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Synthesis and Evaluation of Conformationally Constrained Peptide Analogues as the Src SH3 Domain Binding Ligands

Synthesis and Evaluation of Conformationally Constrained Peptide Analogues as the Src SH3 Domain Binding Ligands

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Abstract.

Src kinase activity is regulated by the interaction of SH3 domain with protein sequences that are rich in proline residues. Identification of more potent SH3 domain binding ligands that can regulate Src kinase activity is a subject of major interest. Conformationally constrained peptides have been previously used for improving the binding potency of the Src SH2 domain binding peptide ligands and peptide substrates of the substrate-binding site of Src. A series of peptide analogues of Ac-VSLARRPLPPLP (**1**, Ac-VSL-12, K_d = 0.34 μM) were synthesized by introducing conformational constraints to improve the binding affinity towards the Src SH3 domain. Peptides synthesized through cyclization between *N*-terminal to *C*-terminal [VSLARRPLPPLP] or *N*-terminal to side chain flanking residues (i.e., [$^{\beta}$AVS]LARRPLPPLP and [VSLE]RRPLPPLP) exhibited at least 6.4-fold less binding affinity (K_d = 2.19-4.85 μM) when compared to **1**. The data suggest upon *N*-terminal cyclization with *C*-terminal or flanking residues, the interactions of the amino acids in the core RPLPPLP reduce significantly with the residues within the Src SH3 domain. Conformationally constrained peptide V[SLARRPLPPLP] **(5)** was synthesized through cyclization of *C*-terminal to the serine side chain and displayed a comparable binding affinity (K_d = 0.35 μM) towards the Src SH3 domain versus that of **1**. Thus, this template may be used to optimize and generate more potent analogues with higher stability.

1. Introduction

Protein tyrosine kinases (PTKs) catalyze the phosphorylation of specific tyrosine residues in proteins. Src, a member of nonreceptor tyrosine kinases, is involved in many cellular signaling pathways. Src contributes significantly in growth, differentiation, proliferation, development, and motility of many normal cell types [1]. Elevated levels of Src kinase activity are found in different cancer cells [2]. Thus, control or inhibition the Src kinase activity through chemical intervention has become a subject of major interest in drug discovery.

Src kinase contains an *N*-terminal unique domain that constitutes the Src homology (SH)4, followed by SH3 and SH2 regulatory domains, a kinase (catalytic) domain, and a *C*-terminal tail which includes a critical tyrosine residue (Tyr527). SH2 and SH3 regulatory domains mediate protein–protein interactions in cellular signaling cascades [3]. Specificity of interacting proteins and peptides with Src is determined by the chemical properties of the module binding surface of the Src SH3 and SH2 domains, which determines a preference for a common sequence with a range of variability. SH3 domains consist of approximately 60 amino acids and bind specifically to protein sequences that are rich in proline residues [4], whereas SH2 domains bind to protein sequences containing a phosphotyrosine residue [5].

The phosphorylation of the Tyr527 in Src by another PTK, Csk, leads to intramolecular binding of the SH2 domain to the *C*-terminal tail and inactivation of the enzyme (closed state) [4,6]. The Src SH3 domain stabilizes this state by interacting with the linker that connects the SH2 domain to the catalytic domain of the kinase. Src becomes active by autophosporylation of Tyr416 residue on the catalytic domain (open state) [7].

Src uses the SH3 domain to recruit numerous proteins which contain a polyproline motif such as Sam 68 [8], STAT3 [9], and p130cas [10,11]. A Src SH3 domain binding ligand can block the protein–protein interactions between Src and other proteins involved in signal transduction pathways.

Two classes of the Src SH3 binding ligands have been found that bind in opposite orientation. Classes I and II contain the consensus sequences of RXLPPXP and XPPLPXR, respectively [12]. The Src SH3 domain has a highly mobile and flexible region called RT loop containing arginine (R) and threonine (T) residues. RT loop is involved in interacting with the Src SH3 domain binding ligands. The binding capacity of both classes correlates on the positively-charged Arg residue of the ligands interacting with a negatively-charged residue in the RT loop of the Src SH3 domain. Classes I and II bind to the SH3 domain with the *N*-terminus or *C*-terminus of the ligand towards the RT loop, respectively.

Screening of the SH3 domain with c-DNA libraries, phage display, and biased peptide libraries has revealed a minimum recognition sequence, PLPPLP, for binding to the Src SH3 domain. Rickles *et al.* used biased phage display libraries to identify sequence RPLPPLP as the Src SH3 domain binding ligand with the K_d value of 17.7 μM [13]. Addition of 5 more residues (VSLAR) flanking to the *N*-terminal of core ligand RPLPPLP generated peptide $V_1S_2L_3A_4R_5R_6P_7L_8P_9P_{10}L_{11}P_{12}$ (VSL-12; K_d = 0.45 μM) with an improved binding affinity of 39-fold versus the core ligand [14]. The structural complex of the ligand and the Src SH3 domain using NMR showed multiple interactions between the peptide residues in the core region and the Src SH3 domain [14].

Ferguson *et al.* showed a bivalent ligand strategy to increase binding between SEM-5 SH3 domain and its natural ligand. A conjugate of PPPVPPR and a cyclic peptide of 6 amino acid (formed by disulfide bonds between two cysteine residues) improved the binding affinity to

SH3 domain by 1000-fold as compared to natural ligand [15]. Cyclization strategy has been commonly used in developing diagnostic and therapeutic peptidic and peptidomimetic agents [16-19]. Since peptides generally adopt highly flexible conformations in solution, a cyclization approach is used to reduce the conformational freedom of the molecules and improving binding affinity of specific conformation. Conformationally constrained structures become usually more selective in their affinity towards specific receptors and more stable towards proteases. In addition, cyclization often results in higher receptor binding affinity possibly by reducing unfavorable entropic effects [20].

We previously designed conformationally constrained peptides for improving the binding potency of SH2 domain binding peptide ligands [21] and peptide substrates of the substrate-binding site of Src [22], demonstrating significantly higher binding affinity by cyclized peptides compared to the corresponding linear analogues. Herein, we investigated to determine whether the cyclization of the sequence in VSL-12 can enhance the binding affinity of the ligand to the SH3 domain. Three cyclization strategies, head to tail, *C*-terminal to side chain, and *N*-terminal to side chain, were used to synthesize conformationally constrained peptides (Fig. 1).

"Please insert Figure 1 here."

