Supporting Information

OGlcNAcylation and Phosphorylation Have Opposing Structural Effects in tau: Phosphothreonine Induces Particular Conformational Order

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Materials

Fmoc-l-amino acids were purchased from Novabiochem (San Diego, CA), Bachem (San Carlos, CA), or Chem-Impex (Wood Dale, IL). O-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyldironium hexafluorophosphate (HBTU) was purchased from Senn Chemicals (San Diego, CA). Rink amide MBHA resin and diisopropylethylamine (DIPEA) were purchased from Chem-Impex. Ethanedithiol (EDT), acetic anhydride (Ac$_2$O), chloroform-D (CDCl$_3$), phenol, 3% tetrazole in acetonitrile, thiaoanisole, triethylsilane (TES), and trifluoroacetic acid (TFA) were purchased from Acros. Boron trifluoride diethyl etherate (BF$_3$•Et$_2$O), N,N-diisopropylcarbodiimide (DIC), and piperidine were purchased from Aldrich or Acros. Acetonitrile (MeCN), methylene chloride (CH$_2$Cl$_2$), methanol (MeOH), chloroform (CHCl$_3$), tetrahydrofuran (THF), dimethylformamide (DMF), pyridine, ether, sodium chloride, hydrogen peroxide, and acetic acid were purchased from Fisher. O,O'-dibenzyl-$N,N$-diisopropylphosphoramidite, and O,O'-diethyl-$N,N$-diisopropylphosphoramidite were purchased from Toronto Research Chemicals (North York, Ontario). β-d-glucosamine pentaacetate (Ac$_4$-GlcNAc) was purchased from Alfa-Aesar. Silica gel was purchased from SiliCycle (Québec City, Québec). Deionized water was purified by a Millipore Synergy 185 water purification system with a Simpak2 cartridge. Solid-phase phosphorylation and diethylphosphorylation reactions were performed in capped disposable fritted tubes (Image Molding) with gentle mixing on a Barnstead-Thermoline Labquake rotary shaker. CH$_2$Cl$_2$ and MeCN were dried via a column-based solvent purification system (Innovative Technologies, Inc.) and stored over activated molecular sieves (4 Å). All other compounds were used as purchased with no additional purification.

Peptide synthesis

Peptides (0.1 or 0.25 mmol) were synthesized on Rink amide resin via standard Fmoc solid phase peptide synthesis using a Rainin PS3 peptide synthesizer. Prior to the start of peptide synthesis, the resin was swelled in DMF (2 × 15 minutes). Standard amino acid couplings were achieved in 1 h using 4 equivalents of HBTU and 4 equivalents of Fmoc amino acid. Couplings of glycosylated amino acid derivatives were achieved using 1.5 equivalents of glycosylated Fmoc amino acid and 1.5 equivalents HBTU or HATU. Glycosylated amino acid couplings were allowed to react for 3-4 hours. Each cycle of peptide synthesis employed the following four steps: (1) removal of the Fmoc protecting group in 20% piperidine in DMF, 3 × 5 minutes; (2) resin wash in DMF, 5 × 1 minute; (3) amino acid coupling (Fmoc amino acid, HBTU, and 0.05 M DIPEA in DMF; 1 h or 4 h); (4) resin wash with DMF, 3 × 1 minute. After addition of the final residue, the peptide N-terminus was deprotected (20% piperidine in DMF, 3 × 5 minutes) and subsequently acetylated (10% acetic anhydride in pyridine, 5 minutes). To avoid β-elimination of the sugar moiety upon pyridine treatment, glycosylated peptides were acetylated using alternative conditions (0.5 M 1:1 DIC:acetic acid in THF, 1 h). Finally, the resin was washed with DMF (6×) and CH$_2$Cl$_2$ (3×).

The synthesis of peptides Ac-TAProxN-NH$_2$ and Ac-TYProxN-NH$_2$ were accomplished using the previously described automated solid phase peptide synthesis strategy termed proline editing. These peptides were synthesized and purified as described.
All peptides were subjected to TFA cleavage/deprotection reactions (2-4 hours in 84% TFA/4% each of H₂O/phenol/thioanisole/EDT, 92.5% TFA/5% TES/2.5% H₂O, or 92% TFA/4% TES/4% H₂O for non-glycosylated peptides; O-GlcNAcylated peptides are described later), the TFA removed by evaporation, the peptides precipitated with ether, and the precipitates dried. Peptides were dissolved in water or phosphate buffer, filtered, and purified by reverse phase HPLC on a Vydac C18 semi-preparative column (250 x 10 mm, 5-10 µm particle, 300 Å pore) or on a Varian Microsorb MV C18 analytical column (250 x 4.6 mm, 3-5 µm particle, 100 Å pore) using linear gradients of buffer B (20% water, 80% MeCN, 0.05% TFA) in buffer A (98% water, 2% MeCN, 0.06% TFA). Peptides were purified to homogeneity and their purity verified by analytical HPLC reinjection. Peptide identity was characterized by ESI-MS (positive ion mode, unless stated otherwise) on an LCQ Advantage (Finnigan) mass spectrometer.

The concentrations of peptides containing tyrosine were determined by UV-vis spectroscopy based on tyrosine absorbance (ε₂₈₀ = 1280 M⁻¹ cm⁻¹ in water). The τ₁₇₄-₁₈₃ peptides, were quantified using the equation: (µg/mL) = (A₂₁₅ – A₂₂₅) x 144.³ The peptides Ac-KTxPP-NH₂, Ac-KSxPP-NH₂, ptau₂₁₁-２₃₈, τau₂₃₄-２₅₁, ptau₂₃₄-２₅₁(OPO₃²⁻)(pS235), and tau₂₃₄-₂₅₁(OPO₃²⁻)(pS235/pS237) were quantified via ¹H NMR by adding an aqueous solution of maleic acid (500 µM final concentration) as an internal standard to the sample after the CD experiment was performed.

Serine/Threonine Phosphorylation and Diethylphosphorylation

Trityl-protected serine and threonine residues were incorporated at intended sites of chemical phosphorylation and diethylphosphorylation to allow for selective modification of the peptides on resin. To accomplish phosphorylation and diethylphosphorylation, the trityl groups were first deprotected in 2% TFA, 5% TES, and 93% CH₂Cl₂ (3 x 1 minutes, or until the flow-through solution was colorless). For chemical phosphorylation, phosphitylation was accomplished under nitrogen by the addition of tetrazole (1.35 mmol; 3 mL of 3% tetrazole solution in MeCN) and O,O'-dibenzyl-N,N-diisopropylphosphoramidite (500 µL, 1.52 mmol) and allowed to react for 6 hours with mixing (Scheme S1).

Scheme S1. Scheme for chemical phosphorylation of peptides on resin. R = CH₃ (Thr) or R = H (Ser).

The necessary phosphitylation step to achieve chemical diethylphosphorylation was analogous, with the sole difference being the substitution of O,O'-diethyl-N,N-diisopropylphosphoramidite (323 µL, 1.52 mmol) for O,O'-dibenzyl-N,N-diisopropylphosphoramidite (Scheme S2).
**Scheme S2.** Scheme for chemical diethylphosphorylation of peptides on resin. R = CH₃ (Thr) or R = H (Ser).

After phosphitylation, the reaction solution was removed and the resin washed with DMF (3×) and CH₂Cl₂ (3×). Oxidation was achieved in 1 h with mixing with tert-butyl hydroperoxide (4 mL of a 3 M solution in CH₂Cl₂). Following oxidation, the solution was removed and the resin washed with DMF (3×), MeOH (3×), and CH₂Cl₂ (3×). Peptides were then subjected to cleavage/deprotection and purification as described above.

**Synthesis of Fmoc-Thr(O-GlcNAc)-OH (3) and Fmoc-Ser(O-GlcNAc)-OH (4)**

**Scheme S3.** Synthesis of peracetylated 2-acetamido-2-deoxy-β-D-glycosides of Fmoc-Thr-OH and Fmoc-Ser-OH.

The protected Fmoc-Thr(β-D-GlcNAc)-OH and Fmoc-Ser(β-D-GlcNAc)-OH were synthesized via a modification of the methodology of Arsequell *et al.* (*Scheme S3*). After synthesis and purification, Fmoc-Thr(β-D-GlcNAc)-OH and Fmoc-Ser(β-D-GlcNAc)-OH were used as amino acid building blocks in Fmoc solid-phase glycopeptide synthesis. The peracylated GlcNAc (960 mg, 2.5 mmol) (1) was vacuum dried and placed under argon in a flask containing 4 Å molecular sieves. The compound was dissolved in 16 mL of anhydrous CH₂Cl₂. At 0°C, 800 µL (7.64 mmol) of BF₃·Et₂O was added dropwise over 1 minute to the suspension and allowed to react for 12–24 h at room temperature. The reaction was monitored by TLC (10% MeOH in CHCl₃) and visualized using Hanessian’s stain (cerium molybdate). When the formation of the oxazoline (2) was complete, the mixture was cooled to 0°C and 400 µL (2.29 mmol) of DIPEA was added. After 10 minutes of stirring at room temperature, Fmoc-Thr-
OH (867 mg, 2.54 mmol) or Fmoc-Ser-OH (831 mg, 2.54 mmol) was dissolved in a 9 mL solution of 2:1 CH₂Cl₂:MeCN and added to the reaction flask. The reaction mixture was gently stirred then left to stand at room temperature and allowed to react for 48–96 hours. Reaction progress was periodically monitored by TLC (10% MeOH in CHCl₃). A second batch of oxazoline (960 mg, 2.46 mmol), prepared as described above, was added after 24 h to increase yield. The crude reaction mixture was diluted with CH₂Cl₂ (40 mL) and filtered through Celite. The crude solution was neutralized by washing with 100 mL saturated bicarbonate solution and further washed with saturated brine solution (100 mL). The combined aqueous washes were kept for extraction of the product (3 or 4) with 300 mL ethyl acetate because some product was present in the aqueous fractions. The crude product was concentrated and purified via column chromatography (2–10% MeOH in CHCl₃). Product 3 was obtained as a white solid in 23% yield (753 mg) and product 4 was obtained as a white solid in 25% yield (811 mg). The ¹H NMR spectra of products 3 and 4 corresponded to literature values.

**Glycopeptide Synthesis**

Glycosylated Fmoc amino acid couplings were performed for 4 hours. Following solid-phase peptide synthesis, the N-terminal residue was deprotected (20% piperidine in DMF, 3 × 5 minutes) and the N-terminus acetylated (0.5 M 1:1 DIC:acetic acid in THF, 60 min). The protected O-GlcNAcylated peptides were subjected to a 90 minute cleavage/deprotection reaction in 92.5% TFA/5% TES/2.5% H₂O; an abridged reaction time was used to suppress β-elimination of the sugars. TFA was partially removed by evaporation, peptide precipitated with ether, and peptides dissolved in water and purified by HPLC. After HPLC purification, the O-acetyl-protected O-GlcNAcylated peptides were lyophilized to remove solvent. The acetyl-protected OGlcNAc hydroxyls were then subjected to deesterification via NaOMe/MeOH (12 mM, 1.5 mL) for 1 h to generate free hydroxyl groups on the carbohydrate alcohols (Scheme S4). Following 1 h of deprotection, the reaction solution was neutralized with 60 µL of 1 M acetic acid and lyophilized. Dried and deprotected glycopeptides were dissolved in water, filtered, and purified to homogeneity by reverse phase HPLC as previously described.

**Scheme S4.** Solution phase O-acetyl deprotection reaction to yield a final O-GlcNAcylated peptide product. R = CH₃ (Thr) or R = H (Ser).
Characterization data of tau peptides

| Peptide | Sequence | Expected mass | Observed mass |
|---------|----------|---------------|---------------|
| 5 | tau<sub>174-183</sub> Ac-KTPPAPKTPP-NH₂ | 1073.6 | 1096.9 (M+Na)⁺ |
| 6 | tau<sub>174-183</sub> Ac-KpTPPAPKpTPP-NH₂ | 1232.6 | 1256.5 (M+Na)⁺ |
| 7(7') | tau<sub>174-183</sub> Ac-KgTPPAPKgTPP-NH₂ | 1481.8 | 741.0 (M+2H)²⁻ |
| 8 | tau<sub>174-183</sub> Ac-KeTPPAPKeTPP-NH₂ | 1347.6 | 674.4 (M+2H)²⁻ |
| 9 | tau<sub>174-183/T175E, T181E</sub> Ac-KEPPAPKEPP-NH₂ | 1129.6 | 566.0 (M+2H)²⁻ |
| 10 | tau<sub>174-183/T175Tle, T181Tle</sub> Ac-KTlePPAPKTlePP-NH₂ | 1097.9 | 1098.7 (M+H)⁺ |
| 11 | tau<sub>196-209</sub> Ac-GYSSPGSPGTPGSR-NH₂ | 1346.6 | 1346.9 (M+H)⁺ |
| 12 | tau<sub>196-209</sub> Ac-GYSpSPgSPgTPGSR-NH₂ | 1746.7 | 872.5 (M−2H)²⁻ |
| 13(13') | tau<sub>196-209</sub> Ac-GYSgSPGgSPgTPGSR-NH₂ | 1956.2 | 979.0 (M+2H)²⁻ |
| 14 | tau<sub>196-209</sub> Ac-GYSeSPGeSPGeTPGSR-NH₂ | 1754.6 | 878.5 (M+2H)²⁻ |
| 15 | tau<sub>211-219</sub> Ac-YRTPSLPTPP-NH₂ | 1168.6 | 1169.8 (M+H)⁺ |
| 16 | tau<sub>211-219</sub> Ac-YRtTPpSLPpTPP-NH₂ | 1408.6 | 705.6 (M+2H)²⁻ |
| 17(17') | tau<sub>211-219</sub> Ac-YRgTPgSLPgTPP-NH₂ | 1778.2 | 890.2 (M+2H)²⁻ |
| 18 | tau<sub>211-219</sub> Ac-YReTPeSLPeTPP-NH₂ | 1576.6 | 1577.3 (M+H)⁺ |
| 19 | tau<sub>229-238</sub> Ac-YVRTPPKSPSS-NH₂ | 1258.7 | 1259.7 (M+H)⁺ |
| 20 | tau<sub>229-238</sub> Ac-YVRpTPpSPpSS-NH₂ | 1498.7 | 750.7 (M+2H)²⁻ |
| 21(21') | tau<sub>229-238</sub> Ac-YVRgTPgSPgSS-NH₂ | 1868.3 | 935.0 (M+2H)²⁻ |
| 22 | tau<sub>229-238</sub> Ac-YVReTPPeSPeSS-NH₂ | 1666.7 | 834.7 (M+2H)²⁻ |
| 23 | tau<sub>211-238</sub> Ac-RTPSLPTPTREPKKVAVRPKSPSS-NH₂ | 3052.5 | 1527.2 (M+2H)²⁻ |
| 24 | tau<sub>211-238</sub> Ac-RpTPpSLpTPPTREPKKVAVRPpTPPKpSPpSS-NH₂ | 3532.2 | 1178.2 (M+3H)³⁻ |
| 25 | tau<sub>234-251</sub> Ac-KSPSSAKSLQTAPVMP-NH₂ | 1923.2 | 962.3 (M+2H)²⁻ |
| 26 | tau<sub>234-251</sub>(pS235) Ac-KpSPSSAKSLQTAPVMP-NH₂ | 2002.0 | 1002.2 (M+H)²⁻ |
| 27 | tau<sub>234-251</sub>(pS235/pS237) Ac-KpSPSSAKSLQTAPVMP-NH₂ | 2082.0 | 1042.2 (M+H)²⁻ |

Table S1. Tau peptides examined in this study. pS or pT indicates a phosphorylated serine or threonine residue. eS or eT indicates a diethylphosphorylated (OPO₂Et₂) serine or threonine residue. gS or gT indicates an OGlCNAcylated serine or threonine residue. Residue numbers are based on the largest isoform of tau (441 amino acids). The peptides 8', 13', 17', and 21' refer to peptides which are O-acetylated on the sugars prior to deacetylation.
### Characterization data of model peptides

| Peptide          | Expected mass | Observed mass       |
|------------------|---------------|---------------------|
| Ac-KTPP-NH₂      | 482.2         | 505.3 (M+Na)⁺       |
| Ac-KpTPP-NH₂     | 562.2         | 608.2 (M+2Na)⁺      |
| Ac-KgTPP-NH₂     | 685.3         | 708.2 (M+Na)⁺       |
| Ac-KeTPP-NH₂     | 618.3         | 641.3 (M+Na)⁺       |
| Ac-KSPP-NH₂      | 468.2         | 469.3 (M+H)⁺        |
| Ac-KpSPP-NH₂     | 548.2         | 571.2 (M+Na)⁺       |
| Ac-KgSPP-NH₂     | 671.3         | 694.2 (M+Na)⁺       |
| Ac-KeSPP-NH₂     | 604.3         | 627.2 (M+Na)⁺       |
| Ac-GPKTPPGY-NH₂  | 856.4         | 879.2 (M+Na)⁺       |
| Ac-GPKpTPPGY-NH₂ | 936.4         | 959.2 (M+Na)⁺       |
| Ac-GPKgTPPGY-NH₂ | 1059.5        | 551.2 (M+2Na)²⁺     |
| Ac-GPKeTPPGY-NH₂ | 992.4         | 1015.0 (M+Na)⁺      |
| Ac-GPPTPPGY-NH₂  | 825.4         | 848.2 (M+Na)⁺       |
| Ac-GPPpTPPGY-NH₂ | 905.3         | 928.3 (M+Na)⁺       |
| Ac-GPPgTPPGY-NH₂ | 1028.4        | 1051.2 (M+Na)⁺      |
| Ac-GPPeTPPGY-NH₂ | 961.2         | 984.4 (M+Na)⁺       |

**Table S2.** Model peptides examined in this study. pS or pT indicates a phosphorylated serine or threonine residue. eS or eT indicates a diethylphosphorylated (OPO₃Et₂) serine or threonine residue. gS or gT indicates an OGlcNAcylated serine or threonine residue.

**Unmodified tau₁₇₄₋₁₈₃ (5)**

Peptide 5 was purified and characterized as previously described.⁵

**Phosphorylated tau₁₇₄₋₁₈₃ (6)**

Peptide 6 was purified and characterized as previously described.⁵

**O-GlcNAcylated tau₁₇₄₋₁₈₃ (7’, 7)**

O-Acetyl-protected peptide 7’ was purified via semi-preparative HPLC using a linear gradient of 0–45% buffer B in buffer A over 60 minutes: $t_R = 33.4$ min, exp. 1733.8, obs. 867.1 (M²⁺). The deacetylated peptide 7 was purified via analytical HPLC using a linear gradient of 0–35% buffer B in buffer A over 60 minutes: $t_R = 36.1$ min, exp 1481.8, obs. 741.0 (M+2H)²⁺.

**Diethylphosphorylated tau₁₇₄₋₁₈₃ (8)**

Peptide 8 was purified via analytical HPLC using a linear gradient of 0–50% buffer B in buffer A over 60 minutes: $t_R = 37.7$ min, exp 1347.6, obs. 674.4 (M+2H)²⁺.
Peptide 9 was purified via analytical HPLC using a linear gradient of 0–35% buffer B in buffer A over 60 minutes: $t_R = 29.8 \text{ min}$, exp. 1129.6, obs. 566.0 (M+2H)$^{2+}$. 

Peptide 10 was purified and characterized as previously described.$^5$

Peptide 11 was purified and characterized as previously described.$^5$

Peptide 12 was purified via analytical HPLC using a linear gradient of 0–20% buffer B in buffer A over 60 minutes: $t_R = 30.8 \text{ min}$, exp. 1746.7, obs. 872.5 (M$–2$H)$^{2–}$ (negative ion mode).

O-Acetyl-protected peptide 13 was purified via analytical HPLC using a linear gradient of 0–40% buffer B in buffer A over 60 minutes: $t_R = 56.8 \text{ min}$, exp. 2336.9, obs. 1168.3 (M)$^{2+}$. The deacetylated peptide 13 was purified via analytical HPLC using a linear gradient of 0–30% buffer B in buffer A over 60 minutes: $t_R = 33.6 \text{ min}$, exp 1956.2, obs. 979.0 (M+2H)$^{2+}$.

Peptide 14 was purified via semi-preparative HPLC using a linear gradient of 0–35% buffer B in buffer A over 60 minutes: $t_R = 51.8 \text{ min}$, exp. 1754.6, obs. 878.5 (M+2H)$^{2+}$.

Peptide 15 was purified and characterized as previously described.$^5$

Peptide 16 was purified and characterized as previously described.$^5$

O-Acetyl-protected peptide 17 was purified via analytical HPLC using a linear gradient of 0–50% buffer B in buffer A over 60 minutes: $t_R = 53.2 \text{ min}$, exp. 2156.5, obs. 1079.1 (M+2H)$^{2+}$. The deacetylated peptide 17 was purified via analytical HPLC using a linear gradient of 0–45% buffer B in buffer A over 60 minutes: $t_R = 39.6 \text{ min}$, exp 1778.2, obs. 890.2 (M+2H)$^{2+}$.

Peptide 18 was purified via analytical HPLC using a linear gradient of 0–60% buffer B in buffer A over 60 minutes: $t_R = 46.9 \text{ min}$, exp. 1576.6, obs. 1577.3 (M+H)$^+$.

Peptide 19 was purified and characterized as previously described.$^5$

Peptide 20 was purified and characterized as previously described.$^5$
O-GlcNAcylated tau_{229-238} (21', 21)
O-Acetyl-protected peptide 21’ was purified via analytical HPLC using a linear gradient of 0–45% buffer B in buffer A over 60 minutes: \( t_R = 49.3 \text{ min, exp. 2246.6, obs. 1124.2 (M+2H)^2+} \). The deacetylated peptide 21 was purified via analytical HPLC using a linear gradient of 0–30% buffer B in buffer A over 60 minutes: \( t_R = 36.7 \text{ min, exp 1868.3, obs. 935.0 (M+2H)^2+} \).

Diethylphosphorylated tau_{229-238} (22)
Peptide 22 was purified via semipreparative HPLC using a linear gradient of 0–40% buffer B in buffer A over 60 minutes: \( t_R = 46.3 \text{ min, exp. 1666.7, obs. 834.7 (M+2H)^2+} \).

Unmodified tau_{211-238} (23)
Peptide 23 was purified via semipreparative HPLC using a linear gradient of 0–30% buffer B in buffer A over 60 minutes: \( t_R = 44.6 \text{ min, exp. 3052.5, obs. 1527.2 (M+2H)^2+} \).

Phosphorylated tau_{211-238} (24)
Peptide 24 was purified via analytical HPLC using a linear gradient of 0–30% buffer B in buffer A over 60 minutes: \( t_R = 49.3 \text{ min, exp. 3532.2, obs. 1178.2 (M+3H)^3+} \).

