Discovery of Potent and Selective SH2 Inhibitors of the Tyrosine Kinase ZAP-70

Chi B. Vu,† Evelyn G. Corpuz, Taylor J. Merry, Selvaluxmi G. Pradeepan, Catherine Bartlett, Regine S. Bohacek, Martyn C. Botfield, Charles J. Eyermann, Berkley A. Lynch, Ian A. MacNeil, Mary K. Ram, Marie Rose van Schravendijk, Shelia Violette, and Tomi K. Sawyer*

ARIAD Pharmaceuticals, Inc., 26 Landsdowne Street, Cambridge, Massachusetts 02139-4234

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A series of 1,2,4-oxadiazole analogues has been shown to be potent and selective SH2 inhibitors of the tyrosine kinase ZAP-70, a potential therapeutic target for immune suppression. These compounds typically are 200−400-fold more potent than the native, monophosphorylated tetrapeptide sequences. When compared with the high-affinity ζ-1-ITAM peptide (Ac-NQL-pYNELNLGRREE-pYDVLD-NH_2, wherein pY refers to phosphotyrosine) some of the best 1,2,4-oxadiazole analogues are approximately 1 order of magnitude less active. This series of compounds displays an unprecedented level of selectivity over the closely related tyrosine kinase Syk, as well as other SH2-containing proteins such as Src and Grb2. Gel shift studies using a protein construct consisting only of C-terminal ZAP-70 SH2 demonstrate that these compounds can effectively engage this particular SH2 domain.

Introduction

The protein tyrosine kinase ZAP-70 has recently emerged as a potentially useful therapeutic target for immune suppression. T-Cell activation is a complex biochemical process which results from the initial recognition of the T-cell antigen receptor (TCR) with a specific antigen through the major histocompatibility complex (MHC) molecule. This process activates the CD4/CD8-associated Src family tyrosine kinase Lck.[1] Lck is critical for the phosphorylation of specific tyrosine residues within the ϵ- and ζ-chains of the immunoreceptor tyrosine activation motifs (ITAMs).[2] Some ZAP-70 may be associated with the basally phosphorylated ζ-chain in resting T-cells, and some may be additionally recruited upon T-cell stimulation. Productive T-cell activation is characterized by the appearance of a hyperphosphorylated form of the ζ-chain and by phosphorylation and catalytic activation of the ZAP-70 kinase by Lck.[3] Catalytic activation of ZAP-70 kinase is dependent on the double phosphorylation of ζ-ITAM residues and their binding to the tandem Src homology 2 (SH2) domains.[4] This complexation and other downstream signaling events are essential for T-cell proliferation.[5] The importance of ZAP-70 to T-cell function in vivo has been demonstrated by ZAP-70 knockout mice and humans with disrupted ZAP-70. In both cases, T-cell function is absent leading to a severely compromised immune response.[6] The protein tyrosine kinase ZAP-70 thus represents a desirable target for drug discovery because of its role as a necessary regulator of T-cell function and its selective expression in T-cells and natural killer cells.

In terms of molecular structure, ZAP-70 has been shown to consist of two tandem SH2 domains. SH2 domains are relatively small protein units having about 100 amino acids that preferentially bind to specific tyrosine-phosphorylated proteins.[7] These SH2 domains play a significant role in cellular signal transduction by their ability to transmit signals,[8] to act as adaptors between receptors,[9] or to regulate the kinase activity of a specific protein.[10] In ZAP-70, these two SH2 domains, here labeled N- and C-terminal SH2, are joined by an interdomain region consisting of 65 amino acid residues. C-Terminal to the tandem SH2 domains is another interdomain region and a catalytic kinase domain. The crystal structure of the tandem SH2 domains of ZAP-70, when associated with a doubly phosphorylated peptide deriving from the ζ-ITAM unit, has recently been solved.[11] The crystal structure shows that the C-terminal SH2 domain has an independent phosphotyrosine (pY) binding pocket, whereas the binding pocket on the N-terminal SH2 domain has contributions from both the N- and C-terminal SH2 domains. Whether the N-terminal SH2 binding pocket exists as such for unliganded ZAP-70 in the cell or whether it is only formed upon engagement of the C-terminal SH2 domain with a bis-phosphorylated ζ-ITAM motif is not yet known.[12] Therefore, either or both of the SH2 domains remain as valid therapeutic targets. In our approach to inhibit T-cell activation, we would like to use this structural information to aid in the design of nonpeptidic molecules that would bind to either of these SH2 domains.

A fluorescence polarization-based competitive binding assay was utilized to measure the affinity of compounds binding to the tandem ZAP SH2 construct.[13] The doubly phosphorylated native ζ-1-ITAM peptide (Ac-NQL-pYNELNLGRREE-pYDVLD-NH_2) binds with high affinity to the tandem SH2 domains of ZAP-70 and has been shown to have an IC_{50} of 38 nM in our fluorescence polarization (FP) binding assay.[14,15] The actual K_d of the ζ-1-ITAM peptide has been determined by us and others to be around 1−2 nM.[13,14,16] In contrast to the high binding affinity of the ζ-1-ITAM peptide, monophosphorylated tetrapeptides of the following sequence $pYX_1X_2X_3$ (where X_{1-3} are varying peptide residues, albeit X_3 is typically hydrophobic) bind weakly to ZAP-70 (Table 1).

* Corresponding author. Tel: (617) 494-0400, ext. 205. Fax: (617) 225-2589. E-mail: tomi.sawyer@ariad.com.
† Current address: Biogen, Inc., 14 Cambridge Center, Cambridge, MA 02139.

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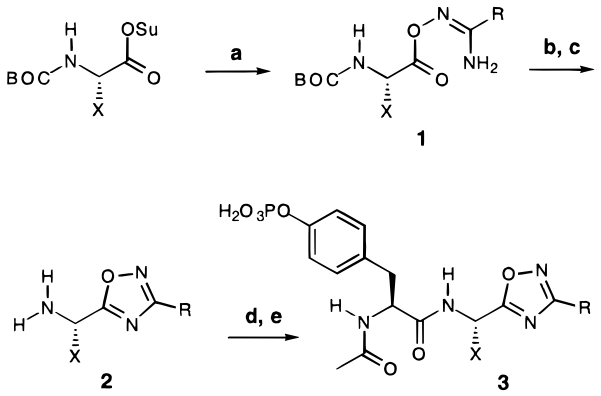
Table 1. SH2 Binding by Various Peptides

Peptides	IC_{50} (μM) ZAP-70	IC_{50} (μM) Syk	IC_{50} (μM) Src
ζ-1-ITAM peptide*	0.038	0.48	20
Ac-pYDVL-NH_2	328	389	261
Ac-pYNEL-NH_2	422	420	237
Ac-pYEEI-NH_2	530	171	6

*ζ-1-ITAM peptide = Ac-NQL-pYNELNLRREE-pYDVLD-NH_2 and pY = Tyr(PO_3H_2). Data taken in part from reference 13.

Scheme 1[a]



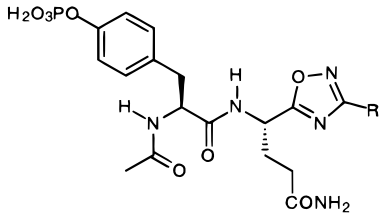
[a] (a) HON=C(NH_2)R, DME (alternatively, any given BOC-amino acid, CH_2Cl_2/DMF, HON=C(NH_2)R, EDC/HOBT/Hunig's base, rt, overnight); (b) pyridine, reflux; (c) TFA, CH_2Cl_2, rt; (d) EDC/HOBT/Hunig's base, Ac-Tyr(PO_3Bzl_2)-OH; (e) 95% TFA, 5% H_2O.

Among the various known single SH2 domain proteins such as Src,[17] tetrapeptides usually bind with better affinity.[18] The growth factor receptor-bound protein-2 (Grb2), an important component of the mitogenic *ras* activation pathway, contains a SH2 domain that interacts strongly with tripeptidic sequences.[19] In fact, a number of effective peptidomimetic or nonpeptidic inhibitors have now been reported for Src SH2[20] and Grb2 SH2.[21] To date, there have been only a few reports on the design and synthesis of ZAP-70 SH2 inhibitors.[22] In this paper, we would like to disclose the synthesis and structure−activity relationship of a series of 1,2,4-oxadiazole analogues as ZAP-70 SH2 inhibitors. Even though these compounds have been designed to be mimetics for the tetrapeptide sequences, many of them are 200−400-fold more potent. Furthermore, this series of compounds displayed an unprecedented level of selectivity (>500-fold) over the closely related tyrosine kinase Syk.

