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Development of a Phosphatase-Stable Phosphotyrosyl Mimetic Suitably Protected for the Synthesis of High-Affinity Grb2 SH2 Domain-Binding Ligands

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Abstract—Synthesis of (*2R*)-2-carboxymethyl-3-(4-(phosphonomethyl)phenyl) proprionic acid (**5**) in *tert*-butyl-protected form (**6**) and its use for the preparation of a Grb2 SH2 domain-directed tripeptide (**8a**) is reported. In extracellular ELISA-based assays, **8a** exhibits potent Grb2 SH2 domain binding affinity ($IC_{50} = 8$ nM). Against cultures of MDA-MB-453 breast cancer cells, which overexpress erbB-2 tyrosine kinase, **8a** is also antimitogenic at concentrations equivalent to those required to inhibit intracellular association of Grb2 protein with phosphorylated $p185^{erbB\text{-}2}$ protein ($IC_{50} = 8$ μM). Analogue **6** may be useful for the preparation of a variety of phosphatase-stable SH2 domain-directed ligands.

Src homology 2 (SH2) domains are homologous modular units that serve critical roles in cellular signal transduction by binding to phosphotyrosyl (pTyr, **1**)-containing sequences.[1] Synthetic ligands that associate with high affinity to specific SH2 domains and thereby block pathogenic signal transduction, are being developed as potential therapeutics for a variety of diseases, including certain cancers.[2,3] Design of such agents is frequently predicated on the binding interactions of pTyr-containing partial sequences of cognate phosphoprotein ligands. Because pTyr residues within these peptides represent significant components of ligand recognition, two major challenges have been development of pTyr mimetics that are both stable to cellular phosphatases and that afford adequate cell membrane permeability.

In extracellular binding assays, phosphonate-based analogues such as phosphonomethyl phenylalanine (Pmp **2**) have been shown to maintain good SH2 domain binding affinity relative to parent pTyr **1** (Fig. 1), while being stable to phosphatases.[4–6] Modification of Pmp by addition of N^{α}-oxalyl functionality (OxoPmp, **3**), has resulted in reasonable inhibitory potency against binding of full length Grb2 to activated growth factor receptor in whole cell assays, where membrane transit of the Pmp-containing ligand is required.[6] In extracellular binding assays, conceptually similar introduction of carboxylic functionality at the α-position of phosphate-containing des-amino pTyr mimetic **4** has also resulted in high affinity binding of ligands directed against the Src[7] and ZAP-70[8,9] SH2 domains. For both OxoPmp **3** and analogue **4**, the design rationale for inclusion of acidic functionality at the α-position was to enhance binding interactions with an Arg67 residue within the pTyr-binding pocket of the Grb2 SH2 domain protein. Combining the structural features of **3** and **4** yields the α-carboxymethyl analogue **5**. Compound **5** may be viewed as a new pTyr mimetic, which displays the phosphatase stability of Pmp (**2**), while potentially affording enhanced binding potency observed with **3** and **4**. Reported herein is the preparation of **5** in protected form bearing *tert*-butyl protection of acidic functionality suitable for peptide synthesis (**6**). It should be noted that previous utilization of **4** as a pTyr mimetic in SH2 domain-directed ligands was achieved in multi-step fashion involving initial incorporation into the peptide ligand of unphosphorylated 'tyrosyl-like' precursor **7** followed by phosphorylation.[7] Title compound **6** is advantageous in containing both α-carboxymethyl and

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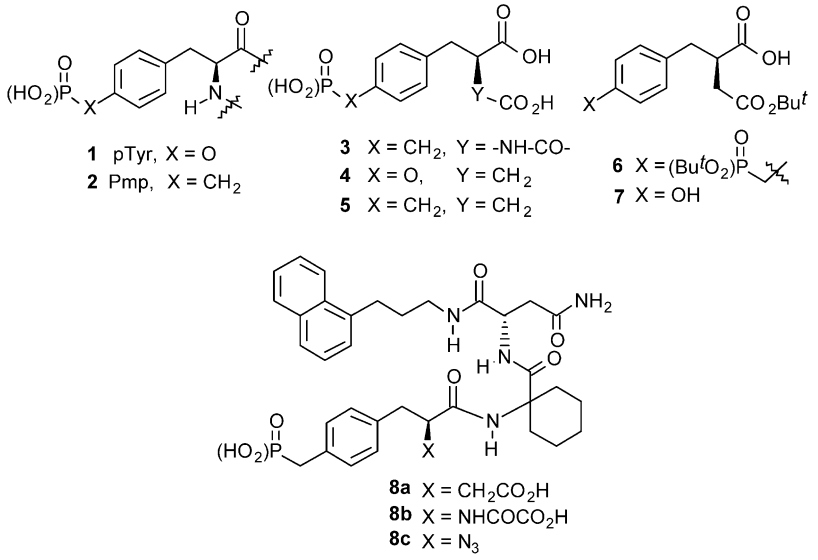


