Design and Synthesis of Non-peptidic Inhibitors for the Syk C-Terminal SH2 Domain Based on Structure-Based In-Silico Screening

Tatsuya Niimi,* Masaya Orita, Miwa Okazawa-Igarashi, Hitoshi Sakashita, Kazumi Kikuchi, Evelyn Ball, Atsushi Ichikawa, Yoko Yamagiwa, Shuichi Sakamoto, Akihiro Tanaka, Shinichi Tsukamoto, and Shigeo Fujita

Yamanouchi Pharmaceutical Co. Ltd., 21 Miyukigaoka, Tsukuba-Shi 305-8585, Japan

Kuniaki Tatsuta,* Yasuhide Maeda, and Ken Chikauchi

Department of Applied Chemistry, School of Science and Engineering, Waseda University, 3-4-1 Ohkubo, Shinjuku-ku, Tokyo 169-8555, Japan

Received July 6, 2001

Structure-based in-silico screening was carried out for the Syk C-terminal SH2 domain. Fragments that could interact with the pY or pY+1 pockets were selected by our in-silico screening. After tethering two fragments bound to these pockets, we have designed and synthesized new compounds that show favorable interaction with the pY+3 pocket. One such compound, having a cyclohexylmalonic acid moiety identified as a novel potent phosphotyrosyl mimetic, exhibited an affinity comparable to that of the monophosphorylated ligand peptide.

Introduction

Syk belongs to a family of hematopoietic cell-specific protein tyrosine kinases that play a critical role in mediating cellular responses activated by the interaction between antigens and antibody receptors;[1] it has therefore emerged as a potentially useful therapeutic target for immune suppression. Syk has two adjacent Src homology 2 (SH2) domains (termed SykNSH2 and SykCSH2). The tandem SH2 domains of Syk bind to protein sequences termed immunoreceptor tyrosine-based activation motifs (ITAM) in which tyrosine residues become phosphorylated upon receptor activation.[2] This SH2-mediated association of Syk with an activated receptor results in the recruitment of Syk into the immunoreceptor subunit and the stimulation of Syk kinase activity. The tertiary structure of the tandem SH2 domains associated with the doubly phosphorylated ITAM has recently been resolved, revealing the flexibility and structural independence between the two SH2 domains.[3] Indeed, the two SH2 domains have not been shown to be equivalent.[4,5] These results suggest that each SH2 domain can play a distinct role and can function as an independent unit. Therefore, either or both of the Syk SH2 domains have the potential to be a key therapeutic target.

Unlike the various known single SH2 domain-containing proteins such as Grb2 and Src, the individual SH2 domains of Syk and of Zap-70, which is closely related to Syk and also contains tandem SH2 domains, have relatively weak affinity (10^{-5} M order) and low selectivity for monophosphorylated peptide.[6] However, the simultaneous interaction of the two SH2 domains with bisphosphorylated peptide confers a high biological affinity (10^{-8} M order) and selectivity.[6] Despite the fact that many effective non-peptidic inhibitors have now been reported for the Grb2[7−9] and Src[10] SH2 domains, there have been only a few reports on the design of Syk and Zap-70 SH2 domain inhibitors. Recently, potent and selective Zap-70 SH2 domain inhibitors have been designed; however, these contain a hydrolyzable phosphate moiety.[11]

Although the phosphotyrosyl residue (pTyr) is a key affinity element for the SH2 domain, its hydrolytic liability and low membrane permeability limit its utilization in inhibitor design. The most potent analogues have employed the phosphonates of phenylalanine.[12] However, achieving efficacy with these compounds in cell-based assays has generally met with only partial success. Alternative compounds based on non-phosphorus pTyr mimetics utilize carboxyl groups to introduce the anionic oxygen of the regular phosphate. Biscarboxyl analogues have generally exhibited reduced binding potency relative to the natural pTyr-containing ligands.[8,13] Recently, however, the biscarboxyl analogue *p*-malonylphenylalanine has been reported as being approximately equivalent to the phosphonate-based pTyr mimetics in binding with the Grb2 SH2 domain.[9]

