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Synthesis and Evaluation of Phosphopeptides Containing Iminodiacetate Groups as Binding Ligands of the Src SH2 Domain

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Abstract

Phosphopeptide pTyr-Glu-Glu-Ile (pYEEI) has been introduced as an optimal Src SH2 domain ligand. Peptides, Ac-K(IDA)pYEEIEK(IDA) (**1**), Ac-KpYEEIEK (**2**), Ac-K(IDA)pYEEIEK (**3**), and Ac-KpYEEIEK(IDA) (**4**), containing 0–2 iminodiacetate (IDA) groups at the *N*- and *C*-terminal lysine residues were synthesized and evaluated as the Src SH2 domain binding ligands. Fluorescence polarization assays showed that peptide **1** had a higher binding affinity ($K_d = 0.6 \mu\text{M}$) to the Src SH2 domain when compared with Ac-pYEEI ($K_d = 1.7 \mu\text{M}$), an optimal Src SH2 domain ligand, and peptides **2–4** ($K_d = 2.9–52.7 \mu\text{M}$). The binding affinity of peptide **1** to the SH2 domain was reduced by more than two fold ($K_d = 1.6 \mu\text{M}$) upon addition of Ni^{2+} ($300 \mu\text{M}$), possibly due to modest structural effect of Ni^{2+} on the protein as shown by circular dichroism experimental results. The binding affinity of **1** was restored in the presence of EDTA ($300 \mu\text{M}$) ($K_d = 0.79 \mu\text{M}$). These studies suggest that peptides containing IDA groups may be used for designing novel SH2 domain binding ligands.

Keywords

phosphopeptides; metal chelation; iminodiacetate group; SH2 domain; UV titration; fluorescence polarization; circular dichroism

1.0. Introduction

Src, one of the first studied non-receptor tyrosine kinases, has been implicated in the genesis and progression of multiple types of human diseases including colon, breast, lung cancers, osteoporosis, and inflammation-mediated bone loss [1,2]. Src tyrosine kinase contains three major domains, in the order from *N*- to *C*-terminal, two regulatory Src homology (SH) domains (SH3 and SH2 domains), and a kinase domain. The Src SH2 domain is a relatively small protein module of approximately 100 amino acids and has extraordinary ability to recognize specifically sequences containing phosphotyrosine residue (pTyr), thereby facilitating phosphorylation-dependent protein–protein interactions that result in signal transduction process [3]. Short peptide inhibitors capable of disrupting these interactions have been designed as useful tools in the mechanistic studies of the Src SH2 domain interactions and as potential

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inhibitors for further pharmaceutical development [4–8]. The Src SH2 domain preferentially binds peptides with the sequence pTyr-Glu-Glu-Ile (pYEEI) with high affinity [9].

The use of conformationally constrained peptides has been shown to be an effective strategy in developing therapeutic peptidic and peptidomimetic agents [10,11]. Constrained peptides are usually more stable towards enzymatic degradations. We previously reported the synthesis and evaluation of a series of covalently bonded conformationally constrained peptide analogues based on the optimal sequence pYEEI as inhibitors of the Src SH2 domain [8]. A number of conformationally constrained peptides showed at least 100-fold increase in the binding affinities to the Src SH2 domain relative to pYEEI and the corresponding linear peptides, respectively. The enhancement in binding affinities was accomplished solely through covalent bonding of *N*-terminal and side chain functional groups, formation of a favorable conformation, reducing unfavorable entropic effects, and/or creating novel bonding interaction between the peptides and the protein. However, the conformational changes were irreversible; the synthesis was cumbersome and challenging; and the preparation of the corresponding linear peptides was necessary for comparative studies.

To minimize one or more of the problems associated with covalent bonding described above, we attempted to introduce the conformational constraints through non-covalent forces by incorporating groups capable of forming of intramolecular hydrogen bindings or chelating to metals.

Metal-chelating groups have been used in the areas of protein purifications [12,13], peptide/protein engineering [14,15], and designing of nucleic acid probes targeting specific DNA molecules [16].

Hochuli and coworkers reported a novel nitrilotriacetic acid quadridentate chelate resin for metal chelate affinity chromatography. It was found that when charged with Ni^{2+} , the resin was able to bind peptides and proteins containing neighboring histidine residues [12].

Hopkins group reported that transition metal could induce a conformational change in the α -helical peptides bearing IDA groups at the *i* and *i*+3 or 4 positions [17]. The cooperative binding of the two IDA groups to one transition metal could cross-link the peptide intramolecularly and stabilize the secondary structure. The non-natural amino acid Fmoc-Lys(IDA)-OH was used in the conventional Fmoc peptide synthesis and introduced into protein-peptide interaction studies of the S-peptide fragment (α -helix rich) of ribonuclease S complex [18].

In a recent report from Syntrix Biosystems, Inc., probes where the *N* and *C* termini of a PNA oligomer are modified with a pair of metal-chelating ligands (IDA or nitrilotriacetic acid, NTA) were described, and it was found that the coordination can force the PNA oligomer into a macrocyclic configuration [16]. The intramolecular chelate dissociates only when the PNA hybridizes to its perfectly matched DNA target.

As part of our ongoing studies of interactions of conformationally constrained phosphopeptides with the Src SH2 domain [8], herein we report the interactions of phosphopeptides containing metal chelating groups with the Src SH2 domain in the presence and absence of metals. Peptide Ac-K(IDA)pYEEIEK(IDA) (1, Figure 1) was structured to incorporate two iminodiacetate groups (IDA), a common metal-chelating ligand, at the *N*- and *C*-terminal lysine residues, flanking the pYEEI motif. The peptide was expected to achieve a constrained conformation through potential intramolecular hydrogen bindings or chelating of the IDA groups with metal ions. The binding affinity of the ligand toward the Src SH2 domain was determined in the absence or presence of Ni^{2+} and/or a metal scavenger. The free amino groups in lysine residues are also strong hydrogen bond donors and metal chelating groups. Three other peptides (Figure 1) containing 0 or 1 IDA group at one of the *N*- or *C*-terminal lysine residues, Ac-KpYEEIEK

(2), Ac-K(IDA)pYEEIEK (3), and Ac-KpYEEIEK(IDA) (4), were also synthesized and evaluated for their binding affinities toward the Src SH2 domains.

2.0. Materials and Methods

2.1. Peptide Synthesis

All reactions were carried out in Bio-Rad polypropylene columns by shaking and mixing, using a VWR rotator, at room temperature. All reagents were purchased from Novabiochem (San Diego, CA), unless otherwise noted.

The peptides (Ac-K(IDA)pYEEIEK(IDA), Ac-KpYEEIEK, Ac-K(IDA)pYEEIEK, Ac-KpYEEIEK(IDA), and Fluorescein-K(IDA)pYEEIEK(IDA)) were synthesized on a Rink amide NovaGel resin employing *N*-(9-fluorenyl)methoxycarbonyl (Fmoc)-based chemistry and Fmoc-L-amino acid building blocks. The synthesis of Fmoc-Lys(IDA)-OH has been previously described [19]. The preparation of peptides containing the IDA moiety was carried out according to the previously described procedure [15] using Fmoc-Lys(IDA(OtBu))-OH as the building block. HBTU and DIPEA (TCI America, Portland, OR) in *N,N*-dimethylformamide (DMF) (VWR, West Chester, PA) were used as coupling and activating reagents, respectively. Fmoc deprotection at each step was carried out using piperidine (EMD, La Jolla, CA) in DMF (20%). After the final coupling, the peptides were *N*-terminally acetylated with acetic anhydride (Sigma-Aldrich, Milwaukee, WI), unless otherwise noted. Assembled peptides were cleaved from the resin with TFA/water/TIS (Sigma-Aldrich) (95:2.5:2.5) for 3 h, hydrolyzed for 19 h after addition of 10% water as described previously (Novabiochem Catalogue 2006/2007, 3.32), dried in vacuum, dissolved in water, and lyophilized on a Labconco FreeZone 6 (Kansas City, MO). The crude peptides were purified by HPLC (Waters Delta Prep 4000, Millipore, Billerica, MA) on a Phenomenex Gemini 10 micron C18 column (Torrance, CA) by elution at 20 mL/min using a gradient of 5–95% acetonitrile (0.1% formic acid) and water (0.1% formic acid) over 30 min. The peptides had a purity of >95% determined by HPLC as shown by analytical HPLC using a similar gradient system and a flow rate of 1 mL/min. The collected fractions were analyzed by LC-MS (Shimadzu LC-10; Waters Micromass Quattro II), and only fractions containing the pure peptide were pooled and lyophilized. All peptides were characterized by LC-MS or SELDI-TOF mass spectrometer.

Ac-Lys(IDA)-pTyr-Glu-Glu-Ile-Glu-Lys(IDA) (1)—The peptide was prepared as described above at 0.3 mmol scale and purified by HPLC in three separate batches. The final overall yield was 141 mg (36%). LC-MS (ESI-TOF) (*m/z*) for C₅₂H₇₈N₉O₂₇P calcd, 1291.5; found, 1291.7 [M]⁺.

