Design, Synthesis and Evaluation of Novel Diazirine Photolabels with Improved Ambient Light Stability and Fluorous-Based Enrichment Capacity

Arun Babu Kumar

University of South Florida, arunaumsc@yahoo.co.in

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Design, Synthesis and Evaluation of Novel Diazirine Photolabels with Improved Ambient Light Stability and Fluorous-Based Enrichment Capacity

by

Arun Babu Kumar

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
Department of Chemistry
College of Arts and Sciences
University of South Florida

Major Professor: Dr. Roman Manetsch
Dr. Mark McLaughlin
Dr. Jon Antilla
Dr. Abdul Malik

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DEDICATION

I would like to dedicate this work to my entire family. The emotional and moral support provided by my parents, wife and grandparents was of vital importance in making this dissertation to fruition. The amusement and laughter provided by my daughter was the much needed relaxation during the lean periods of research.
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ABSTRACT

Photoaffinity labeling is a quintessential technique in studying and analyzing the interaction between a ligand and receptor. Diazirines are one of the important photo-labile moieties used in photoaffinity labeling due to their superior photo labeling characteristics. Herein, we report the investigations we conducted with diazirine photolabels on (a) photochemical aspects leading to enhancement of their ambient light stability and (b) equipping them with fluorous tags to enable fluorous enrichment of labeled proteins. Furthermore, we report a pilot study to develop BACE-1 inhibitors, which have potential to be developed into photoaffinity probes.

3-Trifluoromethyl-3-phenyldiazirine offers good selectivity and protection against pseudolabeling but due to its photo lability, it undergoes decomposition even under ambient light. Thus the laboratory handling, including synthesis, of 3-trifluoromethyl-3-phenyldiazirine is cumbersome and restricted under constant darkness. Herein, we have designed, synthesized and evaluated two photolabels with enhanced stability to ambient light conditions in addition to the good selectivity and protection against pseudolabeling as offered by 3-trifluoromethyl-3-phenyldiazirine. It was also found that the aqueous solubility, a vital physical property for a photolabel, was also improved in the modified ambient light stable photolabels.

Fluorous tags have found wide use in synthetic applications; herein we explore the possibility of its application in photoaffinity studies. We designed, synthesized and conducted photoactivation studies on two fluorous diazirine photolabels. The photoactivation studies unraveled an unanticipated photoreaction when the fluorous tag
was directly connected to the diazirine ring, yielding a fluorous alkene. The more practical photolabel of the two was chosen as the target specific photoaffinity labeling moiety for fluorous proteomics. Upon conducting photolabeling experiments under various conditions, we found that the strong hydrophobic character of the fluorous tag renders the photoaffinity label insoluble in aqueous solutions and significantly alters the binding mode and affinity of the photoaffinity label to its target receptor.

A library of 1,3-disubstituted 2-propanols was combinatorially prepared and tested as small molecule inhibitors of β-secretase (BACE-1). The initial screening of the 1,3-disubstituted 2-propanol library revealed a few low micromolar inhibitors for BACE-1. The compound that showed the best activity was chosen for further SAR studies, which resulted in a potent BACE-1 inhibitor with nanomolar inhibition. Investigation on the selectivity of these compounds for BACE-1 inhibition over cathepsin D revealed that these compound series possess very high selectivity. Furthermore, the physicochemical properties study showed that these compounds possessed the calculated parameters advantageous to cross the blood–brain barrier (BBB).
CHAPTER – 1

Introduction

1.1 Photoaffinity labeling and diazirine as photolabel

Comprehending the molecular interaction between ligands and proteins is of vital importance in numerous fields including chemical biology, drug design and discovery. When crystallization of the protein bound with a ligand is possible, X-ray crystallography is a very valuable technique in understanding the nature of the ligand-protein interaction. NMR is another technique that is applied in understanding the molecular interaction between a ligand and protein. Although these techniques offer valuable information, they require having the protein at a reasonable purity. In comparison to NMR-based studies, for X-ray crystallography it is essential that the protein-ligand complex is crystallizable and the protein-ligand interaction in solution cannot be inferred. In the early 1960s, Westheimer and co-workers first introduced the technique called photoaffinity labeling, in which the photoaffinity label possess a photolabile group.\(^1\) In concept, the photoaffinity label would possess three vital components: (i) a ligand, specific to the target protein to guide the photoaffinity label, (ii) a photolabile moiety, which would form a covalent link between the label and target protein upon photoactivation and (iii) a purification or a detection tag that could be used upon post labeling for purification or detection of labeled protein.\(^2,\)\(^3\) Upon addition of the photoaffinity label to the protein of interest, the label binds to the protein due to the affinity of the ligand and upon photoactivation, the photolabile group forms a reactive moiety which forms a covalent bond with the bound protein (Figure-1.1). The protein that has been cross-linked with the probe can be
subjected to mass spectroscopic analysis to identify the protein and the binding region of the protein.\textsuperscript{2} Since labeling of the protein happens after the ligand of the photoaffinity probe binds to the protein of interest, it is not necessary to have the protein of interest pre-purified. Rather the whole proteome can be subjected to the photolabeling and only the targeted protein would get labeled, which could be purified based on enrichment tag attached to the photoaffinity label.

**Figure 1.1.** Pictorial representation of photoaffinity labeling

Since the days when the concept of photoaffinity labeling was introduced by Westheimer five decades ago, the technique has developed rapidly, finding applications in chemical biology, proteomics, medicinal chemistry, drug design and discovery.\textsuperscript{2-4} The majority of the photoaffinity labeling applications are found in elucidating the biological target of action for potent drugs and identifying the binding pocket or active site. Photoaffinity labeling can also be used for the identification of secondary protein targets that causes toxicity or side effects when binding to a potential drug candidate.\textsuperscript{5} The photolabile groups that are commonly used in photolabeling are diazirines, aryl azides and benzophenones. Upon UV light activation, each of these moieties generates reactive species which can rapidly react with the bound protein forming a covalent linkage, as shown in Scheme 1.1.
Scheme 1.1. Photoactivation and labeling pattern of diazirine, aryl azide and benzophenone photolabels

Each of these photolabels has advantages and disadvantages. In general aryl azides are commonly associated with ease of synthesis and commercial availability, but suffer lower efficiency in photolabeling and the need to photo activate at a wavelength $\lambda < 300$ nm, at which proteins disintegrate. Benzophenones have the advantage of ease of handling, commercial availability and resistance to water deactivation during photolabeling. But its shortcomings such as steric bulk and lipophilicity can alter the binding of the ligand to target receptor. Furthermore, prolonged exposure to UV light is required for photoactivation, which can lead to protein disintegration. Trifluoromethylphenyl diazirines have the convenience of photoactivation at a protein
benign long wavelength UV (λ > 320 nm), shorter UV activation time, robust chemical stability in the dark, predictable photo crosslinking and relatively small steric. But it falls short on synthetic accessibility, since the construction of the diazirine ring takes at least five steps from the starting aryl bromide.

In spite of the synthetic challenge posed by diazirines, they are still one of the preferred photolabeling moieties due to the superior photolabeling characters mentioned above. Diazirines were first identified as potential photolabels by Knowles and co-workers in the 1970s. When a diazirine is photo activated with long wavelength UV light, it generates the desired carbene which can rapidly label the bound receptor due to its high reactivity. In addition the undesired side product the linear diazo intermediate is also formed, which upon further irradiation with UV light (λ > 320 nm) slowly generates the carbene (Scheme 1.2).

Scheme 1.2. Formation of carbene and linear diazo intermediate during photo activation of diazirine

The ratio and stability of the carbene to diazo intermediate is of vital importance because it not only reduces the amount of carbene formation it also can lead to pseudo labeling. Since the linear diazo compound is less reactive than the carbene, upon its formation it would not react with the bound protein immediately. Rather it might
dissociate from the binding pocket and then react non-specifically to other parts of the protein or altogether a different protein due to its sensitivity to nucleophilic attack.\(^\text{14}\) Aromatic diazirines, in which the aromatic ring is directly connected to the diazirine ring, have been previously reported to yield a higher carbene to linear diazo ratio during photoactivation than compared to aliphatic diazirines.\(^\text{14-18}\) In 1980 Brunner and co-workers modified the aromatic diazirines by incorporating a trifluoro moiety on the diazirine carbon, since it renders the undesired side product, the linear diazo species, less susceptible to nucleophilic attack during the photolysis of the diazirine.\(^\text{14}\) When a diazirine is subjected to photo activation it can produce two types of carbenes: (a) a singlet carbene, in which both nonbonding electrons are in the same orbital and (b) a triplet carbene, in which the two electrons occupy two different orbitals. For the purpose of photolabeling, the formation of a singlet carbene is advantageous since it inserts more rapidly with any type of bond (C-H, O-H and N-H) in the near proximity than the triplet carbene.\(^\text{9}\) However, in accordance with the Hund’s rule, the triplet state with two electrons in different orbitals is more stable than the singlet state with two electrons in the same orbital.\(^\text{9}\) This makes the triplet carbene as the ground state and singlet carbene as the excited state with a energy difference of 8 to 10 kcal/mol between them.\(^\text{19-22}\) Phenyl substitution on the diazirine can reduce this energy difference to about 2 to 4 kcal/mol with the triplet carbene still as the ground state.\(^\text{23}\) However, the small energy difference makes the equilibration between the two states very rapid. In fact several studies with phenyl derived diazirines has showed that the majority of the photo activated adduct was formed through the singlet carbene.\(^\text{24-26}\) Due to these reasons, derivatives of 3-trifluoromethyl-3-phenyl-diazirine (Scheme 1.2) have become the most widely used photolabeling moiety among the diazirine photolabels.
1.2 Alzheimer’s disease and BACE-1

Just over a decade ago Alois Alzheimer (1864–1915) identified a type of dementia caused by neurodegeneration in the brain and subsequently named after him as Alzheimer’s disease (AD). Although Alzheimer’s disease was not a major concern in the early days of its discovery, it rapidly gained attention and became one of the most devastating medical phenomenon due to the ever growing life expectancy. AD is reported to be responsible for about 80% of all cases of dementia in patients over 65 years and it has affected over 35 million people globally, with medical care costing over 100 billion annually.\textsuperscript{27, 28} Due to the continuing increase in life expectancy, these figures are predicted to increase rapidly, such that 1 in 85 of the world’s population will be affected by AD in 2050. Although the exact mechanistic pathway for the cause of AD is not understood completely, it is generally accompanied by the deposition of a neurotoxic, 42 amino acid long peptide called amyloid β (Aβ) in the brain.\textsuperscript{29-32} A debate that is as old as the disease itself is whether the deposition of amyloid β in AD affected brain is the cause of the disease or just the symptom of the disease. Amyloid β is produced by the cleavage of the amyloid precursor protein (APP), a trans-membrane protein, by two aspartic acid proteases called β-secretase (BACE-1) and γ-secretase. To stop the production of amyloid β peptide and thereby treating AD, inhibition of BACE-1 and β-secretase is considered to be a viable strategy. One of the most convincing evidence that targeting BACE-1 inhibition is a highly promising strategy in treating AD, is based on the studies with BACE-1 knockout mice.\textsuperscript{33-36} It was found that BACE-1 knockout mice had significantly reduced amyloid β production and lacked the characteristic pathology observed with AD. The BACE-1 knockout mice that were genetically altered to produce excess APP also did not produce significant deposits of amyloid β in the brain, proving that there is no alternate cleavage mechanism to produce
amyloid β in the absence of BACE-1. The BACE-1 knockout mice were examined extensively to evaluate if the removal of BACE-1 gene would cause any physiological side effects on them and found that they were anatomically healthy and no structural change in any vital organs was identified. These data proved that inhibition of BACE-1 is highly likely to result in the reduction of amyloid β production in humans. Further, the lack of side effects in the absence of BACE-1 is a good indication that the inhibition of BACE-1 in humans would not result in any adverse effects.

The discovery of BACE-1 inhibitors has its own set of challenges, since the inhibitors need to occupy an elongated active site, possess selectivity over other aspartic proteases and have physicochemical properties to pass through the blood brain barrier (BBB). The general strategy in finding inhibitors that can occupy the elongated active site of BACE-1 is to replace the analogs of the natural substrate with an in-scissible isostere. Based on this strategy a huge array of potent peptidomimetic inhibitors were developed for BACE-1.\textsuperscript{37-39} But the peptide based inhibitors pose challenges like oral availability, lower stability, higher molecular weight, poor blood brain barrier permeability and higher costs that arise due to the chirality of the peptides. These factors underline the importance of developing small molecular scaffolds for the inhibition of BACE-1. But the first hurdle is to identify small molecules that can efficiently occupy the elongated active site of BACE-1. Although a significant part of the past two decades was dominated by peptide based inhibitors for BACE-1 due to the lack of small molecule inhibitors, recently there have been discoveries of a few new small molecular scaffolds, with BACE-1 inhibitory activity.\textsuperscript{40-45} Aminohydantoins, aminomidazoles, and acylguanidines are selected examples of small molecular scaffolds that have recently showed good potency as inhibitors of BACE-1 (Figure 1.2).\textsuperscript{46-49}
Figure 1.2. Previously reported small molecules with potent BACE-1 inhibition

Though these small molecules possess very potent activity against BACE-1, they can be considered as viable candidates for clinical studies only if they exhibit selectivity over other essential aspartic proteases. Although there are only a limited number of aspartic proteases in mammalian system, cross inhibition of an essential protease by a BACE-1 inhibitor could pose severe side effects. Apart from exhibiting selectivity for BACE-1 the small molecular inhibitors need to possess the essential physicochemical properties to penetrate the blood brain barrier in order to reach BACE-1. Due to these tough requirements there is no small molecule inhibitor of BACE-1 that is available currently or in the immediate future for the treatment of AD.

1.3 Research Aims

1.3.1 Development of ambient light stable 3-trifluoromethyl-3-aryldiazirine photolabel

3-Trifluoromethyl-3-phenyldiazirine photolabels are one of the most preferred photolabeling moieties due to the superior photolabeling properties mentioned earlier. Nevertheless, they still share some of the disadvantages of other photolabels like
instability to ambient light conditions, lipophilicity and limited aqueous solubility. 3-Trifluoromethyl-3-aryldiazirine photolabels have the potential to possess the characteristics of an ideal photolabels if these drawbacks could be eliminated. Our research effort was to design, synthesize and evaluate a novel series of 3-trifluoromethyl-3-aryldiazirine photolabels that could overcome these disadvantages, while retaining the superior photolabeling properties. The design of modified photolabels involves the incorporation of heterocyclic rings in place of the phenyl ring of 3-trifluoromethyl-3-aryldiazirines. Since the ongoing antimalarials research of our lab involves similar heterocyclic residues, the future research in our lab will focus on the application of these enhanced photolabels in identifying the mode of action of these potent antimalarials.

1.3.2 Enhancement of photoaffinity labeling via fluorous enrichment

Fluorous enrichment has found wide application in natural product synthesis, catalysis, microarrays, combinatorial chemistry, peptide labeling and peptide purification. However, their potential application as enrichment tags in photoaffinity labeling is under explored. Our endeavor to explore the application of fluorous enrichment in photoaffinity labeling involved the design, synthesis and photoactivation studies of fluorous tag derivatized diazirine photolabels.

1.3.3 1,3-Disubstituted 2-propanols as small molecule inhibitors of BACE-1

Although the aspartic protease BACE-1 has emerged as one of the leading target towards the treatment of Alzheimer’s disease, there is no viable drug that is available to treat Alzheimer’s disease by inhibiting BACE-1. Extensive and expansive screening is required to identify small molecules that could weather the challenges like potency, selectivity and blood brain permeability. 1,3-Disubstituted 2-propanols have been
reported earlier to be small molecule inhibitors of BACE-1 with moderate activity. Due to
the ease of synthetic accessibility of this molecular scaffold, we decided to conduct a
structure activity relationship (SAR) study on 1,3-disubstituted 2-propanols in inhibiting
BACE-1 and investigate their selectivity for BACE-1 over other aspartic proteases.

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CHAPTER – 2

Bringing 3-trifluoromethyl-3-aryl diazirine photolabeling out of the “dark” age

Photoaffinity labeling is a powerful technique to investigate the substrate interaction with a binding pocket or active site of target receptors which are otherwise unyielding to crystallize. Most notable photoactivatable moieties that are conventionally used in photoaffinity labeling are 3-trifluoromethylphenyl-3-diazirines, benzophenones and phenyl azides.¹ None of these or other photoactivatable moieties fulfill all the requirements of an ideal photolabel² which are (a) ease of synthesis, (b) chemical stability prior to photoactivation, (c) high aqueous solubility, (d) readiness for photoactivation at wavelengths (λ > 320 nm) that will inflict minimal damage to the proteins, (e) capacity to provide a photoactivated intermediate that will react with C–H or X–H bonds without preference and prior to the disengagement of the ligand from the receptor, (f) capability to produce an unambiguous covalent conjugate with receptor, (g) steric compactness which does not alter the binding of the ligand to receptor, and (h) stability to ambient light.¹ ² Since the early 1960s, when Westheimer and co-workers first introduced the technique photoaffinity labeling, the field of photolabeling studies is commonly associated with the cumbersome process of working in darkness. This inconvenience arises due to the photolability of the photoaffinity probes to ambient light conditions.

Trifluoromethylphenyl diazirines and benzophenones are the most commonly used photoactivatable moieties because of their ability to produce reactive intermediates upon photoactivation, which can react with the receptor to give a photoproduct with
predictable uniformity.\textsuperscript{1, 3} Both diazirines and benzophenones are photoactivatable at a UV wavelength higher than 320 nm, while arylazides require activation at wavelengths below 300 nm at which the proteome possibly disintegrates. Despite the advantages of using benzophenone, its use is partially restricted due to the longer irradiation times required for photolabeling, its steric bulk which can alter the ligand binding efficiency to the receptor, and the high lipophilicity of the two phenyl groups which considerably affect the aqueous solubility.\textsuperscript{2, 4} In contrast, diazirines have several advantages such as predictable photolabeling, relative small sterics, chemical stability in the dark, short irradiation time required for photolabeling, and photoactivatability at proteome benign long wavelength UV making diazirines the preferred choice in developing photoaffinity probes.\textsuperscript{3, 4} Nevertheless, diazirines suffer from disadvantages like instability to ambient light conditions and limited aqueous solubility due to the lipophilicity of its functional groups.

