

Published in final edited form as:

Chem Biol Drug Des. 2011 November ; 78(5): 749–756. doi:10.1111/j.1747-0285.2011.01203.x.

Phosphorylation of Enkephalins: NMR and CD Studies in Aqueous and Membrane-Mimicking Environments

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Abstract

Phosphorylation of L-serine-containing enkephalin analogs has been explored as an alternative to glycosylation in an effort to increase blood-brain barrier (BBB) permeability and CNS bioavailability of peptide pharmacophores. Two enkephalin-based peptides were modified for these studies, a set related to DTLES, a mixed μ/δ -agonist, and one related to DAMGO, a highly selective μ -agonist. Each unglycosylated peptide was compared to its phosphate, its mono-benzylphosphate ester, and its β -D-glucoside. Binding was characterized in membrane preparations from CHO cells expressing human μ , δ and κ -opiate receptors (MOR, DOR and KOR). Antinociception was measured in mice using the 55°C tail flick assay. In order to estimate bioavailability, the antinociceptive effect of each opioid agonist was evaluated after intracerebroventricular (*i.c.v.*) or intravenous administration (*i.v.*) of the peptides. Circular dichroism (CD) methods and high field nuclear magnetic resonance (NMR) were used in the presence and absence of sodium dodecylsulfate (SDS) to understand how the presence of a membrane might influence the peptide conformations.

Introduction

Morphine, and its congeners are the most widely prescribed drugs for moderate to severe pain. These “narcotics” produce an array of side effects, including pruritus, miosis, euphoria, sedation, constipation, and respiratory depression. Since their discovery in 1975, much effort has been expended to convert endogenous opioid peptides Met/Leu-enkephalin into analgesics with the hope that they would provide pain relief with reduced side effects. To date, we are aware of only one report of an enkephalin-based peptide being tested as an analgesic in humans. The compound produced potent and fully efficacious analgesia in severe pain states, but required intrathecal administration.¹

Many peptide analogs have been synthesized having greatly improved resistance to enzyme degradation and corresponding increases in plasma stability and serum half-life.^{2,3} The majority of these metabolically stable analogs fail to cross blood-brain barrier (BBB) in appreciable amounts. The BBB is both a physical and metabolic barrier between the CNS

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and the systemic circulation, which serves to regulate and protect the microenvironment of the brain.⁴ The BBB includes tight junctions between the endothelial cells of brain capillaries that behave as a continuous lipid bilayer.⁵

Glycosylation is a post-translational protein modification, which has been shown to increase metabolic stability,⁶ and has been shown to promote BBB penetration of enkephalins to produce CNS activity after peripheral administration in rodents.^{7,8} In some cases these glycosides have been shown to produce antinociceptive effects with greatly reduced side effects compared to traditional small molecules.^{9,10}

Circular dichroism (CD) and nuclear Overhauser studies (NOESY) of these glycosylated enkephalins revealed that they adopt restricted and relatively well-defined conformational ensembles in sodium dodecylsulfate (SDS) micelles, whereas they form predominantly random coil conformations in H₂O.¹¹ In a related study it was shown that glycosylation of the highly MOR selective peptide Tyr-(D)Ala-NMePhe-Gly(OH) (“DAMGO”) improved bioavailability and *in vivo* potency.¹² These MOR selective analogs showed greater potencies than morphine. Theoretically calculated amphipathicities of these glyco-DAMGO analogs correlated with antinociceptive potency (*i.v.* administration), presumably reflecting enhanced transport properties.

In this study we report on phosphorylation as an alternative strategy to enhance peptide stability and BBB penetration. Dass and Mahalaksmi showed that phosphorylated enkephalin was stable to proteolysis.¹³ Further, phosphorylated opioid peptides have been isolated from bovine adrenal medulla, providing further impetus to investigate the effects of phosphorylation on peptide pharmacology.¹⁴ The highly selective MOR agonist DAMGO¹⁵ and the mixed MOR/DOR agonist YtGFL-amide^{16,17} were chosen as the opioid “messages” for this study, which have previously been shown to produce centrally mediated effects upon glycosylation. The chemical structures of the lead peptides and their phosphate derivatives studied are depicted in Figure 1.

