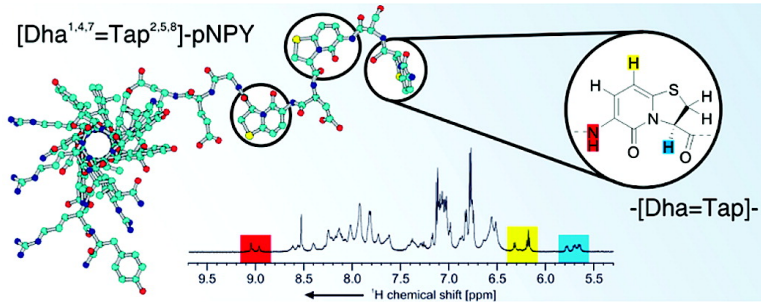
Article

Pyridone Dipeptide Backbone Scan To Elucidate Structural Properties of a Flexible Peptide Segment

Michael Haack, Sebastian Enck, Harald Seger, Armin Geyer, and Annette G. Beck-Sickinger

J. Am. Chem. Soc., **2008**, 130 (26), 8326-8336 • DOI: 10.1021/ja8004495 • Publication Date (Web): 05 June 2008



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES

Published on Web 06/05/2008

Pyridone Dipeptide Backbone Scan To Elucidate Structural Properties of a Flexible Peptide Segment

Michael Haack,[†] Sebastian Enck,[‡] Harald Seger,[‡] Armin Geyer,*[,‡] and Annette G. Beck-Sickinger*[,†]

Institute of Biochemistry, Universität Leipzig, Brüderstr. 34, D-04103 Leipzig, Germany, and Department of Organic Chemistry, Philipps-Universität Marburg, Hans-Meerwein-Str., D-35032 Marburg, Germany

Received January 19, 2008; E-mail: beck-sickinger@unileipzig.de

Abstract: Whereas the C-terminal fragment of neuropeptide Y (NPY) has been structurally well-defined both in solution and as membrane-bound, detailed structural information regarding the proline-rich N-terminus is still missing. The systematic variation of each position by a conformationally constrained pyridone dipeptide building block within the amino terminal segment of NPY leads to a systematic receptor subtype selectivity of the neuropeptide. Thereby, the systematic dipeptide scan proved superior to the traditional L-Ala scan because it showed how to modify the N-terminus in order to obtain increasingly more Y_1 or Y_5 receptor selective ligands. NMR and CD spectroscopic analyses were used to characterize the stepwise rigidification of the N-terminus of NPY when up to three dipeptide building blocks were incorporated by solid-phase peptide synthesis. The pyridone dipeptide increases the hydrophobicity of the amino terminus of NPY, and this allows the tuning of the membrane affinity of NPY. The amphiphilic C-terminal helix of 3-fold-substituted NPY thus becomes visible by selective line broadening in the ^{1}H NMR. Accordingly, we could structurally characterize protein segments that are too flexible for other methods.

Introduction

Neuropeptide Y (NPY) is a linear 36-residue peptide that acts both as a neurotransmitter and as a regulator of hormone secretion.[1–4] Together with the endocrine peptides, peptide YY (PYY) and pancreatic polypeptide (PP), NPY belongs to a family of C-terminally amidated peptides that are involved in the regulation of pharmacologically highly important functions, which recently came into the focus of research.[5–7] These peptides exert most of their biological effects through five rhodopsin-like G protein-coupled Y receptor subtypes.[8–11] Some of them have been found to be overexpressed in human cancer and are therefore promising candidates permitting diagnosis and *in vivo* targeting of tumors by radiolabeled or cytotoxic NPY analogs.[12] Thus, it is of much importance for our understanding beyond receptor function how naturally occurring receptor−ligand systems operate and interact. Crystallization of membrane proteins is still difficult, and the high-resolution structures of bovine rhodopsin and, more recently, of the human β_2 adrenergic receptor still remain the only ones of intact GPCRs so far.[13,14] Whereas MAS NMR analyses appeared for the ligand−receptor complexes of neurotensin and more recently for bradykinin,[15–17] it is still not possible to isolate NPY receptor subtypes as functional complexes, thus foreclosing the direct evaluation of the receptor-bound conformation of NPY. Therefore, structure−activity relationship studies in which the natural peptidic ligand is modified are the basis for the identification of the bioactive conformation of a ligand and for the understanding of its interaction at a distinct receptor subtype.

In 1981 the structure of avian pancreatic polypeptide has been resolved by X-ray crystallography.[18] Also referred to as PP-

[†] Universität Leipzig.
[‡] Philipps-Universität Marburg.

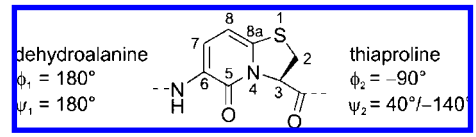
(1) Dumont, Y.; Martel, J. C.; Fournier, A.; St-Pierre, S.; Quirion, R. *Prog. Neurobiol.* **1992**, *38*, 125–67.
(2) Gray, T. S.; Morley, J. E. *Life Sci.* **1986**, *38*, 389–401.
(3) Tatemoto, K. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 2514–8.
(4) Tatemoto, K. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 5485–9.
(5) Batterham, R. L.; Cowley, M. A.; Small, C. J.; Herzog, H.; Cohen, M. A.; Dakin, C. L.; Wren, A. M.; Brynes, A. E.; Low, M. J.; Ghatei, M. A.; Cone, R. D.; Bloom, S. R. *Nature* **2002**, *418*, 650–4.
(6) Tschöp, M. *Nature* **2004**, 430.
(7) Batterham, R. L.; Cowley, M. A.; Small, C. J.; Herzog, H.; Cohen, M. A.; Dakin, C. L.; Wren, A. M.; Brynes, A. E.; Low, M. J.; Ghatei, M. A.; Cone, R. D.; Bloom, S. R. *Nature* **2004**, 430.
(8) Larhammar, D. *Regul. Pept.* **1996**, *62*, 1–11.
(9) Pedrazzini, T.; Pralong, F.; Grouzmann, E. *Cell. Mol. Life Sci.* **2003**, *60*, 350–77.
(10) Gehlert, D. R. *Neuropeptides* **2004**, *38*, 135–40.
(11) Berglund, M. M.; Hipskind, P. A.; Gehlert, D. R. *Exp. Biol. Med. (Maywood)* **2003**, *228*, 217–44.
(12) Körner, M.; Reubi, J. C. *Peptides* **2007**, *28*, 419–25.
(13) Palczewski, K.; Kumasaka, T.; Hori, T.; Behnke, C. A.; Motoshima, H.; Fox, B. A.; Le Trong, I.; Teller, D. C.; Okada, T.; Stenkamp, R. E.; Yamamoto, M.; Miyano, M. *Science* **2000**, *289*, 739–45.
(14) Rasmussen, S. G.; Choi, H. J.; Rosenbaum, D. M.; Kobilka, T. S.; Thian, F. S.; Edwards, P. C.; Burghammer, M.; Ratnala, V. R.; Sanishvili, R.; Fischetti, R. F.; Schertler, G. F.; Weis, W. I.; Kobilka, B. K. *Nature* **2007**, *450*, 383−387.
(15) Luca, S.; White, J. F.; Sohal, A. K.; Filippov, D. V.; van Boom, J. H.; Grisshammer, R.; Baldus, M. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 10706–11.
(16) Luca, S.; Heise, H.; Lange, A.; Baldus, M. *Arch. Pharm. (Weinheim)* **2005**, *338*, 217–28.
(17) Lopez, J. J.; Shukla, A. K.; Reinhart, C.; Schwalbe, H.; Michel, H.; Glaubitz, C. *Angew. Chem., Int. Ed.* **2008**, *47*, 1668–71.
(18) Blundell, T. L.; Pitts, J. E.; Tickle, I. J.; Wood, S. P.; Wu, C.-W. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4175–9.

 10.1021/ja8004495 CCC: $40.75

fold, this structure is characterized by a proline-rich N-terminal part that adopts a polyproline type II helix, which is packed against a well-defined C-terminal α-helix. Both helices are connected *via* a type II β-turn and show an amphiphilic surface. The hydrophobic residues are exposed on the interface area resulting in the interaction of prolines with aromatic side chains of the α-helix through hydrophobic contacts.[19] On the basis of the high homology between PP and NPY, this three-dimensional model was also applied to NPY.[20] Based on NMR data, Darbon et al. have confirmed the hypothesis of the PP-fold, whereas several groups highlighted the existence of dimers.[21–24] Therein, two α-helices which belong to two different molecules face and stabilize each other; the N-terminus remains flexible. The existence of dimers as well as the absence of the N-terminal polyproline helix has finally been confirmed by Bader et al., which characterized the structure of micelle-bound NPY.[25,26] However, due to the lack of amide protons in prolyl residues most of the NMR studies of NPY are limited and only describe the C-terminal part of the molecule in detail. Moreover, the dynamics of this proline-rich part are hard to characterize which made for the description of the N-terminus in the literature to be "flexible". In contrast to the Y_2 receptor subtype, the N-terminal segment is essential for binding at and activation of both Y_1 and Y_5 receptor subtypes.[27–30] In L-Ala scans, the prolyl residues in position 2 and especially in positions 5 and 8 have been found to be important for both affinity and activity by stabilizing the conformation by hydrophobic interactions.[31,32]

Doughty et al. proposed the role of the polyproline helix as to present and orient the functional groups of Tyr^1 for NPY binding site interaction and stabilization of the receptor-active conformation of the α-helix.[33] In 1995 Chu et al. investigated C-terminally truncated NPY fragments by CD and NMR spectroscopy and confirmed the N-terminal contribution to the stability of the C-terminal α-helix.[28,34,35] NPY possesses three prolyl residues within its N-terminal polyproline helix, and consequently, *cis*/*trans* isomerization might occur at the peptidyl−prolyl bonds. Therefore, in search for the bioactive conformation the question arises whether they adopt the *cis* or the *trans* bond conformation when bound to the receptor.[36]

Chart 1[a]



[a] The pyridone dipeptide (Dha=Tap) can be regarded as an N-terminal dehydroalanine (Dha) connected to a C-terminally following thiaproline (Tap). Its incorporation is key to the detailed analysis of peptide segments which are too flexible for characterization and results in a conformational restrained peptide segment, yet leaves the backbone repeat unchanged.

