# Novel $^{99m}$Tc-Labeled Neurotensin Analogues with Optimized Biodistribution Properties

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Two new $^{99m}$Tc-labeled neurotensin(8−13) analogues containing the *retro-N*$^{\alpha}$-carboxymethyl-histidine ((N$^{\alpha}$His)Ac) chelator were synthesized as potential radiopharmaceuticals for visualization of pancreatic carcinoma. To improve the pharmacokinetic properties, (N$^{\alpha}$His)Ac-Arg-NMeArg-Pro-Tyr-Tle-Leu (NT-XII), which is metabolically stabilized at two positions, was further modified. Shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid) was introduced to obtain a more hydrophilic peptide (NT-XVIII), or Tyr$^{11}$ was replaced by 2,6-dimethyltyrosine (Dmt) resulting in a triple-stabilized NT(8−13) analogue (NT-XIX). The latter has the best biodistribution profile.

## Introduction

Radiopharmaceuticals are radionuclide-containing drugs routinely used in nuclear medicine for diagnosis and therapy of various diseases.[1] Single photon emission computed tomography (SPECT) is a nuclear medicine imaging technique that uses $\gamma$ rays and allows visualization of the distribution of a radioactive tracer in a patient's body. The radionuclide $^{99m}$Tc has ideal characteristics ($t_{1/2} = 6$ h, $E_{\gamma} = 140$ keV, 85%) to be used with SPECT, and it is therefore the most used radionuclide in diagnostic nuclear medicine.[2,3] We have developed the *retro-N*$^{\alpha}$-carboxymethyl-histidine chelator [abbreviation (N$^{\alpha}$His)Ac] for the $^{99m}$Tc$^{I}$(CO)$_3$ core, which allows labeling with a very high specific activity.[3]

The neuropeptide neurotensin (NT) is an attractive targeting molecule with potential utility in diagnosis and therapy of ductal pancreatic carcinoma, since it has been observed that NT receptors are highly overexpressed on these tumor cells whereas they are absent in normal pancreas tissue.[4,5] Structure−activity studies have already shown that the C-terminal hexapeptide NT-(8−13), Arg$^8$-Arg$^9$-Pro$^{10}$-Tyr$^{11}$-Ile$^{12}$-Leu$^{13}$, is the minimal sequence required for biological activity.[6] The half-life of NT(8−13) is only a few minutes in both rat and human plasma. We previously reported $^{99m}$Tc(CO)$_3$-labeled NT(8−13) analogues in which the Arg-Arg and Ile-Leu bonds were protected against fast enzymatic degradation.[7,8] One of these analogues is NT-XI ([$^{99m}$Tc(CO)$_3$](N$^{\alpha}$His)Ac-Lys$\Psi$(CH$_2$NH)Arg-Pro-Tyr-Tle-Leu-OH) in which these double stabilizations resulted in a large increase in stability. Its half-life in human plasma could be extended to about 21 days.[9] A first clinical study with this analogue allowed the visualization of the tumor in a patient with a ductal pancreatic carcinoma.[10] However it also showed a rather high accumulation in liver and kidneys for an ideal radiopharmaceutical. The replacement of the Lys$\Psi$(CH$_2$NH)Arg reduced amide bond by an Arg-NMeArg in NT-XII (Table 1) led to a lower kidney uptake and a better tumor-to-kidney ratio, while maintaining receptor affinity and in vitro plasma metabolic stability.[11]

We now report on further improvements of the pharmacokinetic properties of the NT-XII analogue using two strategies.

**Table 1.** Dissociation Constants of $^{99m}$Tc-Labeled NT-Analogues in HT-29 Cells

| peptide | sequence | $K_D$ [nM] |
|---|---|---|
| NT-II[7] | [$^{99m}$Tc(CO)$_3$]-(N$^{\alpha}$His)Ac-Arg-Arg-Pro-Tyr-Ile-Leu | 0.3 ± 0.2 |
| NT-XII | [$^{99m}$Tc(CO)$_3$]-(N$^{\alpha}$His)Ac-Arg-NMeArg-Pro-Tyr-Tle-Leu | 2.0 ± 1.6 |
| NT-XVIII **1** | [$^{99m}$Tc(CO)$_3$]-(N$^{\alpha}$His)Ac-Lys(shikimic)-Arg-NMeArg-Pro-Tyr-Tle-Leu | 4.5 ± 1.7 |
| NT-XIX **2** | [$^{99m}$Tc(CO)$_3$]-(N$^{\alpha}$His)Ac-Arg-NMeArg-Pro-Dmt-Tle-Leu | 15.0 ± 6.0 |

