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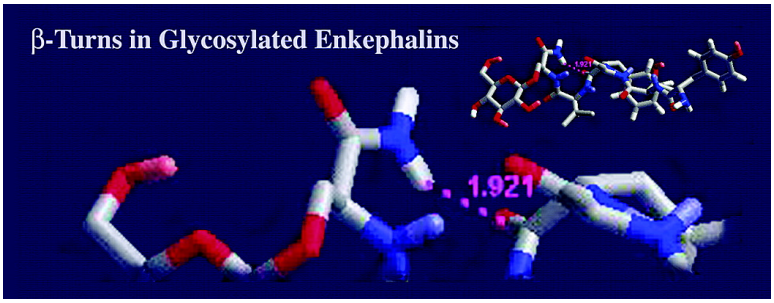
J. Am. Chem. Soc., **2003**, 125 (19), 5823-5831• DOI: 10.1021/ja0268635 • Publication Date (Web): 22 April 2003



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Glycopeptide-Membrane Interactions: Glycosyl Enkephalin Analogues Adopt Turn Conformations by NMR and CD in Amphipathic Media

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Abstract: Four enkephalin analogues (Tyr-D-Thr-Gly-Phe-Leu-Ser-$CONH_2$, **1**, and the related O-linked glycopeptides bearing the monosaccharide β-glucose, **2**, the disaccharide β-maltose, **3**, and the trisaccharide β-maltotriose, **4**) were synthesized, purified by HPLC, and biophysical studies were conducted to examine their interactions with membrane model systems. Glycopeptide **2** has been previously reported to penetrate the blood-brain barrier (BBB), and produce potent analgesia superior to morphine in mice (*J. Med. Chem.* **2000**, *43*, 2586−90 and *J. Pharm. Exp. Ther.* **2001**, *299*, 967−972). The parent peptide and its three glycopeptide derivatives were studied in aqueous solution and in the presence of micelles using 2-D NMR, CD, and molecular mechanics (Monte Carlo studies). Consistent with previous conformational studies on cyclic opioid agonist glycopeptides, it was seen that glycosylation did not significantly perturb the peptide backbone in aqueous solution, but all four compounds strongly associated with 5−30 mM SDS or DPC micelles, and underwent profound membrane-induced conformational changes. Interaction was also observed with POPC:POPE:cholesterol lipid vesicles (LUV) in equilibrium dialysis experiments. Although the peptide backbones of **1**−**4** possessed random coil structures in water, in the presence of the lipid phase they each formed a nearly identical pair of structures, all with a stable β-turn motif at the C-terminus. Use of spin labels (Mn^{2+} and 5-DOXYL-stearic acid) allowed for the determination of the position and orientation of the compounds relative to the surface of the micelle.

Introduction

Exploration of peptide conformation continues to be an important topic in medicinal chemistry, as changes in the secondary structure of peptides have important, yet ill-defined roles in many disease states. Notable among these is Alzheimer's disease, which involves a change in secondary structure of an APP cleavage product,[1] that has been shown to interconvert between a random coil, an α-helix monomer, and a β-sheet oligomer.[2] Similarly, detailed knowledge of glycoprotein and glycopeptide structure and conformation is essential to understand enzymatic catalysis, hormonal control, transport, cell adhesion and cell−cell recognition. NMR and FT-IR studies suggest that O-linked glycosylation of threonine in model peptides can promote secondary structure in $CDCl_3$ and d_6-DMSO,[3] but had earlier been shown to have little or no effect on the aqueous conformations of glycopeptides structurally related to antifreeze glycoproteins.[4]

It is important to distinguish "localized" H-bonding effects observed in both the N-linked (β-GalNAc → N-Asn) glycopeptides that can adopt β-turns upon glycosylation[5] and the O-linked mucins (α-GalNAc → O-Ser/Thr) that form 10-membered glyco-turns reminiscent of β-hairpins,[6] from other more subtle "global" effects of glycosylation.[7] Most studies have concluded that O-linked glycosylation serves to promote β-turn formation,[8] but others have argued that glycosylation promotes

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an extended backbone,[9] or have seen no effect on peptide backbone conformations upon glycosylation.[10] We have argued that glycosylation per se has little or no effect on the peptide backbone conformation in the absence of amide moieties in the sugar,[11] and that the peptide *sequence*, along with the *solvent media*, are the major determinants for backbone conformation, especially with larger peptides (proteins). Short peptides (e.g., endogenous enkephalins[12]) are generally unstructured in solution due to a high degree of flexibility.[13] Studies by several groups have challenged this notion, and suggest that short peptides, even as short as two residues,[14] can display preferred structures in certain environments,[15] particularly in the presence of nonaqueous solvents capable of stabilizing secondary structure.[16] In this study, the conformations of a series of glycosylated enkephalin analogues are examined in the presence and absence of micelles, to understand the origins of the unique in vivo transport properties that are displayed by these potent opioid analgesics.

In mice it has been shown that glycosylated enkephalins show appreciable, yet weakly saturable, transport through the blood−brain barrier (BBB),[17] and that the glycopeptides can bind strongly to opioid receptors in the brain to produce potent analgesia.[18] A series of four glycopeptide enkephalin analogues (Figure 1) were synthesized to examine their conformations in H_2O and in the presence of micelles.[19] It was hoped that the resulting data could be used to rationalize the transport phenomena (BBB penetration) and opioid binding activity (analgesia) of glycosylated enkephalin analogues in vivo. Because the transport process does not appear to be diffusive, or transporter-mediated, an endocytotic event seems likely.[17] If endocytosis occurs without interaction with the membrane (fluid phase endocytosis), no interaction with the membrane would be expected, and if binding to or insertion into the membrane precedes endocytosis (adsorptive endocytosis), then glycopeptide-membrane interactions should be observable.