In this study we investigated a novel and systematic rigidification of the peptide backbone within the N-terminal part of NPY by a pyridone dipeptide chromophore (Chart 1).[37] Side-chain-to-backbone ring structures are incorporated to locally constrain the peptide conformation.[38] The introduction of this N-terminally planar building block reduces the backbone conformational space due to three fixed torsion angles. Moreover, the peptidyl−prolyl bond is fixed in a *trans* conformation. Thereby, the sequence and primary structure of native NPY remain essentially unchanged. Our main focus was to investigate long-range effects of the N-terminus on the C-terminal α-helix in solution which have been determined by CD spectroscopy. Effects on the receptor binding profiles at human Y_1 and Y_5 receptor subtypes have been investigated by radio competition assays. The introduction of the novel pyridone dipeptide provided a more detailed insight into the N-terminal structure free in solution as well as in the presence of SDS micelles by means of NMR spectroscopy. This has become possible since the tertiary amides which typically yield little NMR information are locked within the pyridone ring which even gives information about neighboring amino acid residues due to its ring anisotropy.

Experimental Section

Materials. 1,2-Ethanedithiol, *tert*-butanol, *O*-(7-azobenzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), morpholine, piperidine, thioanisole, trifluoroacetic acid (TFA) (for UV spectroscopy), Pefabloc-SC, sodium dodecyl sulfate (SDS), 2,2,2-trifluoroethanol (TFE), potassium dihydrogen phosphate, disodium hydrogen phosphate, and guanidinium chloride (GdmCl) were obtained from Fluka (Buchs, Switzerland); *N*,*N*-diisopropylethylamine (DIEA) was purchased from Aldrich (Steinheim, Germany). Diethyl ether was obtained from Merck (Darmstadt, Germany), and acetonitrile (ACN), from VWR Prolabo (Darmstadt, Germany). 4-(2′,4′-Dimethoxyphenyl-Fmoc-aminoethyl)-phenoxy resin (Rink amide resin) and N^{α}-Fmoc-protected amino acids were purchased from Novabiochem (Läufelfingen, Switzerland) and Iris Biotech (Marktredwitz, Germany), respectively. *N*,*N*′-Diisopropylcarbodiimide (DIC), *N*-hydroxybenzotriazole (HOBt), and TFA (peptide grade) were purchased from Iris Biotech (Marktredwitz, Germany); *N*,*N*′-dimethylformamide (DMF) was obtained from Biosolve (Valkenswaard, The Netherlands). Cell culture media and supplements as well as trypsin-EDTA, bovine serum albumin (BSA), and phosphate buffered saline (PBS) were obtained from PAA Laboratories (Pasching, Austria). $N^{\epsilon 4}$-[propionyl-^{3}H]-pNPY was purchased from Amersham Biosciences/GE Healthcare (Little

(19) Minakata, H.; Taylor, J. W.; Walker, M. W.; Miller, R. J.; Kaiser, E. T. *J. Biol. Chem.* **1989**, *264*, 7907–13.
(20) Allen, J.; Novotny, J.; Martin, J.; Heinrich, G. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 2532–6.
(21) Darbon, H.; Bernassau, J. M.; Deleuze, C.; Chenu, J.; Roussel, A.; Cambillau, C. *Eur. J. Biochem.* **1992**, *209*, 765–71.
(22) Cowley, D. J.; Hoflack, J. M.; Pelton, J. T.; Saudek, V. *Eur. J. Biochem.* **1992**, *205*, 1099–106.
(23) Mierke, D. F.; Durr, H.; Kessler, H.; Jung, G. *Eur. J. Biochem.* **1992**, *206*, 39–48.
(24) Monks, S. A.; Karagianis, G.; Howlett, G. J.; Norton, R. S. *J Biomol NMR* **1996**, *8*, 379–90.
(25) Bettio, A.; Gutewort, V.; Poppl, A.; Dinger, M. C.; Zschornig, O.; Klaus, A.; Toniolo, C.; Beck-Sickinger, A. G. *J. Pept. Sci.* **2002**, *8*, 671–82.
(26) Bader, R.; Bettio, A.; Beck-Sickinger, A. G.; Zerbe, O. *J. Mol. Biol.* **2001**, *305*, 307–29.
(27) Wahlestedt, C.; Yanaihara, N.; Hakanson, R. *Regul. Pept.* **1986**, *13*, 307–18.
(28) Beck-Sickinger, A. G.; Jung, G. *Biopolymers* **1995**, *37*, 123–42.
(29) Borowsky, B.; Walker, M. W.; Bard, J.; Weinshank, R. L.; Laz, T. M.; Vaysse, P.; Branchek, T. A.; Gerald, C *Regul. Pept.* **1998**, *75−76*, 45–53.
(30) Lundell, I.; Eriksson, H.; Marklund, U.; Larhammar, D. *Peptides* **2001**, *22*, 357–63.
(31) Forest, M.; Martel, J. C.; St-Pierre, S.; Quirion, R.; Fournier, A. *J. Med. Chem.* **1990**, *33*, 1615–9.
(32) Beck-Sickinger, A. G.; Wieland, H. A.; Wittneben, H.; Willim, K. D.; Rudolf, K.; Jung, G. *Eur. J. Biochem.* **1994**, *225*, 947–58.
(33) Doughty, M. B.; Hu, L. *Biopolymers* **1993**, *33*, 1195–206.
(34) Chu, S. S.; Velde, D. V.; Shobe, D.; Balse, P.; Doughty, M. B. *Biopolymers* **1995**, *35*, 583–93.
(35) Cabrele, C.; Beck-Sickinger, A. G. *J. Pept. Sci.* **2000**, *6*, 97–122.
(36) Mutter, M.; Wohr, T.; Gioria, S.; Keller, M. *Biopolymers* **1999**, *51*, 121–8.
(37) Seger, H.; Geyer, A. *Synthesis* **2006**, *3224*, 30.
(38) Freidinger, R. M.; Schwenk Perlow, D.; Veber, D. F. *J. Org. Chem.* **1982**, *47*, 104–9.

Chalf-ont, UK), and scintillation cocktail Optiphase HiSafe, from PerkinElmer (Boston, MA, USA).

Synthesis of Dipeptide Building Block Dha=Tap. The synthesis of (3*R*)-6-amino-5-oxo-2,3-dihydro-5*H*-[1,3]thiazolo[3,2-*a*]pyridine-3-carboxylic acid (Dha=Tap) has been described recently.[37] In brief, bicyclic condensation of D-aldurono-2,5-lactone and L-cystein methylester hydrochloride led to a precursor whose α-hydroxy group had been activated and substituted by azide. Elimination of two hydroxyl groups in the presence of mesylchloride and triethylamine followed by immediate hydrogenation (H_2 and Pd/C) in methanol and subsequent protection with Fmoc-OSu in acetone and acidic ester hydrolysis led to the N^{α}-Fmoc-protected pyridone carboxylic acid.

Peptide Synthesis. Synthesis of porcine NPY and analogs was performed as previously described by automated multiple solid-phase peptide synthesis using the orthogonal Fmoc/^{t}Bu strategy and coupling on Rink amide resin (30 mg, loading 0.6 mmol·g^{-1}) to obtain C-terminally amidated peptides.[39] The Dha=Tap building block and the N-terminally preceding amino acid were introduced by a manual coupling step after activation by HATU. Therefore, the building block (1 equiv) and amino acid (3 equiv), respectively, were dissolved in a solution of HATU (1 equiv/3 equiv) in 300 μL of DMF. DIEA (1 equiv/3 equiv) was added, and after 60 min of incubation and shaking at room temperature, the resin was thoroughly washed and the coupling repeated if necessary. The Fmoc-deprotection step between manual coupling steps was accomplished by 50% morpholine in DMF for 10 min and repeated twice. Final removal of the side-chain protecting groups and cleavage of the peptide from the resin were accomplished simultaneously by using a cleavage cocktail consisting of TFA/thioanisole/1,2-ethanedithiol (90:7:3 v/v/v) within 3 h. The peptides were precipitated from ice-cold diethyl ether, collected by centrifugation, washed four times, and finally, dissolved in *tert*-butanol/water (1:3 v/v) and lyophilized. Purification of the crude peptides was achieved by preparative HPLC on a Shimadzu HPLC system (Shimadzu, Columbia, MD, USA) with a Vydac Protein & Peptides C18 column (218TP1022, 10 μm, 300 Å, 22 mm × 250 mm) using 0.08% TFA in ACN (A) and 0.1% TFA in water (B) as the eluting system (linear gradient of 30 to 60% A over 50 min, flow rate: 10 mL·min^{-1}). Characterization of the peptides was performed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry on a Voyager DE-RP workstation (PerSeptive Biosystems, Framingham, MA, USA) and by analytical reversed-phase HPLC on a Merck-Hitachi D/L-7000 HPLC system (Hitachi, Tokyo, Japan) with a Vydac Protein & Peptides C18 column (218TP54, 5 μm, 300 Å, 4.6 mm × 250 mm) using (A) and (B) as the eluting system (linear gradient of 10 to 60% A over 30 min, flow rate: 0.6 mL·min^{-1}).

Cell Culture. The cell lines SK-N-MC endogenously expressing the human Y_1 receptor subtype and HEC-1-B stably expressing the human Y_5 receptor subtype,[40] respectively, were used to investigate the ability of NPY analogs to compete for the binding of radiolabeled NPY. SK-N-MC cells were cultured in Minimal Essential Medium Eagle (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.2% (v/v) MEM nonessential amino acids (100×), 1 mM sodium pyruvate, and 4 mM L-glutamine. HEC-1-B cells were cultured in MEM supplemented with 10% heat-inactivated FBS, 0.2% (v/v) MEM nonessential amino acids (100×), 1 mM sodium pyruvate, 4 mM L-glutamine, and 400 μg·mL^{-1} G-418 sulfate. Cells were grown as monolayers at 37 °C, 5% carbon dioxide, and 95% relative humidity.

