Supporting Information

In Vitro Selection of Anti-Akt2 Thioether-Macrocyclic Peptides Leading to Isoform-Selective Inhibitors

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Supplementary Figures and Tables

Class	Structure	Name	Inhibition Activity [nM] (K_i or IC_{50})			Cross-Inhibition against Other Kinases [nM]	Ref.[a]
			Akt1	Akt2	Akt3		
I		A674563	11	✓	✓	+ (16–13,000)	[11]
I		A443654	2.5	30	51	+ (6.3–3,600)	[11,12]
I		GSK690693	2	13	9	+ (2–81)	[13]
I		CCT128930	✓	6	✓	+ (120–168)	[14]
I		AT7867	32	17	47	+ (20–85)	[15]
II		perifosine	N.R.	N.R.	N.R.	N.R.	[16]
II	H-AVTDHPDRLWAWEKF-OH	Akt-*in*	18,000 (K_d)	✓	✓	–	[17]
II		API-1	✓	800 (*cell assay*)	✓	–	[18]
II		triciribine	690 (K_d)	✓	✓	–	[19, 20]
II		PIT-1	31,000[b]	✓	✓	+ (40,000–67,000)	[21]
III		MK2206	8	12	65	– (>1,000)	[22]
III		Akti-1	760	24,000	>50,000	– (>50,000)[c]	[24]
III		Akti-2	21,200	325	21,870	– (>50,000)[c]	[24]
III		Akti-1/2	58	210	2,119	+ (3,990)	[24, 25]
IV	RPR-Nval-Y-DAP-Hol	peptide1	112	N.R.	N.R.	+ (3,000-15,000)	[27]
IV	H-ARKRERTYSFGHHA-OH	AKTide-2T	12,000	N.R.	N.R.	N.R.	[28]
IV	H-VELDPEFEPRARERAYAFGH-OH	peptide4	95	✓	✓	+ (12,500)	[29]
IV	Anti-Akt1 scFv	anti-Akt1	✓	✓	✓	N.R.	[30]

[a] All numbered references in this table are cited in the text.
[b] A K_d value was determined by Akt-PH domain competing with PIP3 labeled with tetramethylrhodamine using fluorescence polarization.
[c] Referred in [5] in Supporting Information.

Table 1

Representative Akt inhibitors in class (I)–(IV).

Check mark (√) denotes a comparable inhibitory activity to a K_i or an IC_{50} value reported against an Akt isoform even though the exact values are unavailable in literature. N.R. denotes not reported in literature. Cross-inhibitory action observed against other kinases is shown as + (positive cross-inhibition) or – (no or poor cross-inhibition), and the reported range of the inhibitory potencies is shown in parentheses. "*Cell assay*" indicates an IC_{50} value determined by an appropriate western-blotting analysis of phosphorylation of Akt1 S473 (Akt2 S474) in cells. Since neither K_i nor IC_{50} value is currently available for triciribine, the available K_d value is shown instead. In peptide1, Nval, DAP, and Hol abbreviates norvaline, 2,3-diaminopropionic acid, and homoleucine, respectively.

Primer and Linker	Nucleotide Sequence
P1	5'-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATG-3'
PNNK4	5'-GCTGCCGCTGCCGCTGCCGCA $(MNN)_4$ CATATGTATATCTCCTTCTTAAAG-3'
PNNK5	5'-GCTGCCGCTGCCGCTGCCGCA $(MNN)_5$ CATATGTATATCTCCTTCTTAAAG-3'
PNNK6	5'-GCTGCCGCTGCCGCTGCCGCA $(MNN)_6$ CATATGTATATCTCCTTCTTAAAG-3'
PNNK7	5'-GCTGCCGCTGCCGCTGCCGCA $(MNN)_7$ CATATGTATATCTCCTTCTTAAAG-3'
PNNK8	5'-GCTGCCGCTGCCGCTGCCGCA $(MNN)_8$ CATATGTATATCTCCTTCTTAAAG-3'
PNNK9	5'-GCTGCCGCTGCCGCTGCCGCA $(MNN)_9$ CATATGTATATCTCCTTCTTAAAG-3'
PNNK10	5'-GCTGCCGCTGCCGCTGCCGCA $(MNN)_{10}$ CATATGTATATCTCCTTCTTAAAG-3'
PNNK11	5'-GCTGCCGCTGCCGCTGCCGCA $(MNN)_{11}$ CATATGTATATCTCCTTCTTAAAG-3'
PNNK12	5'-GCTGCCGCTGCCGCTGCCGCA $(MNN)_{12}$ CATATGTATATCTCCTTCTTAAAG-3'
P2	5'-TTTCCGCCCCCCGTCCTAGCTGCCGCTGCCGCTGCCGCA-3'
DNA-PEG-CCPu	$(SPC18)_5$CC(Puromycin) attached at: 5'-CTCCCGCCCCCCGTCC-3'

