koros, A. M. C. & Pettegrew, J. W. Chrysamine-G, a lipophilic analogue of congo red, inhibits A[beta]-induced toxicity in PC12 cells. Life Sciences 63, 1807-1814 (1998).
12. Opazo, C. et al. Radioiodinated clioquinol as a biomarker for Beta-amyloid: Zn2+ complexes in Alzheimer's disease. Aging Cell 5, 69-79 (2006).
13. Cairo, C. W., Strzelec, A., Murphy, R. M. & Kiessling, L. L. Affinity-Based Inhibition of Beta-Amyloid Toxicity. Biochemistry 41, 8620-8629 (2002).
14. Gestwicki, J. E., Crabtree, G. R. & Graef, I. A. Harnessing Chaperones to Generate Small-Molecule Inhibitors of Amyloid {beta} Aggregation. Science 306, 865-869 (2004).
15. Yang, F. et al. Curcumin Inhibits Formation of Amyloid {beta} Oligomers and Fibrils, Binds Plaques, and Reduces Amyloid in Vivo. J. Biol. Chem. 280, 5892-5901 (2005).
16. Blanchard, B. J. et al. Inaugural Article: Efficient reversal of Alzheimer's disease fibril formation and elimination of neurotoxicity by a small molecule. PNAS 101, 14326-14332 (2004).
17. Sato, K., Higuchi, M., Iwata, N., Saido, T. C. & Sasamoto, K. Fluoro-substituted and 13C-labeled styrylbenzene derivatives for detecting brain amyloid plaques. European Journal of Medicinal Chemistry 39, 573-578 (2004).
18. Ishii, K. et al. Chrysamine G and its derivative reduce amyloid [beta]-induced neurotoxicity in mice. Neuroscience Letters 333, 5-8 (2002).
19. Pappolla, M. et al. Inhibition of Alzheimer beta -Fibrillogenesis by Melatonin. J. Biol. Chem. 273, 7185-7188 (1998).
20. Cheng, X. & vanBreemen, R. B. Mass Spectrometry-Based Screening for Inhibitors of Beta-Amyloid Protein Aggregation. Anal. Chem. 77, 7012-7015 (2005).
21. Nesterov, E. E. et al. In vivo optical imaging of amyloid aggregates in brain: design of fluorescent markers. Angew Chem Int Ed Engl 44, 5452-6 (2005).
22. Styren, S. D., Hamilton, R. L., Styren, G. C. & Klunk, W. E. X-34, A Fluorescent Derivative of Congo Red: A Novel Histochemical Stain for Alzheimer's Disease Pathology. J. Histochem. Cytochem. 48, 1223-1232 (2000).
23. Link, C. D. et al. Visualization of fibrillar amyloid deposits in living, transgenic Caenorhabditis elegans animals using the sensitive amyloid dye, X-34. Neurobiology of Aging 22, 217-226 (2001).

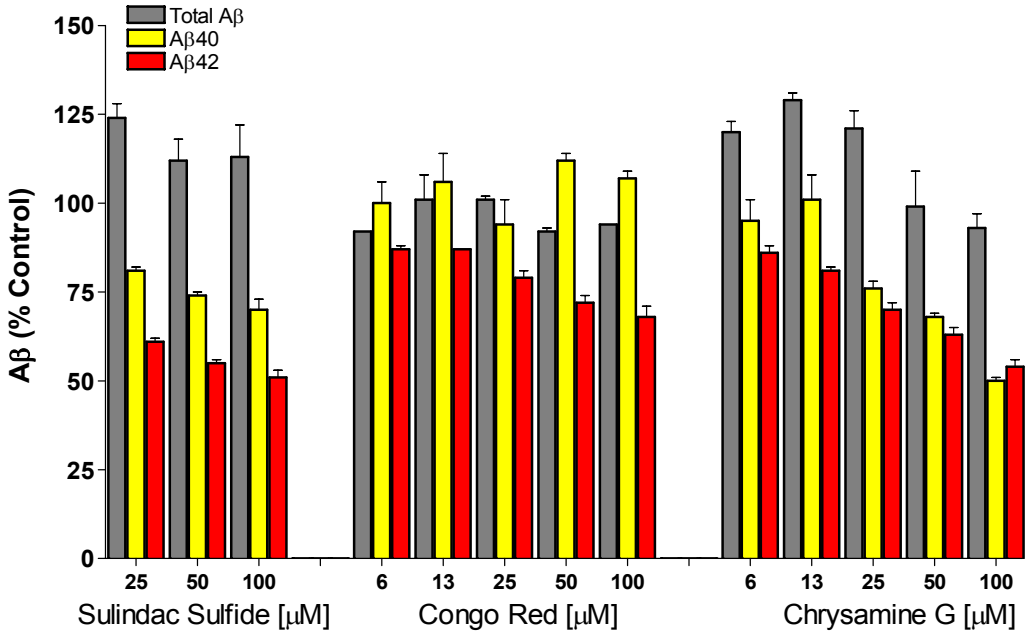




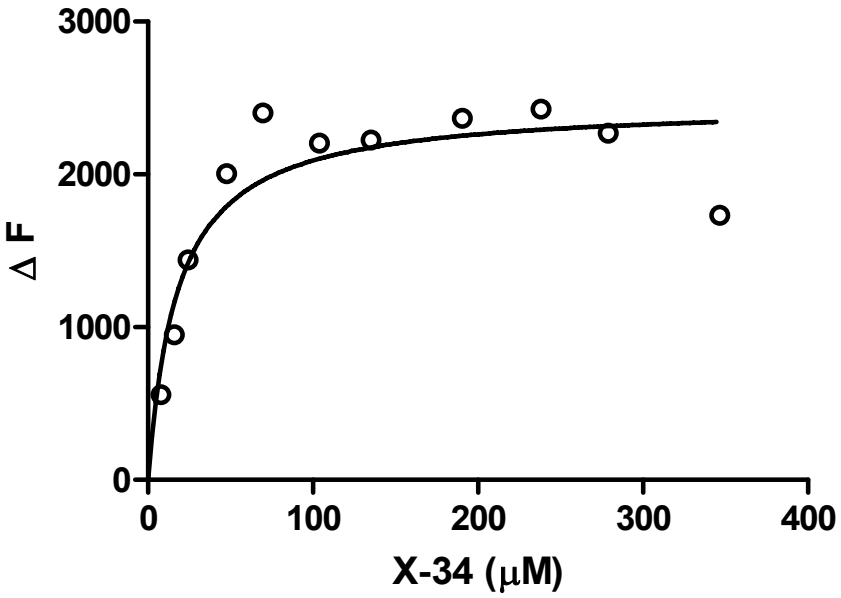




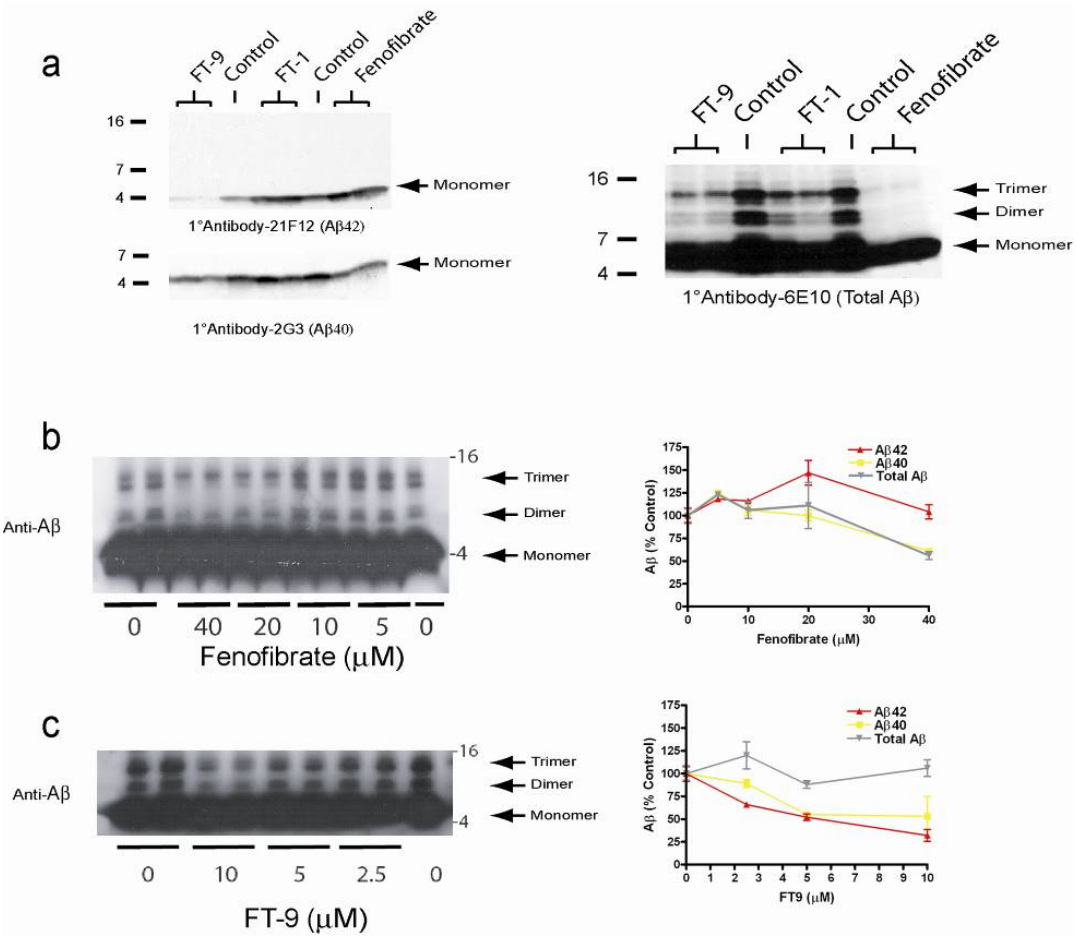
Supplementary Figure 9. Amyloid binding compounds alter the cleavage of Aβ similar to other established γ–secretase modulators. Immunoprecipitation–mass spectrometry studies were conducted on media from H4 APPwt cells grown in the presence of test compounds for 16 hours. Conditions were : control (DMSO, 0.5%), sulindac sulfide (SS; 25 μM), chrysamine g (CG; 25 μM), and X-34 (25 μM). SS was included as a control GSM known to reduce Aβ42 and increase Aβ38. Spectra shown are representative of two experiments with 2-3 replicates each. Identified Aβ peptides based on calculated mass (m/z) are indicated above the peaks. All three compounds consistently decrease levels of Aβ42 but show different propensities to increase shorter Aβ species (33, 34, 37, 38 and 39). Both CG and X-34 raise Aβ33 levels more effectively than SS while this species is nearly undetectable in the control.