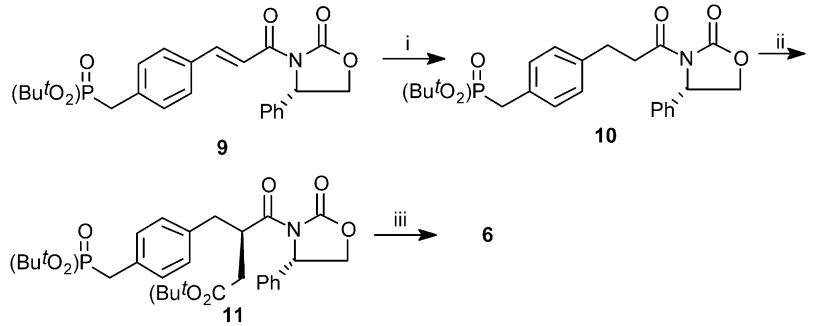
Figure 1. Structures of pTyr and pTyr mimetics.

phosphate-mimicking functionality in a single preformed globally protected reagent. Finally, the utility of **6** for the preparation of SH2 domain-directed peptides is demonstrated by the synthesis of a high affinity Grb2 SH2 domain-binding tripeptide **8a**. The inhibitory profile of **8a** is compared with previously reported OxoPmp-containing **8b** and with α-azido-pTyr mimetic-containing **8c**.

Preparation of **6** relied on chiral induction by Evans' oxazolidinone functionality[10] in a manner similar to that reported for the 4-hydroxy analogue **7**.[9] The approach taken paralleled our recently reported route[11] except that a phenyl oxazolidinone rather than a benzyl oxazolidinone chiral auxiliary was employed (Scheme 1). Common to our current synthesis and this previous approach, 4-phosphonomethyl cinnamoyl **9** was obtained in high yield via Heck reaction[11] and hydrogenated to provide key intermediate **10**. Treatment of **10** with NaHMDS at −78 °C followed by alkylation using *tert*-butyl bromoacetate provided **11** in 78% yield. As observed in the synthesis of related **7**[9] and with the synthesis of the α-azido analogue via electrophilic azidation,[11] chiral induction was high and diastereomerically pure product (as determined by NMR) could be obtained following chromatographic purification. Finally, removal of chiral auxiliary using literature procedures[12] gave the desired product **6** in 86% yield.

In order to demonstrate its synthetic utility, **6** was utilized for the preparation of Grb2 SH2 domain-directed tripeptide mimetic **8a**. Synthesis was similar to that previously desribed to prepare OxoPmp-containing **8b**,[6] except that coupling of **6** using HOBt (*N*-hydroxybenzotriazole) active ester coupling[13] resulted in poor yields (<40%). However, significantly higher yields (92%) could be obtained with HOAt (1-hydroxy-7-azabenzotriazole).[14] Product **8a** was obtained following TFA-mediated deprotection and HPLC purification. Finally, in the first biologically relevant use of an α-azido-containing pTyr mimetic, synthesis of **8c** was achieved in similar fashion, except that in this latter synthesis HOBt active ester coupling provided an adequate yield of desired product (58%).

New phosphatase-stable pTyr mimetic **5** and its globally protected variant **6** have several synthetic advantages over previously reported Pmp-based pTyr mimetics such as OxoPmp (**3**),[6,15] in that functionality at the α-position is introduced complete and protected form in



Scheme 1. Reagents and conditions: (i) 10% Pd·C, H_2, EtOAc (quantitative); (ii) NaHMDS, $BrCH_2CO_2Bu^t$, THF, −78 °C (78% yield); (iii) LiOH, H_2O_2, THF–H_2O (86% yield).

a single step. In contrast, traditional synthesis of functionalized α-amino pTyr mimetics require initial protection of the α-amino groups during residue coupling, followed by deprotection and final amino derivatization with desired biologically relevant groups.

