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Synthesis of Insulin-Regulated Aminopeptidase (IRAP) inhibitors

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Abstract

Synthesis of Insulin-Regulated Aminopeptidase (IRAP) inhibitors

Faith Agalo

The need for alternative cognitive enhancers has risen due to the fact that clinical trial results of the drugs currently approved for treating these disorders have not been satisfactory.

IRAP has become a possible drug target for treating cognitive impairment brought about by Alzheimer's disease, head trauma or cerebral ischemia, among others. This came after the revelation that Angiotensin IV enhances memory and learning. Angiotensin IV, the endogenous ligand of IRAP has been structurally modified with the aim of producing potent IRAP inhibitors. However, the peptidic nature of these inhibitors restricts their use; they are not likely to cross the blood brain barrier.

Other strategies for generating IRAP inhibitors have been through structure-based design and receptor based virtual screening. These drug-like molecules have exhibited positive results in animal studies.

IRAP inhibitors have been identified via a HTS of 10500 low-molecular weight compounds to give the hit based on a spirooxindole dihydroquinazolinone scaffold, with an IC_{50} value of 1.5 μM. In this project, some analogues to this hit compound have successfully been synthesized using a known method, whereas others have been synthesized after additional method development.

The application of the developed method was found to be limited, because poor yield was obtained when a compound with an electron withdrawing substituent on the aniline was synthesized. As a result of this, modification of this method may be required or new methods may have to be developed to synthesize these types of analogues.

Inhibition capability of 5 new spirooxindole dihydroquinazolinones was tested through a biochemical assay. Compound **6e** emerged as the most potent inhibitor in the series, with an IC_{50} value of 0.2 μM. This compound will now serve as a lead compound and should be used as a starting point for future optimization in order to generate more potent IRAP inhibitors.

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Abbreviations

ACH	Acetylcholine
AChEI	Acetylcholinesterase inhibitor
AcOH	Acetic acid
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4 - isoxazolepropionic acid
Ang	Angiotensin
Aβ	amyloid-β
$CDCl_3$	Deuterated Chloroform
Cs_2CO_3	Cesium carbonate
DAD	Diode array Detector
DMSO	Dimethyl sulfoxide
EDDA	Ethylenediamine diacetate
EDG	electron withdrawing group
EMS	Electromagnetic spectrum
Equiv	equivalent
ESI	Electron spray ionization
EtOAc	Ethyl acetate
GLUT4	Glucose transporter type 4
HCOOH	Formic Acid
HD	Huntington's disease
HPLC	High-performance liquid Chromatography
HTS	High-throughput Screening
MeCN	Acetonitril
MeI	iodomethane
mmol	millimoles
MS	Mass spectroscopy
MW	microwave
NFT	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate

NMR	Nuclear Magnetic resonance
PD	Parkinson’s disease
RAS	renin-angiotensin system
SAR	structure activity relationship studies
SP	Senile plaques
TLC	Thin layer chromatography
UV	Ultra violet
XRC	X-ray crystallography

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1 Introduction

1.1 Cognitive disorder

Cognition is a broad term which includes the ability of the brain to perceive, processes and store information so that it can be used at some point in the future.[1] Cognitive domains are sections in the brain associated with memory, executive functions, attention, language and visiospatial skills.[2] A dysfunction of one or several cognitive domains is termed cognitive disorder.[2] Normal aging is associated with some degree of decline in cognitive skills, because the brain structure and function changes.[3] Nonetheless, cognitive impairment symptoms are more pronounced and differ from those seen in age-associated cognitive decline. The impairment can range from mild (mild cognitive impairment) to severe (e.g. dementia).[2] Cognitive dysfunction can be caused by neurodegenerative diseases (Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD)),[1] vascular diseases (diabetes, hypertension),[2] head trauma[4-5] or cerebral ischemia[6], among others. The symptoms of cognitive impairment observed depends on the cause or the part of the brain affected.[1-2]

1.2 Alzheimer's disease

AD is a progressive neurodegenerative disease which was first described by a German psychiatrist, Dr Alois Alzheimers, while examining the brain tissue of his patient who had died of a strange mental illness. He noticed peculiar clumps in the tissues and came to a conclusion that this was a distinct disease of the brain. Following his discovery, research has brought to light the presence of senile plaques (SP) and neurofibrillary tangles (NFT) as the clumps observed by Dr Alzheimer. AD is the major cause of dementia, a form of cognitive decline so severe that it interferes with daily life. Other diseases such as PD, Lewy bodies and vascular diseases can also cause dementia.[7]

1.2.1 Etiology and pathogenesis of Alzheimer's Disease

Epidemiology research has identified old age as the major risk factor for AD. Others include genetics and vascular diseases.[8] Several hypotheses for the cause of AD have been proposed, the most common feature in these hypotheses is the deposition and accumulation of SP and NFT. SP is composed of amyloid-β (Aβ) proteins.[7,9-10] One theory suggests that, due to mutation, the deposition and the consequent aggregation of Aβ induces hyperphosphorylation of tau protein.[9] Tau proteins are found in nerve cells and are known to support the microtubules, whose function is to maintain the structure of the cell, among others.[7] This abnormal phosphorylation of tau proteins makes them unable to be degraded, and hence they accumulate in the never cell as NFT, leading to neuronal dysfunction and death.[9-10] Mitochondrial- and immune dysfunction as well as environmental factors such as exposure to aluminum, diet and viruses have also been hypothesized to cause AD.[10-12] This loss and subsequent death of neurons exhibited in AD causes deficiencies of neurotransmitters like

acetylcholine (ACh), serotonin, and norepinephrine, which in turn give rise to cognitive deficits.[9,13-15]

1.3 Current drugs used for treating cognitive disorders

Currently approved drugs for treating memory and cognitive disorders are mostly AD drugs. These drugs target the cholinergic and glutamatergic system.[16-17]

1.3.1 Cholinergic system

Studies have established that the cholinergic system is important in promoting memory and learning.[18-19] ACh is the principal neurotransmitter in this system and has two receptors: nicotinic and muscarinic. In normal conditions, an action potential causes the release of ACh from the presynaptic terminal of cholinergic neurons. This neurotransmitter binds to its receptors on the post synaptic cleft, generating other secondary signal transduction inside the cell, which eventually increases memory and learning, among other functions. Excess ACh is broken down at the synaptic cleft by cholinesterase.[20] Neuronal loss and death in AD, induces cholinergic deficits. Cholinergic transmission can be increased via cholinesterase inhibition or receptor modulation.[16-17]

1.3.1.1 Cholinesterase inhibitors

Cholinesterase inhibitors (AChEI) are drugs which promote the action of acetylcholine by inhibiting the enzyme which is responsible for its degradation, thus enhancing memory and learning. Donepezil, galantamine, rivastigmine and tacrine are some examples of approved AChEI. Despite their approval, these drugs have demonstrated limited efficacy.[16-17]

1.3.1.2 Acetylcholine receptor modulators

Effort to increase cholinergic transmission via receptor modulation has been futile due to adverse effect generated by the agonist.[16]

1.3.2 Glutamatergic system

Glutamate is the major excitatory neurotransmitter in the brain and has four types of receptors: NMDA, kainate, AMPA and metabotropic receptors.[21] Activation of NMDA receptor by glutamate has been linked to improved memory, learning and synaptic plasticity.[22] On the other hand, overstimulation of glutamate receptor (excitoxicity) due to release of high amount of glutamate induces neuronal degradation via neurotoxicity. Aβ has been postulated to promote the release of glutamate and hence excitoxicity in AD. Likewise, glutamate has also been implicated in the production of Aβ and tau phosphorlylation through a positive feedback loop. The end result of these events is neuronal loss and cognitive deficits.[21-22]

1.3.2.1 NMDA antagonist

NMDA antagonist exerts its effect by blocking the receptor thereby preventing excitotoxicity. One such drug is memantine, a non-competitive antagonist with low to moderate affinity to

the NMDA receptor. Despite its approval for treating cognitive impairment related to AD, its neuroprotective effect is still not clear.[16,22-23]

1.4 Background to the present study

As already stated, the efficacy of the drugs currently being used as cognitive enhancers is unclear; thus new treatment approaches are needed.[16-17,23] The discovery that angiotensin IV (Ang IV) has an effect on learning and memory[24] has given a rise to more research on this peptide.[25-28]

1.4.1 Angiotensin IV

The angiotensin family of peptides has been known to play an important role in controlling the cardiovascular activity and regulating electrolyte/fluid homoestasis in the body through the Renin-angiotensin system (RAS). These peptides are synthesized from their precursor protein angiotensinogen via several enzymatic cleavages. Angiotensin II (Ang II) and angiotensin III (Ang III) are full agonist at the angiotensin II receptor type 1 (AT_1 receptor) and angiotensin II receptor type 2 (AT_2 receptor), where their main function is regulation of the named physiologies (fluid balance and blood pressure).[29] Angiotensin IV (Ang IV) is a hexapeptide formed through enzymatic cleavage of Ang III .[29] This peptide was first considered inactive due to its low affinity for AT_1 and AT_2 receptors, until a separate and distinct binding site for it was discovered. Studies carried out in various animals models have identified the locality of the binding sites in areas of the brain linked to memory and learning. Other binding sites were also found in peripheral organs.[25-26] After the discovery that this hexapeptide enhances memory[24], several confirmatory studies were carried out in various animal models giving the same outcome.[27-28] Consequently, Ang IV and its receptor became a potential new target for drugs intended to treat memory dysfunction.[30]

