Modulation and Characterization of Alzheimer's Disease Associated γ-Secretase

Vom Fachbereich Chemie
der Technischen Universität Darmstadt

Zur Erlangung des akademischen Grades eines

Doctor rerum naturalium (Dr. rer. nat.)

genehmigte
Dissertation

vorgelegt von

Rajeshwar Narlawar

aus Nanded (Indien)

सर्वेऽत्र सुखिनः सन्तु
सर्वे सन्तु निरामयाः।
सर्वे भद्राणि पश्यन्तु
मा कश्चिद् दुःखमाप्नुयात् ॥

May every being be happy,
May every being be free from all diseases,
May every being feel and appear blissful,
May there not be trace of sorrow with any being.

Kathopanishada 2:6:19 1400 B.C.

To my loving parents and my wife

Acknowledgement

First of all I would like to submit my sincere thanks to my thesis guru Prof. Dr. Boris Schmidt for giving me opportunity to pursue my Ph.D. in this elite institute and exposing me to the fascinating world of drug discovery and medicinal chemistry. I am indebted to him for giving me superb guidance, constant encouragement, crystal clear and quality discussions and scientific freedom to implement my ideas. I am ever grateful for getting the opportunity to learn from the best. Without his ideas, remarks and endless interest this work could not have been carried out.

I feel proud and lucky to get an opportunity to work in the company of many wonderful people during the tenure of my doctoral research at the Institute of Organic Chemistry, Darmstadt University of Technology. I would like to express my sincere thanks to my colleagues and lab fellows Ms. Stefanie Baumann, Mr. Mahesh Shioorkar, Mr. Gregor Larbig, Mr. Hannes Braun, Mr. Daniel Kieser, Mr. Ali Taghavi, Mr. Luiz Everson da Silva, and Mrs. Birgit Keenan, for their co-operation and help throughout my doctoral studies.

I owe my warmest thank to Sumaira Umbreen for having been so helpful and for her kind support everyway during the span of my doctoral research. Her advice and valuable discussions in my hard times enabled me to solve many problems, personal and academic.

I would like to express my sincere gratitude to all who have supported, helped, and inspired me during my Ph.D. years at the Institute of Organic Chemistry, TUD.

I wish to thank Dr. Christian Haass, Dr. Karlheinz Baumann, Dr. Harald Steiner, Dr. Marcus Pickhardt, Dr. E. Mandelkow, Dr. Sascha Weggen, Dr. Bharat Aggarwal, Dr. Peter-Michael Kloetzel, Dr. Ulrike Kuckelkorn, Dr. Thomas Kukar, Dr. Wei Wang for their collaboration during this study. My special thanks to Blanca I. Pérez Revaulta for the collaboration and fruitful discussions.

I thank to my amicable MSc mates, Santosh, Sunil, Vishnu, Munfis, Kavita, Nirmala for their friendship over the years and for their constant encouragement to aim higher. I express my special thanks to Prof. Dr. M. S. Shingare for his help, encouragement and support during my early scientific phase.

I owe my deepest gratitude to my parents, my brothers, Shrihari, Dr. Shivakumar and Dipak for their love, endless support and encouragement during these years.

I would like to add a very special note of appreciation to my loving wife, Shilpa and my little princess, Riddhi, for their support, understanding, enduring patience, putting up with my seemingly endless weeks of work, hours in front of the computer, the inconvenience and lost weekends.

Veröffentlichungen

Publikationen:

1. Boris Schmidt, Hannes A. Braun, Rajeshwar Narlawar, Drug Development and PET-diagnostics for Alzheimer's Disease, *Current Medicinal Chemistry*, **2005**, *12*, 1677-1695.

2. Boris Schmidt, Stefanie Baumann, Rajeshwar Narlawar, Hannes A. Braun, Gregor Larbig, Modulators and Inhibitors of β- and γ-Secretase. *Neurodegenerative Diseases*, **2006**, *03*, 290-297.

3. Rajeshwar Narlawar, Blanca Perez Revauelta, Christian Haass, Harald Steiner, Boris Schmidt, Karlheinz Baumann, The Scaffold of the COX-2 Inhibitor Carprofen Provides Alzheimer γ-Secretase Modulators, *Journal of Medicinal Chemistry*, **2006**, *49*, 7588-7591.

4. Rajeshwar Narlawar, Blanca Perez Revauelta, Karlheinz Baumann, Robert Schubenel, Christian Haass, Harald Steiner, Boris Schmidt, *N*-Substituted Carbazolyloxyacetic Acids Modulate Alzheimer Associated γ-Secretase, *Bioorganic and Medicinal Chemistry Letters*, **2007**, *17*, 176-182.

5. Rajeshwar Narlawar, Karlheinz Baumann, Robert Schubenel, Boris Schmidt, Curcumin Derivatives Inhibit or Modulate β-Amyloid Precursor Protein Metabolism, *Neurodegenerative Diseases*, **2007**, in print.

6. Rajeshwar Narlawar, Marcus Pickhardt, Stefanie Leuchtenberger, Sascha Weggen, Karlheinz Baumann, E. Mandelkow, Boris Schmidt, Curcumin Derived Pyrazoles: Dirty Tools or Swiss knives for Alzheimer´s Disease, manuscript submitted to *ChemMedChem*.

7. Rajeshwar Narlawar, Karlheinz Baumann, Christian Czech, Boris Schmidt, The LXR-Agonist TO-901317 can be Tuned from Inverse to Normal Modulation of γ-Secretase by Addition of a Carboxylic Acid and a Lipophilic Anchor, manuscript submitted to *Bioorganic and Medicinal Chemistry Letters*.

Abstract

Alzheimer's disease (AD) is a devastating neurodegenerative disorder that causes the most common form of dementia, affecting approximately 5% of the population over the age of 65 years in Europe. Short-term memory impairment, disorientation, aphasia, and a general cognitive decline are common symptoms early in disease development. According to the World Health Organization, an estimated 37 million people worldwide currently have dementia; AD affects about 18 million of them. In spite of tremendous research efforts, there is no causal therapy for AD. Histopathological hallmarks of AD are the extracellular plaques consisting of the amyloid β-peptide (Aβ) and neurofibrillary tangles which are found in the brains of the AD patients. Aβ is heterogeneously produced by the sequential cleavages of amyloid precursor protein (APP) by the two aspartic proteases: β- and γ-secretase. Intramembrane cleavage by γ-secretase occurs with little sequence specificity, resulting in Aβ fragments of different length, predominantly $A\beta_{40}$, $A\beta_{42}$, and some $A\beta_{38}$. $A\beta_{42}$ being the most aggregatory, and is believed to trigger the amyloid cascade, a pathological series of neurotoxic events, which eventually leads to neurodegeneration and finally AD. γ-Secretase has been an attractive target in many ways for AD therapeutics since it catalyzes the final step in the release of Aβ.

The present work describes the design, synthesis and biological evaluation of γ-secretase modulators, affinity probes and photoaffinity labels. Epidemiological studies have indicated a close association between prolonged use of nonsteroidal anti-inflammaroy drugs (NSAIDs), and reduced risk for AD. Therefore, cyclooxygenase (COX) inhibitors such as flurbiprofen, sulindac sulfoxide and meclofenamic acid were converted into amides and esters and anticipated that the modification of acid functionality would reduce the COX inhibitory activity while increasing the γ-secretase modulatory activity. However, the conversion of the acid moiety of COX inhibitors into their amides or esters resulted either in inverse modulation or inhibition. Further efforts were then focussed on carprofen, a COX-2 inhibitor which is approved for the use in dogs and cows. *N*-substitution of carprofen resulted in potent modulators of γ-secretase, and the compounds displayed little or no effect on γ-secretase cleavage at the ε-site. Knowing about the relevance of *N*-substitution of carprofen and the necessity of the free acid functionality for γ-secretase modulation, a series of *N*-substituted carbazolyloxy acetic acids was synthesized. As anticipated, they turned out to be effective modulators of γ-secretase and displayed little or no effect on γ-secretase cleavage at the ε-site. An analogous derivative of the LXR agonist TO-901317 was synthesized, but the hexafluorocarbinol moiety was replaced by an oxyacetic acid in order to transform it into a modulator of γ-secretase. As expected, the introduction of the acid moiety changed the mode of action from an inverse modulation to normal modulation of γ-secretase. Curcumin has multiple targets in AD. A series of NSAIDs derived affinity labels was synthesized in order to identify and characterize the binding site by immunoprecipitation assay. Flurbiprofen derived photoaffinity label captured active γ-secretase complex in a dose dependant manner, but others either failed to provide a binding partner or resulted in unspecific binding. Flurbiprofen and DAPT derived photoaffnity labels were synthesized in order to investigate the binding site *via* covalent bond formation with the active site on the γ-secretase complex. The photaffnity labelling experiment carried out with a flurbiprofen derived photoaffinity probe revealed that it binds to the *C*-terminal fragment of presenilin (PS). Whereas, DAPT derived photoaffinity label displayed very weak binding to PS, the active site of γ-secretase.

Zusamenfassung

Die Alzheimer-Demenz (AD) ist eine verheerende, neurodegenerative Funktionsstörung, die die häufigste Form der Demenz darstellt und etwa 5% der Bevölkerung über 65 in Europa befällt. Bekannte Symptome in der frühen Krankheitsphase sind die Beeinträchtigung des Kurzzeitgedächtnisses, Orientierungslosigkeit, Aphemie sowie eine generelle Verschlechterung der Wahrnehmung. Laut Schätzungen der Weltgesundheitsbehörde leiden 37 Millionen Menschen weltweit an einer Demenz: 18 Millionen davon an AD. Trotz gewaltiger Forschungsbemühungen gibt es keine Kausaltherapie für die AD. Histopathologische Kennzeichen sind die extrazellulären Plaques, die aus amyloiden β-Peptiden (Aβ) bestehen sowie die Neurofibrillären Bündel, die in den Gehirnen von Alzheimerpatienten gefunden wurden. Aβ wird durch die sequentielle Spaltung des amyloiden Vorläuferproteins (APP) durch zwei Aspartylproteasen, β- and γ-Sekretase, erzeugt. Eine Intramembranspaltung durch die γ-Sekretase erfolgt mit geringer Sequenzspezifität und resultiert in verschiedenen Aβ-Fragmenten unterschiedlicher Länge, vorwiegend Aβ_{40}, Aβ_{42} und etwas Aβ_{38}. Aβ_{42} neigt am stärksten zur Aggregation und man nimmt an, dass es die Amyloidkaskade, eine pathologische Abfolge an neurotoxischen Abläufen, die zur Neurodegeneration und schließlich zur AD führt, auslöst. Die γ-Sekretase stellt durch die Freisetzung von Aβ ein attraktives Zielmolekül für eine Kausaltherapie von AD dar.

Die vorliegende Arbeit beschreibt das Design, die Synthese und die biologische Wirkuntersuchung von γ-Sekretasemodulatoren, Affinitätsproben und Photoaffinitätsmarkern. Epidemiologische Studien implizieren einen engen Zusammenhang zwischen einem längerfristigen Einsatz von nichtsteroidalen Antirheumatika (NSAIDs) und einem verminderten Risiko für AD. Folglich wurden Cyclooxygenaseinhibitoren (COX-Inhibitoren) wie Flurbiprofen, Sulindac-Sulfoxid und Meclofenaminsäure in Amide und Ester umgewandelt und postuliert, dass die Modifizierung der Säurefunktion die COX-inhibierende Aktivität verringert und die γ-Sekretase-modulierende Aktivität erhöht. Die Umwandlung der Säurefunktion dieser COX-Inhibitoren in ihre Amide oder Ester resultierte entweder in inverser Modulation oder in einer vollständigen Hemmung. Weitere Bemühungen wurden schließlich auf Carprofen, einem COX-2-Inhibitor, zugelassen für den Einsatz bei Hunden und Kühen, gerichtet. Eine *N*-Substitution von Carprofen resultierte in potenten Modulatoren der γ-Sekretase, und die Verbindungen zeigten nur eine geringe oder keine Wirkung auf die γ-Sekretase-Spaltung der ϵ-Schnittstelle. Mit dem Wissen um die Bedeutung der *N*-Substitution von Carprofen und der Notwendigkeit der freien Säure auf die γ-Sekretase-Modulation wurde eine Serie an *N*-substituierten Carbazolyloxy-Ethansäuren synthetisiert. Wie erwartet stellten sie sich als effektive Modulatoren der γ-Sekrase heraus und zeigten keine oder nur eine geringe Wirkung auf die γ-Sekretase-Spaltung der ϵ-Schnittstelle. Weiterhin wurde ein analoges Derivat des LXR-Agonisten TO-901317 synthetisiert. Die Hexafluormethanol-Funktion wurde jedoch durch eine Oxyethansäure ersetzt um einen Modulator der γ-Sekretase zu erhalten. Wie erwartet änderte die Einführung der Säurefunktion die Wirkungsweise der γ-Sekretase von inverser zu normaler Modulation. Um die Bindungsstelle durch Immunpräzipitationsexperimente zu bestimmen, wurde eine Serie an von NSAIDs abgeleiteten Affinitätslabeln synthetisiert. Ein von Flurbiprofen abgeleiteter Photoaffinitätsmarker markierte zwar den aktiven γ-Sekretasekomplex dosisabhängig, jedoch gelang wegen unspezifischer Bindung nicht den direkten Bindungspartner zu identifizieren. Flurbiprofen- und DAPT-abgeleitete Photoaffinitätslabel wurden hergestellt um die Bindungsstelle in der Nähe des *aktiven Zentrums* des γ-Sekretasekomplexes zu bestimmen Das Photoaffinitätsexperiment mit einer von Flurbiprofen abgeleiteten Photoaffinitätsprobe zeigte, dass es an das *C*-terminale Fragment von Presenilin (PS) bindet. Die DAPT-abgeleitete Photoaffinitätsprobe zeigte hingegen eine sehr schwache Bindung an PS, der *aktive Zentrum* der γ-Sekretase.

Die Übereinstimmung mit der englischen Zusammenfassung wird bestätigt.

31.5.2007 Darmstadt

Table of Content

Abbreviations

Aβ	β-amyloid peptide
ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
AICD	APP intracellular domain
Ala	alanine
Aph-1	anterior pharynx defective-1
ApoE	apolipoprotein E
APP	amyloid precursor protein
aq.	aqueous
Ar	aryl
BACE	β-site APP cleavage enzyme
Bn	benzyl
BP	benzophenone
Boc	*tert*-butoxy carbonyl
BSA	bovine serum albumin
cat.	catalytic
CBz	carbobenzyloxy
CHAPSO	3-[(3 cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate
CHO	chinese hamster ovary
CNS	central nervous system
Conc	concentration
COX	cyclooxygenase
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
CSF	cerebrospinal fluid
CatD	cathepsin D
CT	computerized tomography
CTF	C-terminal fragment
DCC	1,3-Dicyclohexylcarbodi-imide
CH_2Cl_2	dichloromethane
DIEA	diisopropylethylamine
DMF	*N,N'*-dimethyl formamide
DMAP	*N,N'*-dimethyl amino pyridine
DMSO	dimethyl sulfoxide
EC_{50}	concentration required for obtaining 50% of the maximum response
EDAC	*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimidhyro-chloride
EI	electron impact
ELISA	enzyme- linked immunosorbent assay
Eq.	equivalents
ER	endoplasmic reticulum
ESI-MS	electron spray ionization mass spectroscopy
Et	ethyl
Et_3N	triethylamine
EtOAc	ethyl acetate
EtOCl	ethyl chloroformate
FAD	familial Alzheimer's disease
FRET	fluorescence resonance energy transfer

GSK-3	glycogen kinase synthase-3
h	hour
HDL	high density lipoprotein
HEK	human embryo kidney
HMG-CoA	3-hydroxy-3-methylglutary coenzyme A
HOBt	*N*-hydroxybenzotriazole hydrate
HPLC	high performance liquid chromatography
HTS	high throughput screening
Hz	Hertz
IC_{50}	the half maximal inhibitory Concentration
ICD	intracellular domain
iNOS	inducible nitric oxide synthase
IR	infra red
K_2CO_3	potassium carbonate
$KOBu^t$	potassium tertiary butoxide
LDL	low densitiy lipoprotein
M	molecular ion (molecular mass)
Me	methyl
$MgSO_4$	magnesium sulphate
MHz	mega Hertz
Mp	melting point
MRI	magnetic resonance imaging
MS	mass spectroscopy
NaCl	sodium chloride
NaH	sodium hydride
NaN_3	sodium azide
Na_2SO_4	sodium sulphate
NF-κB	nuclear Factor kappa B
NFT	neurofibrillary tangle
NGF	nerve growth factor
NH_4Cl	ammonium chloride
NICD	notch intracellular domain
NMR	nuclear magnetic resonance
NSAIDs	nonsteroidal anti-inflammatory drugs
NTF	*N*-terminal fragment
PAGE	polyacrylamide gel electrophorosis
Pd	palladium
Pen-2	presenilin ehnancer-2
Ph	phenyl
Phg	phenylglycine
PKC	protein kinase C
PHF	paired helical filament
PPAR	peroxisome proliferator-activated receptor
PPh_3	triphenyl phosphine
$(PPh_3)_4Pd$	tetrakis triphenyl phosphine palladium
PPRE	PPAR-γ responsive element
PMB	*para*-methoxy benzyl
PS1	presenilin-1
PS2	presenilin-2
PyBrop	benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate

RIP	regulated intramembrane proteolysis
RLBA	radioactive ligand binding assay
ROS	reactive oxygen species
RT	room temperature
SAR	structure activity relationship
SDS	sodium dodecyl sulfate
s-APP	secreted form of APP
SF	straight filament
SIFT	scanning for intensely fluorescent target
SPP	signal peptide peptidase
THF	tetrahydrofuran
TLC	thin-layer chromatography
TM	transmembrane
TMD	transmembrane domain
t-Bu	tertiary butyl
tPSA	topological polar surface area
TNF	tumor necrosis factor
Tyr	tyrosine

1. INTRODUCTION

1.1. Alzheimer's Disease

Alzheimer's disease (AD) is a progressive, irreversible neurodegenerative disorder characterized by memory loss, unusual behaviour, personality changes and a decline in thinking abilities. It is the most common form of dementia and accounts for 50–60% of all dementia cases. The prevalence of dementia is below 1% in persons aged 60–64 years.[1] But it shows a dramatic increase with age so that in people aged 85 years or older the occurrence is between 24% and 33% in developed world. In 2001, more than 24 million people had dementia and it is expected to double every 20 years up to 81 million in 2040 because of the increase in life expectancy.[2] Ageing is the most obvious risk factor for AD. Epidemiological studies have indicated several disputed links apart from ageing. Other risk factors which are associated with AD includes: hypercholesterolaemia, hypertension, atherosclerosis, coronary heart disease, smoking, obesity, and diabetes.[3] Several evidences suggest that dietetic intake of homocysteine associated vitamins (vitamin B_{12} and folate), antioxidants such as vitamin C and E, unsaturated fatty acids and moderate alcohol intake, especially wine, ingredients such as curcumin, might lower the risk of AD.[4, 5] The pathological hallmarks of AD are extracellular deposits of aggregated β-amyloid (Aβ) peptide and intracellular neurofibrillary tangles (NFTs). The major proteinaceous component of NFTs are abnormal filaments called straight filaments (SFs) or paired helical filaments (PHFs). The basic protein of these filaments is the microtubule-associated protein tau. Aβ is generated by the sequential proteolytic processing of β-APP by β-secretase and γ-secretase.

1.1.1. Genetics in AD

From a genetic point of view, AD is a heterogeneous disorder with both familial and sporadic forms. Familial AD is an autosomal dominant disorder with onset before age 65 years. The first mutation causing the familial form of the disease was identified in the amyloid precursor protein (APP) gene on chromosome 21.[6] Other families with the familial disease, several additional APP mutations were also found. Mutations in the presenilin 1 (PS1) and presenilin 2 (PS2) genes comprise most cases of familial disease.[7] However, the familial form of AD is very rare with a prevalence below 0·1%. There is a close association between the apolipoprotein E (APOE) ε4 allele and AD.[8] Meta-analysis showed that the APOE ε4 allele increases the risk of AD by 3-fold in heterozygotes and by 15-fold in homozygotes. The APOE ε4 allele controls mainly by adapting age of onset so that each allele copy lowers the age at onset by almost 10 years. APOE is crucial for Aβ deposition. It promotes Aβ fibrillisation and plaque formation. The APOE ε4 allele has been considered to account for most of the genetic risk in sporadic AD.[9] Therefore, the contribution of other competitor genes is probably much less. Several studies have reported weak associations with different candidate genes, but few of them have been confirmed.[10]

1.1.2. Clinical Features and Diagnosis

AD is a slowly progressing disorder, with progressive impairment of memory. The symptoms include aphasia, apraxia, and agnosia, accompanied by general cognitive symptoms, such as impaired judgment, decision-making, and orientation. There are several factors which serve as the basis in the diagnosis of AD such as medical history along with the clinical, neurological, and psychiatric examination. Neuropsychological testing can be helpful to get objective signs of memory disturbances in very primary stages. Neuroimaging is an important diagnostic tool in AD. Computerized tomography (CT) and magnetic resonance imaging (MRI) play a crucial role in the diagnosis of AD to rule out other causes of dementia, such as brain tumour and

subdural haematoma. Cerebral atrophy, visualised as enlarged ventricles and cortical sulci, is also recognized by CT and MRI, but the overlap with typical ageing and other dementias is too large to have any diagnostic significance. Nevertheless, neuroimaging is a valuable tool to detect cerebrovascular disease, such as cerebral infarcts and white-matter lesions, which is of significance to identify AD/vascular dementia. An accurate diagnosis of AD can only be made by neuropathology.[11] Large community-based neuropathology studies revealed that a substantial proportion (20–40%) of non-demented persons have adequate plaques and tangles to warrant a neuropathological diagnosis of AD, yet without symptoms ![12]

1.1.3. Pathogenesis

The histopathological hallmarks in AD are senile or neuritic plaques and neurofibrillary tangles. At the microscopic level these are found (**Figure 1.1**) in the medial temporal lobe structures and cortical areas of the brain, accompanied by a degeneration of neurons and synapses. Several pathogenic mechanisms that trigger these changes have been studied such as: Aβ aggregation and deposition with plaque development, tau hyperphosphorylation with tangle formation, neurovascular dysfunction. Apart from these mechanisms, other mechanisms such as cell-cycle abnormalities, inflammatory processes, oxidative stress, and mitochondrial dysfunction have also been investigated. The discovery of a close relationship between plaque formation and dementia put great focus on the involvement of plaques in the pathogenesis of AD.[13]
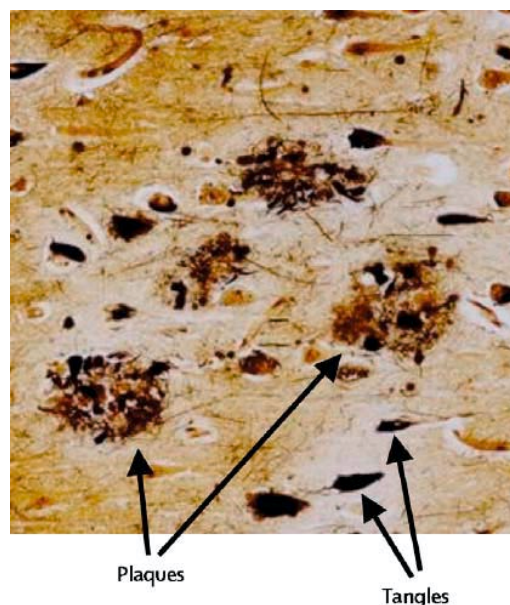
Figure 1.1: Plaques and tangles in the cerebral cortex in AD.
Plaques are extracellular deposits of Aβ which are surrounded by dystrophic neurites, reactive astrocytes, and microglia. $A\beta_{42}$ is the most toxic and fibrilogenic species and forms the central core of plaques. Tangles are intracellular aggregates composed of a hyperphosphorylated form of the microtubule-associated protein tau.[14] (Reprinted from *The Lancet* with permission © copyright 2006 by Elsevir)

1.1.3.1. Tau and neurofibrillary tangles

Neurofibrillary tangles (NFTs) are an additional histopathological hallmark of AD. These are particularly found in cell bodies, apical dendrites as NFT, in distal dendrites as neuropil threads and in the abnormal neurites that are associated with some neuritic plaques. The main

components of these neuritic plaques are abnormal filaments such as straight filaments (SFs) or paired helical filaments (PHFs). The basic component of these filaments is the microtubule-associated protein tau[15] which is widely expressed in the mammalian nervous system. Tau, a normal axonal protein which binds to microtubules through its microtubule-binding domains thus promotes microtubule assembly and stability. Six tau isoforms are produced in the adult human brain and are found in neurofibrillary lesions of AD patients.[16] Tau phosphorylation is controlled by the delicate balance between multiple kinases (eg, GSK-3β and CDK5) and phosphatases (eg, PP-1 and PP-2A).[17] Tau hyperphosphorylation in AD begins intracellularly and leads to sequestration of normal tau and other microtubule-associated proteins. This causes disassembly of microtubules and thus impairs axonal transport, compromising neuronal and synaptic function (**Figure 1.2**). Tau becomes prone to aggregation into insoluble fibrils in tangles, further compromising neuronal function. Tau pathology begins early in the disease process in neurons in the transentorhinal region which later expands to the hippocampus and amygdala, and neocortical association areas. It is still matter of debate whether tau hyperphosphorylation, and tangle formation are a basis or outcome of AD.[18]
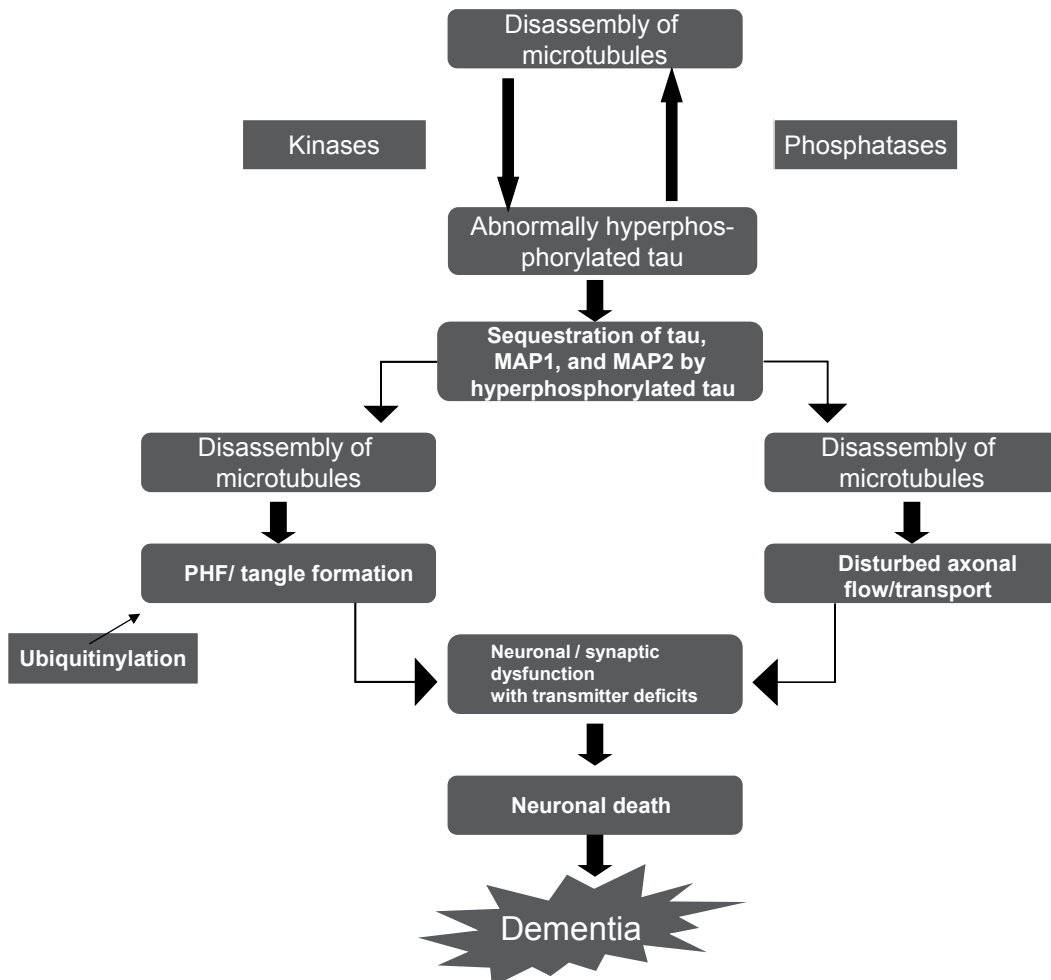
Figure 1.2: Schematic representation of tau hyperphosphorylation and tangle formation.
Tau phosphorylation is controlled by the delicate balance between multiple kinases and phosphatases. Sequestration of normal tau and other microtubule-associated proteins by hyperphosphorylated tau results in the disassembly of microtubules and disturbed axonal transport. Hyperphosphorylated tau becomes prone to aggregation into insoluble PHFs and larger aggregates in tangles.[14] (Modified and reprinted from *The Lancet* with permission © copyright 2006 by Elsevier)

1.1.3.2. Aβ Plaques

Identification of the protein composition of plaques was unsuccessful until the mid-1980s due to the insolubility of plaques. But, the success in purification of plaque cores and the identification of the amino acid sequence of Aβ which is the major constituents of plaques[19] paved the way for the cloning of the APP gene. Aβ is degraded by the peptidases insulin-degrading enzyme, neprilysin, and by endothelin-converting enzyme under normal conditions.[20] Aβ is also cleared from the brain in a process balanced by the efflux. The efflux is mediated by low-density lipoprotein receptor related protein. Influx is mediated by the receptor for advanced glycation end products of Aβ across the blood–brain barrier.[21] The central hypothesis for the cause of AD is the amyloid cascade hypothesis (**Figure 1.3**), which states that an imbalance between the production and clearance of Aβ in the brain is the initiating event and this ultimately leads to neuronal degeneration and dementia.[22] This hypothesis is supported by the finding that the mutations implicated in the familial disease are present in the genes for both the substrate, APP, and the key enzyme, PS for Aβ generation. Most of the APP mutations are found around the secretase sites, and both the APP and PS mutations increase $A\beta_{42}$ production. Soluble Aβ is thought to undergo a conformational change to high β-sheet form, making it prone to aggregate into soluble oligomers and larger insoluble fibrils in plaques. In this process, the fibrillogenic $A\beta_{42}$ isoform triggers the aggregation of other Aβ species.[23]

1.1.4. Amyloid Cascade Hypothesis

It is the most preferred model used to explain the pathogenic events causing AD. The amyloid cascade hypothesis (**Figure 1.3**) states that, an imbalance between the production and clearance of Aβ in the brain is the initiating event which ultimately leads to neuronal degeneration and dementia.

The hypothesis is supported by several evidences and observations.[22]

1. Persons with the Dawns syndrome have an additional copy of the APP gene and thus produce more Aβ.

2. Mutations causing hereditary AD in the APP or PS genes increase production of the longer and more amyloidogenic $A\beta_{42}$ form of the Aβ peptide.

3. The levels of the deposited Aβ associate with cognitive decline and severity of the disease in AD patients and in transgenic animals [24]

4. The Aβ peptide is neurotoxic to several cell lines.

5. Transgenic mice that are knocked out for β-secretase show rescue of memory impairments found in the parental strain with an intact β-secretase gene. This indicates that overexpression of APP does not cause memory deficits and that the production of Aβ and or APP *C*-terminal fragments are required for the demonstration of reduced memory function in the mice.[25]

Tangle formation appears closely linked to Aβ since tau deposition is increased in transgenic mice expressing human mutant tau together with mutant APP as compared to mice expressing mutant tau alone.
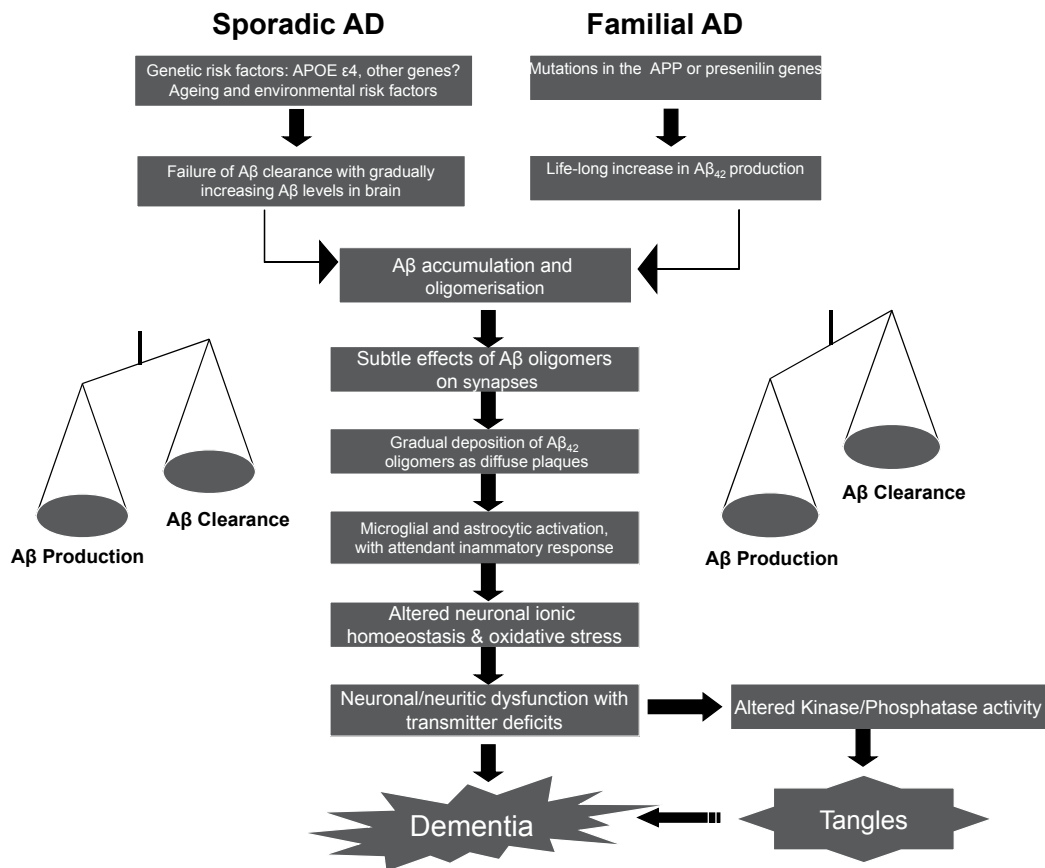
Figure 1.3: Amyloid cascade hypothesis

According to this hypothesis, the central event in the disease pathogenesis is an imbalance between Aβ production and clearance, with increased Aβ production in familial disease and decreased Aβ clearance in sporadic disease. Aβ oligomers could directly inhibit hippocampal long-term potentiation and impair synaptic function, in addition to the inflammatory and oxidative stress caused by aggregated and deposited Aβ. These processes impair neuronal and synaptic function with resulting neurotransmitter deficits and cognitive symptoms. Tau pathology with tangle formation is regarded as a downstream event, but could contribute to neuronal dysfunction and cognitive symptoms.[14] (Modified and reprinted from *The Lancet* with permission ©copyright 2006 by Elsevier)

Moreover, injection of fibrillar Aβ$_{42}$ enhances the tangle formation in tau transgenic mice.[26] The attention has been focused on in which form (fibrills, protofibrills, oligomers or monomers) the amyloid species has the most adverse effects. Several reports blames the oligomeric and protofibrillar aggregates as being the most toxic form of Aβ.[27] Even though the amyloid cascade hypothesis is convincing, there are certain issues which are not clarified by amyloid cascade theory such as, the role of tangles and /or the inflammatory response.[28]

1.1.5. β-Amyloid Precursor Protein

APP is an evolutionary conserved glycoprotein with ubiquitous expression throughout the body and is a type I transmembrane protein. It has a large extracellular *N*-terminal domain and a short *C*-terminal tail projecting into the cytoplasm. APP is produced in the endoplasmic reticulum and trafficked through the secretory pathway to the plasma membrane. APP intracellular domain (AICD) was detected in the nucleus and found to interact with the nuclear adaptor protein Fe65 and the histone acetyltransferase Tip60. AICD has together with these two proteins been reported to activate transcription of a Gal4-receptor gene suggesting a gene regulatory role for APP.

1.1.5.1. APP Processing

Amyloid plaques are deposits composed primarily of insoluble Aβ of approximately 4 kDa generated from APP, which is processed *via* two different pathways: the amyloidogenic and the non-amyloidogenic pathways.

A
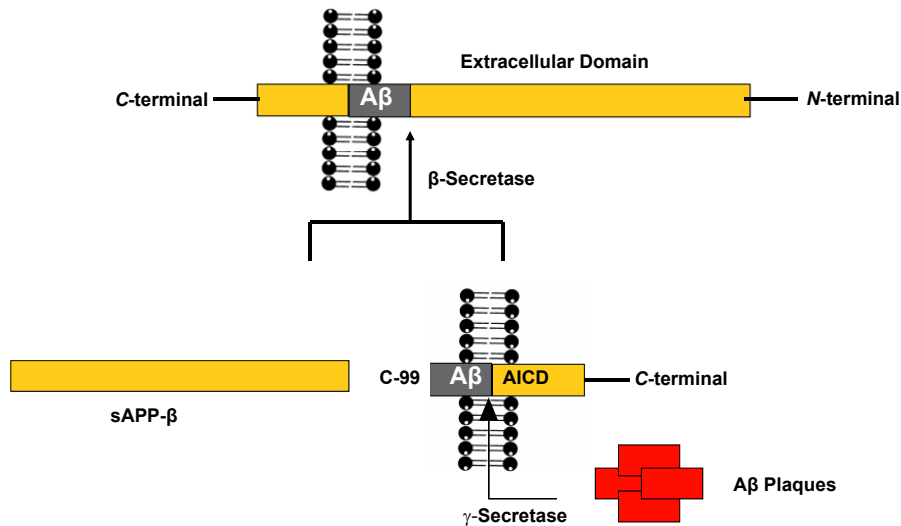
B
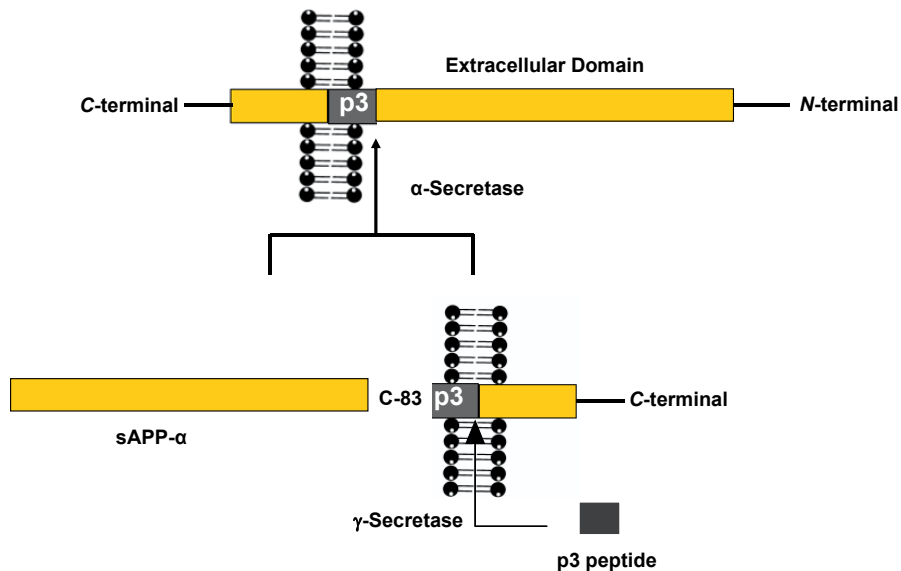
Figure 1.4: Schematic outline of APP processing.
(A) APP is a type I integral membrane protein with a large extra and a short intracellular domain. Cleavage of APP in the endosomal-lysosomal pathway by β-secretase enables the secretion of sAPP-β. Processing of C99 by γ-secretase leads to the release of the Aβ-peptides. (B) Cleavage by α-secretase within the Aβ domain leads to secretion of the soluble sAPP-α and prevents the formation of Aβ-fragments (non-amyloidogenic pathway). The *C*-terminal fragment C83 is processed further by γ-secretase releasing the p3 peptide.

The non-amyloidogenic pathway involves the activity of α-secretase at membrane level (**Figure 1.4 A**).[29] α-Secretase belongs to the family of metalloproteases such as, the tumor necrosis alpha converting enzyme (TACE) and ADAM10. α-Secretase activity cuts within the

sequence of the Aβ, at the residue 17.[30] Non-amyloidogenic processing of APP does not release intact Aβ and the generated fragments p3 peptides, do not aggregate and display amyloidogenic activity. In AD pathology, the production of Aβ is the net result of APP amyloidogenic processing. This involves the activity first of β- then of γ-secretase and requires the internalization of APP from the membrane to the endosomes and the lysosomes (**Figure 1.4 B**).[31] β-secretase, recently identified as the β-site APP-cleaving enzyme (BACE-1),[32] cuts APP at the beginning of the sequence of the Aβ, generating an extracellular soluble fragment called sβ-APP and an intracellular COOH-terminal fragment called C99. Subsequently, γ-secretase cuts C99 at residues 38/40/42 of the Aβ sequence and generates Aβ peptides of various lengths.

1.1.6. Established Therapies for AD

In spite of tremendous research efforts, there is no approved causal treatment for AD today. Current pharmacological approaches related to AD treatment include antioxidant therapy, AChE (acetylcholine esterase) inhibitors, nicotinic and muscarinic agonists, nerve growth factor (NGF), low molecular lipophilic compounds that can activate the neurotrophic factor signalling pathway, anti-inflammatory drugs such as COX-1 and COX-2 inhibitors, drugs which interfere with Aβ formation and deposition, drugs which attenuate Aβ toxicity and food dietary components and ingredients such as curcumin.[33] Some 500 compounds are in development to treat neurodegenerative diseases. At least 10% of these are related to AD. The targets derive from a whole range of sometimes well-known receptors and enzymes: GSK-3, PDE 4 and muscarinic M1. nACh modulators, AChE inhibitors, NMDA modulators, 5-HT agonists and several vaccination projects (e.g. Elan, Cytos Biotechnology) are in advanced stages of research or development.[1] Plaques formation was seen as the main reason for the formation of AD and their removal was one of the therapy goals until 2001. Now, soluble Aβ and early oligomers have to take the blame for most of the Aβ associated effects. AChE inhibitors produce small improvements in cognitive and global assessments, but galanthamine, tacrine, donepezil and rivastigmine do not address the severe mortality in the final stages of AD. Memantine hydrochloride protects the neurons against overactivation of *N*-methyl-*D*-aspartate receptors and was approved for the treatment of moderate to severe AD in 2002 as first of its class. Thus, a causal therapy is still in demand, since no existing therapy effectively stops or cures AD.

1.1.7. β-Secretase

BACE-1 was identified as the β-secretase which cleaves APP and regulates the production of Aβ *in vitro*.[32] *In vivo* experiments confirmed that this enzyme is mainly responsible for initiating the amyloidogenic processing of APP. Particularly, brains of BACE-1 knockout mice had no detectable levels of Aβ and did not show accumulation of APP *C*-terminal fragments (CTF) C99 and C89.[34] Whereas brains of BACE-1 transgenic mice were characterized by increased levels of Aβ and amassing of the CTF.[35] It is a type-1 membrane protein characterized by a large extracellular domain and a short intracellular domain. The two aspartic residues involved in β-secretase activity are located in the extracellular domain. The short intracellular domain contains a sorting sequence (DISLL), which is involved in the trafficking of the protein.[36] Particularly, the LL domain was found to regulate the internalization of the protein from the plasma membrane to the endosomal compartment,[32] and to the lysosomes where BACE-1 is degraded.[37]

1.1.8. γ-Secretase

γ-Secretase mediates the critical step in the release of Aβ from the membrane. γ-Secretase cleavage is unusual, as it carries out the proteolysis within the hydrophobic environment. It generates Aβ peptides of different lengths, indicating a lack of sequence specificity. However, γ-secretase is conformation specific since it selectively cleaves α-helical substrates.[38] The

major forms of Aβ it generates are Aβ$_{38}$, Aβ$_{40}$ and Aβ$_{42}$.[39] The more aggregating and toxic Aβ$_{42}$ corresponds to approximately 5–10% of the total amount of Aβ secreted by cultured cells under normal conditions. Other Aβ peptides of residues 19, 37–39 and 43–48 were also identified but these represent only minor species.[40] The longer the peptides, the more insoluble they are and hence more prone to aggregate and thus seed amyloid deposition. γ-Secretase also mediates the release of AICD by cleaving at the ε-site, nine amino acids away from the γ40 cleavage site.[41] The relationship between γ- and ε-cleavage has been discovered recently, with the isolation of Aβ$_{49}$, which is the the *N*-terminal product of ε-cleavage and thus may be a precursor for secreted Aβ. γ-Secretase processes the APP transmembrane domain (TMD) from its *C*-terminal end by sequential steps and each cleavage occurs on the same side of the α-helix. Biochemical and genetic studies have demonstrated that presenilin (PS), nicastrin, anterior pharynx defective-1 (Aph-1) and presenilin enhancer-2 (Pen-2) form the enzymatically active core of the γ-secretase complex (**Figure 1.5**).[42]

1.1.8.1. Presenilins: PS1 and PS2

Presenilins (PS1 and PS2) control the activity of γ-secretase and are involved in the processing of APP and the production of Aβ.[43] They account for the majority of the cases of inherited familial AD. Levels of Aβ were dramatically decreased in animal models lacking the expression of PS1 but not PS2.[44] This indicates that among the two presenilins, PS1 plays a major role regulating the activity of γ-secretase and hence Aβ. PS1 is an aspartyl protease and its topology is characterized by 9 or 10-membrane spanning domains. The sixth and seventh domains bear the aspartates residues (D257 and D385) which are vital for the production of Aβ. The mutation on the either of these residues abolishes the Aβ generation.[45] It is a mechanism which results in the formation of *N*-terminal and *C*-terminal fragments of PS1. These two fragments contain one of the two critical aspartate residues and remain associated forming a heterodimer which comprises the active PS.[46] PS1 and γ-secretase activity resides in its ability to cleave APP within the plasma membrane at the 38-, 40- and 42- sites of the Aβ sequence. This process is known as Regulated Intra-membrane Proteolysis (RIP). It requires the exchange of water molecules, which are excluded from the hydrophobic lipid bilayer of the cell membrane.[47] The exact mechanism of this process and how it is affected by inherited mutations in PS remains mysterious. Pharmacological γ-secretase activity can be assigned to the two critical aspartate residues in PS, but PS1 alone is not able to coordinate all the processes that lead to its own activation.[48] It has been reported that overexpression of PS1 does not lead to increased levels of its *N*- and *C*-terminal fragments.[49] Therefore, there must be more additional limiting factors which control the endoproteolysis of PS1. Numerous studies performed during the last few years have confirmed that, PS especially PS1, is a part of a tetrameric complex which constitutes the active γ-secretase. The other members of this tetrameric complex are nicastrin, Aph-1 and Pen-2. All of these are membrane inserted proteins which are co-factors in the regulation of γ-secretase dependent APP processing and Aβ formation.

1.1.8.2. Nicastrin

Nicastrin was the first protein of the γ-secretase complex to be identified.[50] The domain of nicastrin involved in the interaction with PS1 has been identified as the *N*-terminal part of its transmembrane domain (TMD).[51] This was further confirmed by the experiments carried out with the transitions-state analogues which bind specifically to active PS. These experiments showed that nicastrin copurifies with the active form of PS1. This indicates that it is an integral part of the active γ-secretase complex.[52] Nicastrin is a large type-1 transmembrane protein (~ 130 kDa) and exists as an immature and a mature protein.[53] Studies performed in order to understand the role of nicastrin revealed that PS1 and nicastrin require each other to become fully mature.[48] These two can be released from the endoplasmic reticulum and be sorted to the plasma membrane where the complex PS1-nicastrin is biologically active.[54]

Due to the large extracellular domain of nicastrin, it was speculated that the role of nicastrin within the γ-secretase complex would be to recognize the substrate by measuring the length of the membrane-inserted end.[55] But, recently it has been reported that nicastrin functions as a γ-secretase substrate receptor and it binds specifically to the *N*-terminal side of the APP and Notch *C*-terminal fragments.
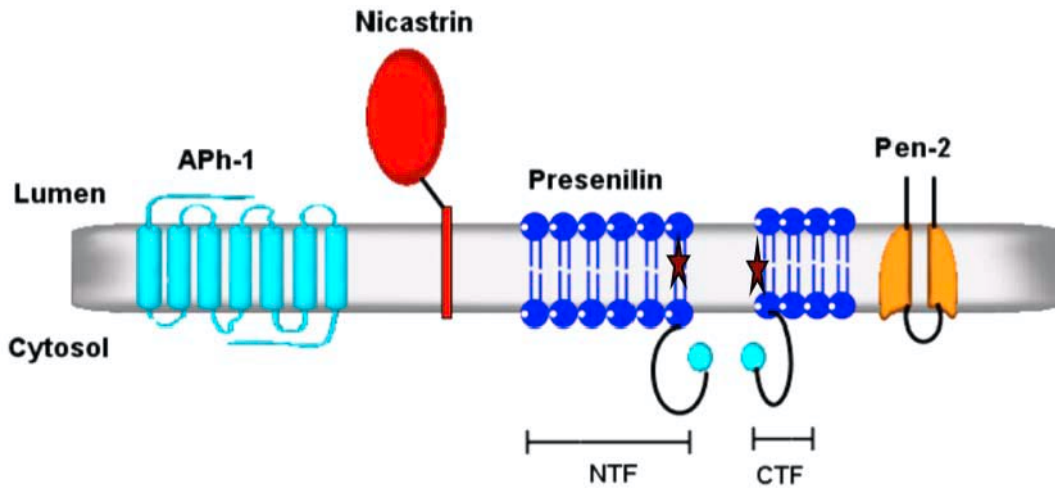

Figure 1.5: Members of the gamma-secretase activity complex.
The γ-secretase complex comprises four integral membrane proteins. **PS** is believed to contain nine to ten TMDs and two critical aspartate residues in transmembrane domains 6 and 7 that are proposed to form the catalytic site of the enzymatic complex. PS becomes proteolytically processed into an NTF with six membrane-spanning domains and a CTF with two transmembrane domains, before becoming inserted into active complexes of high molecular weight. **Nicastrin** comprises a single transmembrane domain and becomes highly glycosylated during maturation of the γ-secretase complex. **Aph-1** is predicted to contain seven transmembrane domains. Nicastrin and Aph-1 can associate as a subcomplex. **Pen-2** contains two transmembrane domains and is required for the endoproteolysis of PS.

1.1.8.3. Anterior pharynx-defective phenotype-1 and Presenilin enhancer-2

Aph-1 (anterior pharynx-defective phenotype) and Pen-2 (presenilin enhancer) were isolated as part of the γ-secretase complex in two different experiments carried out in C. elegans.[56, 57] Aph-1 and Pen-2 are required for Notch signaling and Aβ formation. Aph-1 is required along with PS for the correct localization of the mature form of nicastrin at the cell surface. Aph-1 exists in more than one isoform which may be involved in the formation of the γ-secretase complex. Aph-1-a being the mostly involved in Notch signaling and the processing of APP.[58] Pen-2 is a short two-membrane domain spanning protein and it is formed of approximately 101 amino acids. Pen-2 knockout affects the activity of the γ-secretase complex by altering the Notch-mediated signaling pathway and the processing of APP.[57] The physiological significance of Aph-1 and Pen-2 within the γ-secretase complex is to stabilize mature PS and nicastrin,[59] and to control the endoproteolysis of PS which is crucial for the creation of the active heterodimer.[60] It was revealed that, in absence of PS, Pen-2 is destabilized and degraded in the endoplasmic reticulum as ubiquitinylated protein.[61] The *C*-terminal domain of Pen-2 is involved in the maturation of nicastrin and the formation of the PS heterodimer. Pen-2 associates with the nicastrin/PS1 complex in the endoplasmic reticulum regulating its maturation and sorting to the plasma membrane.
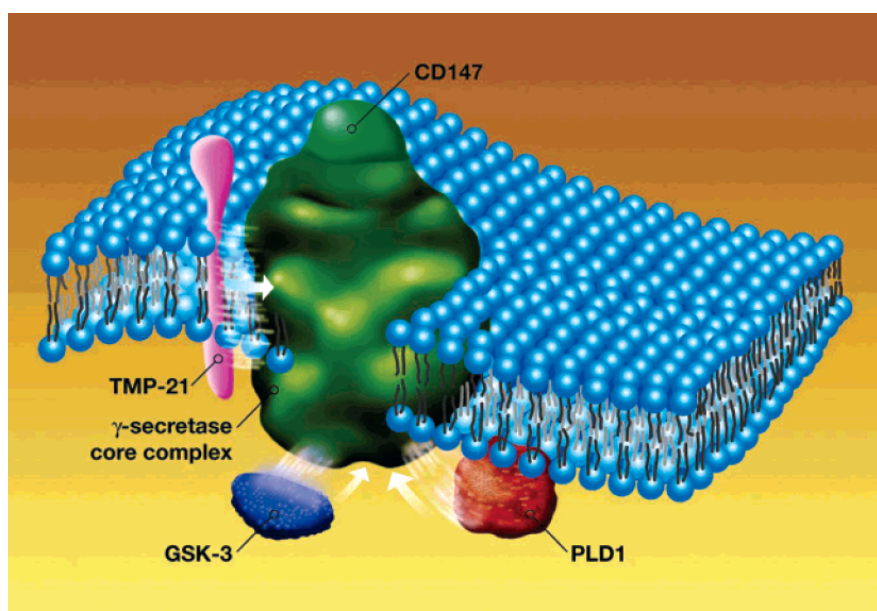
Figure 1.6: γ-Secretase complex and binding partners.
PS, Nicastrin, Aph-1 and Pen-2 are assembled to form the enzymatic core complex. CD147 is an integral regulatory subunit of the native complex. TMP21, PLD1 and GSK3 have been found to modulate γ-secretase activity and transiently or weakly bind to the complex; transient interactions with the complex are depicted by white arrows.[62] (Reprinted from *The Journal of Medicinal Chemistry* with permission © copyright 2007 by the American Chemical Society)

Purification of endogenous γ-secretase complexes from detergent solubilized HeLa cell membranes showed along with the four mandatory components of the enzymatic core it bears an additional integral subunit, the membrane protein CD147. CD147 was found to have a regulatory effect on γ-secretase activity.[63] Apart from CD147, other transient or weak binding partners of the γ-secretase complex, such as glycogen synthase kinase-3 (GSK-3), phospholipase D1 (PLD1) and TMP21 also have been reported (**Figure 1.6**).[62] Although the presence of these binding partners is crucial for the activity of γ-secretase, none of these proteins have been detected in chromatographically purified samples of the complex. CD147 in endogenous γ-secretase complexes was found to down-modulate the production of Aβ-peptides. But, its effect on the processing of other γ-secretase substrates, such as Notch, remains to be investigated.[63] CD147 is a glycosylated type-1 transmembrane protein with a molecular weight of about 50 kDa. It is believed that it has a short cytoplasmic domain about 40 amino acids long, a transmembrane region of approximately 25 amino acids long and a large extracellular region consisting primarily of two immunoglobulin-like (Ig-like) domains. How these four components (PS1, nicastrin, Aph-1 and Pen-2) coordinate γ-secretase activity is still a matter of investigation. Although the catalytic activity of the γ-secretase complex resides in the two aspartate residue in PS1, these four proteins certainly contribute in the regulation of one another's activation and/or maturation. These four protein altogether combine and form the active γ-secretase complex and are obligatory to reconstitute γ-secretase activity in mammalian cells.[64] In addition to APP, the γ-secretase complex processes numerous other type I TM proteins including fragments of Notch, E-cadherin, N-cadherin, CD44, DCC, ErbB4, LRP and nectin-1, which are involved in many physiological and pathological functions.[65]

1.2. γ-Secretase Inhibitors

Special features of the γ-secretase complex hinder crystallisation and thus crystallographic analysis of the enzyme, which is a major obstacle for structure-based drug design. Furthermore, the information available on inhibitor binding sites is still limited. Therefore, all selective, non-peptidic γ-secretase inhibitors had to be provided by high throughput screening (HTS) efforts. Peptidic PS1 inhibitors, like Merck's L-685,458 (**1**, IC_{50} = 17 nM) are potent inhibitors.[66] The semi-peptidic inhibitors have also been comprehensively utilized in a range of discoveries from the Elan/Lilly group.[67-69] DAPT **4** (HEK IC_{50} = 20 nM) was developed from a *N*-dichlorophenylalanine lead and the phenylglycine and the difluoro phenylacetic acid are crucial for activity (**Scheme 1.1**).
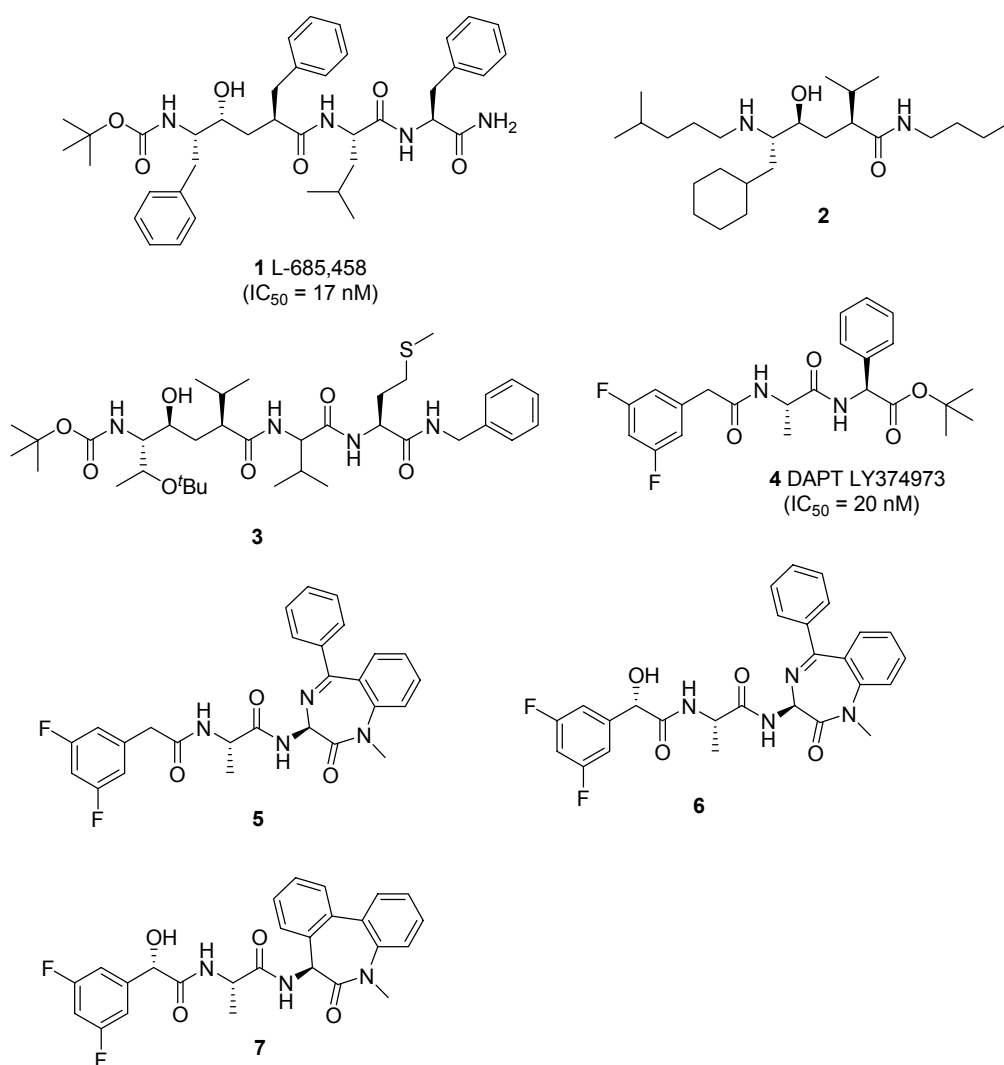
Scheme 1.1: Semi-peptidic γ-secretase inhibitors.

DAPT is not a prodrug, despite the labile nature of tertiary butyl ester, which may be cleaved at the low pH of the gut. It has demonstrated robust efficacy *in vivo* in the PDAPP and Tg2576 mouse models.[70, 71] DAPT has also been shown to cause a phenotype consistent with interference in the Notch signaling pathway in both drosophila and zebra fish embryos.[72] Modification of these early compounds ended up in benzodiazepine-containing analogs such as **5** (IC_{50} 0.3 nM CHO β-APP cells).[73] Further amplification, taking advantage of the

increased activity observed with several metabolites bearing an additional benzylic hydroxyl group, resulted in the discovery of mandelate analogs such as **6** and **7** which displayed high levels of potency in whole cell assays (e.g. **7**; IC_{50} 119 pM – HEK 293 cells overexpressing βAPP695).[74] **7** was further evaluated *in vitro* and *in vivo* as a prototypical, potent, orally active γ-secretase inhibitor: upon oral dosing to 3-5 months old Tg2576 mice, **7** (LY- 411575) gave a dose-dependent reduction of both $A\beta_{40}$ and $A\beta_{42}$ in all compartments studied (cortex, hippocampus, CSF and plasma; 3 hours post-dose) with a minimum effective dose of 1mg/kg (0.3 mg/kg in plasma). Further analyses of the time course of inhibition following a 1 mg/kg dose of **7** exhibited a significant reduction in Aβ levels in cortex, hippocampus and plasma up to 24 hours post-dose although CSF levels were only reduced significantly at 3 and 9 hours post-dose.[75]
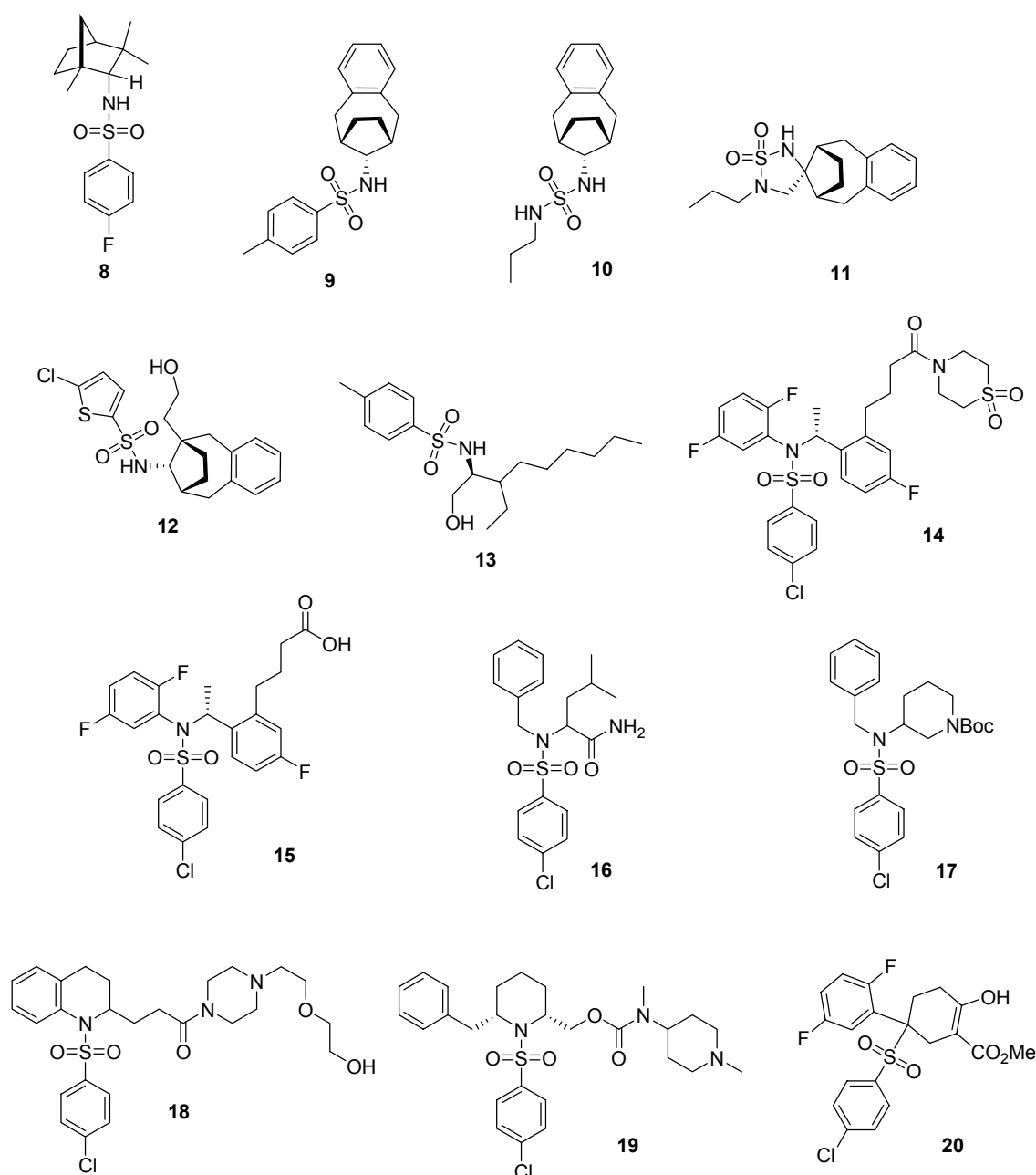
Scheme 1.2: Non-peptidic γ-secretase inhibitors I.

In another study, chronic administration of **7** (LY-411575) to PDAPP mice prior to the onset of plaque deposition resulted in a considerable lowering of brain Aβ levels (by ELISA) and immunohistochemically-determined Aβ burden. In addition, markers of neuritic pathology and glial inflammation were also significantly reduced following this treatment.

Apart from the previously described peptidic and semi-peptidic inhibitors, the efficacy of lipophilic scaffolds based around a sulfonamide moiety (**Scheme 1.2**) have been explored in the recent discoveries.[65] The Amgen group exploited fenchylamine sulfonamides as moderately potent inhibitors. SAR studies revealed that a stereochemical dependence of inhibitory potency with **8** giving optimal activity (IC$_{50}$ 1.8 μM) for the inhibition of Aβ$_{42}$ production in HEK293 cells.[76] The Merck group employed an unusual bicyclo-[4.2.1] sulfonamide scaffold characterized by the toluyl sulfonamide **9**.[77] Related acyclic sulfamides (e.g. **10**) and spirofused cyclic sulfamides (e.g. **11**) have also been reported. More recent disclosures have described a number of more elaborate molecules which bear olefinic substituents, heterocyclic groups, alkynyl groups or substituted oxadiazoles on the benzo-fused moiety. Many of these compounds are claimed to inhibit Aβ production in whole cells showing IC$_{50}$ <1 μM. Additionally, the introduction of bridgehead substituents on to the [4.2.1] core (e.g. **12**) was demonstrated. Wyeth/Arqule groups reported the sulfonamides (**13**) which were prepared from amino alcohols.[78] An alternative series of diaryl sulfonamides **14** and **15** (IC$_{50}$ 7-13 nM solubilized enzyme assay)[79] has also been disclosed by the BMS group.[80]
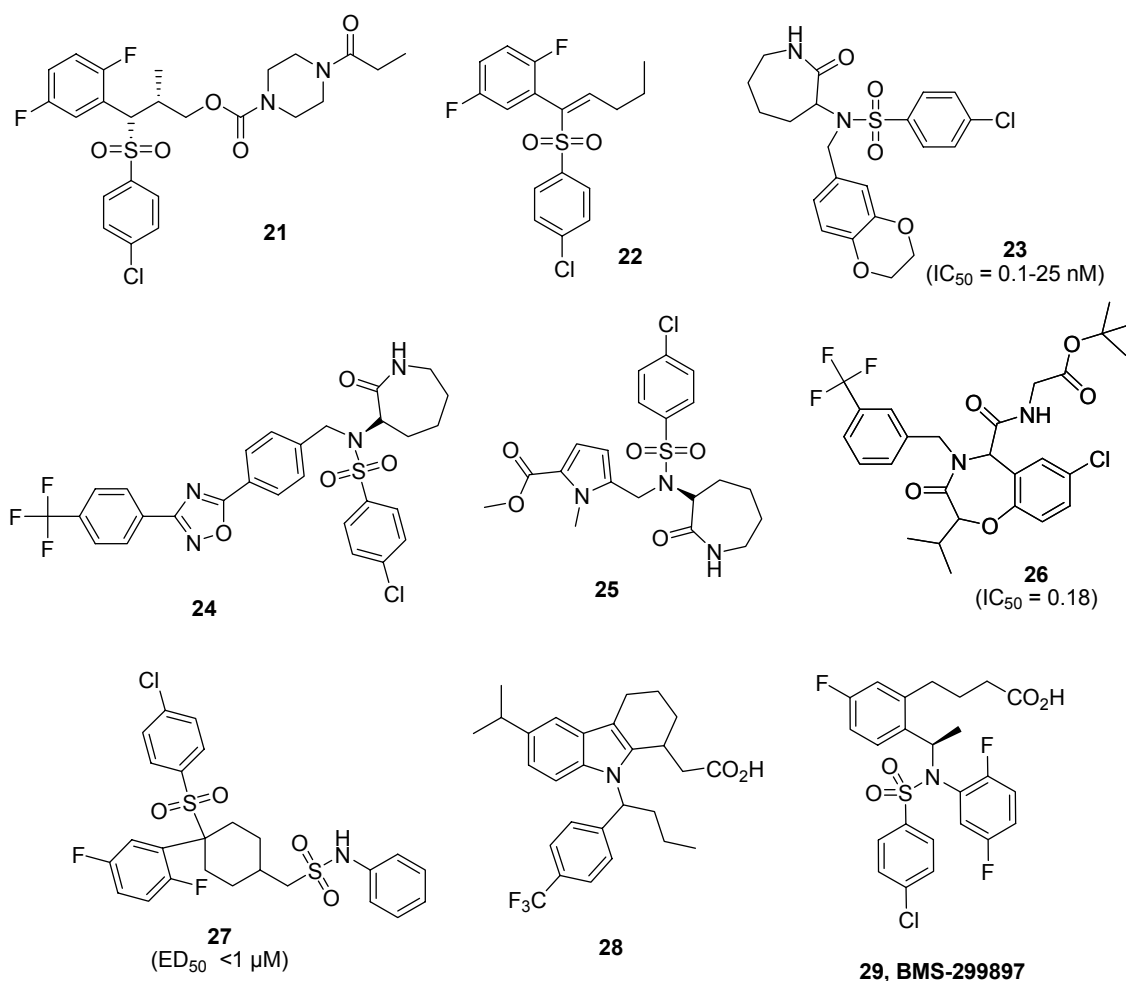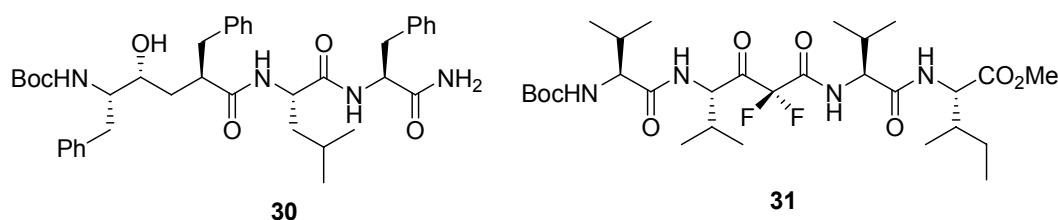

Scheme 1.3: Non-peptidic γ-secretase inhibitors II.

Acetamide derivatives such as **16** also have been reported by the same group. Compound **16** displayed potent inhibitory activity with an IC$_{50}$ <50 nM for inhibition of Aβ secretion in H4 cells. In a series of closely related disclosures, the Schering group employed a variety of sulfonamides, such as piperidine **17**, tetrahydroquinoline **18** (IC$_{50}$ 30-535 nM in DKF167 cells expressing C99) and 2,6-disubstituted piperidine **19** (IC$_{50}$ 0.2 nM). Removal of the nitrogen atom from these sulfonamides resulted in a series of corresponding sulfones. The Merck group has reported cyclohexyl sulfones such as **20** (IC$_{50}$ <100 nM, SH-SY5Y cells) as potent γ-secretase inhibitors.[81-83] Following to this initial discovery of cyclohexyl sulfones, further compounds such as **21** have been claimed by the Bayer AG.[84] Various related sulfone analogs were also claimed in a disclosure by Daiichi Pharm Co. (e.g. **22**, IC$_{50}$ <50 nM, H4 cells). The Elan patent application [85] reported three general synthetic schemes and about 700 tabulated examples. Selected *N*-(oxoazepanyl) benzenesulfonamides showed promising activities, for example, compound **23** inhibited γ-secretase with an IC$_{50}$ within the range of 0.1-25 nM. However, the activities of compound **24** and **25** are unknown (or not reported). Roche's 1,4-benzoxazepin-3-one **26** was prepared by a cyclocondensation of (formylaryloxy)alkanoic acid, amine and isonitrile (**Scheme 1.3**).[86] Compound **26** inhibited γ-secretase with an IC$_{50}$ value of 0.18 (no units given). Additionally, Roche presented malonamide derivatives, where some compounds display IC$_{50}$ values of < 1.0 μM.[87] The aryl cyclohexyl sulfone (**27**),[83] developed by Merck, inhibits the processing of APP by γ-secretase with ED$_{50}$ values of < 1 μM. Elli Lilly optimized their series of dipeptides to challenge the amyloid hypothesis in a clinical setting. A selective γ-secretase modulator (**28**) was reported by MSD,[88] the carboxylic acid seems to be important to achieve the desired ratio of Aβ$_{38}$/Aβ$_{40}$/Aβ$_{42}$. This modulation is distinctly different from inhibition as the total Aβ load may be unaffected. **29** (**BMS-299897**) 2-[(1*R*)-1-[[(4-chlorophenyl) sulfony](2,5-difluorophenyl)amino]ethyl]-5-fluorobenzenepropanoic acid is a potent γ-secretase inhibitor.[89] It was found to reduce Aβ in brain, cerebrospinal fluid (CSF) and plasma in young transgenic mice in a dose and time dependent manner with a significant correlation between brain and CSF Aβ levels. Transgenic mice were used to examine potential side effects due to Notch inhibition which is a crucial issue with γ-secretase inhibitor. **29** (**BMS-299897**) was 15-fold more effective and selective at preventing the cleavage of APP than of Notch *in vitro* (APP IC$_{50}$ of 7.1 nM, Notch IC$_{50}$ of 105.9 nM). No changes in the maturation of CD8+ thymocytes or of intestinal goblet cells were observed in mice treated with **29**.

1.2.1. Additional Cleavage of Substrates by γ-Secretase

The most crucial issue related to γ-secretase inhibition is the identification of a gradually increasing number of additional integral membrane proteins which are processed by the enzyme. Little is known about the functional consequences of inhibiting the processing of these alternative substrates except Notch.

Scheme 1.4: Peptidic γ-secretase inhibitors.

The role of Notch signaling in lymphopoiesis is well known,[90, 91] and most of the reported and confirmed γ-secretase inhibitors such as compounds **30** & **31** (**Scheme 1.4**) impair

thymocyte development in a concentration-dependent manner with phenotypes resulting from a blockage at earlier, immature stage to later stages.[92, 93] Lilly group observed a gastrointestinal toxicity in rats upon dosing the potent γ-secretase inhibitor **23**.[94] Moreover, the histological analysis showed a reduction in absorptive enterocytes, an increase in goblet cells and upregulation of the transcripts for the secreted proteins adipsin, secretin, glucagon-like peptide, cholecystokinin and somatostatin. These observations are in accordance with a disruption of the Hes-1-mediated control of intestinal stem cell fate[95] which sequentially is dependent on the Notch pathway. These observations suggest that the γ-secretase inhibitory effect of **23** may be directly responsible for the observed toxicity. Notch has also been further implicated in maturation in the immune system,[96] memory and learning,[97, 98] and muscular regeneration[99] amongst other pathways.

1.2.2. Interaction of γ-secretase enzyme with different γ-secretase inhibitors

Clarke et al. demonstrated that the two principal classes of inhibitor, aspartyl protease transition state analogues and small molecule non-transition state inhibitors, display fundamental differences in the way they interact with the enzyme.[100] Taking advantage of a γ-secretase enzyme overexpressing cellular system and different radiolabelled γ-secretase inhibitors, it was observed that the maximal binding of nontransition state γ-secretase inhibitors accounts only for half the number of catalytic sites of the recombinant enzyme complex. This characteristic stoichiometry can be best accommodated with a model whereby the non-transition state inhibitors bind to a unique site at the interface of a dimeric enzyme (**Figure 1.7**). Subsequent competition studies confirmed that this site appears to be targeted by the main classes of small molecule γ-secretase inhibitor.
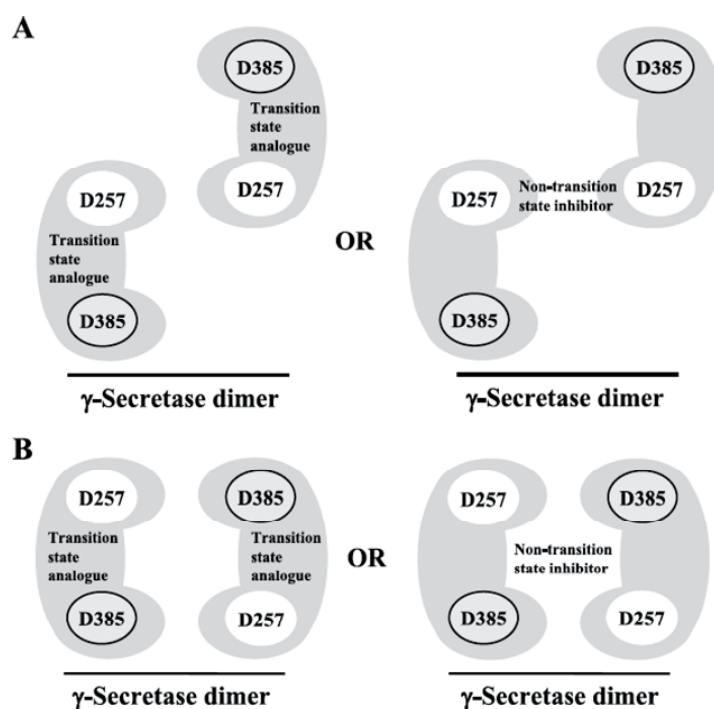
Figure 1.7: Models of the interaction of the γ-secretase enzyme with different types of γ-secretase inhibitor. **(A)** This model is analogous to the dimerization of the G protein-coupled dopamine D2 receptor. It also accounts for the proximity of the two individual PS1-NTF subunits, which harbor the two Asp-257 residues in a dimeric complex. A close alignment of PS1-NTFs in the γ-secretase complex was implied by the generation of PS1-NTF dimmers upon photoaffinity labeling. **(B)** An alternative model of the γ-secretase dimer, envisaging the interaction of the enzyme with the different

types of inhibitor.[100] (Reprinted from *The Journal of Biological Chemistry* © copyright 2006 by The American Society for Biochemistry and Molecular Biology)

Transition state analogue inhibitor binding depends on the presence of two transmembrane aspartate residues that are required for catalysis. Putative binding of the small molecule-type inhibitors to a unique site at the dimer interface could explain the 2:1 stoichiometry observed for the enzyme obtained from γ(NRC-F8)-secretase cells. These interactions are exclusive and lead to noncompetitive antagonism. Essentially, binding of either type of inhibitor to its respective binding site introduces allosteric changes in the enzyme, preventing the binding of the alternative type of inhibitor. NSAID-type γ-secretase modulators target none of these two binding sites but change their conformation. In contrast, the NSAID and γ-secretase modulator, sulindac sulfide displayed noncompetitive antagonism for all types of inhibitor. This finding suggested that non-steroidal anti-inflammatory drug-type γ-secretase modulators target an alternative site on the enzyme, thereby changing the conformation of the binding sites for γ-secretase inhibitors.

It was predicted that there is the presence of at least three binding sites on the γ-secretase enzyme. Each being specifically targeted by a separate class of compounds: transition state analogue inhibitors, non-transition state analogue inhibitors and NSAID-type modulators. [100]

1.2.3. Role of Casin Kinase-1 in Regulation of Aβ

Recently, Flajolet et al. demonstrated using an *in silico* analysis that APP, β-secretase and γ-secretase subunits contain, multiple CK1 consensus phosphorylation sites in their intracellular regions.[101] Many of them are conserved among human, rat and mouse species. Overexpression of constitutively active CK1ε, one of the CK1 isoforms expressed in brain, leads to an increase in Aβ peptide production. The human, rat and mouse amino acid sequences corresponding to amyloid-β related proteins (APP, BACE, PS1, PS2, Aph-1, Pen-2 and nicastrin) were screened for the presence of putative CK1 phosphorylation sites by using different computational tools (e.g., ELM-motifs; http://elm.eu.org, NetPhos 2.0) The *in silico* analysis of intracellular domains in APP, BACE, PS1, PS2, Aph-1, Pen-2 and nicastrin revealed the presence of numerous putative phosphorylation sites, particularly in PS1 and PS2. Moreover, the same *in silico* analysis of the two kinases which have been involved in AD (Cdk5/p35 and GSK3-β) also showed the presence of numerous putative phosphorylation sites for CK1, conserved in human, rat and mouse sequences.
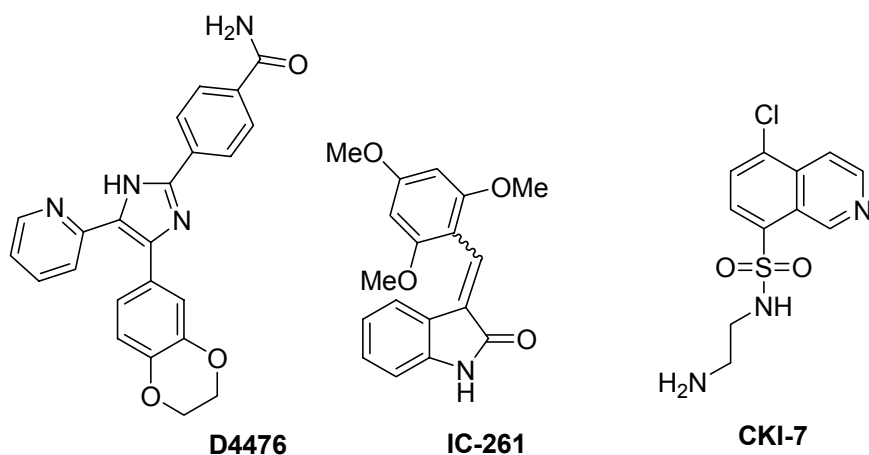
Scheme 1.5: Chemical structures of CK1 inhibitors **D4476**, **IC-261** and **CKI-7**.

The effect of the three different classes of CK1 inhibitors, **IC-261**, **D4476** and **CKI-7** was compared on Aβ peptide production in N2A cells expressing APP-695 (**Scheme 1.5**). Aβ$_{40}$ peptide production was significantly reduced after 3 h of incubation with **IC261** or **D4476** and Aβ$_{42}$ peptide production was significantly reduced after 3 h of incubation with each of the three inhibitors. The reduction was shown by using two different methods: sandwich ELISA and Western blot analysis after immunoprecipitation. None of the CK1 inhibitors inhibited Notch cleavage. Three structurally unrelated CK1 inhibitors significantly decrease Aβ peptide production indicating that the three inhibitors might be acting through a common target, namely CK1. This hypothesis is further supported by the fact that constitutively active CK1ε leads to increased Aβ peptide formation. Altogether, these results indicated the involvement of CK1ε activity in the regulation of the APP γ-secretase cleavage. Notch cleavage was not affected by any of the CK1 inhibitors tested and this indicates that CK1 acts directly or indirectly on the γ-secretase activity to selectively interfere with the APP γ-cleavage site but not at the Notch/APP CK1 ε-cleavage site.

1.3. Non-Steroidal Anti-Inflammatory Drugs and AD

Brain of AD patients show a number of pathological abnormalities such as a profound loss of synapses, profuse reactive gliosis, microglial proliferation and ultimately indication of inflammatory processes.[102] Increasing evidence suggests that a large number of inflammatory mediators are elements of the neuropathology linked with brain degeneration in AD. There are several factors identified in the brain of AD patients which include activated complement proteins, cytokines, chemokines, acute phase reactants, proteases and their inhibitors, proteoglycans, growth factors and various other enzymes. Due to these observations, different inflammatory hypotheses for AD have been proposed. One of these postulates states that, neurodegeneration in AD brain is secondary to an inflammatory response to SPs and NFTs rather than to these hallmarks themselves. Moreover, inflammation initiates the formation of SPs and NFTs and their progressive accumulation which in sequence activates immune reactions that force a self-sustaining "auto destructive" process (**Figure 1.8**). Thus, these hypotheses propose that in both circumstances chronic inflammation has a fundamental role in AD pathogenesis.
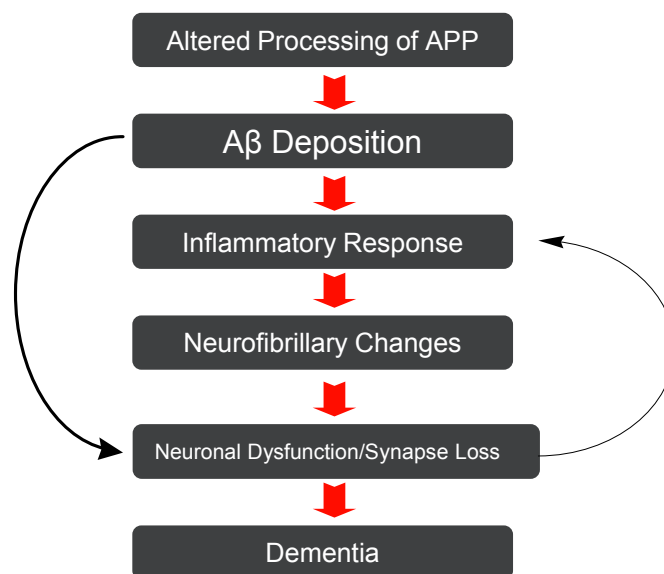

Figure 1.8: Role of inflammation in dementia.

This hypothesis is further supported by the several genetic studies indicating that polymorphisms of some inflammatory genes (i.e., interleukin (IL)-1a; IL-1b; tumor necrosis factor (TNF)-α; α2-macroglobulin, α1- antichimotrypsin) increase the risk of AD.[103] Epidemiological studies demonstrated that use of NSAIDs, may delay or prevent the onset of AD. They slow its progression and reduce the severity of cognitive symptoms.[104, 105] The duration of the treatment is critical for the outcome as long-term treatment is more beneficial compared to the short-term treatment.[104] Moreover, the type of NSAIDs is also crucial for the outcome e.g. ibuprofen, sulindac, indomethacin offers the best protection where as naproxen does not. All this information and the observations supported the rationale for clinical trials of different NSAIDs in AD patients.

1.3.1. Epidemiological Studies

Epidemiological studies indicated that, the long term use of anti-inflammatory drugs may delay the development of AD. For example, patients with rheumatoid arthritis have a lower prevalence rate of AD compared to control patients and this is due to the fact that patients with rheumatoid arthritis usually receive long-term treatment with anti-inflammatory agents including NSAIDs.[106] The results of a co-twin control study[107] and population based cohort studies demonstrated that continuous use of NSAIDs is associated with a delayed onset and reduced risk of AD (**Figure 1.9**).[102] A recent prospective study concluded that long-term NSAID use might reduce the risk of AD, only if such use occurs well before the inception of dementia.[108] Based on these epidemiological observations, neuropathological observations, genetics, *in vitro* and animal experiments, COX activity has been considered a compelling target for therapeutic intervention
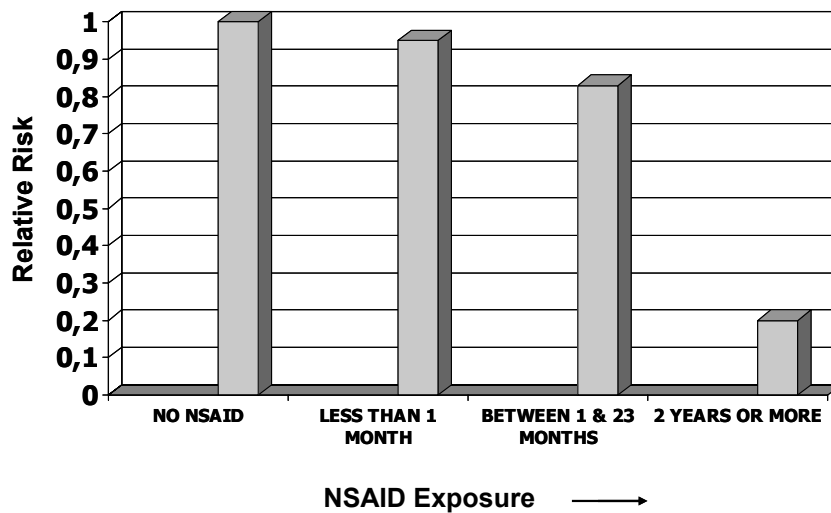
Figure 1.9: Schematic illustration indicating NSAID exposure and the reduced risk of AD.

NSAIDs represent the most widely used therapeutic agents. Conventional NSAIDs reversibly inhibit the COX activity of the enzyme prostaglandin H synthase, which converts arachidonic acid to prostaglandin H2, the immediate precursor of various prostaglandins and thromboxane.[109]

1.3.2. APP Metabolism by NSAIDs

It is well documented that NSAIDs can modulate the secretion of sβ-APP from different neuronal cell lines, astrocytes and neurons *via* a protein kinase C (PKC)-mediated

mechanism.[110] Weggen et al. in their very first study reported three NSAIDs which displayed selective $A\beta_{42}$-lowering activity in a variety of stable transfected cell lines.[111] Two structurally closed compounds, sulindac sulfide and indomethacin lowered $A\beta_{42}$ (IC_{50} = 25–50 μM) whereas the ibuprofen, lowered $A\beta_{42}$ with an IC_{50} of around 250 μM. At maximal non-toxic concentrations, 70–80% $A\beta_{42}$ inhibition was observed without significant reduction of $A\beta_{40}$ levels. Interestingly, this activity was not associated with all NSAIDs and negative results were observed with other commonly prescribed NSAIDs such as naproxen and aspirin as they did not affect either $A\beta_{40}$ or $A\beta_{42}$ levels.[111] Later Eriksen et al. identified other NSAIDs with $A\beta_{42}$-lowering activity such as fenoprofen **36**, flurbiprofen **32** and meclofenamic acid **37**.[112]

32 *R*-Flurbiprofen
$A\beta_{42}$ IC_{50} = 307 μM

33 Sulindac sulfide
$A\beta_{42}$ IC_{50} = 34 μM

34 Indomethacin
$A\beta_{42}$ IC_{50} = 25-50 μM

35 Ibuprofen
$A\beta_{42}$ IC_{50} = 200 μM

36, Fenoprofen

37, Mechlofenamic acid

38 Celecoxib
$A\beta_{42}$ EC_{50} = 10 μM
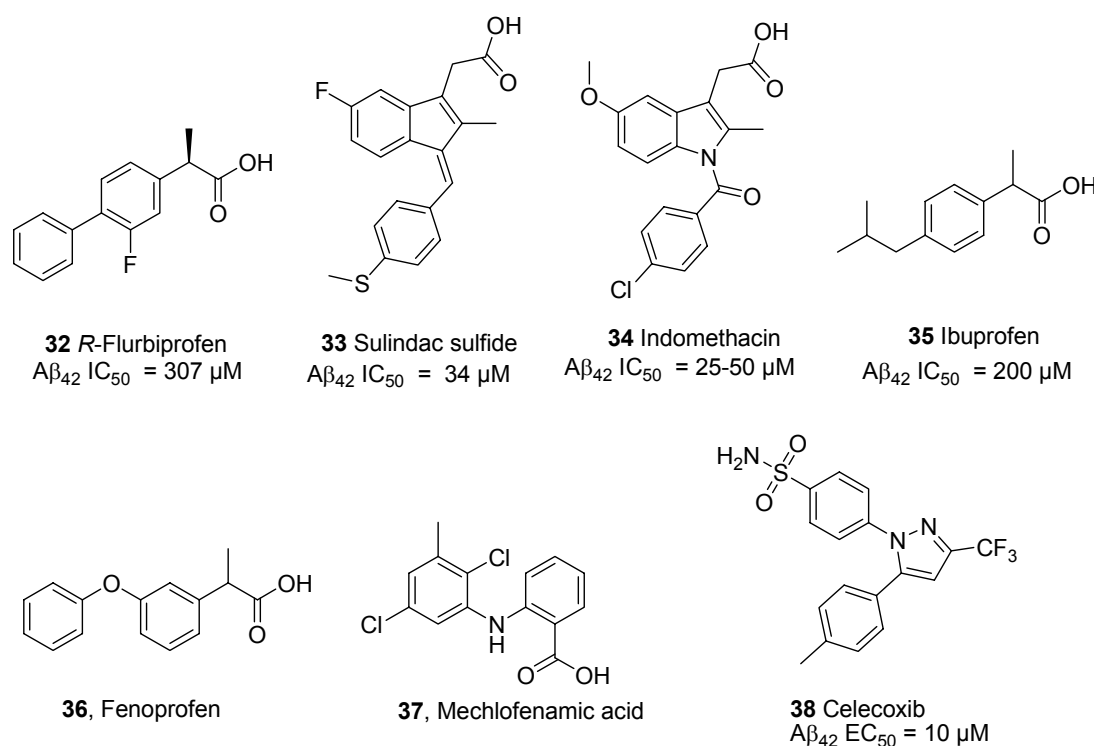
Scheme 1.6: NSAIDs that modulate γ-secretase activity.

