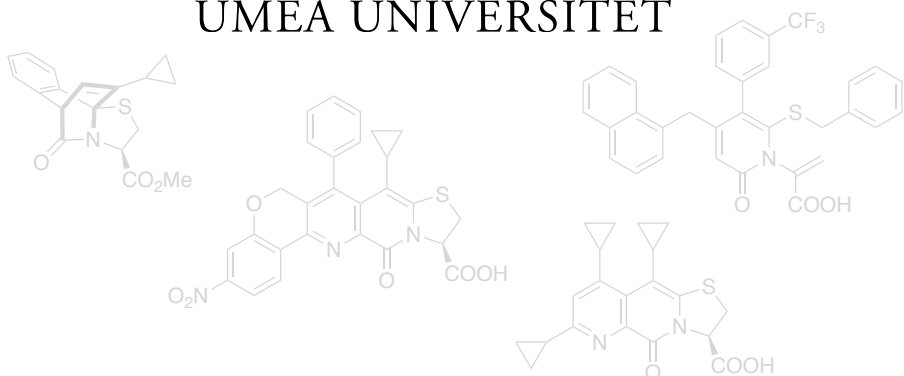
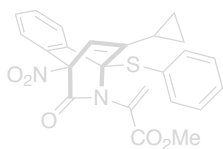




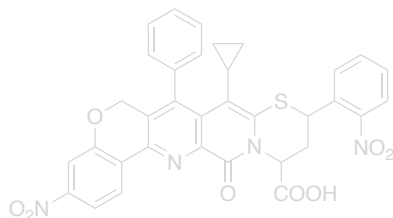
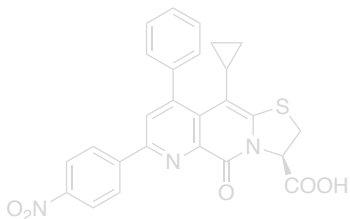
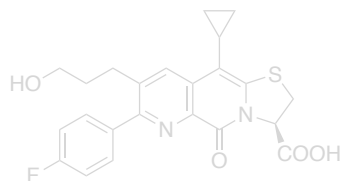
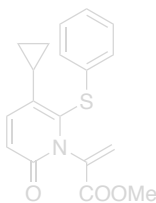
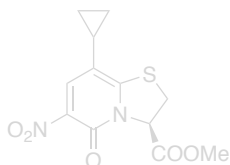
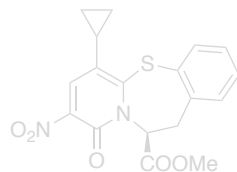
UMEÅ UNIVERSITET



SYNTHESIS OF RING-FUSED PEPTIDOMIMETICS INTERACTING WITH AMYLOID FIBRILS



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"Through endurance we conquer." – Ernest Shackleton

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Abstract

Parkinson's and Alzheimer's disease are the two most common neurological disorders in humans. Both conditions involve progressive death of neurons in the central nervous system, decline in bodily functions and eventually (and invariably), death. So far, no cure exists and the available treatments can only ease symptoms. Despite substantial investments in research, the biomolecular processes are still far from fully understood. However, both diseases are associated with formation of fibrillar protein aggregates called amyloid deposits. Whereas Alzheimer's disease involves aggregation of the Tau and Amyloid β proteins, α -Synuclein fibrilization plays a key role in Parkinson's disease. Although they are chemically distinct, the deposits consist of protein fibres with similar morphology and fold. Small molecules, such as the thiazoline fused 2-pyridones herein presented, can interfere with the formation of amyloid fibres, or bind to them. Besides having potential for diagnostics and treatment, such small molecules constitute valuable tool compounds in future research, to unravel the mechanisms of amyloid formation and pathology. The first step towards successful treatment, diagnostics and prevention of Alzheimer's and Parkinson's disease is understanding the causes and underlying mechanisms better. This thesis narrates the synthesis and development of novel chemical structures: multi ring fused peptidomimetics with the ability to bind mature amyloid fibrils, consisting of α -Synuclein or Amyloid β .

The first project (articles I, III and VI) describes method development for the extension of bicyclic thiazolino 2-pyridones by fusion with aromatic nitrogen heterocycles, which enables the desired amyloid binding properties. Derivatisations of the newly generated central scaffold, and variation of the multiple attached substituents, were subsequently performed in efforts to improve binding strength and solubility, and gain selectivity towards certain fibrils. One of the most promising amyloid fibril binders was evaluated in a human cell line and in mice, and found to be protective against accelerator induced neurotoxicity. One pyrimidine fused compound moreover indicated potent inhibition of Amyloid β aggregation. The second project (articles II, IV and V) focuses on development of methods to modify the thiazoline ring. Ring opening induced by electrophiles generates *N*-alkenyl 2-pyridones but decreases amyloid binding potency. Introduction of a cyclobutane moiety fused with the thiazoline ring is better tolerated, and adds a terminal alkene moiety that can be exploited in future chemical modifications. Expansion of the five membered thiazoline ring to a six membered dihydrothiazine ring, equipped with a nitrophenyl substituent, provides compounds with enhanced fibril binding capacity, which further inhibits Amyloid β fibril formation *in vitro*. Taken together, the synthetic methodologies allow construction and late stage modification of complex fused heterocycles, with several points of variation. Thus, the developed methods may be of future value in our laboratories and elsewhere.

List of Abbreviations

α -Syn	α -Synuclein
Å	Ångström
A β	Amyloid β
A β 40	A β (1–40)
A β 42	A β (1–42)
A β PP	Amyloid β precursor protein
AD	Alzheimer's disease
AFM	atomic force microscopy
ANOVA	analysis of variance
ApoE	apolipoprotein E
Asp	aspartic acid
BBB	blood brain barrier
BODIPY	boron-dipyrromethene
CD	circular dichroism
CNS	central nervous system
cPr	cyclopropyl
Csg	Curlin specific gene
d	day/-s
DBU	1,8-diazabicyclo(4.5.0)undecene
DCE	dichloroethane
DCM	dichloromethane
DDQ	2,3-dichloro-5,6-dicyano- <i>p</i> -benzoquinone
DEA	diethylamine
DFT	density-functional theory
DiBAI-H	di-isobutyl aluminium hydride
DLS	differential light scattering
DMAP	4-(dimethylamino)pyridine
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EAS	electrophilic aromatic substitution
EC ₅₀	half maximal effective concentration
eq.	equivalent/-s
h	hour/-s
HOMO	highest occupied molecular orbital
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
kcal	kilocalory/-ies
LC-MS	liquid chromatography - mass spectrometry

LUMO	lowest un-occupied molecular orbital
Lys	lysine
M	mol/l
<i>m</i> -	meta
min	minute/-s
MM	molecular mechanics
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	magnetic resonance imaging
MS	Molecular sieves
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
MWI	microwave irradiation
NBS	<i>N</i> -bromosuccinimide
NIS	<i>N</i> -iodosuccinimide
NMDA	<i>N</i> -methyl-D-aspartate
NMR	nuclear magnetic resonance
NSAID	non-steroid anti-inflammatory drug
<i>o</i> -	ortho
[O]	oxidant
<i>o.n.</i>	over night
OTf	trifluoromethanesulphonate
<i>p</i> -	para
PBS	phosphate buffered saline
PD	Parkinson's disease
Pd/C	palladium on activated charcoal (10% w/w)
PET	positron emission tomography
<i>r.f.</i>	retention factor
<i>r.t.</i>	room temperature
<i>s</i>	second/-s
SAR	structure-activity relationship
SEM	standard error of mean
SI	supporting information
TBDMS	<i>tert</i> -butyldimethylsilyl-
TEA	triethylamine
TEM	transmission electron microscopy
temp.	temperature
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
ThT	Thioflavin T
TLC	thin layer chromatography
TMEDA	<i>N,N,N',N'</i> -tetramethyl-ethylenediamine
TMS	trimethylsilyl-

triflate	trifluoromethanesulphonate
UV	ultraviolet
v/v	volume ratio
w/w	weight ratio

List of Publications

Articles included in the thesis

Article I: P. Singh, D. E. Adolfsson, J. Ådén, C. Bartens, K. Brännström, A. Olofsson, F. Almqvist, *Pyridine-Fused 2-Pyridones via Povarov and A³ Reactions: Rapid Generation of Highly Functionalized Tricyclic Heterocycles Capable of Amyloid Fibril Binding*, *J. Org. Chem.* **2019**, 84(7), 3887–3903.

Article II: P. Singh, A. G. Cairns, D. E. Adolfsson, J. Ådén, U. H. Sauer, F. Almqvist, *Synthesis of Densely Functionalized N-alkenyl 2-Pyridones via Benzyne-Induced Ring Opening of Thiazolino-Fused 2-Pyridones*, *Org. Lett.* **2019**, 21(17), 6946–6950.

Article III: D. E. Adolfsson, M. Tyagi, P. Singh, A. Deuchmann, J. Ådén, A. L. Gharibyan, S. W. Jayaweera, A. Olofsson, F. Almqvist, *Intramolecular Povarov reactions for the synthesis of Chromenopyridine fused 2-Pyridone Polyheterocycles Binding to α -Synuclein and Amyloid- β fibrils*, Submitted.

Article IV: M. Tyagi, D. E. Adolfsson, P. Singh, J. Ådén, S. Sarkar, A. Kiss, J. Bharate, S. W. Jayaweera, F. Almqvist, *Functionalization of Thiazolino fused 2-Pyridones by thiazoline ring opening and closing: Identification of new Amyloid Binding Heterocycles*, Manuscript.

Article V: D. E. Adolfsson, M. Tyagi, P. Singh, A. Kaur, J. Ådén, J. Bharate, F. Almqvist, *Enhancement of amyloid fibril binding by ring expansion of thiazolino fused 2-pyridone peptidomimetics*, Manuscript.

Article VI: J. Bharate, J. Ådén, A. L. Gharibyan, D. E. Adolfsson, S. W. Jayaweera, K. Vielfort, P. Singh, M. Tyagi, S. Bergström, A. Olofsson, F. Almqvist, *K₂S₂O₈-mediated aerobic oxidative coupling of 6-amino-7-(aminomethyl)-thiazolino-pyridones with aldehydes: Direct access to highly functionalized pyrimidine-fused 2-pyridones with amyloid fibril binding activity*, Manuscript.

Articles not included in the thesis:

* S. W. Jayaweera, D. E. Adolfsson, E. Åberg-Zingmark, K. Brännström, F. Almqvist A. Olofsson, *The β -amyloid interfering property of transthyretin is impaired by cysteine and glutathione conjugation at cysteine 10*, Manuscript

Author contributions

Article I: Minor synthesis, planning and writing.

Article II: Minor synthesis and planning, major writing. *Shared first author. Pardeep Singh, Anderw G. Cairns and Dan E. Adolfsson contributed equally.*

Article III: Major synthesis, planning and writing.

Article IV: Major synthesis, planning and writing. *Shared first author. Mohit Tyagi and Dan E. Adolfsson contributed equally.*

Article V: Major synthesis, planning and writing. *Shared first author. Dan E. Adolfsson and Mohit Tyagi contributed equally.*

Article VI: Minor planning and writing.

Enkel sammanfattning på svenska

Organisk kemi är baserat på kol och väte.

Kemivetenskapen har utvecklats från antikens och medeltidens alkemi och är idag ett brett område. Man särskiljer därför på olika inriktningar såsom Analytisk kemi, Fysikalisk kemi, Oorganisk kemi och Organisk kemi. Det sistnämnda fältet fokuserar på kolbaserad materia, närmare bestämt kemiska föreningar som bl.a. innehåller kol och väte. Här ingår t.ex. lösningsmedel och oljebaserade bränslen, syntetiska textilier, plast, färg, tvål, sprängämnen, bekämpningsmedel, parfym och läkemedel.

2-pyridonerna designades för att "raka" bakterier.

Historiskt så har sjukdomar och andra åkommor ofta behandlats med s.k. folkmedicin. Här ingick många växter som innehåller biologiskt aktiva substanser. Dessa lade grunden till dagens moderna mediciner, som i många fall är helt syntetiska, och ofta säkrare och effektivare än sina föregångare. Många syntetiska mediciner är dock inspirerade av naturligt förekommande kemiska substanser. De tiazolinsammansatta 2-pyridonerna som denna avhandling handlar om är syntetiska ämnen som designats för att likna en β -sträng i ett protein. Genom att "lura" bakterier att de syntetiska molekylerna är detta specifika protein, lyckades man blockera de processer som leder till att fimbrier växer ut. Fimbrierna är proteinfilament som vissa bakterier behöver för att fastna på och invadera sina värdceller. Under utvecklingens lopp upptäckte man att dessa β -sträng-liknande kemiska föreningar kunde hämma bildandet av aggregerat Amyloid β protein, som återfinns i nervsystemet hos patienter med Alzheimers sjukdom.

Amyloid är fibrer som består av proteiner.

Proteiner fyller många livsviktiga funktioner i levande organismer. Dessa byggs upp av endast drygt 20 aminosyror (byggstenar). Sekvensen av dessa byggstenar är avgörande för hur proteinerna veckar sig, och veckningen (3D-strukturen) avgör i sin tur proteinernas biologiska funktion. Men proteiner kan veckas fel, och då få helt andra, oönskade funktioner. I människor känner man i dagsläget till ca 30 proteiner som är särskilt benägna att felveckas. När de veckas fel kan de packa ihop sig till långa fibrer som så småningom bildar stora aggregat, dessa kallas för amyloida plack.

Går det att bota Alzheimers och Parkinsons sjukdomar?

Både Alzheimers och Parkinsons sjukdom är utdragna förlopp där patientens nervceller sakta dör. När de gör det försvinner gradvis somliga av hjärnans livsviktiga funktioner, och till sist dör även patienten. I dagsläget känner man inte till någonting som kan bota dessa sjukdomar, de behandlingar som finns att tillgå kan endast lindra symptomen och ibland fördröja processerna en aning. Trots att dessa sjukdomar har varit kända i över 100 respektive 200 år, och väldigt mycket forskning har genomförts, så förstår man fortfarande mycket lite om orsakerna till insjuknande, och de

biomolekylära sjukdomsprocesserna som ligger bakom. Klart är att båda sjukdomar kan associeras till bildandet av amyloida plack, i eller utanför de påverkade hjärncellerna. I Alzheimers sjukdom är två amyloidbildande proteiner involverade, ett av dem är Amyloid β . Parkinsons sjukdom kopplas i sin tur ihop med proteinet α -Synuclein. Ordningen på aminosyrorna är helt olika för α -Synuclein och Amyloid β , ändå är de respektive amyloid-fibrerna slående lika vad gäller utseende (i elektronmikroskop) och egenskaper. En sådan gemensam egenskap är att somliga kemiska föreningar kan binda in till de amyloida fibrerna. Andra kemiska föreningar kan påverka (skynda på eller fördröja) det förlopp där proteinerna aggregerar. För att i framtiden eventuellt kunna förebygga, bota och diagnosticera Alzheimers respektive Parkinsons sjukdom, behöver vi först bättre förstå orsakerna, och de mekanismer som leder till att hjärnans nervceller dör. Ett sätt är att använda sig av små organiska molekyler som kemiska forskningsverktyg. Kemiska föreningar som binder till amyloida plack har potential att utvecklas till kemiska verktyg som kan diagnosticera Parkinsons eller Alzheimers sjukdom. De har också terapeutisk potential, då de hämmar de aggregerande proteinernas toxiska funktion. Denna doktorsavhandling beskriver metodutveckling, syntes och design av ringsammansatta thiazolin-2-pyridoner med förmåga att binda till amyloida fibrer.

Avhandlingens arbete.

Det första huvudkapitlet beskriver hur de tiazolinsammansatta 2-pyridonerna förlängs med en pyridinring, vilket möjliggör inbindning till amyloida fibrer. Förmågan att binda fibrer är väldigt beroende av hur de små molekylnas centralfragment utrustas med substituent (kemiska extremiteter). Mindre förändringar av molekylnas grundskelett har sedan gjorts för att försöka förbättra deras bindningsförmåga och löslighet i vatten (eller blod). Vi försökte också uppnå förmåga hos molekylna att binda selektivt till en viss sorts Amyloidfibrer. En av de mest lovande kemiska föreningarna, med stark inbindningsförmåga, testades i odlade mänskliga nervceller (cancerceller kan odlas), och även i möss. Den föreningen visade sig kunna hämma celldöd som orsakas av en molekyl som startar eller påskyndar amyloidbildning av α -Synuclein. En annan förening, där pyridinringen bytts ut mot en pyrimidinring (två kväveatomer i ringen, istället för en) hämmar aggregering av Amyloid β i buffertlösning.

Det andra huvudkapitlet fokuserar på förändringar av tiazolinringen. Genom att öppna upp denna ring genom reaktioner med somliga elektrofiler, skapades en användbar funktionell grupp, men föreningarna visade sig tappa en del av sin amyloidbindande förmåga. Att istället förse tiazolinringen med en cyklobutanring var inte alls lika ofördelaktigt. Dessutom blev cyklobutanringarna utrustade med en dubbelbindning utanpå ringen, vilken skulle kunna användas för vidare kemisk modifiering. När tiazolinringen (5-ring) expanderades till en 6-ring, försedd med en viss nitrofenyl-ring, ökade istället förmågan hos de kemiska föreningarna att binda amyloidfibrer. Dessutom hämmar de nya föreningarna aggregering av Amyloid β .

1. Introduction

1.1. Organic chemistry

The science of chemistry originates from alchemy, that was practiced during ancient and medieval times. Alchemy had many objectives, such as the production of medicines, poisons and pure alcohol. The ultimate goals were to find a way to make gold from other materials and to brew potions that granted the consumer with eternal life. The former goal has now been achieved, gold can be made from other elements through nuclear reactions. Alas, the manufacturing cost far surpasses the price of "natural" gold. Although alchemy involved elements of mythology, religion and magic, fundamental experimental methods and laboratory instruments that are used in modern chemistry, were developed.¹⁻⁵

Organic chemistry is the fraction of chemistry that specialises in carbon based chemical compounds. Characteristic products from the field of organic chemistry includes synthetic textiles, paint, petrol, solvents, plastic, soap, perfume, pesticides, explosives and pharmaceuticals.⁶ To be counted as *organic*, a molecule must contain carbon and hydrogen. Compounds like diamond, graphite, carbon dioxide and carbides are counted as *inorganic*. For historical reasons are cyanides and carbonate salts counted as inorganic compounds, although hydrogen cyanide and bicarbonate contains both carbon and hydrogen (*Figure 1.1*). In contrast, urea and methane are counted as organic chemicals.⁷⁻⁸

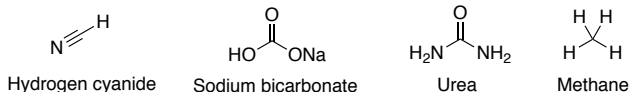
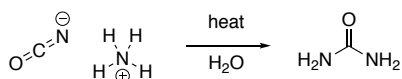


Figure 1.1. The definition of organic versus inorganic is not that clear cut. Hydrogen cyanide and sodium bicarbonate are classified as inorganic, while urea and methane are organic. All these compounds contain carbon and hydrogen.

The modern definition of organic versus inorganic has nothing to do with whether the compound is of biological origin or not. Carbon dioxide can be produced by living organisms through aerobic respiration, and bicarbonate is formed by the pancreas in the human digestive system. Coal deposits found on earth, used by humans as fuels for cooking, heating, smithing, shipping *etc.*, and to produce electricity, were once plants, fossilised during the carboniferous period (360–300 million years ago).⁹ Meanwhile, methane gas is a primordial product from solar nebulae material, and is abundant in space.^{8,10} Until the beginning of the nineteenth century though, the *theory of vitalism* argued that organic chemicals could only be produced by living organisms, through intervention of a *vital force*.⁷ In 1828, Friedrich Wöhler proved this theory wrong. He synthesised urea, a compound produced by animals and excreted in urine, by heating an aqueous solution of ammonium cyanate.¹¹ Ammonium cyanate was considered to be a distinctive inorganic chemical (*Scheme 1.1*). But despite the falsification of the vitalism theory, hydrocarbon-based chemistry is still called organic chemistry. (Today the term organic is often used in a different context. Organic food

and clothes are by all means carbon-hydrogen based, just like their conventional counterparts, but in this context the term organic means *ecological*.)



Scheme 1.1. With Friedrich Wöhler's synthesis of urea from ammonium isocyanate in 1928, the *theory of vitalism* was falsified with definitive evidence.¹¹

In continuation to this theme, about 100 years later, in 1924, Alexander Oparin proposed that life on earth may have evolved from the chemistry of simpler organic molecules which can be formed through abiotic processes.¹² His writings inspired Stanley Miller who together with Harold Urey tested this theory in 1952–53 (*Figure 1.2*). They subjected a mixture of methane, water, hydrogen, carbon dioxide and ammonia, supposed to mimic the "primordial soup" on planet earth before the existence of living organisms, to electric discharges (artificial lightning). They discovered that five different amino acids were formed.^{13–14, N1} Experiments similar to those of 1952–53 have revealed that other simple biomolecules such as adenine and ribose, which are components of RNA, can be formed through abiotic processes.^{15–16} Furthermore, a meteorite that landed in Australia 1969 was found to contain at least 18 different amino acids. 6 of these are also found in living organisms on Earth.^{17–18}

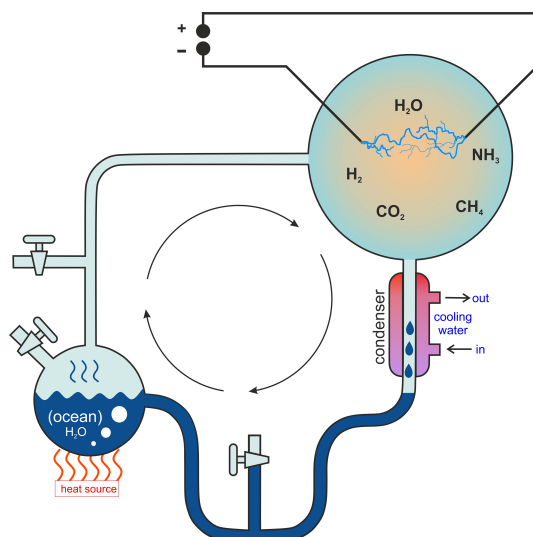


Figure 1.2. Schematic sketch of some apparatus used by Stanley Miller and Harold Urey to show that simple biomolecules can be created from complete lifelessness.¹⁹

1.2. Medicinal chemistry

The compounds discussed in this thesis are of synthetic origin, results of rational drug design. However, before the era of scientific medicine, diseases and other ailments were often treated by what today is referred to as folk medicine, or traditional medicine. Although folk

medicine is often associated with religious confessions, rituals and witchcraft, a significant amount of these early remedies were based on the use of medicinal herbs. This is not at all surprising, given the vast amount of medicinal natural products found in plants, that give noticeable physiological responses when ingested.²⁰ Well known examples include caffeine, cocaine, myristicin and digoxin (*Figure 1.3*).²¹ In fact, folk medicine still see extensive use in all parts of the world, especially in Africa and South-East Asia.²² Even in modern medicine, a large proportion of the drugs used are natural products (isolated or synthesised), derivatives of natural products or natural product inspired.²³

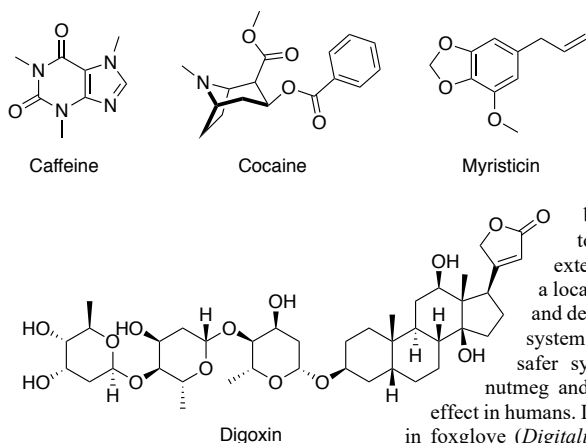


Figure 1.3. Structures of some common medicinal natural products. Caffeine, found in tea leaves and coffee beans for example, is one of the most consumed and socially accepted drugs. Cocaine, found in the leaves of the coca bush, has long been used by native South Americans, but is today infamous for its abuse. It has seen extensive use in medicine and dentistry as a local anaesthetic. Due to the addictiveness and destructive effects on the central nervous system, cocaine has now been replaced with safer synthetic drugs. Myristicin, found in nutmeg and anise, is known for its psychedelic effect in humans. It is also an insecticide. Digoxin, found in foxglove (*Digitalis purpurea*), was previously used to treat various heart disorders, such as atrial fibrillation.

Atropine for example, is used during surgery to decrease salivation and heart rate, to treat glaucoma, an eye disorder, and as an antidote against nerve agent poisoning (Figure 1.4). This drug is simply the synthetically made and racemic form of hyoscyamine, which is found in *Atropa belladonna* (deadly nightshade).²⁴ Paclitaxel, an anticancer drug, was first isolated from the bark of the pacific yew tree in 1971. It is now made semi-synthetically since the amount found naturally is very small.²⁵⁻²⁶ Some of the most well-known modern medicines are the penicillin antibiotics. Penicillin was discovered in extracts from *Penicillium* mould. Many penicillin antibiotics are made *via* a semi synthetic route, where compounds extracted from cultivated mould is purified and then chemically derivatised.²⁷⁻²⁸

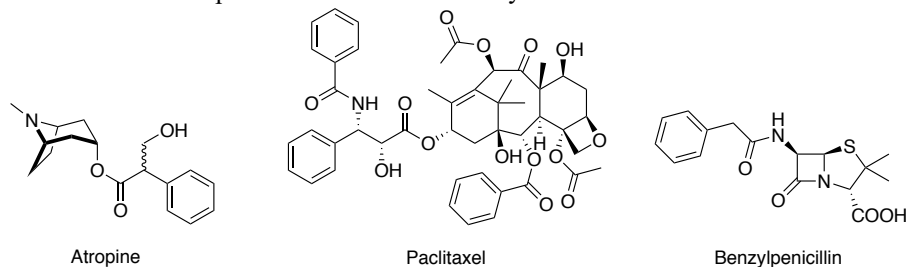
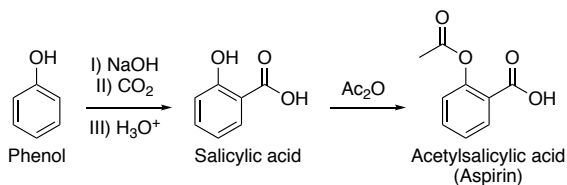


Figure 1.4. Structures of well-known natural products used in modern medicine. Atropine is the synthetic and racemic form of hyoscyamine, (the pure *S*-enantiomer) which is found in nightshade. It is used as antidote against nerve toxins that inhibits acetylcholine esterase, such as some pesticides and agents of chemical warfare. The amounts of Paclitaxel in Pacific yew tree bark are so low that its isolation for medicinal use is unsustainable. It can be made by total synthesis, but a more economical way is to harvest an intermediate from the needles of European yew and modify it chemically. Benzylpenicillin was the first natural penicillin to be marketed (1941). Many of the penicillin antibiotics on the market today are made semi synthetically by modifying natural penicillin produced in mould cultures.

The oldest fully synthetic drug is Aspirin (acetylsalicylic acid). It is made from phenol, a petrochemical product, in two synthetic steps (Scheme 1.2). The name salicylic acid comes from the willow tree family, *Salix*, and salicylic acid was previously extracted from the bark of willow trees. Willow bark have been used

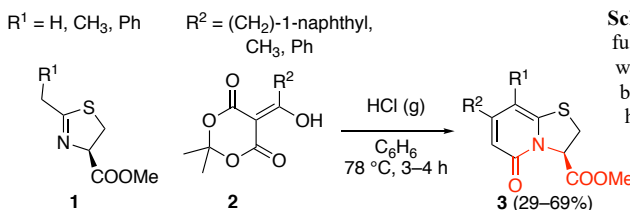
medicinally in ancient Assyria, Egypt and Greece since at least 400 BC.²⁹⁻³⁰ While natural products offer a vast repertoire of biologically active small molecules, their direct use in modern medicine is often accompanied by several drawbacks. This includes limited efficiencies, metabolic instability, high costs of production, limited amounts obtainable from natural sources (exemplified by Paclitaxel), narrow therapeutic intervals (Digoxin), undesired side effects (Cocaine), *etc.* Chemical Synthesis can often supply safer and more effective drugs at a lower cost.^{23, 31-32}



Scheme 1.2. Aspirin is synthesised in two steps from phenol, a product of the oil industry. Salicylic acid is made industrially in the Kolbe-Schmitt process by introducing carbon dioxide under basic conditions. Salicylic acid is then acetylated with acetic anhydride to provide acetylsalicylic acid.

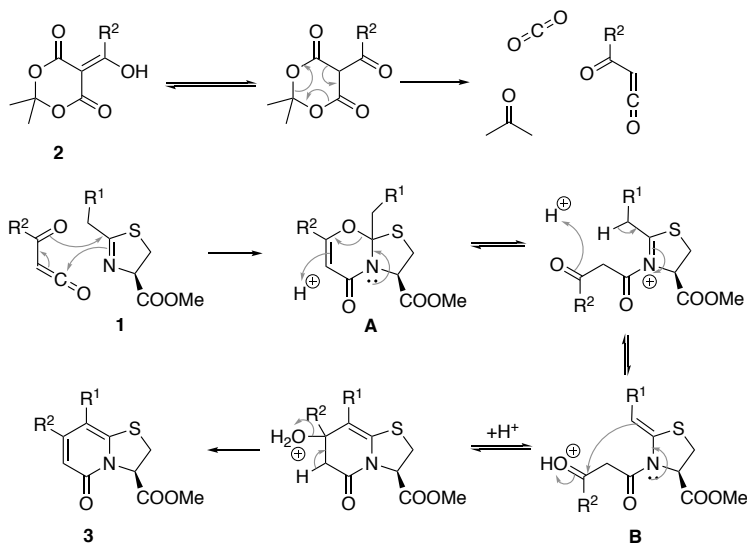
1.3. The legend of the thiazoline fused 2-pyridone peptidomimetics.

The compounds which are the subjects of this thesis are based on the thiazoline fused 2-pyridone **3** (*Scheme 1.3*).³³ This structure was initially designed as a peptidomimetic (the peptidomimetic backbone is highlighted in red) to inhibit the formation of pili on uropathogenic *E. coli*, which causes urinary tract infection.³³⁻³⁴ The pili are extracellular proteinaceous extensions that mediate attachment to and colonisation of host cells.³⁵⁻³⁷ Since the pili are crucial virulence factors, it was perceived that inhibiting their biosynthesis is a reasonable therapeutic strategy.³⁸⁻⁴⁵ **3** was synthesised by a cyclocondensation between thiazoline **1** and acyl ketene, formed *in situ* from Meldrum's acid derivative **2**, at elevated temperatures.^{33, 46} Gratifyingly, after carboxylic acid deprotection, the thiazolino 2-pyridone scaffold was found to possess the desired biological properties and inhibit pilus biogenesis.



Scheme 1.3. Synthesis of thiazoline fused 2-pyridones **3**. The protocol was then improved by replacing benzene with dichloroethane, and heating the mixture to 64 °C for 14 h. The yields were raised to 63–86% and the losses in enantiomeric excess lower.³³

Upon heating Meldrum's acid derivative **2**, an electrocyclic fragmentation generates acetone, carbon dioxide and an acyl ketene (*Scheme 1.4*).⁴⁷ Ketenes are highly reactive species, prone to rapid breakdown through polymerisation reactions.⁴⁸⁻⁵⁰ In presence of thiazoline **1** however, a concerted [4 + 2] cycloaddition is proposed to result in the formation of 1,3-oxazine-4-one intermediate **A**.^{N2} Acid mediated ring opening and proton shift subsequently lead to intermediate **B**. Finally, the ring closes, and upon elimination of water, 2-pyridone **1** is formed.⁵¹⁻⁵²



Scheme 1.4. Proposed mechanism of thiazoline fused 2-pyridone formation. Experiments support formation of 1,3-oxazine-4-one **A**, which under acidic conditions transforms into **3**.⁵¹⁻⁵³

The thiazoline fused 2-pyridone **3**, sometimes just referred to as 2-pyridone, became the central fragment of our peptidomimetic compounds. A lot of derivatisations and modifications have been made on this scaffold in order to improve the potency as "pilicides" to inhibit the virulence of uropathogenic *E. coli*.⁵⁴⁻⁶³ The numbering system used when referring to the various positions of the scaffold, are as follows (*Figure 1.5*): The numbering starts at sulphur, the heaviest atom, and continues clockwise around the fused bicycle. The positions are referred to as "position 7" or "C-7", for example. This numbering should be used carefully, as it can get confounded when compounds are referred to as 2-pyridones. It also changes when the ring system is extended. For historical reasons and for consistency, this numbering system is used throughout the thesis, albeit sparingly.

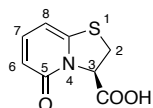


Figure 1.5. The numbering system used for the bicyclic peptidomimetic thiazolino 2-pyridones.

The 2-pyridone moiety is present in a number of biologically active natural products and synthetic compounds, and have thus been studied extensively.⁶⁴⁻⁷⁸ There are examples with antibacterial,^{65, 74-76} antiviral⁷²⁻⁷³ and anticancer properties,^{65-66, 71} as well as compounds that inhibits the angiotensin converting enzyme⁷⁷ and Amyloid β aggregation⁷⁸⁻⁷⁹ (*Figure 1.6*). The first synthesis of a 2-pyridone was reported in 1892.⁸⁰⁻⁸² Since then, numerous and diverse methods for their preparation have been published,⁸³⁻⁹¹ including cycloaddition approaches.

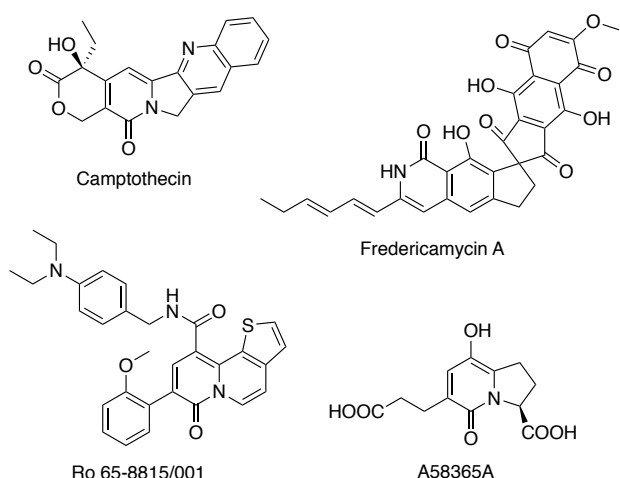


Figure 1.6. Chemical structures of biologically active compounds containing the 2-pyridone motif. The natural products Camptothecin and Fredericamycin A have anticancer properties. The synthetic Ro 65-8815/001 and A58365A are inhibitors of Amyloid β aggregation and angiotensin converting enzyme, respectively.

Since our first preparation of thiazoline fused 2-pyridones in benzene with HCl, the method has been modified to improve yields, minimise loss in enantiomeric purity, shorten reaction times and simplify the practical handling.^{33, 46, 92-93} Compound **4** (Figure 1.7) was among the first potent pilicides to be prepared.³³ Over the years, all of the open positions have been fitted out with different substituents (general structure **5**) and the currently best pilicide **6** has an EC_{50} value of 400 nm in bacterial cell culture.⁹⁴ In addition, fluorescent pilicides bearing coumarin and BODIPY functionalities have been developed.⁹⁵⁻⁹⁶

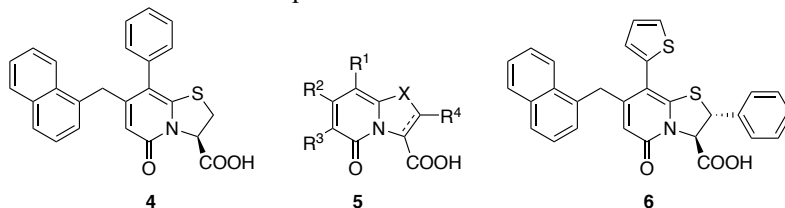


Figure 1.7. Exploration of various substitutions and scaffold modifications in search for active pilicides. The substituents in position C-7 and C-8 have a pronounced effect on activity.^{41, 62, 94} The C-3 carboxylic acid and its stereochemistry is important, but isosteres are tolerated.^{55, 60} Compounds with certain C-6 substituents have increased solubility.^{54, 57} Replacing the sulphur in the thiazoline ring with oxygen is also tolerated, and may be useful in oxidative environments.⁵⁹ C-2 functionalisation of thiazolo and thiazoline fused 2-pyridones, greatly improves pilicide activity.^{45, 58, 61, 63}

The utility of the thiazolino 2-pyridone peptidomimetics is not limited to virulence inhibition of a few Gram-negative bacteria producing pili. In parallel, compounds have been developed against *Chlamydia trachomatis*,⁹⁷⁻¹⁰¹ *Listeria monocytogenes*,¹⁰²⁻¹⁰⁴ *Helicobacter pylori*¹⁰⁵⁻¹⁰⁶ and *Mycobacterium tuberculosis*¹⁰⁷. It was further discovered that certain combinations of sterically demanding C-7 and C-8 substituents enabled the 2-pyridones to interfere with formation of amyloid fibres. Compound **7**, known as FN075 (Figure 1.8), inhibits fibrilization of Amyloid β and CsgA.¹⁰⁸⁻¹⁰⁹ Amyloid β is a short peptide involved in a neurodegenerative disorder

known as Alzheimer's disease,¹¹⁰ while CsgA is the principal constituent of bacterial Curli fibres, which is a functional amyloid produced by *E. coli* (Chapter 1.7).¹¹¹

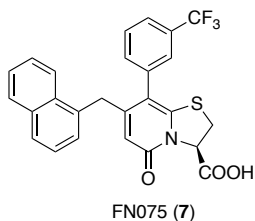


Figure 1.8. The structure of FN075, a compound that was synthesised to inhibit pili production by uropathogenic *E. coli* but found to modulate the aggregation of amyloid forming proteins.