Unmodified tau_{234-251} (25)
Peptide 25 was purified via semipreparative HPLC using a linear gradient of 0–30% buffer B in buffer A over 60 minutes: \( t_R = 57.9 \text{ min, exp. 1923.2, obs. 962.3 (M+2H)^2+} \).

Monophosphorylated tau_{234-251} (pS235) (26)
Phosphorylation of the peptide tau_{234-251} was performed as described in the general procedure on page S3, with a modification to the oxidation step to obtain peptide 26. In order to suppress methionine sulfoxide formation, oxidation was performed for 30 minutes instead of 60 minutes. Peptide 26 was purified via semipreparative HPLC using a linear gradient of 0–20% buffer B in buffer A over 60 minutes: \( t_R = 60 \text{ min, exp. 2002.2, obs. 1002.2 (M+2H)^2+} \).

Diphosphorylated tau_{234-251} (pS235/pS237) (27)
Phosphorylation of the peptide tau_{234-251} was performed as described in the general procedure on page S3, with a modification to the oxidation step to obtain peptide 27. In order to suppress methionine sulfoxide formation oxidation was performed for 30 minutes instead of 60 minutes. Peptide 27 was purified via semipreparative HPLC using a linear gradient of 0–20% buffer B in buffer A over 60 minutes: \( t_R = 58.8 \text{ min, exp. 2082.2, obs. 1042.2 (M+2H)^2+} \).

Ac-KTPP-NH₂ (28)
Peptide 28 was purified via analytical HPLC using a linear gradient of 0–30% buffer B in buffer A over 60 minutes: \( t_R = 18.6 \text{ min, exp. 482.2, obs. 505.3 (M+Na)^+} \).

Ac-KpTPP-NH₂ (29)
Peptide 29 was purified via analytical HPLC using a linear gradient of 0–20% buffer B in buffer A over 60 minutes: \( t_R = 13.1 \text{ min, exp. 562.2, obs. 608.2 (M+2Na)^+} \).
Ac-KT(OGlcNAc)PP-NH₂ (30) (deacetylated)
Peptide 25 was purified via analytical HPLC using a linear gradient of 0–30% buffer B in buffer A over 60 minutes: \( t_R = 47.3 \text{ min} \), exp. 685.3, obs. 708.2 (M+Na)*.

Ac-KT(OPO₃Et₂)PP-NH₂ (31)
Peptide 31 was purified via analytical HPLC using a linear gradient of 0–50% buffer B in buffer A over 60 minutes: \( t_R = 47.3 \text{ min} \), exp. 618.3, obs. 641.3 (M+Na)*.

Ac-KSPP-NH₂ (32)
Peptide 32 was purified via analytical HPLC using a linear gradient of 0–20% buffer B in buffer A over 60 minutes: \( t_R = 14.9 \text{ min} \), exp. 468.2, obs. 469.3 (M+H)*.

Ac-KpSPP-NH₂ (33)
Peptide 33 was purified via analytical HPLC using a linear gradient of 0–20% buffer B in buffer A over 60 minutes: \( t_R = 17.8 \text{ min} \), exp. 548.2, obs. 571.2 (M+Na)*.

Ac-KS(OGlcNAc)PP-NH₂ (34) (deacetylated)
Peptide 34 was purified via analytical HPLC using a linear gradient of 0–15% buffer B in buffer A over 60 minutes: \( t_R = 17.7 \text{ min} \), exp. 671.3, obs. 694.2 (M+Na)*.

Ac-KS(OPO₃Et₂)PP-NH₂ (35)
Peptide 35 was purified via analytical HPLC using a linear gradient of 0–20% buffer B in buffer A over 60 minutes: \( t_R = 36.6 \text{ min} \), exp. 604.3, obs. 627.2 (M+Na)*.

Ac-GPKTPPGY-NH₂ (36)
Peptide 36 was purified via analytical HPLC using a linear gradient of 0–30% buffer B in buffer A over 60 minutes: \( t_R = 36.0 \text{ min} \), exp. 856.4, obs. 879.2 (M+Na)*.

Ac-GPKpTPPGY-NH₂ (37)
Peptide 37 was purified via analytical HPLC using 20 min isocratic 100% buffer A, followed by a linear gradient of 0–30% buffer B in buffer A over 60 minutes: \( t_R = 47.1 \text{ min} \), exp. 936.4, obs. 959.2 (M+Na)*.

Ac-GPKT(OGlcNAc)PPGY-NH₂ (38) (deacetylated)
Peptide 38 was purified via analytical HPLC using a linear gradient of 0–30% buffer B in buffer A over 60 minutes: \( t_R = 35.5 \text{ min} \), exp. 1059.5, obs. 551.2 (M+2Na)²⁺.

Ac-GPKT(OPO₃Et₂)PPGY-NH₂ (39)
Peptide 39 was purified via analytical HPLC using a linear gradient of 0–30% buffer B in buffer A over 60 minutes: \( t_R = 50.9 \text{ min} \), exp. 992.4, obs. 1015.0 (M+Na)*.

Ac-GPPTPGY-NH₂ (40)
Peptide 40 was purified via analytical HPLC using a linear gradient of 0–30% buffer B in buffer A over 60 minutes: \( t_R = 43.2 \text{ min} \), exp. 825.4, obs. 848.2 (M+Na)*.
Ac-GPPpTPPGY-NH$_2$ (41)
Peptide 41 was purified via analytical HPLC using a linear gradient of 0–30% buffer B in buffer A over 60 minutes: $t_R = 38.2$ min, exp. 905.3, obs. 928.3 (M+Na$^+$.)

Ac-GPPT(OGlcNAc)PPGY-NH$_2$ (42) (deacetylated)
Peptide 42 was purified via analytical HPLC using a linear gradient of 0–30% buffer B in buffer A over 60 minutes: $t_R = 41.1$ min, exp. 1028.4, obs. 1051.2 (M+Na$^+$.)

Ac-GPPT(OPO$_3$Et$_2$)PPGY-NH$_2$ (43)
Peptide 43 was purified via analytical HPLC using a linear gradient of 0–30% buffer B in buffer A over 60 minutes: $t_R = 56.6$ min, exp. 961.2, obs. 984.4 (M+Na$^+$.)

Circular dichroism spectroscopy
CD spectra were collected on a Jasco J-810 Spectropolarimeter in a 1 mm cell at 25 °C unless otherwise indicated. Peptide concentrations were 15–450 µM. Solutions contained 25 mM KF in 5 mM phosphate buffer buffer (pH 8.0 or as indicated). Individual scans were made at 1 nm intervals with a 1 nm bandwidth and an averaging time of 4 s. Data represent the average of at least three independent trials. Data were background corrected but were not smoothed. Error bars are shown and indicate standard error.

NMR spectroscopy
NMR spectra of peptides were collected at 298 K or as indicated on a Bruker AVC 600 MHz NMR spectrometer equipped with a triple resonance cryoprobe or a TXI probe. Peptides were dissolved in buffer containing 5 mM phosphate buffer (pH 4.0, 6.5, 7.2, or 8.0) and were internally referenced with TSP. Solutions contained 25 mM NaCl, 100 µM TSP, and 90% H$_2$O/10% D$_2$O. Peptide NMR solution concentrations were 100 µM–1.0 mM. 1–D spectra were collected with a watergate pulse sequence and a relaxation delay of 2–3 s. Watergate TOCSY spectra were recorded for all peptides for resonance assignment. $^3$J$_{\alpha\omega}$ was determined directly from the 1-D spectra. Errors in $^3$J$_{\alpha\omega}$ are estimated to be $\leq$ 0.2 Hz. The calculation of $\phi$ was based on the parametrized Karplus equation, $^3$J$_{\alpha\omega} = 6.51 \cos^2(\phi - 60) - 1.76 \cos(\phi - 60) + 1.6$. The calculation of $\chi_1$ for threonine was based on the parametrized Karplus equation, $^3$J$_{\text{H}1\text{H}2\text{P}} = 4.37 - 1.86 \cos(\chi_1-120) + 3.81 \cos^2(\chi_1-120) - 0.37 \sin(\chi_1-120)$. The calculation of $\chi_2$ ($\chi_2 = C\text{--H}1\text{--O}1\text{--P}$ torsion angle + 120°) was based on the parametrized Karplus equation, $^3$J$_{\text{PH}} = 15.3 \cos^2(\chi_2-120)-6.1 \cos(\chi_2-120) + 1.6$.8
\(^{1}\)H-\(^{13}\)C HSQC (heteronuclear single quantum coherence) spectra were recorded for both the nonphosphorylated and the phosphorylated variants of the peptides tau\(^{174-183}\), tau\(^{211-219}\), tau\(^{229-238}\), Ac-KTPP-NH\(_2\), Ac-KSPP-NH\(_2\), and tau\(^{234-251}\). The peptides were dissolved in 100% D\(_2\)O containing 5 mM potassium phosphate (pH 8.0), 25 mM NaCl, and 100 \(\mu\)M TSP. Peptide concentrations were 500 \(\mu\)M–5 mM. NMR spectra were acquired on samples with natural abundance \(^{13}\)C, using sweep widths of 20833 and 5388 Hz in \(t_1\) and \(t_2\), respectively, 400 \(\times\) 2048 complex data points, 24 scans per \(t_1\) increment and a relaxation delay of 2.0 s. Watergate TOCSY spectra were recorded to confirm resonance assignments. Spectra were recorded with \(^{1}\)H-decoupling using spectral widths of 9 ppm and 130 ppm for peptide tau\(^{174-183}\) and 9 ppm and 80-200 ppm for Ac-KTxPP-NH\(_2\) peptides. Spectral widths of 12 ppm and 80 ppm were used for the \(^{1}\)H-decoupled spectra of Ac-KSxPP-NH\(_2\) peptides. \(^{1}\)H-\(^{13}\)C HMBC spectra were recorded using a modified HSQC experiment with pulse sequence delays based on \(J_{C-H} = 20\) Hz instead of standard HSQC delays based on \(^{1}\)J\(_{C-H} = 145\) Hz. \(^{1}\)J\(_{HaCa}\) coupling constants were measured directly from the HSQC spectra recorded without decoupling and were confirmed by comparison with the differences in chemical shift for the resonance recorded with decoupling. Errors in \(^{1}\)J\(_{HaCa}\) are estimated to be \(\pm 0.5\) Hz. The value of \(\psi\) was calculated on basis of the measured \(^{1}\)J\(_{HaCa}\), with the values of \(\phi\) determined directly from \(^{1}\)J\(_{aN}\) and the parametrized Karplus equation, \(^{1}\)J\(_{HaCa} = 140.3 + 1.4\) sin(\(\psi + 138\)) – 4.1 cos(2(\(\psi + 138\))) + 2.0 cos(2(\(\phi + 30\))).

\(^{1}\)H-\(^{15}\)N HSQC (heteronuclear single quantum coherence) spectra were recorded on samples with natural abundance \(^{15}\)N for both the nonphosphorylated and the phosphorylated variants of the peptides tau\(^{174-183}\), tau\(^{211-219}\), tau\(^{229-238}\), Ac-GPPTPPGY-NH\(_2\) and Ac-KTPP-NH\(_2\). \(^{1}\)H-\(^{15}\)N HSQC spectra were also recorded for the OGlcNAcylated variants of tau\(^{174-183}\) and Ac-KTPP-NH\(_2\). \(^{1}\)H-\(^{15}\)N HSQC spectra were acquired with sweep widths of 6614 Hz in \(t_1\) and 1825 Hz in \(t_2\), respectively, 64 \(\times\) 2048 complex data points, 16 scans per \(t_1\) increment, and a relaxation delay of 2.0 s. A Watergate pulse sequence was used for water suppression. The peptides were dissolved in 90% H\(_2\)O/10% D\(_2\)O containing 5 mM potassium phosphate (pH 8.0), 25 mM NaCl, and 100 \(\mu\)M TSP. Peptide concentrations were 1–2 mM. NMR spectra were recorded at 298 K.

NOESY spectra were acquired with sweep widths of 7183 Hz in \(t_1\) and \(t_2\), 600 \(\times\) 4096 complex data points, respectively, 16 scans per \(t_1\) increment, a relaxation delay of 2.0 s, and a NOESY mixing time of 200 ms.

Fmoc-Thr(Ac\(_3\)-\(\beta\)-d-GlcNAc)-OH and Fmoc-Ser(Ac\(_3\)-\(\beta\)-d-GlcNAc)-OH were dissolved in CDCl\(_3\) at concentrations of 500 \(\mu\)M–2.0 mM and \(^{1}\)H NMR spectra were collected at 296 K on a Bruker DRX 400 MHz NMR spectrometer equipped with a QNP probe.

\(^{31}\)P NMR spectroscopy

\(^{31}\)P NMR spectra were recorded on a Bruker DRX 400 MHz NMR spectrometer equipped with a BBO probe. The peptides were dissolved in buffer containing 5 mM potassium phosphate (pH 8.0) and 25 mM NaCl in 100% D\(_2\)O. To conduct NMR at pH 3.0, the peptide was dissolved in acetate buffer containing 5 mM sodium acetate (pH 3.0) in 100% D\(_2\)O. \(^{31}\)P spectra were collected with 65536 data points and a relaxation delay of 5 seconds. The proton-coupled NMR spectra were recorded at 277, 298, 310, 323, and 338 K at pH 8.0 and at 298 K at pH 3.0. The NMR spectra were internally referenced with 85% H\(_3\)PO\(_4\) (0.00 ppm) using a capillary filled with H\(_3\)PO\(_4\) that was placed in the NMR tube containing the sample.
Figure S1. Top left: CD spectrum of tau_{174-183}(T175E, T181E) (Ac-KEPPAPKEPP-NH$_2$) (9) at pH 8.0, 25 °C; top right: CD spectrum of tau_{174-183}(T175E, T181E) (Ac-KTlePPAPKTlePP-NH$_2$) (10) at pH 8.0, 25 °C; bottom: CD spectra of tau$_{174-183}$ peptides in 5 mM phosphate buffer, 25 mM KF, pH 7.5. Thr: green squares; phosphothreonine: red circles; ThrOGlcNAc: blue diamonds; Thr diethylphosphate: black triangles; Glu: pink open circles; tert-leucine: purple open diamonds. Error bars indicate standard error.
Figure S2. Top left: CD spectrum of \( \text{tau}_{211-238} \) (Ac-RTPSLPTPREPKKVAVRTPPSPSS-NH\(_2\)) (23) at pH 8.0, 25 °C; top right: CD spectrum of \( \text{tau}_{211-238}(\text{OPO}_3^{2-}) \) (Ac-RpTPpSLpTPREPKKVAVVRpTPKPpSpSS-NH\(_2\)) (24) at pH 8.0, 25 °C; bottom left: CD spectrum of \( \text{tau}_{211-238} \) (Ac-RTPSLPTPREPKKVAVRTPPSPSS-NH\(_2\)) (23) at pH 8.0, 0.5 °C; bottom right: CD spectrum of \( \text{tau}_{211-238}(\text{OPO}_3^{2-}) \) (Ac-RpTPpSLpTPREPKKVAVVRpTPKPpSpSS-NH\(_2\)) (24) at pH 8.0, 0.5 °C. The solutions contained 5 mM phosphate buffer and 25 mM KF. Error bars indicate standard errors.
Figure S3. Top left: CD spectrum of tau_{234-251} (Ac-KSPSSAKSRLQTAPVMP-NH₂) (25) at pH 8.0, 25 °C; top right: CD spectrum of tau_{234-251} (Ac-KSPSSAKSRLQTAPVMP-NH₂) (25) at pH 8.0, 0.5 °C; bottom left: CD spectrum of tau_{234-251} (Ac-KSPSSAKSRLQTAPVMP-NH₂) (25) at pH 8.0, 0.5 °C, in 30% TFE; bottom right: CD spectrum of tau_{234-251} (Ac-KSPSSAKSRLQTAPVMP-NH₂) (25) at pH 8.0, 0.5 °C, in 30% TFE. The solutions contained 5 mM phosphate buffer and 25 mM KF. Error bars indicate standard errors.
Figure S4. Top left: CD spectrum of tau\textsubscript{234-251}(pS235) (Ac-KpSPSSAKSLQTAPVPMP-NH\textsubscript{2}) (26) at pH 8.0, 25 °C; top right: CD spectrum of tau\textsubscript{234-251}(pS235) (Ac-KpSPSSAKSLQTAPVPMP-NH\textsubscript{2}) (26) at pH 8.0, 0.5 °C; bottom left: CD spectrum of tau\textsubscript{234-251}(pS235) (Ac-KpSPSSAKSLQTAPVPMP-NH\textsubscript{2}) (26) at pH 8.0, 0.5 °C, containing 30% TFE; bottom right: CD spectrum of tau\textsubscript{234-251}(pS235) (Ac-KpSPSSAKSLQTAPVPMP-NH\textsubscript{2}) (26) at pH 8.0, 0.5 °C, in 30% TFE. The solutions contained 5 mM phosphate buffer and 25 mM KF. Error bars indicate standard errors.
Figure S5. Top left: CD spectrum of tau234-251(pS235/pS237) (Ac-KpSPpSSAKSRLQTAPVPMP-NH$_2$) (27) at pH 8.0, 25 °C; top right: CD spectrum of tau234-251(pS235/pS237) (Ac-KpSPpSSAKSRLQTAPVPMP-NH$_2$) (27) at pH 8.0, 0.5 °C; bottom left: CD spectrum of tau234-251(pS235/pS237) (Ac-KpSPpSSAKSRLQTAPVPMP-NH$_2$) (27) at pH 8.0, 0.5 °C, containing 30% TFE; bottom right: CD spectrum of tau234-251(pS235/pS237) (Ac-KpSPpSSAKSRLQTAPVPMP-NH$_2$) (27) at pH 8.0, 0.5 °C, containing 30% TFE. The solutions contained 5 mM phosphate buffer and 25 mM KF. Error bars indicate standard errors.
**Figure S6.** Top left: CD spectra of tau$_{234-251}$ (Ac-KSPSSAKSRQTAPVPMP-NH$_3$) (25) (green squares), tau$_{234-251}$(pS235) (Ac-KpSPSSAKSRQTAPVPMP-NH$_3$) (26) (magenta open circles), and tau$_{234-251}$(pS235/pS237) (Ac-KpSPpSSAKSRQTAPVPMP-NH$_3$) (27) (red circles), at pH 8.0, 25 °C; top right: CD spectra of tau$_{234-251}$ (Ac-KSPSSAKSRQTAPVPMP-NH$_3$) (25) (green squares), tau$_{234-251}$(pS235) (Ac-KpSPSSAKSRQTAPVPMP-NH$_3$) (26) (magenta open circles), and tau$_{234-251}$(pS235/pS237) (Ac-KpSPpSSAKSRQTAPVPMP-NH$_3$) (27) (red circles), at pH 8.0, 0.5 °C; bottom left: CD spectra of tau$_{234-251}$ (Ac-KSPSSAKSRQTAPVPMP-NH$_3$) (25) (green squares), tau$_{234-251}$(pS235) (Ac-KpSPSSAKSRQTAPVPMP-NH$_3$) (26) (magenta open circles), and tau$_{234-251}$(pS235/pS237) (Ac-KpSPpSSAKSRQTAPVPMP-NH$_3$) (27) (red circles), at pH 8.0, 25 °C containing 30% TFE; bottom right: CD spectra of tau$_{234-251}$ (Ac-KSPSSAKSRQTAPVPMP-NH$_3$) (25) (green squares), tau$_{234-251}$(pS235) (Ac-KpSPSSAKSRQTAPVPMP-NH$_3$) (26) (magenta open circles), and tau$_{234-251}$(pS235/pS237) (Ac-KpSPpSSAKSRQTAPVPMP-NH$_3$) (27) (red circles), at pH 8.0, 0.5 °C containing 30% TFE. Error bars indicate standard error.
Summary of CD data for tau peptides

| Peptide                  | $[\theta]_{228}$, deg cm$^2$ dmol$^{-1}$ | $\lambda$ at local $[\theta]_{\text{max}}$, nm | Local $[\theta]_{\text{max}}$, deg cm$^2$ dmol$^{-1}$ | $\lambda$ at local $[\theta]_{\text{min}}$, nm | Local $[\theta]_{\text{min}}$, deg cm$^2$ dmol$^{-1}$ | $[\theta]_{190}$, deg cm$^2$ dmol$^{-1}$ |
|-------------------------|----------------------------------------|-----------------------------------------------|------------------------------------------------|-----------------------------------------------|------------------------------------------------|-----------------------------------------------|
| Ac-KTPPAPKTPP-NH$_2$    | -452                                   | n.a.                                          | n.a.                                           | 201                                           | -8843                                          | -2040                                         |
| Ac-KpTPPAPKpTPP-NH$_2$  | 339                                    | 228                                           | 339                                            | 201                                           | -13576                                         | -2986                                         |
| Ac-KgTPPAPKgTPP-NH$_2$  | -1121                                  | n.a.                                          | n.a.                                           | 203                                           | -17583                                         | 1426                                          |
| Ac-KeTPPAPKeTPP-NH$_2$  | -1991                                  | n.a.                                          | n.a.                                           | 204                                           | -12390                                         | 1589                                          |
| Ac-KEPPAPKEPP-NH$_2$    | 2                                      | 228                                           | 2                                              | 202                                           | -9949                                          | -2058                                         |
| Ac-KTlePPAPKtlePP-NH$_2$| -1620                                  | n.a.                                          | n.a.                                           | 203                                           | -11300                                         | -2280                                         |
| Ac-GYSSPGSPGTPGSR-NH$_2$| -2468                                  | n.a.                                          | n.a.                                           | 194                                           | -10550                                         | -8154                                         |
| Ac-GYSpSPgSPgTPGSR-NH$_2$| -2323                                  | n.a.                                          | n.a.                                           | 197                                           | -14440                                         | -5508                                         |
| Ac-GYSgSPgSPgTPGSR-NH$_2$| -1370                                  | n.a.                                          | n.a.                                           | 200                                           | -7462                                          | -1384                                         |
| Ac-GYSeSPgSeTPGSR-NH$_2$| -3075                                  | n.a.                                          | n.a.                                           | 206                                           | -6554                                          | 87                                            |
| Ac-YRTPSLPTPP-NH$_2$    | -732                                   | n.a.                                          | n.a.                                           | 201                                           | -17256                                         | -7865                                         |
| Ac-YRpTPpSLpTPP-NH$_2$  | 287                                    | 227                                           | 354                                            | 202                                           | -18151                                         | -4368                                         |
| Ac-YRgTPgSLpTPP-NH$_2$  | -1011                                  | n.a.                                          | n.a.                                           | 204                                           | -15442                                         | 4533                                          |
| Ac-YReTpeSLPeTPP-NH$_2$ | -2661                                  | n.a.                                          | n.a.                                           | 205                                           | -10877                                         | 129                                           |
| Ac-YVRTPPKSPSS-NH$_2$   | -1145                                  | n.a.                                          | n.a.                                           | 200                                           | -21699                                         | -5949                                         |
| Ac-YVRpTPKpSpSS-NH$_2$  | 904                                    | 228                                           | 904                                            | 199                                           | -28500                                         | -9694                                         |
| Ac-YVRgTPKgSpSS-NH$_2$  | -1036                                  | n.a.                                          | n.a.                                           | 204                                           | -15882                                         | 1446                                          |
| Ac-YVReTPKpSeSpSeS-NH$_2$| -2556                                  | n.a.                                          | n.a.                                           | 203                                           | -15532                                         | 2081                                          |
| Ac-RTPSLPTPPREPKKVAVRTPPKSPSS-NH$_2$ | -837 | n.a. | n.a. | 202 | -8247 | -2370 |
| Ac-RpTPpSLpTREPKKVAVRTPPKpSpSS-NH$_2$ | -694 | n.a. | n.a. | 201 | -12097 | -3596 |

