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Incorporation of *ortho*-Carbaboranyl-N_ε-Modified L-Lysine into Neuropeptide Y Receptor Y_1- and Y_2-Selective Analogues

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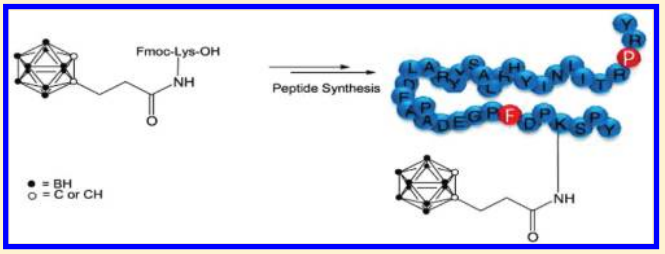
Supporting Information

ABSTRACT: Nontoxic *ortho*-carbaborane is one of the most promising structure for boron neutron capture therapy (BNCT). For directed uptake of *ortho*-carbaborane by tumor cells, receptor-subtype selective neuropeptide Y (NPY) and its derivatives were modified with *ortho*-carbaborane. The derivative [F^7, P^{34}]-NPY has been shown to be a breast cancer selective ligand that binds to the Y_1-receptor subtype, whereas [$Ahx^{(5-24)}$]-NPY selectively addresses Y_2-receptor subtypes that are found in neuroblastoma cells. *ortho*-Carbaboranyl propionic acid was synthesized and linked to the ε-amino group of N_α-Fmoc protected L-lysine. The characterization of the compounds was performed by NMR, IR, and MS studies. The carbaborane-modified amino acid was incorporated into NPY, [F^7, P^{34}]-NPY, and [$Ahx^{(5-24)}$]-NPY by an optimized solid phase peptide synthesis using Fmoc protection. Binding studies and IP accumulation assays confirmed nanomolar affinity and activity of the modified analogues despite of the large carbaborane cluster. Internalization studies revealed excellent and receptor subtype specific uptake of the conjugates into respective cells.



INTRODUCTION

Boron neutron capture therapy (BNCT) is based on the idea of delivering a boron compound to tumor cells and the subsequent irradiation by nonhazardous, thermal neutrons. The latter enter into a nuclear reaction with the ^{10}B isotope, which has a natural abundance of ca. 19.9% and a remarkable capability of capturing thermal neutrons with a capture cross section of ca. 3800 barn.[1,2]

$$^{10}_{5}B + ^{1}_{0}n \rightarrow ^{11}_{5}B \rightarrow ^{7}_{3}Li + ^{4}_{2}He$$

Because of their high ionization potential, the ^7Li and ^4He particles exert a destructive action on cells. Their pathway of action is restricted to the range of ca. 10 μm which coincides with the diameter of a human cell. Both the intracellular production of cytotoxic particles and their limited area of action are the major advantages of BNCT compared to classical chemotherapeutic methods.[1] To date, an increasing number of boron compounds have been synthesized,[1−4] but among these only the *para*-dihydroxy-[^{10}B] boryl-phenylalanine-fructose complex (BPA-Fr) and [^{10}B]-mercaptoundecahydrododecaborate(2−) (BSH) have been studied in clinical trials.[5,6] In the past two decades, mostly compact, boron-rich moieties, e.g., dodecahydrododecaborate(2−) ($B_{12}H_{12}^{2-}$) and dicarba-*closo*-dodecaboranes(12) ($C_2B_{10}H_{12}$), have been functionalized and investigated as boron-delivering agents. Glycosyl-substituents were used to reduce the toxicity and increase the water solubility of compounds containing hydrophobic carbaborane cages.[7−9] They should increase the biological availability by using existing transportation systems but also showed a lack in tumor selectivity.[10] The targeted delivery of ^{10}B into the tumor cells still represents one of the major problems to be solved in BNCT.[11]

Numerous molecules with high affinity and selectivity for tumor cells were found, and they are believed to be used as selective carriers into cancer cells. Peptides, which have been proposed as carrier systems are somatostatin (SST), epidermal growth factor (EGF), neurotensin, substance P, gastrin releasing peptide (GRP), insulin-like growth factor (IGF),[12,13] α-melanocyte stimulating hormone (α-MSH), cholecystokinin (CCK), vascoactive intestinal peptide (VIP), bombesin (BN), and neuropeptide Y (NPY).[14] NPY is a member of the pancreatic polypeptide family and is composed of 36 amino acids. It is one of the most abundant neuropeptides in the brain,[15] binds to four Y-receptor subtypes (Y_1, Y_2, Y_4, and Y_5) in nanomolar concentration and even triggers receptor internalization.[16] The signal transduction of Y-receptors follows a G protein-coupled (GPC) receptor cascade.[17,18]

Reubi et al. investigated the expression of Y-receptors in human breast cancer cells and found a selective overexpression of Y_1-receptor subtypes for more than 90% of the breast tumors and for 100% of all metastatic tumors.[19] Furthermore, they

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reported an alteration of the receptor subtype expression from Y_2-receptors in healthy breast tissue to Y_1-receptors in affected tissue. Recently, successful uptake of a Y_1-receptor selective ^{99m}Tc labeled [F^7, P^{34}]-NPY into breast cancer bearing patients was demonstrated in a diagnostic in vivo approach.[20] The NPY-derivative [F^7, P^{34}]-NPY was shown to preferentially bind to the human Y_1-receptor compared to other Y-receptor subtypes[21] with a high tolerance toward modifications, e.g., labeling with radioactive isotopes.[20,22,23] On the other hand, tissue samples from neuroblastoma show high expression profiles of solely Y_2-receptor subtype[24] and a centrally truncated derivative of neuropeptide Y, [$Ahx^{(5-24)}$]-NPY, displays a high Y_2-receptor selectivity.[25] Therefore, we considered [F^7, P^{34}]-NPY and [$Ahx^{(5-24)}$]-NPY as potent selective carriers for ^{10}B into tumor cells (Table 1). For the first time, we herein describe the combination of BNCT and a tumor selective approach, which uses the Y_1-receptor selective [F^7, P^{34}]-NPY and Y_2-selective [$Ahx^{(5-24)}$]-NPY.

RESULTS

On the basis of our experience with the peptides above, the position lysine 4 can readily be modified without affecting tumor selectivity and receptor internalization.[23] The N_ε-amino group in L-lysine can selectively be converted to yield amides. The moiety attached is placed sufficiently distant (four carbon atoms) from the peptide backbone, and a perturbation of the peptide conformation is not expected. Thus, we chose the position lysine 4 of the peptides for the introduction of *ortho*-carbaborane and designed an *ortho*-carbaborane-modified L-lysine building block, which was subsequently coupled into the peptide sequences by solid phase peptide synthesis using Fmoc/*tert*-butyl protection strategy.

