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Structure-Based Design and Synthesis of Phosphinate Isosteres of Phosphotyrosine for Incorporation in Grb2-SH2 Domain Inhibitors. Part 1

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Abstract—Based on X-ray crystal structure information, mono charged phosphinate isosteres of phosphotyrosine have been designed and incorporated in a short inhibitory peptide sequence of the Grb2-SH2 domain. The resulting compounds, by exploiting additional interactions, inhibit binding to the Grb2-SH2 domain as potently as the corresponding doubly charged (phosphonomethyl)phenylalanine analogue.

Introduction

Inhibiting the binding interactions between the tyrosine kinase growth factor receptors and the Src homology 2 (SH2) domain of the adapter protein Grb2 constitutes a possible strategy in the search for new anticancer drugs.[1] Our efforts in this direction have resulted in the identification of small phosphotyrosine (pTyr)-containing molecules that can efficiently disrupt these interactions in in vitro binding tests.[2–5] However, in the perspective of more advanced pharmacological studies, the phosphate group present in these inhibitors is a liability. The two negative charges of pTyr at physiological pH can in principle impair cell membrane penetration. In addition, the possible hydrolysis of the phosphate ester bond by phosphatases render pTyr-containing compounds unsuitable for cell and in vivo studies. Replacing pTyr in our compounds by a less charged and phosphatase-resistant mimetic—and this without losing high binding affinity for the Grb2-SH2 domain—is therefore desirable. Obtaining stability towards phosphatases without major loss of SH2 binding affinity can be achieved by using a (phosphonomethyl)phenylalanine (Pmp) mimetic.[6,7] However, reducing the number of charges without compromising binding affinity is a far more challenging task since recognition of phosphotyrosyl ligands by SH2 domains critically depends on strong polar interactions involving the three highly charged terminal oxygen atoms of the phosphate group of pTyr.[8] For example, replacement of pTyr, in a given phosphotyrosyl peptide sequence, by monocarboxylic acid mimetics, invariably reduces potency by several orders of magnitude.[9,10]

As reported in this letter, we have addressed these issues by designing mono charged phosphinate mimetics of pTyr whose synthesis is described in Part 2 of this work. The idea underlying this effort was to attenuate the reduction of affinity due to the removal of one charge by exploiting the additional carbon position afforded by a phosphinate group to create new favourable interactions with the SH2 domain. The designed phosphinate isosteres were incorporated in a short prototype inhibitory peptide sequence of the Grb2-SH2 domain to assess their value.

Design/Modelling

We based the design of phosphinate isosteres on the X-ray crystal structure of the Grb2-SH2 domain in complex with a peptide ligand containing Pmp as a replacement for pTyr.[11] Figure 1(a) shows the environment of the phosphonate group of the Pmp moiety in this structure.

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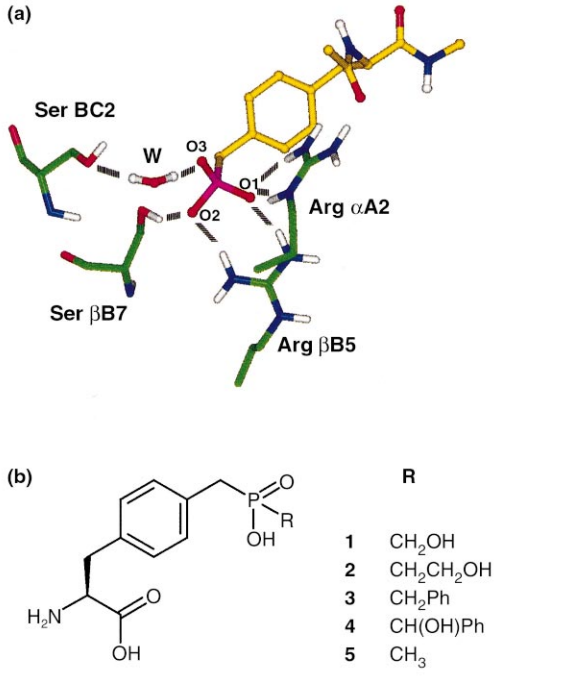


Figure 1. (a) Interactions between the phosphonate group of Pmp and the pTyr binding pocket of Grb2-SH2 observed in a X-ray structure. The hydrogen bonds are represented as dashed lines. (b) Chemical structures of designed isosteres.

