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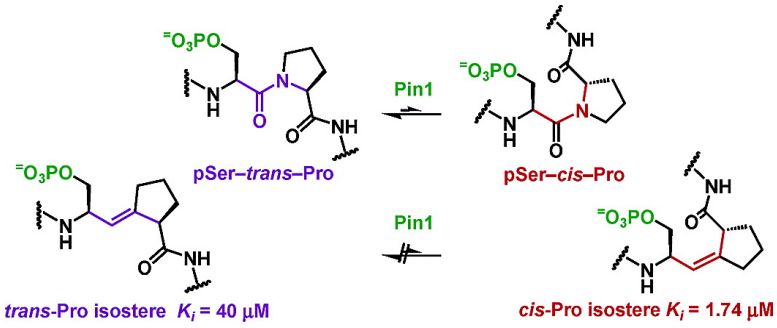

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Conformationally Locked Isostere of PhosphoSer−*cis*-Pro Inhibits Pin1 23-Fold Better than PhosphoSer−*trans*-Pro Isostere

Xiaodong J. Wang, Bailing Xu, Ashley B. Mullins, Freda K. Neiler, and Felicia A. Etzkorn*

Contribution from the Department of Chemistry, Virginia Tech, Blacksburg, Virginia 24061-0212

Received June 18, 2004; E-mail: fetzkorn@vt.edu

Abstract: Stereoisomeric cis and trans substrate analogues for Pin1 were designed and synthesized. The central phosphoSer−Pro core of the Pin1 substrate was replaced by cis and trans amide isosteres in Ac−Phe−Phe−pSer−Ψ[(*Z* and *E*)CH=C]−Pro−Arg−NH_2, **1** and **2**, peptidomimetics. They were synthesized on solid phase in 17% yield for the cis analogue **1**, and 16% yield for the trans analogue **2**. A second trans amide isostere with a C-terminal *N*-methylamide **3** was synthesized in 7% yield. The protease-coupled Pin1 assay showed that all three compounds inhibited the Pin1 peptidyl-prolyl isomerase (PPIase) enzymatic activity. The cis isostere **1** was 23 times more potent ($K_i = 1.74 \pm 0.08$ μM) than its trans counterpart **2** ($K_i = 40 \pm 2$ μM) in competitive inhibition of Pin1. These results suggest that the catalytic site of Pin1 binds cis substrates more tightly in aqueous solution. Antiproliferative activity toward the A2780 human ovarian cancer cell line by the cis and trans analogues correlates with Pin1 inhibition results.

Introduction

The peptidyl-prolyl isomerase (PPIase) parvulin family,[1] together with two other PPIase families, the cyclophilins (CyPs) and FK506 binding proteins (FKBPs), catalyze the cis−trans isomerization of Xaa−Pro amide bonds in proteins.[2,3] PPIases are fascinating enzymes because the reactions they catalyze do not involve bonds breaking or forming, but rather a simple conformational change around Xaa−Pro amide bonds (Figure 1).[4−6] Thus, they act only to reestablish the equilibrium between cis and trans conformations after the equilibrium has been upset in vivo, for example, by protein folding or phosphorylation.[7−9] The activation barrier to the uncatalyzed isomerization of Suc−Ala−Ala−*cis*-Pro−Phe−*p*-nitroanilide (*p*NA) is 19.3 kcal/mol, while the cyclophilin-catalyzed barrier is 16 kcal/mol.[4] There appears to be a single enzymatic transition state between the bound cis and bound trans conformations.[10,11] The raison d'être

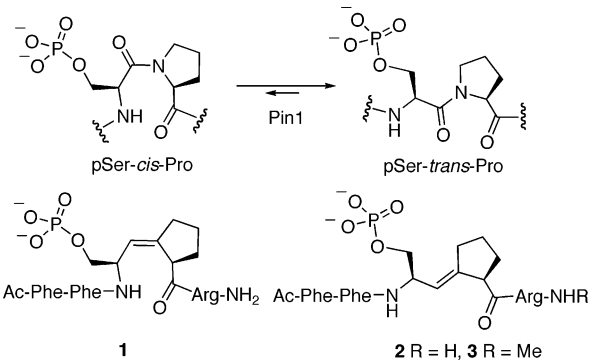

Figure 1. Top: Isomerization of −pSer−Pro− amides by Pin1. Bottom: Conformationally locked inhibitors, cis isostere **1** and trans isosteres **2** and **3**.

of catalysts for such a simple reaction must be simply a need for speed under certain circumstances, for example, rapid protein folding under stress or a cooperative transition in the cell cycle. Indeed, PPIases accelerate the reaction rate by ca. 10^6 s^{-1} over the already rapid thermal isomerization.[6] There must be a biological significance or these enzymes would not have evolved, nor would high cellular concentrations be maintained.[6]

Several features of human Pin1, a member of the parvulins, are unique among PPIases.[1] Unlike CyP and FKBP, which are primarily of interest because they bind the immunosuppressant drugs, cyclosporin and FK506, respectively,[2,3] Pin1 is not known to bind an immunosuppressant. The crystal structure suggests that Pin1 has a strong preference for acidic side chains in the residue preceding proline in substrates.[12] Indeed, Pin1 specifically isomerizes pSer−Pro or pThr−Pro amide bonds in phosphopeptides with selectivities of up to 1300-fold greater

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(k_{cat}/K_m) than the nonphosphorylated peptides.[9] Neither cyclophilin nor FKBP PPIases effectively isomerize peptides with phosphorylated Xaa−Pro moieties.[9]

Pin1 is a significant new cell cycle regulator.[13] Pin1 has been found to regulate mitosis through a simple conformational change, the cis−trans isomerization of pSer/Thr−Pro amide bonds in key cell cycle regulatory proteins,[9] particularly Cdc25 phosphatase,[14,15] the p53 oncogene,[16−18] and the c-Myc oncogene.[19]

Overexpression of Pin1 inhibits the G2-to-M transition, while depletion of Pin1 leads to premature entry into mitosis, followed by apoptosis.[1] Pin1 is present at higher concentrations during mitosis, making it a target primarily in the continuously dividing cells of cancer.[20,21] In addition, Pin1 is overexpressed in a large number of cancer cell types as compared with normal cell types.[22,23]

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. Nonisomerizable Pin1 inhibitors may be useful in elucidating the mechanism of mitosis.

