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Supporting Information

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Stepwise Combinatorial Evolution of An Akt Bisubstrate Inhibitor

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Abbreviations: Mmt, 4-methoxytrityl; BSA, bovine serum albumin; consensus peptide-1, Fmoc-Ala-Arg(Pbf)-Arg(Pbf)-Gly-Ala-Leu-Arg(Pbf)-Gln(Trt)-Ala-NH-$(CH_2)_2$-S-S-Tentagel-Resin; consensus peptide-2, Fmoc-Ala-Arg(Pbf)-Arg(Pbf)-Gly-Dap(Boc)-Leu-Arg(Pbf)-Gln(Trt)-Ala-NH-$(CH_2)_2$-S-S-Tentagel-Resin; consensus peptide-3, Fmoc-Ala-Arg(Pbf)-Arg(Pbf)-Gly-Ala-Leu-Arg(Pbf)-Dap(Boc)-Ala-NH-$(CH_2)_2$-S-S-Tentagel-Resin; consensus peptide-4, [(N-3-Indolylacetyl)-L-isoleucine]-Ala-Arg(Pbf)-Arg(Pbf)-Gly-Dap(Boc)-Leu-Arg(Pbf)-Gln(Trt)-Ala-NH-$(CH_2)_2$-S-S-Tentagel-Resin; consensus peptide-5, Fmoc-Ala-Arg(Pbf)-Arg(Pbf)-Gly-Dap(Mmt)-Leu-Arg(Pbf)-Dap(Boc)-Ala-NH-$(CH_2)_2$-S-S-Tentagel-Resin; consensus peptide-6, [(N-3-Indolylacetyl)-L-isoleucine]-Ala-Arg(Pbf)-Arg(Pbf)-Gly-Dap(3,5-dihydroxynaphthoyl)-Leu-Arg(Pbf)-Dap(Boc)-Ala-NH-$(CH_2)_2$-S-S-Tentagel-Resin; DAG, diacylglycerol; DIPEA, diisopropylethylamine; DMF, dimethyformamide; DMSO, dimethyl sulfoxide; DTT, dithithreitol; EDTA, ethylenediaminetetraacetic acid; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzo-triazole; HPLC, high performance liquid chromatography; MALDI, matrix assisted laser desorption/ionization; MS, mass spectroscopy; Mmt, 4-monomethoxytrityl; NMM, N-methylmorpholine; NMR, nuclear magnetic resonance; Pbf, 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl; Akt, protein kinase B; PS, L-α-phosphatidyl-L-serine; PyBOP, benzotriazole-1-yloxytris-pyrrolidinophosphonium hexafluorophosphate; RP-HPLC, reverse phase high performance liquid chromatograph; SPPS, solid phase peptide synthesis; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Trt, trityl; TSTU, N,N,N',N'-tetramethyl-(succinimido)uranium tetrafluoroborate.

General procedures.

The resins and reagents used for solid phase peptide synthesis, including Tentagel resin, N-9-fluorenylmethyloxycarbonyl (Fmoc)-L-amino acids, N,N,N',N'-tetramethyl-(succinimido)uranium tetrafluoroborate (TSTU), benzotriazole-1-yloxytris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBt), were purchased from Advanced ChemTech. Peptide synthesis grade dichloromethane, N,N-diisopropylethylamine (DIPEA), dimethylformamide (DMF) and trifluoroacetic acid (TFA) were purchased from Fisher and piperidine was obtained from Lancaster. 2-Fmoc-3-(p-methoxytrityl)-diaminopropionic acid [Fmoc-Dap(Mmt)-OH] was prepared from 2-Fmoc-diaminopropionic acid (Fmoc-Dap-OH) and p-anisylchlorodiphenylmethane. Triisopropylsilane (TIS) was purchased from Acros. The 720 carboxylic acids used for the preparation of the peptide libraries were purchased from Aldrich. The reagents for the Akt assay were purchased from Sigma: EGTA (ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid, tetrasodium salt,), $MgCl_2 \cdot 6H_2O$, NaCl, $EDTA \cdot Na_2 \cdot H_2O$ (ethylenediaminetetraacetic acid, disodium salt:dihydrate), Na_3VO_4, Triton®X-100, glycerol except DTT (Dithiothreitol) was purchased from Invitrogen.

The Akt isoforms employed in this study were purchased from Panvera. Radioactive γ-P^{33}-ATP was obtained from AmerSham Biosciences. 96-well (2 mL/well) Uniplates and P81 Cellulose Phosphate Paper Unifilter Plates were obtained from Whatman Inc. Solvent-resistant MultiScreen 96-well (300 μL/well) filter plates, the Multiscreen Resist Vacuum Manifold, and Tape Multiscreen Harvester CL Plates were purchased from Millipore Corporation. Radioactive intensity of the library assays and IC_{50} determinations was detected using a 1450 Microbeta liquid scintillation counter. GraFit Version 5 was used to determine the IC_{50} values.

1D, 2D-^{1}H and ^{13}C NMR spectra of the peptide inhibitors were recorded on a DRx300 MHz Spectrometer in H_2O and DMSO, and chemical shifts are reported in parts per million (ppm) downfield from $(CH_3)_4Si$. The molecular weights of the peptides were analyzed with MALDI (Matrix Assisted Laser Desorption/Ionization) mass spectrometry on the Applied Biosystems Voyager DE. Reverse phase high performance liquid chromatograph (RP-HPLC) was performed on a Waters SD-200 solvent delivery system equipped with a 500 UV/Vis-absorbance detector and recorded on an Apple Macintosh computer using model 600 software (Applied Biosystems Inc.). Chromatographic separations were achieved using linear gradients of buffer B in A (A = 0.1% aqueous TFA; B = 0.1% TFA in CH_3CN) over 50 min at a flow rate of 12 mL/min using a detection wavelength of 218 nm on Delta-Pak C_{18} (300 Å, 15 μm, 3 x 15 cm) column.

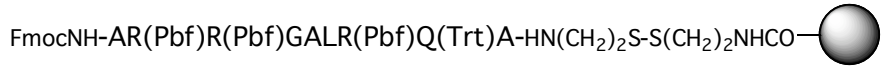
Peptide Synthesis.

Peptides were synthesized using a standard Fmoc solid phase peptide synthesis (SPPS) protocol on an Innova 2000 platform shaker or on an Advanced Chemtech Model 90 Tabletop Peptide Synthesizer.

Synthesis of Fmoc-Ala-Arg(Pbf)-Arg(Pbf)-Gly-Ala-Leu-Arg(Pbf)-Gln(Trt)-Ala-NH-$(CH_2)_2$-S-S-Tentagel-Resin (Resin-peptide A). 5 g of Tentagel S COOH (90 μm, 0.2

mmol/g) and 1.94 g (15 mmol) of DIPEA were successively added to a solution of 1.5 g (5 mmol) of TSTU in 20 mL of DMF. The mixture was shaken for 2 h at ambient temperature. Subsequently, a mixture of 2.25 g (10 mmol) of cystamine dihydrochloride and 2.02 g (20 mmol) of N-methylmorpholine (NMM) in 20 mL of water was slowly added to the Tentagel reaction mixture. Heat was evolved upon addition. Upon cooling to room temperature, the reaction vessel was sealed and shaken overnight. The resin was then drained and washed successively with H_2O (3 x 20 mL), DMF (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL). The free amine substitution level on linker-coupled resin was found to be 0.05 mmol/g.