Some COX-2-specific inhibitors, including celecoxib **38** and other structurally related compounds such as the peroxisome proliferator-activated receptor-γ (PPAR-γ) antagonist, fenofibrate were found to increase $A\beta_{42}$ levels selectively.[113, 114] Furthermore, cell based studies with $A\beta_{42}$-lowering compounds have revealed that γ-secretase modulators do not affect the cleavage of other γ-secretase substrates such as Notch and others.[111, 114-118] Assays with APP-transfected cell lines revealed that, $A\beta_{42}$-lowering NSAIDs do not change APP expression, turnover, internalization or release of the APP ectodomain. Significantly, in contrast to the conventional γ-secretase inhibitors NSAIDs do not cause accumulation of APP *C*-terminal fragments.[111, 114, 115] Some NSAIDs display their Aβ-lowering effect by inhibiting the small GTP-binding protein Rho and its effector, Rho associated kinase (Rock).[119] Interestingly, some NSAIDs could at least *in vitro* shift the β-APP metabolism towards shorter and less fibrillogenic forms of the Aβ peptides (**Figure 1.10**). Ibuprofen **35**, sulindac **33** and indomethacin **34** modulate APP metabolism to explicitly generate less $A\beta_{42}$, the most toxic species which is prone to aggregate and more soluble $A\beta_{38}$ by interfering directly with the activities of β- and γ-secretases which are the two important enzymes in this metabolic pathway. Furthermore, treatment with sulindac sulfide **32**, indomethacin **33** and ibuprofen **35**

(**Scheme 1.6**) did neither impair γ-secretase-mediated NOTCH receptor cleavage and NICD formation nor the generation of the ICDs of APP and the ErbB-4 receptor.[111, 114, 116, 118]
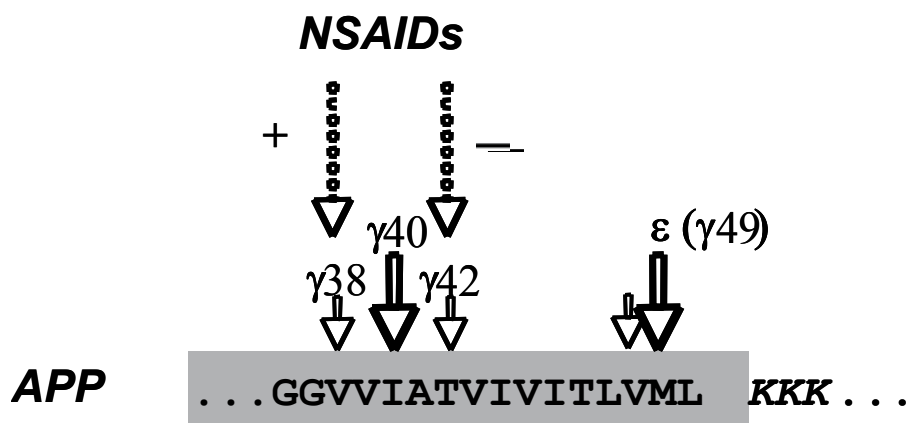

Figure 1.10. APP metabolism by NSAIDs.
Heterogeneous cleavage at the γ-site generates different Aβ species by cleavage at the γ38, γ40 and γ42 sites. A subset of NSAIDs increases the cleavage at the γ38 site while reducing cleavage at the γ42 site.[120] (Reprinted from *The Journal of Medicinal Chemistry* with permission © copyright 2007 by the American Chemical Society)

This consistently unaffected ICDs generation of several γ-secretase substrates indicate that γ-secretase modulators and improved compounds following the similar mode of action may avoid the toxicity which is associated with γ-secretase inhibitors such as **23**. Substantial progress has been made to determine the mechanism of action of Aβ$_{42}$-lowering compounds.

39 Aβ$_{42}$ IC$_{50}$ = 41 μM   **40** Aβ$_{42}$ IC$_{50}$ = 20 μM   **41**, Carprofen

42   **43**
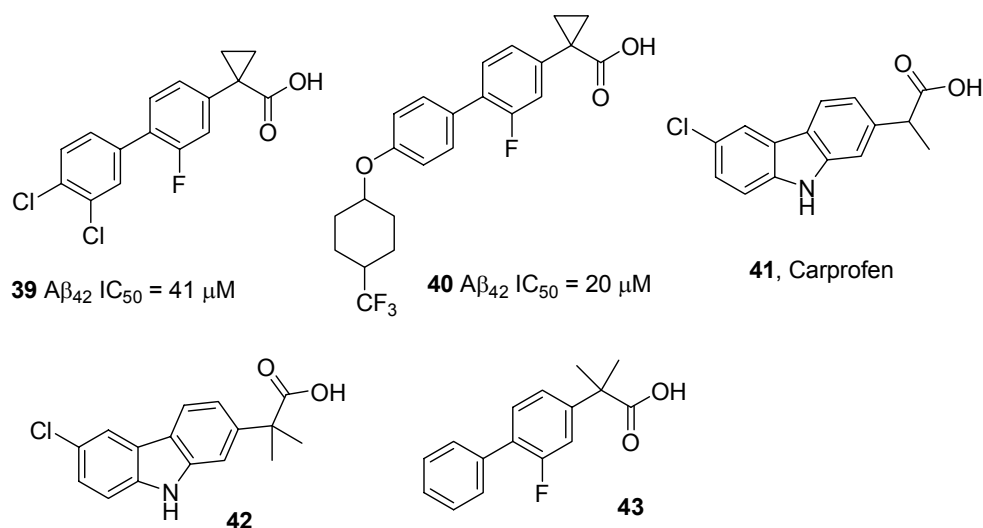
Scheme 1.7: NSAIDs derivatives that modulate γ-secretase.

However, several arguments rule out any involvement of COX in the Aβ$_{42}$-lowering activity such as: only a few NSAIDs display Aβ$_{42}$-lowering activity, whereas all NSAIDs inhibit COX,[111] the Aβ$_{42}$-lowering activity of sulindac sulfide was not impaired in COX-1/-2-deficient cells,[111] and NSAID derivatives have been reported which lower Aβ$_{42}$ devoid of their COX inhibitory activity.[112, 115, 120, 121] Peretto et al. reported novel cyclopropylated

flurbiprofen analogues (**Scheme 1.7**) with potent and selective inhibitory activity on $A\beta_{42}$.[121] Compared to flurbiprofen, the compounds **39**, **40** displayed improved potency on $A\beta_{42}$. Moreover, introduction of the cyclopropyl substituent at the alpha position caused almost complete loss of COX-1 inhibitory activity. In rats, compounds **39**, **40** showed good oral bioavailability and long elimination half-life. Short-term studies in a transgenic mice showed that compounds **39**, **40** decreased plasma $A\beta_{42}$ concentrations significantly. Carprofen **41**, a COX-2 inhibitor approved for the use in dogs, cows and horses is a weak inhibitor of γ-secretase. The geminal dimethyl derivatives of Carprofen and Flurbiprofen (**42**, **43**) displayed 40% and 67% inhibition of $A\beta_{42}$ production respectively in HEK cells at 100 μM concentration [122].

1.3.3. Long-Term and Short-Term Treatment Studies with $A\beta_{42}$-Lowering Compounds

Several long-term and short-term treatment studies in APP-transgenic mouse models of AD have been performed with $A\beta_{42}$-lowering NSAIDs. Even before the $A\beta_{42}$-lowering activity of certain NSAIDs was reported, Lim et al. demonstrated that chronic treatment with high doses of ibuprofen for 6 months strongly reduced both amyloid pathology and inflammatory responses in Tg2576 mice.[123] In animals treated with ibuprofen, the total number and area of $A\beta$ plaques was reduced by 50% while the soluble and insoluble $A\beta$ in the brain was reduced by 30–40%. Significant reductions in the number of plaque-associated activated microglia cells and lowered levels of pro-inflammatory markers were also observed.[123] Indomethacin was found to reduce amyloid pathology in Tg2576 mouse model but celecoxib and nimesulide, did not show significant reductions in amyloid pathology.[124, 125] Short-term treatment studies have demonstrated that $A\beta_{42}$-lowering NSAIDs can lower $A\beta_{42}$ levels in the brains of young, plaque-free APP-transgenic mice. In the initial experiments, 3-month-old Tg2576 mice were orally dosed for 3 days with 50 mg/kg/day of ibuprofen or naproxen.[111] Treatment with ibuprofen resulted in a 39% decrease in SDS-soluble $A\beta_{42}$ without any changes in $A\beta_{40}$ levels, whereas naproxen displayed no effect. Further short term studies have shown *in vivo* $A\beta_{42}$-lowering activity for additional NSAIDs including sulindac sulfide, indomethacin and flurbiprofen.[112] Only a few clinical trials with NSAIDs in AD patients have been conducted to date. In the very first trial with indomethacin, a slowing of the cognitive decline was observed.[126] Severe side effects caused due to the long-term use of NSAIDs hamper the clinical use of NSAIDs, especially in elderly AD patients. Another promising strategy may be the application of specific NSAID enantiomers devoid COX inhibitory activity. A particular interesting compound is the (*R*)-enantiomer of flurbiprofen, which lacks COX inhibitory activity, but is equipotent in reducing $A\beta_{42}$ *in vitro* and *in vivo* compared to (*S*)-flurbiprofen.[112, 115] (*R*)-flurbiprofen is under development for treatment of AD. A phase II clinical trial with duration of 12 months has recently been completed. Results indicated that the drug was well tolerated and positive results were observed with the highest dose (800-mg, twice-daily) in patients with mild AD.

1.3.4. NSAIDs as Agonist of Peroxisome Proliferator-Activated Receptor- γ (PPAR-γ)

Apart from COX inhibition, another concerned mechanism of NSAIDs by which they exert the modulatory activity is the activation of the PPAR-γ. PPAR-γ is a member of the nuclear receptor family of transcription factors. It is expressed in monocytes/macrophage-like cells and its activation is related to the decrease in the expression of inflammatory genes,[127] and the creation of cytokines with potent inflammatory actions (i.e., IL-1, IL-6, TNFα).[128] A few NSAIDs when used at high concentration were found to act as direct PPAR- γ ligands and as a result they reduced cytokine production.[129] *In vitro* studies have demonstrated that indomethacin and ibuprofen, both can activate PPAR-γ in microglia, reduce the $A\beta$-mediated secretion of inflammatory cytokines and neurotoxicity.[130] Since PPAR-γ is involved in AD pathology, it is hypothesized that some of the beneficial effects from the treatment of NSAIDs

in AD may be due to their action on PPAR-γ and subsequent reduction of brain inflammation.[131, 132] But, it is unlikely that this mechanism is involved in the Aβ-lowering effect of NSAIDs, because a direct agonist for PPAR-γ, pioglitazone, failed to affect amyloid formation and deposition in an animal model of AD-like amyloidosis.[133]

1.3.5. Prevention of NF-kB Activation

NF-kB, a transcription factor is involved in the regulation of several cellular target genes.[134] It is expressed throughout in the CNS and is present in neurons and glial cells. It could be a positive regulator of genes whose products mediate acute-phase response, nitric oxide and a full array of inflammatory cytokines. It is well known that NSAIDs prevent the activation of nuclear factor-kB (NF-kB). *In vitro* studies have revealed that various pathogenetic stimuli can activate this factor such as: Aβ peptides and different NSAIDs which can directly prevent this activation with subsequent reduction of the inflammatory responses. NF-kB expression is increased in AD brains and at least *in vitro* this factor can regulate Aβ formation in a neuronal cell line.[135] Recently, it was reported that activated NF-kB is increased in brains of Tg2576 compared with wild type littermates and this enhancement was associated with the deposition of Aβ. Treatment of indomethacin in Tg2576 for seven days revealed that the activity of NF-kB was significantly reduced along with a reduction of reactive astrocytosis.

1.4 Role of Curcumin in AD

Curcumin is a major component of the yellow spice turmeric.[136] This spice is used in traditional diet and as an herbal medicine in India. It is derived from the plant *Curcuma longa* Linn and its preparations have been used to treat various diseases for centuries in Ayurvedic medicines. Curcumin and its constituents are currently undergoing scientific evaluation for their efficacy as anti-inflammatory agents, in preventing and treating cancer[137, 138] for the treatment of human immunodeficiency virus (HIV) infection,[139] and for the treatment of cystic fibrosis.[140] It has been demonstrated that curcumin has anti-oxidant, anti-inflammatory and cholesterol lowering properties. These three properties are believed to play a crucial role in the processes involved in the pathogenesis of AD (**Figure 1.11**). The frequency of AD in India is roughly one-quarter of that in the US in patients between 70 and 79 years and one of the lowest prevalence rates of AD in the world since turmeric consumption is widespread India.

Oxidative damage plays a significant role in the pathogenesis of AD. Increased levels of the oxidized forms of various organic molecules such as lipids, proteins, DNA and carbohydrates have been found in brain, cerebrospinal fluid (CSF), blood and/or urine of AD patients.[141, 142] Oxidative stress is the net effect of unregulated production of reactive oxygen species (ROS), such as hydrogen peroxide, nitric oxide, superoxide and the extremely reactive hydroxyl radicals. Epidemiological studies have indicated an association between dietary antioxidant consumption and a reduced risk for the development of AD.[143] A clinical trial suggested a probable benefit for the anti-oxidant alpha-tocopherol (vitamin E) in slowing the development of established AD.[144] Curcumin displays potent antioxidant activity. Reddy et al. [145] demonstrated that curcumin inhibits lipid peroxidation. Rao et al. [146] reproduced this in a different *in-vitro* model and found that curcumin **44**, demethoxycurcumin **45** and bisdemethoxycurcumin **46** are more potent antioxidants than alpha-tocopherol (**Scheme 1.8**). Curcumin prevents oxidative damage of DNA in mouse fibroblasts.[147] Kim et al. illustrated that the curcuminoids are more potent antioxidants than alpha tocopherol using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical trapping assay.[148]
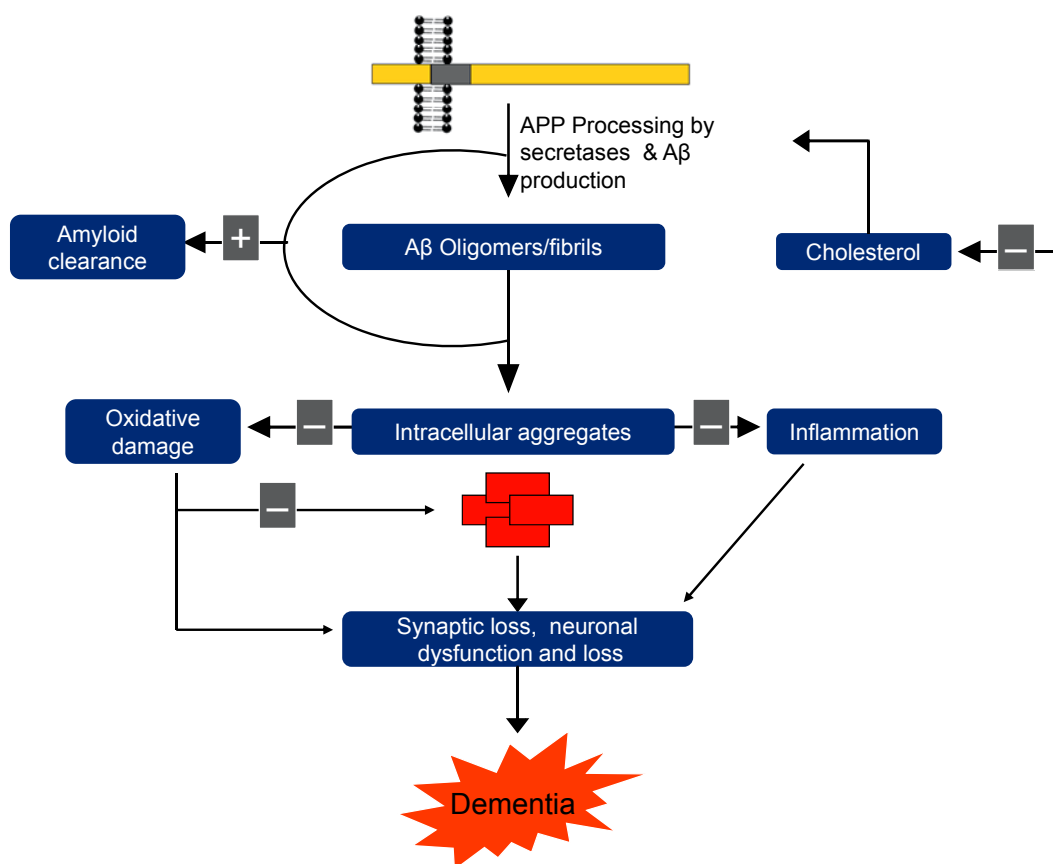
Figure 1.11: Curcumin has multiple targets in AD.

It reduces the Aβ load by inhibiting Aβ$_{42}$ fibril formation and disaggregating preformed fibrils. Curcumin acts as an antioxidant: it reduces oxidative damage, synaptic loss and neuronal dysfunction. Curcumin curtails the damage by inhibiting NF-kB induced *i*NOS, COX-2 and inflammatory cytokinin production by reactive glia. Lastly, curcumin can lower plasma and tissue cholesterol, potentially lowering Aβ production.
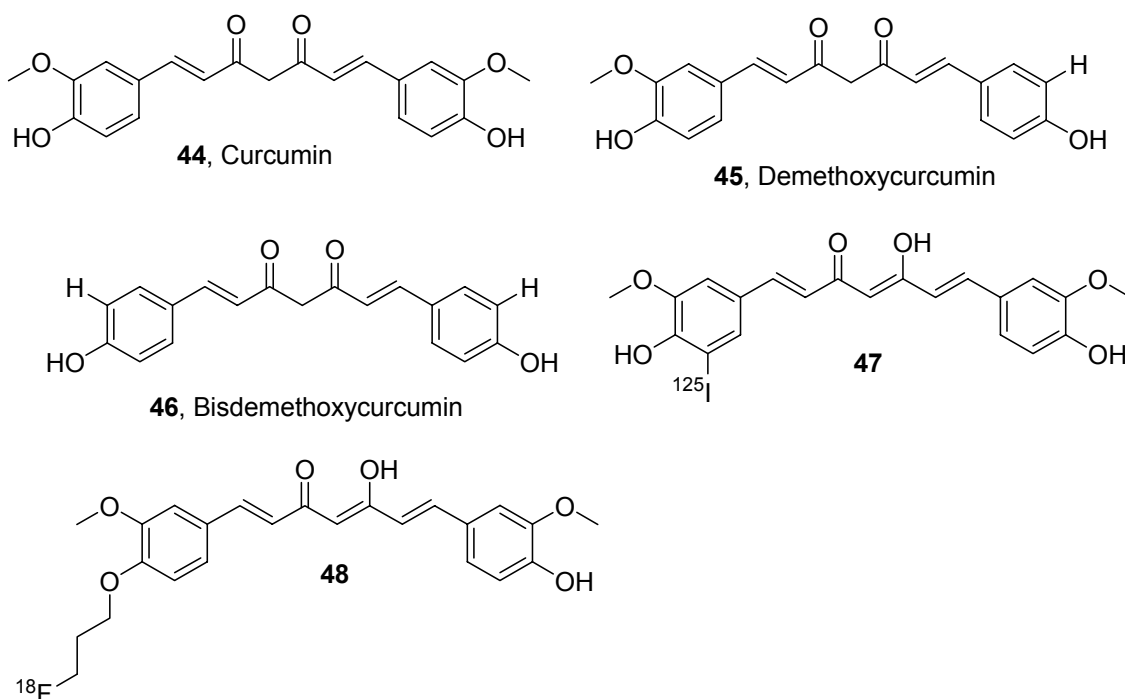
Scheme 1.8: Chemical structures of curcumin, its isomers and curcumin derived radiolabels.

Inflammation is presumed to play a crucial role in AD pathogenesis.[149] Curcumin shows anti-inflammatory effects therefore may have a role in slowing or halting AD. Curcumin inhibits lipoxygenase and COX-2, enzymes which are responsible for the synthesis of the pro-inflammatory leukotrienes, prostaglandins and thromboxanes.[150] It also inhibits AP-1 mediated transcription linked to cytokine regulation *in vitro*,[151] and represses inducible nitric oxide synthase (iNOS) in activated macrophages.[152] These processes are responsible for promotion of inflammation.

Epidemiological studies have indicated that high levels of cholesterol may contribute to the pathogenesis of AD. Persons with high levels of cholesterol have an increased susceptibility to AD apparently influenced by the *APOE* ε-4 genotype. Moreover, AD patients have increased levels of total serum and low density lipoprotein (LDL) cholesterol along with reduced levels of high density lipoprotein (HDL) in their plasma as compared to age match controls. Cholesterol unusually accumulates in the dense cores of Aβ plaques in the brain of AD patients. Epidemiological studies have revealed that use of 3-hydroxy-3- methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors have been associated with a reduced risk of AD.[153] HMG-CoA is involved in the synthesis of cholesterol and cholesterol has an influence on Aβ deposition. It is believed that cholesterol interacts with the amyloid cascade in the pathogenesis of AD. Moreover, generation and clearance of Aβ is regulated by cholesterol. Soni et al. demonstrated that administration of 500 mg of curcuminoids a day for 7 days reduced levels of serum cholesterol and lipid peroxides in healthy volunteers.[154] These observations indicate that there might be an additional mechanism by which curcumin might exert beneficial effects in AD.

It is well documented that toxic effects of Aβ on normal cellular function and viability trigger the pathogenesis of AD. $Aβ_{42}$ has been demonstrated to have adverse effects in a number of cell culture models. Kim et al. demonstrated that curcuminoids protects PC12 rat pheochromocytoma and human umbilical vein endothelial cells from $Aβ_{42}$ injury and speculated that curcumin's antioxidant properties might have mediated this effect.[148] Aggregation of Aβ into fibrils and the subsequent formation of Aβ plaques are speculated to be crucial steps in the pathogenesis of AD. Therefore, these processes are potential targets for AD therapy. Ono et al. carried out an *in vitro* study and measured the effects of curcumin on the formation of Aβ fibrils from $Aβ_{40}$ and $Aβ_{42}$.[155] They found that curcumin inhibits the formation and extension of Aβ fibrils and destabilizes preformed Aβ fibrils in a dose-dependent manner at 0.1-1 μM concentrations. Yang et al. demonstrated that curcumin directly binds small Aβ species to block aggregation and fibril formation, both *in vitro* and *in vivo*.[156] Under aggregating conditions *in vitro*, curcumin inhibited aggregation (IC_{50} = 0.8 μM) and disaggregated fibrillar $Aβ_{40}$ (IC_{50} = 1 μM), indicating favorable stoichiometry for inhibition. Curcumin was found to be a better $Aβ_{40}$ aggregation inhibitor compared to ibuprofen and naproxen and prevented $Aβ_{42}$ oligomer formation and toxicity at 0.1 and 1.0 μM concentration. Therefore, curcumin has primary effects on Aβ aggregation apart from its antioxidant, anti-inflammatory and platelet aggregation inhibiting properties. It has been reported that curcumin has favorable brain permeability and satisfactory Aβ plaque binding properties. Those were predicted from the fluorescence staining of Aβ plaques in brain sections from APPsw transgenic mice administered with curcumin by injection or in diet[156]. Recently Ryu et al. demonstrated that the fluoropropyl-substituted curcumin **47** shows the highest binding affinity (*Ki* 0.07 nM) whereas curcumin exhibited binding affinity for Aβ (1-40) aggregates (*Ki* 0.20 nM). The attachment of iodine to its phenyl ring **48** significantly decreased its binding affinity by 47-fold (**Scheme 1.8**). The radiolabelled fluoropropyl-substituted curcumin was evaluated as a potential probe for Aβ plaque imaging. Partition coefficient measurement and biodistribution in normal mice demonstrated that **48** has a suitable lipophilicity and reasonable initial brain uptake. Metabolism studies also indicated

that [^{18}F] curcumin is metabolically stable in the brain. These results suggested that **47** is a suitable radioligand for Aβ plaque imaging.

Altogether, these properties collectively make curcumin a potential druglike candidate for AD. Widespread use of curcumin as a food additive and relatively small short-term studies in humans advocate its safety and tolerability. However, important information regarding curcumin bioavailability, safety and tolerability especially in an elderly population is still lacking.

1.5. Photoaffinity Labelling

In spite of being over forty years old, photoaffinity labelling is still a useful and reliable method for the structural investigation of receptor binding sites. It remains a classical research area for chemists who design and synthesize new photoactivable reagents. Biochemists and biologists then use these reagents to identify and characterize the target receptors. Investigation of receptor-ligand interactions remains a never-ending challenge for chemists and biologists. Structural exploration of biological receptors is the starting point for a better understanding of how they function and how they can be targeted. Photoaffinity labelling is widely used as a tool for labelling polypeptides with photoactive ligands. In many cases these ligands such as radiolabelled ligands or affinity ligands allow the characterization of functional proteins on sodium dodecyl-sulfate (SDS) gels (**Figure 1.12**). However, only a few of these experiments led to the identification of the amino acid residues labeled by the photosensitive probes. This ultimate step gives important structural information regarding the position of the binding site on the primary structure of a receptor and precise data on the ligand-receptor interaction.
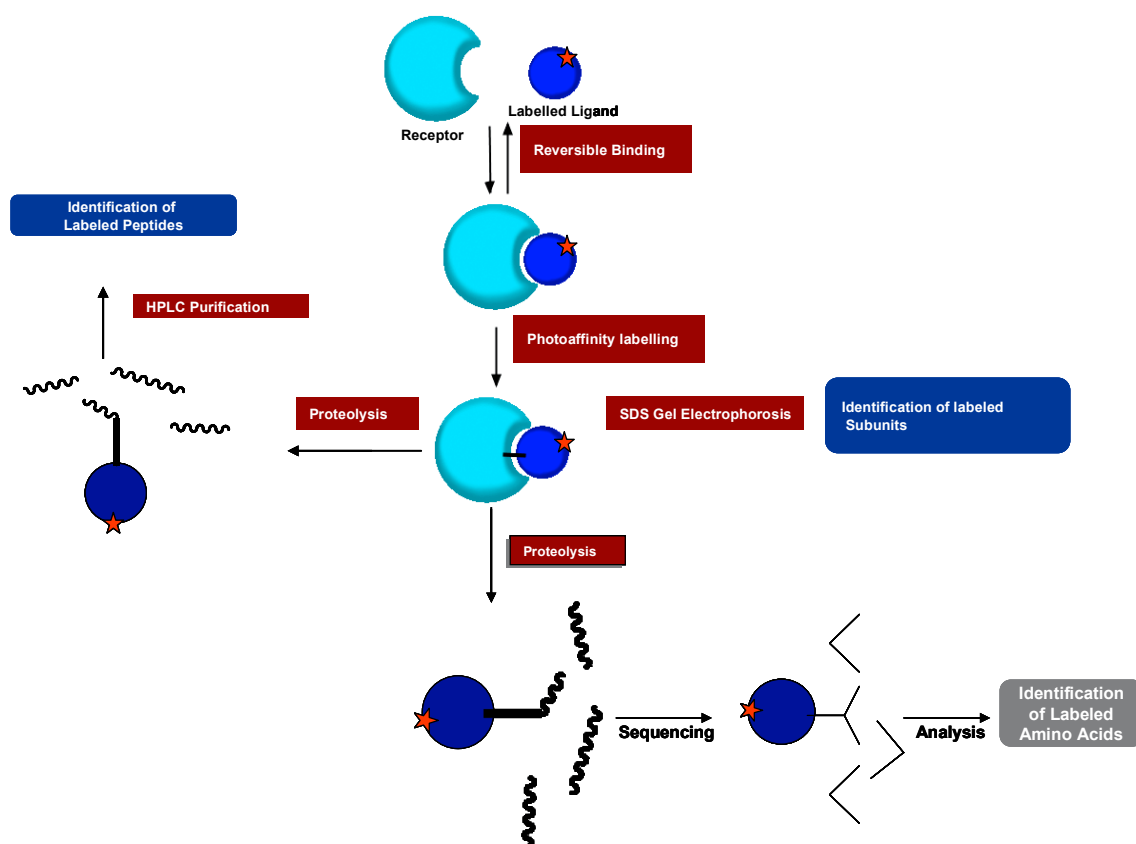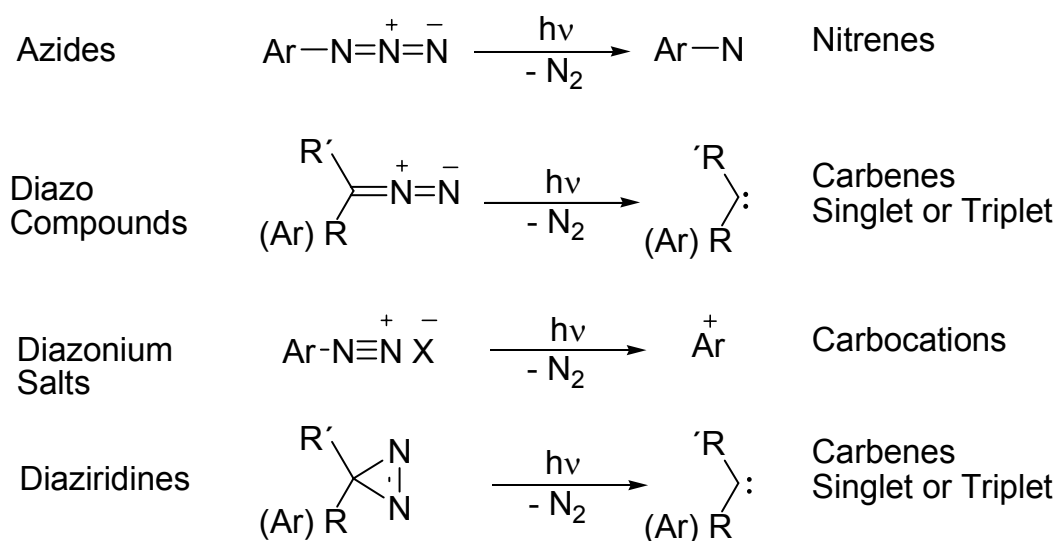

Figure 1.12: Principle and successive steps for the identification of amino acids utilizing photoaffinity labelling technique.

1.5.1. Typical Compounds Used as Photoreative Groups

Commonly used photoreactive groups in photoaffinity labelling are summarized in (**Scheme 1.9**).[157] Aryl azide generates nitrene as a very reactive intermediate species. Diazirine and its linear isomer, diazo, generate carbenes as highly reactive intermediate. Since the discovery of photoaffinity labelling by Westheimer in 1962, many efforts have been devoted to develop various photoreactive groups. One must consider several undesirable features before using azides as photoreactive groups. Notably shorter wavelengths are usually required for azides to achieve effective photodecomposition without significant damage to the bioactive macromolecules. Although the chemistry of intermediate carbenes and nitrenes remains to be investigated,[158] nitrenes are generally treated to be less reactive than carbenes.[159] Furthermore, the short-lived singlet phenylnitrenes rapidly rearrange into more stable ketenimines in which the reactivity is limited to nucleophilic groups.[160] If suitable nucleophiles are not found within the proximity of the ligand-binding site, ketenimines may migrate from the binding pocket to label remote nucleophiles in a nonspecific manner. Perfluorophenylazides can eliminate this undesired side reaction by increasing the energy barrier required for the ketenimine formation.[161, 162] The fluorine substitution on the *ortho* position of an azide group sufficiently increases the lifetime of the highly reactive singlet nitrene to accomplish the desired intermolecular reactions. Cross-linking with nitrenes formally produces nitrogen-carbon bonds or more labile nitrogen heteroatom bonds, whereas carbenes produce the formation of more stable carbon-carbon bonds. The search for replacing the azide photophore led to the discovery of 3-aryl-3-trifluoromethyldiazirines by Brunner et al.[163] Amongst diazirine derivatives, trifluoromethylaryl-diazirines meet most of the criteria required for an ideal photoreactive group. In addition to their excellent chemical stability prior to photolysis, diazirines can be rapidly photolyzed at wavelengths beyond the UV absorbance region of proteins.

Azides	$Ar-N=\overset{+}{N}=\overset{-}{N}$	$\xrightarrow[-N_2]{h\nu}$	$Ar-N$	Nitrenes
Diazo Compounds	$\underset{(Ar)\,R}{\overset{R'}{>}}=\overset{+}{N}=\overset{-}{N}$	$\xrightarrow[-N_2]{h\nu}$	$\underset{(Ar)\,R}{\overset{'R}{>}}:$	Carbenes Singlet or Triplet
Diazonium Salts	$Ar-\overset{+}{N}\equiv\overset{-}{N}\;X$	$\xrightarrow[-N_2]{h\nu}$	$\overset{+}{Ar}$	Carbocations
Diaziridines	$\underset{(Ar)\,R}{\overset{R'}{>}}\!\!\overset{N}{\underset{N}{\mid}}$	$\xrightarrow[-N_2]{h\nu}$	$\underset{(Ar)\,R}{\overset{'R}{>}}:$	Carbenes Singlet or Triplet

Scheme 1.9: Typical compounds used as precursor of reactive species in photoaffinity labelling.

1.5.2 Benzophenones as Photoractive Groups

Benzophenone (BP) photochemistry has attracted the attention of biochemists for more than three decades. The expanding use of BP photoprobes can be attributed to three distinct chemical and biological advantages.

1. They are chemically more stable than diazo esters, aryl azides and diazirines.

2. They can be manipulated at an ambient light and can be activated at 350-360 nm and thus avoid protein damaging wavelengths.
3. They preferentially react with unreactive C–H bonds, even in the presence of solvent water or bulk nucleophiles.

These three properties combine to produce highly efficient covalent modification of the macromolecules. These advantages outweigh the disadvantage of additional bulkiness and hydrophobicity resulting from the introduction of BP group.

1.5.2.1 Photochemistry of benzophenones

Absorption of a proton at ~350 nm results in the promotion of one electron of nonbonding sp^2-like n-orbital on oxygen to an antibonding π^* orbital of carbonyl group. In the diradicaloid triplet state **49**, the electron-deficient oxygen n–orbital is electrophonic and thus interacts with weak C–H σ bonds resulting in hydrogen abstraction to complete its half-filled n-orbital.
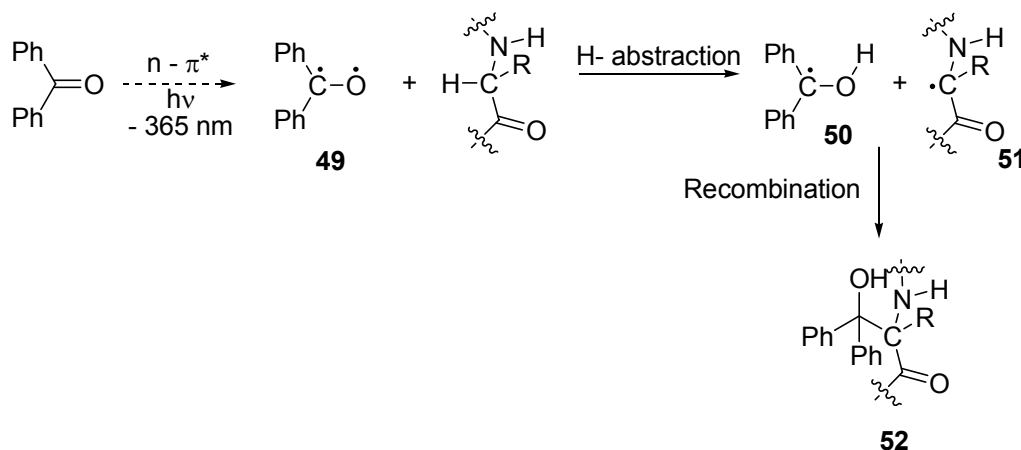
Scheme 1.10: Photochemistry of benzophenone chromophore.

When amines or similar heteroatoms are located near to the excited state carbonyl, an electron transfer step may be followed by proton abstraction from an adjacent alkyl group and a radical 1,2-shift. The ketyl **50** and alkyl radicals **51** that are formed recombine readily to generate a new C–C bond thus produces benzpinacol type compounds such as **52** (**Scheme 1.10**). This basic process, which is also used for remote site functionalization, is based on the observation that the diradicaloid species can only attack geometrically accessible C-H bonds.

There are various factors which controls the activity of BP photoprobe.

1.5.1.2.1. Lifetime

The lifetime of the excited state containing two unpaired electrons is much longer than the singlet state. Therefore, the triplet state may last up to 80-120 μs in the absence of an abstractable H. But, it may be 100 times shorter in the presence of a suitably oriented C–H bond. Increasing concentrations of an H-donor decrease the lifetime proportionally according to the Stern-Volmer equation,

$$\frac{1}{\tau} = \frac{1}{\tau_0} + k_2\,[RH]$$

where τ_0 is t he excited-state lifetime in the absence of RH (H-donor), τ is the excited-state lifetime in the presence of RH (H-donor) and k_2 is the second-order rate coefficient. The triplet state readily relaxes to the ground state if it does not find an H-donor with the required geometry. This relaxation process does not require a scavenger and this constitutes one of the major advantages of this photophore. BP probes relax electronically, maintain their binding and photoactivatable properties and then undergo many excitation- relaxation cycles until a favorable geometry for covalent modification is accomplished. The probability of achieving an optimal geometry for covalent attachment can be further improved by using a flexible linker, although more rigid linkers should give superior data in the topographic mapping of an active site.

1.5.1.2.2. Flexibility and proximity

Intramolecular BP photochemistry with long flexible chains grants multiple sites for attack.[164] Intermolecular reactions are very sluggish, are completely diffusion-controlled and lack the regio-selectivity. In the excited state, the BP ring system is required to be nearly planar, showing significant rigidity. This rigid system can be extended with rigid linkers to relocate the photophore and thus modify the efficiency and site of the resulting attachment. For biochemical experiments, linker size and orientation is critical, since the primary goal is to label the binding site for the ligand while minimizing nonspecific modifications. Typically, the BP photophore in biochemical probes are coupled with active ligands *via* linkers bearing either moderately flexible (1-3 CH_2 units, O, NH) or moderately rigid (C=O, COO) functionalities. In short, BP photochemistry in biochemical systems is most regioselective when the flexibility is limited to what which is necessary to achieve efficient H-abstraction.

1.5.1.2.3. Reactivity and effects of substituents

Besides the geometric factors, the reactivity and the efficiency of covalent attachment strongly depend on the chemical and electronic environment of the photophore and the H-donor moiety. The two major determining factors are: the strength of the bond being broken in the H-donor and the relative stabilities of the resulting alkyl radicals. The homolytic cleavage of C–H bonds, induced by the triplet biradical, is favored over the O–H bonds. In the case of N–H bonds, the electrophilic excited state abstracts an electron first and this event is followed by the H-abstraction from the adjacent C–H group. The most effective H-donors in the biological systems are the backbone C–H bonds in amino acids, polypeptides and carbohydrates. Methylene groups of lipids and amino acid side chains also offer abstractable hydrogens. In the absence of any orientational preference, the reactivity order for C–H bonds is: >NCH, > –SCH, > methine > C=CCH_2 > –CH_2 > –CH_3. The electron rich tertiary centers such as C_γ–H of leucine and C_β–H of valine and the CH_2 groups adjacent to heteroatoms in lysine, argenine and methionene are principally the reactive sites. In photoaffinity probes, BP is either connected to an active ligand or is used to replace a portion of the ligand, partially mimicking its geometry. The substituents on BP can affect the photochemistry dramatically. Electron-withdrawing groups increase the efficiency of H-abstraction. Electron donating groups and electron delocalization into aromatic and conjugated systems cause a partial shift of the electron transition from n–π^* to π–π^*, and the latter triplet state becomes much less reactive toward H-abstraction. Therefore, substituents can affect the steric accessibility, and the introduction of alkyl groups in the *ortho* or *meta* positions of the BP increases the possibility of intramolecular interactions which may reduce the efficacy of the probe.

1.5.1.2.4. Recombination products

Analysis of the site of covalent ligand attachment gives information regarding the exact location of the binding domain. Therefore a stable cross-linked complex is desired. The radical recombination product is suppressed only if a ring is the target, due to the additional

steric hindrance (**Scheme 1.11**). Thus, when proline was found as a main cross-linking site, a dehydroproline moiety **55** arose from a second H-transfer to the ketyl radical, resulting in elimination. Proton abstraction from secondary alcohols can lead to a second ketyl radical, which may form a ketone by the removal of second hydrogen. After recombination, the adduct is generally stable. However, if a glycine residue has been modified, the resulting benzhydrol can undergo elimination (dehydration), giving a highly conjugated species **53**. In general, benzpinacol formation accompanies the heteroradical recombination as a radical dimerization, although in low yield. With laser flash photolysis, the cross-linking can occur on the 2- or 4-position of the phenyl ring, forming highly colored species, referred to as light-absorbing transients **54**.
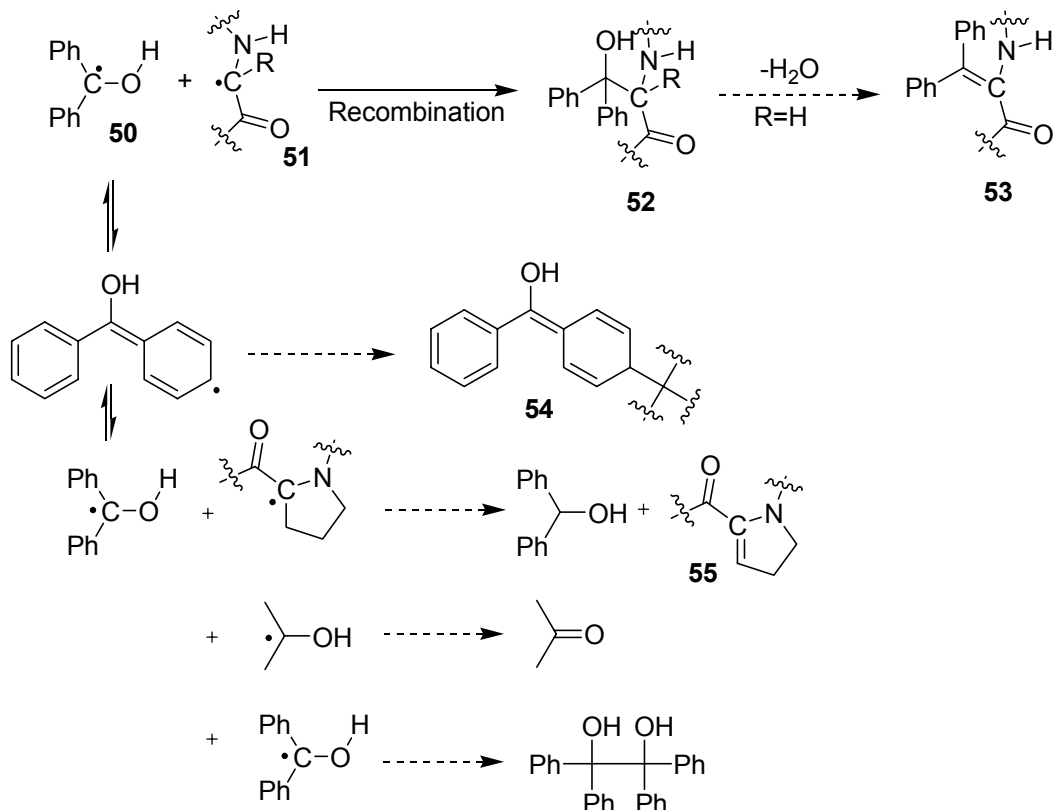
Scheme 1.11: Various radical recombination pathways of the benzophenone chromophore.

2. AIMS OF THIS STUDY

The final and the decisive step in the release of Aβ is carried out by γ-secretase. It is believed that the devastating neurodegenerative process observed in the AD is triggered by the pathological effects of Aβ peptides. $A\beta_{42}$ is the most toxic species of all the Aβ peptides secreted and forms the central core of the plaques. The work presented in this thesis aimed to synthesize novel chemical entities that modulate γ-secretase to secrete less $A\beta_{42}$ and to synthesize affinity or photoaffinity labels to characterize the active γ-secretase complex.

The specific aims in each of the studies were:

1. Synthesis of γ-secretase modulators using the scaffold of selective and nonselective COX inhibitors.

2. Synthesis of γ-secretase modulators using the scaffold of COX-2 selective inhibitors.

3. Synthesis of *N*-substituted carbazolyloxyacetic acids as potential γ-secretase modulators.

4. Synthesis of γ-secretase modulators using the scaffold of LXR-agonists *via* the structural modification.

5. Synthesis of affinity labels derived from the non-selective and selective COX inhibitors for the identification of the binding site *via* immunoprecipitation experiments.

6. Design and synthesis of flurbiprofen derived photoaffinity labels to identify the binding site on the active γ-secretase enzyme complex *via* covalent bond formation.

7. Design and synthesis of DAPT derived photoaffinity labels to identify the binding site and mode of action *via* covalent bond formation with the active site.

3. RESULTS AND DISCUSSION

3.1. Synthesis and Biological Evaluation of NSAIDs Derived γ-Secretase Inhibitors and Modulators.

"The most fruitful basis for the discovery of a new drug is to start with an old drug."

Sir James Black

The term Aβ comprises the peptides of 38-42 amino acids which are generated by the sequential cleavage of the APP by the secretases. $A\beta_{42}$ is more prone to aggregate into fibrils and are critical for formation of plaques. Epidemiological studies have indicated a reduced prevalence of AD among patients using prolonged treatment of NSAIDs. These protective effects of NSAIDs appear directly proportional to the length of treatment and depend on the specific chemical structure of the NSAIDs being used.[105] In spite of these encouraging results, negative results have been reported in all large, long-term, placebo controlled clinical trials which were aimed at reducing inflammation in the brain of AD patients since inflammation has a fundamental role in AD. More recently, it has been reported that some NSAIDs decrease the production of $A\beta_{42}$ *in vitro* and *in vivo*,[111, 112, 114] and impede the progression of $A\beta_{42}$ pathology in transgenic mouse models of AD.[165] The proposed mechanism for this activity is an allosteric modulation of PS1 which is the major component of the γ-secretase complex.[166] They seem to interfere with substrate recognition/cleavage and shift the precision of γ-secretase cleavage from the γ42 to the γ38 site to generate more of $A\beta_{38}$ and less of $A\beta_{42}$.[167, 168] Compounds with reverse shift were reported recently, they result in enhanced $A\beta_{42}$ production.[113] The inhibition of $A\beta_{42}$ is completely independent of their anti-cyclooxygenase activity,[125] and is related to the chemical structure of the NSAIDs but not to the class. Therefore some NSAIDs display modulatory activity (for e.g. ibuprofen, sulindac sulfide, flurbiprofen, indomethacin, diclofenac) where as others do not (naproxen, aspirin, celecoxib). Probably this might be the explanation for the negative outcomes of the large AD trials being carried out so far, since they were performed with compounds (for e.g. naproxen, hydroxychloroquine, dapsone, prednisone, rofecoxib and celecoxib) which are not able to decrease $A\beta_{42}$ production.[169] Unfortunately, the generic use of NSAIDs in AD is hampered by a significant gastrointestinal toxicity associated with COX inhibition. This prompted the identification of new NSAIDs analogues which are capable with potent and selective $A\beta_{42}$ lowering activity but devoid of COX inhibitory activity which may be suitable for chronic use in AD patients.

3.1.1. Flurbiprofen Derivatives as Potential γ-Secretase Modulators

3.1.1.1. Synthesis of flurbiprofen derived amides

Specifically, the initial efforts were concentrated on flurbiprofen since it is one of the most active and studied NSAID so far.[112] The *R*-enantiomer of flurbiprofen is much less active than the *S*-enantiomer on both COX-1 (IC_{50} μM of 44 versus 0.03 μM) and COX-2 (IC_{50} of 123 μM versus 0.91 μM) activity.[121] The *R*-enantiomer (MPC-7869) is reported to be in Phase-III clinical trial for AD in the USA. The IC_{50} of *R*-flurbiprofen on $A\beta_{42}$ (280 μM) is still higher than that on both COX-1 (44 μM) and COX-2 (123 μM). Flurbiprofen **32** (10 and 25 mg/kg/d) elicits non-selective reductions in both $A\beta_{40}$ and $A\beta_{42}$ plasma levels, but was

found to be toxic. It produced small reductions in Aβ_{40} in the cortex at 25 mg/kg/d, but did not affect Aβ levels in the hippocampus or CSF.

Flurbiprofen derivatives were synthesized *via* structural modification of acid moiety in order to increase Aβ_{42} lowering properties and to reduce or lower COX inhibitory activity. Initially flurbiprofen derived amides were synthesized to investigate the impact of amides on the γ- and β-secretase inhibitory activity as depicted in **Scheme 3.1**. The acid moiety of flurbiprofen **32** was coupled with various amines using EDAC and HOBt as coupling reagents in CH$_2$Cl$_2$ to afford the corresponding amides **56** (**Table 3.1**). All the compounds were purified by column chromatography. Only a few of them were characterized by NMR spectroscopy and rest of them were analysed by HPLC.
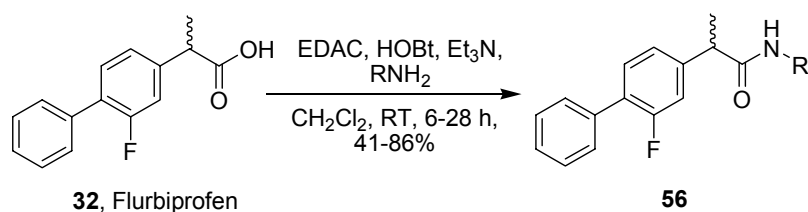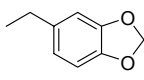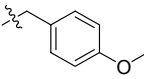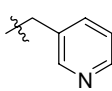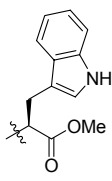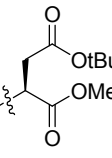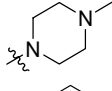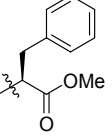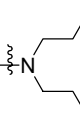

Scheme 3.1: Synthesis of flurbiprofen amides.

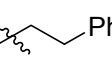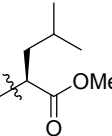The diastereomeric mixture was observed when serine *t*-butyl ester and tyrosine *t*-butyl ester were employed for coupling with racemic flurbiprofen. The diastereomers were separated by column chromatography using ethyl acetate:hexane (2:8) as eluents. (**Scheme 3.2**). The less polar and polar compounds in case of *S*-serine *t*-butyl ester were **56m** and **56n** and in case of *S*-tyrosinol *t*-butyl ester were **56o** and **56p** respectively. The compounds were submitted for the biological experiments without further assignments of absolute stereochemistry.
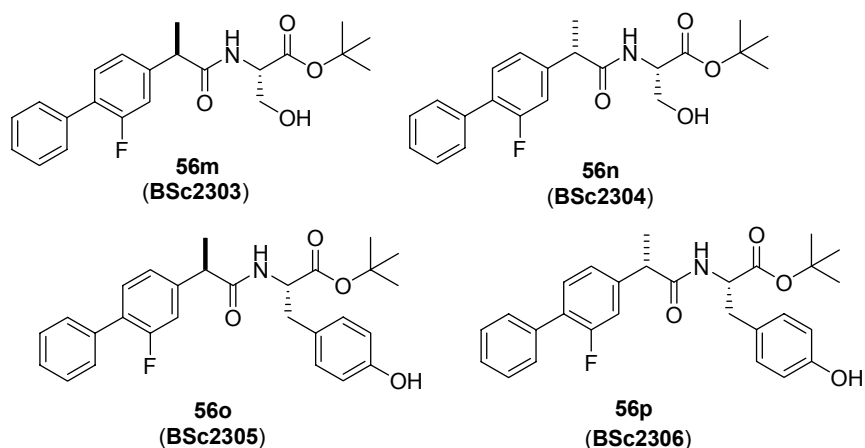
Scheme 3.2: Flurbiprofen derived amides.

Table 3.1: Synthesis of flurbiprofen derived amides.

Entry	Compound	Compound Code	R	Yield %
1	**56a**	**BSc2171**		73
2	**56b**	**BSc2172**		77
3	**56c**	**BSc2222**		61
4	**56d**	**BSc2223**		55
5	**56e**	**BSc2224**		67
6	**56f**	**BSc2226**		64
7	**56g**	**BSc2228**		72
8	**56h**	**BSc2229**		86
9	**56i**	**BSc2230**		41
10	**56j**	**BSc2231**		68
11	**56k**	**BSc2232**		55
12	**56l**	**BSc2233**		58

Compounds **56a** (**BSc2171**) and **56b** (**BSc2172**) were tested for their ability to inhibit or modulate γ-secretase in the Aβ liquid phase electrochemiluminescence (LPECL) assay. Sulindac sulfide was used as the control. Surprisingly, compounds **56a** and **56b** turned out to be inverse modulators of γ-secretase. They affected the γ-secretase cleavage at different

extent. They particularly increased the formation of the most toxic and aggregation prone $A\beta_{42}$ species while reducing the formation of $A\beta_{38}$. The conversion of the acid moiety in to amides changed the mode from modulation to inverse modulation compared to parent compound.
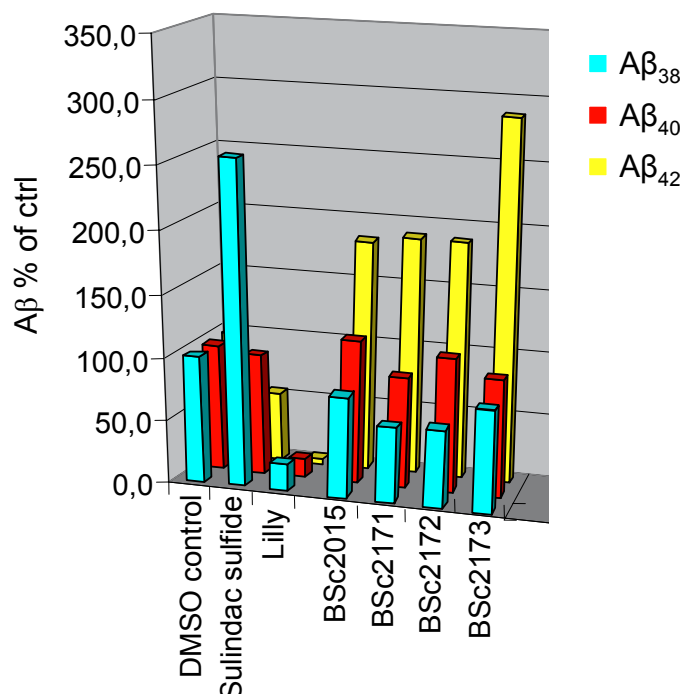

Figure 3.1: Activity report of compounds **56a–b** on γ-secretase.

Compounds **56c–p** (**BSc2222–BSc2233 and BSc2303–BSc2306**) were tested for their potential to inhibit the aspartic protease BACE-1 to rule out upstream cleavage inhibition in the fluorescence resonance energy transfer (FRET) based assay with full length BACE-1,[170] and results are shown in **Table 3.2**. This assay was carried out at Hoffmann-La Roche, Switzerland. FRET is a convenient method in which the fluorescence signal enhancement is observed after the substrate is cleaved by BACE-1, thus separating the quenching moiety (Dabsyl) from the fluorochrome (Lucifer yellow). All enzyme assays were carried out at 20 °C on a FLUOstar (BMG Lab Technologies, D-77656 Offenburg) using 96-well microtiter plates (DYNEX Microfluor 2, Chantilly, VA). The volume of assay was 100 μL. Usually, inhibitors dissolved in DMSO were added into a well followed by the buffer and the enzyme. The concentration of DMSO was kept below 4%. The enzymatic reaction was started by adding the substrate. The fluorescence enhancement was measured at $\lambda_{emission}$ = 520 nm with fluorescence excitation at $\lambda_{excitation}$ = 430 nm. At various inhibitor concentrations reaction kinetics was followed for 30 min. Assays were performed at enzyme concentrations that warranted a linear progression of product formation. A known peptidic inhibitor of the hydroxyl ethylamine class was always included in the experiment as a positive control for inhibition. The results are given as IC_{50} (μM). This assay was found to be very good with known BACE-1 inhibitors, however, only if the compound is water soluble at the assay pH of 4.5. Depending on charge and poor solubility of the compound, this assay may show apparent inhibition that is not specific. To investigate the specificity and selectivity of the synthesized flurbiprofen amides, they were profiled in FRET based assay for BACE-1 and more distant aspartic proteases, cathepsin D (CatD). The activity report is displayed in **Table 3.2**.

None of the compound displayed inhibitory activity in the BACE-1 FRET-assay. Furthermore, the same set of compound was tested in the radioligand binding assay (RLBA) for the ability to inhibit BACE-1. Out of the tested compounds, only **56e**, **56o** and **56p** displayed good inhibitory activity (**Table 3.2**), where as **56d**, **56m** and **56n** displayed moderate inhibitory activity. Rest of the compounds displayed either marginal inhibitory activity or were found to be inactive. Introduction of amines bearing bulky lipophilic groups with polar end groups such as –NH and –OH, increased BACE-1 inhibitory activity (e.g. **56e**, **56m**).

**Table 3.2:** Activity data of flurbiprofen amides on BACE-1.

Entry	Compound	Compound Code	FRET (BACE-1) IC_{50} μM	RLBA (Tween) IC_{50} μM	RLBA (BSA) IC_{50} μM	FRET (CatD) IC_{50} μM
1	**56c**	**BSc2222**	>200	Inactive	Inactive	>200
2	**56d**	**BSc2223**	Inactive	195	Inactive	>200
3	**56e**	**BSc2224**	>200	46	Inactive	34
4	**56f**	**BSc2226**	>200	Inactive	>200	>200
5	**56g**	**BSc2228**	>200	>200	Inactive	Inactive
6	**56h**	**BSc2229**	>200	>200	Inactive	>200
7	**56i**	**BSc2230**	Inactive	201	Inactive	>200
8	**56j**	**BSc2231**	>200	>200	Inactive	>200
9	**56k**	**BSc2232**	>200	>200	Inactive	>200
10	**56l**	**BSc2233**	>200	>200	Inactive	Inactive
11	**56m**	**BSc2303**	Inactive	126	Inactive	129
12	**56n**	**BSc2304**	Inactive	120	Inactive	>200
13	**56o**	**BSc2305**	>200	35	Inactive	9
14	**56p**	**BSc2306**	>200	32	Inactive	11

Moreover, none of the compound displayed inhibitory activity in the RLBA in the presence of bovine serum albumin (BSA). To investigate the specificity and selectivity, compounds **56c–p** were tested in the CatD FRET-assay. Most of the compounds displayed no activity or marginal inhibitory activity in the CatD FRET-assay except the compounds **56e**, **56m**, **56o** and **56p**. The most active compounds were particularly **56o** and **56p** and they displayed the CatD inhibition in the FRET assay at low micromolar concentration.

The Structure Activity Relationship (SAR) revealed that the most active compound in the RLBA (tween) assay were the compounds **56e**, **56o** and **56p** bearing the polar, hydrogen bond donor moiety. But at the same time these compounds lacked selectivity and were found to be equipotent in the CatD FRET-assay. These multipotent aspartic protease inhibitors were abandoned because of the selectivity issue which is very crucial for the drug design. A potential β-secretase inhibitor has to be very selective since aspartic proteases are widely distributed in the body and the treatment of AD will be a long term therapy. Therefore, BACE-1 selectivity is important as the target enzyme resides in the brain, where the closely related ubiquitous enzymes such as CatD are found. BACE-1 inhibitors with poor selectivity against these enzymes may cause undesired side effects.
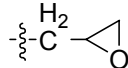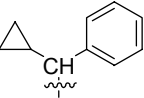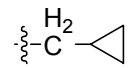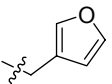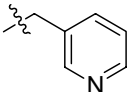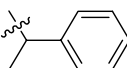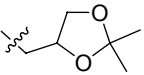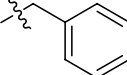
3.1.1.2. Synthesis of flurbiprofen derived esters

Initially, benzyl ester of flurbiprofen **57k** (**BSc2171**) was synthesized in order to investigate the impact of an ester moiety on BACE-1 and γ-secretase inhibitory activity. In the Aβ liquid phase electrochemiluminescence (LPECL) assay, **57k** was found to be an inverse modulator of γ-secretase (**Figure 3.1**). It reduced the formation of $A\beta_{38}$ and increased the formation of $A\beta_{42}$. The flurbiprofen benzyl ester **57k** was tested for its potential to inhibit BACE-1 in the fluorometric BACE activity assay,[171] MDCK cells were used for the assay. Flurbiprofen benzyl ester **57k** displayed good BACE-1 inhibitory activity (IC_{50} = 20 µM). That prompted the synthesis of a series of flurbiprofen 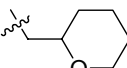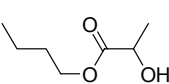derived esters as depicted in **Scheme 3.3**. The acid moiety of flurbiprofen was coupled with various alcohols using EDAC and HOBt as coupling reagents in CH_2Cl_2 at an ambient temperature (**Table 3.3**). The purity of all the compounds was analysed by HPLC and were submitted to the assays for their biological evaluation without further characterization.
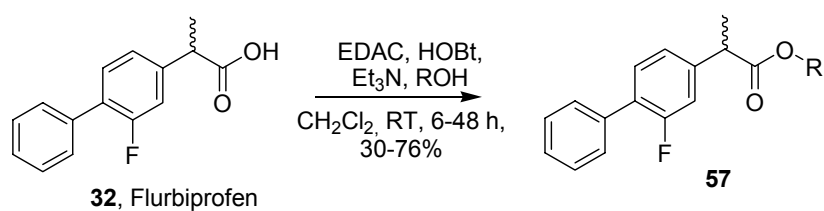
Scheme 3.3: Synthesis of flurbiprofen derived esters.

Table 3.3: Synthesis of flurbiprofen derived esters.

Entry	Compound	Compound Code	R	Yield %
1	**57a**	**BSc2292**		35
2	**57b**	**BSc2293**		43
3	**57c**	**BSc2294**		48
4	**57d**	**BSc2295**		55
5	**57e**	**BSc2297**		65
6	**57f**	**BSc2298**		57
7	**57g**	**BSc2300**		40
8	**57h**	**BSc2301**		34
9	**57i**	**BSc2302**		40
10	**57j**	**BSc2171**		70

A set of compounds **57a–k** (**BSc2292–BSc2302**) was tested for their potential to inhibit BACE-1 in the FRET assay (**Table 3.4**). The assays were performed at Hoffman La-Roche, Basel. Surprisingly, none of the flurbiprofen derived ester displayed significant activity in the FRET assay. The compounds were further analysed for their ability to inhibit BACE-1 in the RLBA (Tween) assay. Only three compounds, **57a** (**BSc2292**), **57e** (**BSc2297**) and **57i** (**BSc2302**) displayed marginal activity in the RLBA (Tween) assay. Most of the compounds were found to be inactive in the RLBA (BSA) assay whereas, compounds **57c** (**BSc2294**) and **57e** (**BSc2297**) displayed very weak inhibitory activity. CatD was used from human liver as obtained commercially (Calbiochem). CatD is a lysosomal aspartyl protease, which is homologous to BACE-1. The CatD assay provides an assessment of relative specificity of inhibitors for BACE-1. None of the flurbiprofen esters exhibited significant inhibitory activity in the CatD assay

.

Table 3.4: Activity report of flurbiprofen esters.

Entry	Compound	Compound Code	FRET (BACE)	RLBA (Tween)	RLBA (BSA)	FRET (CatD)
			IC_{50} µM	IC_{50} µM	IC_{50} µM	IC_{50} µM
1	**57a**	**BSc2292**	>200	179	Inactive	Inactive
2	**57b**	**BSc2293**	>200	>200	Inactive	Inactive
3	**57c**	**BSc2294**	>200	>200	200	Inactive
4	**57d**	**BSc2295**	>200	>200	Inactive	Inactive
5	**57e**	**BSc2297**	>200	95	>200	Inactive
6	**57f**	**BSc2298**	>200	>200	Inactive	Inactive
7	**57g**	**BSc2300**	>200	>200	Inactive	Inactive
8	**57h**	**BSc2301**	>200	>200	Inactive	Inactive
9	**57i**	**BSc2302**	>200	171	Inactive	>200
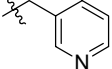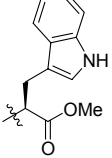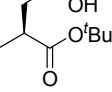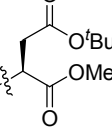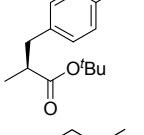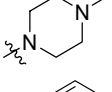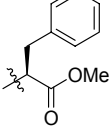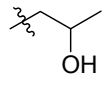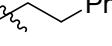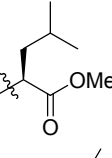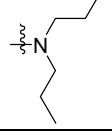
3.1.2. Synthesis of Sulindac Sulfoxide Derived Amides as γ-Secretase Modulators.

Sulindac is a non-steroidal anti-inflammatory drug of the arylalkanoic acid class and useful in the treatment of acute or chronic inflammatory conditions. Sulindac is a prodrug, derived from sulfinylindene that is converted in the body to an active NSAID. More specifically, the agent is converted by liver enzymes to a sulfide which is excreted in the bile and then reabsorbed from the intestine. The exact mechanism of its NSAID properties is unknown, but it is thought to act on enzymes COX-1 and COX-2, inhibiting prostaglandin synthesis. Unlike its active metabolite, sulindac sulfide, sulindac sulfoxide **58** does not inhibit COX-1 and COX-2. Sulindac sulfide **33** is a γ-secretase modulator and reduces Aβ42, the most toxic and fibrillogenic peptide at 40 µM concentration. It was anticipated that transformation of the acid moiety of sulindac sulfoxide into its amide derivatives would turn it into γ-secretase modulator devoid of COX inhibitory activity. Therefore, a series of sulindac sulfoxide derived amides was synthesized as depicted in **Scheme 3.4**.
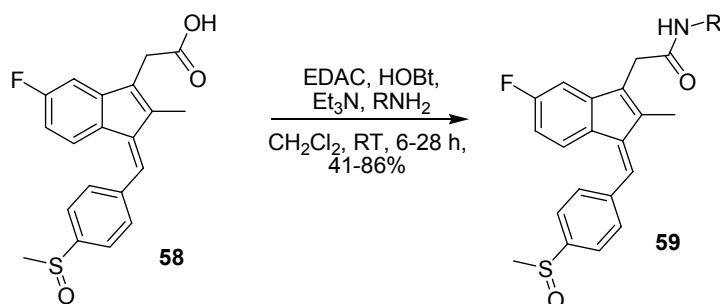
Scheme 3.4: Synthesis of sulindac derived amides.

Table 3.5: Synthesis of sulindac derived amides.

Entry	Compound	Compound Code	R	Yield %
1	**59a**	**BSc2234**		61
2	**59b**	**BSc2235**		55
3	**59c**	**BSc2236**		67
4	**59d**	**BSc2237**		64
5	**59e**	**BSc2238**		72
6	**59f**	**BSc2239**		86
7	**59g**	**BSc2240**		41
8	**59h**	**BSc2241**		68
9	**59i**	**BSc2242**		55
10	**59j**	**BSc2243**		58
11	**59k**	**BSc2244**		75
12	**59l**	**BSc2245**		53

The acid moiety of sulindac sulfoxide **58** was coupled with various amines using EDAC and HOBt as coupling reagents in CH₂Cl₂ at RT to afford amides **59** (**Table 3.5**). The reactions were monitored by TLC and the crude products were purified by column chromatography. The purity of all compounds was analyzed by HPLC and the compounds were submitted for their biological evaluation without further characterization.

A set of compounds **59a–l** (**BSc2234–BSc2245**) was tested in the Aβ liquid phase electrochemiluminescence assay for their potential to modulate γ-secretase activity.
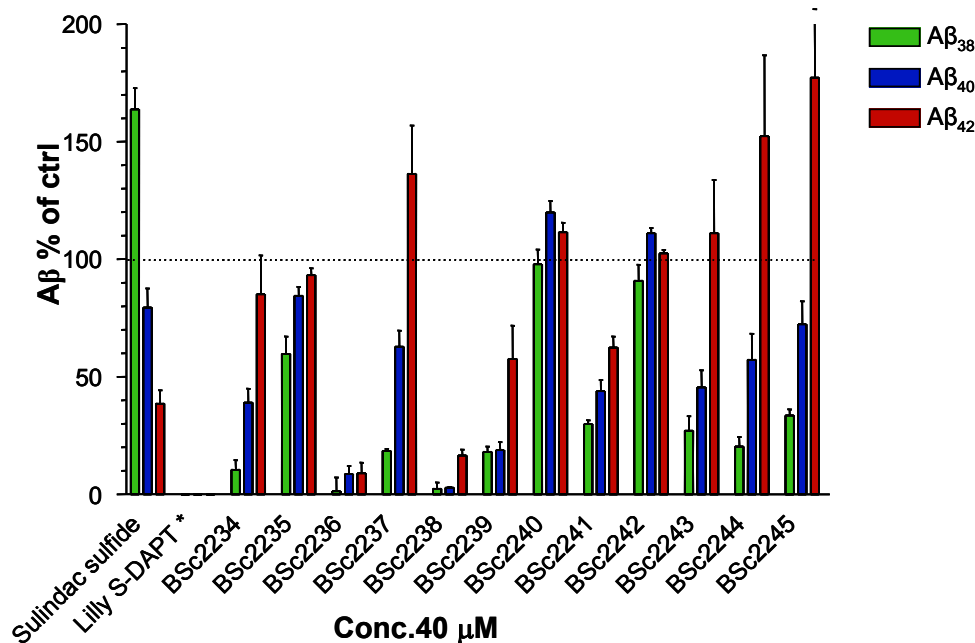
Figure 3.2: Activity data of compounds **59a–l** (**BSc2234–BSc2245**) on γ-secretase.

Sulindac sulfide, an active metabolite of sulindac sulfoxide which is a known modulator of γ-secretase, was used as a standard along with S-DAPT. All the compounds were tested at 40 μM concentration except S-DAPT, which was used at 20 μM concentration. The displayed Aβ levels are the mean of four experiments (**Figure 3.2**). As anticipated, most of the tested compounds displayed γ-secretase modulatory activity. Interestingly, it was found to be an inverse modulatory activity except **59c** and **59i**. The remaining compounds affected the cleavage at the γ38, γ40 and γ42 sites to a different extent and particularly increased the formation of Aβ$_{42}$, while reducing the formation of Aβ$_{38}$. The most active compounds that displayed modulatory activity were particularly, **59d** (**BSc2237**), **59k** (**BSc2344**) and **59l** (**BSc2345**). They significantly increased the formation of Aβ$_{42}$ while reducing the formation of Aβ$_{38}$ and displayed an inverse mode of action compared to typical NSAIDs. Surprisingly, two of the tested compounds, **59c** (**BSc2236**) and **59e** (**BSc2238**), turned out to be full inhibitors of γ-secretase.

Conversion of the acid moiety of sulindac sulfoxide into its corresponding amides turned it into potent, but inverse modulators of γ-secretase. Introduction of amines with lipophilic substituents significantly increased the inverse modulatory activity (e.g. **59d, 59k–l**).On the other hand, the introduction of chiral and bulky amines such as *S*-tryptophan methyl ester and *S*-aspartic acid *t*-butyl ester transformed the sulindac sulfoxide into potent inhibitors of γ-secretase. Introduction of amines with additional polar groups turned it into mild inhibitors of γ-secretase (e.g. **59g**, **59i**). But, none of these compounds displayed the desired straight modulatory activity.

3.1.3. Synthesis of Meclofenamic Acid Derivatives as γ-Secretase Inhibitors.

Meclofenamic acid is a non-selective COX inhibitor. It belongs to the anthranlic class of NSAIDs. It was anticipated that the conversion of the acid moiety of meclofenamic into its amides using various bulky or chiral amines, would turn the nonselective-COX inhibitors meclofenmaic acid into potent γ-secretase inhibitors devoid of COX activity.

Meclofenamic acid derived amide derivatives were synthesized as depicted in **Scheme 3.5**. Sulindac sulfoxide derived amides, **59c** and **59e**, bearing chiral and bulky amines were found to be potent inhibitors of γ-secretase. Therefore, bulky and chiral amines were employed to couple with meclofenamic acid. The acid moiety of meclofenamic acid **37** was converted to the corresponding amides using various amines and EDAC and HOBt as coupling reagents in CH_2Cl_2 at ambient temperature. The reaction was monitored by TLC.
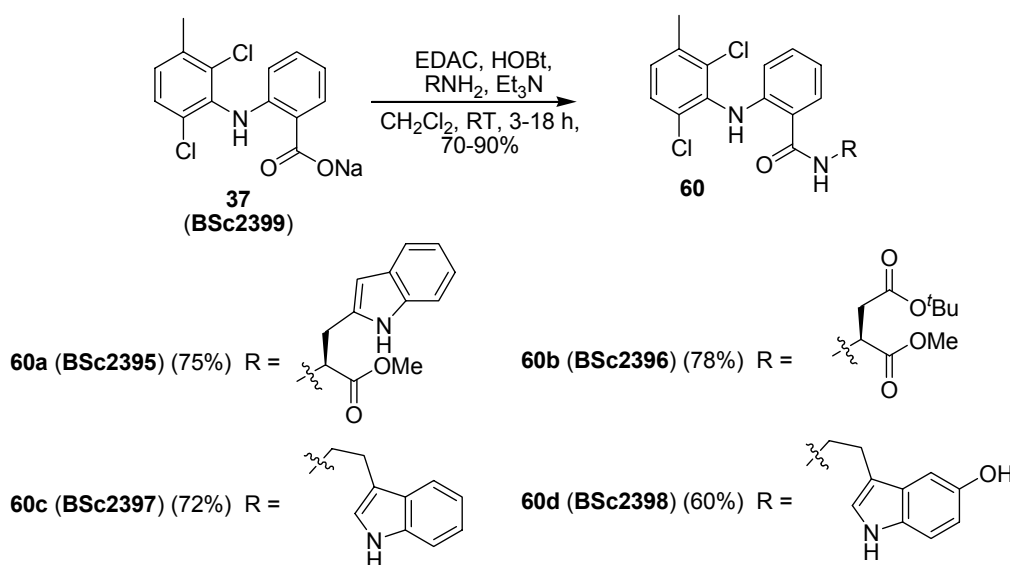
Scheme 3.5: Synthesis of meclofenamic acid derived amide derivatives **60**.

Compounds **60a–d** were tested for their potential to inhibit γ-secretase inhibitors in the Aβ liquid phase electrochemiluminescence assay. All compounds were tested at 20 μM concentration and meclofenamic acid and sulindac sulfide were used as standards (**Figure 3.3**). Meclofenamic acid is known to be a modulator of γ-secretase. But, in the Aβ liquid phase electrochemiluminescence assay it was found to be an inhibitor of γ-secretase (**Figure 3.3**). In contrast to the previous results, conversion of meclofenamic acid into their corresponding amides turned them into γ-secretase modulators except compound **60d**. Compound **60a–c** affected the cleavage at the γ38, γ40 and γ42 sites to a different extent and particularly enhanced the formation of Aβ$_{42,}$ while suppressing the formation of Aβ$_{38}$. The most active γ-secretase modulator was compound **60b** and it increased the formation of Aβ$_{42}$ significantly. In accordance with previous results, only compound **60d** was found to be a potent inhibitor of γ-secretase.
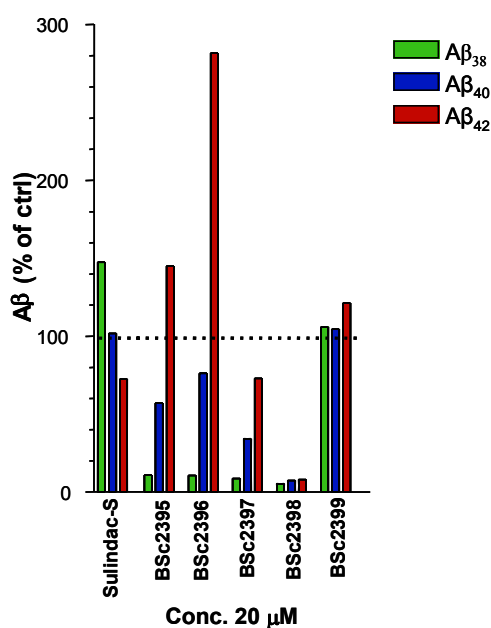
Figure 3.3: γ-Secretase activity data for compounds (**60a–d**).

In conclusion, the conversion of the acid moiety common to the COX inhibitors to the corresponding amides and esters turned them into either γ-secretase inverse modulators or full inhibitors. Derivatization of the acid moiety of flurbiprofen to amides dramatically changed the mode of action on γ-secretase from classical modulation to inverse modulation. Flurbiprofen derived esters **57** and amides **56** were tested for their ability to inhibit BACE-1 but none of the tested compound displayed inhibitory activity on BACE-1 in FRET assay and very few compounds displayed inhibitory activity in the RLBA(Tween) assay (**56o**, **56p**). But these two multipotent compounds lacked the selectivity and displayed the inhibitory activity on other aspartic proteases such as CatD at low micromolar concentration. A similar SAR trend was observed with the amide derivatives of sulindac sulfoxide **59** in the Aβ liquid phase electrochemiluminescence assay. Transformation of sulindac sulfoxide into its amide turned it into an inverse modulator of γ-secretase. But, only compounds **59c** and **59e** were found to be potent inhibitors of γ-secretase. Based on the SAR of compounds **59a–l**, meclofenamic acid derived amides **60a–d** were synthesized. In contrast to the previous results, the conversion of meclofenamic acid into its amide resulted in potent inverse modulators of γ-secretase. Only compound **60d** was found to be an inhibitor of γ-secretase in accordance with the previous results. It is apparent from the SAR that free acid is crucial for the modulatory activity and to preserve the typical NSAID like profile. Any derivatization of the acid moiety either resulted in the loss of modulatory activity or inversion of the modulatory activity. Inhibition was observed as the third mode of action.

3.2. The Scaffold of the COX-2 Inhibitor Carprofen Provides Alzheimer γ-Secretase Modulators

Promising results for the treatment of AD were obtained with some COX-1 inhibitors,[117, 167, 172-174] both *in vitro* and in a prospective, population-based cohort study of 6989 patients.[175] This is not a class effect as the NSAIDs: diclofenac and naproxen do not lower Aβ *in vitro*,[167, 173] and neither naproxen nor rofecoxib (a COX-2 inhibitor) slow cognitive decline in patients with mild-to-moderate AD.[176, 177] The positive clinical results are still in need of a sound rational and an experimental validation.[178] The proof of concept is still missing. NSAIDs, that modulate γ-secretase cleavage of APP, affect the distance between APP and PS1, the catalytic subunit of γ-secretase.[168] Just a few NSAIDs were reported to modulate γ-secretase and an even smaller number of NSAIDs display confirmed modulation at physiological feasible concentrations. Despite these vague, sometimes contradictory results reported for NSAID activity. That encouraged the investigation of NSAIDs as scaffolds for γ-secretase inhibitors. The derivatization of the carboxylic acid common to the COX inhibitors was commenced initially. Although some of the esters and amides displayed moderate inhibition of the cleavage by γ-secretase ($A\beta_{38}$, $A\beta_{40}$, $A\beta_{42}$) this approach was soon abandoned. Few of these initial derivatives displayed increased, unselective inhibition of γ-secretase in comparison to their parent drugs. Most of the carboxylic acid derivatives such as esters and amides resulted in loss of activity. This indicated an important contribution of the carboxylic acid to target affinity. After a brief investigation of flurbiprofen, sulindac sulfoxide and meclofenamic acid analogues, the efforts were focussed on carprofen **53**, which is a COX-2 inhibitor approved for the use in dogs, cows and horses as Imadyl® or Rimadyl®. The selectivity of carprofen versus COX-2$_{canine}$ and COX-1$_{canine}$ is greater than 100:1 (COX-2$_{canine}$ IC_{50}: *R/S*-carprofen 102 nM, *R*-carprofen 5.97 μM, *S*-carprofen 37 nM).[179] Original carprofen (as isolated from Rimadyl® tablets 500 mg) was found to be a weak inhibitor of γ-secretase and reduced $A\beta_{38}$, $A\beta_{40}$ and $A\beta_{42}$ at high concentration. This corresponds to the activity of α-methylated carprofen, which was reported to inhibit $A\beta_{42}$ production by 40% at 100 μM.[122] The readily accessible sulfonamides were inspired by the γ-secretase inhibitor **BMS-299897** and supported by a recent series from Merck Sharp & Dohme.[88, 89]

3.2.1. Synthesis of *N*-sulfonylated carprofen derivatives

Initially *N*-sulfonylated derivatives of carprofen were synthesized as depicted in **Scheme 3.6**. The acid moiety of carprofen was protected as a benzyl ester **61**. *N*-Sulfonylation of **61** was carried out using NaH, *p*-toluene sulfonyl chloride and 3,5-bis(trifluoromethyl) phenyl sulfonyl chloride in THF to afford esters **62**. Subsequent benzyl deprotection of **62** by hydrogenation furnished the desired acids **63**. *N*-tosylated carprofen **63a** was further coupled with various heterocyclic amines in CH_2Cl_2 at RT using EDAC and HOBt as coupling reagents to afford amides **64**. The crude compounds were purified by column chromatography to afford the amides.
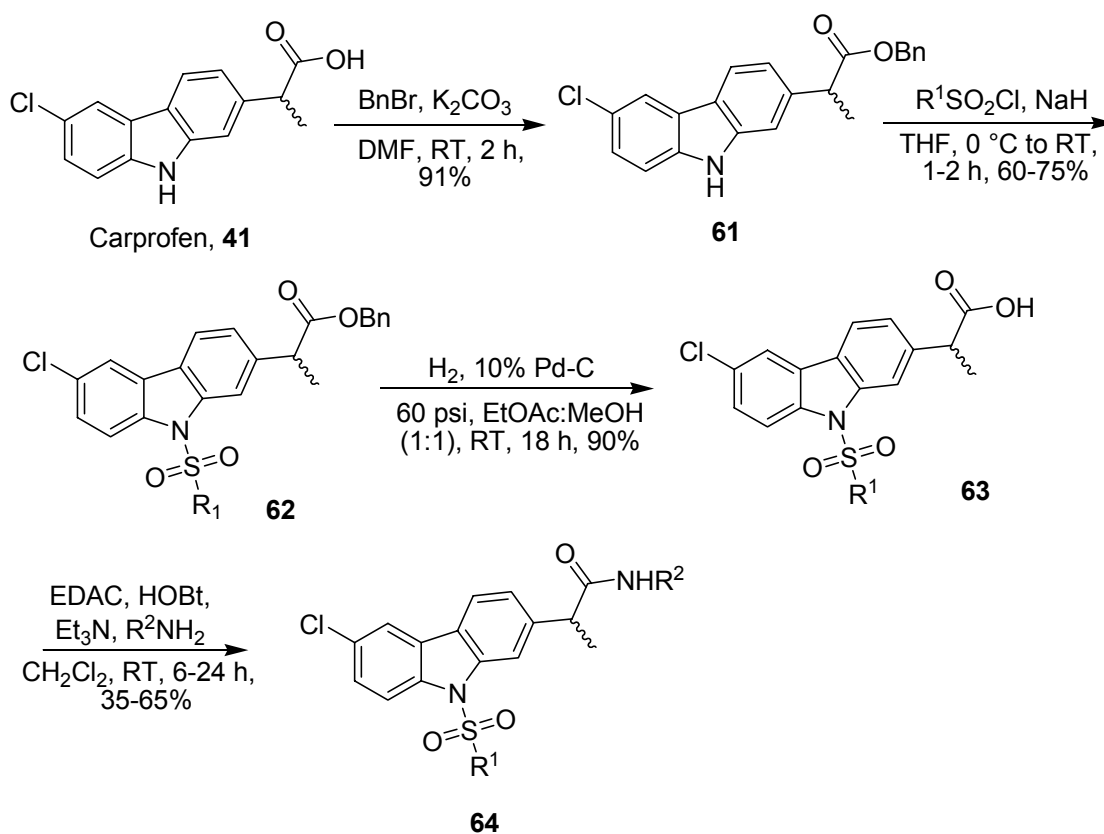
Scheme 3.6: Synthesis of *N*-sulfonylated carprofen amides.

The compounds **62a–b**, **63a–b** and **64a–c** were tested in the Aβ liquid phase electrochemiluminescence assay for their ability to inhibit γ-secretase enzyme activity. The γ-secretase cellular assay was carried out under the supervision of Dr. Karlheinz Baumann at F. Hoffmann La Roche, Basel. Sulindac sulfide was used as standard and all the compounds were tested at 20 μM concentration. These compounds were expected to be inhibitors of γ-secretase activity due to their resemblance to the γ-secretase inhibitor **29** (**BMS-299897**). Serendipitously, *N*-sulfonylated carprofen derivatives, **63a** and **63b**, turned out to be modulators of γ-secretase activity, which control the cleavage pattern of γ-secretase. They affected the cleavage at the γ38, γ40 and γ42 sites to a different extent and particularly suppressed the formation of Aβ$_{42}$, while enhancing the formation of Aβ$_{38}$ and thus showed the

typical profile of effective NSAIDs (**Figure 3.4**). Such modulators may preserve the cleavage of substrates like Notch[42, 180] Notch and a subset of cell-surface receptors and proteins involved in embryonic development, haematopoiesis, cell adhesion and cell/cell contacts.[181] In accordance with previous results, *N*-sulfonylated carprofen benzyl esters **62** and amides derivatives **64** turned out to be inverse modulators of γ-secretase and increased the formation of Aβ$_{42}$ (**Figure 3.4**).

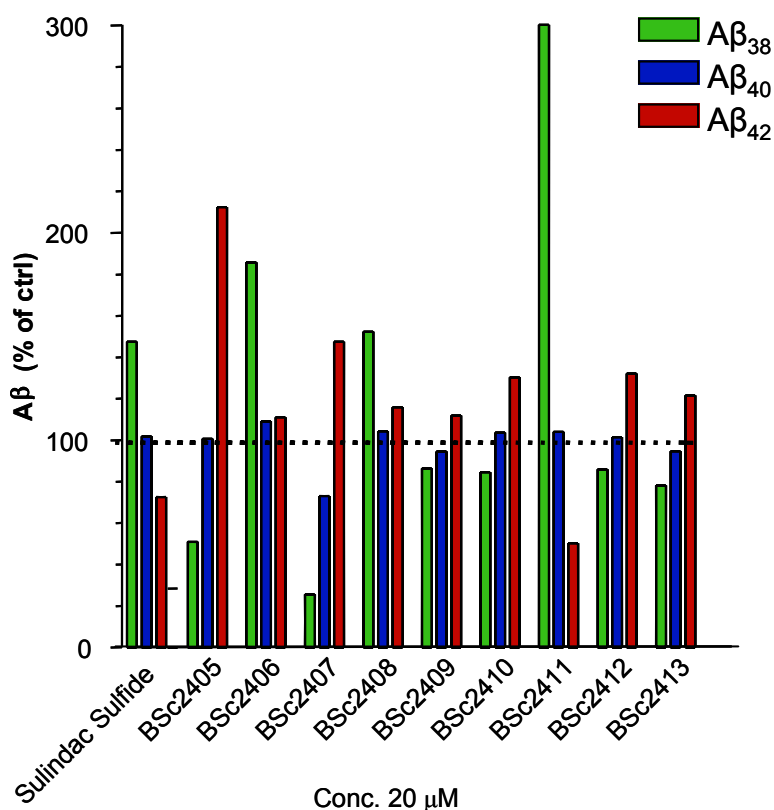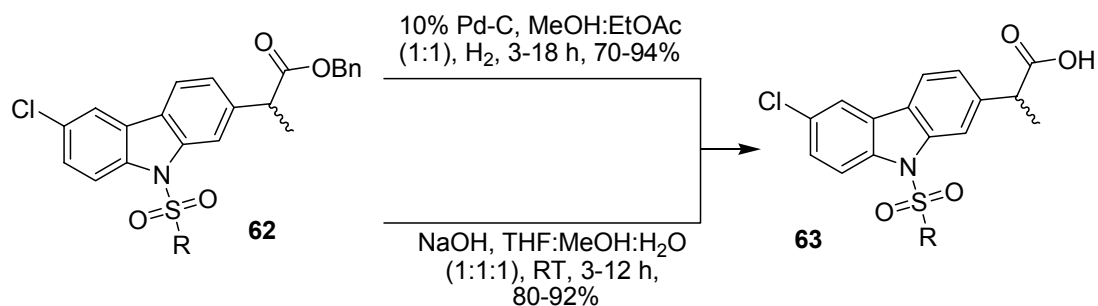**Figure 3.4:** Activity data of *N*-substituted carprofen derivatives **62**, **63** and **64**.

Thus, the initial objective was adapted from inhibition to modulation. The encouraging results obtained from lead compounds **63a** (**BSc2406**) and **63b** (**BSc2411**) prompted the synthesis of a series of *N*-sulfonylated carprofen derivatives.

Scheme 3.7: Synthesis of *N*-sulfonylated carprofen derivatives **63**.

A series of *N*-sulfonylated carprofen derivatives was synthesized using the same methodology as depicted in **Scheme 3.6**. The benzyl group of **62** was removed by either by hydrogenation (**62a–62m**, **62p–62r**) or base hydrolysis in case of **62n–62o**, **62s** due to the presence of the hydrogenation sensitive functionalities (**Scheme 3.7**) to give the acid **63**.

Table 3.6: Activity of carprofen *N*-sulfonamide derivatives.

Entry	Comp. Code	Comp.	R	IC$_{50}$ [µM]		
				Aβ_{38}[a]	Aβ_{40}	Aβ_{42}
1	**Carprofen**	**41**	H	78	133	76
2	**BSc2406**	**63a**	4-Methylphenyl	43	> 100	56
3	**BSc2411**	**63b**	3,5-Bis(trifluoromethyl)phenyl	ND[b]	> 30	11
4	**BSc2826**	**63c**	4,5-Dibromothiophen-2-yl	ND[b]	ND	> 40
5	**BSc2827**	**63d**	3,5-Difluorophenyl	ND[b]	ND	> 40
6	**BSc2829**	**63e**	4-Biphenyl	ND[b]	ND	37
7	**BSc2830**	**63f**	2-Fluorophenyl	ND[b]	ND	> 40
8	**BSc2831**	**63g**	3-Fluorophenyl	ND[b]	ND	> 40
9	**BSc2832**	**63h**	4-Fluorophenyl	ND[b]	ND	> 40
10	**BSc2833**	**63i**	2-Bromophenyl	ND[b]	ND	40
11	**BSc2835**	**63j**	4-Bromophenyl	ND[b]	ND	> 40
12	**BSc2836**	**63k**	Phenyl	ND[b]	ND	> 40
13	**BSc2837**	**63l**	4-Chlorophenyl	ND[b]	ND	> 40
14	**BSc2838**	**63m**	3-Chlorophenyl	ND[b]	ND	> 40
15	**BSc2840**	**63n**	3-Nitrophenyl	ND[b]	ND	39
16	**BSc2841**	**63o**	4-Nitrophenyl	ND[b]	ND	> 40
17	**BSc2842**	**63p**	2,4,6-Tri-*iso*-propylphenyl	0.3	> 20	8.5
18	**BSc3036**	**63q**	4-*n*-Propylphenyl	ND[b]	> 40	> 40
19	**BSc3037**	**63r**	Octyl	ND[b]	> 40	25
20	**BSc3044**	**63s**	4-Cyanophenyl	ND[b]	ND	> 40

[a] EC$_{50}$ values are displayed for Aβ_{38} except compound **41**. The EC$_{50}$ is based on the maximum level with a slope approximating 0.
[b] Maximum effect on Aβ_{38} not observed at 40 µM (except for **41**: 160 µM, **63a**: 200 µM, **63b**:200 µM)

3.2.1. Synthesis of *N*-alkylated carprofen derivatives

N-Alkylated carprofen derivatives were prepared to evaluate the contribution of the sulfonamide moiety in **63r** and the most active derivate **63p**, where the sulfonamide is shielded by isopropyl substituents. The carprofen benzyl ester **61** was alkylated using NaH and RX in THF to yield the ester **65** (**Scheme 3.8**). Subsequent benzyl deprotection by hydrogenation gave the desired *N*-alkylated carprofen **66**. The benzyl deprotection of **65c** was carried out by base hydrolysis due to the presence of hydrogenation sensitive moiety.
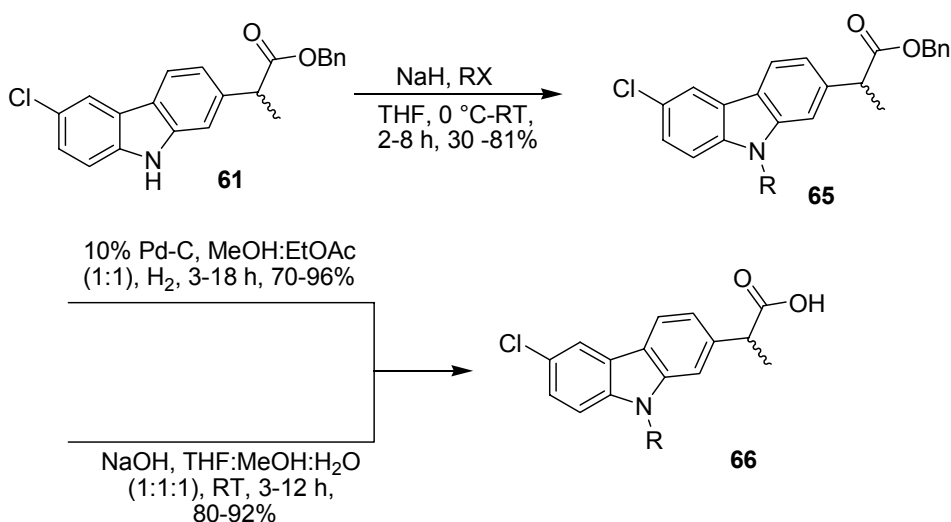
Scheme 3.8: Synthesis of *N*-alkylated carprofen derivatives **66**.

Table 3.7: Activity of *N*-alkylated carprofen derivatives.

Entry	Comp.	R	Code	Activity in μM		
				EC_{50} $A\beta_{38}$	IC_{50} $A\beta_{40}$	IC_{50} $A\beta_{42}$
1	**66a**	3-(Trifluoromethoxy)benzyl	**BSc2901**	ND[a]	> 40	7.5
2	**66b**	3-Methoxybenzyl	**BSc2902**	ND[a]	> 40	39
3	**66c**	3-Nitrobenzyl	**BSc2903**	ND[a]	> 40	22
4	**66d**	Octyl	**BSc3039**	ND[a]	> 40	6.9
5	**66e**	Nonyl	**BSc3040**	8.1	> 40	3.0
6	**66f**	Decyl	**BSc3041**	5.8	> 40	2.9

[a] Maximum effect on $A\beta_{38}$ not observed at 40 μM.