Supplementary Figure 10. Some Amyloid β binding compounds are GSMs. Congo red (CR) and chrysamine G (CG), two of the most well characterized amyloid-β dyes, act as GSMs in cell-free γ-secretase assays. Increasing doses of CR, CG, and sulindac sulfide lowered Aβ42 levels without decreasing total Aβ production. CG and sulindac also lowered Aβ40 at higher doses.



Supplementary Figure 11. X-34 binds to Aβ1-42. Fluorescence titration and saturation binding of Aβ(1-42) with X-34. Incremental increases in fluorescence (ΔF) when Aβ(1-42) was included at the indicated total concentrations of X-34 were fitted to eq. 1 as outlined in the Methods. Fitted parameters were $f_{C\text{-}L}$ = 240 ± 15 μM^{-1} and K_D = 12.1 ± 4.7 μM.



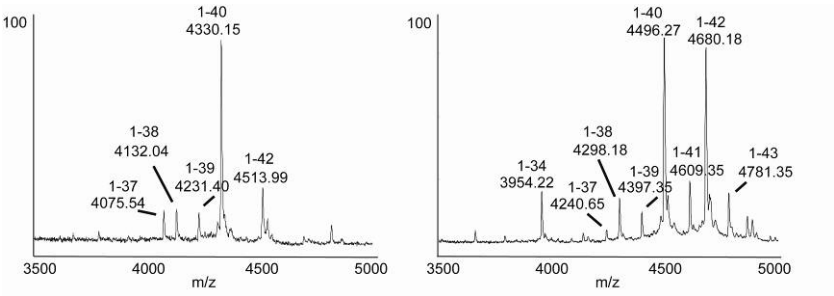
Supplementary Figure 12. γ-secretase modulators decrease the production of cell derived Aβ oligomers. CHO cells stably expressing human APP751 containing the familial Alzheimer's disease mutation V717F (referred to as 7PA2 cells) were incubated for ~16 h in the presence of GSMs. **a)** FT-9 (20μM) lowers Aβ42 while FT-1 (20μM) and fenofibrate (100μM) increase production of Aβ42 in 7PA2 cells after overnight treatment. All three compounds also reduce production of dimeric and trimeric Aβ species as detected by a pan-Aβ antibody (6E10). Aβ40 levels were decreased to varying degrees (26-35%; Li-Cor Odyssey). **b and c)** Because the extended incubation times necessary to reliably detect Aβ oligomers in this assay can be toxic, we carried out additional dose response studies with fenofibrate and FT-9 to verify that decreases in Aβ oligomers levels were not related to toxicity or global inhibition of Aβ production. **b)** IP/western blot analysis of fenofibrate treated 7PA2 cells show decreased Aβ dimers and trimers at doses which increase Aβ42 without decreases in Aβ40 or total Aβ as measured by ELISA (graph on right). **c)** Increasing doses of FT-9 reduced the detection of Aβ oligomeric bands and Aβ42 decreased preferentially with decreases in Aβ40 at higher doses. Total Aβ levels did not decrease suggesting that the observed reduction in oligomers is not a result of inhibition of Aβ production. Aβ species present in conditioned medium were detected by IP using the polyclonal anti-Aβ antibody, DW6 and subsequent immunoblotting with either 6E10 or the 42-specific, anti-Aβ antibody 21F12. Aβ levels in the samples analyzed via IP/western were also quantified via sandwich ELISA. Data presented as percent vehicle control ± s.e.m., n=2.

a

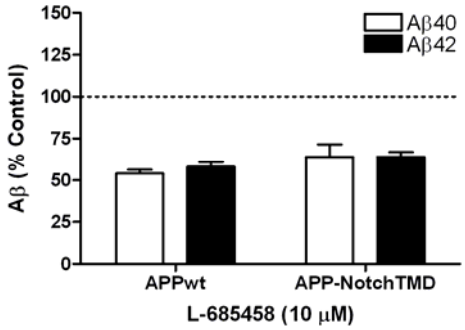
APPwt DAEFRHDSGYEVHHQKLVFFAEDVGSNK**GAIIGL**MVGGVVIATVIVITLVMLK

APP-Notch TMD DAEFRHDSGYEVHHQKLVFFAEDVGSNK**LHFMY**MVGGVVIATVIVITLVMLK

b **APPwt** **APP-Notch TMD**



c



Supplementary Figure 13. Substitution of human Notch sequence in the APP Transmembrane domain (TMD) Changes Sensitivity to Modulation. a) We used site-directed mutagenesis to exchange the analogous region of the human Notch TMD (bold and underlined) for a section (bold and underlined) of wild-type APP (APPwt) we have shown to be labeled by GSMs. The new substrate (APP-Notch TMD) is cleaved by γ-secretase primarily after valine and alanine residues corresponding to 40 and 42 in APPwt (arrows). **b)** Immunopreciptation with Ab9, an N-terminal Aβ, followed by MALDI-mass spectrometry (see Methods) shows the spectrum of Aβ species produced by APPwt (left) and APP-Notch TMD (right). The beginning (1) N-terminal amino acid (AA) to the ending C-terminal AA is shown above each peak, with the molecular weight (m/z) below. 1-40 and 1-42 are detected as the two major species in APP-Notch TMD conditioned media. It should be noted that the mutagenesis strategy to create APP-Notch TMD leads to a single AA deletion relative to APPwt. The correct change in molecular weight caused by the introduced mutations are confirmed by the mass spectrum. For simplicity the peptides are referenced by their C-terminal residues relative to Aβ (40, 42, etc). Representative mass spectra are shown from multiple IP reactions (3) and duplicate spots. **c)** Secretion of Aβ from APPwt and APP-Notch TMD cells is equally inhibited by a transition state GSI. Both cell lines were treated (6 hours) with L-685,458 (10μM), which is hypothesized to target the active site aspartate residues, media was collected, and analyzed for changes in Aβ levels. The 40 and 42 Aβ reactive species from both cell lines were inhibited by L-685,458 with equal sensitivity (no significant difference; 40 $p>0.5$; 42 $p>0.4$; *t* test; n=3).

2. Supplementary Discussion

Our results demonstrate that photoactivatable GSMs do not label γ-secretase but rather, multiple GSMs specifically interact with and label the substrate, making it unlikely that the GSMs we have examined act by targeting the enzyme itself[1]. Notably, the labelling of substrate occurs in the same concentration range over which GSMs shift γ-secretase cleavage of APP, and for one GSM, X-34, modulation of cleavage is shown to correlate well with its affinity for substrate. Further, labelling efficiency correlates with the potency of various GSMs for modulating γ-secretase cleavage of APP and also the differential efficiency that GSMs modulate cleavage of APP as compared to Notch. Moreover, the binding site remains in the secreted Aβ cleavage product, providing a mechanistic link between the GSMs ability to shift cleavage and their tendency to inhibit Aβ aggregation. These data are consistent with our previous finding that mutations within APP influence GSM activity[2] and are reminiscent of and supported by studies showing that pre-treatment of C100Flag with detergent modulates γ-secretase cleavage specificity of the substrate[3].

Several studies have proposed that GSMs lower Aβ42 by allosteric modulation of γ-secretase. A molecular imaging approach has shown that some NSAID GSMs can alter PS and APP conformation[4]. Two other studies report that two NSAIDs with GSM activity display linear non-competitive inhibition for in vitro Aβ42 production by γ-secretase, thus providing evidence of an allosteric mechanism[5, 6]. Our data can be reconciled with an allosteric model of inhibition by proposing that binding of GSMs to APP-CTF induces conformational changes in PS1/γ-secretase when the GSM-bound substrate enters the complex[4]. Indeed, mutations within the transmembrane domain of APP shift the γ-secretase cleavage site and alter PS conformation[7]. Given the low resolution of kinetic studies on γ-secretase it would be very challenging, if not impossible, to distinguish between direct substrate targeting and other types of allosteric targeting of the enzyme based on kinetic analyses[8]. In the Beher *et al* study the observation that sulindac sulphide displays noncompetitive antagonism of a radiolabeled active site directed GSI is also used to support the claim that GSMs target an unknown site on the enzyme[6]. However, these data were generated using crude solubilzed membrane fractions. Because these fractions contain enzyme and substrate and have been shown to contain multiple binding targets for a potent active site directed GSI [9], we believe that this data cannot be used to distinguish between substrate and direct enzyme targeting. Furthermore, the displacement of the GSI is not replicated in photoaffinity studies; NSAID derivatives do not compete for photolabelling of PS1 by a GSI photoprobe and instead actually increase affinity precipitation of PS1[6]. In contrast, we provide multiple lines of evidence to show that various GSMs directly bind substrate. More importantly, we link

substrate binding to activity by showing that alteration of the GSM binding site on APP substrate alters sensitivity to modulation.

