Direct One-Step(18)F-Labeling of Peptides via Nucleophilic Aromatic Substitution

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In Vitro and *in Vivo* Characterization of Novel ^{18}F-Labeled Bombesin Analogues for Targeting GRPR-Positive Tumors

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The gastrin-releasing peptide receptor (GRPR) is overexpressed on a number of human tumors and has been targeted with radiolabeled bombesin analogues for the diagnosis and therapy of these cancers. Seven bombesin analogues containing various linkers and peptide sequences were designed, synthesized, radiolabeled with ^{18}F, and characterized *in vitro* and *in vivo* as potential PET imaging agents. Binding studies displayed nanomolar binding affinities toward human GRPR for all synthesized bombesin analogues. Two high-affinity peptide candidates **6b** ($K_i = 0.7$ nM) and **7b** ($K_i = 0.1$ nM) were chosen for further *in vivo* evaluation. Both tracers revealed specific uptake in GRPR-expressing PC-3 tumors and the pancreas. Compared to **[^{18}F]6b**, compound **[^{18}F]7b** was characterized by superior tumor uptake, higher specificity of tracer uptake, and more favorable tumor-to-nontarget ratios. *In vivo* PET imaging allowed for the visualization of PC-3 tumor in nude mice suggesting that **[^{18}F]7b** is a promising PET tracer candidate for the diagnosis of GRPR-positive tumors in humans.

INTRODUCTION

Bombesin (BBS) is a 14-amino acid neuropeptide that was originally isolated from the skin of the *Bombina* frog and binds with high affinity to gastrin-releasing peptide (GRP) receptors (*1*−*4*). Four subtypes of BBS/GRP receptors have been identified. The bombesin receptor subtype 2 has been shown to be overexpressed on a variety of human tumors, including small-cell lung cancer (SCLC), prostate, breast, gastric, colon, pancreatic, and gastrointestinal cancer (*5*−*9*). Thus, radiolabeled BBS analogues having high affinity for this receptor subtype can be used as radiopharmaceuticals either for the detection or treatment of these cancers (*10*−*12*).

It has been shown that the C-terminal amino acid sequences, Trp8-Ala9-Val10-Gly11-His12-Leu13-Met14-NH_2, are necessary for retaining receptor binding affinity and preserving the biological activity of BBS-like peptides; hence, the N-terminal region of the peptide can be used for radiolabeling. A number of potent BBS analogues have been labeled with various radionuclides, such as $^{99\ m}$Tc, ^{111}In, ^{90}Y, ^{64}Cu, ^{177}Lu, ^{68}Ga, or ^{18}F, for targeting GRPR-expressing cancer cells in both animal models and human subjects (*13*−*25*). Among the available PET nuclides, ^{18}F has ideal characteristics for peptide receptor imaging studies in terms of its half-life (109.7 min) and low β^+ energy (0.64 MeV) (*26*). Current known methods of radiolabeling peptides with ^{18}F rely on using prosthetic groups such as the [^{18}F]succinimidyl 4-fluorobenzoate ([^{18}F]SFB) (*27*), [^{18}F]4-fluorobenzaldehyde (*28, 29*), and thiol-selective ^{18}F-labeling reagents (*30, 31*). Although great improvements have been achieved, the established methods have disadvantages in that the synthetic methods comprise multistep radiosyntheses and time-consuming workup procedures. Therefore, several research groups focus on new synthetic strategies that avoid the use of prosthetic groups. Recent studies have demonstrated the feasibility of labeling organoboron and organosilicon bioconjugates with ^{18}F in a single step (*32*−*35*). In our laboratories, we have successfully labeled bombesin peptides with ^{18}F in one step and applying silicon−fluorine chemistry (*36*). However, the ^{18}F-labeled silicon-based peptides showed relatively low tumor accumulation and retention as well as unfavorable hepatobiliary excretion in nude mice with PC-3 xenografts. This might be due to the introduction of the highly lipophilic di-*tert*-butyl silyl labeling moiety into the peptide. In order to improve the tumor targeting and imaging potential of ^{18}F-labeled bombesin peptides, another approach via ^{18}F-for-$^{+}N(CH_3)_3$ substitution using a less lipophilic benzonitrile labeling moiety has been exploited in our laboratories (*37*). The *in vitro* binding affinity of these fluorinated bombesin peptides toward GRPR were assessed by a competitive displacement assay using [^{125}I]-Tyr^4-bombesin as the radioligand. More than 11-fold higher binding affinity with benzonitrile as the labeling moiety was obtained compared to using di-*tert*-butyl silyl as the labeling moiety with the same peptide sequence (*36*). Therefore, in this study, several new BBS peptides were synthesized and ^{18}F-labeled via the aforementioned ^{18}F-for-$^{+}N(CH_3)_3$ substitution approach. Our efforts have been focused on improving the pharmacokinetics of BBS derivatives by using different linkers, peptide sequences, and non-natural amino acids. Two bombesin analogues, one being positively and another negatively charged, were chosen for further *in vivo* evaluation in nude mice with PC-3 xenografts.

