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Development of L-3-aminotyrosine suitably protected for the synthesis of a novel nonphosphorylated hexapeptide with low-nanomolar Grb2-SH2 domain-binding affinity

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Abstract—Synthesis of orthogonally protected (2*S*)-2-amino-3-(3-amino-4-hydroxy-phenyl)-propionic acid (**10**) suitable for solid phase peptide synthesis and its first use for the preparation of nonphosphorylated Grb2-SH2 domain antagonists (**4a**–**c**) are reported. The 3-aminotyrosine containing sulfoxide-cyclized hexapeptide (**4b**) exhibited potent Grb2-SH2 domain binding affinity with $IC_{50} = 50$ nM, which represents the highest affinity yet reported for a peptide inhibitor against Grb2-SH2 domain with only six residues free of phosphotyrosine or phosphotyrosine mimics. This potent small peptidomimetic **4b** may be representative of a new class of therapeutically relevant Grb2-SH2 domain-directed agents, and acts as a chemotherapeutic lead for the treatment of erbB2-related cancers.
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1. Introduction

Growth factor receptor-bound protein 2 (Grb2) is recognized as an excellent target for drugs in the treatment of cancers because of the early and central role played by Grb2 in the cellular signal transduction pathways.[1] Therapeutic intervention can be approached through blocking the interaction between the phosphotyrosine-containing activated receptors and the Grb2-SH2 domain.[2–5] Recent papers reported significant advances in the use of 'SH2 domain signaling antagonists' in cancer therapy and osteoporosis,[6–9] however, clinical agents operating by this mechanism are lacking, due in part to the unique requirement of anionic phosphate-mimicking functionality for high SH2 domain-binding affinity or the extended peptide nature of most inhibitors, which limits potency in whole cell systems where membrane transit is required.[10,11]

Our approach was derived from a phage library display lead, a nonphosphorylated cyclic peptide comprised of a 9-amino acid long sequence motif, **E[1]LYENVGMY[9]**, flanked by two terminal disulfide linked cysteines.[12,13] This cyclic undecapeptide does not contain phosphotyrosine, yet can specifically bind to the Grb2-SH2 domain with 10–25 μM affinity. Based on the disulfide-cyclized peptide lead, a number of redox-stable thioether-cyclized analogs were developed (Fig. 1), some of which exhibited potent binding affinity and encouraging cellular activity.[14–18] The absence of phosphate or phosphate mimicking functionality in this family of cyclic peptides contributes to the remarkably high specificity and enhanced bioavailability. Thus this class of nonphosphorylated cyclic peptides represents a new promising type of Grb2-SH2 domain binding motif.

Previous SAR studies have disclosed the functional importance of all essential residues in this novel Grb2-SH2 binding motif,[13–18] however, little effort was conducted on the modification of Tyr by introducing additional substituents on the phenyl ring to improve the activity. According to the binding mode of the lead peptide **1** with Grb2-SH2 domain based on molecular