**Table S3.** Summary of CD data for tau peptides. CD data were collected at 0.5 or 25 ºC with 15–200 µM peptide in 5 mM phosphate buffer (pH 8.0) with 25 mM KF. a No local maximum was observed. pS or pT indicates a phosphorylated serine or threonine residue. eS or eT indicates a diethylphosphorylated (OPO$_3$Et$_2$) serine or threonine residue. gS or gT indicates an OGlcnAcylated serine or threonine residue. Tle indicates tert-leucine.
| Peptide                        | $[\theta]_{222}$, deg cm$^2$ dmol$^{-1}$ | $[\theta]_{208}$, deg cm$^2$ dmol$^{-1}$ | $\lambda$ at $[\theta]_{\text{min}}$, nm | $[\theta]_{\text{min}}$, deg cm$^2$ dmol$^{-1}$ | Temp, $^\circ$C | % TFE |
|-------------------------------|--------------------------------------|--------------------------------------|------------------------------------------|-----------------------------------------------|-------------------|-------|
| Ac-KSPSSAKSRLQTAPVMP-NH$_2$  | -2710                                | -9352                                | 199                                      | -16370                                       | 25                | 0     |
| Ac-KpSPSSAKSRLQTAPVMP-NH$_2$ | -4539                                | -13907                               | 200                                      | -22614                                       | 25                | 0     |
| Ac-KpSPpSSAKSRLQTAPVMP-NH$_2$| -3544                                | -16015                               | 199                                      | -25921                                       | 25                | 0     |
| Ac-KSPSSAKSRLQTAPVMP-NH$_2$  | -1709                                | -9640                                | 199                                      | -18781                                       | 0.5               | 0     |
| Ac-KpSPSSAKSRLQTAPVMP-NH$_2$ | -2251                                | -14134                               | 199                                      | -25495                                       | 0.5               | 0     |
| Ac-KpSPpSSAKSRLQTAPVMP-NH$_2$| -2818                                | -21062                               | 200                                      | -37108                                       | 0.5               | 0     |
| Ac-KSPSSAKSRLQTAPVMP-NH$_2$  | -4987                                | -9668                                | 202                                      | -12585                                       | 25                | 30    |
| Ac-KpSPSSAKSRLQTAPVMP-NH$_2$ | -8894                                | -14901                               | 203                                      | -17163                                       | 25                | 30    |
| Ac-KpSPpSSAKSRLQTAPVMP-NH$_2$| -10182                               | -23219                               | 203                                      | -30371                                       | 25                | 30    |
| Ac-KSPSSAKSRLQTAPVMP-NH$_2$  | -4259                                | -10103                               | 201                                      | -13820                                       | 0.5               | 30    |
| Ac-KpSPSSAKSRLQTAPVMP-NH$_2$ | -8292                                | -15166                               | 204                                      | -17710                                       | 0.5               | 30    |
| Ac-KpSPpSSAKSRLQTAPVMP-NH$_2$| -9786                                | -24430                               | 202                                      | -35030                                       | 0.5               | 30    |

*Table S4.* Summary of CD data for tau$_{234-251}$ peptides. CD data were collected at 0.5 or 25 ºC with 15–45 µM peptide in 5 mM phosphate buffer (pH 8.0) with 25 mM KF. pS indicates a phosphorylated serine residue.
CD spectra of model peptides Ac-KTxPP-NH₂

Figure S7. Top left: CD spectrum of Ac-KTPP-NH₂ (28) at pH 8.0, 25 °C; top right: CD spectrum of Ac-KTPP-NH₂ (28) at pH 8.0, 2 °C; bottom left: CD spectrum of Ac-KT(OPO₃⁻)PP-NH₂ (29) at pH 8.0, 25 °C; and bottom right: CD spectrum of Ac-KT(OPO₃⁻)PP-NH₂ (29) at pH 8.0, 2 °C. Error bars indicate standard errors.
Figure S8. Top left: CD spectrum of Ac-KT(OGlcNAc)PP-NH$_2$ (30) at pH 8.0, 25 °C; top right: CD spectrum of Ac-KT(OGlcNAc)PP-NH$_2$ (30) at pH 8.0, 2 °C; bottom left: CD spectrum of Ac-KT(OPO$_3$Et$_2$)PP-NH$_2$ (31) at pH 8.0, 2 °C; and bottom right: CD spectrum of Ac-KT(OPO$_3$Et$_2$)PP-NH$_2$ (31) at pH 8.0, 2 °C. Error bars indicate standard errors.
CD spectra of model peptides Ac-KSxPP-NH$_2$

**Figure S9.** Top left: CD spectrum of Ac-KSPP-NH$_2$ (32) at pH 8.0, 25 °C; top right: CD spectrum of Ac-KSPP-NH$_2$ (32) at pH 8.0, 2 °C; bottom left: CD spectrum of Ac-KS(OPO$_3^{2-}$)PP-NH$_2$ (33) at pH 8.0, 25 °C; and bottom right: CD spectrum of Ac-KS(OPO$_3^{2-}$)PP-NH$_2$ (33) at pH 8.0, 2 °C. Error bars indicate standard errors.
Figure S10. Top left: CD spectrum of Ac-KS(OGlcNAc)PP-NH₂ (34) at pH 8.0, 25 ºC; top right: CD spectrum of Ac-KS(OGlcNAc)PP-NH₂ (34) at pH 8.0, 2 ºC; bottom left: CD spectrum of Ac-KS(OPO₃Et₂)PP-NH₂ (35) at pH 8.0, 2 ºC and bottom right: CD spectrum of Ac-KT(OPO₃Et₂)PP-NH₂ (35) at pH 8.0, 2 ºC. Error bars indicate standard errors.
**Figure S11.** Top left; CD spectra of Ac-KTxPP-NH₂ peptides at 25 ºC; Thr: green squares; phosphothreonine: red circles; ThrOGlcNAc: blue diamonds; Thr diethylphosphate: black triangles; top right; CD spectra of Ac-KTxPP-NH₂ peptides at 2 ºC; Thr: light green squares; phosphothreonine: magenta circles; ThrOGlcNAc: light blue diamonds; Thr diethylphosphate: grey triangles; bottom left; CD spectra of Ac-KSxPP-NH₂ peptides at 25 ºC; Ser: green squares; phosphoserine: red circles; SerOGlcNAc: blue diamonds; Ser diethylphosphate: black triangles; bottom right; CD spectra of Ac-KSxPP-NH₂ peptides at 2 ºC; Ser: light green squares; phosphoserine: magenta circles; SerOGlcNAc: light blue diamonds; Ser diethylphosphate: grey triangles. Data were collected in 5 mM phosphate buffer buffer containing 25 mM KF.
Table S5. Summary of CD data for Ac-KTxPP-NH₂ and Ac-KSxPP-NH₂ peptides. CD data were collected with 100–450 µM peptide in 5 mM phosphate buffer (pH 8.0) containing 25 mM KF at 2 ºC and 25 ºC. *No local maximum was observed.*

| Peptide                  | $[\theta]_{228}$, deg cm² dmol⁻¹ | $\lambda$ at local $[\theta]_{\text{max}}$, nm | local $[\theta]_{\text{max}}$, deg cm² dmol⁻¹ | $\lambda$ at local $[\theta]_{\text{min}}$, nm | $[\theta]_{\text{min}}$, deg cm² dmol⁻¹ | Temp, ºC |
|--------------------------|----------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|----------|
| Ac-KTPP-NH₂              | −2801                            | n.a.                                          | n.a.                                          | 203                                           | −27045                                        | 25       |
| Ac-KT(OPO₃²⁻)PP-NH₂      | 1321                             | 225                                           | 1790                                          | 202                                           | −29165                                        | 25       |
| Ac-KT(OGlcNAc)PP-NH₂     | −1761                            | n.a.                                          | n.a.                                          | 202                                           | −21545                                        | 25       |
| Ac-KT(OPO₃Et₂)PP-NH₂     | −4587                            | n.a.                                          | n.a.                                          | 205                                           | −19258                                        | 25       |
| Ac-KTPP-NH₂              | −1295                            | n.a.                                          | n.a.                                          | 202                                           | −25637                                        | 2        |
| Ac-KT(OPO₃²⁻)PP-NH₂      | 2016                             | 223                                           | 2852                                          | 202                                           | −30878                                        | 2        |
| Ac-KT(OGlcNAc)PP-NH₂     | −602                             | n.a.                                          | n.a.                                          | 202                                           | −15672                                        | 2        |
| Ac-KT(OPO₃Et₂)PP-NH₂     | −4263                            | n.a.                                          | n.a.                                          | 204                                           | −20187                                        | 2        |
| Ac-KSPP-NH₂              | −1638                            | n.a.                                          | n.a.                                          | 202                                           | −39320                                        | 25       |
| Ac-KS(OPO₃²⁻)PP-NH₂      | 8                                | 226                                           | 49                                            | 201                                           | −32565                                        | 25       |
| Ac-KS(OGlcNAc)PP-NH₂     | −1823                            | n.a.                                          | n.a.                                          | 202                                           | −29790                                        | 25       |
| Ac-KS(OPO₃Et₂)PP-NH₂     | −4814                            | n.a.                                          | n.a.                                          | 203                                           | −33453                                        | 25       |
| Ac-KSPP-NH₂              | −920                             | n.a.                                          | n.a.                                          | 201                                           | −38960                                        | 2        |
| Ac-KS(OPO₃²⁻)PP-NH₂      | 774                              | 224                                           | 1278                                          | 201                                           | −33904                                        | 2        |
| Ac-KS(OGlcNAc)PP-NH₂     | −1154                            | n.a.                                          | n.a.                                          | 202                                           | −30043                                        | 2        |
| Ac-KS(OPO₃Et₂)PP-NH₂     | −4043                            | n.a.                                          | n.a.                                          | 203                                           | −33087                                        | 2        |
Table S6. Summary of CD data for Ac-KT<sub>x</sub>PP-NH<sub>2</sub> and Ac-KS<sub>x</sub>PP-NH<sub>2</sub> peptides. CD data were collected with 100–450 µM peptide in 5 mM phosphate buffer (pH 8.0) containing 25 mM KF at 2 ºC and 25 ºC. *No local maximum was observed.*
CD spectra of Ac-GPKTxPPGY-NH₂ peptides

*Figure S12.* Top left: CD spectrum of Ac-GPKTPPGY-NH₂ (36) at pH 6.5, 25 ºC; top right: CD spectrum of Ac-GPKTPPGY-NH₂ (36) at pH 8.0, 25 ºC; and bottom: CD spectrum of Ac-GPKTPPGY-NH₂ (36) at pH 8.0, 2 ºC. Error bars indicate standard errors.
Figure S13. Top left: CD spectrum of Ac-GPKT(OPO$_3$$^{2-}$)PPGY-NH$_2$ (37) at pH 6.5, 25 ºC; top right: CD spectrum of Ac-GPKT(OPO$_3$$^{2-}$)PPGY-NH$_2$ (37) at pH 8.0, 25 ºC; bottom: CD spectrum of Ac-GPKT(OPO$_3$$^{2-}$)PPGY-NH$_2$ (37) at pH 8.0, 2 ºC. Error bars indicate standard errors.
Figure S14. Top left: CD spectrum of Ac-GPKT(OGlcNAc)PPGY-NH$_2$ (38) at pH 6.5, 25 °C; top right: CD spectrum of Ac-GPKT(OGlcNAc)PPGY-NH$_2$ (38) at pH 8.0, 25 °C; bottom: CD spectrum of Ac-GPKT(OGlcNAc)PPGY-NH$_2$ (38) at pH 8.0, 2 °C. Error bars indicate standard errors.
Figure S15. Top left: CD spectrum of Ac-GPKT(OPO$_3$Et$_2$)PPGY-NH$_2$ (39) at pH 6.5, 25 °C; top right: CD spectrum of Ac-GPKT(OPO$_3$Et$_2$)PPGY-NH$_2$ (39) at pH 8.0, 25 °C; bottom: CD spectrum of Ac-GPKT(OPO$_3$Et$_2$)PPGY-NH$_2$ (39) at pH 8.0, 2 °C. Error bars indicate standard error.
CD spectra of peptides Ac-GPPTxPPGY-NH₂

Figure S16. Top left: CD spectrum of Ac-GPPTPPGY-NH₂ (40) at pH 8.0, 25 °C; top right: CD spectrum of Ac-GPPT(OPO₃⁻)PPGY-NH₂ (41) at pH 8.0, 25 °C; bottom left: CD spectrum of Ac-GPPT(OGlcNAc)PPGY-NH₂ (42) at pH 8.0, 25 °C and bottom right: CD spectrum of Ac-GPPT(OPO₃Et₂)PPGY-NH₂ (43) at pH 8.0, 25 °C. Error bars indicate standard error.
Figure S17. Top left; CD spectra of Ac-GPKTxPPGY-NH$_2$ peptides at pH 6.5 at 25 ºC; Thr: green squares; phosphothreonine: red circles; ThrOGlcNAc: blue diamonds; Thr diethylphosphate: black triangles; top right; CD spectra of Ac-GPKTxPPGY-NH$_2$ peptides at pH 8.0 at 25 ºC; Thr: green squares; phosphothreonine: red circles; ThrOGlcNAc: blue diamonds; Thr diethylphosphate: black triangles; bottom left; CD spectra of Ac-GPKTxPPGY-NH$_2$ peptides at 2 ºC; Thr: light green squares; phosphothreonine: magenta circles; ThrOGlcNAc: light blue diamonds; Thr diethylphosphate: grey triangles; bottom right; CD spectra of Ac-GPPTxPPGY-NH$_2$ peptides at pH 8.0 at 25 ºC; Thr: green squares; phosphothreonine: red circles; ThrOGlcNAc: blue diamonds; Thr diethylphosphate: black triangles. Data were collected in 5 mM phosphate buffer buffer containing 25 mM KF.
| Peptide                      | $\theta_{228}$, deg cm$^2$ dmol$^{-1}$ | $\lambda$ at local $\theta_{\text{max}}$, nm | local $\theta_{\text{max}}$, deg cm$^2$ dmol$^{-1}$ | $\lambda$ at local $\theta_{\text{min}}$, nm | $\theta_{\text{min}}$, deg cm$^2$ dmol$^{-1}$ | $\theta_{190}$, deg cm$^2$ dmol$^{-1}$ | Temp, ºC | pH |
|-----------------------------|----------------------------------------|---------------------------------------------|-------------------------------------------------|---------------------------------------------|---------------------------------------------|----------------------------------------|----------|----|
| Ac-GPPTPPGY-NH$_2$          | -230                                   | n.a.                                        | n.a.                                            | 205                                         | -11243                                     | -3448                                  | 25       | 8  |
| Ac-GPPT(OPO$_3^{2-}$)PPGY-NH$_2$ | 272                                   | 231                                         | 449                                             | 206                                         | -16553                                     | -6253                                  | 25       | 8  |
| Ac-GPPT(OGlcNAc)PPGY-NH$_2$ | -252                                   | 232                                         | 112                                             | 205                                         | -17077                                     | -2234                                  | 25       | 8  |
| Ac-GPPT(OPO$_3$Et$_2$)PPGY-NH$_2$ | -1070                                 | n.a.                                        | n.a.                                            | 206                                         | -13342                                     | -3158                                  | 25       | 8  |
| Ac-GPKTPPGY-NH$_2$          | -184                                   | 232                                         | 50                                              | 204                                         | -10285                                     | -4483                                  | 25       | 8  |
| Ac-GPKT(OPO$_3^{2-}$)PPGY-NH$_2$ | 285                                   | 229                                         | 326                                             | 203                                         | -14041                                     | -6134                                  | 25       | 8  |
| Ac-GPKT(OPO$_3^{-2-}$)PPGY-NH$_2$ | 516                                   | 230                                         | 581                                             | 203                                         | -13675                                     | -5736                                  | 25       | 6.5|
| Ac-GPKT(OGlcNAc)PPGY-NH$_2$ | -473                                   | n.a.                                        | n.a.                                            | 203                                         | -11912                                     | -926                                   | 25       | 8  |
| Ac-GPKT(OPO$_3$Et$_2$)PPGY-NH$_2$ | -781                                  | n.a.                                        | n.a.                                            | 205                                         | -6652                                     | -1674                                  | 25       | 8  |
| Ac-GPKTPPGY-NH$_2$          | 183                                    | 230                                         | 253                                             | 203                                         | -13896                                     | -5608                                  | 2        | 8  |
| Ac-GPKT(OPO$_3^{2-}$)PPGY-NH$_2$ | 1560                                  | 227                                         | 1620                                            | 203                                         | -15526                                     | -6728                                  | 2        | 8  |
| Ac-GPKT(OGlcNAc)PPGY-NH$_2$ | 296                                    | 231                                         | 432                                             | 205                                         | -15436                                     | -1656                                  | 2        | 8  |
| Ac-GPKT(OPO$_3$Et$_2$)PPGY-NH$_2$ | -569                                  | n.a.                                        | n.a.                                            | 205                                         | -10956                                     | -2406                                  | 2        | 8  |

Table S7. Summary of CD data for model peptides. CD data were collected with 100–200 µM peptide in 5 mM phosphate buffer (pH 8.0 or 6.5) containing 25 mM KF. $^a$ No local maximum was observed.
$^1$H NMR spectra of all peptides: amide region (stacked spectra) and full spectra of individual peptides.

Analysis of peptides derived from tau$_{174-183}$

$\text{KT}PPAPKTPP$

pH 4

$\text{KT}(\text{OPO}_3^{-2})\text{PPAPKT(OPO}_3^{-2})\text{PP}$

pH 6.5

$\text{KT}(\text{OPO}_3^{-2})\text{PPAPKT(OPO}_3^{-2})\text{PP}$

pH 8

$\text{KT(OGlcNAc)PPAPKT(OGlcNAc)PP}$

pH 4

$\text{KT(OPO }_3^{-2}\text{Et})PPAPKT(OPO}_3^{-2}\text{Et})PP$

pH 4

$\text{KEPPAPKEPP}$

pH 6.6

$\text{KTlePPAPKTlePP}$

pH 4

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure_s18.png}
\caption{$^1$H NMR spectra (amide region) of peptides derived from tau$_{174-183}$. Minor peaks in the NMR spectra are due to the presence of cis amide bonds. pT indicates a phosphorylated threonine residue. eT indicates a diethylphosphorylated (OPO$_3$Et$_2$) threonine residue. gT indicates an OGlcNAcylated threonine residue. Tle indicates tert-leucine. Peptides were dissolved in 5 mM phosphate buffer (pH 4.0, 6.5, or 8.0) at 298 K and were internally referenced with TSP. Solutions contained 25 mM NaCl, 100 $\mu$M TSP, and 90% H$_2$O/10% D$_2$O.}
\end{figure}
Figure S19. $^1$H NMR spectra (amide region) of phosphorylated peptide tau$_{174-183}$ at pH 6.5 (top) and 8 (bottom) at 298 K. Minor peaks in the NMR spectra are due to the presence of cis amide bonds. pT indicates a phosphorylated threonine residue.

The phosphorylated peptide tau$_{174-183}$ showed unusually slow amide exchange at pH 8.0. All five amide protons corresponding to Thr, Ala, and Lys were observed at pH 8.0.