To increase the target-to-nontarget tissue ratios and to enhance the excretion kinetics and blood clearance, the NT analogue was made more hydrophilic, a strategy that has been successful for other peptide-based radiopharmaceuticals.[12,13] Thus the readily available glycomimetic 3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid (shikimic acid) was conjugated to the peptide sequence through a stable amide bond[14] to the side chain of an additional lysine spacer (Figure 1).

Furthermore, our studies of the corresponding $^{18}$F-labeled NT-(8−13) analogues indicated that despite a stabilization of the Arg$^8$-Arg$^9$ and of the Tyr$^{11}$-Ile$^{12}$ peptide bonds, the in vivo stability was still rather low due to cleavage of the Pro$^{10}$-Tyr$^{11}$ peptide bond.[15] We have screened a number of constrained or bulky tyrosine analogues in the NT(8−13) sequence, which indicated that the 2′,6′-dimethyltyrosine[11] (Dmt) analogue had high affinity and improved stability.[16,17] In the present paper, we also report on the results of the triple-stabilized [$^{99m}$Tc(CO)$_3$]-(N$^{\alpha}$His)Ac-Arg-NMeArg-Pro-Dmt-Tle-Leu-OH analogue NT-XIX **2**, which is an interesting candidate as a future radiopharmaceutical.

## Results and Discussion

**Peptide Synthesis and Radiolabeling.** The synthesis of the peptides containing the *retro-N*$^{\alpha}$-carboxymethyl-histidine chelator was performed on a Merrifield polystyrene resin using the standard Boc strategy. For NT-XVIII **1**, two strategies were evaluated, and only the one shown in Scheme 1 proved to be successful. All attempts to first synthesize the peptide containing the chelator and then couple shikimic acid or its triacetylated protected form to the free lysine side chain failed to give the desired peptide in acceptable purity. These experiments showed that the shikimic acid should be coupled to the side chain of the lysine residue before construction of the (N$^{\alpha}$His)Ac chelator.

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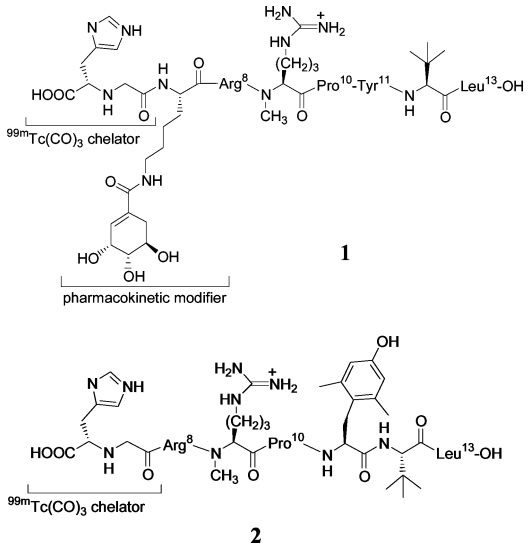
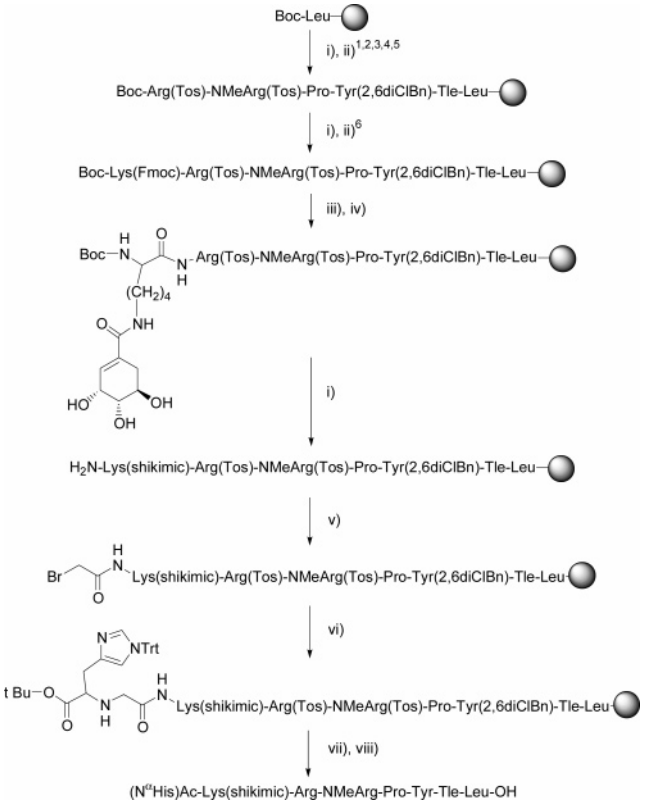