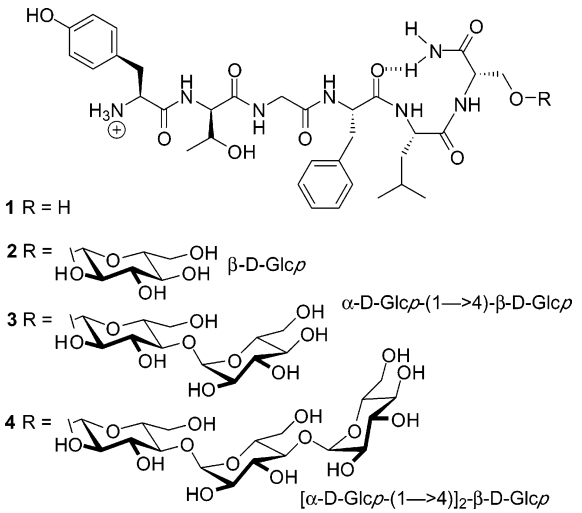
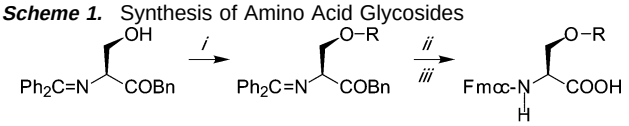


Figure 1. Enkephalin Analogues.

Scheme 1. Synthesis of Amino Acid Glycosides



R—Br	6 Yield (α:β)	7 Yield
a) $(AcO)_4$Glc-Br	32% (5:1)	99%
b) $(AcO)_7$Glc-α(1—>4)-Glc-Br	52% (20:1)	68%
c) $(AcO)_{10}$[Glc-α(1—>4)]$_2$-Glc-Br	61% (20:1)	75%

Reagents: (*i*) Glycosylbromide, AgOTfl, CH_2Cl_2, 4 Å sieves, (*ii*) H_2/Pd−C, CH_3OH, (*iii*) Fmoc-Cl, pyridine, CH_2Cl_2.

Glycopeptide Synthesis. The required glycosyl α-bromides were synthesized using previously published methods,[20] and the glycopeptides were assembled using a modified Fmoc methodology with Rink amide resins.[21] The general synthetic scheme for the Fmoc amino acid glycoside synthesis is outlined below (Scheme 1). Purification of the glycopeptides was accomplished using reversed phase HPLC.

Opioid Binding and Glycopeptide Transport. Although there are several exceptions to the Tyr-Gly-Gly-Phe motif "required" for opioid binding,[22] most specialists agree that a beta turn or a related turn at Gly^2-Gly^3 is required for binding to either the μ- or the δ-opiate receptor.[23] The original structural

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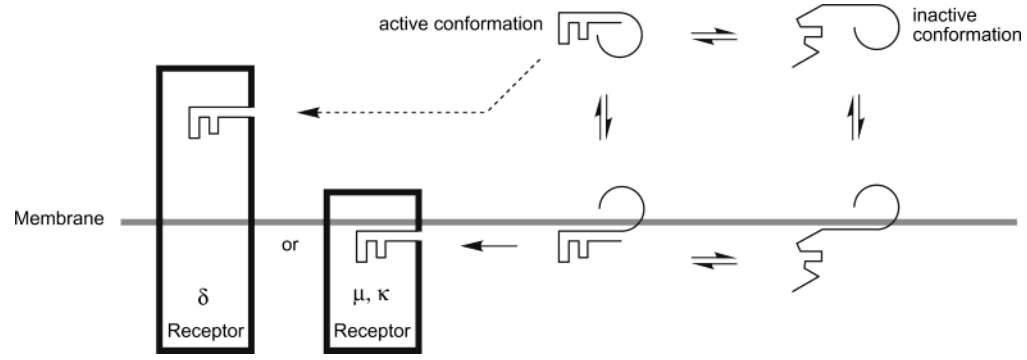

Figure 2. Peptide−Membrane−Receptor Interactions.

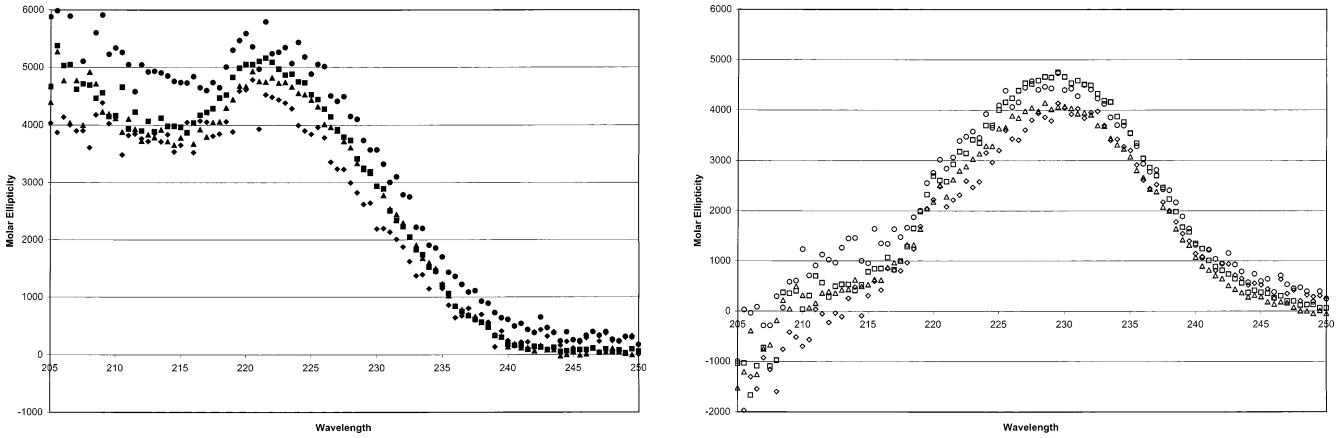

Figure 3. CD plots of compounds **1** (◇), **2** (□), **3** (△), and **4** (○) in H_2O (left, filled figures) and in the presence of 30 mM SDS micelles (right, open figures).

