Pin1 Inhibitors: Towards Understanding the Enzymatic Mechanism

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Abstract

An important role of Pin1 is to catalyze the cis-trans isomerization of pSer/Thr-Pro bonds; as such, it plays an important role in many cellular events through the effects of conformational change on the function of its biological substrates, including Cdc25, c-Jun, and p53. The expression of Pin1 correlates with cyclin D1 levels, which contributes to cancer cell transformation. Overexpression of Pin1 promotes tumor growth, while its inhibition causes tumor cell apoptosis. Because Pin1 is overexpressed in many human cancer tissues, including breast, prostate, and lung cancer tissues, it plays an important role in oncogenesis, making its study vital for the development of anti-cancer agents.

Many inhibitors have been discovered for Pin1, including 1) several classes of designed inhibitors such as alkene isosteres, non-peptidic, small molecular Pin1 inhibitors, and indanyl ketones, and 2) several natural products such as juglone, pepticinnamin E analogues, PiB and its derivatives obtained from a library screen. These Pin1 inhibitors show promise in the development of novel diagnostic and therapeutic anticancer drugs due to their ability to block cell cycle progression. In order to develop potent Pin1 inhibitors, the concept of transition-state analogues was used for the design of three classes of compounds: ketoamide, ketone, and reduced amide analogues.

Specifically, a convergent synthesis of α-ketoamide inhibitors of Pin1 was developed. An α-hydroxyorthothioester derivative of Ser was reacted directly with an aminyl synthon. The reaction was catalyzed by HgO and $HgCl_2$ to form an α-hydroxyamide. Hydrolysis and coupling were combined in one step in 80% yield. Two

diastereomers of a phospho-Ser-Pro α-ketoamide analogue were synthesized. The resulting IC_{50} values of 100 μM and 200 μM were surprisingly weak for the Pin1 peptidyl-prolyl isomerase.

Diastereomeric ketones were synthesized by coupling cyclohexenyl lithium to the serine Weinreb amide, via the Michael addition of a carboxylate synthon. The IC_{50} values of the two ketone diastereomers were determined to be 260 μM and 61 μM, respectively.

Five reduced amide inhibitors for Pin1 were synthesized through a selective reduction using borane. The most potent inhibitor was found to be Fmoc–pSer–Ψ[CH_2N]-Pro–tryptamine, which had an IC_{50} value of 6.3 μM. This represents a 4.5-fold better inhibition for Pin1 than a comparable *cis*-amide alkene isostere. The co-crystal structure of Ac–pSer–Ψ[CH_2N]-Pro–tryptamine bound to Pin1 was determined to 1.76 Å resolution.

Towards understanding the two proposed mechanisms of Pin1 catalysis, nucleophilic-addititon mechanism and twisted-amide mechanism, three classes of Pin1 inhibitors (ketoamide, ketone, and reduced amide analogues) involving a total of nine compounds were synthesized and evaluated. The weak inhibitory activities of ketoamide and ketone analogues do not support the nucleophilic-addition mechanism, while the twisted-amide mechanism of Pin1 catalysis is promising based on the reduced amide inhibitors with good potencies.

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List of Abbreviations

Amino acids

Abu, 2-aminobutyric acid

Ala, alanine

Asn, asparagine

Asp, aspartic acid

Arg, arginine

Bth, β-(3-benzothienyl)-alanine

Cys, cysteine

Gln, glutamine

Glu, glutamic acid

Gly, glycine

His, histidine

Ile, isoleucine

Leu, leucine

Lys, lysine

Met, methionine

NEA, 2-(2-naphthyl)-ethylamine

Nva, norvaline

Phe, phenylalanine

Pro, proline

p, phospho-

Ser, serine

Sta, statine

Thr, threonine

Trp, tryptophan

Tyr, tyrosine

Val, valine

Enzymes and natural products

APP amyloid precursor protein

BACE β-site amyloid precursor protein cleaving enzyme

CCK cholecystokinin

Cdc25C cell division cycle 25 homolog C

CDK cyclin dependent kinase

CsA cyclosporin A

CtA cyclotheonamide A

CyP cyclophilin

DMAP 4-dimethylamino pyridine

fXa factor Xa

FIV the feline immunodeficiency virus

FKBP FK-506 binding proteins

GRF growth-hormone-releasing factor

HCMV human cytomegalovirus

HCV hepatitis C virus

HDM2 human double minute 2

HBV hepatitis B virus

hCyp human cyclophilin

HDACs histone deacetylases

HIV the human immunodeficiency virus

JNK c-Jun *N*-terminal kinase

MDM2 murine double minute 2

NIMA never in mitosis A kinase

NKA neurokinin A

NOS nitric oxide synthase

PED prolyl endopeptidase

Pin1 protein interacting with NIMA #1

PPIase peptidyl-prolyl isomerase

RNase ribonuclease

RNAP II RNA polymerase II

SARS-CoV severe acute respiratory syndrome-associated coronavirus

WW domain WW stands for two tryptophans

Phases of mitosis

G1 preparation for chromosome replication

S DNA replication

G2 preparation for mitosis

M mitosis

Synthesis

Ac acetyl

Bn benzyl

Boc *tert*-butoxycarbonyl

DMAP 4-(dimethylamino)pyridine

DMF *N, N'*-dimethylformamide

EDC 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride

Fmoc fluorenylmethyloxycarbonyl

HOAt 1-hydroxy-7-azabenzotriazole

HOBt 1-hydroxybenzotriazole

LAH lithium aluminum hydride

LDA lithium diisopropylamide

PCC pyridium chlorochromate

THF tetrahydrofuran

TLC thin layer chromatography

Spectrometry

HMQC heteronuclear multiple quantum correlation

COSY correlation spectroscopy

Terms

IC_{50} In determinations of receptor binding affinity of a ligand, the IC_{50} (or EC_{50} - effective concentration 50%) is the concentration required for 50% inhibition

k_{cat} catalyzed rate constant

K_m Michaelis Constant

k_{cat}/K_m enzyme efficiency

SAR structure activity relationships

Chapter 1: Pin1 as an anticancer drug target

This chapter is a review paper entitled Pin1 as an anticancer drug target, published in Drug News & Perspective. I wrote the first draft, and Dr. Felicia Etzkorn edited and rewrote portions of inhibitors of Pin1 in close consultation with me.

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Pin1 as an anticancer drug target

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Abstract

Pin1 specifically catalyzes the cis/trans isomerization of pSer/Thr-Pro bonds, and plays an important role in many cellular events through the effects of conformational change on the function of its biological substrates, including Cdc25, c-Jun, and p53. Pin1 is overexpressed in many human cancer tissues, including breast, prostate, and lung cancer. Its expression correlates with cyclin D1 levels, which contributes to cell transformation. Overexpression of Pin1 promotes tumor growth, while inhibition of Pin1 causes tumor cells apoptosis. Pin1 plays an important role in oncogenesis and may act as an anti cancer target in cancers. Many inhibitors have been discovered for Pin1, including 1) several classes of designed inhibitors: alkene isosteres, reduced amides, indanyl ketones, and 2) the natural products: juglone, pepticinnamin E analogues, PiB and its

derivatives obtained from a library screen. Pin1 inhibitors could be used as a novel type of anticancer drug by blocking cell cycle progression. Therefore, Pin1 represents a new diagnostic and therapeutic anticancer drug target.

Pin1 binds pSer/Thr-Pro motifs

Pin1 (Protein interacting with never-in-mitosis A kinase 1) was discovered in 1996 to be a peptidyl-prolyl isomerase (PPIase) enzyme belonging to parvulin family.[1] Its potential as an anti-cancer target was reviewed in 2003.[2] Pin1 has two domains: a WW domain and a PPIase domain. Both of these domains recognize the phospho-Ser/Thr-Pro bonds present in mitotic phosphoproteins.[3] Pin1 is distinct from two other PPIase families, the cyclophilins and the FK506 binding proteins (FKBPs), since only Pin1 retains PPIase activity on phosphorylated substrates, and phosphorylated substrates are required for Pin1 PPIase activity.[1, 3, 4]

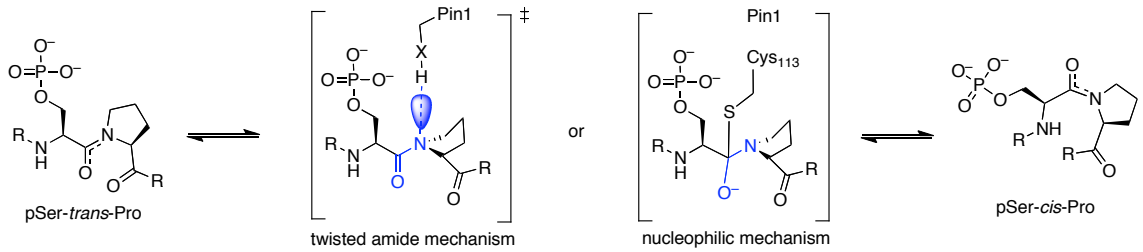
Pin1 enzymatic activity

The PPIase domain catalyzes the interconversion between the pSer/pThr-*cis*- and -*trans*-Pro conformations. The WW domain only binds this motif; it does not catalyze the isomerization. Pin1 causes conformational changes of its substrates only after post-translational phosphorylation. Pin1 catalyzes the *cis-trans* isomerization of pSer/Thr-Pro bonds with its *C*-terminal catalytic PPIase domain, while the function of the WW-domain *in vivo* is not yet well understood. It has been hypothesized that the WW domain might direct Pin1 substrate proteins to the PPIase domain by recognizing and binding pSer/Thr-Pro motifs. Pin1 plays an important role in many cellular events through the conformational change effects on the function of its substrates.[1, 4, 5] The cis-trans

isomerization activity of Pin1 is widely thought to act as a molecular timer in the regulation of the cell cycle.[6]

PPIases accelerate prolyl cis-trans isomerization at a rate of 10^6 s^{-1} faster than the thermal isomerization.[7] A twisted-amide mechanism was proposed for Pin1 based on mechanistic studies of CyP (cyclophilin) and FKBP (FK 506 binding protein).[8, 9] Another proposed mechanism was the nucleophilic catalytic mechanism with a tetrahedral adduct as the intermediate.[5] Neither mechanism has been definitively ruled out.

Scheme 1.1. Hypothetical mechanisms of Pin1.



Regulation of entry into mitosis

Pin1 regulates cell cycle entry into mitosis, the G2 to M transition (G2/M), by catalyzing a conformational change on cell division cycle 25 homolog C (Cdc25C). Cdc25C in turn activates cell division cycle 2 (Cdc2), the central mitotic kinase, by dephosphorylating Thr14 and Tyr15.[10] Recently we have shown that Cdc2 kinase specifically phosphorylates only the Ser-*trans*-Pro conformation of a Cdc25C substrate peptide (S Zhao and FA Etzkorn, unpublished results). Consequently, the essential regulatory role of Pin1 in activating Cdc25C during G2/M must be understood as reestablishing the equilibrium concentration of the catalytically active, pSer-*cis*-Pro form of Cdc25C. Pin1 is also known to regulate the activity of Cyclin D1, another cell-cycle

protein that is active in G1.[11] Because of this cell cycle regulatory activity, inhibition of Pin1 is likely to lead to interesting anti-cancer activities.

Pin1 regulates mitosis by interacting with mitotic phosphorylated proteins including Cdc25C phosphatase, myelin transcription factor 1 (Myt1), polo-like kinase 1 (Plk1),[12] Wee-1 kinase,[13, 14] and Cdc27.[15] Pin1 catalytically creates a conformation change on Cdc25C,[10] and facilitates its dephosphorylation by protein phosphatase 2A (PP2A), thought to be a conformation-specific phosphatase.[13] Pin1 interacts with chromatin specifically at the G2/M transition by modulating the mitotic phosphorylation of topoisomerase IIα through Cdc2/cyclinB, playing a critical role in the chromosome condensation process.[16, 17] S- and M- phase entries are mediated by the stabilization of Emi1 (early mitotic inhibitor) by Pin1 during G2. Emi1 is needed to bring on S- and M-phase entries by stimulating the accumulation of cyclinA and cyclinB.[18]

Pin1 depletion, achieved by three methods: 1) overexpression of Pin1 antisense RNA, 2) overexpression of dominant-negative Pin1, and 3) inhibition of Pin1-PPIase activity by juglone, demonstrated that the PPIase activity of Pin1 is essential for tumor cell survival and entry into mitosis through its targets, mitotic phosphoproteins.[19] The results of Pin1 depletion significantly include apoptosis.[1] Winkler et al found that Pin1 is required for DNA replication checkpoint in *Xenopus laevis* by the depletion of Pin1 causing inappropriate transition of G2 to mitosis or premature mitosis entry.[20]

Other cell cycle checkpoints

Pin1 is not only an important regulator of mitotic phosphoproteins, it is also found to modulate the activities of numerous other proteins. Pin1 positively regulates cyclin D1 function through post-translational stabilization, and maintains cell proliferation.[21, 22] Pin1

regulates the activity of transcriptional factors, including the oncogene transcription factor, AP-1 (activator protein 1), which is comprised of c-Jun and c-Fos,[23, 24] the p53 oncogene,[11, 25, 26] p73,[27] and SRC3 (steroid receptor coactivator 3).[28] Pin1 modulates the structure and function of RNA polymerase II (RNAP II), and plays an important role in mitotic gene silencing and transcription cycling.[29]

DNA damage response

Pin1 interacts with Che1, an RNAP II binding protein, which plays an important role in response to DNA damage and modulates its conformational changes, which are required for Che1/HDM2 (human double minute 2) interaction.[30] Pin1 affects the function of p53 oncogene by regulating the stability of p53 and its transcriptional activity towards the p21 promoter during DNA damage. DNA damage increases the interaction between Pin1 and p53.[11] Pin1 activates p53 in response to DNA damage, and protects p53 from murine double minute 2 (MDM2), an E3 ubiquitin ligase and inhibitor of p53 transcriptional activation.[31, 32]

Pin1 in human cancers

In general, Pin1 is overexpressed in many different cancer cell types.[33] If Pin1 is mainly required in the proliferating cells of cancer, but not absolutely required in normal cells, this overexpression is a good harbinger for Pin1 as an anti-cancer target.[2] On the other hand, if Pin1 activity is required for the health of normally dividing cells, Pin1 inhibitors might have similar side effects to known, non-specific anti-cancer drugs, such as cis-platin. Pin1 has been shown in Pin1-/- knockout mouse studies to be required primarily in development of germ cells, and normal cell proliferation, such as the changes

in mammary glands during pregnancy.[21, 34] Thus, Pin1 is actively under research as a promising anti-cancer drug target.

Breast cancer

Pin1 is expressed 10-fold higher in human breast tumors than in normal tissues, and its levels positively correlate with the Bloom and Richardson grade for breast cancer.[35] Pin1 levels correlate with cyclin D1 overexpression in human breast cancer cells, and Pin1 overexpression elevates cyclin D1 levels in cell lines. Pin1 specifically activates the cyclin D1 promoter through specific sequences such as the AP-1 and /or Ets sites.[35] Cyclin D1 is overexpressed in 50% of patients with breast cancer, and its overexpression contributes to cell transformation, while inhibition of cyclin D1 arrests the growth of tumor cells. Pin1 increases the transcriptional activity of phosphorylated c-Jun toward the cyclin D1 promoter by cooperating with the activated JNK (c-Jun *N*-terminal kinase) of Ha-Ras. These results suggest that overexpression of Pin1 may promote tumor growth. Since inhibition of Pin1 causes tumor cell apoptosis, Pin1 may act as a potent anti-cancer target.[23, 36] Pin1 plays a critical role in tumorigenesis induced by Neu and Ras *in vivo*. Pin1 deletion effectively suppresses the early transformed properties of Ras or Neu transfected mammary epithelial cells (MECs).[37]

Hepatocellular carcinoma (HCC)

Pin1 is overexpressed in more than 50% of HCC.[38] Pin1 overexpression directly contributes to beta-catenin and cyclin D1 overexpression, and leads to beta-catenin accumulation in HCC.[38] Suppression of Pin1 expression by RNA interference (siRNA) in HCC resulted in decreased levels of beta-catenin and cyclin D1.[38] Hepatitis B virus (HBV)

is the most common etiologic agent in HCC. Pin1 overexpression increases the stability of the encoded x-protein of HBV (HBx), and enhances HBx-mediated transactivation.[39]

Non-small cell lung cancer (NSCLC)

Pin1 protein is overexpressed in NSCLC tumor samples, and correlates with the high expression of p53 or MDM2 protein which correlates with cyclin D1 overexpression.[40]

Esophageal squamous cell carcinoma (SCC)

Pin1 is overexpressed in more than 80% of esophageal SCC cell lines, and its overexpression correlates with lymph node metastasis and cyclin D1 overexpression.[41] Pin1 is an independent prognostic factor for esophageal SCC.[41]

Cervical cancer

Pin1 expression is positively correlated to cyclin D1 protein expression in cervical cancer tissues.[42] Pin1 overexpression increases the cyclin D1 protein expression level while Pin1 deletion significantly reduces the cyclin D1 protein expression level in Hela cells.[42] Silencing Pin1 by RNA interference inhibits Hela cell proliferation and causes apoptosis in Hela cells.[42] Pin1 plays an important role in oncogenesis and may act as an anti cancer target in human cervical cancers.

Colorectal cancer

Pin1 overexpression was observed in colorectal cancer cells; 38% of colorectal cancer specimens were detected with high Pin1 expression by Kuramochi et al [43] However, less than 10% of colon adenocarcinoma tissues showed Pin1 overexpression in

the study of Bao et al [33] Although their results are not consistent, that might be due to different methods of determination. Pin1 expression is different in various tumor stages, and its level is higher in a tumor's late stage than that in a tumor's early stage. Pin1 expression is correlated with β-catenin and cyclin D1 expressions, which are related to the development of human colorectal cancer.[43] Pin1 plays an important role in colorectal tumorigenesis.

Prostate cancer (PCa)

Pin1 level is markedly higher in metastatic prostate cancer (PCa) than the normal prostate.[44] Pin1 expresssion accelerates tumor growth by preventing β-catenin coactivation of androgen receptor (AR) and thereby enhancing β-catenin coactivation of Transcription factor 4 (Tcf4) in PCa cells.[44] Pin1 might be a more effective anti cancer drug target compared to androgen ablation therapies for PCa patients.[44]

Thyroid tumors

Pin1 expression correlates closely with the level of cyclin D1 and aberrant beta-catenin expression, which are overexpressed and significant in thyroid tumours.[45]

Oral squamous cell carcinoma (OSCC)

Miyashita et al demonstrated that Pin1 protein and mRNA are overexpressed in OSCC, and Pin1 levels are correlated to cyclin D1 level. That suggests Pin1 relates to oncogenesis of OSCC.[46, 47]

Alzheimer's disease

Tau protein and amyloid precursor protein are two key factors in Alzheimer's disease. The conformation and function of Tau protein can be restored by Pin1 PPIase activity or its dephosphorylation.[48] Liou et al found that Pin1 expression is inversely correlated with predicted neuronal vulnerability and actual neurofibrillary degeneration in Alzheimer's disease.[49] Pin1 is involved promoting amyloid-beta (Aβ) peptides production and regulating amyloid precursor protein processing in the brain.[50-52] Because of these essential activities of Pin1 in the prevention of Alzheimer's disease, any drug targeting Pin1 for cancer treatment might require concurrent and continuing prophylactic treatment to prevent Alzheimer's.

Inhibitors of Pin1 as promising therapies for cancer

In this review, most Pin1 PPIase inhibitors are reviewed to date, including designed inhibitors: 1) alkene isosteres, reduced amides, and ketones, 2) the natural products: Juglone, Pepticinnamin analogues, and PiB and its derivatives. Their inhibition and biological activities as Pin1 inhibitors are also described.

Designed inhibitors

Peptides and peptidomimetics

Fischer and coworkers designed and synthesized a series of substrate-derived inhibitors.[53] Through optimizing binding affinities, and substitution of p-D-Ser for p-L-Ser or a thioxo for the Ser carbonyl, they developed substrate-analogue inhibitors of Pin1 with low micromolar affinities. Inhibitors with low micromolar IC_{50} values are listed in **Table I**.[53]

Table I. Substrate-derived inhibitors of Fischer and coworkers.[53]

Compound	Peptides	IC_{50} for Pin1 (μM)
1	Phe–L–Ser(PO_3H_2)–Ψ[CS–N]–Pro–Phe–*p*NA	4.0 ± 0.5
2	Ac–Ala–Ala–D-Ser(PO_3H_2)–Pro–Leu–*p*NA	1.0 ± 0.1
3	Ac–Ala–Ala–D-Ser(PO_3H_2)–Pro–Arg–*p*NA	3.6 ± 0.3

Wildemann et al identified nanomolar peptidic inhibitors by screening a combinatorial peptide library.[54] Oligopeptides of 5 to 8 residues with non-proteinogenic amino acids and a phospho-D-threonine-pipecolic acid as the central template yielded very potent inhibitors of Pin1 with the lowest known K_i value of 1.2 nM.[54] Moreover, the inhibitors block HeLa cells in the G2/M phase in a dose-dependent manner. Selected peptides with low nanomolar inhibition are listed in Table II.

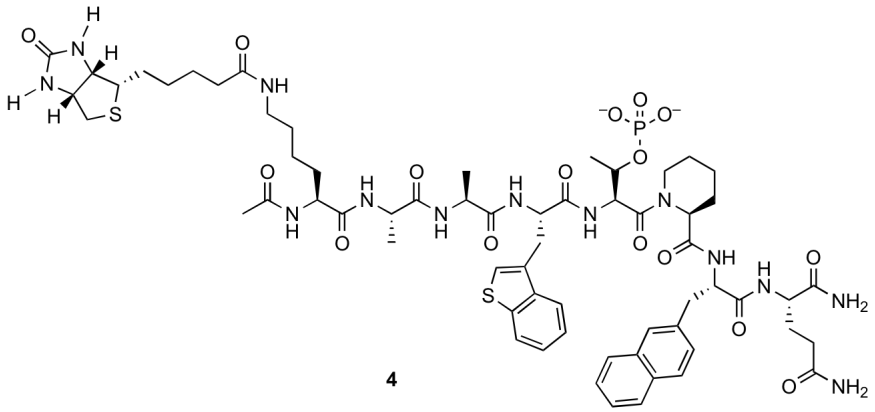


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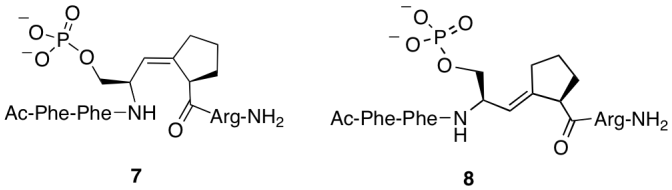
Table II. Low nanomolar peptide inhibitors of Pin1.[54]

Inhibitor	K_i (nM)
Ac–Lys–(*N*-biotinoyl)-Ala–Ala–Bth–D-Thr(PO_3H_2)–Pip–Nal–Gln–NH_2 **4**	1.2
Ac–Phe–D-Thr(PO_3H_2)–Pip–Nal–Gln–NH_2 **5**	18.3
Ac–Lys–(*N*-biotinoyl)-Ala–Ala–Phe–D-Thr(PO_3H_2)–Pip–Nal–Gln–NH_2 **6**	4.8

Substrate analogues with (*Z*)- and (*E*)-alkene isosteres replacing the pSer-*cis*- and *trans*-Pro amides respectively were synthesized by Wang et al[55] It was found that the cis alkene isostere **7** (K_i = 1.74 μM) was 23-fold more potent than the trans alkene isostere **8** (K_i = 40 μM). Antiproliferative activity towards A2780 ovarian cancer cells in vitro of the cis isostere **7** gave an IC_{50} value of 8.3 μM, while the trans isostere **8** had an IC_{50} value of 140 μM.[55] This difference of approximately 20-fold for the *in vitro* and cell-based assay results between the cis and trans isosteres verified that Pin1 was the cellular target for the inhibitors.[55]



7 **8**

Figure 1.1. Alkene isosteres as Pin1 inhibitors.

Cell permeable inhibitors

Two alkene isostere inhibitors, including a phosphorylated prodrug, were synthesized for the inhibition of Pin1 by Zhao and Etzkorn.[56] The *C*- and *N*-terminal aromatic groups were chosen based on the optimal substrate specificity of Pin1.[3] To improve the cell penetration of charged phosphorylated inhibitor **9**, the bis-pivaloyloxymethyl (POM) masking group was used to neutralize the charges on the phosphate.[56] Antiproliferative activity towards A2780 ovarian cancer cells was improved with the bisPOM inhibitor **10** (IC_{50} = 26.9 μM) compared with the charged phosphorylated inhibitor **9** (IC_{50} = 46.2 μM).[56] Again, because the IC_{50} values of the charged inhibitor **9** in the *in vitro* assay and the masked inhibitor **10** in the cell-based

assay were well matched, these results indicated that Pin1 was the cellular target in the antiproliferative activity of the inhibitors.

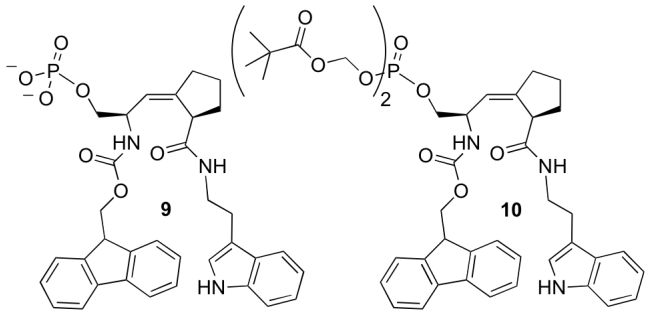
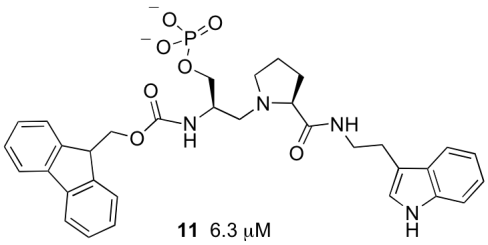


Figure 1.2. Cell permeable inhibitors of Pin1.

Reduced amide inhibitors

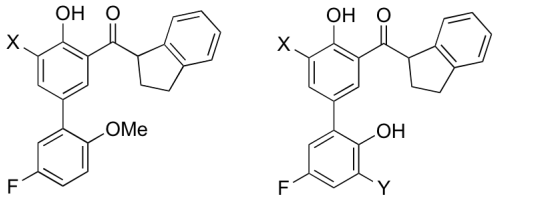
Xu and Etzkorn designed and synthesized a reduced amide peptidomimetic **11** designed to mimic the twisted amide transition state of the PPIase reaction of Pin1.[57] The inhibitor **11**, with the same *C*- and *N*-terminal groups as the alkene isosteres, showed moderate inhibition of Pin1 (IC_{50} = 6.3 μM) that was only slightly improved over the alkene isostere **9**.[57] We concluded that the reduced amide does not behave as a transition state analogue.



Indanyl ketone inhibitors

Daum et al developed a series of aryl indanyl ketone inhibitors of Pin1 **12 – 13** with sub-micromolar inhibition, which are reversible and cell penetrating.[58] These

inhibitors also showed biological activities for p53 and β-catenin, and they are promising anti-cancer drug candidates.[58]



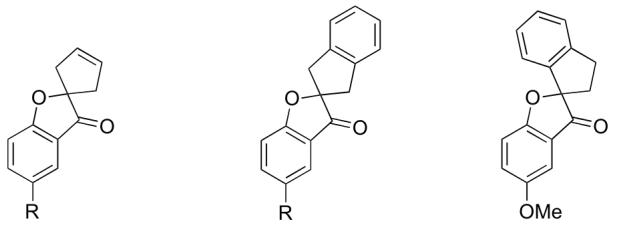
12a X = H, 1.7 μM
12b X = NO_2, 1.1 μM
12c X = NH_2, 4.9 μM

13a X = H, Y = NO_2, 0.5 μM
13b X = H, Y = NH_2, 0.4 μM
13c X = NO_2, Y = NO_2, 0.2 μM

Figure 1.3. Indanyl ketones as inhibitors of Pin1.

Spiroketones

A class of spiroannulated 3-benzofuranones **14 – 16** was synthesized by a very short route.[59] However, they displayed only modest, or even undetectable inhibition of Pin1,[59] and were much worse than the aryl indanyl ketones.[58] Apparently, the ketone is crucial to the inhibitory potency of compounds **12a-c** and **13a-c**. This inhibitor pattern is consistent with a nucleophilic PPIase mechanism first proposed by Noel and coworkers in 1997.[5]



14a R = H, No inhibition
14b R = OMe, K_i = 65 μM

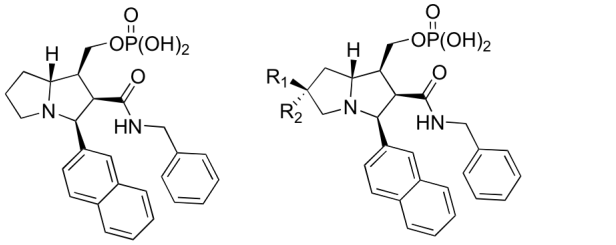
15a R = H, No inhibition
15b R = OMe, K_i = 100 μM

16 K_i = 77 μM

Figure 1.4. Spiroketones as inhibitors of Pin1.

Perhydropyrrolizines

Highly substituted perhydropyrrolizines **17 – 18** were synthesized by Siegrist et al, and found to inhibit Pin1 with K_i values in the micromolar range.[60] ^{15}N, ^{1}H-HSQC-NMR spectroscopy and enzymatic activity assays showed that they bind to both WW domain and PPIase domain of Pin1.[60]



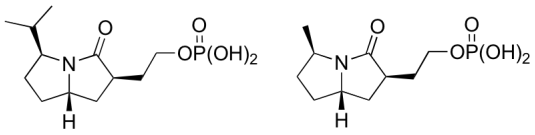
17, K_i = 15 μM

18a R_1 = F, R_2 = H, K_i = 26 μM
18b R_1 = H, R_2 = OMe, K_i = 9 μM

Figure 1.5. Perhydropyrrolizines as inhibitors of Pin1.

WW Domain Ligands

During the development of Pin1 inhibitors **17** and **18**, Siegrist et al found two perhydropyrrolizines that did not inhibit Pin1 PPIase activity, but instead bound to the Pin1 WW domain.[60]



19 **20**

Figure 1.6. Perhydropyrrolizines as Pin1 WW domain ligands.

Smet et al synthesized a series of Ac–pThr–Pro–NH_2 dipeptide analogues **21 – 23** as Pin1 WW domain ligands.[61] They used modified hydrophobic moieties on the *N*-terminus, and proline analogues: (2*R*)-pyrrolidine, (2*S*)-azetidine, and (2*S*)-piperidine etc, and polar mimics of phosphate.[61]

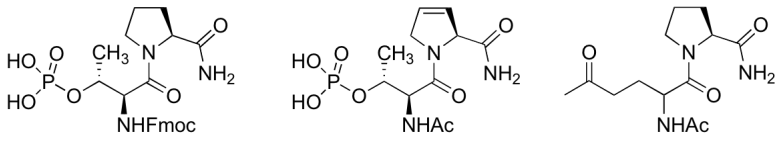


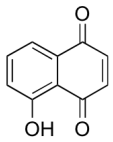
Figure 1.7. Dipeptide analogues as Pin1 WW domain ligands.

Recently, Brautigan and coworkers discovered a new role for Inhibitor 2 (I-2) in the allosteric modification of Pin1 substrate specificity towards mitotic phosphoproteins.[62] I-2 is an ancient inhibitor of protein phosphatase 1 (PP1). [63]

Natural and library screened inhibitors

Juglone

Juglone, 5-hydroxy-1,4-naphthoquinone, specifically and irreversibly inhibits Pin1 by covalently bonding to cysteine residues via Michael addition.[64, 65] It is likely that this compound serves as a general Michael addition trap for thiols. Juglone shows specificity for Pin1 over cyclophilin that does not have an active site thiol.[64, 66] Juglone has also been shown to directly block transcription, possibly through a site on RNA polymerase II, rather than by inhibition of Pin1.[65] A novel class of compounds, naphthalene diimides structurally related to juglone, that did not inhibit topoisomerase I, were found to inhibit Pin1 with IC_{50} values as low as ~1.5 μM.[67]



Inhibition of Pin1 using juglone and repression of Pin1 levels using Pin1 small interfering RNA both decrease accumulation of phosphorylated neurofilament-H (NF)-H

in neurons.[68] Thus, Pin1 catalyzed isomerization of Lys-Ser-Pro residue repeats, which are abundant in NF-H tail domains, regulates NF-H phosphorylation.[68] This indicates that Pin1 inhibition may be an attractive therapeutic target to reduce pathological accumulations of p-NF-H.[68]

Pepticinnamin E derivatives

Three peptidomimetics **24** – **26** derived from the natural product, pepticinnamin E, were developed with IC_{50} values for Pin1 ranging from 7.2 to 0.6 μM.[69] Interestingly, none of these inhibitors contain a pSer/Thr or Pro analogue, but all contain a central D-Tyr-L-Tyr. They were discovered in a high-throughput screen for apoptosis in mammalian transformed cells, and are thought to unfold Pin1 like juglone.[69, 70]

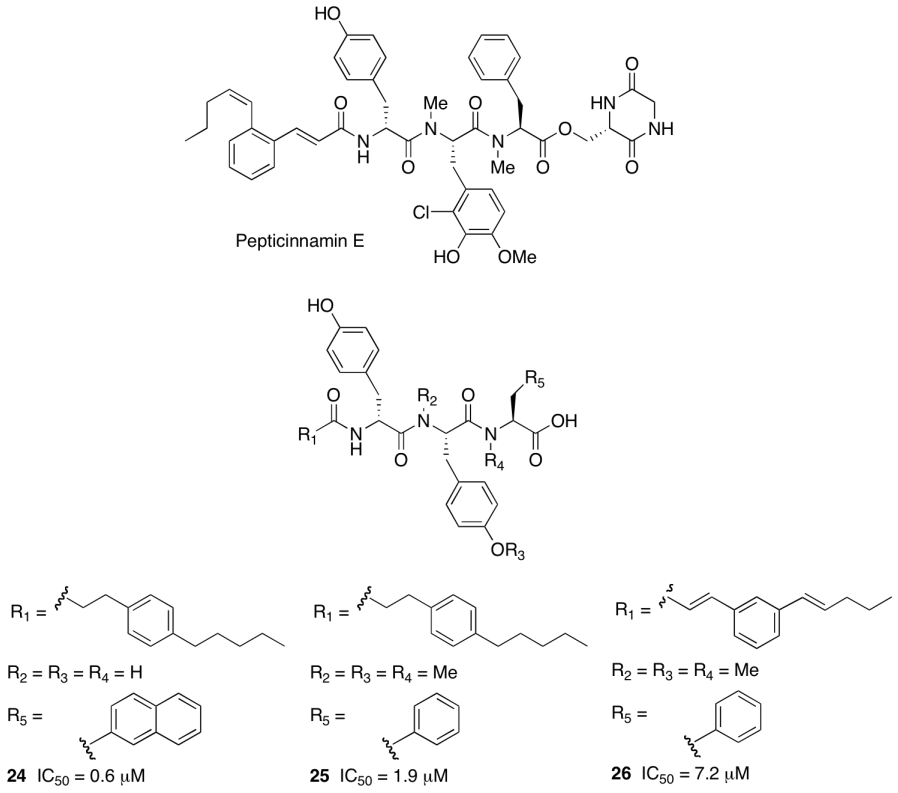
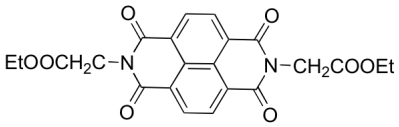


Figure 1.8. Pepticinnamin E derivatives as Pin1 inhibitors.

PiB and its derivatives

Through a chemical compound library screening diethyl-1,3,6,8-tetrahydro-1,3,6,8-tetraoxobenzo[lmn][3,8] phenanthroline-2,7-diacetate, PiB, (IC_{50}, 1.5 μM), and its derivatives were found to inhibit Pin1 PPIase activity *in vitro* with low micromolar IC_{50} values.[67] They also inhibited the growth of several cancer lines.[67] Among them, PiB had the least nonspecific toxicity, and might be used as a novel type of anticancer drug by blocking cell cycle progression.[67]



PiB

Conclusions

Pin1 catalyzes prolyl cis-trans isomerization to function as a molecular timer regulating the cell cycle, cell signaling, gene expression, immune response, and neuronal function.[6] Pin1 interacts with many substrates in a phosphorylation dependent manner, and regulates many cellular events such as cell cycle progression, transformation, and cell proliferation etc. Pin1 is overexpressed in many cancer lines, and plays an important role in oncogenesis.[22] Because of its significant role in cell cycle regulation by a unique mechanism, Pin1 represents an intriguing, although as yet not validated, diagnostic and therapeutic target for cancer.[71, 72] Several promising classes of inhibitors as potential lead compounds have been synthesized, including designed inhibitors, and natural products.

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Chapter 2: α-Ketoamide inhibitors

This chapter is comprised of two parts. Part A features a review of ketoamide analogue inhibitors and synthetic methods for their preparation. Part B presents a paper published in Organic Letters, which concerns the design and synthesis of ketoamide analogue inhibitors of Pin1. I performed all of the experiments from Part B, wrote the Experimental sections, and the first draft of both parts A and B. Dr. Etzkorn edited and rewrote portions of Part A and the paper in Part B in close consultation with me.

Part A: α-Ketoamide Inhibitory Activities and Synthetic Methods

2.1. Introduction

The primary research goal associated with this study was to synthesize potent inhibitors of Pin1 that could serve as anti-cancer drug compounds. α-Ketoamides, ketones and reduced amides (amines) represent three classes of important analogues of amides (Figure 2.1), all of which have been used in the development of potent inhibitors for proteases and other enzymes due to their unique functionalities.

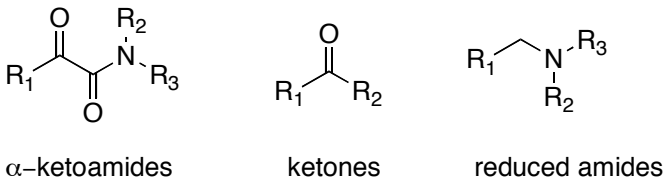
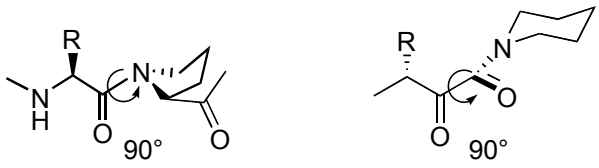


Figure 2.1. General structures of α-ketoamides, ketones and reduced amides

α-Ketoamide, ketone, and reduced amide Ser-Pro analogues are the three classes of inhibitors we designed for Pin1. In this chapter, we focus on understanding and exploring the functionality and inhibitory activities of α-ketoamides, as well as

describing the various synthetic methods used to produce them. The synthesis and characterization of the ketone and reduced amide analogues as inhibitors are described in subsequent chapters.

There have been many studies concerning the α-ketoamide functionality in the design of enzyme inhibitors. Because α-ketoamides have an electron-deficient carbonyl group due to the effect of the neighboring electron-withdrawing amide group, they can serve as transition-state analogues, and allow stable inhibition of catalytic activity. This occurs for serine or cysteine proteases through the formation of a tetrahedral intermediate (gem-diol or hemiketal) with the enzyme upon binding.[1] They may also mimic a "twisted amide" transition-state structure with a torsion angle of 95 to 100º between the carbonyl and the amide groups (Figure 2.2).[2] The "twisted" conformation of α-ketoamide, in the stable structure FK506, mimics the "twisted-amide" transition state to inhibit FKBP. The α-ketoamide functional group has been used in a wide range of enzyme inhibitors to elucidate the mechanisms of enzyme inhibition, including FK506 Binding Protein (FKBP), a peptidyl prolyl cis-trans isomerase (PPIase).



a twisted peptidyl-prolyl amide bond

FK506 substructure

Figure 2.2. α-Ketoamides mimic a "twisted-amide" transition state.[2]

α-Ketoamides are an important class of compounds commonly used as enzyme inhibitors. As such, numerous methods for their synthesis have been reported. We

summarize herein the synthetic methods reported to date, as well as describe synthetic methods that we considered for our pSer-Pro α-ketoamide analogues.

2.2. α-Ketoamide inhibitory activities

2.2.1. HIV and FIV protease inhibitors

Two important aspartyl proteases associated with this study are (1) the human immunodeficiency virus protease (HIV PR), which consists of 99 amino acid residues, and (2) feline immunodeficiency virus (FIV), containing 116 amino acid residues.[3,4] Both of these proteases function as homodimers and feature the same mechanism.[3,4] For example, they both show high specificity for the selective cleavage of Tyr/Phe-Pro amide bonds, making HIV PR in particular an important target for the inhibition of viral replication (Table 2.1.).[3]

Table 2.1. Amino acid sequence of the natural substrates for HIV and FIV proteases about the Y~P cleavage site [3]

	P4	P3	P2	P1	P1′	P2′	P3′	P4′
HIV PR	Ser	Gln	Asn	Tyr	Pro	Ile	Val	Gln
FIV PR	Pro	Gln	Ala	Tyr	Pro	Ile	Gln	Thr

The α-ketoamide core structure **2.1** (Figure 2.3) was found to be a more potent inhibitor of HIV PR ($K_i = 6\ \mu M$)[4] than other isosteric core structures such as hydroxyethylamine[5] and phosphinic acid derivatives.[6] The effects of protecting groups, and the substituents on the proline ring, were studied in an effort to enhance the inhibitory activity of the α-ketoamide core isosteres.[3] The inhibitory activity was improved by a simple modification of the N-terminal protecting group to generate

compound **2.2a**. This compound had a K_i of 214 nM due to the effective interactions between the protecting group and the hydrophobic binding pocket. The substitution of a cis-benzyl ether on C-4 of the proline moiety to generate compound **2.2b** increased binding to a K_i value of 65 nM, which is in agreement with a computer model that predicts attachment of hydrophobic groups to the proline ring moiety would enhance binding.[3] It should be noted, however, that no increased binding was observed with trans-benzyloxy or trans-methoxy groups at the same position.

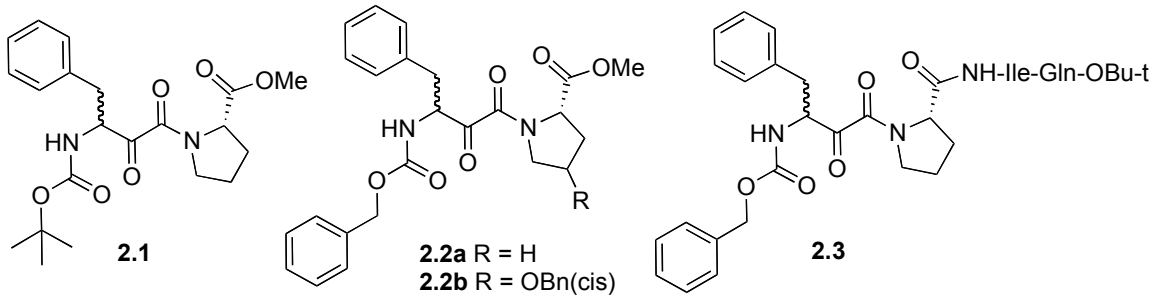
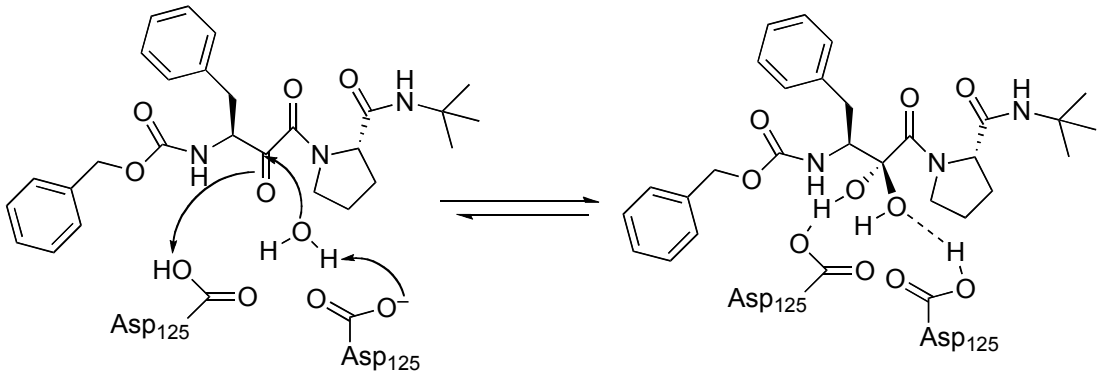


Figure 2.3. α-Ketoamides as HIV and FIV protease inhibitors

The mechanisms associated with the interactions between inhibitor and enzyme were studied. When X-ray was used to determine the structure of the complex between the α-ketoamide **2.2a** and HIV PR, the oxo group of the α-ketoamide was found to be hydrated.[3] Since the ketone moiety of the α-ketoamide remained unhydrated even after incubation in DMSO/D_2O (5:1) for 24h as shown by ^{13}C-NMR studies, hydration of the ketone moiety was hypothesized to be assisted by the active site of HIV PR, as illustrated in Scheme 2.1. However, when these core structures were tested against FIV PR, no significant inhibitory activity was observed—even after conducting some modifications specific to FIV PR. Compound **2.3,** with an IC_{50} of 25 μM and a K_i of 29 μM, was obtained after coupling a side chain to the C-terminus of **2.2a** (Figure 2.3). As a result of

these experiments, it became clear that the FIV PR required additional specific residues between the P4-P4′ sites, which HIV PR did not.[3]

Scheme 2.1. Schematic representation of general acid-general base mechanism for the interaction of inhibitor **2.2a** with HIV PR aspartate groups.[3]



2.2.2. Serine protease inhibitors

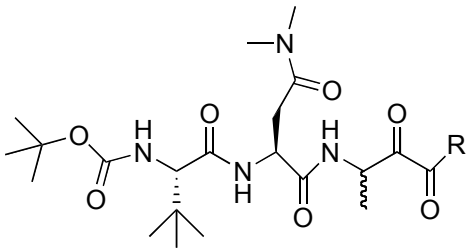
HCMV protease inhibitors

The human cytomegalovirus (HCMV) belongs to the herpes virus family.[7] Research studies have demonstrated this enzyme to be a serine protease with a novel structure compared to other serine proteases. Specifically, it contains a unique catalytic triad consisting of Ser132, His63, and His157.[8] Ogilvie et al. developed several series of HCMV inhibitors that contain an activated carbonyl, including α-ketoamides, based on the amino acid sequences of the two cleavage sites. Compound **2.4a** with an IC_{50} of 0.2 μM represents one of the most potent inhibitors of HCMV protease described thus far.[7]

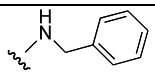
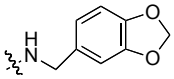
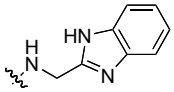
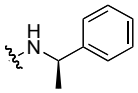
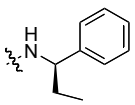
Compounds extending the C-terminus of the α-ketoamides into the S′ pocket improved the binding to the HCMV protease. Compounds **2.4b** and **2.4c** indicated that the C-terminal α-ketoamide moiety affected the inhibition, and that disubstituted amides

were not tolerated at this position (Table 2.2). Compounds **2.4d**, **2.4e** and **2.4f** did not show significant improvements of the inhibition even after making modifications to the C-terminal α-ketoamide moiety. A comparison of **2.4g** to **2.4h** shows that lengthening the side chain of benzyl amide **2.4g** slightly increased the inhibitor potency.[7]

Table 2.2. Effects of extensions into the S′ binding pocket on the activity of α-ketoamide inhibitiors of HCMV [7]



2.4

Compound	R	IC_{50} (μM)
2.4a		0.20 ± 0.05
2.4b	-NHMe	1.1 ± 0.3
2.4c	NMe_2	> 300
2.4d	$NHCH_2CH_2OBn$	0.14 ± 0.03
2.4e		0.10 ± 0.01
2.4f		0.21 ± 0.05
2.4g		0.28 ± 0.04
2.4h		0.11 ± 0.03

Hepatitis C Virus (HCV) NS3 protease inhibitors

HCV, which was first identified in 1989, is the principal etiologic agent of both post-transfusion and non-A/non-B hepatitis.[9, 10] The serine protease, HCV NS3 protein, is becoming one of the most intensively studied and best understood targets. There is a large number of HCV inhibitors already developed or currently in pre-clinical and clinical development.[11, 12] For example, 1,2-dicarbonyl derivatives such as α-ketoamides, α-ketoesters and α-diketones are known to function as serine traps, since they are believed to form a tetrahedral adduct with active site serine hydroxyl groups. Among this group of compounds, the α-ketoamides show particular promise. In fact, much of the development associated with HCV inhibitors is based on α-ketoamide core structures.