1.4.2 Insulin-regulated aminopeptidase (IRAP)

Insulin-regulated aminopeptidase (IRAP) is a type II transmembrane protein belonging to the family of zinc-dependent membrane aminopeptidases. This enzyme was originally cloned and characterized in glucose transporter type 4 (GLUT4) vesicles of muscle and fat cells. GLUT4 is a protein which regulates glucose homeostasis. At elevated levels of insulin, IRAP accompanies GLUT4 to the plasma membrane to normalize the level of glucose. IRAP was found have three domains: a large extracellular catalytic domain, a single transmembrane region and an intracellular domain.[31]

Ang IV binding site was originally referred to as the angiotensin IV (AT_4) receptor, but in 2001 AT_4 receptors in bovine adrenal membranes were purified, sequenced and determined to be IRAP.[32] Likewise, the endogeneous peptide LVV-hemorphin 7 (LVV-H7) has also been described as a ligand to IRAP. These two neuropeptides are said to be competitive inhibitors of IRAP, where they bind at the catalytic site of the enzyme thereby inhibiting its activity.[32-33] The mechanism through which IRAP promotes memory is not clear, though one theory states that the binding of these ligands increases the half-life of IRAP substrates such as,

vasopressin, oxytocin and somatostatin which are known to play a major role in enhancing learning and memory.[34]

1.4.3 Peptide inhibitors

The discovery of IRAP initiated studies wherein, Ang IV was modified through processes such as truncations of peptide bonds, macrocylization and introduction of conformational strains at different amino residues. One of these peptidomimetics IRAP inhibitors (HA-08), synthesized via introduction of constrained macrocyles, exhibited potency 20 times that of Ang IV (Figure 1). The disadvantage of these inhibitors is that they are susceptible to degradation and may not cross the blood brain barrier.[35]

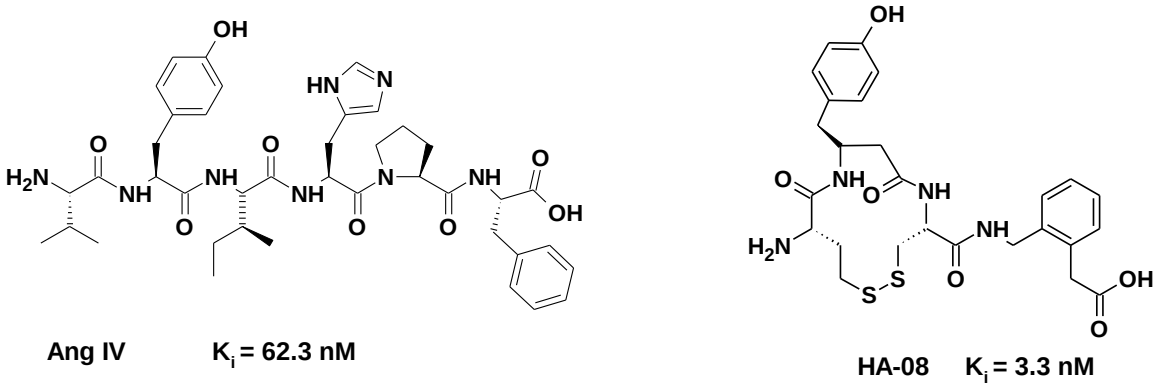
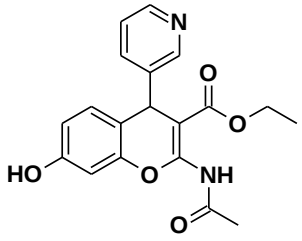


Figure 1: *The structure of Ang IV and the Ang IV peptidomimetic HA-08.*

1.4.4 Non-peptide inhibitors

The limited use of peptidic inhibitors has led to other methods being used to generate IRAP inhibitors, one being structure-based design and receptor based virtual screening.[36] Structure-based design and receptor based virtual screening is a method of designing a drug based on information about the target (protein) compound with help of a computer.[37] Targets are first identified and their 3D structures determined by X-ray crystallography (XRC) or nuclear magnetic resonance (NMR).[37-38] In cases where targets are not known, prediction through homology modeling can be used to generate one.[39] Virtual screening of large library compounds against the indentified target to obtain hit compounds follows. The hit compounds are thereafter purchased or synthesized, tested in bioassays and further optimized to come up with powerful inhibitors.[36-38] Albiston *et al*., used this approach to identify and design benzopyran-based IRAP inhibitors. A total of 1.5million commercially available compounds were screen against a homology model of IRAP. One of the inhibitors (Figure 2) obtained after optimization of the hit compound, demonstrated memory enhancing effect in rats.[36]



HFI-419 K_i **= 0.48 µM**

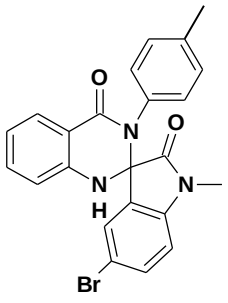
Figure 2: *Benzopyran-based IRAP inhibitors*

1.4.5 High throughput screening (HTS)

High-throughput screening (HTS) has been used in this project to identify non-peptidic inhibitors. Unlike virtual screening, the screening is done *in vitro.* HTS is part of the drug discovery process which encompasses the following units: Target identification and validation, assay development (biochemical or cell based assay) and validation, hit identification via HTS, evaluation and validation of hits to generate leads and optimization of leads to form drug candidates.[40-41]

2 Aim of the present study

A screening campaign of 10500 low molecular weight compounds for their inhibition of the catalytic activity of IRAP has previously been performed resulting in five structurally different inhibitors. One of the hit compounds was the spirooxindole dihydroquinazolinone **1** (Figure 3), with an IC_{50} value of 1.5 µM. Structurally similar compounds to **1** are known to possess a number of medicinal and biological properties such as antitumor, antibiotic analgesic, diuretic, antihistamine, antidepressant, antipyretic, antihypertonic and vasodilating activities.[42]



1

Figure 3: *Structure of one of the hit compounds from the screen*

The aim of this project was to synthesize modified IRAP inhibitors from the hit compound **1** and to continue to investigate the structure activity relationship (SAR) in position R1 and R2 (Figure 4). The SAR was to be accomplished through structural alterations of scaffold (**1**) followed by a measure of the biological activity. The inhibition potency of each compound was to be quantified by their IC_{50} values.

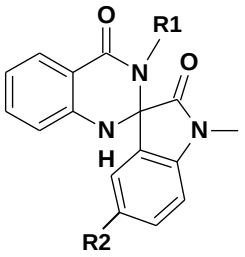


Figure 4: *Positions that were to be investigated*

2.1 Structural modifications

The following are some of the structural changes that were to be performed:

1. Synthesis of analogues of **1** by replacing the bromine at position 5 with other halogens (involves *N*-methylated isatins).
2. Incorporating other amine substituents at position 3'
3. Replacing the methyl group on the phenyl ring (3') with other substituents regarding size, hydrophilicity and electrostatic characteristics (Figure 5).

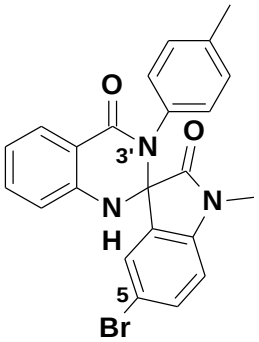


Figure 5: *The sites where the modifications were to be performed*

3 Microwave-assisted synthesis

In this project a microwave equipment was used to perform the synthesis. Microwave-assisted synthesis has transformed synthetic chemistry since its implementation in the mid 80s.[43] Compared to conventional heating such as oil bath, this technique offers the following advantages: increased rate of reaction because higher temperatures can be used, cleaner reactions and higher yield due to less side reactions and reduced consumption of energy since microwaves heat up the sample and not the reaction vessels.[44] Microwaves lie between 0.01 m to 1 m in the electromagnetic spectrum (EMS), corresponding to a frequency of 30 GHz to 0.3 GHz. The reaction heating is nevertheless done at 2.45 GHz.[43-44] Substances with dipole moment, polar or ionic solvents are suitable for use in the microwave, because when irradiated they can generate heat through mechanisms like conduction and dipolar polarization.[45] Microwave synthesis apparatus can have single- or multi-mode cavity. A single mode oven accommodates one vessel at a time, while multiple modes cavities can run many samples at a go.[44] Reaction vessels are always transparent to microwave and are made of

materials such as borosilicate glass or teflon.[43] Some recent advances in microwave–assisted synthesis include enhanced microwave synthesis[46] and microwave heated flow synthesis.[47]

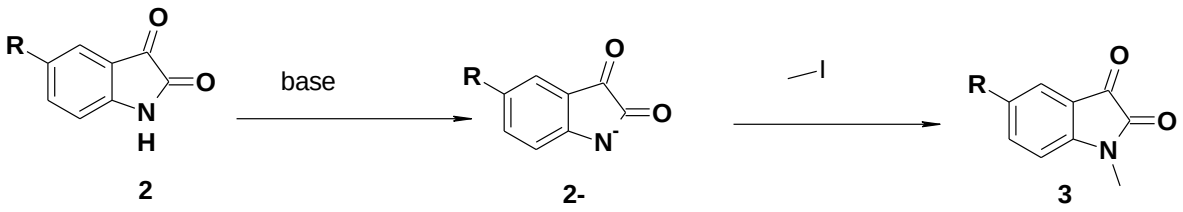
4 N-methylatation of isatins

4.1 S_N2 reaction

The methylations of the isatins follow the S_N2 reaction mechanism. S_N2 is a reaction involving a nucleophile and an electrophilic substrate. A nucleophile is a negatively charged ion or a neutral molecule with unshared electrons, which seeks the positive center in a substrate in a reaction. A substrate contains electrophilic centers with low electron density and a leaving group (LG). LG is a substituent that leaves as a stable molecule or ion during a nucleophilic attack. Halogens in alkyl halides are good LG because they can leave as weak bases and stable ions. Factors that favor S_N2 reactions are unhindered substrate, polar aprotic solvents and strong nucleophiles. Substrates with bulky substituents will hinder the reaction, while polar protic solvents can protonate the nucleophile thereby decreasing its reactivity. The rate of reaction is increased by concentration of both the nucleophile and the substrate.[48]

