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# Labelling of octreotide using $^{76}$Br-prosthetic groups

Ulrika Yngve[1,2], Tanweera S. Khan[2,3], Mats Bergström[2] and Bengt Långström[1,2,*]

[1] *Department of Organic Chemistry, Institute of Chemistry, Uppsala University, Box 531, SE-751 21 Uppsala, Sweden*

[2] *Uppsala University, PET Centre, UAS, SE-751 85 Uppsala, Sweden*

[3] *Department of Medical Sciences, University Hospital, Uppsala, Sweden*

## Summary

A method for labelling the octapeptide octreotide (D–Phe–Cys–Phe–D–Trp–Lys–Thr–Cys–Thr(ol)) with the positron emitting $^{76}$Br ($T_{1/2} = 16$ h) is presented. ε-Boc-protected octreotide was conjugated to *N*−succinimidyl 4-[$^{76}$Br]bromobenzoate **1** and *N*−succinimidyl 5-[$^{76}$Br]bromo-3-pyridinecarboxylate **3** using microwave heating. The conjugates **4** and **5** were isolated in 50–55% radiochemical yield after the removal of the protecting Boc-group. Compound **3** was synthesised from the corresponding trimethylstannyl-precursor in 25% radiochemical yield. The synthesis of methyl-4-[$^{76}$Br] bromobenzimidate **8** in 40% radiochemical yield from the precursor methyl -4-trimethylstannylbenzimidate is also described. Experiments were performed to react **8** with Boc-octreotide but no product was obtained. The binding-properties of $^{76}$Br-conjugates **4** and **5** to meningiomas were investigated using frozen section autoradiography. Compound **5** showed better binding properties than **4**. Copyright © 2001 John Wiley & Sons, Ltd.

**Key Words:** $^{76}$Br; labelled octreotide; prosthetic groups

## Introduction

Radiolabelled analogues of octeotide, a synthetic octapeptide that has shown high selectivity for the somatostatin receptor, are of

*Correspondence to: B. Langstrom, Uppsala University PET centre, UAS, SE-751 85 Uppsala, Sweden.



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interest for the diagnosis and treatment of somatostatin-positive tumours. Many of the labelling methods involve conjugation of metal chelators to the *N*-terminal of the peptide followed by labelling with radiometals such as $^{64}Cu$,[1] $^{67}Ga$,[2] $^{68}Ga$,[3] $^{111}In$,[4] $^{86}Y$[5] and $^{90}Y$[6,7]. Also, Tyr$^3$-octreotide has been labelled with different isotopes of iodine by oxidative iodination of the tyrosine residue.[8] Conjugation of labelled activated esters has been used for the introduction of $^{131}I$ or $^{211}At$[9] and $^{18}F$ has been introduced using conjugation to 2-[$^{18}$F]fluoropropionic acid 4-nitro-phenylester[10] or *N*-succinimidyl 4-[$^{18}$F]fluorobenzoate[11]. Of these various radio-labelled derivatives of octreotide, $^{111}$In-DTPA-(D)Phe$^1$-octreotide (Octreoscan®) and $^{123}$I–Tyr$^3$–octreotide are routinely applied in clinical investigations.[12]

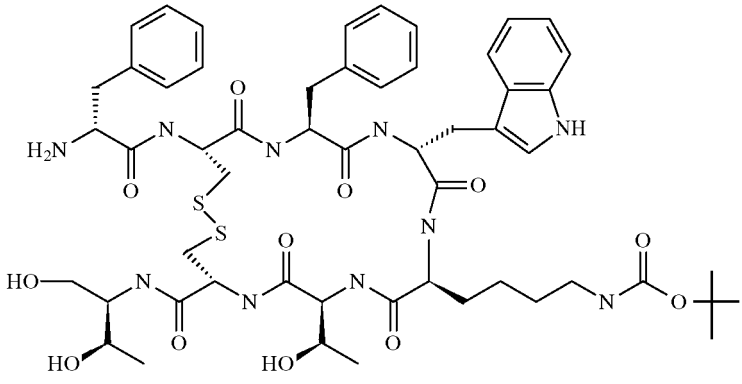
$^{76}Br$ is an alternative to $^{18}F$ and $^{68}Ga$ for labelling radiotracers with applications in positron emission tomography, PET.[13,14] $^{76}Br$ has a half-life of 16.2 h and decays to 55% by positrons and can be produced by the $^{76}Se(p,n)^{76}Br$ reaction. *N*-Succinimidyl 4-[$^{76}$Br]bromobenzoate **1**,[15] *N*-succinimidyl 5-[$^{76}$Br]bromo-3-pyridine-carboxylate **3** and methyl-4-[$^{76}$Br]bromobenzimidate **8** are candidates for incorporating $^{76}Br$ into octreotide through conjugation labelling. The conjugation of compounds **1**,**3** and **8** to octreotide is described in this paper as well as the syntheses of **3** and **8** from the corresponding trimethylstannyl-precursors. The binding properties of the $^{76}$Br-labelled octreotide-conjugates to meningiomas, as determined by frozen section autoradiography, are also presented.