**Figure 1.** Structure of NT-XVIII **1** and of NT-XIX **2**.

**Scheme 1.** Synthesis of NT-XVIII[a]




[a] Reagents and conditions: (i) TFA/$CH_2Cl_2$/2% anisole; (ii)[1] Boc-Tle-OH, DIC, HOBt; (ii)[2] Boc-Tyr(2,6-diClBn), DIC, HOBt; (ii)[3] Boc-Pro-OH, DIC, HOBt; (ii)[4] Boc-NMeArg(Tos)-OH, DIC, HOBt; (ii)[5] Boc-Arg(Tos)-OH, TBTU, DIPEA; (ii)[6] Boc-Lys(Fmoc)-OH, DIC, HOBt; (iii) 20% piperidine/DMF; (iv) shikimic acid, TBTU, HOBt, DIPEA; (v) $BrCH_2COOH$, DIC in DMF; (vi) His(Trt)-OtBu, DIPEA; (vii) TFA/$CH_2Cl_2$/2% TES/2% anisole; (viii) HF(l), anisole, 1 h 0 °C.

So, the synthetic strategy was changed (Scheme 1). In this method, the Fmoc protecting group was first removed from the side chain of lysine, and afterward the unprotected shikimic acid could be coupled. Subsequently, the α-amine of lysine was Boc-deprotected and bromoacylated using DIC as coupling reagent. The use of HOBt has to be avoided to prevent substitution of the bromoacetylated peptide by HOBt. Then a nucleophilic substitution was performed using His(Trt)-OtBu. After precleaving with 50% TFA/$CH_2Cl_2$/anisole/triethylsilane (TES), the peptide was released from the resin by treatment with HF(l). Purification resulted in NT-XVIII **1** with an overall yield of 23%. The synthesis of NT-XIX **2** was performed as described above. The Fmoc-2′,6′-dimethyltyrosine (Dmt) was used without hydroxyl protection. After precleaving, the peptide was released from the resin with HF(l). Purification resulted in NT-XIX with a yield of 20%.

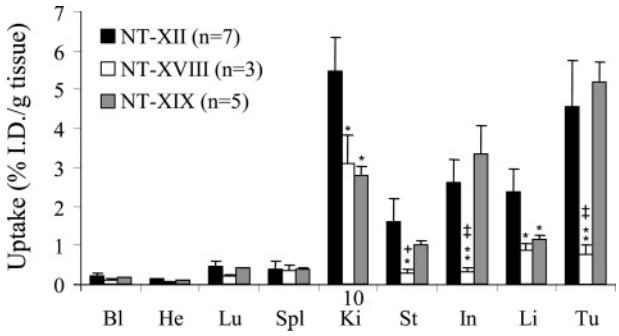



**Figure 2.** Biodistribution in nude mice with HT-29 tumor xenografts 5 h after intravenous injection of the $^{99m}Tc(CO)_3$-labeled analogues (3.5−4 MBq/mouse). Data are % ID/g of tissue sample by reference to total injected dose. Bl = blood; He = heart; Lu = lung; Sp = spleen; Ki = kidney; St = stomach; In = intestine; Li = liver; Tu = tumor; ∗ indicates $P < 0.01$, ∗∗ $P < 0.001$ vs NT-XII, + $P < 0.01$, and ‡ $P < 0.001$ vs NT-XIX using one-way ANOVA + Tukey's multiple comparison test.