References:

1. Dorman, G. & Prestwich, G. D. Benzophenone photophores in biochemistry. Biochemistry 33, 5661-73. (1994).
2. Weggen, S. et al. Evidence That Nonsteroidal Anti-inflammatory Drugs Decrease Amyloid {beta}42 Production by Direct Modulation of {gamma}-Secretase Activity. J. Biol. Chem. 278, 31831-31837 (2003).
3. Fraering, P. C. et al. Purification and characterization of the human gamma-secretase complex. Biochemistry 43, 9774-89 (2004).
4. Lleo, A. et al. Nonsteroidal anti-inflammatory drugs lower A[beta]42 and change presenilin 1 conformation. Nature Medicine 10, 1065-1066 (2004).
5. Takahashi, Y. et al. Sulindac Sulfide Is a Noncompetitive gamma -Secretase Inhibitor That Preferentially Reduces Abeta 42 Generation. J. Biol. Chem. 278, 18664-18670 (2003).
6. Beher, D. et al. Selected Non-steroidal Anti-inflammatory Drugs and Their Derivatives Target {gamma}-Secretase at a Novel Site: Evidence for an allosteric mechanism. J. Biol. Chem. 279, 43419-43426 (2004).
7. Tesco, G. et al. APP substitutions V715F and L720P alter PS1 conformation and differentially affect Abeta and AICD generation. Journal of Neurochemistry 95, 446-456 (2005).
8. Weiss, S. T., McIntyre, N. R., McLaughlin, M. L. & Merkler, D. J. The development of molecular clamps as drugs. Drug Discovery Today 11, 819-824 (2006).
9. Iben, L. G. et al. Signal Peptide Peptidase and {gamma}-Secretase Share Equivalent Inhibitor Binding Pharmacology. J. Biol. Chem. 282, 36829-36836 (2007).

3. Supplementary Methods

General Chemistry Comments

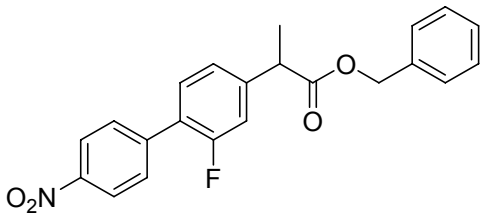
The ^{1}H spectra were recorded on a Bruker AC 300 spectrometer at 300 MHz and Bruker AC 500 spectrometer at 500 MHz. The ^{13}C spectra was recorded on a Bruker AC 300 spectrometer at 75 MHz and Bruker AC 500 spectrometer at 125 MHz. Chemical shifts are reported as ppm downfield from Me_4Si. Mass spectrometry was performed on a Bruker-Franzen Esquire LC mass spectrometer. Flash column chromatography was carried out using Merck silica gel 60 (40-63 and 15-40 μm) and 60G (5-40 μm). Thin-layer chromatography (TLC) was carried out using aluminum sheets precoated with silica gel 60 F254 (0.2 mm; E. Merck). Chromatographic spots were visualized by UV and/or spraying with an acidic, ethanolic solution of *p*-anisaldehyde or an ethanolic solution of ninhydrin followed by heating. For preparative TLC, plates precoated with silica gel 60 F254 (2.0 mm; E. Merck) were used. THF was dried and distilled from sodium and benzophenone. DMF was stored over 3 Å molecular sieves. All other commercial chemicals were used without further purification.

Preparation Flurbiprofen-benzophenone-biotin (Flurbi-BpB)

Benzyl 2-(2-fluoro-4'-nitrobiphenyl-4-yl)propanoate:

A mixture of 2-(2-fluoro-4-biphenyl)propanoic acid (500 mg, 2.59 mmol) and 5 mL of 70% nitric acid was stirred with an efficient stirrer. The suspended solid gradually went into solution during the first 12 hours. The reaction was continued for another 36 hours after which the TLC indicated complete consumption of starting material and formation of two products. The reaction mixture was poured on ice and extracted with CH_2Cl_2 (3×). The combined organic extracts were washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated in vacuo to yield the crude mixture of the *ortho* and *para* nitrated product as an orange gummy mass. MS (ESI): m/z = 312.06 $(M+Na)^+$, 928.3.

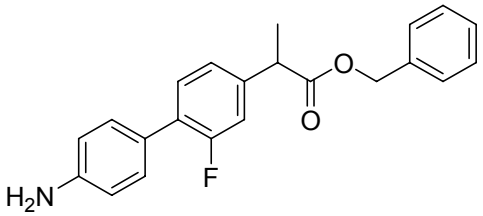
To a stirred solution of nitrated products (750 mg, 2.59 mmol) in anhydrous DMF (15 mL), anhydrous K_2CO_3 (1075 mg, 7.88 mmol) was added and stirred for 30 minutes. Benzyl bromide (0.39 mL, 2.59 mmol) was added to it and stirred for another 3 hours after which TLC indicated complete consumption of the starting material. The reaction mixture was then diluted with ethyl acetate and washed with water. The aqueous layer was extracted with ethyl acetate (3×). The combined organic extract was washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated in vacuo to yield the crude mixture. The crude mixture of the *ortho* and *para* nitrated benzyl ester was purified by column chromatography (ethyl acetate:hexane, 15:85) to afford the title compound as brown gummy mass (250 mg, 32 %). ^{1}HNMR (300 MHz, $CDCl_3$): δ = 8.30 (d, J = 9.00 Hz, 2H), 7.70 (dd, J = 7.20 Hz, J = 2.05 Hz, 2H), 7.42-7.10 (m, 8H), 5.18 (d, J = 15.00 Hz, 1H), 5.12 (d, J = 15.00 Hz, 1H), 3.84 (q, J = 9.00 Hz, 1H), 1.57 (d, J = 9.00 Hz, 3H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.3, 161.4, 158.7, 147.3, 140.6, 140.1, 137.6, 130.6, 130.5, 129.9, 129.8, 129.7, 128.5, 128.3, 128.0, 125.0, 124.1, 124.0, 123.7, 115.8, 66.8, 45.1, 18.3 ppm. MS (ESI): *m/z* 402.25 $(M+Na)^+$.



Benzyl 2-(4'-amino-2-fluorobiphenyl-4-yl)propanoate:

Anhydrous $SnCl_2$ (556 mg, 0.59 mmol) was added to a stirred solution of benzyl 2-(2-fluoro-4'-nitrobiphenyl-4-yl)propanoate (225 mg, 0.59 mmol) in dry ethanol and refluxed for 6 hours. The reaction mixture was then cooled to room temperature and poured on ice. The solution was basified using saturated solution of $NaHCO_3$ and extracted with ethyl acetate (3×). The combined organic extracts were washed with water, brine, dried over anhydrous and evaporated in vacuo to yield the crude product. The crude compound was purified by flash

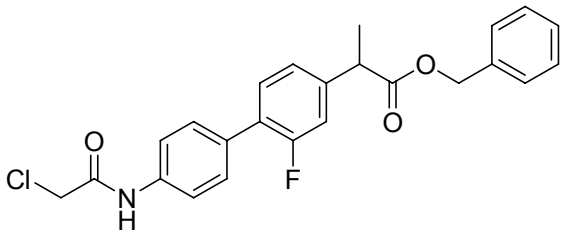
column chromatography (ethyl acetate:hexane, 3:7) to afford the pure product as pale brown gum (120 mg, 57 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.42-7.10 (m, 8H), 7.03.- 6.97 (m, 2H), 6.68 (dd, J = 6.50 Hz, J = 2.10 Hz, 2H), 5.08 (d, J = 15.00 Hz, 1H), 5.02 (d, J = 15.00 Hz, 1H), 3.70 (q, J = 7.00 Hz, 1H), 1.46 (d, J = 7.00 Hz, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 173.9, 161.2, 158.0, 146.0, 140.6, 140.5, 135.8, 130.2, 129.9, 129.9, 129.8, 129.7, 128.5, 128.1, 128.0, 128.7, 125.6, 123.4, 123.3, 115.3, 66.6, 45.0, 18.3 ppm. MS (ESI): *m/z* , 350.21 $(M+H)^+$



Benzyl 2-(4'-(2-chloroacetamido)-2-fluorobiphenyl-4-yl)propanoate:

To a stirred solution of benzyl 2-(4'-amino-2-fluorobiphenyl-4-yl)propanoate (205 mg, 0.58 mmol) in dry CH_2Cl_2, triethylamine (0.098 mL, 0.70 mmol) was added at 0 °C and the mixture stirred for 30 minutes. Chloroacetyl chloride (0.056 mL, 0.70) was added to it drop wise and stirred for another 30 minutes. The reaction mixture was then allowed to attain room temperature and stirred for another 2 hours. It was then diluted with CH_2Cl_2 (100 mL), washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated in vacuo to afford the crude product. The crude compound was purified by column chromatography (ethyl acetate:hexane) to obtain the title compound as colorless solid (240 mg, 95 %). ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.55 (brs, 1H), 7.55 (td, J = 8.50 Hz, J = 1.80 Hz, 2H), 7.47 (td, J = 7.20 Hz, J = 1.70 Hz, 2H), 7.31 (d, J = 8.00 Hz, 1H), 7.29-7.20 (m, 5H), 7.08 (dd, J = 8.00 Hz, J = 1.80 Hz, 2H), 7.04 (dd, J = 11.00 Hz, J = 1.80 Hz, 2H), 5.08 (d, J = 12.50 Hz, 1H), 5.04 (d, J = 12.50 Hz, 1H), 4.14 (s, 2H), 3.72 (q, J = 7.20 Hz, 1H), 1.48 (d, J = 7.20 Hz, 3H) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 175.3, 165.4, 158.0, 137.8, 137.4, 136.5, 135.8, 132.2, 132.1,

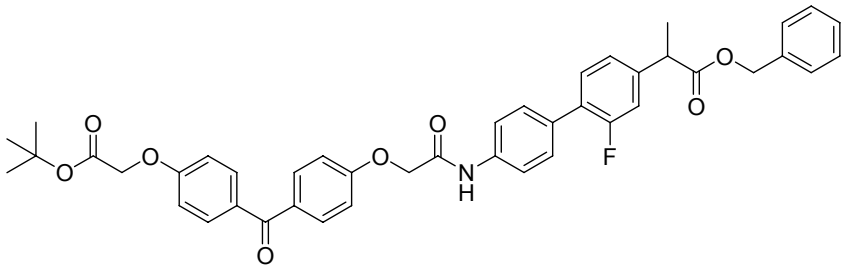
131.3, 131.2, 130.1, 129.8, 129.6, 128.0, 128.7, 125.3, 121.6, 117.0, 116.9, 68.3, 46.6, 44.5, 19.9 ppm. HRMS: calc 426.1272, found 426.1253



Benzyl 2-(4'-(2-(4-(4-(2-tert-butoxy-2-oxoethoxy)benzoyl)phenoxy)acetamido)-2 fluorobiphenyl-4-yl)propanoate:

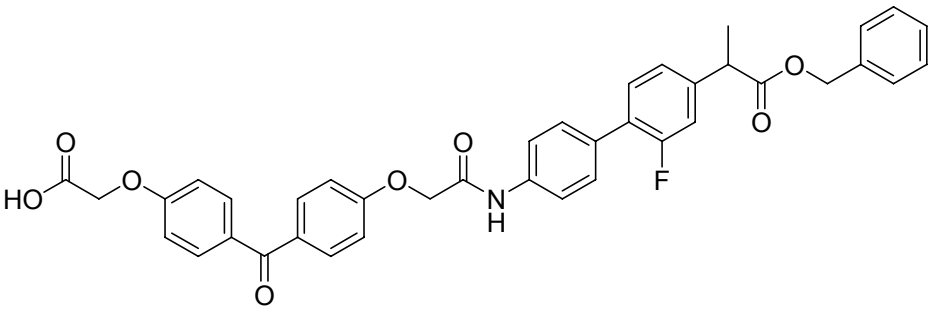
Anhydrous K_2CO_3 (222 mg, 1.60 mmol) and benzyl 2-(4'-(2-chloroacetamido)-2-fluorobiphenyl-4-yl)propanoate (190 mg, 0.44 mmol) were to a stirred solution of tert-butyl 2-(4-(4-hydroxybenzoyl)phenoxy)acetate (176 mg, 0.54 mmol) in dry acetone, and heated to 60-70 °C for 12 hours. The reaction mixture was then cooled to room temperature and filtered. The residue was washed with acetone (3×). The combined organic layer was evaporated in vacuo yield the crude product. The crude product was purified by flash column chromatography (ethyl acetate:hexane, 1:4) to afford the title compound as colorless solid (185 mg, 57 %).

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 8.30 (brs, 1H), 7.84 (dd, J = 7.00 Hz, J = 1.90 Hz, 2H), 7.79 (dd, J = 9.20 Hz, J = 1.90 Hz, 2H), 7.70 (d, J = 8.70 Hz, 2H), 7.56 (dd, J = 8.50 Hz, J = 1.40 Hz, 2H), 7.39 (dd, J = 9.00 Hz, J = 2.40 Hz, 1H), 7.37-7.28 (m, 5H), 7.16 (dd, J = 8.50 Hz, J = 1.90 Hz, 2H), 7.11 (dd, J = 8.80 Hz, J = 1.90 Hz, 2H), 7.07-6.95 (m, 3H), 5.17 (d, J = 12.50 Hz, 1H), 5.10 (d, J = 12.50 Hz, 1H), 4.75 (s, 2H), 4.63 (s, 2H), 3.83 (q, J = 7.20 Hz, 1H), 1.55 (d, J = 7.20 Hz, 3H), 1.52 (s, 9H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 194.2, 173.8, 167.5, 161.4, 159.9, 136.2, 132.5, 132.3, 131.7, 130.6, 129.7, 128.6, 128.3, 128.1, 123.8, 120.2, 115.6, 115.3, 114.4, 114.2, 82.9, 67.6, 66.8, 65.6, 45.1, 28.1, 18.4 ppm. MS (ESI): *m/z* 740.46 $(M+Na)^+$.



2-(4-(4-(2-(4'-(1-(Benzyloxy)-1-oxopropan-2-yl)-2'-fluorobiphenyl-4-ylamino)-2-oxoethoxy)benzoyl)phenoxy)acetic acid:

Trifluoroacetic acid (0.4 mL) was added to a stirred solution of benzyl 2-(4'-(2-(4-(4-(2-tert-butoxy-2-oxoethoxy)benzoyl)phenoxy)acetamido)-2 fluorobiphenyl-4-yl)propanoate (180 mg, 0.25 mmol) in dichloromethane (2 mL) at 0 °C and stirred for 6 hours. It was then evaporated in vacuo to afford the crude acid. The crude acid was purified by acid-base treatment to afford the titled compound as colorless solid (154 mg, 92 %).MS (ESI): *m/z* 684.28 $(M+Na)^+$

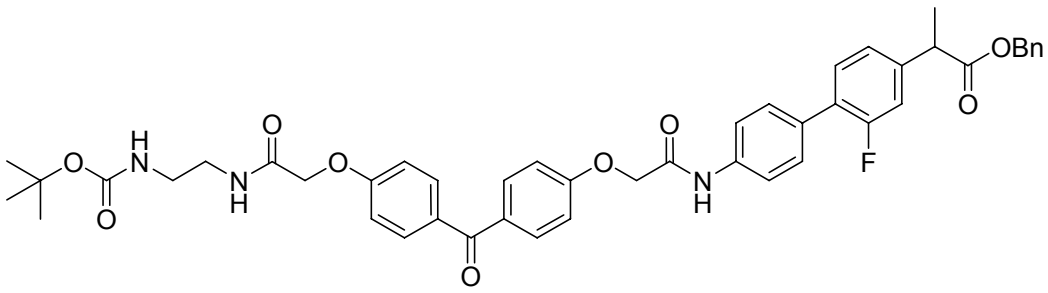


Benzyl 2-(4'-(2-(4-(4-(2-(2-(tert-butoxycarbonylamino)ethylamino)-2-oxoethoxy)benzoyl)phenoxy)acetamido)-2-fluorobiphenyl-4-yl)propanoate:

To a stirred solution of 2-(4-(4-(2-(4'-(1-(benzyloxy)-1-oxopropan-2-yl)-2'-fluorobiphenyl-4-ylamino)-2-oxoethoxy)benzoyl)phenoxy)acetic acid (150 mg, 0.22 mmol) in anhydrous CH_2Cl_2 (2 mL), was added triethylamine (0.032 mL, 0.22 mmol) and stirred at ambient temperature for 10 min Ethyl-3-(3´-dimethylaminopropyl)carbodiimide hydrochloride (48

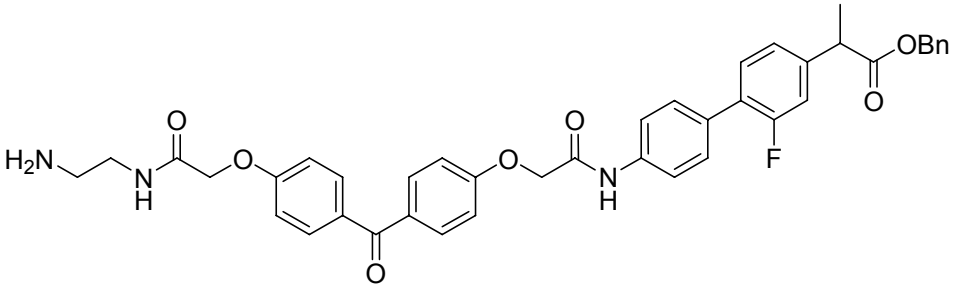
mg, 0.25 mmol) and *N*-hydroxybenzotriazole hydrate (37 mg, 0.27 mmol) were added to it and the resulting solution was stirred at ambient temperature for 30 min until it became clear. A solution of *N*-Boc ethylenediamine (0.043 mL, 0.27 mmol) in CH_2Cl_2 (1 mL) was added to the reaction mixture followed by triethylamine (0.038 mL, 0.27 mmol) and stirred at ambient temperature for 10 h. The reaction mixture was diluted with $CHCl_3$ (100 mL), washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated in vacuo to yield the crude product. The crude product was purified by flash column chromatography (ethyl acetate:hexane, 3:7) to yield the title compound as colorless solid (103 mg, 54 %).

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 8.21 (brs, 1H), 7.88 (dd, *J* = 7.00 Hz, *J* = 2.00 Hz, 2H), 7.74 (dd, *J* = 7.00 Hz, *J* = 2.00 Hz, 2H), 7.61 (td, *J* = 8.50 Hz, *J* = 2.10 Hz, 2H), 7.48 (dd, *J* = 8.50 Hz, *J* = 1.40 Hz, 2H), 7.32-7.19 (m, 6H), 7.08-7.02 (m, 4H), 6.96 (dd, *J* = 7.00 Hz, *J* = 2.00 Hz, 2H), 5.08 (d, *J* = 12.50 Hz, 1H), 5.04 (d, *J* = 12.50 Hz, 1H), 4.98 (brs, 1H), 4.65 (s, 2H), 4.65 (s, 2H), 4.49 (s, 2H), 3.74 (q, *J* = 7.20 Hz, 1H), 3.40 (q, *J* = 6.40 Hz, 2H), 3.30-3.26 (m, 2H), 1.48 (d, *J* = 7.20 Hz, 3H), 1.36 (s, 9H) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 192.1, 171.8, 166.2, 163.6, 158.8, 158.6, 158.1, 156.8, 139.9, 139.8, 134.3, 133.9, 130.6, 130.5, 130.4, 129.8, 128.7, 128.6, 127.8, 126.6, 126.3, 126.1, 121.8, 118.2, 113.6, 113.4, 112.6, 112.5, 78.0, 65.7, 65.3, 64.8, 43.1, 38.7, 38.2, 26.4, 16.4 ppm. HRMS: calc 804.3290, found 804.3222



Benzyl 2-(4'-(2-(4-(4-(2-(2-aminoethylamino)-2-oxoethoxy)benzoyl)phenoxy)acetamido)-2-fluorobiphenyl-4-yl)propanoate:

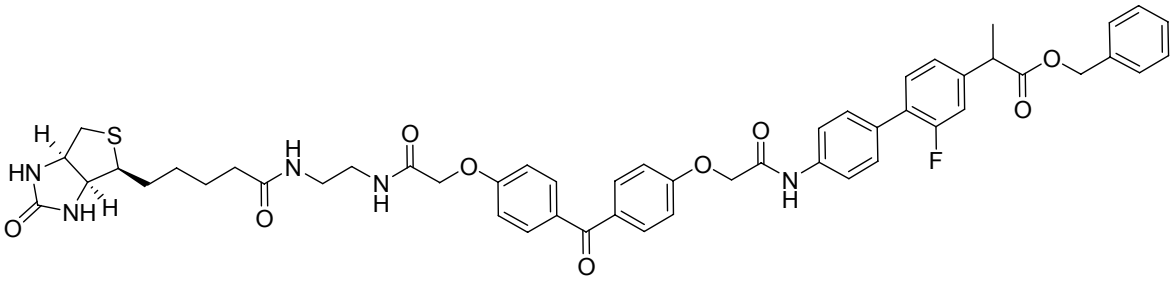
A solution of benzyl 2-(4'-(2-(4-(4-(2-(2-(tert-butoxycarbonylamino)ethylamino)-2-oxoethoxy)benzoyl)phenoxy)acetamido)-2-fluorobiphenyl-4-yl)propanoate (90 mg, 0.11 mmol) in 16 % HCl in dioxane (1 mL) was stirred at ambient temperature for 30 min Dioxane was evaporated in vacuo and lyophilized to obtain the title compound as hydrochloride salt (73 mg, 88 %). The crude compound was used for next step without further purification. **MS** (ESI): *m/z* = 704.4 $(MH)^+$