Results and Discussion

Initial Leads. In our previous work on Src SH2 inhibitors,[23] we identified the 1,2,4-oxadiazole scaffold **3** as a reasonable mimetic for the monophosphorylated tetrapeptide sequence $pYX_1X_2X_3$ found in many SH2 targets. These 1,2,4-oxadiazole analogues were prepared as illustrated in Scheme 1.[24] A desired BOC-protected amino acid, activated as the succinimide, was coupled with the amidoxime derivative HON=C(NH_2)R to afford **1**. In cases where the succinimide was not commercially available, the corresponding acid was coupled directly with the amidoxime in the presence of HOBT/EDC and Hunig's base. The amidoxime, in turn, could be prepared by reacting the corresponding nitrile with hydroxylamine hydrochloride, in the presence of NaOH. Cyclization to the 1,2,4-oxadiazole was carried out by refluxing **1** briefly in pyridine. The BOC group was then removed with TFA, and the resulting amine was coupled with the phosphorylated *N*-acetyltyrosine derivative. Final treatment with TFA then afforded the corresponding phosphate derivative **3**, which could be purified by reversed-phase HPLC. With these 1,2,4-oxadiazole analogues, the native amino acid side chain effectively becomes the pY+1 group. The heterocyclic ring forms a spacer which directs the R group into the lipophilic pY+3 pocket.

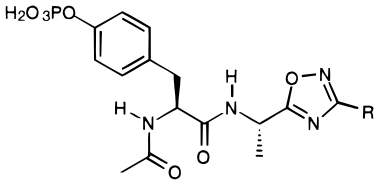
Table 2. SH2 Binding by 1,2,4-Oxadiazole Analogues Derived from L-Glutamine



no.	R	IC_{50} (μM) ZAP-70	IC_{50} (μM) Syk	IC_{50} (μM) Src
4a	CH_2Ph	>500	>500	72
4b	CH_2-(*p*-Me)Ph	65	>500	292
4c	CH_2-(*p*-$CHMe_2$)Ph	36	>500	96
4d	CH_2-(*p*-Cl)Ph	26	>500	205
4e	CH_2-(*m*-Cl)Ph	94	>500	259
4f	CH_2-(*m,p*-Cl_2)Ph	14	>500	206
4g	CH_2-(*o,p*-Cl_2)Ph	184	>500	>500
4h	CH_2-(*o,o,p*-Me_3)Ph	>500	>500	312
4i	CH_2-(1-naphthalene)	55	>500	135
4j	CH_2-(2-naphthalene)	8	>500	274
4k	$(CH_2)_2Ph$	>500	>500	156

In our work, we routinely screened our hits against a variety of SH2-containing proteins such as ZAP-70 and Syk. Compound **4a**, prepared from L-glutamine with a simple benzyl group at the pY+3 position, was identified as one of the more active Src SH2 inhibitors.[23] It also displayed little affinity toward ZAP-70 and Syk. However, we noticed that when the pY+3 group became slightly larger as in **4b** and **4c** that the binding affinity toward Src decreased somewhat (Table 2). At the same time, a substantial gain in ZAP-70 SH2 binding was observed. The selectivity over Syk was unusual. ZAP-70 and Syk belong to the same family of tyrosine kinases. Both of these kinases contain two tandem SH2 domains.[25] Previous work has shown that it is difficult to achieve selectivity between these two SH2-containing proteins even with large peptide sequences.[26] Whereas ZAP-70 is expressed only in T-cells and natural killer cells,[27] Syk is expressed in B-cells, mast cells, polymorphonuclear leukocytes, platelets, macrophages, and immature T-cells.[28] In designing ZAP-70 SH2 inhibitors, a high level of selectivity over Syk as well as other SH2-containing proteins is of special interest. Therefore, we

Table 3. SH2 Binding by 1,2,4-Oxadiazole Analogues Derived from L-Alanine



no.	R	IC_{50} (μM) ZAP-70	IC_{50} (μM) Syk	IC_{50} (μM) Src
5a	CH_2-(*p*-Me)Ph	33	>500	65
5b	CH_2-(*p*-Cl)Ph	14	>500	66
5c	CH_2-(*m*-Cl)Ph	61	>500	68
5d	CH_2-(*m*,*p*-Cl_2)Ph	6	>500	72
5e	CH_2-(2-naphthalene)	5	>500	64
5f	CH_2-(*p*-CF_3)Ph	7	>500	210
5g	CH_2-(*m*-CF_3)Ph	96	>500	115
5h	CH_2-(*p*-F)Ph	72	>500	200
5i	CH_2-(*p*-Br)Ph	7	>500	31
5j	CH_2-(*p*-I)Ph	5	472	18
5k	CH_2-(*m*,*p*-Br_2)Ph	4	>500	39
5l	CH_2-(*m*-Br)-(*p*-Cl)Ph	5	>500	45
5m	CH_2-(*m*-Cl)-(*p*-I)Ph	3	464	50
5n	CH_2-(*m*-Br)-(*p*-I)Ph	3	>500	40

decided to explore this series further to see if we could increase both the binding affinity and the selectivity of this series toward ZAP-70.

Variations at the pY+1 and pY+3 Positions. Keeping L-glutamine as the initial pY+1 amino acid, we continued to scan the pY+3 position with a small number of commercially available nitriles. As shown in Table 2, the *p*-Cl group, as in **4d**, was better than a *p*-Me group. When the Cl group was moved to the *meta* position, as in **4e**, a decrease in binding affinity toward ZAP-70 was observed. However, when a *m*,*p*-Cl_2 group was used, as in **4f**, a gain in ZAP-70 activity was observed. An *ortho* substitution, as in **4g** and **4h**, resulted in a substantial loss of ZAP-70 activity. The *meta*,*para* disubstitution pattern could be replaced with a 2-naphthlene group, as in **4j**. The 1-naphthalene substitution pattern (**4i**), however, was not as favorable. ZAP-70 activity appeared to be optimal with substituted benzyl groups at the pY+3 position. When the benzyl group was homologated, as in **4k**, no improvement in ZAP-70 activity was observed. As discussed previously in our Src SH2 work,[23] straight or branched alkyl chains at the pY+3 position were more favorable for Src activity.

With this small collection of favorable pY+3 groups, we began to scan the pY+1 position. From previous work with Src SH2 inhibitors,[20] we knew that a simple Me group at the pY+1 position (structure **3**, X = Me) could be quite effective. Thus, when L-alanine was replaced as the starting amino acid, the resulting 1,2,4-oxadiazole analogues showed roughly a 2-fold increase in binding affinity (Table 3, compounds **5a**-**5n**). The same SAR that was observed in analogues deriving from L-glutamine was observed here. Namely, a *para* substitution (whether Cl or CF_3) on a benzyl group at

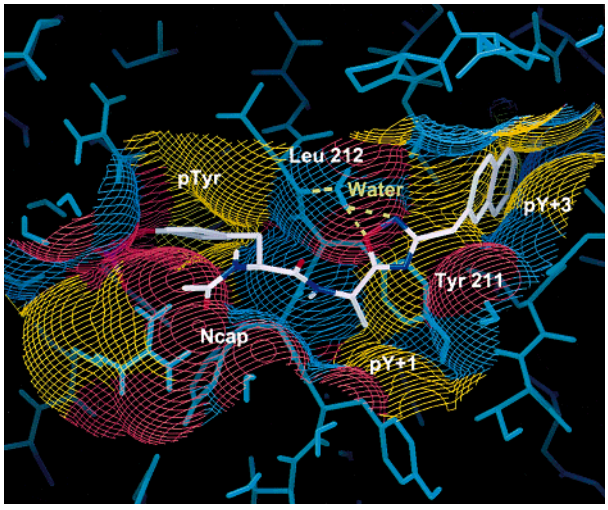


Figure 1. Compound **5e** when docked into the active site of the C-terminal SH2 domain of ZAP-70 using the QXP docking module of the FLO97 molecular modeling program.[29] The active site is represented in the form of a color-coded solvent-accessible surface. The blue, red and yellow areas refer to the hydrogen bond acceptor, hydrogen bond donor, and hydrophobic atoms of the proteins, respectively.

