Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 11, pp. 1209-1214, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00208-9

NOVEL PHOSPHOTYROSINE MIMETICS IN THE DESIGN OF PEPTIDE LIGANDS FOR pp60^{src} SH2 DOMAIN[1]

Aurash Shahripour,*† Mark S. Plummer,† Elizabeth A. Lunney,† Kimberly S. Para,†
Charles J.Stankovic,† John R. Rubin,† Christine Humblet,† James H. Fergus,‡ James S. Marks,‡
Roman Herrera,¶ Susan E. Hubbell,¶ Alan R. Saltiel,¶ and Tomi K. Sawyer†

Departments of Chemistry†, Biochemistry‡, and Signal Transduction¶
Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, Ann Arbor, MI 48105.

Abstract: We have designed and synthesized a series of phosphorylated penta- and tri-peptides of general structures R_x-Glu-Glu-Ile-Glu and R_x-Glu-D-Trp-NH_2, where R_x represents a phosphotyrosine mimetic. These peptides show binding affinity to pp60^{src} SH2 domain in the micromolar range. Data are presented that provide an account of their structure-activity relationships and specificity properties.

Introduction: pp60^{src} (Src) is a nonreceptor tyrosine kinase that interacts with several key signalling proteins via sequence-specific phosphotyrosine (pTyr) mediated binding with its SH2 domain.[2] The Src SH2 domain contains approximately 100 amino acids, and it recognizes endogenous as well as synthetic pTyr-containing proteins and peptides.[3-7] Several cognate phosphoprotein ligands which bind intermolecularly with Src SH2 include middle T antigen,[3] PDGF receptor,[4] EGF receptor,[5] and focal adhesion kinase (FAK).[6]

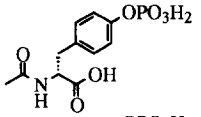
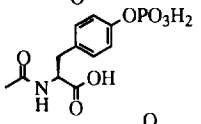
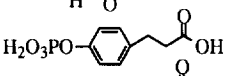
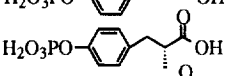
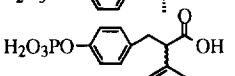
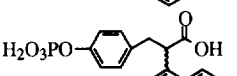
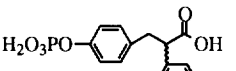
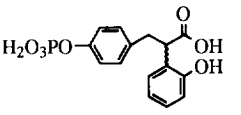
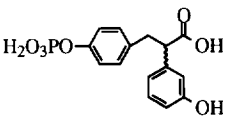
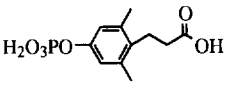
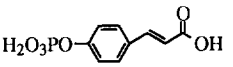
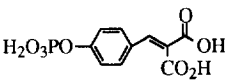
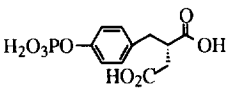
The design of Src SH2-targeted ligands is facilitated by the availability of both NMR and X-ray crystallographic structures for the Src SH2 domain complexed with high affinity, synthetic phosphopeptide ligands.[8-10] We have engaged in studies with pp60^{src} SH2 domain aimed at the discovery of potent peptidomimetic ligands. In particular, the X-ray coordinates from a previously reported[8] structure of the high-affinity phosphopeptide (Glu-Pro-Gln-pTyr-Glu-Glu-Ile-Pro-Ile-Tyr-Leu) bound to the Src SH2 domain provided the initial basis for designing our prototype peptidomimetic ligands. Based on the Src SH2-specific ~pTyr-Glu-Glu-Ile~ sequence[7,9] and known 3-dimensional structures of the native protein and phosphopeptide complexes[8-10] thereof, we have focused drug design on the two key binding pockets of the Src SH2 domain. One of these sites binds the pTyr moiety (P site), and the second binds the hydrophobic side chain of the Ile residue (P+3 site) as shown in Figure 1.

