

# Total Solid-Phase Synthesis of NOTA-Functionalized Peptides for PET Imaging

Brigitte Guérin,<sup>1</sup> Samia Ait-Mohand,<sup>1</sup> Marie-Claude Tremblay,<sup>1</sup> Véronique Dumulon-Perreault,<sup>1</sup> Patrick Fournier,<sup>1</sup> François Bénard<sup>2</sup>

<sup>1</sup>Centre d'imagerie moléculaire de Sherbrooke (CIMS), Department of Nuclear Medicine and Radiobiology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, 3001, 12<sup>th</sup> Avenue North, Sherbrooke, Québec, Canada J1H 5N4. <sup>2</sup>BC Cancer Agency Research Centre, Department of Molecular Oncology, Vancouver, BC, Canada.

*brigitte.guerin2@usherbrooke.ca*

## SUPPORTING INFORMATION

### TABLE OF CONTENTS

- A. Experimental Procedures and Characterization Data
- B. HPLC and Mass Spectra.
- C. Competition Assay Procedure and  $K_i$  Determination

## A. EXPERIMENTAL PROCEDURES AND CHARACTERIZATION DATA

### General Methods

All chemicals and solvents (reagent grade) were used as supplied from the vendors cited below without further purification, unless otherwise noted. Fmoc-protected amino acids were obtained from EMD NovaBiochem® (Gibbstown, NJ, USA) or Chem-Impex International Inc. (Wood Dale, IL, USA). 1,4,7,10-tetraazacyclododecane-1,4,7-tris-*tert*-butyl acetate-10-acetic acid (DOTA(Ot-Bu)<sub>3</sub>OH) and 1,4,7,10-Tetraazacyclododecane-1,4,7-tris-*tert*-butylacetate-10-(N- $\alpha$ -Fmoc-N- $\varepsilon$ -acetamido-L-lysine) (Fmoc-L-Lys-mono-amide-DOTA-tris(*t*-Bu-ester)) were obtained from Macrocyclics (Dallas, TX, USA). 1,4,7 triazacyclononane was obtained from TCI America (Portland, OR, USA). 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) was purchased from Chem-Impex International Inc. NovaSyn® TGR resin was obtained from NovaBiochem®. *N,N*-Diisopropylethylamine (DIEA), thioanisole, triisopropylsilane (TIPS) were obtained from Aldrich Chemical Co. Acetonitrile (MeCN), dichloromethane (DCM), *N,N*-dimethylformamide (DMF), isopropyl alcohol (*i*-Pr-OH) were obtained from Fisher Scientific (Ottawa, ON, CA). DMF was dried over 4 Å molecular sieves at least one week to remove trace amount of amine present in the solvent and filtered before its use. All peptides were purified by using Flash chromatography on a Biotage SP4 system and a C18 column. Purity of the peptides was verified by HPLC and their identity was confirmed by MALDI mass spectrometry or API 3000 LC/MS/MS. Analytical High Performance Liquid Chromatography (HPLC) was performed on an Agilent 1200 system equipped with a Zorbax Eclipse XDB C18 reverse phase column (4.6 × 250 mm, 5 $\mu$ ) and Agilent 1200 series diode array UV/Vis detector. Linear gradient of 0% to 100% acetonitrile in water with 0.1% TFA in 30 min was used. The flow rate was 1mL/min.

## Experimental Procedure

The peptides were synthesized by continuous flow method on a Pioneer Peptide Synthesis System (PerSeptive Biosystems) using the Fmoc strategy and 1 g of NovaSyn® TGR resin with a substitution rate of 0.26 mmol/g. A 2-fold excess of Fmoc-protected amino acids over resin substitution rate was used for coupling. Synthesis was performed using amine free DMF. Fmoc-protected amino acids are activated for coupling with an equimolar amount of HATU, and 2 equivalents of DIEA. Fmoc deprotection was performed in 20% piperidine in DMF and monitored through UV detector at 364 nm. After peptide assembly, the NOTA moiety was synthesized manually on the solid phase under a mechanic agitation. Reaction conditions presented herein have been optimized by monitoring each step (2 to 7, Scheme 1) on analytical reversed-phase HPLC after cleavage of a small amount (10 mg) of the resin-bound peptide with 95% aqueous TFA. The Fmoc deprotection and the coupling step were followed by a Kaiser's test on resin; the resulting colorimetric reaction between resin and ninhydrine indicated the presence of free primary amines after Fmoc deprotection (blue beads) and their absence after coupling (yellow beads).