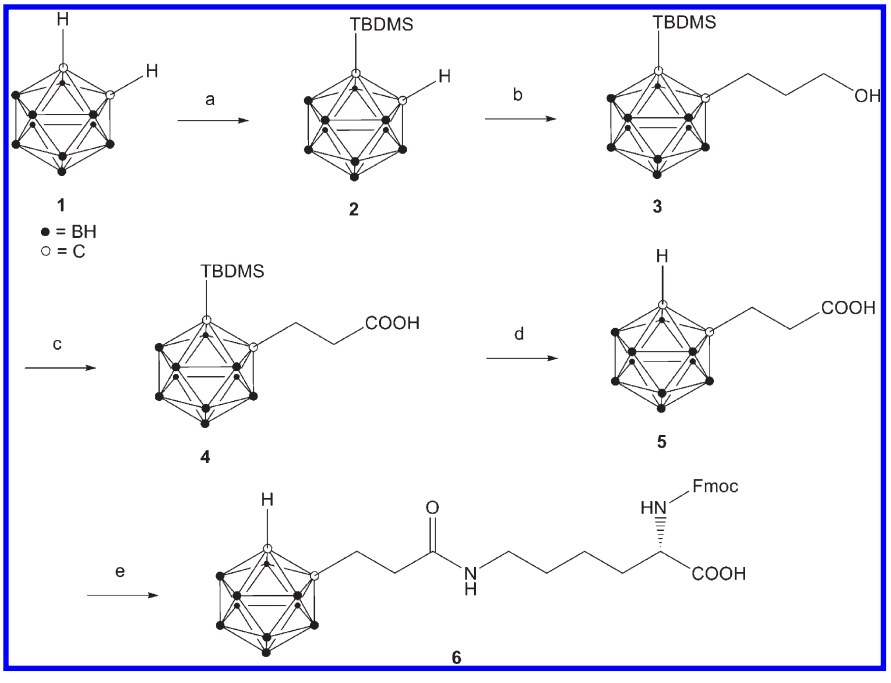
Table 1. Peptide Sequences

peptide	sequence
NPY	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY
[F^7, P^{34}]-NPY	YPSKPDFPGEDAPAEDLARYYSALRHYINLITRPRY
[$Ahx^{(5-24)}$]-NPY	YPSK-**Ahx**-RHYINLITRQRY

Synthetic Procedure. *ortho*-Carbaborane **1** was reacted to yield a carbon TBDMS (TBDMS = Si^tBuMe_2) protected product **2**.[26] **2** was further converted to the alcohol **3**, which was oxidized to give the carboxylic acid **4**. Finally, the TBDMS group was removed to form **5**, *ortho*-carbaboranyl propionic acid (Cpa). The latter has previously been synthesized by other methods, i.e. by cage formation from a substituted alkyne[27−31] or from *ortho*-carbaborane via a Michael addition type reaction.[32] **5** was finally coupled to Fmoc-Lys-OH at the N_ε-amino group to give **6**, (Fmoc-K-N_ε(Cpa)-OH) (Scheme 1). The identity of all compounds was confirmed by ^{1}H, ^{11}B, ^{13}C NMR, IR, and MS studies as well as by elemental analysis. **6** was integrated into the peptide sequences of NPY, Y_1R-selective [F^7, P^{34}]-NPY, and Y_2R-selective [$Ahx^{(5-24)}$]-NPY, respectively.

Peptide Synthesis. Unmodified NPY analogues were synthesized by automated solid phase peptide synthesis. For Fmoc-K-N_ε(Cpa) derivatized peptides, C-termini up to position 5 were synthesized by automated solid phase peptide synthesis. Fmoc-K-N_ε(Cpa)-OH was coupled to the peptide in a manual coupling step at position 4 instead of Fmoc-K-N_ε(Boc)-OH, and subsequent Fmoc-cleavage steps were performed with DBU/DCM (1:1) to protect the piperidine-sensitive carbaborane moiety from decomposition (Scheme 2). After cleavage from the Rink amide resin, peptides were purified up to ≥95% homogeneity by HPLC and identified by MALDI and ESI mass spectrometry

Scheme 1. Synthetic Procedure[a]



[a] Reagents and conditions: (a) *n*-BuLi, toluene−Et_2O (2:1), rt, 2 h, then TBDMS chloride, rt, overnight, 84%; (b) *n*-BuLi, THF, rt, 2 h, then oxetane, rt, overnight, 85%; (c) $NaIO_4$, cat. $RuCl_3 \cdot xH_2O$, CCl_4, MeCN, H_2O (2:2:3), 2 h, rt, 72%; (d) TBAF·$3H_2O$, THF, −78 °C → rt, 1 h, 80%; (e) DCC, HOSu, MeCN, rt, 24 h, then Fmoc-Lys-OH·HCl, DIPEA, DMF, rt, overnight, 79%.

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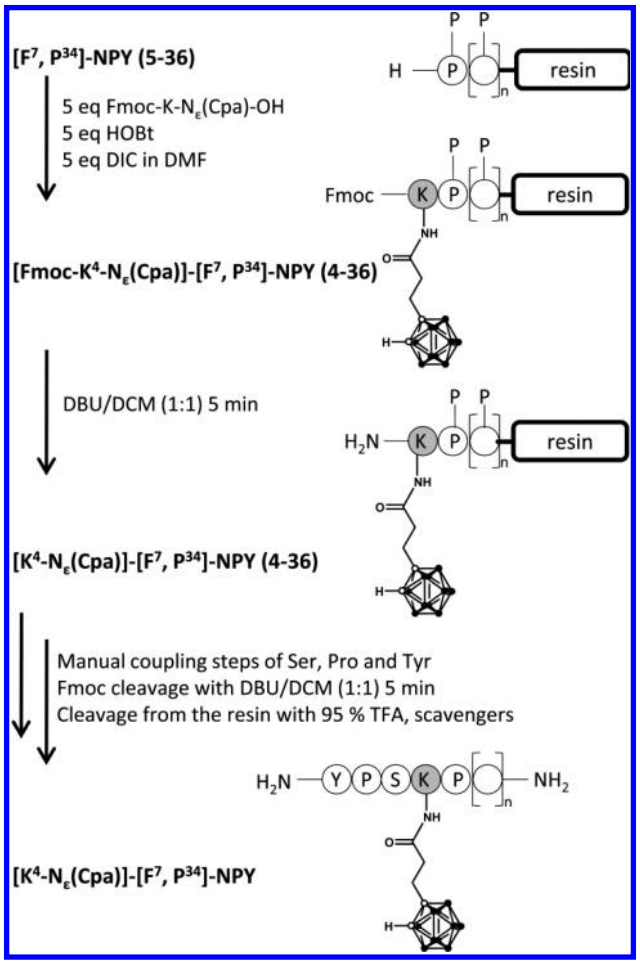
(Figure 1). Because of the isotope ratio of boron with 80.1% ^{11}B and 19.9% ^{10}B in naturally occurring boron, high resolution mass spectra of B_{10} compounds showed a complex isotopic pattern. Figure 1A (middle) displays the isotopic pattern of [K^4-N_ε-(Cpa)]-[F^7, P^{34}]-NPY. Using the Isotope Pattern program (Bruker), a theoretical mass spectrum could be calculated from the molecular formula of the peptide [K^4-N_ε(Cpa)]-[F^7, P^{34}]-NPY, leading to a mass spectrum containing 15 single peaks. The 10 most intense signals (4−13) are visible in the mass spectrum, providing evidence of a successful integration of the carbaborane derivative into the peptide sequence of NPY (Table 2).