Results and Discussion

In vitro binding activity

As shown in Table 1, all four phosphorylated enkephalin derivatives have K_i values of less than 10 nM for binding to the MOR. The phosphate mono-benzyl ester **1b** showed an approximate 2-fold increase in affinity for DOR and KOR compared to phosphate **1a**, while reducing the affinity of the peptide for MOR. Compounds **2a** and **2b** had K_i binding values of less than 5 nM for the MOR. The additional benzyl ester on the phosphate **2b** increased the affinity for DOR ~25-fold relative to **2a**, without affecting the affinity for MOR. These results demonstrate that the addition of a phosphate group to the peptide does not abolish the affinity of either peptide for opioid receptors, and the lipophilic benzyl moiety increased affinity for DOR opioid receptor for both peptide series.

Analgesic activity *in vivo*

The antinociceptive potency of each compound (*i.c.v.* or *i.v.*) is summarized in Table 1. All compounds produced dose-related and time-related antinociceptive effects with full efficacy in the 55°C tail-flick assay following *i.c.v.* administration in mice (Figure 2 and supplemental info). The data indicate that phosphorylation of the mixed δ/μ agonist (DTLES peptide **1a**) and the μ -agonist (DAMGO peptide **2a**) did not alter the antinociceptive activity of the parent peptides significantly. The benzyl esters **1b** and **2b** also showed antinociceptive activity upon *i.c.v.* administration (data not shown, see supplemental info), but not after *i.v.* administration. This result suggests that the lipophilic benzyl esters do not cross the BBB in appreciable amounts. The phosphopeptide data were compared to

glycopeptide data, which revealed that glycosylation of DTLES peptide (glycopeptide **1c**) significantly improved antinociceptive activity upon both *i.c.v.* and *i.v.* administration. However, glycosylation of the DAMGO derivative (glycopeptide **2c**) did not show improved activity compared to the parent peptide. The glycopeptides also showed superior *in vivo* activity compared to the phosphates. There was also a trend for increased duration of action from compound **1** to **1c** and compound **2** to **2c**, respectively.

Circular dichroism analysis

The CD spectra for **1a** and **2a** were recorded in aqueous buffer as well as in the presence of membrane-mimicking SDS micelles (Figure 3). In aqueous buffer the phosphopeptide **1a** displayed two maxima at 220 nm and 205 nm. The CD shape is neither random coil nor corresponding to any particular type of fold. Thus, one can conclude that peptide **1a** probably exists as a conformational ensemble rather than a single conformer in aqueous buffer. A significant change was observed in the CD shape when the spectrum of **1a** was recorded in the presence of SDS micelles; the positive band at 205 nm became a negative band, and the positive maxima at 222 nm shifted to a higher wavelength that reflected an overall conformational change of the molecule. The shape of this CD spectrum is characteristic of a type I/III β -turn conformation as described by Balaram and coworkers.^{18,19} The type I [$\phi_{i+1} = -60^\circ$, $\psi_{i+1} = -30^\circ$, $\phi_{i+2} = -90^\circ$, $\psi_{i+2} \approx 0^\circ$] and type III β -turns [$\phi_{i+1} = -60^\circ$, $\psi_{i+1} = -30^\circ$, $\phi_{i+2} = -60^\circ$, $\psi_{i+2} = -30^\circ$] are closely related and cannot easily be distinguished by CD or NMR. Further, the type III β -turn is essentially a single turn of a 3_{10} helix [$\phi_{i+1} = -60^\circ$, $\psi_{i+1} = -30^\circ$, $\phi_{i+2} = -60^\circ$, $\psi_{i+2} = -30^\circ$]. The NOE patterns (*vide infra*) suggest consecutive turn conformations. Thus, it is probable that the peptide phosphate **1a** adopts a 3_{10} helix conformation in a membrane environment in the presence of SDS micelles, unlike in aqueous buffer where the interconversion of multiple turn types occur.

