Journal of Peptide Science
*J. Peptide Sci.* **8**: 671–682 (2002)
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.428

# Electron Paramagnetic Resonance Backbone Dynamics Studies on Spin-Labelled Neuropeptide Y Analogues

**ANDREA BETTIO,[a] VOLKER GUTEWORT,[b] ANDREAS PÖPPL,[c] MICHAELA C. DINGER,[a] OLAF ZSCHÖRNIG,[b] KLAUS ARNOLD,[b] CLAUDIO TONIOLO[d] and ANNETTE G. BECK-SICKINGER[a]***

[a] Institute of Biochemistry, University of Leipzig, Talstrasse 33, D-04103, Leipzig, Germany
[b] Institute for Medical Physics and Biophysics, University of Leipzig, Liebigstrasse 27, D-04103, Leipzig, Germany
[c] Faculty of Physics, University of Leipzig, Linnestrasse 5, D-04103, Leipzig, Germany
[d] IBC, CNR, Department of Organic Chemistry, University of Padua, Via Marzolo 1, 35131 Padua, Italy

*Received 4 July 2002*
*Accepted 10 July 2002*

Abstract: Neuropeptide Y (NPY) is one of the most abundant peptides in the central nervous system of mammalians. NPY acts by binding to at least five G-protein coupled receptors (GPCRs) which have been named $Y_1$, $Y_2$, $Y_4$, $Y_5$ and $Y_6$. Three spin-labelled NPY analogues containing the nitroxide group of the amino acid TOAC (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid) as a paramagnetic probe were synthesized by solid-phase peptide synthesis. Synthetic problems owing to the sensitivity of nitroxide towards acidic and reducing conditions have been overcome by using a cleavage cocktail that contains anisole and cresol scavengers. Concerning the receptor binding preferences, the analogues [TOAC$^{34}$]-pNPY and [Ala$^{31}$,TOAC$^{32}$]-pNPY showed a marked selectivity for the $Y_5$ receptor, while [TOAC$^{2}$]-pNPY maintained a significant binding also to the $Y_2$ receptor subtype. The modifications of the native peptide structure caused by the introduction of TOAC were examined by circular dichroism. In order to determine the rotational correlation time of the spin probes, electron paramagnetic resonance measurements were performed in solution and in the presence of liposomes. This allowed us to evaluate the backbone dynamics of the different parts of the NPY molecule in the free and membrane bound states. The results of these studies showed that NPY interacts with liposomes by using the *C*-terminal $\alpha$-helix while the *N*-terminal tail retains a flexibility that is comparable to that of the peptide in solution as already shown by NMR studies on DPC micelles. Furthermore, we demonstrated that TOAC-labelling is a valuable tool to investigate changes in the backbone conformation and dynamics. This may be of major importance for peptides and small proteins when they bind to cell membranes. 

Keywords: circular dichroism; EPR; liposomes; neuropeptide Y; NPY; rotational correlation time; spin-labelling; TOAC

Abbreviations: As recommended in *J. Peptide Sci.* **5**: 465–471 (1999) with the following additions and variations; 7 BHK, baby hamster kidney; DPC, dodecylphosphatidylcholine; EDT, 1,2-ethanedithiol; EPR, electron paramagnetic resonance; ESI electron-spray ionization; FT-ICR, Fourier transformation ion cyclotron resonance; GPCR, G-protein coupled receptor; LUV, large unilamellar vesicles; MEM, minimum essential medium; MES, 2-morpholinoethanesulfonic acid; $\alpha$-MSH, $\alpha$-melanocyte stimulating hormone; NPY, neuropeptide Y; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PC, phosphatidylcholine; Pmc, 2,2,5,7,8 pentamethylchroman-6-sulfonyl pNPY, porcine neuropeptide Y; PP, pancreatic polypeptide; PS, phosphatidylserine; PYY, peptide YY; TIS, triisopropylsilane; TOAC, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid.

*Correspondence to: Prof. Dr Annette G. Beck-Sickinger, Institute of Biochemistry, University of Leipzig, Talstrasse 33, D-04103, Leipzig, Germany; e-mail: beck-sickinger@uni-leipzig.de
Contract/grant sponsor: Deutsche Forschungsgemeinschaft; Contract/grant numbers: DFG Be 1264/5-1; DFG Ar 195/8-1.

## INTRODUCTION

NPY consists of 36 amino acids, is *C*-terminally amidated and was isolated from the porcine brain in 1982 [1]. It is one of the most abundant peptides in the central nervous system of mammalians [2], and acts both as a hormone and neurotransmitter. Many important physiological effects are transmitted by NPY, for example the regulation of blood pressure and of food intake, as well as the modulation of its own release and that of other hormones. PP and PYY are two other peptide hormones that belong to the so-called NPY family [3]. They all share the chain length of 36 amino acids, the *C*-terminal amidation and a high homology in the primary structure with NPY. Five distinct receptors, which specifically bind NPY/PP peptides have been cloned so far. They have been named $Y_1$, $Y_2$, $Y_4$, $Y_5$ and $y_6$ and belong to the family of the heptahelical G-protein coupled receptors. A further $Y_3$ receptor has been characterized only pharmacologically and its existence remains unclear [3]. The structure and the dynamics of NPY in solution and in the micelle bound state have been determined by NMR studies [4,5]. In solution the NOE pattern of NPY supports the presence of an amphipathic $\alpha$-helix, which spans residues 13 to 36 [5] while the *N*-terminal part of the peptide does not show any ordered structure. Motional parameters concerning the backbone dynamics were derived from the application of the 'model-free-approach' [6] and indicate that the helical part from residues 17 to 32 is rigid while the 'peripheral' regions of the $\alpha$-helix are more flexible [4]. Furthermore, it was demonstrated that in solution NPY forms dimers in which the hydrophobic face of the aliphatic $\alpha$-helix was used as a dimerization interface [4]. A parallel and an antiparallel orientation of the dimers exist in equilibrium with a partially unfolded monomer. By reducing the NPY concentration in solution the equilibrium shifts towards the unfolded monomer [7]. The results of the NMR studies on NPY bound to DPC micelles led to a three-step model of receptor selection [4]. The peptide binds to membranes as a monomer by using the same hydrophobic face of the $\alpha$-helix as for dimerization [4]. It has been hypothesized that membrane bound NPY moves at the surface of the cell membrane to get into contact with the receptor (Figure 1). In comparison to the structure in solution, the binding to membranes results in an increased rigidity of the *C*-terminal tetrapeptide, which is due to the anchoring of $Tyr^{36}$ residue [4].

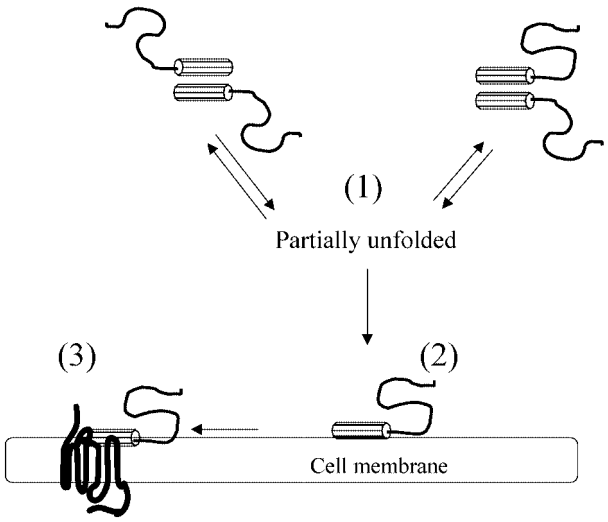



Figure 1 The three-steps model of receptor activation for NPY. Two different types of dimer exist in equilibrium with a partially unfolded monomer in solution (1). The peptide structure in the dimer form is characterized by a *C*-terminal $\alpha$-helix, represented in the picture by a cylinder, and a flexible *N*-terminal tail. The peptide binds to the cell membrane as a monomer (2) and undergoes a two-dimensional diffusion that brings it to bind the receptor (3).