The compounds **63a–63s** and **66a–66f** turned out to be effective modulators of γ-secretase. They affected the cleavage at the γ38, γ40 and γ42 sites to a different extent and particularly suppressed the formation of $A\beta_{42}$, while enhancing the formation of $A\beta_{38}$ and thus showed the typical profile of effective NSAIDs (see **Tables 3.6** and **3.7**).

Detailed results and discussion of these experiments are described in the article attached at the end as Appendix I. i.e.

"Rajeshwar Narlawar, Blanca Perez Revauelta, Christian Haass, Harald Steiner, Boris Schmidt, Karlheinz Baumann, The Scaffold of the COX-2 Inhibitor Carprofen Provides Alzheimer γ-Secretase Modulators, *Journal of Medicinal Chemistry*, **2006**, *49 (26)*, 7588-7591."

3.3. *N*-Substituted Carbazolyloxyacetic Acids Modulate Alzheimer Associated γ–Secretase

Knowing about the relevance of the carboxylic acid and *N*-substitution of carprofen, the scaffolds not included in the patent applications and publications by E. Koo et al. or Stock, or Imbibo, and others were investigated.[182] Carprofen's similarity to the γ-secretase inhibitor **29** (**BMS-299897**) prompted the investigation of *N*-sulfonylated and *N*-alkylated 2-hydroxy carbazolyloxyacetic acids. The corresponding sulfonamides **70a–k** were readily accessible by straightforward synthesis.

3.3.1. Synthesis of *N*-sulfonalyted carbazolyloxyacetic acids

A series of 2-hydroxy carbazole *N*-sulfonamide derivatives was prepared as outlined in **Scheme 3.9**. 2-Hydroxycarbazole **67** was alkylated using anhydrous K_2CO_3 and $ClCH_2CO_2{}^tBu$ in acetone at 60–70 °C to give compound **68**. *N*-sulfonylation of **68** was carried out using NaH, various sulfonyl chlorides in THF to afford *N*-sulfonylated ester **69** (**Table 3.8**). The *t*-butyl deprotection of **69** was accomplished by 20% trifluoroacetic acid in CH_2Cl_2 furnished the desired compounds **70**.
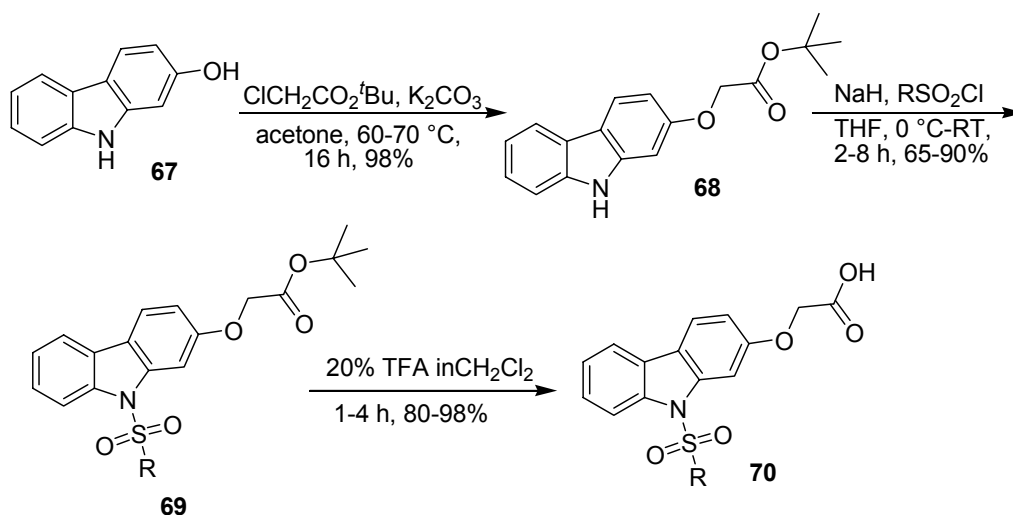
Scheme 3.9: Synthesis of *N*-sulfonylated carbazolyloxyacetic acid derivatives.

Table 3.8: Synthesis of *N*-sulfonylated carbazolyloxyacetic acid derivatives.

Entry	Compound	R
1	**70a**	4-Methylphenyl
2	**70b**	3,5-Bis(trifluoromethyl)Phenyl
3	**70c**	4-Biphenyl
4	**70d**	2,4,6-Tri-*iso*-Propylphenyl
5	**70e**	4-*N*-Propylphenyl
6	**70f**	Octyl
7	**70g**	(1*S*)-1-(Methyl)-7,7-dimethylbicyclo[2.2.1]heptan-2-one
8	**70h**	4-Chlorophenyl
9	**70i**	3,5-Difluorophenyl
10	**70j**	2-Bromophenyl
11	**70k**	3-Nitrophenyl

3.3.2. Synthesis of *N*-alkylated carbazolyloxyacetic acids

N-alkylated carbazolyloxyacetic acids were prepared to evaluate the contribution of the sulfonamide moiety in **70g** and the most active derivative **70d**, where the sulfonamide is shielded by isopropyl substituents. The *N*-alkylated carbazolyloxyacetic acids were synthesized as shown in **Scheme 3.10**. Compound **68** was alkylated using KOBu^t and alkyl halide in dry THF to give *N*-alkylated ester **71**. The *t*-butyl ester hydrolysis was achieved by base hydrolysis to obtain desired *N*-alkylated carbazolyloxyacetic acids as colorless solids **72**.
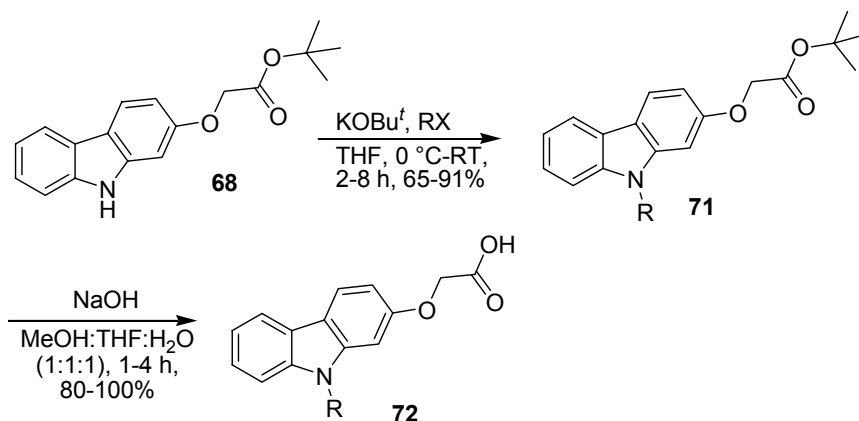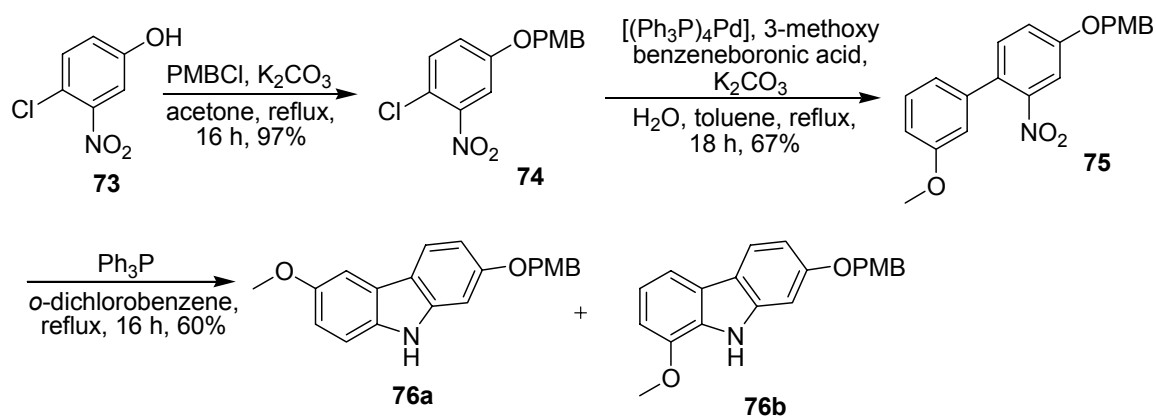

Scheme 3.10: Synthesis of *N*-alkylated carbazolyloxyacetic acids.

It was speculated that the lipophilic substituent anchors the *N*-substituted carbazolyloxyacetic acid in the required orientation within the membrane, thus the maximum tolerated length should be similar to natural phospholipids. To explore this hypothesis, *N*-alkylated carbazolyloxy acetic acids were synthesized using the alkyl halides of length equivalent to the phospholipids such as undecyl, dodecyl, tetradecyl, hexadecyl.

3.3.3. Synthesis of 6-methoxy and 8-methoxy carbazolyloxyacetic acids

The 2-hydroxy-6-methoxy and 2-hydroxy-8-methoxy *N*-sulfonylated carbazolyloxyacetic acids were synthesized in order to investigate the impact of an electron donating substituent on the activity. The synthesis of 6-methoxy and 8-methoxy-2-hydroxy carbazoles was commenced from the commercially available 4-chloro-3-nitrophenol **73** as outlined in **Scheme 3.11**. The phenolic –OH of **73** was protected as a *p*-methoxybenzyl (PMB) ether **74**. Suzuki-Miyaura coupling of **74** with 3-methoxybenzeneboronic acid using the catalyst [(PPh$_3$)$_4$]Pd gave biphenyl **75**, which was then cyclized to its corresponding carbazole by refluxing it in *o*-dichlorobenzene with PPh$_3$. The PPh$_3$ mediated cyclization resulted in two regioisomers, 6-methoxy carbazole, **76a** and 8-methoxy carbazole, **76b** which were separated by flash column chromatography.

N-sulfonylated derivatives of 2-hydroxy-6-methoxy and 2-hydroxy-8-methoxy carbazolyloxyacetic acids were synthesized as depicted in **Scheme 3.12**.

Scheme 3.11: Synthesis of 6-methoxy and 8-methoxy carbazoles.

The PMB group of **76** was deprotected by hydrogenation at 60 psi to get phenol **77**. The free –OH was alkylated using anhydrous K_2CO_3 and $ClCH_2CO_2{}^tBu$ in acetone at 60–70 °C to yield alkylated **78**.
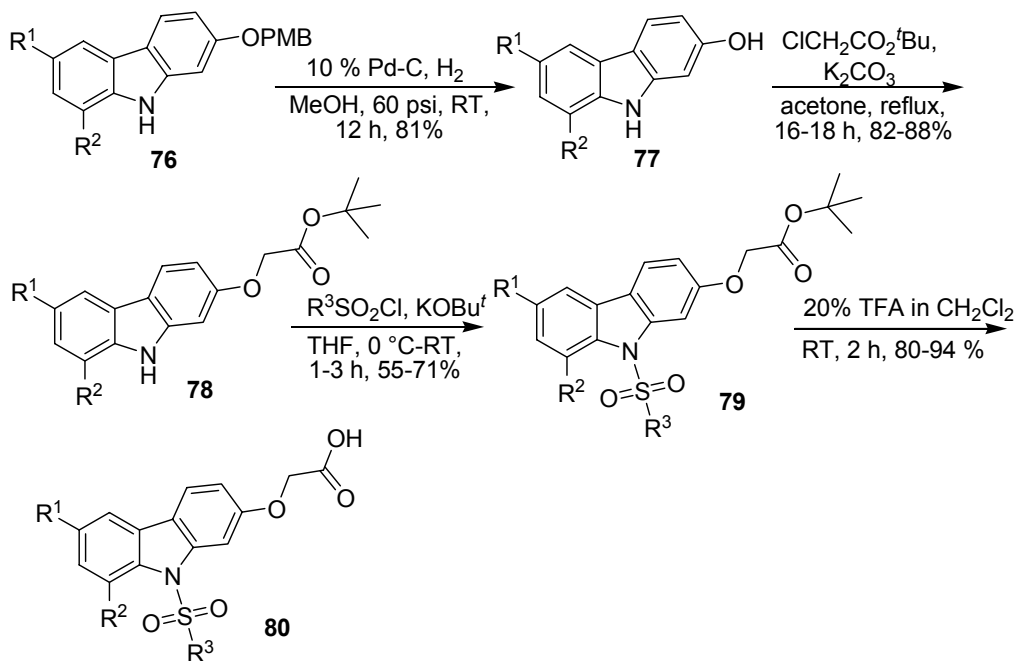
Scheme 3.12: Synthesis of 6-methoxy and 8-methoxy *N*-sulfonylated carbazolyloxyacetic acids.

Table 3.9: Synthesis of 6-methoxy and 8-methoxy *N*-sulfonylated carbazolyloxyacetic acids.

Compound	R^1	R^2	R^3
80a	OMe	H	3,5-(bis-trifluoro)phenyl
80b	OMe	H	4-chlorophenyl
80c	OMe	H	4-biphenyl
80d	OMe	H	2-bromophenyl
80e	H	OMe	4-chlorophenyl
80f	H	OMe	4-biphenyl

N-sulfonylation of **78** was carried out using NaH and R^3SO_2Cl in dry THF to afford *N*-sulfonylated **79** (**Table 3.9**). The *t*-butyl group removal was carried out by 20% TFA in CH_2Cl_2 to furnish the desired compound acids **80**.

Compounds **70a–k** and **72a–g** were tested for their ability to modulate γ-secretase in the Aβ liquid phase electrochemiluminescence assay and turned out to be effective modulators of γ-secretase. They affected the cleavage at the γ38, γ40 and γ42 sites to a different extent and particularly reduced the formation of $A\beta_{42,}$ while increasing the formation of $A\beta_{38}$ (see **Table 3.10** and **Table 3.11**).

Table 3.10: Activity report of *N*-sulfonylated carbazolyloxyacetic acids.

Entry	Compound Code	Compound	Cell Toxicity[c] [μM]	IC$_{50}$ [μM]		
				$A\beta_{38}$[a]	$A\beta_{40}$	$A\beta_{42}$
1	**BSc2913**	**70a**	20	13	>40	39
2	**BSc2915**	**70b**	20	>40	>40	>40
3	**BSc2914**	**70c**	20	13	40	24
4	**BSc2912**	**70d**	20	7.8	19	7.5
5	**BSc3034**	**70e**	>40	ND[b]	>40	38
6	**BSc3033**	**70f**	>40	ND[b]	>100	37
7	**BSc3035**	**70g**	40	14	>40	20
8	**BSc3624**	**70h**	40	32	>40	32
9	**BSc3626**	**70i**	40	13	>40	>40
10	**BSc3627**	**70j**	40	>40	>40	>40
11	**BSc3625**	**70k**	40	16	>40	>40

[a] EC$_{50}$ values are displayed for $A\beta_{38}$.
[b] Maximum effect on $A\beta_{38}$ not observed at 200 μM.
[d] Significant cellular toxicity observed at this concentration. Viability reduction > 20%.

Table 3.11: Activity report of *N*-alkylated carbazolyloxyacetic acids.

Entry	Code	R[c]	Compound	CLogP	Cell toxicity[c] [μM]	IC$_{50}$ [μM]		
						$A\beta_{38}$[a]	$A\beta_{40}$	$A\beta_{42}$
1	**BSc2916**	H	**72a**	2.82	>40	>40	>40	>40
2	**BSc3030**	Octyl	**72b**	7.03	>40	24	>100	19
3	**BSc3031**	Nonyl	**72c**	7.55	>40	30	>100	17
4	**BSc3032**	Decyl	**72d**	8.08	>40	ND	>40	11
5	**BSc3770**	Undecyl	**72e**	8.61	>40	14.6	>40	17.3
6	**BSc3771**	Dodecyl	**72f**	9.14	>40	10.4	>40	15
7	**BSc3772**	Tetradecyl	**72g**	10.2	>40	6.5	>40	8.2
8	**BSc3773**	Hexadecyl	**72h**	11.2	>40	7.3	>40	14.6

[a] EC$_{50}$ values are displayed for $A\beta_{38}$.
[b] Maximum effect on $A\beta_{38}$ not observed at 200 μM.
[c] tPSA for all compounds **72a-h** is 62.9.

The most potent inhibitors of $A\beta_{42}$ were compounds **70d, 70g**, **72c** and **72g**. Compounds **80a–c** showed modulatory activity but required very high concentrations. The compounds **80d–f** turned out to be inverse modulators; they increased $A\beta_{42}$ formation and reduced $A\beta_{38}$ formation at very high concentrations. Introduction of long alkyl chains such as undecyl,

dodecyl, tetradecyl and hexadecyl showed no siginificant improvement in the modulatory activity compared to **72c**, but affected the CLogP dramatically. Compound **72g** was found to be the most active compound amongst the *N*-alkylated carbazolyloxy acetic acids **72**, but the increased CLogP due to the lipophilic substituent tetradecyl makes the blood brain penetration unlikely. The necessary levels for IC_{50}/EC_{50} determination were not reached at 40 μM. The effect of the most potent compounds on γ-secretase cleavage at the ε-site was assessed using an *in vitro* assay that monitors the *de novo* generation of AICD.[183, 184] The formation of AICD was affected by the compounds to various extend.

Detailed results and discussion of these experiments are described in the article attached at the end as Appendix II. i.e.

"Rajeshwar Narlawar, Blanca Perez Revauelta, Karlheinz Baumann, Robert Schubenel, Christian Haass, Harald Steiner, Boris Schmidt, *N*-Substituted Carbazolyloxyacetic Acids Modulate Alzheimer Associated γ-Secretase, *Bioorganic and Medicinal Chemistry Lett*ers, **2007**, *17*, 176-182."

3.4. The LXR-Agonist TO-901317 Can Be Tuned From Inverse to Normal Modulation of γ-Secretase

Most of the reported and confirmed γ-secretase inhibitors are not substrate specific and interfere with Notch processing and signalling. A long term treatment with γ-secretase inhibitors may cause severe gastrointestinal toxicity and interferes with the maturation of B- and T-lymphocytes in mice effects due to inhibition of Notch processing and signalling.[185, 186] But, compounds capable of modulating the active γ-secretase complex by interference with substrate recognition may alter or block Aβ production with little or no effect on Notch cleavage will be ideal candidates for AD therapeutics. The allosteric modulation is particularly attractive target for drug development since it may control the ratio of Aβ fragments while retaining the cleavage of other substrates being processed by γ-secretase. Recent studies suggest that the protease complex contains allosteric binding sites that can alter substrate selectivity and the sites of substrate proteolysis.[187, 188] Epidemiological studies have indicated an association between prolonged use of NSAIDs and reduced risk for AD. Certain NSAIDs (e.g., ibuprofen, indomethacin and sulindac sulfide) can reduce the production of the highly aggregation prone $A\beta_{42}$ peptide and increase the level of $A\beta_{38}$ peptide, independent of their COX inhibitory activity.[167, 173, 189] Enzyme kinetic studies and displacement experiments revealed that the selective NSAIDs can be non-competitive with respective to APP substrate and to a transition-state analogue inhibitor. This indicates that they might be interacting with a site distinct from the active site.[117] *N*-Sulfonylated and *N*-alkylated carprofen and carbazolyloxyacetic acid derivatives that modulate γ-secretase independent of their COX inhibitory activity have been discussed previously.[120, 182]
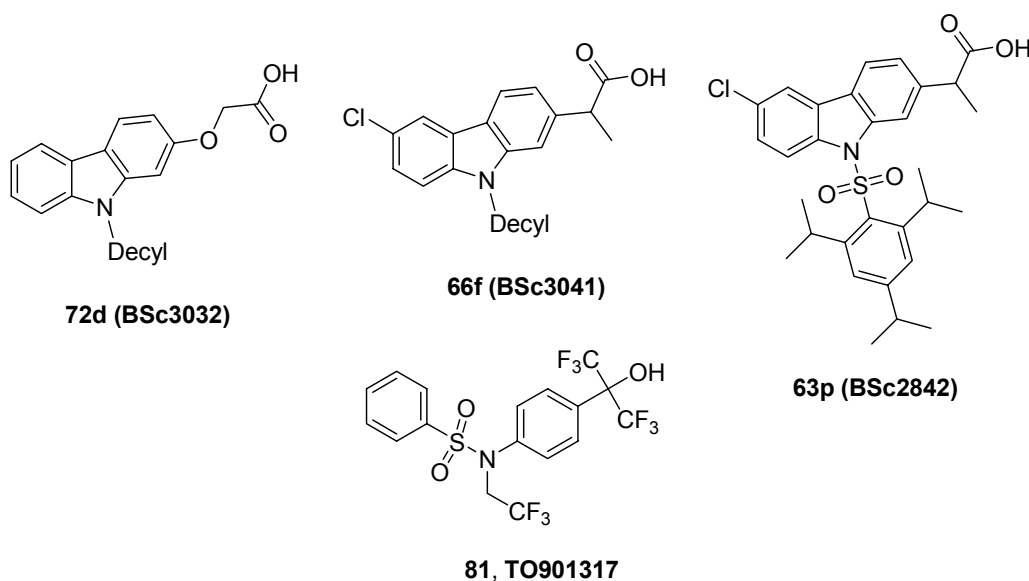

Scheme 3.13: Chemical structures of **72d (BSc3032)**, **66f (BSc3041)**, **63p (BSc2842)** and **TO-901317**.

The most active compounds **63p (BSc2842)**, **66f (BSc3041)** and **72d (BSc3032)**, affected the cleavage at the γ38, γ40 and γ42 sites to a different extent and particularly suppressed the formation of $A\beta_{42}$, while enhancing the formation of $A\beta_{38}$ and thus showed the typical profile of effective NSAIDs (**Scheme 3.13**). Modification of the acid moiety into its amide or ester derivatives resulted in the complete loss of modulatory activity. This indicated an important contribution of the carboxylic acid to target affinity. Recently Czech et al. demonstrated that **TO-901317**,[190] a LXR agonist, interacts directly with the γ-secretase complex to alter the production of different Aβ peptides.[191] Moreover, **TO-901317** directly modulates the site of

cleavage of APP by γ-secretase *in vitro* and displays cholesterol-independent effect on APP processing. It was found to be an inverse modulator of γ-secretase and displayed activity at low micromolar concentration. It selectively increased the formation of Aβ42 and reduced the formation of Aβ38. Riddell et al. reported that LXR agonist, **TO901317**, increased hippocampal ABCA1 and apoE and decreased Aβ42 levels in APP transgenic mice. **TO901317** had no significant effects on levels of Aβ40, full length APP, or the APP processing products. These observations indicate that LXR agonists do not directly inhibit APP processing but rather facilitate the clearance of Aβ42 and may represent a novel therapeutic approach to AD.[190]

In the search for non-peptidic γ-secretase modulators, LXR agonist **TO-901317** analogous compound was synthesized, but the hexafluorocarbinol moiety was replaced by an oxyacetic acid moiety. It was hypothesized that, the replacement of hexafluorocarbinol moiety by an acid moiety would change the mode of action from inverse modulation to normal modulation.

Therefore, the LXR agonist **TO-901317** analogous compound **87** was synthesized as depicted in **Scheme 3.14**. Alkylation of the phenolic –OH of **82** using anhydrous K$_2$CO$_3$ and t-butyl chloroacetate in acetone at 60–70 °C to afford nitro ester **83**. Subsequent nitro group reduction by hydrogenation using 10% Pd-C in ethyl acetate gave amine **84**.
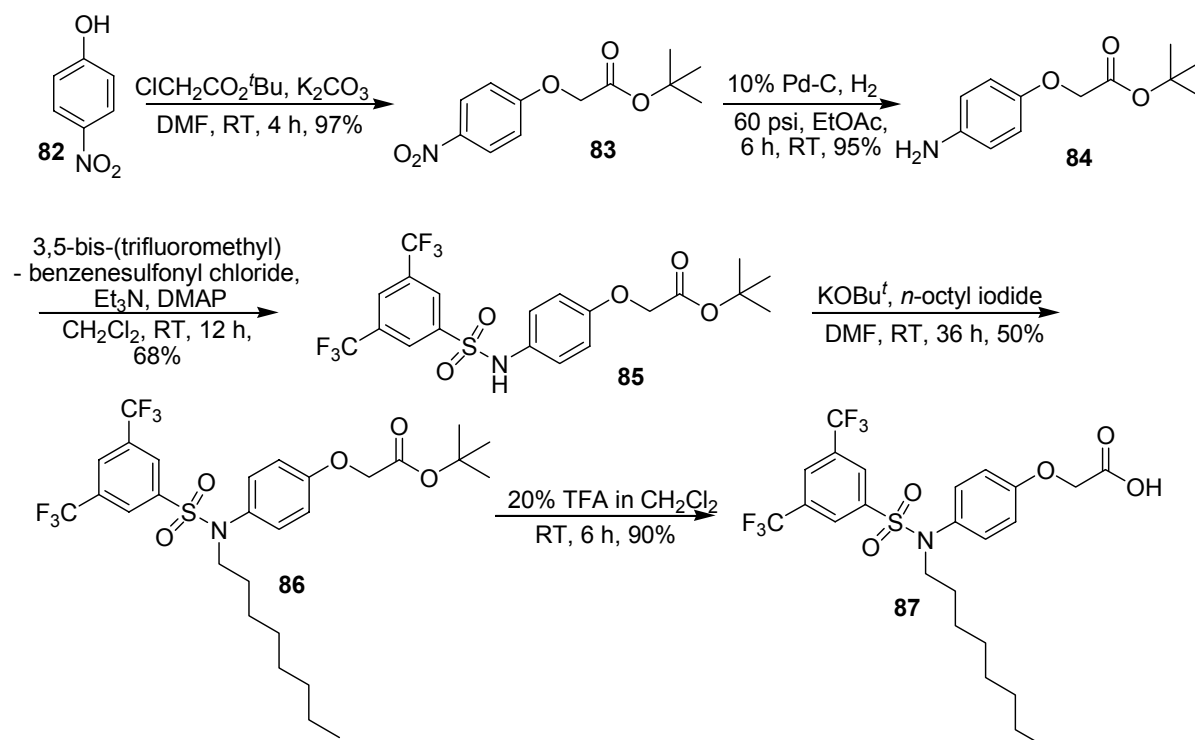
Scheme 3.14: Synthesis of LXR agonist analogous γ-secretase modulator.

N-sulfonylation of amine **84** was carried out using triethyl amine, catalytic DMAP and 3,5-bis-(trifluoromethyl) benzene sulfonyl chloride in CH$_2$Cl$_2$ to afford sulfonamide **85**. *N*-alkylation of sulfonamide **85** was accomplished using KOBu^t and *n*-octyl iodide in DMF provided alkylated product **86** and subsequent acidic cleavage of *t*-butyl ester gave acid **87** as colourless solid.

Compounds **85-87** were tested for their ability to modulate γ-secretase in the Aβ liquid phase electrochemiluminescence assay. As anticipated compounds **85** and **86** turned out to be

inverse modulators of γ-secretase and displayed the inverse modulatory activity in the low micromolar concentration (see **Table 3.12**, **Figure 3.5**).

Table 3.12: Activity report of compounds **85-87**.

Entry	Compound	Compound Code	Cell viability[a] [μM]	Activity in μM				
				IC_{50} $A\beta_{38}$	EC_{50} $A\beta_{38}$	IC_{50} $A\beta_{40}$	IC_{50} $A\beta_{42}$	EC_{50} $A\beta_{42}$
1	**81**	**TO901317**	–	4.5	–	>40	–	3.6
2	**85**	**BSc3850**	>40	>40	–	>40	–	10.6
3	**86**	**BSc3851**	>40	13.3	–	>40	–	6.2
4	**87**	**BSc3769**	40	–	23.7	>40	19.6	–

[a] Significant cellular toxicity observed at this concentration. Viability reduction > 20%.

In accordance with previous results,[120, 182] the ester **85** and **86** affected the cleavage at the γ38, γ40 and γ42 sites to a different extent and suppressed the formation of $A\beta_{38}$, while enhancing the formation of $A\beta_{42}$. *N*-alkylation of sulfonamide **86** dramatically enhanced the γ-secretase modulatory activity (**Table 3.7**). However, compound **87** turned out to be effective modulator of γ-secretase. It reduced particularly the formation of $A\beta_{42}$ and enhanced the formation of less toxic $A\beta_{38}$. The transformation of ester **86** into its free acid **87** changed the mode of action from inverse modulation to normal modulation and displayed a typical NSAID like profile. Moreover, it showed toxicity at 40 μM concentration (**Figure 3.6**). Compound **87** is believed to have no or little effect on the γ-secretase cleavage at ε-site. This confirms the hypothesis that the free acid moiety is crucial for the modulatory activity and for uptake. It is speculated that the lipophilic substituent anchors the *N*-substituted phenoxyacetic acid in the required orientation within the membrane, thus the maximum tolerated length should be similar to natural phospholipids.

In conclusion, replacement of the hexafluorocarbinol moiety of LXR agonist, **TO-901317** by an oxyacetic acid group dramatically changed the mode of action from inverse modulation to modulation. In accordance with previous results, the modification of acid into its derivatives such as ester turned the compounds **85** and **86** into inverse modulators. Introduction of *n*-octyl, a lipophilic substituent on the sulfonamide **85** increased the inverse modulatory activity. Compound **87** with the free acid moiety was found to be a classical modulator of γ-secretase.
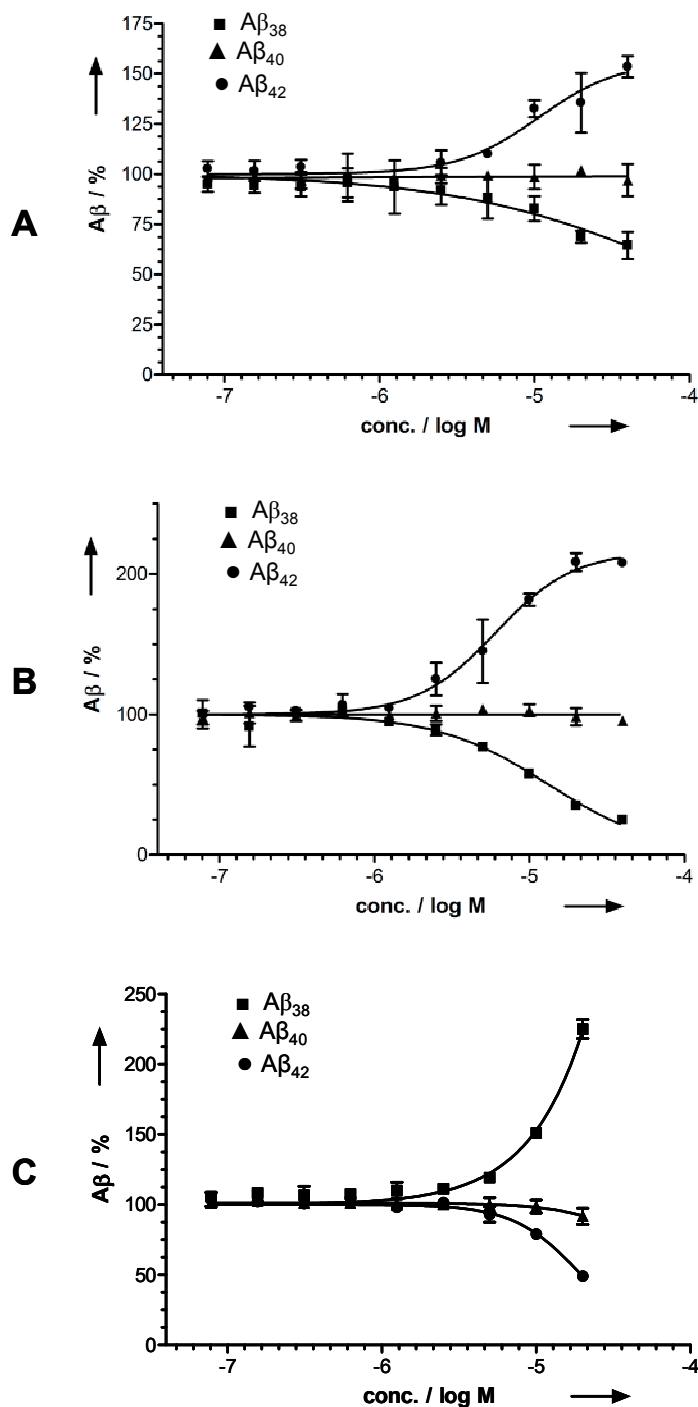
Figure 3.5: Dose response curves; Aβ (% of control). (A) Compound **78**. (B) Compound **79**. (C) Compound **80**.

Again, it is evident from the structure activity relationship data that the free acid moiety is crucial for the PS modulatory activity. This significant modification can be applied to several reported γ-secretase inverse modulators such as celecoxib and rofecoxib and thus applied structural modification may change the mode of action from inverse modulation to modulation.

3.5. Curcumin Derived Pyrazoles and Oxazoles – Swiss Knives or Dirty Tools for AD?

This chapter is removed due to the IP reasons.

3.6. Synthesis and Biological Evaluation of NSAIDs Derived Affinity Labels

γ-Secretase has been an attractive target in many ways for AD therapeutics. But, the interference of most of the reported and confirmed γ-secretase inhibitors with Notch processing and signalling leads to toxicities that rule out their clinical use. Long term treatment with γ-secretase inhibitors causes severe gastrointestinal toxicity and interferes with the maturation of B- and T-lymphocytes in mice. These observed effects are indeed due to inhibition of Notch processing and signalling.[195] Recent studies indicate that the active γ-secretase complex contains allosteric binding sites which may alter the substrate selectivity and the sites of proteolysis. Certain NSAIDs such as ibuprofen, indomethacin and sulindac sulfide can reduce the production of the highly aggregation prone $A\beta_{42}$ peptide and increase the level of $A\beta_{38}$, a pharmacological property that is independent of COX inhibition.[111] The alteration of the proteolytic cleavage site is observed with isolated or purified γ-secretase. This indicates that the compounds interact directly with the γ-secretase complex to exert these effects.[118, 196] Enzyme kinetic studies and displacement experiments suggest that selective NSAIDs can be non-competitive with respect to APP substrate and a transition-state analogue inhibitor, suggesting interaction with a site distinct from the active site. The site of cleavage within the Notch TMD is equally affected, but this slight change does not inhibit the release of the intracellular domain (ICD) and therefore the signalling Notch is not affected.[42] For this reason, these agents may be safer as AD therapeutics compared to the γ-secretase inhibitors that block the active site or the docking site. Surprisingly, the site of proteolytic cleavage by signal peptide peptidase (SPP) can also be modulated by the same NSAIDs that affect γ-secretase. These observations suggest that PS is the site where NSAIDs bind within the γ-secretase complex and that SPP and PS share a conserved binding site for allosteric modulation of substrate cleavage sites. Most of the NSAIDs interfere with the APP mechanism *via* allosteric modulation, but this is not a class effect. Negative results were obtained with some NSAIDs such as aspirin, naproxen.
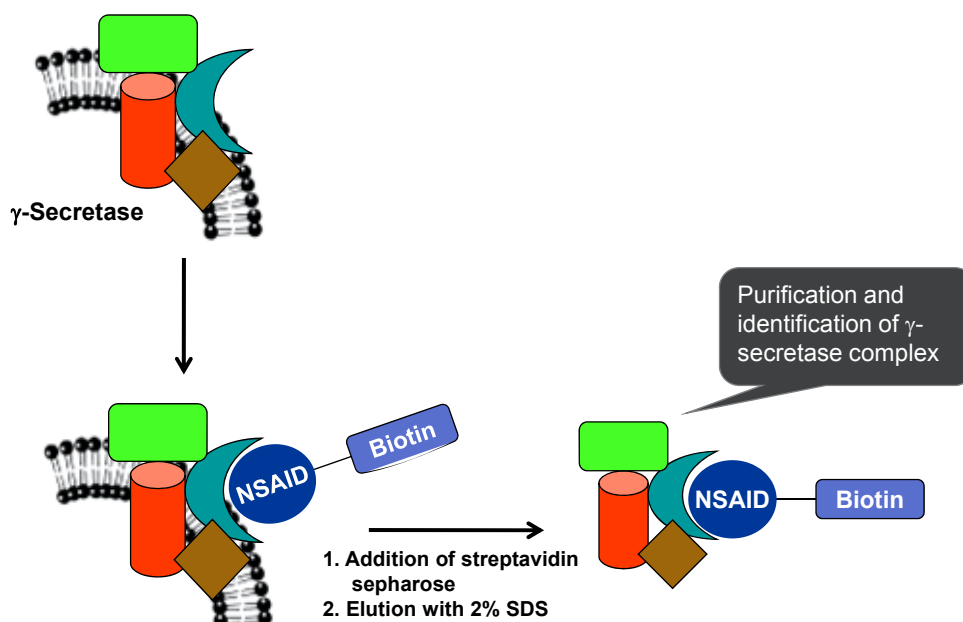

Figure 3.6: Approach to capture the active γ-secretase complex and identify the binding site of NSAID.

It is apparent that certain $A\beta_{42}$-modulating compounds such as NSAIDs target the γ-secretase complex, but the molecular details are still mysterious. One important concern is to identify the interaction partner of $A\beta_{42}$-modulating compounds within the γ-secretase complex.

Although PS seems to be the crucial candidate, but at the same time interaction with any of the components in the γ-secretase complex or with the substrate APP is quiet possible. Even more mysterious issue is how the conformational changes are being persuaded by Aβ$_{42}$-modulating compounds which lead to the observed shift in cleavage specificity from Aβ$_{42}$ to Aβ$_{38}$. One potential explanation is that these compounds induce subtle changes in the presentation of the substrate i.e. APP to the catalytic center in the γ-secretase complex.

To shed light on the binding site of NSAIDs and understand the actual mechanism of modulation, biotinylated NSAIDs were synthesized. Biotin was used to facilitate the isolation and identification of the reversibly labelled adducts *via* their streptavidin-enzyme linked conjugates. It was anticipated that the NSAIDs would bind to the allosteric site on γ-secretase and the biotin tag would help to identify the binding partner *via* immunoprecipitation. Ethylene diamine was used as the spacer to maintain the distance between affinity label and the ligand (**Figure 3.6**).

3.6.1. Synthesis of Biotinylated NSAIDs *via* Structural Modification of Acid Moiety

3.6.1.1. Synthesis of biotinylated flurbiprofen

Initially, biotinylated flurbiprofen was synthesized by the derivatization of acid moiety of flurbiprofen which is common to the COX inhibitors as depicted in **Scheme 3.17**. Flurbiprofen **32** is a classical modulator of γ-secretase and displays the modulatory activity independent to its COX-1 inhibitory activity.[111, 112, 117]
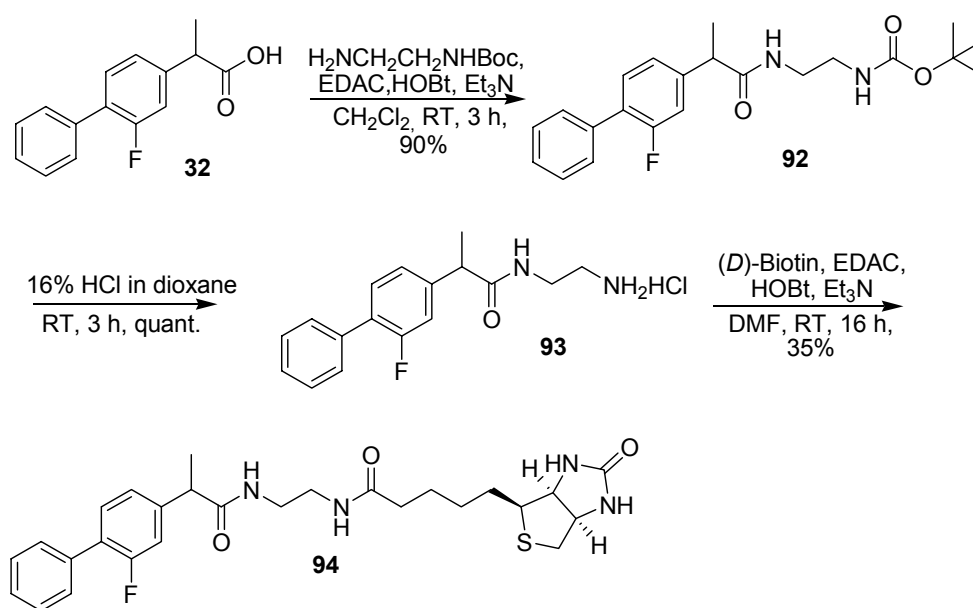
Scheme 3.17: Synthesis of flurbiprofen-biotin **94** (**BSc2174**).

The acid moiety of flurbiprofen was coupled with *N*-Boc ethylene diamine using EDAC and HOBt as coupling reagent to afford the coupled product **92**. *N*-Boc deprotection of compound by 20% TFA in CH$_2$Cl$_2$ gave amine **93** as a trifluoroacetic acid salt. The free amine **93** was then subjected for the condensation with *D*-biotin using EDAC and HOBt as coupling reagent in CH$_2$Cl$_2$. The reaction exhibited poor conversion and yield. It was attributed to the traces of the TFA in amine which remained even after flushing several times with *n*-hexane and drying *in vacuo*. Therefore, the *N*-Boc of **92** deprotected using 16% HCl in dioxane. The reaction displayed 100% conversion on TLC and, yielded the amine as a hydrochloride salt **93**. The

coupling of amine hydrochloride **93** with *D*-biotin using EDAC and HOBt as coupling reagents in DMF went smoothly and gave biotinylated flurbiprofen **94**.

Flurbiprofen-biotin **94** was tested for its ability to modulate γ-secretase in the Aβ liquid phase electrochemiluminescence assay. The assay was performed at Hoffman-La Roche, Basel, by Dr. Karlheinz Baumann. The dose response curve of **94** revealed that it acts as an inverse modulator in the cellular assay (**Figure 3.8**). A classical NSAID suppresses the formation of the most toxic species $A\beta_{42}$ and increase the formation of less toxic $A\beta_{38}$.
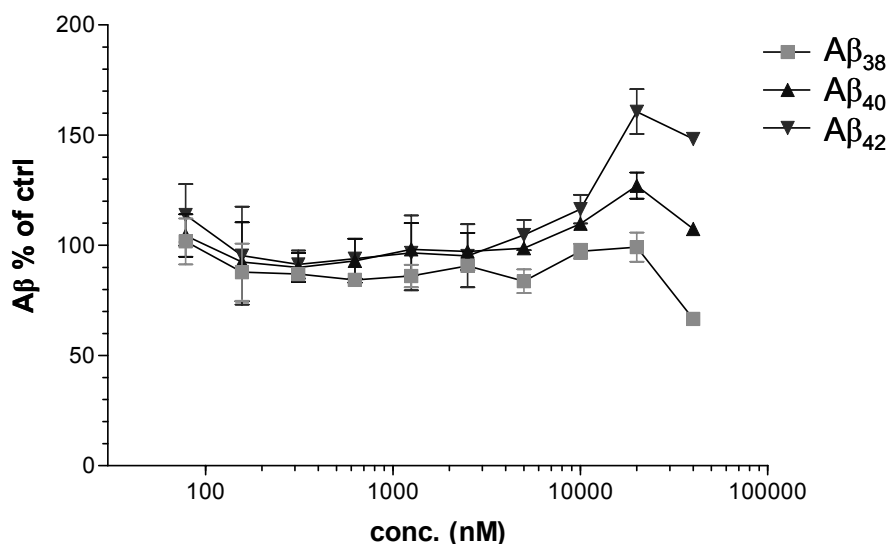

Figure 3.7: Dose response curve for flurbiprofen-biotin **94** (**BSc2174**).

But surprisingly, the conversion of the acid moiety of flurbiprofen into its amide turned it into an inverse modulator of γ-secretase and resulted in an elevation of $A\beta_{42}$ and reduction of $A\beta_{38}$ (**Figure 3.7**). Next, the biotinylated flurbiprofen **94** was tested for its ability to bind and pull down the active γ-secretase complex. The immunoprecipitation experiments were performed at the LMU Munich by Blanca I. Perez Revaulta. It was anticipated that, **94** would bind to the allosteric sites on the active γ-secretase complex and the biotin tag would allow the isolation, since biotin has a very strong affinity with streptavidin. Flurbiprofen-biotin **94** binding was expected to be optimal under solubilization conditions where the enzyme remains in a catalytically active conformation. CHAPSO-solubilized γ-secretase was incubated with the biotinylated affinity ligand for 2 h at 4 °C followed by precipitation of enzyme-inhibitor complexes with streptavidin-coupled magnetic beads. Polypeptides present in the precipitates were characterized by western blotting. Flurbiprofen-biotin was tested for its ability to capture γ-secretase complex at three different concentrations, 100 μM, 250 μM and 500 μM. Further analysis revealed that by combining an incubation of solubilized γ-secretase at an affinity ligand concentration of 500 μM followed by capture using streptavidin beads (**Figure 3.9**) an optimal specific precipitation of the members of active γ-secretase complex, PS1, nicastrin, Aph-1 and pen-2 was observed. Omission of the affinity ligand (**Figure 3.8**) abolished the precipitation of all these polypeptides. The western blots revealed that regardless of having lost its NSAIDs like activity, flurbiprofen-biotin **94** indeed captured the γ-secretase complex in a dose dependant fashion.
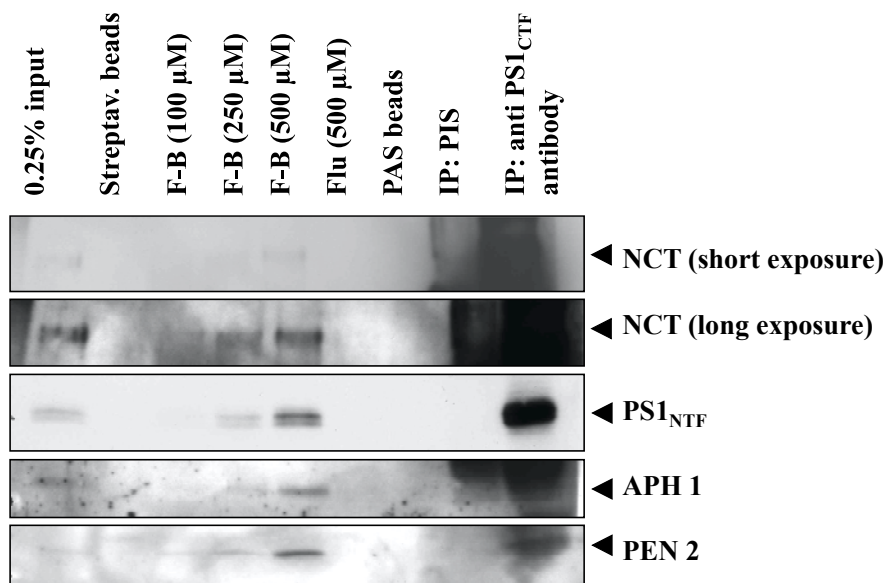
Figure 3.8: F-B **94** captures the γ-secretase complex in dose dependant manner.

One of the crucial issues related to the γ-secretase inhibitors is the interference with other γ-secretase substrates such as Notch and others. Therefore, the effect of flurbiprofen-biotin **94** on γ-secretase cleavage at the ε-site was assessed using an *in vitro* assay that monitors the *de novo* generation of β-amyloid precursor protein intracellular domain (AICD) (**Figure 3.9**). The ε-cleavage of γ-secretase was not inhibited by **94** at low compound concentrations that determined to be modulatory at the γ-secretase site. However, at concentrations where most efficient binding occurred (500 μM), the compound acted as an inhibitor, as monitored by an *in vitro* γ-secretase assay that assessed AICD generation (i.e. γ-secretase cleavage at the ε-site) (**Figure 3.10**).
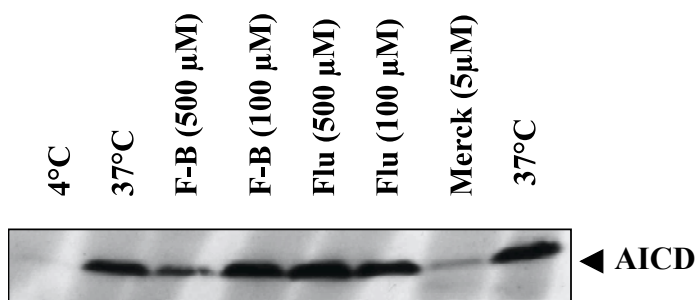

Figure 3.9: Effect of F-biotin **94** and flurbiprofen **32** on γ-secretase cleavage at ε-site.

3.6.1.2. Synthesis of biotinylated flurbiprofen *via* Curtis rearrangement

Biotinylated flurbiprofen was synthesized *via* a Curtis rearrangement. The acid moiety of flurbiprofen was converted to its corresponding urea in order to investigate the impact of urea moiety on the capture efficiency of γ-secretase active complex.

The synthesis commenced from commercially available flurbiprofen **32**. The acid moiety of flurbiprofen **32** was converted to the mixed anhydride using ethyl chloroformate and nucleophilic substitution by azide gave the acyl azide **95**. The acyl azide underwent the Curtis rearrangement to give the isocynate which was trapped *in situ* using *N*-Boc ethylene diamine

to give the urea **96**. *N*-Boc of **96** was deprotected using 16% HCl in dioxane to give the free amine **97** as the hydrochloride salt which was used in the next step without further purification. The coupling reaction of the amine hydrochloride **97** with (*D*)-biotin underwent smoothly using EDAC and HOBt as coupling reagents in DMF to afford the desired biotinylated flurbiprofen **98** as colorless solid (**Scheme 3.18**).
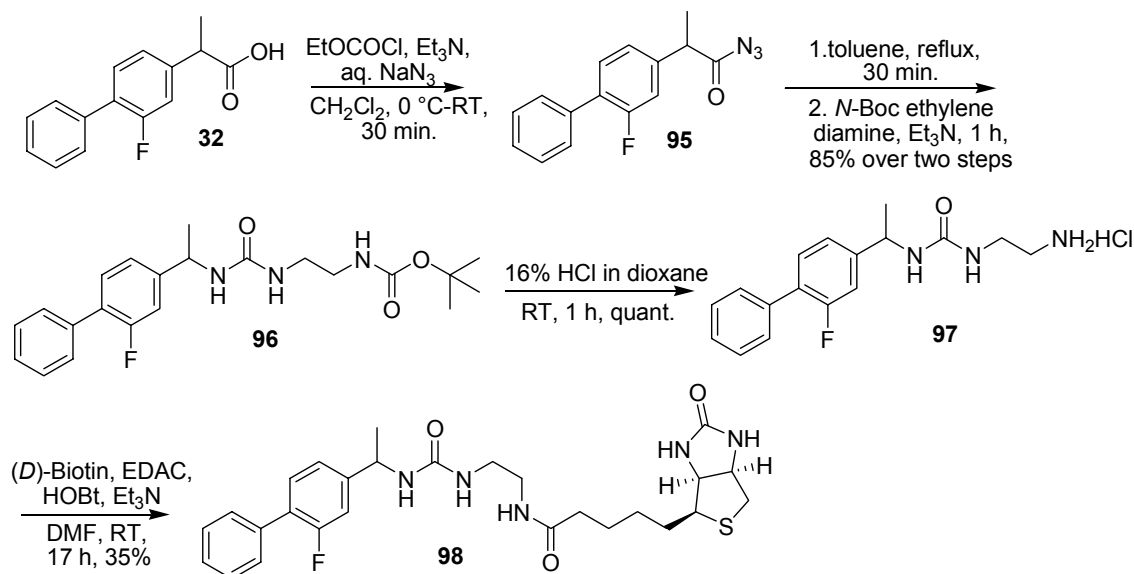
**Scheme 3.18:** Synthesis of flurbiprofen biotin **98** (**BSc2454**) *via* Curtis rearrangement.

The biotinylated flurbiprofen **98** was tested in the Aβ liquid phase electrochemiluminescence assay for its ability to modulate γ-secretase (**Figure 3.12**). The assay results revealed that compound **98** is an inverse modulator of γ-secretase and displayed activity at 40 μM concentration. The immunoprecipation experiments carried out with **98** illustrated that compound does not capture the γ-secretase active complex even at very high concentrations.

Introduction of the biotin *via* functional group transformation into amide or urea resulted in the loss of classical modulation. Instead, the biotinylated probes were found to be inverse modulators of γ-secretase. Even then, the affinity label **98** captured γ-secretase active complex. These results encouraged the synthesis of biotinylated NSAIDs which are known to inhibit or modulate APP metabolism *via* interference with γ-secretase and investigation using immunoprecipation assay for the identification of a binding partner. Therefore, sulindac sulfone, meclofenamic acid, ketoprofen and carprofen were shortlisted due to their ability to modulate or inhibit γ-secretase.

3.6.1.3. Synthesis of biotinylated meclofenamic acid derivative

Meclofenamic acid is a nonselective COX inhibitor and belongs to *N*-arylanthranilic acid derivative which are commonly known as fenamtes. Meclofenamic acid exhibits pharmacologic actions similar to those of aspirin but is 150 times potent that aspirin. It is a potent inhibitor of COX, thereby inhibits the release of prostaglandins. It was found to be an inverse modulator at very high concentration.[112] As discussed previously, meclofenamic acid derived amides were found to be either potent inhibitors or inverse modulators of γ-secretase. Biotinylated meclofenamic acid was synthesized in order to identify the binding partner *via* immunoprecipation. Biotin was introduced *via* the transformation of meclofenamic acid as depicted in **Scheme 3.19**.

Synthesis of biotinylated meclofenamic acid commenced from the commercially available meclofenamic acid sodium salt **37**. The acid of **37** was coupled with *N*-Boc ethylene diamine using EDAC and HOBt in CH_2Cl_2 to get coupled product **99**. *N*-Boc deprotection was accomplished using 16% HCl in dioxane to afford free amine **100** as hydrochloride salt which was used in the next step without further purification. Amine **100** underwent amidation reaction with *D*-biotin using EDAC and HOBt as coupling reagent in DMF. The crude compound was purified by column chromatography using eluents $MeOH:CH_2Cl_2$ (1:9) to obtain biotinylated meclofenamic acid derivative **101** as pale brown solid.
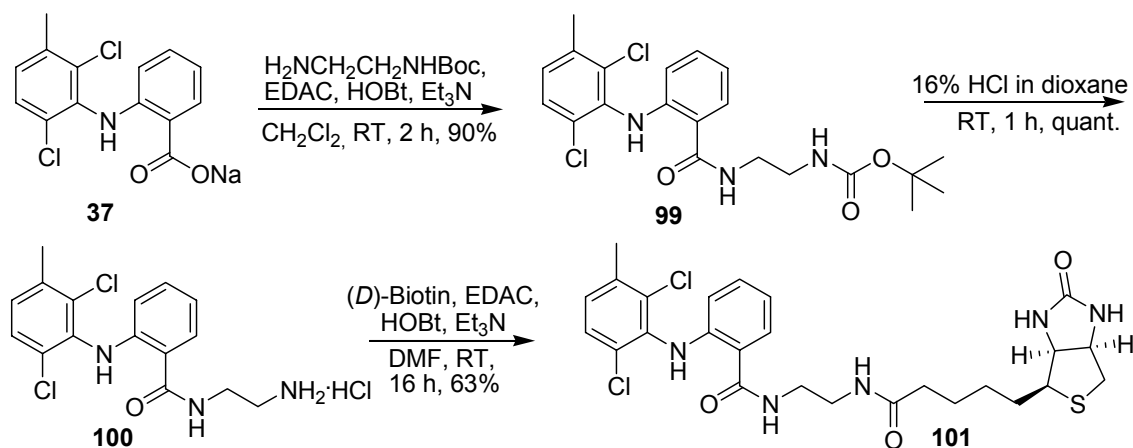
Scheme 3.19: Synthesis of biotinylated meclofenamic acid **101** (**BSc2403**).

The probe **101** was tested for its potential to modulate γ-secretase in the Aβ liquid phase electrochemiluminescence assay. **101** was found to be an inverse modulator of γ-secretase at 40 μM concentration (**Figure 3.10**).

The conversion of meclofenamic acid into its **101** amide turned it into an inverse modulator of γ-secretase. The immunoprecipitation assay of affinity label was carried out to investigate its potential to find a binding partner in immunoprecipitation assay and capture the active γ-secretae complex. Affinity label **101** did not find the binding partner in the immunoprecipitation assay and thus failed to capture the active γ-secretase complex.
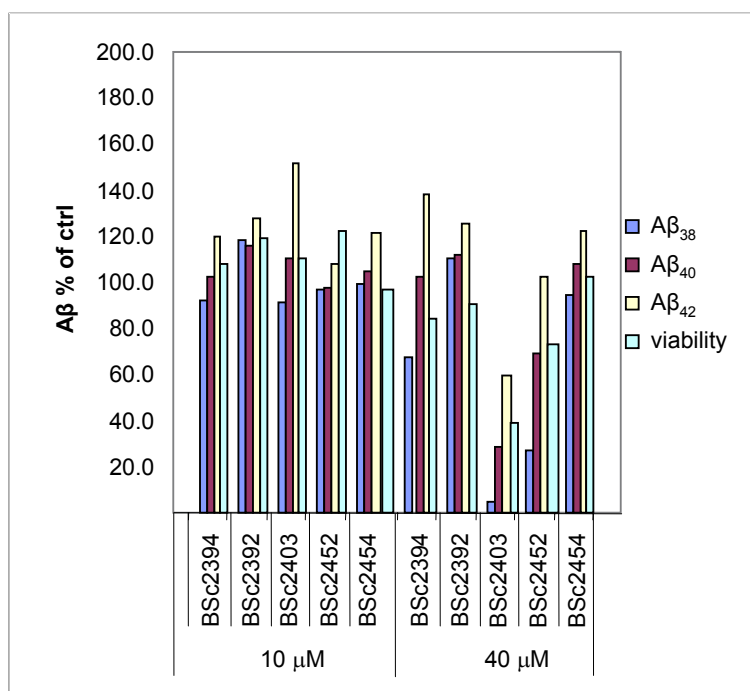
Figure 3.10: Activity report of biotinylated NSAIDs on γ-secretase at 10 μM and 40 μM concentration.

3.6.1.4. Synthesis biotinylated sulindac sulfoxide derivative

Sulindac sulfoxide **102** is a non-selective COX inhibitor. It is a prodrug and its active form, sulindac sulfide **33** displays potent COX inhibitory activity. Sulindac sulfide **33** is a classical modulator of γ-secretase: selectively reduces the formation of Aβ$_{42}$ and increases the formation of Aβ$_{38}$.[111, 112, 116, 117] Sulindac sulfoxide derived affinity label was synthesized as outlined in **Scheme 3.20**. The synthesis began from the commercially available sulindac sulfoxide **102**. The acid **102** underwent coupling with *N*-Boc ethylene diamine using EDAC and HOBt as coupling reagent in CH$_2$Cl$_2$ to achieve compound **103**.
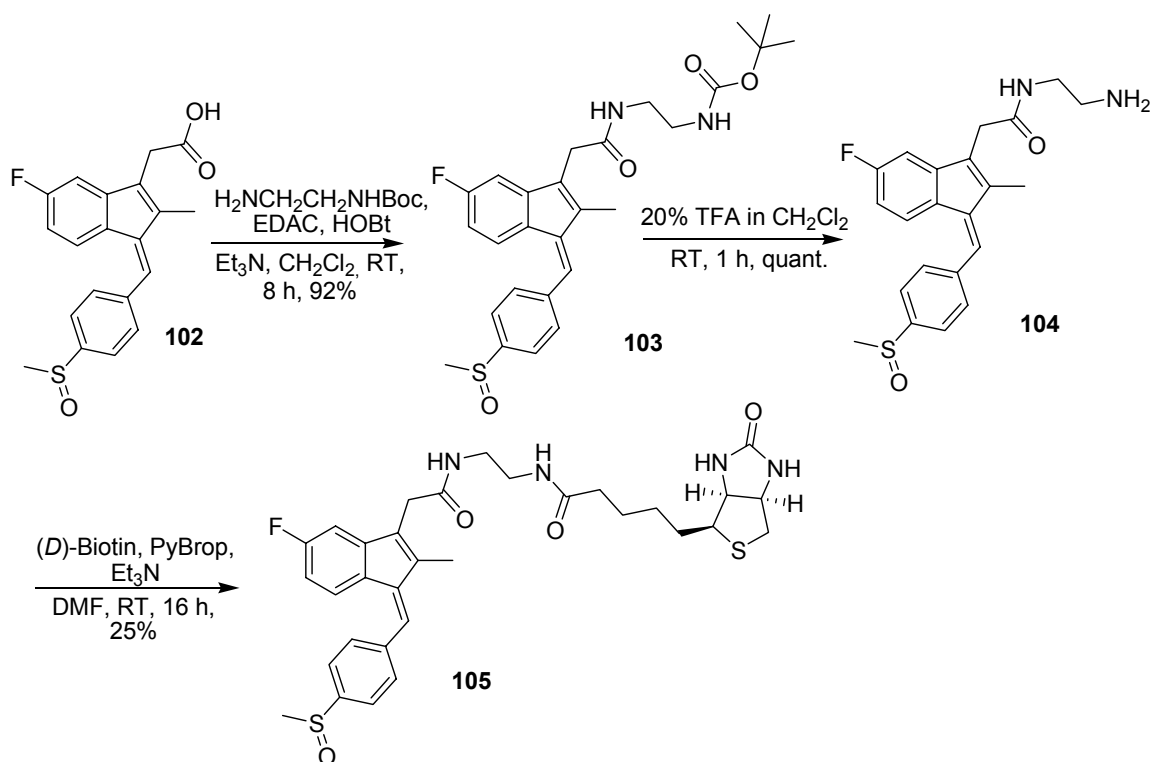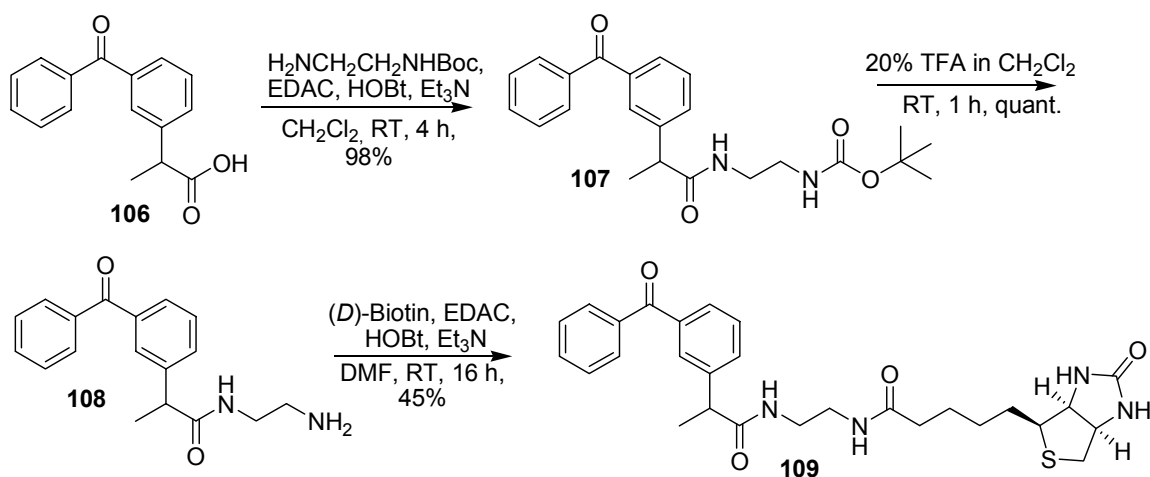
Scheme 3.20: Synthesis of biotinylated sulindac sulfoxide **105** (**BSc2452**).

Initially, the *N*-Boc of **103** was deprotected using 16% HCl in dioxane to obtain the free amine **104** as hydrochloride salt which was used in the next step without further purification. The amine hydrochloride salt **104** was coupled with *D*-biotin in DMF using EDAC and HOBt as coupling reagent. The TLC of the reaction indicated several spots and it made the separation by column chromatography difficult. The formation of impurities was credited to the highly acidic reaction condition used for the cleavage of *N*-Boc. Consequently, *N*-Boc of **103** was deprotected using 20% TFA in CH_2Cl_2 to give amine **104** as TFA salt which was used in the next step without further purification. Amine **104** was coupled with *D*-biotin in DMF using PyBrop as coupling reagent. The TLC indicated complete consumption of starting material and the reaction was much cleaner. The purification by column chromatography gave the sulindac sulfoxide derived affinity label **105** as yellow solid.

The affinity label **105** was tested for its ability to modulate γ-secretase in the Aβ liquid phase electrochemiluminescence assay. Compound **105** was found to be an inverse modulator of γ-secretase and displayed activity at 40 µM concentration. It particularly increased the formation of $A\beta_{42}$, the most toxic species which is prone to aggregate (**Figure 3.10**). Subsequently, the affinity label **105** was tested in immunoprecipitation assay for its ability to find the binding partner and capture the active γ-secretase complex. The assay results indicated that **105** do not capture the γ-secretase active complex even at very high concentrations.

3.6.1.5. Synthesis of biotinylated ketoprofen

Ketoprofen is a COX inhibitor and was found to be an inverse modulator of γ-secretase at high concentration.[112] The chemical strucutre of ketoprofen reveals a photoreactive benzophenone group which can be used directly for the photoaffinity labelling experiment. The biotinylated ketoprofen **109** can be used for the photolabelling experiment, where the benzophenone serves as the photoreactive group and biotin as an affinity tag.

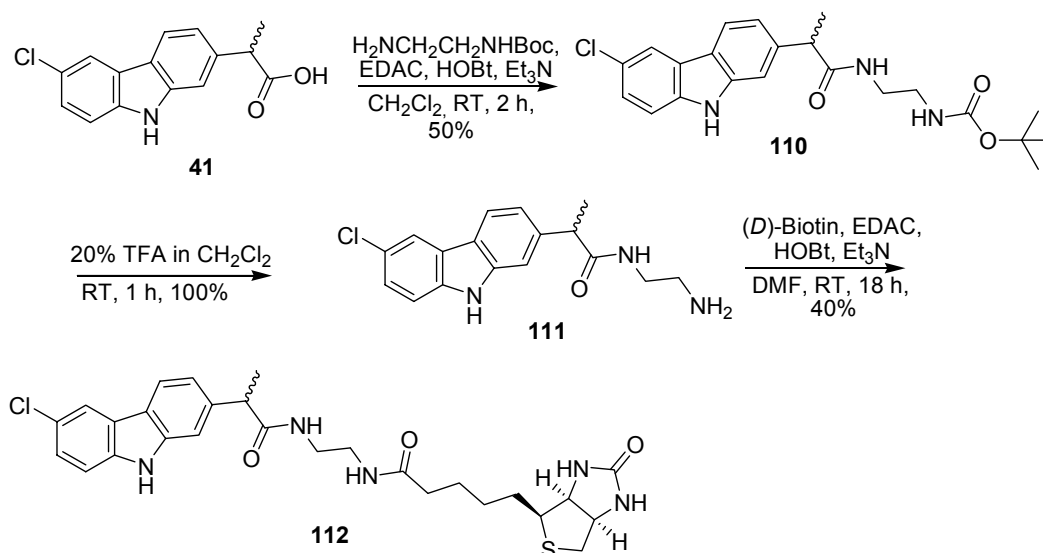**Scheme 3.21:** Synthesis of biotinylated ketoprofen **109** (**BSc2394**).

The biotinylated derivative of ketoprofen was synthesized as illustrated in **Scheme 3.21**. The acid moiety of ketoprofen **106** underwent amidation with *N*-Boc ethylene diamine using EDAC and HOBt as coupling regent in CH_2Cl_2 to afford the coupled product **107**. Subsequent *N*-Boc deprotection using 20% TFA in CH_2Cl_2 gave free the amine **108** as trifluoroacetic acid salt which was used in the next step without further purification. Biotinylation of amine **108** was carried out using *D*-biotin and PyBrop as coupling reagents in DMF to give **109** as a cream colored solid.

Affinity label **109** was for its capability to modulate γ-secretase in the Aβ liquid phase electrochemiluminescence assay. It was found to be an inverse modulator of γ-secretase and displayed the activity at 40 μM concentration. (**Figure 3.10**). The ability of affinity label **109** to bind and capture γ-secrease active complex was tested in immunoprecipitaton assay. Again the experiment did not meet with success.

3.6.1.6. Synthesis of biotinylated carprofen

Carprofen **41** is a COX-2 inhibitor approved for the use in dogs, cows and horses. The selectivity of carprofen versus COX-2canine and COX-1canine is greater than 100:1 (COX-2canine IC_{50}: *R/S*-carprofen 102 nM, *R*-carprofen 5.97 μM, *S*-carprofen 37 nM). Original carprofen (as isolated from 500 mg tablets) was found to be a weak inhibitor of γ-secretase and reduced $Aβ_{38}$, $Aβ_{40}$ and $Aβ_{42}$ at high concentration.[120] Biotinylated carprofen derivative was synthesized as depicted in **Scheme 3.22**.

The acid moiety of carprofen **41** was coupled with *N*-Boc ethylene diamine using EDAC and HOBt as coupling reagents to afford coupled product **110**. *N*-Boc of **110** was deprotected using 20% TFA in CH_2Cl_2 to afford the free amine **111** which was used in the next step without further purification. Subsequently, the amine **111** was coupled with *D*-biotin using EDAC and HOBt as coupling reagents in DMF to afford the affinity label **112** as a cream colored solid.

Scheme 3.22: Synthesis of biotinylated carprofen derivative **112** (**BSc2392**).

Affinity label **112** was tested for its ability to inhibit γ-secretase in the Aβ liquid phase electrochemiluminescence assay. Surprisingly, it was found to be an inverse modulator of γ-secretase (**Figure 3.10**). The transformation of the acid moiety of carprofen into amide changed the mode of action from full blown inhibition to inverse modulation. Furthermore, affinity label **112** was tested in immunoprecipitation assay for its potential to capture active γ-secretase complex. Eventually the affinity label **112** did not reveal any binding in the immunoprecipitation assay.

To summarize, the structure analysis relationship (SAR) of NSAIDs derived affinity labels **94**, **98**, **101**, **105**, **109**, **112** demonstrated that the incorporation of biotin *via* the structural modification of the acid moiety dramatically changed the mode of action from modulation or inhibition to inverse modulation. All the affinity labels selectively increased the formation of $A\beta_{42}$ and reduced the less toxic $A\beta_{38}$ formation. These results confirmed the requirement of the free acid moiety of the NSAIDs which is crucial for the classical modulatory activity and to maintain the typical NSAID like profile. It is presumed that, the free acid might be vital for the uptake. Interestingly, out of all tested affinity labels, only **94** (**BSc2174**) found a binding partner in the immunoprecipitation assay and captured the γ-secretase in the dose dependant fashion despite being an inverse modulator. The optimal concentration required for the γ-secretase capture was 500 μM. Moreover, it did not inhibit the formation of AICD, a typical property exhibited by γ-secretase modulating NSAIDs, even at a very high concentration.

SAR advocated the synthesis of NSAIDs derived affinity labels keeping intact the free acid. As a result, flurbiprofen and carprofen were selected initially.

3.6.2. Synthesis of Biotinylated NSAIDs without perturbing the Acid Moiety

3.6.2.1. Synthesis of biotinylated flurbiprofen with free acid moiety

The synthesis of biotin-flurbiprofen commenced from the commercially available flurbiprofen as outlined in **Scheme 3.23**. Flurbiprofen **32** was nitrated using 70% nitric acid at room temperature for 48 h. The nitration reaction resulted in two regio isomers: *o*-nitrated **113** and *p*-nitrated **114** products.
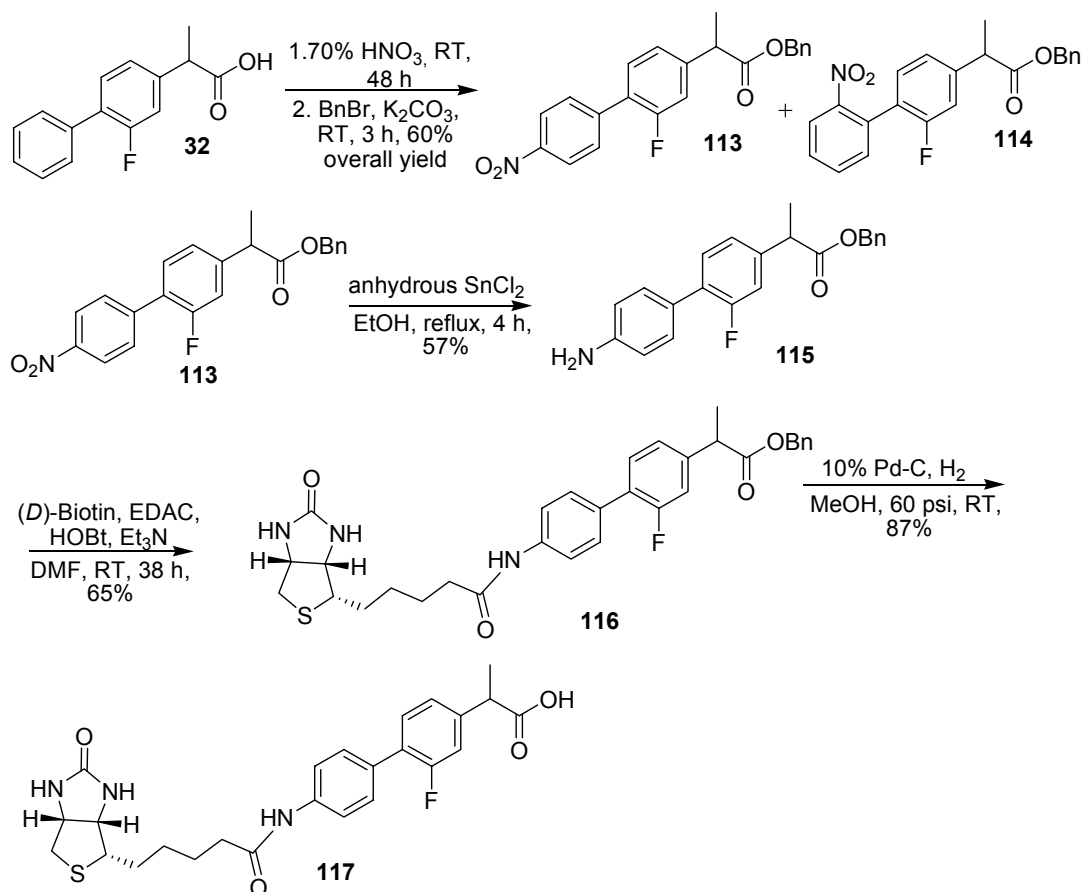
Scheme 3.23: Synthesis of biotinylated flurbiprofen **117** (**BSc2805**).

The purification of the regio isomers at this step was difficult due to an almost similar Rf value on TLC. Therefore, the acid moiety of the nitrated flurbiprofen mixture was protected and the regio isomers **113**, **114** were separated by column chromatography. The nitro group of **113** was chemoselectively reduced to its corresponding amine **115** using anhydrous SnCl$_2$ in ethanol at 80-90 °C. Amine **115** was coupled with *D*-biotin using EDAC and HOBt in DMF to afford biotinylated flurbiprofen benzyl ester **116**. The cleavage of the benzyl group was achieved by hydrogenation at 60 psi in MeOH to afford **117**.
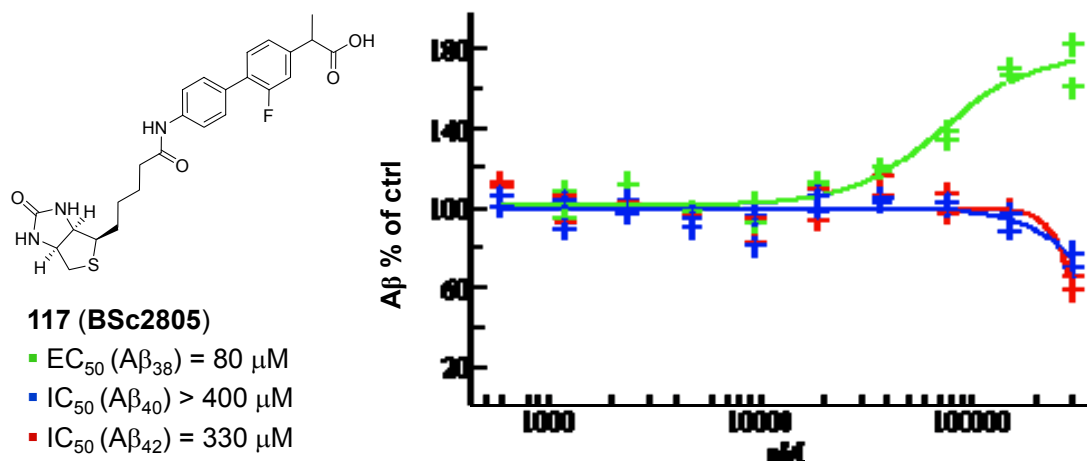

117 (BSc2805)
- EC$_{50}$ (Aβ_{38}) = 80 μM
- IC$_{50}$ (Aβ_{40}) > 400 μM
- IC$_{50}$ (Aβ_{42}) = 330 μM

Figure 3.11: Dose response curve of compound **117** (**BSc2805**).

The probe **117** was tested for its ability to modulate γ-secretase in the Aβ liquid phase electrochemiluminescence assay. As anticipated, the probe **117** (**BSc2805**) turned out to be an effective modulator of γ-seretase. It affected the cleavage of γ38, γ40 and γ42 to a different extent and particularly reduced the formation of Aβ$_{42}$ (**Figure 3.11**).

Affinity label **117** was tested in immunoprecipitation assay for its potential to capture active γ-secretase active complex. The assay revealed the probe **117** did not capture the active γ-secretase complex.

3.6.2.1. Synthesis of biotinylated carprofen *via* *N*-substitution with free acid moiety

Carprofen is a selective COX-2 inhibitor and displays inhibitory activity on γ-secretase. As mentioned in the previous chapter, *N*-substitution of carprofen changed the mode of action from full inhibition to modulation. It was evident from the SAR of *N*-substituted carprofen derivatives that the free acid is crucial for the modulatory activity. Therefore, *N*-substituted carprofen derived labels were designed in order to keep the modulatory activity intact. The synthesis of *N*-sulfonylated carprofen derived affinity label commenced from the previously synthesized intermediate *N*-(4-nitrophenyl sulfonyl) carprofen benzyl ester **62o** as outlined in **Scheme 3.24**. The nitro group of **62o** was chemoselectively reduced to its free amine **118** using anhydrous SnCl$_2$ in ethanol at 80-90 °C. Different methods were employed for the coupling of amine **118** with *D*-biotin. The reaction did not show any indication of product formation even after using various coupling reagents (see **Scheme 3.24**). This was attributed to the low nucleophilicity of aniline **118** which bears an electron withdrawing group at its *para* position.
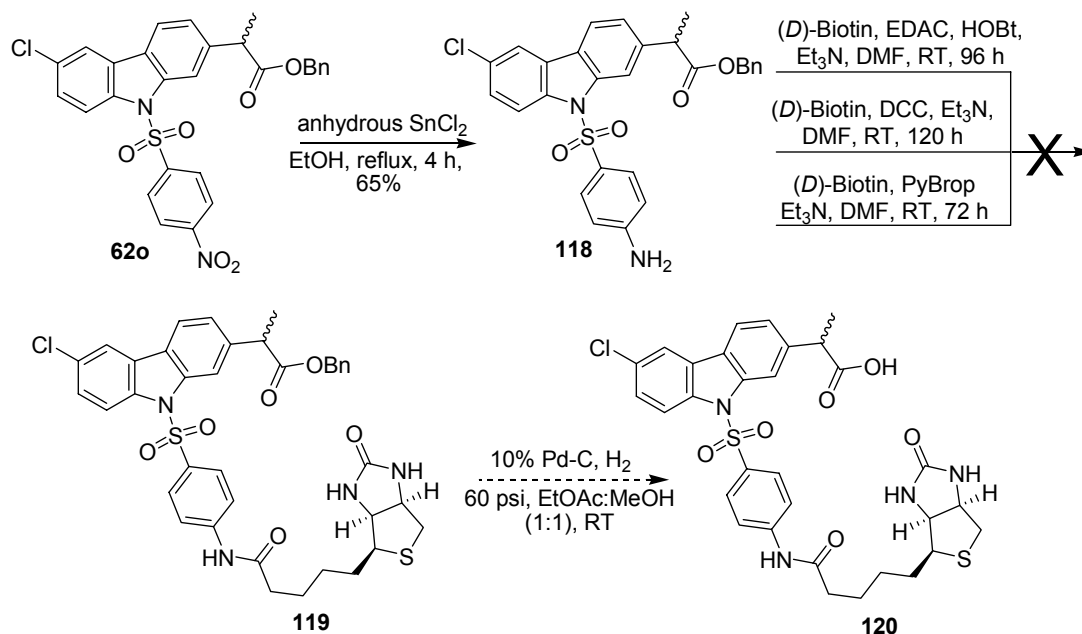
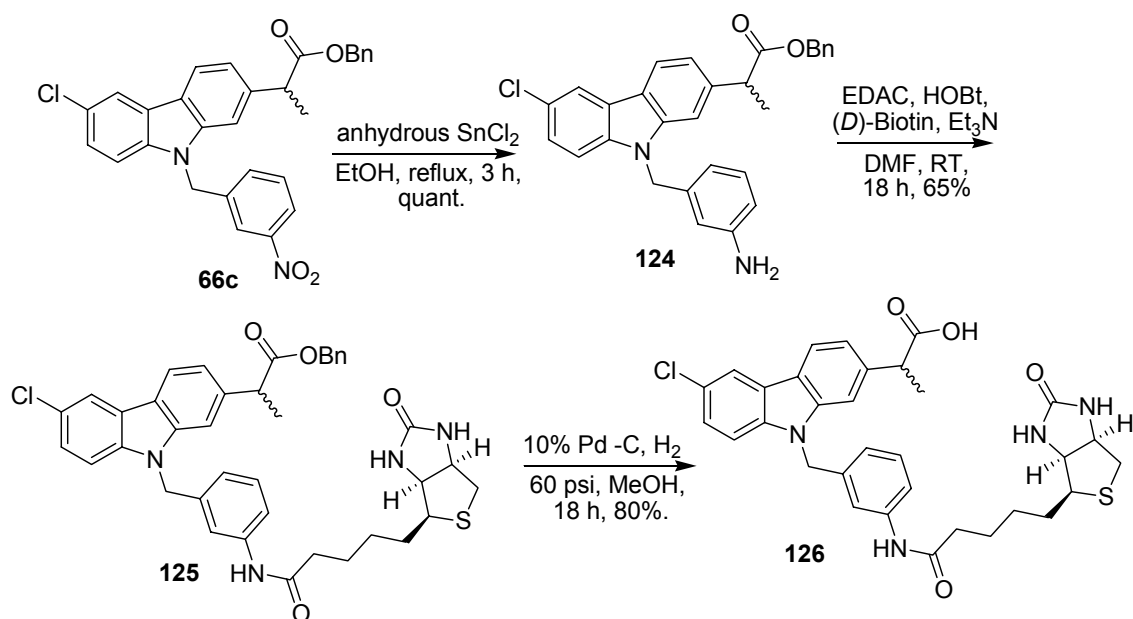**Scheme 3.24:** Synthesis of carprofen derived affinity label **120**.

To overcome this problem, previously synthesized intermediate *N*-(3-nitrophenly sulfonyl) carprofen benzyl ester **62n** was inducted in the synthesis of affinity label **123** as depicted in **Scheme 3.25**.
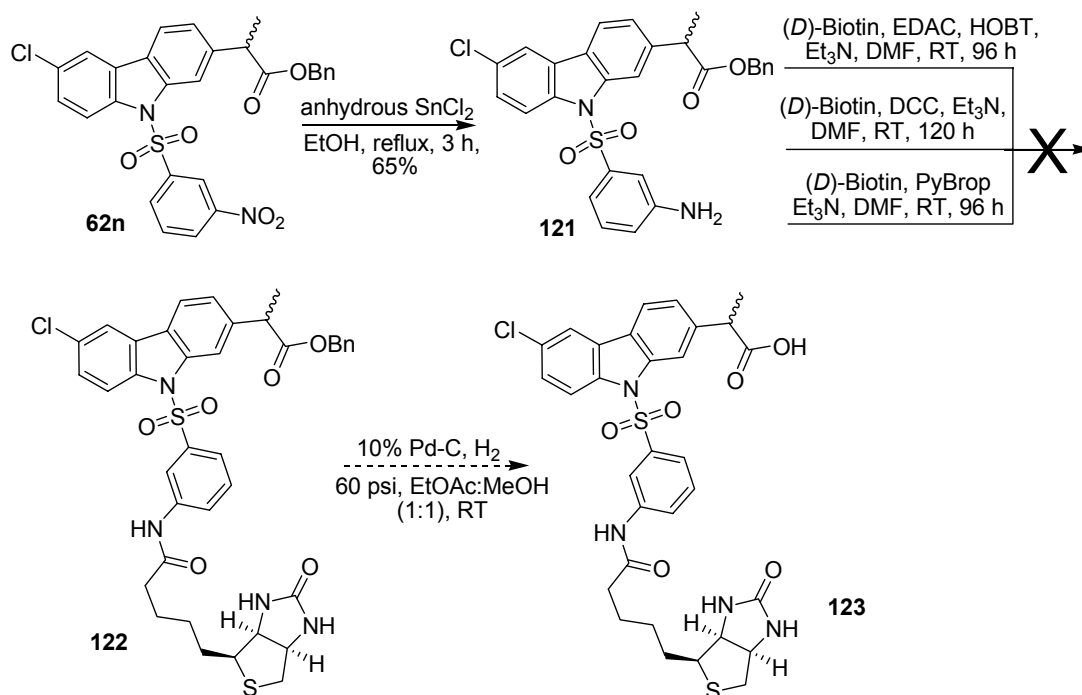
Scheme 3.25: Synthesis of carprofen derived affinity label **116**.

It was presumed that an electon withdrawing group at the *meta* position of amine **121** would not affect the nucleophilicity and thus would facilitate the coupling reaction with *D*-biotin. The chemoselective nitro reduction of intermediate **62n** was carried out using anhydrous $SnCl_2$ to give amine **121**. Amine **121** underwent coupling reaction with *D*-biotin using EDAC and HOBt as coupling reagents in DMF. The reaction did not show any sign of progression even after 96 h. Therefore, other coupling reagents were employed such as DCC and PyBrop. Both of them failed to couple amine **121** with *D*-biotin. It was assumed that sulfonyl group either at *meta* or at *para* position of aniline **121** affects the nucleophilicty dramatically and impedes the amidation reaction with *D*-biotin.

To overcome the nucleophilicity problem of anilines **118** and **121**, previously synthesized intermediate, *N*-(3-nitrobenzyl) carprofen benzyl ester **66c** was opted as a starting material for the synthesis. The synthesis commenced from the intermediate **66c** as illustrated in **Scheme 3.26**. Chemoselective nitro group reduction of *N*-(3-nitrobenzyl) carprofen benzyl ester **66c** was achieved using anhydrous $SnCl_2$ in ethanol to afford amine **124** as pale brown gummy mass. The coupling reaction of amine **124** with *D*-biotin using EDAC and HOBt as coupling reagents in DMF went smoothly and gave the biotinylated ester **125** as colorless solid. The benzyl group cleavage of benzyl ester **125** was achieved by hydrogenation using 10% Pd-C in MeOH at 60 psi to obtain biotinylated carprofen **126**.

Scheme 3.26: Synthesis of biotinylated carprofen derivative **126**.

Affinity label **126** was tested for its ability to modulate γ-secretase in the Aβ liquid phase electrochemiluminescence assay (**Figure 3.12**). As anticipated, compound **126** was found to be a modulator of γ-secretase and affected the cleavage of γ38, γ40 and γ42 to a different extent. It particularly reduced the formation of Aβ$_{42}$ and increased the formation of Aβ$_{38}$. Introduction of a polar end group such as biotin was well tolerated and did not affect the activity yet changed the mode of action from full inhibition to classical modulation. **126** displayed typical NSAID like profile: reduced formation of Aβ$_{42}$, increased formation of Aβ$_{38}$ and no inhibition of γ-secretase cleavage at ε-site. Probe **126** was tested in the immunoprecipitation assay for its ability to capture γ-secretase complex. The immunoprecipitation experiments revealed that **126** did not capture the γ-secretase complex.

126 (BSc2911)
- ■ EC$_{50}$(Aβ$_{38}$) = ND
- ▲ IC$_{50}$(Aβ$_{40}$) = >150 μM
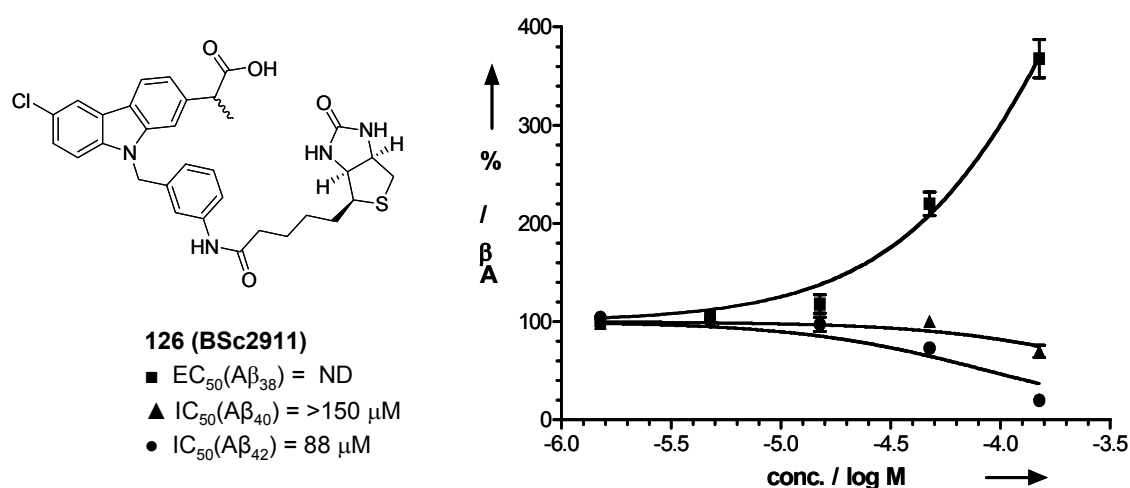- ● IC$_{50}$(Aβ$_{42}$) = 88 μM

Figure 3.12: Dose response curve for compound **126** (**BSc2912**).

To conclude, a series of NSAIDs derived affinity labels (**94**, **98**, **101**, **105**, **109** and **112**) was synthesized *via* structural modification of the common acid moiety. The SAR indicated the

necessity of acid moiety which is believed to be critical for the modulatory activity. The affinity labels (**94**, **98**, **101**, **105**, **109** and **112**) which were synthesized *via* the modification of the acid moiety of NSAIDs resulted in the complete loss of NSAID like profile and turned out to be inverse modulators of γ-secretase. They selectively increased the formation Aβ$_{42}$ and reduced the formation of less toxic Aβ$_{38}$. Out of all tested affinity probes, only **94** captured γ-secretase active complex in dose dependant manner in the immunoprecipitation assay. Despite being an inverse modulator, probe **94** did not inhibit the γ-secretase cleavage at ε-site. Rest of the affinity probes did not capture the γ-secretase complex. Considering the key role of free acid moiety for the γ-secretase modulation, affinity labels **117**, **126** were synthesized without disturbing the free acid moiety of NSAIDs. As anticipated, the incorporation of biotin keeping the free acid moiety intact resulted in the classical modulation of γ-secretase by the affinity probes **117** and **126**. Interestingly, the introduction of biotin in **117** was well tolerated and did not hamper the modulatory activity. However, **117** turned out to be unsuitable bait for the γ-secretase complex although it displayed significant modulation of γ-secretase activity. Carprofen derived affinity probe **126** was found to be a modulator of γ-secretase. The introduction biotin did not hamper the modulatory activity and was well tolerated. Affinity probe **126** did not capture γ-secretase in the immunoprecipitation assay. Robust bait for the successful pull-down of the binding domain within γ-secretase is still unknown.

3.7. Design, Synthesis and Biological Evaluation of Flurbiprofen Derived Photoaffinity Label

γ-Secretase is a multiprotein complex that is assembled from at least four obligatory proteins, PS, nicastrin, Aph-1 and Pen-2 and the PS contains the active site of this enzymatic activity.[197] Flurbiprofen is a COX-1 and COX-2 inhibitor, but displays 30-fold more selectivity towards COX-1. Apart from its COX inhibitory activity it modulates γ-secretase and selectively reduces $A\beta_{42}$ (IC_{50} 305 μM). Cell based studies with $A\beta_{42}$-lowering compounds have demonstrated that γ-secretase modulators do not induce the same molecular side effects as γ-secretase inhibitors in regard to APP processing and effects on other substrates of γ-secretase such as Notch.[111, 115, 117, 118] In contrast, it now seems likely that $A\beta_{42}$-lowering compounds act by direct modulation of γ-secretase activity. This suggested a rather subtle shift in the γ-secretase cleavage pattern with increased production of the shorter $A\beta_{38}$ species at the expense of the longer $A\beta_{42}$ species. Since then, the most convincing evidence for direct γ-secretase modulation was provided by the fact that $A\beta_{42}$-lowering compounds are active in cell-free γ-secretase assays.[112, 116, 117] Compounds that exert activity in these assays are presumed to modulate Aβ production either by direct interaction with the γ-secretase enzyme complex or the substrate APP. One credible explanation for the selective $A\beta_{42}$-lowering activity of NSAIDs is that these compounds change γ-secretase conformation in a similar yet opposite way to PS mutations.
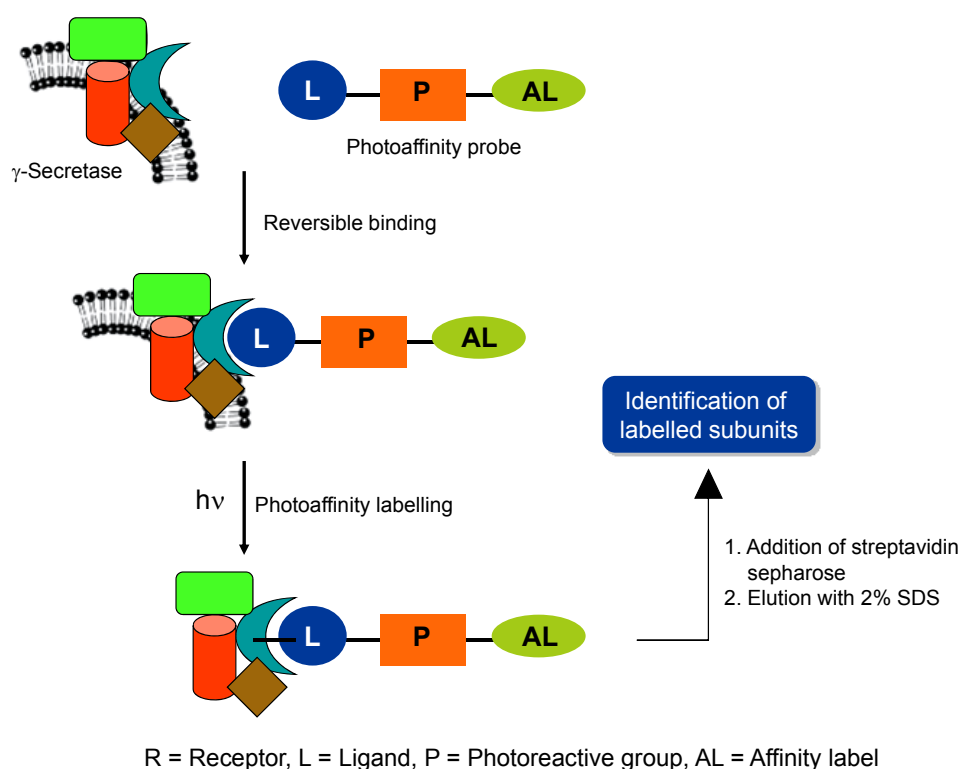

Figure 3.13: Approach to identify the binding site of flurbiprofen on active γ-secretase complex *via* photoaffinity labelling

The elucidation of interacting partners is an immediate entrance into the discovery of medicinal leads. The method of photoaffinity labelling enables the direct probing of target protein through a covalent bond which is photochemically introduced between a ligand and its specific receptor. Thus, photoaffinity labelling enables to identify protein ligand contacts. If the binding site analysis of a target protein is important for defining a particular

pharmacophore, the photoaffinity labelling will give the structural information of the receptor binding domain at micro-level.

