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Kotra Research Group

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Tuteur: Dr. Adrien Rossary

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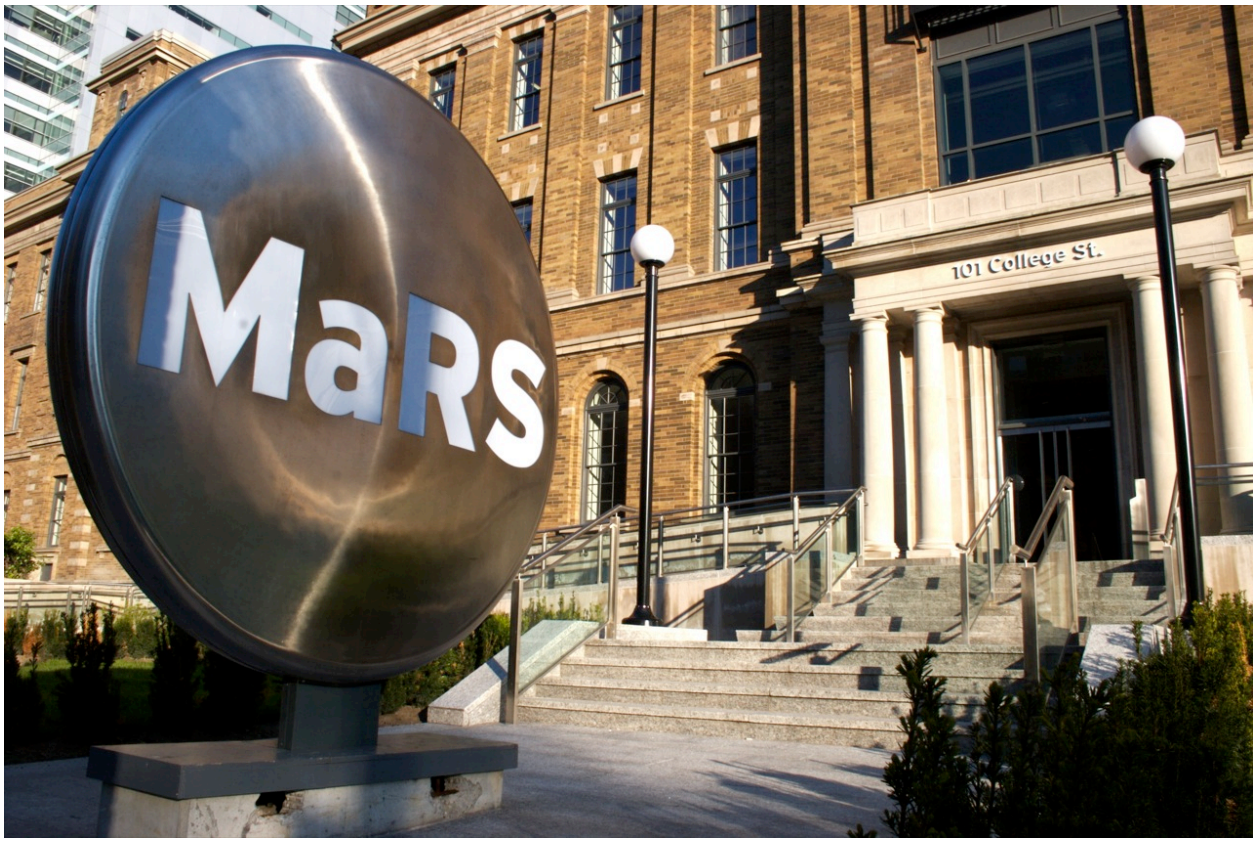


Summary

Abbreviations

BOP: Benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphoniumhexafluorophosphate

Cbz: Carboxybenzyl

DAST: Diethylaminosulfurtrifluoride

DBU: 1,8-Diazabicycloundec-7-ene

DCM: Dichloromethane

DBF: Dibenzofulvene

DIPEA: Diisopropylethylamine

DMF: Dimethylformamide

EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

Fmoc: Fluorenylmethyloxycarbonyl

FP: Falcipain

HOBt: 1-Hydroxybenzotriazole

MaRS: Medical and Related Sciences

THF: Tetrahydrofuran

TMDT: Toronto Medical Discovery Tower

I. Abstract

1. English

Resistance of the *Plasmodium* parasite is common with virtually all currently used antimalarial drugs. Consequently, development of novel therapeutics that will limit the spread of drug-resistant parasites is an urgent need. One attractive target for the development of new drugs is falcipain-2 (FP-2), a major cysteine protease of *Plasmodium falciparum* that mainly acts as a hemoglobinase for amino acid acquisition during the trophozoite stage. Although several FP-2 inhibitors have been developed, no compound has passed the stage of murine studies. Accordingly, research for a FP-2 inhibitor used as an antimarial drug in humans is pivotal. Herein we report the synthesis of a novel peptidomimetic FP-2 inhibitor, structurally determined from in-silico screening at the docking site of FP-2. Additionally, in vitro enzymatic assays were conducted to determine the relative biological activity of the small drug molecules. The described synthesis constitutes a foundational premise for the development of synthetic analogues.

2. Français

Le parasite *Plasmodium* est caractérisé par une résistance fréquente avec la grande majorité des traitements antipaludiques. Il est donc indispensable de développer des moyens thérapeutiques qui limiteront la propagation de parasites résistants. Une cible intéressante pour le développement de nouveaux médicaments est la falcipain-2 (FP-2), une cystéine protéase majeure de *Plasmodium falciparum* qui agit en tant que hémoglobinase pour l'acquisition d'acides aminés lors de l'état de trophozoïte. Bien que plusieurs inhibiteurs de la FP-2 aient été produits, aucun composé n'a passé les études murines. La recherche pour un inhibiteur de la FP-2 utilisable en médecine humaine est donc essentielle. Nous présenterons la synthèse d'un inhibiteur peptidomimétique de la FP-2 dont la détermination est issue d'un screening au niveau du docking site de la FP-2. De plus, des études

enzymatiques in vitro ont été réalisées afin de déterminer l'activité biologique de la molécule. La synthèse décrite ci-dessous constitue un travail préliminaire pour la synthèse d'analogues.

II. Kotra Research Group

1. Presentation

Kotra laboratory is located in the discovery district of Toronto. The name of the institute where the laboratory is situated is MaRS centre, Toronto Medical Discovery Tower. It is a part of the University Health Network that connects four teaching hospitals with the University of Toronto. The medicinal chemistry laboratory is part of the Center for Molecular Design and Preformulations. It has both collaborators in the University Health Network but also from outside, both national (e.g. Canadian Blood Services) and international that are mainly in India.