This structural change is supposed to be part of the appropriate orientation of the $Arg^{33,35}$ residues that are fundamental for receptor binding [8].

The importance of the study of the backbone dynamics for the characterization of the binding of peptides and proteins to cell membranes has already been demonstrated by NMR studies performed with micelles [4,9]. As a model for the study of the interaction of peptide hormones with cell membranes, micelles suffer from two main drawbacks: their reduced dimension (diameter smaller than 20 nm for DPC micelles) and the fact that they are constituted by a phospholipid monolayer. On the other hand, liposomes made of larger vesicles (diameter up to 3 μm) that form double layers of phospholipids cannot be employed in NMR measurements because their dimension produces large rotational correlation times which lead to short transverse relaxation times ($T_2$). This effect results in a broadening of the NMR signals.

Among the various biophysical techniques that can help to elucidate the structure and dynamics of proteins and peptides, electron paramagnetic resonance (EPR) (also termed electron spin resonance) is more and more successfully employed [10,11]. Parameters such as the mobility of the paramagnetic

probe, solvent accessibility and distance between two paramagnetic centres located at different positions can be measured and used to obtain important structural and dynamic information. Furthermore, EPR can be successfully applied to the study of liposomes and liposome-bound peptides [12,13]. The most frequently used paramagnetic centre (spin-label) for biologically relevant systems is the nitroxide radical, which is often attached to the sulphydryl group of a Cys residue [11]. However, this side-chain modification results in a flexible spacer connecting the peptide backbone to the nitroxide spin, whose mobility is therefore frequently too high to reflect the flexibility of the peptide scaffold. The non-proteinogenic amino acid TOAC (Figure 2) has already been extensively used in biochemistry and materials science [14]. This molecule is equipped with a nitroxide radical inserted into the rigid piperidine ring whose carbon atom at position 4 represents the $C^{\alpha}$ atom of the amino acid. The significant reduction of the intrinsic nitroxide flexibility renders TOAC a very suitable tool for the investigation of the mobility of the peptide scaffold by EPR. Detailed conformational analysis on TOAC-containing peptides [10,15–17] has shown that TOAC, like other $C^{\alpha}$-tetrasubstituted $\alpha$-amino acids [18], is a strong stabilizer of $\beta$-turns and $3_{10}/\alpha$-helical structures. The employment of TOAC in solid-phase peptide synthesis has been limited in the past by its low reactivity and the sensitivity of the nitroxide radical to acidic and reducing conditions which are required to cleave the peptide from the resin or to remove protecting groups. The synthesis of a TOAC-labelled $\alpha$-melanocyte stimulating hormone ($\alpha$-MSH) analogue was the first preparation of a bioactive peptide hormone (reported in 1999 [19]). Furthermore a general protocol for the solid-phase peptide synthesis has been recently published [20]. In the present contribution we report our experience on the synthesis of three TOAC-labelled NPY analogues (Figure 3), in which the spin label has been introduced at three different positions. In particular, we developed a protocol for the cleavage of Arg- and TOAC-containing peptides assembled by the Fmoc/*t*Bu strategy to avoid the formation of sulfonated byproducts. One of these analogues ([TOAC$^{34}$]-NPY) has been already successfully employed to probe the dimerization interface of NPY in aqueous solution [4]. In this study we demonstrated that these spin labelled NPY analogues maintain binding properties at the $Y_5$ receptor subtype and that they can be used to obtain information on the dynamics of the different parts of the NPY molecule in solution and when it is bound to liposomes.

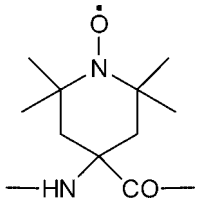



Figure 2 Chemical structure of the amino acid residue TOAC.

## MATERIALS AND METHODS

### Peptide Synthesis

Peptide synthesis was performed by the automated solid-phase method following the Fmoc/*t*Bu strategy. In order to obtain the peptide amide, 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-aminomethyl resin (Rink amide AM resin, Novabiochem, Läufelfingen, Switzerland) was used. The standard coupling steps were performed automatically on a peptide synthesizer (Syro II, MultiSynTech, Bochum, Germany). Each coupling step was repeated twice using 30 mg resin (loading 0.5 mmol/g), a 10-fold molar excess of

| | 1 10 20 30 |
|---|---|
| pNPY | YPSKPDNPG**E**DAPAEDLARYYSALRHYINLITRQRY-$NH_2$ |
| [TOAC$^{2}$]-pNPY | YXSKPDNPG**E**DAPAEDLARYYSALRHYINLITRQRY-$NH_2$ |
| [Ala$^{31}$,TOAC$^{32}$]-pNPY | YPSKPDNPG**E**DAPAEDLARYYSALRHYINLAXRQRY-$NH_2$ |
| [TOAC$^{34}$]-pNPY | YPSKPDNPG**E**DAPAEDLARYYSALRHYINLITRXRY-$NH_2$ |

Figure 3 Amino acid sequences of pNPY and of the three spin-labelled analogues. X = TOAC.

Fmoc-amino acid and diisopropylcarbodiimide/1-hydroxybenzotriazole, and a coupling time of 35 min in 0.6 ml DMF. The Fmoc group was removed by treatment with 20% piperidine in DMF for 20 min. The coupling steps involving the TOAC amino and carboxyl group of TOAC were performed manually as follows: 3 eq of Fmoc-amino acid were activated by 3 eq of HATU and 6 eq of DIPEA. The reaction time was set to 4 h. In order to monitor the completion of the reaction during these critical coupling steps, a small-scale cleavage was performed on a few milligrams of resin and the resulting products were analysed by MS and HPLC. When required, the coupling reaction was repeated. This treatment was especially required in the coupling steps involving the TOAC amino group. Finally, the resin was washed several times with DMF, DCM, methanol, diethyl ether and dried under vacuum. The final cleavage step was performed by suspending the resin in a mixture of TFA/anisole/*p*-cresol 90 : 5 : 5 for 3 h. The crude product was precipitated from cold diethyl ether, centrifuged and washed several times with the same solvent. After drying under nitrogen flow the peptides were dissolved in aqueous ammonia at pH 9 to recover the partly lost radical character, stirred at room temperature for 3 h and lyophilized. Purification was performed by reverse-phase semi-preparative HPLC on a Vydac 218TP510 column ($C_{18,}$ 300 Å pore size, 5 µm particles, 10 × 250 mm, Vydac, Hesperia, CA, USA). The eluting system was 0.08% TFA in acetonitrile (A) and 0.1% TFA in water (B) applying a linear gradient from 30% to 65% A over 30 min at a flow rate of 2.5 ml/min. As the HPLC eluant contains TFA, the basic treatment in aqueous ammonia had to be repeated after the HPLC purification.

## Peptide Analysis

The characterization of the purified products was performed by analytical reverse-phase HPLC on a Vydac 218TP54 column ($C_{18}$, 300 Å pore size, 5 µm particles, 4.6 × 250 mm, Vydac, Hesperia, CA, USA) with the same eluting system and a gradient from 20% to 60% of A over 30 min at a flow rate of 0.6 ml/min. MS analysis was performed on a 7 T APEX II FT-ICR (Bruker Daltonics, Billerica, MA, USA) mass spectrometer and on a Voyager-DE RP (PerSeptive Biosystems, Framingham, USA) MALDI mass spectrometer. Automated Edman degradation was performed by a ABI 473 A protein sequencer (Applied Biosystems, Weiterstadt, Germany). Analytical data are summarized in Table 1.