analogy of the terminal-NH_3^+ and the two aromatic side chains to their counterparts in morphine, et al. is still operative.[24] Cyclic disulfide[25] and cyclic amide[26] enkephalin analogues have been used to enforce these turns, resulting in very potent and selective opioid agonists. Acyclic enkephalin analogues with a D-amino acid substituted for Gly^2 have also been designed to bias the conformational ensemble to obtain greater affinity for opioid receptors and enhanced μ/δ-receptor selectivity.[27]

Because the opioid receptors are G-protein coupled receptors (GPCR) that are bound to the cellular membrane surface, all ligands must come into close contact with the lipophilic surface prior to, or upon binding to the active site (Figure 2). Aqueous conformations of peptides generally differ from those found in mixed media[28] (e.g., micelles, vesicles, cell surfaces), which are probably more biologically relevant.[29] This is the basis of Schwyzer's membrane compartment theory,[30] which suggests that the membrane "catalyzes" ligand−receptor interactions.[31] As pointed out by Max Delbruck some time ago, if the ligand binds to the membrane first, then the "search" for the receptor becomes an efficient 2-dimensional problem, rather than a lengthy 3-dimensional one.[32]

Circular Dichroism Studies. CD was used to examine conformational preferences of compounds **1**−**4** in water, and in the presence (30 mM) of SDS-micelles (Figure 3). From these data, probably dominated by the N-terminal Tyr^1 residue, a number of observations were made. There was a conformational change upon adsorption to the micelle, and the conformational ensembles of **1**−**4** were all very similar in the presence of micelles, regardless of the extent of glycosylation. Virtually identical results were observed in the presence of DPC-micelles (5 mM). In the presence of liposomes (LUV), authentic bilayer aggregates with much less membrane curvature than the micelles, compounds **1**−**4** showed equilibrium constants favoring association with the liposomes ($K_{eq} = 88-280\ mol^{-1}$, Figure 4).[33]

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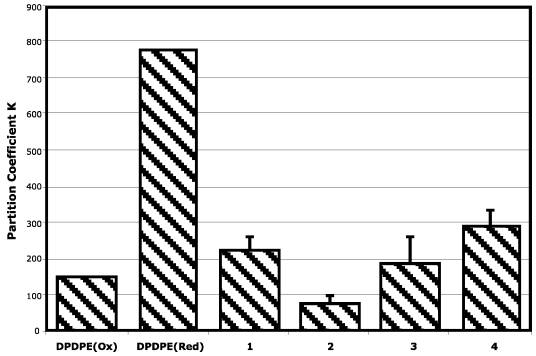

Figure 4. Partition coefficients for the oxidized (S−S) and reduced (SH HS) forms of the δ-selective opioid peptide DPDPE into liposomes are shown along with the partition coefficients for compounds **1**−**4**.

Turn formation has been proposed for short peptides in lipophilic environments, as well as "neo-glycosylated" enkephalin analogues in F_3CCH_2OH.[34] There was more variation in the CD curves (and presumably in the conformations) measured in H_2O than in the presence of micelles. Examination of the CD data showed that the media induced (SDS vs water) *differences* in per residue molar ellipticity at λ_{max} decreased as the degree of glycosylation increased, suggesting the following: (1) the higher glycosylated analogues (**3** and **4**) might form different populations of stable turn structures and random coil structures in H_2O, or (2) glycopeptides **3** and **4** might form more rigid turn structures in the presence of micelles than their lower glycosylated analogues (**1** and **2**). Increased "stiffening" of the peptide backbone upon increased glycosylation has been suggested for other systems.[35] From these CD data, it is impossible to provide detailed information on the glycopeptide conformations, but one can conclude that the conformations displayed by the four compounds are similar in both media.

^{1}H NMR Studies. One and two-dimensional NMR studies in H_2O/D_2O and with deuterated SDS micelles, at varying temperature, were performed on all four enkephalin analogues. Two-dimensional techniques were utilized to fully assign the proton resonances, as well as to provide evidence for secondary structure (Figure 5). The nOe volumes provided conformational constraints for Monte Carlo simulations for peptide **1**, and glycopeptides **2** and **4**. Spin labels were used to determine the orientation of the adsorbed enkephalins with respect to the micelle/water phase boundary, and confirmed by amide-H_2O exchange rates ($\Delta\delta/\Delta T$ plots).

Chemical shift differences (CSD) of the H_α and NH proton resonances clearly indicated changes in the micelle-bound conformational ensembles of **1**−**4** relative to their aqueous conformations. The CSD suggested a turn structure between DThr^2 and Leu^5, as shown by the positive difference at those residues. This was confirmed by nuclear Overhauser effects (nOe), molecular modeling studies (vide infra), and by the temperature dependence of the amide proton shifts ($\Delta\delta/\Delta T$). Nuclear Overhauser effects on the micelle-bound glycopeptides **2**−**4** were more abundant and intense than in H_2O, and when compared to the unglycosylated peptide **1** (Figures 6 and 7). Longer-range nOe's were also observed, suggesting the presence