Llinas-Brunet et al reported two examples of α-ketoamides as HCV protease inhibitors, Ac–Asp–Asp–Ile–Val–Pro–(L/D)-Nva–CONHBn and Ac-Asp–(D)-Asp–Ile–Val–Pro–(L/D)-Nva–CONHBn.[9] The authors found that these two α-ketoamides were better inhibitors of HCV than other peptide sequences containing electrophilic carbonyl groups or the corresponding carboxylic acids. Specifically, Ac–Asp–Asp–Ile–Val–Pro–(L/D)-Nva–CONHBn and Ac-Asp–(D)-Asp–Ile–Val–Pro–(L/D)-Nva–CONHBn were found to effectively inhibit HCV protease with IC_{50} values of 20 μM and 0.64 μM, respectively.[9]

Another α-ketoamide, the hexapeptide **2.5**, was found to effectively inhibit HCV NS3 protease with an IC_{50} value of 2.5 μM. In their study, Han et al designed and prepared a series of α-ketoamides, α-ketoesters, and α-diketones **2.6** based on hexapeptide **2.5** in an attempt to enhance the inhibitory potency by investigating the effect of serine traps (Figure 2.4). [10] The resulting crystal structure showed that HCV NS3

protease displayed a shallow and hydrophobic S1 pocket.[10] Bioassays showed the effectiveness of incorporating allyl and ethyl P1 groups, so the P1 mercaptomethyl group in compound **2.5** was replaced with allyl and ethyl groups in the targets. Among them, the α-ketoamides were found to have the highest affinity for HCV NS3, and compound **2.6a** was found to be a potent inhibitor with an IC_{50} value of 0.42 μM.[10]

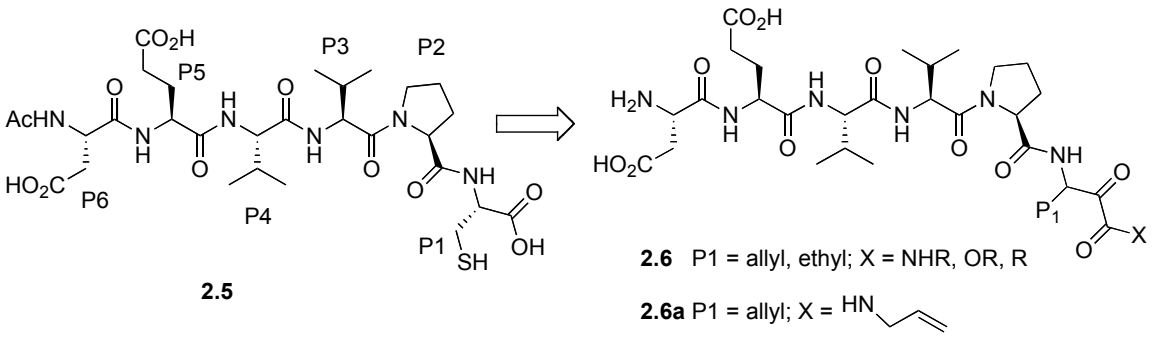


Figure 2.4. α-Ketoamides as HCV NS3 protease inhibitors

During SAR studies to explore primary site interactions using the α-ketoamide moiety as a template, Han et al[13] found glycine carboxylic acid to be the most effective P1' group (Figure 2.5). They also investigated the effect of using carboxylic acid analogues as replacements for glycine, and subsequently developed a series of potent inhibitors for HCV NS3 with IC_{50} values of 20-60 nM.[14]

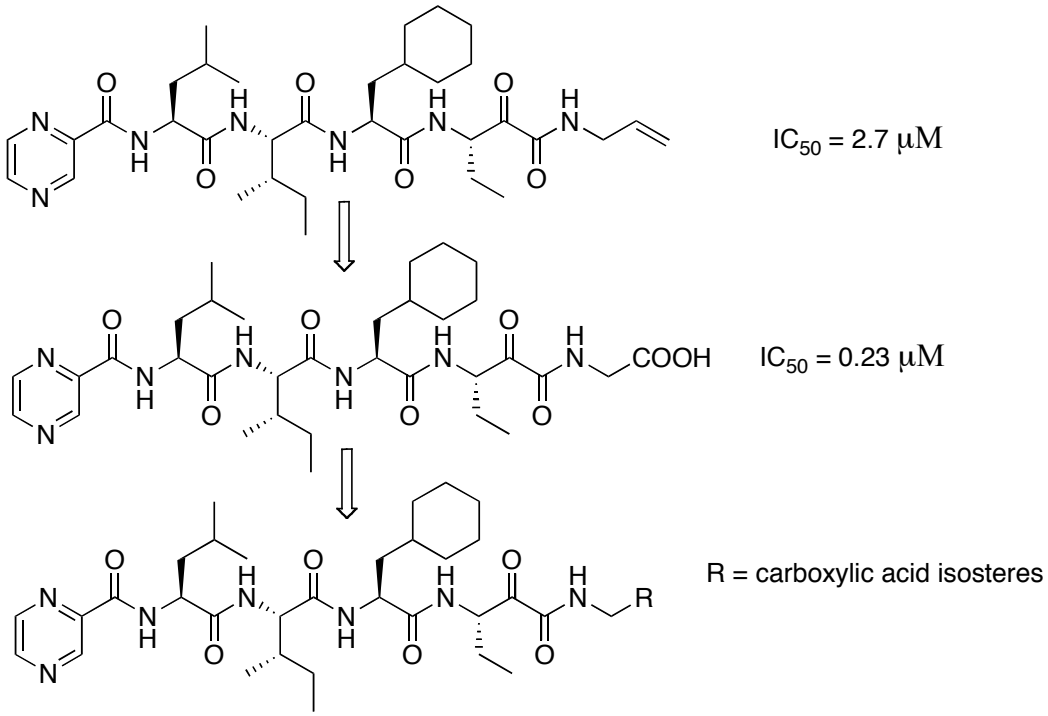
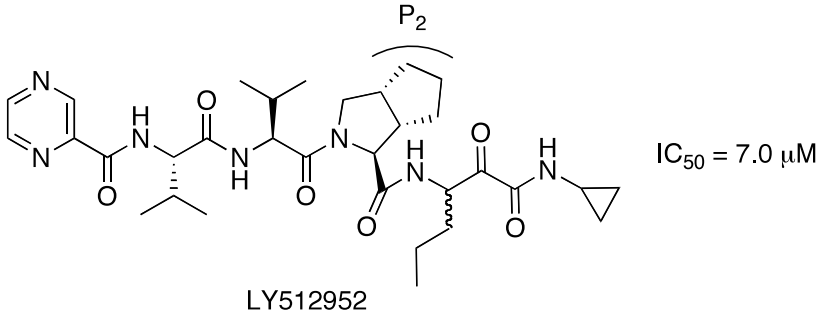


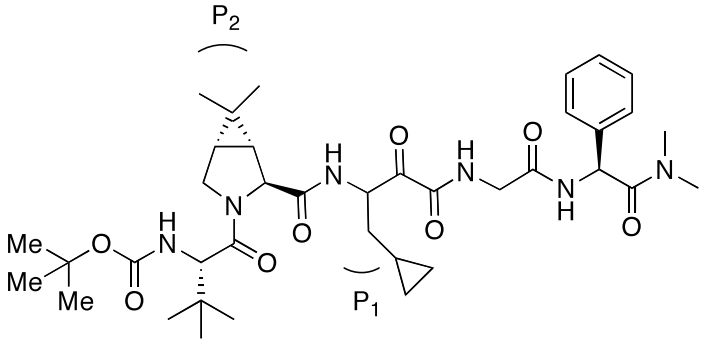
Figure 2.5. SAR study exploring primary site interactions.[13]

Using P_2 modification and α-ketoamide as the core structure, Lilly researchers discovered the HCV protease inhibitor known as LY514962, with a bicycloproline at the P_2 position. Based on their findings, inhibitor LY514952 could lead to a new class of HCV inhibitors with therapeutic potential.[15]



In later studies, Bogen et al discovered a very potent α-ketoamide inhibitor (SCH446211) of the HCV NS3 serine protease by altering the P_2 and P_1 side chains.[16-19]

As a result of these modifications, the oral pharmacokinetic properties of the inhibitor were improved.



SCH446211

Another potent HCV NS3 protease inhibitor SCH503034[20-22] was discovered through an SAR study of compound **2.7** (Figure 2.6), an α-ketoamide, with an excellent activity (K_i = 1.9 nM). In a series of modifications involving amino acid residues of this compound, a subsequent compound known as SCH503034 was obtained with excellent affinity (K_i = 14 nM) (Figure 2.6). The selectivity of human neutrophil elastase (HNE) versus HCV was dramatically increased to 2200 when compared to the selectivity of compound **2.7** (HNE/HCV = 6.8). Given the potency, selectivity, and oral bioavailability of this compound, human clinical trials involving SCH503034 have been initiated. [20-23]

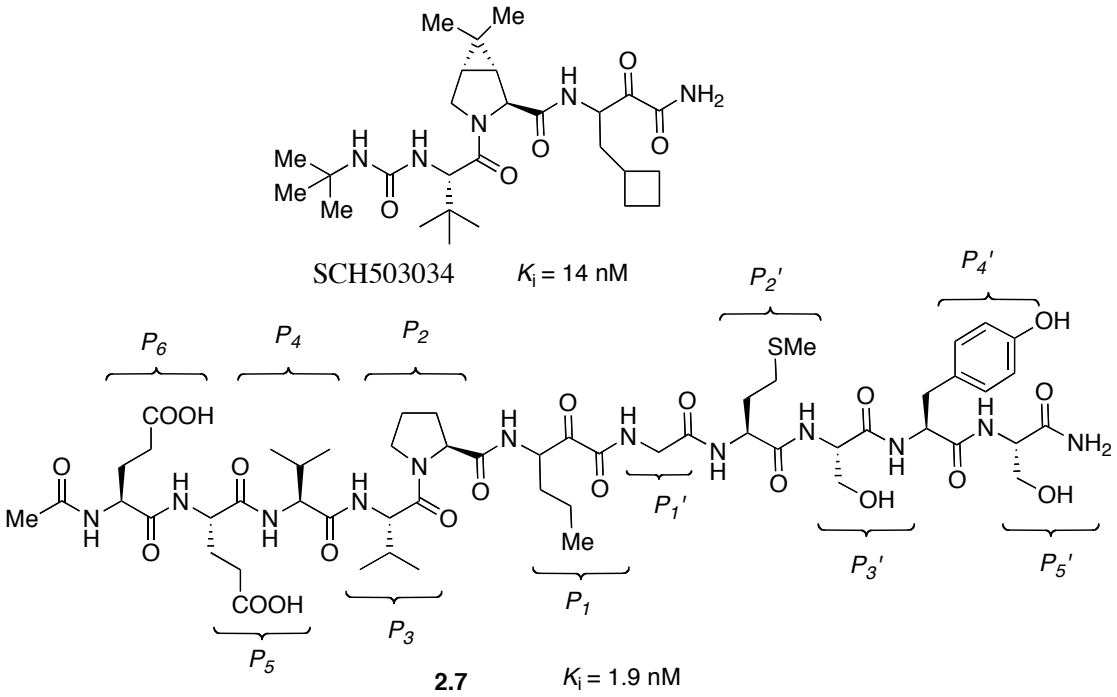


Figure 2.6. SAR study to develop specific inhibitors of HCV.[20-22]

A number of P_2 proline-based macrocyclic α-ketoamide inhibitors were prepared to improve the potency through the close interaction of the macrocycle with the Ala156 methyl residue of the HCV protease.[17, 24] A number of α-ketoamide inhibitors based on a dichlorocyclopropylproline P_2 core **2.8** were also developed.[25] The most promising compound to emerge from these studies was **2.9** that was more potent than SCH503034 (Figure 2.7).[25]

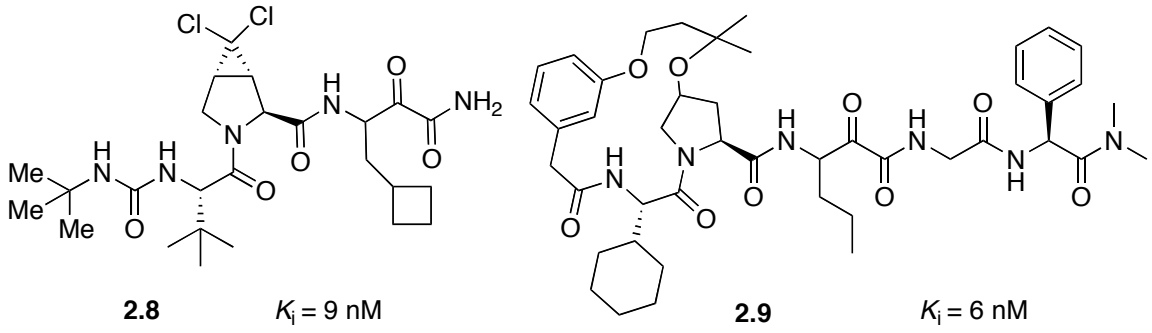
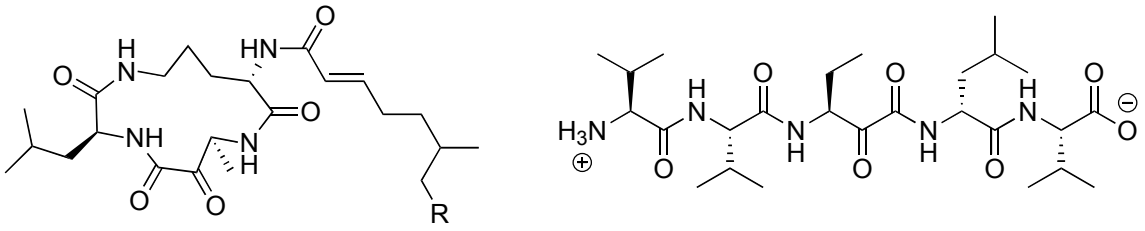


Figure 2.7. P_2 modified HCV inhibitors[25]

Prolyl endopeptidase inhibitors

Prolyl endopeptidase (PED) is a type of serine protease that is inhibited by two noteworthy natural products: eurystatin A **2.10a** and B **2.10b**, which are isolated from *Streptomyces eurythermus* R353-21,[26] and poststatin **2.11** isolated from *Streptomyces viridochromogenes*.[27] Both have been shown to be potent inhibitors of PED (Figure 2.8). They contain an α-ketoamide moiety, which is considered to be the active functional group for enzyme inhibition.



2.10a R =H, eurystatin A
2.10b R = Me, eurystatin B

2.11 Poststatin

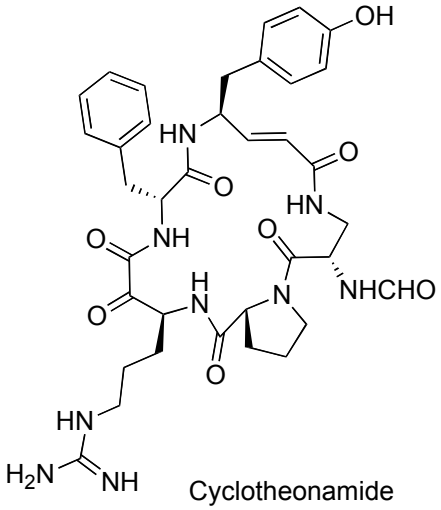
Figure 2.8. α-Ketoamides as PED inhibitors[26, 27]

α-Thrombin and trypsin inhibitors

Thrombin is a trypsin-like terminal serine protease composed of a 259-residue heavy chain and a smaller 36-residue light chain. Thrombin is present in blood and plays a role in thrombosis and hemostasis.[28] Specifically, this serine protease has a "Pro-Arg motif," meaning that the Pro-Arg core of the inhibitor binds in the S2 and S1 subsites.[1, 29,,30]

Cyclotheonamide A (CtA), a 19-membered macrocyclic peptide isolated from the marine sponge *Theonella* sp., was reported as a thrombin inhibitor with an IC_{50} value of 0.1 μM.[29] X-ray crystallography was conducted on a complex of CtA with human α-thrombin, which revealed molecular recognition within the enzyme active site. CtA

contains a Pro-Arg unit, and Arg occupies the S1 specificity pocket, while the Pro occupies the S2 site due to the Pro-Arg motif.[1] The α-ketoamide group serves as a transition-state analogue via the interaction of the electrophilic carbonyl with the hydroxyl group of Ser195 to form a tetrahedral intermediate (hemiketal).[1] In enzyme kinetics studies, CtA was only a moderate inhibitor of human α-thrombin (K_i = 0.18 μM), but was found to be a more effective inhibitor of trypsin (K_i = 23 nM) and streptokinase (K_i = 35 nM).[1]



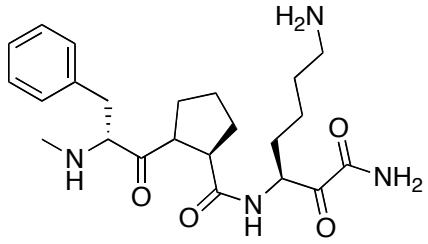
Cyclotheonamide

One important example of a novel class of divalent thrombin inhibitors is known as CVS995, which has a K_i value of 1 pM. CVS995 contains an α-ketoamide moiety that serves as a transition state mimetic, and links an active site binding group to the fibrinogen-binding exosite binding group.[31] When X-ray crystallography was used to examine a complex of CVS995 bound to thrombin, the results showed that the α-ketoamide bridging group forms a tetrahedral transition state mimic with the active site serine and histidine residues in the catalytic triad.

PP-Asp-Pro-Arg-(CO)-Gly-$(Gly)_4$-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Tyr-Leu

CVS995 sequence

Merck research laboratories developed series of α-ketoamide inhibitors of thrombin with K_i values at nanomolar levels, based on its substrate. Although some inhibitors showed only modest oral bioavailability,[32, 33] compound **2.12** had a K_i value of 0.25 nM, making it the most potent of the series. Later, kinetic studies confirmed a two-step pathway for the inhibition of thrombin by α-ketoamide transition state analogue inhibitors. The mechanism involved the formation of the complex followed by the addition of the α-ketoamide to the active site serine residue to form a hemiketal adduct.[34]

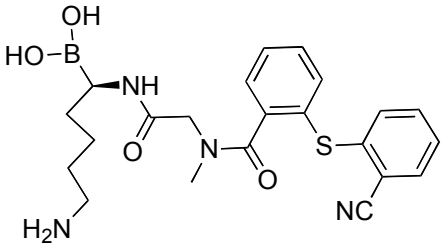


2.12

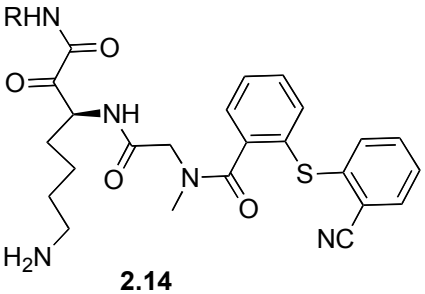
Factor Xa inhibitors

Factor Xa (fXa) plays a crucial role in the coagulation cascade with the proteolytic cleavage of prothrombin to thrombin, making its inhibition an attractive method for preventing thrombosis.[35] A study of the conformationally restricted borolysine compound **2.13** (K_i = 1.1 nM) unexpectedly led to enhanced fXa inhibition following the introduction of a nitrile moiety in the 2′ position of the diaryl thioether.[35] In order to improve the potency and selectivity of the borolysine series by extending inhibitors into the S′ site, boronic acid was replaced by lysine α-ketoamides to generate a series of compounds **2.14**. However, enhancing fXa inhibition was not achieved by extending the ketoamide into the S′ domain (Table 2.3). [35]

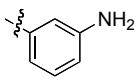
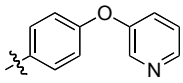
Table 2.3. Inhibition constants for fXa [35]



2.13



2.14

Compound	R	fXa K_i (nM)
2.14a		54
2.14b		120
2.14c		60

2.2.3. Cysteine protease inhibitors

Calpain inhibitors

Calpain is a family of calcium-dependent cysteine proteases. These proteases have been shown to play vital functional roles in cell cycle regulation, synaptic plasticity, and signal transduction—all of which are essential for cell maintenance.[36] There are two distinct types of calpains: calpain I, which requires micromolar concentrations of calcium, and calpain II, which requires millimolar concentrations of calcium. Calpains are linked to a number of physiological processes, making their inhibition potentially useful in the treatment of some degenerative disease states. Many transition-state inhibitors containing an electrophilic carbonyl group have been reported.[37, 38] Researchers have reported that

the mechanism of inhibition involves the formation of a tetrahedral hemithioketal upon reaction of the inhibitor carbonyl group with the active cysteine residue SH; this mimics the transition state of the hydrolysis of amide bond in the enzyme-inhibitor complex.[39, 40] Li et al synthesized a series of α-ketoamides, which they reported to be potent calpain inhibitors. Specifically, they prepared a series of dipeptidyl α-ketoamides with the general structure R_1-L-Leu-D, L-Xaa-CONH-R_2. After being evaluated as inhibitors for cysteine proteases calpain I and calpain II, the best inhibitor for calpain I was identified as compound **2.15a**. Calpain II was found to be more sensitive to these inhibitors than calpain I, making compounds **2.15b**, **2.15c** and **2.15d** the best inhibitors of calpain II (Table 2.4).[39]

Table 2.4. Inhibition constants for calpain I and II

Number	Compound	K_i
2.15a	Z-Leu–Nva–CONH–CH_2-2-pyridyl	19 nM for Calpain I
2.15b	Z-Leu–Abu–CONH–CH_2-CHOH-C_6H_5	15 nM for Calpain II
2.15c	Z-Leu–Abu–CONH–CH_2-2-pyridyl	17 nM for Calpain II
2.15d	Z-Leu–Abu–CONH–CH_2-C_6H_3-(3,5$(OMe)_2$)	22 nM for Calpain II

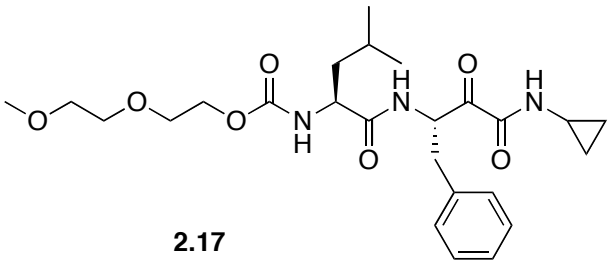
Harbeson et al[41] devised a method for the stereospecific synthesis of α-ketoamides as calpain inhibitors, and prepared a series of compounds with polar groups at the N- and C-termini to make the inhibitors more soluble. The L, L diastereomer **2.16a** was found to be the more effective active inhibitor in comparison to the diastereomer **2.16b** (Table 2.5).[41]

Table 2.5. Inhibition constants for Porcine Calpain I.[41]

Compound	K_i (nM)
Cbz–L-Leu–L-Phe–CONHEt (**2.16a**)	36 ± 2
Cbz–L-Leu–(D,L)-Phe–CONHEt (**2.16a,b**)	89 ± 6
Cbz–L-Leu–D-Phe–CONHEt (**2.16b**)	> 1500

Donkor et al produced a different K_i value for compound **2.16a** (i.e., 0.200 μM)[42] in comparison to Harbeson's value of 36 nM. However, by comparing their own assay results, Donkor's modification of α-ketoamides to α-ketohydroxamates improved the inhibition of porcine erythrocyte calpain I. This outcome suggests that hydrogen bonding and the bulky substituents on the nitrogen atom of the ketoamide affects calpain inhibition.[42]

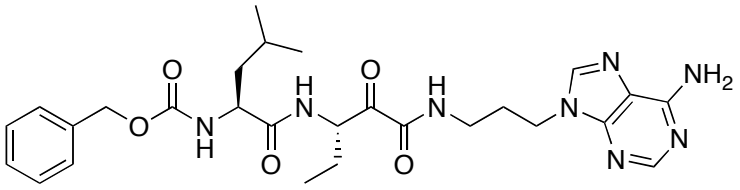
A series of α-ketoamide inhibitors were developed to improve the water solubility and metabolic stability of the corresponding aldehyde inhibitors of calpain.[43-45] By modifying the P3 site with pyridine or amphiphiles such as polyethylene glycol,[44, 45] an amphipathic ketoamide (**2.17)** was discovered with good oral bioavailability, making it a good development candidate for the treatment of retinal diseases.[46]



2.17

As reported by Qian et al, an aromatic-stacking interaction between the adenine moiety and the Trp298 side chain was observed in the co-crystal structure of the α-

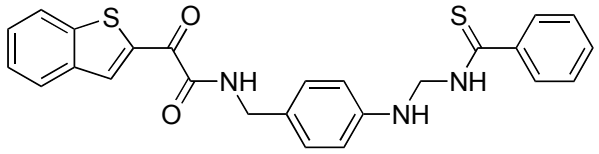
ketoamide inhibitor **2.18** with adenyl primed-side extension.[47] This study provided a direction of the design of potent inhibitors of calpain.



2.18

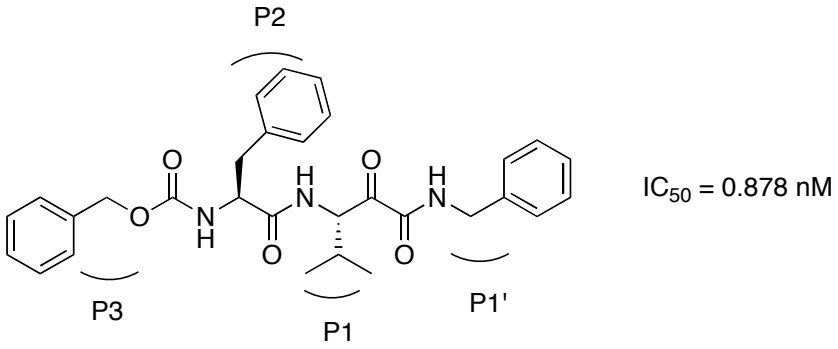
Cruzain inhibitors

Cruzain, which is a major cysteine protease of *Trypanosoma cruzi*, is central for the development and survival of a protozoan parasite that causes Chagas Disease. As such, inhibiting this particular enzyme could be useful in designing anti-parasitic drugs.[48, 49] Baldino et al[48] and Grisostomi et al[49] prepared a series of α-ketoamide derivatives as inhibitors of cysteine proteases such as cruzain, papain and cathepsin B. Specifically, they found that N-monosubstituted α-ketoamides were more potent inhibitors than disubstituted α-ketoamides, which points to the importance of hydrogen bonding interactions in the P1′ subsite. As Baldino et al described, the α-ketoamide **2.19** was found to be an effective inhibitor of certain cysteine proteases such as cruzain and cathepsin B.[48]



2.19

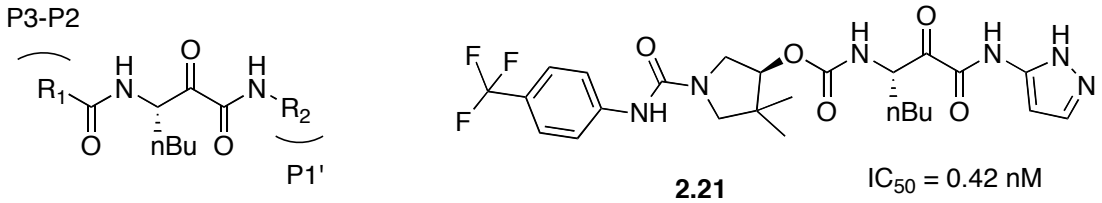
Choe et al developed a series of α-ketoamides as cruzain inhibitors with optimization at the P1, P2, P3, and P1' residues.[50] Some of them showed picomolar potency, and compound **2.20** showed one example.[50]



2.20

Cathepsin K inhibitors

Cathepsin K is a type of collagenolytic, papain-like, cysteine protease that is is predominantly expressed in osteoclasts, which are bone cells that are capable of extracting bone tissue by breaking down its mineralized matrix.[51] Osteoclasts are characterized by high expression of tartrate resistant acid phosphatase (TRAP) and cathepsin K—the latter being essential for bone resorption. Thus, the inhibition of the cathepsin K enzyme is an effective treatment for osteoporosis.[52] A significant amount of research has described the synthesis of inhibitors that are based on α-ketoamides.[53-59] Through modifications on the P1' or P2-P3 subsites (Figure 2.9), a number of compounds have been developed as selective and potent inhibitors of cathepsin K, and compound **2.21** showed nanomolar potency.[54]

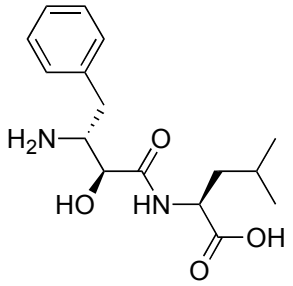


2.21

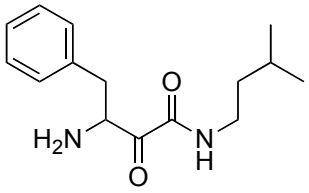
Figure 2.9. Cathepsin K development through modifications on P1' or P2-P3.[54]

2.2.4. Aminopeptidase inhibitors

Bestatin has been shown to be a potent inhibitor of aminopeptidase.[60] In order to study the inhibitory mechanisms of bestatin and its analogues, a ketone analogue of bestatin **2.22** was prepared, which is more electron-deficient than a hydroxy amide. This ketone can be hydrated in an aqueous solution to form a gem-diol that mimics the transition state of the hydrolysis of an amide bond.[60] Thus, 3-amino-2-oxo-4-phenylbutanoic acid amide **2.22** was synthesized as an effective inhibitor of arginyl aminopeptidase (K_i = 1.5 μM), cytosol aminopeptidase (K_i = 1.0 μM) and microsomal aminopeptidase (K_i = 2.5 μM).[60]



Bestatin

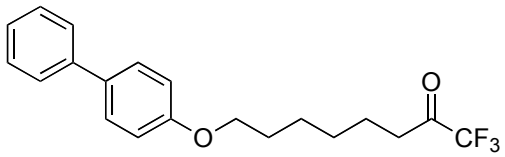


2.22

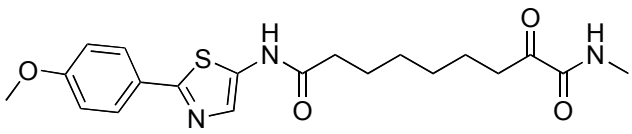
2.2.5. Histone deacetylase inhibitors

Histone deacetylases (HDACs) catalyze the removal of acetyl groups from the ε-amino groups of lysine residues at the N-terminus of nucleosomal histones.[61] HDAC inhibitors have a metal chelating group attached to an aromatic group via a hydrophobic linker. Several HDAC inhibitors have been reported such as trichostatin A (TSA) and suberoyl anilide hydroxamic acid (SAHA). The X-ray crystal structures of TSA and SAHA bound to HDAC clearly show the interactions between the inhibitors and HDAC. First, the metal binding group, a hydroxamic acid moiety, interacts with the active site, zinc. Then, a linker spans the tube-like portion of the binding pocket, after which an

aromatic "capping group" makes contacts with the pocket entrance.[61] Because hydroxamic acids are limited by a short half-life and low oral bioavailability, substitutes for this compound were investigated. Since trifluoromethyl ketones were found to inhibit zinc-dependent enzymes, ketone **2.23** was designed and found to inhibit HDAC with an IC_{50} value of 2.9 μM.[61] A series of α-ketoamides and α-ketoesters were then synthesized with the goal of overcoming the limitation that ketones are easily reduced to the corresponding alcohols in vivo. After optimizing the hydrophobic linker and the aromatic group of α-ketoamides, several ketoamide compounds were found to be potent inhibitors. In particular, compound **2.24** exhibited significant anti-tumor effects in an in vivo tumor model. Abbott scientists replaced the hydroxamate with an α-ketoamide moiety to afforded potent HDAC inhibitors as well.[62]



2.23

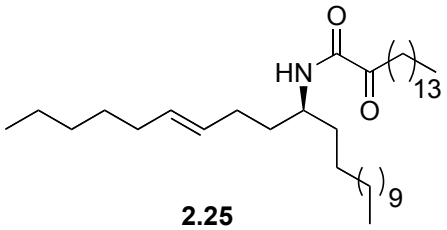


2.24

2.2.6. Pancreatic lipase inhibitors

Lipases, which are water-soluble enzymes that catalyze the hydrolysis of ester bonds in lipid substrates, have a variety of important biotechnological roles to play. For example, pancreatic and gastric lipases are essential in efficient fat digestion; thus, scientists have targeted ways to inhibit these enzymes in order to develop anti-obesity agents.[63, 64] The active site of pancreatic lipase contains Ser-His-Asp, a triad similar to the catalytic triad of serine proteases. As such, a number of active carbonyl groups can be used in the design of effective inhibitors since carbonyl groups such as α-ketoamides, α-ketoesters, and diketones have proven to be potent inhibitors of proteases. For example,

α-ketoamide triglyceride analogues were reported as inhibitors of *Staphylococcus hyicus* lipase. The α-ketoamide compound **2.25** was prepared by incorporating an α-ketoamide group into an optimized lipophilic amino alcohol backbone.[63] Its inhibition of pancreatic lipase was studied through a monomolecular film technique with mixed films of 1,2-dicaprin containing different proportions of compound **2.25**. A 50% decrease in lipase activity was observed at a 0.14 mole fraction of **2.25**.[63]



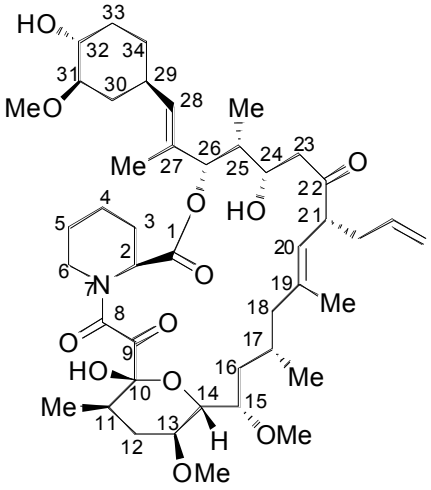
2.25

2.2.7. Peptidyl-prolyl isomerase inhibitors

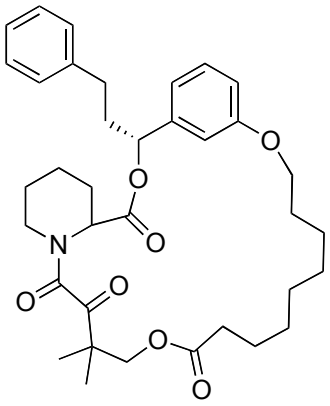
FKBP Rotamase Inhibitors

The FK506 binding protein (FKBP), which was separated and purified from human spleen cells, has been shown to be a PPIase or rotamase.[2] FK506 is a natural product containing a highly electrophilic carbonyl that is adjacent to an acyl-pipicolinyl amide bond. The α-ketoamide has been hypothesized to act in two ways: via a hypothetical tetrahedral-adduct mechanism, which is a covalent interaction, or via a hypothetical twisted-amide mechanism, which is noncovalent. Rosen et al conducted a ^{13}C NMR study of an FKBP and [8,9-^{13}C]-FK506 complex, in which the authors revealed that FKBP inhibition by FK506 is fully reversible. Moreover, Schreiber et al confirmed the absence of any covalent interactions at either the C9 or C8 carbonyl in the FKBP/FK506 complex.[2] These results suggest that the α-ketoamide of FK506 serves as a surrogate of the twisted amide transition state.

Wang and Etzkorn reviewed a series of PPIases inhibitors, including FKBP.[65] As shown in Table 2.6, two α-ketoamide compounds (FK506 and **2.26**) displayed high inhibition properties.



FK506



2.26

Table 2.6. Inhibition constants for FKBP

Compound	Inhibition of FKBP (K_i)
FK506	0.4 nM
2.26	1.0 nM

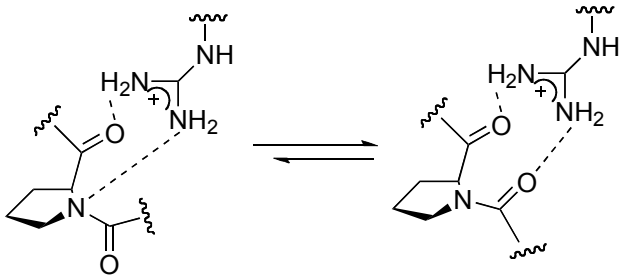
Human Cyclophilin (hCyp-18) Inhibitors

hCyp-18, a member of the human cyclophilin family, plays many significant biological roles. It is involved in the infection of T-cells by HIV-1, and is the receptor of the immunosuppressive undecapeptide drug cyclosporine A.[66] Therefore, it has attracted much interest in designing and synthesizing specific inhibitors.

Although specific mechanisms associated with proline cis-trans isomerization are still somewhat unclear, scientists have ruled out the involvement of a tetrahedral covalent intermediate by identifying the secondary deuterium kinetic isotope effect.[67] Most

research results supported the proposed mechanism of pyramidalization of the nitrogen of pyrrolidine under the assistance of Arg55, as follows: 1) Arg55-assisted deconjugation of the amide via a hydrogen-bond with the guanidinium side chain and pyramidalization of the proline nitrogen, 2) Arg55-assisted rotation of the acyl group via a hydrogen-bond with the guanidinium side chain (See Scheme 2.2).[68-70]

Scheme 2.2. Proposed mechanisms of the hCyp-18 catalyzed isomerization[70]

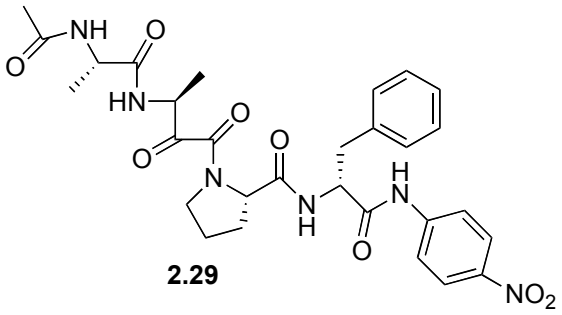


Dugave et al designed and prepared phosphonamide-containing pseudopeptides, such as compound **2.27,** as transition-state analogue inhibitors of hCyp-18.[66] The corresponding α-ketoamide **2.28** was also prepared, but it displayed poor inhibition properties (Table 2.7).[66]

Table 2.7. Inhibition constants for hCyp-18

Compound	K_i ± SD (μM)	IC_{50} ± SD (μM)
2.27 Suc-Ala-GlyΨ(PO_2Et-N)Pro-Phe-pNA	20 ± 5	15 ± 1
2.28 Ac-Ala-GlyΨ(COCO-N)Pro-Phe-pNA	127 ± 7	215 ± 40

Schultz et al developed a ketoamide-containing transition-state hapten analogue inhibitor. Compound **2.29** inhibits both cyclophilin (K_i = 14 μM) and FKBP (K_i = 11 μM).[72]



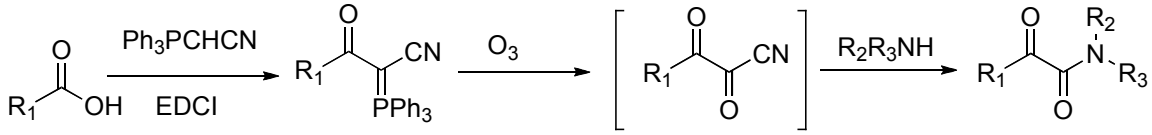
2.29

2.3. Synthetic methods for preparing α-ketoamides

2.3.1. Cyano ylide coupling methodology

Wasserman et al[73] developed a versatile strategy for the formation of α-ketoamides through the use of the (cyanomethylene)triphenylphosphorane. The reaction is shown in Scheme 2.3.

Scheme 2.3. α-Ketoamide synthesis through cyano ylide coupling[73]

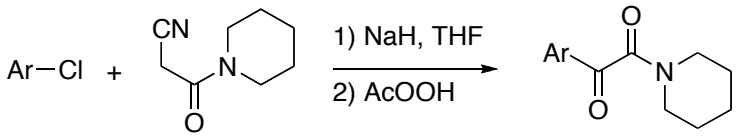


First the carboxylic acid is coupled with (cyanomethylene)triphenylphosphorane in the presence of EDC and DMAP, after which the ylide that is formed is treated with ozone to generate an α,β-diketo nitrile, which is unstable at room temperature. The resulting nitrile is then treated in situ with amines to form α-ketoamides. This method has a number of important applications—for example, in the synthesis of natural products such as eurystatin A and B, poststatin and verongamine.[73-75] Moreover, this cyano ylide coupling methodology has also been applied to various carboxylic acids. The advantage of this method is that the synthesis route is typically short and starting materials are commercially available.

2.3.2. Synthesis from heteroaryl halides

Wang and co-workers developed a one-pot synthesis of nitrogen-containing heteroaryl α-ketoamides (Scheme 2.4).[76] They used the piperidine amide of cyanoacetic acid as a synthon of the α-ketoamide moiety through an S_NAr-type reaction to heteroaryl halides.[76]

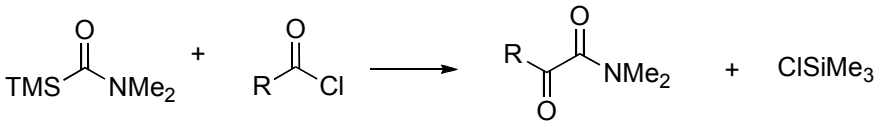
Scheme 2.4. α-Ketoamide synthesis through S_NAr-type reaction[76]



2.3.3. Synthesis from carbamoylsilane and acid chlorides

Recently, Tanaka et al described the reaction of acid chlorides with carbamoylstannane to afford good yields of α-ketoamides under mild conditions.[77] Cunico et al developed a method to synthesize α-ketoamides through the reaction of carbamoylsilane with acid chloride (Scheme 2.5)[78] which avoided the use of toxic organotin compounds.

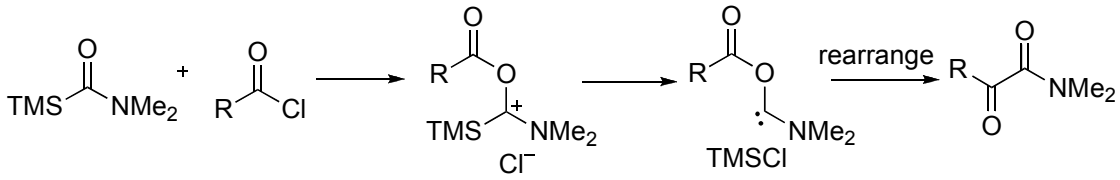
Scheme 2.5. α-Ketoamide synthesis from carbamoylsilane[78]



A mechanism for the formation of α-ketoamides from carbamoylsilane was proposed, as shown in Scheme 2.6. This method was restricted to the synthesis of *N*, *N*-dimethyl α-ketoamides or *N*, *N*-diisopropyl α-ketoamides. The resulting yield ranged from 22–85%, largely depending on the choice of acid chlorides used in the reaction,

which could include aliphatic acid chlorides, olefinic acid chlorides and perfluoro acid chlorides.