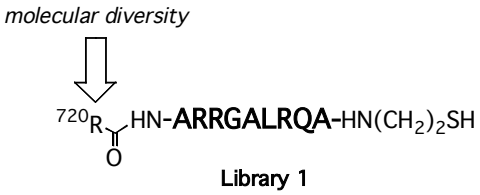
The linker-coupled resin (5 g) was successively submitted to coupling reactions with the required amino acids followed by removal of the Fmoc protecting group via standard conditions (*vide infra*). The Fmoc group was removed with 30% piperidine in DMF and the resin was washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL) and subsequently dried *in vacuo*. The following amino acids were used for the synthesis of the lead sequence: Fmoc-Ala-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH. Each residue was coupled for 2 h, and coupling efficiencies were determined by quantitative ninhydrin reaction. The standard coupling conditions employed 5 eq. of Fmoc-amino acid, 5 eq. of HOBt, 5 eq. of PyBOP, and 10 eq. of NMM in 50 mL DMF with shaking for 2 h. After each coupling step, the resin was successively washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL). The Fmoc protecting group was removed with 30% piperidine in DMF (shaking for 30 min).



Resin-peptide A

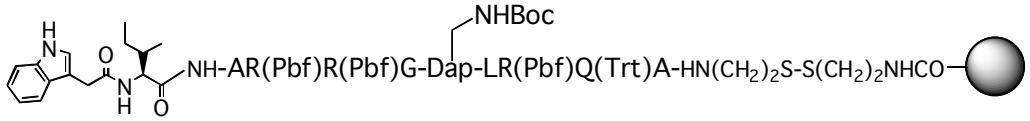
Library 1. The Fmoc group in **Resin-peptide-A** was removed with 30% piperidine in DMF and the resin was washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL) and subsequently dried *in vacuo*. The peptide–bound resin was distributed in 5 mg quantities into individual wells of solvent-resistant MultiScreen™ 96-well filter plates (8 plates total). To each well was added a solution of a carboxylic acid (200 eq.) in 100 μL DMF and a second solution containing PyBOP (200 eq.), HOBt (200 eq.), and NMM (400 eq.) in 100 μL of DMF. A total of 720 different carboxylic acids were employed. The plates were gently shaken overnight, and then each well subjected to a series of washing steps (3 x 200 μL of DMF, 3 x 200 μL of isopropyl alcohol, and 3 x 200 μL of CH_2Cl_2). All the side chain protecting groups were removed via treatment with TFA:H_2O:TIS (95:2.5:2.5) for 3 h at ambient temperature. The resin was washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL), the peptide-nonpeptide conjugates subsequently cleaved from the disulfide-containing resin with 10 mM dithiothreitol (DTT) in 50 mM Tris, pH 7.5 (1 x 200 μL for 1 h and 2 x 150 μL for 1 h each), and filtered into a receiving set of 96-well plates using a vacuum manifold (final volume of 500 μL). The coupling efficiency of the acylation reaction and the purity of peptide-nonpeptide conjugates were assessed via the ninhydrin test and RP-HPLC, respectively. No free *N*-terminal peptide was detected, and >90% of

total ligand was cleaved from the resin with the first DTT cleaving step. The final two DTT washings removed the residual resin-bound peptide. Compound purity was >90% as assessed by HPLC, and the HPLC-purified compounds (i.e. removal of Tris buffer and DTT) were characterized by MALDI-MS. These peptides, containing 720 different groups at the Ala α-amino position in 8 plates, comprise **Library 1**.



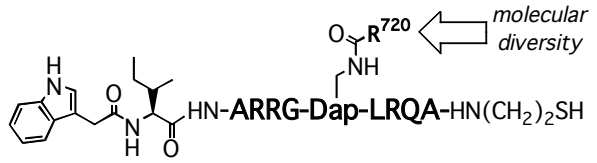
Library 1

Synthesis of [(N-3-Indolylacetyl)-L-isoleucine]-Ala-Arg(Pbf)-Arg(Pbf)-Gly-Dap(Boc)-Leu-Arg(Pbf)-Gln(Trt)-Ala-NH-$(CH_2)_2$-S-S-Tentagel-Resin (Resin-peptide B). Solid phase peptide synthesis was conducted using the Tentagel resin via the protocol described above for **Resin-peptide A**. The following amino acids were sequentially coupled to furnish the desired sequence: Fmoc-Ala-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Dap(Boc)-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH. The Fmoc group at the N-terminus was selectively removed with 40 mL of 30% piperidine in DMF (30 min) and the resulting free amine on the Ala residue was coupled with 1.44 g (5 mmol) of N-(3-indolylacetyl)-L-isoleucine in the presence of 3.25 g (5 mmol) of PyBOP, 0.77 g (5 mmol) of HOBt, and 1.01 g (10 mmol) of NMM in 40 mL of DMF. The reaction mixture was shaken 2 hr. The solvent was removed from the resin and the resin subsequently washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL). The peptide-resin was then used for the preparation of **Library 2**.



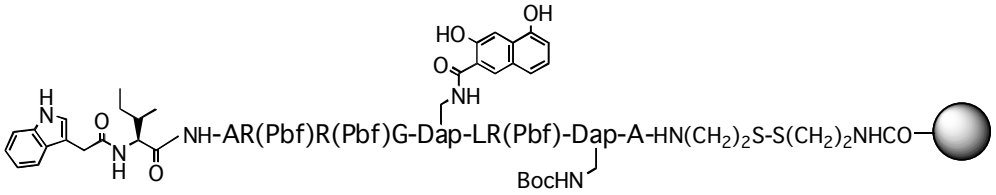
Resin-peptide B

Library 2. The Boc group in **Resin-peptide B** was removed by adding the resin to a 40 mL solution of 25% TFA in CH_2Cl_2. The mixture was shaken for 30 min, the TFA solution drained, and the resin washed with DMF (3 x 20 mL). The peptide-resin at this stage was [N-(3-indolylacetyl)]-Ile-Ala-Arg(Pbf)-Arg(Pbf)-Gly-Dap(NH_2)-Leu-Arg(Pbf)-Gln(Trt)-Ala-NH-$(CH_2)_2$-S-S-Tentagel-Resin. The peptide–bound resin was distributed in 5 mg quantities into individual wells of solvent-resistant MultiScreen™ 96-well filter plates (8 plates total). To each well was added a solution of a carboxylic acid (200 eq.) in 100 μL DMF and a second solution containing PyBOP (200 eq.), HOBt (200 eq.), and NMM (400 eq.) in 100 μL of DMF. A total of 720 different carboxylic acids were employed. The following procedures, as described for **Library 1**, were employed: the resin in each well was coupled with one of 720 different carboxylic acids, the side chain protecting groups were removed, and the peptides were cleaved from the resin to furnish **Library 2**.