Scheme 2. Incorporation of Fmoc-K-N_ε(Cpa) into the Peptide Sequence of [F^7, P^{34}]-NPY by Solid Phase Peptide Synthesis (SPPS)



Receptor Binding and Activity. Receptor binding affinities of all six peptides were tested in radioligand competition assays on an MCF-7 breast adenocarcinoma cell line, which endogenously expresses the Y_1-receptor.[33] In these studies, we could show that the modification of NPY and its hY_1- or hY_2-receptor selective analogues with an *ortho*-carbaboranyl propionic acid moiety on the N_ε-group of lysine 4 decreased peptide affinity toward the receptor from K_I = 0.43 ± 0.1 nM to K_I = 8.4 ± 1.1 nM for [F^7, P^{34}]-NPY and [K^4-N_ε(Cpa)]-[F^7, P^{34}]-NPY, respectively (Figure 2). These values still reflect excellent receptor binding affinities in the nanomolar range for the K^4-N_ε(Cpa)-modified Y_1R-selective peptide. Affinities of the Y_2-receptor subtype selective peptides [$Ahx^{(5-24)}$]-NPY and [K^4-N_ε(Cpa)]-[$Ahx^{(5-24)}$]-NPY indicate that neither of these peptides show a high affinity for the Y_1-receptor.

Peptides were also tested for their ability to activate Y-receptors (Figure 3). For signal transduction assays, COS-7 cells were transiently transfected with the human Y_1- or Y_2-receptor and a chimeric G-protein ($G\alpha_{\Delta 6qi4myr}/G_{qi4}$). After labeling with ^3H myo-inositol, cells were stimulated with peptide solution and intracellular inositol phosphate (IP)-levels were determined by scintillation measurements after anion exchange chromatography. IP accumulation is directly correlated to the magnitude of receptor activation. Carbaborane-modified [F^7, P^{34}]-NPY activates Y_1-receptors (Figure 3A) with the same efficacy and potency as the unmodified Y_1R-selective NPY analogue [F^7, P^{34}]-NPY (EC_{50}: 4.0 ± 2.8 vs 3.0 ± 2.5 nM). In contrast, the Y_2-selective analogue shows a significantly higher EC_{50} value at the Y_1-receptor (271 ± 149 nM), whereas NPY and [K^4-N_ε(Cpa)]-NPY both activate Y_1-receptors at nanomolar concentrations. Neither [F^7, P^{34}]-NPY nor carbaborane-modified [F^7, P^{34}]-NPY were able to fully activate the Y_2R indicated by EC_{50} values of more than 1000 nM (Figure 3B). In comparison, NPY and [K^4-N_ε(Cpa)]-NPY, as well as [$Ahx^{(5-24)}$]-NPY and [K^4-N_ε-(Cpa)]-[$Ahx^{(5-24)}$]-NPY, fully activate the Y_2-receptor with EC_{50} values between 1.6 and 31.6 nM (Table 3). These data clearly indicate the high Y_1-receptor selectivity and affinity of [K^4-N_ε(Cpa)]-[F^7, P^{34}]-NPY and a strong Y_2-receptor selectivity and affinity of [K^4-N_ε(Cpa)]-[$Ahx^{(5-24)}$]-NPY.

Receptor Internalization. To further investigate the data obtained from receptor affinity and activity, microscopic studies visualizing receptor internalization were performed. Receptor localization of cell lines which stably express the human Y_1-receptor C-terminally fused to EYFP (EYFP = enhanced yellow fluorescent protein), the human Y_2-receptor C-terminally fused to EGFP (EGFP = enhanced green fluorescent protein) or which are transiently transfected with the human Y_4- or Y_5-receptor C-terminally fused to EYFP was visualized prior to stimulation and after 60 min stimulation with 1 μM peptide solution (Figure 4). Before stimulation, all Y-receptor subtypes are mainly

Table 2. Analytical Data of the Synthesized Peptides

peptide	M_{calc} [Da]	M_{exp} [Da] $[M + H]^+$	retention time methanol gradient [min]	retention time acetonitrile gradient [min]	purity methanol gradient [%]	purity acetonitrile gradient [%]
NPY	4251.1	4252.2	23.1	39.0	>99	>99
hPP	4179.1	4180.2	23.0	38.5	>99	>99
[K^4-N_ε(Cpa)]-NPY	4451.3	4452.4	26.3	45.2	>99	>99
[F^7, P^{34}]-NPY	4253.1	4254.1	24.2	40.3	>99	>95
[K^4-N_ε(Cpa)]-[F^7, P^{34}]-NPY	4453.3	4454.3	27.4	47.1	>99	>95
[$Ahx^{(5-24)}$]-NPY	2219.2	2220.3	15.3	25.4	>95	>99
[K^4-N_ε(Cpa)]-[$Ahx^{(5-24)}$]-NPY	2419.4	2420.5	21.3	39.2	>99	>99

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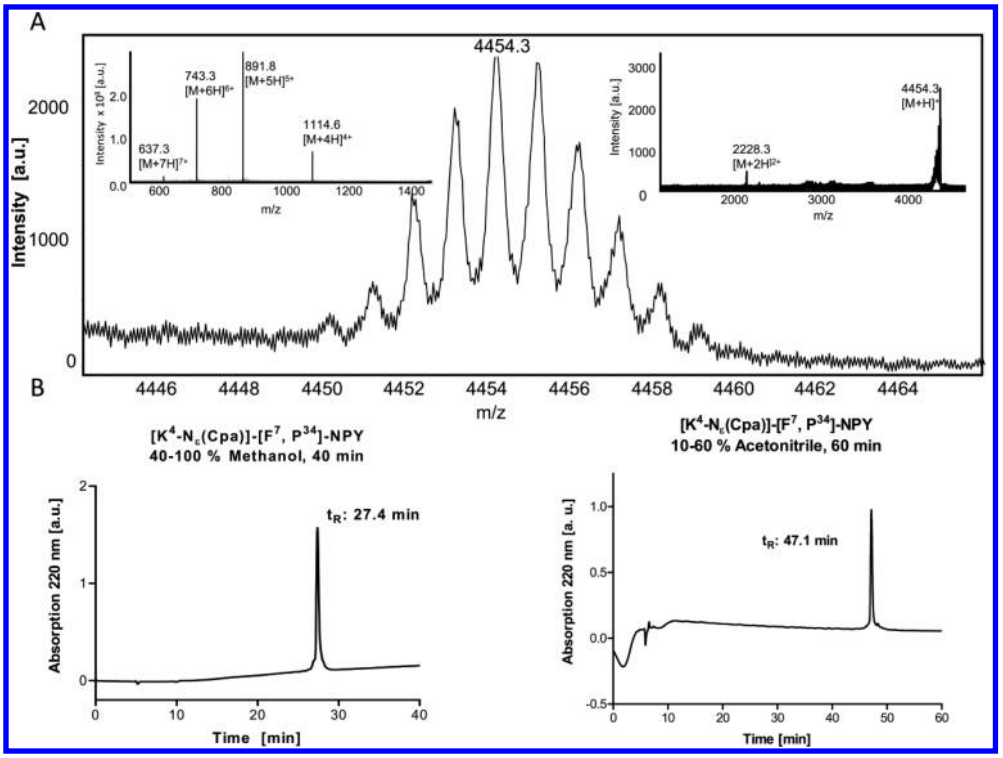

