Supporting Information

Chemical Synthesis of the Multiply Phosphorylated and Biotinylated N-terminal Transactivation Domain of Human p53 (p53TAD)

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I. Materials and methods
All commercial reagents and solvents were used as received. UPLC-MS analyses were performed using a Waters Acquity Ultra Performance LC system equipped with Acquity UPLC® BEH 300 C4, 1.7μm, 2.1 x 100 mm column at a flow rate of 0.3 mL/min. The mobile phase consists of 0.1% formic acid (v/v) and 3.0% acetonitrile (v/v) in water (solvent A) and 0.1% formic acid (v/v) in acetonitrile (solvent B). Preparative HPLC separations were performed using a LabAlliance HPLC solvent delivery system equipped with a Rainin UV-1 detector and (A) a Varian Microsorb 100-5, C18 250x21.4mm column (100 Å pore size) at a flow rate of 16.0 mL/min or (B) a Vydac C18 250x10mm column (218TP1010) at a flow rate of 5.0 mL/min. The mobile phase consists of 0.05% TFA (v/v) in water (solvent A) and 0.04% TFA (v/v) in acetonitrile (solvent B). All circular dichroism (CD) spectra were obtained using an Applied PhotophysicsChirascan™plus CD spectrometer.

II. Chemical synthesis of wild-type p53TAD-Biotin 14

H-MEEPQSDPSVEPPLSQETSDLWKLLPPENNVLSPLPS-Sph 11
(1) Fully protected peptidyl acid Boc-MEEPQSDPSVEPPLSQETSDLWKLLPPENNVLSPLPS-OH 9
Automated peptide synthesis was performed on an Applied Biosystems Pioneer continuous flow peptide synthesizer. Peptides were synthesized under standard automated Fmoc conditions. The deblock solution was a mixture of 100/5/5 of DMF/piperidine/DBU. Fmoc protected amino acid (4.0 eq.), HATU (4.0 eq.) and DIEA (8.0 eq.) were used for the coupling steps. The following amino acids from Chem-Impex International were used: Fmoc-Asp(OMpe)-OH, Fmoc-Glu(Obu)-OH, Fmoc-Phe-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Boc-Met-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Val-OH, Fmoc-Trp(Boc)-OH. Fmoc-Glu(Obu)-Thr(Mc,Mepro)-OH and Fmoc-Ser(tBu)-NovaSyn® TGT resin from EMD Millipore were employed for the synthesis. Upon completion of automated synthesis on a 0.05 mmol scale, the peptide resin was washed into a peptide cleavage vessel with DCM. The resin cleavage was performed by treatment with AcOH/TEF/H2O=95:2.5:2.5 (1) H-Gln(Trt)-Sph 7 EDCI/HOObi CHCl3/TFE (2) TFA:H2O=95:2.5:2.5 (2) Biotin hydrazide 8 EDCI/HOObi CHCl3/TFE NCL 3 M Gn.HCl, 100 mM NaH2PO4, 250 mM TCEP, 40 mM glutathione, pH 6.5, VA-044 (10 eq.), 37°C MFD 6 M Gn.HCl, 300 mM Na2HPO4, 200 mM MPAA, 20 mM TCEP, pH 7.9 3 M Gn.HCl, 300 mM Na2HPO4, 200 mM MPAA, 20 mM TCEP, pH 7.9, VA-044 (10 eq.), 37°C 11, 65% 10, 82% 11+O, 31% 12, 17% 12+O, 19% 11+12 13, 80% 14, 51%
(2) Coupling of H-Gln(Trt)-SPh 7 and Boc-MEEPQSDPSVEPPLSQETFSDLWKLPPENVLSPLPS-OH 9