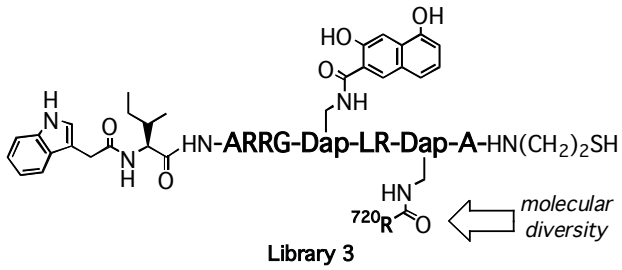
Library 2

Synthesis of [(N-3-Indolylacetyl)-L-isoleucine]-Ala-Arg(Pbf)-Arg(Pbf)-Gly-Dap(3,5-dihydroxynaththoyl)-Leu-Arg(Pbf)-Dap(Boc)-Ala-NH-$(CH_2)_2$-S-S-Tentagel-Resin (Resin-peptide C). Solid phase peptide synthesis was conducted using the Tentagel resin via the protocol described above for **Resin-peptide A**. The following amino acids were sequentially coupled to furnish the desired sequence: Fmoc-Ala-OH, Fmoc-Dap(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Dap(Mmt)-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH. The Fmoc group at the N-terminus of the peptide (5 g resin) was selectively removed with 40 mL of 30% piperidine in DMF (30 min) and the resulting free amine on the Ala residue was coupled with 1.44 g (5 mmol) of N-(3-indolylacetyl)-L-isoleucine in the presence of 3.25 g (5 mmol) of PyBOP, 0.77 g (5 mmol) of HOBt, and 1.01 g (10 mmol) of NMM in 40 mL of DMF. The reaction mixture was shaken 2 hr. The solvent was removed from the resin and the resin subsequently washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL). The Mmt group was selectively removed with 40 mL of AcOH/2,2,2-trifluoroethanol/$ClCH_2CH_2Cl$ (1/2/7) and the resulting free amine on the side chain of the Dap residue was coupled 1.02 g (5 mmol) with 3,5-dihydroxy-2-naphthoic acid in the presence of 3.25 g (5 mmol) of PyBOP, 0.77 g (5 mmol) of HOBt, and 1.01 g (10 mmol) of NMM in 40 mL of DMF. The reaction mixture was shaken overnight. The solvent was removed from the resin and the resin subsequently washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL). The peptide-resin was then used for the preparation of **Library 3**.



Resin-peptide C

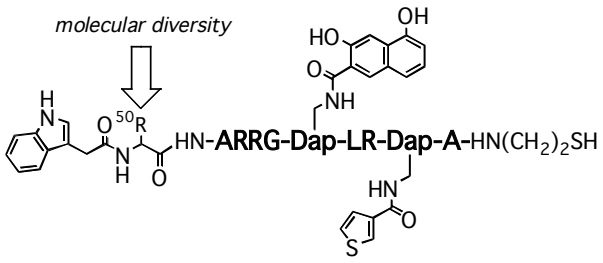
Library 3. The Boc group of ***Resin-peptide C*** was selectively removed by adding the resin to a 40 mL solution of 25% TFA in CH_2Cl_2. The mixture was shaken for 30 min, the TFA solution drained, and the resin washed with DMF (3 x 20 mL). The peptide–bound resin was distributed in 5 mg quantities into individual wells of solvent-resistant MultiScreen™ 96-well filter plates (8 plates total). To each well was added a solution of a carboxylic acid (200 eq.) in 100 μL DMF and a second solution containing PyBOP (200 eq.), HOBt (200 eq.), and NMM (400 eq.) in 100 μL of DMF. A total of 720 different carboxylic acids were employed. The following procedures, as described for **Library 1**, were employed: the resin in each well was coupled with one of 720 different carboxylic acids, the side chain protecting groups were removed, and the peptides were cleaved from the resin to furnish **Library 3**.



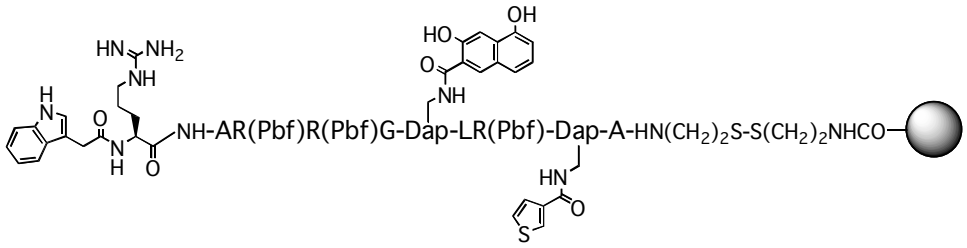
Library 3

Synthesis of (N-3-Indolylacetyl)-(AA)^{50}-Ala-Arg(Pbf)-Arg(Pbf)-Gly-Dap(3,5-dihydroxynaththoyl)-Leu-Arg(Pbf)-Dap(3-thiophenecarboxylic))-Ala-NH-(CH$_2$)$_2$-S-S-Tentagel-Resin (Library 4; Resin-peptide D where AA = Arg). Solid phase peptide synthesis was conducted using the Tentagel resin via the protocol described above for **Resin-peptide A**. The following amino acids were sequentially coupled to furnish the desired sequence: Fmoc-Ala-OH, Fmoc-Dap(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Dap(Mmt)-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH. The N-terminal Fmoc group was removed with 30% piperidine in DMF and the resin was washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL) and subsequently dried in vacuo. The peptide–bound resin was distributed in 5 mg quantities into 50 individual wells of solvent-resistant MultiScreen^{TM} 96-well filter plate. To each well was added a solution of one of 50 different Fmoc-AA-OH (200 eq.) in 100 μL DMF and a second solution containing PyBOP (200 eq.), HOBt (200 eq.), and NMM (400 eq.) in 100 μL of DMF. The plate was gently shaken overnight, and then each well subjected to a series of wash steps (3 x 200 μL of DMF, 3 x 200 μL of isopropyl alcohol, and 3 x 200 μL of CH_2Cl_2). The Fmoc group at the N-terminus on each peptide in each well was selectively removed with 150 μL of 30% piperidine in DMF (30 min) and the resulting free amine coupled with 1.44 mg (5 μmol) of indole-3-acetic acid in the presence of 3.25 mg (5 μmol) of PyBOP, 0.77 mg (5 μmol) of HOBt, and 1.01 mg (10 μmol) of NMM in 100 μL of DMF. The reaction mixture was shaken for 2 hr. The solvent was filtered from each well under reduced pressure and the resin subsequently washed with DMF (3 x 100 μL), isopropyl alcohol (3 x 100 μL), and CH_2Cl_2 (3 x 100 μL). The Mmt group on the first Dap was selectively removed with 40 μL of AcOH/2,2,2-trifluoroethanol/$ClCH_2CH_2Cl$ (1/2/7) and the resulting free amine on the side chain of the Dap residue coupled to 3,5-dihydroxy-2-naphthoic acid (1.02 mg, 5 μmol) in the presence of 3.25 mg (5 μmol) of PyBOP, 0.77 mg (5 μmol) of HOBt, and 1.01 mg (10 μmol) of NMM, in 100 μL of DMF. The reaction mixture was shaken overnight. The solvent was removed from each well under reduced pressure and the resin subsequently washed with DMF (3 x 100 μL), isopropyl alcohol (3 x 100 μL), and CH_2Cl_2 (3 x 100 μL). The Boc group on the second Dap was removed by adding to the resin a 40 μL solution of 25% TFA in CH_2Cl_2. The mixture was shaken for 30 min, the TFA solution drained, and the resin washed with DMF (3 x 100 μL), isopropyl alcohol (3 x 100 μL), CH_2Cl_2 (3 x 100 μL) and subsequently dried *in vacuo*. The resulting free amine on the Dap residue was coupled with 0.32 mg (2.5 μmol) of 3-thiophenecarboxylic acid in the presence of 1.63 mg (2.5 μmol) of PyBop, 0.40 mg (2.5 μmol) of HOBt, and 0.50 mg (5 μmol) of NMM in 100 μL of DMF. The reaction mixture was shaken