In the present study, we describe the design of non-peptidic SykCSH2 inhibitors using structure-based in-silico screening as well as docking studies on the basis of our tertiary structure determined by NMR spectroscopy. The resulting compound exhibited an activity comparable with that of a monophosphorylated hexapeptide. As can be seen from the chemical structure of the most active compound reported here, linking fragments with optimal relative orientation and distance is generally problematic from the viewpoint of chemical synthesis. However, molecular modeling based on the tertiary structure of the SykCSH2 domain described here offsets these problems, thereby facilitating identification of potent lead compounds.

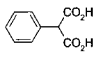
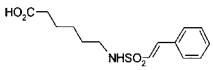
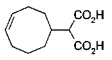
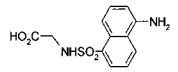
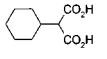
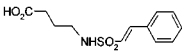
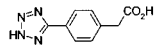
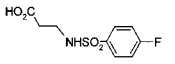
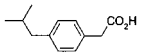
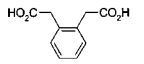
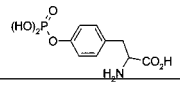
Results and Discussion

Structure Determination for SykCSH2. Resonance assignments and structure determination for SykCSH2 were successfully carried out with a series of triple-resonance experiments. Our NMR studies indicated that SykCSH2 consists of a central antiparallel

* To whom correspondence should be addressed. For T.N.: (phone) 81-298-52-5111; (fax) 81-298-52-5387; (e-mail) niimi@yamanouchi.co.jp. For K.T.: (phone and fax) 81-3-3200-3203; (e-mail) tatsuta@mn.waseda.ac.jp.

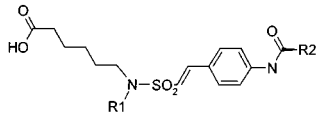
10.1021/jm010313k CCC: $20.00
Published on Web 11/14/2001

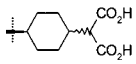
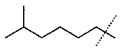
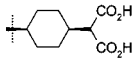
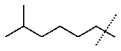
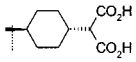
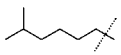
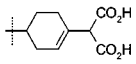
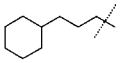
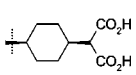
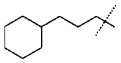
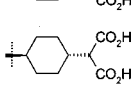
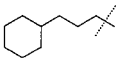
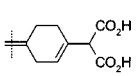
Table 1. Inhibition Activity of Compounds Selected by In-Silico Screening for pY and pY+1 Pockets

compounds		IC50 (mM)	compounds		IC50 (mM)
1		12	7		8.0 (70 %)[b]
2		7.7	8		(39 %)[b]
3		5.9	9		(2.3 %)[b]
4		n.d.[a]	10		(0.9 %)[b]
5		n.d.[a]			
6		n.d.[a]			
pY		14			

[a] n.d.: not detected. [b] Values in parentheses indicate the inhibition ratio at 10 mM compound concentration.

Table 2. Inhibition Activity of Linked Compounds[a]



compounds	R1	R2	IC50 (μM)
11	H		350
12			63
13			69
14			71
15			40
16			38
17			41
pY	VpYTGL		17

[a] All compounds listed above are synthesized as the trisodium salts.