The group is multidisciplinary and works with computer modeling, synthetic medicinal chemistry, conjugation chemistry and biochemistry. Its research is focused on drug discovery which includes drug design, preformulations study and drug delivery.

Currently, the main subjects of study are the design of:

- Inhibitors of various enzymes
    - Falcipain-2, with applications in malaria
    - Orotidine monophosphate decarboxylase, with applications both in cancer and malaria
    - Peptidyl arginine deiminases, with applications in multiple sclerosis.
- Interferon mimetics, with applications in viral infections
- Compounds that inhibit and reverse the degeneration of peripheral neurons that could be use to provide relief in diabetic neuropathy.
- Protein-drug conjugates using cytotoxic drugs for drug delivery purposes.

The architecture of the laboratories at Toronto Medical Discovery Tower (TMDT) is very friendly. There are no walls between the labs of different groups. This facilitates communication and free flow of ideas between students and experienced scientists engaged in different levels of medical research. The facilities at the Center for Molecular Design and Preformulations are shared by the Kotra research group with other research groups of the TMDT.

Furthermore the group is multicultural. It is composed of people originally from Canada, China, India, Italy, Mexico, Peru and Poland. This can bring different methods of thought that helps to tackle a problem from different angles.

2. Intern's position

I worked in the synthetic medicinal chemistry lab of Dr. Lakshmi P. Kotra supervised by Dr. Sai Kumar Chakka whom I assisted.

3. Assignment

My subject of study was the synthesis of novel inhibitors of falcipain-2 as antimalarial agents, using structure-based drug design. I was also in charge of the synthesis of various intermediates and final compounds for other projects to strengthen my synthesis skills. Along with the synthesis I was engaged with senior chemists in the characterization of organic compounds by NMR and LC-MS.

III. Introduction

Malaria is an infectious disease caused by parasites of the genus *Plasmodium*. It is transmitted by the bites of female mosquitoes of the genus *Anopheles*. The parasite first infects the host's hepatocytes then travels to the bloodstream invading erythrocytes and destroying them. Only four species of the genus Plasmodium are specific to humans. *Plasmodium falciparum* is the cause of most severe disease and deaths whereas *P. vivax*, *P. malariae* and *P. ovale* are responsible for milder forms of malaria. One last species, *P. knowlesi*, which was long though specific to simian species, can now be considered as a zoonotic human parasite[1].

The control of malaria relies on two main strategies: preventing malaria infection by biological control, source reduction, prevention of contact with humans (via screens, repellents, mosquito mats and coils) chemoprophylaxis (via antimalarial drugs); and prevention of parasite transmission by drug treatment of malaria patients. Current antimalarial medication includes[2]:

- **Quinoleine core compounds**
  - Natural: quinine
  - Synthetic:
    - 4-aminoquinoleine: chloroquine, amodiaquine
    - 8-aminoquinoleine: primaquine
- **Antifolates**
  - Biguanides: proguanil
  - Diaminopyrimidines: pyrimethamine
  - Sulfonamides: sulfadoxine
- **Arylaminoalcohols**: mefloquine, halofantrine, and lumefantrine
- **Hydroxynaphtoquinones**: atovaquone
- **Artemisinin and its semisynthetic derivatives**: artemether, arteether, artesunate and dihydroartemisinin
- **Antibiotics**: tetracycline, doxycline and clindamycin

Prophylaxis and treatment is becoming ineffective due to drug resistance, which is common with all classes of antimalarial drugs except artemisinin and its semisynthetic derivatives[3]. However, recent studies dating as back as 2008 show artemisinin-resistant strains[4]. This suggests that no therapy is safe from new resistance mechanisms. The alarming increase of antimalarial drug resistance has urged a need to identify and characterize new potential drug targets to develop novel antimalarial therapeutics.

One promising target is falcipain (FP): the best characterized cysteine protease of *Plasmodium falciparum* that shares sequence homology with the papain family[5]. Four falcipains have been characterized so far: falcipain-1 (FP-1), two copies of falcipain-2 (FP-2) that are nearly identical (falcipain-2A previously referred as falcipain-2 and falcipain-2B also referred as falcipain-2') and falcipain-3 (FP-3)[6].

FP-2 and -3 share 67% homology and both of them are located in the food vacuole where they degrade hemoglobin for amino acid acquisition during the erythrocytic cycle[7]. Despite, this similarity, these enzymes have many differences. FP-2's expression starts at the early trophozoite stage whereas FP-3 isn't expressed until the late trophozoite stage[8]. The enzymes also differ on substrate specificity. FP-2 degrades erythrocyte cytoskeleton proteins: ankyrin and band 4.1 which is thought to contribute to schizont rupture[9].On the other side, FP-3 is a more efficient hemoglobinase than FP-2[10].

FP-1 differs from FP-2 and -3 in sequence, activity profile and subcellular localization. Homology between the catalytic domains of FP-1 as compared to FP-2 and -3 is relatively low (about 40%). Studies have shown that FP-1 is capable of hemoglobin hydrolysis but is located in an organelle distinct from the food vacuole[11]. FP-1 has been proposed to play a role in erythrocyte invasion at the merozoite stage[11], which still is a debate topic[12]. For these reasons, most inhibition studies have focused on the key hemoglobinases: FP-2 and -3.

Different chemotypes of falcipain inhibitors have been reported. The most common are peptide-based and peptidomimetic. The oldest example is E-64, a potent cysteine protease inhibitor isolated in 1974 from *Aspergillus japonicus*[13]. Since then, more specific falcipain inhibitors have been synthesized. However, not all inhibitors have a peptide-like or peptidomimetic structure. A recent study showed that non-peptidic compounds extracted from the fruits of *Sorindeia juglandifolia* exhibited inhibiting activity[14]. Unfortunately, not one inhibitor as been selected for human studies.

A series of 10,000 compounds were subjected to in-silico screening at the binding site of the FP-2 enzyme. The results of this screening are confidential. Based on the outcome of the in-silico screening potential candidates with favorable binding profile to the active site FP-2 were selected, purchased and screened for their FP-2 inhibition. After the acquisition of the in-vitro activity data candidates with *Ki'*s more than the standard FP-2 inhibitor were selected for further studies. Based on the essential structural components required for the FP-2 inhibition, a series of compounds were designed and were taken for synthesis. Herein, we will report the synthesis of one of the potential peptidomimetic inhibitor of FP-2.

IV. Materials and Methods

Starting materials were used as obtained from commercial suppliers without further purification or were previously synthesized by Dr. Sai Kumar Chakka (compound B_2). All amino acids used in the described reactions were of the natural levogyre series. No attempts were made to optimize yields.