1.4. Amyloid

Amyloids are intra- or extracellular deposits of aggregated proteins. Most well-known are probably the amyloid deposits found in the brain tissues of patients with neurodegenerative disorders like Alzheimer's and Parkinson's disease. There are over 20 chemically distinct amyloid deposits in humans known to date, of which many are associated with ailments.¹¹²⁻¹¹⁴ These fibrillar deposits have been studied since at least 1639, and have been associated with diseases referred to as "waxy liver" or "spongy spleen", *etc.*¹¹⁵⁻¹¹⁸ It was not until 1854 that the name "amyloid" was introduced to the medicinal field by Rudolph Virchow.^{115, 119} He did not invent this designation himself, it was already in use in the field of botany, but Virchow found that deposits in human tissue stained positive with a solution containing iodine and dilute sulfuric acid. This solution had previously been used to stain matters rich in starch in animals, and Virchow thus thought that the fibrillar deposits found in human organs were polysaccharide derived and similar to cellulose or starch.¹²⁰⁻¹²¹ Friedrich and Kekulé later showed by thorough chemical analysis that a fibrillar structure dissected from spleen tissue actually consisted mainly of proteins. This finding was later supported by Hanssen who digested amyloid with pepsin.¹²²⁻¹²³ But despite the swift falsification of the carbohydrate theory, the name amyloid stuck, and is used still.

1.5. Detecting amyloid fibres

The historically most important dye for staining amyloid is Congo red (*Figure 1.9*).^{117-118, 124-125} Used in textile industry since the 1880:s, Bennhold introduced this dye for amyloid staining in the 1920:s,¹²⁶ its greatest value has been in histology.¹²⁷ It binds strongly and selectively to amyloid fibrils and displays an enhanced green birefringence when visualised under polarised light.¹²⁸ Congo red also has fluorescent properties, which later enabled more sensitive visualisation of amyloid fibrils.¹²⁹ The dye is still used today, although Thioflavin T (ThT) is increasingly preferred due to its higher sensitivity. ThT, introduced by Vassar and Culling in the 1950:s, is a fluorescent dye which selectively binds to amyloid structures and, upon binding, gains enhanced fluorescent properties. In solution, its benzothiazole and aniline rings can rotate freely around the C–C bond connecting them. The preferred conformation is when the rings are out of each others' planes. When bound to an amyloid fibril, the

rotation of the aromatic rings is restricted and the benzothiazole and aniline rings are forced into a coplanar conformation, where an excited state can be maintained.¹³⁰⁻¹³⁶

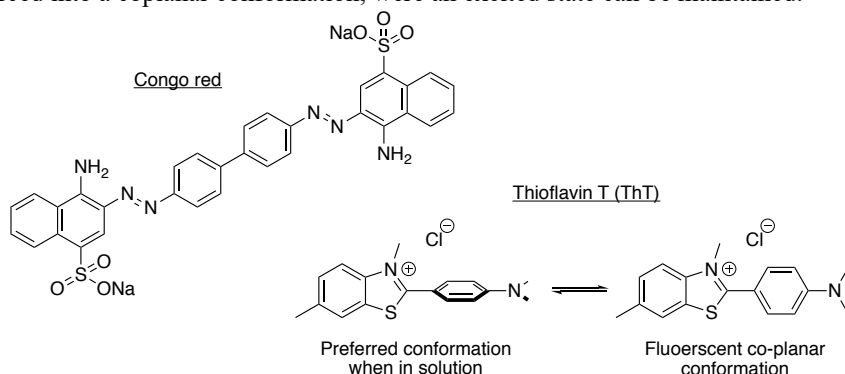


Figure 1.9. Chemical structures of the amyloidophilic dyes Congo red and Thioflavin T. Congo red has been used extensively in histology and even in early diagnostics.¹²⁶ ThT is a fluorescent dye that is used more frequently today. ThT is conformationally restricted when bound to amyloid fibrils. The benzothiazole and aniline rings are forced into a co-planar state, which alters its absorption and emission spectra. Excitation with light of about 440 nm results in emitted light with peak intensity around 480 nm.

With the electron microscope came the possibility to see the fine structure of amyloid deposits (*Figure 1.10*). Long, unbranched rigid fibrils were spotted, 8–10 nm across and often found in bundles, sometimes in association with cell membranes.¹³⁷⁻¹⁴⁰ The characteristic morphology observed when visualised with electron microscopy, and staining with fluorescent dyes, are two very important ways of identifying amyloid fibres.¹⁴¹

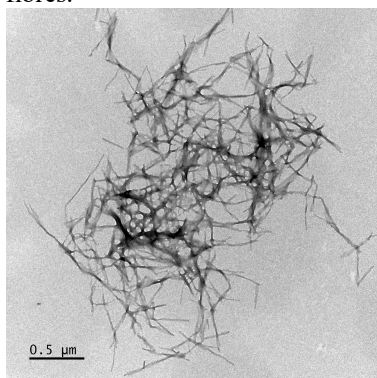
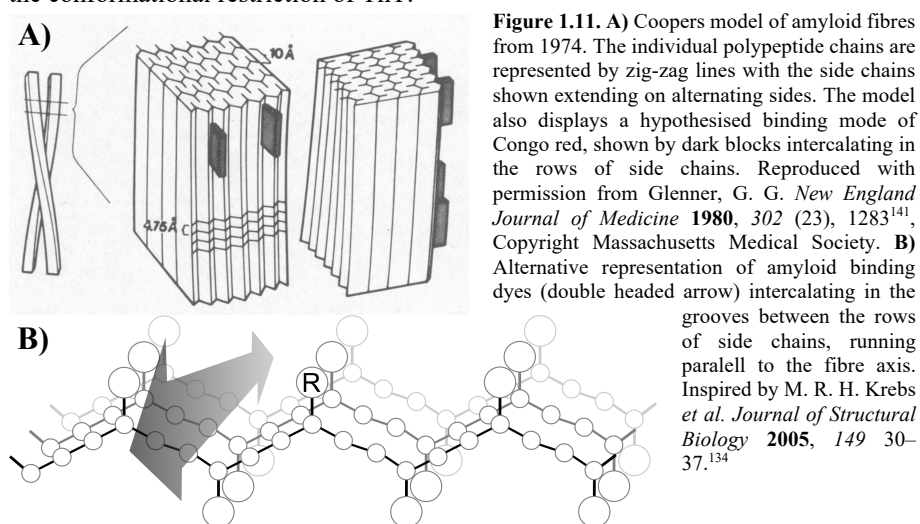


Figure 1.10. Amyloid fibrils of human α -Synuclein seen with a transmission electron microscope (TEM) at 25 000 times magnification. α -Synuclein is the amyloidogenic protein involved in the pathogenesis of Parkinson's disease and other synucleinopathies.¹⁴² The fibrils in the picture were formed *in vitro* from transgenically expressed monomeric human α -Synuclein.

1.6. The structure of amyloid fibrils, and their formation

Within the amyloid fibril, each individual polypeptide chain adopts β -sheet secondary structure and stacks parallel to each other, perpendicular to the fibre axis. The strands are held together by intramolecular hydrogen bonds between the N–H and C=O moieties in the main chains. This unlimited interstrand bonding (cross β -sheet) makes the amyloid fibril an extraordinarily stable quaternary structure. It is likely the most thermodynamically stable form a polypeptide can adopt,¹⁴³⁻¹⁴⁴ and is exceptionally resistant to both denaturation and protease digestion.^{111, 145} Of the around 30 different

human proteins known to form fibrils *in vivo*, all share the same characteristic "amyloid fold" and form unbranched fibrils with a diameter of 8–10 nm and indefinite lengths. The fibril structure is generic, irrespective of amino acid sequence or if the protein aggregates *in vivo* or *in vitro*.^{124-125, 146-148} Amyloid deposits found *in vivo* also contain many other entities though, such as apolipoproteins and proteoglycans, aggregated together with the fibrils of the amyloidogenic protein.^{124, 149} There are several suggestions of how amyloid selective dyes such as Congo red and ThT bind to the fibrils, and more than one mode of binding may be actual simultaneously. A widely considered hypothesis suggests that dyes bind to the amyloid structures by intercalating in narrow grooves between the side chains of amino acid residues (Figure 1.11). This would arrange the small molecules along the fibre axis and explain the conformational restriction of ThT.^{133-134, 141}



Specific proteins are associated with specific diseases and the respective amyloid deposits are often, but not always, located in or close to the tissue that expresses them in highest quantity.¹⁵⁰⁻¹⁵¹ The susceptible proteins do not have any particular amino acid sequence homology, yet all of them adopt the cross β -sheet structure that makes up the characteristic fibrils. It seems that the ability to form these fibrils is intrinsic to polypeptide chains.¹⁵²⁻¹⁵⁴ Indeed, proteins not involved in any disorder, as well as synthetic peptides, have been demonstrated to form amyloid fibrils *in vitro*, when in sufficient concentrations.¹⁵⁵⁻¹⁵⁹ Given the thermodynamic stability of amyloid fibrils, it seems surprising that so few, only some 30 of the more than 31 000 different proteins in the human body, have been found to form fibrils under physiologic conditions, and cause disease.¹⁶⁰ It has been proposed that proteins are "protected" in their native folded states, by kinetic barriers.^{144, 161-163} In folded proteins, much of the polypeptide backbone is buried within the structure, and the interstrand hydrogen bonding found in amyloids is thereby prevented from forming spontaneously.¹⁶⁴⁻¹⁶⁷ In order to be able to form fibrils, the protein must first unfold, at least partially, which

is a process involving high energy transition states.^{144, 167} Proteins in general are thus most vulnerable to aggregation right after their biosynthesis by ribosomes, before they have adopted their native fold, which ultimately is determined by the primary structure. Protein folding is principally spontaneous.¹⁶⁸⁻¹⁷⁰ To aid the folding processes, there are numerous chaperones that assist the new polypeptides to fold correctly.¹⁷¹ There are also chaperones that can correct slightly erroneous folds.¹⁷²⁻¹⁷³ Finally, our cells are equipped with sophisticated quality control mechanisms that degrades misfolded proteins.¹⁷⁴ But many of the known amyloidogenic proteins have a rather loose fold, or are unfolded in their native state. The scarcity of secondary and tertiary structure thus makes these proteins especially vulnerable.¹⁷⁵

1.7. Functional amyloids

Despite being involved in a multitude of disorders, the intrinsic ability of polypeptides to form fibrillar quaternary structures has been taken advantage of by nature. The egg shells of many insects and fish are illustrative examples of biomaterials where amyloid fibrils are key structural components.¹⁷⁶⁻¹⁷⁷ Pigmentation of human skin also involves amyloid. In the melanosomes, an integral membrane protein forms an amyloid structure onto which the melanin pigment attaches.¹⁷⁸⁻¹⁷⁹ *E. coli* and *Salmonella* also make use of amyloid fibrils, for construction of extracellular matrixes needed for bacterial biofilm formation (Figure 1.12).

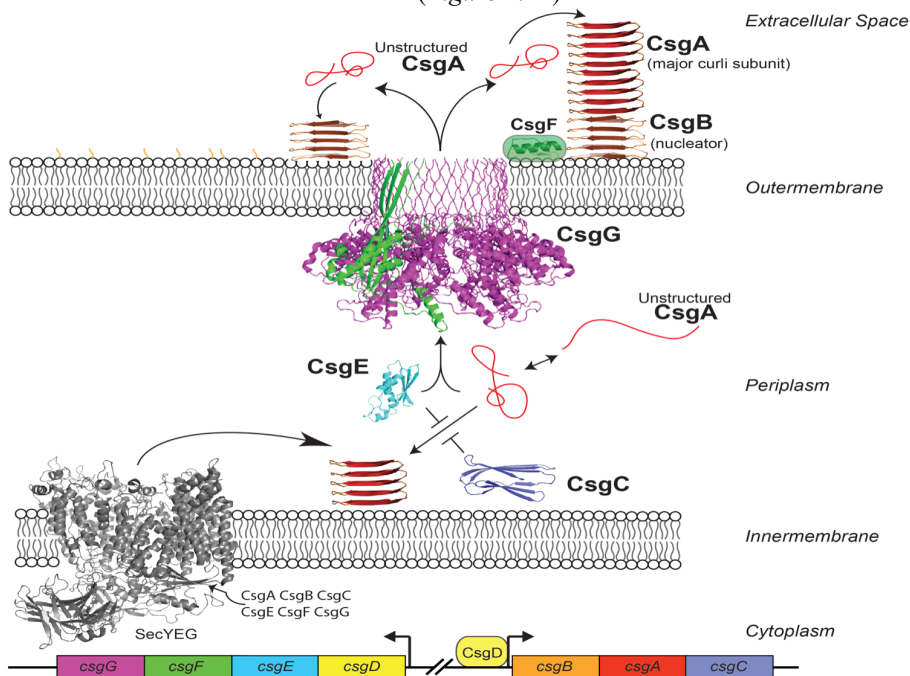


Figure 1.12. The formation of Curli fibres by *E. coli* is a well-designed and regulated process. While inside the cell or in the periplasmic space, the amyloidogenic CsgA is prevented from fibrilization by the chaperones CsgC and CsgE, whereof the former is the most potent *in vitro*. Once exported, CsgA fibrilization is initiated by CsgB. The graphics were kindly provided by Matthew Chapman.

The so called Curli fibres produced by *E. coli* are made up largely by the CsgA protein that the bacteria produce and excrete. The fibre formation is an innovatively regulated process, where proteins attached to the outer cell membrane (CsgB) function as "seeds" to initiate the fibrilization process on the outside of the bacteria.¹¹¹ While inside the outer membrane, CsgA is prevented to aggregate by the chaperones CsgC and CsgE.¹⁸⁰ Interestingly, these bacterial chaperones have been found to modulate the fibrilization of human α -Synuclein *in vitro*. CsgC is a potent inhibitor of α -Synuclein fibril formation, even in sub-stoichiometric amounts,¹⁸⁰ while CsgE accelerates fibril formation.¹⁸¹ Together, these examples again illustrates the inherent ability of polypeptides to form the amyloid fibril quaternary structure, that evolution has put to good use.

1.8. Kinetics of amyloid fibril formation

In addition to the generic amyloid fibril structure, the kinetics of fibril formation *in vitro* is mainly characterised by three features. First, a critical concentration of monomeric protein must be reached for fibril formation to start at all. Below this concentration, the loss in entropy is too unfavourable for multimeric species to form. At higher concentrations, this entropy loss is compensated by the stability of the fibrillar end products. Second, a lag time exists before fibril formation starts, where partial folding and oligomer formation takes place (*Figure 1.13*), and third, the lag time can be eliminated or shortened by addition of pre-formed fibrils or on-path oligomers. The duration of the lag time varies for different proteins, from minutes to days, but is reproducible under given sets of conditions. Factors like monomer concentration, temperature and pH, influence the length of the lag times.^{143, 182-185}

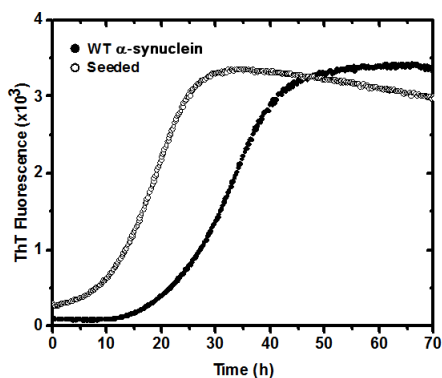


Figure 1.13. *In vitro* fibrilization of human α -Synuclein, the protein associated with Parkinson's disease. The fibril formation was followed with ThT fluorescence. In order to form amyloid fibrils, the monomers must first form oligomers, which takes time. In this setup, the lag time of fibril formation is about 12 h (filled circles). Adding pre-formed fibrils (2.5 % v/v) to the monomers can template fibrilization and ablate the lag time (open circles). Human w.t. α -Synuclein (70 μ M), ThT (20 μ M) and a 2 mm glass bead in phosphate buffered saline (10 mM, pH 7.4) was agitated at 37 °C. Each experiment was performed in triplicates and are here represented as averages.

1.9. Alzheimer's disease and the Amyloid β peptides

The most common and well known human disorder involving formation of amyloid deposits is Alzheimer's disease (AD), which affects about 6% of people over 65 years.¹⁸⁶ Despite being the most prevalent, and certainly the most studied neurodegenerative disorder, it remains as one of the least well understood biomolecular processes of disease. It is especially complicated since it involves two amyloidogenic proteins. AD is named after the physician Alois Alzheimer who first

characterised it in 1906.¹⁸⁷ It involves progressive loss of memory (dementia), speech, orientation, cognitive impairment, and eventually loss of vital functions, resulting in death.¹⁸⁸ It begins characteristically with neuron death in the entorhinal cortex, then continuing to the hippocampus region and the association cortex. Eventually, the cortices controlling sensory and motoric function are affected. Intracellular fibrillary tangles of hyperphosphorylated tau protein and the formation of extracellular plaques, consisting largely of Amyloid β (A β) peptides, are main pathological hallmarks of AD.¹⁸⁹ There are two main isoforms of the A β peptide, which are 40 and 42 amino acid residues long, respectively. The A β 40 isoform is about five times more abundant in the cerebrospinal fluid of healthy subjects without genetic predisposition towards AD. The A β 42 isoform is known to have a lower kinetic stability in its monomeric state and is significantly more "amyloidogenic" and thus possesses higher neurotoxic potential.¹⁹⁰⁻¹⁹¹ The A β peptides are the products of β - and γ -secretases' proteolytic cleavage of the Amyloid β precursor protein (A β PP), which is an integral membrane protein.¹⁹²⁻¹⁹³ The "normal" function of the A β peptides is unknown, but their secretion may be an ancient defence mechanism against pathogens.¹⁹⁴⁻¹⁹⁶ The production of A β peptides, and aggregates thereof, is counterbalanced by enzymes, astrocytes and microglia that clear them.¹⁹⁷⁻²⁰⁵ In addition, A β can be transported out of the brain across the blood brain barrier (BBB) by scavenger receptors, for degradation in the liver.²⁰⁶⁻²⁰⁸ Disturbances in this balance have thus been proposed as possible reasons for the development of AD (late onset).²⁰⁶ While there are several familial versions of early onset AD known, characterised by specific missense mutations in the amino acid sequence of the A β peptide, or elsewhere in the A β PP sequence, the majority of the incidences are sporadic, *i.e.* no known genetic factor is involved. Although, having a relative with AD is one of the highest genetic risk factors, indicating that there are other variations, outside the A β PP gene that comes into play, but are still unknown.²⁰⁹

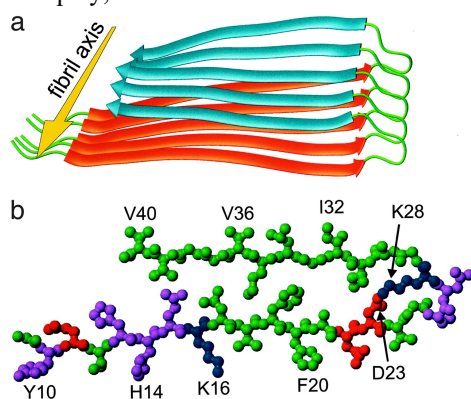


Figure 1.14. Schematic picture of the A β 40 peptide packed into an amyloid fibril (a) and part of the polypeptide chain, with some amino acid residues highlighted (b). Reproduced with permission from Petkova, A. T. *et al. Proc. Nat. Acad. Sci.* **2002**, 99 (26), 16742²¹⁰, Copyright National Academy of Sciences.

The gene encoding A β PP is located on chromosome 21. Interestingly, patients with trisomy 21 (Down syndrome) invariably develop AD if they live to an age of 50 years, and deposits of A β have been found in very young patients, some only 12 years old.²¹¹⁻²¹² Apolipoprotein E (ApoE) is also associated to AD and has been shown to mediate

clearance of A β out of the brain.²¹³⁻²¹⁴ One certain allele of the ApoE gene is a genetic risk factor for developing AD.^{149, 215} Each individual polypeptide chain of A β 40 forms two antiparallel β -strands, with a bend between residue 25 and 29, which is stabilised by an ionic interaction between Asp 23 and Lys 28 (*Figure 1.14*). Removal of this salt bridge by point mutations renders the A β 42 peptide unable to form fibrils.²¹⁰

1.10. Parkinson's disease and α -Synuclein

The second most common neurological disorder involving amyloid formation is Parkinson's disease (PD), which affects around 2% of the population older than 60 years.²¹⁶ It is named in honour of James Parkinson, who in 1817 described cases of a progressive movement disorder.²¹⁷⁻²¹⁸ It involves gradual loss of motoric function and patients suffer from tremors, rigidity and slow limb movements.¹⁴² There is an increasing amount of evidence supporting that the neuron cell death is caused by the aggregation of α -Synuclein.²¹⁹⁻²²⁰ Once started, this process can transmit from cell to cell and spread through the nervous system. It is hypothesised to begin in the peripheral nervous system and reach *medulla oblongata* via the vagus nerve, or from the olfactory bulb.²²¹⁻²²³ It eventually affects *substantia nigra*, where the death of dopaminergic neurons are greatest in the *substantia nigra pars compacta*.²²⁴ The pathological hallmarks include the formation of Lewy bodies, intracellular inclusions containing aggregates of α -Synuclein (α -Syn), named after Fritz J. H. Lewy who in 1912 studied these particles with microscopy.²²⁴ α -Syn is a 140 residue long polypeptide which exists in two states. In association with cell membranes of synaptic vesicles it adopts an α -helical secondary structure, whereas it is unfolded in its native monomeric state in the cytosol.²²⁵⁻²²⁷ Its intrinsically unstructured nature presumably makes it prone to self-aggregation.²²⁸⁻²²⁹ It can be found throughout the CNS, where its concentration is highest in the pre-synaptic terminals of the nerve cells.²²⁰ It plays a role in trafficking of dopamine vesicles and is thought to be involved in enabling of synaptic plasticity.²³⁰ In addition to PD, this protein is associated with a few other neurological diseases, collectively called synucleinopathies.²³¹ *In vivo*, the fibrils subsequently form the intracellular inclusions. There are few known genetic factors for PD, and environmental factors such as oxidative stress and toxins seems to be more relevant.¹⁴² Nonetheless, there are familial cases reported, and a few naturally occurring mutants in the otherwise well conserved sequence of α -Syn are known and associated with early onset.²³² In addition, mutations in other genes have been correlated with synucleinopathies.²³³

1.11. Biomolecular mechanisms underlying pathology

There are many theories attempting to explain the biomolecular disease processes inherent to AD, PD and many other amyloid associated disorders. An early and initially very intuitive explanation was the *loss of function hypothesis*. It argues that aggregation of the liable proteins leads to clearance of its native monomers, thereby rendering it unable to perform its function and the neuron cells die as a result. Loss of function may explain other conformational diseases well. Cystic fibrosis and many

types of cancer result from the loss of important functions (of an ion channel and the tumour suppressor protein p53, respectively) and are caused by mutations.²³⁴ For AD and PD however, the loss of function theory seems insubstantial. For example, results from knock out studies in animals stipulate that the functions of both α -Syn and A β are non-vital.²³⁵⁻²³⁶ Contrarywise, the *gain of toxic function hypothesis* states that the fibrils exert a toxic effect on the cells, either directly by mechanically damaging the cells or indirectly by binding vital proteins, transcription factors, nutrients, metal ions or signal substances *etc.*²³⁷ It has further been proposed that the fibrils can catalyse the formation of reactive oxygen species that are toxic.²³⁸⁻²³⁹ A theory that has gained much support implies that the principal neurotoxic agents are intermediate species, soluble and insoluble oligomers formed along the way from native monomers to fibrils.^{151, 240-241} Moreover, oligomers are several orders of magnitude smaller and thus more mobile than mature fibrils. This means that oligomers are more able to interact with cell structures such as cytoskeleton and cell membranes, and cause damage.²⁴² Oligomers of amyloid forming proteins can form ion channels in lipid bilayers, with similar mechanisms of action as bacterial toxins produced by *Bacillus anthracis* and *Clostridium perfringens*, for example. The *channel theory* implies that the toxic function is exerted by oligomers that permeabilize cell membranes. This could cause leakage of vital ions out of cells, disturbance in the K⁺/Na⁺ balance and influx of Ca²⁺, which could be toxic to the cells. Depolarisation of mitochondrial membranes may further lead to depletion of the cells energy reserves, and leakage of enzymes from lysosomes and peroxisomes may lead to digestive and oxidative damage.²⁴³⁻²⁴⁷ Importantly, not all kinds of oligomers appear to be toxic. Certain soluble oligomers lack toxicity and are off-pathway to fibril formation.^{220, 248-249}

Experiments with transgenic animals have shown that expression of human amyloidogenic proteins leads to disease-like results that correlates well with the symptoms observed in human patients.²⁵⁰ Conversely, evidence also support the hypothesis that native α -Syn has a neuroprotective effect against oxidative stress, when administered in moderate amounts. The loss of this function by its aggregation may make the cells more susceptible.²⁵¹ However, oligomers are neurotoxic, while the end products (Lewy body inclusions) may actually have a protective effect, by sequestering toxic fibrils.²⁵² Amyloid plaques can moreover be found in both healthy and affected tissue.²⁵³ Toxicity of A β fibrils have been demonstrated,²⁵⁴ while amorphous aggregates made by dissociating fibrils in PBS, were shown to be non-toxic.²⁵⁵ Investigations have revealed that the amount of soluble A β (monomers and oligomers together) correlates better with synapse loss in AD patients than the amounts of amyloid deposits.^{242, 256} A mutation in α -Syn that leads to accelerated formation of oligomers, but slower fibril formation, is pathogenic.²⁵² Likewise, patients with the arctic familial variant of AD have decreased amounts of amyloid deposits, compared to patients expressing wild type A β . But the arctic mutant A β peptides show faster formation of oligomers.²⁵⁷ It has been demonstrated with both A β , α -Syn and other amyloid forming proteins that Ca²⁺ selective pores can be formed

in lipid bilayers *in vitro*. The channels could be blocked with Al^{3+} and their formation can be inhibited by Congo red.²⁴⁴ $A\beta$ has been shown to form channels in both rat and human neuron cell lines, and disrupt Ca^{2+} homeostasis.²⁵⁸⁻²⁵⁹ As more and more research data have suggested that oligomers exert the principal neurotoxic effect, the scientific community has approached a kind of consensus about this.^{151, 250}

1.12. Therapeutic approaches

Both AD and PD are severe neurological disorders without cures, that kill 100% of their victims. The lengthy processes involve significant morbidity and AD is among the costliest diseases in developed countries. The worldwide costs of dementia to society was estimated to \$818 billion annually, in 2015.²⁶⁰ The approved medications available today can only alleviate the symptoms to some degree, for a shorter time, and often exhibit significant side effects. Patients with AD can be treated with acetylcholinesterase inhibitors such as Tacrine, Donepezil and Rivastigmine (*Figure 1.15*). These drugs increase the non-amyloidogenic $A\beta$ PP processing. They also delay the breakdown of acetylcholine and thereby compensate for the loss of cholinergic stimulation, which is a result of neuron cell death. The effects are modest and temporal. Side effects include headache, nausea, vomiting, dizziness, sleep disturbances, constipation and diarrhea.²⁶¹⁻²⁶⁵ Complementary to the acetylcholinesterase inhibitors is Memantine, which is an *N*-methyl-D-aspartate (NMDA) receptor antagonist. It blocks the glutaminergic pathway and prevents neuron overstimulation, which can lead to cell death. Overstimulation of the NMDA receptors can occur in patients with both AD and PD.

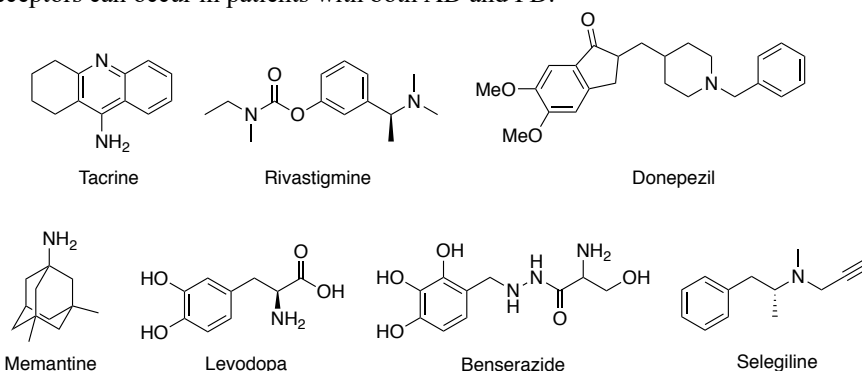


Figure 1.15. Some approved drugs for symptomatic treatment of AD and PD. Tacrine, Rivastigmine and Donepezil are acetylcholinesterase inhibitors used in AD therapy. Memantine is a NMDA receptor antagonist that prevents glutaminergic overstimulation and neuron cell death. It is used by both AD and PD patients. Levodopa is the main treatment given to PD patients and Benserazide is a dopa decarboxylase inhibitor administered together with levodopa. Benserazide does not cross the BBB and inhibits Levodopa to dopamine conversion only peripherally. Although these drugs offer symptomatic treatment and, in some cases, delay the disease progression, they do not offer a cure and all victims eventually die.

Memantine shows modest improvement and retards both disease processes to some degree, with minimal side effects.²⁶⁶ Due to their high oxygen metabolism, neuron cells are especially exposed to oxidative stress and damage. Vitamin E which is a

potent antioxidant is therefore often prescribed or recommended to patients with AD and PD.^{239, 267} PD patients have long been treated with levodopa, which is a precursor to dopamine that can cross the BBB. It compensates for the decrease in dopamine levels in the brain that results from the death of the dopamine producing neuron cells. It alleviates the motor dysfunction symptoms, which are results of the low dopamine levels in the brain.¹⁴² Only a small fraction of the administered levodopa crosses the BBB, and the majority is converted to dopamine in the rest of the body. This results in side effects like nausea and vomiting. To combat this problem, levodopa is administered together with Carbidopa or Benserazide which inhibits the systemic metabolism of levodopa. Involuntary movements is another common side effect to develop. Levodopa also inhibits α -Syn fibrilization. Unfortunately it seems to favour the formation of toxic oligomers instead.²⁶⁸ Related strategies include dopamine agonists and drugs such as Selegiline which inhibit enzymes degrading dopamine. Selegiline also has an inhibitory effect on the formation of α -Synuclein fibrils, and luckily favours the formation of non-toxic oligomers.²⁶⁹ PD patients can also undergo surgery to implant electrical neurostimulators, that improve motoric functions through electrical stimulation of certain brain areas.²⁷⁰

The mechanisms that trigger the onsets of these diseases are far from fully understood. There are several genetic factors, especially for AD, but environmental factors have substantial impact too. Oxidative stress is widely considered.²⁷¹⁻²⁷² Oxidative stress may be a result of aging and it has been proposed that those processes which protect cells against oxidative stress lose activity with aging.²⁷³ Antioxidants are hence considered protective. Inflammation is another theory. The use of non-steroid anti-inflammatory drugs (NSAID) appears to be protective against both AD and PD.²⁷⁴⁻²⁷⁵ Recent theories argue that AD may be initiated by the inflammatory responses that occur in the brain upon infection by *Porphyromonas gingivalis*, a common cause of periodontal infections.²⁷⁶ Similarly, disturbances in the gut flora may be linked to PD. Both inflammation and microbial metabolites with neuromodulatory potential can impact human neurological health and may even be a direct cause of disease onset.^{223, 277} Other studies further suggest that certain viral infections may be linked to PD.²⁷⁸ Methyl-phenyl-tetrahydropyridine (MPTP) (*Figure 1.16*) has initiated PD in very young individuals^{142, 269} and exposure to some pesticides (e.g. Paraquat, Rotenone, Dieldrin and Diethyldithiocarbamate) is considered as a risk factor.²⁷⁹⁻²⁸³ Individuals with a history of head trauma are more likely to develop both AD and PD. A history of depression or hypertension is another risk factors for AD and people with obesity, diabetes or high cholesterol levels also suffers from increased incidence. Moderate amounts of alcohol (in particular red wine), curcumin (found in turmeric), ginkgo leaves and certain flavonoids may be protective against AD. Caffeine intake is negatively correlated with both AD and PD. Interestingly, tobacco smoking is a significant risk factor for development of AD, but seems to be protective against PD. Activities that are intellectually stimulating such as language learning, reading and playing musical instruments are regarded as protective against AD. Also physical

activities and social interactions are beneficial. Physical activity is further a protective factor against PD and is a common therapy for PD patients.^{110, 188, 248, 270, 284-286}

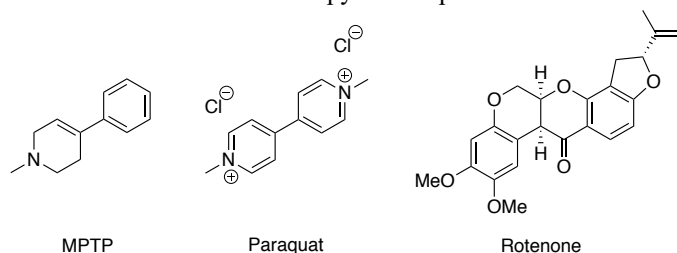


Figure 1.16. Structure of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), Paraquat and Rotenone. MPTP is a by-product in the synthesis of Desmethyprodine, a synthetic opioid analgesic, that is toxic to dopaminergic neurons. Paraquat is a non-selective herbicide which is now banned in several countries. There is a link between Paraquat exposure and PD. Rotenone is a naturally occurring pesticide that inhibits the electron transport chain and is selectively toxic against insects and fish. It has been shown to induce PD-like symptoms in rodents and is a suspected cause in humans.

A lot of research efforts have been undertaken in this field and many therapeutic strategies against these neurological disorders have been considered over the years. One early strategy was dissolution of the amyloid deposits. However, as more recent insight into the biomolecular mechanisms has revealed that oligomeric intermediates have much higher neurotoxic potential, these efforts have been redirected.^{151, 250} Rational drug design has been hampered by lack of reliable structure models of the intended drug targets, oligomers are challenging to study due to their transient nature. The fibrillar end products are insoluble and non-crystalline, and their non-canonical structure makes classical drug-target interaction models irrelevant. However, since the beginning of the 21:st century, solid state NMR studies have started to provide data of useful resolution.²¹⁰ For AD there have been considerable efforts to develop inhibitors against the β - and γ -secretases responsible for the production of the A β peptides.²⁸⁷⁻²⁹⁰ Studies with antibodies against A β monomers, soluble oligomers and mature fibrils have shown significant promise. Antibodies against oligomers are able to recognise oligomers of several amyloidogenic proteins, and ameliorate toxicity.²⁴⁸ Peptides that are complementary to the central hydrophobic region of A β have also been investigated deeply. They act by addition to the growing fibrils' ends and prevent further elongation.^{248, 291} Several natural products with neuroprotective effects have been identified. Among these, polyphenols are likely the most studied.²⁴⁸ Baicalein for example (Figure 1.17), a flavonoid found in certain species of skullcap (*Scutellaria baicalensis*) and used in traditional Chinese remedies, has therapeutic potential. Baicalein, and especially its oxidised quinone form that can undergo imine formation with a lysin side chain of α -Syn, has been shown to inhibit fibril formation and even disaggregate mature fibrils. It induces the formation of spherical soluble oligomers of α -Syn that are non-toxic and off pathway (cannot proceed to fibres). The oligomers even display an inverse seeding effect when added to monomeric α -Syn in fibrilization reaction mixtures. Baicalein is further an antioxidant.²⁹²⁻²⁹⁴ (–)-epigallocatechin gallate is another flavonoid antioxidant, which can be found in green

tea, and has neuroprotective potential. It can interfere with both α -Syn and A β amyloid formation and reduce toxicity in several cell- and animal models. It can also remodel fibrils into smaller non-toxic oligomers and further prevents formation of pores in lipid membranes. (–)-epigallocatechin gallate binds to both monomers and oligomers of α -Synuclein, prevents formation of β -sheets and instead induce formation of non-toxic oligomers.²⁹⁵⁻²⁹⁸

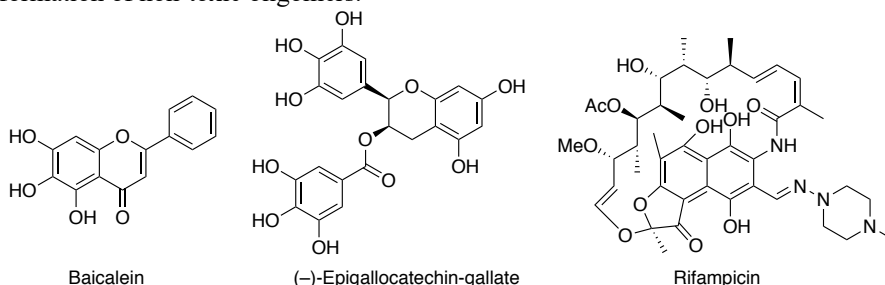


Figure 1.17. Structure of some natural products with amyloid modulating properties. Baicalein is found in the skullcap *Scutellaria baicalensis* and (–)-epigallocatechin gallate in green tea. Rifampicin is an antibiotic commonly used to treat Mycobacterial infections such as tuberculosis and leprosy.