Scheme 2.6. Proposed mechanism for the formation of α-ketoamides from carbamoylsilane [78]

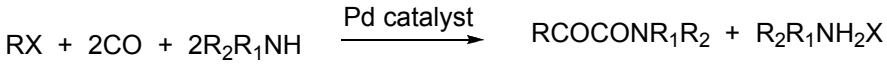


2.3.4. Transition-metal catalysis

Palladium-catalyzed double carbonylation reactions

Palladium-catalyzed double carbonylation of organic halides affords α-ketoamides in the presence of CO and amines (Scheme 2.7).[79-84]

Scheme 2.7. α-Ketoamide synthesis through palladium catalysis[79-84]



The catalytic mechanism proposed by Ozawa et al is shown in Figure 2.10.[82] This method can be used for a number of applications and typically produces good yields. A variety of organic halides such as aryl halides, alkyl halides and alkenyl halides, as well as most types of amines (e.g., proline[85]) can be used in this process. It has not yet been applied to the synthesis of dipeptide α-ketoamides.

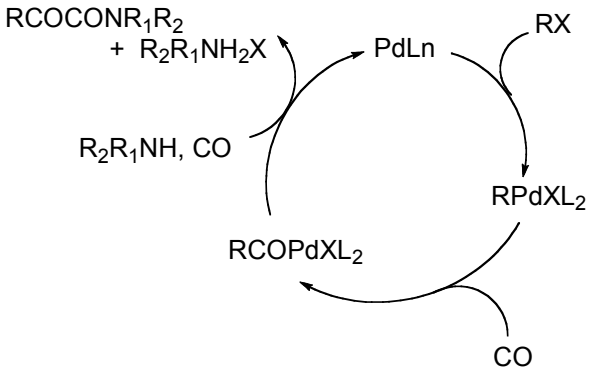
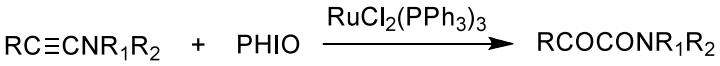


Figure 2.10. Palladium catalytic mechanism cycle[82]

Ruthenium-catalyzed reactions from alkynyl amines and PhIO

Mueller et al investigated the oxidation of alkynyl amines with PhIO under the catalysis of $RuCl_2(PPh_3)_3$, which was used to produce α-ketoamides at room temperature (Scheme 2.8).[86] Yields ranged from 40–80%, even when several kinds of substituted alkynyl amines were used. One disadvantage associated with this type of ruthenium-catalyzed reaction is the need to first synthesize the alkynyl amines as starting materials.

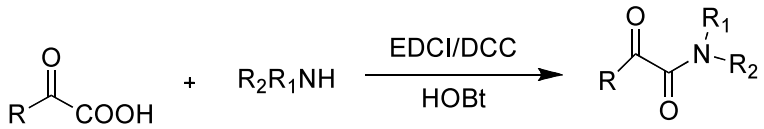
Scheme 2.8. α-Ketoamide synthesis through ruthenium catalysis[86]



2.3.5. Amidation of α-keto acids with amines

During their synthesis of cysteine inhibitors, Li et al coupled α-keto acid derivatives, which were hydrolyzed from α-keto esters, with amines to prepare a series of α-ketoamides. This coupling was performed using EDCI or DCC and HOBt.[39] In a related experiment, Chiou et al used commercially available α-keto acids to couple with primary amines to form α-ketoamides by the mixed carbonic anhydride method (Scheme 2.9).[64]

Scheme 2.9. α-Ketoamide synthesis through α-keto acid coupling[64]



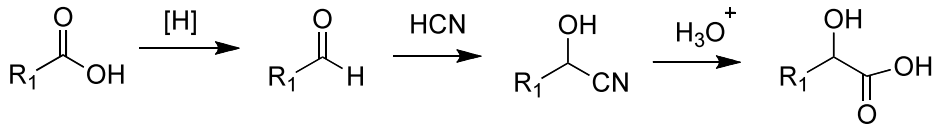
Recently, Bode and co-workers reported a method for synthesizing C-terminal peptide α-ketoacid via cyanosulfur ylide, which they used for amide-forming ligation.[87] As they reported, however, decarbonylation occurred during the coupling of the ketoacids and amines. Moroever, the usefulness of this method is limited by the stability and commercial availability of α-ketoacids.

2.3.6. α-Ketoamides from α-hydroxy acids

The synthesis of α-hydroxy acids

There are numerous methods for synthesizing α-hydroxy amides,[88, 89] the most common one being the coupling of α-hydroxy acids with amines. There are also a variety of ways to synthesize α-hydroxy acids. Scheme 2.10 shows one common method used in most of the α-ketoamide inhibitors reviewed above, which is via cyanide addition to aldehydes. The cyanide source can be KCN, NaCN[41] or Et_2AlCN.[90]

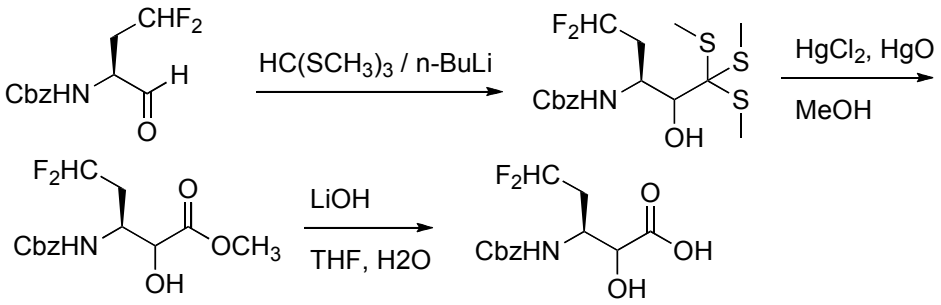
Scheme 2.10. Formation of α-hydroxy acids



Another method that uses aldehydes to produce α-hydroxy acids involves the incorporation of orthothioesters (Scheme 2.11).[91] Specifically, the aldehyde is treated with tris(methylthio) methyl lithium followed by $HgCl_2$/HgO to produce α-hydroxyl

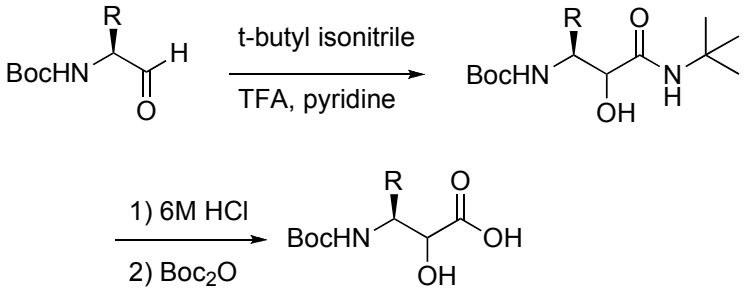
esters. Subsequent hydrolysis of the α-hydroxyl esters affords the corresponding α-hydroxyl acids.

Scheme 2.11. Formation of α-hydroxy acids via orthothioesters[91]



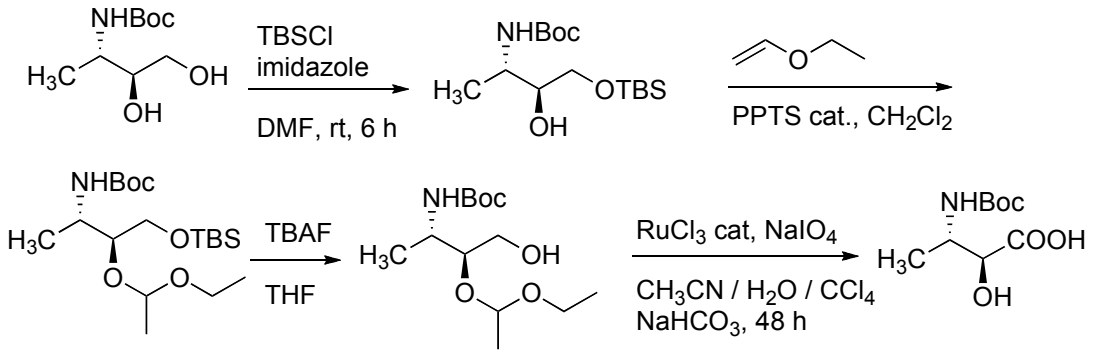
Similarly, a Passerini reaction can also be used to produce α-hydroxyl acids through the addition of aldehydes (Scheme 2.12).

Scheme 2.12. Formation of α-hydroxy acids through Passerini reaction



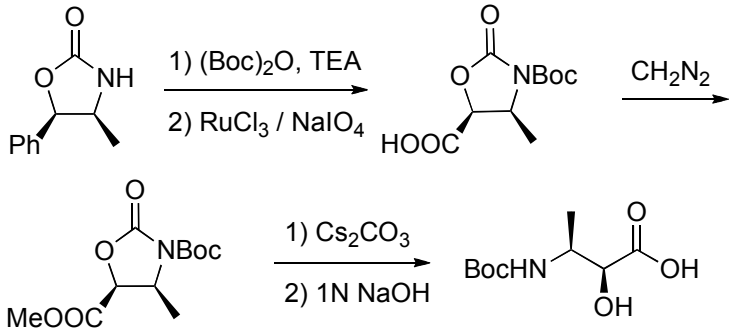
Although the stereochemistry of the carbon attached to hydroxyl group does not matter for the subsequent oxidation to ketoamide, there are ways to enantiomerically synthesize pure α-hydroxyl acids (Scheme 2.13). For example, Riera et al were able to use a diol to prepare α-hydroxyl acid in enantiomerically pure form through the orthogonal protection of the two hydroxyl groups and selective deprotection.[92]

Scheme 2.13. Synthesis of enantiomerically pure α-hydroxyl acids



Alternatively, Ma et al [72] started with oxazolidinone to afford enantiomerically pure α-hydroxyl acid too (Scheme 2.14).[72]

Scheme 2.14. Synthesis of enantiomerically pure α-hydroxyl acids through oxazolidinone[72]



The oxidation of α-hydroxy amides

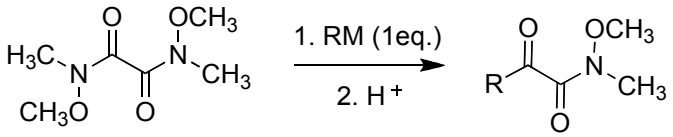
The oxidation of α-hydroxy amides is widely used in the synthesis of α-ketoamide inhibitors of enzymes.[93] Most of the synthetic routes used to produce these important inhibitors involve an oxidation step—typically via Dess-Martin, TEMPO or PDC oxidation. Although it is a common method used in peptide α-ketoamide synthesis, the synthetic route for producing α-hydroxy acids can be protracted. Moreover, the drawback of this method with respect to the oxidation step is the epimerization of the

chiral center adjacent to the ketone group, which causes the formation of a mixture of diastereomers. Harbeson et al, however, reported an oxidation by TEMPO/hypochlorite without epimerization.[41]

2.3.7. From *N*, *N*'-dimethoxy-*N*, *N*'-dimethylethanediamide with Grignard reagents

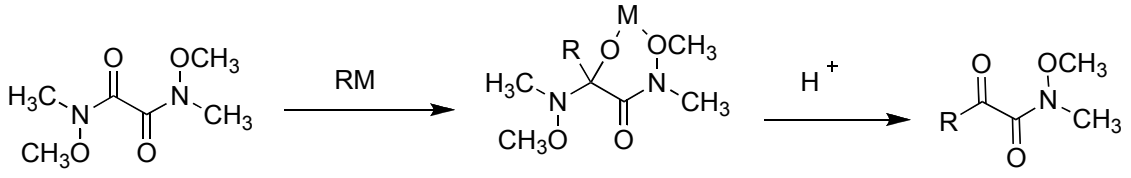
Sibi et al reported the synthesis of α-ketoamides from a 1,2-dicarbonyl-*N*, *N*′-dimethoxy-*N*, *N*′-dimethylethanediamide undergoing nucleophilic displacements with Grignard reagents (Scheme 2.15).[94]

Scheme 2.15. α-Ketoamide synthesis through diamides[94]



A mechanism is shown in Scheme 2.16. It should be noted that the key step in this reaction is the formation of a chelated intermediate, and the keto function will not be released until the hydrolysis.[94]

Scheme 2.16. Proposed mechanism for α-ketoamide synthesis through diamides[94]

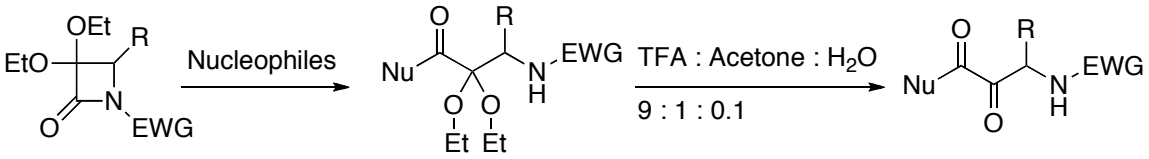


2.3.8. Azetidinone ring-opening

Khim and Nuss discussed how both primary and secondary amines work as nucleophiles in an efficient manner to open the azetidinone rings to form α-ketal amides,

followed by hydrolysis to afford α-ketoamides (Scheme 2.17).[95] As shown below, the electron withdrawing groups (EWG) can be *p*-toluenesulfonyl (*p*-Ts), *p*-nitrobenzenesulfonyl (*p*-NBS), or an allyloxycarbonyl (Alloc) group.

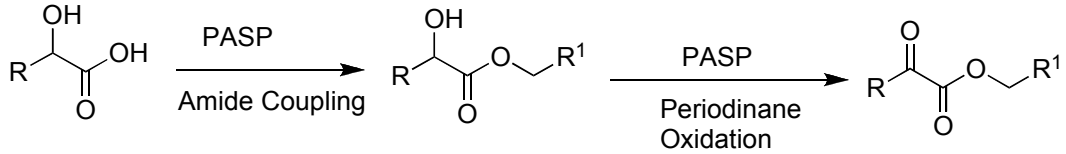
Scheme 2.17. α-Ketoamide synthesis through azetidinone ring opening[95]



2.3.9. Polymer-assisted solution-phase (PASP) library synthesis

South et al developed a method for the library synthesis of α-ketoamides using polymer-assisted solution-phase (PASP) chemistry.[96] This synthetic route features two steps. In the first step, an α-hydroxy acid was coupled with an amine in the PASP media.[96] In the second step, PASP resins were used for the Dess-Martin periodinane oxidation of α-hydroxyamides to α-ketoamides (Scheme 2.18). Products of high purity could then be obtained after simple filtration and evaporation. This research demonstrated a high-yielding (overall yield range: 50–90%), simple -purification library synthesis of α-ketoamides.[96]

Scheme 2.18. α-Ketoamide synthesis through polymer-assisted solution-phase[96]



2.3.10. Resin-bound synthesis of peptide analogues for screening as protease inhibitors

Meldal et al reported a method for the preparation of resin-bound internal α-ketoamide peptides on solid support for protease inhibitor screening.[97] The acid-labile carbonyl functionality of the α-ketoamide was protected by 1,2-ethane dithiol. The 1,3-dithiolane, which is fully compatible with standard solid-phase peptide synthesis conditions and can be easily removed by NBS in 10% aqueous acetone, was incorporated into the peptide synthesized on SPOCC-1500—a type of superpermeable organic combinatorial chemical resin.[97] This method is very useful in the solid phase synthesis of peptide α-ketoamides.

2.4. Conclusions

The syntheses and inhibitory activities of a number of α-ketoamides were reviewed. α-Ketoamides inhibit several proteases, such as serine proteases, cysteine proteases and PPIase enzymes. α-Ketoamides contain an electrophilic carbonyl group that is affected by the neighboring electron-withdrawing amide group, and they can be easily hydrated to form gem-diols or hemiketals. The tetrahedral intermediate mimics the transition state of the hydrolysis of amide bonds. The orthogonal α-ketoamide can also be a transition state analogue for PPIases because it is a surrogate of a twisted amide.

There are many synthetic methods for generating α-ketoamides, including the cyano ylide coupling methodology, synthesis from carbamoylsilane and acid chlorides, transition-metal-catalyzed double carbonylation, amidation of α-keto acids with amines, oxidation from α-hydroxyl amides, and Grignard reagent nucleophilic addition. A solid

phase synthesis for a library of α-ketoamides was also reviewed, which may be useful for developing a variety of inhibitors for biological applications, such as anti-cancer drugs.

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Part B: Synthesis and bioassay of α-ketoamide inhibitors of Pin1

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Convergent synthesis of α-ketoamide inhibitors of Pin1

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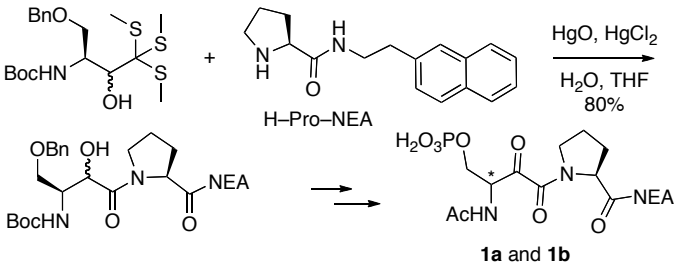
ABSTRACT



A convergent synthesis of α-ketoamide inhibitors of Pin1 is described. An α-hydroxyorthothioester derivative of Ser was reacted directly with an amine synthon. The reaction was catalyzed by HgO and $HgCl_2$ to form α-hydroxyamide. Thus, hydrolysis and coupling were combined in one step with 80% yield. Two diastereomers of a phospho-Ser-Pro α-ketoamide analogue were synthesized. The IC_{50} values of 100 μM and 200 μM were surprisingly weak for Pin1 peptidyl-prolyl isomerase.

α-Ketoamides have been widely used in developing inhibitors of peptidases,[1] HDACs[2,3] and peptidyl prolyl isomerases (PPIases).[4] One of the best studied immunosuppressant drugs, FK506 has an α-ketoamide, and it is a transition state

analogue inhibitor of FK506 binding proteins (FKBPs).[5] α-Ketoamides have been used to inhibit several classes of proteases, such as serine proteases,[6-10] cysteine proteases[11-14] and HIV and FIV proteases.[15]

α-Ketoamides have an electron-deficient carbonyl due to the effect of the neighboring electron-withdrawing amide. α-Ketoamide containing inhibitors have been used to elucidate the mechanisms of enzymes, because they serve as transition state analogues to inhibit enzyme catalytic activity. This occurs for serine or cysteine proteases through the formation of a tetrahedral intermediate analogue (gem-diol or hemiketal) with the enzyme upon binding.[16] FK506, a macrolide with an α-ketoamide functional group, has a dihedral angle of 95° to 100° between the two carbonyl groups.[5] NMR studies of a ^{13}C-labeled structure and a crystal structure of FK506 showed that the two carbonyls were orthogonal both in bound and unbound structures, indicating that FK506 acts as a "twisted amide" transition state analogue to inhibit FKBPs.[5]

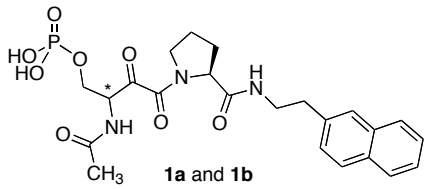
Pin1, a PPIase enzyme belonging to the parvulin family, specifically recognizes pSer/pThr–Pro containing substrates, and regulates many cellular events such as cell cycle progression, transformation, and cell proliferation.[17,18] Pin1 is overexpressed in many cancer cell lines, and plays an important role in oncogenesis.[19,20] Pin1 is a potential diagnostic and therapeutic anti-cancer target.[21-23] Thus, we are interested in developing potent and specific inhibitors of Pin1.[24,25]

Since α-ketoamides are an important class of enzyme inhibitors, numerous synthetic methods have been reported, including: cyano ylide coupling,[26] transition-metal-catalysis,[27] amidation of α-keto acids with amines,[11,28] oxidation of α-hydroxyamides with Dess-Martin periodinane[6] or TEMPO,[29,30] and nucleophilic addition

of a Grignard reagent to a Weinreb amide.[31] A solid-phase method for synthesis of α-ketoamides has been developed.[32] In this study, we have developed a convergent route to synthesize α-ketoamides by coupling a complex amine directly to an α-hydroxyorthothioester to form an α-hydroxyamide, which was then oxidized to an α-ketoamide with the Dess-Martin periodinane.[33]

We have reviewed Pin1 inhibitors, including synthetic inhibitors, and natural products.[4,23] α-Ketoamides are an important class of inhibitor for PPIases (e.g. FK506); the co-crystal structure of FK506 with FKBP was the main evidence cited to support the "twisted amide" transition state for PPIases.[5,34] But α-ketoamides have not yet been reported as Pin1 inhibitors. Our impetus for synthesizing these challenging phosphorylated α-ketoamides was to investigate the ability of these compounds to inhibit Pin1, the only PPIase that regulates the cell cycle.

α-Ketoamides **1a** and **1b** were designed based on the specificity of Pin1 for the pSer/pThr–Pro motif.[18] An acetyl was chosen for the *N*-terminus, since co-crystal structures of Pin1-inhibitor complexes showed no electron density for *N*-terminal residues, and to improve water solubility over Fmoc derivatives.[25,35,36] An aromatic naphthyl was introduced at the *C*-terminus based on substrate and inhibitor sequence specificities.[18,36,37]

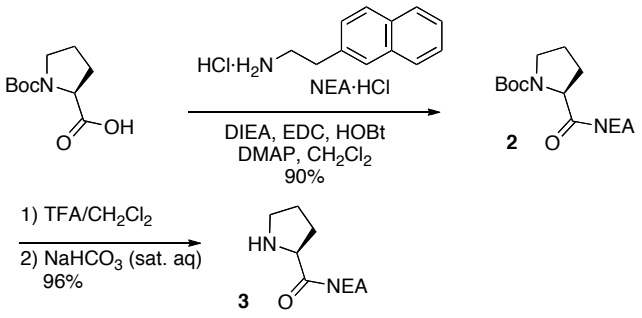


1a and **1b**

Boc–Pro–OH and 2-(2-naphthyl)-ethylamine (NEA) were coupled with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide·HCl (EDC) to give Boc–Pro–NEA **2** (Scheme

2.19). Free amine **3** was prepared by TFA deprotection of **2**, followed by neutralization with aqueous $NaHCO_3$.

Scheme 2.19. Synthesis of H–Pro–NEA **3**.

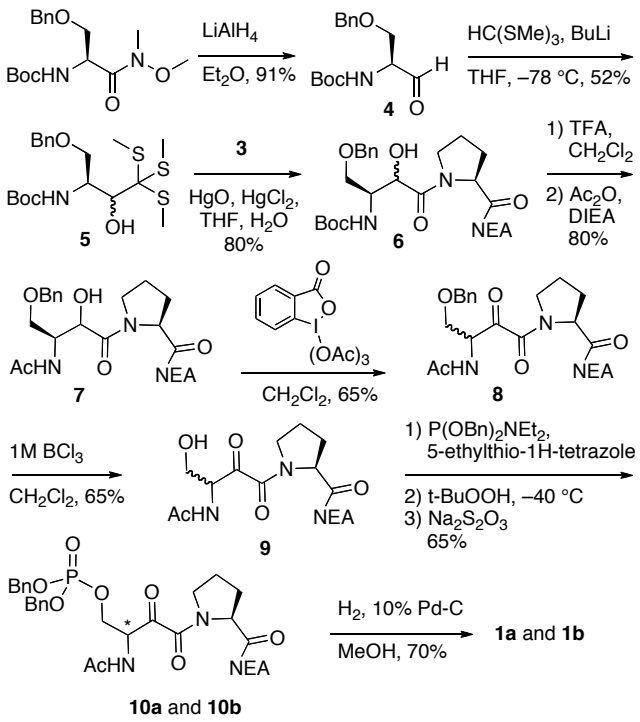


Aldehyde **4** was obtained by $LiAlH_4$ reduction of the Weinreb amide of protected Ser (Scheme 2.20).[38] Lithio tris-methylthiomethane was added to aldehyde **4** to give orthothioester **5**.[39] The two diastereomers of orthothioester **5** were not separated at this stage, since the newly formed stereocenter would be eliminated in the subsequent oxidation of alcohol **7** to ketone **8**. Orthothioester **5** was converted directly to α-hydroxyamide **6** by aminolysis.

The Boc group of **6** was removed with TFA, and the resulting amine was selectively acetylated with one equivalent of acetic anhydride in the presence of the free hydroxyl to give **7** (Scheme 2.20). Alcohol **7** was oxidized with the Dess-Martin reagent to form α-ketoamide **8**.[6,33] Although the Dess-Martin oxidation is known to produce the least epimerization alpha to electrophilic ketones,[6,40,41] epimerization occurred either during oxidation or chromatography, but the diastereomers (1:1) were not separated at this stage. This epimerization occurs easily due to the strongly electron-withdrawing ketoamide group, and has been observed in several syntheses of α-ketoamides.[15,29] Epimerization of α-ketoesters has been observed to occur in solution upon standing for one week.[41] Both

phospho-L-Thr and phospho-D-Thr containing inhibitors of Pin1 have been reported, and the D-Thr inhibitors were more potent.[36,37] We wanted to test both diastereomers of **1** for inhibition, so we did not attempt to improve the stereochemical purity of α-ketoamide **8**.

Scheme 2.20. Synthesis of inhibitors **1a** and **1b**.



The benzyl on the side chain of serine was removed with BCl_3 to give alcohol **9**.[42,43] Reverse-phase, semi-preparative HPLC purification gave a mixture of diastereomers of **9** in a 1:1 ratio as determined by ^{1}H NMR (Supporting Information). The 86 ppm ^{13}C NMR peak was assigned as the hydrated ketone carbon based on the literature, and the assignment below for **10a**.[1] The ketone carbonyl stretch did not appear in the IR at ca. 1720 cm^{-1}, which also indicates hydration of the ketone.

Alcohol **9** was phosphorylated with dibenzyl *N*,*N*-diethylphosphoramidite, followed by oxidation with *t*-BuOOH to produce dibenzylphosphate **10**.[24,44] The diastereomers of **10** were isolated by reverse-phase semi-preparative HPLC. The ketone

carbonyl for both diastereomers **10a** and **10b** appeared in the IR, but not in the ^{13}C NMR. The 84 ppm peak in the ^{13}C, and the 4.74 ppm peak in the ^{1}H NMR were assigned by HMBC as the hydrated ketone (Supporting Information). The ketone carbonyl stretch appeared in the IR at 1723 cm^{-1}, but much weaker than expected, indicating partial hydration.

Hydrogenolysis of each diastereomer of **10** under neutral conditions furnished two diastereomers of the final compound, **1a** and **1b**. Compounds **1a** and **1b** showed no ketone carbonyl in both the ^{13}C and IR spectra. Since we found that both diastereomers were weak Pin1 inhibitors, *vide infra*, we chose not to determine the absolute stereochemistry of the inhibitors.

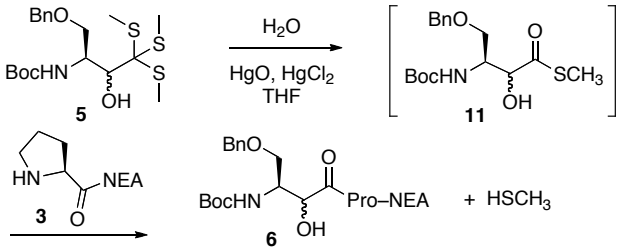
In our method, orthothioester **5** was directly reacted with the fully formed right half of the molecule, the amine H–Pro–NEA **3**, as the nucleophile to produce amide **6** (Scheme 2.20). Orthothioester **5** was used as a carboxylic acid synthon instead of a nitrile to avoid the toxicity of NaCN or KCN. In addition, conditions for the hydrolysis of a nitrile are incompatible with amides.[29]

This synthesis of the target compound was convergent compared with previous linear syntheses of α-ketoamides via α-hydroxy acids. Previously, orthothioesters were either hydrolyzed to a carboxylic acid and coupled with an amine, or methanolyzed to a methyl ester.[39,45,46] Methyl esters require the additional step of hydrolysis before coupling, while α-hydroxy acids are difficult to isolate and purify. Instead, the H–Pro–NEA amine was coupled in one step to the α-hydroxyorthothioester. We attempted ozonolysis of a cyano ylide to form the α-ketoamide functionality using Wasserman's method.[9] However, due to the instability of the α-ketoamide in subsequent Pro ester hydrolysis and

coupling to NEA, we chose to use aminolysis of thioester **5** with amine **3** to form the complete skeleton prior to oxidation to the ketoamide.

The aminolysis was catalyzed by HgO and $HgCl_2$, similar to hydrolysis of orthothioesters.[39,45] Thus, the α-hydroxyamide **6** was formed without additional coupling reagents or steps. We propose that the key step in the reaction involves Hg(II)-catalyzed hydrolysis of the orthothioester to a methyl thioester intermediate **11** (Scheme 2.21).[45,47] The THF used as the solvent was wet, which provided the water for the hydrolysis. Aminolysis with the amine nucleophile present in situ then gave amide **6** directly. Thus, hydrolysis and coupling were combined in a single step with 80% yield. Although the overall yield in this 12-step synthesis is not high (6% for the 9 linear steps), the aminolysis to form the key intermediate for α-ketoamides should be very useful in a variety of syntheses.

Scheme 2.21. Proposed mechanism for hydroxyamide **6** formation.



The α-chymotrypsin protease-coupled assay, with succinyl–Ala–Glu–*cis*-Pro–Phe–*p*-nitroanilide (*p*NA) as the substrate, was used to measure inhibition of Pin1 by the two diastereomers of **1**.[24] The absorbance of *p*NA released from the PPIase trans product by chymotrypsin was recorded continuously at 390 nm by UV/Vis. The IC_{50} values of the two diastereomers were determined to be 100 ± 20 μM and 200 ± 20 μM.

Surprisingly, neither diastereomer was a potent inhibitor of Pin1. These inhibitors

were less potent than a similarly substituted, ground-state analogue, Fmoc–pSer–Ψ[(*Z*)CH=C]–Pro–(2)-*N*-(3)-ethylaminoindole (IC_{50} = 28 μM).[25] Since the α-ketoamide of FK506 acts as a transition state analogue to inhibit FKBPs,[5] we expected more potent inhibition of Pin1 by at least one of the α-ketoamide stereoisomers. There are two proposed mechanisms for Pin1 catalysis: the twisted-amide mechanism,[5,48,49] and the Cys-113 tetrahedral intermediate mechanism.[50] The hemiketal of an α-ketoamide has been shown to act as a tetrahedral transition-state analogue for a serine protease.[16] We anticipated that the ketoamide could act as a potent electrophile if the Cys-113 thiol of Pin1 were positioned for nucleophilic addition. The twisted amide mechanism is not supported by this poor inhibition either. Although a hydrated ketone would preclude either Cys-113 nucleophilic addition, or the orthogonal carbonyl conformation necessary to mimic the twisted amide transition state, as in FK506, the hydration is reversible. The ketone state should be accessible for binding to the enzyme if it were a favorable analogue of the transition state.

Although the two diastereomers of ketoamides were weak inhibitors of Pin1, we have provided a convergent method for synthesizing α-ketoamide peptidomimetics, which is a very useful class of enzyme inhibitors, especially for proteases. Many α-ketoamides have been developed as drugs, and our convergent method contributes to the available methods for their synthesis.

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Supporting Information Available: Experimental procedures, HPLC and spectroscopic data for compounds **1**-**10**, and inhibition data for compound **1**. This

material is available free of charge *via* the Internet at http://pubs.acs.org.

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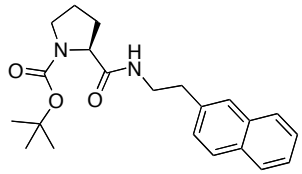
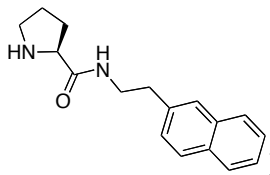
Experimental Section

General Procedures. Unless otherwise indicated, all reactions were carried out under dry N_2 in flame-dried glassware. THF was distilled from sodium-benzophenone, and CH_2Cl_2 was dried by passage through dry alumina. Anhydrous DMF (99.8%), MeOH, and DIEA were used directly from sealed bottles. TFE (99+%) was distilled from sodium before use. LiCl (99+%) was dried under vacuum at 150 °C for 24 h. Brine (NaCl), $Na_2S_2O_3$ and NH_4Cl refer to saturated aqueous solutions, and $NaHCO_3$ refers to a 5% aqueous solution, unless otherwise noted. Flash chromatography was performed on 230–400 mesh silica gel with reagent grade solvents. Analytical HPLC were obtained on a C18 4.6 × 50 mm column with 10% CH_3CN/H_2O for 3 min followed by a 10% to 90% CH_3CN/H_2O gradient over 6 min unless otherwise noted. HPLC results are reported as retention time, integrated % purity. NMR spectra were obtained at ambient temperature in $CDCl_3$ unless otherwise noted. ^{1}H-, ^{13}C-, and ^{31}P-NMR spectra were obtained at 500, 125, and 162 MHz, respectively, unless otherwise noted. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane (TMS). Data are reported as follows: chemical shift, multiplicity: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), broad singlet (br s) coupling constants *J* in Hz, and integration. In ^{13}C-NMR spectral listings, minor rotamers are labeled as (mr), and minor diastereomers are labeled as (md).



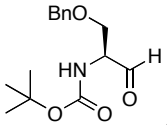
Boc–Pro–2-(2-naphthyl)ethylamine (NEA) 2. 2-(2-Naphthyl)ethylamine hydrochloride (3.00 g, 14.4 mmol) and DIEA (5.58 g, 43.2 mmol)

were dissolved in CH_2Cl_2 (200 mL) and DMF (20 mL). Boc–Pro–OH (3.42 g, 15.9 mmol), EDC (3.30 g, 17.3 mmol), HOBt (2.65 g, 17.3 mmol) and DMAP (0.53 g, 4.3 mmol) were added. After stirring at rt for 24 h, the reaction mixture was diluted with EtOAc (500 mL), and washed with water (2 × 200 mL), 1N HCl (3 × 200 mL), $NaHCO_3$ (3 × 200 mL) and brine (200 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. Crystallization from EtOAc yielded Boc–Pro–NEA **2** (4.3 g, 81%) as white needle crystals. Anal. HPLC, 7.4 min, 99.6% pure; mp 180-182; ^{1}H NMR δ 7.79 (m, 3H), 7.62 (s, 1H), 7.43 (m, 1H), 7.46 (m, 1H), 7.32 (dd, *J* = 1.2, 8.5, 1H), 6.90 (br s, 0.5H), 6.10 (br s, 0.5H), 4.22 (m, 1H), 3.66 (app septet, *J* = 6.9, 1H), 3.58 (m, 1H), 3.4 (br s, 0.5H), 3.26 (br s, 1.5H), 2.98 (m, 1H), 2.96 (app septet, 1H), 2.32 (br s, 0.5H), 2.08 (br s, 1H), 1.80 (br s, 2.5), 1.36 (s, 9H). ^{13}C NMR (DMSO-d_6) δ 172.4, 172.0 (mr), 153.6 (mr), 153.3, 137.1 (mr), 137.0, 133.1, 131.7, 127.7, 127.6 (mr), 127.5 (mr), 127.43, 127.41, 127.3, 126.8 (mr), 126.6, 125.95, 125.89 (mr), 125.3, 78.5 (mr), 78.3, 59.9, 59.6 (mr), 46.6 (mr), 46.4, 39.9, 35.4, 35.2 (mr), 31.0, 30.0 (mr), 28.1 (mr), 28.0, 23.8 (mr), 23.1; HRMS (EI$^+$) calcd for $C_{22}H_{28}N_2O_3$ [M]$^+$ *m/z* = 368.2100, found *m/z* = 368.2107.

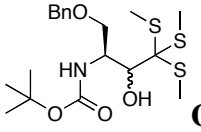


H–Pro–NEA 3. Boc–Pro–NEA **3** (4.3 g, 12 mmol) was dissolved in CH_2Cl_2 (100 mL), and a mixture of TFA (50 mL) and water (1 mL) was added. The reaction was stirred at rt for 2 h. After evaporation, the residue was dissolved in $CHCl_3$ (200 mL), and washed with $NaHCO_3$ (100 mL). The aqueous layer was extracted with $CHCl_3$ (2 × 80 mL). The organic layers were combined, dried over Na_2SO_4,

and filtered. Evaporation provided H–Pro–NEA **3** (3.0 g, 96%) as a white powder. Anal. HPLC, 4.8 min, 98.7% pure; ^{1}H NMR δ 7.78 (m, 3H), 7.68 (br s, 1H), 7.63 (s, 1H), 7.46 (dt, *J* = 1.6, 6.9, 1H), 7.43 (dt, *J* = 1.6, 6.9, 1H), 7.34 (dd, *J* = 1.8, 8.4, 1H), 3.68 (dd, *J* = 5.2, 9.4, 1H), 3.64 (dt, *J* = 13.4, 6.8, 1H), 3.53 (dt, *J* = 13.1, 7.0, 1H), 2.97 (t, *J* = 7.0, 2H), 2.90 (dt, *J* = 6.9, 10.2, 1H), 2.74 (dt, *J* = 6.2, 10.2, 1H), 2.08 (m, 1H), 1.87 (m, 1H), 1.86 (s, 1H), 1.62 (app quint, *J* = 6.9, 2H); ^{13}C NMR δ 175.3, 136.8, 133.7, 132.3, 128.3, 127.8, 127.5, 127.4, 127.3, 126.2, 125.5, 60.7, 47.3, 40.0, 36.1, 30.8, 26.2; HRMS (EI^{+}) calcd for $C_{17}H_{20}N_2O$ [M]$^{+}$ *m/z* = 268.1576, found *m/z* = 268.1579.

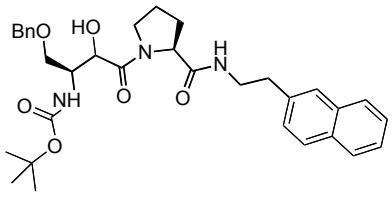


Aldehyde 4.[1] By a modification of the method of Dragulescu et al[1] To a suspension of $LiAlH_4$ (31 mg, 0.83 mmol) in Et_2O was added a solution of Boc–Ser(OBn)–$N(CH_3)OCH_3$,[2] (0.20 g, 0.59 mmol) in Et_2O (5 mL) below 5 °C. After 30 min, EtOAc (20 mL) was added slowly, and then 5% $KHSO_4$ (20 mL) was added. The organic layer was washed with 1N HCl (3 × 20 mL), saturated $NaHCO_3$ (3 × 20 mL), and brine (20 mL). The organic phase was dried with Na_2SO_4, filtered, and evaporated to give aldehyde **4** (150 mg, 91%) as a slightly yellow oil, which was used without further purification. ^{1}H NMR δ 9.63 (s, 1H), 7.36 (m, 5H), 5.42 (d, *J* = 6.3, 1H), 4.53 (d, *J* = 12, 1H), 4.49 (d, *J* = 12, 1H), 4.32 (dt, *J* = 7.3, 3.4, 1H), 4.00 (dd, *J* = 3.2, 9.7, 1H), 3.71 (dd, *J* = 4.1, 9.7, 1H), 1.46 (s, 9H); ^{13}C NMR δ 199.3, 155.8, 137.4, 128.7, 128.1, 127.8, 80.4, 73.7, 68.0, 60.2, 28.4.



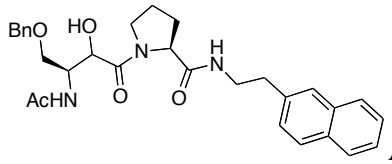
Orthothioester 5. Lithio tris-methylthiomethane ($LiC(SMe)_3$) was generated at –78°C by the addition of *n*-butyl lithium (2.5 M in hexane, 0.53 mL, 1.3

mmol) to a solution of $HC(SMe)_3$ (204 mg, 1.32 mmol). The mixture was stirred at –78°C for 2 h. A solution of aldehyde **4** (185 mg, 0.662 mmol) in THF (5 mL) was added slowly to the $LiC(SMe)_3$ at –78°C. The reaction was stirred at –78°C for 1 h, and quenched with NH_4Cl (30 mL). After extraction with EtOAc (50 mL), the organic layer was washed with NH_4Cl (30 mL), brine (30 mL), and dried over Na_2SO_4. After filtration and evaporation, the residue was purified by chromatography with 17% EtOAc/hexanes to give orthothioester **5** (150 mg, 52%) as a colorless oil. Anal. HPLC, 6.1 min, 96.0%; ^{1}H NMR δ 7.29 (m, 5H), 5.38 (d, J = 7.2, 1H), 4.60 (d, J = 11.7, 1H), 4.52 (d, J = 11.7, 1H), 4.41 (m, 1H), 4.11 (m, 1H), 3.58 (m, 2H), 3.29 (br s, 1H), 2.22 (s, 0.9H), 2.19 (s, 8.1H), 1.44 (s, 9H). (Most minor diastereomer peaks were too small to integrate.) ^{13}C NMR δ 155.6, 138.1, 128.6 (md), 128.5, 128.0 (md), 127.8, 127.7, 81.6 (md), 79.4, 77.4, 74.8, 74.3 (md), 73.7 (md), 73.1, 72.0, 71.9 (md), 51.3 (md), 49.6, 28.6, 28.5 (md), 14.3 (md), 14.0. MS (ESI^+) calcd for $C_{19}H_{31}NO_4S_3 \cdot Na$ $[M + Na]^+$ m/z = 456.1, found m/z = 456.3.



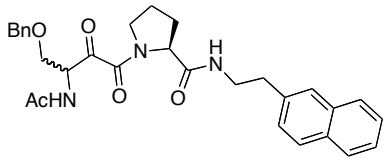
α-Hydroxyamide 6. Orthothioester **5** (600 mg, 1.38 mmol) and H–Pro–NEA **3** (556 mg, 2.07 mmol) were dissolved in wet THF (100 mL), and HgO (1.49 g, 6.90 mmol) and $HgCl_2$ (600 mg, 2.21 mmol) were added. The suspension was stirred at rt for 2 h, filtered through Celite, and the Celite was washed with EtOAc (500 mL). The filtrate was washed with 1N HCl (3 × 150 mL), $NaHCO_3$ (3 × 150 mL), and brine (150 mL). The organic layer was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by chromatography with 66% EtOAc/hexanes to give one major diastereomer of α-hydroxyamide **6** (0.60 g, 80%). Anal. HPLC, 5.0 min,

98.5%; ^{1}H NMR (60 °C) δ 7.77 (app t, *J* = 7.6, 3H), 7.60 (s, 1H), 7.42 (app t, *J* = 6.1, 1H), 7.38 (app t, *J* = 6.6, 1H), 7.30 (m, 6H), 6.78 (br s, 1H), 4.66 (d, *J* = 9.7, 1H), 4.56 (d, *J* = 12, 1H), 4.51 (d, *J* = 12, 1H), 4.36 (d, *J* = 5.5, 1H), 4.32 (d, *J* = 7.4, 1H), 4.10 (m, 1H), 3.62 (m, 2H), 3.52 (m, 4H), 3.23 (m, 1H), 2.97 (m, 2H), 2.30 (m, 1H), 1.86 (m, 3H), 1.37 (s, 9H); ^{13}C NMR δ 172.7 (mr), 172.0, 170.9, 170.7 (mr), 156.5 (mr), 155.7, 138.1, 136.5, 133.65 (mr), 133.62, 132.3, 128.59 (mr), 128.57, 128.28, 128.23 (mr), 127.9, 127.77, 127.75 (mr), 127.68, 127.5, 127.46 (mr), 127.36, 127.33, 127.2 (mr), 126.21, 126.16 (mr), 125.6, 125.5 (mr), 80.5 (mr), 79.9, 73.4, 73.1 (mr), 69.1, 68.7 (mr), 68.3, 67.8 (mr), 61.8 (mr), 61.3, 52.6 (mr), 50.3, 46.7 (mr), 46.4, 40.63 (mr), 40.58, 35.9, 35.1 (mr), 32.1 (mr), 28.36 (mr), 28.34, 27.3, 24.8, 20.9 (mr); MS (ESI^{+}) calcd for $C_{33}H_{41}N_3O_6 \cdot Na$ [M + Na]$^{+}$ *m*/*z* = 598.3, found *m*/*z* = 598.5.