It is well known that flurbiprofen binds to an allosteric site on the active γ-secretase active complex and exerts modulatory activity. It was presumed that flurbiprofen derived photoaffinity label would reversibly bind to the active γ-secretase complex and form a covalent bond formation with the active γ-secretase complex upon photoactivation. Then, addition of streptavidin sepharose followed by the elution with 2% SDS would provide the information regarding the binding partners in detail (**Figure 3.13**).

Flurbiprofen derived probe was synthesized in order to identify the binding site *via* photoaffnity labelling followed by the detection using immunoprecipation assay (**Figure 3.14**). Benzophenone was used as a photoreactive group since it has certain advantages such as: stability, selectivity, allows manipulation at ambient light and activation at 350-360 nm which avoids protein damage. Biotin was used as a non-radioactive tag since it has great affinity with avidin and streptavidin. Thus, the labelled protein fragments can be isolated by means of their interaction with avidin or streptavidin and isolated them by affinity chromatography. Ethylene diamine was used as spacer to maintain the distance between affinity label and the photoreactive group.
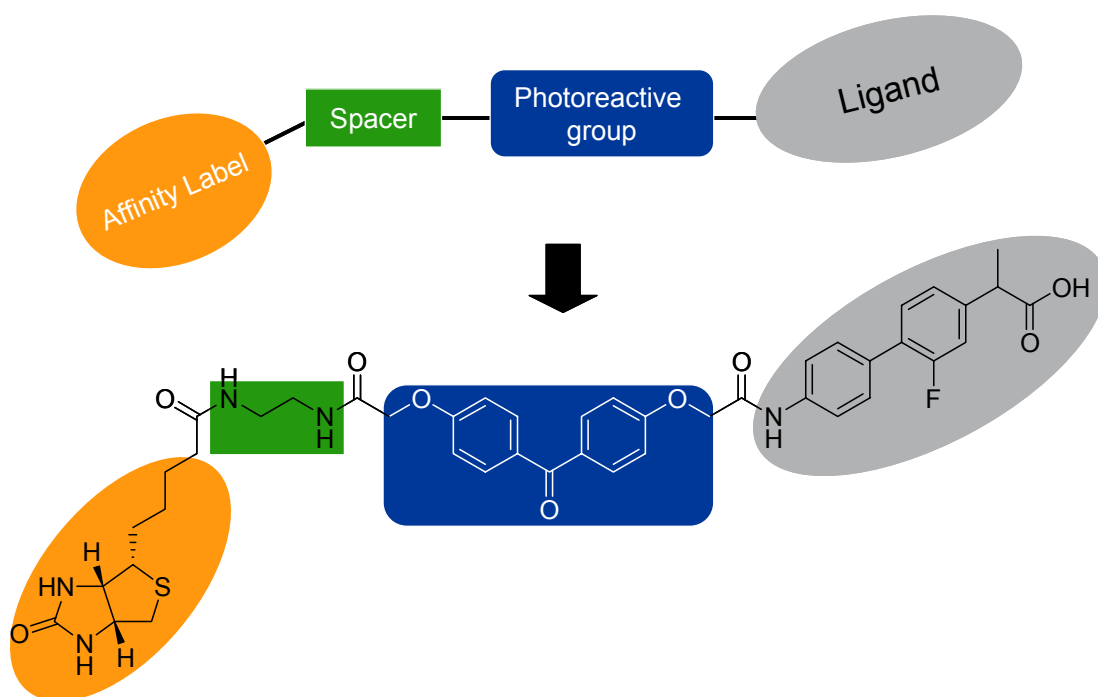

**Figure 3.14:** Design of flurbiprofen derived photoaffinity probe.

The synthesis of flurbiprofen derived photoaffinity label commenced from the previously synthesized intermediate **113** as illustrated in **Scheme 3.27**. The nitro group of **113** was reduced chemoselectively to amine **115** using anhydrous SnCl$_2$. Amine **115** was converted to α-chloro acetamide **128** using chloroacetyl chloride in CH$_2$Cl$_2$. Benzophenone **128** was alkylated using anhydrous K$_2$CO$_3$ and α-chloroacetamide **127** in acetone at 60–70 °C to give compound **129** which was purified by crystallization as colorless solid. The deprotection of the *t*-butyl group **129** was accomplished using 20% trifluoroacetic acid in CH$_2$Cl$_2$. The deprotection reaction went smoothly and the product **131** was purified by crystallization to afford the acid. Different coupling reagents were employed for coupling of acid **130** with *N*-

Boc ethylene diamine such as: PyBrop, DCC and EDAC. The best results were obtained when EDAC and HOBt were used as coupling reagents in CH_2Cl_2 and gave product **131** as colorless solid. *N*-Boc deprotection of compound **131** resulted in the multiple products as indicated by TLC when 16% HCl in dioxane was used. Therefore, *N*-Boc was deprotected using 20% TFA in CH_2Cl_2 to give amine **131** as a trifluoroacetic acid salt (**Scheme 3.28**). Amine **132** was coupled with *D*-biotin in DMF using PyBrop as coupling reagent to afford biotinylated **133** as pale yellow solid. The benzyl ester cleavage of compound **133** was carried out by hydrogenation at 60 psi in MeOH to obtain the acid **134** as pale yellow solid.
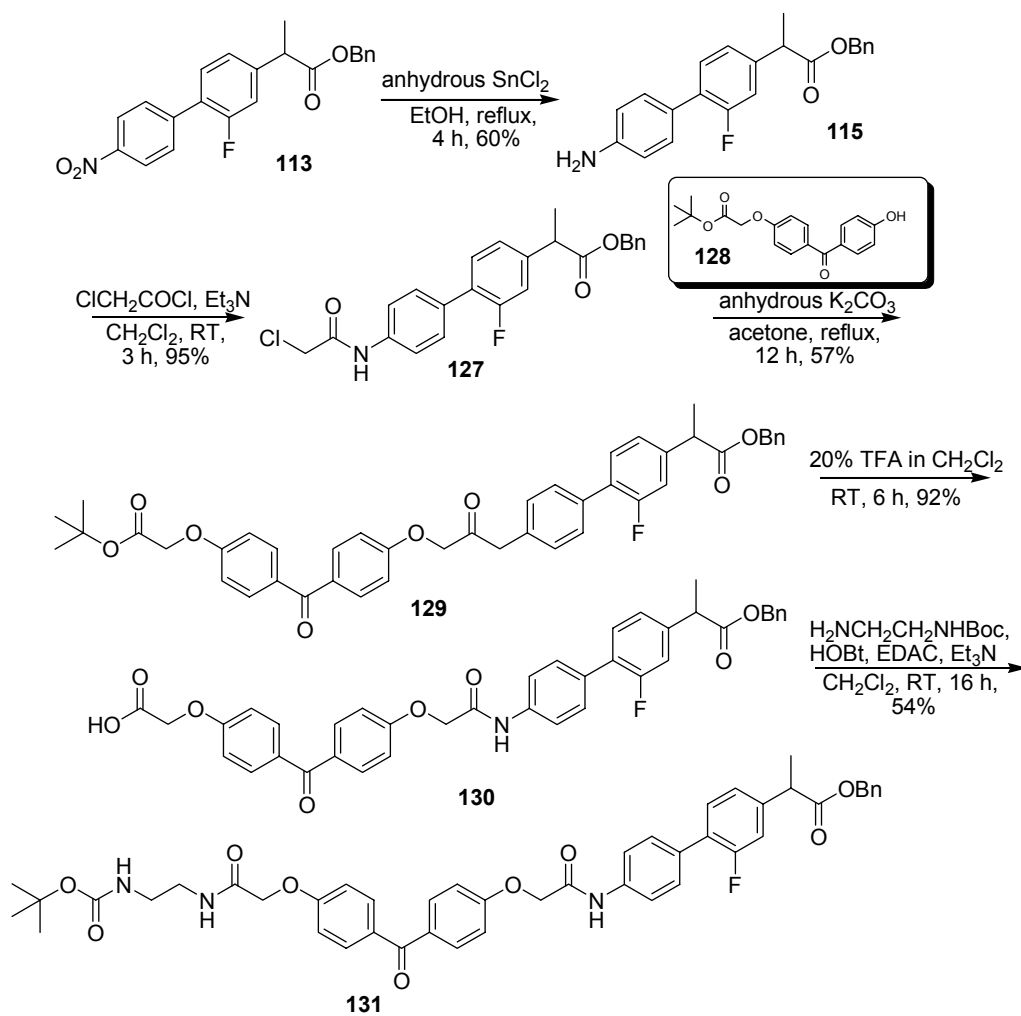
Scheme 3.27: Synthesis of intermediate **131**.

Probe **134** was tested for its potential to modulate γ-secretase in the Aβ liquid phase electrochemiluminescence assay. It was found to be a modulator of γ-secretase and selectively reduced the formation of $A\beta_{42}$ and increased the formation of $A\beta_{38}$. The introduction of benzophenone and biotin neither affected the activity significantly nor changed the mode of action (**Figure 3.15**). Photoaffinity labelling experiments with **134** are being carried out by Dr. Thomas Kukar, Mayo Clinic, Florida, USA. The preliminary data with the flurbiprofen photoaffinity probe revealed that **134** is able to crosslink to the APP-CTF substrate (recombinant C100).
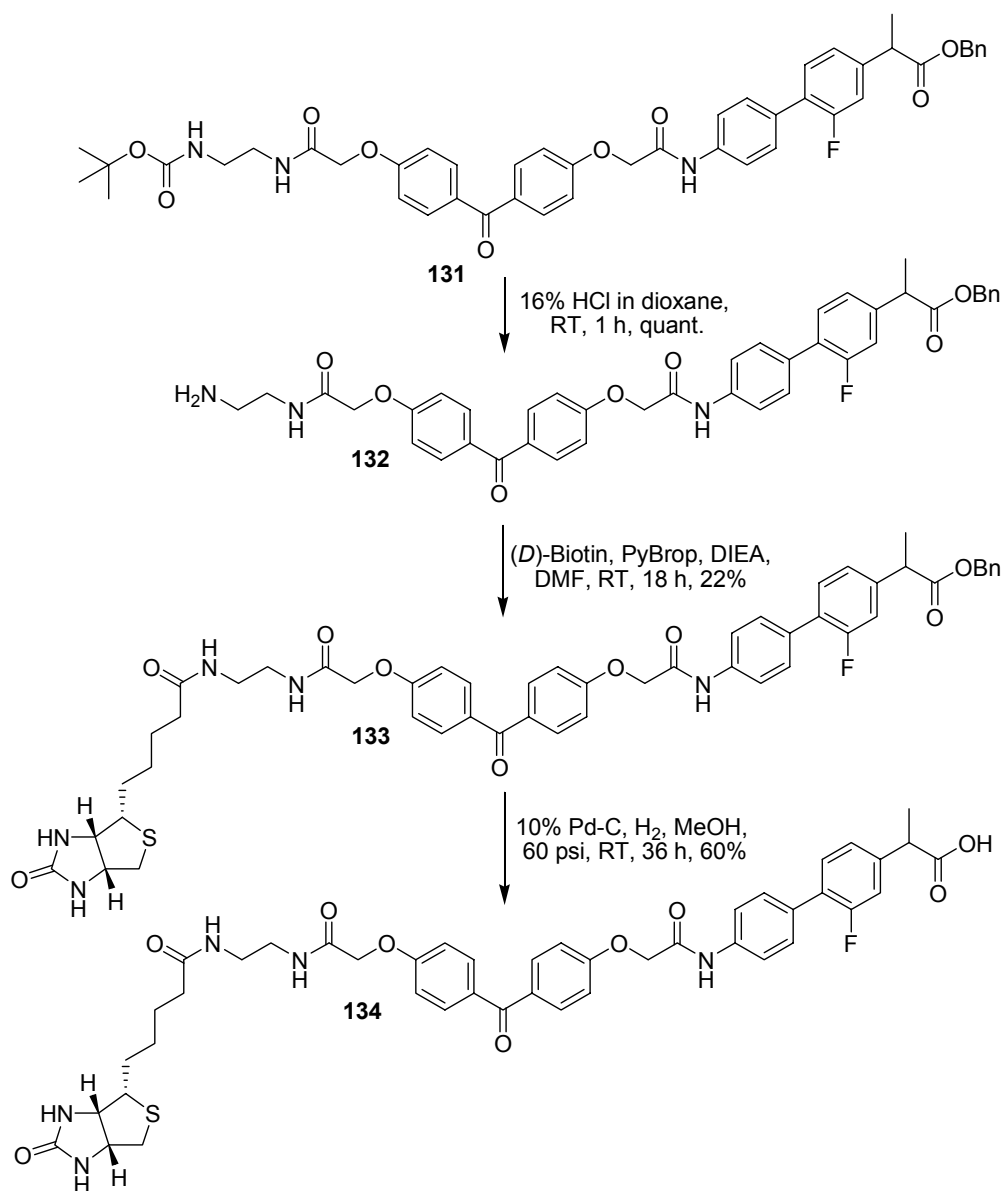
Scheme 3.28: Synthesis of photoaffinity label **134**.

This labelling can be competed away with NSAIDs that lower Aβ$_{42}$ levels. The identification of the binding site of flurbiprofen on active γ-secretase complex *via* photoaffinity labelling with photoaffinity probe **134** is ongoing.
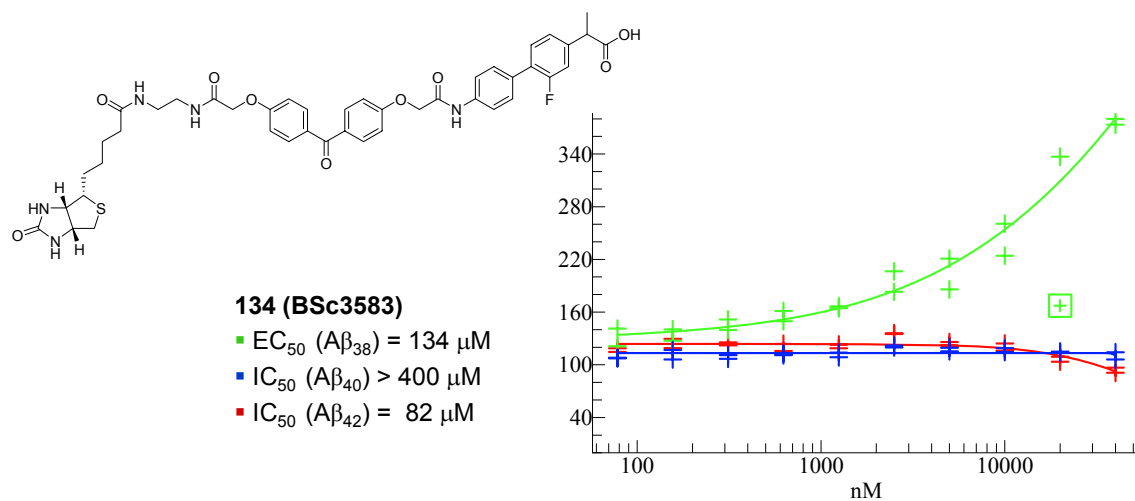
134 (BSc3583)

- EC$_{50}$ (Aβ_{38}) = 134 μM
- IC$_{50}$ (Aβ_{40}) > 400 μM
- IC$_{50}$ (Aβ_{42}) = 82 μM

Figure 3.15: Dose response curve of compound **134** (**BSc3583**).

3.8. Design, Synthesis and Biological Evaluation of DAPT Derived Photoaffinity Label.

PS is a membrane protein which undergoes endo-proteolytic processing within its putative loop region generating *N*-terminal and *C*-terminal fragments (PS-NTF and PS-CTF). It is believed to consist of six and two to four TMDs such as PS1 and PS2. PS expression is absolutely required for γ-secretase activity. Inactivation of PS1 in neurons obtained from PS1 knock-out mice leads to a dramatic reduction of γ-secretase activity and an accumulation of the corresponding substrates, β-APP CTFs. PS alone can not mediate γ-secretase activity. Its overexpression in cell culture systems and transgenic mice did not lead to an increase in Aβ production. In order to achieve this, co-expression of PS1 together with other transmembrane proteins nicastrin, Aph-1 and Pen-2 is required, allowing reconstitution of γ-secretase activity in yeast or other eukaryotic cells.[42] Combined biochemical evidence suggests that all these membrane proteins assemble into the enzymatically active γ-secretase complex with PS at its catalytic center. As a result of complex assembly, PS undergoes endoproteolysis into the active form of the enzyme. It is still unknown how exactly the substrate is hydrolyzed within the membranous hydrophobic environment which requires the presence of water molecule bound to the enzyme.

Despite tremendous research efforts, much is not known about how γ-secretase carries out proteolytic activities in the hydrophobic environment. Various photoaffinity probes were employed using the known and confirmed γ-secretase inhibitors to investigate the mode of action and to identify the binding site of γ-secretase inhibitors. Peptidic PS1 inhibitors, like Merck's **L-685,458** (**135**, IC_{50} = 17 nM) is a potent inhibitor of γ-secretase. It was proposed that compound **135** acts as a direct transition-state analogue of the $Aβ_{40}$ and $Aβ_{42}$ cleavage sites (**Scheme 3.29**). The basic structure of **135** was linked to biotin and photoreactive fragments *N*- or *C*-terminally leading to **L-852,505** (**136**) and **L-852,646** (**137**), which were suitable for labelling studies. In spite of the attachment of photoreactive benzophenones, **136** and **137** preserved inhibitory activity (IC_{50} < 1 nM). Biotin was used to facilitate the isolation and identification of the reversibly labelled adducts *via* their streptavidin-enzyme linked conjugates. Photolysis in the presence of solubilised γ-secretase and isolation on a biotin-specific streptavidin-agarose gel provided a protein of 20 kD linked to **L-852,505** (**136**). This fragment was shown to be the *C*-terminal fragment of PS1 (PS1-CTF) by specific antibodies. In a control experiment, the binding of **136** to wild type PS1 was negative. However, the binding to the deletion construct PS1ΔE9, which lacks the cytosolic E9 loop, was positive.[198] Useful information resulted from the photolysis of **L-852,646** (**137**) in the presence of solubilised γ-secretase. This resulted in the isolation of a 34 kD fragment, which was assigned to be an *N*-terminal fragment of PS1. Elan's semi-peptidic γ-secretase inhibitor DAPT (**4**, IC_{50}=20 nM) was developed from *N*-dichlorophenylalanine lead. The SAR showed that the phenylglycine and the difluoro phenylacetic acid moieties are crucial for the activity.[199, 200] DAPT has displayed very good activity *in vivo* at relatively high doses. The subcutaneous application to mice in a dosage of 100 mg/kg, resulted in a 50% reduction of cortical Aβ levels within 3 hours. 40% Aβ reduction was observed at the dosage of 100 mg/kg orally, again after 3 hours, but no brain levels of DAPT were reported for the latter study.[201] Several preclinical studies carried out with DAPT found that it displays *in vivo* toxicity since DAPT affects the Notch pathway at higher levels (100 to1000-fold).[202, 203] Kan et al. reported the solid phase supported synthesis of DAPT derived photoaffinity label **138**.[204] The synthesis of **138** took more than 18 steps.
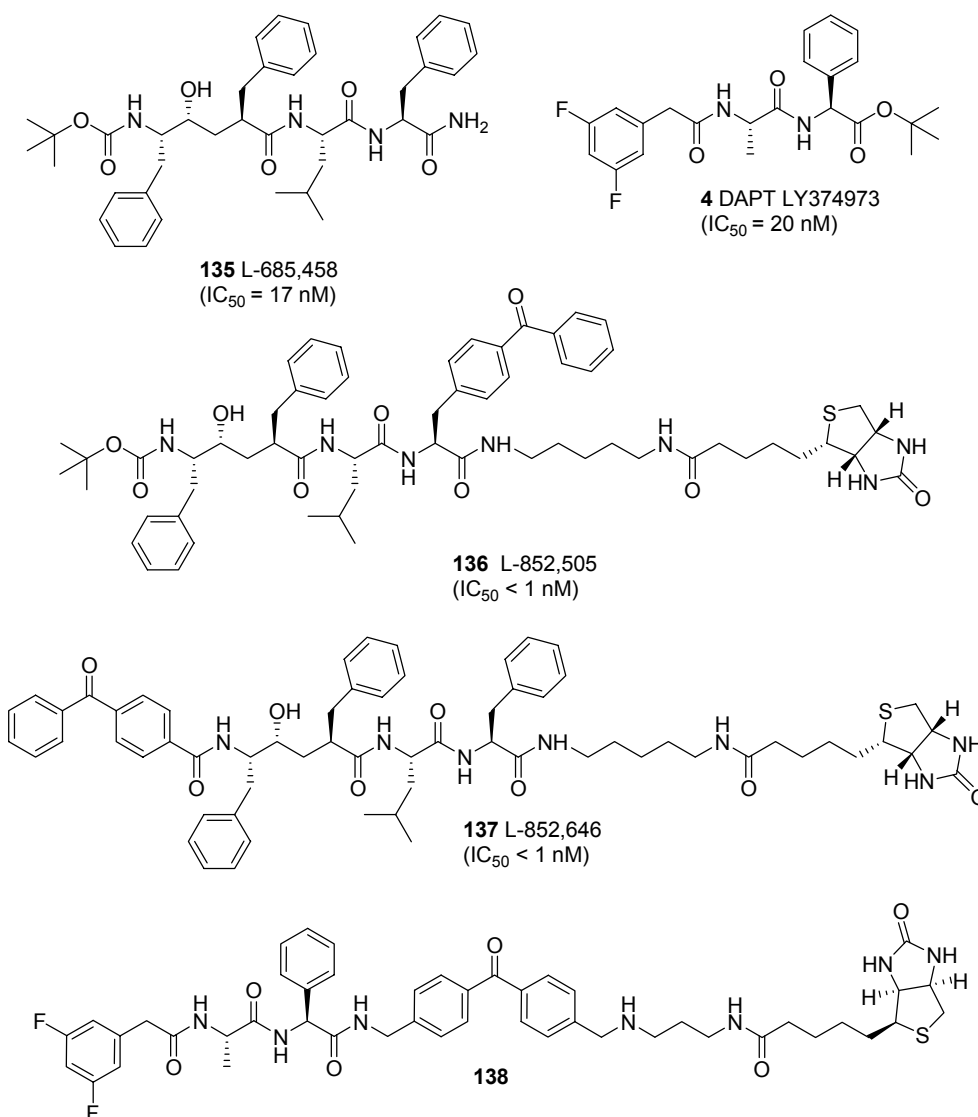
Scheme 3.29: γ-Secretase inhibitors derived photoaffinity probes.

The binding mode of DAPT to γ-secretase is not fully known. DAPT and its analogues do not differentiate between the substrates such as APP and Notch. Therefore it is important to understand the mechanism of action and identify the binding modes of Notch selective inhibitors.

3.8.1. Design and retrosynthetic analysis of DAPT derived photoaffinity label

The DAPT derived photoaffinity label **139** was designed to elucidate the binding mode at more detail as depicted in **Figure 3.16**. The photoreactive group and the affinity label were introduced at *C*-terminal of DAPT since the difluoroacetic acid and phenyl glycine moieties are crucial for the γ-secretase inhibitory activity. Benzophenone was selected as a photoreactive group due to its chemical stability and reactivity. A biotin moiety was incorporated in the photoaffinity probe to facilitate isolation and identification of the adducts.
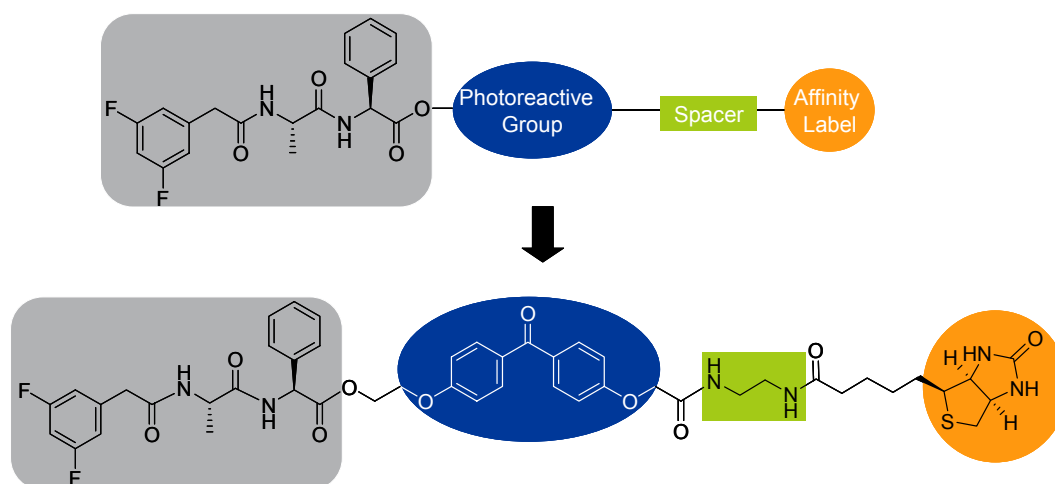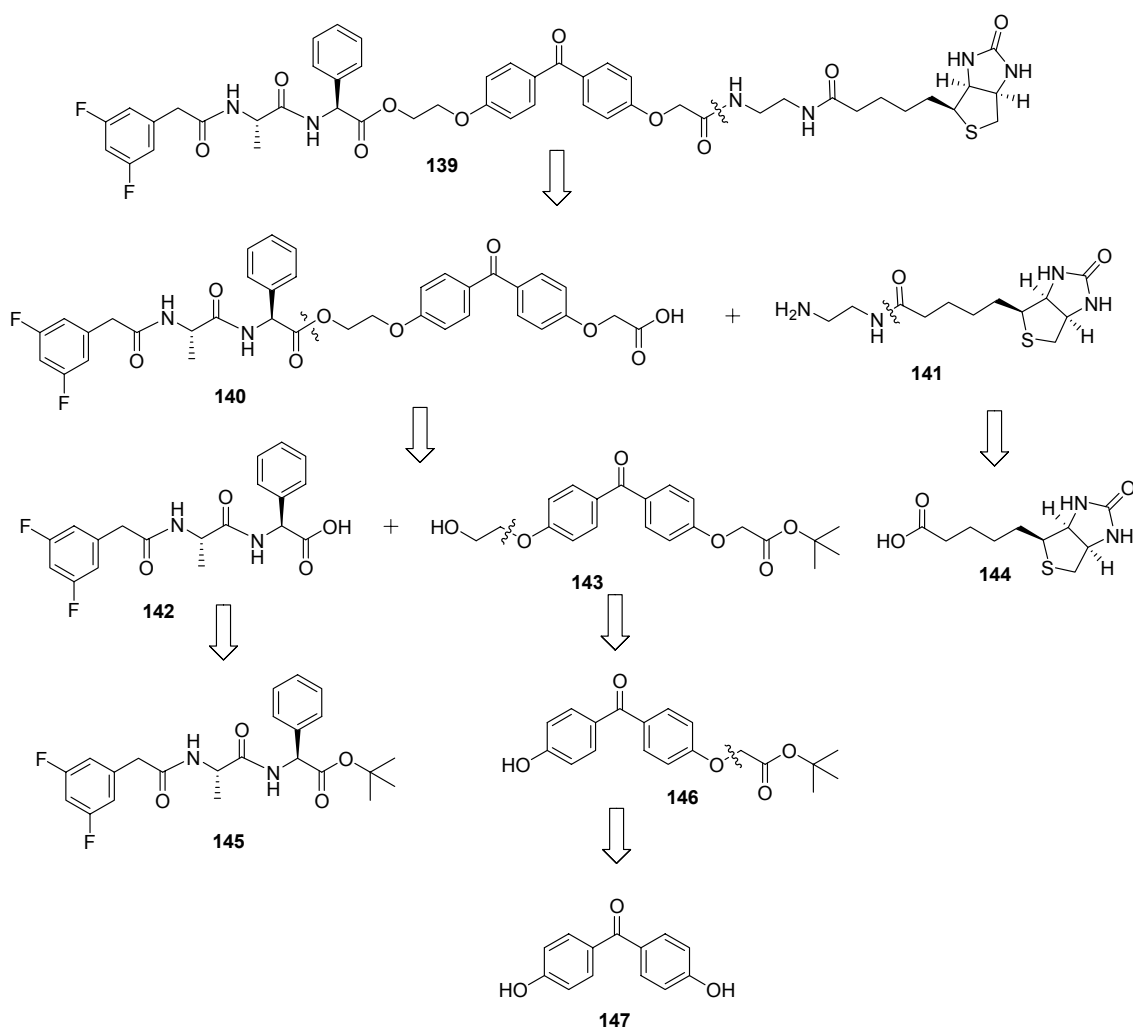
Figure 3.16: Design of DAPT derived photoaffinity label.

The retrosynthetic analysis of DAPT derived photoaffinity label **140** is depicted in **Scheme 3.30**.

Scheme 3.30: Retrosynthetic analysis of DAPT derived probe **139**.

The amide bond of **140** can be constructed by peptide coupling of amine **141** and acid **142**. The ester bond of **141** can be formed by esterification of acid DAPA **142** and alcohol **143**. The amine **141** can be synthesized by two step synthesis from commercially available *D*-biotin and *N*-Boc ethylene diamine. The acid **143** can be derived from DAPT. The alcohol **144** can be synthesized from commercially available 4,4′-hydroxy benzophenone **148** by sequential alkylation of phenolic –OH using *t*-butyl chloroacetate and 2-chloro ethanol respectively.

3.8.2. Synthesis of photoaffinity probe 139.

The synthesis of probe **139** was commenced with the synthesis of DAPA **143** as illustrated in **Scheme 3.31**. The peptide coupling of Z-Ala **148** and Phg-*t*Bu **149** was achieved using EDAC and HOBt as coupling reagents in CH$_2$Cl$_2$ to give the coupled product **150**. Z-deprotection of **150** was achieved by hydrogenation in MeOH at 60 psi to afford amine **151**. Amine **151** was coupled with 3,5-difluorophenyl acetic acid using EDAC and HOBt as coupling reagents in CH$_2$Cl$_2$ to obtain DAPT **145** as colorless solid. The *t*-butyl cleavage of DAPT was accomplished using 20% trifluoroacetic acid in CH$_2$Cl$_2$ to give DAPA **142** as colorless solid.
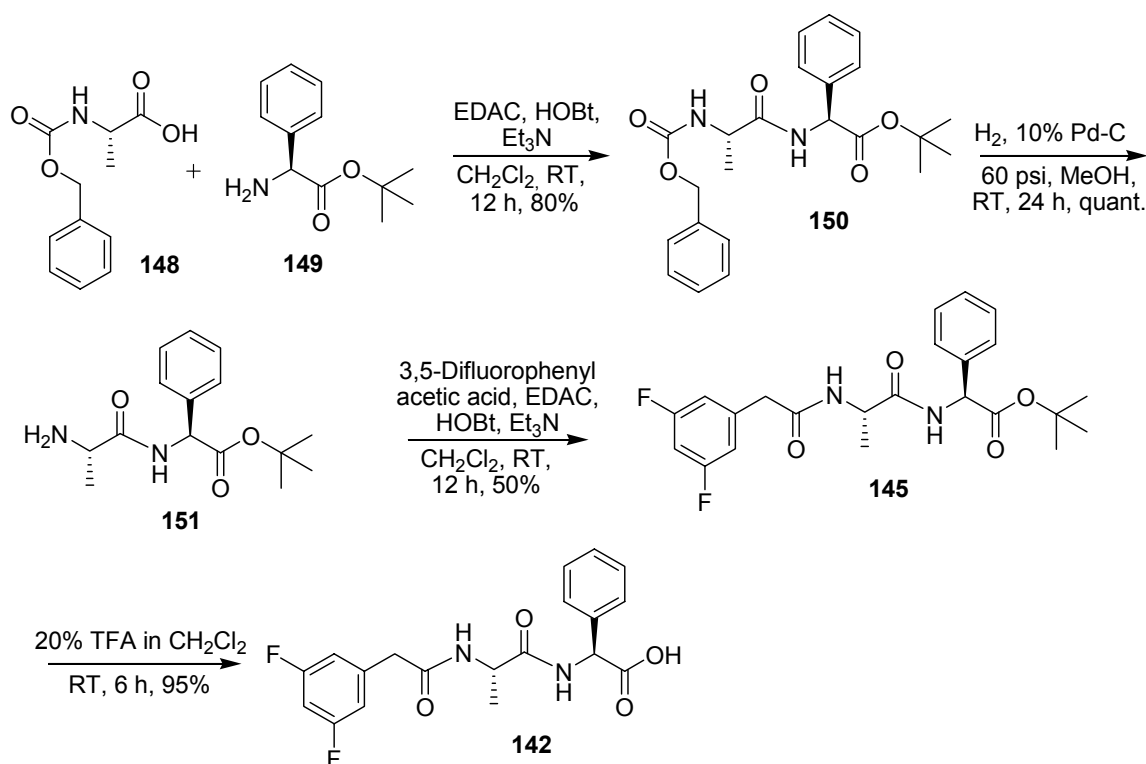
Scheme 3.31: Synthesis of DAPA **142**.

The synthesis of building block **143** commenced from the commercially available 4,4′-hydroxy benzophenone **147** as depicted in **Scheme 3.32**. One of the phenolic –OH of **147** was alkylated using *t*-butyl chloroacetate and anhydrous K$_2$CO$_3$ in dry acetone at 60–70 °C. The reaction yielded a mixture of mono- and di-alkylated product along with the unreacted starting material. This mixture was separated by column chromatography to afford the mono-alkylated product **146**. The phenolic –OH **146** of was alkylated using 2-chloroethanol and anhydrous K$_2$CO$_3$ in acetone at 60–70 °C to give the primary alcohol **143** in 60% yield.
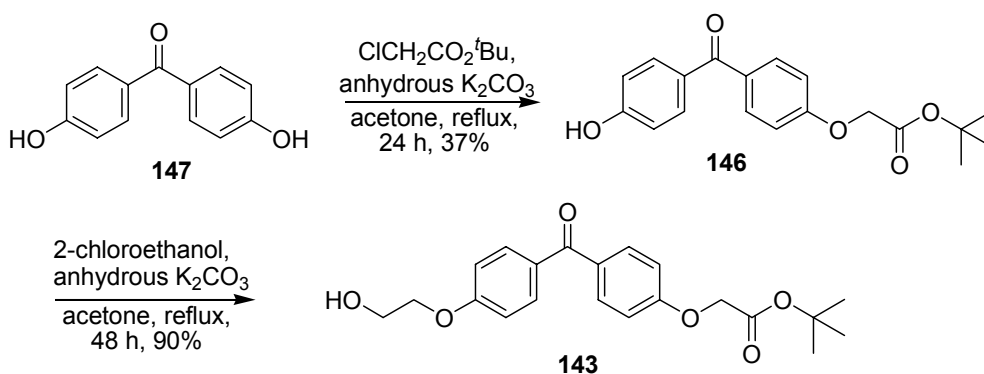
Scheme 3.32: Synthesis of building block **143**.

The synthesis of amine **141** commenced from the commercially available *D*-biotin **144**. *N*-Boc ethylene diamine was coupled with *D*-biotin using EDAC and HOBt as coupling reagents in DMF to afford the product **152**. The isolation of **152** from the reaction mixture and the purification by column chromatography was tedious due to its high polarity and poor solubility in CHCl₃, CH₂Cl₂ and EtOAc. *N*-Boc deprotection **152** was achieved using 20% TFA in CH₂Cl₂ to obtain amine **141** as trifluoroacetic acid salt which was used in the next step without further purification (**Scheme 3.33**).
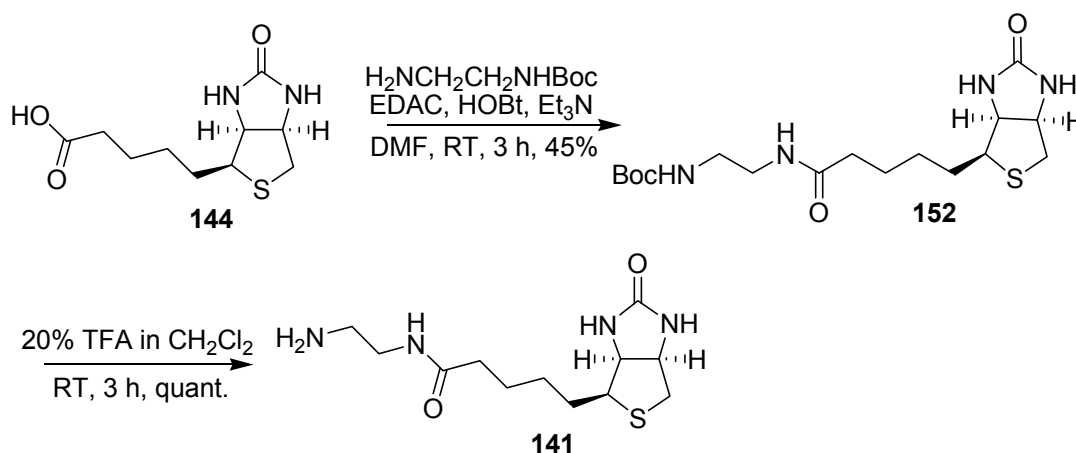

Scheme 3.33: Synthesis of amine **141**.

Different methods were employed for the synthesis of ester **153** as depicted in **Scheme 3.34**. EDAC and HOBt were used initially for the esterification of DAPA **142** with alcohol **143** in CH₂Cl₂ at room temperature. The TLC did not indicate the formation of any new spot apart from the starting material even after 120 h. This prompted the use of different coupling reagent such as DCC and PyBrop. But they helped little as TLC indicated no formation of product even after 120 h. Yamaguchi lactonization is a classical method for the synthesis of macrolactones and esters. This methodology was employed for the esterification of DAPA **142** using the alcohol **143** and 2,4,6-trichlorobenzoyl chloride. The TLC indicated no formation of a product. This advocated the conversion of the acid into mixed anhydride using solid phase supported *p*-toluene sulfonyl chloride and solid phase supported DIEA as base. Nucleophilic attack of alcohol **143** at the carbonyl group of mixed anhydride was anticipated to afford the desired ester. But, even after 7 days, the TCL indicated no formation of product **153**.

1. EDAC, HOBt, DMAP,
   Et$_3$N, CH$_2$Cl$_2$, RT, 120 h

2. DCC, DMAP, Et$_3$N,
   CH$_2$Cl$_2$, RT, 120 h

3. 2,4,6-trichlorobenzoyl chloride,
   DIEA, THF, RT, then DMAP,
   CH$_2$Cl$_2$, RT, 120 h

4. PyBrop, Et$_3$N, CH$_2$Cl$_2$, RT,
   120 h

5. ⬤—TsCl   ⬤—DIEA
   CH$_2$Cl$_2$, RT, 7 days
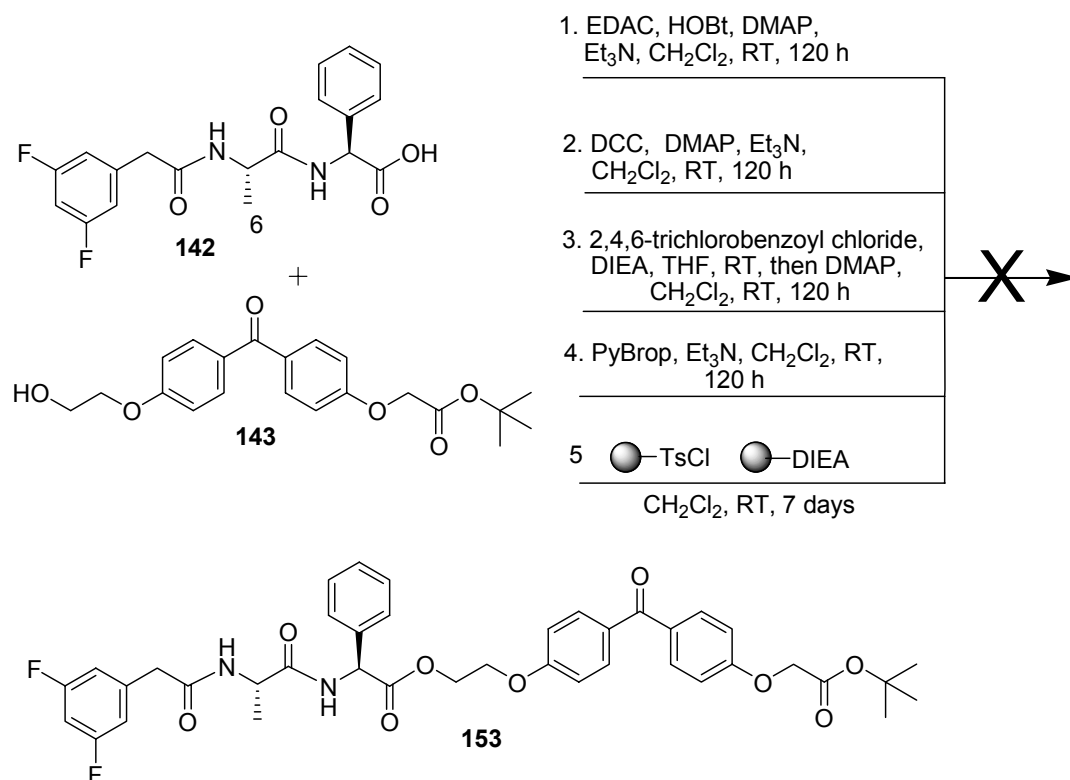
Scheme 3.34: Different methods employed for the synthesis of ester **153**.

The unusual un-reactivity of alcohol **143** to undergo esterification impelled to convert it into a good leaving group such as –Br or Tos. Therefore, the primary alcohol **143** was tosylated using *p*-toluene sulfonyl chloride, pyridine and catalytic DMAP in DMF to afford **154** as outlined in **Scheme 3.35**. The reaction was very sluggish and TLC indicated 50% conversion even after 7 days.
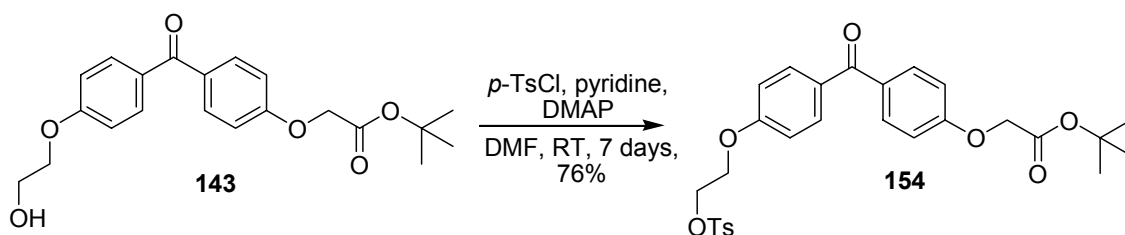
Scheme 3.35: Synthesis of compound **154**.

The ester **153** was synthesized by nucleophilic substitution using DAPA **142**, tosylated alcohol **154** and anhydrous K$_2$CO$_3$ in DMF as illustrated in **Scheme 3.36**. The reaction went smoothly and the desired ester **153** was purified by column chromatography as a colorless gummy mass. The *t*-butyl ester of **153** was cleaved using 20% TFA in CH$_2$Cl$_2$ to get acid **140** as colorless solid. The acid **140** was coupled with amine **141** using EDAC and HOBt as coupling reagents in DMF to afford the photoaffinity label **139** as cream colored solid.
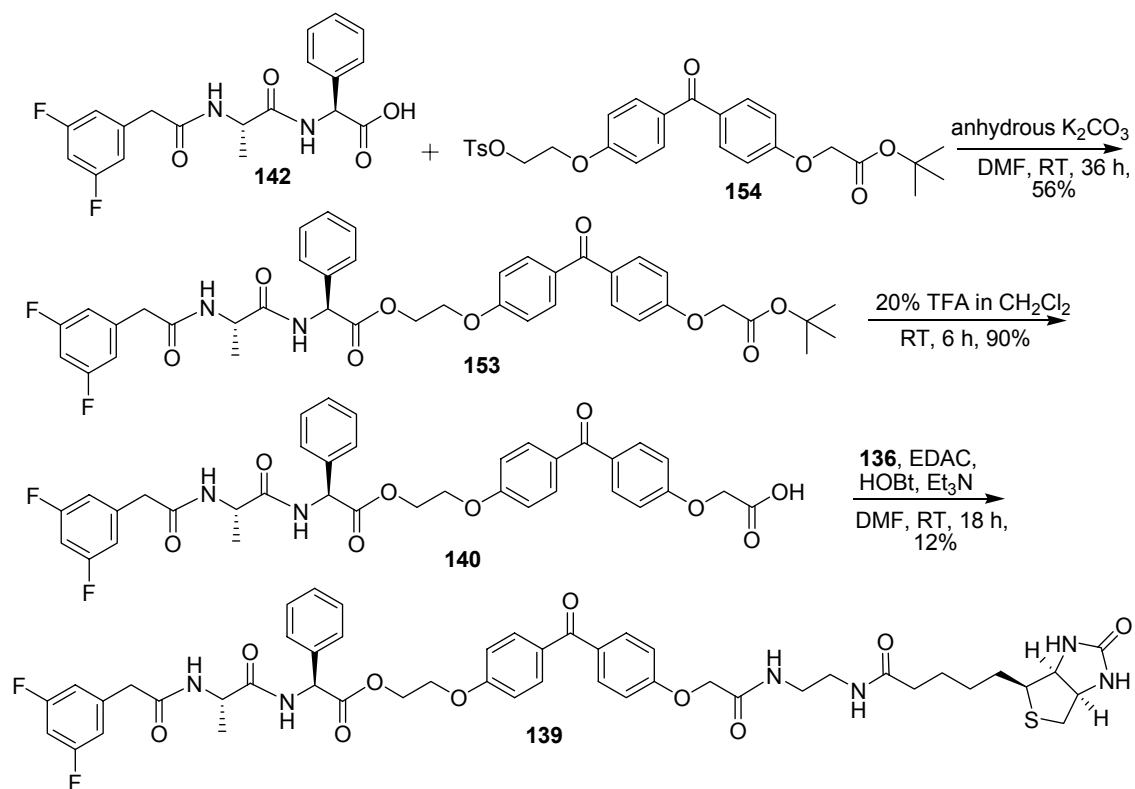
Scheme 3.36: Synthesis of photoaffinity label **139**.

The final step in the synthesis of photoaffinity label **139** gave very poor yield. In order to improve the yield, the synthetic strategy was modified as outlined in **Scheme 3.37**. Acid **140** was coupled with *N*-Boc ethylene diamine using EDAC and HOBt as coupling reagents in CH₂Cl₂ to afford **155**. *N*-Boc deprotection of **155** was performed using 16% HCl in dioxane to get amine **156** as hydrochloride salt which was used in the next step without further purification. Amine **156** was coupled with *D*-biotin using EDAC and HOBt as coupling reagents in DMF to afford the photoaffinity label **139** as pale brown solid. As anticipated, the modification in the synthetic strategy improved the yield significantly in the final step. The introduction of biotin affected the solubility and the polarity dramatically of **139** . The low solubility of photoaffinity label in CHCl₃ and CH₂Cl₂ made the isolation of the crude compound difficult from the reaction mixture. The isolated crude compound was purified by column chromatography using CHCl₃:MeOH (4:1) as eluents to afford the desired photoaffinity label as pale brown solid.
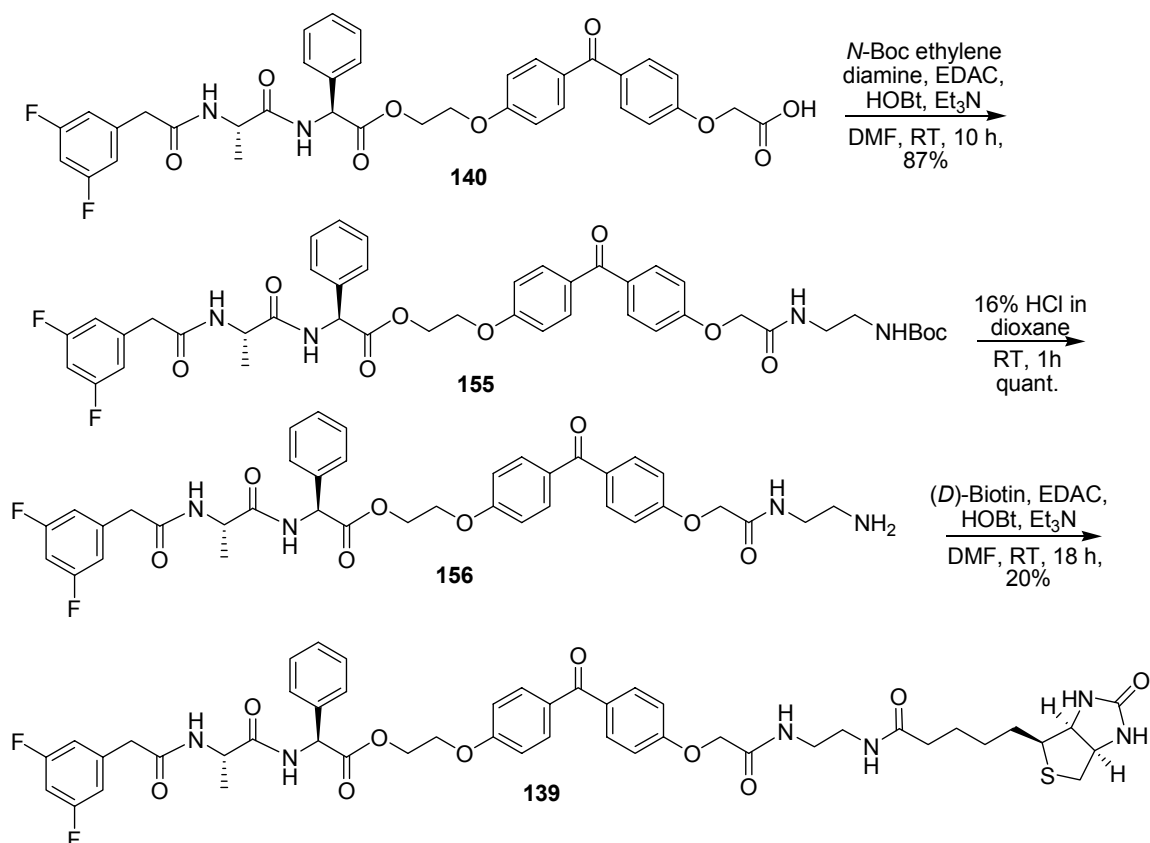
Scheme 3.37: Modified synthesis of photoaffinity label **139**.

3.8.3. Synthesis of biotinylated benzophenone.

The biotinylated benzophenone **162** was synthesized to use as a standard for the photoaffinity labelling experiment. The synthesis started from the commercially available 4-hydroxy benzophenone **157** as depicted in **Scheme 3.38**. The phenolic –OH of **157** was alkylated using *t*-butyl chloroacetate and anhydrous K_2CO_3 in acetone at 60–70 °C. The crude product was purified by crystallization to afford the *t*-butyl ester **158** as colorless solid. The *t*-butyl ester cleavage of **158** was achieved using 20% TFA in CH_2Cl_2 at room temperature and the crude product was purified by crystallization to get the acid **159** as colorless solid. The acid **159** was coupled with *N*-Boc ethylene diamine using EDAC and HOBt as coupling reagents in CH_2Cl_2 at room temperature and further purification by column chromatography gave the coupled product **160** as colorless solid. *N*-Boc cleavage of **160** was accomplished using 16% HCl in dioxane to obtain amine hydrochloride **161** which was used in the next step without further purification. The amine **161** was coupled with *D*-biotin using EDAC and HOBt as coupling reagents in DMF at ambient temperature. Further purification by column chromatography using MeOH:CHCl₃ (1:9) gave the biotinylated benzophenone **162** as cream colored solid.
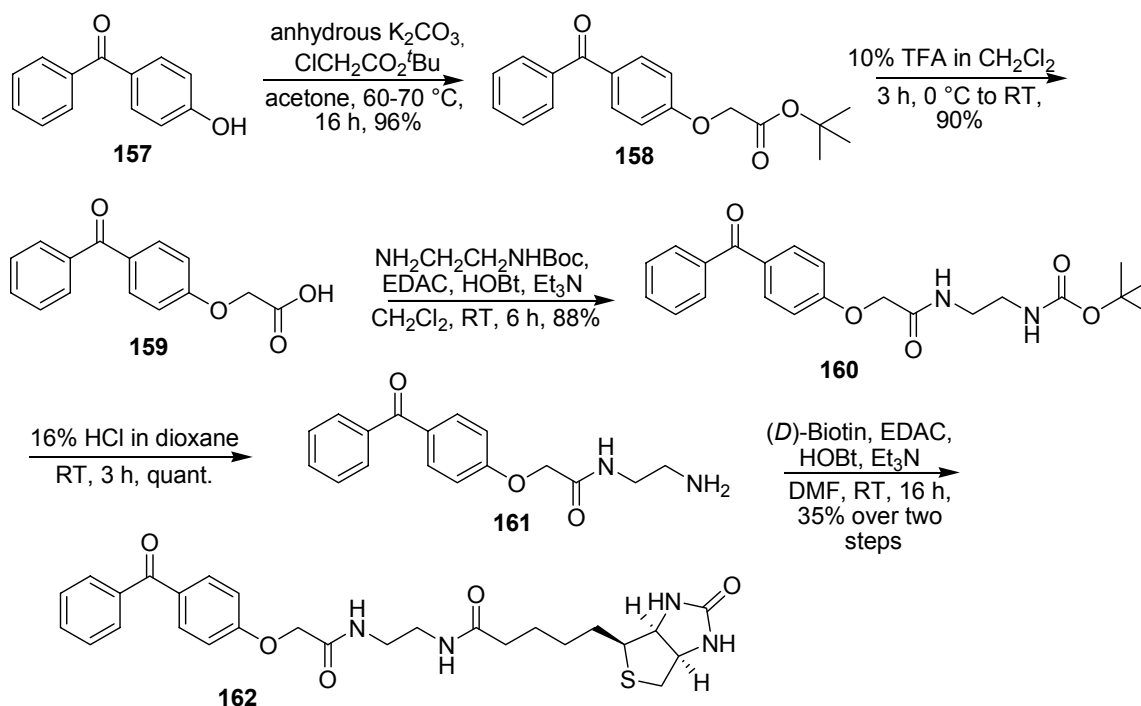
Scheme 3.38: Synthesis of biotinylated benzophenone **162**.

3.8.4. Biological activity report

The photoaffinity labelling experiment was carried out in collaboration with Dr. Harald Steiner, LMU Munich, Germany and Dr. Takeshi Iwatsubo, Japan. The interaction of the photoaffinity probes with γ-secretase was studied using solubilized γ-secretase to circumvent complications arising from limited uptake by cells of the biotinylated photoreactive compounds.
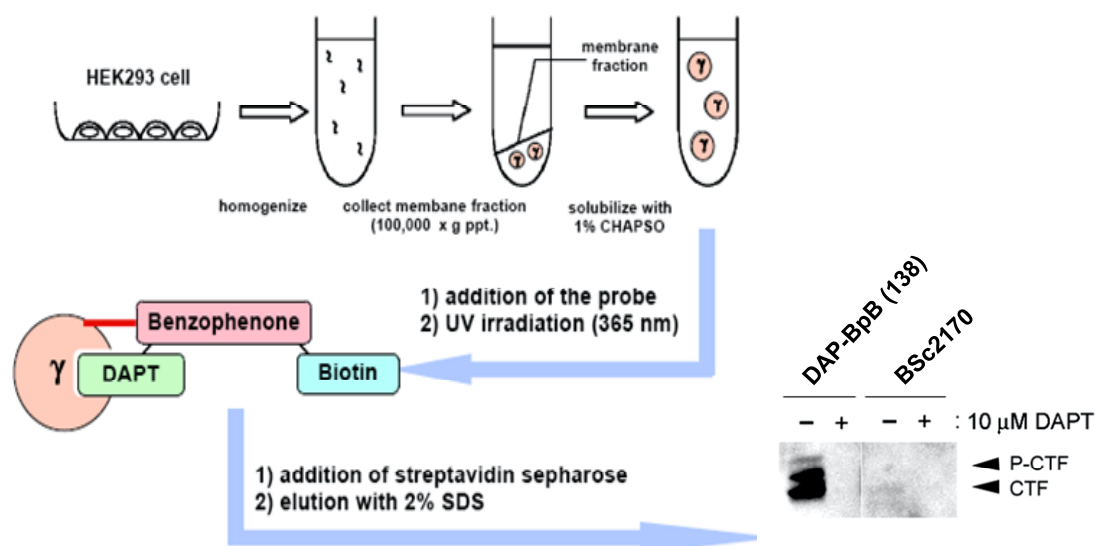

Figure 3.17: Schematic representation of the typical photoaffinity labelling experiment carried out with **139**.

The photoaffinity probe **139** was photoactivated in the presence of solubilized γ-secretase to covalently label the active site of their aspartyl protease targets (**Figure 3.17**). Following

photoactivation, the reaction mixture was treated with CHAPSO buffer to increase the accessibility of the biotin group in the photolabelled proteins to streptavidin. The biotin-linked photolabelled proteins were isolated from the reaction mixture by absorption on streptavidin-agarose beads. The covalently labelled proteins were then eluted from the beads, subjected to SDS±polyacrylamide gel electrophoresis (SDS±PAGE) and visualized by immunoblotting using an anti-biotin antibody. In the immunoblot analysis covalently labelled biotinylated species was obtained. The photoaffinity labelling experiment were performed in comparison with previously reported DAPTBpB **138**.[204]

139 (BSc2170)

- $IC_{50}(A\beta_{38})$ = 0.89 μM
- $IC_{50}(A\beta_{40})$ = 1.04 μM
- $IC_{50}(A\beta_{42})$ = 1.24 μM
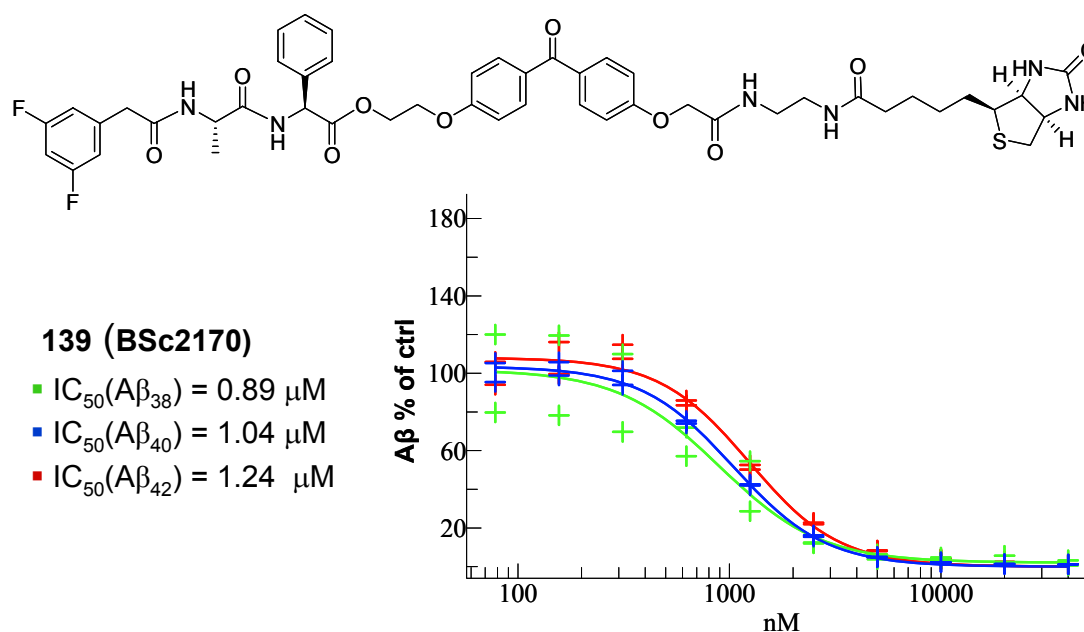
Figure 3.18: Dose response curve for **139** (**BSc2170**).

145 (DAPT)

- $IC_{50}(A\beta_{38})$ = 0.043 μM
- $IC_{50}(A\beta_{40})$ = 0.051 μM
- $IC_{50}(A\beta_{42})$ = 0.055 μM
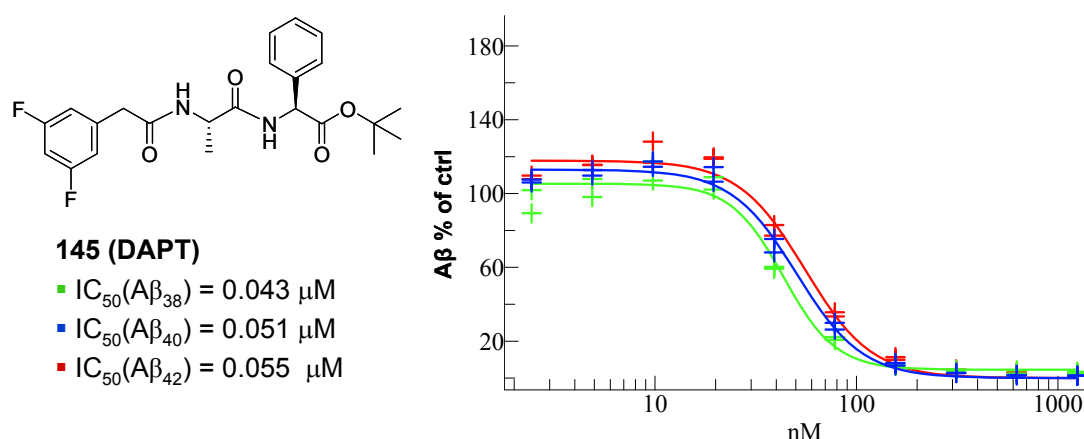
Figure 3.19: Dose response curve for DAPT.

The photoaffinity probe **139** indeed labelled the active site of the PS, but it was very weak binding compared to the DAPTBpB **138**. The photoaffinity probe DAPTBpB **138** labelled the PS-CTF and displayed strong binding.

It was presumed that the introduction of the photoreactive group and an affinity label might have affected the ability to bind dramatically. Therefore, **139** was tested for its potential to

inhibit γ-secretase in the Aβ liquid phase electrochemiluminescence assay. Photoaffinity probe **139** was found to be a potent inhibitor of γ-secretase and displayed the inhibitory activity at nanomolar concentration. The introduction of benzophenone and biotin indeed reduced the inhibitory activity by 20-fold compared to DAPT (**Figure 3.18**, **3.19**).

The resemblance of compound **162** with the ketoprofen derived affinity probe **109** and biotinylated fenofibrate, advocated investigating the ability of **162** to modulate γ-secretase in the Aβ liquid phase electrochemiluminescence assay. As anticipated, the biotinylated benzophenone **162** was found to be an inverse modulator of γ-secretase (**Figure 3.20**). It affected the cleavage of γ38, γ40 and γ42 to a different extent and particularly increased the formation of $A\beta_{42}$.
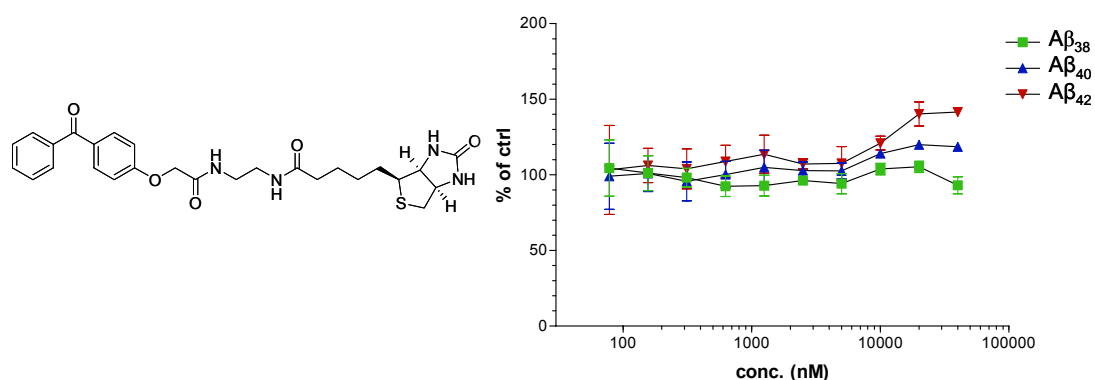

Figure 3.20: Dose response curve for **162** (**BSc2253**).

In conclusion, DAPT derived photoaffinity label **139** was synthesized in solution phase by a straight forward synthesis compared to the simultaneously reported photoaffinity label **139**. Photoreactive group, benzophenone and an affinity label, biotin were introduced at the *C*-terminal of DAPT since the 3,5-difluoro phenyl and phenyl glycine moieties were crucial for the inhibitory activity. As anticipated, the photoaffinity label **139** preserved the γ-secretase inhibitory activity, but introduction of benzophenone and biotin moieties reduced the γ-secretase inhibitory activity 20-fold compared to parent DAPT. To test the ability of **139** to label the active site of γ-secretase *via* covalent bond formation, the photoaffinity labelling experiment was performed. A very weak covalent bond formation with the PS, the active site of γ-secretase was observed when the photoaffinity labelling experiment was performed using **139**.

4. SUMMARY

The final and critical cleavage in the APP processing which generates Aβ is carried out by γ-secretase. γ-Secretase activity can be controlled either by the inhibition of the active site of PS1 or by interference with complex assembly or substrate recognition, the latter resulting from allosteric modulation or inhibition. Most of the reported and confirmed γ-secretase inhibitors are not substrate specific and inhibit the processing of other γ-secretase substrates equally. Long term treatment with γ-secretase inhibitors causes severe gastrointestinal toxicity and interferes with the maturation of B- and T-lymphocytes in mice. These effects are due to inhibition of Notch processing and signalling.[195] But, compounds which are capable of modulating the active γ-secretase and no effect on Notch would be ideal candidates for AD therapeutics. The allosteric mechanisms are particularly attractive targets for drug development, since they may control the ratio of the Aβ fragments: $A\beta_{38}$, $A\beta_{40}$ and $A\beta_{42}$, while retaining the cleavage of other substrates.

4.1. Synthesis of NSAIDs Derived γ-Secretase Inhibitors and Modulators

NSAIDs offer the best solution to the substrate selectivity problem which is the major obstacle in development of AD therapeutics. NSAIDs derived secretase inhibitors were synthesized in order to reduce the COX inhibitory activity and increase the γ-secretase modulatory activity. Flurbiprofen, sulindac sulfoxide and meclofenamic acid were short listed and derivatization of the carboxylic acid common to these COX-1 inhibitors to its esters and amides was carried out. Flurbiprofen amides were found to be inverse modulators of γ-secretase (**56a–b**) in the Aβ liquid phase electrochemiluminescence assay. Flurbiprofen amides (**56c–p**) did not display inhibitory activity on BACE-1 in FRET and in RLBA assay. A series of flurbiprofen derived esters was synthesized and tested for their ability to inhibit BACE-1 in FRET and RLBA assay. The conversion of the acid moiety of sulindac sulfoxide into its various amides (**59a–l**) resulted in either full inhibition (**59c**, **59e**) or inverse modulation (**59d**, **59k**, **59l**). The classical modulatory activity was abolished due to the derivatization of the acid moiety. Meclofenamic acid derived amides (**60a–d**) were synthesized and tested for their potential to inhibit γ-secretase in Aβ liquid phase electrochemiluminescence assay. But, most of the compounds (**60a-c**) turned out to be inverse modulators of γ-secretase. They affected the γ-secretase cleavage at different extent and particularly increased the formation of most toxic and aggregation prone $A\beta_{42}$ species while reducing the formation $A\beta_{38}$. Only compound **60d** was found to be inhibitor of γ-secretase. Thus, few of these initial derivatives displayed increased, unselective inhibition of γ-secretase in comparison to their parent drugs. Most of NSAID carboxylic acid derivatives resulted in loss of activity. This indicated an important contribution of the carboxylic acid to target affinity.

4.2. Synthesis of *N*-Substituted Carprofen Derivatives as γ-Secretase Modulators

After a brief investigation of sulindac analogues, the efforts were focused on carprofen, which is a COX-2 inhibitor approved for the use in dogs, cows and horses. *N*-sulfonylated carprofen derived amides and ester were synthesized and were expected inhibit γ-secretase due to their similarity with **BMS-299897**. But serendipitously, the *N*-substitution of carprofen turned it into a potent γ-secretase modulator and thus initial objective was adapted from inhibition to modulation. In accordance with previous results, *N*-substituted carprofen amides **64** and esters **62** were found to be inverse modulators of γ-secretase. A series of *N*-sulfonylated carprofen derivatives was synthesized to optimize the lead compound **63b**. *N*-Alkylated carprofen derivatives **66** were prepared to evaluate the contribution of the sulfonamide moiety in **63r**

and the most active derivative **63p**, where the sulfonamide is shielded by isopropyl substituents.

The compounds **63a–63s** and **66a–66f** turned out to be effective modulators of γ-secretase. They affected the cleavage at the γ38, γ40 and γ42 sites to a different extent and particularly suppressed the formation of $A\beta_{42}$, while enhancing the formation of $A\beta_{38}$ and thus showed the typical profile of effective NSAIDs. The most active compounds were particularly **63p** ($A\beta_{42}$ IC_{50} = 8.5 μM), **66a** ($A\beta_{42}$ IC_{50} = 7.5 μM) and **66f** ($A\beta_{42}$ IC_{50} = 2.9 μM). The introduction of a single lipophilic substituent, which may vary from arylsulfone to alkyl substituents, transformed the COX-2 inhibitor carprofen into a γ-secretase modulator and improved potency 10-fold or more. Thus, several compounds caused the selective reduction of $A\beta_{42}$ and an increase of the less aggregatory $A\beta_{38}$. The most active compounds (**63p**, **66a** and **66f**) are more potent than the best reported NSAIDs and they are devoid of COX-1 and COX-2 activity at the critical concentration; thus, they do not interfere with the delicate COX-1/COX-2 balance. If affected at all, the ε-cleavage of γ-secretase was inhibited at much higher compound concentrations than those determined to be modulatory at the ε-site. The compounds are therefore expected to have little or no impact on γ-secretase mediated signalling *via* the AICD or *via* ICDs of other γ-secretase substrates. The most active compounds **63p**, **66a** and **66f** are the potential lead candidates for the further optimization.

4.2. Synthesis *N*-Substituted Carbazolyloxyacetic Acids as Substrate Specific γ-Secretase Modulators

Knowing about the relevance of carprofen's similarity with **BMS-299897** and *N*-substitution of carprofen, *N*-Sulfonylated and *N*-alkylated carbazolyloxyacetic acids were synthesized. It was speculated that the lipophilic substituent anchors the *N*-substituted carbazolyloxyacetic acid in the required orientation within the membrane, thus the maximum tolerated length should be similar to natural phospholipids. To explore this hypothesis, *N*-alkylated carbazolyloxy acetic acids were synthesized using the alkyl halides of length equivalent to membrane phospholipids such as undecyl, dodecyl, tetradecyl and hexadecyl. As anticipated, *N*-sulfonylated compounds **70a–k** and *N*-alkylated compounds **72a–g** turned out to be effective modulators of γ-secretase. They affected the cleavage at the γ38, γ40 and γ42 sites to a different extent and particularly reduced the formation of $A\beta_{42}$, while increasing the formation of $A\beta_{38}$. The most active compounds were **70d** ($A\beta_{42}$ IC_{50} = 7.5 μM), **72d** ($A\beta_{42}$ IC_{50} = 11 μM), **72e** ($A\beta_{42}$ IC_{50} = 8.2 μM) and displayed activity at low micromolar concentration. The introduction of a lipophilic substituent, which may vary from arylsulfone to alkyl, turned 2-carbazolyloxyacetic acids into potent γ-secretase modulators. This resulted in the selective reduction of $A\beta_{42}$ and an increase of the less aggregatory $A\beta_{38}$ fragment by several compounds (e.g. **70d**, **72d**, **72e**). Some *in silico* parameters of these lead candidates are close to the range of drug-like compounds, but the lipophilic substituents cause a dramatic increase of the CLogP. Introduction of an electron donating group such as methoxy, at position 6 and 8 of *N*-sulfonylated carbazolyloxyacetic acids either decreased or inversed the modulatory activity. The most active compounds displayed activity on APP overexpressing cell lines in the low micromolar range and little or no effect on the γ-secretase cleavage at the ε-site. If affected at all, the ε-cleavage of γ-secretase was inhibited at much higher compound concentrations than those determined to be modulatory at the ε-site. Therefore, these lead compounds are ideal ones for further optimization since they fulfil the criteria for an ideal compound for AD therapy: reduction of toxic $A\beta_{42}$ levels, no effect on the other cleavages such as AICD and no COX inhibitory activity.

4.3. Structurally Modified Analog of LXR-Agonist TO-901317 as Modulator of γ-Secretase

It was very much evident from the SAR of *N*-substituted carprofen derivatives[120] and *N*-substituted carbazolyloxyacetic acids[182] that the free acid is crucial for the modulatory activity. LXR-agonist **81** (**TO-901317**) was found to be an inverse modulator of γ-secretase. It increased selectively the formation of Aβ42 and reduced the formation of less aggregatory Aβ38 in cellular assay. Knowing about the relevance of free acid moiety and modulation of γ-secretase, an analog of **81** was synthesized, but the hexafluorocarbinol moiety was replaced by an oxyacetic acid moiety. As anticipated, the replacement of the hexafluorocarbinol moiety by an oxyacetic acid group dramatically changed the mode of action from inverse modulation to normal modulation of γ-secretase. Compound **81** (Aβ42 IC$_{50}$ = 19.6 μM) was found to be a modulator of γ-secretase and reduced selectively the formation of Aβ42, while increasing the formation of Aβ38 in Aβ liquid phase electrochemiluminescence assay. In consistence with previous results, the ester derivative of **87** was found to be an inverse modulator of γ-secretase. This confirms the hypothesis that the free acid moiety is mandatory for the modulatory activity for γ-secretase. This significant structural modification can be applied to the known inverse modulators of γ-secretase such as celecoxib and rofecoxib. That might change their mode of action from inverse modulation to normal modulation of γ-secretase.

4.4. Curcumin derived heterocycles: Swiss knives for AD

4.5. Synthesis and Biological Evaluation of NSAIDs Derived Affinity Probes

A set of biotinylated NSAIDs was synthesized to identify the binding identify the binding partner *via* immunoprecipitation. The synthesis of biotinylated NSAIDs was commenced with the commercially available COX and COX-2 inhibitors such as flurbiprofen, ketoprofen, meclofenamic acid, sulindac sulfoxide and carprofen. Initially the carboxylic acid common with NSAIDs was derivatized for biotin incorporation which resulted in the loss of activity or inversion in modulation. They affected the cleavage at the γ38, γ40 and γ42 sites to a different extent and particularly increased the formation of Aβ42, while increasing the formation of Aβ38. Amongst the tested affinity labels (**94**, **98**, **101**, **105**, **109** and **112**), only **94** was able to capture γ-secretase complex in dose dependant manner but failed to pull down the active complex. The SAR advocated the synthesis of the affinity probes without disturbing acid moiety of the NSAIDs to conserve the modulatory activity. Therefore, the biotinylated flurbiprofen **117** and *N*-substituted carprofen **126** were synthesized keeping the acid moiety intact. As anticipated, the introduction of biotin in the presence of the acid moiety resulted in the modulation of γ-secretase. An immunoprecipitation assay carried out with affinity probe **117** indicated that the biotin bound unspecifically. **117** turned out to be unsuitable bait for the pull down of γ-secretase active complex.

4.6. Synthesis and Biological Evaluation of Flurbiprofen and DAPT derived Photoaffinity Probes

Flurbiprofen and DAPT derived photoaffinity probes (**134** and **139**) were synthesized in order to identify the binding site on γ-secretase and their mode of action. Photoaffinity labelling experiments carried out with the photoaffinity probe **139** revealed that it binds covalently to PS, but the observed binding was very weak. Moreover, the introduction of a photoreactive group and the affinity tag did not reduce the γ-secretase inhibitory activity significantly. But, the photoaffinity labelling experiment carried out with flurbiprofen derived photoaffinity probe **134** disclosed that it binds to the *C*-terminal fragment of PS, on the enzyme of the

active γ-secretase complex. The identification of the binding site of flurbiprofen on active γ-secretase complex *via* photoaffinity labelling with photoaffinity probe **134** is ongoing.

5. EXPERIMENTAL

5.1 Instrumentation and General Experimental Considerations

5.1.1. Thin layer chromatography (TLC)

Thin-layer chromatography (TLC) was carried out using aluminum sheets precoated with silica gel 60 F_{254} (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 F_{254} (2.0 mm; E. Merck) were used.

5.1.2. Column chromatography

Flash column chromatography was carried out using Merck silica gel 60 (40-63 and 15-40 μm) and 60G (5-40 μm).

5.1.3. Melting points

Measurements below 200 °C were determined on a Büchi 510 melting point apparatus and above 200 °C were carried out on a Kofler-Heiztischmikroskop apparatus and are uncorrected.

5.1.4. NMR spectroscopy

The ^{1}H spectra were recorded on a Bruker AC 300 and AC 500 spectrometer at 300 and 500 MHz. The ^{13}C spectra was recorded on Bruker AC 300 and AC 500 spectrometer at 75 and 125 MHz. Chemical shifts are reported as % values (ppm) downfield from Me_4Si.

5.1.5. Mass spectrometry

Mass spectrometry was performed on a Bruker-Franzen Esquire LC mass spectrometer.

5.1.6. Drying of solvents

THF was dried and distilled from sodium and benzophenone under nitrogen prior to use. DMF was stored over 3 Å molecular sieves. All other commercial chemicals were used without further purification. CH_2Cl_2 and Et_3N were distilled from CaH_2 under nitrogen prior to use. Amino acid derivatives were bought from Fluka Chemie (Switzerland), NovaBiochem (Switzerland), or Bachem (Switzerland). All other chemicals were of reagent quality and used as obtained from the manufacturers. Reactions were carried out in Ar when necessary.

5.2. Synthesis of NSAID Derivatives as Potential γ-Secretase Inhibitors and Modulators.

5.2.1. General procedure for peptide coupling using EDAC as coupling reagent

1.0 equiv. Et$_3$N was added to a solution of 1.0 equiv. of acid in CH$_2$Cl$_2$ which was followed by the addition of 1.0 equiv. ethyl-3-(3′-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and 1.2 equiv. HOBt (*N*-hydroxybenzotriazole hydrate) and stirred at RT until it became clear. 1.2 equiv. Et$_3$N and 1.2 equiv. amine were added to it simultaneously. The reaction mixture was kept on stirring at RT for 2-24 h and monitored by TLC. The reaction mixture was diluted with CH$_2$Cl$_2$. The organic phase was washed sequentially with NaHCO$_3$, (0.1 N) HCl and brine simultaneously. The organic phase was dried over anhydrous Na$_2$SO$_4$ and the solvent was evaporated *in vacuo* to afford the crude product which was further purified by column chromatography to obtain the pure product.

5.2.2. General procedure for esterification using EDAC as coupling reagent

1.0 equiv. Et$_3$N was added to a solution of 1.0 equiv. of acid in CH$_2$Cl$_2$ which was followed by the addition of 1.0 equiv. ethyl-3-(3′-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, 1.2 equiv. HOBt (*N*-hydroxybenzotriazole hydrate) and stirred at RT until it became clear. 1.2 equiv. Et$_3$N and 1.2 equiv. alcohol was added to it simultaneously. The reaction mixture was kept on stirring at RT for 6–48 h and monitored by TLC. The reaction mixture was diluted with CH$_2$Cl$_2$. The organic phase was washed sequentially with NaHCO$_3$, (0.1 N) HCl and brine simultaneously. The organic phase was dried over anhydrous Na$_2$SO$_4$ and the solvent was evaporated *in vacuo* to afford the crude product which was further purified by column chromatography to obtain the pure product.

5.2.3. Synthesis of Flurbiprofen Derivatives

5.2.3.1. Synthesis of flurbiprofen derived amides.

5.2.3.1.1 *N*-(Benzo[*d*][1,3]dioxol-5-ylmethyl)-2-(2-fluorobiphenyl-4-yl)propanamide (56a)
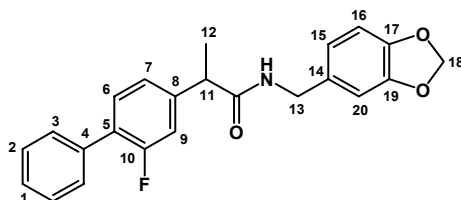
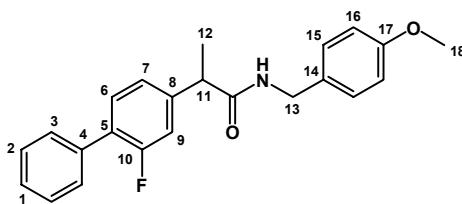The title compound was synthesized by following the general procedure mentioned in **5.2.1.**

Reactants: 2-(2-fluoro-4-biphenyl)propanoic acid (100 mg, 0.4 mmol), EDAC (94 mg, 0.5 mmol), HOBt (69 mg, 0.5 mmol), Et$_3$N (125 μL, 0.9 mmol), (benzo[*d*][1,3]dioxol-6-yl)methanamine (75 mg, 0.5 mmol).

Yield: 115 mg, 75%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.45 (dd, *J* = 6.6 Hz, *J* = 1.5 Hz, 2H; H-3), 7.40-7.27 (m, 5H; H-1, H-2, H-6, H-7), 7.07 (m, 1H; H-9), 6.65-6.55 (m, 3H; H-15, H-16, H-20), 5.84 (s, 2H; H-18), 5.69 (brs, 1H; NH), 4.26 (dd, *J* = 14.7 Hz, *J* = 5.8 Hz, 1H; H-13), 4.21 (dd, *J* = 14.7 Hz, *J* = 5.8 Hz, 1H; H-13′), 3.53 (q, *J* = 7.2 Hz, 1H; H-11), 1.49 (d, *J* = 7.2 Hz, 3H; H-12) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 173.3 (C-11), 159.1 (C-10), 148.0 (C-17, C-19), 142.7 (C-8), 142.6 (C-4), 135.4 (C-14), 131.1 (C-5), 130.1 (C-6), 129.0 (C-2), 128.5 (C-3), 127.8 (C-1), 123.6 (C-7), 121.0 (C-15), 115.5 (C-9), 115.2 (C-16), 108.3 (C-20), 101.1 (C-18), 46.7 (C-11), 43.6 (C-13), 18.7 (C-12) ppm.

5.2.3.1.2 2-(2-Fluorobiphenyl-4-yl)-*N*-(4-methoxybenzyl)propanamide (56b)

The title compound was synthesized by following the general procedure mentioned in **5.2.1.**

Reactants: 2-(2-fluoro-4-biphenyl)propanoic acid (100 mg, 0.4 mmol), EDAC (94 mg, 0.5 mmol), HOBt (69mg, 0.5 mmol), Et$_3$N (125 µL, 0.9 mmol), 4-methoxybenzyl amine (67 mg, 0.5 mmol).

Yield: 119 mg, 80%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.45 (dd, *J* = 7.0 Hz, *J* = 1.4 Hz, 2H; H-3), 7.38-7.25 (m, 4H; H-1, H-2, H-6), 7.08-7.02 (m, 4H; H-H-7, H-9, H-15), 6.75 (dd, *J* = 8.8 Hz, *J* = 2.4 Hz, 2H; H-16), 5.66 (brs, 1H; NH), 4.32 (dd, *J* = 14.6 Hz, *J* = 5.6 Hz, 1H; H-13), 4.26 (dd, *J* = 14.6 Hz, *J* = 5.6 Hz, 1H; H-13´), 3.70 (s, 3H; H-18), 3.49 (q, *J* = 7.6 Hz, 1H; H-11), 1.49 (d, *J* = 7.6 Hz, 3H; H-12) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 173.3 (C-11), 159.1 (C-10), 158.2 (C-17), 142.8 (C-8), 142.7 (C-4), 135.4 (C-14), 131.1 (C-5), 130.3 (C-6), 129.5 (C-2), 129.0 (C-15), 128.5 (C-3), 127.8 (C-1), 123.7 (C-7), 115.5 (C-9), 114.1 (C-16), 55.3 (C-18), 46.7 (C-11), 43.3 (C-13), 18.7 (C-12) ppm.
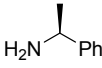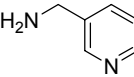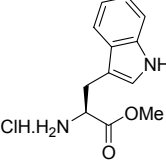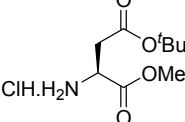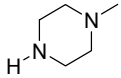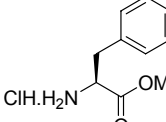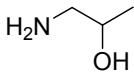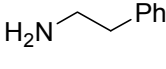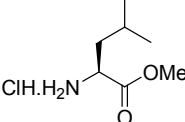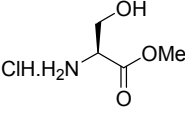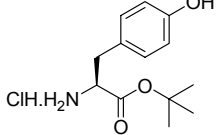
MS (EI): *m/z* = 363 (M$^+$, 23%), 199 (20%), 121 (100%)

5.2.3.1.3 Compound 56c–56p

Compounds **56c–56p** were synthesized using the general procedure mentioned in **5.2.1**.

Reactants: 2-(2-fluoro-4-biphenyl)propanoic acid (40 mg, 0.16 mmol), EDAC (38 mg, 0.2 mmol), HOBt (27 mg, 0.2 mmol), Et$_3$N (125 µL, 0.36 mmol), Amine (0.2 mmol). The amount of various amines used and the yield is depicted in the **Table 5.1**.

Table 5.1: Structure and amount of various amines used in the synthesis of compounds **56c–p**.

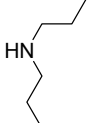| Compound	Amount	Amine	Yield
56c	25 µL		37 mg (61%)
56d	20 µL		30 mg (55%)
56e	50 mg		48 mg (67%)
56f	49 mg		49 mg (64%)
56g	22 µL		38 mg (72%)
56h	36 mg		57 mg (86%)
56i	16 µL		20 mg (41%)
56j	25 µL		38 mg (68%)
56k	29 mg		33 mg (55%)
56l	27 µL		31 mg (58%)
56m & 56n	24 mg		20 mg (**48m**) 18 mg (**48n**) 60% combine
56o & 56p	47 mg		28 mg (**48o**) 25 mg (**48p**) 70% combine

5.2.3.2. Synthesis of flurbiprofen derived esters.
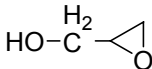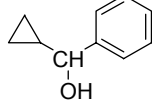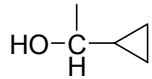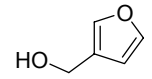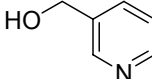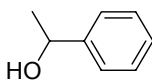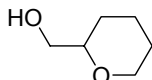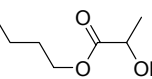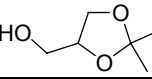
5.2.3.2.1. Flurbiprofen derived esters 57a–57i

Compound **57a–57i** were synthesized by following the general procedure mentioned **in 5.2.2**.

Reactants: 2-(2-fluoro-4-biphenyl)propanoic acid (40 mg, 0.16 mmol), EDAC (38 mg, 0.2 mmol), HOBt (27 mg, 0.2 mmol), Et₃N (125 μL, 0.36 mmol), Alcohol (0.2 mmol).

The amount of various alcohols used and the yield is depicted in the **Table 5.2**.

Table 5.2: Structure and amount of various alcohols used in the synthesis of compounds **57a–i**.

Entry	Compound	Amount	Alcohol	Yield
1	**57a**	16 μL		16 mg (35%)
2	**57b**	17 μL		26 mg (43%)
3	**57c**	16 μL		24 mg (48%)
4	**57d**	15 μL		30 mg (55%)
5	**57e**	16 μL		35 mg (65%)
6	**57f**	20 μL		32 mg (57%)
7	**57g**	21 μL		22 mg (40%)
8	**57h**	25 μL		20 mg (34%)
9	**57i**	21 μL		23 mg (40%)

5.2.3.2.1. Benzyl 2-(2-fluorobiphenyl-4-yl)propanoate (57j)
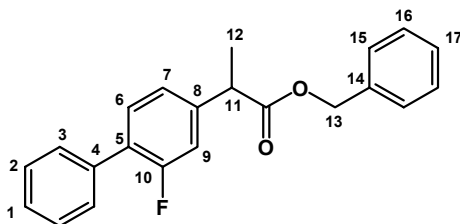
The title compound was synthesized by following the general procedure mentioned in **5.2.2**.

Reactants: 2-(2-fluoro-4-biphenyl)propanoic acid (100 mg, 0.4 mmol), EDAC (94 mg, 0.5 mmol), HOBt (69 mg, 0.5 mmol), Et$_3$N (125 µL, 0.9 mmol), benzyl alcohol (53 mg, 0.5 mmol).

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.46-7.41 (m, 2H; H-3), 7.37-7.18 (m, 9H; H-1, H-2, H-5, H-15, H-16, H17), 7.07-7.01 (m, 2H; H-7, H-9), 5.08 (d, J = 6.4 Hz, 1H; H-13), 5.04 (d, J = 6.4 Hz, 1H; H-13´), 3.72 (q, J = 7.2 Hz, 1H; H-11), 1.47 (d, J = 7.2 Hz, 3H; H-12) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 173.9 (C-11), 161.4 (C-10), 141.8 (C-14), 141.7 (C-8), 135.9 (C-4), 130.9 (C-5), 130.8 (C-6), 129.5 (C-2), 129.0 (C-16), 128.6 (C-3), 128.3 (C-15), 128.1 (C-1), 127.7 (C-17), 126.4 (C-7), 115.5 (C-9), 66.8 (C-13), 45.1 (C-11), 18.4 (C-12) ppm.

MS (EI): m/z = 334 (M$^+$, 38%), 199 (100%), 91 (28%).
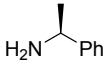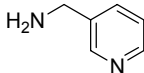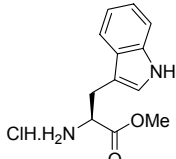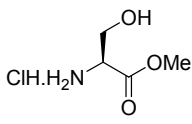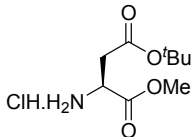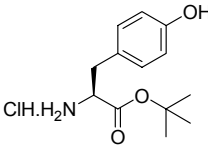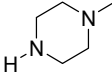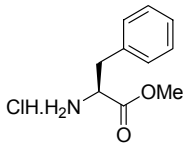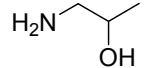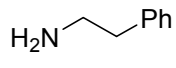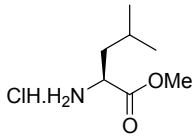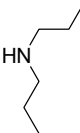
5.2.4. Synthesis of Sulindac Sulfoxide Derived Amides.

Compounds **59a–59l** were synthesized by following the general procedure mentioned in **5.2.1**.

Reactants: 2-((1Z)-1-(4-(Methylsulfinyl)benzylidene)-5-fluoro-2-methyl-1H-inden-3-yl)acetic acid (60 mg, 0.17 mmol), EDAC (36 mg, 0.19 mmol), HOBt (27 mg, 0.2 mmol), Et$_3$N (52 µL, 0.45 mmol), Amine (0.2 mmol).

The amount of various amines used and the yield is depicted in the **Table 5.3**.

Table 5.3: Structure and amount of various amines used in the synthesis of compounds **59a–59l**.

Entry	Compound	Amount	Amines	Yield
1	**59a**	26 μL		47 mg (61%)
2	**59b**	21 μL		41 mg (55%)
3	**59c**	52 mg		67 mg (67%)
4	**59d**	33 mg		49 mg (64%)
5	**59e**	50 mg		70 mg (72%)
6	**59f**	48 mg		83 mg (86%)
7	**59g**	21 mg		30 mg (41%)
8	**59h**	37 mg		59 mg (68%)
9	**59i**	16 μL		38 mg (55%)
10	**59j**	26 μL		44 mg (58%)
11	**59k**	30 mg		61 mg (75%)
12	**59l**	28 μL		39 mg (53%)

5.2.4. Synthesis of Meclofenamic Acid Derived Amides.

5.2.4.1. (*S*)-Methyl 2-(2-(2,6-dichloro-3-methylphenylamino)benzamido)-3-(1*H*-indol-3-yl)propanoate (60a)
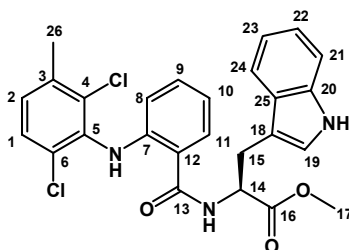
The title compound was synthesized by following the general procedure mentioned in **5.2.1**.

