SYNTHESIS AND EVALUATION OF PHOSPHORAMIDE MUSTARD PRODRUGS FOR SITE-SPECIFIC ACTIVATION

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ABSTRACT OF THE THESIS

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By PRATHIMA SURABHI

Thesis Director: Professor Longqin Hu, Ph.D.

Advanced cancers after metastasis need to be treated systemically using chemotherapy or radiation, which are often associated with debilitating side effects. To improve the selectivity of the chemotherapeutic agents and reduce systemic toxicity, prodrugs with tumor targeted activation mechanisms are being developed. Efforts in this project were focused on designing phosphoramide mustard analogues incorporating two types of tumor-specific activation mechanisms: one by the reductive activation mechanism by *Escherichia coli* nitroreductase and the other by the proteolytic activation mechanism by prostate-specific antigen (PSA) in prostate cancer cells. 5-Nitropyridyl-2-methyl phosphoramide mustard was designed and synthesized to be a substrate of *E. coli* nitroreductase. In cell culture assays it showed an IC_{50} value of 9 nM in Chinese hamster V79 cells transfected to express *E. coli* nitroreductase as compared to 75 μM in the control V79 cells that do not express *E. coli* nitroreductase. These results suggest that 5-nitropyridyl-2-methyl phosphoramide mustard is a good candidate for use in combination with nitroreductase for enzyme prodrug therapy.

For proteolytic activation by (PSA) in prostate cancer cells, we designed a peptide conjugate by linking a substrate peptide sequence to phosphoramide mustard through a

flexible linker that can be modified to better accommodate the unique structural requirements for binding to and catalysis by the target enzyme and thus improve the tumor selectivity of phosphoramide mustard. Pyridine was introduced to modulate the electron-density, to optimize the stability and rate of 1,6-elimination upon proteolytic cleavage. A new set of conditions were developed for selenocarboxylate/azide amidation reaction in order to avoid the use of unstable basic nuclephilic amine intermediates during the synthesis of the peptide conjugated analogue. Under the new amidation conditions, the half-life of the selenocarboxylate was increased by 27-fold at room temperature as compared to the previous amidation method using DMSO as a co-solvent. Excellent yields were obtained with electron-deficient azides and much improved yields were obtained with electron-rich azides upon mild heating. The peptide conjugate, glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-pyridyl-2-methyl phosphoramide mustard, was successfully synthesized using our new amidation strategy and was found to be stable with a half-life >5 days at pH 7.4 and 8.0. It was shown to be a good substrate for PSA with a half-life of 46.8 min at an enzyme/substrate molar ratio of 1/100. However, the peptide conjugate did not show any selective toxicity towards PSA-expressing LNCaP cells with IC_{50} values of 209 μM and 204 μM against PSA-expressing LNCaP and non-PSA-expressing DU 145 cell lines, respectively. The low cytotoxicity against LNCaP cells may be due to the inability of the released species to penetrate the tumor membranes. Further studies are underway to identify the activated species released and to design approaches to improve its membrane permeability.

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I would like to extend a very special thanks to all my family members and friends without whom my graduate study would not be possible.

DEDICATION

I would like to dedicate this thesis to my parents.

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ABBREVIATIONS

Ac	Acetyl
ADEPT	Antibody-directed enzyme prodrug therapy
12ADT	12-Aminododecanoyl-thapsigargin
Ala	Alanine
Asn	Asparagine
ALD	Aldophosphoramide
Chg	Cyclohexylglycine
CPA	Cyclophosphamide
CuCN	Copper cyanide
CYP450	Cytochrome P-450 enzyme
DCC	N,N'-Dicyclohexylcarbodiimide
DIC	N,N'-Diisopropylcarbodiimide
DIEA	Diisopropyl ethylamine
DEA	Diethyl amine
DMAP	4-Dimethylaminopyridine
DMF	N,N'-Dimethyl formamide
Dox	Doxorubicin
DTD	DT-diaphorase
Fm	9-Fluorenylmethyl
FMN	Flavine mononucleotide
Fmoc	9-Flourenylmethoxycarbonyl
GDEPT	Gene-directed enzyme prodrug therapy
Gln	Glutamine
HBTU	2-(1-H-Benzotriazol-1-yl-)-1,1,3,3-tetramethyluronium hexafluorophosphate
HEX	2-Hexenepyranoside
His	Histidine
HOBt	N-Hydroxybenzotriazole

HOSu	N-Hydroxysuccinimide (NHS)
Hyp	Hydroxyproline
i.p.	Intraperitoneal
KCN	Potassium cyanide
Leu	Leucine
LNCaP	Lymph node carcinoma of the prostate
Lys	Lysine
MTD	Maximum tolerated dose
Mu	Morpholinyl carbonyl
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NMP	N-Methylpyrrolidone
NTR	Nitroreductase
Phe	Phenylalanine
PSA	Prostate-specific antigen
Ser	Serine
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
Suc	Succinyl
TBAB	Tetrabutyl ammonium bromide
TBACN	Tetrabutyl ammonium cyanide
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
Tyr	Tyrosine
TG	Thapsigargin
VIN	Vinblastine

INTRODUCTION

I. Prodrugs for tumor-targeted chemotherapy

Cancer is one of the most dreadful diseases and is the second leading cause of death in the United States.[1] It is a disease characterized by uncontrolled cell division and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue (invasion) or by migration of cells to distant sites (metastasis). A series of acquired or inherited mutations damage genetic information that define the cell functions and remove normal control of cell division. Cancer can be treated by surgery, radiation, chemo and biological therapies. However, the choice of treatment depends on the disease state. Localized tumors may be treated by surgery but advanced cancers need radiation or chemotherapy. In spite of the availability of a wide range of therapies, achieving tumor selectivity is still a challenge.

Conventional chemotherapy

Most tumor cells do not have biological targets which are completely foreign to the host and hence anticancer agents rely primarily on the uncontrolled proliferation of cells at the tumor site to exert their action. Based on their mechanism of action, anticancer agents have been classified as alkylating agents, antimetabolites, mitosis inhibitors, hormones and DNA complexing agents.[2,3] However, most anticancer agents are associated with debilitating side effects. For example, anthracycline family of anticancer drugs are associated with severe cardiotoxicity[4] and DNA complexing agents affect normal cells of

gastrointestinal tract and bone marrow.[5] The side effects limit the maximum dose of anticancer agents to below the minimum amount required to kill all viable tumor cells. This results in tumor relapse and induced drug resistance.[6]

Targeted prodrug therapy

In order to improve the selectivity of the chemotherapeutic agents, new anticancer agents with novel mechanisms of action are being developed. Another strategy is to improve the selectivity of existing drugs via the prodrug approach and the latter has attracted much attention.[6] A targeted prodrug is formed by conjugating a cytotoxic agent with a tumor-specific trigger. Thus the resulting prodrug would not affect the normal cells in a systemic administration as the cytotoxic property of the parent drug is masked. When the prodrug reaches the tumor site, it is activated through a biochemical mechanism such as hypoxia or enzymatic action in solid tumors, leading to the release of parent drug and anticancer activity.[6,7]

Solid tumors differ from normal tissues in a number of important aspects due to the differences between the two vasculatures. Blood vessels in tumors are often highly abnormal having distended capillaries with leaky walls and sluggish flow as opposed to the regular, ordered vasculature of normal tissues. These differences can cause potential problems for cancer treatment. For example, hypoxia in solid tumors contributes to resistance to radiotherapy and to some chemotherapeutic agents. However, these differences can also be exploited for selective cancer treatment.[7] Hypoxia provides a reductive microenvironment and this can be effectively targeted by prodrugs acting via

bioreductive activation mechanisms.[8] Several prodrugs containing quinones, nitroaromatic or N-oxide structures are selective towards hypoxic cells and are in various stages of clinical trials. Tirapazamine, porfiromycin are few examples. [9,10]

Many endogenous enzymes such as DT-diaphorase,[11] E-glucoronidase,[12,13] azoreductase,[14] acid phosphatase[15] are reported to be over-expressed in some tumor cells. These enzymes could serve as major targets for activation of prodrugs. Some cytotoxic agents linked to enzyme-specific substrates are much less toxic to normal cells and restore their cytotoxicity upon enzyme catalysis at tumor sites.[16] Recently, several proteases such as plasmin, plasminogen activator, matrix metalloproteinases, prostate specific antigen (PSA) have also been used as prodrug-converting enzymes and targets for peptide-based prodrugs in the treatment of cancer.[6]

Exogenous enzymes have also been delivered to tumor cells as prodrug activating enzymes through antibody-directed enzyme prodrug therapy (ADEPT) and gene-directed enzyme prodrug therapy (GDEPT). In the above therapies, an exogenous enzyme is selectively delivered to the tumor site by genetic fusion or chemical conjugation to a tumor-specific antibody or by enzyme gene delivery systems. The delivered enzyme is responsible for the activation of the prodrug at the tumor site. Several enzyme/prodrug systems are under development for ADEPT and GDEPT. One such example is nfsB gene product of *Escherichia coli* which is an oxygen-insensitive flavoprotein containing nitroreductase (NTR).[17]

Advantages of targeted prodrug therapy

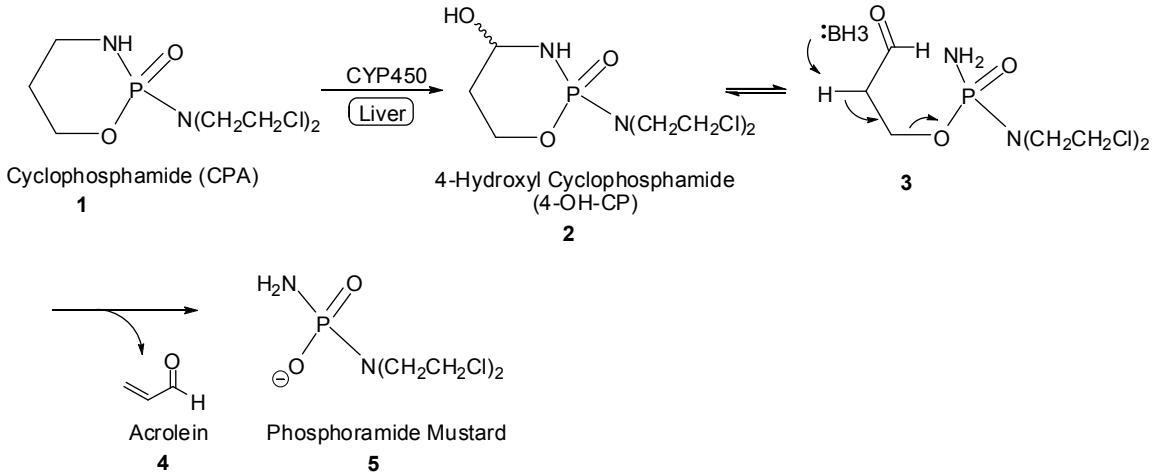
Tumor-targeted prodrug therapy has several advantages over conventional chemotherapy. Improved selectivity to tumor cells should decrease systemic toxicity of the parent drug to a great extent. Prodrugs can be administered in much higher doses than the parent drug and can be continuously and effectively converted to the parent drug at the desired site of action. As a result, the parent drug concentration at the tumor site could be much higher than that achieved via conventional chemotherapy and this effect is called as amplification effect.[18,19] In the targeted prodrug therapy, the cytotoxic agent could be released heterogeneously by cells having the activation mechanism. Upon activation it not only affects the cells with activation mechanism but also affects cells surrounding them which do not possess the activation mechanism. Such an effect is called as bystander effect.[20] Targeted prodrug therapy could lead to much improved therapeutic index of existing anticancer agents.[21]

Cyclophosphamide as a prodrug for cancer

Cyclophosphamide was first discovered about forty years ago and is one of the most successful anticancer drugs developed over the last few decades and is effective against a variety of human cancers. Cyclophosphamide is effective against both cycling and non-cycling cells and hence is one of the few drugs effective against slow-growing solid tumors like prostate cancer.[22]

Mechanism of action of cyclophosphamide

Cyclophosphamide is activated by cytochrome P-450 (CYP450) enzyme in the liver followed by the release of activated phosphoramide mustard and acrolein.[23-26] As shown in scheme 1, CYP450 converts cyclophosphamide (**1**) to 4-hydroxycyclophosphamide (**2**) which undergoes ring opening to form aldophosphoramide (**3**) which undergoes base catalyzed β-elimination to form phosphoramide mustard (**5**) and acrolein (**4**). Phosphoramide mustard is the final alkylating agent responsible for cross-linking the interstranded DNA.[27,28] Acrolein (**4**) is highly cytotoxic to cultured tumor cells but is not responsible for the cytotoxicity of cyclophosphamide.[29,30] It is in fact responsible for hemorrhagic cystitis which is a life threatening side effect associated with cyclophosphamide.



Scheme 1. Mechanism of action of cyclophosphamide

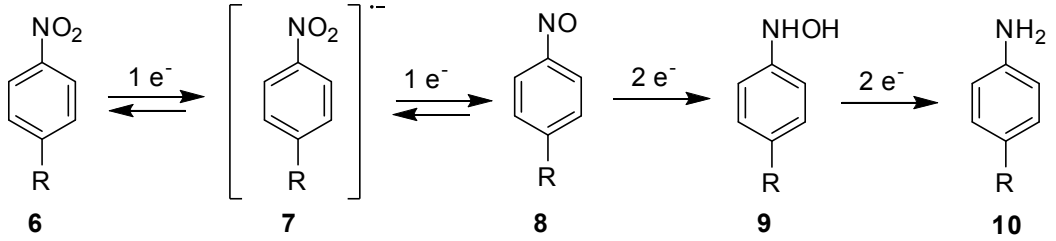
Analogues of cyclophosphamide

The interest in developing more selective cyclophosphamide type drugs led to the discovery of many cyclic and acyclic phosphoramidate prodrugs incorporating a variety of tumor-specific activation mechanisms that distinguish malignant cells from normal cells. Based on the finding, that pH at the tumor site is decreased due to aerobic glycolysis, Tietze's group synthesized and tested an acid-sensitive prodrug 2-hexenopyranoside of aldophosphoramide. This compound was found to release aldophosphoramide after a 24 to 48 h exposure to pH 6.2 medium. This development was encouraging but the exposure time required for the activation of drug was excessive.[31] Borch and his team reported the design and synthesis of hypoxia-selective nitroheterocyclic phosphoramides. They also reported the synthesis of several series of phosphoramidate prodrugs such as napthoquinone series, benzimidazolequinone series,[32] 2-substituted and 3-substituted indolequinone series. The above series of compounds target DT-diaphorase (DTD), an enzyme overexpressed in some tumors.[33,34]

Our efforts were focused on the design of phosphoramide mustard analogues incorporating two types of tumor-specific activation mechanisms; one is the reductive activation mechanism by *Escherichia coli* nitroreductase and second is the proteolytic activation mechanism by prostate-specific antigen (PSA) in prostate cancer cells.

II. Prodrugs activated by *Escherichia coli* nitroreductase

E. coli Nitroreductase (NTR) has been proposed as a prodrug converting enzyme in ADEPT and GDEPT. NTR is a flavin mononucleotide containing enzyme. It is capable of reducing a variety of aromatic nitro groups to their corresponding hydroxylamines in the presence of cofactor NADH or NADPH.[35,36] Reduction of nitro group to hydroxylamine or amine initiates a very large electronic change and provides an efficient "switch" that can be used to trigger cytotoxicity. As shown in scheme 2,[37] nitro group is a strong electron withdrawing group (Hammett σ_p = 0.78) and reduces the oxidation potential of the phosphorinane ring system toward hepatic CYP450 oxidation. The nitro group of nitroaromatics **6** undergoes a 4-electron reduction to form the corresponding hydroxylamine **9** (Hammett σ_p = -0.34) or a 6-electron reduction to form the corresponding amine **10** (Hammett σ_p = -0.66). After this massive electronic "switch" in a single metabolic step ($\Delta\sigma_p$ = 1.12 and 1.44), the cytotoxic portion in the prodrug is turned on and exerts its cytotoxic effect.[38] In scheme 2, the first reduction step producing the nitroradical anion **7** may be easily back scavenged by oxygen to form the parent compound in normal cells whereas the same step is responsible for the selective cytotoxicity of nitroaromatic prodrugs against hypoxic tumor cells.[39]



Scheme 2. Reduction of nitroaromatics to hydroxylamines and amines

Dinitroaziridinylbenzamides, dinitrobenzamide mustards, 4-nitrobenzyl carbamates, and nitroindolines (Figure 1) are the four classes of prodrugs that have been described for activation by NTR. Of the above four classes, the first two are considered to be most promising when used in conjunction with NTR.[37,40,41]

Dinitroaziridinylbenzamide CB1954 (**11**) is currently in phase II clinical trials in conjunction with virally-delivered NTR enzyme. It has been reported to have > 1000× selectivity in cell lines transfected with NTR,[35,42] and potent, long-lasting tumor growth inhibition. CB1954 demonstrated bystander effect in a mouse xenograft model with NTR-transfected tumors.[43]

Dinitrobenzamide SN 23862 (**12**), a mustard analog of CB1954 (**11**) is rapidly reduced by NTR and is not reduced by mammalian DTD and hence has enhanced tumor selectivity. It has been reported to have 63-fold selectivity, and its bromo-mustard analogue 2500-fold selectivity towards NTR transfected cell lines.[44,45]

Nitrobenzyl carbamate **13** is a useful prodrug form to incorporate a number of cytotoxic amines such as aniline mustards, enediynes, and anticancer antibiotics like doxorubicin, mitomycin C and actinomycin D. These prodrugs release their parent cytotoxic amine following exposure to NTR in the presence of NADH or NADPH cofactor.[41]

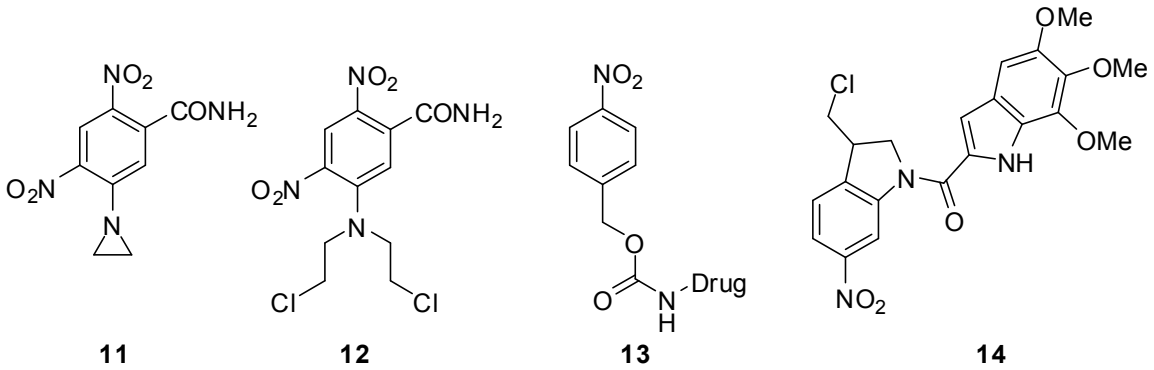


Figure 1. Prodrugs for activation by *E. coli* nitroreductase (NTR)

Duocarmycin analogs contain nitroindoline moiety **14** in their structure, which acts as a substrate for NTR. These compounds bind in the minor groove of DNA and form adducts by alkylation.[46]

Our group has designed and synthesized a novel and superior class of nitroaryl phosphoramides (Figure 2) as potential prodrugs for nitroreductase-mediated enzyme-prodrug therapy. All analogues were found to be stable in phosphate buffer at pH 7.4 and 37 °C and were all good substrates of *E. coli* nitroreductase with half-lives between 2.9 and 11.9 min. It was inferred from SAR studies that, only the analogues with a benzylic oxygen *para* to the nitro group showed significant selective cytotoxicity in NTR-expressing cells. These results suggest that not only good substrate activity but also the benzylic oxygen is essential for the reductive activation of 4-nitrobenzylphosphoramide mustard analogues by *E. coli* nitroreductase. The low IC_{50} and the high selectivity of some of the analogues in *E. coli* nitroreductase-expressing cells indicated their potential to become a drug candidate in enzyme-prodrug therapy. These

nitrobenzylphosphoramide mustards were found to have low cytotoxicity before reduction and were converted to phosphoramide mustard or like reactive species upon bioreduction. The species released upon bioreduction were responsible for cross-linking DNA strands and thus leading to cytotoxicity. It was also confirmed that these analogues exhibit excellent bystander effects which will be critical to the success of any GDEPT. 4-Nitrobenzyl phosphoramide mustard (**17**) showed the best bystander effect with a TE_{50} value of 3.3% compared to 4.5% for 5-aziridinyl-2,4-dinitrobenzamide. This result suggested that hydroxylamine intermediate could penetrate through cellular membranes and deliver the cytotoxic phosphoramide mustard to neighboring tumor cells. The excellent biological activity of these analogues correlated well with the substrate activity for *E. coli* nitroreductase and the expected high cytotoxicity of the reactive species released upon reduction.[6,47] Of all the analogues the acyclic 4-nitrobenzyl phosphoramide mustard (**17**) was found to be the best cytotoxic agent towards nitroreductase-expressing SKOV3 and V79 cells exhibiting 5,600 to 170,000-fold selectivity and an IC_{50} as low as 0.4 nM. One of our objectives in this project is to make further structural modifications to acyclic nitroarylphosphoramide mustard prodrugs and to further improve their water solubility while maintaining high stability and selectivity. For this we designed and synthesized a new analogue **18** with pyridine moiety in the structure which would modulate the electron density due to its electron-withdrawing nature. The compound **18** is proposed to have the same mechanism of action as 4-nitrobenzyl phosphoramide mustard (**17,** Scheme 3). The nitrogen in the pyridine is also expected to increase the polarity and hence the water solubility of the compound and help in increasing the bioavailability. We are trying to achieve a good balance between the stability of the

compound prior to activation and its membrane permeability/ drug release/ activation kinetics after enzyme catalysis.