Figure S20. $^{31}$P NMR spectrum of phosphorylated peptide tau$_{174-183}$($\text{OPO}_3^{2-}$) at pH 8.0 at 298 K in 5 mM phosphate buffer containing 25 mM NaCl. The NMR spectrum was internally referenced with 85% $\text{H}_3\text{PO}_4$ (0 ppm) using a capillary filled with $\text{H}_3\text{PO}_4$ located in the NMR tube containing the sample.
Full \textsuperscript{1}H NMR spectra of peptides derived from tau\textsubscript{174-183}
| Peptide          | δ, H<sup>N</sup> | 3<sup>J</sup>αN | δ, H<sub>α</sub> | δ, H<sub>β</sub> | 3<sup>J</sup>αH<sub>β</sub> |
|------------------|------------------|------------------|------------------|------------------|------------------|
| tau<sub>174–183</sub> (pH 4.0) |                  |                  |                  |                  |                  |
| T                | 8.22             | 7.3              | 4.58             | 4.11             | 8.1              |
| T                | 8.29             | 7.3              | 4.59             | 4.13             | n.d.<sup>a</sup> |
| K                | 8.35             | 6.9              | 4.33             | 1.84, 1.77       | n.d.             |
| K                | 8.40             | 5.5              | 4.34             | 1.83, 1.73       | n.d.             |
| A                | 8.51             | 6.8              | 4.59             | 1.37             | n.d.             |
| tau<sub>174–183</sub>(OPO<sub>3</sub>H<sup>+</sup>) (pH 3.5) |                  |                  |                  |                  |                  |
| pT               | 8.38             | 6.7              | 4.66             | 4.47             | n.d.             |
| pT               | 8.45             | 6.3              | 4.64             | 4.47             | n.d.             |
| K                | 8.32             | 7.0              | 4.31             | 1.83, 1.74       | n.d.             |
| K                | 8.49             | 6.7              | 4.29             | 1.84, 1.78       | n.d.             |
| A                | 8.40             | 5.5              | 4.57             | 1.37             | n.d.             |
| tau<sub>174–183</sub>(OPO<sub>3</sub>Et<sub>2</sub>) (pH 6.5) |                  |                  |                  |                  |                  |
| pT               | 8.96             | 4.3              | 4.51             | 4.37             | n.d.             |
| pT               | 9.08             | 4.2              | 4.55             | 4.38             | 7.6              |
| K                | 8.29             | 7.0              | 4.32             | 1.83, 1.75       | n.d.             |
| K                | 8.40             | 5.5              | 4.32             | 1.84, 1.78       | n.d.             |
| A                | 8.45             | 6.8              | 4.59             | 1.37             | n.d.             |
| tau<sub>174–183</sub>(OPO<sub>3</sub>GlcNAc) (pH 4.0) |                  |                  |                  |                  |                  |
| gT               | 7.96             | 7.5              | 4.58             | 4.13             | 7.4              |
| gT               | 7.97             | 7.5              | 4.58             | 4.10             | n.d.             |
| K                | 8.33             | n.a.             | 4.28             | 1.83, 1.75       | n.d.             |
| K                | 8.45             | 7.0              | 4.30             | 1.82, 1.71       | n.d.             |
| A                | 8.35             | n.d.             | 4.58             | 1.36             | n.d.             |
| tau<sub>174–183</sub>(OPO<sub>3</sub>Et<sub>2</sub>) (pH 4.0) |                  |                  |                  |                  |                  |
| eT               | 8.28             | n.d.             | 4.87             | 4.66             | n.d.             |
| eT               | 8.42             | n.d.             | 4.87             | 4.66             | 7.1              |
| K                | 8.28             | n.d.             | 4.33             | 1.81, 1.71       | n.d.             |
| K                | 8.42             | n.d.             | 4.32             | 1.82, 1.75       | n.d.             |
| A                | 8.33             | 5.2              | 4.57             | 1.36             | n.d.             |
| tau<sub>174–183</sub>(T175E, T181E) (pH 6.5) |                  |                  |                  |                  |                  |
| E                | 8.37             | 5.8              | 4.60             | 2.30, 2.04       | n.d.             |
| E                | 8.46             | 6.6              | 4.59             | 2.31, 2.04       | n.d.             |
| K                | 8.29             | 7.1              | 4.30             | 1.81, 1.72       | n.d.             |
| K                | 8.44             | 7.0              | 4.29             | 1.82, 1.76       | n.d.             |
| A                | 8.38             | 7.0              | 4.59             | 1.37             | n.d.             |
| tau<sub>174–183</sub>(T175Tle, T181Tle) (pH 4.0) |                  |                  |                  |                  |                  |
| Tle              | 7.88             | 7.9              | 4.58             | n.a.<sup>b</sup> | n.d.             |
| Tle              | 7.95             | 8.8              | 4.57             | n.a.             | n.d.             |
| K                | 8.35             | n.d.             | 4.32             | 1.79, 1.70       | n.d.             |
| K                | 8.54             | 7.4              | 4.32             | n.d.             | n.d.             |
| A                | 8.37             | n.d.             | 4.58             | 1.36             | n.d.             |

Table S8. Summary of 1H NMR data for peptides derived from tau<sub>174–183</sub>. Data were collected with 100–200 µM peptide in 5 mM phosphate buffer (pH 3.5, 4.0, 6.5 or 8) with 25 mM NaCl. pT indicates a phosphorylated threonine residue. eT indicates a diethylphosphorylated (OPO<sub>3</sub>Et<sub>2</sub>) threonine residue. gT indicates an OGlCNacylated threonine residue. Tle indicates <i>tert</i>-leucine.<sup>a</sup> n.d. = not determined due to spectral overlap.<sup>b</sup> n.a. = not applicable.
Figure S21. $^1$H-$^{15}$N HSQC spectrum of peptide tau$_{174-183}$ at pH 4.0 in 5 mM phosphate buffer containing 25 mM NaCl at 298 K.
Figure S22. $^1$H-$^{15}$N HSQC spectrum of peptide tau$_{174-183}$(OPO$_3$H$^-$) at pH 4.0 in 5 mM phosphate buffer containing 25 mM NaCl at 298 K.
Figure S23. $^1$H-$^{15}$N HSQC spectrum of peptide tau$_{174-185}$($\text{OPO}_3^{2-}$) at pH 7.5 in 5 mM phosphate buffer containing 25 mM NaCl at 298 K.

Figure S23. $^1$H-$^{15}$N HSQC spectrum of peptide tau$_{174-185}$($\text{OPO}_3^{2-}$) at pH 7.5 in 5 mM phosphate buffer containing 25 mM NaCl at 298 K.
**Figure S24.** $^1$H-$^{15}$N HSQC spectrum of peptide tau$_{174-183}$ (OGlcNAc) at pH 4.0 in 5 mM phosphate buffer containing 25 mM NaCl at 298 K.
Figure S25. $^1$H-$^1$N HSQC spectra of peptides $\tau_{174-183}$ at pH 4.0 (green), $\tau_{174-183}(\text{OPO}_3^{2-})$ (red), $\tau_{174-183}(\text{OPO}_3\text{H}^-)$ (magenta), and $\tau_{174-183}(\text{O}Glc\text{NAc})$ (blue) at 298 K. Data were collected with 2–3 mM peptide in 5 mM phosphate buffer (pH 4.0 or 7.5) with 25 mM NaCl.

Table S9. Summary of $^1$H-$^1$N HSQC NMR data for peptides derived from $\tau_{174-183}$. Data were collected with 2–3 mM peptide in 5 mM phosphate buffer (pH 4.0 or 7.5) with 25 mM NaCl.
Figure S26. $^1$H-$^{13}$C HSQC spectrum of peptides tau$^{174-183}$ at pH 4.0 in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K.
Figure S27. $^1$H-$^{13}$C HSQC spectrum of peptides tau$_{174-183}$($OPO_3^{2-}$) at pH 8.0 in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K.
Figure S28. $^1$H-$^{13}$C HSQC spectra of peptides tau$_{174-183}$ at pH 4.0 (green) and tau$_{174-183}$($OPO_3^{2-}$) (red) at pH 8.0 in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K.
Figure S29. Partial NOESY spectrum of peptide tau_{174-183}(OPO_{3}^{2-}) at pH 8.0 in 5 mM phosphate buffer containing 25 mM NaCl at 298 K.
Figure S30. $^1$H-$^{13}$C HMBC spectra (alpha proton region) of peptides tau$_{174-183}$ at pH 4.0 (green) and tau$_{174-183}$(OPO$_3^{2-}$) (red) at pH 8.0 in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K.
Figure S31. $^1$H-$^{13}$C HMBC spectra (full spectra) of peptides tau$_{174-183}$ at pH 4.0 (green) and tau$_{174-183}$($OPO_3^{2-}$) (red) at pH 8.0 in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K.
| Ac-KTPPAPKTPP-NH<sub>2</sub> | Ac-KpTPPAPKpTPP-NH<sub>2</sub> |
|-------------------------|-------------------------|
| residue | 13<sup>C</sup> δ, ppm | residue | 13<sup>C</sup> δ, ppm |
| Lys, α | 53.4, 53.4 | Lys, α | 53.1, 53.1 |
| Thr, α | 57.0, 57.0 | pThr, α | 58.1, 58.4 |
| Pro, α | 58.9, 60.1 | Pro, α | 58.9, 60.0 |
| Ala, α | 47.5 | Ala, α | 47.5 |
| Thr, β | 66.9, 66.9 | pThr, β | 70.0, 70.0 |
| Pro, δ | 47.7, 48.3 | Pro, δ | 47.7, 48.6 |
| Lys, ε | 39.1, 39.1 | Lys, ε | 39.1, 39.1 |
| Pro, γ | 28.1, 28.1 | Pro, γ | 28.2, 28.2 |
| Lys, β | 30.4, 30.4 | Lys, β | 30.5, 30.5 |
| Pro, β | 29.4, 29.4 | Pro, β | 29.3, 29.3 |
| Lys, γ | 26.2, 26.2 | Lys, γ | 25.9, 25.9 |
| Lys, δ | 22.1, 22.1 | Lys, δ | 21.7, 21.7 |
| Ala, β | 15.2 | Ala, β | 15.2 |
| Thr, γ | 18.7, 18.7 | pThr, γ | 17.8, 17.8 |
| Lys, CO | 174.1, 174.1 | Lys, CO | 173.6, 173.9 |
| Thr, CO | 169.6, 169.6 | pThr, CO | 169.7, 169.7 |
| 176.9, 173.8, | | 176.8, 173.6, |
| Pro, CO | n.d.<sup>a</sup> | Pro, CO | 171.8 |
| Ala, CO | 172.9 | Ala, CO | 172.9 |
| Ac, CO | 174.2 | Ac, CO | 174.0 |

Table S10. Summary of 1H-13C HSQC NMR data for peptides tau<sub>174–183</sub> at pH 4.0 and tau<sub>174–183</sub>(OPO<sub>3</sub><sup>2−</sup>) at pH 8.0, 298 K. Data were collected with 2–3 mM peptide in 5 mM phosphate buffer with 25 mM NaCl. pThr indicates a phosphorylated threonine residue. <sup>a</sup>n.d. indicates not determined because peak is in the H<sub>2</sub>O peak.
Analysis of peptides derived from tau\textsubscript{196–209}

**Figure S32.** \textsuperscript{1}H NMR spectra (amide region) of peptides derived from tau\textsubscript{196–209}. Minor peaks in the NMR spectra are due to the presence of cis amide bonds. Peptides were dissolved in 5 mM phosphate buffer (pH 4.0, 6.5, or 8.0) and were internally referenced with TSP. Solutions contained 25 mM NaCl, 100 µM TSP, and 90% H\textsubscript{2}O/10% D\textsubscript{2}O. pS or pT indicates a phosphorylated serine or threonine residue. eS or eT indicates a diethylphosphorylated (OPO\textsubscript{3}Et\textsubscript{2}) serine or threonine residue. gS or gT indicates an OGlcNAcylated serine or threonine residue.
Figure S33. $^1$H NMR spectra (amide region) of phosphorylated peptide tau$_{196-209}$ at pH 6.5 (top) and pH 8.0 (bottom). Peptides were dissolved in 5 mM phosphate buffer and were internally referenced with TSP. Solutions contained 25 mM NaCl, 100 µM TSP, and 90% H$_2$O/10% D$_2$O. Minor peaks in the NMR spectra are due to the presence of cis amide bonds. pS or pT indicates a phosphorylated serine or threonine residue.

The phosphorylated peptide tau$_{196-209}$ showed broad amide peaks indicative of faster amide proton exchange rates at pH 8.0 compared to pH 6.5. This observation is in contrast to other phosphorylated tau peptide, which showed more resolved amide protons at pH 8.0 and than at pH 6.5, suggesting less ordered structure seen in these glycine-rich peptides, which did not exhibit PPII structure, than in the other tau peptides investigated. Nonetheless, the amide protons were still observed at higher pH for phosphorylated peptide tau$_{196-209}$.
Full $^1$H NMR spectra of peptides derived from tau$_{196-209}$
| Peptide         | $\delta$, $H^N$ | $^3J_{\alpha N}$ | $\delta$, $H_\alpha$ | $\delta$, $H_\beta$ |
|-----------------|-----------------|------------------|-----------------------|-----------------------|
| **tau$_{196-209}$ (pH 4.0)** |                 |                  |                       |                       |
| G               | 8.47            | n.d.             | 3.96                  | n.a.                  |
| G               | 8.47            | n.d.             | 3.96                  | n.a.                  |
| G               | 8.57            | n.d.             | 3.98                  | n.a.                  |
| G               | 8.57            | n.d.             | 3.98                  | n.a.                  |
| Y               | 8.11            | 6.3              | 4.60                  | 3.06, 2.97            |
| S               | 8.18            | 5.8              | 4.43                  | 3.88, 3.87            |
| S               | 8.18            | 5.8              | 4.43                  | 3.88, 3.87            |
| S               | 8.23            | 5.8              | 4.45                  | 3.85, 3.79            |
| S               | 8.23            | 5.8              | 4.45                  | 3.85, 3.79            |
| T               | 8.07            | 7.2              | 4.63                  | 4.19                  |
| R               | 8.41            | 5.9              | 4.34                  | 1.90, 1.77            |
| **tau$_{196-209}$ (OPO$_3$$^{3-}$) (pH 6.5)** |                 |                  |                       |                       |
| G               | 8.24            | n.d.             | 3.86                  | n.a.                  |
| G               | 8.32            | n.d.             | 3.99                  | n.a.                  |
| G               | 8.32            | n.d.             | 3.99                  | n.a.                  |
| G               | 8.66            | n.d.             | 4.00                  | n.a.                  |
| Y               | 8.11            | 7.3              | 4.62                  | 3.09, 2.95            |
| S               | 8.25            | n.d.             | 4.68                  | n.d.                  |
| S               | 8.36            | 6.5              | 4.92                  | 4.11                  |
| pS              | 8.45            | 7.3              | 4.69                  | 4.22, 4.10            |
| pS              | 8.49            | 5.5              | 4.90                  | 4.22, 4.11            |
| pT              | 8.50            | 4.4              | 4.53                  | n.d.                  |
| R               | 8.39            | 7.7              | 4.34                  | 1.96, 1.80            |
| **tau$_{196-209}$ (OPO$_3$$^{2-}$) (pH 8.0)** |                 |                  |                       |                       |
| G               | 8.29            | n.d.             |                       |                       |
| G               | 8.35            | n.d.             |                       |                       |
| G               | 8.35            | n.d.             |                       |                       |
| G               | 8.63            | n.d.             |                       |                       |
| Y               | 8.06            | n.d.             |                       |                       |
| S               | 8.23            | n.d.             |                       |                       |
| S               | 8.28            | n.d.             |                       |                       |
| pS              | 8.69            | n.d.             |                       |                       |
| pS              | 8.78            | n.d.             |                       |                       |
| pT              | 9.67            | n.d.             |                       |                       |
| R               | 8.54            | n.d.             |                       |                       |
| **tau$_{196-209}$ (OGlcNAc) (pH 4.0)** |                 |                  |                       |                       |
| G               | 8.21            | n.d.             | 3.86                  | n.a.                  |
| G               | 8.28            | n.d.             | 3.95                  | n.a.                  |
| G               | 8.37            | n.d.             | 4.00                  | n.a.                  |
| G               | 8.61            | n.d.             | 3.96                  | n.a.                  |
| Y               | 8.08            | 7.0              | 4.61                  | 3.06, 2.97            |
### Table S11.

Summary of $^1$H NMR data for peptides derived from tau$_{196-209}$. Data were collected with 100–200 μM peptide in 5 mM phosphate buffer with 25 mM NaCl. pS or pT indicates a phosphorylated serine or threonine residue. eS or eT indicates a diethylphosphorylated (OPO$_3$Et$_2$) serine or threonine residue. gS or gT indicates an OGlCNacylated serine or threonine residue. *a* n.a. = not applicable. *b* n.d. = not determined due to spectral overlap or exchange broadening.

|   |   |   |   |
|---|---|---|---|
| S | 8.16 | n.d. | 4.57 3.69, 3.56 |
| gS | 8.18 | n.d. | 4.59 3.72, 3.57 |
| gS | 8.28 | n.d. | 4.57 3.64, 3.55 |
| gT | 8.19 | n.d. | 4.70 4.27 |
| R | 8.37 | 6.8 | 8.31 1.93, 1.78 |

**tau$_{196-209}$(OPO$_3$Et$_2$) (pH 4.0)**

|   |   |   |   |
|---|---|---|---|
| G | 8.21 | n.d. | 3.85 n.a. |
| G | 8.34 | n.d. | 3.99 n.a. |
| G | 8.42 | n.d. | 3.98 n.a. |
| G | 8.47 | n.d. | 3.96 n.a. |
| Y | 8.03 | 7.3 | 4.63 3.08, 2.96 |
| S | 8.22 | n.d. | 4.45 3.85 |
| S | 8.25 | 7.6 | 4.49 3.82 |
| eT | 8.43 | n.d. | 4.91 n.d. |
| eS | 8.48 | n.d. | 4.99 4.39, 4.26 |
| eS | 8.36 | n.d. | 5.04 4.40, 4.24 |
| R | 8.42 | n.d. | 4.35 1.93, 1.79 |
Analysis of peptides derived from tau\textsubscript{211–219}

\begin{center}
\begin{figure}
\includegraphics[width=\textwidth]{hnrmspectra}
\caption{\textsuperscript{1}H NMR spectra (amide region) of peptides derived from tau\textsubscript{211–219}. Minor peaks in the NMR spectra are due to the presence of cis amide bonds. Peptides were dissolved in 5 mM phosphate buffer (pH 4.0, 6.5, or 8.0) and were internally referenced with TSP. Solutions contained 25 mM NaCl, 100 \(\mu\)M TSP, and 90\% \(\text{H}_2\text{O}/10\% \text{D}_2\text{O}. \) pS or pT indicates a phosphorylated serine or threonine residue. eS or eT indicates a diethylphosphorylated (OPO\textsubscript{3}Et\textsubscript{2}) serine or threonine residue. gS or gT indicates an OGlcNAcylated serine or threonine residue.}
\end{figure}
\end{center}

Figure S34.
Figure S35. $^1$H NMR spectra (amide region) of phosphorylated peptide tau$_{211-219}$ at pH 6.5 (top) and 8 (bottom). Minor peaks in the NMR spectra are due to the presence of cis amide bonds. Peptides were dissolved in 5 mM phosphate buffer and were internally referenced with TSP. Solutions contained 25 mM NaCl, 100 µM TSP, and 90% H$_2$O/10% D$_2$O. pS or pT indicates a phosphorylated serine or threonine residue.

As observed previously for phosphorylated peptide tau$_{174-183}$, the amide protons were observed to be in slow exchange and were more resolved at pH 8.0 compared to pH 6.5.

Figure S36. $^{31}$P NMR spectrum of phosphorylated peptide tau$_{211-219}$ at pH 8.0, 298 K in 5 mM phosphate buffer containing 25 mM NaCl. The NMR spectrum was internally referenced with 85% H$_3$PO$_4$ (0 ppm) using a capillary filled with H$_3$PO$_4$ located in the NMR tube containing the sample.
Full $^1$H NMR spectra of peptides derived from tau$_{211-219}$

YRTPSLPTPP

$\delta$, ppm

YRT(OPO$_3^{-2-}$)$\times$PS(OPO$_3^{-2-}$)LPT(OPO$_3^{-2-}$)PP

$\delta$, ppm

YRT(OPO$_3^{2-}$)$\times$PS(OPO$_3^{2-}$)LPT(OPO$_3^{2-}$)PP

$\delta$, ppm

YRT(OGlcNAc)$\times$PS(OGlcNAc)LPT(OGlcNAc)PP

$\delta$, ppm

YRT(OPO$_3^{2-}$)$\times$PS(OPO$_3^{2-}$)LPT(OPO$_3^{2-}$)PP

$\delta$, ppm
| peptide       | δ, H\(^{1}H\) | \(^{3}J_{\alpha N}\) | δ, H\(\alpha\) | δ, H\(\beta\) | \(^{3}J_{H\alpha H\beta}\) |
|--------------|----------------|----------------|----------------|----------------|----------------|
| **tau\(_{211-219}\) (pH 4.0)** |                |                |                |                |                |
| Y            | 8.26           | 6.3            | 4.48           | 2.96, 2.96     |                |
| R            | 8.12           | 7.7            | 4.37           | 1.75, 1.66     |                |
| T            | 8.21           | 6.4            | 4.48           | 4.15           |                |
| S            | 8.41           | 6.5            | 4.44           | 3.85, 3.85     |                |
| L            | 8.32           | 7.1            | 4.68           | 1.69           |                |
| T            | 8.34           | 7.0            | 4.55           | 4.11           | 7.0            |
| **tau\(_{211-219}\) (OPO\(_{3}H\)) (pH 4.0)** |                |                |                |                |                |
| Y            | 8.27           | 6.4            | 4.42           | 2.99, 2.88     |                |
| R            | 7.87           | 8.0            | 4.30           | 1.70, 1.61     |                |
| pT           | 8.39           | 6.6            | 4.65           | 4.49           | n.d.           |
| pS           | 8.57           | 4.5            | 4.49           | 4.11, 4.11     | n.d.           |
| L            | 8.31           | 7.4            | 4.69           | 1.67, 1.58     |                |
| pT           | 8.58           | 6.7            | 4.54           | 4.11           | n.d.           |
| **tau\(_{211-219}\) (OPO\(_{3}^{-2}\)) (pH 6.5)** |                |                |                |                |                |
| Y            | 8.29           | 6.3            | 4.44           | 3.00, 2.89     |                |
| R            | 7.88           | 8.0            | 4.32           | 1.72, 1.63     |                |
| pT           | 8.42           | 5.9            | 4.68           | 4.53           | n.d.           |
| pS           | 8.62           | n.d.           | 4.53           | 4.14, 4.14     | n.d.           |
| L            | 8.32           | n.d.           | n.d.           | n.d.           |                |
| pT           | 8.62           | 2.9            | 4.53           | 4.53           | n.d.           |
| **tau\(_{211-219}\) (OPO\(_{3}^{-2}\)) (pH 8.0)** |                |                |                |                |                |
| Y            | 8.29           | 6.1            | 4.44           | 2.99, 2.89     |                |
| R            | 7.65           | n.d.           | 4.27           | 1.68, 1.52     |                |
| pT           | 9.39           | 3.7            | 4.41           | 4.28           | n.d.           |
| pS           | 9.17           | 5.5            | 4.42           | 4.09, 3.97     | n.d.           |
| L            | 8.44           | 7.5            | 4.70           | 1.70, 1.61     |                |
| pT           | 9.66           | 3.1            | 4.30           | 4.30           | n.d.           |
| **tau\(_{211-219}\) (OGlNAC) (pH 4.0)** |                |                |                |                |                |
| Y            | 8.26           | 6.1            | 4.32           | 2.91, 2.77     |                |
| R            | 7.86           | 8.3            | 4.26           |                |                |
| gT           | 7.93           | 4.2            | 4.34           | 4.06           | n.d.           |
| gS           | 8.15           | 7.8            | 4.54           | 3.54, 3.43     | n.d.           |
| L            | 8.17           | 5.3            | 4.69           | n.d.           |                |
| gT           | 8.03           | 5.7            | 4.53           | 4.11           | 6.6            |
| **tau\(_{211-219}\) (OPO\(_{3}Et\)) (pH 4.0)** |                |                |                |                |                |
| Y            | 8.19           | 7.0            | 4.49           | 2.96, 2.96     |                |
| R            | 8.10           | 7.7            | 4.37           |                |                |
| eT           | 8.19           | 7.0            | 4.82           | 4.47           | 8.0            |
| eS           | 8.48           | 7.3            | 4.62           | 4.35, 4.35     | n.d.           |
| L            | 8.18           | 7.7            | 4.71           | n.d.           |                |
| eT           | 8.29           | 6.8            | 4.74           | 4.70           | n.d.           |