4.2 Reaction mechanism for the *N*-Methylation of isatin

The reaction starts with the deprotonation of the halogenated isatin (**2**) by the base Cs_2CO_3, generating the isatin anion (**2-**). This anion then acts as a nucleophile, attacking the electrophilic carbon of iodomethane and at the same time iodine leaves and compound **3** is formed (Scheme 1).[49]



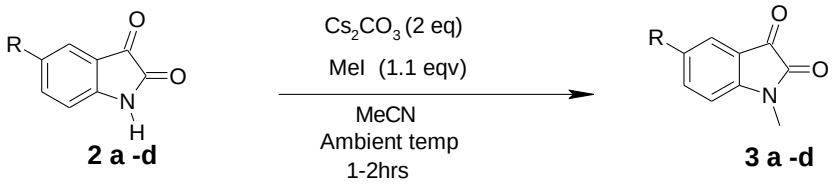
2 **2-** **3**

Scheme 1: alkylation of isatin

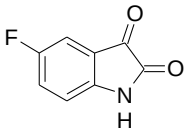
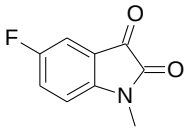
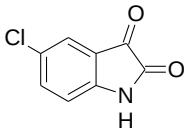
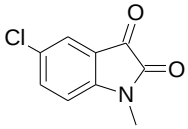
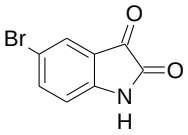
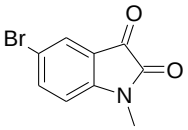
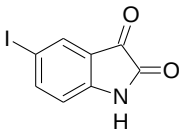
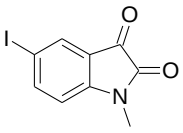
4.3 Method and Results

The synthetic route for the alkylations and the results are displayed in table 1. Compounds **3a, 3b and 3c** were obtained in good yields and used in the next step without further purification. Compound **3c** was impure and had to be purified. The purification was challenging because a solvent system suitable for the separation was not obtained. Hence, only a portion of it was isolated after purification leading to a lower yield.

Table 1 : *N*-methylation of isatin



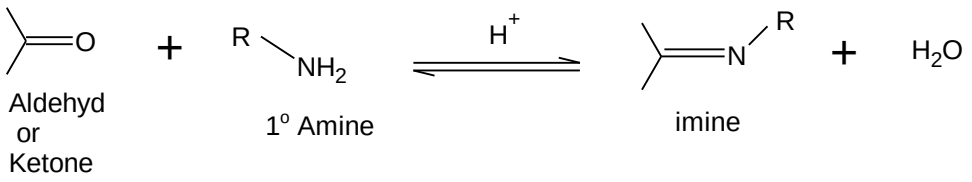
2 a -d **3 a -d**

Entry	Isatin	Product	Yield
1	 **2a**	 **3a**	**92%**
2	 **2b**	 **3b**	**96%**
3	 **2c**	 **3c**	**87%**
4	 **2d**	 **3d**	**58%**

5 Synthesis of halogenated analogues of 1

5.1 Imine formation

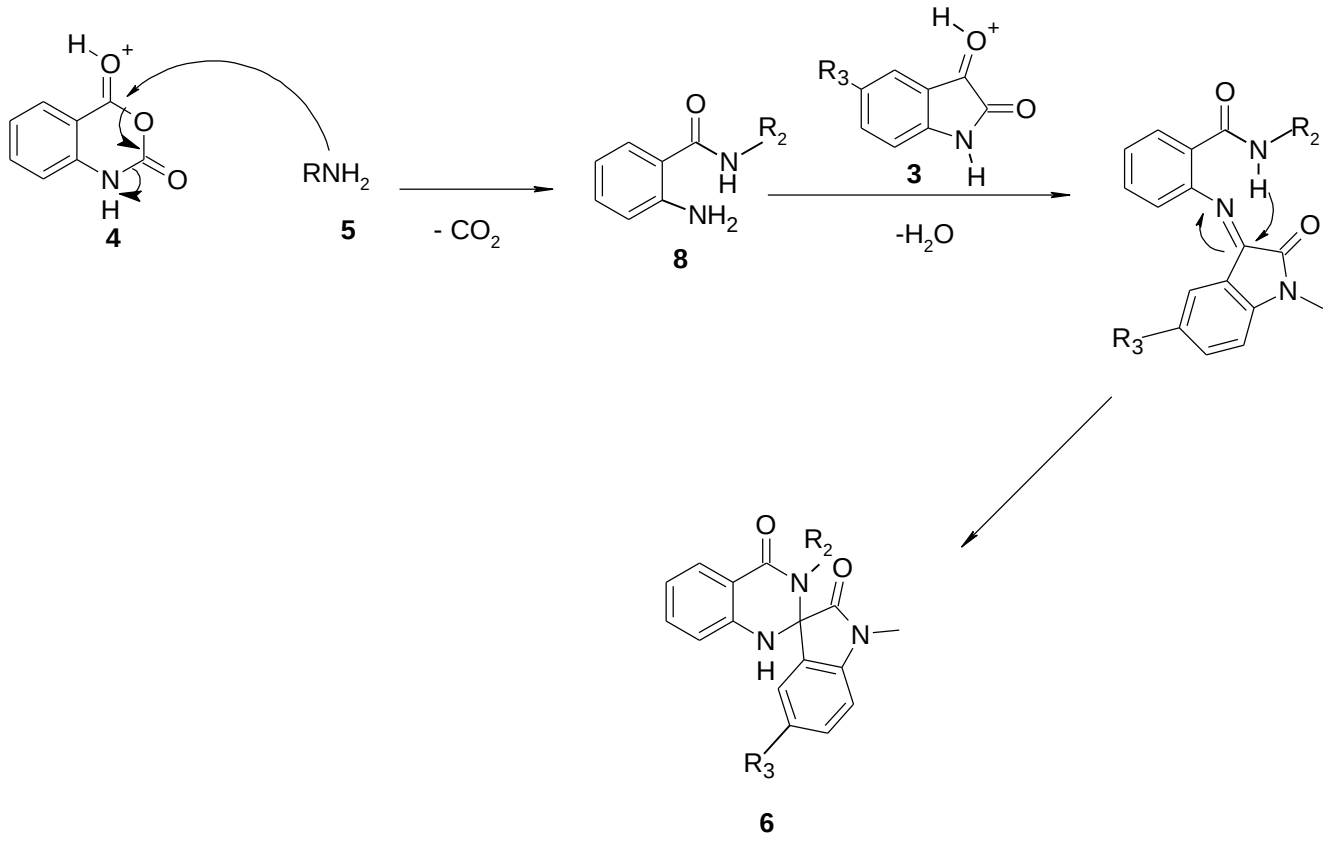
One main reaction that was important for the formation of the halogenated analogues was imine formation. An imine is formed from a primary amine and an aldehyde or ketone as shown in Scheme 2. The reaction is always catalyzed by an acid. The reaction is slow at very high pH, but fastest at pH 4 and 5. The acid converts a poor LG (an –OH group) to a good one (an $-OH_2^+$ group), hence elimination of water.[48]



Scheme 2: imine formation

5.2 Reaction mechanism

The reaction mechanism for synthesizing the halogenated derivates (**6a, b, d**) and other analogues of **1** (**6e-f**) which are synthesized in this project, proceeded through the following steps (Scheme 3): An acid catalyzes the reaction by protonating one of the carbonyl oxygen of **4**, making it more electrophilic. This process facilitates the neucleophilic attack by **5,** followed by a decarboxylation to form the intermediate (**8**). Another protonation ensues, this time on **3**. This makes it possible for **8**, which is equipped with a neucleophilic primary amine to attack the most electrophilic carbon in **3** and water is eliminated**.** An imine is subsequently formed by the reaction of the primary amine in **8** and the ketone in **3**. The imine undergoes intramolecular cyclization to give **6**.[50]



Scheme 3: Reaction mechanism for the formation of 6

5.3 Method

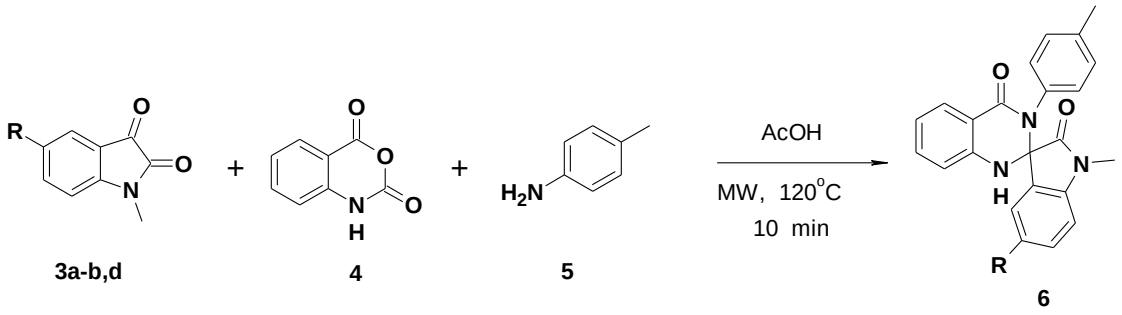
The synthesis of the halogenated derivatives was performed according to the scheme in Table 2. It involves a condensation reaction of the methylated isatin (**3,** 1 equiv) with isatoic anhydride (**4,** 1.2 equiv) and p-toulidine (**5,** 1.2 equiv) using acetic acid as a catalyst and solvent to form **6**. Structurally similar compounds similar to **6** have been synthesized by conventional reflux techniques, which have longer reaction times.[42,50-51] The optimum reaction time, the solvent and catalyst to be used had been developed in earlier studies. The irradiation temperature was determined in this project and it involved comparing the yield obtained after synthesizing one of the derivatives at two irradiation temperatures. The temperatures to be evaluated were 120^{o} C and 150^{o}C. The reaction that gave the highest yield could then be used to synthesize other halogenated compounds.