## Results and discussion

*N*-Succinimidyl 4-[$^{76}$Br]bromobenzoate **1** has previously been used for labelling of proteins and oligonucleotides modified with an aminolinker in the 5′-position.[15] Using this method for labelling with $^{76}Br$, analogous to one of the methods for [$^{18}$F]fluorine-labelling, was our first choice when looking for procedures to label octreotide.

Conjugation of compound **1** and ε-Boc-octreotide using mixtures of buffers and DMF gave low radiochemical yields even at relatively high concentrations of octreotide (10 mg/ml, 8 μmol/ml as compared to 0.7 μmol/ml used in conjugation with oligonucleotides).[15] Due to the good solubility of ε-Boc-octreotide in organic solvents, the

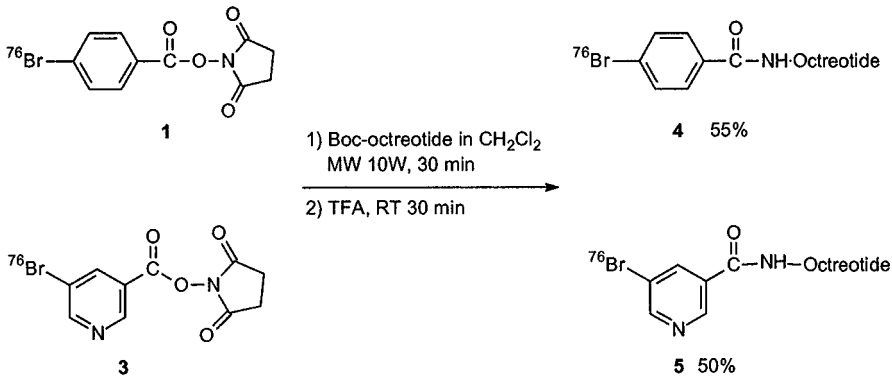


ε-Boc-Octreotide

conjugation reaction was also performed in solvents such as DMF, DMSO, acetonitrile, methanol and dichloromethane with or without the addition of various bases such as triethylamine, 4-(dimethylamino)pyridine (DMAP) and 1,4-diazabicyclo[2.2.2]octane (DABCO). Conventional heating as well as microwave heating were applied to the samples, but the radiochemical yield did not exceed 20%. The use of dichloromethane as the solvent and microwave heating resulted in 70–90% labelling of ε-Boc-octreotide after 30 min using 5 mg/ml of ε-Boc-octreotide (Scheme 1). The Boc-protective group was removed by adding TFA to reaction mixture. After 30 min, the solvents were removed by gas purge and the product **4** was isolate by semi-preparative HPLC in 55% radiochemical yield. The advantage of using dichloromethane as a solvent was that the Boc-protecting group could be removed by the addition of TFA to the reaction mixture. As a comparison, the method presented for conjugation of the $^{131}$I and $^{211}$At-analogues of **1** to octreotide required two HPLC-separations and one solid phase extraction work-up in addition to extra activating reagents used.[9] The specific radioactivity for compound **1** had earlier been determined 20–200 GBq/μmol[15,16] and the specific radioactivity for **4** was determined to 2 GBq/μmol starting from 70 MBq $^{76}$Br.

