Facile and Selective Nanoscale Labeling of Peptides in Solution by Using Photolabile Protecting Groups

Norman Koglin, Manja Lang, Robert Rennert, and Annette G. Beck-Sickinger*

Institute of Biochemistry, University of Leipzig, Brüderstrasse 34, D-04103 Leipzig, Germany

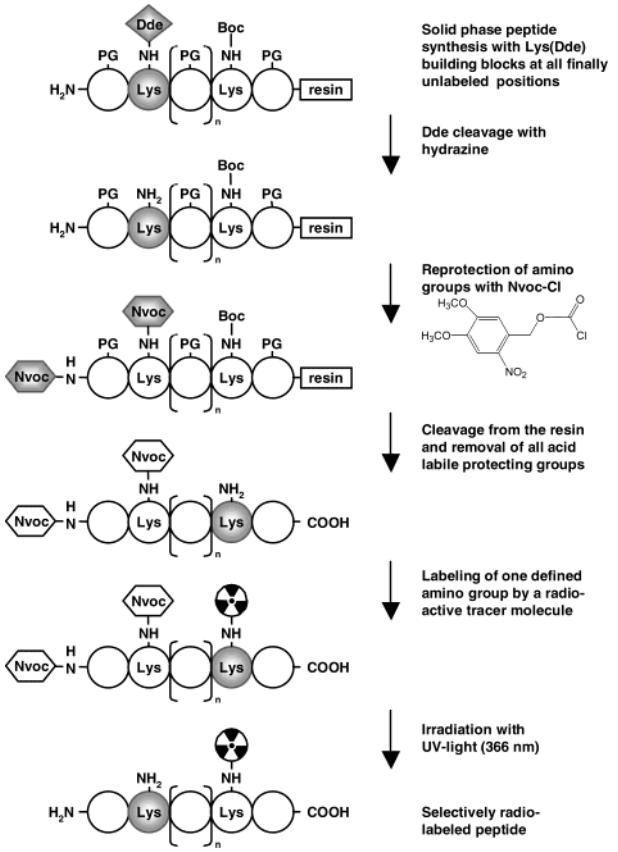
Received June 6, 2003

Abstract: For the selective labeling of peptides, a novel strategy was developed that combines the advantages of solid-phase peptide synthesis with the flexibility of labeling reactions in solution. To direct a label at a distinct position within the peptide sequence, other reactive positions are blocked with photolabile protecting groups that could be easily removed after the labeling reaction. Therefore selective labeling may become feasible for the first time even in nanomol amounts.

Introduction. For many bioactivity studies in medicine, research, and pharmaceutical industry, there is an increasing demand for new and selectively labeled biomolecules. Particularly in the postgenomic age many so far unknown proteins and receptors need to be further characterized. Among them, cell surface receptors and particularly G-protein-coupled receptors with their significant physiological roles still represent the most attractive targets for research and application.[1,2] As peptide/protein interactions are mainly addressed in the first characterization step, many novel labeled peptide ligands are required.

Up to now two strategies have been applied to obtain radiolabeled peptides: (a) direct and (b) indirect labeling. The direct labeling approach is widely used for the oxidative introduction of ^{125}I-atoms into His- or Tyr-residues.[3] This reaction is mainly performed on free peptides in solution and is very often nonselective depending on the particular sequences. The indirect labeling approach can be carried out both in solution and on resin bound peptides via a widely used acylation reaction with *N*-hydroxysuccinimide (OSu)-based activated compounds. Thereby a small chemical group containing the radioactive isotope(s) reacts only with primary amino groups.[4] If the peptide is resin bound, a selective labeling will be possible for this reaction after selective deprotection of one distinct amino group within the sequence or the N-terminus. But for resin-bound labeling, enormous amounts of radioactivity have to be used because the nanoscale handling of resins is impossible. However, for many biological studies even a few nanomoles of a radioligand are sufficient. Furthermore, impurities derived from solid-phase peptide synthesis may also be labeled and require significant effort on the purification in a radioactive environment. Therefore the tracer should be added as a last step during synthesis in order to have an effective and easy-to-handle protocol. In case of labeling in solution the required amounts of peptide and radioactivity can be dispensed easier because free and already purified peptides are used. But if there is more than one suitable residue within the sequence (e.g., several Tyr residues for the oxidative ^{125}I-incorporation or several Lys residues for acylation), no selective labeling will occur. It may result in difficult separation of differently labeled products causing a considerable loss in biological activity especially when the essential residues are also affected.[5]

Scheme 1. Synthesis and Labeling of Partially Nvoc-Protected Peptides with Only One Amino Group Accessible for the Labeling Reagent[a]



[a] Boc, *tert*-butyloxycarbonyl; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl; Nvoc-Cl, 6-nitroveratryloxy-carbonyl chloride; PG, acid labile protecting group.