2. Materials and Methods

2.1. Abbreviations

Symbols and abbreviations for amino acids and peptides are in accord with the recommendations of the IUPAC-IUB Commission on Nomenclature (*J. Biol. Chem.* 1972, *247*, 977). Other abbreviations used are: AcOH, acetic acid; Boc, *tert*-butyloxycarbonyl; *t*Bu, *tert*-butyl; DIC, *N*,*N*′-diisopropylethylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; Dmab, 4-[[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino]phenyl]methyl] ester); Flu, 5(6)-carboxyfluorescein; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate); HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; Mtt, 4-methyltrityl; NMM, *N*-methylmorpholine; Pbf, (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl); 2-Ph*i*Pr, 2-phenylisopropyl; PyAOP, 7-azabenzotriazol-1-yloxy-tripyrrolidinophosphonium hexafluorophosphate; SELDI-TOF, surface-enhanced laser desorption/ionization time-of-flight; *t*Bu, *tert*-butyl; trt, trityl; TFA, trifluoroacetic acid; TIS, triisopropylsilane. .

2.2. Materials

All reagents used for bacterial culture and protein expression were purchased from Fisher Scientific. Glutathione S transferase (GST) was purchased from Sigma-Aldrich Chemical Co (Milwaukee, WI). All the reagents for peptide synthesis were purchased from the Novabiochem. Fmoc-amino acids, H-Pro-2-chlorotrityl chloride resin, coupling reagents, and Fmoc-amino acid building blocks were purchased from Novabiochem. Other chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI).

2.3. General

All reactions were carried out in Bio-Rad polypropylene columns by shaking and mixing using a Glass Col small tube rotator in dry conditions or on a PS3 automated peptide synthesizer (Rainin Instrument Co., Inc.) at room temperature unless otherwise stated. In general, all peptides were synthesized by the solid-phase synthesis strategy employing Fmoc-based chemistry and Fmoc-L-amino acid building blocks. HBTU and DIPEA in DMF were used as coupling and activating reagents, respectively. Fmoc-deprotection at each step was carried out in the presence of piperidine in DMF two times (20% v/v, 10x volume as compared with resin) followed by washing with DMF. Final cleavage of the peptides from the solid support was achieved by using reagent R (TFA/thioanisole/1,2-ethanedithiol/anisole 90:5:3:2 v/v/v/v, volume 5x compared to dried resin) for 2 h. Crude peptides were precipitated by addition of cold diethyl ether (Et_2O), separated, washed by centrifugation (washed with diethyl ether, 3 × 50 mL and centrifuged at 4000 rpm for 5 min), and were purified by preparative reverse-phase HPLC (Shimadzu LC-8A preparative liquid chromatograph) on a Phenomenex-Gemini C18 column (10 µm, 250 × 21.2 mm). The peptides were separated by eluting the crude peptide at 12.0 mL/min using a gradient of 5-65% acetonitrile (0.1% TFA) and water (0.1% TFA) over 60 min, and then, they were lyophilized. Chromatograms were recorded at 220 nm using a UV detector. The purity of final products (>95%) was confirmed by HPLC. The chemical structures of compounds were determined by a SELDI-TOF mass spectrometer on a Ciphergen protein chip instrument using α-cyano-4-hydroxycinnamic as a matrix. Details of procedures are presented below. All of the amino acids in Ac-$V_1S_2L_3A_4R_5R_6P_7L_8P_9P_{10}L_{11}P_{12}$ (**1**) were numbered on the basis of their positions relative to the *N*-terminal valine residue (V1).

2.3.1. Peptide Synthesis

2.3.1.1. Ac-$V_1S_2L_3A_4R_5R_6P_7L_8P_9P_{10}L_{11}P_{12}$ (Ac-VSL-12, 1) and Fluorescein-VSLARRPLPPLP (2, Flu-VSL-12). H-Pro-2-chlorotrityl chloride resin (**11**, 0.91 g, 0.40 mmol, 0.44 mmol/g) was swollen under dry nitrogen using anhydrous DMF for about 15 min. The excess of the solvent was filtered off. The swelling and filtration steps were repeated for 2 more times before the coupling reactions. Fmoc-Leu-OH (1.47 g, 4.16 mmol) was coupled to the *N*-terminal of proline-trityl resin using HBTU (1.52 g, 4.0 mmol) and DIPEA (1.45 mL, 8.8 mmol) in DMF (5 mL) by mixing for 1.5 h. After the coupling was completed (confirmed by Kaiser test), the reaction solution was filtered off and the resin was collected by filtration and washed with DMF (7 × 25 mL), followed by Fmoc-deprotection using piperidine in DMF (20% v/v, 25 mL, 2 times, 5 min and 10 min). The resin was washed with DMF (7 × 25 mL). The subsequent amino acids, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ser(O*t*Bu)-OH, and Fmoc-Val-OH, respectively, were coupled in a similar manner. Fmoc-deprotection at *N*-terminal was carried out in the presence of piperidine in DMF (20% v/v, 25 mL, 2 × 10 min) to afford **12**. The resin was collected by filtration and washed with DMF (10 × 25 mL). Resin **12** was divided into two portions, each with 0.2 mmol scale: (1) To the first portion of the resin (0.2 mmol) was added Ac_2O (945 µL, 10 mmol) and DIPEA (1.74 mL, 10.6 mmol) in DMF (6 mL). The mixture was shaken at room temperature for 45 min. (2) The second portion of resin **12** was labeled with carboxyfluoresceine at *N*-terminal. 5(6)-Carboxyfluorescein (0.38 g, 1 mmol) was preactivated with PyAOP (0.52 g, 1 mmol) and DIPEA (0.35 mL, 2.1 mmol) in DCM/DMF (1:9 v/v, 5 mL) for 10 min and was added to the second portion of resin **12**. The mixture was shaken at room temperature for 3.5 h. The completion of the coupling was monitored by Kaiser test. The excess

of the reagents were washed with DMF (10 × 25 mL). Both portions were washed with DMF, DCM, DMF-MeOH, and MeOH, respectively (each with 2 × 25 mL) to remove the remaining of reagents and impurities, The resins were dried in vacuum for 24 h. Freshly prepared cleavage cocktail, reagent R, TFA/thioanisole/1,2-ethanedithiol/anisole (90:5:3:2 v/v/v/v, 10 mL), was added to the resins. The mixtures were shaken at room temperature for 2 h. The resins were collected by filtration and washed with another 2 mL of cleavage cocktail. Combined filtrates were evaporated to a minimum volume under dry nitrogen. After precipitation in Et_2O, the crude peptides were lyophilized and purified by using a semi-preparative reverse-phase HPLC. Ac-VSL-12 (**1**): SELLDI-TOF (m/z) [$C_{63}H_{108}N_{18}O_{15}$]: calcd., 1356.82; found 1357.79, $[M + H]^+$, 1380.69 $[M + Na]^+$. Flu-VSL12 **(2):** SELDI-TOF (m/z) [$C_{82}H_{118}N_{18}O_{20}$]: calcd., 1674.88; found, 1675.82 $[M + H]^+$, 1697.87 $[M + Na]^+$.