Figure 1. Analytical Data of [K^4-N$_\varepsilon$(Cpa)]-[F^7, P^{34}]-NPY. (A) MALDI-TOF mass spectrum with an experimental mass $[M + H]^+$ of 4454.3 Da (calculated mass of [K^4-N$_\varepsilon$(Cpa)]-[F^7, P^{34}]-NPY: 4453.3 Da ($C_{200}H_{303}N_{53}O_{56}B_{10}$)). The enlargement of the MALDI-TOF mass spectrum shows the complex isotope pattern of the peptide. Peaks 4−13 of 15 theoretical peaks are visible. The ESI mass spectrum displays the 4-, 5-, 6-, and 7-fold charged molecule. (B) RP-HPLC chromatogram using a gradient of 40−100% MeOH (0.08% TFA) in water (0.1% TFA) in 40 min (t_R = 27.4 min) (left) and analytical RP-HPLC chromatogram with a gradient of 10−60% ACN (0.08% TFA) in water (0.1% TFA) over 60 min (t_R = 47.1 min) (right).

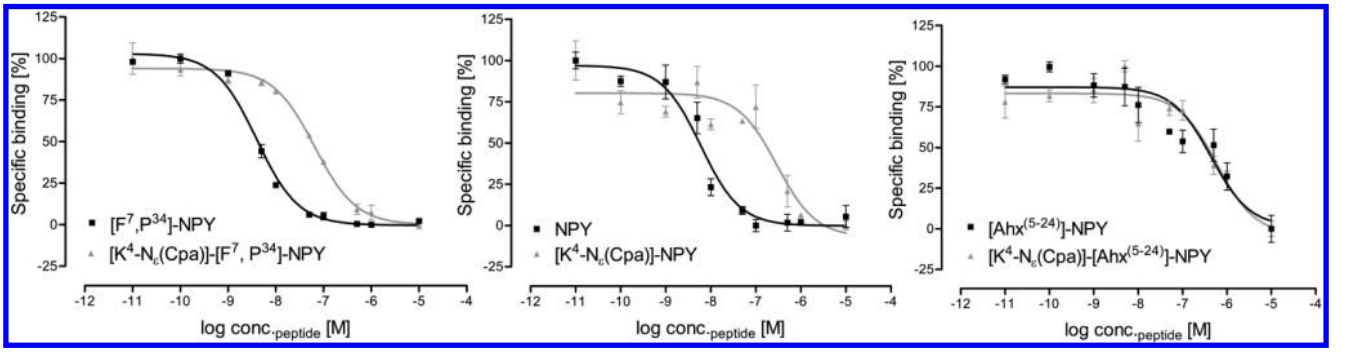

Figure 2. Affinity Studies of Carbaborane-Modified NPY Analogues on the Y$_1$-Receptor. In vitro binding curves of all peptides to MCF-7 breast adenocarcinoma cells (Y$_1$R) indicate a slight loss in receptor activity but still nanomolar affinities for [K^4-N$_\varepsilon$(Cpa)]-modified NPY analogues.

located in the cell membrane; some are detected inside the cytoplasm which is due to the regular protein turnover inside a living cell. For the hY$_1$R, both the universal ligand NPY and the Y$_1$-receptor selective [F^7, P^{34}]-NPY promote microscopically observable receptor internalization. Fluorescent receptors are no longer located in the plasma membrane but are found in vesicles inside the cytoplasm. [K^4-N$_\varepsilon$(Cpa)]-NPY and [K^4-N$_\varepsilon$(Cpa)]-[F^7, P^{34}]-NPY were able to activate Y$_1$-receptors in the same range and induce internalization of these receptors. In contrast, [Ahx$^{(5-24)}$]-NPY and [K^4-N$_\varepsilon$(Cpa)]-[Ahx$^{(5-24)}$]-NPY stimulated Y$_1$-receptors exhibit a full membrane localization, verifying the high receptor subtype selectivity of carbaborane−NPY conjugates. Wild-type NPY, [Ahx$^{(5-24)}$]-NPY, and their [K^4-N$_\varepsilon$-(Cpa)]-modified analogues induced receptor mediated endocytosis at the Y$_2$-receptor, whereas Y$_1$-receptor selective [F^7, P^{34}]-NPY and [K^4-N$_\varepsilon$(Cpa)]-[F^7, P^{34}]-NPY did not lead to any visible effect in receptor localization compared to nonstimulated receptors. The Y$_4$-receptor shows internalization after stimulation with NPY, [K^4-N$_\varepsilon$(Cpa)]-NPY, and hPP, which is the natural ligand of this receptor,[34,35] but not for any of the Y$_1$- or Y$_2$-receptor selective peptides. These are still mainly located in the cell membrane after 60 min of stimulation with peptides, therefore, no significant internalization takes place. None of the peptides induce internalization of the human Y$_5$-receptor, which is localized in the cell membrane after stimulation. This receptor is known to internalize more slowly and to a lesser extent than any of the other Y-receptor subtypes.[16]