2.1 Design of an ambient light stable diazirine photolabel

The photolability of the diazirines arises from the ring strain in the three member ring system. Thus, we hypothesize that imparting a certain degree of stability to the diazirine ring system will increase its stability towards less energetic ambient light conditions while retaining the capability for photoactivation with UV light ($\lambda = 320$ to 400 nm). As reported by Liu and coworkers, the stability of three member cyclic structures can be enhanced by substituting them with electron withdrawing groups.\textsuperscript{5} Since ring strains of small cyclic structures can be reduced by removing the electron density,\textsuperscript{5, 6} we focused on stabilizing the diazirines by this means. One of the substituents on the original diazirine photolabel is the trifluoro group which is already a strong electron withdrawing group. Therefore, the phenyl group should be substituted by an electron withdrawing moiety. Previous studies have indicated that aromatic diazirines produce
higher carbene to diazo ratios when compared to aliphatic diazirines.\textsuperscript{3, 7-11} Therefore, it is imperative to find an electron withdrawing aromatic residue to replace the phenyl group to keep the carbene to diazo ratio unaffected. By keeping the above considerations in mind we chose pyridine and pyrimidine substituents as suitable electron withdrawing replacements for the phenyl ring. It has been reported earlier that electron withdrawing groups can also render the unwanted diazo intermediate less susceptible to nucleophilic attack.\textsuperscript{3} So in addition to stabilizing the diazirine ring the electron withdrawing aromatic rings will reduce the reactivity of diazo intermediates thereby lowering the unwanted pseudolabeling. With this understanding, we designed photolabel 1 with a pyridine ring and photolabel 2 with a pyrimidine ring in place of the phenyl ring (Figure 2.1). For comparative studies, we also prepared the conventional photolabel 3 as reported previously.\textsuperscript{12}

![Figure 2.1](image.png)

**Figure 2.1.** Design of ambient light stable 3-trifluoromethyl-3-aryl diazirine

### 2.2 Synthesis of modified 3-trifluoromethyl-3-aryl diazirine photolabels 1 and 2

The synthesis of the modified photolabel 1 began with the t-butylidimethylsilyl (TBS) protection of alcohol 4 to silyl ether 5 followed by the lithiation with n-butyllithium, which was treated with methyl trifluoroacetate to obtain ketone 6 (Scheme 2.1). Ketone 6 was converted to the tosyl oxime 7 by reaction with hydroxyl amine hydrochloride and sodium acetate followed by a tosylation with tosyl chloride. Tosyl oxime 7 can be subjected to a cyclization using liquid ammonia to give diaziridine 8, which can be oxidized using freshly made silver(I) oxide to diazirine followed by silyl deprotection to
get the desired modified photolabel 1. The synthesis of the modified photolabel 2 started with alcohol 9 and was subjected to the same synthetic route that was used for 1. During the course of the synthesis we found that the yield for the conversion of the ketone to tosyl oxime was very poor yielding, only about 10% of product. After several modifications of the tosylation reaction, we found that the protection of the alcohol as t-butyldiphenylsilyl (TBDPS) ether instead of TBS ether resulted in better yield at sub-zero reaction conditions. The modified photolabel 2 was synthesized from the resultant tosyl oxime 12 by the use of similar synthetic strategy that was employed in the synthesis of 1.

Scheme 2.1. Synthesis of modified photolabels 1 and 2

2.3 Photoactivation studies to compare photolabels 1 and 2 against original photolabel 3

As shown in Figure 2.2, upon photoactivation the diazirine is converted to the singlet carbene, which in turn forms an insertion product with the solvent, and the linear
diazoo compound. Due to the high reactivity of the carbene, it can rapidly react with the bound receptor before the activated photoaffinity probe dissociates. The linear diazo compound is the undesired side product due to its lower reactivity, thereby leading to pseudolabeling upon dissociation from the binding receptor. Thus, the ratio of carbene to linear diazo ratio is of quintessential importance when evaluating the efficiency of diazirine photolabels. To compare this ratio between the conventional 3-trifluoromethyl-3-aryl diazirine photolabel 3 and modified photolabels 1 and 2, they were subjected to photoactivation studies according to the previously established method.\textsuperscript{14-16} A solution of diazirine photolabel in methanol-d\(_4\) was subjected to UV light irradiation (\(\lambda > 320\) nm) and the progress of the photoactivation was monitored by \(^{19}\)F NMR spectroscopy.

\textbf{Figure 2.2.} Photoactivation path of diazirine photolabels

\textbf{Figure 2.3.} Photoactivation product distribution at different time intervals of UV exposure
As evident from the photoactivation product distribution chart (Figure 2.3), the exchange of the phenyl ring in the 3-trifluoromethyl-3-phenyl diazirine by the electron withdrawing pyridine or pyrimidine ring does not affect the carbene to linear diazo intermediate ratio. Across the control 3 and the modified photolabels 1 and 2, the linear diazo intermediate was fairly constant at about 30%. This indicates that the photolabeling efficiency, with respect to the ratio of carbene to linear diazo ratio, will not be affected by switching the phenyl moiety with pyridine or pyrimidine ring system.

2.4 Ambient light stability of modified photolabels 1 and 2

We subjected the modified 3-trifluoromethyl-3-aryl diazirine photolabels 1 and 2 to an ambient light stability test along with the conventional 3-trifluoromethyl-3-phenyl diazirine photolabel 3 to determine the effectiveness of our modifications to increase the light stability of diazirine photolabels. Solutions of the conventional diazirine 3 and modified photolabels 1 and 2 in methanol-d₄ was exposed to ambient light conditions, directly under a linear fluorescent lamp, and the rate of decomposition was observed over a period of one month using ¹⁹F NMR spectroscopy. After 7 days of exposure to ambient light, the conventional diazirine 3 had already undergone significant photodecomposition, as shown by the circled ¹⁹F NMR signal in Table 2.1. In contrast, during the same period of ambient light exposure the modified photolabel 1 had undergone very negligible photodecomposition while the modified photolabel 2 had virtually remained unaffected. We observed that modified photolabels 1 and 2 showed significant resistance against photodecomposition even after one month of ambient light exposure, while the conventional photolabel 3 had undergone heavy decomposition. As estimated by ¹⁹F NMR (Figure 2.4), only about 25% of the conventional photolabel 3 was unaffected after a month of ambient light exposure. On the contrary, about 80% of the modified photolabel 1 was intact and over 90% of the modified photolabel 2 was

22
unaffected upon the same duration of ambient light exposure. This data shows that our hypothesis of increasing the ambient light stability of diazirine photolabel by stabilizing the diazirine ring with electron withdrawing substitution has been proven to be effective.

Table 2.1. Comparison of ambient light stability of modified trifluoromethylaryl diazirines versus the conventional trifluoromethylphenyl diazirine

<table>
<thead>
<tr>
<th>Duration of ambient light exposure</th>
<th>0 day</th>
<th>7 days</th>
<th>31 days</th>
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<tbody>
<tr>
<td><img src="image1.png" alt="Diagram" /></td>
<td><img src="image2.png" alt="Diagram" /></td>
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<td><img src="image11.png" alt="Diagram" /></td>
<td><img src="image12.png" alt="Diagram" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Duration of ambient light exposure</th>
<th>% diazirine intact</th>
<th>% diazirine intact</th>
<th>% diazirine intact</th>
</tr>
</thead>
<tbody>
<tr>
<td>time (days)</td>
<td>3 OH</td>
<td>1 OH</td>
<td>2 OH</td>
</tr>
<tr>
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<tr>
<td>31</td>
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<td>79.4</td>
<td>90.1</td>
</tr>
</tbody>
</table>

Figure 2.4. Amount of diazirine in percentage as estimated by \(^{19}\)F NMR, intact upon exposure to ambient light at different time intervals
2.5 Enhancement of aqueous solubility

An important physical property that is desired in an ideal photolabel is a good aqueous solubility, because the photolabeling experiments are usually conducted in aqueous buffer solutions. The conventional 3-trifluoromethyl-3-phenyl diazirine photolabel is non-polar and thus limited in its aqueous solubility. Since pyridine and pyrimidine are highly soluble in water, replacement of the non-polar phenyl group with these moieties is bound to increase the aqueous solubility. Aqueous solubility studies at pH = 7.4 (Table 2.2) revealed that the conventional photolabel with phenyl moiety had only limited solubility in water, whereas the modified photolabels with pyridine and pyrimidine moieties were soluble even at the upper limits of detection (10 mM).

Table 2.2. Comparison of aqueous solubility of modified aromatic diazirines versus the conventional aromatic diazirine (*upper detection limit)

<table>
<thead>
<tr>
<th></th>
<th>Aqueous solubility (pH = 7.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Diazirine 1" /></td>
<td>$2.94 \pm 0.08 \text{ mM}$</td>
</tr>
<tr>
<td><img src="image2" alt="Diazirine 2" /></td>
<td>$&gt; 10 \text{ mM}^*$</td>
</tr>
<tr>
<td><img src="image3" alt="Diazirine 3" /></td>
<td>$&gt; 10 \text{ mM}^*$</td>
</tr>
</tbody>
</table>
2.6 Design and synthesis of photoaffinity labels based on modified trifluoromethylaryl diazirine photolabels 1 and 2

In spite of the above mentioned advantageous physical properties, the modified trifluoromethylaryl diazirine photolabels 1 and 2 are useful only if they are functional as photolabels in tagging proteins. To verify the practicality of using diazirines 1 and 2 as photolabels, we designed photoaffinity labels that would tag Concanavalin A (Con A), a plant lectin that binds mannose. Since Con A binds to mannose, the photoaffinity label should contain a mannose residue and a biotin moiety for post labeling enrichment and identification. We designed the photoaffinity labels 14, 15 and 16 based on the photolabels 3, 1 and 2 respectively. Compound 14 is the control as a conventional probe containing 3-trifluoromethyl-3-phenyl diazirine moiety.

The synthesis of photoaffinity labels 14, 15 and 16 was achieved by a convergent route, in which biotin amine 20, carboxylic acid derived mannose 23, and diazirines 24, 25 and 26 were synthesized separately as key intermediates and coupled with others at the last steps of the synthesis. Biotin amine 20 was synthesized as reported earlier and the free base was generated by using the basic resin Amberlite IRA-402 (OH form). Benzyl ester 22 was synthesized by a boron trifluoride coupling of D-mannose pentaacetate with the corresponding alcohol in moderate yield. Benzyl ester 22 was exclusively α-mannoside as confirmed by the $^1$H NMR coupling constants. The free carboxylic acid 23 was synthesized by benzyl deprotection of 22 using Pd/C under hydrogen in good yield. The bromides 24 and 25 were synthesized from the corresponding phenyl diazirine 3 and pyridinyl diazirine 1 via Apple reaction. In contrast the Apple reaction did not yield the bromide 26 with pyrimidinyl diazirine 2. However the reaction of pyrimidinyl diazirine 2 with phosphorus tribromide yielded the desired bromide 26.
Scheme 2.2. Synthesis of intermediates 20, 23, 24, 25 and 26

On the convergent step of the synthesis, bromide 24, 25 or 26 was treated with an excess of primary amine 20 to obtain the desired secondary amine 27, 28 or 29. The corresponding secondary amine was subjected to an EDC coupling with carboxylic acid 23 to get the amide 30, 31 or 32, which was deacylated using a catalytic amount of sodium methoxide in methanol to furnish the photoaffinity label 14, 15 or 16.
Scheme 2.3. Synthesis of photoaffinity label 14, 15 and 16

2.7 Photolabeling of Concanavalin A (Con A) with photoaffinity label 14, 15 and 16

To evaluate whether the modified photolabels 1 and 2 will be as functional as the conventional photolabel 3 in labeling proteins, photoaffinity labels 14, 15 and 16 were tested to tag Con A. The control C, which contains Con A in acetate buffer, shows up with coomassie stain but does not make any visible spot in the biotin specific western blot (Figure 2.5). Whereas P1, P2 and P3 which has Con A along with photoaffinity labels 14, 15 or 16 respectively and irradiated with UV light (> 320 nm) is visible with both coomassie stain and western blot. Samples S1, S2 and S3 which has Con A along with photoaffinity labels 14, 15 or 16 respectively but not irradiated with UV light (> 320 nm) shows up only with coomassie stain and not on western blot, proving that the Con A is tagged only upon photo activation of the photoaffinity probes. These data illustrate that
the modified photolabels 1 and 2 are capable of tagging proteins upon photoactivation, just as efficiently as the conventional diazirine photolabel 3.

![Concanavalin A Table](image)

<table>
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<tr>
<th>Probe</th>
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<th>Phenyl</th>
</tr>
</thead>
<tbody>
<tr>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
</tr>
</tbody>
</table>

**Figure 2.5.** Coomassie stain and western blot analysis of control and photolabeled Con A

Since mannose is the natural ligand of Con A, addition of mannose x prior to photolabeling should inhibit the binding of the photoaffinity label to Con A and thereby impede the photolabeling. The suppression of photolabeling in the presence of mannose is vital because it indicates that the labeling is occurring based on the binding of the ligand to the target protein. In the presence of mannose ligand we observed that the photolabeling is severely suppressed (Figure 2.6), indicating that the photolabeling occurs only upon the binding of the mannose ligand of photoaffinity label to Con A.
<table>
<thead>
<tr>
<th>Probe</th>
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<th>100 folds mannose</th>
<th>1000 folds mannose</th>
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<tbody>
<tr>
<td>Pyridinyl</td>
<td></td>
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<td></td>
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<tr>
<td>Pyrimidinyl</td>
<td></td>
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</tbody>
</table>

**Figure 2.6.** Biotin antibody Western blot of photolabeling with photoaffinity labels 15 and 16, P2 and P3 respectively, in the presence of mannose.

In Summary, our hypothesis of developing ambient light stable photolabels by stabilizing the diazirine ring with an electron withdrawing group was proven to be effective. We have developed two modified diazirine photolabels 1 and 2 with enhanced stability to ambient light and increased aqueous solubility. Finally the practical functionality of the modified photolabels 1 and 2 as photoaffinity label for tagging proteins was demonstrated to be as efficient as the conventional photolabel 3.

### 2.8 Experimental

Commercially available reagents and solvents were used without further purification. Thin layer chromatography (TLC) was performed using EMD silica gel 60-F plates and spots were visualized using UV light. Purification by flash chromatography was done using EMD silica gel (230 – 400 mesh). NMR experiments were done on a Varian Inova 400 MHz spectrometer (\(^1\)H at 400, \(^{13}\)C at 100 and \(^{19}\)F at 376 MHz), an Inova 500 MHz spectrometer (\(^1\)H at 500 and \(^{13}\)C at 125 MHz) or an Inova 600 MHz spectrometer (\(^1\)H at 600 and \(^{13}\)C at 150 MHz) and the data was processed using MestReNova. Chemical shifts are reported in ppm with the solvent peak as an internal standard for \(^1\)H and \(^{13}\)C, while trifluorotoluene was used as external standard for \(^{19}\)F NMR spectroscopy. Coupling constants are reported in Hz. High resolution mass spectrometry was performed on Agilent 6540 Ultra-High-Definition (UHD) Q-TOF LC-MS with electrospray ionization. Orel Instruments housing with Osram 150 W XBO xenon short-arc lamp, fitted with a Schott WG-320 filter to eliminate UV lights below 320 nm,
was used for the photoactivation studies. For Western blot analysis Anti-Biotin–Peroxidase antibody produced in goat purchased from Sigma (A4541) was used. Aqueous solubility was determined according to the previously reported HPLC method. Preparative HPLC was conducted using Agilent Eclipse XDB-C18 PN 990967-202 column with gradient 10% to 50% of acetonitrile in water with 0.05% TFA over 15 min followed by 100% acetonitrile for 5 min (5 ml min⁻¹).

**5-bromo-2-(((tert-butyldimethylsilyl)oxy)methyl)pyridine (5)** To a solution of (5-bromopyridin-2-yl)methanol (3.65 g, 19.4 mmol) in dry dichloromethane (DCM) (50 mL), *tert*-butyldimethylsilyl chloride (3.22 g, 21.3 mmol) and imidazole (2.90 g, 42.6 mmol) were added and stirred overnight at room temperature (RT). The reaction mixture was quenched with saturated ammonium chloride and extracted with DCM (3 times). The combined organic layers were dried with anhydrous sodium sulfate and concentrated. The resultant crude was subjected to flash chromatography with silica and 10% ethyl acetate in hexane to give 5 (5.29 g, 90%). Rᵣ 0.51 (10% ethyl acetate in hexanes). δₜ (400 MHz, CDCl₃) 8.54 (d, J = 2.4 Hz, 1H), 7.80 (dd, J = 8.4 Hz, J = 2.3 Hz, 1H), 7.40 (d, J = 8.4 Hz, 1H), 4.76 (s, 2H), 0.94 (s, 9H), 0.10 (s, 6H). δₓ (101 MHz, CDCl₃) 160.1, 149.7, 139.3, 121.6, 118.6, 65.7, 26.0, 18.4, -5.3. HRMS (ESI⁺) for [M + H]⁺; calculated: 302.0570, found: 302.0577 (error = 2.3 ppm).