Rifampicin is a semi synthetic antibiotic that was found to decrease the amounts of amyloid deposits in the brains of leprosy patients using the drug. Its mechanism of action appears to be similar to that of Baicalein and epigallocatechin-gallate. Rifampicin inhibits α -Syn and A β aggregation, but also binds to mature fibrils. It attenuates toxicity of pre-formed A β 42 fibrils through mechanisms not fully understood.²⁹⁹⁻³⁰³ Other small molecules that bind to the fibrillar end products have also demonstrated therapeutic potentials.²⁴⁸ As mentioned, Congo red has been reported to prevent formation of ion channels through cell membranes. It has been proposed that amyloid binding small molecules stabilise the mature fibrils and prevent the reversible formation of toxic oligomers.^{248, 304-305} It has also been suggested that compound binding inhibit adhesion of fibrils to cell surfaces.³⁰⁶ In accordance, natural or synthetic small molecules that binds to mature amyloid fibrils, consequently have therapeutic potential.²⁵⁵

1.13. Diagnostication

There is also a great need for methods to diagnosticate patients with amyloid related disorders. Historically, the neurological diseases were diagnosed from the symptoms the patients suffered from, usually at a late stage. The specific diseases were confirmed *post mortem* by detection of amyloid deposits in histological samples.¹⁵¹ Systemic amyloidosis can be diagnosed *pre mortem* with biopsies taken from the involved organs, in a relatively non-invasive manner. AD and PD are usually diagnosed through behavioural and functional assessments and brain tissue examination is required for definitive confirmation. Consequently, these diseases are often diagnosed in late stages and initial symptoms are often mistaken for normal ageing. Ideally, the neurological disorders should be diagnosed early, through non-

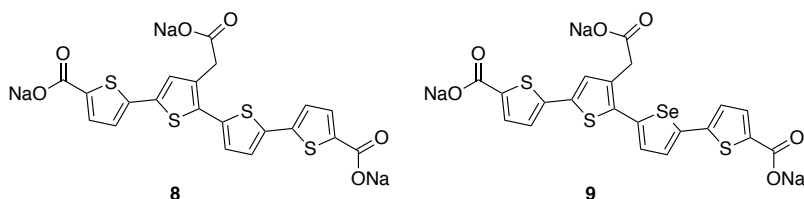


Figure 1.19. Structure of luminescent conjugated oligothiophene **8** and thiophene-selenophene co-oligomer **9** with amyloid binding and fluorescent properties. Variable emission spectra are obtained depending on the conformation of the amyloid ligands. These fluorescent dyes can hence be used to differentiate between A β and Tau fibrils, or amyloid fibrils of different age.

1.14. Thiazoline fused 2-pyridones as modulators of fibril formation

Since discovering the ability of FN075 to inhibit A β fibrilization *in vitro*,¹⁰⁸ it has been subject of extensive investigations. A study of FN075 and other structurally related thiazolino fused 2-pyridones demonstrated that FN075 inhibits the Curli biogenesis in *E. coli*. FN075 is also able to inhibit the fibril formation of CsgA, the main constituent of Curli fibres, *in vitro*.¹⁰⁹ Since Curli fibres are virulence factors which are crucial for bacterial biofilm formation, FN075 and similar 2-pyridones would thus be potential antibacterial agents. Furthermore, given the vast biological differences between humans and *E. coli*, these results taken together pointed to a generic inhibition mechanism upon amyloid fibril formation, exerted by FN075.¹⁰⁹ It was thus initially surprising to observe that FN075 accelerated formation of fibrils by α -Syn *in vitro* (Figure 1.20).³¹⁷⁻³¹⁸ The modulation of amyloid fibril formation by FN075, which has a peptidomimetic backbone, may thus be dependent on the primary structure of amyloidogenic polypeptides. FN075 induces the formation of spherical oligomers of CsgA, that are not amyloidogenic.³¹⁷ Contrariwise, it decreases the lag time of α -Syn fibrilization *in vitro* in a dose-dependent manner, by triggering formation of oligomers that are amyloidogenic.^{249, 317}

The bacterial chaperone CsgC, found in *E. coli* expressing CsgA, efficiently inhibits fibrilization of both bacterial CsgA and human α -Syn.¹⁸⁰ Conversely, A β is unaffected by this chaperone. CsgA and α -Syn have a sequence motif in common which is not present in A β . Further studies with far-UV CD spectroscopy into the aggregation procedure of α -Syn indicated that FN075 accelerates the transformation of random coil species (monomers or oligomers) into oligomers containing β -sheet secondary structure, which are aggregation competent.³¹⁸ These oligomers readily disrupt vesicles *in vitro*, more efficiently than mature fibrils, indicating cytotoxic effect. Injection of FN075 into the striatum of w.t. mice causes death of dopaminergic neurons in *substantia nigra* after 3 months, and sensorimotor compromise after 6 months.²³⁶ Moreover, transgenic fruit flies (*Drosophila melanogaster*) expressing human α -Syn that were fed with FN075 had shorter life span than flies fed with a non-amyloidogenic 2-pyridone, or only with vehicle.³¹⁹

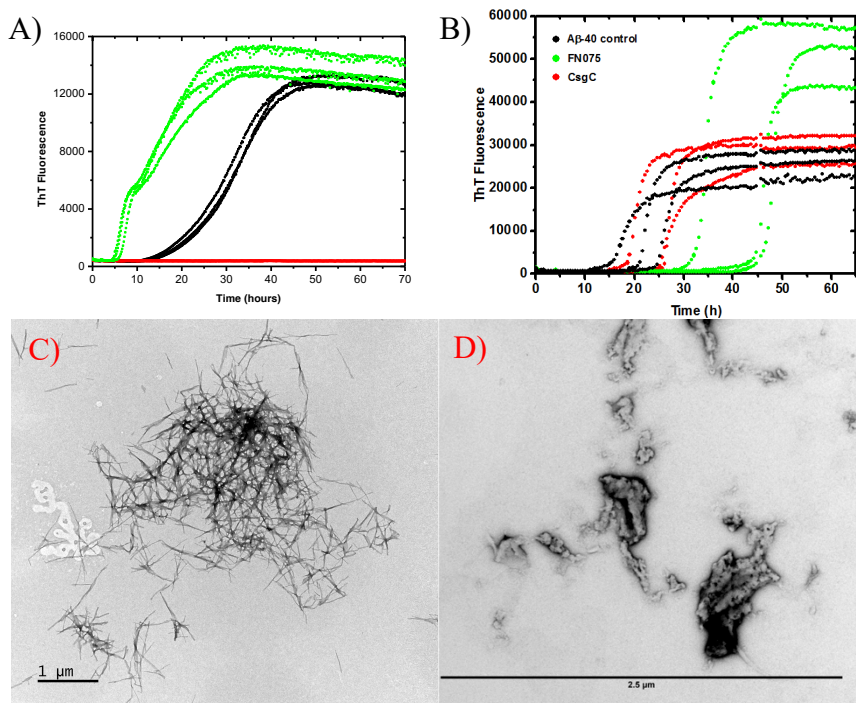


Figure 1.20. FN075 induces the formation of off-pathway, non-amyloidogenic oligomers of A β and CsgA, but accelerates α -Syn fibrillization by triggering formation of on-pathway amyloidogenic oligomers. **A)** ThT trace of α -Syn *in vitro* fibrillization in the absence of compound (black traces) and presence of FN075 (green traces), and bacterial chaperone CsgC, which inhibits fibrillization (red traces)¹⁸⁰. In the absence of compounds, the lag time is about 12 h. FN075 (100 μ M) accelerates the formation of aggregation prone oligomers and the lag time is decreased as a result. Even at a sub-stoichiometric concentrations, CsgC (14 μ M) inhibits fibril formation throughout the experiment. **B)** ThT trace of A β 40 fibrillization *in vitro* without (black) and with FN075 (green). CsgC has no effect on A β fibre formation (red). **C)** Transmission electron microscopy picture of α -Syn fibrils. TEM reveals no difference in appearance of α -Syn fibrils formed in the absence and presence of FN075.³¹⁸ **D)** No fibrils, only amorphous aggregates can be found with TEM upon incubation of monomeric α -Syn with CsgC.

In light of the amyloid modulating properties of FN075, several analogues were synthesised and evaluated, whereof a few showed similar activity of inhibiting CsgA fibrillization (Figure 1.21).³²⁰⁻³²² Extension of the peptidomimetic backbone (compound **10** and **11**) and rigidification of the structure by extension to a tricyclic ring system (**11**) retained the activity against CsgA. Replacement of the thiazoline by a sultam ring slightly enhanced the inhibiting effect (**12**). Introduction of an acetylene spacer between the *m*-CF₃-phenyl substituent and the scaffold (**13**) was also tolerated. Interestingly, bromination of **13** in position C-6 reversed the modulatory effect and decreased the lag time of CsgA fibrillization. The phenyl substituted brominated analogue **14** exerts slightly more acceleration.

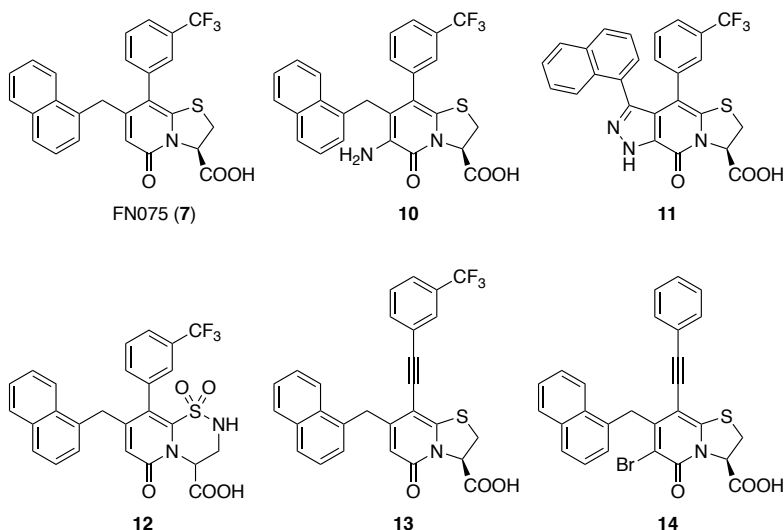


Figure 1.21. FN075 analogues with extended peptidomimetic backbone (**10–11**), sultam fused 2-pyridone (**12**) and analogues with acetylene spacer (**13–14**). Compounds **10–13** inhibit CsgA aggregation, while the brominated compound **14** accelerates it. Compound **10** and **12** inhibits α -Syn aggregation as well.²⁴⁹ The pyrazole fused thiazolino 2-pyridone **11** had limited aqueous solubility.^{249, 320}

Also α -Syn aggregation is inhibited by the amino functionalised FN075 analogue **10** and the sultam fused 2-pyridone **12** (in contrast to FN075, which accelerates α -Syn aggregation).²⁴⁹ Oligomers harvested from the fibrilization reaction mixtures moreover inhibited aggregation of α -Syn, when added in a 1:100 ratio to monomers in new fibrilization mixtures at the start (they were "anti-seeding"). When compound **12** was injected into the striatum of mice, no neuron loss or motoric dysfunction was observed.²³⁶

The utility of FN075 and related analogues as fibrilization modulators, highlighted their potential as chemical tool compounds to gain further insight into the molecular mechanisms underlying human neurodegenerative diseases. The methoxy functionalised FN075 analogue **15** (Figure 1.22) was found to accelerate α -Syn aggregation *in vitro*, almost as effectively as FN075.³²³ Acetoxymethyl ester analogue **16** was thus synthesised, with ¹¹C labelling, for evaluation in PET applications. While the carboxylic acid functionality in FN075 is crucial for its biological activity as α -Syn aggregation accelerator,¹⁰⁸ it certainly hampers BBB permeability. The acetoxymethyl ester functionality grants **16** with the ability to cross the BBB, as well as being a labile functional group *in vivo*, that can be hydrolysed upon BBB passage and exert its biological activity. Intravenous injection of ¹¹C labelled **15** and compound **16** into rhesus monkeys, and subsequent PET analysis, revealed notably higher signals from the brains of the monkeys injected with the ester **16**.³²³ The monkeys were healthy and had thus no amyloid fibril deposits in their brain. It is further uncertain whether compound **15** has sufficient affinity for monomeric α -Syn.

Still, the result highlights that thiazoline fused 2-pyridones as acetoxymethyl esters can cross the BBB and be visualised with PET.

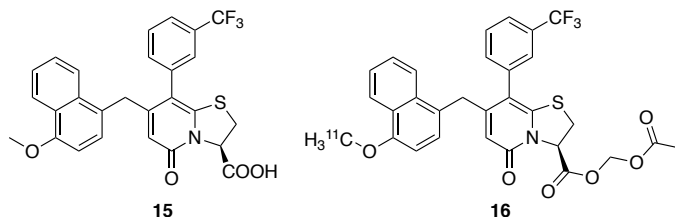


Figure 1.22. Structure of FN075 analogues **15** and **16** for *in vivo* PET analysis.

1.15. Multi ring fused 2-pyridone peptidomimetics

Our group has also reported the construction of indole-, benzoquinoline-, and benzothienopyridine fused thiazolino 2-pyridones **17–19** (Figure 1.23).³²⁴ Based on the diverse biological activities of FN075 and its analogues **10** and **11**, it was hypothesised that further rigidification through annulation of the aromatic ring systems would retain or improve the fibrilization modulating properties, and in addition grant the peptidomimetics with fluorescent properties. Rewardingly, the scaffolds **18** and **19** contained both examples that seemed to inhibit the aggregation and examples that accelerated α -Syn aggregation, although none as efficiently as FN075. In addition, all three scaffolds displayed fluorescence, scaffold **18** having the highest quantum yields (1–24%).

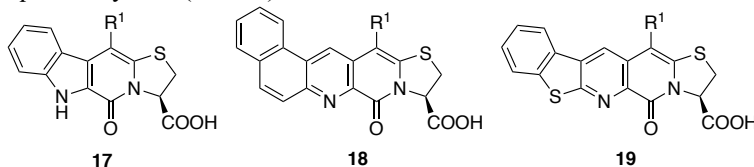


Figure 1.23. Fluorescent indole, benzoquinoline, and benzothienopyridine annulated thiazolino 2-pyridones. Examples of scaffold **18** and **19** include compounds with both accelerating and inhibiting effect on α -Syn fibril formation *in vitro*.

2. Objectives

The aim of the work presented in this doctoral thesis is to establish synthetic methods and produce novel chemical compounds. In addition, the purpose of the molecules generated by the developed methods, is interaction with amyloid fibrils and their formation. The goal is to develop chemical tool compounds that can be used to unravel the biomolecular mechanisms that leads to neurodegenerative disorders such as Alzheimer's and Parkinson's disease.

The thiazoline fused 2-pyridone peptidomimetics, if fitted with certain substitution patterns or rigidified through extension of the heterocyclic ring system, provide compounds with amyloid modulating properties. Different structures can inhibit or accelerate the fibril formation by amyloidogenic proteins associated with Alzheimer's and Parkinson's disease. Herein I describe how multi ring-fused 2-pyridones with new core structures were designed and synthesised, and their application in biological systems. New and existing thiazoline fused 2-pyridones have been extended and altered through strategic chemical transformations to prepare compounds with potent ability to bind amyloid fibrils. Synthetic procedures, including multi component reactions for extension of the fused ring systems, as well as thiazoline ring modification and ring opening, were developed to prepare these biologically active compounds. Their use in human cell lines and mice indicates full ablation of neurotoxicity, caused by a protein selective chemical accelerator of amyloid fibril formation.

3. Synthesis of poly-heterocycles based on the thiazoline fused 2-pyridone scaffold that binds to α -Synuclein and Amyloid β fibrils

Article I, III and VI.

3.1. Background – Modulators of α -Synuclein fibril formation

From the preceding experiments with multi-ring fused 2-pyridones³²⁴ we concluded that the pyridine fused thiazolino 2-pyridone central unit was crucial for enabling the amyloid interfering effects of these annulated heterocycles (*Figure 3.1*). We hence decided to construct a new tricyclic scaffold containing these features, including the extended peptidomimetic backbone (highlighted in red). A tricyclic scaffold containing a pyridine fused with the 2-pyridone, had previously been constructed in our group.³²⁵ However, that isomer lacked the extended peptidomimetic backbone. We further desired a scaffold with multiple points of variation for gaining structure activity relationship (SAR) information in the event that the structures should be active modulators of amyloid fibril formation.

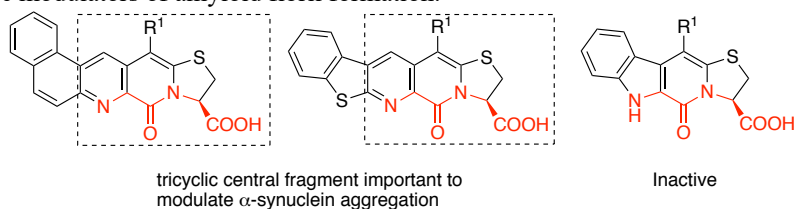
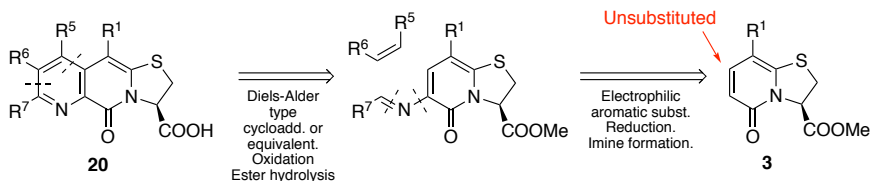


Figure 3.1. Conclusion from the previously performed study with multi-ring fused 2-pyridone peptidomimetics.³²⁴ The peptidomimetic backbones are highlighted with red colour.

3.2. Initial attempts

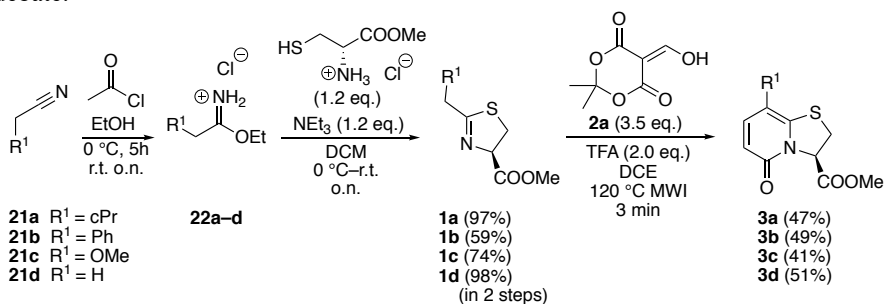
We swiftly realised that the desired tricyclic central fragment **20** was accessible from our bicyclic thiazolino 2-pyridone scaffold **3**, deprived of C-7 substituent (*Scheme 3.1*). No thiazolino 2-pyridones without C-7 substituent had previously been prepared in our laboratories.



Scheme 3.1. Retrosynthetic disassembly of the desired tricyclic central fragment **20**.

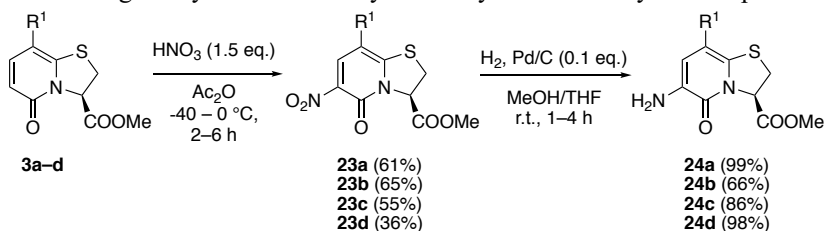
Nevertheless, the bicyclic scaffold **3a–d** was successfully made by cyclocondensation of thiazoline **1a–d**^{33, 46} (*Scheme 3.2*) with formyl Meldrum's acid **2a**³²⁶ according to our already established procedure,⁹³ with small modifications.³²⁷ The Thiazolines themselves were made in two steps from commercially available R¹-substituted

acetonitriles **21a–d**. The first step is an acidic Pinner reaction,^{328–330} the iminoether **22** is then allowed to react with cysteine methyl ester hydrochloride, to provide the thiazolines **1a–d**.^{331–332} The C-3 stereocenter hence comes from the chiral pool. The formyl Meldrum's acid derivative **2a** was made from Meldrum's acid *via* a modified literature procedure.^{326–327} Meldrum's acid itself is easily made from malonic acid and acetone,^{N3} or obtained commercially. Since its discovery by Andrew Meldrum in 1908,^{333–334} the rationale behind its acidity has been a matter of investigation and debate.^{335–337}



Scheme 3.2. Synthesis of bicyclic thiazoline fused 2-pyridone key intermediates.³²⁷

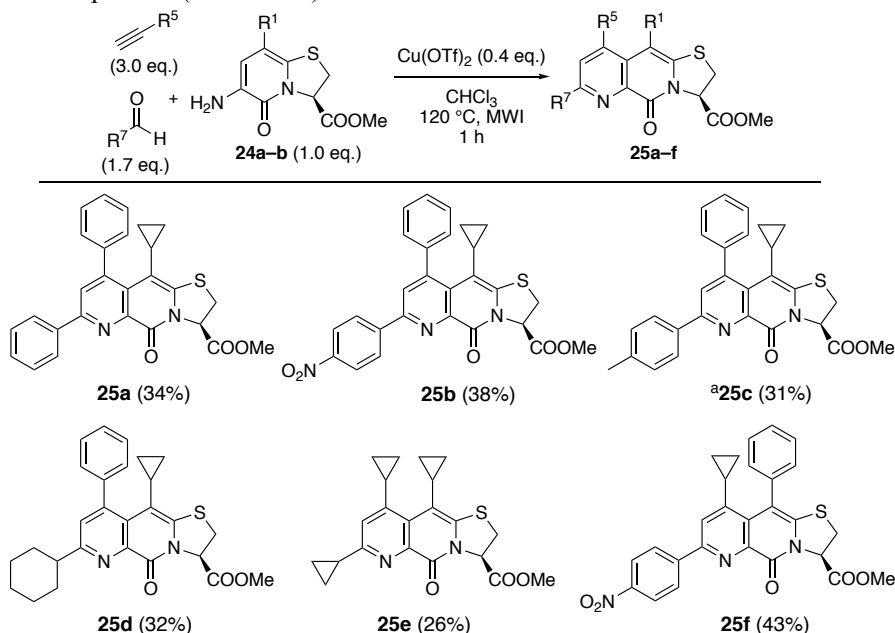
The bicyclic 2-pyridones **3a–d** were nitrated at position C-6 and subsequently reduced to the corresponding amines **24a–d** (Scheme 3.3). The amination also worked to extend the peptidomimetic backbone by one atom. Reduction of the newly introduced nitro functionality by catalytic hydrogenation proceeded smoothly at room temperature and gave clean conversion.^{N4} The catalyst was removed during workup by filtration. The solvent was evaporated from the clear, dark brown solution to give the desired product as an ebony black solid, which was pure according to NMR data and used for the following reaction steps without further purification. At this juncture it should be mentioned that chromatographic purification afforded light brown or light grey solid products. Albeit NMR spectroscopy revealed no significant increase in purity, purification of these products dramatically increased the yields of subsequent reactions. Unfortunately, this was not discovered until most of the work in *article 1* was completed and the yields reported therein thus understates the utility of the methods. The amines **24a–d** have been confirmed unstable on silica though, and their purification negatively affects overall yields in synthesis of tricyclic compounds.



Scheme 3.3. C-6-amination of the bicyclic scaffold **3**.³²⁷

The nitration procedure was later modified by replacing acetic anhydride with DCM and TFA.³³⁸ The modified procedure involved a simpler workup and provided cleaner conversions, removing the need for chromatographic purification after this step.

With the amino pyridones **24a–d** in hand we turned our attention to transformations that would extend the bicyclic structures into a tricyclic, pyridine fused framework. The first method we developed employed the A³-reaction, followed by intramolecular cyclisation. Its unusual name derives from the initials of the three reactants, Aldehyde, Alkyne and Amine.^{339–349} In our case, the amine component was represented by amino 2-pyridones **24a–b**. Commercially available aldehydes and alkynes made up the other two components (*Scheme 3.4*).

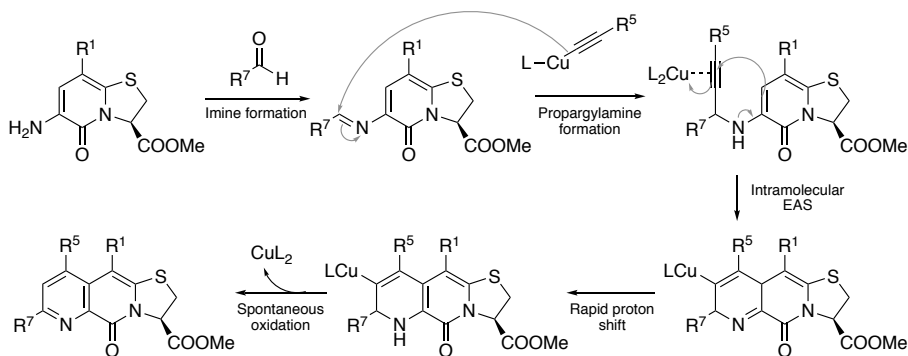


Scheme 3.4. General scheme of the employed A³-reaction and the synthesised analogues. The reactions were carried out with 0.25–0.4 mmol of **24a–b**. ^aThe reaction time was extended to 2 h.

The reactions supplied the desired products after 1–2 h microwave heating at 120°C , workup and purification. Both phenyl- and cyclopropyl acetylene were tolerated as alkyne component. Similarly, both aromatic benzaldehydes and cyclic aliphatic aldehydes were used successfully. Electron poor *p*-nitro benzaldehyde was tolerated well, but a mildly electron donating *p*-methyl substituent slowed down the reaction (**25c**). Strongly electron donating *para* substituents were not tolerated. With *p*-anisaldehyde, only traces of desired product was detected in the resulting complex reaction mixture after 4 h heating.

The details of the A³ reaction is proposed to be as follows (*Scheme 3.5*). Initially, an imine is formed from amine and aldehyde, to which the alkyne is added in a copper

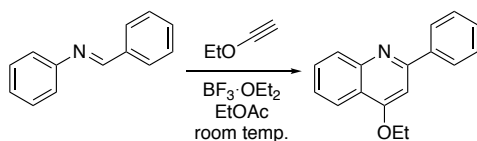
mediated propargylamine formation. The propargylamine subsequently undergoes intramolecular electrophilic aromatic substitution (EAS). Being only one step away from aromaticity, the resulting heterocycle spontaneously oxidises to the pyridine fused 2-pyridone and the desired tricyclic central fragment results.^{339, 341} Alternatively, the process may proceed as a [4 + 2] cycloaddition with inverse electron demand.³⁴²



Scheme 3.5. Stepwise dissection of the A³ reaction.

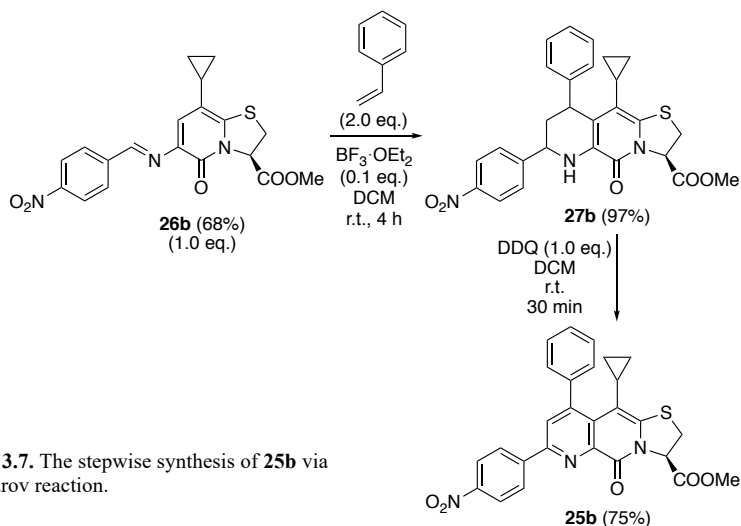
3.3. Method development

We were encouraged by the simplicity of which the framework was constructed *via* the A³ reaction, with three points of variation simultaneously, but limited by the somewhat poor yields and the complexity of the resulting reaction mixtures, which sometimes proved challenging to purify. We hence sought a milder way to synthesise the tricyclic scaffold and considered the Lewis acid catalysed Povarov reaction. The Povarov reaction is mechanistically somewhat similar to the A³ coupling. It relies on the reaction between an electron poor 2-aza diene and an electron rich π -bond (*Scheme 3.6*). Since the initial work by Povarov and co-workers in the 1960:s,³⁵⁰⁻³⁵⁶ this reaction has been used extensively in organic synthesis.³⁵⁷⁻³⁷⁰



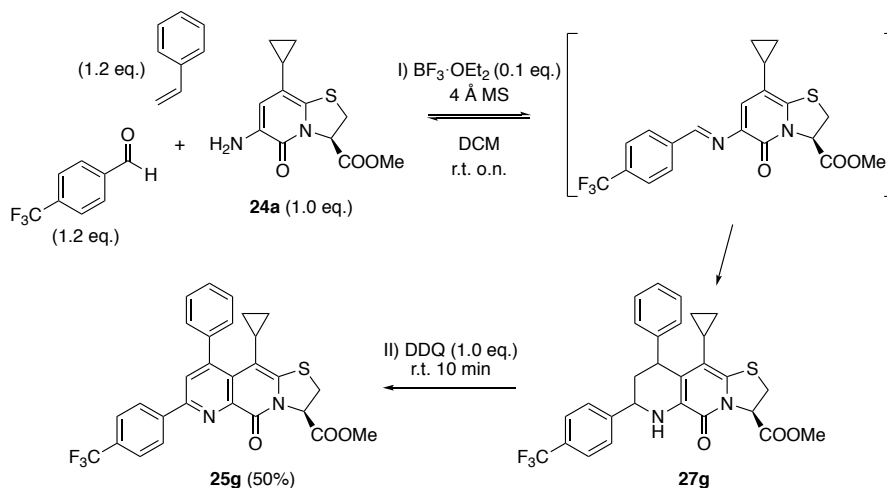
Scheme 3.6. The first Povarov reaction, described by Povarov in 1963. It employed the imine of benzaldehyde and aniline as 2-aza diene and ethoxyacetylene. It was catalysed by boron trifluoride.

In our laboratory, the obvious 2-aza diene was imine **26b**, made from of **24a** (1.0 eq.) and *p*-nitro benzaldehyde (1.2 eq.) (*Scheme 3.7*). After 1 h, the precipitate was collected by filtration. Imine **26b** was then prompted to react with styrene. After initial attempts with Yb(OTf)₃ had proven futile, BF₃·OEt₂ was found to effectively catalyse the transformation to **27b**. The newly formed tetrahydropyridine ring is two steps away from aromaticity and **27b** was stable enough to be isolated, but was observed to slowly oxidise under air to **25b**. This oxidation was effectively expediated by DDQ.



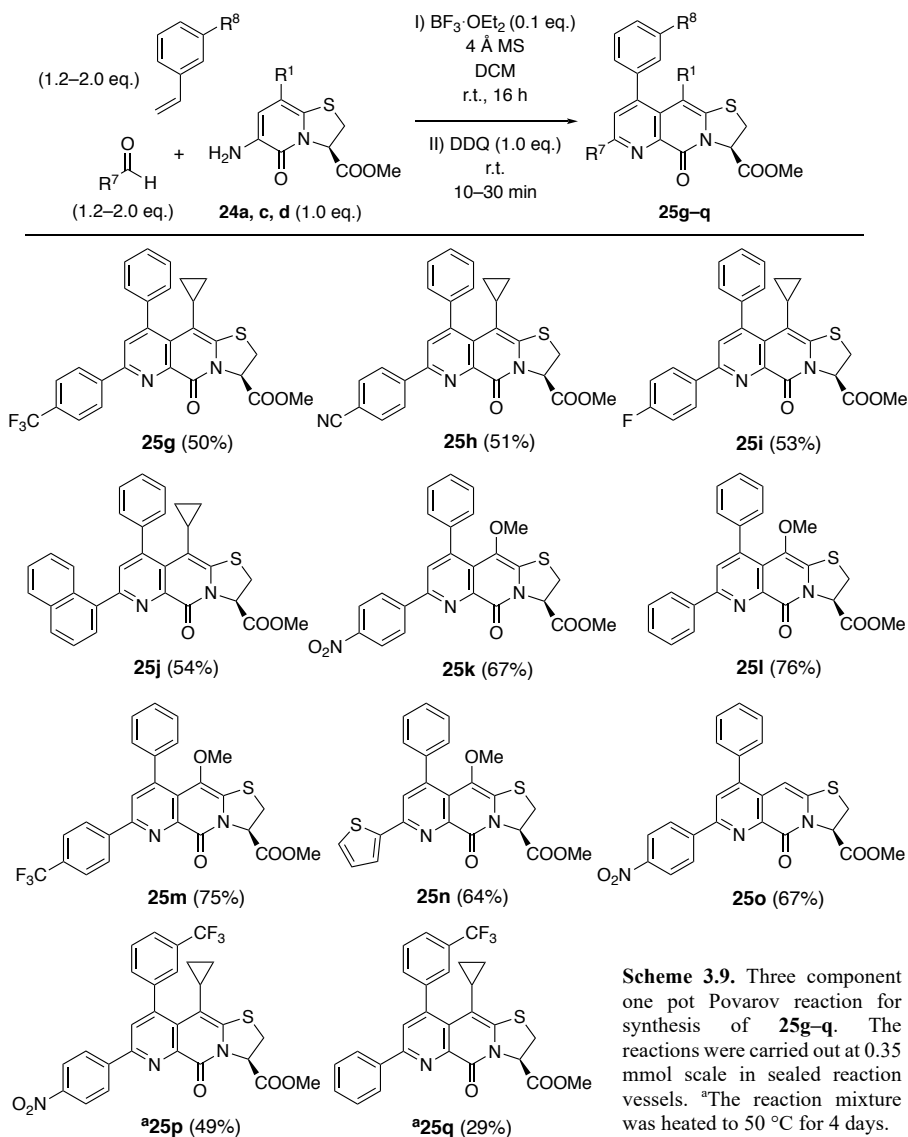
Scheme 3.7. The stepwise synthesis of **25b** via the Povarov reaction.

Invigorated by the fruitful outcome of the stepwise Povarov reaction, we next investigated whether it was possible to perform the above transformation in a one pot approach. Styrene, *p*-(trifluoromethyl) benzaldehyde and amino 2-pyridone **24a** were dissolved in DCM (Scheme 3.8). To trap the water formed in the initial imine formation step and drive the equilibrium forward, 4 Å molecular sieves were added. $\text{BF}_3 \cdot \text{OEt}_2$ was then added to the stirred solution. A colour change from light red to dark purple was observed. Thin layer chromatography (TLC) showed an intense new spot that was yellow-orange under daylight, stained pink-orange when developed with *p*-anisaldehyde dip, and turns brown if left undeveloped under air, just like the spot of amine **24a** does. The spots of the amine and aldehyde had also faded significantly. Upon stirring the mixture over night, TLC no longer showed amine or imine. Instead, three new spots had formed. LC-MS indicated that the major two, least polar spots were the diastereoisomers of adduct **27g**. The last, weakest spot, was the fully oxidised product (**25g**). DDQ rapidly completed the oxidation of **27g** to **25g**, which was subsequently isolated.

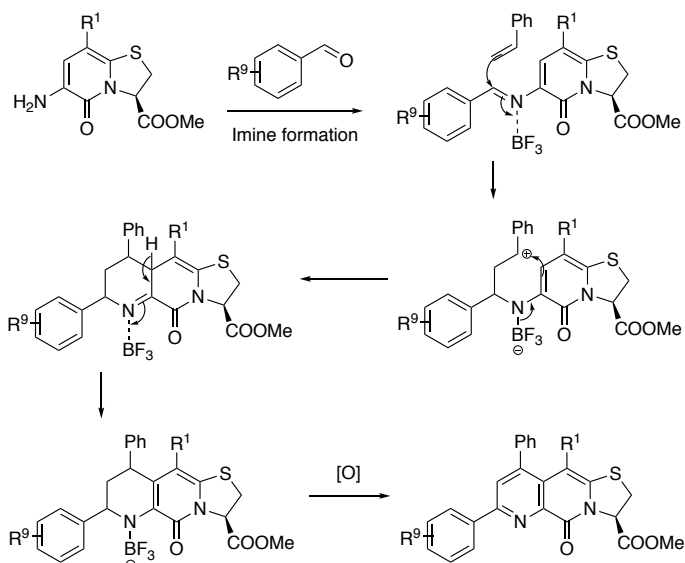


Scheme 3.8. One pot synthesis of **25g** via Povarov reaction, carried out with 0.35 mmol of **24a**.

These results stimulated us to construct a focused library of analogues, made from amines **24a–d** and various aldehydes, with styrenes as the alkene component (*Scheme 3.9*). Gratifyingly, the reaction worked with all combinations tested. DDQ was added to rapidly furnish the desired compound in the same pot, in 50–76% yield after purification. With *m*-CF₃ styrene, the reaction was considerably slower, presumably due to the somewhat lower electron density of the alkene. At r.t. the synthesis of **25p** took 4 days to finish and was only isolated in 16% yield. **25q** was isolated in 4% after 11 days stirring. The nitro group on the benzaldehyde renders the intermediate imine more electrophilic and reactive, and its lower lying LUMO partly compensates for the low-lying HOMO of the alkene in *m*-CF₃ styrene. Reaction times and yields for these two examples were greatly improved by raising the reaction temperature to 50 °C.

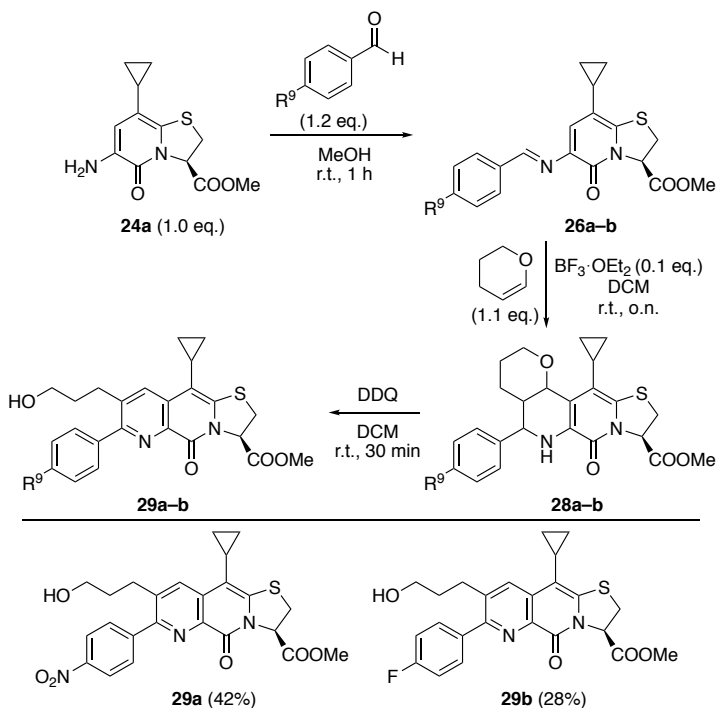


Although Povarov initially described the course as a concerted [4+2] cycloaddition with inverse electron demand, more recent evidence supports a stepwise mechanism (*Scheme 3.10*). The Lewis acid catalyses imine formation and the following Mannich-like reaction. The cascade ends with intramolecular EAS trapping the benzylic carbocation.^{359-361, 371-377} The subsequent oxidation is, as mentioned above, spontaneous but slow under air or oxygen, yet expedited with an auxiliary oxidant, *e.g.* DDQ.