The CD behavior of μ -selective **2a** was completely different from **1a**, and was very similar to the published CD spectrum of DAMGO reported by Doi and colleagues.²⁰ Interestingly, the conformation of the phosphopeptide **2a** was independent of the solvent; the peptide displayed more or less similar spectrum in both aqueous buffer as well SDS micelles. There appeared to be a correlation between the CD shape and receptor selectivity as noted by Doi *et al.* While the MOR-selective peptides related to **1** showed negative bands in the range of 210–230 nm, the mixed MOR-DOR peptide phosphates related to **2** showed positive bands.

NMR Analysis of 1a

The MOR-DOR-agonist phosphopeptide **1a** was subjected to ^1H 2D NMR analysis in $\text{D}_2\text{O}/\text{H}_2\text{O}$ buffered to pH 5.5, and in the presence of SDS micelles to obtain additional conformational information. Standard ROESY experiments yielded good quality spectra in $\text{D}_2\text{O}/\text{H}_2\text{O}$, but failed in the presence of the high M.W. SDS micelles, which required standard NOESY to produce good quality spectra.

The fingerprint regions (C^αH Vs. NH) of the NOESY/ROESY spectra for phosphopeptide **1a** are shown in Figure 4. In water buffered to pH 5.5, the observation of consecutive $d_{\text{NN}}(i, i+1)$ ROEs in the central Gly(3)-Leu(5) segment, along with the lack of any long-range ROEs indicated the presence of localized β -turn conformations.^{21,22} This was further confirmed by the observation of a single medium-range $d_{\alpha\text{N}}(i, i+2)$ ROE between Thr(2) and Phe(4), as well as the diminished temperature coefficient (-4.29 ppb/ K°) for the Phe(4) amide protons. Additional medium- and long-range NOEs characteristic of turn and helical conformations were observed in SDS micelles. Many NOEs between side-chain protons of different amino acids were observed (Supplementary data). The NOEs Leu5 $\text{C}^\delta\text{H} \leftrightarrow$ Phe4 aromatic protons and Leu5 $\text{C}^\delta\text{H} \leftrightarrow$ Tyr1 aromatic protons were especially crucial as they

indicated proximity ($<5\text{\AA}$), and the side-chains might orient on the same side of the backbone to create a hydrophobic core.

The observation of such a large number of NOEs as shown in Figure 5 for such a short peptide sequence was remarkable. Two consecutive medium-range $d_{\alpha\text{N}}(i, i+3)$ NOEs Thr(2) $\text{C}^{\alpha}\text{H}\leftrightarrow$ Leu(5)NH and Gly(3) $\text{C}^{\alpha}\text{H}\leftrightarrow$ Ser(6)NH strongly supported a helical type fold. This NOE is also observed in β -turn conformations. Therefore, it is also possible the phosphopeptide adopts two consecutive turns as in a short 3_{10} -helix conformation.²³ Additional support for the helical type fold comes from the negative deviation of $\text{C}^{\alpha}\text{H}$ from the random coil values (see the discussion below).

Information on hydrogen bonds can be obtained from the amide temperature coefficients ($\Delta\delta/\Delta T$) of the amide bonds. The temperature coefficients for **1a** in H_2O and in the presence of SDS micelles were obtained in 5°C increments starting at 20°C (Supplementary Data). The $\Delta\delta$ -values for all the amide protons were more negative than -4.6 ppb/K° in water, indicating that the backbone was freely solvated and no intramolecular hydrogen bonds were present.²⁴ In SDS micelles the values for all the amide protons became positive, and the C-terminal amide protons showed temperature coefficients more positive than the hydrogen bond threshold value of -4.6 ppb/K° . The diminished temperature coefficient values of amide protons of Phe(4), Leu(5), Ser(6) and one (but only one) of the terminal NH_2 protons in SDS micelles indicated the presence of a hydrogen bond or a high degree of solvent shielding.

