Design, Syntheses, and Bioactivities of Conformationally Locked Pin1 Ground State Inhibitors

by

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Abstract

Pin1 (protein interacting with NIMA 1) is a peptidyl-prolyl isomerase involved in mitosis. As a potential anti-cancer drug target, Pin1 interacts and regulates the activity of an increasing number of cell cycle enzymes by an unknown mechanism. These cell cycle enzymes include Cdc25, Cdc27, Cyclin D1, Myt1, Wee1, NIMA, Cdc2, Plk1 and c-Myc. Recent research has revealed that Pin1 is overexpressed in a variety of cancer cell lines and Pin1 inhibitors inhibit proliferation activity of several cancer cells overexpressing Pin1. The most potent Pin1 inhibitors identified so far are in the micromolar range and no pharmacophore has been identified.

In order to assist the understanding of the biological function of Pin1 using molecular probes, two amide isosteres of Ser-*trans*-Pro and Ser-*cis*-Pro dipeptides were designed and stereoselectively synthesized. The conformationally locked Ser–*trans*–Pro mimic, Boc–SerΨ[(*E*)CH=C]Pro–OH, was synthesized through the use of an Ireland-Claisen [3,3]-sigmatropic rearrangement in nine steps with 13% overall yield from a serine derivative. The Ser-*cis*-Pro mimic, Boc–SerΨ[(*Z*)CH=C]Pro–OH, was synthesized through the use of a Still-Wittig [2,3]-sigmatropic rearrangement in 11 steps with an overall yield of 20% from the same starting material.

Conformationally locked peptidomimetics, including two exactly matched peptidomimetics, Ac–Phe–Phe–pSer–Ψ[*(E)*CH=C]Pro–Arg–NH_2 and Ac–Phe–Phe–pSer–Ψ[*(Z)*CH=C]Pro–Arg–NH_2, were synthesized from these Ser-Pro isosteres using Fmoc SPPS. A protocol for in vitro Pin1 inhibition assay was established for measuring the inhibition constant for these peptidomimetics. A conformationally locked cis peptidomimetic inhibits Pin1 with a K_i of 1.7 μM, 23-fold more potent than its trans counterpart, illustrating the preference of Pin1 for a cis amide bond in its PPIase domain. The A2780 ovarian cancer cell antiproliferation activity of these peptidomimetics parallels their respective Pin1 inhibition data. This research provides a start toward more drug-like Pin1 inhibitor design. Gly–*trans*–Pro isosteres were synthesized using the Ireland-Claisen route. The construction of a non-peptidic (*Z*)-alkene library for Pin1 inhibition was attempted using the Ser-*cis*-Pro mimic, Boc–SerΨ[(*Z*)CH=C]Pro–OH as the core.

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List of Abbreviations

1. Amino acids

Ala, A = alanine

Asn, N = asparagine

Asp, D = aspartic acid

Arg, R = arginine

Cys, C = cysteine

Gln, Q = glutamine

Glu, E = glutamic acid

Gly, G = glycine

His, H = histidine

Ile, L = isoleucine

Leu, L = leucine

Lys, K = lysine

Met, M = methionine

Phe, F = phenylalanine

Pro, P = proline

p = phospho-

pS-P = phosphoSer-Pro

Ser, S = serine

Thr, T = threonine

Trp, W = tryptophan

Tyr, Y = tyrosine

Val, V = valine

Xaa = any naturally occurring amino acid

Yaa = any naturally occurring amino acid

2. Enzymes

CCK = cholecystokinin

CDK = cyclin dependent kinase

CsA = cyclosporin A

CyP = cyclophilin

DMAP = 4-dimethylamino pyridine

FKBP = FK-506 binding proteins

HIV = the human immunodeficiency virus

NIMA = never in mitosis A kinase

Pin1 = protein interacting with NIMA #1

PPIase = peptidyl-prolyl isomerase

RNase = ribonuclease

TFH = thyroliberin

WW domain = WW stands for two tryptophans

3. Phases of mitosis

G1 = preparation for chromosome replication

S = DNA replication

G2 = preparation for mitosis

M = mitosis

4. Synthesis

Ac = acetyl

Bn = benzyl

Boc = *tert*-butoxycarbonyl

Cbz, Z = benzyloxycarbonyl

DCC = *N, N*-dicyclohexylcarbodiimide

Dde = 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl

DMAP = 4-(dimethylamino)pyridine

DMF = *N, N'*-dimethylformamide

EDC = 1-[3-(dimethylammino)propyl]-3-ethylcarbodiimide hydrochloride

HATU = *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate

HBTU = *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate

HOAt = 1-hydroxy-7-azabenzotriazole

HOBt = 1-hydroxybenzotriazole

LAH = lithium aluminum hydride

LDA = lithium diisopropylamide

Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl

Pht = phthalyl

TBAF = tetrabutylammonium fluoride

TBS = *tert*-butyldimethylsilyl

THF = tetrahydrofuran

TIS = triisopropyl silane

TMSCl = chlorotrimethylsilane

PTLC = preparative thin layer chromatography

TLC = thin layer chromatography

SAR = atructure activity relationships

5. Spectrometry

ATR = attenuated total reflection

HMQC = heteronuclear multiple quantum correlation

NOESY = Nuclear Overhauser and Exchange Spectroscope

6. Terms

IC_{50} = In determinations of receptor binding affinity of a ligand using a competitive binding curve, 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

M_r = relative molecular mass, the mass of a molecule relative to the mass of one-twelfth of the mass of a mole of carbon atoms.

Chapter 1. Introduction and Background

1.1. Biology of the peptidyl-prolyl isomerase Pin1

1.1.1. c*is*-Proline

Proline is the only dialkylated amine among the 20 naturally occurring amino acids, thus it forms tertiary amides at the N-terminal side of the residue in a peptide chain or in proteins. The other 19 naturally occurring amino acids contain primary amines, which lead to secondary amides. Amide bonds are planar due to the C=N double bond character, thus exist in discrete cis and trans conformations.[1] The barrier ($\Delta G^{\neq}$) to interconversion of the isomers at room temperature is between 18 kcal/mol to 21 kcal/mol.[2] Secondary amides exist almost exclusively in the trans conformation due to the steric interactions between the two extended side chains (> 99.9% trans), while the cis conformation of Xaa-Pro amide bonds is much more favorable than other amide bonds. N-alkylation reduces the steric advantage of the trans conformation, although the trans conformation is still favored energetically (Figure 1.1). Recently, R. T. Raines and co-workers have shown that an electronic effect—an $n \rightarrow \pi^*$ interaction between the oxygen of the amide bond and the subsequent carbonyl carbon on the C-termini—substantially stabilizes the trans conformer of prolyl amides (Figure 1.2).[3] It is found that 10-30% of prolyl amides exist in the cis conformation in small peptides[4] and approximately 6% in proteins of known structure.[5]

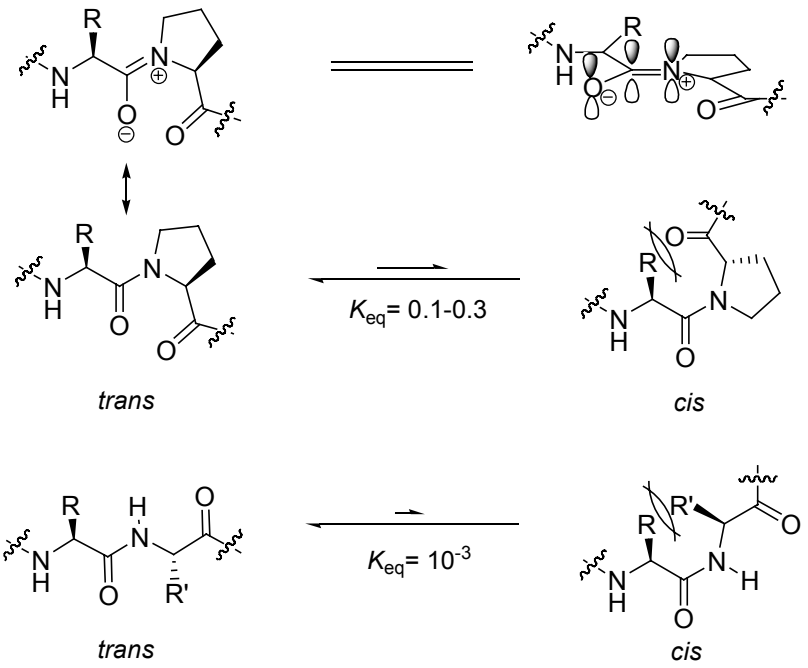


Figure 1.1. Xaa-Pro isomerization (top) vs. Xaa-Xaa isomerization (bottom)

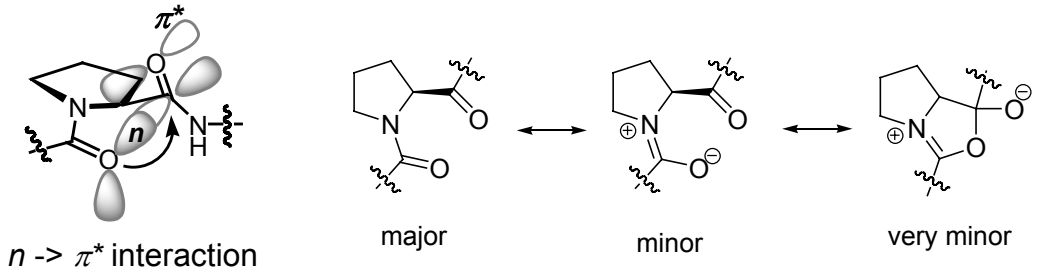


Figure 1.2. An $n \rightarrow \pi^*$ interaction contributes to the stability of the *tran*-Pro.[3]

Left: Molecular orbital showing $n \rightarrow \pi^*$ interaction. Right: the very minor resonance structure on the far right arises from the hyperconjugative delocalization of an $n \rightarrow \pi^*$ interaction.[3]

Since thermal cis-trans amide isomerization (seconds) is much slower than protein folding (milliseconds), the occurrence of cis-prolines complicates protein folding when a particular prolyl amide must isomerize before a protein can reach its native folded

structure.[4,6-9] On the other hand, cis-proline and trans-proline have a great effect on the morphology and the activity of a protein. Brandts et al. proposed that prolyl amide isomerization could be the slow steps in protein folding.[4] Brandts suggested that only those unfolded molecules that contain every proline in the proper conformation could fold to the native state and all protein molecules containing any proline in the incorrect conformation would need to isomerize. The experimentally investigated proteins for this model for slow folding steps include bovine pancreatic trypsin inhibitor,[9,10] dihydrofolate reductase,[11] ribonuclease T1 (RNase T1),[7,12] and cellular retinoic acid-binding protein I (CRABPI).[13]

1.1.2. Peptidyl-prolyl isomerases

Since thermal cis-trans amide isomerization is slow compared to other biological processes, peptidyl-prolyl isomerases (PPIases) have evolved to accelerate the cis-trans isomerization of prolyl amide bonds. Those PPIases fall into three categories: the cyclophilins, the FK-506 binding proteins (FKBPs) and the parvulins.[14,15] Cyclophilin (CyP) refers to the entire class of PPIases that bind the immunosuppressant drug cyclosporin A (CsA) and FKBP refers to the class of PPIases that bind the immunosuppressant drug FK-506. The third class of PPIases, namely parvulins,[16] does not bind immunosuppressant drugs. The PPIases catalyze the cis-trans isomerization of Xaa-Pro amide bonds in folded or unfolded proteins with high efficiency,[17-20] and are implicated in many biological processes.[21-23] CyP and FKBP are involved in immunosuppression (CyP and FKBP),[14,24] neuroprotection/neuroregeneration activities (CyP and FKBP),[25-27] Ca^{2+}-mediated intracellular signaling (FKBP),[28] chaperone

activities (CyP),[29] and HIV infection (CyP).[30] Human Pin1, a member of the recently discovered parvulin PPIase family, has been shown to play an important role in cancer[31] and Alzheimer's disease[32] through regulation of the cell cycle.[20,23]

1.1.3. Pin1

Pin1 (protein interacting with NIMA #1), a PPIase discovered in 1996, belongs to the parvulins.[20] Pin1 is a human protein that has a molecular mass (M_r) of 18,245 Da. and was first discovered in a yeast two-hybrid system by interaction with NIMA (never in mitosis A) kinase that is essential for progression through mitosis in *Aspergillus nidulans*.[20] Pin1 contains 168 amino acid residues with two domains that both recognize the same phosphoSer-Pro or phosphoThr-Pro motif: a 39-residue N-terminal WW (tryptophan tryptophan) domain and a C-terminal PPIase domain. The WW domain is a small protein-protein interaction domain present in a variety of cell signaling proteins unrelated to each other.[33] Defining characteristics of the WW domain are the presence of two invariant tryptophans, one near each terminus, a proline near the C-terminus and a cluster of aromatic residues located centrally in the primary sequence. Hypotheses for the WW domain function in Pin1 include substrate recognition, interaction with anchoring proteins for subcellular localization, and facilitation of nuclear import.[20] The C-terminus of Pin1 contains motifs characteristic of the newly discovered parvulin family of PPIases, which share little similarity with either the cyclophilins or the FKBPs,[16,34] but are conserved throughout eukaryotic cells. Unlike CyP and FKBP, Pin1 has an essential native biological function in the regulation of mitosis. Pin1 is the only PPIase found to be essential for cell growth[9,20] and restores the function of the essential parvulin Ess1/Ptf1

in budding yeast.[35,36] Pin1 both negatively regulates entry into mitosis and is required for normal progression through mitosis in human cells and yeast.[20] Depletion of Pin1/Ess1 from yeast or Hela cells induces mitotic arrest and nuclear fragmentation, whereas Hela cells overexpressing Pin1 arrest in the G2 (preparation for mitosis) phase of the cell cycle, indicating that overexpression of Pin1 inhibits the G2/M transition.[20]

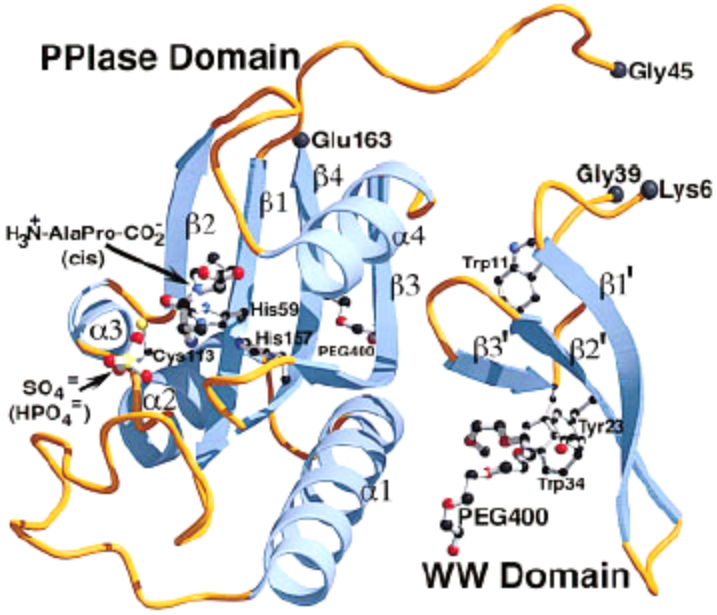


Figure 1.3. X-ray crystal structure of Pin1[37]

Pin1 is unique among the PPIases since it binds substrates in a phosphorylation dependent manner.[37,38] The X-ray crystal structure of Pin1 has been solved to 1.35 Å resolution with an Ala-Pro dipeptide bound in the PPIase domain in the cis conformation.[38] The presence of a specific tightly bound SO_4^{2-} ion in the crystal structure suggests that Pin1 possesses a strong preference for acidic side chains in the residue N-terminal to proline in substrates (Figure 1.3).[38] Indeed, it was found that Pin1 specifically recognizes the phosphoSer-Pro (pS-P) or phosphoThr-Pro (pT-P) amide bonds present in

mitotic phospho-proteins, with up to 1300-fold selectivity over the non-phosphorylated sequences. Neither the cyclophilin nor the FKBP families effectively catalyze the isomerization of peptides with pS/pT-P moieties.[38] Thus phosphorylation of S/T-P renders the prolyl-peptidyl bond resistant to the catalytic action of the conventional PPIases, suggesting the need for a different enzyme to catalyze this reaction.[38] In addition to the high selectivity for phosphorylated sequences, preference was also seen for an arginine at the +1 position and aromatic residues in the –1 through –3 positions around the central pS/pT-P motif.[38] Screening of a chemically synthesized peptide library against Pin1 PPIase activity revealed the best peptide substrate to be WFYpSPR-*p*NA (k_{cat}/K_m[39] = 2×10^7 $M^{-1}S^{-1}$).[38] Pin1 shares the same substrate specificity as the monoclonal antibody MPM2 that specifically recognizes a large number of the mitotic phosphoproteins involved in cell cycle regulation. An increasing number of the cell cycle proteins interacting with Pin1 have been recognized, the most important ones being NIMA kinase, Cdc25 phosphatase, Cdc2 kinase, Plk1 phosphatase, Wee1 phosphatase, Cdc27 phosphatase,[23] the p53 oncogen,[40-42] the c-Myc oncogene,[43] and retinoic acid receptor (RAR).[44]

According to the simplest model of enzyme kinetics, the enzyme catalytic reaction is divided into two processes: $E + S \overset{K_s}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P$. (E: enzyme; S: substrate; ES: enzyme-substrate complex; P: product.). The enzyme and the substrate first combine to give an enzyme-substrate complex, ES. This processes is rapid and reversible. The complex then cleaves to give product in a second step with a first-order rate constant k_{cat} (the turnover number). The reaction rate is given by $v = \frac{[E][S]k_{cat}}{K_m + [S]}$ .....[1], where K_m is equal to the dissociation constant of the enzyme-substrate complex *K*s. When the substrate concentration is low, the reaction rate is simplified to $v = (k_{cat}/K_m)[E][S]$ .....[2], where k_{cat}/K_m is an apparent second-order rate constant that refers to the properties and the reactions of the free enzyme and free substrate.

1.1.4. Regulation of the cell cycle

The cell cycle is characterized by four defined stages including preparation for chromosome replication (G_1), DNA replication (S), preparation for mitosis (G_2), and mitosis (M) (Figure 1.4). Not all populations of cells are dividing. After mitosis, some cells enter the next cell cycle, some enter a quiescent stage (G_0) and are capable of re-entering the cell cycle under certain stimulation, and some become terminally differentiated cells that are destined to die without dividing. The principle of cell cycle regulation is the appropriately timed structural modification of proteins through phosphorylation/dephosphorylation, and ubiquitin-mediated protein degradation. The G2/M transition is governed by the activation of the cyclin dependent kinase Cdc2/cyclinB complex (Figure 1.5). The activity of Cdc2/cyclinB is negatively regulated by phosphorylation by the Wee1 and Myt1 protein kinases, and positively regulated by the dephosphorylation of Thr-14 and Tyr-15 by Cdc25 phosphatase.[45] When the kinase Cdc2/cyclinB is phosphorylated it is inactive, and cell division cannot occur. The activated phosphatase Cdc25 removes two inactivating phosphates from Cdc2, and the cell enters mitosis.

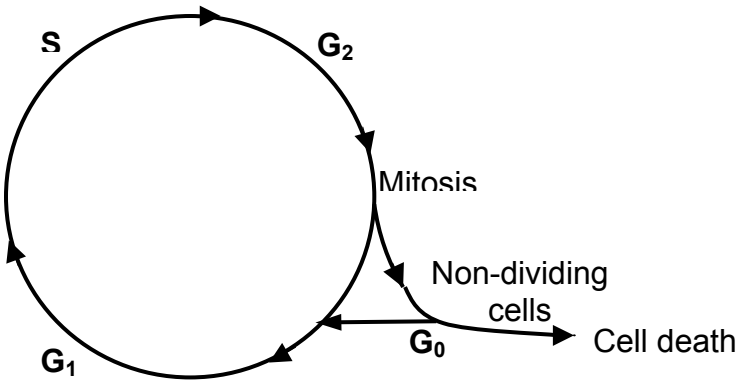


Figure 1.4. The cell Cycle

A two-step mechanism for mitotic regulation of Cdc25 has been proposed, namely kinase phosphorylation followed by a Pin1-dependent conformational change.[46] Pin1 acts as a conformational switch, turning mitosis on and off through isomerization of the phosphoThr-Pro or phosphosSer-Pro amides of Cdc25, thus controlling whether Cdc25 exists in an active conformation.[47] It is unknown whether Pin1 acts as an enzyme to shift the equilibrium between *trans*- and *cis*- phosphoSer-Pro isomers of Cdc25 or Pin1 acts on Cdc25 as a chaperone by binding one phosphoSer-Pro isomer more tightly during the G2/M transition leading to mitosis.

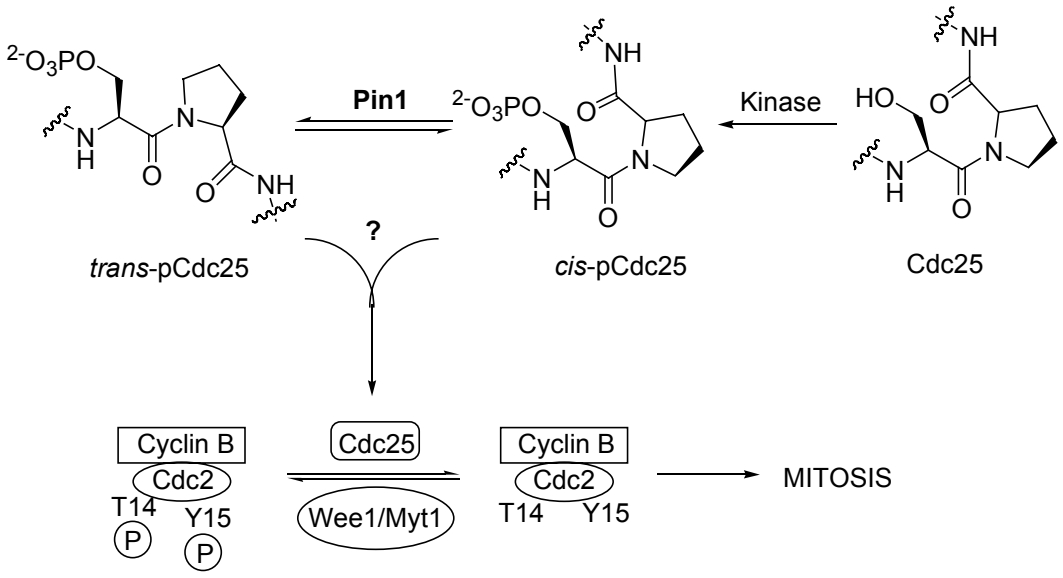


Figure 1.5. The role of Pin1 and its substrate, Cdc25, in mitosis

1.1.5. Significance to cancer

Cell cycle checkpoints in the regulatory pathway control the timing and order of important cell cycle events such as DNA replication and chromosome segregation to ensure the duplication of cells proceeds with high fidelity.[48] Loss of checkpoints results in genetic instability and can be an important early step in the progression of cancer.[48,49] Cell cycle checkpoints exist in both G1 and G2 phases. Cdc25C was identified as a potential target of checkpoint control in human cells during the G2 to M phase transition responding the DNA damage,[50-52] although it is still unclear how Cdc25 is normally activated during mitosis. Thus the investigation of how Cdc25 is activated during the G2/M transition may address a fundamental question in cancer biology. To understand the role of Pin1 as a regulator of Cdc25 and the G2/M transition thus is important in understanding the activition of mitosis.

Although the concentration of Pin1 does not fluctuate during the cell cycle (the total cellular Pin1 concentration in Hela cells was estimated to be ~0.5 μM),[47] Pin1 is present at higher concentrations in cycling cells over the non-dividing cells, making it a target primarily in the continuously dividing cells of cancer.[53,54] In addition, Pin1 is overexpressed in a large number of cancer cell types as compared with normal cell types.[31,55]

Among cell cycle regulatory proteins, the enzymatic reaction catalyzed by Pin1 is unique. Many cell cycle regulatory enzymes are kinases that catalyze phosphate transfer to proteins from ATP, phosphatases that catalyze the dephosphorylation of proteins, histone acetyl transferases (HATs), or deacetylases (HDACs). These are all large classes of enzymes with essential roles in both resting and dividing cells. The mechanisms of

many of these reactions are chemically quite similar, so targeting specific family members is difficult. Although much progress has been made in the design of specific inhibitors for kinases, phosphatases, and HDACs, the unique phosphorylation-dependent PPIase reaction catalyzed by Pin1 on pSer/Thr-Pro sequences makes it a very attractive target for anti-cancer drugs. Because of the essential native function of Pin1, inhibition of this enzyme should target only dividing cells and the rapidly dividing cells of cancer should be particularly susceptible. By understanding how Pin1 activates Cdc25, and the mechanism of Pin1 PPIase activity, it may be possible to prepare transition state inhibitors of PPIase activity. Such inhibitors might represent new anti-cancer drug leads.

1.2. Ground state Xaa-Pro mimics

Synthetic replacement of the amide bonds with a surrogate that would lock the conformation of Pro either cis or trans would address proline's role in molecular recognition. Since conformationally constrained proline peptidomimetics can serve as functional probes for Pin1 to illustrate its conformation specificity and how it acts on Cdc25, designs of conformationally constrained dipeptide mimics are reviewed here.

Conformationally constrained proline peptidomimetics have been designed by various strategies, which include heterocyclic aromatic mimics, bicyclic lactam mimics, isosteres and equilibrium-shifted mimics.[56]

1.2.1. Heterocyclic aromatic mimics

The most common strategy for cis amide bond peptide mimics is the use of heterocyclic aromatic rings, such as tetrazoles,[57-63] triazoles,[64] pyrazoles[64] and pyrroles,[65] to constrain the amide bond in a cis conformation. Since the 1,5-substituted tetrazole ring

system as a cis amide bond surrogate was proposed by Marshall et al. in 1982,[57] many tetrazole dipeptide mimics were synthesized and incorporated into peptide contexts and the biological activities were tested. Lu and Johnson reported the synthesis of compound **1** as a dipeptide mimic of **2** by direct modification of the amide bond of the corresponding dipeptide **2** with PCl_5 and HN_3.[58] But the success of this reaction was found to be dependent upon the amino protecting group employed and also upon the amino acid sequence of the starting dipeptide. Racemization at the α–carbon of the N-terminal amino acid residue occurred during the formation of the tetrazole dipeptide analogue. Subsequently, Zabrocki et al. reported stereoselective synthesis of the tetrazole mimic by the addition of quinoline to the reaction medium.[59] This procedure overcame the problems in the former synthesis and allowed the incorporation of the analogues into longer peptides with maintenance of chiral integrity. The 1,5-tetrazole analogue of Trp-Nle, which has one of the bulkiest of amino acid side chains, is also accessible with high optical purity and was synthesized in such a way that the tetrazole dipeptide could be selectively deprotected, enabling the elongation of the peptide chain to be achieved either from the N-terminus or from the C-terminus.[60]

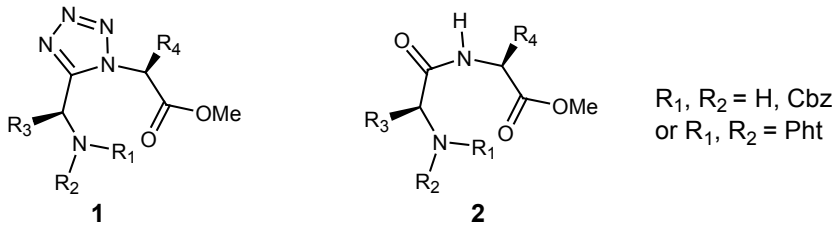
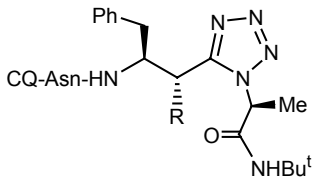


Figure 1.6. Tetrazole *cis*-dipeptide mimics

The analysis of the crystal structure of a cyclic dipeptide, cyclo[L-Phe-Ψ(CN_4)-L-Ala], showed that approximately 88% of the conformations accessible to the *cis* isomer of a dipeptide are also available to peptides in which the amide bond is replace by a 1,5-disubstituted tetrazole ring that locks the amide bond in *cis* conformation.[62]

The 1,5-disubstituted tetrazole peptide analogues were incorporated into longer peptides with maintenance of chiral integrity as conformational probes. Results for the incorporation of tetrazole dipeptide analogues into biologically active peptides, such as thyroliberin,[57] bradykinin,[59] cholecystokinin (CCK)[61] and somatostatin[63] have been reported. Marshall reported replacing an amide bond in thyroliberin (TFH) with a tetrazole, but the analogue was not biologically active, indicating that either the tetrazole was not a good *cis*-amide mimic or the *trans*-amide was the active form of the hormone.[57] Bradykinin mimics incorporating the tetrazole constraint showed no activity in rat uterus or bovine brain membrane binding assays, providing evidence for *trans*-Pro as the active conformation.[59] Hruby and coworkers replaced the Trp-Met amide bond of a cholecystokinin (CCK) pentapeptide with the tetrazole mimic. Interestingly, the tetrazole mimic had decreased affinity for both CCK-A and CCK-B receptors, but protected precursors displayed remarkable selectivity for A vs. B.[61] The somatostatin hexapeptide mimic of somatostatin-14 was found to be 83% as potent as that of somatostatin-14.[63]

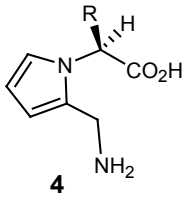
Abell et al. combined together the two basic strategies of peptidomimetics, isosteric replacement and conformational restriction, and reported the first design and synthesis of a cis conformationally restricted isostere **3** as an HIV-1 protease inhibitor with a potency range of IC_{50} 51-300 μM (Figure 1.7).[64]



3 R= H, OH, OAc

Figure 1.7. Conformationally restricted isostere tetrazole peptide mimics

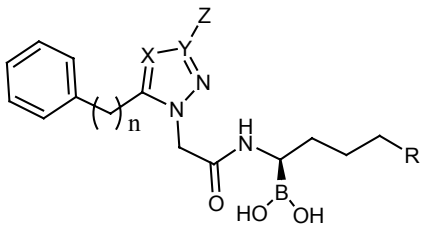
Other aromatic rings also were used as constrained cis peptide mimics, such as 1,2-disubstituted pyrrole **4** Gly-Ψ[C_4N]-Ala, Gly-Ψ[C_4N]-Gly, Gly-Ψ[C_4N]-Leu.[65] A series of pyrazoles and 1,2,4-triazoles **5** as surrogates for cis amide bonds in boronate ester thrombin inhibitors was made and showed good inhibition ranging from 0.8 nM to 42 nM.[66] A notable example is the simple racemic o-aminomethylphenylacetic acid derivative **6**.[67,68] One of the resulting analogs had an IC_{50} of 23 nM in a growth hormone release inhibition assay versus 0.3 nM for native somatostatin.[68]



4

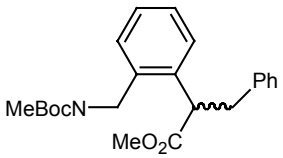
R= Me, Gly-Y[C_4N]-Ala
H, Gly-Y[C_4N]-Gly
CH_2CHMe_2, Gly-Y[C_4N]-Leu

Figure 1.8. Pyrrole peptide mimics



X, Y = CH, N, n =1, 2

5



6

Figure 1.9. Pyrazole, Triazole Mimics and Phenyl Mimic

1.2.2. Bicyclic lactam mimics

Another common strategy for conformationally defined peptide mimics is to use a linkage Y to tether the two amino acids, thus forming a lactam or bicyclic lactam for proline, locking the amide bond in cis or trans conformation. An advantage of this strategy is that it affords cis as well as trans dipeptide mimics. For proline, the cis mimic is formed when Y is between the two α-carbons of both amino acids, while trans mimic is formed when Y is between α-carbon of Xaa and the δ-carbon of Pro, as shown in Figure 1.10.

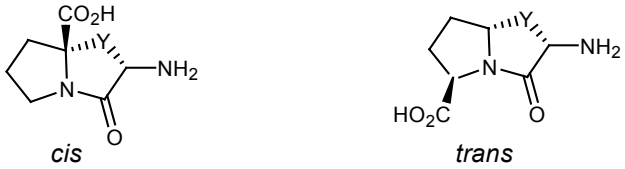
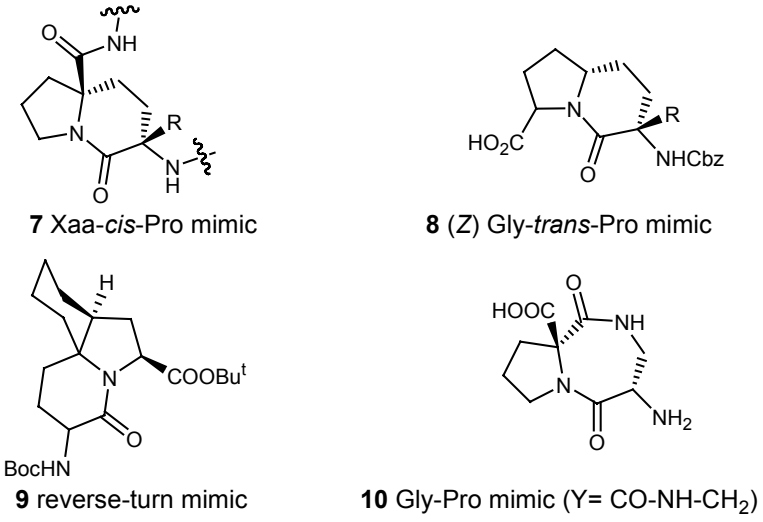


Figure 1.10. Bicyclic lactam Xaa-Pro mimetics

According to molecular modeling, the cis-Xaa-Pro mimic **7**, in which an ethylene linker is used (Y = CH_2CH_2), allows retention of the peptide backbone and amino acid side chains in positions closely similar to those likely to occur in native Xaa-*cis*-Pro type VI turn conformation.[69] The synthesis and characterization of Gly-*cis*-Pro-OH mimic **7** (R = H) were described by two groups as a type VI turn dipeptide mimic starting from optically pure (*R*)-2-allylproline.[69,70] The mimic was incorporated into analogues of cis-Gly^6-Pro^7-bradykinin using Fmoc-solid phase method.[69] Those analogues were 102-103 fold less active than the known bradykinin antagonist D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8] bradykinin (Hoe 140). Given the fact that the tetrazole cis dipeptide antagonist showed no activity, the active conformation of bradykinin was suggested to comprise a C-terminal β-

turn with the Ser[6]-*trans*-Pro[7] geometry. The corresponding Cbz protected Gly-*trans*-Pro mimic **8** was also made as a *β*-turn type II mimic by applying the Schoellkopf bislactam-ether methodology starting from dimethyl-(2*S*,8*S*)-2,8-diamino-5-oxononane-1,9-dicarboxylate.[71]



7 Xaa-*cis*-Pro mimic

8 (*Z*) Gly-*trans*-Pro mimic

9 reverse-turn mimic

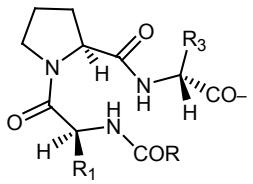
10 Gly-Pro mimic (Y= CO-NH-CH_2)

Figure 1.11. *cis*- and *trans*-Gly-Pro mimics

Germanas et al. reported a very similar synthesis of bicyclic lactam Gly-Pro and the analogue was subsequently modified at the N-terminus to introduce alkyl groups stereoselectively (compound **7**, R = Bn, *i*-Pr, CH_2CH_3, CH_3, $CH_2C_{10}H_7$, H).[72] The various analogues were evaluated for binding to cyclophilin A and shown to have K_d's ranging from 5 μM for R = Bn to > 500 μM.[72] Recently a synthetic scheme was developed to prepare compound **9** as a reverse-turn dipeptide mimic by ring-closing metathesis of a suitable diallylated proline derivative as a key step. It was prepared as both *cis*- and *trans*-fused stereoisomers of *N*-Boc-L-octahydroindole-2-carboxylic acid (l-Oic) methyl

ester, in which the tetrahydropyrrole ring was fused by a cyclohexane ring.[73] This was the first reported practical synthesis of the trans-fused isomer.

Compound **10** is an example of a lactam-methylene (Y = CO-NH-CH_2) linker. It was synthesized non-stereoselectively from L-aminoalanine and diethyl-2,2-pyrolidinedicarboxylate.[74] An attractive feature of this linker is that the lactam provides additional constraint to the bicyclic framework. But no incorporation to peptide or biological activity was reported for this mimic. N^2-, N^3-substituted fused 1,2,5-triazepine-3, 6-diones **12** differs from the parent system **11** by just two H atoms, but fixes the stereochemistry of the acyl proline amide bond in its cis- or (*E*)-configuration through the linkage between the N^{α}-atom of the residue preceding Pro and the Nα-atom of the residue following Pro.[75] The mimic of tetrapeptide Z-Ala-Gly-Pro-Phe(OMe) **13** was synthesized in low yield, yet demonstrated the possibility of extending with defined stereochemistry and sequence both the N-terminus and C-terminus of the 1,2,5-triazepine-3,6-dione dipeptide mimics.[76]



11 Xaa-*cis*-Pro-Yaa

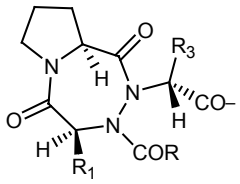


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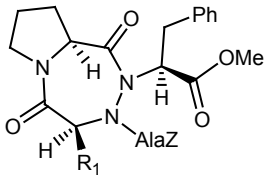
Figure 1.12. N^2-, N^3-Substituted fused 1,2,5-triazepine-3, 6-diones

Other non-proline lactam dipeptide mimics were also synthesized. A β-lactam mimic of an N-methyl cis-amide of the natural product bouvardin **14** was designed and synthesized by Boger and Myers. The peptide mimic was constrained to cis conformation

by the linkage between the two α-carbons. A cyclic peptide mimic containing the β-lactam was 25,000-fold less potent than deoxybouvardin in cytotoxic activity.[77]



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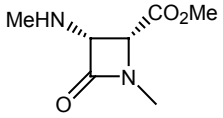

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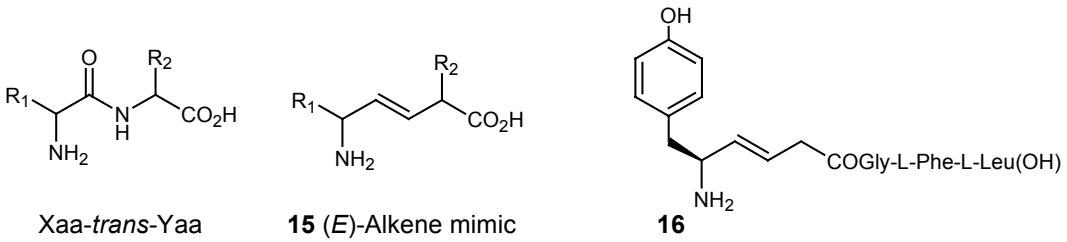
1.2.3. Amide bond bioisosteres

One of the ideal peptide bond surrogates is the olefinic moiety because of the similar geometrical disposition of substituents attached to either of these functional groups. The olefinic group has been successfully employed in a number of different peptides as the trans configuration of the peptide bond. Hann and Sammes reported a method for the generation of the system (R_2=H) **15**.[78] An analogue **16** of Leu^5-enkephalin was synthesized by using L-tyrosine as one of the starting material and condensation of the amino aldehyde with an ylide as the key step to build up trans C-C double bond.[78] The high activity of the analogue (IC_{50} = 4.6 nM at 0 ºC vs. IC_{50} = 3.1nM for Leu^5-enkephalin) indicates that the presence of the amide group is not essential for activity while the trans disposition is important for the bioactivity.[78,79] A similar system with Boc-protected Gly-Xaa dipeptides were synthesized by starting with *trans*-hex-3-enedioic acid and by using a modified Curtius reaction to transform one carboxylic acid group to an amine.[80] Various other strategies have also been used to synthesize (*E*)-alkene dipeptide mimics for application to Leu-enkephalin and substance P analogs.[81] These alkene polypeptide mimics possess biological potencies varying from 0.1% to 300% of those of

the parent polypeptides. This indicates that the trans geometry plays a large part in contributing to the biological activity.[81]



Xaa-*trans*-Yaa **15** (*E*)-Alkene mimic **16**

Figure 1.13. *E*-Alkene dipeptide mimic

The (*E*)-alkene mimics of *D*-Ala-*L*-Ala, *D*-Ala-*D*-Ala, *D*-Ala-*D*-Leu **17** were made by Whitesell and Lawrence through an Ireland-Claisen rearrangement reaction, which is unique in that absolute stereochemistry is controlled at both centers by asymmetric induction, providing a general and flexible route to *E*-alkene mimics.[82] Although no biological activities were reported, mimics of the trans (9, 10) MeLeu-MeLeu amide bond alkene mimics of cyclosporin A were designed and synthesized as conformational probes.[83] While (*E*)-alkene **18** was synthesized by a Still-Wittig rearrangement, (*Z*)-alkene **19** was synthesized by a Wittig reaction.[83]

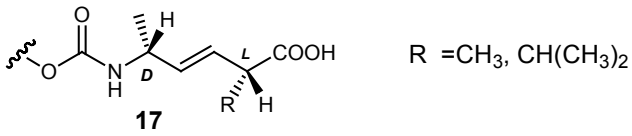


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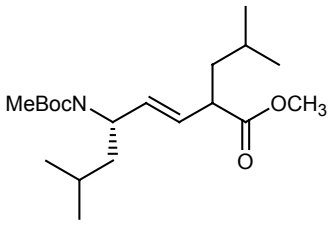
Figure 1.14. Stereospecific Ala-Ala and Ala-Leu *E*-alkene mimics



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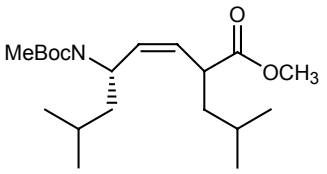
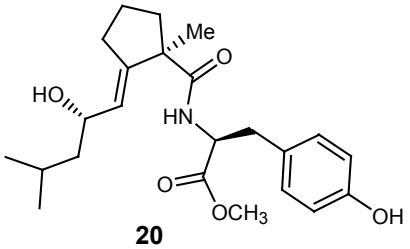


19

Figure 1.15. *trans*- and *cis*-Leu-Leu mimic

A trans-proline mimic tripeptide **20** was synthesized using the Wittig reaction to form the (*E*)-alkene and stereoselective alkylation was used to add the leucine side chain.[84] The methyl group at the prolyl α-position was introduced for synthetic feasibility. It proved to be an inhibitor of the prolyl isomerase activity of recombinant hFKBP12 with an inhibition constant (K_i) of 8.6μM.[84] Another *trans*-proline mimic **21** as inhibitor of dipeptidyl peptidase IV (CD26) was synthesized non-stereoselectively. The desired *Z*-isomer was separated by chromatography as a mixture of two pairs of enantiomers and the two pairs of enantiomers were further separated by chromatography.[85] Note that *Z* corresponds to trans-Pro due to the F priority. The pair that contains the natural stereochemistry was found to be more potent as expected.[86]



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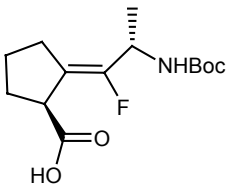
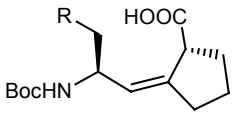


21

Figure 1.16. Proline *E*-Alkene dipeptide mimics

While the cis olefin group is an ideal cis amide bond mimic, potential isomerization of the cis *β, γ*–unsaturated carbonyl system to the more stable *α, β*-unsaturated system limited the use of this particular amide bond surrogate. The only reported conformationally rigid (*Z*)-alkene proline isostere was compound **22**, which was completely stereo-selectively synthesized with high overall yield by Scott Hart in our group by using L-Ala (R = H) as starting material.[87] The second stereocenter was induced by the stereocenter on alanine and the alkene *Z*-geometry was obtained using the Still-Wittig [2, 3]-sigmatropic rearrangement. It was synthesized in a form suitable for incorporation into peptidomimetics and the alanine-proline mimic was incorporated to a tetrapeptide mimic Suc-Ala-Ala-Ψ[(*Z*)CH=C]-Pro-Phe-pNA. The peptidomimetic showed inhibition of PPIase human cyclophilin with an IC_{50} of 6.5 μM.[88] The serine mimic was synthesized in the same way as the alanine mimic using benzyl for oxygen protection with both lower stereoselectivity and *Z*-geometry selectivity and lower overall yield.[89]



22

R = H, Boc-Ala-Y[(Z)CH=C]-L-Pro
= OH, Boc-Ser-Y[(Z)CH=C]-L-Pro

Figure 1.17. Ala-*cis*-Pro and Ser-*cis*-Pro *Z*-alkene mimic**s**

1.2.4. Equilibrium-shifted mimics

Rather than rigidly locking the amide bond isostere to cis or trans conformation, another strategy for design of peptide mimics is incorporating steric factors to shift the amide bond equilibrium so that it favors *cis* or *trans* more than the corresponding

dipeptide. Because the equilibrium between cis and trans still exists, we call it "equilibrium-shifted" peptide mimics. The peptides containing proline variants **23** Aze (Azetidine 2-carboxylic acid) and **24** Pip (pipecolic acid) were found to exist in 40% cis for **23**, greater than that for proline (cis 23%), and 21% cis for **24**.[90] The cis content of other peptides containing proline analogues such as **25**, **26**, **27** and **28** were also determined by dynamic NMR spectroscopy, which was 28% (R = H) and 33% (R = CH_3) for **25**, 38% (R = H) and 33% (R = CH_3) for **26**, 19% for **27** and 8% for **28**.[90]

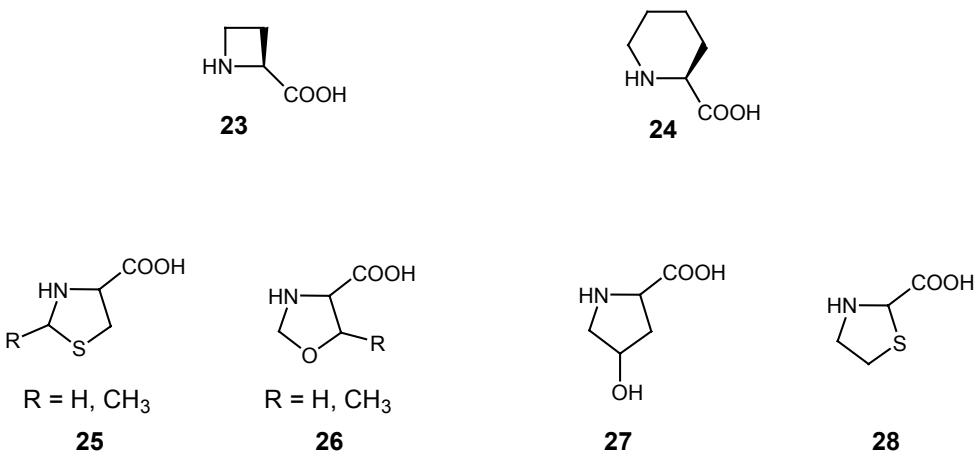
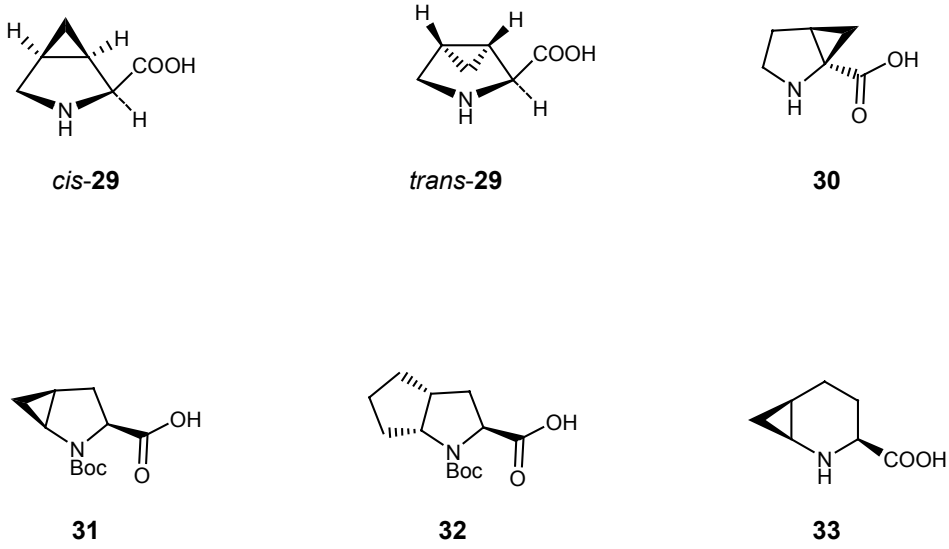


Figure 1.18. Monocyclic proline variants

Bicyclic cyclopropylpyrrolidine such as 3,4-methanoproline 29,[91] 2,3-methanoproline 30,[92,93] 4,5-methanoproline 31[94] as proline mimics were synthesized. In general, a N-acetyl-N'-methylamide containing 2,3-methanoproline 30 exhibits a somewhat greater preference for the cis conformation than does the prolineamide.[95] X-ray analysis showed considerable "flattening" of the pyrrolidine ring of 4,5-methanoproline 31 compared to N-Boc-L-proline[94] and that 31 adopted a cis orientation in the solid state. Other bicyclic

proline mimics 32 and 33 were also synthesized.[94] The replacement of L-proline by conformationally altered ring variants is expected to have important consequences in biological recognition, in cis-trans conformational changes, in the susceptibility of the secondary amide bonds to enzyme cleavage and related processes.