**NOTA unit at the N-terminal position:** Functionalization of the amino terminus of the peptide-resin was provided through the use of the appropriate  $\alpha$ -bromo carboxylic acid activated as the symmetric anhydride.<sup>1</sup> At 0 °C, the bromoacetic acid (5 equiv) was dissolved in 2 mL of DCM and EDC (2.5 equiv) was added to the cold solution. After 15 minutes of stirring, the mixture was diluted in 2mL of DMF and added to the partially protected peptide on resin pre-swelled with DCM. After 60 min of coupling, the resin was washed with DMF (3  $\times$ ), MeOH (3  $\times$ ), DMF (3  $\times$ ), MeOH (3  $\times$ ) and DCM (3  $\times$ ). The bromide was then displaced by the amine function of the 1,4,7 triazacyclononane (5 equiv) in anhydrous DCM following the method of Meares.<sup>2</sup> The bromoacetylated peptide on resin

---

(1) Robey, F. A.; Fields, R. L. *Anal. Biochem.* **1989**, *177*, 373–377.  
(2) Peterson, J. J.; Pak, R. H.; Meares, C. F. *Bioconjugate Chem.* **1999**, *10*, 316–320.

was treated with a solution of 1.4.7 triazacyclononane (5 equiv) in anhydrous DCM (2 mL). For this reaction, a mechanic agitation was maintained for 3 h at room temperature. After coupling, the resin was washed with DMSO (3  $\times$ ), DMF (3  $\times$ ), MeOH (3  $\times$ ) and DCM (3  $\times$ ). The cyclen peptide resin pre-swelled with DCM was then alkylated with *tert*-butyl 2- bromoacetate (3 equiv) in the presence of DIPEA (3 equiv) in dry NMP (2 mL). The resin was washed with DMF (3  $\times$ ), MeOH (3  $\times$ ), DMF (3  $\times$ ), MeOH (3  $\times$ ) and DCM (3  $\times$ ) after 2 h of coupling. During the cyclen alkylation step, a considerable amount of overalkylation was observed with longer reaction time and/or more than three equivalents of *tert*-butyl 2-bromoacetate. Finally, peptides were deprotected and cleaved from the polymer support by treatment with a cocktail of TFA:H<sub>2</sub>O:thioanisole (92:2:6) for 3 h. The resin was removed by filtration and washed with TFA. Combined filtrates were added dropwise to ethyl ether (10 mL of ether/mL of TFA) maintained at 0 °C. The precipitated crude peptides were centrifuged, and the ether solution was decanted. Crude peptides were purified by flash chromatography on a Biotage SP4 system using C18 Cartridge. Purity of the peptides was verified by analytical reversed-phase HPLC. Peptide identity was confirmed by MALDI-TOF MS or by API 3000 LC/MS/MS.

**NOTA unit on a lysine side chain.** For the selective NOTA functionalization on lysine side chain, we used a (ivDde)Lys/*N*-terminal Boc protecting group strategy since Fmoc is readily deprotected under conditions used for Dde cleavage. After the last coupling, the ivDde on a Lysine side chain of the protected peptide on resin was cleaved by using treatments with a solution of 4% hydrazine hydrate in DMF (3  $\times$  5 min) and the resin was washed with DMF (3  $\times$ ), MeOH (3  $\times$ ), DMF (3  $\times$ ), MeOH (3  $\times$ ) and DCM (3  $\times$ ). Depending of the peptide sequence, Fmoc-Ahx-OH can be introduced on Lysine side chain using PyBOP/HOBt activation. The Fmoc-Ahx-OH (2 equiv) was dissolved in 2 mL of DMF, DIEA (2 equiv) at 0 °C and PyBOP (2 equiv) were added to the cold solution. After 15 minutes of stirring, the mixture was added to the partially protected peptide on resin pre-swelled with