EXPERIMENTAL PROCEDURES

General. All chemicals unless otherwise stated were purchased from Sigma-Aldrich or Merck and used without further purification. Fmoc-aminoacids were purchased from IRIS Biotech except Fmoc-Statine, FA01010, and Fmoc-Ala(SO_3Na)−OH), which were purchased from NeoMPS (now Polypeptide). The solvents were of HPLC quality. Peptide syntheses were carried out using Rink-Amide resin (0.68 mmol/g) following the standard Fmoc strategy (*29*). All amino acid residues were,

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if not further specified, L-amino acid residues. FA01010 denotes (4*R*,5*S*)-4-amino-5-methylheptanoic acid, statine (Sta) denotes (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid, Ala(SO_3H) denotes cysteine sulfonic acid, and Ava denotes 5-aminopentanoic acid. High-performance liquid chromatography (HPLC) analyses were performed using an ACE C18 column (50 × 4.6 mm, 3 μm) under the indicated conditions. Analytical HPLC chromatograms were obtained using an Agilent 1100 system with Gina software, equipped with UV multiwavelength and Raytest Gabi Star detectors. Semipreparative HPLC purifications were carried out using a semipreparative ACE C18 column (250 × 10 mm, 5 μm) under the indicated conditions. The semipreparative HPLC system used was a Merck-Hitachi L6200A system equipped with a Knauer variable wavelength detector and an Eberline radiation detector.

Solid-Phase Peptide Synthesis. Peptide synthesis was carried out using Fmoc-Rink Amide-linker functionalized polystyrene resin (0.68 mmol/g). The peptide chain was elongated in cycles of Fmoc deprotection, followed by coupling of the subsequent Fmoc amino acid. Acid-labile side chain protecting groups were used for the following amino acids: Fmoc-His(Trt)−OH, Fmoc-Trp(Boc)−OH, and Fmoc-Gln(Trt)−OH. Fmoc deprotection was achieved by shaking a suspension of the resin in 20% piperidine in DMF for 5 min and repeatedly doing so for another 20 min. The resin was subsequently washed with DMF (2×), CH_2Cl_2 (2×), and DMF (2×). A solution of Fmoc-Xaa−OH (Xaa = amino acid, 4 equiv), HBTU (*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, 4 equiv), HOBT (1-hydroxybenzotriazole, 4 equiv), DIPEA (*N*,*N*′-di-*iso*-propylethylamine, 8 equiv) in DMF was added to the resin-bound free amine peptide and shaken for 90 min at room temperature. This step was repeated with a reaction time of 60 min, and the resin was washed with DMF (2×), CH_2Cl_2 (2×), and DMF (2×). The two steps of Fmoc-deprotection and amino-acid addition were then repeated until the final elongation of the desired peptide was achieved. The peptides were typically prepared starting with 147 mg (0.1 mmol) of the resin. The amounts of reagents and building blocks in all subsequent reactions were calculated on the basis of this amount.

Procedure for the Synthesis of (4-Trimethylammonium-3-cyano-benzoyl)-Functionalized Peptide Trifluoroacetate Salts (Precursor Compounds **1a**−**7a***).* Representative procedure: *N*-methylmorpholine (NMM, 22 μL, 0.2 mmol) was added to a solution of 2-cyano-4-carboxyl-phenyl)-trimethylammonium triflate salt (71 mg, 0.2 mmol) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (66 mg, 0.2 mmol) in DMF (2 mL). The mixture was added to the resin-bound, side chain protected, Fmoc-deprotected peptide (0.1 mmol, based on the initial resin loading) which was prepared by following the general procedures described above. The reaction mixture was shaken intensively for 4 h. The resin was then filtered and washed with DMF (3 × 4 mL) and CH_2Cl_2 (3 × 4 mL). The coupling step was repeated. Thereafter, the resin was treated with a mixture of trifluoroacetic acid, distilled water, phenol, and tri*iso*propylsilane (85/5/5/5, 1.5 mL) for 3 h. The mixture was added to ice cold methyl *tert*-butylether, and the precipitate was separated by centrifugation. Water was added to the pellet, and the supernatant was lyophilized. The residue was purified by preparative RP-18 HPLC-MS with a gradient of 5−30% acetonitrile in 20 min and 0.1% trifluoroacetic acid as cosolvent. The desired fraction was collected and lyophilized, and the product was analyzed by HPLC-MS.