Reaction progress was determined by thin layer chromatography using EMD 250µm silica gel plates with an appropriate solvent system, usually a mixture of methanol in dichloromethane or of ethyl acetate in hexanes. Revelation was achieved by short wave UV (254nm) when possible. If the compound was not UV active, iodine or ninhydrine were used.

Flash column chromatography was performed on Biotage Isolera Four using Silicycle mesh 230-400 (40-63µm) silica gel columns.

Proton nuclear magnetic resonance (^{1}H NMR) was recorded on a Bruker Ultrashield 400 Plus in $CDCl_3$ or deuterated DMSO.

1. Compound B_3

To a solution of compound B_1 (1.00g, 2.58mmol) in anhydrous DCM (5.0mL), compound B_2 (0.504g, 2.58mmol) and HOBt (0.523g, 3.87mmol) were added before cooling the reaction mixture to 0°C. The EDC crosslinker (0.742g, 3.87mmol) and DIPEA (1.00g, 7.74mmol, 1.35mL) were then added to the mixture. The resulting mixture was stirred overnight (about sixteen hours) while allowing it to come back to room temperature. The compound was then directly adsorbed on silica gel. The solvent was evaporated before being purified by flash chromatography using ethyl acetate in hexanes. Pure compound eluded at 40% ethyl acetate in hexanes. The different fractions containing the compound were then gathered and compound was concentrated *in vacuo*.

2. Compound B

A solution of compound B_3 (1.02g, 1.81mmol) in 20% piperidine in DMF (10mL) was stirred at 0°C for five minutes. The reaction mixture was then diluted in ethyl acetate and washed with water. The organic phase was separated and dried with anhydrous sodium sulfate. Compound was then concentrated *in vacuo*.

3. Compound A_1

To a solution of the unprotected amino acid (2.00g, 15.2mmol) dissolved in 1,4-dioxane (40mL) and water (20mL) cooled to 0°C, a solution of sodium bicarbonate (3.80g, 45.7mmol) in water (20mL) followed by carboxybenzyl chloride (2.60g, 15.2mmol, 2.17mL) were added. The resulting mixture was stirred for one hour at 0°C and one hour at room temperature. The reaction mixture was then diluted with ethyl acetate and washed with a 10% water solution of hydrochloric acid. Organic phase was separated and dried in anhydrous sodium sulfate. Compound was then concentrated *in vacuo*.

4. Compound A_3

To a solution of compound A_1 (2.00g, 7.54mmol) in anhydrous DMF (10mL), compound A_2 (1.23mg, 7.54mmol), BOP (3.34g, 7.54mmol) and DIPEA (4.48g, 37.8mmol, 6.56mL) were added. The mixture was stirred for three hours at room temperature. The reaction mixture was then diluted in ethyl acetate and washed with water. The organic phase was separated and dried with anhydrous sodium sulfate. Compound was then concentrated *in vacuo*.

5. Compound A_4

To a solution of compound A_3 (1.40g, 3.82mmol) in freshly made anhydrous DCM (10.0mL), the DAST reagent (1.23g, 7.64mmol) was added at -74°C. The resulting mixture was stirred for 1 hour at -78°C and 3 hours at room temperature. The reaction mixture at then diluted in ethyl acetate and washed with a saturated solution of sodium bicarbonate in water and brine. Organic layer was separated and dried with anhydrous sodium sulfate. Solvent was then evaporated and crude compound was adsorbed on silica gel. Compound was purified by flash chromatography using ethyl acetate in hexanes. Pure compound eluded at 50% ethyl acetate in hexanes. The different fractions containing the compound were gathered and concentrated *in vacuo*.

6. Compound A_5

To a solution of compound A_4 (1.04g, 2.99mmol) in anhydrous DCM (5.0mL), bromotrichloromethane (771mg, 3.89mmol) and DBU (539mg, 3.59mmol) was added in a dropwise manner at 0°C under nitrogen gas and stirred for 16 hours at room temperature. Reaction mixture was then diluted in ethyl acetate and washed with a saturated water solution of ammonium chloride. Organic phase was separated, dried with anhydrous sodium sulfate and concentrated *in vacuo* to afford crude compound. Compound was then purified by flash chromatography using ethyl acetate in hexanes. Pure compound eluded at 30% ethyl acetate in hexanes. The different fractions containing the compound were gathered and concentrated *in vacuo*.

7. Compound A_6

To a solution of compound A_5 (620mg, 1.79mmol) in methanol (20.0mL), palladium on carbon (200mg) was added before connecting the mixture to a source of hydrogen gas and stirring for 20 minutes at room temperature. The suspension was then filtered through Celite and filtrate was recovered. Compound was then concentrated *in vacuo*.

8. Compound A_7

To a solution of compound A_6 (100mg, 0.471mmol) in anhydrous DCM (5mL), DMAP (catalytic amount) and DIPEA (182mg, 1.41mmol, 245µL) were added at 0°C under nitrogen gas. The R_1 substituted acyl chloride was then added and the resulting mixture was stirred for 16 hours at room temperature. Reaction mixture was then diluted in DCM and washed with a saturated water solution of sodium bicarbonate. Organic phase was separated and dried with anhydrous sodium sulfate. Compound was then purified by flash chromatography using ethyl acetate in hexanes. Pure compound did not come out with this solvent system. Column was thus flushed with a 10% of methanol in DCM. The different fractions containing the compound were gathered and concentrated *in vacuo*. TLC indicated that one impurity was present.

9. Compound A

To a solution of compound A_7 (110mg, 0.356mmol) in tetrahydrofuran/methanol (4:2) (5mL), lithium hydroxide (42.0mg, 1.76mmol) was added and the resulting mixture was stirred for 16 hours. Solvent was then evaporated and crude compound was diluted at 0°C in a 10% water solution of HCl to acidify the mixture to a pH of 4. Solvent was then evaporated and crude compound was diluted in a 10% methanol solution in DCM and washed with water. The organic phase was separated and dried with anhydrous sodium sulfate. Compound was then concentrated *in vacuo*.

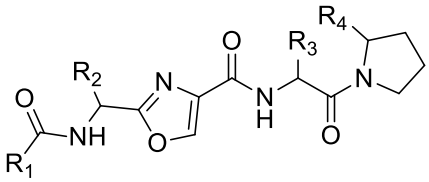
10. Final compound: FP-2 inhibitor

To a solution of compound A (40.0mg, 0.128mmol) in anhydrous DMF (1.0mL), compound B (46.2mg, 0.134mmol), BOP (56.6mg, 0.128mmol) and DIPEA (83.0mg, 0.640mmol, 11.2µL) were added. The mixture was stirred for three hours at room temperature. The reaction mixture was then diluted in ethyl acetate and washed with water. The organic phase was separated and dried with anhydrous sodium sulfate. Compound was then purified by flash chromatography using ethyl acetate in

hexanes. Pure compound eluded at 100% ethyl acetate in hexanes. The different fractions containing the compound were gathered and concentrated *in vacuo*.