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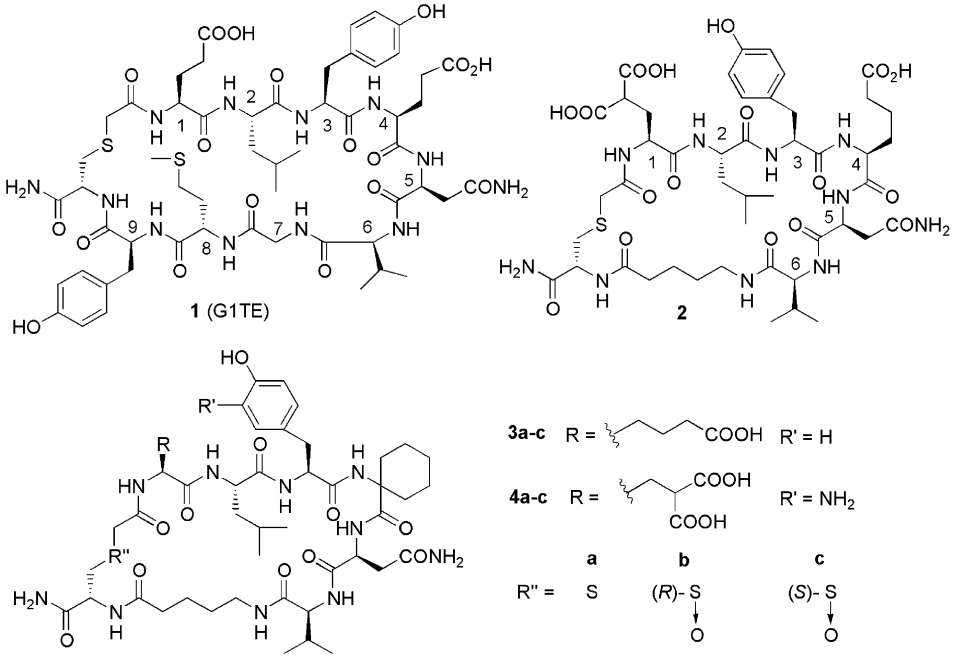


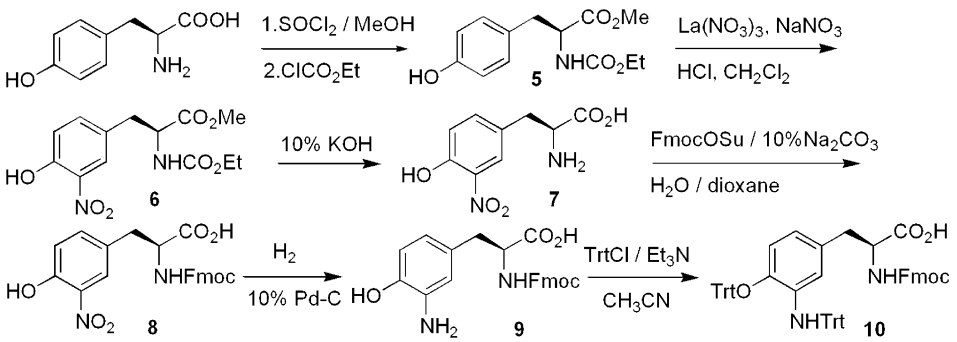
Figure 1. The structures of synthetic nonphosphorylated peptide ligands binding to the Grb2-SH2 domain.

modeling,[14] the Glu[1] carboxyl group together with Tyr[3] interacts with Arg67 (αA-helix) and Arg86 (βC-strand) residues and the three Ser residues 88, 90, and 96 within the binding pocket. We assumed that putting electron donating substituents such as amino group at the 3′-position of Tyr, as a way of increasing the electronegativity of the aromatic ring, would increase the ring π-interaction with the Arg86 and/or Arg67 side chain of the SH2 domain binding cavity; on the other hand, the electron donating group itself would undergo additional hydrogen-bond interactions within the pTyr-binding pocket. This is a brand new strategy distinct from any approaches reported previously to improve the selectivity and affinity. As reported herein, the 3-aminotyrosine-containing nonphosphorylated cyclic peptide **4b** (Fig. 1) has been found to exhibit low-nanomolar Grb2-SH2 domain-binding potency ($IC_{50} = 50$ nM) with only 6 a.a. long motif of **Gla[1]-Leu-(3′-NH$_2$Tyr)-Ac6c-Asn-Val[6]**, without any phosphotyrosine or phosphotyrosine mimics, providing a novel template for the development of nonpTyr containing Grb2-SH2 domain antagonists as potential therapeutics for certain cancers.

2. Synthesis

Although L-3-aminotyrosine in free form is commercially available, a new strategy was developed to synthesize L-3-aminotyrosine in protected form bearing N- and O-trityl protections in the side chain and N-α-Fmoc protection at the backbone suitable for the solid phase peptide synthesis.