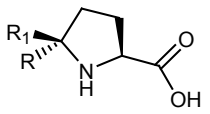


cis-**29** *trans*-**29** **30**

31 **32** **33**

Figure 1.19. Bicyclic proline variants

Steric effects on the trans-cis equilibrium of amides N-terminal to proline were explored with 5-tert-butylprolines by Lubell.[95] Enantio-pure 5-tert-butylprolines **34** were synthesized and can be introduced into a variety of peptides via standard coupling techniques.[96]



R = *tert*-butyl, R_1=H or
R_1 = *tert*-butyl, R=H

34 5-*tert*-Butylproline

(2*S*, 5*R*)-5-*tert*-Butylproline was introduced to an Ala-Pro dipeptide mimic **35**.[97] The relative populations of prolyl *cis*- and *trans*-amide isomers were measured in different solvents. Although the *trans*-Ala-Pro was favored, the *cis*-amide conformation is favored in the corresponding peptide mimic. The 5-*tert*-butylproline residues induce the mimic to adopt a type VIa β-turn conformation, which is independent of solvents.[97]

1.3. Summary

The newly discovered peptidyl-prolyl isomerase Pin1 is a very attractive novel target for potential cancer chemotherapeutics because of its unique role in mitosis signal transduction. It is suggested that Pin1 regulates mitosis by interaction with Cdc25, probably through catalysis of peptidyl-prolyl isomerization of Cdc25. Since conformationally rigid cis and trans prolyl peptide mimics may serve as functional probes for Pin1 PPIase activity, conformationally-constrained proline dipeptide mimics and alkene amide isosteres were reviewed above. Various kinds of mimics such as heterocyclic aromatic mimics, bicyclic lactam mimics, isosteres and "equilibrium-shifted mimics" have been designed and synthesized. Although syntheses of some cis or trans dipeptide alkene mimics were reported for other purposes, no proline alkene mimic had been synthesized stereospecifically or regioselectively prior to our work. The existing synthetic methods of alkene isosteres may help in the synthesis of cis and trans dipeptide proline mimics for Pin1 inhibition.

1.4. Proposed research outline

Pin1 may regulate Cdc25 in several ways: 1) as an enzyme to catalyze a conformational change of phosphoCdc25, 2) as a chaperone to protect a specific conformation of Cdc25 during activation, or 3) as a cofactor to stoichiometrically bind and regulate Cdc25 during mitosis. Through several designed experiments, peptides containing non-isomerizable *cis*- or *trans*-proline isosteres will eventually differentiate between these mechanisms and cast light on the active conformation of Cdc25, the substrate of Pin1. By understanding how Pin1 interacts with its targets and the PPIase enzymatic mechanism we will be able to design better mechanism-based inhibitors that may lead to anti-cancer drugs.

We proposed that Pin1 would bind either the *cis*- or *trans*-phosphoSer-Pro mimic more tightly, indicating the preferred conformer in vivo. Though the work of this thesis, we: 1) designed a general stereoselective route for synthesis of the *E*-alkene isostere of any Xaa-Pro, particularly *E*-alkene of Ser-Pro for Pin1 inhibition. 2) developped a Pin1 inhibition assay that is capable of measuring the steady state constants and determining the inhibition pattern (competitive, non-competitive, or uncompetitive), and 3) addressed the conformation specificity of Pin1 for its phosphoSer-Pro substrate. Specific steps to achieve our goals were:

1. To synthesize non-isomerizable phosphoSer-*cis*- and *trans*-Pro Pin1 substrate mimics.

As reviewed above in the section on conformationally constrained dipeptide mimics, various Xaa-Pro mimics have been synthesized, but none of these proline mimics are as

closely isosteric with the native peptides as the alkene mimics Boc-Ser-Ψ[(*Z*)CH=C]-L-Pro and Boc-Ser(OBn)-Ψ[(*E*)CH=C]-L-Pro.

The cis mimic synthesis was completed by Scott Hart in our group by a Still-Wittig rearrangement.[89] The remaining task was scaling-up the synthesis of the cis mimic to a larger amount for peptide synthesis, the synthesis of the exactly matched *trans*-Pro mimic, and the incorporation of these mimics into peptidomimetics.

AcFFpSPR-*p*NA is a good Pin1 substrate according to the research on sequence specificity of Pin1.[38] It was synthesized by phosphitylation and oxidation on resin in our group,[98] which are different from the previous method in that the Ser side chain was protected as the *tert*-butyl dimethyl silyl ether. Ser-Pro mimics, Fmoc-Ser-Ψ[(*Z*)CH=C]-Pro-OH and Fmoc-Ser-Ψ[(*E*)CH=C]-Pro-OH, were incorporated into peptide mimics according to the above solid phase synthesis method and to be assayed for Pin1 PPIase inhibition .

2. Measure Pin1 PPIase inhibition (K_i).

CyP, FKBP and Pin1 share the intriguing PPIase enzyme activity, catalyzing the cis-trans isomerization of Xaa-Pro amide bonds. The substrates used to measure PPIase activity are tetrapeptides with a C-terminal chromophore, *p*-nitroanilide. The chromophore is rapidly cleaved from the Xaa-trans-Pro conformation by the coupling enzyme, chymotrypsin, to release p-nitroaniline (λ_{max} 390nm) while the cis form is unreactive.[17,99,100] The assay of cis-trans specificity for Pin1 used Daniel Rich's method.[100] In this assay, steady-state conditions can be achieved, permitting measurement of the mode of inhibition: competitive, noncompetitive, uncompetitive or mixed. Although Ac-WFYpSPR-pNA is the best substrate of Pin1, we used the commercial

available substrate Suc-AEPF-*p*NA or synthetic AcFFpSPR-pNA as substrate for simplicity. We measured the separate kinetic parameters K_m for the substrate of Pin1 at high concentration of *cis*-Pro substrate where the steady state assumption is valid. Full inhibition constants for the (*Z*) and (*E*) Ac-FFpSΨ[CH=C]PR-OMe (or -NH_2, or -NMe) substrate analogs for Pin1 were measured and the mode of inhibition determined by Cleland's program.[101] The better inhibition can be considered to indicate the preference of Pin1 for cis or trans substrate.

Chapter 2. Synthesis of the Ser–*trans*–Pro isostere and Scaling up Synthesis of the Ser–*cis*–Pro isostere

2.1. Design of Ser–Pro mimic

Since we want to demonstrate the difference of the binding affinities for the two possible conformers of the Pin1 substrate, a pair of conformationally locked substrate analogues of Ser–Pro was designed based on the alkene amide bond isosteres. Sterically, the alkene bond is a good peptide bond surrogate because of the similar geometrical disposition of substituents attached to either of these functional groups. Previously, Scott A. Hart in our group conducted molecular modeling to compare the structure of the (*Z*)-alkene and the Ala–*cis*–Pro amide bond. A Monte Carlo conformational search and minimization by MacroModel v. 3.5a showed that the lowest energy structure of the dipeptide mimic Ac–AlaΨ[(*Z*)CH=C]–Pro–NHMe overlaid with the corresponding dipeptide with a root-mean-square deviation of 0.17 Å (Figure 2.1).[87] Although this modeling is performed with Ala–*cis*–Pro, little effect on this structure similarity is expected with any other amino acid proceeding proline. A peptidomimetic, **35**, containing Ala–*cis*–Pro isostere was successfully synthesized and inhibited the PPIase activity of cyclophilin with an IC_{50} of 6.5 μM, demonstrating that the simple alkene isosteres of prolyl amide bonds are good mimics and produce potent PPIase inhibitors.[88]

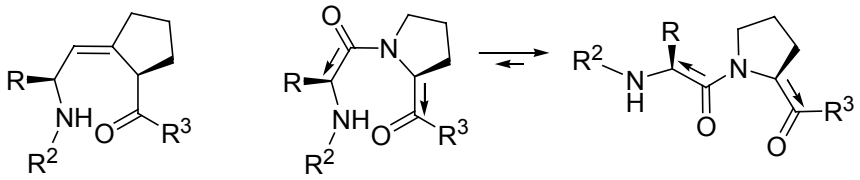
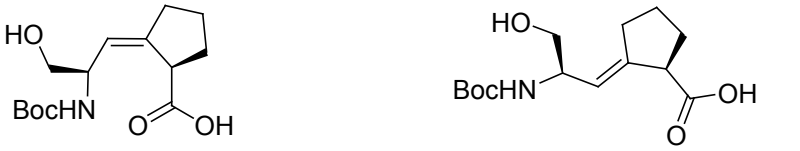


Figure 2.1. Design of the *cis*- and *trans*-Pro mimics

The significance of Pin1 in cell cycle regulation, as well as the previous success in the synthesis and bioactivities of the Ala–Pro (*Z*)-alkene isostere, encouraged us to synthesize the Ser-*cis*-Pro alkene isostere[89] and to develop a synthetic route for the synthesis of the corresponding Ser–*trans*–Pro alkene isostere (Figure 2.2).



Boc-Ser-Ψ[(*Z*)CH=C]-Pro-OH Boc-Ser-Ψ[(*E*)CH=C]-Pro-OH

Figure 2.2. (*Z*)- and (*E*)-alkene Ser-Pro isostere

As for the (*Z*)-alkene isosteres, we desired to design a general synthetic route to *trans*-Pro alkene isoteres that would allow any amino acid to precede proline in the trans dipeptide isosteres. Although our focus originally was to synthesize Pin1 substrate analogues, we expected to synthesize other *trans*-Pro isosteres for a variety of projects, because trans-Pro is also implicated in interesting protein structures, as in type II *β*-turns.[102] Because optically active amino acids are versatile synthons for stereoselective synthesis, starting with the optically pure amino acid N-terminal to Pro in the mimic not only provides the source for stereocontrol in the synthesis, but also imparts generality to the synthesis of any Xaa-Pro alkene mimic. The last requirement in the design of the synthetic route is the (*E*)-alkene selectivity. Generally, an intermolecular Wittig reaction would build the exocyclic carbon-carbon double bonds with less stereocontrol. An intramolecular rearrangement, however, goes through a stereocontrolled transition state, and thus forms the product stereoselectively. While a Claisen rearrangement always

favors the (*E*)-alkene due to steric hindrance, the Still-Wittig reaction may favor either the (*Z*) or the (*E*) alkene, depending upon the solvent, the temperature, and the steric properties of the molecule.[103]

N-Boc-*O*-benzyl-L-serine, used in Merrifield peptide synthesis,[104] was chosen as the starting material for the syntheses of both Ser-*cis*-Pro and Ser-*trans*-Pro mimics. Because Ser is so highly functionalized, significant challenges and side reactions were encountered during the synthesis of these particular Pro mimics. The Ser–*cis*–Pro alkene isostere was successfully synthesized by Scott A. Hart using the Still-Wittig route to construct the (*Z*)-alkene mimic of Ala-*cis*-Pro.[89] The scale-up of this synthesis will be reported herein along with the synthesis of the Ser-trans-Pro alkene isostere.

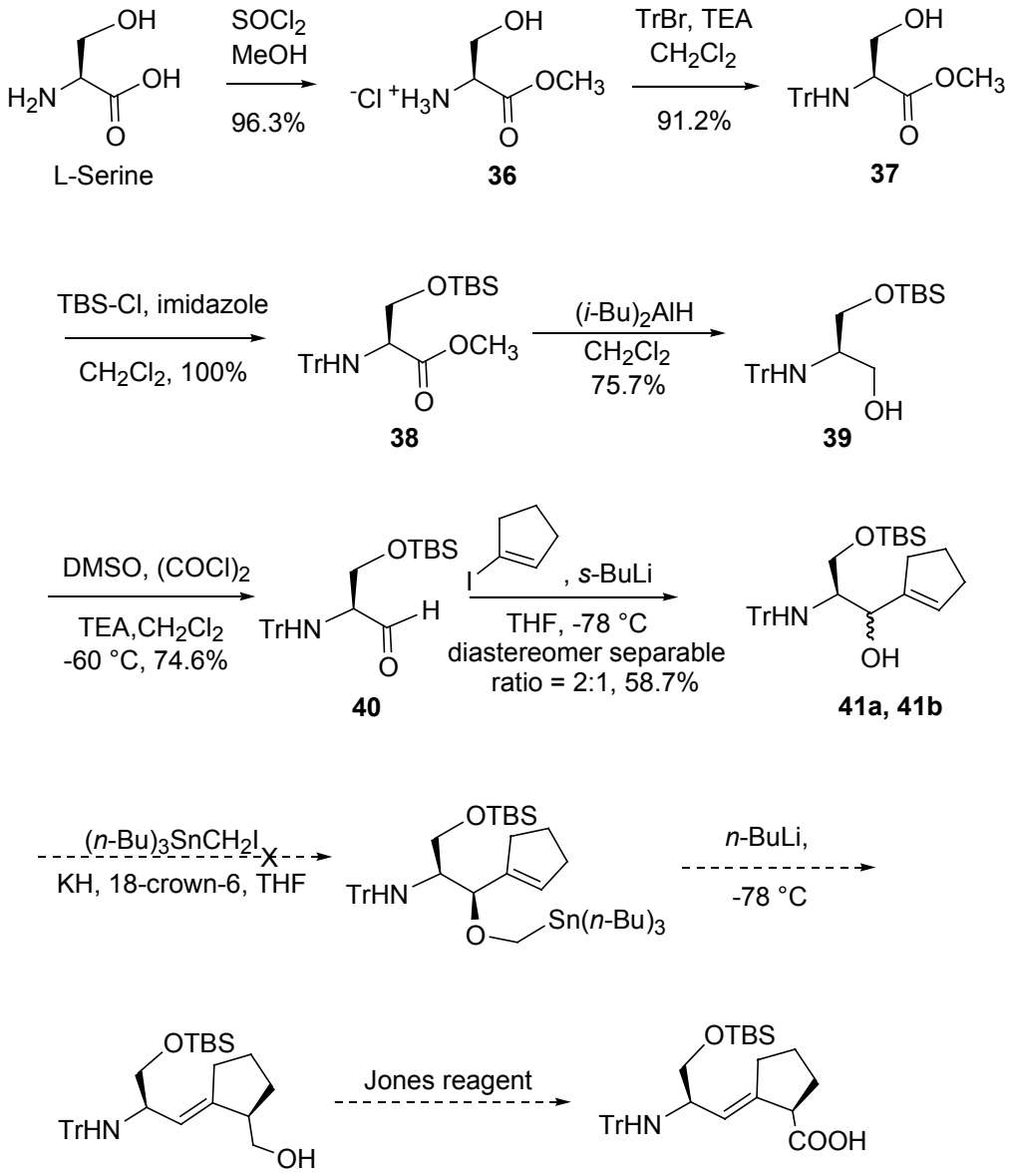
2.2. Initial attempts using a Still-Wittig route to the (*E*)-Alkene Ser–*trans*–Pro isostere

To synthesize the Ser-*trans*-Pro isostere in a form suitable for peptide synthesis, the first route attempted utilized a Still-Wittig reaction as a key step. The newly discovered solvent-dependent stereoselectivity of the Still-Wittig rearrangement of the Ser-Pro mimic precursor[103] would have enabled us to make (*E*)-alkene mimic. The *N*-trityl, *O*-*tert*-butyldimethylsilyl (TBS) protected isostere Trt–Ser(TBS)–Ψ[(*E*)CH=C]–Pro–OH was chosen as the synthetic target for the following two reasons: 1) the TBS group was desired for peptide synthesis to prevent formation of branched peptides, and 2) the trityl group is stable under basic and nucleophilic reaction conditions in the proposed route and can be easily switched to Fmoc for peptide synthesis.

The synthetic route is shown in Scheme 2.1. Beginning with L-Serine, Fisher esterification followed by trityl protection using trityl bromide gave 88% yield (in two

steps) of the crystalline trityl serine methyl ester **37**, which in turn was protected on the serine side chain with the TBS group. Usually, partial reduction of an ester to an aldehyde by hydrides has problems with over reduction to afford the primary alcohol, and usually requires carefully controlled, low temperature addition of one equivalent hydride to an appropriate ester. Although the bulky reducing reagent DIBAL is known as one of the reagents for partial reduction of esters,[105,106] the reaction of one equivalent DIBAL with the methyl ester **38** in toluene at –78 °C gave a mixture of the alcohol, the aldehyde, and the starting material. Reduction using 2.1 equivalent DIBAL gave a mixture of the corresponding alcohol (75.7%) and the aldehyde (19.7%), which were easily separated by flash chromatography. Subsequent oxidation of the alcohol by Swern oxidation[107] gave clean aldehyde **40**. Trityl protected amino aldehydes are known to have excellent stereochemical stability under these conditions due to steric shielding of the α-proton of the amino acid.[108] Alcohol **41** was synthesized by condensation of cyclopentenyl lithium with aldehyde **40**. As expected, the trityl protected α-amino aldehyde **40** did not give the Felkin-Ahn product selectively, but a pair of diastereomers, whose ratio was approximately 1:2. This was because the chelation controlled transition state and the Felkin-Ahn transition state both contributed to this reaction. Due to the formation of byproducts, the best yield observed for this reaction was 58.7% (21.1% and 37.6% for each of the diastereomers). The major byproduct was characterized by ^{1}H NMR, ^{13}C NMR, HMQC, and low resolution MS as the pair of diols in which TBS protection was removed.

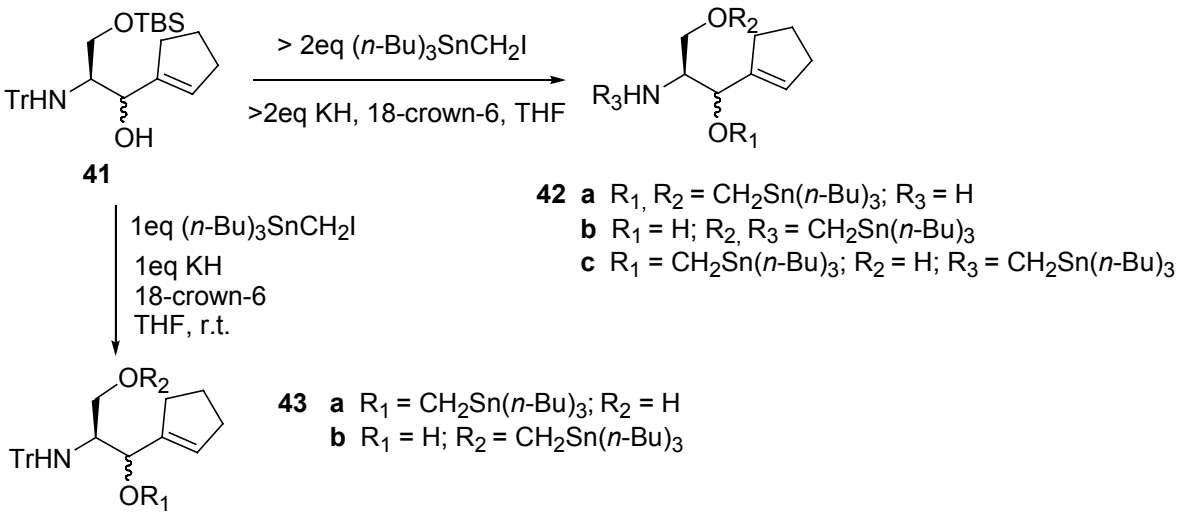
Scheme 2.1. Proposed Still-Wittig route to the (*E*)-Alkene Ser-*trans*-Pro isostere



Without proving the stereochemistry, **41a** and **41b** were submitted to the stannylation reaction. The reaction, however, failed to give the Still-Wittig rearrangement precursor. When about 3 equivalent KH and 3 equivalent $ICH_2Sn(n\text{-}Bu)_3$ were used for the reaction, both trityl and TBS group were removed to give exclusively a product containing two $-CH_2Sn(n\text{-}Bu)_3$ groups, which were characterized by ^1H NMR, ^{13}C NMR, HMQC and low resolution MS. The product could be **42a**, **42b** or **42c**. The yield of **42** from **41a** and **41b**

was 82% and 79.5%, respectively (Scheme 2.2). Trityl alcohol was also isolated in this reaction and recognized by MS and ^{1}H NMR. When exactly one equivalent KH and one equivalent $ICH_2Sn(n\text{-}Bu)_3$ were used for the reaction, TBS was removed and the structural isomers **43a** and **43b** were obtained, each containing one stannane group.

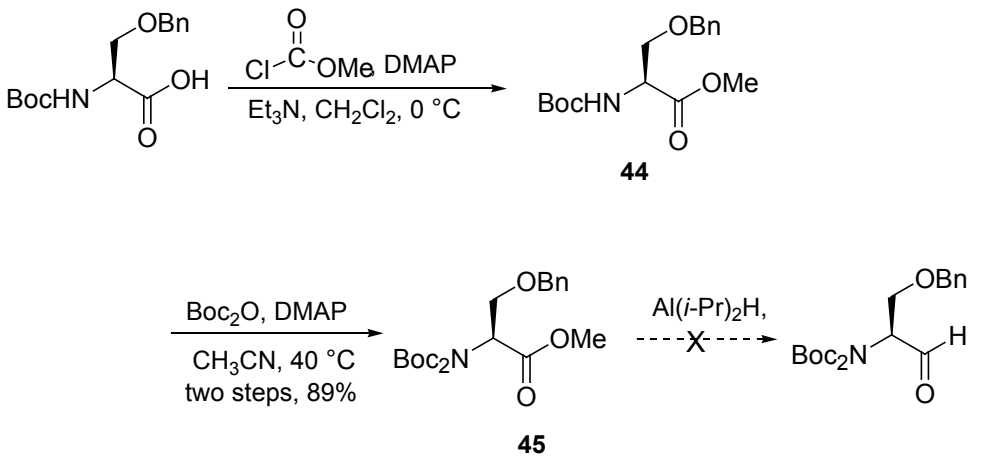
Scheme 2.2. Stannylation



Both trityl and TBS protection group clearly were not stable enough to go through all the reaction conditions in this route. Indeed, migration of the TBS protecting group in alcohols is a known problem under strong basic conditions,[109] though it was unknown if it was the migration that caused the instability of TBS in our reaction. The instability of trityl was not only observed in the reaction from **41** to stannane **42**, but also in the storage of alcohol **41.** It decomposed quantitatively to give trityl alcohol within one month at – 20 °C and within two days at room temperature. In the literature, the trityl protecting group was not a commonly used protecting group for an amine for a long synthetic route.

Trityl and TBS protection were abandoned completely then. We also reconsidered the stereochemical outcome in the condensation step with cyclopentenyl lithium. Hart's work showed the successful synthesis of the tribenzyl protected Ser–*trans*–Pro alkene mimic. Since Hart could not remove the first benzyl from the amine, which was critical for removing the second benzyl to put Fmoc on, we decided to attempt diBoc or BocBn diprotection for the amine.

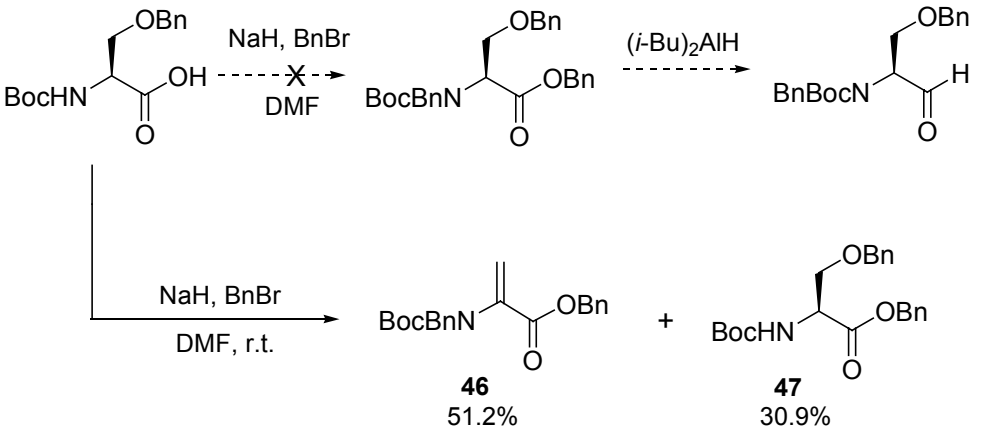
Scheme 2.3. DiBoc protection



N-Boc-*O*-benzyl-serine was easily methylated under basic conditions (Et_3N) with methyl chloroformate[110] and the second Boc was put on by Grehn's method[111] with a two-step yield of 89%. Our first attempt to partially reduce the methyl ester **45** to the aldehyde utilized $(i\text{-Pr})_2AlH$ in THF. THF was chosen as the solvent because we expected that the chelation property of THF to $(i\text{-Pr})_2AlH$ would reduce the activity of $(i\text{-Pr})_2AlH$ and improve its selectivity to give good yield of the partially reduced product, the aldehyde, instead of the alcohol. But no reaction occurred until the temperature was

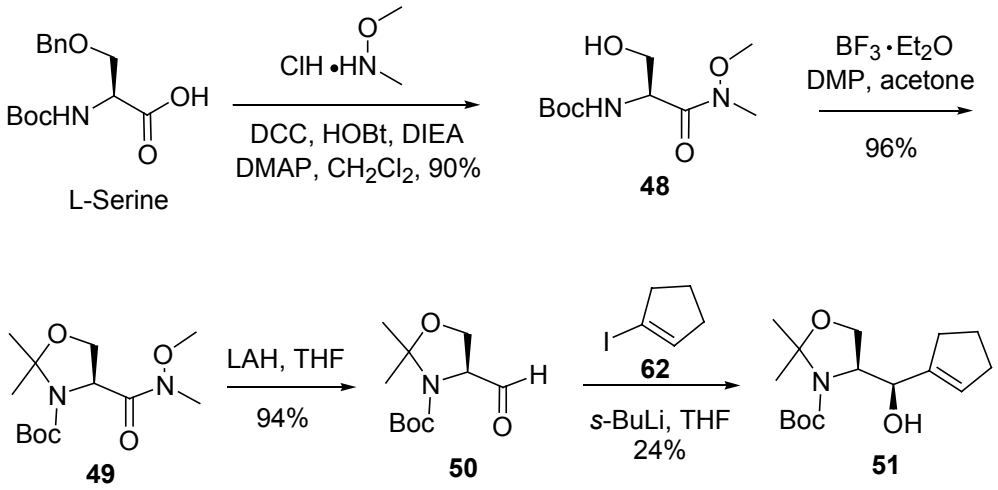
raised to room temparature and then one Boc was removed. The solvent toluene produced no product either. It was suspected that the carbonyl was too hindered by the α-(*N*,*N*-diBoc) group to be reduced.

Scheme 2.4. BnBoc protection



We tried to put benzyl on Boc protected amino acid by using NaH as the base and DMF as the solvent,[112-114] but only the β-elimination product and the *N*-Boc benzyl ester were isolated, indicating that this method for *N*-benzyl protection was not practical. Although methods of synthesizing optically pure *N*-Boc,Bn α-amino aldehyde exist[115] in literature, we decided that it was a good time to investigate a completely different route shown in Scheme 2.6, the Ireland-Claisen rearrangement route. The *E*-alkene was not favorable in Still-Wittig rearrangement for *N*-diprotected precursor.[98]

Scheme 2.5. Protection using a cyclic link through an acetonide



Another diprotection route (Scheme 2.5) was tried utilizing a cyclic link between the amino and the side chain hydroxyl through an acetonide. The aldehyde **50** was synthesized according to Campbell's method [16]. The addition of cyclopentenyl lithium was successful from the integration of the peaks in ^{1}H NMR, but the ^{1}H NMR was complex due to Boc rotamers at room temperature. Although the alcohol **51** can be used for the Still-Wittig rearrangement as well as the Ireland-Claisen rearrangement, this route was abandoned because of the success of the route shown in Scheme 2.6.

2.3. Ireland-Claisen rearrangement route to (*E*)-alkene Ser–*trans*–Pro isostere

The Ireland-Claisen rearrangement goes through a six-membered ring transition state to produce an (*E*)-alkene product regardless of the stereochemistry of the precursor. The (*R*) stereochemistry of the allylic carbon in the precursor, however, dictates the stereochemistry of the allylic carbon in the product. The key step in the Ireland-Claisen rearrangement in the synthesis of the Ser–*trans*–Pro isostere is shown in Figure 2.3. In our system, the bulky group CH(NHBoc)(CH_2OBn) took the equatorial position and the small group H took the axial position in the transition state, this ensured an (*R*)

configuration of the allylic carbon in the product. The stereochemistry of the carbon alpha to the carboxylic acid was not controlled (shown in Figure 2.3 as *R* or *S*).[116]

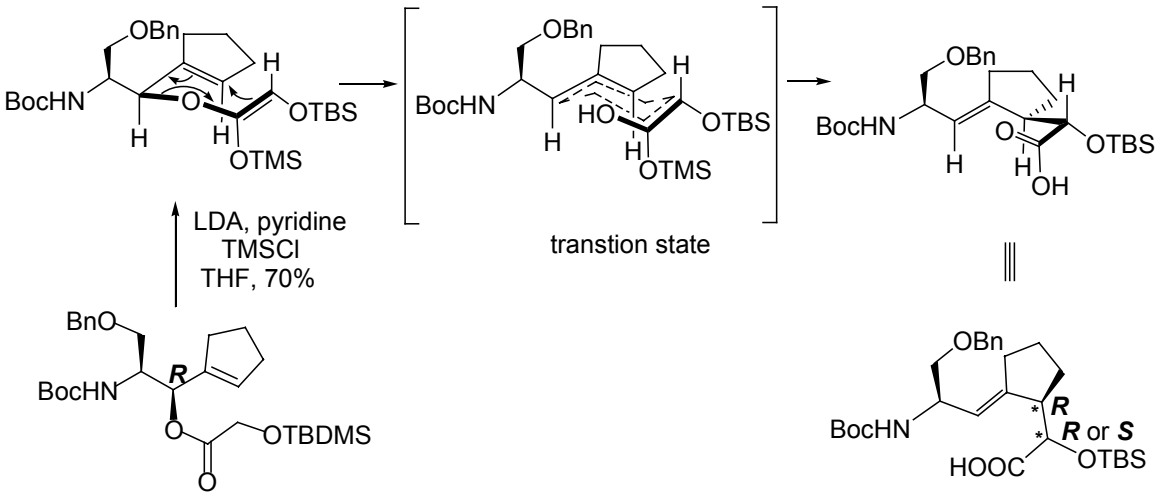


Figure 2.3. Transition state of the Ireland-Claisen rearrangement.

A major advantage of the Ireland-Claisen route over the previous Still-Wittig route is that a pair of (*Z*) and (*E*) alkene is almost certainly to be formed in the Still-Witting route, while (*E*)-alkene would be produced exclusively via the Ireland-Claisen route. In order to synthesize the allylic alcohol precursor mainly in the *R* configuration, a stereoselective reduction of a ketone was necessary. It was expected that the chiral induction could be achieved through the reduction of a optically pure α-amino ketone, derived from the natural amino acid, following either the Felkin-Ahn rule (without chelation) or Cram rule (with chelation) with different amine protecting groups (Figure 2.4).

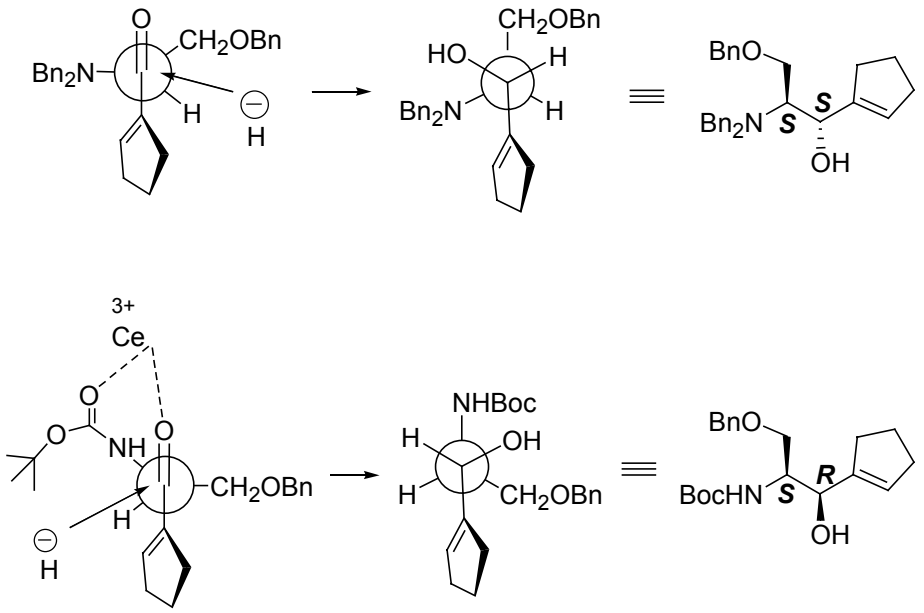
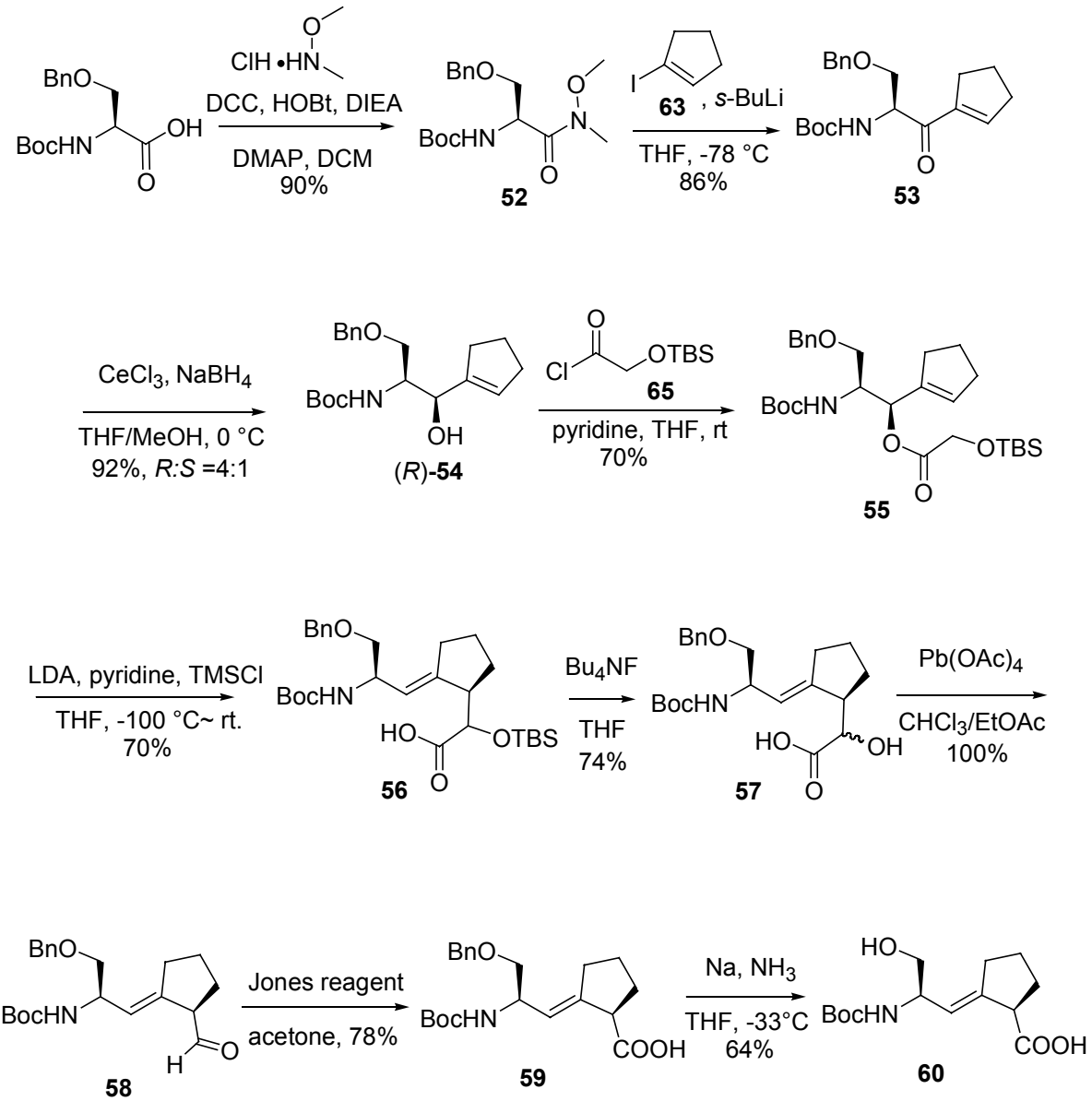


Figure 2.4. Felkin-Ahn reduction (top) vs. chelation controlled reduction (bottom)

The Ireland-Claisen rearrangement proved to be very successful at producing the desired (*E*)-alkene, both in stereoselectivity and in yield (Scheme 2.6). Weinreb amide[117] **52** was prepared easily from *N*-Boc-*O*-benzyl-L-serine by a coupling with *N*,*O*-dimethyl hydroxylamine hydrochloride with 90% yield.[118] Condensation of **52** with cyclopentenyl lithium derived from iodide **62** gave the desired ketone **53** in 86% yield. The reaction was difficult to bring to completion, even with excess cyclopentenyl lithium, probably due to deprotonation of the carbamate. The yield was increased to 86% by adding three equivalents of cyclopentenyl lithium in portions.

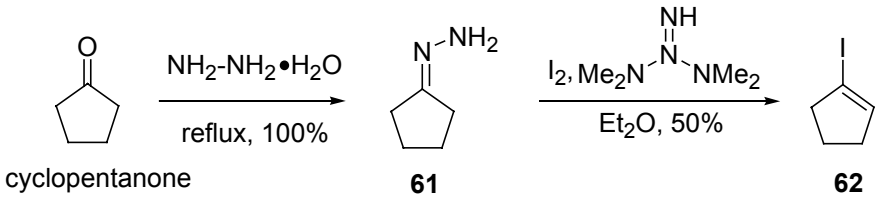
Scheme 2.6. Synthesis of Boc–Ser–Ψ[(*E*)CH=C]Pro–OH by Ireland-Claisen rearrangement.



The chelation-controlled Luche reduction[119] of ketone **53** gave a 4:1 mixture of diastereomers in good yield (92%). The minor diastereomer was removed by precipitation. The major diastereomer of **54** proved to be the desired (*S*,*R*)* by derivatization as the oxazolidinones.[98,120,121] The reagent 1-iodo-cyclopentene[122] **62** was

prepared most easily by the method of Barton[123] in two steps with 50% overall yield from cyclopentanone.

Scheme 2.7. Synthesis of reagent cyclopentenyl iodine



Alcohol **54** was readily transformed to the Ireland-Claisen precursor ester **55**[124] by the reaction with *t*-butyldimethylsilyloxyacetyl chloride, prepared according to the published

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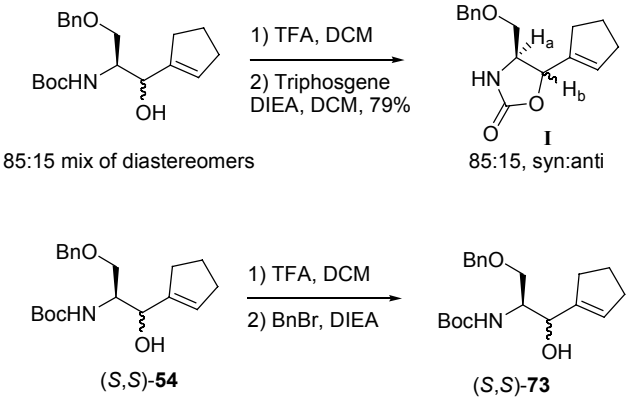
* Stereochemistry of **54** and **73**: The method of Mosher et al.[119] was used to identify the stereochemistry of the allylic alcohol **54** (Scheme 2.16). The original (*S*)-*N*-Boc-*O*-benzyl serine stereocenter was assumed to be stable. The absolute stereochemistry of alcohol **73** was demonstrated by derivatization of an 85:15 mixture of diastereomers of **54** from the Luche reduction (Scheme 2.6), as the oxazolidinones **I**. The major diastereomer was identified as the syn isomer (*S*,*R*)-**54** (oxazolidinone **I** $^3J_{HaHb}$ = 8.0 Hz). The stereochemistry of **73** proved to be the desired (S,S) by derivatizing the minor diastereomer from the Luche reduction, (S,S)-15 (oxazolidinone **I** $^3J_{HaHb}$ = 5.4 Hz), as the tribenzyl protected alcohol **73**, whose ^{1}H NMR matched exactly the tribenzyl alcohol **73** synthesized from ketone **72**.



procedure.[125] The Ireland-Claisen rearrangement of ester **55** was the key step in our synthesis of the Ser-*trans*-Pro mimic (Scheme 2.6). The standard Ireland-Claisen procedure[124,126] was not successful and only starting material was recovered. Activation of TMSCl by pyridine was necessary.[116] The intermediate TBS-protected alcohol was unstable towards silica gel, but subsequent removal of the TBS protecting group by *t*-butyl ammonium fluoride (TBAF) in THF gave the α–hydroxy acid **57** as a stable product. The crude ^{1}H NMR of **57** showed three minor diastereomers in addition to the major isomer, but the stereochemistry at the alcohol center is eliminated by oxidation in the next step. After oxidation, the major diastereomer of **59** was isolated readily by chromatography. The NOESY spectrum of **59** showed the (*E*)-alkene as the only product of the rearrangement (Figure 2.5).

The remaining task then was to degrade one carbon and to oxidize the resulting product to the desired acid without affecting the exocyclic alkene. Initial attempts using the Jones oxidation[127] to oxidatively decarboxylate the α-hydroxy acid **57** and oxidize the resulting aldehyde in one step afforded only small amounts of the desired acid **59** (10% yield). The major product identified was the α,β–unsaturated ketone, probably resulting from fast allylic oxidation and subsequent C-C bond cleavage of the 1,2-diol (Scheme 2.8). This ketone was directly analogous to the ketone side product from Jones oxidation of the doubly protected *N*-Boc, *N*-benzyl (*Z*)-alkene **77** (Scheme 2.8). The (*Z*) ketone product was minimized by adding an excess of the Jones reagent to the alcohol and keeping the reaction at 0 °C.

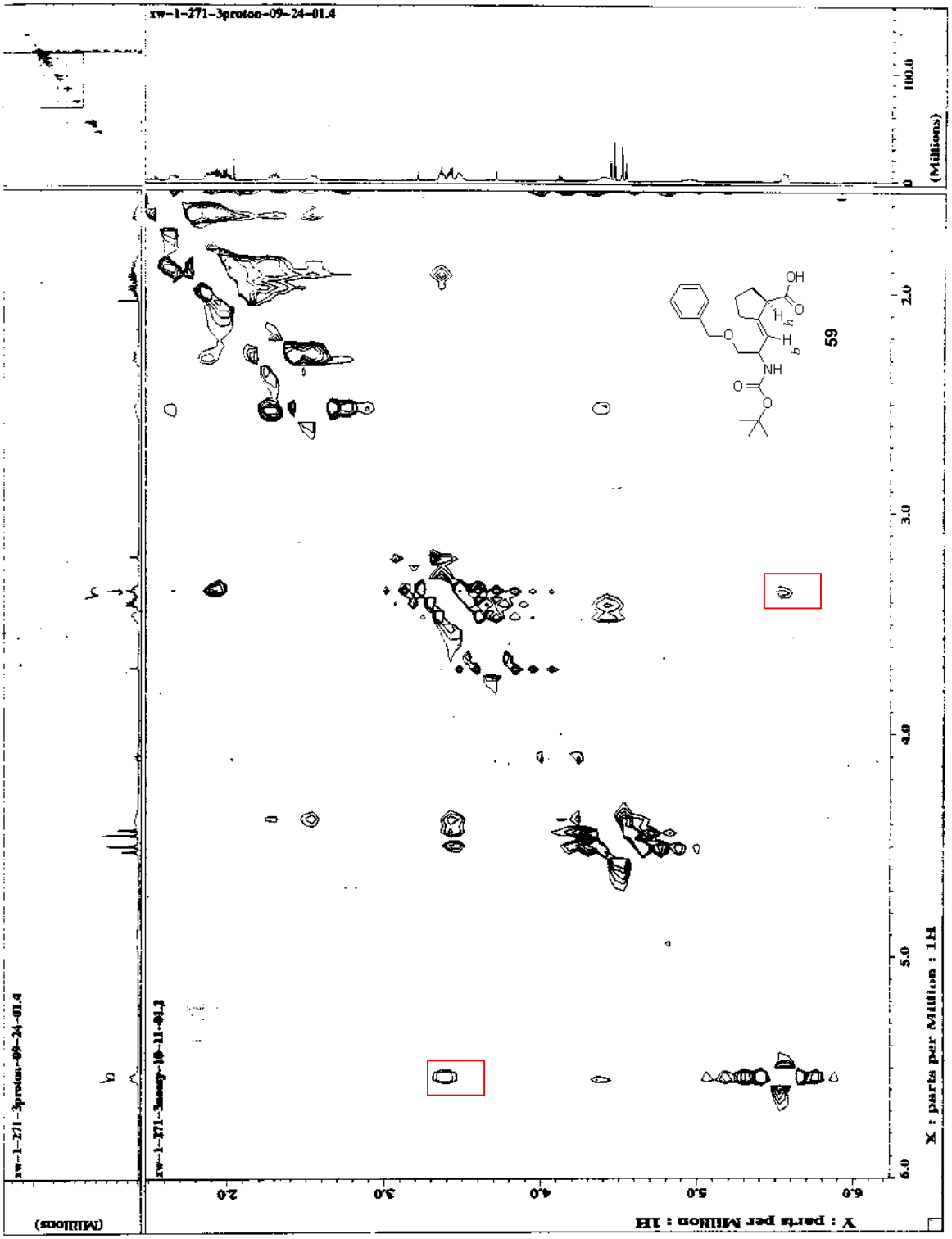
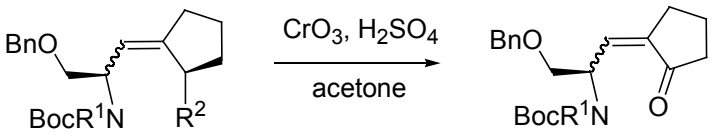


Figure 2.5. NOESY spectrum of acid **59**. Crosspeak b↔h is boxed.

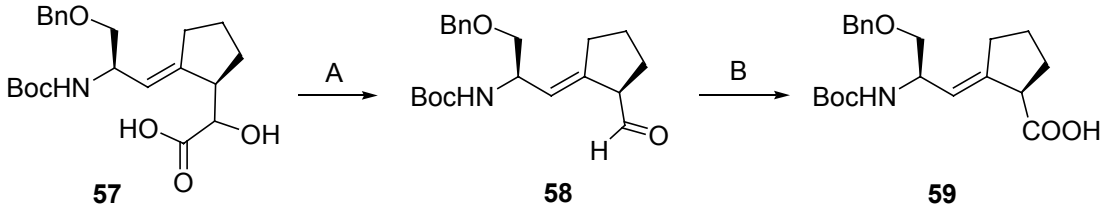
Scheme 2.8. Ketone Side Product from Jones Oxidation



57 R^1 = H, R^2 = CH(OH)COOH, (*E*)
77 R^1 = Bn, R^2 = CH_2OH, (*Z*)

Many reagents of oxidative cleavage of α–hydroxy acids exist in the literature, such as sodium periodate,[128] tetrabutyl ammonium periodate,[129] and lead (IV) tetraacetate,[130] but all cases were to obtain acids conjugated with either a aromatic ring or an α,β-alkene. The conjugation could be a driving force for those reactions, which raised the concern whether the oxidation would proceed smoothly for the non-conjugated acid **57**, as well as whether the oxidation would affect the exocyclic, β,γ-unsaturated alkene. For decarboxylation of α–hydroxy acid **57**, Lead (IV) tetraacetate was the only reagent that cleanly gave high yields of the β,γ-unsaturated aldehyde. The results are summarized in Table 2.1.

Scheme 2.9. Oxidation of α–hydroxy acid **57**



57 **58** **59**

Table 2.1. Reaction A (cleavage of α–hydroxy acid **57**)

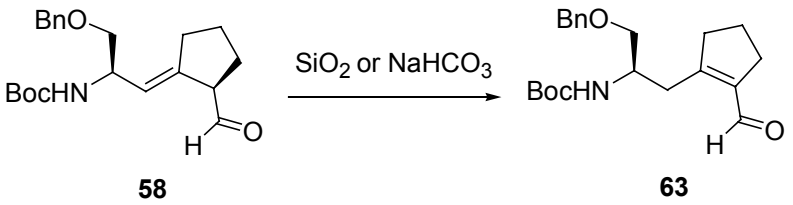
Run	Reagent	Solvent	Product	Yield
1	$NaIO_4$	THF:H_2O (3:2)	No reaction	--
2	$NaIO_4$	Acetone:AcOH:H_2O (4:2:1)	No reaction	--
3	Bu_4NIO_4	$CHCl_3$	Complicated poducts	--
4	$Pb(OAc)_4$	EtOAc/$CHCl_3$	Aldehyde **58**	100%

Table 2.2. Reaction B (oxidation of **58**)

Run	Reagent	Solvent	Product	Yield
1	Jones	Acetone	Acid **59**	78%
2	$NaClO_2$/NaH_2PO_4/ 2-methyl butene	H_2O/*t*-BuOH	No desired product	--

Sodium periodate has been used for oxidative cleavage of diols,[131,132] α-hydroxy ketones,[133] and α–hydroxy carboxylic acids.[128] It gave high yields and clean ketones or aldehydes as products for cleavage of aromatic α–hydroxy acids in the presence of phase transfer reagents.[134] In our case, sodium periodate gave no reaction at room temperature and gave complicated products when the reaction was heated to 60 °C. It was suspected that the low solubility of sodium periodate in organic layer made the reaction difficult. The solubility of quaternary ammonium salts in many organic solvents provided advantages in terms of high reaction rates, low reaction temperatures, and an absence of side reactions. Tetrabutyl ammonium periodate allowed homogeneous reaction in non-aqueous solvents and usually gave a better result than sodium periodate.[129,135,136] While in our case, as in the heated reaction with sodium periodate, butyl ammonium periodate gave complicated products. Lead (IV) tetraacetate,[130] however, gave β,γ-unsaturated aldehyde **58,** quantitatively.

Scheme 2.10. Isomerization of the β, γ-unsaturated aldehyde **58**.



