Letters

X-ray Structure of Citrate Bound to Src SH2 Leads to a High-Affinity, Bone-Targeted Src SH2 Inhibitor

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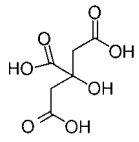
Introduction. Numerous studies provide compelling evidence that the protein tyrosine kinase pp60^{c-Src} (Src) plays a crucial role in osteoclast-mediated bone resorption[1,2] and, moreover, that the SH2 domain of Src specifically regulates osteoclast activity.[3,4]

With the expectation that blocking the SH2 domain of Src with high-affinity ligands might attenuate the excessive signal transduction in bone resorption diseases such as osteoporosis, we have been searching for general ways to improve upon the phosphotyrosine-containing peptides known to bind weakly to Src SH2. To be useful, such ligands need to contain a nonhydrolyzable phosphotyrosine mimic incorporated into a nonpeptidic scaffold. Features that confer specificity for bone would be an additional desirable property for the use of these compounds as therapeutic agents in the treatment of osteoporosis.

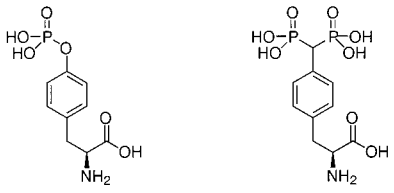
We were afforded a gratuitous starting point toward this goal when one of us (M.H.) determined the crystal structure of Src SH2 (in the absence of an inhibitor) at 1.5 Å resolution and found that the protein, when crystallized from citrate buffer, contained a well-complexed citrate ion in the phosphotyrosine-binding pocket. We now describe how this Src SH2/citrate X-ray structure led us to design and synthesize a nonpeptidic SH2 ligand that contains a phosphotyrosine mimic with bone-targeting properties.

Design. Previous X-ray and NMR structural studies provide detailed knowledge about the three-dimensional structure of a number of different SH2 domains complexed with peptidic, phosphotyrosine (pY)-containing ligands. For example, the structure of the cognate peptide, Ac-pTyr-Glu-Glu-Ile (pYEEI),[5] bound to Lck SH2 was determined at 1.0 Å resolution,[6] and the structure of the low-affinity peptide, pTyr-Val-Pro-Met-Leu (pYVPML), bound to Src SH2 was determined at a resolution of 1.5 Å. [7] These structures show that pY occupies a binding pocket composed of arginine, lysine, serine, and threonine residues. The phosphate group forms numerous hydrogen bonds and salt bridges with these residues (see Figure 1A). Replacement of pY in pYEEI with F abolishes binding affinity, suggesting that the interactions between pY and the residues of its binding pocket are largely responsible for the peptide's binding affinity.[8]



citrate

Like pY in the Lck SH2/pYEEI and Src SH2/pYVPML structures, citrate when bound to Src SH2 forms numerous salt bridges and hydrogen bonds with side chain and backbone protein atoms (Figure 1B). However, citrate forms two **additional** interactions not observed in the SH2/peptide crystal structures: a salt bridge with Lys 206 and a hydrogen bond with the backbone NH of Thr 182. Thus, the pY-binding pocket offers hydrogen-bonding opportunities in addition to those utilized by the natural pY-containing ligands. Taking advantage of this finding, we designed novel SH2 ligands that incorporate the known interactions of pY as well as the additional interactions revealed by the citrate structure. This suggested the replacement of pY (**1**) by diphosphonomethylphenylalanine (dpmF, **2**).



1 **2**

phosphotyrosine (pY), **1**

diphosphonomethyl-phenylalanine (dpmF), **2**

To determine whether the SH2 domain of Src could accommodate this novel amino acid in place of pY, dpmF was flexibly docked into the pY pocket of the Src SH2/citrate crystal structure using the conformational searching/energy minimization procedure of FLO96.[9,10] The docking results indicate that, like the citrate, the two phosphonates of dpmF can form an extensive network of hydrogen bonds (shown in Figure 1C). One of the phosphonates of dpmF occupies a position close to that adopted by the phosphate of pYEEI and pYVPML and forms the equivalent series of salt bridges. The second phosphonate of dpmF forms hydrogen bonds with Ser 180 and the NH of Glu 181 which are also observed in the Src SH2/pYVPML X-ray structure. However, this second phosphonate also forms the **additional** interactions observed in the citrate structure.