Analogue **6** was designed to be particularly useful for the preparation of SH2 domain-directed ligands. High binding affinity has been demonstrated for related phenylphosphate-containing pTyr mimetic **4** against Src-family SH2 domains.[7–9] An extracellular ELISA-based assay[16] was employed in the current study to examine the potency of **6** as a Grb2 SH2 domain-directed pTyr mimetic. For this purpose, **6** was utilized for the construction of Grb2 SH2 domain ligand **8a**, which is structurally similar to previously reported OxoPmp (**3**)-containing **8b**. In this system platform **8a** provides high Grb2 SH2 domain-binding affinity ($IC_{50}=8$ nM), which is essentially identical to OxoPmp-containing **8b** ($IC_{50}=9$ nM) (Fig. 2). Of note, the importance of α-functionality is evidenced by the approximately 3 orders of magnitude loss of potency observed in α-azido-containing analogue **8c** ($IC_{50}=5$ μM).

Of particular importance for SH2 domain-directed ligands, is their ability to function in whole cell assays where cell membrane transport is an issue. As originally reported, introduction of α-acidic functionality through oxolylation of Pmp not only increased Grb2 SH2 domain affinity in extracelluar binding assays, but also enhanced intracelluar Grb2 binding potency in whole cell systems.[6] In order to examine the ability of **6** to function as a pTyr mimetic in a cellular context, whole cells were treated with inhibitors at various concentrations. Cells were then lysed, and the binding of full-length Grb2 to phosphorylated $p185^{erbB\text{-}2}$ protein was evaluated using immunoprecipitation and Western blotting techniques.[17] These experiments indicated that **8a** is able to block the association of Grb2 with $p185^{erbB\text{-}2}$ with an IC_{50} value of 10 μM. This is similar to the value obtained for OxoPmp-containing **8b** ($IC_{50}=3$ μM). Consistent with results of extracellular ELISA-based results, α-azido-containing analogue **8c** ($IC_{50}>30$ μM) was significantly less potent in these whole cell assays.

Synthetic Grb2 SH2 domain ligands may potentially afford new approaches toward anticancer therapies by disconnecting growth factor receptors from downstream mitogenic signalling pathways. One measure of the net effectiveness of these inhibitors is their ability to block growth factor-induced mitogenesis. We have previously utilized MDA-MB-453 breast cancer cells, which are dependent on erbB-2 pathways in order to examine the antimitogenic effects of Grb2 SH2 domain-binding ligands.[18] As shown in Figure 3, treatment of this cell line in culture with parent OxoPmp-containing **8b** elicits a dose-dependent anti-mitogenic ($IC_{50}=5$ μM). That this effect is related to signal transduction blockade and not nonspecific cytotoxicity, is evidenced by the fact that treatment of a related breast cancer cell line (MDA-MB-231), which does not share dependency on erbB-2 signalling, results in no effect on cell growth up to 25 μM concentrations.[19] Consistent with extracellular Grb2 SH2 domain binding affinities shown in Figure 2 and with intracellular binding affinities, tripeptide **8a** also exhibits antimitogenic potency against MDA-MB-453 cells which is nearly identical to parent OxoPmp ($IC_{50}=8$ μM; Fig. 3). As a final note, significantly reduced potency of α-azido containing **8c** was observed ($IC_{50}>50$ μM). Since analogue **8c** differs from **8a** and **8b** only in substitution at the α-position, its poor antimitogenic activity further indicates the importance of functionality at the α-carbon for SH2 domain binding, as well as for whole cell effects. The reasons for the enhanced cellular potency of ligands containing OxoPmp (**3**) and α-carboxymethyl-containing **6** are unknown.