Table S12. Summary of \(^{1}H\) NMR data for peptides derived from tau\(_{211-219}\). Data were collected with 100–200 \(\mu M\) peptide in 5 mM phosphate buffer with 25 mM NaCl. pS or pT indicates a phosphorylated serine or threonine residue. eS or eT indicates a diethylphosphorylated (OPO\(_{3}Et\)) serine or threonine residue. gS or gT indicates an OGlNACylated serine or threonine residue. \(^{a}\)n.d. = not determined due to spectral overlap.
**Figure S37.** $^1$H-$^{15}$N HSQC spectrum of peptide tau$_{211-219}$ at pH 4.0 at 298 K in 5 mM phosphate buffer containing 25 mM NaCl.
Figure S38. $^1$H-$^{15}$N HSQC spectrum of peptide tau$_{211-219}$(OPO$_3$H$^-$) at pH 4.0 at 298 K in 5 mM phosphate buffer containing 25 mM NaCl.
Figure S39. $^1$H-$^{15}$N HSQC spectrum of peptide tau$_{211-219}$(OPO$_3^{2-}$) at pH 7.9 at 298 K in 5 mM phosphate buffer containing 25 mM NaCl.
Figure S40. $^1$H-$^{15}$N HSQC spectra of peptides tau$_{211-219}$ at pH 4.0 (green), tau$_{211-219}$(OPO$_3^{2-}$) (red) (pH 7.9), and tau$_{211-219}$(OPO$_3H^-$) (magenta) (pH 4.0) at 298 K. Data were collected with 2–3 mM peptide in 5 mM phosphate buffer (pH 4.0 or 7.9) with 25 mM NaCl.
| peptide | $\text{Thr}_N$, ppm | $\text{Ser}_N$, ppm | others | pH, Temp |
|---------|----------------|----------------|--------|----------|
| Ac-YRTPSLPTPP-NH$_2$ | 117.8, 118.6 | 116.5 | 124.2 (Arg), 125.4 (Leu), 126.6 (Tyr), 107.1 (carboxamide) | 4.0, 298 K |
| Ac-YRT(OPO$_3^-$)PS(OPO$_3^-$)LPT(OPO$_3^-$)PP-NH$_2$ | 117.4, 120.9 | 116.6 | 125.2 (Arg), 125.0 (Leu), 125.6 (Tyr), 107.4 (carboxamide) | 4.0, 298 K |
| Ac-YRT(OPO$_3^{2-}$)PS(OPO$_3^{2-}$)LPT(OPO$_3^{2-}$)PP-NH$_2$ | 121.8, 125.7 | 119.4 | 125.2 (Arg), 125.1 (Leu), 127.0 (Tyr), 107.4 (carboxamide) | 7.9, 298 K |

*Table S13.* Summary of $^1$H-$^{15}$N HSQC NMR data for peptides derived from tau$_{211-219}$. Data were collected with 2–3 mM peptide in 5 mM phosphate buffer (pH 4.0 or 7.9) with 25 mM NaCl.
Figure S41. $^1$H-$^{13}$C HSQC spectrum of peptide tau$_{211-219}$ at pH 4.0 in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K.
Figure S42. $^1$H-$^1$C HSQC spectrum of peptide tau$_{211-219}$($\text{OPO}_3^{2-}$) at pH 8.0 in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K.
Figure S43. $^1$H-$^{13}$C HSQC spectra of peptides tau$_{211-219}$ at pH 4.0 (green) and tau$_{211-219}$(OPO$_3^{2-}$) (red) (pH 8.0) at 298 K. Data were collected with 1 mM peptide in 5 mM phosphate buffer containing 25 mM NaCl.
**Table S14.** Summary of $^1$H-$^{13}$C NMR data for peptides derived from tau$_{211-219}$. Data were collected with 1 mM peptide in 5 mM phosphate buffer with 25 mM NaCl. pSer or pThr indicates a phosphorylated serine or threonine residue.
Figure S44. $^1$H NMR spectra (amide region) of peptides derived from tau$_{229-238}$. Minor peaks in the NMR spectra are due to the presence of cis amide bonds. Peptides were dissolved in 5 mM phosphate buffer (pH 4.0, 6.5, or 8.0) and were internally referenced with TSP. Solutions contained 25 mM NaCl, 100 µM TSP, and 90% H$_2$O/10% D$_2$O. pS or pT indicates a phosphorylated serine or threonine residue. eS or eT indicates a diethylphosphorylated (OPO$_3$Et$_2$) serine or threonine residue. gS or gT indicates an OGlcNAcylated serine or threonine residue.
Figure S45. $^1$H NMR spectra (amide region) of phosphorylated peptide tau_{229-238} at pH 8.0, 7.3, and 6.5 at 298 K or as indicated. Minor peaks in the NMR spectra are due to the presence of cis amide bonds. Peptides were dissolved in 5 mM phosphate buffer and were internally referenced with TSP. Solutions contained 25 mM NaCl, 100 $\mu$M TSP, and 90% H$_2$O/10% D$_2$O. pS or pT indicates a phosphorylated serine or threonine residue.

Figure S46. $^{31}$P NMR spectrum of phosphorylated peptide tau_{229-238} at pH 8.0 at 298 K in 5 mM phosphate buffer containing 25 mM NaCl. The NMR spectrum was internally referenced with 85% H$_3$PO$_4$ (0 ppm) using a capillary filled with H$_3$PO$_4$ located in the NMR tube containing the sample.
Full $^1$H NMR spectra of peptides derived from tau$_{229-238}$ at pH 6.5 or 8, 298 K

YVRTPPKSPSS

YVRT(OPO$_3^{2-}$)PPKS(OPO$_3^{2-}$)PS(OPO$_3^{2-}$)S

YRT(OPO$_3^2$)PS(OPO$_3^2$)LPT(OPO$_3^2$)PP

YVRT(OGlcnAc)PPKS(OGlcnAc)PS(OGlcnAc)S

YVRT(OPO$_3^{2-}$)PPKS(OPO$_3^{2-}$)PS(OPO$_3^{2-}$)S
Table S15. Summary of $^1$H NMR data for peptides derived from tau$_{229-238}$. Data were collected with 100–200 µM peptide in 5 mM phosphate buffer containing 25 mM NaCl. pS or pT indicates a phosphorylated serine or threonine residue. eS or eT indicates a diethylphosphorylated (OPO$_3$Et$_2$) serine or threonine residue. gS or gT indicates an OGlcNAcylated serine or threonine residue. "n.d. = not determined due to spectral overlap.
Figure S47. $^1$H-$^{15}$N HSQC spectrum of peptide tau$_{229-238}$ at pH 4.0 at 298 K in 5 mM phosphate buffer containing 25 mM NaCl.
Figure S48. $^1$H-$^{15}$N HSQC spectrum of peptide tau$_{229-238}$($OPO_3H^-$) at pH 4.0 at 298 K in 5 mM phosphate buffer containing 25 mM NaCl.
Figure S49. $^1$H-$^{15}$N HSQC spectrum of peptide tau$_{229-238}$($\text{OPO}_3^{2-}$) at pH 8.0 at 298 K in 5 mM phosphate buffer containing 25 mM NaCl. The missing resonance contour for S($\text{OPO}_3^{2-}$) could not be determined because of rapid amide exchange at pH 8.0.
Figure S50. $^1$H-$^{15}$N HSQC spectra of peptides tau$_{229-238}$ at pH 4.0 (green), tau$_{229-238}$(OPO$_3^2$) (red) (pH 8.0), and tau$_{229-238}$(OPO$_3$H$^-$) (magenta) (pH 4.0) at 298 K. Data were collected with 2–3 mM peptide in 5 mM phosphate buffer (pH 4.0 or 8) with 25 mM NaCl.
| peptide | \( \text{Thr}_N \), ppm | \( \text{Ser}_N \), ppm | others | pH, temp |
|---------|----------------|----------------|--------|---------|
| Ac-YVRTPPKSPSS-NH\(_2\) | 118.4 | 115.6,118.9, 121.7 | 118.9 (Lys), 4.0, 298 K |
| Ac-YVRT(OPO\(_3^-\))PPKSP(OPO\(_3^-\))PS(OPO\(_3^-\))S-NH\(_2\) | 122.4 | 115.9 (pS), 118.2 (pS), 118.3 (S) | 118.0 (Lys), 4.0, 298 K |
| Ac-YVRT(OPO\(_3^2^-\))PPKSP(OPO\(_3^2^-\))PS(OPO\(_3^2^-\))S-NH\(_2\) | 124.0 | 122.2 (pS) | 122.3 (Lys), 8.0, 298 K |
| | | | 124.8 (Val), 125.3 (Arg), 126.6 (Tyr), 108.9 (carboxamide) | |

*Table S16.* Summary of \(^1\text{H}-^{15}\text{N}\) HSQC NMR data for peptides derived from tau\(_{229-238}\). Data were collected with 2–3 mM peptide in 5 mM phosphate buffer (pH 4.0 or 8) with 25 mM NaCl. pS indicates a phosphorylated serine residue.
**Figure S51.** $^1$H-$^{13}$C HSQC spectrum of peptide tau$_{229-238}$ at pH 4.0 at 298 K in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K.
Figure S52. $^1$H-$^{13}$C HSQC spectrum of peptide tau$_{229-238}$($\text{OPO}_3^{2-}$) at pH 8.0 at 298 K in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K.
**Figure S53.** $^1$H-$^{13}$C HSQC spectra of peptides tau$_{229-238}$ at pH 4.0 (green) and tau$_{229-238}$(OPO$_3^{2-}$) (red) (pH 8.0) at 298 K. Data were collected with 2 mM peptide in 5 mM phosphate buffer containing 25 mM NaCl.
| Ac-YVRTPPKSPSS-NH₂ | Ac-YVRpTPPKpSPpSS-NH₂ |
|---------------------|------------------------|
| residue            | $^{13}$C δ, ppm        | residue            | $^{13}$C δ, ppm        |
| Lys, α             | 55.4                   | Lys, α             | 55.6                   |
| Thr, α             | 57.0                   | pThr, α            | 57.7                   |
| Ser, α             | 53.6, 53.6             | pSer, α            | 53.1, a n.d.           |
| Ser, α             | 53.3                   | Ser, α             | 55.3                   |
| Val, α             | 59.1                   | Val, α             | 59.0                   |
| Pro, α             | 60.1, 60.8, 60.8       | Pro, α             | 60.1, 60.8, 60.8       |
| Tyr, α             | 59.0                   | Tyr, α             | 59.0                   |
| Arg, α             | 55.4                   | Arg, α             | 53.2                   |
| Ser, β             | 60.7                   | pSer, β            | 62.5, 63.2             |
| Ser, β             | 61.0, 61.0             | Ser, β             | 61.0                   |
| Thr, β             | 66.9                   | pThr, β            | 70.4                   |
| Val, β             | 29.4                   | Val, β             | 29.4                   |
| Tyr, β             | 36.2                   | Tyr, β             | 36.2                   |
| Lys, β             | 24.5                   | Lys, β             | 24.6                   |
| Arg, β             | 24.5                   | Arg, β             | 24.6                   |
| Pro, β             | 24.6, 24.6, 24.6       | Pro, β             | 24.6, 24.6, 24.6       |
| Pro, δ             | 47.8, 48.2, 48.2       | Pro, δ             | 48.1, 48.1, 48.5       |
| Lys, δ             | 26.2                   | Lys, δ             | 26.2                   |
| Arg, δ             | 30.4                   | Arg, δ             | 30.5                   |
| Lys, ε             | 39.2                   | Lys, ε             | 39.2                   |
| Arg, ε             | 40.6                   | Arg, ε             | 40.5                   |
| Pro, γ             | 28.1, 29.4, 29.4       | Pro, γ             | 28.2, 29.5, 29.5       |
| Lys, γ             | 22.0                   | Lys, γ             | 21.6                   |
| Arg, γ             | 24.5                   | Arg, γ             | 24.1                   |
| Val, γ             | 17.8, 18.3             | Val, γ             | 17.7, 18.3             |
| Thr, γ             | 18.8                   | pThr, γ            | 18.0                   |
| Ac                 | 21.5                   | Ac                 | 21.6                   |

Table S17. Summary of $^1$H-$^{13}$C NMR data for peptides derived from tau$_{229-238}$. Data were collected with 2 mM peptide in 5 mM phosphate buffer containing 25 mM NaCl. pSer or pThr indicates a phosphorylated serine or threonine residue. a n.d. = not determined due to spectral overlap with residual water peak.
Analysis of peptides derived from tau\textsubscript{211–238}

Ac-RTPSLPTPPTREPKKVAVVRTPPKSPSS-NH\textsubscript{2}

**Figure S54.** \textsuperscript{1}H NMR spectra (amide region) of peptides derived from tau\textsubscript{211–238}. Minor peaks in the NMR spectra are due to the presence of cis amide bonds. Peptides were dissolved in 5 mM phosphate buffer (pH 6.0, 7.0, 7.6, or 7.9) and were internally referenced with TSP. Solutions contained 25 mM NaCl, 100 µM TSP, and 90% H\textsubscript{2}O/10% D\textsubscript{2}O. pS or pT indicates a phosphorylated serine or threonine residue.
Full $^1$H NMR spectra of peptides derived from tau$_{211-238}$ at 298 K

tau$_{211-238}$
298 K, pH 6

ptau$_{211-238}$
pH 7.0, 298 K

ptau$_{211-238}$
298 K, pH 7.6

ptau$_{211-238}$
298 K, pH 7.9
Figure S55. TOCSY spectra (amide region) of peptides tau$_{211-238}$ (green) at pH 6.0 and tau$_{211-238}$(OPO$_3$$^{2-}$) (red) at pH 7.9 at 298 K. Minor peaks in the spectrum are due to the presence of cis amide bonds.
| Peptide | $\delta$, H$^N$ | $^3J_{\alpha N}$ | $\delta$, H$_\alpha$ | $\delta$, H$_\beta$ |
|---------|----------------|-----------------|-----------------|-----------------|
| **Tau$_{211-238}$ (pH 6)** | | | | |
| E       | 8.57           | n.d.            | 4.66            | 1.65, 1.55      |
| K       | 8.56           | n.d.            | 4.30            | 1.80, 1.68      |
| K       | 8.56           | n.d.            | 4.30            | 1.80, 1.68      |
| K       | 8.56           | n.d.            | 4.30            | 1.80, 1.68      |
| S       | 8.45           | n.d.            | 4.58            | 3.88, 3.88      |
| R       | 8.44           | n.d.            | 4.42            | 1.82, 1.73      |
| R       | 8.44           | n.d.            | 4.42            | 1.82, 1.73      |
| S       | 8.42           | n.d.            | 4.47            | 3.87, 3.87      |
| T       | 8.40           | n.d.            | 4.58            | 4.34            |
| R       | 8.39           | n.d.            | 4.42            | 1.82, 1.73      |
| V       | 8.35           | n.d.            | 4.12            | 2.04            |
| T       | 8.33           | n.d.            | 4.56            | 4.42            |
| S       | 8.31           | n.d.            | 4.43            | 3.88, 3.88      |
| T       | 8.30           | n.d.            | 4.56            | 4.42            |
| A       | 8.30           | n.d.            | 4.52            | 1.25            |
| S       | 8.28           | n.d.            | 4.45            | 3.90, 3.93      |
| V       | 8.22           | n.d.            | 4.11            | 2.05            |
| V       | 8.20           | n.d.            | 4.09            | 2.02            |
| T       | 8.20           | n.d.            | 4.25            | 4.14            |
| L       | 8.14           | n.d.            | 4.38            | 2.01            |
| **Tau$_{211-238}$ (OPO$_3^{2-}$) (pH 7.9)** | | | | |
| pT      | 9.76           | 3.1$^b$         | 4.38            | 4.31            |
| pT      | 9.59           | 3.7$^b$         | 4.39            | 4.29            |
| pS      | 9.22           | 5.3             | 4.47            | 4.08, 4.13      |
| pT      | 9.21           | 4.7$^b$         | 4.47            | 4.35            |
| pS      | 9.15           | 5.5             | 4.43            | 3.99, 4.10      |
| pS      | 9.07           | 5.3             | 4.75            | 3.98, 4.08      |
| K       | 8.43           | n.d.            | 4.32            | 1.79, 1.72      |
| K       | 8.43           | n.d.            | 4.32            | 1.79, 1.72      |
| K       | 8.43           | n.d.            | 4.32            | 1.79, 1.72      |
| R       | 8.36           | n.d.            | 4.41            | 1.89, 1.77      |
| R       | 8.36           | n.d.            | 4.41            | 1.89, 1.77      |
| R       | 8.36           | n.d.            | 4.41            | 1.89, 1.77      |
| T       | 8.26           | n.d.            | 4.57            | 4.35            |
| S       | 8.25           | n.d.            | 4.35            | 3.94, 3.88      |
| V       | 8.19           | n.d.            | 4.07            | 2.05            |
| V       | 8.19           | n.d.            | 4.07            | 2.03            |
| L       | 8.18           | n.d.            | 4.36            | 1.66            |
| V       | 8.14           | n.d.            | 4.06            | 2.04            |
| A       | n.d.           | n.d.            | 4.13            | 0.94            |
| E       | n.d.           | n.d.            | n.d.            | n.d.            |

*Table S18*. Summary of $^1$H NMR data for peptides derived from tau$_{211-238}$. Data were collected with 200-500 µM peptide in 5 mM phosphate buffer containing 25 mM NaCl. pS or pT indicates a phosphorylated serine or threonine residue. $^a$ n.d. indicates not determined due to spectral overlap or peak broadening. $^b$ Data were obtained at pH 7.0 at 298 K.
Analysis of peptides derived from tau_{234-251}

Ac-KSPSSAKSRLQTAPVPMP-NH₂, Ac-KpSPSSAKSRLQTAPVPMP-NH₂, and Ac-KpSPpSSAKSRLQTAPVPMP-NH₂,

Figure S56. ^1^H NMR spectra (amide region) of peptides derived from tau_{234-251}. Minor peaks in the NMR spectra are due to the presence of cis amide bonds. Peptides were dissolved in 5 mM phosphate buffer (pH 4.0, 7.2, or 7.9) and were internally referenced with TSP. Solutions contained 25 mM NaCl, 100 μM TSP, and 90% H₂O/10% D₂O. pS indicates a phosphorylated serine residue.
Full $^1$H NMR spectra of peptides derived from tau$_{234-251}$ at pH 6.5, 7.4, or 7.9

Ac-KSPSSAKSRLQTAPVPMP-NH$_2$
298 K, pH 6.5

Ac-KS(OPO$_3^-$)PSSAKSRLQTAPVPMP-NH$_2$
298 K, pH 7.4

Ac-KS(OPO$_3^-$)PS(OPO$_3^-$)SAKSRLQTAPVPMP-NH$_2$
298 K, pH 7.9
| peptide               | δ, H<sup>1</sup>N | δ, Hα | δ, Hβ          |
|----------------------|-------------------|-------|----------------|
| **tau<sub>234–251</sub> (pH 6)** |                   |       |                |
| K                    | 8.33              | n.d.  | 1.82, 1.72     |
| S                    | 8.48              | n.d.  | 3.89, 3.89     |
| S                    | 8.52              | n.d.  | 3.86, 3.94     |
| S                    | 8.26              | n.d.  | 3.89, 3.89     |
| A                    | 8.23              | n.d.  | 1.63           |
| K                    | 8.27              | n.d.  | 1.89, 1.78     |
| S                    | 8.46              | n.d.  | 3.94, 3.89     |
| R                    | 8.39              | n.d.  | 1.90, 1.79     |
| L                    | 8.26              | n.d.  | 2.10           |
| Q                    | 8.43              | n.d.  | 2.13, 2.02     |
| T                    | 8.17              | 7.7   | 4.20           |
| A                    | 8.23              | n.d.  | 1.63           |
| V                    | 7.84              | n.d.  | 1.98           |
| M                    | 8.46              | n.d.  | 2.11, 1.97     |
| **tau<sub>234–251(pS235)</sub> (pH 7.4)** |                   |       |                |
| K                    | 8.30              | n.d.  | 1.88, 1.81     |
| pS                   | 8.80              | n.d.  | 4.16, 4.05     |
| S                    | 8.55              | n.d.  | 3.99, 3.91     |
| S                    | n.d               | n.d.  | n.d            |
| A                    | 8.23              | n.d.  | 1.64           |
| K                    | 8.22              | n.d.  | 1.88, 1.81     |
| S                    | 8.19              | n.d.  | 3.91, 3.91     |
| R                    | 8.31              | n.d.  | 1.90, 1.80     |
| L                    | 8.25              | n.d.  | 2.08           |
| Q                    | 8.41              | n.d.  | 2.14, 2.01     |
| T                    | 8.16              | n.d.  | 4.19           |
| A                    | 8.23              | n.d.  | 1.64           |
| V                    | 7.84              | n.d.  | 1.97           |
| M                    | 8.47              | n.d.  | 2.10, 1.97     |
| **tau<sub>234–251(pS235/pS237)</sub> (pH 7.9)** |                   |       |                |
| K                    | 8.10              | n.d.  | 1.90, 1.83     |
| pS                   | 9.34              | n.d.  | 4.10, 4.10     |
| S                    | 8.39              | n.d.  | 3.94, 3.94     |
| pS                   | 9.15              | n.d.  | 4.10, 4.00     |
| A                    | 8.23              | n.d.  | 1.47           |
| K                    | 8.24              | n.d.  | 1.80, 1.70     |
| S                    | 8.19              | n.d.  | 3.95, 3.89     |
| R                    | 8.24              | n.d.  | 1.91, 1.79     |
| L                    | 8.26              | n.d.  | 2.08           |
| Q                    | 8.37              | n.d.  | 2.14, 2.02     |
| T                    | 8.14              | n.d.  | 4.21           |
| A                    | 8.23              | n.d.  | 1.47           |
| V                    | 7.84              | n.d.  | 0.92           |
| M                    | 8.46              | n.d.  | 2.11, 1.99     |

