Supplementary Information (SI) for Organic & Biomolecular Chemistry.

Supplementary Information

Affinity Emhancement of Polo-like Kinase 1 Polo-box Domain-binding Peptides by *N*-Methylation and Lipidation

Kohei Tsuji,[†,*] Kenan Bai,[†] Xueyuan Huang,[†] Hirokazu Tamamura

Department of Medicinal Chemistry, Laboratory for Biomaterials and Bioengineering, Institute of Integrated Research, Institute of Science Tokyo, 2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062, Japan

[†] These authors contributed equally to this work.

*Correspondence to K. Tsuji, E-mail: ktsuji.mr@tmd.ac.jp; Fax: +81-3-5280-8039

Contents

I. SYNTHETIC PROCEDURES

II. BIOLOGICAL EVALUATION

SCHEMES

TABLE

I. General methods for synthesis and characterization of compounds

I-I. General methods for synthesis

All reactions utilizing air- or moisture-sensitive reagents were performed in dried glassware under an atmosphere of nitrogen or argon (Ar), using commercially supplied solvents and reagents purchased from Sigma-Aldrich, Tokyo Chemical Industry Co., Ltd. (TCI), FUJIFILM Wako Pure Chemical Corporation, KANTO CHEMICAL CO.,INC., NACALAI TESQUE, INC., WATANABE CHEMICAL INDUSTRIES, LTD., KOKUSAN CHEMICAL Co.,Ltd., BLDpharm, Ambeed, Combi-Blocks, and Matrix Scientific without further purification unless otherwise noted. Preparative RP-HPLC was performed using a Cosmosil 5C$_{18}$-AR-II column (20 × 250 mm, Nacalai Tesque, Inc., Japan) on a JASCO PU-2086 plus, PU-2087 plus, and PU-4086-Binary (JASCO Corporation, Ltd., Japan) in a linear gradient of MeCN containing 0.1% trifluoroacetic acid (TFA, Solvent B) in H_2O containing 0.1% (v/v) TFA (Solvent A) at a flow rate of 10 cm^3 min^{-1}, and eluting products were detected by UV at 220 nm using JASCO UV-2075 plus and UV-4075 (JASCO Corporation, Ltd., Japan).

I-II. Characterization methods

Low-resolution mass spectra were recorded on a Bruker Daltonics micrOTOF focus in the positive detection mode. For analytical HPLC, a Cosmosil 5C$_{18}$-AR-II column (4.6 × 250 mm, Nacalai Tesque, Inc.) was employed with a linear gradient of MeCN containing 0.1% TFA (Solvent B) in H_2O containing 0.1% TFA (Solvent A) at a flow rate of 1.0 cm^3 min^{-1} on a PU-2089 plus (JASCO Corporation, Ltd.), and eluting products were detected by UV at 220 nm using JASCO UV-2075 plus.

I-III. General Fmoc-based solid phase peptide synthesis

Peptides were synthesized using NovaSyn® TGR resin (0.23 or 0.25 mmol/g) and 2-chlorotrityl chloride resin (Cl-Trt(2-Cl) resin, 1.36 or 1.40 mmol/g). 9-Fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase peptide synthesis (SPPS) were performed using the following side chain protected amino acids: O^{t}Bu for Glu, ^{t}Bu for Ser, benzyloxy (Bzl) for pThr.

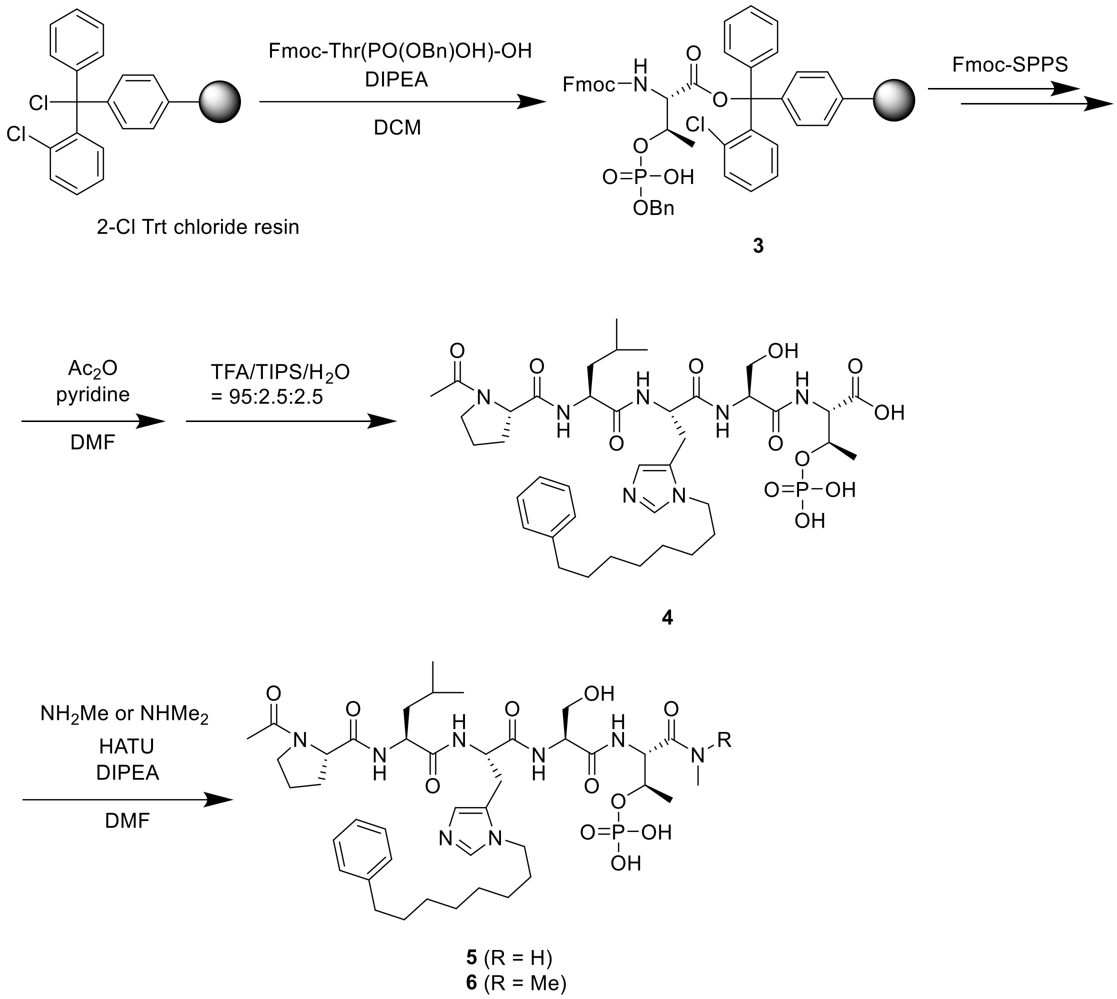
The Cl-Trt(2-Cl) resin was swellen with dichloromethane (DCM) for 30 min with shaking. After the resin swelling, Fmoc-Thr{PO(OBn)OH}-OH (1.0 equiv. based on resin loading) and *N*,*N*-diisopropylethylamine (DIPEA, 4.0 equiv. based on resin loading) in DCM (3.0 mL) was added to the resin, and the mixture was shaken for 2 h. The solution was filtered off, and the resulting resin was washed with DCM and dried under reduced pressure. A portion of the resin was treated with 20% piperidine in *N*,*N*-dimethylformamide (DMF), and the resin loading was calculated from the UV absorbance of the dibenzofulvene-piperidine adduct formed by piperidine treatment at 290 nm.

In the manual peptide synthesis procedures, each cycle of SPPS involves (i) 20 min shaking for Fmoc removal (20% piperidine in DMF) and (ii) 60 min to overnight coupling (Fmoc-amino acid (Fmoc-AA-OH) (2.0 or 4.0 equiv.), 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU, 0.95 mole-equivalents relative to the amino acid) and DIPEA (2.0 mole-equivalents relative to the amino acid) in DMF. Coupling was monitored by the Kaiser ninhydrin test. The coupling step was repeated (double coupling) using a mixture of Fmoc amino acid (1.0 equiv.), HATU (0.95 equiv.), and DIPEA (2.0 equiv.) in DMF, if needed. The elongated peptide resins were treated with Ac_2O (20 equiv.) and pyridine (20 equiv.) in DMF for 45 min for acetylation if necessary. Deprotection of the ivDde group on the Lys ε-amine group was performed by treatment with 2% (v/v) hydrazine monohydrate in *N*-methylpyrrolidone (NMP, two times for 3 h to overnight each). The resin was coupled with Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-miniPEG-OH, 2.0 equiv. based on resin loading) using HATU (1.9 equiv. relative to the amino acid) and DIPEA (4.0 equiv. relative to the amino acid) at room temperature for 1.5 h with shaking, Fmoc-Glu(O^{t}Bu)-OH (2.0 equiv. based on resin loading) using HATU (1.9 equiv. relative to the amino acid) and DIPEA (4.0 equiv. relative to the amino acid) at room temperature for 2 h with shaking, and stearic acid (2.0 equiv. based on resin loading) using HATU (1.9 equiv. relative to the amino acid) and DIPEA (4.0 equiv. relative to the amino acid) at room temperature for 2 h with shaking, or 18-(*tert*-butoxy)-18-oxooctadecanoic acid (2.0 equiv. based on resin loading) using HATU (1.9 equiv. relative to the amino acid) and DIPEA (4 equiv. relative to the amino acid) at room temperature for 2 h with shaking.