In order to decrease the lipophilicity of the labelled peptide, the use of two different prosthetic groups, *N*-succinimidyl 5-[$^{76}$Br]bromo-3-pyridinecarboxylate **3** (Scheme 2) and methyl-4-[$^{76}$Br]bromobenzimidate **8** (Scheme 3), that would give protonated octreotide-conjugates at physiological pH were investigated. Imidates can react with amines to

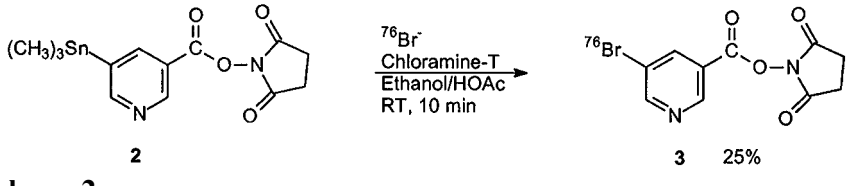
 




**Scheme 1**

form amidines (Scheme 4), which are protonated at physiological pH and the original charge on the peptide can be retained.[17] Iminoesters are reported to be less reactive towards amines than succinimidyl-esters but they are also less sensitive to hydrolysis.[18] For example, methyl-4-hydroxybenzimidate has been radioiodinated and conjugated to proteins[19] and a fluorinating reagent for proteins, 3-[$^{18}$F]fluoro-5-nitrobenzimidate, has been reported[20].

*N*-Succinimidyl 5-[$^{131}$I]iodo-3-pyridinecarboxylate[21] and *N*-succinimidyl 5-[$^{211}$At]astato-3-pyridinecarboxylate[22] and *N*-succinimidyl 5-[$^{211}$Ar]astato-3-pyridinecarboxylate[22] have been used for labelling of monoclonal antibodies and recently used for the labelling of octreotide.[9] *N*-Succinimidyl-3-pyridinecarboxylate **2** and the reference *N*-succinimidyl 5-bromo-3-pyridinecarboxylate were prepared according to the literature.[21,23] An advantage using *N*-succinimidyl 5- [$^{76}$Br]bromo-3-pyridinecarboxylate **3** is that the same conjugation conditions could be applied for reactions using *N*-succinimidyl [$^{76}$Br]bromobenzoate as prosthetic group (Scheme 1).

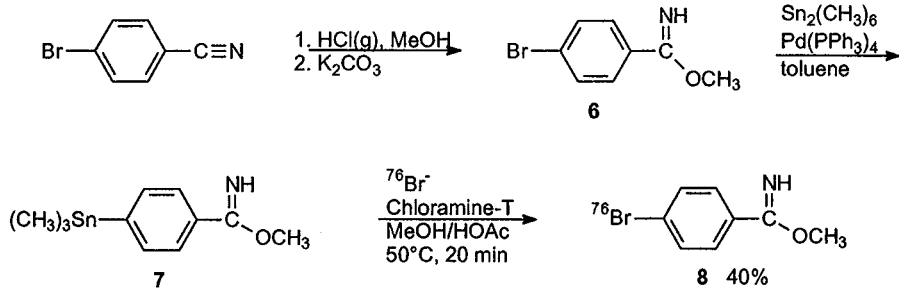



**Scheme 2**

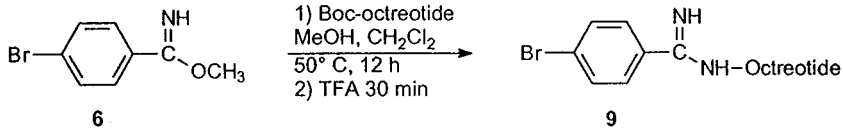
Compound **2** was radiobrominated in 25% radiochemical yield using chloramine-T as an oxidant at room temperature (Scheme 2). All [$^{76}$Br]bromide was consumed according to HPLC-analysis and a very lipophilic labelled side-product was formed, probably due to a minor impurity in stannyl-compound **2**. The use of hexane: ethyl acetate as a mobile phase for HPLC-purification of compound **3** required the addition of 2% acetic acid to the mobile phase. The remaining acetic acid caused problems in the conjugation reaction, giving hydrolysis of **3**, so the mobile phase was changed to dichloromethane containing 1% methanol. Compound **3** was conjugated to ε-Boc-octreotide using the same conditions as for **1** to give a Boc-protected octreotide conjugate of **5** (Scheme 1). Deprotection in TFA followed by HPLC-purification resulted in a 50% radiochemical yield of the labelled octreotide **5** (calculated from **3**). The specific radioactivity for the labelled octreotide **5** was determined to be 1 GBq/μmol starting from 40 MBq $^{76}$Br.