We have developed a novel strategy for a rapid and selective labeling of peptides independent of their sequence by the use of arbitrary amounts of tracer and peptide as shown in Scheme 1. For this purpose we applied an OSu-activated radioactive tracer molecule to modify only one distinct amino group while keeping all other amino groups within the sequence blocked by photolabile Nvoc protecting group(s). In the last step these photolabile protecting group can be easily removed by UV light to obtain selectively radiolabeled peptides in nanomole amounts. Here we demonstrate the usefulness of the technique by synthesizing four different selectively labeled peptide hormones.

Results and Discussion. Automated solid-phase peptide synthesis (SPPS) was performed applying orthogonal 9-fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl strategy. Moreover all Lys side chain amino groups that finally will not carry the label are protected by the Dde

* To whom correspondence should be addressed. Phone: +49 341 97 36900. Fax: +49 341 9736909. E-mail: beck-sickinger@uni-leipzig.de.

10.1021/jm0341251 CCC: $25.00
Published on Web 09/09/2003

Table 1. Analytical Data of Nvoc Containing Peptides and "Cold" Propionylated (*prop.*) Reference Analogues

peptide	molecular mass [Da] calcd	molecular mass [Da] obsd	retention time[a] [min]
N^{α}-Nvoc-pNPY	4492.9	4493.3 (±1.6)[b]	21.3
prop.-K^4-pNPY	4309.7	4308.7[c]	20.5
N^{α}-Nvoc-[$K^{26,27}$(N^{ϵ}-Nvoc)]-hPTH(1−34)-NH_2	4834.4	4836.9 (±2.2)[b]	28.0
[*prop.*-K^{13}]-hPTH (1−34)-NH_2	4172.8	4171.7[c]	17.9
N^{α}-Nvoc-[K^4]-hPP	4420.0	4422.2 (±2.2)[b]	24.5
[*prop.*-K^4]-hPP	4234.1	4234.3[c]	22.0
N^{α}-Nvoc-[K^{35}(N^{ϵ}-Nvoc)]-hαCGRP(8−37)	3604.0	3605.9 (±2.4)[b]	24.0
[*prop.*-K^{24}]-hαCGRP(8−37)	3183.7	3182.8[c]	20.7

[a] Retention times from analytical RP-HPLC. [b] Molecular masses of Nvoc-containing peptides by ESI-MS. [c] Molecular masses by MALDI-MS.

protecting group during the synthesis. After complete peptide synthesis of any desired sequence, the Dde protecting group was selectively removed by using a solution of hydrazine in DMF.[6] The peptide remains still bound to the resin and all acid labile protecting groups (including the protecting group for the amino group to be labeled later) remain fully protected under these conditions.

If the incorporation of the radiolabel is desired at the N-terminus, this amino group will also have to be protected with an acid labile protecting group. This can be easily achieved either by direct incorporation of the N-terminal residue as an N^{α}-Boc protected amino acid or by acylation of the free N-terminal amino group with $(Boc)_2O$ after the last coupling step. In the next step all amino groups that should not be modified by the labeling reagent later were reprotected with Nvoc-Cl to introduce the photolabile protecting group.[7−9] This novel methodology circumvents the synthesis and use of special N^{ϵ}-Nvoc protected derivatives during the coupling steps.[10] Moreover if desired, the N-terminal amino group will also be protected in the same step without employing any further N^{α}-Nvoc protected amino acids.

The Nvoc-containing peptide was cleaved from the resin by using TFA under simultaneous removal of all acid labile protecting groups leaving only the photolabile Nvoc protecting groups bound to the peptide. These partially Nvoc-protected peptides have been analyzed and characterized by standard techniques as well as purified to homogeneity. After purification, all Nvoc-containing peptides showed a purity >95%.