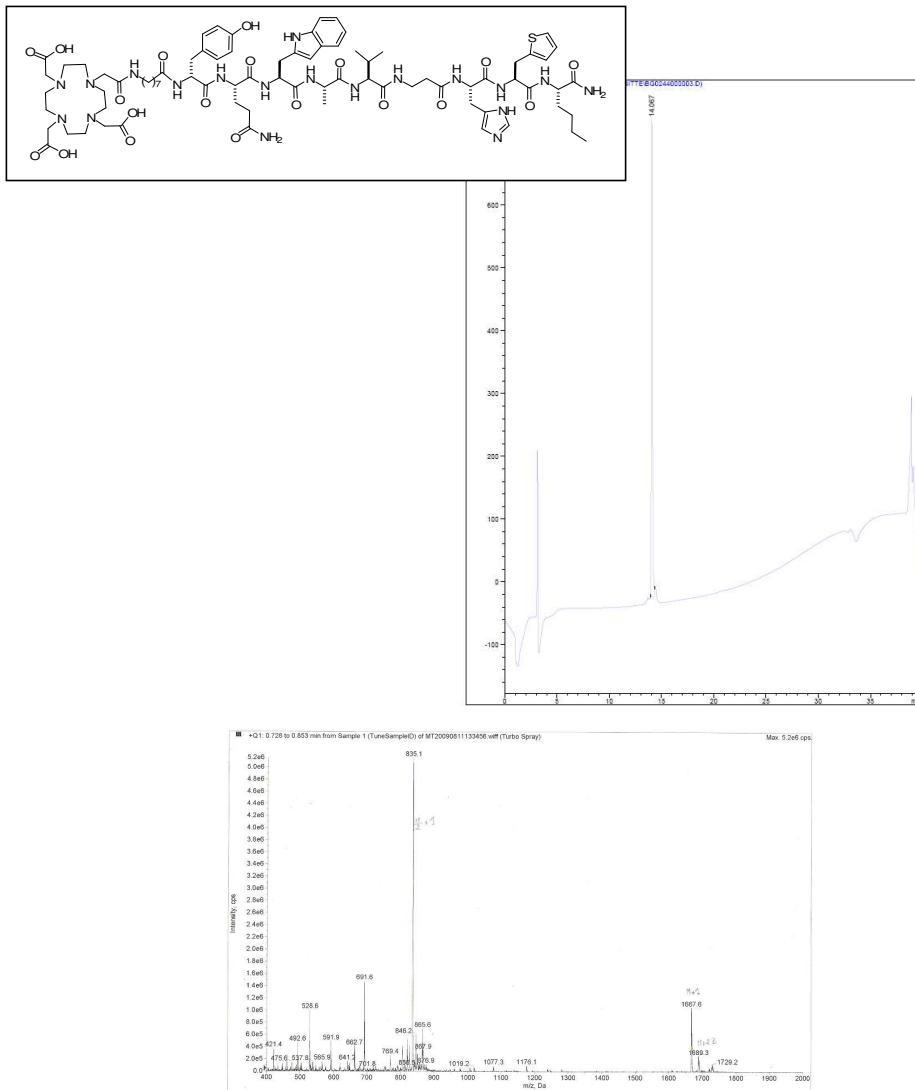
DCM and mixed with HOBt (2 equiv) and DIEA (2 equiv). For this reaction, a mechanical agitation was maintained for 2 h at room temperature. The resin was washed with DMF (3  $\times$ ), MeOH (3  $\times$ ), DMF (3  $\times$ ), MeOH (3  $\times$ ) and DCM (3  $\times$ ). The Fmoc deprotection and the coupling step were followed by a Kaiser's test on resin; the resulting colorimetric reaction between resin and ninhydrine indicated the presence of free primary amines after Fmoc deprotection (blue beads). The partially protected peptide on resin was bromoacetylated by the method of Robey and the NOTA peptide was prepared, cleaved from the resin, purified and characterized as described above for peptide having NOTA unit at the *N*-terminal position.