Table 2

Sequences of primers and DNA-PEG-CCPu used in this study.

SPC18 indicates a hexaethylene glycol chain referred to as Spacer 18.

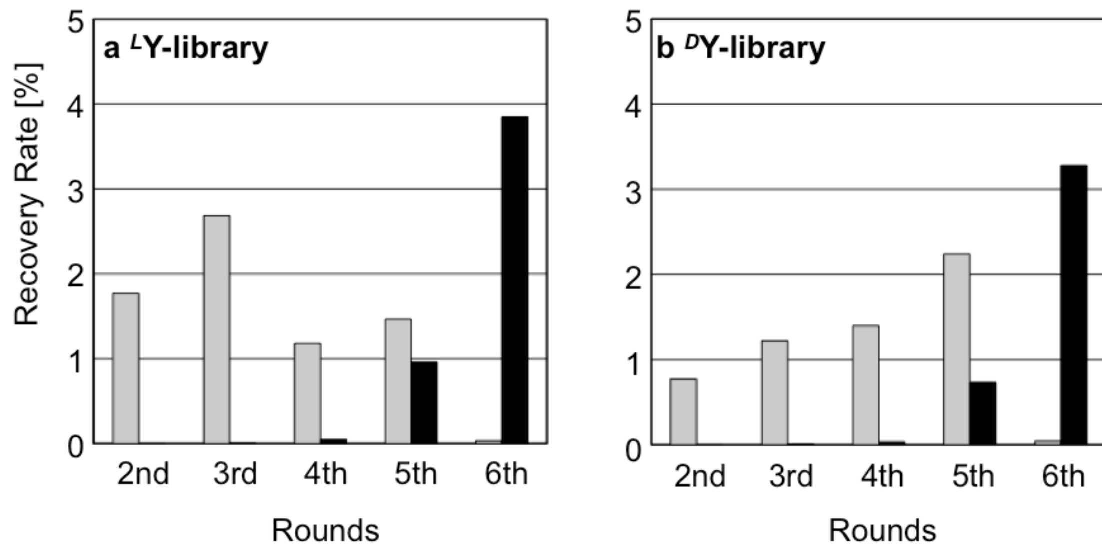
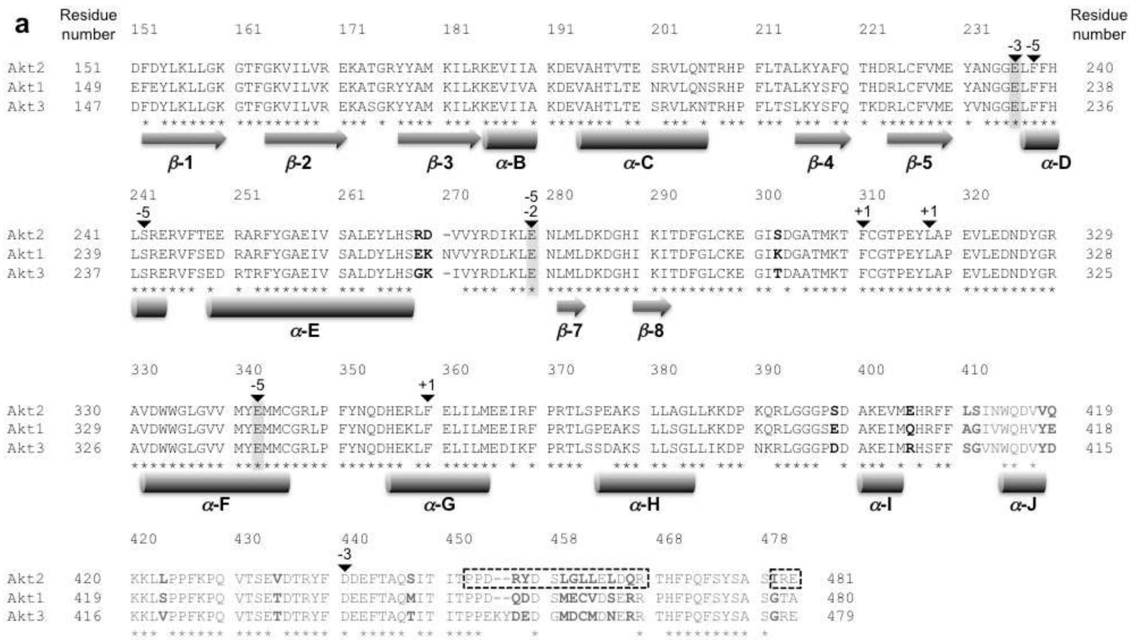