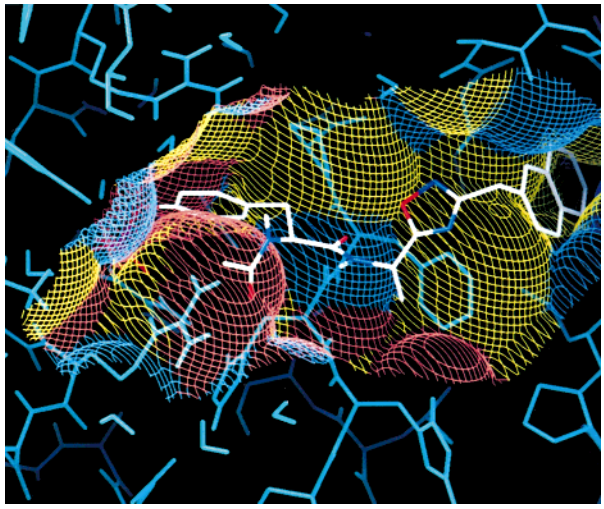


Figure 2. Compound **5e** when docked into the active site of the N-terminal SH2 domain of ZAP-70.

the pY+3 was better than the *meta* substitution (compounds **5b**, **5c, 5f**, **5g**). At the *para* substitution, the following order of activity was observed: I > Br = CF_3 > Cl > Me > F. Note that even though Br was comparable to CF_3 in ZAP-70 activity, the latter group showed better selectivity over Src. As observed previously with analogues derived from pY+1 Gln, the *m*,*p*-Cl_2 substitution was favorable, and the resulting compound **5d** showed comparable ZAP-70 activity to the 2-naphthalene derivative **5e**. The observed preference for a *meta*,*para* substitution pattern or the 2-naphthalene group at the pY+3 position can be rationalized by molecular modeling. Based on the ZAP-70 crystal structure, it can be hypothesized that the higher affinity of the 2-naphthyl and *meta*,*para* substituted benzyls is due not only to the many hydrophobic contacts but also to the displacement of a bound water molecule in the pY+3 pocket at the C-terminal SH2 domain of ZAP-70. Figures 1 and 2 show compound **5e** when it is docked into the active site at the C- and N-terminal SH2

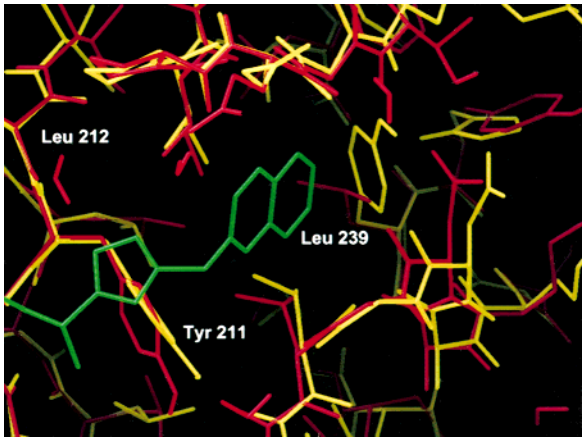


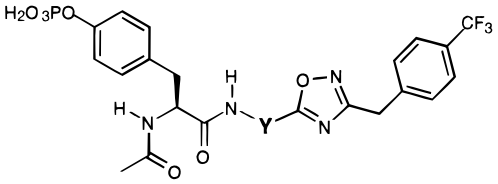
Figure 3. pY+3 pocket at the C-terminal SH2 domain of ZAP-70 and Syk. The protein residues for ZAP-70 are shown in red, and those for Syk are shown in yellow. Compound **5e** is shown partially in green.

domain of ZAP-70, respectively. At the C-terminal SH2 domain, it appears that the 1,2,4-oxadiazole ring could provide water-mediated hydrogen bonds to Leu 212 (Figure 1, water-mediated hydrogen bonds labeled in light green). Compounds **5k**−**5n** were made using the favorable *meta*,*para* substitution pattern along with the observed order of activity of the various halogen groups. Compounds **5k**−**5n** were all better than compound **5d**, the *m*,*p*-Cl_2-substituted analogue. However, the gain in binding affinity was not substantial. We therefore preferred the *m*,*p*-Cl_2 substitution pattern found in **5d** because of its lower molecular weight. In all of these examples, a remarkably high level of selectivity over Syk was maintained. The *m*,*p*-Cl_2, the *p*-CF_3, and the 2-naphthalene substitution patterns were the three most preferred ones for the pY+3 position. Virtual screening of a set of commercially available nitriles using the QXP program[29] identified the 2-naphthyl group as the highest scoring substituent for the pY+3 pocket of C-terminal ZAP-70, primarily due to the large number of hydrophobic contacts it makes with the hydrophobic residues defining the pY+3 pocket.

The high selectivity of this series for ZAP-70 over Syk is most likely due to differences in the pY+3 pocket. The ZAP-70 pY+3 pocket is larger than the Syk pY+3 pocket; ZAP-70 has Leu 239, while Syk has the larger Tyr 244 residue (Figure 3). Thus, the ZAP-70 pY+3 pocket can accommodate the naphthyl substituent, while the Syk pocket is too small for this substituent. Src and ZAP-70 both have a Leu residue in the pY+3 pocket, and therefore some Src activity is observed for the naphthyl and related derivatives.

We have prepared a number of different 1,2,4-oxadiazole analogues derived from pY+1 L-Abu, L-Leu, L-Asp, and L-Trp and have not observed an improvement in ZAP-70 activity over the series deriving from L-Ala (selected data are shown in Table 4, compounds **9**−**11**). However, significant loss of ZAP-70 activity was observed when a *gem*-dimethyl group was used at the pY+1, as in **6**. Conformational analysis of **6** using both molecular mechanics[30] and AM1 semiempirical studies suggests that the low-energy conformations of **6** will place the *pro*-*S*-methyl group in steric conflict with the phenyl group of Tyr 211 (Figure 1). Similar loss in activity was observed when glycine (**7**) was used as the

Table 4. Modifications at the pY+1 Position



no.	Y	IC_{50} (μM) ZAP-70	IC_{50} (μM) Syk	IC_{50} (μM) Src
6	-C(Me_2)-	228	>500	489
7	-CH_2-	128	>500	>500
8	-CH_2CH_2-	>500	>500	>500
9	-(*S*)-$CHCH_2CONH_2$	43	>500	>500
10	-(*S*)-$CHCH_2$-(3-indole)	32	>500	92
11	-(*S*)-$CHCH_2CH_3$	14	>500	98

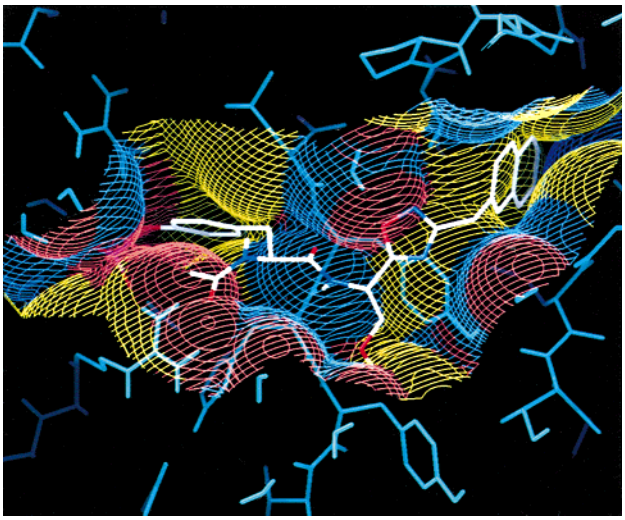


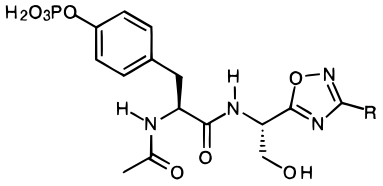
Figure 4. Compound **12c** when docked into the active site at the C-terminal SH2 domain of ZAP-70.

starting amino acid. With β-alanine as in **8**, complete loss of ZAP-70 activity was observed. This was perhaps due to the lack of conformational constraint as well as to the lack of contact with the hydrophobic surface at the pY+2 position.

Molecular modeling was used to probe for additional interactions at the pY+1 position. As shown in Figure 4, the oxadiazole derived from the noncharged serine residue forms complementary interactions with the pY+1 residues. Table 5 (compounds **12a**−**12g**) lists a number of 1,2,4-oxadiazole analogues that were made from L-serine. Consistent with the prediction from modeling, all of the pY+1 Serine-derived analogues were slightly more potent and some showed roughly a 2-fold increase over the corresponding analogues deriving from L-alanine. Again the same SAR trend was observed, and the order of ZAP-70 activity of compounds **12a**−**12g** was as expected. The remarkably high selectivity over Syk was also maintained throughout the series.