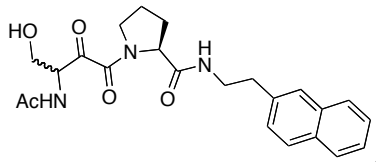
Acetyl α-hydroxyamide 7. Boc α-hydroxyamide **6** (96 mg, 0.17 mmol) was dissolved in CH_2Cl_2 (15 mL), and TFA (8 mL) was added. The mixture was stirred at rt for 1 h. The reaction mixture was then concentrated under reduced pressure. The ammonium salt obtained was then dissolved in CH_2Cl_2 (15 mL), and acetic anhydride (17 mg, 0.17 mmol) and DIEA (50.0 mg, 0.39 mmol) were added. The reaction mixture was stirred at rt for 1 h. After dilution with CH_2Cl_2 (50 mL), the mixture was washed with 1N HCl (2 × 20 mL), 1N NaOH (2 × 20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4. Filtration followed by evaporation yielded **7** (68 mg, 80%) as a yellow oil. Anal. HPLC, 5.7 min, 95.9%; IR (film) 3295 (N–H), 1639 (amide C=O), 1534 (N–H), 1451 (C–N), 1095 (C–O) cm^{-1}; ^{1}H NMR δ 8.27 (t, *J* = 5.5, 0.2H), 7.78 (m, 3H), 7.67 (s, 0.2H), 7.62 (s, 0.8H), 7.43 (m, 2H), 7.33 (m, 5H),

6.64 (t, J = 5.5, 0.8H), 5.64 (d, J = 9.3, 0.8H), 5.61 (d, J = 10.2, 0.2H), 4.58 (d, J = 12.1, 0.8H), 4.53 (d, J = 12.1, 0.8H), 4.44 (m, 2.4H), 4.26 (m, 1H), 3.76 (m, 1H), 3.64-3.50 (m, 3.8H), 3.47 (dd, J = 5.5, 9.1, 1H), 3.37 (dd, J = 5.5, 9.1, 0.2H), 3.32 (m, 0.8H), 3.23 (m, 0.2H), 3.05 (m, 0.4H), 2.99 (ddd, J = 7.0, 13.9, 20.9, 0.8H), 2.92 (ddd, J = 7.0, 13.9, 20.9, 0.8H), 2.30 (m, 0.2H), 2.21 (m, 0.8H), 1.95 (s, 0.6H), 1.93 (m, 1H), 1.89 (s, 2.4H), 1.66 (m, 2H); ^{13}C NMR δ 172.6 (mr), 171.7, 171.4 (mr), 171.0, 170.7 (mr), 170.4, 137.9, 137.7 (mr), 136.7 (mr), 136.5, 133.67 (mr), 133.63, 132.35, 132.32 (mr), 128.6, 128.3, 128.1 (mr), 128.05 (mr), 127.97, 127.82 (mr), 127.78, 127.74, 127.5, 127.42 (mr), 127.38, 127.31, 127.2 (mr), 126.2, 126.1 (mr), 125.6, 125.5 (mr), 73.5, 73.2 (mr), 68.9, 68.6 (mr), 68.1, 67.9 (mr), 61.8 (mr), 61.2, 51.8 (mr), 49.0, 46.9 (mr), 46.6, 40.8 (mr), 40.6, 35.8, 35.4 (mr), 32.2 (mr), 27.7, 24.8, 23.3, 21.1 (mr); MS (ESI$^+$) calcd for $C_{30}H_{36}N_3O_5$ [M + H]$^+$ m/z = 518.2, found m/z = 518.8.



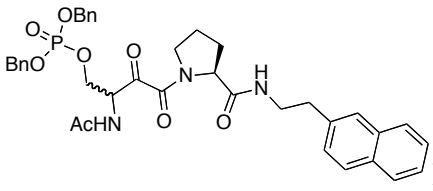
α-Ketoamide 8. Acetyl α-hydroxyamide **7** (40 mg, 0.077 mmol) was dissolved in CH_2Cl_2 (4 mL), and Dess-Martin periodinane (1,1,1-Tris(acetyloxy)-1,1-dihydro-1,2-benziodoxol-3-(1H)-one) (0.3 M in CH_2Cl_2, 0.80 mL, 0.24 mmol) was added dropwise. The reaction mixture was stirred at rt for 3 h. EtOAc (50 mL), 10% $NaHCO_3$ (25 mL), and $Na_2S_2O_3$ (25 mL) were added. After separation, the aqueous layer was extracted with EtOAc (2 × 30 mL). The combined organic layers were dried with Na_2SO_4. After concentration, the residue was purified by chromatography in EtOAc to give a pair of diastereomers of α-ketoamide **8** (26 mg, 65%) as a yellow oil. Anal. HPLC, 4.9 min, 97.7%; IR (film) 3300 (N–H), 1729 (ketone C=O), 1640 (amide C=O), 1534 (N–H), 1438 (C–N), 1100 (C–O) cm^{-1}; ^{1}H NMR δ 7.78 (m, 3H), 7.65 (s,

0.4H), 7.62 (s, 0.4H), 7.59 (s, 0.2H), 7.43 (m, 2H), 7.31 (m, 4H), 7.23 (m, 2H), 6.70 (t, *J* = 5.4, 0.4H), 6.52 (t, *J* = 5.4, 0.4H), 6.36 (d, *J* = 7.2, 0.1H), 6.27 (d, *J* = 6.0, 0.5H), 6.22 (d, *J* = 6.0, 0.4H), 6.04 (t, *J* = 5.4, 0.1H), 5.88 (t, *J* = 5.4, 0.1H), 5.33 (m, 0.1H), 5.11 (m, 0.8H), 4.67 (dd, *J* = 2.3, 8.1, 0.1H), 4.45 (m, 3H), 4.09 (dd, *J* = 4.2, 9.8, 0.1H), 3.94 (dd, *J* = 5.4, 9.8, 0.4H), 3.89 (dd, *J* = 6.3, 10.2, 0.1H), 3.88 (dd, *J* = 5.0, 10.2, 0.4H), 3.76 (dd, *J* = 4.6, 10.2, 0.1H), 3.68 (ddd, *J* = 3.6, 10.0, 16.1, 1H), 3.65-3.35 (m, 3.9H), 2.96 (m, 2H), 2.24 (m, 1H), 2.01 (s, 0.3H), 2.00 (s, 1.2H), 1.96 (s, 1.2H), 1.94 (s, 0.3H), 1.94-1.68 (m, 3H); ^{13}C NMR δ 196.0 (mr), 195.2, 195.0, 194.8 (mr), 171.5 (mr), 171.4 (mr), 170.5 (mr), 170.42, 170.36, 170.33, 170.2, 163.2, 163.1, 162.8 (mr), 137.5 (mr), 137.33, 137.28 (mr), 137.24, 136.5, 136.4, 136.3 (mr), 133.70, 133.66, 132.4, 132.3, 128.7, 128.5 (mr), 128.4, 128.30, 128.25 (mr), 128.20, 128.1 (mr), 127.98, 127.95, 127.91 (mr), 127.78, 127.74, 127.66, 127.61 (mr), 127.55 (mr), 127.4, 127.36, 127.33, 127.26 (mr), 127.17 (mr), 126.35 (mr), 126.29, 126.1, 125.72 (mr), 125.67, 125.5, 73.8, 73.7, 73.5 (mr), 69.8 (mr), 69.3, 69.2, 69.0 (mr), 61.8 (mr), 61.5 (mr), 60.6, 57.2, 56.7 (mr), 56.2, 47.9, 47.3 (mr), 46.9 (mr), 41.0, 40.9, 40.75 (mr), 40.71 (mr), 35.84, 35.75, 35.68 (mr), 31.7 (mr), 31.2 (mr), 28.1, 27.7, 24.9, 24.7, 23.2 (mr), 23.0, 22.8, 22.7 (mr), 21.9 (mr), 21.8 (mr). MS (ESI$^+$) calcd for $C_{30}H_{33}N_3O_5$·Na [M + Na]$^+$ *m*/*z* = 538.2, found *m*/*z* = 538.6.



Alcohol 9. α-Ketoamide **8** (20.0 mg, 0.039 mmol) was dissolved in CH_2Cl_2 (3 mL). The solution was cooled to –78 ºC, and BCl_3 (1 M in CH_2Cl_2, 0.6 mL, 0.6 mmol) was added dropwise. The reaction mixture was stirred at –78 ºC and warmed to 0 ºC over 1.5 h. The reaction mixture was cooled to –78 ºC, and quenched with MeOH (1 mL). The solution was diluted with $CHCl_3$ (25 mL), and washed

with 1N HCl (15 mL). The aqueous layer was extracted with $CHCl_3$ (2 × 10 mL). The combined organic phase was dried with Na_2SO_4. After filtration and evaporation, the residue was purified by semi-preparative HPLC to provide **9** as a white solid after lyophilization (11 mg, 65%). Anal. HPLC, 4.8 min, 98.5%; IR (film) 3300 (O–H, N–H), 1648 (C=O), 1534 (N–H), 1410 (C–N), 1125 (C–O) cm^{-1}; ^1H NMR δ 7.77 (m, 1H), 7.73 (d, *J* = 8.2, 1H), 7.72 (d, *J* = 7.7, 1H), 7.56 (s, 0.5H), 7.54 (s, 0.5H), 7.42 (m, 2H), 7.32 (app t, *J* = 1.9, 0.5H), 7.31 (app t, *J* = 1.9, 0.5H), 6.46 (d, *J* = 8.9, 0.5H), 6.37 (d, *J* = 9.3, 0.5H), 5.18 (br s, 0.5H), 5.11 (br s, 0.5H), 4.49 (dd, *J* = 7.0, 9.5, 0.5H), 4.39 (m, 0.5H), 4.34 (dd, *J* = 7.0, 9.8, 0.5H), 4.28 (dt, *J* = 3.8, 9.5, 0.5H), 4.19 (ddd, *J* = 5.6, 9.4, 13.2, 0.5H), 3.98 (ddd, *J* = 5.2, 10.2, 13.0, 0.5H), 3.94 (dd, *J* = 3.3, 12.1, 0.5H), 3.70 (dd, *J* = 3.4, 12.0, 0.5H), 3.65 (m, 0.5H), 3.58 (m, 1H), 3.49 (m, 2H), 3.42 (m, 0.5H), 3.15-2.84 (m, 3H), 2.44 (m, 0.5H), 2.38 (m, 0.5H), 2.00 (m, 1H), 1.96 (s, 1.5H), 1.95 (s, 1.5H), 1.83 (m, 2H); ^{13}C NMR δ 170.8, 170.5, 167.0, 166.8, 166.0, 165.4, 136.7, 136.4, 133.6, 133.5, 132.3, 128.39, 128.37, 128.0, 127.81, 127.76, 127.73, 127.6, 127.54, 127.51, 127.2, 126.1, 125.6, 86.2, 61.2, 61.1, 60.2, 60.0, 59.9, 54.3, 54.1, 53.8, 46.6, 46.1, 44.4, 44.2, 44.0, 43.6, 36.4, 36.1, 29.5, 23.5, 23.4, 23.1, 23.0, 22.8, 22.7, 22.5; HRMS (ESI^+) calcd for $C_{23}H_{27}N_3O_5 \cdot Na$ $[M + Na]^+$ *m/z* = 448.1848, found *m/z* = 448.1826.

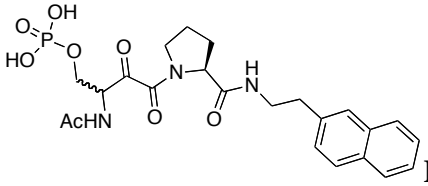


Dibenzylphosphate 10. To a solution of **9** (20.0 mg, 0.047 mmol) in THF (4 mL) was added 5-ethylthio–1H–tetrazole (18 mg, 0.14 mmol) and dibenzyl diethylphosphoramidite (45 mg, 0.14 mmol) at rt, and the mixture was stirred at rt for 14 h. The mixture was cooled to –40 °C and a solution of *tert*-butyl

hydroperoxide in decane (0.043 mL, 5–6 M, 0.2 mmol) was added slowly and the mixture was stirred at –40 °C for 10 min, then at rt for 40 min. The reaction was cooled to –40 °C and quenched by addition of $Na_2S_2O_3$ (20 mL). The mixture was extracted with EtOAc (3 × 25 mL) and dried over Na_2SO_4. The oil obtained by concentration under vacuum was purified by semi-preparative HPLC on a 5 μm C18 column, 19 × 100 mm, with gradient 5% MeOH/H_2O for 3 min followed by 5% to 90% MeOH/H_2O over 10 min, at 254 nm, to separate a pair of diastereomers of **10** as white solids (10 mg each, combined yield 65%). **10a**: Anal. HPLC, 5.3 min, 97.4%; IR (film) 1723 (ketone C=O), 1674 (amide C=O), 1264 (P–O–C), 1011 (C–O) cm^{-1}; ^1H and ^{13}C NMR See Table 2.8 below. HRMS (ESI^+) calcd for $C_{37}H_{41}N_3O_8P$ $[M + H]^+$ m/z = 686.2631, found m/z = 686.2633. **10b**: Anal. HPLC, 5.8 min, 96.1%; IR (film) 1732 (w. ketone C=O), 1667 (amide C=O), 1264 (P–O–C), 1008 (C–O) cm^{-1}; ^1H NMR δ 7.75 (m, 3H), 7.54 (br s, 1H), 7.42 (m, 2H), 7.29 (m, 11H), 6.24 (d, J = 9.3, 1H), 4.98 (m, 5H), 4.51 (ddd, J = 3.9, 7.4, 9.6, 1H), 4.29 (dd, J = 7.2, 9.4, 1H), 4.16 (ddd, J = 7.4, 9.8, 11.2, 1H), 4.06 (ddd, J = 5.6, 9.5, 13.1, 1H), 3.98 (ddd, J = 3.9, 8.8, 11.2, 1H), 3.48 (m, 1H), 3.39 (m, 1H), 3.32 (ddd, J = 6.1, 9.5, 13.1, 1H), 2.97 (ddd, J = 5.5, 9.3, 12.8, 1H), 2.88 (ddd, J = 5.9, 9.5, 12.8, 1H), 2.35 (m, 1H), 1.86 (m, 3H), 1.85 (s, 3H); ^{13}C NMR δ 170.6, 167.1, 164.7, 136.4, 135.6 (d, $^3J_{P\text{-}C}$ = 6.4), 135.5 (d, $^3J_{P\text{-}C}$ = 6.9), 133.6, 132.3, 128.9, 128.8, 128.15, 128.13, 128.07, 127.8, 127.7, 127.5, 126.1, 125.6, 84.7, 69.86 (d, $^2J_{P\text{-}C}$ = 5.7), 69.85 (d, $^2J_{P\text{-}C}$ = 5.8), 64.8 (d, $^2J_{P\text{-}C}$ = 5.4), 60.1, 53.5 (d, $^3J_{P\text{-}C}$ = 5.0), 46.1, 43.7, 36.1, 29.6, 23.1, 22.5; HRMS (ESI^+) calcd for $C_{37}H_{41}N_3O_8P$ $[M + H]^+$ m/z = 686.2631, found m/z = 686.2644.

Table 2.8. ^{1}H NMR and ^{13}C NMR assignments of Compound **10a**.

Assignments	^{1}H NMR	^{13}C NMR
a	1.86 (s, 3H)	22.8
b	6.21 (br s, 1H)	
c	4.61 (ddd, J = 4.6, 7.2, 9.0, 1H)	54.0 (d, $^{3}J_{P\text{-}C}$ = 5.6)
d	4.03 (m, 1H), 3.97 (m, 1H)	65.1 (d, $^{2}J_{P\text{-}C}$ = 5.6)
e	4.95 (m, 4H)	69.81 (d, $^{2}J_{P\text{-}C}$ = 5.9), 69.76 (d, $^{2}J_{P\text{-}C}$ = 6.0)
f	4.39 (dd, J = 7.2, 9.2, 1H)	46.6
g/g'	2.37 (m, 1H), 1.96 (m, 1H)	29.4
h/h'	1.96 (m, 1H), 1.77 (m, 1H)	23.2
i	3.58 (ddd, J = 6.9, 9.8, 11.7, 1H), 3.46 (m, 1H)	60.0
j/j'	3.97 (m, 1H), 3.46 (m, 1H)	44.3
k	2.99 (ddd, J = 5.0, 10.1, 12.6, 1H), 2.86 (ddd, J = 5.9, 10.1, 12.6, 1H)	36.4
l		170.7
m		84.5
n		165.0
o		166.5
p/q/r	7.79 (d, J = 8.6, 1H), 7.74 (d, J = 8.5, 1H), 7.73 (d, J = 7.2, 1H), 7.57 (s, 1H), 7.42 (m, 2H), 7.32 (m, 11H)	136.6, 135.6 (d, $^{3}J_{P\text{-}C}$ = 6.6), 135.5 (d, $^{3}J_{P\text{-}C}$ = 7.1), 133.6, 132.3, 128.9, 128.8, 128.1, 127.8, 127.7, 127.5, 127.4, 126.1, 125.5



Phosphate 1a. To solid diastereomer **10a** (9.0 mg, 0.013 mmol) and 10% Pd/C (5 mg) under N_2 was added MeOH (5 mL). The reaction was stirred under H_2 (1 atm) at rt for 2 h. The reaction mixture was filtered through Celite, and washed with MeOH. The oil obtained by concentration under vacuum was purified by semi-preparative HPLC on a 5 μm C18 column, 19 × 100 mm, with 5% CH_3CN/H_2O for 3 min followed by a gradient of 5% to 50% CH_3CN/H_2O over 10 min, and UV detection at 254 nm, to give **1a** as a white powder (5.0 mg, 70%). The other isomer was

prepared the same way to give **1b** as a white powder (5.0 mg, 70%). **1a**: Anal. HPLC on a C18 4.6 × 50 mm column with 5% CH_3CN/H_2O for 3 min followed by a gradient of 5% to 50% CH_3CN/H_2O over 10 min, flow rate 1.0 mL/min, with UV detection at 254 nm, 6.3 min, 99.9% purity (Broad peak. No acid was used as the buffer eluant due to instability of ketoamide phosphate **1a**.); $[\alpha]^{25}_D$ –107.1 (*c* 0.20, MeOH); IR (film) 3333 br (O–H, N–H), 1663 (amide C=O), 1437 (C–N), 1017 (C–O) cm^{-1}; ^1H NMR (CD_3OD) δ 7.79 (dd, *J* = 1.4, 7.4, 1H), 7.76 (d, *J* = 7.9, 2H), 7.61 (s, 1H), 7.44 (dd, *J* = 1.5, 6.8, 1H), 7.42 (dt, *J* = 1.4, 6.2, 1H), 7.40 (dt, *J* = 1.4, 6.8, 1H), 7.36 (dd, *J* = 1.6, 8.6, 1H), 4.58 (dd, *J* = 3.6, 8.2, 1H), 4.52 (dd, *J* = 6.9, 9.9, 1H), 4.08 (m, 1H), 3.94 (ddd, *J* = 8.0, 8.0, 11.2, 1H), 3.89 (ddd, *J* = 6.2, 10.1, 12.9, 1H), 3.65 (ddd, *J* = 5.1, 10.3, 12.9, 1H), 3.55 (m, 2H), 2.98 (ddd, *J* = 6.2, 10.4, 12.6), 2.87 (ddd, *J* = 5.1, 10.0, 12.6, 1H), 2.32 (m, 1H), 1.94 (m, 2H), 1.90 (s, 3H), 1.76 (m, 1H); ^{13}C NMR (CD_3OD) δ 173.7, 169.4, 166.8, 138.0, 135.0, 133.7, 128.9, 128.7, 128.6, 128.5, 128.3, 126.9, 126.4, 87.1, 64.7 (d, $^2J_{P\text{-}C}$ = 4.3), 60.9, 55.8 (d, $^3J_{P\text{-}C}$ = 7.3), 47.2, 44.9, 37.1, 30.4, 23.3, 22.6; ^{31}P NMR (CD_3OD) δ 1.0; HRMS (ESI^+) calcd for $C_{23}H_{28}N_3O_8P{\cdot}Na$ $[M + Na]^+$ *m/z* = 528.1512, found *m/z* = 528.1516. **1b**: Anal. HPLC (same conditions as **1a**), 5.4 min, 97.6% purity (Broad peak. No acid was used as the buffer eluant due to instability of ketoamide phosphate **1b**.); $[\alpha]^{25}_D$ –23.1 (*c* 0.25, MeOH); IR (film) 3299 (O–H, N–H), 1657 (amide C=O), 1446 (C–N), 1069 (C–O) cm^{-1}; ^1H NMR (CD_3OD) δ 7.79 (d, *J* = 7.5, 1H), 7.76 (dd, *J* = 2.7, 7.2, 2H), 7.63 (s, 1H), 7.43 (dd, *J* = 1.1, 6.6, 1H), 7.41 (d, *J* = 1.9, 1H), 7.38 (m, 1H), 4.46 (m, 1H), 4.28 (dd, *J* = 6.2, 10.6, 1H), 4.22 (m, 1H), 4.00 (m, 2H), 3.58 (m, 3H), 3.02 (ddd, *J* = 5.7, 10.6, 12.4, 1H), 2.92 (m, 1H), 2.32 (m, 1H), 1.95 (m, 2H), 1.92 (s, 3H), 1.63 (m, 1H); ^{13}C NMR (CD_3OD) δ 173.8, 169.3, 165.8, 138.2, 135.0, 133.7, 128.9, 128.7, 128.6, 128.5, 128.2,

126.9, 126.3, 87.5, 63.9, 61.4, 56.5, 46.9, 44.9, 37.0, 31.2, 22.9, 22.6; ^{31}P NMR (CD_3OD) δ 1.2; MS (ESI^+) calcd for $C_{23}H_{29}N_3O_8P$ $[M + H]^+$ m/z = 506.2, found m/z = 506.3.

Pin1 inhibition assay. The Pin1 inhibition assay was performed at 4 °C in 35 mM HEPES pH 7.8 in a total assay volume of 1.2 mL as published.[3] Inhibitor **1a**, 20 μL of stock to give final concentrations of 12 μM, 50 μM, 0.10 mM, 0.20 mM, 0.40 mM, 0.81 mM in 2:1 DMSO:H_2O, and inhibitor **1b**, 20 μL of stock to give final concentrations of 7.0 μM, 36 μM, 72 μM, 0.14 mM, 0.29 mM, 0.43 mM, 0.57 mM, 1.1 mM in 2:1 DMSO:H_2O, were pre-equilibrated with enzyme in HEPES at 4 °C for 15 min. The Pin1 final concentration in the assay was 67 nM. α-Chymotrypsin (final concentration of 6 mg/mL) was added, the cuvette was inverted 2 times to mix, followed by addition of succinyl–Ala–Glu–Pro–Phe-*p*NA (10 μL, 8.6 mM in dry 0.5 M LiCl/trifluoroethanol, final [cis] = 34 μM) with inversion 3 times to initiate the reaction. For each concentration, the assay was performed in duplicate. The plot of % Inhibition vs. log [I] (μM) produced a sigmoidal curve by fitting all of the experimental data to a dose-response curve using TableCurve (version 3 for win32) (Figures 2.11 and 2.12). The IC_{50} values were derived from the fitted equation at 50% inhibition of enzyme activity.

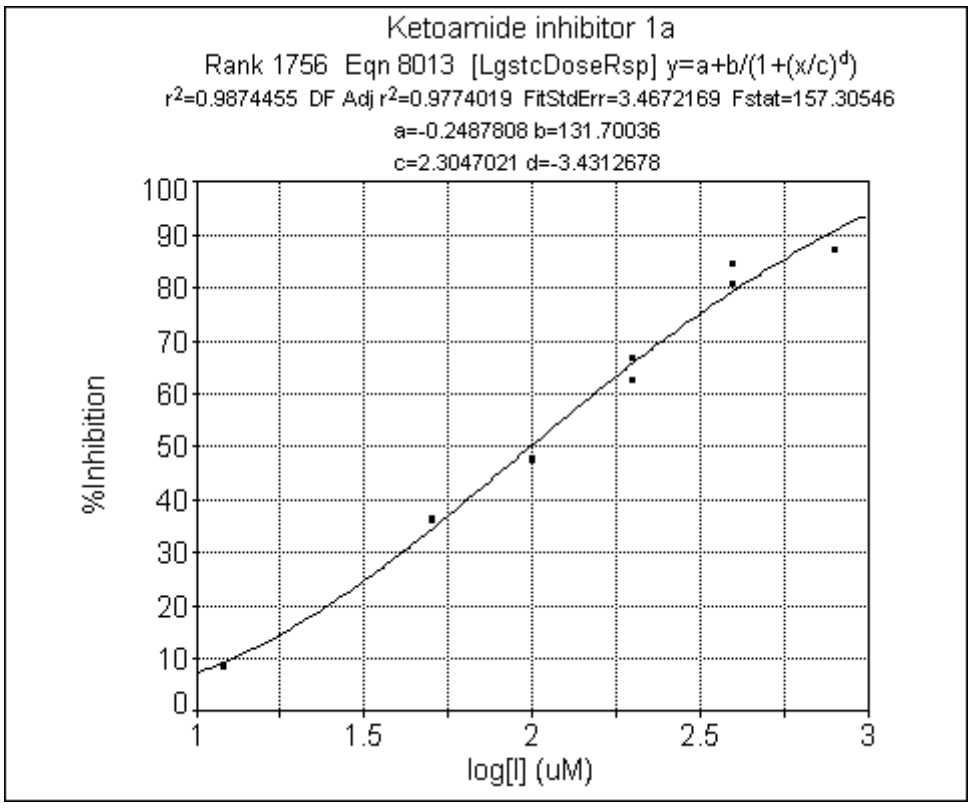


Figure 2.11. Dose response curve for inhibition of Pin1 by compound **1a**. IC_{50} = 100 ± 20 μM.

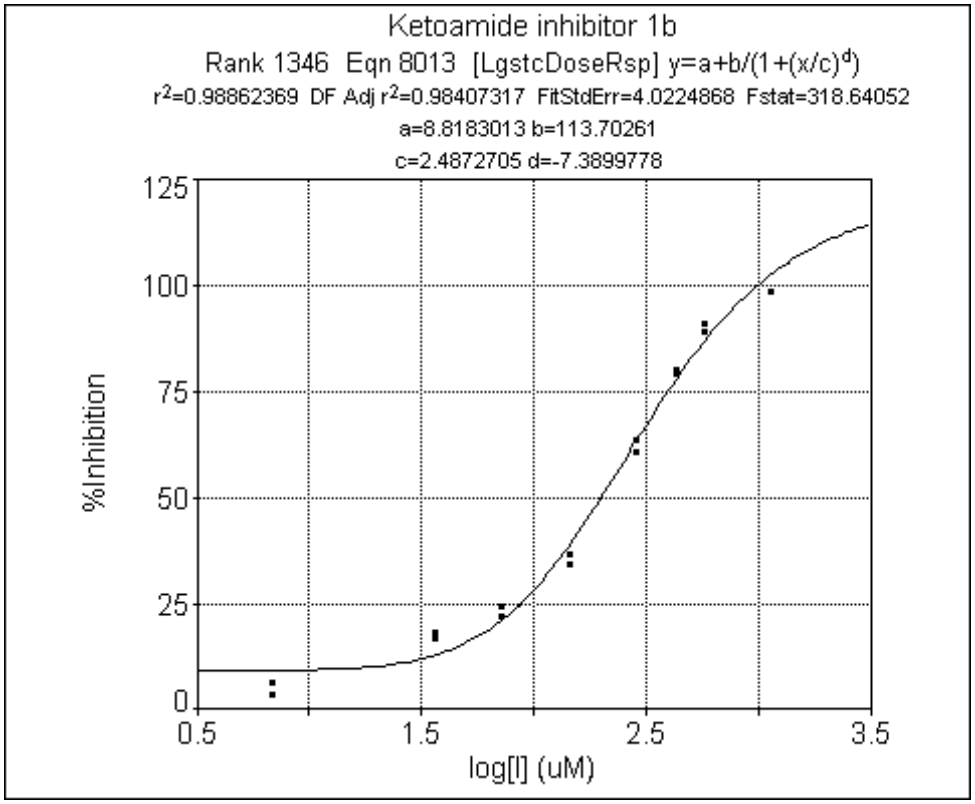


Figure 2.12. Dose response curve for inhibition of Pin1 by compound **1b**. IC_{50} = 200 ± 20 μM.

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3. Wang, X. J.; Xu, B.; Mullins, A. B.; Neiler, F. K.; Etzkorn, F. A., *J. Am. Chem. Soc*. **2004,** *126*, 15533-15542.

Chapter 3: Ketone inhibitors

This chapter includes two parts: A) a literature review on ketone inhibitors and synthetic methods, and B) a manuscript to be submitted, that concerns the design and synthesis of ketone isostere inhibitors of Pin1. I performed all of the synthesis and bioassay experiments from Part B, and Dr. Carla Slebodnick (Virginia Tech Crystallography Laboratory) determined X-ray crystal structures of compounds **6a** and **6b** from Part B. I wrote the first drafts of Part A, the manuscript and experimental section of Part B, and Dr. Etzkorn edited them in close consultation with me.

Part A: Literature review on ketone isostere inhibitors

Ketones have an electrophilic carbonyl group, which could covalently bond to the active site residue of the enzymes. Ketone analogues were designed as Pin1 inhibitors for our development of the potent inhibitors and mechanistic studies on Pin1. In this part, we review the design of ketone analogue inhibitors, their inhibition to proteases, and their use in studying enzyme mechanism.

3.1. Introduction

In the design of potent and specific inhibitors, there are two components that contribute to inhibitory affinity. One component is based on the substrate, providing the specificity of the inhibitor for the enzyme active site. The other component is based on the catalytic chemistry in the active site of the enzyme. Based on substrate specificity, inhibitors are designed by incorporation of a reactive functionality or a stable structure. Inhibitors with a reactive functional group modify an active site residue and inactivate the enzyme. Inhibitors that incorporate a stable structure mimic the transition state of the

catalytic reaction by the enzyme and inhibit the enzyme. The concept of transition-state (TS) analogues is very effective as a basis for designing potent enzyme inhibitors. TS inhibitors can also provide mechanistic information regarding enzyme catalysis.

Aldehydes have been developed as very potent inhibitors for many proteases, but as therapeutic agents, they are limited by metabolic liabilities. Thus ketones, that partially retain the reactivity of the aldehyde carbonyl moiety, have been widely used as analogues of aldehydes for inhibition of many proteases, including serine proteases, cysteine proteases, and aspartyl proteases. This is not a comprehensive review of compounds with the ketone functionality used as inhibitors in all fields. We mainly focus the ketone compounds whose carbonyl functionality contributes as an important feature to the inhibitory activities. The synthesis of ketones is also reviewed.

3.2. Ketone inhibitory activities

Ketones have been widely used in the development of inhibitors. Many potent inhibitors have been found for specific proteases, including cysteine proteases and serine proteases, and for PPIases. Ketones as Pin1 inhibitors have been discussed in Chapter 1.

3.2.1. Cysteine protease inhibitors

Many ketones have been developed as inhibitors of cysteine proteases, including cathepsin K, caspase and calpain.

Cathepsin K inhibitors

Cathepsin K is a cysteine protease of the papain superfamily. It is selectively and abundantly expressed within the osteoclast.[1] It is a promising target for the treatment of osteoporosis.[1] Ketones have been widely used as a class of compounds in substitution for

aldehydes as cathepsin K inhibitors.[1-3] Aldehyde-based inhibitors as therapeutic agents are limited by metabolic liabilities. Ketone-based inhibitors can retain the reactive nature of the aldehyde carbonyl moiety, and circumvent these liabilities. Marquis and co-workers developed several series of acyclic **3.1** and cyclic ketone-based inhibitors **3.2**, and azepanone-based inhibitors **3.3** of cathepsin K.[1-3] Some example compounds of those two series are shown in Figure 3.1.

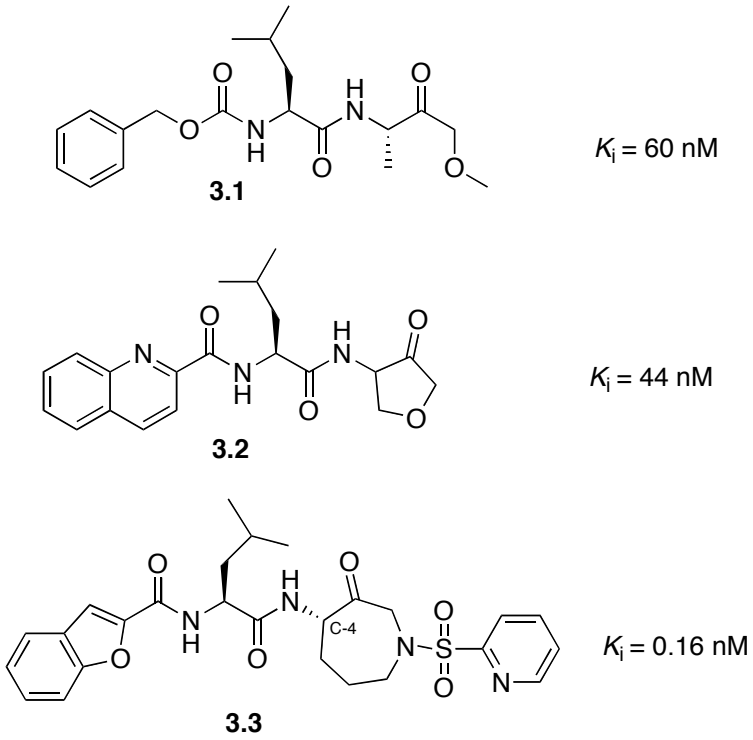
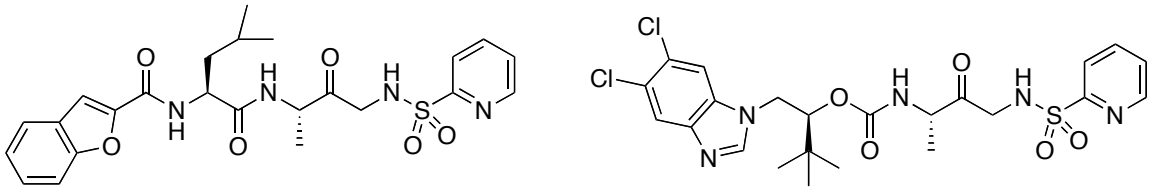


Figure 3.1. Cathepsin K inhibitors.[1-3]

Azepanone-based inhibitor **3.3** improved the configurational stability of the C-4 stereocenter compared to cyclic ketone-based inhibitor **3.2**. The oral bioavailability of **3.3** (42%) was much improved over acyclic and cyclic ketone based inhibitors. The improvements in oral bioavailability might be due to the locked and stable configuration that locks out substrate conformations susceptible to metabolizing enzymes.[3]

Barrette et al developed a series of ketone inhibitors for cathepsin K with even improved oral bioavailability. By eliminating two amide bonds and incorporating a constrained P_3 peptidomimetic fragment, the oral bioavailability of ketone **3.4** [3] was much improved, while the potent inhibitory activity was maintained.[4] Compound **3.5** is another example of that series.



IC_{50} = 7.5 nM

3.4

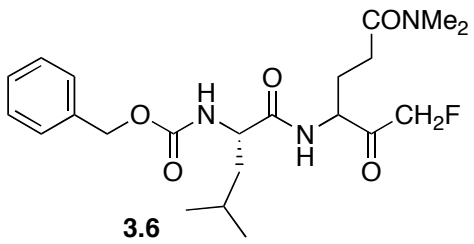
IC_{50} = 1.4 nM

3.5

Figure 3.2. Cathepsin K inhibitors with improved oral bioavailability.[4]

Severe acute respiratory syndrome-associated coronavirus (SARS-CoV) inhibitors

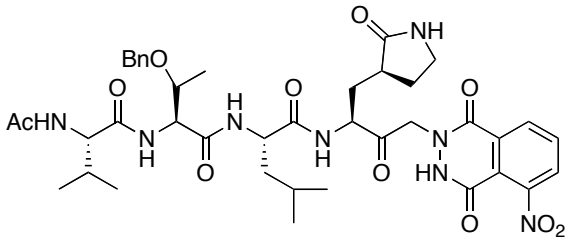
Unlike common cysteine proteases, SARS-CoV 3CL protease has a Cys-His catalytic dyad.[5] Several ketone-based compounds were developed as SARS inhibitors, which exploited the electrophilicity of the ketone functionality. They included keto-glutamine analogues, glutamate or glutamine peptides containing a trifluoromethyl ketone group, glutaminyl fluoromethyl ketones, and trifuoromethyl ketones.[5] Trifluoromethyl ketones (TFMKs) have often been used as inhibitors of serine and cysteine proteases. The carbonyl carbon of TFMK is highly electrophilic due to the presence of three highly electronegative fluorines.[5] Zhang et al designed and synthesized dipeptidyl *N*, *N*-dimethyl glutaminyl fluoromethyl ketones as SARS-CoV inhibitors. Compound **3.6** was found to be a potent inhibitor with low toxity in cells, and the EC_{50} value was 2.5 μM.[6]



3.6

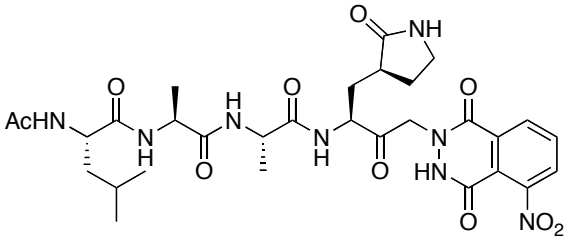
Figure 3.3. SARS-CoV inhibitor.[6]

A series of keto-glutamine analogues with a phthalhydrazido group at the α-position were developed as reversible inhibitors of SARS 3CL, and sub-micromolar inhibitors (e.g. compound **3.7**) were obtained.[7] A series of keto-glutamine analogues bearing a phthalhydrazide moiety (e.g. compound **3.8**) were also found to be good inhibitors of Hepatitis A virus (HAV) in the low micromolar range.[8]



IC_{50} = 0.60 μM

3.7



IC_{50} = 1.6 μM

3.8

Figure 3.4. Phthalhydrazide containing inhibitors for SARS 3CL and HAV.[7 8]

Caspase inhibitors

Caspase is a member of the cysteine protease family. It plays an essential role in apoptosis.[9] A series of dipeptidyl aspartyl fluoromethylketones **3.9** were synthesized as caspase-3 inhibitors by Wang and co-workers.[9] They found Val was the best P_2 amino acid through SAR studies, and free Asp in P_1 was important.[9]

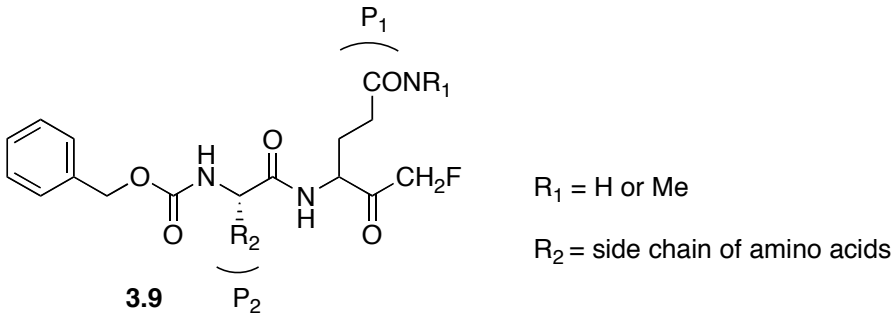


Figure 3.5. Caspase inhibitors.[9]

Calpain I inhibitors

Calpain I, a calcium-activated neutral protease, belongs to a family of intracellular cysteine proteases. Calpain I has been implicated in nervous system disorders.[10]A series of fluoromethyl ketone dipeptides (**3.10**) were developed by Chatterjee and coworkers as recombinant human calpain I inhibitors.[10] SAR studies established that Leu is preferred at the P_2 site.[10]

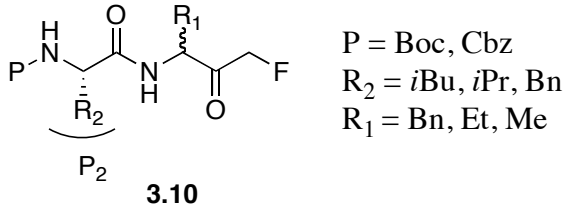


Figure 3.6. Calpain inhibitors.[10]

3.2.2. Serine protease inhibitors

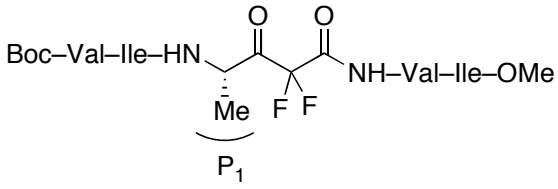
Due to the hydroxyl group in the active site residue of serine proteases, many ketones have been developed as inhibitors of serine proteases, including thrombin and γ-secretases.

Thrombin inhibitors

Thrombin, a trypsin-like serine protease, plays an essential role in hemostasis and thrombosis.[11] A crystal structure of thrombin in complex with a bivalent peptidyl pyridinium methyl ketone was solved.[11] The inhibitor, (D)-cyclohexylalanine-Pro-Arg-($CH_2N^+C_5H_4CH_2CO$)-$(Gly)_4$-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-cyclohexylalanine-(D)Glu (coded P596), forms a reversible covalent complex with thrombin.[11]

Alzheimer's γ-secretase inhibitors

γ-Secretases, belonging to the family of aspartyl proteases, catalyze the final step in the amyloid *β*-protein (A*β*) biosynthesis from the amyloid precursor protein (APP).[12] A substrate-based difluoroketone **3.11** was developed as a more specific inhibitor of γ-secretase in APP-transfected cells,[12] compared to the peptide aldehyde inhibitors. [13-15]



3.11

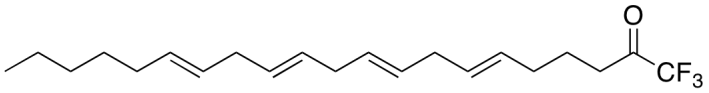
Figure 3.7. γ-Secretase inhibitor[12]

Moore et al found that sterically bulky groups (such as *sec*-butyl or cyclohexylmethyl) in the P_1 position increased the γ-secretase inhibitory potency.[16]

3.2.3. Phospholipase inhibitors

Phospholipases A_2 (PLA_2) catalyze the hydrolysis of fatty esters to form free fatty acids and a lysophospholipid.[17] Cytosolic human phospholipase A2 (cPLA2) was found in the cytosol of cells showing no sequence homology with any of the secreted

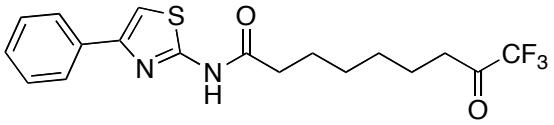
enzymes.[17] A trifluoromethyl ketone analogue of arachidonic acid ($AACOCF_3$) was developed as a potent and slow-binding inhibitor of the cPLA2.[17]



$AACOCF_3$

3.2.4. HDACs inhibitors

Trifluoromethyl ketones were developed as HDAC inhibitors. Sub-micromolar inhibitors were obtained after modification. Compound **3.12** showed antiproliferative effects against the HT 1080 and MDA 435 cell lines.[18]



IC_{50} = 0.31 μM

3.12

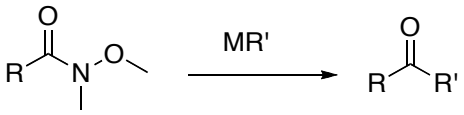
3.3. Synthesis of ketones

The synthetic methods of ketones are briefly reviewed here. The ketone functional group is usually formed through the oxidation of alcohols or the addition of organo metallic reagents to Weinreb amides.

3.3.1. Synthesis of ketones from Weinreb amides[10]

Due to the unique chelation between Weinreb amides and metals, the ketone products are not released until acidic hydrolysis.[19] In this way ketones can be obtained as products instead of alcohols. RCOOLi and RCOOCl[20] can also be used as substrates to make ketones due to their high reactivities (Scheme 1). Here, MR' can be BrMgR' or LiR'.

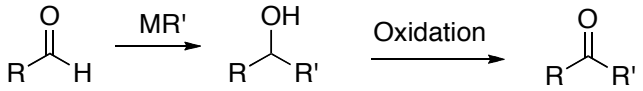
Scheme 3.1. Synthesis of ketones from Weinreb amides.



3.3.2. Synthesis of ketones from alcohols

Aldehydes can be reacted with metallic reagents, such as organomagnesium reagents, and organozinc reagents, to form alcohols, which can then be oxidized by pyridium chlorochromate (PCC) or Dess-Martin periodinane[16, 21] to form the desired ketones (Scheme 2).

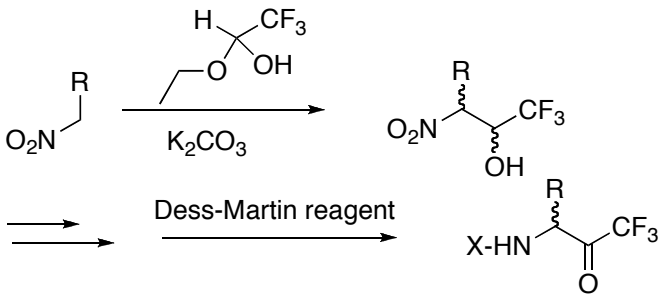
Scheme 3.2. Synthesis of ketones from aldehydes.



3.3.3. Synthesis of ketones from nitro and cyano derivatives

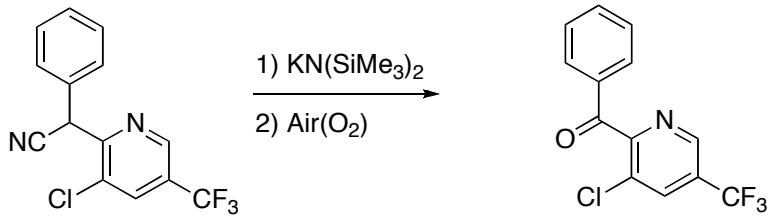
Scheme 3 shows a common method for synthesizing trifluoroketone derivatives.[5] The α proton of nitro derivatives was removed by a base, and the carbon anion formed acted as a nucleophile to attack the hemiacetal derived from the aldehyde to form trifluoromethyl alcohol, which was then oxidized to trifluoroketone derivatives.[5]

Scheme 3.3. Synthesis of ketones from nitro derivatives.[5]



Khanapure and coworkers deprotonated cyano derivatives with potassium bishexamethylsilazide, followed by air oxidation to synthesize ketone analogues (Scheme 4).[20]

Scheme 3.4. Synthesis of ketones from cyano derivatives.[20]



3.4. Conclusions

The inhibitory activities and selected synthetic methods of ketones have been reviewed. Ketones inhibit many proteases such as serine proteases, cysteine proteases, and aspartyl proteases. Ketones contain an electrophilic carbonyl group, which may covalently bind to the active site residue and inactivate enzymes. Ketones are widely used as pharmaceutical agents in some cases to substitute for aldehyde inhibitors due to their resistance to metabolism. We chose to design ketones in the development of Pin1 inhibitors as tetrahedral intermediate analogues.