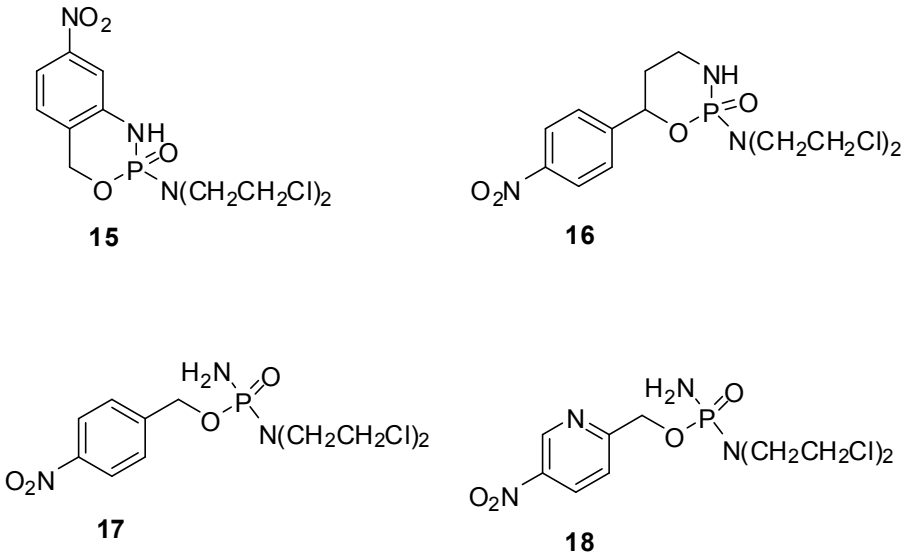
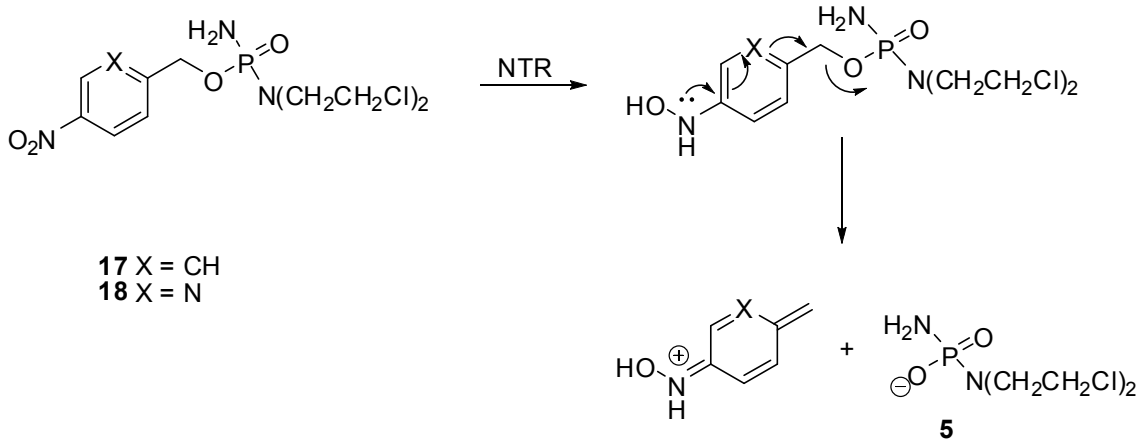


Figure 2. 4-Nitroaryl phosphoramide mustard analogues

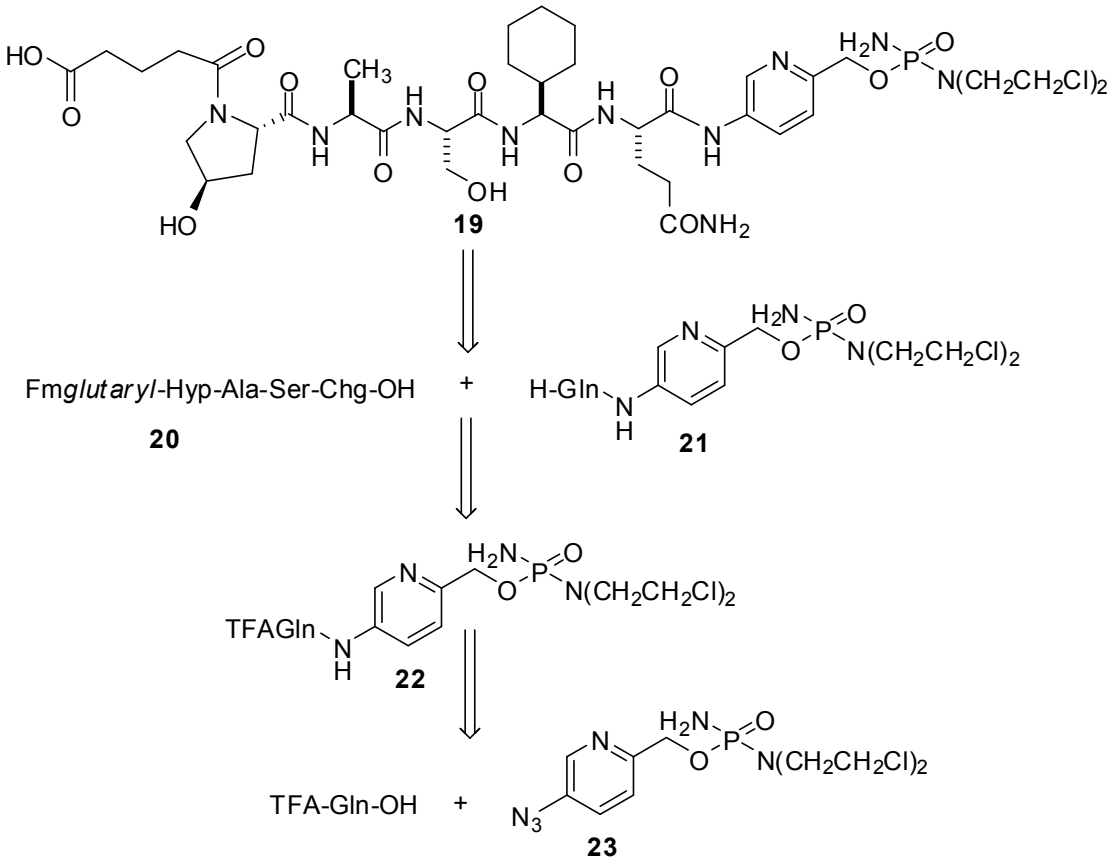


Scheme 3. Mechanism of activation of 4-nitroarylmethyl phosphoramide mustard (**17** & **18**)

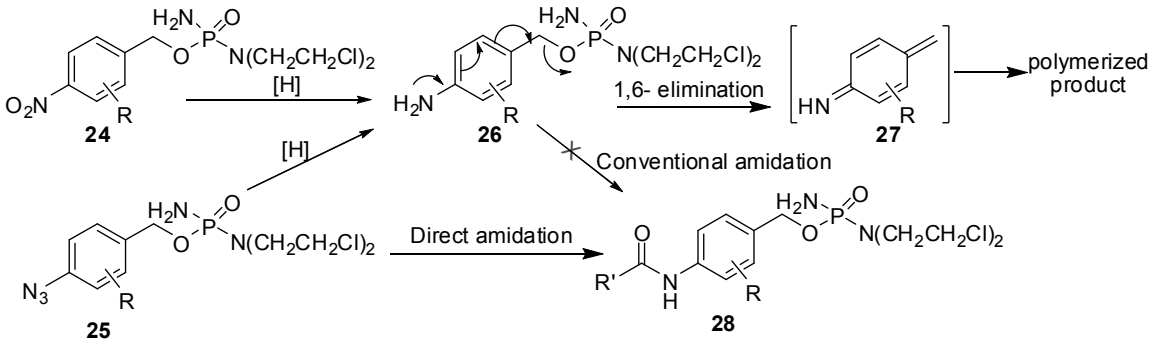
III. Efficient amidation from selenocarboxylate and aromatic azides

We wanted to replace the nitro group in 4-nitroarylmethyl phosphoramide mustard analogues with peptide amido group to obtain prodrugs that can be activated by specific endogenous proteases releasing phosphoramide mustard into the tumor site. We designed and synthesized **19** to be a substrate for PSA and selectively release phosphoramide mustard in prostate cancer cells. Upon cleavage by PSA at the glutamine site, the aromatic amino group in the released species is free and undergoes spontaneous 1,6-elimination, releasing the cytotoxic phosphoramide mustard.

According to the retro synthetic pathway shown in Scheme 4, **22** was obtained via an amidation reaction. Amide bonds are usually formed through the reaction of an activated carboxylic acid with a free amine.[48,49] However, not all functional groups are compatible with the presence of a free amine. It is believed[50] that reduction of nitro and azido groups (compounds **24** and **25**, Scheme 5) failed to give the desired amide product **28**. Instead the amine intermediate **26** underwent 1,6-elimination to form quinonimine methide **27** followed by polymerization. Whereas, direct amidation of azide **25** without reduction to the amine intermediate **26**, resulted in the formation of the desired amide product **28**. In-order to overcome the problem of 1,6-elimination during the synthesis of peptide-conjugated phosphoramide mustard analogue, we had to develop a new amidation reaction without the use of unstable basic nucleophilic amine intermediates.



Scheme 4. Retrosynthesis of peptide-conjugated phosphoramide mustard



Scheme 5. Direct amidation of azides avoids 1,6-elimination and forms the desired amide product.

It has been reported that azides which are neither basic nor nucleophilic and can be easily transformed into various nitrogen-containing functional groups have been used to directly form amide bonds. The amidation of a carboxylic acid with an azide in the presence of a trialkyl phosphine,[51-53] the thio acid/azide amidation,[54-56] and the selenocarboxylate/azide amidation[49,57] and Staudinger-type ligation are few examples.[58-63]

The modified Staudinger ligation,[62,63] involves the formation of an amide bond starting from an azide and a phosphine-linked ester/thio ester in the form of R'COO-$C_{n=1,2}$-PR_2 or R'COS-$C_{n=1,2}$-PR_2. The reaction involves a 5 or 6-membered intramolecular transacylation of an iminophosphorane,[64] followed by hydrolysis of the intermediate to form an amide. Staudinger method has been successfully used in N-glycosylation,[65-70] peptide ligation[71-74] and lactamization.[75]

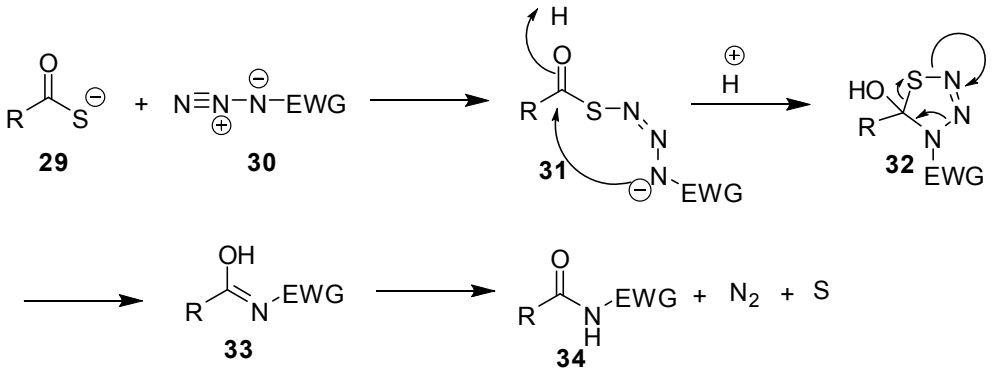
However, the Staudinger ligation favors only aliphatic azides. For aromatic azides with electron-withdrawing substituents, the reactions are reported to be very slow and may also fail.[76] The major drawback of Staudinger-type ligation is that when the reaction fails or is incomplete, hydrolysis of the iminophosphorane intermediate occurs in aqueous medium or during aqueous workup leading to the formation of the corresponding amine thus eliminating the possibility of recovering the azide starting material.[50]

Just and coworkers were the first ones to report the reaction of a thio acid with an organic azide to give the corresponding amide.[54] It was proposed as a conventional nucleophilic acylation reaction between thio acid and the amine formed through a rapid *in situ*

reduction of the azide. Hydrogen sulfide regenerated during the acylation reaction was responsible for the reduction of the azide.[55]

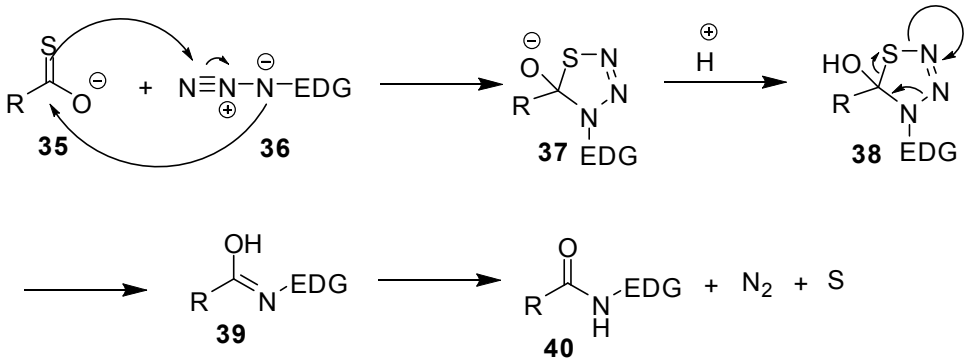
Recently, Williams' group presented an explanation that excluded the formation of an amine as the reactive intermediate and revised the mechanism to proceed via the formation of a thiatriazoline intermediate followed by a retro-[3+2] cycloaddition to form the desired amide product.[56] Presence of bases such as 2,6-lutidine accelerated the reaction. Depending upon the electronic properties of the azide used, two different mechanisms have been proposed for the formation of the thiatriazoline intermediate. For electron-deficient azides, a linear adduct is formed via the bimolecular union of the terminal nitrogen of the azide **30** with sulfur of the thiocarboxylate **29** and cyclization of this adduct **31** gives a thiatriazoline **32** (Scheme 6). For relatively electron-rich azides, the thiatriazoline **38** is formed via an anion-accelerated [3+2] cycloaddition reaction between the thio acid **35** and the azide **36** (Scheme 7).[56] The reaction is fast and selective and hence is considered to be a good synthetic method.

The thio acid/azide reaction works well if the azide is electron deficient. However, sterically hindered and electron-rich azides require high concentration of reactants, reflux temperatures and several hours in order to acheive good conversion yields.[56]



EWG= Electron withdrawing group

Scheme 6. Mechanism of Williams' thio acid/azide amidation for electron-deficient azide.[56]



EDG= Electron donating group

Scheme 7. Mechanism of Williams' thio acid/azide amidation for electron-rich azide.[56]

It was of interest to explore the selenocarboxylate/ azide amidation reaction in which the selenocarboxylate generated *in situ* has been reported to be a good reagent for the introduction of selenium into organic compounds and for efficient amidation.[57] Selenium and sulfur share similar chemical properties but as the selenium atom is larger and more polarizable, it is more nucleophilic than sulfur. It has been reported that selenophenolate

reacts ~7 times faster than thiophenolate in an S_N2 displacement reaction with methyl iodide.

Selenocarboxylates have greater reactivity as compared to thio acids. The enhanced reactivity of selenocarboxylates would accelerate the reaction, shorten the reaction time, and potentially lower the reaction temperature. For example, a sterically hindered 2-azidopiperidine derivative was entirely unreactive towards thioacetic acid in refluxing chloroform after 12 h in the presence of 2,6-lutidine, while it reacted with selenoacetic acid to form the corresponding acetamide in 75% yield under the same reaction conditions.[57]

Selenocarboxylic acids are readily oxidized to diacyl diselenides upon exposure to air and are unstable to heat. However, the corresponding alkali metal and piperidinium salts of aromatic selenocarboxylates are relatively stable.[77,78] For example, potassium 4-methylbenzeneselenocarboxylate remained unchanged when it was exposed to air for 5 h. Most aromatic selenocarboxylate salts can be stored at –17 °C for at least one month under oxygen free conditions.[79]

Selenocarboxylates can be synthesized by the treatment of trimethylsilyl selenocarboxylates with alkali metal fluorides,[77,78] the reaction of carboxylic acids with Woollin's reagent in refluxing toluene,[79] the reaction of acyl chlorides with alkali metal selenides,[80] and the reaction of diacyl selenides with alkali metal hydroxide.[81] However, the above methods have disadvantages. For example, trimethylsilyl selenocarboxylic

esters are highly moisture sensitive, and high reaction temperatures used in case of woolin's reagent would cause racemization of chiral centers in amino acids and peptides.[50]

For our initial efforts,[49] we used potassium selenocarboxylates to react with azides because alkali metal salts of selenocarboxylates were known to be relatively stable.[82] However, to better solubilize potassium selenocarboxylates and potassium methoxide used to prepare selenocarboxylates,[83] we used DMSO as a co-solvent for the reaction. The mild oxidizing property of DMSO accelerated the decomposition of selenocarboxylates, which adversely affected the yield of product formation. Using an HPLC assay, we found that the half-life of selenocarboxylate was only 25 min under the mixed solvent conditions of DMSO and ethyl acetate at room temperature.[49] With electron-deficient azides and excess (2 eq) potassium selenocarboxylate, the reaction was fast and the short half-life of selenocarboxylate did not present a problem. When electron-rich azides were used, the reactions under the same conditions required longer time to complete. The poor stability of selenocarboxylate under these reaction conditions led to much lower conversion yields. Our aim was to overcome the above problems and develop a new set of conditions to increase the solubility and stability of selenocarboxylates and achieve good conversion yields. The new amidation method was critical for the successful synthesis of the peptide-conjugated analogue.

IV. Prodrugs activated by prostate-specific antigen (PSA)

Prostate cancer is the most common type of cancer in men in the United States, and is responsible for more male deaths than any other cancer, except lung cancer. Prostate cancer can be treated effectively when the cancer is confined to prostate gland.[84] However, there is no effective treatment when it has reached the stage of metastasis. Surgery, radiation therapy, hormonal therapy, occasionally chemotherapy, or combinations of these are employed to treat prostate cancer.

Hormonal therapy can be employed for initial stabilization or regression of the disease. However, within a couple of years most patients develop androgen-refractory prostate cancer with a life expectancy of about three years.[85,86] Recently developed chemotherapy regiments offer improved survival to men with advanced prostate cancer.[87,88] Microtubule inhibitors like estramustine phosphate, taxane/taxoids showed significant decrease in serum PSA levels in patients with advanced androgen-independent prostate cancer. Novel methods are being developed to target cytotoxic agents specifically to the prostate cancer cells through mechanisms specific to prostate cancer cells, and the use of PSA as a prodrug converting enzyme is one of them.[89]

Properties of prostate-specific antigen (PSA)

Prostate-specific antigen (PSA) is a 237-amino acid glycoprotein and a member of the kallikrein family of serine proteases with both antigenic and enzymatic activity. PSA is selectively expressed in prostate tissues. It is a serological marker used in the early

detection of prostate cancer and is used to monitor the therapeutic response in treating the disease. Prostate cancer patients have increased serum PSA levels due to cellular PSA "leak" caused by the absence of a basement membrane that normally separates the prostate gland from surrounding stroma. The absence of basement membrane leads to invasive growth of the cancer.[90,91]

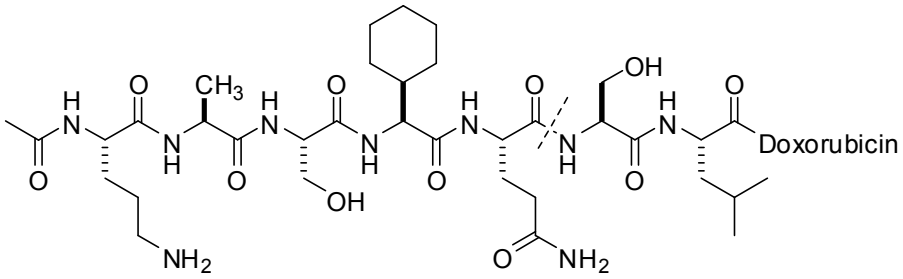
Only PSA present in prostate tissue is enzymatically active. PSA leaked into the blood stream forms stable inactive complexes with serum protease inhibitors such as α_1-antichymotrypsin and α_2-macroglobulin. The concentration of these inhibitors is 10^5 to 10^6 folds higher than serum PSA levels leading to the complete entrapment of any active PSA released from prostate tissue.[92] The PSA levels in blood were found to correlate with the disease state of prostate. Higher blood PSA levels could represent metastatic prostate cancer.[93] PSA has no kallikrein's trypsin like activity but it shows chymotryptic-like activity and prefers a glutamine residue at the P1 site, which is unique for an endopeptidase.[94]

Due to the prostate tissue-specific production, localized activity, inactivation by serum protease inhibitors in blood and increased production in malignant prostate cancer cells, PSA could be used as an effective prodrug-converting enzyme for tumor targeted therapy against advanced prostate cancer.