H-Gln(Trt)-SPh 7 (5.2 mg, 0.01 mmol, 1.0 eq.) and fully-protected peptide Boc-MEEPQSDPSVEPPLSQETFSDLWKLPPENVLSPLPS-OH 9 (69 mg, 0.011 mmol, 1.1 eq.) were dissolved in 200 μL of CHCl₃/TFE (3:1). At -10 °C, HOOBt (1.8 mg, 0.011 mmol, 1.1 eq.) and EDCI (1.7 mg, 0.011 mmol, 1.1 eq.) were added. The mixture was stirred at -10 °C for 1 min and then stirred at room temperature for 2.5 h. The solvent was removed with compressed air. 1.0 mL of H₂O/AcOH (20:1) was added and the mixture was sonicated and centrifuged. The supernatant was discarded and the residue was stirred in 10 mL of TFA/TIS/H₂O (95:2.5:2.5) at room temperature for 1 hour. Then, the solvent was removed with compressed air. The oily residue was precipitated with diethyl ether and centrifuged to give a white pellet. After the ether was decanted, the solid was stirred in 10 mL of MeCN/H₂O/AcOH (20:20:1) for 7 h at room temperature. Then the mixture was lyophilized overnight to get 59 mg of crude product. 30 mg of crude product was dissolved in 2 mL of MeCN/H₂O (1:1) for HPLC purification. After HPLC purification with linear gradient 32-52% B in 30 mins (Varian C18 column, 230 nm wavelength) and lyophilization, 5.64 mg pure product 11 was obtained in 19% yield and 9.40 mg of oxidized product 11+O was also obtained in 31% yield.

Figure S1-1. LC-MS traces and ESI-MS of peptide 11. MS (ESI) calculated for 11 C₁₉₆H₂₉₇N₄₅O₆₄S₂, [M+2H]²⁺ m/z = 2185.5405 Da, [M+3H]³⁺ m/z = 1457.3603 Da.
Figure S1-2. LC-MS traces and ESI-MS of peptide 11+O. MS (ESI) calculated for 11+O \( \text{C}_{196}\text{H}_{297}\text{N}_{45}\text{O}_{65}\text{S}_{2}, \) \([\text{M+2H}]^{2+} \text{m/z} = 2193.5380 \text{Da}, [\text{M+3H}]^{3+} \text{m/z} = 1462.6920 \text{Da}.

H-CMDDLMLSPDDIEQWFTEDPGPDEAPRMPEAA-NNHHBiotin 12

(1) Fully protected peptidyl acid Boc-CMDDLMLSPDDIEQWFTEDPGPDEAPRMPEAA-OH 10

The peptide synthesis was performed as previously described. The following amino acids from Chem-Impex International were used: Fmoc-Ala-OH, Boc-Cys(Trt)-OH, Fmoc-Asp(OMpe)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Met-OH, Fmoc-Pro-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH. Fmoc-Ala-NovaSyn® TGT resin from EMD Millipore was employed for the synthesis. Upon completion of automated synthesis on a 0.05 mmol scale, the peptide resin was washed into a peptide cleavage vessel with DCM. The resin cleavage was performed by treatment with AcOH/TFE/DCM (1:1:8) for 2 x 10 mL x 45 mins to yield the fully protected peptidyl acid Boc-CMDDLMLSPDDIEQWFTEDPGPDEAPRMPEAA-OH 10. The solvent was removed with compressed air. The oily residue was precipitated with diethyl ether and centrifuged to give a white pellet. After the ether was decanted, the solid was dissolved in 10 mL of MeCN/H₂O (1:1) and was lyophilized to dryness. 222 mg of crude peptide 10 was obtained in 82% yield.

(2) Coupling of Boc-CMDDLMLSPDDIEQWFTEDPGPDEAPRMPEAA-OH 10 and Biotin hydrazide 8

Biotin hydrazide 8 (2.6 mg, 0.01 mmol, 1.0 eq.) and fully-protected peptide Boc-CMDDLMLSPDDIEQWFTEDPGPDEAPRMPEAA-OH 10 (59 mg, 0.011 mmol, 1.1 eq.) were dissolved in 200 μL of CHCl₃/TFE (3:1). At -10 °C, HOOBt (1.8 mg, 0.011 mmol, 1.1 eq.) and EDCI (1.7 mg, 0.011 mmol, 1.1 eq.) were added. The mixture was stirred at -10 °C for 1 min and then stirred at room temperature for 2.5 h. The solvent was removed with compressed air. 1.0 mL of H₂O/ACOH (20:1) was added and the mixture was sonicated and centrifuged. The supernatant was discarded and the residue was stirred in...
10 mL of TFA/TIS/H$_2$O (95:2.5:2.5) at room temperature for 1 hour. Then, the solvent was removed with compressed air. The oily residue was precipitated with diethyl ether and centrifuged to give a white pellet. After the ether was decanted, the solid was stirred in 10 mL of MeCN/H$_2$O/AcOH (20:20:1) for 7 h at room temperature. Then the mixture was lyophilized overnight to get 54 mg of crude product. 50 mg of crude product was dissolved in 4 mL of MeCN/H$_2$O (1:1) for HPLC purification. After HPLC purification with linear gradient 27-47% B in 30 mins (Varian C18 column, 230 nm wavelength) and lyophilization, 8.25 mg pure product 12 was obtained in 17% yield and 9.32 mg of oxidized product 12+O was also obtained in 19% yield. The peptide 12 has three Met and the three mono-Met(O) peptides 12+O could be separated from each other as shown in Figure S2-2. (the top three LCMS are for the three mono-Met(O) peptides 12+O, individually and the bottom one is the LCMS of the combination of the three peptides 12+O).