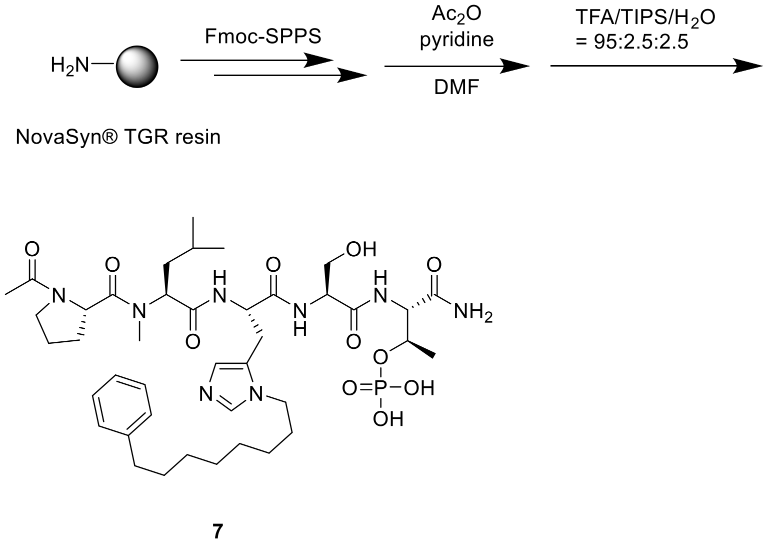
After construction of protected peptides on the resins, the resins were extensively washed with DMF, dichloromethane (DCM), and Et_2O and then dried *in vacuo*. The protected peptide was cleaved from the resin with the deprotection of all the protecting groups on their side chain functional groups by treatment with a mixture of TFA/triisopropylsilane (TIPS)/H_2O = 95:2.5:2.5 (v/v), at room temperature for 2 h. The resin was filtered off and washed with TFA (3 times). The volatile was removed by nitrogen gas flow, and the residue was precipitated by the addition of cold Et_2O. After centrifugation, the supernatant was removed. The precipitate was washed with cold Et_2O (3 times). The crude peptide was purified by preparative RP-HPLC, if necessary. The purified peptide was identified by ESI-TOF MS and analytical RP-HPLC and was lyophilized to obtain as white powder.

The crude peptide carboxylic acid was coupled with methylamine (3.0 equiv. based on resin loading) using HATU (2.85 equiv. relative to based on resin loading) and DIPEA (6.0 equiv. based on resin loading) at room temperature for 1.5 h with shaking, or dimethylamine (3.0 equiv. based on resin loading) using HATU (2.85 equiv. relative based on resin loading) and DIPEA (6.0 equiv. relative to based on resin loading) at room temperature for 2 h with shaking. The obtained crude peptide was purified by preparative RP-HPLC. The purified peptide was identified by ESI-TOF MS and analytical RP-HPLC and was lyophilized to obtain as white powder.

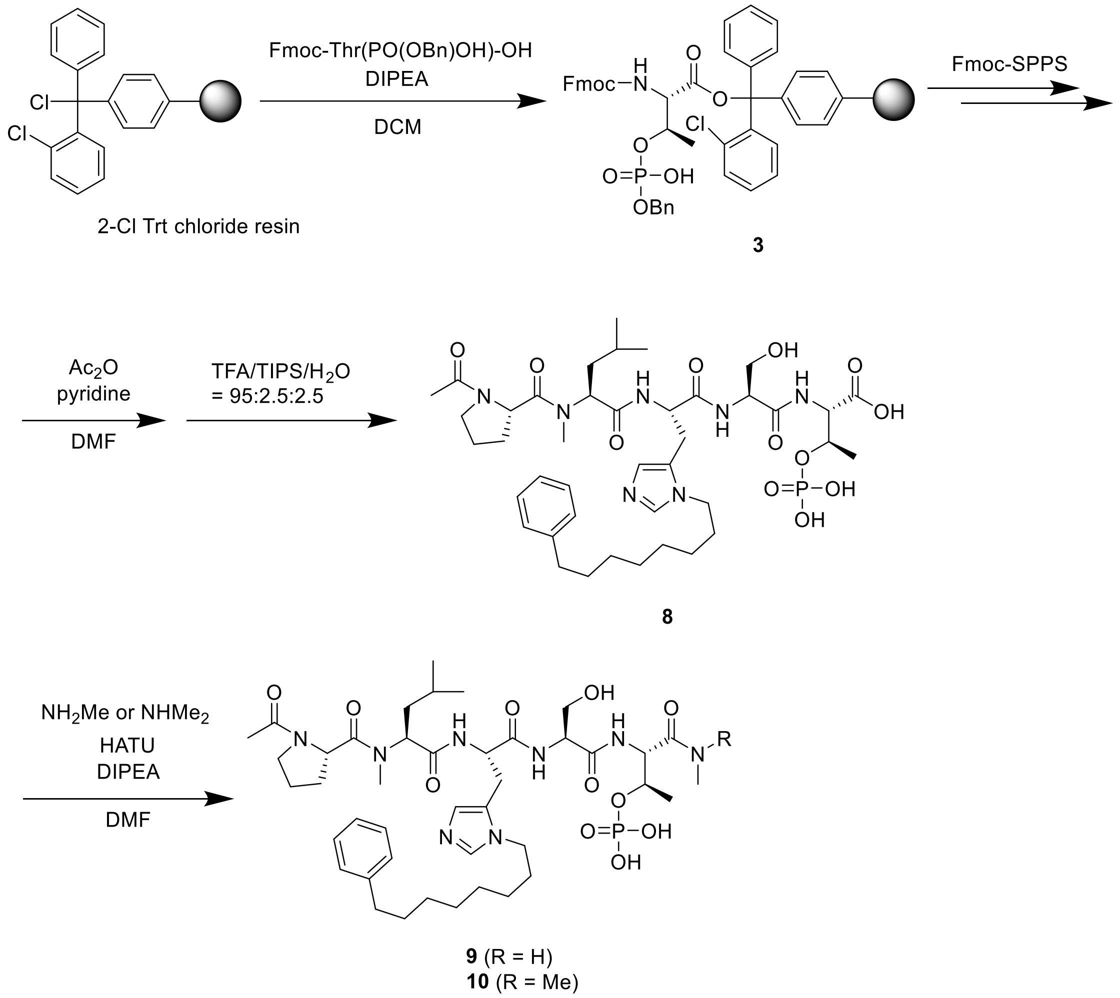
I-IV. Synthesis of C-terminal carboxylic acid peptide 4 and *N*-methylated peptides 5–10



Scheme S1. Synthesis of peptides **4**, **5**, and **6**.