Syntheses of Fluorinated Peptides (Reference Compounds **1b**−**7b***).* Representative procedure: A solution of 3-cyano-4-fluorobenzoic acid (66 mg, 4 equiv) (*37*), HBTU (*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, 151 mg, 4 equiv), HOBT (1-hydroxybenzotriazole, 61 mg, 4 equiv), and DIPEA (*N*,*N*′-di-*iso*-propylethylamine, 70 μL, 4 equiv) in DMF (2 mL) was added to the resin-bound side chain protected peptide (0.1 mmol, based on the initial resin loading) which was prepared by following the general procedures described above. The reaction mixture was shaken intensively for 4 h. The resin was then filtered and washed with DMF (3 × 4 mL) and CH_2Cl_2 (3 × 4 mL). Thereafter, the resin was treated with a mixture of trifluoroacetic acid, distilled water, phenol, and tri*iso*propylsilane (85/5/5/5, 1.5 mL) for 3 h. The mixture was added to ice cold methyl *tert*-butylether, and the precipitate was separated by centrifugation. Water was added to the pellet, and the supernatant was lyophilized. The residue was purified by preparative RP-18 HPLC-MS with a gradient of 5−50% acetonitrile in 20 min and 0.1% trifluoroacetic acid as cosolvent. The desired fraction was collected and lyophilized, and the product was analyzed by HPLC-MS.

Radiolabeling. No-carrier added [^{18}F] fluoride was produced via the ^{18}O (p, n) ^{18}F nuclear reaction by irradiation of enriched [^{18}O] water. [^{18}F]Fluoride was immobilized on an anion-exchange cartridge (QMA light, Waters, AG) and eluted with a solution of Kryptofix $K_{2.2.2}$ (5 mg), Cs_2CO_3 (2.3 mg), or K_2CO_3 (1 mg) in acetonitrile (1.5 mL) and water (0.5 mL). The fluoride was dried by azeotropic distillation of acetonitrile at 110 °C under vacuum with a stream of nitrogen. The azeotropic drying was repeated three times with acetonitrile (3 × 1 mL). To the dried [^{18}F] fluoride complex, the TMA-based bombesin peptide precursor was added (2 mg, in 150 μL of DMSO). The reaction solution was heated at the indicated reaction temperatures. An aliquot was taken from the reaction mixture and analyzed by analytical HPLC. The identity of the tracer was confirmed by coinjection of the authentic standard on the same analytical HPLC system ACE C18 column (50 × 4.6 mm, 3 μm). Eluting conditions: 10 mM K_2HPO_4 in water (solvent A), 10 mM K_2HPO_4 in water/acetonitrile 3/7 (solvent B); 0−7.0 min, 5−95% B; 7.0−7.1 min, 95−100% B; 7.1−8.8 min, 100% B; 8.8−9.0 min, 100−5% B. The flow rate was 2 or 1.8 mL/min. Semipreparative HPLC purifications were carried out using a semipreparative ACE C18 column (250 × 10 mm, 5 μm). Elution system: 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile/water 9/1 (solvent B).

3-Cyano-4-[^{18}F]fluoro-benzoyl-Ava-Gln-Trp-Ala-Val-Gly-His(3Me)-Sta-Leu-NH$_2$ ([^{18}F]**1b***).* A solution of compound **1a** (2 mg) in anhydrous DMSO (150 μL) was added to a reaction vial containing the dry K[^{18}F]F/$K_{2.2.2}$ complex. After heating at 50 °C for 15 min, the crude reaction mixture was analyzed using an ACE C18 column. The ^{18}F-incorporation determined by HPLC was 77% (t_R = 4.92 min).

3-Cyano-4-[^{18}F]fluoro-benzoyl-Arg-Ava-Gln-Trp-Ala-Val-Gly-His(3Me)-Sta-Leu-NH$_2$ ([^{18}F]**2b***).* A solution of compound **2a** (2 mg) in anhydrous DMSO (150 μL) was added to a reaction vial containing the dry Cs[^{18}F]F/$K_{2.2.2}$ complex (3.4 GBq). After heating at 70 °C for 15 min, the crude reaction mixture was analyzed using ACE C18 column. The ^{18}F-incorporation determined by HPLC was 67% (t_R = 4.87 min). The reaction mixture was diluted with water (4 mL) and injected onto an ACE semipreparative HPLC column (0.1% TFA in water (solvent A); B, 0.1% TFA in acetonitrile/water 9/1 (solvent B), 0−2.0 min, 20% B; 2.0−22.0 min, 20−60% B; 22.0−23.0 min, 60−100% B; 23.0−28.0 min, 100% B; flow rate, 3 mL/min), and the product was collected (1.1 GBq). The decay corrected radiochemical yield of the isolated product was around 47%, and radiochemical purity was greater than 99%.