5.4 Results and discussion

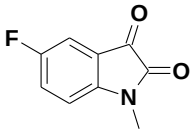
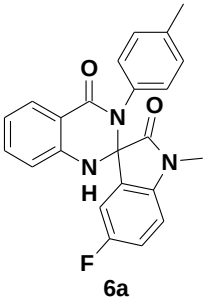
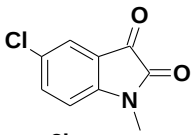
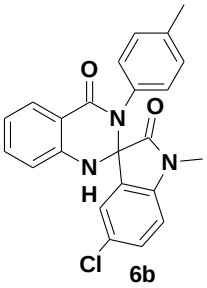
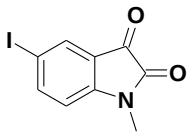
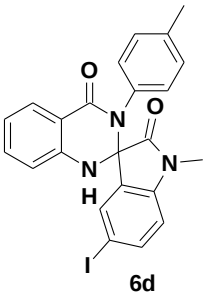
All the reactions were accomplished via the scheme in Table 4. The reaction time, solvent and catalyst were acquired from an earlier study. The irradiation temperature was to be determined by comparing the yields of two reactions performed at 120^{o}C and 150^{o}C. The results suggested that the 120^{o}C reaction was more suitable as it gave a yield of 52% as compared to the 150^{o}C whose yield was 45%. 120^{o}C was then used as the irradiation

temperature for the synthesis of the analogues. The products precipitated from the reaction mixture upon cooling to room temperature. The workup and purification procedures involved, filtering the precipitates from the reaction mixture, the obtained precipitates were thereafter resuspended in warm ethanol and left to cool to room temperature. The supernatant was thereafter filtered off and the precipitate washed with a small amount of chilled ethanol and pentane. The yields; 66% for **6a**, 65% for **6b** and 53% for **6d** are not as high given that the workup procedures explained above were so simple. The loss of product can be attributed to side reactions which formed by-products, poor handling of precipitate during the workup and purification steps. Using too much chilled ethanol may also have dissolved some of the product.

Table 2: Synthesis of **6a, 6b** and **6d**



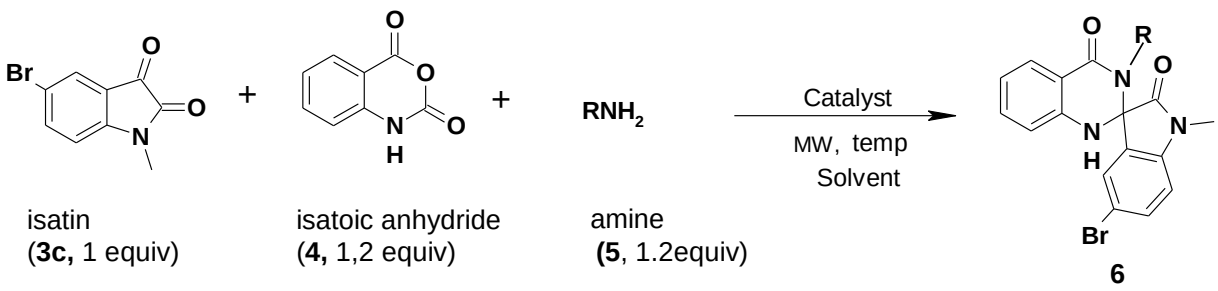
3a-b,d **4** **5** **6**

Entry	Isatin	Product	Yield
1	 **3a**	 **6a**	**66%**
2	 **3b**	 **6b**	**65%**
3	 **3d**	 **6d**	**53%**

6 Other modifications

6.1 Method development

In order to synthesize other analogues of **1**, a method had to be developed since the nucleophiles were rendered inactive by protonation by the solvent. As a starting point, the internet was scoured for methods used to synthesize structurally similar compounds.[42,50-51] These methods were then tried out in the microwave then optimized by changing reaction conditions such as temperature, reaction time and solvent (one reaction condition was changed at a time, while keeping the others constant). The objective was to develop a method that is simple, clean, and offers easy isolation and purification procedures, thus giving good yields. The synthetic route for all the one pot three-component methods attempted and the equivalent amounts of the reactant are shown in Scheme 4.



Scheme 4: Synthetic route and equiv amounts used for synthesis of 6e-f

6.2 Optimization results and discussion for the synthesis of 6e

Table 3 gives the results of the optimization procedures attempted. From the output, it is clear that a method that could give a pure product in good yield was not obtained in this study. An attempt was made to isolate and purify the traces of product seen in method 2 and 3, via flash column chromatography. The process turned out to be challenging, because the number of spots on the TLC plate, together with their position to each other (R_f range less than 0.2-0.3), made it difficult to come up with a suitable solvent system to use as an eluent. To succeed with this isolation, repeated purifications needed to be done and this would result in poor yields. Due this reasoning, methods 2 and 3 were not further purified and were hence abandoned.

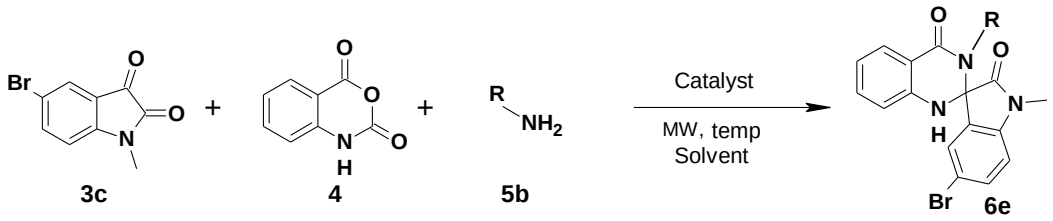
It was also not possible to establish an optimum temperature or time for the all the reactions carried out in the microwave, because increasing reaction time (from 10 min to 40min) or temperature (from 120^{o}C to150^{o}C) did not result in complete consumption of the limiting

reactant (**3c**). Its peak was still visible in the LC-MS report and this was going to impact on the yield had the methods been used.

Since the microwave reactions were unsuccessful, conventional reflux heating were tried (method 7 and 8). The results were not any better. The reactions were not clean, had multiple by-products and were not going to be easy to handle during the workup and purification steps.

Important information gained from the methods in table 6 was the presence of **8b** (intermediate) at the end of most of reactions. The peak of this intermediate was more pronounced compared to other peaks in several LC-MS reports. This gave the view that not much reaction was taking place after the formation of this intermediate. Thus, a decision was made to carry out the reaction in two steps. The first step involved synthesizing **8b**, then using it in the next step to form **6e**.

Table 3 : Results of the optimization reactions



Method	catalyst	solvent	time (min)	temp (^oC)	results (LC-MS & TLC)
1	EDDA 20% mole	water	10-20	120	no product
2	EDDA 20% mole	ethanol	10-40	120	traces of product, SM many spots on TLC plate
3	EDDA 20% mole	ethanol	10-20	150	"
4	20% AcOH (2 ml)	ethanol	10	150	no product
5	1% TFA (0.1ml)	ethanol	10-20	150	no product
6	50% AcOH (5 ml)	toulene	10	150	no product
7	EDDA 20% mole	ethanol	480 (8hrs)	80	traces of product, SM many spots on TLC plate
8	$SnCl_2$ (1:4[a])	ethanol	350 (5hr)	80	no product

[a] = Ratio of **3c** and $SnCl_2$

SM = starting materials

6.3 Optimization results and discussion for the synthesis of 8b

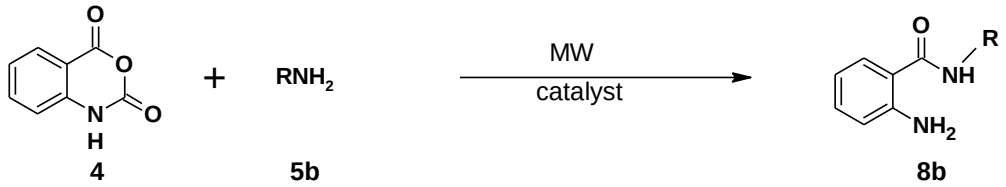
A summary of the results obtained after optimization is displayed in Table 4. In order to come up with a suitable procedure for the synthesis of **8b,** several methods were investigated. Acetic acid (AcOH) was used as a catalyst and solvent in method 1 and the reaction progress monitored by TLC and LC-MS after 5 minutes. The reaction temperature was set to 150^{o}C. LC-MS confirmed traces of the desired product at the end of the reaction. The TLC plate showed 4 spots: one spot belonging to the limiting reactant, the other to the product and the rest to the byproducts. These spots provided evidence that the reaction had most likely not gone to completion; therefore a number of different products had formed. The reaction time needed to be increased.