**1-(6-(((tert-butyldimethylsilyl)oxy)methyl)pyridin-3-yl)-2,2,2-trifluoroethanone (6)** To a solution of 5 (4.10 g, 13.6 mmol) in diethyl ether (60 mL) in an argon back flushed flask, *n*-butyllithium (6.5 mL of 2.5 M solution in hexane) was slowly added at -78°C and left to stir. After 30 minutes methyl trifluoroacetate (2.09 g, 16.3 mmol) was added and stirred at -78°C for 2 hours and warmed to RT. The reaction was quenched with saturated ammonium chloride and extracted with ethyl acetate (3 times). The combined organic layers were dried with anhydrous sodium sulfate and concentrated. The
resultant crude was subjected to flash chromatography with alumina and 4% methanol in DCM to give 6 (3.3 g, 76%). R$_f$ 0.45 (6% methanol in DCM). δ$_H$ (400 MHz, CDCl$_3$) 9.14 (s, 1H), 8.35 (d, J = 7.8 Hz, 1H), 7.74 (d, J = 7.9 Hz, 1H), 4.90 (s, 2H), 0.96 (s, 9H), 0.14 (s, 6H). δ$_C$ (101 MHz, CDCl$_3$) 179.8 (q, J = 36.6 Hz), 169.0 (s), 150.5 (q, J = 2.7 Hz), 138.1 (q, J = 1.9 Hz), 124.4 (s), 120.2 (s), 116.5 (q, J = 291.0 Hz), 66.07 (s), 26.0 (s), 18.5 (s), -5.3 (s). δ$_F$ (376 MHz, CDCl$_3$) -72.30 (s). HRMS (ESI’) for [M + H]$^+$; calculated: 320.1288, found: 320.1297 (error = 2.8 ppm).

1-(6-((tert-butyldimethylsilyl)oxy)methyl)pyridin-3-yl)-2,2,2-trifluoroethanone O-tosyl oxime (7) A suspension of hydroxylamine hydrochloride (0.36 g, 5.18 mmol) and sodium acetate trihydrate (0.93 g, 6.83 mmol) in ethanol (5 mL) was stirred for 10 minutes and allowed to settle. The supernatant of the above mixture was transferred to a solution of 6 (0.55 g, 1.72 mmol) in ethanol (2 mL) and refluxed for 16 hours. Upon cooling to RT the reaction was worked up with a water/DCM extraction (3 times) and the combined organic layers were dried with anhydrous sodium sulfate and concentrated. The concentrated crude was back flushed with argon and dry DCM (5 mL) was added followed by pyridine (273 mg, 3.45 mmol) and 4-dimethylaminopyridine (DMAP) (21.0 mg, 0.17 mmol). The solution was cooled to 0°C and p-toluenesulfonyl anhydride (619 mg, 1.90 mmol) was added slowly and stirred at the same temperature for 30 minutes followed by 2 hours at RT. The reaction was quenched with water and extracted with DCM (3 times). The combined organic layers were dried with anhydrous sodium sulfate and concentrated. The resultant crude was subjected to flash chromatography with silica and 10% ethyl acetate in hexanes to give 7 (0.51 g, 61%). R$_f$ 0.37 (10% ethyl acetate in hexanes). δ$_H$ (400 MHz, CDCl$_3$) 8.51 (s, 1H), 7.86 (d, J = 8.2 Hz, 2H), 7.76 (m, 1H), 7.65 (m, 1H), 7.36 (d, J = 8.0 Hz, 2H), 4.84 (s, 2H), 2.44 (s, 3H), 0.94 (s, 9H), 0.12 (s, 6H). δ$_C$ (101 MHz, CDCl$_3$) 165.3, 151.8 (q, J = 34.3 Hz), 147.9, 146.5, 136.9, 131.1, 130.1,
129.3, 119.8, 119.6 (q, J = 277 Hz), 119.4, 65.9, 25.9, 21.8, 18.4, -5.3. δF (376 MHz, CDCl3) -66.81 (s). HRMS (ESI⁺) for [M + H]⁺; calculated: 489.1486, found: 489.1497 (error = 2.2 ppm).

**2-(((tert-butyldimethylsilyl)oxy)methyl)-5-(3-(trifluoromethyl)diaziridin-3-yl)pyridine (8)** To a solution of 7 (1.28 g, 2.62 mmol) in dry diethyl ether (15 mL) at -50°C, ammonia gas was bubbled and condensed till the volume increased by 15 mL. This solution was stirred vigorously at -50°C overnight and the ammonia was allowed to evaporate by removing the cold bath. After warming to RT the reaction mixture was extracted between water/brine (4:1) and ether (3 times) and the combined organic layers were dried with anhydrous sodium sulfate and concentrated. The concentrated crude was subjected to flash chromatography with silica and 15% ethyl acetate in hexanes to give 8 (0.82 g, 94%). Rf 0.33 (20% ethyl acetate in hexanes). δH (400 MHz, CDCl3) 8.65 (s, 1H), 7.89 (d, J = 8.2 Hz, 1H), 7.53 (d, J = 8.2 Hz, 1H), 4.79 (s, 2H), 2.89 (d, J = 8.7 Hz, 1H), 2.43 (d, J = 8.7 Hz, 1H), 0.92 (s, 9H), 0.09 (s, 6H). δC (101 MHz, CDCl3) 163.6, 148.3, 136.7, 126.0, 123.4 (q, J = 278 Hz), 119.7, 65.8, 56.6 (q, J = 36.7 Hz), 25.9, 18.4, -5.38. δF (376 MHz, CDCl3) δ -75.73 (s). HRMS (ESI⁺) for [M + H]⁺; calculated: 334.1557, found: 334.1568 (error = 3.3 ppm).

**5-((3-(trifluoromethyl)-3H-diazirin-3-yl)pyridin-2-yl)methanol (1)** To a solution of 8 (0.13 g, 0.39 mmol) in dry diethyl ether (4.0 mL), freshly prepared Ag2O (by adding a solution of sodium hydroxide to a solution of silver nitrate) (0.45 g, 1.94 mmol) was added and stirred overnight at RT. The reaction was filtered, the filtrate was concentrated and to which THF (2 mL) was added and redissolved. To it a 1M solution of tetrabutylammonium fluoride (TBAF) in THF (0.47 mL) was added drop wise at RT and stirred till the completion of reaction as indicated by TLC. The reaction was extracted between brine and ethyl acetate (3 times) and the combined organic layers
were dried with anhydrous sodium sulfate and concentrated. The resultant crude was purified by flash column chromatography with silica and 20% ethyl acetate in hexanes to give 1 (64.4 mg, 76%). R_{f} 0.46 (50% ethyl acetate in hexanes). δ_{H} (400 MHz, CDCl_{3}) 8.43 (s, 1H), 7.56 (d, J = 8.2 Hz, 1H), 7.34 (d, J = 8.3 Hz, 1H), 4.79 (s, 2H), 3.63 (s, 1H). δ_{C} (101 MHz, CDCl_{3}) 161.1, 147.1, 135.2, 124.2, 121.9 (q, J = 275 Hz), 120.4, 64.2. δ_{F} (376 MHz, CDCl_{3}) -65.68 (s). HRMS (ESI^+) for [M + H]^+; calculated: 218.0536, found: 218.0537 (error = 0.46 ppm).

5-bromo-2-(((tert-butyldiphenylsilyl)oxy)methyl)pyrimidine (10) To a solution of (5-bromopyrimidin-2-yl)methanol\textsuperscript{13} (5.93 g, 31.4 mmol) in dry dichloromethane (DCM) (90 mL), tert-butyldiphenylsilyl chloride (10.3 g, 37.6 mmol) and imidazole (5.13 g, 75.3 mmol) were added and stirred overnight at room temperature (RT). The reaction mixture was quenched with saturated ammonium chloride and extracted with DCM (3 times). The combined organic layers were dried with anhydrous sodium sulfate and concentrated. The resultant crude was subjected to flash chromatography with silica and 3% ethyl acetate in hexane to give 10 (12.2 g, 91%). R_{f} 0.60 (10% ethyl acetate in hexanes). δ_{H} (400 MHz, CDCl_{3}) 8.74 (s, 2H), 7.76 – 7.69 (m, 4H), 7.45 – 7.32 (m, 6H), 4.89 (s, 2H), 1.10 (s, 9H). δ_{C} (101 MHz, CDCl_{3}) 167.0, 157.8, 135.8, 133.4, 129.9, 127.8, 118.7, 67.1, 26.9, 19.5. HRMS (ESI^+) for [M – phenyl]^+; calculated: 349.0366, found: 349.0374 (error = 2.3 ppm).

1-(2-(((tert-butyldiphenylsilyl)oxy)methyl)pyrimidin-5-yl)-2,2,2 trifluoroethanone (11) To a solution of 10 (10.0 g, 23.4 mmol) and tetramethylethlenediamine (TMEDA) (3.53 g, 30.4 mmol) in THF (195 mL) in an argon back flushed flask, n-butyllithium (10.3 mL of 2.5 M solution in hexanes) was added at -110°C (ethanol and liquid N\textsubscript{2} bath) very slowly. After 3 minutes, methyl trifluoroacetate (5.99 g, 46.8 mmol) was added and stirred at -110°C for 30 minutes and slowly warmed to RT. The reaction was quenched
with saturated ammonium chloride and extracted with ethyl acetate (3 times). The combined organic layers were dried with anhydrous sodium sulfate and concentrated. The resultant crude was subjected to flash chromatography with alumina and 5% methanol in DCM to give 11 (7.81 g, 75%). Rf 0.40 (5% methanol in DCM). HRMS (ESI+) for [M + H₃O]⁺ (since 11 exists as geminal diol); calculated: 463.1659, found: 463.1664 (error = 1.1 ppm).

1-(2-(((tert-butyldiphenylsilyl)oxy)methyl)pyrimidin-5-yl)-2,2,2-trifluoroethanone O-tosyl oxime (12) A suspension of hydroxylamine hydrochloride (7.04 g, 101 mmol) and sodium acetate trihydrate (23.6 g, 173 mmol) in ethanol (128 mL) was stirred vigorously for 10 minutes and allowed to settle. The clear supernatant (100 mL) of the above mixture was transferred to a flask with 11 (6.44 g, 14.5 mmol) and refluxed for 40 hours. Upon cooling to RT the reaction was worked up with water/DCM extraction (3 times) and the combined organic layers were dried with anhydrous sodium sulfate and concentrated. The concentrated crude was back flushed with argon and dry DCM (48 mL) was added, followed by DMAP (178 mg, 1.45 mmol). After cooling the solution to -50°C, N,N-Diisopropylethylamine (DIPEA) (2.06 g, 15.9 mmol) was added followed by part-wise addition of p-toluenesulfonyl chloride (3.03 g, 15.9 mmol) and the reaction temperature was increased slowly in such a way that it reached 0°C in 2 hours. The reaction was quenched with water and extracted with DCM (3 times). The combined organic layers were dried with anhydrous sodium sulfate and concentrated. The resultant crude was subjected to flash chromatography with silica and 10% ethyl acetate in hexanes to give 12 (3.73 g, 42%). Rf 0.38 (10% ethyl acetate in hexanes). δₜ (600 MHz, CDCl₃) 8.76 (s, 2H), 7.91 (m, 2H), 7.77 – 7.73 (m, 4H), 7.45 – 7.36 (m, 8H), 5.02 (s, 2H), 2.50 (s, 3H), 1.15 (s, 9H). δₜ (151 MHz, CDCl₃) 171.5, 156.4, 149.2 (q, J = 35.1 Hz), 146.9, 135.8, 134.9, 133.2, 130.2, 129.9, 129.7, 129.5, 127.9, 119.4 (q, J = 277
Hz), 67.2, 26.9, 21.9, 19.5. HRMS (ESI') for [M + Na]^+; calculated: 636.1571, found: 636.1587 (error = 2.5 ppm).

2-(((tert-butyldiphenylsilyl)oxy)methyl)-5-(3-(trifluoromethyl)diaziridin-3-yl)pyrimidine (13) To a solution of 12 (1.74 g, 2.83 mmol) in dry diethyl ether (15 mL) at -50°C, ammonia gas was bubbled and condensed till the volume increased by 15mL. This solution was stirred vigorously at -50°C overnight and the ammonia was allowed to evaporate by removing the cold bath. After warming to RT the reaction mixture was extracted between water/brine (4:1) and ether (3 times) and the combined organic layers were dried with anhydrous sodium sulfate and concentrated. The concentrated crude was subjected to flash chromatography with silica and 15% ethyl acetate in hexanes to give 13 (881 mg, 68%). Rf 0.31 (20% ethyl acetate in hexanes). δ_H (600 MHz, CDCl₃) 8.92 (s, 2H), 7.74 (m, 4H), 7.43 – 7.39 (m, 2H), 7.38 – 7.34 (m, 4H), 4.99 (s, 2H), 2.92 (d, J = 8.8 Hz, 1H), 2.30 (d, J = 8.8 Hz, 1H), 1.12 (s, 9H). δ_C (151 MHz, CDCl₃) δ 170.4, 156.9, 135.8, 133.3, 129.9, 127.8, 124.2, 123.0 (q, J = 278 Hz), 67.3, 55.2 (q, J = 37.5 Hz), 26.9, 19.5. HRMS (ESI') for [M + H]^+; calculated: 459.1823, found: 459.1833 (error = 2.2 ppm).

(5-(3-(trifluoromethyl)-3H-diazirin-3-yl)pyrimidin-2-yl)methanol (2) To a solution of 13 (790 mg, 1.72 mmol) in dry diethyl ether (20 mL), freshly prepared Ag₂O (by adding solution of sodium hydroxide to a solution of silver nitrate) (1.90 g, 8.20 mmol) was added and stirred overnight at RT. The reaction was filtered and the filtrate was concentrated to which THF (7 mL) was added and redissolved. To it a 1M solution of tetrabutylammonium fluoride (TBAF) in THF (1.9 mL) was added drop wise at RT and stirred till the completion of reaction as indicated by TLC. The reaction was extracted between brine and ethyl acetate (3 times) and the combined organic layers were dried with anhydrous sodium sulfate and concentrated. The resultant crude was purified by
flash column chromatography with silica and 25% ethyl acetate in hexanes to give 2 (317 mg, 85%). Rf 0.48 (50% ethyl acetate in hexanes). δH (400 MHz, CD3OD) 8.76 (s, 2H), 4.80 (s, 2H). δC (101 MHz, CD3OD) δ 170.3, 155.8, 121.6, 121.5 (q, J = 274 Hz), 64.2, 25.5 (q, J = 42.5 Hz). δF (376 MHz, CD3OD) -67.8 (s).

N-(6-aminohexyl)-5-((4S)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (20) The amine 20 was synthesized as outlined by Kottani and co-workers. Upon the BOC deprotection the excess trifluoroacetic acid (TFA) and DCM were removed in the rotavap followed by high vacuum for 6 hours. Amberlite IRA-402 was freshly activated by stirring the resin with 5% NaOH for 10 minutes, followed by filtration and washing the resin with water. Upon removal of the excess TFA, the residue was redissolved in water (2 mL per 100 mg of residue) and to this solution freshly prepared Amberlite IRA-402 (OH⁻ form) was added in small portions with vigorous stirring. The addition of Amberlite IRA-402 was continued till the pH of the solution was found to be neutral as indicated by pH paper. The resin was filtered off using syringe filter and the water layer was freeze dried to get 20 as a free base. δH (400 MHz, CD3OD) 4.49 (ddd, J = 7.9, 5.0, 0.9 Hz, 1H), 4.30 (dd, J = 7.9, 4.5 Hz, 1H), 3.28 – 3.07 (m, 4H), 2.98 – 2.83 (m, 1H), 2.76 – 2.54 (m, 3H), 2.19 (t, J = 7.4 Hz, 2H), 1.83 – 1.23 (m, 15H). HRMS (ESI⁺) for [M + H]⁺; calculated: 343.2162, found: 343.2164 (error = 0.58 ppm)

(2R,3R,4S,5S,6S)-2-(acetoxymethyl)-6-(2-(benzyloxy)-2-oxoethoxy)tetrahydro-2H-pyran-3,4,5-triy1 triacetate (22) To a solution of mannose pentaacetate (0.50 g, 1.28 mmol) and benzyl 2-hydroxyacetate (425 mg, 2.56 mmol) in dry DCM (7 mL) at 0°C, boron trifluoride etherate (0.8 mL) was added slowly and let to stir overnight at RT. The reaction was quenched by the drop wise addition of aqueous saturated sodium bicarbonate solution and extracted between DCM and aqueous saturated sodium...
bicarbonate solution (3 times). The combine organic layers were dried with anhydrous sodium sulfate and concentrated under reduced pressure. The concentrated crude was subjected to flash chromatography with silica and 30% ethyl acetate in hexanes to give **22** (0.433 g, 68%). R<sub>t</sub> 0.56 (50% ethyl acetate in hexanes). δ<sub>H</sub> (600 MHz, CDCl<sub>3</sub>) 7.31 – 7.23 (m, 5H), 5.32 – 5.27 (m, 2H), 5.26 – 5.20 (m, 1H), 5.11 (d, J = 3.0 Hz, 2H), 4.88 (d, J = 1.4 Hz, 1H), 4.24 (d, J = 16.5 Hz, 1H), 4.17 (dd, J = 12.3, 5.0 Hz, 1H), 4.13 (d, J = 16.4 Hz, 1H), 4.10 – 4.05 (m, 1H), 3.97 (dd, J = 12.3, 2.4 Hz, 1H), 2.07 (s, 3H), 2.00 (s, 3H), 1.96 (s, 3H), 1.91 (s, 3H). δ<sub>C</sub> (63 MHz, CDCl<sub>3</sub>) 170.3, 169.5, 169.5, 168.7, 135.0, 128.4, 128.3, 128.2, 97.7, 68.9, 68.7, 66.6, 65.6, 64.4, 62.0, 20.5, 20.45, 20.42, 20.39. HRMS (ESI<sup>+</sup>) for [M + NH<sub>4</sub>]<sup>+</sup>; calculated: 514.1919, found: 514.1930 (error = 2.1 ppm).