Starting from commercially available L-Tyr-OH, as outlined in Scheme 1, we prepared **5** in quantitative yield by esterification of the acidic functionality with thionyl chloride in methanol followed by protection of the amino group with ethyl chloroformate in an aqueous $NaHCO_3$ solution. Selective nitration at the 3′-position of **5** was accomplished in 85% yield by using $La(NO_3)_3$ as an homogeneous catalyst and $NaNO_3$/HCl as the nitration reagent.[19] Treatment of **6** with 10% potassium hydroxide followed by reprotection with Fmoc-OSu provided N-α-Fmoc-3′-nitro-tyrosine **8**. Hydrogenation of the nitro group of **8** catalyzed by Pd/C and then protection of the amino group and hydroxy group by trityl chloride[20] afforded the 3-aminotyrosine **10** in



Scheme 1. Synthetic route toward the L-3-aminotyrosine suitably protected for the solid phase peptide synthesis.

orthogonal protection suitable for the solid phase peptide synthesis.[21]

With the 3′-aminotyrosine suitably protected for the peptide synthesis, we chose a recently developed 6-residue cyclic peptide ligand (Fig. 1, **2**) as the template for further elaboration. This new class of small peptides bearing an ω-amino carboxylic acid linker is advantageous in possessing smaller ring size and less peptide character but equal potency compared to the parent peptide **1**.[22]

Peptides **3a**–**c** and **4a**–**c**[23] were synthesized in a similar procedure described previously.[14,22] The linear peptide precursors were synthesized manually by means of Fmoc based solid phase peptide synthesis,[24] starting with Fmoc-PAL resin[25] for establishing the C-terminal carboxamide. After assembly of the cyclic peptide, the oxidation of the thioether linkage into sulfoxide was readily achieved using 5% H_2O_2 aqueous solution. The resulting sulfoxide diastereoisomers were easily separated by RP-HPLC. The stereochemistry of the sulfoxide diastereoisomers was identified by CD spectra.[16] The faster eluting sulfoxide diastereomer was found to be *R*-configured, and the slower eluting sulfoxide was the *S*-configured analog. All final products were purified by RP-HPLC, and the identity was assessed by mass spectral analyses.

3. Results and discussion

On the basis of the fact that the Grb2-SH2 domain requires the phosphopeptide ligand to bind to it in a unique β-turn fashion,[26] our previous effort of introducing a turn-inducing amino acid such as α-aminocyclohexanecarboxylic acid (Ac6c) in position 4 of G1TE (**1**) analogs proved very successful.[16] The peptide with substitution of Glu[4] with Ac6c loses charge interactions with Ser141 and Arg142, yet gains hydrophobic Van der Waals interactions with Gln106 and Phe108 of the Grb2-SH2 domain. In addition, the 3_{10} helix turn, with the torsion angles $\phi = -62.1°$, $\psi = -34.3°$, and $\omega = -170.4°$, induced by Ac6c is also a favored motif for the improved peptide binding to the Grb2-SH2 domain. In current work, this favorable influence rendered by Ac6c was applied to the new motif of cyclic hexapeptide **2** (Table 1). When replacing Glu[4] with Ac6c, the resulting peptide exhibited an enhanced potency by 2-fold (**3a**, $IC_{50} = 11.4\,\mu M$) relative to its parent peptide (**2**, $IC_{50} = 23.3\,\mu M$). We rationalized that the incorporation of the turn-inducing amino acid into the 6-residue cyclic peptide series **3** and **4** would help constrain the backbone conformation into the favorable β-bend conformation fitting into the binding pocket preferentially. Further modification was undertaken on the cyclization linkage. When the thioether-cyclized peptide **3a** was oxidized into the relatively more rigid *R*-configured sulfoxide **3b**,[22] the binding affinity was slightly improved (**3b**, $IC_{50} = 9.8\,\mu M$). However, the *S*-configured sulfoxide **3c** was much less active (**3c**, $IC_{50} = 84.5\,\mu M$) than its precursor thioether analog **3a**, probably due to the disfavored change of the peptide backbone conformation caused by the repulsion of the sulfoxide functional group with the carbonyl group of the C-terminal Cys, as was observed in the G1TE analogs with 9 a.a. long sequence.[16]