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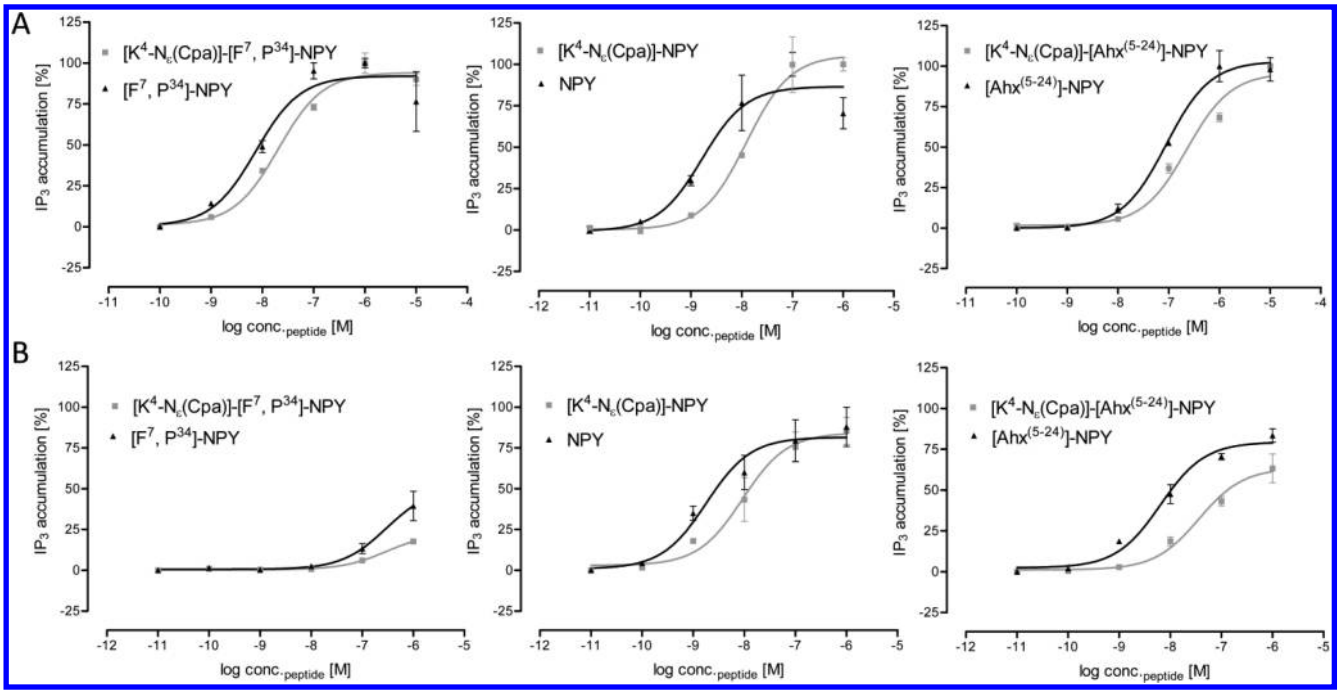

Figure 3. Activity studies of carbaborane-modified NPY analogues on the Y_1-receptor. (A) Signal transduction assays were performed with all peptides on the Y_1-receptor. Y_1-receptor selective [K^4-N_ε(Cpa)]-modified NPY analogues activate the receptor in nanomolar concentrations. (B) Signal transduction assays were performed with all peptides on the Y_2-receptor. Y_1-receptor selective [K^4-N_ε(Cpa)]-modified NPY analogues are not able to induce full receptor activation, whereas the carbaborane-modified Y_2-receptor analogue leads toward high receptor activation.

Table 3. Receptor Binding and Receptor Activation of Carbaborane-NPY analogues of Y_1R and Receptor Activation of Y_2R

peptide	K_I hY_1R [nM]	EC_{50} hY_1R [nM]	EC_{50} hY_2R [nM]
NPY	1.2 ± 0.5	2.1 ± 1.0	1.6 ± 0.8
[K^4-N_ε(Cpa)]-NPY	35.4 ± 6.6	14.2 ± 4.1	10.7 ± 4.4
[F^7, P^{34}]-NPY	0.43 ± 0.1	3.0 ± 2.5	>1000
[K^4-N_ε(Cpa)]-[F^7, P^{34}]-NPY	8.4 ± 1.1	4.0 ± 2.8	>1000
[$Ahx^{(5-24)}$]-NPY	46.8 ± 9.2	116 ± 60	5.2 ± 1.3
[K^4-N_ε(Cpa)]-[$Ahx^{(5-24)}$]-NPY	77.8 ± 39.4	271 ± 149	32 ± 4.2

In summary, the Cpa-modified peptides show increased K_I values compared to those of unmodified analogues. These findings can be explained by higher K_{off} rates for carbaborane containing peptides. Still, these compounds retain their ability to activate their corresponding receptor as demonstrated in receptor activation studies. Most importantly, the Cpa-modified peptides trigger receptor subtype specific internalization, which is a major prerequisite for intracellular boron accumulation. These findings confirm the possibility of synthesizing carbaborane modified neuropeptide Y analogues, which show high affinity and activity at only one Y-receptor subtype.

DISCUSSION

On the basis of the knowledge about different expression profiles of Y-receptor subtypes in neuroblastoma, breast cancer tissue, and normal breast tissue, Fmoc-K-N_ε(Cpa)-OH was synthesized and subsequently integrated into NPY analogues that selectively bind to specific receptor subtypes overexpressed in cancerous tissue. Receptor binding affinities, receptor activation, and ligand-mediated internalization of Y-receptors were evaluated in this study.

The idea of using peptides for drug delivery and as therapeutics is not new. An increasing number of synthetic peptide therapeutics have reached the pharmaceutical markets up to now or are tested in clinical trials.[36] A receptor-independent way of cargo delivery for a cytotoxic substance using cell-penetrating peptides as drug shuttles was shown previously.[37] Furthermore, the same cell-penetrating peptide could be applied for intracellular delivery of plasmid DNA into primary cells.[38] One disadvantage of drug delivery by cell penetrating peptides is an unselective cellular targeting mechanism[39] of these peptides compared to receptor selective ligands. The advantages of receptor targeting peptide therapeutics are their high receptor binding affinities, fast body clearance, high tumor-to-background ratios,[40] nontoxic metabolic cleavage products,[41] and relatively low manufacturing costs.[36] For example, a completely synthetic somatostatin analogue, octreotide, is in use to treat patients suffering from acromegaly[42] and very malignant gastroenteropancreatic neuroendocrine tumors.[43] Radiolabeled receptor selective neuropeptides and their receptors are applicable tools for peptide receptor radiotherapy[44] and in vivo imaging studies.[45]

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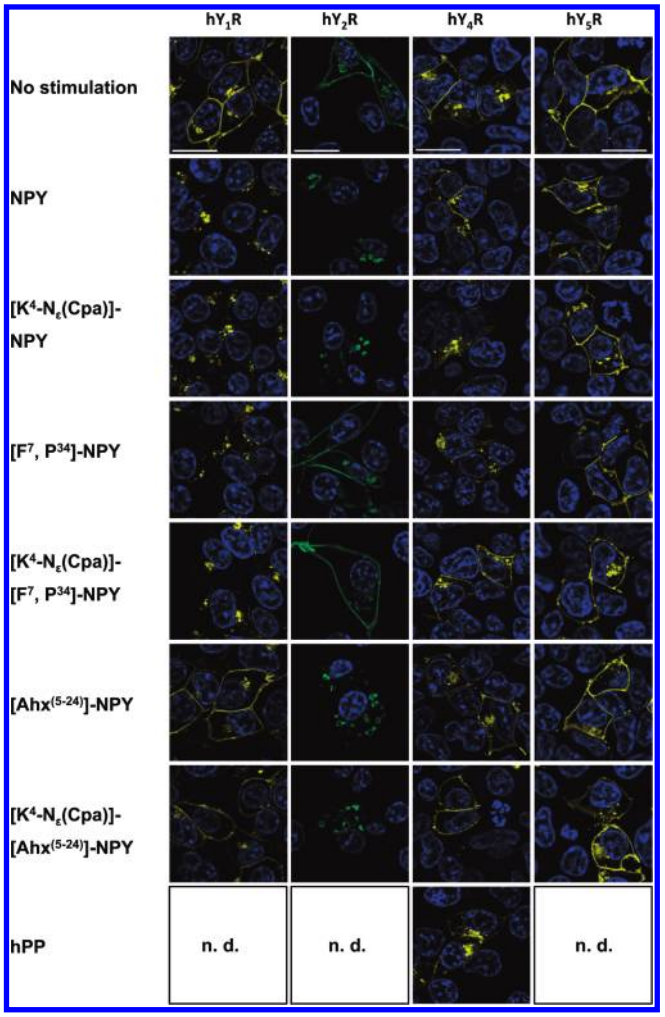