Benzyl 2-(2-fluoro-4'-(2-(4-(4-(2-oxo-2-(2-(5-((3a*R*,4*R*,6a*S*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)ethylamino)ethoxy)benzoyl)phenoxy)acetamido)biphenyl-4-yl)propanoate:

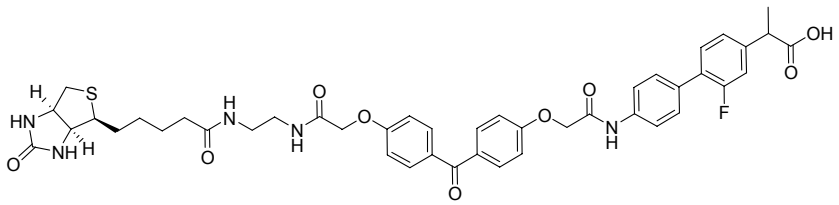
To a stirred solution of D-biotin (33 mg, 0.13 mmol) in anhydrous DMF (2 mL), was added diisopropylethylamine (0.024 mL, 0.13 mmol) and stirred at ambient temperature for 10 min PyBrop (70 mg, 0.15 mmol) was added to it and the resulting solution was stirred at ambient temperature for 30 min until it became clear. A solution of benzyl 2-(4'-(2-(4-(4-(2-(2-aminoethylamino)-2-oxoethoxy)benzoyl)phenoxy)acetamido)-2-fluorobiphenyl-4-yl)propanoate (80 mg, 0.11 mmol) in DMF (1 mL) was added to the reaction mixture followed by diisopropylethyl amine (0.071 mL, 0.40 mmol) and stirred at ambient temperature for 18 h. The reaction mixture was diluted with $CHCl_3$ (100 mL), washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated in vacuo to yield the crude product. The crude product was purified by flash column chromatography (CH_2Cl_2:MeOH, 95:5) to afford the title compound as brownish solid (22 mg, 22 %).

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 9.90 (brs, 1H), 8.05 (brs, 1H), 7.80-7.71 (m, 6H), 7.65 (brs, 1H), 7.50-7.36 (m, 5H), 7.16-7.07 (m, 5H), 6.10 (brs, 1H), 5.14 (d, *J* = 12.50 Hz, 1H), 5.08 (d, *J* = 12.50 Hz, 1H), 4.78 (s, 2H), 4.55 (s, 2H), 4.40-4.34 (m, 1H), 4.22-4.15 (m, 1H), 3.85 (q, *J* = 7.20 Hz, 1H), 3.24-3.12 (m, 4H), 3.10-3.01 (m, 1H), 2.80 (dd, *J* = 12.60 Hz, *J* = 5.00 Hz, 1H), 2.65 (d, *J* = 12.60 Hz, 1H), 2.13 (t, *J* = 7.30 Hz, 2H), 1.76-1.55 (m, 4H), 1.52 (d, *J* = 7.20 Hz, 3H), 1.43-1.35 (m, 2H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 192.1, 176.0, 174.8, 166.2, 162.4, 158.5, 158.4, 158.1, 156.6, 139.6, 139.5, 134.1, 133.5, 130.5, 130.4, 130.3, 129.6, 128.5, 128.4, 127.5, 126.3, 126.1, 126.0, 121.6, 118.2, 113.5, 113.2, 112.1, 112.4, 78.0, 65.7, 65.3, 64.8, 63.7, 62.0, 57.7, 43.1, 41.8, 38.7, 38.2, 38.1, 30.3, 29.8, 27.2, 27.1, 17.4 ppm. MS (ESI): *m/z* = 952.4 $(M+Na)^+$, 928.3 $(M–H)^+$.

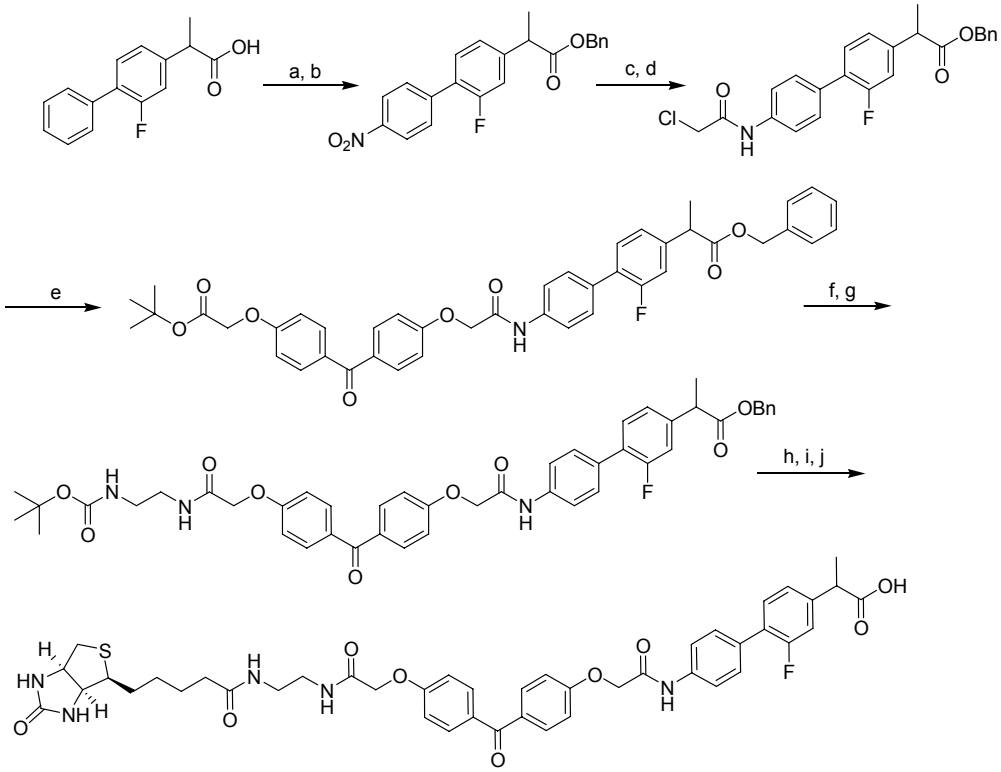


2-(2-fluoro-4'-(2-(4-(4-(2-oxo-2-(2-(5-((3a*R*,4*R*,6a*S*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)ethylamino)ethoxy)benzoyl)phenoxy)acetamido)biphenyl-4-yl)propanoic acid (Flurbi-BpB): To a stirred solution of benzyl 2-(2-fluoro-4'-(2-(4-(4-(2-oxo-2-(2-(5-(2-oxohexahydro-1*H*thieno[3,4-*d*]imidazol-4yl)pentanamido)ethylamino)ethoxy) benzoyl)phenoxy)acetamido)biphenyl-4yl)propanoate, (22 mg, 0.02 mmol) in MeOH (5 mL) was added 10% Pd-C (50 % w/w, 11 mg) and the resulting solution was kept for hydrogenation at baloon pressure at ambient temperature for 18 h. Reaction was filtered through Celite bed and the Celite bed was washed several times with warm MeOH. The combined organic extract was concentrated to yield the crude product. The crude compound was purified by flash column chromatography (CH_2Cl_2:Methanol, 95:5) to obtain the title compound as a lemon colored solid. (12 mg, 60 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.76-

7.58 (m, 8H), 7.45 (brs, 1H), 7.41 (brs, 1H), 7.34-7.26 (m, 2H), 7.24-7.18 (m, 2H), 7.10-6.80 (m, 5H), 4.70 (s, 2H), 4.48 (s, 2H), 4.40-4.33 (m, 1H), 4.22-4.16 (m, 1H), 4.05 (q, J = 7.20 Hz, 1H), 3.38-3.22 (m, 4H), 3.08-3.01 (m, 1H), 2.78 (dd, J = 12.90 Hz, J = 4.80 Hz, 1H), 2.66 (d, J = 12.90 Hz, 1H), 2.10 (t, J = 7.20 Hz, 2H), 1.66-1.49 (m, 4H), 1.47 (d, J = 7.20 Hz, 3H), 1.37-1.28 (m, 2H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 192.1, 176.0, 174.8, 166.2, 162.4, 158.5, 158.4, 158.1, 156.6, 139.6, 139.5, 134.0, 133.7, 133.4, 133.4, 132.8, 131.0, 124.9, 121.8, 121.0, 115.8, 115.6, 68.3, 65.3, 64.8, 63.3, 61.0, 57.0, 41.5, 40.8, 40.1, 37.0, 36.1, 30.3, 29.8, 27.2, 27.1, 15.1 ppm. MS (ESI): m/z = 862.4 $(M+Na)^+$, 838.3 $(M–H)^+$.



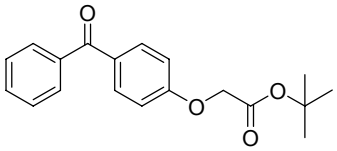
Scheme 1. Preparation of Flurbiprofen-benzophenone-biotin (Flurb-BpB).