Table 1 Analytical and Binding Data for the TOAC-Labelled NPY Analogues[a]

| Peptide | M.W. (a.m.u.) | | $K_i$ (nM) |
|---|---|---|---|
| | Theor. | Exp. | |
| [TOAC$^{2}$]-pNPY | 4351.20 | 4351.28 | 6.5 ± 2.6 |
| [Ala$^{31}$,TOAC$^{32}$]-pNPY | 4305.16 | 4305.20 | 141 ± 98 |
| [TOAC$^{34}$]-pNPY | 4320.19 | 4320.24 | 40 ± 26 |

[a] Molecular weights were measured by ESI FT-ICR-MS. Binding assays on BHK cells selectively expressing the $rY_5$ receptor were performed by competition assays as reported in the Materials and Methods. Each experiment was repeated three times. $IC_{50}$ values were determined by competition curves. $K_i$ values were determined by Scatchard plot. The $K_i$ value for NPY is 17 ± 14 nM.

## Binding Assays

SK-N-MC cells (neuroblastoma, $hY_1$) were cultivated in MEM with Earle's salts supplemented with 10% (v/v) fetal calf serum, 4 mM L-glutamine, 0.2 mM non-essential amino acids and 1 mM sodium pyruvate. SMS-KAN cells (neuroblastoma, $hY_2$) were grown in 50% Dulbecco's modified Eagle medium/50% nutrient mix Ham's F12 with 15% fetal calf serum, 4 mM L-glutamine and 0.2 mM non-essential amino acids. BHK cells stably transfected with $rY_5$-receptors were cultured in Dulbecco's modified Eagle medium containing 10% fetal calf serum and 0.05% geneticin. Cells were grown to confluency at 37 °C and 5% $CO_2$. Cells were resuspended in incubation buffer minimum essential medium (MEM) containing 0.1% bacitracin, 50 mM pefabloc SC and 1% bovine serum albumin. 200 µl of the suspension containing $3.0 \times 10^6$ cells/ml was incubated with 25 µl of a 10 nM solution of $^{3}$H-propionyl-NPY (specific activity: 2.59 TBq/mmol) and 25 µl of NPY in a concentration range of 1 µM to 1 pM. Non specific binding was defined in the presence of 1 µM unlabelled NPY. After 1.5 h at room temperature, the incubation was terminated by centrifugation at 1600×**g** and 4 °C for 5 min. The pellets were washed once with 400 µl PBS, centrifuged and resuspended in 100 µl PBS. The cell suspension was mixed with 3 ml scintillation cocktail and radioactivity was measured in a ß-counter. The $IC_{50}$ value was calculated with the software Prism 3.0. For determination of the $K_i$ values the Cheng–Prusoff equation and a $K_d$ value of 2.4 nM were used. Each measurement was performed in triplicate and $K_i$ ± SEM is given in Table 1.

### Circular Dichroism

Circular dichroism spectra were recorded on a J-715 spectropolarimeter (Jasco, Tokyo, Japan). Quartz cells with a path of 2 mm were used. The peptides were dissolved in acetate buffer at pH 3.2. The exact peptide concentration was determined by UV absorption. Each measurement was repeated four times and the resulting signals were averaged. The response time was set to 2 s with a scan speed of 20 nm/min; sensitivity was set to 20 mdeg and step resolution to 0.2 nm. High frequency noise was reduced using a low-pass Fourier-transform filter. The mean residue molar ellipticity $[\theta]_R$ was expressed in deg cm$^2$ dmol$^{-1}$.

### EPR Measurements

EPR measurements of the TOAC-labelled NPY analogues were performed on a Bruker ESP 380 E FT/CW X-band EPR spectrometer at 25 °C using a field modulation amplitude of 0.05 mT and a microwave power of 3.6 mW. The samples were placed in a quartz glass flat cell and measured in a $TE_{102}$ cavity. For the analysis of the rotational correlation times the line widths of the three nitrogen hyperfine lines in the EPR spectra of the nitroxide radicals were determined by using the NIEHS public EPR software tool [21].

EPR measurements on liposome-bound NPY analogues at different temperatures were performed with a Magnettec MS100 spectrometer using a microwave frequency of 9.45 GHz. Measurements at 130° K were performed on a Bruker (ESP 300 E).

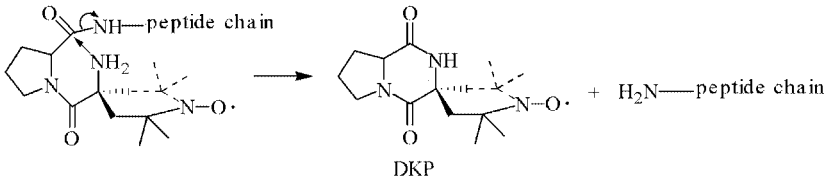
Large unilamellar vesicles (LUV) were produced according to the procedure described by Hope and coworkers [22]. Ox brain phosphatidylserine and egg yolk phosphatidylcholine were obtained from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. The phospholipid concentration was determined using the inorganic phosphate assay [23].

## RESULTS

### Peptide Synthesis

The synthesis of the TOAC-labelled NPY analogues was performed by the solid-phase methodology following the Fmoc/*t*Bu strategy as previously described [4]. The major problems for the incorporation of TOAC into the growing chain was the hindered coupling of the amino acid following TOAC, the sensitivity of TOAC towards acid- and thiol-containing scavengers and the 2,5-dioxopiperazine (DKP) formation by the H-TOAC-Pro-sequence (Scheme 1) during peptide assembly. The best results for coupling were obtained by using a three-fold excess of the efficient activating agent HATU [24]. This finding is in agreement with the conclusion recently reported by Martin *et al.* [20].

Acidic conditions such as 90% TFA are required for the cleavage of the peptide from the resin. However, under these conditions the TOAC nitroxide radical becomes protonated. This species can be partly reduced to the corresponding amine in the presence of reducing agents such as thiols [25]. Whereas the protonation of the nitroxide can be reverted towards the radical form by treatment with an aqueous base as already reported [20,26], the use of reagents such as thioanisole, thiocresol and EDT as scavengers in the cleavage cocktail results in a partially irreversible reaction of the nitroxide radical (Figure 4A). This is a major problem in the synthesis of Arg-containing peptides like NPY. As the Arg residues of the NPY sequence, in particular those located in the *C*-terminal part, are crucial for the maintenance of the biological activity [8], we had to develop a cleavage method compatible with the concomitant presence of TOAC and Arg residues. Several cleavage conditions were tested by varying the components of the scavenger mixture, the nature of the Arg side-chain protecting group and the reaction time, because irreversible Arg sulfonation was frequently found when no thiols were used in the cleavage cocktail [27]. Cleavage of the peptides by treatment with a cocktail composed of TFA/anisole 90 : 10 over 3 h resulted in mono-and bis-sulfonated adducts




Scheme 1 Formation of a 2,5-dioxopiperazine (DKP) adduct from the *N*-terminal peptide sequence H-TOAC-Pro-Xxx-.