Results and Discussion: A radiolabeled analog of the above parent phosphopeptide was used to develop a binding assay (*vide infra*) for testing compounds described in this report. A series of pTyr mimetics (**1A-1M**, Table 1) were substituted into a potent pentapeptide **2**, Ac-pTyr-Glu-Glu-Ile-Glu (IC_{50} = 0.50 μM), which has been previously reported.[7] Inversion of the chirality of the pTyr in compound **2** to give **3** led to a six-fold loss in activity. Relative to pentapeptide **2**, removal of the N-terminal acetamide group (compound **4**) resulted in a seven-fold decreased binding affinity. The α-methyl substituted analog **5** was less potent than the des-amino derivative **4**, implying that more than chirality at this center is important.

The three-dimensional structures[8-10] of pTyr-containing peptides complexed with Src SH2 domain, demonstrate that the phosphate group is tightly bound within its binding pocket by multiple cationic (Arg-155,

Table 1. Structure–activity relationship of pentapeptides having pTyr replacements.

R_X–Glu–Glu–Ile–Glu

Pentapeptide Analog No.	R_x Structure (Free acid intermediate)	R_x No.	IC_{50} (μM)[a]
2		1A	0.50
3		1B	2.80
4		1C	3.40
5		1D	20.0
6		1E	1.90 (Isomer 1)
7			9.30 (Isomer 2)
8		1F	7.30 (Isomer 1)
9			2.60 (Isomer 2)
10		1G	2.80 (Isomer 1)
11			4.50 (Isomer 2)
12[b]		1H	1.40
13		1I	2.10 (Isomer 1)
14			3.30 (Isomer 2)
15		1J	4.50
16		1K	5.50
17		1L	5.0
18		1M	0.35

[a]Values are the average of at least two determinations. [b]Isomers were inseparable; 1:1 mixture of diastereomers.

Arg–175, and Lys–203) and hydrogen–bonding interactions (Glu–178, Ser–187, and Thr–179) as shown in Figure 1. We, therefore, designed conformationally–constrained and anionically–modified analogs of the pTyr residue to accommodate these characteristics. The positively charged Arg–155 has been shown[8] to provide simultaneous recognition of both the phosphate group and the aromatic ring of the pTyr residue. The α–phenyl and α–naphthyl substituted analogs, **6–11**, were designed to possibly augment π/cation interactions with Arg–155. Since Arg–155 also forms an ion pair with the phosphate group, we further designed pTyr analogs having α–(o–hydroxy)phenyl or α–(m–hydroxy)phenyl substituents (compound **12–14**) to explore the possibility of additional intermolecular hydrogen bonding with Arg–155. The corresponding pentapeptides **12–14**, however, did not display increased affinity relative to the reference phenyl derivative **6**. Nevertheless, the α–phenyl and α–(1–naphthyl) substitutions of the pTyr–acetamide moiety did provide analogues exhibiting Src SH2 binding in a stereochemically–selective manner. When possible, these diastereoisomers (pTyr α–center) were separated by reversed–phase HPLC. As reported previously,[11] the Ac–D–pTyr derivative **3** was five–fold less potent than the corresponding L–isomer **2**. A similar trend is observed with the α–phenyl derivatives **6** and **7**, in which the more potent isomer **6** has five fold increased binding affinity relative to **7**. Interestingly, this trend was not observed in the α–(m–hydroxyphenyl) series, where the separated diastereomers **13** and **14** were equipotent, again suggesting the importance of chirality and ring substitution at this center. In light of the critical

nature of pTyr interaction, rotationally–restrained analogues **15** (2,6–dimethyl restricts rotation of the bond between the β–carbon and phenyl ring) and **16** (a double bond removes a degree of freedom at the α–carbon–β–carbon bond), were synthesized. These restraints, however, provided no increase in binding affinity relative to the phenyl derivative **4**. Compound **16** was further modified by addition of an α–carboxyl group (compound **17**) to possibly form an ionic interaction with Arg–155, but no increase in binding affinity was observed. The α–carboxymethyl analog **18** was the most potent of this series implying an electrostatic interaction with Arg–155 as shown in Figure 2.