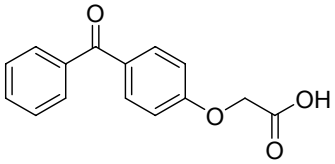
Reagents and conditions: (a) 70% HNO_3, room temp. 48 h.; (b) BnBr, K_2CO_3, DMF, room temp. 3 h, 32 %.; (c) $SnCl_2$, dry EtOH, reflux, 6 h, 57 %.; (d) Chloroacetyl chloride, Et_3N, CH_2Cl_2, 0 °C to room temp, 3 h, 95 %; (e)tert-butyl 2-(4-(4-hydroxybenzoyl)phenoxy)acetate, K_2CO_3, acetone, 60-70 °C, 12 h, 57 %.; (f) 20 % TFA in CH_2Cl_2, room temp., 6 h, 92%.; (g) EDCI, HOBt, *N*-boc ethylene diamine, CH_2Cl_2, room temp, 10 h, 54 %.; (h) 16 % HCl in dioxane, 0.5 h, room temp., 88 %.; (i) D-biotin, EDCI, HOBt, Et_3N, DMF, room temp., 22 %.; (j) 10 % Pd-C, MeOH, H_2, room temp., 60 %.

Preparation of benzophenone-biotin (BpB)

Tert-butyl 2-(4-benzoylphenoxy)acetate: Anhydrous K_2CO_3 (980 mg, 7.56 mmol) was added to a stirred solution of 4-hydroxybenzophenone (500 mg, 2.52 mmol) in acetone (6 mL) and stirred at ambient temperature for 30 min tert-butyl chloroacetate (4.31 mL, 5.04 mmol) was added to it and heated to 60-70 °C for 12 h. Reaction mixture was cooled to room temperature and filtered. The residue was washed with acetone (3×). Combined organic extract was evaporated in vacuo and purified by crystallization to yield the title compound as a colorless solid (750 mg, 97 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.69 (td, J = 7.00 Hz, J = 2.00 Hz, 2H), 7.61 (td, J = 7.00 Hz, J = 1.60 Hz, 2H), 7.50 (tt, J = 7.00 Hz, J = 1.60 Hz, 1H), 7.37 (tt, J = 7.00 Hz, J = 1.60 Hz, 2H), 6.89 (td, J = 7.00 Hz, J = 2.00 Hz, 2H), 4.58 (s, 2H), 1.39 (s, 9H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 199.5, 172.2, 164.1, 141.9, 135.4, 135.0, 134.2, 133.1, 132.3, 118.2, 66.6, 29.1 ppm.

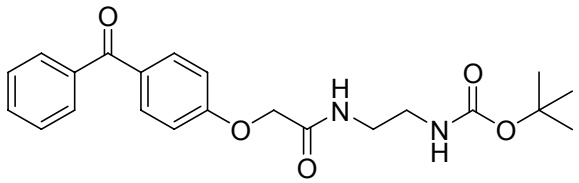


Preparation of 2-(4-benzoylphenoxy)acetic acid: Trifluoroacetic acid (0.4 mL) was added to a stirred solution of tert-butyl 2-(4-benzoylphenoxy)acetate (500 mg, 1.60 mmol) in CH_2Cl_2 (2 mL) at room temperature and stirred for 5 h. Reaction was monitored by TLC. The reaction mixture was evaporated in vacuuo and purified by crystallization to afford the desired product as colorless solid (375 mg, 91 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.75 (td, J = 6.90 Hz, J = 2.00 Hz, 2H), 7.67 (td, J = 6.90 Hz, J = 1.60 Hz, 2H), 7.50 (tt, J = 6.90 Hz, J = 1.60 Hz, 1H), 7.40 (tt, J = 6.90 Hz, J = 1.60 Hz, 2H), 6.92 (td, J = 6.90 Hz, J = 2.00 Hz, 2H), 4.62 (s, 2H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 200.0, 174.2, 165.3, 142.8, 136.4, 136.0, 134.7, 133.6, 132.1, 118.1, 68.8 ppm.



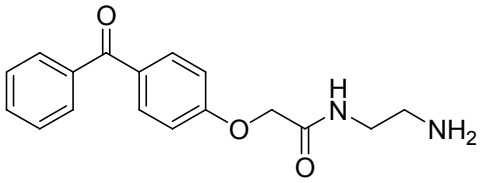
Preparation of tert-butyl 2-(2-(4-benzoylphenoxy)acetamido)ethylcarbamate:

To a stirred solution of 2-(4-benzoylphenoxy)acetic acid (200 mg, 0.78 mmol) in anhydrous CH_2Cl_2 (5 mL), was added triethylamine (0.109 mL, 0.78 mmol) and stirred at ambient temperature for 10 min Ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (180 mg, 0.94 mmol) and *N*-hydroxybenzotriazole hydrate (127 mg, 0.94 mmol) were added to it and the resulting solution was stirred at ambient temperature for 30 min until it became clear. A solution *N*-Boc-ethylenediamine (150 mg, 0.94 mmol) was added to the reaction mixture followed by triethylamine (0.130 mL, 0.94 mmol) and stirred at ambient temperature for 12 h. The reaction mixture was diluted with CH_2Cl_2 (100 mL), washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated in vacuo to yield the crude product. The crude product was purified by flash column chromatography (ethyl acetate:hexane, 95:5) to obtain the title compound as colorless solid (208 mg, 69 %). ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.78 (td, J = 6.70 Hz, J = 2.30 Hz, 2H), 7.68 (dd, J = 7.00 Hz, 2H), 7.50 (tt, J = 7.40 Hz, J = 1.70 Hz, 1H), 7.42 (tt, J = 7.30 Hz, J = 1.40 Hz, 2H), 6.97 (td, J = 8.80 Hz, J = 2.40 Hz, 2H), 4.94 (brs, 1H), 4.52 (s, 2H), 3.41 (q, J = 5.50 Hz, 2H), 3.31-3.21 (m, 2H), 1.34 (s, 9H) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 195.5, 168.2, 160.6, 157.0, 141.6, 138.0, 132.7, 132.2, 131.5, 129.8, 128.3, 114.3, 67.2, 40.7, 40.1, 28.4 ppm.

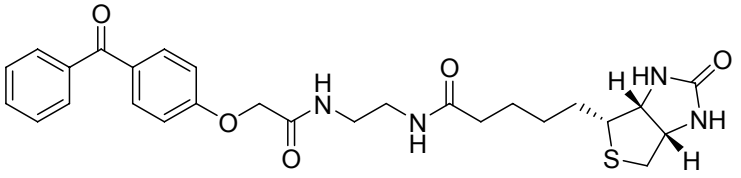


Preparation of *N*-(2-aminoethyl)-2-(4-benzoylphenoxy)acetamide:

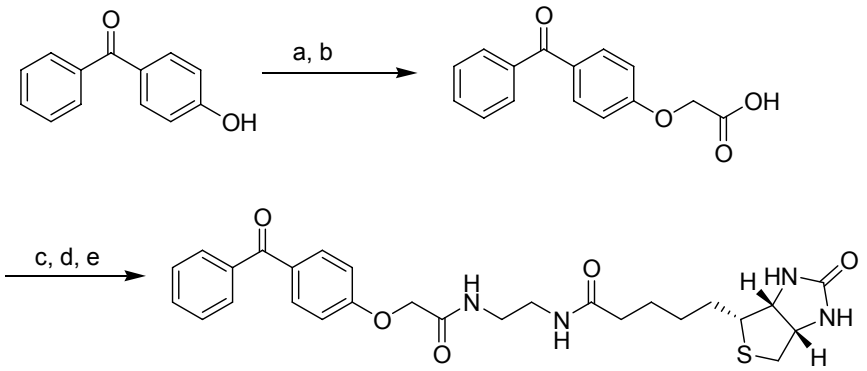
A solution of tert-butyl 2-(2-(4-benzoylphenoxy)acetamido)ethylcarbamate (158 mg, 0.39 mmol) in 18 % HCl in dioxane was stirred at room temperature for 30 min The reaction mixture was evaporated in vacuo and lyophilized to yield the crude amine as hydrochloride which was used in the next step without further purification. MS (ESI): m/z = 321.2 $(M+Na)^+$



***N*-(2-(2-(4-benzoylphenoxy)acetamido)ethyl)-5-((3a*R*,4*R*,6a*S*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide (BpB):** To a stirred solution of D-biotin (78 mg, 0.32 mmol) in anhydrous DMF (2 mL), was added triethylamine (0.033 mL, 0.32 mmol) and stirred at ambient temperature for 10 min Ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (77 mg, 0.4 mmol) and *N*-hydroxybenzotriazole hydrate (54 mg, 0.4 mmol) were added to it and the resulting solution was stirred at ambient temperature for 30 min until it became clear. A solution of *N*-(2-aminoethyl)-2-(4-benzoylphenoxy)acetamide (128 mg, 0.4 mmol) in DMF (1 mL) was added to the reaction mixture followed by triethylamine (0.041 mL, 0.4 mmol) and stirred at ambient temperature for 18 h. The reaction mixture was diluted with $CHCl_3$ (100 mL), washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated in vacuo to yield the crude product. The crude product was purified by flash column chromatography (CH_2Cl_2:MeOH, 95:5) to afford the title compound as brownish solid (42 mg, 20 %). ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 8.07 (brs, 1H), 7.81 (d, J = 8.80 Hz, 2H), 7.70 (dd, J = 8.20 Hz, J = 1.00 Hz, 2H), 7.55 (t, J = 7.40 Hz, 1H), 7.49 (t, J = 8.80 Hz, 2H), 7.08 (d, J = 8.80 Hz, 2H), 4.58 (s, 2H), 4.48-4.44 (m, 1H), 4.28-4.22 (m, 1H), 3.42-3.28 (m, 4H), 3.13-3.06 (m, 1H), 2.85 (dd, J = 12.80 Hz, J = 4.90 Hz, 1H), 2.65 (d, J = 12.80 Hz, 1H), 2.17 (t, J = 7.40 Hz, 2H), 1.70-1.48 (m, 4H), 1.43-1.33 (m, 2H) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 197.9, 176.7, 170.8, 162.6, 139.2, 134.1, 133.8, 132.5, 131.1, 129.8, 127.6, 115.9, 68.4, 63.4, 61.6, 57.0, 41.6, 41.0, 40.9, 37.0, 29.8, 29.5, 26.9 ppm. MS (ESI): *m/z* = 547.3 $(M+Na)^+$

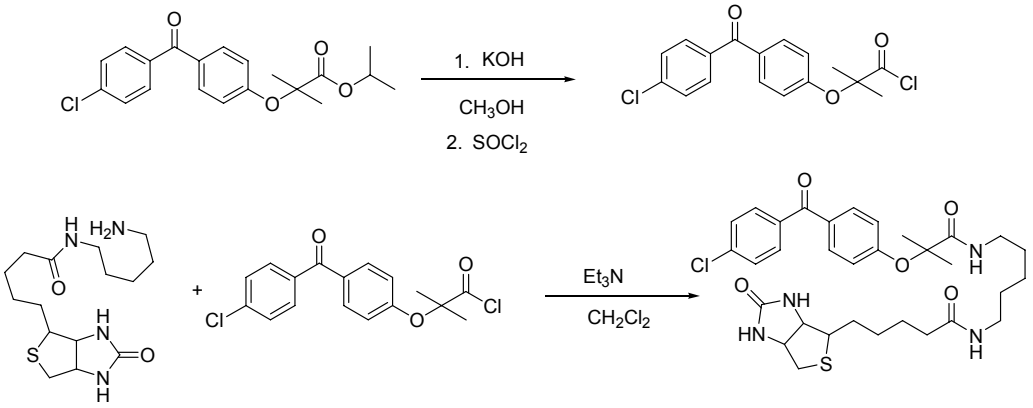


Scheme 2. Preparation of benzophenone-biotin (BpB).

