Modified, cyclic dodecapeptide analog of neuropeptide Y is the smallest full agonist at the human Y_2 receptor

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Abstract In order to stabilize the C-terminal dodecapeptide of neuropeptide Y (NPY) we replaced Leu[28] and Thr[32] by Lys and Glu, respectively, and subsequently linked these residues by lactamization. This peptide analog of NPY shows a more than 100-fold increase in affinity compared to the C-terminal linear dodecapeptide in receptor binding studies performed at human neuroblastoma cells SMS-KAN, which exclusively express the Y_2 receptor subtype. Signal transduction was investigated by measuring Ca^{2+} current inhibition in human SH-SY5Y cells and cyclic [Lys[28]-Glu[32]] NPY Ac-25–36 and NPY were shown to be equipotent in this assay. Thus, this molecule is the smallest Y_2 receptor selective full agonist of NPY. Using 2D-NMR experiments and molecular modelling techniques, the structures of the linear and cyclic peptides have been investigated and significant differences have been found, which may explain the improvement in biological activity. Thus, a model of the bioactive conformation of NPY at the human Y_2 receptor is suggested.

Key words: Neuropeptide Y; Peptide synthesis; Receptor binding; Bioactive conformation; NMR

1. Introduction

Neuropeptide Y (NPY) is a 36-peptide amide which exhibits a high homology with the pancreatic polypeptide (PP) and peptide YY (PYY) in both sequence and 3D structure [1,2]. It was isolated from pig brain and sequenced in 1982 [3]. NPY is one of the most abundant neurohormones in the mammalian peripheral and central nervous systems. Receptor competition binding studies using analogs or segments of NPY, PYY and PP revealed that at least four receptor subtypes (Y_1, Y_2, Y_3, Y_4) exist [4,5]. The Y_1, Y_2, and Y_4 receptors have been cloned recently and it could be shown that they all belong to the G-protein coupled receptor family. Whereas NPY shows high affinity to Y_1 and Y_2 receptors, PP is supposed to be the endogenous ligand of the Y_4 receptor [5].

Y_2 receptors are predominantly localized on the presynaptic membrane of postganglionic, sympathetic neurons of the peripheral nervous system. In addition, Y_2 receptors have been identified in the proximal tubuli of kidneys, in the parasympathetic neurons and in red cells of rats using autoradiography. Centrally, the Y_2 receptor is more dominant than the Y_1 subtype. In the hippocampus of rats only Y_2 receptors are found as well as at the nerve ends of stimulating neurons which are synaptically bound to the hippocampus CA1 neurons. Y_2 receptors mediate the inhibitory effects of NPY on, amongst others, ion excretion in the rat small intestine, adrenaline release in the mesenteric arteries and bronchial contraction in guinea pig. One of the most important activities of the neurohormone – as shown with NPY knock-out mice – is the control of neural excitability, because it suppresses glutamate release from neurons in the hippocampus [6]. Furthermore, elevation of memory retention of rats and modulation of other neurotransmitters have been reported (for recent reviews, see [4,7,8]).

Cell lines exclusively expressing the Y_2 receptor were identified, and signal transduction was characterized as inhibition of cAMP accumulation (LN319 astrocytoma cells) [9] or inhibition of N-type Ca^{2+} channel current amplitudes (SH-SY5Y neuroblastoma cells) [10,11]. In each case the effect was pertussis toxin sensitive, which suggests the involvement of inhibitory G-protein.

Structure activity studies of neuropeptide Y and analogs at the Y_2 receptor revealed that the entire molecule is not required for full activity. So far, the 24-mer peptide NPY(13–36) is most frequently used for the testing of Y_2 receptor mediated actions [4]. We and others have shown recently that smaller centrally or N-terminally truncated analogs such as [Ahx[5–24]] NPY are full Y_2 receptor agonists [9] or show at least good binding properties [12–15]. In addition, the L-Ala scan of NPY 1–36 suggests that only the C-terminal amino acids are involved in receptor binding and we speculated that the N-terminal residues only serve for the stabilization of the molecule [16]. We have now succeeded in developing a small molecule, which only consists of the C-terminal 12 amino acids, and which has been stabilized by an intramolecular lactam bridge. This synthetic cyclopeptide shows high, selective Y_2 receptor affinity and activity, which is comparable to the activity of the native agonist. In addition, we solved the 3D solution structure of this molecule using 2D NMR techniques and compared it with the linear, inactive peptide. We also propose a model for the bioactive conformation of NPY at the Y_2 receptor.