overnight. The solvent was removed from the resin and the resin subsequently washed with DMF (3 x 100 μL), isopropyl alcohol (3 x 100 μL), and CH_2Cl_2 (3 x 100 μL). All the side chain protecting groups in each well were removed via treatment with TFA:H_2O:TIS (95:2.5:2.5) for 3 h at ambient temperature. The resin was washed with DMF (3 x 100 μL), isopropyl alcohol (3 x 100 μL), and CH_2Cl_2 (3 x 100 μL). The peptide-nonpeptide conjugates were subsequently cleaved from the disulfide-containing resin with 10 mM dithiothreitol (DTT) in 50 mM Tris, pH 7.5 (1 x 200 μL for 1 h and 2 x 150 μL for 1 h each) and then filtered into a receiving multi-well plate using a vacuum manifold (final volume of 500 μL).



Library 4

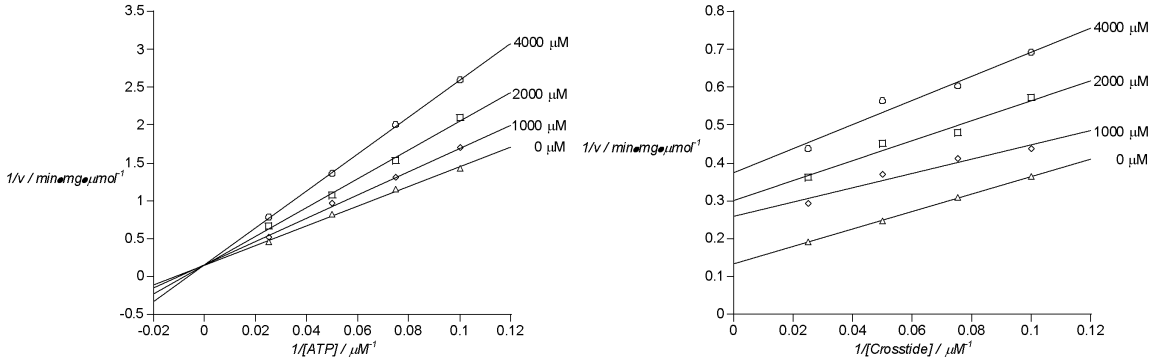


Resin-peptide D

Synthesis and characterization of 1. Peptide **1** was synthesized using the Tentagel resin via the protocol described above for **Resin-peptide A**. The following amino acids were used for the synthesis: Fmoc-Ala-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Dap(Boc)-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH (2.5 g peptide-resin). The Fmoc group was removed with 40 mL of 30% piperidine in DMF and the resin was subsequently mixed with a solution of Ac_2O (0.51 g, 5 mmol) in CH_2Cl_2 (40 mL). Then 1 g (10 mmol) of NMM was added and the mixture was shaken for 1 h. The resin was washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL) and subsequently dried *in vacuo*. The Boc group was removed by adding the resin to a 40 mL solution of 25% TFA in CH_2Cl_2. The mixture was shaken for 30 min, the TFA solution drained, and the resin washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL). The resin was subsequently mixed with a solution of Ac_2O (0.51 g, 5 mmol) in CH_2Cl_2 (40 mL). Then 1 g (10 mmol) of NMM was added and the mixture was shaken for 1 h. The resin was washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL). All the side chain protecting groups were removed by treatment with TFA:H_2O:TIS (95:2.5:2.5) for 3 h at ambient temperature. The peptide was cleaved from

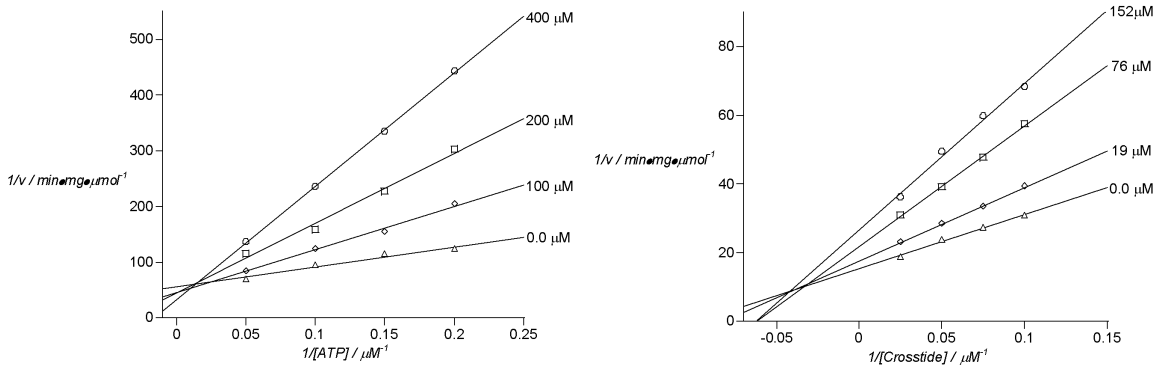
the disulfide-containing resin with 10 mM DTT in 50 mM Tris, pH 7.5 (3 x 10 mL for 1 h each). The crude material was purified by RP-HPLC to furnish 7 mg of **1** as a white solid. ^{1}H NMR (300 MHz, D_2O, ppm) 4.20 -4.25 (m, 8H), 4.00 (m, 1H), 3.87 (s, 2H), 3.66 (s, 1H), 3.33 (m, 2H), 3.12 – 3.16 (m, 2H), 2.59 (m, 2H), 2.30 (m, 2H), 2.05 (m, 1H), 1.95 (s, 3H), 1.70 – 1.77 (m, 8H), 1.55 – 1.56 (m, 10H), 1.30 – 1.33 (m, 8H), 0.78 – 0.87 (m, 13H). MALDI-MS (m/z) calculated for $C_{50}H_{93}N_{21}O_{12}S$ (M^+) 1212.47, found 1212.60.

Inhibition Patterns Versus of 1 Variable ATP and Crosstide (Arg-Pro-Arg-Thr-Ser-Ser-amide) Substrates.