Two of the terminal phosphonate oxygen atoms, O1 and O2, are involved in multiple electrostatic interactions with residues of the phosphotyrosine binding pocket. In particular, as previously observed in other SH2 domain structures, salt bridges are formed with Arg βB5 and Arg αA2.[12] In contrast, the third terminal oxygen atom, O3, does not make any direct interaction with the SH2 domain. It only forms a water mediated hydrogen bond with the side chain of Ser BC2. This led to the idea of removing one charge of the phosphonate group by replacing O3 by a carbon atom. We reasoned that the phosphinate analogue thus obtained would still have the capacity to form the salt bridge interactions with the arginines. In addition, the new carbon position would allow the introduction of substituents to try to compensate, by direct interaction with the SH2 domain, the loss of the water mediated hydrogen bond with Ser BC2 and the overall weakening of electrostatic interactions resulting from the removal of one negative charge. Compounds **1** and **2** (Fig. 1(b)) were the first phosphinate isosteres designed along this line. With these compounds, we sought to create a direct hydrogen bond with Ser BC2 by attaching a hydroxy group in the terminal position of the chain on the phosphorus atom. Modelling[13] indicated that spacers of one or two methylene units between the phosphorus atom and the terminal hydroxy group would be adequate to position the hydroxy oxygen atom within hydrogen bonding distance of the side chain of Ser BC2. However, as illustrated in Figure 2(a), the two carbon spacer was considered to be more attractive because it positioned the hydroxy group at the exact location of the water molecule mediating the hydrogen bond between the

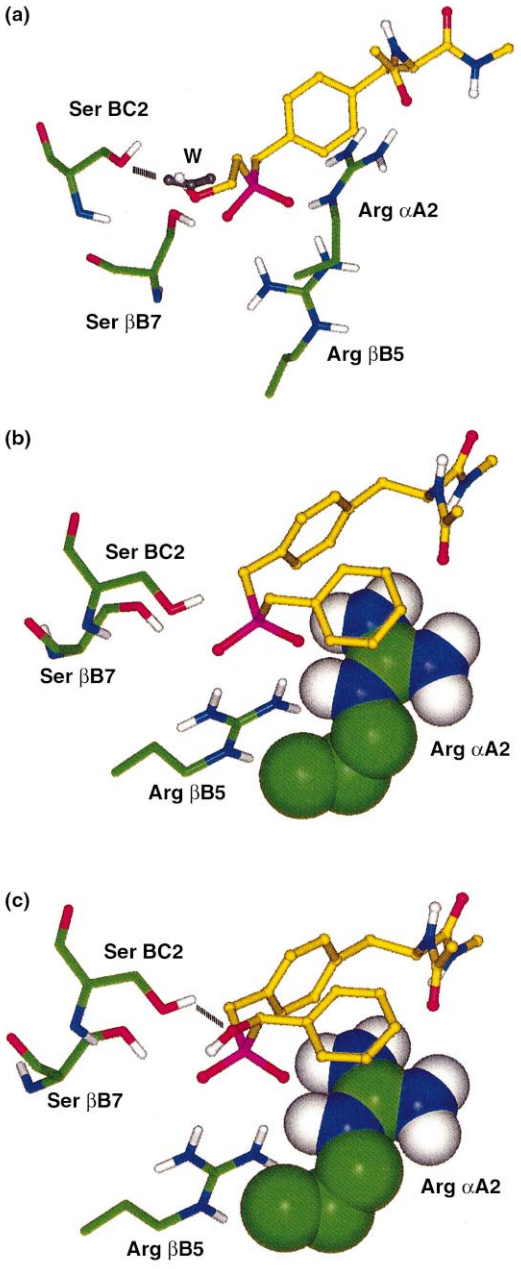


Figure 2. Models of phosphinate isosteres **2** (a) **3** (b) and **4** (c) interacting with the phosphotyrosine pocket residues. Hydrogen bonds to Ser BC2 are indicated as dashed lines. In **4**, the absolute stereochemistry of the carbon bearing the hydroxy substituent must be *R* to form the hydrogen bond with Ser BC2.

phosphonate O3 atom and Ser BC2 in the X-ray structure. We speculated that this modification would lead to a displacement of the water molecule, with the associated entropic advantage, without disrupting a hydrogen bonding network involving other crystallographically determined water molecules located at the bottom of the phosphotyrosine pocket.