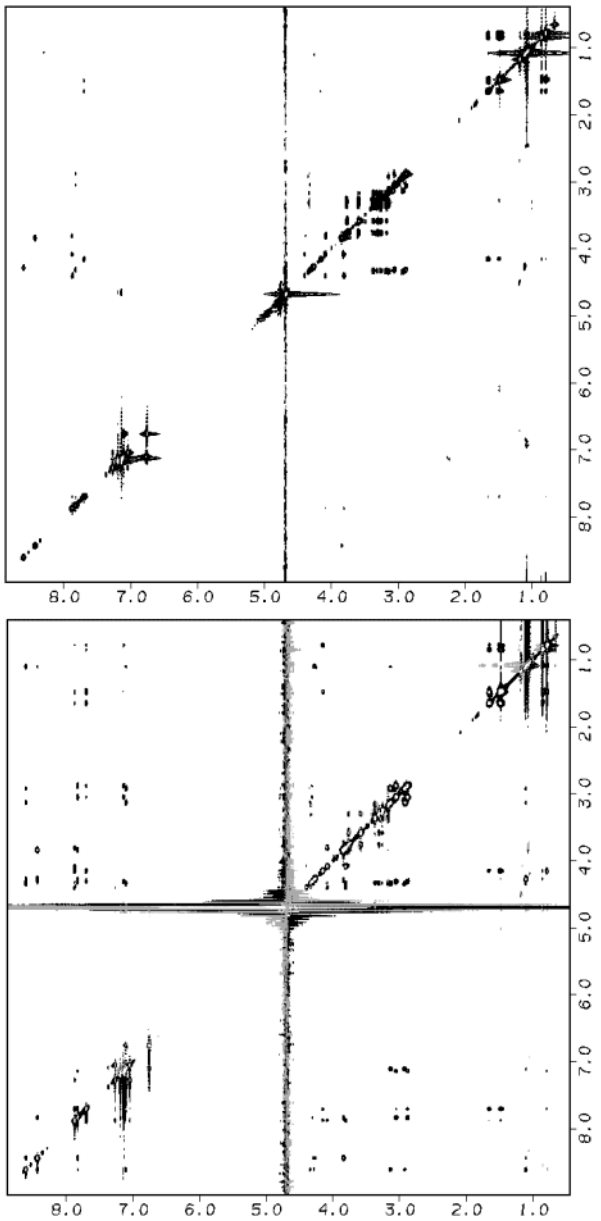

Figure 5. 600 MHz TOCSY Spectrum of glycopeptide **2** at pH 4.5 at 298 °K in the presence of d_{25} SDS micelles (above), and a NOESY spectrum of the same sample of **2** (below).

of more stable secondary structures in the presence of micelles. Peptide **1** showed far fewer nOe's than **2**−**4**, indicating that the peptide is more flexible than **1**, even when adsorbed to a micelle. It is important to note that the long correlation times observed with all of the compounds were consistent with adsorption to a micelle, and not with a molecule tumbling freely within a micelle.

Metal-based radicals and nitroxyl-based spin-labels have used for determining the position of larger peptides in micelles.[36] The cross-peaks of protons exposed to aqueous exterior have previously been shown to broaden or disappear due to paramagnetic broadening by Mn^{2+}. Similar broadening effects for protons near the carbons 1−5 of the SDS are seen with use of 5-doxyl stearic acids.[37] Both were utilized in these studies and

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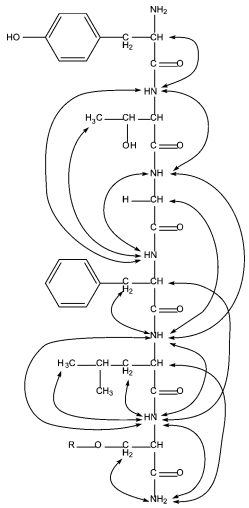

Figure 6. Representative nOe's observed for **1**−**4** in the presence of deuterated SDS micelles.

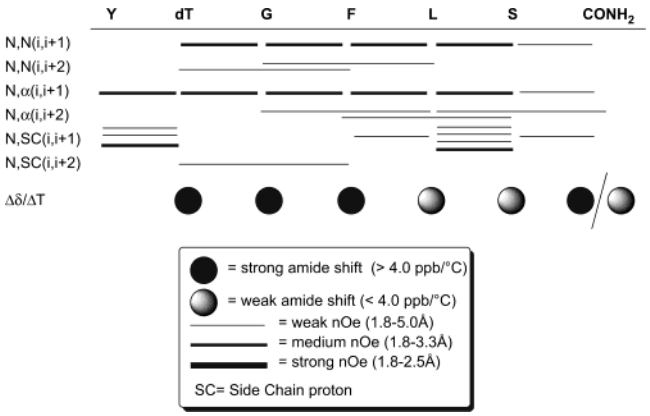

Figure 7. Summary of nuclear Overhauser effects (nOe) and amide proton exchange data for glycopeptide **2** in the presence of deuterated SDS micelles. Data for glycopeptides **3** and **4** were very similar.

showed which protons were exposed to the aqueous exterior and which were confined to the hydrophobic interior of the micelle.

For enkephalins **1**−**4** the NH/H_α region disappeared with either Mn^{2+} or DOXYL stearic acid, suggesting that the backbones of all four of the compounds lie close to the surface of the micelle. In the presence of 5-DOXYL, the lipophilic side chains of Tyr^1, Phe^4, and Leu^5 showed greatly diminished cross-peaks in TOCSY experiments with all 4 enkephalins (Figure 8). The lipophilic side-chains of peptide **1** were not as greatly influenced by the 5-DOXYL stearic acid as the lipophilic side-chains of glycopeptides **2**−**4**. Although one might assume this is due to decreased interaction with the micelle for **1**, this is not consistent with the observed amide exchange rates (vide infra), and may be due to one of several causes: (1) Peptide **1** may experience more efficient relaxation within the micelle due to increased mobility, relative to the more constrained glycopeptides **2**−**4**, thus diminishing the effect of the radical on the protons of **1**; and/or (2) The side chains of **1** may penetrate to a greater depth within the micelle (5-DOXYL only affected the SDS peaks to a depth of 5 carbons); or (3) The 5-DOXYL results do in fact indicate a weaker (ion pairing) association between **1** and the micelle, and that the exchange rate data simply indicate that the micelles induce a greater relative change in the various confomer populations of peptide **1** than glycopeptides **2**−**4**, which are more structured in water than **1** to begin with.

Conversely, the Ser^6 β-glucoside cross-peaks were diminished by the water soluble Mn^{2+} for glycosides **2**, **3**, and **4**, but this effect was much weaker for the more distal α-glycosides in **3** and **4**. This is consistent with the notion that the micelles act as ion exchangers, increasing the effective concentration of Mn^{2+} at their surface near the sulfonate headgroups. The amount of peak broadening of the glycosides increased with increasing concentrations of Mn^{2+}, but the concentration could not be increased enough to completely eliminate the more distal glycoside cross-peaks without concomitant disruption of the micelles by the divalent ions. The N-terminal-NH_3^+ group was assumed to have an ionic association with the-SO_{3-} headgroups of the micelle in all cases.