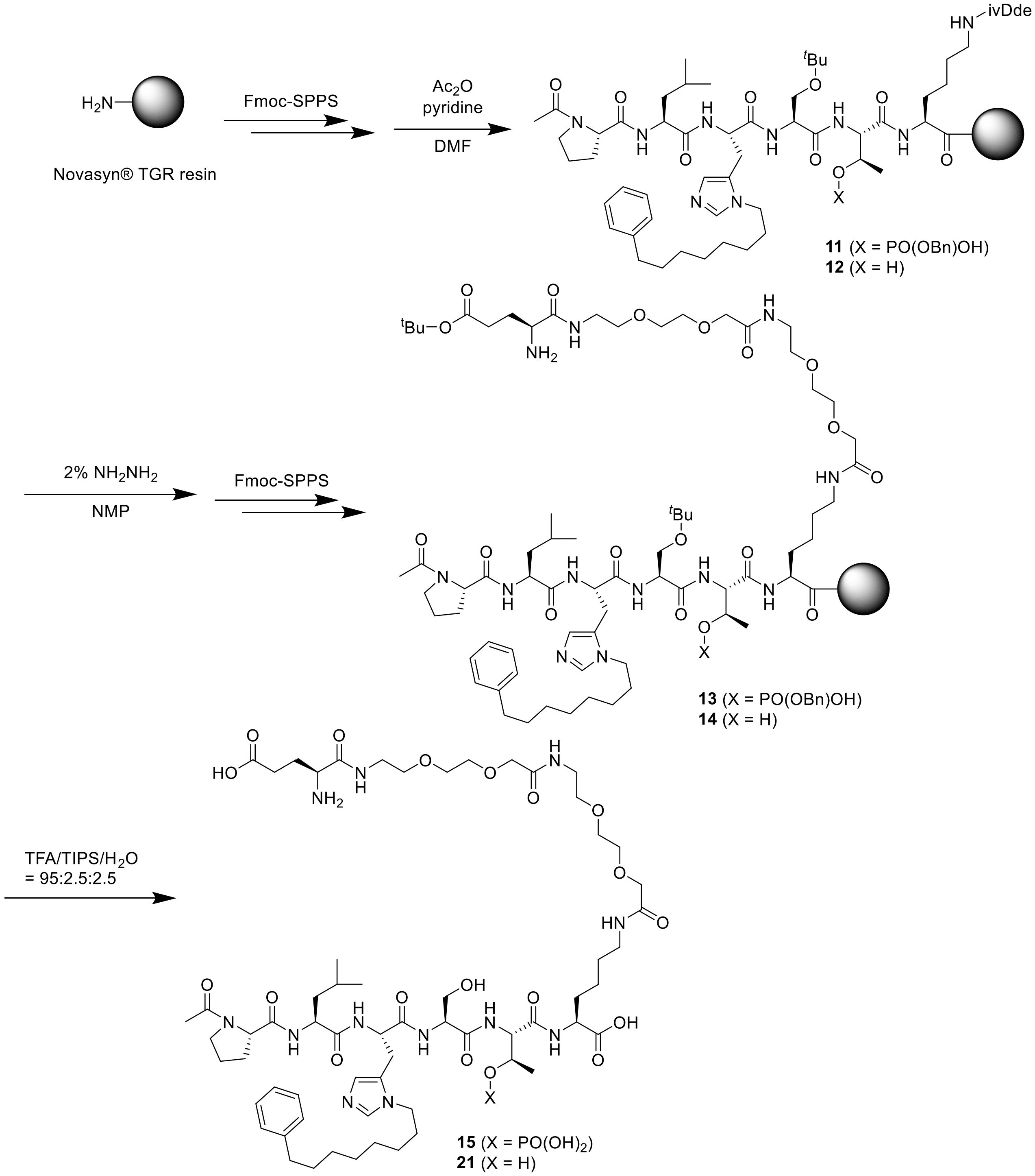
Scheme S2. Synthesis of peptide **7**.



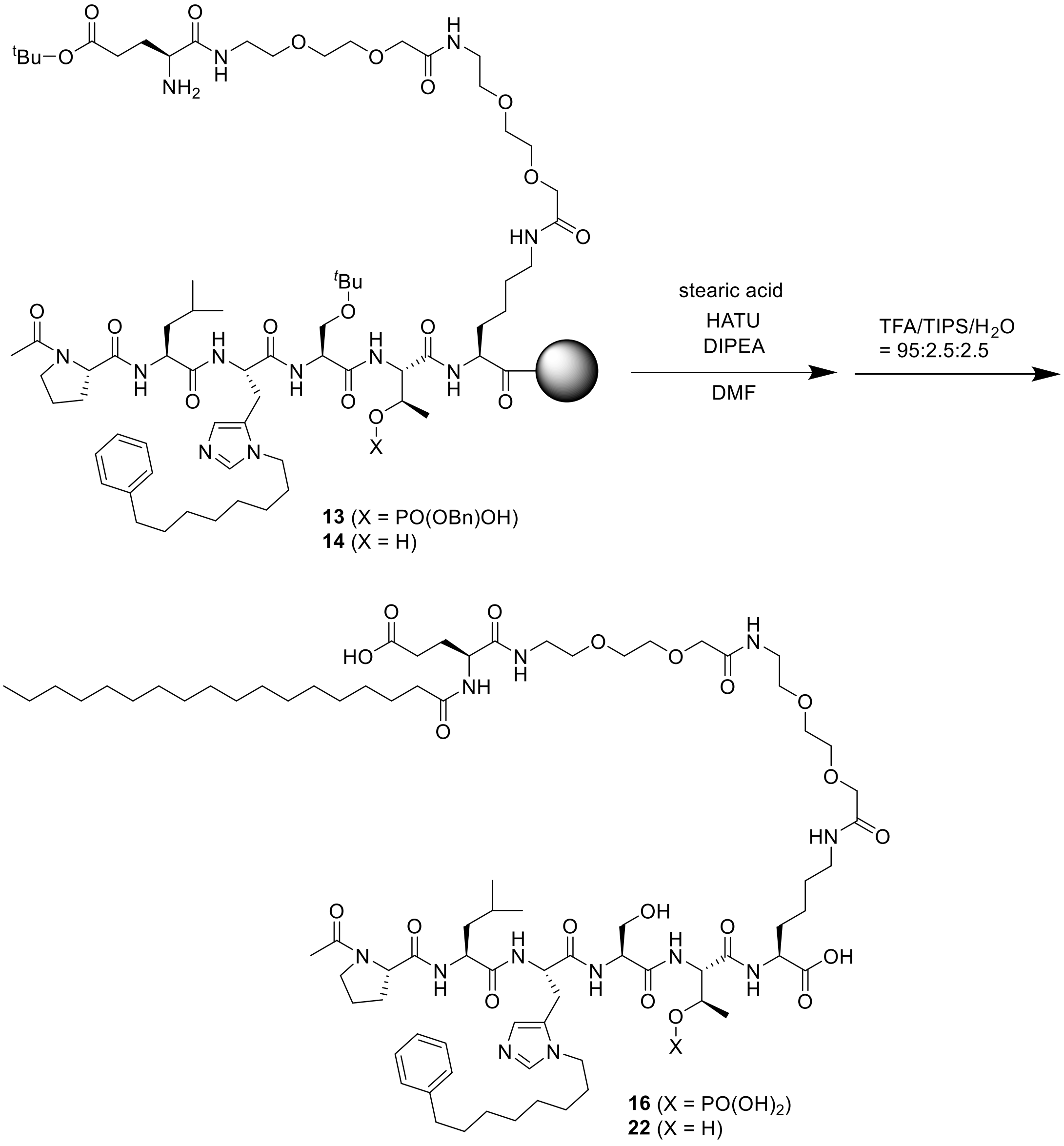
Scheme S3. Synthesis of peptides **8**, **9**, and **10**.

Peptides **4–10** were synthesized following the method mentioned in section **I-III** (Schemes S1–S3), and characterization data of the synthesized peptides were summarized in Table S1. The results of peptides' purity check by analytical RP-HPLC were shown in section **I-VI**.

I-V. Synthesis of lipidated peptides 15–26



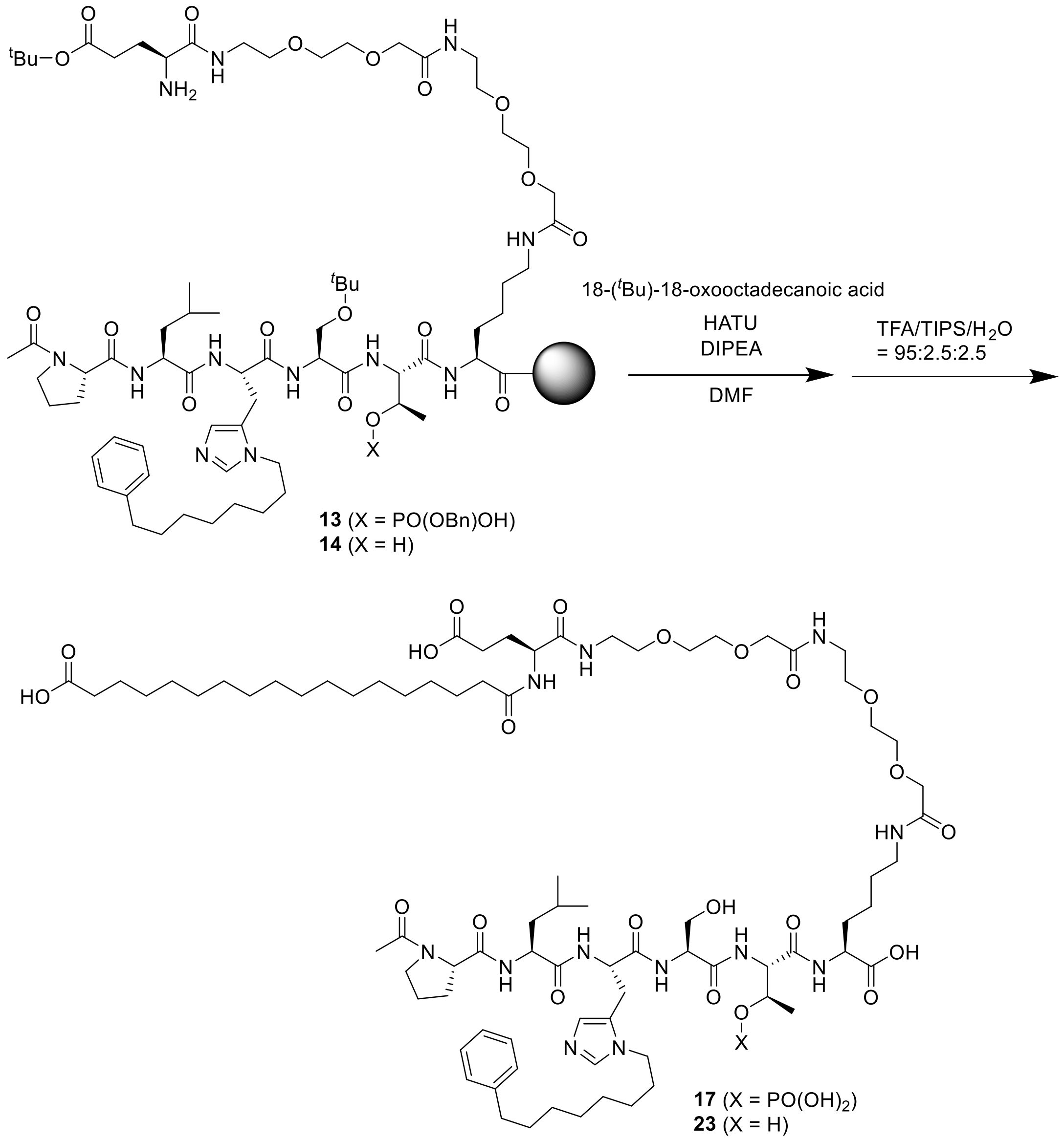
Scheme S4. Synthesis of peptides **15** and **21**.