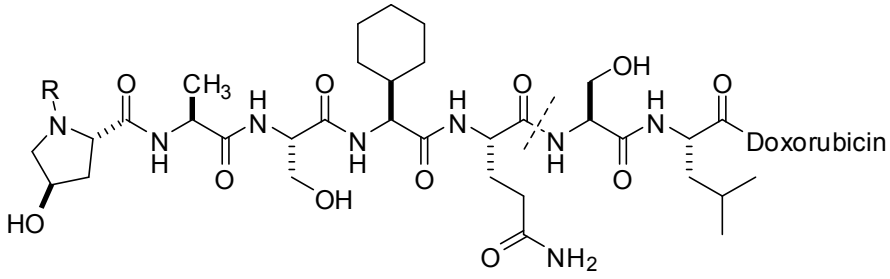
Peptide sequences specific for PSA cleavage

Issacs and coworkers from John Hopkins university reported two peptide sequences, H-Ser-Lys-Leu-Gln- and H–His-Ser-Ser-Lys-Leu-Gln- with good specificity for PSA.[94] The conjugates containing these peptide sequences were stable in human serum over 24 h and showed moderate PSA activity. However, they were not affected by extracellular proteases like chymotrypsin, trypsin, urokinase, plasmin, thrombin, hK1 and tissue plasminogen activator. The histidine containing hexapeptide has increased water solubility and has been used to conjugate several cytotoxic agents as potential prodrugs for PSA activation.

Garsky and coworkers from Merck designed and screened several short peptide sequences in order to couple doxorubicin conjugates for PSA activation.[95] These conjugates were selectively cleaved by PSA and showed selective cytotoxicity against PSA-expressing LNCaP cells. It was found that the heptapeptide doxorubicin conjugate **41** (Figure 3) was the best substate for PSA with a half life of 10 min at an enzyme/substrate molar ratio of 1/100. However, it had an unexpected hypotensive effect associated with it. New analogues were developed to overcome this side effect. It was observed that the capping groups in these conjugates played a key role in the proteolytic process. When the acetyl group of **42a** was replaced by succinyl group the resulting conjugate **42b** had a longer half-life of 55 min. When replaced by glutaryl group the resulting conjugate **42c** had a half-life of 30 min with improved water solubility and hence was used for biological assays.



41



42a R = CH_3CO
42b R = $HOOC(CH_2)_2CO$
42c R = $HOOC(CH_2)_3CO$

Figure 3. Peptide sequences specific for PSA cleavage (----- indicates the cleavage site)

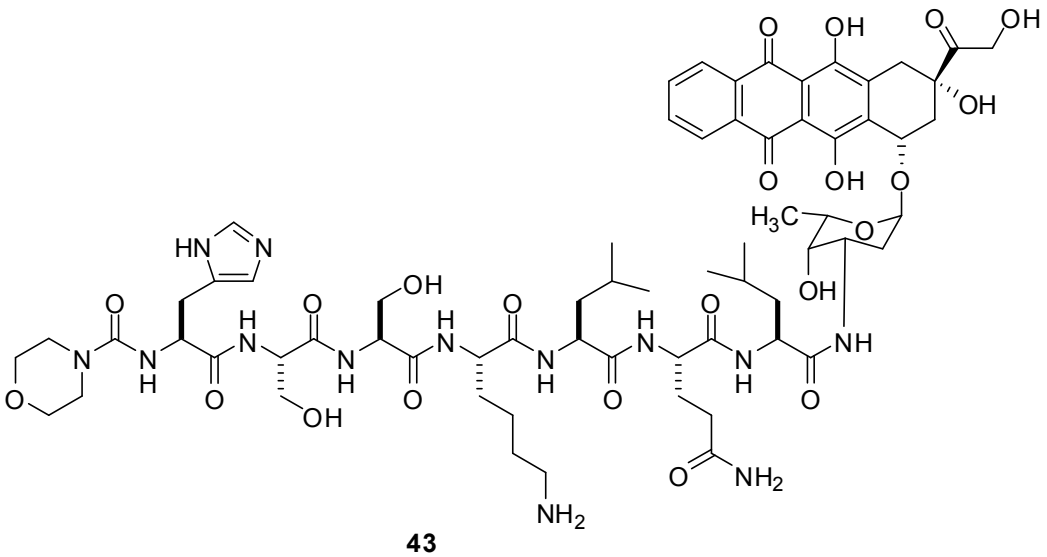
Prodrugs developed for activation by PSA

Several cytotoxic agents have been directly coupled to peptides and demonstrated good selectivity and cytotoxicity against prostate cancer cells. These compounds were easy to synthesize and served as good proof of concept studies. Preclinical assay data indicate that some of these agents showed promising results in reducing circulating PSA levels and tumor weight. The cytotoxic agents used were natural products like doxorubicin, vinblastine and thapsigargin.

Doxorubicin, a topoisomerase II inhibitor and a DNA intercalating cytotoxic agent has been used in the treatment of various cancers.[96] However, its cardiotoxicity and myelotoxicity limited its application. Consequently, doxorubicin prodrugs for tumor-specific activation and improved therapeutic index have been sought.

For example, the heptapeptide conjugate **43** (Figure 4) showed enhanced activity in prostate cancer cell lines containing active PSA and over 90% hydrolysis upon a 72 h exposure to PSA-expressing LNCaP cells. There was no significant systemic toxicity when administered at four times the 100% lethal doxorubicin equivalent dose.[97]

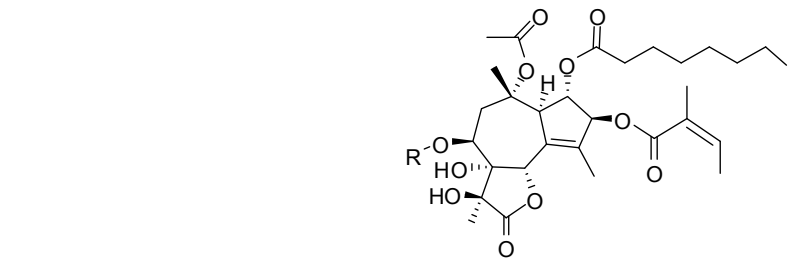
Another heptapeptide doxorubicin conjugate **42c** (Figure 3) was reported to have over 20-fold selective cytotoxicity against PSA-expressing LNCaP cells relative to the non-PSA-expressing DuPRO cells. It was metabolized to H-Leu-Dox and doxorubicin in cell culture assays.[98] Upon intraperitoneal administration, the peptide conjugate was found to have lesser cardiotoxic effect, body weight loss and death rate of mice when compared to the direct injection of doxorubicin. The conjugate reduced the serum PSA levels by 95% and tumor weight by 87% at a dose below the maximum tolerated dose (MTD) of doxorubicin while doxorubicin alone was ineffective at its MTD.



43

Figure 4. Doxorubicin conjugate for PSA activation.

Thapsigargin (TG, **44**, Figure 5) is a natural product that inhibits the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump and can induce apoptosis in both quiescent and proliferating cells existing in metastatic prostate cancer.[99-101] Upon systemic administration, TG causes significant host toxicity due to the presence of SERCA pump in almost all cells. This led to the development of prodrug 12-aminododecanoyl-TG (12ADT, **45**, Figure 5) conjugated to a heptapeptide. The conjugate **46** showed over 100-fold higher cytotoxicity against PSA-expressing LNCaP cells than against non-PSA-expressing cells. Continuous subcutaneous administration of the conjugate over 40 days resulted in nearly complete cessation of tumor growth without any systemic toxicity.



44 (TG): R = $CO(CH_2)_2CH_3$
45 (12ADT): R = $H_2N(CH_2)_{10}COO$

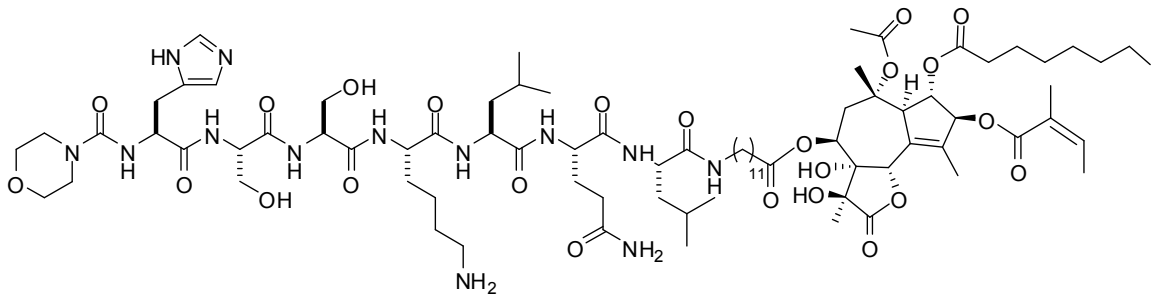


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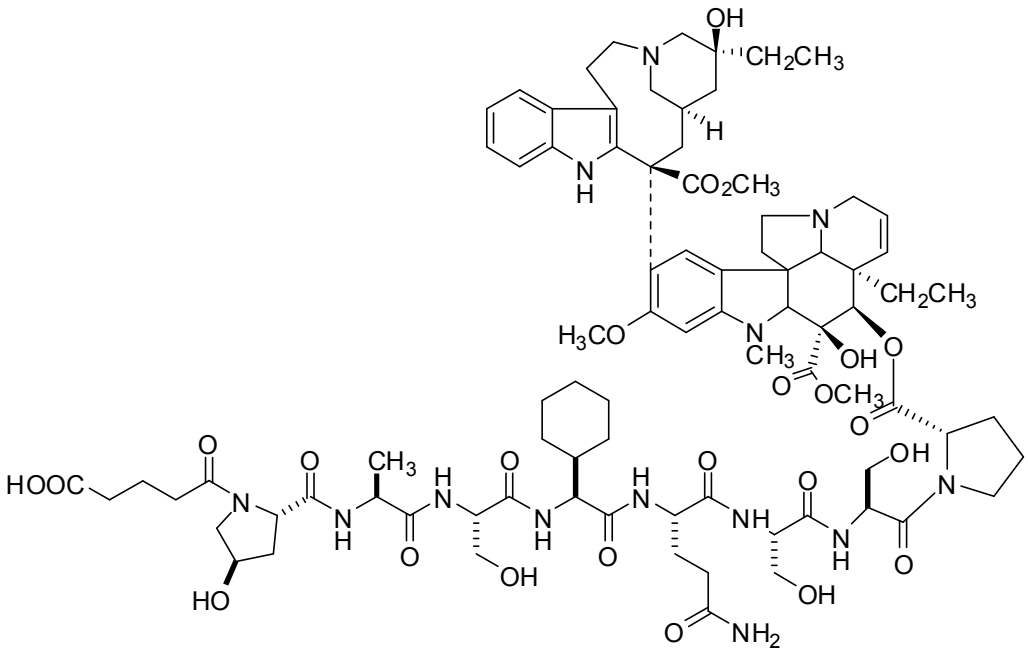
Figure 5. Chemical structures of TG, 12ADT and Mu-His-Ser-Ser-Lys-Leu-Gln-Leu-12ADT.



47

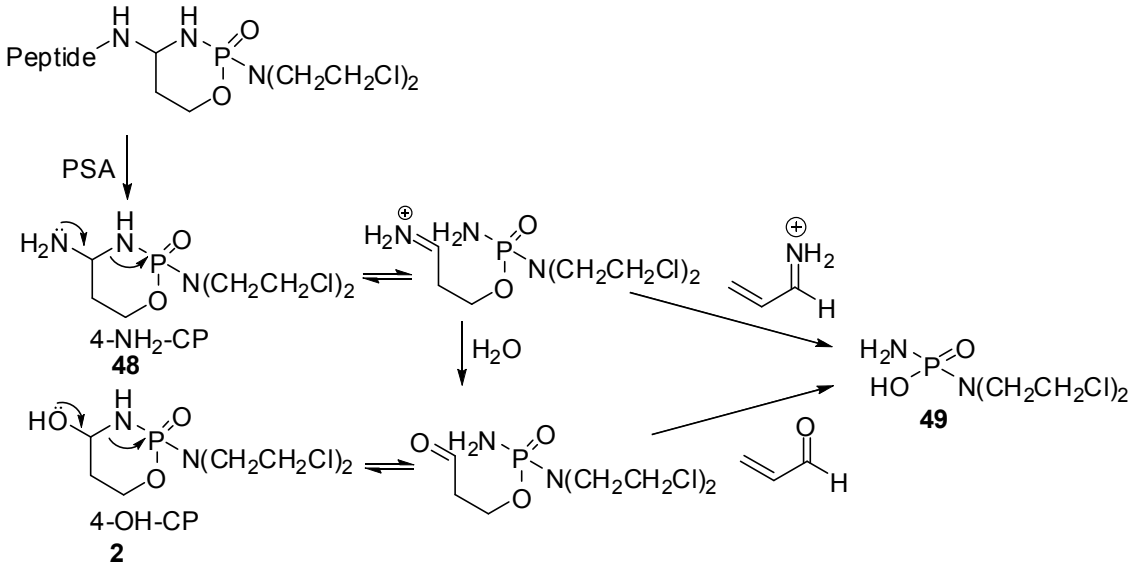
Figure 6. Chemical structure of glutaryl-Hyp-Ser-Ser-Gln-Ser-Ser-Pro-des-acetyl vinblastine.

Vinblastine, a vinca alkaloid is an antimicrotubule cytotoxic agent independent of p53 protein. This makes it a drug of choice to treat metastatic prostate cancer which is always characterized by p53 mutations.[102] An octapeptide conjugate **47** (Figure 6) of des-acetyl vinblastine was reported to be entirely stable in human plasma over 6 h and showed 10 to 30-fold selective cytotoxicity towards PSA-expressing LNCaP cells. It was effective in reducing the serum PSA level by 99% and tumor weight by 85% when administered at a dose just below its MTD.

The above mentioned prodrugs developed for activation by PSA have good activities against prostate cancer but also have the following disadvantages. Due to their bulky

structures, a spacer of 1-3 aminoacids needs to be inserted between the cytotoxic agent and the peptide. Else, the conjugates would not be efficiently cleaved by PSA.[102-104] Thus, release of the parent drug from these conjugates requires post PSA-cleavage processes mediated by non-specific esterase or aminopeptidase to remove the linker. These post PSA-cleavage processes are not always efficient and can lead to the slow release of cytotoxic agents in certain conjugates.[102] According to Lipinski "rules of 5", ideally compounds with drug-like properties should not have molecular weights over 500 daltons. However, the peptide conjugates mentioned above have very high molecular weights, over thousand daltons which is undesirable.[105]

Our group used cyclophosphamide as a prodrug lead based on its advantages over other cytotoxic agents like doxorubicin. Its small molecular size, low molecular weight, and well established structure activity relationship make it a lead molecule for the development of peptide conjugated prodrugs for activation by PSA. Our group was the first to report 4-aminocyclophosphamide (4-NH_2-CPA, **48**) as a prodrug moiety of phosphoramide mustard. As shown in Scheme 8, our design takes advantage of the structural similarity of 4-NH_2-CPA (**48**) to 4-hydroxycyclophosphamide (**2**), which is the enzymatic C-4 oxidation product of cyclophosphamide in liver and is responsible for the release of cytotoxic phosphoramide mustard (**49**) from cyclophosphamide and the chemical instability of a gem-diamine.



Scheme 8. Mechanism of activation of peptide conjugated amino cyclophosphamides.

Chemically, 4-NH_2-CPA (**48**) is a gem-diamine, which is known as a masked aldehyde. Monoacyl or monocarbamyl gemdiamines are labile to acid- or base-catalyzed elimination reactions while N,N'-Diacylated or dicarbamylated gem-diamines are stable compounds widely used in "retro-inverso" peptide mimetics. These characteristics of gem-diamines suggest the release of phosphoramide mustard (**49**) under physiological conditions and its stabilization through peptide conjugation.[6]

Peptide-conjugated phosphoramide mustard prodrugs

Our next effort is focused towards the synthesis of peptide-conjugated phosphoramide mustard prodrugs for activation by PSA. PSA enzyme is sensitive to structural features C-terminal to the cleavage site and hence we are developing new traceless linker systems with structural flexibility that could accommodate for the various structural requirements of PSA enzyme. The linkers are designed to facilitate membrane permeation and

intracellular release of the drug upon cleavage by the target enzyme. Cleavage of the N-terminal substrate peptide will afford a free electron-donating/nucleophilic aromatic amine that can be used as a trigger for subsequent drug release. The amine is neutral under physiological pH and thus the linker-drug conjugate is neutral and capable of permeating through the cellular membrane. Whereas, inside the tumor cells the pH is likely to be acidic, this would promote the intracellular release of the cytotoxic phosphoramide mustard species. Phosphoramide mustard is acidic with pK_a around 0.5 and hence will be completely inonized and trapped inside the target tumor cells, leading to the desired antiproliferative activity. The small size of phosphoramide mustard will also provide more room for the attachment of peptide and linker and further structural modifications to obtain prodrugs with "drug-like" properties. It has already been established that acyclic 4-nitrobenzyl phosphoramide mustard (**17**) analogue is the best cytotoxic agent towards nitroreductase-expressing SKOV3 and V79 cells with 5,600 to 170,000-fold selectivity and an IC_{50} as low as 0.4 nM with improved bystander effect. Hence, we thought replacement of the nitro group in nitro aryl phosphoramide mustard analogues, with peptide amido group would yield prodrugs that can be activated by PSA and release the cytotoxic phosphoramide mustard selectively to prostate cancer cells. Also we can take advantage of the electron-deficient nature of pyridine to stabilize the conjugate and increase the selectivity of the prodrug to PSA producing prostate cancer cells. Preliminary studies conducted in our lab suggested that the peptide glutaryl-Hyp-Ala-Ser-Chg-Gln sequence was a good substrate for PSA and hence we used the same sequence for the new conjugate (**19**). For the preparation of the peptide-conjugated analogue **19**, we developed a new amidation reaction using selenocarboxylate and azides,

thus avoiding the use of unstable basic nuclephilic amine intermediates. The aim of our current research is to synthesize a peptide-conjugated phosphoramide mustard analogue and obtain a right balance between the stability of the conjugate prior to activation and the membrane permeability/drug release/activation kinetics after proteolytic cleavage. The combination of site-specific activation and tumor targeting of cancer cells would lead to the development of a new generation of targeted activation and intracellular drug delivery system for the treatment of advanced cancer.

Objectives

This thesis has the following three specific objectives,

Objective 1. To synthesize and evaluate 5-nitro-pyridyl-2-phosphoramide mustard as a substrate for *E.coli* nitroreductase.

Objective 2. To develop an efficient selenocarboxylate/azide amidation method that would avoid the use of unstable amine intermediates for the synthesis of peptide-conjugated phosphoramide mustard prodrugs.

Objective 3. To synthesize glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-pyridyl-2-methyl phosphoramide mustard and evaluate its chemical stability, substrate activity for PSA and antiproliferative activity against PSA-expressing prostate cancer cell lines.

