Peptide Mimetics

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Addressing Protein–Protein Interactions with Small Molecules: A Pro-Pro Dipeptide Mimic with a PPII Helix Conformation as a Module for the Synthesis of PRD-Binding Ligands**

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Interactions of so-called proline-rich motif-recognizing domains (PRDs) with proteins containing proline-rich motifs (PRMs) are widely utilized by nature and are involved in several relevant processes, such as tyrosine kinase receptor signaling,[1–3] endocytosis,[4] cytoskeletal rearrangements,[5,6] transcription,[7] and splicing.[8,9] In recent years, some PRDs were identified as putative therapeutical targets that can possibly be addressed by synthetic small molecules.[7] An example is the Fyn-SH3 domain, which is involved in the regulation of enzymatic activity and the assembly of signaling complexes.[8]

A common property of all PRMs is that they preferentially form a left-handed polyproline type II (PPII) helix with an overall shape resembling a triangular prism (Figure 1).[9] This structural element has a helical pitch of 9.3 Å, three residues per turn, and typical torsion angles Φ of −75° and Ψ of 145°.[10] Of note, the PPII helix is characterized by a complete lack of main-chain hydrogen bonding patterns.

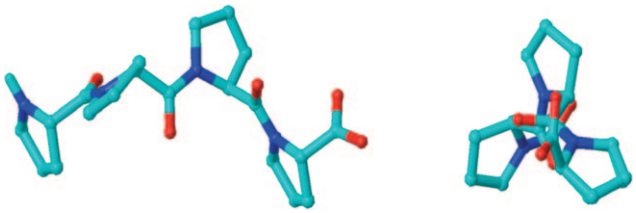

Figure 1. An ideal PPII helix formed by four L-proline units as seen from the side (left) and along the helix axis (right). C cyan, N blue, O red.

Structural studies have shown that the recognition of PRMs by the respective domains (PRDs) requires the polyproline region to adopt a PPII conformation. Moreover, the interaction is mostly stabilized by formation of a hydrogen bond between a conserved tryptophane (located at the domain surface) and a carbonyl oxygen at the backbone of the central PRM of the peptide ligand.[11]

In the course of our research into the development of small molecules that specifically interfere with intracellular protein–protein interactions, we searched for a conformationally defined diproline mimic in a PPII helix conformation that could be incorporated into peptide chains or related modular constructs. To the best of our knowledge, related attempts in other laboratories[12] have not led to the development of systems with satisfying properties to date.

By investigating molecular models, we envisioned that the PPII helix could possibly be stabilized by an appropriate C_2 bridge between two adjacent proline residues. Computer-aided modeling then suggested that the diproline analogue **X**, generated by formal introduction of a "rigid" vinylidene bridge, adopts a single preferred conformation that almost perfectly mimics a Pro-Pro unit of a PPII helix (Figure 2). Herein we describe the stereoselective synthesis of this novel compound (as a Fmoc-protected derivative) and its successful incorporation as a Pro-Pro substitute in the core motif of SH3 domain-binding peptides.

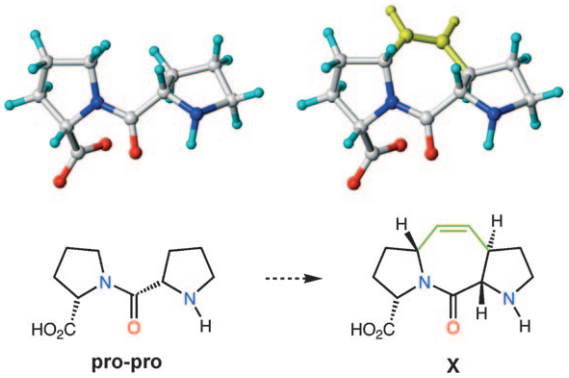

Figure 2. Diproline in the PPII helix conformation (left) and the designed, conformationally restricted analogue **X** (right). The backbone angles of **X** ($\Phi = -78°$ and $\Psi = 146°$) correspond to an ideal PPII helix.