Evidence for the orientation of the glycopeptides **2**−**4** (Figure 13) within the micelle is also supported by temperature-dependent chemical shift studies[38] of the amides. When the amide shift $\Delta\delta/\Delta T$ data from water was compared to $\Delta\delta/\Delta T$ data from micelles (Figure 9), it was obvious that a conformational change had occurred, and that the NH exchange rates were differentially affected by the micelles.[39] The temperature dependencies of the observed amide exchange rates for glycopeptides **2**−**4** were quite similar in the presence of micelles, but diverged in water, with the Phe^4 and Leu^5 residues showing *decreasing* exchange rates with *increasing* glycosylation. The C-terminal amides were particularly diagnostic, providing strong evidence for turn structures. For each of the compounds the chemical shift of the syn N−H showed a large temperature dependence, while the anti N−H did not, suggesting involvement in an intermolecular hydrogen bond. Peptide ***1*** showed slower exchange rates at the Phe^4 and Leu^5 residues of the glycopeptides shifts within the micelles, again supporting the notion that the peptide **1** may be inserted more deeply into the micelle interior than the glycosylated analogues **2**−**4**.

Monte Carlo Studies. Nuclear Overhauser (nOe) constraints for each of the four compounds, obtained from 2-D NMR in per-deuterated SDS micelles, were used as the basis for molecular modeling studies using the MacroModel 7.1 package. Using water as a solvent, 10 000 random conformers were generated and minimized by the conjugate gradient method. Initially, results showed a high degree of hydrogen bonding between the N-terminus and the carbohydrate hydroxyls. Although thermodynamically most favorable *in vacuo*, this interaction was eliminated for two reasons. First, because the ^1H NMR measurements were made at pH 4.5, the amine was likely to be protonated and therefore not capable of hydrogen bonding. Also, the glycopeptide was observed to interact with the micelle, which has negatively charged headgroups that can pair with the protonated amine in an ionic fashion. The 50 lowest energy conformers were then examined for each compound.

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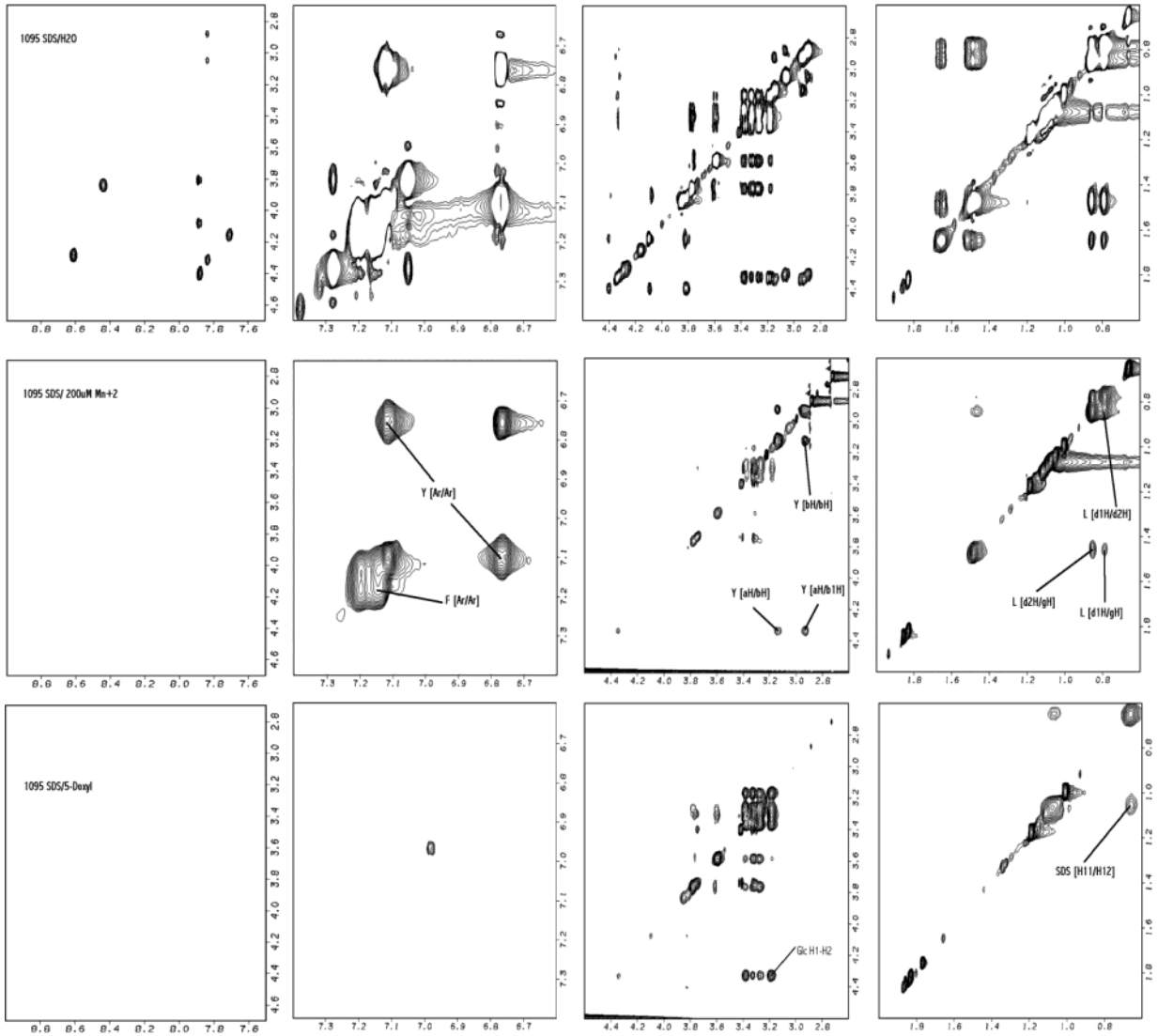

Figure 8. Effect of Radicals on TOCSY Spectra. Glycopeptide **2** with SDS micelles (top row), with 200 mM Mn^{2+} (middle) and 5-DOXYL stearic acid (bottom). Preserved resonances (labeled) are in a phase not be affected by the phase-specific radical probe (Mn^{2+} or DOXYL).