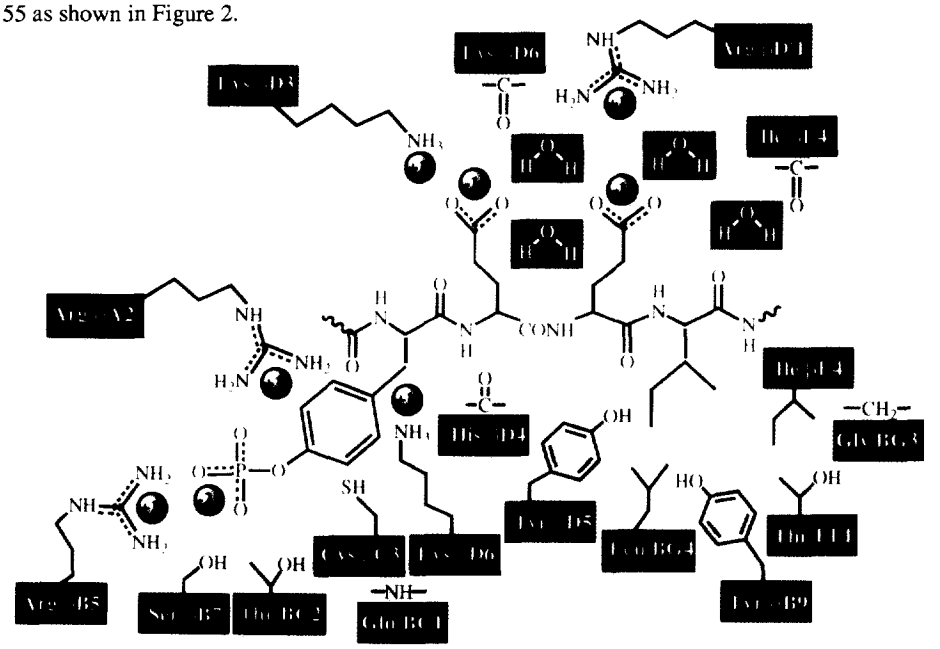


Figure 1. Schematic diagram showing interaction of ~pTyr–Glu–Glu–Ile~ complexed with Src SH2 and illustrating the two key binding sites for pTyr and Ile, respectively.

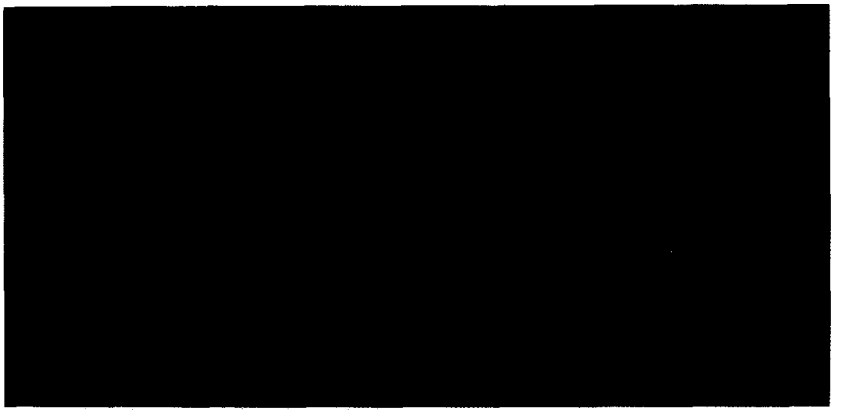


Figure 2. Stereoview of the Ac–pTyr mimetic (R_x = 1M) in analog **18** modeled[12] as a complex with Src SH2. The bifurcated hydrogen bond with Arg–155 is shown in white.