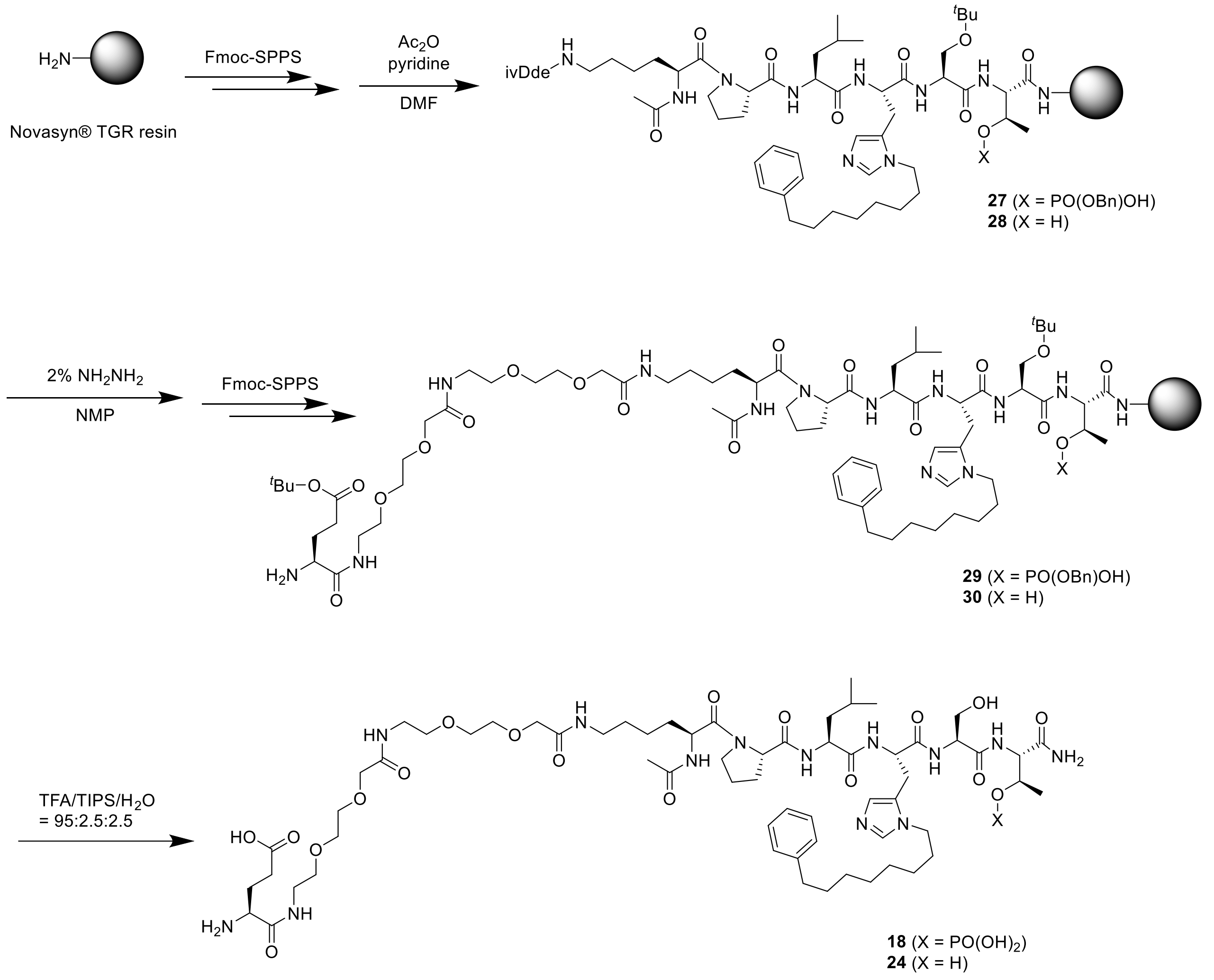
13 (X = PO(OBn)OH)
14 (X = H)

16 (X = $PO(OH)_2$)
22 (X = H)

Scheme S5. Synthesis of peptides **16** and **22**.



Scheme S6. Synthesis of peptides **17** and **23**.

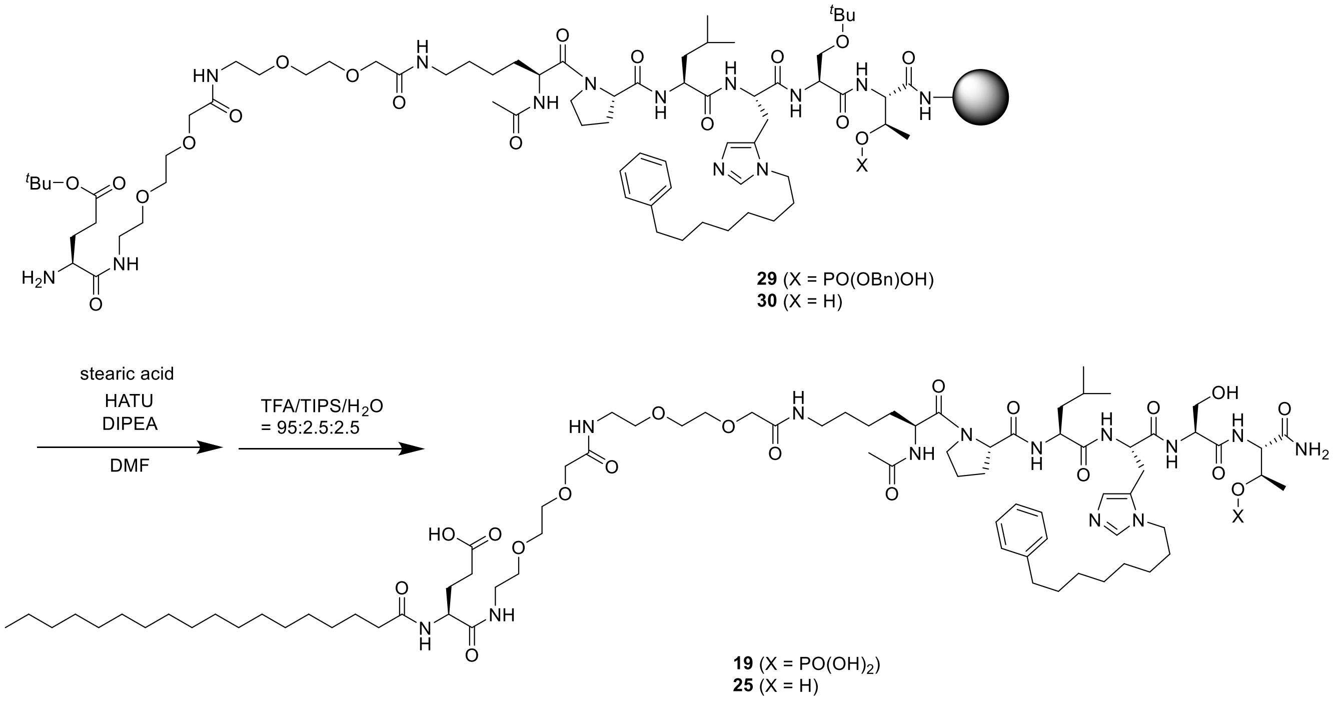


27 (X = PO(OBn)OH)
28 (X = H)

29 (X = PO(OBn)OH)
30 (X = H)