**2-(((2S,3S,4S,5R,6R)-3,4,5-triacetoxy-6-(acetoxymethyl)tetrahydro-2H-pyran-2-yl)oxy)acetic acid (23)** To a solution of **22** (1.22 g, 2.46 mmol) in ethyl acetate (8 mL), palladium on carbon (10%) (0.25 g) was added and subjected to hydrogenation at 60 PSI for 18 hours in a hydrogenator. After the reaction was complete as indicated by LC-MS, it was filtered using a syringe filter and the resultant filtrate was concentrated and subjected to preparative HPLC using reverse phase separation to yield the desired **23** (0.70 g, 70%). δ<sub>H</sub> (600 MHz, CD<sub>3</sub>OD) 5.35 (dd, J = 3.4, 1.7 Hz, 1H), 5.32 (dd, J = 10.1, 3.4 Hz, 1H), 5.28 - 5.23 m, 1H), 4.95 (d, J = 1.7 Hz, 1H), 4.31 – 4.27 (m, 1H), 4.26 – 4.21 (m, 2H), 4.20 – 4.17 (m, 1H), 4.13 – 4.09 (m, 1H), 2.14 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 1.96 (s, 3H). δ<sub>C</sub> (63 MHz, CD<sub>3</sub>OD) 172.8, 172.4, 171.6, 171.5, 99.2, 70.7, 70.5, 70.4, 67.1, 65.3, 63.5, 20.7, 20.64, 20.6. HRMS (ESI<sup>+</sup>) for [M + NH<sub>4</sub>]<sup>+</sup>; calculated: 424.1449, found: 424.1450 (error = 0.24 ppm).

**3-(4-(bromomethyl)phenyl)-3-(trifluoromethyl)-3H-diazirine (24)** To a solution of (4-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl)methanol (95.0 mg, 0.439 mmol) in dry DCM (1.5 mL), triphenylphosphine (134 mg, 0.51 mmol) and carbon tetrabromide (168 mg,
0.51 mmol) were added at RT and stirred overnight. The reaction was quenched with the addition of pentane and filtered. The filtrate was concentrated, the resultant crude was subjected to flash chromatography with silica column and 5% ether in pentane was used as eluent. The pure fractions as identified by TLC were combined and concentrated. Due to the volatile nature of the product only mild vacuum should be employed to remove the solvents to yield 24 (112 mg, 91%). \( R_f = 0.61 \) (10% ethyl acetate in hexanes). \( \delta_r \) (400 MHz, CD\(_2\)Cl\(_2\)) 7.45 (d, \( J = 8.3 \) Hz, 2H), 7.20 (d, \( J = 8.3 \) Hz, 2H), 4.50 (s, 2H). \( \delta_c \) (101 MHz, CD\(_2\)Cl\(_2\)) 139.7, 129.4, 129.0, 126.8 (q, \( J = 1.3 \) Hz), 122.0 (q, \( J = 275 \) Hz), 32.1, 28.2 (q, \( J = 40.4 \) Hz). \( \delta_f \) (376 MHz, CD\(_2\)Cl\(_2\)) -65.67 (s).

**2-(bromomethyl)-5-(3-(trifluoromethyl)-3H-diazirin-3-yl)pyridine (25)** To a solution of 1 (59.0 mg, 272 \( \mu \)mol) in dry DCM (0.8 mL), triphenylphosphine (79.0 mg, 300 \( \mu \)mol) and carbon tetrabromide (99.0 mg, 299 \( \mu \)mol) were added at RT and stirred overnight. The reaction was quenched with the addition of pentane and filtered. The filtrate was concentrated, the resultant crude was subjected to flash chromatography with silica column and 5% ether in pentane was used as eluent. The pure fractions as identified by TLC were combined and concentrated. Due to the volatile nature of the product only mild vacuum should be employed to remove the solvents to yield 25 (71.0 mg, 93%). \( R_f = 0.54 \) (10% ethyl ether in pentane). \( \delta_c \) (400 MHz, CD\(_2\)Cl\(_2\)) 8.44 (d, \( J = 2.4 \) Hz, 1H), 7.57 (dd, \( J = 8.3, 2.4 \) Hz, 1H), 7.49 (d, \( J = 8.3 \) Hz, 1H), 4.55 (s, 2H). \( \delta_c \) (101 MHz, CD\(_2\)Cl\(_2\)) 159.0, 148.2 (q, \( J = 1.6 \) Hz), 135.9 (q, \( J = 1.2 \) Hz), 125.1, 123.7, 122.3 (q, \( J = 274 \) Hz), 33.5, 27.7 (q, \( J = 41.8 \) Hz). \( \delta_f \) (376 MHz, CD\(_2\)Cl\(_2\)) -65.99 (s). HRMS (ESI\(^+\)) for [M + H]\(^+\); calculated: 279.9692, found: 279.9688 (error = 1.4 ppm).

**2-(bromomethyl)-5-(3-(trifluoromethyl)-3H-diazirin-3-yl)pyrimidine (26)** To a solution of 2 (30.0 mg, 137 \( \mu \)mol) in dry DCM (1.5 mL) at 0°C, phosphorus tribromide (44.4 mg, 164 \( \mu \)mol) was added slowly. The reaction was slowly warmed to RT and stirred
overnight. The reaction was quenched with dropwise addition of saturated aqueous sodium bicarbonate and extracted between DCM and aqueous sodium bicarbonate (3 times). The combined organic layers were dried with anhydrous sodium sulfate and concentrated. Due to the volatile nature of the product only mild vacuum should be employed to remove the solvents to yield 26. The product obtained was used without further purification for the next step.

5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(6-((4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)amino)hexyl)pentanamide (27) To a solution of 20 (53.0 mg, 0.155 mmol) in dry DMF (1.5 mL), a solution of 24 (20 mg, 71.7 µmol) in DMF (0.5 mL) was added slowly at RT and stirred for 3 hours. The reaction mixture was diluted with acetonitrile (10 mL) and subjected to reverse phase preparative HPLC purification. The fractions with the desired compound were collected and concentrated by removal of acetonitrile on a rotary evaporator and freeze drying the resultant aqueous solution to yield a white powder of 27 (10.1 mg, 22 %) as a TFA salt. This salt was used for the next step without further purification.

(2R,3S,4S,5S,6S)-2-(acetoxymethyl)-6-(2-oxo-2-((6-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)hexyl)(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)amino)ethoxy)tetrahydro-2H-pyran-3,4,5-triyli triacetate (30) To a solution of TFA salt of 27 (10.0 mg, 15.7 µmmol) in DMF (1 mL), 23 (16.0 mg, 39.4 µmmol), EDCI (7.1 mg, 37.0 µmmol) and DMAP (5.0 mg, 40.9 µmmol) were added at RT and stirred overnight. The reaction mixture was diluted with acetonitrile (10 mL) and subjected to reverse phase preparative HPLC purification. The fractions with the desired compound were collected and concentrated by removal of acetonitrile on a rotary evaporator and freeze drying the resultant aqueous solution to yield 30 (7.5 mg, 51%) as white powder. The proton NMR indicated the existence of 30 as two rotamers. δ_H (600
MHz, CD$_3$OD) 7.37 (d, J = 8.1 Hz, 2H), 7.26 (d, 8.1 Hz, 2H), 5.40 – 5.17 (m, 3H), 4.99 (d, J = 1.5 Hz, 1H), 4.68 - 4.60 (m, 2H), 4.52 – 4.46 (m, 2H), 4.43 – 4.21 (m, 3H), 4.21 – 4.09 (m, 2H), 4.04 – 3.97 (m, 1H), 3.36 – 3.32 (m, 1H), 3.29 – 3.25 (m, 1H), 3.23 – 3.17 (m, 1H), 3.17 – 3.10 (m, 2H), 2.92 (ddd, J = 12.7, 4.9, 3.7 Hz, 1H), 2.70 (d, J = 12.7 Hz, 1H), 2.21 – 2.16 (m, 2H), 2.16 – 2.11 (m, 3H), 2.08 – 1.98 (m, 6H), 1.98 – 1.94 (m, 3H), 1.77 – 1.52 (m, 6H), 1.51 – 1.39 (m, 4H), 1.36 - 1.26 (m, 4H). HRMS (ESI$^+$) for [M + Na]$^+$; calculated: 951.3392, found: 951.3418 (error = 2.7 ppm).

5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(6-(N-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)-2-(((2S,3S,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)acetamido)hexyl)pentanamide (14)

To a solution of 30 (6.2 mg, 6.67 µmol) in dry methanol (0.5 mL), 25% sodium methoxide in methanol (40 µL) was added and let to stir for 3 hours at RT. The reaction was quenched with the addition of 0.1% TFA in methanol solution (5 mL) and subjected to reverse phase preparative HPLC purification. The fractions with the desired compound were collected and concentrated by removal of acetonitrile on a rotary evaporator and freeze drying the resultant aqueous solution to yield 14 (2.5 mg, 49%).

The proton and carbon NMR indicated the existence of 14 as two rotamers. δ$_H$ (600 MHz, CD$_3$OD) 7.34 (d, J = 7.2 Hz, 2H), 7.22 (d, J = 7.2 Hz, 2H), 4.85 (d, J = 1.6 Hz, 1H), 4.61 (d, J = 8.6 Hz, 2H), 4.49 – 4.38 (m, 2H), 4.32 – 4.25 (m, 1.5H), 3.93 (dd, J = 3.4, 1.7 Hz, 0.5H), 3.86 – 3.81 (m, 1H), 3.76 – 3.70 (m, 1H), 3.69 – 3.41 (m, 3H), 3.26 – 3.21 (m, 1H), 3.20 – 3.15 (m, 1H), 3.14 – 3.09 (m, 2H), 2.93- 2.88 (m, 1H), 2.67 (d, J = 8.7 Hz, 1H), 2.16 (t, J = 7.3 Hz, 2H), 1.74 – 1.37 (m, 10H), 1.32 – 1.24 (m, 5H). HRMS (ESI$^+$) for [M + Na]$^+$; calculated: 783.2969, found: 783.2973 (error = 0.51 ppm).

5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(6-(((5-(3-(trifluoromethyl)-3H-diazirin-3-yl)pyridin-2-yl)methyl)amino)hexyl)pentanamide (28) To a solution of 20
(70.0 mg, 205 μmol) in dry DMF (2 mL), a solution of 25 (20 mg, 71.4 μmol) in DMF (0.5 mL) was added slowly at RT and stirred for 3 hours. The reaction mixture was diluted with acetonitrile (10 mL) and subjected to reverse phase preparative HPLC purification. The fractions with the desired compound were collected and concentrated by removal of acetonitrile on a rotary evaporator and freeze drying the resultant aqueous solution to yield white powder of 28 (13.0 mg, 29 %) as a TFA salt. This salt was used for the next step without further purification.

(2R,3R,4S,5S,6S)-2-(acetoxymethyl)-6-(2-oxo-2-((6-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)hexyl)((5-(3-(trifluoromethyl)-3H-diazirin-3-yl)pyridin-2-yl)methyl)amino)ethoxy)tetrahydro-2H-pyran-3,4,5-triy triacetate (31)

To a solution of the TFA salt of 28 (7.0 mg, 11.0 μmmol) in DMF (1 mL), 23 (11.2 mg, 27.6 μmmol), EDCI (5.0 mg, 26.1 μmmol) and DMAP (3.5 mg, 28.6 μmmol) were added at RT and stirred overnight. The reaction mixture was extracted between brine and ethyl acetate (3 times) and the combined organic layer was dried with anhydrous sodium sulfate and concentrated. The resultant crude was subjected to reverse phase preparative HPLC purification. The fractions with the desired compound were collected and concentrated by removal of acetonitrile on a rotary evaporator and freeze drying the resultant aqueous solution to yield 31 (7.5 mg, 73%) as a white powder. The proton NMR indicated the existence of 31 as two rotamers in 1:1 ratio. δH (600 MHz, CDCl3) 8.45 (s, 0.5H), 8.40 (s, 0.5H), 7.61 (d, J = 8.0 Hz, 0.5H), 7.57 (d, J = 8.0 Hz, 0.5H), 7.37 (d, J = 8.0 Hz, 0.5H), 7.27 (d, J = 8.0 Hz, 0.5H), 6.77 - 6.67 (m, 0.5H), 6.29 (s, 0.5H), 6.13 (s, 0.5H), 5.37 – 5.24 (m, 2.5H), 5.19 - 5.13 (m, 1H), 5.01 - 4.88 (m, 1H), 4.73 - 4.55 (m, 3H), 4.43 – 4.31 (m, 3H), 4.29 - 4.20 (m, 1H), 4.15 – 3.96 (m, 2H), 3.41 – 3.11 (m, 5H), 2.99 - 2.87 (m, 1H), 2.77 (t, J = 13.6 Hz, 1H), 2.32 – 2.17 (m, 2H), 2.14 (d, 3H), 2.10 - 2.02 (m, 6H), 1.98 (d, 3H), 1.77 - 1.56 (m, 5H), 1.54 – 1.39 (m, 5H), 1.35 - 1.21
(m, 4H). $\delta_c$ (126 MHz, CDCl$_3$) 174.04, 173.82, 170.87, 170.79, 170.18, 170.12, 169.84, 169.79, 168.91, 168.43, 158.43, 157.78, 148.03, 146.87, 138.05, 125.09, 124.95, 124.68, 122.91, 122.73, 120.93, 120.72, 97.86, 97.77, 69.34, 69.25, 69.23, 69.16, 69.15, 69.06, 65.98, 65.87, 65.15, 62.45, 62.40, 60.90, 60.79, 55.47, 51.87, 50.35, 48.06, 46.76, 40.48, 40.41, 39.52, 39.42, 35.67, 35.55, 29.38, 29.18, 28.67, 27.95, 27.86, 27.83, 27.79, 27.35, 27.06, 27.01, 26.57, 26.52, 26.26, 25.49, 20.97, 20.92, 20.88, 20.80, 20.77, 20.73. HRMS (ESI$^+$) for [M + H]$^+$: calculated: 930.3525, found: 930.3532(error = 0.75 ppm).

5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(6-(N-((5-(3-(trifluoromethyl)-3H-diazirin-3-yl)pyridin-2-yl)methyl)-2-(((2S,3S,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)acetamido)hexyl)pentanamide (15)

To a solution of 31 (7.5 mg, 8.06 µmol) in dry methanol (0.9 mL), 25% sodium methoxide in methanol (10 µL) was added and let to stir for 3 hours at RT. The reaction was quenched with the addition of 0.1% TFA in methanol solution (3 mL) and subjected to reverse phase preparative HPLC purification. The fractions with the desired compound were collected and concentrated by removal of acetonitrile on a rotary evaporator and freeze drying the resultant aqueous solution to yield 15 (3.8 mg, 62%).

$\delta_h$ (500 MHz, CD$_3$OD) 8.49 (s, 0.5H), 8.43 (s, 0.5H), 7.78 – 7.68 (m, 1H), 7.49 – 7.40 (m, 1H), 4.86 (s, 4H), 4.74 - 4.70 (m, 2H), 4.53 - 4.36 (m, 3H), 4.34 - 4.28 (m, 1H), 3.98 – 3.82 (m, 2H), 3.80 – 3.52 (m, 5H), 3.42 - 3.33 (m, 2H), 3.25 - 3.12 (m, 3H), 2.97 - 2.89 (m, 1H), 2.74 - 2.68 (m, 1H), 2.25 - 2.16 (m, 2H), 1.79 – 1.56 (m, 5H), 1.56 – 1.39 (m, 5H), 1.38 - 1.24 (m, 4H). $\delta_c$ (126 MHz, CD$_3$OD) 175.97, 175.94, 171.92, 171.55, 166.09, 160.74, 159.97, 148.90, 148.29, 137.03, 136.90, 125.45, 125.02, 123.26, 123.25 (q, J = 274 Hz), 123.05, 101.44, 75.37, 75.21, 72.40, 72.36, 71.72, 68.58, 68.48, 65.72, 65.17, 63.39, 63.01, 62.88, 61.62, 57.02, 52.50, 51.77, 47.89, 41.04, 40.15, 36.81, 30.29,
30.25, 29.76, 29.55, 29.51, 28.22, 27.60, 27.50, 27.41, 26.91. δF (376 MHz, CD3OD) - 67.44 (s), -67.53 (s). HRMS (ESI+) for [M + Na]+; calculated: 784.2922, found: 784.2924 (error = 0.25 ppm).

5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(6-(((5-(3-(trifluoromethyl)-3H-diazirin-3-yl)pyrimidin-2-yl)methyl)amino)hexyl)pentanamide (29) To a solution of 20 (70.0 mg, 205 μmol) in dry DMF (1.5 mL), a solution of 26 (137 μmol) in DMF (0.5 mL) was added slowly at RT and stirred for 6 hours. The reaction mixture was diluted with acetonitrile (10 mL) and subjected to reverse phase preparative HPLC purification. The fractions with the desired compound were collected and concentrated by removal of acetonitrile on a rotary evaporator and freeze drying the resultant aqueous solution to yield white powder of 29 (37.2 mg, 42%) as a TFA salt. This salt was used for the next step without further purification.

(2R,3S,4S,5S,6S)-2-(acetoxymethyl)-6-(2-oxo-2-((6-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)hexyl)((5-(3-(trifluoromethyl)-3H-diazirin-3-yl)pyrimidin-2-yl)methyl)amino)ethoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (32) To a solution of the TFA salt of 29 (15 mg, 23.4 μmmol) in DMF (2 mL), 23 (33.6 mg, 82.7 μmmol), EDCI (15.9 mg, 82.9 μmmol) and DMAP (13.6 mg, 111 μmmol) were added at RT and stirred 3 hours. The reaction mixture was extracted between brine and ethyl acetate (3 times) and the combined organic layer was dried with anhydrous sodium sulfate and concentrated. The resultant crude was subjected to reverse phase preparative HPLC purification. The fractions with the desired compound were collected and concentrated by removal of acetonitrile on a rotary evaporator and freeze drying the resultant aqueous solution to yield 32 (6.3 mg, 29%) as a white powder. δH (600 MHz, CD3OD) 8.75 (s, 1H), 8.70 (s, 1H), 5.39 – 4.96 (m, 4H), 4.54 – 4.38 (m, 3H), 4.34 – 3.99 (m, 6H), 3.50 – 3.37 (m, 2H), 3.23 – 3.11 (m, 3H), 2.93 (dt, J = 12.4, 4.5 Hz, 1H), 2.71
(d, J = 12.4 Hz, 1H), 2.19 (t, J = 7.3 Hz, 2H), 2.14 (d, 3H), 2.09 – 2.02 (m, 6H), 1.95 (d, 3H), 1.77 – 1.27 (m, 14H). HRMS (ESI\(^+\)) for [M + Na\(^+\)]; calculated: 953.3297, found: 953.3305 (error = 0.84 ppm).