2. Materials and methods

2.1. Materials

Fmoc-protected amino acids and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-tetrafluoroborate (TBTU) were obtained from NovaBiochem (Läufelfingen, Switzerland), aminomethylated polystyrene from Rapp Polymere (Tübingen, Germany) diisopropylcarbodiimide and thiocresol from Aldrich, 1-hydroxybenzotriazole (HOBt), diisopropylethylamine, trifluoroacetic acid, thioanisole and piperidine from Fluka and dimethylformamide (p.a. grade), diethyl ether, aceto-

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Abbreviations: NPY, neuropeptide Y; Fmoc, fluoren-9-ylmethoxycarbonyl; Ahx, 6-aminohexanoic acid

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nitrile and *tert.*-butyl alcohol from Merck. HEPES, Tris, bovine serum albumin, bacitracin, gentamycin and pentamethylsulfonylfluoride were purchased from Sigma, polyethylene imine and Fmoc-aminomethyldimethoxyphenoxy valeric acid from Serva, sodium pyruvate, minimum essential medium with Earle's salts and Ham's F12/50% Dulbecco's modified Eagle medium from Gibco, fetal calf serum, glutamine and non-essential amino acids from Boehringer Mannheim. EDTA and all salts for preparing the buffers were either from Fluka or from Merck. [^{125}I]Bolton Hunter NPY was ordered from Anawa (Zürich, Switzerland) and its specific activity was 81 TBq/mmol. [^{3}H]Propionyl NPY (specific activity 3.2 TBq/mmol) was purchased from Amersham.

2.2. Peptide synthesis, purification and analysis

The linear peptide was synthesized by automated, multiple solid phase peptide synthesis using a robot system (Syro, MultiSynTech, Bochum) and applying Fmoc/tBu strategy as described previously [17].

The N-terminus of the peptide was acetylated while it was still fully protected and bound to the resin. The linear peptide amide was cleaved with trifluoroacetic acid/thioanisole/thiocresol within 3 h, collected by centrifugation and lyophilized from water. For cyclization the unprotected but N-terminal acetylated dodecapeptide amide (1 eq) was dissolved in *N,N*-dimethylformamide (2 mM), 1-hydroxy-benzotriazole (3 eq), TBTU (3 eq) and diisopropylethylamine (1% v/v) were added. After shaking for 2 h the solvent was removed at reduced pressure. The residue was dissolved in trifluoroacetic acid and precipitated from cold diethyl ether. The product was collected by centrifugation and resuspended twice in diethyl ether. Finally it was lyophilized from water. The cyclic product was purified by preparative HPLC on a Nucleosil C-18 column and eluted with 21% acetonitrile.

The peptide was investigated by analytical HPLC (Nucleosil C-18 column, 5, 1200×3 mm, Merck-Hitachi, Darmstadt), purity was >98%. Correct mass was identified by ion-spray mass spectrometry (API III, Sciex, Toronto): M_{exp}: 1698.6 ± 1.0 amu (M_{th}: 1698.9 amu). Amino acid analysis was as expected Leu 1.00 (1), Arg 3.20 (3), His 1.11 (1), Tyr 1.89 (2), Ile 0.95 (1), Asx 1.10 (1), Lys 0.89 (1), Glx 2.06 (2).

2.3. NMR experiments and molecular modelling

The 3D structure of the cyclic [Lys^{28}-Glu^{32}] NPY Ac-25–36 and the linear peptide [Lys^{28},Glu^{32}] NPY Ac-25–36 was obtained from 2D [^{1}H]NMR experiments using 80 ms [^{1}H,^{1}H]TOCSY, [^{1}H,^{1}H]DQF-COSY and 300 ms [^{1}H,^{1}H]ROESY experiments in standard procedure [18]. Whereas both peptides are unstructured in aqueous solution, upon addition of TFE-d_3, which is known to stabilize structures involving hydrogen bonds [19], medium- and long-range NOEs become visible. Therefore, spectra were recorded on 3 mM solutions of the peptides in 30% TFE-d_3/H_2O at 600 MHz with solvent suppression being achieved through low-power preirradiation. Spectra had to be run at 35°C since the cyclic peptide displayed broad lines for the residues involved in the ring presumably due to conformational exchange at lower temperatures. In addition to the distance constraints derived from the ROESY experiment, $^{3}J(H^{N}H^{C\alpha})$ scalar coupling constants were extracted from the ROESY spectrum using the INFIT method [20]. Distance and dihedral angle constraints were used as input for distance geometry calculations computed with the DIANA package [21] The conformational space spanned by the 10 lowest energy DIANA structures is displayed in Fig. 3.