**Figure S2-1.** LC-MS traces and ESI-MS of peptide 12. MS (ESI) calculated for 12 C$_{164}$H$_{245}$N$_{41}$O$_{57}$S$_{5}$, [M+2H]$^{2+}$ m/z = 1931.3069 Da, [M+3H]$^{3+}$ m/z = 1287.8712 Da.
Figure S2-2. LC-MS traces and ESI-MS of peptide 12+O. MS (ESI) calculated for 12+O C_{164}H_{245}N_{41}O_{57}S_{5}, [M+2H]^2+ m/z = 1939.3043 Da, [M+3H]^3+ m/z = 1293.2029 Da.
NCL procedure of peptides 11 and 12
Peptide thioester 11 (0.41 μmol, 1.79 mg, 2.05 mM, 1.0 eq.) and the biotin containing peptide 12 (0.41 μmol, 1.58 mg, 2.05 mM, 1.0 eq.) were dissolved in 200 μL of ligation buffer (6 M Gn-HCl, 300 mM Na₂HPO₄, 200 mM MPAA, 20 mM TCEP, pH 7.9). The reaction mixture was stirred at room temperature and monitored by UPLC-MS. After 9 h, the reaction was quenched by adding 15 mg of MESNA, 0.8 mL of MeCN/H₂O/AcOH (20:20:1) and 15 mg of TCEP. Then the mixture was purified directly by HPLC. After HPLC purification with linear gradient 40-60% B in 30 mins (Varian C18 column, 230 nm wavelength) and lyophilization, 2.68 mg pure product 13 was obtained in 80% yield.

**Figure S3.** LC-MS traces and ESI-MS of peptide 13. MS (ESI) calculated for 13 C₃₅₄H₅₃₆N₈₆O₁₂₁S₆, [M+4H]⁴⁺ m/z = 2030.9189 Da, [M+5H]⁵⁺ m/z = 1624.9351 Da.

MFD procedure of peptide 13
The thiol-substituted peptide 13 (0.16 μmol, 1.30 mg, 0.4 mM, 1.0 eq.) was dissolved in 400 μL of buffer (pH = 6.5) containing 3 M Gn-HCl, 100 mM NaH₂PO₄, 250 mM TCEP and 40 mM glutathione. Then VA-044 (1.6 μmol, 0.52 mg, 4.0 mM, 10 eq.) was added and the mixture was stirred at 37°C. The reaction was monitored by UPLC-MS. After 2 hours, upon consumption of the starting material, the reaction was quenched by adding 0.6 mL of MeCN/H₂O/AcOH (20:20:1). Then the mixture was purified directly by HPLC. After HPLC purification with linear gradient 35-55% B in 30 mins (Varian C18 column, 230 nm wavelength) and lyophilization, 0.66 mg of pure product 14 could be obtained in 51% yield.
**Figure S4.** LC-MS traces and ESI-MS of peptide 14. MS (ESI) calculated for 14 $\text{C}_{354}\text{H}_{536}\text{N}_{86}\text{O}_{121}\text{S}_5$, [M+4H]$^4+ \text{m/z} = 2022.9259 \text{Da}$, [M+5H]$^5+ \text{m/z} = 1618.5407 \text{Da}$.