5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(6-((5-(3-(trifluoromethyl)-3H-diazirin-3-yl)pyrimidin-2-yl)methyl)-2-(((2S,3S,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)acetamido)hexyl)pentanamide (16)

To a solution of 32 (6.8 mg, 7.30 μmmol) in dry methanol (1 mL), 25% sodium methoxide in methanol (10 μL) was added and let to stir for 3 hours at RT. The reaction was quenched with the addition of 0.1% TFA in methanol solution (3 mL) and subjected to reverse phase preparative HPLC purification. The fractions with the desired compound were collected and concentrated by removal of acetonitrile on a rotary evaporator and freeze drying the resultant aqueous solution to yield 16 (3.7 mg, 66%). δ\(_{\text{H}}\) (600 MHz, CD\(_3\)OD) 8.76 (s, 1H), 8.71 (s, 1H), 4.83 – 4.78 (m, 3H), 4.51 – 4.35 (m, 3H), 4.31 (dd, J = 7.7, 4.5 Hz, 1H), 3.94 – 3.81 (m, 1.5H), 3.78 – 3.53 (m, 4H), 3.49 – 3.36 (m, 2.5H), 3.23 – 3.12 (m, 3H), 2.93 (ddd, J = 12.7, 5.0, 3.1 Hz, 1H), 2.71 (dd, J = 12.7, 3.0 Hz, 1H), 2.19 (t, J = 7.4 Hz, 2H), 1.78 – 1.56 (m, 5H), 1.55 – 1.41 (m, 5H), 1.41 – 1.26 (m, 5H). HRMS (ESI\(^+\)) for [M + Na\(^+\)]; calculated: 763.3055, found: 763.3074 (error = 2.5 ppm).

**General procedure for photolabeling**

To a solution of Con A (0.5 mg) in 5 ml of acetate buffer (10 mM, pH=5) with calcium chloride (1 mM), manganese (II) chloride (1 mM) and sodium chloride (200 mM), the photoaffinity label solution (10 mM) in methanol (2 μL) was added at 0°C. This solution was incubated in the dark at 0°C for 10 minutes and the sample was transferred
to a disposable cuvette and photoactivated for 10 minutes. The resultant sample can be used for SDS gel and Western blot analysis or stored at -20°C.

**General Procedure for Western blot**

Upon running the SDS gel, the PVDF membrane and gel was shaken in transfer buffer for 15 minutes. The holder cassette was placed opened in a shallow vessel such that the black panel is lying flat on the bottom of the vessel. Fiber pad presoaked with transfer buffer was placed on the black panel of the holder cassette and a presoaked filter paper was placed over it. The equilibrated gel was carefully placed over the filter paper to avoid any air bubble getting trapped between the layers. Then the PVDF membrane was laid over the gel with care towards avoiding any air pocket between the layers. A presoaked filter paper was placed over the membrane followed by a filter pad. The resultant sandwich was firmly secured and the cassette was closed. The cassette holder was placed in the tank such that the black panel of the holder is on the black panel electrode. Insert the ice pack on the other side of the tank and place the tank on a magnetic stirrer. The tank was filled to the top row of circles in the cassette with transfer. The magnetic stirrer was turned on and the lid was closed with black wire to black panel, red wire to red panel. The unit was connected to a power supply and ran at constant voltage of 70V for 60 minutes. The membrane was carefully removed and the membrane was blocked with 5% (w/v) NFDM (non-fat dry milk) in tris saline Tween-20 buffer (TBS-T) for 1 hour at room temperature. The blocked membrane was incubated overnight on a shaker at 4°C with anti-biotin–peroxidase antibody in TBS-T buffer containing 1% NFDM. The membrane was washed five times (10 minutes each time on a shaker) with TBS-T buffer. The membrane was incubated for 5 minutes at room temperature with SuperSignal West Pico chemiluminescence substrate as per manufacturer’s protocol.
2.9 References


of the National Academy of Sciences of the United States of America 2006, 103, 13917-13921.

CHAPTER – 3

Design, synthesis and photoactivation studies of fluorous photolabels

This chapter is published in Organic and Biomolecular Chemistry (2011, 9, 6284–6292).

The full article is available in the appendix-1 of this dissertation.

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CHAPTER – 4

Photolabeling with fluorous photoaffinity tag

Recently fluorous tags are widely adopted as a purification handle in combinatorial chemistry, natural product synthesis, catalysis, peptide labeling and peptide purification among others.\(^1\) The driving force behind fluorous affinity separation is the unusually strong interaction between fluorous tags.\(^2\) A comparatively big molecule attached to a small fluorous tag can be purified from other non-fluorinated compounds by using fluorous-functionalized silica gel column due to the fluorous interaction. Fluorous tags are commercially available, inexpensive, robust under most of the strenuous reaction conditions, and do not confound mass spectral interpretation due to its stability against fragmentation.\(^3\) These physiochemical properties make fluorous affinity enrichment an ideal candidate for use as a purification technique in proteomics. Peters and coworkers introduced the concept of “fluorous proteomics” in which a peptide covalently linked to a fluorous tag was separated from a complex peptide mixture via fluorous solid phase extraction (FSPE) with fluorous-functionalized silica gel as the stationary phase.\(^3\) Since then, fluorous tags have been proven to be an effective enrichment moiety for peptides and small molecules of biological origin.\(^4\)\(^,\)\(^5\) Pohl coworkers designed fluorous based carbohydrate microarrays for biological screening where intact protein, concanavalin A (Con-A), was concentrated on a fluorous slide using a ligand containing a mannose moiety for the binding to Con-A and a fluorous chain for the non-covalent attachment of the ligand to the fluorous slide.\(^4\) These two and
other similar approaches demonstrate the potential of fluorous-based techniques for peptide and protein enrichment.\(^6\)

### 4.1 Photolabeling Con A with photolabel 1

We wanted to explore the possibility of extending the outstanding enrichment properties of fluorous tag to photoaffinity labeling. As discussed in Chapter-3, the photoaffinity label 1 (Figure 4.1) is designed with α-mannose as the guiding ligand to target the photolabeling towards Concanavalin A (Con A), a mannose binding lectin. Using the photoaffinity label 1, we wanted to investigate if we could photolabel Con A and purify the labeled Con A by fluorous enrichment. As described in the Figure 4.2, the mannose ligand in the photolabel would bind to Con A due to the natural affinity. Upon irradiation of this protein-photolabel complex with UV light (\(\lambda=320\) nm) the diazirine generates a carbene, which would react with the amino acid residue of the bound Con A. The covalent bond formed will link Con A with the fluorous tag, which could be used in the fluorous enrichment of the photolabeled protein.

![Figure 4.1. Fluorous photoaffinity label 1](image)

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Figure 4.2. Representation of the concept behind photolabeling of Con A with photolabel 1

4.2 Approach-1: In-solution photoaffinity labeling of Con A

Hamachi and co-workers have successfully conducted in-solution photoaffinity labeling of Con A with a photoaffinity label containing mannose ligand and diazirine photolabel. Using their photolabeling conditions, the photoaffinity label 1 was subjected to in-solution photolabeling with Con A. Upon irradiation with UV light (λ=320 nm) the sample was subjected to SDS gel electrophoresis followed by coomassie staining and found that there is no visible change between the sample (Con A solution photoactivated with photoaffinity label 1) and control (Con A solution photoactivated without 1). Considering that the photolabeling of Con A with the photolabel increases the net molecular weight by insignificant amount, the identical appearances of the sample and control is understandable. All the samples and controls were subjected to MALDI-TOF MS analysis before and after trypsin digest. Due to the high molecular weight of the whole protein, we were unable to obtain the MALDI mass spectral data for the undigested Con A. But the trypsin digested samples were analyzable by the MALDI-TOF and the mass spectral data showed identical peaks for the samples with photoaffinity label 1 and the control with no photoaffinity label. These spectral data were indicative that there were no labeled peptides after the trypsin digest, suggesting that the photoaffinity label 1 was not tagging Con A during photoactivation. Upon addition of the photolabel to the aqueous buffer containing Con A, we were able to visually see a cloudy
appearance of photoaffinity label 1 precipitating out of the buffer solution. In one of the experiments we centrifuged the precipitated photoaffinity label and then subjected to photoactivation and in another experiment the photoactivation was done without removing the precipitated photoaffinity label. In both cases there was no photolabeling of Con A with 1 happening as indicated by MALDI-TOF analysis. We infer that this inefficiency in photolabeling is arising due to the very high hydrophobicity of the fluorous tag, which causes the photoaffinity label 1 to possess very poor aqueous solubility, leading to its crashing out of the buffer solution before the photoactivation. Further studies with Tween 20 (0.1%) in the buffer solution to aid the aqueous solubility of photoaffinity label 1 did not help in either increasing the solubility or photolabeling of Con A.

4.3 Approach-2: Photoaffinity labeling of Con A on fluorous derived slide

Pohl and co-workers were able to concentrate Con A on fluorous derived glass slides just by using mannose-Con A affinity and fluorous tag interactions. In their experiment, fluorous tagged mannose was spotted on a fluorous derivatized glass slide, where the surface of the glass slide is chemically modified to possess covalently bound fluorous tags. Due to the fluorous interactions, the mannose ligand is non-covalently bound to the glass slide. When a solution of Con A was incubated over the mannose ligand spotted glass slide, Con A binds to the mannose ligand. Even after the excess solution is washed off with buffer, Con A was still bound to the glass slide due to its affinity for mannose ligand, where in turn the mannose is bound the glass slide due to the fluorous interaction (Figure 4.3). Since the Con A that they used was previously tagged with fluorescent tag, the binding of Con A on to the glass slide was visualized by fluorescence imaging of the glass slide.
**Figure 4.3.** Enrichment of Con A on fluorous glass slide using fluorous tagged mannose\(^4\)

We hypothesized that if we coat the glass slide with the photoaffinity label 1 instead of the fluorous tagged mannose and photo-activate upon Con A binding, it would result in photolabeling of Con A (Figure 4.4). This technique does not involve dissolution of fluorous tagged mannose in the aqueous buffer, but instead the fluorous tagged mannose is coated on the surface of the glass slide. Since the solubility of the fluorous tagged mannose is not a requirement for this requirement, we envisioned that the poor aqueous solubility of photoaffinity label 1 would not be a disadvantage.

**Figure 4.4.** Design of photoaffinity labeling experiment using fluorous derivatized glass slide

The photolabeling experiment was conducted on the fluorous derivatized glass slide to overcome the solubility problem of photoaffinity label 1. Upon extracting the glass surface with methanol and concentration followed by MALDI-TOF analysis, it was
evident that the photolabeling was not successful. The fluorous tagged mannose that Pohl and co-workers reported had a long linker that separated the fluorous tag from the mannose ligand, but the photoaffinity label 1 is close to the mannose ligand. It is evident that the strong hydrophobic character of the fluorous tag is significantly inhibiting the binding of mannose to Con A, which in turn impedes the photolabeling.

In conclusion, although fluorous tags possess very attractive characteristics in terms of separation, commercial availability and chemical stability, its strong hydrophobic character prohibits its use in aqueous solutions and has a very strong influence on altering the binding capacity of the ligand. Since photoaffinity labeling involves aqueous solution in every step of the way, starting from proteome solution to subsequent post labeling analysis, the strong hydrophobic character of fluorous tags would be a significant disadvantage to overcome.

4.4 Experimental

An Orel Instruments housing with an Osram 150 W XBO xenon short-arc lamp, fitted with a Schott WG-320 filter to eliminate UV lights below 320 nm, was used for the photoactivation studies. Commercially available reagents and solvents were used without further purification. Con A (Type VI) from Sigma Aldrich was used for photolabeling. A Bruker Daltonics Autoflex was used for MALDI-TOF mass spectrometric analysis. Fluorous modified glass slides were purchased from Fluorous technologies incorporated and used without any modification.
4.4.1 General Procedure

Trypsin Digest

To the protein sample (20 µL), ammonium bicarbonate buffer (pH = 8.5) (150 µL) was added. To this solution trypsin (1:45, trypsin to protein w/w ratio) was added and incubated at 37°C for 18 hours. Upon completion of digestion, aqueous formic acid solution (5%) was added and used for subsequent MALDI-TOF analysis.

MALDI-TOF sample preparation

The digested protein sample (1 µL) and a saturated solution of α-hydroxy cinnamic acid in water:acetonitrile:TFA (49.95:49.95:0.10) mixture (1 µL) were plated on a MALDI sample plate and mixed evenly. The mixture was allowed to dry on the MALDI plate by exposure to ambient air and then subjected to MALDI-TOF analysis.

4.4.2 Approach-1: In-solution photoaffinity labeling with 1

Photolabeling without centrifugation

Photolabeling Sample To a solution of Con A (7.5 mg, 0.30 µmol) in 0.5 ml of acetate buffer (10 mM, pH=5) with calcium chloride (1 mM), manganese (II) chloride (1 mM) and sodium chloride (200 mM), the photoaffinity label 1 (1.1 mg, 1.2 µmol) was added as a solution in DMF (20 µL) at 0°C. This solution was incubated in the dark at 0°C for 1 hour and then bubbled gently with nitrogen to remove oxygen. The sample was then transferred to disposable cuvette and irradiated for 1 hour. The resultant sample was trypsin digested and subjected to MALDI-TOF analysis.

Control To a solution of Con A (7.5 mg, 0.30 µmol) in 0.5 ml of acetate buffer (10 mM, pH=5) with calcium chloride (1 mM), manganese (II) chloride (1 mM) and sodium
chloride (200 mM), DMF (20 μL) was added at 0°C. This solution was incubated in the dark at 0°C for 1 hour and then bubbled gently with nitrogen to remove oxygen. The sample was then transferred to disposable cuvette and irradiated for 1 hour. The resultant sample was trypsin digested and subjected to MALDI-TOF analysis.

**Photolabeling with centrifugation**

**Photolabeling Sample** To a solution of Con A (7.5 mg, 0.30 μmol) in 0.5 ml of acetate buffer (pH=5) with calcium chloride (1 mM), manganese (II) chloride (1 mM) and sodium chloride (200 mM), the photoaffinity label 1 (1.1 mg, 1.2 μmol) was added as a solution in DMF (20 μL) at 0°C. This solution was incubated in the dark at 0°C for 1 hour and then bubbled gently with nitrogen to remove oxygen. At this stage centrifugation was done to remove the precipitated out photoaffinity label. The supernatant was gently transferred to disposable cuvette and irradiated for 1 hour. The resultant sample was trypsin digested and subjected to MALDI-TOF analysis.

**Control** To a solution of Con A (7.5 mg, 0.30 μmol) in 0.5 ml of acetate buffer (pH=5) with calcium chloride (1 mM), manganese (II) chloride (1 mM) and sodium chloride (200 mM), DMF (20 μL) was added at 0°C. This solution was incubated in the dark at 0°C for 1 hour and then bubbled gently with nitrogen to remove oxygen. At this stage centrifugation was done to remove the precipitated out photoaffinity label. The supernatant was gently transferred to disposable cuvette and irradiated for 1 hour. The resultant sample was trypsin digested and subjected to MALDI-TOF analysis.

**4.4.3 Photoaffinity labeling of Con A on fluorous derived glass slide**

**Preparation of Photoaffinity label 1 coated fluorous glass slide** A solution of photoaffinity label 1 (1.0 mg, 1.0 μmol) in methanol-water mixture (6:4, 50 μL) was evenly applied over the fluorous modified glass slide and dried.
Preparation of control fluorous glass slide without photolabel A methanol-water mixture (6:4, 50 μL) was evenly applied over the fluorous modified glass slide and dried.

Photolabeling on fluorous modified glass slide Con A solution (20 μM) in HEPES buffer (10 mM, pH=7.5) with calcium chloride (1 mM), manganese (II) chloride (1 mM) and sodium chloride (100 mM) was gently applied on Photoaffinity label 1 coated fluorous glass slide. After 10 minutes of incubation the plate was exposed to UV light for 30 minutes and the plate was gently rinsed with deionized water to remove the excess Con A. Subsequently the plate was washed with methanol and the washings were collected and concentrated. The resultant sample was trypsin digested and subjected to MALDI-TOF analysis.

Control-fluorous glass slide without photolabel Con A solution (20 μM) in HEPES buffer (10 mM, pH=7.5) with calcium chloride (1 mM), manganese (II) chloride (1 mM) and sodium chloride (100 mM) was gently applied on control fluorous glass slide without photolabel. After 10 minutes of incubation the plate was exposed to UV light for 30 minutes and the plate was gently rinsed with deionized water to remove the excess Con A. Subsequently the plate was washed with methanol and the washings were collected and concentrated. The resultant sample was trypsin digested and subjected to MALDI-TOF analysis.

4.5 References


CHAPTER – 5

Synthesis and structure–activity relationship studies of 1,3-disubstituted 2-propanols as BACE-1 inhibitors

5.1 Previous findings on BACE-1 inhibitors: advancements and hurdles

With over 26 million patients worldwide, Alzheimer’s disease (AD) is considered to be the neurological disease with largest unmet medical demand. AD is currently characterized as an unpreventable, incurable and terminal disease. The direct cost of caring for AD patients is estimated to be about 200 billion dollars annually in US alone. Unless there is a development is AD treatment, these figures are estimated to drastically soar to over 1.1 trillion dollars by 2050.\(^1\) The general consensus in the cause of this disease is the ‘amyloid cascade hypothesis’ which claims that the typical pathology observed in AD is caused due the deposition of the neurotoxic peptide called amyloid β.\(^2\) The production of this amyloid β is achieved by the action of aspartic proteases, BACE-1 (β-secretase) and γ-secretase, on the amyloid precursor protein (APP). Thus it is a general consensus that the production of the neurotoxic peptide amyloid β can be shut down or slowed by inhibiting these protease targets. Since there have been precedence in successfully targeting other proteases for disease-modifying therapy, inhibiting BACE-1 and γ-secretase were considered as viable targets for the treatment of AD.