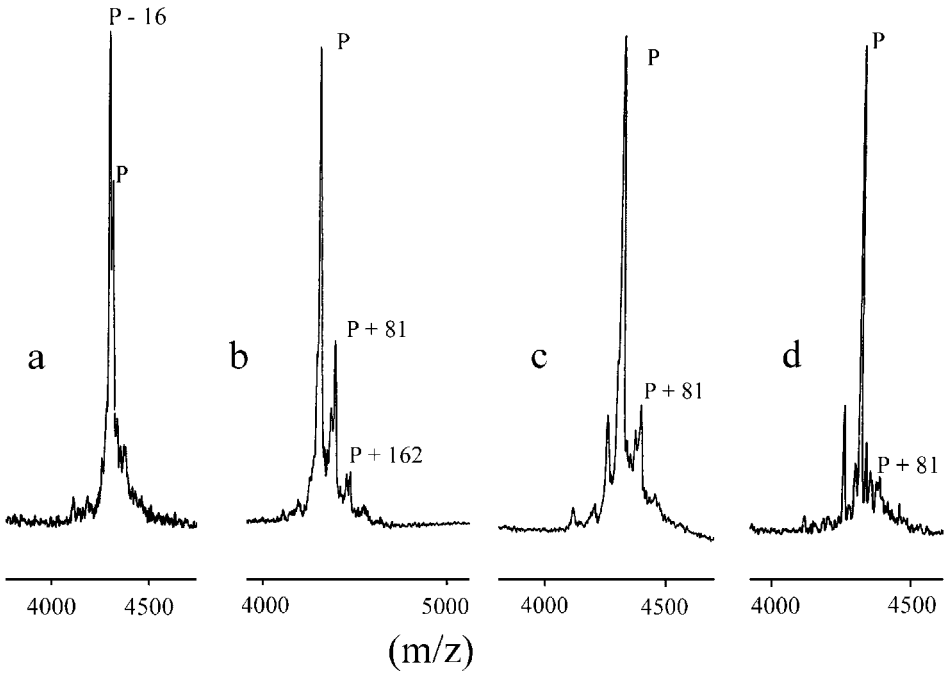



Figure 4 MALDI-MS spectra of the crude [Ala$^{31}$,TOAC$^{32}$]-pNPY obtained by using different cleavage conditions. TFA/thioanisole/thiocresol 90 : 5 : 5 (A); TFA/anisole 90 : 10 (B); TFA/TIS/water 90 : 5 : 5 (C); TFA/anisole/cresol 90 : 5 : 5 (D). P = desired product ($[M + H]^+ = 4306$); P − 16 = byproduct derived from nitroxide reduction ($[M + H]^+ = 4290$); P + 81 = sulfonated byproduct ($[M + H]^+ = 4387$); P + 162 = bisulfonated byproduct ($[M + H]^+ = 4468$).

which could not be separated from the desired product by reverse phase HPLC, but which were clearly identified by mass spectrometry (Figure 4B). No significant differences were observed between the use of Pmc and Pbf for Arg protection. The highest yield of the desired product was obtained by addition of an aliquot of cresol to the scavenger mixture (Figure 4D). The treatment with TFA/anisole/cresol 90 : 5 : 5 for 3 h led to crude peptides showing only traces of sulfonated and reduced-nitroxide-containing byproducts (Figure 4D), whereas sulfonated products were found upon TFA/TIS/water 90 : 5 : 5 treatment (Figure 4C). A basic treatment in aqueous ammonia at pH 9–10 was always performed after the peptide cleavage from the resin in order to restore the integrity of the nitroxide radical. The analytical data reported in Table 1 proved the recovery of the nitroxyl radical character. The optimized synthetic protocol allowed us to obtain the crude TOAC peptide with a satisfactory purity (Figure 5).

During the synthesis of the TOAC-containing NPY analogues we identified a further severe problem. In order to prepare [TOAC$^4$]-NPY the fragment 5–36 was synthesized by automated solid-phase method

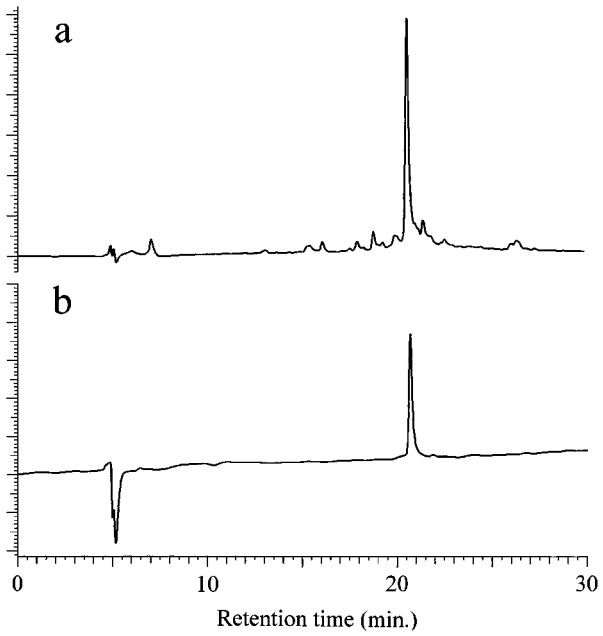



Figure 5 Analytical reverse-phase HPLC profiles of the crude (a) and purified (b) peptide [TOAC$^{34}$]-pNPY using a $C_{18}$ column and a gradient from 20% to 60% of acetonitrile in water over 30 min at the flow rate of 0.6 ml/min.

and Fmoc-TOAC-OH [28] was coupled manually to the free amino group of $Pro^5$ according to the protocol described in the Material and Methods section. The reaction proceeded to completion as demonstrated by the analysis performed after cleavage of some milligrams of resin. $Ser^3$ was coupled manually, followed by automated coupling of $Pro^2$ and $Tyr^1$. Surprisingly, a lack of 294 amu was found in the mass spectrum of the final product. Automated Edman degradation showed the sequence H-$Tyr^1$-$Pro^2$-$Ser^3$-$Asp^6$-$Asn^7$-$Pro^8$-$Gly^9$-$Glu^{10}$-, instead of the expected H-$Tyr^1$-$Pro^2$-$Ser^3$-$TOAC^4$-$Pro^5$-$Asp^6$-$Asn^7$-$Pro^8$-. The absence of the $TOAC^4$ and $Pro^5$ residues in the final product cannot be due to an unsuccessful coupling of Fmoc-Pro-OH and Fmoc-TOAC-OH as the MS analysis performed after each of the two corresponding coupling reactions revealed complete coupling. This unexpected result can only be explained in terms of 2,5-dioxopiperazine formation that leads to loss of the TOAC-Pro dipeptide (Scheme 1). 2,5-Dioxopiperazine formation has already been described in the literature for the related H-Aib-Pro- [29] and H-($\alpha$Me)Phe-Pro-sequences [30]. Accordingly, TOAC was incorporated in position 2 instead of position 4.

## Binding Assays

We tested the binding properties of the TOAC-containing peptides at the $Y_1$, $Y_2$ and $Y_5$ receptors subtypes by using SK-N-MC, SMS-KAN and BHK cells, respectively. The results show a marked preference for the $Y_5$ receptor for the peptides [$TOAC^{34}$]-NPY and [$Ala^{31}$,$TOAC^{32}$]-NPY and $IC_{50} >$ 1 μM at the $Y_1$ and $Y_2$ receptor. The specific binding of [$TOAC^2$]-NPY at the $Y_2$ and $Y_5$ receptors was in the same range as NPY, whereas it was inactive at the $Y_1$ receptor. The binding curve of [$TOAC^2$]-pNPY at the $Y_5$ receptor is shown in Figure 6. A comparison of the $K_i$ values shown in Table 1 indicates that the introduction of the TOAC residue in the *N*-terminal part of the sequence is better tolerated than in proximity to the *C*-terminus.