The 10 DIANA structures were then energy minimized using the forcefield of AMBER4.1 [22] with the standard parameter set parm94. The peptide was centered in a 7.5 Å thick shell of TIP3P water molecules [23] without positional constraining of the solvent molecules, discarding any water atom closer than 1.75 Å to any solute atom and assuming a dielectric constant of 1. During the minimization the lactam bond was held fixed through additional distance constraints. A residue-based cutoff of 10 Å, a secondary cutoff of 15 Å and the all-atom mode were applied. The structure of the solvated peptide was energy minimized by 1000 steps of steepest descent followed by a conjugate gradient minimization procedure until the rms gradient of the potential energy was less than 0.02 kcal/mol·Å. Finally, the quality of the minimized structures was checked by means of the program PROCHECK [24].

2.4. Membrane preparation and receptor binding

SMS-KAN cells and SK-N-MC cells were grown in 50% nutrient mixture Ham's F12/50% Dulbecco's modified Eagle medium with 15% fetal calf serum, 2 mM glutamine, non-essential amino acids, 1% gentamycin at 37°C and 5% CO_2 until they were confluent. Membrane suspensions were prepared as described previously [16].

2.5. Receptor binding

For binding studies membrane suspensions (100 µl) obtained from SMS-KAN (Y_1) or SK-N-MC (Y_2) cells were incubated with 30 pM [^{125}I]Bolton Hunter NPY (^{125}I-BH-NPY) or 1.2 nM [^{3}H]propionyl-NPY (^{3}H-NPY) and different concentrations of the peptide in a total volume of 250 µl for 2 h at room temperature as described recently [16]. Non-specific binding was defined in the presence of 1 µM NPY. Half maximal inhibition of the specific binding of the ^{125}I-BH-NPY and the ^{3}H-NPY of two to three separate experiments is given as the K_i value (mean ± S.E.M.). Eleven concentrations of the competitors were chosen to bracket the K_i value and to cover six orders of magnitude. All competitor curves were fitted with a monophasic binding profile suggesting a single binding site.

2.6. Biological assays

Whole-cell Ca^{2+} channel currents were recorded in SH-SY5Y cells using the amphotericin B perforated-patch technique as previously described [10], using 10 mM Ba^{2+} as charge carrier. Cells were perfused with a solution of composition: NaCl 110 mM, CsCl 5 mM, $MgCl_2$ 0.6 mM, $BaCl_2$ 10 mM, HEPES 5 mM, glucose 10 mM, tetraethylammonium chloride (TEA) 20 mM (pH 7.4, 21–24°C). Patch electrodes (resistances 2–8 M) were filled with a solution of CsCl 120 mM, TEA 20 mM, EGTA 10 mM, $MgCl_2$ 2 mM, HEPES 10 mM. Amphotericin B was also present at a concentration of 240 µg/ml (from a stock solution of 60 mg/ml made in dimethylsulfoxide) and experiments were performed at low light intensity. Cells were voltage clamped at −80 mV and Ca^{2+} channel currents were evoked by 200 ms step depolarizations, applied at a frequency of 0.2 Hz. Current amplitudes were measured at their peak following leak subtraction as previously described [10], and residual capacitance currents have been omitted for illustrations.

3. Results and discussion

The cyclic analog of the C-terminal segment [Lys^{28}-Glu^{32}] NPY Ac-25–36 was synthesized and a high affinity to human Y_2 receptors on SMS-KAN cells was found (Table 1). This is 20-fold increased compared to the linear peptide NPY Ac-25–36 and more than 100-fold increased compared to [Lys^{28},Glu^{32}] NPY Ac-25–36, the linear peptide with the same replacements as the cyclic one. In contrast, Y_1 receptor binding was reduced by more than 4 orders of magnitude compared to NPY and Y_1 selective [Pro^{34}] NPY. The cyclic peptide was obtained by lactamization of Lys and Glu after replacement of Leu^{28} and Thr^{32} of the native sequence (Fig. 1). These residues had been identified by means of the L-alanine scan not to contribute to the binding of the peptide [16]. Furthermore, activity studies have been performed: as previously described [10], bath applications of NPY cause concentration-dependent, reversible inhibitions of Ca^{2+} channel current amplitudes in SH-SY5Y human neuroblastoma cells