2.3.1.2. [VSLARRPLPPLP] (3, head to tail cyclization). The linear peptide was assembled on the H-Pro-2-chlorotrityl chloride resin (**11**, 0.44 mmol/g) in 0.41 mmol scale as described above. After the final deprotection of the *N*-terminal Fmoc group, the protected peptide was cleaved from trityl resin **12** in the presence of the cleavage cocktail AcOH/TFE/DCM (1:2:7 v/v/v, 40 mL) for 1 h at room temperature to yield **13**. The resin was collected by filtration and washed with TFE/DCM (2:8 v/v, 2 × 10 mL). The combined filtrates were evaporated in vacuum. To the residue was added hexane (2 × 25 mL) to remove the acetic acid from the mixture. The crude protected peptide was dried in vacuum overnight. Examining a small portion of the crude showed a major peak of the protected peptide by the HPLC. Thus, the crude was used directly for the cyclization. The dried crude linear protected peptide was dissolved in the DMF/DCM (5:1 v/v, 60 mL). HOAt (223 mg, 1.64 mmol, 4 equiv) and DIC (290 µL, 1.86 mmol, 4.5 equiv.) were added to the mixture and the solution was stirred for 4 h. After completion of the cyclization, as shown by the SELDI-TOF, the solvents were removed under reduced pressure on a rotary evaporator. The crude cyclic protected peptide was dried overnight in vacuum before the final cleavage. Freshly prepared cleavage cocktail, reagent R, TFA/thioanisole/1,2-ethanedithiol/anisole (90:5:3:2 v/v/v/v, 10 mL) was added to dried the cyclic protected peptide. The mixture was stirred at room temperature for 2 h. The cleavage cocktail was concentrated to a minimum volume under reduced pressure by a rotary evaporator. After precipitation of crude peptide in cold Et_2O and centrifugation, the crude peptide was lyophilized and purified by using a semi-preparative reverse-phase HPLC. SELLDI-TOF (m/z) [$C_{61}H_{104}N_{18}O_{13}$]: calcd., 1296.80; found, 1297.91 $[M + H]^+$, 1320.12 $[M + Na]^+$, 1336.01 $[M + K]^+$.

2.3.1.3. Ac-VS$_{Ac}$LARRPLPPLP (4). The linear peptide was assembled on the H-Pro-2-chlorotrityl chloride resin (**11**, 0.45 g, 0.20 mmol, 0.44 mmol/g) on a PS3 automated peptide synthesizer. HBTU (4 equiv) and NMM (0.40 M) in DMF were used as coupling and activating reagents. Piperidine in DMF (20% v/v) was used as the deprotecting reagent. The sequence was assembled using double coupling with Fmoc-Amino Acid-OH building blocks (4 equiv.) as compared to the resin. The building block used in the synthesis were Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ser(OTrt)-OH, and Fmoc-Val-OH, respectively. Deprotection of the Fmoc group at *N*-terminal of the sequence with piperidine in DMF (20% v/v) afforded **14**. A small portion of the resin was cleaved. The crude was used for confirming the molecular weight by SELDI-TOF indicating the presence of the linear sequence. The resin-attached peptide was mixed with the cleavage cocktail (TFA/DCM/TIS, 1:94:5 v/v/v) (4 ×15

mL, 10 min.) under mild conditions. The resin was collected by filtration and washed with dry DCM (5 × 25 mL). Collected combined filtrates were evaporated under reduced pressure. Partially protected peptide was dried in vacuum and was treated with Ac_2O (945 µL, 10 mmol) in DMF (6 mL) in the presence of DIPEA (1.74 mL, 10.6 mmol) at room temperature for 30 min. The solvents were removed under reduced pressure on a rotary evaporator and the crude protected peptide was dried overnight before the final cleavage. Freshly prepared cleavage cocktail, TFA/thioanisole/1,2-ethanedithiol/anisole (90:5:3:2 v/v/v/v, 10 mL) was added to the mixture and the solution was stirred at the room temperature for 2 h. The excess of the solvents were removed under reduced pressure using a rotary evaporator. After precipitation of the crude peptide in cold Et_2O and centrifugation, the crude peptide was lyophilized and purified by using a semi-preparative reverse-phase HPLC. SELLDI-TOF (m/z) [$C_{61}H_{104}N_{18}O_{13}$]: calcd., 1367.84; found, 1368.81 $[M + H]^+$, 1391.03 $[M + Na]^+$, 1406.72 $[M + K]^+$.

2.3.1.4. V[SLARRPLPPLP] (5, *C*-terminal to side chain cyclization). The linear peptide was assembled on H-Pro-2-chlorotrityl chloride resin (**11**, 0.91 g, 0.40 mmol, 0.44 mmol/g) as described above on a PS3 automated peptide synthesizer. Boc-Val-OH was used in place of the regular Fmoc-Val-OH at position 1. After assembling the linear peptide chain, using building blocks Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ser(OTrt)-OH, and Boc-Val-OH, a small portion of the resin was cleaved, and the molecular weight was confirmed by SELDI-TOF. The resin-attached peptide was mixed with the cleavage cocktail, TFA/DCM/TIS (1:94:5 v/v/v, 5 × 25 mL, 10 min.) to deprotect the trityl group from the side chain of the serine and to cleave the partially protected peptide from the resin to afford **15**. The resin was collected by filtration and was washed with dry DCM (5 × 25 mL). The combined filtrates were evaporated under reduced pressure. The partially protected crude peptide was dried in vacuum overnight. A small portion of the crude showed a major peak of protected peptide by HPLC. Thus, the crude was used directly for the cyclization. The crude peptide was dried and dissolved in DMF/DCM (5:1 v/v, 60 mL). HOBt (109 mg, 0.8 mmol), HBTU (303 mg, 0.8 mmol), DIC (125 µL, 0.8 mmol), and DIPEA (697 µL, 4.2 mmol) were added to the solution. The mixture was stirred slowly at room temperature for 8 h. After completion of the cyclization, as shown by SELDI-TOF, the solvents were removed on a rotary evaporator. The crude cyclic protected peptide was dried overnight in vacuum before the final cleavage. Freshly prepared cleavage cocktail, reagent R, TFA/thioanisole/1,2-ethanedithiol/anisole (90:5:3:2 v/v/v/v, 10 mL), was added to dried cyclic protected peptide. The mixture was stirred at room temperature for 2 h. After 2 h, the cleavage cocktail was concentrated under reduced pressure to minimum volume by a rotary evaporator. After precipitation of the crude peptide in cold Et_2O and centrifugation, the crude peptide was lyophilized and purified by using a semi-preparative reverse-phase HPLC. SELDI-TOF (m/z) [$C_{61}H_{104}N_{18}O_{13}$]: calcd., 1296.80; found 1297.92 $[M + H]^+$, 1320.13 $[M + Na]^+$, 1336.02 $[M + K]^+$.