Reactants: 2-(2,6-dichloro-3-methylphenylamino)benzoic acid (60 mg, 0.18 mmol), EDAC (94 mg, 0.22 mmol), HOBt (69 mg, 0.22 mmol), Et$_3$N (62 µL, 0.45 mmol), Tryptophan methyl ester hydrochloride (58 mg, 0.22 mmol).

Yield: 75 mg, 75%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 9.11 (brs, 1H; NH), 8.09 (brs, 1H; NH), 7.47 (d, J = 7.8 Hz, 1H; H-11), 7.21-7.17 (m, 2H; H-1, H-9), 7.11-7.06 (m, 2H; H-21, H-24), 7.03-6.93 (m, 3H, H-19, H-22/23), 6.69 (d, J = 7.5 Hz, 1H; H-8), 6.61 (t, J = 7.0 Hz, 1H; H-10), 6.29 (d, J = 8.4 Hz, 1H; H-2), 5.08-5.03 (m, 1H; H-14), 3.64 (s, 3H; H-17), 3.46-3.41 (m, 2H; H-15), 2.31 (s, 3H; H-26) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 172.5 (C-16), 169.0 (C-13), 145.5 (C-7), 136.4 (C-20), 136.2 (C-5), 135.7 (C-3), 135.0 (C-4), 133.3 (C-9), 130.2 (C-11), 127.8 (C-1), 127.7 (C-25), 126.8 (C-6), 123.0 (C-19), 122.3 (C-23), 121.3 (C-2), 121.2 (C-22), 119.8 (C-8), 119.4 (C-24), 118.4 (C-10), 114.8 (C-21), 111.0 (C-18), 53.4 (C-14), 52.6 (C-17), 30.7 (C-15), 20.7 (C-26) ppm.

5.2.4.2. (*S*)-Di-tert-butyl 2-(2-(2,6-dichloro-3-methylphenylamino)benzamido)succinate (60b)
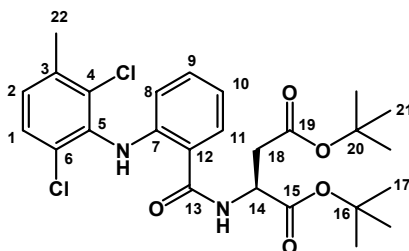
The title compound was synthesized by following the general procedure mentioned in **5.2.1**.

Reactants: 2-(2,6-dichloro-3-methylphenylamino)benzoic acid (60 mg, 0.18 mmol), EDAC (94 mg, 0.22 mmol), HOBt (69 mg, 0.22 mmol), Et$_3$N (62 µL, 0.45 mmol), Asp(O^{t}Bu)^{t}Bu·HCl (56 mg, 0.22 mmol).

Yield: 83 mg, 78%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 9.27 (brs, 1H; NH), 7.45 (dd, J = 7.8 Hz, J = 1.4 Hz, 1H; H-11), 7.17-7.12 (m, 2H; H-8/9), 6.98 (d, J = 8.0 Hz, 1H; H-1), 6.71 (t, J = 7.0 Hz, 1H; H-10), 6.32 (d, J = 8.4 Hz, 1H; H-2), 4.84-4.79 (m, 1H; H-14), 2.92 (dd, J = 7.0 Hz, J = 4.4 Hz, 1H; H-18), 2.82 (dd, J = 7.0 Hz, J = 4.4 Hz, 1H; H-18′), 2.31 (s, 3H; H-22), 1.41 (s, 9H; H-21), 1.38 (s, 9H; H-17) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 170.5 (C-19), 170.1 (C-15), 168.5 (C-13), 145.7 (C-7), 136.4 (C-5), 135.8 (C-3), 133.4 (C-4), 132.3 (C-9), 130.3 (C-11), 127.7 (C-1), 127.2 (C-6), 122.0 (C-2), 120.1 (C-12), 118.0 (C-8), 114.7 (C-10), 82.5 (C-20), 81.7 (C-16), 49.4 (C-14), 37.6 (C-18), 28.1 (C-21), 28.0 (C-17), 20.7 (C-22) ppm.

5.2.4.3. *N*-(2-(1*H*-Indol-3-yl)ethyl)-2-(2,6-dichloro-3-methylphenylamino)benzamide (60c)
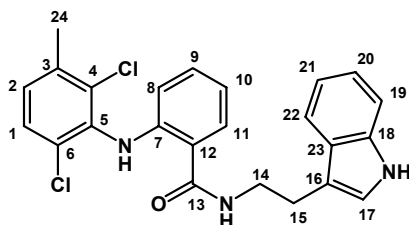
The title compound was synthesized by following the general procedure mentioned in **5.2.1**.

Reactants: 2-(2,6-dichloro-3-methylphenylamino)benzoic acid (60 mg, 0.18 mmol), EDAC (94 mg, 0.22 mmol), HOBt (69 mg, 0.22 mmol), Et₃N (62 µL, 0.45 mmol), tryptamine (37 mg, 0.22 mmol).

Yield: 64 mg, 72%.

¹H NMR (300 MHz, CDCl₃): δ = 9.21 (brs, 1H; NH), 8.04 (brs, 1H, NH), 7.57 (d, *J* = 7.8 Hz, 1H; H-11), 7.28 (d, *J* = 7.8 Hz, 1H; H-1), 7.22-6.95 (m, 7H; H-8/9, H-17, H-19/20/21/22), 6.62 (t, *J* = 7.8 Hz, 1H; H-10), 6.29 (dd, *J* = 8.2 Hz, 1H; H-2), 6.13 (brs, 1H, NH), 3.72 (q, *J* = 6.4 Hz, 2H; H-14), 3.03 (t, *J* = 6.4 Hz, 2H; H-15), 2.31 (s, 3H; H-24) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 169.6 (C-13), 145.2 (C-7), 136.4 (C-18), 135.8 (C-5), 135.1 (C-3), 135.0 (C-4), 133.2 (C-9), 131.9 (C-11), 130.1 (C-23), 127.8 (C-1), 126.6 (C-6), 122.4 (C-17), 122.3 (C-21), 121.4 (C-2), 120.8 (C-12), 120.4 (C-20), 119.6 (C-8), 118.8 (C-22), 118.0 (C-10), 114.8 (C-19), 111.4 (C-16), 42.3 (C-14), 25.3 (C-15), 20.7 (C-24) ppm.

5.2.4.4. 2-(2,6-Dichloro-3-methylphenylamino)-*N*-(2-(5-hydroxy-1*H*-indol-3-yl)ethyl)benzamide (60d)
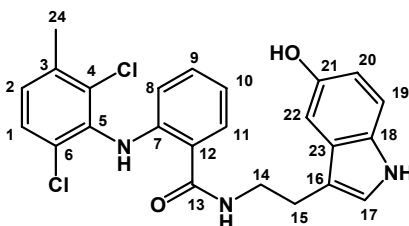
The title compound was synthesized by following the general procedure mentioned in **5.2.1**.

Reactants: 2-(2,6-dichloro-3-methylphenylamino)benzoic acid (60 mg, 0.18 mmol), EDAC (94 mg, 0.22 mmol), HOBt (69 mg, 0.22 mmol), Et₃N (62 µL, 0.45 mmol), seritonin hydrochloride (48 mg, 0.22 mmol).

Yield: 55 mg, 60%.

¹H NMR (300 MHz, CDCl₃): δ = 8.78 (brs, 1H; NH), 7.25 (dd, *J* = 7.8 Hz, *J* = 1.2 Hz, 1H; H-11), 7.22 (d, *J* = 8.2 Hz, 1H; H-1), 7.13-7.07 (m, 3H; H-9, H-17, H-19), 6.99-6.93 (m, 2H; H-8, H-22), 6.81 (brs, 1H; NH), 6.69 (d, *J* = 8.8 Hz, 1H; H-20), 6.64 (t, *J* = 7.0 Hz, 1H; H-10), 6.27 (dd, *J* = 8.4 Hz, 1H; H-2), 3.65 (q, *J* = 6.9 Hz, 2H; H-14), 2.97 (t, *J* = 6.9 Hz, 2H; H-15), 2.31 (s, 3H H-24) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 169.9 (C-13), 150.1 (C-21), 144.8 (C-7), 136.4 (C-5), 135.6 (C-3), 135.2 (C-4), 133.0 (C-9), 131.8 (C-18), 131.4 (C-23), 129.9 (C-11), 126.6 (C-1), 125.8

(C-6), 123.2 (C-17), 122.3 (C-2), 121.4 (C-12), 120.8 (C-8), 119.6 (C-10), 118.0 (C-19), 114.7 (C-20), 113.0 (C-16), 113.7 (C-22), 39.9 (C-14), 25.2 (C-15), 20.5 (C-24) ppm

5.3. Synthesis of Carprofen Derived γ-Secretase Modulators

5.3.1. Synthesis of compound 61, 62, 63, 65 and 66

The synthetic procedures and spectral data of compounds **61**, **62**, **63**, **65** and **66** is published in the supplementary material of the article "The Scaffold of the COX-2 Inhibitor Carprofen Provides Alzheimer γ-Secretase Modulators, *Journal of Medicinal Chemistry*, **2006**, *49 (26)*, 7588-7591." The material is available free of charge via the Internet at http://pubs.acs.org.

5.3.2. Synthesis of 2-(6-Chloro-9-tosyl-9*H*-carbazol-2-yl)propanoic acid amides (64)

5.3.2.1. (2*S*)-Methyl 2-(2-(6-chloro-9-tosyl-9*H*-carbazol-2-yl)propanamido)-3-(1*H*-imidazol-4-yl)propanoate (64a)
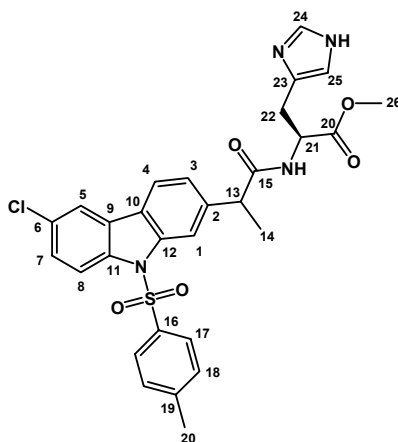
The title compound was synthesized by following the general procedure mentioned in **5.2.1**.

Reactants: 2-(6-Chloro-9-tosyl-9*H*-carbazol-2-yl)propanoic acid (**63a**) (25 mg, 0.06 mmol), EDAC (14 mg, 0.07 mmol), HOBt (9.5 mg, 0.7 mmol), Et$_3$N (15 μL, 0.13 mmol), His-OMe·HCl (12 mg, 0.07 mmol).

Yield: 23 mg, 70%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 8.22 (d, *J* = 1.2 Hz, 1H; H-5), 8.17 (d, *J* = 8.9 Hz, 1H; H-4), 7.74 (d, *J* = 2.1 Hz, 1H; H-1), 7.71 (d, *J* = 8.0 Hz, 1H; H-8), 7.58 (dd, *J* = 8.6, *J* = 1.9 Hz, 2H; H-17), 7.48 (s, 1H; H-24), 7.37 (dd, *J* = 8.8, *J* = 2.1 Hz, 1H; H-3), 7.27 (dd, *J* = 8.3, *J* = 1.4 Hz, 1H; H-7), 7.03 (d, *J* = 8.0 Hz, 2H; H-18), 6.80 (s, 1H; H-26), 4.72-4.64 (m, 1H; H-21), 3.84 (q, *J* = 7.2 Hz, 1H; H-13), 3.71 (s, 3H; H-28), 3.06-2.94 (m, 2H; H-22), 2.19 (s, 3H; H-20), 1.53 (d, *J* = 7.2 Hz, 3H; H-14) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 172.7 (C-15), 172.3 (C-20), 145.3 (C-16), 139.1 (C-12), 137.0 (C-10), 135.6 (C-24), 135.1 (C-19), 134.3 (C-18), 129.7 (C-17), 127.7 (C-6), 126.5 (C-11), 122.5 (C-5), 121.9 (C-3), 120.2 (C-4), 119.8 (C-7), 119.4 (C-26), 116.3 (C-8), 114.6 (C-1), 104.6 (C-9), 54.5 (C-26), 45.9 (C-13), 30.1 (C-22), 21.4 (C-20), 18.7 (C-14) ppm.

MS (ESI): *m/z* = 601.2 (M + Na)$^+$.

5.3.4.2. 2-(6-Chloro-9-tosyl-9*H*-carbazol-2-yl)-*N*-(3-methylisothiazol-5-yl)propanamide (64b)
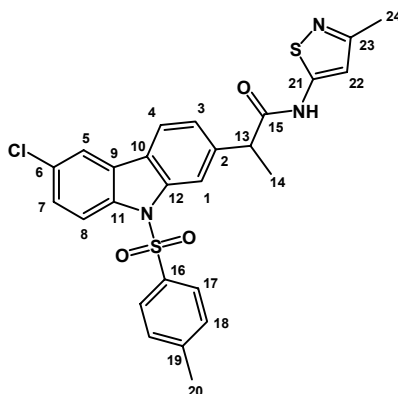
The title compound was synthesized by following the general procedure mentioned in **5.2.1**.

Reactants: 2-(6-Chloro-9-tosyl-9*H*-carbazol-2-yl)propanoic acid (**63a**) (25 mg, 0.06 mmol), EDAC (14 mg, 0.07 mmol), HOBt (9.5 mg, 0.07 mmol), Et₃N (15 μL, 0.13 mmol), 3-methylisothiazol-5-amine (10.6 mg, 0.07 mmol).

Yield: 13 mg, 40%.

¹H NMR (300 MHz, CD₃OD): δ = 8.21(d, J = 1.2 Hz, 1H; H-5), 8.14 (d, J = 8.9 Hz, 1H; H-4), 7.71 (d, J = 2.1 Hz, 1H; H-1), 7.67 (d, J = 8.0 Hz, 1H; H-8), 7.54 (dd, J = 8.6, J = 1.9 Hz, 2H; H-17), 7.34 (dd, J = 8.8, J = 2.1 Hz, 1H; H-3), 7.29 (s, 1H, H-22), 7.23 (dd, J = 8.3, J = 1.4 Hz, 1H; H-7), 7.03 (d, J = 8.0 Hz, 2H; H-18), 3.84 (q, J = 7.2 Hz, 1H; H-13), 2.41 (s, 3H; H-24), 2.19 (s, 3H; H-20), 1.53 (d, J = 7.2 Hz, 3H; H-14) ppm.

¹³C NMR (75 MHz, CD₃OD): δ = 172.7 (C-15), 166.5 (C-23), 148.1 (C-21), 145.1 (C-16), 139.3 (C-12), 137.2 (C-10), 134.5 (C-18), 130.7 (C-17), 127.9 (C-6), 126.4 (C-11), 122.1 (C-5), 121.0 (C-3), 120.1 (C-4), 119.3 (C-7), 116.1 (C-8), 114.0 (C-1), 107.2 (C-22), 104.1 (C-9), 45.9 (C-13), 24.4 (C-20), 19.1 (C-24), 18.3 (C-14) ppm.

MS (ESI): m/z = 549.9 (M + Na)⁺.

5.3.4.3. 2-(6-Chloro-9-tosyl-9*H*-carbazol-2-yl)-*N*-(1*H*-1,2,4-triazol-3-yl)propanamide (64c)
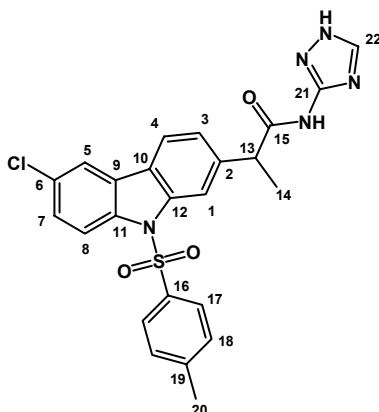
The title compound was synthesized by following the general procedure mentioned in **5.2.1**.

Reactants: 2-(6-Chloro-9-tosyl-9*H*-carbazol-2-yl)propanoic acid (**63a**) (25 mg, 0.06 mmol), EDAC (14 mg, 0.07 mmol), HOBt (9.5 mg, 0.07 mmol), Et₃N (15 μL, 0.13 mmol), 1*H*-1,2,4-triazol-3-amine (10.6 mg, 0.07 mmol).

Yield: 9 mg, 32%.

^{1}H NMR (300 MHz, CD$_3$OD): δ = 8.31 (s, 1H; H-22), 8.19 (d, J = 1.2 Hz, 1H; H-5), 8.14 (d, J = 8.9 Hz, 1H; H-4), 7.70 (d, J = 2.1 Hz, 1H; H-1), 7.68 (d, J = 8.0 Hz, 1H; H-8), 7.49 (dd, J = 8.6, 1.9 Hz, 2H; H-17), 7.31 (dd, J = 8.8, J = 2.1 Hz, 1H; H-3), 7.25 (dd, J = 8.3, J = 1.4 Hz, 1H; H-7), 7.06 (d, J = 8.0 Hz, 2H; H-18), 3.79 (q, J = 7.2 Hz, 1H; H-13), 2.16 (s, 3H; H-20), 1.55 (d, J = 7.2 Hz, 3H; H-14) ppm.

^{13}C NMR (75 MHz, CD$_3$OD): δ = 172.7 (C-15), 155.6 (C-21), 154.9 (C-22), 145.1 (C-16), 139.7 (C-12), 137.6 (C-10), 134.5 (C-18), 130.4 (C-17), 127.9 (C-6), 126.1 (C-11), 122.8 (C-5), 121.2 (C-3), 120.1 (C-4), 119.2 (C-7), 116.8 (C-8), 114.5 (C-1), 104.8 (C-9), 46.2 (C-13), 22.4 (C-20), 18.6 (C-14) ppm.

**MS (ESI):** m/z = 516.1 (M + Na)$^+$.

5.4. Synthesis of *N*-Substituted Carbazolyloxyacetic Acids

5.4.1. Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (68)
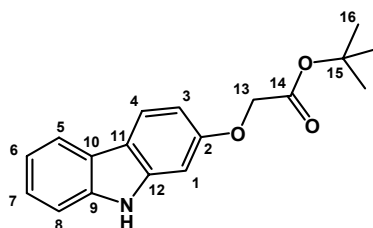
Anhydrous K$_2$CO$_3$ (2.26 g, 16.38 mmol) was added to a stirred solution of 4-hydroxy carbazole (1 g, 5.46 mmol) in acetone (15 mL) and stirred at ambient temperature for 30 min. T-butyl chloroacetate (0.94 mL, 6.55 mmol) was added to it and heated to 60–70 °C for 12 h. Reaction was cooled to room temperature and filtered. The residue was washed with acetone (3×). Combined organic extract was evaporated *in vacuo* and purified by crystallization (EtOAc:hexane) to yield the title compound as a colorless solid (1.6 g, 95%)

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.99 (brs, 1H; NH), 7.96-7.93 (m, 2H; H-4/5), 7.38-7.35 (m, 2H; H-7/8), 7.24-7.21 (m, 1H; H-6), 6.92 (d, J = 3.0 Hz, 1H; H-1), 6.87 (dd, J = 9.0, J = 1.5 Hz, 1H; H-3), 4.61 (s, 2H; H-13), 1.51 (s, 9H; H-16) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 168.3 (C-14), 157.3 (C-2), 139.6 (C-12), 124.7 (C-9), 123.7 (C-11), 122.7 (C-6), 121.0 (C-7), 120.5 (C-4), 119.7 (C-5), 110.8 (C-3), 108.3 (C-10), 96.2 (C-1), 82.3 (C-15), 66.4 (C-13), 28.0 (C-16) ppm.

5.4.2. Synthesis of *N*-sulfonylated of tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate derivatives (69)

5.4.2.1. General procedure for *N*-sulfonylation of carbazole derivatives

NaH (60% dispersion, 1.2 eq.) was combined with THF (5 mL/mmol) and cooled to 0 °C. Tert-butyl 2-(9*H*-carbazol-6-yloxy)acetate (**68**) (1 eq.) was added to the stirring slurry in portions. After 30 minutes R-sulfonyl chloride (1.2 eq.) dissolved in THF was added and the reaction was stirred at ambient temperature for 6–24 h. The heterogeneous reaction mixture was quenched with NH$_4$Cl (sat. aq.), diluted with EtOAc and then washed sequentially with H$_2$O and brine (×2). The organic extract was dried over anhydrous Na$_2$SO$_4$ and evaporated *in vacuo* to give crude compound. The crude compound was purified by flash column chromatography using (EtOAc:hexane, 1:9) to afford the desired product.

5.4.2.2. Tert-butyl 2-(9-tosyl-9*H*-carbazol-2-yloxy)acetate (69a)
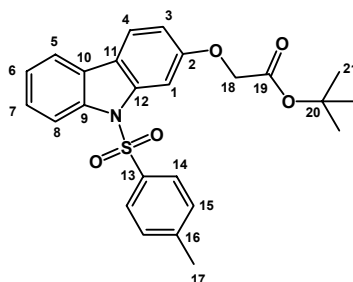
The title compound was synthesized by following the general procedure mentioned in **5.4.2.1**.

Reactants: Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), toluene sulfonyl chloride (167 mg, 0.88 mmol), NaH (36 mg, 0.88 mmol).

Yield: 210 mg, 55%.

^{1}H NMR (300 MHz, CDCl₃): δ = 8.18 (d, *J* = 8.2 Hz, 1H; H-5), 7.80 (d, *J* = 2.2 Hz, 1H; H-1), 7.72-7.68 (m, 2H; H-4, H-8), 7.59 (d, *J* = 8.3 Hz, 2H; H-14), 7.32 (dt, *J* = 7.3, *J* = 1.3 Hz, 1H; H-7), 7.24 (dt, *J* = 7.6, *J* = 1.0 Hz, 1H; H-6), 7.02 (d, *J* = 8.2 Hz, 2H; H-15), 6.92 (dd, *J* = 8.5 Hz, *J* = 2.3 Hz, 2H; H-3), 4.58 (s, 2H; H-18), 1.52 (s, 9H; H-21) ppm.

^{13}C NMR (75 MHz, CDCl₃): δ = 168.1 (C-19), 158.2 (C-2), 145.0 (C-16), 139.7 (C-12), 135.1 (C-13), 129.8 (C-15), 127.7 (C-14), 126.6 (C-9), 126.4 (C-11), 124.1 (C-6), 120.7 (C-4/7), 119.4 (C-5), 115.3 (C-3), 113.1 (C-8), 105.9 (C-10), 97.9 (C-1), 82.8 (C-20), 66.4 (C-18), 28.3 (C-21), 21.6 (C-17) ppm.

5.4.2.3. Tert-butyl 2-(9-(3,5-bis(trifluoromethyl)phenylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (69b)

The title compound was synthesized by following the general procedure mentioned in **5.4.2.1**.

Reactants: Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), 3,5-bis(trifluoromethyl)benzene sulfonyl chloride (280 mg, 0.88 mmol), NaH (36 mg, 0.88 mmol).

Yield: 260 mg, 61%.

^{1}H NMR (300 MHz, CDCl₃): δ = 8.18 (d, *J* = 8.2 Hz, 1H; H-5), 8.07 (s, 2H; H-14, H-18), 7.82 (s, 1H; H-16), 7.74 (s, 1H; H-1), 7.64-7.65 (m, 2H; H-4, H-8), 7.38 (t, *J* = 7.7 Hz, 1H; H-7), 7.21 (t, *J* = 7.6 Hz, 1H; H-6), 6.96 (d, *J* = 8.6 Hz, 1H; H-3), 4.58 (s, 2H; H-21), 1.52 (s, 9H; H-23) ppm.

^{13}C NMR (75 MHz, CDCl₃): δ = 167.8 (C-22), 158.7 (C-2), 139.9 (C-13), 138.1 (C-12), 133.2 (C-15), 132.8 (C-17), 127.4 (C-14), 127.2 (C-18), 126.8 (C-16), 125.3 (C-9), 124.0 (C-19/20), 123.7 (C-11), 121.2 (C-6), 120.4 (C-4/7), 119.8 (C-5), 115.4 (C-3), 114.1 (C-8), 109.3 (C-10), 101.3 (C-1), 82.8 (C-23), 66.4 (C-21), 28.3 (C-24) pm.

5.4.2.4. Tert-butyl 2-(9-(biphenyl-4-ylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (69c)

The title compound was synthesized by following the general procedure mentioned in **5.4.2.1**.

Reactants: Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), 4-biphenyl sulfonyl chloride (222 mg, 0.88 mmol), NaH (36 mg, 0.88 mmol).

Yield: 205 mg, 55%.

¹H NMR (500 MHz, CDCl₃): δ = 8.21 (d, J = 8.3 Hz, 1H; H-5), 7.85 (d, J = 2.3 Hz, 1H; H-1), 7.76 (td, J = 8.6 Hz, J = 2.0 Hz, 2H; H-14), 7.72 (d, J = 8.6 Hz, 2H; H-18), 7.37 (td, J = 8.7 Hz, J = 2.0 Hz, 2H; H-15), 7.40-7.25 (m, 7H; H-4, H-6/7/8, H-19/20), 6.95 (dd, J = 8.6 Hz, J = 2.3 Hz, 1H; H-3), 4.60 (s, 2H; H-21), 1.46 (s, 9H; H-24) ppm.

¹³C NMR (125 MHz, CDCl₃): δ = 168.1 (C-22), 158.2 (C-2), 145.0 (C-16), 139.7 (C-12), 138.6 (C-13), 135.1 (C-17), 129.8 (C-19), 128.9 (C-15), 128.8 (C-14), 127.8 (C-20), 127.6 (C-18), 126.7 (C-9), 126.6, 124.4 (C-11), 122.1 (C-6), 120.7 (C-4/7), 119.4 (C-5), 115.3 (C-3), 113.1 (C-8), 105.1 (C-10), 100.9 (C-1), 82.8 (C-23), 66.4 (C-21), 28.3 (C-24) ppm.

5.4.2.5. Tert-butyl 2-(9-(2,4,6-triisopropylphenylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (69d)

The title compound was synthesized by following the general procedure mentioned in **5.4.2.1**

Reactants: Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), 2,4,6-triisppropylbenzene sulfonyl chloride (266 mg, 0.88 mmol), NaH (36 mg, 0.88 mmol).

Yield: 130 mg, 31%.

¹H NMR (500 MHz, CDCl₃): δ = 7.80-7.75 (m, 2H; H-4/5), 7.59 (m, 1H; H-8), 7.31 (d, J = 2.2, 1H; H-1), 7.23-7.18 (m, 2H; H-6/7), 7.06 (s, 2H; H-15, H-17), 6.90 (dd, J = 8.6 Hz, J = 2.2 Hz, 1H; H-3), 4.39 (s, 2H; H-29), 4.11- 4.06.(m, 2H; H-19, H-27), 2.85-2.76 (m, 1H; H-23), 1.42 (s, 9H; H-32), 1.15 (d, J = 6.9 Hz, 6H; H-24/25), 0.94 (d, J = 6.7 Hz, 12H; H-20/22, H-26/28) ppm.

¹³C NMR (125 MHz, CDCl₃): δ = 165.5 (C-30), 156.6 (C-16), 153.1 (C-2), 150.1 (C-14/18), 138.5 (C-12), 137.5 (C-13), 125.0 (C-9), 124.7 (C-11), 124.0 (C-15/17), 123.0 (C-6), 121.8 (C-7), 119.6 (C-4), 118.2 (C-5), 113.5 (C-3), 113.1 (C-8), 110.9 (C-10), 98.4 (C-1), 81.4 (C-

31), 65.2 (C-29), 33.1 (C-23), 28.4 (C-32), 27.0 (C-21/27), 23.2 (C-20/22/26/28), 22.4 (C-24/25) ppm.

5.4.2.6. Tert-butyl 2-(9-(4-propylphenylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (69e)
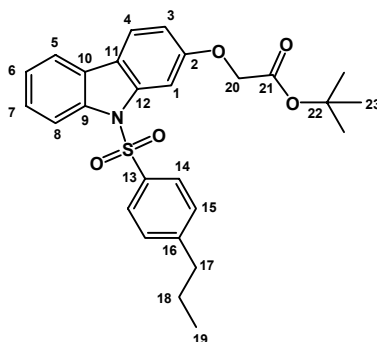
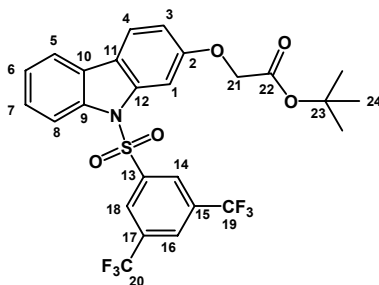The title compound was synthesized by following the general procedure mentioned in **5.4.2.1**.

Reactants: Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), 4-*n*-propylbenzene sulfonyl chloride (157 µL, 0.88 mmol), NaH (36 mg, 0.88 mmol).

Yield: 197 mg, 56%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 8.18 (d, *J* = 9.0 Hz, 1H; H-5), 7.81 (d, *J* = 3.0 Hz, 1H; H-1), 7.72-7.69 (m, 2H; H-4, H-8), 7.63 (d, *J* = 9.0 Hz, 2H; H-14), 7.36-7.24 (m, 2H; H-6/7), 7.03 (d, *J* = 9.0 Hz, 2H; H-15), 6.93 (dd, *J* = 9.0 Hz, *J* = 3.0 Hz, 1H; H-3), 4.59 (s, 2H; H-20), 2.42 (t, *J* = 9.0 Hz, 2H; H-17),1.53-1.40 (m, 11H; H-18, H-23), 0.77 (t, *J* = 7.5 Hz, 3H; H-19) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 167.9 (C-21), 158.1 (C-2), 149.4 (C-16), 139.5 (C-12), 135.1 (C-13), 129.0 (C-15), 128.5 (C-14), 126.4 (C-9), 124.2 (C-11), 122.9 (C-6), 120.7 (C-4/7), 119.2 (C-5), 115.1 (C-3), 113.9 (C-8), 109.7 (C-10), 100.7 (C-1), 82.6 (C-22), 66.2 (C-20), 37.8 (C-17), 28.1 (C-23), 23.8 (C-18), 13.7 (C-19) ppm.

5.4.2.7. Tert-butyl 2-(9-(octylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (69f)
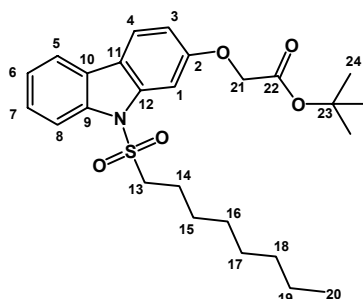
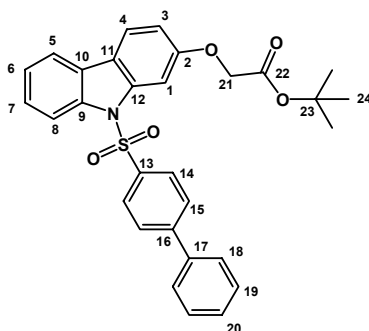The title compound was synthesized by following the general procedure mentioned in **5.4.2.1**.
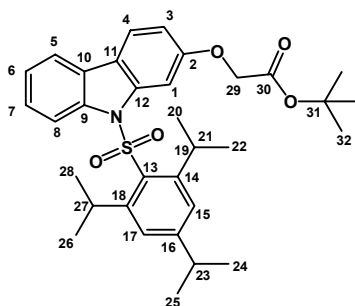
Reactants: Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), octane sulfonyl chloride (225 mg, 0.88 mmol), NaH (36 mg, 0.88 mmol).

Yield: 185 mg, 52%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 8.02 (dd, *J* = 9.0 Hz, *J* = 1.5 Hz, 1H; H-), 7.82-7.72 (m, 2H; H-4, H-8), 7.61 (d, *J* = 3.0 Hz, 1H; H-1), 7.34-7.29 (m, 2H; H-6/7), 6.97 (dd, *J* = 9.0 Hz, *J* = 3.0 Hz, 1H; H-1), 4.56 (s, 2H; H-21), 3.07 (t, *J* = 9.0 Hz, 2H; H-13), 1.56-1.52 (m, 2H; H-14), 1.45 (s, 9H; H-24), 1.21-1.02 (m, 10H; H-15/16/17/18/19), 0.75 (t, *J* = 9.0 Hz, 3H; H-20) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 167.7 (C-22), 158.0 (C-2), 139.7 (C-12), 126.3 (C-9), 125.8 (C-11), 123.9 (C-6), 120.8 (C-7), 119.9 (C-4), 119.4 (C-5), 114.4 (C-3), 113.7 (C-8), 110.0 (C-10), 94.5 (C-1), 82.5 (C-23), 66.0 (C-21), 52.5 (C-13), 31.5 (C-18), 28.9 (C-24), 28.8 (C-16), 28.0 (C-17), 27.9 (C-15), 22.7 (C-14), 22.4 (C-19), 13.9 (C-20) ppm.

5.4.2.8. **Tert-butyl** **2-(9-(((1S)-7,7-dimethyl-2-oxobicyclo[2.2.1]heptan-1-yl)methylsulfonyl)-9H-carbazol-2-yloxy)acetate (69g)**

The title compound was synthesized by following the general procedure mentioned in **5.4.2.1**.

Reactants: Tert-butyl 2-(9H-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), camphor sulfonyl chloride (220 mg, 0.88 mmol), NaH (36 mg, 0.88 mmol).

Yield: 220 mg, 58%.

¹H NMR (300 MHz, CDCl₃): δ = 8.17 (d, J = 9.0 Hz, 1H; H-5), 7.92 (dd, J = 9.0 Hz, J = 3.0 Hz, 1H; H-4), 7.79 (d, J = 9.0 Hz, 1H; H-8), 7.45-7.39 (m, 2H; H-6/7), 7.07 (dd, J = 7.5 Hz, J = 1.5 Hz, 1H; H-1), 4.67 (s, 2H; H-23), 3.53 (d, J = 15 Hz, 1H; H-13), 3.01 (d, J = 15 Hz, 1H; H-13′), 2.63-2.59 (m, 1H; H-16), 2.52-2.41 (m, 1H; H-16′), 2.17-2.10 (m, 2H; H-19), 1.94 (d, (d, J = 18 Hz, 1H; H-17), 1.88-1.70 (m, 2H; H-18), 1.50 (s, 9H; H-26), 1.17 (s, 3H; H-21), 0.82 (s, 3H; H-22) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 213.8 (C-15), 167.8 (C-24), 158.1 (C-2), 139.4 (C-12), 128.4 (C-9), 127.4 (C-11), 123.9 (C-6), 120.8 (C-7), 120.0 (C-4), 119.4 (C-5), 114.4 (C-3), 113.8 (C-8), 100.7 (C-10), 95.2 (C-1), 82.4 (C-25), 66.2 (C-23), 58.2 (C-14), 48.7 (C-13), 42.9 (C-16), 37.4 (C-20), 32.6 (C-17), 29.7 (C-18), 28.5 (C-26), 26.9 (C-19), 19.9 (C-21), 19.7 (C-22) ppm.

5.4.2.9. Tert-butyl 2-(9-(4-chlorophenylsulfonyl)-9H-carbazol-2-yloxy)acetate (69h)

The title compound was synthesized by following the general procedure mentioned in **5.4.2.1**.

Reactants: Tert-butyl 2-(9H-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), 4-chlorobenzene sulfonyl chloride (185 mg, 0.88 mmol), NaH (36 mg, 0.88 mmol).

**Yield**: 205 mg, 60%.

¹H NMR (300 MHz, CDCl₃): δ = 8.14 (d, J = 9.0 Hz, 1H; H-5), 7.78 (d, J = 3.0 Hz, 1H; H-1), 7.73-7.69 (m, 2H; H-4, H-8), 7.63 (dd, J = 7.5 Hz, J = 1.5 Hz, 2H; H-14), 7.36-7.26 (m,

2H; H-6/7), 7.21 (dd, *J* = 9.0 Hz, *J* = 1.5 Hz, 2H; H-15), 6.92 (dd, *J* = 9.0 Hz, *J* =3.0 Hz, 1H; H-3), 4.59 (s, 2H; H-17), 1.47 (s, 9H; H-20) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 167.8 (C-18), 158.1 (C-2), 140.5 (C-16), 139.7 (C-12), 138.4 (C-13), 130.9 (C-15), 129.3 (C-14), 125.8 (C-9), 124.6 (C-11), 122.4 (C-6), 120.4 (C-7), 120.1 (C-4), 119.4 (C-5), 115.1 (C-8), 113.8 (C-3), 107.8 (C-10), 100.9 (C-1), 82.7 (C-19), 66.1 (C-17), 28.1 (C-20) ppm.

5.4.2.10. Tert-butyl 2-(9-(3,5-difluorophenylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (69i)
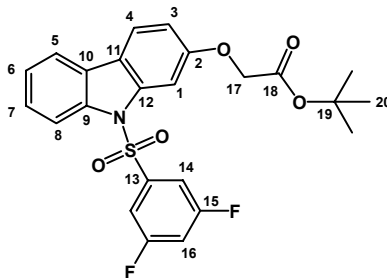
The title compound was synthesized by following the general procedure mentioned in **5.4.2.1**

Reactants: Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), 3,5-difluorobenzene sulfonyl chloride (187 mg, 0.88 mmol), NaH (36 mg, 0.88 mmol).

Yield: 190 mg, 55%.

¹H NMR (300 MHz, CDCl₃): δ = 8.12 (d, *J* = 9.0 Hz, 1H; H-5), 7.76-7-71 (m, 3H; H-1, H-4, H-8), 7.37-7.22 (m, 4H; H-6/7, H-14), 6.97 (dd, *J* = 9.0 Hz, *J* = 1.5 Hz, 1H; H-3), 6.85-6.80 (m, 1H; H-16), 4.59 (s, 2H; H-17), 1.48 (s, 9H; H-20) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 167.7 (C-18), 164.2 (C-15), 158.2 (C-2), 140.4 (C-13), 139.2 (C-12), 126.6 (C-9), 124.7 (C-11), 122.7 (C-6), 120.9 (C-7), 120.7 (C-4), 119.5 (C-5), 115.0 (C-3), 113.4 (C-8), 109.9 (C-10), 100.8 (C-1), 82.7 (C-19), 66.2 (C-17), 28.1 (C-20) ppm.

5.4.2.11. Tert-butyl 2-(9-(2-bromophenylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (69j)
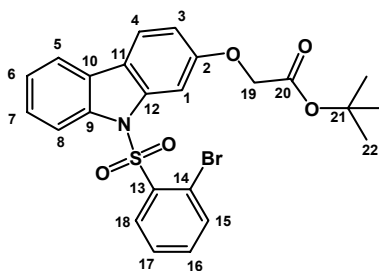
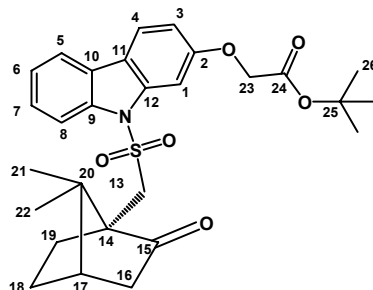The title compound was synthesized by following the general procedure mentioned in

Reactants: Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), 2-bromobenzene sulfonyl chloride (225 mg, 0.88 mmol), NaH (36 mg, 0.88 mmol).

Yield: 205 mg, 55%.

¹H NMR (300 MHz, CDCl₃): δ = 7.83-7.77 (m, 4H; H-5, H-1, H-16, H-18), 7.56-7.53 (m, 2H; H-4, H-17), 7.36-7.23 (m, 4H; H-6/7/8, H-16), 6.95 (dd, *J* = 7.5 Hz, 1.5 Hz, 1H; H-3), 4.50 (s, 2H; H-19), 1.49 (s, 9H; H-22) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 167.8 (C-20), 157.1 (C-2), 140.1 (C-13), 138.7 (C-12), 135.9 (C-16), 134.3 (C-14), 131.2 (C-18), 127.6 (C-17), 125.9 (C-9), 125.5 (C-11), 123.7 (C-

6), 120.9 (C-15), 120.8 (C-7), 119.5 (C-4), 119.4 (C-5), 114.7 (C-3), 113.7 (C-8), 100.7 (C-10), 96.5 (C-1), 82.5 (C-21), 66.1 (C-19), 28.1 (C-22) ppm.

5.4.2.6. Tert-butyl 2-(9-(3-nitrophenylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (69k)
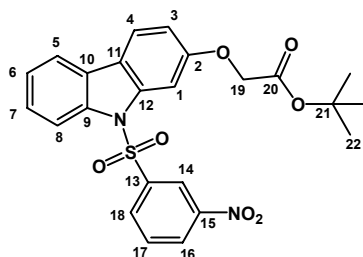
The title compound was synthesized by following the general procedure mentioned in

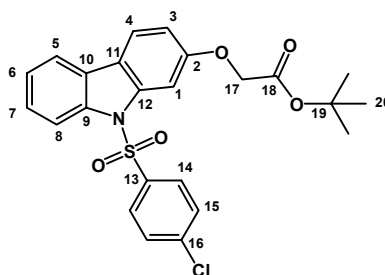**Reactants:** Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), 3-nitrobenzene sulfonyl chloride (195 mg, 0.88 mmol), NaH (36 mg, 0.88 mmol).

Yield: 215 mg, 60%.

¹H NMR (300 MHz, CDCl₃): δ = 8.70 (s, 1H; H-14), 8.43-8.25 (m, 2H; H-16, H-18), 8.04 (d, *J* = 9.0 Hz, 1H; H-5), 7.87 (d, *J* = 3.0 Hz, 1H; H-1), 7.81-7.76 (m, 2H; H-4, H-8), 7.55-7.34 (m, 3H; H-6/7, H-17), 7.04 (dd, *J* = 9.0 Hz, *J* = 3.0 Hz, 1H; H-3), 4.68 (s, 2H; H-19), 1.62 (s, 9H; H-22) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 167.8 (C-20), 158.3 (C-2), 147.8 (C-15), 139.1 (C-13), 137.9 (C-12), 135.5 (C-18), 131.7 (C-17), 128.5 (C-16), 125.2 (C-9), 124.7 (C-11), 122.8 (C-6), 121.8 (C-14), 120.8 (C-4/7), 119.5 (C-5), 115.1 (C-3), 113.6 (C-8), 108.8 (C-10), 100.9 (C-1), 82.7 (C-21), 66.1 (C-19), 28.1 (C-22) ppm.

5.4.3. Synthesis of *N*-sulfonylated 2-(9*H*-carbazol-2-yloxy)acetic acids (70)

5.4.3.1. General procedure for *t*-butyl ester cleavage of *N*-substituted tert-butyl 2-(9*H*-carbazol-2-yloxy)acetates

A solution of *t*-butyl ester in 20% trifluoroacetic acid in CH₂Cl₂ (5 mL/mmol) was stirred at ambient temperature for 4 to 12 h. After completion of reaction (TLC), solvent was evaporated *in vacuo* to get crude product. The crude acid was purified by crystallization (EtOAc:hexane) to afford the desired product.

5.4.3.2. 2-(9-Tosyl-9*H*-carbazol-2-yloxy)acetic acid (70a)
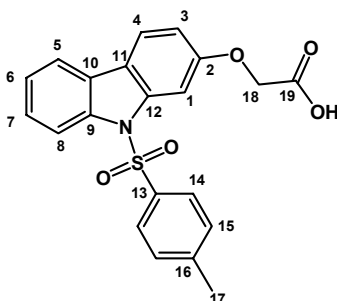
The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-Tosyl-9*H*-carbazol-2-yloxy)acetate (100 mg, 0.22 mmol), 20% trifluoroacetic acid in CH₂Cl₂ (5 mL).

Yield: 78 mg, 90%.

¹H NMR (300 MHz, CDCl₃): δ = 8.15 (d, J = 8.2 Hz, 1H; H-5), 7.80 (d, J = 7.0 Hz, 1H; H-4), 7.73-7.68 (m, 2H; H-6/7), 7.58 (d, J = 8.5 Hz, 2H; H-14), 7.32 (dt, J = 7.8, 1.2 Hz, 1H; H-8), 7.26 (d, J = 8.5 Hz, 2H; H-15), 7.14 (d, J = 1.3 Hz, 1H; H-1), 6.93 (dd, J = 8.6 Hz, 1.3 Hz, 1H; H-3), 4.62 (s, 2H; H-18) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 172.6 (C-19), 159.4 (C-2), 146.7 (C-16), 139.8 (C-12), 135.8 (C-13), 131.0 (C-15), 127.8 (C-14), 127.6 (C-9), 125.5 (C-11), 122.1 (C-6), 120.7 (C-7), 119.8 (C-4), 116.3 (C-5), 113.9 (C-3), 111.2 (C-8), 102.3 (C-10), 97.2 (C-1), 66.8 (C-18), 22.3 (C-17) ppm.

MS (ESI): m/z = 418.1 (M+Na)⁺.

5.4.3.3. 2-(9-(3,5-Bis(trifluoromethyl)phenylsulfonyl)-9*H*-carbazol-2-yloxy)acetic acid (70b)

The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-(3,5-bis(trifluoromethyl)phenylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (100 mg, 0.174 mmol), 20% trifluoroacetic acid in CH₂Cl₂ (5 mL).

Yield: 85 mg, 95%.

¹H NMR (500 MHz, CDCl₃): δ = 8.99 (s, 2H; H-14), 8.95 (s, 1H; H-16), 8.75 (d, J = 6.7 Hz, 1H; H-5), 8.63 (d, J = 8.4 Hz, 1H; H-4), 8.5 (m, 2H; H-6/7), 8.23 (d, J = 7.6 Hz, 1H; H-8), 8.15 (d, J = 1.4 Hz, 1H; H-1), 7.82 (dd, J = 8.8 Hz, J = 1.4 Hz, 1H; H-3), 4.68 (s, 2H; H-19) ppm.

¹³C NMR (125 MHz, CDCl₃): δ = 171.8 (C-20), 158.7 (C-2), 139.9 (C-13), 139.2 (C-12), 135.4 (C-15), 131.0 (C-16), 127.4 (C-14), 127.2 (C-17/18), 126.8 (C-9), 125.3 (C-11), 124.0 (C-6), 121.2 (C-7), 120.4 (C-4), 119.8 (C-5), 115.4 (C-3), 114.1 (C-8), 105.8 (C-10), 96.8 (C-1), 72.4 (C-19) ppm.

5.4.3.4. 2-(9-(Biphenyl-4-ylsulfonyl)-9*H*-carbazol-2-yloxy)acetic acid (70c)

The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-(biphenyl-4-ylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (100 mg, 0.194 mmol), 20% trifluoroacetic acid in CH₂Cl₂ (5 mL).

Yield: 82 mg, 93%.

¹H NMR (500 MHz, CDCl₃): δ = 8.20 (d, J = 8.4 Hz, 1H; H-5), 7.82-7.82 (m, 5H; H-4, H-14/15), 7.52 (dd, J = 6.8 Hz, J = 1.8 Hz, 2H; H-18), 7.41-7.37(m, 3H; H-6/7/8), 7.32-7.26 (m, 4H; H-1, H-19/20), 6.96 (dd, J = 8.6 Hz, J = 1.2 Hz, 1H; H-3), 4.73 (s, 2H; H-21) ppm.

¹³C NMR (125 MHz, CDCl₃): δ = 172 (C-22), 158.2 (C-2), 145.0 (C-16), 139.7 (C-12), 138.6 (C-13), 135.1 (C-17), 129.8 (C-19), 129.1 (C-15), 128.8 (C-14), 128.1 (C-18), 127.9 (C-20), 126.7 (C-9), 126.4 (C-11), 124.1 (C-6), 120.7 (C-7), 119.4 (C-4), 118.2 (C-5), 115.3 (C-3), 113.1 (C-8), 107.2 (C-10), 95.7 (C-1), 60.3 (C-21) ppm.

MS (ESI): m/z = 480.1 (M+Na)⁺.

5.4.3.5. 2-(9-(2,4,6-Triisopropylphenylsulfonyl)-9*H*-carbazol-2-yloxy)acetic acid (70d)

The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-(2,4,6-triisopropylphenylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (100 mg, 0.177 mmol), 20% trifluoroacetic acid in CH₂Cl₂ (5 mL).

Yield: 86 mg, 95%.

¹H NMR (300 MHz, CDCl₃): δ = 7.80-7.75 (m, 2H; H-15, H-17), 7.62-7.57 (m, 1H; H-5), 7.23-7.18 (m, 2H; H-4, H-8), 7.15-7.09 (m, 2H; H-6/7), 6.92-6.87 (m, 2H; H-1, H-3), 4.45 (s, 2H; H-29), 4.05- 3.95.(m, 2H; H-21, H-27), 2.86-2.76 (m, 1H; H-23), 1.15 (d, J = 6.9 Hz, 6H; H-24/25), 0.93 (d, J = 6.7 Hz, 12H; H-20/21, H-26/28) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 169.5 (C-30), 159.1 (C-2), 156.0 (C-16), 152.6 (C-14/18), 139.9 (C-12), 130.2 (C-13), 127.2 (C-9), 126.4 (C-11), 125.5 (C-15/17), 122.4 (C-6), 122.1 (C-7), 120.7 (C-4), 117.5 (C-5), 114.7 (C-3), 113.2 (C-8), 100.7 (C-10), 93.2 (C-1), 67.0 (C-29), 35.8 (C-23), 30.9 (C-21/27), 25.2 (C-20/22/26/28), 24.5 (C-24/25) ppm.

MS (ESI): m/z = 430.2 (M+Na)⁺.

5.4.3.6. 2-(9-(4-Propylphenylsulfonyl)-9*H*-carbazol-2-yloxy)acetic acid (70e)
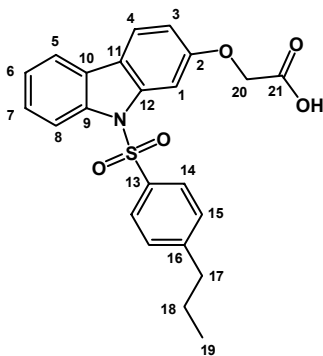
The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-(biphenyl-4-ylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (100 mg, 0.2 mmol), 20% trifluoroacetic acid in CH_2Cl_2 (5 mL).

Yield: 85 mg, 97%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 8.17 (d, *J* = 8.0 Hz, 1H; H-5), 7.81 (d, *J* = 8.4 Hz, 1H; H-4), 7.69 (d, *J* = 8.4 Hz, 2H; H-14), 7.62 (d, *J* = 8.4 Hz, 1H; H-8), 7.34-7.23 (m, 2H; H-6/7), 7.12 (d, *J* = 2.4 Hz, 1H; H-1), 7.04 (d, *J* = 8.7 Hz, 2H; H-15), 6.93 (dd, *J* = 8.7 Hz, 2.4 Hz, 1H; H-1), 4.66 (s, 2H; H-20), 2.41 (t, *J* = 7.8 Hz, 2H; H-17),1.51-1.34 (m, 2H; H-18), 0.76 (t, *J* = 7.5 Hz, 3H; H-19) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 175.2 (C-21), 153.5 (C-2), 143.3 (C-16), 138.8 (C-12), 135.8 (C-13), 129.9 (C-15), 128.9 (C-14), 125.8 (C-9), 124.5 (C-11), 123.1 (C-6), 120.1 (C-7), 119.7 (C-4), 118.8 (C-5), 113.4 (C-3), 111.4 (C-8), 104.8 (C-10), 95.8 (C-1), 69.7 (C-20), 41.6 (C-17), 27.6 (C-18), 17.4 (C-19) ppm

MS (ESI): *m/z* = 446.2 (M+Na$^+$).

5.4.3.7. 2-(9-(Octylsulfonyl)-9*H*-carbazol-2-yloxy)acetic acid (70f)
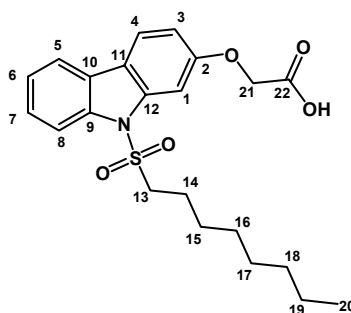
The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

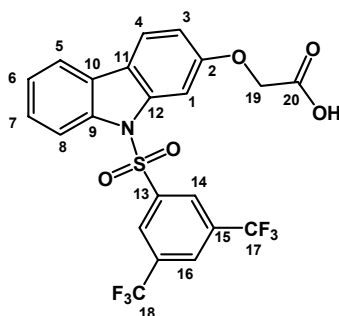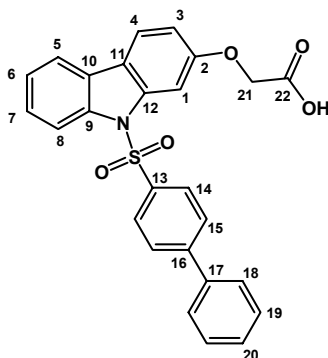**Reactants:** Tert-butyl 2-(9-(octylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (100 mg, 0.21 mmol), 20% trifluoroacetic acid in CH_2Cl_2 (5 mL).

Yield: 82 mg, 90%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 8.01 (dd, *J* = 9.0 Hz, 1.5 Hz, 1H; H-5), 7.51-7.22 (m, 4H; H-4, H-6/7/8), 6.95 (d, *J* = 1.5 Hz, 1H; H-1), 6.86 (dd, *J* = 7.5 Hz, *J* = 1.5 Hz, 1H; H-3), 4.82 (s, 2H), 4.23 (t, *J* = 7.5 Hz, 2H; H-13), 1.91-1.82 (m, 2H; H-14), 1.40-1.21 (m, 10H; H-15/16/17/18/19), 0.87 (t, *J* = 6.0 Hz, 3H; H-20) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 172.8 (C-22), 156.8 (C-2), 140.8 (C-12), 129.0 (C-9), 124.8 (C-11), 122.7 (C-7), 121.2 (C-4), 119.9 (C-5), 113.7 (C-3), 113.1 (C-8), 106.6 (C-10), 95.0 (C-1), 65.5 (C-21), 43.1 (C-13), 31.7 (C-18), 29.3 (C-16), 29.1 (C-17), 28.8 (C-15), 24.5 (C-19), 22.6 (C-14), 14.0 (C-20) ppm.

MS (ESI): *m/z* = 440.2 (M+Na)$^+$.

5.4.3.8. 2-(9-(((1*S*)-7,7-Dimethyl-2-oxobicyclo[2.2.1]heptan-1-yl)methylsulfonyl)-9*H*-carbazol-2-yloxy)acetic acid (70g)
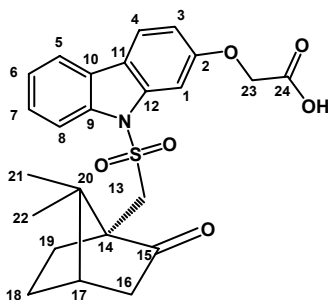
The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**

Reactants: Tert-butyl 2-(9-(((1*S*)-7,7-dimethyl-2-oxobicyclo[2.2.1]heptan-1-yl)methylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (100 mg, 0.19 mmol), 20% trifluoroacetic acid in CH_2Cl_2 (5 mL).

Yield: 79 mg, 89%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 8.07 (d, *J* = 7.5 Hz, 1H; H-5), 7.83-7-77 (m, 2H; H-4, H-8), 7.73 (d, *J* = 2.1 Hz, 1H; H-1), 7.39-7.30 (m, 2H; H-6/7), 7.01 (dd, *J* = 8.5 Hz, 2.2 Hz, 1H; H-3), 4.67 (s, 2H; H-23), 3.42 (d, *J* = 15 Hz, 1H; H-13), 2.91 (d, *J* = 15 Hz, 1H; H-13′), 2.52-2.41 (m, 1H; H-16), 2.33-2.22 (m, 1H; H-16′), 2.09-1.95 (m, 2H; H-17/19), 1.87 (m, 1H; H-19′), 1.78-1.66 (m, 1H; H-18), 1.48-1.33 (m, 1H; H-18′), 1.07 (s, 3H; H-21), 0.72 (s, 3H; H-22) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 214.8 (C-15), 175.3 (C-24), 161.9 (C-2), 142.3 (C-12), 128.3 (C-9), 127.9 (C-11), 124.8 (C-6), 121.0 (C-7), 120.3 (C-4), 118.2 (C-5), 116.6 (C-3), 113.2 (C-8), 104.1 (C-10), 95.5 (C-1), 69.7 (C-23), 62.2 (C-14), 46.7 (C-13), 42.3 (C-16), 37.5 (C-20), 33.7 (C-17), 29.3 (C-18), 27.5 (C-19), 23.6 (C-21), 23.5 (C-22) ppm.

MS (ESI): *m/z* = 478.2 (M+Na)$^+$.

5.4.3.9. 2-(9-(4-Chlorophenylsulfonyl)-9*H*-carbazol-2-yloxy)acetic acid (70h)
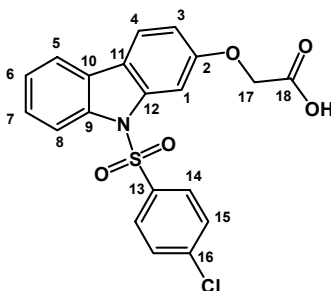
The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-(4-chlorophenylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (100 mg, 0.21 mmol), 20% trifluoroacetic acid in CH_2Cl_2 (5 mL).
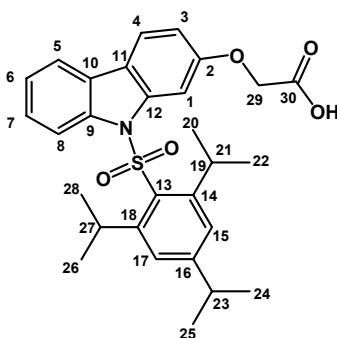
Yield: 86 mg, 97%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 8.15 (d, *J* = 7.8 Hz, 1H; H-5), 7.78 (d, *J* = 8.1 Hz, 1H; H-4), 7.72-7.69 (m, 2H; H-14), 7.65-7.62 (m, 2H; H-15), 7.29-7.20 (m, 4H; H-1, H-6/7/8), 6.95 (dd, *J* = 8.1 Hz, 2.3 Hz, 1H; H-3), 4.69 (s, 2H; H-17) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 174.8 (C-18), 161.8 (C-2), 144.8 (C-16), 141.1 (C-12), 137.6 (C-13), 131.9 (C-15), 131.1 (C-14), 126.3 (C-9), 125.3 (C-11), 122.7 (C-6), 120.3 (C-7), 118.9 (C-4), 116.8 (C-3), 113.1 (C-8), 104.9 (C-10), 97.3 (C-1), 64.5 (C-17) ppm.

5.4.3.10. 2-(9-(3,5-Difluorophenylsulfonyl)-9*H*-carbazol-2-yloxy)acetic acid (70i)
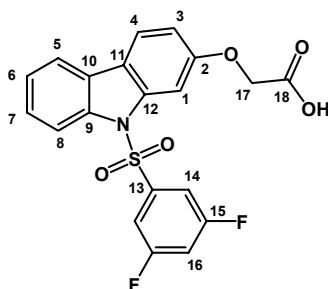
The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-(3,5-difluorophenylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (100 mg, 0.21 mmol), 20% trifluoroacetic acid in CH_2Cl_2 (5 mL).

Yield: 79 mg, 90%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 8.12 (d, *J* = 8.1 Hz, 1H; H-5), 7.77-7-72 (m, 3H; H-4, H-14), 7.37-7.27 (m, 5H; H-1, H-6/7/8), 6.97 (dd, *J* = 8.6 Hz, 2.2 Hz, 1H; H-3), 6.91-6.81 (m, 1H; H-16), 4.68 (s, 2H; H-17) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 174.7 (C-18), 164.7 (C-16), 161.9 (C-2), 144.1 (C-13), 141.9 (C-12), 128.6 (C-9), 124.8 (C-11), 123.4 (C-6), 120.1 (C-7), 119.5 (C-4), 118.8 (C-5), 116.9 (C-3), 114.2 (C-8), 105.9 (C-10), 94.4 (C-1), 69.5 (C-17) ppm.

5.4.3.11. 2-(9-(2-Bromophenylsulfonyl)-9*H*-carbazol-2-yloxy)acetic acid (70j)

The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-(2-bromophenylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (100 mg, 0.19 mmol), 20% trifluoroacetic acid in CH_2Cl_2 (5 mL).

Yield: 71 mg, 80%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.97 (d, *J* = 8.1 Hz, 1H; H-18), 7.80-7.51 (m, 3H; H-4, H-5, H-15), 7.58-7.50 (m, 2H; H-8, H-17), 7.38-7.23 (m, 4H; H1, H-6, H-7, H-16), 6.95 (dd, *J* = 8.5 Hz, *J* = 2.2 Hz, 1H; H-3), 4.59 (s, 2H; H-19) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 174.7 (C-20), 161.4 (C-2), 143.7 (C-13), 139.8 (C-12), 135.2 (C-16), 131.5 (C-15), 129.9 (C-18), 128.7 (C-17), 127.6 (C-9), 124.8 (C-15), 123.6 (C-6), 120.3 (C-7), 119.6 (C-4), 118.6 (C-5), 116.3 (C-3), 111.5 (C-8), 104.7 (C-10), 97.2 (C-1), 69.6 (C-19) ppm.

5.4.3.6. 2-(9-(3-Nitrophenylsulfonyl)-9*H*-carbazol-2-yloxy)acetic acid (70k)

The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-(3-nitrophenylsulfonyl)-9*H*-carbazol-2-yloxy)acetate (100 mg, 0.2 mmol), 20% trifluoroacetic acid in CH_2Cl_2 (5 mL).

Yield: 81 mg, 92%.

**^{1}H NMR (300 MHz, CDCl$_3$):** δ = 8.55 (d, J = 1.9 Hz, 1H; H-14), 8.19-8.12 (m, 2H; H-5, H-18), 8.01-7.86 (m, 1H; H-16), 7.81 (d, J = 2.28 Hz, 1H; H-1), 7.73-7.70 (m, 2H; H-4, H-17), 7.50 (t, J = 8.0 Hz, 1H; H-8), 7.39-7.25 (m 2H; H-6/7), 6.97 (dd, J = 8.6 Hz, 2.3 Hz, 1H; H-3), 4.71 (s, 2H; H-19) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 174.7 (C-20), 162.0 (C-2), 151.8 (C-15), 142.9 (C-13), 141.8 (C-12), 135.7 (C-18), 132.1 (C-17), 128.6 (C-16), 127.9 (C-16), 127.5 (C-9), 124.8 (C-11), 122.1 (C-6), 121.2 (C-14), 120.1 (C-7), 119.5 (C-4), 118.1 (C-5), 115.1 (C-3), 113.6 (C-8), 104.5 (C-10), 99.3 (C-1), 69.5 (C-19) ppm.

MS (ESI): m/z = 446.2 (M+Na$^+$).

5.4.4. Synthesis of *N*-alkylated tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate derivatives (71)

5.4.4.1. General procedure for *N*-alkylation of tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate derivatives

KOBu^t (1.1 eq.) was added to a stirred solution of THF (5 mL/mmol) tert-butyl 2-(9*H*-carbazol-6-yloxy)acetate (**68**) (1 eq.) at 0 °C. After 30 minutes alkyl halide (1.2 eq.) dissolved in THF was added. The reaction mixture was stirred at ambient temperature for 6–24 h. The heterogeneous reaction mixture was quenched with NH$_4$Cl (sat. aq), diluted with EtOAc and then washed sequentially with H$_2$O and brine. The organic extract was dried over anhydrous Na$_2$SO$_4$ and evaporated *in vacuo* to give crude compound. The crude compound was purified by flash column chromatography (EtOAc:hexane, 1:9).

5.4.4.2. Tert-butyl 2-(9-octyl-9*H*-carbazol-2-yloxy)acetate (71a)
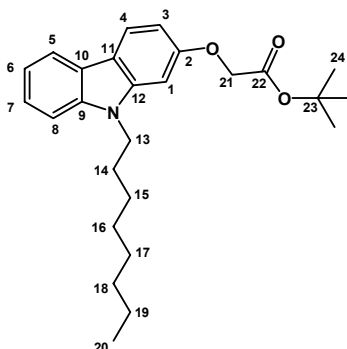
The title compound was synthesized by following the general procedure mentioned in **5.4.4.1**.

Reactants: Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), 1-iodooctane (160 µL, 0.88 mmol), KOBu^t (98 mg, 0.88 mmol).

Yield: 260 mg, 90%.

¹H NMR (300 MHz, CDCl₃): δ = 7.93 (d, *J* = 9.0 Hz, 1H; H-5), 7.88 (d, *J* = 9.0 Hz, 1H; H-4), 7.32-7.28 (m, 2H; H-6/7), 7.14-7.10 (m, 1H; H-8), 6.82 (d, *J* = 3.0 Hz, 1H; H-1), 6.75 (dd, *J* = 9.0 Hz, *J* = 1.5 Hz, 1H; H-3), 4.56 (s, 2H; H-21), 4.14 (t, *J* = 7.5 Hz, 2H; H-13), 1.81-1.72 (m, 2H; H-14), 1.43 (s, 9H; H-24), 1.32-1.12 (m, 10 H; H-15/16/17/18/19), 0.79 (t, *J* = 7.5 Hz, 3H; H-20) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 167.2 (C-22), 156.2 (C-2), 139.7 (C-12), 126.9 (C-9), 125.5 (C-11), 123.5 (C-6), 121.8 (C-7), 120.2 (C-4) 118.6 (C-5), 117.9 (C-3), 116.6 (C-8), 107.4 (C-10), 93.7 (C-1), 81.2 (C-23), 65.4 (C-21), 52.1 (C-13), 30.8 (C-18), 28.4 (C-24), 28.1 (C-14), 27.8 (C-16), 27.0 (C-17), 26.3 (C-15), 21.5 (C-19), 13.0 (C-20) ppm.

5.4.4.3. Tert-butyl 2-(9-nonyl-9*H*-carbazol-2-yloxy)acetate (71b)

The title compound was synthesized by following the general procedure mentioned in **5.4.4.1**.

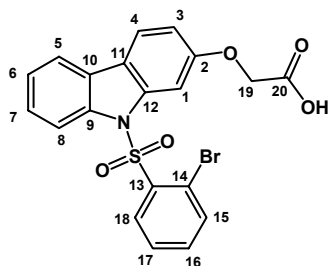**Reactants:** Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), 1-bromononane (168 µL, 0.88 mmol), KOBuᵗ (98 mg, 0.88 mmol).

Yield: 248 mg, 80%.

¹H NMR (300 MHz, CDCl₃): δ = 7.93 (d, *J* = 9.0 Hz, 1H; H-5), 7.89 (d, *J* = 9.0 Hz, 1H; H-4), 7.32-7.28 (m, 2H; H-6/7), 7.14-7.10 (m, 1H; H-8), 6.83 (d, *J* = 3.0 Hz, 1H; H-1), 6.75 (dd, *J* = 9.0 Hz, *J* = 1.5 Hz, 1H, H-3), 4.56 (s, 2H; H-21), 4.14 (t, *J* = 7.5 Hz, 2H; H-13), 1.82-1.71 (m, 2H; H-14), 1.43 (s, 9H; H-25), 1.36-1.13 (m, 12H; H-15/16/17/18/19/20), 0.80 (t, *J* = 7.5 Hz, 3H; H-21) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 168.2 (C-23), 157.2 (C-2), 141.6 (C-12), 127.5 (C-9), 125.8 (C-11), 124.5 (C-6), 122.8 (C-7), 121.0 (C-4), 119.6 (C-5), 118.9 (C-3), 117.6 (C-8), 108.4 (C-10), 94.7 (C-1), 82.2 (C-24), 66.4 (C-22), 53.1 (C-13), 31.8 (C-19), 29.4 (C-24), 29.2 (C-17), 28.8 (C-25), 27.3 (C-16/18), 26.8 (C-15), 22.6 (C-20), 14.0 (C-21) ppm.

5.4.4.4. Tert-butyl 2-(9-decyl-9*H*-carbazol-2-yloxy)acetate (71c)

The title compound was synthesized by following the general procedure mentioned in **5.4.4.1**

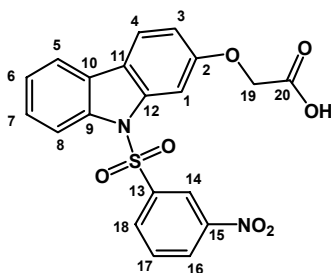**Reactants:** Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), 1-bromodecane (183 µL, 0.88 mmol), KOBu^t (98 mg, 0.88 mmol).

Yield: 272 mg, 85%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.93 (d, J = 9.0 Hz, 1H; H-5), 7.89 (d, J = 9.0 Hz, 1H; H-4), 7.31-7.28 (m, 2H; H-6/7), 7.14-7.10 (m, 1H; H-8), 6.83 (d, J = 1.5 Hz, 1H; H-1), 6.75 (dd, J = 9.0 Hz, J = 1.5 Hz, 1H; H-3), 4.56 (s, 2H; H-21), 4.14 (t, J = 7.5 Hz, 2H; H-13), 1.82-1.71 (m, 2H; H-14), 1.43 (s, 9H; H-26), 1.37-1.12 (m, 14H; H-15/16/17/18/19/20/21), 0.80 (t, J = 6.0 Hz, 3H; H-22) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 168.2 (C-23), 157.2 (C-2), 141.6 (C-12), 127.8 (C-9), 125.7 (C-11), 124.5 (C-6), 122.9 (C-7), 121.3 (C-4), 119.4 (C-5), 118.6 (C-3), 117.4 (C-8), 108.1 (C-10), 94.7 (C-1), 82.2 (C-25), 66.4 (C-23), 53.1 (C-13), 31.8 (C-10), 29.5 (C-14), 29.4 (C-17), 29.2 (C-18), 28.8 (C-26), 28.0 (C-16/19), 27.3 (C-15), 22.6 (C-21), 14.0 (C-22) ppm.

5.4.4.5. Tert-butyl 2-(9-undecyl-9*H*-carbazol-2-yloxy)acetate (71d)

The title compound was synthesized by following the general procedure mentioned in **5.4.4.1**.

Reactants: Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), 1-bromoundecane (196 µL, 0.88 mmol), KOBu^t (98 mg, 0.88 mmol).

Yield: 210 mg, 65%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.91 (d, J = 7.7 Hz, 1H; H-5), 7.88 (d, J = 8.5 Hz, 1H; H-4), 7.33-7.27 (m, 2H; H-6/7), 7.14-7.09 (m, 1H; H-8), 6.82 (d, J = 2.1 Hz, 1H; H-1), 6.75 (dd, J = 8.6 Hz, J = 2.1 Hz, 1H; H-3), 4.56 (s, 2H; H-21), 4.13 (t, J = 7.5 Hz, 2H; H-13), 1.82-1.72 (m, 2H; H-14), 1.43 (s, 9H; H-27), 1.34-1.11 (m, 16H; H-15/16/17/18/19/20/21/22), 0.80 (t, J = 6.0 Hz, 3H; H-23) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 168.2 (C-25), 157.2 (C-2), 141.6 (C-12), 127.7 (C-9), 125.7 (C-11), 124.5 (C-6), 122.8 (C-7), 121.0 (C-4), 119.6 (C-5), 118.8 (C-3), 117.6 (C-8), 108.4 (C-10), 94.7 (C-1), 82.2 (C-26), 66.4 (C-24), 53.1 (C-13), 31.8 (C-21), 29.5 (C-14), 29.4 (C-17/19), 29.2 (C-18), 28.8 (C-16), 28.3 (C-27), 28.0 (C-20), 27.3 (C-15), 22.6 (C-22), 14.0 (C-23) ppm.

5.4.4.6. Tert-butyl 2-(9-dodecyl-9*H*-carbazol-2-yloxy)acetate (71e)

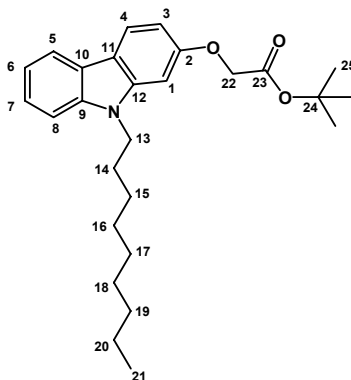The title compound was synthesized by following the general procedure mentioned in **5.4.4.1**.

Reactants: Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), 1-bromododecane (211 μL, 0.88 mmol), KOBu^t (98 mg, 0.88 mmol).

Yield: 207 mg, 61%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.92 (d, *J* = 7.8 Hz, 1H; H-5), 7.89 (d, *J* = 8.6 Hz, 1H; H-4), 7.32-7.26 (m, 2H; H-6/7), 7.14-7.09 (m, 1H; H-8), 6.83 (d, *J* = 2.0 Hz, 1H; H-1), 6.76 (dd, *J* = 8.4 Hz, *J* = 2.2 Hz, 1H; H-3), 4.56 (s, 2H; H-21), 4.13 (t, *J* = 7.5 Hz, 2H; H-13), 1.81-1.71 (m, 2H; H-14), 1.43 (s, 9H; H-28), 1.41-1.12 (m, 18H; H-15/16/17/18/19/20/21/22/23), 0.80 (t, *J* = 6.0 Hz, 3H; H-24) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 168.2 (C-26), 157.2 (C-2), 141.6 (C-12), 127.7 (C-9), 125.7 (C-11), 124.5 (C-6), 122.8 (C-7), 121.0 (C-4), 119.6 (C-5), 118.8 (C-3), 117.6 (C-8), 108.4 (C-10), 94.7 (C-1), 82.2 (C-27), 66.4 (C-25), 53.1 (C-13), 31.9 (C-22), 29.5 (C-14), 29.4 (C-17/19), 29.3 (C-18/20), 28.8 (C-16), 28.3 (C-28), 28.0 (C-21), 27.3 (C-15), 22.6 (C-23), 14.1 (C-24) ppm.

5.4.4.7. Tert-butyl 2-(9-tetradecyl-9*H*-carbazol-2-yloxy)acetate (71f)

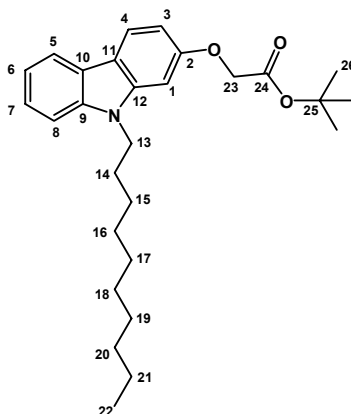The title compound was synthesized by following the general procedure mentioned in **5.4.4.1**.

Reactants: Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), 1-bromotetradecane (239 μL, 0.88 mmol), KOBu^t (98 mg, 0.88 mmol).

Yield: 227 mg, 63%.

¹H NMR (300 MHz, CDCl₃): δ = 7.91 (d, J = 7.8 Hz, 1H; H-5), 7.88 (d, J = 8.5 Hz, 1H; H-4), 7.34-7.26 (m, 2H; H-6/7), 7.13-7.08 (m, 1H; H-8), 6.82 (d, J = 2.2 Hz, 1H; H-1), 6.74 (dd, J = 8.5 Hz, J = 2.2 Hz, 1H; H-3), 4.56 (s, 2H; H-21), 4.13 (t, J = 7.5 Hz, 2H; H-13), 1.81-1.71 (m, 2H; H-14), 1.43 (s, 9H; H-30), 1.41-1.12 (m, 22H; H-15/16/17/18/19/20/21/22/23/24/25), 0.80 (t, J = 6.0 Hz, 3H; H-26) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 168.2 (C-28), 157.2 (C-2), 141.6 (C-12), 127.7 (C-9), 125.7 (C-11), 124.5 (C-6), 122.8 (C-7), 121.0 (C-4), 119.6 (C-5), 118.8 (C-3), 117.6 (C-8), 108.4 (C-10), 94.7 (C-1), 82.2 (C-29), 66.4 (C-27), 53.1 (C-13), 31.9 (C-24), 29.7 (C-14), 29.6 (C-23), 29.5 (C-22), 29.4 (C-17/19), 29.3 (C-18/20), 28.8 (C-16), 28.5 (C-30), 28.0 (C-21), 27.3 (C-15), 22.6 (C-25), 14.1 (C-26) ppm.

5.4.4.8. Tert-butyl 2-(9-hexadecyl-9*H*-carbazol-2-yloxy)acetate (71g)

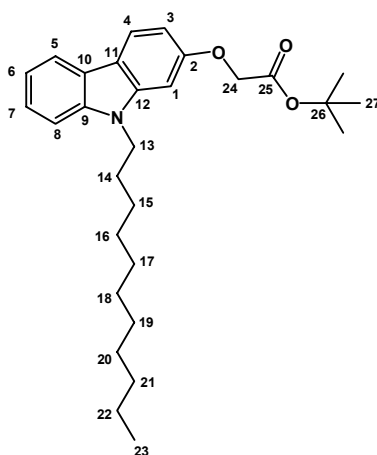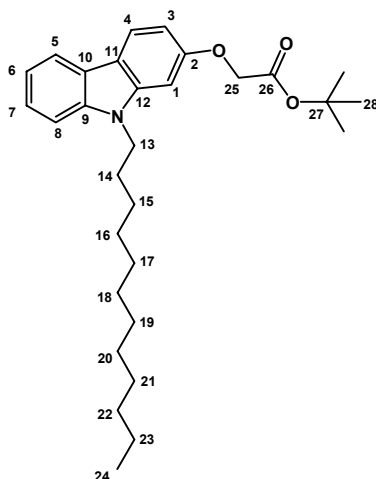The title compound was synthesized by following the general procedure mentioned in **5.4.4.1**.

Reactants: Tert-butyl 2-(9*H*-carbazol-2-yloxy)acetate (**68**) (200 mg, 0.74 mmol), (200 mg, 0.73 mmol), 1-bromohexadecane (268 µL, 0.88 mmol), KOBu^t (98 mg, 0.88 mmol).

Yield: 204 mg, 53%.

¹H NMR (300 MHz, CDCl₃): δ = 7.91 (d, J = 7.6 Hz, 1H; H-5), 7.88 (d, J = 8.4 Hz, 1H; H-4), 7.31-7.27 (m, 2H; H-6/7), 7.14-7.09 (m, 1H; H-8), 6.82 (d, J = 2.0 Hz, 1H; H-1), 6.75 (dd, J = 8.5 Hz, J = 2.2 Hz, 1H; H-3), 4.56 (s, 2H; H-21), 4.13 (t, J = 7.5 Hz, 2H; H-13), 1.81-1.71 (m, 2H; H-14), 1.43 (s, 9H; H-30), 1.40-1.12 (m, 26H; H-15/16/17/18/19/20/21/22/23/24/25/26/27), 0.80 (t, J = 6.0 Hz, 3H; H-28) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 167.2 (C-30), 156.2 (C-2), 140.6 (C-12), 127.7 (C-9), 125.7 (C-11), 123.5 (C-6), 121.8 (C-7), 120.0 (C-4), 118.6 (C-5), 117.8 (C-3), 116.6 (C-8), 107.4 (C-10), 93.7 (C-1), 81.2 (C-31), 65.4 (C-29), 52.1 (C-13), 30.9 (C-26), 28.7 (C-14), 28.6 (C-23/25), 28.5 (C-22/24), 28.4 (C-17/19), 28.3 (C-32), 28.3 (C-18/20), 27.8 (C-16), 27.0 (C-21), 26.3 (C-15), 21.6 (C-27), 13.1 (C-28) ppm.

5.4.5. Synthesis of *N*-alkylated 2-(9*H*-carbazol-2-yloxy)acetic acids (72)

5.4.5.1. 2-(9-Octyl-9*H*-carbazol-2-yloxy)acetic acid (72a)
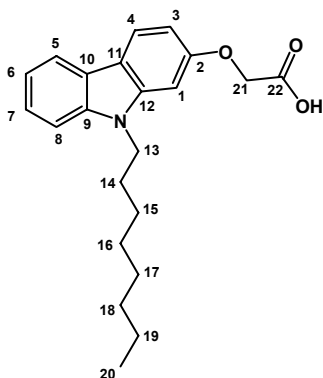
The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-octyl-9*H*-carbazol-2-yloxy)acetate (**71a**) (100 mg, 0.24 mmol), 20% trifluoroacetic acid in CH_2Cl_2 (5 mL).

Yield: 82 mg, 95%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 8.02 (d, *J* = 6.8 Hz, 1H; H-5), 7.83-7.80 (m, 2H; H-4, H-8), 7.65 (d, *J* = 2.1 Hz, 1H; H-1), 7.35-7.30 (m, 2H; H-6/7), 6.98 (dd, *J* = 8.6 Hz, 2.1 Hz, 1H; H-3), 4.65 (s, 2H; H-21), 3.09 (t, *J* = 8.0 Hz, 2H; H-13), 1.62-1.51 (m, 2H; H-14), 1.21-1.11 (m, 10H; H-15/16/17/18/19), 0.75 (t, *J* = 7.0 Hz, 3H; H-20) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 174.6 (C-22), 161.7 (C-2), 142.5 (C-12), 130.2 (C-8), 127.8 (C-11), 124.7 (C-6), 124.0 (C-7), 123.3 (C-4), 118.2 (C-5), 116.1 (C-3), 113.2 (C-8), 104.4 (C-10), 97.2 (C-1), 69.4 (C-21), 56.4 (C-13), 35.3 (C-18), 32.5 (C-14), 32.4 (C-16), 31.7 (C-17), 26.6 (C-15), 22.3 (C-19), 17.7 (C-20) ppm.

5.4.5.2. 2-(9-Nonyl-9*H*-carbazol-2-yloxy)acetic acid (72b)
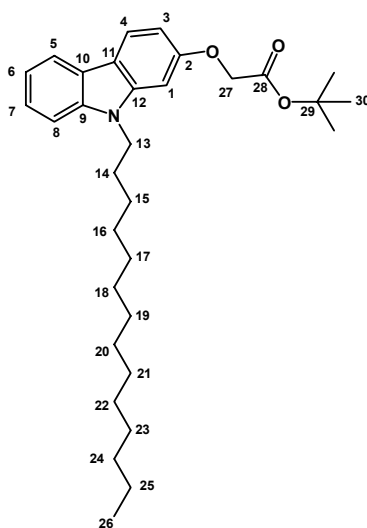

The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-nonyl-9*H*-carbazol-2-yloxy)acetate (**71b**) (100 mg, 0.23 mmol), 20% trifluoroacetic acid in CH_2Cl_2 (5 mL).

Yield: 74 mg, 86%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.92 (d, *J* = 7.7 Hz, 1H; H-5), 7.87 (d, *J* = 8.8 Hz, 1H; H-4), 7.32-7.29 (m, 2H; H-1, H-8), 7.11 (t, *J* = 7.8 Hz, 1H; H-6), 6.86-6.84 (m, 1H; H-7), 6.75 (dd, *J* = 8.0 Hz, *J* = 2.3 Hz, 1H; H-3), 4.67 (s, 2H; H-21), 4.14 (t, *J* = 7.2 Hz, 2H; H-13), 1.91-

1.80 (m, 2H; H-14), 1.36-1.02 (m, 12H; H-15/16/17/18/19/20), 0.78 (t, J = 7.0 Hz, 3H; H-21) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 175.1 (C-23), 160.9 (C-2), 144.6 (C-12), 128.5 (C-9), 126.6 (C-11), 124.9 (C-6), 123.4 (C-7), 122.7 (C-4), 121.5 (C-5), 113.3 (C-3), 110.7 (C-8), 105.5 (C-10), 98.8 (C-1), 69.9 (C-22), 60.4 (C-13), 35.6 (C-19), 33.2 (C-14), 32.1 (C-17), 31.0 (C-16), 30.6 (C-18), 29.5 (C-15), 26.5 (C-20), 17.8 (C-21) ppm.

MS (ESI): m/z = 390.2 (M+Na)$^{+}$.

5.4.5.3. 2-(9-Decyl-9*H*-carbazol-2-yloxy)acetic acid (72c)
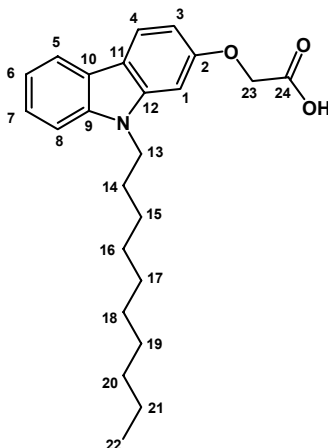
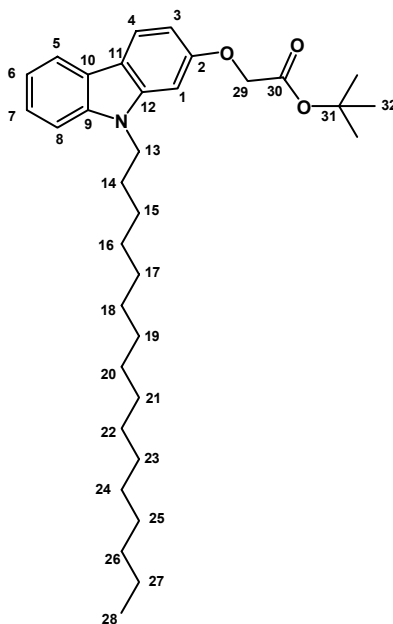The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-decyl-9*H*-carbazol-2-yloxy)acetate (**71c**) (100 mg, 0.22 mmol), 20% trifluoroacetic acid in CH$_2$Cl$_2$ (5 mL).

Yield: 77 mg, 89%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 8.03-7.98 (m, 2H; H-4/5), 7.44-7.38 (m, 2H; H-1, H-8), 7.24-7.20 (m, 2H; H-6/7), 6.94 (d, J = 1.5 Hz, 1H; H-1), 6.85 (dd, J = 7.5 Hz, J = 1.5 Hz, 1H; H-3), 4.82 (s, 2H; H-23), 4.23 (t, J = 7.5 Hz, 2H; H-13), 1.91-1.80 (m, 2H; H-14), 1.46-1.19 (m, 14H; H-15/16/17/18/19/20/21), 0.88 (t, J = 7.5 Hz, 3H; H-22) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 172.7 (C-24), 156.4 (C-2), 141.5 (C-12), 128.8 (C-9), 124.8 (C-11), 122.7 (C-6), 121.2 (C-7), 119.7 (C-5), 119.5 (C-3), 118.2 (C-8), 108.5 (C-10), 95.0 (C-1), 65.6 (C-23), 53.1 (C-13), 31.8 (C-28), 29.5 (C-17/18), 29.2 (C-16), 28.8 (C-19), 27.3 (C-15), 22.6 (C-21), 14.8 (C-22) ppm.

MS (ESI): m/z = 404.3 (M+Na)$^{+}$.

5.4.5.4. 2-(9-Undecyl-9*H*-carbazol-2-yloxy)acetic acid (72d)
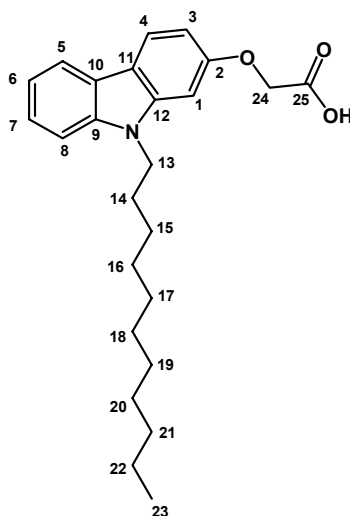
The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-undecyl-9*H*-carbazol-2-yloxy)acetate (**71d**) (100 mg, 0.22 mmol), 20% trifluoroacetic acid in CH_2Cl_2 (5 mL).

Yield: 75 mg, 86%.

^{1}H NMR (300 MHz, CDCl_3): δ = 7.91 (d, *J* = 7.0 Hz, 1H; H-5), 7.86 (d, *J* = 8.5 Hz, 1H; H-4), 7.33-7.27 (m, 2H; H-6/7), 7.14-7.09 (m, 1H; H-8), 6.82 (d, *J* = 2.0 Hz, 1H; H-1), 6.78 (dd, *J* = 8.5 Hz, *J* = 2.0 Hz, 1H; H-3), 4.59 (s, 2H; H-21), 4.15 (t, *J* = 7.5 Hz, 2H; H-13), 1.81-1.72 (m, 2H; H-14), 1.32-1.11 (m, 16H; H-15/16/17/18/19/20/21/22), 0.79 (t, *J* = 6.0 Hz, 3H; H-23) ppm.

^{13}C NMR (75 MHz, CDCl_3): δ = 177.9 (C-25), 157.2 (C-2), 141.6 (C-12), 127.7 (C-9), 125.7 (C-11), 124.8 (C-6), 122.8 (C-7), 121.3 (C-4), 119.8 (C-5), 119.1 (C-3), 117.6 (C-8), 108.8 (C-10), 95.0 (C-1), 66.4 (C-24), 53.1 (C-13), 31.8 (C-21), 29.5 (C-14), 29.4 (C-17/19), 29.2 (C-18), 28.8 (C-16), 28.0 (C-20), 27.3 (C-15), 22.6 (C-22), 14.0 (C-23) ppm.

5.4.5.5. 2-(9-Dodecyl-9*H*-carbazol-2-yloxy)acetic acid (72e)

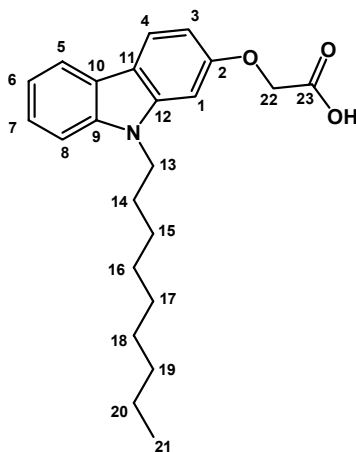The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-dodecyl-9*H*-carbazol-2-yloxy)acetate (**71e**) (100 mg, 0.21 mmol), 20% trifluoroacetic acid in CH_2Cl_2 (5 mL).

Yield: 79 mg, 90%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.92 (d, J = 7.2 Hz, 1H; H-5), 7.85 (d, J = 8.4 Hz, 1H; H-4), 7.32-7.28 (m, 2H; H-6/7), 7.13-7.09 (m, 1H; H-8), 6.81 (d, J = 2.1 Hz, 1H; H-1), 6.77 (dd, J = 8.6 Hz, J = 2.1 Hz, 1H; H-3), 4.60 (s, 2H; H-21), 4.14 (t, J = 7.5 Hz, 2H; H-13), 1.80-1.71 (m, 2H; H-14), 1.31-1.11 (m, 18H; H-15/16/17/18/19/20/21/22/23), 0.79 (t, J = 6.0 Hz, 3H; H-24) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 178.2 (C-26), 156.2 (C-2), 140.6 (C-12), 127.9 (C-9), 126.4 (C-11), 125.4 (C-6), 123.4 (C-7), 121.9 (C-4), 119.8 (C-5), 119.3 (C-3), 118.6 (C-8), 109.3 (C-10), 96.1 (C-1), 66.4 (C-25), 54.1 (C-13), 32.1 (C-22), 29.9 (C-14), 29.6 (C-17/19), 29.5 (C-18/20), 29.0 (C-16), 28.7 (C-28), 28.3 (C-21), 27.8 (C-15), 22.8 (C-23), 13.7 (C-24) ppm.

5.4.5.6. 2-(9-Tetradecyl-9*H*-carbazol-2-yloxy)acetic acid (72f)
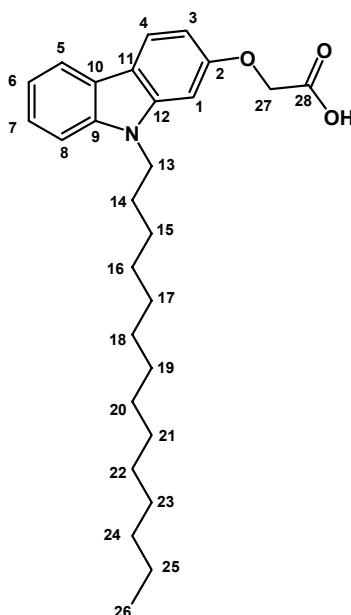
The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-tetradecyl-9*H*-carbazol-2-yloxy)acetate (**71f**) (100 mg, 0.2 mmol), 20% trifluoroacetic acid in CH$_2$Cl$_2$ (5 mL).

Yield: 81 mg, 92%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.92 (d, J = 7.0 Hz, 1H; H-5), 7.84 (d, J = 8.5 Hz, 1H; H-4), 7.32-7.28 (m, 2H; H-6/7), 7.13-7.08 (m, 1H; H-8), 6.82 (d, J = 2.2 Hz, 1H; H-1), 6.77 (dd, J = 8.6 Hz, J = 2.2 Hz, 1H; H-3), 4.60 (s, 2H; H-21), 4.14 (t, J = 7.6 Hz, 2H; H-13), 1.82-1.70 (m, 2H; H-14), 1.33-1.11 (m, 22H; H-15/16/17/18/19/20/21/22/23/24/25), 0.79 (t, J = 6.0 Hz, 3H; H-26) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 178.2 (C-28), 157.5 (C-2), 141.1 (C-12), 128.5 (C-9), 126.7 (C-11), 125.5 (C-6), 123.5 (C-7), 122.0 (C-4), 120.1 (C-5), 119.3 (C-3), 118.3 (C-8), 108.1 (C-10), 96.7 (C-1), 66.4 (C-27), 54.1 (C-13), 31.6 (C-24), 29.8 (C-14), 29.7 (C-23), 29.6 (C-22), 29.5 (C-17/19), 29.4 (C-18/20), 28.9 (C-16), 28.6 (C-30), 28.3 (C-21), 27.8 (C-15), 22.8 (C-25), 13.6 (C-26) ppm.