*Table S19.* Summary of <sup>1</sup>H NMR data for peptides derived from tau<sub>234–251</sub>. Data were collected with 2 mM peptide in 5 mM phosphate buffer containing 25 mM NaCl. pS indicates a phosphorylated serine residue. <sup>a</sup>n.d. indicates not determined due to spectral overlap.
Figure S57. $^1$H-$^1$C HSQC spectrum of peptide tau$_{234-251}$ at pH 4.0 at 298 K in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K.
Figure S58. $^1$H-$^{13}$C HSQC spectrum of peptide tau$_{234-251}$ (pS235) at pH 8.0 at 298 K in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K. pS indicates a phosphorylated serine residue.
Figure S59. $^1$H-$^{13}$C HSQC spectrum of peptide tau$_{234-251}$(pS235/pS237) at pH 8.0 at 298 K in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K. pS indicates a phosphorylated serine residue.
Figure S60. $^1$H-$^{13}$C HSQC spectra of peptides tau$_{229-238}$ at pH 4.0 (green), tau$_{234-251}$ (pS235) at pH 8.0 (magenta), and tau$_{234-251}$ (pS235/pS237) (red) at pH 8.0. Data were collected at 298 K with 2 mM peptide in 5 mM phosphate buffer containing 25 mM NaCl.
| KSPSSAKSRLQTAPVPMP | KpSPSSAKSRLQTAPVPMP | KpSPpSSAKSRLQTAPVPMP |
|---------------------|---------------------|---------------------|
| residue             | $^{13}$C δ, ppm     | residue             | $^{13}$C δ, ppm     | $^{13}$C δ, ppm     |
| Lys, α              | 53.5, 53.4          | Lys, α              | 53.8, 53.4          | Lys, α              | 53.7, 53.4 |
| 60.7,               |                     |                     | 60.4,               |                     | 60.8,         |
| Ser, α              | 60.3, 60.1          | Ser, α              | 60.2, 60.1          | Ser, α              | 60.1,         |
|                     | 60.1,               |                     |                     |                     |               |
| Ser, α              | 60.1, 57.0, 55.6,   | pSer, α             | 60.5, 56.9, 56.1,   | pSer, α             | 60.3, 57.0,   |
|                     | 55.6,               |                     | 55.8, 55.5,         |                     | 55.7,         |
| Pro, α              | 55.6,               | Pro, α              | 55.6,               | Pro, α              | 55.7,         |
| Ala, α              | 47.7,               | Ala, α              | 47.7,               | Ala, α              | 47.7,         |
| Arg, α              | 52.4,               | Arg, α              | 52.4,               | Arg, α              | 52.4,         |
| Leu, α              | 53.0,               | Leu, α              | 52.9,               | Leu, α              | 53.0,         |
| Gln, α              | 53.6,               | Gln, α              | n.d.,               | Gln, α              | 52.4,         |
| Thr, α              | 58.8,               | Thr, α              | 58.9,               | Thr, α              | 58.8,         |
| Val, α              | 60.2,               | Val, α              | 60.2,               | Val, α              | 60.3,         |
| Met, α              | 50.0,               | Met, α              | 50.4,               | Met, α              | 50.3,         |
| Lys, β              | 26.8, 60.9, 60.9,   | Lys, β              | 26.8, 60.9, 60.9,   | Lys, β              | 26.8,         |
|                     | 60.9,               |                     | 60.9,               |                     | 60.9,         |
| Ser, β              | 60.9,               | Ser, β              | 60.1,               | Ser, β              | 62.3,         |
| Ser, β              | 60.9,               | pSer, β             | 62.2,               | pSer, β             | 61.7,         |
|                     | 24.6,               |                     | 24.6,               |                     | 24.6,         |
|                     | 24.6,               |                     | 24.6,               |                     | 24.6,         |
| Pro, β              | 24.6,               | Pro, β              | 24.6,               | Pro, β              | 24.6,         |
|                     | 24.6,               |                     | 24.6,               |                     | 24.6,         |
|                     | 16.3,               |                     | 16.3,               |                     | 16.3,         |
| Ala, β              | 15.4,               | Ala, β              | 15.4,               | Ala, β              | 15.4,         |
| Arg, β              | 29.6,               | Arg, β              | 29.6,               | Arg, β              | 29.6,         |
| Leu, β              | 39.5,               | Leu, β              | 39.5,               | Leu, β              | 39.5,         |
| Gln, β              | 29.8,               | Gln, β              | 29.8,               | Gln, β              | 29.8,         |
| Thr, β              | 67.0,               | Thr, β              | 67.0,               | Thr, β              | 67.0,         |
| Val, β              | 29.4,               | Val, β              | 29.4,               | Val, β              | 29.4,         |
| Met, β              | 29.3,               | Met, β              | 29.3,               | Met, β              | 29.3,         |
| Lys, γ              | 22.0,               | Lys, γ              | 22.0,               | Lys, γ              | 22.0,         |
| Pro, γ              | 29.2,               | Pro, γ              | 29.2,               | Pro, γ              | 29.2,         |
| Arg, γ              | 24.3,               | Arg, γ              | 24.3,               | Arg, γ              | 24.3,         |
| Leu, γ              | 22.0,               | Leu, γ              | 22.0,               | Leu, γ              | 22.0,         |
| Gln, γ              | 31.0,               | Gln, γ              | 31.0,               | Gln, γ              | 31.0,         |
| Thr, γ              | 18.8,               | Thr, γ              | 18.8,               | Thr, γ              | 18.8,         |
| Val, γ              | 20.6,               | Val, γ              | 20.6,               | Val, γ              | 20.6,         |
|        |        |        |
|--------|--------|--------|
|        | 47.7,  | 47.7,  |
|        | 47.7,  | 47.7,  |
| Pro, δ | 48.8,  | Pro, δ |
|        | 48.8   | 48.8,  |
|        |        | 48.8   |
| Leu, δ | 17.5   | Leu, δ |
|        |        | 17.5   |
| Met, δ | 18.2   | Met, δ |
|        |        | 18.2   |
| Arg, δ | 29.3   | Arg, δ |
|        |        | 29.3   |
| Arg, ε | 40.5   | Arg, ε |
|        |        | 40.5   |
| Lys, ε | 39.1   | Lys, ε |
|        |        | 39.1   |
| NHAc   | 14.2   | NHAc   |
|        |        | 14.2   |

**Table S20.** Summary of $^1$H-$^{13}$C NMR data for peptides derived from tau_{234-251}. Data were collected with 2 mM peptide in 5 mM phosphate buffer containing 25 mM NaCl. pSer indicates a phosphorylated serine residue. *"n.d." indicates not determined due to spectral overlap with residual water peak.
Figure S61. $^1$H NMR spectra (amide region) of Ac-KTxPP-NH$_2$ peptides at 298 K. Minor peaks in the NMR spectra are due to the presence of cis amide bonds. Peptides were dissolved in 5 mM phosphate buffer (pH 4.0, 6.5, or 8.0) and were internally referenced with TSP. Solutions contained 25 mM NaCl, 100 µM TSP, and 90% H$_2$O/10% D$_2$O.
pH-dependent NMR spectra of peptide Ac-KpTPP-NH$_2$

Figure S62. $^1$H NMR spectra (amide region) of Ac-KpTPP-NH$_2$ peptides. Minor peaks in the NMR spectra are due to the presence of cis amide bonds. Peptides were dissolved in 5 mM phosphate buffer (pH 4.0, 6.5, or 8.0) and were internally referenced with TSP. Solutions contained 25 mM NaCl, 100 µM TSP, and 90% H$_2$O/10% D$_2$O.
Full $^1$H NMR spectra of Ac-KTxPP-NH$_2$ peptides

Ac-KTPP-NH$_2$
298 K, pH 6.5

Ac-KT(OPO$_3^{2-}$)PP-NH$_2$
298 K, pH 6.5

Ac-KT(OPO$_3^{2-}$)PP-NH$_2$
298 K, pH 8
Ac-KT(OPO$_3^{2-}$)PP-NH$_2$
278 K, pH 6.5

Ac-KT(OPO$_3^{2-}$)PP-NH$_2$
278 K, pH 8

Ac-KT(OGlcNAc)PP-NH$_2$
298 K, pH 6.5

Ac-KT(OPO$_3^{2-}$Et$_2$)PP-NH$_2$
298 K, pH 6.5
Due to the unusually slow exchange of the amide protons at pH 8.0 for the peptide Ac-KT(OPO$_3^{-}$)PP-NH$_2$, $^1$H NMR experiments were conducted with increased salt (NaCl) concentrations to identify whether lysine electrostatic interactions were the basis of slow exchange. A very modest upfield shift of the Thr and Lys amide proton was observed with increased NaCl. The rest of the protons throughout the peptide remained essentially unperturbed. The peaks appear to be more resolved at higher salt concentrations. Strikingly, the Thr amide proton at 1 M NaCl concentration at pH 8.0 was still observed to be in slow exchange. These observations are consistent with a dynamic hydrogen bond between the phosphate and Thr backbone amide ordering the peptide and reducing backbone amide exchange rates, potentially via n→π* interactions stabilizing structure. These data are not consistent with a lysine-phosphate electrostatic interaction driving the structure. Notably, experiments were conducted in 5 mM phosphate buffer.

Figure S63. Full $^1$H NMR spectra of peptide Ac-KT(OPO$_3^{-}$)PP-NH$_2$ in 5 mM phosphate buffer containing 25 mM, 125 mM, 225 mM, 325 mM, or 1 M NaCl.
Figure S64. $^1$H NMR spectra (amide region) of peptide Ac-KT(OPO$_3^{2-}$)PP-NH$_2$ in 5 mM phosphate buffer containing 25 mM, 125 mM, 225 mM, 325 mM, or 1 M NaCl at pH 8.0.
$^1$H NMR spectra of Ac-KT(OPO$_3^{2-}$)PP-NH$_2$ at higher temperatures

To examine the possibility of hydrogen bonding between the backbone amide and the side-chain phosphate, $^1$H NMR experiments were conducted at elevated temperatures. As expected, with increasing temperature the Thr amides exhibited faster exchange rates, and at 338 K no amide resonances were observed. Temperature-dependent NMR experiments at 323 and 338 K were conducted on an AV 400 MHz instrument equipped with a BBO probe. 1-D spectra were collected with a watergate pulse sequence with 65536 acquisition data points and a relaxation delay of 3 s.

![Figure S65. $^1$H NMR spectra (amide region) of peptide Ac-KT(OPO$_3^{2-}$)PP-NH$_2$ in 5 mM phosphate buffer containing 25 mM NaCl at 278, 298, 308, 323, and 338 K.](image)
Figure S66. Full $^1$H NMR spectra of peptide Ac-KT(OPO$_3^{2-}$)PP-NH$_2$ in 5 mM phosphate buffer containing 25 mM NaCl at 278, 298, 308, 323, and 338 K.
**Figure S67.** $^1$H NMR spectra (H$\alpha$ region) of peptides Ac-KTPP-NH$_2$ and Ac-KT(OPO$_3^{2-}$)PP-NH$_2$ at pH 8.0 in 100% D$_2$O (eliminating HN-H$\alpha$ coupling due to amide H-D exchange) showing the coupling between Thr H$\alpha$ and H$\beta$ protons.

$$T_{H\alpha} \ J_{H\alpha H\beta} = 6.4 \text{ Hz}$$

**Figure S68.** $^1$H NMR spectra of peptides Ac-KT(OGlcNAc)PP-NH$_2$ and Ac-KT(OPO$_3^{2-}$Et$_2$)PP-NH$_2$ at pH 6.5 in 100% D$_2$O showing the coupling between Thr H$\alpha$ and H$\beta$ protons.

$$T_{H\alpha} \ J_{H\alpha H\beta} = 7.4 \text{ Hz}$$
Summary of NMR data for peptides Ac-KT<sub>x</sub>PP-NH<sub>2</sub>

| peptide                  | pH     | δ, H<sup>N</sup> | <sup>3</sup> J<sub>αN</sub> | δ, H<sub>α</sub> | δ, C<sub>α</sub> | δ, C<sub>co</sub> | others        |
|--------------------------|--------|------------------|------------------|----------------|----------------|----------------|---------------|
| Ac-KTPP-NH<sub>2</sub>   | 4.0    | 2.03             |                  |                |                |                |               |
| Lys                      |        | 8.32             | 6.8              | 4.33           | n.d.           | n.d.           | 2.99, 1.81, 1.71, 1.43 |
| Thr                      |        | 8.28             | 7.3              | 4.58           | n.d.           | n.d.           | 4.11, 1.25    |
| Pro3                     |        | n.a.             | n.a.             | 4.38           | n.d.           | n.d.           | 3.82, 3.67, 2.32, 2.30, 2.05, 1.93 |
| Pro4                     |        | n.a.<sup>a</sup> | n.a.             | n.d.<sup>b</sup> | n.d.           | n.d.           | 3.91, 3.70, 2.38, 2.07, 2.02, 1.92 |
| Ac-                      |        |                  |                  |                |                |                | 2.03          |

| Ac-KTPP-NH<sub>2</sub>   | 6.5    | 298 K            |                  |                |                |                |               |
| Lys                      |        | 8.34             | 6.8              | 4.34           | 53.5           | 174.2          | 3.00, 1.82, 1.73, 1.44 |
| Thr                      |        | 8.30             | 7.2              | 4.59           | 57.1           | 169.5          | 4.12, 1.28    |
| Pro3                     |        | n.a.             | n.a.             | 4.40           | 60.2           | 176.7          | 3.84, 3.69, 2.31, 2.07, 1.95 |
| Pro4                     |        | n.a.             | n.a.             | 4.72           | 58.9           | n.d. 3.92, 3.71, 2.39, 2.09, 2.03, 1.91 |
| Ac-                      |        |                  |                  |                |                |                | 2.05          |

| Ac-KTPP-NH<sub>2</sub>   | 6.5    | 278 K            |                  |                |                |                |               |
| Lys                      |        | 8.49             | 6.9              | 4.33           | n.d.           | n.d.           | 3.00, 1.82, 1.72, 1.45 |
| Thr                      |        | 8.48             | 6.8              | 4.58           | n.d.           | n.d.           | 4.11, 1.28    |
| Pro3                     |        | n.a.             | n.a.             | 4.38           | n.d.           | n.d.           | 3.85, 3.69, 2.35, 2.32, 2.06, 1.96 |
| Pro4                     |        | n.a.             | n.a.             | 4.74           | n.d.           | n.d.           | 3.95, 3.71, 2.40, 2.09, 1.93 |
| Ac-                      |        |                  |                  |                |                |                | 2.04          |

| peptide                  | pH     | δ, H<sup>N</sup> | <sup>3</sup> J<sub>αN</sub> | δ, H<sub>α</sub> | others        |
|--------------------------|--------|------------------|------------------|----------------|---------------|
| Ac-KT(OGlcNAc)PP-NH<sub>2</sub> | 6.5    | 298 K            |                  |                |               |
| Lys                      |        | 8.34             | 7.0              | 4.31           | 3.01, 1.83, 1.73, 1.45 |
| Thr                      |        | 8.01             | 6.4              | 4.59           | 4.13, 1.26    |
| Pro3                     |        | n.a.             | n.a.             | 4.40           | 3.82, 3.68, 2.32, 2.06, 1.95 |
| Pro4                     |        | n.a.             | n.a.             | 4.72           | 3.95, 3.69, 2.39, 2.08, 2.04, 1.94 |
| Ac-                      |        |                  |                  |                | 2.03          |

| Ac-KT(OPO<sub>3</sub>Et<sub>2</sub>)PP-NH<sub>2</sub> | pH 4.0 | 298 K | δ, H<sup>N</sup> | <sup>3</sup> J<sub>αN</sub> | δ, H<sub>α</sub> | others        |
|-----------------------------------------------------|--------|-------|------------------|------------------|----------------|---------------|
| Lys                                                  |        | 8.30  | 7.0              | 4.32             | 3.16, 1.80, 1.72, 1.36 |
| Thr                                                  |        | 8.47  | 8.4              | 4.88             | 4.67, 1.45     |
| Pro3                                                 |        | n.a.  | n.a.             | 4.40             | 3.84, 3.69, 2.32, 2.07, 1.96 |
| Pro4                                                 |        | n.a.<sup>a</sup> | n.a.             | 4.64             | 3.86, 3.74, 2.39, 2.10, 2.04, 1.96 |
| Ac-                                                  |        |       |                  |                  | 2.04          |

<sup>a</sup>n.a. = not applicable. <sup>b</sup>n.d. = not determined. <sup>c</sup>Data obtained at pH 8.0.

Table S21. Summary of <sup>1</sup>H NMR data for peptides Ac-KT<sub>x</sub>PP-NH<sub>2</sub>.

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| peptide            | $\delta$, H$^1$ | $^3$J$\alpha$N | $\delta$, H$\alpha$ | $\delta$, C$\alpha$ | $\delta$, C$\text{co}$ | others          |
|-------------------|----------------|----------------|----------------------|---------------------|----------------------|----------------|
| **Ac-KT(OPO$_3^-$)PP-NH$_2$ pH 4.0 298 K** |               |                |                      |                     |                      |                 |
| Lys               | 8.31           | 7.8            | 4.31                 | n.d.                | n.d.                 | 3.00, 1.83, 1.74, 1.68, 1.45 |
| Thr               | 8.46           | 6.8            | 4.63                 | n.d.                | n.d.                 | 4.45, 1.37       |
| Pro3              | n.a.           | n.a.           | 4.41                 | n.d.                | n.d.                 | 3.82, 3.67, 2.31, 2.05, 1.95 |
| Pro4              | n.a.           | n.a.           | n.d.                 | n.d.                | n.d.                 | 3.99, 3.73, 2.38, 2.10, 2.02, 1.90 |
| Ac-               |                |                |                      |                     |                      | 2.04            |
| **Ac-KT(OPO$_3^{-2-}$)PP-NH$_2$ pH 6.5 298 K** |               |                |                      |                     |                      |                 |
| Lys               | 8.27           | 7.0            | 4.32                 | n.d.                | n.d.                 | 3.03, 1.82, 1.75, 1.53 |
| Thr               | 9.39           | 3.1            | 4.44                 | n.d.                | n.d.                 | 4.31, 1.37       |
| Pro3              | n.a.           | n.a.           | 4.41                 | n.d.                | n.d.                 | 3.85, 3.68, 2.32, 2.06, 1.95 |
| Pro4              | n.a.           | n.a.           | n.d.                 | n.d.                | n.d.                 | 4.08, 3.73, 2.41, 2.11, 2.04, 1.93 |
| Ac-               |                |                |                      |                     |                      | 2.05            |
| **Ac-KT(OPO$_3^{-2-}$)PP-NH$_2$ pH 8.0 298 K** |               |                |                      |                     |                      |                 |
| Lys               | 8.24           | 6.8            | 4.32                 | 53.6                | 173.8                | 3.03, 1.81, 1.75, 1.47 |
| Thr               | 9.73           | 3.5            | 4.36                 | 57.1                | 169.7                | 4.26, 1.37       |
| Pro3              | n.a.           | n.a.           | 4.40                 | 60.2                | 176.9                | 3.85, 3.68, 2.32, 2.06, 1.95 |
| Pro4              | n.a.           | n.a.           | 4.70                 | 59.0                | n.d.                 | 4.11, 3.75, 2.40, 2.11, 2.05, 1.93 |
| Ac-               |                |                |                      |                     | 174.3                | 2.05            |
| **Ac-KT(OPO$_3^{-2-}$)PP-NH$_2$ pH 6.5 278 K** |               |                |                      |                     |                      |                 |
| Lys               | 8.41           | 6.9            | 4.14                 | n.d.                | n.d.                 | 2.86, 1.65, 1.58, 1.31 |
| Thr               | 9.57           | 3.1            | 4.27                 | n.d.                | n.d.                 | 4.14, 1.22       |
| Pro3              | n.a.           | n.a.           | 4.42                 | n.d.                | n.d.                 | 3.87, 3.70, 2.34, 2.07, 1.96 |
| Pro4              | n.a.           | n.a.           | 4.74                 | n.d.                | n.d.                 | 4.11, 3.74, 2.42, 2.03, 1.92 |
| Ac-               |                |                |                      |                     |                      | 2.04            |
| **Ac-KT(OPO$_3^{-2-}$)PP-NH$_2$ pH 8.0 278 K** |               |                |                      |                     |                      |                 |
| Lys               | 8.39           | 6.8            | 4.13                 | n.d.                | n.d.                 | 2.86, 1.70, 1.57, 1.31 |
| Thr               | 9.85           | 3.5            | 4.20                 | n.d.                | n.d.                 | 4.09, 1.21       |
| Pro3              | n.a.           | n.a.           | 4.40                 | n.d.                | n.d.                 | 3.85, 3.67, 2.32, 2.06, 1.95 |
| Pro4              | n.a.           | n.a.           | 4.74                 | n.d.                | n.d.                 | 4.12, 3.74, 2.41, 2.11, 2.05, 1.90 |
| Ac-               | n.a.           | n.a.           | n.d.                 | n.d.                |                      | 2.04            |

*Table S22.* Summary of $^1$H NMR data for peptides Ac-KT$_x$PP-NH$_2$. $^a$n.a. = not applicable. $^b$n.d. = not determined due to overlap with water peak.
Table S23. Summary of $^1$H NMR data for peptides Ac-KTxPP-NH$_2$ at 298 K. $^a$NMR at 278 K.

| Peptide          | $\delta$, H$^N$ | $J_{\alpha N}$, $\delta$, H$_{\alpha}$ | $\delta$, H$_{\beta}$ | $\delta$, H$^N$ | $J_{\alpha N}$, $\delta$, H$_{\alpha}$ | $\delta$, Lys$_{\text{others}}$ | pH     |
|------------------|-----------------|------------------------------------------|------------------------|-----------------|------------------------------------------|-----------------------------|--------|
| KTPP             | 8.28            | 7.3                                      | 4.58                   | 4.11            | 8.32                                     | 6.8                          | 4.33   | 2.99, 1.81, 1.71, 1.43, 4.0 |
| KT(OPO$_3^{3-}$)PP | 8.46            | 6.1                                      | 4.63                   | 4.45            | 8.31                                     | 7.0                          | 4.31   | 3.00, 1.83, 1.74, 1.68, 1.45, 4.0 |
| KT(OPO$_3^{2-}$)PP | 9.73            | 3.5                                      | 4.27                   | 4.14            | 8.24                                     | 6.8                          | 4.32   | 3.03, 1.81, 1.75, 1.47, 8.0 |
| KT(OPO$_3^{2-}$)PP$^a$ | 9.85            | 3.5                                      | 4.20                   | 4.09            | 8.39                                     | 6.8                          | 4.13   | 2.86, 1.70, 1.57, 1.31, 8.0 |
| KT(OGlcNAc)PP    | 8.01            | 6.4                                      | 4.59                   | 4.13            | 8.34                                     | 7.0                          | 4.31   | 3.01, 1.83, 1.73, 1.45, 6.5  |
| KT(OPO$_3$Et$_2$)PP | 8.47            | 8.4                                      | 4.88                   | 4.67            | 8.30                                     | 7.0                          | 4.32   | 3.16, 1.80, 1.72, 1.36, 4.0  |

Figure S69. Partial ROESY spectrum of peptide Ac-KT(OPO$_3^{2-}$)PP-NH$_2$ at pH 8.0 in 5 mM phosphate buffer containing 25 mM NaCl at 298 K. These data were used to assign the proline residues via the expected C$_{\alpha}$(i) $\rightarrow$ HN(i+1) ROEs with the C-terminal carboxamide.
Figure S70. $^1$H-$^{15}$N HSQC spectrum of peptide Ac-KTPP-NH$_2$ at pH 6.5 in 5 mM phosphate buffer containing 25 mM NaCl at 298 K.
Figure S71. $^1$H-$_{15}$N HSQC spectrum of peptide Ac-KT(OPO$_3$H$^-$)NH$_2$ at pH 4.0 in 5 mM phosphate buffer containing 25 mM NaCl at 298 K.
Figure S72. $^1$H-$^{15}$N HSQC spectrum of peptide Ac-KT(OPO$_3^{2-}$)NH$_2$ at pH 7.2 in 5 mM phosphate buffer containing 25 mM NaCl at 298 K.
Figure S73. $^1$H-$^{15}$N HSQC spectra of peptides Ac-KTPP-NH$_2$ at pH 6.5 (green), Ac-KT(OPO$_3^{2-}$)PP-NH$_2$ (red), and Ac-KT(OPO$_3$H$^-$)NH$_2$ (magenta) at 298 K. Data were collected with 1-2 mM peptide in 5 mM phosphate buffer (pH 4.0, 6.5, or 7.2) with 25 mM NaCl.