2.3.1.5. [$^{\beta}$AVS]LARRPLPPLP (6, *N*-terminal to side chain cyclization). The linear peptide was synthesized on a PS3 automatic peptide synthesizer as described above using Fmoc-Pro-Wang resin (**16**, 0.35 mmol, 0.51 mmol/g). HBTU (4 equiv) and NMM (0.37 M) in DMF were used as coupling and activating reagents. Piperidine in DMF (20% v/v) was used as the deprotecting reagent. The sequence was assembled using double coupling with Fmoc-Amino Acid-OH building blocks (4 equiv.) as compared to the resin. The building block used in the synthesis were Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Pro-OH,

Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ser(OTrt)-OH, Fmoc-Val-OH, and Fmoc-β-Ala-OH, respectively. After deprotection of the Fmoc group at the end of *N*-terminal of the sequence with piperidine in DMF (20% v/v), a small portion of the resin was cleaved and the molecular weight of the peptide was confirmed by SELDI-TOF. The trityl group from the side chain of the serine was removed under mild conditions (TFA/DCM/TIS, 1:94:5 v/v/v, 30 mL, 4 × 10 min.) to yield **17**. The resin was collected by filtration and was washed with dry DCM (5 × 50 mL). The resin was dried in vacuum for 30 min. To the resin-attached peptide was added, DMF/DCM (60 mL, 5:1 v/v), HOAt (190.5 mg, 1.4 mmol, 4 equiv), and DIC (245 µL, 1.57 mmol, 4.5 equiv) and the mixture was shaken at room temperature for 8 h. The completion of cyclization was confirmed by Kaiser test as well as by SELDI-TOF. The resin was collected by filtration. After washing the resin by DMF, DCM, and MeOH, respectively (each 5 × 30 mL), the resin was dried in vacuum for 24 h. Freshly prepared cleavage cocktail, reagent R, TFA/thioanisole/1,2-ethanedithiol/anisole (90:5:3:2 v/v/v/v, 12 mL), was added to dried resin-attached peptide. The mixture was shaken at room temperature for 2 h. After filtration, the filtrate was concentrated to a minimum volume under reduced pressure by a rotary evaporator. After precipitation of the crude peptide in cold Et_2O and centrifugation, the crude peptide was lyophilized and purified by using semi-preparative reverse-phase HPLC. SELDI-TOF (m/z) [$C_{61}H_{104}N_{18}O_{13}$]: calcd., 1367.84; found, 1368.81 $[M + H]^+$, 1391.03 $[M + Na]^+$, 1406.71 $[M + K]^+$

2.3.1.6. Ac-VSLK$_{Ac}$RRPLPPLP (7). The linear peptide was assembled on the H-Pro-2-chlorotrityl chloride resin (**11**, 0.45 g, 0.20 mmol, 0.44 mmol/g) as described above on a PS3 automated peptide synthesizer. Fmoc-Lys (Mtt)-OH was used in place of Fmoc-Ala-OH at position 4. Assembling of the linear peptide chain was carried out using building blocks, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys (Mtt)-OH, Fmoc-Leu-OH, Fmoc-Ser(O*t*Bu)-OH, and Fmoc-Val-OH, respectively. After deprotection of the Fmoc group at the end of *N*-terminal of the sequence, a small portion of resin **18** was cleaved. The crude was monitored with SELDI-TOF confirming the molecular weight of the linear sequence. Resin **18** was mixed with the cleavage cocktail, (TFA/DCM/TIS) (2:93:5 v/v/v, 15 mL, 5 × 15 min.), to deprotect the Mtt group from the side chain of the lysine and to cleave the partially protected peptide from the resin. The resin was collected by filtration and was washed with dry DCM (5 × 25 mL). Collected combined filtrates were evaporated in vacuum. The partially protected peptide was dried in vacuum and was treated with Ac_2O (945 µL, 10 mmol) and DIPEA (1.74 mL, 10 mmol) in DMF (6 mL). The mixture was shaken at room temperature for 30 min. The solvents were removed under reduced pressure on a rotary evaporator and the crude was dried overnight before the final cleavage. Freshly prepared cleavage cocktail, TFA/thioanisole/1,2-ethanedithiol/anisole (90:5:3:2 v/v/v/v, 10 mL), was added to the crude and the mixture was stirred at the room temperature for 2 h. The excess of the solvent was removed using a rotary evaporator. After precipitation of the crude peptide in cold Et_2O, and centrifugation, the crude peptide was lyophilized and purified by using a semi-preparative reverse-phase HPLC. SELDI-TOF (m/z) for [$C_{68}H_{117}N_{19}O_{16}$]: calcd, 1455.89; found, 1456.61 $[M + H]^+$, 1478.90 $[M + Na]^+$, 1494.81 $[M + K]^+$.

2.3.1.7. VSL[KRRPLPPLP] (8, *C*-terminal to side chain cyclization). The linear peptide was assembled on H-Pro-2-chlorotrityl chloride resin (**11**, 0.91 g, 0.40 mmol, 0.44 mmol/g) as described above on a PS3 automated peptide synthesizer. Fmoc-Lys(Mtt)-OH and Boc-Val-OH