Weinreb amide substrates or aldehydes are often used for the synthesis of ketones. The Weinreb amide synthetic strategy was applied to our synthesis of ketone isosteres as Pin1 inhibitors.

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Part B: Synthesis and bioassay of ketone inhibitors of Pin1

Ketone peptidomimetics as Pin1 inhibitors provide evidence against a nucleophilic addition mechanism

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Abstract

Ketone inhibitors of Pin1, the cell cycle regulatory peptidyl-prolyl isomerase (PPIase), were designed as electrophilic acceptors for the active-site Cys113 nucleophile. The IC_{50} values of two diastereomers were determined to be **1a**, 260 μM, and *rac*-**1b**, 61 μM, indicating that the mechanism of Pin1 PPIase is not likely to be a nucleophilic addition-rotation-elimination.

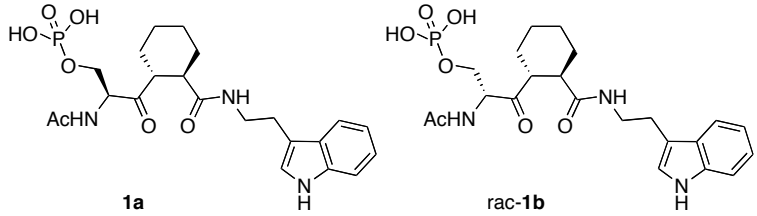
Protein interacting with never-in-mitosis A kinase 1 (Pin1) was discovered in 1996 to be a peptidyl-prolyl isomerase (PPIase) enzyme belonging to the parvulin family.[1] Pin1 has two domains: a WW domain and a PPIase domain. Both of these domains recognize the phospho-Ser/Thr-Pro bonds present in mitotic phosphoproteins.[2] Pin1 is distinct from two other PPIase families, the cyclophilins and the FK506 binding proteins (FKBPs), since Pin1 only retains PPIase activity on phosphorylated substrates.[3] Pin1 catalyzes prolyl cis-trans isomerization to function as a molecular timer regulating the cell cycle, cell signaling, gene expression, immune response, and neuronal function.[4] Pin1 is overexpressed in many cancer lines, and plays an important role in oncogenesis.[5] Because of its significant role in cell cycle regulation by a unique mechanism, Pin1 represents an intriguing diagnostic and therapeutic target for cancer.[6, 7] Several promising

classes of Pin1 inhibitors have been synthesized as potential lead compounds, including designed inhibitors,[8-12] and natural products.[13, 14]

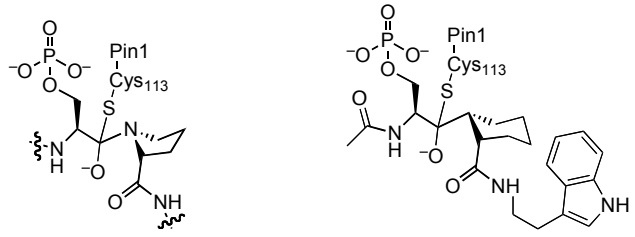
Ketones have been widely used as a class of compound analogues to aldehydes or carboxylic acids as inhibitors of many proteases including serine proteases, cysteine proteases,[15, 16] and aspartyl proteases.[17, 18] Ketone peptidomimetics have not yet been developed as mechanism-based inhibitors of PPIases, except for juglone, which is a non-specific inhibitor of Pin1 through Michael addition to a thiol of Pin1, resulting in unfolding.[13] Daum et al developed a series of aryl indanyl ketone inhibitors of Pin1, and the best inhibition was 0.2 μM.[8] Those inhibitors are reversible and cell penetrating, and they also showed biological activities for p53 and β-catenin. There are two proposed mechanisms for Pin1 catalysis: the twisted-amide mechanism[19, 20] and the tetrahedral transition-state mechanism.[21] Daum et al proposed that the aryl indanyl ketones mimic the transition state of the twisted amide mechanism, based on the conformation in one crystal structure.[8] We recently published two α-ketoamides as Pin1 inhibitors, but the weak inhibition values did not support either proposed mechanism.[22]

In the design of potent and specific inhibitors, there are two components contributing to inhibitory affinity: 1) the design, based on the substrate, provides the specificity of the inhibitor for the enzyme active site, and 2) incorporation of a reactive functionality, that covalently modifies an active site residue and inactivates the enzyme (mechanism-based inhibitor), or a stable structure, that mimics the transition state of the catalytic reaction by the enzyme (transition-state analogue). The concept of transition-state (TS) analogues is very effective as a basis for designing potent enzyme inhibitors, and can provide mechanistic information regarding enzyme catalysis.[23] We designed

tetrahedral-intermediate inhibitors **1a** and **1b** of Pin1 based on the second of the two proposed mechanisms.



Ketone analogues **1a** and **1b** were designed based on peptide inhibitor specificities.[24] The amide bond between the Ser-Pip was replaced with a ketone by changing the N atom to a C–H. Ketones are more electrophilic than amides, and were expected to modify the active site thiol to form a tetrahedral intermediate (hemithioketal) and inhibit the enzyme as tetrahedral-intermediate analogues (Figure 3.8). Tryptamine was coupled to the C-terminus, since Pin1 prefers aromatic residues there.[2, 10, 24] An acetyl was used at the N-terminus because X-ray crystal structures of our bound inhibitors showed no electron-density for residues N-terminal of pSer.[25] In addition, the acetyl group improved the water solubility of the inhibitors for enzyme assays. The six-membered piperidyl Pro analogue was chosen based on the improved inhibition of peptides with a Pip residue.[10, 24]

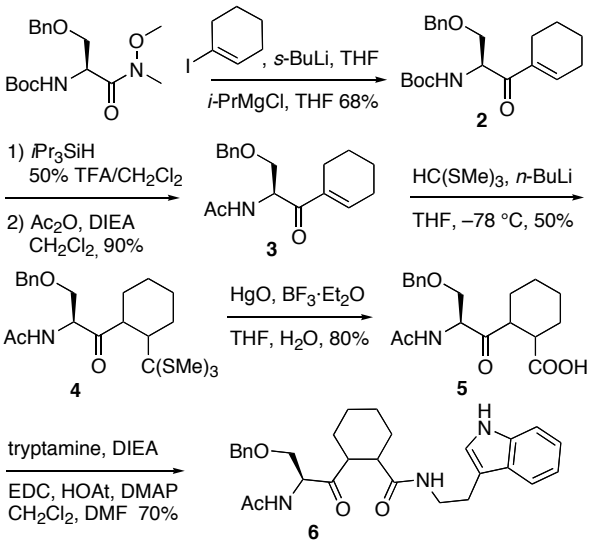


Tetrahedral-Adduct Mechanism Ketones as Tetrahedral-Intermediate Analogues

Figure 3.8. Ketones as tetrahedral-intermediate analogues.

In the synthesis of ketones **1**, addition of an alkenyl lithium reagent to a Weinreb amide was used to form the ketone functionality (Scheme 3.5). Cyclohexanone was reacted with hydrazine mono-hydrate to form the hydrazone, which was reacted with iodine in the presence of 1,1,3,3-tetramethylguanidine to generate 1-iodocyclohexene, analogous to the synthesis of 1-iodocyclopentene.[26] α,β-Unsaturated ketone **2** was obtained by the addition of cyclohexenyl lithium to the Weinreb amide, prepared by coupling Boc–Ser(OBn)–OH with *N,O*-dimethylhydroxylamine. [26] The lithium reagent was prepared *in situ* by treating 1-iodocyclohexene with *s*-BuLi.

Scheme 3.5. Synthesis of ketone intermediate **6**.



We first attempted the reaction of Boc-protected α, β-unsaturated ketone **2** with lithium trimethylthiomethane, however a cyclic carbamate was formed as the major product instead of the desired orthothioester. We have used similar cyclic carbamates in stereochemical proofs.[26] The Boc group was then removed with TFA, and the ammonium salt formed was acetylated with acetic anhydride to give ketone **3**. The carbamate ring-closure did not happen with the acetyl amide. Michael addition to form orthothioester **4**

was accomplished with lithium trimethylthio methide.[27] After Michael addition, two major diastereomers of the orthothioester were obtained as a mixture. Hydrolysis of orthothioester **4** in a mixture of THF and H_2O with $BF_3 \cdot Et_2O$ and HgO gave carboxylic acid **5**.[27] Without further purification, acid **5** was coupled to tryptamine with EDC to generate the ketone peptidomimetic **6**. The two diastereomers were separated by silica flash chromatography.

Each diastereomer was crystallized from a mixture of EtOAc and hexanes at room temperature. The major diastereomer crystallized as colorless needles. The stereochemistry was determined by X-ray crystallography, and the absolute configuration was determined to be SRR (**6a)**, with the L-Ser configuration intact (Figure 3.9). The minor diastereomer **6b** crystallized as colorless plates. From the crystal structure, it was found that the stereocenter of the serine analogue was epimerized to D/L-Ser, and the minor diastereomer was obtained as a racemic mixture. The absolute configurations were assigned as RRR and SSS (rac-**6b**). The benzyl group was modeled with positional disorder. The chiral center adjacent to the carbonyl functionality was easily epimerized due to the electron-withdrawing effect. We suspect the diastereoselectivity gave the anti-Ser trans-cyclohexyl compound **6a** as the major isomer due to thermodynamic control in the reaction.

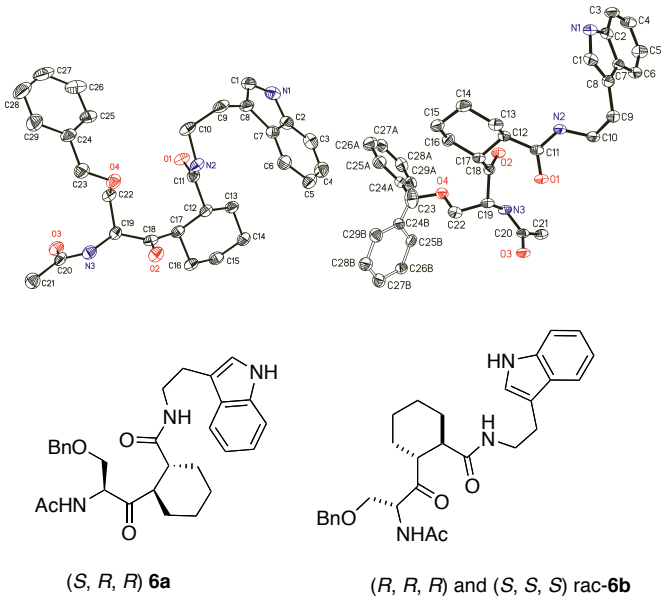
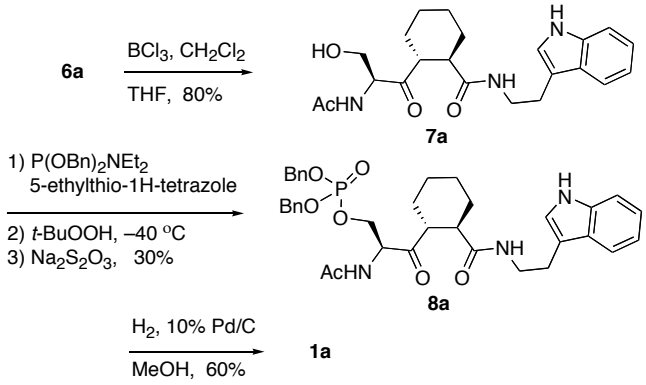


Figure 3.9. Stereochemistry from X-ray structures of compounds **6a** and **6b.**

The two diastereomers were created separately to result in the final compounds **1a** and **1b**. Scheme 3.6 shows only the reactions to give the major diastereomer. The synthesis of diastereomer **1b** is described in the Supporting Information. The major diastereomer **6a** was treated with BCl_3 to remove the benzyl group and form alcohol **7a**. Phosphorylations were attempted with dibenzyl, di-*tert*-butyl or dicyanoethyl phosphoramidite to form phosphate **8a**, and phosphates with di-tert butyl or di-cyanoethyl as the protecting groups instead of benzyl groups. None of the phosphates were stable on a silica gel column, and β-elimination products were obtained after chromatography. TFA deprotection of crude di-*tert*-butyl phosphate, and NH_4OH deprotection of crude dicyanoethyl phosphate both gave β-elimination products. Thus, the dibenzylphosphate was chosen to carry through to the final products **1a** and **1b**.

Scheme 3.6. Phosphorylation to give final product **1a**.



Hydrogenation of the crude dibenzyl phosphate went very slowly at atmospheric pressure of hydrogen. This might be due to poisoning of the Pd catalyst. The complicated crude mixture made the final purification very difficult. Thus, dibenzyl phosphate **8a** was purified by reverse-phase semi-preparative HPLC. With pure dibenzyl phosphate, hydrogenation worked very well, and gave a very clean final product **1a**.[22]

The α-chymotrypsin protease-coupled assay was used to evaluate inhibition of Pin1 by compounds **1a** and **1b**. [9] The substrate was succinyl–Ala–Glu–Pro–Phe–*p*NA, and the absorbance of *p*NA released from PPIase trans product was recorded at 390 nm by UV/Vis. The IC_{50} values of the two diastereomers, **1a** and **1b**, were determined to be 260 ± 32 μM and 61 ± 8 μM. The stereochemistry affected the inhibition, since isomer **1b** was about three times more potent than isomer **1a**. This is in agreement with potent inhibition of Pin1 by D-Ser containing peptide inhibitors of Pin1.[10] Since isomer **1b** is racemic, the best possible inhibition constant for a single enantiomer could be as low as 30 μM, which is comparable to a similar ground-state alkene isostere inhibitor we have reported.[12] A similar reduced amide analogue was 5-fold better than even the best possible ketone in this set.[28] The ketones were designed as tetrahedral-intermediate

analogues based on the tetrahedral transition-state mechanism. Based on the poor inhibition by ketones **1a** and **1b**, they clearly do not behave as such. These results do not support the tetrahedral transition-state, or nucleophilic addition-rotation-elimination mechanism, in agreement with the results of our α-ketoamide inhibitors.[22]

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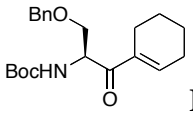
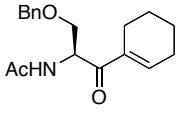
Experimental Section

General Procedures. Unless otherwise indicated, all reactions were carried out under dry N_2 in flame-dried glassware. THF was distilled from sodium-benzophenone, and CH_2Cl_2 was dried by passage through dry alumina. Anhydrous DMF (99.8%), MeOH, and DIEA were used directly from sealed bottles. TFE (99+%) was distilled from sodium before use. LiCl (99+%) was dried under vacuum at 150 °C for 24 h. Brine (NaCl), $Na_2S_2O_3$, $NaHCO_3$, and NH_4Cl refer to saturated aqueous solutions, and HCl refers to a 1N aqueous solution, unless otherwise noted. Flash chromatography was performed on 230–400 mesh silica gel with reagent grade solvents. Analytical HPLC were obtained on a C18 4.6 × 50 mm column with 10% CH_3CN/H_2O for 3 min followed by a 10% to 90% CH_3CN/H_2O gradient over 6 min unless otherwise noted. HPLC results are reported as retention time, integrated % purity. NMR spectra were obtained at ambient temperature in $CDCl_3$ unless otherwise noted. ^{1}H-, ^{13}C-, and ^{31}P-NMR spectra were obtained at 500, 125, and 162 MHz, respectively, unless otherwise noted. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane (TMS). Data are reported as follows: chemical shift, multiplicity: singlet (s), doublet (d), triplet (t), multiplet (m), broad singlet (br s), coupling constants *J* in Hz, and integration.



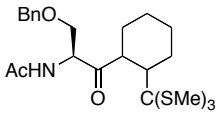
Boc ketone 2. To a solution of 1-iodocyclohexene (5.50 g, 26.4 mmol) in THF (60 mL) at –40 °C was added *s*-butyllithium (1.4 M in cyclohexane, 37.8 mL, 52.9 mmol). The mixture was stirred at –40 °C for 3 h. Boc–Ser(OBn)-N(OMe)Me Weinreb amide (5.96 g, 17.6 mmol) was dissolved in THF (60 mL) in another round-bottom flask and cooled to –78 °C, *i*-PrMgCl (2.0 M in THF, 8.64 mL, 17.3 mmol) was

then added dropwise. The Weinreb amide solution was stirred at –78 °C for 1 h. The corresponding cyclohexenyllithium generated was added *via* canula at –78 °C to the Weinreb amide solution. After stirring at –78 °C for 1h, the reaction was warmed to rt and stirred at rt for 12 h, and then quenched with NH_4Cl (80 mL). The resulting mixture was diluted with water (40 mL) and EtOAc (100 mL). The aqueous layer was extracted with EtOAc (100 mL). The organic layers were combined, and washed with NH_4Cl (2 × 80 mL), $NaHCO_3$ (80 mL), and brine (80 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated. The crude product was then purified by chromatography on silica (eluent: 8% EtOAc/hexanes) to yield ketone **2** (4.3 g, 68%) as a colorless oil. Anal. HPLC, 254 nm, 7.3 min, 98.2%; ^{1}H NMR δ 7.28 (m, 5H), 6.91 (m, 1H), 5.59 (d, J = 8.3, 1H), 5.13 (dt, J = 4.4, 8.3, 1H), 4.54 (d, J = 12.4, 1H), 4.42 (d, J = 12.4, 1H), 3.68 (dd, J = 4.4, 9.3, 1H), 3.66 (dd, J = 4.4, 9.6, 1H), 2.36 (m, 1H), 2.21 (m, 2H), 2.12 (m, 1H), 1.62 (m, 4H), 1.44 (s, 9H); ^{13}C NMR δ 197.8, 155.5, 141.8, 137.8, 137.4, 128.4, 127.7, 127.6, 79.8, 73.1, 71.3, 54.3, 28.4, 26.2, 23.4, 21.8, 21.5; ESI$^+$ HRMS: calcd for $C_{21}H_{29}NO_4$·Na $[M + Na]^+$ m/z = 382.1994, found m/z = 382.1998.



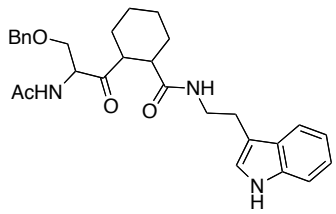
Acetyl ketone 3. Boc ketone **2** (1.5 g, 4.2 mmol) was dissolved in CH_2Cl_2 (20 mL), and iPr$_3$SiH (0.2 mL) and TFA (20 mL) were added. The mixture was stirred at rt for 0.5 h. The reaction mixture was then concentrated under reduced pressure. The residue was triturated with hexanes (3 × 25 mL). After evaporation in vacuo for 2 h, the ammonium salt obtained was then dissolved in CH_2Cl_2 (20 mL), and Ac_2O (2 mL) and DIEA (2 mL) were added. The reaction mixture was stirred at rt for 1 h. After dilution with CH_2Cl_2 (30 mL), the mixture was washed with HCl (2 × 25 mL), 1N NaOH

(2 × 25 mL), and brine (25 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated. The residue was purified by flash chromatography on silica (step gradient: 25% then 50% EtOAc /hexanes) to yield **3** (1.1 g, 90%) as a pale yellow oil. Anal. HPLC, 254 nm, 5.1 min, 100%; ^{1}H NMR δ 7.35-7.20 (m, 5H), 6.93 (m, 1H), 6.62 (br, 1H), 5.42 (m, 1H), 5.52 (d, *J* = 12.3, 1H), 4.40 (d, *J* = 12.3, 1H), 3.70 (m, 2H), 2.39-2.04 (m, 4H), 2.02 (s, 3H), 1.62 (m, 4H); ^{13}C NMR δ 197.3, 169.8, 142.3, 137.7, 137.2, 128.5, 127.9, 127.6, 72.2, 71.1, 53.3, 26.2, 23.44, 23.38, 21.8, 21.5; ESI$^+$HRMS: calcd for $C_{18}H_{24}NO_3$ $[M + H]^+$ *m/z* = 302.1756, found *m/z* = 302.1760.



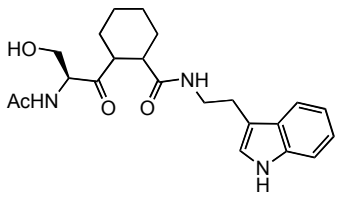
Orthothioformate 4. *n*-Butyllithium (2.5 M in hexane, 6.81 mL, 17.0 mmol) was added dropwise to a solution of *tris*(methylthio)methane (2.68 g, 17.0 mmol) dried over 4 Å molecular sieves in THF (65 mL) at –78 °C. The solution was stirred at –78 °C for 2 h, and then a solution of the acetyl ketone **3** (0.790 g, 2.62 mmol) dried over 4 Å molecular sieves in THF (50 mL) was added dropwise *via* cannula. The reaction mixture was stirred at –78 °C for 2 h, and then quenched with NH_4Cl (80 mL). The resulting mixture was extracted with EtOAc (3 × 150 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated. The crude product was then purified by chromatography on silica (step gradient: 0% then 20% EtOAc/hexanes) to yield the orthothioformate **4** in mixture of two diastereomers (0.60 g, 50%) as a colorless oil. The mixture was used for the next reaction without separation. The partial mixture of diastereomers was separated for characterization. Major diastereomer: ^{1}H NMR δ 7.30 (m, 5H), 6.60 (d, *J* = 7.5, 1H), 5.26 (ddd, *J* = 3.4, 4.2, 7.6, 1H), 4.52 (d, *J* = 12.1, 1H), 4.49 (d, *J* = 11.8, 1H), 3.89 (dd, *J* = 3.3, 10.2, 1H), 3.79 (dd, *J* = 4.4, 9.9, 1H), 3.11 (ddd, *J* = 3.8,

10.5, 11.5, 1H), 2.39 (ddd, J = 3.6, 10.4, 11.8), 2.09 (m, 1H), 2.07 (s, 9H), 2.02 (s, 3H), 1.82 (m, 1H), 1.73 (m, 1H), 1.63 (m, 2H), 1.38 (m, 1H), 1.31 (m, 1H), 1.22 (m, 1H), 1.06 (m, 1H); ^{13}C NMR δ 207.0, 169.4, 137.9, 128.5, 127.9, 127.8, 75.8, 73.4, 68.9, 59.5, 51.5, 48.0, 31.6, 28.6, 25.6, 25.1, 23.6, 15.2; HRMS: calcd for $C_{22}H_{33}NO_3S_3{\cdot}Na$ $[M + Na]^+$ m/z = 478.1520, found m/z = 478.1530.



Ac–Ser(OBn)–Ψ[C(O)CH]–Pip–(2)-*N*-(3)-ethylaminoindole 6. A mixture of compound **4** (0.30 g, 0.66 mmol) and HgO (0.70 g, 3.2 mmol) was suspended in 4:1 THF:H_2O (45 mL), and $BF_3{\cdot}Et_2O$ (1.2 mL, 9.6 mmol) was added. The mixture was stirred at rt for 3 h. The reaction mixture was diluted with water (10 mL) and extracted with EtOAc (3 × 50 mL). The resulting organic layer was dried over Na_2SO_4, filtered and evaporated. The residue was filtered through silica to remove HgO, and the solvent was evaporated in vacuo. The crude carboxylic acid **5** was dissolved in a mixture of CH_2Cl_2 (100 mL) and DMF (15 mL), and tryptamine (0.27 g, 1.7 mmol), EDC (0.32 g, 1.68 mmol), HOAt (0.25g, 1.6 mmol), DMAP (50 mg, 0.4 mmol) and DIEA (0.37 g, 2.9 mmol) were added. The reaction was stirred at rt for 16 h. The mixture was diluted with EtOAc (400 mL), washed with water (3 × 150 mL), HCl (3 × 150 mL), $NaHCO_3$ (3 × 150 mL) and brine (150 mL). The crude product was then purified by chromatography on silica (step gradient: 0% then 30% EtOAc/hexanes) to yield two diastereomers. The major diastereomer (0.12 g, 36%) and the minor diastereomer (60 mg, 18%) were obtained as colorless oils first and then solidified. Both solids were recrystallized from a mixture of EtOAc:hexanes 1:2 to determine the

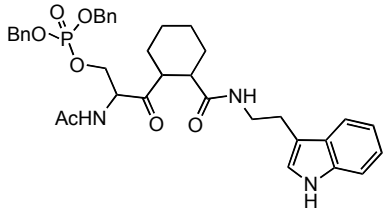
stereochemistry. Major isomer **6a**: Anal. HPLC, 254 nm, 5.0 min, 98.9%; ^{1}H NMR δ 8.10 (br, 1H), 7.57 (dd, *J* = 0.7, 7.8, 1H), 7.37 (dt, *J* = 0.8, 8.2, 1H), 7.31-7.21 (m, 5H), 7.20 (dt, *J* = 1.2, 7.5, 1H), 7.11 (dt, *J* = 0.9, 7.5, 1H), 7.02 (d, *J* = 2.2, 1H), 6.30 (d, *J* = 7.5, 1H), 5.60 (t, *J* = 5.8, 1H), 4.84 (dt, *J* = 4.2, 7.4, 1H), 4.58 (d, *J* = 11.8, 1H), 4.48 (d, *J* = 11.8, 1H), 3.86 (dd, *J* = 3.9, 9.9,1H), 3.76 (dd, *J* = 4.4, 9.9, 1H), 3.44 (m, 2H), 3.05 (ddd, *J* = 3.3, 10.7, 12.4, 1H), 2.84 (m, 2H), 2.37 (m, 1H), 2.13 (m, 1H), 1.99 (s, 3H), 1.80 (m, 3H), 1.49 (m, 1H), 1.26 (m, 2H), 1.08 (m, 1H); ^{13}C NMR δ 209.5, 174.7, 170.0, 137.9, 136.5, 128.5, 128.0, 127.9, 127.4, 122.4, 122.3, 119.6, 118.9, 113.1, 111.3, 73.3, 68.4, 60.0, 49.2, 45.8, 39.4, 29.7, 29.5, 25.7, 25.4, 25.3, 23.3; ESI^{+} HRMS: calcd for $C_{29}H_{35}N_3O_4$·Na $[M + Na]^+$ *m/z* = 512.2525, found *m/z* = 512.2530. Minor isomer **6b**: Anal. HPLC, 254 nm, 5.1 min, 96.3%; ^{1}H NMR δ 8.25 (br, 1H), 7.56 (d, *J* = 8.0, 1H), 7.36 (d, *J* = 8.3, 1H), 7.35-7.25 (m, 5H), 7.19 (ddd, *J* = 1.1, 7.2, 8.1, 1H), 7.11 (ddd, *J* = 1.0, 7.4, 8.0, 1H), 7.00 (d, *J* = 2.2, 1H), 6.75 (d, *J* = 7.7, 1H), 5.56 (t, *J* = 5.8, 1H), 4.86 (dt, *J* = 4.0, 7.4, 1H), 4.49 (d, *J* = 12.0, 1H), 4.46 (d, *J* = 12.1, 1H), 3.92 (dd, *J* = 3.3, 9.9, 1H), 3.75 (dd, *J* = 4.1, 9.9, 1H), 3.48 (q, *J* = 6.4, 2H), 3.01 (dt, *J* = 3.0, 11.1, 1H), 2.86 (m, 2H), 2.33 (m, 1H), 2.01 (s, 3H), 1.78 (m, 4H), 1.43 (m, 1H), 1.19 (m, 3H); ^{13}C NMR δ 210.9, 174.5, 170.1, 137.8, 136.5, 128.5, 127.9, 127.88, 127.4, 122.4, 122.2, 119.5, 118.8, 112.9, 111.4, 77.4, 73.4, 68.5, 58.8, 47.8, 47.7, 39.6, 30.0, 28.6, 25.3, 25.1, 23.4; ESI^{+}HRMS: calcd for $C_{29}H_{36}N_3O_4$ $[M + H]^+$ *m/z* = 490.2706, found *m/z* = 490.2710.



Ac–Ser–Ψ[C(O)C]–Pip–(2)-*N*-(3)-ethylaminoindole 7. Ac–Ser(OBn)–Ψ[C(O)CH]–Pip–(2)-*N*-(3)-ethylaminoindole **6a** (48 mg, 0.098 mmol)

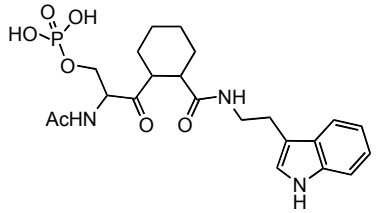
was dissolved in CH_2Cl_2 (8 mL). The solution was cooled to –78 °C, and BCl_3 (1 M in CH_2Cl_2, 1.2 mL) was added dropwise. The reaction mixture was stirred at –78 °C and warmed to 0 °C over 1.5 h. The reaction mixture was cooled to –78 °C, and MeOH (0.5 mL) and aq. HCl (2N, 5 mL) were added. The solution was diluted with EtOAc (150 mL), and washed with HCl (30 mL), 5% aq. $NaHCO_3$ (30 mL), and brine (30 mL). After filtration and evaporation, the residue was purified on silica (step gradient: %0 then 5% isopropanol/EtOAc). The product **7a** was obtained as a colorless oil (31 mg, 80%). ^{1}H NMR δ 8.20 (s, 1H), 7.59 (d, *J* = 7.5, 1H), 7.40 (t, *J* = 1.0, 1H), 7.38 (t, *J* = 0.8, 1H) 7.22 (app. tt, *J* = 7.3, 1.1, 1H), 7.14 (app. tt, *J* = 7.3, 1.2, 1H), 7.06 (d, *J* = 2.1, 1H), 6.50 (d, *J* = 7.3, 1H), 5.75 (t, *J* = 5.6, 1H), 4.87 (m, 1H), 4.39 (d, *J* = 11.8, 1H), 4.14 (m, 1H), 3.81 (m, 1H), 3.55 (dd, *J* = 13.2, 6.4, 1H), 3.49 (dd, *J* = 12.8, 6.4, 1H), 3.04 (dt, *J* = 3.4, 11.6, 1H), 2.91 (dt, *J* = 0.9, 6.4, 1H), 2.52 (dt, *J* = 3.3, 11.6, 1H), 2.06 (m, 1H), 2.02 (s, 3H), 1.81 (m, 3H), 1.42 (m, 1H), 1.26 (m, 2H), 1.06 (m, 1H); ^{13}C NMR (100 MHz) δ 210.4, 176.0, 170.0, 136.5, 127.3, 122.5, 122.4, 119.7, 118.8, 112.7, 111.4, 63.4, 59.5, 48.7, 45.9, 39.9, 30.4, 29.2, 25.68, 25.67, 25.2, 23.4. ESI$^+$HRMS: calcd for $C_{22}H_{30}N_3O_4$ $[M + H]^+$ *m/z* = 400.2236, found *m/z* = 400.2260. By the same procedure, the minor isomer **7b** was obtained as an oil (20 mg, 80%). ^{1}H NMR δ 8.39 (s, 1H), 7.56 (d, *J* = 7.9, 1H), 7.37 (d, *J* = 8.0, 1H), 7.25 (br s, 1H), 7.20 (dt, *J* = 1.1, 7.6, 1H), 7.12 (dt, *J* = 0.8, 7.5, 1H), 7.03 (s, 1H), 5.86 (t, *J* = 5.1, 1H), 4.53 (ddd, *J* = 2.7, 4.6, 7.4, 1H), 4.08 (br s, 1H), 3.93 (dd, *J* = 2.4, 11.8, 1H), 3.73 (dd, *J* = 4.4, 11.8, 1H), 3.53 (dd, *J* = 6.6, 13.2, 1H), 3.48 (dd, *J* = 6.6, 13.2, 1H), 3.00 (dt, *J* = 3.2, 11.3, 1H), 2.90 (t, *J* = 6.6, 2H), 2.48 (dt, *J* = 3.3, 11.7, 1H), 2.08 (s, 3H), 1.90 (d, *J* = 3.2, 1H), 1.76 (m, 3H), 1.40 (m, 1H), 1.21 (m, 2H), 1.09 (m, 1H); ^{13}C NMR δ 213.9, 175.8, 170.9, 136.6, 127.3, 122.5, 122.4, 119.6, 118.7, 112.6,

111.5, 63.2, 60.3, 48.2, 47.3, 40.0, 30.3, 28.8, 25.6, 25.4, 25.2, 23.3; ESI$^+$HRMS: calcd for $C_{22}H_{29}N_3O_4$·Na $[M + Na]^+$ m/z = 422.2056, found m/z = 422.2063.



Ac–Ser(PO(OBn)$_2$)–Ψ[C(O)CH]–Pip–(2)-*N*-(3)-ethylaminoindole 8. To a solution of **7a** (33 mg, 0.083 mmol) in THF (10 mL) was added 5-ethylthio–1*H*–tetrazole (32 mg, 0.25 mmol) and dibenzyl diethylphosphoramidite (0.087 mL, 0.25 mmol) at rt. The mixture was stirred at rt for 16 h. The mixture was cooled to –40 °C, and a solution of 5–6 M *tert*-butyl hydroperoxide in decane (61 μL, 0.33 mmol) was added dropwise and the mixture was stirred at –40 °C for 10 min, then at rt for 30 min. The reaction was cooled to –40 °C and quenched by $Na_2S_2O_3$. The mixture was diluted with EtOAc (80 mL), and washed with HCl (20 mL), 5% of $NaHCO_3$ (aq., 20 mL), brine (20 mL), and dried over Na_2SO_4. Concentration in vacuo provided a residue, which was purified by semi-preparative HPLC on a 5 μm C18 column, 19 × 100 mm, with gradient 10% CH_3CN/H_2O for 3 min followed by 10% to 90% CH_3CN /H_2O over 10 min, at 254 nm, to afford **8a** as a colorless oil (20 mg, 36%). ^{1}H NMR (CD_3OD) δ 7.53 (dt, J = 7.9, 1.0, 1H), 7.32 (m, 11H), 7.06 (ddd, J = 1.1, 7.0, 8.1, 1H), 7.01 (s, 1H), 6.97 (ddd, J = 0.9, 7.0, 8.0, 1H), 5.04 (d, J = 6.6, 2H), 5.02 (d, J = 6.1, 2H), 4.93 (dd, J = 3.8, 6.8, 1H), 4.53 (ddd, J = 3.9, 6.9, 10.8, 1H), 4.16 (ddd, J = 7.1, 8.2, 11.1, 1H), 3.37 (m, 2H), 2.95 (ddd, J = 3.2, 10.8, 12.2, 1H), 2.85 (m, 2H), 2.51 (m, 1H), 2.10 (m, 1H), 1.94 (s, 3H), 1.86 (m, 1H), 1.78 (m, 2H), 1.32 (m, 3H), 1.10 (m, 1H); ^{13}C NMR δ 209.5, 177.2, 173.2, 138.1, 137.1, 137.0, 129.73, 129.68, 129.23, 129.19, 128.8, 123.5, 122.3, 119.6, 119.3, 113.2, 112.2, 71.0, 67.0 (d, $^4J_{P\text{-}C}$ = 5.0), 59.1 (d, $^4J_{P\text{-}C}$ =

7.5), 50.6, 47.3, 41.3, 31.1, 30.2, 26.6, 26.5, 26.3, 22.4; ^{31}P NMR (202 MHz): δ –0.40; ESI$^+$HRMS: calcd for $C_{36}H_{43}N_3O_7P$ $[M + H]^+$ m/z = 660.2839, found m/z = 660.2846. By the same procedure, the minor isomer **8b** was obtained as an oil (17 mg, 37%). ^{1}H NMR δ 8.49 (s, 1H), 7.54 (d, J = 7.7, 1H), 7.36 (d, J = 8.0, 1H), 7.33 (m, 10H), 7.18 (ddd, J = 1.1, 7.1, 8.1, 1H), 7.10 (ddd, J = 1.0, 7.0, 8.0, 1H), 6.98 (d, J = 2.2, 1H), 6.91 (d, J = 8.3, 1H), 5.50 (t, J = 5.8, 1H), 5.02 (m, 4H), 4.88 (ddt, J = 1.6, 4.2, 8.0, 1H), 4.32 (ddd, J = 4.1, 6.4, 10.5, 1H), 4.27 (ddd, J = 4.7, 6.3, 10.4, 1H) 3.58 (ddt, J = 6.0, 7.7, 13.5, 1H), 3.43 (ddt, J = 5.5, 6.5, 13.8, 1H), 2.95 (dt, J = 3.2, 11.2, 1H), 2.86 (m, 2H), 2.37 (dt, J = 3.5, 11.6, 1H), 1.94 (s, 3H), 1.84 (d, J = 11.8, 1H), 1.74 (m, 3H), 1.39 (m, 1H), 1.16 (m, 3H); ^{13}C NMR δ 209.5, 174.7, 170.5, 136.6, 135.9, 135.88, 135.8, 128.74, 128.71, 128.69, 128.13, 128.11, 127.4, 122.5, 122.2, 119.5, 118.7, 112.6, 111.5, 69.60 (d, $^3J_{P\text{-}C}$ = 3.9), 69.55 (d, $^3J_{P\text{-}C}$ = 3.8), 65.4 (d, $^4J_{P\text{-}C}$ = 5.4), 57.1 (d, $^3J_{P\text{-}C}$ = 9.1), 47.4, 47.1, 39.5, 30.0, 28.7, 25.4, 25.3, 25.2, 23.2; ^{31}P NMR: δ –0.53; ESI$^+$HRMS: calcd for $C_{36}H_{43}N_3O_7P$ $[M + H]^+$ m/z = 660.2839, found m/z = 659.2842.



Ac–Ser(PO(OH)$_2$)–Ψ[C(O)CH]–Pip–(2)-*N*-(3)-ethylaminoindole 1. Dibenzyl phosphate **8a** (14 mg, 0.021 mmol) and 10% Pd-C (7 mg) were dissolved in MeOH (8 mL). The reaction was stirred under H_2 (1 atm) at rt for 2 h. The reaction mixture was filtered through Celite, and washed with MeOH. After evaporation, the residue was purified by semi-preparative HPLC on a 5 μm C18 column, 19 × 100 mm, with gradient 5% CH_3CN/H_2O for 3 min followed by 5% to 90% CH_3CN /H_2O over 6 min, at 254 nm, to provide **1a** as a white solid after lyophlization (8.0 mg,

78%). Anal. HPLC, 254 nm, (gradient: 5% B for 3 min, then 5–90% B over 6 min), 6.0 min, 99.9%; ^{1}H NMR (CD_3OD) δ 7.54 (d, J = 7.7, 1H), 7.32 (d, J = 8.2, 1H), 7.07 (m, 2H), 6.99 (ddd, J = 0.9, 7.0, 8.0, 1H), 4.92 (m, 1H), 4.35 (m, 1H), 4.18 (m, 1H), 3.40 (m, 2H), 3.04 (dt, J = 3.0, 11.4, 1H), 2.89 (m, 2H), 2.52 (ddd, J = 3.4, 10.5, 12.2, 1H), 2.22 (dd, J = 2.2, 13.2, 1H), 2.00 (s, 3H), 1.83 (m, 3H), 1.35 (m, 3H), 1.11 (m, 1H); ^{13}C NMR (CD_3OD) δ 209.8, 177.7, 173.2, 138.1, 128.8, 123.6, 122.2, 119.5, 119.3, 113.2, 112.2, 65.5, 59.2 (d, $^{3}J_{P\text{-}C}$ = 12), 50.4, 47.1, 41.2, 31.2, 30.3, 26.8, 26.6, 26.2, 22.3; ^{31}P NMR (CD_3OD) δ –1.44; ESI^{+} HRMS calcd for $C_{22}H_{31}N_3O_7P$ $[M + H]^{+}$ m/z = 480.1900, found m/z = 480.1906. By the same procedure, the minor isomer **1b** was obtained as a white powder (5.5 mg, 70%). Anal. HPLC, 254 nm, (gradient: 5% B for 3 min, then 5–90% B over 6 min), 6.8 min, 99.1%; ^{1}H NMR (DMSO-d_6): δ 10.82 (br s, 1H), 8.25 (br s, 1H), 7.89 (t, J = 5.6, 1H), 7.50 (d, J = 7.8, 1H), 7.31 (d, J = 7.7, 1H), 7.10 (d, J = 1.5, 1H), 7.04 (t, J = 7.4, 1H), 6.96 (t, J = 7.2, 1H), 4.67 (m, 1H), 4.14 (m, 1H), 3.96 (m, 1H), 3.24 (m, 2H), 2.99 (t, J = 10.4, 1H), 2.73 (m, 2H), 2.36 (dt, J = 3.6, 11.3, 1H), 1.94 (m, 1H), 1.87 (s, 3H), 1.82 (m, 1H), 1.68 (m, 2H), 1.24 (m, 3H), 1.02 (m, 1H); ^{13}C NMR (CD_3OD): δ 210.5, 177.2, 173.1, 138.1, 128.8, 123.6, 122.2, 119.5, 119.3, 113.3, 112.2, 65.4, 60.0, 49.4, 49.3, 41.2, 31.3, 29.3, 26.5, 26.4, 26.2, 22.6; ^{31}P NMR (DMSO-d_6): δ –2.93; ESI^{+} MS calcd for $C_{22}H_{31}N_3O_7P$ $[M + H]^{+}$ m/z = 480.19, found m/z = 480.18.

Pin1 inhibition assay. The Pin1 inhibition assay was performed at 4 °C in 35 mM HEPES pH 7.8 in a total assay volume of 1.2 mL as published. Inhibitors were dissolved in 2:1 DMSO:H_2O and 20 μL of stock was added to give final concentrations of **1a**: 12 μM, 50 μM, 0.10 mM, 0.20 mM, 0.40 mM, 0.81 mM, and **1b**: 10 μM, 20 μM, 40 μM, 60 μM, 0.12 mM, 0.24 mM, 0.48 mM, were pre-equilibrated with Pin1 in HEPES

at 4 °C for 15 min. The Pin1 final concentration in the assay was 67 nM. The final concentration of succinyl–Ala–Glu–[cis]Pro–Phe-*p*NA was 34 μM. For each concentration, the assay was performed in duplicate. The plot of % Inhibition vs. log [I] (μM) produced a sigmoidal curve by fitting all of the experimental data to a dose-response curve using TableCurve (version 3 for win32) (Figures 3.10 and 3.11). The IC_{50} values were derived from the fitted equation at 50% inhibition of enzyme activity.

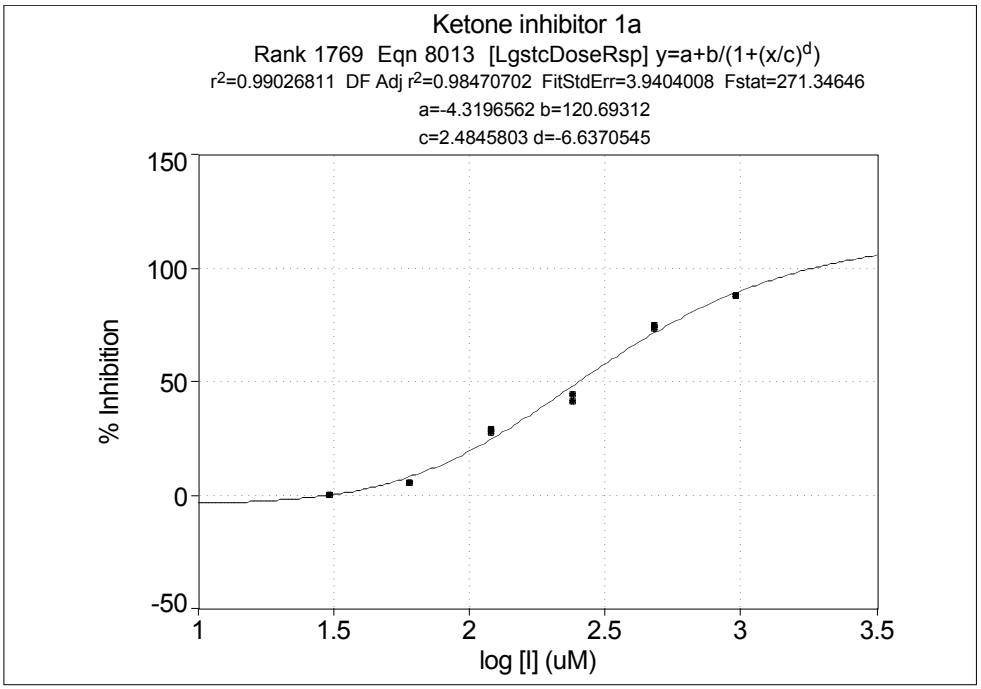


Figure 3.10. Dose response curve for inhibition of Pin1 by **1a**. IC_{50} = 260 ± 30 μM

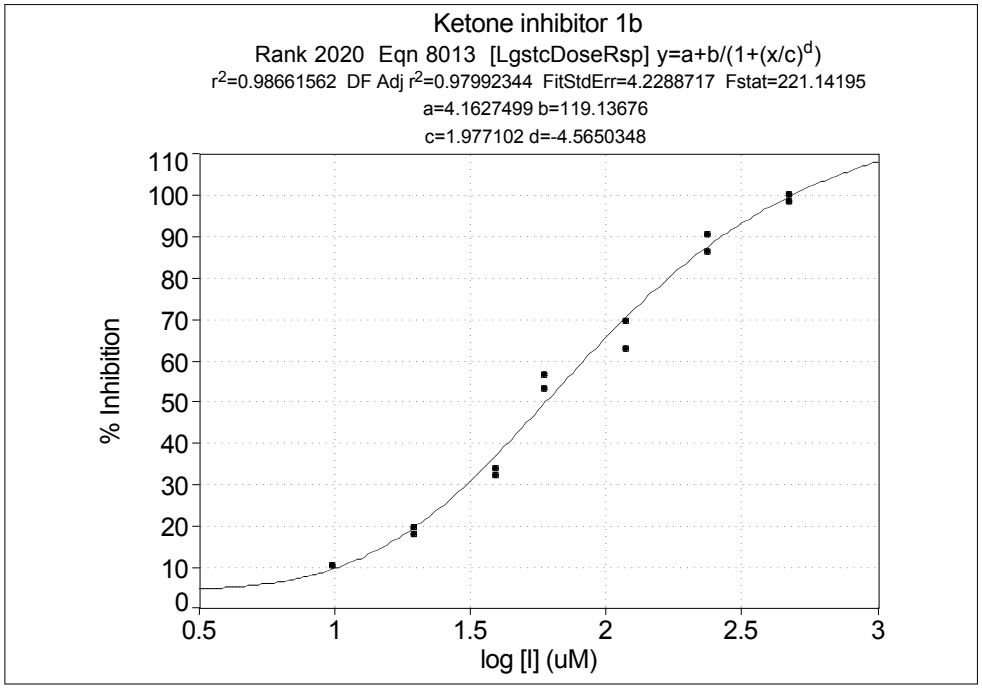


Figure 3.11. Dose response curve for inhibition of Pin1 by **1b**. IC_{50} = 61 ± 8 μM.