In the syntheses of methyl-4-bromobenzmidate **6**, different methods were investigated. The highest yield (74%) was obtained by bubbling HCl (g) in a methanol solution of 4-bromobenzonitrile (Scheme 3).[24] Bubbling HCl (g) into a solution of 4-bromobenzonitrile and methanol in equimolar amounts in diethyl ether[25] or the use of 4-bromobenzonitrile and sodium methoxide[26] gave lower yields. The stannyl-compound **7** was prepared in 40% yield using a palladium catalysed metallation reaction.[27] Compound **7** was radiobrominated and isolated in 40% radiochemical yield using chloramine-T as an oxidant (Scheme 3). Since the conjugation of **8** to octreotide failed, no further optimisation studies were performed. Removal of the solvent from the purified compound **8** had to be done with care since the radioactivity in the vessel containing **8** decreased when purging with helium gas after all




**Scheme 3**




6 9

**Scheme 4**

solvent was removed. Adding a small amount of high boiling solvent, for example DMF, solved this problem.

The conjugation between methyl-4-bromobenzimidate **6** and ε-Boc-octreotide worked well using a large excess of the imidate **6** in methanol/dichloromethane at 70°C overnight (Scheme 4). The use of these conditions in the labelling synthesis was not successful. Using microwave heating and dichloromethane as in the *N*-succinimidyl bromobenzoate case, left the methyl-4-[$^{76}$Br]bromobenzimidate **8** unreacted. A variety of conditions using mixtures of borate buffer and DMF or DMSO at different temperatures were tested. The use of organic solvents left compound **8** unreacted. With water as solvent, **8** was consumed, probably due to hydrolysis. Addition of different organic bases was also explored, but no product was observed. The reactivity of compound **8** was tested using BSA (10 mg/ml) in borate buffer pH 8.7. The radiochemical yield, determined by size exclusion chromatography, was 10%.

Meningiomas have high affinity binding sites specific to somatostatin (SRIH) receptors,[28] whereas heart lacks specific binding. High specific binding of the tracer $^{76}$Br-octreotide **5** was observed in meningioma tissue, whereas $^{76}$Br-octreotide **4** only demonstrated non-specific binding (Figure 1). The non-specific binding, observed in meningioma

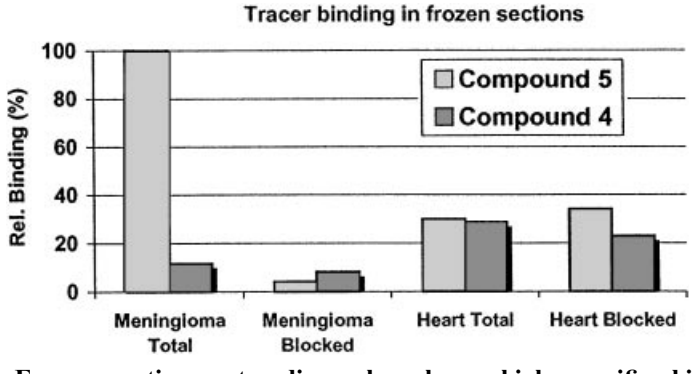



**Figure 1. Frozen section autoradiography shows high specific binding in meningioma with $^{76}$Br-octreotide 5 in comparison to $^{76}$Br-octreotide 4**

after blocking and in heart tissue, was of similar magnitude with the two tracers. The difference in the behaviour of the two tracers might be due to the difference in lipophilicity.[11] The pyridine-moiety in compound **5** is protonated at physiological pH, retaining the positive charge originating from the *N*-terminal of octreotide and lowering the lipophilicity compared to **4**.

## Experimental

### *General*

All chemicals used were commercially available and used without further purification unless otherwise indicated. Boc-octreotide and octreotide were a gift from Novartis Pharma Ag, Basel, Switzerland. Methanol was refluxed over Mg and $I_2$ and distilled onto molecular sieves under $N_2$-atmosphere. HCl (g) was dried by passing it through sulphuric acid (conc.) and a drying tower containing $CaCl_2$. Deionised water (18.2 MΩ) and 99.5% ethanol were used for labelling reactions.