were used in place of Fmoc-Ala-OH and Fmoc-Val-OH at positions 1 and 4, respectively. The peptide assembly was carried out using building blocks Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys (Mtt)-OH, Fmoc-Leu-OH, Fmoc-Ser(O*t*Bu)-OH, and Boc-Val-OH to yield **19**. After completion of the sequence, a small portion of the resin was cleaved. The crude showed the molecular weight of the linear sequence using SELDI-TOF. Resin **19** was mixed with the cleavage cocktail, TFA/DCM/TIS (2:93:5 v/v/v, 15 mL, 5 × 15 min.), to deprotect the Mtt group from the side chain of the lysine and to cleave the partially protected peptide from the resin to yield **20**. The resin was washed with dry DCM (5 × 25 mL) and collected combined filtrates were evaporated under reduced pressure. After drying the partial protected crude peptide, the cyclization of the side chain with the *C*-terminal was achieved in the solution phase. The crude was dried and dissolved in DMF/DCM (5:1 v/v, 60 mL). HOAt (223 mg, 1.64 mmol) and DIC (290 µL, 1.86 mmol) were added and the mixture was stirred for 8 h. After completion of the cyclization, as shown by the SELDI-TOF, the solvents were removed on a rotary evaporator. The crude cyclic protected peptide was dried overnight before the final cleavage. Freshly prepared cleavage cocktail reagent R, TFA/thioanisole/1,2-ethanedithiol/anisole (90:5:3:2 v/v/v/v, 10 mL), was added to dried cyclic protected peptide. The solution was stirred at room temperature for 2 h. The cleavage cocktail was concentrated to a minimum volume under reduced pressure by using a rotary evaporator. After precipitation of the crude peptide in cold Et_2O, and centrifugation, the crude peptide was lyophilized and purified by using a semi-preparative reverse-phase HPLC. SELDI-TOF (m/z) for $[C_{61}H_{104}N_{18}O_{13}]$: calcd., 1354.70; found, 1356.11 $[M+H]^+$, 1378.31 $[M + Na]^+$, 1393.70 $[M + K]^+$.

2.3.1.8. VSLERRPLPPLP (9) and [VSLE]RRPLPPLP (10, *N*-terminal to side chain cyclization). The linear peptide was assembled on H-Pro-2-chlorotrityl chloride resin (**11**, 0.91 g, 0.40 mmol, 0.44 mmol/g) as described above on a PS3 automated peptide synthesizer. Fmoc-Glu(ODmab)-OH was used in the place of Fmoc-Ala-OH at position 4. The Fmoc group of the *N*-terminal was removed with piperidine (20%) to yield **21**. The Dmab group from the side chain of the glutamic acid was removed using hydrazine hydrate in DMF (2%, 3 × 25 mL) for 10 min to afford **22**. The resin-attached peptide was washed with the excess of the solvents, DMF, DCM, and DMF, respectively (each 3 × 35 mL) and was dried in vacuum overnight. The resin was divided into two portions. The first portion was used for the synthesis of the linear peptide by cleavage using reagent R to yield **9** as described below. The second part of **22** was used for the cyclization between *N*-terminal and the COOH group of the side chain of glutamic acid. The resin was swollen in the dry DMF under nitrogen. The excess of solvent was removed and to the resin was added DMF/DCM (5:1 v/v, 60 mL), HOAt (112 mg, 0.82 mmol), and DIC (145 µL, 9.3 mmol). The mixture was agitated for 12 h at room temperature. After completion of the cyclization, as shown by SELDI-TOF by cleavage of a few resin beads, the solvents were removed by filtration. The resin was washed with excess of solvents, DMF, DCM, and MeOH (3 × 35 mL, each), respectively, and dried in vacuum overnight. Freshly prepared cleavage cocktail, TFA/thioanisole/1,2-ethanedithiol/anisole (90:5:3:2 v/v/v/v, 10 mL), was added to dried cyclic and linear peptide resins and the mixture was shaken at room temperature for 2 h. The resins were collected by filtration and washed with another 2 mL of cleavage cocktail. Combined filtrates of both peptides were evaporated in separate flask to a minimum volume amount under dry nitrogen. After precipitation of the crude peptide in cold Et_2O, and centrifugation, the crude peptide was lyophilized and purified by using semi-preparative reverse-phase HPLC. **9**: SELDI-

TOF (m/z) for [$C_{63}H_{108}N_{18}O_{16}$]: calcd., 1372.82; found, 1374.0 $[M + H]^+$; 1395.91 $[M + Na]^+$. **10**: SELDI-TOF (m/z) for [$C_{61}H_{104}N_{18}O_{13}$]; calcd., 1355.63; found, 1356.64 $[M + H]^+$; 1378.70 $[M + Na]^+$.

2.3.2. SH3 domain protein purification. The Chicken Src SH3 domain was expressed as a glutathione *S*-transferase fusion protein in *E. coli* DH5α cells using the plasmid pGEX-4T1. Upon sequence verification, a 500 mL culture of cells in LB and ampicillin were grown at 37 ºC while shaking at 250 rpm. When an OD_{600} of about 0.6 was achieved, the culture was diluted with another 500 mL of fresh LB and ampicillin and expression was induced with 400 μM IPTG. The induction was for 8 h at 250 rpm and 20 ºC. Harvesting of the cells by centrifugation was followed by freeze-thaw cycle and re-suspension in fresh PBS buffer pH 7.4. The cells were then sonicated and the lysate was cleared by centrifugation. The lysate was incubated for a period of one hour with approximately 1 mL glutathione-agarose at 4 ºC. This lysate was then passed through a column and washed with 40 mL each of PBS, 50 mM Tris-Cl pH 7.4, and 50 mM Tris-Cl pH 8.0. The elution of purified GST-Src SH3 fusion protein was then carried out with 10 mM reduced glutathione in 50 mM Tris pH 8.0. All buffers used were chilled and 0.1% β-mercaptoethanol was added fresh prior to use. Eluted fractions were qualified and quantified by Bradford assay and SDS-PAGE, respectively (Fig. 2). These fractions, ranging from 2.25 to 7.50 g/L in concentration, were aliquoted and stored in the elution buffer at -20 ºC

"Please insert Figure 2 here."

2.3.3. Fluorescence polarization assay

To determine the binding of VSL12 towards the Src SH3 domain, fluorescein-labeled VSL12 was synthesized and purified by HPLC as described above. The fluorescence polarization (FP) binding assay was carried out as described previously [23]. Flu-VSL12 (120 nM) was kept constant in the presence of increasing SH3 domain concentration in 50 mM Tris pH 8.0 buffer. FP was then detected using a PerkinElmer LS55 Luminescence Spectrophotometer at 25 ºC. Wavelengths utilized were 485 nM for excitation and 530 nM for emission. The net change in FP as a function of the SH3 domain concentration was fit to the following equation: $FP = FP_{max} \times [SH3] / (K_d + [SH3])$, where FP_{max} was the maximum polarization value at saturation and K_d was the dissociation constant of VSL12 binding to Src SH3 domain. Data analysis was carried out using LabFit software.

The binding of unlabeled Ac-VSL12 **(1)** to compete against Flu-VSL12 **(2)** in binding to the Src SH3 domain was evaluated. The initial competition screening tested the effect of 500 nM unlabeled derivative on the binding of Flu-VSL12 to the Src SH3 domain. The concentration of Flu-VSL12 and SH3 domain were 120 nM and 1 μM, respectively. Subsequently, a second round of screening was carried out with 2 μM unlabeled derivative in the presence of 2 μM of Src SH3 and 120 nM of Flu-VSL12.

