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# DESIGN AND SYNTHESIS OF SH3 DOMAIN BINDING LIGANDS: MODIFICATIONS OF THE CONSENSUS SEQUENCE XPpXP

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**Abstract:** Spirolactam-based Pro-Pro mimetics incorporated in the consensus sequence XPpXP, lead to effective nonpeptidic ligands of SH3 domains. © 1998 Elsevier Science Ltd. All rights reserved.

Src homology 3 (SH3) domains are small protein modules that mediate protein–protein interactions in many vital cellular signal-transduction pathways.[1] The conserved protein units of SH3 domains contain sequences of 50–100 amino acids and bind to proline-rich protein ligands ($K_D$ 5–100 μM) via shallow hydrophobic pockets on the domain's surface. The ligands form a poly-proline type II (PPII) left-handed α-helix containing a consensus sequence generalized as XPpXP, where X is an aliphatic amino acid and p is preferred as proline to maintain the helix.

Research in nonpeptidic ligands of SH3 domains is limited. Elegant studies preformed in the Schreiber laboratory illustrate the feasibility of incorporating nonpeptidic elements in SH3 domain ligands.[2a–c] Initial work using biased combinatorial libraries and molecular modeling revealed effective replacements for the N-terminus of PLPPLP. For example, peptidomimetics of VSLARR bound in the specificity pocket (Figure 1).[2] Extension of this approach to the C-terminus led to tripeptide mimetics that maintain similar hydrophobic interactions as the C-terminal PLP sequence.[2c] Combination of both replacements provided a ligand (AB) with affinity ($K_D$ ~ 1.6 μM) comparable to the canonical 12-mer peptide, yet retaining only three original amino acid residues (see insert).[2c] Herein we describe a highly

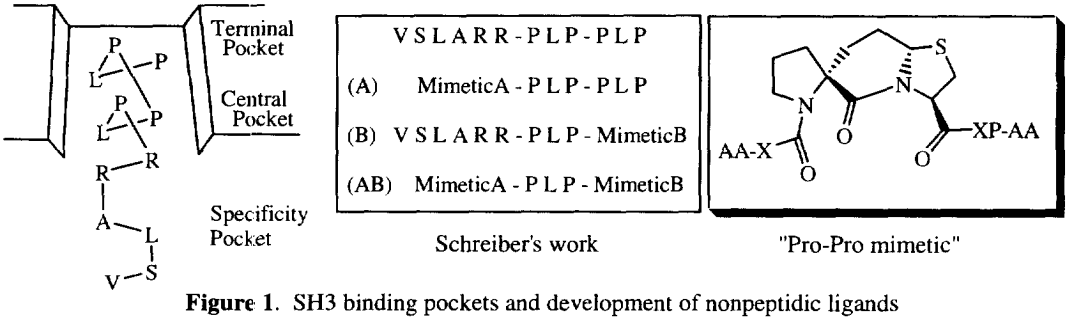


**Figure 1.** SH3 binding pockets and development of nonpeptidic ligands

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constrained spirolactam PP (or PX) mimetic of the PLPPLP sequence, which represents a novel scaffold for the assembly of nonpeptidic SH3 ligands. Nonapeptides that incorporate the spirolactam design have been prepared for the SH3 domain of Lyn, a tyrosine kinase implicated in transmembrane signal transduction through the high affinity IgE receptor (FcεRI) and a potential drug target for allergic asthma.[3]

In considering practical ligands for SH3 domains, our strategy was to enhance binding at the central PLPPLP pocket with a conformationally constrained mimetic and then truncate the usual decapeptide-size ligands to smaller molecules. This approach required a rigid central template amenable to further elaboration using molecular modeling and combinatorial chemistry methods. We noticed that the bound conformation of the proline-rich peptide, RLP2, with Src SH3[4] was different from a minimized unbound conformation of a PP-based fragment, presumably as a consequence of A(1,3) strain between Cα-H and $C_5$-N of adjacent prolines.[5] However, we thought that the bound conformation could be "captured" in the constrained spirobicyclic or spirotricyclic systems, **1** and **2,** respectively, by introducing a carbon chain between the two proline residues (Figure 2). The bicyclic system is effectively a Pro-Gly mimetic arising from the translocation of proline ring $C_3$-$C_5$ carbons to Cα of the adjacent proline residue. This process eliminates two chiral centers and results in a less demanding synthesis.