As with all published NT analogues from the same series, yields higher than 95% were reached after radiolabeling with $^{99m}Tc(CO)_3$. In the HPLC analyses, one main peak was obtained, which corresponded to the radiolabeled peptides. In general, pertechnetate and tricarbonyl represented 0−5% and 0−1%, respectively, of the total activity, and they were not detectable after purification.

**In Vitro Binding Assays.** The dissociation constant ($K_D$) was determined in HT-29 cells (Table 1). The binding was very specific for all the analogues (nonspecific binding represented less than 1% of the total binding). Compared to NT-XII, the addition of Lys(shikimic acid) in NT-XVIII **1** reduced the affinity by a factor of 2, whereas the substitution of $Tyr^{11}$ by Dmt in NT-XIX **2** lead to a 7.5 times lower affinity.

**Metabolic Stability.** The metabolic stability of the labeled analogues was determined in human plasma obtained from healthy volunteers and in HT-29 cells using a concentration of peptide of 0.2−0.5 nM.[8−10] Analysis by HPLC indicated a high plasma stability for all analogues with little degradation within 24 h. Stability in the cells was considerably lower for NT-XII and NT-XVIII **1** with half-lives of approximately 6.5 and 2.5 h, respectively. In contrast, NT-XIX **2** showed a much higher stability in HT-29 cells. After 24 h incubation at 37 °C, 70% of the radiolabeled NT-XIX **2** was still intact.

**Biodistribution Studies.** In vivo biodistribution results are shown in Figure 2. The introduction of the shikimic acid resulted in a 2-fold reduction in kidney and liver uptake in comparison with $^{99m}Tc(CO)_3$-NT-XII. At 5 h postinjection, the accumulation in the kidneys for $^{99m}Tc(CO)_3$-NT-XVIII **1** was 3.1% ID/g (ID = injected dose) and in the liver 0.9% ID/g. This is significantly lower than the 5.5% and 2.4% ID/g in kidneys and liver, respectively, obtained with $^{99m}Tc(CO)_3$-NT-XII. $^{99m}Tc(CO)_3$-NT-XVIII **1** showed, however, a much lower tumor uptake than $^{99m}Tc(CO)_3$-NT-XII, 0.6% vs 4.6% ID/g. The uptake in intestine, a NT receptor-positive tissue, was also much lower for $^{99m}Tc(CO)_3$-NT-XVIII **1** than for $^{99m}Tc(CO)_3$-NT-XII (0.3% and 2.6% ID/g, respectively). These lower uptakes are likely the combination of a lower affinity and a lower in vivo stability.

The analogue $^{99m}$Tc(CO)$_3$-NT-XIX **2** showed a much better biodistribution profile (Figure 2). Compared to $^{99m}$Tc(CO)$_3$-NT-XII, kidney and liver uptake were also significantly reduced with values comparable to those of $^{99m}$Tc(CO)$_3$-NT-XVIII **1**. Interestingly, tumor uptake of $^{99m}$Tc(CO)$_3$-NT-XIX **2** (5.2% ± 0.5% ID/g) was slightly higher than that of $^{99m}$Tc(CO)$_3$-NT-XII (4.6% ± 1.2% ID/g). Tumor-to-blood ratios were similar for NT-XII and NT-XIX (21.4 ± 4.0 and 28.4 ± 2.9, respectively) and much higher than that of NT-XVIII (6.8 ± 2.6). Tumor-to-liver ratios were better for NT-XIX than for NT-XII and NT-XVIII (4.5 ± 0.7, 2.1 ± 0.1, and 0.9 ± 0.2, respectively). Finally, an interesting tumor-to-kidney ratio of 1.9 ± 0.1 was obtained for NT-XIX, 2- and 10-fold higher than those of NT-XII and NT-XVIII (0.9 ± 0.3 and 0.2 ± 0.1, respectively). Thus, despite its lower receptor affinity, the analogue NT-XIX **2** showed the highest tumor uptake as well as the lowest kidney and liver uptake. This can be ascribed to its higher in vivo metabolic stability, since a correlation between peptide stability and tumor uptake ratios has been demonstrated for NT analogues.[9] This metabolic stability may also explain the intestinal uptake, a NT receptor-positive tissue. The biodistribution profile of NT-XIX **2** was better than that previously reported for other NT-analogues from the same series. Furthermore, tumor-to-kidney ratios were higher than those of the $^{111}$In-DTPA-NT analogue 17 reported by Achilefu et al.[18] or of the $^{111}$In-DTPA and $^{111}$In-DOTA-NT analogues reported by de Visser et al.[19]