### III. Chemical synthesis of mono-phosphorylated p53TAD-Biotin 18

H-MEEPQSDPVEPPLSQETFSDLWKLPENVLSPLSQ-SPh 16

(1) Fully protected peptidyl acid
Automated peptide synthesis was performed on an Applied Biosystems Pioneer continuous flow peptide synthesizer. Peptides were synthesized under standard automated Fmoc conditions. The deblock solution was a mixture of 100/5/5 of DMF/piperidine/DBU. Fmoc protected amino acid (4.0 eq.), HATU (4.0 eq.) and DIEA (8.0 eq.) were used for the coupling steps. The following amino acids from Chem-Impex International were used: Fmoc-Asp(OMpe)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Boc-Met-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Ser(PO(OBzl)OH)-OH, Fmoc-Val-OH, Fmoc-Trp(Boc)-OH, Fmoc-Glu(OtBu)-Thr(ψMe,Mepro)-OH and Fmoc-Ser(tBu)-NovaSyn \textsuperscript{®} TGT resin from EMD Millipore were employed for the synthesis. Upon completion of automated synthesis on a 0.05 mmol scale, the peptide resin was washed into a peptide cleavage vessel with DCM. The resin cleavage was performed by treatment with AcOH/TFE/DCM (1:1:8) for 2 x 10 mL x 45 mins to yield the fully protected peptidyl acid Boc-MEEPQSDPSVEPPLSQETFS(PO(OBzl)OH)DLWKLLPENNVLSPLPS-OH. The solvent was removed with compressed air. The oily residue was precipitated with diethyl ether and centrifuged to give a white pellet. After the ether was decanted, the solid was dissolved in 10 mL of MeCN/H\textsubscript{2}O (1:1) and was lyophilized to dryness. 181 mg of crude peptide was obtained in 57% yield.

Coupling of H-Gln(Trt)-SPh and Boc-MEEPQSDPSVEPPLSQETFS(PO(OBzl)OH)DLWKLLPENNVLSPLPS-OH

H-Gln(Trt)-SPh (8.8 mg, 0.017 mmol, 1.0 eq.) and fully-protected peptide Boc-MEEPQSDPSVEPPLSQETFS(PO(OBzl)OH)DLWKLLPENNVLSPLPS-OH (120 mg, 0.019 mmol, 1.1 eq.) were dissolved in 350 \textmu L of CHCl\textsubscript{3}/TFE (3:1). At \(-10^\circ\text{C},\) HOOBt (3.1 mg, 0.019 mmol, 1.1 eq.) and EDCI (2.9 mg, 0.019 mmol, 1.1 eq.) were added. The mixture was stirred at \(-10^\circ\text{C}\) for 1 min and then stirred at room temperature for 2.5 h. The solvent was removed with compressed air. Then 1.0 mL of H\textsubscript{2}O/AcOH (20:1) was added and the mixture was sonicated and centrifuged. The supernatant was discarded and the residue was dissolved in 5mL of stirred in 10 mL of MeCN/H\textsubscript{2}O (1:1) and lyophilized overnight to get 110 mg of crude product. 20mg of crude peptide was stirred in 10mL of TFA/TIS/H\textsubscript{2}O (95:2.5:2.5) at room temperature for 6 hours (the cleavage step was monitored by LCMS until the Bzl group of Ser(PO(OBzl)OH) was totally removed). Then the solvent was removed with compressed air. The oily residue was precipitated with diethyl ether and centrifuged to give a white pellet. After the ether was decanted, the solid was stirred in 4 mL of MeCN/H\textsubscript{2}O/AcOH (20:20:1) for 10 h at room temperature. Then the mixture was directly purified by HPLC with linear gradient 30-50% B in 30 mins (Varian C18 column, 230 nm wavelength). After lyophilization, 0.49 mg pure product was obtained in 4% yield and 5.57 mg of oxidized product was also obtained in 40% yield.
Figure S5-1. LC-MS traces and ESI-MS of peptide 16. MS (ESI) calculated for 16 $C_{196}H_{298}N_{45}O_{67}PS_2$, [M+2H]$^{2+}$ m/z = 2225.5237 Da, [M+3H]$^{3+}$ m/z = 1484.0158 Da.

Figure S5-2. LC-MS traces and ESI-MS of peptide 16+O. MS (ESI) calculated for 16+O $C_{196}H_{298}N_{45}O_{68}PS_2$, [M+2H]$^{2+}$ m/z = 2233.5212 Da, [M+3H]$^{3+}$ m/z = 1489.3474 Da.