All newly synthesized VSL12 derivatives **(3-10)** were then fully evaluated in triplicate, for their binding to the Src SH3. With Flu-VSL12 at 120 nM, VSL12 and SH3 domain were used at constant concentrations of 1 μM. The peptides were used at concentrations of 0, 0.25, 0.5, 1.0, 2.0, and 4.0 μM. The binding buffer was 50 mM Tris pH = 8.0. The K_d was then determined for the competing ligand by plotting the FP as a function of the increasing concentration of the unlabeled derivative and fitting the curve to the equation: $FP = A \times ([SH3]_t \times [Probe]_t \times K_{d2})/(K_{d1} \times K_{d2} + K_{d1} \times [Pept]_t + [SH3]_t \times K_{d2})$, where K_{d1} is the dissociation constant of Flu-VSL12, as

determined above and K_{d2} is the dissociation constant of the unlabeled VSL12 derivative. *A* is a conversion factor between the concentration of the probe-SH3 complex and the FP value. $[SH3]_t$ and $[Probe]_t$ were total SH3 and Flu-VSL12 concentrations. $[Pept]_t$ is the total concentration of unlabeled competing ligand.

3. Results and Discussion

3.1. Chemistry

The presence of proline residue at the carboxy terminus of a peptide during solid-phase peptide synthesis has been associated with high levels of diketopiperazine formation [24]. Thus, the synthesis was carried out on a 2-chlorotrityl chloride resin. The bulkiness of the trityl linker minimized diketopiperazine formation during tripeptide formation.

Scheme 1 shows the synthesis of Ac-VSL12 (Ac-VSLARRPLPPLP, **1**), the fluorescein-labeled VSL12 (Fluorescein-VSLARRPLPPLP, Flu-VSL-12, **2**)**,** and the conformationally constrained peptide **(3)** resulted from head to tail cyclization. To synthesize compound **1** and its carboxyfluorescein derivative **(2)**, H-Pro-2-chlorotrityl chloride resin **11** was used and the linear sequence was assembled on the resin using Fmoc/*t*Bu solid-phase peptide synthesis methodology (Scheme 1). The Fmoc group at the end of the synthesis was removed by using piperidine (20% v/v) in DMF to yield **12.** Resin-attached peptide **12** was capped by using acetic anhydride in DMF and underwent cleavage and deprotection to afford **1**. Alternatively, resin **12** was conjugated with the 5(6)-carboxyfluorescein in the presence of PyAOP/HOAt/DMF in DMF, which after final cleavage and deprotection gave fluorescent-labeled peptide **2**. The fluorescent probe was used for determination of the binding affinity between the Src SH3 domain and synthesized compounds.

The synthesis of conformationally constrained peptide [VSLARRPLPPLP] **(3)** by head to tail cyclization was performed by using resin **12**. The cleavage in the presence of AcOH/TFE/DCM (1:2:7 v/v/v) for 1 h at room temperature afforded the linear protected peptide **13** with free *N*- and *C*-terminals. Head to tail cyclization was carried out in the presence of HOAt/DIC in DMF/DCM. Deprotection with reagent R and purification afforded conformationally constrained peptide **3** (Scheme 1).

"Please insert Scheme 1 here."

The synthesis of diacetylated linear peptide Ac-VS$_{Ac}$LARRPLPPLP **(4)** is depicted in Scheme 2. Fmoc-Ser(Trt)-OH at position 2 was assembled as the building block on resin-attached peptide while synthesizing the sequence of the VSL-12 on H-Pro-2-chlorotrityl resin **(11)**. The Fmoc group was removed from the *N*-terminal to yield **14**. The deprotection of the trityl group and cleavage from the resin in the presence of cocktail (TFA/DCM/TIS) (1:94:5 v/v/v) afforded the partially protected peptide. The *N*-terminal of the peptide and side chain of serine were capped with acetyl group in the presence of acetic anhydride and DIPEA in DMF at room temperature to give **4**. Because of capped serine residue, **4** cannot be used to perform cyclization between *C*-terminal and side chain of serine.

To cyclize the peptide through *C*-terminal to side chain cyclization, Boc protected valine (Boc-Val-OH) and Fmoc-Ser(Trt)-OH were used at positions 1 and 2 while assembling the peptide on resin **11**. Thus, no capping step was required before the cyclization. The deprotection of the serine side chain and cleavage from the resin were achieved by using a cleavage cocktail,

TFA/DCM/TIS (1:94:5 v/v/v), to yield the partially protected peptide **15**, which underwent cyclization in the presence of HOBt, HBTU, DIC, DIPEA in DMF/DCM (5:1, v/v) at room temperature for 8 h. After cyclization between *C*-terminal and side chain of serine, the final deprotection was achieved by using reagent R to afford compound V[SLARRPLPPLP] **(5)** (Scheme 2).

"Please insert Scheme 2 here."

N-terminal valine to side chain serine cyclization failed when 2-chlorotrityl resin was used due to simultaneous deprotection of serine side chain and cleavage of partially protected peptide from the trityl resin in one step. Because of high sensitivity of trityl resin to acid, Fmoc-pro-Wang resin (**16)** was used for the synthesis (Scheme 3). After every coupling, a small amount of resin was cleaved to monitor the progress of the reaction by confirming the molecular weight of the peptide using SELDI-TOF. The cyclization between *N*-terminal valine and the side chain of serine was not successful. β-Alanine was assembled at the *N*-terminal of linear peptide. After Fmoc-deprotection, the trityl group from the side chain of serine was deprotected using a cleavage cocktail, TFA/DCM/TIS (1:94:5 v/v/v), to afford **17**. The cyclization between free amino group of β-alanine and the side chain of serine at position 3 was carried out in the presence of HOAt, DIC, and DMF/DCM (5:1 v/v) at room temperature for 8 h. The final cleavage afforded [$^{\beta}$AVS]LARRPLPPLP **(6)**.

"Please insert Scheme 3 here."

The synthesis of diacetylated linear peptide Ac-VSLK$_{Ac}$RRPLPPLP **(7)** was started using Fmoc solid-phase synthesis protocol on 2-chlorotrityl resin **11** using Fmoc-Lys(Mtt)-OH in place of alanine at position 4 (Scheme 4). After the assembly of the peptide, the Fmoc group was removed from the *N*-terminal to yield **18**. Deprotection of the MTT group followed by the cleavage of the partially protected peptide from the resin was achieved in the presence of cleavage cocktail, TFA/DCM/TIS (2:93:5 v/v/v). Capping the peptide with acetic anhydride, followed by deprotection by Reagent R afforded the diacetylated peptide Ac-VSLK$_{Ac}$RRPLPPLP **(7)**.