V. Results

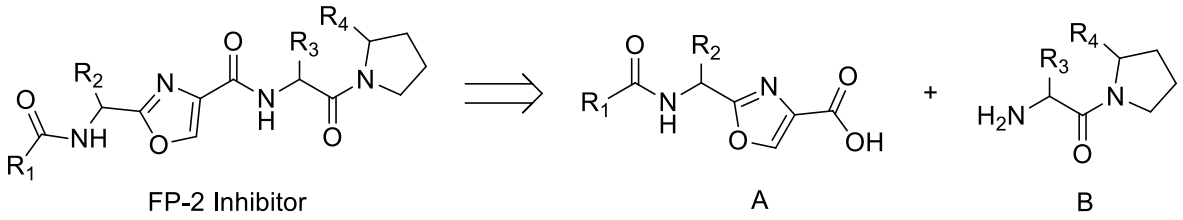
Our study will focus on the synthesis of one peptidomimetic falcipain-2 inhibitor. Figure 1 shows the semi-developed formula of said compound.



Chemical Formula: $C_{34}H_{42}FN_5O_6$
Molecular Weight: 635.73

Figure 1. Peptidomimetic falcipain-2 inhibitor.R_1, R_2, R_3 and R_4 represent moieties that have been censored.

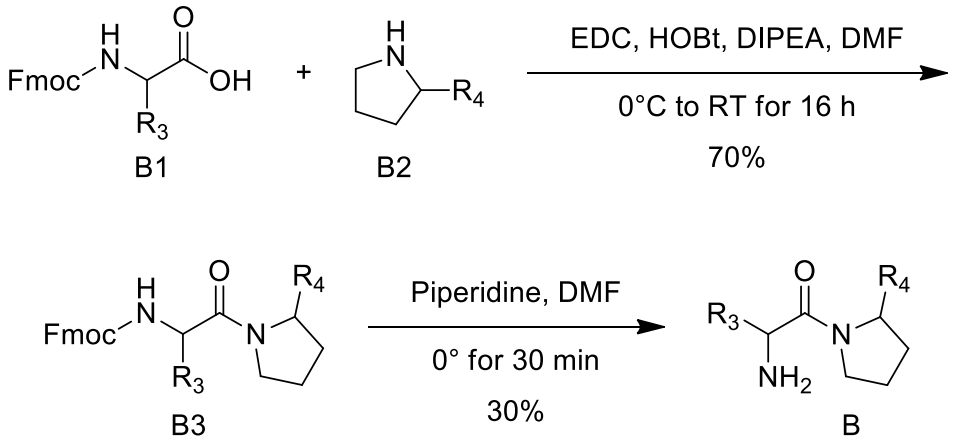
After many trials, one synthetic route for the FP-2 inhibitor was developed. Using retrosynthetic analysis, the compound was divided into two simpler compounds by breaking a peptide bound (Figure 2).



FP-2 Inhibitor A B

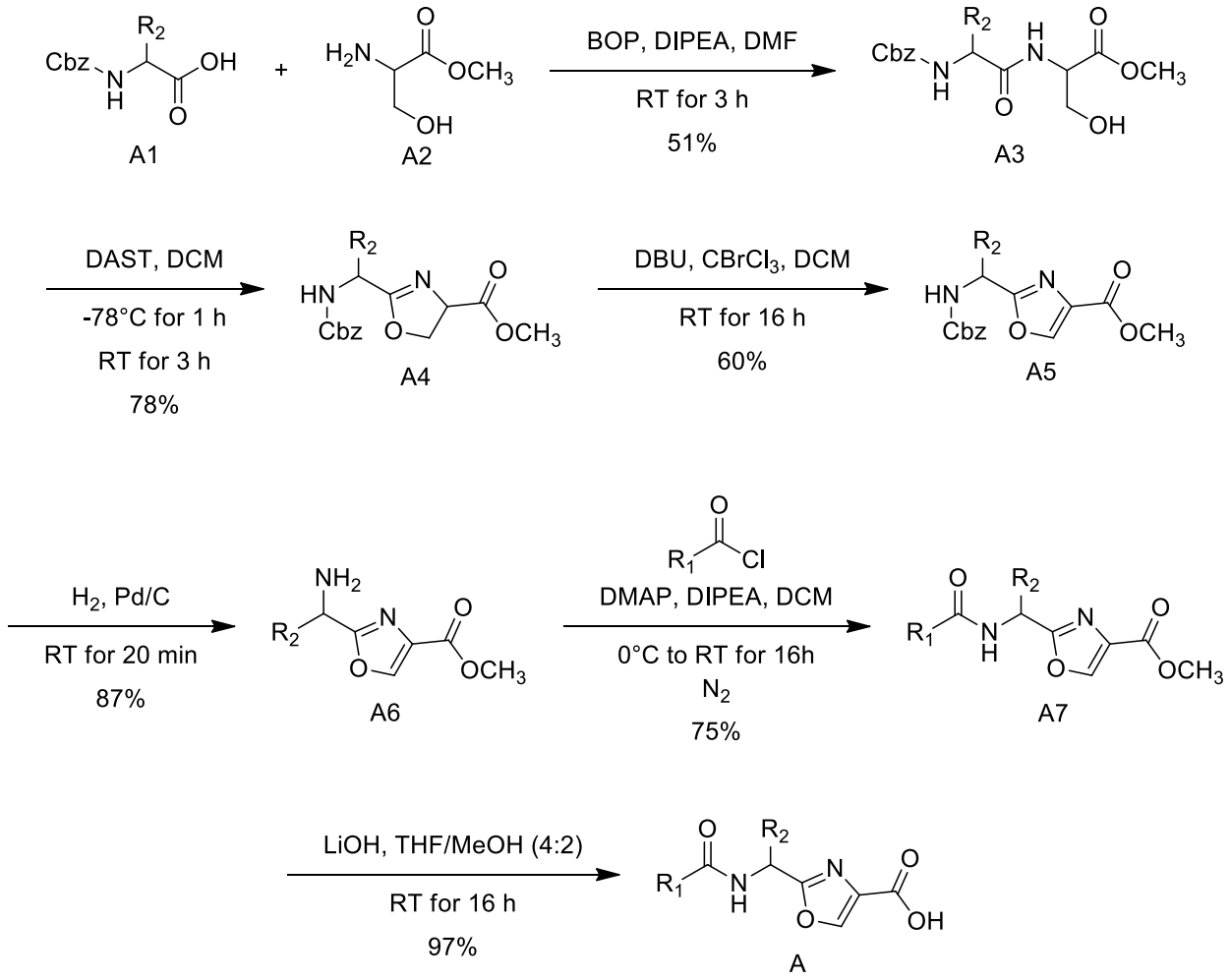
Figure 2. Retrosynthetic analysis of FP-2 inhibitor.