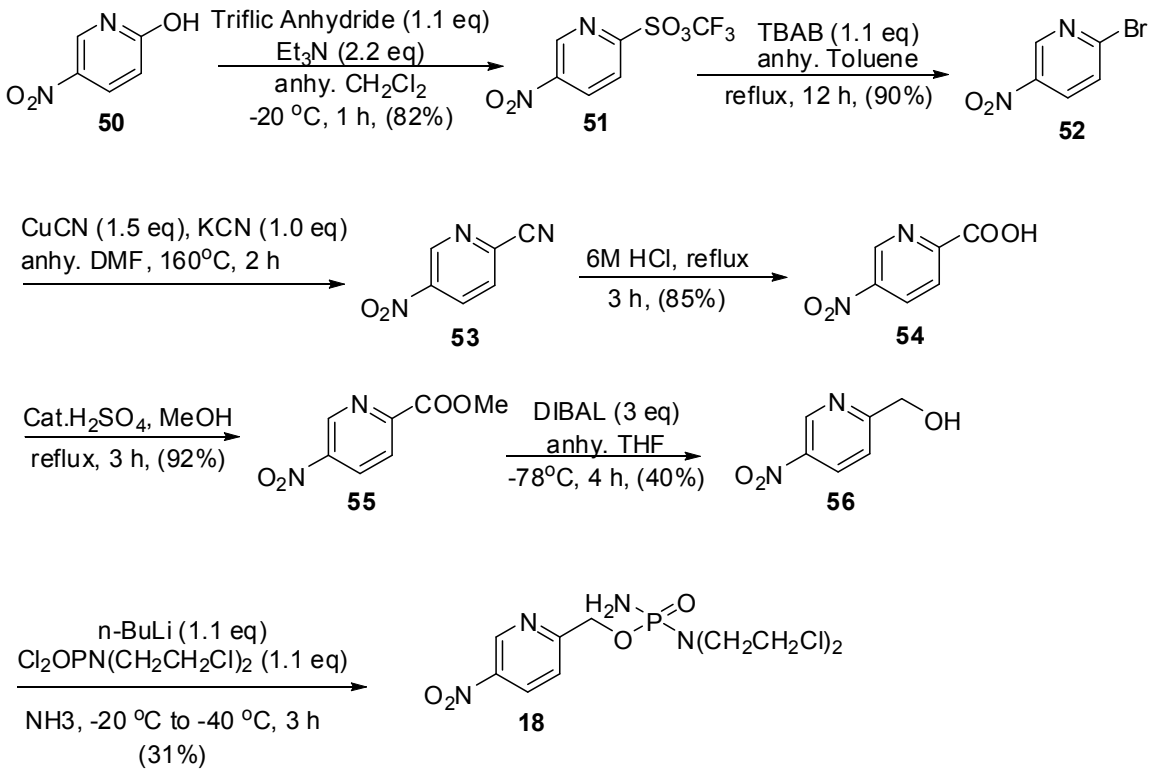
RESULTS AND DISCUSSION

I. Synthesis and evaluation of 5-nitropyridyl-2-methyl phosphoramide mustard (18)

A. Synthesis

The synthesis of 5-nitropyridyl-2-methyl phosphoramide mustard (**18**) was accomplished in 7 steps starting from 2-hydroxy-5-nitropyridine (**50**) as shown in Scheme 9. 2-(Trifluoromethanesulfonyl)oxy-3-nitropyridine (**51**) was prepared as trifluoromethanesulfonyl is a good leaving group. The synthesis was done in the presence of 1.1 eq triflic anhydride and 2.2 eq triethyl amine in anhy. methylene chloride at –20 ^{o}C.[106] The reaction was completed in 1 h and gave the product as a pale yellow solid in 82% yield.

Treatment of 2-(trifluoromethanesulfonyl)oxy-3-nitropyridine with 1.1 eq TBACN in refluxing toluene was attempted to introduce the cyano group to directly form **53** but the reaction failed to yield the desired product. The failure of this reaction can be attributed to the hygroscopic nature of TBACN and the absorbed water reacted with the active triflate leading to its hydrolysis. As this reaction failed, we prepared 2-bromo-5-nitropyridine (**46**) by nucleophilic substitution reaction. 2-(Trifluoromethanesulfonyl)oxy-3-nitropyridine (**51**) was treated with 1.1 eq TBAB in refluxing toluene to introduce the bromo group. High temperature was employed to shorten the reaction time and aid in the solubility of TBAB. TBAB was chosen as the source of bromide ion as it is easy to handle and less hazardous than other reagents.[106] The reaction was very clean and gave the product as a white solid in 90% yield.



Scheme 9. Synthesis of 5-nitropyridyl-2-methyl phosphoramide mustard (**18**)

The next step was the nucleophilic displacement reaction in which the bromide was displaced by cyanide.[107] This was achieved by heating 2-bromo-5-nitropyridine **(52)** in anhy. DMF in the presence of 1.5 eq CuCN and 1.0 eq KCN at 160 ^{o}C for 2 h. The use of high temperature was a key parameter for the success of this reaction. The workup of this reaction is very important. Acid workup was avoided as it would generate toxic hydrogen cyanide. Hence, we used potassium phosphate buffer which would prevent the formation of any hydrogen cyanide.[107] The precipitate formed during workup was extracted several times with ethyl acetate in order to recover the entire product. The product **53** was sufficiently pure and used for the next step without any further purification.

The cyano group was converted to acid group by acid hydrolysis using 6 M HCl solution. The reaction was conducted at reflux temperature and complete hydrolysis was achieved in about 3 h and the product **54** precipitated out upon cooling.[107] The yield after 2 steps was 85%.

The acid **54** was converted into methyl ester **55** via acid catalyzed esterification process. The esterification was rapid and complete in methanol in the presence of catalytic amount of sulfuric acid under reflux conditions giving the product as a yellow solid in 92% yield.

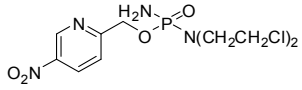
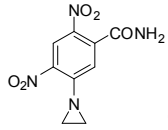
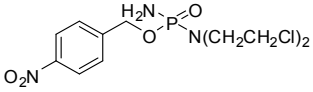
The methyl ester **55** was reduced to the corresponding alcohol **56** using 3 eq. of DIBAL as a reducing agent. The reaction was performed in anhydrous THF and at –78 ^{o}C to prevent the reduction of nitro group. The yield after column purification was 40%.

For the synthesis of **18,** n-BuLi was used to deprotonate the alcohol **56** and to this bis(2-chloroethyl) phosphoramidic dichloride was added. The reaction was carried out at –40 ^{o}C for 3 h and then ammonia gas was bubbled for 15 min to form the desired product in 31% yield after purification.[47] The overall yield of the synthesis after 7 steps was 7%.

B. Antiproliferative assay

The 5-nitropyridyl-2-methyl phosphoramide mustard (**18**) synthesized was evaluated for its antiproliferative action against cell lines expressing *E. coli* nitroreductase (T116) or human quinone oxidoreductase enzyme NQO1 (hDT7). The cells were Chinese hamster V79 cells that were transfected with a bicistronic vector encoding for the *E. coli* nitroreductase or the human quinone oxidoreductase protein and puromycin resistance protein as the selective marker. V79 cells transfected with only the vector (F179) were used as the controls for the assay. CB1954 (**11**), and 4-nitrobenzyl phosphoramide mustard (**17**) were used as a control for the experiment. The cells were exposed for 72 h to the test compound, CB1954 and, 4-nitrobenzyl phosphoramide mustard (**17**). From the results obtained (Table 1), it was found that the new analogue had an IC_{50} of 74.7 μM towards the control F179 cells and had an IC_{50} value of 9 nM and 8300-fold selectivity towards nitroreductase expressing T116 cells. When compared to 4-nitrobenzyl phosphoramide mustard (**17**),[47] the test compound had lower cytotoxicity and lower selectivity. However, when compared to the CB1954, the test compound had higher cytotoxicity towards NTR-expressing cells. These results suggest that 5-nitropyridyl-2-methyl phosphoramide mustard (**18**) is activated by *E. coli* nitroreductase to release phosphoramide mustard and exhibited highly selective cytotoxicity to *E. coli* nitroreductase-expressing cells.

Table 1. Antiproliferative assay of CB1954, 4-nitrophenyl phosphoramide mustard (**17**) and 5-nitropyridyl-2-methyl phosphoramide mustard (**18**)

Compound	IC_{50}[a] F179 (NTR-)	IC_{50}[a] T116 (NTR+)	Ratio[b] (F179/T116)
 **18**	75	0.009	8,300
 CB1954	254	0.033	7,700
 **17**	67	0.0004	170,000

[a] IC_{50} values are the concentration required to reduce the cell number to 50% of the control.

[b]Ratio of IC_{50} values as an indication of activation by *E.coli* nitroreductase.

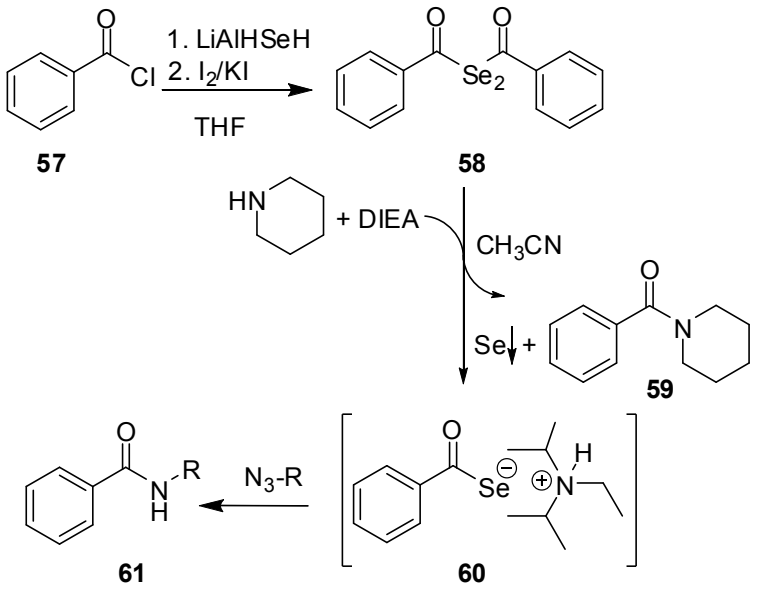
II. Efficient amidation from selenocarboxylate and aromatic azides

For the synthesis of peptide-conjugated phosphoramide mustard analogue, we developed a new amidation method that would avoid the basic, nucleophilic amine intermediates. Selenocarboxylates have greater reactivity as compared to thio acids. The enhanced reactivity of selenocarboxylates would accelerate the reaction, shorten the reaction time, and potentially lower the reaction temperature. Hence we were interested in developing the selenocarboxylate/azide amidation method which would prevent the *in situ* reduction of the azido group and form the desired amide bond.

For our initial efforts,[49] we used potassium selenocarboxylates to react with azides because alkali metal salts of selenocarboxylates were known to be relatively stable.[82] However, to better solubilize potassium selenocarboxylates and potassium methoxide used to prepare the selenocarboxylates,[83] we used DMSO as a co-solvent for the reaction. The mild oxidizing property of DMSO accelerated decomposition of selenocarboxylates, which adversely affected the yield of product formation. Using an HPLC assay, we found that the half-life of selenocarboxylate was only 25 min under the mixed solvent conditions of DMSO and ethyl acetate at room temperature.[49] With electron-deficient azides and excess (2 eq) potassium selenocarboxylate, the reaction was fast and the short half-life of selenocarboxylate did not present a problem. When electron-rich azides were used, the reactions under the same conditions required longer time to complete. The poor stability of selenocarboxylate under these reaction conditions led to much lower conversion yields. Our efforts then focused on the use of organic amine salts of selenocarboxylates and on finding conditions to improve the solubility and stability of

selenocarboxylates and to increase the reaction yields with electron-rich azides. We used diisopropylethylammonium benzeneselenocarboxylate (**60**) as a model substrate and monitored its subsequent direct amidation with azides in a much improved set of reaction conditions.

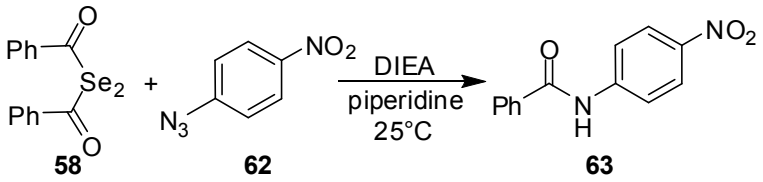
In our previous method,[49] diacyl selenides were used to prepare selenocarboxylates. Here, we used a diacyl diselenide instead, as diacyl diselenides are more stable than diacyl selenides. As shown in Scheme 10, dibenzoyl diselenide (**58**) was prepared from benzoyl chloride (**57**) upon treatment with LiAlHSeH in THF and subsequent oxidation with iodine and potassium iodide.[108,109] The dibenzoyl diselenide was easily purified by silica gel flash column chromatography and can be stored for several months at -20 °C without significant decomposition. To prepare selenocarboxylate, dibenzoyl diselenide was dissolved in acetonitrile and then treated under argon with 1 eq of diisopropylethylamine (DIEA) and 1 eq of piperidine to form the selenium metal, the stable *N*-benzoylpiperidine (**59**) and diisopropylethylammonium benzeneselenocarboxylate (**60**). The DIEA benzeneselenocarboxylate (**60**) was used directly without purification to react with various azides to form amide products **61** in a one-pot process. Since azides do not react with diacyl diselenide, they can be dissolved together prior to the addition of DIEA and piperidine to simplify operation and to improve the product yields. Because of increased stability of DIEA benzeneselenocarboxylate as discussed later, we were also able to decrease the amount of selenocarboxylate from the earlier 2 eq excess to only 1.2 eq for electron-deficient azides.



Scheme10. *In situ* generation of benzeneselenocarboxylate and subsequent amidation with azides

We selected 4-nitrophenyl azide (**62**), one of the most electron-deficient aromatic azides that reacted quickly with selenocarboxylate, to explore different solvent systems for the amidation reaction. As shown in Table 2, aqueous organic solvent containing 50% water (entries 1-3) gave good yields of the amide product suggesting that the amidation reaction does not require anhydrous conditions. However, the presence of too much water would adversely affect the solubility of the organic azides in the reaction medium, resulting in heterogeneity of the reaction mixture. Even for electron-deficient 4-nitrophenyl azide (**58**), it took 3 h for the reaction to complete in 50% aqueous methanol and isopropanol (entries 1 and 2, Table 2). When acetonitrile was used, the solubility of the azide improved and the reaction was complete within 1 h in 50% acetonitrile (entry 3, Table 2). The same reaction, if run in neat acetonitrile, took less than 30 min to complete (entry 4,

Table 2. Effect of solvents on reaction time and yield[a]



Entry	Solvent system	Reaction time (h)	Yield (%)[b]
1	CH_3OH/H_2O (1:1, v/v)	3	75
2	$(CH_3)_2CH_2OH/H_2O$ (1:1,v/v)	3	73
3	CH_3CN/H_2O (1:1, v/v)	1	83
4	CH_3CN (neat)	0.5	95
5	$CHCl_3/CH_3OH$ (1:1, v/v)	2	78

[a] General conditions: 4-Nitrophenyl azide **62** (0.2 mmol), dibenzoyl diselenide **58** (0.24 mmol), DIEA (0.24 mmol), and piperidine (0.24 mmol) in the given solvent system at room temperature
[b] Isolated yield (%)

Table 2). Mixed solvents of chloroform and methanol slowed down the amidation reaction and lowered the yield (entry 5, Table 2). Hence, neat acetonitrile was selected as the solvent of choice for the amidation reaction.

The stability of DIEA benzeneselenocarboxylate in acetonitrile was measured using an HPLC assay via the reaction of selenocarboxylate with *p*-toluenesulfonyl azide (**69**), the fastest amidation reaction in Table 3. When DIEA benzeneselenocarboxylate (**60**) was incubated with 2 eq of *p*-toluenesulfonyl azide (**69**) in acetonitrile at room temperature or 55 °C, complete and quantitative amidation was achieved in less than 5 min as monitored by HPLC. To monitor the stability of DIEA benzeneselenocarboxylate (**60**), dibenzoyl diselenide (**58**) was mixed with 1.2 eq of DIEA and 1.2 eq of piperidine at room temperature under argon. The DIEA benzeneselenocarboxylate formed was then allowed

to stand at room temperature or at 55 °C and aliquots were combined with 2 eq of *p*-toluenesulfonyl azide (**69**). The amide product, *N*-(*p*-toluenesulfonyl) benzamide (**84**), was analyzed to determine the amount of DIEA benzeneselenocarboxylate remained in solution. Figure 8 shows the stability of DIEA benzeneselenocarboxylate at 25 °C and 55 °C. The half-life of DIEA benzeneselenocarboxylate was found to be 11.3 h at 25 °C. This is an improvement of 27 times in stability if compared to the half-life of 25 min under the previous conditions when DMSO was used as a co-solvent.[49] Even when the temperature was raised to 55 °C, the half-life of the selenocarboxylate was 1.4 h under our present conditions.

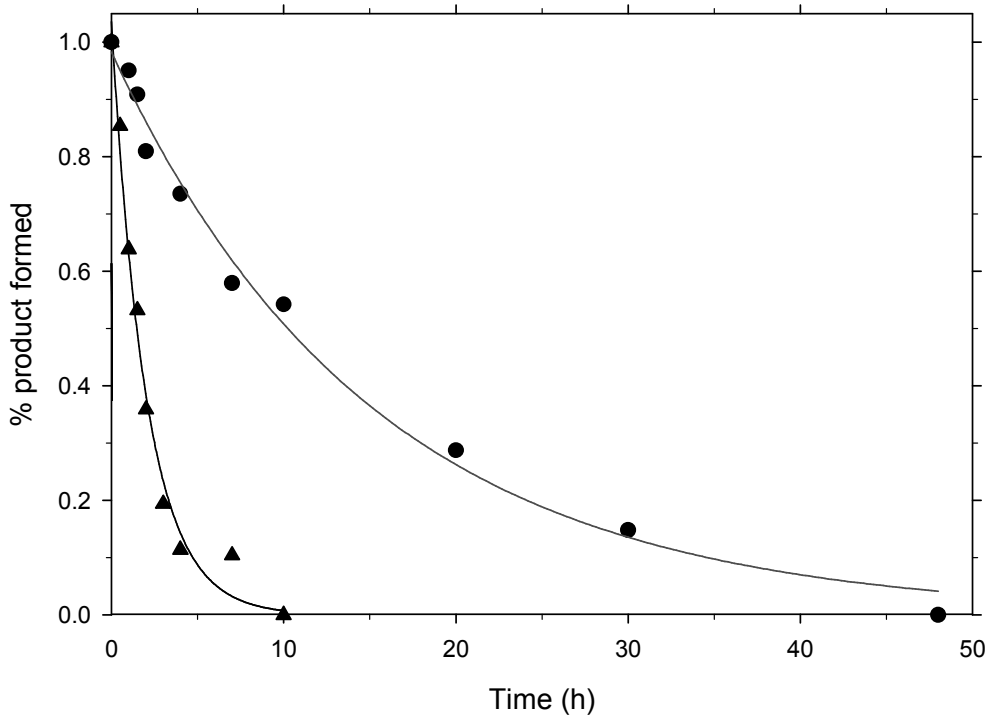


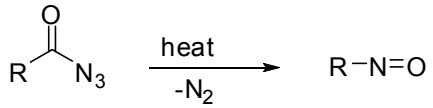
Figure 7. Stability of DIEA benzeneselenocarboxylate at 25 °C (●) and 55 °C (▲) as monitored by conversion to N-(*p*-toluenesulfonyl) benzamide (**84**) in an HPLC assay.

After we confirmed the increased stability of DIEA benzeneselenocarboxylate under our new reaction conditions, a variety of azides (**62**, **64**-**78**) were used to explore the effect of this improved stability on the amidation reaction. For electron-deficient azides, we found as shown in Table 3 that using 1.2 eq of selenocarboxylate were sufficient to give excellent conversion rates to the corresponding amides with isolated yields between 87%-96%. For electron-rich azides that are less reactive, 2 eq of selenocarboxylate and mild heating were used to obtain good yields based on azides. The position of substitution on the phenyl azide also affects the rate of amidation. When the nitro group was in the *para* position (entry 1, Table 3), the reaction was complete within 30 min. When the nitro group was moved to the *ortho* position, the reaction was slower and required 1 h to complete (entry 2, Table 3), suggesting the presence of steric effects in addition to the electronic effects on the amidation reaction. Since DIEA selenocarboxylate is more stable in acetonitrile than the corresponding potassium salt in the previous DMSO-containing solvent system, we were able to raise the reaction temperature to 55 °C to further speed up the amidation reactions for less reactive azides. The phenyl azide and *p*-methoxyphenyl azide gave amidation yields of only 25% and 7%, respectively in the DMSO-containing solvent system.[49] Under our new conditions, the same azides gave a much improved yield of 81% and 65%, respectively (entries 10 and 11, Table 3).