In the methods (2-6) that followed, the reaction time was increased to 10 min, and ethanol was, however, used as a solvent. The percentage of the catalyst (AcOH) used in the methods were 40%, 20%, 10%, 5% and 2% respectively. At the end of each reaction, LC-MS confirmed that the methods had produced the desired product, but the spots on the TLC plate varied. While method 2 (40% AcOH) and 6 (2% AcOH) showed 3 spots, the other methods (3-5) had only two spots. These results indicated that methods 3-5 formed one byproduct, because the other spot belonged to the product. On the other hand, method 2 and 6 had each produced 2 byproducts; therefore it was easier to isolate and purify the products formed when using methods 3-5. Isolation and purification of the product formed in method 3 yielded of 81% **8b**.

The trend was a little different when toluene was used as a solvent (methods 7-10). In these approaches, lowering the percentage of the catalyst i.e. 40%, 20%, 10% and 2% respectively, gave better results. Method 10 (2% AcOH) was purified via flash chromatography yielding 77% of **8b**.

Any method that produced a byproduct (method 3-5 and 8-10) with toluene or ethanol as the solvent can be used to give **8b**. Nevertheless, using a non polar solvent such as toluene in a microwave can be ineffective, especially in a case where there are no reactants in the reaction mix that can generate heat.[45] The yields presented in Table 4 (method 5 and 10) were calculated after purification by chromatography. In most cases the product (**8b**) was used in the next step without further purification. Method 5 was chosen to synthesize the intermediates (**8b-d)**. Even though the results from method 5 and 10 are similar (in terms of the number of spots on TLC plates), a higher yield was obtained in the former method. Moreover, the use of ethanol as a solvent was more effective in this case, because toluene sometimes gave errors in the microwave when the irradiation temperature for a reaction could not be attained. The optimum time for the synthesis of **8b** can vary from 5-10 min, though 10 min gave better results. The irradiation temperature used was 150^{o}C. Reactions at 120 ^{o} C were not tested.

Table 4: Optimization results for the synthesis of 8b



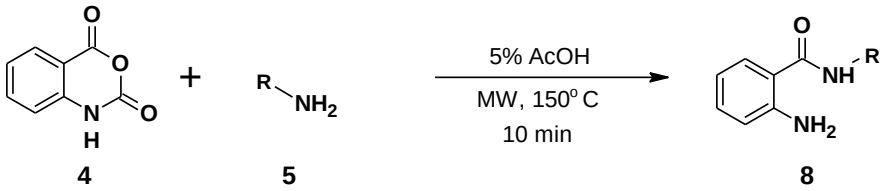
4 5b 8b

method	catalyst	solvent	time (min)	temp (^{o}C)	LC-MS & TLC	yield
1	AcOH	AcOH	5	150	Product present 4 spot on TLC plate	
2	40% AcOH	ethanol	10	150	Product formed 3 spots on TLC plate	
3	**20% AcOH**	**ethanol**	**10**	**150**	**Product formed 2 spots on TLC plate**	
4	**10% AcOH**	**ethanol**	**10**	**"**	**"**	
5	**5% AcOH**	**ethanol**	**10**	**"**	**"**	**81%**
6	2% AcOH	ethanol	10	150	Product formed 3 spots on TLC plate	
7	40% AcOH	toulene	10	150	Product formed 3 spots on TLC plate	
8	**20% AcOH**	**toulene**	**10**	**150**	**Product formed 2 spots on TLC plate**	
9	**10% AcOH**	**"**	**10**	**"**	**"**	
10	**2% AcOH**	**"**	**10**	**"**	**"**	**77%**

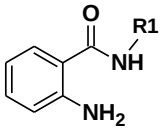
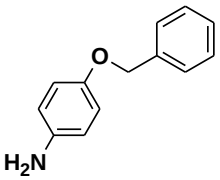
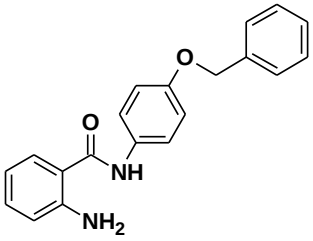
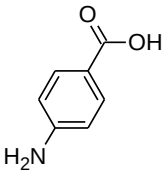
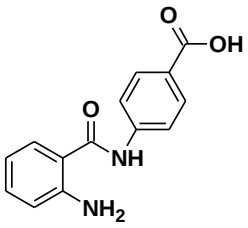
6.4 Results for the synthesis of 8b-d using the developed method

The results of the synthesis of **8b-d** are given in Table 5. 83% of **8b** and 88% of **8c** were obtained. **8d** had a low yield equivalent to 13%.The most probable reason for the poor yield can be linked to the electron withdrawing effect of the carboxylic acid substituent in **5d.** The substituent withdraws the electrons towards itself**,** making the amine less reactive as a nucleophile.

Table 5: synthesis of 8b-d using the method developed



4 5 8

entry	amine	intermediate	yield
1	 5b	 8b	83%
2	 5c	 8c	88%
3	 5d	 8d	13%

6.5 Optimization results and discussion of the of Synthesis of 6

In this optimization step, 5-bromo isatin was used instead of the methylated analog. This was done to avoid preparation of the methylated analog for each optimization procedure. Table 6 shows the synthetic route and optimization procedures used to develop a method for the synthesis of compounds **6e** and **6f.**

When toluene was used as a solvent, there were no noticeable changes observed in the LC-MS and TLC results, as the amount of the catalyst (AcOH) was increased from 20% to 40% (methods 1and 2). According to the LC-MS report the product had been successfully synthesized, but there were also traces of the limiting reactant and other byproducts. Some peaks in the LC-MS report disappeared when the reaction time was increased from 10 minutes to 30 minutes, but the limiting reactant's peak was still present. Furthermore, the two

methods were not clean, considering the fact that there was a black precipitate left on the filter during the workup procedures. Analysis of this precipitate revealed the presence of the product and other byproducts. The filtrate also contained traces of the product. Judging from the number of spots and their position on the TLC plate, together with the fact that the black precipitate was insoluble in most organic solvents, isolation and purification of the product was going to be difficult. Based on this reasoning, the method was abandoned.

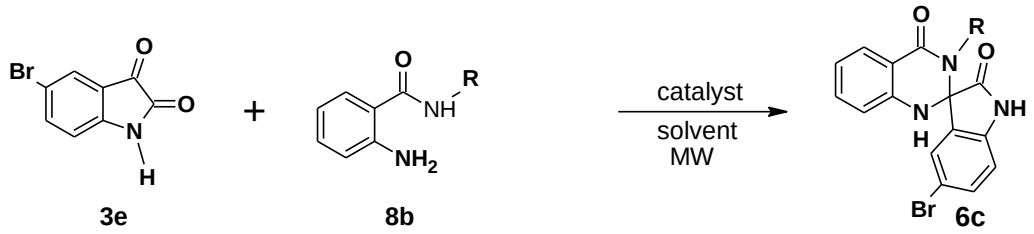
Using ethanol as a solvent (method 3) and 20% acetic acid as a catalyst did not produce a black precipitate however, this method was similar to the toluene reactions mentioned above, in terms of the number of spots on the TLC plate and the presence of the limiting reactant after 40 minutes of irradiation. This method was abandoned at this stage due to the presence of limiting reactant in the reaction mix (i.e. after 40 min reaction time), which could impact on the yield.

In the next optimization steps (methods 4-6) stronger catalysts, namely TFA and HCl, were in the reactions. The aim was to determine whether the limiting reactants could be consumed, so that better yields are obtained.

LC-MS and TLC analysis were performed after every 10 minutes of the reaction. By comparing the results (from TLC and LC-MS) of methods 4 (10% TFA) and 5 (5% TFA) to that of method 6, it could be concluded that there was no noticeable difference when TFA or HCl (1 drop) were used as catalyst. This outcome also suggested that the optimum time of the reaction lies between 30-40 min, because during this period, there is no significant change in terms of peaks in LC-MS report or spots on TLC plate. The yield was calculated to 25% for method 5 and 32 % for method 6 after purification.

The peak for the limiting reactant was still visible after 30 minutes in both methods. The workup procedures were similar to those used in purifying **6a, 6b** and **6d** due to the fact that products precipitated. These two methods were chosen and used to synthesize **6e**.