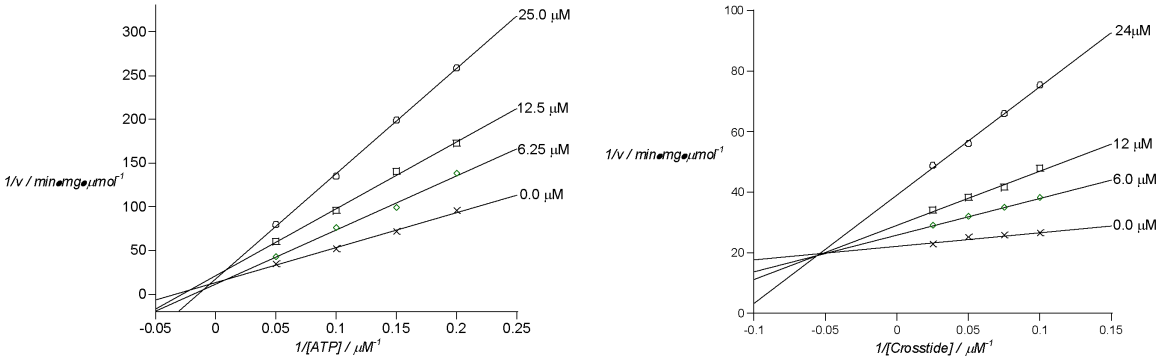
Synthesis and characterization of 5. The Fmoc group in **Resin-peptide A** (2.5 g resin) was removed with 40 mL of 30% piperidine in DMF and the resulting free amine on the Ala residue was coupled with 0.72 g (2.5 mmol) of N-(3-indolylacetyl)-L-isoleucine in the presence of 1.63 g (2.5 mmol) of PyBop, 0.40 g (2.5 mmol) of HOBt, and 0.50 g (5 mmol) of NMM in 25 mL of DMF. The reaction mixture was shaken overnight. The solvent was removed from the resin and the resin subsequently washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL). The peptide was cleaved from the disulfide-containing resin with 10 mM DTT in 50 mM Tris, pH 7.5 (3 x 10 mL for 1 h each). The crude material was purified by RP-HPLC to furnish 10 mg of Compound **5** as a white solid. ^{1}H NMR (300 MHz, D_2O, ppm) 7.54 (d, *J = 7.77 Hz*, 1H, Ar-H), 7.44 (d, *J = 8.15 Hz*, 1H, Ar-H), 7.25 (s, 1H, Ar-H), 7.18 (t, *J = 7.23 Hz, 7.14 Hz*, 1H, Ar-H), 7.08 (t, *J = 7.23 Hz, 7.51 Hz*, 1H, Ar-H), 3.85 (2H, CH_2CO) for **3-indolylacetyl group**; 4.20 – 4.25 (m, 7H), 4.10 (m, 2H), 3.76 (s, 2H), 3.25 (m, 2H), 3.10 (m, 4H), 2.85 (m, 2H), 2.58 (m, 2H), 2.28 (m, 2H), 2.02 (m, 1H), 1.99 (m, 1H), 1.68 – 1.72 (m, 5H), 1.52 – 1.54 (m, 8H), 1.25 – 1.33 (m, 14H), 0.77 – 0.86 (m, 12H). MALDI-MS (m/z) calculated for $C_{58}H_{98}N_{22}O_{12}S$ (M^+) 1327.60, found 1327.87.

Inhibition Patterns of 5 Versus Variable ATP and Crosstide (Arg-Pro-Arg-Thr-Ser-Ser-amide) Substrates.



Synthesis and characterization of 6. The Boc group in **Resin-peptide B** (2.5g) was removed by adding the resin to a 40 mL solution of 25% TFA in CH_2Cl_2. The mixture was shaken for 30 min, the TFA solution drained, and the resin washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL) and subsequently dried *in vacuo*. The resulting free amine on the Dap residue was coupled with 0.51 g (2.5 mmol) of 3,5-dihydroxy-2-naphthoic acid in the presence of 1.63 g (2.5 mmol) of PyBop, 0.40 g (2.5 mmol) of HOBt, and 0.50 g (5 mmol) of NMM in 25 mL of DMF. The reaction mixture was shaken overnight. The solvent was removed from the resin and the resin subsequently washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL). All the side chain protecting groups were removed by treatment with TFA:H_2O:TIS (95:2.5:2.5) for 3 h at ambient temperature. The peptide was cleaved from the disulfide-containing resin with 10 mM DTT in 50 mM Tris, pH 7.5 (3 x 10 mL for 1 h each). The crude material was purified by RP-HPLC to furnish 9 mg of **6** as a white solid. ^{1}H NMR (300 MHz, D_2O, ppm) 8.22 (s, 1H, Ar-H), 7.47 (s, 2H, Ar-H), 7.46 (d, J = 7.69 Hz, 2H, Ar-H), 7.23 (m, 2H, Ar-H), 7.13 (d, J = 7.77 Hz, 1H, Ar-H), 7.04 (d, J = 7.59 Hz, 1H, Ar-H), 6.94 (d, J = 7.47 Hz, 1H, Ar-H) for 3-indolyl and 3,5-naphthoyl group; 4.52 (m, 1H), 4.15 (m, 6H), 3.95 (s, 2H), 3.80 (s, 2H), 3.72 (m, 2H), 3.29 (m, 3H), 2.92 (m, 3H), 2.55 (m, 2H), 1.92 (m, 4H), 1.64 (m, 8H), 1.49 (m, 8H), 1.27 (m, 5H), 1.19 (m, 4H), 0.78 (m, 6H), 0.69 (m, 7H). MALDI-MS (m/z) calculated for $C_{69}H_{105}N_{23}O_{15}S$ (M^+) 1528.78, found 1527.51.

Inhibition Patterns of 6 Versus Variable ATP and Crosstide (Arg-Pro-Arg-Thr-Ser-Ser-amide) Substrates.