The effect of fluoro substitution on aromatic azide is also interesting. Electron-withdrawing groups are expected to stabilize the transition state through delocalization of the negative charge on the nitrogen, and thus facilitate amidation.[49] Fluorine substitution

at the *meta* position as in compound **76** improved the amidation yield from 56% to 78% (entries 13 *vs* 14, Table 3). However, when fluoro substitution was moved to the *ortho* position, the yield of the corresponding amide was reduced to only 37% (entry 15, Table 3). This was surprising, but could be explained by the different electronic effects of fluoro substitution at the *meta* and *ortho/para* positions. Fluorine has electron-withdrawing inductive effects (σ_I = 0.50) at the *meta* position and has both electron-withdrawing inductive and electron-donating resonance effects at the *ortho/para* positions ($\sigma_R°$ = -0.31).[110] Apparently due to the electron-donating resonance effect of fluoro substituent in the *ortho* position, delocalization of the negative charge on the nitrogen in the transition state was probably hindered, thereby lowering the reaction yield.[49]

Directly introducing electron-withdrawing groups adjacent to azido such as sulfonyl and phosphoryl gave excellent yields of *N*-acyl sulfonamide **85** and *N*-acyl phosphoramide **86** with only 1.2 eq of selenocarboxylate (entries 7 and 8, Table 3). Reaction of sulfonyl azide with selenocarboxylate was the fastest reaction with near quantitative yield (entry 7, Table 3). Acyl azide such as benzoyl azide (**71**) gave poorer yield. With 2 eq of selenocarboxylate, the isolated yield was only 51% at 55 °C. This was due to the instability of the acyl azide itself that led to a side reaction, Curtius rearrangement, during the amidation process. The Curtius rearrangement is the thermal decomposition of carboxylic azides to produce an isocyanate.

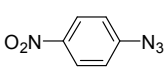
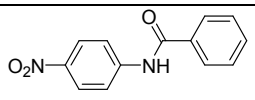
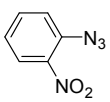
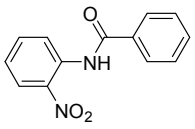


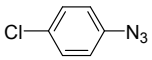
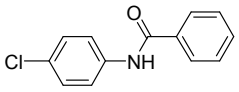
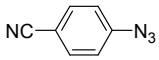
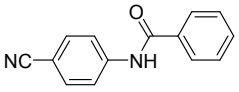
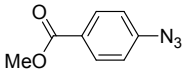
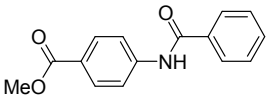
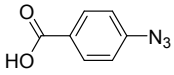
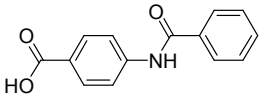
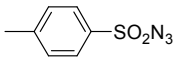
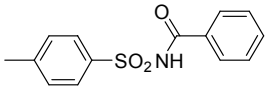
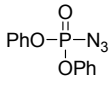
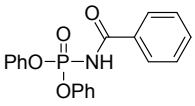
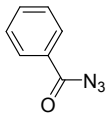
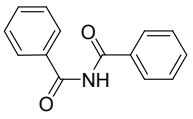
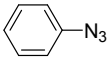
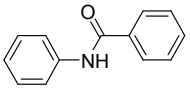
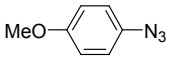
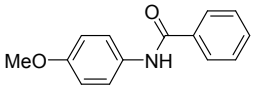
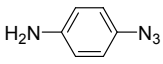
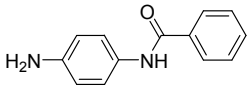
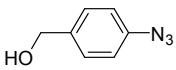
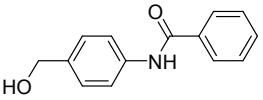
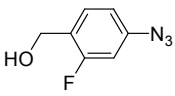
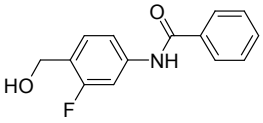
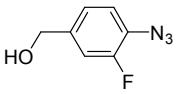
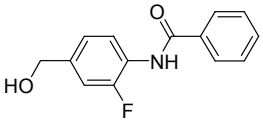
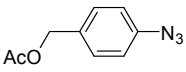
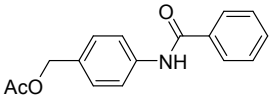
Scheme 11. Curtius rearrangement

Consistent with the mechanism proposed,[49] the reaction is compatible with the presence of free amines and alcohols (entries 12-15, Table 3). The formation of 4-benzoylaminobenzyl acetate (**93**) from 4-azido benzyl acetate (**78**, entry 16, Table 3) indicated the formation of amide without the prior reduction of azide to its corresponding amine as we reported previously.[49]

At the same time other selenocarboxylate/azide amidation conditions were also being developed in our lab. A facile one-pot procedure for the coupling of carboxylic acid and azide via selenocarboxylate and selenatriazoline had been developed and successfully applied to the coupling of amino acids and peptides with azides. The half-life of benzeneselenocarboxylate was found to be 16 h and 10 h in THF at 25 °C and 55 °C, respectively. This new one-pot procedure avoided the waste of the carboxylate equivalent when a diacyl diselenide or diacyl selenide. Significantly improved yields were also achieved for the less reactive azides because of the dramatically increased stability of selenocarboxylates and the procedure was easy to handle.[50] Hence we used this one-pot procedure for the synthesis of our target peptide conjugate **19**.

Table 3. Amidation reaction of selenocarboxylates with azides[a]

Entry	Azide starting material	Amide product	Selenocarboxyl -ate(eq)[b]	Temperature (°C)	Time (h)	Yield (%)[c]
1	 **62**	 **63**	1.2	25	0.5	95
2	 **64**	 **79**	1.2	25	1.0	93

3	 **65**	 **80**	1.2	25	2.0	95
4	 **66**	 **81**	1.2	25	1.0	96
5	 **67**	 **82**	1.2	25	2.0	87
6	 **68**	 **83**	1.2	25	1.0	89
7	 **69**	 **84**	1.2	25	0.08	96
8	 **70**	 **85**	1.2	25	3.0	88
9	 **71**	 **86**	2.0	55	12[d]	51
10	 **72**	 **87**	2.0	55	6.0	81
11	 **73**	 **88**	2.0	55	6.0	65
12	 **74**	 **89**	2.0	55	6.0	54
13	 **75**	 **90**	2.0	55	12[d]	56
14	 **76**	 **91**	2.0	55	6.0	78
15	 **77**	 **92**	2.0	55	12[d]	37
16	 **78**	 **93**	2.0	55	6.0	68

[a] General conditions: Azide (0.2 mmol), diacyl diselenide (0.24 or 0.4 mmol), DIEA (0.24 or 0.4 mmol), piperidine (0.24 or 0.4 mmol), acetonitrile (8 mL). [b] equivalents of selenocarboxylate formed *in situ*. [c] isolated yield (%) of the amide product. [d] Reactions were left overnight (~ 12 h) for convenience.

III. Synthesis and evaluation of glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-pyridyl-2-methyl phosphoramide mustard (19)

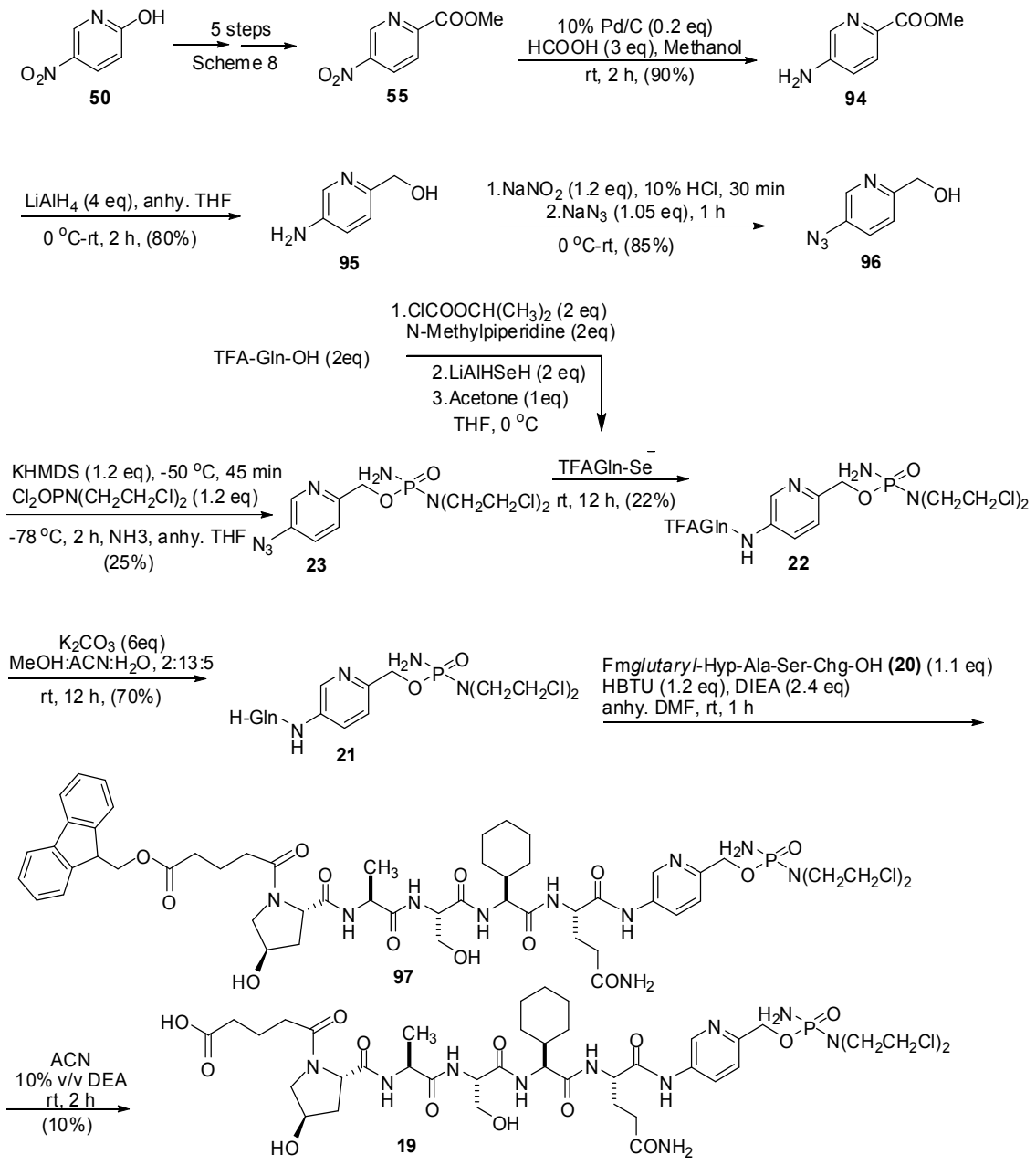
A. Synthesis

The synthesis of the target compound started from 2-hydroxy-5-nitropyridine (**50**) as shown in Scheme 12. The reduction of nitro group in **55** to amino group was accomplished by catalytic reduction in the presence of 0.2 eq Pd/C and 3 eq of formic acid in methanol. Using this method, the reaction was complete in about 2 h and formed the desired product **94** in 90% yield.

In the next step, the methyl ester **94** was reduced to the corresponding alcohol **95** using 4 eq lithium aluminium hydride in anhy. THF to obtain the desired product in 80% yield. The amino group was converted to azido group by diazotization in 10% w/w HCl solution followed by treatment with sodium azide. The reaction was complete in 1 h and gave **96** as a brown oil in 85% yield.[111]

For the synthesis of **23** we used 1.2 eq KHMDS as the base for deprotonation, and 1.2 eq bis(2-chloroethyl) phosphoramidic dichloride was added and the reaction was carried out at -78 ^{o}C for 2 h under nitrogen. The desired product was formed but was very minor and the starting material left unreacted was recovered. In order to push the reaction, the temperature was increased to -50 ^{o}C and after 2 h it was observed that the major product formed was the side product (Figure 8) and the minor product was the desired product. In our next attempt the deprotonation was performed at -50 ^{o}C for 45 min and the

reaction mixture was then cooled to -78 ^{o}C before the addition of bis (2-chloroethyl) phosphoramidic dichloride and the reaction was allowed to continue at -78 ^{o}C for 2 h



Scheme 12. Synthesis of glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-pyridyl-2-methyl phosphoramide mustard (**19**)

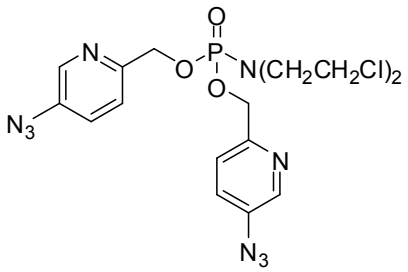


Figure 8. Side product formed during the synthesis of 5-azido pyridyl-2-methyl phosphoramide mustard (**23**)

under nitrogen. Under these conditions the side product formation was reduced and the desired product formation increased. The temperature was then increased up to -60 ^{o}C in- order to push the reaction forward but it was observed that the side product was the major product again. Thus, temperature played a very important role in this reaction and the starting material could be recovered. The isolated yield of the product was 25% and the yield based on the recovered starting material was 65%.

For the synthesis of **22,** the selenocarboxylate generation and amidation were carried out successfully in one pot.[50] After the generation of selenocarboxylate, the unreacted $LiAlH_4$ was destroyed by adding acetone in order to prevent the reduction of the azido group in **23** to amino group. Then the azide **23** was added to the selenocarboxylate and stirred at room temperature for 12 h to get the desired product in 22% yield.

The deprotection of TFA was accomplished in the presence of excess potassium carbonate using a mixed solvent system of MeOH/ACN/H_2O. The reaction was

completed successfully at room temperature in about 12 h and gave the desired product **21** in 70% yield.

The peptide **20** required for the next step was synthesized using Fmoc chemistry. The resin used for the peptide assembly was 4-hydroxymethylphenoxy wang resin. C-Terminal amino acid was loaded onto the resin using DMAP/DIC protocol. The subsequent Fmoc amino acids were activated and coupled using HOBt/HBTU/DIEA. The side chain hydroxyl group of Ser was protected by t-butyl group and the secondary hydroxyl group in hydroxyproline (Hyp) was not protected. Following completion of the assembly on the resin support, the N-terminal Fmoc group was removed via the standard 20% piperidine/NMP protocol. After each coupling and deprotection step, Kaiser test was performed to determine the completeness of the above processes. Deprotection and removal of the peptide from the resin support was effected by 95% TFA in CH_2Cl_2. The fluorenyl methyl ester (OFm) protecting group remained intact under these conditions while the t-butyl group was removed. After cleavage, the peptide was purified by reverse phase HPLC and purity was confirmed by LC-MS.

The peptide **20** was activated by 1.2 eq HBTU and DIEA to give the activated ester which was reacted with the H-Gln-NH-pyridyl-2-phosphoramide mustard (**21**) in NMP to form the desired peptide conjugated product **97**. The product was precipitated and directly used for the next step. The deprotection of Fm group was achieved using 10% v/v DEA in ACN at room temperature in 2 h and formed the target compound **19** which was purified by reverse phase HPLC. The final target compound was purified using HP1090 system on a Phenomenex C_{18} column (10 μm, 10 x 250 mm). Initially10% -

50% ACN/H_2O containing 0.1% TFA was used as the mobile phase for HPLC purification. To our surprise it was found that the desired product was not present in any of the fractions collected from HPLC. However, the crude sample before HPLC purification had the desired product as the major peak upon LCMS analysis. Hence, we thought the compound might be sensitive to TFA in the mobile phase used for HPLC purification. Then we used a mobile phase containing 0.1% formic acid, similar to the one used for LCMS analysis and even then we were unable to find the desired product in the fractions collected after HPLC purification. This indicated that the target compound was unstable under acidic conditions. The target compound was incubated in mobile phase containing 0.1% TFA and 0.1% formic acid separately, and the incubated mixtures were analysed using LCMS. It was observed that the target compound disappeared in 30 min and 1.5 h, in mobile phases containing 0.1% TFA and 0.1% formic acid respectively. Hence, we decided to use a neutral buffer like ammonium acetate in the mobile phase. When the target compound was incubated in a mobile phase containing 10 mM ammonium acetate, upon LCMS analysis it was observed that there was no significant change in the desired product even after 24 h. Hence, the mobile phase used for the final purification was, Solvent A, H_2O/10 mM ammonium acetate; solvent B, 80% ACN/H_2O/10 mM ammonium acetate. The gradient used was 27%-30% B in 15 min. The major peak now collected from HPLC corresponded to the desired target compound and it was reconfirmed using LCMS.

A. Chemical stability

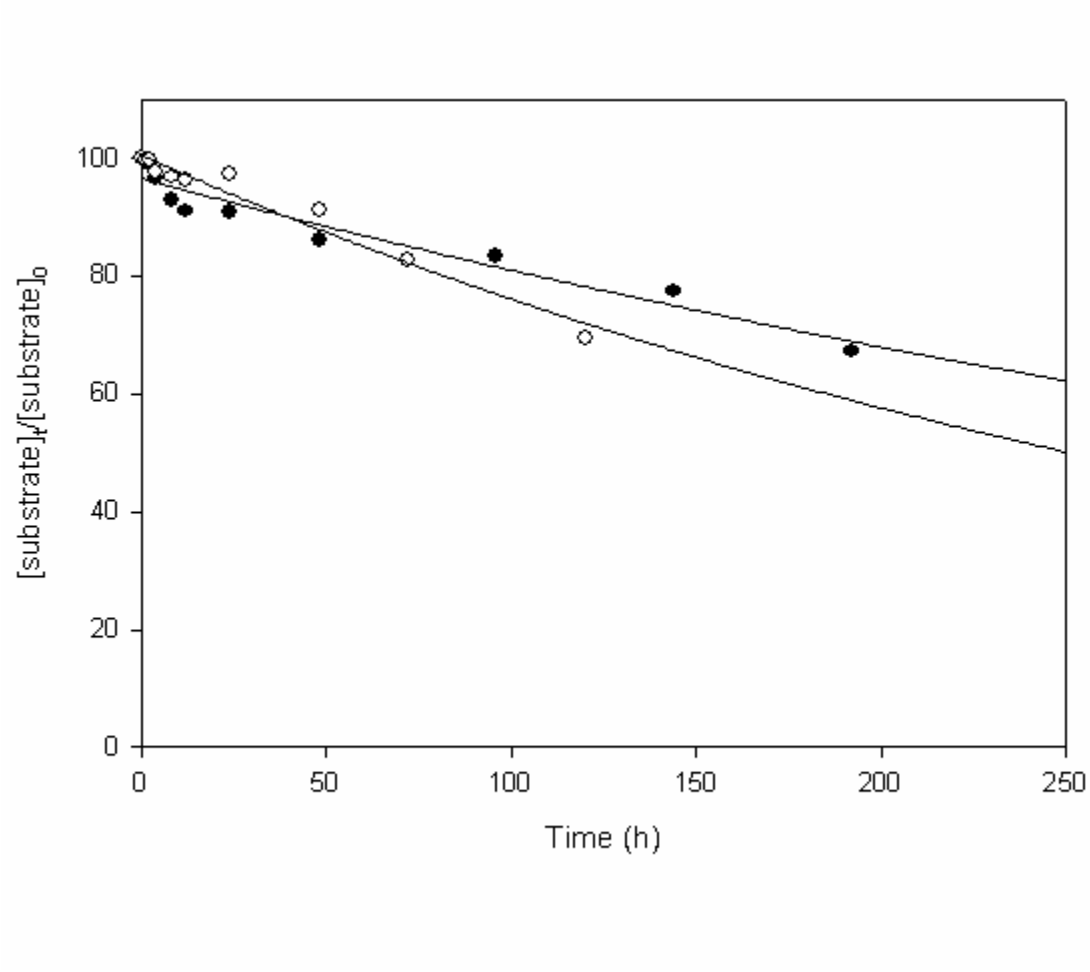


Figure 9. Stability of peptide conjugated pyridyl phosphoramide mustard **19** in Hank's buffer; pH 7.4 (●) and PSA assay buffer; pH 8.0 (o) at 37°C.

The stability of glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-pyridyl-2-methyl phosphoramide mustard (**19**) was determined under conditions employed for both cell culture assay and PSA enzyme assay. Cell culture assay is done in Hank's buffer at pH 7.4 and PSA enzyme assay is done in buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 0.1% Tween® 20 at pH 8.0. The substrate was incubated in hank's buffer and the PSA assay buffer separately at 37 °C. Aliquots were withdrawn at different time intervals and stability was determined by reverse phase HPLC analysis. Half-life was calculated based

on the disappearance of the substrate. It was observed that the target compound **19** was stable in both hank's buffer (pH 7.4) and PSA assay buffer (pH 8.0) with a half life > 5 days (Figure 9).

B. Substrate activity for PSA

The peptide conjugate, glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-pyridyl-2-methyl phosphoramide mustard (**19**) was evaluated as a substrate for PSA at an enzyme/substrate molar ratio of 1/100. Substrate stock solution was added to PSA buffer solution and the reaction was initiated by adding the above solution to PSA. Aliquots were withdrawn at various time intervals and quenched with acetonitrile (20%) and stored frozen prior to HPLC analysis. The half life was calculated based on the disappearance of the substrate. The peptide doxorubicin conjugate, glutaryl-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox (**42c**) of known substrate activity for PSA was used as a control.[95]

As shown in Figure 10, the half-life of the target compound **19** was found to be 47 min and that of doxorubicin conjugate **42c** was 20 min. From the data obtained it was evident that the target compound was cleaved by PSA. However, the substrate concentration did not reach zero upon hydrolysis of the target compound. This may be due to inactivation of PSA enzyme upon release of the activated alkylating agent.