The C^{α} proton chemical shift values are sensitive to the secondary structure of proteins. Sykes and coworkers²⁵ created a chemical shift index based on this principle which allowed us to get the first indication of specific secondary structural elements present in the peptides comparable to CD. Generally, the observation of consecutive negative deviations from random coil (upfield-shifted $\text{C}^{\alpha}\text{H}$ resonances) indicates a helical conformation. The N-terminal segment residues displayed negative deviations in water, whereas in SDS micelles the C-terminal residues also showed negative deviations. It is not possible to assign a particular secondary structure conformation based solely on these values because the peptide is not long enough, but the differences show that the conformation of phosphopeptide **1a** is very sensitive to the solvent milieu.

In order to obtain the 3D structures of the peptide phosphates a Stochastic conformational search was applied using distance restraints calculated from NOE data. Examination of low energy structures from the calculation suggests there are two major family of conformers possible for the phosphates. The lowest-energy structures from both the families are shown in Figure 6. The backbone dihedral angle of residues in the middle segment Gly(3), Phe(4) and Leu(5) are helical ($\Phi = -60^\circ$ and $\Psi = -30^\circ$) in one family. At least four residues are necessary to describe a α -helical turn. Therefore, it is possible to describe a 3_{10} type helical fold for this region. Since dihedral values are the same for both type III β -turns and 3_{10} helices, this region can also be described as two type III β -turn – one centered at Gly(3)-Phe(4) dipeptide segment and another centered at Phe(4)-Leu(5) – fused together. Both the family of structures are somewhat closely related to each other. The major difference is dihedral value of Leu(5) residue. The other family has only one type III β -turn centered at Gly(3)-Phe(4) dipeptide segment. The two different yet related family of conformers could be reason for the mixed δ/μ receptor selectivity of this phosphopeptide.

Effect of Phosphorylation on Peptide Backbone Conformation

The CD and NMR data of the phosphopeptides were compared with data of parent peptides to examine the effect of phosphorylation on peptide conformation. The CD spectrum of **1a** (Supplementary data) is significantly different from the parent peptide **1** indicating

phosphorylation induced conformational change. Comparison of NOE summary (data not shown- see supplemental info) of phosphopeptide **1a** with parent unphosphorylated peptide **1c** reveals phosphorylation stabilizes the peptide structure. Many additional NOEs appear in the NOESY spectrum of **1a**. The observation of consecutive $d_{\alpha N}(i, i+2)$ and $d_{NN}(i, i+2)$ NOEs for **1a** clearly suggest consecutive turns throughout the peptide length which is in contrast to **1** where only few of those NOEs were observed in the middle of the segment. Moreover, the observation of two consecutive medium-range $d_{\alpha N}(i, i+3)$ NOEs Thr(2) $C^{\alpha}H \leftrightarrow$ Leu(5)NH and Gly(3) $C^{\alpha}H \leftrightarrow$ Ser(6)NH, which are not observed for **1**, strongly support consecutive turns or short helical type fold for **1a**. Additional NMR parameters such as CSI values²⁵ of C^{α} protons and temperature coefficient data (data not shown - see supplemental info) also indicate the structure stabilization by the phosphate group attached to the serine side-chain. In the case of phospho-enkephalin analog, C^{α} protons of all the residues display negative deviation from the random coil value, and most of the C-terminal amide protons are solvent shielded. This indicates they might all be involved in hydrogen bonds. However, the phosphorylation did not alter the conformation of DAMGO analog **2a**. The CD and NMR data did not show any significant differences in the conformation by the attachment of phosphate group unlike the phospho-enkephalin analog.

Conclusions

Phosphorylation may provide a useful method to enhance water solubility and effective biodistribution of peptide drugs, but may be less effective than the corresponding serine glucosides, at least for these two short enkephalin sequences. Phosphorylation also stabilized the consecutive type III β -turn, a 3_{10} type helical conformation in the middle segment of the phosphate analog of DTLES sequence, but did not significantly affect the conformation of the DAMGO analog.