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.[12] X-ray crystal structures of human CyPA (hCyPA) complexes with Xaa−Pro peptides show a preference for *cis*-Pro amides.[24−26] 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,[27] in agreement with the observation of cis substrate in the X-ray structures.

Alkenes as amide isosteres have been shown to be effective inhibitors of PPIases. We have shown that hCyPA is inhibited by an Ala−*cis*-Pro (*Z*)-alkene peptidomimetic (IC_{50} = 6.5 μM).[28,29] FKBP is inhibited by a Leu−*trans*-Pro (*E*)-alkene isostere (IC_{50} = 8.6 μM).[30] The synthesis and biological activities of alkene analogues and PPIase inhibitors have been reviewed.[31,32] The first reported inhibitor of Pin1 and yeast homologues, Ptf1 and Ess1, was the natural product juglone (5-hydroxy-1,4-naphthoquinone), found by screening pure secondary metabolites against the enzymatic activity of *E. coli* parvulin.[33] Pentapeptides centered on phosphoserine were found to be the optimal substrates for Pin1 PPIase activity.[34] Interestingly, incorporation of D-Ser instead of the natural L-Ser results in Pin1 inhibition (IC_{50} = 1 μM).[34] Substitution of the amide preceding Pro with a thioxo amide produced a 4 μM Pin1 inhibitor.[34]

In this work, three conformationally locked Pin1 substrate isosteres, Ac−Phe−Phe−pSer−Ψ[(*Z* and *E*)CH=C]−Pro−Arg−NH_2, **1** and **2**, and *N*-methylamide, **3**, were designed, synthesized, and assayed for Pin1 PPIase inhibition (Figure 1). These peptidomimetics were based on the selectivity of Pin1 for aromatic amino acids at the N-terminus and basic residues at the C-terminus.[9] Previously, we synthesized the core alkene isosteres of Ser−*cis*-Pro and Ser−*trans*-Pro dipeptides stereoselectively through a Still−Wittig rearrangement and an Ireland−Claisen rearrangement, respectively.[35,36] Otaka et al. reported an alternate synthesis of a similar Ser−*trans*-Pro isostere.[37] We now report the solid-phase synthesis of the (*Z*)- and (*E*)-alkene peptidomimetics, the inhibition constants (K_i), and the competitive mode of inhibition of **1** and **2** for Pin1.[38,39] Antiproliferative activity toward ovarian cancer cells in vitro is also reported. These inhibitors provide evidence to establish Pin1 as an anticancer drug target.

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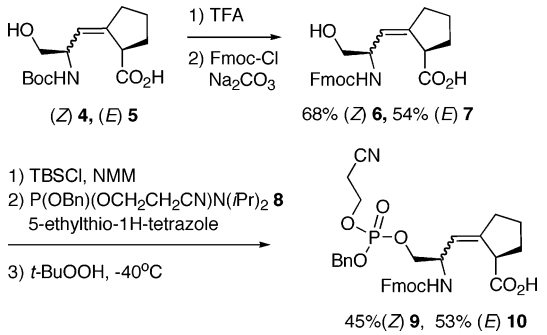
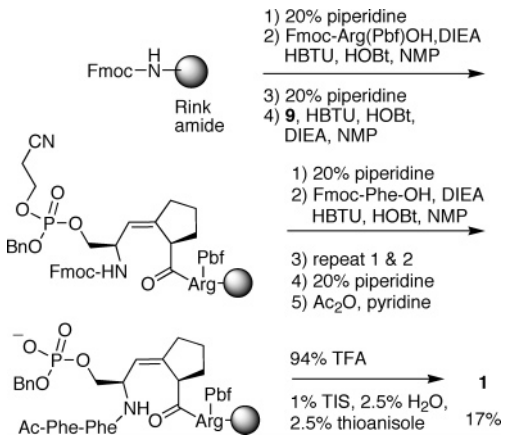
Scheme 1. Protection and Phosphorylation of Fmoc-Ser-*cis*- and -*trans*-Pro-OH Amide Isosteres



Scheme 2. Synthesis of Cis Isostere Peptidomimetic **1**



Results and Discussion

Synthesis of Isosteric Cis and Trans Peptidomimetics 1 and 2. We previously reported the syntheses of a pair of stereoisomeric alkene analogues of Ser−*cis*-Pro and Ser−*trans*-Pro dipeptides.[36] We now report the incorporation of cis **4** and trans **5** isosteres (Scheme 1) into peptidomimetics, Ac−Phe−Phe−pSer−Ψ[(*Z* and *E*)CH═C]−Pro−Arg−NH_2, **1** and **2**, as Pin1 substrate analogue inhibitors (Figure 1). The matched stereoisomeric pair of compounds was synthesized to permit direct, quantitative comparison of the affinity (K_i) of each inhibitor for the Pin1 PPIase catalytic site. Both cis and trans pentapeptide analogues were made with the C-termini as carboxamides and 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.