Figure 1

The course of enrichment of a) ^{L}Y- and b) ^{D}Y-libraries.

The recovery rates in the graphs represent the fraction of the eluted cDNAs from mRNA complexes binding to either the Akt2-beads (black bars) or the non-immobilized beads at the last pre-clear process, *i.e.* negative selection (gray bars) over the amount of the input mRNA complexes. After the 5th round, the non-immobilized beads were also washed three times before elution in the same manner as the Akt2-immobilized beads.



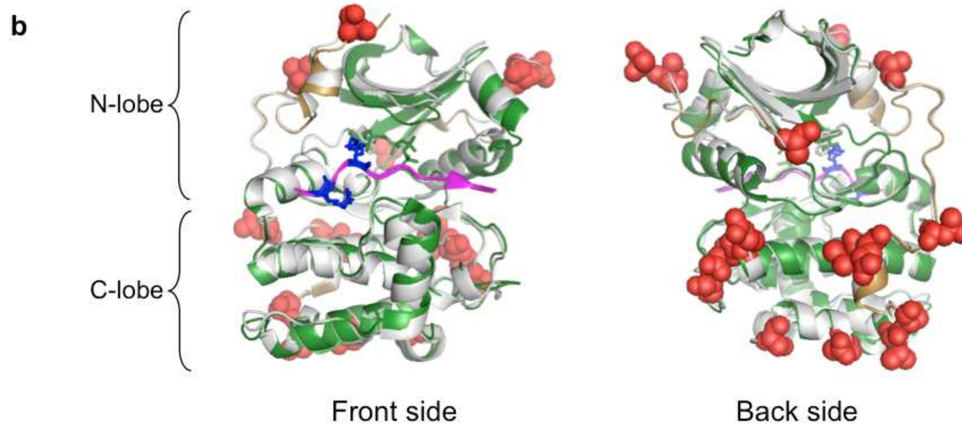


Figure 2

a) Sequence alignment of the kinase domain and C-terminal region of Akt isoforms by Clustal W.

The sequences are based on human Akt1 (accession number: P31749), human Akt2 (P31751), and human Akt3 (Q9Y243), obtained from UniprotKB. Asterisks indicate identical amino acid residues in human Akt isoforms. Residues in Akt2 kinase domain (152–409) and C-terminal region (410–481) are shown in black and gray, respectively. Gray cylinders and arrows represent α-helix and β-sheet structures in Akt2 (PDB ID: 1O6K), respectively. Unique or less similar residues in Akt2 over Akt1/3 are bold, corresponding to the residues shown in red sphere atoms in Supplementary Figure 2b.

Residues 452–467 and 479–481 of Akt2 in open dotted-square in the alignment are invisible in the structure due to the disordered regions. Arrowheads indicate the key residues in Akt2 that interact with a GSk3β peptide (GRPRTTSFAE) bound to Akt2 in Supplementary Figure 2b. The superscripts, –5, –3, –2, and +1, above the arrowheads indicate residual positions in a GSk3β peptide that interacts with these residues in Akt2. The conserved glutamate residues in Akt isoforms interacting with the two arginine residues at –5 and –3, in a GSk3β peptide are highlighted in gray.

b) Crystal structures of Akt1-ΔPH and Akt2-ΔPH superimposed.