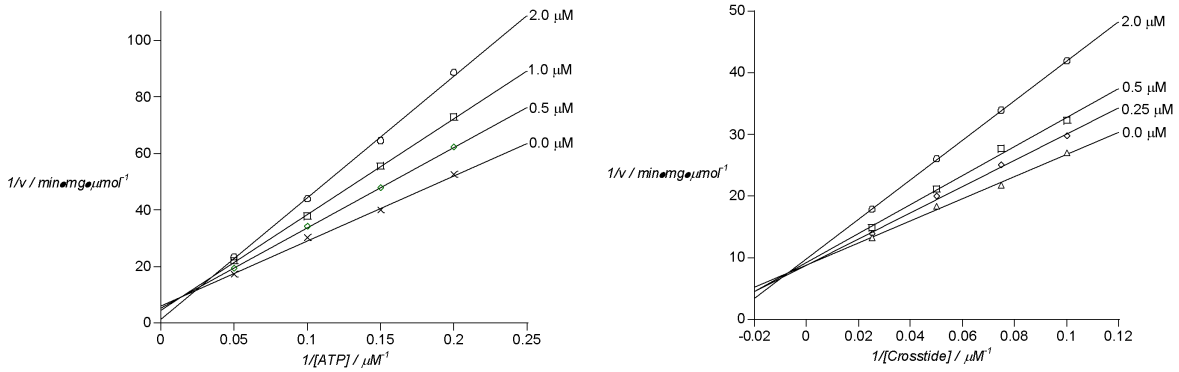


Synthesis and characterization of the dimethoxy analogue of 6. The Boc group in **Resin-peptide B** (2.5g) was removed by adding the resin to a 40 mL solution of 25% TFA in CH_2Cl_2. The mixture was shaken for 30 min, the TFA solution drained, and the resin washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL) and subsequently dried in vacuo. The resulting free amine on the Dap residue was coupled with 0.58 g (2.5 mmol) of 3,5-dimethoxy-2-naphthoic acid in the presence of 1.63 g (2.5 mmol) of PyBop, 0.40 g (2.5 mmol) of HOBt, and 0.50 g (5 mmol) of NMM in 25 mL of DMF. The reaction mixture was shaken overnight. The solvent was removed from the resin and the resin subsequently washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL). All the side chain protecting groups were removed by treatment with TFA:H_2O:TIS (95:2.5:2.5) for 3 h at ambient temperature. The peptide was cleaved from the disulfide-containing resin with 10 mM DTT in 50 mM Tris, pH 7.5 (3 x 10 mL for 1 h each). The crude material was purified by RP-HPLC to furnish 10 mg of the dimethoxy analogue of **6** as a white solid. ^{1}H NMR (300 MHz, D_2O, ppm) 8.26 (s, 1H, Ar-H), 7.69 (s, 2H, Ar-H), 7.48 (d, *J = 7.80 Hz*, 2H, Ar-H), 7.40 (m, 2H, Ar-H), 7.35 (d, *J = 7.20 Hz*, 1H, Ar-H), 7.19 (d, *J = 6.00 Hz*, 1H, Ar-H), 7.03 (m, 1H, Ar-H) for 3-indolyl and naphthoyl group; 4.50 (m, 1H), 4.20 (m, 6H), 3.90 (s, 2H), 3.82 (s, 2H), 3.69 (m, 2H), 3.30 (m, 3H), 2.90 (m, 3H), 2.83 (s, 6H), 2.50 (m, 2H), 1.91 (m, 4H), 1.54 (m, 8H), 1.46 (m, 8H), 1.28 (m, 5H), 1.20 (m, 4H), 0.80 (m, 6H), 0.72 (m, 7H). MALDI-MS (m/z) calculated for $C_{71}H_{109}N_{23}O_{15}S$ (M^+) 1556.84, found 1557.73.

Synthesis and characterization of 7. The Boc group in **Resin-peptide C** (2.5g) was removed by adding the resin to a 40 mL solution of 25% TFA in CH_2Cl_2. The mixture was shaken for 30 min, the TFA solution drained, and the resin washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL) and subsequently dried *in vacuo*. The resulting free amine on the Dap residue was coupled with 0.32 g (2.5 mmol) of 3-thiophenecarboxylic acid in the presence of 1.63 g (2.5

mmol) of PyBop, 0.40 g (2.5 mmol) of HOBt, and 0.50 g (5 mmol) of NMM in 25 mL of DMF. The reaction mixture was shaken overnight. The solvent was removed from the resin and the resin subsequently washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL). All the side chain protecting groups were removed by treatment with TFA:H_2O:TIS (95:2.5:2.5) for 3 h at ambient temperature. The peptide was cleaved from the disulfide-containing resin with 10 mM DTT in 50 mM Tris, pH 7.5 (3 x 10 mL for 1 h each). The crude material was purified by RP-HPLC to furnish 10 mg of **7** as a white solid. ^{1}H NMR (300 MHz, DMSO, ppm) 10.14 (1H, indolyl-NH), 7.54 (1H, Ar-H), 7.45 (1H, Ar-H), 7.27 (1H, Ar-H), 7.20 (1H, Ar-H), 7.10 (1H, Ar-H), 3.80 (2H, CH_2CO) for 3-indolylacetyl group; 8.14 (1H, s, CONH), 4.04 (1H, $C_\alpha H$), 1.80 (1H, $C_\beta H_1$), 1.41 (2H, $C_\gamma H_2$), 1.14 (3H, $C_\gamma H_3$), 0.80 (3H, $C_\delta H_3$) for Ile-1 from N-teminus; 8.28 (1H, s, CONH), 4.19 (1H, $C_\alpha H$), 1.27 (3H, $C_\beta H_3$) for Ala-2; 7.72 (1H, s, CONH), 4.05 (1H, $C_\alpha H$), 1.52 (1H, $C_\beta H_1$), 1.31 (1H, $C_\beta H_1$), 1.23 (2H, $C_\gamma H_2$), 2.77 (2H, $C_\delta H_2$), 6.89 ($N^\varepsilon H$) for Arg-3; 8.18 (1H, s, CONH), 4.27 (1H, $C_\alpha H$), 1.70 (1H, $C_\beta H_1$), 1.55 (1H, $C_\beta H_1$), 1.44 (2H, $C_\gamma H_2$), 2.94 (2H, $C_\delta H_2$), 7.04 ($N^\varepsilon H$) for Arg-4; 8.51 (1H, s, CONH), 3.90 (2H, $C_\alpha H_2$) for Gly-5; 8.44 (1H, s, CONH), 4.57 (1H, $C_\alpha H$), 3.90 (1H, $C_\beta H$), 3.87 (1H, $C_\beta H$) for Dap-6; 9.50 (1H, CONH of –Dap-Aryl), 8.31 (1H, Ar-H), 7.54 (1H, Ar-H), 7.45 (1H, Ar-H), 7.27 (1H, Ar-H), 7.00 (1H, Ar-H) for 3,4-dihydroxynaphthoyl group; 8.18 (1H, s, CONH), 4.35 (1H, $C_\alpha H$), 1.54 (2H, $C_\beta H_2$), 1.43 (6H, $C_\gamma H_6$), 0.70 (3H, $C_\delta H_3$) for Leu-7; 8.17 (1H, s, CONH), 4.14 (1H, $C_\alpha H$), 1.68 (1H, $C_\beta H_1$), 1.58 (1H, $C_\beta H_1$), 1.41 (2H, $C_\gamma H_2$), 2.87 (2H, $C_\delta H_2$), 6.95 ($N^\varepsilon H$) for Arg-8; 8.16 (1H, s, CONH), 4.55 (1H, $C_\alpha H$), 3.67 (2H, $C_\beta H_2$), for Dap-9; 8.26 (1H, CONH), 8.00 (1H, Ar-H), 7.45 (1H, Ar-H), 7.36 (1H, Ar-H) for 2-thiophenyl group; 8.24 (1H, s, CONH), 4.25 (1H, $C_\alpha H$), 1.34 (3H, $C_\beta H_3$) for Ala-10 (C-terminus); 8.04 (1H, s, CON<u>H</u>CH_2), 3.34 (2H, $C_\alpha H_2$, alpha from NH), 2.66 (2H, $C_\beta H_2$). MALDI-MS (m/z) calculated for $C_{72}H_{105}N_{23}O_{15}S$ (M^+) 1596.88, found 1596.19.