Fmoc chemistry is more widely used than Boc chemistry in solid-phase peptide synthesis[40,41] and permits complicated modifications of the peptide. Because attempts at peptide syntheses using Merrifield chemistry[42] did not yield satisfactory results, both mimics, Boc−Ser−Ψ[(*Z*)CH═C]−Pro−OH, **4** and Boc−Ser−Ψ[(*E*)CH═C]−Pro−OH, **5**, were reprotected as the Fmoc-carbamates **6** and **7** (Scheme 1). Deprotections of Boc by acidolysis were carried out in the presence of triethylsilane as a carbocation scavenger, greatly improving the yields.[43] Reactions with Fmoc−Cl were conducted by adding saturated Na_2CO_3 intermittently to maintain the pH between 8 and 9, giving the Fmoc-protected compounds **6** and **7** with two-step yields of 68% and 54%, respectively.

Phosphorylation via a building block approach was found to give the best results in each case, although global phosphorylation to give the *N*-methylcarboxamide **3** was also successful (Figure 1). The unsymmetrical phosphoramidite, *O*-benzyl-*O*-β-cyanoethyl-*N*,*N*-diisopropylphosphoramidite, **8**, was originally used as a phosphorylation reagent for the synthesis of a glycolipid.[44] 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.[45] The dipeptide analogues were phosphorylated with **8** in a one-pot reaction according to published procedures with minor modifications.[44,46] Each Fmoc-protected isostere was treated with 1 equiv each of TBSCl[47] and NMM,[47] which selectively blocked the carboxyl group and left the side-chain hydroxyl group free. Phosphitylation by **8** and 5-ethylthio-1*H*-tetrazole, followed by oxidation with *tert*-butyl hydroperoxide, gave the protected phosphodipeptide isosteres **9** and **10** in 45% and 53% yield, respectively (Scheme 1). No isomerization of the β,γ-unsaturated acids to the more stable α,β-unsaturated acids occurred during these reactions. Activated ester conditions that do cause this isomerization will be described.

Peptide synthesis incorporating the block-phosphorylated cis isostere **9** into pentapeptide analogue **1** was successful using the Rink amide MBHA resin[47] with HBTU/HOBt[47] as the coupling reagents in NMP[47] (Scheme 2).[48,49] The side chain of arginine was protected with the Pbf[47] group. One attempt to couple cis isostere **7** with the alcohol side chain unprotected was unsuccessful because the activated ester cyclized to form the corresponding seven-membered lactone.

Originally, trans analogue **2** was synthesized by the same procedure used for the synthesis of **1**, except that only 0.7 equiv of the (*E*)-alkene building block **10** was used. 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.[50] This suggested that the HOBt activated ester of (*E*)-alkene **10** isomerized to the α,β-unsaturated carbonyl compound. Indeed, intermediate **7** was shown

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(47) Abbreviations: TBSCl = *tert*-butyldimethylsilyl chloride, NMM = *N*-methyl morpholine, Rink amide MBHA = 4-(2′,4′-dimethoxylphenyl-Fmoc-aminomethyl)-penoxyacetamido-norleucyl-4-methylbenzhydrylamine, HBTU = *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole, NMP = *N*-methylpyrrolidinone, Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, HOAt = 1-hydroxy-7-azabenzotriazole, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, TBAF = tetra-*n*-butylammonium fluoride, TFE = trifluoroethanol, MCA = 7-(4-methylcoumaryl)amide, DIEA = *N*,*N*-diisopropylethylamine, TIS = triisopropylsilane, HEPES = 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, EDC = *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide, TRIS = tris(hydroxymethyl)-aminomethane, DTT = dithiothreitol.
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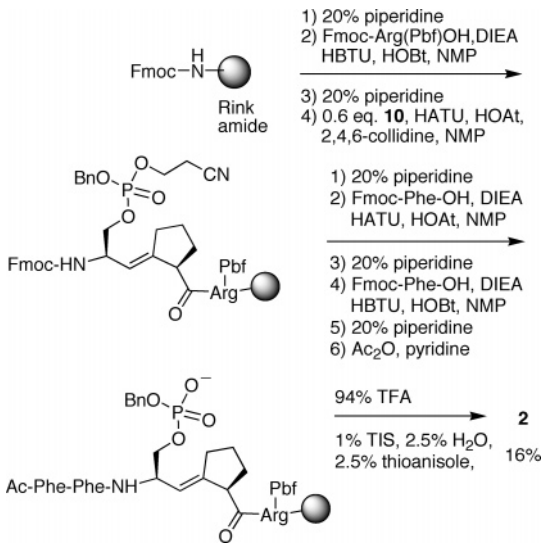
Scheme 3. Synthesis of Trans Isosteric Peptidomimetic **2**