Compound B was synthesized in two steps (Scheme 1). Stereospecific coupling of a fluorenylmethyloxycarbonyl (Fmoc) protected amino acid (B_1) with a pyrrolidine derivative (B_2) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt) as reagents formed peptidic compound (B_3) with 70% yield. Use of a 20% piperidine solution in dimethylformamide (DMF) formed desired compound B but reaction was characterized by a yield of only 30%.



Scheme 1. Synthesis of compound B.

Synthesis of compound A (Scheme 2) was more complicated notably due to the presence of oxazole ring. Non-stereospecific coupling between a Cbz protected amino acid (A_1) with serine methyl ester (A_2) using benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphoniumhexafluorophosphate (BOP) reagent produced compound A_3. Cyclization was then achieved by the use of diethylaminosulfurtrifluoride (DAST) reagent and permitted the formation of the unstable oxazoline ring found in compound A_4. Use of 1,8-diazabicycloundec-7-ene (DBU) and bromotrichloromethane formed the stable oxazole ring found in compound A_5. The Cbz deprotection to afford compound A_6 without oxazole reduction was then achieved with the use of hydrogen gas with a large amount of palladium on carbon. Coupound A was then finally obtained by coupling with the acyl chloride followed by saponification of the methyl ester with lithium hydroxyde.



Scheme 2. Synthesis of compound A.

The FP inhibitor was finally obtained by non-stereospecific coupling reaction between compounds A and B using the BOP reagent. (Scheme 3).

Scheme 3. Coupling of compounds A and B to afford FP-2 inhibitor.

VI. Discussion

1. Choice of peptide coupling reagents

Two reagent systems were used for peptide bond formation. The EDC reagent used with HOBt permitted the conservation of stereochemistry whereas the BOP reagent would be responsible for racemization of the compound. Use of EDC with HOBt was thus crucial in the presence of a stable chiral center such as the nitrogen atom found in the pyrrolidine ring of our FP-2 inhibitor. However, the use of EDC/HOBt compared to BOP represents a five-fold increase in reaction time. Coupling of compound A_1 with L-serine methyl ester (A_2) generates a chiral center on the amide nitrogen. However, after formation of the oxazoline, the chirality is destroyed justifying the use of the speedy BOP reagent. The final stage coupling reaction is also responsible of the formation of a chiral center that would justify the use of EDC/HOBt. Nevertheless, the nitrogen inversion phenomenon makes the isolation of one specific stereoisomer impossible justifying once more the use of the BOP reagent.

2. Choice of protecting groups

Protection of the α-amino group is necessary for reduction of nucleophilicity. Here, we have experimented with two popular protection groups, Cbz and Fmoc.

Cbz has been largely favored in liquid-phase peptide synthesis for its simple introduction, removal conditions and minor side-reactions. One problem we faced with the use of this protector was the selective deprotection of the Cbz dipeptide A_5. Removal of Cbz is usually achieved by catalytical hydrogenation employing hydrogen gas in the presence of a catalyzer such as palladium on carbon at room temperature for several hours. These conditions could interfere with the oxazole ring aromaticity. Accordingly, we decided to use a large amount of palladium on carbon catalyzer to reduce the reaction time to 20 minutes. The deprotection was thus selective and achieved with respectable yield (87%).

Fmoc is one of the most popular and most well established α-amino protectors. Its deprotection is realized by non hydrolytic base treatment. These mild conditions are achieved by a variety of organic amines in polar solvents, most commonly 20-50% piperidine in a DMF solution. One of the byproducts of this reaction is dibenzofulvene (DBF). In the case of solid-phase peptide synthesis, the unreacted base and DBF can be easily washed off the resin. However, in the case of liquid-phase peptide synthesis, the removal of the side products are much more subtle which as limited its use since DBF is nor volatile nor water soluble. Furthermore, DBF is a reactive electrophilic reagent (Michael acceptor) that may react with the deprotected amine thus compromising yield which may explain the low yield (30%) obtain for the deprotection of compound B_3.

3. Oxazole ring synthesis

The biggest problem in the synthesis of the described compound was the formation of oxazoline ring. Different protocols used with compounds at different stages of synthesis were tried but all except the one described in Results produced the desired compound A_4. Our first trials used Mitsonobu reaction conditions (triphenylphosphine and diethyl azodicarboxylate) based on the works of Galeotti and al.[15] but results were unfruitful. Based on more recent literature, we used the DAST reagent with different compounds[16]. Nevertheless, only the Cbz protected compound (A_3) produced the oxazoline ring. The initial inhibitor that we planned to synthesize contained an oxazoline ring in its structure. Unfortunately, this structure was found to be unstable and compound A_4 was degraded during its acylation into compound A_5. We thus decided to aromatize this oxazoline ring into the much more stable oxazole ring that we find in our FP-2 disclosed in figure 1.