5.4.5.7. 2-(9-Hexadecyl-9*H*-carbazol-2-yloxy)acetic acid (72g)
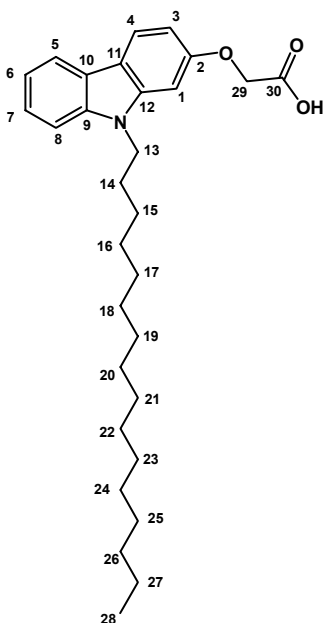
The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-hexadecyl-9*H*-carbazol-2-yloxy)acetate (**71g**) (100 mg, 0.19 mmol), 20% trifluoroacetic acid in CH_2Cl_2 (5 mL).
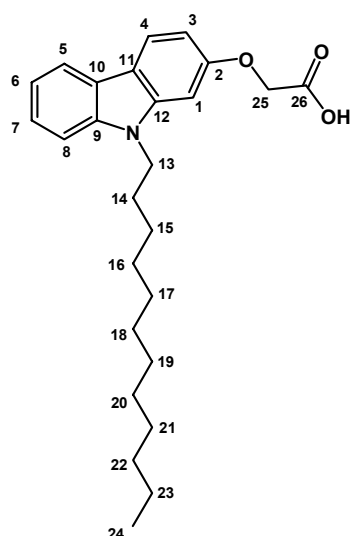
Yield: 84 mg, 95%.

¹H NMR (300 MHz, CDCl₃): δ = 7.91 (d, *J* = 7.2 Hz, 1H; H-5), 7.85 (d, *J* = 8.6 Hz, 1H; H-4), 7.32-7.28 (m, 2H; H-6/7), 7.13-7.09 (m, 1H; H-8), 6.81 (d, *J* = 2.1 Hz, 1H; H-1), 6.76 (dd, *J* = 8.6 Hz, *J* = 2.1 Hz, 1H; H-3), 4.61 (s, 2H; H-21), 4.15 (t, *J* = 7.6 Hz, 2H; H-13), 1.81-1.70 (m, 2H; H-14), 1.35-1.10 (m, 26H; H-15/16/17/18/19/20/21/22/23/24/25/26/27), 0.80 (t, *J* = 6.4 Hz, 3H; H-28) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 178.2 (C-30), 156.4 (C-2), 141.4 (C-12), 127.5 (C-9), 125.5 (C-11), 123.6 (C-6), 121.9 (C-7), 120.6 (C-4), 119.2 (C-5), 117.9 (C-3), 116.5 (C-8), 107.8 (C-10), 95.5 (C-1), 65.4 (C-29), 52.1 (C-13), 31.5 (C-26), 29.7 (C-14), 29.6 (C-23/25), 29.5 (C-22/24), 29.4 (C-17/19), 29.3 (C-18/20), 28.8 (C-16), 28.0 (C-21), 27.3 (C-15), 22.6 (C-27), 13.8 (C-28) ppm.

5.4.6. Synthesis of 6-methoxy and 8-methoxy *N*-sulfonylated carbazolyloxyacetic acids.

5.4.6.1. 1-((4-Chloro-3-nitrophenoxy)methyl)-4-methoxybenzene (74)
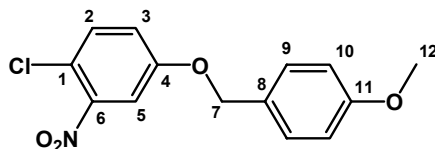
Anhydrous K_2CO_3 (9.56 g, 69 mmol) was added to a stirred solution of 4-chloro-3-nitro phenol (4 g, 23 mmol) in acetone (50 mL) and stirred at ambient temperature for 30 min. *p*-Methoxy benzyl chloride (3.3 mL, 24.2 mmol) was added to it and heated to 60–70 °C for 12 h. The reaction mixture was cooled to RT and filtered. The residue was washed with acetone (3×). Combined organic extract was evaporated *in vacuo* and purified by crystallization (EtOAc:hexane) to yield the title compound as a pale yellow solid (6.3 g, 93%)

¹H NMR (300 MHz, CDCl₃): δ = 7.64 (d, J = 3.0 Hz, 1H; H-5), 7.33 (d, J = 8.9 Hz, 1H; H-2), 7.26 (td, J = 8.8 Hz, 2.5 Hz, 2H; H-9), 7.03 (dd, J = 8.9 Hz, 3.0 Hz, 1H; H-3), 6.86 (td, J = 8.8 Hz, 2.5 Hz, 2H; H-10), 4.95 (s, 2H; H-7), 3.81 (s, 3H; H-12) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 159.9 (C-11), 159.7 (C-4), 149.6 (C-6), 132.4 (C-8), 129.3 (C-2), 127.2 (C-9), 120.6 (C-3), 118.3 (C-1), 114.2 (C-10), 111.5 (C-5), 70.7 (C-7), 55.3 (C-12) ppm.

5.4.6.2. 3'-Methoxy-4-(4-methoxybenzyloxy)-2-nitrobiphenyl (75)
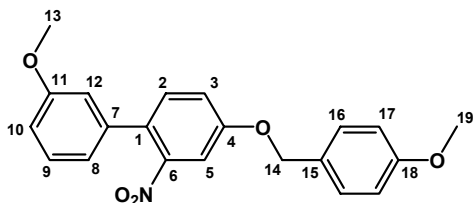
A mixture of the 1-((4-chloro-3-nitrophenoxy)methyl)-4-methoxybenzene (**67**) (1 g, 3.41 mmol), 3-methoxy phenylboronic acid (0.623 g, 4.09 mmol) and 2 M (aq.) K₂CO₃ (0.95 g, 6.83 mmol) was taken up in toluene (10 mL) and sparged with bubbling N₂ for 5 min. At that time, Pd(PPh₃)₄ (0.08 g, 0.07 mmol) was added and sparging continued for an additional 10 min before the flask was closed and the contents heated to reflux. Upon complete consumption of the halogen starting material (24 h), the reaction was cooled, filtered and washed with EtOAc (~ 400 mL). The organic mixture was washed with H₂O (2 × 50 mL) and brine, dried over anhydrous Na₂SO₄ and evaporated *in vacuo*. Chromatography of the residue gave the pure product as colorless solid (0.75 g, 56%).

¹H NMR (300 MHz, CDCl₃): δ = 7.35 (d, J = 3.3 Hz, 1H; H-5), 7.30-7.18 (m, 4H; H-2/3, H-8/9), 7.10 (dd, J = 8.5 Hz, J = 2.6 Hz, 1H; H-10), 6.88-6.73 (m, 5H; H-12, H-16/17), 4.99 (s, 2H; H-14), 4.06 (s, 3H; H-13), 4.03 (s, 3H; H-19) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 159.8 (C-11), 159.6 (C-4), 158.2 (C-18), 149.6 (C-6), 138.5 (C-7), 132.6 (C-15), 130.1 (C-9), 129.7 (C-3), 129.3 (C-16), 127.7 (C-1), 120.4 (C-3), 119.1 (C-8), 114.3 (C-17), 113.7 (C-10), 113.3 (C-12), 110.0 (C-5), 69.9 (C-14), 55.3 (C-13), 55.2 (C-19) ppm.

MS (EI): *m/z* = 365 (16%, M⁺), 335 (7%), 265 (31%), 263 (100%), 244 (22%), 198 (16%).

5.4.6.3. 2-(4-Methoxybenzyloxy)-6-methoxy-9*H*-carbazole & 7-(4-Methoxybenzyloxy)-1-methoxy-9*H*-carbazole (76)

2-Nitrobiphenyl derivative (0.74 g, 2 mmol) and PPh₃ (1.33 g, 5 mmol) was taken up in 1,2-dichlorobenzene (*o*-DCB) (6 mL) under N₂ and heated to reflux, with vigorous stirring. The reaction was stopped upon complete consumption of the nitrobiphenyl starting material (6 h), at which point the reaction was cooled and the solvent stripped *in vacuo*. The residue was chromatographed (EtOAc:hexane, 1:9) directly to separate the two regio isomers formed i.e. 6-methoxy carbazole as colorless solid (0.3 g, 44%) and 8-methoxy carbazole as colorless solid (0.2 g, 30%).

5.4.6.3.1. 6-Methoxy-2-(4-methoxybenzyloxy)-9*H*-carbazole (76a)
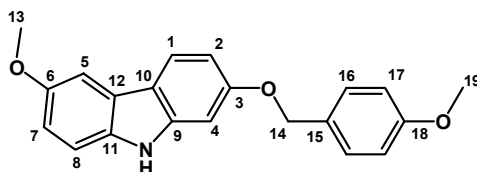
¹H NMR (300 MHz, CDCl₃): δ = 8.09 (brs, 1H; NH), 7.83 (d, J = 6.0 Hz, 1H; H-1), 7.51 (d, J = 9.0 Hz, 1H; H-8), 7.35-7.21 (m, 3H; H-5, H-16), 7.03 (d, J = 7.5 Hz, 1H; H-7), 6.91 (d, J

= 1.5 Hz, 1H; H-4), 6.88-6.82 (m 2H; H-17), 6.76 (dd, *J* = 7.5 Hz, *J* = 1.5 Hz, 1H; H-2), 4.99 (s, 2H; H-14), 3.92 (s, 3H; H-13), 3.73 (s, 3H; H-19) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 158.4 (C-18), 157.1 (C-6), 156.3 (C-3), 134.4 (C-15), 132.5 (C-12), 128.5 (C-11), 128.2 (C-16), 127.5 (C-9), 123.4 (C-1), 118.3 (C-17), 116.7 (C-8), 113.9 (C-2), 111.1 (C-7), 108.5 (C-10), 104.2 (C-5), 95.0 (C-4), 69.2 (C-14), 54.4 (C-13), 54.2 (C-19) ppm.

5.4.6.3.2. 1-Methoxy-7-(4-methoxybenzyloxy)-9*H*-carbazole (76b)
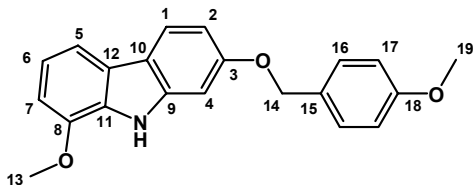
^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.81 (d, *J* = 9.0 Hz, 1H; H-1), 7.40 (d, *J* = 1.5 Hz, 1H; H-4), 7.32-7.29 (m, *J* = 9.0 Hz, 1.5 Hz, 2H; H-16), 7.27-7.18 (m, 2H; H-5/6), 6.89 (dd, *J* = 7.5 Hz, *J* = 1.5 Hz, 1H; H-3), 6.85 (dd, *J* = 9.0 Hz, *J* = 1.5 Hz, 2H; H-17), 6.81 (d, *J* = 9.0 Hz, 17), 4.99 (s, 2H; H-14), 3.84 (s, 3H; H-13), 3.73 (s, 3H; H-19) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 158.4 (C-18), 157.1 (C-3), 144.4 (C-8), 139.3 (C-15), 134.4 (C-12), 128.4 (C-16), 128.2 (C-11), 127.5 (C-9), 123.8 (C-6), 121.7 (C-1), 118.3 (C-17), 116.7 (C-2), 113.0 (C-5), 109.9 (C-7), 107.5 (C-10), 101.7 (C-4), 69.3 (C-14), 55.1 (C-13), 54.3 (C-19) ppm.

5.4.6.4. 6-Methoxy-9*H*-carbazol-2-ol (77a)
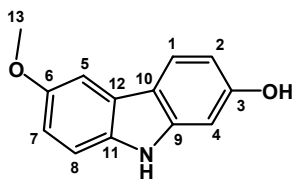
10% Pd-C (20% w/w, 60 mg) was added to the solution of 6-methoxy-2-(4-methoxybenzyloxy)-9*H*-carbazole (**76a**), (300 mg, 0.9 mmol) in MeOH (5 mL) and the resulting suspension was kept under H$_2$ at 60 psi, at ambient temperature for 24 h. The reaction mixture was filtered through the celite bed and the celite bed was washed several times with warm MeOH. The combined organic extract was evaporated *in vacuo* to yield the crude product. The crude compound was purified by flash column chromatography (EtOAc:hexane, 3:7) to obtain the title compound as a cream colored (165 mg, 86%).

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.79 (d, *J* = 8.0 Hz, 1H; H-1), 7.55 (brs, 1H; NH), 7.50 (dd, *J* = 1.8 Hz, *J* = 7.8 Hz, 1H; H-8), 7.04 (d, *J* = 1.8 Hz, 1H; H-5), 6.88 (d, *J* = 1.7 Hz, 1H; H-4), 6.80 (dd, *J* = 7.6 Hz, *J* = 1.6 Hz, 1H; H-2), 6.69 (dd, *J* = 8.4 Hz, *J* = 2.2 Hz, 1H; H-7), 3.97 (s, 3H; H-13) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 157.1 (C-6), 148.9 (C-3), 134.3 (C-12), 130.2 (C-11), 125.9 (C-9), 122.0 (C-1), 118.8 (C-2), 113.0 (C-8), 109.6 (C-7), 106.1 (C-10), 102.3 (C-5), 97.9 (C-4), 56.5 (C-13) ppm.

MS (EI): *m/z* = 213 (68%, M$^+$), 198 (49%), 170 (71%), 144 (9%), 119 (18%), 43 (100%).

5.4.6.5. 8-Methoxy-9*H*-carbazol-2-ol (77b)
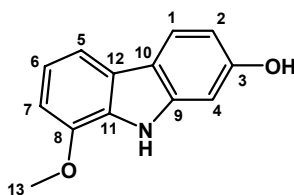
10% Pd-C (20% w/w, 40 mg) was added to the solution of 1-methoxy-7-(4-methoxybenzyloxy)-9*H*-carbazole (**76b**), (200 mg, 0.6 mmol) in MeOH (5 mL) and the resulting suspension was kept under H_2 at 60 psi, at ambient temperature for 24 h. The reaction mixture was filtered through the celite bed and the celite bed was washed several times with warm MeOH. The combined organic extract was evaporated *in vacuo* to yield the crude product. The crude compound was purified by flash column chromatography (EtOAc:hexane, 3:7) to obtain the title compound as a cream colored (115 mg, 90%).

MS (EI): *m/z* = 213 (71%, M$^+$), 198 (44%), 170 (67%), 144 (12%), 43 (100%).

5.4.6.6. Tert-butyl 2-(3-methoxy-9*H*-carbazol-7-yloxy)acetate (78a)
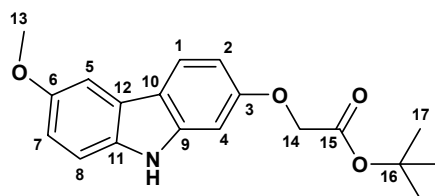
Anhydrous K_2CO_3 (242 mg, 1.8 mmol) was added to a stirred solution of 6-methoxy-9*H*-carbazol-2-ol (**77a**) (130 mg, 0.6 mmol) in acetone (5 mL) and stirred at ambient temperature for 30 min. *T*-butyl chloroacetate (105 μL, 0.75 mmol) was added to it and heated to 60–70 °C for 12 h. Reaction was 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 (190 mg, 95%)

^{1}H NMR (300 MHz, CDCl$_3$): *δ* = 8.09 (brs, 1H; NH), 7.84 (d, *J* = 8.6 Hz, 1H; H-1), 7.51 (d, 7.8 Hz, 1H; H-8), 7.07 (d, *J* = 1.8 Hz, 1H; H-5), 6.87 (d, *J* = 2.2 Hz, 1H; H-4), 6.80-6.76 (m, 2H; H-2, H-7), 4.53 (s, 2H; H-14), 3.93 (s, 3H; H-13), 1.42 (s, 9H; H-17) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): *δ* = 168.2 (C-15), 157.2 (C-6), 155.4 (C-3), 140.2 (C-12), 129.7 (C-11), 124.3 (C-9), 121.2 (C-1), 113.9 (C-8), 111.4 (C-2), 110.2 (C-7), 108.4 (C-10), 105.2 (C-5), 96.3 (C-4), 82.3 (C-16), 66.4 (C-14), 55.5 (C-13), 28.0 (C-17) ppm.

5.4.6.7. Tert-butyl 2-(1-methoxy-9*H*-carbazol-7-yloxy)acetate (78b)
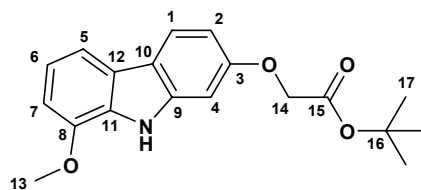
Anhydrous K_2CO_3 (148 mg, 1 mmol) was added to a stirred solution of 8-methoxy-9*H*-carbazol-2-ol (**77b**) (75 mg, 0.35 mmol) in acetone (5 mL) and stirred at ambient temperature for 30 min. *T*-butyl chloroacetate (62 μL, 0.42 mmol) was added to it and heated to 60–70 °C for 12 h. Reaction was cooled 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 (115 mg, 90%)

¹H NMR (300 MHz, CDCl₃): δ = 7.90 (brs, 1H; NH), 7.81 (d, J = 8.5 Hz, 1H; H-1), 7.38 (d, 8.5 Hz, 1H; H-5), 7.19 (d, J = 1.5 Hz, 1H; H-4), 6.90 (t, J = 8.7 Hz, 1H; H-6), 6.81 (d, J = 8.5 Hz, 1H; H-7), 6.76 (dd, J = 8.5 Hz, J = 1.5 Hz, 1H; H-3), 4.52 (s, 2H; H-14), 3.74 (s, 3H; H-13), 1.42 (s, 9H; H-17) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 167.2 (C-15), 156.3 (C-3), 147.4 (C-8), 138.4 (C-12), 130.5 (C-11), 124.3 (C-9), 123.2 (C-6), 119.9 (C-1), 114.0 (C-2), 113.6 (C-5), 109.9 (C-7), 107.0 (C-10), 95.1 (C-4), 81.3 (C-16), 65.3 (C-14), 55.0 (C-13), 28.2 (C-17) ppm.

MS (EI): m/z = 327 (22%, M⁺), 271 (68%), 212 (26%), 94 (48), 59 (51%), 43 (100%).

5.4.6.8. Tert-butyl 2-(9-(3,5-bis(trifluoromethyl)phenylsulfonyl)-6-methoxy-9*H*-carbazol-2-yloxy)acetate (79a)
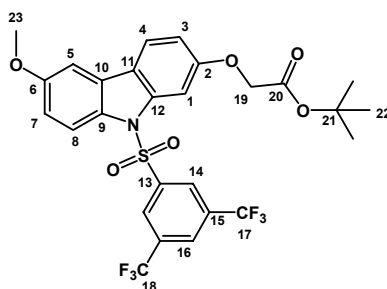
The title compound was synthesized by following the general procedure mentioned in **5.4.2.1**.

Reactants: Tert-butyl 2-(3-methoxy-9*H*-carbazol-7-yloxy)acetate (**78a**) (40 mg, 0.12 mmol), 3,5-bis(trifluoromethyl)phenyl sulfonyl chloride (46 mg, 0.14 mmol), KOBuᵗ (17 mg, 0.13 mmol).

Yield: 44 mg, 60%.

¹H NMR (300 MHz, CDCl₃): δ = 8.23 (s, 2H; H-14), 7.97 (s, 1H; H-16), 7.79 (d, J = 8.4 Hz, 1H; H-4), 7.73 (d, J = 8.7 Hz, 1H; H-8), 7.37 (d, J = 1.0 Hz, 1H; H-6), 7.22 (d, J = 1.1 Hz, 1H; H-1), 7.00 (dd, J = 8.5 Hz, 1.5 Hz, 1H; H-7), 6.77 (dd, J = 7.2 Hz, J = 1.2 Hz, 1H; H-3), 4.59 (s, 2H; H-19), 1.43 (s, 9H; H-22) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 167.7 (C-20), 158.2 (C-6), 155.5 (C-2), 142.2 (C-13), 140.5 (C-12), 133.1 (C-16), 129.1 (C-5), 128.7 (C-14), 127.7 (C-9), 125.6 (C-17/18), 123.5 (C-11), 121.0 (C-4), 113.3 (C-8), 111.0 (C-3), 109.8 (C-7), 107.7 (C-10), 102.7 (C-1), 82.6 (C-21), 66.1 (C-19), 55.2 (C-23), 28.0 (C-22) pm.

MS (ESI): m/z = 626.1 (M+Na)⁺.

5.4.6.9. Tert-butyl 2-(9-(4-chlorophenylsulfonyl)-6-methoxy-9*H*-carbazol-2-yloxy)acetate (79b)
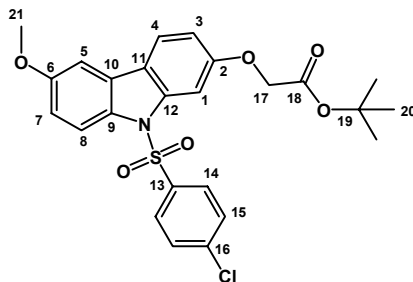
The title compound was synthesized by following the general procedure mentioned in **5.4.2.1**.

Reactants: Tert-butyl 2-(3-methoxy-9*H*-carbazol-7-yloxy)acetate (**78a**) (40 mg, 0.12 mmol), 4-chlorophenyl sulfonyl chloride (31 mg, 0.14 mmol), KOBuᵗ (17 mg, 0.13 mmol).

Yield: 42 mg, 70%.

¹H NMR (300 MHz, CDCl₃): δ = 7.81 (d, J = 8.4 Hz, 1H; H-4), 7.72-7.64 (m, 3H; H-8; H-14), 7.37-7.30 (m, 3H; H-5, H-15), 7.20-7.14 (m, 1H; H-1), 6.97 (dd, J = 8.7 Hz, J = 2.4 Hz, 1H; H-7), 6.76 (dd, J = 8.7 Hz, J = 1.0 Hz, 1H; H-3), 4.58 (s, 2H; H-17), 1.44 (s, 9H; H-20) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 166.8 (C-18), 156.9 (C-6), 154.2 (C-2), 140.7 (C-16), 138.3 (C-12), 138.2 (C-13), 130.1 (C-15), 129.5 (C-14), 126.3 (C-9), 125.8 (C-11), 121.9 (C-4), 116.4 (C-8), 114.4 (C-3), 109.8 (C-7), 104.6 (C-10), 96.1 (C-1), 81.5 (C-19), 65.0 (C-17), 54.7 (C-21), 28.1 (C-20) ppm.

5.4.6.10. Tert-butyl 2-(9-(biphenyl-4-ylsulfonyl)-6-methoxy-9*H*-carbazol-2-yloxy)acetate (79c)

The title compound was synthesized by following the general procedure mentioned in **5.4.2.1**.

Reactants: Tert-butyl 2-(3-methoxy-9*H*-carbazol-7-yloxy)acetate (**78a**) (40 mg, 0.12 mmol), 4-biphenyl sulfonyl chloride (37 mg, 0.14 mmol), KOBu^t (17 mg, 0.13 mmol).

Yield: 46 mg, 70%.

¹H NMR (300 MHz, CDCl₃): δ = 7.88 (d, J = 2.2 Hz, 1H; H-5), 7.80 (d, J = 8.8 Hz, 2H; H-14), 7.72 (d, J = 8.7 Hz, 1H; H-4), 7.55 (td, J = 6.6 Hz, J = 1.9 Hz, 2H; H-15), 7.48 (dd, J = 6.6 Hz, J = 1.8 Hz, 2H; H-18), 7.40-7.31 (m, 4H; H-8, H-19/20), 7.19-7.14 (m, 1H; H-1), 6.97 (dd, J = 8.6 Hz, J = 2.2 Hz, 1H; H-7), 6.76 (d, J = 7.2 Hz, 1H; H-3), 4.59 (s, 2H; H-21), 3.65 (s, 3H; H-27), 1.41 (s, 9H; H-24) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 166.8 (C-22), 156.9 (C-6), 154.3 (C-2), 143.3 (C-16), 141.7 (C-12), 139.8 (C-13), 138.5 (C-17), 130.1 (C-19), 129.3 (C-15), 128.9 (C-14), 128.2 (C-18), 127.5 (C-20), 127.1 (C-9), 126.2 (C-11), 125.9 (C-4), 1139 (C-8), 111.2 (C-3), 110.8 (C-7), 104.2 (C-10), 95.2 (C-1), 81.5 (C-23), 65.4 (C-21), 54.7 (C-27), 28.6 (C-24) ppm.

5.4.6.11. Tert-butyl 2-(9-(2-bromophenylsulfonyl)-6-methoxy-9*H*-carbazol-2-yloxy)acetate (79d)

The title compound was synthesized by following the general procedure mentioned in **5.4.2.1**.

Reactants: Tert-butyl 2-(3-methoxy-9*H*-carbazol-7-yloxy)acetate (**78a**) (40 mg, 0.12 mmol), 2-bromophenyl sulfonyl chloride (38 mg, 0.14 mmol), KOBu^t (17 mg, 0.13 mmol).

Yield: 34 mg, 52%.

¹H NMR (300 MHz, CDCl₃): δ = 8.25 (dd, J = 8.0 Hz, J = 1.7 Hz, 1H; H-18), 7.89 (d, J = 2.2 Hz, 1H; H-5), 7.76 (d, J = 8.5 Hz, 1H; H-15), 7.60 (dd, J = 7.9 Hz, 1H; H-4), 7.49-7.31 (m, 3H; H-8, H-16/17), 7.24-7.14 (s, 1H; H-1), 6.98 (dd, J = 8.6 Hz, J = 2.3 Hz, 1H; H-7), 6.69 (d, J = 6.8 Hz, 1H; H-3), 4.58 (s, 2H; H-19), 3.45 (s, 3H; H-21), 1.43 (s, 9H; H-23) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 167.9 (C-20), 157.6 (C-6), 155.3 (C-2), 144.6 (C-13), 141.8 (C-12), 138.2 (C-16), 135.1 (C-15), 133.6 (C-18), 131.4 (C-17), 128.9 (C-9), 126.7 (C-11), 125.0 (C-14), 122.1 (C-4), 115.5 (C-8), 113.3 (C-3), 110.8 (C-7), 107.8 (C-10), 103.2 (C-5), 95.2 (C-1), 82.4 (C-22), 66.1 (C-19), 55.0 (C-21), 28.1 (C-23) ppm.

5.4.6.6. Tert-butyl 2-(9-(4-chlorophenylsulfonyl)-8-methoxy-9*H*-carbazol-2-yloxy)acetate (79e)

The title compound was synthesized by following the general procedure mentioned in **5.4.2.1**.

Reactants: Tert-butyl 2-(1-methoxy-9*H*-carbazol-7-yloxy)acetate (**78b**) (18 mg, 0.055 mmol), 4-chlorophenyl sulfonyl chloride (14 mg, 0.066 mmol), KOBu^t (7.5 mg, 0.06 mmol).

Yield: 19 mg, 70%.

¹H NMR (300 MHz, CDCl₃): δ = 8.07-8.04 (m, 1H; H-4), 7.79-7.72 (m, 1H; H-5), 7.63-7.52 (m, 2H; H-15), 7.41-7.29 (m, 2H; H-16), 7.16-7.09 (m, 1H; H-1), 6.96-6.87 (m, 3H; H-3, H-6/7), 4.59 (s, 3H; H-17), 3.80 (s, 3H; H-21), 1.47 (s, 9H; H-20) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 168.8 (C-18), 154.9 (C-2), 148.2 (C-8), 140.7 (C-16), 137.3 (C-12), 136.2 (C-13), 130.4 (C-15), 129.7 (C-14), 125.3 (C-9), 125.2 (C-11), 123.7 (C-6), 121.9 (C-4), 114.4 (C-3), 113.4 (C-5), 109.8 (C-7), 107.6 (C-10), 95.1 (C-1), 80.5 (C-19), 66.1 (C-17), 55.2 (C-21), 28.4 (C-20) ppm.

5.4.6.13. Tert-butyl 2-(9-(biphenyl-4-ylsulfonyl)-8-methoxy-9*H*-carbazol-2-yloxy)acetate (79f)

The title compound was synthesized by following the general procedure mentioned in **5.4.2.1**.

Reactants: Tert-butyl 2-(1-methoxy-9*H*-carbazol-7-yloxy)acetate (**78b**) (18 mg, 0.055 mmol), 4-biphenyl sulfonyl chloride (17 mg, 0.066 mmol), KOBu^t (7.5 mg, 0.06 mmol).

Yield: 23 mg, 80%.

¹H NMR (300 MHz, CDCl₃): δ = 8.10 (d, J = 9.0 Hz, 1H; H-4), 7.81 (d, J = 1.8 Hz, 1H; H-1), 7.73-7.63 (m, 3H; H-5, H-14), 7.42-7.28 (m, 7H; H-15, H-18/19/20), 7.17-7-12 (m, 1H; H-6), 6.97-6.91 (m, 2H; H-3, H-7), 4.60 (s, 2H; H-21), 3.80 (s, 3H; H-27), 1.42 (s, 9H; H-24) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 168.8 (C-22), 155.3 (C-2), 147.3 (C-8), 142.3 (C-16), 140.7 (C-12), 139.1 (C-13), 137.9 (C-17), 129.7 (C-19), 129.1 (C-15), 128.6 (C-14), 128.1 (C-18), 127.8 (C-20), 127.6 (C-9), 127.1 (C-11), 125.9 (C-4), 124.1 (C-6), 113.9 (C-3), 111.6 (C-5), 109.8 (C-7), 106.2 (C-10), 96.2 (C-1), 81.8 (C-23), 66.4 (C-21), 55.4 (C-27), 28.3 (C-24) ppm.

5.4.6.14. 2-(9-(3,5-Bis(trifluoromethyl)phenylsulfonyl)-6-methoxy-9*H*-carbazol-2-yloxy)acetic acid (80a)

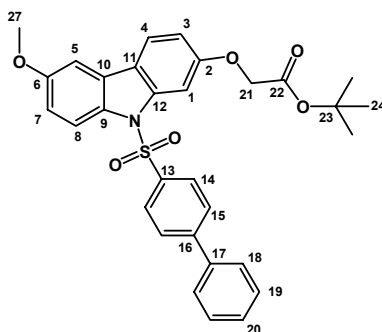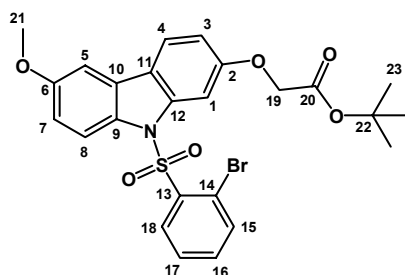The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-(3,5-bis(trifluoromethyl)phenylsulfonyl)-6-methoxy-9*H*-carbazol-2-yloxy)acetate (**79a**) (25 mg, 0.04 mmol), 20% trifluoroacetic acid in CH₂Cl₂ (2 mL).

Yield: 19 mg, 90%.

¹H NMR (300 MHz, CDCl₃): δ = 8.25 (s, 2H; H-14), 8.01 (s, 1H; H-16), 7.81 (d, J = 8.6 Hz, 1H; H-4), 7.75 (d, J = 8.8 Hz, 1H; H-8), 7.41 (d, J = 1.5 Hz, 1H; H-6), 7.24 (d, J = 1.6 Hz, 1H; H-1), 7.05 (dd, J = 8.8 Hz, J = 1.8 Hz, 1H; H-7), 6.81 (dd, J = 8.2 Hz, J = 1.5 Hz, 1H; H-3), 4.65 (s, 2H; H-19), 3.54 (s, 3H; H-23) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 174.7 (C-20), 157.2 (C-6), 156.5 (C-2), 143.2 (C-13), 140.1 (C-12), 133.6 (C-16), 129.7 (C-5), 128.9 (C-14), 128.1 (C-9), 125.8 (C-17/18), 123.9 (C-11), 121.6 (C-4), 113.7 (C-8), 111.9 (C-3), 110.5 (C-7), 108.3 (C-10), 104.7 (C-1), 96.6 (C-1), 66.9 (C-19), 56.6 (C-23) pm.

MS (ESI): m/z = 626.1 (M+Na)⁺.

5.4.6.15. 2-(9-(4-Chlorophenylsulfonyl)-6-methoxy-9*H*-carbazol-2-yloxy)acetic acid (80b)
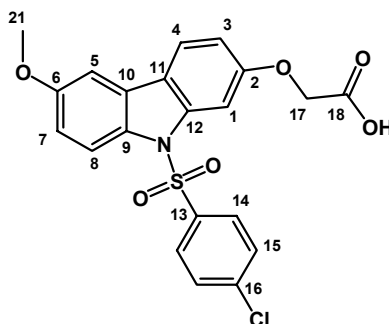
The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-(4-chlorophenylsulfonyl)-6-methoxy-9*H*-carbazol-2-yloxy)acetate (**79b**) (25 mg, 0.05 mmol), 20% trifluoroacetic acid in CH₂Cl₂ (2 mL).

Yield: 20 mg, 94%.

¹H NMR (300 MHz, CDCl₃): δ = 7.91 (s, 1H; H-5), 7.80 (d, J = 1.5 Hz, 1H; H-1), 7.59 (d, J = 8.7 Hz, 2H; H-14), 7.44 (d, J = 9.0 Hz, 1H; H-4), 7.21 (d, J = 8.7 Hz, 2H; H-15), 7.10-6.84 (m, 3H; H-3, H-7, H-8), 6.66 (dd, J = 8.7 Hz, J = 1.5 Hz, 1H; H-3), 4.54 (s, 2H; H-17) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 173.8 (C-18), 157.1 (C-6), 155.1 (C-2), 141.4 (C-16), 138.3 (C-12), 138.1 (C-13), 130.3 (C-15), 129.6 (C-14), 126.4 (C-9), 125.9 (C-11), 122.2 (C-4), 117.1 (C-8), 115.1 (C-3), 109.9 (C-7), 105.1 (C-10), 97.5 (C-1), 66.5 (C-17), 55.2 (C-21) ppm.

5.4.6.16. 2-(9-(Biphenyl-4-ylsulfonyl)-6-methoxy-9*H*-carbazol-2-yloxy)acetic acid (80c)

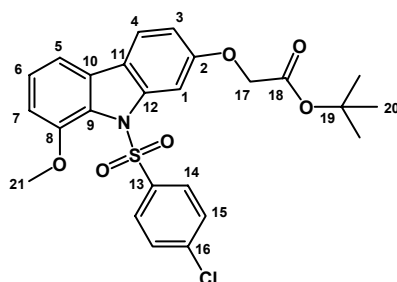The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-(biphenyl-4-ylsulfonyl)-6-methoxy-9*H*-carbazol-2-yloxy)acetate (**79c**) (25 mg, 0.045 mmol), 20% trifluoroacetic acid in CH₂Cl₂ (2 mL).

Yield: 21 mg, 95%.

¹H NMR (300 MHz, CDCl₃): δ = 7.89 (d, J = 2.2 Hz, 1H; H-5), 7.74 (d, J = 8.6 Hz, 2H; H-14), 7.57 (d, J = 8.6 Hz, 1H; H-4), 7.50 (d, J = 8.6 Hz, 2H; H-15), 7.41 (dd, J = 7.8 Hz, J = 2.2 Hz, 2H; H-18), 7.32-7.20 (m, 4H; H-8, H-19/20), 7.11-7.08 (s, 1H; H-1), 6.91 (dd, J = 8.8 Hz, J = 2.1 Hz, 1H; H-7), 6.69 (d, J = 7.6 Hz, 1H; H-3), 4.57 (s, 2H; H-21), 3.59 (s, 3H; H-27) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 174.8 (C-22), 156.3 (C-6), 155.1 (C-2), 142.6 (C-16), 141.1 (C-12), 139.3 (C-13), 138.1 (C-17), 130.4 (C-19), 129.4 (C-15), 128.3 (C-14), 127.8 (C-18), 127.2 (C-20), 126.9 (C-9), 126.1 (C-11), 125.4 (C-4), 113.2 (C-8), 111.1 (C-3), 109.2 (C-7), 106.1 (C-10), 96.5 (C-1), 66.2 (C-21), 56.1 (C-27) ppm.

5.4.6.17. 2-(9-(2-Bromophenylsulfonyl)-6-methoxy-9*H*-carbazol-2-yloxy)acetic acid (80d)

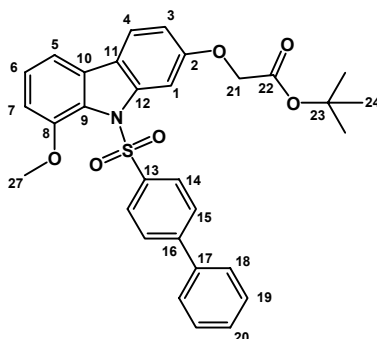The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

Reactants: Tert-butyl 2-(9-(2-bromophenylsulfonyl)-6-methoxy-9*H*-carbazol-2-yloxy)acetate (**79d**) (20 mg, 0.036 mmol), 20% trifluoroacetic acid in CH₂Cl₂ (2 mL).

Yield: 14 mg, 80%.

¹H NMR (300 MHz, CDCl₃): *δ* = 8.25 (dd, *J* = 8.0 Hz, *J* = 1.7 Hz, 1H; H-18), 7.91 (d, *J* = 2.2 Hz, 1H; H-5), 7.77 (d, *J* = 8.6 Hz, 1H; H-15), 7.61 (d, *J* = 7.9 Hz, 1H; H-4), 7.51-7.29 (m, 3H; H-8, H-16/17), 7.24-7.14 (s, 1H; H-1), 6.98 (dd, *J* = 8.6 Hz, *J* = 2.3 Hz, 1H; H-7), 6.69 (d, *J* = 6.8 Hz, 1H; H-3), 4.69 (s, 2H; H-19), 3.45 (s, 3H; H-21) ppm.

¹³C NMR (75 MHz, CDCl₃): *δ* = 175.9 (C-20), 156.2 (C-6), 155.5 (C-2), 145.1 (C-13), 140.2 (C-12), 137.5 (C-16), 134.8 (C-15), 132.8 (C-18), 131.1 (C-17), 129.4 (C-9), 127.1 (C-11), 125.6 (C-14), 122.3 (C-4), 115.6 (C-8), 113.1 (C-3), 110.1 (C-7), 106.1 (C-10), 101.3 (C-5), 97.1 (C-1), 66.5 (C-19), 56.1 (C-21) ppm.

5.4.6.18. 2-(9-(4-Chlorophenylsulfonyl)-8-methoxy-9*H*-carbazol-2-yloxy)acetic acid (80e)

The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.
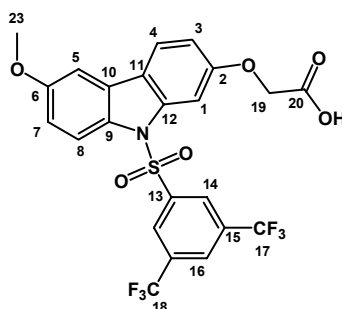
Reactants: Tert-butyl 2-(9-(4-chlorophenylsulfonyl)-8-methoxy-9*H*-carbazol-2-yloxy)acetate (**79e**) (15 mg, 0.03 mmol), 20% trifluoroacetic acid in CH₂Cl₂ (2 mL).

Yield: 12 mg, 93%.

¹H NMR (300 MHz, CDCl₃): *δ* = 8.08-8.05 (m, 2H; H-4/5), 7.81 (m, 1H; H-1), 7.70-7.52 (m, 3H; H-6, H-14), 7.41-7.30 (m, 2H; H-15), 7.15-7.10 (s, 1H; H-1), 6.97-6.84 (m, 2H; H-3, H-7), 4.59 (s, 3H; H-17), 3.80 (s, 3H; H-21) ppm.

¹³C NMR (75 MHz, CDCl₃): *δ* = 174.5 (C-18), 156.1 (C-2), 148.5 (C-8), 140.5 (C-16), 136.9 (C-12), 136.1 (C-13), 130.1 (C-15), 129.2 (C-14), 125.1 (C-9), 124.8 (C-11), 122.9 (C-6), 122.1 (C-4), 113.9 (C-3), 111.9 (C-5), 110.1 (C-7), 106.9 (C-10), 96.3 (C-1), 66.5 (C-17), 56.3 (C-21) ppm.

5.4.6.19. 2-(9-(Biphenyl-4-ylsulfonyl)-8-methoxy-9*H*-carbazol-2-yloxy)acetic acid (80f)
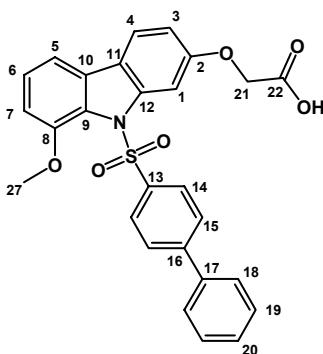
The title compound was synthesized by following the general procedure mentioned in **5.4.3.1**.

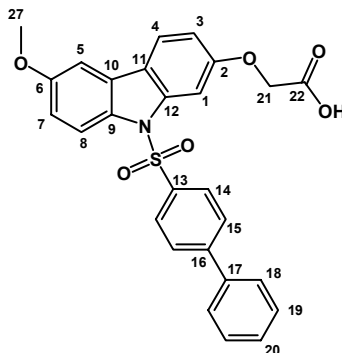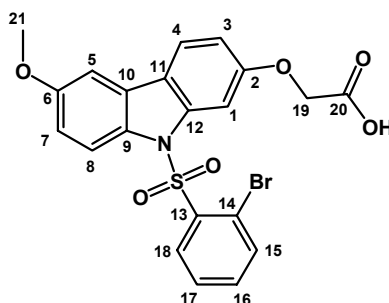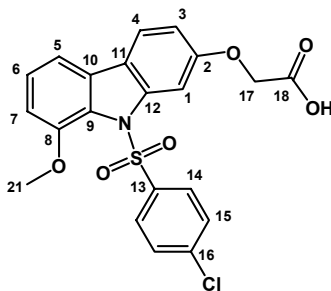**Reactants:** Tert-butyl 2-(9-(biphenyl-4-ylsulfonyl)-8-methoxy-9*H*-carbazol-2-yloxy)acetate (**79f**) (15 mg, 0.027 mmol), 20% trifluoroacetic acid in CH₂Cl₂ (2 mL).

Yield: 12 mg, 90%.

¹H NMR (300 MHz, CDCl₃): *δ* = 8.13 (d, *J* = 9.0 Hz, 1H; H-4), 7.85 (d, *J* = 1.5 Hz, 1H; H-1), 7.71 (d, *J* = 9.0 Hz, 2H; H-14), 7.67 (d, *J* = 9.0 Hz, 1H; H-5), 7.42 (d, *J* = 9.0 Hz, 2H; H-

15), 7.41-7.30 (m, 6H; H-6, H-18/19/20), 6.98 (d, J = 9.0 Hz, 1H; H-7), 6.94 (d, J = 9.0 Hz, J = 1.5 Hz, 1H; H-3), 4.76 (s, 2H; H-21), 3.81 (s, 3H; H-27) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 176.8 (C-22), 156.1 (C-2), 148.1 (C-8), 141.3 (C-16), 140.1 (C-12), 138.8 (C-13), 137.6 (C-17), 129.3 (C-19), 128.4 (C-15), 128.1 (C-14), 127.6 (C-18), 127.1 (C-20), 126.9 (C-9), 126.4 (C-11), 125.5 (C-4), 125.1 (C-6), 113.5 (C-3), 111.2 (C-5), 108.3 (C-7), 105.2 (C-10), 97.2 (C-1), 66.8 (C-21), 56.1 (C-27) ppm.

5.5. Synthesis of LXR Agonist TO-901317 Analogous γ-Secretase Modulator

5.5.1. Tert-butyl 2-(4-nitrophenoxy)acetate (83)
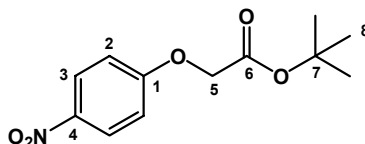
Anhydrous K$_2$CO$_3$ (2.26 g, 16.38 mmol) was added to a stirred solution of 4-nitro phenol (1 g, 7.19 mmol) in acetone (15 mL) and stirred for 30 min. at ambient temperature. *T*-butyl chloroacetate (1.13 mL, 7.91 mmol) was added to it and heated to 60–70 °C for 16 h. Reaction was monitored by TLC. Reaction 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 yellowish solid (1.73 g, 95%).

^{1}H NMR (300 MHz, CDCl$_3$): δ = 8.02 (d, J = 9.0, 2H; H-3), 6.90 (d, J = 9.0, 2H; H-2), 4.41 (s, 2H; H-5), 1.42 (s, 9H; H-8) ppm.

5.5.2. Tert-butyl 2-(4-aminophenoxy)acetate (84)
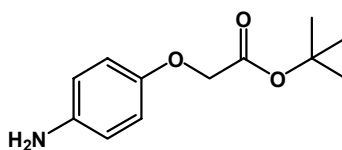
10% Pd-C (300 mg, 20% w/w) was added to a stirred solution of tert-butyl 2-(4-nitrophenoxy)acetate (**83**) methanol (20 mL) and the suspension was kept under H$_2$ at 60 psi at room temperature for 6 h. The reaction was monitored by TLC and the ninhydrin was used for the visualisation. The reaction mixture was filtered through the celite bed. The celite bed was washed with warm methanol (3×). Combined organic extracts were evaporated *in vacuo* to yield the title compound as pale brown solid. The crude product was used for the next step without further purification.

5.5.3. Tert-butyl 2-(4-(3,5-bis(trifluoromethyl)phenylsulfonamido)phenoxy)acetate (85)
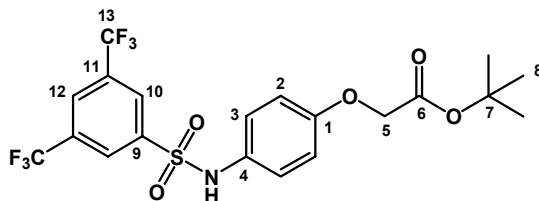
Triethyl amine (150 μL, 1.08 mmol) and catalytic DMAP (22 mg, 0.18 mmol) were added to a stirred solution of tert-butyl 2-(4-aminophenoxy)acetate (**84**) (200 mg, 0.89 mmol) in CH$_2$Cl$_2$ and stirred at an ambient temperature for 30 min. 3,5-Bis-(trifluoromethyl) phenyl sulfonyl chloride (342 mg, 1.08 mmol) was then added to it and stirred for another 18 h. Reaction was monitored by TLC. The reaction mixture was diluted with CH$_2$Cl$_2$, washed with H$_2$O, dried over anhydrous Na$_2$SO$_4$ and evaporated *in vacuo* to afford the crude compound.

The crude compound was purified by column chromatography (EtOAc:hexane, 2:8) to afford the title compound as cream colored solid (150 mg, 35%).

^{1}H NMR (300 MHz, CDCl$_3$): δ = 8.03 (s, 2H; H-10), 7.95 (s, 1H; H-12), 6.88 (dd, J = 9.0 Hz, J = 3.0 Hz, 2H; H-3), 6.70 (dd, J = 9.0 Hz, J = 3.0 Hz, 2H; H-2), 4.40 (s, 2H; H-5), 1.41 (s, 3H; H-8) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 167.8 (C-6), 157.0 (C-1), 141.7 (C-9), 132.9 (C-11), 132.4 (C-4), 127.5 (C-12), 126.3 (C-10), 124.1 (C-13), 120.5 (C-3), 115.5 (C-2), 82.8 (C-7), 65.8 (C-5), 27.9 (C-8) pm.

5.5.4. Tert-butyl 2-(4-(*N*-octyl-3,5-bis(trifluoromethyl)phenylsulfonamido)phenoxy)acetate (86)
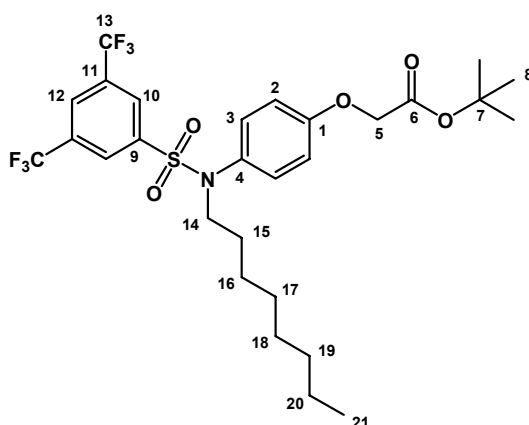
KOBu^t (27 mg, 0.22 mmol) was added to a stirred solution of tert-butyl 2-(4-(3,5-bis(trifluoromethyl)phenylsulfonamido)phenoxy)acetate (**85**) (100 mg, 0.2 mmol) and stirred at room temperature for 30 min. To it octyl iodide (44 µL, 0.24 mmol) was added and stirred for another 48 h at an ambient temperature. Reaction was monitored by TLC. The reaction mixture was diluted with EtOAc (50 mL) and washed with water. The aqueous phase was extracted with EtOAc (3×). The combined organic extract was washed with water, brine, dried over anhydrous Na$_2$SO$_4$ and rotary evaporated to afford the crude product. The product was purified by column chromatography (EtOAc:hexane, 0.5:9.5) to obtain the title compound as colorless gummy mass (40 mg, 33%).

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.98 (s, 2H; H-10), 7.93 (s, 1H; H-12), 6.84 (d, J = 9.0 Hz, 2H; H-3), 6.77 (d, J = 9.0 Hz, 2H; H-2), 4.43 (s, 2H; H-5), 3.43 (t, J = 7.5 Hz, 2H; H-14), 1.52-1.49 (m, 2H; H-15), 1.42 (s, 9H; H-8), 1.34-1.12 (m, 10H; H-16/17/18/19/20), 0.80 (t, J = 7.5 Hz, 3H; H-21) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 167.5 (C-6), 157.9 (C-1), 141.2 (C-9), 132.8 (C-11), 132.4 (C-4), 127.8 (C-12), 126.5 (C-10), 124.4 (C-13), 120.8 (C-3), 115.4 (C-2), 82.6 (C-7), 65.8 (C-5), 51.3 (C-14), 30.8 (C-19), 28.9 (C-8), 28.5 (C-15), 28.1 (C-17), 28.0 (C-18), 26.2 (C-16), 22.5 (C-20), 14.0 (C-21) ppm.

5.5.5. 2-(4-(*N*-Octyl-3,5-bis(trifluoromethyl)phenylsulfonamido)phenoxy)acetic acid (87)
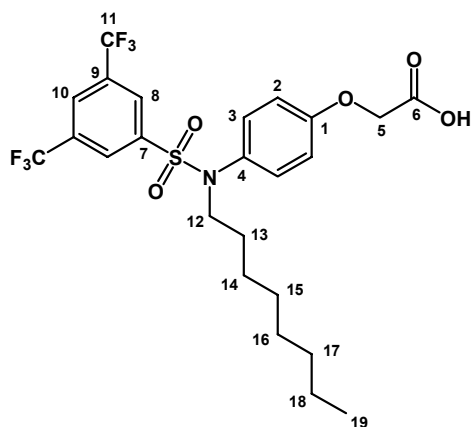
Tert-butyl 2-(4-(*N*-octyl-3,5-bis(trifluoromethyl)phenylsulfonamido)phenoxy)acetate (**86**) (30 mg, 0.05 mmol) was added to a solution of 20% TFA in CH_2Cl_2 and stirred at room temperature for 4 h. Reaction was monitored by TLC. Solvent was evaporated *in vacuo* and flushed with hexane several times. The crude compound was purified by crystallization (EtOAc) to afford the title compound as cream colored solid (21 mg, 77%).

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.97 (s, 1H; H-10), 7.91 (s, 2H; H-8), 6.90-6.76 (m, 4H; H-2, H-3), 4.40 (s, 2H; H-5), 3.40 (t, J = 7.5 Hz, 2H; H-12), 1.37-1.28 (m, 2H; H-13), 1.24-1.11 (m, 10H; H-14/15/16/17/18), 0.78 (t, J = 7.0 Hz, 3H; H-19) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 173.5 (C-6), 155.9 (C-1), 140.2 (C-7), 131.7 (C-9), 132.4 (C-4), 124.8 (C-8), 123.4 (C-11), 122.5 (C-3), 114.5 (C-2), 64.3 (C-5), 50.1 (C-12), 30.6 (C-17), 28.7 (C-6), 28.0 (C-13), 27.1 (C-15), 25.2 (C-16), 22.8 (C-14), 21.5 (C-18), 13.0 (C-19) ppm.

5.6 Synthesis of Curcumin Derived Oxazole and Pyrazoles Derivatives

The synthetic procedures and spectral data of curcumin derived oxazoles and pyrazoles are removed due to the IP reasons.

5.7. Synthesis of NSAIDs Derived Affinity Labels.

5.7.1. Tert-butyl 2-(2-(2-fluorobiphenyl-4-yl)propanamido)ethylcarbamate (92)
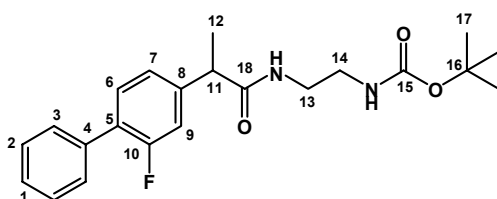
The title compound was synthesized by following the general procedure mentioned in **5.2.1**.

Reactants: 2-(2-fluoro-4-biphenyl)propanoic acid (150 mg, 0.61 mmol), EDAC (118 mg, 0.74 mmol), HOBt (83 mg, 0.74 mmol), Et$_3$N (189 µL, 0.14 mmol), *N*-Boc ethylene diamine (116 µL, 0.5 mmol).

Yield: 213 mg, 90%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.44 (dd, *J* = 7.0 Hz, *J* = 1.4 Hz, 2H; H-3), 7.37-7.25 (m, 4H; H-1, H-2, H-6), 7.09-7.01 (m, 2H; H-7, H-9), 6.45 (brs, 1H; NH), 4.93 (brs, 1H; NH), 3.49 (q, *J* = 7.0 Hz, 1H; H-11), 3.24-3.05 (m, 4H; 13-H, 14-H), 1.47 (p, *J* = 7.0 Hz, 3H; H-12), 1.33 (s, 9H; H-17) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 174.3 (C-18), 158.2 (C-10), 157.1 (C-15), 142.9 (C-8), 135.5 (C-4), 131.1 (C-5), 130.0 (C-6), 129.0 (C-2), 128.5 (C-3), 127.9 (C-1), 123.6 (C-7), 115.5 (C-9), 79.8 (C-16), 46.6 (C-11), 41.3 (C-13), 40.0 (C-14), 28.4 (C-16), 18.6 (C-17) ppm.

5.7.2. *N*-(2-Aminoethyl)-2-(2-fluorobiphenyl-4-yl)propanamide (93)
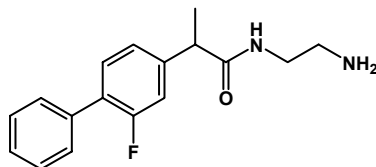
A solution of compound tert-butyl 2-(2-(2-fluorobiphenyl-4-yl)propanamido)ethylcarbamate (**92**) (120 mg, 0.3 mmol) in 16% HCl in dioxane (1 mL) was stirred at room temperature for 1 h. Reaction was monitored by TLC and then the reaction mixture was evaporated *in vacuo* and lyophilized to afford the title compound as hydrochloride salt which was used in the next step without any purification.

5.7.3. *N*-(2-(2-(2-Fluorobiphenyl-4-yl)propanamido)ethyl)-5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide (94)
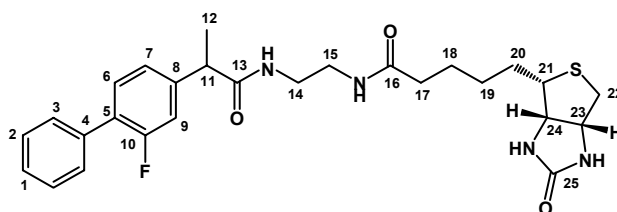
Et₃N (54 µL, 0.38 mmol) was added to a stirred solution of *D*-biotin (94 mg, 0.38 mmol) in anhydrous DMF (3 mL) and stirred at ambient temperature for 10 min. To it ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (88 mg, 0.46 mmol) and *N*-hydroxybenzotriazole hydrate (52 mg, 0.46 mmol) were added and the resulting solution was stirred at ambient temperature for 30 min. until it became clear. A solution of *N*-(2-aminoethyl)-2-(2-fluorobiphenyl-4-yl)propanamide (**93**) as hydrochloride salt (100 mg, 0.3 mmol) in DMF (1 mL) was added to the reaction mixture followed by Et₃N (129 µL, 0.9 mmol) and stirred at ambient temperature for 16 h. The reaction mixture was diluted with CHCl₃ (100 mL), washed sequentially with water, 0.2 N HCl, H₂O, 2 N NaOH, H₂O, brine and dried over anhydrous Na₂SO₄ and evaporated *in vacuo* to yield the crude product. The crude product was purified by flash column chromatography (CHCl₂:MeOH, 95:5) to afford the title compound as colorless solid (55 mg, 35%).

¹H NMR (500 MHz, CDCl₃): δ = 8.09 (brs, 1H; NH), 7.81 (brs, 1H; NH), 7.56-7.36 (m, 6H; H-1, H-2, H-3, H-6), 7.27-7.19 (m, 2H; H-7, H-9), 6.41 (brs, 1H; NH), 6.38 (brs, 1H; NH), 4.37-4.28 (m, 1H; H-24), 4.16-4.08 (m, 1H; H-23), 3.64 (q, *J* = 7.2 Hz, 1H; H-11), 3.18-3.02 (m, 5H; H-14, H-15, H-21), 2.80 (dd, *J* = 6.8 Hz, *J* = 5.0 Hz, 1H; H-22), 2.58 (d, *J* = 6.8 Hz, 1H; H-22′), 2.20 (t, *J* = 7.2 Hz, 2H; H-17), 1.68-1.42 (m, 6H; H-18, H-19, H-20), 1.38 (d, *J* = 7.0 Hz, 3H; H-12) ppm.

¹³C NMR (125 MHz, CDCl₃): δ = 172.8 (C-16), 171.2 (C-13), 162.7 (C-25), 160.3 (C-10), 136.9 (C-8), 136.4 (C-4), 130.4 (C-5), 130.1 (C-6), 129.8 (C-2), 128.6 (C-3), 128.1 (C-1), 123.7 (C-7), 114.9 (C-9), 60.9 (C-24), 59.1 (C-23), 55.2 (C-21), 44.6 (C-11), 41.8 (C-22), 39.5 (C-14), 39.3 (C-15), 35.1 (C-17), 28.1 (C-20), 27.9 (C-18), 25.1 (C-19), 18.5 (C-12) ppm.

MS (EI): *m/z* = 56.5 (M⁺, 64%), 452.5 (71%), 270 (40%), 227.2 (84%), 199.2 (100%)

5.7.4. 2-(2-Fluorobiphenyl-4-yl)propanoyl azide (95)
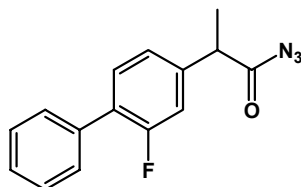
Flurbiprofen (200 mg, 0.82 mmol) was dissolved in THF (5 mL) under N₂ and cooled to –20 °C. After addition of EtOCOCl (86 µL, 1.14 mmol) and Et₃N (125 µL, 0.9 mmol), the reaction mixture was stirred at –20 °C for 20 min. The resulting white suspension was allowed to warm to –10 °C and treated with aq. NaN₃ solution (133 mg, 2.05 mmol). The mixture was stirred for 15 min, diluted with AcOEt, washed with brine, dried over anhydrous NaSO₄ and evaporated *in vacuo* to give the expected acyl azide as colorless solid, which was used in the next step without further purification.

5.7.5. Tert-butyl 2-(3-(1-(2-fluorobiphenyl-4-yl)ethyl)ureido)ethylcarbamate (96)
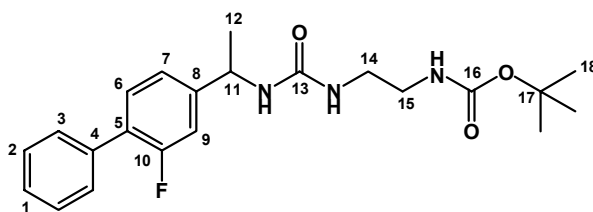
Toluene was added under Ar to a flask containing 2-(2-Fluorobiphenyl-4-yl)propanoyl azide (**95**) and the resulting solution was heated to 65 °C under stirring. After the N_2 gas evolution had stopped (15 min.), *N*-Boc ethylene diamine (128 µL, 0.9 mmol) and pyridine (Et$_3$N (125 µL, 0.9 mmol)) were added. The mixture was stirred for 30 min at 65 °C and then cooled to room temperature. Tolune was removed *in vacuo* and the crude compound was purified by column chromatography (EtOAc:hexane, 2:8) to afford the title compound as colorless solid (280 mg, 85%).

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.42 (td, *J* = 8.3 Hz, *J* = 1.2 Hz, 2H; H-3), 7.37-7.24 (m, 4H; H-1, H-2, H-6), 7.09-7.02 (m, 2H; H-7, H-9), 5.40 (brs, 2H, NH), 5.05 (brs, 1H, NH), 4.82 (p, *J* = 7.0 Hz, 1H; H-11), 3.24-3.09 (m, 4H; H-14, H-15), 1.41 (d, *J* = 7.0 Hz, 3H; H-12), 1.34 (s, 9H; H-18) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 161.5 (C-13), 158.1 (C-10), 157.0 (C-16), 146.5 (C-8), 135.6 (C-4), 129.1 (C-6), 129.0 (C-2), 128.2 (C-3), 127.6 (C-1), 122.0 (C-7), 113.7 (C-9), 79.7 (C-17), 49.4 (C-11), 41.2 (C-14), 40.8 (C-15), 28.4 (C-17), 23.1 (C-18) ppm.

5.7.6. 1-(2-Aminoethyl)-3-(1-(2-fluorobiphenyl-4-yl)ethyl)urea (97)
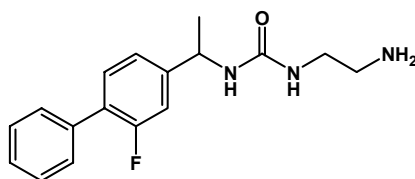
A solution of compound tert-butyl 2-(3-(1-(2-fluorobiphenyl-4-yl)ethyl)ureido)ethylcarbamate (**96**) (100 mg, 0.25 mmol) in 16% HCl in dioxane (1 mL) was stirred at room temperature for 1 h. Reaction was monitored by TLC The reaction mixture was evaporated *in vacuuo* and lyophilized to afford the title compound as hydrochloride salt which was used in the next step without any purification.

5.7.7. *N*-(2-(3-(1-(2-Fluorobiphenyl-4-yl)ethyl)ureido)ethyl)-5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide (98)
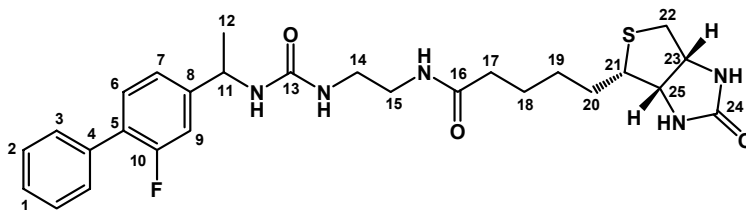
Et$_3$N (43 µL, 0.32 mmol) was added to a stirred solution of *D*-biotin (76 mg, 0.32 mmol) in anhydrous DMF (3 mL) and stirred at ambient temperature for 10 min. To it ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (72 mg, 0.38 mmol) and *N*-hydroxybenzotriazole hydrate (51 mg, 0.38 mmol) were added and the resulting solution was stirred at ambient temperature for 30 min. until it became clear. A solution of 1-(2-aminoethyl)-3-(1-(2-fluorobiphenyl-4-yl)ethyl)urea hydrochloride (**97**) (85 mg, 0.25 mmol) in DMF (1 mL) was added to the reaction mixture followed by Et$_3$N (217 µL, 1.56 mmol) and

stirred at ambient temperature for 17 h. The reaction mixture was diluted with $CHCl_3$ (100 mL), washed with water, 0.2 N HCl, H_2O, 2 N NaOH, H_2O, brine and 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 colorless solid (46 mg, 35%).

^{1}H NMR (500 MHz, CDCl$_3$): δ = 7.42 (dd, J = 8.2 Hz, J = 1.6 Hz, 2H; H-3), 7.34 (tt, J = 7.2 Hz, J = 1.6 Hz, 2H; H-2), 7.32-7.30 (m, 1H; H-6), 7.26 (t, J = 7.4 Hz, J = 1.6 Hz, 1H; H-1), 7.08 (dd, J = 8.0 Hz, J = 1.6 Hz, 1H; H-7), 7.03 (dd, J = 11.8 Hz, J = 1.6 Hz, 1H; H-9), 4.79-4.71 (m, 1H; H-11), 4.41-4.36 (m, 1H; H-24), 4.20-4.15 (m, 1H; H-23), 3.20-3.13 (m, 1H; H-21), 2.80 (dd, J = 6.8 Hz, J = 5.0 Hz, 1H; H-22), 2.64 (d, J = 6.8 Hz, 1H; H-22′), 2.04 (t, J = 7.2 Hz, 2H; H-17), 1.65-1.54 (m, 4H; H-18, H-20), 1.37 (d, J = 7.0 Hz, 3H; 12-H) 1.36-1.28 (m, 2H; H-19) ppm.

^{13}C NMR (125 MHz, CDCl$_3$): δ = 176.8 (C-16), 166.2 (C-13), 160.8 (C-24), 160.5 (C-10), 148.8 (C-8), 137.4 (C-4), 130.5 (C-5), 130.0 (C-6), 129.2 (C-2), 128.5 (C-3), 128.2 (C-1), 123.5 (C-7), 114.8 (C-9), 63.6 (C-25), 62.0 (C-23), 57.3 (C-21), 51.4 (C-11), 41.8 (C-22), 41.1 (C-14), 39.1 (C-15), 37.1 (C-17), 29.7 (C-20), 27.1 (C-18), 26.8 (C-19), 24.2 (C-12) ppm.

5.7.8. Tert-butyl 2-(2-(2,6-dichloro-3-methylphenylamino)benzamido)ethylcarbamate (99)
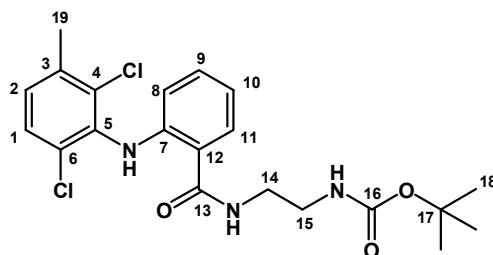
The title compound was synthesized by following the general procedure mentioned in **5.2.1**.

Reactants: 2-(2,6-dichloro-3-methylphenylamino)benzoic acid sodium salt (100 mg, 0.32 mmol), EDAC (73 mg, 0.38 mmol), HOBt (51 mg, 0.38 mmol), Et$_3$N (52 μL, 0.45 mmol), *N*-Boc ethylene diamine (60 μL, 0.38 mmol).

Yield: 128 mg, 90%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 9.43 (brs, 1H; NH), 7.49 (dd, J = 8.0 Hz, J = 1.4 Hz, 1H; H-11), 7.21 (d, J = 8.2 Hz, 1H; H-1), 7.19 (m, 1H; H-9), 6.97 (d, J = 8.2 Hz, 1H; H-8), 6.68 (t, J = 7.0 Hz, 1H; H-10), 6.29 (d, J = 8.2 Hz, 1H; H-2), 5.05 (t, J = 7.0 Hz, 1H; NH), 3.48 (q, J = 6.0 Hz, 2H; H-14), 3.32 (q, J = 6.0 Hz, 2H; H-15), 2.32 (s, 3H; H-19), 1.36 (s, 9H; H-18) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 170.1 (C-13), 157.5 (C-16), 145.6 (C-7), 136.4 (C-5), 135.8 (C-3), 133.3 (C-4), 132.0 (C-9), 128.1 (C-11), 127.7 (C-1), 127.1 (C-6), 122.0 (C-2), 120.1 (C-12), 118.1 (C-18), 114.7 (C-10), 80.0 (C-17), 41.7 (C-15), 40.0 (C-14), 28.4 (C-18), 20.7 (C-19) ppm.

5.7.9. *N*-(2-Aminoethyl)-2-(2,6-dichloro-3-methylphenylamino)benzamide (100)
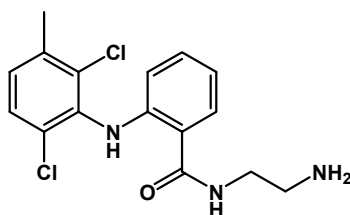
A solution of compound tert-butyl 2-(2-(2,6-dichloro-3-methylphenylamino)benzamido)ethylcarbamate (**99**) (100 mg, 0.3 mmol) in 16% HCl in dioxane (1 mL) was stirred at room temperature for 1 h. Reaction was monitored by TLC and then the reaction mixture was evaporated *in vacuo* and lyophilized to afford the title compound as hydrochloride salt which was used in the next step without any purification.

5.7.10. 2-(2,6-Dichloro-3-methylphenylamino)-*N*-(2-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)ethyl)benzamide (101)
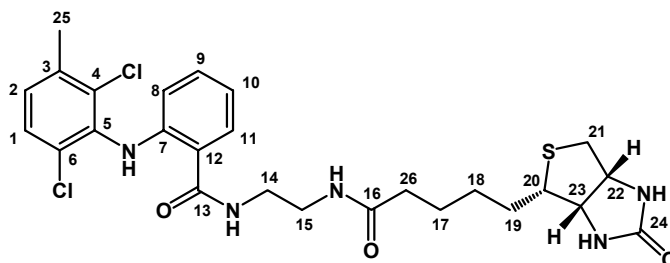
Et₃N (52 μL, 0.38 mmol) was added to a stirred solution of biotin (91 mg, 0.38 mmol) in anhydrous DMF (3 mL) and stirred at ambient temperature for 10 min. To it ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (86 mg, 0.45 mmol) and *N*-hydroxybenzotriazole hydrate (61 mg, 0.3 mmol) were added and the resulting solution was stirred at ambient temperature for 30 min. until it became clear. A solution of *N*-(2-aminoethyl)-2-(2,6-dichloro-3-methylphenylamino)benzamide hydrochloride (**100**) (101 mg, 0.28 mmol) in DMF (1 mL) was added to the reaction mixture followed by Et₃N (49 μL, 0.35 mmol) and stirred at ambient temperature for 16 h. The reaction mixture was diluted with CHCl₃ (100 mL), washed with water, 0.2 N HCl, H₂O, 2 N NaOH, H₂O, brine and dried over anhydrous Na₂SO₄ and evaporated *in vacuo* to yield the crude product. The crude product was purified by flash column chromatography (DCM: MeOH, 95:5) to afford the title compound as colorless solid (106 mg, 63%).

¹H NMR (300 MHz, CDCl₃): δ = 7.49 (dd, *J* = 7.8 Hz, *J* = 1.4 Hz, 1H; H-11), 6.98 (d, *J* = 8.2 Hz, 1H; H-1), 6.88 (t, *J* = 7.0 Hz, 1H; H-9), 6.78 (dd, *J* = 8.2 Hz, *J* = 1.0 Hz, 1H; H-8), 6.48 (t, *J* = 7.0 Hz, 1H; H-10), 6.01 (d, *J* = 8.2 Hz, 1H; H-2), 4.08-4.04 (m, 1H; H-22), 3.88-3.84 (m, 1H; H-23), 3.24-3.14 (m, 2H; H-15), 3.15-3.08 (m, 2H; H-14), 2.74-2.68 (m, 1H; H-20), 2.52 (dd, *J* = 6.8 Hz, *J* = 5.0 Hz, 1H; H-21), 2.35 (d, *J* = 6.8 Hz, 1H; H-21′), 2.07 (s, 3H; H-25), 1.89 (t, *J* = 7.2 Hz, 2H; H-16), 1.60-1.39 (m, 4H; H-17/19); 1.10-1.03 (m, 2H; H-18) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 175.1 (C-15), 170.5 (C-13), 164.7 (C-24), 144.9 (C-7), 136.2 (C-5), 135.3 (C-3), 135.0 (C-4), 134.8 (C-9), 130.7 (C-11), 128.7 (C-1), 127.6 (C-6), 121.5 (C-2), 120.5 (C-12), 117.7 (C-8), 114.9 (C-10), 61.6 (C-23), 59.9 (C-22), 55.2 (C-20), 41.5 (C-21), 39.8 (C-14), 39.4 (C-15), 38.8 (C-26), 28.0 (C-19), 27.8 (C-18), 25.5 (C-17), 19.8 (C-25) ppm.

MS (ESI): *m/z* = 586.1 (M+Na)⁺, 562.1 (M–H)⁺

5.7.11. (*Z*)-Tert-butyl 2-(2-(5-fluoro-2-methyl-1-(4-(methylsulfinyl)benzylidene)-1*H*-inden-3-yl)acetamido)ethylcarbamate (103)

The title compound was synthesized by following the general procedure mentioned in **5.2.1**.

Reactants: Sulindac sulfoxide (100 mg, 0.28 mmol), EDAC (65 mg, 0.34 mmol), HOBt (42 mg, 0.3 mmol), Et$_3$N (86 µL, 0.62 mmol), *N*-Boc ethylene diamine (50 µL, 0.3 mmol).

Yield: 128 mg, 92%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.65 (d, *J* = 8.4 Hz, 2H; H-13), 7.59 (d, *J* = 8.4 Hz, 2H; H-12), 7.10-7.16 (m, 2H; H-2, H-10), 6.82 (d, *J* = 8.8 Hz, 1H; H-5), 6.51 (d, *J* = 8.8 Hz, 1H; H-6), 6.15 (brs, 1H; NH), 4.85 (brs, 1H; NH), 3.40 (s, 2H; H-16), 3.50-3.11 (m, 4H; H-18, H-19), 2.73 (s, 3H; H-15), 2.14 (s, 3H; H-9), 1.32 (s, 9H; H-22) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 170.0 (C-17), 165.1 (C-1), 161.8 (C-20), 145.5 (C-3), 141.6 (C-14), 139.6 (C-23), 138.8 (C-11), 135.5 (C-8), 132.5 (C-7), 130.3 (C-4), 128.9 (C-6), 128.4 (C-12), 123.9 (C-13), 111.3 (C-6), 111.0 (C-2), 79.8 (C-21), 43.9 (C-16), 40.9 (C-19), 40.3 (C-18), 33.7 (C-18), 28.4 (C-22), 10.6, (C-9) ppm.

5.7.6. (*Z*)-*N*-(2-aminoethyl)-2-(5-fluoro-2-methyl-1-(4-(methylsulfinyl)benzylidene)-1*H*-inden-3-yl)acetamide (104)
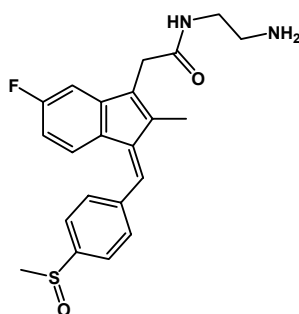
A solution of compound (*Z*)-tert-butyl 2-(2-(5-fluoro-2-methyl-1-(4-(methylsulfinyl)benzylidene)-1*H*-inden-3-yl)acetamido)ethylcarbamate (**103**) (100 mg, 0.2 mmol) in 20% TFA in CH$_2$Cl$_2$ (3 mL) was stirred at room temperature for 1 h. Reaction was monitored by TLC. The reaction mixture was then evaporated *in vacuo* and lyophilized to afford the title compound as trifluoroacetic acid salt which was used in the next step without further purification.

5.7.13. *N*-(2-(2-((*Z*)-5-Fluoro-2-methyl-1-(4-(methylsulfinyl)benzylidene)-1*H*-inden-3-yl)acetamido)ethyl)-5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide (105)
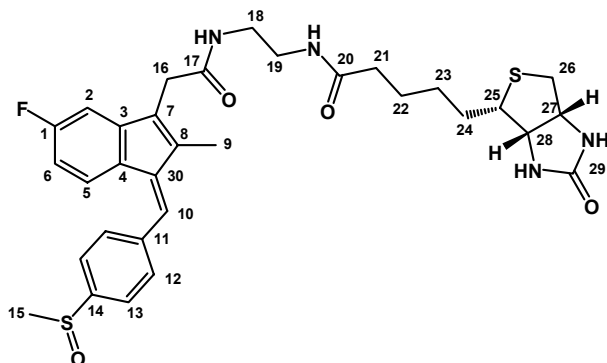
Et$_3$N (16 µL, 0.29 mmol) was added to a stirred solution of *D*-biotin (71 mg, 0.29 mmol) in anhydrous DMF (3 mL) and stirred at ambient temperature for 10 min. To it PyBrop (mg, 0.32 mmol) was added and the resulting solution was stirred at ambient temperature for 30 min. A solution of (*Z*)-*N*-(2-aminoethyl)-2-(5-fluoro-2-methyl-1-(4-(methylsulfinyl)benzylidene)-1*H*-inden-3-yl)acetamide (**104**) (100 mg, 0.23 mmol) in DMF (1 mL) was added to the reaction mixure followed by Et$_3$N (161 µL, 1.16 mmol) and stirred at ambient temperature for 14 h. The reaction mixture was diluted with CHCl$_3$ (100 mL), washed sequentially with water, 0.2 N HCl, H$_2$O, 2 N NaOH, H$_2$O, brine and dried over anhydrous Na$_2$SO$_4$ and evaporated *in vacuo* to yield the crude product. The crude product was purified by flash column chromatography (CHCl$_2$:MeOH, 95:5) to afford the title compound as brownish solid (36 mg, 25%).

^{1}H NMR (500 MHz, CD$_3$OD): δ = 7.70 (d, *J* = 8.6 Hz, 2H; H-13), 7.63 (d, *J* = 8.6 Hz, 2H; H-12), 7.11-7.18 (m, 2H; H-2, H-10), 6.85 (d, *J* = 8.8 Hz, 1H; H-5), 6.55 (d, *J* = 8.8 Hz, 1H; H-6), 4.44-4.37 (m, 1H; H-27), 4.24-4.16 (m, 1H; H-28), 3.41 (s, 2H; H-16), 3.51-3.12 (m, 4H; H-18, H-19), 2.85-2.79 (m, 1H; H-25), 2.75 (s, 3H; H-15), 2.72 (dd, *J* = 6.8 Hz, *J* = 5.0 Hz, 1H; H-26), 2.48 (d, *J* = 6.8 Hz, 1H; H-26′), 2.16 (s, 3H; H-9), 1.66-1.42 (m, 4H; H-22, H-24), 1.34-1.25 (m, 2H; H-23) ppm.

^{13}C NMR (125 MHz, CD$_3$OD): δ = 175.0 (C-20), 170.1 (C-17), 166.6 (C-29), 161.8 (C-1), 145.6 (C-3), 141.8 (C-14), 139.5 (C-23), 138.8 (C-11), 135.4 (C-8), 132.7 (C-7), 130.4 (C-4), 129.2 (C-6), 128.5 (C-12), 124.2 (C-13), 114.9 (C-6), 111.7 (C-2), 61.9 (C-28), 58.1 (C-27), 57.3 (C-25), 45.3 (C-15), 41.8 (C-26), 40.7 (C-19), 40.1 (C-18), 37.4 (C-21), 27.0 (C-24), 26.1 (C-22), 25.9 (C-23), 16.6 (C-9) ppm.

5.7.14. Tert-butyl 2-(2-(3-benzoylphenyl)propanamido)ethylcarbamate (107)
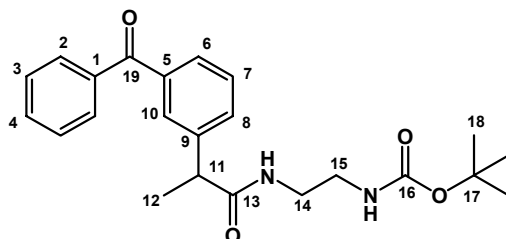
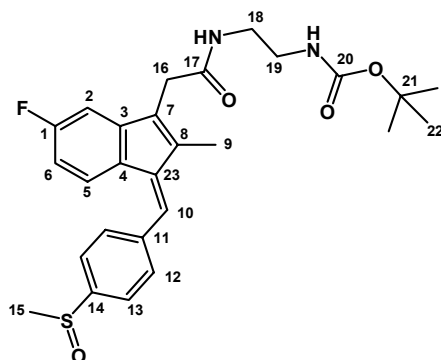The title compound was synthesized by following the general procedure mentioned in **5.2.1**.

**Reactants:** Ketoprofen (150 mg, 0.6 mmol), EDAC (136 mg, 0.7 mmol), HOBt (96 mg, 0.7 mmol), Et$_3$N (182 µL, 0.13 mmol), *N*-Boc ethylene diamine (112 µL, 0.7 mmol).

Yield: 230 mg, 98%.

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.72 (td, J = 7.0 Hz, J = 1.8 Hz, 2H; H-2), 7.67 (t, J = 1.8 Hz, 1H; H-4), 7.57 (dt, J = 7.8 Hz, J = 1.4 Hz, 1H; H-8), 7.51 (d, J = 6.0 Hz, 2H; H-6, H-10), 7.43-7.19 (m, 3H; H-3, H-7), 6.32 (brs, 1H; NH), 4.89 (brs, 1H; NH), 3.54 (q, J = 7.2 Hz, 1H; H-11), 3.31-3.15 (m, 4H; H-14, H-15), 1.45 (d, J = 7.2 Hz, 3H; H-12), 1.32 (s, 9H; H-18) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 196.6 (C-19), 174.3 (C-13), 159.2 (C-18), 141.9 (C-5), 138.0 (C-1), 137.5 (C-9), 132.6 (C-8), 132.1 (C-4), 131.6 (C-10), 130.1 (C-2), 129.2 (C-6), 129.0 (C-7), 128.7 (C-3), 79.8 (C-17), 47.0 (C-11), 41.1 (C-14), 40.3 (C-15), 28.4 (C-18), 18.8, (C-12) ppm.

5.7.15. *N*-(2-Aminoethyl)-2-(3-benzoylphenyl)propanamide (108)
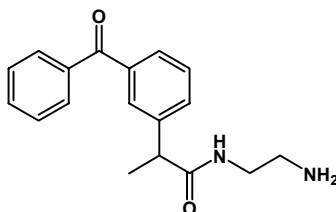
A solution of compound tert-butyl 2-(2-(3-benzoylphenyl)propanamido)ethylcarbamate (**107**) (110 mg, 0.28 mmol) in 20% TFA in CH$_2$Cl$_2$ (3 mL) was stirred at room temperature for 1 h. Reaction was monitored by TLC. The reaction mixture was then evaporated *in vacuo* and lyophilized to afford the title compound as trifluoroacetic acid salt which was used in the next step without purification.

5.7.16. *N*-(2-(2-(3-Benzoylphenyl)propanamido)ethyl)-5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide (109)
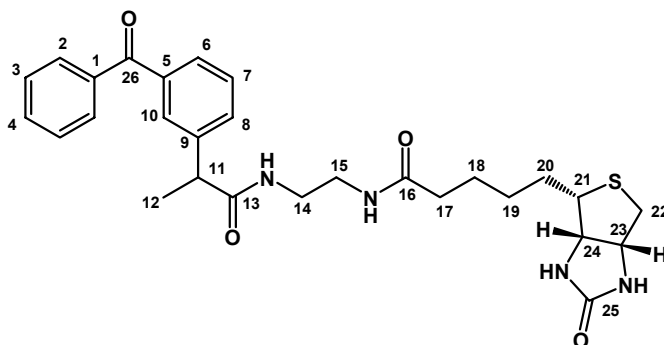
Et$_3$N (39 μL, 0.28 mmol) was added to a stirred solution of *D*-biotin (85 mg, 0.28 mmol) in anhydrous DMF (3 mL) and stirred at ambient temperature for 10 min. To it ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (80 mg, 0.41 mmol), *N*-hydroxybenzotriazole hydrate (56 mg, 0.41 mmol) were added and the resulting solution was stirred at ambient temperature for 30 min. until it became clear. A solution of *N*-(2-aminoethyl)-2-(3-benzoylphenyl)propanamide (**108**) (92 mg, 0.28 mmol) in DMF (1 mL) was added to the reaction mixture followed by Et$_3$N (49 μL, 0.35 mmol) and stirred at ambient temperature for 16 h. The reaction mixture was diluted with CHCl$_3$ (100 mL), washed sequentially with water, 0.2 N HCl, H$_2$O, 2 N NaOH, H$_2$O, brine and dried over anhydrous Na$_2$SO$_4$ and evaporated *in vacuo* to yield the crude product. The crude product was purified by flash column chromatography (CH$_2$Cl$_2$:MeOH, 95:5) to afford the title compound as brownish solid (65 mg, 45%).

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.91 (brs, 1H; NH), 7.86-7.81 (m, 3H; H-2/4), 7.72-7.65 (m, 3H; H-6/8/10), 7.60-7.50 (m, 3H; H-3/7), 4.56-4.51 (m, 1H; H-22), 4.35 (m, 1H; H-23), 3.77 (q, J = 7.6 Hz, 1H; H-11), 3.46-3.38 (m, 4H; H-14/15), 3.25-3.19 (m, 1H; H-21), 2.97

(dd, *J* = 6.8 Hz, *J* = 5.0 Hz, 1H; H-22), 2.78 (d, *J* = 6.8 Hz, 1H; H-22′), 2.17 (t, *J* = 7.4 Hz, 2H; H-17), 1.82-1.62 (m, 4H; H-18/20), 1.57 (d, *J* = 7.6 Hz, 3H; H-12), 1.49-1.39 (m, 2H; H-19) ppm.

¹³C NMR (75 MHz, CDCl₃): *δ* = 197.2 (C-26), 175.3 (C-16), 174.8 (C-13), 141.8 (C-5), 141.6 (C-1), 137.4 (C-9), 132.5 (C-8), 132.1 (C-4), 131.4 (C-10), 130.2 (C-2), 129.7 (C-6), 129.1 (C-7), 128.6 (C-3), 61.7 (C-24), 59.9 (C-23), 55.3 (C-21), 46.0 (C-11), 39.8 (C-22), 39.1 (C-14), 39.0 (C-15), 35.3 (C-17), 28.1 (C-20), 27.8 (C-19), 25.1 (C-18), 18.5, (C-12) ppm.

MS (ESI): *m/z* = 545.3 (M+Na)⁺

5.7.17. Tert-butyl 2-(2-(6-chloro-9*H*-carbazol-2-yl)propanamido)ethylcarbamate (110)
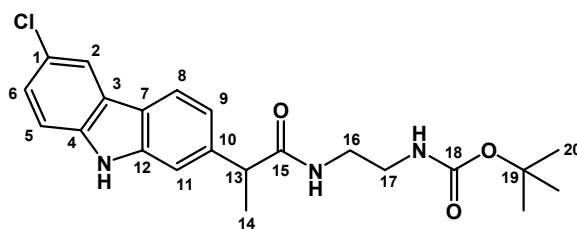
The title compound was synthesized by following the general procedure mentioned in **5.2.1**.

Reactants: Carprofen (100 mg, 0.38 mmol), EDAC (89 mg, 0.46 mmol), HOBt (63 mg, 0.46 mmol), Et₃N (118 µL, 0.84 mmol), *N*-Boc ethylene diamine (73 µL, 0.46 mmol).

Yield: 75 mg, 50%.

¹H NMR (500 MHz, CD₃OD): *δ* = 7.88 (d, *J* = 1.9 Hz, 1H; H-2), 7.82 (d, *J* = 8.6 Hz, 1H; H-8), 7.29 (d, *J* = 1.1 Hz, 1H; H-11), 7.20-7.16 (m, 2H; H-5/6), 7.07 (dd, *J* = 8.0 Hz, *J* = 1.5 Hz, 1H; H-9), 3.60 (q, *J* = 7.0 Hz, 1H; H-13), 3.20-3.14 (m, 2H; H-16), 3.08-3.03 (m, 2H; H-17), 1.47 (d, *J* = 7.5 Hz, 3H; H-14), 1.26 (s, 9H; H-20) ppm.

¹³C NMR (125 MHz, CD₃OD): *δ* = 173.1 (C-15), 165.1 (C-18), 137.4 (C-12), 131.3 (C-7), 127.1 (C-10), 127.1 (C-1), 125.6 (C-4), 124.1 (C-2), 120.7 (C-9), 119.1 (C-8), 118.1 (C-6), 113.5 (C-5), 110.5 (C-11), 106.3 (C-3), 80.6 (C-19), 39.5 (C-17), 38.6 (C-16), 28.6 (C-20) ppm.

5.7.18. *N*-(2-Aminoethyl)-2-(6-chloro-9*H*-carbazol-2-yl)propanamide (111)
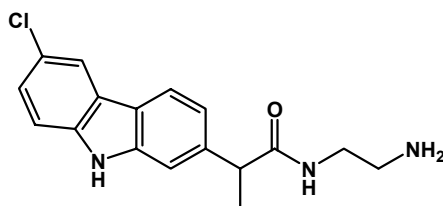
A solution of compound tert-butyl 2-(2-(6-chloro-9*H*-carbazol-2-yl)propanamido)ethylcarbamate (**110**) (50 mg, 0.12 mmol) in 20% TFA in CHCl₂ (1 mL) was stirred at room temperature for 1 h. Reaction was monitored by TLC. The reaction mixture was then evaporated *in vacuo* and lyophilized to afford the title compound as trifluoroacetic acid salt which was used in the next step without further purification.

5.7.19. *N*-(2-(2-(6-Chloro-9*H*-carbazol-2-yl)propanamido)ethyl)-5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide (112)
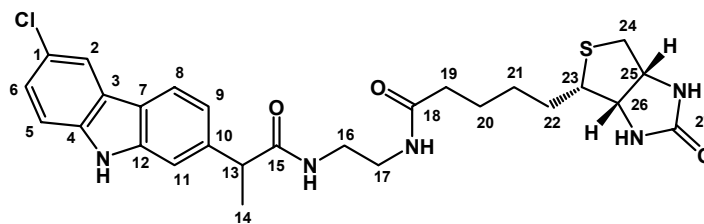
To a stirred solution of (*D*)-biotin (37 mg, 0.15 mmol) in anhydrous DMF (2 mL), Et$_3$N (21 µL, 0.15 mmol) was added and stirred at ambient temperature for 10 min. To it, ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (35 mg, 0.18 mmol), *N*-hydroxybenzotriazole hydrate (25 mg, 0.18 mmol) were added and the resulting solution was stirred at ambient temperature for 30 min. until it became clear. A solution of *N*-(2-aminoethyl)-2-(6-chloro-9*H*-carbazol-2-yl)propanamide (**111**) (42 mg, 0.12 mmol) in DMF (1 mL) was added to the reaction mixture followed by Et$_3$N (17 µL, 0.12 mmol) and stirred at ambient temperature for 18 h. The reaction mixture was diluted with CHCl$_3$ (100 mL), washed sequentially with water, 0.2 N HCl, H$_2$O, 2 N NaOH, H$_2$O, brine and dried over anhydrous Na$_2$SO$_4$ and evaporated *in vacuo* to yield the crude product. The crude product was purified by flash column chromatography (CHCl$_2$:MeOH, 95:5) to afford the title compound as brownish solid (25 mg, 40%).

^{1}H NMR (300 MHz, CD$_3$OD): δ = 7.63 (d, *J* = 1.9 Hz, 1H; H-2), 7.59 (d, *J* = 8.2 Hz, 1H; H-8), 7.07 (d, *J* = 7.6 Hz, 1H; H-5), 7.03 (s, 1H; H-11), 6.97 (dd, *J* = 8.6 Hz, *J* = 1.9 Hz, 1H; H-6), 6.80 (d, *J* = 8.0 Hz, 1H; H-9), 4.10-4.07 (m, 1H; H-25), 3.87-3.74 (m, 1H; H-26), 3.41 (q, *J* = 7.2 Hz, 1H; H-13), 3.32-3.92 (m, 4H; H-16, H-17), 2.66-2.56 (m, 1H; H-23), 2.52 (dd, *J* = 6.8 Hz, *J* = 4.8 Hz, 1H; H-24), 2.37 (d, *J* = 6.8 Hz, 1H; H-24′), 1.87 (t, *J* = 7.2 Hz, 2H; H-19), 1.22 (d, *J* = 7.2 Hz, 3H; H-14), 1.05-0.99 (m, 4H; H-20, H-22), 0.84-0.75 (m, 2H; H-21) ppm.

^{13}C NMR (75 MHz, CD$_3$OD): δ = 176.3 (C-18), 173.1 (C-15), 165.1 (C-27), 139.5 (C-12), 132.3 (C-7), 127.5 (C-10), 126.5 (C-1), 125.0 (C-4), 123.7 (C-2), 122.7 (C-9), 119.9 (C-8), 119.1 (C-6), 113.5 (C-5), 111.5 (C-11), 109.3 (C-3), 61.6 (C-26), 59.9 (C-25), 55.3 (C-23), 44.7 (C-13), 41.5 (C-24), 39.8 (C-17), 38.9 (C-16), 38.6 (C-19), 28.0 (C-22), 24.9 (C-21), 24.1 (C-20), 17.9 (C-14) ppm.

5.7.20. Benzyl 2-(2-fluoro-4'-nitrobiphenyl-4-yl)propanoate (113)
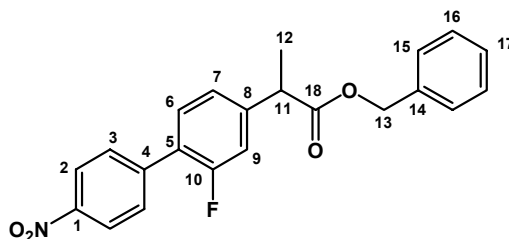
A mixture of 2-(2-fluoro-4-biphenyl)propanoic acid (500 g, 2.59 mmol) and 5 mL of 70% nitric acid was stirred with an efficient stirrer. The suspended solid gradually went into solution during the first 12 hours. The reaction was continued for another 48 h after which the TLC indicated complete consumption of starting material and formation of two products. The reaction mixture was poured on ice and extracted with CH$_2$Cl$_2$ (×3). The combined organic extracts were washed with water, brine, dried over anhydrous Na$_2$SO$_4$ and evaporated *in vacuo* to yield the crude mixture of the *ortho* and *para* nitrated product as an orange gummy mass.

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 h after which TLC indicated the complete consumption of 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 esters was purified by column chromatography (EtOAc:hexane, 2:8) to afford the title compound as brown gummy mass (250 mg, 32%).