To further explore the utility of the above pTyr mimetics several were incorporated into a series of C–terminally modified phosphopeptides. Specifically, the design of tri–peptides such as Ac–pTyr–Glu–D–Trp–NH_2 (compound **20**) were based on modeling prediction[13] that suggested a C–terminal D–amino acid directly following the P+1 Glu residue might provide a novel scaffold strategy to afford molecular recognition at the hydrophobic P+3 binding pocket. As evident with analog **20** (IC_{50} = 4.0 μM) this design strategy was shown to be successful. Modifications of the N–terminal Ac–pTyr moiety of **20** by several R_x groups are summarized in Table 2. In general, this series of the peptide analogues showed a four– to ten–fold reduction in binding activity as compared to **20**. Nevertheless, similar structure–activity relationships were

Table 2. Structure–activity relationship of tripeptide having pTyr replacements.

R_X–Glu–D–Trp–NH_2

Tripeptide Analog No.	R_x No. (See Table 1)	IC_{50} (μM)[a]	Tripeptide Analog No.	R_X No. (See Table 1)	IC_{50} (μM)[a]
20	1A	4.0	**24**[b]	1F	30.0
21	1C	21.3	**25**[b]	1G	40.0
22[b]	1D	13.4	**26**	1J	30.0
23[b]	1E	28.0	**27**	1K	30.0

[a] Values are the average of at least two determinations. [b] Isomers were inseparable; 1:1 mixture of diastereomers.

observed for this series of tripeptide analogues as compared with the aforementioned pentapeptides. Specifically, removal of the acetamide group of **20** to give compound **21** resulted in a five–fold loss of binding affinity. The conformationally–constrained analogs (**22**–**25**) and rotationally–restrained analogs (**26** and **27**) showed decreased binding potencies relative to the corresponding compounds in the pentapeptide series. These compounds, however, were tested as an inseparable mixture of diastereoisomers. Overall, our results indicate that N–acetyl group removal or replacement can be accomplished without complete lose of activity. Such information may be critical to the design of metabolically–stable derivatives.

Compound **6** was assessed (relative to **2**) to determine if modifying the pTyr moiety affected SH2 binding selectivity. The binding affinities of these two analogues were examined at concentrations ≤ 100 μM against Src, Abl, Grb2, Syp (N–terminal), and PLCγ1 (C–terminal) SH2 protein constructs and are summarized in Table 3. Selectivity towards Src SH2 domain was generally observed, compound **2** did exhibit binding to Abl SH2 albeit about ten–fold less potent relative to Src SH2.

Table 3. Comparative IC_{50} or % inhibition of selected compounds to block binding of various SH2 domain constructs to PDGF receptor kinase.

	IC_{50} (μM) or % Inhibition @ 100 μM				
Compound	Src	Abl	Grb2	Syp(N)	PLC(C)
2	1.35	15.30	45.25%	>100	>100
6	7.35	>100	>100	>100	>100

Conclusion: We have prepared several Ac–pTyr mimetics as novel N–terminal groups in a series of penta– and tri–peptide ligands for Src SH2. A systematically–modified series of such compounds were investigated to determine the extent to which designed functionalities, including aromatic and carboxyalkyl groups, might provide

effective replacements as predicted from molecular modeling studies. This structure–activity study extends previous reports[11,14] that have detailed the systematic examination of potential phosphate–mimetics (e.g.,–OSO_3H, –$CF_2PO_3H_2$, and $CH(CO_2H)_2$) of the pTyr residue in a pentapeptide template. Such information is critical for the rational design of peptidomimetic and nonpeptide second–generation ligands which are potent, specific, and metabolically–stable to provide an opportunity to explore that cellular effects of Src SH2 blockade in signal transduction pathways.