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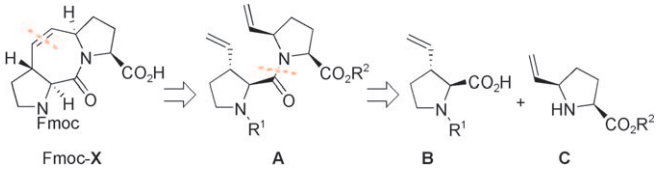
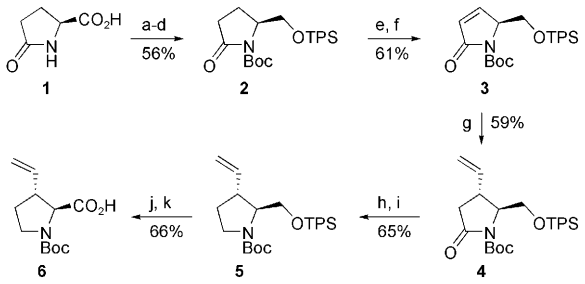
Our strategy for the synthesis of the target molecule Fmoc-**X** is outlined in Scheme 1. As a key step, we intended to use a ruthenium-catalyzed ring-closing metathesis reaction to form the central seven-membered ring. The required dipeptide intermediate **A** would in turn be derived from appropriately protected vinylproline building blocks **B** and **C**; the synthesis of these is however not trivial.[13,14]



Scheme 1. Retrosynthesis of Fmoc-**X**. Fmoc = fluorenylmethoxycarbonyl.

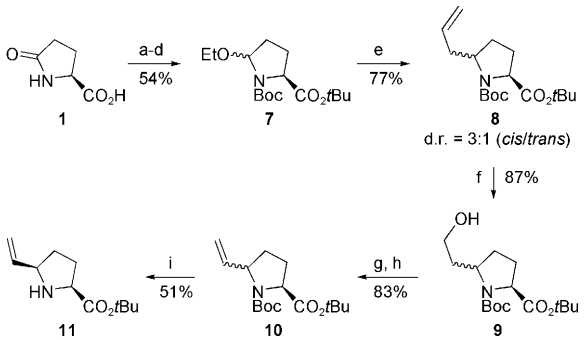
As building block **B**, the Boc-protected *trans*-3-vinylproline **6** was synthesized starting from L-pyroglutamic acid (**1**) by the sequence shown in Scheme 2.[15] First, conversion of the carboxylic acid into a TPS-protected alcohol followed by *N*-



Scheme 2. Synthesis of the type **B** building block **6**: a) $SOCl_2$, EtOH, RT, 15 h, 99%; b) $NaBH_4$, LiCl, THF, RT, 15 h, 73%; c) TPSCl, imidazole, DMF, RT, 15 h, 87%; d) Boc_2O, DMAP, MeCN, RT, 15 h, 89%; e) LiN(TMS)$_2$, PhSeCl, THF, −78 °C, 2 h, 78%; f) O_3, CH_2Cl_2, −78 °C, 15 h, 78%; g) CH_2=CHMgBr, CuBr·SMe_2, TMSCl, THF/Et_2O, −78 °C, 2 h, 59%; h) $LiBHEt_3$, THF, −78 °C, 1 h; i) Et_3SiH, BF_3·OEt_2, CH_2Cl_2, −78 °C, 2 h, 65% (2 steps); j) TBAF, THF, RT, 15 h, 89%; k) Jones reagent, acetone, RT, 2 h, 74%. Boc = *tert*-butoxycarbonyl, DMAP = 4-(*N*,*N*-dimethylamino)pyridine, TBAF = tri-*n*-butylammonium fluoride, TMS = trimethylsilyl, TPS = *tert*-butyldiphenylsilyl.

Boc protection afforded **2**, which was subsequently transformed to the α,β-unsaturated derivative **3** through α-selenylation and oxidation-induced elimination using ozone. The vinyl group was then introduced by 1,4-addition of a cuprate reagent to give the *trans*-configurated pyrrolidone **4** as a pure diastereomer. Reduction of **4** with $LiEt_3BH$ gave the corresponding "lactamol", which on ionic hydrogenation (Et_3SiH, BF_3·Et_2O) afforded the deoxygenated compound **5** in 65% yield (two steps). Finally, fluoride-mediated cleavage of the silyl protecting group and Jones oxidation gave rise to **6** in 9% overall yield (11 steps).