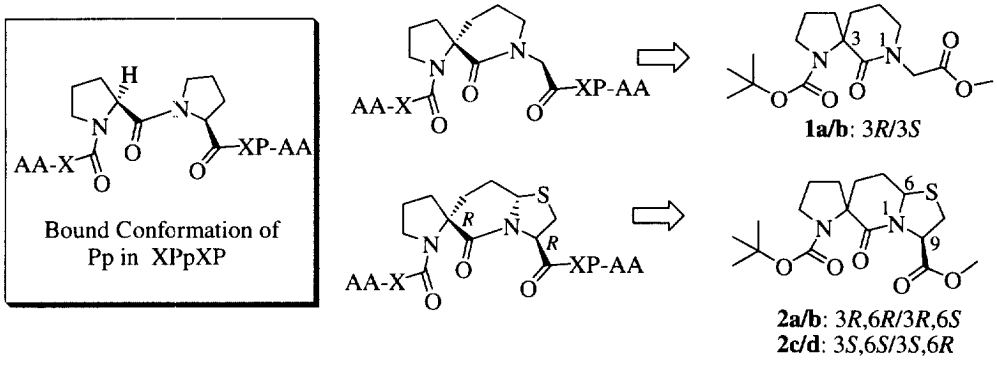


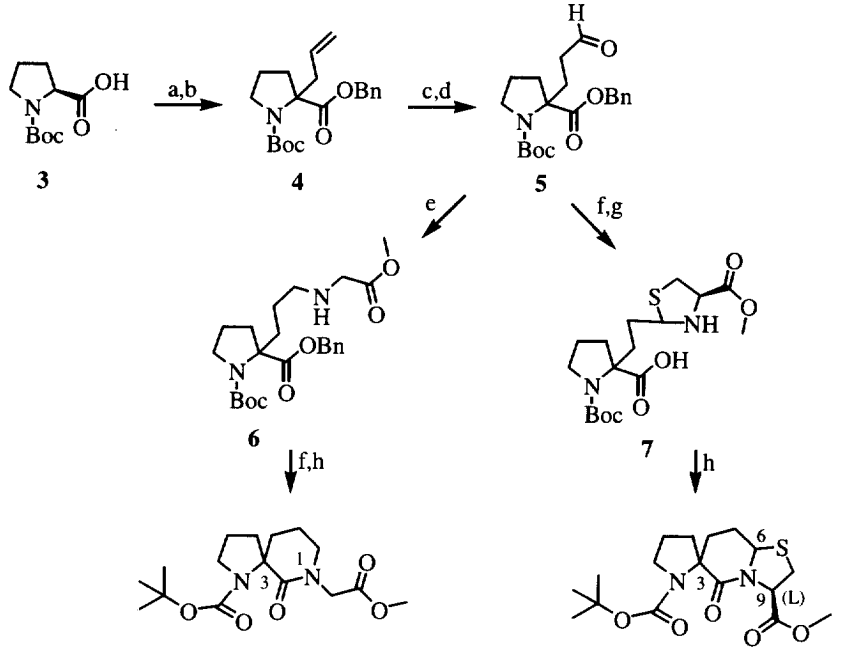

**Figure 2.** Constraining of the Pro-Pro consensus sequence (XPpXP) to mimic the bound conformation

Appropriate spirolactam stereochemistry is required to properly mimic the Pro-Pro fragment. (3*R*)-Bicyclic **1** and (3*R*,6*R*,9*R*)- or (3*R*,6*S*,9*R*)-tricyclic **2a/b** possess the desired stereochemistry and adopt the requisite secondary structure. Modeling of these isomers reveal complete overlap with bound PLPPLP in an extended conformation. Corresponding tricyclic spirolactams with (3*S*) stereochemistry adopt a β-turn conformation[6] whereas both bicyclic isomers (**1a** or **1b**) can adopt either extended or β-turn conformations; the latter are not expected to lead to productive binding.[5]

**Chemistry:** Spirolactams were generated from a common aldehyde intermediate **5** (Scheme 1). Commercial Boc-proline **3** was protected as the benzyl ester and alkylated with allyl bromide to give olefin