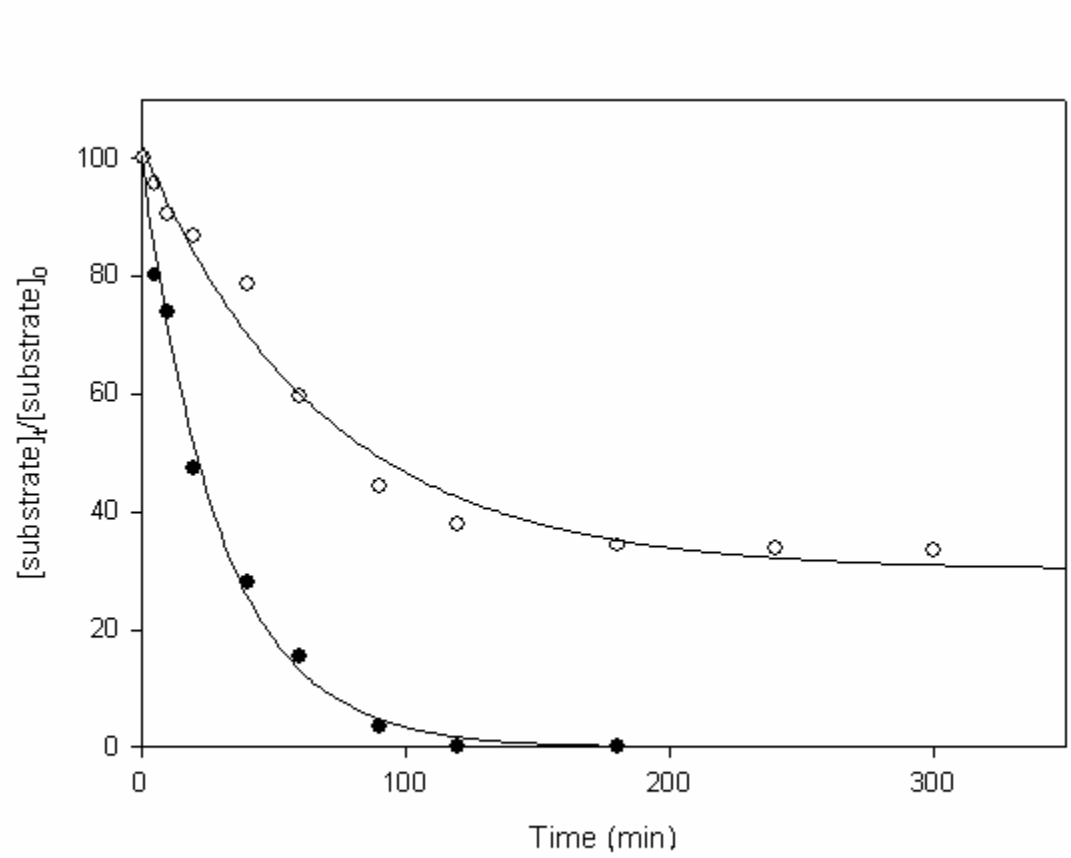


Figure 10. The disappearance of peptide doxorubicin conjugate (●) and peptide pyridyl phosphoramide mustard conjugate (o) during cleavage by PSA. The substrates were incubated with PSA by an enzyme/substrate molar ratio of 1/100 in 50 mM Tris/HCl buffer, 10 mM $CaCl_2$, 0.1% Tween-20, pH 8.0, 37 °C.

C. *In vitro* antiproliferative activity in prostate cancer cell lines

The antiproliferative activity of glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-pyridyl-2-methyl phosphoramide mustard (**19**) was investigated by a standard MTT assay using an androgen-sensitive PSA–producing human prostate cancer cell line (LNCaP) and an androgen-insensitive human prostate cancer cell line (DU 145) that does not produce PSA. Cells were split at 80% confluence following trypsinization and subcultured at 1:6 and medium changed every 72 h. The harvested cells were washed twice with HBSS (Hank's buffered saline solution) and resuspended in fresh serum-free culture medium containing 2% v/v TCM and plated into 96-well microtiter plates. A serial dilution of target compound was prepared and was added to the cells after over night incubation. Medium alone was used a as negative control. The compound was evaluated thrice and MTT assay was performed 3 days after the addition of target compound. The absorbance at 570 nm (reference 650 nm) was read using a Dynatech MR5000 microtiter plate reader. Wells containing medium alone were used as blanks. The IC_{50} inhibition concentration was calculated for both cell lines. The MTT assay was performed at least twice. The peptide doxorubicin conjugate, glutaryl-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox (**42c**) of known antiproliferative activity for PSA was used as a control. As recorded in Table 4, the average IC_{50} against LNCaP cells was 209 μM and against DU 145 was 204 μM and there was no selectivity towards PSA expressing LNCaP cells. From the PSA assay result it is known that the peptide conjugate **19** is a good substrate of PSA. In spite of being a good substrate for PSA, the cytotoxicity against LNCaP cells was low. This may be due to the inability of the released species to penetrate the tumor cells. After PSA

cleavage the nitrogen of the pyridine ring might be positively charged which might have prevented the alkylating agent from penetrating the cell membrane of the tumor cells. Further investigations are underway to identify the species released and to design approaches to improve its membrane permeability.

Table 4. Antiproliferative assay of glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-pyridyl-2-methyl phosphoramide mustard **(19)**

Compound		IC50 (μM)[a]		Ratio[b]
		LNCaP	DU 145	(DU 145/LNCaP)
42c		10 ± 2	961 ± 82	96
19	Assay 1	177 ± 3	218 ± 4	1
19	Assay 2	240 ± 5	190 ± 1	0.8
19	Average	209 ± 45	204 ± 20	1

[a] IC_{50} values are the concentration required to reduce the cell number to 50% of the control.
[b]Ratio of IC_{50} values as an indication of activation by PSA.

SUMMARY

We were successful in our efforts to synthesize and evaluate phosphoramide mustard analogues incorporating two types of tumor-specific activation mechanisms; one is the reductive activation mechanism by *Escherichia coli* nitroreductase and second is the proteolytic activation mechanism by prostate-specific antigen (PSA) in prostate cancer cells. We were trying to achieve a good balance between the stability of the compound prior to activation and its membrane permeability/drug release/activation kinetics after enzyme catalysis or bioreduction. The 5-nitropyridyl-2-methyl phosphoramide mustard (**18**) synthesized showed an IC_{50} value of 9 nM and the compound had 8300-fold selectivity towards nitroreductase-expressing cells. These results suggest that 5-nitropyridyl-2-methyl phosphoramide mustard (**18**) is a good candidate for use in combination with nitroreductase for enzyme prodrug therapy. In our research we investigated the effect of linking a substrate peptide sequence to an anticancer drug through a flexible linker that can be modified to better accommodate the unique structural requirements for binding to and catalysis by the target PSA enzyme and thus improve the tumor selectivity of phosphoramide mustard. Pyridine was introduced to modulate the electron-density, to optimize the stability and rate of 1,6-elimination upon proteolytic cleavage. A series of steps were involved in the synthesis of the peptide conjugated phosphoramide mustard analogue. We developed a new set of conditions for the selenocarboxylate/azide amidation reaction required for the synthesis of the peptide conjugated phosphoramide mustard. The new conditions present an attractive, alternative method to the conventional nucleophilic acylation when an amide bond needs to be

formed without going through an amine intermediate. The half-life of the selenocarboxylate was increased by 27-fold at room temperature. Excellent yields were obtained with electron-deficient azides and much improved yields were obtained with electron-rich azides upon mild heating. Thus, these new conditions represent a significant improvement over the earlier conditions used to couple selenocarboxylates with azides. The glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-pyridyl-2-methyl phosphoramide mustard (**19**) was synthesized and evaluated for chemical stability, substrate activity towards PSA and antiproliferative activity. The results showed that the peptide conjugate was stable at pH 7.4 and 8.0 with half life >5 days. It is a good substrate for PSA with a half life of 47 min at an enzyme/substrate molar ratio of 1/100. In spite of having good substrate activity towards PSA the peptide conjugate exhibited low cytotoxicity with an IC_{50} value of 209 μM and was not selective towards PSA expressing LNCaP cell lines. The low cytotoxicity and lack of selectivity towards LNCaP cells might be due to the inability of the activated drug to penetrate the cell membranes. Further studies are underway to improve the cytotoxicity and selectivity of the analogue.

EXPERIMENTAL

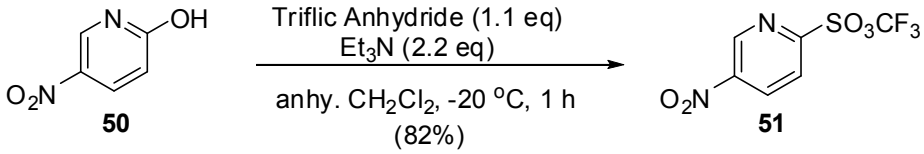
General methods

Moisture sensitive reactions were performed in oven-dried glassware under a positive pressure of argon or nitrogen. Air and moisture sensitive materials were transferred by means of a cannula or syringe under an argon or nitrogen atmosphere. Solvents used were either ACS reagent grade or HPLC grade. Tetrahydrofuran was dried over sodium/benzophenone. N, N-dimethyl formamide was dried over 4 Å molecular sieves for at least one week prior to use or used new (Aldrich). Dichloromethane was dried using Solvtec system. Unless otherwise stated, all reactions were stirred using a magnetic stirrer and monitored by thin-layer chromatography (TLC) using 0.25 mm Whatmann precoated silica gel plates and LC-MS. Flash column chromatography was performed using silica gel (Merck 230-400 mesh) manually or using Teledyne ISCO CombiFlash Companion Automated Flash Chromatographic System with prepacked silica gel columns. Unless otherwise noted, yield refers to chromatographically and spectroscopically (^{1}H NMR) homogenous material. All reagents purchased were either ACS grade or better and used without any further purification. n-BuLi was used after standardization using diphenylacetic acid. Melting points were determined on a Mel-Temp capillary apparatus and are uncorrected. Infrared spectra were recorded with Thermo-Nicolet Avatar 360 FTIR spectrometer and the absorbance is reported in reciprocal centimeters (cm^{-1}). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 spectrometer at ambient temperature and calibrated using residual undeuterated solvents as an internal reference. Chemical shifts (200 MHz for ^{1}H and 50 MHz for ^{13}C) are reported in parts per million. Coupling constants (*J* values) are given in

hertz (Hz). The following abbreviations were used to explain the multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad.

Analytical LC-MS data and kinetic study data were obtained using Shimadzu LCMS-2010 system equipped with a Chromolith speed ROD C_{18} reversed phase column (5 μm, 4.6 x 50 mm). Solvent A was 0.1% HCOOH/H_2O, Solvent B was 0.1% HCOOH/CH_3CN, and the gradient was 10-90% B in 10 min at a flow rate of 1 mL/min. HPLC analysis was performed on HP1090 HPLC system or Gilson HPLC system using the following columns: (1) System I: HP1090 system equipped with Waters symmetry C_{18} column (3.5 μm, 4.6 x 150 mm); (2) System II: HP1090 system equipped with Phenomenex C_{18} column (10 μm, 10 x 250 mm); (3) System III: Gilson HPLC system equipped with Phenomenex C_{18} column (10 μm, 10 x 250 mm).

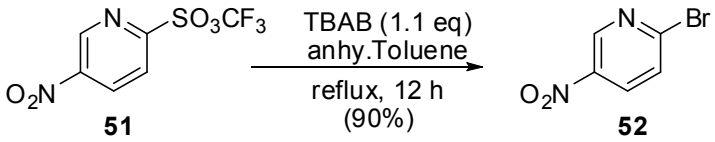
2-(Trifluoromethanesulfonyl)oxy-3-nitropyridine (51)



2-Hydroxy-5-nitropyridine (10 g, 71.4 mmol) and triethyl amine (21.8 mL, 157 mmol) were dissolved in anhy. CH_2Cl_2 (100 mL) and cooled to –20 °C. Then triflic anhydride (13.2 mL, 78.5 mmol) was added slowly and the reaction mixture was stirred for 1 h at –20 °C until the starting material disappeared. The product was extracted CH_2Cl_2 (75 mL x 3). Organic fractions were washed with water, brine, dried over sodium sulfate and concentrated under vacuum. Product was purified by flash column chromatography (0-100% gradient of ETOAc/hexane). The product was a pale yellow solid (15 g, 82%); mp

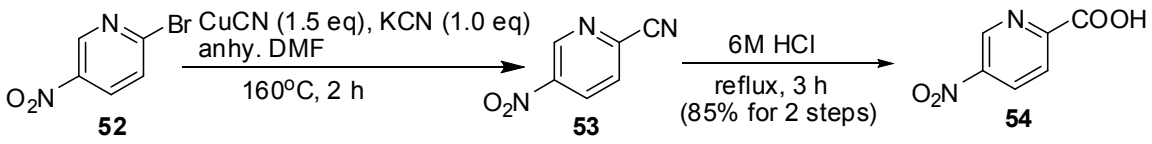
79-82 ^{o}C (lit. mp 82-83 ^{o}C)[106]; IR (KBr, cm^{-1}): 1535, 1355; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 9.23 (d, 1H, J = 2.8 Hz), 8.68 (dd, 1H, J = 2.6, 9 Hz), 7.37 (d, 1H, J = 8.8 Hz); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 158.3, 145.0, 144.1, 136.5, 128.1, 121.7, 115.5, 115.4, 109.0.

2-Bromo-5-nitropyridine (52)



TBAB (16.4 g, 51 mmol) and 2-(trifluoromethanesulfonyl)oxy-3-nitropyridine (12.5 g, 46.1 mmol) were dissolved in anhy. toluene (10 mL) and the mixture was refluxed overnight (12 h) under nitrogen. Then toluene was removed under reduced pressure and the residue was dissolved in ethyl acetate (50 mL). Organic fractions were washed with water, brine, dried over sodium sulfate and concentrated under vacuum. Product was purified by flash column chromatography (0-100% gradient of ETOAc/hexane). The product was a white solid (8.2 g, 90%); mp 137-139 ^{o}C (lit. mp 139-141 ^{o}C)[106]; IR (KBr, cm^{-1}): 1511, 1354; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 9.19 (d, 1H, J = 2.4 Hz), 8.31 (dd, 1H, J = 2.6, 8.5 Hz), 7.70 (d, 1H, J = 8.8 Hz); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 148.3, 145.6, 143.8, 133.0, 128.7.

5-Nitropicolinic acid (54)

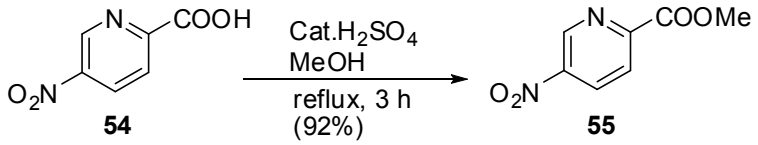


A suspension of CuCN (5.3 g, 60 mmol) and KCN (2.6 g, 40 mmol) in anhydrous DMF (50 mL) was heated to 160 °C. A solution of 2-bromo-5-nitropyridine (8 g, 40 mmol) in anhydrous DMF (40 mL) was added to the above suspension and the reaction mixture was stirred under nitrogen for 2 h at 160 °C until the starting material disappeared. The reaction mixture was cooled to room temperature and diluted with ethyl acetate (100 mL). It was then added to $NaHPO_4$ buffer (165 mL) and stirred for 20 min. The brown solid was filtered off and the filtrate was extracted with ethyl acetate (50 mL x 3). Organic fractions were washed with water, brine, dried over sodium sulfate and concentrated under vacuum. The light yellow solid product **53** was sufficiently pure and was directly used for the next step. mp 48-49 °C (lit. mp 51-52 °C)[107]; IR (KBr, cm^{-1}): 1524, 1354; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 9.48 (dd, 1H, J = 0.8, 2.6 Hz), 8.65 (dd, 1H, J = 2.6, 8.8 Hz), 7.96 (dd, 1H, J = 0.8, 8.4 Hz); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 146.1, 145.0, 138.2, 132.4, 128.8, 115.5.

2-Cyano-5-nitropyridine was suspended in 6 M HCl (50 mL) and stirred at reflux temperature for 3 h until the starting material disappeared. The reaction mixture was cooled to room temperature and stirred in an ice bath for 15 min when the product precipitated as a light brown solid. The precipitate was washed with cold water and dried under vacuum to give **54** (5.6 g, 85%); mp 204-206 °C (lit. mp 208-210 °C)[112]; IR (KBr, cm^{-1}): 1524, 1356; ^{1}H NMR (DMSO-d_6, 200 MHz): δ 9.42 (d, 1H, J = 2.2 Hz), 8.72 (dd,

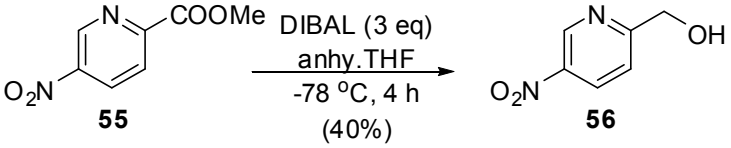
1H, J = 2.6, 8.6 Hz), 8.25 (d, 1H, J = 8.8 Hz); ^{13}C NMR (DMSO-d_6, 50 MHz): δ 164.7, 152.7, 145.6, 144.6, 133.0, 125.3; LC-MS (ESI$^-$): 166.9 $[M-H]^-$.

Methyl 5-nitropicolinate (55)



The starting material, 5-nitropicolinic acid (5 g, 30 mmol) was dissolved in methanol (50 mL) and catalytic amount of H_2SO_4 was added and the mixture was refluxed for 3 h. Then the reaction mixture was cooled and methanol was removed under reduced pressure. Residue was extracted with ethyl acetate (50 mL x 3), washed with water, brine, dried over sodium sulfate and concentrated under vacuum to yield **55** as a yellow solid (5 g, 92%); mp 152-154 ^{o}C (lit. mp 150-153 ^{o}C)[112]; IR (KBr, cm^{-1}): 1740, 1522, 1354; ^{1}H NMR (DMSO-d_6, 200 MHz): δ 9.44 (d, 1H, J = 2.6 Hz), 8.75 (dd, 1H, J = 2.6, 8.4 Hz), 8.28 (d, 1H, J = 8.8 Hz) 3.93 (s, 3H); ^{13}C NMR (DMSO-d_6, 50 MHz): δ 163.7, 151.4, 145.7, 144.7, 133.1, 125.4; LC-MS (ESI$^+$): 183.1 $[M+H]^+$.

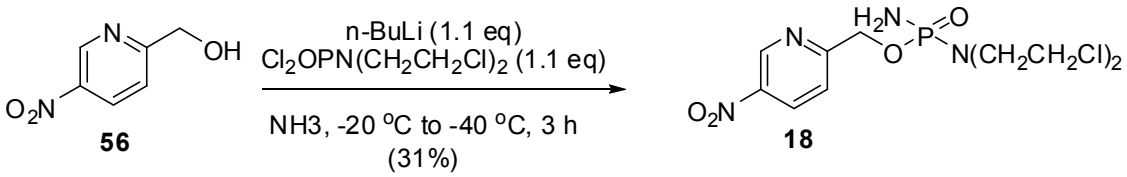
(5-Nitropyridin-2yl)methanol (56)



DIBAL (583 µL, 3.27 mmol) was added to methyl 5-nitropicolinate (200 mg, 1.09 mmol) drop wise at –78 ^{o}C and the mixture was stirred for 4 h at –78 ^{o}C. The reaction was quenched with 1 N HCl (20 mL) and diluted with methylene chloride (50 mL). The

organic phase was washed with water and brine and dried over sodium sulfate and concentrated under vacuum. The product was purified by flash column chromatography (hexane/ ETOAc, 3:1). The product obtained was a white solid (65 mg, 40%); mp 98-99 °C; IR (KBr, cm^{-1}): 3380, 1524, 1353; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 9.38 (brs, 1H), 8.48 (dd, 1H, J = 2.6, 8.4 Hz), 7.53 (d, 1H, J = 8.4 Hz), 4.90 (s, 2H), 3.28 (bs, 1H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 165.0, 143.5, 130.9, 119.6, 63.6; LC-MS (ESI^+): 155.0 $[M+H]^+$.