**NCL procedure of peptide 16+O and 12+O**

Peptide thioester 16+O (0.43 μmol, 1.94 mg, 2.17 mM, 1.0 eq.) and the biotin containing peptide 12+O (0.43 μmol, 1.68 mg, 2.17 mM, 1.0 eq.) were dissolved in 200 μL of ligation buffer (6 M Gn-HCl, 300 mM Na$_2$HPO$_4$, 200 mM MPAA, 20 mM TCEP, pH 7.9). The reaction mixture was stirred at room temperature and monitored by UPLC-MS. After 24 h, the reaction was quenched by adding 15 mg of MESNA, 0.8 mL of MeCN/H$_2$O/AcOH (20:20:1) and 15 mg of TCEP. Then the mixture was purified directly by HPLC. After HPLC purification with linear gradient 32-52% B in 30 mins (Varian C18 column, 230 nm wavelength) and lyophilization, 2.24 mg pure product 17+2O was obtained in 63% yield.
Figure S6. LC-MS traces and ESI-MS of peptide 17+2O. MS (ESI) calculated for 17+2O $\text{C}_{354}\text{H}_{537}\text{N}_{86}\text{O}_{126}\text{PS}_{6}$, [M+4H]$^{4+}$ m/z = 2058.9080 Da, [M+5H]$^{5+}$ m/z = 1647.3264 Da.

**MFD procedure of peptide 17+2O**

The thiol-substituted peptide 17+2O (0.21 μmol, 1.69 mg, 0.26 mM, 1.0 eq.) was dissolved in 800 μL of buffer (pH = 6.5) containing 3 M Gn-HCl, 100 mM NaH$_2$PO$_4$, 250 mM TCEP and 40 mM glutathione. Then VA-044 (2.1 μmol, 0.68 mg, 2.6 mM, 10 eq.) was added and the mixture was stirred at 37°C. The reaction was monitored by UPLC-MS. After 2 hours, upon consumption of the starting material, the reaction was quenched by adding 1.2 mL of MeCN/H$_2$O/AcOH (20:20:1). Then the mixture was purified directly by HPLC. After HPLC purification with linear gradient 35-55% B in 30 mins (Varian C18 column, 230 nm wavelength) and lyophilization, 1.35 mg of pure product 18+2O could be obtained in 80% yield.
Figure S7. LC-MS traces and ESI-MS of peptide 18+20. MS (ESI) calculated for 18+20 C_{354}H_{537}N_{86}O_{126}PS_{5}, [M+4H]^4+ m/z = 2050.9150 Da, [M+5H]^5+ m/z = 1640.9320 Da.

Met(O)-reduction of peptide 18+20
The Met-oxidized peptide 18+20 (0.08 μmol, 0.65 mg, 0.25 mM, 1.0 eq.) was dissolved in 325 μL of 10% AcOH solution. Then N-methylmercaptoacetamide (7.9 μmol, 0.83 mg, 24 mM, 100 eq.) was added and the mixture was stirred under Argon at 37°C. The reaction was monitored by UPLC-MS. After 48 hours, upon consumption of the starting material, the mixture was purified directly by HPLC. After HPLC purification with linear gradient 35-55% B in 30 mins (Varian C18 column, 230 nm wavelength) and lyophilization, 0.26 mg of pure product 18 could be obtained in 40% yield.

Figure S8. LC-MS traces and ESI-MS of peptide 18. MS (ESI) calculated for 18 C_{354}H_{537}N_{86}O_{124}PS_{5}, [M+4H]^4+ m/z = 2042.9175 Da, [M+5H]^5+ m/z = 1634.5340 Da.
IV. Chemical synthesis of Penta-phosphorylated P53TAD-Biotin 24

(1) Fully protected peptidyl acid
Boc-MEEPQSDPSVEPPLSQETSDWLKPPENNVLSPLPSQ-SPh

Automated peptide synthesis was performed on an Applied Biosystems Pioneer continuous flow peptide synthesizer. Peptides were synthesized under standard automated Fmoc conditions. The deblock solution was a mixture of 100/5/5 of DMF/piperidine/DBU. Fmoc protected amino acid (4.0 eq.), HATU (4.0 eq.) and DIEA (8.0 eq.) were used for the coupling steps. The following amino acids from Chem-Impex International were used: Fmoc-Asp(OMpe)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Boc-Met-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Ser(PO(OBzl)OH)-OH, Fmoc-Thr(PO(OBzl)OH)-OH, Fmoc-Val-OH, Fmoc-Trp(Boc)-OH. Fmoc-Ser(tBu)-NovaSyn® TGT resin from EMD Millipore were employed for the synthesis. Upon completion of automated synthesis on a 0.05 mmol scale, the peptide resin was washed into a peptide cleavage vessel with DCM. The resin cleavage was performed by treatment with AcOH/TFE/DCM (1:1:8) for 2 x 10 mL x 60 mins to yield the fully protected peptidyl acid Boc-MEEPQSDPSVEPPLSQETSDWLKPPENNVLSPLPSQ-SPh. The solvent was removed under vacuum. The oily residue was dissolved in 10 mL of MeCN/H2O (1:1) and was lyophilized to dryness. 210 mg of crude peptide 19 was obtained in 64% yield.