The kinase domain and C-terminal region of Akt1-ΔPH (144–478 in light gray ribbon; PDB ID:3CQW) and Akt2-ΔPH (kinase domain region; 146–409 in green colored ribbon, C-terminal region; 410-479 in sand colored ribbon; PDB ID:1O6K) (*1, 2*). A substrate peptide, GSK3β, is shown in magenta. Two arginine residues at the positions –5 and –3 in a GSK3β peptide are shown as blue stick atoms. Kinase inhibitor and ATP analog in Akt1 and Akt2 are shown as gray and green stick atoms adjacent to the ATP binding pocket. Unique or less similar residues in Akt2 compared to Akt1 (and Akt3) are shown as red sphere atoms. All structures were drawn by the PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.

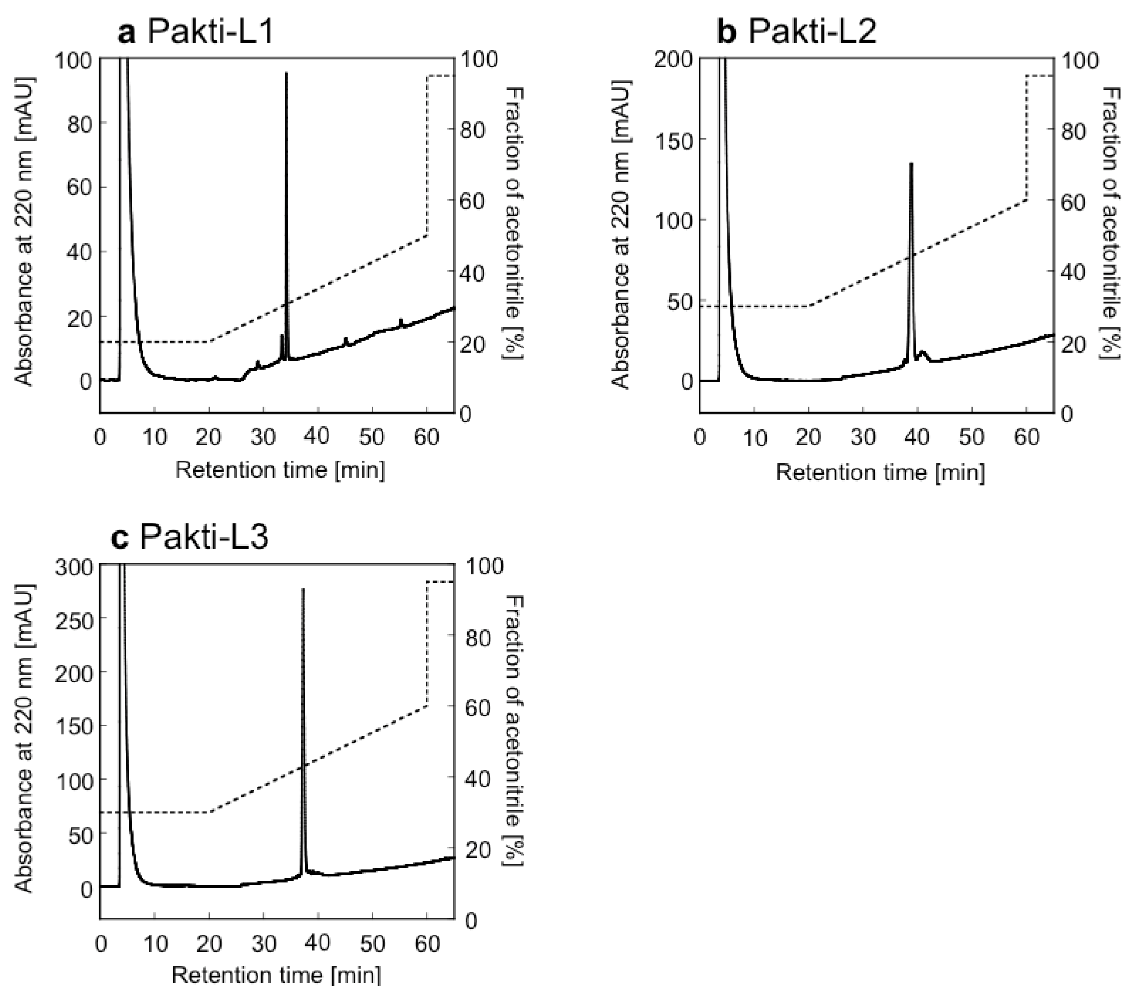


Figure 3.