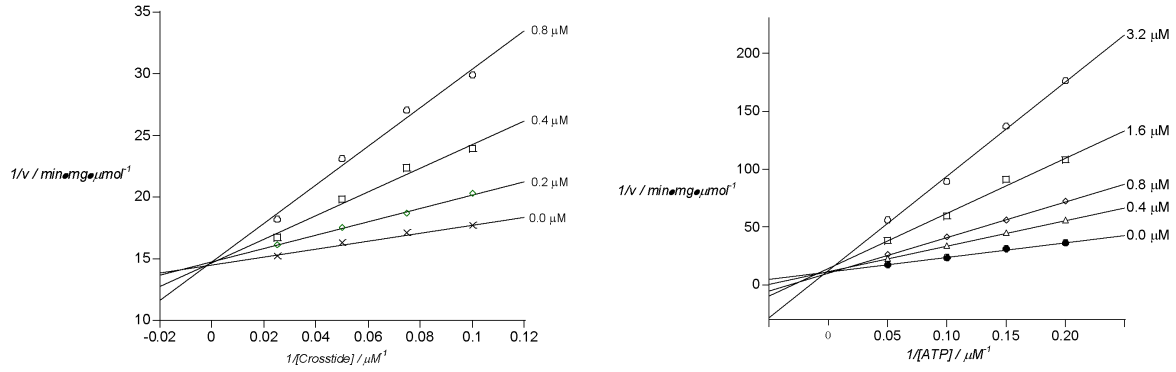
Inhibition Patterns of 7 Versus Variable ATP and Crosstide (Arg-Pro-Arg-Thr-Ser-Ser-amide) Substrates.



Synthesis and characterization of compound 8. The Fmoc group at the N-terminus of ***Resin-peptide D*** (5 g resin) was selectively removed with 40 mL of 30% piperidin in DMF (30 min) and the resulting free amine on the Ala residue was coupled with 3.24 g (5 mmol) of Fmoc-Arg(Pbf)-OH in the presence of 3.25 g (5 mmol) of PyBOP, 0.77 g (5 mmol) of HOBt, and 1.01 g (10 mmol) of NMM in 40 mL of DMF. The reaction mixture was shaken 2 hr. The solvent was removed from the resin and the resin subsequently washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL). The Fmoc group at the N-terminus was selectively removed with 40 mL of 30% piperidin in DMF (30 min) and the resulting free amine on the Arg residue was coupled with 0.87 g (5 mmol) of indol-3-acetic acid in the presence of 3.25 g (5 mmol) of PyBOP, 0.77 g (5 mmol) of HOBt, and 1.01 g (10 mmol) of NMM in 40 mL of DMF. The reaction mixture was shaken 2 hr. The solvent was removed from the resin and the resin subsequently washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL). The Mmt group was selectively removed with 40 mL of AcOH/2,2,2-trifluoroethanol/$ClCH_2CH_2Cl$ (1/2/7) and the resulting free amine on the side chain of the Dap residue was coupled 1.02 g (5 mmol) of 3,5-dihydroxy-2-naphthoic acid in the presence of 3.25 g (5 mmol) of PyBOP, 0.77 g (5 mmol) of HOBt, and 1.01 g (10 mmol) of NMM in 40 mL of DMF. The reaction mixture was shaken overnight. The solvent was removed from the resin and the resin subsequently washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL). The Boc group was removed by adding the resin to a 40 mL solution of 25% TFA in CH_2Cl_2. The mixture was shaken for 30 min, the TFA solution drained, and the resin washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL) and subsequently dried in vacuo. The resulting free amine on the Dap residue was coupled with 0.32 g (2.5 mmol) of 3-thiophenecarboxylic acid in the presence of 1.63 g (2.5 mmol) of PyBop, 0.40 g (2.5 mmol) of HOBt, and 0.50 g (5 mmol) of NMM in 25 mL of DMF. The reaction mixture was shaken overnight. The solvent was removed from the resin and the resin subsequently washed with DMF (3 x 20 mL), isopropyl alcohol (3 x 20 mL), and CH_2Cl_2 (3 x 20 mL). All the side chain protecting groups were removed by treatment with TFA:H_2O:TIS (95:2.5:2.5) for 3 h at ambient temperature. The peptide was cleaved from the disulfide-containing resin with 10 mM DTT in 50 mM Tris, pH 7.5 (3 x 10 mL for 1 h each). The crude material was purified by RP-HPLC to furnish 12 mg of **8** as a white solid. ^{1}H NMR (300 MHz, DMSO, ppm) 10.16 (1H, indolyl-NH), 7.55 (1H, Ar-H), 7.46 (1H, Ar-H), 7.28 (1H, Ar-H), 7.22 (1H, Ar-H), 7.11 (1H, Ar-H), 3.79 (2H, CH_2CO) for 3-indolylacetyl group; 8.34 (1H, s, CONH), 4.22 (1H, $C_\alpha H$), 1.79 (1H, $C_\beta H_1$), 1.71 (1H, $C_\beta H_1$), 1.50 (2H, $C_\gamma H_2$), 3.03 (2H, $C_\delta H_2$), 7.04 ($N^\varepsilon H$) for Arg-1 from N-teminus; 8.27 (1H, s, CONH), 4.19 (1H, $C_\alpha H$), 1.29 (3H, $C_\beta H_3$) for Ala-2; 7.72 (1H, s, CONH), 4.10 (1H, $C_\alpha H$), 1.57 (1H, $C_\beta H_1$), 1.38 (1H, $C_\beta H_1$), 1.31 (2H, $C_\gamma H_2$), 2.85 (2H, $C_\delta H_2$), 6.98 ($N^\varepsilon H$) for Arg-3; 8.24 (1H, s, CONH), 4.29 (1H, $C_\alpha H$), 1.72 (1H, $C_\beta H_1$), 1.50 (1H, $C_\beta H_1$), 1.44 (2H, $C_\gamma H_2$), 2.94 (2H, $C_\delta H_2$), 6.99 ($N^\varepsilon H$) for Arg-4; 8.53 (1H, s, CONH), 3.86 (1H, $C_\alpha H_1$) 3.79 (1H, $C_\alpha H_1$) for Gly-5; 8.50 (1H, s, CONH), 4.56 (1H, $C_\alpha H$), 3.89 (1H, $C_\beta H$), 3.85 (1H, $C_\beta H$) for Dap-6; 9.09 (1H, CONH of –Dap-Aryl), 8.34 (1H, Ar-H), 7.55 (1H, Ar-H), 7.48 (1H, Ar-H), 7.28 (1H, Ar-H), 7.01 (1H, Ar-H) for 3,4-dihydroxynaphthoyl group; 8.14 (1H, s, CONH), 4.33 (1H, $C_\alpha H$), 1.55 (2H, $C_\beta H_2$), 1.47 (6H, $C_\gamma H_6$), 0.71 (3H, $C_\delta H_3$) for Leu-7; 8.15 (1H, s, CONH), 4.14 (1H, $C_\alpha H$), 1.70 (1H, $C_\beta H_1$), 1.60 (1H, $C_\beta H_1$), 1.42 (2H, $C_\gamma H_2$), 2.91 (2H, $C_\delta H_2$), 6.99 ($N^\varepsilon H$) for Arg-8; 8.19 (1H, s, CONH), 4.59 (1H,