β-sheet (βA−βG) and two α-helices (αA and αB) on each side of the central core region and its overall protein conformation is similar to the previously reported structure of SykCSH2 complexed with peptide ligand.[3,14] The pY pocket, which recognizes the pTyr residue in the peptide ligand, is a positively charged region formed by αA, βB, the BC loop, βC, and βD, in particular, Argα A2, Argβ B5, Argβ B7, Alaβ C3, Hisβ D4, Tyrβ D5, and Argβ D6. The pY+1 pocket is a shallow depression formed by Lysβ D1, Leuβ D3, and Tyrβ D5. The pY+3 pocket is a hydrophobic groove formed by Ileβ E4, Tyrβ D5, GlyEF3, GlyBG3, and LeuBG4, which lies between βD, βE, and the BG loop. We tried to design new SykCSH2 inhibitors by in-silico screening based on our protein structure as described below.

In-Silico Screening and Competitive Binding Assay. We have recently reported the discovery of novel inhibitors of macrophage migration inhibitory factor using in-silico screening.[15] To find inhibitors of the SykCSH2 domain, a similar in-silico screening approach using the three-dimensional structure of the SykCSH2 domain was applied to our proprietary chemical inventory. In a preliminary trial using the program DOCK4.0.1,[16] interesting compounds could not be found because they all partially occupied the three major SH2 domain pockets pY, pY+1, and pY+3. We therefore tried a different approach in which we (1) carried out separate in-silico screenings for each of the three binding sites to identify low molecular weight fragments, (2) selected fragments suitable for interaction with key residues in the pocket by visual inspection, and (3) designed molecules by linking fragments using both molecular modeling and the structure information obtained from NMR studies. For each binding pocket of SykCSH2, several hundred compounds were selected that showed high force field scores in three types of scoring functions of DOCK4.0.1: DOCK energy scoring, DOCK chemical scoring, and DOCK contact scoring. During the selection of compounds, unfavorable fragments, such as reagents and chemically unstable compounds, were removed using an in-house program.

Key protein residues in the pY and pY+1 pockets are Arg residues (Argα A2, Argβ B5, Argβ B7, and Argβ D6) binding to the pTyr and a Lys residue (Lysβ D1) binding to the glutamic acid residue, respectively.[3,14] Some compounds and peptide inhibitors known as Src SH2 inhibitors contain a carboxyl group, and it has been reported that this carboxyl group locates and specifically interacts with the pY or pY+1 pocket.[13,17] Because it is highly possible that the key residues in these pockets interact with the carboxyl group, we have focused our study on those molecules containing a carboxyl group available to the pY and pY+1 pockets.

Competitive binding assays using surface plasmon resonance to evaluate the ability to bind to SykCSH2 were carried out mainly on those compounds bearing a carboxylic group as determined by visible inspection. Compounds **1**−**3** contained a malonic acid moiety, and compound **1** has previously been reported to function as a pTyr mimetic, inhibiting the Grb2 SH2 domain.[9] These compounds all had a more potent binding affinity than phosphotyrosine itself (IC_{50} = 14 mM, Table 1). In particular, compound **3** was the most active compound among compounds **1**−**6** and would be a potent pTyr mimetic candidate. Compounds **7**−**10**, selected as candidates to bind to the pY+1 pocket of SykCSH2, contained a carboxylic group that was predicted to interact with Lysβ D1, mimicking the anionic oxygen of the hydrophilic residue at the pY+1 position in the docking study (Table 1). Compounds **7** and **8** exhibited weak binding affinity to SykCSH2. Compounds **9** and **10**, which have chemical structures similar to those of **7** and **8**, respectively, also showed very weak binding affinity (less than 5% inhibition at 10 mM). Compounds selected in the silico screening for the pY+3 pocket showed very weak affinities. This may be due to the fact that the pY+3 pocket consisted of hydrophobic residues, thereby lacking key residues for characteristic interaction with inhibitors.