Molecular modeling also suggested that a longer hydrophobic side chain at the pY+1 could be used to place a hydrophobic group near Tyr 209 (Figure 5). Table 6 lists additional analogues that were made from L-homophenylalanine (compounds **13a** and **13b**). As

Table 5. SH2 Binding by 1,2,4-Oxadiazole Analogues Derived from L-Serine



no.	R	IC_{50} (μM) ZAP-70	IC_{50} (μM) Syk	IC_{50} (μM) Src
12a	CH_2-(*p*-Cl)Ph	11	>500	149
12b	CH_2-(*m,p*-Cl_2)Ph	4	>500	75
12c	CH_2-(2-naphthalene)	2	>500	99
12d	CH_2-(*p*-CF_3)Ph	4	>500	282
12e	CH_2-(*p*-F)Ph	31	>500	132
12f	CH_2-(*p*-Br)Ph	7	>500	32
12g	CH_2-(*p*-I)Ph	4	452	37

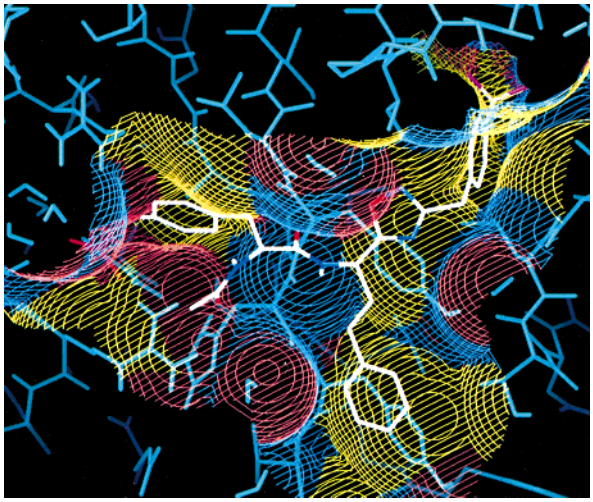
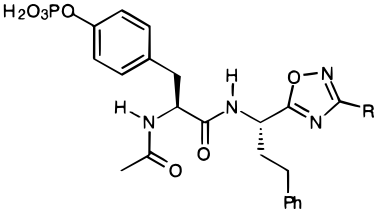


Figure 5. Compound **13a** when docked into the active site at the C-terminal SH2 domain of ZAP-70.

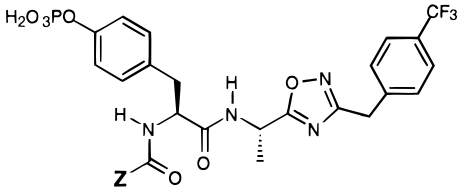
Table 6. SH2 Binding by 1,2,4-Oxadiazole Analogues Derived from L-Homophenylalanine



no.	R	IC_{50} (μM) ZAP-70	IC_{50} (μM) Syk	IC_{50} (μM) Src
13a	CH_2-(*p*-CF_3)Ph	5	323	95
13b	CH_2-(2-naphthalene)	3	288	78
13c	CH_2-(*m,p*-Cl_2)Ph	2	250	60

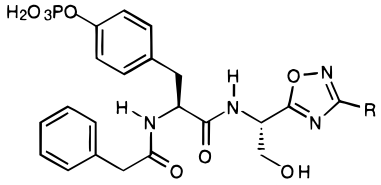
predicted from modeling, these analogues were more potent than the corresponding analogues deriving from either L-Gln or L-Ala. The smaller than expected improvement in activity may be due to the increased

Table 7. SH2 Binding by 1,2,4-Oxadiazole Analogues with Varying N-Caps



no.	Z	IC_{50} (μM) ZAP-70	IC_{50} (μM) Syk	IC_{50} (μM) Src
14a	CH_2-Ph	3	>500	123
14b	CH_2-(2-thiophene)	5	>500	143
14c	CH_2-(*m,p*-F_2)Ph	6	397	130
14d	CH_2-(*p*-OMe)Ph	6	>500	87
14e	CH_2-(2-naphthalene)	3	302	49
14f	CH_2-(3-indole)	5	433	62

Table 8. SH2 Binding by 1,2,4-Oxadiazole Analogues Derived from L-Serine and *N*-Phenylacetyl-L-tyrosine



no.	R	IC_{50} (μM) ZAP-70	IC_{50} (μM) Syk	IC_{50} (μM) Src
15a	CH_2-(*p*-CF_3)Ph	3	318	161
15b	CH_2-(*m,p*-Cl_2)Ph	2	>500	35
15c	CH_2-(2-naphthalene)	1	>500	68

hydrophobic interactions being offset by placing a large hydrophobic group in the mostly solvent-exposed pY+1 region. The analogues deriving from L-homo-Phe were comparable in activity to those derived from pY+1 L-Ser. Note that even though the analogues derived from L-homo-Phe were still selective over Syk, their selectivity was not as high as what had been observed with analogues deriving from L-Ala or L-Ser.

Modifications at the pTyr N-Terminal Moiety. Additional modeling suggested that an aromatic group at the *N*-acetyl portion of these 1,2,4-oxadiazole analogues could offer some beneficial contact with the Arg residue that forms cation−π interactions with the pTyr phenyl ring. A number of analogues having a constant pY+1 and pY+3 group and varying N-caps were prepared (Table 7, compounds **14a**−**14f**).[31] The *N*-phenylacetyl group (compound **14a**) offered roughly a 2-fold increase in binding affinity over the *N*-acetyl group (compound **5f**). Other electron-rich aromatic groups did not seem to increase the ZAP-70 activity significantly, although some did offer more improvement in Src activity (compounds **14d**−**14f**).

We then installed the phenylacetyl N-cap onto a small number of analogues deriving from L-serine (Table 8, compounds **15a**−**15c**). In most cases, a 2-fold increase in binding affinity was obtained. Compound **15c** was the most active one. It had an IC_{50} of 1 μM against ZAP-

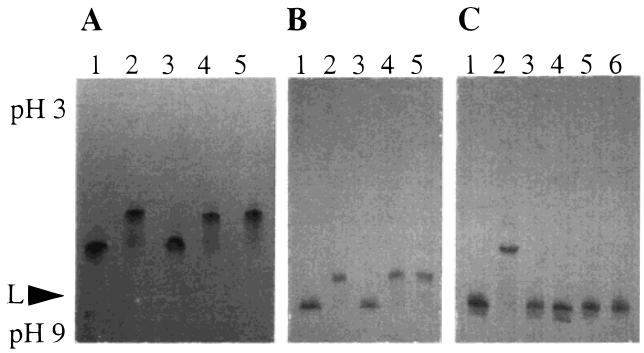


Figure 6. Gel shift assay showing binding of active compounds to the isolated C-terminal ZAP-70 SH2 domain. (A) Tandem ZAP-70 SH2: lane 1, protein alone; lanes 2−5, protein with compounds **14a**, **8**, **12d**, and **15c**, respectively. (B) Same as gel A but with the isolated C-terminal ZAP-70 SH2 domain. (C) Src SH2: lane 1, protein alone; lane 2, protein with Ac-pYEEI-NH_2 peptide; lanes 3−6, protein with compounds **14a**, **8**, **12d**, and **15c**, respectively. Technical notes: L indicates the load position where the 1-μL samples were deposited on the flatbed gel surface, while pH 3 and pH 9 indicate the final pH's at the two ends of the gel when the pH gradient is fully focused; focusing was terminated prematurely in this experiment to avoid dissociation of the complexes.

Table 9. Grb2 SH2 Binding by Selected 1,2,4-Oxadiazole Analogues

no.	IC_{50} (μM) Grb2	no.	IC_{50} (μM) Grb2	no.	IC_{50} (μM) Grb2
4f	441	**5f**	>500	**13b**	>500
4j	327	**12b**	>500	**13c**	340
4a	456	**12c**	479	**15a**	>500
5d	>500	**12d**	>500	**15b**	263
5e	>500	**13a**	>500	**15c**	368

70 and was highly selective over Syk and Src. Thus far, the SH2 binding affinities shown in Tables 2−8 are for ZAP-70, Syk, and Src. We also tested most of these analogues against Grb2. As shown in Table 9, this series of 1,2,4-oxadiazole analogues, in general, had an excellent selectivity over this particular SH2-containing protein. This is not unexpected as the known SH2 inhibitors of Grb2 adopt a β-turn binding conformation which is inaccessible for this series of 1,2,4-oxadiazoles. With respect to ZAP-70 SH2, compound **15c** was about 400-fold more potent than the native tetrapeptide sequences, and it was only about 20-fold less active than the doubly phosphorylated ζ-1-ITAM peptide.