Table 6: Optimization results for the synthesis of **6c**



method	catalyst	solvent	temp (°C)	time (min)	details	yield
1	20% AcOH	toulene	150	30	dirty reaction	
2	40% AcOH	toulene	150	30	dirty reaction	
3	20% AcOH	ethanol	150	30-40	clean but limiting reactant present	
4	10% TFA	ethanol	150	10-50	same as 5 and 6	
5	**5% TFA**	**ethanol**	**150**	**10-40**		**25%**
6	**1drop HCl**	**ethanol**	**150**	**10-30**		**32%**

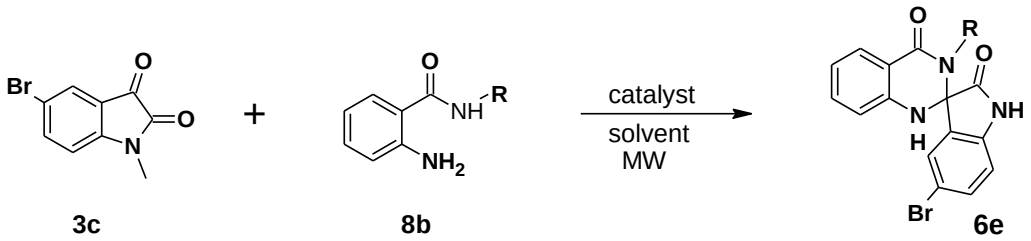
6.6 Results of the Synthesis of 6e using the method developed

The results in Table 7 demonstrate that the methods tested behaved differently when *N*-methylated bromoisatin (**3c)** was used; hence further optimization was required. The modification entailed a slight reduction in the amount of catalyst (see methods 3 and 4).

A black precipitate which was insoluble in most organic solvents and not easy to handle formed with 1 drop of HCl as a catalyst (method 1). After filtration and analysis (LC-MS) of this precipitate, traces of **6e** could be seen in both the precipitate and the filtrate. Since the isolation and purification of the product was going to be difficult given the nature of the precipitate, this synthesis approach was discontinued.

TFA reactions (methods 2-4) were cleaner with no black precipitates observed at the end of the reaction. On the other hand, methods 2 and 3 were not easy to purify. Purification through flash chromatography was not an option because these precipitates were insoluble in various organic solvents. A reduction of TFA by 1% (method 4) gave a precipitate which was easier to purify, yielding 40% of **6e**. This method was selected and used to synthesize **6e-f**.

Table 7: Optimization: Synthesis of **6e**



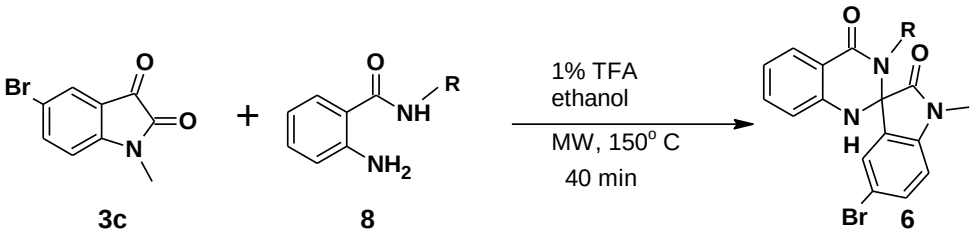
3c **8b** **6e**

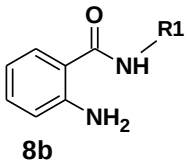
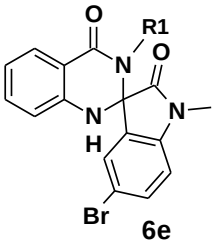
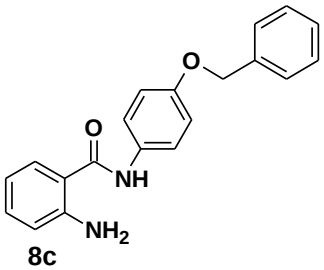
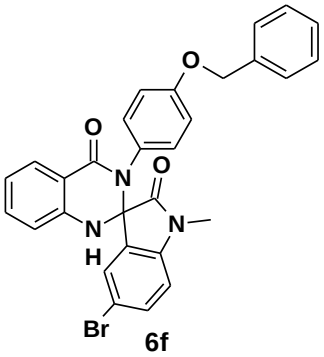
method	catalyst	solvent	temp (° C)	time (min)	details	yield
1	5% TFA	ethanol	150	30-40	Purification difficult	
2	1drop HCl	ethanol	150	30	reaction not clean black precipitate formed	
3	2% TFA	ethanol	150	30-40	Purification difficult	
4	**1% TFA**	**ethanol**	**150**	**30-40**		**40%**

6.7 Synthesis of 6e-f using the developed method

The developed method was used to synthesize **6e** and **6f** according to Table 8. The two products were obtained in 55% and 79% isolated yield, respectively. Since this reaction was scaled up, a higher yield was achieved for **8b** compared to that from the method development (see Table 7, method 4). Loss of product during the workup steps together with the formation of byproducts may have contributed to the lower yield of compound **6e.**

Table 8: Synthesis of 6e and 6f using the developed method



entry	8	6	yield
1	 8b	 6e	55%
2	 8c	 6f	79%

7 Biological evaluation

The inhibitory activities against IRAP of the synthesized analogues were evaluated by a biochemical assay. This assay estimated the potency of a substance by measuring the biological response that it produces. The potency is quantified by inhibition concentration 50 (IC_{50}) values, which is the concentration of the inhibitor which reduces a biological activity by 50%. This value can be derived from a concentration-response curve, where activity or response is plotted as a function of the inhibitor concentration. More potent inhibitors will have lower values. IC_{50} values can vary with changes in assay conditions such as pH, ionic strength of the solution and temperatures. Furthermore, the substrate concentration used for inhibitors of different types (competitive, non-competitive, and uncompetitive) can also impact on the IC_{50} value.[52]

7.1 Initial SAR study results

Initial SAR studies have revealed that having bromine in position 5 (Figure 6) is important for activity. Removal of bromine decreases the activity 10 fold, moving it from position 5 to the 7 gives an inactive compound and substituting it with different aryl ,vinyl and methyl groups results in weaker or inactive compounds. *N*-methyl group at position 1 is also important for inhibition, because removing it decreases the activity by more than 10-fold. Finally, a large group at the para position of the aniline (position 3') seems beneficial for activity.

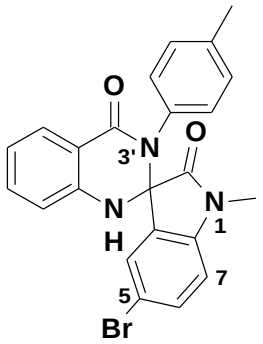
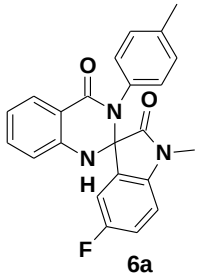
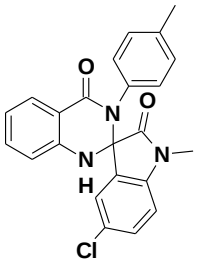
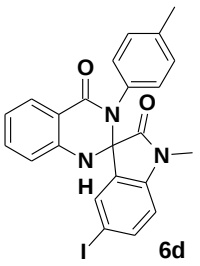
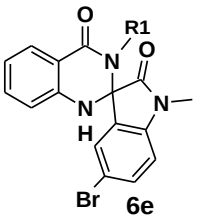
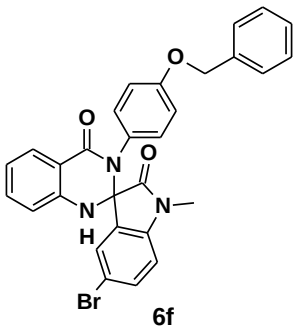


Figure 6: *Structure of the hit compounds with positions where SAR studies were performed indicated*

7.2 Biological assay results

All the analogues of hit compound **1** (**6a-6b** and **6d-f**) synthesized in this project were evaluated for their inhibitory activity against IRAP. The potency of each compound was quantified by the IC_{50} value. The lower this value the more potent it was. Table 9 presents the results of the assay. The results concur with the previous SAR studies that demonstrated that halogens together with the other important groups (see figure 6) mentioned in these studies are important for activity. Furthermore, halogen bonding could potentially be one of the major interactions between this part of the analogues (Figure 6, position 5) and IRAP. Halogen bonding is an interaction between halogen atom (acting as a Lewis acid) and electron donors such as a Lewis base. The strength of the interaction increases down the periodic table, with iodine having the strongest and fluorine the weakest. In other words, the strength of the halogen bonding increases with the size and polarizability of the halogen atom.[53] This type of bonding may explain why compound **6d** exhibited a higher potency (IC_{50} of 1 µM) than compound **6a** (IC_{50} of 6.4 µM). Compound **6f** also displayed inhibitory activity against IRAP however, the exact IC_{50} value was hard to determine due to the high lipophilicity of the compound which caused it to precipitate in the assay buffer especially at higher concentrations. Out of the 5 compounds investigated, compound **6e** emerged as the most potent with the lowest $1C_{50}$ value of 0.2 µM. The results suggest that **6e** is now the best inhibitor in the series of the analogues of **1**. This compound can now be a lead compound whose structure can be modified in order to come up with even better inhibitors of IRAP.

Table 9 : Biological evalution results

Entry	Structure	IC_{50}
1	 6a	6.4 µM
2	 6b	2.5 µM
3	 6d	1.0 µM
4	 6e	0.20 µM
5	 6f	Incomplete curve

8 Conclusion and Outlook

Cognitive enhancers aimed at treating memory dysfunctions brought about by head trauma, cerebral ischemia and diseases such as AD, PD or HD are required because the efficacy of currently approved drugs for treatment are such disorders are still unclear.

The discovery that inhibition of IRAP by Ang IV promotes learning and memory has made it a potential new target for drug intervention. Structurally modified analogues of Ang IV have been synthesized, and have proven to be potent inhibitors. However, these analogues are peptidic in character and may not cross the blood brain barrier.