$^1$H NMR and $^{13}$C NMR spectra were recorded on a Varian XL-300 (300 MHz) or Varian Gemini (200 MHz) spectrometer with chloroform-$d_1$ as solvent and as internal standard. Thin-layer chromatography (TLC) was performed using DC-Alufolien Kiselgel 60 $F_{254}$ plates (Merck, Darmstadt, Germany). Ultraviolet (UV) absorbance was visualised using short- and long-wave ultraviolet light. A Micro Well 10 (Labwell AB, Uppsala, Sweden) was used for the microwave heating. Microinsert vials (0.2 ml) placed in 1.5 ml glass vials equipped with a screw cap and double septa were used for the reactions in the microwave oven. The reported radiochemical yields are uncorrected with respect to decay. Autoradiographic imaging was performed using a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA) and data was analysed by ImageQuant.

### *Liquid chromatography*

Analytical liquid chromatography separations were performed using a Beckman (Fullerton, CA, USA) System (a 126 pump and 166 UV detector) with a $\beta^+$-flow detector in series. Data collection was performed using the Beckman System Gold Noveau Chromatography Software Package. Semi-preparative HPLC was performed using

equipment from Waters (Milford, MA, USA); a modified M 6000 A pump, a 440 UV detector with a $\beta^+$-flow detector in series. The UV-absorbance was measured at 280 nm and the retention-times of the radioactive compounds were matched with those of characterised standards.

Electrospray ionisation mass spectroscopy, ESI-MS, was performed using a Fisons Platform (Micromass, Manchester, UK). A post-column split was used and 1% of the flow was delivered to the electrospray probe.

The following columns and mobile phases were used:

System 1: Ultrasphere ODS C-18 HPLC column (Beckman) 250 × 4.6 mm ID, 5 μm, flow 1.5 ml/min, A = 10 mM TFA, B = MeCN.

(1a) 20% B 0–7 min, linear gradient 20–90% B 7–15 min, linear gradient 90–20% 18–21 min.
(1b) 10% B 0–2 min, linear gradient 10–50% B 2–22 min, linear gradient 50–80% B 25–28 min, linear gradient 80–10% 30–34 min.
(1c) 20% B 0–7 min, linear gradient 20–70% B 7–15 min, linear gradient 70–20% B 18–21 min.

System 2: Ultrasphere Silica HPLC-column (Beckman) 25 × 10 mm ID, 5 μm, flow 4 ml/min.

(2a) hexane : ethyl acetate : acetic acid 70 : 30 : 0.1 isocratic elution,
(2b) hexane : ethyl acetate : acetic acid 70 : 30 : 2 isocratic elution,
(2c) dichloromethane : methanol 100 : 1 isocratic elution.

System 3: Ultrasphere ODS C-18-column (Beckman) 250 × 10 min ID, 5 μm, flow 4 ml/min, A = 50 mM phosphate buffer pH 7, B = MeCN, 10% B 0–2 min, linear gradient 10–50% B 2–22 min, linear gradient 50–80% 35–38 min, 80% B 38–40 min.

*Production of $^{76}$Br*

$^{76}$Br was produced via the $^{76}$Se(p, n)$^{76}$Br nuclear reaction using a 16 MeV $H^+$ cyclotron (Scanditronix, Uppsala, Sweden) and an $^{76}$Se-enriched $Cu_2Se$-target.[13] The $^{76}$Br was released from the target-pellet by a thermochromatographic method and deposited on the walls of a piece of Teflon-tubing. The [$^{76}$Br]bromide was recovered from the tubing by rinsing with water and the resulting $^{76}$Br-solution was passed through a C-18 SPEC (Ansys Inc., Irvine, CA, USA), evaporated to dryness and redissolved in the solvent of choice prior to use (15).

*Synthesis of N-succinimidyl 5-[$^{76}$Br]bromo-3-pyridinecarboxylate* **3**

0.5–1 mg of stannyl-compound **2**, [$^{76}$Br]bromide in ethanol (200 µl), chloramine-T (10 mg/ml, 20 µl) and acetic acid (2 µl) were mixed and left at room temperature for 10 min. The solvent was evaporated and the product **3** was isolated in 25% radiochemical yield using normal phase HPLC, system 2b $R_t = 15.5$ min or system 2c $R_t = 12.8$ min. Analysis by HPLC system 1 c gave $t_R = 11.8$ min.