To further improve the binding affinity as well as the pharmacological potential of the projected SH2 ligands, it was deemed essential to reduce the peptide character of the portion linking the dpmF headgroup to the hydrophobic C-terminal group present in peptide ligands such as pYEEI. Lunney et al.[11,12] had shown that a

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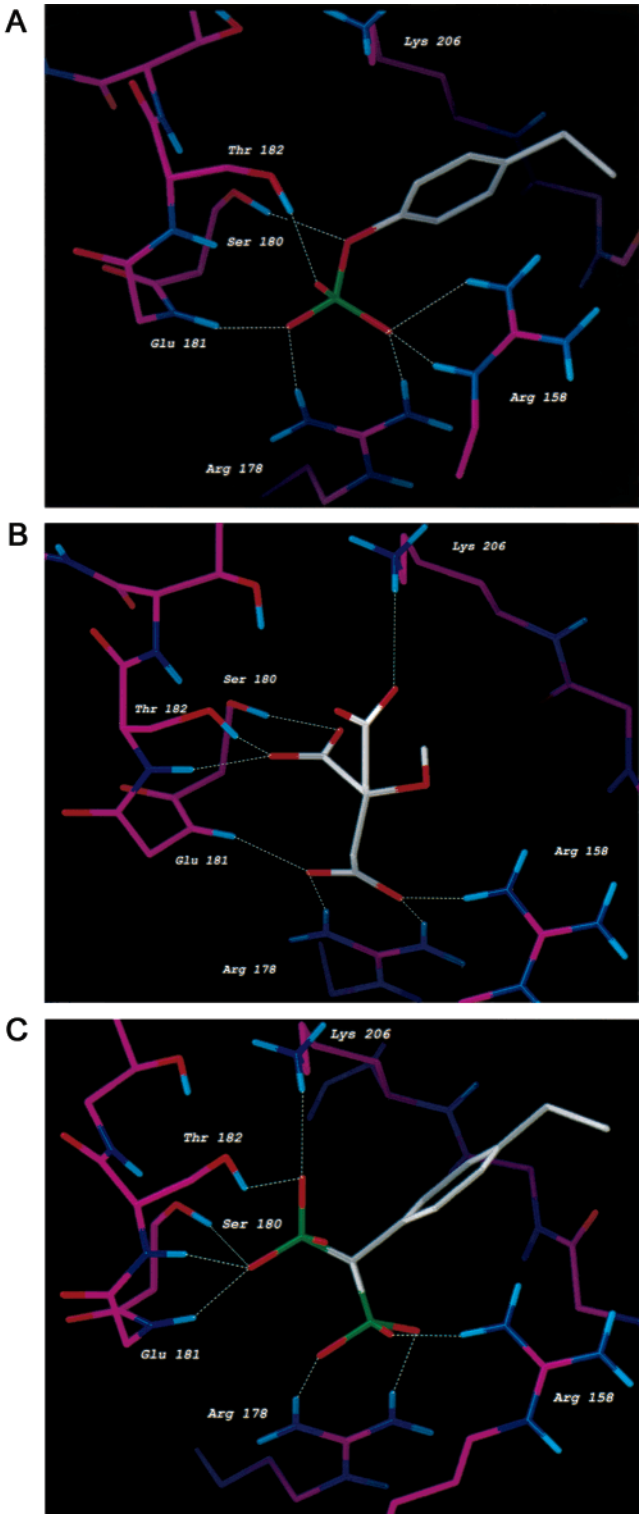


Figure 1. (A) Hydrogen bonds formed by phosphotyrosine observed in the X-ray structure of pTyr-Val-Pro-Met-Leu bound to the SH2 domain of Src. Hydrogen bonds are shown with dotted lines. Atoms are colored as follows: oxygen, red; nitrogen, blue; hydrogen, light blue; carbon atoms of the protein, purple; carbon atoms of the ligand, white; phosphorus, green. (B) Hydrogen bonds formed by citrate ion bound to Src SH2 from the Src SH2/citrate X-ray structure. (C) Hydrogen bonds formed by dpmF with Src SH2 predicted by molecular modeling studies.

benzamide, as in **3**, can serve as such a linker. Therefore, we modeled molecule **4** which exploits both the dpmF and benzamide/cyclohexyl moieties.