Table 1
Affinity of NPY and analogs to human neuroblastoma cells selectively expressing Y_1 or Y_2 receptors

	SMS-KAN (Y_2) (K_i [nM])	SK-MC (Y_1) (K_i [nM])
NPY	0.04 ± 0.01	0.2 ± 0.05
[Pro^{34}] NPY	15.3 ± 0.9	0.3 ± 0.04
NPY Ac-25–36	21 ± 0.9	>10000
[Lys^{28}-Glu^{32}] NPY Ac-25–36	>1000	>10000
cyclo[Lys^{28}-Glu^{32}] NPY Ac-25–36	0.9 ± 0.3	>10000

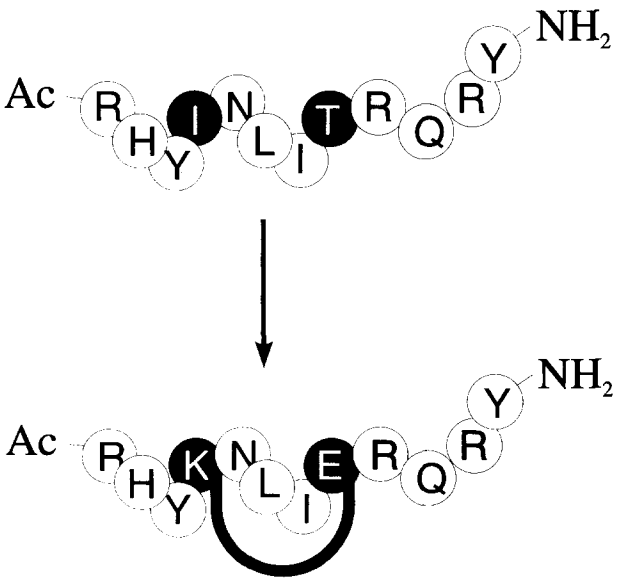

Fig. 1. Scheme of the acetylated C-terminal segment of neuropeptide Y and [Lys^{28}-Glu^{32}] NPY Ac-25–36. Exchanged residues and lactam bridge are marked in gray.

as shown in Fig. 2A. This inhibitory effect is mediated by Y_2 receptors coupled to pertussis toxin sensitive G-proteins. The concentration-response relationship for inhibition of Ca^{2+} channel current amplitude NPY is given by filled circles (Fig. 2C). Like NPY, [Lys^{28}-Glu^{32}] NPY Ac-25–36 also reversibly inhibits the currents in SH-SY5Y cells (Fig. 2B), and the potency of [Lys^{28}-Glu^{32}] NPY Ac-25–36 was similar to that of NPY (Fig. 2C, open circles). These results indicate that [Lys^{28}-Glu^{32}] NPY Ac-25–36 is a full agonist at Y_2 receptors in the human neuroblastoma cell line SH-SY5Y and therefore the lowest molecular weight analog of NPY known to date.

In the initial studies characterizing NPY receptor subtypes [4], the Y_2 receptor was distinguished from the Y_1 receptor by the fact that the former can recognize C-terminal segments of the hormone. Further investigations showed that activity is maintained in the C-terminal moiety, such as NPY(16–36) [25]. These results led us to speculate that a modified segment of NPY Ac-25–36 could be sufficient for Y_2 receptor recognition. The decrease of binding capacity of [Lys^{28}-Glu^{32}] NPY Ac-25–36 compared to NPY could be due to the lack of stability under the conditions of the binding assay as a different behavior of C-terminal analogs has been reported before [26].

The linear peptide [Lys^{28},Glu^{32}] NPY Ac-25–36 and the cyclopeptide [Lys^{28}-Glu^{32}] NPY Ac-25–36 have been investigated by 2D-NMR experiments in order to characterize their structure. NMR data reveal sequential H^N-H^N NOE contacts at the C-terminus for both the linear and the cyclized peptide. These close contacts are indicative of a C-terminal helical winding of the backbone, which for both peptides starts at Asn^{29} and extends to residue Tyr^{36} for the linear peptide and to Gln^{34} for the cyclic peptide (Fig. 3), where it is followed by a turn. However, NOEs between residues from the C- and N-terminus such as between the sidechains of residues His^{26} and Tyr^{36} have only been detected for the cyclic analog. Using the distances obtained from the ROESY experiment a 3D model has been built up. The results suggest that the biologically active conformation of the C-terminal part of NPY is very similar to the tertiary structure of [Lys^{28}-Glu^{32}] NPY Ac-25–36. Further evidence for the C-terminal helix derives from CD