5-Nitropyridyl-2-methyl phosphoramide mustard (18)



n-BuLi (175 µL, 0.35 mmol) was added to (5-nitropyridin-2yl) methanol (50 mg, 0.32 mmol) dissolved in anhydrous THF (10 mL) at –40 °C and stirred for 30 min under nitrogen. To the reaction mixture was added Bis(2-chloroethyl)phosphoramidic dichloride (91 mg, 0.35 mmol) and the mixture was stirred at –40 °C for 3 h. Then ammonia gas was bubbled into the reaction mixture for 15 min. Solvent was removed via a rotovap and product was extracted with CH_2Cl_2 (50 mL x 3). Organic fractions were washed with water, brine, dried over sodium sulfate and concentrated under vacuum. Residue was subjected to flash column chromatography (CH_2Cl_2/CH_3OH, 9:1) to give **18** a brown semisolid (35 mg, 32%); ^{1}H NMR ($CDCl_3$, 200 MHz): δ 9.35 (d, 1H, J = 2.2), 8.48 (dd, 1H, J = 2.2, 8.4 Hz), 7.64 (d, 1H, J = 8.8 Hz), 5.08-5.33 (m, 2H), 3.54-3.67 (m, 4H), 3.43-3.51 (m, 4H), 3.20 (bs, 2H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 162.9 (d, J = 8.0),

144.7, 143.5, 132.0, 121.2, 66.7 (d, J = 4.2), 49.0 (d, J = 4.6), 42.4; LC-MS (ESI^+): 357.1 $[M+H]^+$, 379.1 $[M+H+ Na]^+$. HRMS (FAB) *m/z* calc'd for $C_{10}H_{15}Cl_2N_4O_4P$ $[M+H]^+$ 357.0286, found 357.0281.

General procedure for the preparation of amides:

Diacyldiselenide (0.24 mmol) and azide (0.2 mmol) were dissolved in deaerated acetonitrile (5 mL) and stirred at room temperature under an argon atmosphere. To this was added DIEA (0.24 mmol), followed by piperidine (0.24 mmol). The mixture was stirred at room temperature or heated at 55 °C depending on the azide. The reaction was monitored using TLC and LCMS. Upon completion, the mixture was concentrated and dissolved in ethyl acetate and filtered through a celite pad to remove selenium powder. The ethyl acetate phase was washed with aqueous saturated $NaHCO_3$, water and concentrated to dryness. The crude product was then purified by silica gel flash column chromatography.

***N*-(4-Nitrophenyl)benzamide (63)**

The product (46 mg) was obtained after flash column chromatography (CH_2Cl_2/MeOH, 0.05:10) in 95% isolated yield. mp 196-198 °C; IR (KBr, cm^{-1}): 3337, 1656, 1507, 1345; ^1H NMR (DMSO-d_6, 200 MHz): δ 10.81 (s, -NH), 8.28 (d, 2H, J = 7.4 Hz), 8.08 (d, 2H, J = 8.4 Hz), 7.99 (d, 2H, J = 8.4 Hz), 7.55-7.64 (m, 3H); ^{13}C NMR (DMSO-d_6, 50 MHz): δ 166.1, 145.4, 142.4, 134.1, 132.1, 128.4, 127.8, 124.7, 119.8; LC-MS (ESI^+): 243.1 $[M+H]^+$, 284.1 $[M+H+ CH_3CN]^+$.

***N*-(2-Nitrophenyl)benzamide (79)**

The product (45 mg) was obtained after flash column chromatography (Hexane/EtOAc, 5:1) in 93% isolated yield. mp 96-97 °C; IR (KBr, cm^{-1}): 3363, 1685, 1502, 1342; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 11.34 (bs, NH), 9.00 (dd, 1H, J = 1.4 Hz, J = 8.6 Hz), 8.27 (dd, 1H, J = 1.4, 8.5 Hz), (m, 2H), 7.48-7.75 (m, 4H), 7.17-7.25 (m, 1H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 164.9, 135.3, 134.5, 133.3, 131.8, 128.2, 126.5, 125.0, 122.4, 121.3; LC-MS (ESI^+): 243.1 $[M+H]^+$, 284.1 $[M+H+ CH_3CN]^+$.

***N*-(4-Chlorophenyl)benzamide (80)**

The product (44 mg) was obtained after flash column chromatography (Hexane/EtOAc, 2:1) in 95% isolated yield. mp 190-191 °C; IR (KBr, cm^{-1}): 3337, 1638; ^{1}H NMR (acetone-d_6, 200 MHz): δ 7.98 (dd, 2H, J = 1.4, 8.0 Hz), 7.86-7.90 (m, 2H), 7.50-7.54 (m, 3H) 7.37 (dd, 2H, J = 1.8, 6.8 Hz); ^{13}C NMR (acetone-d_6, 50 MHz): δ 166.3, 139.3, 139.2, 136.0, 132.5, 129.4, 129.3, 128.9, 122.5, 122.4; LC-MS (ESI^+): 232.0 $[M+H]^+$.

***N*-(4-Cyanophenyl)benzamide (81)**

The product (43 mg) was obtained after flash column chromatography (CH_2Cl_2/MeOH, 0.05:10) in 96% isolated yield. mp 167-168 °C; IR (KBr, cm^{-1}): 3352, 2228, 1661; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 8.14 (s, 1H, -NH), 7.76-7.87 (m, 4H), 7.48-7.76 (m, 5H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 165.0, 141.1, 133.3, 132.5, 131.6, 128.1, 126.3, 119.1, 117.8, 106.4; LC-MS (ESI^+): 223.1 $[M+H]^+$, 257.1 $[M+H+ CH_3CN]^+$.

Methyl-4-benzamidobenzoate (82)

The product (45 mg) was obtained after flash column chromatography (Hexane/EtOAc, 1:1) in 87% isolated yield. mp 162-163 °C; IR (KBr, cm^{-1}): 3378, 1716, 1670; ^1H NMR ($CDCl_3$, 200 MHz): δ 8.03 (dd, 2H, J = 1.8, 7.0 Hz), 7.87 (dd, 2H, J = 1.4, 4.9 Hz), 7.72 (dd, 2H, J = 2, 7.0 Hz), 7.47-7.70 (m, 3H), 3.89 (s, 3H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 166.6, 165.8, 142.1, 134.6, 132.1, 130.9, 128.9, 127.1, 125.9, 119.2, 52.0; LC-MS (ESI^+): 256.0 $[M+H]^+$, 297.0 $[M+H+ CH_3CN]^+$.

4-Benzamidobenzoic acid (83)

The product (43 mg) was obtained after flash column chromatography (Hexane/EtOAc, 1:1) in 89% isolated yield. mp 285-286 °C; IR (KBr, cm^{-1}): 3317, 1678; ^1H NMR (DMSO-d_6, 200 MHz): δ 10.52 (s, -COOH), 7.92 (brs, 6H), 7.53-7.57 (brm, 3H); ^{13}C NMR (DMSO-d_6, 50 MHz): δ 167.0, 165.9, 143.1, 134.6, 131.7, 130.1, 128.3, 127.7, 125.6, 119.4; LC-MS (ESI^-): 240.1$[M-H]^-$, 258.1 $[M-H+H_2O]^-$.

***N*-(4-Toluenesulfonyl)benzamide (84)**

The product (53 mg) was obtained after flash column chromatography (Hexane/Acetone, 1:3) in 96% isolated yield. mp 115-116 °C; IR (KBr, cm^{-1}): 3309, 1709; ^1H NMR ($CDCl_3$, 200 MHz): δ 9.52 (brs, -NH), 8.05 (d, 2H, J = 8.4 Hz), 7.83 (d, 2H, J = 7.4 Hz), 7.32-7.58 (m, 5H), 2.43 (s, 3H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 164.4, 145.2, 135.4, 133.4, 131.1, 129.68, 128.8, 128.7, 127.9, 21.7; LC-MS (ESI^-): 274.1 $[M-H]^-$.

Diphenyl *N*-benzoylphosphoramidate (85)

The product (62 mg) was obtained after flash column chromatography (Hexane/Acetone, 4:1) in 88% isolated yield. mp 125-126 °C; IR (KBr, cm^{-1}): 3139, 1693; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 9.57 (brs, -NH), 7.98 (d, 2H, J = 8.6 Hz), 7.56 (t, 1H, J = 7.8 Hz), 7.39 (dd, 2H, J = 7.6, 8.0 Hz), 7.1-7.43 (m, 10H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 167.5 (d, J = 3.4 Hz), 150.0 (d, J = 6.8 Hz), 133.0, 132.1 (d, J = 11 Hz), 129.7, 128.5, 128.4, 125.6, 125.5, 120.5, 120.4; LC-MS (ESI^+): 354.1 $[M+H]^+$, 417.2 $[M+H+Na+CH_3CN]^+$.

N-benzoyl benzamide (86)

The product (12 mg) was obtained after flash column chromatography (Hexane/EtOAc, 2:1) in 51% isolated yield. mp 144-145 °C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 9.26 (brs, -NH), 7.84 (dd, 4H, J = 1.4, 8.0 Hz), δ 7.40-7.60 (m, 6H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 166.7, 133.3, 133.0, 128.7, 127.9; LC- MS (ESI^+): 226.1 $[M+H]^+$, 289.1 $[M+H+Na+CH_3CN]^+$.

N-phenylbenzamide (87)

The product (32 mg) was obtained after flash column chromatography (Hexane/EtOAc, 10:3) in 81% isolated yield. mp 161-162 °C; IR (KBr, cm^{-1}): 3343, 1655; ^{1}H NMR (CD_3OD, 200 MHz): δ 7.84 (dd, 2H, J = 1.8, 4.0 Hz), 7.62 (dd, 2H, J = 1.6, 5 Hz), 7.38-7.61 (m, 4H), 7.22-7.33 (m, 2H), 7.03-7.11 (m, 1H); ^{13}C NMR (CD_3OD, 50 MHz): δ 168.9, 139.9, 136.3, 132.8, 129.8, 129.6, 128.6, 125.6, 122.4; LC-MS (ESI^+): 198.0 $[M+H]^+$, 239.0 $[M+H+CH_3CN]^+$.

***N*-(4-Methoxyphenyl)benzamide (88)**

The product (30 mg) was obtained after flash column chromatography (Hexane/EtOAc, 2:1) in 65% isolated yield. mp 152-154 °C; IR (KBr, cm^{-1}): 3330, 1647; ^1H NMR (acetone-d_6, 200 MHz): δ 9.40 (brs, NH), 7.97 (dd, 2H, J = 1.4, 7.8 Hz), 7.72 (d, 2H, J = 8.6 Hz), 7.42-7.59 (m, 3H), 6.91 (dd, 2H, J = 2.4, 6.8 Hz), 3.78 (s, 3H, -OCH_3); ^{13}C NMR (acetone-d_6, 50 MHz): δ 165.7, 156.9, 136.3, 133.3, 131.9, 129.0, 125.0, 122.4, 114.4, 55.5; LC-MS (ESI^+): 228.1$[M+H]^+$, 269.1 $[M+H+CH_3CN]^+$.

***N*-(4-Aminophenyl)benzamide (89)**

The product (23 mg) was obtained after flash column chromatography (Hexane/EtOAc, 2:1) in 54% isolated yield. ^1H NMR (CD_3OD, 200 MHz): δ 7.89 (dd, 2H, J = 8.0 Hz, J = 1.8 Hz), 7.42-7.52 (m, 3H), 7.37 (dd, 2H, J = 2.0, 7.0 Hz), 6.73 (dd, 2H, J = 1.8, 8.0 Hz); ^{13}C NMR (CD_3OD, 50 MHz): δ 168.6, 145.9,136.3, 132.6, 130.4, 129.5, 128.4, 124.2, 116.6; LC-MS (ESI^+): 213.1 $[M+H]^+$, 254.1 $[M+H+CH_3CN]^+$.

***N*-(4-Hydroxymethylphenyl)benzamide (90)**

The product (26 mg) was obtained after flash column chromatography (Hexane/EtOAc, 2:1) in 56% isolated yield. mp 149-150 °C; ^1H NMR (CD_3OD, 200 MHz): δ 7.94 (dd, 2H, J = 2.0, 8.4 Hz), 7.67 (dd, 2H, J = 1.8, 7.8 Hz), 7.47-7.60 (m, 3H), 7.37 (d, 2H, J = 8.4 Hz), 4.61 (s, 2H, -C$\underline{H_2}$OH). ^{13}C NMR (CD_3OD, 50 MHz): δ 168.9, 139.0, 136.3, 132.9, 129.6, 128.6, 128.5, 122.2, 64.9; LC-MS (ESI^+): 228.1 $[M+H]^+$, 269.1 $[M+H+CH_3CN]^+$.

***N*-(3-Fluoro-4-Hydroxymethylphenyl)benzamide (91)**

The product (38 mg) was obtained after flash column chromatography (Hexane/EtOAc, 1:1) in 78% isolated yield. mp 120-121 °C; IR (KBr, cm^{-1}): 3298, 1654; ^{1}H NMR (CD_3OD, 200 MHz): δ 7.86 (dd, 2H, J = 1.6, 8 Hz), 7.36-7.63 (m, 6H); ^{13}C NMR (CD_3OD, 50 MHz): δ 167.5, 162.8, 158.0, 134.8, 131.6, 129.3, 128.5, 128.3, 127.3, 116.0 (d, J = 3.1 Hz), 107.5 (d, J = 27 Hz), 67.8; LC-MS (ESI^+): 246.0 $[M+H]^+$, 287.0 $[M+H+CH_3CN]^+$.

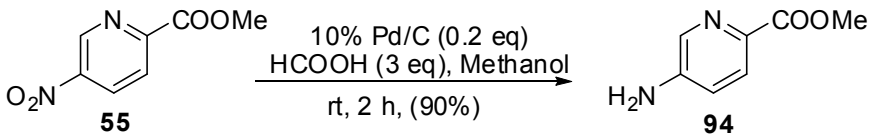
***N*-(2-Fluoro-4-Hydroxymethylphenyl)benzamide (92)**

The product (18 mg) was obtained after flash column chromatography (Hexane/EtOAc, 1:1) in 37% isolated yield. mp 108-110 °C; IR (KBr, cm^{-1}): 3327, 1647; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 8.42 (t, 1H), 8.04 (bs, -NH), 7.44-7.52 (m, 3H), 7.13-7.20 (m, 3H), 4.67 (s, 2H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 165.4, 155.2, 150.4, 137.8, 134.5, 132.2, 128.9, 127.1, 122.9 (d, J = 3.05 Hz), 121.8, 113.4 (d, J = 19.7), 64.4; LC-MS (ESI^+): 246.0 $[M+H]^+$, 287.0 $[M+H+CH_3CN]^+$.

4-Benzamidobenzyl acetate (93)

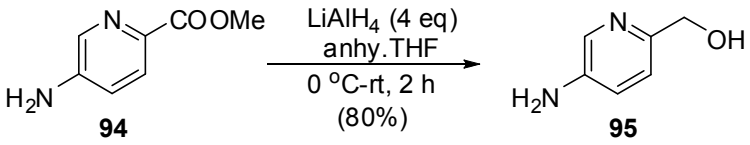
The product (37 mg) was obtained after flash column chromatography (Hexane/EtOAc, 1:1) in 68% isolated yield. mp 126-127 °C; IR (KBr, cm^{-1}): 3332, 1732, 1655; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.93 (brs, 1H, -NH), 7.67 (d, 2H, J = 8.4 Hz), 7.40-7.54 (m, 3H), 7.34 (d, 2H, J = 8.4 Hz), 5.06 (s, 2H), 2.07 (s, 3H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 171.0, 165.7, 138.0, 134.9, 132.1, 131.9, 129.3, 128.8, 127.0, 120.2, 65.9, 21.0; LC-MS (ESI^+): 270.1 $[M+H]^+$, 311.1 $[M+H+CH_3CN]^+$.

Methyl 5-aminopicolinate (94)



Methyl 5-nitropicolinate (5 g, 27.4 mmol) and 10% Pd/C (583 mg, 5.5 mmol) and formic acid (3 mL, 82.2 mmol) were suspended in methanol (500 mL) and the reaction mixture was flushed with 3 cycles of nitrogen followed by 3 cycles of hydrogen and stirred for 2 h at room temperature under hydrogen balloon. The mixture was filtered through celite and methanol was removed by a rotovap. The residue was subjected to flash column chromatography (10% CH_3OH/CH_2Cl_2) to give the product **94** as a white solid (3.7 g, 90%); mp 85-87 ^oC; IR (KBr, cm^{-1}): 3347, 1715; ^1H NMR ($CDCl_3$, 200 MHz): δ 8.14 (brs, 1H), 7.94 (d, 1H, J = 8.0 Hz), 6.92 (dd, 1H, J = 3.0, 8.4 Hz), 5.26 (brs, 2H), 3.90 (s, 3H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 165.7, 145.7, 137.3, 136.6, 126.5, 120.0, 52.3; LC-MS (ESI^+): 153.1 $[M+H]^+$.

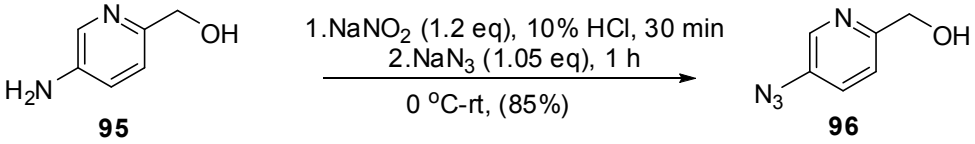
(5-Aminopyridin-2yl)methanol (95)



The starting material, methyl 5-aminopicolinate (3.4 g, 22.3 mmol) was dissolved in anhy. THF (100 mL) and cooled to 0 ^oC. Then $LiAlH_4$ (3.4 g, 89.2 mmol) was added and mixture stirred at room temperature for 2 h until the reaction was complete. Then 3.4 mL of water was added to the reaction mixture dropwise with stirring in an ice bath followed by the addition of 3.4 mL, 15% sodium hydroxide solution and 10.2 mL water. This

formed a granular precipitate of all the unreacted $LiAlH_4$ which was filtered through a celite pad using acetonitrile as the solvent. Then the solvent was rotovaped and product was purified by flash column chromatography using 10% $MeOH/CH_2Cl_2$ containing 0.1% NH_4OH as eluent. Product obtained was a brown solid (2.2 g, 80%); mp 170-172 ^oC; IR (KBr, cm^{-1}): 3319; ^1H NMR (DMSO-d_6, 200 MHz): δ 7.86 (d, 1H, J = 2.2 Hz), 7.05 (d, 1H, J = 8.4 Hz), 6.93 (dd, 1H, J = 2.6, 8.4 Hz), 5.16 (brs, 3H), 4.39 (s, 2H); ^{13}C NMR (DMSO-d_6, 50 MHz): δ 148.8, 143.3, 135.0, 120.9, 120.7, 64.2; LC-MS (ESI^+): 124.9 $[M+H]^+$.

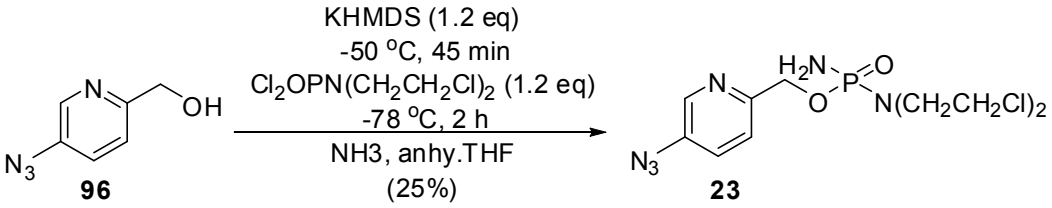
(5-Azidopyridin-2yl)methanol (96)



(5-Aminopyridin-2yl)methanol (1 g, 8 mmol) was dissolved in 10% HCl (76 mL) and cooled to 0 ^oC. Then sodium nitrite solution (663 mg, 9.6 mmol, 15 mL water) was added drop wise and stirred for 30 min at 0 ^oC. Then sodium azide solution (550 mg, 8.4 mmol, 18 mL water) was added drop wise at 0 ^oC and mixture stirred at room temperature for 1 h. The reaction mixture was basified with 5% sodium carbonate solution and extracted with ethyl acetate (50 mL x 3). Organic fractions were washed with water, brine, dried over sodium sulfate and concentrated under vacuum. Product was purified by flash column chromatography (10% $MeOH/CH_2Cl_2$). Product obtained was a brown oil (1 g, 85%); IR (KBr, cm^{-1}): 3240, 2115; ^1H NMR ($CDCl_3$, 200 MHz): δ 8.25 (d, 1H, J = 1.4 Hz), 7.28-7.36 (m, 2H), 4.70 (s, 2H), 3.43 (brs, 1H); ^{13}C NMR

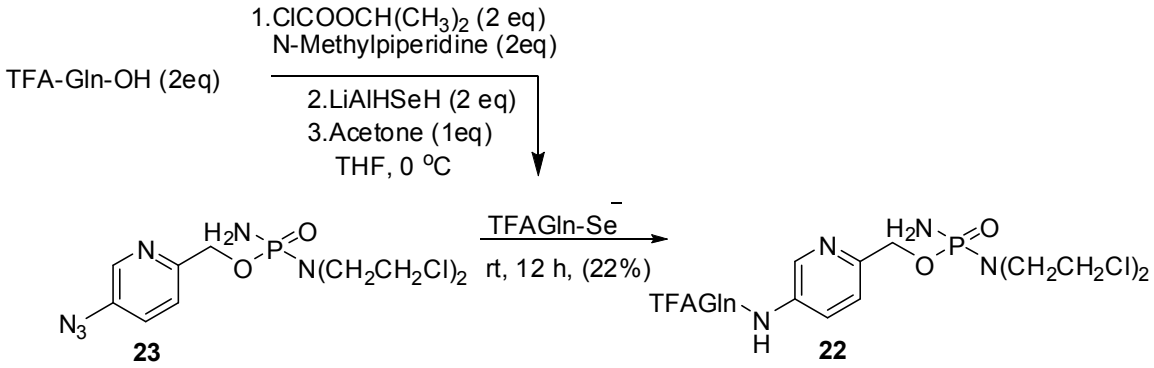
($CDCl_3$, 50 MHz): δ 155.9, 139.9, 136.0, 126.8, 121.3, 64.0; LC-MS (ESI^+): 151.0 $[M+H]^+$.