Gel Shift Studies. As discussed above, molecular modeling suggests that these 1,2,4-oxadiazole analogues could engage either the C- or N-terminal SH2 domain of ZAP-70 (Figures 1 and 2). The published crystal structure[11] suggests that ligands that engage the C-terminal SH2 domain may be studied using the isolated C-terminal domain, whereas ligands engaging the N-terminal SH2 domain would require either the tandem SH2 domain protein or intact ZAP-70 for binding studies. We, therefore, purified the isolated C-terminal SH2 domain and developed a gel shift assay to evaluate binding of compounds to this domain (Figure 6). Protein was mixed with excess compound at concentrations (30−130 μM protein) where the protein should be fully complexed, given the IC_{50}'s of the active compounds in the ZAP-70 SH2 binding assay ($IC_{50} \leq 5$ μM). The complexes were loaded on isoelectric focusing gels and run for a short time to allow all species to migrate some distance toward the position of their isoelectric point on the gel. The theoretical pI's of the uncomplexed proteins used were tandem ZAP-70 SH2 domains 7.3, C-terminal SH2 domain 8.6, and Src SH2 domain 8.9. As the compounds are negatively charged, the complexes would be expected to migrate toward a more acidic position on the gel than the uncomplexed protein. By using only a very short run time (≤5 min), which was insufficient to achieve full isoelectric focusing, dissociation of the complexes was minimized and binding of compound was detected as a shift in the protein band on the gel. All three compounds tested that had IC_{50}'s ≤ 5 μM (compounds **12d**, **14a**, and **15c**) shifted the migration position of both the tandem ZAP-70 SH2 domain protein and the C-terminal SH2 domain, whereas a compound that had an $IC_{50} \geq 500$ μM (compound **8**) showed no shift (Figure 6A,B). None of the compounds gave a shift with Src SH2 (Figure 6C), consistent with their lack of binding to Src SH2 apparent from the fluorescence polarization assay. In contrast, the peptide Ac-pYEEI-NH_2 showed a strong gel shift with Src SH2 (Figure 6C), which is consistent with its high negative charge and known affinity for Src SH2 and indicates that the Src SH2 was fully active and capable of binding a suitable ligand. Therefore, the gel shifts seen with ZAP-70 SH2's reflect ZAP-70-specific binding. The data show that all three active compounds tested are able to bind to the C-terminal SH2 domain of ZAP-70 in the absence of the N-terminal domain. The gel shift assay cannot distinguish whether the compounds might be able to bind to both domains but clearly rules out exclusive binding to the N-terminal domain for the compounds tested.

Conclusion

In summary, we have described the modification of an initial Src SH2 lead into a series of potent and selective ZAP-70 SH2 inhibitors. These compounds represent a significant improvement in both potency and selectivity over known cognate tetrapeptide ligands. Some of the most potent analogues are approximately 1 order of magnitude less active than the native ζ-1-ITAM peptide. This series of 1,2,4-oxadiazole analogues is highly selective over a number of SH2-containing proteins such as Syk, Src, and Grb2. Preliminary gel shift studies have ruled out the possibility that these compounds are engaging only the N-terminal SH2 domain of ZAP-70. Our current understanding is that these compounds are engaging at the minimum the C-terminal SH2 domain of ZAP-70 or, perhaps, both SH2 domains. Future structural studies are expected to confirm the binding mode of these compounds. This series of 1,2,4-oxadiazole analogues provides compelling evidence that potent and selective ZAP-70 SH2 inhibitors can be designed from a monodentate template as opposed to the larger bidentate structure of the ζ-1-ITAM peptide. From a medicinal chemistry perspective, a monodentate mimetic with low molecular weight represents a significant step forward in the design of compounds with more drug-like properties. Currently,

these leads are providing the basis for our future designs of ZAP-70 inhibitors for cellular studies. For effective cellular activity, the phosphate group will have to be replaced with a cell-permeable and phosphatase-resistant functional group. Such studies will be disclosed in due course.

Experimental Section

General Information. All proton and phosphorus nuclear magnetic resonance spectra were determined in the indicated solvent using a Bruker 300 MHz machine with the appropriate internal standard. Low-resolution MS were performed on a Micromass Platform 2/single quadrupole/electrospray. Unless indicated otherwise, reagent-grade chemicals and solvents were purchased from either Aldrich, Lancaster, or Burdick & Jackson. All amino acids, and the appropriate coupling reagents, were purchased from either Bachem or Advanced ChemTech. Analytical HPLC analysis was carried out using a HP series1050, with either a C8 or C18 reverse-phase column from Vydac. Preparative HPLC was carried out using either a Hitachi LC4200 or Dynamax SD200, both equipped with UV/visible detector. Preparative HPLC columns were packed with either C8 or C18. HPLC-grade solvents (H_2O and CH_3CN) were buffered with 0.1% TFA.

All substituted benzyl cyanides were commercially available with the exception of 3,4-dibromobenzyl cyanide (used to prepare **5k**), 3-bromo-4-chlorobenzyl cyanide (used to prepare **5l**), 3-chloro-4-iodobenzyl cyanide (used to prepare **5m**), and 3-bromo-4-iodobenzyl cyanide (used to prepare **5n**).

3-Bromo-4-iodobenzyl Cyanide. Methyl 4-iodobenzoate (4.80 g, 18.2 mmol) was dissolved in 25 mL of concentrated sulfuric acid and NBS (3.25 g, 18.2 mmol) was added in a single portion at room temperature. The resulting reaction mixture was stirred at room temperature for 15 h. It was then poured carefully into crushed ice and extracted with EtOAc. The combined organic layers were washed with saturated aqueous $NaHCO_3$ and brine, dried (Na_2SO_4), and concentrated under reduced pressure to afford 6.20 g of methyl 3-bromo-4-iodobenzoate (99%). This was dissolved in 150 mL of THF and NaOH (880 mg, 1.2 equiv) was added as a solution in 50 mL of H_2O. The resulting reaction mixture was stirred at room temperature for 3 h. The volatile solvent was removed under reduced pressure. The aqueous layer was washed once with Et_2O, acidified to pH 2 with 3 N HCl, and extracted with EtOAc. The combined organic layers were washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure to yield 5.0 g of 3-bromoiodobenzoic acid (84%). This acid was dissolved in 100 mL of anhydrous THF and cooled to 0 °C. Borane in THF (18.4 mL of a 1.0 M solution in THF) was added at 0 °C and the resulting reaction mixture was stirred at that temperature for 10 min. It was then warmed to room temperature and stirred for 24 h. The reaction was cooled to 0 °C and quenched with dilute 10% aqueous citric acid. The mixture was extracted with EtOAc. The combined organic layers were washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. Purification by chromatography (1:1 hexanes/EtOAc) afforded 4.2 g of 3-bromo-4-iodobenzyl alcohol (88%). This alcohol (4.2 g, 13.4 mmol) was dissoved in 150 mL of CH_2Cl_2 along with triethylamine (1.14 mL, 1.1 equiv). The solution was cooled to 0 °C and methanesulfonyl chloride (2.43 mL, 1.3 equiv) was added. The reaction was stirred at 0 °C for 30 min and then warmed to room temperature and stirred for an additional 30 min. It was quenched with brine and extracted with EtOAc. The combined organic layers were washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure to give the mesylate derivative. This was immediately dissolved in 150 mL of methyl ethyl ketone and NaI (6.0 g, 3.0 equiv) was added. The reaction was stirred under reflux for 3 h. It was then cooled to room temperature, diluted with H_2O, and concentrated under reduced pressure. The resulting aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure to obtain 3-bromo-4-iodobenzyl iodide. This material was dissolved in 80 mL of DMF and KCN (2 equiv) was added. The resulting reaction mixture was stirred at 50 °C for 15 h. It was then cooled to room temperature, diluted with H_2O, and extracted with EtOAc. The combined organic layers were washed with H_2O and brine, dried (Na_2SO_4), and concentrated under reduced pressure. Purification by chromotography (9:1 hexanes/EtOAc) afforded 1.5 g of 3-bromoiodobenzyl cyanide as a light-yellow oil (35% from 3-bromo-4-iodobenzyl alcohol): ^{1}H NMR ($CDCl_3$) δ 3.75 (s, 2 H), 7.10 (d, 1 H, J = 8.0 Hz), 7.60 (s, 1 H), 7.90 (d, 1 H, J = 8.0 Hz).

3,4-Dibromobenzyl Cyanide. Methyl 4-bromobenzoate was subjected to the same reaction conditions detailed above in order to obtain methyl 3,4-dibromobenzoate. This ester (5.0 g, 17 mmol) was dissolved in 150 mL of anhydrous Et_2O and added to a suspension of $LiAlH_4$ (650 mg, 1 equiv) in 100 mL of Et_2O at 0 °C. The reaction mixture was warmed to room temperature and stirred for 30 min. It was then cooled to 0 °C and carefully quenched first with 0.5 mL of MeOH and then with 2 mL of H_2O. The resulting mixture was diluted with 3 N HCl and extracted with EtOAc. The combined organic layers were washed with dilute citric acid and brine, dried (Na_2SO_4), and concentrated under reduced pressure. Purification by chromatography (1:1 hexanes/EtOAc) afforded 4.2 g of 3,4-dibromobenzyl alcohol. This alcohol was subjected to the same reaction sequence outlined above in order to obtain 3,4-dibromobenzyl cyanide as a light-yellow oil: ^{1}H NMR ($CDCl_3$) δ 3.70 (s, 2 H), 7.15 (d, 1 H, J = 8.0 Hz), 7.60 (s, 1 H), 7.65 (d, 1 H, J = 8.0 Hz).

3-Bromo-4-chlorobenzyl Cyanide. Methyl 4-chlorobenzoate was subjected to the same reaction conditions outlined above in order to obtain 3-bromo-4-chlorobenzyl cyanide as a light-yellow oil: ^{1}H NMR ($CDCl_3$) δ 3.75 (s, 2 H), 7.20 (d, 1 H, J = 8.0 Hz), 7.50 (d, 1 H, J = 8.0 Hz), 7.65 (s, 1 H).