VII. Conclusion

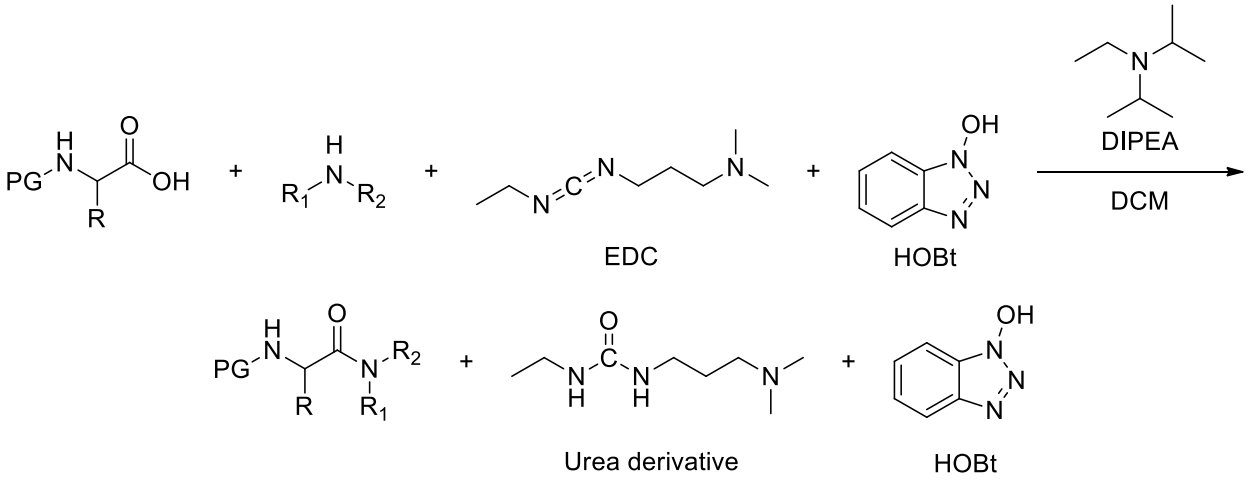
The synthetic schemes described in results show the synthesis of one FP-2 inhibitor. However, these are also a preliminary work for the synthesis similar designed compounds whether they be peptide-based or peptidomimetic. For example, the synthesis of a peptide-based compound may be achieved by simple removal of the cyclization and ring oxidation steps in scheme 2. Furthermore, the pyrrolidine ring in compound B can be replaced by an imidazole ring by simple modification of compound. Once the desired designed compounds are synthesized, they will then be screened in-vitro for their FP-2 inhibition. Based on the results of the in-vitro screening further modifications on the pharmacophore will be attempted until we nail down to a potential FP-2 inhibitor with a low *Ki* and desirable physiochemical properties (solubility, pKa, log P). Next stage of the project includes the *in vivo* animal and eventually human stage 1 and stage 2 studies with a lead FP-2 inhibitor. Hopefully, one of the synthesized compounds will become a marketed drug.