A screening campaign of 10500 low molecular weight molecules has produced 5 hit compounds, one of them being spirooxindole dihydroquinazolinone **1**. In order to continue with the SAR studies, analogues of **1** were synthesized. While a method had been developed for the synthesis of the halogenated analogues (**6a, 6b and 6c**) from a previous study, synthesis of other analogues (**6e-f)** required method development.

The method developed offered the following advantages: clean, simple workup and purification procedures and fair yields. Better yields can be obtained by being careful when performing the workup steps. The flexibility of the method is limited, because an attempt to synthesize a compound with an electron withdrawing substituent gave poor yield. This implies that the synthesis of compound containing certain groups might require modification of the developed method or a new method needed to be development. Using a Lewis acid to catalyze the reactions is one approach that can be tested in a new method.

The biological evaluation results demonstrate that **6e** is the best inhibitor in the series with an IC_{50} value of 0.2 μM. This outcome suggests that the structure of **6e** can be used as a new lead compound when performing any future optimization so as to come up with more potent inhibitors. The solubility of these spirooxindole compounds need to be improved to make it possible for more advanced in vitro/in vivo studies to be performed. One way of enhancing solubility can be through incorporation of hydrophilic substituent in to the molecules.

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10 Experimental data

General

All reagents and solvents were purchased from Sigma Aldrich and used as supplied without further purification. The microwave assisted synthesis was done in Biotage® Initiator+ microwave using Biotage vials. The vials were available in the following sizes: 0.2-0.5 mL, 0.5-2 mL, 2-5 mL and 10-20 mL. The choice of vial depended on the volume of the reaction. Microwave apparatus had a single mode heating cavity. TLC was done on aluminum sheets precoated with silica gel 60 F-254 plates and visualized with UV-light. The plates were obtained from Sigma Aldrich. Flash chromatography was done on silica gel 60 (40-63 µM). Analytical HPLC-MS was performed on a Dionex UltiMate 3000 HPLC system with Bruker Amazon SL ion trap mass spectrometer and detection by UV(DAD) and MS(ESI+), using a Phenomenex Kinetex C18 column (50 x3.0 mm, 2.6 µM particle size, 100 Å pore size) and a flow rate of 1.5 mL/min. A gradient of H_2O/MeCN/0.005% HCOOH was used. ^1H and ^{13}C NMR were obtained on a Varian Mercury spectrometer (^1H: 400 MHz, ^{13}C: 101 MHz) using [D_6] DMSO or $CDCl_3$. The NMR spectra were recorded at 25^oC.

General procedure for the *N*-methylation of isatins

In a round- bottomed flask containing a magnetic stirrer, isatin (1 equiv) and Cs_2CO_3 (2 equiv) were dissolved in MeCN (0.05M). MeI (1.1 equiv) was then added in one portion from a syringe. The reaction mixture was stirred at ambient temperature for 2 h; the reaction progress was monitored by TLC (EtOAc:toluene (1:4→3:2)). After the reaction was complete, the solvent was removed under reduced pressure and the residue extracted with water and EtOAc (2x50 ml). The organic layers were combined, washed with brine, dried over $MgSO_4$, filtered and concentrated. Flash chromatography ((EtOAc:toluene (1:4→3:2)) was used for the products that required purification, otherwise, they were used in the next step without further purification. The identity and purity of the product was confirmed via LC-MS and NMR.

5-fluoro-1-methylindoline-2,3-dione (3a)

Following the general procedure, the product was synthesized from 5-fluoro-isatin **2a** (500 mg, 3.03 mmol), Cs_2CO_3 (1970 mg, 6.06 mmol) and MeI (0.21 ml, 3.33mmol) to give **3a** as reddish brown powder (500 mg, 92%).^1H NMR (400 MHz, $CDCl_3$) δ 7.36–7.29 (m, 2H), 6.86 (dd, *J*=8.0, 0.8, 1H), 3.25 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 182.8, 160.3, 158.2, 147.7, 124.9, 124.7, 118.20, 112.4, 26.2. LC-MS: *m/z*=179.81 (M^+ + 1)

5-chloro-1-methylindoline-2,3-dione (3b)

Following the general procedure, the product was synthesized from 5-chloro-isatin **2b** (500mg, 2.76 mmol), Cs_2CO_3 (1800 mg, 5.51 mmol) and MeI (0.19 ml, 3.03mmol) to obtain **3b** as a yellow product (520 mg, 96%).^1H NMR (400 MHz, DMSO-d_6) δ 7.72 (dd, *J*=8.4, 2.3 Hz, 1H), 7.59 (dd, *J*=2.3, 0.4 Hz, 1H), 7.18 (dd, *J*=8.4, 0.4 Hz, 1H), 3.13 (s, 3H). ^{13}C NMR (101 MHz, dmso) δ 182.3, 158.0, 149.9, 137.0, 127.3, 123.7, 118.8, 112.3, 26.1. LC-MS: *m/z* =195.80 (M^+ + 1)

Preparation of of 5-bromo-1-methylindoline-2,3-dione (3c).

Following the general procedure, the product was synthesized from 5-bromo-isatin **2c** (500 mg, 2.21 mmol), Cs_2CO_3 (1440 mg, 4.42 mmol) and MeI (0.15 ml, 2.43 mmol) to obtain **3c** as a reddish brown powder (460 mg, 87%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.85 (dd, *J*=8.4, 2.1 Hz, 1H), 7.69 (d, *J*=2.1 Hz, 1H), 7.12 (d, *J*=8.4 Hz, 1H), 3.12 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 182.6, 158.2, 150.7, 140.2, 126.9, 119.6, 115.3, 113.1, 26.5. LC-MS: *m/z*=240.05 (M^+ + 1).

Preparation of 5-iodo-1-methylindoline-2,3-dione (3d).

Following the general procedure, the product was synthesized from 5-iodo-isatin **2c** (500 mg, 1.83 mmol), Cs_2CO_3 (1200 mg, 3.70 mmol) and MeI (0.13 ml, 2.01mmol). **3d** was obtained as reddish brown powder **3d** (520 mg, 58%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 (dd, *J*=8.2, 1.8 Hz, 1H), 7.88 (dd, *J*=1.8, 0.5 Hz, 1H), 6.70 (dd, *J*=8.2, 0.5 Hz, 1H), 3.24 (s, 3H). ^{13}C NMR (101 MHz, $cdcl_3$) δ 182.0, 158.0, 151.0, 147.0, 134.0, 119.2, 112.2, 86.2, 26.5. LC-MS: *m/z*=287.78 (M^+ + 1)

General procedure for the synthesis of 6a, 6b and 6d

A mixture of **3** (1 equiv), **4** (1.2 equiv), **5a** (1.2 equiv) and 0.1M of AcOH in a suitable microwave vial containing a magnetic stirrer, was heated in the microwave for 10 min at 120^{o}C. LC-MS and TLC were used to confirm the identity and purity of the products. After completion of the reaction, the reaction mix was left to cool to room temperature. The workup and purification procedures included the following activities: filtering the precipitates from the reaction mix, re-suspending the obtained precipitates in warm ethanol then leaving it to cool to room temperature, filtering off the supernatant and finally washing the precipitate with a small amount of chilled ethanol and pentane.

5-fluoro-1-methyl-3´-(p-toly)-1´*H*-spiro[indole-3,2´-quinazoline]-2,4´(3´*H*)-dione (6a).

Following the general procedure, the product was synthesized from **3a** (200 mg, 1.12 mmol), **4** (220 mg, 1.34 mmol) and **5a** (120 mg, 1.34 mmol) in 11 ml AcOH to give **6a** in form of a white powder (300 mg, 66%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.68 (dt, *J*=7.8, 1.6, 1.4, 1H), 7.63-7.57 (m, 2H), 7.32 (ddd, *J*=7.3, 6.5, 1.6, 1H), 7.16-7.09 (m, 1H), 7.02 (d, *J*=8.4, 2H), 6.91-6.83 (m, 3H), 6.78 (ddd, *J*=7.4, 6.9, 0.8, 1H), 6.69 (d, *J*=8.0, 1H), 3.02 (s, 3H), 2.18 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.9, 163.4, 159.6, 157.1, 145.8, 139.4, 137.1, 135.1, 133.7, 129.1, 128.4, 127.5, 117.9, 117.4, 117.2, 114.5, 114.3, 114.1, 110.1, 76.4, 26.1, 20.6. LC-MS: *m/z*=388.09 (M^+ + 1)

5-chloro-1-methyl-3´-(p-toly)-1´*H*-spiro[indole-3,2´-quinazoline]-2,4´(3´*H*)-dione (6b).

Following the general procedure, the product was synthesized from 3**b** (200 mg, 1.02 mmol), **4** (200 mg, 1.23 mmol) and **5a** (110 mg, 1.23 mmol) in 10 ml AcOH to give **6b** as a white powder (270 mg, 65%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.73 (dd, *J*=2.2, 0.4 Hz, 1H), 7.68 (dd, *J*=7.7, 1.6 Hz, 1H), 7.62 (s, 1H), 7.36-7.30 (m, 2H), 7.04-7.00 (m, 2H), 6.91 (dd, *J*=8.5, 0.4 Hz, 1H), 6.88-6.83 (m, 2H), 6.78 (ddd, *J*=7.8, 7.4, 1.2 Hz, 1H), 6.68 (ddd, *J*=8.2, 1.2, 0.5 Hz, 1H), 3.02 (s, 3H), 2.19 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.7, 163.4, 145.8,

142.1, 137.1, 135.1, 133.7, 130.8, 129.3, 128.6, 127.6, 126.9, 126.3, 118.0, 114.5, 114.1, 110.6, 76.1, 26.1, 20.6. LC-MS: *m/z*=403.11(M^+ + 1)

5-iodo-1-methyl-3´-(p-toly)-1´*H*-spiro[indole-3,2´-quinazoline]-2,4´(3´*H*)-dione (6d).