(2) Coupling of H-Gln(Trt)-SPh and Boc-MEEPQSDPSVEPPLSQETSDWLKPPENNVLSPLPSQ-SPh

H-Gln(Trt)-SPh

S13
Boc-MEEPQSDPSVEPPLS(PO(OBzl)OH)QET(PO(OBzl)OH)FS(PO(OBzl)OH)DLWKLLPENNVLSPLPS-OH 19 (145 mg, 0.022 mmol, 1.1 eq.) were dissolved in 400 μL of CHCl₃/TFE (3:1). At -10°C, HOOBt (3.6 mg, 0.022 mmol, 1.1 eq.) and EDCI (3.4 mg, 0.022 mmol, 1.1 eq.) were added. The mixture was stirred at -10°C for 1 min and then stirred at room temperature for 2.5 h. The solvent was removed under vacuum. Then 2.0 mL of H₂O/AcOH (20:1) was added and the mixture was sonicated and centrifuged. The supernatant was discarded and the residue was stirred in 2 mL of H₂O/AcOH (20:1) for 12 h at room temperature. Then the mixture was directly purified by HPLC with linear gradient 30-50% B in 30 mins (Varian C18 column, 230 nm wavelength). After lyophilization, 28.1 mg of oxidized product 21+O was obtained in 30% yield.

Figure S9. LC-MS traces and ESI-MS of peptide 21+O. MS (ESI) calculated for 21+O C₁₉₆H₃₀₀N₄₅O₇₄P₃S₂, [M+2H]²⁺ m/z = 2313.4875 Da, [M+3H]³⁺ m/z = 1542.6583 Da.

H-CMDDLMLS(PO₃H₂)PDDIEQWFT(PO₃H₂)EDPGPDEAPRMPEAA-NHNHBiotin 22
(1) Fully protected peptidyl acid
Boc-CMDDLMLS(PO(OBzl)OH)PDDIEQWFT(PO(OBzl)OH)EDPGPDEAPRMPEAA-OH 20

The peptide synthesis was performed as previously described. The following amino acids from Chem-Impex International were used: Fmoc-Ala-OH, Boc-Cys(Trt)-OH, Fmoc-Asp(OMpe)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Met-OH, Fmoc-Pro-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(PO(OBzl)OH)-OH, Fmoc-Thr(PO(OBzl)OH)-OH, Fmoc-Trp(Boc)-OH. Fmoc-Ala-NovaSyn® TGT resin from EMD Millipore was employed for the synthesis. Upon completion of automated synthesis on a 0.05 mmol scale, the peptide resin was washed into a peptide cleavage vessel with DCM. The resin cleavage was performed by treatment with AcOH/TFE/DCM (1:1:8) for
2 x 10 mL x 60 mins to yield the fully protected peptidyl acid Boc-CMDDLMLS(PO(OBzl)OH)PDDIEQWFT(PO(OBzl)OH)EDPGPDEAPRMPEAA-OH. The solvent was removed under vacuum. The oily residue was precipitated with diethyl ether and centrifuged to give a white pellet. After the ether was decanted, the solid was dissolved in 10 mL of MeCN/H2O (1:1) and was lyophilized to dryness. 215 mg of crude peptide 20 was obtained in 76% yield.

(2) Coupling of Boc-CMDDLMLS(PO(OBzl)OH)PDDIEQWFT(PO(OBzl)OH)EDPGPDEAPRMPEAA-OH 20 and Biotin hydrazide 8