## Conclusions

The introduction of the hydrophilic shikimic acid moiety through a lysine linker into the double-stabilized NT(8−13) sequence did result in the expected lower kidney and liver accumulation. However the receptor affinity and especially tumor uptake were also reduced. The replacement of the Tyr$^{11}$ residue by the bulky 2′,6′-dimethyltyrosine, despite resulting in a decreased receptor affinity, maintained the accumulation in the tumor at the same level as in NT-XII, which may be ascribed to its higher metabolic stability. Moreover, kidney and liver uptake were also reduced by a factor of 2. Therefore, NT-XIX **2** is the most promising analogue for further evaluation.

## Experimental Section

**General.** The Boc-protected L-amino acids used in solid-phase peptide synthesis (SPPS), Boc-Tyr(2,6diClBn)-OH, Boc-Pro-OH, Boc-Arg(Tos)-OH, and Boc-Lys(Fmoc)-OH, and the preloaded Merrifield resin were obtained from NovaBiochem (Läufelfingen, Switzerland). Boc-NMeArg(Tos)-OH and His(Trt)-OtBu were purchased from Bachem (Bubendorf, Switzerland), and Fmoc-Dmt-OH was obtained from RSP Amino Acid LLC (Shirley, MA). Diisopropylcarbodiimide (DIC), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU), 1-hydroxybenzotriazole (HOBt), trifluoroacetic acid (TFA), dichloromethane, and Boc-Tle-OH were provided by Fluka (Bornem, Belgium). Triethylsilane (TES), piperidine, bromoacetic acid, and shikimic acid were obtained from Aldrich (Bornem, Belgium); anisole, ethyl diisopropylamine (DIPEA), and dimethylformamide were obtained from Acros (Geel, Belgium). Solid-phase peptide synthesis (SPPS) was performed on a semi-automated peptide synthesizer SP640B (Labortec, Bubendorf, Switzerland). Purification of the peptides was done on a semipreparative high-performance liquid chromatography (HPLC) system (Gilson) on a reverse phase (RP) $C_{18}$ column (DiscoveryBIO SUPELCO Wide Pore, 25 cm × 2.21 cm, 5 μm) with a linear gradient (3−80% $CH_3CN$ in $H_2O$, containing 0.1% TFA, in 30 min) and a flow rate of 20 mL/min with UV detection at 215 nm. The peptides were analyzed by HPLC on a RP column using the conditions given in Table 2. Mass spectra were recorded on a VG Quatro II spectrometer (electrospray ionization, ES-MS) using MassLynx 2.22 software for data analysis. For thin-layer chromatography (TLC), plastic silica-coated plates with $F_{254}$ indicator were used from Merck (Darmstadt, Germany), using EtOAc/*n*-BuOH/AcOH/$H_2O$ 1:1:1:1 as eluent. To reveal the spots, the plates were treated with a permanganate solution, containing $KMnO_4$ (3 g), $K_2CO_3$ (20 g), 5% aqueous NaOH (5 mL), and $H_2O$ (300 mL).

**Table 2.** Analytical Data for NT-XVIII, NT-XIX, and NT-XII

| compd | % purity | MW calcd *m*/*z* | ES-MS[a] [M + H$^+$] | HPLC $t_R$ (min), UV trace: system 1[b] | HPLC $t_R$ (min), UV trace: system 2[c] |
|---|---|---|---|---|---|
| NT-XII | >97 | 1025.6 | 1026.6 | 12.3 | 17.8 |
| | | [M + 2H$^+$]/2 = 513.8 | 514.3 | | |
| NT-XIX | >98 | 1053.6 | 1054.6 | 11.9 | 17.7 |
| | | [M + 2H$^+$]/2 = 527.8 | 527.9 | | |
| NT-XVIII | >99 | 1309.7 | 1310.7 | 11.3 | 12.6 |
| | | [M + 2H$^+$]/2 = 655.8 | 656.1 | | |