3-Chloro-4-iodobenzyl Cyanide. 3-Chloroiodotoluene (Transworld; 4.92 g, 19.5 mmol) was dissolved in 150 mL of CCl_4 and NBS (3.5 g, 1 equiv) was added. The reaction mixture was stirred under reflux while being irradiated with an intense visible lamp. After 6 h, the reaction mixture was cooled to room temperature and filtered. The filtrate was concentrated under reduced pressure to afford 6.4 g of 3-chloro-4-iodobenzyl bromide. This material was dissolved in 150 mL of methyl ethyl ketone and NaI (3 equiv) was added. The reaction mixture was stirred under reflux for 3 h. It was then cooled to room temperature, diluted with H_2O, and concentrated under reduced pressure. The aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. The resulting 3-chloro-4-iodobenzyl iodide was dissolved in 150 mL of DMF and KCN (2 equiv) was added. The reaction mixture was stirred at 50 °C for 15 h. It was then cooled to room temperature, diluted with H_2O, and extracted with EtOAc. The combined organic layers were washed with H_2O and brine, dried (Na_2SO_4), and concentrated under reduced pressure. Purification by chromatography (9:1 hexanes/EtOAc) afforded 1.2 g of 3-chloro-4-iodobenzyl cyanide as a light-yellow oil: ^{1}H NMR ($CDCl_3$) δ 3.80 (s, 2 H), 7.00 (d, 1 H, J = 8.0 Hz), 7.55 (s, 1 H), 7.95 (d, 1 H, J = 8.0 Hz).

General Procedure A for Preparing Amidoximes. In a typical procedure, an appropriately substituted benzyl cyanide (40−50 mmol) was dissolved in 80 mL of absolute EtOH. A solution containing hydroxylamine hydrochloride (1.2 equiv) and NaOH (1.2 equiv) in 20 mL of H_2O was added and the resulting reaction mixture was stirred under reflux for 2 days. The reaction mixture was then cooled to room temperature and concentrated under reduced pressure to remove the volatile solvent. The resulting residue was diluted with 50 mL of H_2O and extracted with EtOAc. The combined organic layers were washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure to afford the desired amidoxime. If necessary, these amidoximes could be purified by chromatography over silica gel using a mixture of ethyl acetate and hexanes. In most cases, the amidoximes obtained were of high purity and no purification was needed.

General Procedure B for Preparing Amine Derivatives 2 (BOC-amino acids already activated as the succinimide). In a typical procedure, 1 mmol of a given BOC-protected amino acid, suitably preactivated as the succinimide, was dissolved in 7 mL of ethylene glycol dimethyl ether along with 1 equiv of the desired amidoxime. The resulting reaction mixture was stirred at room temperature for 15 h. It was then concentrated under reduced pressure. The resulting residue was taken up in EtOAc, washed with dilute aqueous $NaHCO_3$ and brine, dried (Na_2SO_4), and concentrated under reduced pressure to give the intermediate product **1**. This material was dissolved in 4 mL of pyridine and heated under reflux for 30 min. The reaction mixture was cooled to room temperature, diluted with EtOAc, and washed repeatedly with 10% aqueous citric acid to remove all pyridine. The organic layer was then washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure to give the BOC-protected 1,2,4-oxadiazole analogue. If necessary, this intermediate could be purified by chromatography using a mixture of EtOAc and hexanes. Otherwise, this BOC-protected 1,2,4-oxadiazole analogue was dissolved in 6 mL of 25% TFA in CH_2Cl_2 and allowed to stand at room temperature for 3 h. It was then concentrated under a stream of N_2. The resulting residue was taken up in EtOAc and washed with saturated aqueous $NaHCO_3$. The organic layer was washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure to afford the amine derivative **2**. This material was used immediately for the amide coupling without further purification.

General Procedure C for Preparing Amine Derivatives 2 (unactivated BOC-amino acids). In a typical procedure, a given BOC-amino acid (1 mmol) was dissolved in 2 mL of dry DMF and 6 mL of CH_2Cl_2 along with 1.2 equiv of a desired amidoxime. HOBT (1.2 mmol) and EDC·HCl (1.2 mmol) were added, followed by 1.5 mmol of diisopropylethylamine (Hunig's base). The resulting reaction mixture was stirred at room temperature for 15 h. It was then concentrated under reduced pressure and redissolved in 50 mL of EtOAc. The organic layer was washed with dilute aqueous $NaHCO_3$ and brine, dried (Na_2SO_4), and concentrated under reduced pressure to give the intermediate product **1**. This material was dissolved in 4 mL of pyridine and heated under reflux for 30 min. The reaction mixture was cooled to room temperature, diluted with EtOAc, and washed repeatedly with 10% aqueous citric acid to remove all pyridine. The organic layer was then washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure to afford the BOC-protected 1,2,4-oxadiazole analogue. If necessary, this intermediate could be purified by chromatography using a mixture of EtOAc and hexanes. Otherwise, it could be treated with 25% TFA in CH_2Cl_2 in the same manner as detailed in procedure A to afford the amine derivative **2**. This amine was used immediately for the amide coupling without further purification.

General Procedure D for Preparing Phosphate Derivatives of the General Structure 3. In a typical procedure, Ac-Tyr(PO_3Bzl_2)-OH (0.3−0.5 mmol) was dissolved in 5 mL of CH_2Cl_2 and 2 mL of dry DMF along with 1.2 equiv of the desired amine derivative **2**. HOBT (1.2 equiv) and EDC·HCl (1.2 equiv) were added at room temperature. After 1.5 equiv of Hunig's base was added, the resulting reaction mixture was stirred at room temperature for 4 h. It was then concentrated under reduced pressure. The resulting residue was taken up in EtOAc and washed successively with dilute aqueous $NaHCO_3$, H_2O, 10% aqueous citric acid, and brine. The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The resulting residue was dissolved in 95% TFA and 5% H_2O and allowed to stand at room temperature for 3 h. It was then concentrated under a stream of N_2. The gummy residue was precipitated with Et_2O and then washed with Et_2O to give the crude phosphate derivative. This phosphate derivative, in turn, could be purified by reverse-phase HPLC using a mixture of H_2O and CH_3CN that had been buffered with 0.1% TFA.

1,2,4-Oxadiazole Analogues Derived from L-Glutamine (Table 2, compounds 4a−4j). All analogues listed in Table 2 were prepared according to the preparation of compound **4j** using the appropriate amidoxime. **Preparation of compound 4j:** The desired amidoxime was prepared according to the above general procedure A using commercially available 2-(cyanomethyl)naphthalene. This amidoxime was then reacted with BOC-Gln-OSu according to the general procedure B in order to obtain the 1,2,4-oxadiazole amine. This amine derivative was coupled with Ac-Tyr(OPO_3Bzl_2)-OH according to the general procedure D in order to obtain the final product **4j**. A portion of the product was purified by preparative HPLC: ^{1}H NMR (DMSO) δ 1.65 (s, 3 H), 2.40−2.90 (m, 2 H), 4.20 (s, 2 H), 4.30−4.40 (m, 1 H), 4.90−5.0 (m, 1 H), 6.70 (br s, 1 H), 6.90 (d, 2 H, J = 8.1 Hz), 7.10 (d, 2 H, J = 8.1 Hz), 7.20 (br s, 1 H), 7.40−7.80 (m, 7 H), 8.0 (d, 1 H, NH, J = 8.3 Hz), 8.60 (d, 1 H, NH, J = 7.6 Hz); ^{31}P NMR (DMSO) δ −0.95; MS 594 (M − H); high-resolution MS calcd for $C_{28}H_{30}N_5O_8P$ 596.1912 (M + H), found 596.1915; HPLC (analytical, Kromasil C8, 10 min run) retention time = 3.51 min (gradient elution: 0 min, 10% aq CH_3CN; 0.5 min, 10% aq CH_3CN; 8 min, 90% aq CH_3CN; 9 min, 90% aq CH_3CN; 9.1 min, 10% aq CH_3CN).