Chapter 4: Reduced amide inhibitors

This chapter includes two parts: A) a literature review on reduced amide inhibitors and the synthetic methods, and B) a manuscript to be submitted, that concerns the design and synthesis of reduced amide analogue inhibitors of Pin1. I performed all of the synthesis and bioassay experiments from Part B, Dr. Felicia Etzkorn did crystallization of inhibitor **2** with Pin1, and collected the data. Dr. Yan Zhang (Department of Chemistry and Biochemistry, University of Texas, Austin) processed the X-ray data. I wrote the first drafts of Part A, the manuscript and experimental section of Part B, and Dr. Etzkorn edited them in close consultation with me.

Part A: Literature review of reduced amide inhibitors

Reduced amides (tertiary amines) have an sp^3 nitrogen, which is pyramidal, and have hydrogen bonding ability. Reduced amide analogues were designed as Pin1 inhibitors for development of potent inhibitors and mechanistic studies of Pin1. In this part, we review the design of reduced amide analogue inhibitors, their inhibition of proteases, and their use in studying enzyme mechanism.

4.1. Introduction

We anticipated that studies to better understand the molecular basis for enzyme-inhibitor specificities will provide a means to design inhibitors that are less susceptible to the development of resistance.[1] The $\Psi[CH_2NH]$ moiety introduces unique properties over the amide bond, such as protease resistance, increased flexibility and increased H-bond donor ability.[1] That makes $\Psi[CH_2NH]$ moieties interesting for developing potent inhibitors and mechanistic studies.

4.2. Reduced amides inhibitory activities

Reduced amide peptidomimetics have been widely used as analogues of amides in the development of inhibitors. They are found to inhibit many proteases and enzymes.

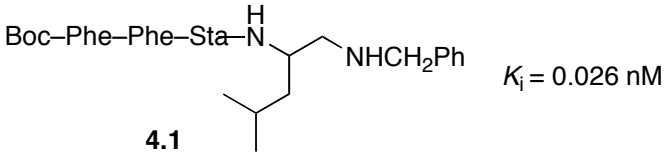
4.2.1. Aspartyl proteases inhibitors

Many reduced amide analogues have been developed as inhibitors of aspartyl proteases, which include renin protease, β-secretase and HIV protease.

Renin inhibitors

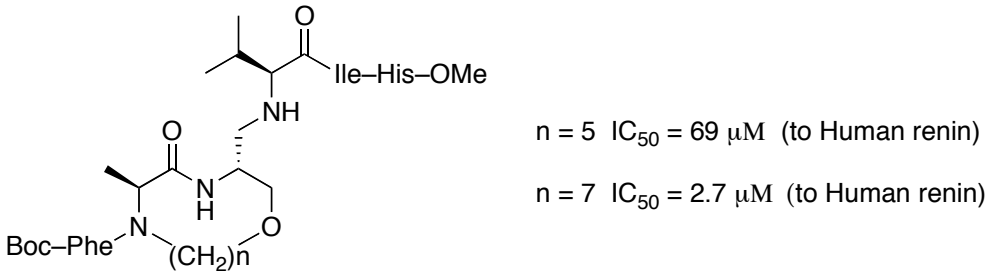
The renin enzyme plays a key role in the blood pressure regulating renin-angiotensin cascade.[2] Renin inhibitors have been widely developed, including one very important class of compounds, which replaces the scissile amide bond with the reduced moiety $–CH_2-NH-$, usually called a reduced amide or amine. Through the reduction of Leu-Leu scissile bond of a series of octapeptides, a series of reduced amides were produced as potent and selective inhibitors of canine plasma renin.[2] Szelke et al also applied the same approach in developing human plasma renin inhibitors.[2] With very high inhibitory potency at the nanomolar level, they believed the reduced moiety $–CH_2NH–$ acted as a non-hydrolyzable analogue of the tetrahedral transition state of peptide-bond hydrolysis.[2]

Evans and coworkers reported a uniquely potent renin inhibitor with the modified reduced amide structure from a tetrapeptide, in which the C-terminal amide bond was reduced to the corresponding amine **4.1**.Their study also showed that **4.1** was bound effectively by some unanticipated plasma component.[3]



4.1

Two cyclic compounds were developed as renin inhibitors with high specificity and metabolic stability.[4] These compounds were synthesized with ring sizes of 12 and 14 and a reduced amide replacing the amide bond at the active site. The cyclic compounds **4.2** were resistant to cleavage by chymotrypsin. Sham and coworkers also found that the 10-membered-ring compound was inactive against both human and mouse renins due to a conformation in solution that is inconsistent with binding.[4]

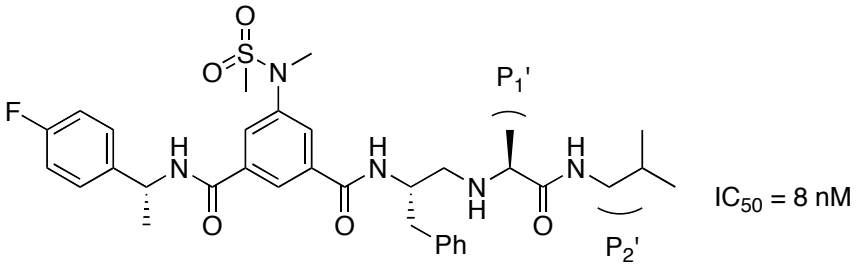


4.2

β-Site amyloid precursor protein cleaving enzyme (BACE-1) inhibitors

β-Secretase (BACE-1) is an aspartyl protease that plays a role in the rate-limiting step in the generation of Aβ peptide fragments.[5] Aβ monomers can form neurotoxic oligomers and result in Alzheimer's disease (AD). [5] Thus, BACE-1 has been a target for treatment of AD. Based on the application of $\Psi[CH_2NH]$ in the development of potent inhibitors of aspartyl proteases, Coburn and coworkers developed a series of reduced amide isostere inhibitors of BACE-1.[5] These substrate-based reduced amide inhibitors all contained a non-cleavable peptide surrogate. With an SAR study at the P_1' and P_2' positions, they obtained inhibitors with low nanomolar enzymatic and cellular potencies.[5] The crystal structure of one reduced amide **4.3**, in complex with BACE-1, revealed the

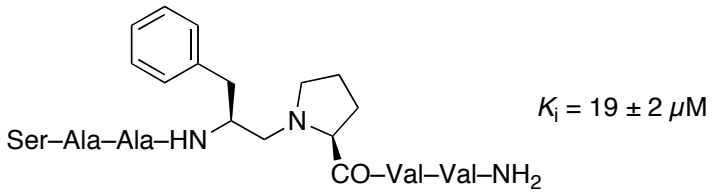
nitrogen atom of the reduced amide bond shared a hydrogen bond with only half of the active site dyad, specifically Asp228.[5]



4.3

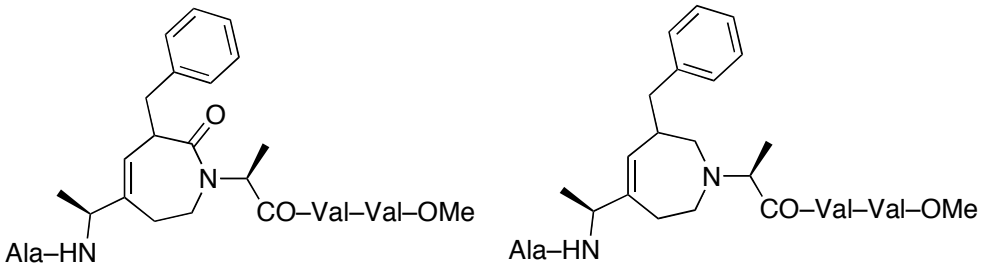
HIV protease inhibitors

Dreyer and coworkers studied several classes of analogues of a heptapeptide substrate as HIV-1 protease inhibitors, in which the scissile dipeptide bond was modified to form statines, hydroxyethylene isosteres, phosphinic acids, a reduced amide isostere, and a difluoroketone.[6] It was found that the reduced amides were relatively weak inhibitors. The reduced amide inhibitor **4.4** was the best among them.



4.4

C_7 mimetics were designed to mimic a γ-turn conformation, and were used to probe substrate conformational requirements in binding to HIV-1 protease (Figure 4.1).[7] Reduction of the amide bond of C_7 mimetic **4.5** resulted in a constrained reduced amide mimetic **4.6**, which was 44-fold better than the linear reduce-amide **4.3**.[7]



K_i = 147 ± 4 μM

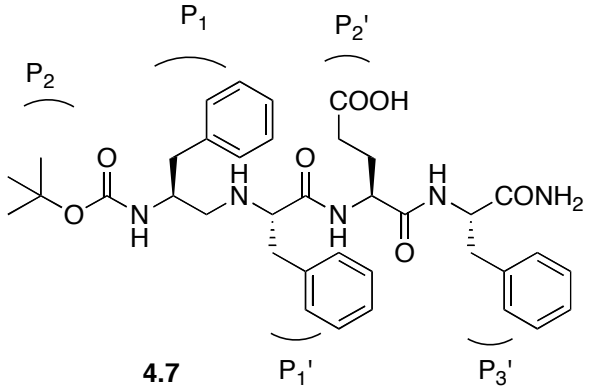
4.5

K_i = 0.43 ± 0.07 μM

4.6

Figure 4.1 Cyclic inhibitors of HIV protease[7]

Based on the substrate specificity, a series of reduced amide inhibitors of HIV protease were synthesized.[8, 9] Aromatic Phe residues were prefered in the P_1, P_1'and P_3' positions, Bulky groups were required in the P_2 position. Introduction of a Glu into the P_2' position yielded a potent inhibior **4.7**, Boc-Phe-Ψ[CH_2-NH]-Phe-Glu-Phe-OMe (K_i = 0.2 nM).[8, 9]



4.7

P_1'

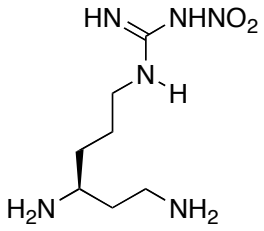
P_3'

K_i = 0.2 nM

4.2.2. Neuronal nitric oxide synthase inhibitors

Nitric oxide synthase (NOS) enzymatically produces nitric oxide (NO) from arginine in numerous tissues and cell types.[10] There are three isoforms of this enzyme, inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS).[10] Selective inhibition of these isoforms of NOS could be useful in the therapeutic treatment of certain

diseases due to the overproduction of NO.[10] Hah et al synthesized a series of reduced amide bond pseudodipeptide analogues as inhibitors of NOS.[10] The reduced amide analogues either preserved or improved the potency for nNOS, and selectively inhibited nNOS significantly over eNOS and iNOS. [10] Among those compounds, the most potent nNOS inhibitor **4.8** also showed the highest selectivity.[10]

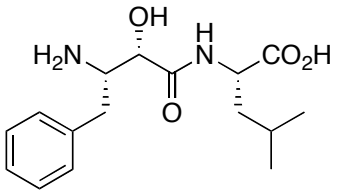


K_i = 120 nM

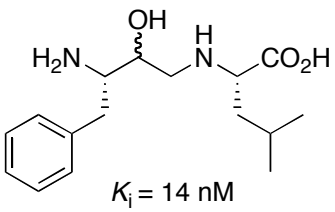
4.8

4.2.3. Aminopeptidase inhibitor

Aminopeptidases catalyze the hydrolysis of the amino-terminal residue from a peptide chain. Bestatin was isolated as a natural product that potently inhibits aminopeptidase enzymes.[11] In order to study the inhibition mechanism of the enzymes by bestatin, Harbeson et al synthesized a bestatin analogue by replacing the amide bond of bestatin with the reduced amide group. That modification yielded a potent inhibitor of arginine aminopeptidase **4.9** (mixture of two diastereomers) (Figure 4.2).[11] Kinetic studies of the inhibitor showed evidence that the inhibitor binds to the S1' and S2' subsites on the enzyme.[11]



Bestatin



K_i = 14 nM

4.9

Figure 4.2 Modification of Bestatin.[11]

4.2.4. Neurokinin A (NKA) NK2 receptor

The mammalian tachykinin, neurokinin A, produces its physiological and pharmacological effects through binding to NK_2, a tachykinin receptor.[12] Antagonists of the receptor are promising therapeutic agents for some disorders such as asthma, urinary incontinence, and inflammation etc.[12] Harbeson and coworkers used a reduced amide bond replacement for converting $NKA_{4\text{-}10}$ into an NK_2 antagonist because of the therapeutic potential of antagonists.[12] They found [$\Psi(CH_2NH)^9$-Leu^{10}]$NKA_{4\text{-}10}$ **4.10** showed significant affinity for the NK_2 receptor and weak partial agonist activity.[12] After $\Psi[CH_2NH]$ alkylation and replacement of Leu^{10} with Phe, the most potent compound **4.11**, [βAla^8,$\Psi(CH_2N(CH_2)_2CH_3)^9$,$Phe^{10}$]$NKA_{4\text{-}10}$ was obtained, which also showed partial agonist activity (Figure 4.3). Although it is not clear how the replacement affects the activity, the $\Psi[CH_2NH]$ moiety obviously increased the conformational freedom.[12]

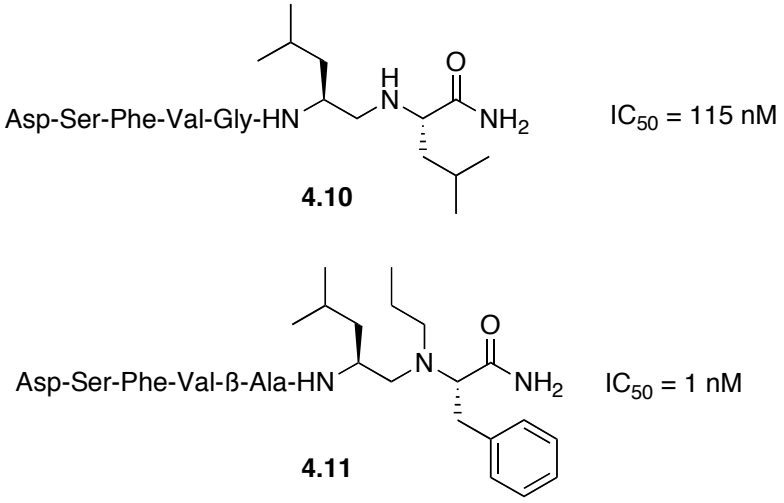
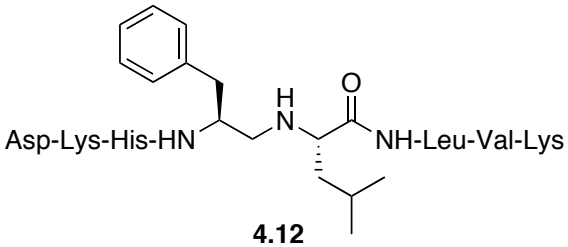


Figure 4.3 Reduced amides as NK2 inhibitors[12]

4.2.5. Cysteine protease B inhibitors

A solid-phase library was synthesized to screen inhibitors of recombinant cysteine

protease B.[13] The split-mix method was applied to form a one-bead–two-compound library. The library contained about 3,400 octapeptides with a centrally located reduced-amide bond.[13] The reduced-amide bond was introduced by reductive amidation of resin-bound amines with amino aldehydes. From the analysis of the hits, the substrate sequence specificities were determined. Most of the compounds inhibited the enzyme at micromolar concentrations, except for a few at nanomolar concentrations. One inhibitor **4.12**, Asp–Lys–His–Phe–Ψ[CH_2NH]Leu–Leu–Vla–Lys (K_i = 1 μM) was studied for antiparasite efficacy with an IC_{50} of 50 μM.[13]



4.12

4.2.6. Growth hormone-releasing factor (1-29)

Hocart and coworkers applied reduced amides to the study of the antagonistic and histamine releasing activities of a luteinizing hormone-releasing hormone.[14, 15] They also developed reduced-amide analogues of growth-hormone-releasing factor (GRF) (1-29), and studied their agonist activities.[14, 15]

Although incorporation of the reduced amide bond in different positions caused different activities, there were no potent analogues developed in their studies (Table 4.1).[15]

Table 4.1. Biological potencies of reduced peptide bond GRF(1-29)NH_2[15] analogues

Analogue	% potency
GRF(1-29)NH_2	100
$Tyr^1\Psi[CH_2NH]Ala^2$	0.12
$Ala^2\Psi[CH_2NH]Asp^3$	0.13
$Asp^3\Psi[CH_2NH]Ala^4$	1.6
$Ala^4\Psi[CH_2NH]Ile^5$	0.02
$Ile^5\Psi[CH_2NH]Phe^6$	< 0.01
$Phe^6\Psi[CH_2NH]Thr^7$	0.13
$Thr^7\Psi[CH_2NH]Asn^8$	0.02
$Tyr^{10}\Psi[CH_2NH]Arg^{11}$	0.39

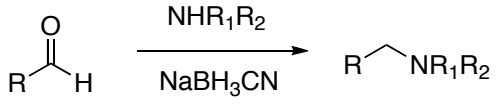
4.3. Synthetic methods for reduced amides

The synthetic methods of reduced amides are briefly reviewed here. The synthesis of reduced amides is usually through a reduction reaction. Two commonly used reduction reactions are discussed below.

4.3.1. Synthesis by reductive amination

Amination and reduction is one commonly used method to synthesize the reduced amide, either through solution phase[1, 5, 7] or solid-phase synthesis.[13-15] The general reaction is shown in Scheme 1.

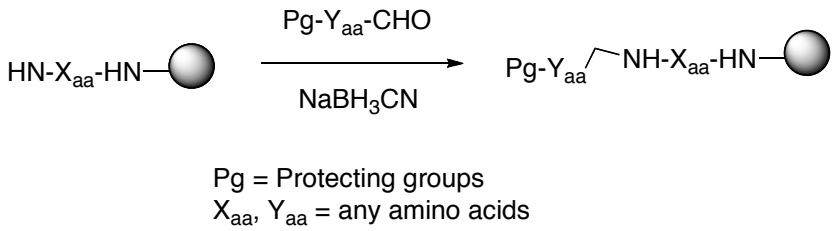
Scheme 4.1. Reductive amination method to synthesize reduced amides.



For reduced amide peptide isosteres, reductive amination with a protected amino

acid aldehyde in the presence of $NaBH_3CN$ furnishes reduced amide isosteres. A general method for solid-phase synthesis is shown in Scheme 4.2. [13-15]

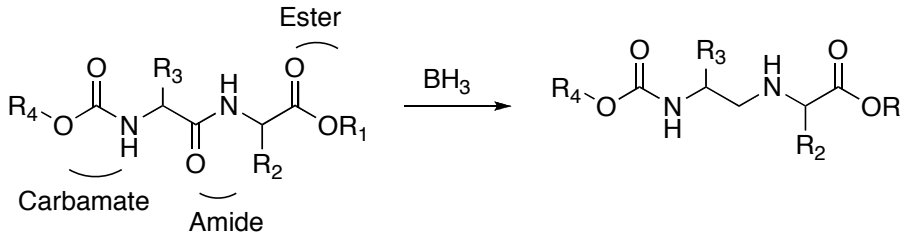
Scheme 4.2. Reductive amination on solid phase.[13-15]



4.3.2. Synthesis through selective borane reduction

Another common method to synthesize reduced amides is selective reduction of the amide bonds with borane (Scheme 3). Due to different reactivity, amide bonds can be selectively reduced to amines in the presence of carbamates and esters.[16] This is the method that was used for the synthesis of our Pin1 inhibitors.

Scheme 4.3. Selective reduction of amides by borane.



4.4. Conclusions

The inhibitory activities and synthetic methods of reduced amides were reviewed. With the non-hydrolysable functional group and increased hydrogen bond donor activity, reduced amides have been developed as inhibitors for many proteases such as aspartyl proteases and HIV proteases. Due to the unique features of reduced amides, such as a

pyramidal nitrogen and increased hydrogen bonding ability over amides, it is very important to apply the reduced amides to the development of Pin1 inhibitors, based on the proposed twisted-amide mechanism of Pin1 catalysis.

The synthesis of reduced amides included reductive amination and selective reduction by borane, and we applied the borane reduction method to the synthesis of the important intermediates of our reduced amide inhibitors of Pin1.

Reference:

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Part B: Synthesis and bioassay of reduced amide inhibitors of Pin1

Inhibition and Structure of Reduced Amides with the Peptidyl-Prolyl Isomerase Pin1

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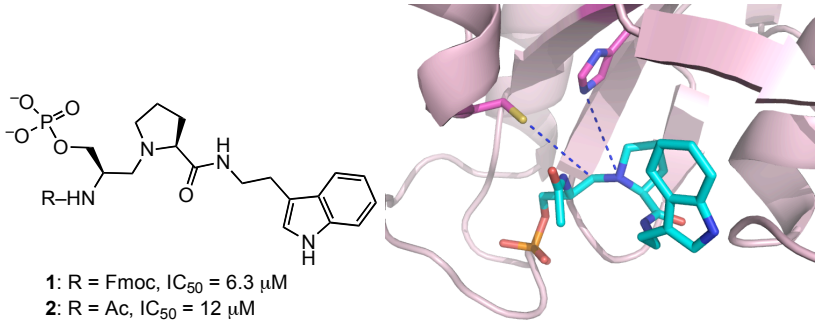


Abstract. Reduced amide inhibitors **1** and **2** for Pin1 were designed and synthesized. Inhibitor **1** had an IC_{50} value of 6.3 μM for Pin1, which is 4.5-fold better inhibition for Pin1 than our comparable *cis*-amide alkene isostere. The change of Fmoc to Ac improved aqueous solubility for structural determination, and resulted in a similar IC_{50} value of 12 μM for Pin1. The co-crystal structure of **2** bound to Pin1 was determined to 1.76 Å resolution. The structure revealed the lack of an expected hydrogen bond between the tertiary amine and any Pin1 active site residue, providing some evidence against a hydrogen-bond assisted twisted amide mechanism.

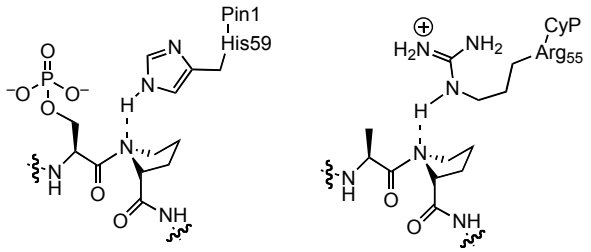
Pin1, a peptidyl-prolyl isomerase (PPIase) enzyme in the parvulin family, along with the well known immunophilin PPIases, human cyclophlin A (hCyPA) and FK506 binding protein (FKBP), catalyze the cis-trans isomerization of Xaa-Pro amide bonds.[1-3]

Pin1 is distinct from CyP and FKBP, because it has several demonstrated roles in regulating cell cycle progression.[4, 5] Pin1 regulates the cooperative transition of cells from G2 to M phase by interaction with a variety of cell cycle proteins, including Cdc25C phosphatase, which regulates the activity of CDK1, the central mitotic kinase.[6, 7] Pin1 is also known to regulate the function of cyclin D1, another cell-cycle protein that is active in G1, through direct interaction with cJun, an oncogene.[8] Pin1 specifically recognizes the phosphoSer/Thr–Pro amide bonds present in mitotic phosphoproteins.[6, 9] Inhibition of Pin1 is likely to lead to interesting anti-cancer activities. We present here reduced amide inhibitors of the PPIase activity of Pin1.

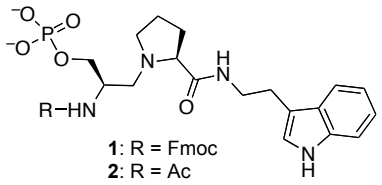
PPIases accelerate prolyl cis-trans amide isomerization at a rate of ca. 10^6 s^{-1} faster than the thermal isomerization.[10] A twisted amide mechanism has been proposed based on mechanistic studies of CyP and FKBP.[4, 11-14] It was observed that the ketone carbonyl of inhibitor FK506 was orthogonal to the amide plane in the bound conformation, so FK506 was proposed to act as a transition state analogue of the twisted amide to inhibit FKBP.[15-17] FK506 acts as a very potent inhibitor of FKBP, which affirms this interpretation.[18] Normal secondary kinetic isotope effects for CyP substrates deuterated on the N-terminal side of the Xaa-Pro amide also supported the twisted amide mechanism.[19-21]

The concept of a transition-state analogue is very effective as a basis for designing potent enzyme inhibitors.[22] In the proposed twisted amide mechanism, the nitrogen is pyramidal and could act as a hydrogen bond acceptor from donors in the active sites of the enzymes (Figure 4.4).[4, 14] In an inhibitor, a reduced amide would act as a stable hydrogen bond acceptor analogous to the transition state.

Figure 4.4. Twisted amide mechanism proposed for Pin1 and hCyPA.



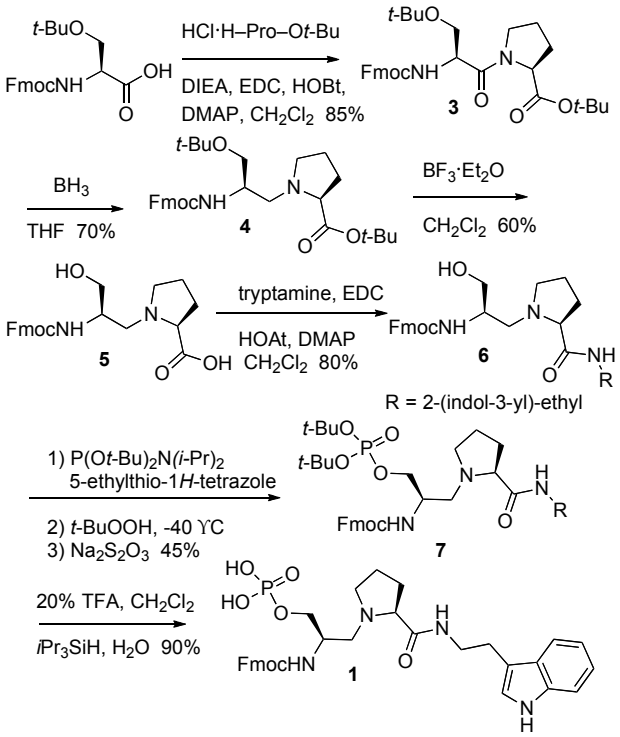
Aromatic residues are preferred on both the C-terminus and N-terminus of Pin1 substrates.[6, 23] Cis and trans alkene analogues, Ac–Phe–Phe–pSer–Ψ[CH=C]-Pro–Arg–NH_2, synthesized in our lab, were found to be good inhibitors of Pin1.[24] Both crystal structures of the inhibitor-Pin1 complexes showed that the electron density for the two N-terminal phenylalanines was missing.[25] The reduced amides, Fmoc–pSer–Ψ[CH_2N]-Pro–tryptamine **1**, and Ac–pSer–Ψ[CH_2N]-Pro–tryptamine **2**, were designed as Pin1inhibitors. The truncated analogues were designed with Fmoc or Ac at the N-terminus and tryptamine at the C-terminus of the pSer–Pro core. The Fmoc group was chosen because it acted as a protecting group for the amine during the whole synthesis, and it could be analogous to the aromatic residues on the N-terminus. The substitution of Ac for Fmoc was made to improve aqueous solubility for X-ray structure determination.



The reduced amide isostere concept was first reported in the development of potent inhibitors of human renin.[26] Since then, reduced amide peptide isosteres have been used widely in the development of inhibitors for aspartyl proteases such as human renin[26-28] and HIV.[29-31] Reduced amide isosteres were also exploited in the design and synthesis

of potent inhibitors for β-site amyloid precursor protein cleaving enzyme (BACE-1),[32] neurokinin A NK2 receptor,[33] neuronal nitric oxide synthase,[34] cysteine protease B,[35] and dipeptidyl peptidase IV,[36] among others.

Scheme 4.4. Synthesis of reduced amide **1**.



The key step in the synthesis was selective reduction of the amide in the presence of both an ester and a carbamate (Scheme 4.4). Fmoc–Ser(*t*-Bu)–OH was coupled to H–Pro–O*t*-Bu to form amide **3** using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide·HCl (EDC), *N*,*N*-diisopropyl ethylamine (DIEA), and *N*-hydroxybenzotriazole (HOBt). Amide **3** was reduced selectively with borane to form the reduced amide **4**.[37, 38] Both *t*-Bu groups were removed with $BF_3{\cdot}Et_2O$ to form intermediate **5**, although the deprotection has been reported only for a *t*-Bu ester.[39] Free acid **5** was coupled selectively to tryptamine using EDC, 1-hydroxyl-7-azabenzo-triazole (HOAt), and DMAP in the presence of the free hydroxyl group to give amide **6** in 80% yield. The yield was high due

to the greater nucleophilicity of the amine than the alcohol, and faster coupling with HOAt than HOBt activated esters.[40, 41]

The first attempted phosphorylation using ammonium *tert*-butyl *H*-phosphonate was unsuccessful.[42] Phosphorylation to give **7** was accomplished by using di-*tert*-butyl diisopropyl-phosphoramidite followed by oxidation with *tert*-butyl hydroperoxide.[43, 44] Deprotection with TFA released the final phosphorylated Pin1 inhibitor **1**.

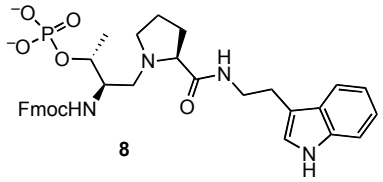
During enzyme assay of Pin1 inhibitor **1**, and the attempt at co-crystallization of the inhibitor with Pin1, it was found that the solubility of the inhibitor **1** was very poor. Because of the poor solubility of the Fmoc-containing inhibitor **1**, we were unable to soak the inhibitor into crystals of Pin1 for structure determination. Because of the lack of N-terminal residues in electron density maps of Pin1-peptide inhibitor co-crystal structures, we believed the Fmoc group on the N-terminus would not be essential for the inhibition or binding. Thus we put acetyl on the N-terminus of inhibitor **2** to improve the solubility and soak into the enzyme crystals more efficiently.

Three additional compounds were designed and synthesized as Pin1 inhibitors. Compound **8** was designed since pThr–Pro is also a specified substrate motif of Pin1. In order to improve the inhibitory activity, compound **9** was designed based on Fischer's potent nanomolar peptidomimetic inhibitors,[45] by replacing Pro with Pip. Two diastereomers **9a** and **9b** were obtained with the use of racemic pipecolic acid. Similar strategies were used for the synthesis of inhibitors **8** and **9**. Detailed schemes and methods were provided in the experimental section.

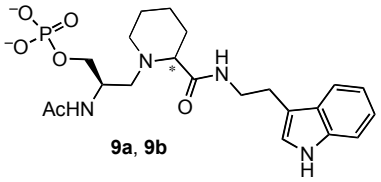
The α-chymotrypsin protease-coupled assay was used to evaluate inhibition of Pin1.[24, 46, 47] The Pin1 substrate was succinyl–Ala–Glu–*cis*-Pro–Phe–*p*NA at a

concentration of 34 μM, with a K_m [cis] value of 180 mM. The absorbance of *p*NA released from the trans product was recorded at 390 nm by UV/Vis. The assay results for all of the target compounds are listed in Table 4.2.

Table 4.2. Protease coupled assay results for compounds **1**, **2**, **8**, **9a**, **and 9b**.



8



9a, **9b**

	Pin1 Inhibitor	IC_{50} (μM)
1	Fmoc–pSer–Ψ[CH_2N]-Pro–tryptamine	6.3 ± 0.4
2	Ac–pSer–Ψ[CH_2N]-Pro–tryptamine	12 ± 2
8	Fmoc–pThr–Ψ[CH_2N]-Pro–tryptamine	30 ± 2
9a	Ac–pSer–Ψ[CH_2N]-(∗)Pip–tryptamine	16 ± 2
9b	Ac–pSer–Ψ[CH_2N]-(∗)Pip–tryptamine	189 ± 17

Compound **1** was found to be the most potent inhibitor among the reduced amides. The fact that compound **1** is a better inhibitor than compound **8** implies that Pin1 may prefer pSer–Pro to pThr–Pro. Replacement of the Fmoc at the N-terminus in compound **1** with an Ac to give compound **2** indeed improved the water solubility. This modification reduced the inhibitory activity only slightly. The aromatic residue probably contributes only to non-specific binding. Replacement of the Pro in compound **2** with a racemic Pip gave a pair of diastereomers **9a** and **9b**, however, even the more potent one, **9a**, is slightly worse than **2**, indicating that the six-membered ring is not a better choice than its five-membered counterpart in this case. Therefore we decided not to expend more effort to

determine the stereochemistry. However, compound **9a** is approximately 12-fold more potent than its diastereomer **9b**, showing the importance of the α-carbon configuration on the Pip ring. We suspect that **9a** has the native-like stereochemistry by comparison with the L-Pro derivative **2**.

The design of the inhibitors was based on the proposed hydrogen-bonded twistase mechanism of PPIases, but they are not as potent as we expected.[4, 12, 14] One possible reason is that the mechanism of the Pin1 PPIase reaction may not include the transitory formation of hydrogen bond to a tertiary amine as expected from mutagenesis,[4, 11] and computational studies[13, 14] of other members of the PPIase family. Several other factors may contribute to the lower-than-expected inhibition of the reduced amide inhibitors: 1) The flexibility of the reduced amide would cause these inhibitors to have an entropic penalty upon binding; 2) The carbonyl of the rotatable amide is missing from the inhibitors compared to the proposed twisted amide transition state. In addition, the Fmoc and tryptaminyl groups of **1** flanking the rotatable bond were not optimized for inhibition of Pin1. Although Pin1 inhibitors **1** and **2** may not be as potent as a transition-state analogue ought to be, they are more potent than the analogous alkene ground-state isostere.

The reduced amide, Fmoc–pSer–Ψ[CH_2N]-Pro–tryptamine, **1** is 4.5-fold more potent than the analogous (*Z*)-alkene inhibitor of Pin1, Fmoc–pSer–Ψ[(*Z*)CH=C]-Pro–tryptamine (IC_{50} = 28.3 μM).[44] The most potent Pin1 inhibitors known to date are the D-Thr containing peptide analogues of Fischer and coworkers.[45] The octapeptide, Ac–Lys(*N*-biotinoyl)–Ala–Ala–Bth–D-Thr(PO_3H_2)–Pip–Nal–Gln–NH_2 had a K_i value of 1.2 nM, and the pentapeptide analogue, Ac–Phe–D-Thr(PO_3H_2)–Pip–Nal–Gln–NH_2 had a K_i

value of 18.3 nM. However, neither diastereomer of compound **9** was a better inhibitor than **1** or **2** with the modification of the Pro to Pip.

Crystallization and Structural Determination

We expected hydrogen bonding between the nitrogen of the reduced amide and the active site residues of Pin1 enzyme, which would make the reduced amide inhibitors act as transition state analogues. However, the inhibition affinity of these two inhibitors was not high enough to be considered as the transition state mimics. To better understand the inhibitory mechanism, we solved high-resolution crystal structures of Pin1 complex with inhibitor **2**.

Table 4.3. Data collection and refinement statistics.

Crystal Data	A3
Space Group	$P3_121$
Unit Cell	
a (Å)	68.6
b (Å)	68.8
c (Å)	79.8
$\alpha=\beta$ (°)	90.0
γ (°)	120
Data collection	
X-ray source	APS[a]
Resolution (Å) [b]	1.76 (48-1.76)
R_{sym} (%)	4.8 (47.2)
Completeness (%)[b]	93.9 (82.6) %
Refinement statistics	
R-work (%)	22.1
R-free (%)	25.7

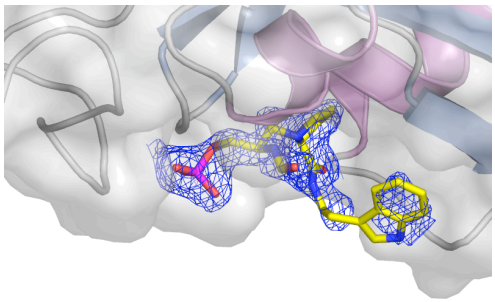
RMS Bond lengths (Å)	0.018
RMS Bond angles (°)	1.751
No. of ligand or cofactor molecules [c]	1 RED
	1 PE4
No. of water molecules	92
Average B overall (Å^2)	31.2

[a] Argonne National Laboratories

[b] The values in parentheses are for the highest resolution shell

3D Structure of the Pin1-Inhibitor 2 Complex

The structure of Pin1-inhibitor **2** complex was determined and refined to 1.76 Å resolution (Table 4.3). Inhibitor **2** is bound to the Pin1 PPIase domain. The phosphate moiety is bound in the basic cavity formed by the residues of Lys63, Arg68 and Arg69 of Pin1. The prolyl ring is cradled in a hydrophobic pocket formed by the side chains of Leu122, Met130, and Phe134. No hydrogen bonding was detected between the prolyl nitrogen and any active site residude, such as Cys113, His59 or His157.



Conclusions

The reduced amides **1** and **2** represent a new class of PPIase inhibitors with improved potency over our similar alkene isostere inhibitor of Pin1.[44] The X-ray crystal structure of **2** bound in the active site of Pin1 shows no hydrogen bond between any

active site residue and the tertiary amine nitrogen. This explains the lack of potency, which is lower than expected for a transition-state analogue inhibitor. These isosteres are much easier to synthesize, which may allow the reduced amide motif to be exploited as a core design element for optimized Pin1 inhibition.

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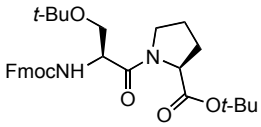
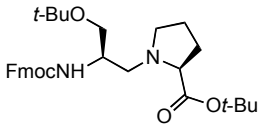
Experimental Section

General Procedures Unless otherwise indicated, all reactions were carried out under N_2 in flame-dried glassware. THF was distilled from sodium-benzophenone, and CH_2Cl_2 was dried by passage through dry alumina. Anhydrous DMF (99.8%), MeOH, and DIEA were used directly from sealed bottles. TFE (99+%) was distilled from sodium before use. LiCl (99+%) was dried under vacuum at 150 °C for 24 h. Brine (NaCl), $NaHCO_3$ and NH_4Cl refer to saturated aqueous solutions unless otherwise noted. Flash chromatography was performed on 230–400 mesh silica gel with reagent grade solvents. NMR spectra were obtained at ambient temperature in $CDCl_3$ unless otherwise noted. ^{1}H-, ^{13}C-, and ^{31}P-NMR spectra were obtained at 500, 125, and 162 MHz, respectively, unless otherwise noted. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane (TMS). Data are reported as follows: chemical shift, multiplicity: singlet (s), doublet (d), triplet (t), quadruplet (q), quintuplet (qt), multiplet (m), coupling constants *J* in Hz, and integration. In ^{13}C-NMR spectral listings, minor rotamers are labeled (m). Analytical HPLC were obtained on a C18 4.6 × 50 mm column, with 10% to 90% CH_3CN/H_2O gradient over 7 min at 254 nM, and the retention time and purity are listed, unless otherwise noted.



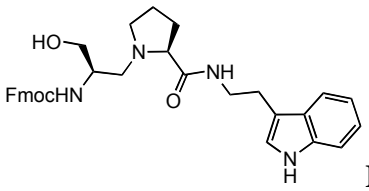
Fmoc–Ser(O*t*Bu)–Pro–O*t*Bu, 3. A solution of DIEA (1.0 g, 7.8 mmol) and HCl·H–Pro–O*t*Bu (1.3 g, 5.3 mmol) in DMF (5 mL) was added to a solution of Fmoc–Ser(O*t*Bu)–OH (2.0 g, 5.2 mmol), EDC (1.2 g, 6.3 mmol), and HOBt (1.0 g, 6.3 mmol) in DMF (15 mL). The mixture was stirred at rt for 14 h, then diluted

with EtOAc (80 mL), and washed with 1 M HCl (30 mL), and 5% aq $NaHCO_3$ (20 mL). The organic layer was then dried over Na_2SO_4, filtered, and evaporated. The crude product was purified by chromatography (30 to 50% of EtOAc in hexanes) to yield **3** (2.0 g, 80%) as a colorless oil. Anal. HPLC (on a normal phase MetaSil AQ 5µ 4.6 × 50 mm Si column, using 5% *i*-PrOH in Hexanes isocratic, with a flow rate 1.0 mL/min), 2.0 min, 99%. $[\alpha]^{25}_{D} = -48.6$ (*c* 1, MeOH). ^{1}H NMR: δ 7.74 (d, *J* = 7.2, 2H), 7.59 (d, *J* = 6.9, 2H), 7.38 (t, *J* = 7.6, 2H), 7.27 (m, 2H), 5.74 (d, *J* = 7.7, 0.4H), 5.71 (d, *J* = 8.0, 0.6H), 4.92 (dd, *J* = 1.6, 8.3, 0.4H), 4.65 (dt, *J* = 6.4, 8.2, 0.6H), 4.51 (ddd, *J* = 4.9, 7.8, 10.1, 0.4H), 4.35 (m, 2.4H), 4.19 (dd, *J* = 7.7, 15.9, 1H), 3.85 (ddd, *J* = 5.2, 7.5, 9.7, 0.6H), 3.65 (m, 1.4H), 3.58 (dd, *J* = 4.7, 6.4, 1H), 3.52 (m, 0.6H), 3.36 (dd, *J* = 8.4, 10.0, 0.6H), 2.23 (m, 1H), 2.12 (m, 2H), 1.90 (m, 2H), 1.46 (s, 5H), 1.42 (s, 4H), 1.20 (s, 5H), 1.13 (s, 4H). ^{13}C NMR: δ 171.1 (m), 170.9, 170.2 (m), 169.3, 156.0, 155.3 (m), 144.01 (m), 143.99, 143.90, 143.87 (m), 141.31 (m), 141.30, 127.7, 127.1 (m), 125.24, 125.21 (m), 119.99 (m), 119.98, 82.5 (m), 81.1, 73.7 (m), 73.6, 67.1, 67.0 (m), 64.0 (m), 62.8, 59.93 (m), 59.89, 53.1, 52.7 (m), 47.3, 47.17 (m), 47.16, 46.3 (m), 30.7 (m), 29.1, 28.0, 27.9 (m), 27.4 (m), 27.3, 24.8 (m), 22.5. HRMS (FAB$^+$): calcd for $C_{31}H_{41}N_2O_6$ $[M + H]^+$ *m/z* =537.2965, found *m/z* = 537.2979.



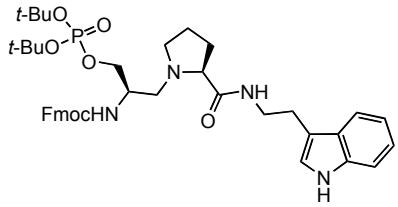
Fmoc–Ser(O*t*Bu)–Ψ[CH_2N]–Pro–O*t*Bu, 4. Fmoc–Ser(O*t*Bu)–Pro–O*t*Bu **3** (1.27 g, 2.37 mmol) in dry THF (10 mL) was added to a solution of borane (1.00 M in THF, 3.94 mL, 3.94 mmol) at 0 °C over a period of 15 min. After the addition was complete, the resulting mixture was stirred at rt for 24 h. Then the reaction mixture was cooled to 0 °C, and MeOH (5 mL) was added slowly. The solvents

were evaporated, and the residue was purified by flash chromatography (step gradient: 0 to 25% of EtOAc in hexanes) to yield **4** (1.26 g, 65%) as a colorless oil. Anal. HPLC (on a normal phase MetaSil AQ 5μ 4.6 × 50 mm Si column, using 5% *i*-PrOH in Hexanes isocratic, with a flow rate 1.0 mL/min), 2.0 min, 93%. $[\alpha]^{25}_{D} = -27.0$ (*c* 1, MeOH). ^{1}H NMR: δ 7.75 (d, *J* = 7.5, 2H), 7.66 (t, *J* = 6.6, 2H), 7.39 (t, *J* = 7.4, 2H), 7.29 (tt, *J* = 1.4, 7.4, 2H), 5.96 (d, *J* = 6.0, 1H), 4.34 (m, 2H), 4.24 (t, *J* = 7.1, 1H), 3.74 (m, 1H), 3.59 (m, 1H), 3.41 (m, 1H), 3.21 (m, 2H), 2.80 (m, 2H), 2.59 (d, *J* = 6.5, 1H), 2.06 (m, 1H), 1.85 (m, 3H), 1.47 (s, 9H), 1.19 (s, 9H). ^{13}C NMR: δ 174.2, 156.3, 144.2, 141.3, 127.6, 127.0, 125.3, 120.0, 80.7, 73.0, 67.5, 66.6, 61.2, 55.5, 54.7, 51.0, 47.4, 29.6, 28.1, 27.6, 23.9. HRMS (FAB$^+$): calcd for $C_{31}H_{43}N_2O_5$ $[M + H]^+$ *m/z* = 523.3172, found *m/z* = 523.3169.