Binding Assays. For radioligand binding assay cells were grown to confluence, detached by trypsination with EDTA, and resuspended in incubation buffer consisting of MEM, 50 μM Pefabloc-SC, and 1% BSA. All binding experiments were performed in triplicates and repeated two to four times independently. For saturation analysis 200 μL of cell suspension containing (1−2) × 10^5 cells were incubated with 25 μL of a solution of $N^{\epsilon 4}$-[propionyl-^{3}H]-pNPY in 1% BSA and of increasing concentration (0.2−160 nM). Nonspecific binding was determined by competition of radioligand when adding 25 μL of a solution of 10 μM unlabeled pNPY in 1% BSA; for total-binding 25 μL of a 1% BSA solution were added to give a final volume of 250 μL. In competitive binding experiments 200 μL of cell suspension containing (3−6) × 10^5 cells were incubated with 25 μL of 10 nM radioligand solution and 25 μL of a solution of unlabeled NPY analog at various concentrations in the range between 10 pM and 10 μM. After shaking for 90 min at room temperature, the incubation was terminated by centrifugation for 5 min at 4 °C and 2000 × *g*. The pellets were washed two times with 400 μL of ice-cold PBS, centrifuged, and finally resuspended in 100 μL of PBS. After scintillation cocktail had been mixed with the pellets, bound radioactivity was determined using a liquid scintillation analyzer (Packard Tricarb 2900). Binding data were analyzed with GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA, USA). Saturation curves were fitted to a one-site binding model, and K_d values were calculated. In competition experiments a one-site competition model was used for curve fitting; obtained IC_{50} values were converted into K_I values using the equation of Cheng and Prusoff and the respective K_d values obtained in saturation analysis before.[41]

Signal Transduction Assay. Signal transduction of selected analogs was studied by cotransfection of COS-7 cells with the Y_1 receptor subtype and chimeric G proteins as described before.[42,43] Thereby, EC_{50} and E_{max} values were obtained from concentration−response curves.

Circular Dichroism Spectroscopy. The circular dichroism (CD) spectra of 2.5−10 μM peptide solutions buffered with 10 mM phosphate buffer pH 7, in 50% TFE and in 117 mM SDS, respectively, were measured in the far ultraviolet region from 250 to 190 nm using a Jasco J-715 spectropolarimeter. Cuvettes with a 2 and 5 mm path length as well as the following parameters were used: 20 nm·min^{-1} scanning speed, 4 s response, 0.2 nm step resolution, 2 nm bandwidth, 20 °C temperature . The final spectra were averaged from three baseline-corrected scans and smoothed using the Savitzky−Golay method. Since the helical content f_H of a peptide is proportional to its mean residue ellipticity (MRE) at 222 nm, calculation of the fractional helicity was done using equation $f_{H,obs} = ([\Theta]_{obs} - [\Theta]_C)/([\Theta]_H - [\Theta]_C)$, where $[\Theta]_{obs}$ is the experimentally observed MRE at 222 nm. Values for $[\Theta]_H$ and $[\Theta]_C$, corresponding to maximal helicity and to the completely random coil state assuming a two-state transition processes for NPY, have been experimentally determined by increasing the content of TFE and GdmCl, respectively, to be −24 400 and 870 deg·cm^2·dmol^{-1} at 20 °C.[44−48] To avoid the problem of errors in the peptide concentration that, in turn, may lead to significant errors in the calculated helical content, two additional parameters, R_1 and R_2, have been used. Each is a ratio of intensities in the CD spectrum and therefore independent of the peptide concentration, but both are sensitive measures of the helical content which is present in a peptide that is in equilibrium between α-helical and random coil conformations.[49] R_1 and R_2 were calculated using equations $R_1 = [\Theta]_{190-195nm}/[\Theta]_{207-210nm}$ and $R_2 = [\Theta]_{222nm}/[\Theta]_{207-210nm}$, respec-

(39) Lang, M.; Soll, R. M.; Durrenberger, F.; Dautzenberg, F. M.; Beck-Sickinger, A. G. *J. Med. Chem.* **2004**, *47*, 1153–60.
(40) Moser, C.; Bernhardt, G.; Michel, J.; Schwarz, H.; Buschauer, A. *Can. J. Physiol. Pharmacol.* **2000**, *78*, 134–42.
(41) Cheng, Y.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099–108.
(42) Kostenis, E. *Trends Pharmacol. Sci.* **2001**, *22*, 560–4.
(43) Merten, N.; Lindner, D.; Rabe, N.; Römpler, H.; Mörl, K.; Schöneberg, T.; Beck-Sickinger, A. G. *J. Biol. Chem.* **2007**, *282*, 7543–51.
(44) Woody, R. W. In *The Peptides, Vol. 7*; Hruby, V. J. , Ed.; Academic Press: New York, 1985; pp 15−114..
(45) Chang, C. F.; Zehfus, M. H. *Biopolymers* **1998**, *46*, 181–93.
(46) Marqusee, S.; Robbins, V. H.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5286–90.
(47) Tanford, C. *Adv. Protein Chem.* **1968**, *23*, 121–282.
(48) Tanford, C. *Adv. Protein Chem.* **1970**, *24*, 1–95.
(49) Bruch, M. D.; Dhingra, M. M.; Gierasch, L. M. *Proteins* **1991**, *10*, 130–9.

Table 1. Characterization of Peptides

no.	peptide	sequence	HPLC elution[a]	mass $[M+H]^+$ (m/z)	
				calcd	found
NPY	NPY	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY-NH_2	46 %	4254.7	4254.6
I	[Dha[1]=Tap[2]]-NPY	⌐⌐SKPDNPGEDAPAEDLARYYSALRHYINLITRQRY-NH_2	46 %	4188.7	4188.7
II	[Dha[2]=Tap[3]]-NPY	Y⌐⌐KPDNPGEDAPAEDLARYYSALRHYINLITRQRY-NH_2	47 %	4264.8	4264.9
III	[Dha[4]=Tap[5]]-NPY	YPS⌐⌐DNPGEDAPAEDLARYYSALRHYINLITRQRY-NH_2	47 %	4223.7	4224.3
IV	[Dha[5]=Tap[6]]-NPY	YPSK⌐⌐NPGEDAPAEDLARYYSALRHYINLITRQRY-NH_2	47 %	4236.8	4236.7
V	[Dha[7]=Tap[8]]-NPY	YPSKPD⌐⌐GEDAPAEDLARYYSALRHYINLITRQRY-NH_2	46 %	4237.7	4237.7
VI	[Dha[8]=Tap[9]]-NPY	YPSKPDN⌐⌐EDAPAEDLARYYSALRHYINLITRQRY-NH_2	48 %	4294.8	4295.0
VII	[Dha[12]=Tap[13]]-NPY	YPSKPDNPGED⌐⌐AEDLARYYSALRHYINLITRQRY-NH_2	46 %	4280.8	4280.9
VIII	[Dha[1,4]=Tap[2,5]]-NPY	⌐⌐S⌐⌐DNPGEDAPAEDLARYYSALRHYINLITRQRY-NH_2	47 %	4157.6	4157.6
IX	[Dha[2,5]=Tap[3,6]]-NPY	Y⌐⌐K⌐⌐NPGEDAPAEDLARYYSALRHYINLITRQRY-NH_2	48 %	4246.8	4246.9
X	[Dha[1,7]=Tap[2,8]]-NPY	⌐⌐SKPD⌐⌐GEDAPAEDLARYYSALRHYINLITRQRY-NH_2	50 %	4171.7	4171.3
XI	[Dha[2,8]=Tap[3,9]]-NPY	Y⌐⌐KPDN⌐⌐EDAPAEDLARYYSALRHYINLITRQRY-NH_2	50 %	4304.8	4304.7
XII	[Dha[4,7]=Tap[5,8]]-NPY	YPS⌐⌐D⌐⌐GEDAPAEDLARYYSALRHYINLITRQRY-NH_2	48 %	4206.7	4206.6
XIII	[Dha[5,8]=Tap[6,9]]-NPY	YPSK⌐⌐N⌐⌐EDAPAEDLARYYSALRHYINLITRQRY-NH_2	48 %	4276.8	4276.6
XIV	[Dha[1,4,7]=Tap[2,5,8]]-NPY	⌐⌐S⌐⌐D⌐⌐GEDAPAEDLARYYSALRHYINLITRQRY-NH_2	50 %	4140.6	4140.3
XV	[Dha[2,5,8]=Tap[3,6,9]]-NPY	Y⌐⌐K⌐⌐N⌐⌐EDAPAEDLARYYSALRHYINLITRQRY-NH_2	50 %	4286.8	4287.2
XVI	NPY_{1-9}	YPSKPDNPG-NH_2	n. d.	973.5	973.5
XVII	NPY_{1-13}	YPSKPDNPGEDAP-NH_2	n. d.	1385.4	1385.5
XVIII	[Dha[1,4,7]=Tap[2,5,8]]-NPY_{1-13}	⌐⌐S⌐⌐D⌐⌐GEDAP-NH_2	n. d.	1271.3	1271.5
XIX	NPY_{10-36}	EDAPAEDLARYYSALRHYINLITRQRY-NH_2	n. d.	3298.7	3298.8
XX	[Dha[12]=Tap[13]]-NPY_{12-36}	⌐⌐AEDLARYYSALRHYINLITRQRY-NH_2	n. d.	3078.5	3078.8
XXI	NPY_{14-36}	AEDLARYYSALRHYINLITRQRY-NH_2	n. d.	2884.5	2884.5

[a] elution of the peptide at % A (0.08% TFA in acetonitrile); ⌐⌐ dipeptide building block Dha=Tap. (Chart 1)

tively, as well as the local minima and maxima of the molar ellipticity in the given wavelength range.