Analytical reverse-phase HPLC profiles of the purified peptides, a) Pakti-L1, b) L2, and c) L3.

The gradient patterns of the acetonitrile solution are represented by dotted lines.

Peptide	Formula	Calculated Mass [Da] m/z			Measured Mass [Da] m/z		
		$[M+H]^+$	$[M+2H]^{2+}$	$[M+3H]^{3+}$	$[M+H]^+$	$[M+2H]^{2+}$	$[M+3H]^{3+}$
Pakti-L1	$C_{76}H_{130}N_{25}O_{19}S^+$	1728.97	864.99	576.99	1728.97	864.99	576.99
Pakti-L2	$C_{94}H_{144}N_{25}O_{18}S^+$	1943.08	972.05	648.37	-	972.04	648.37
Pakti-L3	$C_{91}H_{137}N_{24}O_{19}S^+$	1902.02	951.51	634.68	1902.02	951.51	634.68

Table 3.

The characterization of the purified peptides.

The ESI mass spectra of the peak fraction of the purified Pakti-L1, L2, and L3 in the RP-HPLC profiles were analyzed by flow injection (direct ESI-MS in positive mode, Thermo Scientific Exactive Mass Spectrometer).

Supplementary Notes

Chemical Synthesis of *N*-Chloroacetyl-*L*-tyrosine-cyanomethyl Ester (ClAc^{L}Y-CME) and *N*-Chloroacetyl-*D*-tyrosine-cyanomethyl Ester (ClAc^{L}Y-CME)

To a mixture of *L*-tyrosine (2.17 g, 12 mmol) and $NaHCO_3$ (2.02 g, 24 mmol) in 34 mL of H_2O, *N*-hydroxysuccinimide 1-chloroacetate (1.53 g, 8 mmol) in 1,4-dioxane was added and then the mixture was stirred at room temperature for 2.5 h. After the reaction, the mixture was evaporated to remove 1,4-dioxane and the solution was washed by ethyl acetate (20 mL x 2). The water layer was acidified by 1M HCl, the solution was extracted with ethyl acetate (30 mL x 2), and the organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue (ClAc^{L}Y-OH) was mixed with *N*,*N*-diisopropylethylamine (DIPEA) (2.8 mL, 16.4 mmol) and chloroacetonitrile (1.6 mL) in 5.5 mL of *N*,*N*-dimethylformamide (DMF), and the reaction mixture was stirred at room temperature for 16 h. After the reaction, diethyl ether was added and the solution was washed with 1 M HCl three times, sat. $NaHCO_3$ three times, and brine once, and the organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography to give ClAc^{L}Y-CME (80.8 mg, 23%): ^{1}H NMR (300 MHz, DMSO-d_6, δ): 9.25 (s, 1H), 8.73 (d, J = 7.2 Hz, 1H), 7.01 (d, J = 8.4 Hz, 2H), 6.66 (d, J = 8.7 Hz, 2H), 4.99 (s, 2H), 4.48 (m, 1H), 4.07 (s, 2H), 2.92 (m, 2H). The high-resolution mass spectrum (HRMS) was measured by flow injection (direct ESI-MS in negative mode, Thermo Exactive spectrometer) because of severe positive ionization. HRMS (ESI) calculated for $C_{13}H_{12}ClN_2O_4^-$: ([M-H]$^-$) m/z 295.0491, found: m/z 295.0495.

ClAc^{D}Y-CME was synthesized and analyzed by the same procedure with ClAc^{L}Y-CME: ^{1}H NMR (300 MHz, DMSO-d_6, δ): 9.26 (s, 1H), 8.72 (d, J = 7.2 Hz, 1H), 7.01 (d, J = 8.4 Hz, 2H), 6.66 (d, J = 8.7 Hz, 2H), 4.98 (s, 2H), 4.48 (m, 1H), 4.07 (s,

2H), 2.92 (m, 2H). HRMS (ESI) calculated for $C_{13}H_{12}ClN_2O_4^-$: ([M-H]$^-$) m/z 295.0491, found: m/z 295.0496.