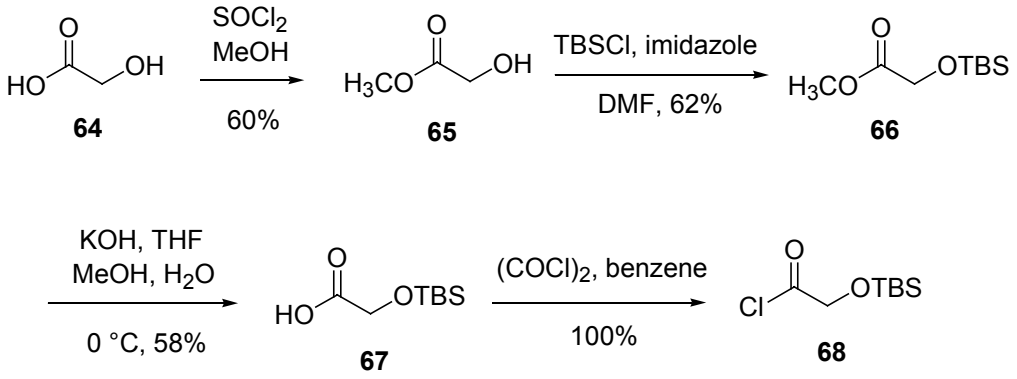
58 **63**

The β,γ-unsaturated aldehyde was oxidized further without purification. Isomerization of the β,γ-unsaturated aldehyde to the more stable α,β-unsaturated aldehyde occurred readily during basic work-up (aqueous $NaHCO_3$) or silica gel purification, so the aldehyde was handled as little as possible. Jones oxidation of the aldehyde yielded the corresponding β,γ-unsaturated carboxylic acid **59**, without loss of the acid sensitive Boc group. As an alternative to the Jones oxidation, sodium chlorite led only to decomposition of the aldehyde.[137] The ketone side product from allylic oxidation was not observed in this oxidation of the aldehyde. The β,γ-unsaturated acid **59** is stable towards isomerization under aqueous acidic or basic conditions. A single diastereomer of **59** was obtained after chromatography. The (*E*)-alkene stereochemistry of **59** was demonstrated by NOESY (Figure 2.5). The benzyl protection on oxygen was successfully removed with Na/NH_3 to give the (*E*)-alkene Boc–Ser–Ψ[(*E*)CH=C]Pro–OH **60** in a form suitable for peptide synthesis using Boc chemistry.

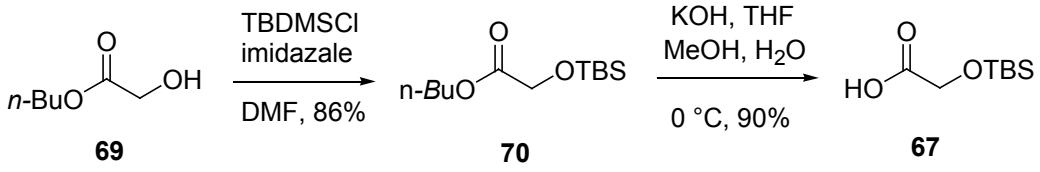
The reagent *tert*-butyldimethylsilyloxyacetyl chloride, used in the Ireland-Claisen rearrangement, was synthesized via two synthetic routes, as illustrated in Scheme 2.11 and Scheme 2.12. Initially the synthesis started with glycolic acid **64**. The carboxylic acid was protected with methyl using Fisher esterification. Then the alcohol was protected by the *tert*-butyldimethylsilyl ether on the free hydroxyl group, followed by the hydrolysis to give *tert*-butyldimethylsilyloxyacetic acid **67**, which in turn reacted with oxalyl chloride

in benzene to form the acid chloride **68** (Scheme 2.11). An attempt to directly protect the hydroxyl group of **64** with TBS in the presence of the carboxylic acid produced a mixture of the TBS ether, the TBS ester, and the fully protected product. Those three products were difficult to separate. A recently published procedure using commercially available butyl glycolic ester yielded a better result (Scheme 2.12).[125]

Scheme 2.11. Synthesis of reagent *tert*-butyldimethylsilyloxyacetyl chloride



Scheme 2.12. An efficient route to *tert*-butyldimethylsilyloxyacetyl chloride



2.4. Scaling up the synthesis of the Ser-cis-Pro alkene isostere

The synthesis of the cis-Pro isostere utilized a [2,3]-sigmatropic Still-Wittig rearrangement as the key step. The possible transition states of the Still-Wittin rearrangement are shown in Figure 2.6. When the substituent R' is bulky, it prefers the

pseudoaxial position, minimizing the steric interactions with the vinylic substituent R. With the R' group taking the axial position in the transition state, the product formed is the (*Z*)-alkene.

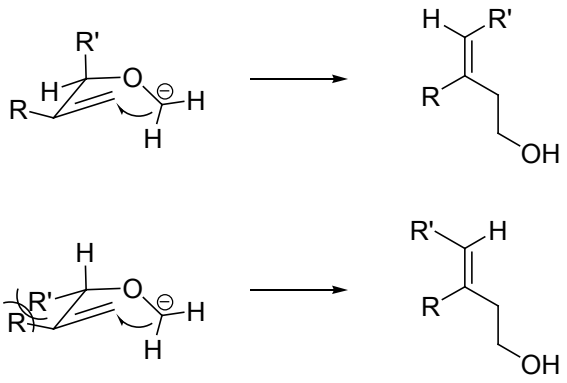
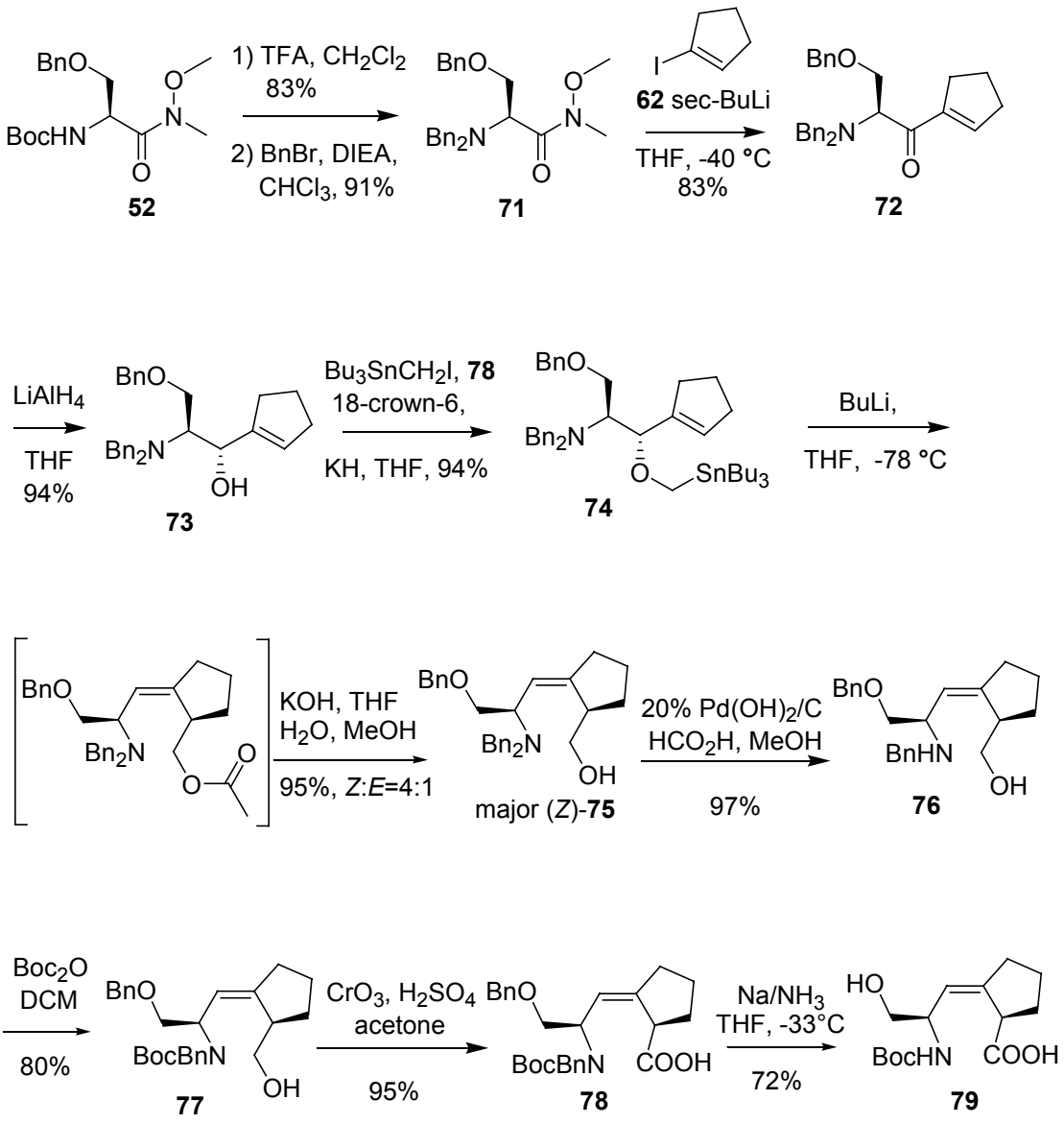


Figure 2.6. Possible transition states of the Still-Wittig rearrangement. Favored transition state is shown (top) along with the less favored transition state (bottom).

Starting from the Weinreb amide **52**, the Boc protection was removed by 25% TFA in dichloromethane to give the free amine, which could be easily purified by flash chromatography because of its high polarity. Condensation of the Weinreb amide **71** with cyclopentenelithium generated in situ stops at the ketone stage. Reduction of ketone **72** with 1.2 equivalents of lithium aluminum hydride went though a Felkin-Ahn transition state to give a single diastereomer (*S*, *S*)-alcohol **73**, as demonstrated by ^{1}H NMR of the crude product.[121] The iodomethyltributyltin reagent was prepared by the method of Steiz et al (Scheme 2.14).[138] The intermediate chloromethyltributyltin **80** was obtained by addition of methanesulfonyl chloride to tributylstannylmethoxide resulting from addition reaction of tributylstannane with lithium diisopropylamide and paraformaldehyde. The halogen exchange reaction of **80** with sodium iodide yields 90% of the reagent iodomethyltributyltin **81**. The purity of the tributyltin methyliodide affectted the yield of

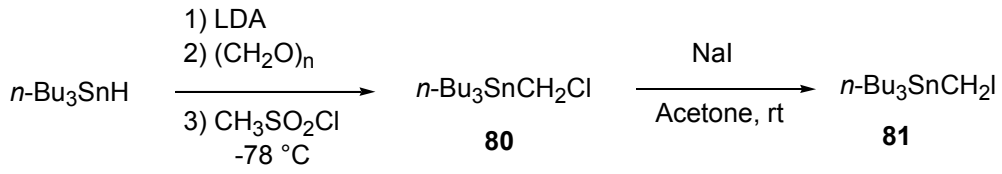
the tributylstannylmethyl ether **74** dramatically. A impure tin reagent resulted in either no reaction or decreased reaction yields of 35-70%, while over 90% yield was guaranteed when using pure tin reagent.

Scheme 2.13. Synthesis of Boc–Ser–Ψ[(*Z*)CH=C]–Pro–OH by Still-Wittig Rearrangement



The stannine ether intermediate **74** was formed by a substitution reaction in 94% yield. The Still-Witting rearrangement of **74** gave different ratios of the (*Z*)-alkene to (*E*)-alkene under different reaction conditions such as the reaction temperature and the amount and concentration of the base, *n*-BuLi. The (*Z*)-alkene to (*E*)-alkene ratio was 4:1 (76% yield vs. 19% yield), as indicated by integration of the ^{1}H NMR of the crude acetate product of (*Z*)**-75** and (*E*)**-75**. This ratio matches exactly the calculated ratio at 3-21G* level using *ab initio* calculations.[103]

Scheme 2.14. Synthesis of the iodomethyltributyltin reagent

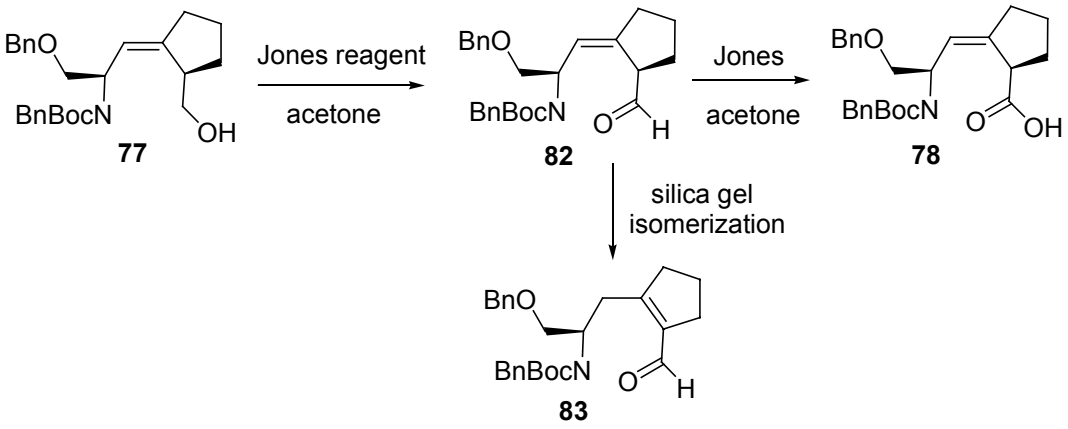


With (*Z*)**-76** in hand, monodebenzylation by catalytic transfer hydrogenation with formic acid on Pearlman's catalyst went smoothly without affecting the benzyl ether or the alkene bond. After reprotection of the amine using di-*tert*-butyl dicarbonate, Jones oxidation of the doubly protected Boc-benzylamine **77** produced acid **78** in 95% yield. An excess of the Jones reagent were added to the reaction to avoid the formation of a ketone byproduct. Finally, Birch reduction removed both benzyl protective group on the amine and on the hydroxyl to yield Boc–Ser–Ψ[(*Z*)CH=C]–Pro–OH **79**.

When performing a 6.8 g scale Jones oxidation with half an hour reaction time, the reaction unexpectedly generated 60% of the aldehyde **82** and 37% of the acid **78** (Scheme 2.15). Similar to the corresponding trans aldehyde, the aldehyde **82** underwent

isomerization upon silica gel purification to form the α,β-unsaturated aldehyde **83**, at rate much slower than the trans aldehyde. Aldehyde **82** could be resubmitted to Jones oxidation to produce the desired acid **78**. This showed the Jones oxidation of the alcohol **77** went through an aldehyde intermediate and the reaction could be stopped at the aldehyde stage.

Scheme 2.15. Formation of an aldehyde intermediate



2.5. Conclusions

Two amide isosteres of Ser-*trans*-Pro and Ser-*cis*-Pro dipeptides were designed and stereoselectively synthesized with high yields to be incorporated into peptide inhibitors of the phosphorylation-dependent peptidyl prolyl isomerase Pin1, an essential regulator of the cell cycle. The conformationally locked Ser–*trans*–Pro mimic, Boc–SerΨ[(*E*)CH=C]Pro–OH, was synthesized through the use of an Ireland-Claisen [3,3]-sigmatropic rearrangement in nine steps with 13% overall yield from the natural amino acid serine. The Ser-*cis*-Pro mimic, Boc–SerΨ[(*Z*)CH=C]Pro–OH, was synthesized through the use of an Still-Wittig [2,3]-sigmatropic rearrangement in 11 steps with an overall yield of 20% from the same starting material. The synthesis of the Ser-*cis*-Pro

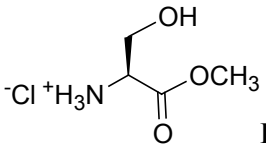
mimic, Boc–SerΨ[(*Z*)CH=C]Pro–OH, was scaled up to obtain grams of the mimic for the synthesis of peptidomimetics (Chapter 3), as well as for the construction of a non-peptidic (*Z*)-alkene library for Pin1 inhibition (Chapter 5). Both iosteres were synthesized in a properly protected form that can be manipulated for the peptide synthesis.

The Ireland-Claisen rearrangement route was high yielding and highly stereoselective and served as a general synthetic route for constructing any Xaa–*trans*–Pro isostere starting from the corresponding optically pure amino acid (Xaa). The generality of this route has been demonstrated in the synthesis of a Gly–*trans*–Pro isotere (Chapter 6).

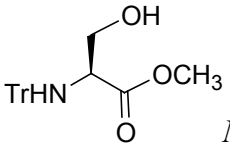
Experimental

General. Unless otherwise indicated, all reactions were carried out under N_2 in flame-dried glassware. THF, toluene, and CH_2Cl_2 were dried by passage through alumina. Anhydrous (99.8%) DMF was purchased from Aldrich and used directly from SureSeal^{TM} bottles. Dimethyl sulfoxide (DMSO) was anhydrous and dried with 4Å molecular sieves. Triethylamine (TEA) was distilled from CaH_2 and $(COCl)_2$ was distilled before use each time. Diisopropylethylamine (DIEA) was distilled from CaH_2 under a N_2 atmosphere. Brine (NaCl), $NaHCO_3$ and NH_4Cl refer to saturated aqueous solutions. Flash chromatography was performed on 32-63 μm or 230-400 mesh, ASTM, EM Science silica gel with reagent grade solvents. Melting points were determined with a Thomas Hoover Capillary Melting Point Apparatus and were uncorrected. NMR spectra were obtained at ambient temperature in $CDCl_3$ unless otherwise noted. Proton (500 MHz) and carbon-13 (125 MHz) NMR spectra were measured on a JEOL NMR

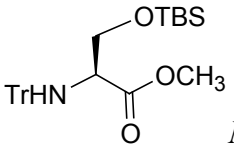
spectrometer. ^{1}H NMR spectra are reported as chemical shift (multiplicity, coupling constant in Hz, number of protons).



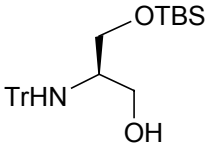
L-Serine methyl ester hydrochloride (36). To 150 mL of MeOH at 0 °C was added $SOCl_2$ (21 mL, 300 mmol) over 5 min, followed by L-serine (10.5 g, 100 mmol). After stirring for 24 h at rt, the solvent was removed and recrystallization of the residue from MeOH/Et_2O gave 14.97 g (yield 96.3%) white crystals. m.p. 160 °C (decomposed). ^{1}H NMR (DMSO-d_6) δ 8.59 (s, 3H), 5.63 (t, 1H), 4.09 (t, 1H), 3.82 (d, 2H), 3.74 (s, 3H). ^{13}C NMR (DMSO-d_6) δ 169.0, 60.0, 54.9, 53.3.



***N*-Triphenylmethyl-L-serine methyl ester (37).** L-Serine methyl ester hydrochloride **36** (6.22 g, 40 mmol) was dissolved in CH_2Cl_2 (250 mL) containing TEA (8.08 g, 11.13 mL, 80 mmol). Triphenylmethyl bromide (12.92 g, 40 mmol) was added and the reaction was allowed to stir 24 h at rt. After diluting with 250 mL CH_2Cl_2, the reaction was washed with water (100 mL), saturated aq. NaCl (100 mL × 2), dried on $MgSO_4$, and concentrated. Chromatography on silica gel with 30% EtOAc in hexane gave 13.18 g (91.2%) white crystals. m.p. 146-147 °C. ^{1}H NMR (DMSO-d_6) δ 7.43-7.19 (m, 15H), 4.94 (t, 1H), 3.59 (m, 1H), 3.41(m, 1H), 3.20(m, 1H), 3.13 (s, 3H), 2.80 (d, 1H). ^{13}C NMR (DMSO-d_6) δ 174.3, 146.4, 129.0, 128.4, 126.9, 70.5, 64.7, 58.8, 51.7.

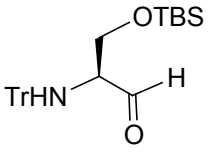


***N*-Triphenylmethyl-*O*-*tert*-butyldimethylsilyl-L-serine methyl ester (38).** *N*-Triphenylmethyl-L-serine methyl ester **37** (2.315 g, 6.4 mmol) and imidazole (1.30 g, 19.2 mmol) were dissolved in 100 mL CH_2Cl_2. *tert*-Butyldimethylsilyl chloride (TBS-Cl, 1.16 g, 7.69 mmol) was added. The reaction was stirred for 1 h. The solvent was removed and the residue was dissolved in 200 mL EtOAc , washed with saturated aq. NH_4Cl (50 mL × 2), saturated aq. $NaHCO_3$ (50 mL × 2), brine (50 mL) and dried on $MgSO_4$. Chromatography on silica gel with 10% EtOAc in hexane gave 3.072 g (yield 100%) white crystals. m.p. 90 -91°C. ^{1}H NMR δ 7.51-7.10 (m, 15H), 3.90 (m, 1H), 3.64 (t, 1H), 3.45 (m, 1H), 3.18 (s, 3H), 2.70 (d, 1H), 0.86 (s, 9H), 0.03 (d, 6H). ^{13}C NMR δ 186.0, 174.5, 146.1, 128.9, 127.9, 126.5, 70.8, 66.2, 58.4, 51.5, 25.8, 18.3, -5.5

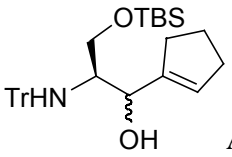


***N*-Triphenylmethyl-*O*-*tert*-butyldimethylsilyl-L-serinol (39).** *N*-Triphenylmethyl-*O*-*tert*-butyldimethylsilyl-L-serine methyl ester **38** (2.0 g, 4.2 mmol) in 100 mL CH_2Cl_2 was dried over 3 Å molecular sieves for 1 h. The solution was transferred to a dry flask and cooled to –78 °C. (*i*-Bu)$_2$AlH (1.5 M in toluene, 5.9 mL, 8.82 mmol) was added slowly and stirred for 3 h. The reaction was quenched with saturated aq. $NaHCO_3$ 50 mL at –78 °C. The reaction mixture was rapidly stirred and was warmed to rt. HCl (1 N, 50 mL) was added. The reaction was extracted with CH_2Cl_2 100 mL, washed with water (70 mL × 2), dried on $MgSO_4$ and concentrated. Toluene was removed azeotropically with MeOH. Chromatography on silica gel with 3% EtOAc in

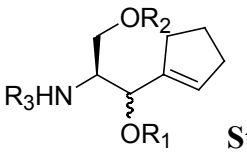
hexane then 5% EtOAc in hexane gave 0.369 g (yield 19.7%) aldehyde **40** as yellowish syrup and 1.424 g (yield 75.7%) alcohol **39** as yellowish syrup. For *N*-triphenylmethyl-*O*-*tert*-butyldimethylsilyl-L-serinol **39**: ^{1}H NMR δ 7.58-7.15 (m, 15H), 3.42 (dd, 1H), 3.33 (dd, 1H), 3.07 (dd, 1H), 2.92 (dd, 1H), 2.76 (m, 1H), 2.36 (br, s, 2H), 0.86 (s, 9H), -0.017, -0.025 (d, 6H). ^{13}C NMR δ 146.9, 128.8, 128.0, 126.5, 71.0, 64.9, 63.8, 53.9, 25.9, 18.3, -5.4. *N*-Triphenylmethyl-*O*-*tert*-butyldimethylsilyl-L-serinal **40**: ^{1}H NMR δ 9.26 (d, 1H), 7.51-7.15 (m, 15H), 3.71 (dd, 1H), 3.36 (t, 1H), 3.19 (dd, 2H), 0.84 (s, 9H), -0.02 (d, 6H). ^{13}C NMR δ 205.0, 146.1, 128.8, 128.7, 128.1, 70.9, 64.2, 63.1, 25.9, 18.3, -5.5.



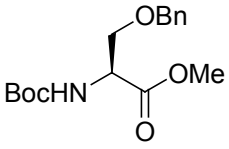
***N*-Triphenylmethyl-*O*-*tert*-butyldimethylsilyl-L-serinal (40).** Oxalyl chloride (0.44 g, 3.45 mmol) in 15 mL CH_2Cl_2 was cooled to –60 °C. DMSO (0.59 g, 0.54 mL, 7.53 mmol) was added over 5 min, then stirred for 10 min. *N*-triphenylmethyl-*O*-*tert*-butyldimethylsilyl-L-serinol **39** in 10 mL CH_2Cl_2 was added over 5 min, then stirred for 15 min. Et_3N (1.59 g, 2.2 mL, 15.69 mmol) was added over 5 min. The bath was removed and 15 mL H_2O was added then stirred for 10 min. The aqueous layer was extracted with $CHCl_3$ 20 mL. The organic layers were combined and washed with 1 N HCl (10 mL), water (10 mL), Na_2CO_3 (10 mL) and water (10 mL) and dried on $MgSO_4$. Chromatography on silica gel with 5% EtOAc in hexane gave 1.045 g (yield 74.6%) of a yellowish syrup. ^{1}H NMR δ 9.26 (d, 1H), 7.51-7.15 (m, 15H), 3.71 (dd, 1H), 3.37 (t, 1H), 3.21 (dd, 1H), 1.51 (br, s, 1H), 0.84 (s, 9H), -0.02 (d, 6H). ^{13}C NMR δ 205.0, 146.1, 128.8, 128.7, 128.1, 70.9, 64.2, 63.1, 25.9, 18.3, -5.5.



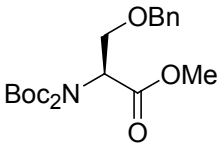
Alcohol (41). 1-Iodocyclopentene (0.474 g, 2.5 mmol) was dissolved in 10 mL THF and cooled to –40 °C, *s*-BuLi (1.3 M in cyclohexane, 1.9 mL, 2.5 mmol) was added and the reaction mixture was stirred at –40 °C for 2 h. Aldehyde **40** (0.446 g, 1 mmol) in 5 mL THF was added to the reaction slowly at –78 °C. After stirring at –78 °C for 2 h, the reaction was quenched with NH_4Cl (10 mL), diluted to 60 mL with EtOAc, washed with NH_4Cl (10 mL × 2), brine (10 mL), dried on $MgSO_4$ and concentrated. Chromatography with 3% EtOAc in hexane followed by 5% EtOAc in hexane eluted a pair of diastereomers as colorless syrup. The first diastereomer was 0.109 g and the second one was 0.193 g, total yield 58.7 %. The first diastereomer: ^{1}H NMR δ 7.61, 7.59 (d, 6H), 7.35-7.16 (m, 9H), 5.51 (d, 1H), 3.77 (br, m, 1H), 3.69-3.66 (dd, 1H), 3.44-3.41(dd, 1H), 3.08 (s, 1H), 2.94 (br, s, 1H), 2.64 (d, 1H), 2.22 (m, 2H), 1.90-1.70 (m, 4H), 0.92 (s, 9H), 0.09 (s, 3H), 0.05 (s, 3H). ^{13}C NMR δ 146.9, 145.7, 128.9, 128.0, 127.9, 127.3, 126.5, 124.5, 74.6, 71.4, 65.5, 53.9, 32.6, 32.1, 25.9, 23.6, 18.2, -5.4, -5.6. The second diastereomer: ^{1}H NMR δ 7.55, 7.53 (d, 6H), 7.30-7.16 (m, 9H), 5.66 (s, 1H), 4.23, 4.22 (d, 1H), 2.98, 2.96 (d, 1H), 2.78, (t, 1H), 2.65 (br, s, 1H), 2.47-2.44 (dd, 1H), 2.36-2.20 (m, 3H), 2.09-2.04 (m, 1H), 1.90-1.74 (m, 2H), 0.80 (s, 9H), -0.11 (s, 3H), -0.13 (s, 3H). ^{13}C NMR δ 146.7, 144.9, 129.0, 128.4, 128.0, 126.6, 71.0, 61.2, 55.4, 32.3, 31.1, 25.9, 23.3, 18.1, -5.4, -5.6.



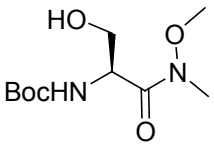
Stannane (42): A suspension of KH (30% in mineral oil, 10 mg, 0.249 mmol) in 1 mL THF was added dropwise to a mixture of alcohol **41b** (40.2 mg, 0.078 mmol) and 18-crown-6 (39.9 mg, 0.151 mmol) in 3 mL THF. The resulting solution was added to 3 mL THF with $ICH_2Sn(n\text{-}Bu)_3$ (84 mg, 0.195 mmol) in THF. The reaction was stirred for 1 h at rt, quenched with MeOH, diluted to 30 mL with EtOAc, washed with NH_4Cl (10 mL), $NaHCO_3$ (10 mL), brine (10 mL), dried on $MgSO_4$ and concentrated. Chromatography on silica gel with 4% EtOAc in hexane gave 47.3 mg of an orange liquid, yield 79.5% (the yield was 82% when **41a** was used as starting material). The product from **41b**: ^{1}H NMR δ 5.62 (s, 1H), 3.71-3.66 (dd, 3H), 3.50, 3.49 (d, 1H), 3.36, 3.34 (d, 1H), 3.22 (dd, 1H), 3.10 (dd, 1H), 2.88 (m, 1H), 2.34 (m, 2H), 2.22 (m, 1H), 2.13 (m, 1H), 1.87 (m, 2H), 1.48 (m, 12H), 1.28 (m, 12H), 0.86 (m, 30H). ^{13}C NMR δ 142.4, 130.1, 85.5, 77.2, 62.6, 59.1, 53.5, 32.2, 30.1, 29.3, 29.3, 27.4, 23.5, 13.8, 9.1, 9.0. MS (FAB^+, normal ion M/e^+): 767.5, 765.5, 763.3; 709.4, 707.4, 705.4; 591.4, 387.3, 179.1, 177.1. The product from **41a**: ^{1}H NMR δ 5.66 (s, 1H), 3.68 (m, 3H), 3.54 (d, 1H), 3.43 (dd, 1H), 3.36, 3.35 (d, 1H), 3.29 (dd, 1H), 2.88 (b, 1H), 2.36 (m, 2H), 2.20 (m, 2H), 1.88 (m, 2H), 1.84 (m, 12H), 1.28 (m, 12H), 0.87 (m, 30H). ^{13}C NMR δ 142.5, 130.7, 85.2, 76.8, 62.4, 59.3, 52.7, 32.3, 30.7, 29.3, 29.3, 27.4, 27.4, 23.4, 13.8, 13.8, 9.1, 9.0. MS (FAB^+, normal ion M/e^+): 767.5, 765.5; 709.4, 707.4, 705.4; 591.4, 387.3, 292.5, 181.1, 179.1, 177.1.



Ester (44). *N*-Boc-*O*-benzyl-L-serine (2.953 g, 10 mmol) was dissolved in CH_2Cl_2 (40 mL) containing Et_3N (1.51 g, 15 mmol) and the mixture was cooled to 0 °C. 4-dimethylamino pyridine (DMAP, 0.13 g, 1.0 mmol) was added, followed by methyl chloroformate (1.134 g, 12 mmol). The reaction mixture was stirred at rt for 1h then diluted with 300 mL CH_2Cl_2, washed with sat. $NaHCO_3$ (50 mL × 2), dried on $MgSO_4$ and concentrated. Chromatography with 30% EtOAc in hexane on silica gel gave 3.09 g of a yellowish oil, yield 100%. ^{1}H NMR δ 7.20-7.40 (m, 5H), 5.40 (d, 1H), 4.53 (dd, 2H), 4.44 (m, 1H), 3.85 (dd, 1H), 3.74 (s, 3H), 3.67 (dd, 1H), 1.41 (s, 9H).

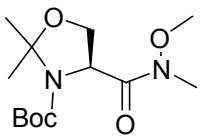


Ester (45). Ester **44** (3.09 g, 10 mmol) and DMAP (0.246 g, 2 mmol) were dissolved in 40 mL acetonitrile, followed by $(Boc)_2O$ (4.37 g, 20 mmol). The reaction mixture was stirred at 40 °C for 16 h and the solvent was removed in vacuum evaporator. The residue was purified on silica gel column with 10% EtOAc in Hexane to give 3.645 g yellowish oil, yield 89%. ^{1}H NMR δ 7.32-7.26 (m, 5H), 5.29-5.25 (dd, 1H), 4.62-4.46 (dd, 2H), 4.10-4.06 (dd, 1H), 3.95-3.90 (dd, 1H), 3.71 (s, 3H), 1.50 (s, 18H). ^{13}C NMR δ 169.8, 152.3, 138.2, 128.5, 127.8, 83.4, 73.0, 68.8, 57.9, 52.4, 28.2.

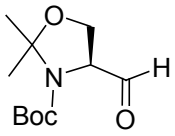


Weinreb amide (48). *N*-Boc-L-serine (8.20 g, 40.0 mmol), DIEA (10.4 g, 80.0 mmol), and *N*,*O*-dimethylhydroxylamine hydrochloride (5.82 g, 60.0 mmol) were

dissolved in CH_2Cl_2 (160 mL) and cooled to 0 °C. DCC (9.08g, 44mmol) and 1-hydroxy-1*H*-benzotriazole (HOBt, 6.74 g, 44.0 mmol) were added. After 16 h, the reaction mixture was filtered, washed with NH_4Cl (3 × 80 mL), brine (80 mL), dried on $MgSO_4$ and concentrated. Chromatograhpy on silica gel with 40% EtOAc in hexanes yielded 9.4 g white solid, yield 95% . ^{1}H NMR δ 6.50 (br, s, 1H), 5.44 (d, 1H), 4.78 (s, 1H), 3.82 (d, 2H), 3.76 (s, 3H), 3.20 (s, 3H), 1.42 (s, 9H).

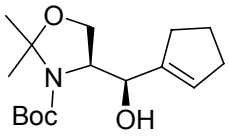


Weinreb amide (49). N-Boc-serine Weinreb amide **48** (9.4 g, 38 mmol) was dissolved in acetone (160 mL) and 2, 2-dimethoxypropane (DMP, 50 mL) and BF_3•Et_2O (about 0.4 mL) was added until there was a permanent color change (colorless to dark yellow) and the reaction was stirred for 90 min. Et_3N (1.0 mL) was added to quench the reaction and the solvent was evaporated to give a white solid which was purified by silica gel column with 30% EtOAc in hexane to give 11.1 g (yield 96%) white solid. ^{1}H NMR δ 4.78 (dd, 0.5H), 4.71 (dd, 0.5H), 4.18 (ddd, 1H), 3.97-3.92 (ddd, 1H), 3.73 (s, 1.5H), 3.69 (s, 1.5H), 3.20 (s, 3H), 1.69 (s, 1.5H), 1.68 (s, 1.5H), 1.55 (s, 1.5H), 1.51 (s, 1.5 H), 1.48 (s, 4.5H), 1.40 (s, 1.5H).

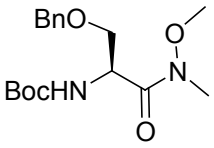


Aldehyde (50). Weinreb amide **49** (389 mg, 1.35 mmol) was dissolved in anhydrous THF (4.0 mL) and cooled to 0 °C and 1.0M $LiAlH_4$ (26 mg, 0.68 mmol) in THF (0.68 mL) was added dropwise and then the mixture was stirred for 2 h. The reaction was quenched carefully by sat. aq. $KHSO_4$ (2.7 mL) at –15 °C. The solution was

diluted with Et_2O (7 mL) and stirred vigorously for 30 min. The organic layer was dried on $MgSO_4$ and concentrated to give 292 mg (yield 94%) colorless liquid. ^1H NMR (DMSO-d_6, 120°C) δ 9.55 (d, 1H), 4.35 (ddd, 1H), 4.10 (dd, 1H), 4.04 (m, 1H), 1.55 (s, 3H), 1.15 (s, 3H), 1.43 (s, 9H).

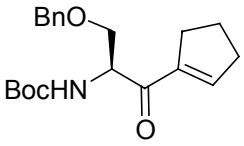


Alcohol (51). 1-Iodocyclopentene (291 mg, 1.5 mmol) was dissolved in 3 mL THF and cooled to –40 °C, *s*-Buli (1.3 M in cyclohexane, 0.23 mL, 3.0 mmol) was added and stirred for 2 h. The resulting mixture was transfer to a solution of aldehyde **50** (229 mg, 1.0 mmol) in 2 mL THF at –78 °C, stirred at –20 °C for 2 h then quenched with 1.5 mL NH_4Cl, diluted to 30 mL with EtOAc, washed with NH_4Cl (4 mL × 2), $NaHCO_3$ (4 mL), brine (4 mL), dried on $MgSO_4$ and concentrated. Chromatography with 15% EtOAc in hexane on silica gel yielded 70 mg (yield 24%) white solid. ^1H NMR ($CDCl_3$ δ): no resoluble NMR available.

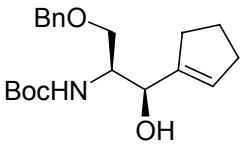


Boc-Ser(OBn) Weinreb amide (52).[118] *N*-Boc-Ser(OBn)-OH (2.95 g, 10.0 mmol), *N,O*-dimethylhydroxylamine hydrochloride (1.85 g, 20.0 mmol) and DIEA (5.2 g, 40 mmol) were dissolved in 1:1 CH_2Cl_2/DMF (100 mL) and cooled to 0 °C. 1-Hydroxy-1H-benzotriazole (HOBt, 1.84 g, 12.0 mmol), DCC (2.48 g, 12.0 mmol) and DMAP (ca. 30 mg) were added and the reaction was stirred for 24 h. The reaction was filtered to remove dicyclohexylurea and concentrated. The resulting slurry was diluted

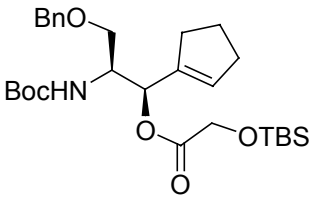
with 150 mL ethyl acetate and washed with NH_4Cl (2 × 50 mL), $NaHCO_3$ (2 × 50 mL) and brine (50 mL). The organic layer was dried on $MgSO_4$ and concentrated. Chromatography on silica with 30% EtOAc in hexane gave 3.04 g (90%) of **52** as a colorless syrup. ^{1}H NMR δ 7.35-7.23 (m, 5H), 5.42 (d, J = 8.5, 1H), 4.87 (br, s, 1H), 4.56 (d, J = 12.5, 1H), 4.49 (d, J = 12.5, 1H), 3.71 (s, 3H), 3.66 (m, 2H), 3.17 (s, 3H), 1.43 (s, 9H).



Ketone (53). To a solution of 1-iodocyclopentene **62** (7.59 g, 39.1 mmol) in 100 mL THF at -40 °C was added *s*-BuLi (1.3 M in cyclohexane, 60 mL, 78 mmol). The reaction was stirred at –40 °C for 3 h to generate cyclopentenyl lithium. Then the mixture was added via syringe in three portions to a solution of Weinreb amide **52** (4.41 g, 13.0 mmol) in THF (50 mL), dried over 3 Å molecular sieves for 3 h, at –78 °C. The mixture was stirred for 3 h at –78 °C, quenched with NH_4Cl (20 mL), diluted with EtOAc (200 mL), washed with NH_4Cl (2 × 50 mL), $NaHCO_3$ (50 mL), brine (50 mL), dried over $MgSO_4$ and concentrated. Chromatography on silica with 8% EtOAc in hexane, then 12% EtOAc in hexane, gave 3.88 g (86%) of ketone **53** as a yellowish oil. ^{1}H NMR δ 7.34-7.22 (m, 5H), 6.79 (m, 1H), 5.57 (d, J = 10.5, 1H), 5.00 (m, 1H), 4.54 (d, J = 12.4, 1H), 4.43 (d, J = 12.0, 1H), 3.71 (d, J = 4.4, 2H), 2.62 (m, 1H), 2.54 (m, 3H), 2.00-1.82 (m, 2H), 1.44 (s, 9H). ^{13}C NMR δ 195.0, 155.5, 145.5, 143.3, 137.7, 128.4, 127.8, 127.6, 79.8, 73.2, 71.1, 56.4, 34.3, 31.0, 28.4, 22.5. Anal. Calcd. for: $C_{20}H_{27}O_4N$: C, 69.54; H, 7.88; N, 4.05. Found: C, 69.54; H, 7.74; N, 4.01.

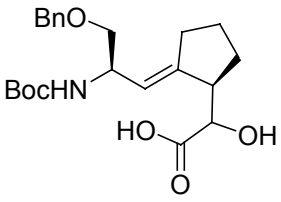


Alcohol (54). Ketone **53** (3.78 g, 11.0 mmol) was dissolved in 2.5:1 THF/MeOH (125 mL) and cooled to 0 °C. $CeCl_3$ (4.91 g, 13.2 mmol) was added, followed by $NaBH_4$ (0.84 g, 22 mmol). After stirring 2 h at 0 °C, the reaction was quenched with NH_4Cl (50 mL), diluted with EtOAc (200 mL), washed with NH_4Cl (2 × 100 mL), brine (100 mL), dried on $MgSO_4$ and concentrated. Chromatography on silica with 15% EtOAc in hexane yielded 3.49 g (92%) of a white solid as a 4:1 mixture of diastereomers. m.p. 67-68 °C. The major diastereomer was isolated by recrystallization from EtOAc/*n*-hexane. ^{1}H NMR δ 7.36-7.28 (m, 5H), 5.65 (m, 1H), 5.35 (d, J = 8.4, 1H), 4.51 (d, J = 11.6, 1H), 4.42 (d, J = 12.0, 1H), 4.33 (br, s, 1H), 3.84 (br, s, 1H), 3.71-3.68 (dd, J = 3.4, 13.4, 1H), 3.60-3.55 (dd, J = 2.6, 9.4, 1H), 3.18 (d, J = 8.4, 1H), 2.35-2.20 (m, 4H), 1.87 (m, 2H), 1.44 (s, 9H) ^{13}C NMR δ 155.9, 144.7, 137.6, 128.7, 128.2, 128.1, 126.7, 79.7, 74.1, 74.0, 70.6, 52.1, 32.4, 28.6, 23.9. Anal. Calcd for: $C_{20}H_{29}O_4N$: C, 69.14; H, 8.41; N, 4.03. Found: C, 69.42; H, 8.54; N, 4.12.



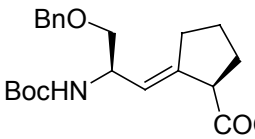
Ester (55). To a solution of alcohol **54** (3.26 g, 9.38 mmol) and pyridine (2.28 mL, 28.2 mmol) in THF (4 mL) was added a solution of *tert*-butyldimethylsilyloxyacetyl chloride[124] (2.05 g, 9.40 mmol) in THF (4 mL) dropwise at 0 °C. The reaction was stirred for 3 h at rt then diluted with 30 mL Et_2O, washed with 0.5 N HCl (2 × 20 mL), $NaHCO_3$ (10 mL), brine (10 mL), dried on $MgSO_4$ and concentrated. Chromatography with 4% EtOAc in hexanes on silica gave 3.48 g (70%)

of ester **55** as a yellow oil. ^{1}H NMR δ 7.35-7.28 (m, 5H), 5.67 (s, 1H), 5.58 (d, *J* = 8.0, 1H), 4.83 (d, *J* = 9.4, 1H), 4.51 (d, *J* = 11.9, 1H), 4.42 (d, *J* = 11.9, 1H), 4.16 (s, 2H), 4.04 (m, 1H), 3.55 (dd, *J* = 3.5, 9.4, 1H), 3.48 (dd, *J* = 3.3, 9.5, 1H), 2.41 (m, 1H), 2.33-2.21 (m, 3H), 1.83 (m, 2H), 1.40 (s, 9H), 0.90 (s, 9H), 0.07 (s, 6H). ^{13}C NMR *δ* 170.6, 155.3, 139.9, 138.0, 130.2, 128.5, 127.8, 127.7, 79.5, 73.3, 72.6, 68.5, 61.8, 51.0, 32.4, 31.6, 28.4, 25.8, 23.2, 18.4, -5.4. Anal. Calcd for: $C_{28}H_{45}NO_4Si$: C, 64.70; H, 8.73; N, 2.69. Found: C, 64.58; H, 8.89; N, 2.69.



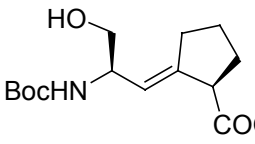
***α*-Hydroxy acid (57).** To a solution of diisopropylamine (3.3 mL, 24 mmol) in THF (40 mL) was added *n*-butyl lithium (2.5 M in hexane, 8.6 mL, 22 mmol) at 0 °C. The mixture was stirred for 15 min to generate LDA. Then a mixture of chlorotrimethyl silane (7.52 mL, 59.2 mmol) and pyridine (5.22 mL, 64.6 mmol) in THF (15 mL) was added dropwise to the LDA solution at –100 °C. After 5 min, a solution of ester **55** (2.83 g, 5.38 mmol) in THF (18 mL) was added dropwise and the reaction was stirred at –100 °C for 25 min then warmed slowly to rt over 1.5 h and stirred at rt for 1.5 h. The reaction was quenched with 1 N HCl (70 mL) and the aqueous layer was extracted with Et_2O (2 × 150 mL). The organic layer was dried on $MgSO_4$ and concentrated to give 1.98 g (crude yield 70%) colorless glassy oil. Without further purification, the product was dissolved in 10 mL THF. Tetrabutylammonium fluoride (2.8 g, 11 mmol) in THF (10 mL) was added at 0 °C, stirred at 0 °C for 5 min then at rt for 1 h. The reaction was quenched with 0.5 N HCl (50 mL), extracted with EtOAc (100

mL), dried on $MgSO_4$ and concentrated. Chromatography with 50% EtOAc in hexane on silica gave 1.16 g (52%) of α-hydroxy acid **57** as a colorless foam. ^{1}H NMR (DMSO-d_6) δ 7.36-7.24 (m, 5H), 6.84 (d, J = 7.35, 1H), 5.28 (d, J = 7.80, 1H), 4.50 (d, J = 11.9, 1H), 4.44 (d, J = 12.2, 1H), 4.31 (br, s, 1H), 3.84 (d, J = 6.0, 1H), 3.40-3.32 (m, 2H), 3.27 (dd, J = 5.1, 10.1, 1H), 2.70-2.61 (m, 1H), 2.41-2.37 (m, 1H), 2.17-2.10 (m, 1H), 1.74-1.67 (m, 2H), 1.55-1.42 (m, 2H), 1.37 (s, 9H). ^{13}C NMR (DMSO-d_6) δ 175.3, 155.7, 145.4, 139.2, 128.7, 127.9, 127.8, 121.6, 78.0, 74.0, 72.5, 72.3, 50.5, 47.6, 30.0, 29.6, 28.8, 24.6. Anal. Calcd for: $C_{22}H_{31}NO_6$: C, 65.17; H, 7.71; N, 3.45. Found: C, 65.03; H, 7.80; N, 3.47.



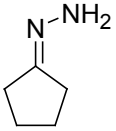
Acid (59). Lead tetraacetate (2.69 g, 6.06 mmol) in $CHCl_3$ (13.5 mL) was added dropwise to a solution of acid **57** (2.28 g, 5.51 mmol) in EtOAc (81 mL) at 0 °C. The reaction was stirred for 10 min then quenched with ethylene glycol (8 mL), diluted with EtOAc (150 mL), washed with H_2O (4 × 15 mL), brine (15 mL), dried on Na_2SO_4 and concentrated to give 2.02 g (100% crude yield) aldehyde **58** as yellowish oil. ^{1}H NMR δ 9.38 (d, J = 2.8, 1H), 7.36-7.27 (m, 5H), 5.39 (dd, J = 2.2, 8.6, 1H), 4.95 (d, J = 7.1, 1H), 4.55 (d, J = 12.2, 1H), 4.47 (d, J = 12.2, 1H), 4.41 (br, s, 1H), 3.50 (dd, J = 4.3, 9.3, 1H), 3.43 (dd, J = 5.0, 9.4, 1H), 3.25 (m, 1H), 2.55 (m, 1H), 2.24 (m, 1H), 1.99 (m, 1H), 1.86 (m, 1H), 1.72 (m, 2H), 1.43 (s, 9H). The product was dissolved in acetone (140 mL) and cooled to 0 °C. Jones reagent (2.7 M H_2SO_4, 2.7 M CrO_3, 4 mL, 11 mmol) was added dropwise. The reaction was stirred at 0 °C for 0.5 h and quenched with isopropyl alcohol (12 mL) and stirred for 10 min. The precipitate was filtered out and the

solvent was evaporated. The residue was extracted with EtOAc (3 × 200 mL), washed H_2O (50 mL), brine (50 mL), dried on Na_2SO_4 and concentrated. Chromatography on silica with 30% EtOAc in hexane gave 1.65 g (78%) of acid **59** as a colorless oil. ^{1}H NMR δ 7.30 (m, 5H), 5.55 (d, J = 6.7, 1H), 4.93 (br, s, 1H), 4.53 (d, J = 12.1, 1H), 4.51 (d, J = 12.1, 1H), 4.39 (br, s, 1H), 3.47 (dd, J = 3.5, 9.2, 1H), 3.41 (dd, J = 5.3, 9.6, 1H), 3.36 (t, J = 7.0, 1H), 2.54 (m, 1H), 2.29 (m, 1H), 2.04-1.84 (m, 3H), 1.66 (m, 1H), 1.43 (s, 9H). ^{13}C NMR δ 179.9, 155.6, 143.8, 138.2, 128.5, 127.7, 127.6, 122.6, 79.4, 73.1, 72.1, 50.4, 49.5, 30.1, 29.4, 28.5, 25.1. IR (cm^{-1}): 3000-2800 (br), 1701 (s), 1162, 731, 697. HRMS calcd for $C_{21}H_{29}NO_5$ (MH^+) m/z = 376.2124, found m/z = 376.2133.

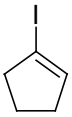


Boc–Ser–Ψ[(*E*)CH=C]Pro–OH (60). NH_3 (35 mL) was distilled, allowed to warm to reflux (–33 °C) and Na (ca. 330 mg, 14 mmol) was added until a deep blue solution was sustained. Acid **59** (575 mg, 1.50 mmol) in THF (13 mL) was added directly to the Na/NH_3 solution via syringe. After stirring 15 min at reflux, the reaction was quenched with NH_4Cl (20 mL), then allowed to warm to rt. NH_4Cl (40 mL) was added, and the mixture was extracted with $CHCl_3$ (5 × 30 mL). The aqueous layer was acidified with 1 N HCl and extracted with $CHCl_3$ (6 × 50 mL). The $CHCl_3$ layer was dried on $MgSO_4$ and concentrated to give 280 mg (64%) of the acid as a yellowish oil. ^{1}H NMR (DMSO-d_6) δ 6.66 (d, J = 7.4, 1H), 5.31 (dd, J = 2.1, 8.7, 1H), 4.61 (br, s, 1H), 4.06 (s, 1H), 3.27 (dd, J = 7.1, 10.8, 1H), 3.20 (dd, J = 5.7, 10.5, 1H), 3.16 (m, 1H), 2.39 (m, 1H), 2.22 (m, 1H), 1.80 (m, 3H), 1.52 (m, 1H), 1.36 (s, 9H). ^{13}C NMR δ 175.4,

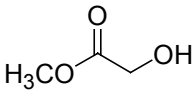
155.7, 143.6, 122.5, 78.0, 64.0, 52.9, 49.6, 30.1, 29.5, 28.8, 25.0. HRMS calcd for $C_{14}H_{23}NO_5$ (MH^+) *m/z* = 286.1654, found *m/z* = 286.1661.