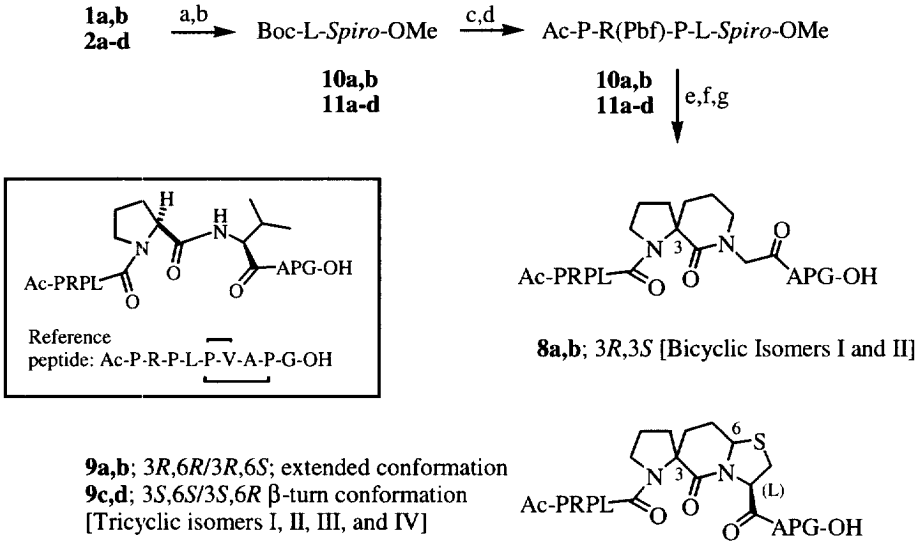
**4**. Hydroboration/oxidation followed by Swern oxidation gave aldehyde **5**. This aldehyde was then subjected to reductive alkylation with glycine methyl ester to afford amine **6** or acid deprotected and then condensed with cysteine methyl ester to generate the thiazolidine **7**. Amine **6** was separated by chromatography from dialkylated amine byproduct, subjected to hydrogenolysis, and then cyclized with EDCI or 2-chloro-N-methyl pyridinium iodide to spirolactams **1a,b**. Using similar cyclization conditions, thiazolidine **7** cyclized to give four diastereomers **2a–d**, which were separated by silica gel column chromatography. α-Alkylation of proline with stereochemical control is known,[7] but we proceeded to incorporate unassigned stereoisomers **1** and **2** into nonapeptides targeting Lyn.

A reference nonapeptide, Ac-PRPLPVAPG-OH, which corresponds to the C-terminal proline-rich region in p85 that is recognized by Lyn,[8] and a related 18-mer peptide possessing additional residues required for BIAcore analysis, were prepared for comparative purposes.[9] Six nonapeptides, **8a,b** and **9a–d**, were prepared with spirolactams **1a,b** and **2a–d**, respectively, replacing Pro-Val in the reference peptide (Scheme 2). Since amino acids other than proline occur regularly in PPII helices,[10] replacement of the Pro-Val sequence, instead of Pro-Pro, provides a reasonable evaluation of the spirolactam mimetics.




**Scheme 1:** (a) i) $Cs_2(CO_3)$, MeOH ii) BnBr, DMF (87%); (b) LDA, Allyl bromide, (65%); (c) i) $BH_3$·THF ii) $KOH/H_2O_2$ (90%); (d) Swern oxid. (90%); (e) Gly-OMe, DIPEA, MeOH,then $NaBH_4$ (78%); (f) $H_2$, $Pd(OH)_2/C$ (90-94%); (g) Cys-OMe, NaOH (aq.) (94%); (h) EDCI, NMM (85-90%).

Nonapeptides **8a** and **8b**, embodying the bicyclic spirolactam were synthesized as a diastereomeric mixture and separated at the protected nonapeptide stage, whereas nonapeptides **9a–d** incorporating the tricyclic spirolactam were prepared individually. A two-step procedure to couple the N-terminal tetrapeptide fragment, Ac-PR(Pbf)PL-OH, with the spirolactam was necessary in order to reduce the amount of isomerization observed during a direct coupling. In the event, **1a,b** and **2a–d** were deprotected with TFA and coupled with Boc-Leu-$NH_2$ to the tripeptides **10a,b** and **11a–b** (Scheme 2). A reduced yield (40%) was noted during the coupling reaction with two tricyclic isomers, presumably the (3*S*) isomers as a consequence of β-turn conformations of the spirolactams. The yield was improved to 60% by using Boc-Leu-F in the coupling reaction. Deprotection with TFA and coupling with Ac-PR(Pbf)P-OH generated hexapeptides **12a,b** and **13a–d**. Methyl ester hydrolysis and coupling with the tripeptide, $H_2N$-APG-OtBu, generated the protected nonapeptides. The two bicyclic isomers (3*S*) and (3*R*) were separated on silica gel at this stage. The six nonapeptides were treated with TFA to produce the desired single peptides **8a,b** and **9a–d**.[11] Stereochemical assignment at C-3 in the tricyclic spirolactams is tentative and is based on the observed activities (vide infra). The stereochemistry at C-6 remains unassigned. Based on molecular models, the (*R*) or (*S*) stereochemistry at C-6 does not dramatically alter the overall conformation and is not expected to affect binding.