## Circular Dichroism

In order to investigate the effect of the introduction of TOAC on the NPY structure in solution, CD spectra of the TOAC-labelled NPY analogues and of the native pNPY were recorded in acetate buffer at pH 3.2 at a peptide concentration of 0.2 mM (Figure 7). The typical CD spectrum of NPY is characterized by a positive maximum

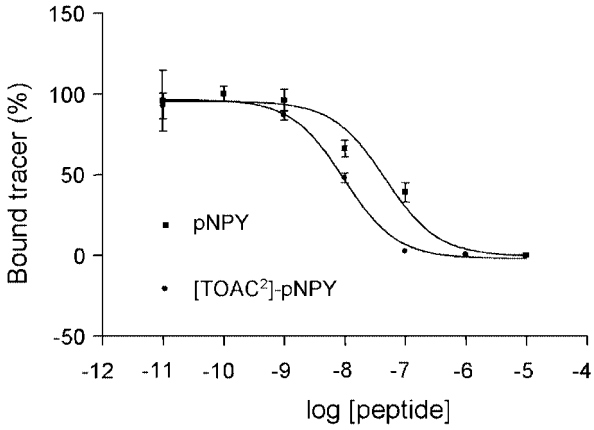



Figure 6 Binding curves of pNPY (■) and [$TOAC^2$]-pNPY (●) at the $Y_5$ receptor.

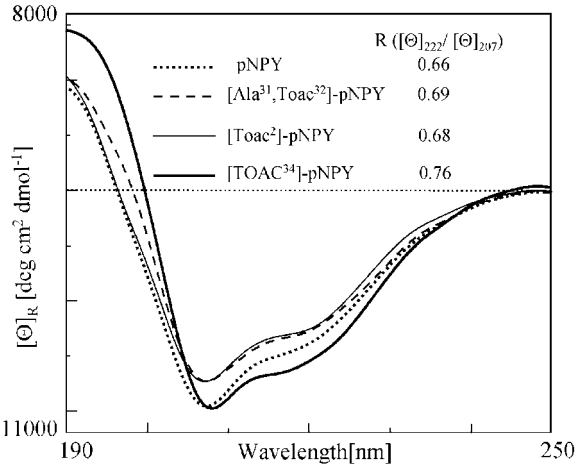



Figure 7 CD spectra of native pNPY and of the TOAC-labelled NPY analogues at the concentration of 0.2 mM and at pH 3.2. The reported R values represents the ratios between the molar ellipticity values at 222 nm and 207 nm.

at 190 nm, a negative band at 207 nm and a negative shoulder at about 220 nm [31]. A similar profile is observed also in the TOAC-containing peptides, although significant differences regarding the band intensities were found. The analogue [$Ala^{31}$,$TOAC^{32}$]-pNPY shows a diminished intensity of the negative bands and its spectrum is very similar to that of [$TOAC^2$]-pNPY, whereas the analogue [$TOAC^{34}$]-pNPY displays more intense bands. A useful parameter for the interpretation of the CD spectra produced by helical peptide structures in solution is the ratio between the molar ellipticities at 222 nm and 207 nm ($R = [\Theta]_{222}/[\Theta]_{207}$) [32]. It has been demonstrated that

this value is approximately 1 for $\alpha$-helices while it decreases to 0.4 in $3_{10}$-helices [33]. Figure 7 reports the $R$ values corresponding to the four peptides obtained from the CD spectra. While $R$ varies only from 0.66 to 0.69 for pNPY, [TOAC$^2$]-pNPY and [Ala$^{31}$,TOAC$^{32}$]-pNPY, it becomes significantly higher (0.76) for [TOAC$^{34}$]-pNPY. This result can be interpreted with an increased content of $\alpha$-helical structure for this analogue.

## EPR Measurements in Solution

The mobility of the nitroxide spin label was evaluated by measuring the corresponding rotational correlation times ($\tau_R$). In order to avoid the effect of intermolecular spin–spin interactions on the ESR spectra, which may result from dimerization of NPY and would produce additional line broadening components, the experiments were performed at 0.1 mM solutions of spin-labelled NPY analogues containing also 0.9 mM pNPY in acetate buffer (pH 3.2). Under these conditions each spin-labelled NPY molecule should form a dimer with an unlabelled one. The pH value of 3.2 was chosen to allow the comparison with the NMR data obtained under the same conditions [4,5] and because of the decreased solubility typical for NPY at higher pH values. The rotational correlation times ($\tau_R$) were calculated from the linewidths and the corresponding spin Hamiltonian parameters [17] according to the Redfield theory [34] which is valid within the limit of the fast motional narrowing region ($10^{-11}$ s $\leq \tau_R \leq 10^{-9}$ s).

The rotational correlation times for the three TOAC-labelled peptides in acetate buffer at pH 3.2 (Table 2) are located between 0.53 and 1.17 ns, at the border between the fast motional narrowing region ($10^{-11}$ s $\leq \tau_R \leq 10^{-9}$ s) in which the Redfield theory can be used and the slow motional regime ($10^{-9}$ s $\leq \tau_R \leq 10^{-8}$ s) in which the full stochastic Liouville equation [35] should be applied. In order to compare the degree of rotational freedom of the spin probe in the three different positions of the NPY molecule, we applied the Redfield theory for all peptides instead of the Liouville treatment. According to this approach two distinct values of rotational correlation times ($\tau_B$ and $\tau_C$) can be determined [36]. If the rotation of the spin probe is isotropic the rotational correlation times $\tau_B$ and $\tau_C$ should be equal for the same peptide. If this is not the case, then the motion will be anisotropic. Accordingly, anisotropic rotation was observed for the spin label of all three NPY analogues (Table 2) as it was observed in the case of the TOAC-labelled $\alpha$-MSH analogue (acetyl-TOAC$^0$-$\alpha$-MSH) [19]. The hyperfine constant $a_0$ was equivalent for all three peptides.

Table 2 EPR Data for the TOAC-Labelled NPY Analogues[a]

| Peptide | $\tau_B$ (ns) | $\tau_C$ (ns) | $a_0$ (mT) |
|---|---|---|---|
| [TOAC$^{34}$]-pNPY | 0.88 | 1.13 | 1.61 |
| (in solution) | ±0.09 | ±0.13 | ±0.08 |
| [Ala$^{31}$,TOAC$^{32}$]-pNPY | 0.93 | 1.17 | 1.61 |
| (in solution) | ±0.09 | ±0.12 | ±0.08 |
| [TOAC$^2$]-pNPY | 0.54 | 0.66 | 1.61 |
| (in solution) | ±0.03 | ±0.04 | ±0.08 |
| [TOAC$^2$]-pNPY | 0.53 | 0.87 | n.d |
| (bound to liposomes) | ±0.03 | ±0.05 | |

[a] Measurements were performed at 25 °C. Only rotational correlation time values calculated using the Redfield theory are reported. Measurements in solution were performed with 0.1 mM labelled NPY and 0.9 mM pNPY in acetate buffer at pH 3.2. Measurements with liposomes were carried out with a peptide concentration of 0.2 mM in the presence of 10 mM PS/PC (molar ratio PS/PC 1 : 3) in 10 mM MES buffer in the presence of 100 mM NaCl at pH 6.0.