3-Cyano-4-[^{18}F]fluoro-benzoyl-Arg-βAla-Gln-Trp-Ala-Val-Gly-His(3Me)-Sta-Leu-NH$_2$ ([^{18}F]**3b***).* A solution of compound **3a** (2 mg) in anhydrous DMSO (150 μL) was added to a reaction vial containing dry K[^{18}F]F/$K_{2.2.2}$ complex (1.49 GBq). After heating at 50 °C for 15 min, the crude reaction mixture was

analyzed using an ACE C18 column. The ^{18}F-incorporation determined by HPLC was 56% (t_R = 4.78 min). The reaction mixture was diluted with water (4 mL) and injected onto an ACE semipreparative HPLC (0−5 min, 29% B; 5−25 min, 29−34% B; flow rate 3 mL/min). The desired ^{18}F-labeled product was collected (150 MBq, 21.1% d.c. RCY) with a specific activity of 72.5 GBq/μmol.

*3-Cyano-4-[^{18}F]fluoro-benzoyl-Arg-Ser-Gln-Trp-Ala-Val-Gly-His(3Me)-Sta-Leu-NH$_2$ (***[^{18}F]4b***).* A solution of compound **4a** (2 mg) in anhydrous DMSO (150 μL) was added to a reaction vial containing dry K[^{18}F]F/K$_{2.2.2}$ complex (2.9 GBq). After heating at 50 °C for 15 min, the crude reaction mixture was analyzed using ACE C18 column. The ^{18}F-incorporation determined by HPLC was 35% (t_R = 4.94 min). The reaction mixture was diluted with water (4 mL) and injected onto an ACE semipreparative HPLC (0−2 min, 35% B; 2−22 min, 35−55% B; flow rate 3 mL/min). The desired ^{18}F-labeled product was collected (28 MBq, 4% d.c. RCY).

*3-Cyano-4-[^{18}F]fluoro-benzoyl-Arg-Ava-Gln-Trp-Ala-Val-Gly-His-FA01010-Leu-NH$_2$ (***[^{18}F]5b***).* A solution of compound **5a** (2 mg) in anhydrous DMSO (150 μL) was added to a reaction vial containing dry K[^{18}F]F/K$_{2.2.2}$ complex (11 GBq). After heating at 70 °C for 15 min, the crude reaction mixture was analyzed using ACE C18 column. The ^{18}F-incorporation determined by HPLC was 63% (t_R = 5.51 min).

*3-Cyano-4-[^{18}F]fluoro-benzoyl-Arg-Ava--Gln-Trp-Ala-Val-NMeGly-His-Sta-Leu-NH$_2$ (***[^{18}F]6b***).* Radiolabeling based on the published procedure (*37*).

*3-Cyano-4-[^{18}F]fluoro-benzoyl-Ala(SO$_3$H)-Ava-Gln-Trp-Ala-Val-NMeGly-His-Sta-Leu-NH$_2$ (***[^{18}F]7b***).* A solution of compound **7a** (2 mg) in anhydrous DMSO (150 μL) was added to a reaction vial containing dry Cs[^{18}F]F/K$_{2.2.2}$ complex (20 GBq). After heating at 90 °C for 15 min, the crude reaction mixture was analyzed using ACE C18 column. The ^{18}F-incorporation determined by HPLC was 76% (t_R = 4.30 min). The reaction mixture was diluted with water (3 mL) and injected onto an ACE semipreparative HPLC column (0.1% TFA in water (solvent A), B, 0.1% TFA in acetonitrile/water 9/1 (solvent B), 0−2.0 min, 20% B; 2.0−22.0 min, 20−60% B; 22.0−23.0 min, 60−100% B; flow rate, 3 mL/min), and the product was collected (2.1 GBq). The decay corrected radiochemical yield of the isolated product was around 14%, and radiochemical purity was greater than 99%. Specific activity was 77 GBq/μmol at the end of the synthesis.