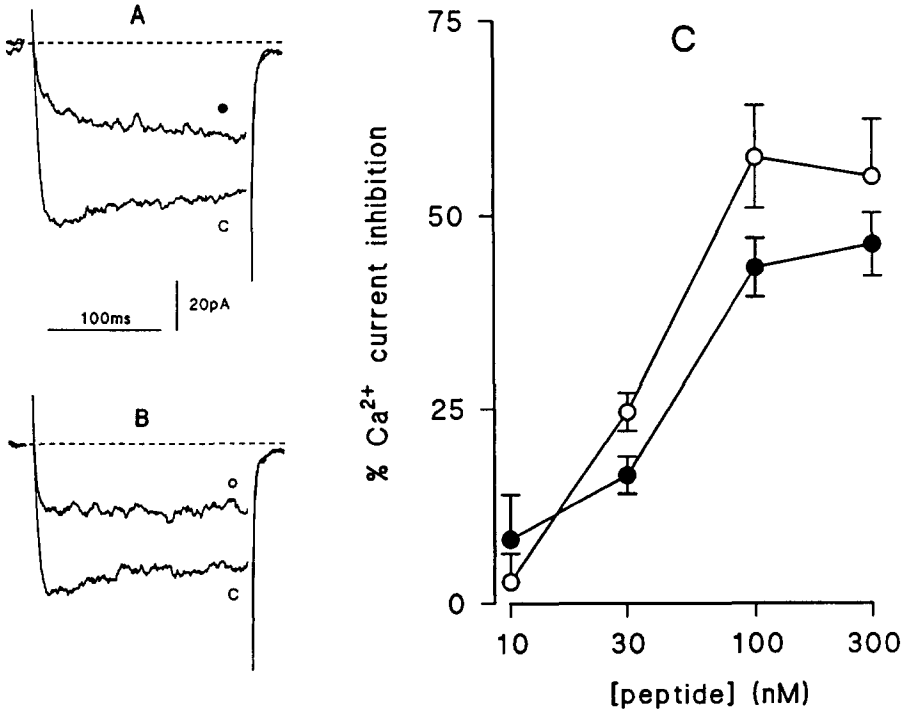

Fig. 2. Example currents evoked in a SH-SY5Y cell by step depolarizations from −80 to 0 mV before (c) and during bath application of 100 nM NPY (A, ●) or [Lys^{28}-Glu^{32}] NPY Ac-25–36 (B, ○). Scale bars apply to both A and B. (C) Log concentration-response curves indicating mean percentage inhibition of peak inward current amplitudes caused by NPY (●) and [Lys^{28}-Glu^{32}] NPY Ac-25–36 (○). Each point is the mean with vertical standard error bars taken from 4–14 cells in each case.

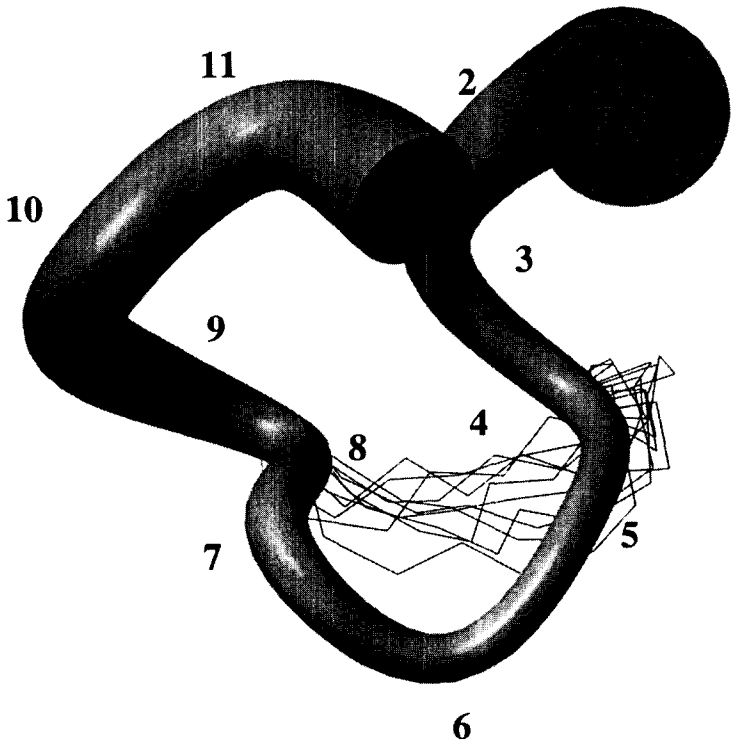

Fig. 3. Superposition of backbone atoms of residues 4–8 of the energy minimized DIANA structures. The less well defined carbon atoms of the lactam bridge are indicated by thin lines, whereas for the rest of the backbone atoms the envelope of the structure bundle is displayed. The drawing was prepared using the program MOLMOL [32].