## B. HPLC AND MASS SPECTRA

### DOTA-Aoc-BBN(6-14)

Peptide	Mass Calcd	Mass Found <sup>a</sup>	Yield (%)	Purity (%) <sup>b</sup>	K <sub>i</sub> (nM)
DOTA-Aoc-BBN(6-14)	1667	1668	11	93	2.2 ± 1.4 <sup>c</sup>

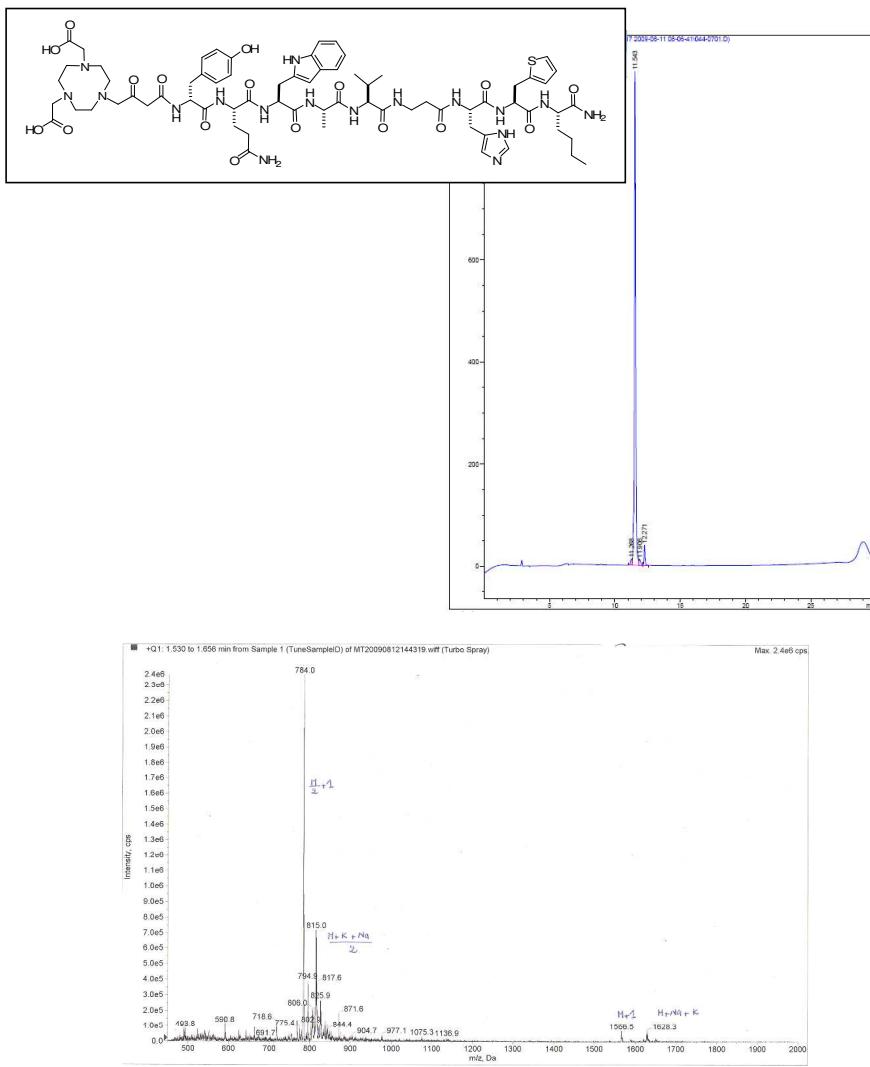
<sup>a</sup>Masses were measured via MALDI mass spectrometry using Micromass ToF Spec 2F. <sup>b</sup>Purity was determined by HPLC analysis. <sup>c</sup>Affinities for GRPR were determined using [<sup>125</sup>I-Tyr<sup>4</sup>]Bombesin in PC3 cells.



### NOTA-Aoc-BBN(6-14)

Peptide	Mass Calcd	Mass Found <sup>a</sup>	Yield (%)	Purity (%) <sup>b</sup>	K <sub>i</sub> (nM)
NOTA-Aoc-BBN(6-14)	1566	1567	16	93	2.2 ± 1.4 <sup>c</sup>