Table 1. Grb2-SH2 domain inhibitory activity of the cyclic hexapeptides[a]

Compound	IC_{50} (μM)[b]
2	23.3 ± 2.7[c]
3a	11.4 ± 0.9
3b	9.8 ± 3.2
3c	84.5 ± 15.5
4a	0.16 ± 0.09
4b	$\mathbf{0.050} \pm 0.010$
4c	1.08 ± 0.25

[a] The competitive binding affinity of ligands for the Grb2-SH2 domain protein was assessed using biacore surface plasmon resonance (SPR) methods. IC_{50} values were determined by mixing the inhibitor with recombinant Grb2-SH2 protein and measuring the amount of binding at equilibrium to an immobilized SHC(pTyr-317) phosphopeptide in a manner similar to that reported previously.[12]

[b] Values are means of at least two independent experiments, and are expressed as the concentration at which half-maximal competition was observed (IC_{50}). Standard deviation is given in parentheses.

[c] The value was previously reported[22] and used here as a reference.

We have found that the Glu[1] side chain in G1TE (**1**) compensates for the absence of Tyr[3] phosphorylation in retaining effective binding to the Grb2-SH2 domain.[14] Replacement of Glu[1] with γ-carboxyglutamic acid (Gla) prominently improves binding affinity. As suggested by molecular modeling,[14,18] the side chains of Gla[1], and Tyr[3] both point toward the same direction within the partially vacant pTyr-binding pocket and form an intramolecular hydrogen bond between the hydroxyl group of Tyr[3] and one of the carboxyl groups of Gla[1]. Based on these findings, we developed a fully elaborated cyclic hexapeptide **4a** with Gla in position 1 and 3-aminotyrosine in position 3. The introduction of an electron donating amino group at the 3′-position of Tyr, in order to afford electron-rich aromatic ring and more hydrogen-bond donor, resulted in a significant enhancement of the Grb2-SH2 domain binding affinity (**4a**, $IC_{50} = 0.16\,\mu M$). As expected, when the thioether linkage of **4a** was oxidized into the *R*-configured sulfoxide to give inhibitor **4b**, the binding affinity was remarkably improved, with an $IC_{50} = 50\,nM$. This analog is 3.2-fold more potent than **4a** and 466-fold more potent than the parent peptide **2**. Consistently, the *S*-configured sulfoxide analog **4c** ($IC_{50} = 1.08\,\mu M$) was 6.7 times less potent than its thioether counterpart. Notably, compound **4b** is one of the most potent and smallest nonphosphorus-containing Grb2-SH2 domain antagonists reported to date.

The gain in activity obtained with **4b** could be ascribed to the strengthening interactions with the Grb2-SH2 domain endowed by the 3-aminotyrosine. The electron donating substituents, such as 3′-amino group of Tyr could increase the π-interaction of the electron-rich ring with the Arg86 and/or Arg67 side chain of the Grb2 SH2 binding pocket; furthermore, the 3′-amino group could form an intramolecular hydrogen bond with one of the carboxyl groups of Gla[1]. Such an intramolecular

H-bond locks the side chain conformation of the pTyr-site binding motif of **4** series and directs the malonyl groups of Gla[1] to the pTyr-binding site of Grb2-SH2 protein.