Ac-Lys-pTyr-Glu-Glu-Ile-Glu-Lys (2)—The peptide was prepared as described above at 0.095 mmol scale and purified by HPLC. The final overall yield was 38 mg (38%). LC-MS (ESI-TOF) (*m/z*) for C₄₄H₇₀N₉O₁₉P calcd, 1059.5; found, 1059.6 [M]⁺.

Ac-Lys(IDA)-pTyr-Glu-Glu-Ile-Glu-Lys (3)—The peptide was prepared as described above at 0.085 mmol scale and purified by HPLC. The final overall yield was 12 mg (12%). LC-MS (ESI-TOF) (*m/z*) for C₄₈H₇₄N₉O₂₃P calcd, 1175.5; found, 1176.0 [M + H]⁺.

Ac-Lys-pTyr-Glu-Glu-Ile-Glu-Lys(IDA) (4)—The peptide was prepared as described above at 0.085 mmol scale and purified by HPLC. The final overall yield was 25 mg (25%). LC-MS (ESI-TOF) (*m/z*) for C₄₈H₇₄N₉O₂₃P calcd, 1175.5; found, 1175.9 [M + H]⁺.

Fluorescein-Lys(IDA)-pTyr-Glu-Glu-Ile-Glu-Lys(IDA) (5)—The peptide was prepared as described above at 0.063 mmol scale. After the final Lys(IDA) coupling and Fmoc deprotection, 5,6-carboxyfluorescein succinimidyl ester (33 mg, 0.070 mmol) and DIPEA (0.066 mL, 0.25 mmol) were added to the swelled resin in DMF (1 mL) and the mixture was shaken overnight in light-protected column. After the solution was drained, the resin was washed with DMF (3 × 20 mL), and the fluorescein coupling was repeated overnight. After the solution was drained, the resin was washed with DMF (4 × 20 mL). The peptide was cleaved and purified by HPLC as described above. All the fractions were protected from light. The final overall yield was 11 mg (11%). LC-MS (ESI-TOF) (m/z) for $C_{71}H_{86}N_9O_{32}P$ calcd, 1607.5; found, 1609.2 $[M + H]^+$; MS (SELDI-TOF) (m/z) for $C_{71}H_{86}N_9O_{32}P$ calcd, 1607.5; found, 1606.3 $[M - 1]^+$, 1629.2 $[M + Na - 1]^+$, 1645.7 $[M + K - 1]^+$,

2.2. UV Titration Assay

All spectra were collected on a Beckman Coulter DU-800 UV-Vis spectrophotometer with a scan rate of 120 nm/s, a slit width of 2 mm, and using 1 cm UV-Cuvette semi micro cells at pH = 8.5 from 210 to 310 nm. Difference titration experiments were performed by subtracting the signal of a cuvette containing only buffer solution from the signal of a similar solution containing 10 μ M $NiCl_2$ after adding equivalent aliquots of the peptide solution to both cuvettes. The extinction coefficients of the complexes were calculated from the initial slopes of the titration curves and were based on the total metal concentration. In another set of experiments, the K_d values were determined by monitoring the changes at 225 nm absorbance of solutions of 10 μ M peptide upon addition of aliquots of $NiCl_2$ up to a final concentration of 10 μ M.

2.3. Expression and Purification of the Src SH2 Domain

Bacterial DH5 α cells containing recombinant plasmid pGEX-Src-SH2 domain were used to inoculate 500 mL of Luria-Bertani (LB) culture medium. After 10–12 h culture at 37 °C, the bacterial culture (OD_{600nm} around 1.0) was cooled down to 25 °C and diluted by another 500 mL of LB medium. Then 0.4 mM isopropyl β -D-isogalactopyranoside (IPTG) was added to induce the production of the fusion protein for 7 h at 25 °C. After induction, the bacterial cells were harvested by centrifugation at 5000 rpm for 5 min at 4 °C. Then bacterial pellets were resuspended in 50 mL 1X PBS and sonicated for 15 sec. Resuspension and sonication were repeated 5 times. All cell debris, including insoluble proteins, were removed by centrifugation at 20,000 rpm for 30 min. The supernatant was mixed and incubated with 0.1 g of glutathione-agarose resin for 1 h and loaded into purification column. When all liquid portions flowed through the column, 1 or more column volumes of PBS was used to wash the glutathione-agarose resin, which had already bound to the fusion protein GST-tagged Src SH2 domain, until no proteins can be detected in the flow-through by Bradford protein reagent. In order to adjust the pH of the resin from 7.4 to 8.0, another 1 volume of 50 mM Tris buffer (pH 7.4) and 1 volume of 50 mM Tris buffer (pH 8.0) were used to wash the beads. Reduced L-glutathione (10 mM) in 50 mM Tris (pH 8.0) buffer was used to elute the fusion protein. All purification steps were carried out at 4 °C. Protein concentrations were determined by Bradford protein assay using bovine serum albumin as a standard.

2.4. Fluorescence Polarization Binding Assay

2.4.1. Competitive Fluorescence Polarization Binding Assay in Presence of the Src SH2 Domain—All peptides were tested as competitors of the fluorescent probe F-GpYEEI for binding affinity to the Src SH2 domain using fluorescent polarization (FP) competitive binding assay as described below. FP intensities were measured at 25 °C in a disposable glass tube (volume of 600 μ L) using a Perkin-Elmer LS 55 luminescence spectrometer equipped with an FP apparatus. The excitation and emission wavelengths were

set at 485 and 535 nm, respectively. For the competition assay, final concentrations of 80 nM fluorescent probe F-GpYEEI, 750 nM Src SH2 domain, phosphate buffer (20 mM, pH 7.3, 100 mM NaCl, 2 mM DTT, 0.1% BSA), water, and various concentrations (0 – 100 μ M) of each competitor peptide were used. The order of addition to each glass tube (600 μ L) was (i) buffer, (ii) water, (iii) fluorescent probe, (iv) Src SH2 domain, and (v) competitor peptide. A blank control (with the Src SH2 domain but without a peptide) and a background control (without both the Src SH2 domain and the peptide) were used. The inhibition percentage of fluorescence probe binding to the Src SH2 domain by the sample was calculated by the following equation:

$$\left(1 - \frac{FP_{\text{blk}} - FP_{\text{s}}}{FP_{\text{blk}} - FP_{\text{bgd}}}\right) \times 100$$

where FP_{blk} is the fluorescent polarization value of the blank control. FP_{s} is the fluorescent polarization value of the sample (peptide), and FP_{bgd} is the fluorescent polarization value of the background control. The inhibition percentages of the various concentrations of the assayed peptides were plotted, and the K_{d} value (the concentration of peptides at which the binding site on Src SH2 domain is half occupied) was calculated using LAB Fit Curve Fitting Software 7.2. The reported K_{d} values are the mean of three separate determinations with a standard deviation of less than 5%.

2.4.2. Competitive Fluorescence Polarization Binding Assay in Presence of the Src SH2 Domain and Different Metal Ions—The assays were performed as described above with the exception that the metal ions with different final concentrations [NiCl_2 (2, 5, 10, 50, 100, 200, 300 and 500 μ M), CoCl_2 , CdCl_2 , CuCl_2 , and ZnSO_4 (2 and 300 μ M)], were added after addition of the competitor peptides. In another set of assay, EDTA (300 μ M) was also used after the addition of the same concentration of Ni^{2+} and incubated for 10 min.

2.4.3. Saturation Binding Assay Using Fluorescein-Labeled Peptide 5—Fluorescein-labeled peptide **5** (80 nM) was used in the presence of Ni^{2+} alone at different concentrations (0.08, 0.16, 0.32, 0.64, 1, 3, 10, 30, 100, 200, 300, 400, and 500 μ M). In another experiment, F-GpYEEI (80 nM) was used in the place of peptide **5** as a control peptide. The same assay was carried out in the presence of a similar amount of EDTA added to each concentration point of Ni^{2+} described above. The Src SH2 domain had a fixed concentration of 0.75 μ M in all experiments.

2.5. Circular Dichroism (CD) Spectroscopy

All CD spectra were recorded in 1 cm path length cuvette on a nitrogen-flushed JASCO J-810 spectropolarimeter interfaced with a 25 $^{\circ}\text{C}$ water bath by averaging six consecutive scans. All spectra were recorded with a 4 ms response and a bandwidth of 1 nm. CD spectra were measured with the spectropolarimeter using a 50 nm.min⁻¹ scan speed. All spectra were corrected for background by subtraction of appropriate blanks. The data are represented in the 185–265 nm spectral range.