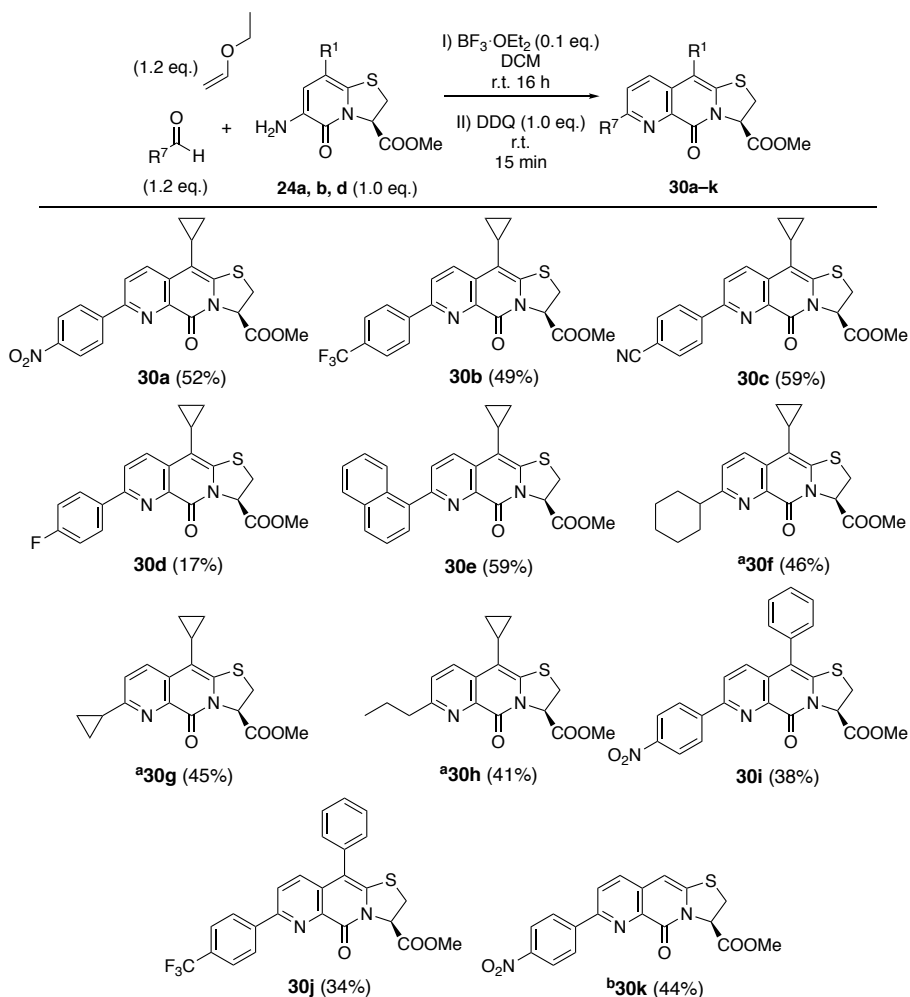
Scheme 3.10. Mechanistic details of the Povarov reaction.

After having observed slower reactions with an electron poor alkene, we replaced styrene with 5,6-dihydropyran. Aware of the potential for mesomeric contributions by the oxygen's lone pair electrons, we expected faster reactions with dihydropyran. We performed the reactions in a stepwise manner (*Scheme 3.11*). Instead of dehydrogenation, alkoxide was eliminated during oxidation, opening up the tetrahydropyran ring and giving the straight chain alcohols **29a–b** in poor overall yields.



Scheme 3.11. Stepwise Povarov reaction for synthesis of **28a–b**.^{N5} Scale: 0.35 mmol **24a**.

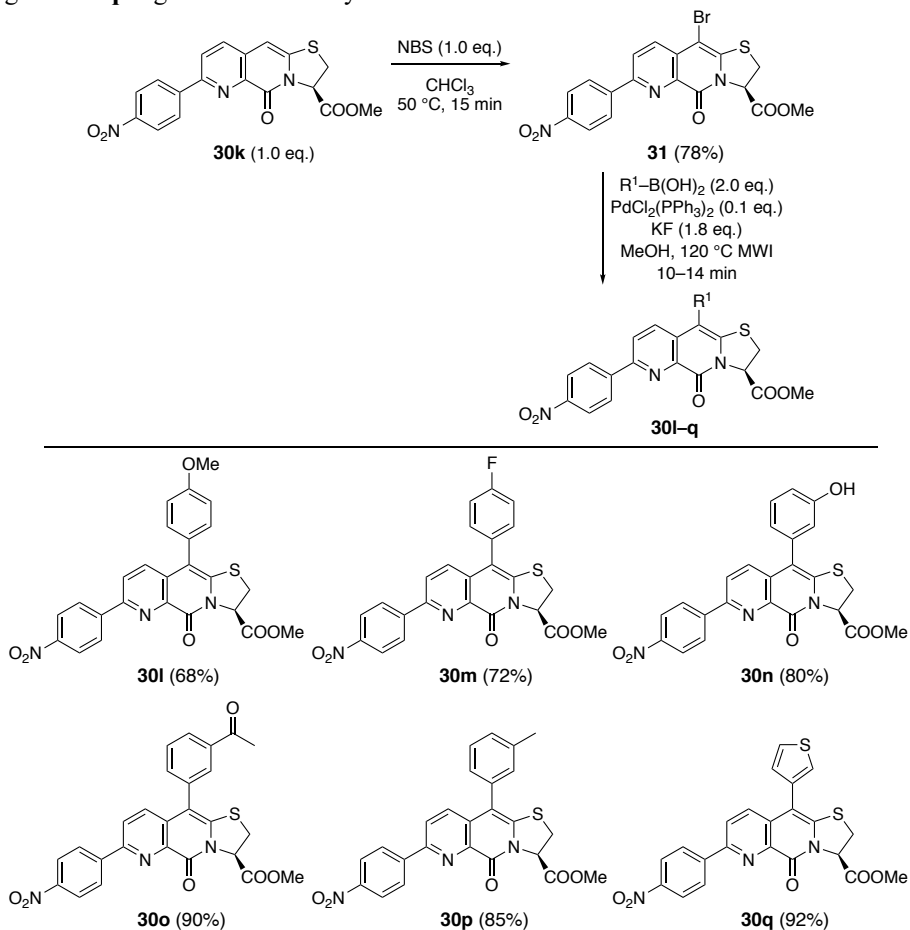
With the alkoxide elimination in mind, we next thought of an alkyl vinyl ether as dienophile. If the alkoxide was eliminated during oxidation³⁶² as in the synthesis of **29**, we would be able to synthesise analogues with a less substituted pyridine ring. As this would expand the scope of the reaction as well as the potential SAR, we allowed amino 2-pyridones **24a**, **b** and **d** to react with various aldehydes and ethyl vinyl ether (*Scheme 3.12*). The products **30a–k** were furnished in moderate yields. **30k** was synthesised on gram scale (5.30 mmol) to verify the scalability of the procedure. The reactions with ethyl vinyl ether proceeded more rapidly compared to the preceding reactions with styrene. The oxidation of the intermediate adducts was faster as well, especially when employing an aliphatic aldehyde component. Full conversion to the desired products **30f–h** was observed after 16 h. Some un-oxidised adducts remained for the other examples, and DDQ was added to complete the conversion.



Scheme 3.12. Three component one pot Povarov reactions with ethyl vinyl ether employed as alkene component. The reactions were performed at 0.33 mmol scale. ^aPovarov adduct oxidised spontaneously under air, no DDQ added. ^bSynthesised on 1.2 g (5.30 mmol) scale.

The biological properties of these compounds will be discussed in detail in the following section of this chapter. But since the compounds which were made was constantly being evaluated, we knew at this point that the *p*-nitrophenyl substituent was beneficial for biological activity versus α -Synuclein fibrils. We therefore saw the opportunity for a late stage introduction of R^1 substituents to **30k**, where this position had remained un-functionalised throughout the synthesis from acetonitrile **21d** (Scheme 3.2). That would give us access to a focused library of compounds with *p*-nitrophenyl functionality as R^7 substituent, and enable us to explore SAR in the position of R^1 . We therefore investigated a halogenation approach, followed by Suzuki coupling³⁷⁸⁻³⁷⁹ with various aromatic boronic acids (Scheme 3.13). **30k** was

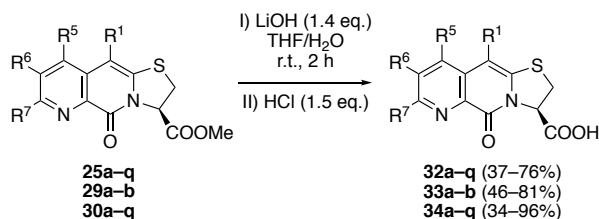
regioselectively brominated and **31** was subsequently coupled with boronic acids to give **30l–q** in good to excellent yields.



Scheme 3.13. Late stage functionalisation with various aromatic R^1 substituents *via* bromination and Suzuki coupling. The bromination was performed at 2.1 mmol scale, the Suzuki couplings at 0.13 mmol scale.^{N5}

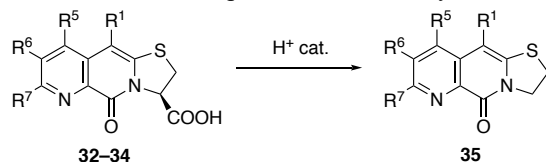
3.4. Biological evaluation – Effects on α -Synuclein and Amyloid β

The carboxylic acid is essential for biological activity of the thiazoline fused 2-pyridone scaffolds versus α -Syn, as previously established.¹⁰⁸ During synthesis, the carboxylic acid is protected as a methyl ester, which upon complete construction of the remaining structure is deprotected through saponification. The tricyclic fused aromatic compounds **25a–q**, **29a–b** and **30a–q** were all hydrolysed to their corresponding carboxylic acids **32a–q**, **33a–b** and **34a–q** (Scheme 3.14).



Scheme 3.14. Deprotection of the desired carboxylic acid functional group by basic hydrolysis of the methyl ester protecting group.

The lithium carboxylates were neutralised with hydrochloric acid upon complete reaction, extracted and purified with preparative reverse phase HPLC (H₂O/MeCN + 0.75% HCOOH) to provide the pure carboxylic acids after lyophilisation. We observed that some of our acids were unstable and prone to decarboxylation under the acidic conditions (*Scheme 3.15*).^{N6} After understanding that some compounds were acid labile, we developed a modified protocol in order to synthesise these compounds pure. Instead of hydrochloric acid, pre-washed Amberlyst 15 was used to quench the basic reaction mixtures.³⁸⁰ Upon workup, the residue was triturated with diethyl ether twice, to furnish the pure desired carboxylic acid.



Scheme 3.15. Some final products were unstable and underwent decarboxylation under the acidic conditions of the reverse phase chromatographic purification.

Initially, each of the compounds with general structure **32–34** was probed against monomeric human α -Syn *in vitro*. The mixtures of monomeric α -Syn, compound (**32–34**) and Thioflavin T (ThT) were agitated with a 2 mm glass bead in buffer at physiological temperature and pH. The formation of amyloid fibres was followed by ThT fluorescence. Monomeric α -Syn slowly aggregates to form amyloid fibrils. Agitation with a glass bead greatly improves reproducibility between experiments^{318, 381-382} As previously discussed, the formation of α -Syn amyloid fibrils goes from monomers *via* oligomeric states, which then proceed to mature fibrils.²²⁰ The formation of these intermediate oligomers takes some time, and not until the concentration of oligomeric species is high enough, fibres can be formed. This behaviour explains the observed fluorescence lag time. The fibril formation starts, rather abruptly, after 10–20 h of agitation, a time interval which is reproducible under the given conditions and specific for α -Syn (*Figure 3.2*).

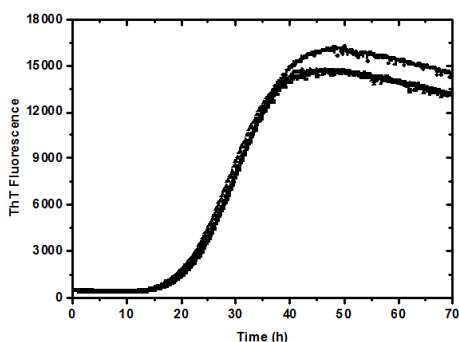


Figure 3.2. Typical ThT fluorescence curve for α -Syn *in vitro* fibrilization. Monomeric α -Syn (70 μ M) and ThT (20 μ M) is mixed in PBS (10 mM, pH 7.4) with DMSO (100 μ M) and a 2 mm glass bead in a 96-well plate, and incubated with agitation at 37 $^{\circ}$ C. The fluorescence (480 nm) is measured every 5 min. Fibre formation starts after about 12 h. Lag time often varies a little bit between protein batches and experiments but is reproducible within the same microtiter plate. The fluorescence intensity reached in the plateau phase, after about 40 h, is less reproducible and often varies between individual wells of the same plate, as seen in this and other figures.

When performing the experiments, each plate has three wells with only α -Synuclein (no compound) as a control and for comparison. Note how the lag time, about 12 h, is reproducible between the three replicates. Another three replicates are incubated with CsgC, the bacterial chaperone which catalytically prevents aggregation and was discussed in the introduction chapter, this as a negative control (*Figure 1.20A*). Observe that there is no increase at all in fluorescence. This implies that there are no fibrils in the samples, which has been verified with TEM and AFM.^{181, 327} A positive control is included as well, FN075, the previously described compound that accelerates the formation of α -Syn amyloid fibres. The lag time is much shorter, 4 h, compared to the background control with only α -Syn.

All compounds not having *p*-nitrophenyl as R^7 substituent of the tricyclic scaffold were inactive (*Figure 3.3*). The compounds that did contain the *p*-nitrophenyl group on the other hand, had effect on the ThT fluorescence intensity in the plateau phase, while not affecting the lag time.

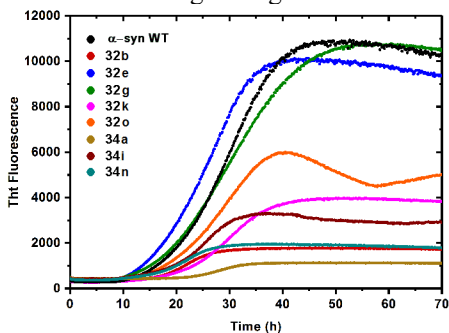
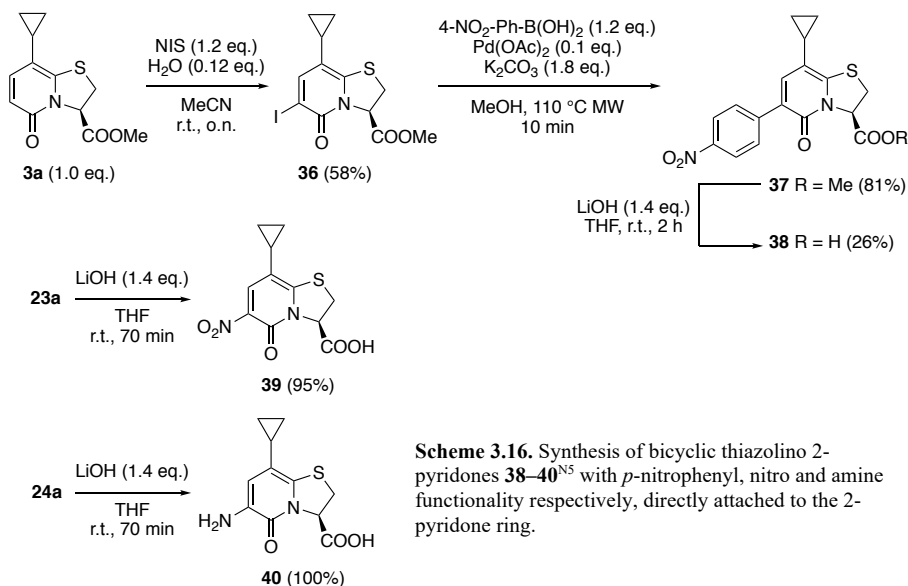


Figure 3.3. ThT traces for a selection of inactive (32e, g) and active (32b, k, o, 34a, i, n) compounds (100 μ M). Each experiment was performed as triplicates and herein represented as average. The active compounds were thought of as inhibitors since we regarded the fluorescence intensity to be proportional to the amount of fibrils present.

Since the active compounds all were equipped with the *p*-nitrophenyl group as R^7 -substituent, we naturally suspected that the activity depended on this functionality. The corresponding bicyclic 2-pyridone **38**, with a *p*-nitrophenyl group attached directly to the pyridone ring was thus prepared (*Scheme 3.16*). In addition, we hydrolysed the intermediate **23a** to make C-6 nitro functionalised compound **39**. We further prepared the aminopyridone **40** from the corresponding intermediate (**24a**).



Scheme 3.16. Synthesis of bicyclic thiazolino 2-pyridones **38–40**^{N5} with *p*-nitrophenyl, nitro and amine functionality respectively, directly attached to the 2-pyridone ring.

These compounds did not display any activity versus α -Syn (*Figure 3.4*), which stressed the importance of the pyridine ring for the observed biological activity.

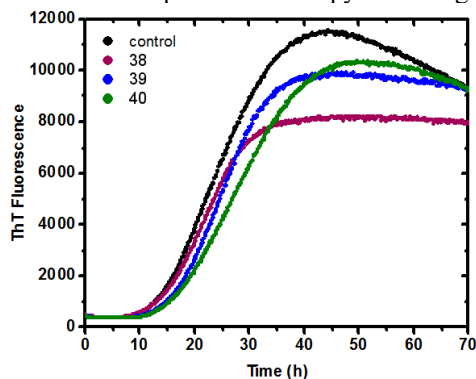
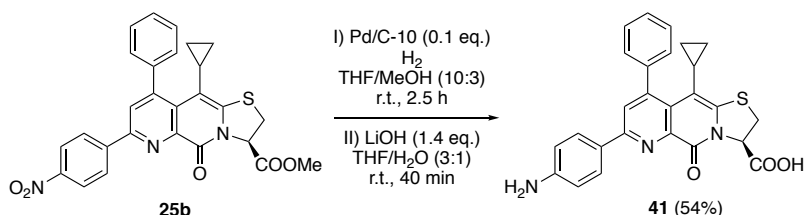


Figure 3.4. The bicyclic thiazolino 2-pyridone **38** equipped with a *p*-NO₂-phenyl group directly on the 2-pyridone ring does not display any significant effect on the ThT-fluorescence intensity, indicating that the pyridine ring is important for activity. Likewise, **39** and **40** with nitro and amine functionalisation is inactive in the assay. Each trace represents the average of three identical experiments.

The nitro functionality is somewhat special compared to other functional groups commonly used in medicinal chemistry, it is rarely encountered in nature and no good isostere is known.³⁸³⁻³⁸⁶ In an effort to begin assessing its role in amyloid binding pyridine fused thiazolino 2-pyridones, the nitro group in compound **25b** was reduced to the corresponding amine in compound **41** (*Scheme 3.17*).^{N7} The reduction resulted in a major loss of activity, indicated by ThT-fluorescence upon fibre formation (*Figure 3.5*, blue trace), compared to the corresponding compound **32b** with a nitro group (yellow trace).



Scheme 3.17. Reduction of the nitro group in compound **25b**, which is crucial for activity of this core scaffold.^{N5}

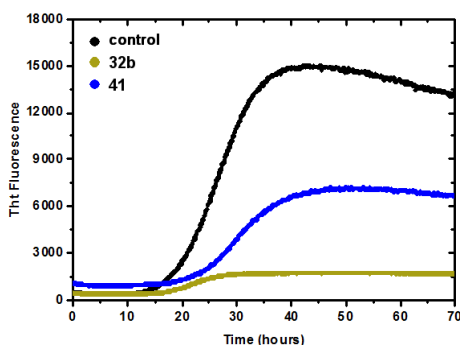


Figure 3.5. ThT fluorescence as a function of time during *in vitro* fibrilization of α -Syn in the absence (black trace) and presence (blue trace) of compound **41**. For comparison, the results with compound **32b** is reproduced (yellow trace). All experiments were performed in triplicates and are herein represented as averages.

The wells where compound **32b**, **f**, **k**, **o**, **p**, **34a**, **i** and **k-o**, were incubated together with α -Syn, showed a significantly lower ThT fluorescence. The degree varied from adequate (*e.g.* **32o**) to excellent (*e.g.* **34a**). These compounds were regarded as "inhibitors" since we thought that they inhibited the formation of amyloid fibrils, indicated by reduced fluorescence. To test this hypothesis, samples were taken from the reaction mixtures of a selection of compounds at the endpoint of the fibrilization experiments. The samples were applied to copper grids and then visualised with TEM (*Figure 3.6*). There were plenty of fibres to be found in the control samples. In the wells where the chaperone CsgC had been present, no fibrillar structures could be visualised. What little that could be seen was small, amorphous, diffuse and scarcely distributed. To our surprise, there were plentiful fibrils to be found in the samples where **34a** and **34n** had been present during fibrilization, despite the low intensities of the fluorescence signals.

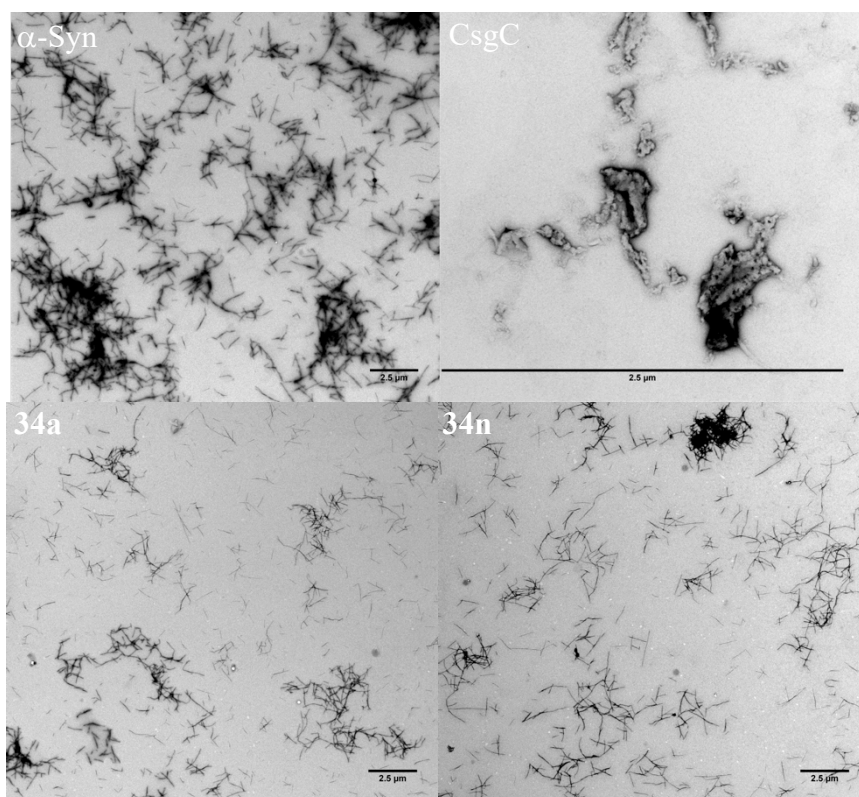


Figure 3.6. TEM pictures of the reaction mixtures from control experiments and selected compounds. CsgC, but not compound **34a** or **34n**, inhibits formation of amyloid like fibrils.

3.5. Amyloid fibril binding

The fibrilization curves (*Figure 3.3*) looked like the fluorescence traces of inhibitors previously published by ourselves and others.^{302, 324} Pre-eminently, fibres were found upon TEM visualisation. Furthermore, differential light scattering (DLS) experiments with compound **34a** did not suggest any modulatory activity and the length of the lag phase also indicates that fibrilization starts in a similar manner as without any compound present. Could the compounds which displayed lower fluorescence intensity in the fibrilization screening, quench the ThT fluorescence somehow? If the 2-pyridones absorbed the exciting light (440 nm) or the emitted light (480 nm), the lower ThT fluorescence could be explained. The "active" compounds were all equipped with a *p*-nitrophenyl substituent and had a characteristic intense yellow colour. Indeed, the absorbance spectrum of the active compound **34a** revealed a significantly higher absorptivity at 440 nm than the inactive **32a** (*Figure 3.7*). However, both control compounds **38** and **39**, which were not active in the ThT assay, had intense yellow colour and displayed similar absorptivities as **34a**.

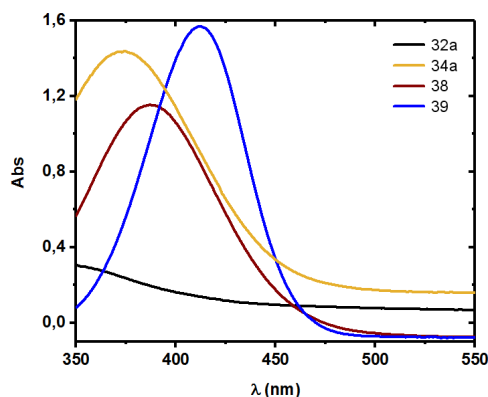


Figure 3.7. Absorbance spectra for compound **32a**, **34a**, **38** and **39**. The latter three compounds, with nitrophenyl or nitro groups, have higher absorptivity around 440 nm (the wavelength used to excite ThT) than **32a**. However, **38** and **39** does not lead to a decreased ThT fluorescence in the *in vitro* assay (Figure 21.6). Thus, incomplete ThT excitation is not a sufficient explanation for the lower fluorescence intensity observed in the presence of **34a**.

Could the compounds bind to the amyloid fibril structure, in a similar way as ThT does, and compete out ThT? This phenomenon has been reported earlier.^{302, 312, 387} To test for binding, the fibrilization experiments were repeated in a modified setup (Figure 3.8). α -Syn was allowed to form amyloid fibrils in the absence of compounds. When the plate had been agitated for 70 h, it was taken out of the plate reader and the compounds were added. Upon resuming the incubation, we noticed how the fluorescence intensities dropped, rather abruptly. Compounds with *p*-NO₂-phenyl groups quenched a significant amount of the ThT-fluorescence, compared to compounds not equipped with *p*-NO₂-phenyl groups.

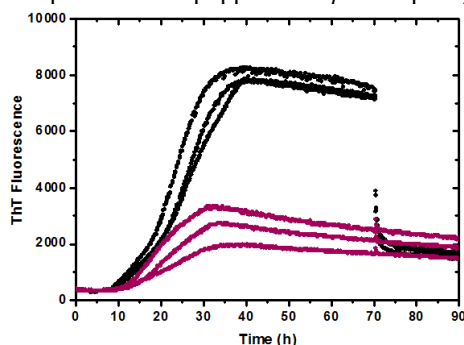


Figure 3.8. ThT fluorescence versus time for fibril formation of α -Syn, in the absence (black) and presence of **32f** (sanguine). After 70 h, when the fibrilization had reached the plateau phase, **32f** was added to the mature fibrils, represented by the black traces. The fluorescence intensity decreased as **32f** bound to the amyloid fibrils and displaced ThT.

Our hypothesis was verified. The compound did indeed displace the bound ThT from the amyloid fibre, it did not inhibit its formation as previously thought. The question immediately rose whether the compounds in our previous publications were merely binding to the fibrils too, instead of preventing α -Syn amyloid fibre formation. Alas, a quick inspection reveals that the amount of fibrils identified by TEM, AFM, CD and DLS is significantly lower for compounds therein called inhibitors,^{249, 324} and so these compounds should still be recognised as inhibitors.

Although our pyridine fused peptidomimetics were intended as inhibitors of fibril formation, amyloid binding is a feature of high scientific relevance, as earlier described. Compounds displaying amyloid binding have been shown to decrease cell toxicity, and further have potential as diagnostic tool compounds (Chapter 1.12). We

hence evaluated all active binders according to the same method as with compound **32f**. The results are displayed as the percentage of fluorescence retained upon addition of each compound to mature α -Syn fibrils, compared to the intensity of the control experiment, after 70 h (Figure 3.9). The compounds were also probed for their binding properties to Amyloid β (A β 40) fibrils in a similar manner.³²⁷ Comparable results were obtained, the compounds that bind α -Syn, also bind to A β 40, with about the same qualitative trends in binding strength. Minor differences in binding ability between different analogues and the different amyloids could be observed, but no selectivity can be claimed at this point.

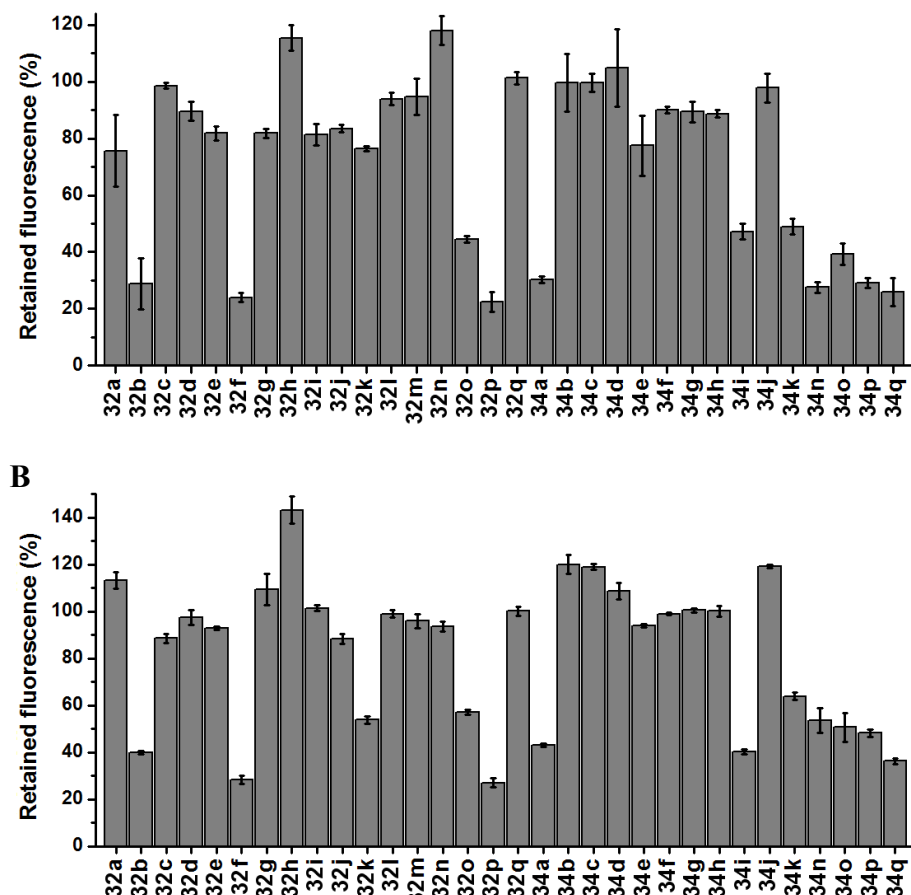


Figure 3.9. Bar chart representation of the retained fluorescence upon addition of each compound to mature α -Syn (A) and A β 40 (B) fibrils. The retained intensities are normalised to the same control experiment (average of triplicates) and the retained intensity for inactive compounds can therefore appear to be >100% in this depiction. Compound **33a–b**, **34l–m** were not included in this study. **B)** ThT was excited with monochromatic light with a wavelength of 430 nm. The fluorescence emission was recorded at 485 nm.

Resembling structure activity relationships were observed for both α -Syn and A β 40 fibrils. With the *p*-NO₂-phenyl R⁷ substituent in place, a methoxy group as R¹

substituent appears unfavourable, evident by lower binding capability compared to compounds with cyclopropyl, phenyl and hydrogen as R¹ substituents.

3.6. Activity of α -Syn binding compounds in cells and mice

Aware of the therapeutic potentials of small molecules that binds to amyloid fibrils (Chapter 1.12), we proceeded with evaluations of the most promising compound (**34a**) in a human neuron cell line (SH-SY5Y). These cells express α -Syn and are susceptible to conditions that trigger its fibrilization.³⁸⁸ Rewardingly, the neuron cells were protected from the toxic effect of FN075 if they were administered with **34a** before FN075 treatment (Figure 3.10).

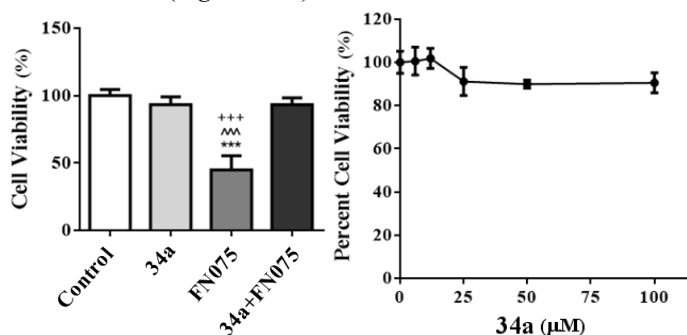


Figure 3.10. A) Survival of SH-SY5Y cells upon treatment with fibrilization accelerator FN075 (50 μ M) and amyloid fibril binder **34a** (25 μ M). MTT cell viability assay, 22 h upon addition of compounds, was used to quantify changes in mitochondrial respiration as an indicator of cell death. Treatment with the binder **34a** alone has no significant effect on MTT cell viability. FN075 treatment causes about 50% reduction in viability. Conversely, cells that were pre-treated with **34a** (25 μ M) 2 h before FN075 (50 μ M) addition, showed no significant drop in viability after 22 h ($F(4,23) = 13.01$, $p < 0.001$). Statistical analysis was conducted using a One-way ANOVA, followed by a Tukey's multiple comparisons *post-hoc* test. Data are presented as means and SEMs. $n = 4-6$, *** $p < 0.001$ vs Control, ^^ $p < 0.001$ vs **34a**, +++ $p < 0.001$ vs **34a**+FN075. **B)** SH-SY5Y cell toxicity evaluation of **34a**. SH-SY5Y cells were treated with **34a** (0–100 μ M) for 24 hours.

We further carried forth with experiments in mice. By inducing the fibrilization of α -Syn, FN075 creates a mouse model for Parkinson's disease. We wanted to see whether the compound **34a** had any effect on the neurotoxicity induced by accelerating α -Syn aggregation with FN075. Wild type mice were injected with compound (or DMSO for control) into the *substantia nigra* 6 months prior to sensorimotor assessment. The evaluations included a "sticky note test" where a small piece of adhesive was fastened on the nose of the mouse.²³⁶ The mouse was subsequently released, and the time taken for the mouse to notice and remove the sticker, was measured (Figure 3.11). This test examines their sense and motoric function. Mice that were injected with compound **34a** was indistinguishable from the control group injected with DMSO. Mice injected with FN075 took longer time to notice and remove their stickers. These results indicated that the mice subjected to FN075 injection were functionally compromised, and was in agreement to previously published data.²³⁶ But mice that were injected with **34a**, 2 weeks prior to FN075 injection, did not develop any symptoms according to this evaluation.

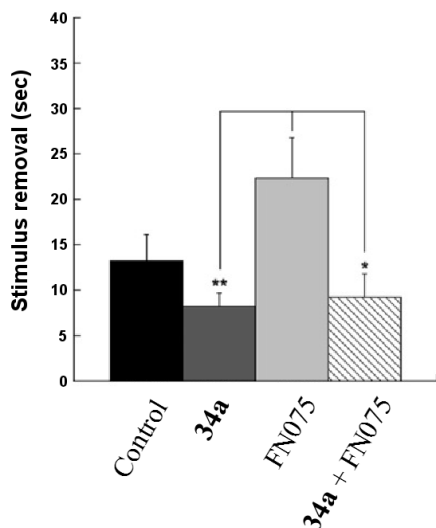


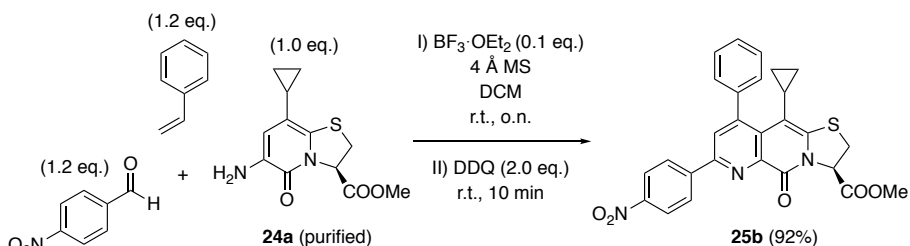
Figure 3.11. Results of adhesive removal test. Mice were injected with substances in the *substantia nigra* (2 μ l of a 1 mM solution in 0.9% NaCl with 10% DMSO) 6 months before evaluation. The control mice were injected with solvent (0.9% NaCl with 10% DMSO). Mice injected with FN075 display a significant motoric dysfunction, unless pre-treated with **34a**. If a mouse did not remove the stimulus, it was removed by the examiner after 60 s.

3.7. Presumptions

There are more than a single hypothesis that may explain the biological functions of pyridine fused thiazolino 2-pyridones. First, although ThT does not produce enhanced fluorescence in the presence of oligomers *in vitro*, there is not ground to exclude the possibility that a compound like **34a**, which binds mature fibrils, can interact with aggregation prone oligomers. And while not able to prevent aggregation *in vitro*, it may interfere with the toxic function of the oligomers *in vivo*. Second, while an increasing amount of evidence supports the theory of oligomers being the principal neurotoxic species, there are also evidence of α -Syn fibrils having a toxic effect *in vivo*.^{220, 250} One hypothesis argue that the fibrils exert their toxic effect by sequestering the cell of vital components,²³⁷ others state that amyloid fibrils catalyse the formation of reactive oxygen species.²³⁸⁻²³⁹ A compound that binds to mature fibrils may prevent such events. Finally, the fibrillar end products are in equilibrium with oligomers and monomers. Small molecule binding could stabilise the fibrils thermodynamically, shifting the equilibrium away from toxic oligomers.^{248, 304}

3.8. Yield improvement of Povarov reactions

A total of 37 pyridine fused thiazolino 2-pyridones were synthesised with the A³ and Povarov reactions under the established conditions. Afterwards it was discovered that the key intermediates, the amino functionalised thiazolino 2-pyridones **24a–d**, were diluted with an unknown contamination that was invisible to TLC, ¹H and ¹³C NMR spectroscopy. As described (*Chapter 3.2*), chromatographic purification transformed the ebony black solid into a light brown one. Povarov reaction with black **24a** supplied products with yields in the range 50–54% with styrene (*Scheme 3.9*). Purified **24a** bestowed a substantial increase in reaction yield (*Scheme 3.18*). All yields up to this point represents use of un-purified amines, while all yields from here and onward, are results with purified amines.