4. Conclusions

In this study, we developed a new strategy to synthesize orthogonally protected 3-L-aminotyrosine suitable for solid phase peptide synthesis and successfully incorporated it into a new structural motif effectively binding to Grb2-SH2 domain with only 6-amino acids sequence. The 3-aminotyrosine containing sulfoxide-cyclized hexapeptide (**4b**) exhibited potent Grb2-SH2 domain binding affinity with an $IC_{50} = 50$ nM, which represents the highest affinity yet reported for a hexapeptide inhibitor against Grb2-SH2 domain free of phosphotyrosine or phosphotyrosine mimics. The interaction between the 3′-amino group of Tyr^3 and one of the carboxyl groups of Gla^1 might stabilize the favorable conformation for the cyclic peptides binding to Grb2-SH2 domain; on the other hand, the electron donating ability of the amino group at the 3′-position of Tyr might contribute to an increasing interaction between the electron-rich phenyl ring with the Arg86 and/or Arg67 side chain of the Grb2-SH2 domain binding cavity. This fully elaborated small peptidomimetic **4b** with low-nanomolar Grb2-SH2 domain-binding affinity and reduced peptide nature provides a novel template for the development of nonpTyr containing Grb2-SH2 domain antagonists, and potentially may find value in the chemical therapeutics of erbB2-related cancers.

