Traceless Solid-Phase Synthesis and Evaluation of reversible Methylene Azide Inhibitors for Cysteine Proteases

Lee M.Y.¹ and Yao S.Q.²

Department of Chemistry, Faculty of Science, National University of Singapore
10 Kent Ridge Road, Singapore 117546

ABSTRACT

Cysteine proteases account for 26% of human endopeptidase enzymes and they play crucial roles in diseases, immune defense, inflammation and apoptosis. The development of potent and selective cysteine protease inhibitors has been an increasingly active area of research as many cysteine protease inhibitors with reactive electrophiles have failed clinical trials. In addition, better understanding of these protein-protein network and apoptosis pathway can be achieved by using photoreactive peptides/proteins.

INTRODUCTION

Under the class of cysteine proteases are the caspases, which play key roles in both the cytokine maturation and apoptosis. As the over expression of these enzymes results in neurodegenerative diseases, the design of potent and selective inhibitors against the caspases will be useful in developing potential treatment against these neurodegenerative diseases. Studies have known that caspases have a near absolute requirement for an aspartic acid at the P₁ position of their substrate/inhibitor and the S₄ subsite is crucial in determining their specificity.

The goal of this project is to synthesize a library of azidomethylene cysteine protease inhibitors using the traceless solid-phase synthesis. Each inhibitor consists of an azidomethylene warhead, a pyridyl linker and a P₄ variable acid block (Figure 1). The use of an azidomethylene unit, which is less reactive and more stable than the aldehyde unit, is proposed to be able to confer potent and reversible inhibition of the enzyme. Besides, through extended tethering, the pyridyl linker was found to enhance the potency of the inhibitors via effective binding to the S₂ and S₃ pocket of the enzyme active site.

Another aim of this project is to synthesize the photoreactive phenylalanine analogue, L-4-[3-( trifluoromethyl)-3H-diazirin-3-yl]-phenylalanine (TmdPhe), which can be incorporated into peptides and proteins (Figure 2). The P₁ aspartic acid residue first binds to the S₁ pocket of the caspase active site. Upon UV irradiation, the diazirine unit will generate a reactive carbene that form a permanent cross-linkage with the active site. A biotin tag was incorporated at the N-

---

¹ Student
² Associate Professor
terminus of the peptide chain for detection of the photolabeled protein. These photoreactive peptides/proteins are powerful tools in the identification of interactions in protein networks.

Figure 2. Peptide chain incorporating an unnatural amino acid photo-cross-linker (TmdPhe) and a biotin tag (left); Structure of TmdPhe (right)

RESULTS AND DISCUSSION

Synthesis of Azidomethylene Warhead

Fmoc-protected amino acids were first reduced to their respective Fmoc-protected alcohols (Scheme 1). The alcohol functional group was then converted to azide via the Mitsunobu reaction. Finally, Fmoc deprotection gives the methylene azide warheads in good to high yields.

Synthesis of Pyridyl Linker

Scheme 2. Synthesis route of the 2,5-Disubstituted Nicotinic Acid 11

Scheme 1. Synthesis route of the azido amines 4a-d
The synthesis of the pyridyl linker (Scheme 2) started with the protection of the carboxylic acid group to form a methyl ester. After which, a benzylic bromination followed by an azidation reaction were carried out to form a free primary amine. Base catalyzed hydrolysis of the methyl ester then regenerates the carboxylic group. Finally, Fmoc protection of the amino group will give the final pyridyl linker. The overall yields were good except for the benzylic bromination step, which was low yielding as a result of the formation of di- or tri- brominated side products. Also, due to substantial product loss during the purification of 11, the subsequent traceless solid-phase synthesis was carried out using three other linkers.

Traceless Solid-Phase Synthesis

![Scheme 3. Traceless solid-phase synthesis route of azidomethylene inhibitors](image)

Using the high-throughput traceless solid-phase synthesis (Scheme 3), an azidomethylene library of 249 compounds was constructed. The first step involves the reductive amination of the compounds 4a-d with the PL-FMP resin, followed by the coupling of the linker 13a-c to the secondary amino group of 12a-d. Deprotection of the Fmoc-protected amino group of the compound 14 generates the free primary amine, which was then coupled with various carboxylic acids, sulfonyle chlorides and isocyanates building blocks. Finally, cleavage of the compounds from the resin using TFA gives the final methylene azide inhibitors 9, 10 and 11 in high yields and purity of greater than 90% in most of the cases.

Synthesis of TmdPhe

Although the synthesis of the TmdPhe has yet been completed (Scheme 4), the yields for the first three steps of the synthesis were generally high. The diazirine unit was observed to be photodegradation under normal light conditions. Hence, care should be taken to carry out subsequent steps in the dark.
CONCLUSION

A library of 249 member azidomethylene cysteine protease inhibitors has been successfully synthesized using the high-throughput traceless solid-phase synthesis. Each inhibitor comprises of an azidomethylene warhead (leu, phe, asp or lys), a linker (phenyl, cyclohexane or acylclic hexane linker) and an acid block (carboxylic acids, sulfonyl chlorides or isocyanates). In addition, upon successful synthesis of the photoreactive phenylalanine analogue, TmdPhe, the immediate goal is to incorporate it into peptides or proteins targeting caspases to study the protein-protein interactions.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Associate Professor Yao Shao Qin, and all my lab mates for their advice and continued support throughout the course of my experiments.

REFERENCES