In particular BACE-1 gained considerable attention upon the research on mice deleted with BACE-1 gene. It was found that the deletion of BACE-1 gene in mice
improved their cognitive performance, displayed only minor behavioral change and did not result in any alteration of the antonym of vital organs.\textsuperscript{3-5} These results were indicative that inhibition of BACE-1 could be targeted as disease-modifying therapy towards the treatment of AD with minimal side effects. The crystal structure studies have revealed that BACE-1 active site possess a long cleft with two aspartic acid residues aligned to hydrolyze the substrate.\textsuperscript{6,7} On either side of the aspartic acid residues there are sub-pockets positioned to align and recognize the substrate.

Since transition state isostere inhibitors of renin and HIV protease have made to the clinical trial stage, it was theorized that BACE-1 inhibitors can also designed based on transition state analogs. Hence the first generation of BACE-1 inhibitors was peptidic inhibitors mimicking the transition state of the natural substrate of BACE-1. The initial transition state isosteres OM00-3 and OM99-2 (Figure 5.1) with $K_i$ constants of 0.3 nmol/L and 1.6 nmol/L, respectively, were found to be potent inhibitors of BACE-1.\textsuperscript{8,9} Studies on the crystal structure of these inhibitors bound with BACE-1 furnished atomic level interaction details, which were useful in designing the future generations of BACE-1 inhibitors. The development of second generation BACE-1 inhibitors were mainly concentrated towards (a) reduction of molecular weight to enhance blood brain barrier (BBB) penetration (b) improve selectivity over other aspartic proteases. Initial attempts to reduce the molecular weight by extensive deletion of the peripheral residues in OM99-2 resulted in drastic loss of activity.\textsuperscript{10,11} Upon careful considerations and inclusion of some of the deleted residues yielded inhibitors with nmol/L activity, resulting in scaffolds with average molecular weight of 700 Da.\textsuperscript{11} Further, understanding the structural correlation behind differential inhibition with divergent structural scaffolds led to the design of highly selective inhibitors.\textsuperscript{12} Though very high potency and good selectivity was achieved by transition state isosteres peptidic inhibitors, their higher molecular weight restricted the
blood brain barrier permeability and ensued the interest in development of small molecule inhibitors for BACE-1.

![Chemical structures OM99-2 and OM00-3](image)

**Figure 5.1.** Transition state isostere inhibitors of BACE-1

The general strategy that was adopted in finding small molecule inhibitors for BACE-1 was high-throughput screening. Acylguanidine inhibitors for BACE-1 was developed through this approach and the lead compound was subjected to structure activity relationship (SAR) to yield the hit compound (Figure 5.2) with IC<sub>50</sub> of 110 nmol/L.<sup>13</sup> Studies on the crystal structure of the acylguanidine analog bound with BACE-1 revealed that the guanidine residue is involved in a hydrogen bonding with the catalytic aspartyl residues. Further studies revealed that the acylguanidine analogs possess poor selectivity over other aspartic proteases and poor BBB penetration.<sup>14</sup> Aminoquinazoline compound showed an activity of \( K_i = 11 \text{ nmol/L} \), an impressive potency for a small molecule inhibitor of BACE-1.<sup>15</sup> The co-crystallization of aminoquinazoline derived inhibitor with BACE-1 revealed that the guanidine residue in the inhibitor was involved in a network of hydrogen bonding with both the catalytic aspartic acid residues in the active site. Recently aminohydantoins has been studied extensively for their activity against BACE-1 by Malamas and co-workers.<sup>16-18</sup> According to the crystal structure studies the imidazole ring moiety forms hydrogen bonding with both the catalytic aspartic acid
residues in the binding pocket. These series of compounds exhibited better BBB penetration and reasonably good selectivity towards structurally related proteases like renin, cathepsin D and pepsin. Although there have been significant advancements in finding potent inhibitors for BACE-1, there has been little progress in developing a drug that could treat AD by the inhibition of BACE-1. The bottlenecks in the development of BACE-1 inhibitors to disease-modifying drugs in treatment of AD are the need to develop inhibitor that could penetrate the blood brain barrier and to design inhibitors that can exhibit high selectivity over other essential aspartyl proteases.

![Small molecule inhibitors for BACE-1](attachment:figure5.png)

**Figure 5.2.** Small molecule inhibitors for BACE-1

### 5.2 Results and discussion

The conceptual design, results and discussion for this chapter is published in Bioorganic and Medicinal Chemistry Letters, 22, (2012) 4740–4744 (DOI: 10.1016/j.bmcl.2012.05.072).

The full article is available in the appendix-2 of this dissertation.

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http://www.rsc.org
Design, synthesis and photoactivation studies of fluorous photolabels†

Arun Babu Kumar, Jordan Micheal Anderson and Roman Manetsch*

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Two fluorous diazirine photolabels were designed, synthesized and subjected to photoactivation studies. The photoactivation studies revealed an unexpected photo-reaction when the fluorous tag was directly connected to the diazirine ring, leading to the formation of a fluorous alkene. The more efficient photolabel of the two was identified as a flexible precursor for target specific photoaffinity labels for fluorous proteomics by adding appropriate ligands depending on the target protein subset. As a proof of feasibility, mannose residues were added to the photolabel making it a potential photoaffinity label to tag proteins that bind mannose.

Introduction

In proteomics, fractionation techniques are widely applied and usually involve an affinity-based enrichment step prior to mass spectrometry analysis. Biotin is one of the most common affinity tags taking advantage of the very strong biotin-streptavidin interaction. Immobilized metal ion affinity chromatography (IMAC), lectin affinity chromatography and enrichment of an alkyne-tagged probe via the Huisgen 1,3-dipolar cycloaddition are alternative affinity-based separations which have recently found broad use. Nevertheless, there is a dire need for additional fractionation techniques complementing the current ones that suffer from shortcomings such as impermeability, incomplete elution from affinity column and complication of MS/MS spectral interpretation due to undesired fragmentation of the affinity tag.

Fluorous tags have emerged as a useful purification tool in catalysis,4 combinatorial chemistry,5 natural product synthesis,6 microarrays7 peptide labeling, peptide purification and proteomics.8,9 Fluorous tags are commercially available, relatively inexpensive, highly inert to most reaction conditions and do not complicate MS/MS spectral interpretation due to their stability against fragmentation in the mass spectrometer.10 Recently, the concept of fluorous proteomics has been introduced since fluorous tags have proven to be an effective enrichment strategy for peptides, proteins and small molecules of biological origin.11-14

Photoaffinity labeling has been a valuable biochemical tool for studying the interaction between a ligand and its receptor. Although there has been significant success in the use of fluorous tags in the enrichment of peptides12,15 and proteins,16 there has been limited research in the development of fluorous photoaffinity probes. Song and Zhang reported the synthesis of the first class of fluorous photolabeling agents, in which the photoactive diazirine ring is incorporated on the fluorous tag (Fig. 1).15 Meanwhile, Burkard and coworkers reported the design and synthesis of fluorinated photoaffinity probe attached to V-ATPase inhibitors.16 Although these previous works showed the efficiency of fluorous probe–fluorescent stationary phase interaction, its use in protein labeling experiments has not been reported thus far.

![Previously reported fluorous photolabel.15](image1)

Fig. 1 Previously reported fluorous photolabel.15

![Design of fluorous photolabels 1 and 2.](image2)

Fig. 2 Design of fluorous photolabels 1 and 2.

Herein, we describe the design and synthesis of two diazirine-containing fluorous photolabels, 1 and 2 (Fig. 2). Photolabel 1, which is closely related to the previously reported photolabel,9 consists of a diazirine ring directly installed in the fluorous tag, while in probe 2, the diazirine ring and the fluorous tag are apart from each other.

Furthermore, we report photoactivation studies of diazirines 1 and 2. These studies demonstrate that fluorous probe 1 unexpectedly eliminates to form a fluorous alkene as the major product when the fluorous chain is directly connected to the diazirine ring.
These results suggest that probe 1 or a fluorinated photoaffinity probe design similar to the probe developed by Song and Zhang probably leads to deactivation upon UV irradiation, rendering it unavailable to tag the protein. In contrast, photolabel 2 with its fluorine tag remote from the diazirine ring undergoes photoactivation cleanly to the desired product. Therefore, photolabel 2 can serve as a flexible precursor for the synthesis of fluorous and target-specific photoaffinity labels by attaching suitable ligands to the benzyl alcohol groups. As proof of principle, the synthesis and characterization of potential target-specific, mammose-containing photolabel 3 is discussed.

Results and discussion

Design of fluorous photolabels

In photoaffinity labeling, a ligand linked to a photoactive group is bound non-covalently to a specific receptor in the proteome. Upon exposure to light, a covalent bond is formed between the photoactive group and the receptor. The labeled protein is then separated from the unlabeled proteins using the affinity tag that the photolabel is equipped with. Analogously, the design of fluorous photolabels 1 and 2 includes the integration of three essential moieties in one molecule (Fig. 2): (a) alcohol functions for the attachment of ligands that specifically bind to the receptor of interest in a proteome mixture, (b) a diazirine ring for the photoinduced covalent cross linking between the photolabel and its receptors and (c) a fluorous tag for the straightforward isolation of the photolabeled targets from the proteome mixture. Two alcohol residues were incorporated in photolabels 1 and 2 to induce avidity effect by covalent attachment of two ligands.

Synthesis of photolabels

For the synthesis of photolabel 1 (Scheme 1), diol 4 was protected as its tert-butyldimethyl silyl (TBS) ether 5. Lithiation of 5 by n-butyllithium followed by the addition of methyl perfluorohepantionate gave ketone 6 in moderate yield. Ketone 6 was converted to the corresponding oxime 7 and subsequently tosylated to the tosyl oxime 8 in good yield. The attempts to form the diaziridine via direct nucleophilic attack of tosyl oxime 8 with ammonia failed. The bulky nature of the two TBS groups and the fluorous tag in 8 could be the possible reason for the unexpected failure. Thus a synthetic route was devised involving a TBS deprotection prior to the diaziridine ring formation. The tosyl oxime 8 was treated with HF for TBS deprotection. Then, the cyclopropane step with ammonia yielded the desired diaziridine, which was oxidized using iodine to give the diazirine 1 in 49% yield over three steps.

The synthesis of photolabel 2 began with the preparation of aryl iodide 9 through the diazo intermediate derived from dimethylamineethanol. The aryl iodide 9 was subjected to Miyaura borylation followed by hydrolysis to give the boronic acid 10 (Scheme 2). In parallel, 3-bromo-5-iodobenzonic acid was refluxed with diphenyl phosphazyl azide (DPPA) and triethylamine to generate the corresponding aryl isocyanate, which underwent Curtius rearrangement with tert-butyl alcohol to give the Boc-protected amine 11. The boronic acid 10 was coupled with the iodide 11 via Suzuki cross-coupling reaction, followed by Boc deprotection under acidic condition to give amine 12 in good yield over two steps. Amine 12 was converted to its corresponding anilinesulfonic acid using sodium nitrite and HBFOX, which was followed by Heck coupling of the anilinesulfonic acid with (perfluoroethyl)acetylene to give the alkene 13.

The next crucial step was the selective reduction of the electron-deficient carbon-carbon double bond of the aryl bromide 13 via catalytic hydrogenation. Alkynyl-substituted aryl bromides undergo dehydrobromination under the widely used hydrogenation conditions of hydrogen and palladium on charcoal (Pd/C). Catalytic hydrogenation of perfluorinated alkynyl-aryl bromides to the corresponding saturated aryl bromides has been previously reported to succeed with rhodium on charcoal (Rh/C) only at high hydrogen pressure of 50 bar. Similarly, alkynyl-substituted aryl bromides have been reported to be hydrogenated with Adam’s catalyst (Pd/C). However, previous attempts to reduce perfluorinated alkenes under similar reaction conditions failed due to the electron-deficient double bond. After exploring a number of reaction conditions, Pd/C in ethyl acetate at 40 psi (2.75 bar) hydrogen pressure was found to provide the

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maximal yield of 14. Other combinations of solvent and hydrogen pressures resulted in the formation of debrominated product or the unreacted alkene. This catalytic hydrogenation is an interesting find due to the aforementioned importance of the molecules with fluorine tags and allyl-bromide residues that help in further derivatization. Using these optimized reaction conditions, alkene 13 was hydrogenated to the diester 14 in 83% yield. Diester 14 was subjected to LAH reduction and the resulting diol was protected as the TBS ether 15 in good yield. Compound 15 was reacted with n-butyllithium followed by methyl trifluoroacetate to furnish ketone 16 in moderate yield. Treatment of ketone 16 with hydroxylamine hydrochloride and sodium acetate followed by tosylation furnished tosyl oxime 17 in moderate yield. The tosyl oxime 17 was cyclized with liquid ammonia to the diaziridine 18 in good yield. Oxidation of the diaziridine 18 using AgO followed by TBS deprotection yielded the desired photolabel 2 in good yield (Scheme 3).

Photoactivation studies of photolabels 1 and 2

The photoactivation reactions were conducted in deuterated methanol with UV light irradiation (λ > 320 nm) and the reactions were monitored by 19F-NMR at various time intervals. As expected from previous studies, upon irradiation, diazirine 2 cleanly reacted within 5 min to methyl ether 19 and linear diazo compound 20 (Fig. 3). On further exposure to UV light, the linear diazo compound 20 was converted to the methyl ether 19. 19F-NMR and LCMS (Fig. 4, A—right and B—bottom) of the final sample indicated that ether 19 was the only major product. Further purification and analysis of the photo irradiated final sample confirmed the formation of one single product 19. The photoactivation of photolabel 1, which has structural similarity with the work reported by Song and Zhang, was investigated next (Scheme 4). Close observation of the chemical shift of the trifluoromethyl group in 19F-NMR (Fig. 4, A—left) over the course of the photoreaction suggested the decomposition of photolabel 1 into multiple products. HPLC analysis (Fig. 4, B—top) of the photoirradiated sample identified three distinct peaks. The two most abundant photoproducts were isolated by reverse phase HPLC and subjected to HRMS. The mass of one of the minor products matched the predicted photoactivation product, ether 21. However, the major product had a mass corresponding to a molecular formula of C8H8D5F3O5, which led to the conclusion that it was 21—DF. Further characterization using 1D and 2D NMR (19F-gCOSY, 19F-gNOESY, Fig. 5) indicated that the major photoactivation product with the mass equal to 21—DF is the polyfluorinated alkene 22. In recent literature, a combination of 19F-gCOSY and 19F-gNOESY has proved to be an efficient method for structural analysis of perfluorinated straight chains. 19F-gCOSY shows the JHF correlations whereas 19F-gNOESY shows both JHF and JHF correlations. By using these two techniques in tandem, sequencing of straight chain fluorous tag is made straightforward and reliable. As shown in Fig. 5, 19F-gNOESY of 22 gives JHF and JHF correlations while 19F-gCOSY shows only the JHF correlations except for the vinyl fluorine due to its connection to a sp2 carbon. The third minor photoproduct was not characterized due to its very low availability.

It has been suggested that photoaffinity labeling is highly dependent on the generation of a reactive intermediate ideally undergoing clean insertion reactions. If the lifetime of the reactive intermediate is too long and the ligand-protein exchange rate is high, then the photolabel dissociates from the binding site and possibly attaches to parts of the protein other than the binding site or even other proteins, resulting in an unspecific labeling also termed pseudo-affinity labeling. In order to avoid pseudo-affinity labeling, the photochemically generated intermediate should be highly reactive, very short lived and not susceptible to intramolecular rearrangements to much less reactive species. The unexpected outcome of the photoreaction of probe 1 suggests that the predicted carbene-insertion reaction is not the major pathway and thus probe 1 has potential for pseudoaffinity labeling. These
(A) $^{19}$F NMR (zoomed at CF$_2$ signal of fluorous tag) of 1 (left) and 2 (right) at different time intervals of UV irradiation (B) HPLC trace of 1 (top) and 2 (bottom) before (left) and after (right) UV irradiation.

Scheme 4 Photoactivation pattern of the photolabel 1 and 2.

![Chemical Structures](image)

Fig. 5 The correlations observed with $^{19}$F-gCOSY and $^{19}$F-NOESY of photoprod 22.

results clearly distinguish compound 1 from the conventional trifluoromethyl Diazirine probe 2.

**Synthesis of mannose photoaffinity probe 3**

The photoactivation studies suggest that photolabeling agent 1 and the previously reported photolabels (Fig. 1), in which the diazirine ring is directly incorporated on the fluorous chain, would possibly lead to poor and/or nonspecific protein labeling and consequently to erroneous or misleading data interpretations. Therefore, for deriving target specific photoaffinity probes, it is essential to use a predictable photolabel like 2. Since proteins that bind to mannose residues occupy a central role in glycochemistry, we decided to assess the derivatization capacity of photolabel 2 using mannose ligands. To achieve glycosylation of the diol 2 with a glycosyl-donor like mannose pentaacetate, it is essential to use boron trifluoride etherate to activate the glycosyl donor. The glycosylation reaction yielded the desired bis-glycosylated product with the intact diazirine ring as the major product. Subsequently, the crude material was subjected to acetate deprotection using sodium methoxide and purified by preparative HPLC to obtain the desired fluorous photolabel 3 in moderate yield over two steps (Scheme 5). The coupling constants and 2D NMR indicated that the bis-glycosylation was diastereoselective, yielding the $\alpha$-anomer exclusively. The alcohols in the photolabel can be further converted to amine and carboxylic acid groups as reported by Song and Zhang, for efficient attachment of various ligands targeting different receptors.