In the accompanying diode array detection spectrum of the HPLC chromatograms the presence of the Nvoc groups could be confirmed by an additional absorption at 350−360 nm. Analytical data of the Nvoc-containing peptides and analytical data of the "cold" propionylated analogues obtained after modification on the resin are shown in Table 1. Subsequently these partially Nvoc-protected peptides possess only one free amino group which then can be selectively labeled by the Bolton−Hunter reaction in solution. Finally the Nvoc groups were easily removed by irradiation with UV light, and after only one HPLC purification step the selectively radiolabeled products were obtained. Adopting this novel strategy we successfully synthesized several selectively radiolabeled peptides in an efficient manner independent of their sequence as shown in Figure 1.

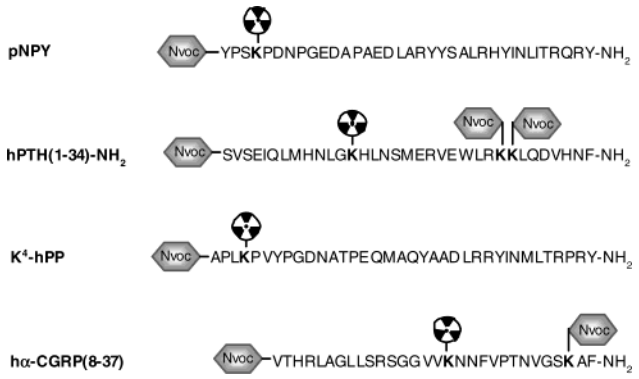


Figure 1. Sequences of selectively radiolabeled and still Nvoc-protected peptides. In the next step the photolabile Nvoc groups will be removed by irradiation with UV light. Finally the radiolabeled peptides will be obtained after only one single HPLC purification step. (NPY, neuropeptide Y; PTH, parathyroid hormone; PP, pancreatic polypeptide; CGRP, calcitonin gene-related peptide)

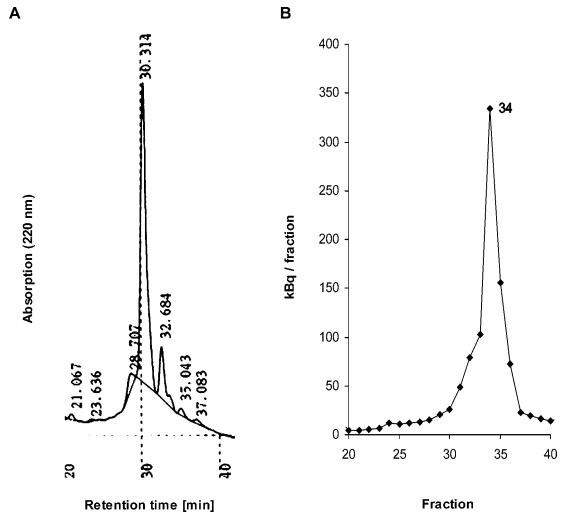


Figure 2. (A) HPLC-chromatogram after ^{3}H-labeling and UV-irradiation of Nvoc-NPY. In this step the labeled product was separated from the nonlabeled peptide and other components from the labeling reaction. The peak with the retention time of 30.3 min corresponds, after the Nvoc removal, to nonlabeled NPY which was added in excess to the labeling reagent. According to the radioactivity profile (B), the peak which was eluted under conditions described in the Experimental Section after 32.7 min (respectively fraction 34) corresponds to ^{3}H-propionyl Lys^4 NPY.

Because of the defined 1:1 stoichiometry (peptide and tracer) of the labeling reaction and the following purification step, in which nonreacted labeling compound and nonlabeled peptide are eliminated, the specific radioactivity of the initially used radiolabel is maintained and transferred to the peptide.

For comparison with an already well characterized and commercially available radioligand and to establish this novel methodology, ^{3}H-propionyl-Lys^4 NPY was synthesized first. Thereby the Lys^4 side chain was selectively radiolabeled while the N-terminal amino group was blocked with the Nvoc group. After Nvoc cleavage and purification selectively radiolabeled ^{3}H-NPY was obtained after only one purification step as shown in Figure 2.