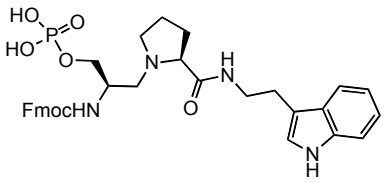
Fmoc–Ser–Ψ[CH$_2$N]–Pro–(2)-*N*-(3)-ethylaminoindole, 6. Fmoc–Ser(O*t*Bu)–Ψ[CH$_2$N]–Pro–O*t*Bu **4** (250 mg, 4.78 mmol) was dissolved in CH_2Cl_2 (8 mL), and then $BF_3{\cdot}Et_2O$ (0.8 mL) was added. The reaction mixture was stirred at rt for 20 min. After evaporation, the residue was purified by chromatography (gradient: 10 to 25% of MeOH in CH_2Cl_2) to yield **5** (108 mg, 55%) as a colorless oil. HRMS: calcd for $C_{23}H_{27}N_2O_5$ $[M + H]^+$ m/z = 411.1920, found m/z = 411.1932. Fmoc–Ser–Ψ[CH$_2$N]–Pro–OH **5** (55 mg, 0.13 mmol), tryptamine (26 mg, 0.16 mmol), HOAt (25 mg, 0.16 mmol), and DMAP (5.0 mg, 0.04 mmol) were dissolved in DMF (3 mL), and EDC (31 mg, 0.16 mmol) was added. The reaction mixture was stirred at rt for 14 h, then diluted with EtOAc (50 mL) and washed with water (25 mL × 3) followed by brine (15 mL). The organic layer was dried over Na_2SO_4. After filtration and

evaporation, the residue was purified by flash chromatography using 9% MeOH/EtOAc) to yield **6** as a colorless oil (35 mg, 47%). $[\alpha]^{25}_{D} = -28.7$ (*c* 1, MeOH). ^{1}H NMR: δ 8.10 (br s, 1H), 7.78 (d, *J* = 7.6, 2H), 7.58 (m, 3H), 7.41 (t, *J* = 7.4, 2H), 7.33 (t, *J* = 7.6, 2H), 7.15 (d, *J* = 8.1, 1H), 7.08 (qt, *J* = 7.0, 2H), 6.94 (d, *J* = 2.3, 1H), 4.67 (d, *J* = 8.8, 1H), 4.46 (t, *J* = 5.7, 2H), 4.19 (t, *J* = 6.1, 1H), 3.57 (m, 2H), 3.47 (m, 1H), 3.18 (m, 2H), 3.05 (m, 1H), 2.90 (m, 2H), 2.86 (m, 1H), 2.25 (m, 1H), 2.17, (m, 2H), 2.10 (m, 1H), 1.71 (m, 4H), 1.63 (m, 1H). ^{13}C NMR: δ 174.5, 156.3, 144.0, 141.5, 136.3, 127.9, 127.7, 127.3, 125.0, 122.4, 122.3, 120.2, 119.6, 119.0, 113.4, 111.5, 68.4, 66.3, 62.2, 55.3, 53.7, 51.2, 47.4, 39.4, 30.2, 24.9, 24.1. MS (ESI^{+}): calcd for $C_{33}H_{37}N_4O_4$ $[M + H]^{+}$ *m*/*z* = 553.28, found *m*/*z* = 553.32.



Fmoc–Ser(PO(O*t*Bu)$_2$)–Ψ[CH$_2$N]–Pro–(2)-*N*-(3)-ethylaminoindole, 7. To a solution of **6** (85 mg, 0.15 mmol) in THF (4 mL) was added 5-ethylthio–H–tetrazole (60 mg, 0.46 mmol) and di-*tert*-butyl diisopropylphosphoramidite (85 mg, 0.31 mmol) at rt, and the mixture was stirred at rt for 17 h. The mixture was cooled to –40 °C and a solution of 5–6 M *tert*-butyl hydroperoxide in decane (0.11 mL, 0.61 mmol) was added slowly and the mixture was stirred at –40 °C for 1 h. then at rt for 30 min. The reaction was cooled to –40 °C and quenched by addition of saturated aq $Na_2S_2O_3$. The mixture was extracted with EtOAc (20 mL × 2), and the combined organic extracts were washed with water (20 mL), brine (20 mL), and dried over Na_2SO_4. Concentration under vacuum provided a residue, which was purified by flash chromatography to afford **7** as a colorless oil (51 mg, 45%). Anal.

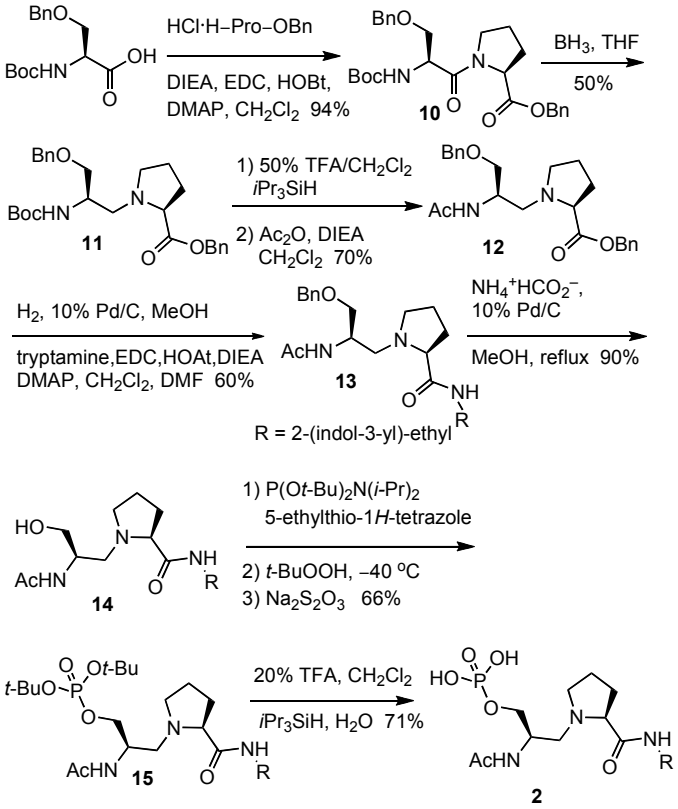
HPLC (on a normal phase MetaSil AQ 5μ 4.6 × 50 mm Si column, using 5% *i*-PrOH in Hexanes isocratic, with a flow rate 1.0 mL/min), 4.0 min, 91%. ^{1}H NMR (400 MHz): δ 9.03 (br, 1H), 7.77 (d, *J* = 7.6, 2H), 7.59 (m, 3H), 7.41 (t, *J* = 7.4, 2H), 7.31 (m, 3H), 7.12 (t, *J* = 7.1, 1H), 7.06 (t, *J* = 7.2, 1H & s, 1H), 5.07 (d, *J* = 8.3, 1H), 4.38 (ddd, *J* = 6,6, 10.8, 15.0, 2H), 4.19 (t, *J* = 6.7, 1H), 3.81 (tt, *J* = 3.6, 7.2, 1H), 3.67 (dt, 2H), 3.56 (m, 1H), 3.38 (ddd, *J* = 5.2, 8.5, 10.6, 1H), 3.02 (m, 2H), 2.95 (m, 2H), 2.43 (dd, *J* = 5.6, 12.4, 1H), 2.33 (m, 2H), 2.09 (m, 1H), 1.80 (m, 1H), 1.71 (m, 1H), 1.56 (m, 1H), 1.48 (d, *J* = 2.0, 18H). ^{13}C NMR (100 MHz): δ 174.3, 156.1, 143.9, 141.4, 136.5, 127.8, 127.2, 125.15, 125.11, 122.3, 122.0, 120.1, 119.3, 119.0, 113.0, 111.6, 83.4, 69.2, 66.7, 66.2, 56.8, 54.2, 50.7, 47.3, 39.2, 30.3, 29.9, 24.8, 24.3. ^{31}P NMR: δ -8.43. HRMS (FAB^{+}): calcd for $C_{41}H_{54}N_4O_7P$ $[M + H]^+$ *m/z* = 745.3730, found *m/z* = 745.3751.

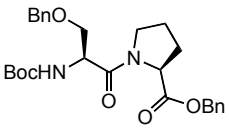


Fmoc–Ser(PO(OH)$_2$)–Ψ[CH$_2$N]–Pro–(2)-*N*-(3)-ethylaminoindole, 1. To a solution of **7** (43 mg, 0.058 mmol) in anhydrous CH_2Cl_2 (2 mL) was added a mixture of TFA (0.5 mL), H_2O (0.1 mL) and *i*Pr$_3$SiH (0.02 mL), and the reaction was stirred at rt for 1 h. The solvent was removed under vacuum, and the residue was purified by semi-preparative HPLC to provide **1** as a white solid after lyophlization (35 mg, 95%). Anal. HPLC (using solvent A, 0.1% TFA in H_2O, and solvent B, 0.1% TFA in CH_3CN, 254 nm), 8.7 min, 97%. $[\alpha]^{25}_{D} = -4.4$ (*c* 1, MeOH). ^{1}H NMR (CD_3OD): δ 7.74 (dd, *J* = 2.8, 7.3, 2H), 7.62 (d, *J* = 7.3, 1H), 7.52 (dd, *J* = 7.7, 13.5, 2H), 7.34 (m, 3H), 7.26 (t, *J* = 7.5, 1H), 7.22(t, *J* = 7.6, 1H), 7.05 (m, 2H), 6.94 (t, *J* = 7.4, 1H), 4.44 (dd, *J* = 6.4, 10.6, 1H), 4.31 (dd, *J* = 7.1, 10.5, 1H), 4.15 (m, 2H), 4.08 (m, 1H), 3.94 (m,

1H), 3.89 (m, 1H), 3.68 (m, 1H), 3.52 (m, 2H), 3.39 (d, J = 11.5, 1H), 3.28 (d, J = 13.0, 1H), 3.16 (m, 1H), 2.95 (m, 2H), 2.36 (m, 1H), 2.04 (m, 1H), 1.86 (m, 2H). ^{13}C NMR (CD_3OD): δ 168.6, 158.0, 145.3, 145.0, 142.6, 138.1, 128.8, 128.2, 126.2, 126.1, 123.7, 122.4, 120.9, 119.7, 119.3, 112.8, 112.4, 68.7, 68.0, 66.6, 57.6, 56.7, 41.8, 30.8, 26.0, 24.2. ^{31}P NMR (CD_3OD): δ 0.29. MS (ESI^+) calcd for $C_{33}H_{37}N_4O_7P$ $[M]^+$ m/z =632.24 found m/z = 632.27.

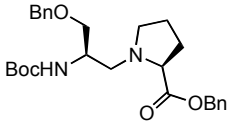
Scheme 4.5. Synthesis of Ac-pSer-Ψ[CH_2N]-Pro-tryptamine inhibitor **2**.





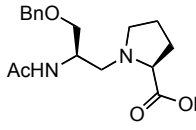
Boc–Ser(OBn)–Pro–OBn, 10. Boc–L–Ser(OBn)–OH (2.00 g, 6.77 mmol), HCl·H–Pro–OBn (1.80 g, 7.45 mmol), and DIEA (2.63 g, 20.3 mmol) were dissolved in CH_2Cl_2 (100 mL) and cooled to 0 °C, and EDC (1.55 g, 8.12 mmol), HOBt

(1.24 g, 8.12 mmol) and DMAP (227 mg, 2.03 mmol) were added. The mixture was stirred at rt for 48 h, then diluted with 20 mL CH_2Cl_2. The mixture was washed with 1M HCl (50 mL × 2), $NaHCO_3$ (50 mL × 2), and Brine (50 mL). The organic layer was then dried over Na_2SO_4, filtered and evaporated. The crude product was purified by flash chromatography (step gradient: 25 to 50% of EtOAc in hexanes) to yield **10** (3.0 g, 94%) as a colorless oil. Anal. HPLC (210 nM), 10.4 min, 99%. $[\alpha]^{25}_D = -56.9$ (*c* 1, MeOH). ^{1}H NMR: δ 7.32 (m, 10H), 5.41 (d, *J* = 8.6, 0.82H), 5.37 (d, *J* = 8.5, 0.18H), 5.21 (d, *J* = 12.1, 1H), 5.10 (d, *J* = 12.5, 1H), 4.84 (dd, *J* = 2.1, 8.2, 0.18H), 4.70 (m, 0.82H), 4.58 (dd, *J* = 3.8, 8.4, 1H), 4.51 (d, *J* = 11.7, 1H), 4.44 (d, *J* = 11.9, 1H), 3.69 (m, 2H), 3.61 (m, 2H), 2.19 (m, 1H), 1.97 (m, 3H), 1.42 (s, 7.4H), 1.40 (s, 1.6H). ^{13}C NMR: δ 171.8 (m), 171.7, 170.2 (m), 169.5, 155.4, 154.8 (m), 138.0, 137.9 (m), 135.8, 135.5(m), 128.7 (m), 128.6, 128.44(m), 128.40, 128.33, 128.27 (m), 128.2, 127.8(m), 127.72, 127.67, 127.6(m), 79.8, 79.7 (m), 73.4 (m), 73.2, 71.7 (m), 70.6, 67.5 (m), 66.9, 59.3 (m), 59.2, 52.1, 51.5 (m), 47.1, 46.5 (m), 30.8 (m), 29.1, 28.41, 28.38 (m), 24.9, 22.4 (m). HRMS (FAB$^+$): calcd for $C_{27}H_{35}N_2O_6$ $[M + H]^+$ *m/z* = 483.24951, found *m/z* = 483.24969.

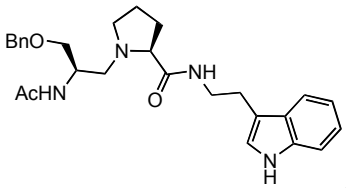


Boc–Ser(OBn)–Ψ[CH$_2$N]–Pro–OBn, 11. Boc–Ser(OBn)–Pro–OBn **10** (3.00 g, 6.22 mmol) was dissolved in dry THF (14 mL), a solution of borane (1 M in THF, 12.4 mL, 12.4 mmol) was added dropwise at 0 °C. After the addition was complete, the resulting mixture was stirred at 0 °C for 2 h and at rt for 24 h. Then the reaction was cooled to 0 °C, and methanol (10 mL) was added slowly. The resulting mixture was evaporated and purified by flash chromatography (eluent: 25% EtOAc in hexane) to yield **11** (1.4 g, 50%) as a colorless oil. Anal. HPLC, 11.2 min, 97%. $[\alpha]^{25}_D = -$

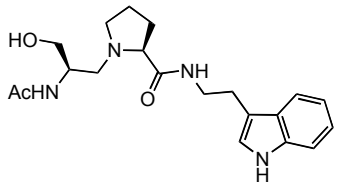
34.4 (*c* 1, MeOH). ^{1}H NMR: δ 7.31 (m, 10H), 5.15 (m, 1H), 5.13 (d, *J* = 12.3, 1H), 5.09 (d, *J* = 12.3, 1H), 4.51 (d, *J* = 11.8, 1H), 4.46 (d, *J* = 11.7, 1H), 3.79 (m, 1H), 3.67 (m, 1H), 3.55 (dd, *J* = 5.0, 9.2, 1H), 3.32 (dd, *J* = 5.6, 8.6, 1H), 3.17 (dt, *J* = 4.1, 8.0, 1H), 2.86 (dd, *J* = 8.0, 12.4, 1H), 2.65 (m, 1H), 2.52 (m, 1H), 2.08 (m, 1H), 1.90 (m, 2H), 1.80 (m, 1H), 1.43 (s, 9H). ^{13}C NMR: δ 174.3, 155.7, 138.5, 136.1, 128.6, 128.4, 128.2, 127.7, 127.6, 79.1, 73.2, 70.0, 66.6, 66.3, 55.7, 54.1, 49.7, 29.5, 28.5, 23.9. HRMS (FAB^{+}): calcd for $C_{27}H_{37}N_2O_5$ [M + H]$^{+}$ *m/z* = 469.2702, found *m/z* = 469.2709.



Ac–Ser(OBn)–Ψ[CH$_2$N]–Pro–OBn, 12. Boc–Ser(OBn)–Ψ[CH$_2$N]–Pro–OBn **11** (500 mg, 1.07 mmol) was dissolved in CH_2Cl_2 (10 mL) and a solution of TFA (10 mL) and *i*Pr$_3$SiH (0.1 mL) was added. The mixture was stir at rt 2 h. The reaction mixture was then concentrated under reduced pressure. The ammonium salt obtained was then dissolved in CH_2Cl_2 (5 mL) with DIEA (0.6 mL), and acetic anhydride (0.6 mL) was added. The reaction mixture was stir at rt for 14 h. After concentration, the residue was purified by by flash chromatography (eluent: EtOAc) to yield **12** (0.30 g, 70%). Anal. HPLC (210 nM), 9.4 min, 99%. $[\alpha]^{25}_{D} = -27.4$ (*c* 1, MeOH). ^{1}H NMR: δ 7.34 (m, 10H), 6.67 (d, *J* = 7.3, 1H), 5.15 (d, *J* = 12.1, 1H), 5.10 (d, *J* = 12.2, 1H), 4.54 (d, *J* = 11.9, 1H), 4.46 (d, *J* = 11.9, 1H), 4.07 (m, 1H), 3.68 (dd, *J* = 4.4, 9.2, 1H), 3.48 (dd, *J* = 7.0, 9.2, 1H), 3.37 (m, 1H), 3.17 (m, 1H), 2.86 (dd, *J* = 4.0, 13.0, 1H), 2.74 (dd, *J* = 6.0, 12.8, 1H), 2.54 (m, 1H), 2.17 (m, 1H), 1.94 (s, 3H), 1.86 (m, 3H). ^{13}C NMR: δ 175.1, 170.1, 138.4, 135.9, 128.7, 128.5, 128.4, 128.3, 127.9, 127.8, 73.3, 69.4, 67.2, 66.6, 55.6, 55.5, 48.7, 30.0, 24.3, 23.4. HRMS (FAB^{+}): calcd for $C_{24}H_{31}N_2O_4$ [M + H]$^{+}$ *m/z* = 411.2284, found *m/z* = 411.2273.

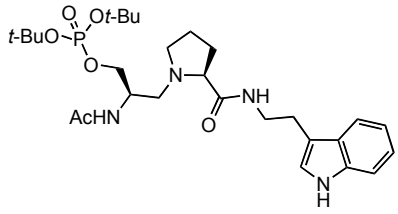


Ac–Ser(OBn)–Ψ[CH_2N]–Pro–(2)-*N*-(3)-ethylaminoindole, 13. Ac–Ser(OBn)–Ψ[CH_2N]–Pro–OBn **12** (0.15 g, 0.36 mmol) was dissolved in MeOH (5 mL), and 10% Pd/C (30 mg) was added. Then the reaction was stirred at rt under H_2 from a balloon for 2 h. After filtration through Celite, washing with MeOH, and evaporation, the crude oil and tryptamine (60 mg, 0.38 mmol) were dissolved in a mixture of CH_2Cl_2 (10 mL) and DMF (5 mL), and then HOAt (58 mg, 0.38 mmol), DMAP (10 mg, 0.09 mmol), and EDC (72 mg, 0.38 mmol) were added. The reaction mixture was stirred at rt for 24 h, then diluted with CH_2Cl_2 (30 mL). The mixture was washed with water (20 mL) and Brine (25 mL). The organic layer was dried over Na_2SO_4. After filtration and evaporation, the residue was purified by flash chromatography (step gradient: 6% to 17% MeOH in EtOAc) to yield **13** as a light yellow oil (85 mg; 60%). Anal. HPLC (210 nM), 8.5 min, 96%. ^{1}H NMR: δ 8.43 (s br, 1H), 7.56 (m, 2H), 7.32 (m, 4H), 7.16 (m, 3H), 7.08 (dt, *J* = 0.9, 7.4, 1H), 6.97 (d, *J* = 2.4, 1H), 5.60 (d, *J* = 8.1, 1H), 4.18 (d, *J* = 11.8, 1H), 4.13 (d, *J* = 11.9, 1H), 3.96 (dtt, *J* = 3.0, 6.0, 9.0, 1H), 3.65 (dq, *J* = 6.6, 13.2, 1H), 3.40 (m, 1H), 3.15 (dd, *J* = 3.0, 9.6, 1H), 3.13 (dd, *J* = 3.4, 9.6, 1H), 3.05 (m, 2H), 3.01 (dt, *J* = 6.2, 14.4, 1H), 2.88 (dt, *J* = 7.2, 14.4, 1H), 2.56 (dd, *J* = 8.7, 12.1, 1H), 2.37 (dd, *J* = 6.2, 12.1, 1H), 2.34 (dt, *J* = 6.8, 9.5, 1H), 2.12 (m, 1H), 1.87 (s, 3H), 1.82 (m, 1H), 1.75 (m, 1H), 1.64 (m, 1H). ^{13}C NMR: δ 174.6, 169.8, 137.9, 136.5, 128.6, 128.0, 127.6, 122.2, 122.1, 119.4, 113.3, 111.4, 73.1, 69.2, 68.9, 56.7, 54.4, 48.5, 39.4, 30.4, 25.4, 24.5, 23.5. HRMS (FAB$^+$): calcd for $C_{27}H_{35}N_4O_3$ [M + H]$^+$ *m/z* = 463.27092, found *m/z* = 463.27051.



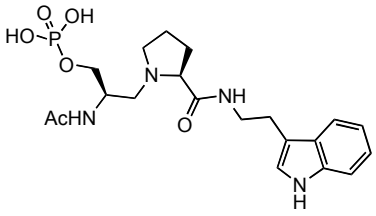
Ac–Ser–Ψ[CH_2N]–Pro–(2)-*N*-(3)-ethylaminoindole,

14. Ac–Ser(OBn)–Ψ[CH_2N]–Pro–(2)-*N*-(3)-ethylaminoindole **13** (125 mg, 0.270 mmol) was dissolved in MeOH (8 mL), and 10% Pd/C (50 mg) and ammonium formate (120 mg) were added. The reaction was refluxed for 8 h. Filtrated through Celite, washing with MeOH, and flash chromatography purification (17% MeOH in EtOAc) gave **14** as a lightly yellow oil (100 mg, 100%). Anal. HPLC (210 nM), 7.0 min, 94%. ^{1}H NMR: δ 8.58 (br, 1H), 7.60 (d, *J* = 8.1, 1H), 7.37 (d, *J* = 8.1, 1H), 7.35 (m, 1H), 7.20 (t, *J* = 7.6, 1H), 7.10 (t, *J* = 7.4, 1H), 7.00 (s, 1H), 5.97 (br, 1H), 3.84 (m, 1H), 3.65 (m, 1H), 3.53 (m, 1H), 3.28 (m, 2H), 3.03 (m, 2H), 2.93 (m, 2H), 2.45 (m, 1H), 2.24 (m, 2H), 2.09 (m, 1H), 1.91 (s, 3H), 1.74 (m, 3H). ^{13}C NMR (CD_3OD, 100MHz): δ 177.2, 173.1, 138.1, 128.8, 123.5, 122.4, 119.6, 119.4, 113.1, 112.2, 69.6, 62.9, 57.1, 55.4, 51.9, 41.0, 31.3, 26.1, 25.0, 22.7. HRMS (FAB$^+$): calcd for $C_{20}H_{29}N_4O_3$ [M + H]$^+$ *m*/*z* = 373.22397, found *m*/*z* = 373.22684.



Ac–Ser(PO(O*t*Bu)$_2$)–Ψ[CH_2N]–Pro–(2)-*N*-(3)-ethylaminoindole, 15. To a solution of **14** (70 mg, 0.19 mmol) in THF (8 mL) was added 5-ethylthio–H–tetrazole (98 mg, 0.75 mmol) and di-*tert*-butyl diisopropylphosphoramidite (0.12 mL, 0.38 mmol) at rt, and the mixture was stirred at rt for 15 h. The mixture was cooled to –40 °C and a solution of 5–6 M *tert*-butyl hydroperoxide in decane (0.15 mL, 0.75 mmol) was added slowly and the mixture was

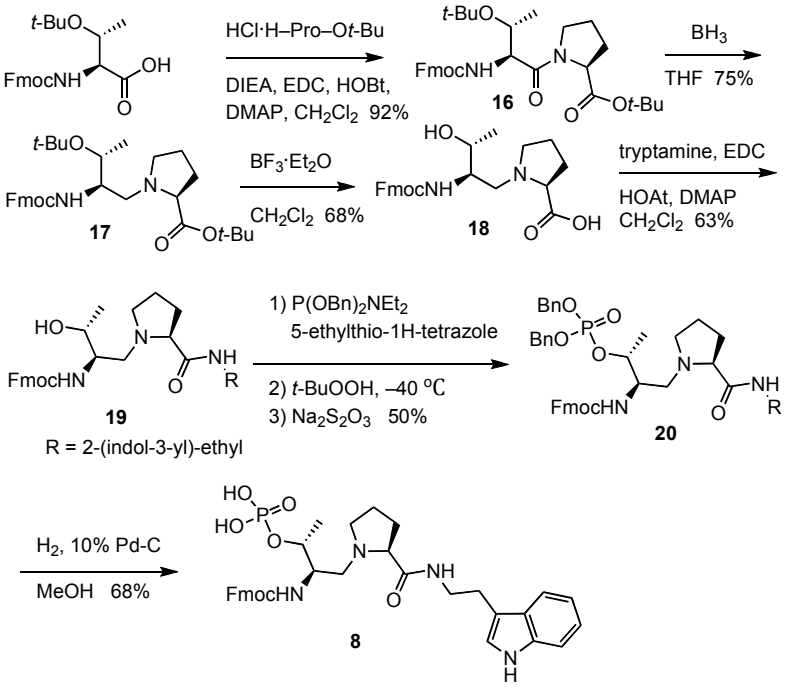
stirred at rt for 30 min. The reaction was cooled to –40 °C and quenched by addition of saturated aq $Na_2S_2O_3$. The mixture was extracted with CH_2Cl_2 (20 mL × 2), and the combined organic extracts were dried over Na_2SO_4. Concentration under vacuum provided a residue, which was purified by flash chromatography (step gradient: 9 to 17% of EtOAc in MeOH) to afford **15** as a colorless oil (70 mg, 66%). Anal. HPLC, 254 nm, 8.5 min, 98%. $[\alpha]^{25}_D$ = +21.6 (*c* 1, MeOH). ^{1}H NMR: δ 9.01 (s, 1H), 7.64 (d, *J* = 8.0, 1H), 7.38 (m, 2H), 7.19 (m, 1H), 7.10 (m, 2H), 6.07 (d, *J* = 7.9, 1H), 3.90 (m, 1H), 3.79 (m, 1H), 3.63 (m, 2H), 3.47 (dt, *J* = 5.2, 10.4, 1H), 3.10 (m, 1H), 3.01 (m, 3H), 2.47 (dd, *J* = 5.8, 12.2, 1H), 2.37 (m, 2H), 2.09 (m, 1H), 1.84 (s, 3H), 1.80 (m, 1H), 1.73 (m, 1H), 1.57 (m, 1H), 1.50 (d, *J* = 6.4, 18H). ^{13}C NMR: δ 174.5, 170.1, 136.6, 127.8, 122.4, 122.1, 119.4, 119.0, 113.1, 111.5, 83.53 (d, *J* = 4.2), 83.47 (d, *J* = 4.2), 69.2, 66.4 (d, *J* = 5.7), 56.4, 54.4, 49.1 (d, *J* = 4.8), 39.2, 30.4, 30.03 (d, *J* = 4.3), 29.99 (d, *J* = 4.3), 25.0, 24.5, 23.4. ^{31}P NMR: δ –7.86. MS (ESI$^+$): calcd for $C_{28}H_{45}N_4O_6P$ $[M + H]^+$ *m*/*z* = 565.3, found *m*/*z* = 565.3.

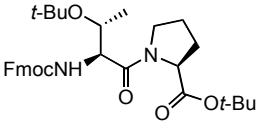


Ac–Ser(PO(OH)$_2$)–Ψ[CH$_2$N]–Pro–(2)-*N*-(3)-ethylaminoindole, 2. To a solution of **13** (30 mg, 0.053 mmol) in CH_2Cl_2 (2 mL) was added a mixture of TFA (0.5 mL), H_2O (0.01 mL) and *i*Pr_3SiH (0.01 mL), and the reaction was stirred at rt for 1 h. The solvent was removed under vacuum, and the residue was purified by semi-preparative HPLC to provide **2** as a white solid after lyophilization (17 mg, 71%). Anal. HPLC with gradient 10% to 10% B over 7 min (A, 0.1% TFA in H_2O, B, 0.1% TFA in CH_3CN), 6.4 min, 97%. $[\alpha]^{25}_D$ = –12.3 (*c* 1, MeOH). ^{1}H NMR

(DMSO-d_6, 400 MHz): δ 10.9 (s, 1H), 8.38 (br, 1H), 8.04 (d, *J* = 6.0, 1H), 7.56 (d, *J* = 7.6, 1H), 7.33 (d, *J* = 8.0, 1H), 7.15 (s, 1H), 7.05 (t, *J* = 7.4, 1H), 6.97 (t, *J* = 7.4, 1H), 4.11 (m, 1H), 3.80 (m, 2H), 3.62 (m, 1H), 3.39 (m, 3H), 3.07 (m, 1H), 2.85 (m, 4H), 2.18 (m, 1H), 1.83 (s, 3H), 1.71 (m, 3H). ^{13}C NMR (CD_3OD): δ 173.8, 168.7, 138.2, 128.8, 123.8, 122.4, 119.6, 119.3, 112.8, 112.4, 68.8, 66.2, 57.4, 56.8, 48.2, 41.7, 30.8, 26.0, 24.2, 22.8. ^{31}P NMR (DMSO-d_6): δ 0.62. HRMS (FAB^+): calcd for $C_{20}H_{30}N_4O_6P$ $[M + H]^+$ *m/z* = 453.1903, found *m/z* = 453.1912.

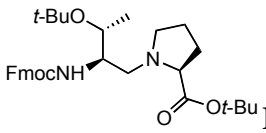
Scheme 4.6. Synthesis of Fmoc-pThr-Ψ[CH_2N]-Pro-tryptamine inhibitor **8**.





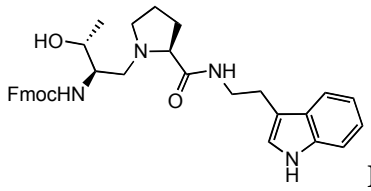
Fmoc–Thr(O*t*Bu)–Pro–O*t*Bu, 16. A solution of DIEA (0.49 g, 3.8 mmol) and HCl·H–Pro–O*t*Bu (0.52 g, 2.5 mmol) in CH_2Cl_2 (25 mL) was added to a solution of Fmoc–Thr(O*t*Bu)–OH (1.0 g, 2.5 mmol), EDC (0.58g, 3.0 mmol), and HOBt (0.46 g, 3.0 mmol) in CH_2Cl_2 (25 mL). The mixture was stirred at rt for 13 h,

and then washed with 1 M HCl (30 mL), 5% aq $NaHCO_3$ (30 mL) and brine (30 mL). The organic layer was then dried over Na_2SO_4, filtered, and evaporated. The crude product was purified by chromatography (20% of EtOAc in hexanes) to yield **16** (1.3 g, 92%) as a colorless oil. ^{1}H NMR: δ 7.75 (d, *J* = 7.7, 2H), 7.59 (m, 2H), 7.38 (m, 2H), 7.29 (m, 2H), 5.95 (d, *J* = 6.9, 0.4H), 5.75 (d, *J* = 7.8, 0.6H), 5.21 (dd, *J* = 3.4, 7.0, 0.4H), 4.45 (dd, *J* = 5.2, 7.8, 0.6H), 4.35 (m, 3H), 4.20 (t, *J* = 7.2, 1H), 3.92 (m, 1.6H), 3.72 (m, 1H), 3.54 (dt, *J* = 8.2, 16.6, 0.4H), 2.19 (m, 1H), 2.05-1.84 (m, 3H), 1.47 (s, 5.4H), 1.42 (s, 3.6H), 1.27 (s, 3.6H), 1.23 (s, 5.4H), 1.21 (d, *J* = 6.2, 1.8H), 1.07 (d, *J* = 6.6, 1.2H). ^{13}C NMR: δ 171.5 (m), 171.2, 169.0, 168.3 (m), 155.9, 155.3 (m), 144.1, 143.9 (m), 127.7, 127.1, 125.31, 125.26 (m), 120.0, 82.5 (m), 81.3, 75.1 (m), 74.6, 70.9 (m), 69.2, 67.0, 66.9 (m), 60.14 (m), 60.06, 57.6, 57.3 (m), 48.0, 47.2, 46.6 (m), 30.7, 30.5 (m), 29.2 (m), 28.4, 28.0, 27.9 (m), 25.0, 22.3(m), 19.2, 17.6 (m). HRMS (ESI$^+$): calcd for $C_{32}H_{43}N_2O_6$ $[M + H]^+$ *m/z* = 551.3121, found *m/z* = 551.3134.



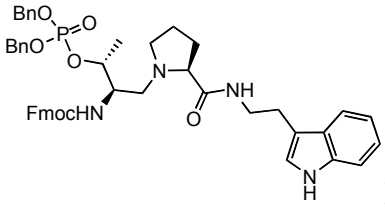
Fmoc–Thr(O*t*Bu)–Ψ[CH_2N]–Pro–O*t*Bu, 17. Fmoc–Thr(O*t*Bu)–Pro–O*t*Bu **16** (1.14 g, 2.08 mmol) in dry THF (15 mL) was added to a solution of borane (1.00 M in THF, 4.15 mL, 4.15 mmol) at 0 °C over a period of 15 min. After the addition was complete, the resulting mixture was stirred at rt for 13 h. Then the reaction mixture was cooled to 0 °C, and MeOH (5 mL) was added slowly. After evaporation, the residue was purified by flash chromatography (12% EtOAc/hexanes) to yield **17** (0.44 g, 40%) as a colorless oil, and 0.54 g (47%) of the starting material was recovered. ^{1}H NMR: δ 7.76 (m, 2H), 7.61 (m, 2H), 7.39 (m, 2H), 7.31 (m, 2H), 5.28 (d, *J* = 8.3, 1H), 4.36 (d, *J* = 7.3, 2H), 4.25 (t, *J* = 7.2, 1H), 3.99 (m, 1H), 3.62 (m, 1H), 3.25

(dd, J = 4.1, 8.7, 1H), 3.06 (m, 1H), 2.85 (dd, J = 6.0, 12.4, 1H), 2.67 (q, J = 7.5, 1H), 2.55 (dd, J = 7.3, 12.3, 1H), 2.04 (m, 1H), 1.83 (m, 3H), 1.44 (s, 9H), 1.20 (s, 9H), 1.13 (d, J = 6.4, 3H). ^{13}C NMR: δ 156.9, 144.5, 141.5, 127.8, 127.2, 125.4, 124.8, 120.2, 66.7, 66.6, 66.3, 65.3, 55.5, 55.4, 53.2, 50.5, 47.5, 29.7, 29.0, 28.3, 23.5, 20.1. HRMS (ESI$^+$): calcd for $C_{32}H_{45}N_2O_5$ $[M + H]^+$ m/z = 537.3328, found m/z = 537.3325.



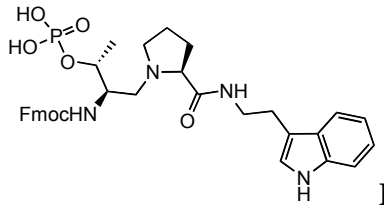
Fmoc–Thr–Ψ[CH_2N]–Pro–(2)-*N*-(3)-ethylaminoindole, 19. Fmoc–Thr(O*t*Bu)–Ψ[CH_2N]–Pro–O*t*Bu **17** (240 mg, 4.47 mmol) was dissolved in CH_2Cl_2 (8 mL), and then $BF_3{\cdot}Et_2O$ (0.8 mL), iPr$_3$SiH (10 μL) and H_2O (10 μL) were added. The reaction mixture was stirred at rt for 1 h. After evaporation, the residue was purified by chromatography (step gradient: 10% then 25% MeOH/CH_2Cl_2) to yield **18** (130 mg, 68%) as a colorless oil. Fmoc–Thr–Ψ[CH_2N]–Pro–OH **18** (130 mg, 0.31 mmol) was dissolved in DMF (10 mL), and EDC (72 mg, 0.37 mmol), HOAt (58 mg, 0.37 mmol) and DMAP (10.0 mg, 0.092 mmol) were added followed by tryptamine (60 mg, 0.37 mmol). The reaction mixture was stirred at rt for 12 h, diluted with EtOAc (60 mL) and washed with water (40 mL). The aqueous solution was extracted with EtOAc (30 mL). The combined organic solution was washed with $NaHCO_3$ (30 mL), water (30 mL) followed by brine (30 mL). The organic layer was dried over Na_2SO_4. After filtration and evaporation, the residue was purified by flash chromatography using EtOAc to yield **19** as a colorless oil (110 mg, 63%). ^{1}H NMR: δ 7.92 (s, 1H), 7.78 (d, J = 7.5, 2H), 7.59 (t, J = 6.4, 3H), 7.41 (t, J = 7.1, 2H), 7.33 (t, J = 7.4, 3H), 7.13 (m, 3H), 6.94 (s, 1H), 4.55 (d, J = 8.2, 1H), 4.49 (d, J = 6.0, 2H), 4.20 (t, J = 6.0, 2H), 3.64 (app.

sextet, J = 6.5, 1H), 3.55 (m, 2H), 3.36 (m, 1H), 3.07 (dt, J = 6.2, 14.8, 1H), 2.93 (m, 2H), 2.88 (dt, J = 6.3, 15.1, 1H), 2.34 (t, J = 10.8, 1H), 2.18 (m, 2H), 2.12 (m, 1H), 1.81-1.65 (m, 4H), 0.83 (d, J = 6.4, 3H). ^{13}C NMR: δ 156.6, 144.1, 144.0, 141.5, 136.3, 127.9, 127.6, 127.3, 125.0, 122.6, 122.4, 120.2, 119.7, 119.0, 113.7, 111.6, 68.4, 66.2, 65.2, 56.2, 54.3, 53.6, 47.5, 39.0, 30.1, 25.2, 24.0, 19.9. HRMS (ESI$^+$): calcd for $C_{34}H_{39}N_4O_4$ [M + H]$^+$ m/z = 567.2971, found m/z = 567.2981.



Fmoc–Thr(PO(OBn)$_2$)–Ψ[CH$_2$N]–Pro–(2)-*N*-(3)-ethylaminoindole, 20. To a solution of **19** (60 mg, 0.11 mmol) in THF (3 mL) was added 5-ethylthio–1*H*–tetrazole (41 mg, 0.32 mmol) and di-benzyl diethylphosphoramidite (67 mg, 0.21 mmol) at rt, and the mixture was stirred at rt for 16 h. The mixture was cooled to –40 °C and a solution of 5–6 M *tert*-butyl hydroperoxide in decane (0.077 mL, 0.42 mmol) was added slowly and the mixture was stirred at –40 °C for 10 min, then at rt for 30 min. The reaction was cooled to –40 °C and quenched by addition of saturated aq $Na_2S_2O_3$. The mixture was diluted with EtOAc (60 mL), and washed with water (20 mL × 2), brine (20 mL), and dried over Na_2SO_4. Concentration under vacuum provided a residue, which was purified by flash chromatography using EtOAc as the eluent to afford **20** as a colorless oil (44 mg, 50%). ^{1}H NMR: δ 8.37 (s, 1H), 7.77 (d, J = 7.4, 1H), 7.65 (d, J = 7.7, 1H), 7.58 (d, J = 7.4, 1H), 7.56 (d, J = 7.4, 1H), 7.40 (t, J = 7.6, 2H), 7.36-7.24 (m, 13H), 7.12 (t, J = 7.4, 1H), 7.05 (t, J = 7.4, 1H), 6.98 (d, J = 2.0, 1H), 5.06 (dd, J = 9.0, 11.6, 1H), 5.01 (dd, J = 8.5, 11.8, 1H), 4.97 (dd, J = 10.4, 11.9, 1H), 4.92 (dd, J = 9.9, 11.8, 1H), 4.50 (d, J = 9.6, 1H), 4.46 (d, J = 6.3, 2H),

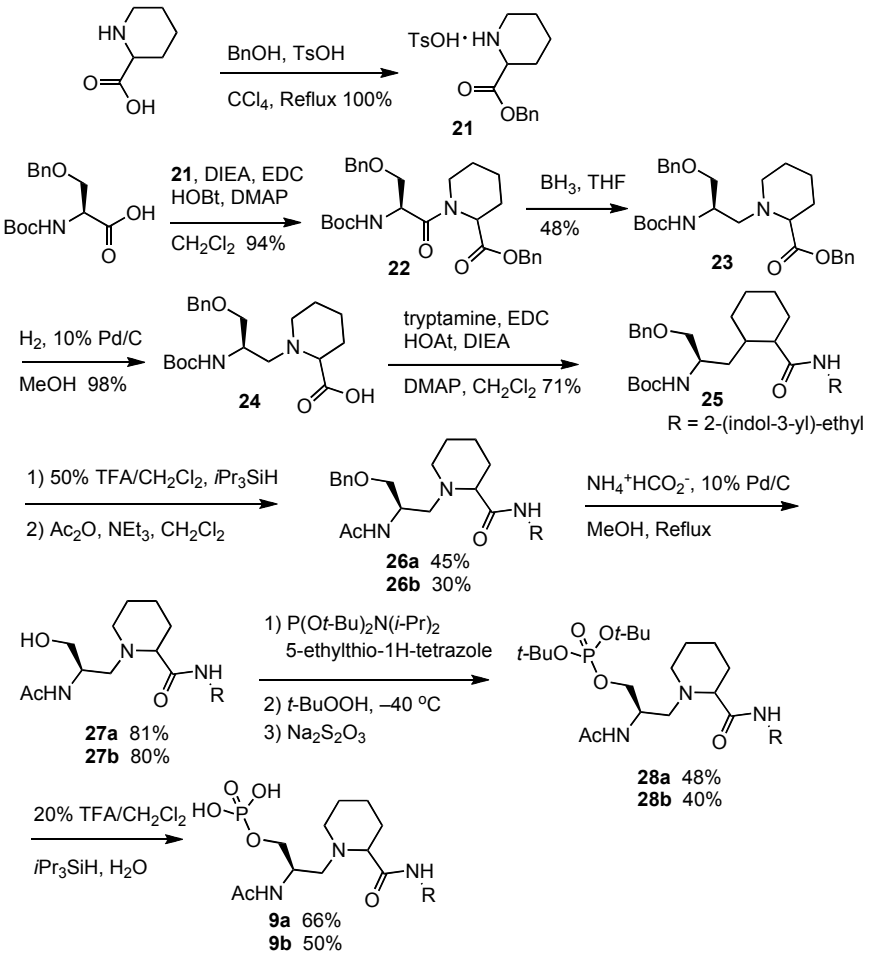
4.35 (m, 1H), 4.18 (t, J = 6.3, 1H), 3.67 (app. Sextet, J = 6.7, 1H), 3.61-3.52 (m, 1H), 3.48 (m, 1H), 3.06-2.94 (m, 3H), 2.81 (t, J = 7.0, 1H), 2.38 (dd, J = 6.8, 12.6, 1H), 2.22-2.14 (m, 2H), 2.03 (m, 1H), 1.78 (m, 2H), 1.64 (m, 1H), 1.52-1.47 (m, 1H), 1.08 (d, J = 6.3, 3H). ^{13}C NMR: δ 174.5, 156.6, 144.0, 143.9, 141.5, 136.4, 135.8 (d, $^{3}J_{P\text{-}C}$ = 5.1), 135.7 (d, $^{3}J_{P\text{-}C}$ = 6.4), 129.0, 128.9, 128.2, 128.0, 127.9, 127.7, 127.2, 125.01, 124.95, 122.2, 122.0, 120.2, 119.3, 119.0, 113.4, 111.3, 75.5 (d, $^{2}J_{P\text{-}C}$ = 5.8), 69.68 (d, $^{2}J_{P\text{-}C}$ = 5.3), 69.64(d, $^{2}J_{P\text{-}C}$ = 5.0), 69.4, 66.5, 58.0, 55.2, 54.6, 47.5, 39.2, 30.5, 25.2, 24.6, 18.6. ^{31}P NMR (202 MHz): δ 0.22. HRMS (ESI^{+}): calcd for $C_{48}H_{52}N_4O_7P$ $[M + H]^{+}$ m/z =827.3574, found m/z = 827.3579.