Scheme 3.18. Synthesis of compound **25b** at 0.94 mmol scale from pre-purified **24a**. 2 eq. of DDQ was needed to complete the oxidation of the intermediate adduct.

In addition, the Povarov reaction works excellent with alkynes instead of alkenes.^{350, 360, 364} Replacing styrene with phenyl acetylene in the reaction setup above, furnishes **25b** directly, without need for DDQ-oxidation, in similar reaction time. The formation was accompanied with by-products however, one of them in significant amount.

3.9. Further evolutions – Intramolecular Povarov reactions

Most of the pyridine fused tricyclic compounds made so far relied on the developed method. With the one pot, three component Povarov reaction, any of the compounds **32–34** (scaffold **20**) could be produced with ease from thiazoline fused 2-pyridones **24** and various aldehydes and alkene components (*Figure 3.12*). The method enabled rapid construction of the desired tricyclic scaffold with multiple points of variation, allowing installation of different functionalities R^1 , R^5 , R^6 and R^7 in a single synthetic operation.

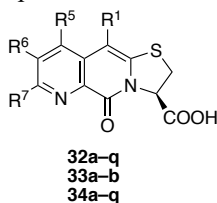
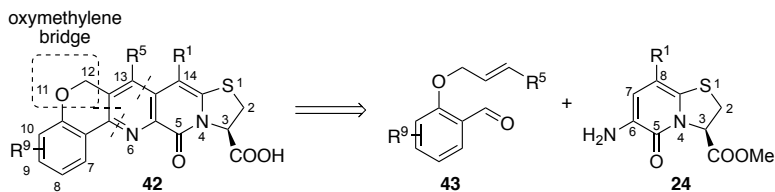


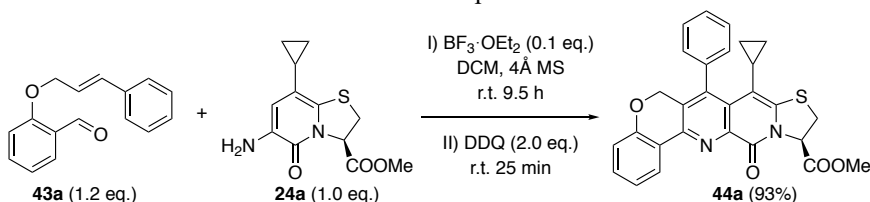
Figure 3.12. Pyridine fused thiazolino 2-pyridone scaffold **20**, with four variable substituents.

Aware of the possibility to perform the three component Povarov reaction in an intramolecular fashion, where two of the reacting components are tethered in one molecule, we envisioned a new, modified scaffold **42** with an oxymethylene link between the R^7 aryl substituent and the pyridine ring (*Scheme 3.19*).^{364, 389–394} The ether linkage was hypothesised to increase aqueous solubility by increased hydrogen bonding, a feature that could also confer selectivity between different fibril structures. The sp^3 -hybridised methylene carbon moreover decreases the planarity of the structures, which also have the potential to increase selectivity between different targets, and alter solubility.³⁹⁵ The new scaffold is hence expected to have improved properties.



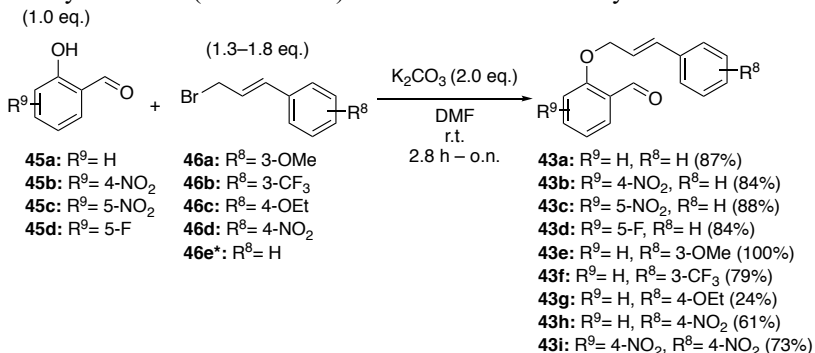
Scheme 3.19. Retrosynthetic strategy devised for construction of a new scaffold with improved pharmacological properties, from thiazoline fused amino 2-pyridone **24** and alkylated salicylaldehyde **43**. The numbering system used on bicyclic thiazolino 2-pyridones is applied on the oxymethylene linked and pyridine fused scaffold **42** as shown.

We perceived that the desired ether linked scaffold **42** would be accessible from amino-2-pyridone **24** and *O*-alkylated salicylaldehyde **43**. We began our work by investigating the feasibility of the reaction between **24a** and *O*-cinnamyl salicylaldehyde **43a** in an intramolecular Povarov setup with boron trifluoride catalysis, according to our established procedure (Scheme 3.20). The starting materials were dissolved in DCM at room temp. To the solution was added $\text{BF}_3 \cdot \text{OEt}_2$.

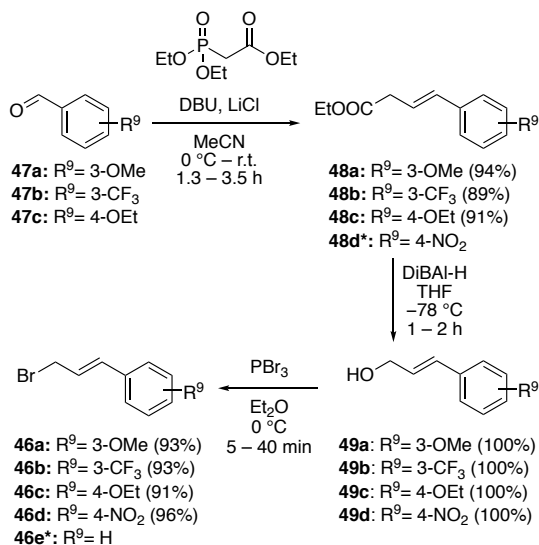


Scheme 3.20. Synthesis of ether bridged, pyridine fused structure **44a** via Lewis acid catalysed intramolecular Povarov reaction of *O*-cinnamyl salicylaldehyde **43a** and amino 2-pyridone **24a**.

We were delighted by the success of the reaction, which delivered the desired product **44a** in excellent yield after purification. To explore the scope of the reaction, we prepared a set of *O*-alkylated salicylaldehydes³⁹⁶⁻³⁹⁸ **43b–i** with different substitution on the salicylaldehyde and cinnamyl rings (Scheme 3.21). The substituted cinnamyl moiety was incorporated through alkylation of salicylaldehyde **45a–d** with the corresponding cinnamyl bromide **46a–e**, prepared in three steps from the benzaldehydes **47a–c** (Scheme 3.22) or obtained commercially.³⁹⁹⁻⁴⁰¹

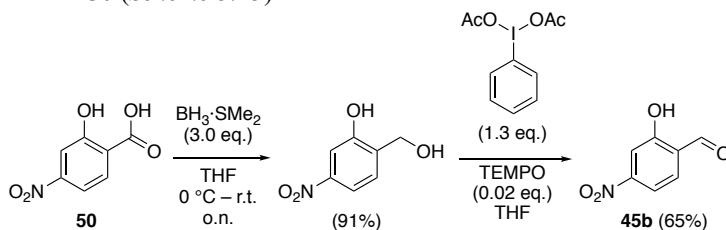


Scheme 3.21. Synthesis of *O*-alkylated salicylaldehydes **43a–i** with various substitution. *This reactant was obtained commercially.



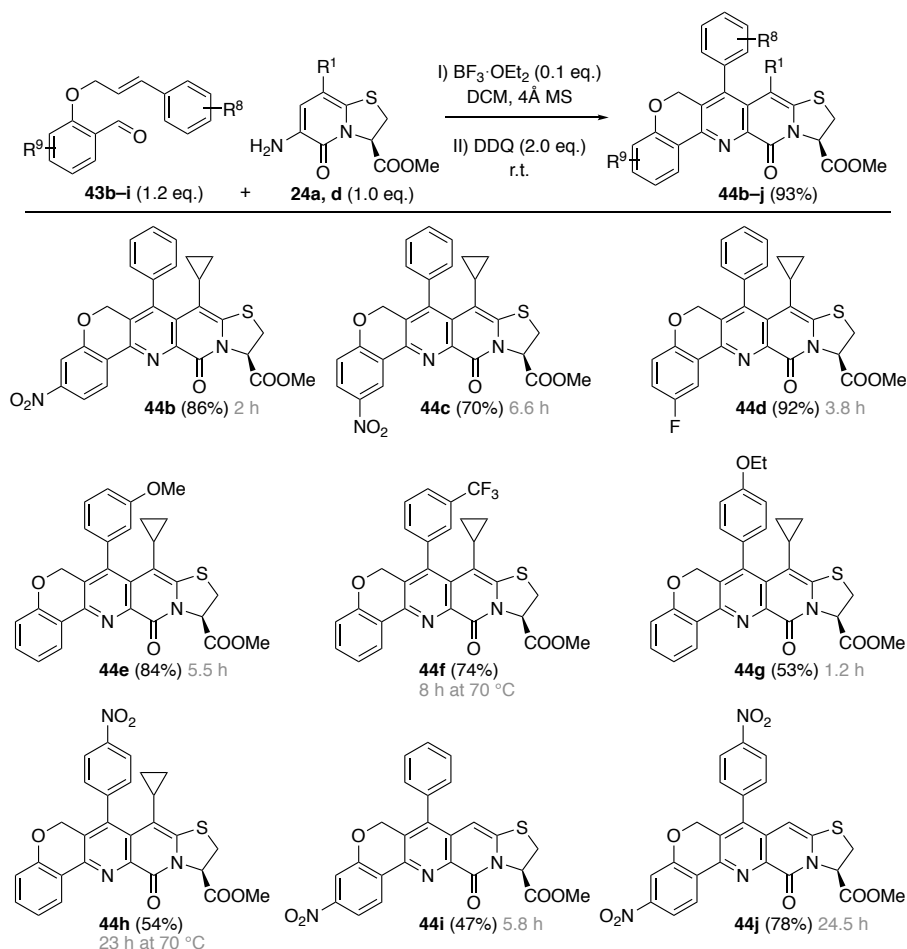
Scheme 3.22. Synthesis of cinnamyl bromides **46a–d** from the corresponding aldehydes via Horner-Wadsworth-Emmons olefination, reduction and bromination. Olefination (10–15 mmol scale): LiCl (1.2 eq.), DBU (1.2 eq.) Triethylphosphonoacetate (1.2 eq.). Reduction (8.5–10.5 mmol scale): DiBAL-H (2.4 eq.). Bromination (4–6 mmol scale): PBr₃ (1.0 eq.) *This reactant was obtained commercially.

Also the 4-nitrosalicylaldehyde **45b** was prepared,⁴⁰² from the corresponding carboxylic acid **50** (Scheme 3.23).



Scheme 3.23. Intermediate **45b** was synthesised by reduction of carboxylic acid **50**, and subsequent oxidation of the alcohol functionality to aldehyde.

With the required components in hand we constructed a small squadron of analogues **44b–j** (Scheme 3.24) to be subject of biological evaluation, upon later deprotection. The method worked for all tested combinations of components. Aware of the importance of the 4-nitrophenyl substituent in scaffold **20**, we continued our efforts with *O*-cinnamyl-4-nitrosalicylaldehyde **43b**. An instant colour change and an expeditious formation of a precipitate upon addition of the Lewis acid indicated rapid formation of the intermediate imine. The reaction mixture initially got so thick so that the magnetic stirring was compromised and the reaction tube had to be shaken. The viscosity gradually decreased, the magnetic stirring could be continued after a while, to eventually become a clear solution. In just 2 h stirring at room temp., TLC-analysis showed reaction completion, whereupon DDQ was added to oxidise the intermediate adducts. **44b** was then isolated in excellent yield. The short reaction time compared to the formation of **44a** parallels the lowering of LUMO of the electrophilic imine, by the electron withdrawing nitro group, which is in conjugation with the imine.^{371, 403-405} Moving the nitro group one step, into *meta* position relative to the aldehyde group, renders the Povarov reaction to **44c** slower (6.6 h) but still faster than for **44a** (9.5 h).

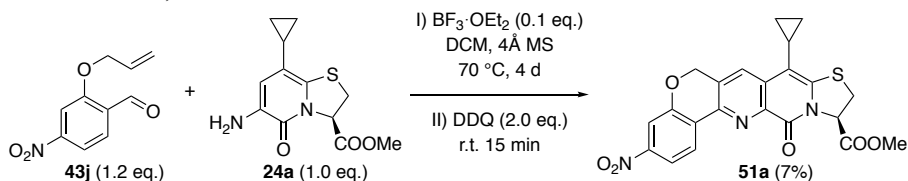


Scheme 3.24. Preparation of compounds **44b–j** through intramolecular Povarov reaction between *O*-alkylated salicylaldehydes **43a–i** and amino 2-pyridones **24a, d**.

The same qualitative trend holds for the fluoro substituent, compound **44d**. Supplying the cinnamyl ring with an electron donating R^8 substituent in *meta* position, compound **44e**, also increases the rate of the Povarov reaction, by raising HOMO and increasing nucleophilicity of the alkene's β -carbon in the cinnamyl moiety. An electron withdrawing trifluoromethyl group as R^8 substituent, compound **44f**, instead works to slow down the Povarov reaction by making the alkene less nucleophilic towards the 2-aza diene. Only minor amounts of adduct was observed after 18 h stirring at room temperature. By raising the reaction temperature to 70 °C, the reaction rate was increased and consumption of the limiting reactant **24a** was evident by TLC after 8 h (amine as well as imine intermediate was consumed). Moving the R^8 substituents to *para* position relative to the alkene, increases their influence by enabling mesomeric contributions. When 4'-ethoxy-substituted *O*-cinnamyl salicylaldehyde **43g** was mixed with amino 2-pyridone **24a** in the intramolecular Povarov reaction, completion

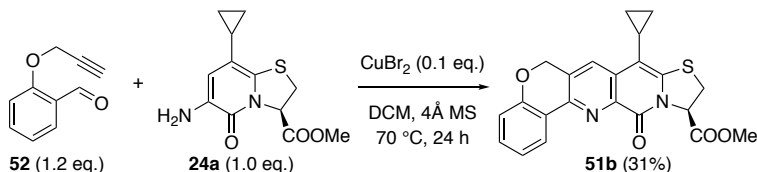
was suggested by TLC analysis after just 1.2 h. Product **44g** was isolated in moderate yield upon oxidation and purification. The reason for significantly lower yield compared to preceding examples seems to be breakdown on silica. A strongly electron withdrawing nitro group as R⁸ substituent increased the reaction time to 23 h at 70 °C. The conversion was less clean and **44h** was isolated in moderate yield after oxidation and purification. The reaction also worked for C-8 unsubstituted amino 2-pyridone **24d** to give **44i–j** in good yields. The LUMO lowering effect of the R⁹ nitro group appears to compensate for some of the R⁸ nitro group's HOMO lowering in **43i**, as the Povarov reaction towards **44j** was complete after 24.5 h at room temperature.

Consolidated by the fruitful outcome so far, we then turned our efforts towards synthesis of R⁵ unsubstituted target molecules **51**. The SAR from the recent study on pyridine fused 2-pyridones indicated that R⁵ unsubstituted analogues had the best α -Syn binding properties. It was approached by Povarov reaction between **24a** and *O*-allyl salicylic aldehyde **43j** (Scheme 3.25). To our dismay, we were only able to isolate petty amounts (7%) of the desired product, even after several days at 70 °C, from the complex reaction mixture. Microwave irradiation, 120 °C for 3 h, shared the same lack of success, **51a** was isolated in 11%.



Scheme 3.25. Unsuccessful attempt to synthesise R⁵ unsubstituted compound **51a** from *O*-allyl 4-nitrosalicylaldehyde **43j**.

We then performed a catalyst and condition screen, trying several Lewis and Brønsted acids [TFA , SnCl_4 , TiCl_4 , FeCl_3 , $\text{Y}(\text{OTf})_3$, $\text{Yb}(\text{OTf})_3$, $\text{La}(\text{OTf})_3$, $\text{Dy}(\text{OTf})_3$, CuCl_2 , $\text{Cu}(\text{OAc})_2$, $\text{Cu}(\text{OTf})_2$, $\text{Cu}(\text{II})\text{TMEDA}$, CuI and CuBr_2], and solvents (DCM, THF and MeCN). The best results were obtained with CuBr_2 and *O*-propargyl salicylaldehyde **52** in DCM at elevated temperatures (Scheme 3.26). **51b** was furnished without the need for auxiliary oxidant, albeit only in 31% and with traces of impurities.

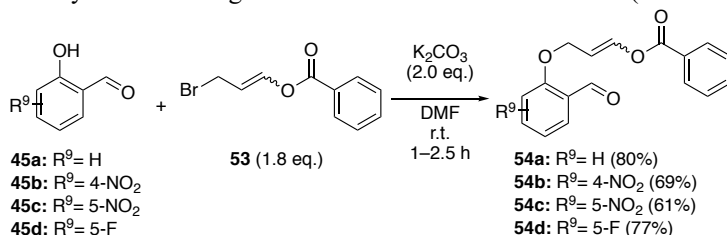


Scheme 3.26. Somewhat successful synthesis of **51b** with *O*-propargyl salicylaldehyde under altered conditions.

Raising the temperature or increasing the catalyst load increased the amounts of by-products even further. At 50 °C, the reaction proceeded much slower, without providing cleaner conversion. Decreasing the catalyst load slowed down the reaction,

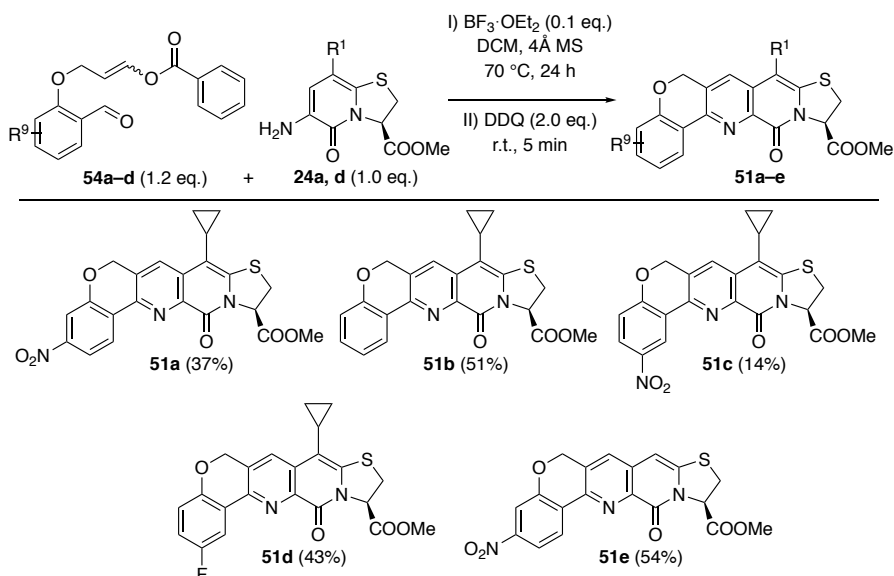
which was far from completion after two days. The method in *Scheme 3.26* was applied for synthesis of **51a**, but failed to provide pure product. The yield was <18%.

With poor yields and complex reaction mixtures, from which isolation of the desired compounds proved challenging, we started to consider other alternatives. Aware of the mechanistic features of the Lewis acid catalysed Povarov reaction, we realised that the use of allyl or propargyl moieties as alkene components would require the reaction to go *via* high energy carbocations, primary and vinyl carbocations respectively, or through a different mechanism.^{360, 389-390, 392, 406-407} Published efforts with terminal alkenes and alkynes in the intramolecular Povarov reaction are often lower yielding as well,^{357, 389, 403-404} although there are fruitful examples too.³⁹¹ With our previous strives in mind, where we had successfully made use of ethyl vinyl ether as alkene component, for the synthesis of R⁵ unsubstituted tricyclic analogues **34a–q**, we naturally thought of employing a similar vinyl moiety as electron donating auxiliary. With 3-bromopropenyl benzoate **53**⁴⁰⁸⁻⁴¹⁰ we managed to *O*-alkylate the salicylic aldehydes **45a–d** to give the desired intermediates **54a–d** (*Scheme 3.27*).



Scheme 3.27. Synthesis of the *O*-propenyl benzoate salicylaldehydes **54a–d** by alkylation of the corresponding salicylaldehydes **45a–d** with 3-bromopropenyl benzoate **53**.

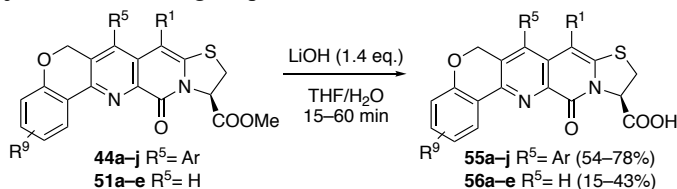
With the alkene component now armed with an electron donating auxiliary, capable of mesomeric contributions, we expected expeditious Povarov reactions between **54a–d** and **24a, d**, but heating the reaction mixtures to 70 °C was required to achieve synthetically useful reaction times (*Scheme 3.28*). Although the yields were modest, with this method we were able to synthesise and isolate the desired compounds **51a–e** pure after 24 h reaction time, followed by oxidation with DDQ at room temp. The low yield of **51c** can be explained by competing side reactions and a complicated purification.



Scheme 3.28. Synthesis of the R^5 unsubstituted analogues **51a-e** using *O*-propenyl benzoate alkylated salicylaldehydes according to established procedure at 70 °C.

3.10. Outcome – An improved scaffold

With the two final sets of compounds in hand, we hydrolysed the methyl ester to deprotect the carboxylic acid (*Scheme 3.29*). **44a-j** and **51a-e** was converted to **55a-j** and **56a-e** through saponification.



Scheme 3.29. Basic hydrolysis of methyl esters to reveal the final active compounds **55a-j** and **56a-e**.

The carboxylic acids **55a-j** and **56a-e** were initially screened in a α -Syn fibrilization assay to detect fibril binding compounds and any amyloid modulating properties (*Figure 3.13*). The complete data from the biological evaluation of compounds **55** and **56** can be found in the supporting information of *article III*. To be certain that fibrils were formed, and the low ThT emission reflected effective competition by the compounds against ThT for binding, rather than inhibition of fibril formation, a sample was taken from the mixture with **56a** (green trace) and visualised with TEM. Fibrils were indeed found.

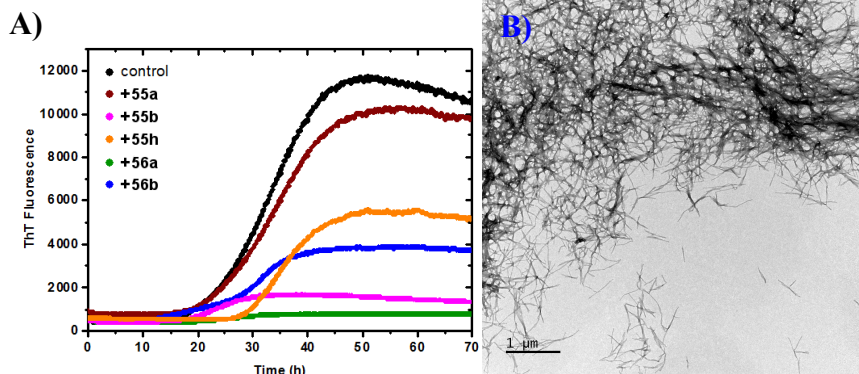


Figure 3.13. A) Representative selection of ThT fluorescence traces. Each compound was evaluated in triplicates and normalised to the average. Compound **55b** and **56a** appears to bind fibrils strongly, while **55a** does not appear to bind to any significant extent. **55h** and **56c** are borderline. B) TEM image of α -Syn fibrils formed in the presence of **56a** (green trace).

The compounds were also screened for A β 40 binding in an equivalent assay (Figure 3.14). The same analogues that bound α -Syn, also binds A β 40 fibrils.

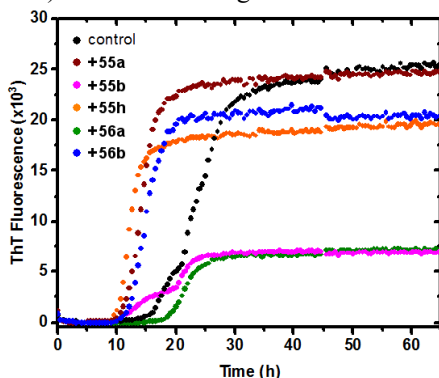


Figure 3.14. Compounds **55a**, **b**, **h** and **56a**, **c** probed for A β 40 fibril binding and modulation of fibril formation. The same qualitative trends were observed as for α -Syn. The full dataset can be found in the supporting information of *article III*. Compound (20 μ M), A β 40 monomers (5 μ M) and ThT (40 μ M) were incubated in PBS buffer (pH 7.4) with DMSO (1%) at 37 $^{\circ}$ C. The plate was agitated only briefly (3 s) before each measurement (every 30 min). The shoulders on the black and pink curves is due to differences in lag time between the three replicates.

The compounds which did not seem to bind fibrils to any significant extent were not evaluated further. All compounds equipped with a nitro group bound to A β 40 fibrils. All of them except **55c** also bound α -Syn fibrils with significant strength. Compounds **55b**, **c**, **h–j** and **56a**, **c**, **e** had a significant effect on the fluorescence intensity, compared to the control experiments, and were investigated further. The compounds were added after 40 h (A β 40) or 70 h (α -Syn), when the ThT fluorescence trace had reached the plateau phase (Figure 3.15). The retained fluorescence is represented in the bar charts (Figure 3.16). Most compounds appear to bind both α -Syn and A β 40 with approximately the same qualitative trends. It is not appropriate to conclude any selectivity at this juncture, since the ratios between compound, ThT and protein monomers differs between the α -Syn and A β assays.

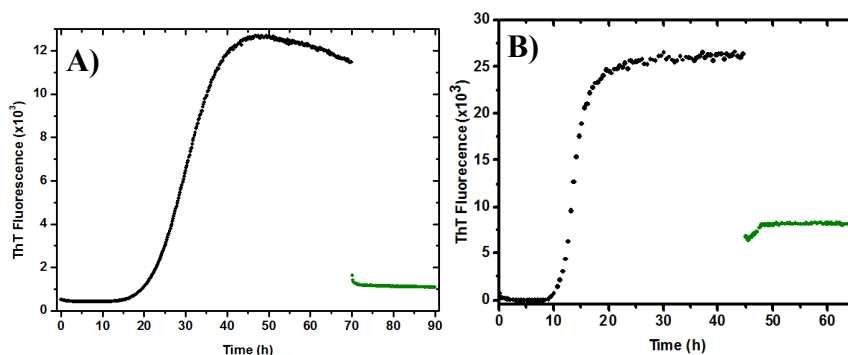


Figure 3.15. Addition of compounds to mature fibrils demonstrates that the compounds bind to the amyloid structure and displaces ThT, which leads to a decrease in fluorescence. Compounds were added to the mixtures when complete amyloid formation was indicated by a steady plateau of ThT fluorescence. All experiments were performed in triplicates. **A)** ThT trace for compound **56a** added to α -Syn fibrils after 70 h. **B)** **56a** added to A β 40 fibrils after 45 h. The full dataset can be found in of *article III* (SI).

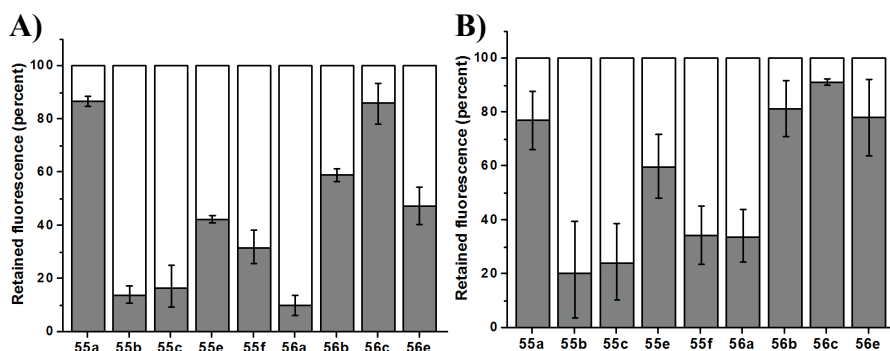


Figure 3.16. Bar chart representation of retained ThT fluorescence after addition of compounds to mature fibrils, compared to the fluorescence intensity in the same wells, 1 h before compound addition. **A)** Compounds added to ThT-bound α -Syn. Bars represent the intensity 5 h after addition **B)** Compounds added to ThT-bound A β 40. Bars represent intensity 15 h after compound addition.

Conclusively, **56a** is the best α -Syn binder among the analogues of the oxymethylene bridged scaffold **42**. The equivalent compound in scaffold **20** without oxymethylene bridge, **34a**, was also the best α -Syn binder in that library (*Figure 3.17*). However, the introduction of the oxymethylene linkage resulted in a minor improvement in α -Syn binding (**55b** and **56a** vs. **32b** and **34a**, *Figure 3.16 and 3.9*)

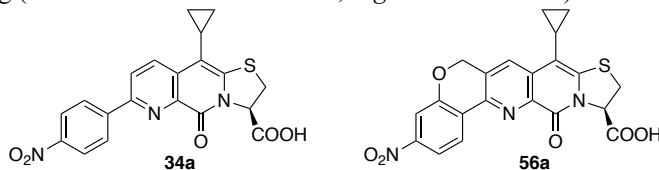


Figure 3.17. The best compounds in both the non-linked and oxymethylene bridged scaffold was otherwise decorated identically, with $R^1 = cPr$, $R^5 = H$ and $R^7 = p\text{-NO}_2\text{-Ph}$.

3.11. Pyrimidine fused homologs

Because of the promising results obtained with the pyridine fused thiazolino 2-pyridones **32** and **34**, the pyrimidine fused homologs **57** and **58** were kindly designed and synthesised by a co-worker in our group (Figure 3.18). The pyrimidine ring is a moiety found in many pharmaceutically active compounds,⁴¹¹⁻⁴¹⁵ including A β aggregation inhibitors,⁴¹⁶ as well as in nucleotide bases. Given the properties of pyridine fused thiazolino 2-pyridones described above, we naturally hypothesised that the pyrimidine fused analogues would have similar properties. We moreover wondered how the introduction of the extra nitrogen would affect properties such as solubility, and perhaps selectivity for different amyloid structures. The route to pyrimidine fused thiazolino 2-pyridones is notably different from the preparation of pyridine fused compounds. The synthesis is described in the attached *article VI* and will not be subject of discussion in this thesis.

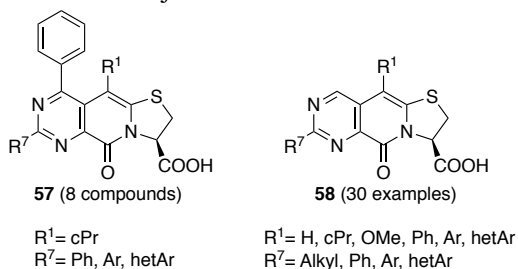
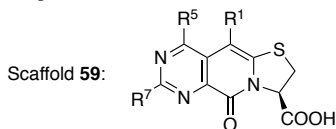


Figure 3.18. General structure of pyrimidine fused thiazolino 2-pyridones **57** and **58**.

In broad strokes, the substituent scope of **57** and **58** is analogous to **32** and **34**. Moreover, the biological activity of the compounds and the SAR:s essentially superimpose, but the pyrimidine fused compounds are somewhat less active than the pyridine fused predecessors. A representative selection of compounds (**57a–c** and **58a–e**) is presented below (Table 3.1, Figure 3.19–3.20). The 4-nitrophenyl group as R⁷ substituent is again important for binding activity and best results are obtained with cyclopropyl as R¹ substituent, but other substituents are tolerated. Notably, the R⁵ phenyl substituent imbues the pyrimidine fused compounds with *better* α -Syn binding ability compared to having the position unsubstituted (**57a** vs. **58a**) (Figure 3.19B). Pyridine fused compounds displayed the *opposite* relationship (**32b** vs. **34a**). Interestingly, **57b** and **c** with 2-naphthyl and methylenedioxyphenyl groups as R⁷ substituent, instead of the *p*-NO₂-phenyl group, is as good as **58e**, the weakest α -Syn binder with *p*-NO₂-phenyl group as R⁷ substituent. The most striking feature however, is that **57b** seems to inhibit A β fibrilization completely, throughout the duration of the experiments (Figure 3.20A), thereby surpassing FN075 in inhibitory efficiency (Figure 1.19B). However, the data is preliminary at the time of writing and more detailed experiments to investigate the inhibitory properties of this compound is underway.

Table 3.1. Structures of selected compounds **57** and **58**.



Compound	R ¹	R ⁵	R ⁷
57a	cPr	Ph	<i>p</i> -NO ₂ -Ph
57b	cPr	Ph	2-naphthyl
57c	cPr	Ph	3,4-methylenedioxyphenyl
58a	cPr	H	<i>p</i> -NO ₂ -Ph
58b	cPr	H	<i>p</i> -CH ₃ -Ph
58c	<i>p</i> -F-Ph	H	<i>p</i> -NO ₂ -Ph
58d	OMe	H	<i>p</i> -NO ₂ -Ph
58e	H	H	<i>p</i> -NO ₂ -Ph

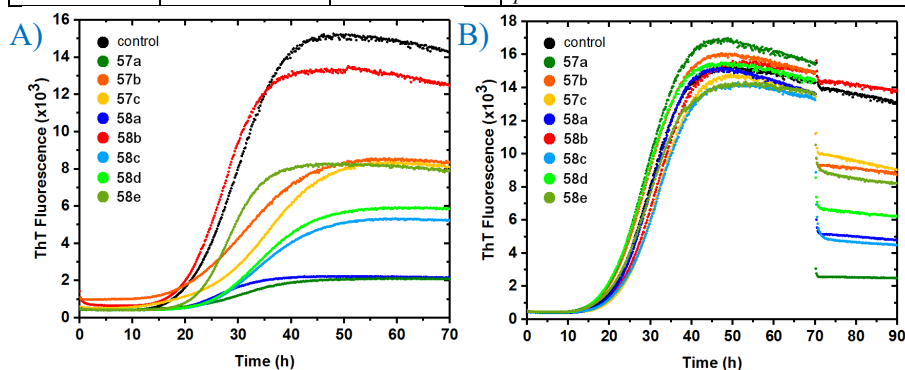


Figure 3.19. Evaluation of selected compounds (**57a–c** and **58a–e**) for α -Syn fibril binding and fibrilization modulation. **A)** Compounds were added at 0 h. **B)** Compounds were added after 70 h. The assays were performed as described in Figure 3.2., 3.3. and 3.8., and traces represent averages of triplicate experiments.

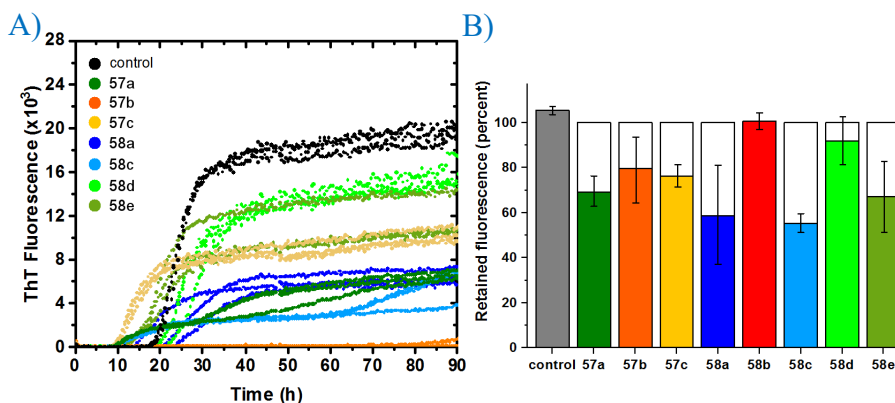


Figure 3.20. Evaluation of **57a–c** and **58a–e** against A β 40. **A)** A β 40 fibrilization assay, compounds were included from start. Each compound was analysed twice, and each experiment is represented individually with a ThT fluorescence trace. **B)** Bar chart of retained ThT fluorescence upon addition of compounds to mature A β 40 fibrils (49 h).

3.12. Ames test – Mutagenicity of multi ring fused compounds

Unfortunately, many nitroaryl compounds are mutagenic and this motif is thus often avoided in drug development, although there are many exceptions.³⁸³⁻³⁸⁶ One way to assess these features is the Ames test for mutagenicity in bacteria.⁴¹⁷ Bacterial strains with mutations in a gene needed for the biosynthesis of histidine is grown in a medium containing the suspected mutagen. Often are several bacterial strains with different mutations, such as critical point mutations or frame shifts, employed. Enzymes extracted from liver are also included, to screen any mutagenic metabolites of the respective compounds as well. If a compound or its metabolites are able to induce mutations that restores the histidine biosynthesis function, the bacteria is able to grow in media lacking this amino acid. A compound is regarded as mutagenic if it significantly increases the number of colonies that can grow in a medium lacking histidine. Obviously, mutagenicity is not a desired property of potential therapeutic drug candidates. Compound **57a** and **58a** were evaluated in an Ames test and found to be mutagenic (*Figure 3.21*). Rewardingly though, compound **57b** without nitro groups, but with potent activity as A β aggregation inhibitor, was not mutagenic.