The synthesis of the *cis*-5-vinylproline ester **11** (as building block **C**) was performed as shown in Scheme 3. Starting again from L-pyroglutamic acid (**1**), intermediate **7** was prepared as a mixture of diastereomers by common functional-group



Scheme 3. Synthesis of the type **C** building block **11**: a) $HClO_4$, *t*BuOAc, RT, 15 h, 72%; b) Boc_2O, DMAP, MeCN, RT, 15 h, 84%; c) DIBALH, CH_2Cl_2, −78 °C, 2 h, 93%; d) PPTS, EtOH, RT, 15 h, 96%; e) CH_2=$CHCH_2SiMe_3$, BF_3·OEt_2, CH_2Cl_2, −78 °C, 30 min, 77%; f) O_3, −78 °C, then $NaBH_4$, CH_2Cl_2/MeOH, RT, 12 h, 87%; g) (*o*-NO_2Ph)SeCN, P(*n*Bu)$_3$, Py, THF, RT, 30 min, 92%; h) O_3, CH_2Cl_2, Δ, 30 min, 90%; i) TMSOTf, CH_2Cl_2, 0 °C, 5 min, then chromatographic separation of diastereomers (SiO_2, CH_2Cl_2/MeOH 25:1), 51%. DIBALH = diisobutylaluminum hydride, PPTS = pyridinium *p*-tolylsulfonate, py = pyridine, OTf = trifluoromethanesulfonate.

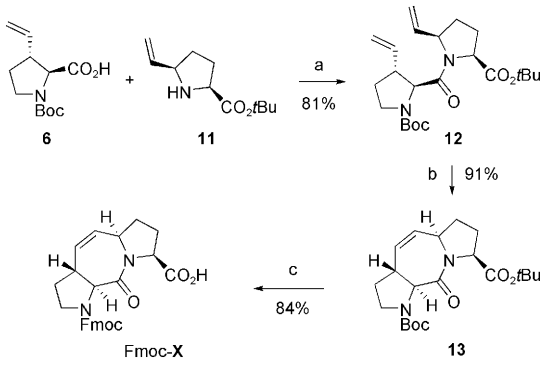
manipulation. Ionic allylation of **7** (allyl-TMS, BF_3·Et_2O) then proceeded in a *cis*-selective manner to afford **8** (d.r. ca. 3:1).[16] Without separation, this mixture was ozonolyzed to give the alcohol **9** after reductive workup. Elimination of the primary OH functionality was then achieved through ozone oxidation of an intermediate *o*-nitrophenylselenide.[17] Treatment of **10** with TMSOTf allowed the selective deprotection (Boc cleavage) of the secondary amino group without affecting the *tert*-butyl ester function.[18] Fortunately, the separation of the *cis*/*trans* diastereomers was possible at this stage with standard flash column chromatography on silica gel, and the desired pure *cis* isomer **11** was obtained in 15% overall yield from **1** (9 steps).

The connection of the two vinylproline building blocks **6** and **11** was then achieved by peptide coupling using PyBOP in the presence of DIPEA.[18] Cyclization of the resulting dipeptide **12** by ring-closing metathesis proceeded smoothly in the presence of 5 mol% of a Grubbs II catalyst.[18] Finally, the *N*-Boc and the *tert*-butyl ester groups were cleaved by treatment of **13** with TFA to give the amino acid **X**, which was directly converted into the target compound Fmoc-**X** (Scheme 4).

Using standard solid-phase Fmoc chemistry,[19] the scaffold **X** was then incorporated as a Pro-Pro substitute into two peptides (WT and LL), which had been previously identified as ligands that bind to the SH3 domain (Table 1).[20]

The binding properties of the **X**-containing peptides (**X**-WT and **X**-LL) to the Fyn SH3 domain were then investigated (in comparison to the parent ligands WT and LL) by means of NMR spectroscopy (^{15}N HSQC[21a] and ^{15}N SOFAST-HMQC[21b]) and isothermal titration calorimetry (ITC).[22]

For all of the ligands, NMR spectra of ^{15}N-Fyn-SH3 were recorded in the absence or presence of the ligands and at different ligand concentrations using a protein (^{15}N-Fyn-SH3) concentration of 0.8 mM and 0.4 mM (for WT and **X**-WT, respectively) or 0.1 mM (for LL and **X**-LL). The low solubility of LL and **X**-LL required the use of DMSO as a co-solvent.