Comparing with the commercially available ^{3}H-NPY, no difference was observed in competitive binding

Table 2. Selectively Radiolabeled Peptide Hormones via Nvoc Strategy: Yield[a] and Affinity at Their Respective Receptor(s) Expressed as IC_{50} Values from Competitive Binding Studies with the Respective Native Ligand[b]

selectively radiolabeled peptide	yield[a] kBq	yield[a] nmol	IC_{50} values [nM] at the respective receptor(s)
[^{3}H-*prop.*-K^4]-pNPY	592	0.2	hY_1: 1.3 ± 1.4 hY_2: 3.7 ± 2.0 hY_5: 5.3 ± 3.7
[^{3}H-*prop.*-K^{13}]-hPTH(1−34)-NH_2	60	0.02	$hPTH_1R$: 2.0 ± 0.5
[^{3}H-*prop.*-K^4]-hPP	260	0.08	rY_4: 0.1 ± 0.07
[^{3}H-*prop.*-K^{24}]-hαCGRP(8−37)	230	0.07	$CGRP_1$: 1.3 ±2.0

[a] Yield of the combined fractions after HPLC purification. [b] To displace the respective radioligand, pNPY, hPTH(1−34)-NH_2, hPP, hαCGRP(8−37) were used as competitor, respectively.

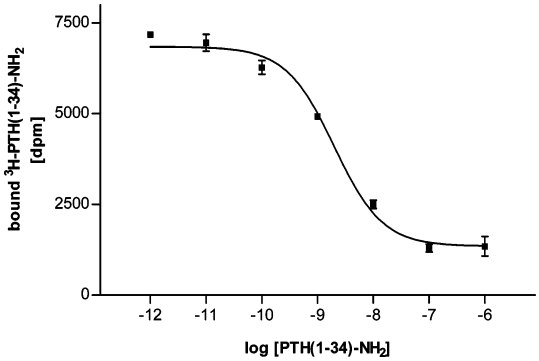


Figure 3. Competitive binding experiment with ^{3}H-PTH(1−34)-NH_2 at the PTH1 receptor in the presence of varying concentrations of PTH(1−34)-NH_2 as competitor. From the resulting displacement curve, an IC_{50} value of 2 nM could be obtained which confirms the suitability of this novel radioligand.

studies with several Y receptor subtypes (Y_1, Y_2, and Y_5) as shown in Table 2 and compared to ref 13. This indicates that the NPY N-terminus that is important for binding to several Y receptor subtypes remained unaffected by the labeling procedure and by the Nvoc cleavage.

In the next example, PTH(1−34)-NH_2 containing four amino groups (N-terminus, Lys^{13}, Lys^{26}, and Lys^{27}) was selectively ^{3}H-propionylated at Lys^{13} whereas all other amino groups were protected with Nvoc groups. After Nvoc cleavage and purification, this novel radioligand proved to be bioactive in a competitive binding assay by using PTH(1−34)-NH_2 as a competitor at the PTH1 receptor. An IC_{50} value of 2 nM was found, indicating a nearly identical behavior of labeled and nonlabeled PTH(1−34)-NH_2 at the PTH1 receptor as shown in Figure 3. Similar to NPY, Lys^4-hPP was selectively labeled at the Lys^4 side chain while the N-terminus was blocked with the Nvoc group. In bioactivity assays a competitive behavior could be observed at the rY_4 receptor.

Furthermore, human α-CGRP(8−37), containing three amino groups (N-terminus, Lys^{24}, and Lys^{35}) was selectively labeled at Lys^{24} and proven to bind to its receptor on SK-N-MC cells.

Conclusion. The method presented here can be widely used for the selective labeling of peptides with *N*-hydroxysuccinimidyl active ester based compounds. Peptide sequences, which could not be labeled selectively in the past because of the presence of several amino groups, are now accessible. Because of the rapid purification after labeling, this novel strategy also allows for the first time the selective incorporation of isotopes with much shorter half-lifes into longer peptide sequences (e.g., for ^{18}F-labeling of peptides for PET biodistribution studies). However, if tracer with much higher specific radioactivity are used, yields might be reduced because of the higher peptide−tracer ratio. Furthermore, the feasibility of nanoscale labeling will make this technique a method of choice even for higly expensive or rare labels such as special fluorescent dyes or toxins if they are insensitive to the UV irradiation used for Nvoc cleavage.