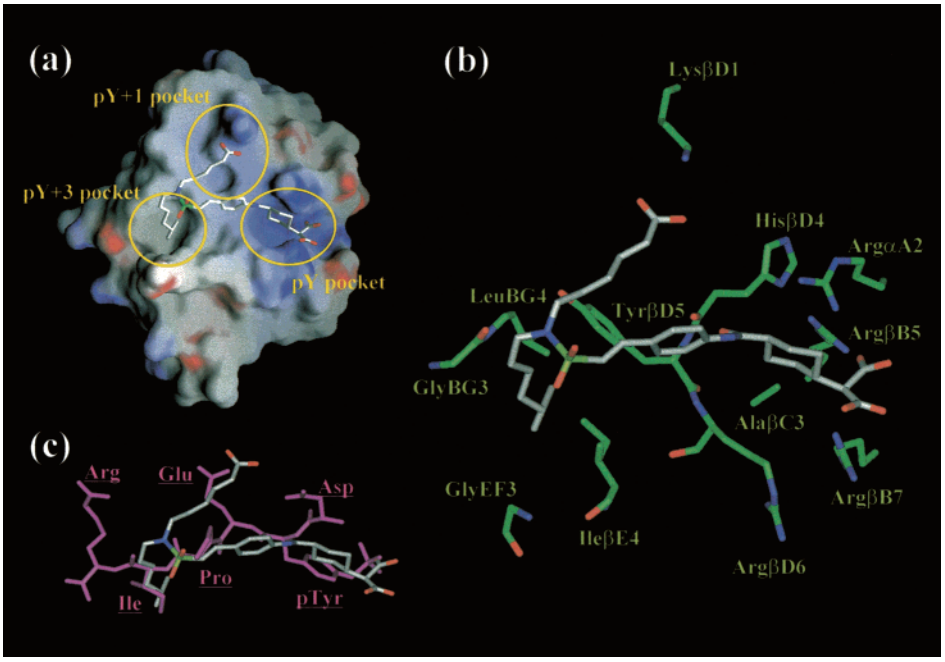


Figure 1. (a) Structural model of compound **12** and the SykCSH2 complex. The molecular surface of SykCSH2 was calculated and displayed using GRASP.[18] The surface is colored according to the local electrostatic potential and is colored deep blue in the most positive regions and deep red in the most negative regions. (b) Highlights of the interaction of compound **12** with SykCSH2 at the pY, pY+1, and pY+3 pockets in the structural model. (c) Superimposition of the crystal structure of phosphopeptide (DpYEPI) complexed with SykCSH2[8] and the model structure of compound **12**.

Design of the Compounds Binding to the pY and pY+1 Pockets. The strategy of tethering two binding moieties together has been applied in the design of some therapeutic proteins. To design linked compounds that bind to the pY and pY+1 pockets of SykCSH2, an NMR study was carried out on a ternary complex composed of unlabled SykCSH2, compound **2**, and compound **7**. Compound **2** was chosen because the characteristic ^{1}H chemical shifts of the 4,5-hydrogens on **2** were separated from those hydrogens on **7** and were good probes for detection of the intermolecular nuclear Overhauser effect (NOE) between **2** and **7**. Weak intermolecular NOEs were observed between the 4,5-hydrogens of **2** and the 4-hydrogen on the phenyl ring of **7** (data not shown). This indicated that compounds **2** and **7** were located such that these hydrogens were in proximity to each other in the ternary complex with SykCSH2. On the basis of this structural information, we designed a linked compound with compound **3**, which was the most potent compound for the pY pocket. As a result, compound **11**, in which the 4-hydrogen of **3** and the 4-hydrogen on the phenyl ring of **7** were linked to one amide bond unit, was synthesized (cis−trans isomers, Table 2). As expected, it exhibited an enhanced binding affinity for SykCSH2 relative to the untethered compounds. Thus, compound **11** was employed as the platform compound in the following analysis.