VIII. Annex: general reactions and proposed mechanisms

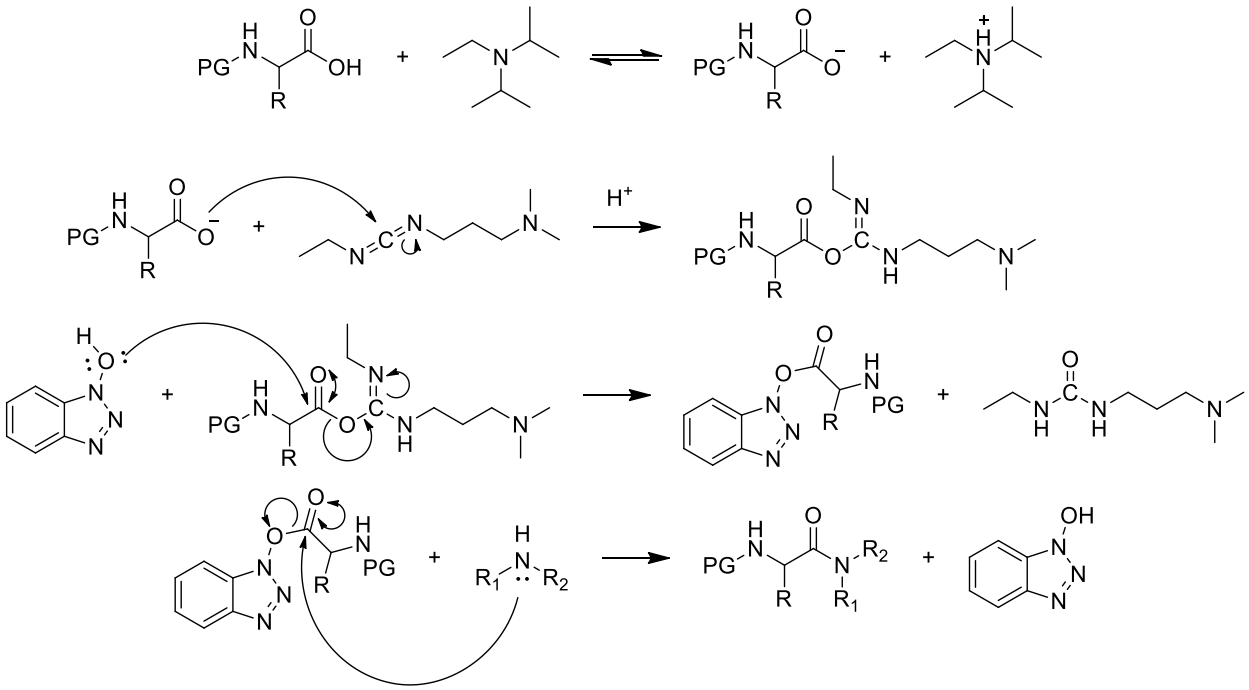
1. Coupling reactions

a. EDC/HOBt coupling

General reaction

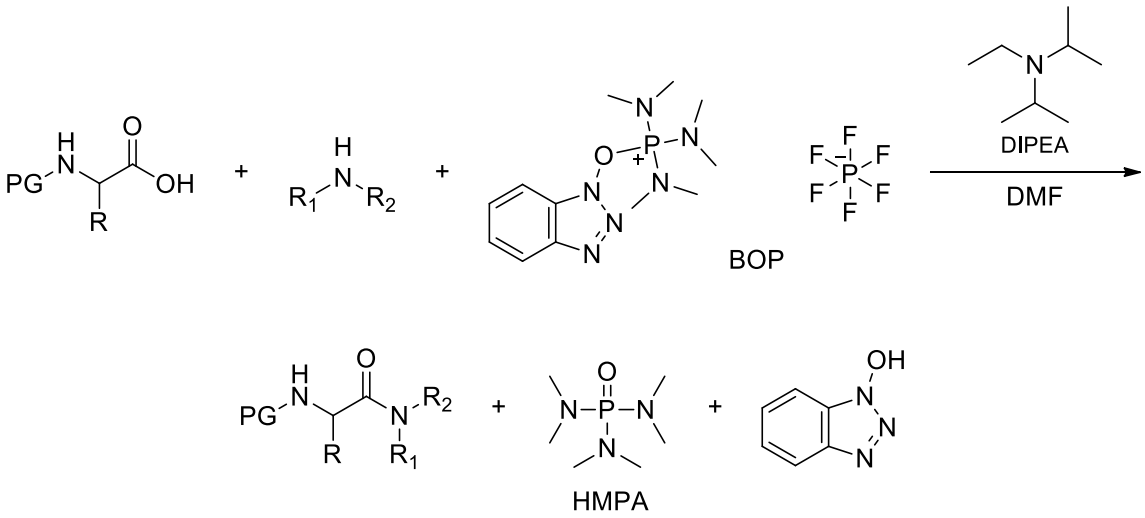


Proposed mechanism

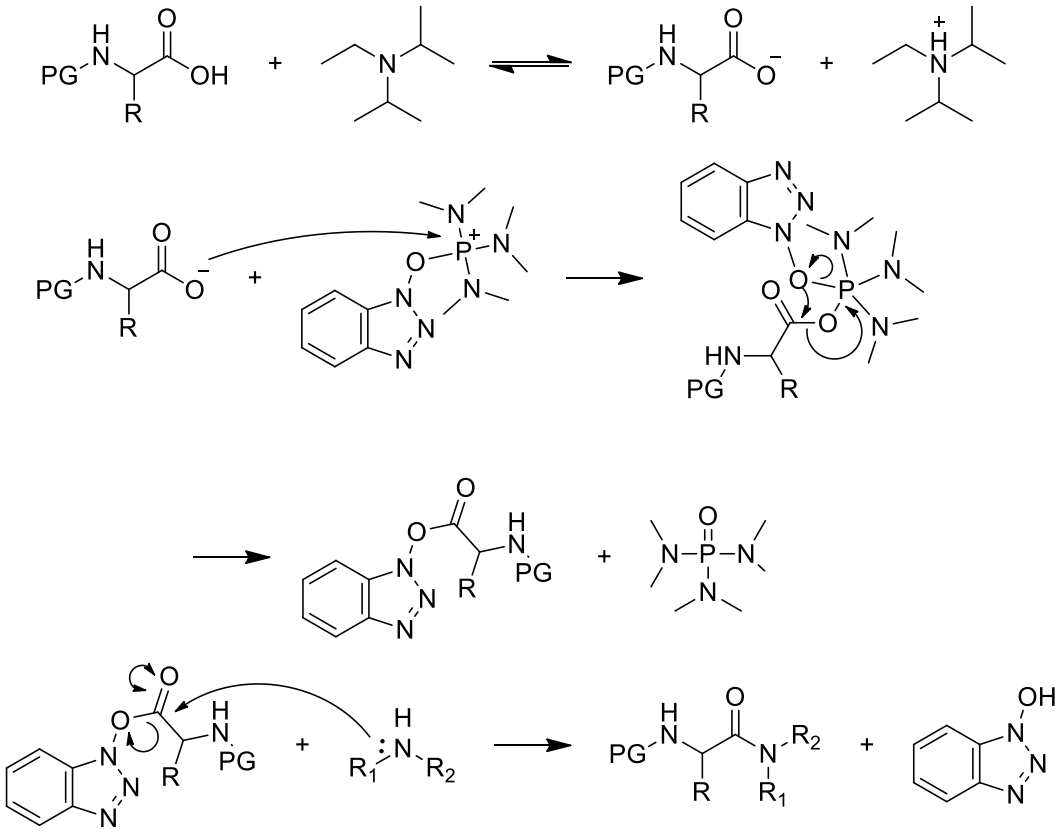


b. BOP coupling

General reaction



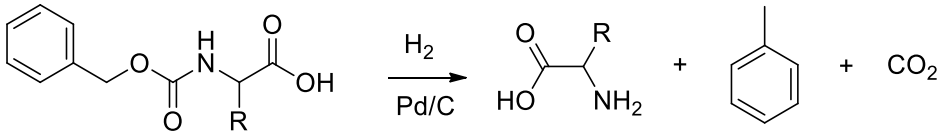
Proposed mechanism



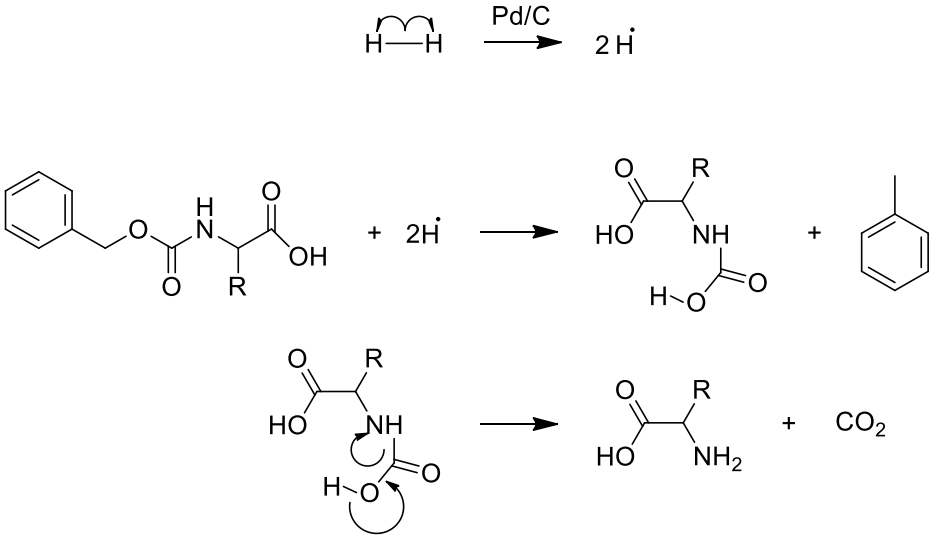
2. Deprotection reactions

a. Cbz deprotection

General reaction

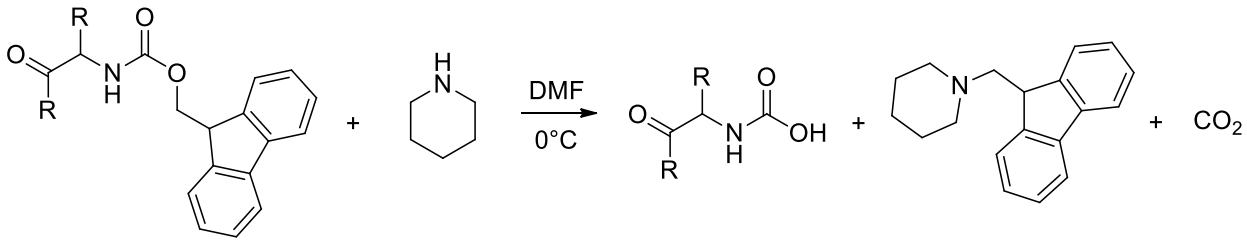


Proposed mechanism