***In Vitro* Binding Affinity Studies.** The receptor binding affinities of bombesin (BBS) analogues **1b**−**7b** were determined in quadruplicate in a scintillation proximity assay (SPA) using cellular membranes transfected with human bombesin 2 receptors (GRPR) from PerkinElmer (RBHBS2M). The membranes and agglutinin-coupled SPA beads type A (PVT PEI Treated Wheatgerm, Amersham Bioscience) were mixed in assay buffer (50 mM Tris/HCl at pH 7.2; 5 mM $MgCl_2$; 1 mM EGTA; protease inhibitor (Roche Diagnostics GmbH; 1 tablet/50 mL); and 0.3% polyethylenemine) to give final concentrations of approximately 20 μg/mL protein and 8 mg/mL PVT-SPA beads. The ligand [^{125}I]-Tyr^4-bombesin (PerkinElmer; specific activity, 81.4 TBq/mmol) was diluted to 0.2 nM in assay buffer. The test compounds were dissolved in DMSO to give 1 mM stock solutions. They were further diluted in assay buffer to 2 pM−300 nM. Nonspecific binding was determined by an excess of 10 μM Tyr^4-bombesin (Sigma).

The assay was then performed as follows: First, 10 μL of compound solution to be tested for binding was placed in white 384 well plates (Lumitrac 200, Greiner). Next, 20 μL of the GRPR/PVT-SPA bead mixture and 20 μL of the ligand solution were added. After 120 min of incubation at room temperature, another 50 μL of assay buffer was added; the plate was sealed and centrifuged for 10 min at 520*g* at room temperature. Signals were measured in Top Count (Perkin-Elmer) for 1 min integration time per well. Nonspecific binding determined by an excess of bombesin was subtracted from total binding to yield the specific binding at each concentration. The IC_{50} and K_i values were calculated by nonlinear regression using *GraFit* 5 data analysis software (Erithacus Software Ltd.).

Lipophilicity: log $D_{7.4}$ Determination. The distribution coefficient D (log $D_{7.4}$) was determined by the "shake flask method" (*38*): the peptide was dissolved in a mixture of phosphate buffer (600 μL, pH 7.4) and *n*-octanol (600 μL) at 20 °C. The sample was equilibrated by shaking for 4 min followed by centrifugation. The concentrations of the peptide in each phase were measured by HPLC-UV absorption. The log D value was calculated as the log ratio of UV peak area at 254 nm in the organic and aqueous phases of the tested compound.

Biodistribution Studies. All animal experiments were performed in compliance with the current version of the German and Swiss law on the Protection of Animals. The biodistribution experiments were performed using nude mice bearing human prostate tumors (PC-3). For the induction of tumor xenografts, PC-3 cells (2×10^6 cells/mouse) were injected subcutaneously and allowed to grow for four to five weeks. Animals were injected intravenously with approximately 177 kBq of **[^{18}F]6b** and 230 kBq of **[^{18}F]7b** (100 μL). The animals were sacrificed at different postinjection time points (from 0.5 to 4 h, n = 3 for each time point). Organs and tissues of interest were collected and weighed. The amount of radioactivity was determined in the γ-counter to calculate the uptake (% injected dose per g tissue). In addition, three mice received 100 μg of bombesin coinjected with the radiolabeled compound and were sacrificed at 1 h postinjection to determine nonspecific uptake.

PET Study. Four nude mice were inoculated subcutaneously with PC-3 tumor cells (see above) next to the right shoulder. Animals were injected intravenously with 10−15 MBq of **[^{18}F]7b** and scanned in a whole body configuration from 60 to 105 min p.i. in the dedicated small-animal PET system quadHIDAC. Two animals were coinjected with 50 μg of bombesin (1 mg/mL), while two animals received a corresponding volume of vehicle. PET data acquisition and monitoring of anesthesia were performed as described elsewhere (*39*). After the termination of the PET scan, animals were sacrificed at 107 min p.i. by decapitation for tissue sampling. PET data were reconstructed in a single 45 min time frame and normalized to the injected dose per body weight.

RESULTS AND DISCUSSION

Peptide Synthesis. On the basis of the amino acid sequence 7−14 of natural bombesin, we have designed and synthesized a series of new BBS analogues. To overcome the rapid degradation of natural BBS, some modifications were introduced at positions 13 and 14. Met14 was replaced by Leu for stabilization against aminopeptidase 3.4.11.1, and Leu13 was replaced by a non-natural amino acid (FA01010 or Sta) to prevent cleavage by neutral endopeptidase 3.4.24.11. Further modifications were the introduction of the methylated versions of His12 or Gly11. Additionally, a polar spacer, such as -Ava-, -Ava-Arg-, -Arg-βAla-, -Arg-Ser-, or -Ala(SO$_3$H)-Ava- was inserted between the labeling moiety and the binding sequence in order to further improve the pharmacokinetics and to avoid interference of the radiolabel with the binding region.