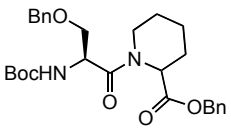


Fmoc–Thr(PO(OH)$_2$)–Ψ[CH$_2$N]–Pro–(2)-*N*-(3)-ethylaminoindole, 8. Dibenzyl phosphate **20** (15 mg, 0.018 mmol) and 10% Pd-C (13 mg) were dissolved in MeOH (2 mL). The reaction was stirred under H_2 (1 atm) at rt for 2 h. The reaction mixture was filtered through Celite, and washed with MeOH. After evaporation, the residue was purified by semi-preparative HPLC to provide **8** as a white solid after lyophilization (8.0 mg, 68%). Anal. HPLC (solvent A: 0.1% HCO_2H/H_2O, solvent B: 0.1% HCO_2H/CH_3CN, gradient 10% B for 3 min, then 10–90% B over 6 min), 8.3 min, 99%. ^{1}H NMR (CD_3OD): δ 7.77 (dd, J = 4.2, 7.6, 2H), 7.65 (d, J = 7.7, 1H), 7.56 (t, J = 6.8, 2H), 7.35 (m, 3H), 7.27 (t, J = 7.6, 1H), 7.24 (t, J = 7.5, 1H), 7.07 (t, J = 7.6, 1H), 7.06 (s, 1H), 6.96 (t, J = 7.4, 1H), 4.46 (d, J = 6.3, 2H), 4.28 (m, 1H), 4.20 (t, J = 6.2, 1H), 4.05 (m, 1H), 3.92 (m, 1H), 3.54 (m, 3H), 3.23 (m, 1H), 3.10 (m, 1H), 3.02-2.94 (m, 2H), 2.32 (m, 1H), 1.98 (m, 1H), 1.83 (m, 2H), 1.17 (d, J = 4.8, 3H). ^{13}C NMR

(CD_3OD): δ 158.7, 145.5, 145.0, 142.6, 138.2, 128.8, 128.7, 128.1, 126.2, 126.1, 123.7, 122.4, 120.9, 119.6, 119.3, 112.9, 112.3, 72.61, 72.57, 68.2, 67.7, 57.6, 57.4, 54.0, 41.6, 31.0, 26.0, 24.4, 18.5. HRMS (ESI^+) calcd for $C_{34}H_{40}N_4O_7P$ $[M + H]^+$ m/z = 647.2635, found m/z = 647.2639.

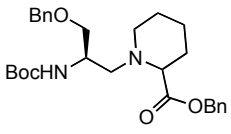
Scheme 4.7. Synthesis of inhibitor **9**.





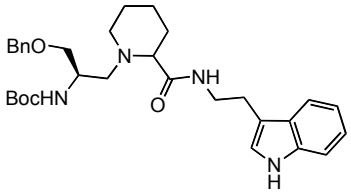
Boc–Ser(OBn)–Pip–OBn, 22. H–Pip–OH (6.25 g, 48.4 mmol) was suspended in CCl_4 (80 mL), and TsOH (11.0 g, 96.8 mmol) and BnOH (40 mL) were added. The reaction was heated at reflux for 24 h. Evaporation and precipitation with

Et_2O (500 mL) yielded TsOH·H–Pip–OBn **21** (17.4 g, 100%) as a white solid. TsOH·H–Pip–OBn **21** (3.50 g, 8.82 mmol) and DIEA (2.64 g, 20.3 mmol) were dissolved in CH_2Cl_2 (80 mL), and Boc–L–Ser(OBn)–OH (2.00 g, 6.78 mmol), EDC (1.55 g, 8.12 mmol), HOBt (1.25 g, 8.12 mmol) and DMAP (0.25 mg, 2.03 mmol) were added. The mixture was stirred at rt for 15 h. The mixture was washed with water (30 mL), 1M HCl (30 mL × 2), $NaHCO_3$ (30 mL × 2), and brine (30 mL). The organic layer was then dried over Na_2SO_4, filtered and evaporated. The crude product was purified by flash chromatography (eluent: 25% EtOAc/hexanes) to yield **22** (3.1 g, 94%) as a colorless oil. ^{1}H NMR (50 ºC): δ 7.27 (m, 10H), 5.49 (br s), 5.40 (m, 1H), 5.15 (m, 2H), 4.90 (m, 1H), 4.48 (m, 2H), 3.87 (m, 1H), 3.63-3.51 (m, 2H), 3.20 (m, 1H), 2.25 (m, 1H), 1.72-1.46 (m, 4H), 1.43 (br s, 9H), 1.26 (m, 1H). ^{13}C NMR: δ 170.8, 170.5, 170.2 (d), 138.0 (d), 137.7, 135.7, 135.6 (d), 128.6, 128.4 (d), 128.33, 128.29 (d), 128.2, 128.06 (d), 127.99, 127.97 (d), 127.89, 127.8, 127.6, 79.67, 77.36 (d), 73.3, 73.1 (d), 71.2, 70.4 (d), 67.0 (d), 66.9, 52.6, 52.4 (d), 50.5 (d), 50.2, 43.7 (d), 43.6, 28.4, 26.6, 25.2 (d), 24.8, 21.0, 20.8 (d). HRMS (ESI$^+$): calcd for $C_{28}H_{37}N_2O_6$ $[M + H]^+$ m/z = 497.2652, found m/z = 497.2655.



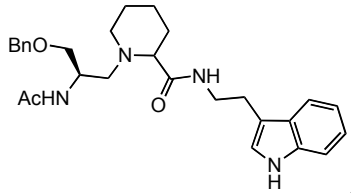
Boc–Ser(OBn)–Ψ[CH_2N]–Pip–OBn, 23. Boc–Ser(OBn)–Pro–OBn **22** (2.80 g, 5.78 mmol) was dissolved in dry THF (12 mL), a solution of borane (1 M in THF, 11.6 mL, 11.6 mmol) was added dropwise at 0 °C. After the addition was complete, the resulting mixture was stirred at 0 °C for 3 h and at rt for 11 h. Then the reaction was cooled to 0 °C, and MeOH (9 mL) was added slowly. The resulting mixture was evaporated and purified by flash chromatography (eluent: 9% EtOAc/hexane) to yield **23** (1.3 g, 48%) as a colorless oil. ^{1}H NMR: δ 7.35-7.27 (m, 10H), 5.14 (m, 2.5H),

4.92 (br, 0.5H), 4.47 (m, 2H), 3.76 (m, 1H), 3.60 (m, 1H), 3.54 (m, 0.5H), 3.42 (m, 0.5H), 3.24 (m, 1H), 3.08 (m, 0.5H), 3.02 (m, 0.5H), 2.64 (m, 1H), 2.50 (m, 1H), 2.32 (m, 1H), 1.81-1.38 (m, 6H), 1.46 (s, 0.5H), 1.44 (s, 0.5H). ^{13}C NMR: δ 173.9, 173.7 (d), 156.0 (d), 155.7, 138.4, 136.1, 128.70, 128.67 (d), 128.48, 128.46 (d), 128.39, 128.36 (d), 128.30 (d), 128.26, 127.8, 127.74, 127.71 (d), 79.2 (d), 79.1, 73.4, 73.2 (d), 70.3, 70.0 (d), 66.2, 66.1 (d), 64.7, 64.2 (d), 57.2 (d), 57.1, 50.6 (d), 50.1, 48.7, 48.4 (d), 29.54, 29.52 (d), 28.58, 28.56 (d), 25.6, 25.5 (d), 22.2, 22.1 (d). HRMS (ESI$^+$): calcd for $C_{28}H_{39}N_2O_5$ [M + H]$^+$ m/z = 483.2859, found m/z = 483.2772.



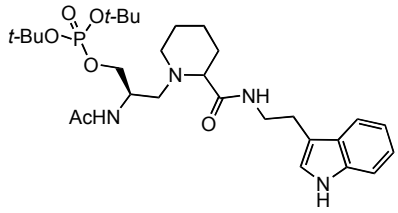
Boc–Ser(OBn)–Ψ[CH$_2$N]–Pip–(2)-*N*-(3)-ethylaminoindole, 25. Boc–Ser(OBn)–Ψ[CH$_2$N]–Pip–OBn **23** (1.0 g, 2.1 mmol) was dissolved in MeOH (50 mL), and 10% Pd/C (200 mg) was added. Then the reaction was stirred at rt under H_2 from a balloon for 2 h. After filtration through Celite and washing with MeOH, evaporation yielded Boc–Ser(OBn)–Ψ[CH$_2$N]–Pip–OH **24** (0.8 g, 98%) as a slightly yellow oil. Without further purification, the crude Boc–Ser(OBn)–Ψ[CH$_2$N]–Pip–OH was dissolved in CH_2Cl_2 (400 mL), and then tryptamine (0.40 g, 2.5 mmol), EDC (0.48 g, 2.5 mmol), HOAt (0.39 g, 2.5 mmol), and DMAP (0.07 mg, 0.63 mmol) were added. The reaction mixture was stirred at rt for 18 h. The mixture was washed with water (150 mL) and brine (150 mL). The organic layer was dried over Na_2SO_4. After filtration and evaporation, the residue was purified by flash chromatography (eluent: 33% EtOAc/hexanes) to yield **25** as a light yellow oil (0.8 g, 71%). ^{1}H NMR: δ 8.25 (br, 0.6H), 8.11 (br, 0.4H), 7.59 (t, J = 7.5, 1H), 7.33-7.15 (m, 7.8H), 7.09 (m, 1H), 7.99 (m, 0.8H),

6.86 (br, 0.4H), 4.66 (d, J = 8.9, 0.4H), 4.32 (m, 2.6H), 3.80 (m, 1.6), 3.60 (m, 0.4H), 3.48 (m, 1H), 3.28-2.88 (m, 5H), 2.73 (m, 1H), 2.50 (m, 0.4H), 2.41 (m, 0.6H), 2.28 (dd, J = 6.6, 12.8, 0.4H), 2.17 (dd, J = 3.8, 13.0, 0.6H), 2.08 (m, 0.4H), 1.88 (m, 1.6H), 1.64-1.24 (m, 15H). ^{13}C NMR (100 mHz): δ 174.8 (d), 174.2, 158.82, 155.78 (d), 138.1, 137.9 (d), 136.45 (d), 136.46, 128.5, 127.91 (d), 127.88, 127.8, 127.7 (d), 127.6, 127.4 (d), 122.2, 122.1 (d), 122.0, 118.93, 119.5, 119.4 (d), 118.88 (d), 113.4 (d), 113.0, 111.4, 111.3 (d), 79.5, 77.4 (d), 73.2, 70.6 (d), 69.6 (d), 67.9, 58.9 (d), 57.1, 52.3, 51.8, 48.7, 47.8 (d), 39.3, 38.9 (d), 30.2 (d), 29.4, 28.6, 28.5 (d), 25.4, 25.2 9 (d), 24.6 (d), 24.3, 23.4 (d), 23.3. HRMS (FAB$^+$): calcd for $C_{31}H_{43}N_4O_4$ [M + H]$^+$ m/z = 535.3284, found m/z = 535.3285.



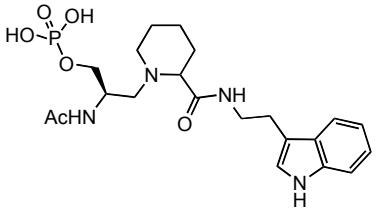
Ac–Ser(OBn)–Ψ[CH$_2$N]–Pip–(2)-*N*-(3)-ethylaminoindole, 26. Boc–Ser(OBn)–Ψ[CH$_2$N]–Pip–(2)-*N*-(3)-ethylaminoindole **25** (120 mg, 0.22 mmol) was dissolved in CH_2Cl_2 (4 mL) and a solution of TFA (4 mL) and iPr$_3$SiH (0.01 mL) was added at 0 °C. The mixture was stir at 0 °C for 30 min, and then at rt for 2 h. The reaction mixture was then concentrated under reduced pressure. The ammonium salt obtained was then dissolved in CH_2Cl_2, and basified with $NaHCO_3$ (40 mL). After separation, the aqueous layer was extracted with CH_2Cl_2 (20 mL × 3). The combined organic layers were washed with brine (20 mL), and dried over Na_2SO_4. After filtration and evaporation, the residue was dissolved in CH_2Cl_2 (5 mL). Ac_2O (46 mg, 0.45 mmol) and Et_3N (46 mg, 0.45 mmol) were added. The reaction mixture was stir at rt for 14 h. After dilution with CH_2Cl_2 (50 mL), the mixture was washed with 0.1 M NaOH

(20 mL × 2) and brine (20 mL), and dried over Na_2SO_4. After filtration and concentration, the residue was purified by flash chromatography with EtOAc to yield two diastereomers **26a** (48 mg, 45%), and **26b** (32 mg, 30%). **26a**: ^{1}H NMR: δ 8.17 (s, 1H), 7.58 (d, *J* = 7.4, 1H), 7.31 (m, 4H), 7.23 (m, 2H), 7.18 (appt. dt, *J* = 1.0, 7.9, 1H), 7.10 (t, *J* = 7.4, 2H), 7.02 (d, *J* = 2.3, 1H), 5.59 (d, *J* = 8.9, 1H), 4.34 (d, *J* = 12.1, 1H), 4.30 (d, *J* = 12.0, 1H), 4.07 (m, 1H), 3.62 (m, 1H), 3.54 (m, 1H), 3.23 (dd, *J* = 3.0, 9.6, 1H), 3.14 (dd, *J* = 3.8, 9.6, 1H), 2.97 (m, 2H), 2.88 (dt, *J* = 3.8, 7.9, 1H), 2.70 (dd, *J* = 3.5, 9.6, 1H), 2.40 (dd, *J* = 8.4, 12.9, 1H), 2.24 (dd, *J* = 6.2, 13.0, 1H), 2.08 (m, 1H), 1.86 (m, 1H), 1.84 (s, 3H), 1.64 (m, 1H), 1.53 (m, 2H), 1.31 (m, 2H). ^{13}C NMR: δ 174.3, 170.0, 137.8, 136.5, 128.6, 128.1, 127.9, 127.6, 122.2, 122.1, 119.5, 118.9, 113.2, 111.4, 69.2, 68.1, 56.4, 52.6, 47.3, 47.2, 38.9, 38.8, 29.6, 25.3, 24.5, 23.3. HRMS (FAB$^+$): calcd for $C_{28}H_{37}N_4O_3$ $[M + H]^+$ *m/z* = 477.2866, found *m/z* = 477.2841. **26b**: ^{1}H NMR: δ 8.05 (br, 1H), 7.57 (d, *J* = 7.9, 1H), 7.38 (m, 2H), 7.32 (m, 3H), 7.28 (dd, *J* = 0.6, 8.0, 1H), 7.17 (dt, *J* = 1.0, 7.2, 1H), 7.09 (dt, *J* = 0.7, 7.4, 1H), 6.92 (d, *J* = 1.8, 1H), 6.62 (t, *J* = 5.2, 1H), 5.43 (br, 1H), 4.45 (d, *J* =11.4, 1H), 4.42 (d, *J* = 12.0, 1H), 4.14 (m, 1H), 3.72 (m, 1H), 3.53 (m, 1H), 3.32 (dd, *J* = 3.0, 9.6, 1H), 3.30 (dd, *J* = 4.4, 9.3, 1H), 3.04 (m, 2H), 2.94 (m, 1H), 2.73 (dd, *J* = 3.6, 9.8, 1H), 2.58 (dd, *J* = 10.6, 12.9, 1H), 2.26 (dd, *J* = 3.8, 13.2, 1H), 1.85 (m, 1H), 1.84 (m, 1H), 1.62 (m, 1H), 1.54 (s, 3H), 1.52 (m, 1H), 1.30 (m, 2H). ^{13}C NMR: δ 174.6, 170.3, 138.0, 136.4, 128.7, 128.2, 128.1, 127.2, 122.4, 122.3, 119.6, 118.7, 113.0, 111.5, 73.5, 70.9, 67.7, 57.9, 51.5, 46.8, 39.2, 29.6, 25.2, 24.3, 23.3, 23.0. HRMS (ESI$^+$): calcd for $C_{28}H_{37}N_4O_3$ $[M + H]^+$ *m/z* = 477.2866, found *m/z* = 477.2848.



Ac–L-Ser(PO(O*t*Bu)$_2$)–Ψ[CH$_2$N]–L-Pip–(2)-*N*-(3)-ethylaminoindole, 28. Ac–L-Ser(OBn)–Ψ[CH$_2$N]–L-Pip–(2)-*N*-(3)-ethylaminoindole **26a** (38 mg, 0.080 mmol) was dissolved in MeOH (5 mL), and 10% Pd/C (20 mg) and ammonium formate (150 mg) were added. The reaction was heated at reflux for 5 h. After filtration through Celite and washing with MeOH, evaporation yielded **27a** (25 mg, 81%) as a slightly yellow oil. To a solution of (25 mg, 0.065 mmol) in THF (5 mL) was added 5-ethylthio–H–tetrazole (34 mg, 0.26 mmol) and di-*tert*-butyl diisopropylphosphoramidite (72 mg, 0.26 mmol) at rt, and the mixture was stirred at rt for 8 h. The mixture was cooled to –40 °C and a solution of 5–6 M *tert*-butyl hydroperoxide in decane (47 μL, 0.26 mmol) was added slowly and the mixture was stirred at rt for 30 min. The reaction was cooled to –40 °C and quenched by addition of saturated aq $Na_2S_2O_3$. The mixture was extracted with CH_2Cl_2 (20 mL × 2), and the combined organic extracts were dried over Na_2SO_4. Concentration under vacuum provided a residue, which was purified by flash chromatography (step gradient: 0% then 9% of *i*PrOH in EtOAc) to afford **28a** as a colorless oil (18 mg, 48%). ^{1}H NMR: δ 9.00 (br, 1H), 7.64 (d, *J* = 7.9, 1H), 7.38 (d, *J* = 7.8, 1H), 7.17 (dt, *J* = 1.2, 7.6, 1H), 7.10 (dt, *J* = 0.8, 7.4, 1H), 6.92 (m, 1H), 5.83 (d, *J* = 8.0), 4.05 (m, 1H), 3.75 (m, 2H), 3.62 (m, 1H), 3.42 (m, 1H), 3.05 (m, 2H), 2.84 (m, 1H), 2.75 (dd, J = 3.4, 8.7, 1H), 2.33 (dd, 1H), 2.27 (dd, 1H), 2.15 (t, 1H), 1.82 (s, 3H), 1.79 (m, 1H), 1.59 (m, 2H), 1.49 (s, 18H), 1.46 (m, 1H), 1.31 (m, 2H). ^{13}C NMR: δ 173.8, 170.2, 136.6, 127.6, 122.4, 122.1, 119.4, 119.0, 113.0, 111.6, 83.4, 67.9, 66.3, 55.7, 51.8, 47.4, 38.8, 30.0, 28.8, 25.2, 24.0, 23.3, 23.1. ^{31}P:

δ -8.53. ^{31}P NMR: δ –8.53. HRMS (FAB$^+$): calcd for $C_{29}H_{48}N_4O_6P$ [M + H]$^+$ *m/z* = 579.3311, found *m/z* = 579.3265. By a similar procedure, **28b** was obtained as a colorless oil (12 mg, 40%). ^{1}H NMR: δ 10.22 (s, 1H), 7.58 (d, *J* = 7.9, 1H), 7.41 (d, *J* = 8.4, 1H), 7.17 (m, 1H), 7.10 (m, 1H), 7.06 (br, 1H), 6.43 (br, 1H), 5.01 (d, *J* = 5.9, 1H), 4.22 (br, 1H), 3,86 (m, 2H), 3.78 (m, 1H), 3.56 (m, 1H), 3.18 (m, 1H), 3.13 (m, 1H), 3.05 (m, 1H), 2.67 (dd, *J* = 3.4, 10.8, 1H), 2.58 (m, 1H), 2.05 (dd, *J* = 3.3, 13.0, 1H), 1.92 (m, 1H), 1.82 (m, 2H), 1.67 (m, 1H), 1.52 (m, 21H), 1.48 (m, 1H), 1.37 (m, 1H), 1.22 (m, 1H). ^{13}C NMR: δ 174.8, 170.6, 136.8, 127.1, 122.6, 122.2, 119.4, 118.7, 112.5, 112.1, 83.5, 68.3, 67.5, 58.1, 52.0, 46.1, 39.2, 30.8, 30.0, 25.0, 24.8, 23.4, 22.3. ^{31}P-NMR: δ -9.26. HRMS (FAB$^+$): calcd for $C_{29}H_{48}N_4O_6P$ [M + H]$^+$ *m/z* = 579.3311, found *m/z* = 579.3351.



Ac–L-Ser(PO(OH)$_2$)–Ψ[CH$_2$N]–L-Pip–(2)-*N*-(3)-ethylaminoindole, 9. To a solution of **28a** (15 mg, 0.026 mmol) in CH_2Cl_2 (4 mL) was added a mixture of TFA (1 mL), H_2O (0.04 mL) and *i*Pr_3SiH (0.04 mL), and the reaction was stirred at rt for 1h. The solvent was removed under vacuum, and the residue was purified by semi-preparative HPLC to provide **9a** as a white solid after lyophilization (8.0 mg, 66%). Anal. HPLC (solvent A: 0.1% HCO_2H/H_2O, solvent B: 0.1% HCO_2H/CH_3CN, gradient 10% B for 3 min, then 10–90% B over 6 min), 6.5 min, 97%. ^{1}H NMR (CD_3OD): δ 7.56 (d, *J* = 7.7, 1H), 7.35 (d, *J* = 8.3, 1H), 7.10 (s, 1H), 7.08 (t, *J* = 7.2, 1H), 7.00 (t, *J* = 7.5, 1H), 4.34 (m, 1H), 3.90 (m, 2H), 3.74 (m, 2H), 3.60 (m, 2H), 3.12 (m, 3H), 2.99 (t, *J* = 6.8, 2H), 2.00 (m, 1H), 1.97 (s, 3H), 1.78 (m, 4H), 1.52 (m, 1H). ^{13}C NMR (CD_3OD): δ 174.1, 168.6, 138.2, 128.8, 123.7, 122.4, 120.0, 119.3, 112.7, 112.4, 65.8, 58.6, 57.4,

54.4, 48.5, 41.2, 29.5, 26.0, 23.7, 22.5, 21.6. ^{31}P NMR (CD_3OD): δ 0.69. HRMS (ESI^+): calcd for $C_{21}H_{32}N_4O_6P$ $[M + H]^+$ *m/z* = 467.2059, found *m/z* = 467.2062. By a similar procedure, **9b** was obtained as a white solid (4 mg, 50%). Anal. HPLC (solvent A: 0.1% HCO_2H/H_2O, solvent B: 0.1% HCO_2H/CH_3CN, gradient 10% B for 3 min, then 10–90% B over 6 min), 6.5 min, 95%. ^{1}H NMR (400 mHz, CD_3OD): δ 7.57 (d, *J* = 7.9, 1H), 7.37 (d, *J* = 8.1, 1H), 7.10 (m, 2H), 7.00 (t, *J* = 7.4, 1H), 4.39 (m, 1H), 3.90 (m, 3H), 3.58 (m, 3H), 3.12 (m, 1H), 2.94 (m, 4H), 2.00 (s, 3H), 1.92 (m, 2H), 1.80 (m, 1H), 1.69 (m, 2H), 1.51 (m, 1H). ^{13}C NMR (CD_3OD): δ 176.0, 169.4, 138.2, 128.7, 123.9, 122.4, 119.6, 119.3, 112.6, 112.5, 67.9, 65.6, 60.8, 53.6, 47.8, 41.0, 30.3, 26.1, 24.2, 22.5, 22.4. ^{31}P NMR (202 MHz, CD_3OD): δ 1.64. HRMS (ESI^+): calcd for $C_{21}H_{32}N_4O_6P$ $[M + H]^+$ *m/z* = 467.2059, found *m/z* = 467.2060.

Pin1 inhibition assay. Pin1 inhibition assay of **1**, **2**, **8**, **9a**, and **9b** was performed as published.[1] All the inhibitors were pre-equilibrated in a 1.0 mL quartz cuvette at 4 °C for 10 min. Inhibitor **1** was assayed at final concentrations of 3.4, 4.5, 6.8, 9.0, 14, 18, 23, 36, 68, with 10 μL of stock in 4:3 DMSO:H_2O. Inhibitor **2** was assayed at final concentrations of 1.5, 4.7, 17.5, 35.0, 116, with 10 μL of stock in 1:1 DMSO: H_2O. The inhibitor **8** was assayed at final concentrations of 1.2 μM, 9.7 μM, 20.0 μM, 39 μM, 78 μM, 0.16 mM, 0.32 mM, 0.47 mM, with 20 μL of stock in 4:3 DMSO: H_2O. Inhibitor **9a** was assayed at final concentrations of 3.5, 7.0, 14, 28, 42, 83, with 10 μL of stock in 1:1 DMSO: H_2O. Inhibitor **9b** was assayed at final concentrations of 39 μM, 78 μM, 0.12 mM, 0.25 mM, 0.50 mM, 1.0 mM, with 10 μL of stock in 1:1 DMSO: H_2O. For each concentration, the assay was performed in duplicate, and all of the data were used for calculation of IC_{50}. The plot of % Inhibition vs. log [I] (μM) produced a sigmoidal curve

by fitting all the experimental data to a dose response curve using TableCurve (version 3 for win32) (Figures 4.5 to 4.9). The IC_{50} values were derived from the curves at 50% inhibition of enzyme activity.

Reference:

1. Wang, X. J.; Xu, B.; Mullins, A. B.; Neiler, F. K.; Etzkorn, F. A. *J Am Chem Soc* **2004**, *126*, 15533.

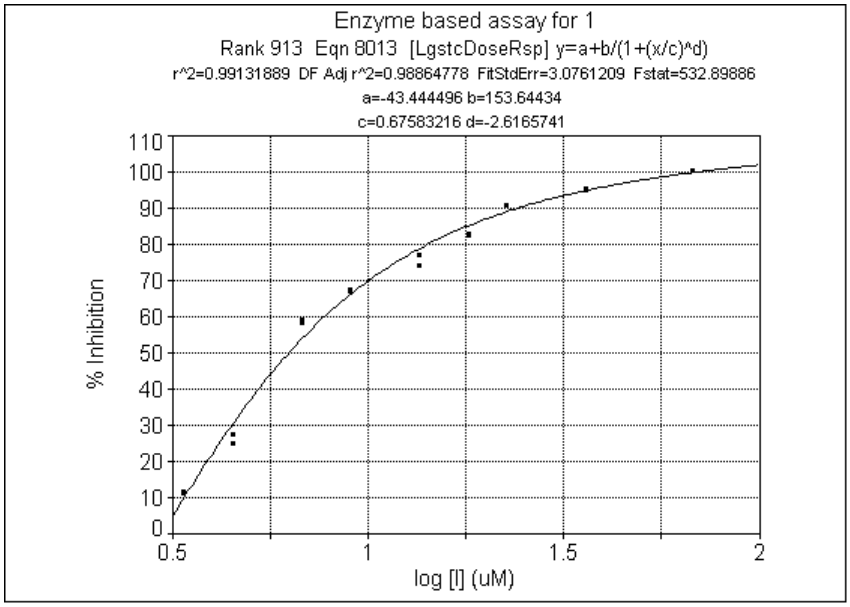


Figure 4.5. Dose response curve for inhibition of Pin1 by Fmoc–Ser($PO(OH)_2$)–Ψ[CH_2N]–Pro–(2)-*N*-(3)-ethylaminoindole **1** (IC_{50} = 6.3 ± 0.4 μM).

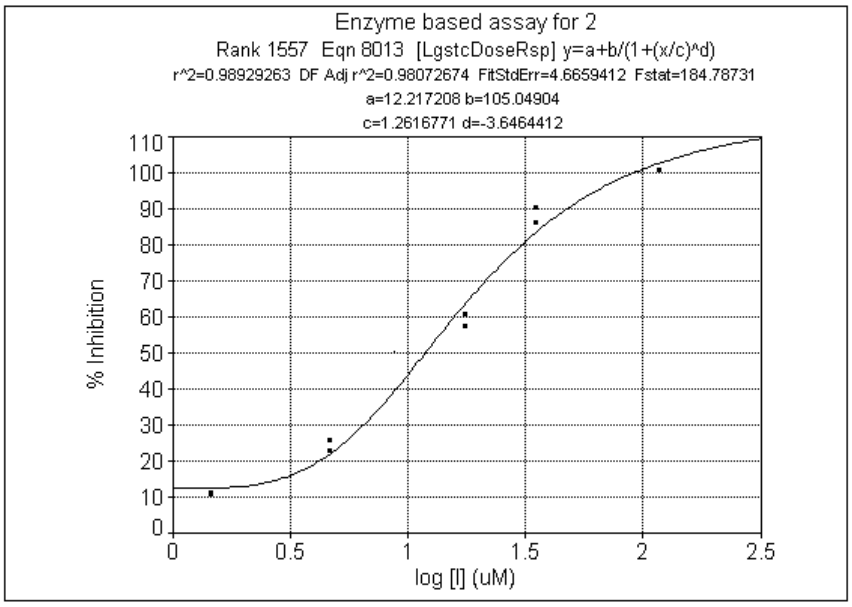


Figure 4.6. Dose response curve for inhibition of Pin1 by Ac–Ser($PO(OH)_2$)–Ψ[CH_2N]–Pro–(2)-*N*-(3)-ethylaminoindole **2** (IC_{50} = 12 ± 2 μM).

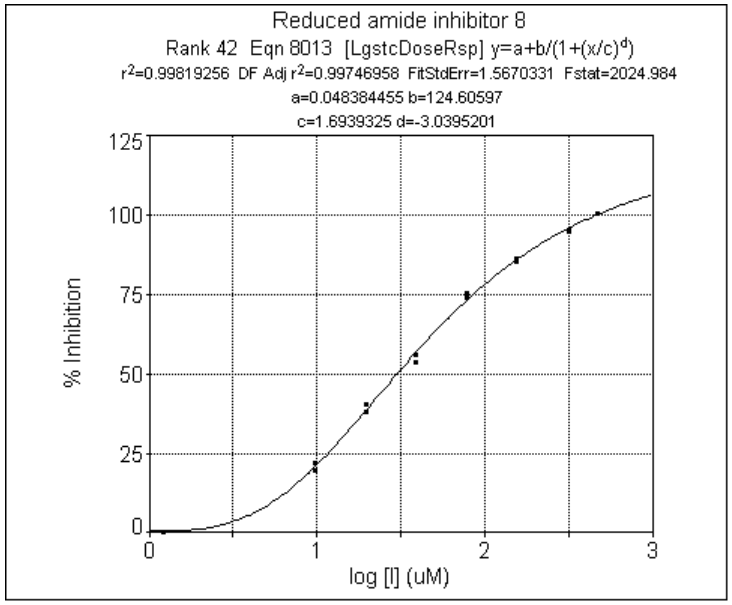


Figure 4.7. Dose response curve for inhibition of Pin1 by Fmoc–Thr($PO(OH)_2$)–Ψ[CH_2N]–Pro–(2)-*N*-(3)-ethylaminoindole **8** (IC_{50} =30 ± 2 μM).

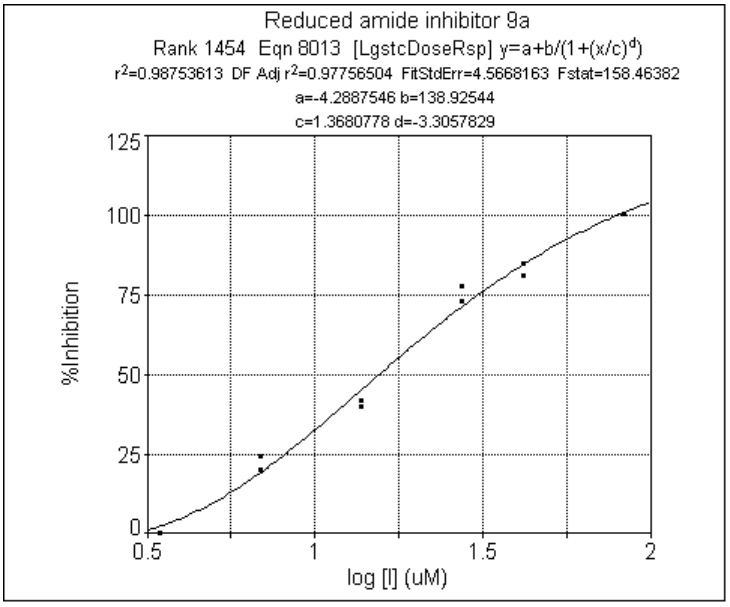


Figure 4.8. Dose response curve for inhibition of Pin1 by Ac–Ser($PO(OH)_2$)–(Ψ[CH_2N]-(L/D)Pip–(2)-*N*-(3)-ethylaminoindole **9a** (IC_{50} =16 ± 2 μM).

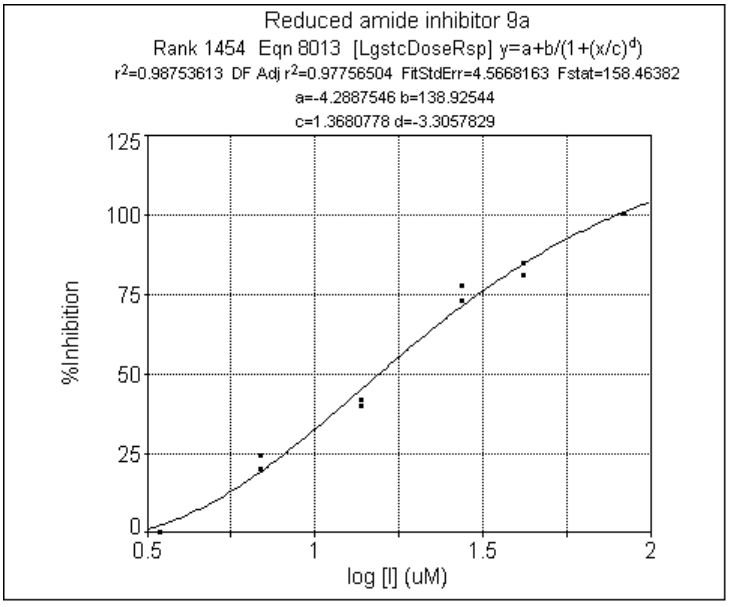


Figure 4.9. Dose response curve for inhibition of Pin1 by Ac–Ser($PO(OH)_2$)–(Ψ[CH_2N]-(D/L)Pip–(2)-*N*-(3)-ethylaminoindole **9b** (IC_{50} =190 ± 20 μM).

Chapter 5: Conclusions and Future Work

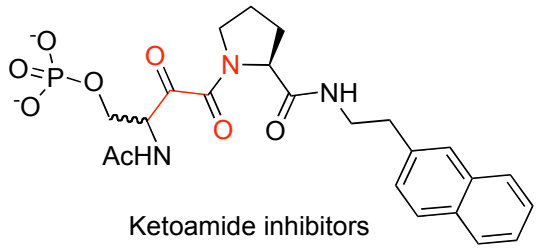
As discussed in Chapter 1, two promising mechanisms are associated with Pin1—the "nucleophilic" mechanism and the "twisted-amide" mechanism. Based on the two mechanisms, three classes of compounds—involving a total of nine inhibitors, were designed and synthesized as Pin1 transition-state analogue inhibitors (Table 5.1). The three classes, which are grouped according to their modified functionalities, are known as ketoamide, ketone, and reduced amide analogues. We subsequently tested each of the nine synthesized Pin1 inhibitors and their IC_{50} values are shown in Table 5.1.

Table 5.1. Protease coupled assay results for all synthesized Pin1 inhibitors.

Class of Compound	Pin1 Inhibitor	IC_{50} (μM)
Ketoamides	Ac–p(∗)Ser–C(O)–Pro–2-(2-naphthyl)-ethylamine	100 ± 10
	Ac–p(∗)Ser–C(O)–Pro–2-(2-naphthyl)-ethylamine	200 ± 20
Ketones	Ac–p(L)-Ser–Ψ[C(O)CH]-(L)Pip–(2)-*N*-(3)-ethylaminoindole	260 ± 30
	Ac–p(L/D)-Ser–Ψ[C(O)CH]-(D/L)Pip–(2)-*N*-(3)-ethylaminoindole	61 ± 8
Reduced amides	Fmoc–pSer–Ψ[CH_2N]-Pro–(2)-*N*-(3)-ethylaminoindole	6.3 ± 0.4
	Ac–pSer–Ψ[CH_2N]-Pro–(2)-*N*-(3)-ethylaminoindole	12 ± 2
	Fmoc–pThr–Ψ[CH_2N]-Pro–(2)-*N*-(3)-ethylaminoindole	30 ± 2
	Ac–pSer–Ψ[CH_2N]-(∗)Pip–(2)-*N*-(3)-ethylaminoindole	16 ± 2
	Ac–pSer–Ψ[CH_2N]-(∗)Pip–(2)-*N*-(3)-ethylaminoindole	190 ± 20

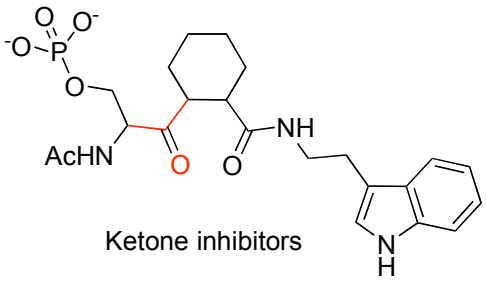
Due to epimerization at the carbon α to the ketone, a pair of diastereomers of Ac–pSer–C(O)–Pro–2-(2-naphthyl)-ethylamine (ketoamide inhibitors) were obtained. We

believe that the better inhibitor is the D-Ser analogue due to previous and simultaneous results obtained in our lab and Fisher's lab, but the alternative cannot be ruled out.



Ketoamide inhibitors

Two isomers of Ac–pSer–Ψ[C(O)CH]Pip–(2)-*N*-(3)-ethylaminoindole (ketone inhibitors) were obtained. The α-carbon of adjacent to the carbonyl functionality was epimerized during the synthesis. The major isomer, Ac–p-(L)-Ser–Ψ[C(O)C]-(L)-Pip–(2)-*N*-(3)-ethylaminoindole was more potent than the Ac–p-(L/D)-Ser–Ψ[C(O)C]-(D/L)Pip–(2)-*N*-(3)-ethylaminoindole (a pair of enantiomers). The stereochemistry was determined by X-ray crystallography through the crystal structures of two intermediates.



Ketone inhibitors

Although ketoamide analogues and ketone analogues were designed as tetrahedral-intermediate analogues of Pin1 due to their ketone functionality with increased electrophilicity over the amides, their weak inhibitory activities did not support nucleophilic catalysis mechanism. With the increased nucleophilicity, the ketoamides or ketones could form the hemithioketals, which mimic the tetrahedral intermediate in the nucleophilic catalysis mechanism. From another aspect, if the ketone of ketoamides were orthogonal to the amide (like FK 506), they could act as the twisted amide analogue in

the "twisted-amide" mechanism. However, these compounds were found to be very weak inhibitors of Pin1. It should also be noted that when we compared the IC_{50} values, we determined that the diastereomers did not affect the inhibitory activity significantly.

Five reduced amide inhibitors were synthesized and evaluated: 1) Fmoc–pSer–Ψ[CH_2N]Pro–(2)-*N*-(3)-ethylaminoindole, 2) Fmoc–pThr–Ψ[CH_2N]Pro–(2)-*N*-(3)-ethylaminoindole, 3) Ac–pSer–Ψ[CH_2N]Pro–(2)-*N*-(3)-ethylaminoindole, and 4/5) Ac–pSer–Ψ[CH_2N]-(∗)-Pip–(2)-*N*-(3)-ethylaminoindole (a pair of separated diastereomers) (Figure 5.1).

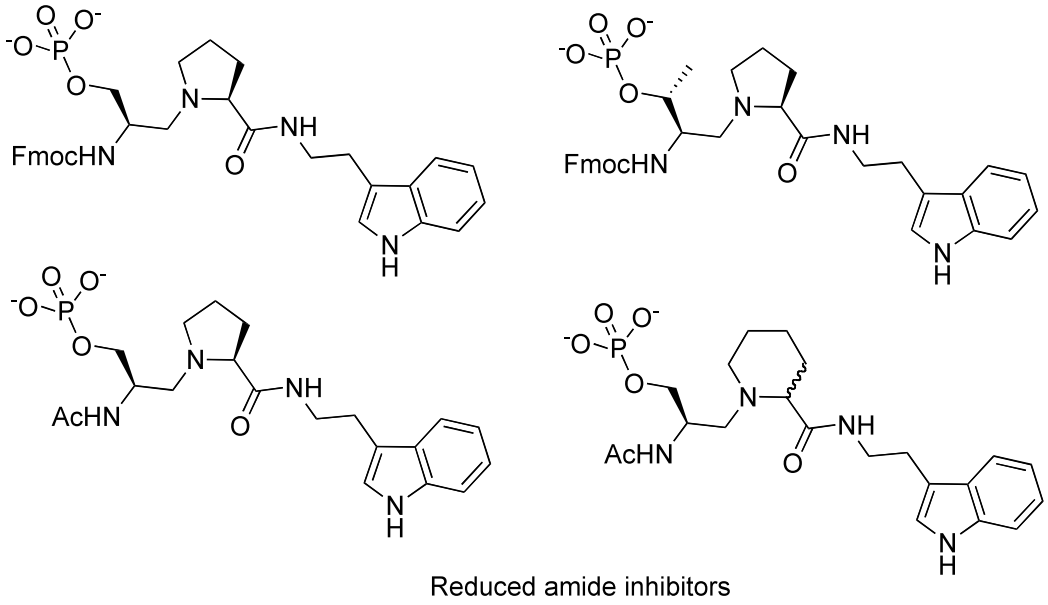


Figure 5.1. The chemical structures of five key reduced amide inhibitors of Pin1.

The reduced amide inhibitors were designed based on a proposed hydrogen-bond assisted twisted-amide mechanism, since the nitrogen atom in a reduced-amide bond is pyramidal. It turns out they are the best non-peptidic small molecule inhibitors we have made, even though, due to the lack of a ketone group and the increased flexibility of the amine, the reduced amide inhibitors are not working as effectively as transition-state

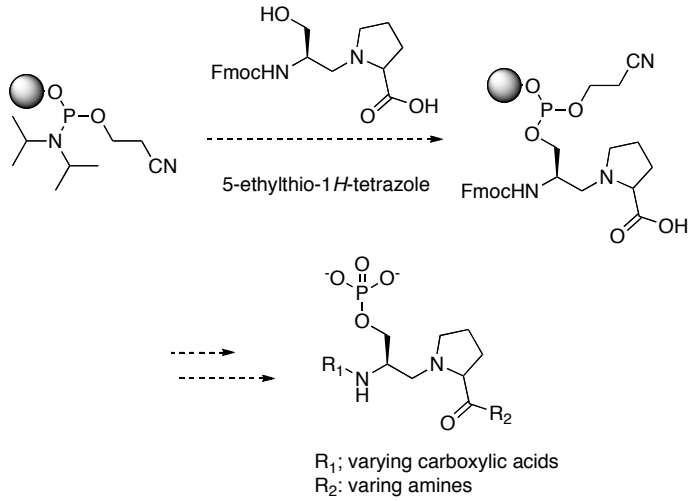
analogues act. When we examined the crystal structure of the Ac–pSer–Ψ[CH_2N]Pro–(2)-*N*-(3)-ethylaminoindole in complex with Pin1, we did not detect any hydrogen bonding between the nitrogen atom and the active site residues of Pin1. With the modification of the Pro ring to Pip ring for increased hydrophobic interaction, the inhibitory activity of Ac–pSer–Ψ[CH_2N]-(∗)-Pip–(2)-*N*-(3)-ethylaminoindole was not improved. Since this modification was not based on the enzyme mechanism, while just structural study on the site interactions, it is not surprising that no improvement was obtained.

Among the five reduced amide inhibitors evaluated for this study, the most potent one was determined to be Fmoc–pSer–Ψ[CH_2N]Pro–(2)-*N*-(3)-ethylaminoindole. Specifically, it was found to be 4.5 times more potent than the analogous (*Z*)-alkene inhibitor of Pin1, Fmoc–pSer–Ψ[(*Z*)CH=C]-Pro–(2)-*N*-(3)-ethylaminoindole (IC_{50} = 28.3 μM), which we synthesized in our lab.[46] It should also be noted that the synthesis of the targeted reduced amide involved just six steps, while the synthesis of Fmoc–pSer–Ψ[(*Z*)CH=C]-Pro–(2)-*N*-(3)-ethylaminoindole required six steps following the synthesis of the Fmoc–Ser–Ψ[(*Z*)CH=C]-Pro–OH.

In the future, since we found that the reduced amides were very good Pin1 inhibitors, as well as relatively easy to synthesize, we designed a library using the reduced amide as the core structure in order to screen the most effective inhibitors of Pin1. Only three steps were needed to synthesize the intermediate Fmoc–Ser–Ψ[CH_2N]Pro–OH, which will then be used as the principal building block for the development of the reduced amide library using solid-phase synthesis (Scheme 5.1). Specifically, using the phosphorylating resin successfully developed in our laboratory

(Xingguo Chen, unpublished results), in a one-step process, we can load the building block to the resin through the hydroxyl group. Various organic acids and amines will be coupled to each flanking *N*- and *C*-termini to develop a reduced amide library of Pin1 catalytic site inhibitors.

Scheme 5.1 Proposed synthesis of a reduced amide library for Pin1.



In conclusion, three classes of Pin1 inhibitors (ketoamide, ketone, and reduced amide analogues) involving a total of nine compounds were synthesized and evaluated. Even though we could not get a solid support on either of Pin1 catalysis mechanism based on the current results, we still can fairly say that the weak inhibitory activities of ketoamide and ketone analogues did not support the nucleophilic catalysis mechanism, while the twisted-amide mechanism of Pin1 catalysis is promising based on the reduced amide inhibitors with good potencies. The reduced amide inhibitor, Fmoc–pSer–Ψ[CH_2N]Pro–(2)-*N*-(3)-ethylaminoindole, was determined to be the most potent among the nine inhibitors. Due to its effectiveness and easy synthesis, a library was designed using the reduced amide as the core structure. Future research will investigate the sequence specificity in order to develop more potent inhibitors of Pin1.