3.0. Results and Disucssion

3.1. Chemistry

Peptides containing 0–2 IDA groups (Figure 1) were synthesized by solid-phase Fmoc-based chemistry employing Rink amide NovaGel resin as the solid support and Fmoc-L-amino acids as building blocks. The synthesis of Fmoc-Lys(IDA)(OtBu)-OH has been previously reported [19]. The synthesis of peptide **1** is shown as a representative example in Scheme 1 using Fmoc-

Lys(IDA(OtBu))-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Ile-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Tyr(H₂PO₃)-OH, and Fmoc-Lys(IDA(OtBu))-OH, respectively, in coupling reactions with the resin. 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N,N*-diisopropylethylamine (DIPEA) in *N,N*-dimethylformamide (DMF) were used as coupling and activating reagents, respectively. Fmoc deprotection at each step was carried out using piperidine in DMF (20%). After the final deprotection, the peptides were capped by *N*-terminal acetylation with acetic anhydride. Then a mixture of trifluoroacetic acid (TFA):water:triisopropylsilane (TIS) (95:2.5:2.5) was used for side chain deprotection of amino acids and cleavage of the synthesized peptide from the resin. Finally, the resin was hydrolyzed for 19 h after addition of 10% water, dried in vacuum, and lyophilized. The crude peptides were purified by preparative reverse-phase HPLC and analyzed by LC-MS using a gradient of 5–95% acetonitrile (0.1% formic acid) and water (0.1% formic acid) at 20 mL/min over 30 min. The purity of final products was confirmed by analytical HPLC (>95%) using a similar gradient system at 1 mL/min. A similar protocol was also used for the synthesis of peptides **2–5** using appropriate Fmoc-amino acid building blocks or 5,6-carboxyfluorescein succinimidyl ester.

3.2. UV Titration Studies

UV Titration studies were carried out to determine whether Ni²⁺ binds to the peptides. Different concentrations of peptide **1** (1 to 100 μM) were incubated with a fixed concentration of 10 μM Ni²⁺ (Figure 2a). In a lower range of peptide concentration (<10 μM) and less than one equivalent of Ni²⁺ concentration, the monitored absorbance was concentration-dependent and increased consistently. However, at higher concentrations of the peptide (>15 μM), the absorbance reached its steady state, suggesting that Ni²⁺ ions were already saturated with peptides.

When the Δε was plotted with different equivalents (from 0 to 10) of peptide **1**, the curve was consistent with the formation of a 1:1 metal-peptide complex (Figure 2b). This indicates that the two IDA groups on the *N*- and *C*-termini can possibly interact with one Ni²⁺ ion and form a cyclic peptide-metal chelation complex. However, other peptide-metal complex species, such as those resulting from intermolecular interactions between the peptide and a Ni²⁺ ion in 1:2 metal-peptide complex, may also be present at higher concentrations of the peptide. The conformation and the binding profile of this cyclic complex were further investigated by using CD and fluorescence polarization studies.

The difference absorption spectra of free and metal-bound cyclic peptide **1** demonstrated that the absorbance at ~225 nm can be used to monitor the formation of peptide-metal complex (Figure 2c). The K_d was determined to be 234.7 nM using the Igor Pro algorithm by plotting the absorbance change at specific wavelength versus concentration of peptide upon addition of aliquots of Ni²⁺ from an aqueous stock.

Peptide **2** containing two unsubstituted lysine residues exhibited the highest binding affinity toward Ni²⁺ (K_d = 97.4 nM) (Table 1) when compared with other peptides. Peptides **3** and **4** also exhibited higher binding affinity toward Ni²⁺ when compared with peptide **1**, possibly due to the presence of at least one free amino group that is a stronger chelating group than a carboxylate. Control peptide Ac-pYEEI does not have a strong chelating group at the *N*- or *C*-termini. Thus, Ac-pYEEI did not exhibit a high binding affinity toward Ni²⁺, suggesting that the high affinity binding between peptide **1** and Ni²⁺ is not due to the interactions with negatively-charged phosphate group.

3.3. Fluorescence Polarization (FP) Binding Assay

3.3.1. Competitive Binding Assay in Presence of the Src SH2 Domain—The binding affinities of the synthesized peptides **1–4** along with Ac-pYEEI against the Src SH2 domain were determined and compared in the presence of the reference peptide probe, F-GpYEEI, using a fluorescence polarization competitive assay. The results expressed as K_d values are shown in Table 2.

Peptide **1** with two IDA groups at the *N*- and *C*-terminal lysine residues showed 2.7-fold higher binding affinity toward the SH2 domain when compared with the optimal sequence Ac-GpYEEI. These data suggest that the carboxylic groups on the IDA groups may have contributed to the binding to the Src SH2 domain binding pockets, possibly through additional hydrogen bonding or electrostatic interactions. Furthermore, the introduction of the two negatively charged IDA groups may have generated a favorable conformational change within modified pYEEI derivative for appropriate binding to the SH2 domain.

The crystal structure of the Src SH2 domain complexed with peptide pYEEI showed two major binding pockets within the domain substrate binding site, the hydrophilic pTyr binding pocket (P site) and the hydrophobic pocket (P+3 site) [3]. The glutamic acid residues (EE) in the P+1 and P+2 positions have minor interactions with amino acids in the SH2 domain. The two lysine residues in peptide **1** are located at the P–1 and P+5 positions, and may have minor interactions with other small binding grooves or other important amino acids outside the P and P+3 sites. Further structural studies are required to identify the key amino acids outside these two major binding pockets that are involved in interactions with IDA groups. That information may provide insights for designing more potent Src SH2 domain binding ligands.

Peptide **2**, which lacks the two IDA groups but has the two lysine residues at both terminals, had a K_d value of 52.70 μ M showing significant decrease in the binding affinity when compared with peptide **1** and Ac-pYEEI. The reduction of binding affinity in **2** suggests that the presence of positively-charged amino acid residues at both *N*- and *C*-termini is detrimental to interactions with the SH2 domain. Incorporation of one IDA group at the *N*- or *C*-terminal in peptides **3** and **4** rescued partially the binding affinity when compared with peptide **2**. However, peptides **3** and **4** showed approximately 5-fold lower binding affinity toward the SH2 domain when compared with that of peptide **1** containing two IDA groups. These data suggest that the presence of two positively-charged lysine residues at either the *N*- or *C*-terminals is deleterious in interactions with the SH2 domain. On the other hand, the presence of one or two IDA groups can provide additional interactions with amino acid residues existing on both sides of the binding pocket regions of the Src SH2 domain leading to enhanced binding affinity of the peptides toward to the SH2 domain.

3.3.2. Competitive Binding Assay in Presence of the Src SH2 Domain and Different Metal Ions—Table 3 shows that by incubating peptide **1** in the presence of different concentrations of Ni^{2+} (0–500 μ M for peptide **1**), the binding affinities toward the Src SH2 domain consistently decrease. The binding affinity of peptide **1** ($K_d = 0.6 \mu$ M) was reduced by more than two fold ($K_d = 1.6 \mu$ M) upon addition of Ni^{2+} (300 μ M). This binding affinity is similar to the K_d value of Ac-pYEEI, suggesting that Ni^{2+} possibly causes some structural effects on the binding pocket of the protein or blocks the interactions of the carboxylate groups with the SH2 domain through the formation of a complex between the two IDA groups and Ni^{2+} .

These data were consistent with the results of UV titration studies showing the formation of a 1:1 complex between peptide **1** and Ni^{2+} with a K_d value of 234.7 nM. In the experimental conditions of the fluorescence polarization assay while free peptide and peptide-metal complex coexist, the binding affinity of peptide **1** is approximately 7-fold lower toward the SH2 domain

than Ni^{2+} . On the other hand, even at the highest concentration of Ni^{2+} (500 μM), the peptide retained a high binding affinity for the Src SH2 domain. It is not clear from these experiments whether the peptide-metal complex binds to the SH2 domain in a similar manner to peptide **1**. One direct method to determine whether peptide **1** and the peptide-metal complex bind to the Src SH2 domain in the cyclized form is to synthesize a covalently cyclized peptide by linking between the two IDA groups or two lysine residues and compare the binding affinity with the peptide **1** in the presence of Ni^{2+} . Unfortunately, the synthesis of the covalently cyclized peptide was not successful by using standard methods. Further studies, such as synthesizing a new class of cyclized peptides with different ring sizes, are required to understand effect of the ring size and the role of the lysine residues in the binding to the Src SH2 domain.

To further prove the effect of the peptide-metal complex formation on the binding affinity of peptide **1** to the Src SH2 domain, FP assay was conducted by adding the same concentration of EDTA (300 μM) to the system containing 300 μM Ni^{2+} . As shown in Table 3, upon adding EDTA to the system, the binding affinity of peptide **1** was almost restored to the original level, changing the K_d value from 1.55 to 0.79 μM . EDTA was a very effective competitive chelating agent with a much higher affinity than peptide **1** for binding to Ni^{2+} . It is possible that EDTA reversed the structural effect of Ni^{2+} on the protein. Another possibility is that most of the peptide in the peptide- Ni^{2+} complex was replaced by EDTA, forcing the peptide **1** to return to the unbounded free state for optimal interaction with the SH2 domain.