Figure 4. Receptor internalization studies on Y_1R, Y_2R, Y_4R, and Y_5R with unmodified and carbaborane-modified NPY analogues. Y-receptor internalization in stable and transient expression systems (HEK293_hY$_1$_EYFP, BHK_hY$_2$_EGFP, HEK293_hY$_4$_EYFP, and HEK293_hY$_5$_EYFP). Nonactivated receptors remain in the cell membrane whereas activated receptors are rapidly internalized into the cell. [K^4-Nε(Cpa)]-modified peptides induce receptor internalization exactly in the same mode as unmodified receptor selective peptides. Bar: 20 μm. nd: not determined.

Surprisingly, references dealing with the concept of modifying peptides by boron-rich moieties are rare. Approaches to incorporate boron rich moieties into peptides are given,[46,47] and in some cases, the resulting conjugates have been evaluated biochemically with promising findings, i.e., the peptides retained their affinity toward receptors.[48−52]

The peptides of the neuropeptide Y family (NPY, pancreatic polypeptide, and peptide YY) and their receptors display an excellent opportunity to target Y-receptor expressing tumor cells. High expression levels of the Y_1-receptor subtype are found in breast cancer tissue,[53] Ewing sarcoma tumors,[54] and in the colon adenocarcinoma cell line HT-29.[55] The tumor expression profile for the Y_2-receptor subtype indicates a high receptor density in brain tumors such as neuroblastoma[24] and glioblastoma,[56] which makes this receptor an interesting target for new therapy approaches.[57]

In the past, we showed that Y_1-receptor expressing cells can be targeted by a daunorubicin−NPY bioconjugate with an acid-labile hydrazone linker, resulting in a cell growth reduction of more than 65% of a SK-N-MC cell line, which belongs to the Ewing sarcoma family of tumors.[58]

It is well-known which amino acid residues in the peptide sequence of NPY are essential for receptor binding.[59] In terms of receptor affinity, the lysine residue on position four is tolerant for modifications. Therefore, an *ortho*-carbaboranyl-modified L-lysine was synthesized and subsequently incorporated into highly Y-receptor selective peptides ([F^7, P^{34}]-NPY and [$Ahx^{(5-24)}$]-NPY) at position four to yield carbaborane-modified NPY analogues. Receptor binding of all prepared bioconjugates and their unmodified analogues was tested on a Y_1R expressing breast adenocarcinoma cell line, revealing a slightly reduced affinity for [K^4-N$_\varepsilon$(Cpa)]-[F^7, P^{34}]-NPY compared to [F^7, P^{34}]-NPY and very low affinity of the Y_2R selective peptides. Receptor activity studies confirmed the results obtained in affinity studies. [K^4-N$_\varepsilon$(Cpa)]-[F^7, P^{34}]-NPY activated Y_1-receptors with low EC_{50} values, whereas no activation of the Y_2-receptor was observed. In contrast, [K^4-N$_\varepsilon$(Cpa)]-[$Ahx^{(5-24)}$]-NPY exhibited high EC_{50} values on the Y_1-receptor and activated Y_2-receptors with EC_{50} values in the nanomolar range. These data indicate that the strong receptor subtype selectivity of *ortho*-carbaborane-modified NPY analogues is maintained even after introducing the *ortho*-carbaboranyl-modified L-lysine into the peptide sequence. Receptor activity studies were supported by fluorescence microscopy investigating the internalization behavior of Y-receptors after peptide stimulation,[16] which showed receptor internalization of a receptor subtype only upon stimulation with the respective agonist or its nonmodified analogue.

CONCLUSIONS

We could prepare the carbaborane-bearing amino acid Fmoc-Lys-N$_\varepsilon$-*ortho*-carbaboranyl propionic acid ((Fmoc-K-N$_\varepsilon$(Cpa)-OH) and have optimized peptide synthesis in order to directly include it into the synthesis of the 36mer peptide amide. Binding and signal transduction assays revealed only a slight loss of affinity and activity despite the size of the carbaborane cluster. Selectivity was maintained and internalization studies showed a clear and selective uptake of the carbaborane-containing analogues of NPY in cells of the respective subtype.

MATERIAL AND METHODS

General Synthetic Methods. All chemical reactions were carried out in an atmosphere of dry nitrogen. The solvents were purified and distilled under nitrogen prior to use.[60] The chemicals were used as purchased. The ^{1}H, ^{11}B, and ^{13}C NMR spectra were recorded on a Bruker AVANCE DRX 400 spectrometer. The chemical shifts of ^{1}H, ^{11}B, and ^{13}C NMR spectra are reported in parts per million at 400.13, 128.38, and 100.63 MHz, respectively, and utilize tetramethylsilane as an internal standard and the referencing to the unified scale.[61] Acidic protons in ^{1}H NMR spectra were proven by exchange after the addition of D_2O. IR spectra were recorded on a Perkin-Elmer system 2000 FT-IR spectrometer, scanning between 400 and 4000 cm^{-1}, using KBr disks. Mass spectra were recorded on a Finnigan MAT MAT8200 spectrometer (EI) or on a VG Analytics ZAB HSQ system (ESI). The measurement of optical rotation was performed on a Krüss Optronic P3002 RS polarimeter. The elemental analyses were carried out in a Hereaus VARIO EL oven. The melting points were measured in sealed tubes.

***N*$_\alpha$-(Fluoren-9′-yl-methyloxycarbonyl)-*N*$_\varepsilon$-{3′′-[1′′′,2′′′-dicarba-*closo*-dodecaboran(12)-1′′′-yl] propanoyl}-L-lysine (Fmoc-K-N$_\varepsilon$(Cpa)-OH), 6.** (A) Activation of **5. 5** (1.00 g, 4.61 mmol) was

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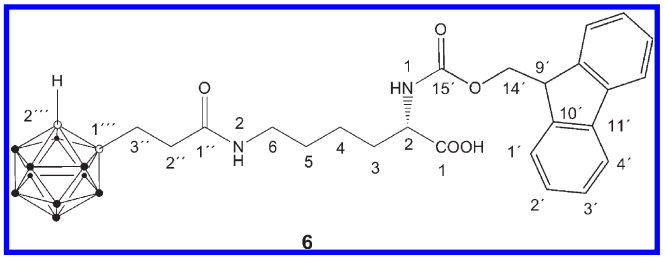

Figure 5. N_α-(Fluoren-9′-yl-methyloxycarbonyl)-N_ε-{3″[1‴,2‴-dicarba-*closo*-dodecaboran(12)-1‴-yl] propanoyl}-L-lysine, (Fmoc-K-N_ε(Cpa)-OH), **6**.