The synthesis of the conformationally constrained peptide VSL[KRRPLPPLP] **(8)** (*C*-terminal to side chain cyclization) was carried out using Boc-Val-OH in place of Fmoc-Val-OH at position 1 to afford **19**. The Mtt group from the side chain of lysine was cleaved and the partially protected peptide was cleaved from the resin in the presence of cleavage cocktail, TFA/DCM/TIS (2:93:5 v/v/v) to yield **20** (Scheme 4). *C*-terminal to side chain cyclization was achieved in the presence of HOAt and DIC in DMF/DCM (5:1 v/v). Final deprotection afforded the conformationally constrained peptide VSL[KRRPLPPLP] **(8)**.

"Please insert Scheme 4 here."

The synthesis of linear peptide VSLERRPLPPLP **(9)** and conformationally constrained peptide [VSLE]RRPLPPLP (**10**, *N*-terminal to side chain cyclization) is shown in Scheme 5. The linear peptide was assembled on resin **11** by using Fmoc-Glu (ODmab)-OH in the place of Fmoc-Ala-OH at position 4. Removing the Fmoc group of the *N*-terminal afforded resin-attached peptide **21**. The Dmab group from the side chain of the glutamic acid was removed using

hydrazine hydrate in DMF (2%) to yield **22**. A portion of resin-attached peptide was cleaved and deprotected to afford compound **9**. Furthermore, another part of resin-attached peptide **22** underwent cyclization between NH_2 group of the *N*-terminal and the side chain COOH group of glutamic acid residue in the presence of HOAt, DIC, and DMF/DCM (5:1 v/v). The final cleavage and deprotection afforded peptide **10**.
"Please insert Scheme 5 here."

3.2. Fluorescent Polarization Binding Assay

The binding affinities of the synthesized peptides **(2-10)** against the Src SH3 domain were examined and compared with the corresponding linear peptide (**1**) (Table 1) using a fluorescence polarization competitive assay [23]. Unlabeled linear probe **1** competed with probe **2** for binding to the Src SH3 domain resulting in the decreased FP values. Ac-VSL-12 **(1)** showed a K_d value of 0.34 μM that was consistent with the reported value [14].

VSL-12 contains a core RPLPPLP that forms a helix upon binding with the Src SH3 domain. VSLAR flanking sequence in VSL-12 was selected from a phage library to increase the binding affinity of the core sequence [14]. There are three major interactions between the core portion of VSL-12 and the Src SH3 domain (i.e. hydrophobic, electrostatic, and hydrogen bonding interactions). Both Leu^8-Pro^9 and Leu^{11}-Pro^{12} moieties bind to hydrophobic pockets containing Tyr^{90}, Tyr^{92}, Tyr^{136}, and Trp^{118} of the Src SH3 domain. Additionally, Arg^6 in RPLPPLP portion forms a salt bridge with Asp^{99} near the RT loop of the SH3 domain. The carbonyl of Arg^6 in VSL-12 and the indole of NH of Trp^{118} forms a hydrogen bond. Side chain NH_2 of Asn^{59} in the SH3 domain make an intermolecular hydrogen bond with the carbonyl of Pro^9 in the ligand. Other intermolecular hydrogen bonding interactions are formed between the carbonyl of Pro^{10} in the ligand and the hydroxyl of Tyr^{136} [14].

The corresponding conformationally constrained peptide **3**, synthesized through head to tail cyclization of VSL-12, showed 10-fold weaker binding affinity (K_d =3.5 μM) when compared with **1**. Decreased binding affinity towards the Src SH3 domain by a non-linear structure **3** may be due to the loss of some of hydrophobic, hydrogen bonding, and/or electrostatic interactions between some residues in the constrained peptide and the Src SH3 domain. The folded structure in **3** may not provide appropriate locations for the side chains of amino acids, Leu-Pro and Arg, in RPLPPLP for maximal interactions with hydrophobic pockets and negatively-charged Asp^{99} of the Src SH3 domain.

Diacetylated peptide **4** with a capped OH group of serine residue at position 2 exhibited a comparable binding affinity (K_d = 0.56 μM) versus that of VSL-12, suggesting that the side chain of serine may be used for the synthesis of constrained peptides without the significant loss of binding affinity. Thus, conformationally constrained peptide **5** was synthesized through *C*-terminal cyclization with the side chain residue of serine at position 2, and showed similar binding affinity (K_d = 0.35 μM) when compared the corresponding linear peptide **1**. These data suggest that cyclization between serine 2 in flanking residues (VSLAR) and *C*-terminal can be tolerated. Flanking residues Leu^3, Ala^4, and Arg^5 of VSL-12 occupy a large pocket surrounded by Trp^{118}, Tyr^{131} and RT loop of the Src SH3 domain [14]. The size of the pocket allows the cyclization with serine 2 in the flanking residues without disturbing the hydrophobic and electrostatic interactions by core RPLPPLP residues.

On the other hand, *N*-terminal cyclization with the side chain of serine residue at position 2 in flanking sequence in conformationally constrained peptide **6** decreased binding affinity (K_d =4.8 μM). These data indicate that use of *N*-terminal residue in generating conformationally

constrained peptides as shown in peptides **3** and **6** was counterproductive, possibly due to critical interaction of *N*-terminal residue valine 1 with the Src SH3 domain. The flanking sequences increase binding affinity of the ligands to SH3 domain and in the case of **6** the cyclization between flanking residues appears to significantly affect the ability of the peptide to bind with the Src SH3 domain.

The placement of a capped lysine residue at position 4 in peptide **7** in place of alanine reduced the binding affinity (K_d =2.2 μM). Similarly, the placement of negatively-charged glutamic acid at position 4 in peptide **9** was not beneficial (K_d =2.7 μM). Flanking residues Ala^4 of VSL-12 occupy a pocket surrounded by Trp^{118}, Tyr^{131} and RT loop of the Src SH3 domain and replacement of the Ala with a large side chains in **7** and **9** was not tolerated. As expected cyclization of *C*-terminal to side chain of lysine residue (K4) in peptide **8** did not improve the binding affinity (K_d =2.9 μM) possibly due to significant change in the structure for appropriate interactions with the SH3 domain.

Cyclization of *N*-terminal to side chain of glutamic acid residue at position 4 (E4) in peptide **10** improved the binding affinity marginally (K_d = 2.2 μM) when compared to the corresponding linear peptide **9**.

Table 1. The binding affinities of peptides towards the Src SH3 domain[a].