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References and notes

1. (a) Smithgall, T. E. *J. Pharmcol. Toxical. Methods* **1995**, *34*, 125–132; (b) Lowenstein, E. J.; Daly, R. J.; Batzer, W. L.; Margolis, B.; Lammers, R.; Ullrich, A.; Skolnik, E. Y.; Bar-Sagi, S.; Schlessinger, J. *Cell* **1992**, *70*, 431–442.
2. Pawson, T.; Gish, G. D.; Nash, P. *Trends Cell Biol.* **2001**, *11*, 504–511.
3. Shakespeare, W. C. *Curr. Opin. Chem. Biol.* **2001**, *5*, 409–415.
4. Muller, G. *Top. Curr. Chem.* **2001**, *211*, 17–59.
5. Feller, S. M.; Tuchscherer, G.; Voss, J. *Leuk. Lymph.* **2003**, *44*, 411–427.
6. Fretz, H.; Furet, P.; Garcia-Echeverria, C.; Rahuel, J.; Schoepfer, J. *Curr. Pharm. Des.* **2000**, *6*, 1777–1796.
7. Gay, B.; Suarez, S.; Caravatti, G.; Furet, P.; Meyer, T.; Schoepfer, J. *Int. J. Cancer* **1999**, *83*, 235.
8. Shi, Z.-D.; Lee, K.; Liu, H.; Zhang, M.; Roberts, L. R.; Worthy, K. M.; Fivash, M. J.; Fisher, R. J.; Yang, D.; Burke, T. R., Jr. *Biochem. Biophys. Res. Commun.* **2003**, *310*, 378–383.
9. Shakespeare, W.; Yang, M.; Bohacek, R.; Cerasoli, F.; Stebbins, K.; Sundaramoorthi, R.; Azimioara, M.; Vu, C.; Pradeepan, S.; Metcalf, C., III; Haraldson, C.; Merry, T.; Dalgarno, D.; Narula, S.; Hatada, M.; Lu, X.; Van Schravendijk, M. R.; Adams, S.; Violette, S.; Smith, J.; Guan, W.; Bartlett, C.; Herson, J.; Iuliucci, J.; Weigele, M.; Sawyer, T. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9373–9378.
10. Bradshaw, J. M.; Mitaxov, V.; Waksman, G. *J. Mol. Biol.* **1999**, *293*, 971–985.
11. Bradshaw, J. M.; Waksman, G. *Adv. Prot. Chem.* **2003**, *61*, 161–210.
12. Oligino, L.; Lung, F.-D. T.; Sastry, L.; Bigelow, J.; Cao, T.; Curran, M.; Burke, T. R., Jr.; Wang, S.; Krag, D.; Roller, P. P.; King, C. R. *J. Biol. Chem.* **1997**, *272*, 29046–29052.
13. Lung, F.-D. T.; Long, Y.-Q.; King, C. R.; Wu, X.-W.; Wang, S.; Roller, P. P. *J. Pept. Res.* **2001**, *57*, 447–454.
14. Long, Y.-Q.; Voigt, J. H.; Lung, F.-D. T.; King, C. R.; Roller, P. P. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2267–2272.
15. Long, Y.-Q.; Yao, Z.-J.; Voigt, J. H.; Lung, F.-D. T.; Luo, J. H.; Burke, T. R., Jr.; King, C. R.; Yang, D.-J.; Roller, P. P. *Biochem. Biophys. Res. Commun.* **1999**, *264*, 902–908.
16. Li, P.; Zhang, M.; Long, Y.-Q.; Peach, M. L.; Liu, H.; Yang, D.; Nicklaus, M.; Roller, P. P. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2173–2177.
17. Long, Y.-Q.; Guo, R.; Luo, J. H.; Yang, D.; Roller, P. P. *Biochem. Biophys. Res. Commun.* **2003**, *310*, 334–340.
18. Li, P.; Zhang, M.; Peach, M. L.; Zhang, X.; Liu, H.; Nicklaus, M.; Yang, D.; Roller, P. P. *Biochem. Biophys. Res. Commun.* **2003**, *307*, 1038–1044.
19. (a) Ma, D.; Tang, W.; Kozikowski, A. P.; Lewin, N. E.; Blumberg, P. M. *J. Org. Chem.* **1999**, *64*, 6366–6373; (b) Ouertani, M.; Girard, P.; Kagan, H. B. *Tetrahedron Lett.* **1982**, *42*, 4315–4318.
20. Mastrolorenzo, A.; Scozzafava, A.; Supuran, C. T. *Eur. J. Pharm. Sci.* **2000**, *11*, 325–332.
21. The physicochemical data for **10**: ^{1}H NMR ($CDCl_3$, 400 MHz): δ ppm 7.8–7.3 (m, 8H), 7.2–7.0 (m, 31H), 6.71–6.69 (dd, 2H, $J = 1.5$ Hz), 5.2 (d, 1H, $J = 8.4$ Hz), 4.6–4.5 (m, 1H), 4.4–4.3 (m, 2H), 4.2 (t, 1H, $J = 6.6$ Hz), 3.2–2.9 (m, 2H); EI-MS m/z (%): 416 (M-2Trt, 6), 238 (M-2Trt-Fmoc, 14), 243 (Trt, 100); $[\alpha]_D^{20}$ −3 ($c = 1.3$, DMF).
22. Long, Y.-Q.; Lung, T. F.-D.; Roller, P. P. *Bioorg. Med. Chem.* **2003**, *11*, 3929–3936.
23. Peptide **4b** was purified by RP-HPLC (Vydac-C18 column, 10×250 mm; flow rate, 2.0 mL/min), eluent A, 0.05% TFA in H_2O, eluent B, 0.05% TFA in 9:1 CH_3CN–water, gradient 20–40% B over 35 min, Rt = 25.6 min; ESI-MS ($M-H^+$) 1077.20 (calcd 1077.20) ($M+Na^+$) 1101.5 (calcd 1101.19); Peptide **4c**, gradient 20–40% B over 35 min, Rt = 28.0 min; ESI-MS ($M-H^+$) 1077.2 (calcd 1077.20) ($M+Na^+$) 1101.5 (calcd 1101.19).
24. Atherton, E.; Sheppard, R. C. *Solid Phase Peptide Synthesis: A Practical Approach*; IRL: Oxford, 1989.
25. (a) Bernatowicz, M. S.; Daniels, S. B.; Köster, H. *Tetrahedron Lett.* **1989**, *30*, 4645–4648; (b) Albericio, F.; Kneib-Cordonier, N.; Biancalana, S.; Gera, L.; Masada, R. I.; Hudson, D.; Barany, G. *J. Org. Chem.* **1990**, *55*, 3730–3743.
26. Rahuel, J.; Gay, B.; Erdmann, D.; Strauss, A.; GarciaEcheverria, C.; Furet, P.; Caravatti, G.; Fretz, H.; Schoepfer, J.; Grutter, M. G. *Nature Struct. Biol.* **1996**, *3*, 586–589.