## EPR Measurements on Liposome-bound NPY

If compared with those of the corresponding peptides alone in solution (Figure 8a), the EPR spectra of the TOAC-NPY analogues recorded in the presence of PS-LUV (Figure 8b) showed two components. The first component displayed three sharp lines characteristic for a nitroxide radical that undergoes rapid rotation and can be attributed to NPY in solution. The broadened lines of the second component are typical of a slow tumbling nitroxide and are produced by the liposome-bound peptide. In order to maximize the binding of NPY to liposomes, experiments aimed at measuring the rotational correlation times were performed with a molar peptide/phospholipid ratio of 1 : 50. A further increase of the lipid content did not show any change of the NPY binding behaviour. A comparison of the shapes of the EPR spectra of the three NPY analogues (Figure 9) shows the location of TOAC in proximity to the *N*-terminal part of the peptide chain as in the analogue [TOAC$^2$]-NPY leads to a much more mobile spin probe and produces a spectrum in which the slow tumbling component is absent (Figure 9c). Furthermore, the correlation time for this analogue becomes smaller than 1 ns at

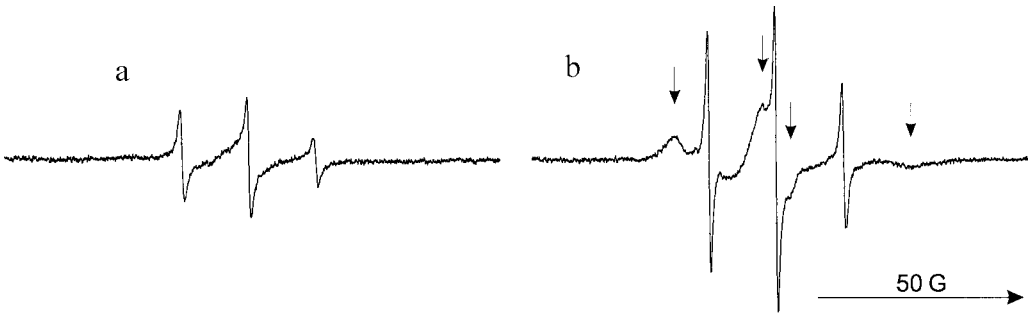



Figure 8 EPR signal of 200 μM [TOAC$^{34}$]-pNPY in the absence (a) and presence (b) of 10 mM PS-LUV. Spectrum (b) shows clearly two signal components referable to a free and a bound component. The bound component is indicated by arrows. Both measurements were obtained at 32 °C in 10 mM MES buffer in the presence of 100 mM NaCl at pH 6.0 (scan width: 120 G; modulation amplitude: 0.4 G).

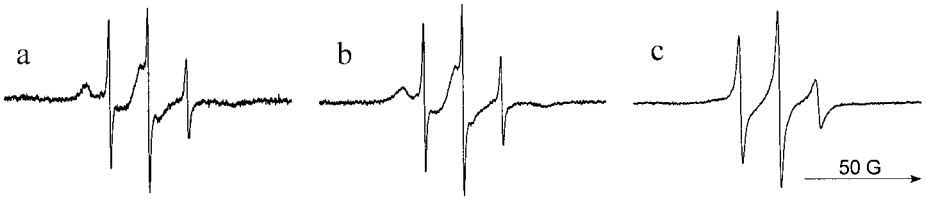



Figure 9 EPR signal of [Ala$^{31}$,TOAC$^{32}$]-pNPY (a), [TOAC$^{34}$]-pNPY (b) and [TOAC$^{2}$]-pNPY (c) at a concentration of 200 μM in the presence of 10 mM PS/PC-LUV (molar ratio PS/PC 1 : 3). All measurements were obtained at 20 °C in 10 mM MES buffer in the presence of 100 mM NaCl at pH 6.0 (scan width: 120 G; modulation amplitude: 0.4 G).

a temperature higher than 20 °C. It can therefore be calculated using the Redfield theory valid in the fast motional narrowing region. This allows the comparison of the $\tau_B$ and $\tau_C$ values in solution and in the bound phase; $\tau_B$ is almost equal in the bound state and in solution, while $\tau_C$ is higher in the bound state. This finding indicates an almost equal mobility but a higher anisotropy in the liposome bound state (Table 2). For the peptides [TOAC$^{31}$, Ala$^{32}$]-pNPY and [TOAC$^{34}$]-pNPY the approximate values of the rotational correlation times for the liposome-bound components were calculated on the temperature range from 0° to 50 °C (Figure 10) by using the full stochastic treatment [34] according to

$$\tau_R \approx (\hbar/\mu_B g A^r_{zz})(1 - A'^{\bullet}_{zz}/A^r_{zz})^{-1/2} \quad (1)$$

where $\mu_B$ represents the Bohr magneton, g is the g-factor [34] and $A^r_{zz}$ represents the rigid limit value of the outer hyperfine splitting $A'_{zz}$. The value of $A^r_{zz}$ was determined from measurements performed at 130°K. In these two cases the $\tau_R$ values are all above 3.5 ns and belong therefore to the slow rotational motion regime. As expected, the values of the rotational correlation times decrease by decreasing the temperature. Furthermore, the correlation times of the peptide [TOAC$^{34}$]-pNPY are larger than those obtained from [TOAC,$^{31}$ Ala$^{32}$]-pNPY in the entire temperature interval. The corresponding values for the peptide [TOAC$^{2}$]-pNPY are the smallest of the set and were calculated using the model proposed for the motional narrowing regime with anisotropic rotation axially symmetric about the nitroxide *z*-axis [34].

## DISCUSSION

The compatibility of TOAC to solid-phase peptide synthesis *via* Fmoc/*t*Bu strategy has already been demonstrated [20]. Here we present a protocol that is useful for the solid-phase synthesis of large (36-residue) Arg-containing TOAC-peptides. To our knowledge, the three NPY analogues presented in this work are the largest peptide, in which TOAC has been introduced to date. Besides carrying a stable nitroxide radical, TOAC has also the property of inducing $\beta$-turns and $3_{10}/\alpha$-helical structures like many other members of the family of the $C^{\alpha}$-tetrasubstituted amino acids [18]. The amino acid Aib ($\alpha$-aminoisobutyric acid), which can be considered as the prototype of this family, has been

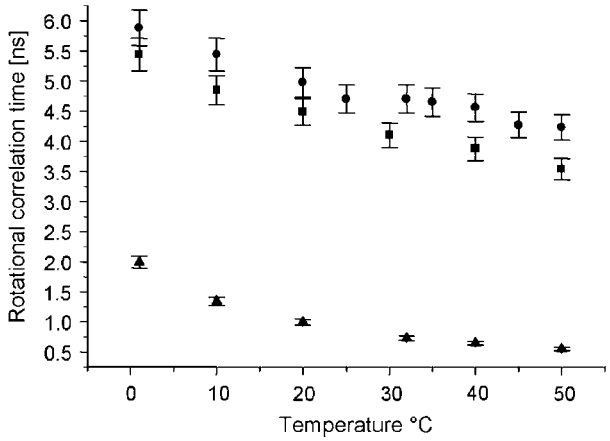



Figure 10 Rotational correlation times of the peptides [TOAC$^{34}$]-pNPY (■), [TOAC$^{2}$]-pNPY (▲) and [Ala$^{31}$,TOAC$^{32}$]-pNPY (●) bound to an artificial membrane made of PS/PC-LUV. For the peptides [TOAC,$^{31}$ Ala$^{32}$]-pNPY and [TOAC$^{34}$]-pNPY the rotational correlation times were calculated using the full stochastic treatment [34]. The values referred to the peptide [TOAC$^{2}$]-pNPY were calculated using the model proposed for the motional narrowing regime with anisotropic rotation which is axially symmetric about the nitroxide *z*-axis [34]. Measurements were performed with a peptide concentration of 200 μM in the presence of 10 mM PS/PC-LUV (PS/PC molar ratio 1 : 3). All measurements were obtained in 10 mM MES buffer in the presence of 100 mM NaCl at pH 6.0 (scan width: 120 G; modulation amplitude: 0.4 G).