Experimental Section. Peptide Synthesis. The peptides were synthesized according to the Fmoc-strategy by automated multiple solid-phase peptide synthesis as described previously.[11] For Lys side chains either Boc- or Dde-protected building blocks were chosen depending on the labeling position. After complete synthesis of the peptide sequence, the Dde protecting group was selectively removed on the resin by using 2% hydrazine in *N*,*N*-dimethylformamide (DMF).[6] For the incorporation of the Nvoc group, 5 equiv of 1-hydroxybenzotriazole (HOBt), 5 equiv of Nvoc-Cl (Sigma-Aldrich), and 10 equiv of *N*,*N*-diisopropylethylamine (DIEA) were dissolved in 1 mL of DMF. The resulting solution was added to DMF preswollen resin and shaken for 3 h at room temperature. "Cold" propionylated analogues were synthesized simultaneously whereas Dde-side chain-protected Lys building blocks were introduced in those positions where the propionyl group should be attached now. After removal of the Dde group, the respective Lys side chain was propionylated by using 10 equiv of propionic acid anhydride and 10 equiv of DIEA in 1 mL of dichloromethane for 15 min.

After all modifications on resin-bound peptides, the ninhydrin assay was performed to ensure the completion of reaction.[12] The resin was washed with DMF, methanol, dichloromethane, and diethyl ether and dried in a vacuum. Finally the peptides were cleaved from the resin with trifluoroacetic acid (TFA) in the presence of scavenger, removing all acid-labile protecting groups simultaneously.[11] After precipitation and washing with ice-cold diethyl ether, the peptides were lyophilized and analyzed by HPLC on a Vydac RP18-column (4.6 × 250 mm; 5 μm/300 Å) by using linear gradients of 10−60% B in A over 30 min and a flow rate of 0.6 mL·min^{-1}. Further purification of the peptides was achieved by preparative HPLC on a RP18 column (Waters, 5 μm, 25 × 300 mm) by using a linear gradient of 20−60% B in A (A = 0.1% TFA in water; B = 0.08% TFA in acetonitrile) over 45 min and a flow rate of 15 mL·min^{-1}. The peptides were further characterized either by ESI-MS for Nvoc-containing analogues or by MALDI-MS for all other peptides.

Radioactive Labeling. For radioactive labeling, an excess of the Nvoc-protected peptide (2 nmol lyophilized aliquot) was dissolved in 10 μL of 0.1% DIEA/DMF or in an aqueous buffer (10 mM borate buffer, pH 8.0), containing varying amounts of acetonitrile depending on solubility of the peptide. *N*-Succinimidyl-[2,3-^{3}H]-propionate, 3.48 TBq/mmol in toluene (Amersham) was used as the labeling agent in silanized tubes. To remove the solvent, a gentle N_2 stream was applied. The dissolved peptide was added to ester residue (1 nmol)

and shaken for 2 h at room temperature. After the labeling reaction, the Nvoc protecting groups were removed by irradiation with UV light (Atlas Fluotest forte, 366 nm, 180 W). The reaction was carried out for 30 min on ice after addition of 10 μL of 50% acetonitrile in 0.1% TFA/water. Afterward the fully deprotected and labeled peptide was purified by HPLC by using an Impaq RP18-column (4.6 × 250 mm; 5 μm/300 Å) and linear gradients (e.g., 30% to 45% B in A over 55 min) at a flow rate of 0.6 mL·min^{-1} (A = 0.1% TFA in water; B = 0.08% TFA in acetonitrile). Aliquots (10 μL) were taken each minute, mixed with scintillation cocktail, and measured on a β-counter. Radioactive fractions corresponding to the desired product according to retention time of the cold-labeled peptide were combined and eluents removed in a vacuum. The radiolabeled peptides were redissolved in 30% acetonitrile and 0.15 M triethylammonium phosphate (pH 3.4) and stored at 4 °C.