Because the Src SH2/citrate structure contained no ligand atoms in the binding site region beyond the phosphate pocket, this structure was found to be

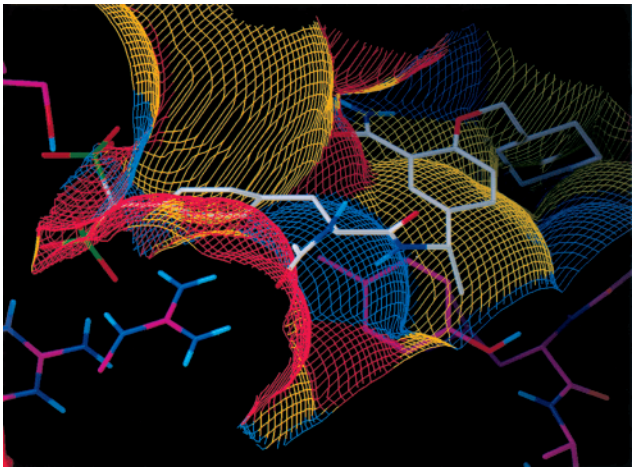
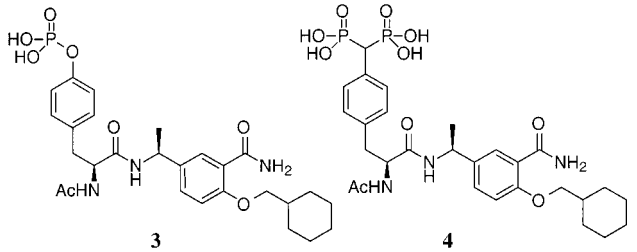


Figure 2. Compound **4** docked into the binding site of Lck SH2. The accessible surface of the binding site is displayed as a mesh, colored to display chemical specificity: hydrophobic regions of the binding site are shown in yellow, hydrogen bond donor regions in red, and hydrogen bond acceptor regions in blue. For optimal van der Waals contact, ligand atoms lie on or close to the mesh. Ligand atoms forming hydrogen bonds with protein atoms penetrate the mesh. Note the oxygen atoms of the dmpF moiety penetrating the mesh as they from hydrogen bonds with atoms of the phosphotyrosine pocket. Generated using the Flo96 software.

unsatisfactory for modeling SH2 ligands. For example, extensive docking studies did not succeed in correctly fitting pYEEI into this binding site. Therefore, we used the high-resolution (1.0 Å) structure of Lck SH2/pYEEI for our modeling studies and conducted conformational searching experiments to simulate the binding of compound **4** to Lck SH2. Figure 2 shows the result of these studies: the dpmF moiety forms the favorable interactions described above; the benzene ring is positioned above Tyr 181 (205 in Src)[13] forming favorable stacking



interactions; the benzamide displaces two water molecules forming hydrogen bonds with the backbone NH of Lys 182 (206 in Src) and the backbone carbonyl of Ile 193 (217 in Src); the ether oxygen forms an intramolecular hydrogen bond with the NH of the benzamide enabling the cyclohexyl side chain to form five hydrophobic contacts as it extends into the pY+3 pocket. Because of the additional interactions formed by the second phosphonate of dpmF, we predicted that **4** would bind to Src SH2 with higher affinity than **3**.

Encouraged by these molecular modeling results, we synthesized compound **4.**

Synthesis. The synthesis of compound **4** has been optimized as outlined in Scheme 1. (Details of the synthetic procedures are given in the Supporting Information.) For comparison we also synthesized compound **5** (see Table 1).

Src SH2 Binding Assay. A fluorescence polarization-based competitive binding assay was used to determine the IC_{50} of our compounds to the Src SH2 domain.[14] Results are shown in Table 1.

Scheme 1. Chemical Synthesis of **4**

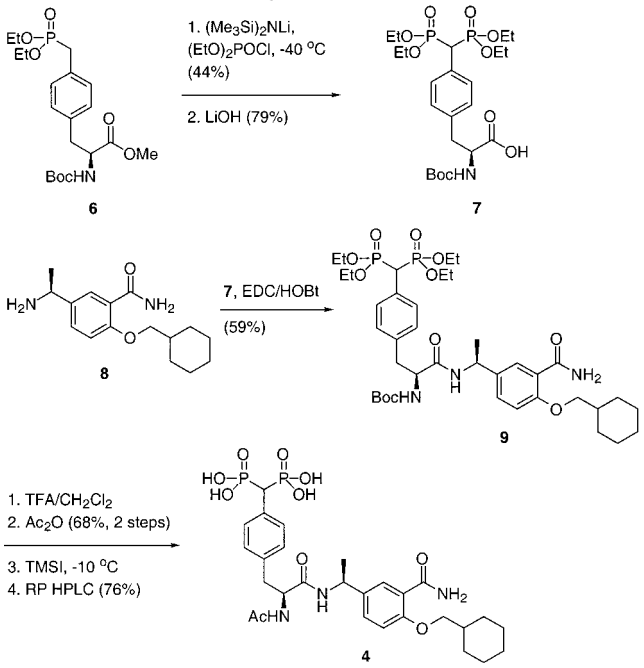
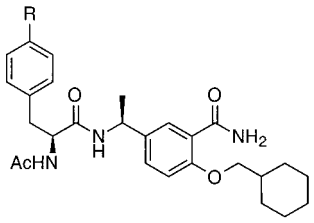
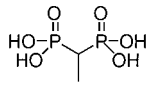