1,2,4-Oxadiazole Analogues Derived from L-Alanine (Table 3, compounds 5a−5n). All analogues listed in Table 3 were prepared according to the preparation of compound **5f** using the appropriate amidoxime. **Preparation of compound 5f:** The desired amidoxime was prepared according to the above general procedure A using commercially available 4-(trifluoromethyl)benzonitrile. This amidoxime was then reacted with BOC-Ala-OSu according to the general procedure B in order to obtain the 1,2,4-oxadiazole amine. This amine derivative was coupled with Ac-Tyr(OPO_3Bzl_2)-OH according to the general procedure D in order to obtain the final product **5f**. A portion of the product was purified by preparative HPLC: ^{1}H NMR (DMSO) δ 1.70 (d, 3 H, J = 8.0 Hz), 1.90 (s, 3 H), 2.60−3.20 (m, 2 H), 4.20 (s, 2H), 4.50−4.60 (m, 1 H), 5.20−5.30 (m, 1 H), 7.25 (d, 2H, J = 8.4 Hz), 7.30 (d, 2 H, J = 8.4 Hz), 7.65 (d, 2 H, J = 8.1 Hz), 7.80 (d, 2 H, J = 8.1 Hz), 8.1 (d, 1 H, NH, J = 8.2 Hz), 8.9 (d, 1 H, NH, J = 7.5 Hz); ^{31}P NMR (DMSO) δ −0.95; MS 555 (M − H); High-resolution MS calcd for $C_{23}H_{24}F_3N_4O_7P$ 557.1413 (M + H), found 557.1428; HPLC (analytical, Kromasil C8, 15 min run) retention time = 7.74 min (gradient elution: 0 min, 10% aq CH_3CN; 2 min, 10% aq CH_3CN; 11 min, 80% aq CH_3CN; 13 min, 80% aq CH_3CN; 13.1 min, 10% aq CH_3CN; 15 min, 10% aq CH_3CN).

1,2,4-Oxadiazole Analogues Listed in Tables 4 and 5. All analogues listed in these tables were prepared according to the general procedure outlined below for compound **12b**, substituting the appropriate BOC-protected amino acid and amidoxime. **Preparation of 12b:** The desired amidoxime was prepared according to the general procedure A using 3,4-dichlorobenzyl cyanide. This amidoxime was then reacted with BOC-Ser-OH according to the general procedure C in order to obtain the 1,2,4-oxadiazole amine. This amine derivative was coupled with Ac-Tyr(OPO_3Bzl_2)-OH according to the general procedure D in order to obtain the final product **12b**. A portion of the product was purified by preparative HPLC: ^{1}H NMR (DMSO) δ 1.80 (s, 3 H), 2.30−2.90 (m, 2 H), 3.60−3.70 (m, 2 H), 3.90 (s, 2 H), 4.30−4.40 (m, 1 H), 4.90−5.00 (m, 1 H), 6.80 (d, 2H, J = 8.2 Hz), 7.10 (d, 2 H, J = 8.0 Hz), 7.30 (d, 1 H, J = 8.0 Hz), 7.50 (d, 1 H, J = 8.0 Hz), 7.55 (s, 1 H); ^{31}P NMR (DMSO) δ −0.95; MS 572 (M − H); High-resolution MS calcd for $C_{22}H_{23}Cl_2N_4O_8P$ 573.0709, found 573.0726; HPLC (analytical, Kromasil C8, 10 min run) retention time = 4.08 min (gradient elution: 0 min, 10% aq CH_3CN; 0.5 min, 10% aq CH_3CN; 8 min, 90% aq CH_3CN; 9 min, 90% aq CH_3CN; 9.1 min, 10% aq CH_3CN).

1,2,4-Oxadiazole Analogues Derived from L-homophenylalanine (Table 6, compounds 13a−13c). All analogues listed in Table 6 were prepared according to the preparation of compound **13c** using the appropriate amidoxime. **Preparation of 13c:** The desired amidoxime was prepared according to the general procedure A using 3,4-dichlorobenzyl cyanide. This amidoxime was then reacted with BOC-homo-Phe-OH according to the general procedure C in

order to obtain the 1,2,4-oxadiazole amine. This amine derivative was coupled with Ac-Tyr(OPO_3Bzl_2)-OH according to the general procedure D in order to obtain the final product **13c**. A portion of the product was purified by preparative HPLC: ^{1}H NMR (DMSO) δ 1.70 (s, 3 H), 1.90−2.05 (m, 2 H), 2.40−2.90 (m, 6 H), 4.00 (s, 2 H), 4.30−4.40 (m, 1 H), 4.85−4.95 (m, 1 H), 6.80−7.60 (m, 9 H), 8.00 (d, 1 H, NH, J = 8.5 Hz), 8.74 (d, 1 H, NH, J = 7.7 Hz); ^{31}P NMR (DMSO) δ −0.95; MS 645 (M − H); high-resolution MS calcd for $C_{29}H_{29}Cl_2N_4O_7P$ 647.1232 (M + H), found 647.1245; HPLC (analytical, Kromasil C8, 10 min run) retention time = 5.29 min (gradient elution: 0 min, 10% aq CH_3CN; 0.5 min, 10% aq CH_3CN; 8 min, 90% aq CH_3CN; 9 min, 90% aq CH_3CN; 9.1 min, 10% aq CH_3CN).

1,2,4-Oxadiazole Analogues Listed in Table 7 (compounds 14a−14f). All analogues listed in this table were prepared according to the preparation of compound **14a** using the appropriate acid chloride. **Preparation of 14a:** The desired 1,2,4-oxadiazole amine was prepared according to the general procedure B using BOC-Ala-OH and the amidoxime deriving from 4-(trifluoromethyl)benzonitrile (general procedure A). In a typical procedure, BOC-Tyr(PO_3Bzl_2)-OH (0.3−0.5 mmol) was dissolved in 5 mL of CH_2Cl_2 and 2 mL of dry DMF along with 1.2 equiv of the desired amine derivative **2**. HOBT (1.2 equiv) and EDC·HCl (1.2 equiv) were added at room temperature. After 1.5 equiv of Hunig's base was added, the resulting reaction mixture was stirred at room temperature for 4 h. It was then concentrated under reduced pressure. The resulting residue was taken up in EtOAc and washed successively with dilute aqueous $NaHCO_3$, H_2O, 10% aqueous citric acid, and brine. The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The resulting residue was dissolved in 95% TFA and 5% H_2O and allowed to stand at room temperature for 3 h. It was then concentrated under a stream of N_2. The gummy residue was precipitated with Et_2O and then washed with Et_2O to give the crude amino phosphate derivative. This amine derivative (0.4 mmol) was then dissolved in 3 mL of DMF. The resulting solution was cooled to 0 °C along with 4 equiv of Hunig's base. Phenylacetyl chloride (1.2 equiv) was then added at 0 °C. The reaction mixture was stirred at 0 °C for 15 min and then warmed to room temperature. After 45 min, the reaction was quenched with 100 μL of 6 N HCl. A portion of this solution was directly purified by reverse-phase HPLC in order to obtain **14a**: ^{1}H NMR (DMSO) δ 1.70 (d, 3 H, J = 8.0 Hz), 2.30−2.70 (m, 2 H), 3.20−3.45 (m, 2 H), 4.10 (s, 2 H), 4.35−4.45 (m, 1 H), 4.90−5.05 (m, 1 H), 6.80−7.80 (m, 13 H), 8.1 (d, 1 H, NH, J = 8.2 Hz), 8.9 (d, 1 H, NH, J = 7.5 Hz); ^{31}P NMR (DMSO) δ −0.95; MS 633 (M + H); High-resolution MS calcd for $C_{29}H_{28}F_3N_4O_7P$ 633.1727 (M + H), found 633.1720; HPLC (analytical, Kromasil C8, 15 min run) retention time = 9.16 min (gradient elution: 0 min, 10% aq CH_3CN; 2 min, 10% aq CH_3CN; 11 min, 80% aq CH_3CN; 13 min, 80% aq CH_3CN; 13.1 min, 10% aq CH_3CN; 15 min, 10% aq CH_3CN).

1,2,4-Oxadiazole Analogues listed in Table 8 (compounds 15a−15c). All compounds listed in Table 8 were prepared according to the general procedure detailed above for the synthesis of compounds **14a**−**14f** using BOC-Ser-OH and the appropriate amidoxime.

Molecular Modeling. A model of the C- and N-terminal SH2 domain of ZAP-70 has been developed based on a high-resolution (1.9 Å) crystal structure of the tandem SH2 domain of ZAP-70 complexed to the doubly phosphorylated ζ-1-ITAM peptide.[11]

To determine if a molecule is likely to bind to either the C- or N-terminal SH2 domain of ZAP-70, the molecule is docked into this binding site model using the QXP docking module of the new FLO97 molecular modeling program.[29] This program uses extensive conformational searching and energy minimization to identify the conformation a molecule is likely to adopt upon binding to SH2. During the docking procedure, most binding site atoms are held fixed. However, those residues known to change conformation upon binding with different ligands are allowed to move freely during the energy minimization step. Other binding site atoms displaying smaller changes in position are constrained with a force constant.

Docked molecules are evaluated using the following criteria: steric fit, hydrophobic and hydrogen-bonding interactions, low molecular mechanics energy, and low internal ligand energy. Replacements of (or hydrogen bonds formed with) crystallographically determined water molecules are also taken into account.