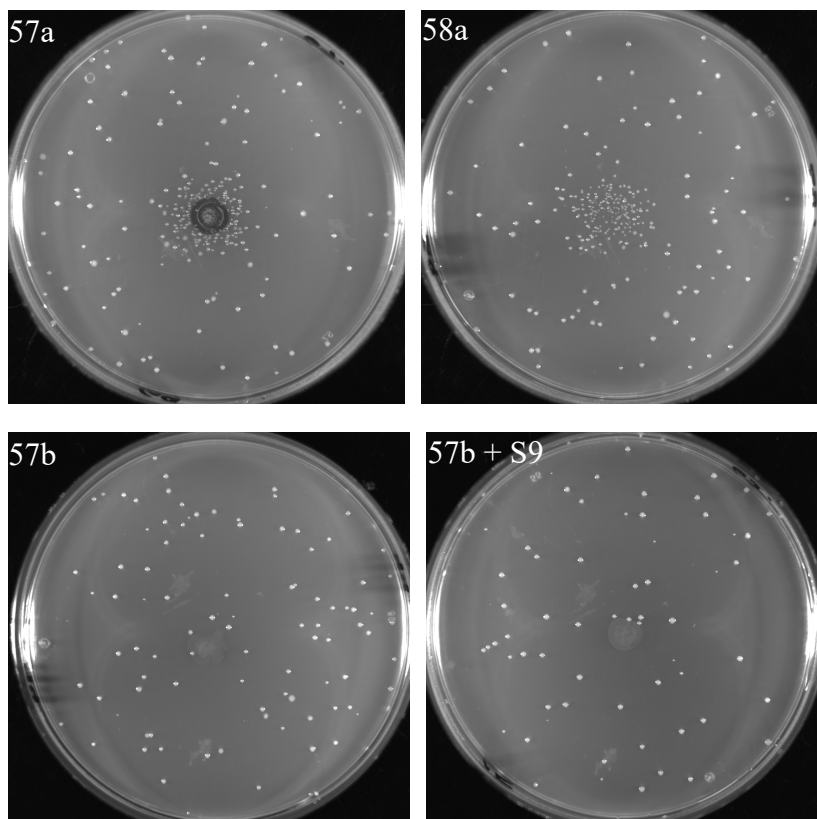


Figure 3.21. Evaluation of mutagenicity with Ames test in *Salmonella typhimurium* strain TA100. Both **57a** and **57b** show mutagenic potential, seen as an increased number of revertants near the center of the Petri plates, where the compounds were added. **57b** does not show any mutagenicity however, neither alone, nor in combination of S9 liver enzyme extract.

3.13. Conclusion

To conclude this chapter, the pyridine fused thiazolino 2-pyridones were designed to modulate α -Syn fibril formation but were instead found to bind mature α -Syn and A β 40 fibrils. Introduction of the oxymethylene linkage slightly improved the binding ability. Addition of the extra nitrogen in pyrimidine fused thiazolino 2-pyridones is associated with a minor loss of binding strength, but one compound is an efficient inhibitor of A β 40 fibrilization. The corresponding pyridine fused homolog, equipped with this substitution pattern (Article IV) displays the same strong inhibitory activity in the initial evaluation. This inhibitory activity is very inspiring as the preliminary data acquired so far indicates that it is stronger than FN075. And while FN075 is neurotoxic (initiates aggregation of α -Syn), **57b** does not appear to affect α -Syn fibril formation and is moreover not flagged as a potential genotoxic compound. Further investigations are needed to assess the potentials of this and structurally related compounds.

4. Ring opening of thiazoline fused 2-pyridone peptidomimetics

Articles II, IV and V

4.1. Preface

From the concluding remarks of chapter 3, we can notice that extension of the 2-pyridone ring with nitrogen heterocycles, and variation of substituents on the resulting scaffolds, have been investigated (Figure 4.1). Conversely, the thiazoline ring has remained un-derivatised. It was thus natural to now contemplate modifications in this region of the molecules. Ideally, modifications that could be carried out at a late stage, on already synthesised structures. And so it was, as benzyne was taken into consideration.

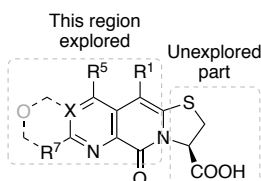


Figure 4.1. Overview of the pyridine/pyrimidine fused thiazolino 2-pyridone structure. While the "left" part (green dotted area) is well explored in the previous chapter, the "right" region of the scaffolds, the thiazoline ring (red dotted area), has remained a constant part of the molecule.

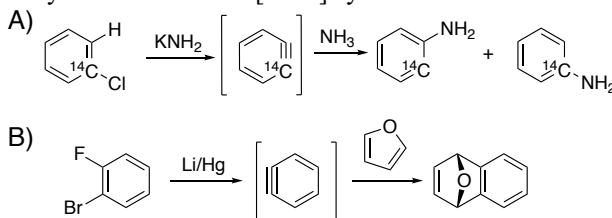
4.2. Benzyne, a highly reactive reagent

o-Benzyne **60**, often called just *benzyne*, is a dehydrogenated analogue of benzene, where one of the double bonds is replaced by a triple bond (Figure 4.2). While conventional alkynes are straight, the triple bond in benzyne (their general name is aryne) is bent and has about 63 kJ/mol of angle strain.⁴¹⁸ Thus, arynes have a low lying LUMO and a tight HOMO-LUMO band gap.⁴¹⁹ Consequently, arynes are excellent electrophiles and readily react in cycloaddition reactions. As a matter of fact, arynes are transient species and has to be generated *in situ*.⁴²⁰⁻⁴²¹



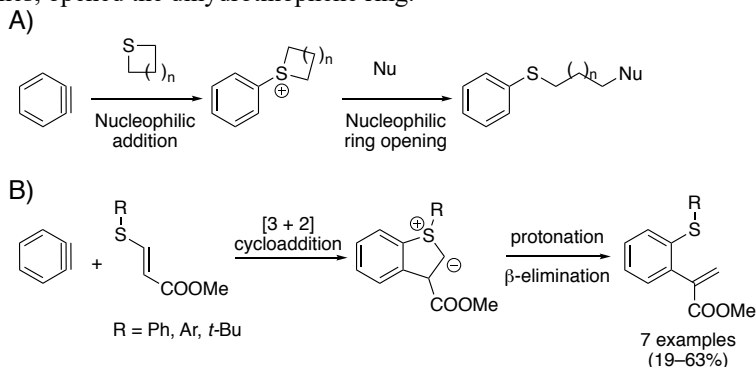
Figure 4.2. *o*-Benzyne, the simplest aryne. Their strained nature makes them highly reactive.

Arynes were postulated as reaction intermediates already in 1902.⁴²² More decisive evidence for their existence came from isotopic labelling experiments done by Roberts *et al.* in the 1950's (Scheme 4.1),⁴²³ and when Wittig and co-workers trapped benzyne with furan in a [4 + 2] cycloaddition.⁴²⁴



Scheme 4.1. Early evidence supporting the existence of benzyne as a reactive intermediate. **A)** Isotope labelled experiments with chlorobenzene and potassium amide. **B)** Trapping of benzyne by cycloaddition with furan.

Both pericyclic reactions with, and nucleophilic addition to, arynes are well established in the literature. Even poor nucleophiles such as sulphides reacts readily with arynes. Reactions between thioethers and arynes were first described by Mertz and others in the 1960's⁴²⁵⁻⁴²⁶ and has since been explored extensively in other laboratories.⁴²⁷⁻⁴³⁹ Of particular interest to us was the ring opening of cyclic sulphides reported by Hoye, Tan and Xu (*Scheme 4.2A*).⁴⁴⁰⁻⁴⁴³ We also noticed Studer's work on [3 + 2] cycloadditions between vinyl sulphides and benzyne (*Scheme 4.2B*).⁴⁴⁴ β -elimination of the adducts, which are structurally related to thiazoline fused 2-pyridones, opened the dihydrothiophene ring.



Scheme 4.2. **A)** Thioether ring opening can be triggered by nucleophilic attack on arynes. **B)** β -elimination of the benzannulated dihydrothiophene, generated upon [3 + 2] cycloaddition between benzyne and vinyl sulphides, provides *o*-vinyl substituted phenyl sulphides.

Given that our model compounds contains both a thioether and a diene system⁴⁴⁵⁻⁴⁵⁰ (*Figure 4.3*), it was of unsought interest to investigate their reactivity with arynes.^{418, 451}

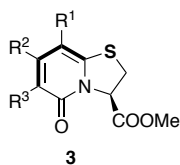
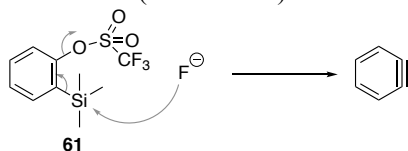


Figure 4.3. The thiazolino fused 2-pyridones contains both a diene system and a sulphide functionality. Both these motifs show reactivity with arynes.

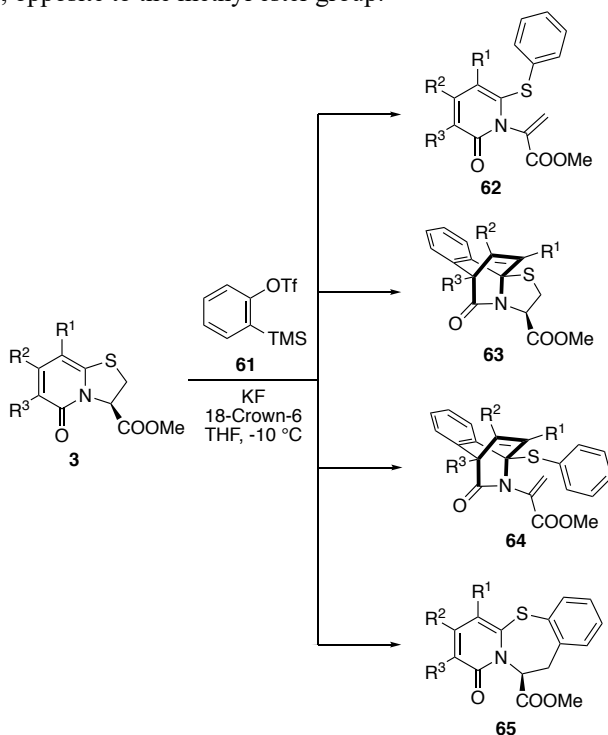
4.3. The eve of strife – Reactions of thiazolino 2-pyridones with benzyne

The first challenge to overcome was the generation of benzyne *in situ*. A multitude of methods to achieve this has been developed. Among the safer and milder ways is the treatment of 2-(trimethylsilyl) phenyl triflate **61** with fluoride (*Scheme 4.3*).⁴⁵²⁻⁴⁵⁴ Initial trials demonstrated that benzyne was conveniently generated at a steady rate from this commercially available precursor, in solution with potassium fluoride and crown ether (18-Crown-6) at sub-ambient temperatures.



Scheme 4.3. Benzyne can be generated from 2-(trimethylsilyl)-phenyl trifluoromethane sulfonate **61** by reaction with a fluoride salt in solution.

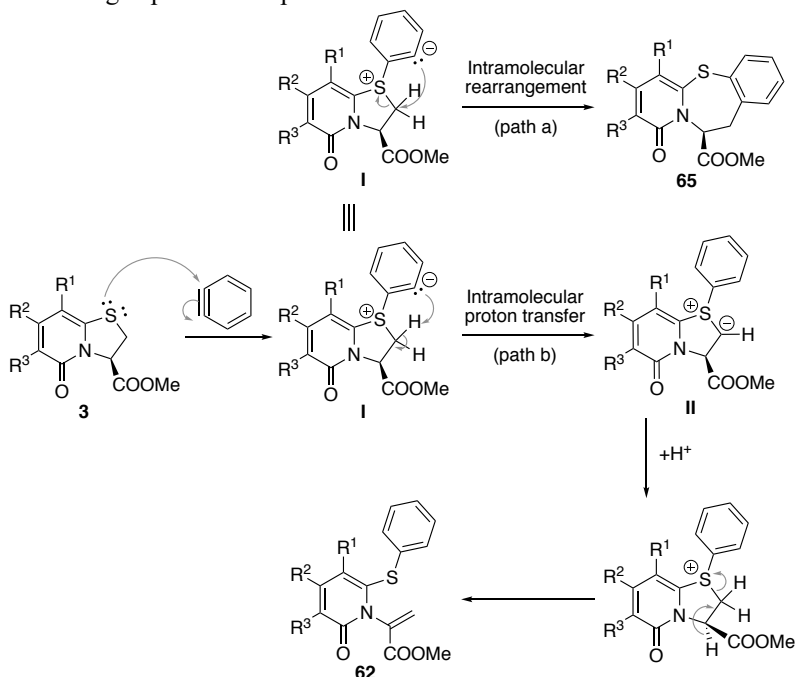
The next thing to explore was how benzyne reacted with thiazoline fused 2-pyridones **3**. As speculated, the reactions often afforded a mixture of the thiazoline ring opened products **62** and the [4 + 2] cycloaddition products **63** (Scheme 4.4). The electronic nature of the substituents R^1 – R^3 influenced the reactivity of **3a**, **c–f**, and the product ratio. Generally, the thiazoline ring opening by thioether attack on benzyne was favoured, but by strategic choice of substituents and altering of the reaction conditions we were able to bias the cycloaddition reaction and get **63g–h** as major products. The reaction with **3i** was unfruitful at reduced temperatures but provided **63i** exclusively when performed at room temp. The electron withdrawing nature of the substituents in **3i** seemingly hampers the nucleophilicity of the sulphur, which becomes unreactive towards benzyne. Similarly, it was observed that 2-pyridones such as **23a**, bearing a strongly electron withdrawing nitro group, does not react to give ring opened product **62j**. Instead **23a** underwent cycloaddition with benzyne to produce the bridged bicycle **63j**, but **63j** was not the isolated end product of this experiment. With the conjugation between the electron withdrawing nitro group and the sulphur broken, the sulphur in **63j** reacted promptly with a second equivalent of benzyne and **64j** resulted. The minor amount of **23a** that did react through thioether attack on benzyne, eventually resulted in the ring expanded product **65j**, through an altered reaction pathway. The structure of **65** was proven with X-ray crystallography. This technique was also used to verify the structure of **63**, and we concluded that benzyne approached **3** only from the least hindered side, opposite to the methyl ester group.⁴⁵⁵



Substrate	Substituents			Products (%)			
	R ¹	R ²	R ³	62	63	64	65
3a	cPr	H	H	40	21	--	--
3c	OMe	H	H	49	--	--	--
3d	H	H	H	50	traces	--	--
3e	H	CH ₂ Cl	H	23	20	--	--
3f	H	Ph	H	25	28	--	--
3g ^a	<i>m</i> -CF ₃ -Ph	CH ₂ -1-naphthyl	H	21	25	--	--
3h ^b	cPr	CH ₂ Cl	H	17	50	--	--
3i ^c	I	CH ₂ -1-naphthyl	Br	--	44	--	--
3j (23a) ^d	cPr	H	NO ₂	--	--	50	18

Scheme 4.4. Reactions between thiazoline fused 2-pyridones **3** and benzyne. **3** (1.0 eq.), 2-(trimethylsilyl)phenyl-triflate **61** (2.5 eq.), KF (2.5 eq.), 18-Crown-6 (2.5 eq.), dry conditions, 0.07 M. The reaction mixtures were stirred at -10 °C until completion was suggested by TLC (16 h–5 days). ^aThe reaction was performed at 0 °C with 18-Crown-6 (3.0 eq.) and 0.3 M of **3g**. ^b**61** (2.0 eq.), 18-Crown-6 (3.0 eq.). The reaction was carried out at r.t. in MeCN. ^cThe reaction was performed at r.t. ^d8% of **23a** remained at work-up.

Based on these observations, and further experiments with deuterium labelled substrate, solvent and added water,⁴⁵⁵ the following mechanism was proposed (Scheme 4.5). Upon attack by the thioether's lone pair electrons on benzyne, the intermediate **I** can undergo an intramolecular rearrangement according to *path a*, to provide the ring expanded compound **65**.

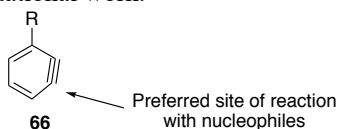


Scheme 4.5. Mechanism proposal for the benzyne induced ring expansion (path a) and ring opening fragmentation (path b).

This pathway appears to be favoured by a strongly electron withdrawing (*i.e.* nitro) R³ substituent. However, intermediate **I** ordinarily undergoes an intramolecular 1,4 proton transfer to generate the ylide intermediate **II**. Upon protonation of **II** by traces of water, acidic α -protons on unreacted **3**, or other proton sources, the resulting sulphonium ion fragments through an intramolecular β -elimination reaction, and *N*-alkenyl 2-pyridone **62** is formed.

4.4. Reactions with substituted benzyne

Substituted arynes are widely employed in chemical synthesis. Special research attention has been given to 3-substituted arynes because of the higher than expected regioselectivity of reaction with nucleophiles (*Figure 4.4*). The origin of this selectivity has been a matter of investigation in the literature, and recently an angle distortion model has gained support as explanation, by Garg and Houk's computational work.⁴⁵⁶⁻⁴⁵⁸



Selectivity trend (R): F>OMe>Cl>Br>I

Figure 4.4. The high selectivity for nucleophilic attack on C-1 originates from the angle distortion caused by inductively electron withdrawing C-3 substituents. The carbon with the larger angle has higher p-character and is thus more electrophilic, while the carbon with the smaller angle has higher s-character and can stabilise an intermediate anion better.

In addition to being regioselective, the 3-substituted benzynes **66** are even more reactive as electrophiles. We further thought that the distorted nature of their HOMO and LUMO orbitals would decrease their propensity to react with the more symmetric diene system in the 2-pyridone ring. Taken together we hypothesised that by using 3-methoxybenzyne, we could favour the thiazoline ring opening reaction over [4 + 2] cycloaddition, which still has been reported to occur with 3-substituted arynes.⁴²¹ 3-methoxybenzyne was generated from precursor **67** (*Figure 4.5*).^{N8}

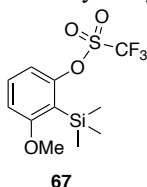
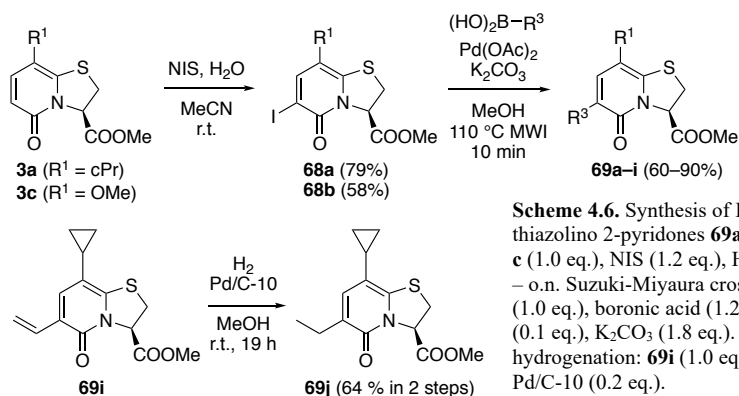


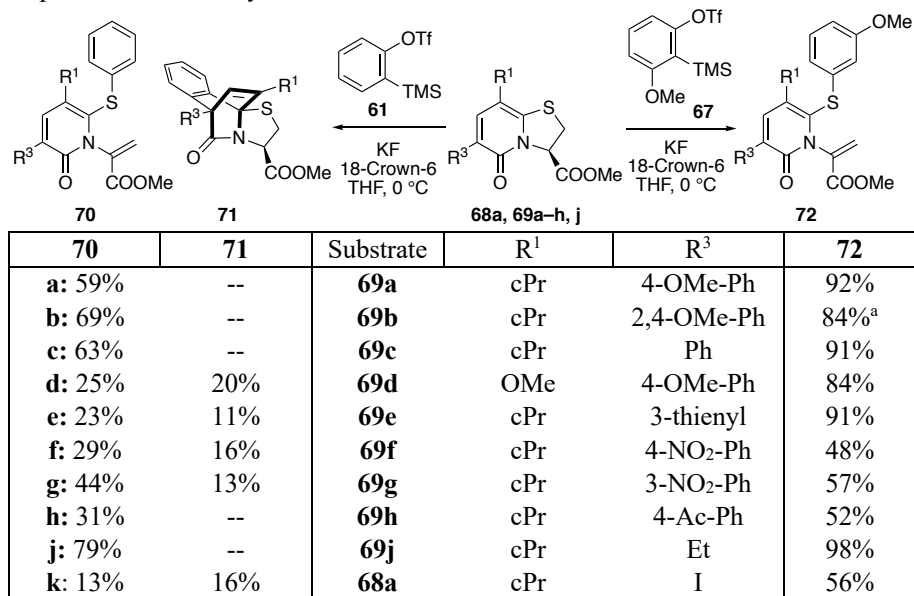
Figure 4.5. 3-methoxy-2-(trimethylsilyl)phenyl trifluoromethanesulfonate **67**.

As stipulated, we found that no [4 + 2] cycloaddition occurred between thiazolino 2-pyridone **3a** and 3-methoxybenzyne, even at room temp. Although more reactive, 3-methoxybenzyne proved to be tempered and useful for synthesis of *N*-alkenyl 2-pyridones, with improved yields. We synthesised a set of thiazolino 2-pyridones **69** with aromatic and aliphatic R³ substituents to be used as substrates for reaction with arynes (*Scheme 4.6*). We subsequently allowed thiazolino 2-pyridones **69a–h, j** and **68a** to react with benzyne and 3-methoxy benzyne respectively (*Scheme 4.7*). We were able to shorten reaction times and improve the outcomes by adjusting the conditions. The stoichiometry of the reagents was adjusted, concentration and temperature were increased. The substrates **69** with aryl or alkyl R³ substituents were more inclined to undergo the ring opening transformation by reaction with benzyne.



Scheme 4.6. Synthesis of R^3 substituted thiazolino 2-pyridones **69a-j**. Iodination: **3a**, **c** (1.0 eq.), NIS (1.2 eq.), H_2O (0.12 eq.), 2 h – o.n. Suzuki-Miyaura cross coupling: **68a-b** (1.0 eq.), boronic acid (1.2 eq.), $\text{Pd}(\text{OAc})_2$ (0.1 eq.), K_2CO_3 (1.8 eq.). Catalytic hydrogenation: **69i** (1.0 eq.), H_2 (1.0 atm.) Pd/C-10 (0.2 eq.).

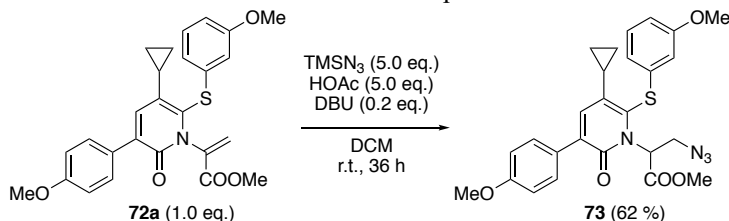
N-alkenyl 2-pyridones **70** were formed exclusively (with one exception) and with good yields when the substrate was fitted with ethyl, phenyl or aryl with electron donating groups, as R^3 substituent. **69d**, bearing a methoxy group as R^1 substituent gave a mixture of ring opened **70d** and cycloaddition product **71d**. The methoxy group likely activates the diene towards cycloaddition. **69e-g**, equipped with aryl groups bearing electron withdrawing substituents, likewise provided a mixture of **70** and **71**. The reaction of **69h** with benzyne was slow and the low yield of **70** in this case seems to reflect just low, rather than competing reactivity. The reaction with the iodinated **68a** resulted in very low yields, slightly favouring cycloaddition. Conclusively, the electronic nature of the R^3 substituent have pronounced effects on the reactivity of the sulphide towards benzyne.



Scheme 4.7. Reactions of thiazoline fused 2-pyridones with benzyne and 3-methoxy benzyne. Thiazoline fused 2-pyridone (0.4 mmol, 1.0 eq.), aryne precursor (1.4 eq.), KF (2.0 eq.), 18-Crown-6 (2.5 eq.), 0.3 M, dry conditions. The reactions were monitored with TLC and worked up when completion was indicated, after 7 h – 2 days. ^aThe reaction was performed at 1.4 mmol scale.

Gratifyingly, 3-methoxy benzyne proved to be unreactive in cycloaddition with the 2-pyridones employed, and more reactive towards the thioether moiety. **72a–h, j, k** were isolated in good to excellent yield as the sole products after less than 24 h of stirring at 0 °C. Electronic factors again had a strong influence on the outcome, with C-6 iodo or electron poor aryl substituents hampering the reactivity and leading to lower yields of the desired products.

To demonstrate that the Michael acceptor in the *N*-alkenyl 2-pyridones indeed works as a Michael acceptor, **72a** was allowed to react with TMS-azide (Scheme 4.8). The 1,4 adduct **73** was isolated after 36 h at room temp.



Scheme 4.8. Addition of azide to the Michael acceptor functionality of *N*-alkenyl 2-pyridone **72a**.

4.5. Ring opening of biologically active compounds

We applied the ring opening transformation on three biologically active compounds. FN075 methyl ester **3g** (Scheme 4.4), benzoquinoline fused thiazolino 2-pyridone **74**^{324, N9} (Scheme 4.9), and pyridine fused **25b** (Scheme 3.4). FN075 accelerate the formation of α -Syn amyloid fibrils³¹⁷ and **32b** (the carboxylic acid corresponding to **25b**) bind to mature fibrils. The carboxylic acids were deprotected (Scheme 4.9) and **76a–c** were evaluated (Figure 4.6) in the α -Syn *in vitro* fibrilization assay described earlier (Chapter 3.4). The ring opening transformation ablated some of the accelerating effect of FN075, and some of the amyloid binding potential of **32b** was lost, but did not affect the inactive benzoquinoline fused compound significantly.

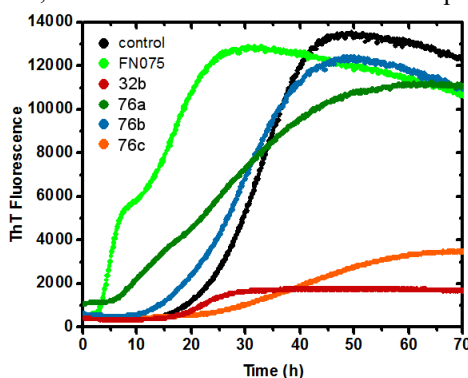
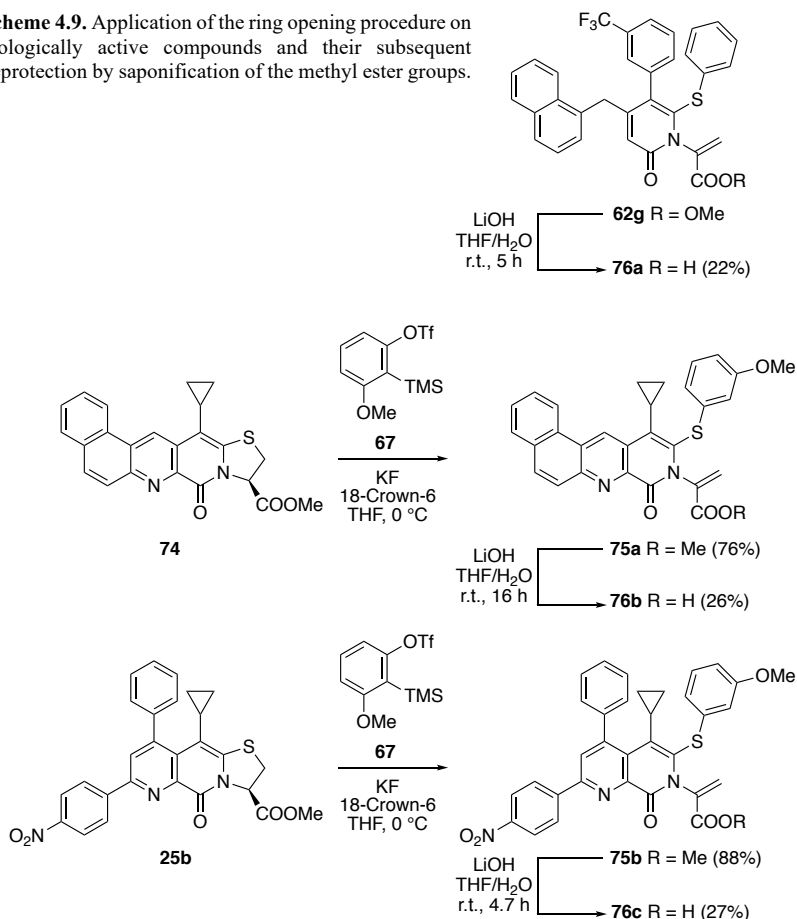


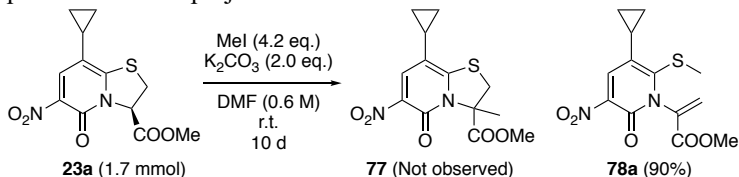
Figure 4.6. The ring opened compounds **76a–c** were evaluated for their abilities to accelerate α -Syn amyloid formation and bind to mature fibrils. The experiments were performed as previously described (Figure 3.2–3.3). **76a** appears to be somewhat weaker as accelerator than FN075. **76c** is likewise less effective as a fibril binder, compared to **32b**. **76b** is inactive, similar to its parent compound **18a** (Chapter 1.15).

Scheme 4.9. Application of the ring opening procedure on biologically active compounds and their subsequent deprotection by saponification of the methyl ester groups.



4.6. A discovery – An unintended thiazoline ring opening

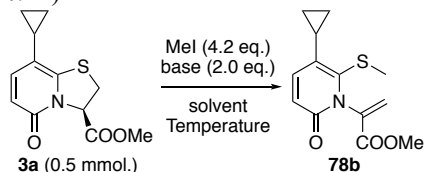
While gathering support for the mechanism of the ring opening reaction, the C-methylated compound **77** was desired (*Scheme 4.10*). In an attempt to methylate the α -carbon of the methyl ester, **23a** was treated with methyl iodide and potassium carbonate.^{N10} The conversion was very clean, but did not afford the desired C-alkylated compound **77**, but the ring opened isomer **78a**, in excellent yield. The need for **77** was later circumscribed, but this discovery, which was not surprising after all, was the prelude to a new project.



Scheme 4.10. Unintended synthesis of thiazoline ring opened compound **78a** through S-alkylation with methyl iodide. 6% of **23a** remained unreacted.

4.7. Thiazoline ring opening with alkyl halides

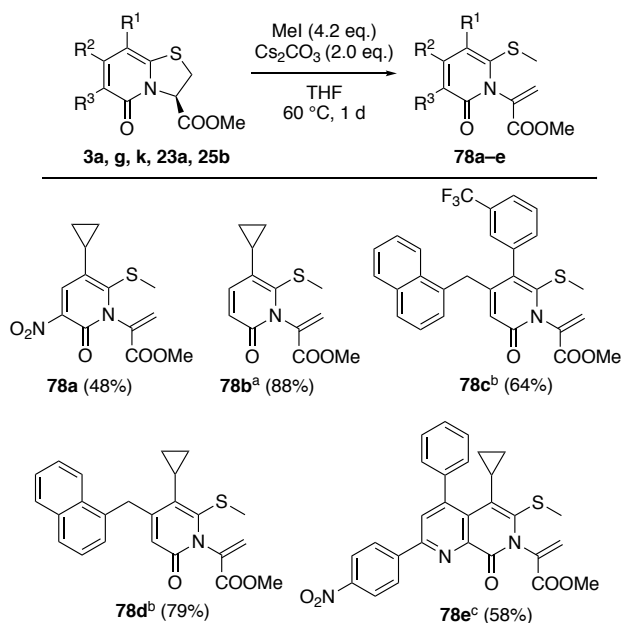
It was decided to elaborate on the thiazoline ring opening with other electrophiles than arynes. **3a** was used as model substrate to establish useful reaction conditions (*Scheme 4.11*).



Scheme 4.11. Screening of reaction conditions to improve the ring opening procedure with methyl iodide. The substrate and amounts of methyl iodide and base were kept constant, while the identity of the base was varied, along with the solvent and temperature.

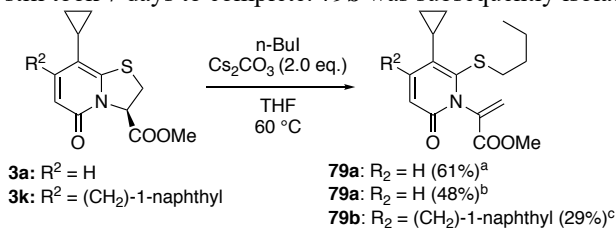
Mirroring the conditions that led to production of **78a** in 90% yield, afforded only 26% of **78b** after 10 days stirring. 69% of **3a** was isolated back. This seemed surprising, given the strong negative influence of electron withdrawing substituents on the reactivity of the thioether moiety with arynes, described above. Raising the temperature to 60 °C notably sped up the reaction, but did not afford completion in 5 days' time. The organic bases DBU and DMAP provided only traces of product. But replacing potassium carbonate with caesium carbonate afforded full, clean conversion to **78b** in just 1 day. DMF could also be replaced with THF as solvent, allowing simpler monitoring and workup of the reactions. The load of methyl iodide could also be decreased to 3 equivalents without extending the reaction time beyond 1 day. However, we later realised that this depended on the reactants, and returned to 4.2 eq. as default electrophile load.

Next, we prompted a set of five thiazolino 2-pyridones, including **3a** and **23a**, to ring open with methyl iodide (*Scheme 4.12*). Intriguingly, the developed procedure afforded full conversion of **23a** in 24 h, but **78a** was isolated in only 48%. **78b** was isolated in 88% yield after 24 h heating with 3 eq. of MeI, as established. Substrates **3g** and **3k** with (CH₂)-1-naphthyl groups as R² substituents needed a high loading of MeI (9.0 eq.) for the reactions to complete within 24 h (**78c–d**). Pyridine fused thiazolino 2-pyridone **25b** required an increase in base (to 3.0 eq.) as well, to complete formation of **78e** in 1 day. Attempting the synthesis of **78c–e** according to the default conditions led to longer reaction times and much lower yields of the desired products. Often the reactions stalled after 3–4 days and did not complete unless supplemented with more reagents. Despite that the conditions needed to be tailored for each individual substrate, the procedures eventually afforded the desired products in good to excellent yields, in just 1 day.



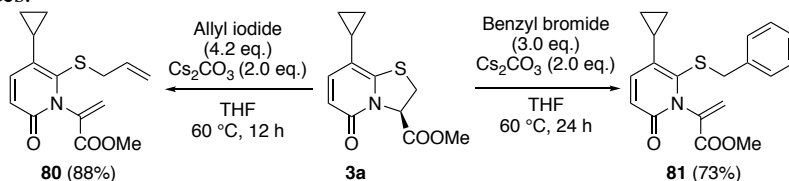
Scheme 4.12. Ring opening of thiazolino 2-pyridones **3a, g, k, 23a** and pyridine fused thiazolino 2-pyridone **25b** with methyl iodide. The reactions were performed at 0.5 mmol scale and 0.3 M in dried THF. ^a3.0 eq. of MeI was used. ^b9.0 eq. MeI was used. ^c5.0 eq. MeI and 3.0 eq. Cs_2CO_3 was used, and this reaction was performed at 0.25 mmol scale.

We continued with *n*-butyl iodide (Scheme 4.13). The reaction of **3a** was notably slower with *n*-BuI than with MeI, likely reflecting the lower reactivity of the former electrophile. **79a** was isolated in 61% after 2 days. Repeating this reaction with 4.2 eq. of butyl iodide led to completion in 1 day, but a slightly lower yield of **79a**. The ring opening of (CH₂)-1-naphthyl decorated analogue **3k** required 9 eq. of *n*-BuI and still took 7 days to complete. **79b** was subsequently isolated in a modest yield.



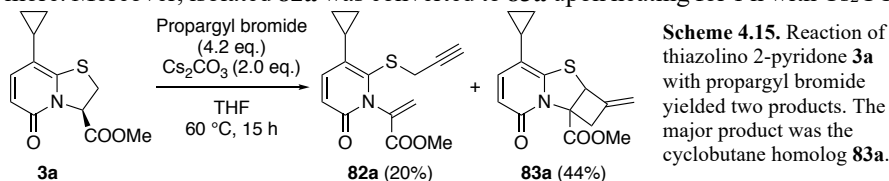
Scheme 4.13. Thiathiazoline ring opening of **3a** and **3k** with *n*-BuI. Reactions were performed at 0.5 mmol scale and 0.3 M in dried THF. ^a3.0 eq. *n*-BuI and 2 days reaction time. ^b4.2 eq. *n*-BuI. The reaction was finished in 1 day. ^c9.0 eq. *n*-BuI was used. The reaction took 7 days to complete.

Use of highly reactive allyl iodide furnished fast conversion to the ring opened product **80** (Scheme 4.14). **3a** was likewise converted to **81** through ring opening with benzyl bromide. Conclusively, the ring opening reaction works well with methyl iodide and related primary, allylic and benzylic electrophiles, but not with cyclic secondary halides.



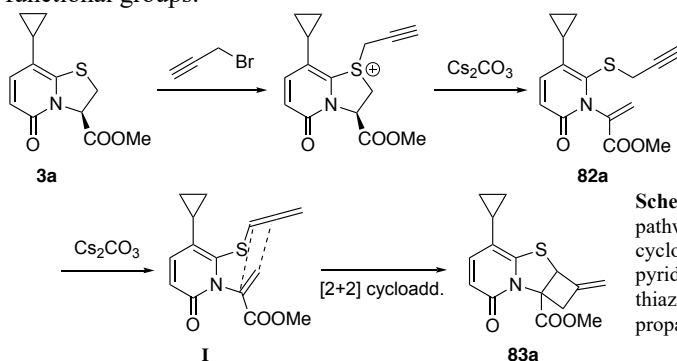
Scheme 4.14. Synthesis of *N*-alkenyl 2-pyridones **80** and **81** by thiathiazoline ring opening with allyl iodide and benzyl bromide, respectively. The reactions were performed at 0.5 mmol scale in dried THF (0.3 M).