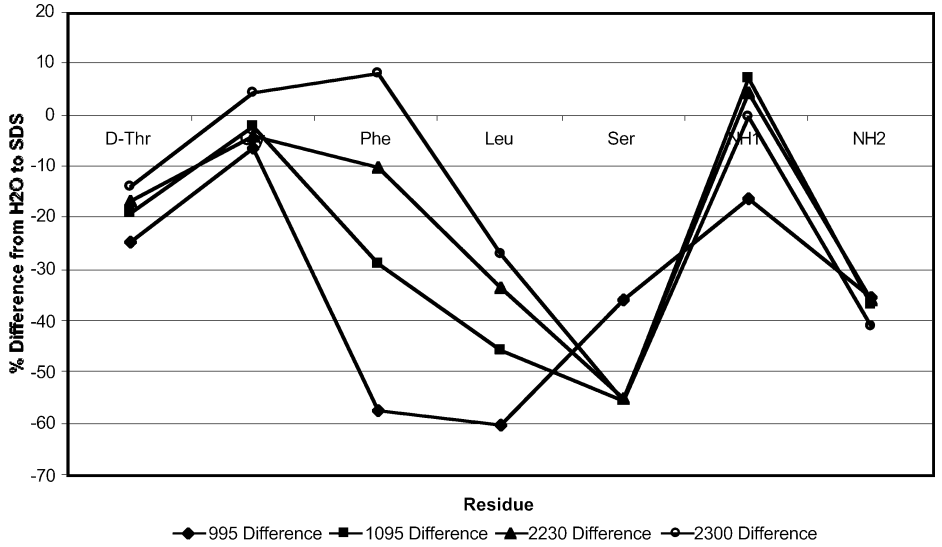

Figure 9. Percent difference in NH exchange rates (H_2O vs SDS) by residue for ***1***, ***2***, ***3***, and ***4***.

Conformers with a maximum RMS deviation from the global minimum lower than 1.0 Å were grouped into a class and overlaid for comparison purposes (Figures 10 and 11). The nOe-constrained Monte Carlo simulations showed two distinct

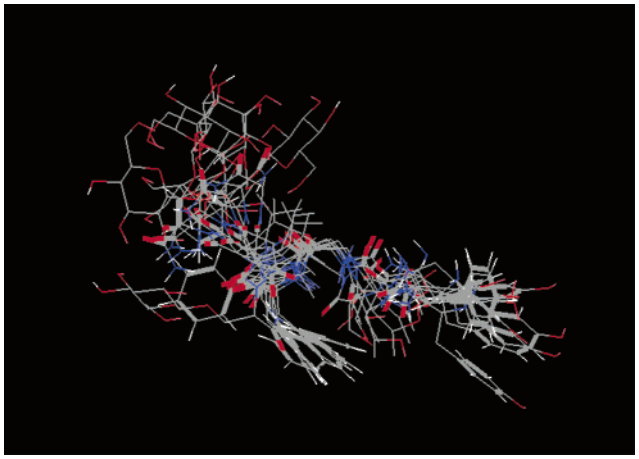

Figure 10. First ensemble (Figure 12, top) of conformations for ***2*** in SDS generated by Monte Carlo calculations, consistent with the observed nOe constraints (9 of lowest 20 conformations).

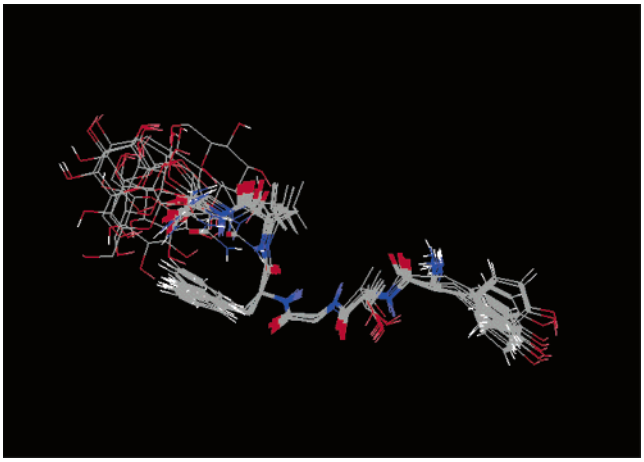

Figure 11. Second ensemble (Figure 12, bottom) of conformations for ***2*** in SDS generated by Monte Carlo calculations, consistent with the observed nOe constraints (10 of lowest 20 conformations).

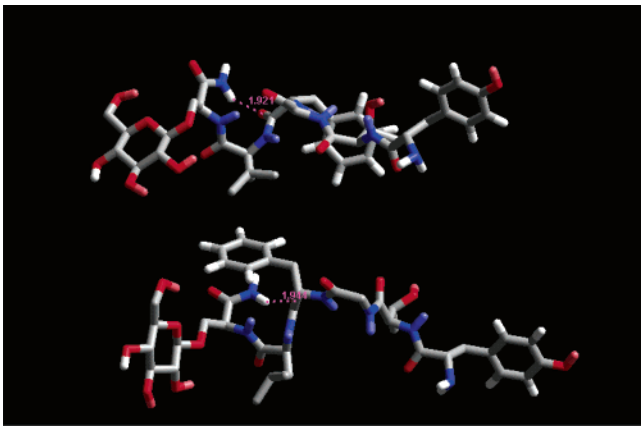

Figure 12. Two representative low energy conformations of ***2*** in SDS generated by Monte Carlo calculations with MacroModel 7.1, with the amide NH ↔ carbonyl C═O (Phe[5]) distances indicated (1.92 Å and 1.94 Å).