Scheme 4. Synthesis of Fmoc-**X**: a) PyBOP, DIPEA, MeCN, RT, 15 h, 81%; b) 5 mol% Grubbs II, CH_2Cl_2, Δ, 20 h, 91%; c) TFA, CH_2Cl_2, RT, 1 h, then FmocCl, K_2CO_3, MeCN, H_2O, RT, 15 h, 86%. DIPEA = diisopropylethylamine, PyBOP = benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate, TFA = trifluoroacetic acid.

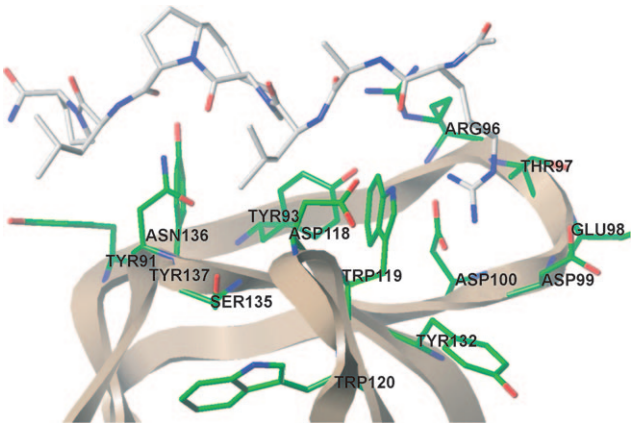


Figure 4. The canonical binding model for the contact between the Fyn-SH3 domain and all ligands investigated, with **X**-WT as an example.

Table 1: Sequences of the peptide ligands investigated.

	Ligand	Sequence[a]
	WT	Ac-Arg-Ala-Leu-Pro-Pro-Leu-Pro-NH_2
	X-WT	Ac-Arg-Ala-Leu-**X**-Leu-Pro-NH_2
	LL	Z^1-Arg-Z^2-Leu-Pro-Pro-Leu-Pro-Z^3-NH_2
	X-LL	Z^1-Arg-Z^2-Leu-**X**-Leu-Pro-Z^3-NH_2

[a] Z^1 = 2-hydroxynicotinoyl; Z^2 = (2*S*)-2-amino-3-(2-hydroxy-2-phenylacetamido)propanoyl; Z^3 = (*S*)-2-amino-3-(2,3-dihydroxybenzamido)propanoyl.

By keeping the DMSO concentration below 4%, comparable spectra could be obtained. As an example, the superimposed ^{15}N HSQC spectra of ^{15}N-Fyn-SH3 for the WT and the **X**-WT ligands are shown in Figure 3.

The NMR measurements clearly show that all four ligands bind in the same mode to the Fyn-SH3 domain.[23] The canonical binding motif (Figure 4) involves residues of the so-called RT loop (Arg96, Thr97, and Asp100), as indicated by a complete disappearance of the backbone NH protons upon addition of either of the four ligands. The same behavior is observed for the NH signals of Trp119 and Trp120. Trp119 contributes to the binding through a hydrogen bond from the ε-NH group of its side chain to the backbone C=O of the second amino acid of the ligand (Ala for WT and **X**-WT; Z^2 for LL and **X**-LL). The backbone NH groups of both tryptophanes are located in proximity of the arginine side chain of the ligand. The binding of the C terminus of the ligands, that is, the modified PPLP motif, mainly involves Tyr91, Tyr93, Asn126, and Tyr137, as indicated by a considerable shift of the corresponding backbone NH signals.

Next, we turned our attention to the ITC measurements. As the LL and the **X**-LL ligands are rather lipophilic and exhibit a limited solubility in aqueous solution (< 1 mM), they were transferred into the ITC cell as a DMSO solution whilst still keeping the overall DMSO concentration in the ITC cell low (usually below 1%).