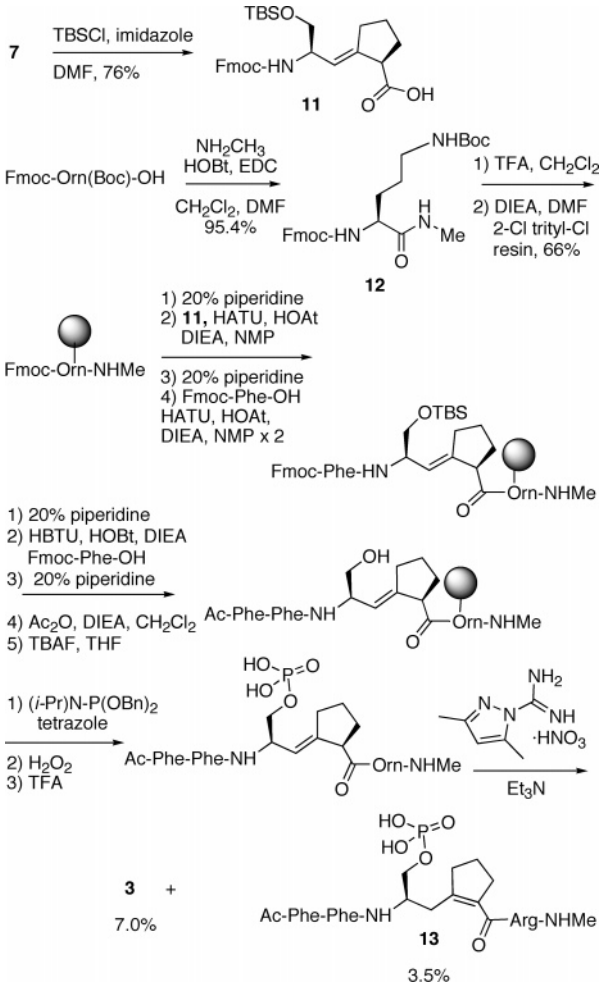


by NMR to isomerize under HBTU/HOBt activation conditions (Supporting Information). Interestingly, there were no signs of isomerization of (*Z*)-alkene **9** under similar conditions. These results are the opposite of disubstituted alkene peptide isosteres in which *cis*-alkenes have been shown to be more susceptible to alkene isomerization than *trans*-alkenes.[51]

Because this alkene isomerization and amino acid racemization have similar mechanisms that are both due to the acidity of the proton α to the activated ester, coupling conditions that minimize racemization would prevent alkene isomerization. Carpino and co-workers have shown that HOAt[47] and its corresponding uronium salt, HATU,[47] are more effective activating reagents in avoiding racemization of amino acids during peptide synthesis.[52−55] When coupling of (*E*)-alkene **10** was conducted with a combination of HATU/HOAt and the hindered base 2,4,6-collidine,[53] the desired peptide mimic **2,** Ac−Phe−Phe−pSer−Ψ[(*E*)CH═H]−Pro−Arg−NH_2, was obtained as the major product in 16% overall yield after HPLC purification (Scheme 3).

Synthesis of *N*-Methylamide Trans Peptidomimetic 3. Prior to the synthesis of the (*Z*)-alkene pentapeptide mimic **1**, we had completed the synthesis of *N*-methylamide **3**. This inhibitor differs only by one methyl group from **2**, but because a direct comparison between exact stereoisomers was desired, we chose to repeat the synthetic route used to make cis mimic **1** as described above. The synthesis of *N*-methylamide **3** is now described. The route used was analogous to the route described by Schutkowski et al. to synthesize phosphorylated *p*-nitroanilide peptide substrates for Pin1.[56]

Scheme 4. Synthesis of Ac−Phe−Phe−pSer−Ψ[(*E*)CH═H]−Pro−Arg−NHMe **3**



Protection of the hydroxyl side chain was conducted in the presence of the free carboxylic acid (Scheme 4). At least 2.5 equiv of TBSCl was 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, **11**, in 76% yield. With building block **11** in hand, Fmoc-based solid-phase peptide synthesis with global phosphorylation was used to produce the trans isosteric inhibitor **3**.

The initial synthetic target was the methyl ester, Ac−Phe−Phe−pSer−Ψ[(*E*)CH═C]−Pro−Arg−OMe, but the Fmoc-ornithine methyl ester could not be loaded onto the resin, probably because of cyclization to the lactam. Guanidinylation at the end of peptide synthesis under basic conditions was expected to have similar problems, so the synthetic target was modified to Ac−Phe−Phe−Ser−Ψ[(*E*)CH═C]−Pro−Arg−NHMe, **3**. The amide bond was more stable than the methyl ester and was not expected to cyclize under peptide synthesis or guanidinylation conditions.[57]

The synthesis of the phosphorylated peptide mimic **3** was carried out on the 2-chlorotrityl chloride resin,[58,59] according

(50) 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, this ^{1}H NMR matches that of **13** obtained in the synthesis of **3**.

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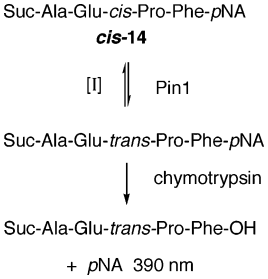
to Schutkowski and co-workers,[56] with two modifications (Scheme 4). First, the dipeptide isostere **11** was incorporated with a TBS protected side chain to avoid acylation of the hydroxyl group. This protection also allowed excess activated amino acids to be used in subsequent coupling steps. Second, 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, **12**, in 95% yield (Scheme 4). 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.[60] 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.[61] Guanidinylation[57] was difficult at room temperature, but went to completion after 7 h at 45 °C as monitored by reverse phase HPLC.

HPLC analysis after guanidinylation showed two major peaks with close retention times of 12.5 and 12.9 min. 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. During solid-phase synthesis, HPLC analysis of the intermediates cleaved from the resin with 10% TFA after each coupling after the trans isostere **11** showed a pair of peaks with very close retention times as major products. Because the alkene proton peak was missing in the first isomer **13** at 12.5 min, it is likely that the trans β,γ-unsaturated isostere **11**, as the HOAt-activated ester, isomerized to form the α,β-unsaturated alkene under these coupling conditions. In the synthesis of primary amide trans isostere **2**, a major side product was the isomerized alkene, yet only the desired β,γ-unsaturated product was obtained using coupling conditions that suppress racemization of activated esters (vide infra). HPLC purification of *N*-methylamide trans isostere **3** was performed on a C4 semipreparative column. The purities of both isomers **3** and **13** were above 97% by HPLC.