Table 1. Src SH2 Binding Properties

Compound	Structure	Src SH2 binding, IC_{50}, μM
Ac-pYEEI-NH_2		5.0
		
R		
3		2.2
4		0.35
5		7.9

Comparison of compound **4** with compound **5** shows that the incorporation of the second phosphonate group in the dpmF headgroup resulted in a 22-fold increase in binding affinity. Thus, the combination of the benzamide moiety with this high-affinity pY mimic resulted in a compound with a 14-fold higher binding affinity than that of the cognate peptide.

Hydroxyapatite Binding. The presence of the diphosphonomethyl group in **4** suggests that the molecule might target to bone in vivo. The bisphosphonate moiety has been shown to bind to bone and is present in a number of drugs used clinically to treat osteoporosis.[15] To evaluate this in vitro, a bone-binding model was developed. A microcrystalline hydroxyapatite adsorption column with phosphate gradient elution was used to measure the affinity of particular bone-targeting groups to hydroxyapatite. Tighter binding compounds elute later in the gradient. Retention time is expressed in terms in K', the number of void volumes required to elute a particular compound.

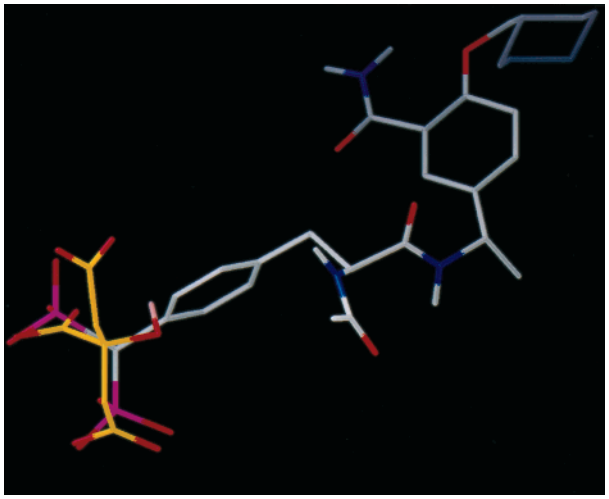


Figure 3. Conformation adopted by compound **4** upon binding to Src SH2 predicted by molecular modeling in green, determined by NMR in yellow, and determined by X-ray crystallography in blue. Prepared by superimposition of binding site atoms.

The hydroxyapatite chromatography results show that neither Ac-pYEEI-NH_2 nor compound **5** have measurable affinity for hydroxyapatite ($K' < 0.1$). On the other hand, compound **4** has a similar affinity for hydroxyapatite ($K' = 3.7$) as alendronate ($K' = 3.6$), a bone-targeted bisphosphonate drug. These results suggest that the diphosphonomethyl moiety of **4** is responsible for its affinity to hydroxyapatite and that **4** will bind to bone with affinity similar as alendronate.

Rabbit Osteoclast Resorption. Because Src has been implicated in the regulation of osteoclast functional activity, we tested the ability of compound **4** to inhibit osteoclast-mediated resorption of dentine. Compound **4** inhibited resorption of dentine with an IC_{50} of 2 μM. This is a significant improvement over a recently reported Src-modifying aldehyde compound (with an IC_{50} of 43μM), also developed in these laboratories.[4] To rule out the possibility that the observed antiresorptive activity of compound **4** was not due to nonspecific association with the bone matrix, we synthesized an analogue containing the dpmF moiety which possesses no affinity for the Src SH2 domain. This molecule did not demonstrate any antiresorptive activity.