Peptide	Sequence	K_d[b]
VSL-12 (**1**)	Ac-VSLARRPLPPLP	0.34
3	[VSLARRPLPPLP]	3.54
4	Ac-VS(Ac)LARRPLPPLP	0.56
5	V[SLARRPLPPLP]	0.35
6	[$^{\beta}$AVS]LARRPLPPLP	4.85
7	Ac-VSLK(Ac)RRPLPPLP	2.21
8	VSL[KRRPLPPLP]	2.96
9	VSLERRPLPPLP	2.70
10	[VSLE]RRPLPPLP	2.19

[a][Src SH3] = 1 μM, [Flu-VSL12, **2**]=120 nM; [b]In general mean of three separate determinations with a standard deviation of less than 5%.

In general, these data provide structure-activity relationships for VSL-12 derivatives. The optimal binding of VSL-12 with the Src SH3 domain occur through delicate hydrophobic, electrostatic, and hydrogen bonding interactions of core and flanking residues of the ligand with specific residues of the protein when the peptide is oriented in an extended form. The Src SH3 domain binding pocket is shallow, thus the design of conformationally constrained peptides for this regulatory domain remains a challenge as residues in cyclized peptides lose some critical interactions. These results suggest that *N*-terminal cyclization reduces the binding affinity towards the Src SH3 domain compared to linear peptide **1** as shown in conformationally constrained peptides **3**, **6**, and **10.** Thus, *N*-terminal valine 1 in VSL-12 cannot be subjected to strain for generating more potent Src SH3 domain binding ligands. Further optimization of conformationally constrained peptide **5** or generating other constrained peptides through side chain to side chain cyclization may result in producing ligands with appropriate orientation and more optimal binding affinity and stability.

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Figure and Scheme Legends

Fig. 1. The chemical structures of the conformationally constrained and linear peptide analogues of VSL12 **(1-10)**.

Fig. 2. SDS-PAGE of GST-Src SH3 fusion protein, stained with Coomassie Blue.

Scheme 1. Synthesis of Ac-VSL-12 **(1)**, Flu-VSL-12 **(2)**, and [VSLARRPLPPLP] **(3)** (head to tail cyclization). Reagents and reaction conditions: (i) (a) Fmoc solid-phase synthesis: coupling of Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ser(O*t*Bu)-OH, and Fmoc-Val-OH, respectively, in the presence of HBTU/DIPEA in DMF with Fmoc-deprotection (20% v/v Piperidine in DMF) after each coupling; (ii) 20% Piperidine in DMF; (iii) Ac_2O, DIPEA, DMF; (iv) reagent R, 2 h; (v) 5(6)-carboxyfluorescein, PyAOP, DIPEA, DCM/DMF(1:9 v/v), 3.5 h; (vi) AcOH/TFE/DCM (1:2:7 v/v/v), 1h and then TFE/DCM (2:8 v/v); (vii) HOAt, DIC, DMF/DCM (5:1 v/v), 2 h.

Scheme 2. Synthesis of Ac-VS(Ac)LARRPLPPLP **(4)** and V[SLARRPLPPLP] (**5**, *C*-terminal to side chain cyclization). Reagents and reaction conditions: (i) Fmoc solid-phase synthesis: coupling of Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ser(OTrt)-OH, and Fmoc-Val-OH, respectively, in the presence of HBTU/DIPEA in DMF with Fmoc-deprotection (20% Piperidine in DMF) after each coupling; (ii) 20% Piperidine in DMF; (iii) (TFA/DCM/TIS) (1:94:5 v/v/v), (iv) Ac_2O DIPEA, DMF; (v) Reagent R, 2 h; (vi) Fmoc solid-

phase synthesis: coupling of Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ser(OTrt)-OH, and Boc-Val-OH, respectively, in the presence of HBTU/DIPEA in DMF with Fmoc-deprotection (20% Piperidine in DMF) after each coupling; (vii) TFA/DCM/TIS (1:94:5 v/v/v), (viii) HOBt, HBTU, DIC, DIPEA in DMF/DCM (5:1 v/v), 8 h.

Scheme 3. Synthesis of [$^{\beta}$AVS]LARRPLPPLP **(6)** (*N*-terminal to side chain cyclization). Reagents and reaction conditions: (i) Fmoc solid-phase synthesis: coupling of Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ser(Trt)-OH, Fmoc-Val-OH, and Fmoc-β-Ala-OH, respectively, in the presence of HBTU/DIPEA in DMF with Fmoc-deprotection (20% piperidine in DMF) after each coupling; (ii) 20% Piperidine in DMF; (iii) TFA/DCM/TIS (1:94:5 v/v/v); (iv) HOAt (4 equiv), DIC (4.5 equiv.), DMF/DCM (5:1 v/v), 8 h; (v) Reagent R.

Scheme 4. Synthesis of Ac-VSLK$_{Ac}$RRPLPPLP **(7)** and VSL[KRRPLPPLP] **8** (*C*-terminal to side chain cyclization). Reagents and reaction conditions: (i) Fmoc solid-phase synthesis: Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys (Mtt)-OH, Fmoc-Leu-OH, Fmoc-Ser(O*t*Bu)-OH, and Fmoc-Val-OH, respectively, in the presence of HBTU/DIPEA in DMF with Fmoc-deprotection (20% Piperidine in DMF) after each coupling; (ii) 20% Piperidine in DMF; (iii) TFA/DCM/TIS (2:93:5 v/v/v); (iv) Ac_2O, DIPEA, DMF; (v) Reagent R, 2 h; (vi) Fmoc solid-phase synthesis: Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys (Mtt)-OH, Fmoc-Leu-OH, Fmoc-Ser(O*t*Bu)-OH, and Boc-

Val-OH, respectively, in the presence of HBTU/DIPEA in DMF with Fmoc-deprotection (20% Piperidine in DMF) after each coupling; (vii) HOAt, DIC, DMF/DCM (5:1 v/v), 8 h.

Scheme 5. Synthesis of VSLERRPLPPLP **(9)** and [VSLE]RRPLPPLP (**10**, *N*-terminal to side chain cyclization): (i) (a) Fmoc solid-phase synthesis: coupling of Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Glu(ODmab)-OH, Fmoc-Leu-OH, Fmoc-Ser(O*t*Bu)-OH, and Fmoc-Val-OH, respectively, in the presence of HBTU/DIPEA in DMF with Fmoc-deprotection (20% Piperidine in DMF) after each coupling; (ii) 20% Piperidine in DMF; (iii) hydrazine hydrate in DMF (2%, 3 × 25 mL), 10 min; (iv) HOAt, DIC, DMF/DCM (5:1 v/v), 12 h; (v) Reagent R, 2h.