Utilization of the pY+3 Pocket. The pY+3 pocket is usually occupied with a hydrophobic amino acid residue at the pY+3 position (Leu in SykCSH2). Although we did not find any fragments that bound to the pY+3 pocket, use of this interaction site was attractive to increase the binding affinity, as demonstrated for other SH2 inhibitors. An isopropyl moiety was incorporated into **11** using four methylene units to mimic the side chain of the Leu residue at the pY+3 position (compounds **12** and **13**, Table 2). The docking model of **12** complexed with SykCSH2 (Figure 1a) suggested that additional favorable contact with the pY+3 pocket should be expected without any perturbations in the contact with the pY and pY+1 pockets when a hydrophobic moiety was attached to the nitrogen on the sulfonamide of **11**. As expected, an increased affinity was observed with **12** (IC_{50} = 63 μM) and **13** (IC_{50} = 69 μM). There was no significant difference observed between cis and trans isomers. Substitution of cyclohexene with cyclohexane of the R2 moiety to eliminate cis−trans isomerism (compound **14)** did not affect the binding activity (IC_{50} = 71 μM). Substitution of the isopropyl moiety with cyclohexane and shortening the linker by one methylene unit showed approximately a 2-fold improvement of inhibitory activity (Table 2, IC_{50} = 40 μM for **15**, IC_{50} = 38 μM for **16**, IC_{50} = 41 μM for **17**), which was comparable with the results of the monophosphorylated peptide (IC_{50} = 17 μM).

Structural Basis of Compounds Designed for Binding to SykCSH2. Parts a and b of Figure 1 show the docking model of compound **12** and SykCSH2. In this model, two carboxylic groups in the malonic acid part of **12** interact with the side chains of ArgαA2, ArgβB5, and ArgβB7, and the cyclohexane ring is located close to HisβD4, AlaβC3, and TyrβD5 in the pY pocket. These interactions are thought to mimic those observed for the phosphorus and benzene rings of pTyr in the crystal strucutre.[3,14] Our competitive binding studies indicated that there were no significant differences in activity between cis−trans isomers of the cyclohexane ring (**12** and **13**, and **15** and **16**). This suggests that the recognition of the ring in the pY pocket may not be rigorous. Previous NMR studies for PLCγ SH2 domains reported that a high degree of mobility on a wide-ranging time scale was observed around the pY pocket, especially in the βB−BC loop.[19] In our NMR study of SykCSH2, this region was poorly constrained

because of the small numbers of observed NOEs and some $^{1}H-^{15}N$ cross-peaks from this region were more broadened than those from other regions, which may result from conformational exchange phenomena. Flexibility of the pY pocket may be the reason that recognition in the pY pocket is allowed.

Crystallographic studies of the SykCSH2 indicated that the methylene part of the glutamic acid of the phosphopeptide at the pY+1 position interacted hydrophobically with LeuβD3, TyrβD5, and LeuBG5, whereas the carboxylic group of the glutamic acid of the phosphopeptide at the pY+1 position was too far from the amine group of LysβD1 to form a hydrogen bond (5.3 Å).[3] Conversely, in our model, the carboxylic group of **12** is located within hydrogen-bonding distance of the amine group of LysβD1 (Figure 1b) because **12** has a methylene chain long enough to interact with the amine group of LysβD1. In fact, compound **9**, which has only three methylene groups, did not show a binding affinity for SykCSH2. The length of the methylene chain of **12** seems to be important for binding to SykCSH2.

The R1 moiety of **12** (5-methylhexyl) and the side chain of Ile at the pY+3 position in the phosphopeptide are well superimposed (Figure 1c). The isopropyl group interacts hydrophobically with IleβE4, GlyEF3, GlyBG3, and LeuBG4. Compounds **15** and **16**, which have a cyclohexane ring instead of an isopropyl group at the pY+3 position, showed a 2-fold improvement of inhibitory activity. Plummer et al. have also reported that cyclohexane rings were effective pY+3 Ile side chain replacements for the Src SH2 domain.[17]

Conclusion

We applied a structure-based in-silico screening as well as a docking simulation to the discovery of SykCSH2 inhibitors. In this trial, cyclohexylmalonic acid was identified as a novel pTyr mimetic. It exhibited a higher affinity ($IC_{50} = 5.9$ mM) than pTyr itself ($IC_{50} = 14$ mM) and phenylmalonic acid ($IC_{50} = 12$ mM). Cyclohexylmalonic acid was also effective in the linked compound. The most active compound, **16**, exhibited activity comparable to that of the monophosphorylated peptide ligand (VpYTGLS). Further optimization of fragments corresponding to each pocket and that of the linker would lead to more potent inhibitors.