18 (X = PO(OH)$_2$)
24 (X = H)

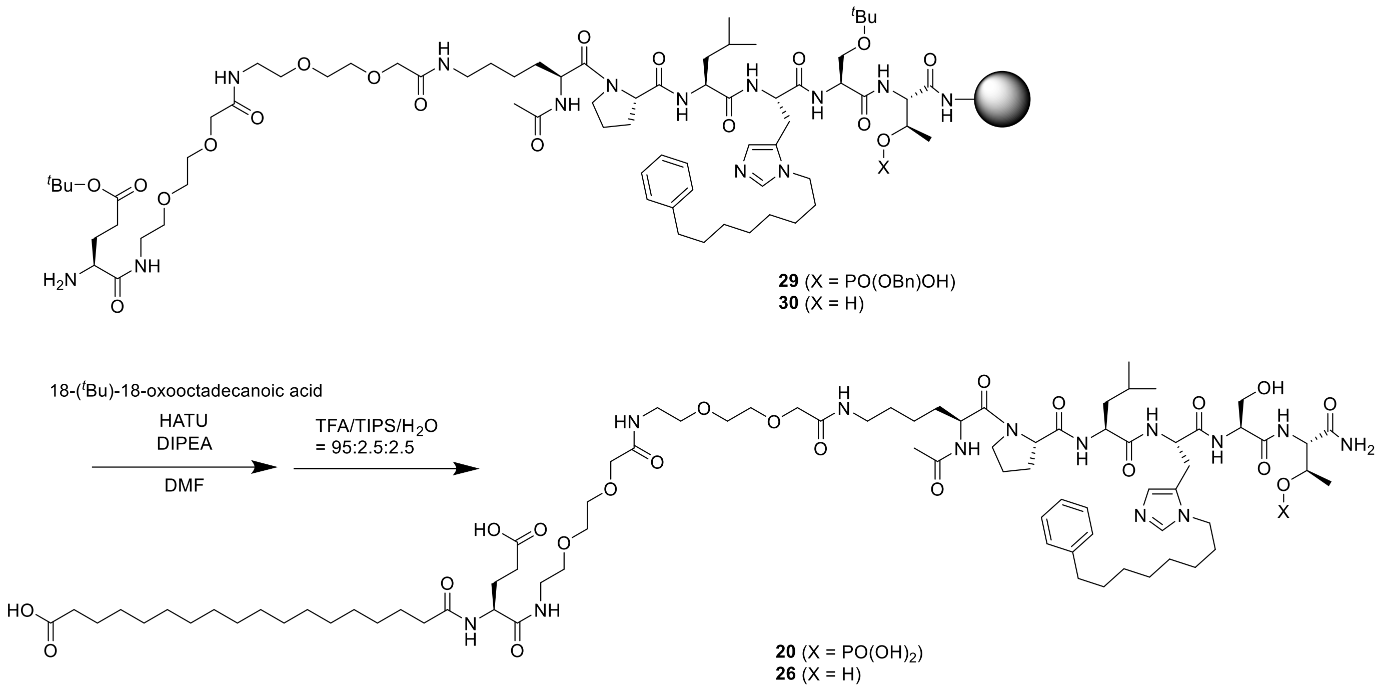
Scheme S7. Synthesis of peptides **18** and **24**.



29 (X = PO(OBn)OH)
30 (X = H)

19 (X = PO(OH)$_2$)
25 (X = H)

Scheme S8. Synthesis of peptides **19** and **25**.



Scheme S9. Synthesis of peptides **20** and **26**.

Peptides **15–26** were synthesized following the method mentioned in section **I-III** (Schemes S4–S9), and characterization data of the synthesized peptides were summarized in Table S1. The results of peptides' purity check by analytical RP-HPLC were shown in section **I-VI**.

Table S1. Characterization data of synthesized peptides **4–10** and **15–26**.

Peptide	Analytical HPLC[a]		Preparative HPLC[b]	*m/z*		Yield[d] (%)
	[c]t_R (min)	Gradient	Gradient	Calcd.	Found	
4	19.4	30% - 50% / 30 min	30% - 50% / 40 min	865.0 $[M + H]^+$	865.0	5.2
5	19.4	30% - 50% / 30 min	30% - 50% / 40 min	878.0 $[M + H]^+$	878.0	6.5
6	19.3	30% - 50% / 30 min	30% - 50% / 40 min	892.0 $[M + H]^+$	892.0	2.1
7	20.0	30% - 50% / 30 min	30% - 50% / 40 min	878.0 $[M + H]^+$	878.0	3.3
8	20.6	30% - 50% / 30 min	30% - 50% / 40 min	879.0 $[M + H]^+$	879.0	6.4
9	21.1	30% - 50% / 30 min	30% - 50% / 40 min	892.0 $[M + H]^+$	892.0	3.2
10	21.4	30% - 50% / 30 min	30% - 50% / 40 min	906.1 $[M + H]^+$	906.1	2.4
15	23.5	25% - 50% / 35 min	25% - 50% / 40 min	1412.6 $[M + 2H]^{2+}$	1412.6	6.2
16	15.2	65% - 95% / 40 min	65% - 95% / 40 min	1679.1 $[M + 2H]^{2+}$	1679.1	8.4

17	28.6	40% - 60% / 40 min	50% - 70% / 40 min	1709.0 $[M + 2H]^{2+}$	1709.0	6.4
18	20.7	40% - 60% / 40 min	25% - 50% / 40 min	1412.6 $[M + 2H]^{2+}$	1412.6	4.6
19	10.9	60% - 80% / 30 min	65% - 95% / 40 min	1679.1 $[M + 2H]^{2+}$	1679.1	7.6
20	25.5	40% - 60% / 40 min	50% - 70% / 40 min	1709.0 $[M + 2H]^{2+}$	1709.0	7.0
21	21.2	25% - 50% / 30 min	25% - 50% / 40 min	1332.6 $[M + 2H]^{2+}$	1332.6	7.4
22	18.1	60% - 80% / 40 min	65% - 95% / 40 min	1599.1 $[M + 2H]^{2+}$	1599.1	8.6
23	18.0	50% - 70% / 40 min	50% - 70% / 40 min	1629.1 $[M + 2H]^{2+}$	1629.1	7.2
24	20.2	25% - 50% / 40 min	25% - 50% / 40 min	1332.6 $[M + 2H]^{2+}$	1332.6	4.9
25	19.0	60% - 80% / 40 min	65% - 95% / 40 min	1599.1 $[M + 2H]^{2+}$	1599.1	4.9
26	25.2	50% - 70% / 40 min	50% - 70% / 40 min	1629.1 $[M + 2H]^{2+}$	1629.1	6.4

0.1% TFA in H_2O (v/v, solvent A) and 0.1% TFA in MeCN (v/v, solvent B) was used for HPLC elution.

[a] Cosmosil 5C_{18}-AR-II analytical column was employed with a linear gradient of solvent B in solvent A.

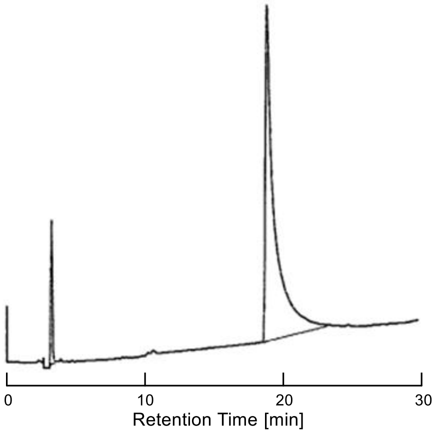
[b] Cosmosil 5C_{18}-AR-II preparative column was employed with a linear gradient of solvent B in solvent A.

[c] Retention time in analytical HPLC analysis.

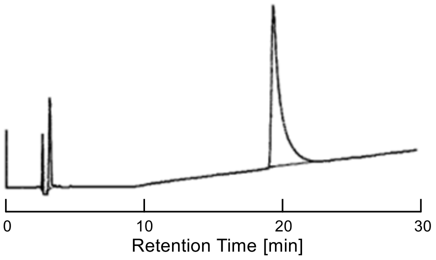
[d] from NovaSyn® TGR resin or Cl-Trt(2-Cl) resin.