The two classes of phosphates differ greatly in their interaction with the SDS micelles, with the more water soluble DAMGO sequence showing a largely electrostatic interaction, while the DTLES sequence¹⁷ is aided by additional lipophilic interactions, particularly the Leu(5) side chain residue.²⁶ The phosphate does not interfere with opioid binding. The lipophilic benzyl phosphate ester moiety altered the μ/δ -selectivity, and blocked distribution to the CNS. Further studies with other peptide sequences may be indicated, and will help establish the generality of these findings.

Materials and Methods

Synthesis and Purification of Phosphopeptides

Amino acids, coupling reagents and Rink-amide resin were purchased from Advanced ChemTech (Louisville, USA). The phosphopeptides were synthesized manually by standard solid-phase methods employing fluorenylmethoxycarbonyl (Fmoc) chemistry on rink amide resin. The choice of side chain protecting groups allowed for removal in a single step at the end of the synthesis while the phosphopeptide is still attached to the resin. The side chain protected amino acids used in the synthesis were Fmoc-DThr(But)-OH, Fmoc-Tyr(But)-OH and Fmoc-Ser(OPO(OBz)OH)-OH. The amide couplings were with HBTU/HOBt/DIPEA. Each coupling was performed in a manual peptide synthesis vessel using DMF as solvent by agitating using N_2 for 90 minutes. The coupling was monitored by the Kaiser ninhydrin test. Fmoc groups were removed with a solution of 2% piperidine and 2% DBU in DMF. Once the phosphopeptide was assembled and the final Fmoc group was removed, the phosphopeptide was cleaved from the resin with a cocktail $F_3CCOOH:Et_3SiH:H_2O:PhOMe:CH_2Cl_2$ (9:0.5:0.5:0.05:1) that also removed the side chain protecting groups. The crude phosphopeptides were precipitated with ice-cold ether, filtered, redissolved in H_2O and lyophilized. The phosphopeptides were purified by RP-HPLC with a

preparative RP(C-18) column using acetonitrile-water gradient containing 0.1% TFA. Homogeneity of the final glycopeptides was assured by analytical RP-HPLC and mass spectrometry.

Receptor Binding Studies

To determine the affinity and selectivity of the peptides for the μ , δ , and κ opioid receptors, Chinese hamster ovary (CHO) cells that stably expressed one type of human opioid receptor were used as previously described.²⁷ Cell membranes were incubated at 25°C with the radiolabeled ligands in a final volume of 1 ml of 50 mM Tris-HCl, pH 7.5. Incubation times of 60 min were used for the μ -selective peptide [³H]DAMGO and the κ -selective ligand [³H]U69,593, and a 3-hr incubation will be used with the δ -selective antagonist [³H]naltrindole. The final concentrations of [³H]DAMGO, [³H]naltrindole, and [³H]U69,593 were 0.25 nM, 0.2 nM, and 1 nM, respectively. Nonspecific binding was measured by inclusion of 10 μ M naloxone for μ and κ binding and 100 mM for δ binding. The binding was terminated by filtering the samples through Schleicher & Scheull No. 32 glass fiber filters using a Brandel 48-well cell harvester. The filters were washed three times with 3 ml of cold 50 mM Tris-HCl, pH 7.5, and were counted in 2 ml of ScintiSafe 30% scintillation fluid (Fisher Scientific, Fair Lawn, NJ). For [³H]U69,593 binding, the filters will be soaked in 0.1% polyethylenimine for at least 30 min before use. IC₅₀ values will be calculated by least squares fit to a logarithm-probit analysis. K_i values of unlabeled compounds will be calculated from the equation $K_i = (IC_{50}) / (1 + S)$ where S = (concentration of radioligand) (K_d of radioligand).²⁸

Antinociceptive testing

Adult, male ICR mice (25–35 g, Harlan Industries, Cleveland, OH) were used for all in vivo experiments. Mice were housed in groups of 4–5 in standard Plexiglas containers with food and water available *ad libitum*. Animals were on a 12-h light/dark cycle (lights on at 07:00). Drug administrations were made via the i.c.v. or i.v. routes of administration. Antinociception was assessed using the 55°C warm-water tail flick assay.²⁹ Mice were tested for baseline latencies by immersing the distal third of the tail into the water. Typical baseline latencies were between 1–2 seconds and any mouse with a baseline over 5 seconds was excluded from further procedures. The test compound was administered at t = 0 min and tail flick latencies were measured at various time points after injection. A maximal score of 100% was given to a mouse that did not respond within 10 seconds to avoid tissue damage. Antinociception was calculated using the following formula: % antinociception = (test latency - baseline latency) / (10 - baseline latency) × 100.