NMR Experiments. The peptides were dissolved in 0.6 mL of H_2O/D_2O (5:1) containing 80 to 100 equiv of perdeuterated sodium dodecyl sulfate (SDS-d25). All measurements were performed on a Bruker Avance DRX 600 spectrometer with a 5 mm BBI probe head. Water suppression was achieved by excitation sculpting with gradients (double watergate DPFGSE sequence).[50] Homonuclear 2D spectra (TOCSY, NOESY) were recorded at 320 K in the phase-sensitive mode as data matrices of 512 (t_1) real × 2048 (t_2) complex data points; 48 scans (TOCSY) and 80 scans (NOESY), respectively, were used per t_1 increment. The used spectral widths were between 6602 and 9014 Hz. Mixing times of 100 ms (TOCSY) and 150 ms (NOESY) were applied. Heteronuclear 2D HSQC experiments of native NPY and monosubstituted derivatives were performed at 300 and 320 K in the phase-sensitive mode with data matrices of 512 (t_1, ^{13}C) real × 2048 (t_2, ^1H) complex data points and 80 scans per t_1 increment. The used spectral width was 7211 (^1H) and 24 146 Hz (^{13}C). The HSQC measurement of [Dha[1,4,7]=Tap[2,5,8]]-NPY was carried out at 320 K with data matrices of 384 (t_1, ^{13}C) real × 2048 (t_2, ^1H) complex data points, 160 scans were performed per t_1 increment, and spectral widths of 8013 (^1H) and 12073 Hz (^{13}C) were chosen. All data were recorded and analyzed using Bruker TopSpin software. The spectra were calibrated on the well-separated SDS-CH_3 signal which appears temperature-independent at 0.772 ppm. This chemical shift is related to sodium 3-(trimethylsilyl)propionic acid as an internal reference with $\delta = 0.00$ ppm.

Results

Peptide Synthesis. All peptides were synthesized on the Rink amide resin by automated solid-phase peptide synthesis using the orthogonal Fmoc/^{t}Bu strategy to obtain C-terminally amidated peptides. For coupling of the N^{α}-Fmoc-protected dipeptide building block Dha=Tap as well as for the N-terminally preceding amino acid, the coupling reagent HATU has been used.[51–53] The excess of amino acid could be reduced from 10-fold and double coupling in automated synthesis to a single manual coupling step with 3-fold excess. Introduction of the dipeptide building block in either a matched or mismatched position led to two sets of peptides with single, double, and 3-fold substitution, respectively. In the matched position, the thiaproline (Tap) moiety of the building block substitutes a prolyl residue within the native NPY sequence (residues 2, 5, 8, and 13). Mismatched substitution led to peptides with the dehydroalanine (Dha) moiety in the position of the native proline. Additionally, to investigate the contribution of N- and C-termini in the CD spectra, various terminally truncated analogs of NPY were synthesized. Final cleavage of the peptides from the resin was achieved by TFA. Subsequently, the peptides were characterized by HPLC and mass spectrometry. Peptides were purified by preparative HPLC to obtain products with >95% purity. The analytical data of the synthesized peptides are shown in Table 1.

Circular Dichroism Spectroscopy. CD studies of NPY as well as of its Dha=Tap containing analogs were performed at concentrations of 5 and 10 μM in 10 mM phosphate buffer at pH 7, 117 mM SDS micelles and in 50% TFE, respectively. The presence of a single isodichroic point at 201 nm in TFE titration of NPY indicated a two-state transition process between random-coil and helical structures and has been assumed for all analogs (data not shown). The CD spectra of all peptides were indicative of a notable amount of α-helical structure in a TFE/water mixture and, to a lower extent, also in SDS micelles. R_1 values are in the range between −1.5 and −1.9 in SDS micelles as well as between −1.8 and −2.1 in TFE/water, respectively (data not shown). The presence of a helical structure is also confirmed by R_2 values which are 0.8 to 0.9 in SDS

(50) Hwang, T. L.; Shaka, A. J. *J. Magn. Reson., Ser. A* **1995**, *112*, 275–9.
(51) Albericio, F.; Bofill, J. M.; El-Faham, A.; Kates, S. A. *J. Org. Chem.* **1998**, *63*, 9678–83.
(52) Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397–8.
(53) Carpino, L. A.; El-Faham, A. *J. Org. Chem.* **1995**, *60*, 3561–4.

Table 2. Receptor Binding Affniities and CD Data of Full Length NPY Analogs

	binding at SK-N-MC Y_1		binding at HEC-1-B Y_5		characteristic CD values in 10 mM phosphate buffer pH 7			
no.	K_I [nM]	relative to NPY[a]	K_I [nM]	relative to NPY[a]	$f_{H,obs}$[b]	R_1[c]	R_2[d]	isodichroic point [nm]
NPY	9.3 ± 3.2	1	23 ± 1.4	1	0.57	−1.5	0.9	201.5
I	78 ± 1	8.4	101 ± 27	4.4	0.61	−2	1.3	202.3
II	99 ± 8	10.6	57 ± 8	2.5	0.22	−1.6	0.8	202.3
III	85 ± 3	9.1	211 ± 19	9.2	0.32	−41.1	21.4	204.6
IV	91 ± 10	9.8	91 ± 6	4	0.48	−1.4	0.9	200.5
V	39 ± 7	4.2	130 ± 5	5.7	0.31	−1.2	0.8	200.5
VI	109 ± 5	11.7	196 ± 12	8.5	0.21	−1.1	0.7	201.5
VII	7.4 ± 1	0.8	30 ± 4	1.3	0.50	−1.5	0.9	201
VIII	180 ± 35	19.4	241 ± 16	10.5	0.60	−2.2	1.4	203
IX	201 ± 9	21.6	77 ± 16	3.3	0.14	−1.5	0.8	203.9
X	76 ± 10	8.2	142 ± 11	6.2	0.12	−1.9	0.6	202.2
XI	342 ± 32	36.8	175 ± 20	7.6	0.12	−1.2	0.6	200.6
XII	92 ± 4	9.9	188 ± 27	8.2	0	−3.4	−1.2	208.4
XIII	213 ± 54	22.9	86 ± 12	3.7	0.12	−1.1	0.7	204.2
XIV	304 ± 62	32.7	286 ± 43	12.4	0.27	−7	5.2	206.4
XV	326 ± 12	35.1	140 ± 18	6.1	0.09	−1.3	0.6	204.5

[a] Relative binding is given by K_I(analog)/K_I(NPY). [b] $f_{H,obs} = ([\Theta E]_{obs} - [\Theta]_C)/([\Theta]_H - [\Theta]_C)$ with $[\Theta]_H = -24\,400$ deg·cm^2·dmol^{-1} and $[\Theta]_C = 870$ deg·cm^2·dmol^{-1}. [c] $R_1 = [\Theta]_{190-195\ nm}/[\Theta]_{207-210\ nm}$. [d] $R_2 = [\Theta]_{222\ nm}/[\Theta]_{207-210\ nm}$.

micelles and 0.9 to 1.0 in TFE/water, respectively. Moreover, all spectra have a minimum near 222 nm and a second minimum between 207 and 210 nm. The helical content of all analogs is in the range of that of native NPY: 64−70% in SDS micelles and 85−95% in 50% TFE, respectively, with respect to the maximal helical content observed for NPY in 100% TFE. This suggests that disturbances of the C-terminal α-helix caused by the N-terminal segment can be prevented by addition of TFE and SDS. Characteristic CD values including ratios R_1 and R_2 as well as fractional helicity f_H and isodichroic points obtained in 10 mM phosphate buffer at pH 7 are summarized in Table 2. Introduction of Dha=Tap at position 4/5 results in a significant loss of helicity as values for R_1 and R_2 are not indicative of an α-helical peptide, with the exception of peptide VIII. In addition, the isodichroic points of peptides III, XII, and XIV are notably shifted to longer wavelengths. In contrast, single substitutions at positions 1/2, 5/6, and 12/13, respectively, do not decrease or even slightly increase the helicity of the respective analog. Accordingly, introduction of Dha=Tap in position 1/2 is able to compensate the helix-destabilizing effect of Dha=Tap in position 4/5 as shown for peptide VIII. Darbon et al. described a structure of two adjacent but discontiguous α-helices comprising residues 15−26 and 28−35,[21] the interdigitation of Dha^1=Tap^2 and Tyr^{27} due to increased hydrophobic interactions might contribute to the observed helix stabilization. The extent of disturbance depends on the exact position of the substitution by Dha=Tap, but no systematic correlation is in evidence. Thereby, two different effects can be observed. Values of the molar ellipticity [Θ] at 222 nm decrease with the number of Dha=Tap substitutions as given by f_H. Independently thereof, the values for R_1 and R_2 describing the shape of the spectrum and the "quality" of the helix are changed. Obviously, the most critical position is 4/5, where substitution led to the most distinct destabilization of the C-terminal helix.

In addition, the CD spectra of several terminally truncated NPY analogs were obtained to distinguish whether the resulting overall CD spectrum results just from the sum of the individual spectra of N- and C-terminus or from a mutual interaction of both termini. Both the measured spectrum of the mixture of the modified N-terminus (peptide XVIII) and native C-terminus (peptide XXI) as well as the calculated spectrum of the two fragments are quite similar and of decreased helical content. The latter can be increased by TFE but without reaching the extent of full-length NPY. In contrast, the 3-fold matched-substituted full-length analog (peptide XIV) exhibits a completely different spectrum in phosphate buffer indicating the loss of α-helical structure, which in turn can be retained in TFE reaching a value equal to that of native NPY (Figure 1). This destabilization might be due to the introduced conformational constraints as well as the increased hydrophobicity of the N-terminus and resultant interactions.