Protected Amino Acids for Peptide Chemical Synthesis

For chemical synthesis of the selected peptides and crosstide-KK, Fmoc-*L*-Ala-OH, Fmoc-*L*-Cys(Trt)-OH, Fmoc-*L*-Asp(OtBu)-OH, Fmoc-*L*-Glu(OtBu)-OH, Fmoc-*L*-Phe-OH, Fmoc-*L*-Gly-OH, Fmoc-*L*-His(Trt)-OH, Fmoc-*L*-Ile-OH, Fmoc-*L*-Lys(Boc)-OH, Fmoc-*L*-Asn(Trt)-OH, Fmoc-*L*-Pro-OH, Fmoc-*L*-Gln(Trt)-OH, Fmoc-*L*-Ser(tBu)-OH, Fmoc-*L*-Thr(tBu)-OH, Fmoc-*L*-Val-OH, Fmoc-*L*-Trp(Boc)-OH, Fmoc-*L*-Tyr(tBu)-OH, Rink Amide AM resin, and HBTU were purchased from Novabiochem; Fmoc-*L*-Leu-OH, Fmoc-*D*-Tyr(tBu)-OH, and HOBt were purchased from Watanabe Chem. Ind. Ltd.; Fmoc-*L*-Arg(Pbf)-OH was purchased from AAPPTec.

Chemical Synthesis of Pakti-L/Pakti-D Peptides and Crosstide-KK

All of the selected peptides and crosstide-KK were synthesized by an automated peptide synthesizer, Syro I (Biotage AB), using the appropriate amount of resin for a 25 μmol reaction scale. After complete extension of the selected peptides, the amino group of N-terminus of the selected peptides was reacted with 250 μL of 0.2 M monochloroacetyl-*N*-hydroxysuccinimide ester in *N*-methyl-2-pyrrolidone (NMP). The beads were washed with 1 mL of dichloromethane three times and air-dried. Pakti-L1–L4, D1, and D2 were digested from half the amount of the beads and Pakti-L5, L6, D3, and crosstide-KK were digested from the beads by adding 1 mL of a solution consisting of trifluoroacetic acid (TFA), triisopropylsilane (TIS), 1,2-ethanedithiole (EDT), and H_2O in either the ratio of TFA : TIS : EDT : H_2O = 95 : 2.5 : 0 : 2.5 or the ratio of TFA : TIS : EDT : H_2O = 92.5 : 2.5 : 2.5 : 2.5. The crude peptides were separated by filtration from the resin and precipitated with the addition of 10 mL of chilled *tert*-butyl methyl ether or diethyl ether. The precipitates were washed with 10 mL of diethyl ether three times and air-dried. The precipitates of the selected peptides were dissolved in 1 mL of dimethylsulfoxide (DMSO) or 50% (v/v) acetonitrile in H_2O containing 0.1% (v/v) TFA. The precipitate of crosstide-KK was dissolved in 1 mL 10% (v/v) acetonitrile in H_2O containing 0.1% (v/v) TFA. For macrocyclization of the selected peptides, the peptide solutions in DMSO were mixed with 200 μL of 1 M Tris buffer or 6 μL of triethylamine (TEA) and incubated at room temperature for 30 min.

The pH of the peptide suspensions was adjusted to acidic conditions with TFA to quench the macrocyclization reaction. Pakti-L1–L3, L6, D1, and D2 were diluted with 20% (v/v) acetonitrile in H_2O containing 0.1% (v/v) TFA. Pakti-D3 was diluted with 30% (v/v) acetonitrile in H_2O containing 0.1% (v/v) TFA. Pakti-L5 was diluted with 35% (v/v) acetonitrile in H_2O containing 0.1% (v/v) TFA. Pakti-L4 was diluted with 40 % (v/v) acetonitrile in H_2O containing 0.1% (v/v) TFA. Crosstide-KK solutions was diluted with 10% (v/v) acetonitrile in H_2O containing 0.1% (v/v) TFA. The diluted peptides were loaded onto a column, COSMOSIL 5C_{18}-AR-300 (Nacalai, Cat No. 37918-31), and purified by HPLC (Gilson), respectively. Using a rotary evaporator, acetonitrile was removed from the peptides, which were subsequently lyophilized. The selected peptides were dissolved into DMSO as stock solution. The stock peptides were diluted with 50% (v/v) acetonitrile in H_2O containing 0.1% (v/v) TFA or 90% (v/v) DMSO in H_2O and their concentrations were determined by using a spectrophotometer. Crosstide-KK was dissolved into H_2O and filtered with a Millex®-LH filter (Millipore, Cat. No. SLLHH13NK). The concentration of crosstide-KK was determined by amino acid analysis.