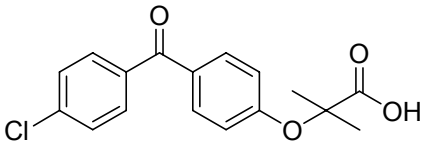


Reagents and conditions: (a) t-butyl chloroacetate, K_2CO_3, acetone, 60-70 °C,.12 h, 97 %.; (b) 20 % TFA in DCM, room temp., 5 h, 91%.; (c) EDCI, HOBt, *N*-boc ethylene diamine, DCM, room temp, 12 h, 69 %.; (d) 16 % HCl in dioxane, 0.5 h, room temp.; (e) D-biotin, EDCI, HOBt, Et_3N, DMF, room temp. 18 h, 22 %.

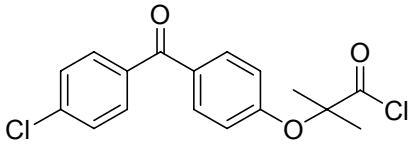
Preparation of Fenofibrate-biotin (Fen-B; Scheme 3).



Preparation of 2-(4-(4-chlorobenzoyl)phenoxy)-2-methylpropanoic acid (fenofibric acid): To a solution of Fenofibrate (5.54 mmol, 2.0 g) dissolved in 110 mL MeOH, was added aq. 1N KOH solution (55.4 mmol, 55.4 mL), and the resulting mixture was refluxed overnight at 70 ºC. The reaction mixture was then cooled in an ice-bath and acidified with conc. HCl. The solid was collected by suction filtered, and dried under high vacuum to give fenofibric acid as a white powder; yield 1.76 g (96%). ^{1}H-NMR: δ (300 MHz, DMSO-d_6): δ 13.20 (s, 1H), 7.73 (d, 4H, *J* = 8.50 Hz), 7.61 (d, 2H, *J* = 8.50 Hz), 6.93 (d,2H, *J* = 8.78 Hz), 1.59 (s, 6H); MS: m/z (ESI) m/z 319.04 (M^+ +1).



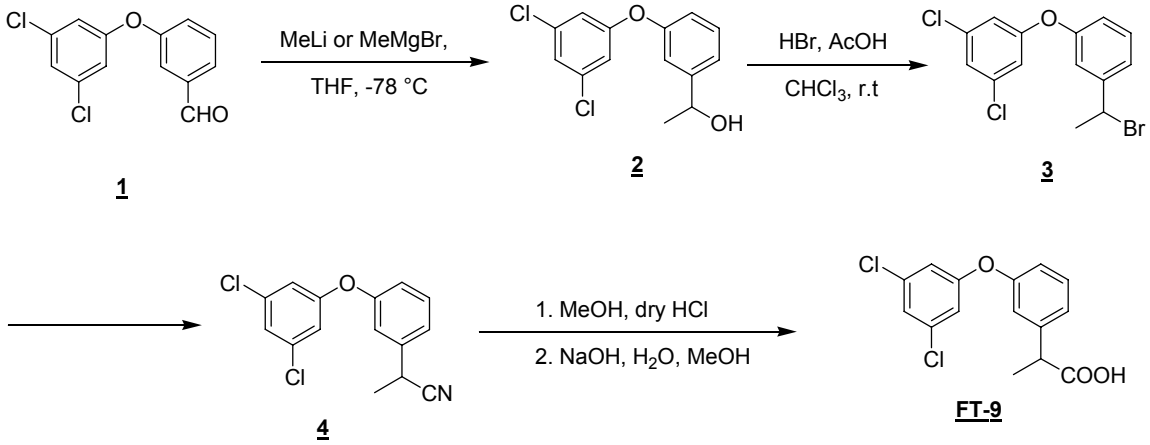
Preparation of 2-(4-(4-chlorobenzoyl)phenoxy)-2-methylpropanoyl chloride (fenofibrate acid chloride): Fenofibric acid (0.63 mmol, 0.2 g) was dissolved in 10 mL of thionyl chloride under nitrogen and the mixture was stirred at r.t for 2h. The progress of reaction was monitored by thin layer silica gel chromatography. The excess SOCl2 was evaporated under reduced pressure to afford fenofibric acid chloride as a white solid in essentially quantitative yield. ^{1}H NMR ($CDCl_3$): δ 7.77 (d, 2H, *J* = 8.84 Hz), 7.71 (d, 2H, *J* = 8.55 Hz), 7.45 (d, 2H, *J* = 8.55 Hz), 6.92 (d, 2H, *J* = 8.84 Hz), 1.74 (s, 6H).



Preparation of N-(5-(2-(4-(4-chlorobenzoyl)phenoxy)-2-methylpropanamido)pentyl)-5-(2-oxo-hexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (Fenofibrate-biotin; Fen-B): Biotin (0.085 mmol, 28 mg) and fenofibrate acid chloride (0.170 mmol, 57 mg) were dissolved in 0.7 mL of anhydrous DCM under an atmosphere of nitrogen. To this solution, triethylamine (0.682 mmol, 69 mg) was added dropwise under nitrogen and the resulting mixture was stirred overnight at r.t. Upon completion of the reaction (TLC monitoring), sat'd aqueous NaHCO3 and water were added and the aqueous phase was extracted with DCM (x3). The combined extracts were sequentially washed with water, and brine, and finally dried over MgSO4. After evaporation of the DCM, a gummy residue was obtained. Purification by silica-gel column chromatography using 10% MeOH/DCM as eluent furnished the product as an off-white gummy solid; yield 54 mg (59.7%). ^{1}H-NMR ($CDCl_3$): δ 7.74 (d, 2H, J = 8.77 Hz), 7.71 (d, 2H, J = 8.53 Hz), 7.46 (d, 2H, J = 8.53 Hz), 6.96 (d, 2H,

J = 8.77 Hz), 6.64 (t, 1H, J = 5.90 Hz), 6.47 (s, 1H), 6.31 (t, 1H, J = 5.58 Hz), 5.61 (s, 1H), 4.50 (m, 1H), 4.29 (m, 1H), 3.27 (m, 2H), 3.14 (m, 3H), 2.89 (dd, 1H, J = 12.80 Hz, J = 4.80 Hz), 2.72 (d, 1H, J = 12.80), 2.18 (t, 2H, J = 7.41 Hz), 1.71-1.42 (m, 16H), 1.24 (m, 2H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 194.80, 174.46, 173.66, 164.58, 159.01, 139.157, 136.41, 132.38, 132.25, 131.88, 129.07, 128.98, 119.78, 118.10, 62.32, 60.75, 55.8, 40.89, 39.68, 39.55, 36.15, 29.51, 29.45, 28.39, 28, 32, 25.90, 25.71, 25.66. HRMS calc. for $C_{32}H_{41}ClN_4O_5S$ (M+H) 629.2559, found 629.2537;

Preparation of 2-(3-(3,5-dichlorophenoxy)phenyl)propanoic acid (FT-9; Scheme 4)



1-(3-(3,5-dichlorophenoxy)phenyl)ethanol (2). To a solution of the aldehyde 1 (2.0 g, 7.49 mmol) in 100 mL of THF, cooled to -78°C, was added dropwise a 3M solution of methyl magnesium bromide (7.49 mL, 22.46 mmol) in diethyl ether under a static atmosphere of nitrogen. After stirring for 30 min, the reaction was quenched with 10% ammonium chloride solution and the cold bath was removed to allow the reaction mixture to come to r.t. The aqueous phase was extracted with ether (x3), and the extracts were combined and washed with water and brine, and dried (MgSO4). After concentration under reduced pressure, a crude oil was obtained that was purified by silica gel chromatography (eluent 20% EtOAc/hexane); yield 1.2 g, 56.6%. ^{1}H-NMR ($CDCl_3$) δ 7.36 (t, 1H, *J* = 7.8 Hz), 7.31-6.81 (m, 6H), 4.91(m, 1H), 1.50 (d, 3H, *J* = 6.4 Hz). MS (ESI) *m/z* 283.05 (M+1, 100%)

1-(3-(1-bromoethyl)phenoxy)-3,5-dichlorobenzene (3). To a solution of the alcohol **2** (5.65 g, 19.95 mmol) in chloroform (150 mL) was added HBr (5.0 mL of a 45%, w/v in acetic acid) dropwise via syringe at r.t. and the resulting solution was stirred for 1-2 hrs while monitoring the reaction by TLC. Upon completion of the reaction, the reaction mixture was washed first with sat'd $NaHCO_3$ and then with brine. After drying the organic phase over $MgSO_4$ and concentration, the crude was directly taken to the next step without purification. ^{1}H-NMR ($CDCl_3$) δ 7.35 (t, 1H, *J* = 7.8 Hz), 7.25 (b dt, 1H), 7.12 (t, 1H, *J* =1.9 Hz), 7.09 (t, 1H, *J* = 1.2 Hz), 6.93 (m, 1H), 6.88 (d, 2H, *J* = 1.2 Hz), 5.16 (q, 1H, *J* =6.9 Hz), 2.03 (d, 3H, *J* = 6.9 Hz). MS (ESI) *m/z* 346.97 (M+1, 100%)