The observation that the resulting CD spectrum is not only given by the sum of the spectra of the individual termini was also found for unmodified NPY_{1-13} (peptide XVII) and the corresponding C-terminal NPY_{14-36} (peptide XXI) as well as for NPY_{1-9} (peptide XVI) and NPY_{10-36} (peptide XIX). Moreover, the spectra reveal that both the 3-fold substituted (Figure 1) and unsubstituted (data not shown) N-termini are unstructured and contribute only to a minor amount to the overall CD spectrum. Single or multiple introduction of Dha=Tap does not alter the CD spectrum of the N-terminus. In contrast to NPY_{14-36}, the helical content of the N-terminally elongated C-terminal fragment NPY_{10-36} can be increased up to that of full-length NPY in TFE. This suggests that an N-terminal truncation of 13 residues will decrease the stability of the C-terminal helix in NPY, which is in agreement with the work of Gurrath et al.[54] A truncation of nine residues does only slightly influence the C-terminal α-helix.

In addition, capping of NPY_{14-36} by Dha=Tap in position 12/13 (peptide XX) only slightly increases the helical content in phosphate buffer and is therefore not sufficient to stabilize the α-helix. The resulting C-terminal fragment shows a helical content comparable to NPY_{14-36} rather than to NPY_{10-36} (Figure 2). Assuming a K_d value of 2 μM, the CD studies have been performed at concentrations in which NPY is monomeric to an extent of 27%.[19,22,55] The data confirm the role of the N-terminus to stabilize the C-terminal α-helix in aqueous solution by hydrophobic interactions.[19,33,56,57] Whereas elongation of NPY_{14-36} by Dha^{12}=Tap^{13} only slightly increases the helical

(54) Gurrath, M.; Bisello, A.; Bottazzo, K.; Chung, C. W.; Mammi, S.; Peggion, E. *J. Pept. Sci.* **1996**, *2*, 176–93.
(55) Nordmann, A.; Blommers, M. J.; Fretz, H.; Arvinte, T.; Drake, A. F. *Eur. J. Biochem.* **1999**, *261*, 216–26.
(56) Cabrele, C.; Wieland, H. A.; Langer, M.; Stidsen, C. E.; Beck-Sickinger, A. G. *Peptides* **2001**, *22*, 365–78.
(57) Hu, L.; Balse, P.; Doughty, M. B. *J. Med. Chem.* **1994**, *37*, 3622–9.

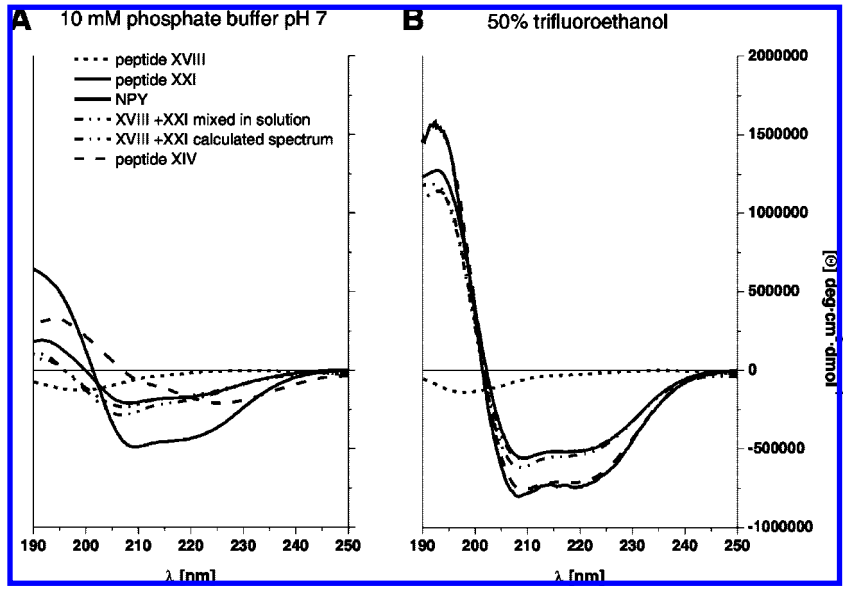


Figure 1. CD spectra of the 3-fold matched-substituted NPY analog (peptide XIV), its modified N-terminus (peptide XVIII), and the corresponding C-terminus (peptide XXI) as well as the experimentally obtained and calculated spectra of the mixture of N- and C-termini in phosphate buffer (A) and 50% trifluoroethanol (B).

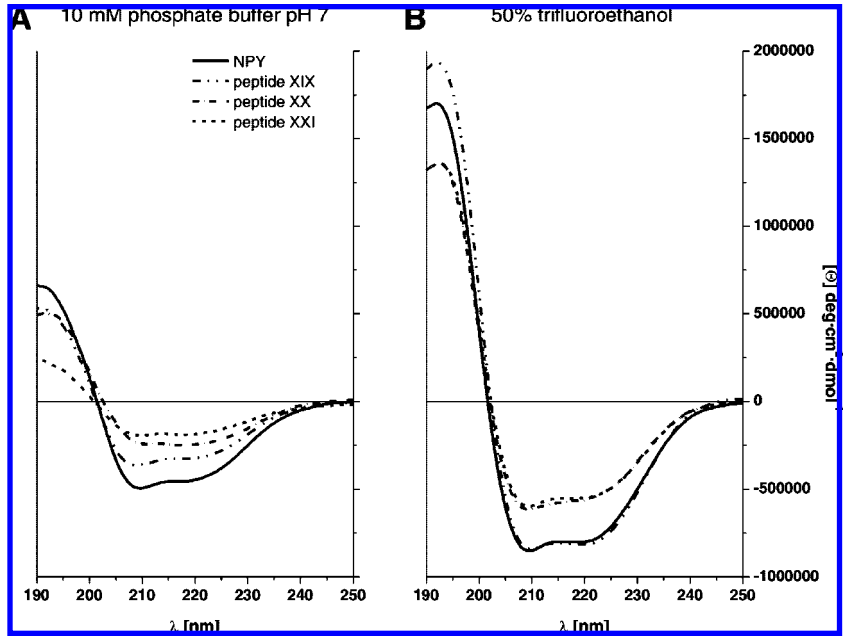


Figure 2. CD spectra of NPY and of the C-terminal NPY fragments NPY_{10-36} (peptide XIX), [Dha^{12}=Tap^{13}]-NPY_{12-36} (peptide XX) and NPY_{14-36} (peptide XXI) in phosphate buffer (A) and 50% trifluoroethanol (B).

content of the C-terminus, in particular Glu^{10} and Asp^{11} may serve as flanking residues and hydrogen bond donors and contribute to the formation and stabilization of the α-helix.[58]

Saturation Binding Assays. By using radiolabeled NPY in concentrations from 20 pM to 16 nM and by applying a one-site binding model for curve fitting, saturation curves have been obtained for SK-N-MC cells that express the Y_1 receptor subtype and HEC-1-B cells that express the Y_5 receptor subtype. K_d values of 3.6 ± 0.1 nM and 7.2 ± 0.4 nM, respectively, have been identified.

Competitive Binding Assays. To further characterize the influence of amino acid substitution by Dha=Tap the binding of all NPY analogs was tested by using a radio competition assay on human cell lines selectively expressing the Y_1 and Y_5 receptor subtype, respectively. IC_{50} values obtained after applying a one-site competition model were converted into K_I values. Binding affinities as well as the relative binding compared to unmodified NPY are summarized in Table 2.

With the exception of peptide VII all analogs show decreased affinities at the Y_1 and Y_5 receptor subtype. Substitutions by Dha=Tap are much better tolerated by the Y_5 receptor subtype which show only a 3- to 12-fold loss of affinity independent of the number of substitutions. Here, the exact position of substitution is rather important. Interestingly, mismatched substitutions of Pro^2 and Pro^5 led to significantly higher affinities than the corresponding matched ones. In contrast, binding at

(58) Presta, L. G.; Rose, G. D. *Science* **1988**, *240*, 1632–41.

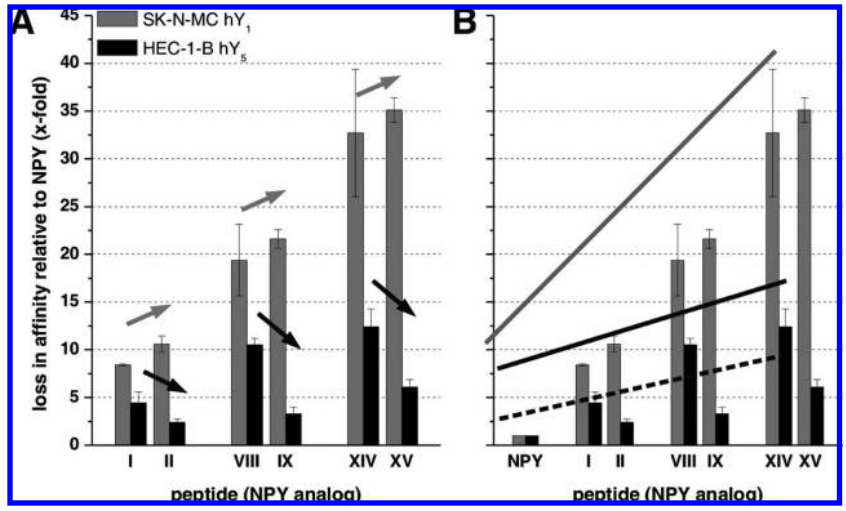


Figure 3. Receptor trends. (A) Receptor-dependent differences of NPY analogs with matched and mismatched substitutions. Gray arrows point up the only minor decreases in Y_1 receptor affinity that are associated with the shift from a matched to a mismatched position of Dha=Tap. Black arrows are indicative of the more distinct and contrary binding behaviour at the Y_5 receptor subtype. (B) Receptor selectivity of NPY analogs indicating that Dha=Tap substitutions are much better tolerated by the Y_5 receptor subtype which in addition shows distinct differences between matched (solid line) and mismatched positions (dashed line).