Circular Dichroism

All of the circular dichroism experiments were carried out on OLIS DSM-20 spectrophotometer equipped with temperature controller. The glycopeptide stock solutions were prepared by weighing the lyophilized powder to make 0.5–1.0 mM solution and the pH was adjusted to the desired value. The samples were prepared by diluting the stock solution to 180 μ M. The reported data are average of four scans recorded between 200 and 250 nm by using integration time of three seconds, a scan step of 0.5 nm in a cell with a path length of 0.1 cm at 25 °C. All the spectra were baseline corrected and were smoothed using KaleidaGraph Ver. 4 software (Synergy Software, USA).

NMR Spectroscopy

All NMR spectra were recorded on a Bruker DRX600 600MHz spectrometer at 298 °K. Glycopeptide concentration for the NMR experiments varied from 2–3 mM. The micelle samples were prepared by dissolving the peptide and 100 equivalent of perdeuterated SDS in

0.6 ml of phosphate buffer (10 mM) containing 10% D₂O. The pH of the each sample was adjusted to 4.5 by using DCl or NaOD as necessary. TSP was added as internal standard for referencing. Two-dimensional nuclear Overhauser enhancement (NOESY)³⁰ and total correlation spectra (TOCSY)³¹ were acquired using standard pulse sequences and processed using XWINNMR (Bruker Inc) and FELIX2000 (Accelrys Inc, San Diego, CA). Mixing time for TOCSY spectra were either 80 or 100ms and for NOESY spectra was 300 ms. All experiments were 750 increments in t₁, 16 or 32 scans each, 1.5 s relaxation delay, size 2 or 4K and the spectral processing was with shifted sine bell window multiplications in both dimensions. The water suppression was achieved by using WATERGATE pulse sequence.³² Coupling constants (³J_{αH-NH}) were measured directly from the 1D spectra wherever possible.

Molecular Modeling

All the structural calculations were done using Molecular Operating Environment (MOE) software 2007.09 (Chemical Computing Inc., Canada). The standard Stochastic Conformational Search protocol was used for generating conformations using AMBER99³³ as the force field with 8 Å cutoff for non-bonded interactions. The Stochastic Conformational Search method in MOE generates conformations by randomly sampling local minima of the potential energy surface similar to the Monte Carlo type routine called Random Incremental Pulse Search (RIPS) described by Raber and coworkers.³⁴ RIPS method generates new molecular conformations by randomly perturbing the position of each coordinate of each atom in the molecule by some small amount, typically less than 2 angstroms, followed by energy minimization. The Stochastic Conformational Search method in MOE is similar in spirit except that it is based upon random rotations of bonds (including ring bonds) instead of Cartesian coordinate perturbation. A distance dependent dielectric constant of 4 was used for the calculations. All the inter-residue distance restraints calculated from the NOESY experiments were applied in the form of flat-bottom potential well with a common lower bound of 2.0 Å and the upper bound were obtained from integral volumes of the NOESY peaks. The NOE integral volumes were classified into strong, medium and weak with 3.0, 4.0 and 5.0 Å as upper bound distance. The starting structure for the calculations were having extended conformation. A total of 10000 conformations were generated with energy cut-off of 25 Kcal, which rejected many final conformers that had energy higher than 25 Kcal of the lowest-energy conformer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the Office of Naval Research (N00014-05-1-0807 & N00014-02-1-0471), the National Science Foundation (CHE-607917) and the National Institutes of Health (NINDS-NS-052727) for support.

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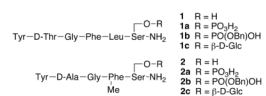


Figure 1.
Chemical structures of the compounds tested in this study.