Acknowledgment. This work was supported financially by Grant-in-Aid for Specially Promoted Research from the Ministry of Education, Science, Sport and Culture, Japan.

Supporting Information Available: Experimental procedures for the protein structure analyses, measurements of binding affinity, and the synthesis of **11**−**17** with analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Kurosaki, T. Molecular mechanisms in B cell antigen receptor signaling. *Curr. Opin. Immunol.* **1997**, *9*, 309−318. (b) Turner, M.; Schweighoffer, E.; Colucci, F.; Di Santo, J. P.; Tybulewicz, V. L. Tyrosine kinase SYK: essential functions for immunoreceptor signalling. *Immunol. Today* **2000**, *21*,148−154.

(2) Bu, J. Y.; Shaw, A. S.; Chan, A. C. Analysis of the interaction of ZAP-70 and syk protein−tyrosine kinases with the T-cell antigen receptor by plasmon resonance. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 5106−5110.

(3) Futterer, K.; Wong, J.; Grucza, R. A.; Chan, A. C.; Waksman, G. Structural basis for syk tyrosine kinase ubiquity in signal transduction pathways revealed by the crystal structure of its regulatory SH2 domains bound to a dually phosphorylated ITAM peptide. *J. Mol. Biol.* **1998**, *281*, 523−537.

(4) Kihara, H.; Siraganian, R. P. Src homology 2 domains of Syk and Lyn bind to tyrosine-phosphorylated subunits of the high affinity IgE receptor. *J. Biol. Chem.* **1994**, *269*, 22427−22432.

(5) Wan, Y.; Kurosaki, T.; Huang, X. Y. Tyrosine kinases in activation of the MAP kinase cascade by G-protein-coupled receptors. *Nature* **1996**, *380*, 541−544.

(6) Ottinger, E. A.; Botfield, M. C.; Shoelson, S. E. Tandem SH2 domains confer high specificity in tyrosine kinase signaling. *J. Biol. Chem.* **1998**, *273*, 729−735.

(7) Schoepfer, J.; Fretz, H.; Gay, B.; Furet, P.; Garcia-Echeverria, C.; End, N.; Caravatti, G. Highly potent inhibitors of the Grb2-SH2 domain. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 221−226.

(8) Yao, Z.-J.; King, C. R.; Cao, T.; Kelley, J.; Milne, G. W. A.; Voigt, J. H.; Burke, T. R., Jr. Potent inhibition of Grb2 SH2 domain binding by non-phosphate-containing ligands. *J. Med. Chem.* **1999**, *42*, 25−35.

(9) Gao, Y.; Luo, J.; Yao, Z. J.; Guo, R.; Zou, H.; Kelley, J.; Voigt, J. H.; Yang, D.; Burke, T. R., Jr. Inhibition of Grb2 SH2 domain binding by non-phosphate-containing ligands. 2. 4-(2-Malonyl)-phenylalanine as a potent phosphotyrosyl mimetic. *J. Med. Chem.* **2000**, *43*, 911−920.

(10) Shakespeare, W. C.; Bohacek, R. S.; Azimioara, M. D.; Macek, K. J.; Luke, G. P.; Dalgarno, D. C.; Hatada, M. H.; Lu, X.; Violette, S. M.; Bartlett, C.; Sawyer, T. K. Structure-Based Design of Novel Bicyclic Nonpeptide Inhibitors for the Src SH2 Domain. *J. Med. Chem.* **2000**, *43*, 3815−3819.