**Enrichment of photoaffinity probe 3 by FSPE**

To evaluate the enriching ability of the photolabel using fluorous interactions a mixture of peptide along with the photolabel 3 was loaded on to a bed of fluorous silica gel in a pipette column (fluorous bed: 40 x 6 mm) (Fig. 6). Initially the loaded fluorous short column was flushed with water to elute out the peptide (Fig. 6, B) and followed by elution with methanol to get the
directly incorporated in the fluorous tag, mainly undergoes a self-deactivating elimination to yield a polyfluorinated alkene. This potential self-deactivation of the transient carbene suggests that photoprobe 1 is not ideal for specific photolabeling of protein or prostate samples. Conversely, photolabel 2 was found to be a reliable photolabel for derivatization with target specific ligands. As a proof of feasibility to derivatize photolabel 2, mannose-containing photoaffinity probe 3 was synthesized and its efficacy in fluorous enrichment was evaluated. On the route to synthesize photolabel 2, we have optimized an efficient and low hydrogen pressure method to hydrogenate a carbon-carbon double bond on a perfluorinated alkynyl-aryl bromide.

**Experimental section**

**General information**

All NMR experiments were performed on a Varian Inova 400 MHz spectrometer (1H at 400, 13C at 100 and 19F at 376 MHz). Chemical shifts (in ppm, δ scale) are reported using solvent peak as internal standard. The ESI high resolution mass spectra were recorded using Agilent G1969A mass detector with time-of-flight (TOF) analyzer. Thin layer chromatography was performed on EMD silica gel 60F plates and the spots were visualized with UV light or iodine stain. Purification of reaction crude was done by flash chromatography using EMD silica gel (230–400 mesh).

The elemental analysis were carried out at Atlantic Microlab, Inc., Georgia. The melting points of compounds were measured on Electrothermal Mel-Temp 3.0. The photolabeling studies were done using Oriel Instruments housing with Osram 150 W XBO xenon short arc lamp. The light source was fitted with a Schott filter WG 320 to eliminate UV lights below 320 nm. The photolabeling studies were done as reported by Brunner and Richards.

(5-Bromo-1,3-phenylene)bis(methylene)bis(oxo)bis(tert-butyl)diamidylsilane (5). To a solution of (5-bromo-1,3-phenylene)diamethanol27 4 (1.21 g, 5.57 mmol) in dry dichloromethane (35 mL), TBSCl (2.10 g, 13.9 mmol) and imidazole (1.13 g, 16.6 mmol) were added and stirred at room temperature for 16 h. The reaction mixture was quenched with sat. ammonium chloride solution and extracted with dichloromethane. The organic layer was dried over anhydrous sodium sulfate and concentrated. The resulting crude was purified by column chromatography (19:1, hexanes and ethyl acetate) to give 5 (2.33 g, 94%) as a colorless oil. Rf 0.72 (19:1 hexanes and ethyl acetate), δH (400 MHz, CDCl3) 7.32 (1H, s), 7.19 (1H, s), 4.70 (4H, s), 0.93 (18H, s), 0.09 (12H, s), δF (100 MHz, CDCl3) 143.9, 127.7, 128.5, 64.5, 26.1, 18.6, -5.0. Elemental analysis (CHN) calculated for C65H145Br2O14Si (%) C, 53.9; H, 8.4; N, 0.0; found C, 54.15; H, 8.4; N, 0.0.

1-(3,5-Bis(tert-butyl)dihydroxyimino)methylphenyl)-2,2,3,3,4,4,5,5,6,6,7,7,7-tridecafluorohexane-1-one (6). To a solution of 5 (1.94 g, 4.35 mmol) in dry diethyl ether (30 mL) under argon, n-butyl lithium (4.35 mL of 2.5 M soin hexane) was added dropwise at ~40 °C. The reaction mixture was warmed to 10 °C and stirred for 2 h, then cooled back to ~50 °C and methyl perfluorooctanoate (6.57 g, 17.4 mmol) was added dropwise. The solution was warmed to room temperature and

**Conclusions**

We have designed and synthesized two multifunctional photolabels 1 and 2. Photoactivation studies with labeling agents 1 and 2 revealed that photolabel 1, in which the diazirine ring is

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sulfate and concentrated under vacuum. This crude was dissolved in methanol (1.5 mL) and triethylamine (60 mg) was added to it, followed by the dropwise addition of iodine solution in methanol (30 mg mL⁻¹) until the color of iodine persisted. The reaction proceeded for another 30 min and the solvent was evaporated, followed by the addition of water and extraction with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate, concentrated and the resulting crude was purified using silica column chromatography (97:3, dichloromethane and methanol) to give I (18.9 mg, 49%) as a white solid. Rf 0.51 (23:1, dichloromethane and methanol). M.p. 69–70 °C. δ₁ (100 MHz, CDCl₃) 7.43 (1H) 7.33 (2H), 4.85 (2H), 4.61 (4H), δ₂ (100 MHz, CDCl₃) 144.9, 130.4, 128.3, 126.3, 119.6 109.0 (m), 64.5, 29.1 (t, J = 26.8 Hz), δ₃ (576 MHz, CDCl₃) –82.85 (3F), –110.95 (2F), –121.50 (2F), –123.39 (2F), –124.28 (2F), –127.77 (2F). HRMS (ESI⁺) for [M + Na⁺]: calculated 519.0387, found 519.0339 (error = –1.84 ppm).

1-tert-Butyl-3-bromo-5-isodophenylcarbamate (11). A solution of 3-bromo-5-iodobenzonic acid (5.0 g, 15.3 mmol) in tert-butanol (25 mL), triethylamine (2.01 g, 19.9 mmol) and diphenylphosphoryl azide (4.63 g, 16.8 mmol) were added and refluxed for 24 h under argon. The reaction mixture was concentrated under vacuum, extracted with diethyl ether, and washed with 10% NaOH solution, water (2 times) and then brine solution. After drying over anhydrous sodium sulfate, the organic layer was concentrated and the crude was purified using silica column chromatography (9:1, hexanes and ethyl acetate) to give II (4.57 g, 75%) as a white solid. Rf 0.72 (4:1, hexanes and ethyl acetate). M.p. 97–98 °C. δ₁ (400 MHz, CDCl₃) 7.65 (1H), 7.54 (1H), 7.48 (1H), 6.44 (1H, s), 1.49 (9H), δ₂ (100 MHz, CDCl₃) 122.2, 140.7, 134.2, 125.8, 123.2, 120.8, 94.3, 81.7, 28.5. HRMS (ESI⁺) for [M + H⁺]: calculated 397.0247, found 397.0242 (error = –1.17 ppm).

3-Dimethyl-5-amino-5-bromophenylsilic acid-3-1,3-dicarboxylate (12). To an argon-bubbled flask, tetradehydrofuran (150 mL), 2 M sodium carbonate solution (45 mL), HIO₄ (10.95 g, 46.0 mmol), H₂O (11.5 g, 28.8 mmol) and bis(triphenylphosphine)palladium(0) dichloride (1.01 g, 1.44 mmol) were added and refluxed for 24 h. The reaction mixture was cooled, extracted with ethyl acetate and washed with water. The organic layer was dried with anhydrous sodium sulfate and concentrated. The resulting crude was purified using silica column chromatography (9:1, hexanes and ethyl acetate) to give the product 12 (5.97 g, 57%). Rf 0.37 (4:1, hexanes and ethyl acetate). M.p. 195–197 °C. δ₁ (400 MHz, CDCl₃) 8.59 (1H, t, J = 1.4 Hz), 8.28 (2H, d, J = 1.9 Hz), 7.03 (1H, t, J = 1.5 Hz), 6.93 (1H, d, J = 1.9 Hz), 6.90 (1H, t, J = 1.7 Hz), 3.98 (9H), δ₂ (100 MHz, CDCl₃) 167.55, 151.93, 142.95, 142.84, 133.03, 132.74, 130.46, 124.72, 119.49, 113.85, 113.23, 53.18. HRMS (ESI⁺) for [M + Na⁺]: calculated 385.9988, found 385.9986 (error = –2.29 ppm).

Dimethyl-3-bromo-5,5-dimethyl-5,5-dihydro-1,3-dicarboxylate (13). To a 50% aqueous solution of tetrafluoroboric acid (70 mL) and water (70 mL) at 0 °C, 12 (5.97 g, 16.4 mmol) was added. A solution of sodium nitrite (1.69 g, 24.6 mmol) in water (10 mL) was added dropwise to the reaction mixture over 10 min and warmed to room temperature.
After 16 h the reaction mixture was filtered and washed with 5% tetrahydrofuran acid followed by ethyl ether. Upon drying the mixture under argon, it was suspended in dimethylacetamide (100 mL) followed by the addition of palladium(t) acetate (55 mg, 245 µmol) and perfluorooctylsulfane (7.94 g, 22.9 mmol). The suspension was warmed to 40 °C and stirred until gas evolution ceased and then stirred for an additional 30 min. The reaction mixture was concentrated, extracted with ether and washed with water followed by drying the organic layer over anhydrous sodium sulfate and concentration under vacuum. The crude was purified using silica column chromatography (17:3, hexanes and ethyl acetate) to give the product 1 as a white solid (7.7 g, 68%). Rf = 0.53 (4:1, hexanes and ethyl acetate). mp: 156-157 °C. δN (400 MHz, CDCl3) = 8.68 (1H, s), 8.39 (2H, s), 7.77 (1H, s), 7.65 (1H, s), 7.60 (1H, s), 7.17 (1H, d, J = 16.0 Hz), 6.36-6.22 (1H, m), 3.97 (6H, s). δC (100 MHz, CDCl3) = 166.1, 142.0, 139.9, 138.20 (t, J = 9.3 Hz), 136.3, 132.4, 131.9, 131.8, 130.5, 130.0, 125.6, 124.0, 123.0, 107.3 (m), 117.0 (t, J = 33.3 Hz), 52.8, 38.7 (376 MHz, CDCl3) = −81.24 (3F), −114.98 (2F), −122.90 (2F), −123.83 (2F), −126.58 (2F). Elemental analysis (CHN, F) calculated for C29H34BrF23O7 (50.9%), C: 41.6, H: 2.0, N: 0.0, F: 35.6; found C: 41.9, H: 1.9, N: 0.0, F: 35.9.

**Dimethyl 3-bromo-5-(3,4,4,5,5,6,6,7,7,8,8,8-triclaburofluoroxy)phthalic anhydride (14)**. To a solution of 13 (1.59 g, 2.30 mmol) in ethyl acetate (20 mL) 10% platinum oxide (26 mg, 115 µmol) was added and the reaction mixture was shaken in a hydrogenator under 40 psi of hydrogen gas. After 3 days, another portion of platinum oxide (26 mg, 115 µmol) was added and the reaction was shaken under 40 psi for another 3 days and the reaction mixture was filtered and concentrated. The crude was purified using silica column chromatography (4:1, hexanes and ethyl acetate) to give the product 14 as a white solid. Rf = 0.25 (4:1 hexanes and ethyl acetate). mp: 77-80 °C. δN (400 MHz, CDCl3) = 8.66 (1H, s), 8.38 (2H, s), 7.66 (1H, s), 7.40 (2H, s), 3.97 (6H, s), 2.96 (2H, dd, J = 10.0, 6.7 Hz), 2.49-2.33 (2H, m). δC (100 MHz, CDCl3) = 166.2, 142.2, 141.8, 134.0, 132.4, 131.6, 131.3, 130.2, 128.9, 126.8, 123.7, 121.6-106.5 (m), 52.8, 33.9 (t, J = 9.2 Hz), 26.5. δF (376 MHz, CDCl3) = −81.24 (3F), −114.98 (2F), −122.90 (2F), −123.83 (2F), −126.58 (2F). Elemental analysis (CHN, F) calculated for C29H34BrF23O7 (50.9%), C: 41.6, H: 2.3, Br: 11.5, F: 35.5; found C: 41.75, H: 2.2, Br: 11.6, F: 35.0, O: 9.5.

**3-bromo-5-(3,4,4,5,5,6,6,7,7,8,8,8-triclaburofluoroxy)biphenyl-3-carboxylic acid** (15). To a suspension of lithium hydride aluminium hydride (211 mg, 5.36 mmol) in dry tetrahydrofuran (9 mL), a solution of 14 (1.288 g, 1.85 mmol) in dry tetrahydrofuran (11 mL) was added dropwise at 0 °C under argon. Another 4 mL of tetrahydrofuran was added through the dropping funnel to rinse down the dropping funnel and warmed to room temperature. After 45 min the reaction was quenched by slow addition of saturated sodium sulfate solution and extracted with ethyl acetate followed by drying the organic layer with anhydrous sodium sulfate and concentrated under vacuum. To the concentrated residue, dichloromethane (12 mL), TBDMSCl (838 mg, 5.56 mmol) and imidazole (757 mg, 11.1 mmol) were added at room temperature and stirred overnight. The reaction was quenched with saturated ammonium chloride solution, extracted with dichloromethane and the organic layer was dried over anhydrous sodium sulfate and concentrated under vacuum. The resulting crude was purified using silica column chromatography (97:3, hexanes and ethyl acetate) (Rf = 0.38, 86%) as a colorless oil. Rf = 0.68 (19:1, hexanes and ethyl acetate). δC (376 MHz, CDCl3) = 7.61 (1H, s), 7.37 (2H, s), 7.34 (1H, s), 7.33 (1H, s), 4.80 (4H, s), 3.02-2.84 (2H, m), 2.55-2.27 (2H, m), 0.96 (18H, s), 0.12 (12H, s). δN (100 MHz, CDCl3) = 144.2, 142.5, 141.8, 139.4, 130.2, 128.9, 127.8, 126.5, 125.8, 121.6, 120.7, 123.6, 123.3, 121.6-107.0 (m), 65.3, 33.1 (t, J = 22.3 Hz), 25.3-27.5 (m), 18.7-5.1. δF (376 MHz, CDCl3) = −114.29 (3F), −123.29 (2F), −123.89 (2F), −126.58 (2F).
0.46 (% 1 hexanes and ethyl acetate), δ(400 MHz; CDCl₃) 7.89 (2H, t, J = 7.5 Hz), 7.45-7.32 (2H, m), 7.16 (1H, s), 4.82 (4H, s), 3.16-2.94 (2H, m), 2.62-2.28 (5H, m), 0.97 (18H, s), 0.13 (12H, t), δ(100 MHz; CDCl₃) 154.3 (q, J = 33.7 Hz), 146.4, 143.2, 142.7, 140.7, 139.4, 131.4, 130.5, 130.1, 129.5, 127.0, 126.0, 125.4, 123.7, 126.1, 27.1-77.7 (7H, m), 65.0, 32.9 (t, J = 22.3 Hz), 28.72-23.11 (m), 21.9, 18.6, -5.2, δ(576 MHz; CDCl₃) -61.93, -67.41 (2F), -71.1, -81.28 (3F), -114.95 (2F), -122.28 (2F), -123.29 (2F), -123.84 (2F), -126.09 (2F).

3-(2,3,5,6-Tetrafluorophenyl)-1-ethyl-4-(4-isopropylphenyl)-1H-pyrrole-2,4,5-triyl-2,4,5-triyl-1H-pyrrole-2,4,5-triyl-2,4,5-triyl-1H-pyrrole (3). To a solution of 2.4 (40 mg, 58.8 µmol) of the product in dry diethyl ether (6 mL), ammonia (10 mL) was added at 0 °C and stirred for 8 h. The reaction mixture was warmed to room temperature and stirred for additional 18 h by evaporation of the solvent. The reaction mixture was washed with dichloromethane and the organic layer was dried over anhydrous sodium sulfate and concentrated. The residue was dissolved in anhydrous methanol (0.4 mL) followed by the addition of 5 M sodium hydroxide solution (40 µL) and stirred at room temperature, under argon, for 3 h. The reaction was quenched with the addition of Amberlyst-15 and filtered. The filtrate was concentrated and the resultant residue was purified by reverse phase semi-prep HPLC. The collected peaks with the desired compound, as identified by LC-MS, were concentrated and lyophilized to give 3 (25.6 mg, 43%) as a white solid. δ(400 MHz; CDCl₃) 7.69 (1H, s), 7.53 (2H, s), 7.48 (1H, s), 7.32 (1H, s), 7.19 (1H, s), 4.88 (2H, d, J = 1.7 Hz), 4.84 (13H, including solvent peak), 4.8 (2H, d, J = 12.0 Hz), 3.91-3.81 (4H, m), 3.79-3.69 (4H, m), 3.67-3.58 (4H, m), 3.10-3.00 (2H, m), 2.65-2.46 (2H, m), δ(100 MHz; CDCl₃) 143.9, 143.1, 141.5, 140.5, 131.2, 130.5, 128.7, 127.5, 126.9, 124.8, 124.2-108.2 (m), 100.9, 75.2, 72.8, 72.3, 69.8, 68.8, 63.1, 33.4 (t, J = 19.2), 29.7 (q, J = 80.4), 27.5, δ(376 MHz; CDCl₃) -67.41 (3F), -82.84 (3F), -115.74 (2F), -123.33 (2F), -124.31 (2F), -124.74 (2F), -127.75 (2F). HRMS (ESI) for [M+Na⁺]: calculated: 1015.1905; found: 1015.1906 (error = 0.1 ppm). Please refer to ESI for H-SCOSY and HMOC.

3-(2,3,4,5,6,7,8,8,8'-Octafluorotriphenyl)1H-pyrrole-2,4,5-triyl-2,4,5-triyl-1H-pyrrole-2,4,5-triyl-1H-pyrrole-2,4,5-triyl-1H-pyrrole (4). To a solution of 1 (100 mg, 0.23 mmol) in dry diethyl ether (6 mL), dimethylformamide (24 µL) was added at 0 °C and stirred for 1 h. The reaction mixture was filtered and concentrated under vacuum to give 4 (53.5 mg, 72%) as a white solid. δ(400 MHz; CDCl₃) 7.63 (1H, s), 7.51 (2H, s), 7.38 (4H, s), 4.69 (4H, s), 3.08-3.01 (2H, m), 2.71-2.42 (2H, m), δ(100 MHz; CDCl₃) 178.59 (3F), -82.85 (3F), -115.79 (2F, m), -123.34 (2F, m), -124.32 (2F, m), -124.79 (2F, m), -127.67 (2F, m), HRMS (ESI) for [M+NH₄⁺]: calculated: 694.1746, found: 694.1718 (error = -0.32 ppm). Please refer to ESI for H-SCOSY and HMOC.