Much to our satisfaction, the ITC data (Table 2) are in accordance to the NMR results and confirm that we were indeed able to replace the diproline unit in WT and LL by our scaffold **X** without destroying their ability of binding to the Fyn SH3 domain. Despite the fact that the **X**-containing ligands show a slightly lower affinity to the target protein in comparison to the parent ligands (as expressed by the K_D and $\Delta G°$ values), our results clearly demonstrate for the first time that the core motif of a PRD-binding peptide can be structurally modified.[11]

A second look at Table 2 reveals that WT, **X**-WT, and LL clearly show the typical thermodynamic signature of an

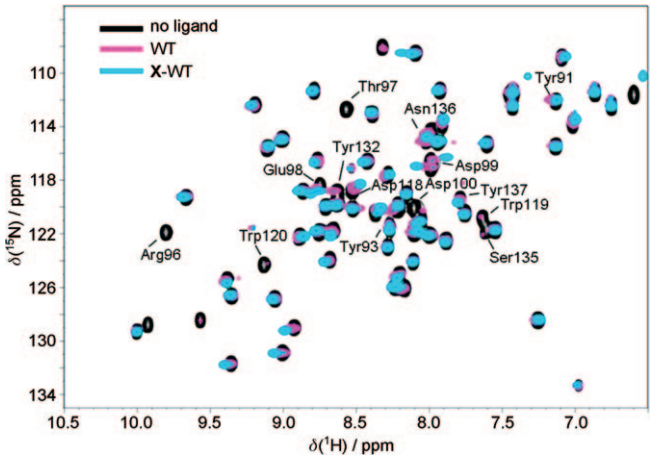


Figure 3. Superimposed ^{15}N HSQC spectra of ^{15}N-Fyn-SH3 (0.8 mM (WT), 0.4 mM (**X**-WT)) without a ligand and in the presence of WT (0.75 mM) or **X**-WT (0.325 mM).

Table 2: Results of the performed ITC measurements (Fyn-SH3).

	WT	**X**-WT	LL	**X**-LL
K_D[a]	18 ± 5	62 ± 13	8 ± 2	27 ± 4
$\Delta G°$[b]	−27.1 ± 0.7	−24.0 ± 0.5	−29.2 ± 0.7	−26.1 ± 0.3
$\Delta H°$[b]	−60 ± 10	−54 ± 13	−58 ± 10	−19 ± 3
$-T\Delta S°$[b]	33 ± 9	30 ± 13	29 ± 10	−7 ± 4

[a] [μM]. [b] [kJ mol^{-1}]; 95% confidence intervals given.

interaction between a SH3 domain and a peptide ligand (that is, a very favorable enthalpic contribution opposed by an unfavorable binding entropy).[24–26] A plausible explanation for this behavior is the formation of a complex hydrogen bond network that is also mediated by water molecules buried at the binding interface.[26]

Although the NMR data suggest a similar binding mode for all four ligands, the different thermodynamic behavior of **X**-LL is complimented by a change of the backbone NH signal of Asn136 (see the Supporting Information, Figure 4). One possible explanation for this different thermodynamic profile could be that the water-mediated binding of the C-terminal region of **X**-LL is changed. It also cannot be excluded that substitution of two prolines of the LL ligand by the scaffold **X** results in secondary interactions between the now-rigid scaffold backbone and the Z residues.[27]

In conclusion, we have developed a stereoselective synthesis of the designed tricyclic Pro-Pro mimetic **X** that exploits ruthenium-catalyzed ring-closing metathesis to assemble the conformationally restrained scaffold from suitable vinylproline building blocks. Moreover, we have demonstrated that this novel PPII helix mimetic indeed fulfills its intended function: When peptide ligands addressing the proline-rich motif-recognizing Fyn-SH3 domain were modified with **X** (that is, by substitution of two adjacent prolines by **X**), the resulting molecules still exhibited pronounced binding properties.

This work shows the general possibility to address comparably flat binding sites ("undrugable targets") at the surface of proteins using rationally designed synthetic small molecules as central modules of quasi peptidic ligands. We are optimistic that this approach will lead to the development of new active compounds with potentially useful pharmaceutical properties.

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[1] R. Aasland, C. Abrams, C. Ampe, L. Ball, M. Bedford, G. Cesareni, M. Gimona, J. Hurley, T. Jarchau, V. Lehto, M. Lemmon, R. Linding, B. Mayer, M. Nagai, M. Sudol, U. Walter, S. Winder, *FEBS Lett.* **2002**, *513*, 141 – 144.

[2] L. Ball, R. Kühne, B. Hoffmann, A. Häfner, P. Schmieder, R. Volkmer-Engert, M. Hof, M. Wahl, J. Schneider-Mergener, U. Walter, H. Oschkinat, T. Jarchau, *EMBO J.* **2000**, *19*, 4903 – 4914.