Experimental, Materials, and Methods

Chemistry: The synthesis of intermediates **1D**, **1M**, and **1E–1I** were carried out using Evans methodology, and Knoevenagel condensation, respectively. Intermediate **1J** was synthesized according to a published procedure,[15] and compound **1N** from the known cyclic anhydride.[16]

Materials. ^{125}I–Radiolabeling of Glu–Pro–Gln–pTyr–Glu–Glu–Ile–Pro–Ile–Tyr–Leu was carried out by Amersham Corporation. Filter plates (0.45 μM hydrophobic PVDF) were purchased from Millipore. Glutathione sepharose 4B beads were purchased from Pharmacia. Each SH2 protein was expressed as a fusion construct of glutathione S–transferase (GST) according to a published procedure.[17]

Src SH2 binding assay. Binding of ^{125}I–labeled phosphopeptide to the GST–SH2 fusion protein was performed in 20 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA and 0.1% NP–40. Assay additions resulted in Src SH2 fusion protein–glutathione sepharose bead complex, 2.8 nM ^{125}I–labeled phosphopeptide and 2% DMSO test sample at the indicated concentration ranging from 0.1 to 100 μM. Binding was carried out at room temperature for 20 minutes while continuously inverting the plate. Bound ^{125}I–labeled phosphopeptide was separated from free by vacuum filtration and washing two times with 100 μL assay buffer/well. The remaining radioactivity was determined by scintillation counting. All measurements were determined in duplicate.

Specificity Testing for Phosphopeptides. The specificity of selected phosphopeptides were determined using an assay consisting of PDGF receptor kinase as the cognate phosphoprotein and ^{35}S–labeled SH2–GST protein fusion constructs. Inhibition of binding of ^{35}S–SH2–GST protein constructs of Src, Abl, Grb2, Syp (N–terminal), and PLC–γ1 (C–terminal) to immobilized phosphorylated PDGF receptor kinase was conducted using a Millipore filter plate (0.45 μM PDVM) in 20 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 10 mM $MgCl_2$, and 0.1% Triton. The binding assay was carried out at room temperature for 30 minutes, and the unbound ^{35}S–SH2–GST protein was separated from that complexed to immobilized PDGF receptor by vacuum filtration. Bound ^{35}S–SH2–GST protein was measured by scintllation counting, and all measurements were determined in duplicate.

Peptide Synthesis. Peptide synthesis was performed using standard solid–phase peptide synthesis methodology based on Fmoc/t–butyl protection strategy[18] employing either an Applied Biosystems (ABI) 431A peptide synthesizer or a manual shaker. The peptides were synthesized according to a described procedure.[19] The peptides were cleaved from the resin using TFA:water:thioanisole (20:1:1, v:v:v). All phosphopeptides were isolated by preparative reversed–phase HPLC, and both analytical data (electrospray mass spectrometry, and ^{31}P, ^{1}H NMR spectroscopy data were satisfactory to confirm the structural integrity of final products.