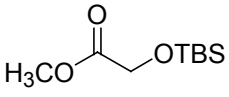
Hydrazone (61). (By the method of Barton et al.[123]) Cyclopentanone (107 mL, 1.2 mol) and hydrozine monohydrate (181 g, 3.6 mol) were combined at rt and heated at reflux for 2 h. The reaction was diluted with 500 mL $CHCl_3$, washed with water (2 × 100 mL), dried on $MgSO_4$ and concentrated to provide 127g (yield 100%) product as a colorless liquid. ^{1}H NMR δ 4.80 (s, 2H), 2.33-2.30 (t, 2H), 2.16-2.12 (m, 2H), 1.85-1.67 (m, 4H).



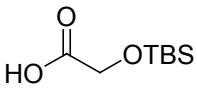
1-Iodocyclopentene (62). (By the method of Barton et al.[123]) To a solution of I_2 (111 g, 440 mmol) in 400 mL Et_2O was added a solution of tetramethylguanidine (275 mL, 2.2 mol) in 200 mL Et_2O slowly (Caution: exothermic!) and stirred for another 1h. A solution of **61** (20 g, 202 mmol) in 100 mL Et_2O was added over 15 min (Cuation: exothermic!) and stirred for another 10 min, reflux for 2 h. The reaction was cooled to rt, filtered to remove the solids, and diluted with 1 L Et_2O, washed with 2 N HCl (3 × 100mL), $Na_2S_2O_3$ (100 mL), brine (100 mL), dried over $MgSO_4$ and concentrated. Chromatography with pure hexane on silica gel gave 19.6 g (yield 50%) slightly orange liquid. ^{1}H NMR δ 6.12-6.10 (m, 1H), 2.64-2.58 (m, 2H), 2.36-2.30 (m, 2H), 1.98-1.90 (m, 2H).



Glycolic acid methyl ester (65). Glycolic acid **64** (7.6 g, 100 mmol) was dissolved in 150 mL MeOH and cooled to 0 °C. $SOCl_2$ (21.7 mL, 300 mmol) was added slowly and the reaction was stirred for 24 h then concentrated. The remaining liquid was dissolved in $CHCl_3$ and concetrated (2 × 25mL). Distillation (bp. 80 °C, aspirator) gave 5.4 g (yield 60%) colorless liquid. ^{1}H MNR δ 4.17 (s, 2H), 3.81 (s, 3H), 2.32 (s, b, 1H).

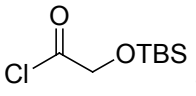


Ester (66): Imidazole (3.92 g, 57.6 mmol) was added to a solution of **65** (2.36 g, 26.2 mmol) in DMF (25 mL), followed by *tert*-butyldimethylsilyl chloride (TBSCl, 4.33 g, 28.8 mmol). The reaction was stirred at rt for 19 h then diluted to150 mL with EtOAc and washed with sat. NH_4Cl (2 × 30 mL), $NaHCO_3$ (30 mL), water (30 mL) dried on $MgSO_4$ and concentrated. The residue was vacuum distilled (50-54 °C, 0.6 Torr) to give 3.78 g colorless liquid, yield 62%. ^{1}H NMR δ 4.23 (s, 2H), 3.71 (s, 3H), 0.90 (s, 9H), 0.09 (s, 6H).

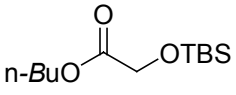


***tert*-Butyldimethylsilyloxyacetic acid (67)** (from methyl ester). To a solution of **66** (3.78 g, 17.7 mmol) in 10 mL THF was slowly added a solution of KOH in 2 mL MeOH and 4 mL H_2O at –10 °C. the mixture was allowed to warm to rt and stirred for 1 h. the reaction was washed with Et_2O (40 mL), the aqueous layer was acidified at 0 °C to pH = 2 with 3 N HCl (1.6 mL concentrated HCl in 4 mL H_2O) and extracted with Et_2O (2 × 40 mL). The organic layer was washed with H_2O (40 mL), brine (40 mL), dried on $MgSO_4$, concentrated and vacuum dried to give 2.03 g (yield 58%) white solid (very

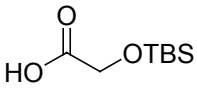
volatile, could be lost from vacuum drying). ^{1}H NMR δ 4.22 (s, 2H), 0.92 (s, 9H), 0.14 (s, 6H).



***tert*-Butyldimethylsilyloxyacetyl chloride (68).** The TBS protected acid **67** (1.9 g, 10 mmol) was dissolved in benzene (25 mL) and a small amount of benzene (10 mL) was distilled out to remove water. Oxalyl chloride (1.32 mL, 15 mmol) was added dropwise, and the reaction mixture was stirred at rt for 40 min and refluxed for 50 min. Excess oxalyl chloride and most of the benzene was removed by distillation at atmospheric pressure to give 2.1 g **68** (100% yield) as slightly yellowish liquid. ^{1}H NMR δ 4.55 (s, 2H), 0.92 (s, 9H), 0.12 (s, 6H).

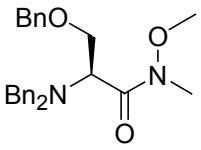


***O*-TBS *n*-butyl glycolate (70)**. To a mixture of *n*-butyl glycolate **69** (13.26 g, 100 mmol) and imidazole (14.96 g, 220 mmol) was added TBSCl (16.50 g, 110 mmol) at 0 °C. after being stirred at rt overnight, distillation yielded 21.03 g (yield 86%) ester **70** as colorless liquid (bp. 75 °C, 0.6 Torr). ^{1}H NMR δ 4.22 (s, 2H), 4.13 (t, 2H), 1.66-1.57 (m, 2H), 1.41-1.33 (m, 2H), 0.91 (s, 12H), 0.09 (s, 6H).

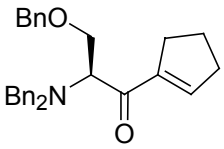


***tert*-Butyldimethylsilyloxyacetic acid (67)** (from *n*-butyl ester). To a solution of the ester **70** (21.0 g, 85.5 mmol) in 50 mL THF was slowly added a solution of KOH (4.82 g, 86 mmol) in methanol (10 mL) and H_2O (20 mL) at –10 °C. the reaction mixture was stirred at 0 °C for 1 h, diluted with 300 mL H_2O, and washed with

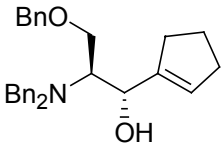
Et_2O (2 × 100 mL). The aqueous layer was acidified with 3 N HCl (a solution of 8.74 mL concentrated HCl in 20 mL H_2O) at 0 °C to pH = 2. This mixture was extracted with Et_2O (2 × 200 mL) and washed with water and brine, dried on $MgSO_4$ and concentrated. 15.4 g (yield 90%) acid **67** was obtained as colorless liquid, which solidified upon cooling. ^{1}H NMR δ 4.22 (s, 2H), 0.92 (s, 9H), 0.14 (s, 6H).



***N*,*N*,*O*-Tribenzyl serine Weinreb amide (71).** *N*-Boc-*O*-benzyl serine Weinreb amide,[118] **48**, (16.1 g, 47.6 mmol) was dissolved in CH_2Cl_2 (225 mL) and TFA (75 mL) was added and stirred 30 min. The mixture was concentrated, then quenched with $NaHCO_3$ until gas evolution ceased. The aqueous mixture was extracted with CH_2Cl_2 (8 × 200 mL), dried on $MgSO_4$, and concentrated. Chromatography on silica with 20% EtOAc in petroleum ether (pet. ether) to remove impurities, followed by product elution with 10% MeOH in EtOAc yielded 9.6 g (83%) of the amine as a clear oil. The amine (9.6 g, 40.3 mmol) was dissolved in CH_2Cl_2 (40 mL), then benzyl bromide (14.2 mL, 20.4 g, 119 mmol) and DIEA (41.4 mL, 30.7 g, 238 mmol) were added. After 4 d at rt, the reaction was diluted with EtOAc (400 mL), washed with NH_4Cl (4 × 100 mL) and brine (100 mL), dried on $MgSO_4$, and concentrated. Chromatography on silica with 5% EtOAc in pet. ether to remove benzyl bromide, then 20% EtOAc in pet. ether to elute the product yielded 15.2 g (91%) of dibenzyl amine **71** as a yellowish oil. ^{1}H NMR δ 7.40-7.17 (m, 15H), 4.56 (d, J = 11.9, 1H), 4.48 (d, J = 11.9, 1H), 4.13 (m, 1H), 3.98-3.84 (m, 4H), 3.76 (d, J = 14.1, 2H), 3.28 (br s, 3H), 3.20 (br s, 3H).

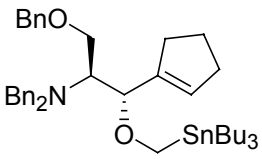


Ketone (72). Cyclopentenyl lithium was generated by adding fresh *s*-BuLi (1.3 M in cyclohexane, 80.0 mL, 104 mmol) to a solution of freshly prepared 1-iodocyclopentene **7** (10.1 g, 52.0 mmol) in THF (400 mL) at –40 °C. The solution was maintained at –40 °C for 2 hr, and Weinreb amide **71** (14 g, 34.7 mmol) in THF (100 mL) was cooled to –40 °C and added slowly via cannula. The mixture was stirred 1 h at –40 °C. The reaction was quenched with NH_4Cl (20 mL), diluted with EtOAc (600 mL), washed with NH_4Cl (3 × 100 mL), brine (100 mL), dried over Na_2SO_4, and concentrated. Chromatography on silica with 5% EtOAc in hexanes yielded 12.8 g (86%) of the ketone **72**. ^{1}H NMR δ 7.39-7.20 (m, 15H), 6.11 (m, 1H), 4.55 (d, J = 12.3, 1H), 4.48 (d, J = 12.3, 1H), 4.24 (app. t, J = 6.6, 1H), 3.90 (d, J = 6.6, 2H), 3.79 (d, J = 13.6, 2H), 3.71 (d, J = 14.1, 2H), 2.59-2.39 (m, 4H), 1.98-1.84 (m, 2H).

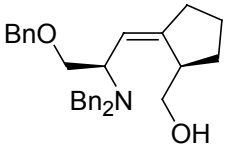


(*S,S*)-Alcohol (73). Ketone **72** (12.0 g, 28.2 mmol) was dissolved in THF (280 mL) and 1 M $LiAlH_4$ in THF solution (33.9 mL, 33.9 mmol) was added. After 1 h, the reaction was quenched with MeOH (50 mL), then NH_4Cl (50 mL), after stiring vigorously for 1 h, the reaction was diluted with EtOAc (500 mL), washed with NH_4Cl (150 mL), and 1 M sodium potassium tartrate (2 × 150 mL). The aqueous layers were extracted with CH_2Cl_2 (3 × 200 mL). The combined organic layers were dried over $MgSO_4$ and concentrated to yield 11.3 g (94%) of alcohol **73** as a clear oil. ^{1}H NMR δ 7.49-7.24 (m, 15H), 5.65 (m, 1H), 4.62 (d, J = 11.9, 1H), 4.53 (d, J = 11.9, 1H), 4.48 (s,

1H), 4.26 (d, J = 10.1, 1H), 4.02 (d, J = 13.2, 2H), 3.80-3.70 (m, 3H), 3.58 (dd, J = 10.6, 3.1, 1H), 3.07 (m, 1H), 2.43-2.17 (m, 3H), 2.00-1.75 (m, 3H).

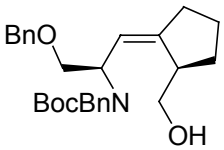


Stannane (74). To a solution of alcohol **73** (9.4 g, 22 mmol) in THF (100 mL), was added 18-crown-6 (6.4 g, 24.2 mmol) in THF (20 mL), KH (1.06 g, 26.4 mmol, 35% suspension in mineral oil) in THF (20 mL), and Bu_3SnCH_2I, purified by silica gel chromatography, (12.9 g, 26.4 mmol) in THF (20 mL), and stirred 30 min at rt. The reaction was quenched with MeOH and diluted with EtOAc (400 mL), washed with NH_4Cl (2 × 100 mL), brine (100 mL), dried on $MgSO_4$, and concentrated. Purification by chromatography on silica with 3% EtOAc in hexanes yielded 15.1 g (94%) of stannane **74** as a clear liquid. ^{1}H NMR δ 7.40-7.26 (m, 15H), 5.60 (br s, 1H), 4.45 (d, J = 12.0, 1H), 4.37 (d, J = 12.0, 1H), 4.05 (d, J = 7.8, 1H), 3.99 (d, J = 13.7, 2H), 3.83 (d, J = 13.7, 2H), 3.74 (dm, J = 9.9, 1H), 3.60 (dd, J = 9.6, 5.7, 1H), 3.53 (dd, J = 9.6, 4.6, 1H), 3.41 (dm, J = 9.6, 1H), 2.99 (m, 1H), 2.40-2.28 (m, 2H), 1.99 (br s, 2H), 1.82 (m, 2H), 1.54 (m, 6H), 1.33 (m, 6H), 0.91 (m, 15H).



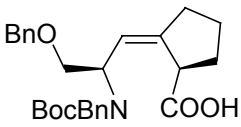
(*Z*)-Alkene (*Z*)-75 and (*E*)-alkene (*E*)-75. Stannane **74** (7.70 g, 10.5 mmol) was dissolved in THF (200 mL) and cooled to –78 °C. *n*-BuLi (2.5 M in hexane, 5.5 mL, 13.7 mmol) was cooled to –78 °C, added slowly via cannula and stirred 1.5 h at –78 °C for 3 h then the reaction was warmed slowly to –50 °C over 1 h. The reaction was

quenched with MeOH and concentrated. The residue was diluted with EtOAc (500 mL), store at rt for 2 h, washed with NH_4Cl (2 × 150 mL), brine (150 mL), dried on Na_2SO_4 and concentrated. ^{1}H NMR revealed the (*Z*) and (*E*) acetate formed exclusively, indicating the acetate formation was quickly finished within 2 h when product was diluted with EtOAc. ^{1}H NMR δ 7.38-7.16 (m, 15H), 5.56 (d, J = 9.2, 0.79 H), 5.45 (d, J = 9.2, 0.22 H), 4.50 (d, J = 3.6, 0.79H), 4.47 (d, J = 2.8, 0.22H), 3.85-3.65 (m, 7H), 3.55-3.43 (m, 2H), 2.75 (m, 1H), 2.37 (m, 1H), 2.26 (m, 1H), 2.09 (s, 0.63), 2.01(s, 2.37). (the peaks at lower ppm mixed with the proton peaks from tetrabutyltin and were not distinguished).The acetates (10.5 mmol) was dissolve in a solution of KOH (589 mg, 10.5 mmol) in 1:2:7 MeOH:H_2O:THF (100 mL) and stirred at rt for 2 h. The reaction was extracted with ether (5 × 100 mL). The ether layer was dried on $MgSO_4$ and concentrated. Chromatography on silica with 15% EtOAc in hexanes yielded 3.48 g (75%) of **(*Z*)-75**, and 0.88 g (19%) of **(*E*)-75** as clear oils. **(*E*)-75**: ^{1}H NMR δ 7.38-7.27 (m, 15H), 5.43 (br d, J = 9.4, 1H), 4.51 (d, J = 12.1, 1H), 4.47 (d, J = 12.1, 1H), 3.84 (d, J = 13.9, 2H), 3.73 (m, 1H), 3.64-3.47 (m, 6H), 2.65 (m, 1H), 2.05 (m, 2H), 1.85 (m, 1H), 1.69 (m, 1H), 1.56 (m, 2H). (*Z*)-**75**: ^{1}H NMR δ 7.38-7.26 (m, 15H), 5.55 (br d, J = 8.7,1H), 4.57 (d, J = 12.2, 1H), 4.53 (d, J = 12.2, 1H), 4.12 (br s, 1H), 3.89 (d, J = 13.3, 2H), 3.79 (m, 1H), 3.67 (m, 4H), 3.33 (m, 1H), 3.27 (m, 1H), 2.53 (m, 1H), 2.31-2.18 (m, 2H), 1.71-1.47 (m, 4H).



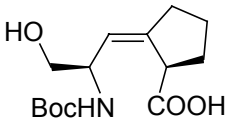
Boc-benzylamine (77). (*Z*)-Alkene **75** (9.1 g, 20.6 mmol), and 20% $Pd(OH)_2$/C (948 mg) were blanketed with argon and MeOH (630 mL) was added,

followed by 96% HCOOH (120 mL). After stirring 60 min, the reaction was filtered immediately through Celite, concentrated, neutralized with solid $NaHCO_3$ until gas evolution ceased, extracted with CH_2Cl_2 (5 × 100 mL), dried over Na_2SO_4, and concentrated to yield 7.0 g (97%) of the monobenzylamine **76** without further purification. ^{1}H NMR δ 7.36-7.30 (m, 10H), 5.50 (br d, J = 8.3, 1H), 4.56 (br d, J = 1.6, 2H), 3.72 (d, J = 11.2, 1H), 3.66-3.60 (m, 3H), 3.55-3.50 (m, 1H), 3.48-3.45 (dd, J = 10.8, 4.3, 1H), 3.41-3.37 (m, 1H), 2.83 (m, 1H), 2.37-2.22 (m, 2H), 1.89-1.85 (m, 1H), 1.64 (m, 1H), 1.54-1.38 (m, 2H). The monobenzylamine (7.0 g, 19.9 mmol) was dissolved in CH_2Cl_2 (300 ml), and di-*tert*-butyldicarbonate (10.84 g, 49.7 mmol) was added and stirred for 17 h. The mixture was concentrated and purification by chromatography on silica with 20% EtOAc in hexanes yielded 7.0 g (78%) of the Bocbenzyl amine **77** as a colorless oil. ^{1}H NMR δ 7.36-7.16 (m, 10H), 5.36 (br d, J = 8.9, 1H), 5.18 (br s, 1H), 4.47-4.37 (m, 4H), 3.48-3.46 (m, 5H), 2.87 (br s, 1H), 2.20 (m, 2H), 1.75 (m, 1H), 1.65 (m, 2H), 1.54 (m, 1H), 1.34 (br s, 9H).

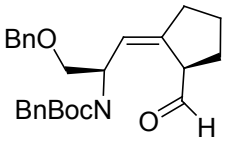


Boc-benzylamino Acid (78). Boc-benzylamine **77** (2.0 g, 4.18 mmol) was dissolved in acetone (200 mL) and cooled to 0 °C. Jones reagent (2.7 M H_2SO_4, 2.7 M CrO_3; 4.5 mL, 12 mmol) was added, and the resulting solution was stirred for 1 h at 0 °C. The reaction was quenched with 2-propanol (50 mL) and stirred for 10 min. The mixture was diluted with water (400 mL), extracted with CH_2Cl_2 (10 × 50 mL), dried on $MgSO_4$, and concentrated. Chromatography on silica with 20% EtOAc in petroleum ether yielded 1.9 g (95%) of the acid **78** as a pale yellow oil. ^{1}H NMR δ 7.34-7.16 (m, 10H),

5.53 (br, d, J = 9.2, 1H), 4.92 (br s, 1H), 4.47-4.27 (m, 4H), 3.69-3.24 (m, 3H), 2.46 (m, 1H), 2.28 (m, 1H), 2.11 (m, 1H), 1.89 (m, 2H), 1.62 (m, 1H), 1.38 (br s, 9H).



Boc–SerΨ[(*Z*)CH=C]Pro–OH (79). NH_3 (ca. 140 mL) was distilled into a round bottom flask at –78 °C and allowed to warm to reflux (–33 °C). Na (ca. 1.4 g, 30.0 mmol) was added until a deep blue solution was sustained. A solution of acid **78** (1.4 g, 3.0 mmol) in THF (50 mL) was added directly to the Na/NH_3 solution slowly via cannula over ca. 5 min. After stirring 45 min at reflux, the reaction was quenched with NH_4Cl (35 mL), then allowed to warm to rt with concentration to ca. 30 mL. The mixture was diluted with NH_4Cl (50 mL), acidified with 1 N HCl to pH 5 and extracted with $CHCl_3$ (10 × 50 mL), dried on $MgSO_4$, and concentrated. Chromatography on silica with 0.5% acetic acid and 3% MeOH in $CHCl_3$ gave 618 mg (72% yield) of white solid. ^{1}H NMR (DMSO-d_6) δ 6.48 (br d, J = 6.2, 1H), 5.20 (d, J = 8.4, 1H), 4.08 (m, 1H), 3.36 (m, 1H), 3.28 (dd, J = 10.6, 5.7, 1H), 3.13 (dd, J = 10.6, 6.6, 1H), 2.20 (m, 2H), 1.81 (m, 2H), 1.67 (m, 1H), 1.47 (m, 1H), 1.31 (s, 9H).



Formation of the Aldehyde (82). Bocbenzyl amine **77** (6.8 g, 15.1 mmol) was dissolved in acetone (340 mL) and cooled to 0 °C. Jones reagent (2.7 M H_2SO_4, 2.7 M CrO_3, 7.0 mL, 19 mmol) was added and stirred 30 min at 0 °C. The reaction was quenched with isopropanol (100 mL) and stirred 5 min. The mixture was diluted with water (400 mL), extracted with CH_2Cl_2 (10 × 100 mL), dried on $MgSO_4$, and

concentrated. Chromatography on silica with 20% EtOAc in pet. ether yielded 2.6 g (37%) of the acid **78** as a pale yellow oil, 2.0 g aldehyde **82** (29.5% yield) as a yellowish oil, and 2.1 g aldehyde **83** (31% yeild). The aldehyde **82** (2.0 g, 4.5 mmol) was dissolved in 200 mL acetone. Jones reagent (2.5 mL, 6.68 mmol) was added to the solution. The reaction was stirred at 0 °C for 1 h and quenched with isopropanol (30 mL) and stirred 5 min. the product was worked up as described above. After silica gel purification, 1.4 g of the acid **78** was obtained (68% yield). ^{1}H NMR for acid **78** δ 7.34-7.16 (m, 10H), 5.53 (br d, *J*=9.2, 1H), 4.92 (br s, 1H), 4.47-4.27 (m, 4H), 3.69-3.24 (m, 3H), 2.46 (m, 1H), 2.28 (m, 1H), 2.11 (m, 1H), 1.89 (m, 2H), 1.62 (m, 1H), 1.38 (br s, 9H).

Chapter 3. Synthesis of conformationally locked peptidomimetics as Pin1 inhibitors

3.1. Design of the peptidomimetics as Pin1 ground state analogues

Pin1 not only recognizes the phosphoSer-Pro motif with a selectivity of up to 1300-fold over the non-phosphorylated form, but also prefers aromatic amino acid residues N-termini to the pSer-Pro and a basic residue such as arginine on the C-terminus. Screening of a chemically synthesized peptide library against Pin1 PPIase activity revealed that the best peptide substrate is Trp–Phe–Tyr–pSer–Pro–Arg–*p*NA ($k_{cat}/K_m = 2 \times 10^7$ $M^{-1}s^{-1}$).[38] The substrate specificity of Pin1 is shown in Table 3.1.

Table 3.1. Sequence specificity of Pin1[38]

Ligand	-4	-3	-2	-1	+1	+2	+3
Pin1	W	F	Y	pS	P	R	L
		Y	I	R		F	I
		F		F			Y
		W		W			

We intended to synthesize a matched stereoisomeric pair of compounds to permit direct, quantitative comparison of the affinity (K_i) of each inhibitor for the Pin1 PPIase catalytic site. These peptidomimetics were designed based on the best substrate for Pin1 to ensure a good binding affinity to allow the meaningful comparison of those two conformations of the substrate, but with some simplification. Pentapeptide mimics Ac–Phe–Phe–pSer–Ψ[(*Z* and *E*)CH=C]–Pro–Arg–NH_2 (or –NHMe or –OMe) were our targets. These cis and trans pentapeptide analogues were designed with the C-termini as carboxamides or esters and with acetylated N-termini to remove charges that could interfere with binding. The acetylated carboxamides are more like the protein substrates of Pin1, in which the charged ends of the protein do not make contact with the enzyme

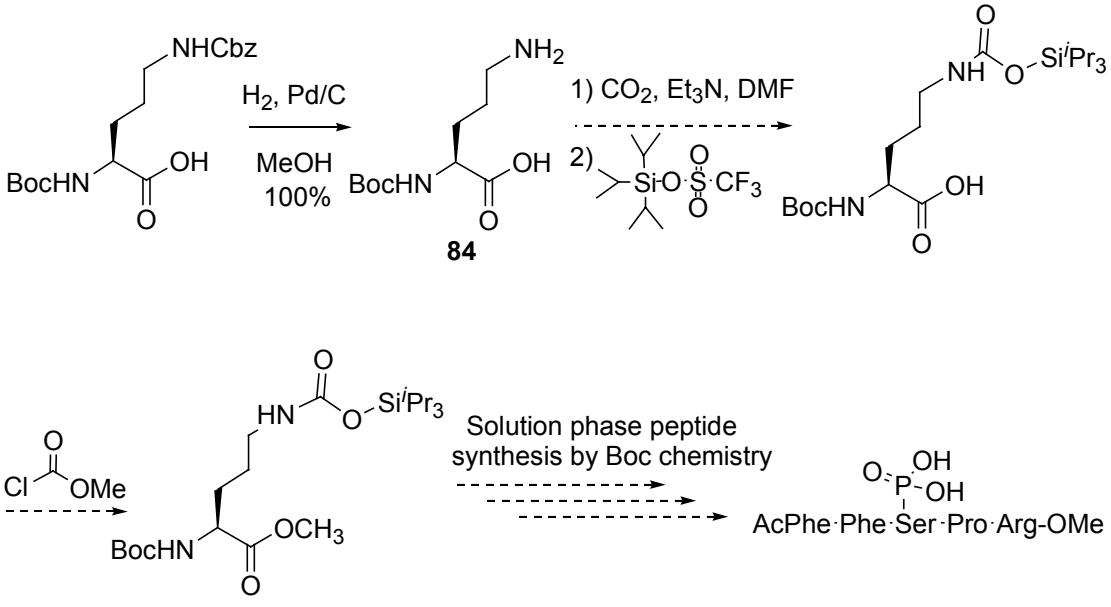
active site. By masking the termini, the charges on the phosphate and Arg guanidinyl groups should be accentuated and ensure the correct mode of binding.

3.2. Boc solid phase peptide synthesis

Synthesis of the inhibitor peptide Ac–Phe–Phe-pSerΨ[(*Z*)CH=C]Pro–Arg–OMe was attempted by Hart,[98] utilizing the Fmoc–Ser(OTBDPS)–Ψ[(*Z*)CH=C]Pro–OH as a building block. Athough mass spectrometry indicated the presence of the desired peptide, the yield was only 0.012% after HPLC purification. The bulky silyl protection on the serine side chain was a likely source of difficulty.[98]

To avoid the bulky silyl protecting group (TBDPS) and changing protecting groups for the dipeptide mimics, we decided to try solution phase peptide synthesis using Boc chemistry.

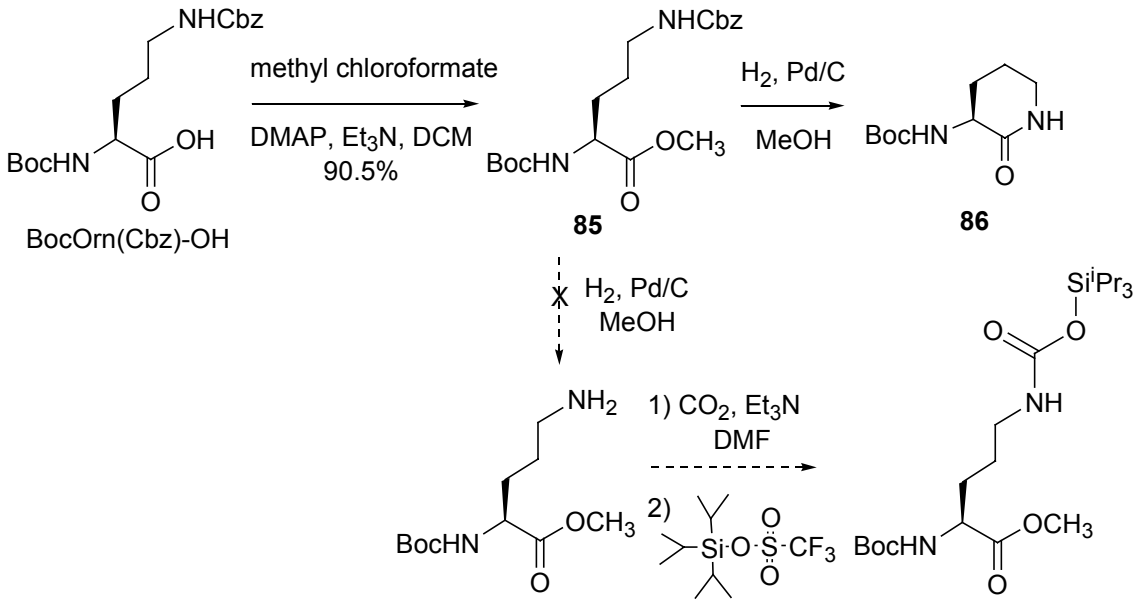
Scheme 3.1. Solution peptide analog synthesis



The triisopropylsilyloxycarbonyl ("Tsoc") protecting group developed recently[139] is orthogonal to Boc, Fmoc and Cbz, thus may be used in both Boc and Fmoc peptide chemistry. Very mild and specific conditions, using fluoride ion in THF at 0˚C for 15-30 min, removes Tsoc.[139] We planned to use Tsoc for the protection of the ornithine side chain. Since there is only one reference about this interesting protecting group, it was necessary to explore its application in peptide synthesis in solution or on solid phase.

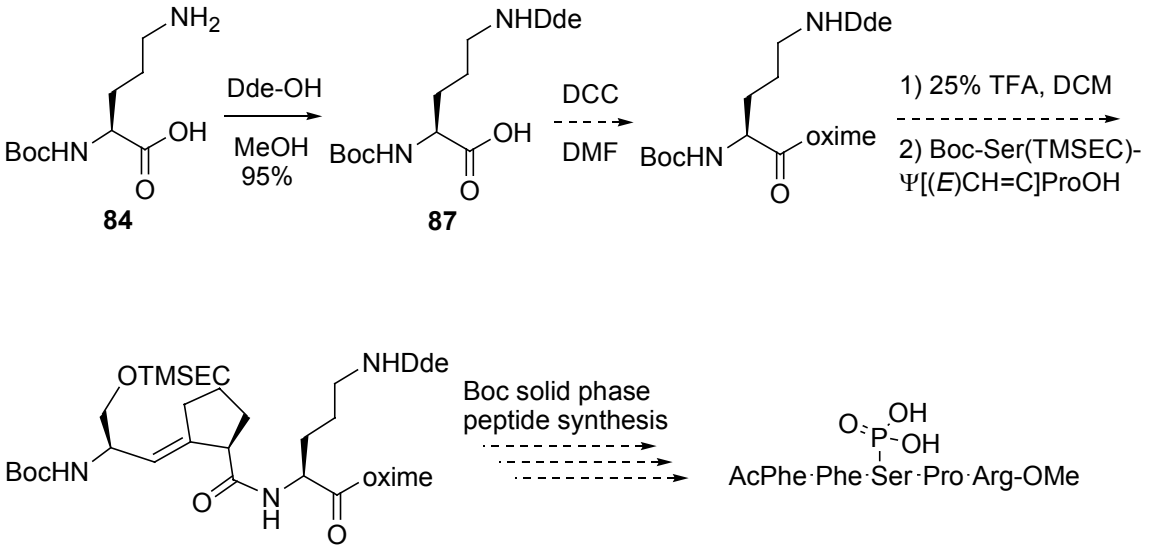
The proposed solution phase peptide synthesis using Tsoc as ornithine side chain protection was outlined in Scheme 3.1. Hydrogenation gave 100% yield of Boc–Orn–OH as a white solid, insoluble in DMF at –78˚C. The protection of **84** by Tsoc was unsuccessful and only starting material was recovered. The low solubility of the Boc–Orn–OH under the reaction conditions was suspected to cause the difficulty of this reaction. This reaction was attempted only once and this route was not exhausted.

Scheme 3.2. Synthesis of Boc–Orn(Tsoc)–OMe



An alternative route for synthesizing Boc–Orn(Tsoc)–OMe is shown in Scheme 3.2. Methylation of Boc–Orn(Cbz)–OH using methyl chloroformate followed by hydrogenation gave a clean product (85% in two steps) identified as a cyclized lactam, **86**, instead of the desired methyl Boc ornithine ester. Because of the position of the amino group in ornithine, the hydrogenation must have been successful and the intramolecular attack on the carbonyl by the free amine occurred sequentially. The cyclization was facilitated by the good leaving group, methoxy, to give the six-membered ring lactam.

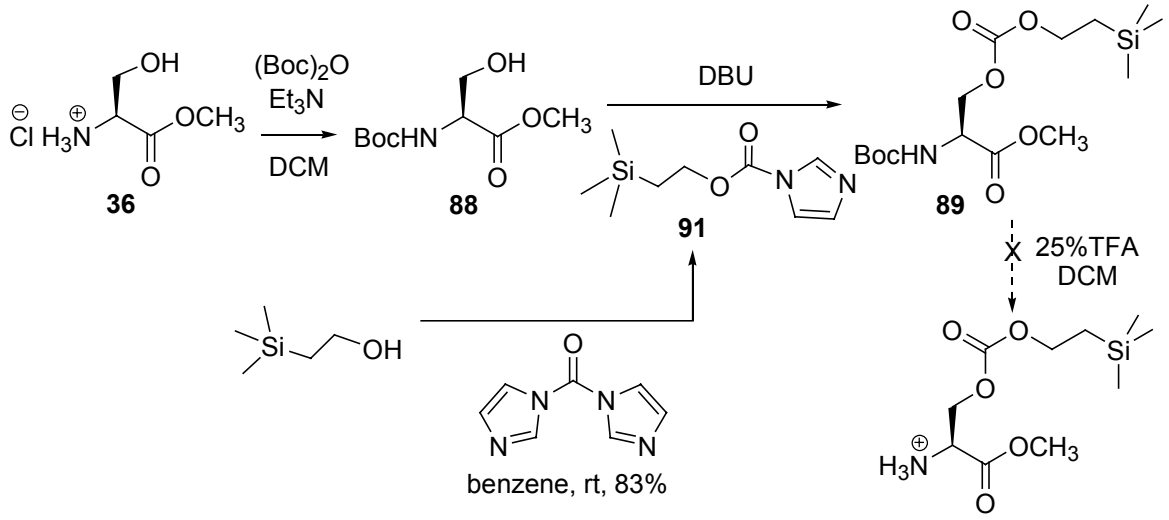
Scheme 3.3. Proposed solid phase peptide synthesis by Boc chemistry



Another synthetic route (Scheme 3.3) using Boc solid phase peptide synthesis required changing the hydroxyl protecting group of the mimics **59** and **79** since Birch

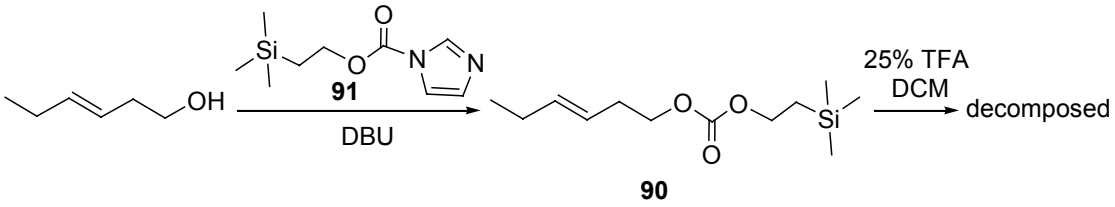
reduction to remove benzyl was expected to be difficult on solid phase support. An interesting silyl protecting group for hydroxyl function is TMSEC, trimethylsilylethoxycarbonyl group.[109] TMSEC was originally used for amine protection.[140] It is readily deprotected by F^- to give gaseous products on cleavage. 2-(trimethylsilyl)ethyl chloroformate and 1-[[2-(trimethylsilyl)ethoxy]carbonyl] imidazole **90**, both easily accessible reagents, can be conveniently used for the protection of hydroxyl function of different substrates.[141,142] The protected product 2-(trimethylsilyl)ethoxycarbonate was stable in 80% acetic acid at 20 °C for over 170 h.[142] It was also reported that the amine protected product 2-(trimethylsilyl)ethoxycarbamate was not stable to TFA.[142] Whether protected hydroxyl groups might be stable to TFA was unknown.

Scheme 3.4. Stability of trimethylsilylethoxy carbonate to TFA



In order to test the stability of the trimethylsilylethoxy carbonate to 25% TFA in CH_2Cl_2, the standard deprotection reagent for Boc chemistry, compounds **89** and **90** were synthesized. Boc-serine methyl ester **89** is relevant to the serine-like part of our mimics. The carboxylic acid was protected as the methyl ester to avoid the problem of a zwitterion if TMSEC could indeed be removed by TFA. A small amount of compound **89** was treated with 25% TFA for 1 h. Unfortunately the ^{1}H NMR of the crude product showed no silyl group in crude products.

Scheme 3.5. ^{1}H NMR study on the stability of trimethylsilylethoxy carbonate to TFA



Compound **90** was synthesized as a model compound with the same alkene geometry and position as the trans mimic **59**, but with no other functionalities to interfere with the test of TMSEC stability. *trans*-3-Hexen-1-ol was protected easily with TMSEC with 95% yield. The stability test was carried out in an NMR tube, since TFA showed only a single peak with a ppm value around 11.5 that would not interfere with peaks from compound **90** and possible products. ^{1}H NMR spectra were measured after combining **90** with TFA and $CDCl_3$ for 20 min, 50 min, 110 min, and 270 min and revealed that silyl group was completely removed in 50 min. The product peaks did not match with *trans*-3-hexen-1-ol, indicating that the product was not the deprotected alcohol but some other

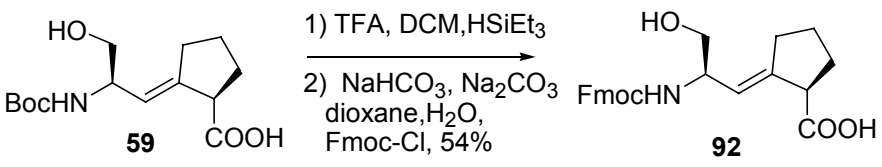
derivative. The conclusion was that TMSEC was not suitable for Boc solid phase peptide synthesis.

Initial synthesis by this route (Scheme 3.3) included deprotection of Boc–Orn(Cbz)–OH and reprotection by Dde on the side chain to give compound **87**. Further synthesis by this route was abandoned because of the susceptibility of TMSEC to TFA. Since the changing of protecting groups is unavoidable, we turned back to the more widely used Fmoc peptide synthesis.

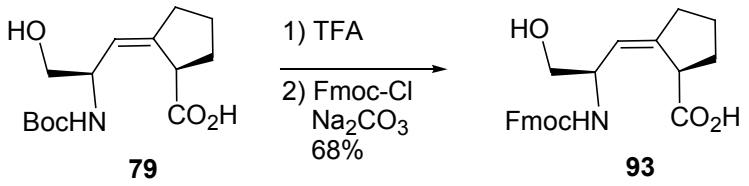
3.3. Preparation for Fmoc solid phase peptide synthesis: changing protecting groups

Fmoc chemistry is more widely used than Boc chemistry in solid-phase peptide synthesis[143,144] and permits complicated modifications of the peptide. Both mimics, Boc–Ser–Ψ[(*E*)CH=C]–Pro–OH, **59** and Boc–Ser–Ψ[(*Z*)CH=C]–Pro–OH, **79**, were reprotected as the Fmoc-carbamates **92** and **93** (Scheme 3.1 and Scheme 3.2). Deprotection of Boc by acidolysis for the trans isostere **59** was carried out in the presence of triethylsilane as a carbocation scavenger, greatly improving the yield from 20% to 54%.[145] Sequential protection by Fmoc was conducted by adding saturated Na_2CO_3 intermittently to dioxane-saturated aqueous $NaHCO_3$ solution[146-148] to maintain the pH between 8 and 9, giving the Fmoc-protected compounds **92** and **93** with two-step yields of 54% and 68%, respectively.

Scheme 3.6. Synthesis of the trans phospho building block



Scheme 3.7. Synthesis of the cis phospho building block

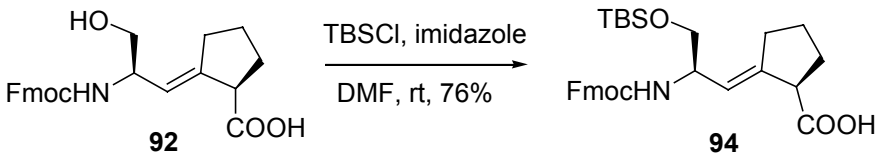


3.4. Synthesis of trans alkene peptidomimetics using 2-Cl trityl-Cl resin

In principle, there are two strategies for the synthesis of phosphopeptides: the building block approach, using preformed protected phosphoamino acids, and the global phosphorylation method that involves post-synthetic phosphorylation of unprotected hydroxyl groups on the solid support.

The peptide synthesis was embarked upon first with the trans isostere using the global phosphorylation approach. We chose to protect the free hydroxyl group on the side chain with *tert*-butyl dimethylsilyl, which is orthogonal to Fmoc. The side chain protection was necessary for both cis and trans mimics because with a free side chain, the trans mimic underwent isomerization quickly in the coupling step (see section 3.6) and the cis mimic cyclized intramlecularly to form a lactone.[Bailing Xu, Felicia A. Etzkorn, personal communication] Protection of the hydroxyl side chain was conducted in the presence of the free carboxylic acid (Scheme 3.8). At least 2.5 equivalents of TBSCl were used to silylate both the side-chain hydroxyl and the carboxyl. The TBS ester of the carboxylic acid was formed temporarily, and the mildly acidic aqueous workup (NH_4Cl) deprotected only the TBS ester to produce the desired Fmoc–Ser(TBS)–Ψ[(*E*)CH=C]–Pro–OH, **94**, in 76% yield.

Scheme 3.8. Synthesis of the TBS protected building block

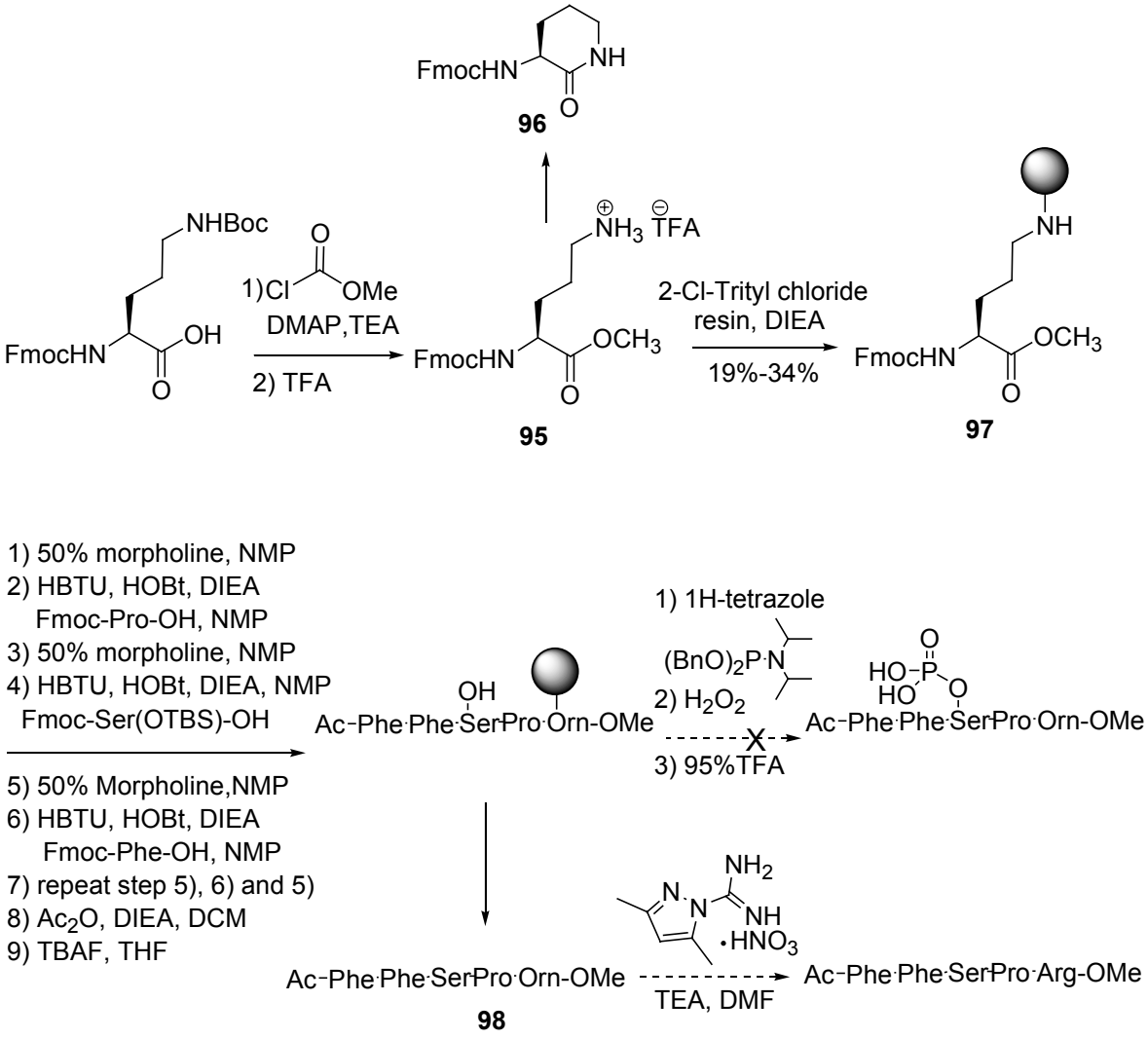


The initial synthetic target was the methyl ester, Ac–Phe–Phe–pSer–Ψ[(*E*)CH=C]–Pro–Arg–OMe. Scheme 3.9 shows the proposed model synthesis of a pentapeptide, Ac–Phe–Phe–pSer–Pro–Arg–OMe. Fmoc–Orn(Boc)–OH was methylated with methyl chloroformate under basic condition (Et_3N) without affecting the base sensitive Fmoc.[109] Without purification of the methyl ester, Boc was deprotected by TFA. Upon addition of a base ($NaHCO_3$) to free the side chain amine from the TFA salt, cyclization occurred to form the lactone **96**. The loading of Fmoc–Orn–OMe was finally carried out as its TFA salt, with DIEA to free the amine in situ (Table 3.2). Still, cyclization was suspected to occur due to the low yields of resin loading (19%-43%). CH_2Cl_2 was found to be a better solvent than THF giving better loading yield (0.48 mmol/g vs. 0.27 mmol/g). Longer reaction time also improved the loading yield. Guanidinylation at the end of peptide synthesis under basic conditions was expected to cause the same cyclization.

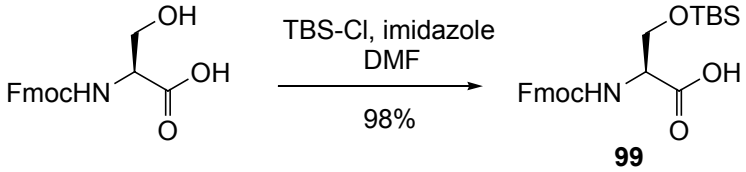
Table 3.2. Loading of Fmoc-Orn(NH_3^+ TFA^-)–OMe onto 2-chlorotrityl chloride resin

	eq. of FmocOrn(OMe) to resin	Solvent	Reaction time (hours)	Loading (mmol/g resin)	Yield
1	2:1	CH_2Cl_2	2	0.31	22%
2	0.5:1	CH_2Cl_2	16	0.48	43%
3	2:1	THF	2	0.27	19%

Scheme 3.9. Model peptide synthesis



Scheme 3.10. Synthesis of Fmoc–Ser(TBS)–OH



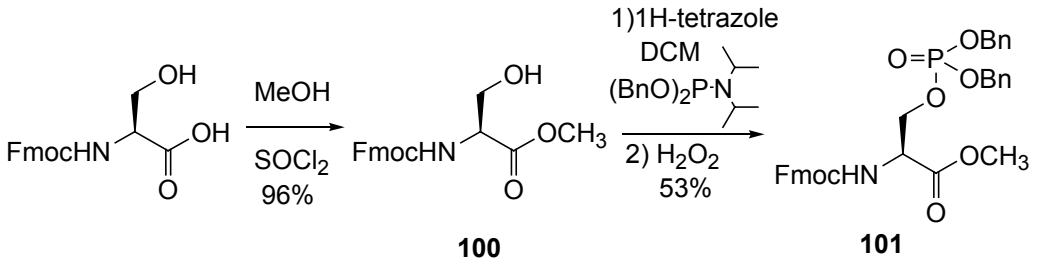
Serine was incorporated with the side chain protected as the TBS ether to match the TBS protected dipeptide mimics (Scheme 3.10). Fmoc deprotection was carried out with 50% morpholine in NMP, following the previous synthesis of Pin1 substrate *p*-

nitroanilide.[149] After phosphorylation on resin with (*N*,*N*-diisopropyl) dibenzylphosphoramidite and oxidation with hydrogen peroxide, the crude peptide was cleaved from the resin with 95% TFA in CH_2Cl_2, conditions under which the benzyl phosphate would be simultaneously deprotected. Unfortunately, MS-FAB (positive) showed that the final product was Ac–Phe–Phe–Ser–Pro–Orn–OMe instead of the corresponding phosphopeptide. Although this Ac–Phe–Phe–Ser–Pro–Orn–OMe was not our target, after guanidinylation it could be used for kinase inhibition studies.[Felicia A. Etzkorn, Grant application]

Two questions were raised at this point: 1) why was the phosphorylation difficult on the resin? 2) How could cyclization be avoided when loading the Fmoc–Orn–OH onto the resin?