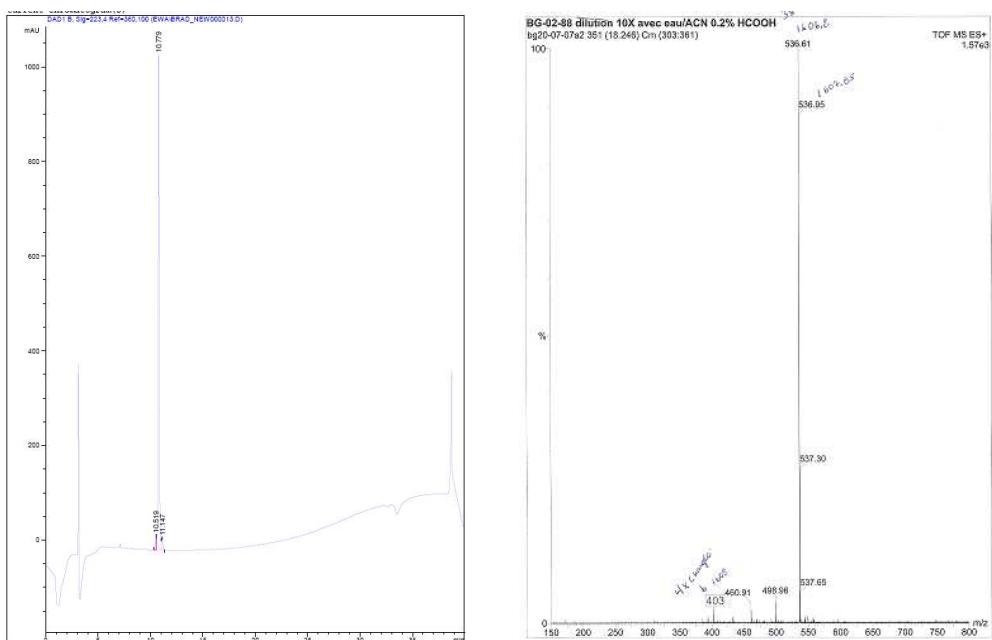
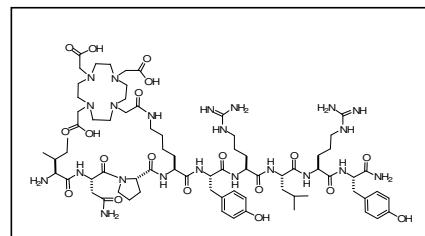
<sup>a</sup>Masses were measured via MALDI mass spectrometry using Micromass ToF Spec 2F. <sup>b</sup>Purity was determined by HPLC analysis. <sup>c</sup>Affinities for GRPR were determined using [<sup>125</sup>I-Tyr<sup>5</sup>]Bombesin in PC3 cells.



**(DOTA)Lys<sup>4</sup>BVD15**

Peptide	Mass		Yield (%)	Purity (%) <sup>b</sup>	K <sub>i</sub> (nM)
	Calcd	Found <sup>a</sup>			
(DOTA)Lys <sup>4</sup> BVD15	1608	1608	12	98	23 ± 13 <sup>d</sup>

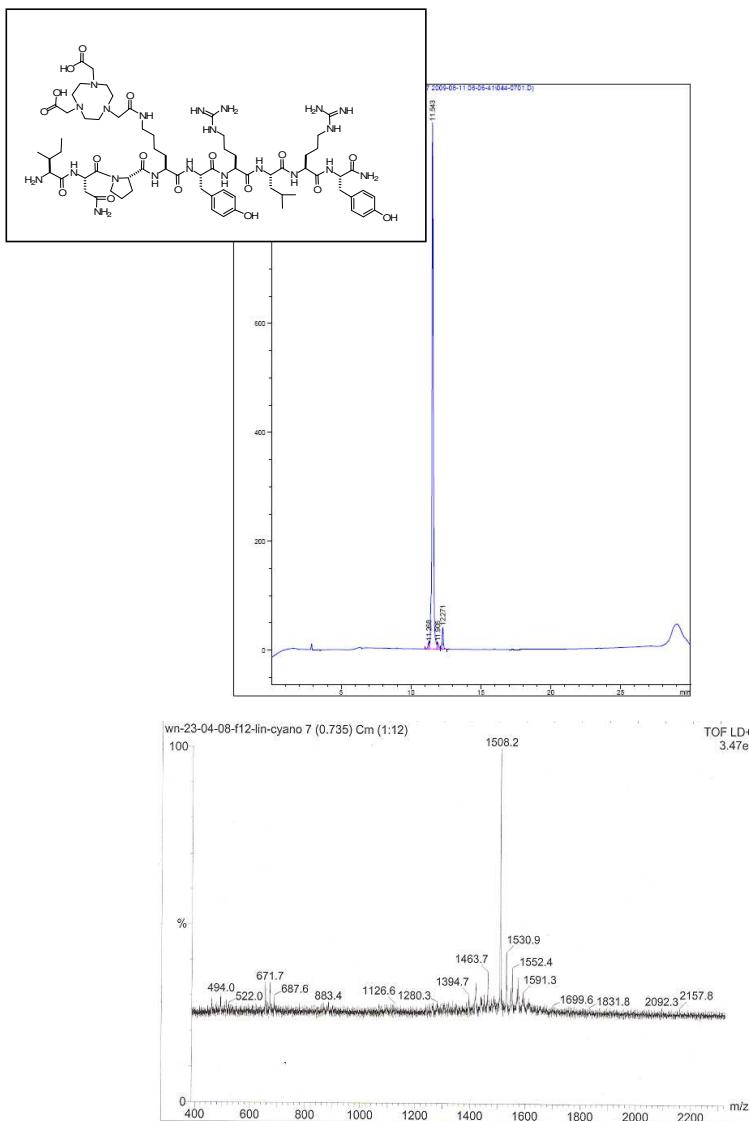
<sup>a</sup>Masses were measured via MALDI mass spectrometry using Micromass ToF Spec 2F. <sup>b</sup>Purity was determined by HPLC analysis. <sup>d</sup>Affinities for Y1 receptors were determined using <sup>125</sup>I-NPY in MCF-7 cells.