backbone conformations for **1**−**4** when adsorbed to a micelle. The first had H-bond stabilization from the amide of D-Thr[2] to the carbonyl of Leu[5], and the second had no such H-bond stabilization (Figure 12). In both cases, a pseudo-β-turn (i + 3rd amino acid = NH_3) was observed from the C-terminal amide to the carbonyl of Phe[4]. Despite the flexibility of the side chains,

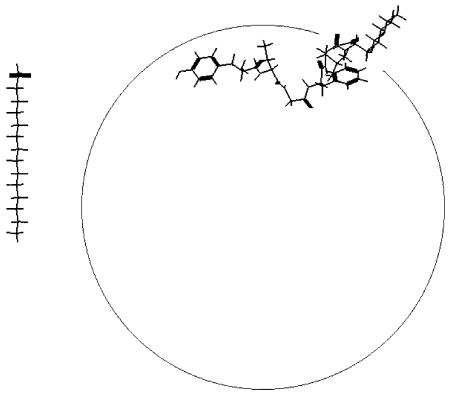

Figure 13. Proposed position and conformation of glycopeptide **2** in an SDS micelle (to scale). A single SDS molecule is shown at left.

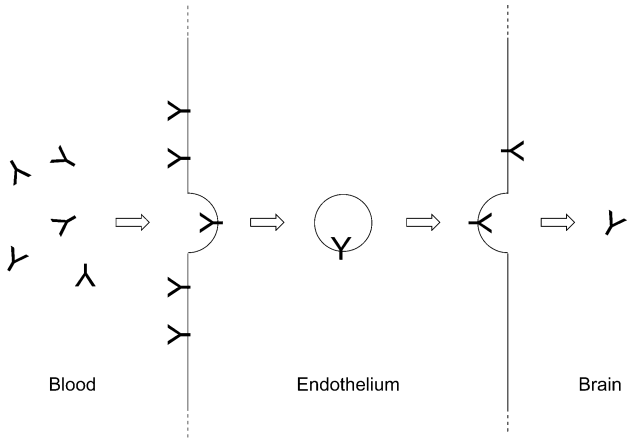

Figure 14. Hypothetical glycopeptide transport scheme. Glycopeptide molecules (Y) can adsorb to the endothelium (capillary lining), penetrate the blood−brain barrier by absorptive endocytosis, followed by exocytosis and migration back into the aqueous phase within the brain.

virtually all of the calculated low-energy conformations fell into one of these two conformational ensembles. The two ensembles differed primarily by rotation about only two dihedral angles in the backbone.

Implications for Glycopeptide Transport Processes. Our hypothesis is that the incorporation of hydrophilic carbohydrate moieties into opioid peptides renders them amphipathic, promoting exchange between lipid and aqueous phases. This can enhance the ability of the resulting glycopeptides to *reversibly* insert into lipid phases, thus allowing for membrane-mediated transport across the endothelial layer (blood−brain barrier) via adsoptive endocytosis and subsequent exocytosis into the brain (Figure 14). Without the carbohydrate moiety a lipophilic opioid peptide can remain within the lipid phase, inhibiting transport and exposing the peptide backbone to enkephalinases and other catabolic peptidases. This "glycosylation strategy" may not be capable of rendering all neurologically active peptides transportable, but has already been validated with a diverse cross-section of peptides and proteins.[40]

Experimental Section

Glycopeptide Assembly and Purification. The 6-residue peptide and glycopeptides were manually synthesized using modified solid-phase FMOC chemistry[41] with HBTU/HOBt promoted peptide coupling (2.0 equiv/2.0 equiv per 1.5 equiv of amino acid). Coupling reaction times varied from 40 to 90 min, and were monitored by the Kaiser

ninhydrin test.[42] The −OAc protecting groups were removed from the carbohydrate with $H_2NNH_2 \cdot H_2O$,[43] and $-OC(CH_3)_3$ side chain protecting groups were cleaved with 90% F_3CCOOH in CH_2Cl_2, which also effected cleavage from the resin. The crude peptides were precipitated with ice-cold ether, filtered, dissolved in water and lyophilized. Purification was carried out on a Perkin-Elmer LC250 HPLC using a preparative-scale (700 × 45 mm) Vydac C_{18} reverse-phase peptide chromatography column. The following conditions were used: a linear AB gradient of CH_3CN/0.1% aq. F_3CCOOH moving from 10 to 50% CH_3CN over 30 min. at a flow rate of 7 mL/min at RT. After preparative HPLC, all fractions were analyzed by analytical HPLC for purity, using a Hewlett-Packard Series II 1040 analytical HPLC, with a linear AB gradient of CH_3CN/0.1% aq. F_3CCOOH moving from 10 of 40% CH_3CN over 40 min. at a flow rate of 1 mL/min at RT. Water used for HPLC purification was triple-filtered, and degassed with argon for 2 h prior to use. HPLC grade CH_3CN was purchased from Fischer Scientific.

NMR Characterization. The purified peptides were characterized by HRMS, ^1H, DQF−COSY, NOESY, and TOCSY[44] NMR. Water suppression in all experiments was achieved using the WATERGATE 3−9−19 pulse sequence with gradients.[45] NMR experiments were performed on Bruker DRX600 (600 MHz), and processed using XwinNmr software (Bruker Inc.) and the Felix2000 package (MSI Inc.). A modified DIPSI 2-rc mixing sequence[46] was used with a TOCSY mixing time of 70 ms, at a spin-lock field of 8.3 kHz. The NOESY mixing time was 100 ms. The TOCSY and NOESY used 88 and 104 transients per FID, respectively, and 400 increments of t_1. Both experiments used a 90°-shifted sine-squared window function in both dimensions. Experiments were conducted at 298°K, and referenced to H_2O at this temperature. Samples were prepared as follows: For glycopeptides in water, 0.5 mg of sample was dissolved in 0.5 mL CD_3COONa/HCl buffer [0.45 mM in H_2O/D_2O (9:1), pH 4.5, 1 mM NaN_3]. For glycopeptide in SDS micelles, 5.5 mg of d_{25} SDS was also added to the mixture, which was sonicated for 5 min prior to the experiments. A stock solution of 2.65 mM $MnCl_2$ was prepared and added to the sample to achieve a total concentration of 200μM in Mn^{2+}. Spin labeled samples were prepared similarly to SDS samples, with a sonication time of 30 min. FAB mass spectral analysis was performed at the University of Arizona Mass Spectrometry Facility. Amide temperature dependence studies were conducted from 25 °C to 40 °C, at 3° increments, allowing 5 min for temperature equilibration. Spectra were referenced to water at that temperature using the formula