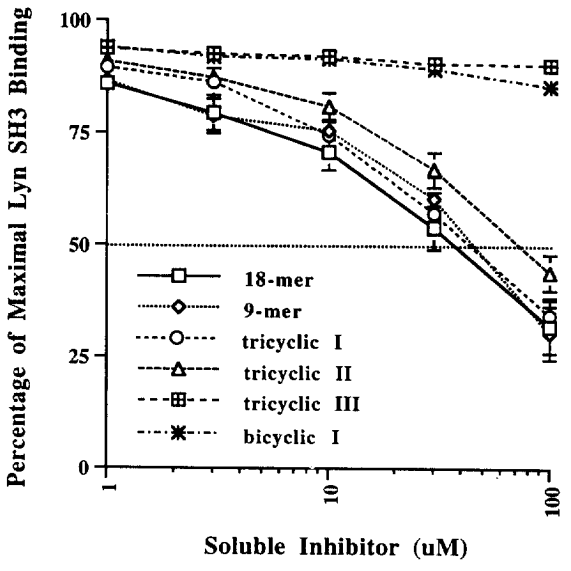



**Scheme 2.** (a) TFA, $CH_2Cl_2$ (92-95%); (b) Boc-Leu-OH, EDCI, NMM, HOBt (45-89%); (c) TFA, $CH_2Cl_2$ (87-95%); d) Ac-PR(Pbf)P-OH, EDCI, NMM, HOBt (85-90%); (e) LiOH/THF/MeOH (82-90%); (f) $H_2N$-APG-OtBu, EDCI, NMM, HOBt (54-74%); (g) TFA, $CH_2Cl_2$ (85-90%)

**Biochemical Results:** An SH3 domain BIAcore binding assay was developed[12] to test the spirolactam's ability to mimic the Pp (or PV) region in nonapeptide ligands. The p85 derived 18-mer peptide was

biotinylated and adhered to the BIAcore chip via strepavidin interactions. A solution of Lyn SH3 domain was then flowed over the chip and the association with the immobilized 18-mer peptide was measured. In parallel experiments the reference peptides, 9-mer or unbiotinylated 18-mer, or the nonapeptides incorporating the spirolactams were added to the SH3 solution before passage over the 18-mer derivatized chip. This allowed analysis of binding reflected by competition for the SH3 domain.

The nonapeptides with tricyclic isomers in a predicted extended conformation, **9a** and **9b**, bound the SH3 domain with an $IC_{50}$ of 46 and 75 μM, respectively; an affinity similar to the canonical peptides (9-mer $IC_{50}$ = 46 μM, 18-mer $IC_{50}$ = 38 μM). These results support the view that the rigid tricyclic structure mimics the bound conformation of XPpPX-containing peptides. Lack of enhanced binding reflects the absence of entropy gain from the structural constrain. The other spirolactam-containing nonapeptides, **8a,b** and **9c,d**, bind to the SH3 domain with lower affinity, likely as a consequence of adopting β-turn conformations.




In summary, we have studied the conformational differences between bound and unbound ligands in the XPpX motif and designed spirolactam-based peptidomimetics, which generate ligands predisposed to bind in extended conformations. Direct replacement of the PV sequence with tricyclic spirolactams **2a** and **2b** results in comparable binding affinities to reference peptides targeted for the SH3 domain of Lyn. This work complements previous efforts to develop nonpeptide ligands of SH3 domains and offers the possibility of designing ligands free of amino acid residues.

**Acknowledgements:** We are indebted to Dr. Chris Pleiman for the development of a high throughput assay in order to study a library of compounds derived from **1a,b** and **2a–d**.