Table S24. Summary of $^1$H-$^{15}$N HSQC NMR data for peptides Ac-KTxPP-NH$_2$ at 298 K.
Figure S74. $^1$H-$^{13}$C HSQC spectrum of peptide Ac-KTPP-NH$_2$ at pH 4.0 in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K.
Figure S75. $^1$H-$^{13}$C HSQC spectrum of peptide Ac-KT(OPO$_3$$^{2-}$)PP-NH$_2$ at pH 8.0 in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K.
Figure S76. $^1$H-$^1$C HSQC spectra of peptides Ac-KT(OPO$_3$$^{2-}$)PP-NH$_2$ (red) at pH 8.0 and Ac-KTPP-NH$_2$ (green) at pH 4.0 in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K.
Figure S77. $^1$H-$^{13}$C HMBC spectra of peptides Ac-KTPP-NH$_2$ (green) and Ac-KT(OPO$_3^{2-}$)PP-NH$_2$ (red) at pH 8.0 in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K. The Pro4 (4) carbonyl carbon chemical shift could not be determined due to spectral overlap of H$_\alpha$ with residual water.
|                  | Ac-KTPP-NH₂ |                  | Ac-KT(OPO₃²⁻)PP-NH₂ |
|------------------|-------------|------------------|-------------------|
| residue          | ¹³C δ, ppm   | residue          | ¹³C δ, ppm        |
| Lys, α           | 53.5        | Lys, α           | 53.1              |
| Thr, α           | 57.1        | pThr, α          | 58.8              |
| Pro3, α          | 58.9        | Pro3, α          | 58.9              |
| Pro4, α          | 60.2        | Pro4, α          | 60.1              |
| Thr, β           | 66.9        | pThr, β          | 69.8              |
| Pro3, δ          | 47.8        | Pro3, δ          | 47.8              |
| Pro4, δ          | 48.4        | Pro4, δ          | 48.7              |
| Lys, ε           | 39.2        | Lys, ε           | 39.2              |
| Pro3, γ          | 28.2        | Pro3, γ          | 28.2              |
| Pro4, γ          | 29.6        | Pro4, γ          | 29.6              |
| Lys, β           | 30.4        | Lys, β           | 30.5              |
| NHAc             | 21.6        | NHAc             | 21.6              |
| Pro3, Pro4, β    | 24.6        | Pro3, Pro4, β    | 24.7              |
| Lys, γ           | 22.1        | Lys, γ           | 21.6              |
| Lys, δ           | 26.3        | Lys, δ           | 25.9              |
| Thr, γ           | 18.7        | pThr, γ          | 18.0              |
| Lys, CO          | 174.2       | Lys, CO          | 173.8             |
| Thr, CO          | 169.5       | pThr, CO         | 169.7             |
| Pro3, CO         | 176.7       | Pro3, CO         | 176.9             |
| Pro4, CO         | n.d.        | Pro4, CO         | n.d.              |
| Ac, CO           | 174.3       | Ac, CO           | 174.3             |

Table S25. Summary of ¹³C NMR data from ¹H-¹³C HSQC and HMBC experiments at 298 K. The spectra were recorded at pH 8.0 in 5 mM phosphate buffer with 25 mM NaCl in 100% D₂O at 298 K. pThr indicates a phosphorylated threonine residue.
| Ac-KTPP-NH₂ residue | ¹³C δ, ppm | Ac-KT(OPO₃²⁻)PP-NH₂ residue | ¹³C δ, ppm |
|---------------------|-----------|-----------------------------|-----------|
| Lys, α              | 53.2      | Lys, α                      | 52.8      |
| Thr, α              | 57.0      | pThr, α                     | 58.5      |
| Pro3, α             | 58.6      | Pro3, α                     | 58.7      |
| Pro4, α             | 59.9      | Pro4, α                     | 59.8      |
| Thr, β              | 66.6      | pThr, β                     | 69.4      |
| Pro3, δ             | 47.6      | Pro3, δ                     | 47.5      |
| Pro4, δ             | 48.1      | Pro4, δ                     | 48.4      |
| Lys, ε              | 38.8      | Lys, ε                      | 38.8      |
| Pro3, γ             | 27.9      | Pro3, γ                     | 28.0      |
| Pro4, γ             | 29.2      | Pro4, γ                     | 29.3      |
| Lys, β              | 30.1      | Lys, β                      | 30.1      |
| Pro3, β             | 21.2      | Pro3, β                     | 21.3      |
| Pro4, β             | 24.4      | Pro4, β                     | 24.5      |
| Lys, γ              | 21.8      | Lys, γ                      | 21.4      |
| Lys, δ              | 25.9      | Lys, δ                      | 25.7      |
| Thr, γ              | 18.4      | pThr, γ                     | 17.7      |

*Table S26.* Summary of ¹³C NMR data from ¹H-¹³C HSQC experiments at 278 K. The spectra were recorded at pH 8.0 in 5 mM phosphate buffer with 25 mM NaCl in 100% D₂O at 278 K. pThr indicates a phosphorylated threonine residue.
$^{31}$P NMR spectra of peptide Ac-KpTPP-NH$_2$

Figure S78. $^{31}$P NMR spectra of peptide Ac-KT(OPO$_3^-$)PP-NH$_2$ at pH 3.0 in 5 mM acetate buffer containing 50 µM phosphate and 250 µM NaCl and at pH 8.0 in 5 mM phosphate buffer containing 25 mM NaCl. The NMR spectra were internally referenced with 85% H$_3$PO$_4$ (0 ppm) using a capillary filled with H$_3$PO$_4$ located in the NMR tube containing the sample.
### Table S27. Summary of $^{31}$P NMR data for peptide Ac-KpTPP-NH$_2$ at pH 3.0 (298 K) and pH 8.0 (277, 298, 310, 323, and 338 K). n.d. = not determined.

| Peptide                  | $\delta$, P$_{\text{trans}}$ | $\delta$, P$_{\text{cis}}$ | $^3J_{\text{PH}, \text{trans}}$ | $^3J_{\text{PH}, \text{cis}}$ | pH | Temp, K |
|--------------------------|-----------------------------|-----------------------------|---------------------------------|-------------------------------|----|---------|
| Ac-KT(OPO$_3$H$^-$)PP-NH$_2$ | 1.37                        | 0.14                        | 8.9                             | 9.1                           | 3.0 | 298     |
| Ac-KT(OPO$_3^{2-}$)PP-NH$_2$ | 4.75                        | n.d.                        | 9.1                             | n.d.                          | 8.0 | 277     |
| Ac-KT(OPO$_3^{2-}$)PP-NH$_2$ | 2.22                        | 1.72                        | 8.7                             | 9.0                           | 8.0 | 298     |
| Ac-KT(OPO$_3^{2-}$)PP-NH$_2$ | 2.21                        | 1.72                        | 8.6                             | 8.8                           | 8.0 | 310     |
| Ac-KT(OPO$_3^{2-}$)PP-NH$_2$ | 2.22                        | 1.72                        | 8.6                             | 8.7                           | 8.0 | 323     |
| Ac-KT(OPO$_3^{2-}$)PP-NH$_2$ | 2.08                        | 1.65                        | 8.5                             | 8.3                           | 8.0 | 338     |

**1H NMR spectra of Ac-KS$_x$PP-NH$_2$ peptides**

**Ac-KSPP-NH$_2$**

298 K, pH 4

**Ac-KS(OPO$_3^-$)PP-NH$_2$**

298 K, pH 3

**Ac-KS(OPO$_3^{2-}$)PP-NH$_2$**

298 K, pH 7.2

**Ac-KS(OGlcNAc)PP-NH$_2$**

298 K, pH 4

**Ac-KS(OPO$_3^{2-}$)PP-NH$_2$**

298 K, pH 4

**Figure S79.** $^1$H NMR spectra (amide region) of peptides Ac-KS$_x$PP-NH$_2$ at 298 K. Minor peaks in the NMR spectra are due to the presence of cis amide bonds. Peptides were dissolved in buffer containing 5 mM phosphate buffer (pH 3.0, 4.0, or 7.2) and were internally referenced with TSP. Solutions contained 25 mM NaCl, 100 µM TSP, and 90% H$_2$O/10% D$_2$O.
pH-dependent NMR of peptide Ac-KpSPP-NH$_2$

Figure S80. $^1$H NMR spectra (amide region) of Ac-KpSPP-NH$_2$. Minor peaks in the NMR spectra are due to the presence of cis amide bonds. Peptides were dissolved in 5 mM phosphate buffer (pH 3.0, 7.2, or 8.0) and were internally referenced with TSP. Solutions contained 25 mM NaCl, 100 μM TSP, and 90% H$_2$O/10% D$_2$O.
Full $^1$H NMR spectra for Ac-KSxPP-NH$_2$ peptides

Ac-KSPP-NH$_2$
298 K, pH 4

Ac-KS(OPO$_3^-$)PP-NH$_2$
298 K, pH 3

Ac-KS(OPO$_3^{2-}$)PP-NH$_2$
298 K, pH 7.2

Ac-KS(OPO$_3^{2-}$)PP-NH$_2$
298 K, pH 8
Ac-KS(OPO$_3$$^{2-}$)PP-NH$_2$
278 K, pH 8

Ac-KS(OGlcNAc)PP-NH$_2$
298 K, pH 4

Ac-KS(OPO$_3$Et$_2$)PP-NH$_2$
298 K, pH 4
Summary of NMR data for peptides Ac-KSxPP-NH$_2$

| Peptide                  | $\delta$, H$_N$ | $^3$J$_{\alpha\alpha}$ | $\delta$, H$_\alpha$ | $\delta$, H$_\beta$ | $\delta$, H$_\gamma$ | $\delta$, H$_\delta$ | pH |
|--------------------------|-----------------|------------------------|----------------------|---------------------|---------------------|---------------------|----|
| KSPP                     | 8.42            | 6.8                    | 4.75                 | 3.89, 3.76          | 8.29                | 7.0                 | 4.32, 2.99, 1.82, 1.72, 1.44 | 4.0 |
| KS(OPO$_3$H$^-$)PP       | 8.54            | 6.5                    | 4.85                 | 4.12, 4.05          | 8.30                | 7.0                 | 4.32 3.00, 1.83, 1.74, 1.43  | 3.0 |
| KS(OPO$_3^{2-}$)PP       | 9.02            | 5.5                    | 4.74                 | 4.07, 3.97          | 8.27                | 7.1                 | 4.35 3.03, 1.85, 1.77, 1.50  | 7.2 |
| KS(OPO$_3^{2-}$)PP       | 9.05            | n.d.                   | 4.73                 | 4.07, 3.97          | 8.27                | 7.1                 | 4.35 3.03, 1.85, 1.77, 1.50  | 8.0 |
| KS(OPO$_3^{2-}$)PP$^a$   | 9.17            | 5.0                    | 4.64                 | 3.96, 3.88          | 8.42                | 6.9                 | 4.24 2.92, 1.75, 1.67, 1.41  | 8.0 |
| KS(OGlcnAC)PP            | 8.43            | 6.4                    | 4.78                 | 4.00, 3.92          | 8.26                | 7.2                 | 4.32 2.99, 1.81, 1.69, 1.43  | 4.0 |
| KS(OPO$_3$Et$_2$)PP      | 8.64            | 7.6                    | 4.99                 | 4.39, 4.19          | 8.30                | 7.1                 | 4.31 2.99, 1.81, 1.72, 1.44  | 4.0 |

Table S28. Summary of $^1$H NMR data for peptides Ac-KSxPP-NH$_2$. The data were collected at 298 K in 5 mM phosphate buffer containing 25 mM NaCl. $^a$ NMR data were collected at 278 K.

| Peptide                  | $\delta$, H$_N$ | $^3$J$_{\alpha\alpha}$ | $\delta$, H$_\alpha$ | $\delta$, H$_\beta$ | $\delta$, H$_\gamma$ | $\delta$, H$_\delta$ | pH |
|--------------------------|-----------------|------------------------|----------------------|---------------------|---------------------|---------------------|----|
| Ac-KSPP-NH$_2$, pH 4.0, 298 K |                  |                        |                      |                     |                     |                     |    |
| Pro3                     | 4.38            |                        |                      |                     |                     |                     |    |
| Pro4                     | n.d.            |                        |                      |                     |                     |                     |    |
| Ac-KS(OPO$_3$H$^-$)PP-NH$_2$, pH 3.0, 298 K |                  |                        |                      |                     |                     |                     |    |
| Pro3                     | 4.39            |                        |                      |                     |                     |                     |    |
| Pro4                     | 4.72            |                        |                      |                     |                     |                     |    |
| Ac-KS(OPO$_3^{2-}$)PP-NH$_2$, pH 7.0, 298 K |                  |                        |                      |                     |                     |                     |    |
| Pro3                     | 4.40            |                        |                      |                     |                     |                     |    |
| Pro4                     | 4.73            |                        |                      |                     |                     |                     |    |
| Ac-KS(OPO$_3^{2-}$)PP-NH$_2$, pH 8.0, 298 K |                  |                        |                      |                     |                     |                     |    |
| Pro3                     | 4.40            |                        |                      |                     |                     |                     |    |
| Pro4                     | 4.73            |                        |                      |                     |                     |                     |    |
| Ac-KS(OPO$_3^{2-}$)PP-NH$_2$, pH 8.0, 278 K |                  |                        |                      |                     |                     |                     |    |
| Pro3                     | 4.40            |                        |                      |                     |                     |                     |    |
| Pro4                     | 4.75            |                        |                      |                     |                     |                     |    |
| Ac-KS(OGlcnAC)PP-NH$_2$, pH 4.0, 298 K |                  |                        |                      |                     |                     |                     |    |
| Pro3                     | 4.37            |                        |                      |                     |                     |                     |    |
| Pro4                     | 4.78            |                        |                      |                     |                     |                     |    |
| Ac-KS(OPO$_3$Et$_2$)PP-NH$_2$, pH 4.0, 298 K |                  |                        |                      |                     |                     |                     |    |
| Pro3                     | 4.37            |                        |                      |                     |                     |                     |    |
| Pro4                     | n.d.$^a$        |                        |                      |                     |                     |                     |    |

Table S29. $^1$H NMR data for proline protons of peptides Ac-KSxPP-NH$_2$. The peptides were dissolved in 5 mM phosphate buffer buffer containing 25 mM NaCl. $^a$ n.d. indicates not determined because the peak is in water peak.
Figure S81. $^1$H-$^1$C HSQC spectrum of peptide Ac-KSPP-NH$_2$ at pH 4.0 in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K.
Figure S82. $^1$H-$^{13}$C HSQC spectrum of peptide Ac-KS(OPO$_3^{2-}$)PP-NH$_2$ at pH 8.0 in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K.
Figure S83. $^1$H-$^{13}$C HSQC spectra of peptides Ac-KS(OPO$_3^{2-}$)PP-NH$_2$ (red) at pH 8.0 and Ac-KSPP-NH$_2$ (green) at pH 4.0 in 5 mM phosphate buffer with 25 mM NaCl in 100% D$_2$O at 298 K.
| Ac-KSPP-NH₂ residue | Ac-KS(OPO₃²⁻)PP-NH₂ residue | ¹³C δ, ppm |
|---------------------|-----------------------------|-----------|
| Lys, α              | Lys, α                      | 53.2      |
| Ser, α              | pSer, α                     | 53.6      |
| Pro3, α             | Pro3, α                      | 60.2      |
| Pro4, α             | Pro4, α                      | 58.9      |
| Ser, β              | pSer, β                      | 61.6      |
| Pro3, δ             | Pro3, δ                      | 47.7      |
| Pro4, δ             | Pro4, δ                      | 48.0      |
| Lys, ε              | Lys, ε                       | 39.1      |
| Pro3, γ             | Pro3, γ                      | 28.0      |
| Pro4, γ             | Pro4, γ                      | 29.5      |
| Lys, β              | Lys, β                       | 26.0      |
| Pro3, β             | Pro3, β                      | 21.6      |
| Pro4, β             | Pro4, β                      | 24.7      |
| Lys, γ              | Lys, γ                       | 21.6      |
| NHAc                | NHAc                         | 23.2      |
| Lys, δ              | Lys, δ                       | 30.6      |

*Table S30.* Summary of ¹³C NMR data from ¹H-¹³C HSQC experiments. The data were collected at pH 4.0 or pH 8.0 in 5 mM phosphate buffer with 25 mM NaCl in D₂O at 298 K. pSer indicates a phosphorylated serine residue.
**Figure S84.** $^1$H NMR spectra (amide region) of peptides Ac-GPPTxPPGY-NH$_2$ at 298 K. The peptide samples were dissolved in 5 mM phosphate buffer with 25 mM NaCl and 100 µM TSP. NMR experiments were conducted at pH 4.0 unless otherwise specified.

**Figure S85.** $^1$H NMR spectrum (amide region) of Ac-GPPT(OPO$_3^{2-}$)PPGY-NH$_2$ in the presence of 2 mM MgCl$_2$ at 298 K. The NMR spectrum is identical to the spectrum obtained in the absence of MgCl$_2$. 
Figure S86. $^1$H NMR spectra (aliphatic region) of Ac-GPPTxPPGY-NH$_2$ peptides at 298 K in D$_2$O. The peptides were dissolved in 5 mM phosphate buffer pH 8.0 with 25 mM NaCl and 100 µM TSP.

The modification on threonine produces structural effects which are evident on the diastereotopic β, γ, and δ protons of proline. In the case of the phosphorylated peptide, the diastereotopic proline δ protons are observed to be more dispersed ($\Delta H_\delta = 0.10$ ppm) compared to the unmodified peptide ($\Delta H_\delta = 0.07$ ppm) and OGlcnAc and diethylphosphoryl (OPO$_3$Et$_2$) peptides ($\Delta H_\delta = 0.06$ ppm). The more sterically demanding diethylphosphorated and O-GlcNAcylated peptides showed less dispersion of the δ protons compared to the phosphorylated peptide. This effect can also be seen on proline H$_\beta$ and H$_\gamma$ protons.
Figure S87. $^1$H NMR spectra (amide region) of peptides Ac-GPPTPPGY-NH$_2$ at pH 6.5 and 8. The peptides were dissolved in 5 mM phosphate buffer (pH 8.0 or 6.5) containing 25 mM NaCl and 100 µM TSP.

As noted previously for all phosphorylated tau peptides as well as the model peptides Ac-KpTPP-NH$_2$ and Ac-KpSPP-NH$_2$, the amide protons of phosphorylated residues were observed to be more downfield and better resolved at pH 8.0 compared to pH 6.5. This downfield shift and better resolved peaks for the amide protons for the phosphorylated residues at pH 8.0 are general for all the peptides analyzed in this study with the lone exception of tau$_{196-209}$, which showed amides in relatively faster exchange (peak broadening and reduced magnitude, as expected for disordered peptides), but nonetheless more downfield than the nonphosphorylated versions.
Figure S88. $^1$H-$^{15}$N HSQC spectrum of peptide Ac-GPPTPPGY-NH$_2$ at pH 4.0 in 5 mM phosphate buffer containing 25 mM NaCl at 298 K.
Figure S89. $^1$H-^{15}N HSQC spectrum of peptide Ac-GPPT(OPO$_3$H$^-$)PPGY-NH$_2$ at pH 4.0 in 5 mM phosphate buffer containing 25 mM NaCl at 298 K.
*Figure S90.* $^1$H-$^{15}$N HSQC spectrum of peptide Ac-GPPT(OPO$_3^{2-}$)PPGY-NH$_2$ at pH 8.0 in 5 mM phosphate buffer containing 25 mM NaCl at 298 K.
**Figure S91.** $^1$H-$^1$N HSQC spectra of peptides Ac-GPPTPPGY-NH$_2$ at pH 4.0 (green), Ac-GPPT(OPO$_3^{2-}$)PPGY-NH$_2$(red) (pH 8.0), and Ac-GPPT(OPO$_2$H$^-$)PPGY-NH$_2$(magenta) (pH 4.0) at 298 K. Data were collected with 1–2 mM peptide in 5 mM phosphate buffer (pH 4.0 or 8.0) with 25 mM NaCl.
| peptide                              | δ, H<sup>N</sup>  | J<sub>αN</sub>, | δ, H<sub>α</sub> | δ, H<sub>β</sub> | δ, H<sup>N</sup>  | Gly | Tyr | Tyr | pH |
|-------------------------------------|-------------------|-----------------|-----------------|-----------------|-------------------|-----|-----|-----|-----|
| GPPTPPGY                            | 8.29              | 7.3             | 4.51            | 4.05            | 8.49              | 8.18| 7.95| 7.3 | 4.0 |
| GPPT(OPO<sub>3</sub>H<sup>-</sup>)PPGY | 8.40              | 6.5             | 4.63            | 4.47            | 8.48              | 8.17| 7.97| 7.5 | 4.0 |
| GPPT(OPO<sub>3</sub><sup>-2</sup>)PPGY | 9.50              | n.d.<sup>a</sup> | 4.42            | 4.30            | 8.46              | 8.20| 7.99| n.d.| 6.5 |
| GPPT(OPO<sub>3</sub><sup>-2</sup>)PPGY | 9.64              | 3.5             | 4.33            | 4.25            | 8.21              | n.d.| 7.99| n.d.| 8.0 |
| GPPT(OPO<sub>3</sub>E<sub>t</sub>2)PPGY | 8.22              | 7.9             | 4.65            | n.d.            | 8.47              | 8.18| 7.97| 7.3 | 6.5 |
| GPPT(OPO<sub>3</sub>E<sub>t</sub>2)PPGY | 8.22              | n.d.            | 4.65            | 4.65            | 8.47              | 8.17| 7.97| n.d.| 7.5 |
| GPPT(OGlcNAc)PPGY                   | 8.01              | n.d.            | 4.51            | 4.07            | 8.45              | 8.18| 7.98| n.d.| 7.5 |
| GPPT(OGlcNAc)PPGY                   | 8.01              | 5.9             | 4.51            | 4.07            | 8.45              | 8.18| 7.98| 7.2 | 6.5 |

Table S31. Summary of 1H NMR data for peptides Ac-GPPT<sub>x</sub>PPGY-NH<sub>2</sub>. Data were collected with 100–200 µM peptide at 298 K in 5 mM phosphate buffer (pH 4.0, 6.5, 7.5, or 8.0) with 25 mM NaCl. <sup>a</sup>n.d. = not determined due to rapid amide exchange.

| peptide                              | Thr<sub>N</sub>, ppm | Gly<sub>N</sub>, ppm | Tyr<sub>N</sub>, ppm | carboxamide, ppm | pH, temp |
|-------------------------------------|-----------------------|----------------------|---------------------|-----------------|----------|
| Ac-GPPTPPGY-NH<sub>2</sub>          | 117.2                 | 109.2                | 114.4               | 120.4           | 108.7    | 4.0, 298 K |
| Ac-GPPT(OPO<sub>3</sub>H<sup>-</sup>)PPGY-NH<sub>2</sub> | 117.3                 | 109.3                | 114.6               | 120.5           | 108.9    | 4.0, 298 K |
| Ac-GPPT(OPO<sub>3</sub><sup>-2</sup>)PPGY-NH<sub>2</sub> | 123.4                 | n.d., 114.7          | n.d.                | n.d.            | 105.5    | 8.0, 298 K |

Table S32. Summary of 1H-15N HSQC NMR data for peptides Ac-GPPT<sub>x</sub>PPGY-NH<sub>2</sub>. Data were collected with 2–3 mM peptide at 298 K in 5 mM phosphate buffer (pH 4.0 or 8.0) with 25 mM NaCl. <sup>a</sup>n.d. = not determined due to rapid amide exchange.
$^1$H NMR spectra of Ac-GPKTxPPGY-NH$_2$ peptides

**Figure S92.** $^1$H NMR spectra (amide region) of peptides Ac-GPKTxPPGY-NH$_2$ at 298 K. The peptide samples were dissolved in 5 mM phosphate buffer buffer pH 4.0 with 25 mM NaCl and 100 µM TSP.
Figure S93. $^1$H NMR spectra (amide region) of peptides Ac-GPKT$_x$PPGY-NH$_2$ at 298 K. The peptide samples were dissolved in 5 mM phosphate buffer with 25 mM NaCl and 100 μM TSP (pH 6.5 unless otherwise indicated).
Figure S94. $^1$H NMR spectra (amide region) of peptides Ac-GPKTxPPGY-NH$_2$ at pH 8.0 (298 K). The peptide samples were dissolved in 5 mM phosphate buffer pH 8.0 with 25 mM NaCl and 100 µM TSP.