Keeping cell permeability considerations in mind, we wondered whether it would be possible to use a purely hydrophobic side chain to create new favourable contacts with the protein. In previous work, we had shown

that the binding affinity of phosphotyrosyl Grb2-SH2 ligands can be dramatically improved by attaching to the N-terminus of pTyr an aromatic moiety able to form a stacking interaction with the guanidinium group of residue Arg αA2.[5] A modelling experiment showed that attachment of a benzyl group to the phosphorus atom, at the position corresponding to O3 in the X-ray structure, allowed the formation of the same stacking interaction. As illustrated in Figure 2(b), this idea was the basis of the design of **3**, a more lipophilic phosphinate isostere compared to **1** or **2**.

Finally, **4** was designed as a combination of both ideas: targeting Ser BC2 for hydrogen bonding and Arg αA2 for stacking. We also envisaged the synthesis of **5** as a reference to assess the success of our strategy aiming at establishing new favourable interactions with the SH2 domain.

Chemistry

The synthesis of the N^{α}-Fmoc derivatives of phosphinate isosteres **1**–**5** is described in the accompanying paper (Part 2). We report here their incorporation in the short prototype sequence Ac-Xxx-Ac_6c-(1*S*,2*R*)-Achc-NH_2 (Xxx = **1**–**5**). This sequence stems from previous work[2,3] where we identified suitable non-proteinogenic amino acid replacements for residues pTyr + 1 and pTyr + 2[14] of the minimal peptide motif recognized by the Grb2-SH2 domain.

The (1*S*,2*R*)-2-amino-cyclohexanecarboxylic acid amide β-amino acid (**12**, Achc) was prepared as depicted in Scheme 1. *rac,cis*-2-Amino-cyclohexanecarboxylic acid (**13**) was synthesised by known procedures[15] using sodium sulfite for the reduction of the *N*-chlorosulfonyl β-lactam[16] and concentrated hydrogen chloride for the hydrolysis of the β-lactam. The *cis* orientation assigned to the racemic **13** is that expected for a dipolar addition of chlorosulfonyl isocyanate with olefinic substrates.[16] Resolution of the racemic mixture was performed by liquid chromatography using a chiral support.[17]

Compounds **6**–**10** were synthesised in solution using standard conditions. The enantiomerically pure cyclic β-amino acid **12** was coupled to N^{α}-Boc-Ac_6c-OH (Ac_6c, 1-aminocyclohexane carboxylic acid) using *N*-ethyl-*N*′-(3-dimethylamino-propyl)-carbodiimide hydrochloride and 1-hydroxybenzotriazole in the presence of *N*-methylmorpholine in *N*,*N*-dimethylformamide. After removal of the *tert*-butyloxycarbonyl protecting group with 4 N HCl in dioxane, the dipeptide was coupled to the required N^{α}-Fmoc amino acids (**1**–**5**) using *O*-(1,2-dihydro-2-*oxo*-1-pyridyl)-*N*,*N*,*N*′,*N*′-tetramethyl-uronium tetrafluoroborate[18] and diisopropylethylamine in *N*-methylpyrrolidinone. Treatment of the Fmoc-protected compounds with a 20% solution of piperidine in *N*,*N*-dimethylacetamide and subsequent acetylation of the free N-terminal amino group with acetic anhydride afforded the target compounds **6**–**10**.[19] Compound **11** was synthesised manually on a Rink amide MBHA resin,[20] employing the Fmoc/*tert*-butyl strategy[21] and coupling protocols previously reported from our group.[2,19,22]

Results and Discussion

Binding affinity was assessed by an ELISA-type assay that measures the ability of a compound to inhibit the binding of the phosphorylated C-terminal intracellular domain of the epidermal growth factor receptor (EGFR) to the Grb2-SH2 domain.[23] IC_{50} values of compounds **6**–**10** in this assay are reported in Table 1. Also included in the table is the IC_{50} value of the reference phosphonate analogue **11**.