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10 μmol) was dissolved in methanol-D4 (5 mL) in a test tube. This solution was subjected to photo irradiation with UV light (>320 nm) and the photostimulated was followed at various time intervals using 1H NMR. After 2 h of UV irradiation the sample was subjected to HPLC purification and the major peak at 12.9 min was collected and concentrated, followed by resynthesis to yield 0.5 (400 MHz; CD3OD) 7.50 (1H, s), 7.25 (2H, s), 4.66 (4H, sh). δ1 (376 MHz; CD3OD) 82.90 (3F, br.), -112.41 (2F, m), -123.81 (2F, m), -124.66 (2F, m), -127.86 (2F, m), -157.96 (1F, m). HRMS (ESI) for [M+Na]+: calculated 501.1121, found: 501.1117 (error = -9.7 ppm). Please refer to ES1 for 19F-COSY and 19F-NOESY.

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Notes and references


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APPENDIX – 2

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Jul 03, 2012

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Synthesis and structure-activity relationship studies of 1,3-disubstituted 2-propanols as BACE-1 inhibitors

Arun Babu Kumar, Jordan Michael Anderson, Anthony Lester Melendez, Roman Manetsch*

Department of Chemistry, University of South Florida, CHE 205, 4202 E. Fowler Ave, Tampa, FL 33620, USA

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ABSTRACT
A library of 1,3-disubstituted 2-propanols was synthesized and evaluated as low molecular weight probes for β-secretase inhibition. By screening a library of 121 1,3-disubstituted 2-propanol derivatives, we identified several compounds inhibiting the enzyme at low micromolar concentrations. The initial hits were optimized to yield potent BACE-1 inhibitors exhibiting an IC₅₀ constant in the nanomolar range. Exploration of the pharmacological properties revealed that these small molecular inhibitors possessed a high selectivity over cathepsin D and desirable physicochemical properties beneficial to cross the blood–brain barrier.

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The protease BACE-1 (also known as β-secretase) is a validated target for the development of inhibitory compounds with potential as a treatment for Alzheimer's disease (AD).1–3 BACE-1 is an aspartic protease which cleaves the amyloid precursor protein (APP), a transmembrane protein, into a 42 amino acid peptide also known as the amyloid β (Aβ).4–6 Though, the exact mechanisms of events leading to AD are still under scrutiny, recent reports suggest that the insoluble Aβ peptide is neurotoxic causing the neurodegeneration typically observed in AD.6,7 Furthermore, it has been shown that BACE-1 knockout mice not only produce Aβ at significantly reduced amounts, but also do not develop the typical pathology of AD.8–10 The inhibition of BACE-1, therefore, is considered to be among the best strategies to reduce the undesired Aβ production and to treat AD.

In general, the development of efficacious BACE-1 inhibitors is difficult due to the requirement that the inhibitory compounds must not only be potent, but also display selectivity over other aspartic proteases and have good physicochemical properties to penetrate the blood–brain barrier.11 Furthermore, the elongated BACE-1 active site dimensions challenge medicinal chemists to design a small molecule that efficiently occupies the active site.12,13

Pepidonometics approach with transition state isosteres has been one of the successful strategy for developing BACE-1 inhibitors.14–16 For example, hydroxethylamines (HEAs) are known to broadly mimic the transition state of aspartyl proteases' substrates14,16 and they have been successfully incorporated in BACE-1 inhibitory compounds.17–23 Macchia and co-workers previously reported the study of a small library of eight hydroxethylamine-containing BACE-1 inhibitors exhibiting IC₅₀ values in the low μM range.13,25 Extensive docking studies with compound 1 suggests that the hydroxyl group tightly interacts with the aspartic acid residue Asp 228 positioning the carbazole moiety in the S1 and S2 subpockets, while the 1-naphthylamine interacts in the S2' pocket via a

![Diagram]

Figure 1. Schematic representation of the main interactions between BACE-1 inhibitor 1 and the BACE-1 active site. Hydrogen bonds are shown as dotted lines.13
hydrogen bond between the nitrogen and tyrosine 198 and Van der Waals interactions with tyrosine Tyr 198, valine Val 69, and isoleucine Ile 126 (Fig. 1).\(^1\)

As these 1,3-disubstituted 2-propanol derivatives possess low 
\(\mu M\) potency, small molecule size and attractive synthetic tractability we decided to conduct an extensive investigation on this molecular scaffold for BACE-1 inhibition. A diverse set of indoles, secondary amines and carbazoles derivatized with an epoxide group was synthesized and further reacted with commercially available thiols to yield a library of 1,3-disubstituted 2-propanols (Fig. 2A). All reactions were performed at 50–100 mg scale and the products were purified by column chromatography and subsequently characterized by \(^1\)H NMR, \(^{13}\)C NMR, and MS. Besides the previously reported carbazole and indole containing scaffolds, the library design was further expanded by a biphenyl (E9), a diphenylmethane (E10) and various secondary amines (E1, E11, E12 and E13). Although not all possible compound combinations were prepared and tested, a selection of 121 1,3-disubstituted 2-propanols was synthesized and tested to provide reliable information about epoxide thiol combinations that yield promising BACE-1 inhibitors. The whole 1,3-disubstituted 2-propanol library of compounds was initially tested at 100 \(\mu M\) concentration using a detergent-containing fluorescence resonance energy transfer (FRET)-based BACE-1 assay, which previously has been successfully used in identifying BACE-1 inhibitors.\(^{24,26}\) Of these 121 compounds, the compounds that showed activity at 100 \(\mu M\) were then screened at both 50 and 25 \(\mu M\) compound concentration. Although this simple screening approach did not provide very precise inhibition data, it provided valuable structure–activity trends important for hit optimization. The majority of the compounds that displayed activity contain at least one extended ring system such as a carbazole, a tetrahydro-carbazole, or a 2-phenyl-indole, while

![Chemical structures](image)

**Figure 2.** Synthesis and screening of a library of 1,3-disubstituted-2-propanols. (A) Preparation of the initial library starting from epoxides and commercially available thiols. Of the 234 possible compounds, 121 analogues have been synthesized. (B) A set of 121 compounds have been tested initially at 100 \(\mu M\) concentration. Compounds displaying 75% inhibition or greater have been further tested for BACE-1 inhibition at 50 and 25 \(\mu M\) concentrations. The inhibition data for the screening at 100, 50, and 25 \(\mu M\) compound concentrations have been given as percent inhibition. Percent inhibition data is the average of two or more independent measurements.

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compounds with an indole ring or small aromatic residues are less potent. Furthermore, compounds containing basic amine were completely inactive, though these comprise aromatic residues.

All compounds that showed an activity of 50% inhibition or greater at 25 μM were then tested at multiple concentrations to establish an inhibitory concentration I_{50}. The inhibitory concentrations were determined for 16 compounds resulting in a range of I_{50} constants from 4.93 to 361.1 μM. The best inhibitor was found to be E7T18 with an I_{50} of 4.93 μM (Table 1), while 2-phenyl-indole E8T18 was slightly less potent. One of the most promi-nent bottlenecks in identifying lead molecules for BACE-1 inhibition is developing inhibitors with selectivity for BACE-1 over other essential asparyl proteases. Compounds displaying I_{50} in the single digit μM range were further assessed for their selectivity to inhibit BACE-1 over cathepsin D. Cathepsin D is an asparyl protease with a catalytic domain similar to BACE-1 and is ubiquitously present in almost all cells. Importantly, none of the tested compounds showed any significant inhibition of cathepsin D up to 300 μM compound concentration rendering the best compounds, E7T18 and E8T18, a selectivity of at least 50 fold. As reported by Schecht and co-workers, lead identification can be compromised by promiscuous compounds that act as noncompetitive inhibitors by aggregate formation leading to false positives. As put forth by Seidler and co-workers, among the important criteria in assessing aggregation-based promiscuity include non-specific activity and high sensitivity to the presence of detergent. The fact that the lead inhibitors show a good selectivity for BACE-1 over cathepsin D and the presence of 0.05% detergent in the assay buffer to disrupt aggregates are good indicators that the lead inhibitors are likely to be valid hits for BACE-1.

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>BACE-I IC_{50} (μM)</th>
<th>Cathepsin D IC_{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7T18</td>
<td>4.93 ± 0.86</td>
<td>&gt;300</td>
</tr>
<tr>
<td>E8T18</td>
<td>361.1 ± 7.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>E8T19</td>
<td>13.0 ± 3.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>E9T10</td>
<td>25.4 ± 6.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>EBT15</td>
<td>10.3 ± 0.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>EBT18</td>
<td>6.05 ± 1.01</td>
<td>&gt;300</td>
</tr>
<tr>
<td>EBT19</td>
<td>9.60 ± 1.20</td>
<td>&gt;300</td>
</tr>
<tr>
<td>EBT19</td>
<td>10.7 ± 2.0</td>
<td>n.d.</td>
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</table>

Next, studies have been undertaken to investigate the linker connecting the carbazole or 2-phenyl-indole to the naphthyl ring. This linker optimization focused on compound E7T18, since it is the most potent compound. Initially, analogues have been synthesized to evaluate whether the alcohol group is important for activity. Complete removal of the hydroxyl group in compound 2 was combined with a decrease in activity (Table 2). Next, the analogues of E7T18 were synthesized independently and tested against BACE-1. The S enantiomer 3 with an I_{50} of 5.39 μM seems to bind to BACE-1 with an affinity similar to the racemic compound E7T18, whereas the R enantiomer 4 was found to be slightly less active. Importantly considering the standard deviations to be in the range of 0.8-1.1 μM, the small differences between the I_{50} of compounds E7T18, 3 and 4 support the previous docking studies by Macchia and co-workers, in which the hydroxyl group of H5A inhibitor interacts with Asp 238 independently of the alcohol's stereochemistry. With the idea to improve the interaction between the compound's linker and BACE-1, other groups were introduced as replacements of the alcohol. The potency dramatically dropped for ketone 5 possibly due to an unfavorable interaction between the negatively charged Asp 238 and the ketone's dipole moment. Similarly, primaryamines 6 and 7, as well as piperazine derivative 8 did not improve the activity against BACE-1.

Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>R and R'</th>
<th>BACE-1 IC_{50} (μM)</th>
<th>Cathepsin D IC_{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>R = R' = H</td>
<td>7.52 ± 1.13</td>
<td>&gt;300</td>
</tr>
<tr>
<td>3</td>
<td>R = OH, R' = H, S-configuration</td>
<td>5.39 ± 0.81</td>
<td>&gt;300</td>
</tr>
<tr>
<td>4</td>
<td>R = H, R' = OH, R-configuration</td>
<td>7.04 ± 1.02</td>
<td>&gt;300</td>
</tr>
<tr>
<td>5</td>
<td>R = carboxyl</td>
<td>&gt;80.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>6</td>
<td>R = NH2, R' = H, racemic</td>
<td>15.7 ± 1.4</td>
<td>n.d.</td>
</tr>
<tr>
<td>7</td>
<td>R = NR2CH2NH2, R' = H, racemic</td>
<td>7.38 ± 0.22</td>
<td>&gt;300</td>
</tr>
<tr>
<td>8</td>
<td>R = piperazine, R' = H, racemic</td>
<td>&gt;50.0</td>
<td>n.d.</td>
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<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>BACE-1 IC₅₀ (µM)</th>
<th>Calpain D IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td><img src="image" alt="Structure" /></td>
<td>0.71 ± 0.10</td>
<td>&gt;300</td>
</tr>
<tr>
<td>13</td>
<td><img src="image" alt="Structure" /></td>
<td>&gt;30</td>
<td>n.d.</td>
</tr>
<tr>
<td>14</td>
<td><img src="image" alt="Structure" /></td>
<td>&gt;100</td>
<td>n.d.</td>
</tr>
<tr>
<td>15</td>
<td><img src="image" alt="Structure" /></td>
<td>1.82 ± 0.50</td>
<td>&gt;300</td>
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</table>

Also it can be inferred that the linker chain length of four methylene units is slightly better than three methylene units.

Finally, a set of compounds was prepared, in which the 2-propanol linker was substituted in 1- and 3-positions with two carbazole or two 1-naphthylamine moieties. Surprisingly, the bis-carbazole 12 was very potent with an IC₅₀ of 710 nM, while poor inhibition was determined for bis-naphthyl 13 (Table 4). Consequently, analogues of 12 with 4 carbon linker, 14 and 15, were prepared to probe the optimal linker length and the position of the alcohol group, but they did not improve on the inhibition of BACE-1 over 12 with 3 carbon linker.

Besides potency against BACE-1 and selectivity over off-target proteases such as cathepsin D, useful inhibitors must also have acceptable physicochemical properties to penetrate the blood-brain barrier. A selection of BACE-1 inhibitors has been evaluated for blood-brain barrier permeability according to the rules proposed by Clark and Lobell (Table 5). These rules predict that a compound will possess acceptable blood-brain barrier permeability if the compound’s molecular weight is less than 450 g/mol, the polar surface area (PSA) is < 100 Å², the number of nitrogen and oxygen (N+O) < 6, and the parameter ΔδP(N+O) > 0. Alternatively, using QuickPrep, the partition coefficient logBB, which is defined as the ratio of the steady-state concentration of the compound in the brain over the one in the blood (logBB = log([compound]brain/[compound]blood)), has been computationally calculated for the most promising compounds. Excellent blood-brain permeabilities are predicted for compounds with a logBB > 0.3, while compounds with a logBB < 1.0 are classified to possess very poor blood-brain barrier permeability. Analysis of the most potent compounds against BACE-1 revealed that all analogues are in compliance with the rules dictated by Clark and Lobell. Furthermore, logBB predictions clearly propose that the blood-brain barrier permeability is in the acceptable range for the most potent compounds such as E7T18, 9, and 12. Compound E8T18, a close analogue of E7T18 in which the carbazole ring has been replaced by the 2-phenylindole moiety, has excellent calculated blood-brain barrier permeability. Overall, these predictions are encouraging and underline the potential of the herein described compound series.

In summary, we have conducted a study on a compound series of 1,3-disubstituted 2-propanol for the inhibition of BACE-1. A selected set of 121 compounds has been combinatorially prepared and tested for inhibition of BACE-1 activity and hit compounds such as E7T18, E7T3 and E8T18 have been identified to have IC₅₀ of 493, 530, and 6.06 µM, respectively. For the first time, the selectivity of 1,3-disubstituted 2-propanol to inhibit BACE-1 over other essential aminopeptidase was tested. Promising hit compounds have demonstrated to selectively inhibit BACE-1 over cathepsin D with a selectivity factor of 50 or more. Subsequently, structure-activity relationship studies focusing primarily on analogues of hit compound E7T18 have been undertaken to further refine the initial hit compounds. The screening results including the detailed structure-activity relationship studies suggest that best BACE-1 inhibition is observed if (a) a naphthyl or carbazole ring in combination with an extended aromatic ring system such as a carbazole or a 2-phenylindole is present to occupy the 51, 52, 5′, and 5′′ subpockets, (b) the linker has four methylene units, which is marginally better than the linker with three units, (c) the linker is sterically compact and it can accommodate only one hydroxyl group. Further optimization efforts ultimately lead to the most potent and selective compound, bis-carbazole 12, with an IC₅₀ of 710 nM against BACE-1 and a selectivity of >420 times over cathepsin D.

The structure-activity relationship data with respect to the hydroxyl-containing linker supports the docking model proposed by Macchia for HEA compound series[16] Additional studies are required, however, to fully understand the details of how compounds such as hit E7T18 or bis-carbazole 12 interact with BACE-1 on an atomic-level resolution. Nonetheless, the good potency in conjunction with high selectivity over cathepsin D and good blood-brain barrier permeability predictions make the herein presented

---

Table 5

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW</th>
<th>N+O</th>
<th>PSA</th>
<th>logBB</th>
<th>logBB</th>
<th>logBR</th>
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<tr>
<td>E7T18</td>
<td>388</td>
<td>2</td>
<td>20.4</td>
<td>4.53</td>
<td>-0.03</td>
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<tr>
<td>E7T3</td>
<td>409</td>
<td>2</td>
<td>21.0</td>
<td>5.25</td>
<td>0.374</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>367</td>
<td>1</td>
<td>2.81</td>
<td>6.85</td>
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<tr>
<td>10</td>
<td>397</td>
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<td>20.9</td>
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<tr>
<td>11</td>
<td>413</td>
<td>3</td>
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</tr>
<tr>
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<td>390</td>
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<td>23.2</td>
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<tr>
<td>15</td>
<td>420</td>
<td>4</td>
<td>46.3</td>
<td>2.84</td>
<td>0.001</td>
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All parameters have been calculated using the software QuickPrep (Schrödinger, LLC, New York).
compound series an excellent platform for future development of potent and selective BACE-1 inhibitors.

Experimental section

Compounds were prepared using synthetic procedures as reported in the Supplementary Data. The purity of all the compounds tested for BACE-1 was found to be ≥ 95% via HPLC analysis. Assay for BACE-1 has been done using previously reported methodology.24-30 Cathepsin D activity has been done using FRET-based assay kit from AnaSpec (catalog no. 72170), which was validated by testing with known inhibitor of Cathepsin D. For the testing of compounds against BACE-1 and cathepsin D, measurements were made in triplicates and inhibitory concentrations IC50 are reported with the standard deviation.

Funding

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Supplementary data

Supplementary data (experimental details of the synthesis for all compounds including 1H NMR, 13C NMR, and HR-MS for all tested compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.05.072.

References and notes

APPENDIX – 3

NMR Spectra