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11. **1a/b** *Rf* 0.40 ($SiO_2$; EtOAc); $^1H$ NMR (200 MHz, $CDCl_3$) δ 4.74 & 4.65 (d, 1H, *J* = 17.2 Hz, 1 x α-gly-$CH_2$), 3.71 & 3.66 (s, 3H, $OCH_3$), 3.75–3.18 (m, 5H), 2.62–2.18 (m, 2H), 2.06–1.60 (m, 6H), 1.41 & 1.39 (s, 9H). **2a,b** *Rf* 0.35 ($SiO_2$; 8/2/1 $CH_2Cl_2$/EtOAc/Hexane); $^1H$ NMR (200 MHz, $CDCl_3$) δ 5.39–5.25 (m, 1H), 4.94–4.84 (m, 1H), 3.73 (br s, 3H), 3.75–3.06 (m, 4H), 2.75–2.53 (m, 1H), 2.38–2.08 (m, 2H), 2.08–1.66 (m, 5H), 1.41 (br s, 9H); MS cal'd 370.46, expt $MH^+$ 371.20. **2a,b** *Rf* 0.30 ($SiO_2$; 8/2/1 $CH_2Cl_2$/EtOAc/Hexane); $^1H$ NMR (200 MHz, $CDCl_3$) δ 5.00 (dd, 1H, *J* = 11.0 & 3.20 Hz), 4.88–4.74 (m, 1H), 3.74 & 3.72 (s, 3H), 3.62–3.26 (m, 3H), 3.12 (br t, 1H, *J* = 12.8 Hz), 2.61 (br dt, 1H, *J* = 13.40 & 3.40 Hz), 2.44–2.18 (m, 2H), 2.08–1.62 (m, 5H), 1.42 & 1.40 (s, 9H); MS cal'd 370.46, expt $MH^+$ 371.19. **2c,d** *Rf* 0.38 ($SiO_2$; 8/2/1 $CH_2Cl_2$/EtOAc/Hexane); $^1H$ NMR (200 MHz, $CDCl_3$) δ 5.12–4.90 (m, 2H), 3.76 & 3.73 (s, 3H), 3.75–3.50 (m, 2H), 3.36–3.09 (m, 2H), 2.82–2.64 (m, 1H), 2.38–2.18 (m, 2H), 2.04–1.88 (m, 2H), 1.88–1.68 (m, 3H), 1.49 & 1.43 (s, 9H); MS cal'd 370.46, expt $MH^+$ 371.18. **2c,d** *Rf* 0.20 ($SiO_2$; 8/2/1 $CH_2Cl_2$/EtOAc/Hexane); $^1H$ NMR (200 MHz, $CDCl_3$) δ 4.80 (dd, 1H, *J* = 10.4 & 3.00 Hz), 4.76–4.66 (m, 1H), 3.75 & 3.72 (s, 3H), 3.72–3.29 (m, 3H), 3.12 (br t, 1H, *J* = 10.8 Hz), 2.74–2.40 (m, 1H), 2.36–1.62 (m, 7H), 1.50 & 1.44 (s, 9H); MS cal'd 370.46, expt $MH^+$ 371.21. Mass spectral data for **8a/b**: calcd. 943, found $MH^+$ 944; Mass spectral data for **9a–d**: calcd. 987, found $MH^+$ 988.
12. *Lyn* SH3-GST fusion proteins were generated as previously described,[8] purified from bacterial lysates by glutathione affinity chromatography and eluted by competition. Aggregates were removed by size exclusion chromatography on Sephadex 75 (Pharmacia Biotech, Uppsala, Sweden).
Surface plasmon resonance measurements were performed on a BIAcore 2000 biosensor (BIAcore AB, Uppsala, Sweden). A synthetic, canonical proline-rich peptide derived from phosphatidylinositol 3-kinase p85,[3b] GGGKPRPPRPLPVAPGSS, was immobilized on a prepared streptavidin coated sensor chip (Sensor Chip SA, BIAcore AB) via an N-terminal biotin moiety at a density of 0.05 pmol/$mm^2$. All experiments were conducted at 25 °C in the running buffer HBS (0.01M HEPES, pH 7.4, 0.15 M NaCl, 3.4 mM EDTA, 0.005% surfactant P20). Monomeric *Lyn* SH3-GST fusion protein (1 μM) was injected over the peptide surface at a rate of 30 μL/min. for 4.5 min. Dissociation was monitored during subsequent washing of the chip with running buffer for an additional 5.0 min. Regeneration of the peptide surface was accomplished with a 10 sec pulse of 5 mM NaOH. To test their activity, 0.01–100 μM spirolactam or non-biotinylated canonical peptide was incubated with the fusion protein prior to injection. Data were recorded in real-time as sensorgrams and analyzed subsequently. In preliminary experiments, the canonical peptide had an estimated $K_D$ ~ 10 μM.