Protein Expression and Purification. ZAP-70 tandem SH2: The vector pGEX2KT, identical to pGEX-KT[32] but derived from pGEX-2T instead of pGEX-1, was used to express ZAP-70 SH2's. *E. coli* BL21 cells transformed with pGEX2KT-huZAP(1−259) were grown in BHI medium with 200 mg/L ampicillin and induced with 1 mM IPTG for 5.5 h at 25 °C. Frozen cell paste (40 g) was resuspended in 2 volumes of 10 mM potassium phosphate buffer (pH 7.6) containing 640 mM NaCl, 5 mM DTT (buffer A), and 1 mM Pefabloc (Boehringer-Mannheim) then disrupted using a French pressure cell at 16 000 psi(Aminco). The resulting lysate was diluted 1:1 with buffer A, clarified by centrifugation (35 min at 18 000 g), and passed over a 50-mL glutathione-agarose column (Sigma) equilibrated in buffer A. After washing with buffer A, the GST-fusion protein was eluted with 20 mM Tris (pH 7.6) containing 400 mM NaCl, 20 mM reduced glutathione, and 2 mM DTT. The GST-fusion was digested at 4 °C overnight with human thrombin (Enzyme Research Laboratories, Cat #HT 660) at a ratio of 0.5 μg of thrombin/mg of GST-fusion. When digestion was complete as judged by SDS−PAGE, the pool was diluted 3-fold with 20 mM Tris and 5 mM DTT (pH 7.8) and loaded onto a 40-mL *O*-phospho-L-tyrosine-agarose column (Sigma) equilibrated in 20 mM Tris (pH 7.8) containing 100 mM NaCl and 5 mM DTT. The column was eluted with 4 column volumes (CV) of a gradient of NaCl up to 2 M in the same buffer. The ZAP-70 tandem SH2 fragment eluted at approximately 400 mM NaCl. This eluate was dialyzed into 20 mM Tris with 5 mM DTT (pH 8.0) and loaded on a 10 mL Q Sepharose FF (Sigma) column equilibrated in the same buffer. The column was eluted with 4 CV of a gradient of NaCl up to 1 M in the same buffer. The purified protein (69 mg) was stored at −80 °C in 20 mM Tris buffer (pH 8.0) containing 500 mM NaCl, 5 mM DTT, 0.02% NaN_3, and 10% glycerol.

ZAP-70 C-terminal SH2: *E. coli* BL21 cells transformed with pGEX2KT-huZAP(158−259) were grown as described above except that the medium was 2xYT and the induction temperature 30 °C. The purification through the thrombin digestion was also as described above. The completed thrombin digest was diluted 4-fold with 10 mM Tris pH 7.4 containing 5 mM DTT and loaded on a *O*-phospho-L-tyrosine-agarose column (17 mL) equilibrated in 20 mM Tris (pH 7.4) containing 50 mM NaCl and 5 mM DTT and eluted with NaCl as described above. The eluate was further purified on a Superdex 75 gel filtration column (1.6 × 60 cm; Pharmacia) run at 1 mL/min in in 20 mM Tris buffer (pH 7.4) containing 500 mM NaCl, 5 mM DTT, 5 mM EDTA, and 0.02% NaN_3. The final pool (66 mg from 50 g of frozen cell paste) was stored at −80 °C.

Syk tandem SH2: *E. coli* BL21(DE3) cells transformed with pET-huSyk(5−265) were grown as described for the ZAP-70 tandem SH2. Frozen cell paste (33 g) was resuspended in 50 mM potassium phosphate buffer (pH 7.4) containing 100 mM NaCl, 5 mM DTT 1 mM EDTA, and 1 mM PMSF, and lysed by French pressure cell. The lysate was diluted 1:1 with H_2O and clarified by centrifugation. The supernatant was loaded onto a 40-mL carboxy-sulfon column (J. T. Baker) equilibrated in 10 mM potassium phosphate buffer (pH 6.0) containing 5 mM DTT. The column was washed with the same buffer and eluted with a step gradient of 100 mM potassium phosphate buffer (pH 7.4) containing 5 mM DTT. The protein pool was diluted 5-fold with H_2O to bring the phosphate concentration down to 20 mM, then loaded on a 40-mL *O*-phospho-L-tyrosine-agarose column equilibrated in 10 mM potassium phosphate buffer (pH 7.4) containing 5 mM DTT, and eluted with a 4 CV gradient up to 2 M NaCl in the same

buffer. The purified protein (530 mg) was dialyzed against 20 mM Tris (pH 7.4) containing 100 mM NaCl, 5 mM DTT, and 10% glycerol and stored at −80 °C.

Grb2 SH2: *E. coli* BL21(DE3) cells transformed with pT7-huGrb2(55−152) were grown as described for the ZAP-70 tandem SH2 except that the induction temperature was 30 °C. Frozen cell paste (44 g) was resuspended in 20 mM Tris (pH 7.7) containing 100 mM NaCl, 5 mM DTT, and 1 mM PMSF and lysed by French Pressure cell. The lysate was diluted with an equal amount of 20 mM Tris (pH 7.7) and 5 mM DTT and clarified by centrifugation. The supernatant was loaded on a 40-mL *O*-phospho-L-tyrosine-agarose column equilibrated in 20 mM Tris (pH 7.7) containing 100 mM NaCl and 5 mM DTT and eluted with a 5 CV gradient of up to 2 M NaCl in the same buffer. The protein (144 mg) was dialyzed against 50 mM potassium phosphate (pH 7.4) containing 250 mM NaCl, 5 mM DTT, 10% glycerol, and 0.02% NaN_3 and stored at −80 °C.

Src SH2: huSrc(145−251) was expressed and purified as described previously,[33] except that it should be noted that the bacterial lysate supernatant was diluted approximately 3-fold to reduce the NaCl concentration to <100 mM, prior to loading on the carboxy-sulfon column.

Gel shift assays: ZAP-70 tandem SH2, ZAP-70 C-terminal SH2, and Src SH2 stock solutions (1−2 mg/mL) were mixed with 2 mM working dilutions of compounds in 20 mM HEPES (pH 7.4) (prepared from a 50 mM stock in DMSO) at a 2−10-fold molar ratio of compounds to protein. The peptides Ac-pYEEI-NH_2 and Ac−NQL-pYNELNLGRREE-pYDVLD-NH_2 were used as controls. The mixtures were incubated at 4 °C for 30 min, loaded on pH 3−9 isoelectric focusing gels (Phastgel IEF, Pharmacia), and subjected to isoelectric focusing using a PhastSystem (Pharmacia). The run was stopped after 150 Vh as longer separations resulted in dissociation of the complexes. The gels were fixed and stained for protein with Coomassie Blue as per the manufacturer's instructions.

In vitro binding assays: Fluorescence polarization competitive binding assays were used to measure the IC_{50}'s of compounds binding to the different SH2 domains (complete experimental details and analysis of the ZAP-70 and Syk assays[13] and the Src and Grb2 assays[16] are published separately). Briefly, compounds were assayed as 1:2 dilutions over a range of concentrations from 1000 to 0.04 μM. Results are shown as mean IC_{50} values based on three or more separate experiments. Binding assays were carried out on a Jolly FPM2 96-well plate reader with standard cutoff filters (excitation λ = 485 nm, BP = 22; emission λ = 530 nm, BP = 30). Compounds were serially diluted in buffer solution (100 mM NaCl, 20 mM phosphate, pH 7.4, 10 mM dithiothreitol, 4% DMSO, 1 mM EDTA, and 0.1% bovine γ-globulin) and then added to 25 nM tandem ZAP SH2 domain protein, 25 nM Syk SH2 domain protein, 150 nM Src SH2 domain protein, or 1.25 μM Grb2 SH2 domain protein premixed with 20 nM of the corresponding fluorescein-conjugated peptide. Binding reactions were carried out for 5 min at room temperature. The fluorescein conjugated peptide used to monitor Src SH2 binding was fluoro-pYpYpYIE-NH_2, the probe used to monitor ZAP tandem SH2 binding was fluoro-GpYNELNLGRREEpY-DVL-NH_2, the probe used to monitor Syk binding was fluoro-ApYTGLSTRNQETpYETL-NH_2, and the probe used to monitor Grb2 binding was fluoro-GpYVNV-NH_2. IC_{50}'s were calculated based on the percent binding of the fluorescein-conjugated peptide to the protein with compound addition relative to vehicle-alone control samples. IC_{50}'s measured by the fluorescence polarization method underestimate the actual K_d's of the protein−compound interaction.[13,16] It is possible to calculate K_d's from the measured IC_{50}'s (dependent on the measurements matching certain experimental constraints).[34] From the measured IC_{50}'s reported herein, K_d's can be estimated by dividing by a factor of approximately 20 (e.g. an IC_{50} of 20 μM would represent a K_d of approximately 1 μM). By this calculation, the best ZAP-70 SH2 inhibitors reported herein have K_d's, conservatively estimated, of less than 100 nM.

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