[3] L. Ball, T. Jarchau, H. Oschkinat, U. Walter, *FEBS Lett.* **2002**, *513*, 45 – 52.

[4] I. Geisler, J. Chmielewski, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2765 – 2768.

[5] M. Dustin, M. Olszowy, A. Holdorf, J. Bromley, N. Desai, P. Widder, F. Rosenberger, P. van der Merwe, P. Allen, A. Shaw, *Cell* **1998**, *94*, 667 – 677.

[6] V. Laurent, T. Loisel, B. Harbeck, A. Wehman, L. Gröbe, B. Jockusch, J. Wehland, F. Gertler, M. Carlier, *J. Cell Biol.* **1999**, *144*, 1245 – 1258.

[7] A. Goldstrohm, T. Albrecht, C. Suñé, M. Bedford, M. Garcia-Blanco, *Mol. Cell. Biol.* **2001**, *21*, 7617 – 7628.

[8] M. Kofler, M. Schuemann, C. Merz, D. Kosslick, A. Schlundt, A. Tannert, M. Schaefer, R. Luhrmann, E. Krause, C. Freund, *Mol. Cell. Proteomics* **2009**, *8*, 2461 – 2473.

[9] B. Laggerbauer, S. Liu, E. Makarov, H.-P. Vornlocher, O. Makarova, D. Ingelfinger, T. Achsel, R. Lührmann, *RNA* **2005**, *11*, 598 – 608.

[10] a) C. M. Deber, F. A. Bovey, J. P. Carver, E. R. Blout, *J. Am. Chem. Soc.* **1970**, *92*, 6191 – 6198; b) N. Helbecque, M. H. Loucheux-Lefebvre, *Int. J. Peptide Protein Res.* **1982**, *19*, 94 – 101; c) H. Okabayashi, T. Isemura, S. Sakakibara, *Biopolymers* **1968**, *6*, 323 – 330; d) R. K. Dukor, T. A. Kiederling, *Biopolymers* **1991**, *31*, 1747 – 1761; e) R. K. Dukor, T. A. Kiederling, V. Gut, *Int. J. Peptide Protein Res.* **1991**, *38*, 198 – 203; f) P. M. Cowan, S. McGavin, *Nature* **1955**, *176*, 501 – 503.

[11] a) S. Li, *Biochem. J.* **2005**, *390*, 641 – 653; b) L. Ball, R. Kühne, J. Schneider-Mergener, H. Oschkinat, *Angew. Chem.* **2005**, *117*, 2912 – 2930; *Angew. Chem. Int. Ed.* **2005**, *44*, 2852 – 2869; c) C. Freund, H.-G. Schmalz, J. Sticht, R. Kühne, *Handb. Exp. Pharmacol.* **2008**, *186*, 407 – 429.

[12] a) N. G. Bandur, K. Harms, U. Koert, *Synlett* **2005**, 773 – 776; b) A. Mamai, N. E. Hughes, A. Wurthmann, J. S. Madalengoitia, *J. Org. Chem.* **2001**, *66*, 6483 – 6486; c) A. Mamai, R. Zhang, A. Natarajan, J. S. Madalengoitia, *J. Org. Chem.* **2001**, *66*, 455 – 460; d) P. Tremmel, A. Geyer, *J. Am. Chem. Soc.* **2002**, *124*, 8548 – 8549; e) M. Kümin, L.-S. Sonntag, H. Wennemers, *J. Am. Chem. Soc.* **2007**, *129*, 466 – 467; f) R. S. Erdmann, M. Kümin, H. Wennemers, *Chimia* **2009**, *63*, 197 – 200; g) Y. A. Fillon, J. P. Anderson, J. Chmielewski, *J. Am. Chem. Soc.* **2005**, *127*, 11798 – 11803; h) P. Ruzza, G. Siligardi, A. Donella-Deana, A. Calderan, R. Hussain, C. Rubini, L. Cesaro, A. Olser, A Guiotto, L. A. Pinna, G. Borin, *J. Pept. Sci.* **2006**, *12*, 462 – 471; i) D. J. Witter, S. J. Famiglietti, J. C. Cambier, A. L. Castelhano, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3137 – 3142; j) T. M. Kapoor, A. H. Andreotti, S. L. Schreiber, *J. Am. Chem. Soc.* **1998**, *120*, 23 – 29; k) J. P. Morken, T. M. Kapoor, S. Feng, F. Shirai, S. L. Schreiber, *J. Am. Chem. Soc.* **1998**, *120*, 30 – 36.