A model reaction was run to test whether the phosphorylation would work in solution. Fmoc–Ser–OH was methylated by a Fischer esterification, and the side chain was phosphorylated with (*N*,*N*-diisopropyl)dibenylphosphoramidite in the presence of 1*H*-tetrazole as catalyst, followed by hydrogen peroxide oxidation. The desired product was obtained in 53% yield. Since the model phosphorylation did occur, the global phosphorylation condition on solid phase required optimization.

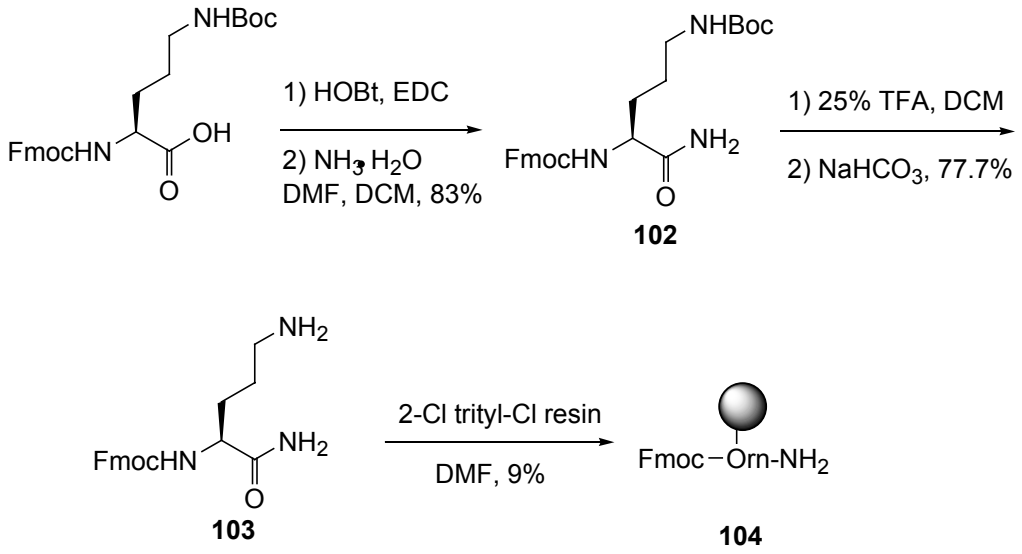
Scheme 3.11. Synthesis of Fmoc-serine(dibenzylphosphate) methyl ester



100 **101**

To avoid cyclization, our target peptide was changed from Ac–Phe–Phe–pSer[Ψ(*E*)CH=C]–Pro–Arg–OMe to Ac–Phe–Phe–pSer[Ψ(*E*)CH=C]–Pro–Arg–NHMe or Ac–Phe–Phe–pSer[Ψ(*E*)CH=C]–Pro–Arg–NH_2. A C-terminal methyl amide or primary amide (–NH_2) is not chromophore that could interfere with the Pin1 assay even though they could be cleaved by proteases. The amide bond is more stable than the methyl ester and was not expected to cyclize under peptide synthesis or guanidinylation conditions.[150]

Scheme 3.12. Synthesis of Fmoc-Orn(NHBoc)-NH_2 and its loading onto resin

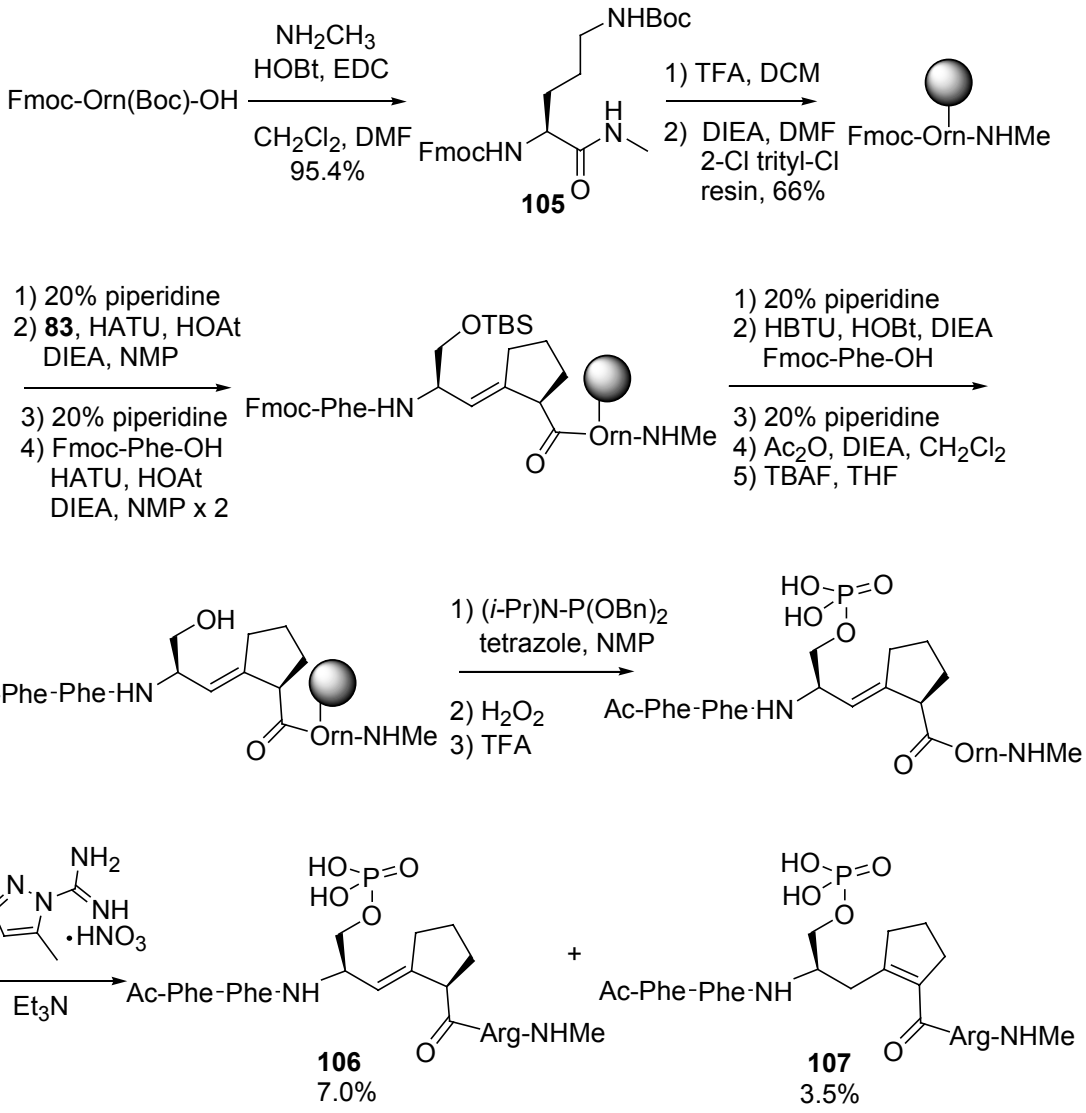


Fmoc-Orn(NHBoc)-NH_2 was made by a two steps sequence[151] (Scheme 3.12) using aqueous ammonium hydroxide that did not remove the base sensitive Fmoc. Compound **103** was not soluble in all solvents attempted including DCM, MeOH, DMSO and DMF, making the first loading onto the resin extremely difficult. UV test of the loading showed that the loading was only 0.14 mmole/g (9% yield). While Fmoc–Orn($NH_3^+ \cdot TFA^-$)–

NHMe was attached to the resin with a loading of 0.48-0.61 mmol/g (Scheme 3.13), which was suitable for peptide synthesis.

The synthesis of the phosphorylated peptide mimic **3** was carried out on the 2-chlorotrityl chloride resin,[152,153] according to Schutkowski and co-workers,[154] with two modifications (Scheme 3.13). First, the dipeptide isostere **94** was incorporated with a TBS protected side chain to avoid acylation of the hydroxyl group. This protection also allowed excess activated amino acids to be used in subsequent coupling steps. Second, 20% piperidine was used to deprotect Fmoc instead of 50% morpholine, because the C-terminal amide of the Pin1 inhibitor was not sensitive to cleavage by 20% piperidine like the *p*-nitroanilides. Fmoc–Orn(Boc)–OH was coupled to methylamine to give Fmoc–Orn(Boc)–NHMe, **88**, in 95% yield (Scheme 3.13). Boc was removed with 25% TFA in CH_2Cl_2, and the resin was loaded using the TFA salt without purification. The loading yield was 66% based on 2-chlorotrityl resin, as determined by the standard UV absorbance method.[155] Amino acid couplings, except for the final phenylalanine, were performed with the activation reagents HATU and HOAt to prevent isomerization or racemization. Silyl protection was removed with TBAF. Global phosphorylation utilized (*N*,*N*-diisopropyl)dibenzylphosphoramidite, followed by hydrogen peroxide oxidation. Guanidinylation[150] was difficult at rt, but went to completion after 7 h at 45 °C as monitored by reverse phase HPLC.

Scheme 3.13. Synthesis of Ac-Phe-Phe-pSer-Ψ[(*E*)CH=C]-Pro-Arg-NHMe



HPLC analysis after guanidinylation showed two major peaks with close retention times of 12.5 and 12.9 min. (HPLC condition: 10%-100% CH_3CN in H_2O with 0.1% TFA over 30 min on a C_{18} reverse phase analytical column at 1.0 mL min^{-1}). These two peaks both had the desired *m*/*z* (771.4 by LC-MS), indicating that the compounds were isomers. The ratio of this pair of peaks varied with each peptide synthesis batch. The

separation of these two peaks was achieved by changing HPLC conditions as shown in Figure 3.1.

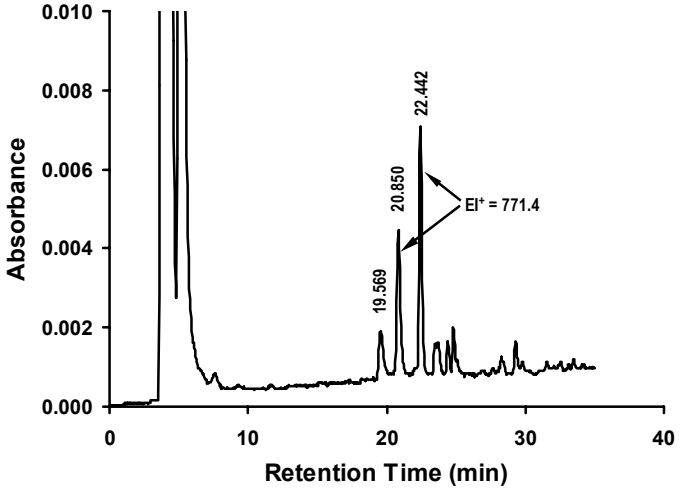


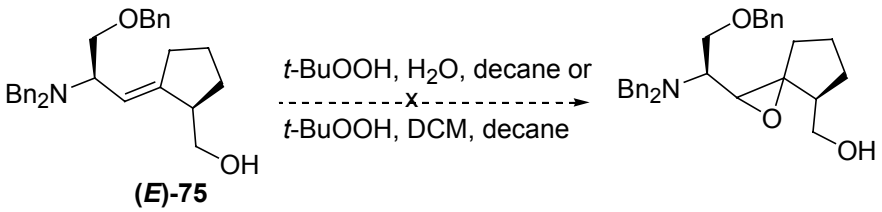
Figure 3.1. HPLC analysis of the crude product.

The two major peaks had the same mass $(M+1)^+$ = 771.4 from LC-MS. HPLC conditions: maintain 20% B for 10 min, then do a gradient from 20% B to 50% B over 25 min on a C_{18} reverse phase analytical column at 1.0 mL min^{-1}. A solvent: H_2O containing 0.1% TFA; B solvent: CH_3CN containing 0.1% TFA.

During solid-phase synthesis, HPLC analysis of the intermediates cleaved from the resin with 10% TFA after each coupling after the trans isostere **94** showed a pair of peaks with very close retention times as major products. Because the alkene proton peak was missing in the first isomer **107** at 20.9 min (Figure 3.1), it is likely that the trans β,γ-unsaturated isostere **94** (Scheme 3.8), as the HOAt-activated ester, isomerized to form the α,β-unsaturated alkene under these coupling conditions. HPLC purification of *N*-methylamide trans isostere **106** was performed on a C_4 semipreparative column. The purities of both isomers **106** and **107** were greater than 97% by HPLC.

During the synthesis of the trans peptide analogues, hydrogen peroxide was chosen as oxidation reagent to oxide the phosphite because we were concerned about the sensitive exoxcylic alkene. Although the synthesis was successful, *tert*-butyl hydroperoxide and *m*CPBA[156-160] were more typical choices for oxidizing phosphite to phosphate on solid phase. *m*CPBA was expected to oxidize the alkene to epoxide, so was not used for oxidizing phosphite to phosphate. The stability of alkene to *t*-BuOOH was tested by a model oxidation of tribenzyl protected alcohol, **(*E*)-75**, the minor isomer from the Still-Wittig rearrangement for the synthesis of Boc–SerΨ[(*Z*)CH=C]–Pro–OH. Compound **(*E*)-75** was combined with *t*-BuOOH in decane and H_2O to form a heterogenous reaction mixture, and the mixture was stirred for 2 hours. At the same time, compound **(*E*)-75** was combined with *t*-BuOOH in decane and DCM to form a homogenous solution, and this solution was stirred also for 2 hours. The starting material were recovered and none of the reaction systems afforded any product. This indicated that the trans alkene was stable to up to 3 mmol/mL of *t*-BuOOH in either H_2O or DCM, the typical concentration for peptide phosplorylation. Indeed, H_2O_2 could be replaced by *t*-BuOOH in peptide mimic solid phase synthesis (see section 3.5).

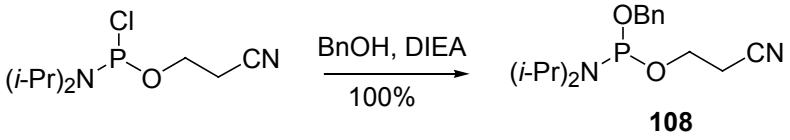
Scheme 3.14. Stability of alkene to *t*-BuOOH



3.5. Synthesis of trans and cis isostere peptidomimetics using Rink amide MBHA resin

Although global phosphorylation to give the *N*-methylcarboxamide **106** was successful, we turned to the building block method due to its success in synthesizing the cis peptidomimetics. Bailing Xu successfully synthesized the cis peptidomimetic Ac–Phe–Phe–pSer–Ψ[(*E*)CH=C]–Pro–Arg–NH_2, **113** (Scheme 3.20), in 17% yield starting from the phosphate building block **110** (Scheme 3.17).[161] These syntheses were improved to synthesize enough of the peptidomimetics for bioassays, NMR study, and X-ray studies. In order to make the comparison of cis and trans Pin1 substrate analogues more meaningful, we embarked on the route to Ac–Phe–Phe–pSer–Ψ[(*E*)CH=C]–Pro–Arg–NH_2 via the same method for making its cis counterpart.

Scheme 3.15. Synthesis of the phosphorylation reagent



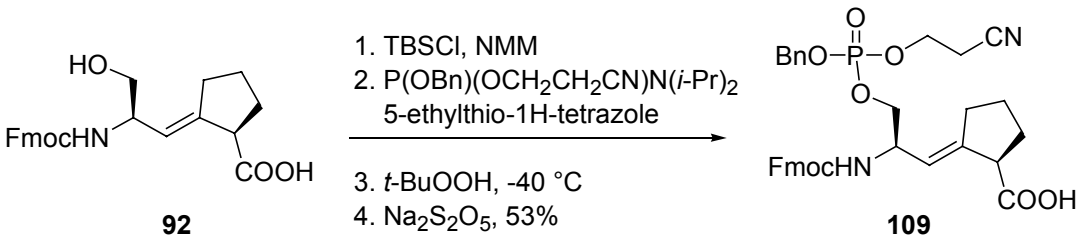
108

The unsymmetrical phosphoramidite, *O*-benzyl-*O*-*β*-cyanoethyl-*N*,*N*-diisopropyl-hosphoramidite, **108**, was originally used as a phosphorylation reagent for the synthesis of a glycolipid (Scheme 3.15).[162] The *β*-cyanoethyl group can be removed by piperidine simultaneously with Fmoc deprotection to leave the phosphate monoanion, which is the most stable form of phosphoserine in peptide synthesis.[163] We employed it in an interassembly procedure during solid phase synthesis to phosphorylate peptide intermediates possessing an N-terminal Fmoc-Ser.[161] The phosphoramidite **108** was

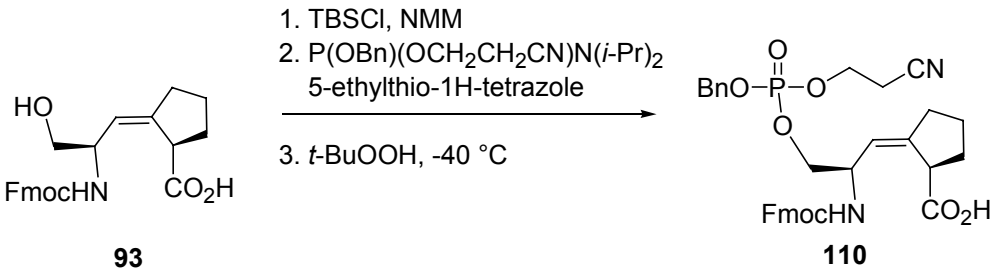
produced in an excellent yield (100%) in a one-step reaction with chloro-*O*-*β*-cyanoethyl-*N*,*N*-diisopropylphosphoamidite mixed with BnOH and DIEA in ether at 0 °C (Scheme 3.15).

The phosphorylation of Fmoc–SerΨ[(*E*)CH=C]–Pro–OH, **92**, and Fmoc–SerΨ[(*Z*)CH=C]–Pro–OH, **93**, was accomplished in a "one pot" reaction according to published procedures with minor modifications (Scheme 3.16).[164] In this procedure, Each Fmoc protected isostere was treated with one equivalent each of TBSCl and NMM, which selectively blocked the carboxyl group and left the side-chain hydroxyl group free. Phosphitylation by **108** and 5-ethylthio-1*H*-tetrazole followed by oxidation with *tert*-butyl hydroperoxide and simple aqueous acid work-up gave the protected phosphodipeptide isosteres **109** and **110** in 45% and 53% yield, respectively (Scheme 3.16 and Scheme 3.17).

Scheme 3.16. Synthesis of trans phosphorylated intermediate



Scheme 3.17. Synthesis of cis phosphorylated intermediate



93 **110**

Originally, the standard Fmoc chemistry using Rink amide MBHA resin with HOBt/HBTU as the coupling reagents and DIEA as base in NMP[165,166] was utilized to synthesize the trans peptide mimic, except that only 0.7 equiv of the (*E*)-alkene building block **109** was used (Scheme 3.18). The side chain of arginine was protected with the Pbf group. Analysis of the crude peptidomimetic by LC-MS showed that the major peak had the desired *m*/*z* of 756.4, but there was no alkene proton peak in the ^{1}H NMR spectra of any of the products after separation by preparative HPLC.[167] This suggested that the HOBt activated ester of (*E*)-alkene **109** isomerized to the α,β-unsaturated carbonyl compound. The major product was the isomerized alkene, **111**, yet only the desired β,γ-unsaturated product **112** (Scheme 3.19, *vida infra*) was obtained using coupling conditions that suppress racemization of activated esters. Indeed, intermediate **92** was shown by NMR to isomerize under HBTU/HOBt activation conditions (Section 3.6). Interestingly, there were no signs of isomerization of (*Z*)-alkene **110** under similar conditions.[161] These results are the opposite of disubstituted alkene peptide isosteres in which *cis*-alkenes have been shown to be more susceptible to alkene isomerization than *trans*-alkenes.[79]

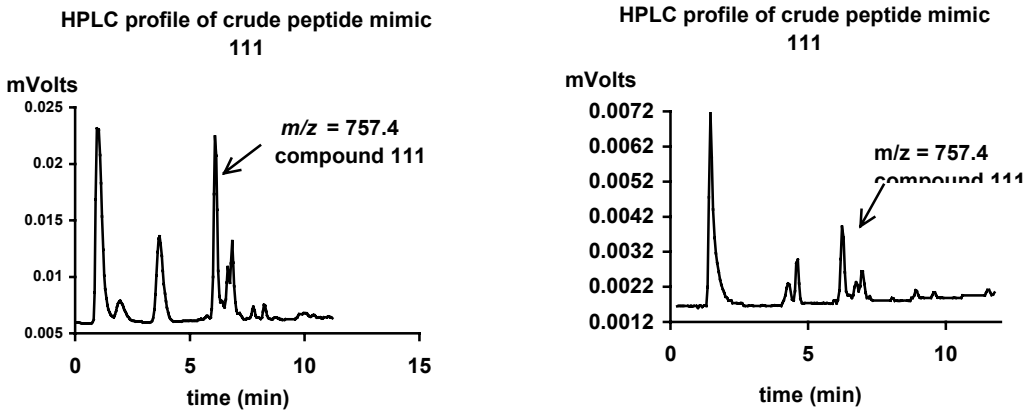
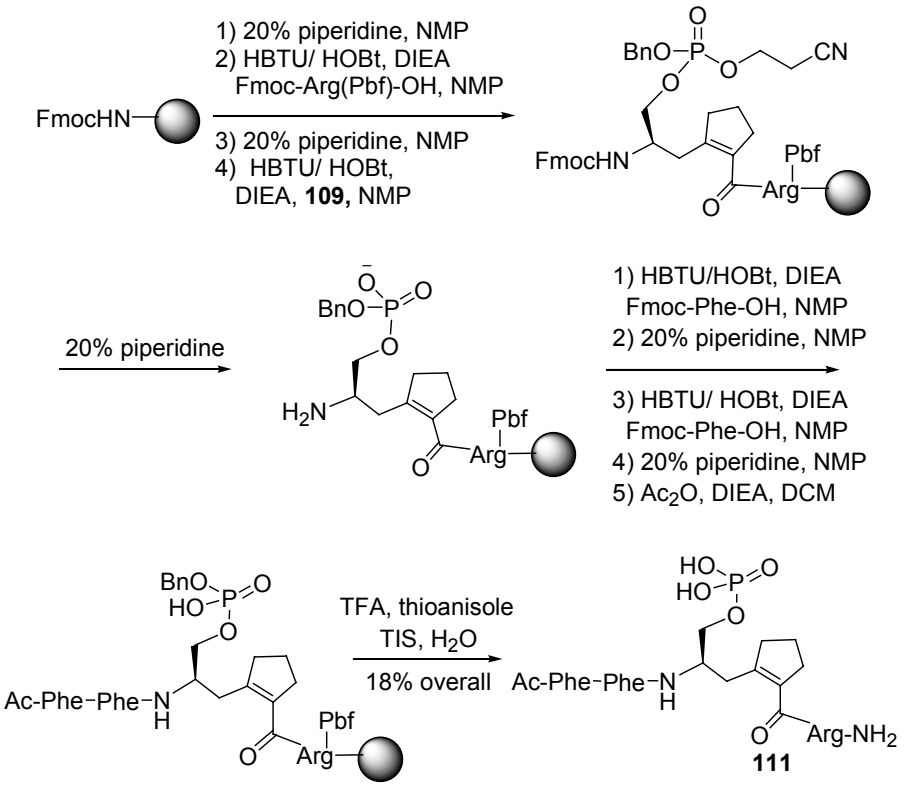


Figure 3.2. The HPLC profile of SPPS of the crude trans pentapeptide mimic **111**.

These two chromatographs are from different reaction batches. HPLC conditions: maintain 15% B for 1 min, then do a gradient from 15% B to 40% B over 10 min on a 100 × 4.6 mm Varian Polaris C_{18} reverse phase analytical column at 2.0 mL min^{-1}. A solvent: H_2O containing 0.1% TFA; B solvent: CH_3CN containing 0.1% TFA.

Scheme 3.18. Solid phase synthesis of compound **111**

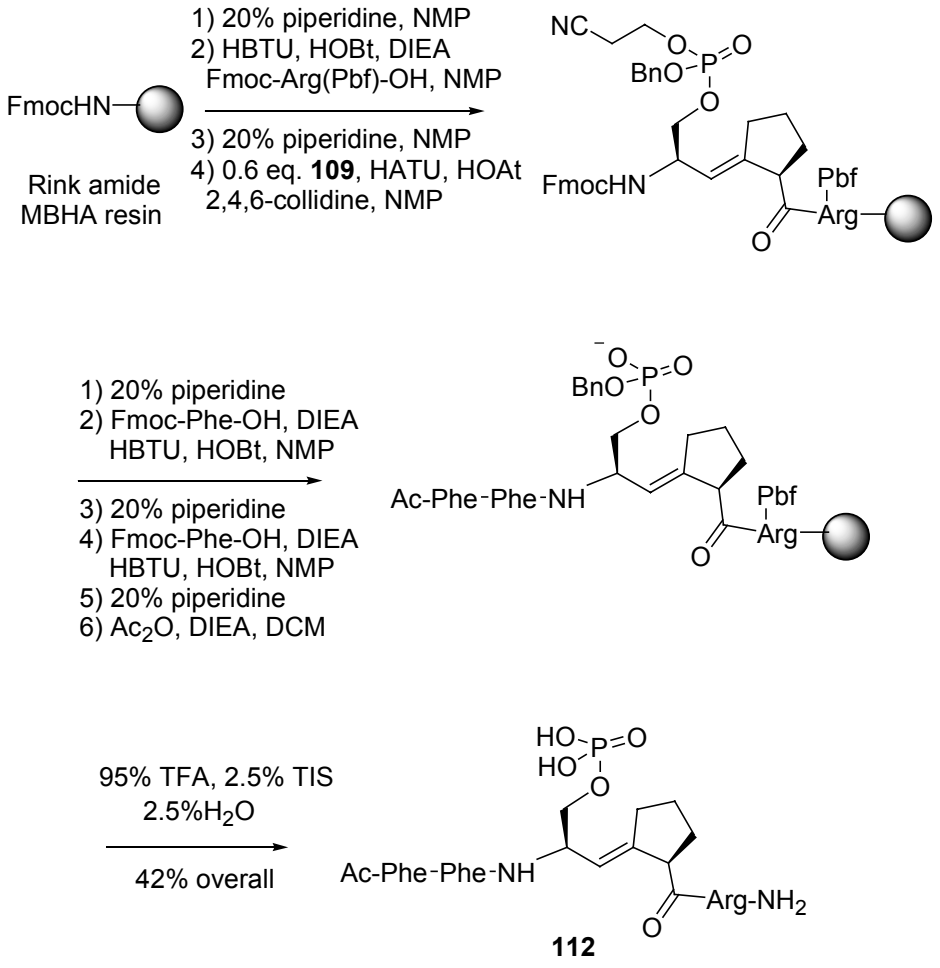


In summary, the double bonds in Boc–Ala–Ψ[(*Z*)CH=C]–Pro–OH, Boc–Ser–Ψ[(*Z*/*E*)CH=C]–Pro–OH were inert to isomerization under fairly strong acid (Jones reagent) and base (NH_3/Na) conditions.[87,121] The double bond in Boc–Ala–Ψ[(*Z*)CH=C]–Pro–OH was also stable both under conditions of amide coupling to the activated ester and toward heating with tertiary amine base.[88] Fmoc–Ser(PO(OBn)(OCH_2CH_2CN))Ψ[(*Z*)CH=C]–Pro–OH did not isomerize under amino acid coupling conditions (HOBt/HBTU and DIEA).[161] On the other hand, Fmoc–Ser (PO (OBn)(OCH_2CH_2CN))Ψ[(*E*)CH=C]–Pro–OH and Fmoc–Ser(TBS)Ψ[(*E*)CH=C]–Pro–OH isomerized rapidly under amino acid coupling conditions to form α,β-unsaturated alkenes. The tendency of isomerization of exocylic alkenes or dipeptide alkene isosteres might be an interesting issue to address by computational chemistry. In the literature, some other (*E*)-alkene dipeptide isosteres were shown to be stable toward isomerization.[168,169]

Because this alkene isomerization and amino acid racemization have similar mechanisms that are both due to the acidity of the proton alpha to the activated ester, coupling conditions that minimize racemization would prevent alkene isomerization. Carpino and co-workers have shown that HOAt and its corresponding uronium salt, HATU, are more effective activating reagents in avoiding racemization of amino acids during peptide synthesis.[170-173] When using optimized SPPS conditions and coupling (*E*)-alkene **109** with a combination of HATU/HOAt and the hindered base 2,4,6-collidine,[171] the desired peptide mimic **112,** Ac–Phe–Phe–pSer–Ψ[(*E*)CH=H]–Pro–Arg–NH_2, was obtained as the major product in 42% overall yield after HPLC purification (Scheme

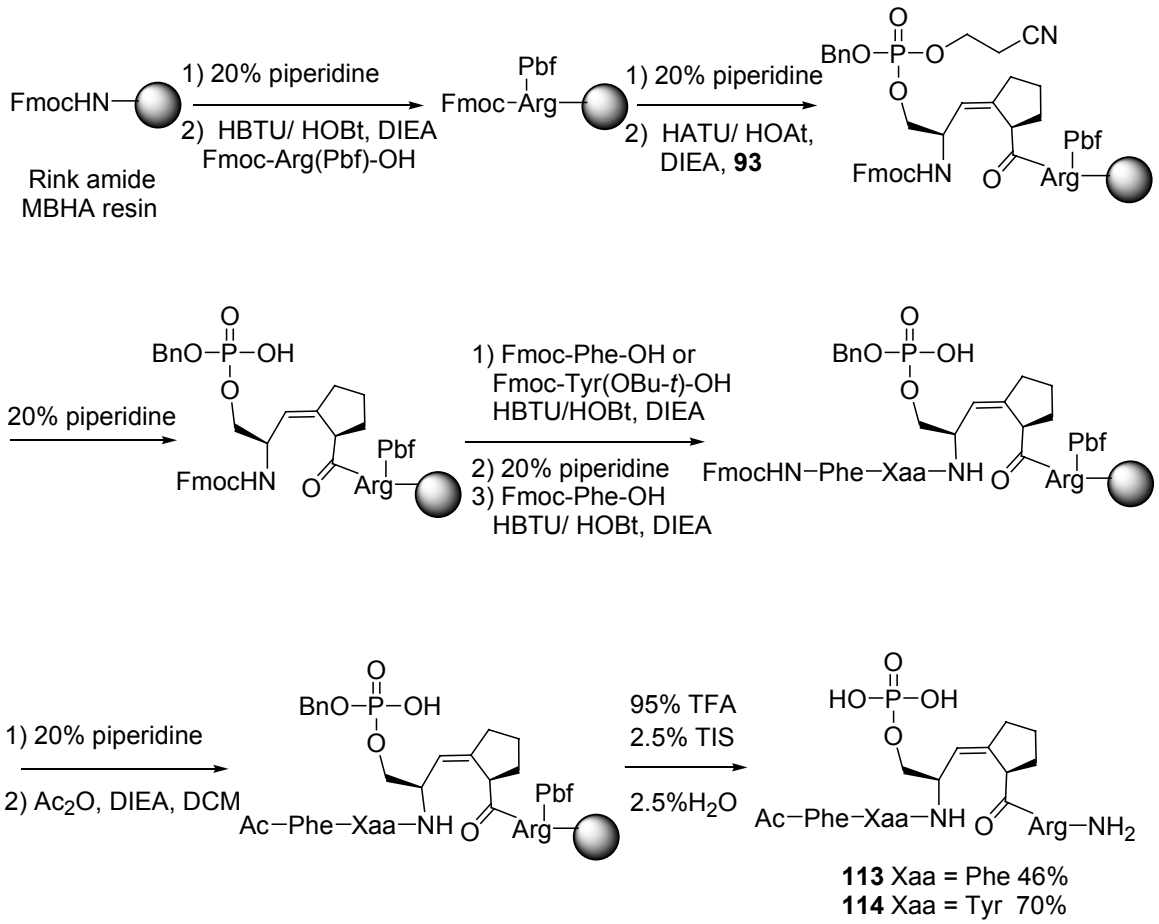
3.19). These optimized conditions include: 1) shorter coupling time, 20 min for each amino acid and 90 min for the dipeptide mimic. Double couplings were conducted for natural amino acids if Kaiser test indicated the first coupling was not quantitative. 2) After coupling the dipeptide building block onto the resin, the cyanoethyl group was removed simultaneously with Fmoc in 20 min using 20% piperidine. 3) Acetic acid washing to remove residual NMP and drying over KOH for overnight were performed just prior to the cleavage of the peptide from the resin. 4) The peptide was cleavage with 95% TFA, 2.5%TIS, and 2.5% water.

Scheme 3.19. Synthesis of the trans peptidomimetic using Rink MHBA resin



Peptide synthesis incorporating the block-phosphorylated cis isostere **110** into cis peptidomimetics Ac–Phe–Phe–pSerΨ[(*Z*)CH=C]–Pro–Arg–NH_2, **113,** and Ac–Phe–Tyr–pSerΨ[(*Z*)CH=C]–Pro–Arg–NH_2, **114,** was successful using the same method as for the synthesis of the trans peptidomimetics (Scheme 3.20). Boc protection on tyrosine hydroxyl group was used as recommended for SPPS methods. After HPLC purification, the peptidomimetics **113** and **114** were obtained as white solids in high purity in ca. 46% and 72% yield, respectively.

Scheme 3.20. Synthesis of cis peptidomimetics using Rink MHBA resin

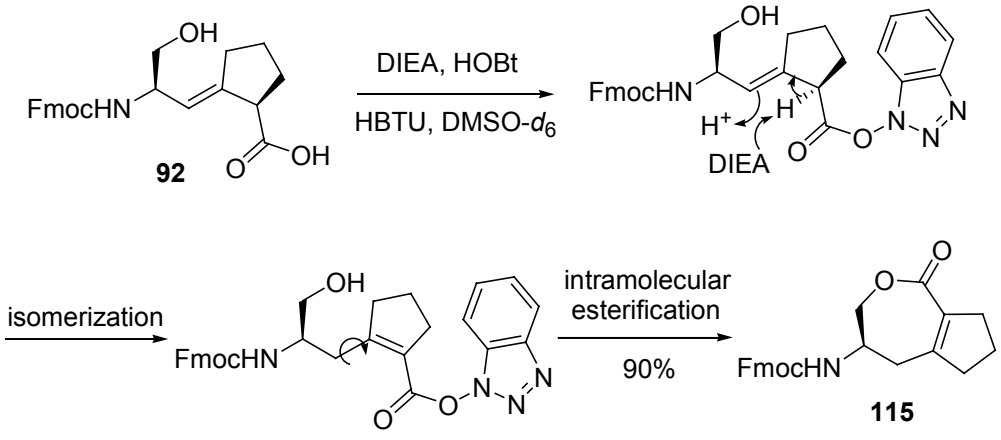


3.6. Isomerization study of the (Z)-alkene and (*E*)-alkene isosteres by ^{1}H NMR

In order to provide solid evidence for the isomerization of the (*E*)-alkene isosteres under normal amino acid coupling conditions, as well as to demonstrate the isomerization reactivity of the (*E*)-alkene isosteres vs. the (*Z*)-alkene isosteres, a milligram scale reaction of Fmoc–SerΨ[(*E*)CH=C]–Pro–OH with standard Fmoc SPPS reagents HOBt, HBTU, and DIEA was run in a NMR tube. The initial plan was to monitor the loss of the proton peak from the exocyclic alkene by recording the ^{1}H NMR every 10 to 20 min. Before HOBt, HBTU and DIEA were added, the ^{1}H NMR of Fmoc–SerΨ[(*E*)CH=C]–Pro–OH **92** in DMSO-d_6 was obtained as the spectrum at time 0 min. Three equivalents of HOBt and HBTU and six equivalents of DIEA were added as in the SPPS of the trans peptidomimetic **111**. The NMR data collected within 20 min showed no trace of the exocyclic alkene proton peak, indicating the isomerization occurred exceptionally fast.

After work-up, a nonpolar solid was separated exclusively and proved to be a Fmoc protected lactone by the ^{1}H and ^{13}C NMR spectra, indicating that Fmoc–SerΨ[(*E*)CH=C]–Pro–OH **92** immediately underwent isomerization upon the addition of coupling reagents, followed by cyclization to form an intramolecular ester **115**.

Scheme 3.21. Isomerization of the (*E*)-alkene isostere and intramolecular ester formation



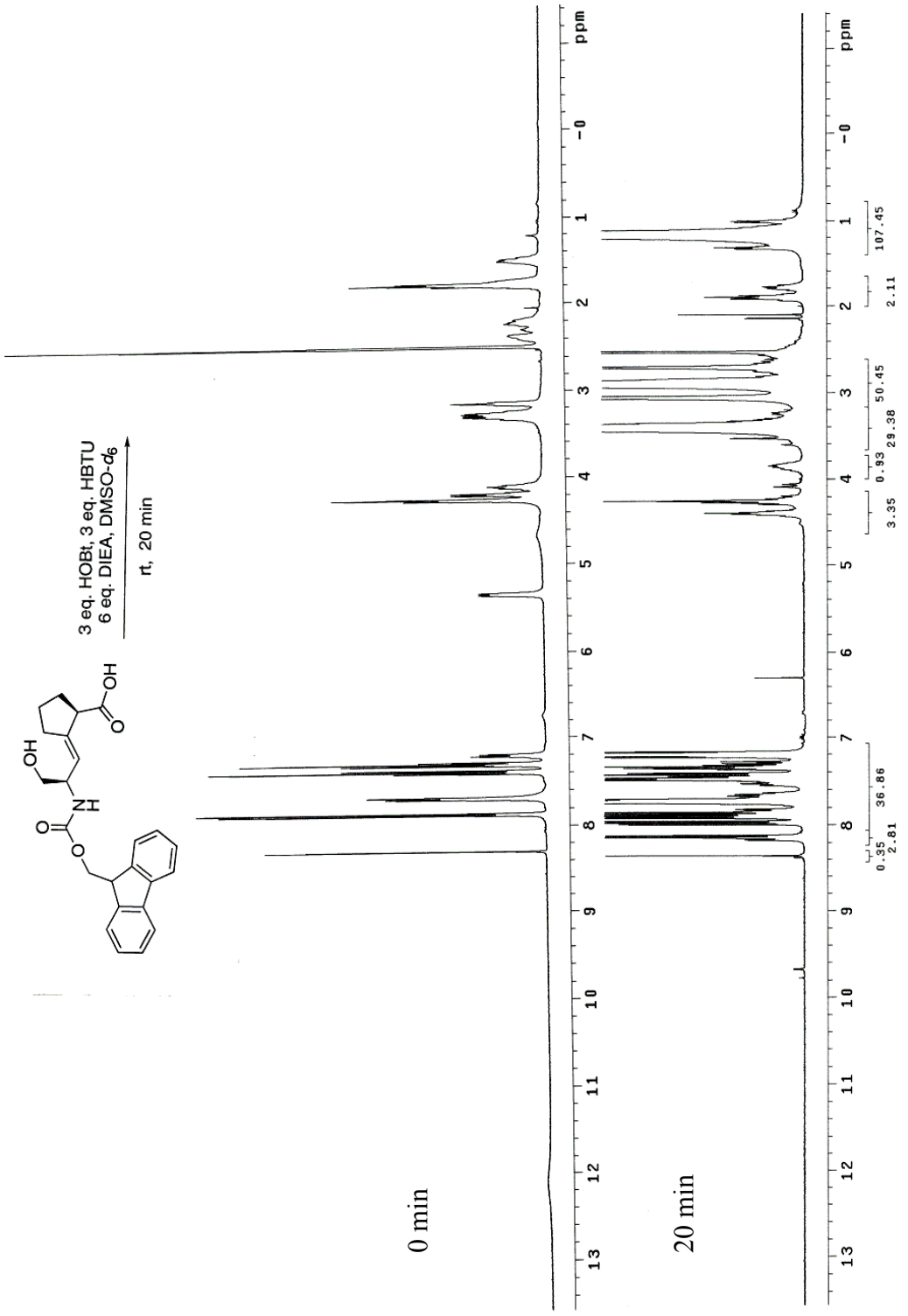


Figure 3.3. ^{1}H MNR study on the isomerization of the trans activated ester

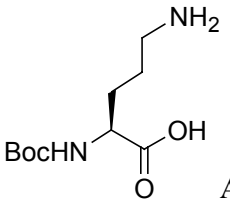
3.7. Conclusions

We designed, synthesized, and assayed a series of conformationally locked inhibitors of the PPIase Pin1, based loosely on the optimal hexapeptide Pin1 substrate Ac–Trp–Phe–Tyr–pSer–Pro–Arg–*p*NA.[36] The central pSer-Pro core of the Pin1 substrate was replaced by (*Z*)-and (*E*)-alkene analogues. They were synthesized on solid-phase resin from Fmoc-protected, phosphorylated building blocks **109** and **110** in yields of 42% for the trans analogue **112,** 46% for the cis analogue **113**, and 70% for the cis analogure**114**. The trans dipeptide isostere **109** was coupled with HATU/HOAt and 2,4,6-collidine to minimize the isomerization of the alkene. Trans *N*-methylamide analogue **106** was also synthesized on solid-phase resin in 7% yield.

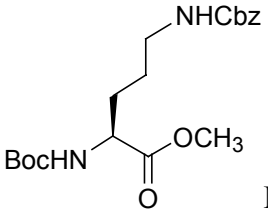
Experimental

General. Unless otherwise indicated, all reactions were carried out under N_2 in flame-dried glassware. THF and CH_2Cl_2 were dried by passage through dry alumina. Anhydrous DMF (99.8%) and NMM were used directly from sealed bottles. Peptide synthesis grade DMF, DIEA, and NMP were purchased. Brine (NaCl), $NaHCO_3$, and NH_4Cl refer to saturated aqueous solutions unless otherwise noted. Flash chromatography was performed on 32-63 μm or 230-400 mesh silica gel with reagent grade solvents. NMR spectra were obtained at ambient temperature in $CDCl_3$ unless otherwise noted. Proton, carbon-13, and phosphorus-31 NMR spectra were obtained at 500, 125, and 162 MHz, respectively, unless otherwise noted. Coupling constants *J* are given in Hz. Analytical HPLC was performed on a 5 μm RP C18 column, 100 × 4.4 mm, semipreparative HPLC on a 5 μm RP C18 column, 100 × 21.2 mm or on a 10 μm RP C4 column 250 × 22 mm, and preparative HPLC on a 100 × 50 mm RP C18 column, using

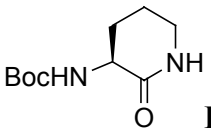
solvents (A) 0.1% TFA in H_2O, and (B) 0.1% TFA in CH_3CN, with UV detection at 220 nm unless otherwise noted.



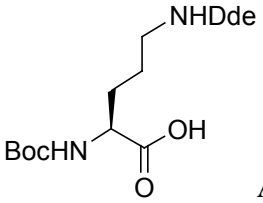
Acid (84): Boc–Orn(Cbz)–OH (183.2 mg, 0.5 mmol) was dissolved in MeOH (5 mL) in a Parr Shaker hydrogenation bottle and degassed with argon for 15 min. Pd/C (10%, 12.5 mg) was added and the solution was shaken for 2 hr at rt under 45 psi of H_2. The catalyst was removed by filtration and the filtrate was concentrated to give 111.3 mg (96.2% yield) deprotected amino acid as a white solid. ^{1}H NMR (D_2O) δ 3.91-3.83 (m, br, 1H), 2.99 (t, J = 7.2, 2H), 1.81-1.64 (m, 4H), 1.41 (s, 9H).



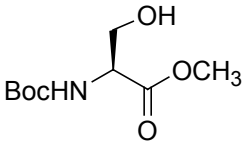
Methyl ester (85): Boc–Orn(Cbz)–OH (183 mg, 0.50 mmol) and triethylamine (102 μL, 0.75 mmol) were dissolved in CH_2Cl_2 (2 mL) and cooled to 0 °C. DMAP (6.1 mg, 0.05 mmol) and methyl chloroformate (56.7 mg, 46 μL, 0.6 mmol) were added. The reaction mixture was stirred for 1 h, diluted with CH_2Cl_2 to 30 mL, washed with sat. aq. NH_4Cl (2 × 10 mL), dried on Na_2SO_4 and concentrated. Chromatography on silica gel with 40% EtOAc in hexane gave 172 mg (91% yield) ester as colorless oil. ^{1}H NMR δ 7.38-7.28 (m, 5H), 5.08 (s, 3H), 4.88 (s, br, 1H), 4.30 (m, 1H), 3.72 (s, 3H), 3.21 (dd, J = 6.60, 12.60, 2H), 1.82 (m, 1H), 1.68-1.50 (m, 3H), 1.43 (s, 9H).



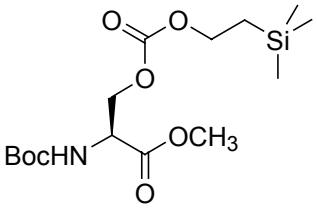
Lactam (86): Ester **85** (157 mg, 0.41 mmol) was dissolved in MeOH (5 mL) in a Parr Shaker hydrogenation bottle and degassed with Ar for 15 min. 10% Pd/C (10.2 mg) was added and the solution was shaken for 2 h at rt. under 45 psi of H_2. The catalyst was removed by filtration on celite and the filtrate was concentrated. Chromatography with 5% MeOH in $CHCl_3$ on silica gel yielded 82 mg (93% yield) lactam as colorless oil. ^{1}H NMR δ 6.73 (s, br, 1H), 5.50 (d, J = 4.8, 1H), 3.99 (m, 1H), 3.28 (m, 2H), 2.41 (m, 1H), 1.91-1.77 (m, 2H), 1.62-1.52 (m, 1H), 1.41 (s, 9H). ^{13}C NMR δ 172.2, 156.1, 79.8, 51.6, 41.9, 28.6, 28.0, 21.2. FAB$^+$-MS = 215 $(M+1/e)^+$, 429 $[2M+1)/e]^+$.



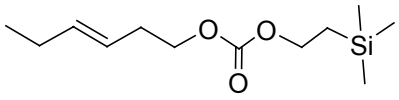
Acid (87): Boc–Orn–OH (116 mg, 0.50 mmol) and 2-acetyldimedone (91 mg, 0.50 mmol) were dissolved in MeOH. After refluxing for 1 h, additional 2-acetyldimedone (91 mg, 0.50 mmol) was added. The reaction mixture was refluxed for an additional 24 h and then the solvent was evaporated. Chromatography on silica gel with 10% MeOH in $CHCl_3$, then 30% MeOH in $CHCl_3$ gave 188 mg (95% yield) of product as colorless glassy oil. ^{1}H NMR δ 5.40 (d, 1H), 4.38 (m, 1H), 3.48 (m, 2H), 2.56 (s, 3H), 2.37 (s, 4H), 2.00 (m, 1H), 1.84 (m, 3H), 1.45 (s, 9H), 1.02 (s, 6H).



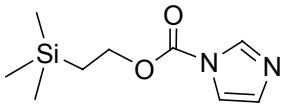
Boc–Ser–OMe (88): Serine methyl ester hydrochloride **36** (311 mg, 2.00 mmol) and Boc_2O (524 mg, 2.4 mmol) were dissolved in CH_2Cl_2 (10 mL). Triethyl amine (202 mg, 2.0 mmol) was added and the reaction mixture was stirred for 4 h at rt. The reaction was diluted with chloroform (20 mL), washed with NH_4Cl (10 mL), $NaHCO_3$ (10 mL), dried over $MgSO_4$, then concentrated. The residue was dissolved in MeOH (10 mL) and washed with hexanes (3 × 10 mL). After MeOH was removed by rotary-evaporation, 0.418 g of a colorless syrup (yield 95.4%) was obtained.



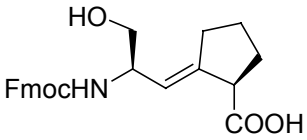
Silyl ethyl carbonate (89): To a solution of imidazolide **91** (106 mg, 0.50 mmol) at 0 °C was added dropwise a solution of methyl Boc-serine ester **88** (110 mg, 0.50 mmol) in CH_2Cl_2 (1 mL). 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU, 15 mg, 0.1 mmol) was added and the reaction was stirred for 0.5 h. HCl (0.2 N, 2.5 mL) was added and the aqueous layer was extracted with EtOAc (3 × 15 mL) and dried on Na_2SO_4. Chromatography on silica gel with 5% EtOAc in hexane, then 10% EtOAc in hexane gave 57 mg (31% yield) of product. ^{1}H NMR δ 5.34 (d, 1 H), 4.54 (m, 1H), 4.47 (dd, J = 11.00, 3.45, 1H), 4.35 (dd, J = 10.90, 3.55, 2H), 4.21-4.18 (m, 2H), 3.74 (s, 3H), 1.42 (s, 9H), 1.04-1.00 (m, 2H), 0.01 (s, 9H).



Carbonate (90): Imidazolide **91** (425 mg, 2.0 mmol) and DBU (64 mg, 0.40 mmol) were dissolved in CH_2Cl_2 (7 mL). 3-*trans*-hexene-1-ol (200 mg, 2.0 mmol) in CH_2Cl_2 (10 mL) was added dropwise at 0 °C. The mixture was stirred for 72 h, then diluted with EtOAc (40 mL), washed with NH_4Cl (2 × 10 mL), brine (10 mL), dried on $MgSO_4$ and concentrated. Chromatography on silica gel with 2% EtOAc in hexane gave 465 mg (yield 95%) of a colorless liquid. ^{1}H NMR δ 5.57-5.49 (m, 1H), 5.37-5.29 (m, 1H), 4.19-4.15 (m, 2H), 4.07 (t, J = 7.0, 2H), 2.33-2.28 (m, 2H), 2.00-1.93 (m, 2H), 1.03-0.99 (m, 2H), 0.92 (t, J = 7.40, 3H), 0.00 (s, 9H).