¹H NMR (300 MHz, CDCl₃): δ = 8.30 (d, J = 9.0 Hz, 2H; H-2), 7.70 (dd, J = 7.2 Hz, J = 2.0 Hz, 2H; H-4), 7.42-7.10 (m, 8H; H-6, ; H-7, ; H-9, ; H-15, ; H-16, ; H-17), 5.18 (d, J = 15.0 Hz, 1H; H-13), 5.12 (d, J = 15.0 Hz, 1H; H-13′), 3.84 (q, J = 9.0 Hz, 1H; H-11), 1.57 (d, J = 9.0 Hz, 3H; H-12) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 174.3 (C-18), 161.4 (C-10), 147.3 (C-1), 140.6 (C-4), 140.1 (C-14), 137.6 (C-8), 130.6 (C-5), 130.5 (C-6), 129.9 (C-16), 129.8 (C-3), 127.7 (C-17), 127.5 (C-15), 125.0 (C-7), 124.1 (C-2), 115.8 (C-9), 66.8 (C-13), 45.1 (C-11), 18.3 (C-12) ppm.

5.7.21. Benzyl 2-(4'-amino-2-fluorobiphenyl-4-yl)propanoate (115)
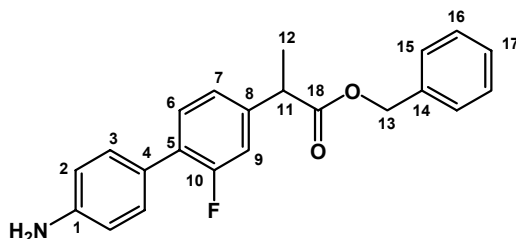
Anhydrous $SnCl_2$ (556 mg, 0.59 mmol) was added to a stirred solution of benzyl 2-(2-fluoro-4'-nitrobiphenyl-4-yl)propanoate (**113**) (225 mg, 0.59 mmol) in dry ethanol and refluxed for 6 h. The reaction mixture was then cooled to room temperature and poured on ice. The solution was basified using 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 column chromatography (EtOAc:hexane, 2:8) to afford the pure product as pale brown gum (120 mg, 57%).

¹H NMR (300 MHz, CDCl₃): δ = 7.42-7.10 (m, 8H; H-6; H-7; H-9; H-15; H-16; H-17), 7.03.- 6.97 (m, 2H; H-3), 6.68 (dd, J = 6.5 Hz, J = 2.1 Hz, 2H; H-2), 5.08 (d, J = 15.0 Hz, 1H; H-13), 5.02 (d, J = 15.0 Hz, 1H; H-13′), 3.70 (q, J = 7.0 Hz, 1H; H-11), 1.46 (d, J = 7.0 Hz, 3H; H-12) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 173.9 (C-18), 161.2 (C-10), 146.0 (C-1), 140.5 (C-8), 130.8 (C-5), 130.2 (C-6), 129.9 (C-16), 129.2 (C-3), 127.8 (C-17), 127.3 (C-15), 126.5 (C-7), 126.4 (C-4), 118.1 (C-9), 117.7 (C-2), 66.6 (C-13), 45.0 (C-11), 18.3 (C-12) ppm

5.7.22. Benzyl 2-(2-fluoro-4'-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)biphenyl-4-yl)propanoate (116)
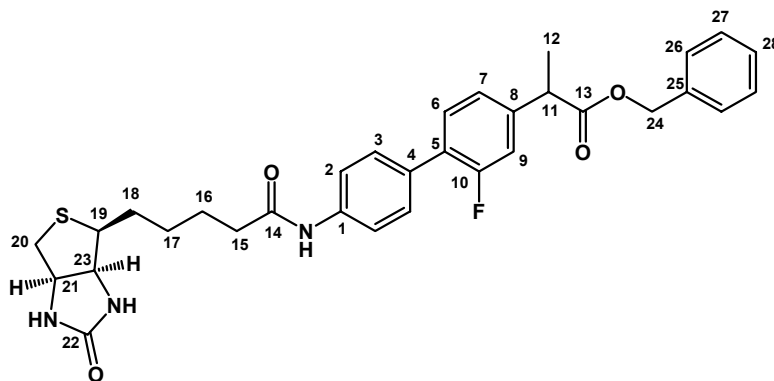
Et$_3$N (18 μL, 0.1281 mmol) was added to a stirred solution of *D*-biotin (32 mg, 0.1281 mmol) in anhydrous DMF (2 mL) and stirred at ambient temperature for 10 min. To it ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (31 mg, 0.1602 mmol), *N*-hydroxybenzotriazole hydrate (22 mg, 0.1602 mmol) were added and the resulting solution was stirred at ambient temperature for 30 min. until it became clear. A solution of benzyl 2-(4'-amino-2-fluorobiphenyl-4-yl)propanoate (**115**) (75 mg, 0.1602 mmol) in DMF (1 mL) was added to the reaction mixture followed by Et$_3$N (23 μL, 0.1602 mmol) and stirred at ambient temperature for 36 h. The reaction mixture was diluted with CHCl$_3$ (100 mL), washed sequentially with water, 0.2 N HCl, H$_2$O, 2 N NaOH, H$_2$O, brine and dried over anhydrous Na$_2$SO$_4$ and evaporated *in vacuo* to yield the crude product. The crude product was purified by flash column chromatography (CH$_2$Cl$_2$:MeOH, 95:5) to afford the title compound as brownish solid (72 mg, 65%).

^{1}H NMR (500 MHz, CD$_3$OD): δ = 7.56 (dd, *J* = 6.8 Hz, *J* = 1.8 Hz, 2H; H-2), 7.40 (dd, *J* = 8.5 Hz, *J* = 1.2 Hz, 2H; H-3), 7.28 (t, *J* = 8.0 Hz, 1H; H-6), 7.25-7.15 (m, 5H; H-26, H-27, H-28), 7.04 (dd, *J* = 8.0 Hz, *J* = 1.6 Hz, 1H; H-7), 6.98 (dd, *J* = 6.0 Hz, *J* = 1.6 Hz, 1H; H-9), 5.04 (s, 2H; H-24), 4.42 (q, *J* = 4.2 Hz, 1H; H-23), 4.24 (p, *J* = 4.1 Hz, 1H; H-21), 3.74 (q, *J* = 7.2 Hz, 1H; H-11), 3.14-3.10 (m, 1H; H-19), 2.83 (dd, *J* = 6.8 Hz, *J* = 5.0 Hz, 1H; H-20), 2.65 (d, *J* = 6.8 Hz, 1H; H-20′), 2.34 (t, *J* = 7.2 Hz, 2H, 15-C), 1.74-1.63 (m, 4H, 16-H, 18-H), 1.62-1.54 (m, 2H, 17-H), 1.44 (d, *J* = 7.2 Hz, 3H, 12-H) ppm.

^{13}C NMR (125 MHz, CD$_3$OD): δ = 176.1 (C-13), 174.8 (C-14), 164.8 (C-22), 160.4 (C-10), 143.2 (C-25), 139.8 (C-1), 137.5 (C-8), 132.8 (C-4), 130.9 (C-5), 130.1 (C-6), 129.8 (C-26), 129.5 (C-27), 129.1 (C-3), 125.3 (C-7), 121.6 (C-2), 116.9 (C-9), 68.4 (C-24), 63.7 (C-23), 62.0 (C-21), 57.3 (C-19), 46.7 (C-11), 41.8 (C-20), 38.2 (C-18), 30.2 (C-16), 29.9 (C-17), 27.1 (C-16), 19.5 (C-12) ppm.

5.7.23.   2-(2-fluoro-4'-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)biphenyl-4-yl)propanoic acid (117)
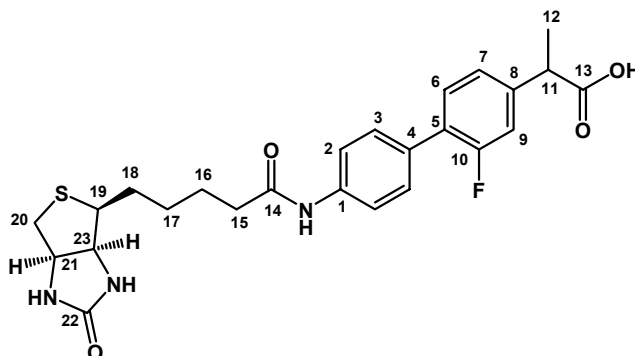
10% Pd-C (30% w/w, 21 mg) was added to a stirred solution of benzyl 2-(2-fluoro-4'-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)biphenyl-4-yl)propanoate (**116**), (70 mg, 0.1 mmol) in MeOH (5 mL) and the resulting solution was kept under H$_2$ at 60 psi for 18 h. Reaction was filtered through the celite bed and the celite bed was washed several times with warm MeOH. The combined organic extract was evaporated *in vacuo* to yield the crude product. The crude compound was purified by flash column chromatography (CH$_2$Cl$_2$:MeOH, 95:5) to afford the title compound as a cream colored solid (52 mg, 87%).

^{1}H NMR (300 MHz, CD$_3$OD): δ = 7.60 (d, *J* = 8.8 Hz, 2H; H-2), 7.40 (d, *J* = 7.2 Hz, 2H; H-3), 7.30 (t, *J* = 8.0 Hz, 1H; H-6), 7.12-7.08 (m, 2H; H-7, H-9), 4.43-4.24 (m, 2H; H-23, H-21), 3.78 (q, *J* = 7.2 Hz, 1H; H-11), 3.15-3.12 (m, 1H; H-19), 2.88 (dd, *J* = 6.8 Hz, *J* = 4.9 Hz, 1H; H-20), 2.66 (d, *J* = 6.8 Hz, 1H; H-20′), 2.36 (t, *J* = 7.2 Hz, 2H; H-15), 1.74-1.64 (m, 4H; H-16, H-18), 1.63-1.54 (m, 2H; H-17), 1.44 (d, *J* = 7.2 Hz, 3H, 12-H) ppm.

^{13}C NMR (75 MHz, CD$_3$OD): δ = 178.2 (C-13), 172.2 (C-14), 165.1 (C-22), 161.1 (C-10), 137.9 (C-1), 136.4 (C-8), 132.4 (C-4), 130.1 (C-5), 129.8 (C-6), 128.8 (C-3), 123.4 (C-2), 119.4 (C-9), 61.4 (C-23), 59.7 (C-21), 55.3 (C-19), 42.9 (C-11), 41.8 (C-20), 36.1 (C-15), 28.1 (C-18), 25.3 (C-16), 25.0 (C-17), 18.0 (C-12) ppm.

5.7.24. Benzyl 2-(9-(3-aminobenzyl)-3-chloro-9*H*-carbazol-7-yl)propanoate (125)
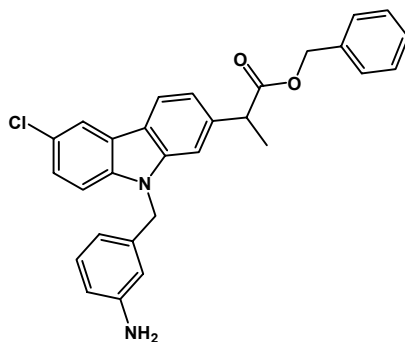
To a stirred solution of compound of benzyl 2-(9-(3-nitrobenzyl)-3-chloro-9*H*-carbazol-7-yl)propanoate (**66c**) (80 mg, 0.16 mmol) in absolute ethanol (3 mL), was added anhydrous SnCl$_2$ (152 mg, 0.8 mmol) and refluxed for 3 h. Reaction was monitored by TLC. The reaction mixture was then cooled to room temperature, basified up to pH:9 using 2N NaOH and extracted with EtOAc. The aqueous layer was extracted with EtOAc (3×). The combined organic extract was washed with H$_2$O, brine, dried over anhydrous Na$_2$SO$_4$ and evaporated *in vacuo* to yield the crude product. The crude compound was used for the next step without further purification.

5.7.25. Benzyl 2-(9-(3-(5-(hexahydro-2-oxo-1*H*-thieno[3,4-d]imidazol-6-yl)pentanamido)benzyl)-3-chloro-9*H*-carbazol-7-yl)propanoate (125)
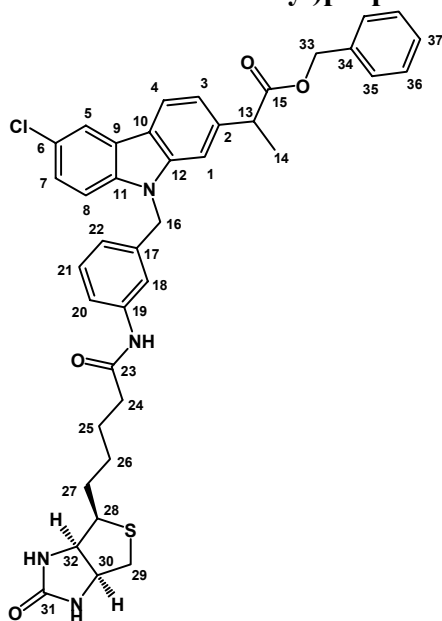
To a stirred solution of *D*-biotin (32 mg, 0.13 mmol) in anhydrous DMF (2 mL), was added Et₃N (18 µL, 0.13 mmol) and stirred at ambient temperature for 10 min. To it ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (31 mg, 0.16 mmol), *N*-hydroxybenzotriazole hydrate (22 mg, 0.16 mmol) were added and the resulting solution was stirred at ambient temperature for 30 min. until it became clear. A solution of benzyl 2-(9-(3-aminobenzyl)-3-chloro-9*H*-carbazol-7-yl)propanoate, **(66c)** (75 mg, 0.16 mmol) in DMF (1 mL) was added to the reaction mixture followed by Et₃N (23 µL, 0.16 mmol) and stirred at ambient temperature for 18 h. The reaction mixture was diluted with CHCl₃ (100 mL), washed sequentially with water, 0.2 N HCl, H₂O, 2 N NaOH, H₂O, brine and dried over anhydrous Na₂SO₄ and evaporated *in vacuo* to yield the crude product. The crude product was purified by flash column chromatography (CH₂Cl₂:MeOH, 95:5) to afford the title compound **11** as brownish solid (72 mg, 65%).

¹H NMR (300 MHz, CDCl₃): δ = 7.93 (d, *J* = 1.2 Hz, 1H; H-5), 7.89 (d, *J* = 8.1 Hz, 1H; H-4), 7.43 (d, *J* = 8.0 Hz, 1H; H-8), 7.29-7.04 (m, 11H; H-1, H-3, H-7, H-18, H-20/21, H-34/35/36), 6.68 (d, *J* = 7.4 Hz, 1H; H-22), 5.32 (s, 2H; H-16), 5.05-4.94 (m, 2H; H-33), 4.38 - 4.40 (m, 1H; H-32), 4.18 - 4.14 (m, 1H; H-30), 3.84 (q, *J* = 7.2 Hz, 1H; H-13), 3.06 - 2.97 (m, 1H; H-28), 2.84 - 2.72 (m, 1H; H-29), 2.62 - 2.53 (m, 1H; H-29′), 2.19 (t, *J* = 7.3 Hz, 2H; H-24), 1.66 - 1.42 (m, 4H; H-25/27), 1.54 (d, *J* = 7.2 Hz, 3H; H-14), 1.42 - 1.30 (m, 2H; H-26) ppm.

¹³C NMR (75 MHz, CDCl₃): δ = 174.8 (C-23), 172.4 (C-13), 164.1 (C-31), 141.5 (C-34), 139.3 (C-19), 139.0 (C-12), 137.6 (C-17), 135.9 (C-10), 129.5 (C-36), 128.5 (C-21), 128.2 (C-6), 127.9 (C-37), 127.6 (C-2), 127.2 (C-35), 125.9 (C-22), 125.5 (C-11), 122.9 (C-5), 122.0 (C-3), 121.9 (C-18), 121.3 (C-4), 120.7 (C-3), 120.0 (C-20), 119.4 (C-8), 117.6 (C-1), 110.2 (C-9), 66.7 (C-33), 61.9 (C-32), 60.6 (C-30), 55.6 (C-28), 46.2 (C-13), 40.4 (C-29), 36.7 (C-24), 28.4 (C-27), 25.4 (C-25/26), 18.8 (C-14) ppm

5.7.26. 2-(9-(3-(5-(Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanamido)benzyl)-3-chloro-9*H*-carbazol-7-yl)propanoic acid (126)
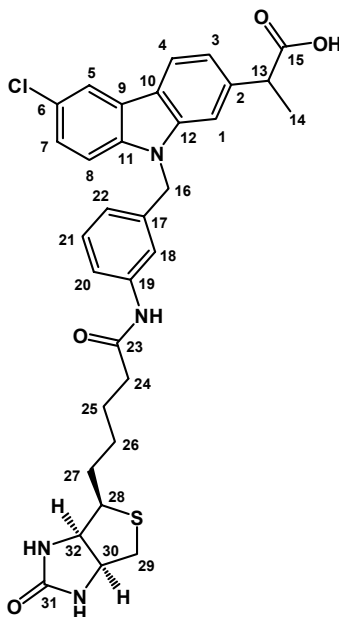
To a stirred solution of benzyl 2-(9-(3-(5-(hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanamido)benzyl)-3-chloro-9*H*-carbazol-7-yl)propanoate, (**125**) (70 mg, 0.1039 mmol) in MeOH (5 mL) was added 10% Pd-C (30% w/w, 21 mg) and the resulting suspension was kept under H_2 at 60 psi for 18 h. The reaction mixture was filtered through the celite bed and the celite bed was washed several times with warm MeOH. The combined organic extract was evaporated *in vacuo* to yield the crude product. The crude compound was further purified by flash column chromatography (CHCl$_3$:MeOH, 95:5) to afford the title compound as a cream colored solid. (48 mg, 80%)

mp: 186.6 °C

^{1}H NMR (500 MHz, CDCl$_3$): δ = 7.93 (s, 1H; H-5), 7.88 (d, *J* = 8.0 Hz, 1H; H-4), 7.52 (s, 1H; H-1), 7.47 (d, *J* = 7.8 Hz, 1H; H-8), 7.34 (s, 1H; H-18), 7.24 – 7.18 (m, 2H; H-7, H-20), 7.16 (d, *J* = 8.1 Hz, 1H; H-3), 7.12 (t, *J* = 7.9 Hz, 1H; H-21), 6.79 (d, *J* = 7.6 Hz, 1H; H-22), 5.38 (s, 2H; H-16), 4.38 - 4.34 (m, 1H; H-32), 4.17 - 4.14 (m, 1H; H-30), 3.84 (q, *J* = 7.2 Hz, 1H; H-13), 3.07 – 3.02 (m, 1H; H-28), 2.79 (dd, *J* = 6.7, 5.0 Hz, 1H; H-29), 2.62 (d, *J* = 5.0 Hz, 1H; H-29′), 2.22 (t, *J* = 7.2 Hz, 2H; H-24), 1.64 - 1.55 (m, 4H; H-25, H-27), 1.42 (d, *J* = 7.2 Hz, 3H; H-14), 1.37 - 1.31 (m, 2H; H-26) ppm.

^{13}C NMR (125 MHz, CDCl$_3$): δ = 177.6 (C-15), 174.6 (C-23), 167.6 (C-31), 141.1 (C-19), 138.8 (C-12), 137.7 (C-17), 135.5 (C-10), 128.8 (C-21), 128.1 (C-2), 127.8 (C-6), 125.8 (C-22), 125.6 (C-11), 122.1 (C-5) (C-3), 121.9 (C-18), 120.1 (C-4), 119.7 (C-7), 118.9 (C-20), 116.3 (C-8), 115.2 (C-1), 107.8 (C-1), 61.6 (C-32), 59.9 (C-30), 55.2 (C-28), 46.0 (C-13), 39.7 (C-29), 36.0 (C-24), 28.0 (C-27), 27.7 (C-25), 25.0 (C-26), 18.8 (C-14) ppm.

MS (ESI): *m/z* = 627.2 (M+Na)$^+$.

5.8. Synthesis of Flurbiprofen Derived Photoaffinity Label.

5.8.1. Benzyl 2-(4'-(2-chloroacetamido)-2-fluorobiphenyl-4-yl)propanoate (127)
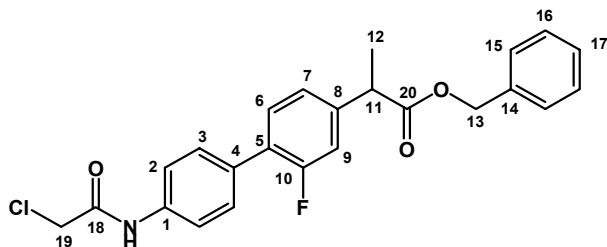
Triethlyl amine (0.098 mL, 0.7 mmol) was added to a stirred solution of benzyl 2-(4'-amino-2-fluorobiphenyl-4-yl)propanoate (**115**) (205 mg, 0.58 mmol) in dry CH_2Cl_2 at 0 °C and stirred for 30 minutes. Chloroacetyl chloride (0.056 µL, 0.7 mmol) was added to it drop wise and stirred for another 30 minutes. The reaction mixture was then allowed to attain the room temperature and stirred for another 2.5 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 (EtOAc:hexane, 2:8) to obtain the title compound as colorless solid (240 mg, 95%).

¹H NMR (500 MHz, CDCl₃): δ = 7.55 (brs, 1H; NH), 7.55 (td, J = 8.5 Hz, J = 1.8 Hz, 2H; H-2), 7.47 (td, J = 7.2 Hz, J = 1.7 Hz, 2H; H-3), 7.31 (d, J = 8.0 Hz, 1H; H-9), 7.29-7.20 (m, 5H; H-15, H-16, H-17), 7.08 (dd, J = 8.0 Hz, J = 1.8 Hz, 1H; H-7), 7.04 (dd, J = 11.0 Hz, J = 1.8 Hz, 1H; H-9), 5.08 (d, J = 6.5 Hz, 1H; H-13), 5.04 (d, J = 6.5 Hz, 1H; H-13′), 4.14 (s, 2H; H-19), 3.72 (q, J = 7.2 Hz, 1H; H-11), 1.48 (d, J = 7.2 Hz, 3H; H-12) ppm.

¹³C NMR (125 MHz, CDCl₃): δ = 175.3 (C-20), 165.4 (C-18), 158.0 (C-10), 141.5 (C-14), 137.8 (C-1), 137.4 (C-8), 133.5 (C-4), 130.8 (C-5), 130.2 (C-6), 130.1 (C-16), 129.0, (C-3) 127.9 (C-15), 126.1 (C-7), 121.6 (C-2), 117.3 (C-9), 68.3 (C-20), 46.6 (C-11), 44.5 (C-19), 19.9 (C-12) ppm.

5.8.2. Benzyl 2-(4'-(2-(4-(4-(2-tert-butoxy-2-oxoethoxy)benzoyl)phenoxy)acetamido)-2 fluorobiphenyl-4-yl)propanoate (129)
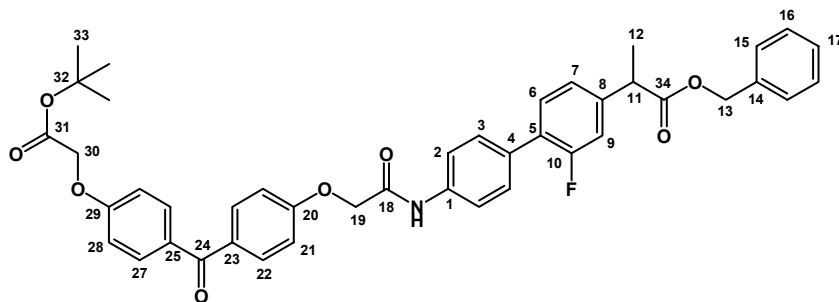
Anhydrous K_2CO_3 (222 mg, 1.6 mmol) and benzyl 2-(4'-(2-chloroacetamido)-2-fluorobiphenyl-4-yl)propanoate (**128**) (190 mg, 0.44 mmol) were added to a stirred solution of tert-butyl 2-(4-(4-hydroxybenzoyl)phenoxy)acetate (176 mg, 0.54 mmol) in dry acetone and heated to 60–70 °C for 12 hours. The reaction mixture was then cooled to room temperature and filtered. The residue was washed with acetone (×3). The combined organic layer was rotary evaporated to yield the crude product. The crude product was purified by column chromatography (EtOAc:hexane, 1:4) to afford the title compound as colorless solid (185 mg, 57%).

¹H NMR (300 MHz, CDCl₃): δ = 8.30 (brs, 1H; NH), 7.84 (dd, J = 7.0 Hz, J = 1.9 Hz, 2H; H-23), 7.79 (dd, J = 9.2 Hz, J = 1.9 Hz, 2H; H-25), 7.70 (d, J = 8.7 Hz, 2H; H-1), 7.56 (dd, J = 8.5 Hz, J = 1.4 Hz, 2H; H-4), 7.39 (dd, J = 9.0 Hz, J = 2.4 Hz, 1H; H-6), 7.37-7.28 (m, 5H;

H-15, H-16, H-17), 7.16 (dd, J = 8.5 Hz, J = 1.9 Hz, 2H; H-20), 7.11 (dd, J = 8.8 Hz, J = 1.9 Hz, 2H; H-29), 7.07-6.95 (m, 2H; H-7, H-9), 5.17 (d, J = 6.5 Hz, 1H; H-13), 5.10 (d, J = 6.5 Hz, 1H; H-13′), 4.75 (s, 2H; H-30), 4.63 (s, 2H; H-19), 3.83 (q, J = 7.2 Hz, 1H; H-11), 1.55 (d, J = 7.2 Hz, 3H; H-12), 1.52 (s, 9H; H-33) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 194.2 (C-24), 173.8 (C-34), 167.5 (C-31), 161.4 (C-20, C-29), 159.9 (C-10), 141.4 (C-14), 139.6 (C-1), 136.2 (C-8), 132.5 (C-26, C-28), 132.3 (C-4), 131.5 (C-22, C-27), 131.7 (C-6), 130.6 (C-6), 129.7 (C-16), 128.6 (C-3), 128.3 (C-17), 128.1 (C-15), 125.8 (C-7), 123.8 (C-2), 118.2 (C-9), 115.6 (C-21), 115.3 (C-28), 82.9 (C-32), 67.6 (C-13), 66.8 (C-19), 65.6 (C-30), 45.1 (C-11), 28.1 (C-33), 18.4 (C-12) ppm.

5.8.3.   2-(4-(4-(2-(4'-(1-(Benzyloxy)-1-oxopropan-2-yl)-2'-fluorobiphenyl-4-ylamino)-2-oxoethoxy)benzoyl)phenoxy)acetic acid (130)
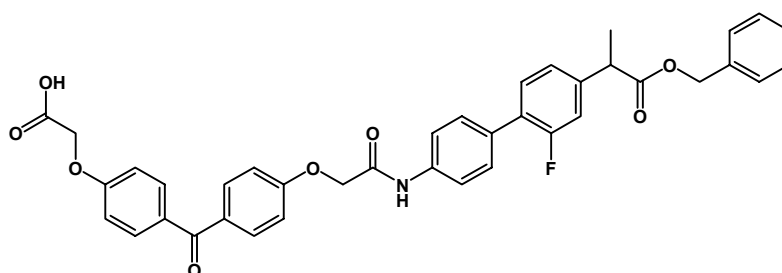
Trifluoroacetic acid (0.4 mL) was added to a stirred solution of benzyl 2-(4'-(2-(4-(4-(2-tert-butoxy-2-oxoethoxy)benzoyl)phenoxy)acetamido)-2   fluorobiphenyl-4-yl)propanoate (**129**) (180 mg, 0.25 mmol) in CH$_2$Cl$_2$ (2 mL) at 0 °C and stirred for 6 hours. It was then evaporated *in vacuo* to afford 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* = 684.3 (M+Na)$^+$.

5.8.4.   Benzyl   2-(4'-(2-(4-(4-(2-(2-(tert-butoxycarbonylamino)ethylamino)-2-oxoethoxy)benzoyl)phenoxy)acetamido)-2-fluorobiphenyl-4-yl)propanoate (131)
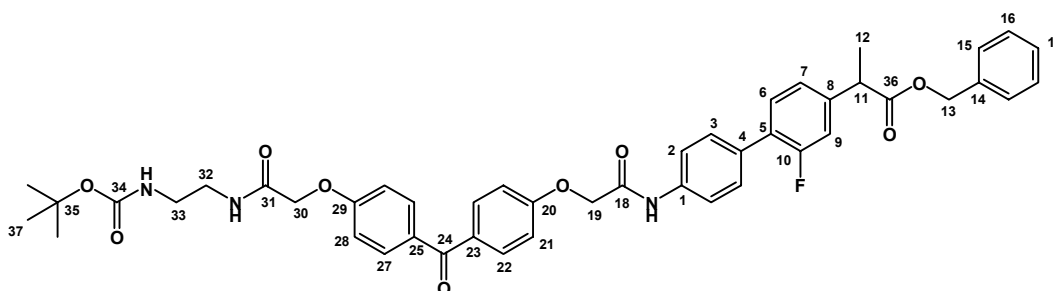
To a stirred solution of 2-(4-(4-(2-(4'-(1-(benzyloxy)-1-oxopropan-2-yl)-2'-fluorobiphenyl-4-ylamino)-2-oxoethoxy)benzoyl)phenoxy)acetic acid (**130**) (150 mg, 0.22 mmol) in anhydrous CH$_2$Cl$_2$ (2 mL), was added Et$_3$N (0.032 mL, 0.22 mmol) and stirred at ambient temperature for 10 min. Ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (48 mg, 0.25 mmol), *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 ethylene diamine (43 μL, 0.27 mmol) in CH$_2$Cl$_2$ (1 mL) was added to the reaction mixture followed by Et$_3$N (0.038 mL, 0.27 mmol) and stirred at ambient temperature for 16 h. The reaction mixture was diluted with CHCl$_3$ (100 mL), washed with water, brine, dried over anhydrous Na$_2$SO$_4$ and evaporated *in vacuo* to yield the crude product. The crude product was purified by flash column chromatography (EtOAc:hexane, 3:7) to yield the title compound as colorless solid (103 mg, 54%)

¹H NMR (500 MHz, CDCl₃): δ = 8.21 (brs, 1H; NH), 7.88 (dd, *J* = 7.0 Hz, *J* = 2.0 Hz, 2H; H-22), 7.74 (dd, *J* = 7.0 Hz, *J* = 2.0 Hz, 2H; H-27), 7.61 (td, *J* = 8.5 Hz, *J* = 2.1 Hz, 2H; H-1), 7.48 (dd, *J* = 8.5 Hz, *J* = 1.4 Hz, 2H; H-4), 7.32-7.19 (m, 6H; H-15, H-16, H-15, H-6), 7.08-7.02 (m, 4H; H-7, H-9, H-21), 6.96 (dd, *J* = 7.0 Hz, *J* = 2.0 Hz, 2H; H-28), 5.08 (d, *J* = 6.5 Hz, 1H; H-13), 5.04 (d, *J* = 6.5 Hz, 1H; H-13´), 4.98 (brs, 1H; NH), 4.67 (s, 2H; H-19), 4.65 (s, 2H; H-30), 3.74 (q, *J* = 7.2 Hz, 1H; H-11), 3.40 (q, *J* = 6.4 Hz, 2H; H-32), 3.30-3.26 (m, 2H; H-33), 1.48 (d, *J* = 7.2 Hz, 3H; H-12), 1.36 (s, 9H; H-37) ppm.

¹³C NMR (125 MHz, CDCl₃): δ = 192.1 (C-24), 171.8 (C-36), 166.2 (C-18), 164.6 (C-29, C-20), 163.6 (C-31), 158.8 (C-34), 158.6 (C-10), 141.1 (C-14), 137.8 (C-1), 136.9 (C-8), 132.8 (C-4), 132.2 (C-23, C-25), 131.5 (C-27), 130.6 (C-5), 130.5 (C-6), 130.4 (C-16), 129.8 (C-3), 128.7 (C-17), 128.6 (C-15), 127.8 (C-7), 121.8 (C-2), 118.2 (C-9), 113.6 (C-21), 113.4 (C-29), 78.0 (C-35), 65.7 (C-13), 65.3 (C-19), 64.8 (C-30), 43.1 (C-12), 38.7 (C-32), 38.2 (C-33), 26.4 (C-37), 16.4 (C-12) ppm.

MS (ESI): *m/z* = 826.5 (M+Na)⁺.

5.8.5. Benzyl 2-(4'-(2-(4-(4-(2-aminoethylamino)-2-oxoethoxy)benzoyl)phenoxy)acetamido)-2-fluorobiphenyl-4-yl)propanoate (132)
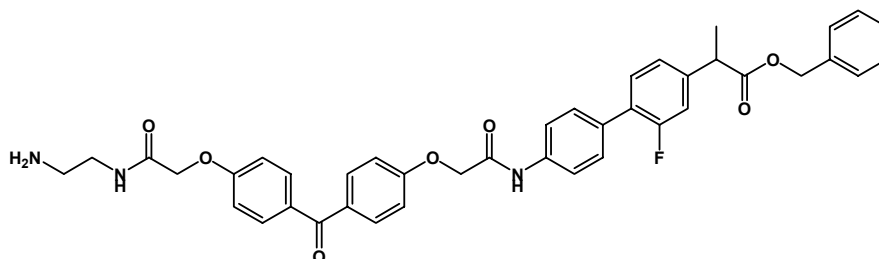
A solution of benzyl 2-(4'-(2-(4-(4-(2-(2-(tert-butoxycarbonylamino)ethylamino)-2-oxoethoxy)benzoyl)phenoxy)acetamido)-2-fluorobiphenyl-4-yl)propanoate (**131**) (90 mg, 0.11 mmol) in 16% HCl in dioxane (1 mL) was stirred at ambient temperature for 1 h. Dioxane was evaporated *in vacuo* and lyophilized to obtain the title compound as hydrochloride salt. The crude compound was used for next step without further purification.

MS (ESI): *m/z* = 704.4 (M+H)⁺.

5.8.6. 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 (133)
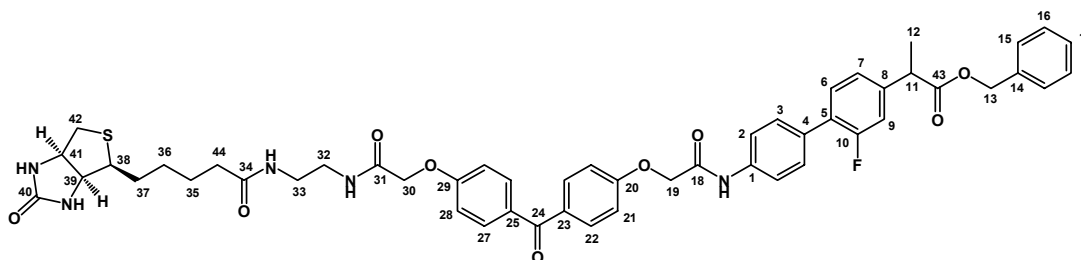
To a stirred solution of *D*-biotin (33 mg, 0.13 mmol) in anhydrous DMF (2 mL), was added DIEA (0.024 mL, 0.13 mmol) and stirred at ambient temperature for 10 min. To it PyBrop (70 mg, 0.15 mmol) was added and the resulting solution was stirred at ambient temperature for 30 min. until it became clear. 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) (**132**) in DMF (1 mL) was added to the reaction mixure followed by di-isopropyl ethyl amine (0.071 mL, 0.4 mmol) and stirred at ambient temperature for 18 h. The reaction mixture was diluted with CHCl₃ (100 mL), washed with water, brine, dried over anhydrous

Na$_2$SO$_4$ and evaporated *in vacuo* to yield the crude product. The crude product was purified by flash column chromatography (CH$_2$Cl$_2$:MeOH, 95:5) to afford the title compound as brownish solid (22 mg, 25%).

^{1}H NMR (300 MHz, CDCl$_3$): δ = 9.90 (brs, 1H; NH), 8.05 (brs, 1H; NH), 7.80-7.71 (m, 6H; H-1, H-22, H-27), 7.65 (brs, 1H; NH), 7.50-7.36 (m, 5H; H-4, H-6, H-7, H-9), 7.16-7.07 (m, 5H; H-15, H-16, H-17), 6.10 (brs, 1H; NH), 5.14 (d, *J* = 6.5 Hz, 1H; 13), 5.08 (d, *J* = 6.5 Hz, 1H; 13′), 4.78 (s, 2H; H-19), 4.55 (s, 2H; H-30), 4.40-4.34 (m, 1H; H-41), 4.22-4.15 (m, 1H; H-42), 3.85 (q, *J* = 7.2 Hz, 1H; H-11), 3.24-3.12 (m, 4H; H-32, H-33), 3.10-3.01 (m, 1H; H-38), 2.80 (dd, *J* = 6.6 Hz, *J* = 5.0 Hz, 1H; H-42), 2.65 (d, *J* = 6.6 Hz, 1H; H-42′), 2.13 (t, *J* = 7.3 Hz, 2H; H-44), 1.76-1.55 (m, 4H; H-37, H-35), 1.52 (d, *J* = 7.2 Hz, 3H; H-12), 1.43-1.35 (m, 2H; H-36) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 192.1 (C-24), 176.0 (C-43), 174.8 (C-34), 166.2 (C-18), 164.2 (C-20), 163.9 (C-29), 162.4 (C-31), 159.5 (C-40), 158.4 (C-10), 142.4 (C-14), 137.6 (C-1), 139.6 (C-8), 132.5 (C-23, C-25), 132.1 (C-4), 131.5 (C-22, C-27), 130.5 (C-5), 130.4 (C-6), 130.3 (C-16), 129.6 (C-3), 128.5 (C-15), 128.4 (C-17), 127.5 (C-7), 121.6 (C-2), 118.6 (C-9), 114.2 (C-21, C-28), 65.7 (C-13), 65.3 (C-30), 64.8 (C-19), 63.7 (C-39), 62.0 (C-41), 57.7 (C-38), 43.1 (C-11), 41.8 (C-42), 38.7 (C-32), 38.2 (C-33), 37.1 (C-44), 29.8 (C-37), 27.2 (C-36), 27.1 (C-35), 17.4 (C-12) ppm.

MS (ESI): *m/z* = 952.4 (M+Na)$^+$, 928.3 (M–H)$^+$.

5.8.7. 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 (134)
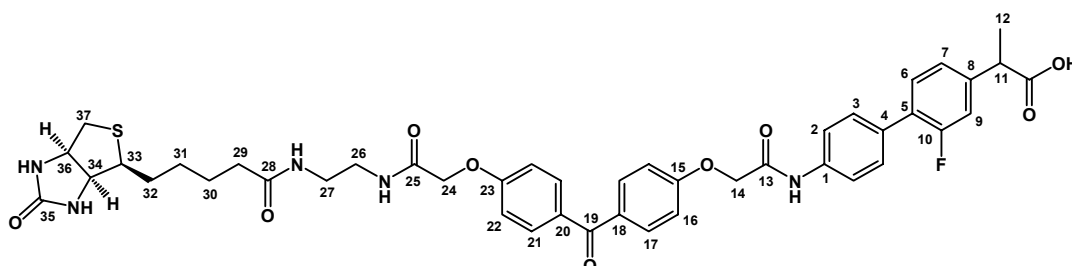
To a stirred solution of benzyl 2-(2-fluoro-4'-(2-(4-(4-(2-oxo-2-(2-(5-(2-oxohexahydro-1*H* thieno[3,4-*d*]imidazol-4yl)pentanamido)ethylamino)ethoxy)benzoyl)phenoxy)acetamido) biphenyl-4yl)propanoate (**133**) (22 mg, 0.02 mmol) in MeOH (5 mL) was added 10% Pd-C (50% w/w, 11 mg) and the resulting suspension was kept under H$_2$ at 60 psi at ambient temperature for 36 h. The reaction mixture was filtered through the celite bed and the celite bed was washed several times with warm MeOH. The combined organic extract was evaporated *in vacuo* to yield the crude product. The crude compound was purified by flash column chromatography (CH$_2$Cl$_2$:MeOH, 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, 6H; H-21, H-17, H-2), 7.45 (brs, 1H; NH), 7.41 (brs, 1H; NH), 7.34-7.26 (m, 2H; H-3), 7.24-7.18 (m, 2H; H-6, H-7), 7.10-6.80 (m, 5H; H-9, H-16, H-22), 4.70 (s, 2H; H-14), 4.48 (s, 2H; H-24), 4.40-4.33 (m, 1H; H-36), 4.22-4.16 (m, 1H; H-34), 4.05 (q, *J* = 7.2 Hz, 1H, H-11), 3.38-3.22 (m, 4H; H-26, H-27), 3.08-3.01 (m, 1H; H-33), 2.78 (dd, *J* = 6.9 Hz, *J* = 4.8 Hz, 1H; H-37), 2.66 (d, *J* = 6.9 Hz, 1H; H-37′), 2.10 (t, *J* = 7.2 Hz, 2H; H-29), 1.66-1.49 (m, 4H; H-30, H-32), 1.47 (d, *J* = 7.2 Hz, 3H; H-12), 1.37-1.28 (m, 2H; H-31) ppm.

^{13}**C NMR (75 MHz, CDCl$_3$):** δ = 192.1 (C-19), 176.0 (C-11), 174.8 (C-28), 166.2 (C-13), 164.2 (C-23, C-15), 159.5 (C-35), 158.4 (C-10), 139.6 (C-1), 137.5 (C-8), 134.0 (C-4), 132.7 (C-20, C-18), 131.4 (C-17, C-21), 130.4 (C-5), 129.8 (C-6), 128.8 (C-3), 124.9 (C-7), 121.8 (C-2), 117.8 (C-9), 115.6 (C-16, C-22), 65.3 (C-14), 64.8 (C-24), 63.3 (C-36), 61.0 (C-34), 57.0 (C-33), 44.5 (C-11), 40.8 (C-37), 40.1 (C-27), 37.0 (C-26), 36.1 (C-29), 30.3, (C-32), 27.2 (C-31), 27.1 (C-30), 15.1 (C-12) ppm.

MS (ESI): m/z = 862.4 (M+Na)$^+$, 838.3 (M–H)$^+$

5.9. Synthesis of DAPT Derived Photoaffinity Label.

5.9.1. Z-Ala-Phg-O(*t*-Bu) (150)
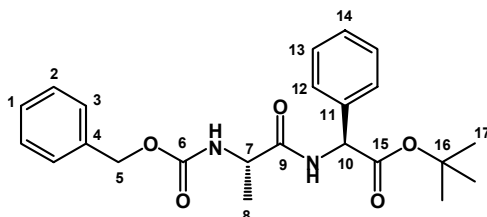
Et$_3$N (0.46 mL, 3.33 mmol) was added to a solution of Z-alanine (0.75 g, 3.33 mmol) in CH$_2$Cl$_2$ (15 mL) followed by the addition EDAC (0.77 g, 4 mmol, HOBt (0.54 g, 4 mmol) and stirred at RT until it became clear. Et$_3$N (0.46 mL, 3.33 mmol) and Phg-O(*t*-Bu)·HCl (0.65 g, 2.67 mmol) was added to it simultaneously. The reaction mixture stirred at RT for 12 h. The reaction mixture was then diluted with CH$_2$Cl$_2$ (300 mL). The organic phase was washed with NaHCO$_3$, (0.1 N) HCl and brine simultaneously. The organic phase was dried over anhydrous Na$_2$SO$_4$ and the solvent was evaporated *in vacuo* to afford the crude product which was further purified by column chromatography (EtOAc:hexane, 3:7) to get the pure product as colorless solid (1.1 g, 80%).

^{1}**H-NMR (300 MHz, DMSO- D_6):** δ = 8.52 (d, J = 7.4 Hz, 1H; NH,), 8.47 (d, J = 7.0 Hz, 1H; NH), 7.42–7.33 (m, 10H; H-1, H-2, H-3, H-12, H-13, H-14), 5.26 (d, 1H, J = 7.3 Hz, 1H; H-10), 5.32 (s, 2H; H-5), 4.41–4-37 (m, 1H; H-7), 1.35 (s, 9H; H-17), 1.11 (d, J = 7.0 Hz; 3H, H-8) ppm.

^{13}**C-NMR (75 MHz, DMSO- D_6):** δ = 175.0 (C-15), 169.3 (C-9), 164.7 (C-6), 138.7 (C-4), 136.9 (C-8), 128.5 (C-9), 128.0 (C-11), 127.9 (C-2), 127.4 (C-1), 127.0 (C-3, C-10), 81.2 (C-13), 65.8 (C-5), 56.5 (C-10), 52.2 (C-7), 27.6 (C-17), 19.5 (C-8) ppm.

5.9.2. Ala-Phg-O(*t*-Bu) (151)
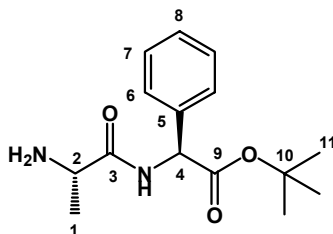
10% Pd-C (20% w/w, 0.6 g) was added to the solution of Z-Ala-Phg-O(*t*-Bu), (3 g, 7.3 mmol) in EtOH (50 mL) and the resulting suspension was kept under H$_2$ at 60 psi, at ambient temperature for 24 h. The reaction mixture was filtered through the celite bed and the celite bed was washed several times with warm EtOH. The combined organic extract was evaporated *in vacuo* to yield the crude product. The crude compound was was used in the next step without further purification.

¹H-NMR (300 MHz, DMSO- *D₆*): δ = 8.50 (d, *J* = 7.3 Hz, 1H; NH), 7.41–7.37 (m, 5H; H-6, H-7, H-8), 5.22 (d, *J* = 7.3 Hz, 1H; H-4); 4.38–4-35 (m, 1H; H-2), 1.33 (s, 9H; H-11), 1.13 (d, *J* = 7.2 Hz, 3H, H-1) ppm.

MS (EI): *m/z* = 278 (M⁺, 40%), 235 (100%), 215 (12%), 205 (78%).

5.9.3. (*S*)-Tert-butyl 2-((S)-2-(2-(3,5-difluorophenyl)acetamido)propanamido)-2-phenylacetate (DAPT) (145)
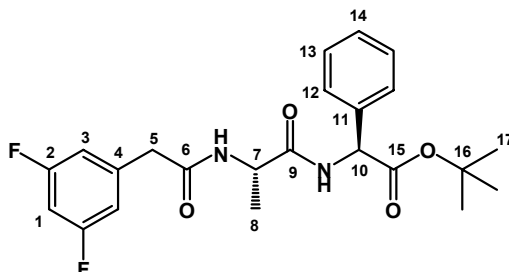
Et₃N (1.18 mL, 8.55 mmol) was added to a solution of 3,5-difluoro phenylacetic acid (1.47 g, 8.55 mmol) in CH₂Cl₂ (30 mL) followed by the addition EDAC (1.8 g, 9.4 mmol), HOBt (1.27 g, 9.4 mmol) and stirred at RT until it became clear. Et₃N (1.18 mL, 8.55 mmol) and Ala-Phg-O(*t*-Bu) (1.9 g, 6.84 mmol) was added to it simultaneously. The reaction mixture stirred at RT for 12 h. The reaction mixture was then diluted with CH₂Cl₂ (500 mL). The organic phase was washed with NaHCO₃, (0.1 N) HCl and brine. The organic phase was dried over anhydrous Na₂SO₄ and the solvent was evaporated *in vacuo* to afford the crude product which was further purified by column chromatography (CH₂Cl₂:MeOH, 9:1) to get the pure product as colorless solid (1.47 g, 50%).

¹H-NMR (300 MHz, DMSO- *D₆*): δ = 8.48 (d, *J* = 7.0 Hz, 1H; NH), 8.32 (d, *J* = 7.1 Hz 1H; NH), 7.42–7.33 (m, 5H; H-12, H-13, H-14), 7.06 (m, 1H; H-1), 7.00-6.93 (m, 2H; H-3), 5.44 (d, *J* = 7.0 Hz, 1H; H-10), 4.61–4-58 (m, 1H; H-7), 3.54 (s, 2H, H-5), 1.40 (s, 9H; H-17), 1.21 (d, *J* = 7.0 Hz, 3H, H-8) ppm.

¹³C-NMR (75 MHz, DMSO- *D₆*): δ = 172.5 (C-15), 171.3 (C-9), 170.8 (C-6), 164.8 (C-2), 138.1 (C-4), 136.9 (C-11), 129.0 (C-12), 128.8 (C-13), 127.4 (C-14), 113.1 (C-3), 103.4 (C-1), 81.0 (C-16), 56.3 (C-10), 50.2 (C-7), 41.4 (C-5), 27.8 (C-17), 18.5 (C-8) ppm.

5.9.4. (*S*)-2-((*S*)-2-(2-(3,5-Difluorophenyl)acetamido)propanamido)-2-phenylacetic acid (DAPA) (142)
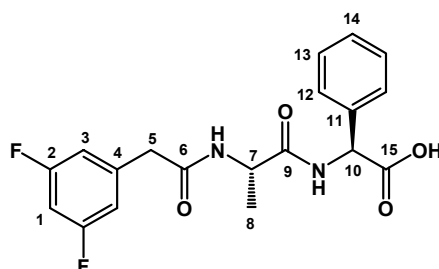
Trifluoroacetic acid (0.6 mL) was added to a stirred solution of DAPT (**145**) (100 mg, 0.23 mmol) in CH₂Cl₂ (3 mL) at 0 °C and then allowed to warm at an ambient temperature. The reaction mixture was stirred for 6 h and evaporated *in vacuo* to afford the crude product. The crude product was purified by crystallization (CHCl₃:MeOH) to obtain the title compound as colorless solid (82 mg, 95%).

¹H-NMR (300 MHz, DMSO- *D₆*): δ = 8.60 (d, *J* = 7.4 Hz, 1H; NH), 8.36 (d, *J* = 7.6 Hz, 1H; NH), 7.39–7.32 (m, 5H; H-12, H-13, H-14), 7.08 (m, 1H; H-1), 7.01–6.95 (m, 2H; H-3), 5.31

(d, *J* = 7.4 Hz, 1H; H-10,), 4.44 (q, 1H, *J* = 7.2 Hz; H-7), 3.51 (s, 2H; H-5), 1.25 (d, *J* = 7.0 Hz, 3H; H-8,) ppm.

5.9.5. Tert-butyl 2-(4-(4-hydroxybenzoyl)phenoxy)acetate (146)
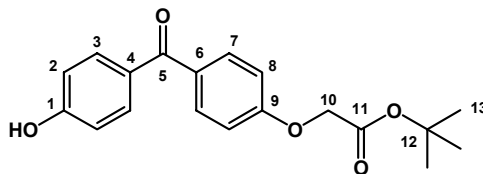
Anhydrous K$_2$CO$_3$ (1.3 g, 9.34 mmol) was added to a stirred solution of bis(4-hydroxyphenyl)methanone (2 g, 9.33 mmol) in acetone (40 mL) and stirred at ambient temperature for 30 min. *T*-butyl chloroacetate (1.4 mL, 9.8 mmol) was added to it and heated to 60–70 °C for 24 h. Reaction was cooled to room temperature and filtered. The residue was washed with acetone (×3). Combined organic extract was evaporated *in vacuo* and purified by column chromatography (EtOAc:hexane, 2:8) to yield the title compound as a colorless solid (1.1 g, 37%)

¹H NMR (300 MHz, CDCl₃): *δ* = 7.70 (td, *J* = 8.8 Hz, *J* = 2.0 Hz, 2H; H-7), 7.64 (td, *J* = 8.8 Hz, *J* = 2.0 Hz, 2H; H-3), 6.91 (td, *J* = 8.8 Hz, *J* = 2.4 Hz, 2H; H-8), 6.82 (td, *J* = 8.8 Hz, *J* = 2.4 Hz, 2H; H-2), 4.52 (s, 2H; H-10), 2.97 (brs, 1H; NH), 1.42 (s, 9H; H-13) ppm.

¹³C NMR (75 MHz, CDCl₃): *δ* = 195.3 (C-5), 167.8 (C-11), 161.3 (C-9), 161.0 (C-1), 132.7 (C-4), 132.2 (C-6), 131.6 (C-3), 131.2 (C-7), 115.1 (C-2), 114.1 (C-8), 83.0 (C-12), 65.5 (C-10), 28.0 (C-13) ppm.

6.9.6. Tert-butyl 2-(4-(4-(2-hydroxyethoxy)benzoyl)phenoxy)acetate (143)
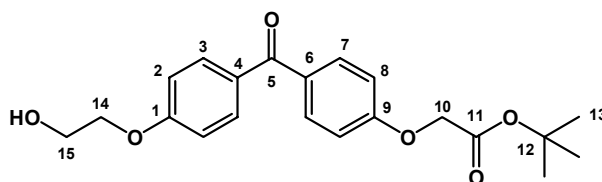
Anhydrous K$_2$CO$_3$ (630 mg, 4.55 mmol) was added to a stirred solution of tert-butyl 2-(4-(4-hydroxybenzoyl)phenoxy)acetate (**146**) (500 mg, 1.52 mmol) in acetone 15 mL) and stirred at ambient temperature for 30 min. 2-Chloro ethanol (507 µL, 7.6 mmol) was added to it and heated to 60–70 °C for 48 h. The reaction 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 (EtOAc:hexane) to yield the title compound as a colorless solid (510 mg, 90%)

¹H NMR (300 MHz, CDCl₃): *δ* = 7.70 (td, *J* = 8.8 Hz, *J* = 2.4 Hz, 4H; H-3, H-7), 7.40 (brs, 1H; OH), 6.91-6.84 (m, 4H; H-2, H-8), 4.51 (s, 2H; H-10), 4.11 (t, *J* = 4.5 Hz, 2H; H-14), 3.94 (t, *J* = 4.5 Hz, 2H; H-15), 1.43 (s, 9H; H-13) ppm.

¹³C NMR (75 MHz, CDCl₃): *δ* = 194.5 (C-5), 167.5 (C-11), 162.1 (C-9), 161.1 (C-1), 132.3 (C-4), 132.2 (C-6), 131.5 (C-7), 130.9 (C-3), 114.1 (C-2), 114.0 (C-9), 82.9 (C-12), 69.5 (C-14), 65.5 (C-15), 61.3 (C-10), 28.1 (C-13) ppm.

5.9.7. Tert-butyl 2-(5-((3a*R*,4*R*,6a*S*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)ethylcarbamate (151)
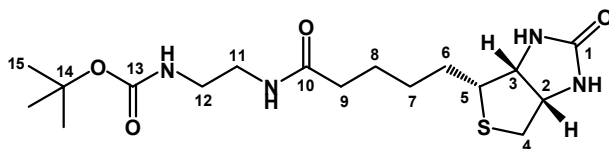
Et$_3$N (28 μL, 0.2 mmol) was added to a solution of *D*-biotin (50 mg, 0.2 mmol) in DMF (4 mL) followed by the addition EDAC (48 g, 0.24 mmol) and HOBt (34 mg, 0.24 mmol) and stirred at RT until it became clear. Et$_3$N (260 μL, 1.8 mmol) and *N*-Boc ethylene diamine (40 μL, 0.22 mmol) was added to it simultaneously. The reaction mixture stirred at RT for 3 h. The reaction mixture was then diluted with EtOAc (100 mL) and washed with brine. The organic phase was dried over anhydrous Na$_2$SO$_4$ and the solvent was evaporated *in vacuo* to afford the crude product which was further purified by column chromatography (EtOAc:hexane, 4:6) to get the title compound as colorless solid (35 mg, 45%).

^{1}H NMR (300 MHz, CDCl$_3$): δ = 4.50-4.40 (m, 1H; H-3), 4.30-4.20 (m, 1H; H-2), 3.80 (brs, 4H; NH), 3.20 (q, *J* = 5.5 Hz, 2H; H-12), 3.11 (q, *J* = 5.5 Hz, 2H; H-11), 3.08-3.02 (m, 1H; H-5), 2.85 (dd, *J* = 6.8 Hz, *J* = 4.6 Hz, 1H; H-4), 2.68 (d, *J* = 6.8 Hz, 1H; H-4′), 2.13 (t, *J* = 7.2 Hz, 2H; H-9), 1.68-1.54 (m, 4H; H-6- H-8), 1.42-1.11 (m, 11H; H-7, H-15) ppm

^{13}C NMR (75 MHz, CDCl$_3$): δ = 172.1 (C-10), 165.2 (C-1), 156.9 (C-13), 62.0 (C-3), 60.4 (C-2), 55.5 (C-5), 41.5 (C-4), 40.2 (C-12), 39.7 (C-11), 35.6 (C-9), 29.6 (C-6), 27.9 (C-15), 25.4 (C-7), 25.2 (C-8) ppm.

MS (ESI): *m/z* = 409.2 (M+Na)$^+$.

5.9.8. *N*-(2-Aminoethyl)-5-((3a*R*,4*R*,6a*S*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide (141)
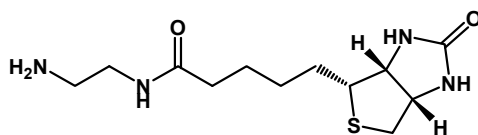
A solution of tert-butyl 2-(5-((3a*R*,4*R*,6a*S*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)ethylcarbamate (**151**) (25 mg, 0.06 mmol) in 20% TFA in CH$_2$Cl$_2$ was stirred at room temperature for 3 h. The reaction mixture was then evaporated *in vacuo* and lyophilized to afford the title compound as trifluoroacetic acid salt which was used in the next step without purification.

MS (ESI): *m/z* = 309.3 (M+Na)$^+$.

5.9.9. Tert-butyl 2-(4-(4-(2-(tosyloxy)ethoxy)benzoyl)phenoxy)acetate (154)
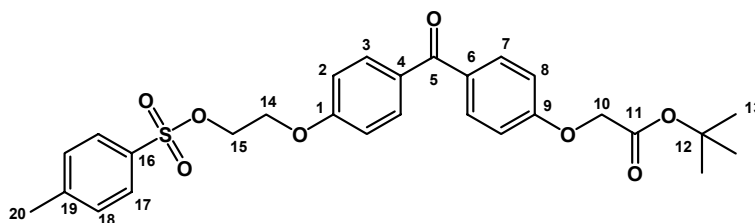
Pyridine (220 μL, 2.7 mmol) and *p*-toluene sulfonyl chloride (385 mg, 2 mmol) were added to a stirred solution of tert-butyl 2-(4-(4-(2-hydroxyethoxy)benzoyl)phenoxy)acetate (**143**) (500 mg, 1.35 mmol) in CH$_2$Cl$_2$ (10 mL) and stirred at ambient temperature for 7 days. Reaction was monitored by TLC. Upon completion of reaction, it was diluted with CH$_2$Cl$_2$ (100 mL). Organic phase was washed with water, 0.1 N HCl, dried over anhydrous Na$_2$SO$_4$, evaporated *in vacuo* and purified by column chromatography (EtOAc:hexane, 2.5:7.5) to afford the title compound as a colorless gummy mass (540 mg, 76%)

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.74 (d, *J* = 8.8 Hz, 2H; H-17), 7.71-7.64 (m, 4H; H-3, H-7), 7.28 (d, *J* = 8.2 Hz, 2H; H-18), 6.88 (d, *J* = 8.8 Hz, 2H; H-8), 6.78 (d, *J* = 8.8 Hz, 2H; H-2), 4.52 (s, 2H; H-10), 4.33 (t, *J* = 4.4 Hz, 2H; H-14), 4.15 (t, *J* = 4.4 Hz, 2H; H-15), 2.38 (s, 3H; H-20), 1.42 (s, 9H; H-13) ppm.

^{13}C NMR (75 MHz, CDCl₃): δ = 195.5 (C-5), 167.5 (C-11), 161.2 (C-9, C-1), 145.2 (C-19), 135.8 (C-16), 132.2 (C-6), 131.8 (C-4), 131.5 (C-7), 130.9 (C-3), 130.3 (C-18), 128.1 (C-17), 114.2 (C-8), 114.1 (C-2), 82.9 (C-12), 67.9 (C-14), 65.6 (C-15), 60.5 (C-10), 28.1 (C-13), 21.7 (C-20) ppm.

5.9.10. (*S*)-2-(4-(4-(2-Tert-butoxy-2-oxoethoxy)benzoyl)phenoxy)ethyl 2-((*S*)-2-(2-(3,5-difluorophenyl)acetamido)propanamido)-2-phenylacetate (153)
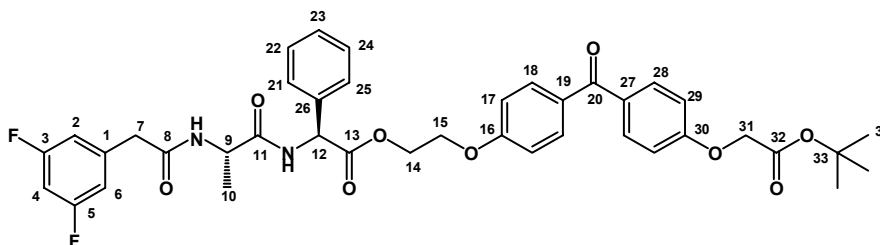
Anhydrous K₂CO₃ was added (110 mg, 0.8 mmol) to a stirred solution of (*S*)-2-((*S*)-2-(2-(3,5-difluorophenyl)acetamido)propanamido)-2-phenylacetic acid (**142**) (100 mg, 0.27 mmol) in anhydrous DMF (4 mL) and stirred for 30 min. at ambient temperature. A solution of tert-butyl 2-(4-(4-(2 (tosyloxy)ethoxy)benzoyl)phenoxy)acetate (**154**) in dry DMF (1 mL) was added to it and the reaction mixture was stirred at ambient temperature for another 36 h. The reaction mixture was then diluted with ethyl acetate (100 mL). The aqueous layer was extracted with ethyl acetate (×3). The combined organic extract was washed with water, brine, dried over anhydrous Na₂SO₄ and evaporated *in vacuo* to afford the crude product. The crude product was purified by column chromatography (EtOAc:hexane, 1:4) to yield the title compound as colorless gummy mass (108 mg, 56%).

^{1}H NMR (500 MHz, CDCl₃): δ = 7.70 (d, J = 8.8 Hz, 2H; H-28), 7.67 (d, J = 8.8 Hz, 2H; H-18), 7.26-7.22 (m, 5H; H-21, H-22, H-23, H-24, H-25), 6.89 (d, J = 8.8 Hz, 2H; H-29), 6.78 (d, J = 8.8 Hz, 2H; H-17), 6.71-6.63 (m, 3H; H-2, H-4, H-6), 6.05 (d, J = 7.8 Hz, 1H; H-12), 5.48 (q, J = 7.0 Hz, 1H; H-9), 4.52 (s, 2H; H-31), 4.52-4.22 (m, 4H; H-14, H-15), 4.12 (s, 2H; H-7), 3.47 (brs, 1H; NH), 3.41 (brs, 1H; NH), 1.42 (s, 9H; H-34), 1.26 (d, J = 7.0 Hz, 3H; H-10) ppm.

^{13}C NMR (125 MHz, CDCl₃): δ = 197.5 (C-20), 172.5 (C-11), 172.4 (C-8), 169.4 (C-32), 166.2 (C-3, C-5), 165.5 (C-30), 162.1 (C-16), 141.2 (C-1), 137.1 (C-26), 133.8 (C-27), 132.2 (C-28), 131.9 (C-19), 131.5 (C-18), 130.9 (C-25, C-21), 129.9 (C-22, C-24), 127.6 (C-23), 115.3 (C-29), 114.6 (C-17), 113.4 (C-2, C-6), 104.2 (C-4), 82.9 (C-33), 67.8 (C-15), 65.6 (C-14), 64.5 (C-31), 57.1 (C-12), 50.1 (C-9), 39.8 (C-7), 28.9 (C-34), 16.4 (C-10) ppm.

MS (ESI): m/z = 753.3 (M+Na)⁺, 729.2 (M–H)⁺

5.9.11. 2-(4-(4-(2-((*S*)-2-((*S*)-2-(2-(3,5-Difluorophenyl)acetamido)propanamido)-2-phenylacetoxy)ethoxy)benzoyl)phenoxy)acetic acid (140)

Trifluoroacetic acid (0.2 mL) was added to a stirred solution of (*S*)-2-(4-(4-(2-tert-butoxy-2-oxoethoxy)benzoyl)phenoxy)ethyl 2-((*S*)-2-(2-(3,5 difluorophenyl)acetamido)propanamido)-2-phenylacetate (**153**) (100 mg, 0.137) in CH₂Cl₂ at 0 °C and then allowed to warm at an ambient temperature. The reaction was stirred for 6 h and then evaporated *in vacuo* to afford

the crude product. The crude product was purified by crystallization (EtOAc) to obtain the title compound as colorless solid (83 mg, 90%).

MS (ESI): m/z = 697.2 (M+Na)$^+$, 673.1 (M–H)$^+$.

5.9.6. **(S)-2-(4-(4-(2-(2-(Tert-butoxycarbonylamino)ethylamino)-2-oxoethoxy)benzoyl)phenoxy)ethyl 2-((S)-2-(2-(3,5-difluorophenyl)acetamido)propanamido)-2-phenylacetate (155)**
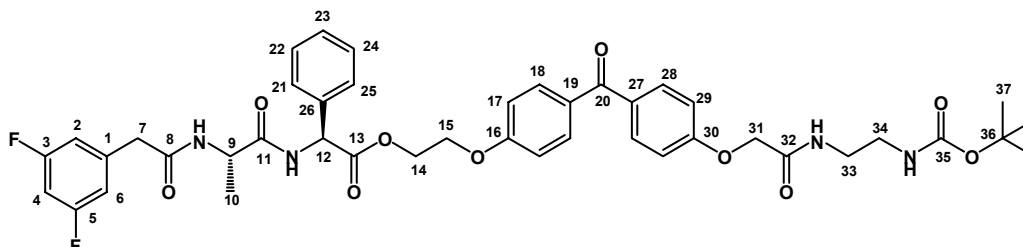
To a stirred solution of 2-(4-(4-(2-((S)-2-((S)-2-(2-(3,5-difluorophenyl)acetamido)propanamido)-2 phenylacetoxy)ethoxy)benzoyl)phenoxy)acetic acid (**140**) (80 mg, 0.11 mmol) in anhydrous CH$_2$Cl$_2$ (4 mL), was added Et$_3$N (16 μL, 0.11 mmol) and stirred at ambient temperature for 10 min. Ethyl-3-(3´-dimethylaminopropyl)carbodiimide hydrochloride (25 mg, 0.13 mmol) and *N*-hydroxybenzotriazole hydrate (18 mg, 0.22 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 ethylene diamine (21 μL, 0.13 mmol) in CH$_2$Cl$_2$ (1 mL) was added to the reaction mixture followed by Et$_3$N (19 μL, 0.13 mmol) and stirred at ambient temperature for 10 h. The reaction mixture was diluted with CHCl$_3$ (50 mL), washed with water, brine, dried over anhydrous Na$_2$SO$_4$ and evaporated *in vacuo* to yield the crude product. The crude product was purified by flash column chromatography (EtOAc:hexane, 3:7) to yield the title compound as colorless solid (90 mg, 87%).

^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.74 (d, J = 8.8 Hz, 2H; H-28), 7.67 (d, J = 8.2 Hz, 2H; H-18), 7.27-7.22 (m, 5H; H-21, H-22, H-23, H-24, H-25), 6.95 (d, J = 8.8 Hz, 2H; H-29), 6.78 (d, J = 8.8 Hz, 2H; H-17), 6.69-6.61 (m, 3H; H-2, H-4, H-6), 6.18-6.12 (m, 1H; H-12), 5.45 (m, 1H; H-9), 4.80 (brs, 1H; NH), 4.58 (s, 2H; H-31), 4.46-4.40 (m, 2H; H-15), 4.14-4.08 (m, 2H; H-14), 3.48 (s, 2H; H-7), 3.43-3.38 (m, 2H; H-33), 3.31-3.24 (m, 2H; H-34), 1.70 (brs, 2H; NH), 1.37 (s, 9H; H-37), 1.30 (d, J = 7.0 Hz, 3H; H-10) ppm.

^{13}C NMR (75 MHz, CDCl$_3$): δ = 196.8 (C-20), 172.2 (C-11), 172.1 (C-13), 169.6 (C-32), 165.9 (C-3, C-5), 165.2 (C-30), 162.2 (C-16), 156.6 (C-35), 141.4 (C-1), 137.5 (C-26), 133.3 (C-27), 132.1 (C-19), 131.2 (C-28), 130.7 (C-18), 129.5 (C-21, C-25), 129.2 (C-22, C-24), 127.8 (C-23), 115.6 (C-29), 114.9 (C-17), 113.3 (C-2, C-6), 105.1 (C-4), 80.2 (C-36), 69.1 (C-15), 67.6 (C-14), 64.3 (C-31), 57.1 (C-12), 50.1 (C-9), 41.2 (C-7), 40.5 (C-34), 40.1 (C-33), 28.4 (C-37), 17.4 (C-10) ppm.

MS (ESI): m/z = 839.4 (M+Na)$^+$, 815.2 (M–H)$^+$

5.9.13. (S)-2-(4-(4-(2-(2-Aminoethylamino)-2-oxoethoxy)benzoyl)phenoxy)ethyl 2-((S)-2-(2-(3,5-difluorophenyl)acetamido)propanamido)-2-phenylacetate (156)
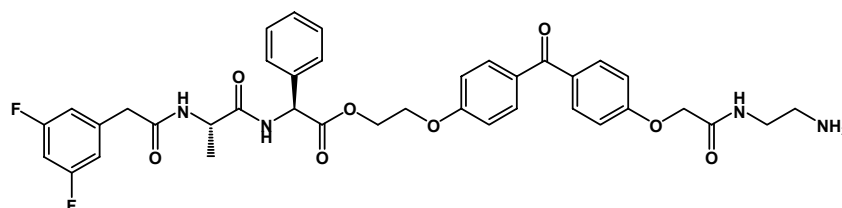
A solution of compound (*S*)-2-(4-(4-(2-(2-(tert-butoxycarbonylamino)ethylamino)-2-oxoethoxy)benzoyl)phenoxy)ethyl 2-((*S*)-2-(2-(3,5-difluorophenyl)acetamido)propanamido)-2-phenylacetate (**155**) (80 mg, 0.1 mmol) in 16% HCl in dioxane (1 mL) was stirred at room temperature for 1 h. The reaction was monitored by TLC. The reaction mixture was evaporated *in vacuo* and lyophilized to afford the title compound as hydrochloride salt which was used in the next step without purification.

MS (ESI): *m/z* = 717.3 (M+H)$^+$.

5.9.14. (*S*)-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)ethyl 2-((*S*)-2-(2-(3,5-difluorophenyl)acetamido)propanamido)-2-phenylacetate (139)
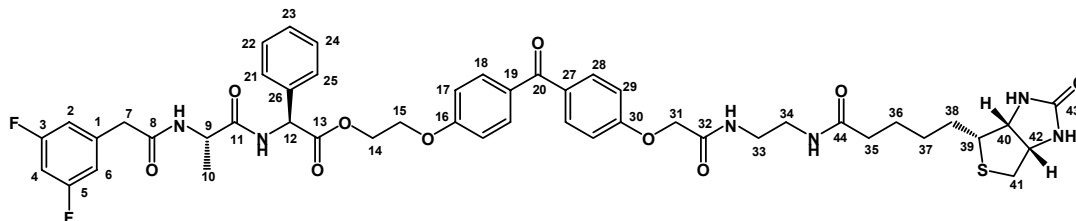
To a stirred solution of *D*-biotin (30 mg, 0.12 mmol) in anhydrous DMF (2 mL), was added Et$_3$N (17 μL, 0.12 mmol) and stirred at ambient temperature for 10 min. Ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (28 mg, 0.15 mmol), *N*-hydroxybenzotriazole hydrate (20 mg, 0.15 mmol) were added to it and the resulting solution was stirred at ambient temperature for 30 min. until it became clear. A solution of (S)-2-(4-(4-(2-(2-aminoethylamino)-2-oxoethoxy)benzoyl)phenoxy)ethyl 2-((S)-2-(2-(3,5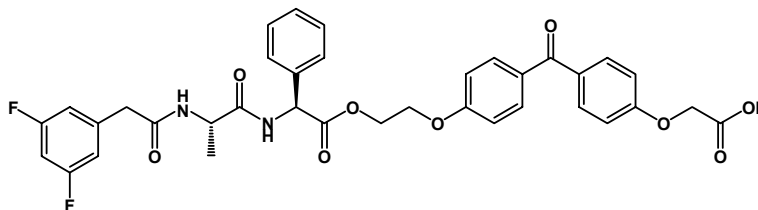-difluorophenyl)acetamido)propanamido)-2-phenylacetate (**156**) (80 mg, 0.1 mmol) in DMF (1 mL) was added to the reaction mixture followed by Et$_3$N (68 μL, 0.5 mmol) and stirred at ambient temperature for 18 h. The reaction mixture was diluted with CHCl$_3$ (100 mL), washed with water, brine, dried over anhydrous Na$_2$SO$_4$ and evaporated *in vacuo* to yield the crude product. The crude product was purified by flash column chromatography (CH$_2$Cl$_2$:MeOH, 95:5) to obtain the title compound as a pale brown solid (20 mg, 20%).

^{1}H NMR (300 MHz, CD$_3$OD): δ = 7.68 (d, *J* = 8.0 Hz, 2H; H-28), 7.64 (dd, *J* = 8.2 Hz, *J* = 1.6 Hz, 2H; H-18), 7.29-7.18 (m, 5H; H-21, H-22, H-23, H-24, H-25), 7.02 (d, *J* = 8.8 Hz, 2H; H-29), 6.89-6.75 (m, 4H; H-2, H-6, H-17), 6.67 (m, 1H; H-4), 5.40 (d, *J* = 1.4 Hz, 1H; H-12), 4.50 (s, 2H; H-31), 4.46-4.31 (m, 5H; H-9, H-14, H-15), 4.20-4.12 (m, 2H; H-40, H-42), 3.43 (s, 2H; H-7), 3.34-3.26 (m, 4H; H-33, H-34), 3.08-3.02 (m, 1H; H-39), 2.78 (dd, *J* = 6.8 Hz, *J* = 4.8 Hz, 1H; H-41), 2.58 (d, *J* = 6.8 Hz, 1H; H-41′), 2.10 (t, *J* = 7.2 Hz, 2H; H-28), 1.66-1.39 (m, 4H; H,36, H-38), 1.36-1.17 (m, 5H; H-10, H-37) ppm.

^{13}C NMR (75 MHz, CD$_3$OD): δ = 196.8 (C-20), 172.7 (C-44), 172.2 (C-11), 172.1 (C-13), 171.9 (C-32), 169.6 (C-43), 165.9 (C-3, C-5), 163.7 (C-30), 162.5 (C-16), 141.2 (C-1), 138.1 (C-26), 133.5 (C-27), 132.8 (C-19), 131.8 (C-28), 130.9 (C-18), 129.8 (C-21, C-25), 128.8 (C-22, C-24), 128.7 (C-23), 115.8 (C-29), 115.5 (C-17), 113.1 (C-2, C-6), 105.2 (C-4), 69.2 (C-15), 68.3 (C-31), 64.5 (C-14), 63.5 (C-40), 61.8 (C-42), 58.4 (C-39), 56.7 (C-12), 50.0 (C-9), 41.2 (C-7), 41.0 (C-41), 40.6 (C-33), 39.9 (C-34), 36.9 (C-35), 29.8 (C-38), 26.8 (C-37), 26.5 (C-36), 18.3 (C-10) ppm.

MS (ESI): *m/z* = 943.4 (M)$^+$, 965.4 (M+Na)$^+$

6. REFERENCES

[1]   B. Schmidt, H. A. Braun, R. Narlawar, *Curr. Med. Chem.* **2005**, *12*, 1677.

[2]   C. Mount, C. Downton, *Nat. Med.* **2006**, *12*, 780.

[3]   R. Mayeux, *Ann. Rev. Neurosci.* **2003**, *26*, 81.

[4]   J. A. Luchsinger, M. X. Tang, Y. Stern, S. Shea, R. Mayeux, *Am. J. Epidemiol.* **2001**, *154*, 635.

[5]   A. Luchsinger Jose, M.-X. Tang, S. Shea, R. Mayeux, *Arch. Neurol.* **2003**, *60*, 203.

[6]   A. Goate, M. C. Chartier-Harlin, M. Mullan, J. Brown, F. Crawford, L. Fidani, L. Giuffra, A. Haynes, N. Irving, *Nature* **1991**, *349*, 704.

[7]   R. Sherrington, E. I. Rogaev, Y. Liang, E. A. Rogaeva, G. Levesque, M. Ikeda, H. Chi, C. Lin, G. Li, K. Holman, *Nature* **1995**, *375*, 754.

[8]   E. H. Corder, A. M. Saunders, W. J. Strittmatter, D. E. Schmechel, P. C. Gaskell, G. W. Small, A. D. Roses, J. L. Haines, M. A. Pericak-Vance, *Science* **1993**, *261*, 921.

[9]   J. Raber, Y. Huang, J. W. Ashford, *Neurobiol. Aging* **2004**, *25*, 641.

[10]   J. A. Prince, H. Zetterberg, N. Andreasen, J. Marcusson, K. Blennow, *Neurology* **2004**, *62*, 2116.

[11]   G. McKhann, D. Drachman, M. Folstein, R. Katzman, D. Price, E. M. Stadlan, *Neurology* **1984**, *34*, 939.

[12]   D. A. Snowdon, L. H. Greiner, J. A. Mortimer, K. P. Riley, P. A. Greiner, W. R. Markesbery, *J. Am. Med. Assoc.* **1997**, *277*, 813.

[13]   G. Blessed, B. E. Tomlinson, M. Roth, *Br. J. Psychiatry* **1968**, *114*, 797.

[14]   K. Blennow, M. J. de Leon, H. Zetterberg, *Lancet* **2006**, *368*, 387.

[15]   M. Goedert, C. M. Wischik, R. A. Crowther, J. E. Walker, A. Klug, *Proc. Natl. Acad. Sci. U. S. A.* **1988**, *85*, 4051.

[16]   M. Goedert, M. G. Spillantini, N. J. Cairns, R. A. Crowther, *Neuron* **1992**, *8*, 159.

[17]   K. Iqbal, A. Del C. Alonso, S. Chen, M. O. Chohan, E. El-Akkad, C.-X. Gong, S. Khatoon, B. Li, F. Liu, A. Rahman, H. Tanimukai, I. Grundke-Iqbal, *Biochim. Biophys. Acta* **2005**, *1739*, 198.

[18]   M. Goedert, M. Hasegawa, R. Jakes, S. Lawler, A. Cuenda, P. Cohen, *FEBS Lett.* **1997**, *409*, 57.

[19]   C. L. Masters, G. Simms, N. A. Weinman, G. Multhaup, B. L. McDonald, K. Beyreuther, *Proc. Natl. Acad. Sci. U. S. A.* **1985**, *82*, 4245.

[20]   J. A. Carson, A. J. Turner, *J. Neurochem.* **2002**, *81*, 1.

[21]   R. E. Tanzi, R. D. Moir, S. L. Wagner, *Neuron* **2004**, *43*, 605.

[22]   J. Hardy, D. J. Selkoe, *Science* **2002**, *297*, 353.

[23]   J. T. Jarrett, E. P. Berger, P. T. Lansbury, Jr., *Biochemistry* **1993**, *32*, 4693.

[24]   J. Näslund, V. Haroutunian, R. Mohs, K. L. Davis, P. Davies, P. Greengard, J. D. Buxbaum, *J. Am. Med. Assoc.* **2000**, *283*, 1571.

[25]   M. Ohno, E. A. Sametsky, L. H. Younkin, H. Oakley, S. G. Younkin, M. Citron, R. Vassar, J. F. Disterhoft, *Neuron* **2004**, *41*, 27.

[26]   J. Goetz, F. Chen, J. van Dorpe, R. M. Nitsch, *Science* **2001**, *293*, 1491.

[27]   D. M. Walsh, I. Klyubin, V. Fadeeva Julia, K. Cullen William, R. Anwyl, S. Wolfe Michael, J. Rowan Michael, J. Selkoe Dennis, *Nature* **2002**, *416*, 535.

[28]   P. L. McGeer, M. Schulzer, E. G. McGeer, *Neurology* **1996**, *47*, 425.

[29]   S. Parvathy, E. H. Karran, A. J. Turner, N. M. Hooper, *FEBS Lett.* **1998**, *431*, 63.

[30]   J. Nunan, D. H. Small, *Essays Biochem.* **2002**, *38*, 37.

[31]   R. G. Perez, S. Soriano, J. D. Hayes, B. Ostaszewski, W. Xia, D. J. Selkoe, X. Chen, G. B. Stokin, E. H. Koo, *J. Biol. Chem.* **1999**, *274*, 18851.

[32]   R. Vassar, B. D. Bennett, S. Babu-Khan, S. Kahn, E. A. Mendiaz, P. Denis, D. B. Teplow, S. Ross, P. Amarante, R. Loeloff, Y. Luo, S. Fisher, J. Fuller, S. Edenson, J. Lile, M. A. Jarosinski, A. L. Biere, E. Curran, T. Burgess, J. C. Louis, F. Collins, J. Treanor, G. Rogers, M. Citron, *Science* **1999**, *286*, 735.

[33]   R. Narlawar, K. Baumann, R. Schubenel, B. Schmidt, *Neurodegen. Dis.* **2007** *in print*.

[34]   L. Pastorino, A. F. Ikin, S. Lamprianou, N. Vacaresse, J. P. Revelli, K. Platt, P. Paganetti, P. M. Mathews, S. Harroch, J. D. Buxbaum, *Mol. Cell. Neurosci.* **2004**, *25*, 642.

[35]   U. Bodendorf, S. Danner, F. Fischer, M. Stefani, C. Sturchler-Pierrat, K.-H. Wiederhold, M. Staufenbiel, P. Paganetti, *J. Neurochem.* **2002**, *80*, 799.

[36]   I. Hussain, D. Powell, D. R. Howlett, D. G. Tew, T. D. Meek, C. Chapman, I. S. Gloger, K. E. Murphy, C. D. Southan, D. M. Ryan, T. S. Smith, D. L. Simmons, F. S. Walsh, C. Dingwall, G. Christie, *Mol. Cell. Neurosci.* **1999**, *14*, 419.

[37]   Y. H. Koh, C. A. F. von Arnim, B. T. Hyman, R. E. Tanzi, G. Tesco, *J. Biol. Chem.* **2005**, *280*, 32499.

[38]   S. F. Lichtenthaler, R. Wang, H. Grimm, S. N. Uljon, C. L. Masters, K. Beyreuther, *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 3053.

[39]   D. Beher, J. D. J. Wrigley, A. P. Owens, M. S. Shearman, *J. Neurochem.* **2002**, *82*, 563.

[40]   C. Esh, L. Patton, W. Kalback, T. A. Kokjohn, J. Lopez, D. Brune, A. J. Newell, T. Beach, D. Schenk, D. Games, S. Paul, K. Bales, B. Ghetti, E. M. Castano, A. E. Roher, *Biochemistry* **2005**, *44*, 13807.

[41]   A. Weidemann, S. Eggert, F. B. M. Reinhard, M. Vogel, K. Paliga, G. Baier, C. L. Masters, K. Beyreuther, G. Evin, *Biochemistry* **2002**, *41*, 2825.

[42]   M. S. Wolfe, *Biochemistry* **2006**, *45*, 7931.

[43]   A. L. Brunkan, A. M. Goate, *J. Neurochem.* **2005**, *93*, 769.

[44]   P. Mastrangelo, P. M. Mathews, M. A. Chisti, S. D. Schmidt, Y. Gu, J. Yang, M. J. Mazzella, J. Coomaraswamy, P. Horne, B. Strome, H. Pelly, G. Levesque, C. Ebeling, Y. Jiang, R. A. Nixon, R. Rozmahel, P. E. Fraser, P. St. George-Hyslop, G. A. Carlson, D. Westaway, *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 8972.

[45]   W. T. Kimberly, W. Xia, T. Rahmati, M. S. Wolfe, D. J. Selkoe, *J. Biol. Chem.* **2000**, *275*, 3173.

[46]   A. Capell, J. Grunberg, B. Pesold, A. Diehlmann, M. Citron, R. Nixon, K. Beyreuther, D. J. Selkoe, C. Haass, *J. Biol. Chem.* **1998**, *273*, 3205.

[47]   M. S. Brown, J. Ye, R. B. Rawson, J. L. Goldstein, *Cell* **2000**, *100*, 391.

[48]   D. Selkoe, R. Kopan, *Ann. Rev. Neurosci.* **2003**, *26*, 565.

[49]   G. Thinakaran, D. R. Borchelt, M. K. Lee, H. H. Slunt, L. Spitzer, G. Kim, T. Ratovitsky, F. Davenport, C. Nordstedt, *Neuron* **1996**, *17*, 181.

[50]   G. Yu, M. Nishimura, S. Arawaka, D. Levitan, L. Zhang, A. Tandon, Y. Q. Song, E. Rogaeva, F. Chen, T. Kawarai, A. Supala, L. Levesque, H. Yu, D. S. Yang, E. Holmes, P. Milman, Y. Liang, D. M. Zhang, D. H. Xu, C. Sato, E. Rogaev, M. Smith, C. Janus, Y. Zhang, R. Aebersold, L. S. Farrer, S. Sorbi, A. Bruni, P. Fraser, P. St George-Hyslop, *Nature* **2000**, *407*, 48.

[51]   A. Capell, C. Kaether, D. Edbauer, K. Shirotani, S. Merkl, H. Steiner, C. Haass, *J. Biol. Chem.* **2003**, *278*, 52519.

[52]   W. P. Esler, W. T. Kimberly, B. L. Ostaszewski, T. S. Diehl, C. L. Moore, J. Y. Tsai, T. Rahmati, W. Xia, D. J. Selkoe, M. S. Wolfe, *Nat. Cell Biol.* **2000**, *2*, 428.

[53]   D. Edbauer, E. Winkler, C. Haass, H. Steiner, *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 8666.

[54]   C. Kaether, A. Capell, D. Edbauer, E. Winkler, B. Novak, H. Steiner, C. Haass, *EMBO J.* **2004**, *23*, 4738.

[55]   G. Struhl, A. Adachi, *Mol. Cell* **2000**, *6*, 625.

[56]   C. Goutte, M. Tsunozaki, V. A. Hale, J. R. Priess, *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 775.

[57]   R. Francis, G. McGrath, J. Zhang, D. A. Ruddy, M. Sym, J. Apfeld, M. Nicoll, M. Maxwell, B. Hai, M. C. Ellis, A. L. Parks, W. Xu, J. Li, M. Gurney, R. L. Myers, C. S. Himes, R. Hiebsch, C. Ruble, J. S. Nye, D. Curtis, *Dev. Cell* **2002**, *3*, 85.

[58]   L. Serneels, T. Dejaegere, K. Craessaerts, K. Horre, E. Jorissen, T. Tousseyn, S. Hebert, M. Coolen, G. Martens, A. Zwijsen, W. Annaert, D. Hartmann, B. De Strooper, *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 1719.

[59]   H. Steiner, E. Winkler, D. Edbauer, S. Prokop, G. Basset, A. Yamasaki, M. Kostka, C. Haass, *J. Biol. Chem.* **2002**, *277*, 39062.

[60]   W.-j. Luo, H. Wang, H. Li, B. S. Kim, S. Shah, H.-J. Lee, G. Thinakaran, T.-W. Kim, G. Yu, H. Xu, *J. Biol. Chem.* **2003**, *278*, 7850.

[61]   A. Bergman, E. M. Hansson, S. E. Pursglove, M. R. Farmery, L. Lannfelt, U. Lendahl, J. Lundkvist, J. Naeslund, *J. Biol. Chem.* **2004**, *279*, 16744.

[62]   S. Zhou, H. Zhou, P. J. Walian, B. K. Jap, *Biochemistry* **2007**, *46*, 2553.

[63]   S. Zhou, H. Zhou, P. J. Walian, B. K. Jap, *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 7499.

[64]   W. T. Kimberly, M. J. LaVoie, B. L. Ostaszewski, W. Ye, M. S. Wolfe, D. J. Selkoe, *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 6382.

[65]   I. Churcher, D. Beher, *Curr. Pharma. Des.* **2005**, *11*, 3363.

[66]   M. S. Shearman, D. Beher, E. E. Clarke, H. D. Lewis, T. Harrison, P. Hunt, A. Nadin, A. L. Smith, G. Stevenson, J. L. Castro, *Biochemistry* **2000**, *39*, 8698.

[67]   J. E. Audia, T. C. Britton, J. J. Droste, B. K. Folmer, G. W. Huffman, V. John, L. H. Latimer, T. E. Mabry, J. S. Nissen, W. J. Porter, J. K. Reel, E. D. Thorsett, J. S. Tung, J. Wu, **2001**, US 6191166.

[68]   J. E. Audia, V. John, L. H. Latimer, S. L. McDaniel, J. S. Nissen, E. D. Thorsett, J. S. Tung, **2002**, WO 2002047671.

[69]   J. E. Audia, T. E. Mabry, J. S. Nissen, S. L. McDaniel, **1999**, WO 9932453.

[70]   H. F. Dovey, V. John, J. P. Anderson, L. Z. Chen, P. De Saint Andrieu, L. Y. Fang, S. B. Freedman, B. Folmer, E. Goldbach, E. J. Holsztynska, K. L. Hu, K. L. Johnson-Wood, S. L. Kennedy, D. Kholodenko, J. E. Knops, L. H. Latimer, M. Lee, Z. Liao, I. M. Lieberburg, R. N. Motter, L. C. Mutter, J. Nietz, K. P. Quinn, K. L. Sacchi, P. A. Seubert, G. M. Shopp, E. D. Thorsett, J. S. Tung, J. Wu, S. Yang, C. T. Yin, D. B. Schenk, P. C. May, L. D. Altstiel, M. H. Bender, L. N. Boggs, T. C. Britton, J. C. Clemens, D. L. Czilli, D. K. Dieckman-McGinty, J. J. Droste, K. S. Fuson, B. D. Gitter, P. A. Hyslop, E. M. Johnstone, W. Y. Li, S. P. Little, T. E. Mabry, F. D. Miller, B. Ni, J. S. Nissen, W. J. Porter, B. D. Potts, J. K. Reel, D. Stephenson, Y. Su, L. A. Shipley, C. A. Whitesitt, T. Yin, J. E. Audia, *J. Neurochem.* **2001**, *76*, 173.

[71]   T. A. Lanz, C. S. Himes, G. Pallante, L. Adams, S. Yamazaki, B. Amore, K. M. Merchant, *J. Pharmacol. Exp. Ther.* **2003**, *305*, 864.

[72]   A. Geling, H. Steiner, M. Willem, L. Bally-Cuif, C. Haass, *EMBO Rep.* **2002**, *3*, 688.

[73]   D. Seiffert, J. D. Bradley, C. M. Rominger, D. H. Rominger, F. Yang, J. E. Meredith, Jr., Q. Wang, A. H. Roach, L. A. Thompson, S. M. Spitz, J. N. Higaki, S. R. Prakash, A. P. Combs, R. A. Copeland, S. P. Arneric, P. R. Hartig, D. W. Robertson, B. Cordell, A. M. Stern, R. E. Olson, R. Zaczek, *J. Biol. Chem.* **2000**, *275*, 34086.

[74]   H. D. Lewis, B. I. P. Perez Revuelta, A. Nadin, J. G. Neduvelil, T. Harrison, S. J. Pollack, M. S. Shearman, *Biochemistry* **2003**, *42*, 7580.

[75]   T. A. Lanz, J. D. Hosley, W. J. Adams, K. M. Merchant, *J. Pharmacol. Exp. Ther.* **2004**, *309*, 49.

[76]   G. M. Rishton, D. M. Retz, P. A. Tempest, J. Novotny, S. Kahn, J. J. Treanor, J. D. Lile, M. Citron, *J. Med. Chem.* **2000**, *43*, 2297.

[77]   P. C. Belanger, I. J. Collins, J. C. Hannam, T. Harrison, S. J. Lewis, A. Madin, E. G. McIver, A. J. Nadin, J. G. Neduvelil, M. S. Shearman, A. L. Smith, T. J. Sparey, G. I. Stevenson, M. R. Teall, **2001**, WO 2001070677.

[78]   A. F. Kreft, D. C. Cole, K. R. Woller, J. R. Stock, G. Diamanitis, D. M. Kurbrak, K. M. Kutterer, W. J. Moore, D. S. Casebier, **2002**, WO 2002057252.

[79]   G. Tian, S. V. Ghanekar, D. Aharony, A. B. Shenvi, R. T. Jacobs, X. Liu, B. D. Greenberg, *J. Biol. Chem.* **2003**, *278*, 28968.

[80]   D. W. Smith, B. Munoz, K. Srinivasan, C. P. Bergstrom, P. V. Chaturvedula, M. S. Deshpande, D. J. Keavy, W. Y. Lau, M. F. Parker, C. P. Sloan, O. B. Wallace, H. H. Wang, **2000**, WO 2000050391.

[81]   J. L. Castro Pineiro, I. Churcher, K. Dinnell, T. Harrison, S. Kerrad, A. J. Nadin, P. J. Oakley, A. P. Owens, D. E. Shaw, M. R. Teall, B. J. Williams, S. Williams, **2002**, WO 20010821.

[82]   K. M. J. Brands, A. J. Davies, P. J. Oakley, J. P. Scott, D. E. Shaw, M. R. Teall, **2004**, WO 2004013090.

[83]   I. Churcher, T. Harrison, S. Kerrad, P. J. Oakley, D. E. Shaw, M. R. Teall, S. Williams, **2004**, WO 2004031137.

[84]   M. Hendrix, K. Baumann, R. Grosser, G. Koenig, V. Duesterhus, J. Krueger, **2003**, WO2003059335.

[85]   M. Neitzel, M. S. Dappen, J. Marugg, **2005**, WO 2005042489.

[86]   G. Galley, R. A. Goodnow, J.-U. Peters, **2004**, US 2004235819.

[87]   A. Flohr, G. Galley, R. Jakob-Roetne, E. A. Kitas, J.-U. Peters, W. Wostl, **2005**, US 20050075327.

[88]   D. Beher, M. Bettati, G. D. Checksfield, I. Churcher, V. A. Doughty, P. J. Oakley, A. Quddus, M. R. Teall, J. D. Wrigley, **2005**, WO 2005013985.

[89]   D. M. Barten, V. L. Guss, J. A. Corsa, A. Loo, S. B. Hansel, M. Zheng, B. Munoz, K. Srinivasan, B. Wang, B. J. Robertson, C. T. Polson, J. Wang, S. B. Roberts, J. P. Hendrick, J. J. Anderson, J. K. Loy, R. Denton, T. A. Verdoorn, D. W. Smith, K. M. Felsenstein, *J. Pharmacol. Exp. Ther.* **2005**, *312*, 635.