All peptides in Ac-GPKTxPPGY-NH$_2$ series were analyzed at pH 8.0 to examine amide exchange. All peptides except the phosphorylated peptide Ac-GPKT(OPO$_3^{2-}$)PPGY-NH$_2$ showed rapid amide exchange with water at pH 8.0.
The effect of threonine side chain modification was propagated through the length of the peptide, as is evident from chemical shift differences in the Hβ, Hγ, and Hδ protons of proline. The phosphorylated peptide notably showed greater dispersion of the diastereotopic Hδ protons than any of the other peptides.
Summary of NMR data for peptides Ac-GPKTxPPGY-NH₂

| peptide                        | δ, H⁴⁻δ, Hα | δ, Hβ, Hγ | δ, H⁴⁻δ, Hα | δ, Lys (others) | δ, H⁴⁻δ, Hα | δ, Hβ, Tyr | δ, H⁴⁻δ, Hα | pH  |
|--------------------------------|-------------|-----------|-------------|----------------|-------------|------------|-------------|-----|
| GPKT(OGlcNAc)PPGY             | 8.14        | 4.52      | 4.05        | 1.20           | 8.52        | 4.37       | 3.00, 1.84, 1.75, 1.68 | 7.94, 4.48 | 3.06, 2.97 | 8.48, 8.19 | 4.0 |
| GPKT(OPO₃H⁺)PPGY              | 8.35        | 4.63      | 4.45        | 1.34           | 8.52        | 4.30 / 3.00 | 1.84, 1.78, 1.69, 1.47 | 7.97, 4.50 | 3.08, 2.95 | 8.46, 8.20 | 3.0 |
| GPKT(OPO₃⁻²)PPGY              | 9.32        | 4.31      | 4.23        | 1.35           | 8.46        | 4.33       | 3.03, 1.84, 1.77, 1.48 | 8.00, 4.51 | 3.09, 2.97 | 8.48, 8.22 | 6.5 |
| GPKT(OPO₃₂⁻)PPGY              | 9.72        | 4.34      | 4.24        | 1.35           | 8.53        | 4.34       | 3.04, 1.83, 1.74, 1.49 | 7.97, 4.39 | 3.08, 2.93 | 8.20, 8.0  | 6.5 |
| GPKT(OPO₂Et₂)PPGY             | 8.28        | 4.59      | n.d.        | 1.39           | 8.47        | 4.34 / 2.99 | 1.82, 1.74, 1.67, 1.43 | 7.95, 4.47 | 3.05, 2.98 | 8.46, 8.17 | 4.0 |
| GPKT(OPO₂Et₂)PPGY             | 8.29        | 4.60      | 4.60        | 1.40           | 8.50        | 4.36       | 3.01, 1.84, 1.75, 1.45 | 7.97, 4.49 | 3.07, 3.00 | 8.48, 8.18 | 6.5 |
| GPKT(OGlcNAc)PPGY             | 7.96        | 4.53      | 4.06        | 1.19           | 8.49        | 4.33       | 3.01, 1.82, 1.75, 1.46 | 7.97, 4.48 | 3.05, 2.97 | 8.43, 8.18 | 4.0 |
| GPKT(OGlcNAc)PPGY             | 7.98        | 4.55      | 4.07        | 1.21           | 8.50        | 4.34       | 3.02, 1.84, 1.76, 1.46 | 7.99, 4.49 | 3.06, 2.98 | 8.45, 8.19 | 6.5 |

Table S33. Summary of ¹H NMR data of peptides Ac-GPKTxPPGY-NH₂ at 298 K. n.d. indicates not determined due to spectral overlap or fast amide exchange.

| peptide                        | δ, H⁴⁻³JαN, | δ, Hα | δ, H⁴⁻δ, Hα | δ, H⁴⁻³JαN, | δ, Hα | δ, H⁴⁻δ, Hα | δ, H⁴⁻³JαN, | δ, Hα | pH  |
|--------------------------------|-------------|-------|-------------|-------------|-------|-------------|-------------|-------|-----|
| Ac-GPKTPPPGY-NH₂                | 8.14        | 7.4   | 4.52        | 4.05        | 8.52 | 7.2         | 4.37        | 7.94, 4.48 | 7.2 | 4.48 | 4.0 |
| Ac-GPKT(OPO₃H⁺)PPGY-NH₂         | 8.35        | 6.7   | 4.63        | 4.45        | 8.52 | 6.8         | 4.30        | 7.97, 4.50 | 7.4 | 4.50 | 3.0 |
| Ac-GPKT(OPO₃⁻²)PPGY-NH₂         | 9.32        | n.d.  | 4.31        | 4.23        | 8.46 | 6.8         | 4.33        | 8.00, 4.51 | 7.4 | 4.51 | 6.5 |
| Ac-GPKT(OPO₃₂⁻)PPGY-NH₂         | 9.72        | 3.5   | 4.34        | 4.24        | 8.43 | n.d.        | 4.34        | 7.97, n.d.  | 4.39 | 8.0  | 6.5 |
| Ac-GPKT(OPO₂Et₂)PPGY-NH₂        | 8.28        | 8.3   | 4.59        | n.d.        | 8.47 | 7.3         | 4.34        | 7.95, 4.47 | 7.2 | 4.47 | 4.0 |
| Ac-GPKT(OPO₂Et₂)PPGY-NH₂        | 8.29        | 8.3   | 4.60        | 4.60        | 8.50 | 7.3         | 4.36        | 7.97, 4.49 | 7.3 | 4.49 | 6.5 |
| Ac-GPKT(OGlcNAc)PPGY-NH₂        | 7.96        | 6.7   | 4.53        | 4.06        | 8.49 | 7.1         | 4.33        | 7.97, 4.48 | 7.2 | 4.48 | 4.0 |
| Ac-GPKT(OGlcNAc)PPGY-NH₂        | 7.98        | 6.5   | 4.55        | 4.07        | 8.50 | 6.8         | 4.34        | 7.99, n.d.  | 4.49 | 6.5  | 6.5 |

Table S34. ¹H NMR data (³JαN values and key proton chemical shifts) for peptides Ac-GPKTxPPGY-NH₂ at 298 K. n.d. = not determined due to spectral overlap or fast amide exchange.
Analysis of steric vs stereoelectronic effects in Ac-TYProxN-NH₂ and Ac-TAProxN-NH₂ peptides

To understand the conformational effects of phosphorylation via model peptides used to quantify steric and stereoelectronic effects of substituents², the peptides Ac-TYProxN-NH₂ were synthesized (Prox = 4-substituted prolines). Peptides were post-synthetically modified via proline editing to introduce 4R-phosphate, 4S-phosphate, 4R-diethylphosphate, and 4S-diethylphosphate on 4-hydroxyproline. To eliminate the effects on structure due to potential interactions with the hydrophobic tyrosine residue, the peptides Ac-TAProxN-NH₂ were also synthesized and post-synthetically modified to generate peptides with modified prolines with 4R-phosphate, 4S-phosphate, 4R-diethylphosphate, and 4S-diethylphosphate substitutions. All peptides thus synthesized were analyzed via ¹H NMR spectroscopy for their conformational effects based upon steric and stereoelectronic effects.² The phosphorylated peptides were also analyzed as a function of pH to interrogate the effects as a function of phosphate protonation state. The data from ref. 2 on Ac-TYProxN-NH₂ peptides and Ac-TAProxN-NH₂ peptides, other than pH-dependent Ac-TAProxN-NH₂ phosphorylated hydroxyprolines, which were not reported, indicate a smaller magnitude of stereoelectronic effect for diethylphosphates than observed in hydroxyprolines or phosphorylated hydroxyprolines. This result is in contrast to the expectation that the hydroxyproline diethylphosphates should be the most electron-withdrawing, and thus have the largest stereoelectronic effects. Indeed, the diethylphosphorylated serine and threonine residues had the most downfield-shift Hβ chemical shifts of all peptides examined, consistent with the expected greater electron-withdrawing effect of the diethylphosphates. These data support the concept that the diethylphosphate functions significantly sterically, with the steric effect at least partially counterbalancing the stereoelectronic effect in these peptides, as was also seen in the larger ³Jα for serine and threonine diethylphosphates (Table 1).

pH-dependent NMR data on Ac-TAProxN-NH₂ phosphorylated hydroxyprolines (below) were consistent with data on Ac-TYProxN-NH₂ phosphorylated hydroxyprolines (ref. 2), indicating that the stereoelectronic effects of phosphorylation, whether as a monoanionic phosphate or a dianionic phosphate, are relatively similar to those of a hydroxy group. Thus, the structural effects observed due to phosphorylation are not likely to be due to a change in the stereoelectronic effect of a hydroxyl group versus a phosphate.

The diastereotopic β-protons of proline (Hβ) are more dispersed in the 4R configuration relative to 4S configuration. A characteristic splitting pattern is observed for the two diastereotopic β-protons (Hβ). A reversal in the splitting pattern based upon the 4R or 4S configuration on the Cγ of proline indicated reversal in ring pucker preferences. Similar observations based upon sterics and stereoelectronics were described recently on a large series of 4-substituted prolines in Ac-TYProxN-NH₂ and Ac-TAProxN-NH₂ peptide context.²
pH-dependent $^1$H NMR analysis for peptides Ac-TAP(4R-OPO$_3$$^{2-}$)N-NH$_2$ and Ac-TAP(4S-OPO$_3$$^{2-}$)N-NH$_2$

Figure S96. pH-dependent $^1$H NMR spectra (amide region) of peptide Ac-TAP(4R-OPO$_3$$^{2-}$)N-NH$_2$. 
Figure S97. pH-dependent $^1$H NMR spectra (aliphatic region) of peptide Ac-TAP(4R-OPO$_3$$\text{O}$$^2$$^-$$)N-NH$_2$. 
Figure S98. pH-dependent $^1$H NMR spectra (amide region) of peptide Ac-TAP(4S-OPO$_3^{2-}$)N-NH$_2$. 
Figure S99. pH-dependent $^1$H NMR spectra (aliphatic region) of peptide Ac-TAP(4S-OPO$_3$$^-$$^-$/2$^-$)N-NH$_2$. 
| X = | $k_{\text{trans/cis}}$ | $\Delta G_{\text{trans/cis}}$ | $\Delta \Delta G_{\text{trans/cis}}$ | $^3J_{\alpha\text{N}}$ | $^3J_{\delta\text{N}}$ | $\delta, \text{H}^\text{N}$ | $\delta, \text{H}^\text{N}$ | pH |
|-----|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----|
| 4R-OPO$_3$H$^-$ | 21.1 | -1.80 | -0.40 | 6.1 | 5.7 | 8.31 | 8.44 | 8.68 | 2.0 |
| 4S-OPO$_3$H$^-$ | 8.2 | -1.25 | 0.15 | 5.0 | 5.6 | 8.44 | 8.51 | 8.18 | 2.0 |
| 4R-OPO$_3$H$^-$ | 18.2 | -1.72 | -0.32 | 5.9 | 5.7 | 8.32 | 8.45 | 8.69 | 4.0 |
| 4S-OPO$_3$H$^-$ | 8.5 | -1.27 | 0.13 | 4.8 | 6.0 | 8.42 | 8.49 | 8.17 | 4.0 |
| 4R-OPO$_3$^{-/2-} | 21.0 | -1.80 | -0.40 | 6.1 | 5.7 | 8.31 | 8.45 | 8.68 | 5.6 |
| 4S-OPO$_3$^{-/2-} | 8.6 | -1.27 | 0.13 | 4.9 | 5.6 | 8.42 | 8.49 | 8.17 | 5.6 |
| 4R-OPO$_3$^{-/2-} | 18.5 | -1.73 | -0.33 | n.a.$^b$ | 4.9 | 8.53 | 8.43 | 8.73 | 7.0 |
| 4S-OPO$_3$^{-/2-} | 8.9 | -1.29 | 0.11 | n.a. | n.a. | 8.37 | 8.47 | 8.19 | 7.0 |
| 4R-OPO$_3$^{-/2-} | n.d.$^a$ | n.d. | n.d. | n.a. | n.a. | n.a. | n.a. | n.a. | 8.8 |
| 4S-OPO$_3$^{-/2-} | 7.5 | -1.19 | 0.21 | n.a. | n.a. | n.a. | n.a. | n.a. | 8.8 |

Table S35. Summary of pH-dependent $^1$H NMR data of peptides Ac-TAP(4$R$-OPO$_3$^{-/2-})N-NH$_2$ and Ac-TAP(4$S$-OPO$_3$^{-/2-})N-NH$_2$. $^a$ n.d. = not determined due to spectral overlap. $^b$ n.a. = not applicable.
### Summary of serine and threonine NMR chemical shift data across all peptides as a function of residue and post-translational modification

| peptide        | ThrOPO⁺ | ThrOPO⁻ | ThrOPO H | SerOPO⁺ | SerOPO⁻ | SerOPO H | Thr     | Ser    | ThrOGlcNAc | SerOGlcNAc | ThrOPO Et | SerOPO Et |
|----------------|---------|---------|----------|---------|---------|----------|---------|-------|------------|------------|-----------|-----------|
| tau33_33     | 9.73    | 9.06    | 8.45     | 9.67    | 8.78    | 8.49     | 8.07    | 8.22  | 7.97       | 8.19       | 8.28      | 8.43      |
| tau33_34     | 9.58    | 9.96    | 8.36     | 9.67    | 8.78    | 8.49     | 8.07    | 8.22  | 7.96       | 8.19       | 8.28      | 8.43      |
| tau33_35     | 9.42    | 9.53    | 8.38     | 9.21    | 9.05    | 8.74     | 8.69    | 8.25  | 8.40       | 8.59       | 8.69      | 8.59      |
| KTPP         | 9.73    | 9.39    | 8.46     | 9.05    | 8.74    | 8.59     | 8.30    | 8.42  | 8.01       | 8.43       | 8.47      | 8.64      |
| mean standard dev | 0.12   | 0.40     | 0.07     | 0.21   | 0.21    | 0.09     | 0.11   | 0.12  | 0.10       | 0.10       | 0.12      | 0.12      |

**Table S36.** Summary of serine and threonine amide NMR chemical shift data for all peptides.

| peptide        | ThrOPO⁺ | ThrOPO⁻ | ThrOPO H | SerOPO⁺ | SerOPO⁻ | SerOPO H | Thr     | Ser    | ThrOGlcNAc | SerOGlcNAc | ThrOPO Et | SerOPO Et |
|----------------|---------|---------|----------|---------|---------|----------|---------|-------|------------|------------|-----------|-----------|
| tau33_33     | 4.39    | 4.55    | 4.67     | 4.53    | 4.99    | 4.63     | 4.59    | 4.58  | 4.45       | 4.70       | 4.59      | 4.91      |
| tau33_34     | 4.37    | 4.51    | 4.64     | 4.53    | 4.99    | 4.63     | 4.59    | 4.58  | 4.45       | 4.70       | 4.59      | 4.91      |
| tau33_35     | 4.41    | 4.68    | 4.63     | 4.42    | 4.53    | 4.42     | 4.55    | 4.43  | 4.57       | 4.67       | 4.67      | 4.67      |
| KTPP         | 4.27    | 4.44    | 4.63     | 4.73    | 4.65    | 4.65     | 4.75    | 4.78  | 4.86       | 4.69       | 4.69      | 4.69      |
| mean standard dev | 0.05   | 0.10     | 0.10     | 0.17   | 0.18    | 0.09     | 0.12   | 0.09  | 0.13       | 0.11       | 0.11      | 0.11      |

**Table S37.** Summary of serine and threonine Hα NMR chemical shift data for all peptides.

| peptide        | ThrOPO⁺ | ThrOPO⁻ | ThrOPO H | SerOPO⁺ | SerOPO⁻ | SerOPO H | Thr     | Ser    | ThrOGlcNAc | SerOGlcNAc | ThrOPO Et | SerOPO Et |
|----------------|---------|---------|----------|---------|---------|----------|---------|-------|------------|------------|-----------|-----------|
| tau33_33     | 4.28    | 4.37    | 4.48     | 4.11    | 4.13    | 4.10     | 4.66    | 4.40  | 4.40       | 4.66       | 4.66      | 4.66      |
| tau33_34     | 4.26    | 4.38    | 4.47     | 4.11    | 4.13    | 4.10     | 4.66    | 4.40  | 4.40       | 4.66       | 4.66      | 4.66      |
| tau33_35     | 4.30    | 4.53    | 4.42     | 4.09    | 4.11    | 4.11     | 4.35    | 4.35  | 4.35       | 4.35       | 4.35      | 4.35      |
| KTPP         | 4.28    | 4.31    | 4.45     | 4.12    | 4.13    | 4.13     | 4.67    | 4.41  | 4.41       | 4.41       | 4.41      | 4.41      |
| mean standard dev | 0.02   | 0.11     | 0.14     | 0.06   | 0.05    | 0.04     | 0.06   | 0.06  | 0.06       | 0.06       | 0.06      | 0.06      |

**Table S38.** Summary of serine and threonine Hβ NMR chemical shift data for all peptides.
### Table S39. Summary of serine and threonine $^{15}$N NMR chemical shift data for all peptides.

| Peptide  | ThrOPQ$_{\text{N}}$ | ThrOPQ$_{\text{C}}$ | ThrOPQ$_{\text{O}}$ | SerOPQ$_{\text{N}}$ | SerOPQ$_{\text{C}}$ | SerOPQ$_{\text{O}}$ | Thr | Ser | ThrGlcNAC |
|----------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|-----|-----|------------|
| Tau$_{14-163}$ | 125.1 | 118.5 | 118.2 | 118.8 |
| Tau$_{14-163}$ | 125.6 | 119.0 | 118.3 | 118.8 |
| Tau$_{156-209}$ | 121.8 | 117.4 | 119.4 | 116.6 | 117.8 | 116.5 |
| Tau$_{211-219}$ | 125.7 | 120.9 | 118.6 |
| Tau$_{220-229}$ | 124.0 | 122.4 | 122.2 | 118.2 | 118.4 | 115.6 |
| Tau$_{220-229}$ | 115.9 | 118.9 | 121.7 |
| KTPP            | 124.8 | 119.4 | 118.3 |
| KSPP            | 123.4 | 117.3 | 117.2 |
| GPPTPPTYG       | 124.3 | 119.3 | 120.8 | 116.9 | 118.1 | 118.2 | 118.8 |
| GPKTTPPGY       | 1.4 | 1.8 | 2.0 | 1.2 | 0.5 | 2.7 | 0.0 |

| Peptide  | $^{13}$C Thr$_{\alpha}$ | $^{13}$C Thr$_{\beta}$ | $^{13}$C Thr$_{\gamma}$ | $^{13}$C Thr$_{\epsilon}$ | $^{13}$C Ser$_{\alpha}$ | $^{13}$C Ser$_{\beta}$ | $^{13}$C Thr$_{\epsilon}$ | $^{13}$C Thr$_{\epsilon}$ | $^{13}$C SadO | $^{13}$C SadO | $^{13}$C SadO | $^{13}$C SadO |
|----------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|-------------|-------------|-------------|-------------|
| Tau$_{14-163}$ | 58.1 | 70.0 | 17.8 | 169.7 | 57.0 | 66.9 | 18.7 | 169.6 |
| Tau$_{14-163}$ | 59.4 | 70.0 | 17.8 | 169.7 | 57.0 | 66.9 | 18.7 | 169.6 |
| Tau$_{156-209}$ | 58.8 | 69.9 | 113.9 | 52.2 | 63.0 | 57.1 | 66.8 | 18.7 | 52.5 | 60.9 |
| Tau$_{211-219}$ | 58.6 | 119.1 | 82.5 | 53.1 | 63.2 | 57.0 | 66.9 | 18.8 | 53.6 | 60.7 |
| Tau$_{220-229}$ | 57.7 | 70.4 | 18.0 | 169.7 | 53.6 | 61.6 | 57.1 | 66.9 | 18.7 | 169.5 | 53.6 | 60.2 |
| KTPP            | 58.8 | 69.6 | 18.0 | 169.7 | 53.6 | 61.6 | 57.1 | 66.9 | 18.7 | 169.5 | 53.6 | 60.2 |
| KSPP            | 58.8 | 70.0 | 17.9 | 169.7 | 53.6 | 61.6 | 57.1 | 66.9 | 18.7 | 169.5 | 53.6 | 60.2 |
| GPPTPPTYG       | 58.4 | 70.0 | 17.9 | 169.7 | 53.0 | 62.6 | 57.1 | 66.9 | 18.7 | 169.6 | 53.3 | 60.7 |
| GPKTTPPGY       | 0.4 | 0.2 | 0.1 | 0.0 | 0.7 | 0.7 | 0.1 | 0.1 | 0.0 | 0.1 | 0.6 | 0.4 |

### Table S40. Summary of serine and threonine $^{13}$C NMR chemical shift data for all peptides.

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