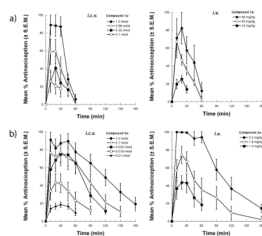


Figure 2. Antinociceptive i.c.v. and i.v. dose- and time-response curves for phosphopeptides **1a** and **2a** in the mouse 55°C warm water tail-flick test. Bars represent SEMs for each data point.

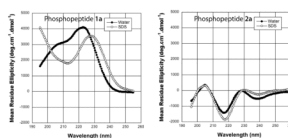


Figure 3.
CD spectra of phosphopeptides **1a** and **2a** in aqueous buffer and SDS micelles measured at pH 5.5 and 25°C.

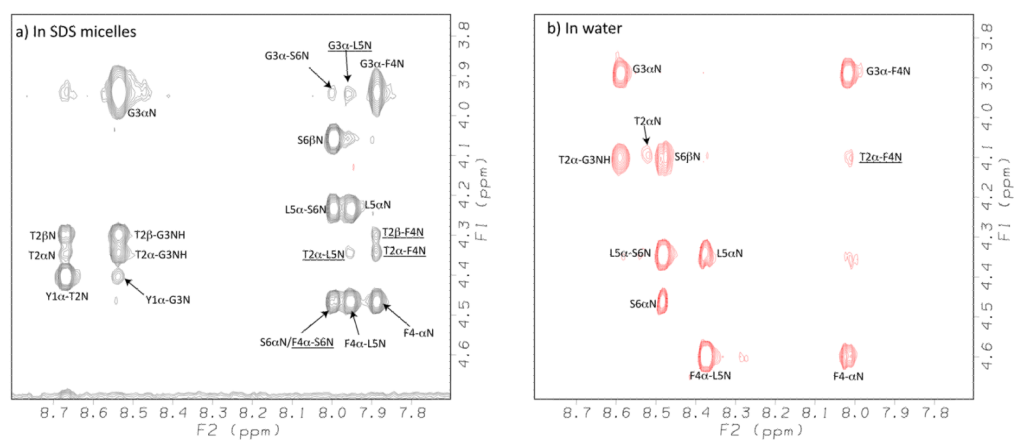


Figure 4. Fingerprint regions ($C^{\alpha}H-NH$) of NOESY/ROESY spectra for the phosphopeptide **1a** in a) SDS micelles and b) water measured at pH 5.5 and 25°C using a mixing time of 300 ms. The medium- and long-range NOE correlations are underlined.

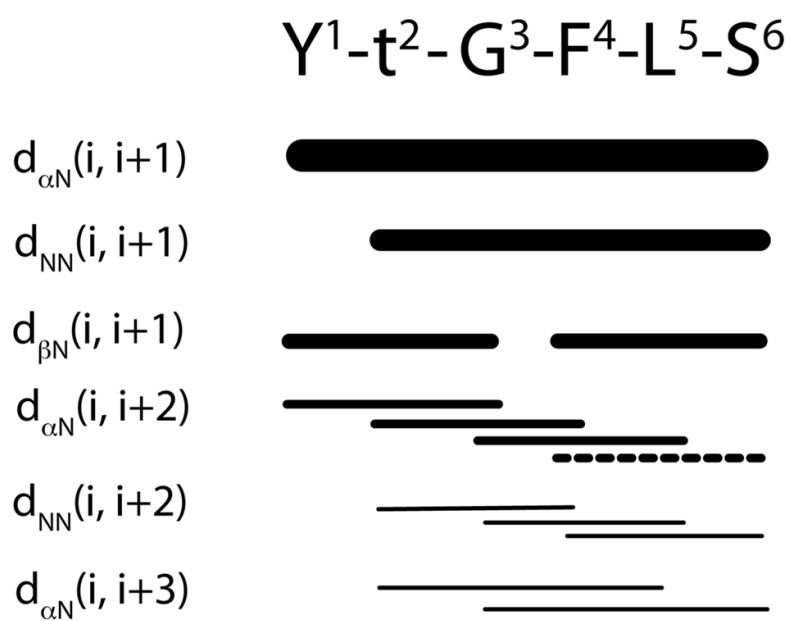


Figure 5. NOE summary for the phosphopeptide **1a** in SDS micelles measured at pH 5.5 and 25 °C. The line thickness is approximately proportional to the intensity of the observed NOE cross peaks. The dashed line indicates the signal overlap of the cross-peaks.