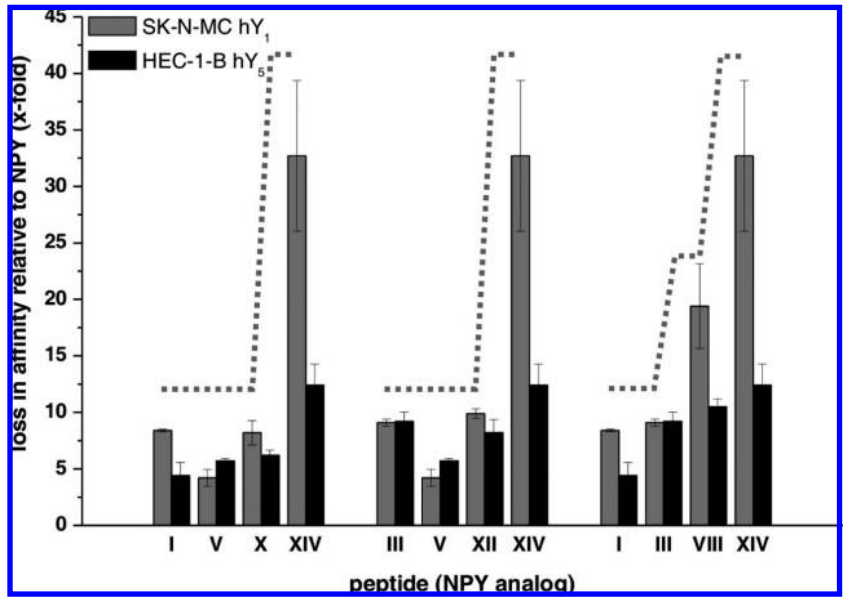


Figure 4. Loss in affinity of the 3-fold matched-substituted analog (peptide XIV) is mainly due to the concomitant introduction of Dha=Tap in positions 1/2 and 4/5 which on its own (peptide VIII) led to a K_I value that results additively from those of the singly substituted analogs (peptides I and III). In contrast, concomitant introduction of the pyridone dipeptide in positions 1/2 and 4/5, respectively, as well as in position 7/8 did not result in increased K_I values (peptides X and XII) compared to the singly substituted peptides (peptides I and III).

the Y_1 receptor subtype is characterized by its dependence of the number of substitutions. A single introduction of Dha=Tap led to an average 10-fold loss of affinity. With few exceptions regarding position 7/8, additional substitutions result in NPY analogs that lost affinity up to 37-fold. Y_1 receptor substitutions in a matched position have unexceptionally led to higher affinities than mismatched substitutions of the same prolyl residue. Thereby, the differences in binding affinities between matched and mismatched are only minor when Pro^2 and Pro^5, respectively, were substituted individually or in combination. The differences are more distinct (up to 5-fold) when Pro^8 was involved in single or multiple substitutions, respectively. The receptor-dependent differences of the substitution in either a matched or mismatched position are shown in Figure 3.

Table 3. Signal Transduction Assay of Selected Analogs

no.	EC_{50} [nM]	EC_{50} relative to NPY[a]	E_{max} [dpm]	E_{max} relative to NPY
NPY	2	1	11297	1
I	30	15	9476	0.84
V	20	10	11457	0.93
VI	158	79	10530	1.01

[a] Relative values are given by EC_{50}(analog)/EC_{50}(NPY) and E_{max}(analog)/E_{max}(NPY).

At both receptor subtypes the binding affinities of peptides of the matched set are less influenced by substitution of Asn^7-Pro^8. Comparing peptides I/X and III/XII, respectively, the K_I values do not increase due to the additional substitution in position 7/8. In fact, the increase in K_I values of the 3-fold substituted analog (peptide XIV) is rather influenced by the

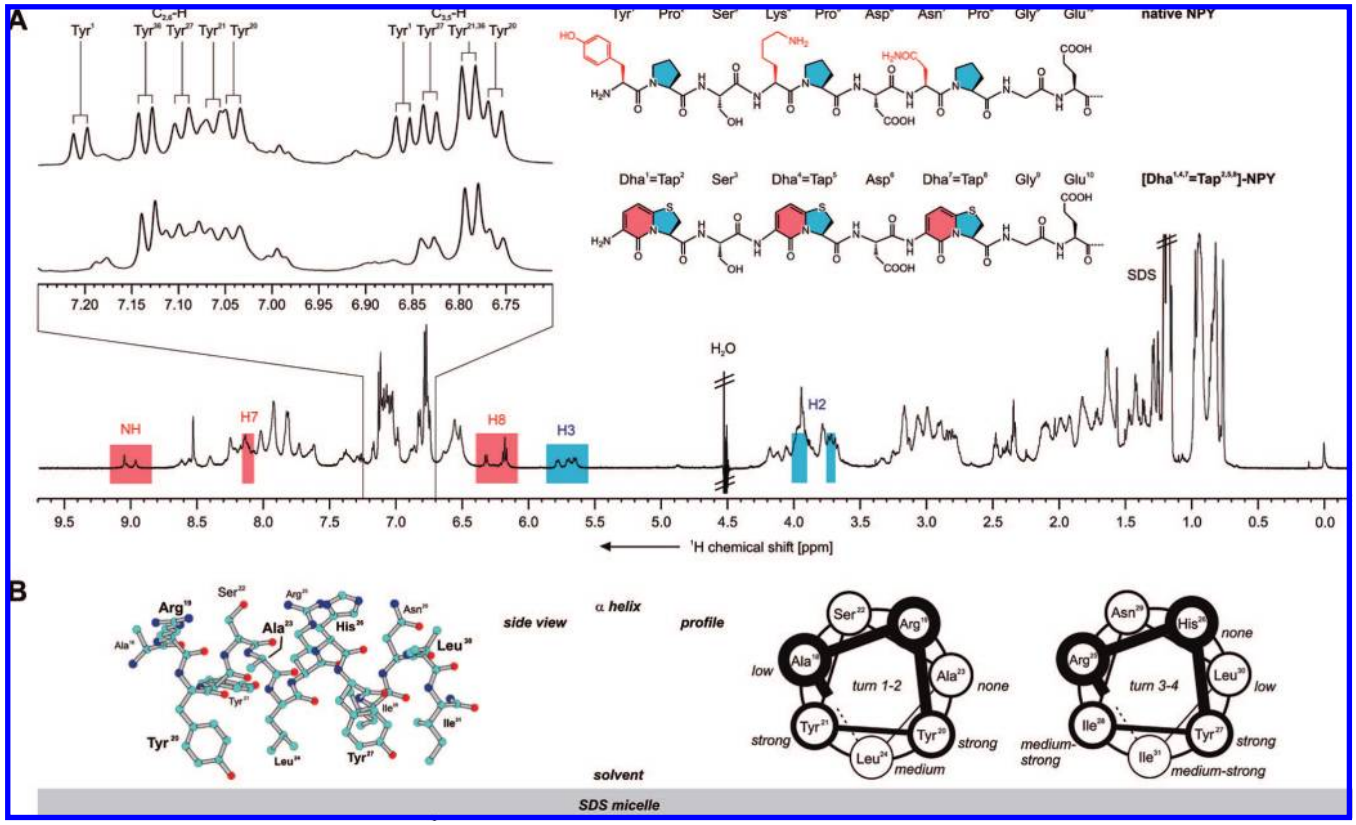


Figure 5. A selective line broadening within the ^{1}H NMR spectrum was observed after increasing the hydrophobicity of the N-terminus. (A) Depiction of the Tyr region of the ^{1}H NMR spectra (320 K, H_2O/D_2O 5:1, 100 equiv of SDS-d25) of native NPY (top) and peptide XIV (bottom) and the respective N-termini (residues 1−10). While the signals of the Tyr^{20}, Tyr^{21}, and Tyr^{27} side chains appear broadened in peptide XIV, this is not observed for the C-terminal Tyr^{36}. For the native NPY in the same solvent environment this is not observed for any of the Tyr side chains. This results from a stronger interaction of the more lipophilic peptide with the SDS detergent. As in the complete ^{1}H NMR spectrum of peptide XIV, two signals of the Dha and one signal of the Tap units are greatly separated from the crowded regions. This leads to an advantageous simplification of NMR data evaluation. (B) The line broadening data of residues located in the α-helical section coincides with the α-helix orientation on the micelle surface postulated by Bader et al.[26] A part of the α-helical segment of NPY (residues 18−31) is shown perpendicular to (B left) and along the helical axis (B right), together with the observed extents of line broadening.

concomitant substitution of Pro^2 and Pro^5, which was especially found to be more impressive for the Y_1 receptor subtype (Figure 4).

Introduction of the pyridone dipeptide building block not only leads to a more hydrophobic N-terminus and to the loss of possible side chain interactions of the substituted amino acid residues preceding (matched) or following (mismatched) the prolyl residue within the native sequence. Moreover, new interactions based on π-stacking interactions due to the aromatic building block might become possible. We have previously investigated the contribution of each amino acid side chain of NPY to the receptor binding by exchanging systematically each single residue by L-Ala. The most sensitive positions within the N-terminal segment have been found to be Pro^2 and Pro^5 at the Y_1 receptor, since their replacement provoked the most detrimental loss of affinity up to 500-fold and 1000-fold, respectively.[32,59] Not even the simultaneous substitution of all three prolyl residues and their neighboring residues resulted in such a decrease in affinity. At the Y_5 receptor subtype, residues Pro^5 and Pro^8 have been found to be the positions that are most sensitive to replacement by L-Ala within the N-terminus. Again, substitution by Dha=Tap did not exceed a 12-fold loss in affinity in comparison to the 70-fold loss in affinity for [Ala^8]-NPY.

Signal Transduction Assay. Results of the signal transduction assay that has been performed for selected analogs are shown in Table 3. Values of E_{max} are given with regard to NPY and are indicative of receptor activation in an agonistic manner by the Dha=Tap substituted NPY analogs.