used in our group to obtain the first $Y_5$-receptor-selective agonist [Ala$^{31}$,Aib$^{32}$]-pNPY [31]. Ala$^{31}$-Aib$^{32}$ was determined to be the key motif for $Y_5$ receptor selectivity [31]. Structural investigation performed by 2D NMR revealed the presence of an *i*, *i*+3 hydrogen bond typical of a $\beta$-turn between residues 28 and 31. As this $\beta$-turn interrupts the $\alpha$-helical *i*, *i*+4 hydrogen bond repeat, the *C*-terminal pentapeptide appears to be devoid of ordered structure. Accordingly, the CD spectrum of [Ala$^{31}$,Aib$^{32}$]-pNPY shows a decreased intensity of the positive band at 190 nm and of the negative bands at 207 and 220 nm. This feature is compatible with a decreased $\alpha$-helical content and the presence of a $\beta$-turn structure. The paramagnetic nitroxide group broadens the NMR signals and does not allow NMR structural analysis to be performed on TOAC-labelled peptides. However, the CD spectrum of [Ala$^{31}$,TOAC$^{32}$]-pNPY (Figure 7) also show a decrease of $\alpha$-helical structure when compared with that of native pNPY. A further confirmation that TOAC exhibits a similar conformation to Aib is the marked preference of [Ala$^{31}$,TOAC$^{32}$]-pNPY for the $Y_5$ receptor. The analogue [TOAC$^{34}$]-pNPY possesses a value of the ratio between the molar ellipticity at 222 nm and at 207 nm $R = [\Theta]_{222}/[\Theta]_{207}$ (0.76), which is the highest among the investigated peptides (Figure 7) indicating a higher $\alpha$-helical content. Although the NOE pattern of the 2D NMR spectrum of NPY is consistent with an $\alpha$-helix extended up to the *C*-terminus [5], NMR relaxation data demonstrate that the *C*-terminal tetrapeptide is much more flexible than the central part of the $\alpha$-helix encompassing residues 18–32 [4]. The increased helicity in [TOAC$^{34}$]-pNPY might be due to an increased rigidity in the *C*-terminal segment promoted by TOAC. The discussion of the structure of [TOAC$^{2}$]-pNPY is more complex as TOAC is located in the *N*-terminal unordered region. It is therefore unlikely that the $C^{\alpha}$ -tetrasubstituted amino acid can modify the $\alpha$-helical structure located in the *C*-terminal part. However, as the chiroptical properties of NPY are determined by the coexistence of $\alpha$-helical and unordered structure, it is possible that the introduction of TOAC at position 2 may influence the local dynamics of the flexible *N*-terminal tail. Among the three TOAC labelled NPY, [TOAC$^{2}$]-pNPY is the only one that shows a significant binding potency at the $Y_2$ receptor. This finding is in agreement with previous work indicating that the *N*-terminal part of the molecule is not important for the binding [37] and the introduction of unnatural residues therein is well tolerated. Concerning the binding to the $Y_5$ receptor, the $K_i$ value determined for [TOAC$^{2}$]-pNPY ($6.5 \pm 2.6$ nM) (Table 1) is similar to that determined for pNPY ($17 \pm 14$ nM) and even slightly lower while the corresponding values for [Ala$^{31}$,TOAC$^{32}$]-pNPY ($141 \pm 98$) and [TOAC$^{34}$]-pNPY ($40 \pm 26$) are significantly higher. This could be due to the fact that the residues located in proximity to the *C*-terminus may not be substituted without a considerable loss of binding potency.

The rotational correlation times of the analogue [TOAC$^{2}$]NPY in solution are smaller than those of the analogues [Ala$^{31}$,TOAC$^{32}$]-NPY and [TOAC$^{34}$]-NPY. This result is in full agreement with the model of NPY structure in solution derived by NMR measurements [4,5], in which the *N*-terminal part of the molecule is flexible while the *C*-terminal part (except the last four residues which are also flexible) adopts an $\alpha$-helical conformation and is involved in hydrophobic interactions with a second NPY molecule. The correlation times of the peptide [TOAC$^{34}$]-NPY are slightly smaller than those obtained for [Ala$^{31}$,TOAC$^{32}$]-NPY. In [TOAC$^{34}$]-NPY

the spin probe is located within the *C*-terminal tetrapeptide which, according to NMR measurements, has a higher flexibility compared with the 'transition region' (residues $Ala^{12}$ to $Asp^{16}$) between the $\alpha$-helix and the *N*-terminal flexible part [4]. Although the CD measurements indicate that the introduction of a TOAC residue in position 34 causes an increased rigidity in the *C*-terminal segment, the mobility of the spin probe is higher in position 34 than 32. The identical hyperfine constant values ($a_0$) indicate that the spin probe, although being located at different positions of the peptide backbone, is affected by the same microenvironment.

The analysis of the mobility of the spin probe in the liposome bound peptides indicates that the *N*-terminal part is as flexible as in solution, while the *C*-terminal part is much more rigid. This behaviour has been observed over a wide temperature interval which ranges from 10° to 50 °C (Figure 10). An identical behaviour has been described for pNPY bound to micelles [4]. From these results we can conclude that NPY adopts a similar binding mode for liposomes and micelles, as in both cases the hydrophobic face of the $\alpha$-helix binds to the membrane while the *N*-terminal tail is not directly involved in any peptide–membrane interaction and remains flexible. The fact that the spin label is slightly more flexible in position 34 than in position 32 may be explained by the hypothesis that residue 32 is directly anchored to the membrane while the side chain of residue 34 points towards the bulk solvent.

In conclusion, we have demonstrated that TOAC-labelling is a powerful method for the investigation of the backbone dynamics of polypeptides in the free and membrane-bound states. The three-step model of receptor selection [38,39] highlights the importance of the ligand binding to cellular membranes as the decisive step for receptor recognition and selection. The TOAC-labelled NPY analogues allowed us to investigate to what extent the phospholipid composition of the liposome can influence the structure and dynamics of the membrane bound ligand and whether this phenomenon, in turn, alters receptor selectivity. Furthermore, the preserved binding properties at the $Y_5$ receptor can be employed to perform backbone dynamics studies on the receptor-bound ligand. TOAC can be easily used in solid-phase peptide synthesis and now also with Arg–containing peptides. Further investigations aimed at the introduction of TOAC in longer peptides and proteins by native chemical ligation are currently in progress in our group.

## Acknowledgements

We would like to thank Professor F. Formaggio for helpful discussion concerning the synthesis of the TOAC peptides. We are grateful to Dr J. Bär for Edman sequencing, Dr A. Sinz and Mrs R. Oehme for the FT-ICR measurements, Mrs Regina Schaaf for MALDI-MS and Mrs Hella Späte for technical support in cell culture.