$C_\alpha H$), 3.70 (2H, $C_\beta H_2$), for Dap-9; 8.31 (1H, CONH), 7.98 (1H, Ar-H), 7.47 (1H, Ar-H), 7.38 (1H, Ar-H) for 2-thiophenyl group; 8.22 (1H, s, CONH), 4.25 (1H, $C_\alpha H$), 1.34 (3H, $C_\beta H_3$) for Ala-10 (C-terminus); 8.06 (1H, s, CON<u>H</u>CH_2), 3.34 (2H, $C_\alpha H_2$, alpha from NH), 2.62 (2H, $C_\beta H_2$). MALDI-MS (m/z) calculated for $C_{72}H_{106}N_{26}O_{15}S$ (M^+) 1639.91, found 1640.03.

Inhibition Patterns of 8 Versus Variable ATP and Crosstide (Arg-Pro-Arg-Thr-Ser-Ser-amide) Substrates.



Synthesis and characterization of compounds 9 - 10. Both peptides were prepared in a fashion analogous to that of peptide **1**. However, instead of N-terminal acetylation, the peptides were acylated with indole-3-acetic acid, and the side chain protecting groups subsequently removed with a cocktail of TFA (95%), TIS (2.5%) and water (2.5%) for 3 h at ambient temperature. The resin was sequentially washed with DMF, 2-propanol and CH_2Cl_2. The peptides were cleaved from the resin with 20 mM DTT solution for 3 h (2x). Purification was achieved via HPLC as described above. MALDI-MS (m/z) calculated for compound **9** $C_{56}H_{95}N_{21}O_{11}S$ (M^+) 1270.5, found 1271.1. MALDI-MS (m/z) calculated for compound **10** $C_{55}H_{92}N_{22}O_{12}S$ (M^+) 1285.5, found 1286.5.

Akt Assays.

General. The peptide Arg-Pro-Arg-Thr-Ser-Ser-amide (crosstide) was used as the Akt substrate.

Akt screen. 20 μL of 12.5 μM peptide inhibitor candidate (from each well of **Libraries 1** - **4**) was added to individual wells of 96 multiwell assay plates containing 20 μL assay buffer [20 mM HEPES (pH 7.4), 37.5 μM substrate, 10 mM DTT, 10 mM $MgCl_2 \cdot 6H_2O$, 0.5 mM EGTA·Na, and 250 μM cold ATP supplemented with 330 μCi/96-well plate (3 μCi/well) [γ-^{33}P] ATP for radioactive detection. 10 μL of an enzyme buffer solution, containing 20 mM Tris (pH 7.5), Akt α (0.1 μg/well), 150 mM NaCl, 2.0 mM DTT, 0.01% Triton®X-100, 0.1 mM Na_3VO_4, 20% glycerol, and 0.5 mM EDTA·4Na·$2H_2O$, was added to initiate the reaction. Total reaction volume in each well was 50 μL. After a 10-min incubation at 30 °C, 100 μL of 6% phosphoric acid was added to each well to stop the reaction (total volume: 150 μL). Following an additional 5 min

incubation at ambient temperature, 75 μL from each reaction well was transferred into each well of a Unifilter (P81 cellulose phosphate paper) assay plate and washed four times with 0.1% phosphoric acid in water. ScintiSafe 30% solution was added to each well and ^{33}P-incorporation measured by scintillation counting with a 1420 MicroBeta^{TM} TriLux & MicroBeta JET (Perkin Elmer). Lead compounds from each library were combined into a single 96 well plate and re-assayed as described above in order to identify the best inhibitor.

IC_{50} determinations of resynthesized inhibitor leads. Assays were performed in triplicate at pH 7.5 and thermostatically maintained at 30 ºC using a Boekel constant temperature device. 20 μL assay buffer solution, containing 20 mM Hepes (pH 7.4), 37.5 μM crosstide, $MgCl_2 \cdot 6H_2O$ (10 mM), EGTA·Na (0.5 mM), cold ATP (250 μM), supplemented with 330 μCi/plate [γ–^{33}P]ATP for radioactive detection, were added to 20 μL of a solution containing inhibitor lead at various concentrations (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512 μM). 10 μL enzyme buffer solution containing 20 mM Tris (pH 7.5), Akt (~0.1 μg/well), NaCl (150 mM), DTT (2.0 mM), Triton®X-100 (0.01%), Na_3VO_4, glycerol (20%), and $EDTA \cdot 4Na \cdot 2H_2O$ (0.5 mM) was added to initiate the reaction. Reactions and their work-up were carried out as described above. The IC_{50} values for pure compounds as inhibitors were calculated based on the experimental data using GraFit (Erithacus Software Limited).

K_i determinations versus variable crosstide concentration. 20 μL of inhibitor (concentration = 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 μM) was added to a 20 μL assay buffer containing 20 mM HEPES (pH 7.4), crosstide (concentrations = 10, 13.3, 20, and 40 μM), 10 mM $MgCl_2 \cdot 6H_2O$, 0.5 mM EGTA·Na, and 250 μM cold ATP supplemented with 3.0 μCi/well [γ-^{33}P]ATP for radioactive detection. 10 μL enzyme diluted buffer containing 20 mM Tris (pH 7.5), Akt (0.1 μg/well), 150 mM NaCl, 2.0 mM DTT, 0.01% Triton®X-100, 0.1 mM Na_3VO_4, 20% glycerol, and 0.5 mM $EDTA \cdot 4Na \cdot 2H_2O$ was added to initiate the reaction. Total reaction volume in each well was 50 μL. All experiments were performed in triplicate. Subsequent assay workup and scintillation counting were performed as described above. K_i values were calculated using KaleidGraph (Synergy Software).

K_i determinations versus variable ATP concentration. 20 μL of inhibitor (concentration = 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 μM) was added to a 20 μL assay buffer containing 20 mM HEPES (pH 7.4), 37.5 μM crosstide, 10 mM $MgCl_2 \cdot 6H_2O$, 0.5 mM EGTA·Na, and cold ATP (concentrations = 5, 6.6, 10, and 20 μM), supplemented with 0.2 μCi/well [γ-^{33}P]ATP for radioactive detection. 10 μL enzyme diluted buffer containing 20 mM Tris (pH 7.5), Akt α (0.1 μg/well), 150 mM NaCl, 2.0 mM DTT, 0.01% Triton®X-100, 0.1 mM Na_3VO_4, 20% glycerol, and 0.5 mM 0.5 mM $EDTA \cdot 4Na \cdot 2H_2O$ was added to initiate the reaction. Total reaction volume in each well was 50 µL. All experiments were performed in triplicate. Subsequent assay workup and scintillation counting were performed as described above.