NMR Spectroscopy. Figure 5A shows the complete ^{1}H NMR spectrum of peptide XIV in the membrane mimetic environment of SDS micelles. One single signal set is obtained because of the absence of *cis*/*trans* isomerism around tertiary amide bonds. Relevant resonance signals of the bicyclic lactam appear in empty sections of the ^{1}H NMR between 5.5 and 6.5 ppm as well as around 9 ppm which is helpful for the further signal assignment and the later identification of relevant NOEs. Furthermore, expansions of the aromatic region of the ^{1}H NMR spectrum of peptide XIV and of native NPY are shown. The most striking feature resulting from a comparison of both spectra is a selective line broadening which is limited only to certain residues, yet over almost the whole peptide. This shows a distinctive long-range effect of the Dha=Tap units over more than 20 residues. Such a line broadening is expected for hydrophobic molecules which associate with the SDS micelles, and it therefore scales with the increasing hydrophobicity of the NPY analogs of Table 1. It is very pronounced for peptide XIV where the hydrophobic N-terminus is completely associated with the micelles and therefore experiences the line broadening. Well resolved resonances are detected for every third to fourth amino acid between Leu^{17} and Ile^{31} while the terminal four amino acids are again well resolved. Figure 5B shows an interpretation of this observation by analysis of the amphiphilic

(59) Eckard, C. P.; Cabrele, C.; Wieland, H. A.; Beck-Sickinger, A. *Molecules* **2001**, *6*, 448–67.

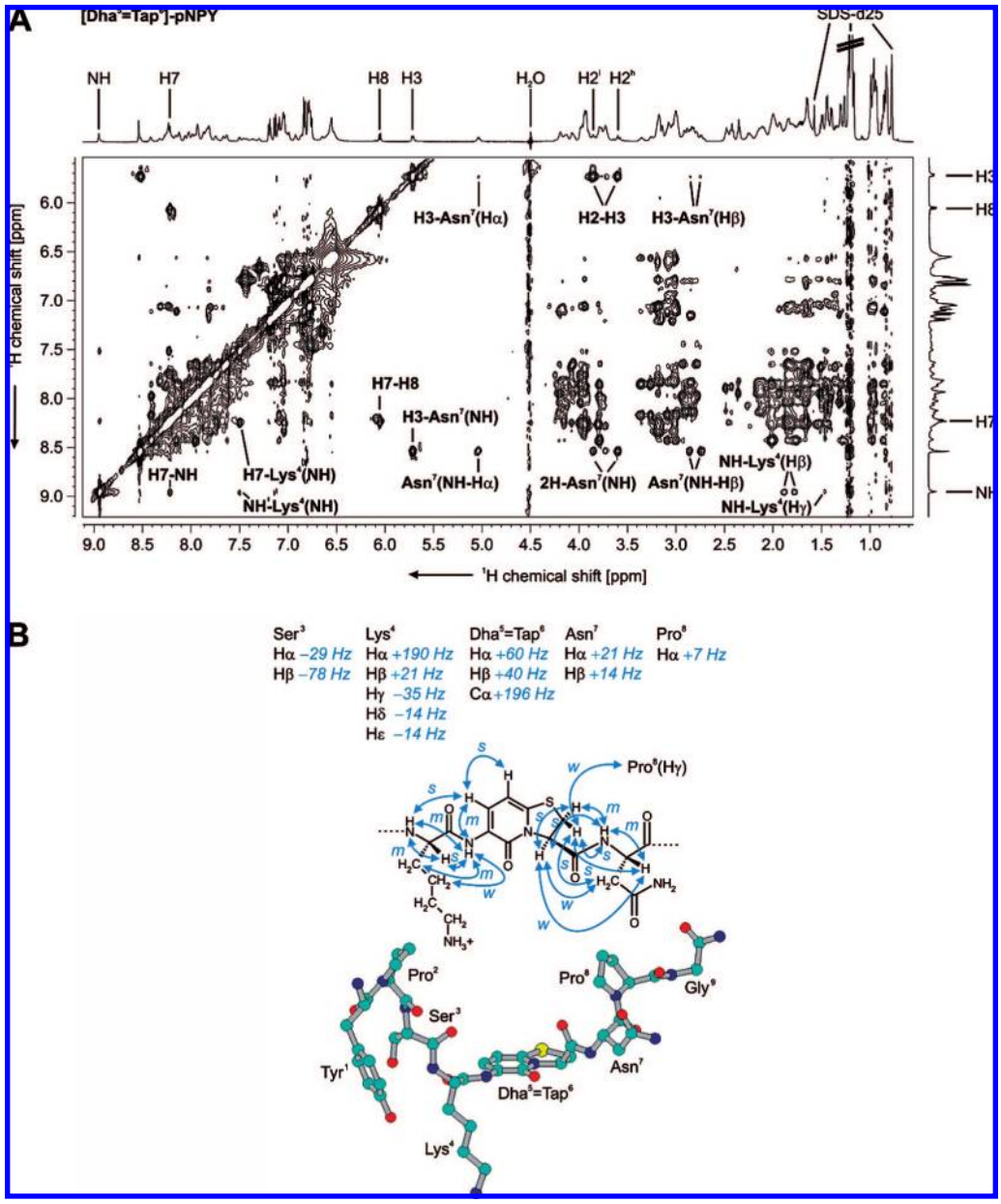


Figure 6. NOE based structure elucidation of the N-terminus of peptide IV. (A) Region of the NOESY spectrum of peptide IV (320 K, H_2O/D_2O 5:1, 90 equiv of SDS-d25) with assigned NOE within the Dha=Tap unit and to the adjoining residues. (B) Depiction of the changes in chemical shifts (in Hz) compared to native pNPY which result from the shielding or deshielding effect of the aromatic pyridone ring. Below, the structure of the N-terminus (residues 1−9), calculated on basis of the shown NOEs, is depicted. NOEs are assigned as *s* (strong), *m* (medium), and *w* (weak).

helical section of NPY. In agreement with the orientation of the α-helix on the membrane according to Bader et al.,[26] the residue selective broadening of NMR signals in the case of the 3-fold substituted peptide XIV is limited to residues located toward the surface. These results assume that the NMR signal broadening of peptide XIV is caused by a slower chemical exchange, namely through the slower micelle on ↔ off equilibrium of this more hydrophobic peptide compared to native NPY.

The location of some Dha=Tap NMR signals outside the crowded spectral regions facilitates data evaluation of 2D NMR spectra, as can be seen from the depiction of a NOESY region of peptide IV (Figure 6A). The NOE assignments are based on the nearly complete identification of backbone and side chain resonances, and the local NOE pattern around the Dha=Tap dipeptide together with the NOE based calculated structure of the peptide segment (Figure 6B) gives an impression of the local environment of the pyridone dipeptide. The aromatic character of the pyridone ring has considerable shielding or deshielding effects on neighboring amino acids, yielding valuable information on the geometry of the continuing backbone relative to the aromatic Dha=Tap plain. Long-range chemical shift variations drop below measuring uncertainty after Glu^{10} even for the 3-fold substituted peptide XIV. Selected NMR data of the N-terminal stretch of peptide XIV and a computer-generated model are shown in Figure 7. Three Dha=Tap dipeptide building blocks exert significantly stronger shielding or deshielding effects on Gly^9 and Glu^{10} than one dipeptide building block.

For the completely assigned NPY-mimics we observed that the pronounced extended character of the N-terminal eight amino acids scales with these de/shielding effects. The more rigidified

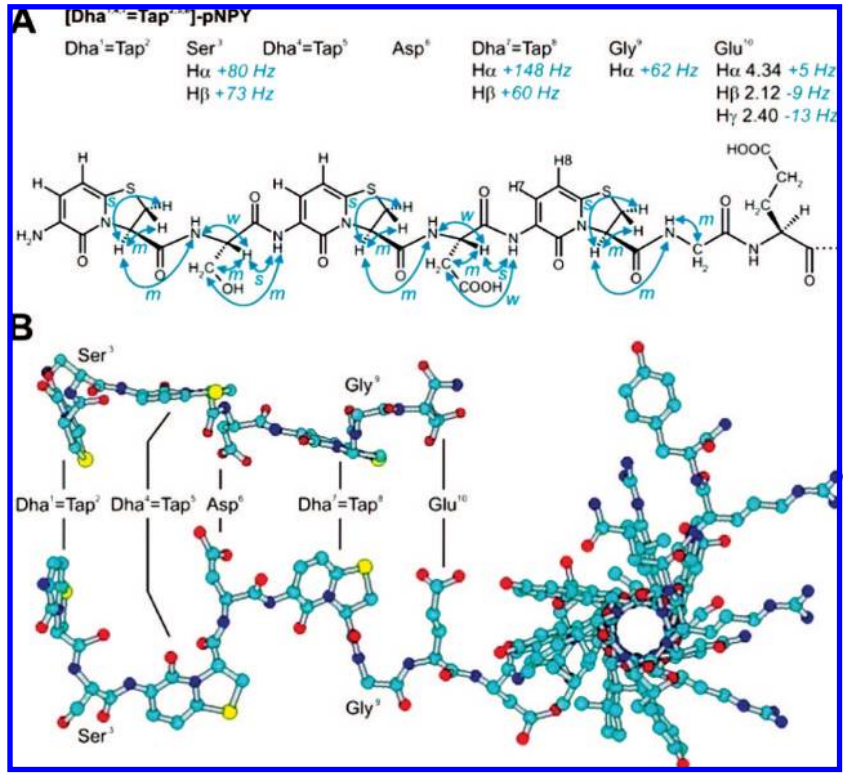


Figure 7. NOE based structure elucidation of the 3-fold substituted N-terminus of peptide XIV. (A) Depiction of the changes in chemical shifts (in Hz) compared to native NPY and of NOEs (s = strong, m = medium, w = weak). (B) The NOE based calculated structure of the N-terminal segment (residues 1−10) shows a remarkable in-plane orientation of the [Dha^4=Tap^5] and [Dha^7=Tap^8] bicyclic systems. This matches very well with the increased membrane affinity of the 3-fold substituted peptide (Figure 5), as this conformation of the N-terminus effects an optimal coordination of the hydrophobic pyridones to the membrane. Below, the calculated structure associated to the whole NPY is shown (view along the α-helical axis).

the N-terminus, the more pronounced the kink at Gly^9 which serves as a joint between the N-terminus and the subsequent helical part.