Analogue **5** represents a new phosphatase-stable variant of the previously disclosed pTyr mimetic **4**, which may also be viewed as an analogue of OxoPmp (**3**). The preparation of **5** in protected form provides a new reagent

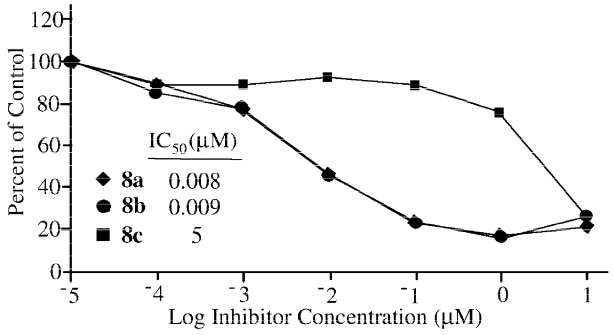


Figure 2. Extracellular ELISA assay of Grb2 SH2 domain binding.

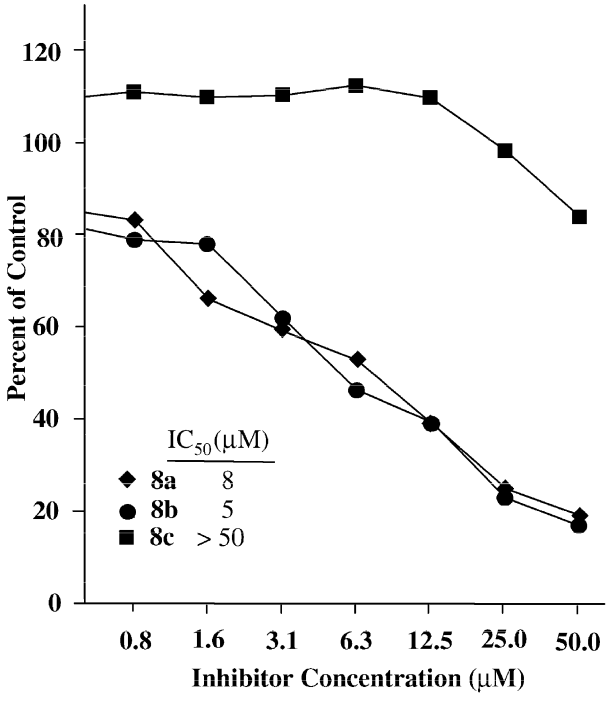


Figure 3. Survival of MDA-MB-453 breast cancer cells following treatment with inhibitors.

(**6**) for the facile synthesis of SH2 domain ligands, which may exhibit high affinity in both extracellular and whole cell systems. As such, compound **6** may represent a useful tool for the preparation of signal transduction antagonists and potentially, may find value in the development of anticancer therapeutics.

Acknowledgements

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16. A biotinated phosphopeptide encompassing a Grb2 SH2 domain-binding sequence derived from SHC protein, was bound at 25 ng/mL to 96-well plates coated with streptavidin (Roche Diagnostics GmbH #1645692) overnight. Nonspecific interactions were inhibited by 5% bovine serum albumin containing TBS. Samples of recombinant purified Grb2 SH2-GST fusion protein were pre-incubated with serial dilutions of inhibitors, then added into each well. After extensive washing with 0.1% bovine serum albumin in TBS, bound Grb2 SH2 domain was detected using anti-GST antibodies and goat anti-mouse antibody conjugated to alkaline phosphatase. Quantitation of bound alkaline phosphatase was achieved by a colorimetric reaction employing *p*-nitrophenylphosphate as substrate.
17. ErbB-2 over-expressing breast cancer cells, MDA-MB-453, were treated with inhibitors (30, 10, 3, 1, 0 μM) for 4 h in serum-free IMEM medium. Cells were washed twice with PBS to remove inhibitor, then cell lysates were prepared using 1% Triton X-100 in PBS containing 0.2 mM $NaVO_4$. Grb2 and associated Grb2-binding proteins were immunoprecipitated from each lysate (250 μg) with anti-Grb2 antibodies and collected using protein G Agarose (Roches Diagnostics GmbH #1243233). Immunoprecipitated proteins were separated by SDS-PAGE on 4–20% Tris–glycine gradient gels (Invitrogen/Novex), and pTyr-containing proteins were detected by western blotting using anti-phosphotyrosine antibodies (Santa Cruz #sc-7020) and visualized with ECL (Amersham). Membranes were subsequently stripped and Grb2 proteins were re-probed with an antibody recognizing the total Grb2 protein (Santa Cruz #sc-8034) as an internal immunoprecipitation control. A major tyrosine phosphorylated protein in these cells is the p185 erbB-2.
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