[13] For selected general reviews on peptide mimetics, see: a) L. Belvisi, L. Colombo, L. Manzoni, D. Potenza, C. Scolastico, *Synlett* **2004**, 1449 – 1471; b) A. Grauer, B. König, *Eur. J. Org. Chem.* **2009**, 5099 – 5111.

[14] For other work on peptide mimetic synthesis by ring-closing metathesis, see: a) L. M. Beal, K. D. Moeller, *Tetrahedron Lett.* **1998**, *39*, 4639 – 4642; b) J. Einsiedel, H. Lanig, R. Waibel, P. Gmeiner, *J. Org. Chem.* **2007**, *72*, 9102 – 9113.

[15] a) C. Herdeis, H. P. Hubmann, *Tetrahedron: Asymmetry* **1992**, *3*, 1213 – 1221; b) C. Herdeis, H. P. Hubmann, H. Latter, *Tetrahedron: Asymmetry* **1994**, *5*, 351 – 354; c) N. Lunglois, R. Z. Andriamialisoa, *Tetrahedron Lett.* **1991**, *32*, 3057 – 3058; d) C. Acevedo, E. Kogut, M. A. Lipton, *Tetrahedron Lett.* **2001**, *57*, 6353 – 6359; e) P. Somfai, H. Ming He, D. Tanner, *Tetrahedron Lett.* **1991**, *32*, 283 – 286.

[16] a) F. Schülzchen, Dissertation, FU Berlin **2000**; b) O. Hara, K. Sugimoto, K. Maikno, Y. Hamada, *Synlett* **2004**, 1625 – 1627; Schülzchen (Ref. [16a]) separated the *cis* and *trans* diastereomers of **8** by preparative HPLC. In contrast, we further converted this mixture and separated the diastereomers at the stage of **11** by simple flash chromatography on silica gel.

[17] P. A. Grieco, S. Gilman, M. Nishizawa, *J. Org. Chem.* **1976**, *41*, 1485 – 1486.

[18] M. M. Fernández, A. Diez, M. Rubiralta, E. Montenegro, N. Casamitjana, *J. Org. Chem.* **2002**, *67*, 7587 – 7599, and references therein.

[19] I. Coin, M. Beyermann, M. Bienert, *Nat. Protoc.* **2007**, *2*, 3247 – 3256.

[20] H. Li, D. S. Lawrence, *Chem. Biol.* **2005**, *12*, 905 – 912.

[21] a) G. Bodenhausen, D. J. Ruben, *Chem. Phys. Lett.* **1980**, *69*, 185 – 189; b) P. Schanda, B. Brutscher, *J. Am. Chem. Soc.* **2005**, *127*, 8014 – 8015.
[22] A. Velazquez-Campoy, S. A. Leavitt, E. Freire in *Protein-Protein Interactions: Methods and Applications* (Ed.: H. Fu), Humana, Totowa, **2004**.
[23] The close similarity of the ^{15}N HSQC spectra for WT/**X**-WT and the ^{15}N SOFAST-HMQC for LL/**X**-LL (see Supporting Information) justifies that only the WT/**X**-WT spectra are discussed here in detail.
[24] J. P. Demers, A. Mittermaier, *J. Am. Chem. Soc.* **2009**, *131*, 4355 – 4367.
[25] J. C. Ferreon, V. J. Hilser, *Biochemistry* **2004**, *43*, 7787 – 7797.
[26] A. Palencia, E. S. Cobos, P. L. Mateo, J. C. Martínez, *J. Mol. Biol.* **2004**, *336*, 527 – 537.
[27] For the binding of LL to Fyn SH3, Li et al. (Ref. [20]) reported a dissociation constant of 25 nM at 4 °C, which was determined by equilibrium dialysis using fluorimetric detection of the highly fluorescent LL ligand. Because the LL ligand is highly lipohilic, it may possibly bind to the dialysis cassette in an unspecific manner. In fact, our own measurements showed that the LL ligand binds to Fyn SH3 only with a K_D in the μM range.