**(NOTA)Lys<sup>4</sup>BVD15**

Peptide	Mass Calcd	Mass Found <sup>a</sup>	Yield (%)	Purity (%) <sup>b</sup>	K <sub>i</sub> (nM)
(NOTA)Lys <sup>4</sup> BVD15	1507	1508	30	97	23 ± 13 <sup>d</sup>

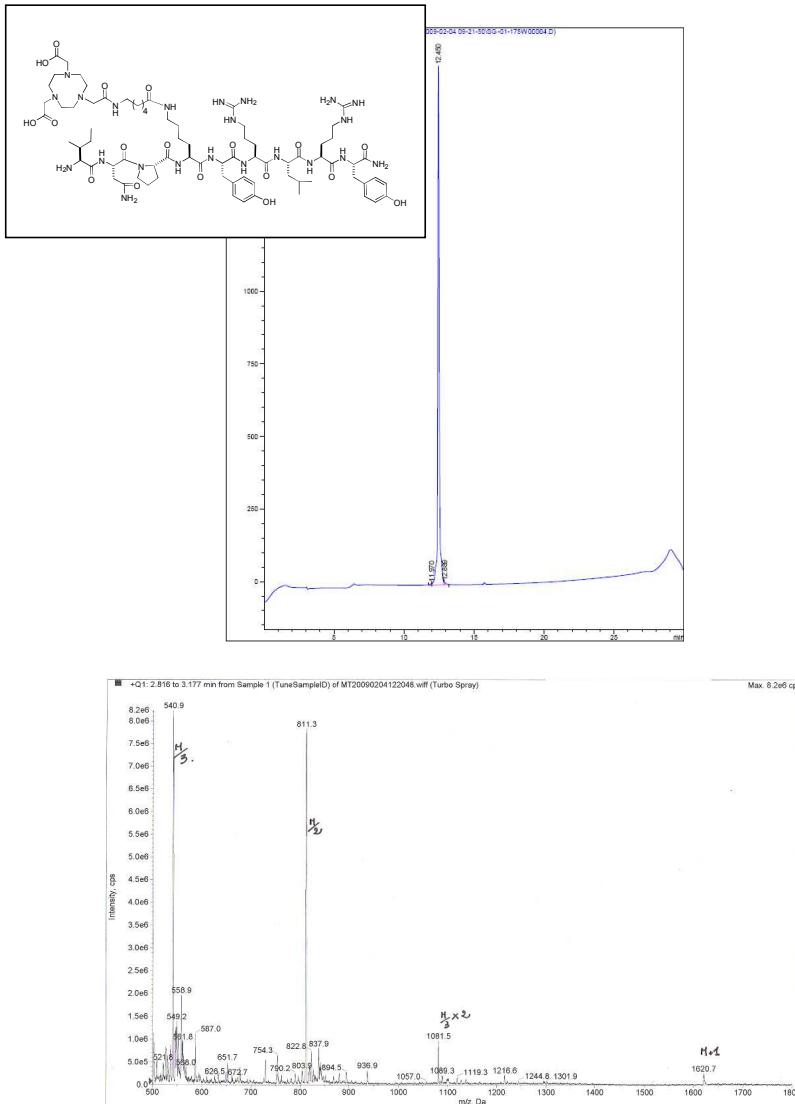
<sup>a</sup> Masses were measured via MALDI mass spectrometry using Micromass ToF Spec 2F. <sup>b</sup> Purity was determined by HPLC analysis. <sup>d</sup> Affinities for Y1 receptors were determined using <sup>125</sup>I-NPY in MCF-7 cells.