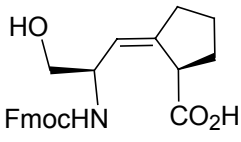


1-[[2-(trimethylsilyl)ethoxy]carbonyl]imidazole (91):[141] Carbonyl-diimidazole (CDI, 3.89 g, 24.0 mmol) was added to a solution of 2-(trimethylsilyl)ethanol (2.87 mL, 20 mmol) in benzene (20 mL). The resulting mixture was stirred at rt for 1 h then diluted with CH_2Cl_2 (200 mL), then washed quickly with ice-cold water. The organic layer was filtered and concentrated. Chromatography on silica gel with 1:1 hexane-Et_2O gave 3.54 g (yield 83%) of product as white crystals. ^{1}H NMR δ 8.12 (s, 1H), 7.41 (t, J = 1.38, 1H), 7.05 (dd, J = 1.60, 0.95, 1H), 4.51-4.48 (m, 2H), 1.18-1.15 (m, 2H), 0.08 (s, 9H).



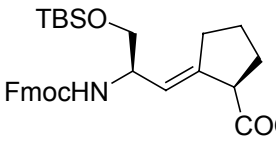
Fmoc–Ser–Ψ[(*E*)CH=C]–Pro–OH (92). Boc–Ser–Ψ[(*E*)CH=C]–Pro–OH, **59** (0.72 g, 2.5 mmol), was dissolved in a mixture of TFA (4.0

mL) and CH_2Cl_2 (12.0 mL). Triethylsilane (0.98 mL, 6.2 mmol) was added via syringe. The reaction mixture was stirred for 30 min, and the solvent was evaporated. The remaining TFA and triethylsilane were removed under vacuum at rt. Without further purification, the crude product was dissolved in a mixture of dioxane (10 mL), $NaHCO_3$ (5 mL), and saturated aqueous Na_2CO_3 (5 mL). The mixture was cooled to 0 °C. Fmoc-Cl (764 mg, 2.95 mmol) was added slowly, and the reaction was stirred at 0 °C for 3 h. H_2O (20 mL) was added, and the mixture was washed with $CHCl_3$ (3 × 20 mL). The aqueous layer was acidified with 2 N HCl to pH 3 and extracted with $CHCl_3$ (5 × 30 mL). The combined organic layers were dried with $MgSO_4$ and concentrated. Chromatography on silica gel with 2% MeOH, 5% MeOH, then 10% MeOH in $CHCl_3$ gave 650 mg (54%) of **92** as a white foam, mp 55-56 °C. ^{1}H NMR (DMSO-d_6) δ 12.16 (br s, 1H), 7.88 (d, J = 7.4, 2H), 7.71 (d, J = 7.4, 2H), 7.41 (t, J = 7.5, 2H), 7.32 (t, J = 7.6, 3H), 5.30 (d, J = 8.3, 1H), 4.67 (br s, 1H), 4.25-4.18 (m, 3H), 4.10 (m, 1H), 3.33 (dd, J = 10.8, 7.1, 1H), 3.22 (dd, J = 10.6, 6.0, 1H), 2.97 (m, 1H), 2.30 (m, 1H), 2.19 (m, 1H), 1.85 (m, 1H), 1.76-1.63 (m, 2H), 1.45 (m, 1H). ^{13}C NMR (DMSO-d_6) δ 175.3, 156.2, 144.5, 144.0, 141.2, 128.1, 127.5, 125.7, 121.9, 120.6, 65.8, 63.8, 53.5, 49.5, 47.3, 30.0, 29.5, 24.9. HRMS calcd. for $C_{24}H_{26}NO_5$ (MH^+) m/z = 408.1811, found m/z = 408.1812.



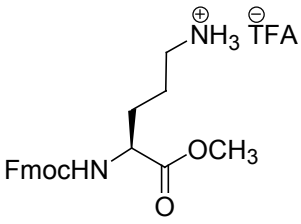
Fmoc–Ser–Ψ[(*Z*)CH=C]–Pro–OH (93). Boc–Ser–Ψ[(*Z*)CH=C]–Pro–OH, **79** (114 mg, 0.40 mmol), was dissolved in a solution of 1:3 TFA:CH_2Cl_2 (10 mL) at 0 °C. The reaction mixture was stirred for 45 min at rt and the solvent was evaporated. The remaining TFA was removed under vacuum at rt. Without further

purification, the crude product was dissolved in a 10% aqueous Na_2CO_3 (2.0 mL), then cooled to 0 °C. A solution of Fmoc-Cl (114 mg, 0.44 mmol) in dioxane (2.0 mL) was added slowly to the above reaction mixture and stirred at rt for 3 h. The reaction mixture was diluted with H_2O (30 mL) and extracted with ether (2 × 20 mL). The aqueous layer was acidified with 1 N HCl to pH 3, and extracted with EtOAc (3 × 30 mL) and CH_2Cl_2 (3 × 30 mL). The combined organic layer was dried over $MgSO_4$ and concentrated to give 120 mg of the crude product. Chromatography with 0.5% acetic acid and 5% MeOH in CH_2Cl_2 eluted 94 mg (58%) product as a white solid. ^{1}H NMR (DMSO-d_6) δ 12.1 (br s, 1H), 7.87 (d, J = 7.6, 2H), 7.71 (d, J = 7.6, 2H), 7.40 (app. t, J = 7.4, 2H), 7.32 (app. t, J = 7.4 2H), 7.12 (d, J = 7.6, 1H), 5.31 (d, J = 9.2, 1H), 4.65 (br s, 1H), 4.24-4.17 (m, 4H), 3.44 (m, 1H), 3.38 (dd, J = 10.6, 5.4, 1H), 3.24 (m, 1H), 2.31 (m, 1H), 2.22 (m, 1H), 1.88 (m, 2H), 1.74 (m, 1H), 1.53 (m, 1H). ^{13}C NMR (DMSO-d_6) δ 175.2, 155.3, 144.0, 143.9, 143.0, 140.7, 127.6, 127.0, 125.3, 122.2, 120.0, 65.3, 63.7, 52.4, 46.7, 45.4, 33.4, 31.1, 24.1. HRMS calcd. for $C_{24}H_{26}NO_5$ (MH^+) m/z = 408.1811, found m/z = 408.1806.

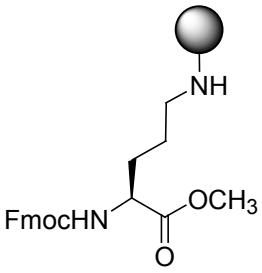


Fmoc–Ser(TBS)–Ψ[(*E*)CH=C]–Pro–OH (94). Fmoc–SerΨ[(*E*)CH=C]–Pro–OH, **92** (465 mg, 1.12 mmol) and imidazole (381 mg, 5.60 mmol) were dissolved in DMF (4.0 mL), and TBSCl (422 mg, 2.80 mmol) was added. The mixture was stirred for 16 h, and then NH_4Cl (20 mL) was added. The mixture was stirred for an additional 50 min, and then diluted with EtOAc (30 mL), washed with NH_4Cl (2 × 10 mL), dried with $MgSO_4$, and concentrated. Chromatography on silica gel with 0.1% acetic acid/30% EtOAc/hexanes gave 450 mg (76%) of **94** as a colorless foam.

mp 62-63 °C. ^{1}H NMR (DMSO-d_6) δ 7.88 (d, J = 7.4, 2H), 7.68 (d, J = 7.4, 2H), 7.41 (t, J = 7.5, 2H), 7.31 (t, J = 7.2, 2H), 7.28 (d, J = 8.5, 1H), 5.37 (d, J = 7.6, 1H), 4.27 (m, 2H), 4.16 (m, 2H), 3.50 (dd, J = 10.1, 6.7, 1H), 3.40 (dd, J = 9.9, 6.7, 1H), 3.17 (t, J = 7.1, 1H), 2.35 (m, 1H), 2.26 (m, 1H), 1.80 (m, 3H), 1.53 (m, 1H), 0.82 (s, 9H), -0.01 (d, J = 2.8, 6H). ^{13}C NMR (DMSO-d_6) δ 175.2, 156.2, 144.6, 144.5, 141.3, 128.1, 127.6, 125.8, 121.3, 120.7, 65.9, 65.3, 53.1, 49.6, 47.3, 30.1, 29.7, 26.3, 25.0, 18.5, -4.8, -4.9. Anal. Calcd for $C_{30}H_{39}NO_5Si$: C, 69.06; H, 7.53; N, 2.68. Found: C, 68.98; H, 7.62; N, 2.70.



Methyl ester (95). Fmoc–Orn(Boc)–OH (0.91 g, 2 mmol) and triethylamine (0.4 mL, 3 mmol) were dissolved in CH_2Cl_2 (8 mL) and cooled to 0°C. DMAP (25 mg, 0.2 mmol) and methyl chloroformate (227 mg, 2.4 mmol) were added, The reaction mixture was stirred for 2 h and diluted with CH_2Cl_2 to 100 mL, washed with sat. aq. NH_4Cl (3 × 20 mL), dried on Na_2SO_4 and concentrated. ^{1}H NMR δ Without further purification, the crude product was dissolve in 25% TFA in CH_2Cl_2 (18 mL) and stirred for 10 min. the reaction mixture was concentrated to give 0.964 g (100% crude yield) TFA salt as a pale yellow oil. ^{1}H NMR δ



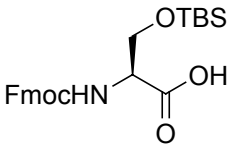
Fmoc–Orn–OMe on 2-chlorotrityl chloride resin (97). Fmoc–Orn($NH_3^+TFA^-$)–OMe **95** (465.2 mg, 1 mmol) was dissolved in dry CH_2Cl_2 (14 mL) containing DIEA (3.48 mL, 20 mmol). The mixture was added to 2-chloro tritylchloride resin (1.4 mmol/g, 1.43 g, 2 mmol). The reaction was vortex shaken 16 h then washed with 17:2:1 CH_2Cl_2:MeOH:DIEA (3 × 5 mL), DMF (2 × 5mL), DCM (3 × 5 mL), dried in vacuo over KOH overnight. Loading 0.48 mmol/g (yield 43%).

Fmoc loading UV test was carried out on Agilent 8453A UV/VIS spectrophotometer. Loaded resin (5.9 mg and 7.1 mg) was weighed into two 10 mL volumeric flask and freshly prepared 20% piperidine in DMF was added to 10 mL. After 5 h, the solutions were agitated and 1 mL of each solution was transferred to a pair of 1 cm UV cuvettes separately and the UV absorbance was measured at λ= 290 nm. The UV absorbance of the same 20% piperidine solution was measured as blank. Fmoc loading was calculated by[174]:

$$\text{Fmoc loading} = (\text{mg of resin per mL}) \times (A_{abs}\text{-}A_{ref})/1.65$$

$$\varepsilon = 1.65 \text{ for Fmoc at } \lambda = 290 \text{ nm}$$

A_{abs}-A_{ref} were 1.38 and 1.73, respectively. Average loading was 0.48 mmol/g (yield 43 ± 2.9%).

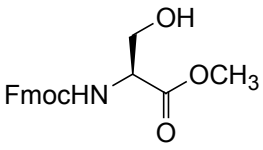


Fmoc–Ser(TBS)–OH (99): Fmoc-serine (1.05 g, 3.2 mmol) and imidazole (1.09 g, 16.0 mmol) were dissolved in DMF (6.4 mL) and cooled to 0 °C. *tert*-Butyl dimethylsilylchloride (1.21 g, 8.0 mmol) was added and the reaction mixture was stirred for 3 h. The reaction was diluted with CH_2Cl_2 to 200 mL, washed with NH_4Cl (3 × 40 mL), H_2O (40 mL), dried on Na_2SO_4 and concentrated. Chromatography on silica gel with 30% EtOAc in hexanes to elute impurities then 5% MeOH in $CHCl_3$ yielded 1.39 g (yield 98%) of a colorless oil. ^{1}H NMR δ 7.76 (d, J = 7.6, 2H), 7.61 (t, J = 8.6, 2H), 7.39 (t, J = 7.5, 2H), 7.30 (t, J = 7.5, 2H), 5.66 (d, J = 8.3, 1H), 4.46 (m, 1H), 4.37 (m, 2H), 4.25 (m, 1H), 4.14 (dd, J = 2.6, 10.2, 1H), 3.89 (dd, J = 3.7, 10.1, 1H), 0.89 (s, 9H), 0.07 (d, J = 5.3, 6H).

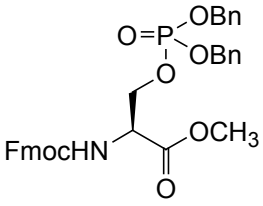
Peptide Ac–Phe–Phe–Ser–Pro–Orn–OMe (98): Fmoc–Orn–OMe on resin (200 mg) was swelled in NMP for 2 h, then the peptide was elongated by standard Fmoc solid phase methods as follows: N-terminus deprotections were performed with 50% morpholine in NMP (2 × 20 min); washes between steps were performed with NMP (5 ×); couplings were performed with 3 eq of Fmoc amino acids for 30 min in DMF (0.5 mL)/NMP (0.5 mL), activated with 3 eq HOBt, 3 eq HBTU and 6 eq DIEA and were double coupled. Free amines were capped with 10% Ac_2O/ 10% DIEA in CH_2Cl_2 for 5 min after completion of each double coupling. Serine was incorporated as the Fmoc-Ser(OTBS)-OH to prevent side chain acylation. The completed peptide was deprotected at the N-terminus and the final acetylation was carried out for 10 min.

$Bu_4N^+ \cdot F^-$ in THF (1.0 M THF, 0.18 mL, 0.18 mmol) and 0.5 mL THF were added to the resin and shaken for 16 hr. After washing with CH_2Cl_2 (5 ×), (*N*,*N*-diisopropyl)dibenzyl-phosphoramidite (104 mg, 0.30 mmol) and tetrazole (70 mg, 1.0 mmol) were dissolved in 1:1 CH_2Cl_2:THF (1 mL) and added to the resin. After shaking for 10 h, aqueous 30% H_2O_2 (0.6 mL) in CH_2Cl_2 (1.4 mL) was added and the mixture was shaken 2 h. The resin was washed with CH_2Cl_2 (5 ×), shrunk with MeOH and dried in vacuo overnight.

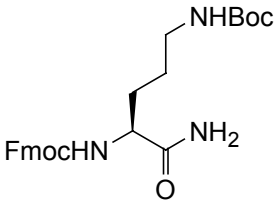
The dried resin was treated with 95% TFA in CH_2Cl_2 (2 mL) 3 h. FAB(+)-MS indicated the presence of Ac–Phe–Phe–Ser–Pro–Orn–OMe (m+1) = 667.3, but not Ac–Phe–Phe–phosphoSer–Pro–Orn–OMe (m+1) = 747.3.



Fmoc-Ser methyl ester (100): To 4 mL of MeOH at 0 °C was added $SOCl_2$ (1.5 mL, 3.0 mmol) dropwise, followed by Fmoc-Serine (327 mg, 1.0 mmol). After stirring for 5 h at rt, the solvent was removed and chromatography with 5% MeOH in CH_2Cl_2 on silica gel gave 327 mg (yield 96%) of a white solid. m.p. 111-112 °C. ^{1}H NMR δ 7.76 (dd, J = 0.95, 7.6, 2H), 7.60 (m, 2H), 7.40 (td, J = 7.5, 2H), 7.32 (tt, J = 7.4, 2H), 5.72 (d, J = 6.9, 1H), 4.44 (m, 3H), 4.23 (t, J = 6.9, 1H), 4.00 (dd, J = 2.3, 11.0, 1H), 3.93 (dd, J = 2.1, 10.8, 1H) 3.79 (s, 3H), 2.20 (br, s, 1H).

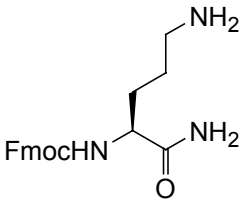


Phosphate (101): Methyl ester **100** (68 mg, 0.20 mmol), dibenzyldiisopropylphosphoramidite (138 mg, 0.40 mmol), and 1H-tetrazole (46 mg, 0.66 mmol) were dissolved in CH_2Cl_2 (3 mL) and stirred for 40 min. Aqueous 30% H_2O_2 (0.10 mL, 0.66 mmol) was added and stirred for 1 h, and the reaction was quenched with sodium thiosulfate (10%, 1 mL), diluted with $CHCl_3$ (25 mL), washed with $NaHCO_3$ (2 × 10 mL), dried on $MgSO_4$, and concentrated. Chromatography with 20% EtOAc in hexanes to elute impurities, then 40% EtOAc in hexanes gave 64 mg (53%) of a white solid. m.p. 129-130 °C. ^{1}H NMR δ 7.76 (d, J = 7.6, 2H), 7.60 (dd, J = 4.8, 7.1, 2H), 7.39 (t, J = 7.5, 2H), 7.32 (m, 12H), 5.78 (d, J = 8.3, 1H), 5.02 (m, 4H), 4.54 (m, 1H), 4.42 (m, 2H), 4.32 (dd, J = 7.3, 10.5, 1H), 4.23 (m, 2H), 3.72 (s, 3H). ^{13}C NMR δ 169.4, 155.9, 143.9, 143.8, 141.4, 135.6 (dd, J = 2.9, 6.7), 128.8, 128.7, 128.1 (d, J = 1.9), 127.8, 127.2, 125.2 (d, J = 6.7), 120.1, 69.8 (dd, J = 3.4, 5.3), 67.4, 67.4 (d, J = 4.8), 54.5 (d, J = 7.7), 53.0, 47.1. ^{31}P NMR δ -0.869. MS-FAB(+): calcd. $(M+1)^+$/e=602.2 Found. $(M+1)^+$/e=602.2.

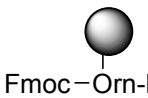


Amide (102): Fmoc–Orn(Boc)–OH (5.46 g, 12 mmol) was dissolved in a mixture of DMF (12 mL) and CH_2Cl_2 (48 mL) and cooled to 0 °C. HOBt (2.11 g, 14.4 mmol) and EDC (2.76 g, 14.4 mmol) were added. After stirring 40 min, 28% aqueous NH_4OH (1.2 mL, 12 mmol) was added and the reaction mixture was stirred

for another 1 h. The reaction was diluted to 200 mL with $CHCl_3$, $NaHCO_3$ was added (100 mL), and the white precipitate was removed by filtration. Recrystallization with MeOH and $CHCl_3$ gave 4.51 g (yield 83%) of a white solid. ^{1}H NMR (DMSO-d_6) δ 7.89 (d, J = 7.6, 2H), 7.73 (d, J = 7.4, 2H), 7.43-7.37 (m, 3H), 7.33 (t, J = 7.4, 2H), 7.29 (s, 1H), 7.00 (s, 1H), 6.82 (t, J = 5.2, 1H), 4.23 (m, 3H), 3.91 (m, 1H), 2.91 (m, 2H), 1.61 (m, 1H), 1.50 (m, 1H), 1.37 (s, 9H). ^{13}C NMR (DMSO-d_6) δ 174.5, 156.5, 156.2, 144.5, 144.4, 141.3, 128.2, 127.6, 125.9, 120.7, 78.0, 66.2, 54.8, 47.2, 29.9, 28.9, 26.8.

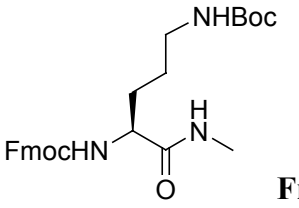


Amine (103): Amide **102** (3.63 g, 8.0 mmol) was dissolved in 25% TFA in CH_2Cl_2 (32 mL) and stirred 1.5 h at rt. The solvent was evaporated. The residue was neutralized with saturated aqueous $NaHCO_3$ until gas generation ceased. The white solid was washed with H_2O (50 mL), $CHCl_3$ (50 mL), and recrystalized with MeOH (cal. 25 mL) to give 2.83 g (yield 100%) of a white solid. ^{1}H NMR (DMSO-d_6) δ 7.90-7.82 (m, 3H), 7.73 (d, 1H), 7.42-7.29 (m, 6H), 6.97 (s, 1H), 6.89 (s, 1H), 6.72 (t, 1H), 4.22 (m, 2H), 3.89 (m, 1H), 3.05 (t, 1H), 2.92 (m, 2H), 1.63-1.30 (m, 4H).

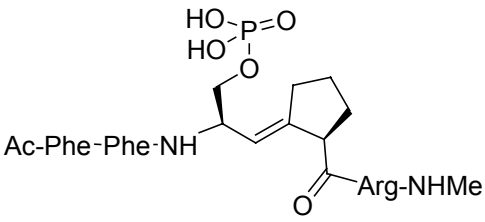


Fmoc-Orn-NH_2 on 2-Chlorotrityl chloride resin (104): Fmoc–Orn–NH_2 **103** (0.82 g, 2.3 mmol) was slurried in dry DMF (6.6 mL) (insoluble) and the white solid and DMF mixture was added to 2-chloro tritylchloride resin (1.5 mmol/g, 0.66 g, 1 mmol). The reaction was vortex shaken 8 h at rt then washed with 17:2:1

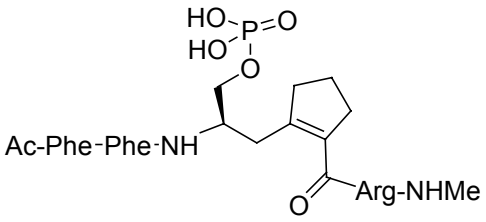
CH_2Cl_2:MeOH:DIEA (3 ×), MeOH until the white solid was washed away, then DMF (2 ×), DCM (3 ×), dried in vacuo over KOH to give **104** with a loading of 0.14 mmol/g (yield 9%). Loading was determined by the same UV test as compound **97**.



Fmoc–Orn(Boc)–NHMe (105). Fmoc–Orn(Boc)–OH (4.55 g, 10.0 mmol), HOBt (1.84 g, 12.0 mmol), and EDC (2.30 g, 12.0 mmol) were dissolved in 4:1 CH_2Cl_2:DMF (50 mL). After the mixture was stirred for 30 min, methylamine (2.0 M in THF, 10.0 mL, 20.0 mmol) was added and the mixture was stirred for another 45 min. The mixture was diluted with $CHCl_3$ (50 mL), washed with $NaHCO_3$ (3 × 20 mL), dried over Na_2SO_4, and concentrated. Recrystallization from 4:1 Et_2O: MeOH (cal. 60 mL) gave 4.2 g (90%) of **105** as a white solid. mp 101-102 °C. ^{1}H NMR δ 7.75 (d, J = 7.6, 2H), 7.58 (m, 2H), 7.38 (t, J = 7.3, 2H), 7.29 (t, J = 7.5, 2H), 6.62 (br s, 1H), 5.72 (d, J = 7.1, 1H), 4.74 (br s, 1H), 4.37 (m, 3H), 4.19 (t, J = 7.0, 1H), 3.38 (m, 1H), 3.06 (m, 1H), 2.78 (d, J = 4.4, 3H), 1.78 (br s, 1H), 1.63-1.46 (m, 3H), 1.43 (s, 9H). ^{13}C NMR δ 172.7, 156.9, 156.5, 144.0, 143.9, 141.4, 127.8, 127.2, 125.2, 120.1, 79.5, 67.0, 53.5, 47.3, 39.0, 30.4, 28.5, 26.7, 26.3.



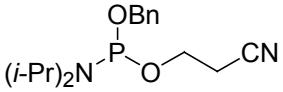
and



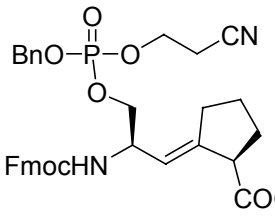
Ac–Phe–Phe–pSer–Ψ[(*E*)CH=C]–Pro–Arg–NHMe (106) and isomer (107). Fmoc–Orn(Boc)–NHMe, **105** (1.1 g, 2.4 mmol), was dissolved in a mixture of CH_2Cl_2 (7.5 mL) and TFA (2.5 mL) and stirred for 1 h, and then the solvent was removed in vacuo. The residue was triturated with ether, and the ether was removed by pipet. After drying in vacuo, the white solid was dissolved in DMF (5 mL), neutralized with DIEA (2.1 mL, 4.5 mmol), and stirred for 1 h. The resulting mixture was added to 2-chlorotrityl chloride resin[152,153] (900 mg, 1.5 mmol/g, 1.35 mmol) preswelled with CH_2Cl_2. The resin was shaken for 14 h, washed with 17:2:1 CH_2Cl_2:MeOH:DIEA (3 × 7 mL), CH_2Cl_2 (3 × 7 mL), DMF (2 × 7 mL), and CH_2Cl_2 (2 × 7 mL), and dried in vacuo over KOH to give 1.3 g (66% yield, loading[155] 0.67 mmol/g). The Fmoc–Orn–NHMe loaded resin (200 mg, 0.67 mmol/g, 0.13 mmol) was swelled in NMP (3 × 2 mL) and treated with 20% piperidine in NMP (3 × 3 mL) for 10 min each. After the resin was washed with NMP (5 × 4 mL), a solution containing Fmoc–Ser(TBS)–Ψ[(*E*)CH=C]–Pro–OH, **94** (70 mg, 0.13 mmol), HATU (153 mg, 0.40 mmol), HOAt (55 mg, 0.40 mmol), and DIEA (0.14 mL, 0.80 mmol) in 1:1 DMF/NMP (1 mL) was added to the resin. Double coupling was performed for 0.5 h each with vortex agitation. After the resin was washed with NMP, Fmoc was cleaved with 20% piperidine/NMP (3 × 2 mL) 10 min each. Fmoc–Phe–OH (156 mg, 0.40 mmol), HATU (153 mg, 0.40 mmol), HOAt (55 mg, 0.40 mmol), and DIEA (0.14 mL, 0.80 mmol) in 1:1 DMF:NMP (1 mL) were double coupled for 3 h each. Coupling of the second Fmoc-Phe-OH was carried out as for the first one, with a shorter coupling time (30 min each). Ac_2O capping with 10% Ac_2O, 10% DIEA in CH_2Cl_2 (2 mL) was carried out after each coupling for 5 min, and the final capping was conducted for 10 min. $Bu_4N^+F^-$ (1.0 M in THF, 1.3 mL, 1.3 mmol) diluted with THF (0.9 mL), was

added to the resin, and the reaction was shaken 3 h. After the resin was washed with CH_2Cl_2 (5 × 4 mL), (*N*,*N*-diisopropyl)dibenzylphosphoramidite (463 mg, 1.34 mmol) and tetrazole (375 mg, 5.36 mmol) were dissolved in NMP (1.8 mL) and added to the resin. After shaking under N_2 for 2 h, NMP was removed in vacuo. Aqueous 30% H_2O_2 (0.6 mL) in CH_2Cl_2 (1.0 mL) was added. The resin was shaken for an additional 2 h and then washed with CH_2Cl_2 (5 × 4 mL), shrunk with MeOH, and dried in vacuo. The dried resin was treated with a mixture of 95% TFA, 1% H_2O, and 4% CH_2Cl_2 (1.5 mL) for 4 h, rinsed with CH_2Cl_2, and the solution was concentrated. Ether was added to the oily residue to precipitate the product as a white solid. After the ether solution was removed via a pipet, the solid was dissolved in 1:1 DMF:DMSO (3 mL). Triethylamine (0.14 mL) and 3,5-dimethylpyrazole-1-carboxamidine nitrate (145 mg, 0.721 mmol) were added. The reaction was stirred at 45 °C for 7 h. Water (10 mL) was added to the crude product and lyophilized. Analytical HPLC (RP C_{18} 250 × 4.4 mm, 10% B for 1 min, 10- 100% B over 30 min, 210 nm) showed two major products with retention times of 12.5 and 12.9 min. The separation of these two peaks was enhanced by changing the HPLC conditions. Elution at 20 mL/ min with 20% B for 10 min, then increasing to 50% B over 25 min on the C4 semipreparative column, gave **107** (20.0 min, 3.4 mg, 3.5%) and **106** (21.8 min, 6.9 mg, 7.0%) as white solids. Analytical data for **107**: HPLC RP C_{18} 250 × 4.4 mm, 20% B for 10 min, 20-50% B over 25 min, 210 nm, ret. time 23.9 min, purity 97.3%. The alkene peak of **107** was missing in the ^{1}H NMR. ^{1}H NMR (DMSO-d_6) δ 8.36 (d, J = 6.9, 1H), 8.07 (d, J = 8.0, 2H), 8.03 (d, J = 8.3, 1H), 7.95 (d, J = 4.6, 1H), 7.69 (d, J = 7.1, 1H), 7.26-7.14 (m, 13H), 6.58 (br s, 2H), 4.44 (m, 2H), 4.28 (m, 1H), 3.97 (m, 1H), 3.75-3.64 (m, 2H), 3.09 (dd, J = 14.5, 7.1, 2H), 3.03 (m, 1H), 2.96-2.89 (m, 2H), 2.76 (dd, J =

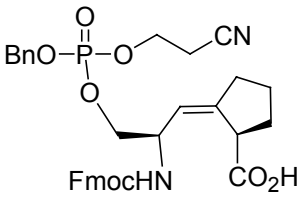
13.6, 9.0, 1H), 2.69-2.63 (m, 2H), 2.59 (m, 3H), 2.37 (m, 2H), 1.77- 1.42 (m, 11H). ^{31}P NMR (DMSO-d_6) δ -0.41. MALDI-TOF calcd. for $C_{36}H_{52}N_8O_9P$ (MH^+) m/z = 771.4, found m/z = 770.4. Analytical data for **106** (21.8 min): HPLC RP C_{18} 250 × 4.4 mm, 20% B for 10 min, 20-50% B over 25 min, 210 nm, ret. time 24.8 min, purity 99.0%. ^{1}H NMR (DMSO-d_6) δ 8.39 (br s, 1H), 8.15 (d, J = 8.3, 1H), 8.08 (d, J = 8.3, 1H), 7.97 (m, 2H), 7.25-7.15 (m, 14H), 6.58 (br s, 1H), 5.40 (d, J = 8.5, 1H), 4.45 (m, 3H), 4.22 (dd, J = 14.1, 8.8, 1H), 3.71 (m, 2H), 3.26 (t, J = 6.8, 2H), 3.10 (m, 1H), 3.01 (dd, J = 13.7, 4.5, 2H), 2.93 (dd, J = 13.9, 4.0, 1H), 2.82 (dd, J = 13.6, 8.8, 1H), 2.67 (dd, J = 13.8, 10.3, 1H), 2.58 (d, J = 3.9, 3H), 2.38 (m, 1H), 2.21 (m, 1H), 1.90-1.38 (m, 11H). ^{31}P NMR (DMSO-d_6) δ -0.43. MALDI-TOF calcd. for $C_{36}H_{52}N_8O_9P$ (MH^+) m/z = 771.4, found m/z = 770.3. HRMS calcd. for $C_{36}H_{52}N_8O_9P$ (MH^+) m/z = 771.3595, found m/z = 771.3555.



***O*-Benzyl-*O*-β-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (108).** This is a modification of the method of Crich.[162] A solution of BnOH (433 mg, 4.0 mmol) and DIEA (1.4 mL, 8.0 mmol) in ether (4 mL) was added to an ice-cooled (0 °C) solution of chloro-*O*-β-cyanoethyl-*N,N*-diisopropylphosphoramidite (950 mg, 4.0 mmol) in ether (6 mL). The solution was stirred for 2 h at rt and the salt was removed by filtration. The filtrate was concentrated to give 2.26 g **108** (with DIEA, 100%)) as a light yellow oil, which was used in the next step without further purification. ^{1}H NMR δ 7.23-7.38 (m, 5H), 4.76-4.64 (m, 2H), 3.85 (m, 2H), 3.66 (m, 2H), 2.62 (t, J = 6.3, 2H), 1.20 (t, J = 6.5, 12H). ^{31}P NMR δ 148.5.

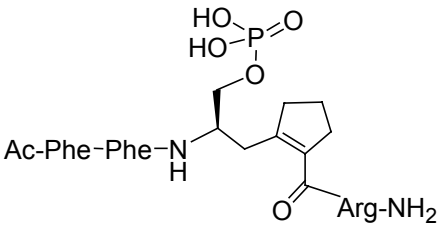


Fmoc–Ser(PO(OBn)(OCH$_2$CH$_2$CN))–Ψ[(*E*)CH=C]–Pro–OH (109). To a stirred solution of Fmoc–SerΨ[(*E*)CH=C]–Pro–OH, **92** (204 mg, 0.50 mmol), in THF (5 mL) was added *N*-methylmorpholine (51 mg, 0.50 mmol), followed by TBSCl (75 mg, 0.50 mmol). After 30 min, a solution of **108** (308 mg, 1.0 mmol) in THF (2 mL) was added, followed by 5-ethylthio-1*H*-tetrazole (260 mg, 2.0 mmol) in one portion. The reaction mixture was stirred for 2 h at rt, then cooled to -40 °C, and *tert*-butyl hydroperoxide (5 M in decane, 0.4 mL, 2.0 mmol) was added. The cold bath was removed. After being stirred for 30 min, the mixture was again cooled to 0 °C, and 5 mL of 10% aqueous $Na_2S_2O_5$ was added. The mixture was transferred for separation using ether (80 mL). The organic layer was washed with 10% aqueous $Na_2S_2O_5$ (10 mL), brine (10 mL), dried over $MgSO_4$, and concentrated. Chromatography on silica gel with 5% MeOH in CH_2Cl_2 gave 170 mg (53%) of **109** as a colorless syrup. 1H NMR (DMSO-d_6) δ 12.15, 7.88 (d, J = 7.6, 2H), 7.68 (d, J = 6.9, 2H), 7.54 (d, J = 8.7, 1H), 7.42-7.29 (m, 9H), 5.40 (d, J = 7.1, 1H), 5.04 (dd, J = 3.5, 7.6, 2H), 4.39 (m, 1H), 4.30 (m, 2H), 4.21 (m, 1H), 4.11 (m, 2H), 3.91 (m, 1H), 3.85 (m, 1H), 3.20 (m, 1H), 2.87 (m, 2H), 2.35 (m, 1H), 2.25 (m, 1H), 1.85-1.76 (m, 3H), 1.55 (m, 1H). ^{13}C NMR (DMSO-d_6) δ 174.5, 155.6, 145.6, 143.8 (d, J_{PC} = 13.4), 140.7, 135.8, 128.5, 128.4, 127.9, 127.6, 127.0, 125.1, 120.1, 119.0, 118.2, 68.7, 67.9, 65.4, 62.2, 50.6, 49.1, 46.7, 29.5, 29.0, 24.3, 19.0 (d, J_{PC} = 7.7). ^{31}P NMR (DMSO-d_6) δ -1.81, -1.84. HRMS calcd. for $C_{34}H_{36}N_2O_8P$ (MH^+) m/z = 631.2209, found m/z = 631.2198.

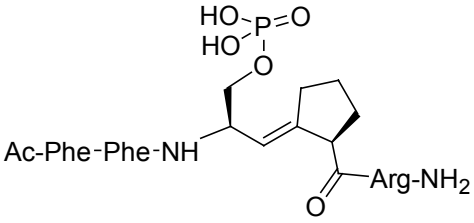


Fmoc–Ser(PO(OBn)(OCH$_2$CH$_2$CN))–Ψ[(*Z*)CH=C]–Pro–OH (110). NMM (25.3 mg, 0.25 mmol) was added to a stirred solution of Fmoc–Ser–Ψ[(*Z*)CH=C]–Pro–OH, **93** (100 mg, 0.25 mmol), in THF (2 mL), followed by *tert*-butyldimethylsilyl chloride (TBSCl) (41.5 mg, 0.27 mmol). After 30 min, a solution of *O*-benzyl-*O*-*β*-cyanoethyl-*N*,*N*-diisopropylphosphoramidite, **108** (154 mg, 0.5 mmol) in THF (1 mL) was added, followed by 5-ethylthio-1*H*-tetrazole (130 mg, 1.0 mmol) in one portion. The reaction mixture was stirred for 3 h at rt, then cooled to –40 °C, and *tert*-butyl hydroperoxide (5 M in decane, 100 μL, 0.5 mmol) was added. The cold bath was removed. After being stirred for 30 min, the mixture again was cooled to –40 °C, and 4 mL of 10% aqueous $Na_2S_2O_5$ was added. The mixture was transferred for separation using ether (2 × 40 mL). The organic layer was dried over $MgSO_4$, and concentrated. Chromatography on silica gel with 10% acetone in CH_2Cl_2 to remove impurities, then 1% acetic acid and 10% acetone in CH_2Cl_2 eluted 96 mg (62%) of **110** as a colorless syrup. ^{1}H NMR (DMSO-d_6) δ 12.19 (br s, 1H), 7.90 (d, J = 7.6, 2H), 7.69 (d, J = 7.2, 2H), 7.50 (d, J = 7.6, 1H), 7.43-7.29 (m, 9H), 5.37 (d, J = 8.4, 1H), 5.04 (dd, J = 3.8, 7.8, 2H), 4.50 (m, 1H), 4.29-4.20 (m, 3H), 4.13 (m, 2H), 3.96-3.87 (m, 2H), 3.43 (t, J = 6.0, 1H), 2.88 (m, 2H), 2.31 (m, 1H), 2.24 (m, 1H), 1.87 (m, 3H), 1.73 (m, 1H). ^{13}C NMR (DMSO-d_6) δ 174.9, 155.3, 145.1, 143.9 (d, J_{PC} = 8.3), 140.7, 135.8 (dd, J_{PC} = 2.3, 6.8), 128.5, 128.4, 127.8 (d, J_{PC} = 3.1), 127.6, 127.1, 125.2 (d, J_{PC} = 3.8), 120.1, 119.6, 118.2 (d, J_{PC} = 1.5), 68.7 (d, J_{PC} = 5.3), 68.3, 65.5, 62.3 (dd, J_{PC} = 2.3, 5.3), 50.2 (d, J_{PC} = 8.5), 46.6, 45.5,

33.5, 31.0, 24.1, 19.0 (d, J_{PC} = 7.6). ^{31}P NMR (DMSO-d_6) δ -1.76. HRMS calcd. for $C_{34}H_{36}N_2O_8P$ (MH^+) m/z = 631.2209, found m/z = 631.2216.

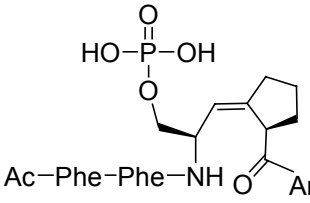


(*E*)-alkene isomer (111). General procedure for peptide synthesis is detailed with the synthesis of **114**. The solid phase synthesis of **111** was performed in a similar manner except standard Fmoc peptide synthesis reagents HOBt/HBTU (3 eq. relative to **109**) and DIEA (3 eq. relative to **109**) were used for the coupling of (*E*)-alkene building block **109**. The main product was obtained as 12.5 mg (18%) of a white solid. ^{1}H NMR (DMSO-d_6) δ 8.38 (d, J = 5.7, 1H), 8.07 (d, J = 7.3, 1H), 8.02 (d, J = 8.3, 1H), 7.59 (d, J = 7.4, 1H), 7.44 (s, 1H), 7.26-7.14 (m, 15H), 4.44 (m, 2H), 4.28 (m, 1H), 3.97 (br s, 1H), 3.73 (br s, 1H), 3.67 (br s, 1H), 3.26 (m, 2H), 3.15-2.35 (m, 8H), 1.71 (m, 9H), 1.51 (m, 2H). HRMS calcd. for $C_{35}H_{50}N_8O_9P$ (MH^+) m/z = 757.3438, found m/z) 757.3413. Except for the *N*-methyl amide at 2.59 ppm, this ^{1}H NMR matches that of **107** obtained in the synthesis of **106**.



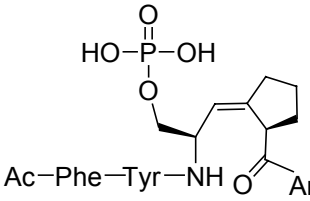
Ac–Phe–Phe–pSer–Ψ[(*E*)CH=C]–Pro–Arg–NH$_2$ (112). General procedure for peptide synthesis is detailed with the synthesis of **114**. The solid phase synthesis of **112** was performed in a similar manner except the synthesis was

conducted on a smaller scale (12 mg of building block, 0.019 mmol) and 2,4,6-collidine was used as base instead of DIEA. The crude product was purified by semipreparative C_{18} HPLC at 15 mL/min, 15% to 30% B over 22 min. Purified **2** (9.3 mg, 16%) eluted at 11.9 min as a white solid. Purity > 99% by analytical HPLC (2 mL/min, 15% B for 1 min, 15 to 40% B over 10 min, ret. time 8.8 min). ^{1}H NMR (DMSO-d_6) δ 11.26 (br s, 2H), 8.15 (d, J = 7.4, 1H), 8.11 (d, J = 8.0, 1H), 8.00 (d, J = 8.5, 1H), 7.98 (d, J = 8.3, 1H), 7.76 (br s, 1H), 7.42 (s, 1H), 7.26-7.15 (m, 11H), 7.08 (s, 1H), 6.85 (br s, 2H), 5.32 (d, J = 7.4, 1H), 4.49 (m, 2H), 4.42 (m, 1H), 4.22 (m, 1H), 3.74 (m, 2H), 3.28 (app. t, J = 7.0, 1H), 3.11 (m, 2H), 3.00 (dd, J = 13.9, 4.9, 1H), 2.91 (dd, J = 14.0, 4.4, 1H), 2.83 (dd, J = 13.9, 8.6, 1H), 2.66 (dd, J = 13.9, 10.0, 1H), 2.33 (m, 1H), 2.22 (m, 1H), 1.83 (m, 2H), 1.76-1.66 (m, 2H), 1.74 (s, 3H), 1.56-1.41 (m, 4H). ^{13}C NMR (DMSO-d_6) δ 173.5, 173.3, 171.0, 169.9, 169.4, 156.7, 147.3, 138.0, 137.4, 129.3, 129.0, 128.0, 126.2, 126.1, 119.4, 66.4, 54.1, 53.7, 51.7, 49.8, 49.0, 40.3, 37.7, 37.1, 29.7, 29.4, 29.2, 25.1,24.8, 22.4. ^{31}P NMR (DMSO-d_6) δ -1.216. HRMS calcd. for $C_{35}H_{50}N_8O_9P$ (MH^+) m/z = 757.3438, found m/z = 757.3475.



Ac–Phe–Phe–pSer–Ψ[(*E*)CH=C]–Pro–Arg–NH$_2$ (113). The solid phase synthesis of **113** was performed in a manner similar to that for **114,** except the synthesis was conducted on a smaller scale (20 mg of building block, 0.032 mmol). Purified **113** was eluted at 12.9 min isocratically with 20% B at 50 mL/min as a white solid (12 mg, 46%). Purity > 99% by analytical HPLC (2 mL/min, 10% B for 1

min, 10 to 90% B over 10 min, ret. time 6.5 min). ^{1}H NMR (DMSO-d_6) δ 8.15 (d, J = 8.0, 1H), 8.02 (d, J = 7.2, 1H), 7.98 (d, J = 8.4, 2H), 7.62 (br s, 1H), 7.32-7.03 (m, 17H), 5.23 (d, J = 8.4, 1H), 4.55 (m, 1H), 4.41 (m, 2H), 4.19 (m, 1H), 3.83 (m, 1H), 3.66 (m, 1H), 3.52 (t, 1H), 3.09 (m, 2H), 3.01 (dd, J = 4.0, 14.0, 1H), 2.88 (dd, J = 4.0, 14.0, 1H), 2.79 (dd, J = 10.0, 14.0, 1H), 2.67 (dd, J = 10.0, 14.0, 1H), 2.32 (m, 1H), 2.24 (m, 1H), 1.85 (m, 2H), 1.75-1.68 (m, 5H), 1.63 (m, 1H), 1.49 (m, 3H). ^{13}C NMR (DMSO-d_6) δ 173.6, 172.8, 171.2, 170.5, 169.7, 156.7, 145.3, 137.9, 137.7, 129.2, 129.0, 128.1, 128.0, 126.2, 120.2, 66.4, 54.1, 52.1, 49.2, 46.3, 40.5, 37.6, 36.8, 34.0, 31.7, 28.9, 24.9, 24.2, 22.4. ^{31}P NMR (DMSO-d_6): δ 0.081. HRMS calcd. for $C_{35}H_{50}N_8O_9P$ (MH^+) m/z = 757.3438, found m/z = 757.3476.



Ac–Phe–Tyr–pSer–Ψ[(*Z*)CH=C]–Pro–Arg–NH$_2$ (114). Manual solid-phase synthesis of **114** was performed in 5 mL disposable polypropylene columns by standard Fmoc chemistry. Rink amide MBHA resin[165] (156 mg, 0.10 mol, 0.64 mmol/g) was swelled in CH_2Cl_2 (1 × 3 mL, 10 min) and NMP (3 mL, 10 min). Amino acids (Arg, Tyr, and Phe) were either coupled once (Tyr) or double coupled (Arg and Phe). For each amino acid coupling cycle, the N-protecting Fmoc group was removed with 20% piperidine in NMP (2 × 3 mL, 10 min each). After the mixture was washed with NMP (5 × 3 mL, 1 min each), and CH_2Cl_2 (5 × 3 mL, 1 min each), a solution of amino acid, Fmoc–Arg(Pbf)–OH (193 mg, 0.30 mmol) or Fmoc–Tyr(*t*Bu)–OH (138 mg, 0.30 mmol) or Fmoc–Phe–OH (117.0 mg, 0.30 mmol), HBTU[166] (114 mg,

0.30 mmol), HOBt (46 mg, 0.30 mmol), and DIEA (78 mg, 0.60 mmol) in NMP (3 mL) were added to the resin and shaken for 20 min. Double coupling was conducted when the Kaiser test[175] indicated the coupling was not quantitative. For the coupling of the dipeptide isostere, Fmoc–Ser(PO(OBn)(OCH_2CH_2CN))–Ψ[(*Z*)CH=C]–Pro–OH, **110** (44 mg, 0.070 mmol), HATU (0.07 mmol), HOAt (0.07 mmol) and DIEA (0.21 mmol) were added to the resin**,** followed by a solution of **110** in 3 mL NMP. The reaction was agitated for 90 min. The resin was washed with NMP (5 × 3 mL, 1 min each), CH_2Cl_2 (5 × 3 mL, 1 min each), and NMP (5 × 3 mL, 1 min each). The peptide was capped with 10% Ac_2O, 10% DIEA in CH_2Cl_2 (3 mL) for 15 min. The cyanoethyl group was removed with 20% piperidine in NMP simultaneously with Fmoc (2 × 3 mL, 10 min each). Acetylation of the N-terminus was carried out with 10% Ac_2O, 10% DIEA in CH_2Cl_2 (4 mL) for 30 min. The resin then was washed with CH_2Cl_2 (5 × 4 mL, 1 min each), acetic acid (5 × 4 mL, 1 min each), MeOH (5 × 3 mL, 1 min each), and ether (3 × 3 mL, 1 min each) and dried in vacuo over KOH overnight.

The dried resin was treated with a mixture of 95% TFA, 2.5% H_2O, 2.5% triisopropylsilane (TIS) (4 mL) for 4 h, filtered, and rinsed with TFA. The combined solutions were concentrated to a small volume. The crude product was triturated with ether (50 mL), and dried in vacuo to give 80 mg of crude peptide **114**. A 40 mg portion of the crude product was purified by preparative HPLC on a 100 × 212 mm Varian Polaris C_{18} RP column (10 μ). 20 mg (yield 72%) of the product was eluted as a white solid. ^{1}H NMR (DMSO-d_6) δ 9.19 (br, s, 1H), 8.14 (d, J = 8.0, 1H), 7.97 (d, J=7.4, 1H), 7.94 (d, J = 7.4, 1H), 7.87 (d, J = 8.3, 1H), 7.57 (br, s, 1H), 7.40-6.80 (m, 13H), 6.65 (d, J = 8.5, 2H), 5.23 (d, J = 7.6, 1H), 4.54 (m, 1H), 4.41 (m, 1H), 4.34 (m, 1H), 4.19 (dd, J = 7.8,

13.3, 1H), 3.83 (m, 1H), 3.67 (m, 1H), 3.51 (t, J = 6.0, 1H), 3.11 (m, 1H), 2.89 (m, 1H), 2.67 (m, 1H), 2.34 (m, 1H), 2.22 (m, 1H), 1.85 (m, 2H), 1.72 (m, 6H), 1.63 (m, 1H), 1.49 (m, 3H). ^{13}C NMR (DMSO-d_6) δ 173.6, 172.8, 171.1, 170.7, 169.6, 156.7, 155.8, 145.3, 138.0, 130.1, 129.0, 128.0, 127.7, 126.1, 120.2, 114.9, 66.4, 54.4, 54.0, 52.1, 49.2, 46.3, 40.5, 36.8, 33.9, 31.6, 28.8, 24.9, 24.1, 22.4. ^{31}P NMR (DMSO-d_6): δ -1.012. MS-ESI(+) calcd for $C_{35}H_{50}N_8O_{10}P$ (MH^+) m/z = 773.3, found m/z = 773.6.

Kinetic study of the isomerization of Fmoc–SerΨ[(*E*)CH=C]–Pro–OH (92) and characterization for ester (115). Fmoc–SerΨ[(*E*)CH=C]–Pro–OH **92** (15 mg, 0.037 mmol) was dissolved in 0.6 ml DMSO-d_6 in a NMR tube. A ^{1}H NMR of the above solution was taken at rt. HOBt (16.9 mg, 0.11 mmol) and HBTU (41.9 mg, 0.11 mmol) were added to the NMR tube, followed by DIEA (28.5 mg, 0.22 mmol). The reaction was shaken gently for 2 min to completely dissolve and mix the reagents. A ^{1}H NMR was taken immediately. The data collection was completed 20 min later from the exact time HOBt was added. The proton peak from the alkene was missing in the 2nd ^{1}H NMR spectra, indicating that the dipeptide mimic **92** was quantitatively isomerized to the α,β-unsaturated alkene. The reaction was diluted with 0.1 N HCl aq. (1 mL) and extracted with ethyl acetate (3 × 3 mL). The organic layer was dried over $MgSO_4$ and concentrated. Chromatography with 30% ethyl acetate in hexanes gave 13 mg (90.2 yield) of a white solid, compound **115**. ^{1}H NMR δ 7.76 (d, J = 7.6, 2H), 7.57 (d, J=7.6, 2H), 7.40 (t, J = 7.4, 2H), 7.32 (t, J = 7.4, 2H), 5.20 (d J = 6.7, 1H), 2.85-2.70 (m, 3H), 2.57 (m, 3H), 1.87 (m, 2H). ^{13}C NMR (DMSO-d_6) δ 167.1, 155.7, 153.2, 143.7, 141.4, 128.4, 127.8, 127.1, 125.0, 120.0, 69.0, 66.9, 48.0, 47.2, 40.6, 37.0, 35.7, 21.2.