Nonradioactive peptides were synthesized by solid phase peptide synthesis (SPPS) following standard Fmoc strategies. Coupling of the resin-bound peptide with 3-cyano-4-fluoro-benzoic acid or 3-cyano-4-trimethylamino-benzoic acid afforded the corresponding nonradioactive (^{19}F) peptide standards and

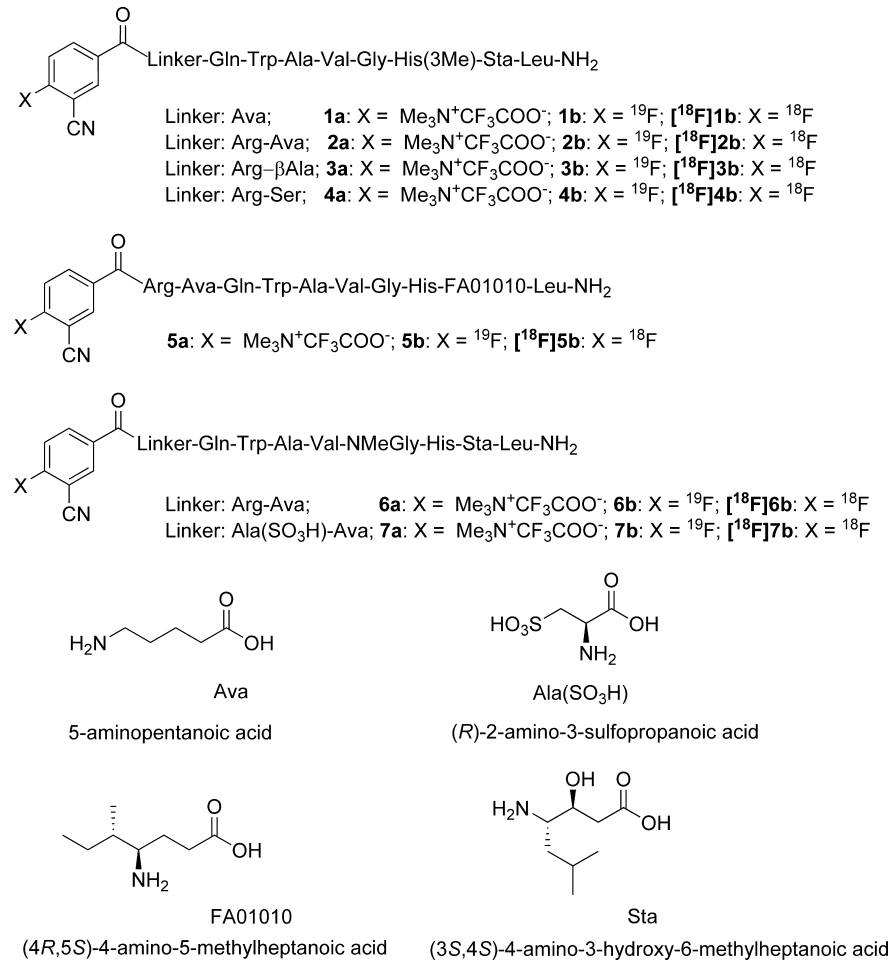

Figure 1. Synthesized bombesin analogues and structures of non-natural amino acids.

Table 1. Mass Spectrometric Analysis of Synthesized Bombesin Peptide Analogues and Their Yields[a]

peptides	molecular formula	calculated	found	yield (%)
1a	$C_{63}H_{93}N_{16}O_{12}$ (M^+)	1265.7	M^+: 1265.5	11
1b	$C_{60}H_{84}FN_{15}O_{12}$	1225.6	$[M + H]^+$: 1226.6	10
2a	$C_{69}H_{105}N_{20}O_{13}$ (M^+)	1421.8	$[(M + H)/2]^+$: 711.8	13
2b	$C_{66}H_{96}FN_{19}O_{13}$	1381.7	$[M + H]^+$: 1383.1	3
3a	$C_{67}H_{101}N_{20}O_{13}$ (M^+)	1393.8	M^+: 1393.8	22
3b	$C_{64}H_{92}FN_{19}O_{13}$	1353.7	$[M + H]^+$: 1354.8	5
4a	$C_{67}H_{101}N_{20}O_{14}$ (M^+)	1409.8	$[(M + H)/2]^+$: 705.7	20
4b	$C_{64}H_{92}FN_{19}O_{14}$	1369.7	$[(M + 2H)/2]^+$: 686.6	12
5a	$C_{68}H_{103}N_{20}O_{12}$ (M^+)	1391.8	M^+: 1391.9	8
5b	$C_{65}H_{94}FN_{19}O_{12}$	1351.7	$[M + H]^+$: 1352.9	12
6a	$C_{69}H_{105}N_{20}O_{13}$ (M^+)	1421.8	$[(M + H)/2]^+$: 711.9	11
6b	$C_{66}H_{96}FN_{19}O_{13}$	1381.7	$[(M + 2H)/2]^+$: 692.4	8
7a	$C_{66}H_{98}N_{17}O_{16}S$ (M^+)	1416.7	M^+: 1417.4	17
7b	$C_{63}H_{89}FN_{16}O_{16}S$	1376.6	$[M + H]^+$: 1378.0	34