Biotin hydrazide 8 (4.2 mg, 0.016 mmol, 1.0 eq.) and fully-protected peptide Boc-CMDDLMLS(PO(OBzl)OH)PDDIEQWFT(PO(OBzl)OH)EDPGPDEAPRMPEAA-OH 20 (100 mg, 0.018 mmol, 1.1 eq.) were dissolved in 340 μL of CHCl₃/TFE (3:1). At -10°C, HOOBt (2.9 mg, 0.018 mmol, 1.1 eq.) and EDCI (2.8 mg, 0.018 mmol, 1.1 eq.) were added. The mixture was stirred at -10°C for 1 min and then stirred at room temperature for 2.5 h. The solvent was removed under vacuum. 1.0 mL of H2O/AcOH (20:1) was added and the mixture was sonicated and centrifuged. The supernatant was discarded and the residue was stirred in 2mL of TFA/TIS/H2O (95:2.5:2.5) at room temperature for 5 hours (the cleavage step was monitored by LCMS until the Bzl group of Ser(PO(OBzl)OH) and Thr(PO(OBzl)OH) was totally removed). Then the mixture was precipitated by adding dropwise to 20mL of cold ether and centrifuged to give a white pellet. After the ether was decanted, the solid was stirred in 10 mL of MeCN/H2O/AcOH (20:20:1) for 12 h at room temperature. Then the mixture was directly purified by HPLC with linear gradient 27-47% B in 30 mins (Varian C18 column, 230 nm wavelength). After lyophilization, 9.5 mg of product 22 was obtained in 15% yield.

Figure S10. LC-MS traces and ESI-MS of peptide 22. MS (ESI) calculated for 22 C₁₆₄H₂₄₇N₄₁O₆₃P₂S₅, [M+2H]²⁺ m/z = 2011.2732 Da, [M+3H]³⁺ m/z = 1341.1821 Da.

NCL procedure of peptides 21+O and 22
Peptide thioester $21+\text{O}$ (2.17 μmol, 10.04 mg, 2.4 mM, 1.2 eq.) and the biotin containing peptide $22$ (1.81 μmol, 7.30 mg, 2.0 mM, 1.0 eq.) were dissolved in 900 μL of ligation buffer (6 M Gn·HCl, 300 mM Na₂HPO₄, 200 mM MPAA, 20 mM TCEP, pH 7.9). The reaction mixture was stirred at room temperature and monitored by UPLC-MS. After 20 h, the reaction was quenched by adding 15 mg of MESNA, 1 mL of H₂O/AcOH (20:1) and 15 mg of TCEP. Then the mixture was extracted by ethyl acetate (1 mL x 3 times) to remove MPAA. The aqueous layer was purified directly by HPLC. After HPLC purification with linear gradient 32-52% B in 30 mins (Varian C18 column, 230 nm wavelength) and lyophilization, 6.66 mg of oxidized product $23+\text{O}$ was obtained in 43% yield.

**Figure S11.** LC-MS traces and ESI-MS of peptide $23+\text{O}$. MS (ESI) calculated for $23+\text{O} \text{C}_{354}\text{H}_{541}\text{N}_{86}\text{O}_{137}\text{P}_{5}\text{S}_{6}$, [M+4H]$^{4+}$ m/z = 2134.8756 Da, [M+5H]$^{5+}$ m/z = 1708.1004 Da.

**MFD procedure of peptide $23+\text{O}$**

The thiol-substituted peptide $23+\text{O}$ (0.52 μmol, 4.48 mg, 0.26 mM, 1.0 eq.) was dissolved in 2 mL of buffer (pH = 6.5) containing 3 M Gn·HCl, 100 mM NaH₂PO₄, 250 mM TCEP and 40 mM glutathione. Then VA-044 (5.2 μmol, 1.68 mg, 2.6 mM, 10 eq.) was added and the mixture was stirred at 37°C. The reaction was monitored by UPLC-MS. After 6 hours, upon consumption of the starting material, the reaction was quenched by adding 2 mL of MeCN/H₂O/AcOH (20:20:1). Then the mixture was purified directly by HPLC. After HPLC purification with linear gradient 32-52% B in 30 mins (Varian C18 column, 230 nm wavelength) and lyophilization, 2.52 mg of product $24+\text{O}$ could be obtained in 57% yield.
Met(O)-reduction of peptide 24+O

The Met-oxidized peptide 24+O (0.27 μmol, 2.26 mg, 0.25 mM, 1.0 eq.) was dissolved in 1.1 mL of 10% AcOH solution. Then N-methylmercaptoacetamide (27 μmol, 2.84 mg, 25 mM, 100 eq.) was added and the mixture was stirred under Argon at 37°C. The reaction was monitored by UPLC-MS. However, neither the starting material nor the product could be detected by UPLC-MS.