I-VI. Analytical HPLC data for the synthesized peptides

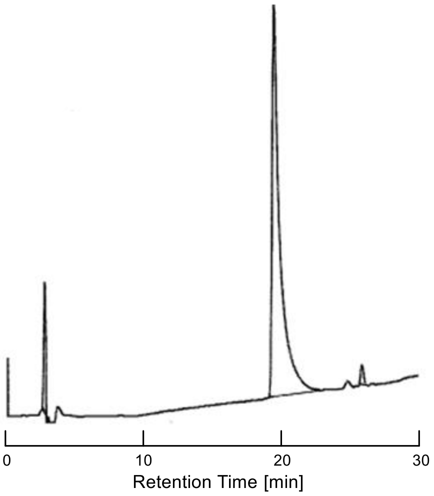
Peptide **4**



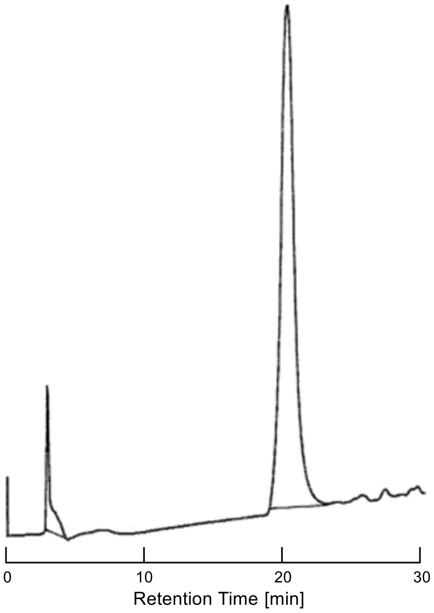
Peptide **5**



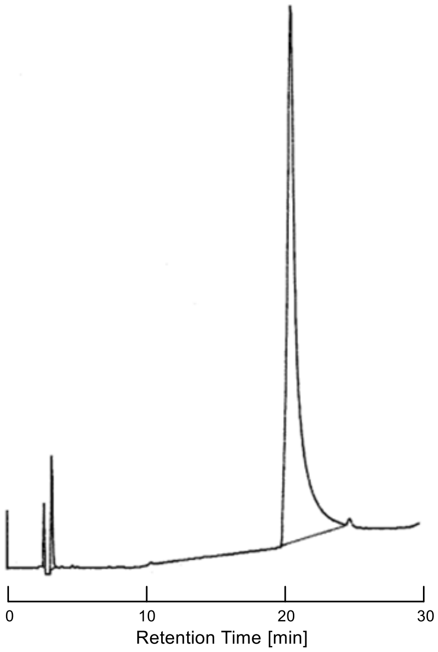
Peptide **6**



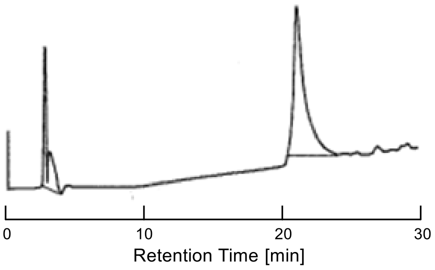
Peptide **7**



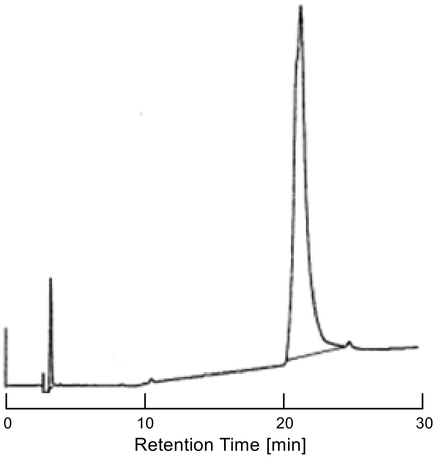
Peptide **8**



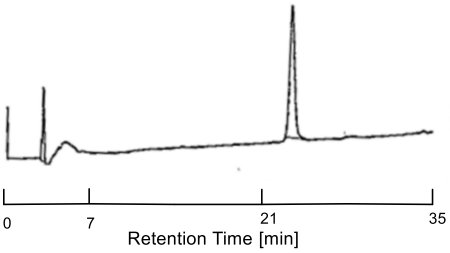
Peptide **9**



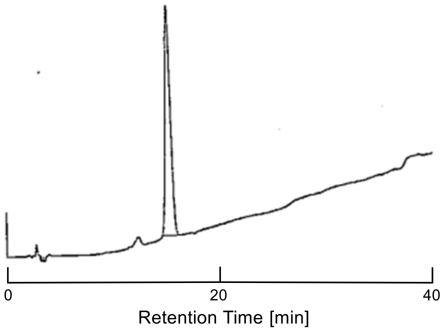
Peptide **10**



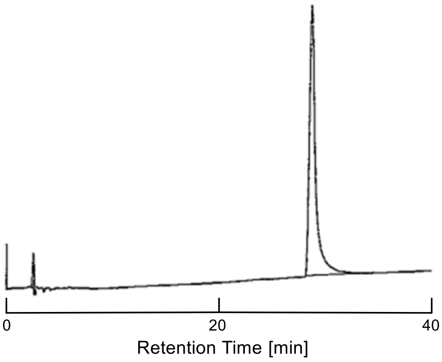
Peptide **15**



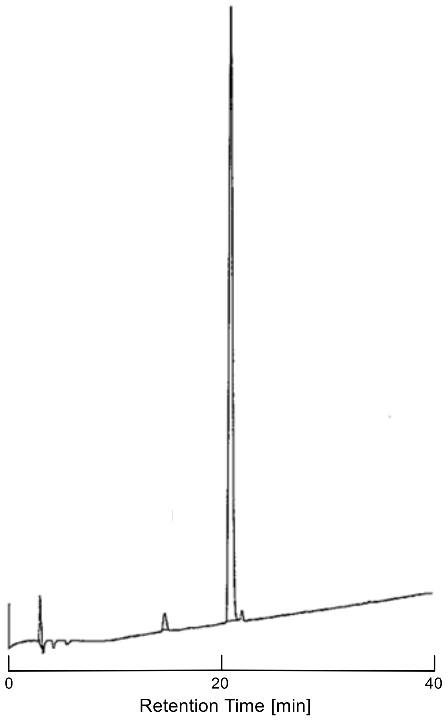
Peptide **16**



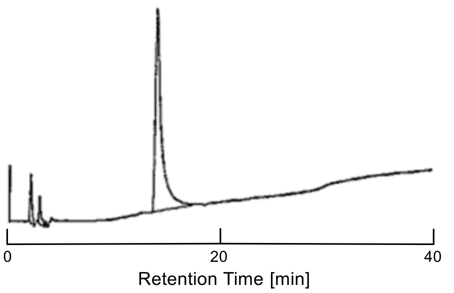
Peptide **17**



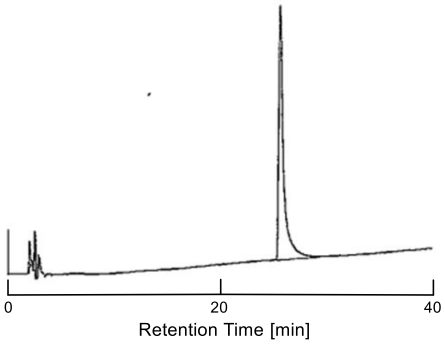
Peptide **18**



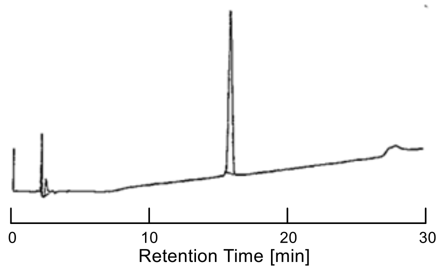
Peptide **19**



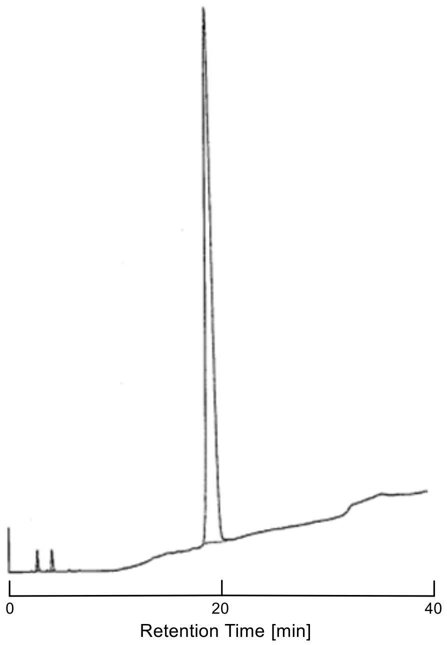
Peptide **20**



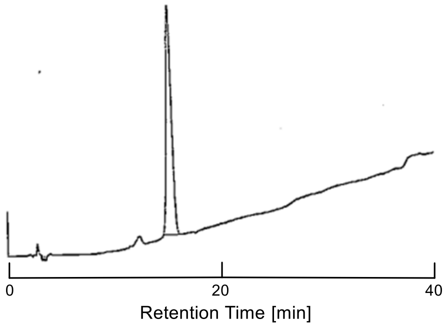
Peptide **21**



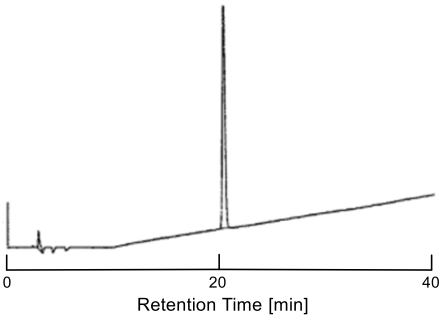
Peptide **22**



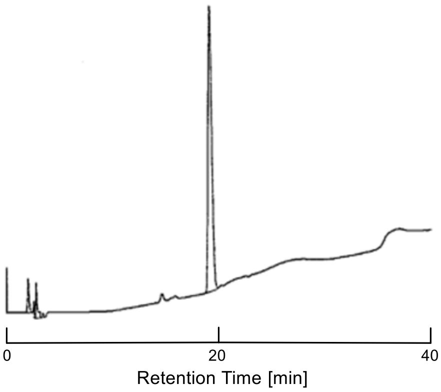
Peptide **23**



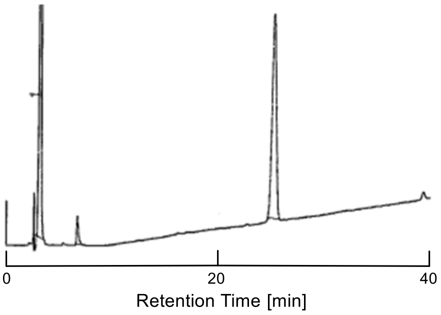
Peptide **24**



Peptide **25**



Peptide **26**



II. BIOLOGICAL EVALUATION

II-I. Results from fluorescence polarization (FP) assays and MTT assays for *N*-methylated peptides

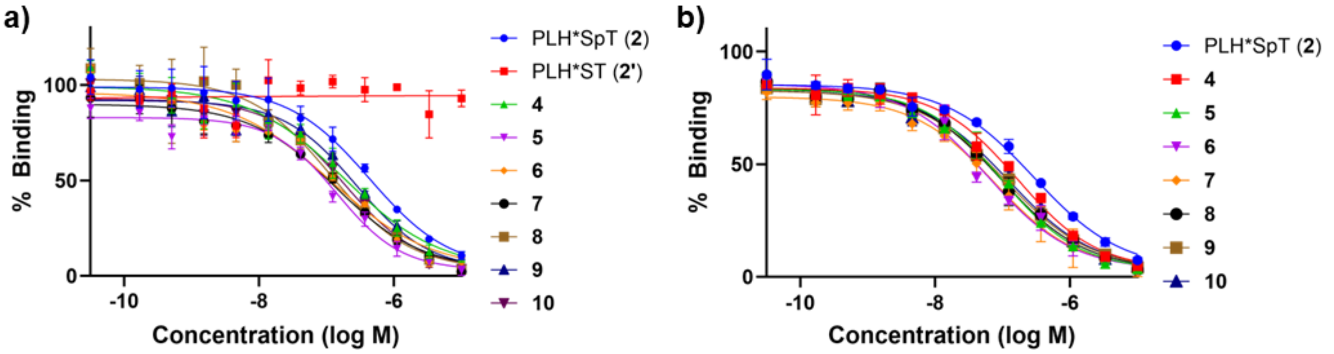


Fig. S1. Results from fluorescence polarization (FP) assays shown in Table 1, which measured the ability of Plk1 inhibitors to compete with FITC-mini-PEG-PLH*SpT for binding to full-length Plk1. The X axis represents inhibitor concentration (log M) and the Y axis represents relative probe binging