*Synthesis of N-succinimidyl 4-[$^{76}$Br]bromobenzoyl-octreotide* **4**

*N*-Succinimidyl 4-[$^{76}$Br]bromobenzoate **1** was prepared as described previously.[15] The solvent was evaporated by a gas purge in a 0.2 ml microinsert vial. ε-Boc-octreotide (0.25 mg) in dichloromethane (50 µl) was added to the residue containing **1** and the mixture was heated by microwaves, 10 W for 30 min to give 70–90% of labelled Boc- protected octreotide-conjugate of **4** (determined by HPLC-analysis). TFA (50 µl) was added and the reaction was left at room temperature for 30 min. The solvents were removed under a stream of nitrogen, the residue was redissolved in water: acetonitrile 80 : 20 and 4-[$^{76}$Br]bromobenzoyl octreotide **4** was isolated in 55% radiochemical yield (calculated from **1**) using semi-preparative HPLC system 3, $R_t = 30.5$ min. The products were analysed using HPLC-system 1b. Boc-**4** $t_R = 29.3$ min, compound **4** $t_R = 24.1$ min.

The identity of the peaks in the chromatogram was confirmed using LC–MS, HPLC-system 1b, after addition of carrier *N*-succinimidyl 4-bromobenzoate in the conjugation reaction. LC-MS (ESI+) : (Boc-**4**) $m/z = 1303$ [M + H$^+$], compound **4** $m/z = 1203$ [M + H$^+$].

*Synthesis of 5-[$^{76}$Br]bromo-3-pyridinecarboxy-octreotide* **5**

Using the same conditions as in the synthesis of **4**, compound **5** was isolated in 50% radiochemical yield (calculated from **3**) using HPLC-system 3, $t_R = 24.4$ min. Analysis by HPLC system 1b: (Boc-**5**) $t_R = 28.6$ min, compound **5** $t_R = 22.0$ min. LC–MS: (Boc-**5**) $m/z = 1304$ [$M$ + H$^+$], 652 [M + 2H$^+$]; compound **5** $m/z = 602.7$ [M + 2H$^+$].

*Synthesis of methyl-4-bromobenzimidate* **6**

4-Bromobenzonitrile (1.96 g, 10.8 mmol) was dissolved in dry methanol (100 ml) and the mixture was cooled on ice. Dry hydrogen chloride was bubbled through the solution for 3 h and the reaction mixture was left under nitrogen atmosphere at room temperature overnight. The methanol was evaporated *in vacuo*. The solid was recrystallised from

methanol : ether to give (2.0 g, 74%) of the hydrochloride of **6**. $^1$H NMR (200 MHz): $\delta$ 8.3 (m, 2 H), 7.7(m, 2 H), 4.53 (s,3H), 1,8(s, 2 H).

The hydrochloride of compound **6** was dissolved in 1 M $K_2CO_3$ and extracted with ether, dried over $MgSO_4$ and evaporated to give **6**. $^1$H NMR (300 MHz): $\delta$ 7.6 (m, 2 H), 7.5 (m, 2 H), 3.9 (2, 3 H), 3.9 (s, 3 H). $^{13}$C NMR: $\delta$ 167, 132, 121, 128, 126, 53. IR ($CHCl_3$) 3024 (N=H stretch). MS (ESI+): $m/z = 215.0$ $[M+H]^+$ calculated M = 214.0.

*Synthesis of methyl-4trimethylstannylbenzimidate 7*

Compound **6** (0.50 g, 2.34 mmol) was dissolved in dry toluene. Hexamethylditin (1.34 g, 4.1 mmol) was added followed by tetrakis (triphenylphosphine)palladium(0), $Pd(PPh_3)_4$, (46 mg, 0.04 mmol). The mixture was stirred under nitrogen atmosphere at 60°C for 2 h. The temperature was raised to 75°C and left for 20 h. The solvent was removed *in vacuo* and the product was purified on silica, eluting with pentane containing 2% triethylamine until the unreacted tin eluted, followed by pentane: ethyl acetate 10 : 2 containing 2% triethylamine giving compound **7** in 40% yield. $^1$H NMR (300 MHz): $\delta$ 7.75 (m, 2 H), 7.55 (m, 2 H). 3.95 (s, 3 H), 0.35 (s, 9 H). $^{13}$C NMR (75 MHz): $\delta$ 168, 146, 136, 132, 126, 53, −9.6.