[90]   T. Washburn, E. Schweighoffer, T. Gridley, D. Chang, B. J. Fowlkes, D. Cado, E. Robey, *Cell* **1997**, *88*, 833.

[91]   J. C. Pui, D. Allman, L. Xu, S. DeRocco, F. G. Karnell, S. Bakkour, J. Y. Lee, T. Kadesch, R. R. Hardy, J. C. Aster, W. S. Pear, *Immunity* **1999**, *11*, 299.

[92]   B. K. Hadland, N. R. Manley, D. Su, G. D. Longmore, C. L. Moore, M. S. Wolfe, E. H. Schroeter, R. Kopan, *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 7487.

[93]   P. Doerfler, M. S. Shearman, R. M. Perlmutter, *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 9312.

[94]   G. H. Searfoss, W. H. Jordan, D. O. Calligaro, E. J. Galbreath, L. M. Schirtzinger, B. R. Berridge, H. Gao, M. A. Higgins, P. C. May, T. P. Ryan, *J. Biol. Chem.* **2003**, *278*, 46107.

[95]   J. Jensen, E. E. Pedersen, P. Galante, J. Hald, R. S. Heller, M. Ishibashi, R. Kageyama, F. Guillemot, P. Serup, O. D. Madsen, *Nat. Genet.* **2000**, *24*, 36.

[96]   G. J. McKenzie, L. L. Young, E. Briend, J. R. Lamb, M. J. Dallman, B. R. Champion, *Semin. Cell Dev. Biol.* **2003**, *14*, 127.

[97]   R. M. Costa, T. Honjo, A. J. Silva, *Curr. Biol.* **2003**, *13*, 1348.

[98]   A. Presente, R. S. Boyles, C. N. Serway, J. S. de Belle, A. J. Andres, *Proc. Natl Acad. Sci. U. S. A.* **2004**, *101*, 1764.

[99]   I. M. Conboy, M. J. Conboy, G. M. Smythe, T. A. Rando, *Science* **2003**, *302*, 1575.

[100] E. E. Clarke, I. Churcher, S. Ellis, J. D. Wrigley, H. D. Lewis, T. Harrison, M. S. Shearman, D. Beher, *J. Biol. Chem.* **2006**, *281*, 31279.

[101] M. Flajolet, G. He, M. Heiman, A. Lin, A. C. Nairn, P. Greengard, *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 4159.

[102] D. Pratico, J. Q. Trojanowski, *Neurobiol. Aging* **2000**, *21*, 441.

[103] P. L. McGeer, E. G. McGeer, *Arch. Neurol.* **2001**, *58*, 1790.

[104] B. A. In't Veld, A. Ruitenberg, A. Hofman, L. J. Launer, C. M. van Duijn, T. Stijnen, M. M. B. Bretteler, B. H. C. Stricker, *N. Engl. J. Med.* **2001**, *345*, 1515.

[105] M. Etminan, S. Gill, A. Samii, *Br. Med. J.* **2003**, *327*, 128.

[106] A. V. K. O. B. Yermakova, M., *Neurobiology of Aging* **2001**, *22*, 823.

[107] W. J. Lukiw, N. G. Bazan, *J. Neurosci. Res.* **1997**, *50*, 937.

[108] P. P. Zandi, J. C. Breitner, J. C. Anthony, *Expert Opin. Pharmacother.* **2002**, *3*, 365.

[109] R. N. Dubois, S. B. Abramson, L. Crofford, R. A. Gupta, L. S. Simon, L. B. Van De Putte, P. E. Lipsky, *FASEB. J.* **1998**, *12*, 1063.

[110] Y. Avramovich, T. Amit, M. B. H. Youdim, *J. Biol. Chem.* **2002**, *277*, 31466.

[111] S. Weggen, J. L. Eriksen, P. Das, S. A. Sagi, R. Wang, C. U. Pietrzik, K. A. Findlay, T. E. Smith, M. P. Murphy, T. Bulter, D. E. Kang, N. Marquez-Sterling, T. E. Golde, E. H. Koo, *Nature* **2001**, *414*, 212.

[112] J. L. Eriksen, S. A. Sagi, T. E. Smith, S. Weggen, P. Das, D. C. McLendon, V. V. Ozols, K. W. Jessing, K. H. Zavitz, E. H. Koo, T. E. Golde, *J. Clin. Invest.* **2003**, *112*, 440.

[113] T. Kukar, M. P. Murphy, J. L. Eriksen, S. A. Sagi, S. Weggen, T. E. Smith, T. Ladd, M. A. Khan, R. Kache, J. Beard, M. Dodson, S. Merit, V. V. Ozols, P. Z. Anastasiadis, P. Das, A. Fauq, E. H. Koo, T. E. Golde, *Nat. Med.* **2005**, *11*, 545.

[114] L. Gasparini, L. Rusconi, H. Xu, P. del Soldato, E. Ongini, *J. Neurochem.* **2004**, *88*, 337.

[115] T. Morihara, T. Chu, O. Ubeda, W. Beech, G. M. Cole, *J. Neurochem.* **2002**, *83*, 1009.

[116] Y. Takahashi, I. Hayashi, Y. Tominari, K. Rikimaru, Y. Morohashi, T. Kan, H. Natsugari, T. Fukuyama, T. Tomita, T. Iwatsubo, *J. Biol. Chem.* **2003**, *278*, 18664.

[117] D. Beher, E. E. Clarke, J. D. J. Wrigley, A. C. L. Martin, A. Nadin, I. Churcher, M. S. Shearman, *J. Biol. Chem.* **2004**, *279*, 43419.

[118] S. Weggen, J. L. Eriksen, S. A. Sagi, C. U. Pietrzik, T. E. Golde, E. H. Koo, *J. Biol. Chem.* **2003**, *278*, 30748.

[119] Y. Zhou, Y. Su, B. Li, F. Liu, J. W. Ryder, X. Wu, P. A. Gonzalez-DeWhitt, V. Gelfanova, J. E. Hale, P. C. May, S. M. Paul, B. Ni, *Science* **2003**, *302*, 1215.

[120] R. Narlawar, B. I. Perez Revuelta, C. Haass, H. Steiner, B. Schmidt, K. Baumann, *J. Med. Chem.* **2006**, *49*, 7588.

[121] I. Peretto, S. Radaelli, C. Parini, M. Zandi, L. F. Raveglia, G. Dondio, L. Fontanella, P. Misiano, C. Bigogno, A. Rizzi, B. Riccardi, M. Biscaioli, S. Marchetti, P. Puccini, S. Catinella, I. Rondelli, V. Cenacchi, P. T. Bolzoni, P. Caruso, G. Villetti, F. Facchinetti, E. DelGiudice, N. Moretto, B. P. Imbimbo, *J. Med. Chem.* **2005**, *48*, 5705.

[122] N. Stock, B. Munoz, J. D. J. Wrigley, M. S. Shearman, D. Beher, J. Peachey, T. L. Williamson, G. Bain, W. Chen, X. Jiang, R. St-Jacques, P. Prasit, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2219.

[123] G. P. Lim, F. Yang, T. Chu, P. Chen, W. Beech, B. Teter, T. Tran, O. Ubeda, K. H. Ashe, S. A. Frautschy, G. M. Cole, *J. Neurosci.* **2000**, *20*, 5709.

[124] P. T. Jantzen, K. E. Connor, G. DiCarlo, G. L. Wenk, J. L. Wallace, A. M. Rojiani, D. Coppola, D. Morgan, M. N. Gordon, *J. Neurosci.* **2002**, *22*, 2246.

[125] S. Sung, H. Yang, K. Uryu, E. B. Lee, L. Zhao, D. Shineman, J. Q. Trojanowski, V. M. Y. Lee, D. Pratico, *Am. J. Pathol.* **2004**, *165*, 2197.

[126] J. Rogers, L. C. Kirby, S. R. Hempelman, D. L. Berry, P. L. McGeer, A. W. Kaszniak, J. Zalinski, M. Cofield, L. Mansukhani, P. Willson, *Neurology* **1993**, *43*, 1609.

[127] M. Ricote, A. C. Li, T. M. Willson, C. J. Kelly, C. K. Glass, *Nature* **1998**, *391*, 79.

[128] C. Jiang, A. T. Ting, B. Seed, *Nature* **1998**, *391*, 82.

[129] D. Bishop-Bailey, T. D. Warner, *FASEB J.* **2003**, *17*, 1925.

[130] C. K. Combs, D. E. Johnson, J. C. Karlo, S. B. Cannady, G. E. Landreth, *J. Neurosci.* **2000**, *20*, 558.

[131] Y. Kitamura, S. Shimohama, H. Koike, J. i. Kakimura, Y. Matsuoka, Y. Nomura, P. J. Gebicke-Haerter, T. Taniguchi, *Biochem. Biophys. Res. Comm.* **1999**, *254*, 582.

[132] S. Brune, H. Koelsch, U. Ptok, M. Majores, A. Schulz, R. Schlosser, M. L. Rao, W. Maier, R. Heun, *J. Neural Transm.* **2003**, *110*, 1041.

[133] Q. Yan, J. Zhang, H. Liu, S. Babu-Khan, R. Vassar, A. L. Biere, M. Citron, G. Landreth, *J. Neurosci.* **2003**, *23*, 7504.

[134] M. J. May, S. Ghosh, *Immunol.Today* **1998**, *19*, 80.

[135] M. P. Mattson, S. Camandola, *J. Clin. Invest.* **2001**, *107*, 247.

[136] M. Ringman John, A. Frautschy Sally, M. Cole Gregory, L. Masterman Donna, L. Cummings Jeffrey, *Curr. Alz. Res.* **2005**, *2*, 131.

[137] G. J. Kelloff, J. A. Crowell, E. T. Hawk, V. E. Steele, R. A. Lubet, C. W. Boone, J. M. Covey, L. A. Doody, G. S. Omenn, P. Greenwald, W. K. Hong, D. R. Parkinson, D. Bagheri, G. T. Baxter, M. Blunden, M. K. Doeltz, K. M. Eisenhauer, K. Johnson, G. G. Knapp, D. G. Longfellow, W. F. Malone, S. G. Nayfield, H. E. Seifried, L. M. Swall, C. C. Sigman, *J. Cell. Biochem. Suppl.* **1996**, *26*, 54.

[138] B. B. Aggarwal, A. Kumar, A. C. Bharti, *Anticancer Res.* **2003**, *23*, 363.

[139] A. J. Vlietinck, T. De Bruyne, S. Apers, L. A. Pieters, *Planta Medica* **1998**, *64*, 97.

[140] M. E. Egan, M. Pearson, S. A. Weiner, V. Rajendran, D. Rubin, J. Gloeckner-Pagel, S. Canny, K. Du, G. L. Lukacs, M. J. Caplan, *Science* **2004**, *304*, 600.

[141] H. Tohgi, T. Abe, K. Yamazaki, T. Murata, E. Ishizaki, C. Isobe, *Neurosci. Lett.* **1999**, *269*, 52.

[142] D. Pratico, C. M. Clark, V. M. Lee, J. Q. Trojanowski, J. Rokach, G. A. FitzGerald, *Ann. Neurol.* **2000**, *48*, 809.

[143] M. J. Engelhart, M. I. Geerlings, A. Ruitenberg, J. C. van Swieten, A. Hofman, J. C. M. Witteman, M. M. B. Breteler, *J. Am. Med. Assoc.* **2002**, *287*, 3223.

[144] M. Sano, C. Ernesto, R. G. Thomas, M. R. Klauber, K. Schafer, M. Grundman, P. Woodbury, J. Growdon, C. W. Cotman, E. Pfeiffer, L. S. Schneider, L. J. Thal, *N. Engl. J. Med.* **1997**, *336*, 1216.

[145] A. C. Reddy, B. R. Lokesh, *Mol. Cell. Biochem.* **1992**, *111*, 117.

[146] Sreejayan, M. N. Rao, *J. Pharm. Pharmacol.* **1994**, *46*, 1013.

[147] C. A. Shih, J. K. Lin, *Carcinogenesis* **1993**, *14*, 709.

[148] D. S. Kim, S. Y. Park, J. K. Kim, *Neurosci. Lett.* **2001**, *303*, 57.

[149] H. Akiyama, S. Barger, S. Barnum, B. Bradt, J. Bauer, G. M. Cole, N. R. Cooper, P. Eikelenboom, M. Emmerling, B. L. Fiebich, C. E. Finch, S. Frautschy, W. S. Griffin, H. Hampel, M. Hull, G. Landreth, L. Lue, R. Mrak, I. R. Mackenzie, P. L. McGeer, M. K. O'Banion, J. Pachter, G. Pasinetti, C. Plata-Salaman, J. Rogers, R. Rydel, Y. Shen, W. Streit, R. Strohmeyer, I. Tooyoma, F. L. Van Muiswinkel, R. Veerhuis, D. Walker, S. Webster, B. Wegrzyniak, G. Wenk, T. Wyss-Coray, *Neurobiol. Aging* **2000**, *21*, 383.

[150] H. P. Ammon, H. Safayhi, T. Mack, J. Sabieraj, *J. Ethnopharmacol.* **1993**, *38*, 113.

[151] Y. X. Xu, K. A. Pindolia, N. Janakiraman, R. A. Chapman, S. C. Gautam, *Hematopathol. Mol. Hematol.* **1998**, *11*, 49.

[152] M. H. Pan, S. Y. Lin-Shiau, J. K. Lin, *Biochem. Pharmacol.* **2000**, *60*, 1665.

[153] L. Puglielli, B. C. Ellis, L. A. M. Ingano, D. M. Kovacs, *J. Mol. Neurosci.* **2004**, *24*, 93.

[154] K. B. Soni, R. Kuttan, *Indian J. Physiol. Pharmacol.* **1992**, *36*, 273.

[155] K. Ono, K. Hasegawa, H. Naiki, M. Yamada, *J. Neurosci. Res.* **2004**, *75*, 742.

[156] F. Yang, P. Lim Giselle, N. Begum Aynun, J. Ubeda Oliver, R. Simmons Mychica, S. Ambegaokar Surendra, P. Chen Pingping, R. Kayed, G. Glabe Charles, A. Frautschy Sally, M. Cole Gregory, *J. Biol. Chem.* **2005**, *280*, 5892.

[157] Y. Hatanaka, H. Nakayama, Y. Kanaoka, *Rev. Heteroatom Chem.* **1996**, *14*, 213.

[158] A. P. Scott, M. S. Platz, L. Radom, *J. Am. Chem. Soc.* **2001**, *123*, 6069.

[159] C. R. Kemnitz, W. L. Karney, W. T. Borden, *J. Am. Chem. Soc.* **1998**, *120*, 3499.

[160] M. S. Platz, *Acc. Chem. Res.* **1995**, *28*, 487.

[161] W. L. Karney, W. T. Borden, *J. Am. Chem. Soc.* **1997**, *119*, 3347.

[162] K. A. H. Chehade, H. P. Spielmann, *J. Org. Chem.* **2000**, *65*, 4949.

[163] J. Brunner, *Annu. Rev. Biochem.* **1993**, *62*, 483.

[164] M. A. Winnik, *Chem. Rev.* **1981**, *81*, 491.

[165] D. Praticò, *Curr. Med. Chem.* **2005**, *5*, 111.

[166] Y. Matsuoka, M. Picciano, B. Malester, J. LaFrancois, C. Zehr, J. M. Daeschner, J. A. Olschowka, M. I. Fonseca, M. K. O'Banion, A. J. Tenner, C. A. Lemere, K. Duff, *Am. J. Pathol.* **2001**, *158*, 1345.

[167] S. Weggen, J. L. Eriksen, P. Das, S. A. Sagi, R. Wang, C. U. Pietrizik, K. A. Findlay, T. E. Smith, M. P. Murphy, T. Bulter, D. E. Kang, N. Marquez-Sterling, T. E. Golde, E. H. Koo, *Nature* **2001**, *414*, 212.

[168] A. Lleo, O. Berezovska, L. Herl, S. Raju, A. Deng, B. J. Bacskai, M. P. Frosch, M. Irizarry, B. T. Hyman, *Nat. Med.* **2004**, *10*, 1065.

[169] Z. Xiang, L. Ho, S. Yemul, Z. Zhao, P. Pompl, K. Kelley, A. Dang, W. Qing, D. Teplow, G. M. Pasinetti, *Gene Expr.* **2002**, *10*, 271.

[170] F. Gruninger-Leitch, D. Schlatter, E. Kung, P. Nelbock, H. Dobeli, *J. Biol. Chem.* **2002**, *277*, 4687.

[171] A. Capell, L. Meyn, R. Fluhrer, D. B. Teplow, J. Walter, C. Haass, *J. Biol. Chem.* **2002**, *277*, 5637.

[172] S. Weggen, J. L. Eriksen, S. A. Sagi, C. U. Pietrzik, T. E. Golde, E. H. Koo, *J. Biol. Chem.* **2003**, *278*, 30748.

[173] J. L. Eriksen, S. A. Sagi, T. E. Smith, S. Weggen, P. Das, D. C. McLendon, V. V. Ozols, K. W. Jessing, K. H. Zavitz, E. H. Koo, T. E. Golde, *J. Clin. Invest.* **2003**, *112*, 440.

[174] Y. Takahashi, I. Hayashi, Y. Tominari, K. Rikimaru, Y. Morohashi, T. Kan, H. Natsugari, T. Fukuyama, T. Tomita, T. Iwatsubo, *J. Biol. Chem.* **2003**, *278*, 18664.

[175] B. A. in 't Veld, A. Ruitenberg, A. Hofman, L. J. Launer, C. M. van Duijn, T. Stijnen, M. M. B. Breteler, B. H. C. Stricker, *N. Engl. J. Med.* **2001**, *345*, 1515.

[176] S. Aisen Paul, A. Schafer Kimberly, M. Grundman, E. Pfeiffer, M. Sano, L. Davis Kenneth, R. Farlow Martin, S. Jin, G. Thomas Ronald, J. Thal Leon, *J. Am. Med. Assoc.* **2003**, *289*, 2819.

[177] S. A. Reines, G. A. Block, J. C. Morris, G. Liu, M. L. Nessly, C. R. Lines, B. A. Norman, C. C. Baranak, *Neurology* **2004**, *62*, 66.

[178] J. L. Cummings, *N. Eng. J. Med.* **2004**, *351*, 110.

[179] A. P. Ricketts, K. M. Lundy, S. B. Seibel, *Am. J. Vet. Res.* **1998**, *59*, 1441.

[180] S. Artavanis-Tsakonas, M. D. Rand, R. J. Lake, *Science* **1999**, *284*, 770.

[181] G. Evin, M. F. Sernee, C. L. Masters, *CNS Drugs* **2006**, *20*, 351.

[182] R. Narlawar, B. I. Perez Revuelta, K. Baumann, R. Schubenel, C. Haass, H. Steiner, B. Schmidt, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 176.

[183] M. Sastre, H. Steiner, K. Fuchs, A. Capell, G. Multhaup, M. M. Condron, D. B. Teplow, C. Haass, *EMBO J.* **2001**, *2*, 835.

[184] G. Larbig, A. Zall, B. Schmidt, *Helv. Chim. Acta* **2004**, *87*, 2334.

[185] G. H. Searfoss, W. H. Jordan, D. O. Calligaro, E. J. Galbreath, L. M. Schirtzinger, B. R. Berridge, H. Gao, M. A. Higgins, P. C. May, T. P. Ryan, *J. Biol. Chem.* **2003**, *278*, 46107.

[186] G. T. Wong, D. Manfra, F. M. Poulet, Q. Zhang, H. Josien, T. Bara, L. Engstrom, M. Pinzon-Ortiz, J. S. Fine, H.-J. J. Lee, L. Zhang, G. A. Higgins, E. M. Parker, *J. Biol. Chem.* **2004**, *279*, 12876.

[187] E. Czirr, S. Weggen, *Neurodegener. Dis.* **2006**, *3*, 298.

[188] S. Leuchtenberger, D. Beher, S. Weggen, *Curr. Pharm. Des.* **2006**, *12*, 4337.

[189] S. Weggen, L. Eriksen Jason, A. Sagi Sarah, U. Pietrzik Claus, V. Ozols, A. Fauq, E. Golde Todd, H. Koo Edward, *J. Biol. Chem.* **2003**, *278*, 31831.

[190] D. R. Riddell, H. Zhou, T. A. Comery, E. Kouranova, C. F. Lo, H. K. Warwick, R. H. Ring, Y. Kirksey, S. Aschmies, J. Xu, K. Kubek, W. D. Hirst, C. Gonzales, Y. Chen, E. Murphy, S. Leonard, D. Vasylyev, A. Oganesian, R. L. Martone, M. N. Pangalos, P. H. Reinhart, J. S. Jacobsen, *Mol. Cell. Neurosci.* **2007**, *34*, 621.

[191] C. Czech, M. P. Burns, L. Vardanian, A. Augustin, H. Jacobsen, K. Baumann, G. W. Rebeck, *J. Neurochem.* **2007**, *101*, 929.

[192] J. M. Ringman, S. A. Frautschy, G. M. Cole, D. L. Masterman, J. L. Cummings, *Curr. Alz. Res.* **2005**, *2*, 131.

[193] U. Bertsch, K. F. Winklhofer, T. Hirschberger, J. Bieschke, P. Weber, F. U. Hartl, P. Tavan, J. Tatzelt, H. A. Kretzschmar, A. Giese, *J. Virol.* **2005**, *79*, 7785.

[194] I. Khlistunova, J. Biernat, Y. Wang, M. Pickhardt, M. von Bergen, Z. Gazova, E. Mandelkow, E. M. Mandelkow, *J. Biol. Chem.* **2006**, *281*, 1205.

[195] G. T. Wong, D. Manfra, F. M. Poulet, Q. Zhang, H. Josien, T. Bara, L. Engstrom, M. Pinzon-Ortiz, J. S. Fine, H.-J. J. Lee, L. Zhang, G. A. Higgins, E. M. Parker, *J. Biol. Chem.* **2004**, *279*, 12876.

[196] P. C. Fraering, W. Ye, J.-M. Strub, G. Dolios, M. J. LaVoie, B. L. Ostaszewski, A. van Dorsselaer, R. Wang, D. J. Selkoe, M. S. Wolfe, *Biochemistry* **2004**, *43*, 9774.

[197] C. Haass, *EMBO J.* **2004**, *23*, 483.

[198] C. McLendon, T. Xin, C. Ziani-Cherif, M. P. Murphy, K. A. Findlay, P. A. Lewis, I. Pinnix, K. Sambamurti, R. Wang, A. Fauq, T. E. Golde, *FASEB* **2000**, *14*, 2383.

[199] H. F. Dovey, V. John, J. P. Anderson, L. Z. Chen, P. De Saint Andrieu, L. Y. Fang, S. B. Freedman, B. Folmer, E. Goldbach, E. J. Holsztynska, K. L. Hu, K. L. Johnson-Wood, S. L. Kennedy, D. Kholodenko, J. E. Knops, L. H. Latimer, M. Lee, Z. Liao, I. M. Lieberburg, R. N. Motter, L. C. Mutter, J. Nietz, K. P. Quinn, K. L. Sacchi, P. A. Seubert, G. M. Shopp, E. D. Thorsett, J. S. Tung, J. Wu, S. Yang, C. T. Yin, D. B. Schenk, P. C. May, L. D. Altstiel, M. H. Bender, L. N. Boggs, T. C. Britton, J. C. Clemens, D. L. Czilli, D. K. Dieckman-McGinty, J. J. Droste, K. S. Fuson, B. D. Gitter, P. A. Hyslop, E. M. Johnstone, W. Y. Li, S. P. Little, T. E. Mabry, F. D. Miller, B. Ni, J. S. Nissen, W. J. Porter, B. D. Potts, J. K. Reel, D. Stephenson, Y. Su, L. A. Shipley, C. A. Whitesitt, T. Yin, J. E. Audia, *J. Neurochem.* **2001**, *76*, 173.

[200] J. E. Audia, T. C. Britton, J. J. Droste, B. K. Folmer, G. W. Huffman, V. John, L. H. Latimer, T. E. Mabry, J. S. Nissen, **1998**. WO 9822494.

[201] T. A. Lanz, C. S. Himes, G. Pallante, L. Adams, S. Yamazaki, B. Amore, K. M. Merchant, *J. Pharmacol. Exp. Ther.* **2003**, *305*, 864.

[202] A. Geling, H. Steiner, M. Willem, L. Bally-Cuif, C. Haass, *EMBO Rep.* **2002**, *3*, 688.

[203] B. K. Hadland, N. R. Manley, D.-M. Su, G. D. Longmore, C. L. Moore, M. S. Wolfe, E. H. Schroeter, R. Kopan, *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 7487.

[204]   T. Kan, Y. Tominari, Y. Morohashi, H. Natsugari, T. Tomita, T. Iwatsubo, T. Fukuyama, *Chem. Commun.* **2003**, 2244.

7. APPENDIX

I.   Rajeshwar Narlawar, Blanca Perez Revauelta, Christian Haass, Harald Steiner, Boris Schmidt, Karlheinz Baumann, The Scaffold of the COX-2 Inhibitor Carprofen Provides Alzheimer γ-Secretase Modulators, *Journal of Medicinal Chemistry*, **2006**, *49*, 7588-7591.

II. Rajeshwar Narlawar, Blanca Perez Revauelta, Karlheinz Baumann, Robert Schubenel, Christian Haass, Harald Steiner, Boris Schmidt, *N*-Substituted Carbazolyloxyacetic Acids Modulate Alzheimer Associated γ-Secretase, *Bioorganic and Medicinal Chemistry Lett*ers, **2007**, *17*, 176-182.

Scaffold of the Cyclooxygenase-2 (COX-2) Inhibitor Carprofen Provides Alzheimer γ-Secretase Modulators

Rajeshwar Narlawar,[†] Blanca I. Pérez Revuelta,[‡] Christian Haass,[‡] Harald Steiner,[‡] Boris Schmidt,*,[†] and Karlheinz Baumann[§]

Clemens Schöpf-Institute of Chemistry and Biochemistry, Darmstadt University of Technology, Petersenstrasse 22, D-64287 Darmstadt, Germany, Department of Biochemistry, Laboratory for Alzheimer's and Parkinson's Disease Research, Adolf-Butenandt-Institute, Ludwig-Maximilians-University, Schillerstrasse 44, D-80336 Munich, Germany, and Preclinical Research CNS, Pharmaceuticals Division, F. Hoffmann-La Roche Ltd., Building 70/345, CH-4070 Basel, Switzerland

Abstract: N-Sulfonylated and N-alkylated carprofen derivatives were investigated for their inhibition and modulation of γ-secretase, which is associated with Alzheimer's disease. The introduction of a lipophilic substituent transformed the COX-2 inhibitor carprofen into a potent γ-secretase modulator. Several compounds (e.g., **9p**, **11f**) caused selective reduction of Aβ$_{42}$ and an increase of Aβ$_{38}$. The most active compounds displayed activities in the low micromolar range and no effect on the γ-secretase cleavage at the ε-site.

Despite tremendous progress in understanding Alzheimer's disease (AD), there remains the challenge to develop agents for its therapy. Approved drugs such as acetylcholinesterase inhibitors and memantine hydrochloride offer symptomatic treatment, but they do not address the basic pathology of the disease: deposition of amyloid plaques and development of neurofibrillary tangles. The metabolism of the β-amyloid precursor protein (APP), which is cleaved by the aspartic proteases β-secretase and γ-secretase, results in the generation and pathological deposition of the 40 and 42 amino acid long peptides Aβ$_{40}$ and Aβ$_{42}$ (Figure 1). These fragments are the major components of amyloid fibrils.[1,2]

Promising results for the treatment of AD were obtained with some cyclooxygenase-1 (COX-1) inhibitors,[3−7] both in vitro and in a prospective, population-based cohort study of 6989 patients.[8] This is not a class effect because nonsteroidal anti-inflammatory drugs (NSAIDs) (e.g., diclofenac (2-[2-(2,6-dichlorophenyl)aminophenyl]ethanoic acid) and naproxen ((+)-(*S*)-2-(6-methoxynaphthalen-2-yl)propanoic acid)) do not lower Aβ in vitro,[3,5] and neither naproxen nor rofecoxib (4-(4-methylsulfonylphenyl)-3-phenyl-5*H*-furan-2-one, a COX-2 inhibitor) slows cognitive decline in patients with mild-to-moderate AD.[9,10] The positive clinical results are still in need of a sound rationale and experimental validation.[11] The proof of concept is still missing. NSAIDs that modulate γ-secretase cleavage of APP affect the distance between APP and presenilin 1, the catalytic subunit of γ-secretase.[12] They seem to interfere with substrate recognition/cleavage and shift the precision of γ-secretase cleavage from the γ42 to the γ38 site (Figure 1) to generate more Aβ$_{38}$ and less Aβ$_{42}$.[3,12] Compounds with reverse shift were reported recently, and these enhanced Aβ$_{42}$ produc-

* To whom correspondence should be addressed. Phone: (+49) 6151-163075. Fax: (+49) 6151-163278. E-mail: schmidt_boris@t-online.de.
    † Darmstadt University of Technology.
    ‡ Ludwig-Maximilians-University.
    § F. Hoffmann-La Roche Ltd.
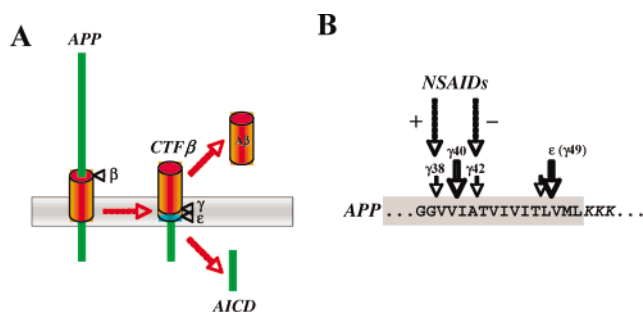
Figure 1. Schematic representation of APP processing and the modulation of γ-secretase cleavage by NSAIDs. (A) APP is first processed by β-secretase to generate the C-terminal fragment CTFβ, which is subsequently cleaved within its transmembrane domain at two principle sites, γ and ε, by γ-secretase to release Aβ and AICD. (B) Heterogeneous cleavage at the γ-site generates different Aβ species by cleavage at the γ38, γ40, and γ42 sites. A subset of NSAIDs increases the cleavage at the γ38 site while reducing cleavage at the γ42 site.

Scheme 1. Effect of NSAIDs (**1**−**4**) on Aβ Levels as Reported by Weggen[3] and Beher[7] and the Structure of BMS-299897 (**5**)

1 *R*-Flurbiprofen
Aβ$_{42}$ IC$_{50}$ = 307 μM

2 Sulindac Sulfide
Aβ$_{42}$ IC$_{50}$ = 34 μM

3 Indomethacin
Aβ$_{42}$ IC$_{50}$ = 25-50 μM

4 Ibuprofen
Aβ$_{42}$ IC$_{50}$ = 200 μM

5 BMS-299897
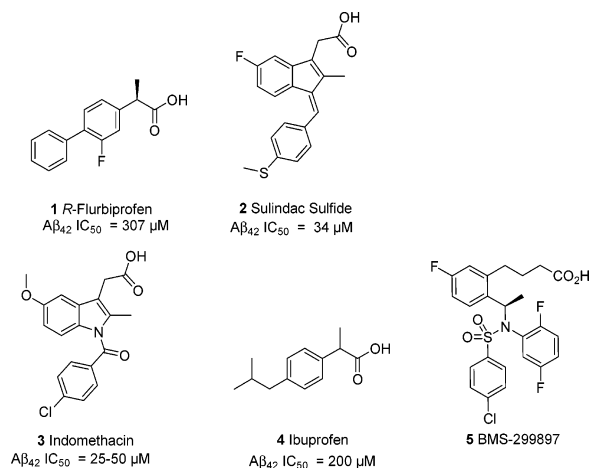
tion.[13] Noncompetitive antagonism indicates an allosteric mechanism of action.[7] Flurbiprofen ((±)-2-(3-fluoro-4-phenylphenyl)-propanoic acid, **1**) (10 and 25 mg kg^{-1} d^{-1}) elicits nonselective reductions in Aβ$_{1−40}$ and Aβ$_{1−42}$ plasma levels but was found to be toxic. It produced small reductions in Aβ$_{1−40}$ in the cortex at 25 mg kg^{-1} d^{-1} but did not affect Aβ levels in the hippocampus or cerebrospinal fluid. Cyclopropylated flurbiprofen analogues without COX activity display improved potency on γ-secretase inhibition.[14] Contrary to previous reports, sulindac sulfide (2-((5*Z*)-1-(4-(methylthio)benzylidene)-5-fluoro-2-methyl-1*H*-inden-3-yl)acetic acid, **2**) and ibuprofen (2-(4-isobutyl-phenyl)propanoic acid, **4**) were found to be neither toxic nor efficacious at doses up to 50 mg kg^{-1} d^{-1}.[15] The kinetics of Aβ formation in the presence of the two NSAIDs and the displacement of an active site directed inhibitor support allosteric, noncompetitive modes of action of sulindac sulfide (**2**)[6] and *R*-flurbiprofen (**1**, Scheme 1) at low concentrations.[7] This resulted in selective inhibition of Aβ$_{42}$ production. However, both NSAIDs shift their modes of action from modulation to complete, nonselective inhibition of γ-secretase at high concentrations. Unfortunately, NSAID derivatives have escaped photoaffinity labeling techniques so far, except for biotinylated fenofibrate which labelled the C-terminal fragment of APP.[16]
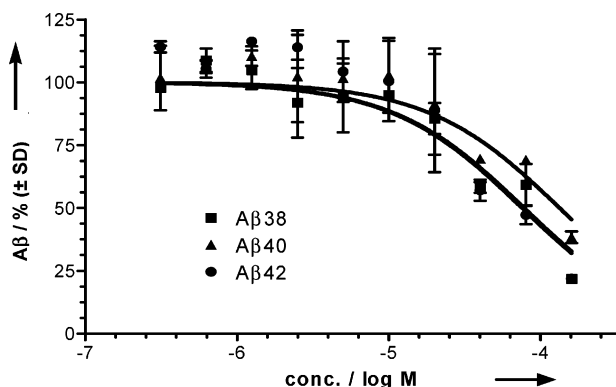
Figure 2. Dose response curve of carprofen (**6**).

In summary, just a few NSAIDs (**1**−**4**, Scheme 1) were reported to modulate γ-secretase, and an even smaller number of NSAIDs display confirmed modulation at physiological concentrations. Despite these vague, sometimes contradictory results reported for NSAID activity, we and others[13,17] were encouraged to investigate NSAIDs as scaffolds for γ-secretase inhibitors. We commenced with the derivatization of the carboxylic acid common to the COX-1 inhibitors. Furthermore, we included COX-2 inhibitors that are different from the coxib class such as carprofen (($\pm$)-2-(3-chloro-9H-carbazol-7-yl)-propanoic acid, **6**) and etodolac (2-(1,8-diethyl-1,3,4,9-tetra-hydropyrano[3,4-b]indol-1-yl)acetic acid) because they are structurally closer to COX-1 than to COX-2 inhibitors. Although some of the esters and amides displayed moderate inhibition of the cleavage by γ-secretase (Aβ_{38}, Aβ_{40}, Aβ_{42}), this approach was soon abandoned. Few of these initial derivatives displayed increased, unselective inhibition of γ-secretase in comparison to their parent drugs. Most of the approximately 150 NSAID carboxylic acid derivatives (e.g., **7**) resulted in loss of activity. This indicated an important contribution of the carboxylic acid to target affinity. After a brief investigation of sulindac analogues we focused on carprofen, which is a COX-2 inhibitor approved for the use in dogs, cows, and horses. The selectivity of carprofen versus COX-2$_{canine}$ and COX-1$_{canine}$ is greater than 100:1 (COX-2$_{canine}$ IC$_{50}$: R/S-carprofen 102 nM, R-carprofen 5.97 μM, S-carprofen 37 nM).[18] Original carprofen (as isolated from 500 mg tablets) was found to be a weak inhibitor of γ-secretase and reduced Aβ_{38}, Aβ_{40}, and Aβ_{42} at high concentration (Figure 2). This corresponds to the activity of α-methylated carprofen, which was reported to inhibit Aβ_{42} production by 40% at 100 μM.[17] This is in contrast to the inverse modulation of the COX-2 inhibitors that was reported recently.[13] The readily accessible sulfonamides were inspired by the γ-secretase inhibitor **5** (BMS-299897) and supported by a recent series from Merck Sharp & Dohme.[19,20] An analogue series of carprofen N-sulfonamides was prepared as outlined in Scheme 2. The acid functionality of carprofen was protected as a benzyl ester **7**. N-Sulfonylation of **7** was carried out using NaH and a sulfonyl chloride in THF. The benzyl group of **8** was removed by hydrogenation (**8a**−**m,p**−**r**) or base hydrolysis (**8n**−**o,s**) to give the acid **9**. We expected these compounds to be inhibitors of γ-secretase activity because of their resemblance to the γ-secretase inhibitor **5**. Serendipitously, they turned out to be modulators of γ-secretase activity, which control the cleavage pattern of γ-secretase. Such modulators may preserve the cleavage of substrates like Notch; thus, we adopted our initial objectives from inhibition to modulation. Notch−ligand interaction is a highly conserved mechanism that regulates specific cell fate during development.[21,22]

Scheme 2[a]
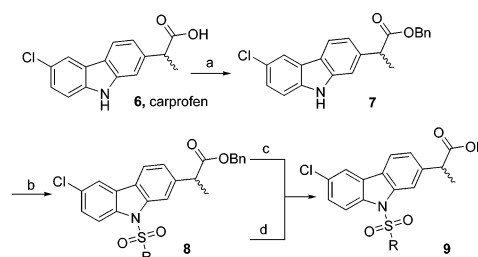
[a] Reagents: (a) BnBr, K$_2$CO$_3$, DMF, room temp, 1.5 h, 97%; (b) NaH, RSO$_2$Cl, THF, 0 °C to room temp, 2−8 h, 22−84%; (c) 10% Pd−C, MeOH/EtOAc (1:1), H$_2$, 3−18 h, 70−94%; (d) NaOH, THF/MeOH/H$_2$O (1:1:1), room temp, 3−12 h, 80−92%.

Scheme 3[a]
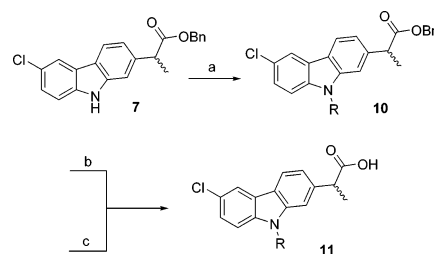
[a] Reagents: (a) NaH, RX, THF, 0 °C to room temp, 2−8 h, 30−81%; (b) 10% Pd−C, MeOH/EtOAc (1:1), H$_2$, 3−18 h, 70−96%; (c) NaOH, THF/MeOH/H$_2$O (1:1:1), room temp, 3−12 h, 80−92%.

Scheme 4[a]
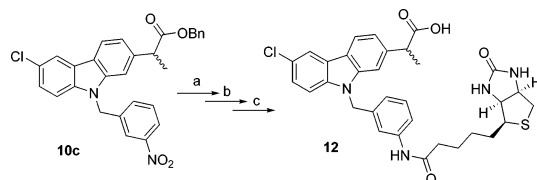
[a] Reagents: (a) SnCl$_2$, EtOH, reflux, 3 h, 90%; (b) EDCl, HOBT, biotin, Et$_3$N, DMF, room temp, 16 h, 65%; (c) 10% Pd−C, MeOH, H$_2$, room temp, 18 h, 90%.

N-Alkylated carprofen derivatives were prepared (Scheme 3) to evaluate the contribution of the sulfonamide moiety in **9r** and the most active derivative **9p**, where the sulfonamide is shielded by isopropyl substituents. The carprofen benzyl ester **7** was alkylated using NaH and RX in THF to yield the ester **10**. Subsequent benzyl deprotection by hydrogenation gave the desired N-alkylated carprofen **11**. The benzyl deprotection of **10c** was carried out by base hydrolysis.

The biotinylated carprofen derivative **12** was synthesized as depicted in Scheme 4 to identify the binding partner via immunoprecipitation.

To evaluate the compounds for their potency in modulating γ-secretase activity, we used the Aβ liquid-phase electro-chemiluminescence assay to measure Aβ isoforms.[27] γ-Secretase cleavage activity at the ϵ-site was monitored by de novo production of AICD in vitro using the previously reported assay.[25]

Compounds **9a**−**s**, **11a**−**f**, and **12** turned out to be effective modulators of γ-secretase. They affected the cleavage at the γ38, γ40, and γ42 sites to a different extent and particularly suppressed the formation of Aβ_{42} while enhancing the formation of Aβ_{38} and thus showed the typical profile of effective NSAIDs (see Tables 1 and 2, Figures 3 and 4). Compounds **9b,p** and **11a,e,f** were the most potent inhibitors of Aβ_{42}. Interestingly, the modulatory activity was preserved in the biotinylated **12**, which may therefore be used as an affinity reagent for

Table 1. Activity of Carprofen N-Sulfonamide Derivatives

|			IC$_{50}$ (μM)		
entry	compd	R	Aβ_{38}[a]	Aβ_{40}	Aβ_{42}
1	**6**	H	78	133	76
2	**9a**	4-methylphenyl	43	>100	56
3	**9b**	3,5-bis(trifluoromethyl)phenyl	ND[b]	>30	11
4	**9c**	4,5-dibromothiophen-2-yl	ND[b]	ND	>40
5	**9d**	3,5-difluorophenyl	ND[b]	ND	>40
6	**9e**	4-biphenyl	ND[b]	ND	37
7	**9f**	2-fluorophenyl	ND[b]	ND	>40
8	**9g**	3-fluorophenyl	ND[b]	ND	>40
9	**9h**	4-fluorophenyl	ND[b]	ND	>40
10	**9i**	2-bromophenyl	ND[b]	ND	40
11	**9j**	4-bromophenyl	ND[b]	ND	>40
12	**9k**	phenyl	ND[b]	ND	>40
13	**9l**	4-chlorophenyl	ND[b]	ND	>40
14	**9m**	3-chlorophenyl	ND[b]	ND	>40
15	**9n**	3-nitrophenyl	ND[b]	ND	39
16	**9o**	4-nitrophenyl	ND[b]	ND	>40
17	**9p**	2,4,6-triisopropylphenyl	9.3	>20	8.5
18	**9q**	4-n-propylphenyl	ND[b]	>40	>40
19	**9r**	octyl	ND[b]	>40	25
20	**9s**	4-cyanophenyl	ND[b]	ND	>40

[a] EC$_{50}$ values are displayed for Aβ_{38} except **6**. The EC$_{50}$ is based on the maximum level with a slope approximating 0. [b] Maximum effect on Aβ_{38} not observed at 40 μM (except for **6**, 160 μM; **9a**, 200 μM; and **9b**, 200 μM).

Table 2. Activity of N-Alkylated Carprofen Derivatives.

			activity (μM)		
			EC$_{50}$	IC$_{50}$	IC$_{50}$
entry	compd	R	Aβ_{38}	Aβ_{40}	Aβ_{42}
1	**12**		ND[a]	>150	88
2	**11a**	3-(trifluoromethoxy)benzyl	ND[a]	>40	7.5
3	**11b**	3-methoxybenzyl	ND[a]	>40	39
4	**11c**	3-nitrobenzyl	ND[a]	>40	22
5	**11d**	octyl	ND[a]	>40	6.9
6	**11e**	nonyl	8.1	>40	3.0
7	**11f**	decyl	5.8	>40	2.9

[a] Maximum effect on Aβ_{38} not observed at 40 μM (except for **12**, 150 μM).

γ-secretase. We observed from another series that N-alkylation of carbazole with shorter *n*-alkyl chains such as *n*-butyl and *n*-hexyl diminishes the γ-secretase modulatory activity (unpublished results). The observed differences for octyl, nonyl, or decyl substitution are small and so is the difference for alkylsulfone amides of equivalent length (see **11e** versus **9r**). The sulfone amides display an increased topological polar surface area (tPSA) from 49 to approximately 93 Å^2, which makes blood−brain barrier penetration less likely.[23] The calculated increase in tPSA is only partially compensated by a small reduction of the clogP (− 0.5) in comparison to the analogue alkyl derivatives. This favors the alkyl derivatives over the sulfone amides for further investigation in animal models. The N-benzylated or N-arylsulfonated derivatives benefit from fluorinated substituents (**9b**, **11a**) in the meta position. Ortho fluorination as in (**9f**) did not alter the activity in comparison to the parent sulfonamide (**9k**). The replacement of the methoxy group in **11b** by a trifluoromethoxy resulted in a 5-fold improvement of the IC$_{50}$ (Aβ_{42}). This fluorine-derived enhancement may be due to lipophilicity or polar interactions. We speculate that the lipophilic substituent anchors the N-substituted carprofen in the required orientation within the membrane; thus, the maximum tolerated length should be similar to those of natural phospholipids. The effect of the most potent compounds on γ-secretase cleavage at the ϵ-site was assessed by an in vitro assay that monitors the de novo generation of the APP intracellular domain (AICD).[24,25] The generation of AICD was
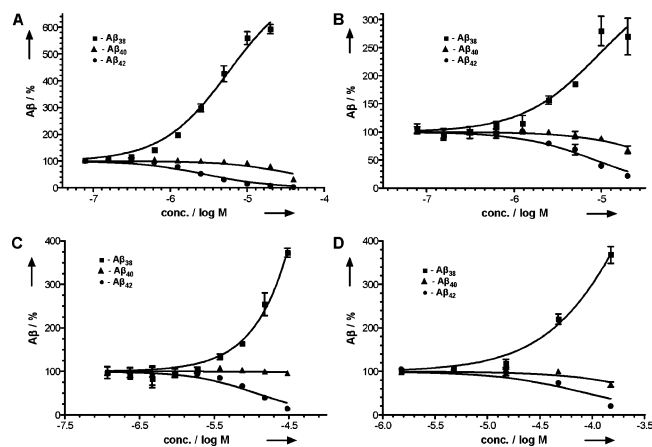
Figure 3. Dose response curves for the most active carprofen derivatives (Aβ % of control): (A) **11f**; (B) **9p**; (C) **9b**; (D) **12**; (■) Aβ_{38}; (▲) Aβ_{40}; (●) Aβ_{42}).
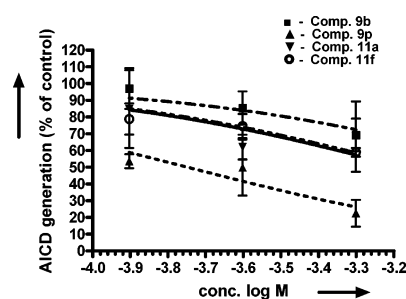
Figure 4. Dose−response curves for carprofen derivatives (**9b**, **9p**, **11a** and **11f**) on in vitro AICD generation. Results are the average of three independent experiments. Error bars indicate the standard error of the mean.

affected by the compounds to various extent (Figure 4). However, consistent with previous results,[3,7] generally much higher compound concentrations than those determined to be modulatory were required to inhibit the ϵ-cleavage. One of the most active carprofen derivatives **11f** was selected for evaluation in COX-1 and COX-2 assays to rule out COX-1 or COX-2 mediated effects at the necessary concentrations for γ-secretase modulation. The assays were performed at CEREP (www.cerep.com) using indomethacin as a standard for COX-1 and NS398 (N-(2-(cyclohexyloxy)-4-nitrophenyl)methanesulfonamide) for COX-2. **11f** displayed no activity on COX-1 and only marginal activity on COX-2 at 10 μM. No toxicity was observed at 40 μM in H4 cells except for **9p** and **12**, which significantly decreased viability at 40 μM, respectively (data not shown).

In conclusion, the introduction of a single lipophilic substituent, which may vary from arylsulfone to alkyl substituents, turns the COX-2 inhibitor carprofen into a γ-secretase modulator and improved potency 10-fold or more. Thus, several compounds (e.g., **9p**, **11f**) caused the selective reduction of Aβ_{42} and an increase of the less aggregatory Aβ_{38}. The most active compounds are more potent than the best reported NSAIDs, and they are devoid of COX-1 and COX-2 activity at the critical concentration; thus, they do not interfere with the delicate COX-1/COX-2 balance. Some of the sulfonamides are comparable in potency to the best N-alkylated analogues (**11b**), but the 50% increase of the tPSA (**9p**: 93.4 Å^2) makes a penetration of the blood−brain barrier less likely. Therefore, the N-alkylated derivatives are favored over the sulfonamides for further investigations. The properties of these N-alkylated lead candidates are close to the range of approved drugs or preclinical candidates except for their clogP.[26] The carboxylic acid group

may interfere with uptake, but the tPSA of **11b** is just 62.9 Å^2. This compares favorably to lumiracoxib ({2-[(2-chloro-6-fluorophenyl)amino]-5-methylphenyl}acetic acid, tPSA = 58 Å^2). The more polar **11c** has similar properties (tPSA = 96.1, clogP = 5.83) as the carboxylic acid **5** (clogP = 5.92, tPSA = 93.4 Å^2).[19] The lipophilic substituents cause amphiphilic properties of the carboxylic acids, which may interact with membranes. However, the placement of a polar end group, as in **12**, weakened but did not reverse the modulatory effect. Again, we favor the N-alkylated derivatives for the investigation of potential membrane interactions, as they allow the incorporation of phospholipids analogues and membrane disrupting fragments.

If affected at all, the ϵ-cleavage of γ-secretase was inhibited at much higher compound concentrations than those determined to be modulatory at the γ-site (Figure 4). The compounds are therefore expected to have little or no impact on γ-secretase-mediated signaling via the AICD or via intracellular domains of other γ-secretase substrates. However, the interaction site of these compounds has not been identified yet. The improvement of potency and the investigation of in vivo activity are subject to further investigations.

Supporting Information Available: Synthetic procedure and spectral data for the tested compounds and experimental procedure for assay. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Haass, C. Take Five—BACE and the γ-Secretase Quartet Conduct Alzheimer's Amyloid β-Peptide Generation. *EMBO J.* **2004**, *23*, 483—488.

(2) Churcher, I.; Beher, D. γ-Secretase as a Therapeutic Target for the Treatment of Alzheimer's Disease. *Curr. Pharm. Des.* **2005**, *11*, 3363—3382.

(3) Weggen, S.; Eriksen, J. L.; Das, P.; Sagi, S. A.; Wang, R.; Pietrizik, C. U.; Findlay, K. A.; Smith, T. E.; Murphy, M. P.; Bulter, T.; Kang, D. E.; Marquez-Sterling, N.; Golde, T. E.; Koo, E. H. A Subset of NSAIDs Lower Amyloidogenic Aβ_{42} Independently of Cyclooxygenase Activity. *Nature* **2001**, *414*, 212—216.

(4) Weggen, S.; Eriksen, J. L.; Sagi, S. A.; Pietrzik, C. U.; Golde, T. E.; Koo, E. H. Aβ_{42}-Lowering Nonsteroidal Anti-Inflammatory Drugs Preserve Intramembrane Cleavage of the Amyloid Precursor Protein (APP) and ErbB-4 Receptor and Signaling through the APP Intracellular Domain. *J. Biol. Chem.* **2003**, *278*, 30748—30754.

(5) Eriksen, J. L.; Sagi, S. A.; Smith, T. E.; Weggen, S.; Das, P.; McLendon, D. C.; Ozols, V. V.; Jessing, K. W.; Zavitz, K. H.; Koo, E. H.; Golde, T. E. NSAIDs and Enantiomers of Flurbiprofen Target γ-Secretase and Lower Aβ_{42} in Vivo. *J. Clin. Invest.* **2003**, *112*, 440—449.

(6) Takahashi, Y.; Hayashi, I.; Tominari, Y.; Rikimaru, K.; Morohashi, Y.; Kan, T.; Natsugari, H.; Fukuyama, T.; Tomita, T.; Iwatsubo, T. Sulindac Sulfide Is a Noncompetitive γ-Secretase Inhibitor That Preferentially Reduces Aβ_{42} Generation. *J. Biol. Chem.* **2003**, *278*, 18664—18670.

(7) Beher, D.; Clarke, E. E.; Wrigley, J. D. J.; Martin, A. C. L.; Nadin, A.; Churcher, I.; Shearman, M. S. Selected Non-Steroidal Anti-Inflammatory Drugs and Their Derivatives Target γ-Secretase at a Novel Site: Evidence for an Allosteric Mechanism. *J. Biol. Chem.* **2004**, *279*, 43419—43426.

(8) in 't Veld, B. A.; Ruitenberg, A.; Hofman, A.; Launer, L. J.; van Duijn, C. M.; Stijnen, T.; Breteler, M. M. B.; Stricker, B. H. C. Nonsteroidal Antiinflammatory Drugs and the Risk of Alzheimer's Disease. *N. Engl. J. Med.* **2001**, *345*, 1515—1521.

(9) Aisen, P. S.; Schafer, K. A.; Grundman, M.; Pfeiffer, E.; Sano, M.; Davis, K. L.; Farlow, M. R.; Jin, S.; Thomas, R. G.; Thal, L. J. Effects of Rofecoxib or Naproxen vs Placebo on Alzheimer Disease Progression: A Randomized Controlled Trial. *J. Am. Med. Assoc.* **2003**, *289*, 2819—2826.

(10) Reines, S. A.; Block, G. A.; Morris, J. C.; Liu, G.; Nessly, M. L.; Lines, C. R.; Norman, B. A.; Baranak, C. C. Rofecoxib: No Effect on Alzheimer's Disease in a 1-Year, Randomized, Blinded, Controlled Study. *Neurology* **2004**, *62*, 66—71.

(11) Cummings, J. L. Continuing Medical Education: Alzheimer's Disease. *N. Engl. J. Med.* **2004**, *351*, 110.

(12) Lleo, A.; Berezovska, O.; Herl, L.; Raju, S.; Deng, A.; Bacskai, B. J.; Frosch, M. P.; Irizarry, M.; Hyman, B. T. Nonsteroidal Anti-Inflammatory Drugs Lower Aβ_{42} and Change Presenilin 1 Conformation. *Nat. Med.* **2004**, *10*, 1065—1066.

(13) Kukar, T.; Murphy, M. P.; Eriksen, J. L.; Sagi, S. A.; Weggen, S.; Smith, T. E.; Ladd, T.; Khan, M. A.; Kache, R.; Beard, J.; Dodson, M.; Merit, S.; Ozols, V. V.; Anastasiadis, P. Z.; Das, P.; Fauq, A.; Koo, E. H.; Golde, T. E. Diverse Compounds Mimic Alzheimer Disease-Causing Mutations by Augmenting Aβ_{42} Production. *Nat. Med.* **2005**, *11*, 545—550.

(14) Peretto, I.; Radaelli, S.; Parini, C.; Zandi, M.; Raveglia, L. F.; Dondio, G.; Fontanella, L.; Misiano, P.; Bigogno, C.; Rizzi, A.; Riccardi, B.; Biscaioli, M.; Marchetti, S.; Puccini, P.; Catinella, S.; Rondelli, I.; Cenacchi, V.; Bolzoni, P. T.; Caruso, P.; Villetti, G.; Facchinetti, F.; DelGiudice, E.; Moretto, N.; Imbimbo, B. P. Synthesis and Biological Activity of Flurbiprofen Analogues as Selective Inhibitors of β-Amyloid$_{1-42}$ Secretion. *J. Med. Chem.* **2005**, *48*, 5705—5720.

(15) Lanz, T. A.; Fici, G. J.; Merchant, K. M. Lack of Specific Amyloid-beta(1—42) Suppression by Nonsteroidal Anti-Inflammatory Drugs in Young, Plaque-Free Tg2576 Mice and in Guinea Pig Neuronal Cultures. *J. Pharmacol. Exp. Ther.* **2005**, *312*, 399—406.

(16) Kukar, T.; Ladd, T.; Bann, M.; Fraering, P.; Kache, R.; Fauq, A.; Wolfe, M.; Koo, E. H.; Golde, T. E. P4-289: Mechanistic Insights into the *in vitro* and *in vivo* Modulation of Aβ_{42} Levels and Amyloid Pathology by NSAIDS and Related Compounds. *Alzheimer's and Dementia* **2006**, *2*, S601—S602.

(17) Stock, N.; Munoz, B.; Wrigley, J. D. J.; Shearman, M. S.; Beher, D.; Peachey, J.; Williamson, T. L.; Bain, G.; Chen, W.; Jiang, X.; St-Jacques, R.; Prasit, P. The Geminal Dimethyl Analogue of Flurbiprofen as a Novel Aβ_{42} Inhibitor and Potential Alzheimer's Disease Modifying Agent. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2219—2223.

(18) Ricketts, A. P.; Lundy, K. M.; Seibel, S. B. Evaluation of Selective Inhibition of Canine Cyclooxygenase 1 and 2 by Carprofen and Other Nonsteroidal Anti-Inflammatory Drugs. *Am. J. Vet. Res.* **1998**, *59*, 1441—1446.

(19) Barten, D. M.; Guss, V. L.; Corsa, J. A.; Loo, A.; Hansel, S. B.; Zheng, M.; Munoz, B.; Srinivasan, K.; Wang, B.; Robertson, B. J.; Polson, C. T.; Wang, J.; Roberts, S. B.; Hendrick, J. P.; Anderson, J. J.; Loy, J. K.; Denton, R.; Verdoorn, T. A.; Smith, D. W.; Felsenstein, K. M. Dynamics of β-Amyloid Reductions in Brain, Cerebrospinal Fluid, and Plasma of β-Amyloid Precursor Protein Transgenic Mice Treated with a γ-Secretase Inhibitor. *J. Pharmacol. Exp. Ther.* **2005**, *312*, 635—643.

(20) Beher, D.; Bettati, M.; Checksfield, G. D.; Churcher, I.; Doughty, V. A.; Oakley, P. J.; Quddus, A.; Teall, M. R.; Wrigley, J. D. Preparation of Tetrahydrocarbazole-1-Alkanoic Acids for the Treatment of Alzheimer's Disease and Related Conditions. WO 2005013985.

(21) Selkoe, D.; Kopan, R. Notch and Presenilin: Regulated Intramembrane Proteolysis Links Development and Degeneration. *Annu. Rev. Neurosci.* **2003**, *26*, 565—597.

(22) Kopan, R.; Ilagan, M. X. γ-Secretase: Proteasome of the Membrane? *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 499—504.

(23) Ertl, P.; Rohde, B.; Selzer, P. Fast Calculation of Molecular Polar Surface Area as a Sum of Fragment-Based Contributions and Its Application to the Prediction of Drug Transport Properties. *J. Med. Chem.* **2000**, *43*, 3714—3717.

(24) Sastre, M.; Steiner, H.; Fuchs, K.; Capell, A.; Multhaup, G.; Condron, M. M.; Teplow, D. B.; Haass, C. Presenilin-Dependent γ-Secretase Processing of β-Amyloid Precursor Protein at a Site Corresponding to the S3 Cleavage of Notch. *EMBO J.* **2001**, *2*, 835—841.

(25) Larbig, G.; Zall, A.; Schmidt, B. Inhibitors Designed for Presenilin 1 Utilizing by Means of Aspartic Acid Activation. *Helv. Chim. Acta* **2004**, *87*, 2334—2340.

(26) Boehm, H.-J., Schneider, G., Eds.; *Virtual Screening for Bioactive Molecules*; Wiley-VCH: Weinheim, Germany, 2000.

(27) Brockhaus, M.; Grunberg, J.; Rohrig, S.; Loetscher, H.; Wittenburg, N.; Baumeister, R.; Jacobsen, H.; Haass, C. Caspase-Mediated Cleavage Is Not Required for the Activity of Presenilins in Amyloidogenesis and NOTCH Signaling. *NeuroReport* **1998**, *9*, 1481—1486.

N-Substituted carbazolyloxyacetic acids modulate Alzheimer associated γ-secretase

Rajeshwar Narlawar,[a] Blanca I. Pérez Revuelta,[b] Karlheinz Baumann,[c] Robert Schubenel,[c] Christian Haass,[b] Harald Steiner[b] and Boris Schmidt[a,*]

[a]*Clemens Schöpf-Institute of Chemistry and Biochemistry, Darmstadt University of Technology, Petersenstr. 22, D-64287 Darmstadt, Germany*
[b]*Laboratory for Alzheimer's and Parkinson's Disease Research, Ludwig Maximilian University München, Schillerstr. 44, D-80336 München, Germany*
[c]*F. Hoffmann-La Roche Ltd, Pharmaceuticals Division, Preclinical Research CNS, Bldg. 70/345, CH-4070 Basel, Switzerland*

Abstract—*N*-Sulfonylated and *N*-alkylated carbazolyloxyacetic acids were investigated for the inhibition and modulation of the Alzheimer's disease associated γ-secretase. The introduction of a lipophilic substituent, which may vary from arylsulfone to alkyl, turned 2-carbazolyloxyacetic acids into potent γ-secretase modulators. This resulted in the selective reduction of $A\beta_{42}$ and an increase of the less aggregatory $A\beta_{38}$ fragment by several compounds (e.g., **7d** and **8c**). Introduction of an electron donating group at position 6 and 8 of *N*-substituted carbazolyloxyacetic acids either decreased the activity or inversed modulation. The most active compounds displayed activity on amyloid precursor protein (APP) overexpressing cell lines in the low micromolar range and little or no effect on the γ-secretase cleavage at the ε-site.
© 2006 Elsevier Ltd. All rights reserved.

Alzheimer's disease (AD) is a devastating neurodegenerative disorder that causes the most common form of dementia, affecting approximately 5% of the population over the age of 65 years in Europe. Short-term memory impairment, disorientation, aphasia and a general cognitive decline are common symptoms early in disease development. As the disease progresses, spatial and motor abilities are affected and the patient becomes bedridden and completely dependent on the caretaker. According to the World Health Organization, an estimated 37 million people worldwide currently have dementia; AD affects about 18 million of them.[1]

Pathological lesions and plaques consisting of the amyloid β-peptide (Aβ) are found in the brains of the AD patients. Aβ is heterogeneously produced by the sequential cleavages of amyloid precursor protein (APP) by the two aspartic proteases: β- and γ-secre-

tase. Cleavage at the Aβ N-terminus is executed by the β-site cleaving enzyme (BACE).[2,3] The following intramembrane cleavage by γ-secretase occurs with little sequence specificity, resulting in Aβ fragments of different length, predominantly $A\beta_{40}$, $A\beta_{42}$ and some $A\beta_{38}$. $A\beta_{42}$ being the most aggregatory. The C-terminal fragments generated by α- and β-secretase cleavage, C83 and C99, are cleaved within their transmembrane domains to produce the p3 peptide. The functional γ-secretase complex requires the correct assembly of at least five components: presenilin-1 (PS1), nicastrin, anterior pharynx defective-1 (Aph-1), presenilin enhancer-2 (Pen-2) and the substrate.[4–10] PS1, a catalytic subunit of the γ-secretase complex, harbours two aspartates in the transmembrane domains 6 and 7 that define a novel active site closely resembling that of other recently identified aspartic proteases.[11–14] PS1 acts as a heterodimer of N- and C-terminal fragments (NTF, CTF) that derive from autoproteolysis. γ-Secretase activity can be controlled by the inhibition of the active site of PS1 or by interference with complex assembly or substrate recognition, the latter resulting in allosteric modulation or inhibition. The allosteric mechanisms are particularly attractive targets for drug development as they may control the ratio of the Aβ

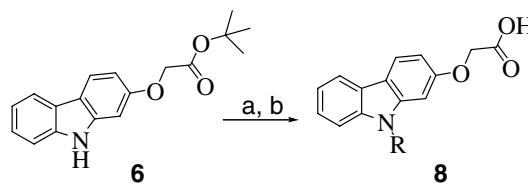fragments: Aβ$_{38}$, Aβ$_{40}$, and Aβ$_{42}$, while retaining the cleavage of other substrates. Most of the reported and confirmed γ-secretase inhibitors are not substrate specific and inhibit the processing of other γ-secretase substrates equally,[15] for example, Notch and other.[16]

Epidemiological studies indicated an association between nonsteroidal anti-inflammatory drugs (NSAIDs)
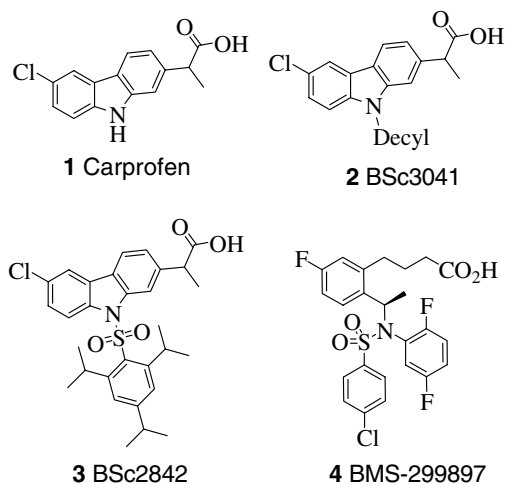
Scheme 1. Carprofen, the most active carprofen derivatives and BMS-299897.

Scheme 3. Synthesis of N-alkylated carbazolyloxyacetic acids and **8d**. Reagents and conditions: (a) KO^{t}Bu, RX, THF, 0 °C rt, 2–8 h, 80–91%; (b) NaOH, MeOH/H$_2$O (1:1) rt, 2 h, 92–97%.

Entry	Compound	R
1	**8a**	Octyl
2	**8b**	Nonyl
3	**8c**	Decyl
4	**8d**	H
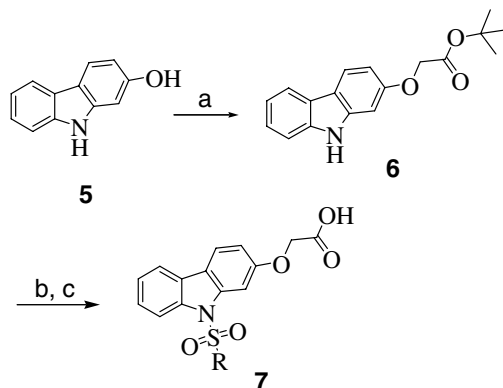
Entry	Compound	R
1	**7a**	4-methylphenyl
2	**7b**	3,5-bis(trifluoromethyl)phenyl
3	**7c**	4-biphenyl
4	**7d**	2,4,6-tri-*iso*-propylphenyl
5	**7e**	4-*n*-propylphenyl
6	**7f**	Octyl
7	**7g**	(1S)-1-(methyl)-7,7-dimethylbicyclo[2.2.1]heptan-2-one
8	**7h**	4-chlorophenyl
9	**7i**	3,5-difluorophenyl
10	**7j**	2-bromophenyl
11	**7k**	3-nitrophenyl

Scheme 2. Synthesis of *N*-sulfonylated carbazolyloxyacetic acid derivatives. Reagents and conditions: (a) ClCH$_2$CO$_2^t$Bu, K$_2$CO$_3$, acetone, 60–70 °C, 16 h, 100%; (b) NaH, RSO$_2$Cl, THF, 0 °C rt, 2–8 h, 80–100%; (c) 20% TFA in DCM, 1–4 h, 80–100%.

use and a reduced risk for the development of AD.[17–20] Recently, we reported N-sulfonylated and N-alkylated derivatives (**2**, **3**) of carprofen (Scheme 1) that modulate γ-secretase and selectively reduce Aβ$_{42}$.[21,22] This is accompanied by an increase of the less aggregatory Aβ$_{38}$ species. Carprofen **1** is a COX-2 inhibitor (COX = cyclooxygenase) approved for the use in dogs, cows and horses as Imadyl® or Rimadyl®. The selectivity of carprofen versus COX-2$_{canine}$ and COX-1$_{canine}$ is

greater than 100:1 (COX-2$_{canine}$ IC$_{50}$/*R*/*S*-carprofen 102 nM, *R*-carprofen 5.97 μM, *S*-carprofen 37 nM).[23] Original carprofen (as isolated from Rimadyl® tablets 500 mg) was found to be a weak inhibitor of γ-secretase and reduced Aβ$_{38}$, Aβ$_{40}$ and Aβ$_{42}$ at high concentrations. Knowing about the relevance of the carboxylic acid and N-substitution of carprofen, we decided to investigate scaffolds not included in the patent applications and publications by Koo et al.[24] or Stock,[25] or Imbimbo[26] and others.[27,28] Carprofen's similarity to the γ-secretase inhibitor BMS-299897 (Scheme 1) prompted us to investigate N-sulfonylated and N-alkylated 2-hydroxy carbazolyloxyacetic acids. The corresponding sulfonamides **7a**–**k** were readily accessible by straightforward synthesis.

Scheme 4. Synthesis of 6-methoxy and 8-methoxy carbazoles. Reagents and conditions: (a) PMBCl, K$_2$CO$_3$, acetone, reflux, 16 h, 97%; (b) [(Ph$_3$P)$_4$Pd], 3-methoxybenzene-boronic acid, K$_2$CO$_3$, H$_2$O, toluene, reflux, 18 h, 67% (c) Ph$_3$P, o-dichlorobenzene, reflux, 16 h, 60%.

A series of 2-hydroxy carbazole N-sulfonamide derivatives was prepared as outlined in Scheme 2. 2-Hydroxycarbazole **5** was alkylated using anhydrous K$_2$CO$_3$ and ClCH$_2$CO$_2^t$Bu in acetone at 60–70 °C to give compound **6**. N-Sulfonylation of **6** was carried out using NaH, various sulfonyl chlorides in THF and subsequent t-butyl deprotection by 20% trifluoroacetic acid in dichloromethane furnished the desired compound **7**.

N-Alkylated carbazolyloxyacetic acids were prepared to evaluate the contribution of the sulfonamide moiety in
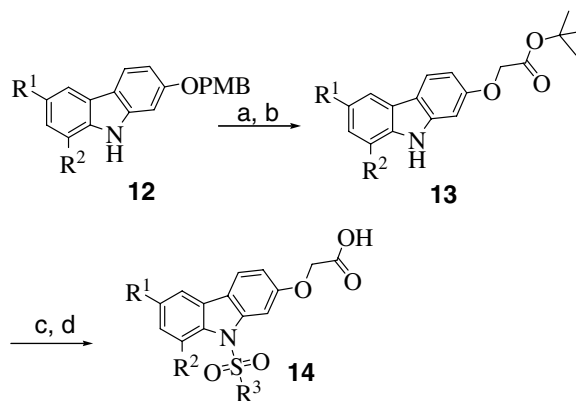
Entry	Compound	R^1	R^2	R^3
1	**14a**	OMe	H	3,5-(bis-trifluoro)phenyl
2	**14b**	OMe	H	4-chlorophenyl
3	**14c**	OMe	H	4-biphenyl
4	**14d**	OMe	H	2-bromophenyl
5	**14e**	H	OMe	4-chlorophenyl
6	**14f**	H	OMe	4-biphenyl

Scheme 5. Synthesis of 6-methoxy and 8-methoxy N-sulfonylated carbazolyloxyacetic acids. Reagents and conditions: (a) 10% Pd–C, H$_2$, MeOH, 60 psi, rt, 12 h, 81%; (b) ClCH$_2$CO$_2^t$Bu, K$_2$CO$_3$, acetone, reflux, 16–18 h, 82–88%; (c) R^{3}SO$_2$Cl, NaH, THF, 0 °C rt, 1–3 h, 55–71%; (d) 20% TFA in DCM, rt, 2 h, 80–94%.

Table 1. Activity report of N-alkylated and N-sulfonylated carbazolyloxyacetic acids

Entry	Compound	Cell toxicity[d] (μM)	IC$_{50}$ (μM)		
			Aβ_{38}[a]	Aβ_{40}	Aβ_{42}
1	**7a**	20	13	>40	39
2	**7b**	20	>40	>40	>40
3	**7c**	20	13	40	24
4	**7d**	20	7.8	19	7.5
5	**7e**	>40	ND[b]	>40	38
6	**7f**	>40	ND[b]	>40	37
7	**7g**	40	14	>40	20
8	**7h**	40	32	>40	32
9	**7i**	40	13	>40	>40
10	**7j**	40	>40	>40	>40
11	**7k**	40	16	>40	>40
12	**8a**	>40	24	>40	19
13	**8b**	>40	30	>40	17
14	**8c**	>40	ND[b]	>40	11
15	**8d**	>40	>40	>40	>40
16	**2**	—	5.8	>40	2.9
17	**3**	—	9.3	>20	8.5
18	**4**[c]	—	—	—	0.0071

[a] EC$_{50}$ values are displayed for Aβ_{38}.
[b] Maximum effect on Aβ_{38} not observed at the highest concentration tested.
[c] IC$_{50}$ value is displayed for total Aβ.
[d] Significant cellular toxicity observed at this concentration. Viability reduction > 20%.

7g and the most active derivate **7d**, where the sulfonamide is shielded by isopropyl substituents. The N-alkylated carbazolyloxyacetic acids were synthesized as shown in Scheme 3. Compound **6** was alkylated using KO^{t}Bu and alkyl halide in dry THF. Subsequent deprotection of t-butyl group by base hydrolysis provided desired N-alkylated carbazolyloxyacetic acid **8**.

We synthesized the 2-hydroxy-6-methoxy and 2-hydroxy-8-methoxy N-sulfonylated carbazolyloxyacetic acids to investigate the impact of an electron donating substituent on the activity. We commenced the synthesis of 6-methoxy and 8-methoxy-2-hydroxy carbazoles from the commercially available 4-chloro-3-nitrophenol **9** as outlined in Scheme 4. The –OH functionality of **9** was protected as a *para*-methoxybenzyl (PMB) ether **10**. Suzuki–Miyaura coupling of **10** with 3-methoxybenzeneboronic acid using the catalyst [(PPh$_3$)$_4$]Pd gave biphenyl **11**, which was then cyclized to its corresponding carbazole by refluxing it in *o*-dichlorobenzene with PPh$_3$. The PPh$_3$ mediated cyclization resulted in two regioisomers, 6-methoxy carbazole, **12a** and 8-methoxy carbazole, **12b** which were separated by flash column chromatography.

N-Sulfonylated derivatives of 2-hydroxy-6-methoxy and 2-hydroxy-8-methoxy carbazolyloxyacetic acids were synthesized as depicted in Scheme 5. The PMB group of **12** was deprotected by hydrogenation at 60 psi and the free –OH was alkylated using anhydrous K$_2$CO$_3$ and ClCH$_2$CO$_2^t$Bu in acetone at 60–70 °C to yield alkylated **13**. N-Sulfonylation of **13** was carried out using NaH and R^{3}SO$_2$Cl in dry THF and subsequent t-butyl group removal by

20% TFA in DCM furnished the desired compound **14**.

Compounds **7a–k** and **8a–c** turned out to be effective modulators of γ-secretase. They affected the cleavage at the γ38, γ40 and γ42 sites to a different extent, and particularly reduced the formation of Aβ_{42}, while increasing the formation of Aβ_{38} (see Table 1 and Fig. 1).[33] The most potent inhibitors of Aβ_{42} were compounds **7d**, **7g**, and **8c**. Compounds **14a–c** showed modulatory activity but required very high concentrations. Compounds **14e** and **14f** showed some indications to be inverse modulators, they increased Aβ_{42} formation and reduced Aβ_{38} formation at very high concentrations (see Fig. 2). The necessary levels for IC$_{50}$/EC$_{50}$ determination were not reached at 40 μM. The effect of the most potent compounds on γ-secretase cleavage at the ϵ-site was assessed using an in vitro assay that monitors the de novo generation of β-amyloid precursor protein intracellular domain (AICD).[29,30] The formation of AICD was affected by the compounds to various extent (Fig. 3). However, much higher compound concentrations than those determined to be modulatory were required to inhibit the ϵ-cleavage. One of the most active N-sulfonylated carbazolyloxyacetic acids (**7d**) was selected for evaluation in COX-1 and COX-2 assays to rule out COX-1 or COX-2 mediated effects at the concentrations necessary for γ-secretase modulation. The assays were performed at CEREP (www.cerep.com) using indomethacin as a standard for COX-1 and NS398 for COX-2. The compound **7d** displayed no activity on COX-1 and COX-2 at 10 μM concentration. Only a few compounds within this structural class displayed cell toxicity in H4 cells at the highest concentration tested (40 μM). It is very likely that this toxicity is caused by unique structural properties of single compounds.[34,35]

The introduction of a single lipophilic substituent, which may vary from arylsulfone to alkyl, turns the N-substituted carbazolyloxyacetic acids into a potent γ-secretase modulator. Some in silico parameters of these lead candidates are close to the range of drug-like compounds,[31] but the lipophilic substituents cause a dramatic increase of the clog$P$. The carboxylic acid may interfere with uptake, but the total polar surface area of **8c** is just 62.9 Å^2, this compares to COX189 (lumiracoxib, tPSA = 58 Å^2). The more polar compound **7c** displays similar properties (clog$P$ = 6.426, tPSA = 107.5 Å^2) as the carboxylic acid BMS-299897 (clog$P$ = 5.92, tPSA = 93.4 Å^2).[27] Further increases in chain length will worsen this property, but the determination of the maximum length may provide information on drug localisation or trafficking. We speculate that the lipophilic substituent anchors the N-substituted carbazolyloxyacetic acid in the required orientation within the membrane, thus the maximum tolerated length should be similar to natural phospholipids. This hypothesis will be explored in the next series. The desired selective reductions of Aβ_{42} and the accompanying increases of the less aggregatory Aβ_{38} fragment are displayed by several compounds
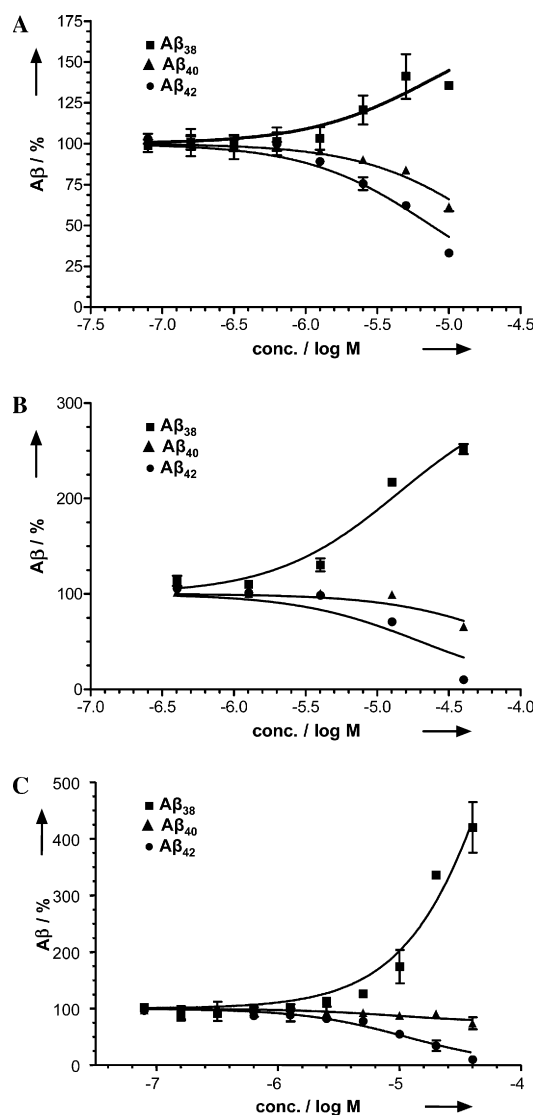
Figure 1. Dose–response curves for the most active N-substituted carbazolyloxyacetic acid derivatives; Aβ (% of control). (A) Compound **7d**. (B) Compound **7g**. (C) Compound **8c**.
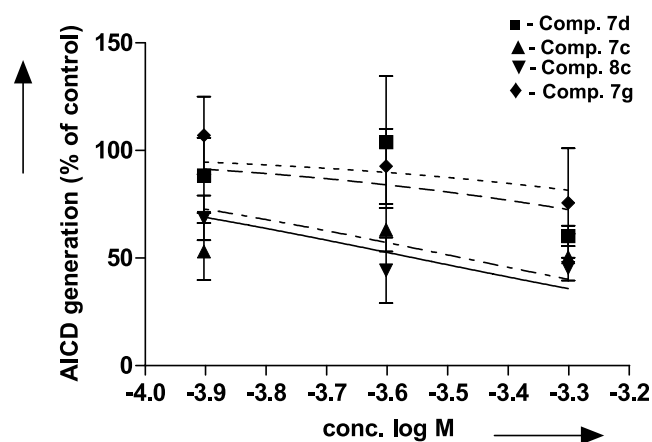

Figure 3. Dose–response curves for the most active N-substituted carbazolyloxyacetic acids (**7d**, **7c**, **8c**, and **7g**) on in vitro AICD generation. Results are the average of three independent experiments. Error bars indicate the standard error of the mean.

(e.g., **7d** and **8c**). The introduction of an electron donating substituent at 6- and 8-position of N-substituted carbazolyloxyacetic acids either decreased the modulatory activity (**14a–14c**) or inversed modulatory activity (**14d–14f**). If affected at all, the ε-cleavage of γ-secretase was inhibited at much higher compound concentrations than those determined to be modulatory at the γ-site (Fig. 3). The compounds are thus expected to have little or no impact on γ-secretase-mediated signalling via the AICD or via intracellular domains (ICDs) of other γ-secretase substrates.

The present data qualify the scaffold as a lead structure for γ-secretase modulation, but do not justify extensive investigation of the current compounds in animals. Improvements of potency, solubility and the investigation in neuronal cells are required prior to the investigation of in vivo activity.
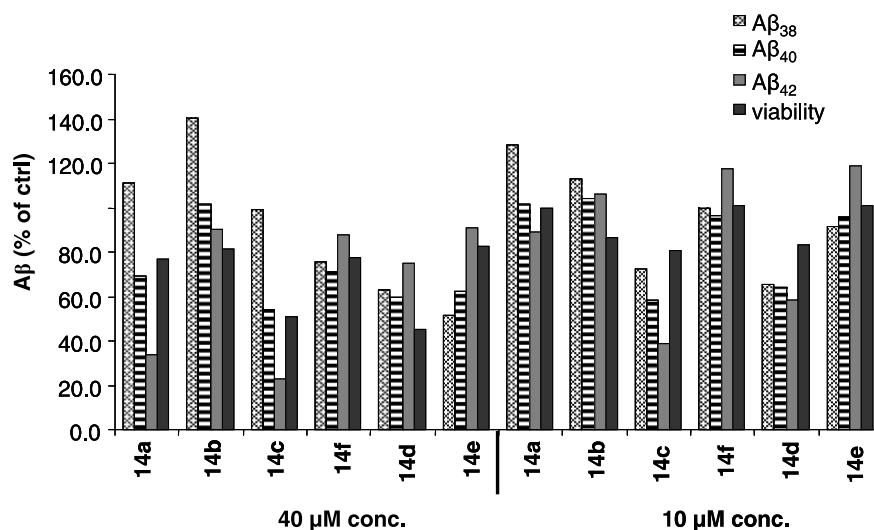

Figure 2. Activity of the compounds **14a–14f**.

References and notes

1. Mount, C.; Downton, C. *Nat. Med.* **2006**, *12*, 780.
2. Schmidt, B. *ChemBioChem* **2003**, *4*, 366.
3. Schmidt, B.; Braun, H. A.; Narlawar, R. *Curr. Med. Chem.* **2005**, *12*, 1677.
4. Chung, H.-M.; Struhl, G. *Nat. Cell Biol.* **2001**, *3*, 1129.
5. Goutte, C.; Tsunozaki, M.; Hale, V. A.; Priess, J. R. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 775.
6. Francis, R.; McGrath, G.; Zhang, J.; Ruddy, D. A.; Sym, M.; Apfeld, J.; Nicoll, M.; Maxwell, M.; Hai, B.; Ellis, M. C.; Parks, A. L.; Xu, W.; Li, J.; Gurney, M.; Myers, R. L.; Himes, C. S.; Hiebsch, R.; Ruble, C.; Nye, J. S.; Curtis, D. *Dev. Cell* **2002**, *3*, 85.
7. Zhou, S.; Zhou, H.; Walian, P. J.; Jap, B. K. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 7499.
8. Gu, Y.; Sanjo, N.; Chen, F.; Hasegawa, H.; Petit, A.; Ruan, X.; Li, W.; Shier, C.; Kawarai, T.; Schmitt-Ulms, G.; Westaway, D.; St. George-Hyslop, P.; Fraser, P. E. *J. Biol. Chem.* **2004**, *279*, 31329.
9. Takasugi, N.; Tomita, T.; Hayashi, I.; Tsuruoka, M.; Niimura, M.; Takahashi, Y.; Thinakaran, G.; Iwatsubo, T. *Nature* **2003**, *422*, 438.
10. Kimberly, W. T.; LaVoie, M. J.; Ostaszewski, B. L.; Ye, W.; Wolfe, M. S.; Selkoe, D. J. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 6382.
11. De Strooper, B.; Annaert, W.; Cupers, P.; Saftig, P.; Craessaerts, K.; Mumm, J. S.; Schroeter, E. H.; Schrijvers, V.; Wolfe, M. S.; Ray, W. J.; Goate, A.; Kopan, R. *Nature* **1999**, *398*, 518.
12. Wolfe, M. S.; Xia, W.; Ostaszewski, B. L.; Diehl, T. S.; Kimberly, W. T.; Selkoe, D. J. *Nature* **1999**, *398*, 513.
13. Esler, W. P.; Kimberly, W. T.; Ostaszewski, B. L.; Diehl, T. S.; Moore, C. L.; Tsai, J.-Y.; Rahmati, T.; Xia, W.; Selkoe, D. J.; Wolfe, M. S. *Nat. Cell Biol.* **2000**, *2*, 428.
14. Steiner, H.; Kostka, M.; Romig, H.; Basset, G.; Pesold, B.; Hardy, J.; Capell, A.; Meyn, L.; Grim, M. L.; Baumeister, R.; Fechteler, K.; Haass, C. *Nat. Cell Biol.* **2000**, *2*, 848.
15. Churcher, I.; Beher, D. *Curr. Pharm. Des.* **2005**, *11*, 3363.
16. Evin, G.; Sernee, M. F.; Masters, C. L. *CNS Drugs* **2006**, *20*, 351.
17. in 't Veld, B. A.; Ruitenberg, A.; Hofman, A.; Launer, L. J.; van Duijn, C. M.; Stijnen, T.; Breteler, M. M. B.; Stricker, B. H. C. *N. Engl. J. Med.* **2001**, *345*, 1515.
18. McGeer, P. L.; Schulzer, M.; McGeer, E. G. *Neurology* **1996**, *47*, 425.
19. Breitner, J. C.; Welsh, K. A.; Helms, M. J.; Gaskell, P. C.; Gau, B. A.; Roses, A. D.; Pericak-Vance, M. A.; Saunders, A. M. *Neurobiol. Aging* **1995**, *16*, 523.
20. Aisen, P. S.; Luddy, A.; Durner, M.; Reinhard, J. F., Jr.; Pasinetti, G. M. *J. Neurol. Sci.* **1998**, *161*, 66.
21. Schmidt, B.; Baumann, S.; Narlawar, R.; Braun, H. A.; Larbig, G. *Neurodegen. Dis.* **2006**, *3*, in press, doi:10.1159/000095269.
22. Narlawar, R.; Blanca, P. R.; Steiner, H.; Haass, C.; Schmidt, B.; Baumann, K. *J. Med. Chem.* **2006**, *49*, accepted for publication.
23. Ricketts, A. P.; Lundy, K. M.; Seibel, S. B. *Am. J. Vet. Res.* **1998**, *59*, 1441.
24. Koo, E. H. M.; Golde, T. E.; Galasko, D. R. WO 2001078721, 2001.
25. Stock, N.; Munoz, B.; Wrigley, J. D. J.; Shearman, M. S.; Beher, D.; Peachey, J.; Williamson, T. L.; Bain, G.; Chen, W.; Jiang, X.; St-Jacques, R.; Prasit, P. *Bioorg. Med. Chem. Lett.* **2005**, *16*, 2219.
26. Peretto, I.; Radaelli, S.; Parini, C.; Zandi, M.; Raveglia, L. F.; Dondio, G.; Fontanella, L.; Misiano, P.; Bigogno, C.; Rizzi, A.; Riccardi, B.; Biscaioli, M.; Marchetti, S.; Puccini, P.; Catinella, S.; Rondelli, I.; Cenacchi, V.; Bolzoni, P. T.; Caruso, P.; Villetti, G.; Facchinetti, F.; DelGiudice, E.; Moretto, N.; Imbimbo, B. P. *J. Med. Chem.* **2005**, *48*, 5705.
27. Barten, D. M.; Guss, V. L.; Corsa, J. A.; Loo, A.; Hansel, S. B.; Zheng, M.; Munoz, B.; Srinivasan, K.; Wang, B.; Robertson, B. J.; Polson, C. T.; Wang, J.; Roberts, S. B.; Hendrick, J. P.; Anderson, J. J.; Loy, J. K.; Denton, R.; Verdoorn, T. A.; Smith, D. W.; Felsenstein, K. M. *J. Pharmacol. Exp. Ther.* **2005**, *312*, 635.
28. Beher, D.; Bettati, M.; Checksfield, G. D.; Churcher, I.; Doughty, V. A.; Oakley, P. J.; Quddus, A.; Teall, M. R.; Wrigley, J. D. WO 2005013985, 2005.
29. Sastre, M.; Steiner, H.; Fuchs, K.; Capell, A.; Multhaup, G.; Condron, M. M.; Teplow, D. B.; Haass, C. *EMBO* **2001**, *2*, 835.
30. Larbig, G.; Zall, A.; Schmidt, B. *Helv. Chim. Acta* **2004**, *87*, 2334.
31. Boehm, H.-J.; Schneider, G. *Virtual Screening for Bioactive Molecules*; Wiley-VCH: Weinheim/New York, 2000.
32. Brockhaus, M.; Grunberg, J.; Rohrig, S.; Loetscher, H.; Wittenburg, N.; Baumeister, R.; Jacobsen, H.; Haass, C. *Neuroreport* **1998**, *9*, 1481.
33. *Aβ liquid phase electrochemiluminescence (LPECL) and AICD assays.* To evaluate the compounds for their potency in modulating γ-secretase activity, we used the LPECL assay to measure Aβ isoforms. APP-overexpressing cell lines were generated by stably transfecting human neuroglioma H4 cells obtained from ATCC (Accession No. CRL-1573 or HTB-148) with human APP695 in vector pcDNA3.1. The biotinylated Aβ specific antibodies 6E10 or 4G8 were used (Signet Laboratories) as capture antibodies. The C-terminal specific Aβ antibodies BAP15, BAP24 and BAP29 were generated as described previously.[32] Antibodies were labelled with TAG electro-chemiluminescent label according to manufacturer's protocol (Bioveris). Labelled antibodies were purified from unincorporated label using a PD-10 column (Pharmacia) and stored in phosphate-buffered saline (PBS) containing 0.1% sodium azide at 4 °C for several weeks or at −80 °C for long-term storage. Cells were plated in 96-well plates (30–60,000 cells/200 µL/well) and allowed to adhere for 2 h. Compounds were dissolved in DMSO (vehicle) and further diluted in cell culture medium to the desired concentration. Hundred microlitres of compound containing media was added to the cells and incubated for 20–24 h at 37 °C. Before use, M-280 paramagnetic beads (Bioveris) were diluted with assay buffer (50 mM Tris, 60 mM NaCl, 0.5% BSA and 1% Tween 20, pH 7.4). Fifty microlitres of conditioned culture medium from the plated cells were incubated with 50 µL of beads and 25 µL of each labelled antibody (6E10-bio and BAP29-TAG for detection of $A\beta_{1-38}$, 6E10-bio and BAP24-TAG for detection of $A\beta_{40}$, 6E10-bio and BAP15-TAG for detection of $A\beta_{42}$; for detection of total Aβ 4G8-bio and 6E10-TAG were used) in a final volume of 250 µL for 3 h at room temperature with gentle shaking. Synthetic $A\beta_{38}$, $A\beta_{40}$ and $A\beta_{42}$ peptides (Bachem) were used to generate standard curves. These Aβ peptides were solubilised in DMSO at a concentration of 1 mg/mL, aliquoted and

stored frozen at −80 °C. Immediately before use, they were diluted in culture media to 16–2000 pg/mL. Electro-chemiluminescence was quantified using an M-Series M8 analyzer (Bioveris). IC_{50}/EC_{50} values were calculated using GraphPad Prism ver4 software. EC_{50} values can only be calculated when the dose–response curve had reached a stable plateau at the maximum applied concentration. γ-Secretase activity was monitored by de novo production of AICD (APP intracellular domain) in vitro by the previously reported assay.[30]

34. *Cell toxicity assay.* Cell viability was measured in the corresponding cells after removal of the media for the Aβ assays by a colourimetric cell proliferation assay (CellTiter 96TM AQ assay, Promega) utilizing the bioreduction of MTS (Owen's reagent) to formazan according to the manufacturer's instructions. Briefly, after removal of the supernatant for Aβ assays 80 μL of fresh cell culture medium was added to each well and incubated for 1 h before 20 μL MTS/PES solution was added. The optical density was recorded at 490 nm after 1 h incubation at 37 °C. Cell viability was expressed as % of untreated control.

35. Barltrop, J. A.; Owen, T. C.; Cory, A. H.; Cory, J. C. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 611.

Curriculum Vitae

Name: Rajeshwar Narlawar

Date of Birth: 05th July 1977.

Place of Birth: Nanded, India

Nationality: Indian

Marital status: Married

Permanent Address: Shri Ganesh, Barhali Road, Mukhed-431715, Nanded, India

Academic career

July 1994 – June
1998
BSc in Chemistry, Botany, and Zoology
Institution: Dr. B. A. Marathwada University, Aurangabad, India.

June 1998 – May
2000
MSc Specialization in Organic Chemistry
Institution: Dr. B. A. Marathwada University, Aurangabad, India.

Since June 2003
Doctoral thesis under the supervision of Prof. Dr. Boris Schmidt at Darmstadt University of Technology, Darmstadt, Germany.

Industrial experience

Worked as a 'Senior Chemist' in "Drug Discovery Research, Dr. Reddy´s Laboratories, India" from 1st November, 2000 to 1st September, 2002.

Language proficiencies

English, Marathi, Hindi, German (bridging level),

Sanskrit (basic understanding).

Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich meine Dissertation selbstständig und nur mit den angegebenen Hilfsmitteln angefertigt und noch keinen Promotionsversuch unternommen habe.

Rajeshwar Narlawar
Darmstadt, den