measurements (data not shown). In order to estimate the extent to which the peptides are structurally ordered, we compared their vicinal H^N-$H^{C\alpha}$ scalar coupling constants. Vicinal H^N-$H^{C\alpha}$ scalar coupling constants mostly close to 6.5 Hz for the case of the linear peptide indicate a low population of the helical structure in spite of the detected sequential amide ROE contacts. In contrast, coupling constants of [Lys[28]-Glu[32]] NPY Ac-25–36 close to 5.5 Hz for the helical segment or 8.9 Hz for the residues involved in the turn indicate that the structure displayed in Fig. 3 is much higher populated, not only in the rigid cycle, but also at both termini. In good agreement with this structure, Y_2 receptor affinity could be correlated with helicity [27].

A comparison of the 3D model of NPY (Fig. 4A) to that of [Lys[28]-Glu[32]] NPY Ac-25–36 (Fig. 4B) reveals that both peptides display a hairpin-like structure, in which the N-terminus covers one side of the C-terminal helix. We propose that this feature is necessary for biological function. Evidence stems from the fact that residues of the N-terminal part of NPY can be substituted without loss of biological activity. Furthermore, the size, the orientation and the position of the lactam ring play an important role (Rist et al., unpublished data) suggesting that the orientation of the C-terminal helix with respect to the N-terminus is of major importance. This is supported by the observation that the major difference between the inactive linear and the active cyclic peptide was the orientation of the C-terminal amino acids Arg[35]-Tyr[36]-NH_2: whereas for the linear peptide this dipeptide is part of the helix, in the case of the cyclic derivative the last two residues are involved in a bend which orients the C-terminus towards the N-terminus.

Our 3D-structure of [Lys[28]-Glu[32]] NPY Ac-25–36 is in good agreement with previous structure activity studies: Pro[34] would disturb this conformation, thus reducing Y_2 affinity. So the Y_1 receptor selectivity of all Pro[34] analogs can be explained [28]. Analogs of Ac-25–36 with replacement of Asn[29], Leu[30] or Ile[31] by helix reducing amino acids (Gly, D-aa) have been found inactive [29] and large hydrophobic residues active [30]. In addition, molar Y_2 receptor affinity was

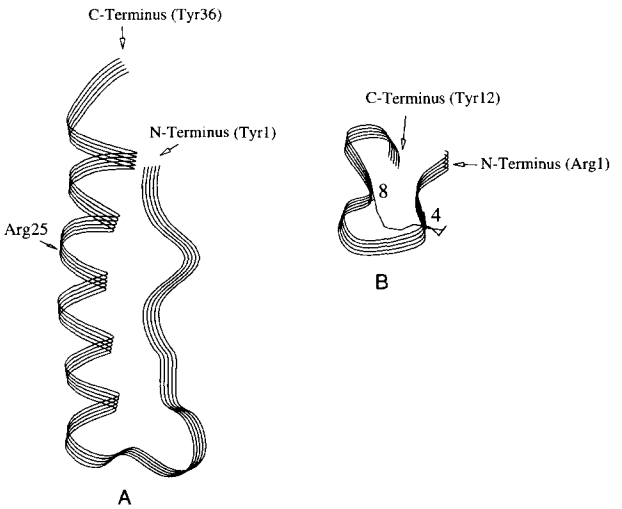

Fig. 4. Ribbon representation of NPY (Y_2) (left) [16] and the NMR mean structure of [Lys[28]-Glu[32]] NPY (right). The N-terminal Arg^1 of [Lys[28]-Glu[32]] NPY corresponds to Arg^{25} in the sequence of NPY. The lactam bridge is shown as a thin line. The drawing was prepared by means of program InsightII (Biosym Inc., San Diego, CA).

found for C-terminal analogs with replacement of Glu^{34} by Leu and Thr^{32} by Tyr [31].

Our predictions have been confirmed in the present study since we have succeeded in developing a cyclic dodecapeptide exhibiting high receptor affinity and agonistic properties as strong as NPY itself. The 3D structure of the cyclic peptide was found to be stable and could be well characterized.

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