Similarly the addition of Ni^{2+} ions (0, 2 and 300 μM) to peptides **2–4** slightly affected the K_d values. These data suggest that the addition of Ni^{2+} did not significantly change the pattern of interactions for peptides **2–4** with the Src SH2 domain and the changes were believed to be caused by Ni^{2+} addition only. The Ni^{2+} ion itself may have caused some structural effects on the Src SH2 domain that may have eventually affected the binding with the peptides. This phenomenon was shown in the CD experiments described later.

To test the hypothesis, other types of transition metals were selected, including Co^{2+} , Cd^{2+} , Cu^{2+} , and Zn^{2+} , and used in similar experimental conditions (Table 4). Interestingly, only Co^{2+} showed similar binding patterns when compared to Ni^{2+} at 2 and 300 μM . All other metals, Cd^{2+} , Cu^{2+} , and Zn^{2+} , showed deleterious effects in the assay at both 2 and 300 μM conditions. Even at 2 μM of Cd^{2+} , Cu^{2+} , and Zn^{2+} , the FP values revealed that there were no binding interactions of F-GpYEEI to the Src SH2 domain, possibly due to the partial or complete denaturation/ structural changes of the Src SH2 domain and/or changing the conformation of the protein binding sites in the presence of these metals. It is not clear why this type of protein structural modifications do not happen in the presence of Ni^{2+} and Co^{2+} , but could be related to the physicochemical properties of the metals and their differences in interactions with the SH2 domain.

3.3.3. Saturation Binding Assay Using Fluorescein-Labeled Peptide 5—

Fluorescein-labeled peptide **5** (Figure 1) was synthesized and used as a probe instead of F-GpYEEI to study the interactions of peptide containing two IDA groups with Ni^{2+} in the presence of EDTA or the Src SH2 domain. Several different FP studies were performed, such as assays of peptide **5** with Ni^{2+} , peptide **5** with Ni^{2+} in the presence or absence of same concentration of EDTA, and peptide **5** with Ni^{2+} in the presence of a fixed concentration of the Src SH2 domain. F-GpYEEI with Ni^{2+} was used as a control.

As shown in Figure 3, the control peptide F-GpYEEI did not show any dramatic change in FP values upon addition of Ni^{2+} even at high concentration of 500 μM . This result was expected since Ac-pYEEI had very weak affinity toward Ni^{2+} as described above. However, peptide **5** showed a significant increase in FP values in the presence of Ni^{2+} when compared with F-

GpYEEI, suggesting the formation of peptide-metal complex in the assay condition. To explain this observation, the same concentration of EDTA was added to the assay system. In the presence of the metal scavenger, the FP values showed no significant changes at different concentrations of Ni^{2+} , meaning that EDTA disrupted the complex formation of peptide **5** through chelating with Ni^{2+} .

As described above, the interaction between peptide **1** and the Src SH2 domain was slightly disrupted upon the addition of high concentrations of Ni^{2+} (K_d changed from 0.63 to 1.55 μM). Peptide **5** showed a similar binding disruption pattern in the presence of the Src SH2 domain as shown by the reduced fluorescence polarization values with increasing Ni^{2+} concentrations (Figure 3). Similarly, fluorescent-labeled peptide **5** was still able to bind to the Src SH2 domain even at 500 μM of Ni^{2+} . The presence of the fluorescein group at the *N*-terminal of peptide **5** may have partially changed the conformation of the optimal interaction with the SH2 domain.

3.4. Circular Dichroism (CD) Spectroscopy Study

3.4.1. CD Studies of Peptides with Different Concentration of Ni^{2+} in the Absence of the Src SH2 Domain—Peptide **1** showed similar folded conformations at different concentrations of Ni^{2+} (0–500 μM) (Figure 4). The peptide showed a significantly folded structure in the absence of the metal based on the ellipticity values and retained its secondary structure in the presence of Ni^{2+} . This result was not surprising since the binding affinity of peptide **1** to the SH2 domain was slightly decreased in the presence of Ni^{2+} . In other words, Ni^{2+} did not disrupt the conformation of peptide **1** significantly for interaction with the SH2 domain. This may explain why peptide **1** itself was able to bind to the Src SH2 domain with a higher binding affinity than Ac-pYEEI. The addition of two IDA groups may have assisted the peptide to adopt a favorable conformation for the interaction with the Src SH2 domain. The addition of Ni^{2+} partially disrupted the interaction of IDA groups with the SH2 domain, but it did not change the conformation of the peptide since the folding of the peptide was not disturbed. Peptide **1** appears to have an appropriate folded structure in the absence of Ni^{2+} and addition of the metal did not change the secondary structure during the interaction with the SH2 domain.

Peptide **2** exhibited a much lower binding affinity to the SH2 domain because of the importance of the IDA groups versus the positively-charged amino groups. Peptide **2** was folded much less compared to **1** in the absence of the metal based on the ellipticity values. Addition of 5 μM Ni^{2+} to **2** enhanced the ellipticity values. As shown in Table 3, the binding affinity of **2** increased in the presence of 300 μM of Ni^{2+} ($K_d = 39.3 \mu\text{M}$), suggesting the folding of this peptide in the presence of the high concentration of the metal may have assisted the interaction with the SH2 domain. Peptide **2** showed more folding in the presence of Ni^{2+} , but compared to peptide **1**, ellipticity values were lower even at the highest concentrations of Ni^{2+} . This was mainly because the amino groups of peptide **2** can also chelate to Ni^{2+} .

Peptides **3** and **4** showed similar pattern to **1** with a minor increase of ellipticity in the presence of Ni^{2+} . However, their ellipticity values were higher than peptide **2**. In general, it appears that peptides **1**, **3** and **4** have secondary structures with higher ellipticities than that of peptide **2** and their secondary structures are not affected significantly in the presence of Ni^{2+} .

3.4.2. CD Studies of the Src SH2 Domain (Fixed Concentration) in the Presence of Different Concentrations of Ni^{2+} —Slight enhancement in ellipticity of the Src SH2 domain was observed in the presence of Ni^{2+} , suggesting that this metal affects the secondary structure of Src SH2 domain (Figure 5). Because of there are several types of secondary structures in the Src SH2 domain, including α -helices and β -sheets, the peaks are broader from 200–230 nm with a minimum at 210 nm.

3.4.3. CD Studies of Fixed Concentration of Peptides and the Src SH2 Domain with Different Concentrations of Ni²⁺—Similar equivalents of the peptides and SH2 domain were used with different concentrations of Ni²⁺ in similar ratios as the other experiments described above (Figure 6). In general, the changes in the secondary structures appear to be from the effect of Ni²⁺ on the Src SH2 domain and not because of the interaction of the peptide with the Src SH2 domain. Comparing the spectra with those of the Src SH2 domain alone in the presence of Ni²⁺ (Figure 5) shows that the differences are not significant. These data were consistent with the binding affinity data that showed lower binding affinity in the presence of Ni²⁺, possibly because of the changes in the secondary structure of the Src SH2 domain at high concentrations of the metal. Peptides **1**, **3**, and **4** showed some ellipticity enhancement after 1–1.5 μ M of Ni²⁺. At higher concentrations of Ni²⁺, there was no significant change for the peak with the minima of 210 nm. Peptide **2** exhibited larger changes up to 1.5 μ M of Ni²⁺.

4.0. Conclusions

Herein, we introduced novel peptides containing 0–2 IDA groups at the side chains of lysine residues flanking the optimal sequence of the Src SH2 domain ligand pYEEI to introduce conformational constraints through non-covalent forces, such as intramolecular hydrogen bindings or metal chelation. UV titration studies demonstrated that all four peptides were capable of chelating with one Ni²⁺ ion possibly by formation of a cyclic complex. However, only peptide **1** showed 2.7-fold higher binding affinity toward the SH2 domain when compared with the optimal sequence Ac-GpYEEI. Although CD experiments did not show significant changes in the structure of the peptide in the absence or presence of Ni²⁺, the increased affinity when compared to the reference peptide Ac-pYEEI indicated that the addition of IDA groups at the two terminals may have already caused the formation of a favorable conformation for peptide **1** for binding to the Src SH2 domain. The non-covalent interactions between the two IDA groups within peptide **1** could explain the lack of dramatic effect of Ni²⁺ ion on the ligand binding affinity toward SH2 domain. Ni²⁺ could serve as a bridge to bring the two IDA groups together, achieving similar conformational effect as that via direct inter-IDA group interactions in the absence of Ni²⁺. Furthermore, based on the CD experimental data, Ni²⁺ caused the structural disruption on the protein and thus modest effect on the peptide-SH2 domain binding interaction. FP assays showed a 2 fold decrease of the binding affinity for the Src SH2 domain for peptide **1** when Ni²⁺ was added to the system at high concentration of 300–500 μ M. Nonetheless, the addition of EDTA dropped the K_d value back to the almost original level, suggesting the disruption is reversible. In future studies, optimized substrate sequences from other protein families may be used to construct IDA containing substrate peptides. With further optimization, this class of peptides may serve as potential SH2 domain binding ligands.