based on the FP (Ex: 485 nm, Em: 528 nm) of no inhibitor (100%) and blank (no protein, 0%). Data points represent average ± SEM from at least two independent experiments and fit using non-linear regression in GraphPad Prism 10. (a,b) Experiment 1 or 2, respectively.

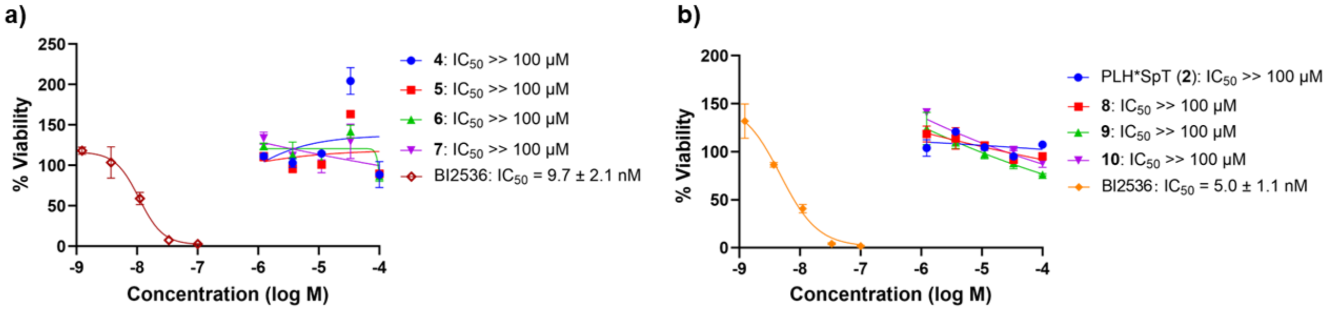


Fig. S2. Results from MTT assays, which measured the viability of HeLa cells with treatment of the Plk1 inhibitors **2**, **4–10**, and BI2536. The X axis represents inhibitor concentration (log M) and the Y axis represents relative cell viability based on the absorbance intensity at 565 nm of no inhibitor (100%) and blank (no cells, 0%). Data points and IC$_{50}$ values represent average ± SEM from two independent experiments and fit using non-linear regression in GraphPad Prism 10. (a) The results of the MTT assays of **4–7**; (b) the results of the MTT assays of **2** and **8–10**.

II-II. Results from FP assays and MTT assays for lipidated peptides

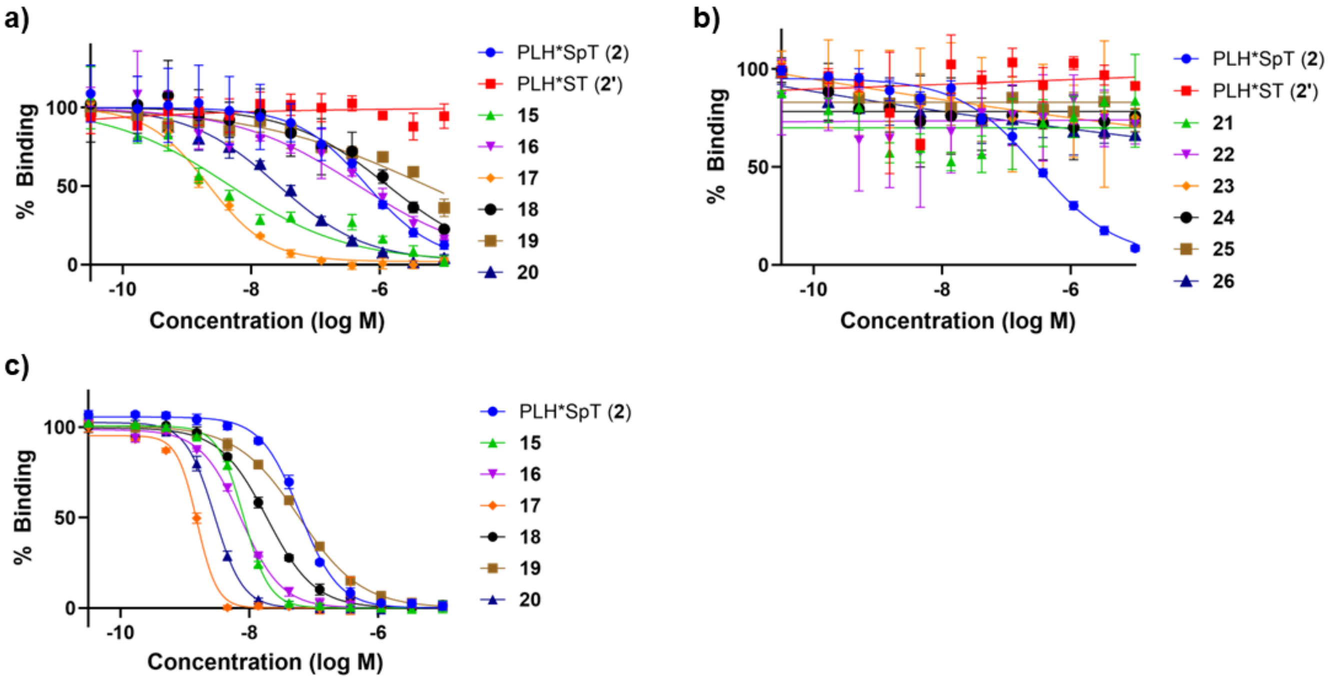


Fig. S3. Results from fluorescence polarization (FP) assays, which measured the ability of Plk1 inhibitors to compete with (a,b) FITC-mini-PEG-PLH*SpT or (c) FITC-mini-PEG-FDPPLHSpTA for binding to full-length Plk1. The X axis represents inhibitor concentration (log M) and the Y axis represents relative probe binging based on the FP (Ex: 485 nm, Em: 528 nm) of no inhibitor (100%) and blank (no protein, 0%). Data points represent average ± SEM from three independent experiments and fit using non-linear regression in GraphPad Prism 10.