**(NOTA-Ahx)Lys<sup>4</sup>BVD15**

Peptide	Mass Calcd	Mass Found <sup>a</sup>	Yield (%)	Purity (%) <sup>b</sup>	K <sub>i</sub> (nM)
(NOTA-Ahx)Lys <sup>4</sup> BVD15	1620	1621	36	95	5.6 ± 4.4 <sup>d</sup>

<sup>a</sup>Masses were measured via MALDI mass spectrometry using Micromass Tof Spec 2F. <sup>b</sup>Purity was determined by HPLC analysis. <sup>c</sup>Affinities for Y1 receptors were determined using <sup>125</sup>I-NPY in MCF-7 cells.



### C. COMPETITION ASSAY PROCEDURE AND $K_i$ DETERMINATION

**Cell culture.** One human breast cancer (MCF-7) and one prostate cancer (PC-3) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). They were cultured in DMEM media for MCF-7 and HAM'S F-12 for PC-3, complemented with 10% FBS, 2mM L-glutamine, 1 U/ml penicillin G, 1 $\mu$ g/ml streptomycin and 2  $\mu$ g/ml amphotericin B.

**Receptor Binding Assays.** Competition assays are performed in 24-well plates using the two cell lines described earlier. The cells were cultured until near confluence and the medium was replaced by 400  $\mu$ L of reaction medium (RPMI complemented with 2 mg/mL BSA, 4,8 mg/mL HEPES, 1 U/mL penicillin G and 1 $\mu$ g/mL streptomycin). For the assay, equal volumes of radioactive and non radioactive ligands were added. Concentrations of radioactive ligands were  $10^{-11}$  M for [ $^{125}$ I-Lys<sup>4</sup>]Neuropeptide Y (74 TBq/mmol; Perkin Elmer Life science Products, Boston, MA) and  $10^{-12}$  M for [ $^{125}$ I-Tyr<sup>4</sup>]Bombesin (74 TBq/mmol; Perkin Elmer Life science Products, Boston, MA). Increasing concentrations ( $10^{-5}$  to  $10^{-14}$ ) of the NPY or GRP ligand of interest were added to consecutive wells. The plates were incubated for 40 min at 37 °C with agitation. After the incubation, the reaction medium was removed and the cells were washed three times with PBS at room temperature. The cells were harvested and the bounded  $^{125}$ I-peptide was counted in a gamma counter (Cobra II auto-gamma counter, Packard, MN). Finally, the data were analysed with GraphPad Prism Software to determine the  $IC_{50}$  value. The  $K_i$  was calculated from the  $IC_{50}$  using the equation of Cheng and Prusoff (Cheng Y., Prusoff W. H., *Biochem. Pharmacol.* 22: 3099-3108, 1973). The  $K_d$  values for the radioligands have been determined from experiments done under similar conditions.

**Deleted:** inhibition constant ( $K_i$ )

$$K_i = \frac{IC_{50}}{1 + \frac{[radioligand]}{K_d}}$$

$K_d = 5.2 \times 10^{-10} \text{ M}$  for [ $^{125}\text{I}$ -Lys<sup>4</sup>]Neuropeptide Y, see Balasubramaniam, A.; Dhawan, V. C.; Mullins, D. E.; Chance, W.T.; Sheriff, S.; Guzzi, M.; Prabhakaran, M.; Parker E. M. *J. Med. Chem.* **2001**, *44*, 1479.

$K_d = 1.5 \times 10^{-10} \text{ M}$  for [ $^{125}\text{I}$ -Tyr<sup>4</sup>]Bombesin, see Aprikian, A.G., Han, K., Chevalier, S., Bazinet, M., Viallet, J. *J. Mol. Endocrinol.* **1996**, *16*, 297.