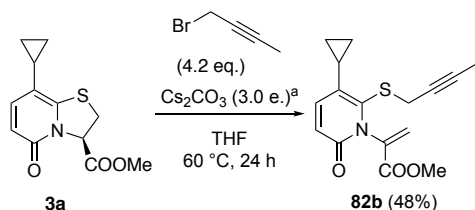
We then treated **3a** with propargyl bromide and expected the ring opened compound **82a** as sole product (*Scheme 4.15*). But two products were isolated, and the major product was found to be the cyclobutane fused homolog **83a**, with an exocyclic terminal alkene attached. Repeating this experiment and letting the mixture stir for 23 h, gave almost exclusively **83a**. The minor amounts of **82a** present at this time, was consumed upon adding 1 eq. of caesium carbonate and heating the mixture for 1 h more. Moreover, isolated **82a** was converted to **83a** upon heating for 1 h with Cs₂CO₃.



4.8. Cut and glue – Thiazoline ring opening and reformation

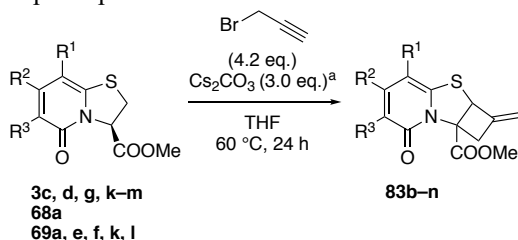
We believe that the formation of **83a** from **82a** goes via an *in situ* formed allene,⁴⁵⁹⁻⁴⁶⁶ according to the following mechanism (*Scheme 4.16*). *N*-alkenyl 2-pyridone **82a** forms first, by the thioether alkylation and subsequent fragmentation of the sulfonium ion, as previously described (*Scheme 4.5*). Then, a base catalysed allene formation *in situ* provides intermediate **I**, which undergoes a [2 + 2] cycloaddition. The terminal alkyne proton appears necessary for allene formation under these conditions, as **82b**, formed by treatment of **3a** with 1-bromo-2-butyne, does not proceed with ring closure (*Scheme 4.17*). Although, allene formation from internal alkynes under basic conditions is reported in the literature.⁴⁶⁷ The cycloaddition reforms the recently opened thiazoline ring, now fused with a cyclobutane moiety, which is equipped with a terminal alkene. This terminal alkene can potentially be used as a reactive handle to perform further late stage modifications of the scaffold, to supply it further with functional groups.





Scheme 4.17. Control experiment with 1-bromo-2-butyne. This electrophile induces the thiazoline ring opening but **82b** does not continue along the cascade in *Figure 4.15*, likely because it does not have the terminal alkyne proton needed for allene formation under these conditions. *2.0 eq. included from start, 1.0 eq. added after 23 h.

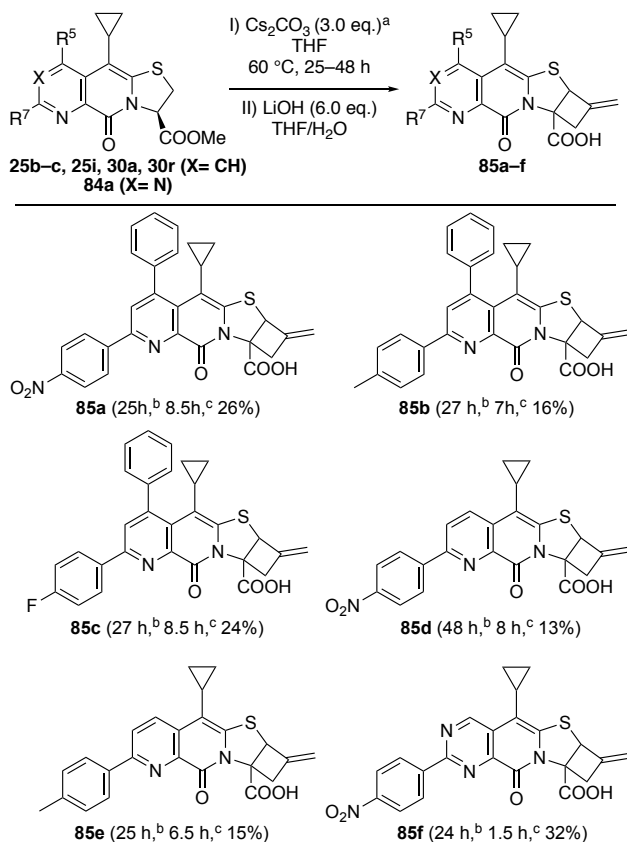
To elaborate on this transformation further, a set of 14 more bicyclic thiazolino 2-pyridones were treated with propargyl bromide according to the established procedure (*Scheme 4.18*). **83b–m** were promptly supplied in 40–77% yield after one day's reaction time, workup and purification.



Substrate	R ¹	R ²	R ³	Product
3c	OMe	H	H	83b (50%)
3d	H	H	H	83c (48%)
3g	<i>m</i> -CF ₃ -Ph	(CH ₂)-1-naphthyl	H	83d (59%)
3k	cPr	(CH ₂)-1-naphthyl	H	83e (49%)
3l	H	(CH ₂)-1-naphthyl	H	83f (41%)
3m	NMe ₂	(CH ₂)-1-naphthyl	H	83g (58%)
68a	cPr	H	I	83h (58%)
69a	cPr	H	<i>p</i> -OMe-Ph	83i (74%)
69e	cPr	H	3-thiophenyl	83j (65%)
69f	cPr	H	<i>p</i> -NO ₂ -Ph	83k (68%)
69k	cPr	H	<i>p</i> -CH ₃ -Ph	83l (77%)
69l	cPr	H	3-furyl	83m (66%)

Scheme 4.18. Cyclobutane homologation of bicyclic thiazolino 2-pyridones **3**, **68** and **69** by a "cut and glue" method with propargyl bromide. All reactions were performed at 0.5 mmol scale at 0.3 M in dried THF. *2.0 eq. included from start, 1.0 eq. added after 23 h.

We also applied this method for homologation of tricyclic pyridine/pyrimidine fused thiazolino 2-pyridones **25b–c**, **25i**, **30a**, **30r** and **84a** (*Scheme 4.19*). The matter was complicated since the ester group was observed to trans-esterify. Hence, a mixture of the methyl and propargyl esters were isolated after a rough purification, which was subjected to saponification forthwith. The carboxylic acid **85a–f** were obtained in 13–26% over two steps.



Scheme 4.19. Synthesis of cyclobutane extended pyridine/pyrimidine fused thiazolino 2-pyridones. The reactions were performed at 0.5 mmol scale in dried THF (0.3 M). The yields given are the overall yields over the two steps. ^a2.0 eq. Cs₂CO₃ included from start, 1.0 eq. added after 23 h. ^bReaction time for cyclobutanation. ^cReaction time for ester hydrolysis. ^dAfter 23 h was added 2 eq. Cs₂CO₃ and additionally 4.2 eq. propargyl bromide.

The pyrimidine fused compounds **85a–f** above were evaluated for α -Syn binding properties (Figure 4.7). Rewardingly, the incorporation of this cyclobutane and alkene moiety was tolerated. The ability to displace bound ThT from mature fibrils was negatively affected only to a very small extent. By comparison, ring opened S-methyl analogue **86**, prepared in 27% by saponification of **78e**, lost a considerable amount of its fibril binding ability. This observation parallels with its aryl counterpart **76c**, ring opened with 3-methoxy benzyne (Scheme 4.9 and Figure 4.6).

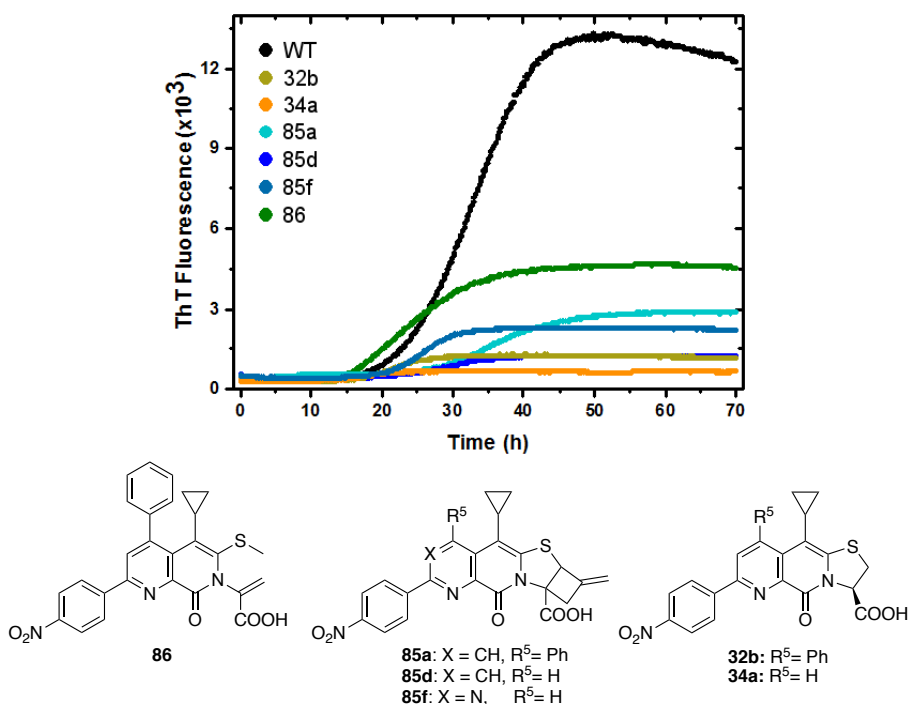


Figure 4.7. ThT fluorescence traces (average of three replicates) for ring opened (**86**) and cyclobutane fused (**85a, d, f**) amyloid fibril binding compounds. The "parent compounds" **32b** and **34a** are included for comparison. The ring opening ablates some of the fibril binding ability, but incorporation of the cyclobutane and alkene moieties is better tolerated.

The *N*-alkenyl 2-pyridones **78c–d**, **79b** and cyclobutane fused compounds **83d–e** with (CH₂)-1-naphthyl substituents were likewise hydrolysed to their corresponding carboxylic acids **88a–e** (Figure 4.8A) and their ability to interfere with α -Syn amyloid formation was probed. In the interest of solubility, **88b** and **e** were prepared as imidazolium carboxylate salts, by addition of equimolar amount of imidazole to the purified carboxylic acids.

Incorporation of the cyclobutane moiety had a very small effect on the acceleration ability of FN075 (Figure 4.8B), and the lag time of α -Syn fibrilization was not notably longer in the presence of **88b** (blue trace) than FN075 (light green). The ring opened **88a** on the other hand have lower accelerating effect (dark green). The structurally related compound **89**, known as C10, does not affect the lag time of human w.t. α -Syn *in vitro* and is commonly used as a negative control.¹⁰⁹ Additionally, both ring opening with *n*-BuI (**88d**) and addition of the cyclobutane + alkene moiety (**88e**) grants α -Syn aggregation accelerating properties, while **88c** remains inactive.

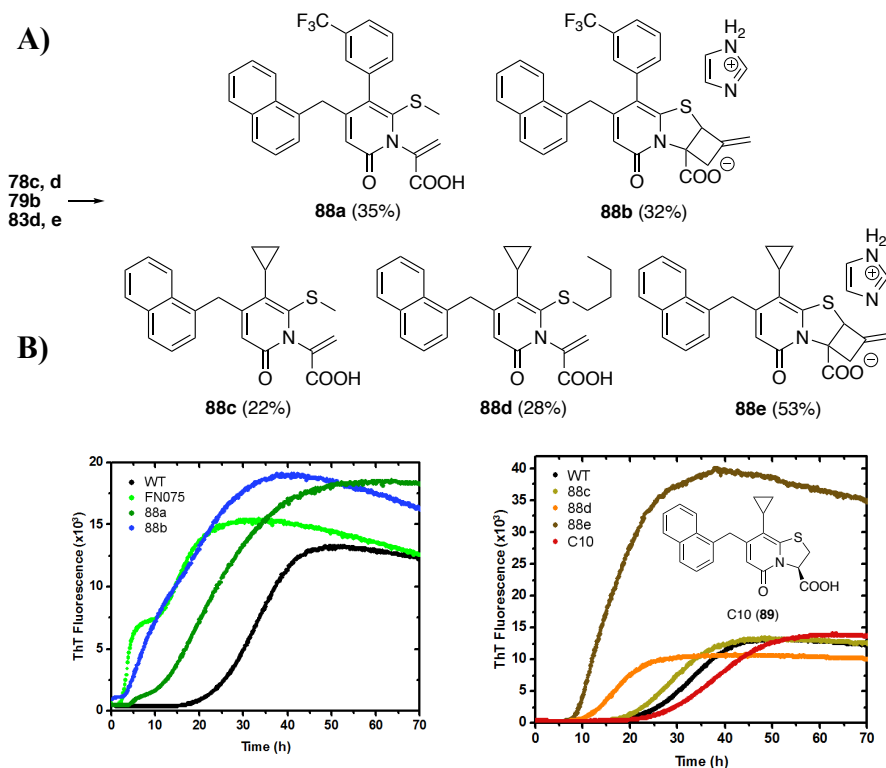
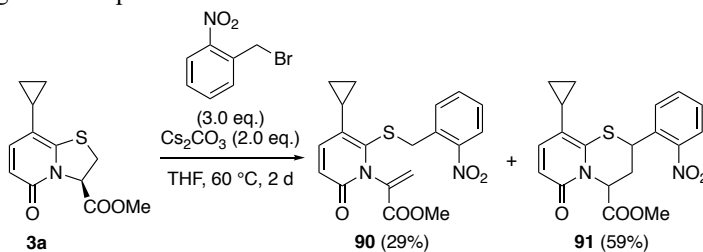


Figure 4.8. **A)** Ester hydrolysis for deprotection of carboxylic acids **88a–e**. **88a,c,d**: I) LiOH (6.0 eq.), THF/H₂O, r.t. II) HCl (7.0 eq., 1M). **88b,e**: I) LiOH (6.0 eq.), THF/H₂O, r.t., II) HCl (7.0 eq., 1M). III) Imidazole (1.0 eq.), MeOH, r.t., 24 h. **B)** The compounds were evaluated for their ability to accelerate α -Syn amyloid formation *in vitro*. Observe the different scaling of the y-axes. Each trace represents the average of triplicate experiments. *Left*: FN075 analogues. The cyclobutane moiety was well tolerated (**88b**), while the ring opened analogue **88a** is a notably weaker accelerator. *Right*: Of the ring opened compounds, **88c** was found inactive, as the parent compound, while **88d** became a decent accelerator. Cyclobutane fused analogue **88e** was also found to accelerate fibril formation. The reason for the extensive ThT fluorescence intensity, compared to other experiments, is unknown, but the phenomenon is reproducible.

4.9. Open and close – Thiazoline ring expansion

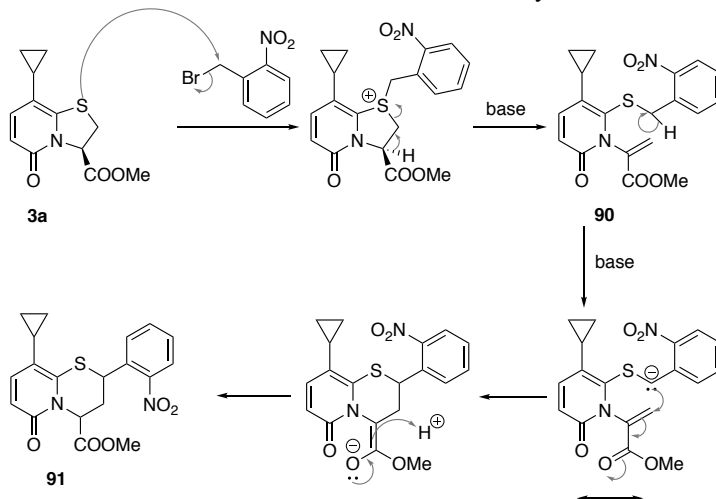
As shown in *scheme 4.14.*, the ring opening reaction worked excellent with benzyl bromide. In continuation, we also attempted to ring open **3a** with 2-nitrobenzyl bromide (*Scheme 4.20*). Similar to the reaction with propargyl bromide, we observed formation of multiple products. In addition to ring opened **90**, we isolated compound **91** as a pair of separable diastereoisomers. The isolated **90** was treated with Cs₂CO₃ (2.0 eq.) in THF at 60 °C, and was found to fully convert into **91** in 6 h. Further, each of the isolated diastereomers of **91** was likewise treated with Cs₂CO₃. We found that an equilibrium was reached between the diastereomers. Finally, the synthesis was repeated with 5 eq. 2-nitrobenzyl bromide and 3 eq. of Cs₂CO₃. After 24 h, **3a** was consumed and a mixture of **90** and **91** was observed by TLC. After 3 days stirring at 60 °C we could no longer detect **90**. Stirring for 1 more day did not visibly alter the relative intensity of the two spots on TLC, corresponding to the two diastereomers.

91 was subsequently isolated in 79% yield with a diastereomeric ratio of 1:2.3, favouring the more polar isomer.



Scheme 4.20. Attempts to ring open the thiazoline ring with 2-nitrobenzyl bromide succeeded. But the ring opened product **90** continued reacting to form the ring-closed dihydro[1,3]thiazine isomer **91** in the same pot.

It appears as the electron withdrawing nitro group renders the benzylic protons just acidic enough to be abstracted by Cs_2CO_3 under the reaction conditions. An intramolecular Michael addition then forms the dihydrothiazine fused 2-pyridone **91** (Scheme 4.21). The corresponding ring opening with benzyl bromide (Scheme 4.14) did not continue along this pathway upon extended reaction times. Treatment of **81** with LiHMDS did however afford 13% of the dihydrothiazine isomer.

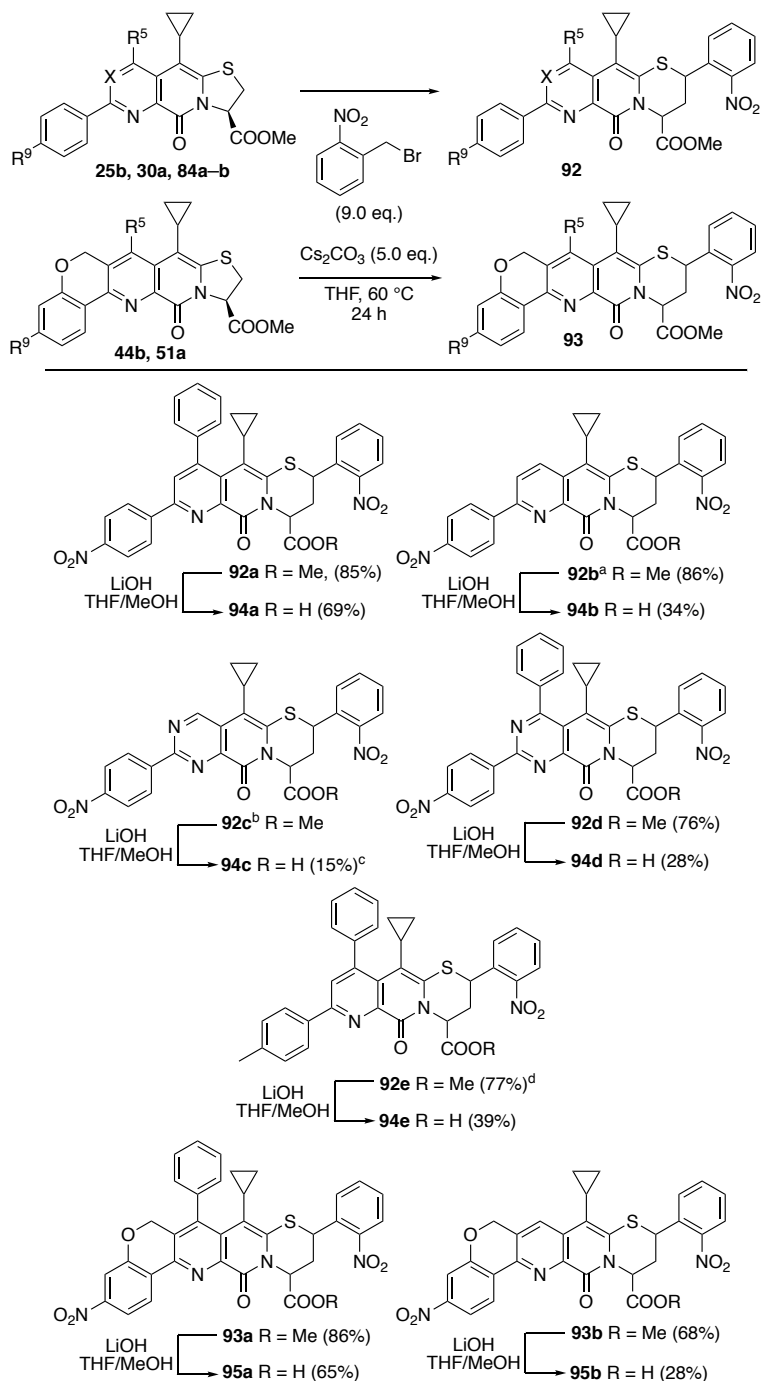


Scheme 4.21. Proposed mechanism for the ring expansion of thiazoline fused to dihydrothiazine fused 2-pyridones via ring opening and re-closure.

We saw the potential of this thiazoline ring expansion protocol to introduce nitroaryl substituents, and explore effects of structural diversity in this region of the scaffold. Thus, we applied the procedure to pyridine and pyrimidine fused thiazolino 2-pyridones (Scheme 4.22), as well as (CH_2) -1-naphthyl equipped pyridones (Scheme 4.23), which interacts with amyloid fibrils and their formation, respectively. With 5 eq. of 2-nitrobenzyl bromide and 3 eq. of Cs_2CO_3 , **92a** and **93a** was prepared in 69 and 63% respectively, after 3 days. At this time, significant quantities of starting material remained but TLC visualised no further reaction. Full conversion, shorter reaction times and higher yields were obtained by raising the load of the electrophile and base, to 9 and 5 eq., respectively. These conditions were hence established as the

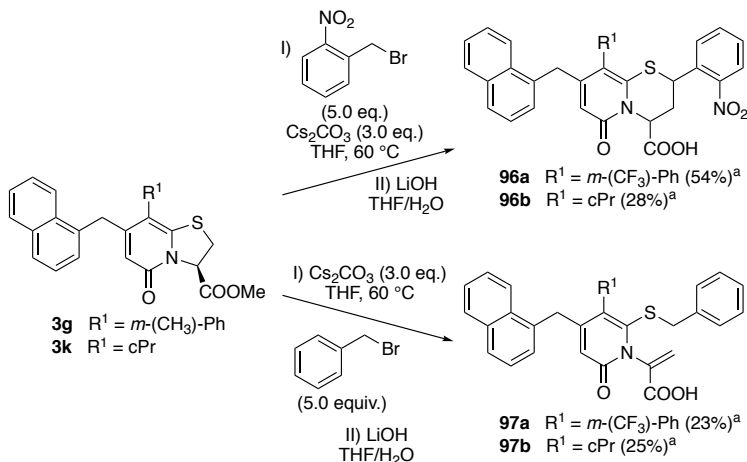
general synthetic procedure for preparation of **92a–e** and **93a–b**. All compounds were isolated as mixtures of diastereomers. Compared to **91**, whose diastereomers were easily separable by flash column chromatography, the diastereomers of **92a–e** and **93a–b** were less well resolved in the mobile phase system employed. A small amount of each diastereomer could however be isolated pure, enough to be subject of spectroscopic characterisation. The vast majority was eluted as a diastereomeric mixture and hydrolysed as such, to produce the corresponding compounds **92a–e** and **93a–b** with deprotected carboxylic acids. Reverse phase chromatography (C-18; MeCN/H₂O) offered no separation of the diastereomeric pair. Experiments with **91** showed that the pure diastereomers interconverted partially during saponification. The minor diastereomer of **91** gave a diastereomeric mixture of the resulting carboxylic acids, 1:0.23 in favour of the same diastereomer. The major diastereomer of **91** likewise interconverted, albeit to a lesser extent, the diastereomeric ratio was 0.03:1 upon complete hydrolysis. Nevertheless, this behaviour indicated that separation of diastereomers before ester hydrolysis would likely be futile. Trials with chiral reverse phase chromatography is on-going. Since the two diastereoisomers are in equilibrium with each other under basic conditions it is natural to assume that the major diastereoisomer of the dihydrothiazine fused compounds has the *o*-nitrophenyl and ester (or carboxylic acid) groups on opposite faces (*anti*). Indeed, molecular mechanics (MM) calculations indicate a 3.5 kcal/mol energy difference between the *syn* and *anti* diastereomers, with *anti* having the lowest energy. Density-functional theory (DFT) calculations at the BLYP-D3 (6–31G**) level support a 2.4 kcal/mol lower energy of the *anti* diastereomer.^{N12}

Unfortunately, when **25b** and **44b** was ring opened with benzyl bromide, the desired products were unstable.^{N11}



Scheme 4.22. Thiiazoline ring expansion of pyridine and pyrimidine fused compounds. All syntheses were carried out at 0.25 mmol scale in dried THF (0.3 M). The pure **92a-e** and **93a-b** were hydrolysed with LiOH (6.0 eq.). ^a2-nitrobenzyl bromide (5.0 eq.), Cs₂CO₃ (3.0 eq.). ^b2-nitrobenzyl bromide (11.0 eq.), Cs₂CO₃ (7.0 eq.). ^cIn two steps. ^dThe reaction mixture was stirred for 3 d. Diastereomeric ratios (higher

r.f./lower r.f.): **92a** (1.0:1.2), **92b** (1.0:1.0), **92d** (2.5:1.0), **92e** (1.3:1.0), **93a** (1.0:1.0), **93b** (1.3:1.0). Diastereomeric ratios for carboxylic acids: **94a** (1.0:2.0), **94b** (1.4:1.0), **94c** (4.0:1.0), **94e** (1.0:2.0), **95a** (1.7:1.0), **95b** (1.0:2.0).



Scheme 4.23. Ring opening and ring expansion of bicyclic thiazolino 2-pyridones **3g** and **3k**. The reactions were performed with 0.5 mmol of **3** in dried THF (0.3 M). The intermediates were hydrolysed to the corresponding methyl esters with LiOH (6.0 eq.). HCl (7.0 eq.) was added upon complete saponification.^aOverall yields from **3**.

For ring expansion of the bicyclic thiazolino 2-pyridones **3g** and **3k** bearing (CH₂)-1-naphthyl groups, 5 eq. of 2-nitrobenzyl bromide and 3 eq. of the base was sufficient to afford full transformation into the dihydrothiazine fused analogues. The reactions did not furnish clean conversion however, and the desired compounds proved challenging to purify with flash column chromatography. Hence, the partially purified products were promptly treated with lithium hydroxide to deprotect carboxylic acids **96a–b**, which were easily isolated pure by reverse phase chromatography. Compared to the pyridine/pyrimidine fused compounds (Scheme 4.22), **3g** and **3k** were effortlessly ring opened with benzyl bromide (Scheme 4.23). Again, chromatographic purifications were complicated by the formation of side products, and the impure esters were upon partial purification hydrolysed to carboxylic acids **97a–b**.

4.10. Transcend – Improved amyloid fibril binders

Ring expansion of the (CH₂)-1-naphthyl equipped bicyclic peptidomimetics, from thiazoline to dihydrothiazine **96a–b**, did not have any new effect on their ability to accelerate α -Syn fibril formation, compared to previous ring opened and cyclobutane fused compounds **88a–e** (Figure 4.9). No major conclusions about the effects of the modifications to FN075 can be made at this point. The modified analogues **96a** and **97a** display a slower increase in ThT fluorescence intensity but lag times are similar. Ring expansion of C10 (**89**) appears to have the same effect as ring opening and cyclobutane incorporation, but stronger. **96b** gain a considerable ability to accelerate fibril formation, **97b** is slightly more modest.

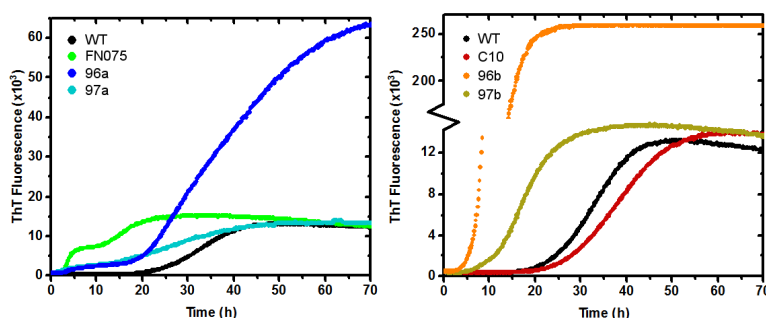


Figure 4.9. Evaluation of ring opened/expanded analogues of FN075 and C10. *Left:* FN075 analogues. The lag times does not differ significantly between FN075, **96a** and **97a**. *Right:* C10 analogues. In comparison with the parent molecule, both **96b** and **97b** are good accelerators of α -Syn fibrilization. The massive intensity of the ThT fluorescence gained for the latter compound is of unknown cause. All traces represent the average of the results from three replicates.

The ring expansion of pyridine and pyrimidine fused peptidomimetics on the other hand, underwent a clear improvement of ThT-displacement ability from α -Syn fibrils (*Figure 4.10 left*). Ring expanded analogues (**94a** and **95a**) of compounds **32b** (*Figure 3.3*) and **55b** (*Figure 3.13*), which were already decent α -Syn fibril binders, eliminated the ThT-fluorescence almost completely. Compound **32c**, which was a weak binder, also underwent improvement upon ring expansion to dihydrothiazine analog **94e**. The rest of compounds **94–95** gave similar results in the ThT assay as **94a** and **95a** in *Scheme 4.20*. (see *Article V*). Since the levels of ThT fluorescence corresponding to **94a** and **95a** are so low, it is from *Figure 4.10 left* not possible to judge if these compounds bind to mature fibrils or act as fibrilization inhibitors. In the $A\beta_{40}$ fibrilization assay however, it seems like **94a**, **94e** and **95a** inhibits fibril formation (*Figure 4.10 right*).

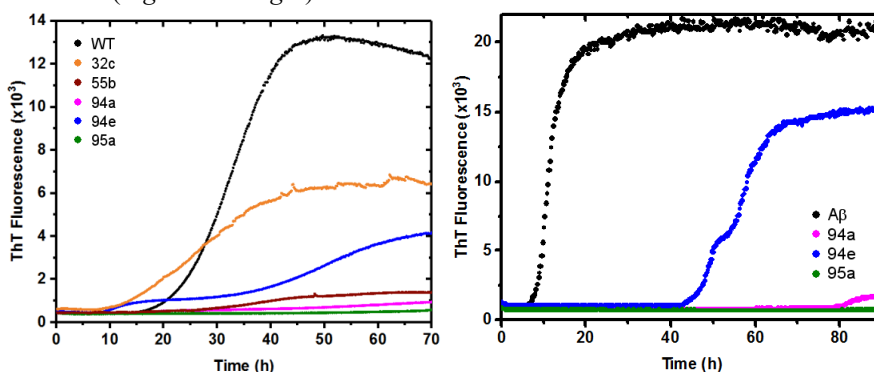


Figure 4.10. Evaluation of ring expanded pyridine fused compounds **94a**, **94e** and **95a**. *Left:* Evaluation against α -Syn. The low levels of ThT fluorescence in the presence of **94a** and **95a**, and the non-typical curvature of the trace corresponding to **94e**, makes it difficult to deduce whether the compounds are fibrilization inhibitors and/or mature fibril binders. *Right:* Evaluation against $A\beta_{40}$. **94a** and **94e** are clearly inhibiting $A\beta$ fibrilization by extension of the lag time.

94a and **95a** were thus added to mature fibrils to measure ThT-displacement (*Figure 4.11*). Both compounds effectively displaced ThT from mature α -Syn and A β 40 fibrils, thereby confirming potent amyloid fibril binding. Further experiments are needed to determine if **94a** and **95a** also inhibit fibril formation.

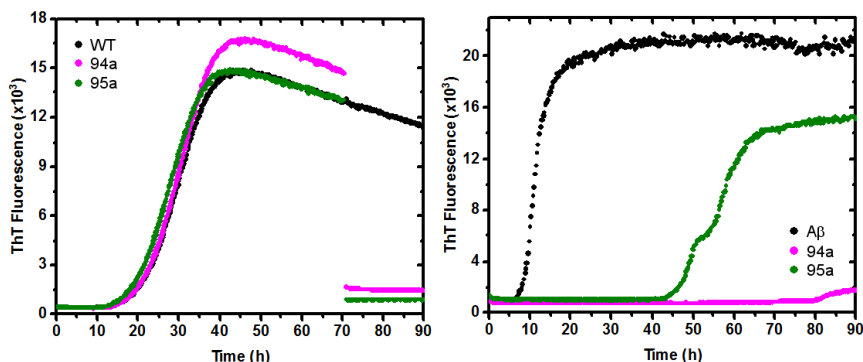


Figure 4.11. Addition of **94a** and **95a** to mature amyloid fibrils. The results clearly confirm amyloid binding, but does not tell if the compounds also affect fibril formation. *Left:* Addition to α -Syn fibrils. *Right:* Addition to A β 40 fibrils.

In addition, upon completion of the α -Syn fibrilization experiments represented by *Figure 4.10 left*, samples were visualised with TEM, and fibrils were found (*Figure 4.12*)

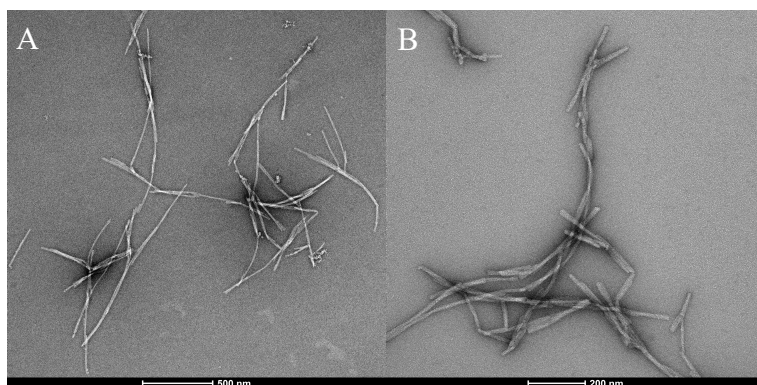


Figure 4.12. Transmission electron micrographs of α -Syn fibrils formed *in vitro* in the presence of dihydrothiazine fused compounds. The presence of these amyloid binding compounds does not affect the appearance of the amyloid fibrils, indicated by a control experiment. **A)** α -Syn fibrils formed in the presence of **94a** at 22 000 x magnification. **B)** Fibrils formed in presence of **95a**, 45 000 x.

Interestingly, samples with A β 40 visualised with TEM upon complete incubation with **95a** (*Figure 4.10 right*) reveals both fibrillar and amorphous structures (*Figure 4.13A*). The control experiment where A β fibrils were allowed to form in the absence of compound shows only fibrils upon TEM inspection (*Figure 4.13B*).

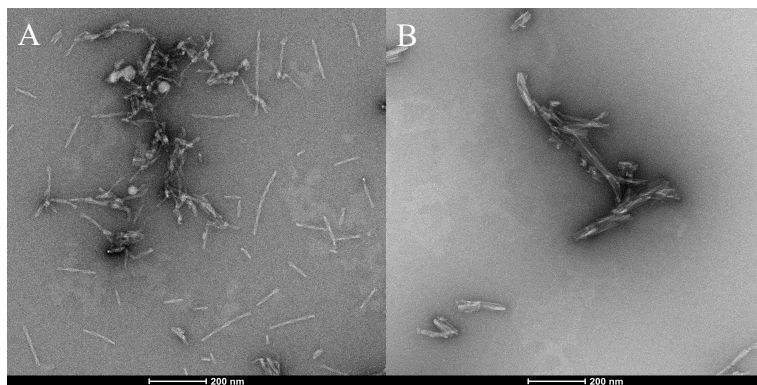


Figure 4.13. Transmission electron microscopy pictures of A β 40 fibrils formed *in vitro*, at 45 000 x. **A)** Fibrils formed in presence of **95a**. **B)** Fibrils formed in the absence of compound (control).

Conclusively, pyridine and pyrimidine fused thiazolino 2-pyridones equipped with the *p*-NO₂-phenyl substituent were considerably improved as amyloid fibril binders by expansion of the thiazoline ring.

5. Summary and concluding remarks

5.1. Conclusions

Methods have been established for the preparation of complex fused heterocycles, with a number of variable substituents, which can be used to tune biological activity. The construction of the pyridine fused scaffolds (with or without oxymethylene bridge) works by extension of thiazoline fused 2-pyridones. The methods used for pyridine ring formation employ three component reactions, with simultaneous formation of several C–C and C–N bonds. Methods have also been developed for thiazoline ring opening and ring expansion, and addition of a cyclobutane unit fused with the thiazoline. These transformations allow late stage modification of thiazoline fused heterocycles. Taken together, the established procedures can be of future use in our laboratories, for continued development of biologically active 2-pyridone based structures, and also be adapted to different compounds by others.

Over 100 2-pyridone based final compounds have been successfully synthesised and evaluated against α -Syn and/or A β 40, for modulation of fibril formation or mature fibril binding. Additionally, about 80 thiazolino 2-pyridones have been prepared for purposes other than biological testing, including exploration of chemical reactivity. The pyridine fused scaffold **20**, with multiple points of variation (*Figure 5.1*) was developed in an effort to find chemical tool compounds which could interfere with α -Syn amyloid formation. Analogs equipped with the *p*-NO₂-phenyl motif, such as **34a**, initially appeared to inhibit fibrilization *in vitro*, but was eventually demonstrated to bind mature amyloid fibrils of α -Syn and A β 40. **34a** was further evaluated in a human cell line as well as mice. It was shown to be protective against FN075 induced cell death and neurodegenerative damage of sensorimotor functions, respectively. The conditions induced by FN075 are similar to the pathology observed in patients suffering from Parkinson's disease.