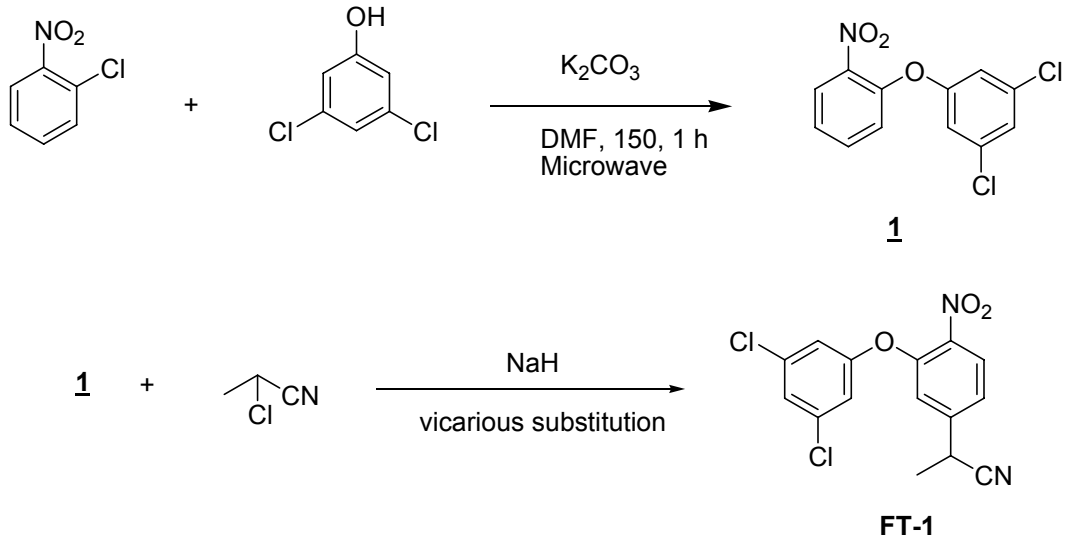
2-(3-(3,5-dichlorophenoxy)phenyl)propanenitrile (4). The crude bromide **3** (6.6 g, 19.07 mmol) was dissolved in 30 mL of dry DMF. To this solution, sodium cyanide (4.67 g, 95.0 mmol) was added and the resulting suspension was stirred at r.t. overnight in the dark at which point the TLC showed completion of reaction. Water was added and the aqueous phase was extracted with ether (x3). The ether extracts were combined and washed sequentially with water and brine. The partially dried extract was then dried over $MgSO_4$ and concentrated to give an oil that was purified over silica gel to give the pure cyanide as a colorless oil in essentially quantitative yield. ^{1}H-NMR ($CDCl_3$) δ 7.40 (t, 1H, *J* = 7.9 Hz), 7.21 (bd, 1H, *J* = 7.7 Hz), 7.10 (t, 1H, *J* =1.5 Hz), 7.04 (bt, 1H, *J* = 2.1 Hz), 6.97 (dm, 1H, *J* = 8.1 Hz), 6.87 (d, 2H, *J* = 1.9 Hz), 3.90 (q, 1H, *J* =7.2 Hz), 1.65 (d, 3H, *J* = 7.2 Hz). MS (ESI) *m/z* 292.08 (M+1, 100%).

2-(3-(3,5-dichlorophenoxy)phenyl)propanoic acid (FT-9). The cyanide **4** (5.5 g, 18.83 mmol) was dissolved in methanol (300 mL) and the solution was cooled to 0°C. Dry HCl gas was bubbled to saturation through this solution. This acidic mixture was left stirred overnight at r.t. The MeOH was evaporated to 1/3 of its original volume. Water was added and the aqueous phase was extracted with ether (x3). The ether extracts were combined and washed sequentially with water and brine. The extract was finally dried over $MgSO_4$, filtered, and

concentrated to give an oily residue that was purified over silica gel to furnish the methyl ester as a colorless oil in essentially quantitative yield. Some runs had to be purified by silica gel chromatography (elution with 15% EtOAc/hexane).

A 2N-NaOH solution (50 mL) was added to the ester (6.11 g, 18.79 mmol) dissolved in MeOH (100 mL) and the resulting mixture was stirred at r.t for 12 h. After the completion of reaction, the MeOH was evaporated under reduced pressure and the aqueous phase was extracted three times with EtOAc. The extracts were combined, washed with water and brine as usual, and dried ($MgSO_4$). Filtration and evaporation of EtOAc gave an oily residue which solidified when kept in freezer for several hours. ^{1}H-NMR ($CDCl_3$) δ 7.34 (t, 1H, *J* = 7.9 Hz), 7.15 (bt, 1H, *J* = 7.5, 1.5 Hz), 7.08 (t, 1H, *J* =1.5 Hz), 7.03 (bt, 1H, *J* = 1.5 Hz), 6.92 (dm, 1H, *J* = 8.0 Hz), 6.87 (d, 2H, *J* = 1.5 Hz), 4.12 (q, 1H, *J* =7.0 Hz), 1.53 (d, 3H, *J* = 7.0 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 180.71, 159.05, 156.11, 142.48, 136.03, 13.66, 124.31, 123.65, 119.60, 118.91, 117.33, 45.54, 18.45. HRMS: calc. for $C_{15}H_{11}Cl_2O_3$ (M-H) 309.0091, found 309.0019.

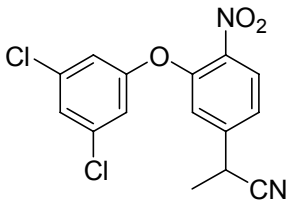
Preparation of 2-(3-(3,5-dichlorophenoxy)-4-nitrophenyl)propanenitrile (FT-1; Scheme 5)



1,3-dichloro-5-(2-nitrophenoxy)benzene (1). A 20-ml microwave Carious tube was charged with 3,5-dichlorophenol (1.1 g, 6.70 mmol), K_2CO_3 (1.16 g, 8.38 mmol), and 2-Cl-nitrobenzene (0.88 g, 5.59 mmol). The tube was sealed, thoroughly mixed by shaking, and irradiated at 150 °C for 1h in a Biotage microwave instrument. After the tube was cooled, n-

butanol (6 mL) was added to the contents of the Carius tube and the mixture was agitated vigorously with metallic spatula. Next, water was added and the contents of the tube were transferred to an Erlenmeyer flask. This brown mixture was acidified with 2N-HCl and stirred for at least one hour. Extraction of the mixture with n-butanol (X3) was followed by combining the extracts and drying ($MgSO_4$). The organic phase was filtered and evaporated with a rotary evaporator which resulted in a dark colored mass. The pure product **1** was purified on silica gel by eluting with 10% EtOAc/hexane to give the biaryl ether **1** as a gum; yield 1.30 g, 82%. ^{1}H-NMR ($CDCl_3$) δ 8.05 (d, 1H, *J* = 8.5 Hz), 7.37 (dd, 1H, *J* = 2.5, 1.9 Hz), 7.17 (t, 1H, *J* = 1.8 Hz), 7.12 (d, 1H, *J* = 1.8 Hz), 6.89 (d, 2H, *J* = 1.9 Hz), 3.97 (q, 1H, *J* = 7.3 Hz), 1.66 (d, 3H, *J* = 7.3 Hz).

2-(3-(3,5-dichlorophenoxy)-4-nitrophenyl)propanenitrile (FT-1). A 100-mL round-bottom flask was charged with biphenyl ether **1** (3.28 g, 11.55 mmol), 2-chloropropionitrile (1.02 mL, 11.55 mmol), and 50 mL of dry DMF under nitrogen. In a separate container, potassium t-butoxide (2.59 g, 23.1 mmol) was dissolved in 50mL of DMF at 0°C. Next, the contents of the first flask were transferred dropwise to the second via cannula. The resulting dark colored mixture was stirred at 0 °C for 30 min following which the reaction mixture was allowed to warm to r.t over 1h. After stirring for a further 30 min at r.t, the reaction was quenched with 10% HCl. Standard work-up with ether extraction and drying of ether layers with $MgSO_4$ afforded a colored residue which was chromatographed over silica gel (elution with 15% EtOAc/hexane) to afford **FT-1** as a thick oil. ^{1}H-NMR ($CDCl_3$) δ 8.02 (dd, 1H, *J* = 8.2, 1.6 Hz), 7.62 (dt, 1H, *J* = 8.2, 1.6 Hz), 7.34 (dt, 1H, *J* = 8.2, 1.6 Hz), 7.15 (t, 1H, *J* = 1.6 Hz), 7.13 (dd, 1H, *J* = 8.2, 1.6 Hz), 6.89 (d, 2H, *J* = 1.6 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 149.56, 144.80, 136.53, 127.50, 125.22, 123.79, 121.09, 120.16, 118.47, 117. 33, 115.58, 31.47, 21.45. HRMS: calc. for $C_{15}H_9Cl_2N_2O_3$ (M-H), 334.9996, found 334.9977.



The compounds **X-34**[1] and **AOI987**[2] were synthesized according to published procedures. The γ-secretase inhibitor **LY-411,575** was first synthesized according to a published patent[3] and larger quantities were made using an improved synthetic strategy[4].

References:

1. Sellarajah, S. et al. Synthesis of Analogues of Congo Red and Evaluation of Their Anti-Prion Activity. J. Med. Chem. 47, 5515-5534 (2004).
2. Hintersteiner, M. et al. In vivo detection of amyloid-[beta] deposits by near-infrared imaging using an oxazine-derivative probe. Nat Biotech 23, 577-583 (2005).
3. Wu, J. et al. Preparation of cycloalkyl, lactam, lactone and related compounds for inhibiting b-amyloid peptide release and/or its synthesis. PCT Int Appl WO9828268 A2, 889 (1998).
4. Fauq, A. H., Simpson, K., Maharvi, G. M., Golde, T. & Das, P. A multigram chemical synthesis of the [gamma]-secretase inhibitor LY411575 and its diastereoisomers. Bioorganic & Medicinal Chemistry Letters 17, 6392-6395 (2007).