Chapter 4. Pin1 in vitro assay and bioactivities of the conformationally locked peptidomimetics

4.1. Introduction — substrate conformation specificity of PPIases

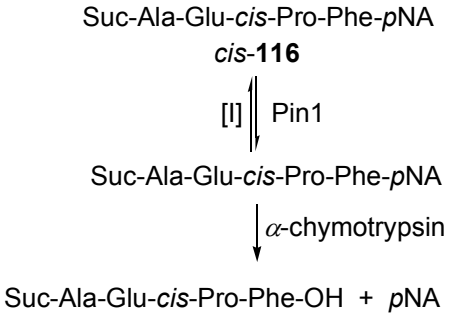
Despite the considerable amount of information provided by investigations on the biology, mechanism, substrates, and inhibitors for PPIases, fundamental questions remain to be answered. What is the conformational specificity for the protein or peptide substrate? Do the PPIases bind one conformational isomer of the substrate tighter than the other? X-ray crystallography has provided the most straightforward evidence of conformational preference for PPIase substrates. Pin1 was first crystallized with the Ala-*cis*-Pro dipeptide bound in the active site, although the specificity for Ala is poor relative to Ser or Thr preceding the Pro.[37] No evidence has been reported for Pin1 specificity for the cis or trans conformation of a longer peptide substrate. X-ray crystal structures of human CyPA (hCyPA) complexes with Xaa-Pro peptides show a preference for *cis*-Pro amides.[176-179] In solution, dynamic NMR showed that the trans to cis isomerization rate was 1.45 times faster than the cis to trans rate for hCyPA and the affinity of the cis substrate was 4-fold greater than the trans isomer,[180] in agreement with the observation of cis substrate in the X-ray structures. Three-dimensional structures of FKBP complexes are limited to FBKP/FK506 or FKBP/FK506 analogues.

An alternative method of elucidating cis/trans substrate conformation specificity is to synthesize conformationally constrained substrate analogues as molecular probes and determine their binding affinity or inhibition constant for the enzyme. This method provides conformational evidence in an environment closer to biological systems. Our hypothesis was that Pin1 would bind either the *cis*- or *trans*-phosphoSer-Pro mimic more

tightly, indicating the preferred conformer in solution. The (*E*/*Z*) alkene analogues of Pin1 substrate serve not only as rationally designed conformational probes, but also as lead compounds for designing Pin1 inhibitors. A protocol of full inhibition assays for Pin1 was needed to determine the inhibition pattern and for measuring the Pin1 inhibition constants (K_i).

4.2. Pin1 in vitro inhibition assay

Scheme 4.1. Pin1 PPIase Inhibition Assay



Several PPIase inhibition assays have been developed.[100,181,182] We adapted the protease-coupled assay of Rich and co-workers for CyP and FKBP to Pin1 (Scheme 4.1).[100] The proteases, chymotrypsin and trypsin, specifically cleave the amide bond between the P_2' and P_3' positions of Xaa-*trans*-Pro-containing peptides.[183] Thus every *cis*-Pro has to isomerize before it can be cleaved. This conformational specificity was manipulated to measure the activity of PPIases.[100] In this case, the *p*-nitroanilide group was cleaved from the commercially available peptide substrate Suc–Ala–Glu–Pro–Phe–*p*NA (Scheme 4.1). The release of the pNA by chymotrypsin cleavage can be directly monitored by UV-VIS spectrometry at 390 nm (or higher wavelength when the concentration of the substrate was higher). We utilized a large excess of α-chymotrypsin (60 mg/mL) to ensure that the cleavage step is very fast; thus the first step, Glu–Pro

isomerization is rate limiting and the rate of release of the *p*NA is equal to the isomerization rate of the Glu-*cis*-Pro amide to the Glu-*trans*-Pro amide. This rate includes both thermal isomerization rate and the Pin1-catalyzed isomerization rate.

4.2.1. Enzyme kinetics and mathematical basis for protease-coupled assay

The enzyme-catalyzed reaction in the simplest model is illustrated in Figure 4.1. Under steady state conditions, and in the case that the spontaneous reaction rate is slow ($k_3 << k_{cat}$), the kinetic equation is represented by Equation (4.1).

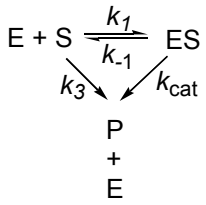


Figure 4.1. Enzyme catalyzed reaction

$$\frac{d[\mathrm{ES}]}{dt} = k_1 \cdot [\mathrm{S}][\mathrm{E}] - k_{-1} \cdot [\mathrm{ES}] - k_{cat} \cdot [\mathrm{ES}] = 0 \quad \text{.....(4.1)}$$

$$[\mathrm{ES}] = \frac{k_1 \cdot [\mathrm{S}][\mathrm{E}]}{k_{-1} + k_{cat}}$$

$$[\mathrm{E}] = [\mathrm{E}]_0 - [\mathrm{ES}] = [\mathrm{E}]_0 - \frac{k_1 \cdot [\mathrm{S}][\mathrm{E}]}{k_{-1} + k_{cat}}$$

$$[\mathrm{E}] + \frac{k_1 \cdot [\mathrm{S}][\mathrm{E}]}{k_{-1} + k_{cat}} = [\mathrm{E}]_0$$

$$\frac{k_{-1} + k_{cat} + k_1 \cdot [\mathrm{S}]}{k_{-1} + k_{cat}} \cdot [\mathrm{E}] = [\mathrm{E}]_0$$

$$[\mathrm{E}] = \frac{(k_{cat} + k_{-1}) \cdot [\mathrm{E}]_0}{k_{-1} + k_{cat} + k_1 \cdot [\mathrm{S}]}$$

$$v = k_{cat} \cdot [\mathrm{ES}] = k_{cat} \cdot \frac{k_1 \cdot [\mathrm{S}][\mathrm{E}]}{k_{-1} + k_{cat}} = \left(k_{cat} \cdot \frac{k_1 \cdot [\mathrm{S}]}{k_{-1} + k_{cat}} \right) \cdot \left[\frac{(k_{cat} + k_{-1}) \cdot [\mathrm{E}]_0}{k_{-1} + k_{cat} + k_1 \cdot [\mathrm{S}]} \right]$$

$$= \frac{k_{cat} \cdot k_1 \cdot [\mathrm{E}]_0 \cdot [\mathrm{S}]}{k_{-1} + k_{cat} + k_1 \cdot [\mathrm{S}]} = \frac{k_{cat} \cdot [\mathrm{E}]_0 \cdot [\mathrm{S}]}{\frac{k_{-1} + k_{cat}}{k_1} + [\mathrm{S}]}$$

Since $V_{\max} = k_{cat} \cdot [\mathrm{E}]_0$ and $K_m = \frac{k_{-1} + k_{cat}}{k_1}$

$$v = \frac{V_{\max} \cdot [S]}{K_m + [S]} \text{.....(4.2)}$$

For peptidyl-prolyl isomerization, the spontaneous thermal isomerization rate is fast. When considering the thermal isomerization,

$v = k_{cat} \cdot [ES] + k_3 \cdot [S]$, where k_3 is the thermal isomerization first order rate constant.

$$v = k_{cat} \cdot [ES] + k_3 \cdot [S] = \frac{V_{\max} \cdot [S]}{K_m + [S]} + k_3 \cdot [S] \text{.....(4.3)}$$

When $K_m >> [S]$, the reaction appears to be a first order reaction with an apparent first order rate constant k_{obs}.

$$v = \frac{V_{\max} \cdot [S]}{K_m + [S]} + k_3 \cdot [S] \approx \frac{V_{\max} \cdot [S]}{K_m} + k_3 \cdot [S] = \left(\frac{V_{\max}}{K_m} + k_3 \right) \cdot [S] = k_{obs} \cdot [S] \text{.....(4.4)}$$

where $k_{obs} = \left(\frac{V_{\max}}{K_m} + k_3 \right)$.

In a typical protease-coupled assay developed for PPIase inhibition, the reaction rate is monitored by the release of a chromophore, nitroaniline. The isomerization reaction is illustrated in Figure 4.2.

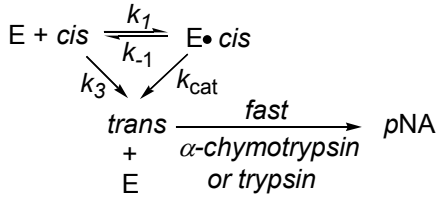


Figure 4.2. PPIase catalyzed cis-trans isomerization

The peptidyl-prolyl isomerization rate is very fast (thermal isomerization is usually complete in 90 sec at 0 °C, catalysized reaction $k_{cat}/K_m = 1 \times 10^6 \sim 1 \times 10^7$), this prohibited us to use the 10% of the reation in the linear reagion to measure the reaction rate because: 1) only few data could be obtained in 9 sec (10% of 90 sec); 2) the thermal

isomerization is too fast and it has to be subtracted from the Pin1 assay to obtain the rate solely contributed from enzyme catalysis; 3) practically, at least 6-8 sec was needed to mix the assay solution after the PPIase reaction was inigiated and before the progress of the reaction was monitored by UV-Vis spectrometry.

When K_m >> [S] (the concentration of the substrate, which is equal to the concentration of Suc–Ala–Glu–*cis*–Pro–Phe–*p*NA, [*cis*]) from (4.4),

$$v = \left(\frac{V_{\max}}{K_m} + k_3 \right) \cdot [cis] = k_{obs} \cdot [\mathrm{c}is] = \frac{d[trans]}{dt} = \frac{d[p\mathrm{NA}]}{dt}$$

$$d[p\mathrm{NA}] = k_{obs} \cdot [cis] \cdot dt$$

$$d[p\mathrm{NA}] = k_{obs} \cdot ([\mathrm{C}]_0 - [p\mathrm{NA}]) \cdot dt$$

$$\frac{d[p\mathrm{NA}]}{([\mathrm{C}]_0 - [p\mathrm{NA}])} = k_{obs} \cdot dt$$

$$\int_{[trans]_0}^{[p\mathrm{NA}]} \frac{d[p\mathrm{NA}]}{([\mathrm{C}]_0 - [p\mathrm{NA}])} = \int_0^t k_{obs} \cdot dt$$

$$-\ln\left(\frac{[\mathrm{C}]_0 - [p\mathrm{NA}]}{[\mathrm{C}]_0 - [trans]_0}\right) = -\ln\left(\frac{[\mathrm{C}]_0 - [p\mathrm{NA}]}{[cis]_0}\right) = k_{obs} \cdot t$$

$$[p\mathrm{NA}] = [\mathrm{C}]_0 - [cis]_0 \cdot e^{(-kobs \cdot t)} \quad \text{.....(4.5)}$$

Where [*p*NA] is the concentration of the chromophore nitroaniline, $[cis]_0$ is the initial concentration of cis component of the peptide substrate, [*cis*] is the concentration of cis conformer present in solution, $[trans]_0$ is the initial concentration of trans component of the peptide substrate, [*trans*] is the concentration of trans conformer present in solution, $[\mathrm{C}]_0$ is the total concentration of the peptide substrate (including both Suc–Ala–Glu–*cis*–Pro–Phe–*p*NA and Suc–Ala–Glu–*trans*–Pro–Phe–*p*NA).

As a result, the UV absorbance progress curve of [*p*NA] can be fit into a first order exponential equation:

$$A = A_1 + A_2 \cdot e^{(-kobs \cdot t)}, \qquad A_1 \neq A_2$$

where A represents [pNA], A_1 represents [S]$_0$ and $-A_2$ represents [cis]$_0$. A_1, A_2 and k_{obs} can be derived from the resulting plots of the data. The initial velocity can be derived from k_{obs}.

In a typical protease-coupled PPIase inhibition assay with a UV-Vis spectrometer monitoring the release of pNA, in order to make the absorbance in the reasonable range of 0-2 AU, the concentration of the cis peptide substrate is always far less than K_m (for Pin1 K_m is about 120 μM,[184] for FKPB 520 μM,[100] for CyP A 980 μM[100]). This makes the first order exponential rate analysis always applicable.

In an assay to measure the kinetic constant K_m or the inhibition constant K_i, the cis substrate concentration has to approach at least K_m. Since the prerequisite of $K_m >>$ [cis] (where [cis] is the actual substrate concentration, in this case, the cis conformer of the peptide Suc–Ala–Glu–Pro–Phe–pNA), the first order exponential model no longer exists, and the absorbance curve must be analyzed by a different model derived from the kinetic equation:

$$v = \frac{d[trans]}{dt} = \frac{d[p\mathrm{NA}]}{dt} = \frac{V_{\max} \cdot [cis]}{K_m + [cis]} + k_3 \cdot [cis] \quad \text{.....(4.6)}$$

Integration of equation (4.6) yielded:

$$\frac{V_{\max}}{k_3} \ln\left(1 - \frac{[p\mathrm{NA}] - [trans]_0}{[cis]_0 + K_m + \frac{V_{\max}}{k_3}}\right) + K_m \cdot \ln\left(1 - \frac{[p\mathrm{NA}] - [trans]_0}{[cis]_0}\right) + k_3 \cdot t \cdot \left(K_m + \frac{V_{\max}}{k_3}\right) = 0 \quad \text{....(4.7)}$$

The thermal background rate k_3 is a constant when the temperature is constant and k_3 can be measured independently in the absence of PPIases. The three parameters [$trans$]$_0$, $\frac{V_{\max}}{k_3}$ and K_m can be determined though nonlinear least-squares curve fitting.

Enzyme catalyzed initial velocity can be calculated from following equation:

$$v_{0,\,enzyme} = \frac{V_{max} \cdot [cis]_0}{K_m + [cis]_0} \quad \text{.....(4.8)}$$

The non-linear curve fit of $v_{0,\,\text{enzyme}}$ *vs*. $[cis]_0$ with varying concentration of an inhibitor will give the inhibition constant K_i. The plot will also demonstrate the inhibition pattern (noncompetitive, competitive or uncompetitive) of the inhibitor.[101]

4.2.2. Pin1 in vitro inhibition assay –determining conditions, K_m, IC_{50}, and K_i

The best substrate for Pin1 inhibition assay would be a peptide containing a pSer/pThr-Pro motif, for example Ac–Typ–Phe–Tyr–pSer–Pro–Arg–pNA (k_{cat}/K_m = 20,160 mM^{-1}).[38] A similar peptide AcFFpSPR-pNA synthesized in our lab [Scott A. Hart, unpublished result] contains the pSer/pThr-Pro motif, arginine, and the aromatic residue N-terminal to phosphoSer. Although AcFFpSPR-*p*NA has a high k_{cat}/K_m [Xiaodong J. Wang, Scott A. Hart, Etzkorn F. A. unpublished result], the commercially available peptide Suc-AEPF-*p*NA is a good enough substrate for Pin1 (k_{cat}/K_m = 3,410 mM^{-1}) and can be obtained in large quantity. The glutamic acid contains a negative charge on the side chain, which mimics a phospho-serine. An advantage of this sequence over the best Pin1 substrate is that the C-terminal phenylalanine makes it a suitable substrate for chymotrypsin, rather than trypsin, which may proteolyze Pin1 to a greater extent.[181] Because trypsin recognizes Xaa–*trans*–Pro–Arg–*p*NA, it may cleave $–NH_2$ from our trans inhibitors when cleaving Pin1 substrates.

In this coupled assay, chymotrypsin is used in excess (60 mg/mL, 51 units/mg) to ensure proteolysis is not rate limiting. Indeed, doubling the amount of chymotrypsin led to no increase in the rate. Although chymotrypsin cleaves to the C-terminal side of Phe

residues, it is highly unlikely to cleave our inhibitors because of the specificity of chymotrypsin for Xaa–*trans*–Pro–Phe sequences.

In the aqueous solution, the Glu–Pro amide bond in the substrate is about 90% trans, and thus 90% of the peptide would be cleaved at the onset of the assay. This leaves a very narrow assay window (10% of 2 AU, which is 0.2 AU) and leads to an unfavorable signal-to-noise ratio. A typical progression curve is shown schematically in Figure 4.3 A. Rich and coworkers solved this problem by using TFE or THF containing 0.47 M LiCl as the substrate solvents to increase the cis isomer of the peptide substrate up to 70%.[185] This higher concentration of the cis peptide substrate at the onset of the isomerization allows the measurement of K_m and K_i.[185] In our assay, TFE containing 0.47 M LiCl was used as the substrate solvent and the population of the cis conformer was about 50%.

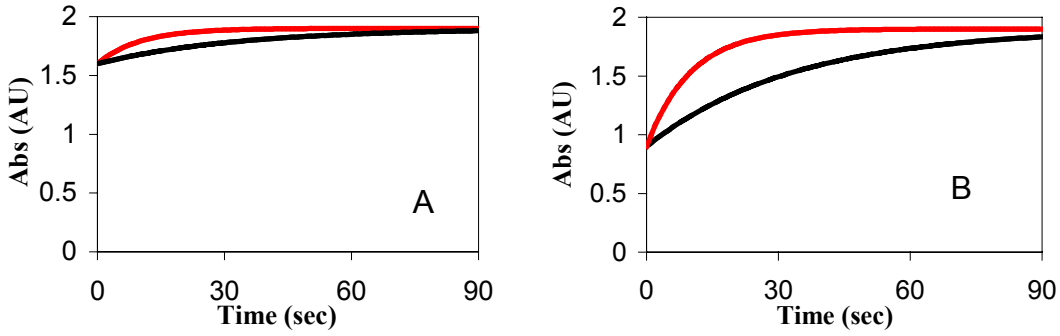


Figure 4.3. Schematic representation of the PPIase assay.

A: 10% cis When peptide substrate is dissolved in aqueous solution. B: 50% cis in TFE with 0.47 M LiCl was employed as peptide substrate solvent. Black line: thermal isomerization; red line: enzymatic isomerization.

Instead of 50 mM HEPES with 100 mM NaCl buffer typically used for the hCyPA assay,[186] 35 mM HEPES pH 7.8 was used as the Pin1 buffer because Pin1 activity

dramatically decreases with increasing ionic strength.[149] For substrate **116**, Pin1 has maximum activity at pH 6; either increasing or decreasing pH results in decreased Pin1 activity.[37] We conducted the Pin1 inhibition assay at pH 7.8 to make sure the inhibitor existed in the diionized phosphate form, which mimics the actual form of the substrate recognized by Pin1 at physiological pH. Such a pH value did compromise the Pin1 activity with the substrate **116**, but because a full kinetic inhibition analysis varying both substrate and inhibitors was to be performed, the catalytic efficiency was acceptable (k_{cat}/K_m) 496 mM^{-1} s^{-1}).

A typical cis-trans isomerization of the substrate is complete in 90 seconds. A Pin1 concentration (final concentration 67 nM) was chosen so that the assay in the absence of any inhibitor was complete in 40 seconds. In order to obtain IC_{50} values, the concentrations of Pin1 and substrate were kept constant. The reported K_m value of 120 μM for Suc–Ala–Glu–Pro–Phe–MCA[184] was a good estimate of the K_m value for Suc–Ala–Glu–Pro–Phe–*p*NA to determine which equation we would use to fit the absorbance curve. Thus the concentration of Suc–Ala–Glu–*cis*–Pro–Phe–*p*NA was assumed to be far less than K_m when it was less than 50 μM, so for all IC_{50} measurements, the substrate concentration utilized was below 50 μM. The inhibitor was pre-incubated with Pin1 in the buffer for 2 min at 4°C. The absorbance curve at λ = 510 nm was subtracted from the progress curve at 390 nm to correct for the background. The resulting curve was fit to equation $A = A_1 + A_2 \cdot e^{(-kobs \cdot t)}$ by non-linear curve fitting. The percent inhibition was calculated using the equation:

$$\%inhibition = \frac{100 \times (k_{obs,\,I} - k_3)}{k_{obs,\,Pin1} - k_3} \text{.....(4.9)} .$$

where $k_{obs,\ Pin1}$ is the first order rate constant in the presence of Pin1, but without inhibitor, and k_3 is the thermal isomerization rate. $k_{obs,\ I}$ is the first order rate constant in the presence of both Pin1 and inhibitors.

The plot of percent inhibition vs. the logarithmic of the inhibitor concentration produced a sigmoid curve (Figure 4.4). The calculated values of IC_{50} were obtained by fitting experimental data to either a hyperbolic curve or a dose response curve (95% confidence level) by non-linear regress.

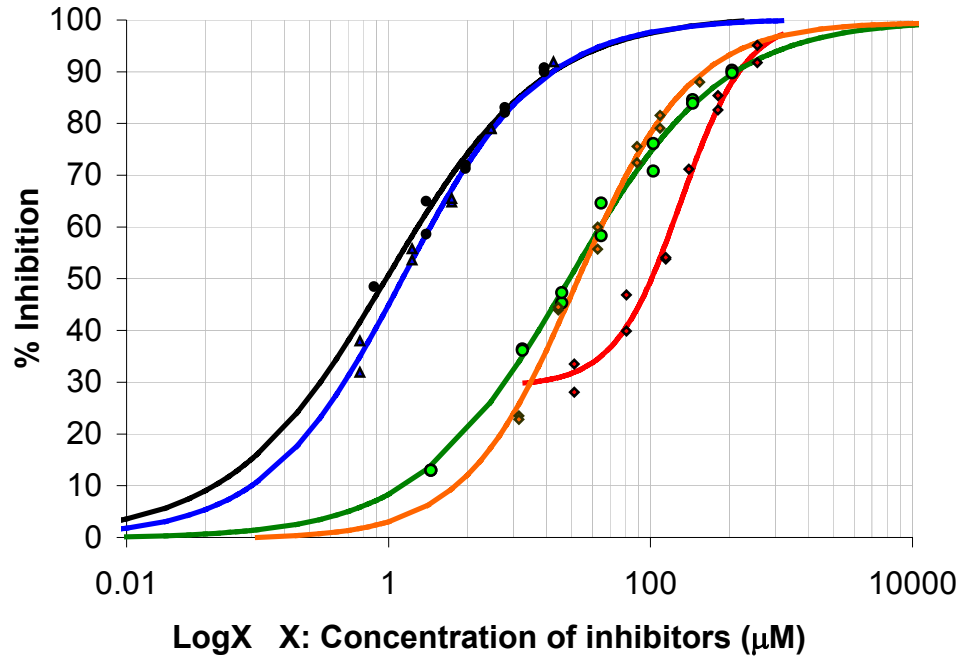


Figure 4.4. Dose response curve.

Black: cis primary amide **114,** IC_{50} = 0.97 ± 0.09 μM; Blue: cis primary amide **113,** IC_{50} = 1.3 ± 0.2 μM; Green: trans methyl amide **106,** IC_{50} = 24. ± 3 μM; Orange: trans primary amide **112,** IC_{50} = 28. ± 3 μM; Red: the isomer **107,** IC_{50} = 102 ± 20 μM. All assays were conducted under the same experimental conditions. Cis peptidomimetics were about 20 fold more potent than the trans peptidomimetics. The control compound,

107, with the alkene bond in the improper position was about 4-fold less potent than the trans compounds and around 100-fold less potent than the cis compounds.

The determination of the Michaelis constant (K_m) for the substrate ***cis*-116** and the measurement of the inhibition constants for cis isostere **113** and trans isostere **112** were conducted by the method of Kofron.[100] The K_m value for ***cis*-116** was 183 ± 9 μM, which is in agreement with the published K_m value of Suc–Ala–Glu–*cis*–Pro–Phe–MCA (120 μM).[184]

For the inhibition constants (K_{is}), data analysis was performed as previously described in section 4.2.1.[100] The resulting initial velocity, substrate concentrations, and inhibitor concentrations were evaluated using Cleland's programs[101] to determine the inhibition constants and the inhibition patterns. Thus, the inhibition patterns were determined to be competitive for both trans isostere **112** and cis isostere **113**. The hyperbolic and double reciprocal plots are shown in Figure 4.5. The competitive inhibition constants (K_{is}) are given from Cleland's program and summarized in Table 4.1.

Table 4.1. Inhibition of Pin1

Compounds	IC_{50} (μM)	K_{is} (μM)	A2780 IC_{50} (μM)
106. Ac–Phe–Phe–pSer–Ψ[*(E)*CH=C]Pro–Arg–NHMe	24. ± 3	ND[a]	ND[a]
107. (Endocyclic alkene isomer of **106**)	102 ± 20	ND[a]	ND[a]
112. Ac–Phe–Phe–pSer–Ψ[*(E)*CH=C]Pro–Arg–NH_2	28. ± 3	40. ± 2	140 ± 10
113. Ac–Phe–Phe–pSer–Ψ[*(Z)*CH=C]Pro–Arg–NH_2	1.3 ± 0.2	1.74 ± 0.08	8.3 ± 0.5
114. Ac–Phe–Tyr–pSer–Ψ[*(Z)*CH=C]Pro–Arg–NH_2	0.97 ± 0.09	ND[a]	ND[a]

[a] ND = not determined.

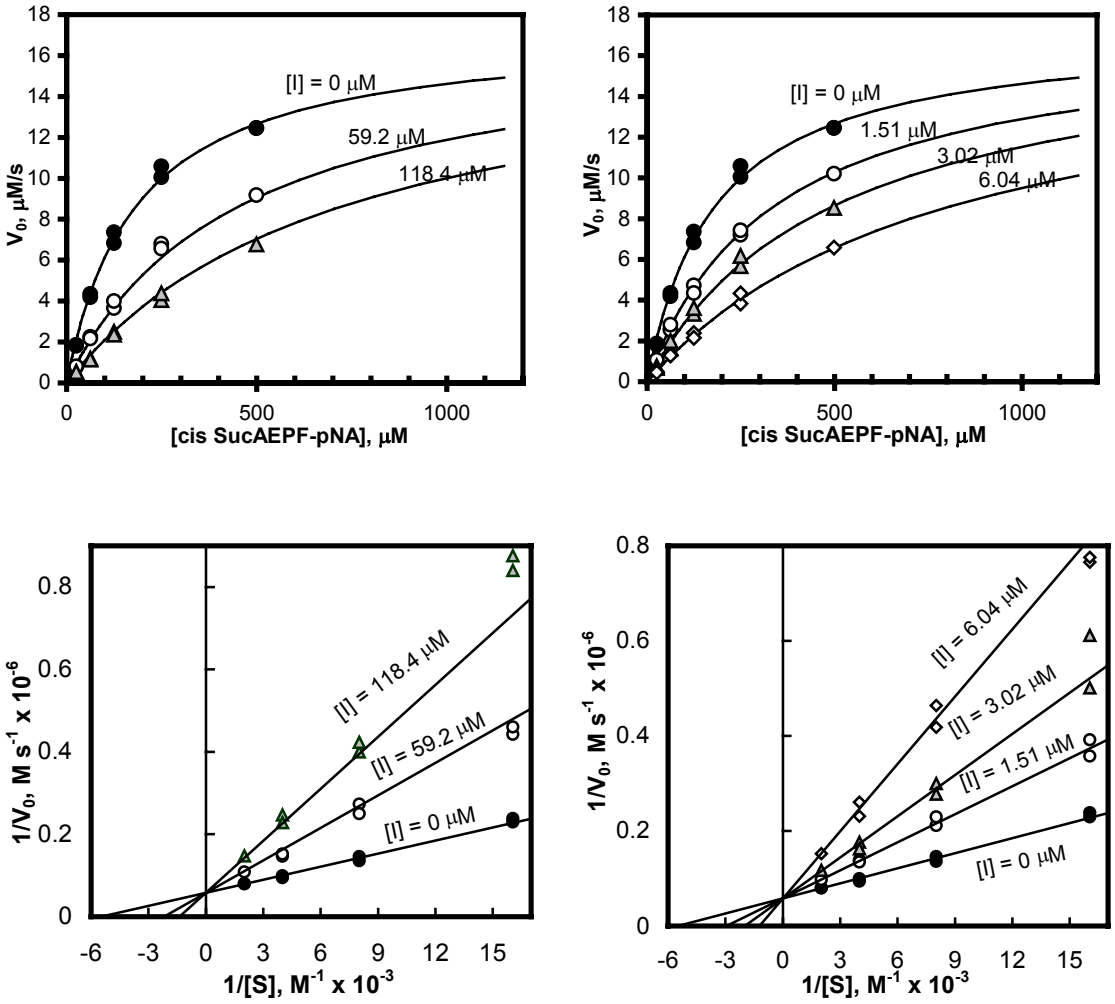


Figure 4.5. Hyperbolic and double reciprocal plots of the competitive inhibition of human Pin1 by trans **112** (left) and cis **113** (right) isosteres.

The K_{is}, K_m, and V_{max} values were calculated from Clelend's programs.

4.2.3 A2780 Ovarian Cancer Cell Assay

Compounds **112** and **113** were assayed for antiproliferative activity against A2780 ovarian cancer cells as previously reported.[187,188] Results from the antiproliferative assay gave an IC_{50} value of 8.3 μM for the cis isostere **113** and 140 μM for the trans isostere **112** (Table 4.1). Thus, the cis isostere **113** is 17-fold more potent than the corresponding trans isostere **112**.

4.3. Implications of Pin1 Inhibition by Conformationally Locked Substrate Analogues.

The cis and trans amide isosteres are both competitive inhibitors, indicating that they both bind in the PPIase domain catalytic site of human Pin1. Both the trans isostere and the cis isostere bound to Pin1 in its active site in the X-ray crystal structure.[Jessie Zhang, Joseph Noel, unpublished results] This is in agreement with our expectation for the cis amide analogue.[37] The noncatalytic WW domain of Pin1 has been demonstrated to bind the pSer/pThr–*trans*–Pro motif in both the X-ray[189] and the solution NMR[190] structures. Trans isostere **112**, however, binds to the catalytic domain, as demonstrated by competitive inhibition, probably because the sequence of amino acids flanking the pSer-Pro core recognition element of a substrate (or inhibitor) also plays an important role in Pin1 binding and catalysis.[38] The flanking sequence specificity of the WW domain is different from that of the catalytic domain.[38,189] We speculate that the aromatic C-terminal and basic N-terminal amino acids in inhibitor **112** direct it into the catalytic domain of Pin1.

The competitive inhibition constant (K_{is}) of the trans inhibitor **112** is 23 times higher than its cis counterpart **113**. The fact that cis isostere **113** inhibits Pin1 activity much more than trans isostere **112** suggests: (1) the catalytic domain of Pin1 binds the cis analogue more tightly than the trans analogue, and (2) in aqueous solution, Pin1 preferentially binds the cis substrate. Our results are in agreement with X-ray structures of Pin1 and hCyPA.[37,176,177,191] The more hydrophobic *N*-methylamide of inhibitor **106** did not improve the inhibition of Pin1 significantly over the primary amide **112** (Table 4.1). The IC_{50} values are within experimental error of each other (24. ± 3 and 28. ± 3

μM). The poor inhibition by compound **107** shows that the location of the double bond is also essential for inhibition and that the correctly placed alkenes are truly isosteres of the cis and trans prolyl amides. Taken together, these results suggest that, although pSer–*cis*–Pro and pSer–*trans*–Pro are both Pin1 substrates, Pin1 may facilitate the trans to cis isomerization in biological processes more efficiently by lowering the energy barrier of the trans to cis interconvertion more than the cis to trans interconvertion (Figure 4.6).[192]

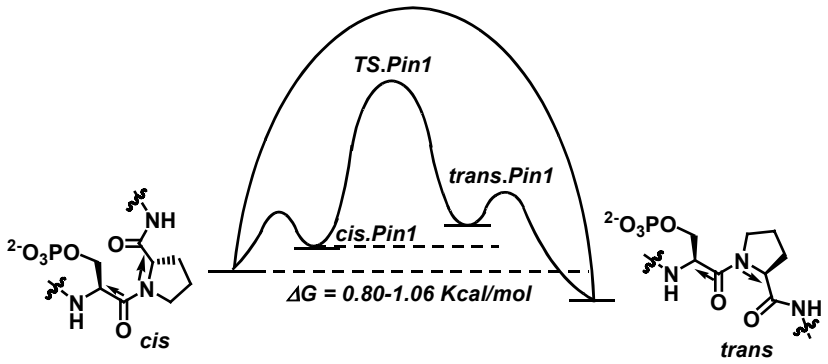


Figure 4.6. Energy diagram of cis-trans isomerization

The cis isostere **113** inhibits proliferation of A2780 ovarian cancer cells 17-fold better than the trans isostere **112**, comparable to the 21-fold difference in inhibition of Pin1 PPIase activity (Table 4.1). This suggests that Pin1 may be the primary target that accounts for the antiproliferative activity of the inhibitors. The IC_{50} values for A2780 antiproliferation are close to the Pin1 IC_{50} values, about a 5-fold difference for both **112** and **113**. It is certainly possible that these inhibitors are susceptible to either proteases or phosphatases in the cell culture assay, which might explain the differences in activities from the Pin1 inhibition assays. Further modifications of these inhibitors to make them less peptidic and less susceptible to phosphatases will improve their potential as drug

leads. Other Pin1 inhibitors identified by screening have been shown to inhibit a cancer cell line that overexpresses Pin1 with IC_{50} values in agreement with Pin1 enzyme inhibition.[184] The ovarian cancer cell line growth inhibition results increase the significance of Pin1 as a potential anti-cancer drug target.

4.4. Conclusions

A protease-coupled assay for CyP and FKBP was adapted to assay a series of substrate analogues as Pin1 inhibitors. These substrate analogues inhibited Pin1 with IC_{50} values ranging from sub-micromolar to hundreds of micromolar, with the cis analogue Ac–Phe–Tyr–pSer–Ψ[*(Z)*CH=C]Pro–Arg–NH_2, **114**, being the most potent. We demonstrated that both the trans and the cis alkene pentapeptide analogues **112** and **113** are competitive inhibitors of Pin1 by this protease-coupled assay. The fact that the (*Z*)-alkene **113** was a more potent competitive inhibitor (K_{is} = 1.74 ± 0.08 μM) than the (*E*)-alkene **112** (K_{is} = 39.8 ± 2.4 μM) suggests that Pin1 binds the cis substrate more tightly at the catalytic site. Indeed both the trans isostere and the cis isostere bound to Pin1 in its active site in the X-ray crystal structure.[Jessie Zhang, Joseph Noel, unpublished results] These two Pin1 inhibitors also inhibited A2780 ovarian cancer cell growth in vitro with IC_{50} values of 8.3 ± 0.5 μM for the cis analogue **113** and 140 ± 10 μM for the trans analogue **112**. This suggests that Pin1 could be the target that accounts for the antiproliferative activity against the human ovarian cancer cell line. The two (*Z*)-alkene inhibitors, **113** and **114**, are among the most potent inhibitors found for Pin1 so far, and they are neither a nonspecific thiol capture agent such as juglone nor an ordinary peptide. Nonpeptidic compounds possessing the core Ser-*cis*/*trans*-Pro isosteres are quite

promising as Pin1 inhibitors, as tools for investigating Pin1 regulation of mitosis, and as anti-cancer drug leads.

Although the anti-proliferative activity of these peptidomimetics against the ovarian cancer cell line parallels their Pin1 inhibition activity, the question remains whether the growth inhibition of the cancer cell line is solely a result of Pin1 inhibition or whether some other enzymes or biological processes are involved. Since our inhibitors are substrate analogues of pSer-Pro, they may be susceptible to other enzymes such as proteases and phosphatases. In collaboration with Dr. Anthony R. Means at Duke University Medical Center, the best Pin1 inhibitor **114** will be used to identify whether Pin1 was the true target in the cell based assay.

Experimental

General .TFE (99+%) was distilled from sodium before use. LiCl (99+%) was dried in vacuo at 150 °C for 24 h. The human His6-Pin1 DNA plasmid was a generous gift from Professor P. Todd Stukenberg (University of Virginia). UV-Vis absorbance readings were collected on an Agilent 8453 UV spectrophotometer. Curve fitting was conducted using TableCurve (version 3 for win 32) software.

Determination of the Michaelis Constant K_m for the Human Pin1 Substrate. Determination of the steady-state kinetic parameters for the human Pin1 substrate Suc-Ala-Glu-Pro-Phe-*p*NA was performed as described for hCyPA and FKBP.[38] Human Pin1 was assayed at varied cis substrate **116** concentrations ranging from 25 to 500 μM. The concentration of the cis substrate was determined by the UV absorbance of *p*NA (ε =

12,250 at 390 nM) after cleavage by α-chymotrypsin. The cis component of the substrate was approximately 51%. The assay buffer (1.05 mL of 35 mM HEPES, pH 7.8[193] at 0 °C; final concentration 31 mM HEPES) and Pin1 (10 μL of a 8.0 μM stock solution, in 20 mM Tris-HCl aqueous solution, pH 7.8, concentration measured by Bradford assay, final concentration 67 nM) were preequilibrated in the spectrometer until the temperature reached 4.0 °C. The thermal isomerization rate constant (k_3) was determined in the absence of Pin1. Immediately before the assay was started, 120 μL of ice-cooled α-chymotrypsin solution (60 mg/mL in 1 mM HCl; final concentration 6 mg/mL) was added. Additional substrate solvent (0.47 M LiCl/TFE) was added as needed to bring the total volume of substrate and cosolvent to 10 μL. The peptide substrate Suc–Ala–Glu–Pro–Phe–*p*NA, dissolved in dry 0.47 M LiCl/TFE, was added to the cuvette via syringe, and the solution was mixed vigorously by inversion three times. The final volume in a semi-micro 1.0 cm path length polystyrene cell was 1.2 mL. After a mixing delay of 6-8 s, the progress of the reaction was monitored at 4 °C by absorbance at 390 nM for 90 s.

IC_{50} Measurements of Pin1 Inhibitors. The assay buffer (1.05 mL of 35 mM HEPES, pH 7.8; final concentration 31 mM HEPES), Pin1 (10 μL of stock solution), and inhibitors (10 μL of varying concentrations in 1:3 DMSO:H_2O) were preequilibrated in the cuvette at 4 °C for 10 min. The concentrations of the inhibitors were determined by hydrolysis in constant-boiling HCl in a sealed tube at 110 °C for 23 h followed by HPLC separation (10-90% B over 10 min) and phenylalanine integration as compared with a series of phenylalanine solutions of known concentration. Immediately before the assay was started, 120 μL of ice-cooled chymotrypsin solution (60 mg/mL in 0.001 M HCl;

final concentration 6 mg/mL) was added. The peptide substrate Suc–Ala–Glu–Pro–Phe–*p*NA (10 μL) in 0.47 M LiCl/TFE was added via syringe, and the reaction was mixed as before. The cis substrate concentrations [cis] for measuring the IC_{50} values were 43.2 μM for **112**, **113** and **114**, and 36.0 μM for **106** and **107**. The calculated values of IC_{50} were obtained by fitting experimental data to either a hyperbolic curve or a dose response curve (95% confidence level) by TableCurve (version 3 for win 32).

***K*$_i$ Measurements for Pin1 Inhibitors.** Assays were performed as described for the determination of IC_{50}. The cis substrate concentrations were 25.6, 62.3, 125, 249, and 499 μM. The final concentrations of the (*Z*)-alkene inhibitor **113** were 1.51, 3.02, and 6.04 μM, and the final concentrations of the (*E*)-alkene inhibitor **112** were 58.2 and 118.4 μM. When [*cis*] ≥ 50 μM, the absorbance curves at 390 nM, 420 nM or 445 nM (depending on the substrate concentration; higher concentration used higher wavelength) were fit to equation (4.7) by TableCurve (version 3 for win 32) software:

$$\frac{V_{\max}}{k_3}\ln\left(1-\frac{[p\mathrm{NA}]-[trans]_0}{[cis]_0+K_m+\frac{V_{\max}}{k_3}}\right)+K_m\cdot\ln\left(1-\frac{[p\mathrm{NA}]-[trans]_0}{[cis]_0}\right)+k_3\cdot t\cdot\left(K_m+\frac{V_{\max}}{k_3}\right)=0 \quad \text{....(4.7)}$$

and the initial velocity was calculated from equation (4.8):

$$v_{0,\,enzyme}=\frac{V_{\max}\cdot[cis]_0}{K_m+[cis]_0} \quad \text{.....(4.8)}$$

The resulting initial velocity, substrate concentrations and inhibitor concentrations were evaluated using Cleland's programs[101] to determine the inhibition constants and the inhibition pattern.

Competitive inhibition was fit to equation (4.9):

$$v = \frac{V_{max}[cis]_0}{K_m(1+[I]/K_{is})+[cis]_0} \text{.....(4.9)}$$

Noncompetitive inhibition was fit to equation (4.10):

$$v = \frac{V_{max}[cis]_0}{K_m(1+[I]/K_{is})+[cis]_0(1+[I]/K_{ii})} \text{.....(4.10)}$$

Uncompetitive inhibition was fit to equation (4.11):

$$v = \frac{V_{max}[cis]_0}{K_m+[cis]_0(1+[I]/K_{ii})} \text{.....(4.11)}$$

The fit with the lowest average residual least square root (SIGMA) is usually the best fit. With hyperbolic fit, the SIGMA values of cis isostere **113** for competitive, noncompetitive, and uncompetitive inhibition patterns were 0.1827, 0.1862 and 0.8561, respectively. The SIGMA values of the trans isostere **112** for competitive, noncompetitive, and uncompetitive inhibition patterns were 0.2776, 0.2829 and 09484, respectively. The data for both **112** and **113** fit best to equation (4.9) leading to the conclusion that both inhibitors are competitive. Even though the data fit well to equation (4.10) for noncompetitive inhibition, when K_{ii} is very large the noncompetitive component of equation (4.10) goes to zero and the result resembles equation (4.9) for competitive inhibition. For both **112** and **113**, the calculated value of K_{ii} was infinity.

A2780 Bioassay. Antiproliferative activity against the A2780 human ovarian cancer cell line was measured as published.[187,188] The concentrations of **113** used were 18, 9.0, 4.5, 2.2, 1.1, 0.56, 0.28, and 0.14 μM (repeated at 7.9 μM in triplicate), and the concentrations of **112** were 200, 100, 51, 25, 13, 6.3, 3.1, and 1.5 μM in triplicate.

Chapter 5. Construction of a cis alkene library for Pin1 inhibition

5.1. Introduction

The exciting initial biological results for our simple peptide bioisosteres led us to synthesis and screen more potent Pin1 inhibitors as potential anti-cancer drug leads. Although a sub-micromolar Pin1 inhibitor, the most potent Pin1 inhibition in the literature, Ac–Phe–Tyr–pSerΨ[(*Z*)CH=C]–Pro–Arg–NH_2, **114**, was synthesized, it is not potent enough as a potential drug lead. Those substrate-based peptide analogues mimic the natural substrate of enzymes, thus are less specific. For example, those analogues are susceptible to other enzymes such as proteases and phosphatases because of their phosphoSer-Pro sequence. In addition, the structures of those peptide analogues could be very flexible, which makes the computational modeling difficult.

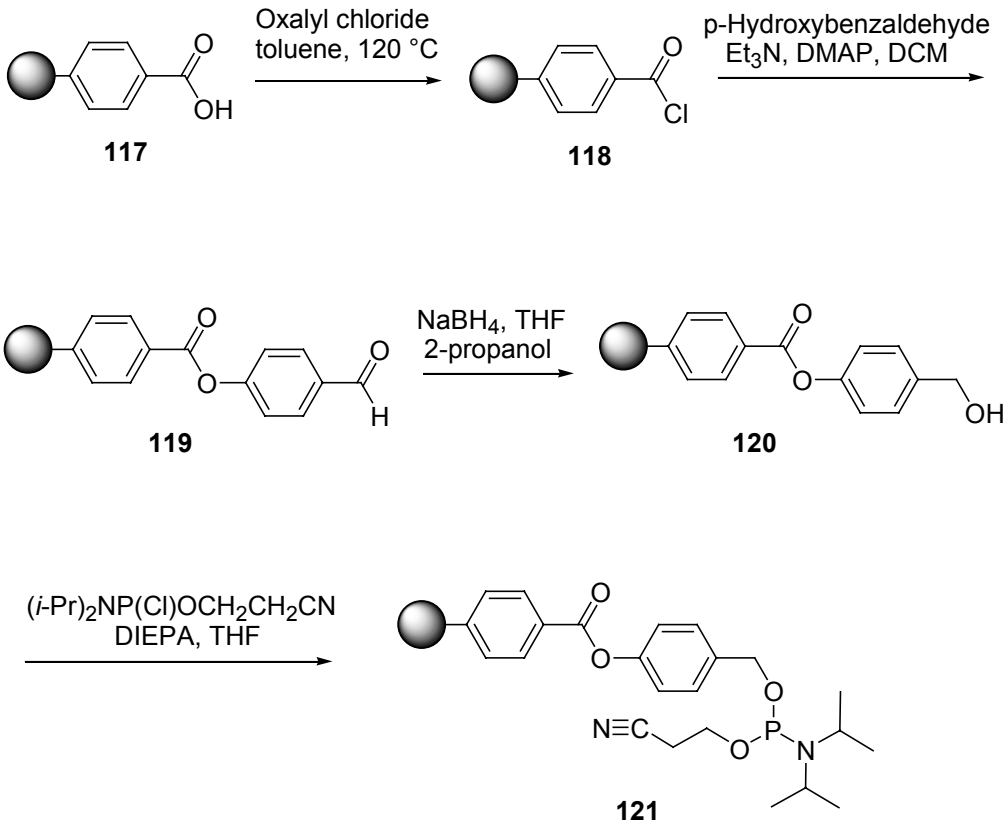
In the X-ray crystal structure of Pin1 complexed with either the trans isostere, **112**, or the cis isostere, **113**, the central phosphoSerΨ[(*E*)CH=C]Pro or phosphoSerΨ[(*Z*)CH=C]Pro motif was well resolved, but the amino acid residues flanking the dipeptide isosteres were disordered. This leaves a great space for improving the inhibition of (*Z*)-alkene derivatives. By synthesizing a compound library by the changing structure flanking the core of SerΨ[(Z)CH=C]Pro, we expect to find better Pin1 inhibitors and establish the structure-activity relationship (SAR) for the PPIase domain of Pin1. Because of the *Z*-configuration of the alkenes, this substrate-based library is expected to prefer the catalytic site over the WW domain binding site. These potential inhibitors are less peptidic and more "drug-like". A diverse synthetic method capable of yielding a large number of compounds rapidly is desired. A parallel solid-phase synthesis using semi-automated equipment meets such a requirement.

5.2. Construction of a (Z)-alkene library for Pin1 inhibition

A hypothesis was established that by changing functional groups flanking the central pSer–*cis*–Pro alkene, the compounds formed will have different Pin1 PPIase inhibition activity. Our design of the synthetic route was to first immobilize the core Ser–*cis*–Pro isostere onto a resin via its free hydroxyl group on the side chain, then couple it with a variety of amines and acids using peptide coupling reagents to the C-terminus and N-terminus, respectively. The phosphitylating resin **121**[194] provides a simple synthetic procedure. The synthesis of the functionalized resin **121** involves a 4-step sequence starting from the commercially available carboxylic acid polystyrene resin **117**.

Carboxylic acid polystyrene resin **117** was transformed to acid chloride **118** by refluxing with oxalyl chloride in dry toluene for 24 h. The resin was then coupled with p-hydroxybenzaldehyde to form ester **119** with aldehyde functionality. Reduction of the aldehyde **119** with sodium borohydride produced alcohol **120**, which was then reacted with *O*-benzyl-*O*-*β*-cyanoethyl-*N,N*-diisopropylphosphoramidite to afford the immobilized phosphorylating reagent **121**. The yield of each step was not calculated since there is not a simple method to calculate the yield of solid phase reagent. The presence of modified resins **118**, **119** and **120** was confirmed by FT-IR spectra. The formation of the resin **121** was confirmed by a weak CN resonance at 2250 cm^{-1} in its FT-IR spectra. The CN resonance was weaker than usual probably because the functional group was on resin and we were using the simplified sampling method (diamond ATR) for IR.

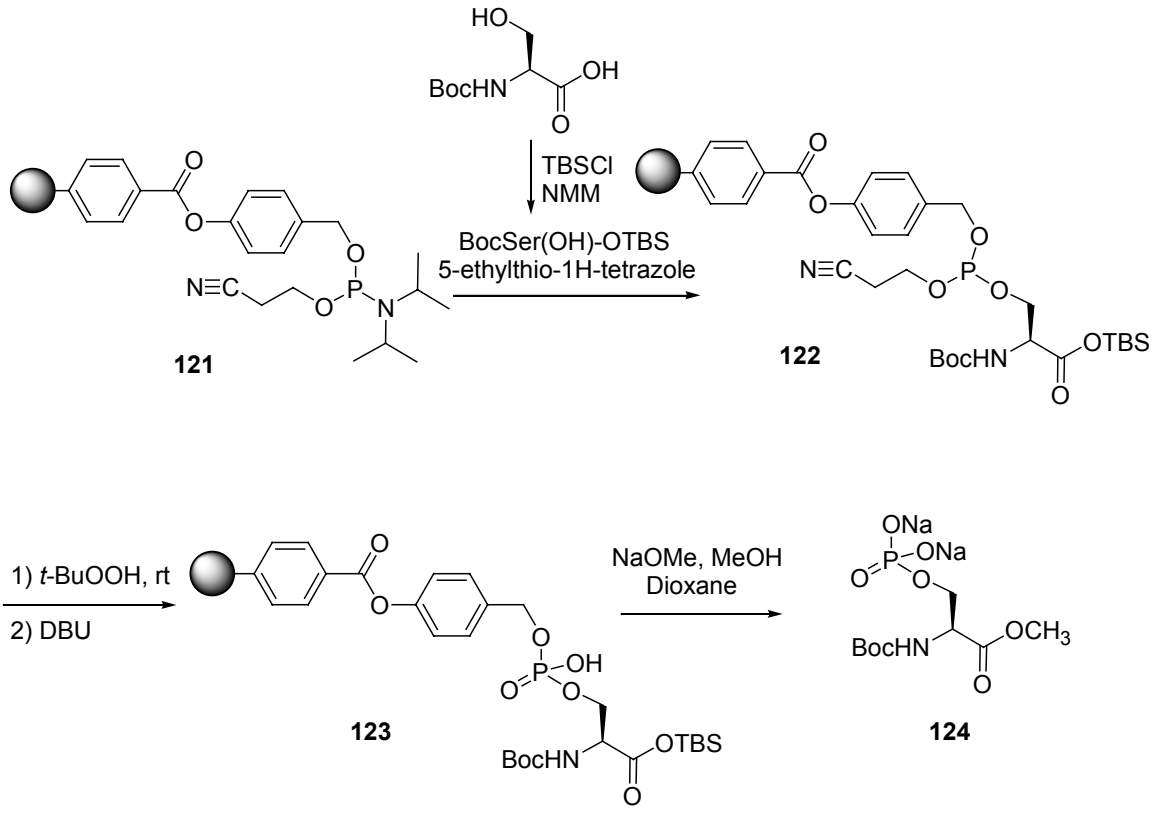
Scheme 5.1. Synthesis of the solid phase phosphorylating reagent



To test the feasibility of synthesizing the Pin1 inhibitor library using this resin, a model phosphorylation reaction was conducted with Boc–Ser–OH since we plan to use the Boc protected dipeptide isostere as the starting material for the solid phase synthesis. The free carboxylic acid of Boc–Ser–OH was temporarily protected by *tert*-butyl dimethyl silyl ester in the presence of the base *N*-methylmorpholine. Then this reaction mixture was added to resin **121** in one pot and agitated for 24 h. Without characterization of the intermediates, the resin was oxidized with *tert*-butyl hydroperoxide and the cyanoethyl group was deprotected by DBU. The phosphorylated product was cleaved by freshly prepared sodium methoxide. A major product was obtained after flash

chromatography. The ^{31}P NMR spectrum revealed two peaks around 0 ppm. The product was not identified due to the difficulty of assigning its ^{1}H NMR spectrum, as well as the lack of informative MS data (the product still contained a large excess of the sodium ions).