Discussion and Conclusion

The molecular conformation of NPY in solution and of the N-terminal segment in particular has been extensively and controversially debated in the literature. Two classes of structural models have been proposed so far. The first is derived from NMR analysis of human NPY and [Leu^{31},Pro^{34}]-NPY and closely resembles the hairpin-like fold found in the crystal structure of dimeric avian pancreatic polypeptide (aPP).[18,21,60] In this model, the N-terminus is packed against the C-terminal α-helix, which in turn is stabilized by hydrophobic interactions. This solution structure has also been found for bovine pancreatic polypeptide (bPP) and peptide YY (PYY).[61,62] Based on NMR data of NPY in water and TFE, in the second model the N-terminal segment is fully flexible, whereas the C-terminus (residues 11−36 in water and 19−34 in TFE, respectively) is in an α-helical conformation.[22–24,63] Only few authors dealt with the conformation of NPY fragments. Whereas N-terminal segments comprising the proline-rich region have been found to be unstructured and biologically inactive regarding affinity as well as activity, the Y_2 receptor subtype-specific fragment NPY_{13-36} is completely α-helical.[34,64]

In this study, NPY has been the subject of the first systematic N-terminal backbone scan known so far. Herein, a pyridone-based dipeptide building block has been used as a backbone conformational constraint that limits the degrees of freedom and constrains the synthetic peptide into a more distinct conformation. This concept not only plays an important role for the design of peptidomimetics in the drug development process but also is a well-known approach for enhancing receptor-selectivity and modulating efficacy.[38,65] Moreover, the appearance of additional signals within a hitherto empty area of the NMR spectrum significantly simplifies the signal assignment. It was therefore possible to investigate the structure of the N-terminal segment as part of the whole NPY molecule by means of NMR spectroscopy in more detail. The building block can be easily introduced by means of solid-phase peptide synthesis. This concept can therefore be extended onto many other peptidic ligands that bear prolyl residues within their sequence. Those residues usually complicate NMR signal assignments due to the lack of amide protons, and structural information has not been available so far.

Since conformational studies in solution do not permit any definite conclusions with regard to the receptor-bound conformation, especially of the *cis*/*trans* peptidyl−prolyl bond conformation within the N-terminus, receptor binding studies have been performed. Their results indicate that both receptor

(60) Khiat, A.; Labelle, M.; Boulanger, Y. *J. Pept. Res.* **1998**, *51*, 317–22.
(61) Li, X. A.; Sutcliffe, M. J.; Schwartz, T. W.; Dobson, C. M. *Biochemistry* **1992**, *31*, 1245–53.
(62) Keire, D. A.; Kobayashi, M.; Solomon, T. E.; Reeve, J. R. *Biochemistry* **2000**, *39*, 9935–42.
(63) Saudek, V.; Pelton, J. T. *Biochemistry* **1990**, *29*, 4509–15.
(64) Arvidsson, K.; Jarvet, J.; Allard, P.; Ehrenberg, A. *J. Biomol. NMR* **1994**, *4*, 653–72.
(65) Perdih, A.; Kikelj, D. *Curr. Med. Chem.* **2006**, *13*, 1525–56.

subtypes tolerate conformational constraints introduced by the pyridone dipeptide, which fixes the N-terminal peptidyl−prolyl bond in the *trans* conformation.

Comparison of the loss in receptor affinity of the Dha=Tap substituted analogs with results obtained from L-Ala scan studies reveals that the prerequisites of the peptide ligand seem to be based on hydrophobic interactions rather than on interactions of the side chains and their orientation.[32,59] The binding affinity to the Y_1 receptor decreased with the increasing number of substitutions by Dha=Tap, whereas the exact position is crucial for Y_5 receptor binding. Those binding biases result in a significant selectivity of the NPY analogs for either the Y_1 or Y_5 receptor subtype. Exemplarily investigated analogs show receptor activation in an agonistic manner, which is assumed to be true for all analogs. In order to distinguish the contributions of hydrophobicity and the *cis*/*trans* peptidyl−prolyl bond conformation for receptor binding and activation, respectively, further studies are currently undertaken using proline analogs that induce or force the *cis*-conformation. However, the obtained data shed light on the topological requirements of different Y receptor subtypes and might therefore be useful for the design of new ligands of pharmacological interest.

In addition, structural studies of terminal fragments as well as substituted NPY analogs using CD spectroscopy clearly reveal a significant mutual interaction of both N- and C-termini. Thereby, the C-terminal α-helix of native NPY is stabilized by the N-terminus. These findings are in agreement with the work of Forest et al. and Beck-Sickinger et al. and can only be explained if one assumes a solution structure similar to the PP-fold in which both termini get into close proximity.[31,32]

The pyridone ring leads to a bathochromic shift of the amide chromophores of the N-terminal part of NPY resulting in the effective decoupling of the CD response of the N- and C-terminal halves of NPY. Here, the pyridone ring is a beneficial backbone chromophore which does not alter the peptide backbone repeat of $(N-C^{\alpha}-CO-)_n$ or the distance between the N- and C-terminus of NPY. Depending on the number and exact position, the introduction of the pyridone building block led to a more or less distinct destabilization of the C-terminal α-helix. Thereby, the helicity did not correlate with the observed receptor binding profile. Interestingly, the disturbance of the helical structure observed in phosphate buffer can be revoked by TFE and in the presence of SDS micelles, respectively. All analogs show the same structure as unmodified NPY, which can be considered as a prerequisite for receptor binding and activation.

No NOEs are visible across the pyridone ring of the Dha=Tap dipeptide, an observation which underlines the extended rigid character of Dha=Tap. This dipeptide completely locks three backbone torsions and allows only two preferred conformations for Ψ_2. The examination of the selective line broadening of the amino acids incorporated in membrane association allows for the direct observation of the amphiphilic helix without the necessity of performing relaxation measurements.

The systematic dipeptide scan led to systematic trends in the NMR data of the examined NPY mimics. The membrane association which was extensively studied by Bader et al.[26] is directly visible in the ^{1}H NMR of peptide XIV. Three Dha=Tap dipeptides significantly reduce the flexibility of the N-terminus. As NPY tends to be globular, the most efficient way to achieve this is the kink at the joint Gly^9. This bending is especially pronounced in peptide XIV which becomes visible in the chemical shifts of the Gly^9-Glu^{10} segment. The rigidified N-terminal peptide segment rotates relatively independently of the C-terminal helix. Ten snapshots from a vacuum molecular dynamics simulation are shown in Figure 8. Under vacuum simulation conditions the globular trend is additionally pronounced and results in a smooth back-folding of the N-terminus which is very reminiscent of the proposed receptor-bound conformation.[59] Starting structures with Pro^{13} in the *cis*-configuration do not converge in a comparable fashion.

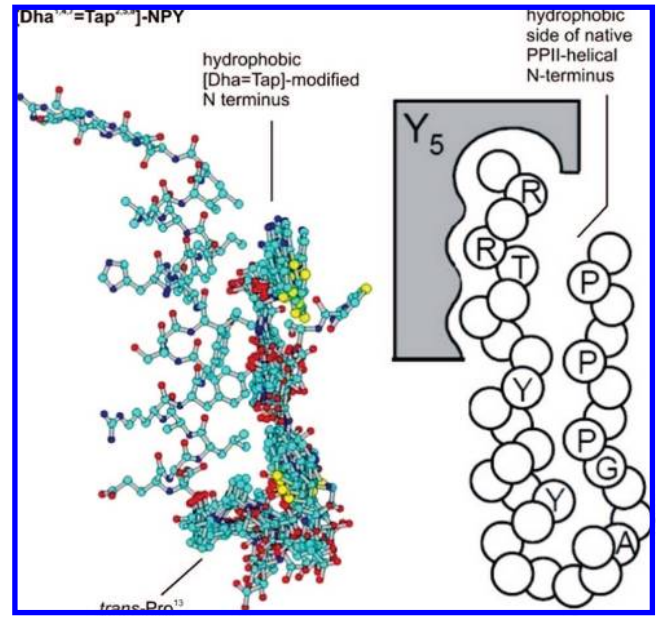


Figure 8. The NOE-based calculated structure of the 3-fold substituted N-terminal fragment of peptide XIV (Figure 7) was frozen, and the whole peptide was calculated. The simulations showed a strong trend of a back-folding onto the α-helix. Compared to the postulated hY$_5$-bound conformation of NPY,[59] there is a noticeable similarity of both structures. The modified N-terminus seems to mimic the hydrophobic side of the PPII-helix formed by the native segment.

The systematic dipeptide scan within the peptide sequence which contains three proline residues proved superior to the traditional L-Ala scan because it shows a direction of how to modify the N-terminus in order to obtain increasingly more Y_1 or Y_5 selective ligands. Dha=Tap is an advantageous label for NMR spectroscopy where it brings signal dispersion into crowded spectral regions. UV and CD spectroscopy profit from the extension of the amide chromophore of the peptide backbone toward an aromatic heterocyclic ring system.

Acknowledgment. This paper is dedicated to Professor Manfred Mutter on the occasion of his 65th birthday. The authors thank Doris Haines and Kristin Löbner for their technical assistance in peptide synthesis, Regina Reppich for recording mass spectra, and Jan Stichel for the signal transduction assays. Financial support of the DFG (Be 1264/9-1, SFB 610) and of the Studienstiftung des Deutschen Volkes (a scholarship for S.E.) and the donation of chemicals by Evonik Degussa GmbH are gratefully acknowledged.

Supporting Information Available: Complete ref 6 as well as method and results of structure calculation and molecular modeling. This material is available free of charge via the Internet at http://pubs.acs.org.

JA8004495