Structure Determination. To confirm the predicted binding mode of **4**, the structure of its complex with Src SH2 was determined by NMR and that of its complex with the mutated Lck SH2 (S164C) by X-ray crystallography. The experimentally determined binding conformations of **4** proved to be very similar to those predicted by molecular modeling (Figure 3). NMR experiments revealed 46 NOEs between compound **4** and the protein. These clearly indicate that the dmpF of compound **4** occupies the pY pocket and that the cyclohexyl moiety binds in the pY+3 pocket.

The X-ray structures of the Lck SH2/**4** complex reveals that the dpmF binds, as predicted, with the phenyl ring in a position very similar to that of pY in the pYEEI complex. The benzamide group is stacked against the benzene ring of Tyr 181 (205 in Src). The

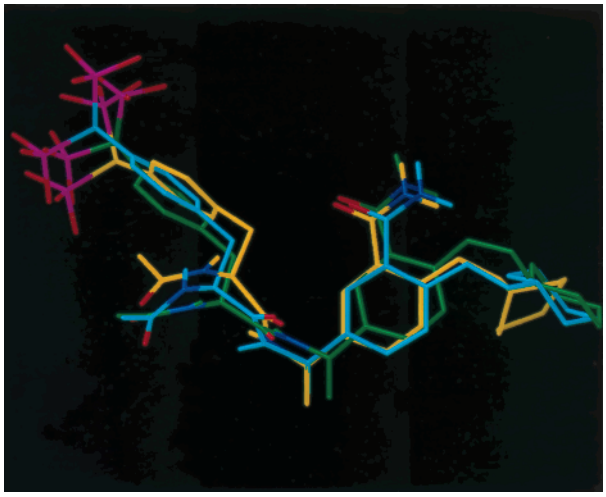


Figure 4. Comparison between citrate and compound **4** from the crystal structure of Src SH2/citrate and Lck SH2 (S164C)/**4**, constructed by superimposition of the binding site atoms.

benzamide carbonyl oxygen is in a position occupied by a water molecule in the pYEEI structure and forms a hydrogen bond with the backbone NH of Lys 182 (206 in Src). There are also two water molecules in this vicinity, and the temperature factors of these waters are as low as the backbone chain of Lck SH2, indicating that they are highly localized and held together by a number of strong hydrogen bonds. There are two direct and four water-mediated hydrogen bonds between the benzamide group and the protein. The cyclohexyl group forms extended contacts with the pY+3 pocket although it does not extend as deeply into the pY+3 pocket as the Ile side chain of pYEEI. An unexpected difference between the Lck SH2/**4** structure and that of previous SH2/ligand complexes is the open conformation of the BC loop. However, the crystal structure of a close analogue of compound **4** displays the BC loop in a position similar to that found in the Src SH2/citrate structure, forming hydrogen bonds with the second phosphate as predicted by our model.[16] NMR results also clearly indicate that the BC loop is in contact with the dpmF group of compound **4**. Thus, we conclude that the open loop conformation observed in the X-ray structure is due to crystal packing effects.

Another difference between the predicted ligand-binding mode and the X-ray structure is the presence in the X-ray structure of a water molecule between the NH_2 group of the benzamide and the carbonyl of Ile 193. As no water molecules are present in our binding site model, the NH_2 group of the benzamide was predicted to form a direct, weak hydrogen bond with the carbonyl of Ile 193, instead of the water-mediated hydrogen bond observed in the X-ray structure.

The superposition of complexes of Lck SH2 with compound **4** predicted by molecular modeling and determined by X-ray crystallography are shown in Figure 3. The binding mode of compound **4** determined by NMR has also been superimposed. Figure 4 shows a close-up of the superposition of the X-ray structures of the dpmF moiety from the Lck SH2/**4** complex and the Src SH2/citrate complex.

Conclusion. A crystal structure of the citrate ion bound to Src SH2 revealed that the phosphotyrosine pocket of Src SH2 offers hydrogen-bonding opportunities in addition to those displayed by natural pY-containing ligands. Molecular modeling predicted that a geminal bisphosphonate could form interactions similar to those of citrate. Incorporation of this moiety into a nonpetidic ligand for Src SH2 resulted in a compound which had 14 times greater binding affinity than the cognate (natural) peptide, possessed bone-binding properties, and inhibited osteoclast-mediated resorption of dentine. X-ray and NMR structures of the compound bound to Src and Lck SH2 agreed with molecular modeling predictions. These findings should provide a new paradigm for the design of agents to treat osteoporosis.

Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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