Abbreviations

IDA, iminodiacetate; F-GpYEEI, fluorescein-labeled GpYEEI; FP, fluorescence polarization; CD, circular dichroism..

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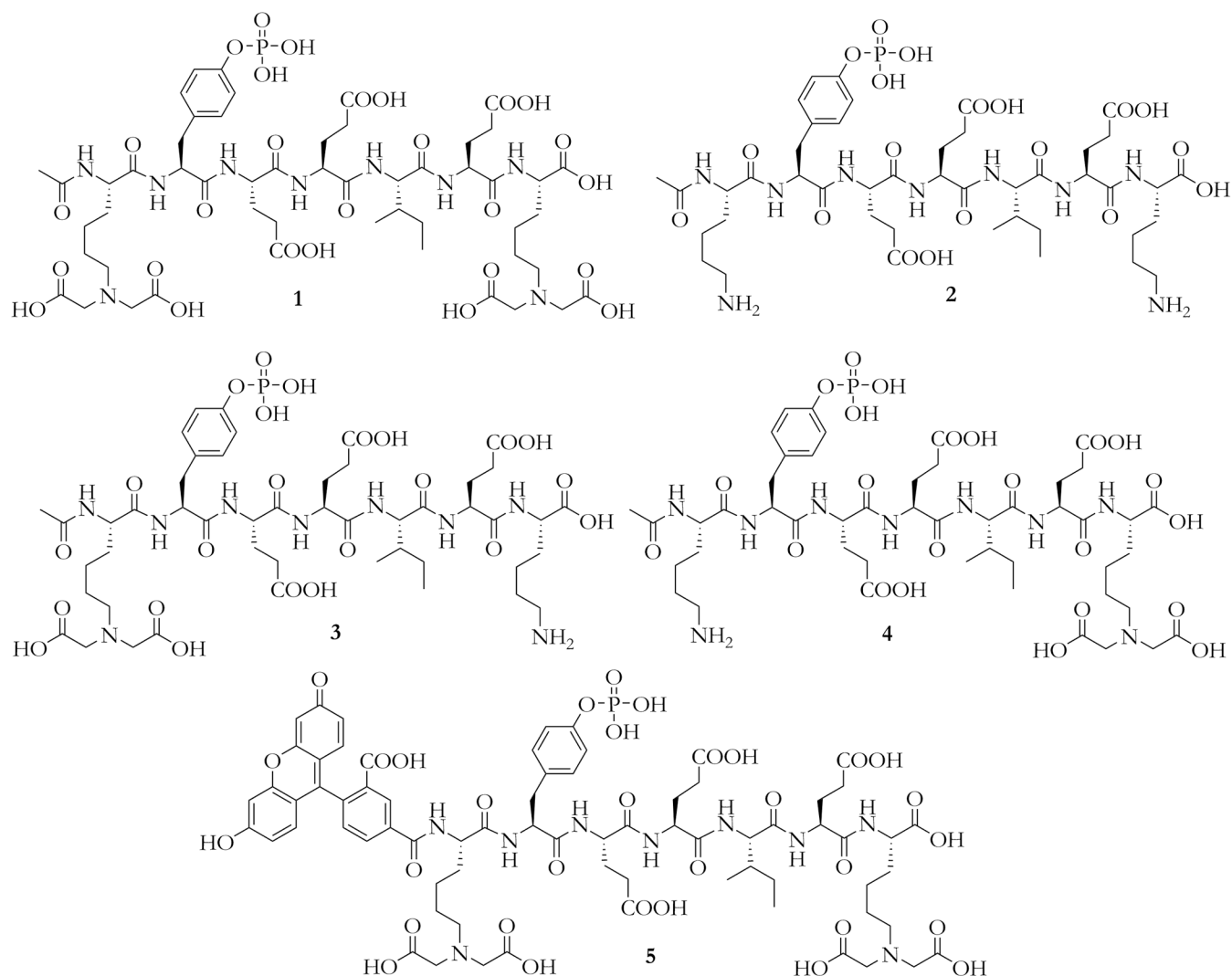
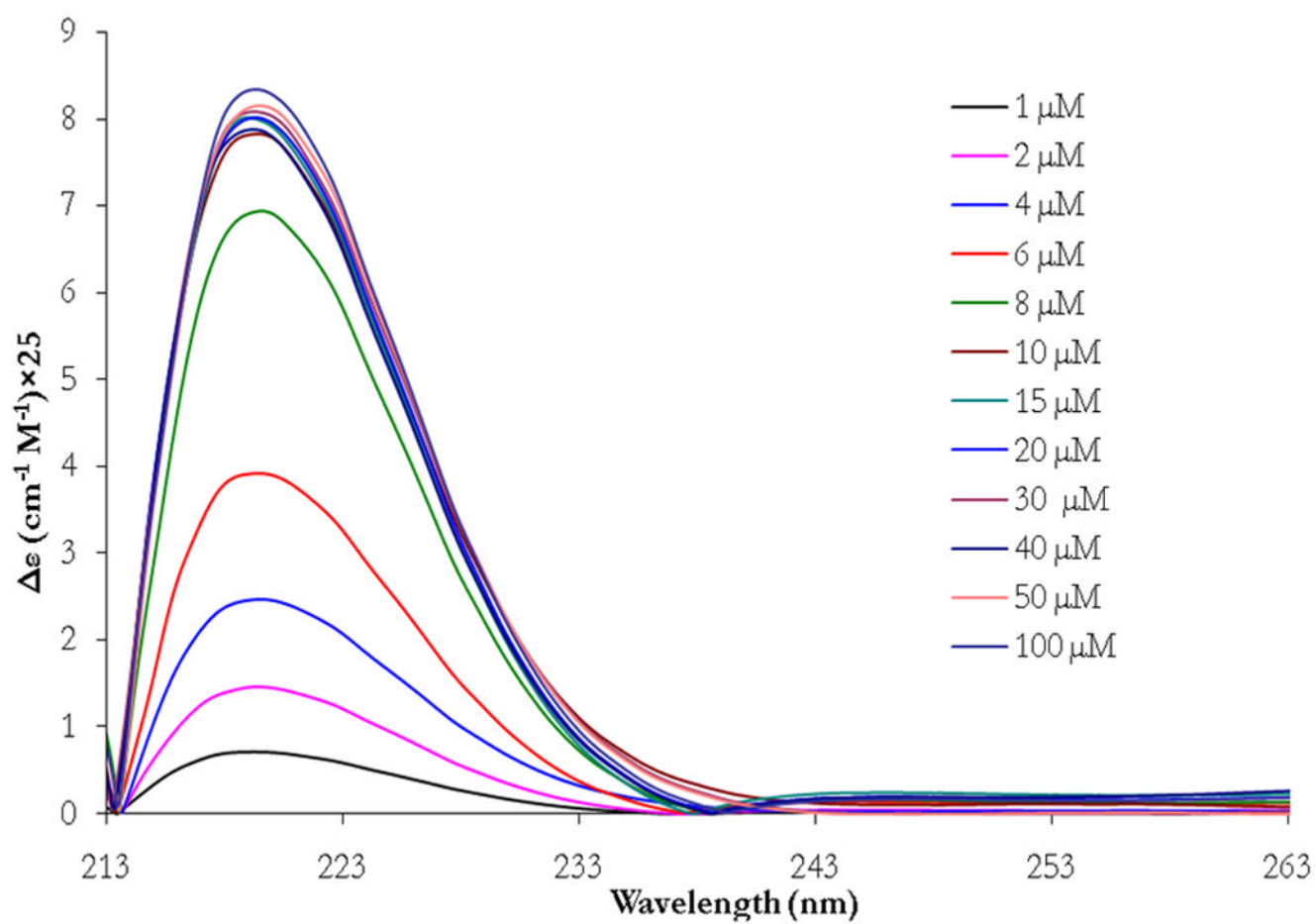
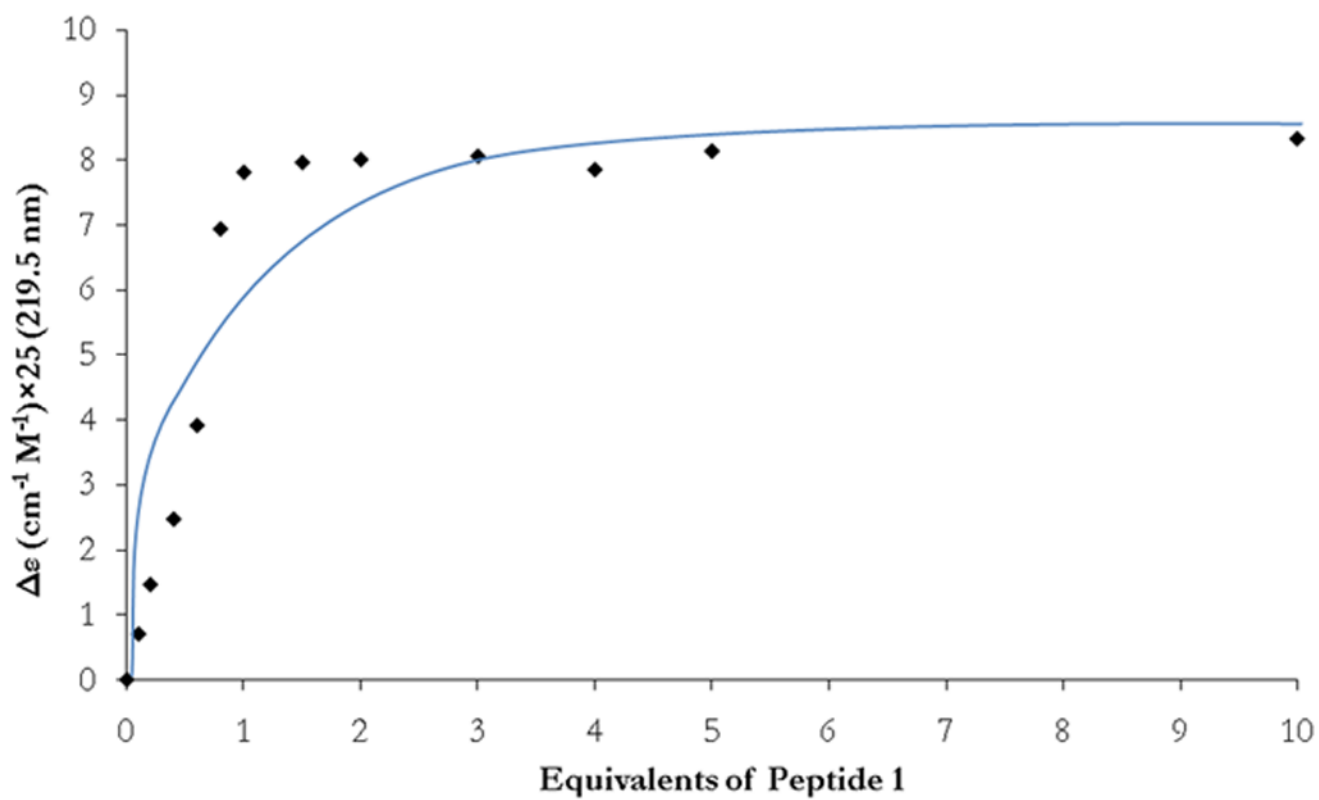