References

1. A preliminary disclosure of this work has appeared at the Fourteenth American Peptide Symposium, June 18–23, **1995**. The Ohio State University, Columbus, Ohio, U. S.A.
2. Margolis, B. *Cell Growth Differ.* **1992**, 3, 73.
3. Courtneidge, S. A.; Goutebroze, L.; Cartwright, A.; Heber, A.; Sherneck, S.; Funteun, J. *J. Virology* **1991**, 65, 3301.
4. Mori, S.; Ronnstrand, L.; Yokote, K.; Engstrom, A.; Courtneidge, S. A.; Claesson–Welsh, L.; Heldin, C–H. *EMBO J.* **1993**, 12, 2257.
5. Luttrell, D. K.; Lee, A.; Lansing, T. J.; Crosby, R. M.; Jung, K. D.; Willard, D.; Luther, M.; Rodriguez, M.; Berman, J.; Gilmer, T. M. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, 91, 83.
6. Xing, Z.; Chen, H–C.; Nowlen, J. K.; Taylor, S. J.; Shalloway, D.; Guan, J–L. *Mol. Biol. Cell* **1994**, 5, 413.
7. Songyang, Z.; Shoelson, S. E.; Chaudhuri, M.; Gish, G.; Pawson, T.; Haser, W. G.; King, F.; Roberts, T.; Ratnofsky, S.; Lechleider, R. J.; Neel, B. G.; Birge, R. B.; Fajardo, J. E.; Cou, M. M.; Hanafusa, H.; Schaffhausen, B.; Cantley, L. C. *Cell* **1993**, 72, 767.
8. Waksman, G.; Kominos, D.; Roberson, S. C.; Pant, N.; Baltimore, D.; Birge, R. B.; Cowburn, D.; Hanafusa, H.; Mayer, B. J.; Overduin, M.; Resh, M. D.; Rios, C. B.; Silverman, L.; Kuriyan, J. *Nature* **1992**, 358, 646.
9. Waksman, G.; Shoelson, S. E.; Pant, N.; Cowburn, D.; Kuriyan, J. *Cell* **1993**, 72, 779.
10. Xu, R. X.; Word, M.; Davis, D. G.; Rink, M. J.; Willard, D. H.; Gampe, R. T. *Biochemistry* **1995**, 34, 2107.
11. Gilmer, T.; Rodriguez, M.; Jordon, S.; Crosby, R.; Alligood, K.; Green, M.; Kimery, M.; Wagner, C.; Kinder, D.; Charifson, P.; Hassell, A. M.; Willard, D.; Luther, M.; Rusnak, D.; Sternback, D. D.; Mehrotra, M.; Peel, M.; Shampine, L.; Davis, R.; Robins, J.; Patel, I. R.; Kassel, D.; Burkhart, W.; Moyer, M.; Bradshaw, T.; Berman, J. *J. Biol. Chem.* **1994**, 269, 31711.
12. Molecular modeling was carried out using the Sybyl software, version 6, commercially available from Tripos Associates, Inc., 1699 Hanley Rd., Suite 303, St. Louis, MO 63144. The previously reported[9] X–ray crystallographic structure of Glu–Pro–Gln–pTyr–Glu–Glu–Ile–Pro–Ile–Tyr–Leu complexed with Src SH2 provided the initial structure for this study.
13. Plummer, M. S.; Shahripour, A.; Lunney, E. A.; Para, K. S.; Vara Prasad J. V. N.; Singh, J.; Stankovic, C. J.; Humblet, C.; Fergus, J. H.; Marks, J. S.; Sawyer, T. K.; *Drug Des. Discovery*. in press.
14. (a) Burke, T. R., Jr.; Smyth, M. S.; Otaka, A.; Nomizu, M.; Roller, P. P.; Wolf, G.; Case, R.; Shoelson, S. E. *Biochemistry* **1994**, 33, 6490. (b) Ye, B.; Akamatsu, M.; Shoelson, S. E.; Wolf, G.; Peraldi, S. G.; Yan, X.; Roller, P. P.; Burke, T. R*J. Med. Chem.* **1995**, 38, 4270.
15. Bhattacharya, K. K.; Pal, P.; Ghosh, K.; Sen, P. K. *Indian J. Chem.* **1980**, 19B, 191.
16. Chaturvedi, D. N.; Kulkarni, R. A. *Indian J. Chem.* **1979**, 17B, 534.
17. Zhu, G.; Decker, S. J.; Mayer, B. J.; Saltiel, A. R. *J. Biol. Chem.* **1993**, 268, 1775.
18. Fields, G. G.; Noble, R. L. *Int. J. Pept. Prot. Res.* **1990**, 35, 161.
19. McNamara, D. J.; Dobrusin, E. M.; Zhu, G.; Decker, S. J.; Saltiel, A. R. *Int. J. Pept. Prot. Res.* **1993**, 42, 240.

(*Received in USA* 11 *March* 1996; *accepted* 23 *April* 1996)