[a] Peaks observed in the ES-MS spectra. [b] RP-HPLC with UV-detection (215 nm); column, Supelco Discovery Bio wide pore ($C_{18}$, 5 μm, 250 mm × 4.6 mm); solvent A = 0.1% TFA (v/v) and solvent B = $CH_3CN$ containing 0.1% TFA (v/v); linear gradient, 3% B to 100% B in 30 min; flow rate, 1 mL/min. [c] RP-HPLC with UV-detection (215 nm); column, Supelco Discovery Bio wide pore ($C_{18}$, 5 μm, 250 mm × 4.6 mm), solvent A = 0.1% TFA (v/v) and solvent B = $CH_3OH$ containing 0.1% TFA (v/v); linear gradient, 3% B to 100% B in 30 min; flow rate, 1 mL/min.

**Synthesis of NT-XII.** SPPS was performed on a Merrifield polystyrene resin. *tert*-Butyloxycarbonyl main-chain protected amino acids were used. The Boc-deprotection was performed in a mixture of TFA/$CH_2Cl_2$/anisole (49/49/2) (5 min + 20 min). After filtration of the TFA mixture and neutralization by washing with 20% DIPEA/$CH_2Cl_2$, the couplings were performed by using 3 equiv of protected amino acid and DIC (3 equiv) in the presence of HOBt (3 equiv). For the coupling of Boc-Arg(Tos)-OH to the NMe-Arg, the coupling reagent TBTU was used in 4-fold excess together with 4 equiv of the protected amino acid and 8 equiv of DIPEA. The completeness of the couplings was checked with the ninhydrin or NF 31 color tests.[20] After Boc deprotection of Arg$^8$ the α-amine was bromoacylated by adding DIC (5 equiv) and bromoacetic acid (5 equiv) in DMF to the resin. After 15 min, the reagents were removed, the resin was washed, and His(Trt)-OtBu (3 equiv) was dissolved in DMF and added to the resin together with DIPEA (3 equiv). The nucleophilic substitution was left shaking for 24 h at room temperature. After precleaving with TFA/$CH_2Cl_2$/anisole/TES (48:48:2:2) during 5 and 25 min, the peptide was cleaved from the resin by treatment with HF(l) for 1 h at 0 °C. HPLC purification of the peptide gave an overall yield of 23%. (TLC, $R_f$ = 0.08). Analytical data of the compound are presented in Table 2.

**Synthesis of NT-XVIII 1 and NT-XIX 2.** The synthesis of these peptide sequences was performed as described for NT-XII. For NT-XVIII, Boc-Lys(Fmoc)-OH was coupled to the arginine, and the lysine side chain was Fmoc deprotected with 20% piperidine in DMF (2 × 10 min), after which the shikimic acid (1 equiv) was coupled by using TBTU (1 equiv), HOBt (1 equiv) and DIPEA (1 equiv) in DMF. This coupling was complete after 1 h shaking at room temperature. The reagents were removed, and the resin was washed several times with DMF, $^i$PrOH, DMF, and $CH_2Cl_2$ to swell the resin again. After Boc deprotection, the α-amino group of lysine was bromoacylated, followed by substitution with His(Trt)-OtBu as described for NT-XII. For NT-XIX, Fmoc-2′,6′-dimethyltyrosine was used without hydroxyl protection. Before cleavage of the peptides from the resin with HF(l) and anisole (1 h in ice-bath), a precleaving was performed with 50% TFA/$CH_2Cl_2$/2% anisole/2% TES (5 min + 25 min). After cleavage from the resin, the peptide was dissolved in acetic acid and lyophilized, and after purification by preparative HPLC, NT-XVIII **1** was obtained in an overall yield

of 23% (TLC, $R_f = 0.05$), and NT-XIX **2** was obtained in 20% yield (TLC, $R_f = 0.06$). The analytical data are presented in Table 2.

**Radiolabeling and Biological Evaluation.** Radiolabeling with $^{99m}Tc(CO)_3$, receptor binding assays, metabolic stability determinations, and biodistribution studies were performed as previously described.[8,9] Further details are reported in the Supporting Information.

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**Supporting Information Available:** Methods for radiolabeling, receptor binding assays, metabolic stability determinations, and biodistribution studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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