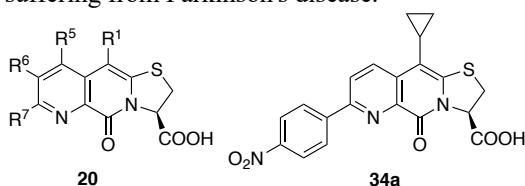
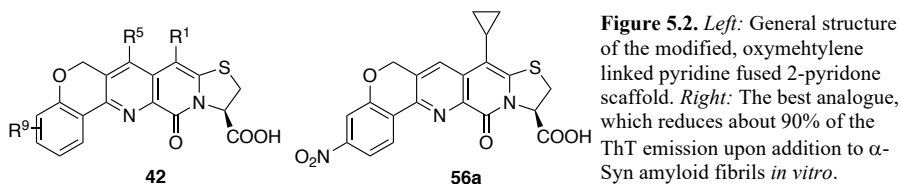
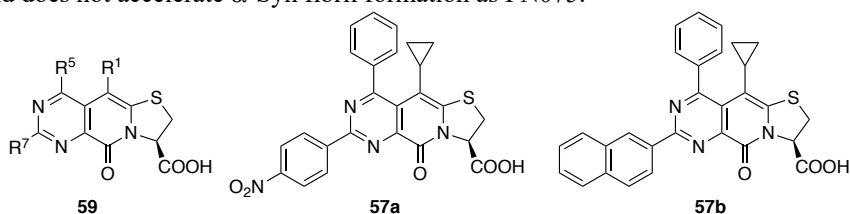


Figure 5.1. *Left:* General structure of the pyridine fused thiazolino 2-pyridones, a central fragment with up to four variable substituents. *Right:* The best example in this set of compounds. Addition to mature α -Syn fibrils quenches about 85% of the ThT-fluorescence by its displacement.

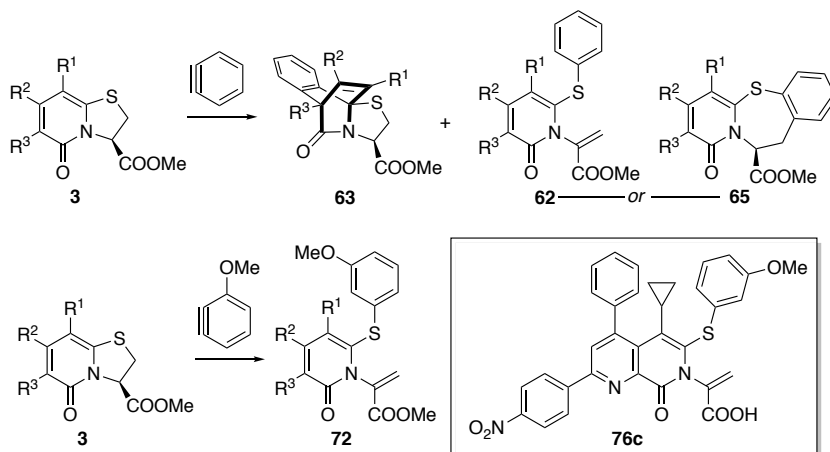
Scaffold **42** (*Figure 5.2*) with an oxymethylene bridge, that further rigidifies the structure by fusing the R⁷-aryl and pyridine rings together, was designed and synthesised. The sp³ hybridised methylene carbon was estimated to reduce planarity of the structures, a feature which may confer selectivity between different fibrils. Scaffold **42** did not gain any selectivity, but the ability to bind α -Syn fibrils was slightly improved. The placement of the R⁹ nitro group had a dramatic effect on the compounds' biological activity. Moving the nitro group from position *para* to *meta*, with regard to the pyridine ring, resulted in a major loss of binding capacity.



The pyrimidine fused scaffold **59** (Figure 5.3) generally had slightly reduced ability to bind α -Syn fibrils compared to the pyridine fused predecessor **20**. However, one example, compound **57b**, inhibits A β 40 fibrilization more efficiently than FN075. This compound thus constitutes a potential starting point for development of A β selective inhibitors of amyloid fibril formation. Any therapeutic potential though, is highly dependent on whether **57b** induces formation of toxic or non-toxic oligomers. **57b** does not display any mutagenic potential as the nitro functionalised analog **57a**, and does not accelerate α -Syn fibril formation as FN075.

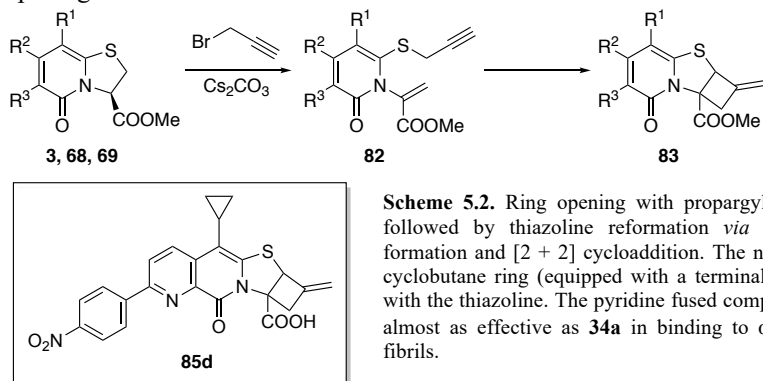


In the meantime, thiazolino 2-pyridones were found to react with benzyne, by cycloaddition with the 2-pyridone ring's diene system, as well as by thioether attack on aryne (Scheme 5.1). The latter mode of reactivity led to thiazoline ring opening, or in rare cases, expansion to a seven membered ring through aryne insertion. With 3-methoxybenzyne, only the thioether attack on aryne pathways were reactive. The thiazoline ring opening protocol was applied on a pyridine fused compound to synthesise **76c**. **76c** lost a significant amount of its ability to displace ThT from α -Syn fibrils compared to its parent compound **32b**, with the thiazoline ring intact.



Scheme 5.1. Thiazoline fused 2-pyridones displayed multiple modes of reactivity with *o*-benzynes. [4 + 2] cycloaddition competed with thioether attack. The latter mode mostly provided *N*-alkenyl pyridones through thiazoline ring opening. 3-methoxybenzynes on the other hand, reacts exclusively through thioether attack. Applied to amyloid fibril binder **32b**, the thiazoline ring opening produced **76c**, which displayed a moderate loss of α -Syn binding activity *in vitro*.

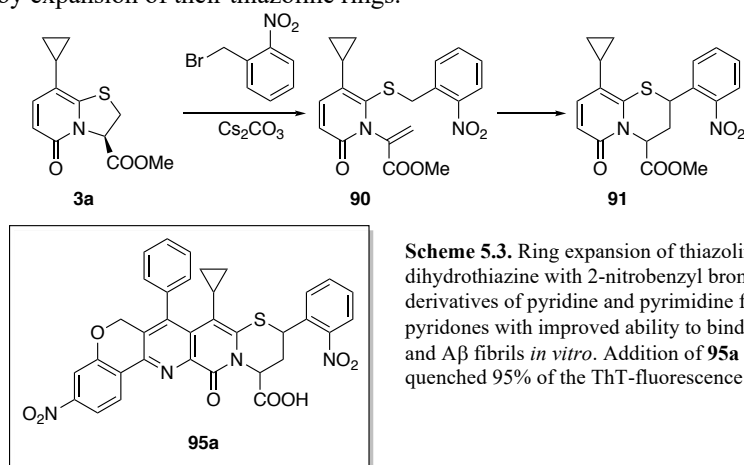
The thiazoline ring opening could also be carried out with alkyl and benzyl halides. When the thiazoline ring was opened by reaction with propargyl bromide, the immediate product underwent *in situ* allene formation, followed by an intramolecular [2 + 2] cycloaddition (Scheme 5.2). The result was reformation of the thiazoline ring, now fused with a cyclobutane ring, bearing an exocyclic terminal alkene, a handle for further chemical transformations. Although this transformation was associated with a minor loss of amyloid binding activity, it was better tolerated than the thiazoline ring opening.



Scheme 5.2. Ring opening with propargyl bromide was followed by thiazoline reformation *via in situ* allene formation and [2 + 2] cycloaddition. The net result was a cyclobutane ring (equipped with a terminal alkene) fused with the thiazoline. The pyridine fused compound **85d** was almost as effective as **34a** in binding to α -Syn amyloid fibrils.

Similarly, when thiazolino 2-pyridones were treated with 2-nitrobenzyl bromide, the ring opened compound **90** was not the final product (Scheme 5.3). A benzylic proton could be abstracted, and subsequent ring closure through intramolecular Michael addition, generated compound **91**. This reaction was likewise applied to amyloid fibril binding 2-pyridone based compounds. Both pyridine and pyrimidine fused thiazolino

2-pyridones, equipped with the *p*-NO₂-phenyl substituent, was considerably improved by expansion of their thiazoline rings.



Scheme 5.3. Ring expansion of thiazoline to dihydrothiazine with 2-nitrobenzyl bromide generated derivatives of pyridine and pyrimidine fused 2-pyridones with improved ability to bind mature α -Syn and A β fibrils *in vitro*. Addition of **95a** to α -Syn fibrils quenched 95% of the ThT-fluorescence.

No clear structural model exists of the binding sites on the fibrils, that ThT and the above described compounds compete for. However, an hypothesised mode of binding in shallow grooves along the fibre's axis, originally made public by Cooper in 1974,¹⁴¹ has gained support. According to this hypothesis, flat heterocycles such as Congo red, ThT and multi ring fused 2-pyridone peptidomimetics fits neatly in the grooves between the rows of side chains (*Figure 5.4*). The bottom of these grooves is estimated to be hydrophobic, while the top can be flanked by polar functionalities on hydrophilic side chains, such as lysine, arginine and glutamic acid. A closer look on the multi ring fused 2-pyridone **95a** reveals a region of mostly hydrophobic motifs, in the upper part as conventionally drawn (*Figure 5.5*). The lower part on the other hand contains several functionalities capable of polar interactions, such as nitro, aniline, carboxylic acid, and the amide incorporated in the 2-pyridone.

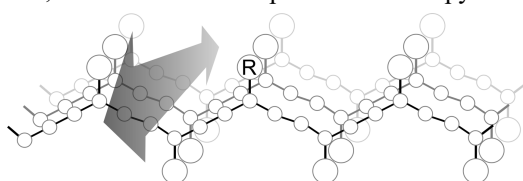


Figure 5.4. Amyloid binding dyes, represented by the double headed arrow is hypothesised to bind in the grooves between rows of side chains, parallel to the axis of the fibre. Inspired by M. R. H. Krebs *et al. Journal of Structural Biology* **2005**, 149 30–37.¹³⁴

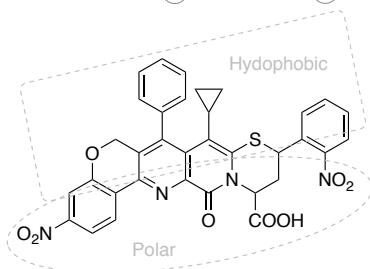


Figure 5.5. Compound **95a**. The top part of this structure is relatively hydrophobic, while the bottom part contain many polar functional group.

This hypothesis could explain why the introduction of the *o*-NO₂-phenyl group, that accompanies the described ring expansion, improves the ability to bind amyloid fibrils.

5.2. Future prospects

Small molecules which can bind selectively to protein specific amyloid deposits have great potentials as diagnostic tool compounds (*Chapter 1.13*). Despite the structural similarity between different fibrils (fibre morphology, cross β -sheet fold, Congophilia, *etc.*), are there subtle distinguishing features that can be exploited to gain selective fibril binding. As mentioned, organic molecules which can distinguish between fibril types by differences in fluorescence emission spectra, have been developed. Moreover, compounds that shows selectivity towards binding A β over other fibrils have been reported. The same is true for α -Syn.³¹² The conditions under which the compounds herein presented were evaluated, differ substantially between the two fibrils. These parameters have been tailored independently for each protein, since α -Syn and A β have different characteristic fibrilization kinetics. In order to make reliable comparisons of affinities to the different fibrils, the testing conditions must be comparable.

The α -Syn binding potency of the dihydrothiazine and pyridine fused 2-pyridones **94a** and **95a** are unprecedented by any 2-pyridone based peptidomimetics in our collections. While the fibril binding properties have been confirmed by ThT displacement from mature α -Syn fibrils, it remains to investigate whether these two compounds also inhibit α -Syn fibril formation. From the shapes of the ThT traces in *Figure 4.10A* one may speculate that the lag times could be extended. Further, **94a** and **95a** do indeed extend the lag time of A β 40 fibrilization, although this observation has been made with other 2-pyridone based peptidomimetics as well. It is not unlikely that dihydrothiazine fused compounds have a dual function, *i.e.* acting as both binders and inhibitors. Rifampicin, baicalein and (–)-epigallocatechin gallate (*Figure 1.17*) have been reported to both bind mature amyloid fibrils and inhibit their formation. The latter two natural products moreover have the ability to disaggregate pre-formed amyloid fibrils. To examine whether the dihydrothiazine fused compounds affect the lag time of α -Syn fibrilization, the ThT concentration could perhaps be increased, as a first step. In addition, CD measurements, TEM, DLS, gel electrophoresis and size exclusion chromatography may provide useful information.

The promising results from the initial tests with pyridine fused compound **34a** in cells (*Figure 3.10*) have motivated us to continue this study. A selection of the best compounds has thus been delivered to our co-workers and will be subjects of more extensive investigations shortly. If the compounds prove to be protective to the human neuron cell line, against FN075 induced toxicity, we desire to continue evaluating their therapeutic potential in animal models. Moreover, the need for diagnostic tool compounds is also great. PET radiotracers have shown diagnostic value by A β plaque imaging in live human patients (*Chapter 1.13*). The thiazoline fused 2-pyridone

scaffold has already demonstrated potential in a PET study with non-human primates (Compound **15** and **16**, Chapter 1.14). It therefore seems natural to consider a potent amyloid fibril binder from our collections, as an acetoxymethyl ester prodrug.

Finally, amyloid formation is not only a pathological process. As briefly discussed in Chapter 1.7, the Curli fibres produced by *E.coli* are bacterial functional amyloids that enables surface attachment and biofilm formation. Consequently, these are bacterial virulence factors, and biofilm formation presents several challenges in human medicine and food industry. Biofilm formation allows bacteria to persist sanitation of equipment, evade host immune system and even resist antibiotic treatment.^{109, 320} Inhibiting biofilm formation with small molecules presents a strategy to treat problematic and recurring infections caused by *E.coli* and other biofilm forming bacteria, such as the *Salmonella* species. Thiazolino fused 2-pyridone peptidomimetics have previously been demonstrated to inhibit aggregation of CsgA, the major Curli subunit, both *in vitro* and *in vivo* (Chapter 1.14). Selected pyridine and pyrimidine fused thiazolino 2-pyridones presented herein have been submitted to our collaborator Matthew Chapman's research group, to be evaluated against CsgA.

Returning to the synthesis of multi ring fused, 2-pyridone based peptidomimetic heterocycles, we are encouraged by the improvements in amyloid fibril binding observed with the dihydrothiazine fused compounds **94a** and **95a**. We do not yet have spectroscopic (NOESY) or crystallographic evidence for which of the two diastereomers (major, more polar or minor, less polar) that is the *syn* and which is the *anti*-isomer. Further, since **94–95** were evaluated as diastereomeric mixtures, we are curious about which of the four stereoisomers that has the strongest amyloid binding activity, or if they are similar in efficiency. We are also curious whether the improvement derives from the ring expansion itself, or the introduction of the *o*-nitrophenyl substituent. Derivatives with alternative substitution of the C-2 aryl group are also of interest, to establish further SAR in this region. Possible ways to explore the effects of chemical changes in this region of the pyridine/pyrimidine fused 2-pyridone based peptidomimetics is to exploit the C-2 functionalisation procedures that previously have been established in our labs.

Just mentioned are only a handful possible avenues of investigation, and the suggested chemical transformations represent just a few ideas. The 2-pyridone peptidomimetics have time and again demonstrated the power of rational drug design, and their potential, and continue to show new structural variabilities in our labs. More specifically, the development of 2-pyridone based amyloid binding heterocycles have not reached roads end. The ideas put forward in the final chapter of this thesis is only meant as a possible starting point of future research. The incidence of Alzheimer's and Parkinson's disease is only estimated to increase as human civilisations continue to develop. All victims invariably die, treatments available today can only alleviate the symptoms, and occasionally retard the progression to some extent. Despite huge research efforts, the causes for disease and their exact mechanisms of action are still far from understood. If these questions could be answered, the chances to prevent,

perhaps even cure the diseases, would improve dramatically. Without treatments, the loss of productivity, life quality and collective experience will continue, and the vast economic burdens upon human societies will keep rising. The total worldwide cost that derives from dementia exceeds 800 billion dollar annually,²⁶⁰ Alzheimer's and Parkinson's disease are the two most common causes of dementia. With that said, the efforts to unravel the secrets of Alzheimer's and Parkinson's disease must continue. Molecular and chemical biology, natural product based drug discovery and rational drug design all need to work in tandem to solve the issues.

"Success flourishes only in perseverance — ceaseless, restless perseverance." – Manfred von Richthofen, the Red Baron.

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"Continuous effort - not strength or intelligence - is the key to unlocking our potential." – Winston Churchill

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7. Notes

N1. Later analysis of the archived mixtures revealed 22 amino acids.⁴⁶⁸

N2. A 1,3-oxazine-4-one have been isolated after the reaction between Meldrum's acid derivative **3** and an imine, and then converted into the corresponding 2-pyridone under acidic conditions.⁵¹⁻⁵² But other mechanisms under acidic conditions cannot be excluded.^{33, 51}

N3. To a solution of malonic acid (750 mmol) in acetic anhydride (90 ml) was added H₂SO₄ (2.25 ml). The resulting mixture was cooled in ice/water bath, whereupon acetone (810 mmol) was added dropwise. The reaction mixture was stirred for 20 min and then left o.n. at 4 °C without stirring. The crystalline precipitate was harvested by filtration and washed with small portions of cold water. The crude product was then dissolved in acetone and precipitated by adding the double volume of water to the solution. The purified product was dried thoroughly by passing air through the precipitate. Then dried under vacuum for several days, until no more ice was found in the liquid nitrogen trap, or lyophilised.

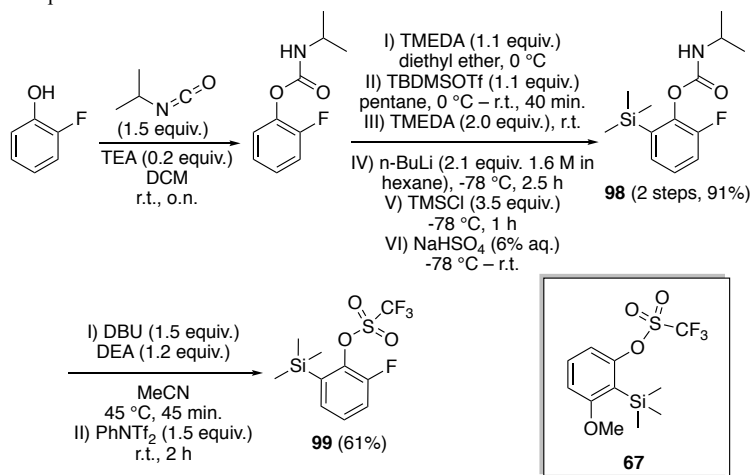
N4. Addition of a few drops of acetic acid to slower reactions completed the turnover.

N5. See experimental procedure and data in Appendix.

N6. One sign of this breakdown was a pair of triplets, around 3.5 and 4.5 ppm respectively, in the ¹H-NMR-spectra, corresponding the vicinal methylene hydrogens of the decarboxylated scaffold **35**. In the ¹³C-spectra, most of the aromatic carbons had minor "acolyte signals" just up- or down-field of the parent peak. TLC, when eluted with magic mix in EtOAc, visualised a non-polar minor spot together with the otherwise pure desired product, that had the same characteristic colour under daylight or near-UV (365 nm) irradiation, as the major spot. Upon thorough drying of the TLC-plate under high vacuum and development with bromocresol green stain, the minor spot did not stain green as the major carboxylic acid parent compound spot. Further, when an impure sample was analysed with LC-MS, the mass corresponding to decarboxylation (–44 amu) was found prominent in the minor UV-peak of the chromatogram.

N7. The reduction of **25b** was carried out in the same manner as for the preparation of **24a–d** that has been described previously. The crude amine intermediate was hydrolysed according to published procedures.³²⁷

N8. We first prepared, 2-fluoro-6-(trimethylsilyl)phenyl triflate **99**, a precursor to 3-fluorobenzynes, by a 3-step procedure from 2-fluorophenol (*Scheme 7.1*).⁴⁵⁶ Unfortunately, 3-fluorobenzynes proved challenging to work with. Its rate of formation from **99**, and consumption through side reactions, was fast, and the yields of the desired products were low.



Scheme 7.1. Preparation of 2-fluoro-6-(trimethylsilyl)phenyl trifluoromethanesulfonate **99**. 3-methoxy-2-(trimethylsilyl)phenyl trifluoromethanesulfonate **67** was obtained commercially.

N9. The effect of the corresponding carboxylic acid is very modest and has previously been regarded as negligible.³²⁴

N10. Normally, strong bases are used for α -deprotonation of carbons adjacent to only one anion stabilising group, such as carbonyl.

N11. Ring opened **25b** (isolated in 39%) slowly broke down when stored at -20°C in solid form, or in heptane/EtOAc mixture under air at r.t. 2D-TLC confirmed a slow decay, incomplete when the plate was stored o.n. at r.t. An NMR sample in chloroform solution showed significant decay in a few h time, but surprisingly, did not progress o.n. Alas, when saponified, the breakdown was complete, without any desired product indicated by LC-MS in the crude mixture, nor isolated by reverse phase chromatography. Attempts to ring open **44b** was likewise unsuccessful. The consumption of **44b** was slow and provided a multitude of unspecific by-product. Attempts to purify the indistinctive yellow spots visible on TLC afforded minor amounts of a complex mixture, showing two methoxy like signals upon ^1H -NMR analysis, along with lots of junk.

N12. Molecular mechanics: Force field used: OPLS3 (solvent: water). A conformational search was done on both isomers. MM energies of conformers with the lowest energies were compared, and used in the DFT calculations, cut to only contain the tricyclic ring with the substituents forming the chirality. A geometry optimization of the cut conformers was made using BLYP-D3 (basis set: 6-31G**) gas phase, and single point energies of the final geometries was calculated and compared. Software: Molecular mechanic calculations were made using MacroModel (v. 11.6) and quantum mechanics using Jaguar (v. 9.6) within the Schrödinger suite (v. 2017-2).

Results:

	MM (Minima, water) kJ	Difference MM in kcal	DFT (Optimum, gas phase) Hartree	Difference DFT in kcal
Anti	-316.363	-3.50	-	
Syn	-301.716		-	
Anti-cut	-		-1631.928337	-2.40
Syn-cut	-		-1631.924508	

8. Experimental procedure and characterisation data for compounds not included in article I–VI.

General procedure for synthesis of 29a–b:

Aminopyridone **24a** (197 mg, 1.0 eq.) dissolved in MeOH (5 ml) was placed in a small E-flask. Arylaldehyde (1.2 eq.) was added to the stirred solution. The reaction mixture was left stirring at r.t. until TLC indicated reaction completion or almost completion with no further progress between two analyses. The reaction mixture was filtered and the precipitate was washed with heptane (1 ml) and sucked dry, then dried under vacuum for 40–120 min. Imin (0.23–0.25 mmol) was dissolved in DCM (4 ml) in a test tube. 3,4-dihydropyran (1.2 eq.) was added to the stirred solution, followed by $\text{BF}_3\cdot\text{OEt}_2$ (0.1 eq.). The reaction mixture was stirred at room temp. until complete conversion was indicated by TLC analysis. The reaction mixture was worked up and purified with flash column chromatography according to the general procedure for Povarov reactions.³²⁷ The purified adduct (0.19 mmol) was dissolved in DCM (2 ml). DDQ (1.0 eq.) was added to the stirred solution, and the resulting mixture was stirred for 1 h., whereupon TLC was used to confirm complete oxidation. The crude product was worked up and purified with chromatography according to standard procedure.³²⁷

methyl (R)-10-cyclopropyl-8-(3-hydroxypropyl)-7-(4-nitrophenyl)-5-oxo-2,3-dihydro-5H-thiazolo[2,3-g][1,7]naphthyridine-3-carboxylate (29a): The compound was prepared by following the general procedure above. Yellow solid (60

mg, 42%). ¹H NMR (400 MHz, CDCl₃) δ 8.33–8.16 (m, 3H), 7.80–7.67 (m, 2H), 5.74 (dd, *J* = 8.2, 2.1 Hz, 1H), 3.77 (s, 3H), 3.76–3.66 (m, 1H), 3.64–3.51 (m, 3H), 2.96–2.83 (m, 2H), 1.88–1.65 (m, 3H), 1.19–1.02 (m, 2H), 0.72 (dq, *J* = 5.5, 2.3, 1.9 Hz, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 168.7, 159.7, 155.3, 147.6, 146.3, 142.6, 139.2, 138.1, 135.0, 132.5, 130.6, 123.5, 107.8, 62.9, 61.8, 53.4, 33.3, 31.8, 29.4, 10.0, 7.6, 7.5.

methyl (R)-10-cyclopropyl-7-(4-fluorophenyl)-8-(3-hydroxypropyl)-5-oxo-2,3-dihydro-5H-thiazolo[2,3-g][1,7]naphthyridine-3-carboxylate (29b): The compound was prepared by following the general procedure above. Light orange solid (51 mg, 28%) ¹H-NMR (400 MHz, CDCl₃) δ: 0.66–0.76 (m, 2H), 1.02–1.14 (m, 2H), 1.72–1.82 (m, 3H), 2.87 (t, *J* = 7.8 Hz, 2H), 3.51–3.59 (m, 3H), 3.67–3.75 (m, 1H), 3.76 (s, 3H), 5.74 (dd, *J* = 1.3, 7.9 Hz, 1H), 7.06 (apparent triplet, *J* = 8.5 Hz, 2H), 7.49 (dd, *J* = 5.4, 8.4 Hz, 2H), 8.22 (s, 1H). ¹³C-NMR (100 MHz, CDCl₃) δ: 7.5 (CH₂), 7.6 (CH₂), 10.1 (CH), 29.6 (CH₂), 31.8 (CH₂), 33.3 (CH₂), 53.4 (CH₃), 62.0 (CH₂), 62.9 (CH), 107.9 (C), 115.3 (d, *J* = 22 Hz, CH), 131.3 (d, *J* = 8 Hz, CH), 132.3 (CH), 134.4 (C), 135.9 (C), 137.8 (C), 139.3 (C), 141.6 (C), 156.9 (C), 159.8 (C), 162.9 (d, *J* = 248 Hz, C), 168.9 (C). ¹⁹F-NMR-data (376 MHz, CDCl₃) δ: –113.6. HRMS (ESI-TOF): (M+H)⁺ calculated [C₂₄H₂₄FN₂O₄S]⁺: 455.1435 amu, observed 435.1439 amu.

methyl (R)-10-(4-methoxyphenyl)-7-(4-nitrophenyl)-5-oxo-2,3-dihydro-5H-thiazolo[2,3-g][1,7]naphthyridine-3-carboxylate (30l): The compound was prepared from **31** by following the general Suzuki coupling procedure.³²⁷ Yellow solid (18 mg, 68%). ¹H NMR (400 MHz, CDCl₃) δ 8.38–8.24 (m, 4H), 7.95 (d, *J* = 8.6 Hz, 1H), 7.74 (d, *J* = 8.6 Hz, 1H), 7.37–7.28 (m, 2H), 7.04 (tt, *J* = 6.5, 2.7 Hz, 2H), 5.87 (dd, *J* = 8.2, 2.1 Hz, 1H), 3.89 (s, 3H), 3.84 (s, 3H), 3.79–3.70 (m, 1H), 3.55 (dd, *J* = 11.6, 2.2 Hz, 1H). ¹³C NMR (100 MHz, CDCl₃) δ 168.6, 159.9, 159.6, 152.7, 148.4, 144.2, 141.9, 140.0, 134.3, 133.4, 131.8, 131.4, 128.1, 127.1, 124.1, 114.9, 114.6, 110.5, 63.8, 55.5, 53.6, 31.8.

methyl (R)-10-(4-fluorophenyl)-7-(4-nitrophenyl)-5-oxo-2,3-dihydro-5H-thiazolo[2,3-g][1,7]naphthyridine-3-carboxylate (30m): The compound was prepared from **31** by following the general Suzuki coupling procedure,³²⁷ but heated for 12 min. Yellow solid (26 mg, 72%). ¹H NMR (400 MHz, CDCl₃) δ 8.42–8.28 (m, 4H), 7.96 (d, *J* = 8.6 Hz, 1H), 7.68 (d, *J* = 8.6 Hz, 1H), 7.37 (td, *J* = 10.5, 5.5 Hz, 2H), 7.25–7.16 (m, 2H), 5.87 (dd, *J* = 8.3, 2.1 Hz, 1H), 3.85 (s, 3H), 3.80–3.72 (m, 1H), 3.57 (dd, *J* = 11.7, 2.0 Hz, 1H). ¹³C NMR (100 MHz, CDCl₃) δ 168.5, 162.9 (d, *J* = 249 Hz), 159.5, 152.9, 148.5, 144.1, 142.2, 140.0, 134.0, 133.1, 132.5 (d, *J* = 8 Hz), 132.2 (d, *J* = 8 Hz), 130.9 (d, *J* = 4 Hz), 128.2, 124.3, 124.2, 116.6 (d, *J* = 22 Hz), 109.7, 63.8, 53.7, 31.9.

(R)-10-cyclopropyl-8-(3-hydroxypropyl)-7-(4-nitrophenyl)-5-oxo-2,3-dihydro-5H-thiazolo[2,3-g][1,7]naphthyridine-3-carboxylic acid (33a): The compound was prepared by following the general saponification procedure.³²⁷ The reaction was finished in 2 h. Light yellow powder (26 mg, 81%). ¹H NMR [400 MHz, (CD₃)₂SO] δ 8.39–8.33 (m, 2H), 8.32 (s, 1H), 7.90–7.73 (m, 2H), 5.60 (dd, *J* = 8.7, 1.6 Hz, 1H), 3.84 (dd, *J* = 11.8, 8.7 Hz, 1H), 3.59 (dd, *J* = 11.8, 1.6 Hz, 1H), 3.36 (t, *J* = 6.2 Hz, 2H), 2.94–2.67 (m, 2H), 1.82 (dq, *J* = 7.9, 5.7, 2.7 Hz, 1H), 1.75–1.58

(m, 2H), 1.09 (tdq, $J = 13.0, 9.3, 4.4, 3.6$ Hz, 2H), 0.72–0.51 (m, 2H). ^{13}C NMR [100 MHz, $(\text{CD}_3)_2\text{SO}$] δ 169.7, 158.4, 154.4, 147.1, 146.3, 143.1, 139.2, 137.2, 134.4, 131.9, 130.5, 123.4, 106.0, 62.6, 59.9, 33.0, 31.2, 28.9, 9.6, 7.4, 7.2.

(R)-10-cyclopropyl-7-(4-fluorophenyl)-8-(3-hydroxypropyl)-5-oxo-2,3-dihydro-5H-thiazolo[2,3-g][1,7]naphthyridine-3-carboxylic acid (**33b**): The compound was prepared by following the general saponification procedure.³²⁷ Off white, slightly orange solid (15 mg, 46%). ^1H -NMR [600 MHz, $(\text{CD}_3)_2\text{SO}$] δ : 0.55–0.67 (m, 2H), 1.04–1.13 (m, 1H), 1.63–1.69 (m, 2H), 1.78–1.85 (m, 1H), 2.78–2.83 (m, 2H), 3.33–3.38 (m, 2H), 3.57 (dd, $J = 1.1, 11.7$ Hz, 1H), 3.83 (dd, $J = 8.7, 11.7$ Hz, 1H), 4.44–4.52 (bs, 1H), 5.58 (d, $J = 7.7$ Hz, 1H), 7.33 (apparent triplet, $J = 8.8$ Hz, 2H), 7.58 (dd, $J = 5.6, 8.5$), 8.27 (s, 1H), 13.49 (bs, 1H). ^{13}C -NMR [151 MHz, $(\text{CD}_3)_2\text{SO}$] δ : 7.1, 7.2, 7.3, 9.6, 9.7, 27.7, 29.0, 31.1, 32.9, 40.0, 50.6, 59.9, 62.5, 106.0, 115.0 (d, $J = 21$ Hz), 131.1 (d, $J = 8$ Hz), 131.6, 133.9, 136.2, (d, $J = 3$ Hz), 137.0, 139.1, 142.3, 155.7, 158.6, 161.9 (d, $J = 245$ Hz), 169.7. HRMS (ESI-TOF): $(\text{M}+\text{Na})^+$ calculated $[\text{C}_{23}\text{H}_{21}\text{FN}_2\text{NaO}_3\text{S}]$: 463.1098 amu, observed: 463.1101 amu.

(R)-10-(4-methoxyphenyl)-7-(4-nitrophenyl)-5-oxo-2,3-dihydro-5H-thiazolo[2,3-g][1,7]naphthyridine-3-carboxylic acid (**34l**): The compound was prepared by following the general procedure for saponification,³²⁷ but with 1.2 eq. LiOH. Fluffy yellow powder (15 mg, 55%). ^1H NMR [400 MHz, $(\text{CD}_3)_2\text{SO}$] δ 8.48–8.41 (m, 2H), 8.41–8.36 (m, 2H), 8.34 (d, $J = 8.8$ Hz, 1H), 7.67 (d, $J = 8.7$ Hz, 1H), 7.41–7.23 (m, 2H), 7.17–7.05 (m, 2H), 5.73 (dd, $J = 8.7, 1.6$ Hz, 1H), 3.92–3.86 (m, 1H), 3.84 (s, 3H), 3.58 (dd, $J = 11.7, 1.7$ Hz, 1H). ^{13}C NMR [100 MHz, $(\text{CD}_3)_2\text{SO}$] δ 169.6, 159.2, 158.3, 151.1, 147.8, 143.7, 143.0, 139.1, 133.7, 132.8, 131.4, 127.8, 126.9, 124.6, 124.1, 114.8, 114.4, 108.5, 63.6, 55.2, 31.3.

(R)-10-(4-fluorophenyl)-7-(4-nitrophenyl)-5-oxo-2,3-dihydro-5H-thiazolo[2,3-g][1,7]naphthyridine-3-carboxylic acid (**34m**): The compound was prepared by following the general procedure for saponification,³²⁷ but with 1.2 eq. LiOH. Yellow solid (17 mg, 58%). ^1H NMR [400 MHz, $(\text{CD}_3)_2\text{SO}$] δ 8.50–8.28 (m, 5H), 7.64 (d, $J = 8.7$ Hz, 1H), 7.54–7.33 (m, 4H), 5.73 (d, $J = 8.4$ Hz, 1H), 3.89 (dd, $J = 11.8, 8.6$ Hz, 1H), 3.60 (d, $J = 11.7$ Hz, 1H). ^{13}C NMR [100 MHz, $(\text{CD}_3)_2\text{SO}$] δ 169.6, 162.0 (d, $J = 245$ Hz), 158.2, 151.2, 147.8, 143.7, 143.3, 139.0, 133.5, 132.7, 132.5, 131.2, 127.8, 124.7, 124.1, 116.3 (d, $J = 21.8$ Hz), 107.8, 63.6, 31.5.

(R)-6-amino-8-cyclopropyl-5-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyridine-3-carboxylic acid (**40**): The product was prepared from **24a** according to the standard saponification procedure, and purified with preparative reverse phase HPLC,³²⁷ but the quenched reaction mixture was neutralised to pH 7–8 with aq. NaHCO_3 solution before workup and extracted with CHCl_3/IPA 9:1 (8 x 5 ml). Off white, slightly light brown solid (47 mg, 100%). ^1H NMR [600 MHz, $(\text{CD}_3)_2\text{SO}$] δ 6.23 (s, 1H), 5.42 (dd, $J = 8.6, 1.6$ Hz, 1H), 3.80 (dd, $J = 11.8, 8.6$ Hz, 1H), 3.52 (dd, $J = 11.8, 1.6$ Hz, 1H), 1.48 (tt, $J = 8.3, 5.1$ Hz, 1H), 0.85–0.76 (m, 2H), 0.55–0.42 (m, 2H). ^{13}C NMR [151 MHz, $(\text{CD}_3)_2\text{SO}$] δ 169.6, 156.1, 112.9, 62.8, 40.4, 40.1, 31.7, 12.6, 6.3, 6.0.

(*R*)-7-(4-aminophenyl)-10-cyclopropyl-5-oxo-9-phenyl-2,3-dihydro-5*H*-thiazolo[2,3-*g*][1,7]naphthyridine-3-carboxylic acid (**41**): Pd/C-10 (25 mg, 0.10 eq.) was weighed up in a 50 ml rbf and evacuated. Back-filled with nitrogen thrice. MeOH (7 ml) was added and the flask was evacuated. Then back-filled with hydrogen from balloon. Compound **25b** (110 mg, 1.0 eq.) was dissolved in THF (10 ml). The solution was added to the stirred catalyst suspension via syringe, followed by MeOH (3 ml), and stirred at room temp. TLC after 2.5 h showed full conv. The mixture was filtered through packed, DCM+TEA-wet Celite. The Celite was rinsed with MeOH until the eluate was transparent, which was subsequently evaporated, re-dissolved in DCM, loaded onto a samplet and purified with Biotage automated flash column chromatography (25 g, EtOAc/MeOH/TEA 94.5; 5; 0.5% as 20-70 % in heptane. The fractions containing the desired product, significantly contaminated, were combined and evaporated (99 mg). 25 g of the methyl ester was subjected to the standard saponification, workup and purification conditions, to provide the desired product **25b** (13 mg, 54%) as a fluffy, light brown powder. ¹H NMR [400 MHz, (CD₃)₂SO] δ 7.96 (d, *J* = 8.4 Hz, 2H), 7.84 (s, 1H), 7.62 – 7.31 (m, 5H), 6.65 (d, *J* = 8.4 Hz, 2H), 5.59 (dd, *J* = 8.6, 1.9 Hz, 1H), 3.84 (dd, *J* = 11.8, 8.7 Hz, 1H), 3.54 (dd, *J* = 11.7, 2.0 Hz, 1H), 1.10 (s, 1H), 0.28–0.06 (m, 4H).

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