dissolved in MeCN (10 mL). DCC (dicyclohexylcarbodiimide, 1.05 g, 5.09 mmol) and HOSu (*N*-hydroxy succinimide, 0.59 g, 5.09 mmol) were dissolved in MeCN (20 mL). The solution was stirred for 24 h, and the solvent was removed in vacuo. $CHCl_3$ (50 mL) was added under constant heating. The mixture was cooled to ambient temperature, and the solid (dicyclohexylurea) was removed by filtration. The solvent of the filtrate was removed under reduced pressure. The white solid obtained was used without further purification and characterization. (B) Coupling to Fmoc protected L-lysine. The white solid of procedure A and Fmoc-Lys-OH·HCl (3.41 g, 9.25 mmol) were dissolved in DMF (20 mL). DIPEA (diisopropylethyl amine, 1.57 mL, 9.25 mmol) was added, and the solution was stirred for 6 h. A second equivalent of DIPEA (1.57 mL, 9.25 mmol) was added, and the solution was stirred overnight. The mixture was diluted with CH_2Cl_2 (150 mL). The organic phase was extracted with diluted HCl (7 × 50 mL), dried over $MgSO_4$, and the solvent evaporated in vacuo. Column chromatography ($CHCl_3$−MeOH, 24:1, v/v) yielded **6** as a colorless foam (2.10 g, 3.69 mmol, 79%). R_f = 0.10 ($CHCl_3$−MeOH, 9:1, v/v), mp = 84−89 °C. ^{1}H NMR ($CDCl_3$) (for assignment see Figure 5): 1.38−1.85 (m, 6H, CH_2 in C3, C4, C5), 1.20−3.00 (br, 10H, $B_{10}H_{10}CCH$), 2.42, 2.51 (both m, 4H, CH_2 in C2″, C3″), 3.17 (m, 2H, CH_2 in C6), 3.47 (s, 1H, *CH* in C2‴), 4.19 (t, J = 6.6 Hz, 1H, *CH* in C9′), 4.32−4.38 (m, 3H, *CH* in C2, CH_2 in C14′), 5.73 (s, 1H, N*H* in N1), 6.01 (s, 1H, N*H* in N2), 7.28 (t, J = 8.8 Hz, 2H), 7.38 (t, J = 6.6 Hz, 2H, both *CH* in C2′, C3′ and equivalent positions), 7.56 (d, J = 6.8 Hz, 2H), 7.74 (d, J = 7.2 Hz, 2H, both *CH* in C1′, C4′, and equivalent positions), 8.05 (br, s, 1H, COO*H*). ^{13}C{^{1}H} NMR ($CDCl_3$, APT): 22.40 (C4), 28.54 (C5), 31.76 (C3), 32.80, 35.09 (C2″, C3″), 39.31 (C6), 47.06 (C9′), 53.57 (C2), 61.94 (C2‴), 67.19 (C14′), 74.38 (C1‴), 120.10, 125.03, 127.14, 127.87 (all four C1′, C2′, C3′, C4′, and equivalent positions), 141.25, 143.57 (both C10′, C11′, and equivalent positions), 156.49 (C1), 170.89, 175.29 (both C1″, C15′). ^{11}B{^{1}H} NMR ($CDCl_3$): −11.8, −9.5, −5.7, −2.3. IR: 3408, 3338 (m, ν(N−H in amide)), 3058, 2941, 2864 (m, ν(C−H)), 2591 (ν(B−H)), 1709 (s, ν(C═O) in amide), 1655 (s, ν(C═C)), 1450 (m, δ(C−H)), 1248, 1219 (s, ν(C−O)), 760, 740 (s, δ(C−H) out of plane in aryl). MS (ESI−): 565 ([M − H]$^-$, 100%). $[\alpha]_D^{25}$ = 7.8° (1 M in $CHCl_3$). Elemental analysis: calcd C (55.10%), H (6.77%), N (4.94%); found C (55.81%), H (7.19%), N (5.01%).

Materials for Peptide Synthesis and Biological Studies. Fmoc-protected proteinogenic amino acids were obtained from Novabiochem (Läufelfingen, Switzerland), Fluka (Buchs, Switzerland), and Iris Biotech (Marktredwitz, Germany). 4-(2,4-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy (Rink amide) resin was obtained from Novabiochem. DMF, DCM, methanol (MeOH), and diethylether (Et_2O) were purchased from Scharlau (La Jota, Barcelona, Spain). Hydroxybenzotriazole (HOBt), thioanisole, *p*-thiocresol, piperidine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), trifluoroacetic acid (TFA), and *tert*-butanol were purchased from Fluka. *N*,*N*′-Diisopropylcarbodiimide (DIC) was purchased from Aldrich (Buchs, Switzerland). Acetonitrile (ACN) was obtained from Romil (Cambridge, England).

Dulbecco's Modified Eagle Medium (DMEM)/Ham's F 12 (1:1), DMEM high glucose, DMEM with Earl's Salts, Dulbecco's PBS, fetal calf serum (FCS), and trypsin/EDTA were purchased from PAA laboratories (Pasching, Austria). OPTI-MEM reduced serum medium was purchased from Gibco (Life Technologies, Basel, Switzerland). Penicillin, streptomycin, and L-glutamine were obtained from PAA laboratories (Pasching, Austria). Metafectene was obtained from Biontex Laboratories GmbH (Martinsried, Planegg, Germany). Lipofectamine 2000 was purchased from Invitrogen (Darmstadt, Germany). Hygromycin B and Puromycin were purchased from Merck/Calbiochem (Darmstadt, Germany). Hoechst 33342 and lithium chloride (LiCl) were obtained from Sigma-Aldrich (Taufkirchen, Germany). BSA was obtained from Sigma (Buchs, Switzerland), Pefabloc SC from Fluka. Chimeric G-proteins were kindly provided by E. Kostensis. K^4(N_ε-[propionyl-^{3}H])-pNPY and *myo*-[2-^{3}H]-inositol were obtained from GE Healthcare Europe GmbH (Braunschweig, Germany). Scintillation cocktail was purchased from Perkin-Elmer (Rodgau, Germany).