(11) Vu, C. B.; Corpuz, E. G.; Merry, T. J.; Pradeepan, S. G.; Bartlett, C.; Bohacek, R. S.; Botfield, M. C.; Eyermann, C. J.; Lynch, B. A.; MacNeil, I. A.; Ram, M. K.; van Schravendijk, M. R.; Violette, S.; Sawyer T. K. Discovery of Potent and Selective SH2 Inhibitors of the Tyrosine Kinase ZAP-70. *J. Med. Chem.* **1999**, *42*, 4088−4098.

(12) Burke, T. R., Jr.; Smyth, M.; Nomizu, M.; Otaka, A.; Roller, P. P. Preparation of fluoro- and hydroxy-4-phosphonomethyl-D,L-phenylalanine suitably protected for solid-phase synthesis of peptides containing hydrolytically stable analogues of *O*-phosphotyrosine. *J. Org. Chem.* **1993**, *58*, 1336−1340.

(13) Ye, B.; Akamatsu, M.; Shoelson, S. E.; Wolf, G.; Giorgetti-Peraldi, S.; Yan, X. J.; Roller, P. P.; Burke, T. R. L-*O*-(2-malonyl)-tyrosine: A new phosphotyrosyl mimetic for the preparation of Src homology 2 domain inhibitory peptides. *J. Med. Chem.* **1995**, *38*, 4270−4275.

(14) Narula, S. S.; Yuan, R. W.; Adams, S. E.; Green, O. M.; Green, J.; Philips, T. B.; Zydowsky, L. D.; Botfield, M. C.; Hatada, M.; Laird, E. R.; Zoller, M. J.; Karas, J. L.; Dalgarno, D. C. Solution structure of the C-terminal SH2 domain of the human tyrosine kinase Syk complexed with a phosphotyrosine pentapeptide. *Structure* **1995**, *3*, 1061−1073.

(15) Orita, M.; Yamamoto, S.; Katayama, N.; Aoki, M.; Takayama, K.; Yamagiwa, Y.; Seki, N.; Suzuki, H.; Kurihara, H.; Sakashita, H.; Takeuchi, M.; Fujita, S.; Yamada, T.; Tanaka, A. Coumarin and chromen-4-one analogues as tautomerase inhibitors of macrophage migration inhibitory factor: discovery and X-ray crystallography. *J. Med. Chem.* **2001**, *44*, 540−547.

(16) (a) Kuntz, I. D. Structure-based strategies for drug design and discovery. *Science* **1992**, *257*, 1078−1082. (b). Shoichet, B. K.; Stroud, R. M.; Santi, D. V.; Kuntz, I. D.; Perry, K. M. Structure-based discovery of inhibitors of thymidylate synthase. *Science* **1993**, *259*, 1445−1450.

(17) Plummer, M. S.; Lunney, E. A.; Para, K. S.; Shahripour, A.; Stankovic, C. J.; Humblet, C.; Fergus, J. H.; Marks, J. S.; Herrera, R.; Hubbell, S.; Saltiel, A.; Sawyer, T. K. Design of peptidomimetic ligands for the pp60src SH2 domain. *Bioorg. Med. Chem.* **1997**, *5*, 41−47.

(18) Nicholls, A.; Sharp, K. A.; Honig, B. Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins: Struct., Funct., Genet.* **1991**, *11*, 281−296.

(19) Farrow, N. A.; Muhandiram, R.; Singer, A. U.; Pascal, S. M.; Kay, C. M.; Gish, G.; Shoelson, S. E.; Pawson, T.; Forman-Kay, J. D.; Kay, L. E. Backbone dynamics of a free and phosphopeptide-complexed Src homology 2 domain studied by ^{15}N NMR relaxation. *Biochemistry* **1994**, *33*, 5984−6003.

JM010313K