Our strategy to compensate the loss of one negative charge by creating new interactions using a neutral side-chain was successful. The potencies of phosphinate derivatives **7**, **8** and **9** are in the same range as that of the doubly charged phosphonate analogue **11**. Morever, the IC_{50} values follow the trend that was expected from our modelling studies. Replacement of one of the hydroxy groups of the phosphonate moiety of **11** by a simple methyl results in a significant loss of affinity (compound **10**), a consequence of weaker electrostatic

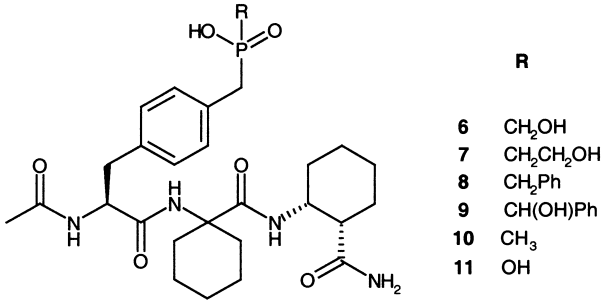
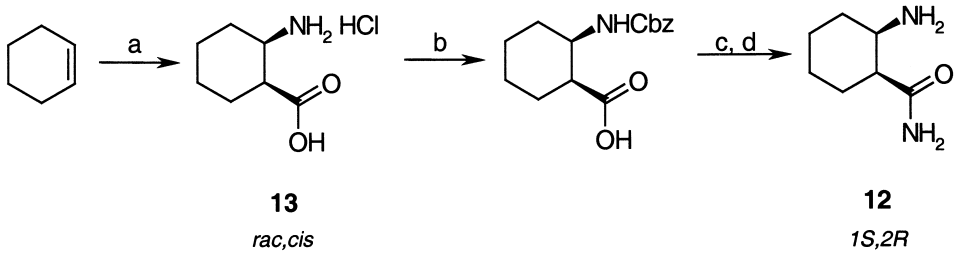


Figure 3. Chemical structures of target compounds.



Scheme 1. Conditions: a, (i) $ClSO_2NCO$, DCM; (ii) Na_2SO_3; (iii) HCl_{concd}, 53% (over three steps); b, (i) Cbz-Cl, Na_2CO_3, dioxane, 79%; chromatographic chiral separation, 66% recovery; c, isobutylchloroformate, TEA, THF, −10 °C then gaseous NH_3, 78%; d, H_2, Pd(C), MeOH, quant.

Table 1. Inhibitory activity of phosphinate derivatives Ac-Phe(4-CH_2-PO_2H-R)-Ac_6c-(1*S*,2*R*)-Achc-NH_2 and reference phosphonopeptide Ac-Pmp-Ac_6c-(1*S*,2*R*)-Achc-NH_2

Compounds	R	IC_{50}, μM[a]
6	CH_2OH	7.6
7	CH_2CH_2OH	1.6
8	CH_2Ph	1.2
9	CH(OH)Ph[b]	0.53
10	CH_3	25.5
11	OH	0.7

[a]Values are means of three experiments.
[b]Mixture of two diastereomers (*R* and *S* at the hydroxy substituted benzylic carbon).

interactions with the SH2 domain. Introduction of an alcohol chain targeting Ser BC2 for hydrogen bonding enhances potency (**6** and **7** compared to **10**). The effect is more pronounced with **7**, in agreement with our hypothesis that a water molecule is displaced. A substantial gain in potency (20 times) is also obtained with the benzyl substituent (**8** compared to **10**), which supports the existence of the designed stacking interaction with Arg αA2. As anticipated, combining the two favourable structural modifications results in the most active derivative **9** of the series.

Conclusion

This work demonstrates that it is possible to find mono charged isosteres of pTyr that give Grb2-SH2 domain inhibitors as potent as that obtained with the classical doubly charged Pmp isostere when incorporated in the same peptide sequence. In addition to bearing only one charge, the resulting compounds reported here do not contain any proteinogenic amino acids. These molecules pave the way for a new generation of Grb2-SH2 domain blockers useful in cell and in vivo studies.

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563.6, $C_{27}H_{40}N_4O_7P_1$; **6**); 577.9 (calcd 577.6, $C_{28}H_{42}N_4O_7P_1$; **7**); 623.6 (calc. 623.7, $C_{33}H_{44}N_4O_6P_1$; **8**); 639.6 (calcd 639.7, $C_{33}H_{44}N_4O_7P_1$; **9**); 549.7 (calcd 549.6, $C_{27}H_{42}N_4O_6P_1$; **10**); and 549.6 (calcd 549.6, $C_{26}H_{38}N_4O_7P_1$; **11**).

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