$$\delta = 5.013 - (°C/°96.9)$$

$\Delta\delta/\Delta T$ calculations were performed by plotting amide shift in ppm against temperature and creating a best-fit line using linear regression. The slope of the linear regression line was the $\Delta\delta/\Delta T$ for that residue in that media.

Preparation of Liposomes and Equilibrium Dialysis Measurements. The following lipids: 1-palmitoyl-2-oleoyl-sn-glycerophosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-phosphatidylethanolamine (POPE), and cholesterol in the molar ratio (65:25:10) were used to prepare lipid vesicles (LUV). The LUV for the equilibrium dialysis meaurements were prepared from 120 mg of this dried lipid mixture, which was hydrated in 4 mL Hepes buffer (150 mM NaCl, 10 mM Hepes, 1 mM NaN_3, 0.1 mM EDTA, pH 7.4). The lipid suspensions were frozen and thawed 10 times to form extended bilayers, which were then extruded under argon pressure at 40 °C. The size distribution of the LUV were determined by quasielastic light scattering (QELS).

The equilibrium dialysis assay developed by Romanowski et al.[47] was used to determine partitioning of the peptide **1**, and glycopeptides **2**−**4** between the aqueous phase and the LUV. The apparatus for equilibrium dialysis consisted of 5 Teflon cells. Each dialysis cell was separated by a cellulose dialysis membrane, average MW cutoff 12 000−14 000. One ml of 0.1 mM peptide solution in Hepes buffer (pH 7.4) is placed on the cis side of the membrane in each cell, and 1 mL of lipid suspension with different concentrations of lipid (12.5 mM, 25 mM, 37.5 mM, and 50 mM) was placed on the trans side of the membrane. The buffer solution was placed in one cell on the trans side of the membrane. Dialysis was performed overnight at 25 °C in a water bath with the 5 cells mounted in a rotor that ensured homogeneity of the peptide and lipid distribution in the dialysis cells. After dialysis, the concentration of the samples **1**−**4** on the cis side (unbound) and the trans side (bound) was determined by measurement of the intrinsic fluorescence (tyrosine residue, 275 nm excitation/305 nm emission). Duplicate measurements were performed for each sample. The data were analyzed using the nonsaturable partitioning model, which yields the partition coefficient

$$I_O/I = K[L]/(2[W] + 1)$$

where I is the intensity of fluorescence (proportional to the unbound concentration), I_O is the intensity of fluorescence in the cell with no lipid on the trans side, [L] is the concentration of lipids, [W] is the concentration of water, and K is the water−membrane partition coefficient. The membrane phase actually includes both the nonpolar interior as well as any possible association of the solute with the lipid headgroups.[33]

Molecular Modeling. 10 000 Random conformers were generated by using the Monte Carlo search algorithm available for the MacroModel 7.1 package.[48] Nontrivial distance constraints were designated to one of three classes (strong: 1.8−2.5 Å, medium: 1.8−3.3 Å, and weak: 1.8−5.0 Å) by measuring the NOE volume and comparing to a rigid standard (Tyr 3,5H-2,6H at 2.5 Å, Serβ1H-β2H at 1.8 Å, and SerαH-SerβH at a max of 3.0 Å) and applied to each structure before minimization. The Amber force field[49] and GBSA solvent model for water were applied to these structures, and a 50 kJ/mole cutoff was used, resulting in rejection of most of the final conformations.

Circular Dichroism. CD studies were performed on Aviv Associates model 60DS, using an Endcal Model RTE4DD water circulator as a temperature control vehicle, with stoppered cells of 1 cm path length. The instrument was calibrated using 10-camphorsulfonic acid. Peptides were dissolved in triple-filtered deionized water. Concentration was determined by UV. Extinction coefficients for all compounds were calculated to be 1405 M^{-1}.[50] Spectra were observed every 0.5 nm from 250 to 205 nm, using a 1.5 nm bandwidth, and averaged over 4 scans.

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All observed spectra were baseline-subtracted and molar ellipticities were determined using the formula[51]

$$[\theta] = [\theta]_{obs}(MRW)/10(lc) \quad (1c)$$

where $[\theta]_{obs}$ is the observed ellipticity in degrees, MRW is mean residue weight, l is the cell path length in centimeters, and *c* is the peptide concentration in mg/mL. The data were then smoothed using Microsoft Excel 2000 moving average curve-fitting, with a period of seven.

Acknowledgment. We thank the Arizona Disease Control Research Commission (94-15), the NSF (CHE-9526909), the Phoenix Chapter of the ARCS Foundation (Spetzler Fellowship) and the ONR (N00014-02-1-0471) for support of this work. We also acknowledge Prof. Michael Cusanovich for the CD measurement, and Dr. Neil E. Jacobsen for assistance with the NMR measurements. We also note with profound sadness the recent passing of our friend and colleague, Prof. David F. O'Brien.

Supporting Information Available: Complete experimental procedures for the synthesis and purification of peptide **1** and glycopeptides **2**−**4**, ^{1}H NMR spectra in SDS and water, nOe assignments, detailed procedures for the CD measurements, details of the Monte Carlo simulations, and proton exchange rate measurements using NMR are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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