5-Azidopyridyl-2-methyl phosphoramide mustard (23)



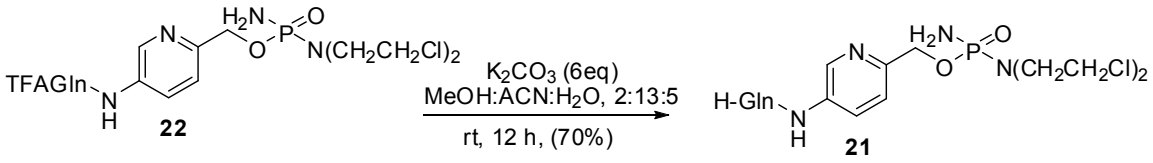
(5-Azidopyridin-2yl)methanol (1 g, 6.6 mmol) was dissolved in anhy. THF (30 mL) and cooled to –50 °C. Then KHMDS (1.67 g, 7.9 mmol) was added and stirred for 30 min at -50 °C. Then the mixture was cooled to -78 °C and bis(2-chloroethyl)phosphoramidic dichloride (2 g, 7.9 mmol) in 70 mL anhy. THF at -78 °C was added and mixture stirred for 2 h at -78 °C. Ammonia gas was bubbled into the reaction mixture for 15 min. THF was rotovaped and product was dissolved in CH_2Cl_2 (50 mL) and washed with water, brine, dried over sodium sulfate and concentrated under vacuum. Product was purified by flash column chromatography (0-100% ETOAc/hexane gradient) to yield a brown oil (580 mg, 25%); ^1H NMR ($CDCl_3$, 200 MHz): δ 8.30 (t, 1H), 7.37 (d, 2H, J = 2.2), 4.92-5.21 (m, 2H), 3.584-3.65 (m, 4H), 3.38-3.50 (m, 4H) 3.19 (bs, 2H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 152.6 (d, J = 6.9), 140.8, 136.7, 126.7, 122.7, 67.1 (d, J = 5.0), 49.3 (d, J = 4.6), 42.5 (d, J = 0.8); LC-MS (ESI^+): 353.1 $[M+H]^+$, 375.1 $[M+H+ Na]^+$. HRMS (FAB) *m/z* calc'd for $C_{10}H_{15}Cl_2N_6O_2P$ $[M+H]^+$ 353.0449, found 353.0444.

TFA-Gln-NH-pyridyl-2-phosphoramide mustard (22)



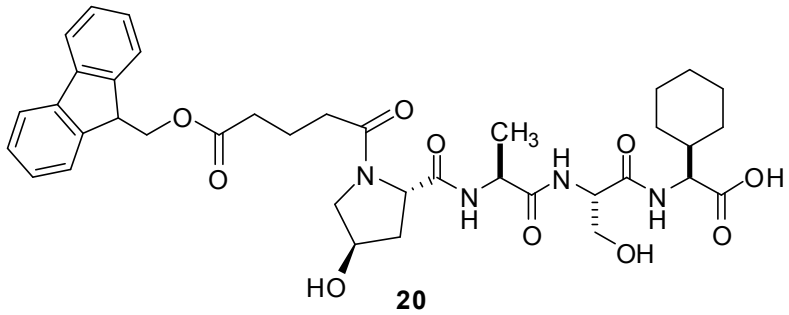
TFA-Gln-OH (275 mg, 1.1 mmol) was activated with isopropyl chloroformate (1.1 mL, 1.1 mmol) and N-methyl piperidine (139 µL, 1.1 mmol) at 0 °C in anhy. THF (10 mL). The selenating reagent was prepared by suspending $LiAlH_4$ (45 mg, 1.1 mmol) in anhy. THF (10 mL) to which selenium (91 mg, 1.1 mmol) was added and stirred for 20 min at 0 °C. The activated TFA-Gln-OH was cannulated into the selenating reagent to produce selenocarboxylate and stirred at 0 °C for 30 min. Then acetone (42 µL) was added and mixture stirred at room temperature for 20 min. Then 5-azidopyridyl-2-methyl phosphoramide mustard (200 mg, 0.57 mmol) was injected into the above reaction mixture and stirred overnight at room temperature. THF was rotovaped and product was purified by flash column chromatography (10% MeOH/ CH_2Cl_2) to give a light yellow solid (70 mg, 22%); ^{1}H NMR (CD_3OD, 200 MHz): δ 8.68 (d, 1H, J = 2.2 Hz), 8.05 (dd, 1H, J = 2.6, 8.4 Hz), 7.46 (d, 1H, J = 8.4Hz), 4.94 (d, 2H, J = 7.8 Hz), 4.47 (q, 1H), 3.54-3.61 (m, 4H), 3.32-3.41 (m, 4H), 2.29-2.40 (m, 2H), 2.04-2.18 (m, 2H); ^{13}C NMR (CD_3OD, 50 MHz): δ 177.4, 171.2, 159.5, 158.8, 153.1, 141.8, 136.2, 129.7, 123.3, 120.2, 114.5, 68.7, 68.0 (d, J = 4.6), 43.1, 32.7, 32.2, 28.1; LC-MS (ESI$^-$): 549.0 $[M-H]^-$.

H-Gln-NH-pyridyl-2-phosphoramide mustard (21)



TFA-Gln-NH-pyridyl-2-phosphoramide mustard (70 mg, 0.125 mmol) was dissolved in a 2:13:5 mixture of MeOH:ACN:H_2O (12 mL). Then 0.5 M potassium carbonate solution (3 mL) was added and the mixture was stirred at room temperature for overnight (12 h). The solvent was rotovaped and product purified by flash column chromatography (30% MeOH/DCM with 1% NH_4OH) to get a white solid (40 mg, 70%); ^{1}H NMR (CD_3OD, 200 MHz): δ 8.72 (brs, 1H), 8.07 (dd, 1H, J = 2.6, 8.6 Hz), 7.48 (d, 1H, J = 8.4 Hz), 4.94 (d, J = 7.6), 4.04 (t, 1H), 3.53-3.61 (m, 4H), 3.32-3.41 (m, 4H), 2.42 (t, 2H), 2.07-2.19 (m, 2H); ^{13}C NMR (CD_3OD, 50 MHz): δ 175.6, 152.3, 140.4, 134.4, 128.3, 122.0, 66.7, 53.5, 41.7, 30.4, 26.9; LC-MS (ESI^+): 455.2 $[M+H]^+$, 477.2 $[M+H+Na]^+$. HRMS (FAB) *m/z* calc'd for $C_{15}H_{25}Cl_2N_6O_4P$ $[M+H]^+$ 455.1130, found 455.1137.

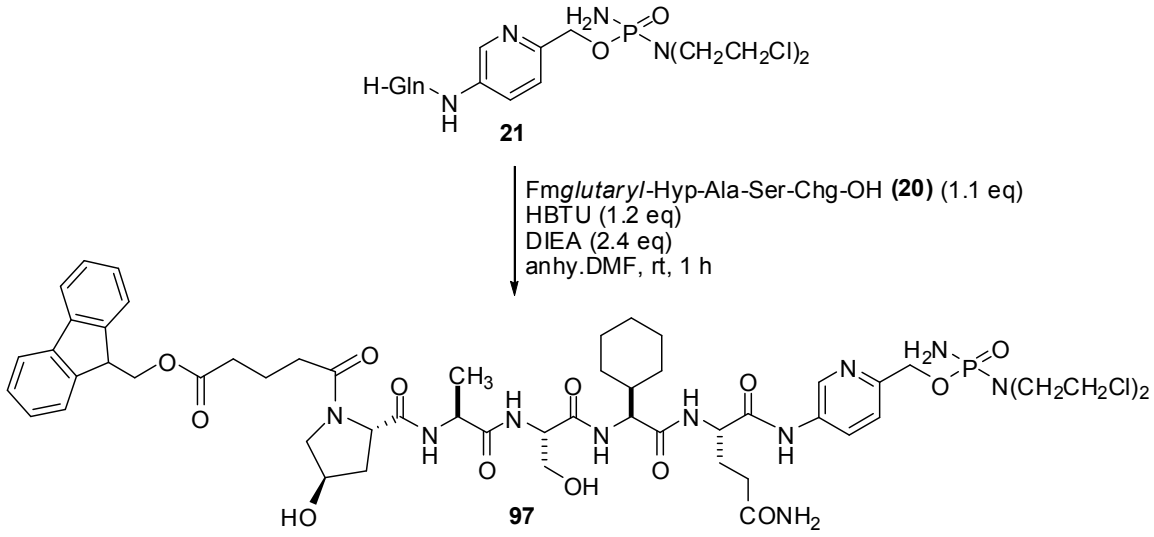
Fm-glutaryl-Hyp-Ala-Ser-Chg-OH (20)



The target peptide was synthesized using standard solid-phase peptide synthesis protocols using Advanced ChemTech-90 model peptide synthesizer. The resin used for the

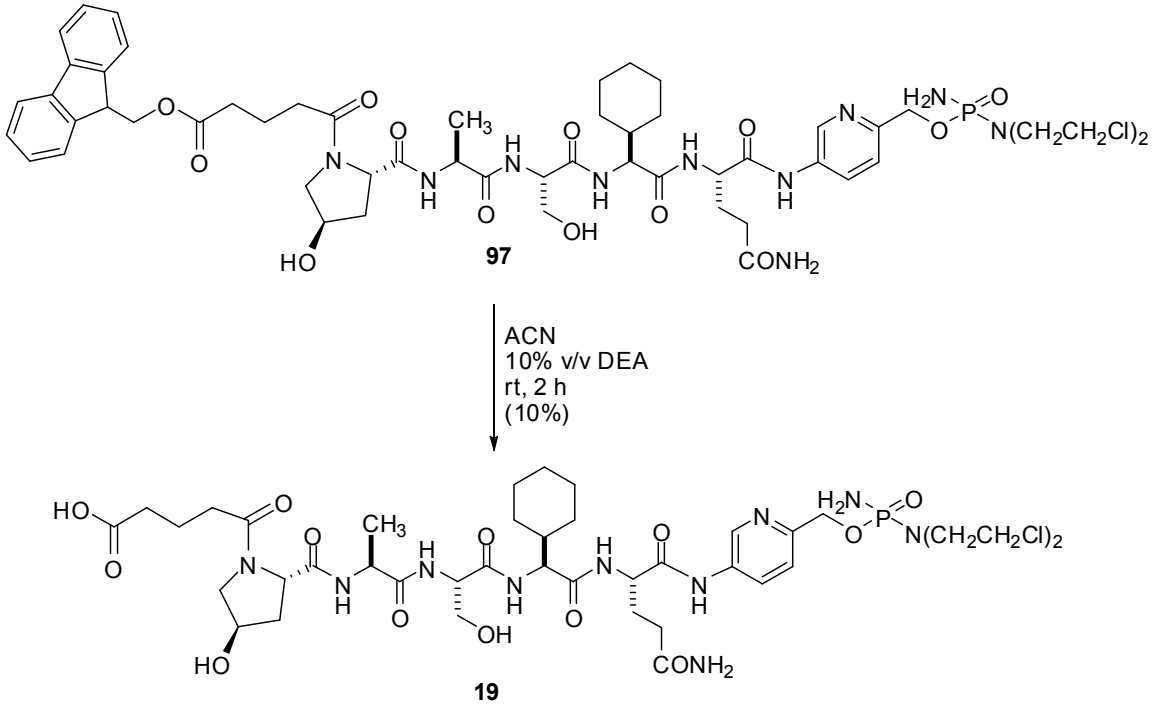
peptide assembly was 4-hydroxymethylphenoxy wang resin (971 mg, 0.78 mmol), purchased from Advanced ChemTech. N^{α}-Fmoc-protected amino acids of the L-configuration and reagents were purchased from Advanced ChemTech. C-Terminal amino acid was loaded onto wang resin using HOBt/DMAP/DIC protocol. The subsequent Fmoc amino acids were activated and coupled using HOBt/HBTU/DIEA, using 3-fold excess of activated protected amino acids and NMP as solvent. The side chain hydroxyl group of Ser was protected by t-butyl group and the secondary hydroxyl group in hydroxyproline (Hyp) was not protected. Following completion of the assembly on the resin support, the N-terminal Fmoc group was removed via the standard 20% piperidine/NMP protocol, followed by washing (using dichloromethane/isopropanol/NMP wash protocol). After each coupling and deprotection step, Kaiser test was performed to determine the completeness of the above processes. Deprotection and removal of the peptide from the resin support was effected by 95% TFA in CH_2Cl_2. The fluorenyl methyl ester (OFm) protecting group remained intact under these conditions. After removal of solvents under reduced pressure, the peptide was purified by preparative HPLC. Gilson HPLC system equipped with Phenomenex C_{18} column (10 μm, 10 x 250 mm). Solvent A, 0.1% TFA/H_2O, solvent B, 0.075% TFA/MeOH. A linear gradient of 30-90% B in 20 min and a flow rate of 12 mL/min were used to elute the peptide and the UV absorbance was monitored at both 220 nm and 280 nm. The fraction at 15 min was collected and lyophilized to dryness to give a white fluffy solid (130 mg, 46%); LC-MS (ESI^-) 719.3 $[M-H]^-$.

Glutaryl(OFm)-Hyp-Ala-Ser-Chg-Gln-NH-pyridyl-2-phosphoramide mustard (97)



The peptide Fm-glutaryl-Hyp-Ala-Ser-Chg-OH (47.5 mg, 0.07 mmol), HBTU (20 mg, 0.05 mmol) were dissolved in 400 µL NMP to which DIEA (18.46 µL, 0.1 mmol) was added and stirred at room temperature for 30 min. Then H-Gln-NH-pyridyl-2-phosphoramide mustard (20 mg, 0.04 mmol) in 400 µL NMP was added to the above mixture and stirred at room temperature for 1 h. Then cold 5% $NaHCO_3$ solution (5 mL) was added drop wise to the reaction mixture to induce precipitation of the product as a white solid. The precipitate was separated by centrifugation and washed with cold water (5 mL x 2) and directly used for the next step. LC-MS (ESI^+): 1157.0 $[M+H]^+$.

Glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-pyridyl-2-methyl phosphoramide mustard (19)



Glutary*l*(OFm)-Hyp-Ala-Ser-Chg-Gln-NH-pyridyl-2-phosphoramide mustard obtained in the previous step was dissolved in 500 µL acetonitrile to which 10% v/v DEA (50 µL) was added and mixture stirred at room temperature for 2 h. The solvent was rotovaped and residue was washed with ether (500 µL x 3). The product precipitate was collected by centrifugation and purified by reverse phase HPLC using HP1090 system equipped with Phenomenex C_{18} column (10 µm, 10 x 250 mm). Solvent A, H_2O/10 mM ammonium acetate; solvent B, 80% ACN/H_2O/10 mM ammonium acetate. The gradient used was 27%-30% B in 15 min. A flow rate of 3 mL/min was used and UV absorbance was monitored at 245 nm and 280 nm. The fraction at 13.2 min was collected and lyophilized to dryness to obtain a white fluffy solid (4 mg, 10%); LC-MS (ESI^-) 977.0 $[M-H]^-$. HRMS (FAB) *m/z* calc'd for $C_{39}H_{61}Cl_2N_{10}O_{13}P$ $[M+H]^+$ 979.3612, found 979.3519.

Stability assay of DIEA benzeneselenocarboxylate

To monitor the stability of DIEA benzeneselenocarboxylate, dibenzoyl diselenide was mixed with 1.2 eq of DIEA and 1.2 eq of piperidine at room temperature under an argon atmosphere. The DIEA benzeneselenocarboxylate formed was then allowed to stand at room temperature or at 55 °C and aliquots were combined with 2 eq of p-toluenesulfonyl azide. The amide product, N-(ptoluenesulfonyl) benzamide, was analyzed to determine the amount of DIEA benzeneselenocarboxylate that remained in solution. The analysis was done using Shimadzu LCMS-2010 system equipped with a Chromolith speed ROD C_{18} reversed phase column (5 μm, 4.6 x 50 mm). Solvent A was 0.1% HCOOH/H_2O, Solvent B was 0.1% HCOOH/CH_3CN, and the gradient was 10-90% B in 10 min, at a flow rate of 1 mL/min.

Stability test in PSA assay buffer and hank's buffer

The peptide conjugate **19** was dissolved in 10 μL DMSO to which 490 μL PSA buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 0.1% Tween® 20) at pH 8.0 or hank's buffer at pH 7.4 was added. The mixture was vortexed and incubated at 37 °C. Aliquots (20 μL) were withdrawn at different time intervals and stored frozen prior to HPLC analysis. HP1090 system equipped with Waters symmetry C_{18} column (3.5 μm, 4.6 x 150 mm) was used. Gradient of 10% - 50% acetonitrile containing 10 mM ammonium acetate buffer at a flow rate of 1 mL/min and a detection wavelength of 245 nm were used for analysis. The half-life was determined based on the disappearance of the peptide conjugate.

Prostate-specific antigen (PSA) assay

PSA was purchased from CALIBIOCHEM. The substrate was mixed with PSA at a molar ratio of 100 to 1 in PSA buffer. Substrate stock solution (5 μL, 10 mM in DMSO) was added to 295 μL PSA buffer solution (50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 0.1% Tween® 20) prewarmed to 37 °C. The reaction was initiated by adding 245 μL of the above solution to 5 μL PSA (100 μg, 2.45 mg/mL). Aliquots (20 μL) were withdrawn at various time intervals and quenched with 5 μL acetonitrile and stored frozen prior to HPLC analysis. The half life was calculated based on the disappearance of thepeptide conjugate.

***In vitro* antiproliferative assay of 5-nitropyridyl-2-methyl phosphoramide mustard**

V79 Chinese hamster lung fibroblasts used for the assay were grown in a monolayer culture in DMEM containing 10% FCS and 4 mM glutamine. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C and subcultured twice weekly by trypsinization. The cells were transfected with a bicistronic vector encoding for the *E.coli* nitroreductase and puromycin resistance protein as the selective marker. The positive clones were collected in growth medium containing 10 μg/mL puromycin and maintained under selective pressure. Cells expressing either *E.coli* nitroreductase (T116) or human quinone oxidoreductase (hDT7) in exponential phase of growth were trypsinized, seeded in 96-well plates at a density of 1000 cells/well, and permitted to recover for 24 h. F179 cells were tranfected with only the vector and were used as controls. Then the medium was replaced with fresh medium containing the co-substrate (100 μM). Serial dilutions of the test compound were prepared and then the cells were

incubated with the test compound at 37 °C for 72 h. The plates were fixed and stained with SRB before reading with optical absorption at 590 nm. Results were expressed as a percentage of control growth and IC_{50} values are the concentration required to reduce the cell number to 50% of the control and were obtained by interpolation.

***In vitro* antiproliferative activity assay in prostate cancer cell lines**

The antiproliferative activity of glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-pyridyl-2-methyl phosphoramide mustard was investigated using a standard MTT assay in an androgen-sensitive PSA–producing human prostate cancer cell line (LNCaP) and an androgen-insensitive human prostate cancer cell line (DU 145) that does not produce PSA. All cell lines were obtained from ATCC (American Type Culture Collection) and were maintained in RPMI 1640 (w/L-Glu and phenol red) supplemented with 10% FBS (Fetal Bovine Serum), with 100 units/mL penicillin G and 100 units/mL streptomycin sulfate. Cells were split at 80% confluence following trypsinization and subcultured at 1:6 and medium changed every 72 h. The harvested cells were washed twice with HBSS (Hank's buffered saline solution) and resuspended in fresh serum-free culture medium containing 2% v/v TCM and plated into 96-well microtiter plates (5,000 cells in 0.1 mL medium/well). A serial dilution (500 μM-10 nM) of target compound was prepared and added to the cells after over night incubation. Medium alone was used as negative control. The compound was evaluated in triplicate and MTT assay was performed 3 days after the addition of target compound. The absorbance at 570 nm (reference 650 nm) was read using a Dynatech MR5000 microtiter plate reader. Wells containing medium alone were used as blanks. The IC_{50} inhibition concentration was calculated for both cell lines.

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