Pin1 Inhibition Assays. Several PPIase inhibition assays have been developed.[34,38,62] We adapted the protease-coupled assay of Rich and co-workers for CyP and FKBP to Pin1 (Scheme 5).[38] This assay is based on the conformational specificity of the proteases chymotrypsin or trypsin that specifically cleave the amide bond between the $P_{2'}$ and $P_{3'}$ positions of Xaa−*trans*-Pro-containing peptides.[63] TFE[47] containing 0.47 M LiCl was used as the substrate solvent to increase the concentration of the cis isomer of the peptide substrate enough to measure K_m and K_i at the onset of the isomerization.[39]

Scheme 5. Pin1 PPIase Inhibition Assay



Inhibitors were preincubated with Pin1 for 10 min at 4 °C. The reaction was initiated by addition of substrate rather than chymotrypsin to maintain the concentration of cis substrate at the start of the reaction.

The commercially available peptide Suc−Ala−Glu−Pro−Phe−pNA, **14** (Scheme 5), was used as the substrate for Pin1 (k_{cat}/K_m = 3410 mM^{-1} s^{-1}).[9] The C-terminal phenylalanine makes it a suitable substrate for chymotrypsin, rather than trypsin which may proteolyze Pin1 to a greater extent.[62] In this coupled assay, the chymotrypsin is used in excess to be sure 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.

Instead of 50 mM HEPES[47] with 100 mM NaCl buffer typically used for the hCyPA assay,[64] 35 mM HEPES pH 7.8 was used as the Pin1 buffer because Pin1 activity dramatically decreases with increasing ionic strength.[65] For substrate **14**, Pin1 has maximum activity at pH 6; either increasing or decreasing pH results in decreased Pin1 activity.[12] 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 **14**, 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}).

The determination of the Michaelis constant (K_m) for the substrate ***cis*-14** and the measurement of the inhibition constants for cis isostere **1** and trans isostere **2** were conducted by the method of Kofron.[38] The K_m value for ***cis*-14** was 183 ± 9 μM, which is in agreement with the published K_m value of Suc−Ala−Glu−*cis*-Pro−Phe−MCA[47] (120 μM).[66] The calculated values of IC_{50} were obtained by fitting to a hyperbolic curve (Table 1).

For the inhibition constants (K_{is}), data analysis was performed according to Kofron et al. (see Supporting Information).[38] The resulting initial velocity, substrate concentrations, and inhibitor concentrations were evaluated using Cleland's programs[67] to determine the inhibition constants and the inhibition patterns. Thus, the inhibition patterns were determined to be competitive for both cis isostere **1** and trans isostere **2**. The hyperbolic and

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Table 1. Inhibition of Pin1 and A2780 Ovarian Cancer Cell Antiproliferation

compounds	IC_{50} (μM)	K_{is} (μM)	A2780 IC_{50} (μM)
1 Ac−Phe−Phe−pSer−Ψ[(*Z*)CH=C]−Pro−Arg−NH_2	1.3 ± 0.2	1.74 ± 0.08	8.3 ± 0.5
2 Ac−Phe−Phe−pSer−Ψ[(*E*)CH=C]−Pro−Arg−NH_2	28 ± 3	40 ± 2	140 ± 10
3 Ac−Phe−Phe−pSer−Ψ[(*E*)CH=C]−Pro−Arg−NHMe	24 ± 3	ND[a]	ND[a]
13 (endocyclic alkene isomer of **3**)	100 ± 20	ND[a]	ND[a]

[a] ND = not determined.

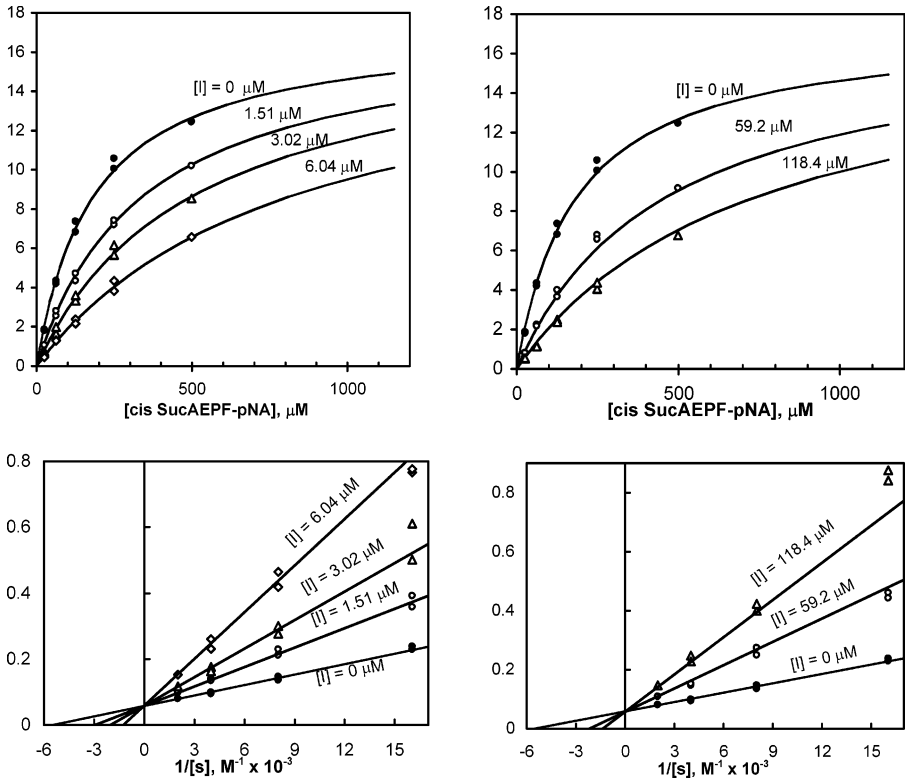

Figure 2. Hyperbolic and double reciprocal plots of the competitive inhibition of human Pin1 by **1** (left) and **2** (right). The K_{is}, K_m, and V_{max} values were calculated with Cleland's programs.[67]

double reciprocal plots are shown in Figure 2. The competitive inhibition constants (K_{is}) are summarized in Table 1.