IR: 2980 cm$^{-1}$ (N=H stretch). LC–MS (ESI+): $m/z$ 299.3 $[M+H]^+$, calculated M = 299.0 (monoisotopic mass) with isotope distribution corresponding to the distribution of Sn.

*Synthesis of methyl-4-[$^{76}$Br]bromobenzimidate 8*

Compound **7** (1.0–1.5 mg) was dissolved in methanol (50 µl). Chloramine-T (20 µl, 10 mg/ml in methanol), acetic acid (2 µl) and [$^{76}$Br] $Br^-$ (150 µl in methanol) were added. The mixture was heated at 50°C for 20 min, and the methanol was removed by a gentle stream of He. The residue was dissolved in hexane: ethyl acetate and the product **8** was isolated in 40% radiochemical yield using semi-preparative HPLC, system 2a, $t_R = 14.5$ min.

The identity and purity of the compound was confirmed using HPLC system 1a, $t_R = 11.3$ min and LC–MS (ESI+): $m/z = 215.0$ $[M+H]^+$.

*Synthesis of 4-bromobenzimidate octreotide 9*

ε-Boc-octreotide (2 mg, 1, 8 µmol) and methyl-4-bromobenzimidate **6** (3.9 mg, 18 µmol) where dissolved in methanol (100 µl) and $CH_2Cl_2$ (100 µl) in a capped vial and heated at 50°C overnight. The

reaction-mixture was analysed by LC-MS, HPLC-system 1b, $t_R = 25.8$ min. LC-MS (ESI+): $m/z = 1302$ $[M+H]^+$.

Deprotection of the ε-Boc-octreotide-conjugate in TFA (50 µl) gave a product **9** with $m/z = 601.3$ $[M+2H]^{2+}$, calculated for bromobenzimidate octreotide **9** 601.2. HPLC-system 1b, $t_R = 19.6$ min.

*Synthesis of [$^{76}$Br]bromobenzimidate albumin*

BSA (10 mg/ml) in borate buffer 0.1 M, pH 8.7 (100 µl) was added to the dried compound **8**. The mixture was heated at 40°C for 1 h and was analysed by size exclusion chromatography using a Fast Desalting HR 10/10 FPLC gel filtration column (Amersham Pharmacia Biotech, Uppsala, Sweden) and phosphate buffer pH 7. Ten percent of the radioactivity was incorporated in the high molecular fraction.

*Frozen section autoradiography*

Frozen human meningiomas and rat heart were cut in 20 µm sections and mounted on microscope slides. Visualization of SRIH receptors was performed as described by Reubi et al.[28] Sections were preincubated for 10 min at room temperature in 50 mM Tris–HCl buffer, pH 7.4, containing 2 mM $CaCl_2$ and 5 mM KCl and washed twice for 2 min in 50 mM Tris–HCl buffer. To define the total binding, sections were incubated for 2 h at room temperature in 170 mM Tris–HCl buffer, pH 7.4, containing 1% BSA, 40 µg bacitracin and 5 mM $MgCl_2$ in the presence of brominated ligand **4** and **5** (500 pM). Non-specific binding was determined by adding 1 µM octreotide. Incubated sections were washed thrice for 5 min in cold incubation buffer containing 0.25% BSA. After a brief dip in distilled water, the sections were dried quickly under a stream of warm air and apposed to a storage phosphor (SP) imaging plate.

For quantification, individual calibration standards were prepared for each section and exposed to same imaging plate.[29] The standard was a 20-µl drop of tracer solution on a thin absorbent paper. Using the SP imaging system, volume of standard and average count/pixel subtracting the background area of each region of interest of a structure was measured.

## Conclusions

*N*-Succinimidyl 4-[$^{76}$Br]bromobenzoate **1** and *N*-succinimidyl 5-[$^{76}$Br]bromo-3-pyridinecarboxylate **3** were used to label Boc-protected

octreotide by microwave heating. The Boc-group was removed by the addition of TFA to the reaction mixture and the products were isolated by HPLC. This method is an improvement over the published use of astato and iodo-analogues of **1** and **3** in the labelling of octreotide. Also, methyl-4-[$^{76}$Br]bromobenzimidate **8** could be synthesized from the corresponding trimethylstannyl-compound. Compound **8** did not react with Boc-octreotide under the conditions examined. Octeotide-conjugate **5** showed better binding-properties to meningiomas than **4** as determined by frozen section autoradiography.

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