Figure 1. Chemical structures of peptides **1–4** containing 0–2 IDA groups and fluorescein-labelled peptide derivative **5**.





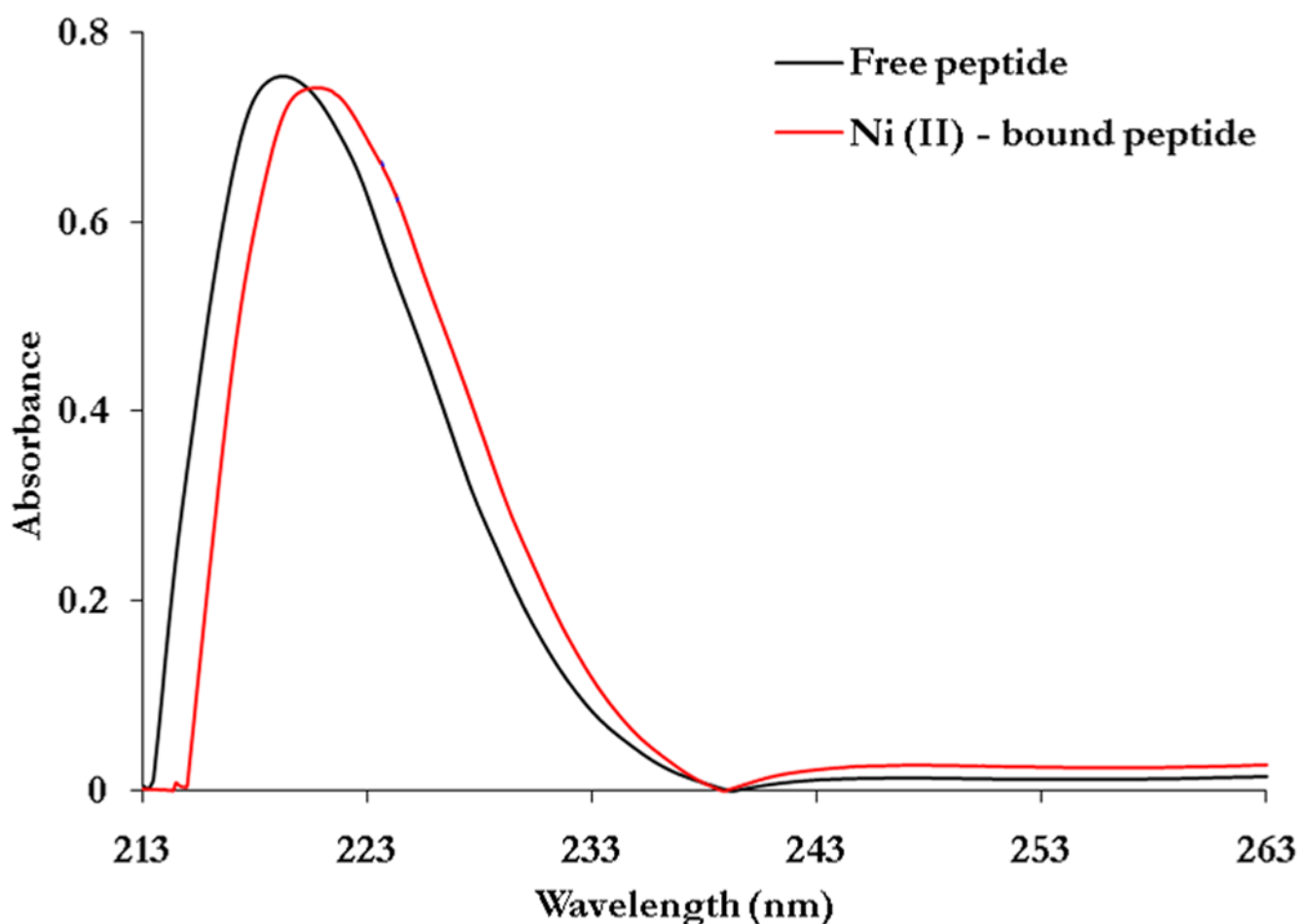


Figure 2.

(a) UV titration of peptide **1** (1–100 μM) with a solution of Ni^{2+} (10 μM). The data are plotted as $\Delta\epsilon$ versus wavelength. (b) The $\Delta\epsilon$ versus equivalents of peptide **1** (0–10 equiv). One equivalent of Ni^{2+} interacts with one equivalent of peptide **1**. (c) Difference absorption spectra of free and metal-bound peptide **1**. Free peptide **1** (10 μM) (black); Ni^{2+} -bound peptide: peptide **1** (10 μM) with NiCl_2 (10 μM) (red).