Following the general procedure, the product was synthesized from **3d** (140 mg, 0.5 mmol), **4** (100 mg, 0.60 mmol) and **5a** (60 mg, 0.60 mmol) in 5 ml AcOH to give **6d** as a white powder (130 mg, 53%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.92 (d, *J*=1.7, 1H), 7.68 (dd, *J*=7.8, 1.4, 1H), 7.64-7.59 (m, *J*=8.4, 1.9, 2H), 7.33 (dt, *J*=7.0, 6.4, 1.6, 1H), 7.02 (d, *J*=8.3, 2H), 6.83 (d, *J*=8.0, 2H), 6.78 (td, *J*=6.9, 0.9, 1H), 6.73 (d, *J*=8.3, 1H), 6.68 (d, *J*=7.8, 1H), 3.01 (s, 3H), 2.19 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.4, 163.4, 145.8, 142.1, 139.4, 137.1, 135.1, 134.4, 133.7, 129.3, 129.1, 127.5, 118.0, 114.5, 114.1, 111.5, 85.7, 76.0, 26.01, 20.57. LC-MS: *m/z*=495.99 (M^+ + 1)

General procedure for 8b-d

A mixture of **4** (1 equiv), **5b-d** (1 equiv) and 5% (0.5 ml) AcOH(9.5 ml) in ethanol (10 ml) in a microwave vial containing a magnetic stirrer was heated in the microwave for 10 minutes at 150^{o}C .Identity, purity and characterization of the product was confirmed by LC-MS,TLC and NMR. If the product was deemed pure, it was used in the next step without further purification, otherwise flash chromatography (EtOAc:toluene (1:4→3:2)) or recrystallization were used to purify the product.

Synthesis of 8b

Following the general procedure the product was synthesized from a mixture of **4 (**0.20 g, 1.20 mmol), **5b** (0.13 ml, 1.20 mmol) and 5% (0.5 ml) AcOH(9.5 ml) in ethanol (10ml) to afford **8b** in form of a white powder (230 mg, 83%).^{1}H NMR (400 MHz, $CDCl_3$) δ 7.16–7.02 (m, 8H), 6.98 (ddd, *J*=8.3, 7.2, 1.4, 1H), 6.46 (dd, *J*=8.2, 0.9, 1H), 6.43–6.38 (m, 1H), 6.10 (s, 1H), 1.80 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.2, 149.0, 139.0, 133.0, 129.0, 128.0, 127.7, 127.21, 117.5, 116.7, 116.0, 43.6. LC-MS: *m/z*=227.04 (M^+ + 1)

2-amino-N-(4-(benzyloxy)phenyl)benzamide (8c)

Following the general procedure the product was synthesized from a mixture of **4 (**200 mg, 1.20 mmol), **5c** (240 mg, 1.20 mmol) and 5% (0.5 ml) AcOH(9.5 ml) in ethanol (10ml) to afford **8c** as a violet powder (340 mg , 88 %).^{1}H NMR (400 MHz, DMSO-d_6) δ 9.87 (s, 1H), 7.64-7.56 (m, 3H), 7.49-7.31 (m, 5H), 7.18 (ddd, *J*=8.4, 7.2, 1.5, 1H), 7.02-6.94 (m, 2H), 6.73 (dd, *J*=8.2, 1.0, 1H), 6.59 (ddd, *J*=8.3, 7.1, 1.1, 1H), 6.30 (s, 2H), 5.09 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.5, 154.4, 149.6, 137.2, 132.5, 131.9, 128.5, 128.4, 127.8, 127.6, 122.1, 116.3, 115.4, 114.6, 69.3. LC-MS: *m/z*=319.30 (M^+ + 1)

4-(2-aminobenzamido)benzoic acid (8d)

Following the general procedure, the product was synthesized from a mixture of **4 (**200 mg, 1.20 mmol), **5d** (160 mg, 1.20 mmol) and 5% (0.5 ml) AcOH(9.5 ml) in ethanol (10ml) to obtain **8d** in form of white crystals (40 mg, 13%). Purification was by recrystallization.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.26 (s, 1H), 8.01-7.81 (m, 6H), 7.74 (ddd, *J*=7.4, 6.6, 1.5, 1H), 6.76 (dd, *J*=7.5, 0.9, 1H), 6.60 (ddd, *J*=7.5, 7.0, 1.0, 1H), 6.36 (s, 2H). LC-MS: *m/z*=257.30 (M^+ + 1)

General procedure for the method developed to synthesize 6c

A mixture of **3e** (1 equiv), **8b** (1.2 equiv), 5% TFA in ethanol in a microwave vial, was heated in the microwave for 40 minutes at 150^{o}C. Identity and purity of the compound was confirmed by LC-MS and TLC. The workup and purification procedures involved, filtering the precipitates from the reaction mix, the obtained precipitates were thereafter resuspended in warm ethanol and let to cool to room temperature. The supernatant was thereafter filtered off and the precipitate washed with a small amount of chilled ethanol and pentane.

Synthesis of 6c

Following the general procedure, the product was synthesized from a mixture of **3e** (236 mg, 1.05 mmol), **8b** (283 mg, 1.25 mmol) and 5% TFA in ethanol in a microwave vial, to give **6c** as a white powder(143mg 32%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.53 (s, 1H), 7.72 (dd, *J*=7.7, 1.2, 1H), 7.52-7.45 (m, 2H), 7.28 (ddd, *J*=7.7, 6.6, 1.5, 1H), 7.20-7.13 (m, 4H), 6.92-6.85 (m, 2H), 6.80-6.74 (m, 2H), 6.63 (d, *J*=8.0, 1H), 4.68 (d, *J*=15.6, 1H), 3.95 (d, *J*=15.6, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.7, 163.8, 145.6, 141.6, 137.5, 133.9, 133.6, 129.3, 128.4, 127.9, 127.5, 127.3, 127.0, 117.9, 114.4, 113.9, 113.6, 112.4, 75.27, 45.8. LC-MS: *m/z*=436.0 (M^+ + 1)

General procedure for 6e & 6f

A mixture of **3c** (1 equiv), **8b-c** (1.2 equiv) and 1% TFA in ethanol in a microwave vial, was heated in the microwave for 40 minutes at 150^{o}C. Identity and purity of the compound was confirmed by LC-MS and TLC. The workup and purification procedures involved, filtering the precipitates from the reaction mix, the obtained precipitates were thereafter resuspended in warm ethanol and let to cool to room temperature. The supernatant was thereafter filtered off and the precipitate washed with a small amount of chilled ethanol and pentane.

Synthesis of (6e)

Following the general procedure, the product was synthesized from, a mixture of **3c** (0.12 g , 0.48 mmol), **8b** (0.13g, 0.48 mmol), and 1% TFA(0.05 ml) in ethanol(4,95 ml) to afford 6e in form of a white powder (130mg, 53%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.76 (dd, *J*=6.7, 1.1, 1H), 7.63 (dd, *J*=8.4, 2.1, 1H), 7.44 (s, 1H), 7.39 (d, *J*=2.0, 1H), 7.27 (ddd, *J*=8.3, 7.1, 1.5, 1H), 7.17-7.12 (m, 3H), 6.97 (d, *J*=8.4, 1H), 6.82-6.76 (m, 3H), 6.62 (d, *J*=8.1, 1H), 4.43-4.31 (m, 2H), 2.78 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.1, 163.9, 145.8, 143.3, 137.0, 134.3, 133.7, 129.1, 128.0, 127.9, 127.8, 127.7, 127.3, 118.3, 114.8, 114.6, 114.3, 111.6, 74.6, 45.7, 26.03. LC-MS: *m/z*=448.0 (M^+ + 1)

3'-(4-(benzyloxy)phenyl)-5-bromo-1-methyl-1'H-spiro[indoline-3,2'-quinazoline]-2,4'(3'H)-dione (6f)

Following the general procedure, the product was synthesized from, a mixture of **3c** (0.12 g , 0.48 mmol),**8c** (0.13g, 0.48 mmol), and 1% TFA (0.05 ml) in ethanol(4.95 ml) to afford **6f** as

a white powder (180mg, 79%).^{1}H NMR (400 MHz, DMSO-d_6) δ 7.84 (d, *J*=1.9, 1H), 7.68 (d, *J*=7.0, 1H), 7.62 (s, 1H), 7.49 (dd, *J*=8.4, 2.1, 1H), 7.42-7.29 (m, 6H), 6.91-6.82 (m, 5H), 6.81-6.76 (m, 1H), 6.68 (d, *J*=8.0, 1H), 4.99 (s, 2H), 3.01 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.5, 163.5, 157.4, 145.8, 142.4, 136.7, 133.8, 133.6, 130.4, 129.0, 128.9, 128.4, 127.87, 127.8, 127.5, 118.0, 114.8, 114.6, 114.5, 114.1, 111.1, 76.2, 69.3, 26.1. LC-MS: *m/z*=541.0 (M^+ + 1)

Biology

The screening campaign and the enzymatic assays were performed at Chemical Biology Consortium Sweden (CBCS) according to the procedure in the experimental section of Borhade *et al*.[54]

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