## REFERENCES

1. Tatemoto K. Neuropeptide Y: complete amino acid sequence of the brain peptide. *Proc. Natl Acad. Sci. USA* 1982; **79**: 5485–5489.
2. Heilig M, Widerlov E. Neuropeptide Y: an overview of central distribution, functional aspects, and possible involvement in neuropsychiatric illnesses. *Acta Psychiatr. Scand.* 1990; **82**: 95–114.
3. Michel MC, Beck-Sickinger AG, Cox H, Doods HN, Herzog H, Larhammar D, Quirion R, Schwartz T, Westfall T. XVI International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol. Rev.* 1998; **50**: 143–150.
4. Bader R, Bettio A, Beck-Sickinger AG, Zerbe O. Structure and dynamics of micelle-bound neuropeptide Y: comparison with unligated NPY and implications for receptor selection. *J. Mol. Biol.* 2001; **305**: 307–329.
5. Monks SA, Karagianis G, Howlett GJ, Norton RS. Solution structure of human neuropeptide Y. *J. Biomol. NMR* 1996; **8**: 379–390.
6. Lipari G, Szabo A. Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. 1. Theory and range of validity. *J. Am. Chem. Soc.* 1982; **104**: 4559–4570.
7. Bettio A, Dinger MC, Beck-Sickinger AG. The neuropeptide Y monomer in solution is not folded in the pancreatic-polypeptide-fold. *J. Protein Sci., in press* 2002; **11**: 1834–1834.
8. Beck-Sickinger AG, Wieland HA, Wittneben H, Willim KD, Rudolf K, Jung G. Complete L-alanine scan of neuropeptide Y reveals ligands binding to Y1 and Y2 receptors with distinguished conformations. *Eur. J. Biochem.* 1994; **225**: 947–958.
9. Papavoine CH, Remerowski ML, Horstink LM, Konings RN, Hilbers CW, van de Ven FJ. Backbone dynamics of the major coat protein of bacteriophage M13 in detergent micelles by $^{15}N$ nuclear magnetic resonance relaxation measurements using the model-free approach and reduced spectral density mapping. *Biochemistry* 1997; **36**: 4015–4026.

10. Hanson P, Martinez G, Millhauser G, Formaggio F, Crisma M, Toniolo C, Vita C. Distinguishing helix conformations in alanine-rich peptides using the unnatural amino acid TOAC and electron spin resonance. *J. Am. Chem. Soc.* 1996; **118**: 271–272.
11. Hubbell WL, Cafiso DS, Altenbach C. Identifying conformational changes with site-directed spin labeling. *Nature Struct. Biol.* 2000; **7**: 735–739.
12. Monaco V, Formaggio F, Crisma M, Toniolo C, Hanson P, Millhauser GL. Orientation and immersion depth of a helical lipopeptaibol in membranes using TOAC as an ESR probe. *Biopolymers* 1999; **50**: 239–253.
13. Veiga MP, Goni FM, Alonso A, Marsh D. Mixed membranes of sphingolipids and glycerolipids as studied by spin-label ESR spectroscopy. A search for domain formation. *Biochemistry* 2000; **39**: 9876–9883.
14. Toniolo C, Crisma M, Formaggio F. TOAC, a nitroxide spin-labelled, achiral C$\alpha$-tetrasubstituted $\alpha$-amino acid, is an excellent tool in material science and biochemistry. *Biopolymers* 1998; **47**: 153–158.
15. Hanson MP, Millhauser G, Formaggio F, Crisma M, Toniolo C. ESR characterization of hexameric, helical peptides using double TOAC spin labeling. *J. Am. Chem. Soc.* 1996; **118**: 7618–7625.
16. Smythe ML, Nakaie CR, Marshall GR. $\alpha$-Helical versus $3_{10}$ conformation of alanine based peptides in aqueous solution: an electron spin resonance investigation. *J. Am. Chem. Soc.* 1995; **117**: 10 555–10 562.
17. Hanson MP, Anderson DJ, Martinez G, Millhauser G, Formaggio F, Crisma M, Toniolo C, Vita C. Electron spin resonance and structural analysis of water soluble alanine-rich peptides incorporating TOAC. *Mol. Phys.* 1998; **95**: 957–966.
18. Toniolo C, Benedetti E. Structures of polypeptides from amino acids disubstituted at the $\alpha$-carbon. *Macromolecules* 1991; **24**: 4004–4009.
19. Barbosa SR, Cilli EM, Lamy-Freund MT, Castrucci AM, Nakaie CR. First synthesis of a fully active spin-labeled peptide hormone. *FEBS Lett.* 1999; **446**: 45–48.
20. Martin L, Ivancich A, Vita C, Formaggio F, Toniolo C. Solid-phase synthesis of peptides containing the spin-labeled 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC). *J. Peptide Res.* 2001; **58**: 424–432.
21. Duling DR. Simulation of multiple isotropic spin-trap EPR-spectra. *J. Magn. Reson. Ser. B* 1994; **104**: 105–110.
22. Hope MJ, Bally MB, Webb G, Cullis PR. Production of large unilamellar vesicles by rapid extrusion procedure. Characterisation of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta* 1985; **812**: 55–65.
23. Chen PS, Toribara TY, Warner H. Microdetermination of phosphorus. *Anal. Chem.* 1956; **28**: 1756–1758.
24. Carpino LA, El-Faham A, Minor CA, Albericio F. Advantageous applications of azabenzotriazole (triazolpyridine)-based coupling reagents to solid-phase peptide synthesis. *J. Chem. Soc. Chem. Commun.* 1994; 201–203.
25. Weinkam RJ, Jorgensen EC. Free radical analogs of histidine. *J. Am. Chem. Soc.* 1971; **93**: 7028–7033.
26. Nakaie CR, Schreier S, Paiva AC. Synthesis and properties of spin-labeled angiotensin derivatives. *Biochim. Biophys. Acta* 1983; **742**: 63–71.
27. Beck-Sickinger AG, Schnorrenberg G, Metzger J, Jung G. Sulfonation of arginine residues as side reaction in Fmoc-peptide synthesis. *Int. J. Peptide Protein Res.* 1991; **38**: 25–31.
28. Marchetto R, Schreier S, Nakaie CR. A novel spin-labeled amino-acid derivative for use in peptide-synthesis-(9-fluorenylmethyloxycarbonyl)-2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid. *J. Am. Chem. Soc.* 1993; **115**: 11 042–11 043.
29. Gerig JT, McLeod RS. Attempted synthesis of 2-methylalanyl-L-prolyl-L-tryptophan. An unexpected result. *J. Org. Chem.* 1976; **41**: 1653–1655.
30. Valle G, Formaggio F, Crisma M, Toniolo C, Boesten WHJ, Polinelli S, Schoemaker HE, Kamphuis J. Crystal structure of cyclo(C$\alpha$-methyl-phenylalanine-proline)(S,S)(R,S) diastereomeric mixture, $C_{15}H_{18}N_2O_2$. *Zeit. Kristallogr.* 1992; **202**: 168–170.
31. Cabrele C, Langer M, Bader R, Wieland HA, Doods HN, Zerbe O, Beck-Sickinger AG. The first selective agonist for the neuropeptide Y $Y_5$ receptor increases food intake in rats. *J. Biol. Chem.* 2000; **275**: 36 043–36 048.
32. Manning MC, Woody RW. Theoretical CD studies of polypeptide helices: examination of important electronic and geometric factors. *Biopolymers* 1991; **31**: 569–586.
33. Toniolo C, Polese A, Formaggio F, Crisma M, Kamphuis J. Circular dichroism spectrum of a peptide $3_{10}$-helix. *J. Am. Chem. Soc.* 1996; **118**: 2744–2745.
34. Marsh D. In *Biological Magnetic Resonance*, Berliner RJ (ed.). Plenum: New York, 1989; Vol. 8, 255–302.
35. Freed JH. In *Spin Labeling. Theory and Applications*, Berliner RJ (ed.). Plenum: New York, 1976; Vol. 1, 53–132.
36. Schreier S, Polnaszek CF, Smith IC. Spin labels in membranes. Problems in practice. *Biochim. Biophys. Acta* 1978; **515**: 395–436.
37. Cabrele C, Beck-Sickinger AG. Molecular characterization of the ligand-receptor interaction of the neuropeptide Y family. *J. Peptide Sci.* 2000; **6**: 97–122.
38. Schwyzer R. 100 years of lock-and-key concept: are peptide keys shaped and guided to their receptors by the target cell membrane? *Biopolymers* 1995; **37**: 5–16.
39. Schwyzer R. Molecular mechanism of opioid receptor selection. *Biochemistry* 1986; **25**: 6335–6342.