[a] Isolated yields (not optimized; single syntheses) based on the amount and loading of commercial Fmoc-Rink-amide resin used for the synthesis.

precursors for ^{18}F-labeling, respectively. The nonradioactive BBS analogues served as a surrogate for ^{18}F-labeled compounds in the determination of binding affinity and as a standard reference for radio HPLC analysis. Figure 1 shows the sequences and the structures of the non-natural amino acid derivatives. All of the newly synthesized bombesin precursors and standard references were purified by reverse-phase HPLC and characterized by mass spectrometry (Table 1).

Radiolabeling. The direct ^{18}F-fluorination protocol developed previously in our laboratories was applied to these new bombesin peptide analogues. The synthetic scheme is depicted in Figure 2 using peptide **7a** as an example. Under the indicated radiolabeling conditions (Table 2), most peptides showed high ^{18}F-labeling efficiency. For peptide **4a**, which contains an Arg-Ser linker, a 29% ^{18}F-incorporation was obtained under mild radiolabeling conditions (50 °C, 15 min). For peptide **7a** containing the Ala(SO_3H)-Ava linker, a similar ^{18}F-labeling efficiency (26%, Table 2, entry 7) was achieved. These results successfully demonstrated that aromatic nucleophilic substitutions of ^{18}F-for-$^{+}$N(CH_3)$_3$ are feasible not only in the presence of histidine, tryptophan, and arginine amino acids but also in the presence of the unprotected acid and hydroxyl functionalities (serine amino acid). In addition, it was found that the ^{18}F-labeling efficiency could be dramatically improved from 26% to 75% by increasing the reaction temperature from 70 to 90 °C (Table 2, entries 7 and 8).

Concerning the stereochemistry of the peptides, similar peptides have been labeled under basic conditions (pH 8−10) and at elevated temperatures (75−100 °C); no racemization of the peptides was observed (*40*−*42*). Because most of our radiolabeling conditions (pH < 8, temperature 50−70 °C) are even milder than those previously reported, peptide racemization is not expected to occur under the above-mentioned radiolabeling conditions (Table 2). In addition, binding analysis of a ^{18}F-labeled peptide containing the same peptide sequence as **7b** revealed K_d and IC_{50} values of 1.43 nM and 0.94 nM, respectively, showing that the ^{18}F-labeled peptide still retains high binding affinity toward GRPR after heating at 90 °C for 10 min.