Reverse-Phase HPLC Profiles of the Purified Peptides

One millimolar Pakti-L1 in DMSO was 50-fold diluted with 20% (v/v) acetonitrile in H_2O containing 0.1% (v/v) TFA, and 1 mM Pakti-L2 and L3 in DMSO were 50-fold diluted with 30% (v/v) acetonitrile in H_2O containing 0.1% (v/v) TFA, respectively. The resultant 40 µM peptide solutions were filtrated with 0.45 µm hydrophilic syringe filter (Millipore Cat. No. SLLHR04NK). 50 µL of the filtrate Pakti-L1 was injected into HPLC equipped with reversed-phase chromatography column, COSMOSIL® 5C_{18}-AR-300 (Nacalai Cat. No. 37914-71), equilibrated in 20% (v/v) acetonitrile in H_2O containing 0.1% (v/v) TFA and eluted with a 0.75% min^{-1} gradient (dotted line) of 99.9% (v/v) acetonitrile in 0.1% (v/v) TFA solution up to 50% (v/v) acetonitrile solution. In the same manner, 50 µL of the filtrates Pakti-L2 and L3 were injected into the same column equilibrated in 30% (v/v) acetonitrile in H_2O containing in 0.1% (v/v) TFA and eluted with a 0.75% min^{-1} gradient (dotted lines) of 99.9% (v/v) acetonitrile solution in 0.1% (v/v) TFA up to a 60% (v/v) acetonitrile solution, respectively. The RP-HPLC profiles were monitored by measuring the absorbance at 220 nm by a diode array

detector (Hitachi L-2455).

Preparation of the Purified Peptides for Their Characterization by ESI-MS

The main fractions of Pakti-L1, L2, and L3 in the analytical RP-HPLC profiles (Supplementary Figure 3) were lyophilized and dissolved with 66.6% (v/v) acetonitrile in H_2O containing 0.02% (v/v) formic acid for Pakti-L1 and 75% (v/v) acetonitrile in H_2O containing 0.01% (v/v) formic acid for Pakti-L2 and L3, respectively.

Primers and Kinases

All the primers used in this study are listed in Supplementary Table S2. Inactive full-length Akt2 protein tagged with N-terminal hexahistidine (His-Akt2, Cat. No. ab42593, EC number: 2.7.11.1) was purchased from Abcam. Active Akt2 tagged with N-terminal hexahistidine and deleted the PH domain (His-Akt2 ΔPH S474D, Cat. No. 14-339) was purchased from Millipore. Active GST-Akt1 ΔPH (Cat. No. 01-101, EC number: 2.7.11.1), GST-Akt2 ΔPH (Cat. No. 01-0102), GST-Akt3 ΔPH (Cat.No. 01-103, EC number: 2.7.11.1), GST-PKA Cα (Cat. No. 01–127, EC number: 2.7.11.11), and GST-SGK (Cat.No. 01-158, EC number: 2.7.11.1) were purchased from Carna Biosciences Inc..

Preparation of Flexizyme and $tRNA^{fMet}_{CAU}$

All primers for preparation of flexizyme and **$tRNA^{fMet}_{CAU}$** were purchased from Operon Biotechnology (Japan). The details were described previously (*3, 4*).

Preparation of $ClAc^{L}Y$-$tRNA^{fMet}_{CAU}$ or $ClAc^{D}Y$-$tRNA^{fMet}_{CAU}$

To prepare $ClAc^{L}Y$-$tRNA^{fMet}_{CAU}$ or $ClAc^{D}Y$-$tRNA^{fMet}_{CAU}$, 40 μM $tRNA^{fMet}_{CAU}$, 600 mM $MgCl_2$, and 5mM $ClAc^{L}Y$-CME or $ClAc^{D}Y$-CME in DMSO were mixed in 100 mM HEPES-KOH (pH 8.0) and incubated on ice for 1 h. After the reaction, one-tenth volume of 3 M sodium acetate (pH 5.2) was added to quench the reaction, and the tRNA was recovered by ethanol precipitation. The pellet was rinsed twice with 70% (v/v) ethanol containing 0.1 M sodium acetate (pH 5.2). The pellet was air-dried and stored at –80 °C. The pellet was dissolved with 1 mM sodium acetate (pH 5.2) before use for in vitro translation reaction.