Peptide Synthesis. Peptides were synthesized by automated solid phase peptide synthesis using the 9-fluorenyl methyloxycarbonyl-*tert*-butyl (Fmoc/tBu) strategy with an automated multiple peptide synthesis robot system (Syro, MultiSynTech, Bochum, Germany) on a Rink amide resin (30 mg with a resin loading of 0.6 mmol/g). A 10-fold excess of amino acid was activated with 10 equiv of DIC and 0.5 M HOBt in DMF. Coupling reactions were performed as double couplings with 40 min for each step. Fmoc-cleavage was achieved with 40% piperidine in DMF for 3 min and repeated with 20% piperidine for 10 min. Cleavage from the resin was performed for all peptides with TFA/thioanisole/*p*-thiocresol (90:5:5). The crude product was purified by HPLC on a Phenomenex Jupiter Proteo C_{18} column, (150 mm × 21.2 mm) with a flow rate of 10 mL/min and a gradient of 40−100% MeOH (0.08% TFA) in water (0.1% TFA) in 40 min. The purified peptide was identified by MALDI and ESI mass spectrometry. For determining the purity of the peptides, analytical reversed-phase high-performance liquid chromatography was performed on a Vydac RP18-column (4.6 mm × 250 mm; 5 μm) and on a Phenomenex Jupiter Proteo C_{18} column (4.6 mm × 250 mm; 5 μm). Eluents consisted of 0.1% trifluoroacetic acid in water and 0.08% trifluoroacetic acid in acetonitrile or methanol. At least two different gradients and systems were applied for each peptide. The flow rate was 0.6 mL/min for both gradient systems. Detection was performed at 220 nm. Peaks were integrated using the Merck Hitachi model D-7000 chromatography data station software (Merck KGaA, Darmstadt) and EZ Chrome Elite Software (Chromtech GmbH, Idstein). The purity of the obtained peptides was ≥95%.

Introduction of Fmoc-K-N_ε(Cpa)-OH into the Peptide Sequence. Coupling of Fmoc-K-N_ε(Cpa)-OH to the resin-bound peptide was performed by manual coupling steps using a 5-fold excess of Fmoc-K-N_ε(Cpa)-OH activated with 5 equiv of DIC and 5 equiv of HOBt in DMF for 12 h. Cleavage of the Fmoc-protecting group was realized by treatment with DBU/DCM (1:1) for 5 min. All subsequent N_α-Fmoc-protected proteinogenic amino acids were coupled by manual coupling steps and Fmoc removed with DBU/DCM (1:1)

Cell Culture. All cell lines were maintained in a humidified atmosphere at 37 °C and 5% CO_2 and were grown to confluence prior to use. COS-7 cells (African green monkey, kidney) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS), 100 units/mL penicillin, and 100 μg/mL streptomycin. MCF-7 cells (human breast adenocarcinoma) were cultured in DMEM/Ham's F 12 containing 10% FCS and 2 mM L-glutamine. HEK293 cells (human embryo kidney) stably transfected with the human Y_1-receptor and C-terminally labeled with EYFP as described elsewhere[16] were cultured in DMEM/Ham's F12 without L-glutamine containing 15% FCS and 100 μg/mL hygromycin B. BHK

cells (baby hamster kidney) stably transfected with the human Y_2-receptor and C-terminally labeled with EGFP were maintained in DMEM containing 10% FCS and 8 μg/mL puromycin. HEK293 cells were cultured in DMEM/Ham's F12 containing 10% FCS.

Receptor Binding Studies. For receptor binding assays, MCF-7 cells were resuspended in incubation buffer (MEM containing 50 mM Pefabloc SC and 1% BSA). Peptide solutions (10^{-5}−10^{-10} M) and 10^{-9} M K^4[N$_\varepsilon$-(propionyl-^{3}H)]-pNPY were prepared in water containing 1% BSA and incubated with 200 μL of cell suspension containing ca. 600000 cells. Incubation was performed for 90 min at room temperature and terminated by centrifugation at 4 °C for 5 min. Cell pellets were washed twice with ice-cold phosphate buffered saline (PBS), centrifuged, and resuspended in 100 μL of ice-cold PBS. The cell suspension was mixed with scintillation cocktail, and radioactivity was measured in a scintillation counter. IC_{50} values of the binding curves were calculated by nonlinear regression on a sigmoidal one-site competition based model by using PRISM 3.0 program (GraphPad Software, San Diego, USA). K_I values were calculated from IC_{50} values using the Cheng−Prusoff equation.[62] A K_D value for ^{3}H-NPY of 0.18 nM was used. Each experiment was performed 2−3 times in triplicate.

Receptor Activation Studies. For signal transduction (inositol phosphate (IP) accumulation) assays, COS-7 cells were seeded into 24-well plates (1.0×10^5 cells/well) and transiently cotransfected with 0.32 μg plasmid DNA encoding the hY_1R or hY_2R C-terminally fused to EYFP and 0.08 μg plasmid DNA coding for the chimeric G protein $G\alpha_{\Delta 6qi4myr}$ (hY_1R) or $G\alpha_{qi4}$ (hY_2R) using Metafectene. The assay for receptor activation has been described before.[63] Cells were stimulated with peptide solution in concentration ranges from 10^{-5} to 10^{-12} M. Intracellular IP levels were determined by anion exchange chromatography as described previously.[64,65] Data were analyzed with PRISM 3.0 program (GraphPad Software, San Diego, USA). EC_{50} values were obtained from concentration−response curves. All signal transduction assays were performed in duplicate and repeated at least two times independently.

Receptor Internalization Studies. HEK293 cells stably transfected with the human Y1-receptor C-terminally fused to EYFP (HEK293_hY1_EYFP) and BHK cells stably transfected with the human Y_2-receptor C-terminally fused to EGFP (BHK_hY_2_EGFP) were seeded into sterile μ-Slide 8 well plates (ibidi GmbH, Martinsried, Germany) and cultured to 80% confluency. HEK293 cells were seeded into sterile μ-Slide 8 well plates and transfected with 0.5 μg plasmid DNA encoding for either the human Y_4- or Y_5-receptor C-terminally fused to EYFP after growing them to 70% confluency using Lipofectamine 2000 transfection reagent.

Prior to ligand stimulation, cells were starved in OPTI-MEM reduced serum medium for 60 min. Cell nuclei were imaged with Hoechst 33342 nuclear stain. Stimulation occurred for 60 min with 1 μM NPY analogue in OPTI-MEM reduced serum medium at 37 °C. Live cell images were obtained with an Axio Observer microscope and ApoTome imaging system (Zeiss, Jena, Germany). Fluorescence images were made and edited with the AxioVision software Release 3.0.

■ ASSOCIATED CONTENT

Supporting Information. Synthesis and spectroscopic data for intermediate stages of N_α-(fluoren-9′-yl-methyloxycarbonyl)-N_ε-{3″-[1‴,2‴-dicarba-*closo*-dodecaboran(12)-1‴-yl]propanoyl}-L-lysine, (Fmoc-K-N_ε(Cpa)-OH), **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

BNCT, boron neutron capture therapy; NPY, neuropeptide Y; PP, pancreatic polypeptide; (h)Y_1R, human Y_1-receptor; Ahx, aminohexanoic acid; Fmoc, fluorenylmethyloxycarbonyl; IP, inositol phosphate; BPA-Fr, boryl-phenylalanine-fructose; BSH, B-mercaptoundecahydrododecaborate(2-); GPCR, G protein-coupled receptor; Cpa, carbaboranyl propionic acid; Boc, *tert*-butoxycarbonyl; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCM, dichloromethane; HPLC, high performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; ESI, electrospray ionization; HOBt, hydroxybenzotriazole; DIC, diisopropyl carbodiimide; DMF, dimethylformamide; TFA, trifluoro acetic acid; EC_{50}, half maximal effective concentration; EYFP, enhanced yellow fluorescent protein; EGFP, enhanced green fluorescent protein

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