We also tried the NH₄I/Me₂S condition to reduce the Met-oxidized peptide 24+O as shown below. The Met-oxidized peptide 24+O (0.10 μmol, 0.92 mg, 1 mM, 1.0 eq.) was dissolved in 0.1 mL of TFA at 0°C. Then NH₄I (2 μmol, 0.29 mg, 20 mM, 20 eq.) and Me₂S (2 μmol, 0.12 mg, 20 mM, 20 eq.) were added and the mixture was stirred at 0°C for 30 mins. The reaction was monitored by UPLC-MS. Again; no desired product could be isolated.
V. New strategy for the chemical synthesis of penta-phosphorylated p53TAD-Biotin

Reduction of H-M(O)EEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQ-SPh

The Met-oxidized peptide 21+O (0.20 μmol, 0.93 mg, 1 mM, 1.0 eq.) was dissolved in 0.2 mL of TFA at 0°C. Then NH4I (4 μmol, 0.58 mg, 20 mM, 20 eq.) and Me2S (4 μmol, 0.25 mg, 20 mM, 20 eq.) were added and the mixture was stirred at 0°C for 30 mins. The reaction was monitored by UPLC-MS. Then the mixture was purified directly by HPLC. After HPLC purification with linear gradient 25-45% B in 30 mins (Vydac C18 column, 230 nm wavelength) and lyophilization, 0.53 mg of product 21 could be obtained in 57% yield.

Figure S13. LC-MS traces and ESI-MS of peptide 21. MS (ESI) calculated for 21 C196H300N45O73P3S2, [M+2H]2+ m/z = 2305.4900 Da, [M+3H]3+ m/z = 1537.3267 Da.
NCL procedure of peptides 21 and 22

Peptide thioester 21 (0.34 μmol, 1.58 mg, 2.3 mM, 1.2 eq.) and the biotin containing peptide 22 (0.29 μmol, 1.15 mg, 1.9 mM, 1.0 eq.) were dissolved in 150 μL of ligation buffer (6 M Gn-HCl, 300 mM Na₂HPO₄, 200 mM MPAA, 20 mM TCEP, pH 7.9). The reaction mixture was stirred at room temperature and monitored by UPLC-MS. After 21 h, the reaction was quenched by adding 15 mg of MESNA, 1 mL of H₂O/AcOH (20:1) and 15 mg of TCEP. Then the mixture was extracted by ethyl acetate (1 mL x 3 times) to remove MPAA. The aqueous layer was purified directly by HPLC. After HPLC purification with linear gradient 26-46% B in 30 mins (Vydac C18 column, 230 nm wavelength) and lyophilization, 0.84 mg of product 23 was obtained in 34% yield.

Figure S14. LC-MS traces and ESI-MS of peptide 23. MS (ESI) calculated for 23 C₃₅₅H₅₄₁N₈₆O₁₃₆P₅S₆, [M+4H]⁴⁺ m/z = 2130.8768 Da, [M+5H]⁵⁺ m/z = 1704.9015 Da.

MFD procedure of peptide 23

The thiol-substituted peptide 23 (0.06 μmol, 0.51 mg, 0.28 mM, 1.0 eq.) was dissolved in 215 μL of buffer (pH = 6.5) containing 3 M Gn-HCl, 100 mM NaH₂PO₄, 250 mM TCEP and 40 mM glutathione. Then VA-044 (0.6 μmol, 0.19 mg, 2.8 mM, 10 eq.) was added and the mixture was stirred at 37°C under Argon. The reaction was monitored by UPLC-MS. After 4 hours, upon consumption of the starting material, the reaction was quenched by adding 0.6 mL of MeCN/H₂O/AcOH (20:20:1). Then the mixture was purified directly by HPLC. After HPLC purification with linear gradient 26-46% B in 30 mins (Vydac C18 column, 230 nm wavelength) and lyophilization, 0.33 mg of product 24 could be obtained in 65% yield.
VI. Circular dichroism (CD) spectra of peptide 14, 18 and 24

The CD spectra were acquired in a 0.5 mm quartz cuvette under nitrogen at a flow rate of 1 L/min. Each peptide was dissolved in 5 mM phosphate buffer with a pH of 7.0 (J. Biol. Chem. 1995, 270:25014-25019). The peptide concentrations for 14, 18 and 24 were 0.4, 0.15 and 0.4 g/L, respectively. CD spectra were obtained at 20 °C with a step of 0.5 nm, 0.5 s per point and a spectral width of 190-260 nm. The spectra are the average of 5 scans with an averaged 5 scan buffer baseline subtracted.

Figure S16. CD spectra of peptide 14, 18 and 24.