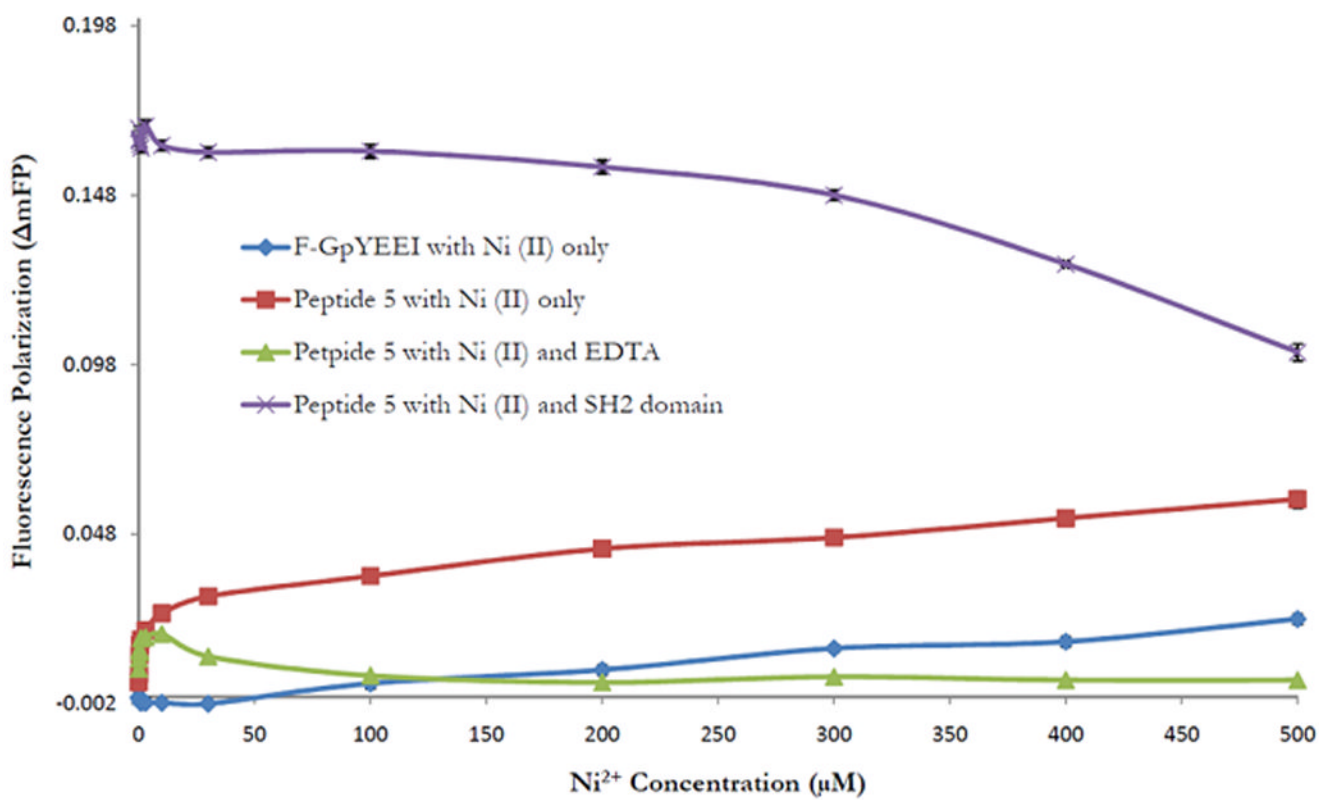


Figure 3.
The fluorescence polarization values in different saturation assay conditions.

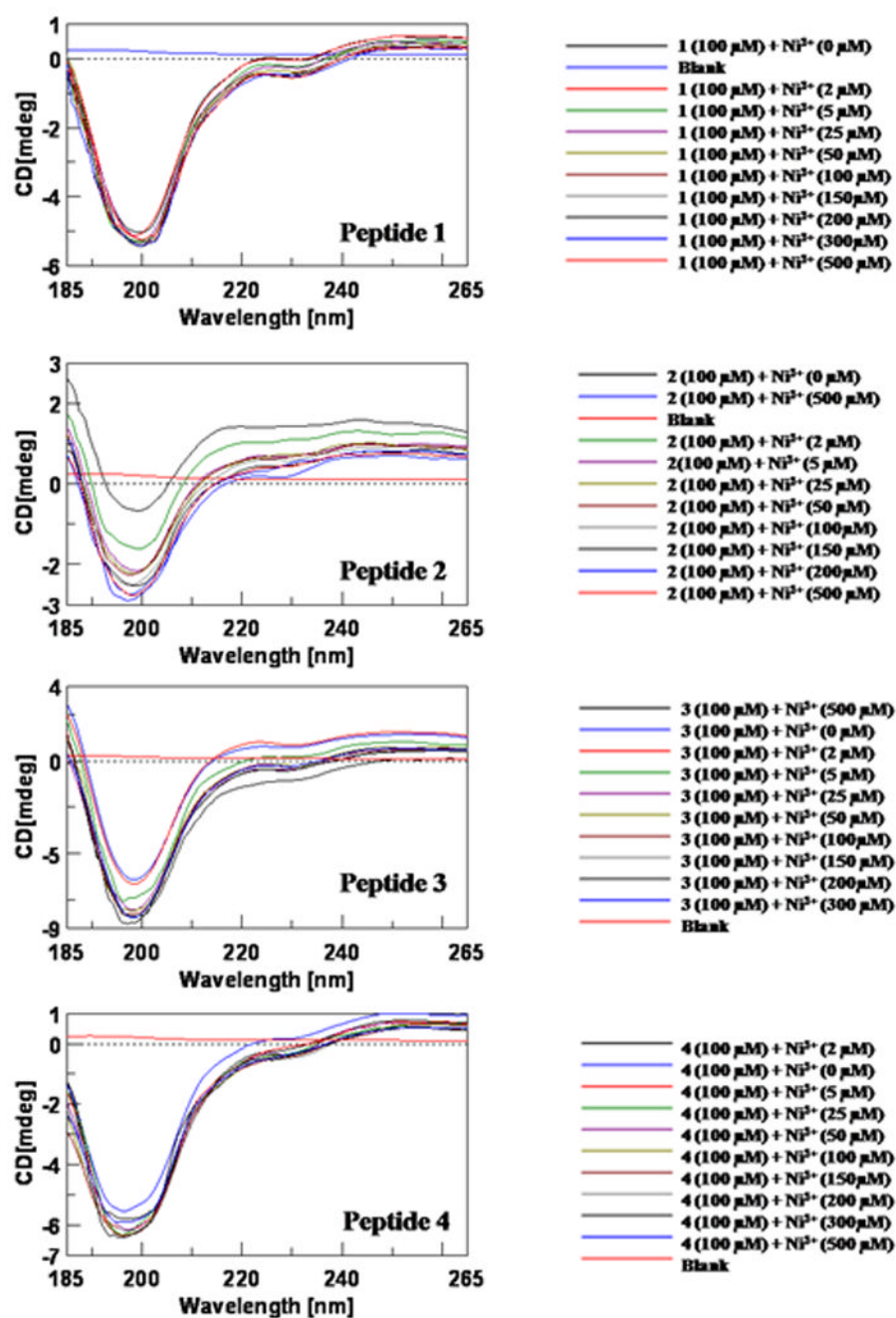


Figure 4.

The CD spectra of peptides **1–4** (100 μ M) in the presence of different concentrations of Ni^{2+} in the absence of the Src SH2 domain.

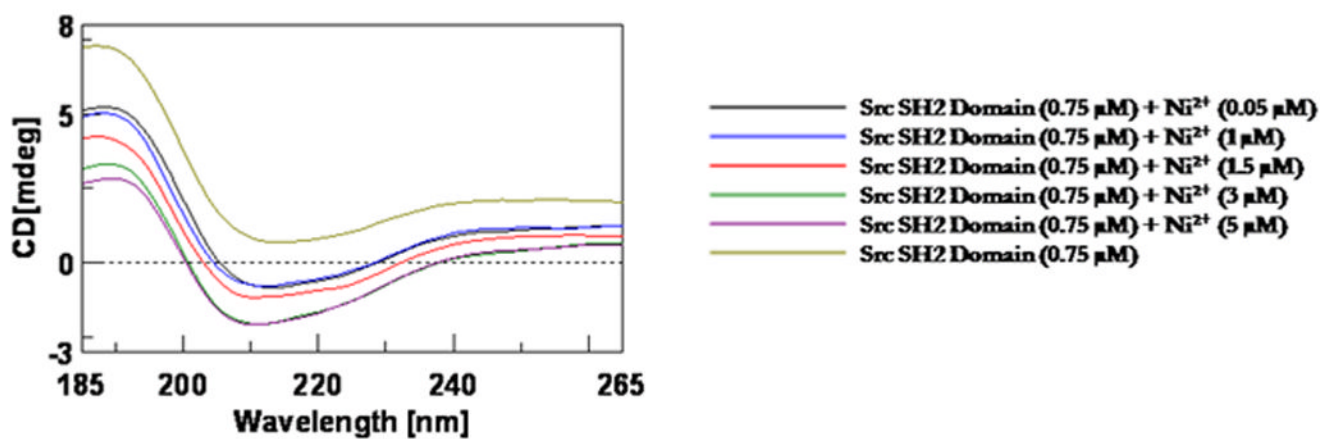


Figure 5.
The CD spectra of different concentration of Ni^{2+} and fixed concentration of Src SH2 domain.