Biological Assays. Cell lines that stably or transiently express one receptor subtype were used for receptor binding assays. For the investigation of ^{3}H-NPY, baby hamster kidney (BHK) cells transiently transfected with the hY_1, hY_2, and hY_5 receptor subtype, respectively, were used as previously described.[13] For ^{3}H-hPP, stably transfected rY_4−BHK cells, grown in DMEM medium, containing 10% fetal calf serum (FCS), 0.5% geneticin, and 0.5% hygromycin were used. The HEK293 cells transiently transfected with the PTH1-receptor were used to test the biological activity of ^{3}H-PTH(1−34)-NH_2. These cells were cultured with Dulbecco's MEM with Glutamax-I containing 10% FCS. SK-N-MC cells were used to study ^{3}H-CGRP. These cells were grown in MEM containing 10% FCS, 2% 200 mM glutamine, 0.2% nonessential amino acids, and 1% sodium pyruvate. All cell lines were grown to confluency at 37 °C and 5% CO_2 in a humidified atmosphere (95%). For competitive binding assays, the cells were removed from the culture flask and resuspended in incubation buffer as described previously.[11] For the investigation of the CGRP receptor, a resuspension buffer containing 50 mM Tris (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 0.1% bacitracin, 50 μM Pefabloc SC, and 1% BSA was used. Binding assays were performed in triplicates as described previously.[11] In brief, 200 μL of the cell suspension containing approximately 600 000 cells were incubated with 25 μL of a 10 nM solution of the radioligand and 25 μL of the respective native ligand as competitor at varying concentrations (10^{-6}−10^{-12} M). After 1.5 h at room temperature, the incubation was terminated by centrifugation. The pellets were washed once with PBS, centrifuged, and resuspended in PBS. After scintillation cocktail was mixed with the pellets, bound radioactivity was determined by using a β-counter.

Acknowledgment. The financial contribution of the Deutsche Forschungsgemeinschaft (Be 1264/3; SFB 610-A1), and the HFSP (RG0045/2000-B) is kindly acknowledged.

References

(1) Marinissen, M. J.; Gutkind, J. S. G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends Pharmacol. Sci.* **2001**, *22*, 368−376.
(2) Civelli, O.; Nothacker, H. P.; Saito, Y.; Wang, Z.; Lin, S. H. et al. Novel neurotransmitters as natural ligands of orphan G-protein-coupled receptors. *Trends Neurosci.* **2001**, *24*, 230−237.
(3) Bolton, A. E. *Radioiodination Techniques, Review 18*, 2nd ed.; Amersham International plc: Sheffield, UK, 1985.
(4) Bolton, A. E.; Hunter, W. M. Labeling of proteins to high specific radioactivities by conjugation to an iodine-125-containing acylating agent. Application to the radiommunoassay. *Biochem. J.* **1973**, *133*, 529−538.
(5) Schmeisser, H.; Kontsekova, E.; Vancova, I.; Mucha, V.; Kontsek, P. Radioiodination of human interferon-a2 interferes with binding of C-terminal specific antibodies. *J. Immunol. Methods* **2000**, *238*, 81−85.
(6) Bycroft, B. W.; Chan, W. C.; Chhabra, S. R.; Teesdale-Spittle, P. H.; Hardy, P. M. A novel amino protection-deprotection procedure and its application in solid-phase peptide synthesis. *J. Chem. Soc., Chem. Commun.* **1993**, *9*, 776−777.
(7) Pillai, V. N. R. Photoremovable protecting groups in organic synthesis. *Synthesis* **1980**, *1*, 1−25.
(8) Dorman, G.; Prestwich, G. D. Using photolabile ligands in drug discovery and development. *Trends Biotechnol.* **2000**, *18*, 64−77.
(9) Fodor, S. P.; Read, J. L.; Pirrung, M. C.; Stryer, L.; Lu, A. T. et al. Light-directed, spatially addressable parallel chemical synthesis. *Science* **1991**, *251*, 767−773.
(10) Rusiecki, V. K.; Warne, S. A. Synthesis of Nα-Fmoc-Nϵ-Nvoc-lysine and use in the preparation of selectively functionalized peptides. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 707−710.
(11) Cabrele, C.; Wieland, H. A.; Koglin, N.; Stidsen, C.; Beck-Sickinger, A. G. Ala(31)-Aib(32): identification of the key motif for high affinity and selectivity of neuropeptide Y at the Y(5)-receptor. *Biochemistry* **2002**, *41*, 8043−8049.
(12) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **1970**, *34*, 595−598.
(13) Dinger, M. C.; Bader, J. E.; Kobor, A. D.; Kretzschmar, A. K.; Beck-Sickinger, A. G. Homodimerization of neuropeptide y receptors investigated by fluorescence resonance energy transfer in living cells. *J. Biol. Chem.* **2003**, *278*, 10562−10571.

JM0341251