A2780 Ovarian Cancer Cell Assay. Compounds **1** and **2** were assayed for antiproliferative activity against A2780 ovarian cancer cells as previously reported.[68,69] Results from the antiproliferative assay gave an IC_{50} value of 8.3 μM for the cis isostere **1** and 140 μM for the trans isostere **2** (Table 1). Thus, the cis isostere **1** is 17-fold more potent than the corresponding trans isostere **2**.

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. This is in agreement with our expectation for the cis amide analogue.[12] The noncatalytic WW domain of Pin1 has been demonstrated to bind the pSer/pThr−*trans*-Pro motif in both the X-ray[70] and the solution NMR[71] structures. Trans isostere **2**, however, binds to the catalytic domain, as demonstrated by competitive inhibition. We think this is 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.[9] The flanking sequence specificity of the WW domain is different from that of the catalytic domain.[9,70] We speculate that the aromatic C-terminal and basic N-terminal amino acids in inhibitor **2** direct it into the catalytic domain of Pin1.

The competitive inhibition constant (K_{is}) of the trans inhibitor **2** is 23 times higher than its cis counterpart **1**. The fact that cis isostere **1** inhibits Pin1 activity much more than trans isostere **2** 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.[12,24−26] The more hydrophobic *N*-methylamide of inhibitor **3** did not improve the inhibition of Pin1 significantly over the primary amide **2** (Table 1). The IC_{50} values are within experimental error of each other (24 ± 3 and 28 ± 3 μM). The poor inhibition by compound **13** 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

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processes more efficiently by raising the ground-state energy of the trans amide isomer more than the cis.[11]

The cis isostere **1** inhibits proliferation of A2780 ovarian cancer cells 17-fold better than the trans isostere **2**, comparable to the 21-fold difference in inhibition of Pin1 PPIase activity (Table 1). This suggests that Pin1 is 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 **1** and **2**. 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 are underway in our labs. 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.[66] The ovarian cancer cell line growth inhibition results increase the significance of Pin1 as a potential anti-cancer drug target.

Experimental Section

General Procedures. 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[47] were used directly from sealed bottles. Peptide synthesis grade DMF, DIEA,[47] and NMP[47] were purchased. TFE[47] (99+%) was distilled from sodium before use. LiCl (99+%) was dried in vacuo at 150 °C for 24 h. Brine (NaCl), $NaHCO_3$, and NH_4Cl refer to saturated aqueous solutions unless otherwise noted. Flash chromatography was performed on 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. The human His_6-Pin1 DNA plasmid was a generous gift from Professor P. Todd Stukenberg (University of Virginia).

Fmoc−Ser−Ψ[(*Z*)CH=C]−Pro-OH, 6. Boc−Ser−Ψ[(*Z*)CH=C]−Pro−OH, **4** (80 mg, 0.28 mmol), was dissolved in TFA (5 mL) and CH_2Cl_2 (5 mL) at 0 °C. The reaction mixture was stirred for 45 min, and the solvent was evaporated. The remaining TFA was removed under vacuum at room temperature. Without further purification, the crude product was dissolved in a 10% aqueous Na_2CO_3 (1.0 mL), then cooled to 0 °C. A solution of Fmoc−Cl (80 mg, 0.31 mmol) in dioxane (1.0 mL) was added slowly and stirred at room temperature for 5 h. The reaction mixture was diluted with H_2O (15 mL) and extracted with ether (2 × 10 mL). The aqueous layer was acidified with 1 N HCl to pH 3, and extracted with EtOAc (3 × 15 mL) and CH_2Cl_2 (3 × 15 mL). The combined organic layer was dried over $MgSO_4$ and concentrated to give 77 mg (68%) of **6** as a white powder. ^{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): 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−Ψ[(*E*)CH=C]−Pro−OH, 7. Boc−Ser−Ψ[(*E*)CH=C]−Pro−OH, **5** (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 room temperature. 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 in $CHCl_3$, 5% MeOH in $CHCl_3$, then 10% MeOH in $CHCl_3$ gave 650 mg (54%) of **7** 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.

***O*-Benzyl-*O*-*β*-cyanoethyl-*N*,*N*-diisopropylphosphoramidite, 8.** This is a modification of the method of Crich.[44] A solution of BnOH (0.52 mL, 5.0 mmol) and DIEA (0.87 mL, 5.0 mmol) in ether (20 mL) was added to an ice-cooled solution of chloro-*O*-*β*-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (1.1 mL, 5.0 mmol) in ether (10 mL). The solution was stirred for 2 h at room temperature, and the salt was removed by filtration. The filtrate was concentrated to give **8** as a light yellow oil (1.48 g, 96%), which was used immediately in the next step without further purification. ^{1}H NMR: δ 7.23−7.38 (m, 5H), 4.71 (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[(*Z*)CH=C]−Pro−OH, 9. NMM (59.7 mg, 0.589 mmol) was added to a stirred solution of Fmoc−Ser−Ψ[(*Z*)CH=C]−Pro−OH, **6** (240 mg, 0.589 mmol), in THF (4 mL), followed by *tert*-butyldimethylsilyl chloride (TBSCl) (97.9 mg, 0.648 mmol). After 30 min, a solution of **8** (364 mg, 1.18 mmol) in THF (2 mL) was added, followed by 5-ethylthio-1*H*-tetrazole (307 mg, 2.36 mmol) in one portion. The reaction mixture was stirred for 2 h at room temperature, then cooled to −40 °C, and *tert*-butyl hydroperoxide (5 M in decane, 240 μL, 1.18 mmol) was added. The cold bath was removed. After being stirred for 30 min, the mixture again was cooled to −40 °C, and 5 mL of 10% aqueous $Na_2S_2O_3$ was added. The mixture was transferred for separation using ether (80 mL). The organic layer was washed with 10% aqueous $Na_2S_2O_3$ (1 × 10 mL) and brine (1 × 10 mL), dried over $MgSO_4$, and concentrated. Chromatography on silica gel with 30−40% acetone in CH_2Cl_2 gave 167 mg (45%) of **9** as a colorless syrup. ^{1}H NMR: 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.