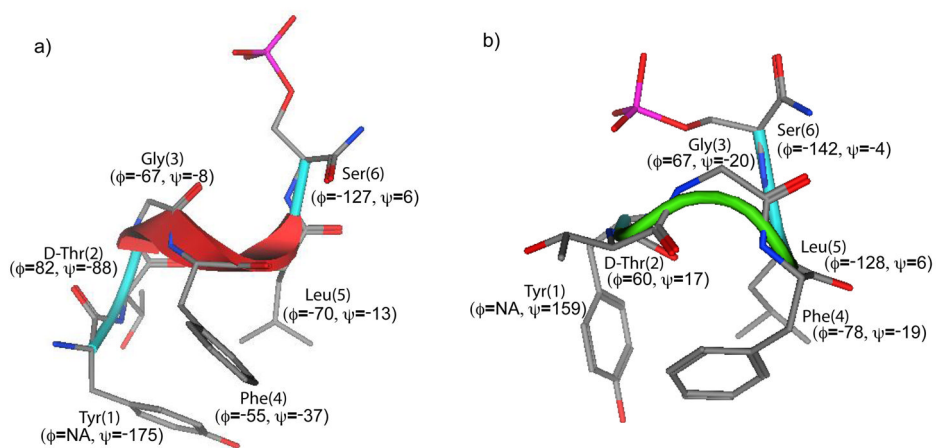


Figure 6. Lowest energy structure of phosphopeptide **1a** from a) Ensemble of helical conformers, and b) Ensemble of turn conformers. The backbone dihedral angle values are indicated in parentheses. Hydrogen atoms have been omitted for clarity.

Table 1

Binding affinities and antinociceptive potencies of the phosphopeptides.

Peptide	K _i (nM ± S.E.)			Ratio	A ₅₀ (95% CI)	
	[³ H]DAMGO MOR	[³ H]Naltrindole DOR	[³ H]U69,593 KOR		<i>i.c.v.</i> (nmol/mouse)	<i>i.v.</i> (μmol/kg)
1	1.4 ± 0.08	4.1 ± 0.41	34 ± 2.2	MOR:DOR: KOR 1:3:15	0.068 (0.054–0.086)	46.4 (35.4–60.7)
1a	3.9 ± 0.19	7.9 ± 0.73	310 ± 66	1:2:79	0.34 (0.22–0.52)	54.1 (48.5–60.2)
1b	8.7 ± 0.93	3.3 ± 0.33	130 ± 6.2	3:1:39	0.093 (0.053–0.16)	30% @ 32.0 mg/Kg
1c	2.4 ± 0.017	7.0 ± 1.2	49 ± 4.3	1:3:20	0.02 (0.01–0.04)	11.4 (8.5–15.2)
2	0.68 ± 0.2	600 ± 44	190 ± 9.3	1:880:220	0.002 (0.0015–0.003)	0.135 (0.106–0.172)
2a	3.8 ± 0.73	1400 ± 180	31% inhib. @ 10 μM	1:370:>1000	0.042 (0.02–0.07)	1.5 (1.3–1.8)
2b	4.2 ± 0.34	55 ± 4.6	570 ± 9.8	1:13:140	0.033 (0.02–0.05)	30% @ 38.1 mg/Kg
2c [†]	1.3 ± 0.14	54% inhib. @ 10 μM	270 ± 2.5	1:>7000:208	0.019 (0.012–0.029)	0.602 (0.470–0.772)

[†]Values are taken from Lowery *et al.*¹²

Peptides were incubated with membranes from CHO cell expressing human opioid receptors. Analgesic potency was evaluated *i.c.v.* and *i.v.* in mice. Details are provided in the experimental section.