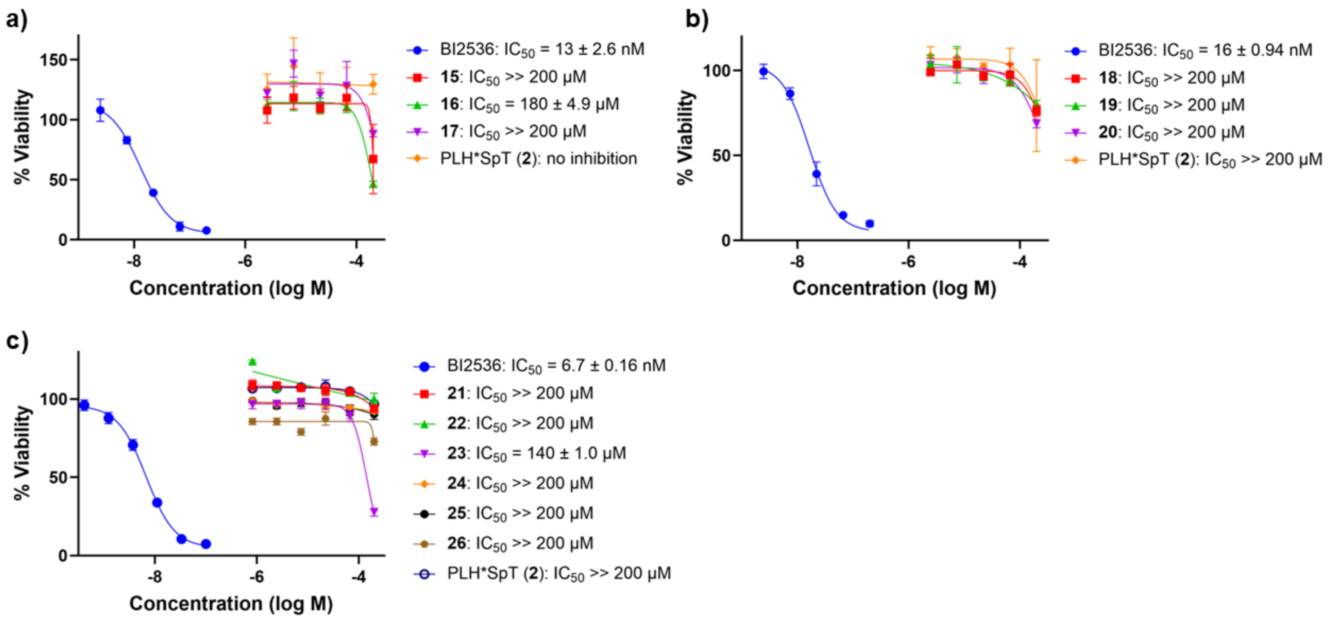
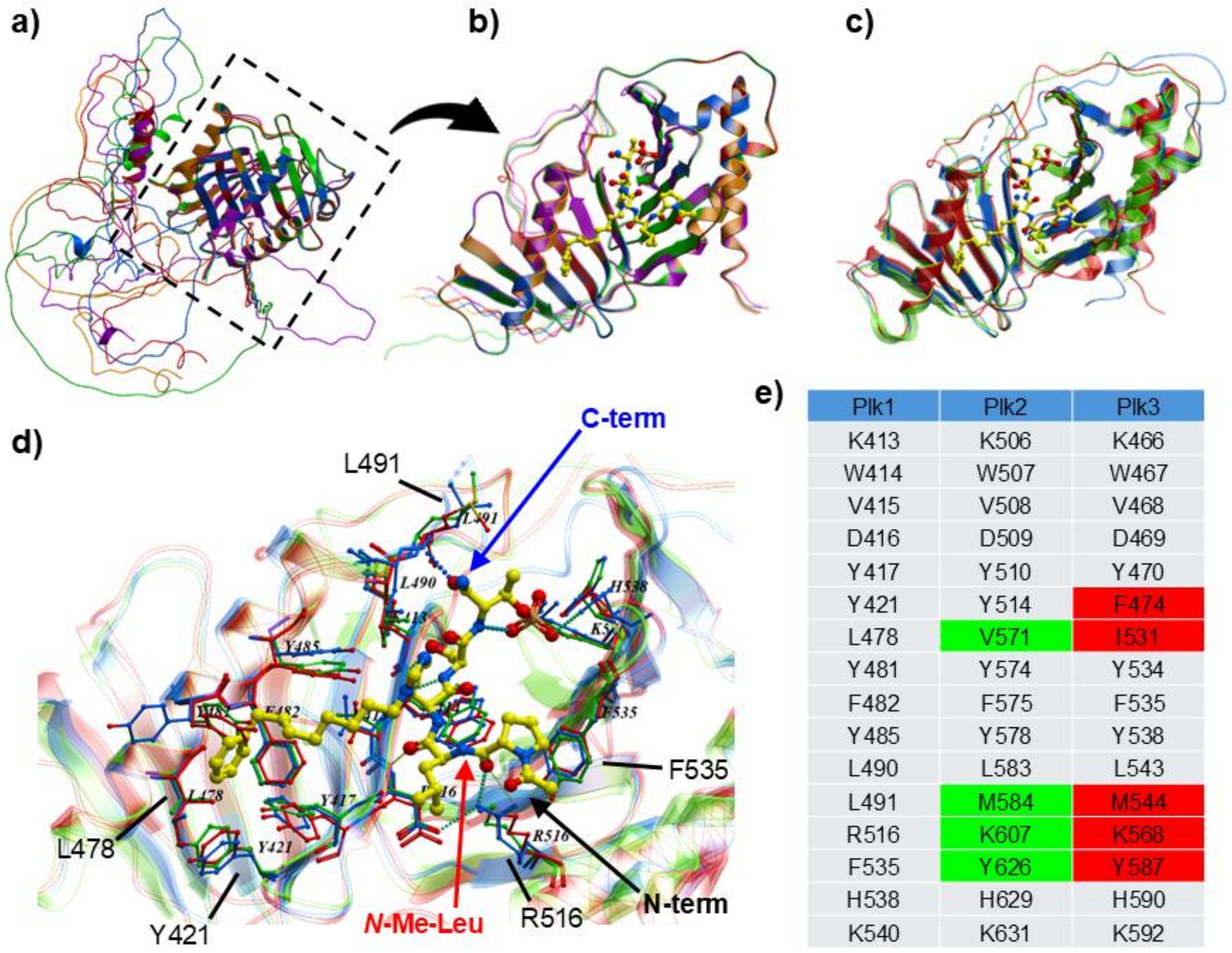


Fig. S4. Results from MTT assays, which measured the viability of HeLa cells with treatment of the Plk1 inhibitors **2**, **15**–**26**, and BI2536. The X axis represents inhibitor concentration (log M) and the Y axis represents relative cell viability based on the absorbance intensity at 565 nm of no inhibitor (100%) and blank (no cells, 0%). Data points and IC_{50} values represent average ± SEM from at least two independent experiments and fit using non-linear regression in GraphPad Prism 10. (a) The results of the MTT assays of **15**–**17**; (b) the results of the MTT assays of **18**–**20**; (c) the results of the MTT assays of **21**–**26**.

III. Representation of Plk1–3 PBD structures



Plk1	Plk2	Plk3
K413	K506	K466
W414	W507	W467
V415	V508	V468
D416	D509	D469
Y417	Y510	Y470
Y421	Y514	F474
L478	V571	I531
Y481	Y574	Y534
F482	F575	F535
Y485	Y578	Y538
L490	L583	L543
L491	M584	M544
R516	K607	K568
F535	Y626	Y587
H538	H629	H590
K540	K631	K592

Fig. S5. Structural representation of Plk1–3 PBDs. (a) Five predicted structures of Plk3 PBD (312D–646A) using ColabFold shown as red, orange, green, blue, and purple ribbons; (b) extended structures within a circle with dash-line in (a) with superimposition of PLH*SpT (**2**, yellow sticks); (c) superimposed structures of PLH*SpT (**2**)-bound Plk1 PBD (PDB: 3RQ7, yellow sticks and a blue ribbon), Plk2 PBD (PDB: 4RS6, a green ribbon), and PlK3 PBD (a red ribbon as shown in (a)); (d) interaction sites of PLH*SpT (**2**) and Plk1 PBD with potining the modification sites on PLH*SpT (**2**); (e) aminoacid residues of PLH*SpT (**2**) interaction site in Plk1 PBD and its corresponding residues of Plk2 and Plk3 PBDs. Green and red highlights showed different aminoacids from Plk1 PBD.