Fmoc−Ser(PO(OBn)(OCH$_2$CH$_2$CN))−Ψ[(*E*)CH=C]−Pro−OH, 10. To a stirred solution of Fmoc−SerΨ[(*E*)CH=C]−Pro−OH, **5** (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 **8** (308 mg, 1.0 mmol) in THF (2 mL) was added, followed by 5-ethylthio-1H-tetrazole (260 mg, 2.0 mmol) in one portion.

The reaction mixture was stirred for 2 h at room temperature, 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 **10** as a colorless syrup. ^{1}H 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.

Ac−Phe−Phe−pSer−Ψ[(*Z*)CH=C]−Pro−Arg−NH$_2$, 1. Manual solid-phase synthesis of **1** was performed in 5 mL disposable polypropylene columns by standard Fmoc chemistry. Rink amide MBHA resin[48] (100 mg, 0.064 mol, 0.64 mmol/g) was swelled in CH_2Cl_2 (1 × 3 mL, 10 min) and NMP (3 mL, 10 min). For each amino acid coupling cycle, the Fmoc group was removed in two steps with 20% piperidine/NMP (4 mL) for 5 and 15 min. After the mixture was washed with NMP (5 × 3 mL, 1 min each), CH_2Cl_2 (5 × 3 mL, 1 min each), MeOH (5 × 3 mL, 1 min each), CH_2Cl_2 (5 × 3 mL, 1 min each), and NMP (5 × 3 mL, 1 min each), a solution of amino acid, Fmoc−Arg(Pbf)−OH (124 mg, 0.192 mmol) or Fmoc−Phe−OH (75.0 mg, 0.192 mmol), HBTU[49] (70.0 mg, 0.192 mmol), HOBt (29.5 mg, 0.192 mmol), and DIEA (67 μL, 0.384 mmol) in NMP (2 mL) were added to the resin and shaken for 1−2 h, until a Kaiser test[72] indicated the coupling was complete. The resin then 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). Fmoc-protected cis mimic **9** (40 mg, 0.077 mmol), HBTU (23 mg, 0.077 mmol), HOBt (10 mg, 0.077 mmol), and DIEA (22 μL, 0.154 mmol) in NMP (2 mL) were added to the resin and shaken for 3 h. The resin was capped with Ac_2O (60 μL, 0.640 mmol) and pyridine (52 μL, 0.640 mmol) for 30 min. The resin was treated with 20% piperidine in NMP (3 × 4 mL, 5 min, 15 min, and 3.5 h) to remove both Fmoc and cyanoethyl groups. Acetylation of the N-terminus was carried out with Ac_2O (60 μL, 0.640 mmol) and pyridine (52 μL, 0.640 mmol) for 30 min. The resin was washed with NMP (5 × 3 mL, 1 min each), CH_2Cl_2 (5 × 3 mL, 1 min each), MeOH (5 × 3 mL, 1 min each), and ether (3 × 3 mL, 1 min each) and dried in vacuo.

The dried resin was treated with a mixture of 94% TFA, 2.5% H_2O, 2.5% thioanisole, and 1% triisopropylsilane (TIS) (1.5 mL) for 3 h, filtered, and rinsed with CH_2Cl_2 and MeOH. The combined solutions were concentrated to a small volume. The crude product was precipitated with ether (50 mL), collected by filtration, and dried in vacuo to give 25 mg (52%) of crude peptide **1**. A 15 mg portion of the crude product was purified by preparative HPLC. Purified **1** was eluted at 12.9 min isocratically with 20% B at 50 mL/min as a white solid (4.9 mg, 17%). 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−Phe−pSer−Ψ[(*E*)CH=C]−Pro−Arg−NH$_2$, 2. The solid-phase synthesis of **2** was performed in a manner similar to that for **1** with the following exceptions. Rink amide MBHA resin[48] (200 mg, 0.13 mmol, 0.64 mmol/g) was used. Washes and deprotections were ca. 4 mL each. To couple the first Fmoc−Phe−OH (147 mg, 0.38 mmol), HATU (146.0 mg, 0.38 mmol) and HOAt (52 mg, 0.38 mmol) were used instead of HBTU and HOBt. Fmoc-protected trans isostere **10** (50 mg, 0.08 mmol), HATU (88 mg, 0.23 mmol), HOAt (32 mg, 0.23 mmol), and 2,4,6-collidine (56 mg, 0.46 mmol) was added to the resin and shaken for 2 h, and then the peptide was capped with 10% Ac_2O, 10% DIEA in CH_2Cl_2 (4 mL) for 30 min. Upon completion of coupling **10**, the resin was treated with 20% piperidine in NMP (3 × 4 mL) for 15 min, 1 h, and 4.5 h to remove Fmoc and cyanoethyl groups. Final acetylation was carried out with 10% Ac_2O, 10% DIEA in CH_2Cl_2 (4 mL) for 30 min. The resin was washed and dried in vacuo as before.