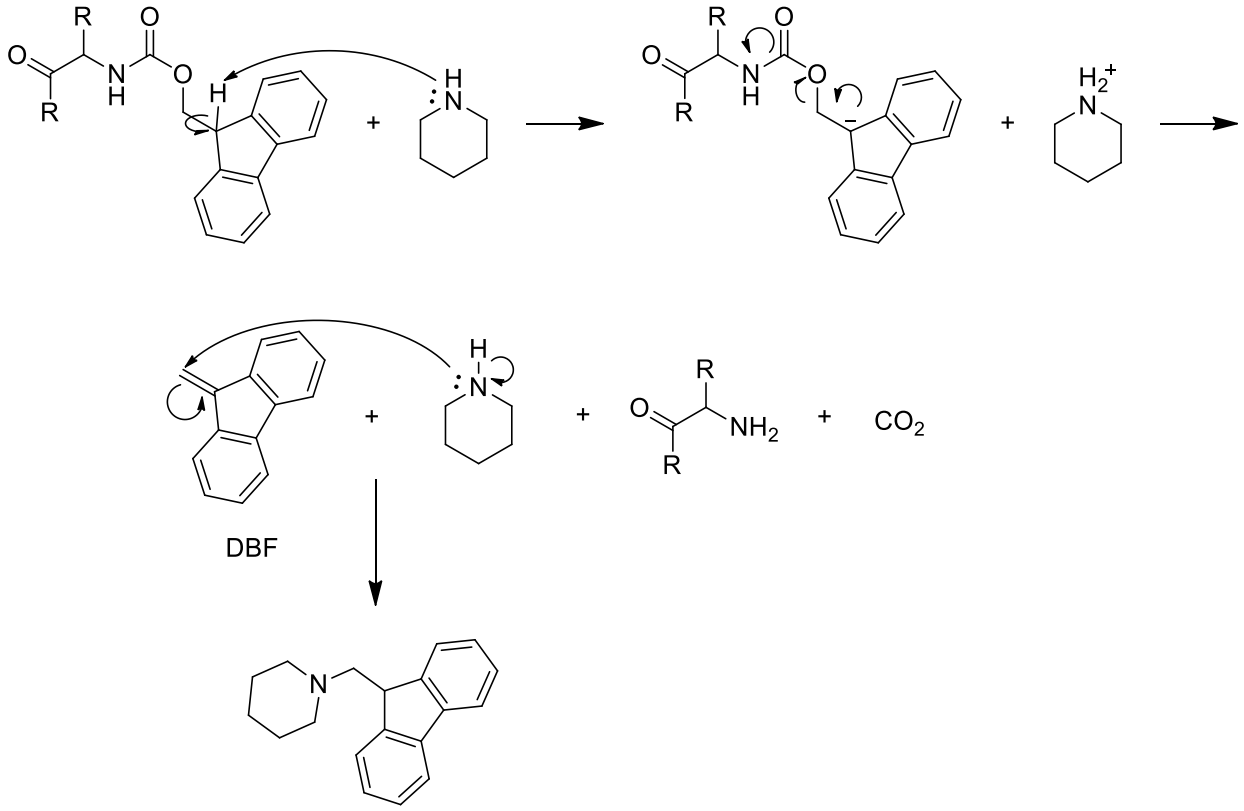


b. Fmoc deprotection

General reaction



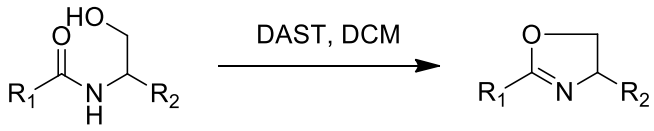
Proposed mechanism



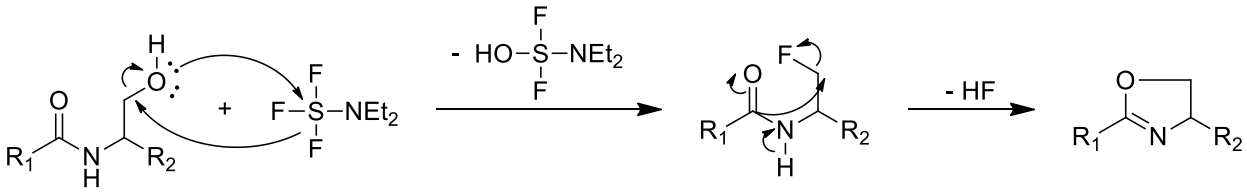
3. Oxazole ring synthesis

c. Oxazoline formation

General reaction

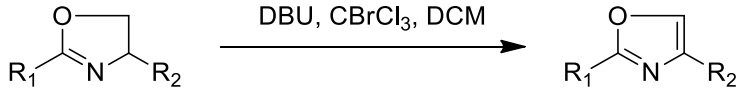


Proposed mechanism

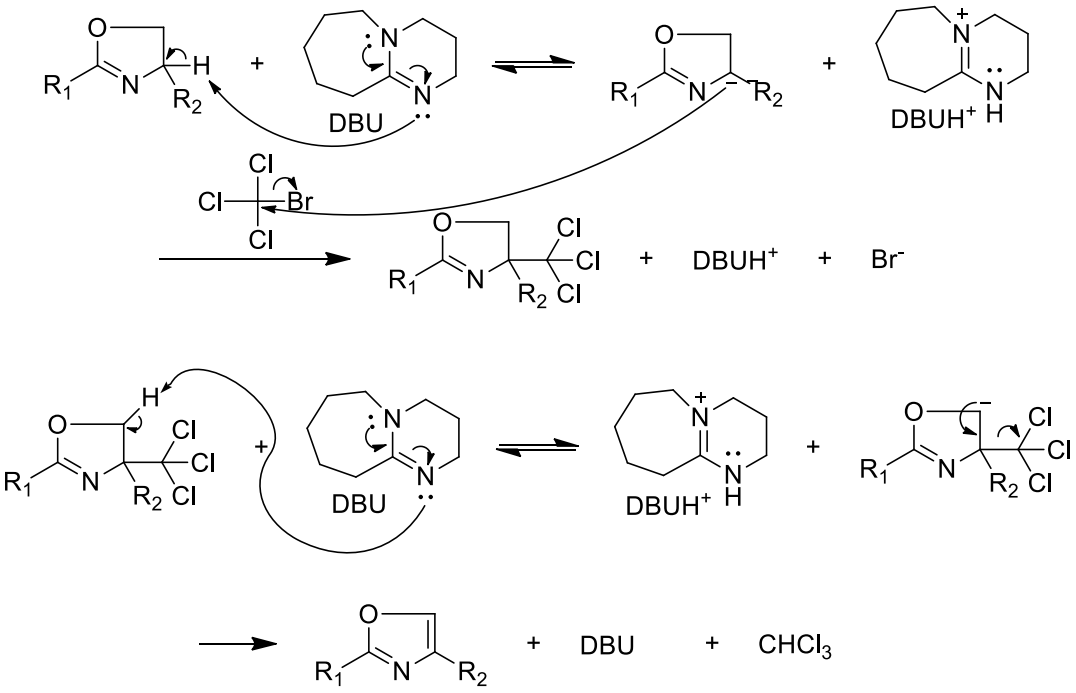


d. Oxazole formation

General reaction



Proposed mechanism



IX. Personal insight and acknowledgements

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