Determination of the Amino Acid Sequences Enriched in the Respective Libraries

The eluted cDNAs from the respective libraries after the 5th and 6th rounds in the enrichment process were cloned in the vector, pGEM-T easy (Promega, Cat. No. A1360) and transformed into the competent cells of *E.coli* strain DH5α. The isolated colonies arbitrarily chosen from the transformants were cultured in 3 mL of LB medium at 37 °C over night. From the cultures, the plasmids were purified using DNA purification kit, and then the coding region of random amino acid sequence was analyzed.

Inhibition Assays for Akt Isoforms and Other Kinases

The inhibitory activities of the peptides, Pakti-L1, L2, and L3, against other Akt isoforms were evaluated using HTRF® KinEASE™ STK2 kit for GST-PKA Cα (0.05 nM) and STK3 kit for GST-Akt1 ΔPH (0.2 nM), GST-Akt2 ΔPH (0.2 nM), GST-Akt3 ΔPH (0.1 nM), and GST-SGK (1 nM), where the concentration of kinases used in the assay is shown in parenthesis. The assays were performed according to the manuals' protocols. The reaction mixtures containing one of the kinases, the substrate peptide STK2 or STK3, and one of the cyclic peptides in 1% (v/v) DMSO or 1% (v/v) DMSO in the kinase buffer were pre-incubated at room temperature. The samples were mixed with 1 mM ATP (f.c. 100 μM) to initiate the reaction, incubated at room temperature for 30 min, and added the detection reagents containing ethylenediaminetetraacetic acid (EDTA) to quench the phosphorylation reaction. After additional incubation at room temperature for 1 h, the intensities of the emitted fluorescence at 620 nm and 668 nm were measured by a plate reader, Flexstation 3 (Molecular Devices Inc.), after excitation at 314 nm. The ratio of the fluorescence at 668 nm to that at 620 nm was defined as phosphorylation activity. The average of the activities in triplicate of the samples without the inhibitory peptide or kinase were defined as the activities, 100% or 0%, respectively. Compared to the activities, 100% and 0%, the average of the phosphorylation activities in triplicate at each concentration of a peptide was normalized as the relative activity. Error bars were calculated as the standard deviations of the relative activities in triplicate.

Determination of IC_{50} Values of the Inhibitory Peptides against Akt Isoforms

The reaction mixture contains 3.68 μL of H_2O, 1 μL of 10× kinase buffer (500 mM Tris-HCl (pH 7.5), 1 mM ethyleneglycoltetraacetic acid (EGTA), and 100 mM

$Mg(OAc)_2$), 1.32 μL of 760 μM crosstide-KK, 2 μL of an inhibitory peptide in 1% (v/v) DMSO or 1% (v/v) DMSO, and 1 μL of 20 nM GST-Akt2 ΔPH, GST-Akt1 ΔPH, or GST-Akt3 ΔPH. The samples were mixed with 1 μL of 1 mM ATP (f.c. 100 μM) spiked with [γ-^{32}P]ATP to initiate the reaction, incubated at 30 °C for 30 min, and spotted on phosphocellulose papers, respectively. The resultant papers were immersed immediately into the adequate volume of 75 mM phosphoric acid and stirred with a magnetic stir bar at room temperature for 5 min. The papers were replaced into fresh 75 mM phosphoric acid and immersed at room temperature for 5 min two more times. The papers were rinsed gently in acetone and air-dried. The intensity of Cherenkov light from the papers was measured by a liquid scintillation counter (Beckman LC6500). The phosphorylation activity at each concentration of an inhibitory peptide was measured in triplicate. The average of the phosphorylation activities of the samples without inhibitory peptides or kinase were defined as the activities of 100% or 0%, respectively. The average of observed activities in triplicate at each concentration of a particular peptide was normalized as the relative activity in which error bars were calculated as the standard deviations of the relative activities in triplicate. IC_{50} values were estimated by the curve fitting using Hill equation by KaleidaGraph (Hulinks Inc.)

References

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