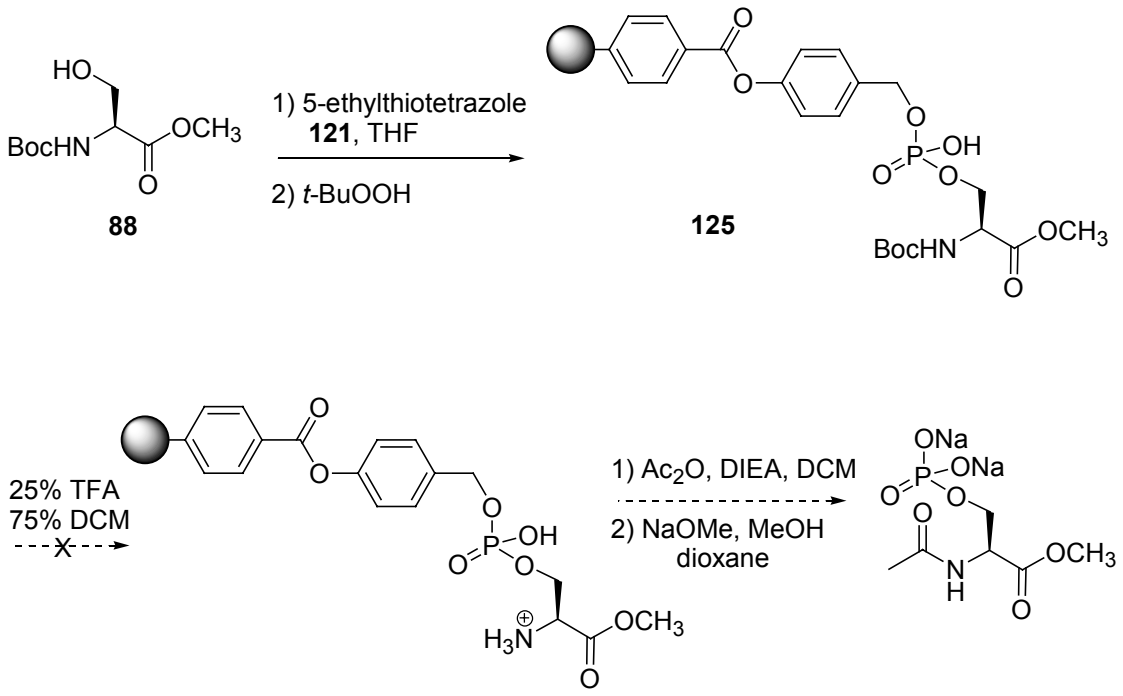
Scheme 5.2. Phosphorylation of Boc–Ser–OH using the solid phase reagent



Our plan for library synthesis was to anchor Boc protected dipeptide to resins via the side chain, couple with amines, then to remove Boc protection followed by coupling with an acid. Because of the difficulty in identifying the product for the previous model reaction, another model synthesis was conducted (Scheme 5.3). This model synthesis was designed to test whether the solid phase linker was stable to TFA and whether the coupling would go smoothly on the resin. Serine protected methyl ester was chosen as

starting material to eliminate any unwanted reaction with the free carboxylic acid functionality. Unfortunately, the solid phase linker is not stable to 25% TFA in DCM, the standard deprotection condition for Boc group. The products cleaved by TFA from the resin are to be analyzed by LC-MS.

Scheme 5.3. Phosphorylation of Boc–Ser–OMe



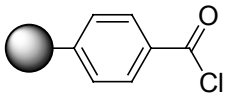
Should this phosphorylating resin method not work, other methods can be attempted. For example, the hydroxyl group on side chain of Boc–SerΨ[(*Z*)CH=C]–Pro–OH **76** can be anchored directly to a resin and cleaved after the coupling of the amines and the acids, then phosphorylated at the end.

5.3. Conclusions

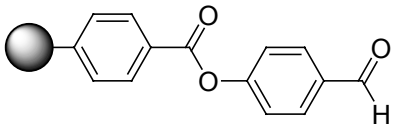
The solid phase phosphorylating reagent **121** was synthesized via a four-step

sequence one several gram scale according to a procedure in the literature,[194] to be used both as phosphorylating reagent and solid phase support for the synthesis of a library of Pin1 inhibitors. Initial studies showed the Boc chemistry using TFA as deprotecting reagent was not suitable for this resin. Changing the protecting group on the dipeptide isostere or changing the deprotection method is necessary.

Experimental

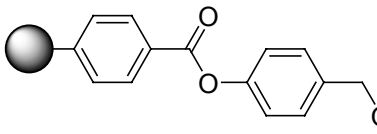


Polymer-bound benzoyl chloride (118). Carboxypolystyrene, **117** (1 g, 100-200 mesh, 1% DVB, 4.27 mmol/g), was suspended in dry toluene (50 mL), stirred slowly and heated at 120 °C for 1 h. Oxalyl chloride (14 mL, 32.9 mmol) was added and refluxing was continued for 24 h. The resin was filtered and washed with dry toluene (20 mL), dry ether (10 mL), dry toluene (20 mL), and dry ether (20 mL) consecutively and dried under vacuum overnight to give 1.08 g (yield 100%) of **118**. IR (cm^{-1}): 1768 (C=O, Acid chloride), 1733 (C=O).

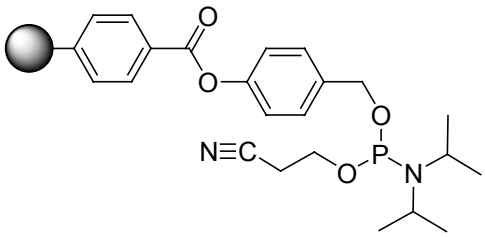


Polymer-bound *p*-hydroxybenzaldehyde (119). *p*-Hydroxybenzaldehyde (3.3 g, 27.0 mmol) and triethylamine (4.1 mL) were added to a swelled solution of resin **118** (1 g, 4.0 mmol/g) in dry dichloromethane (2.5 mL) under N_2. DMAP (27 mg, 0.22 mmol) was added. After stirring for 10 h, DCC was added to the reaction. After 12 h MeOH (0.3 mL) was added to neutralize unreacted benzoyl chloride or benzoic acid. After 12 h further stirring, the resin was filtered and washed successively

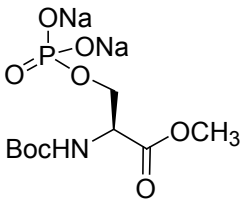
with CH_2Cl_2 (20 mL), THF (10 mL), MeOH (10 mL), THF (10 mL) and dry ether (10 mL) and dried under vacuum overnight to give 1.49 g (yield 100%) of **119**. IR (cm-1): 1695 (C=O, aldehyde), 1736 (C=O, ester).



Polymer-bound *p*-hydroxybenzyl alcohol (120). Sodium borohydride (137 mg, 3.6 mmol) was added slowly to a suspension of resin **119** (0.42 g, 2.9 mmol/g) in *i*Pr-OH alcohol (0.5 mL) and THF (3 mL). After stirring for 4 h, the resin was collected by filtration and washed successively with THF (10 mL), 3% v/v HOAc/dioxane (10 mL), THF (5 mL), MeOH (5 mL), THF (5 mL) and dry ether (5 mL) and dried under vacuum for 1 h to give 0.42 g (yield 100%) of **120**. IR (cm^{-1}): 3415 (br, O-H), 1730 (C=O, ester).



Polymer-bound cyanoethoxy *N,N*-diisopropylamine phosphine (121). 2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.42 mL, 2.4 mmol) was added dropwise to a stirred suspension of **120** (200 mg, 2.9 mmol/g) and DIEA (0.37 mL, 1.6 mmol) in anhydrous THF (2 mL) and stirred for 24 h at rt under N_2 atmosphere. The resin was collected by filtration and washed successively with THF (20 mL), MeOH (10 mL), THF (10 mL) and dry ether (10 mL) and dried under vacuum for 1 h to give 360 mg (yield 100%) of **121**. IR (cm^{-1}): 2250(CN) 1732 (C=O), 1013 (P-O-C).



Phosphorylation of Boc–Ser–OH. Boc–Ser–OH (53 mg, 0.26 mmol) was dissolved in dry THF (3 mL), followed by *tert*-butyl dimethylsilyl chloride and *N*-methylmorpholine (26 mg, 0.26 mmol) under N_2 at rt. The reaction mixture was stirred at rt for 30 min and then was transferred to a 5 mL polystyrene reaction column containing resin **121** (160 mg, 1.60 mmol/g). 5-ethylthio-1H-tetrazole (100 mg, 0.77 mmol) was dissolved in dry DMSO and added to the reaction. The reaction was agitated for 24 h at rt under N_2. The resin was washed successively with THF (10 mL), MeOH (5 mL), THF (10 mL) and dry ether (5 mL) and dried under vacuum for 1 h. *tert*-Butyl hydroperoxide in decane (5-6 M, 150 μL, 0.9 mmol) was added to the resin suspended in THF (3 mL). After 1 h stirring at rt, the resins were washed successively with THF (50 mL), MeOH (20 mL), THF (50 mL) and dry ether (20 mL) and dried under vacuum for 1 h. IR (cm^{-1}): 1730. A solution of 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU, 77 μL, 0.52 mmol) was added to the resins and agitated for 48 h at rt. The resin was washed successively with THF (50 mL), MeOH (20 mL), THF (50 mL) and dry ether (20 mL) and was dried under vacuum for 1 h. Sodium (12 mg, 0.52 mmol) was added to a mixture of dry MeOH (0.6 mL) and dry dioxane (3 mL) to generate a sodium methoxide solution. This solution was added to the resin and shaken for 48 h at rt. Water (1 mL) was added and stirring was continued for another 1 h. Aqueous HCl (0.5 M) was added until the pH was 2-3. The solution was collected and the resin was washed with dioxane (5 mL) and water (1 mL). The solution containing the product was lyophilized directly to give glassy syrup.

Chromatography with 15% methanol and 0.5% formic acid in dichloromethane give 18 mg of colorless oil. ^{1}H NMR of this product was too complicated to assign peaks. ^{31}P NMR (DMSO-d_6): δ 0.66, 0.40.

Chapter 6. Synthesis of Gly–trans–Pro–Hpy isosteres for collagen biomaterial

6.1. Introduction

Collagen is a highly abundant fibrous protein present throughout the animal, constituting 25 percent of all protein in body. Collagen is the scaffolding material found in skin, bones, tendons, cartilage, blood vessels and nearly all organs where it serves to form a matrix for holding and supporting cells. The basic structure of collagen is modified to meet the specialized needs of particular tissues.[195] Basically, collagen contains three polyproline type II helix chains each coiling in a left-handed manner and coiling with each other to form a right-handed super helix.[196] The unique triple helical structure of collagen results from the its primary structure, which can be represented by $(Xaa–Yaa–Zaa)_{300}$, where 10 percent of Xaa is proline, 10-12 percent of Yaa is 4(*R*)-hydroxyproline, and Zaa is typically Gly.[197,198] The presence of Gly at every third amino acid position is one of the most important structural elements of the collagen triple helix, as Gly is the only amino acid small enough to fit into the highly compacted super helix at that position. However, the high occurrence of hydroxyproline and proline in collagen and interchain hydrogen bonds between C=O and N-H groups contribute to stabilization of collagen's unique triple helical structure.[197,198] A typical molecule of collagen consists of around 300 units of Xaa-Yaa-Gly. This highly repeated sequence of collagen makes possible the polymerization of tripeptide monomers to prepare collagen analogues.

The thermodynamic feasibility of both cis and trans conformational isomers of Xaa–Pro and Xaa–Hyp amides leads to a significant challenge for folding collagen peptides.[199-203] In native collagens, globular C-terminal domains initiates triple helix formation,[204] but proline isomerization is rate limiting in collagen folding.[4,13] There are

on average 20-22 amides that can exist in cis or trans in a 300 unit repeat of Xaa–Yaa–Gly, thus the number of possible conformational states of one strand is 1.5×10^6. The wrong cis conformers that must isomerize thus slow down the triple helix formation. Folding of collagen occurs in a processive fashion. Upon the complete formation of the triple helix, all amides exist in the trans conformation that is stablized within the folded protein.

Significant research has been performed regarding both the unique structural features of collagen and its potential biomedical applications.[205] Collagen has been regarded as one of the most useful biomaterials due to its excellent biocompatibility and safety. Major uses of collagen as a biomaterial include applications of collagen in drug delivery systems and in tissue-engineering systems.[205] However, the insufficient supply, poor mechanical strength, and ineffectiveness in the management of the infected sites of the collage-based system make improvements necessary.[206]

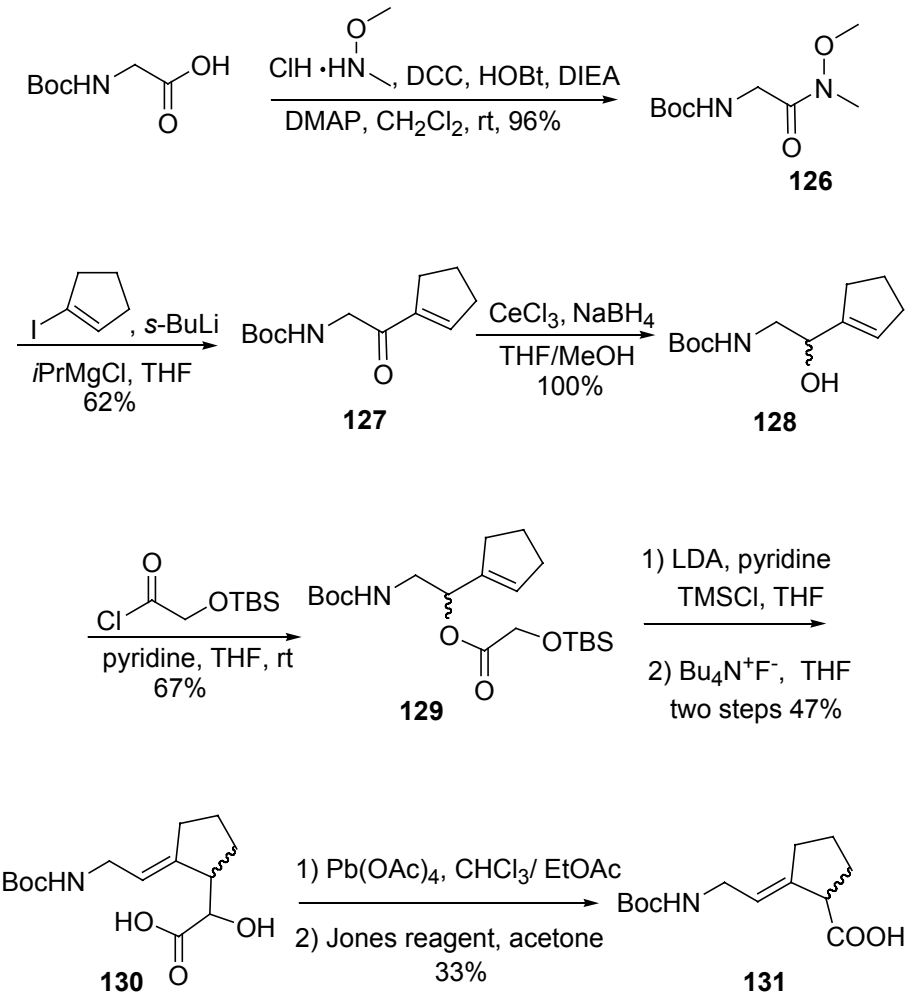
Several researchers have studied mimics of biological collagen, including polypeptides of the type $(Pro–Pro–Gly)_n$ and $(Pro–Flp–Gly)_n$ (where Flp represents 4(*R*)-fluoroproline), and all D-amino acid peptides.[207-209] However, none have prepared collagen mimics in which the amide bonds themselves have been altered. The replacement of the amide by an (*E*)-alkene would have two effects on the structure of the possible collagen mimics. First, the hydrophilic properties and hydrogen bonding sites are completely removed, which may thermodynamically destabilize the triple helix structure. On the other hand, the *E*-alkenes are not isomerable and represent the native conformation of the amides in collagen, which may dynamically facilitate the formation of the triple helixes.

Using amide bond polymerization of the appropriate monomers, we intended to synthesize materials that mimic the biological structure and behavior or collagen, yet are resistant to degradation. Since all proline in collagen exists in the trans conformation,[210,211] a route that affords *E* monomer regio-selectively is desired. Our recent success in Ser–*trans*–Pro (*E*)-alkene isostere synthesis provides a route to the monomer Gly–Ψ[(*E*)CH=C]–Pro–Hyp for initial polymerization studies. Alkene amide bond surrogates provide not only conformational control, but also resistance to peptidases. These synthetic collagen mimics will contribute not only to studies on the stability of collagen-like triple helical structures, but may also provide useful structural biomaterials.

6.2. Synthesis of Gly–*trans*–Pro–Hyp isosteres

The Gly–Pro dipeptide alkene analogues Gly–Ψ[(*E*)CH=C]–Pro–OH, a key intermediate of monomer Gly–Ψ[(*E*)CH=C]–Pro–Hyp for collagen mimic synthesis, has been used to provide conformationally constrained analogues of the Suc–Ala–Gly–Pro–Phe–pNA tetrapeptide substrate for cyclophilin.[212] A comparison of Gly–Ψ[CH=C]–Pro with the corresponding Gly–Ψ[CF=C]–Pro dipeptide isosteres indicated electrostatic interactions of the isosteric alkene bond may be important for cyclophilin inhibition.[212] Their synthetic strategy, either stereoselective or non-stereoselective, however, was never published. The Ireland-Claisen rearrangement strategy for selectively synthesizing Ser–Ψ[(*E*)CH=C]–Pro–OH (Chapter 2), thus was chosen for the monomer Gly–Ψ[(*E*)CH=C]–Pro–Hyp synthesis (Scheme 6.1).

Scheme 6.1. Synthesis of the isostere Gly–Ψ[(*E*)CH=C]–(D, L)–Pro–OH

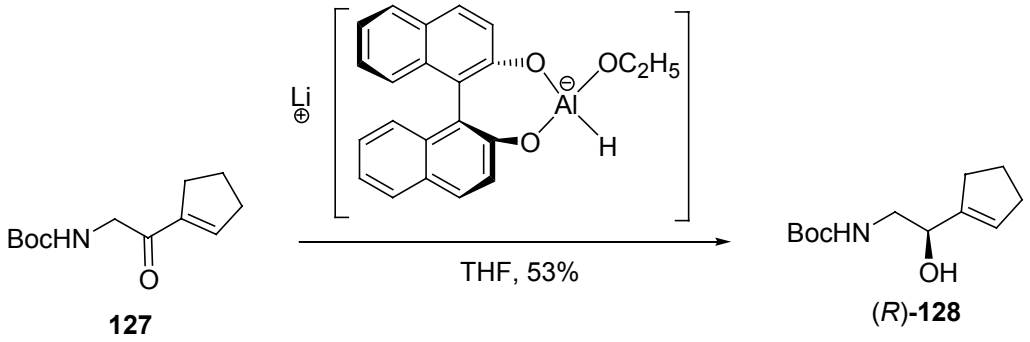


Starting from Boc–Gly–OH, the dipeptide isostere Gly–Ψ[(*E*)CH=C]–(D, L)–Pro–OH, **131**, was synthesized in eight steps with a total yield of 6%. The Boc-glycine Weinreb amide **126** was easily prepared as a white crystal with a yield of 96%. Our previous research employed 2.5 equiv of vinyl iodine regent for making the Boc-Ser ketone derivative, taking into account that deprotonation of the carbamate consumes 1 equivalent of vinyl lithium reagent. This occurs prior to the nucleophilic attack of the vinyl lithium to the Boc-Ser Weinreb amide. Li et al. utilized 1 equivalent of a simple Grignard reagent such as isopropyl magnesium chloride or methyl magnesium chloride to

deprotonate the carbamate and save the expensive and commercially unavailable reagents.[213] The condensation reaction was completed using 1 eq. isopropyl magnesium chloride to deprotonate the Boc carbamate and only 1.5 eq. of vinyl iodine reagent to give 62% of glycine α-Boc aminoketone **127**. The lower yield compared to the yield for synthesizing serine α-Boc aminoketone probably resulted from the instability of glycine α-Boc-aminoketone toward acid or Lewis acid. By standing at room temperature for 3 hours, the colorless elutant of compound **127** from silica chromatography turned yellow, and the solution of **127** in chloroform turned brown from colorless. Unlike serine, glycine is the only natural amino acid without a chiral center, thus we could not induce a chiral center to afford the (*R*) enantiomer of compound **128**. Noyori asymmetric reduction of the ketone **127** using stoichiometric amount of (*S*)-(-)-binaphthol-modified lithium aluminum hydride[214,215] afforded alcohol **128** with a yield of 53%, presumably assigned as (*R*) configuration, according to the reaction mechanism (Scheme 6.2).[214]

Although part of the chiral reagent (*S*)-(-)-binaphthol can be recycled, a more efficient asymmetric reduction using catalytic amount of chiral catalyst is desired. Martin Wills's group has described the enantioselective synthesis of β-hydroxy amines using asymmetric transfer hydrogenation of α-amino ketones.[216,217] In the published example, the best result (99% ee) was obtained with 0.5 mol% $[Ru(cymene)_2]Cl_2$ and 1 mol% TsDPEN ((*1R,2R*)-(-)-N-(4-toluenesulfonyl)-1,2-diphenylethylenediamine) as catalysts and HCO_2H and Et_3N as proton source.[216,217] This provided a practical way for making large amount of Gly–Ψ[(*E*)CH=C]–Pro–Hyp mimicking the L configuration of proline at the α position stereoselectively.

Scheme 6.2. Asymmetric reduction by binaphthol modified lithium aluminum hydride

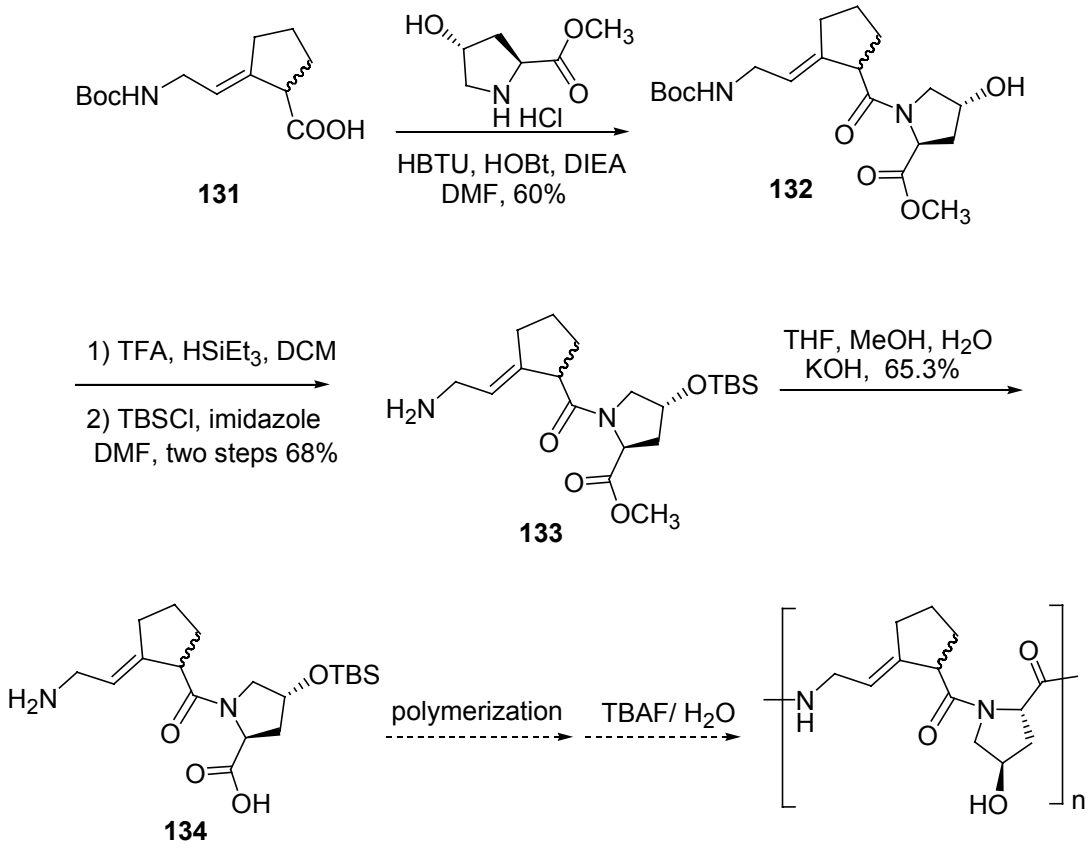


Although asymmetric reduction is possible, at this stage we were more interested in product **128** efficiently, even if it was racemic. Non-enantioselective reduction by the simple reducing reagent $NaBH_4$ afforded alcohol **128** in quantitative yield, which was carried on to afforded two diastereomers of the monomer, **134**, to be separated by HPLC and polymerized.

The remaining steps of the synthesis followed the steps of their Ser–Ψ[(*E*)CH=C]–Pro counterparts, except that a pair of enantiomers was obtained. As an alternative for the reagent *tert*-butyldimethylsilyloxyacetyl chloride, the commercially available reagent 2-benzoxyacetyl chloride could be used to synthesize an ester similar to compound **129** from alcohol **128**. But the deprotection of benzyl without reduction of the alkene is expected to be much more difficult.

To avoid formation of branched polymer, polymerization of protected Gly–Ψ[(*E*)CH=C]–(D,L)–Pro–Hyp–OH is desired. *tert*-Butyldimethylsilyl was a good choice because of the ease of installation and deprotection. Although the TBS protecting group is not orthogonal to Boc, the monomer Gly–Ψ[(*E*)CH=C]–(D,L)–Pro–Hyp(OTBS)–OH, **134**, was successfully synthesized through the strategy shown in Scheme 6.3.

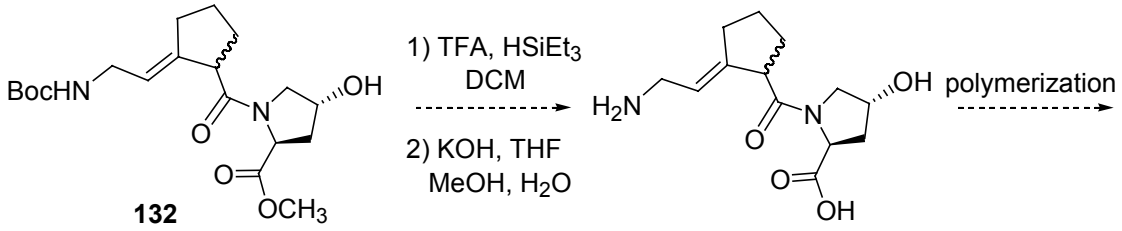
Scheme 6.3. Synthesis of Gly–Ψ[(*E*)CH=C]–(D,L)–Pro–Hyp(OTBS)–OH



Gly–Ψ[(*E*)CH=C]–(D,L)–Pro–OH **131** was coupled with 4-hydroxyl proline methyl ester using HBTU/HOBt. Tripeptide mimic Gly–Ψ[(*E*)CH=C]–(D,L)–Pro–Hyp(OTBS)–OH was obtained in 60% yield. Boc was removed by TFA with triethylsilane as cation scavenger. Without further purification, the TBS protecting group was installed with a two-step yield of 68%. Basic hydrolysis of methyl ester with potassium hydroxide released the carboxylic acid that is ready to polymerize. As expected, HPLC on monomer **134** showed two peaks with close retention times, corresponding to Gly–Ψ[(*E*)CH=C]–(L)–Pro–Hyp(OTBS)–OH and Gly–Ψ[(*E*)CH=C]–(D)–Pro–Hyp(OTBS)–OH. The ratio

of this pair of diastereomers was about 1:1. Scheme 6.4 shows the synthesis of an alternative monomer Gly–Ψ[(*E*)CH=C]–(D,L)–Pro–Hyp(OH)–OH, without any protection on the hydroxyl group. If the polymerization works for side chain unprotected monomer, the synthetic route will be simplified by two steps.

Scheme 6.4. Polymerization of Gly–Ψ[(*E*)CH=C]–(D, L)–Pro–Hyp(OH)–OH

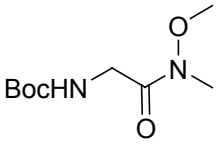


6.3. Conclusions

A pair of dipeptide amide isosteres of Gly–*trans*–Pro, Boc–Gly–Ψ[(*E*)CH=C]–(D,L)–Pro–OH was synthesized using an Ireland-Claisen rearrangement with an overall yield of 6% in 8 steps. This demonstrated the generality of synthesizing any Xaa–Pro isostere using the Ireland-Claisen rearrangement route. Several tripeptide isosteres, Boc–Gly–Ψ[(*E*)CH=C]–(D,L)–Pro–Hyp(OH)–OCH_3, Gly–Ψ[(*E*)CH=C]–(D,L)–Pro–Hyp(OTBS)–OCH_3, Gly–Ψ[(*E*)CH=C]–(D,L)–Pro–Hyp(OTBS)–OH, were all synthesized, to be used in amide polymerization reaction for collagen mimetic material synthesis. Because the synthesis was conducted on a very small scale, improvement of the overall yield for those dipeptide or tripeptide isosteres is expected in future work.

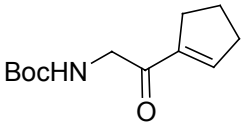
Experimental

General. Unless otherwise indicated, all reactions were carried out under N_2 in flame-dried glassware. THF, toluene, and CH_2Cl_2 were dried by passage through alumina. Anhydrous (99.8%) DMF was purchased from Aldrich and used directly from SureSeal^{TM} bottles. Dimethyl sulfoxide (DMSO) was anhydrous and dried with 4Å molecular sieves. Triethylamine (TEA) was distilled from CaH_2 and $(COCl)_2$ was distilled before use each time. Diisopropylethylamine (DIEA) was distilled from CaH_2 under a N_2 atmosphere. Brine (NaCl), $NaHCO_3$ and NH_4Cl refer to saturated aqueous solutions. Flash chromatography was performed on 32-63 μm or 230-400 mesh, ASTM, EM Science silica gel with reagent grade solvents. Melting points were determined with a Thomas Hoover Capillary Melting Point Apparatus and were uncorrected. NMR spectra were obtained at ambient temperature in $CDCl_3$ unless otherwise noted. Proton (500 MHz) and carbon-13 (125 MHz) NMR spectra were measured on a JEOL NMR spectrometer. ^{1}H NMR spectra are reported as chemical shift (multiplicity, coupling constant in Hz, number of protons).

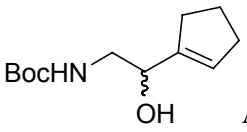


Boc–Gly Weinreb amide (126). Boc–Gly–OH (10.5 g, 60.0 mmol), *N,O*-dimethylhydroxylamine hydrochloride (11.1 g, 120 mmol) and DIEA (31.2 g, 240 mmol) were dissolved in 1:1 CH_2Cl_2/DMF (500 mL) and cooled to 0 °C. 1-Hydroxy-1*H*-benzotriazole (HOBt, 11.0 g, 72.0 mmol), DCC (14.9 g, 72.0 mmol) and DMAP (ca. 100 mg) were added and the reaction was stirred for 24 h. The reaction was filtered to remove dicyclohexylurea and concentrated. The resulting slurry was diluted with ethyl

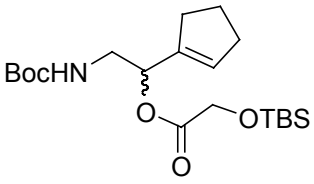
acetate (500 mL) and washed with NH_4Cl (2 × 100 mL), $NaHCO_3$ (2 × 100 mL) and brine (100 mL). The organic layer was dried on $MgSO_4$ and concentrated. Chromatography on silica with 20% EtOAc in hexane gave 12.6 g (96%) of **126** as a colorless plate-like crystal. m.p. 101-102 °C. ^{1}H NMR δ 5.25 (br, s, 1H), 4.07 (d, J = 3.7, 2H), 3.70 (s, 3H), 3.19 (s, 3H), 1.44 (s, 9H).



Ketone (127). To a solution of 1-iodocyclopentene (2.91 g, 15.0 mmol) in 80 mL THF at –40 °C was added *s*-BuLi (1.3 M in cyclohexane, 23 ml, 30 mmol). The reaction was stirred at –40 °C for 3 h to generate cyclopentenyl lithium. In another flask, Boc-glycine Weinreb amide **126** (2.18 g, 10.0 mmol) was dissolved in 20 mL of dry THF, and degassed under N_2. The solution was cooled to –15 to –10 °C and to the resulting slurry was charged with *i*-PrMgCl (2.0 M in THF, 4.9 mL, 9.8 mmol) dropwise at –15 to –5 °C to afford a clear solution. After cooling to –78 °C, the cyclopentenyl lithium solution was added via cannula to the deprotonated Weinreb amide solution. The mixture was stirred for 1 h at –78 °C, quenched with NH_4Cl (10 mL), diluted with EtOAc (100 mL), washed with NH_4Cl (2 × 20 mL), $NaHCO_3$ (20 ml), brine (20 mL), dried over $MgSO_4$ and concentrated. Chromatography on silica with 10% EtOAc in hexane gave 1.40 g (62%) of ketone **127** as a yellowish solid. Ketone **127** decomposed upon standing in solution. ^{1}H NMR δ 6.81 (s, 1H), 5.36 (br, s, 1H), 4.29 (d, J = 4.6, 2H), 2.56 (t, J = 7.7, 4H), 1.92 (m, 2H), 1.43 (s, 9H). ^{13}C NMR δ 192.9, 155.8, 144.6, 143.1, 79.7, 47.5, 34.2, 30.6, 28.4, 22.5. Anal. Calcd. for: $C_{12}H_{19}NO_3$: C, 63.98; H, 8.50; N, 6.22. Found: C, 63.71; H, 8.51; N, 6.15.

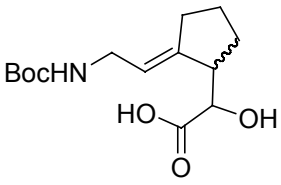


Alcohol (128). Ketone **127** (1.35 g, 6.00 mmol) was dissolved in 2.5:1 THF:MeOH (70 ml) and cooled to 0 °C. $CeCl_3$ (2.69 g, 7.20 mmol) was added, followed by $NaBH_4$ (0.46 g, 12 mmol). After stirring 1 h at 0 °C, the reaction was quenched with NH_4Cl (15 mL), diluted with EtOAc (100 mL), washed with NH_4Cl (2 × 20 mL), brine (20 mL), dried on $MgSO_4$, and concentrated. Chromatography on silica with 20% EtOAc in hexane yielded 1.36 g (100%) of a white solid. ^{1}H NMR δ 5.66 (m, 1H), 4.89 (br, s, 1H), 4.31 (d, J = 5.5, 1H), 3.38 (m, 1H), 3.13 (m, 1H), 2.31 (m, 4H), 1.88 (m, 2H), 1.43 (s, 9H). ^{13}C NMR δ 156.7, 144.6, 126.4, 79.6, 70.9, 45.3, 32.3, 31.9, 28.4, 23.4. Anal. Calcd for: $C_{12}H_{21}NO_3$: C, 63.41; H, 9.31; N, 6.16. Found: C, 63.63; H, 9.47; N, 6.09.



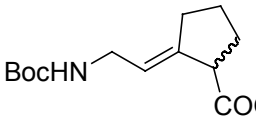
Ester (129). To a solution of alcohol **128** (12 mg, 0.053 mmol) and pyridine (13.3 μL, 0.165 mmol) in THF (0.1 mL) was added a solution of *tert*-butyldimethylsilyloxyacetyl chloride[124] (12 mg, 0.055 mmol) in THF (0.1 mL) dropwise at 0 °C. The mixture was stirred for 0.5 h at rt, then diluted with Et_2O (5 mL), washed with 0.5 N HCl (2 × 0.4 mL), $NaHCO_3$ (1 mL), brine (1 mL), dried on $MgSO_4$ and concentrated. Chromatography with 5% EtOAc in hexanes on silica gave 14.5 g (67%) of ester **129** as colorless oil. ^{1}H NMR δ 5.67 (s, 1H), 5.48 (br, s, 1H), 4.64 (br, s, 1H), 4.24 (s, 2H), 3.43 (m, 1H), 3.33 (m, 1H), 1.87 (m, 2H), 1.45 (s, 9H), 0.90 (s, 9H), 0.08 (s,

6H). ^{13}C NMR δ 171.2, 155.8, 139.9, 128.8, 79.6, 72.8, 61.8, 42.8, 32.4, 32.0, 28.4, 25.9, 25.6, 23.1, -5.4.

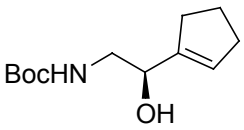


α-Hydroxy acid (130). To a solution of diisopropylamine (0.21 mL, 1.5 mmol) in THF (2.0 mL) was added *n*-butyl lithium (2.5 M in hexane, 0.54 mL, 1.3 mmol) at 0 °C. The mixture was stirred for 15 min to generate LDA. Then a mixture of chlorotrimethyl silane (0.46 mL, 3.7 mmol) and pyridine (0.32 mL, 4.0 mmol) in THF (0.8 mL) was added dropwise to the LDA solution at –100 °C. After 5 min, a solution of ester **129** (136 mg, 0.333 mmol) in THF (1 mL) was added dropwise and the reaction was stirred at –100 °C for 25 min, then warmed slowly to rt over 1.5 h and heated to 45 °C for 1 h. The reaction was quenched with 1 N HCl (5.0 mL) and the aqueous layer was extracted with Et_2O (2 × 7 mL). The organic layer was dried on $MgSO_4$ and concentrated to give 106 mg (crude yield 78%) of a yellowish glassy oil. Without further purification, the oil was dissolved in 0.8 mL THF. TBAF (261 mg, 1.00 mmol) in THF (0.5 mL) was added at 0 °C, stirred at 0 °C for 5 min, and then at rt for 1 h. The reaction was quenched with 0.5 N HCl (2 mL), extracted with EtOAc (5 mL), dried on $MgSO_4$ and concentrated. Chromatography with 5% MeOH in $CHCl_3$ on silica gave 46 mg (52%) of α-hydroxy acid **130** as yellowish oil. ^{1}H NMR (DMSO-d_6) δ 6.81, (br, s, 1H), 5.31 (br, s, 1H), 3.84 (d, J = 5.8, 1H) 3.48 (m, 2H), 3.16 (t, J = 8.5, 1H) 2.64 (m, 1H), 2.27 (m, 1H), 2.12 (m, 1H), 1.70 (m, 2H), 1.58-1.42 (m, 2H), 1.37 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 175.4,

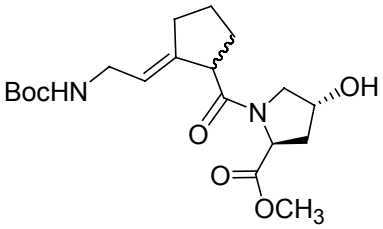
156.0, 144.4, 120.2, 79.7, 78.0, 73.7, 58.1, 47.4, 29.8, 28.9, 24.5, 23.6, 24.5, 23.6, 19.8, 14.1.



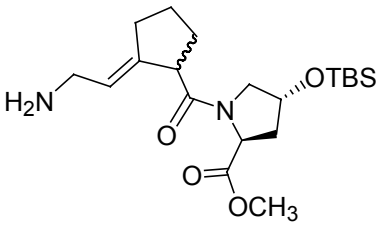
Acid (131). Lead tetraacetate (78 mg, 0.17 mmol) in $CHCl_3$ (0.4 mL) was added dropwise to a solution of α-hydroxy acid **130** (45.6 mg, 0.16 mmol) in EtOAc (2.2 mL) at 0 °C. The reaction was stirred for 10 min, then quenched with ethylene glycol (0.6 mL), diluted with EtOAc (20 mL), washed with H_2O (4 × 2 mL) and brine (2 mL), dried on Na_2SO_4, and concentrated to give 38 mg (100% crude yield) of aldehyde as yellow oil. The product was dissolved in acetone (4.8 mL) and cooled to 0 °C. Jones reagent (2.7 M H_2SO_4, 2.7 M CrO_3; 0.12 mL, 0.32 mmol) was added dropwise. The reaction was stirred at 0 °C for 0.5 h, quenched with isopropyl alcohol (0.5 mL), and stirred for 10 min. The precipitate was removed by filtration, and the solvent was evaporated. The residue was extracted with EtOAc (3 × 5 mL), washed H_2O (1.5 mL) and brine (1.5 mL), dried on Na_2SO_4, and concentrated. Chromatography on silica with 40% EtOAc and 0.1 acetic acid in hexane gave 12.5 mg (31%) of acid **131** as a white solid. ^{1}H NMR (DMSO-d_6) δ 12.16 (br, s, 1H), 6.93 (t, J=5.4, 1H), 5.37 (s, 1H), 3.50 (m, 2H), 3.16 (t, J = 7.3, 1H), 2.29 (m, 1H), 2.22 (m, 1H), 1.80 (m, 3H), 1.55 (m, 1H), 1.37 (s, 9H). ^{13}C NMR (DMSO-d_6) δ 175.3, 156.1, 142.9, 120.8, 78.1, 49.5, 30.1, 29.2, 28.8, 25.0. Anal. Calcd for: $C_{13}H_{21}NO_4$: C, 61.16; H, 8.29; N, 5.49. Found: C, 61.11; H, 8.25; N, 5.48.



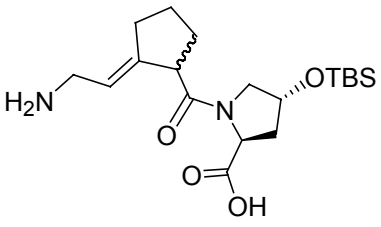
Chiral lithium aluminum hydride reduction to give alcohol *R*-(128): Ethyl alcohol (14 mg, 0.30 mmol) and a (*S*)-binaphthol THF solution were added consecutively to a solution of $LiAlH_4$ in THF (1.0 M, 0.30 mL, 0.30 mmol) dropwise at rt. After stirring at rt for 30 min, the reaction mixture was cooled to –100 °C and a solution of ketone **127** (23 mg, 0.10 mmol) in THF (0.2 mL) was added, stirred at –78 °C for 2 h. The reaction was quenched with a mixture of methanol (50 μL) and H_2O (200 μL) at –78 °C and warmed to rt. Ethyl ether (1.5 mL) was added and stirred for 1 h. The mixture was dried over $MgSO_4$ and concentrated. Chromatography with 30% EtOAc in hexanes gave 12 mg (yield 53%) of a white solid. ^{1}H NMR data were the same as for racemic **128**.



Amide (132): HOBt (192 mg, 1.25 mmol), HBTU (473.8 mg, 1.25 mmol), DIEA (323 mg. 2.5 mmol) and acid **131** (120 mg, 0.5 mmol) were dissolved in DMF (25 mL). 4-(*R*)-Hydroxyproline methyl ester hydrochloride salt (225mg, 1.25 mmol) was added. The reaction mixture was stirred at rt for 1 h, then diluted with EtOAc (75 mL), washed with H_2O (3 × 25 mL), $NaHCO_3$ (25 mL), brine (25 mL), dried on $MgSO_4$ and concentrated. Chromatography with 50% EtOAc in hexanes yielded 110 mg (yield 60%) of colorless syrup.



Amine (133): Amide **132** (110 mg, 0.302 mmol) and triethylsilane (87.79 mg, 0.755 mmol) were dissolved in 25% TFA in DCM and stirred for 0.5 h at rt. Solvent was evaporated. The remaining TFA and triethyl silane were removed under high vacuum. Without further purification, the residue was dissolved in 2 mL CH_2Cl_2, then TBS-Cl (91 mg, 0.604 mmol) and imidazole (82 mg, 1.208 mmol) were added. The reaction mixture was stirred at rt for 4 h then diluted with EtOAc, washed with $NaHCO_3$ (2 × 7 mL), H_2O (7 mL), dried on $MgSO_4$, and concentrated. Chromatography on silica gel with 15% MeOH in $CHCl_3$ gave 81 mg (67.7%) as a colorless oil.



Acid (134): To a solution of amine **133** (80 mg, 0.2 mmol) in THF (1.2 mL) was added slowly a solution of KOH in 1:2 MeOH: H_2O (0.6 mL) at –10 °C. After stirring for 1 h at 0 °C, the reaction was diluted with 5 mL THF, acidified with 1 N HCl (0.21 mL), dried over $MgSO_4$ and concentrated. Chromatography on silica gel with 15% MeOH in $CHCl_3$ gave 50 mg (yield 65.3%) of a colorless oil.

Chapter 7. Conclusions

We designed and stereoselectively synthesized two amide isosteres of Ser-*trans*-Pro and Ser-*cis*-Pro dipeptides with high yields. The conformationally locked Ser–*trans*–Pro mimic, Boc–SerΨ[(*E*)CH=C]Pro–OH, was synthesized through the use of an Ireland-Claisen [3,3]-sigmatropic rearrangement in nine steps with 13% overall yield from the natural amino acid serine. The Ser-*cis*-Pro mimic, Boc–SerΨ[(*Z*)CH=C]Pro–OH, was synthesized through the use of an Still-Wittig [2,3]-sigmatropic rearrangement in 11 steps with an overall yield of 20% from the same starting material. The Ireland-Claisen rearrangement route was high yielding and highly stereoselective and served as a general synthetic route for constructing any Xaa–*trans*–Pro isostere starting from the corresponding optically pure amino acid (Xaa). Gly–*trans*–Pro isoteres were synthesized using the Ireland-Claisen route, to be used to synthesize collagen-like biomaterials.

A series of conformationally locked inhibitors of the PPIase Pin1, based loosely on the optimal hexapeptide Pin1 substrate Ac–Trp–Phe–Tyr–pSer–Pro–Arg–*p*NA,[36] were designed, synthesized, and assayed. These inhibitors were used to study the PPIase mechanism of Pin1, an essential cell cycle regulator, and will be used to assist the understanding the biological function of Pin1 in mitosis. The central pSer-Pro core of the Pin1 substrate was replaced by (*Z*)-and (*E*)-alkene analogues. They were synthesized on solid-phase resin using Fmoc chemistry from Fmoc-protected, phosphorylated building blocks **109** and **110,** and Fmoc-, TBS-protected building block **94.** The trans dipeptide isostere **109** was coupled with HATU/HOAt and 2,4,6-collidine to minimize the isomerization of the alkene. The construction of a non-peptidic (*Z*)-alkene library for

Pin1 inhibition was attempted using the Ser-*cis*-Pro mimic, Boc–SerΨ[(*Z*)CH=C]Pro–OH as the core.

A protease-coupled assay for CyP and FKBP was adapted to assay these Pin1 inhibitors. They inhibited Pin1 with IC_{50} values ranging from sub-micromolar to hundreds of micromolar, with the cis analogue Ac–Phe–Tyr–pSer–Ψ[*(Z)*CH=C]Pro–Arg–NH_2, **114**, being the most potent. We demonstrated that both the trans and the cis alkene pentapeptide analogues **112** and **113** are competitive inhibitors of Pin1 by this protease-coupled assay. The fact that the (*Z*)-alkene **113** was a more potent competitive inhibitor (K_{is} = 1.74 ± 0.08 μM) than the (*E*)-alkene **112** (K_{is} = 39.8 ± 2.4 μM) suggests that Pin1 binds the cis substrate more tightly at the catalytic site. Indeed both the trans isostere and the cis isostere bound to Pin1 in its active site in the X-ray crystal structure.[Jessie Zhang, Joseph Noel, unpublished results] These two Pin1 inhibitors also inhibited A2780 ovarian cancer cell growth in vitro with IC_{50} values of 8.3 ± 0.5 μM for the cis analogue **113** and 140 ± 10 μM for the trans analogue **112**. This suggests that Pin1 could be the target that accounts for the antiproliferative activity against the human ovarian cancer cell line. The two (*Z*)-alkene inhibitors, **113** and **114**, are among the most potent inhibitors found for Pin1 so far, and they are neither a nonspecific thiol capture agent such as juglone nor an ordinary peptide. Nonpeptidic compounds possessing the core Ser-*cis*/*trans*-Pro isosteres are quite promising as Pin1 inhibitors, as tools for investigating Pin1 regulation of mitosis, and as anti-cancer drug leads.

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