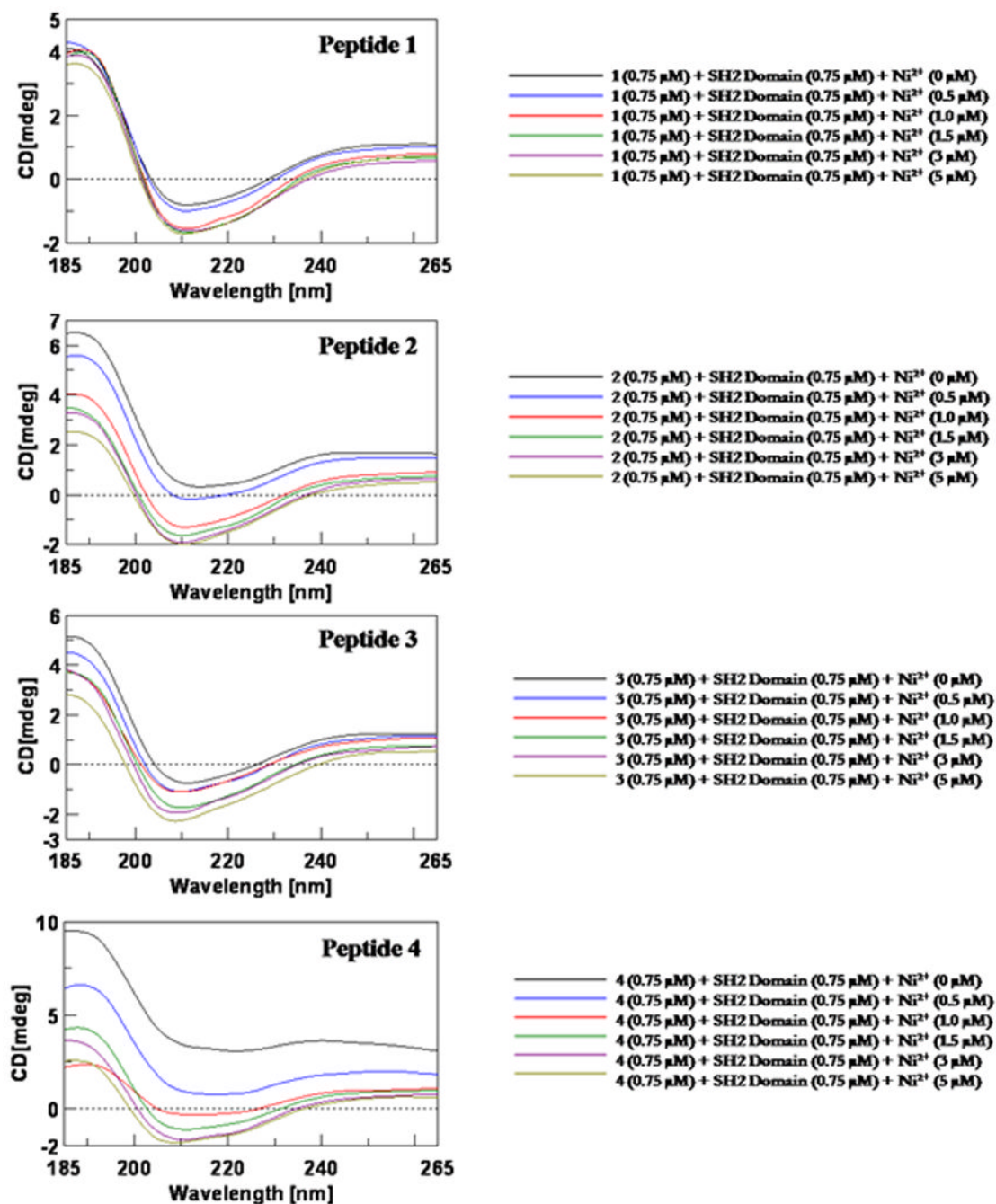
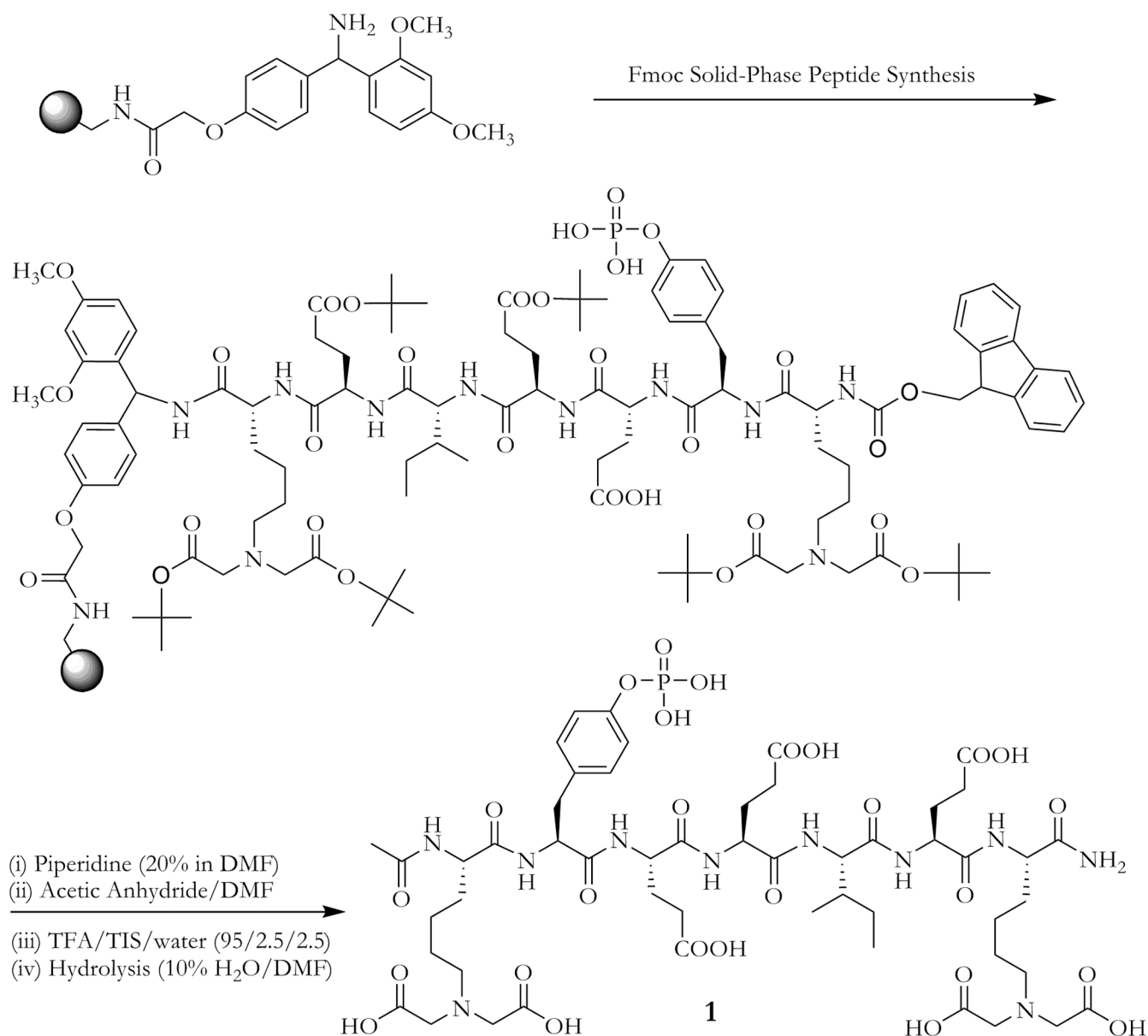


Figure 6.

The CD spectra of fixed concentration of peptides **1–4** and the Src SH2 domain with different concentrations of Ni^{2+} .



Scheme 1.
Solid-phase synthesis of peptide **1**.

Table 1The Binding Affinity of Peptides **1–4** to Ni²⁺.

Peptides	K _d (nM) ^a
Ac-pYEEI	>600
1	234.7
2	97.4
3	153.2
4	161.9

^aK_d: The concentration of peptides (Ac-pYEEI or **1–4**) at which half of them bind to Ni²⁺. Values are means of three experiments with a standard deviation of less than ± 5%.

Table 2

Binding Affinity of Peptides Containing IDA Group and Ac-pYEEI to the Src SH2 domain.

Peptides	K_d (μ M) ^a
Ac-pYEEI	1.70
1	0.63
2	52.70
3	2.96
4	3.35

^a K_d : The concentration of peptides (Ac-pYEEI or **1–4**) at which the binding site at the Src SH2 domain is half occupied. Values are means of three experiments with a standard deviation of less than $\pm 5\%$.

Table 3

Binding Affinity of the Peptides Containing 0–2 IDA Groups (**1–4**) toward the Src SH2 Domain in the Presence of Ni^{2+} .

Compounds	Ni^{2+} (μM)	K_d (μM) ^a
1	0	0.63
1	2	0.69
1	5	1.02
1	10	1.08
1	50	1.18
1	100	1.15
1	200	1.07
1	300	1.55
1	500	1.45
1 (300 μM EDTA)	300	0.79
2	0	52.70
2	2	55.68
2	300	39.30
3	0	2.96
3	2	2.98
3	300	3.70
4	0	3.35
4	2	4.13
4	300	4.01

^a K_d : The concentration of peptides (AcpYEEI or **1–4**) at which the binding site at the Src SH2 domain is half occupied. Values are means of three experiments with a standard deviation of less than $\pm 5\%$.

Table 4

Binding Affinity of Peptide **1** toward the Src SH2 Domain in the Presence of Different Concentration of Metals (Co^{2+} , Cd^{2+} , Cu^{2+} , and Zn^{2+}).

Compounds	K_d (μM) ^a
1 (2 μM Co^{2+})	1.09
1 (with 300 μM Co^{2+})	2.55
1 (with 2 μM Cd^{2+} , Cu^{2+} or Zn^{2+})	NA ^b
1 (with 300 μM Cd^{2+} , Cu^{2+} or Zn^{2+})	NA ^b

^a K_d : The concentration of peptides (AcpYEEI or **1–4**) at which the binding site at the Src SH2 domain is half occupied. Values are means of three experiments with a standard deviation of less than $\pm 5\%$

^bNA = no binding of F-GpYEEI with the Src SH2 domain was observed after metal addition.