The product was cleaved from the dried resin, precipitated with ether, collected, and dried in vacuo as for **1** to give 113 mg of crude product. The crude product was purified by semipreparative C18 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.

Fmoc−Ser(TBS)−Ψ[(*E*)CH=C]−Pro−OH, 11. Fmoc−Ser−Ψ[(*E*)CH=C]−Pro−OH, **7** (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 **11** 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.

Fmoc−Orn(Boc)−NHMe, 12. Fmoc−Orn(Boc)−OH (4.55 g, 10.0 mmol), HOBt (1.84 g, 12.0 mmol), and EDC[47] (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 gave 4.2 g (90%) of **12** 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

(72) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595−598.

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.

Ac−Phe−Phe−pSer−Ψ[(*E*)CH=C]−Pro−Arg−NHMe, 3. Fmoc−Orn(Boc)−NHMe, **12** (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[58,59] (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[60] 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, **11** (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 C18 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 **13** (20.0 min, 3.4 mg, 3.5%) and **3** (21.8 min, 6.9 mg, 7.0%) as white solids. Analytical data for **13**: HPLC RP C18 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 **13** 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 **3** (21.8 min): HPLC RP C18 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.

Expression and Purification of Pin1. The pET28C−His_6-human Pin1 (*Eco*RI/ *Not*1 site, kanamycin resistant) plasmid was transformed into BL21 competent *E. coli* cells (20 min on ice, heat-shocked 2 min at 42 °C). Transformed cells in LB (2 mL) were shaken 4 h at 37 °C and plated on six kanamycin-LB plates, and then incubated for 18 h at 37 °C. Cells were transferred from the plates to 200 mL of LB with 1 mL of kanamycin and incubated 1 h. An aliquot (20 mL) was transferred to LB (10 × 500 mL) and incubated at 37 °C to 170 kLett turbidity. Protein expression was induced with lactose to 1% w/v (5 g each) for 3.5 h. Cells were collected by centrifugation (5000 rpm for 5 min) and stored at −20 °C. Cells were suspended in 50 mM TRIS[47] pH 7.9 and sonicated at 4 °C for 20 min (65% pulse, 1 min on, 1 min off). A fast nickel chelating Sepharose FPLC column was washed with H_2O (5 mL/min over 20 min), and then charged with 100 mM $NiSO_4$ (5 mL/min over 20 min). Binding buffer (0.5 M NaCl, 5 mM imidazole, 20 mM TRIS, pH 7.9) was then loaded onto the column followed by loading of crude protein. The column was washed with 50 mM imidazole, 0.5 M NaCl, 20 mM TRIS, pH 7.9 for about 1 h until there was no absorption at 280 nm. Pin1 was eluted with 250 mM imidazole, 0.5 M NaCl, 20 mM TRIS, pH 7.9. Pin1-containing fractions were collected in a plastic container and verified by SDS-PAGE gel (22.1 kDa). Fractions were dialyzed against 20 mM TRIS pH 7.7, 0.2 mM EDTA, 5 mM DTT,[47] 50 mM KCl, and 5% glycerol overnight at 4 °C to give a final protein concentration of 1.24 mg/mL. Pin1 was pelleted and frozen using liquid N_2 and was stored at −80 °C.

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 **14** 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[33] at 0 °C; final concentration 31 mM HEPES) and Pin1 (10 μL of a 8.0 μM stock solution, 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 0.001 M 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 **1** and **2**, and 36.0 μM for **3** and **13**.

***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 **1** were 1.51, 3.02, and 6.04 μM, and the final concentrations of the (*E*)-alkene inhibitor **2** were 58.2 and 118.4 μM.

A2780 Bioassay. Antiproliferative activity against the A2780 human ovarian cancer cell line was measured as published.[68,69] The concentrations of **1** 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 **2** were 200, 100, 51, 25, 13, 6.3, 3.1, and 1.5 μM in triplicate.

Conclusion

We designed, synthesized, and assayed a pair 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.[9] 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 **9** and **10** in yields of 17% for the cis analogue **1** and 16% for the trans analogue **2**. The trans dipeptide isostere **9** was coupled with HATU/HOAt and 2,4,6-collidine to minimize the isomerization of the alkene. Trans *N*-methylamide analogue **3** was also synthesized on solid-phase resin in 7% yield. We demonstrated that both the cis and the trans alkene pentapeptide analogues **1** and **2** are competitive inhibitors of Pin1 by the protease-coupled assay. The fact that the (*Z*)-alkene **1** was a more potent competitive inhibitor (K_{is} = 1.74 ± 0.08 μM) than the (*E*)-alkene **2** (K_{is} = 39.8 ± 2.4 μM) suggests that Pin1 binds cis substrate more tightly at the catalytic site. 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 **1** and 140 ± 10 μM for the trans analogue **2**. This suggests that Pin1 could be the target that accounts for the antiproliferative activity against the human ovarian cancer cell line. The (*Z*)-alkene inhibitor is among the most potent inhibitors found for Pin1 so far, and it is 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, tools for investigating Pin1 regulation of mitosis, and anti-cancer drug leads.

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Supporting Information Available: HPLC chromatograms for compounds **1**−**3**, **6**, **7**, **9**, **10**, **11**, and **13**. NMR spectra for compounds **1**−**3** and **6**−**13**. This material is available free of charge via the Internet at http://pubs.acs.org.

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