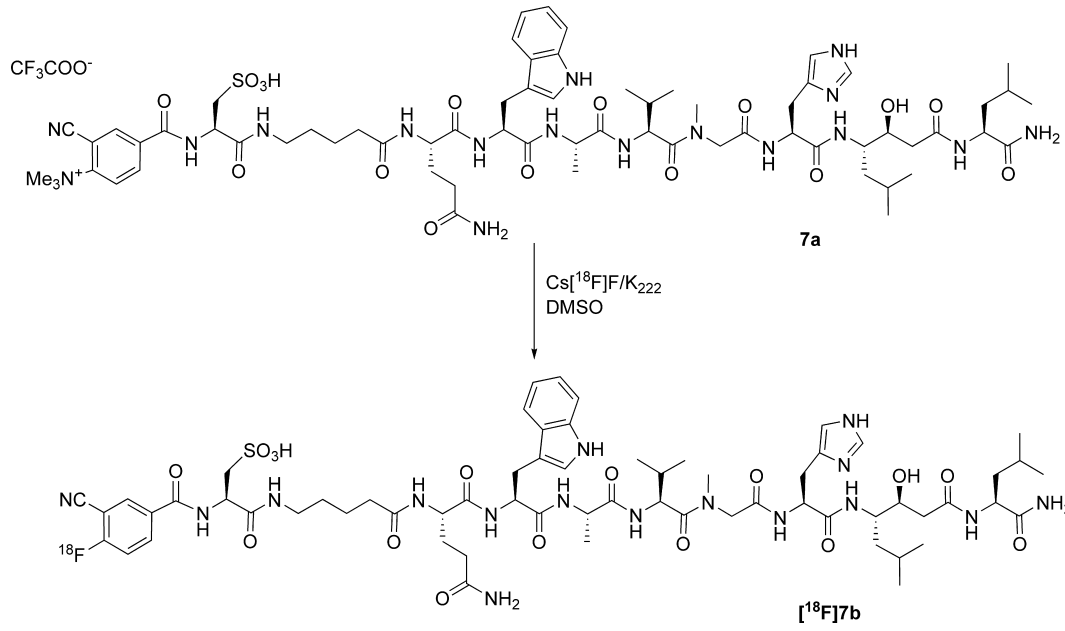

Figure 2. Synthetic scheme for the generation of **[^{18}F]7b**.

Table 2. ^{18}F-Radiolabeling of TMA-Based Bombesin Peptides[a]

entry	compd.	temp (°C)	reaction time	^{18}F-incorporation (% HPLC)	d. c. RCY (%)
1	**1a**	50	15 min	69 ± 8 (n = 2)	n.d.
2	**2a**	70	15 min	69 ± 7 (n = 3)	18%
3	**3a**	70	15 min	56 ± 1 (n = 2)	21%
4	**4a**	50	15 min	29 ± 6 (n = 2)	4%
5	**5a**	70	15 min	63 (n = 1)	n.d.
6	**6a**	70	15 min	51 ± 5 (n = 2)	20%
7	**7a**	70	15 min	26 ± 2 (n = 2)	n.d.
8	**7a**	90	15 min	74 ± 3 (n = 2)	14%

[a] ^{18}F-labeling was carried out in DMSO (150 μL) with a 2 mg amount of precursor using Cs_2CO_3 as the base. Conversion was determined from a radio-HPLC chromatogram representing the percentage of radioactivity area of the product related to the total radioactivity area. n.d.: not determined.

***In Vitro* Receptor Binding Affinity and Distribution Coefficient log $D_{7.4}$.** The binding affinities of the novel bombesin analogues toward the human GRPR were determined by a scintillation proximity assay using human GRPR-transfected cell membranes. All synthesized bombesin analogues showed nanomolar binding affinities toward human GRPR (Table 3). For peptide **1b** with Ava as a linker, a binding affinity of 28 nM was obtained. However, the attachment of one more hydrophilic amino acid Arg to the linker resulted in a more than 100 times higher binding affinity (Table 3; **2b**, K_i = 0.2 nM). Encouraged by these results, Arg-βAla and Arg-Ser were tested with the same peptide sequence. In both cases, binding affinities in the high nanomolar range were obtained (Table 3. K_i = 6.2 nM and 6.4 nM for **3b** and **4b**, respectively). With Arg-Ava as the linker, further investigations on the peptide sequence by exchanging Sta with FA01010 demonstrated that Sta was superior to FA01010 in terms of binding affinity. While the difference between the methylated His or the methylated Gly was not so obvious in this study (Table 3, K_i = 0.2 nM and 0.7 nM for **2b** and **6b**, respectively), the methylated Gly BBS analogue showed a 10-fold higher binding affinity than its corresponding methylated His analogue in our previous studies (*36*). Thus, the most promising peptide sequence toward human GRPR in this study was Gln-Trp-Ala-Val-NMeGly-His-Sta-Leu-NH_2. Further derivatization of the optimal peptide sequence by exchanging the linker Arg-Ava to Ala(SO_3H)-Ava yielded picomolar affinity (K_i = 0.1 nM) of this peptide candidate toward the GRPR.

The log $D_{7.4}$ values of the peptides are in the similar range (−0.8 to −1.4) with respect to the different polar linkers of Arg-Ava, Arg-βAla, or Ala(SO_3H)-Ava and peptide sequences. Compounds **6b** and **7b** gave the same log $D_{7.4}$ value of −1.1 (Table 3).

Biodistribution Studies. The biodistributions of **[^{18}F]6b** and **[^{18}F]7b** were carried out using nude mice bearing subcutaneous human prostate tumors (PC-3). The results are shown in Table 4. For **[^{18}F]6b**, the tumor uptake was 2.36 ± 0.47% ID/g at 30 min after injection, which slightly decreased to 1.80 ± 1.56% ID/g at 60 min postinjection and 1.61 ± 0.23% ID/g at 4 h postinjection. The binding of **[^{18}F]6b** toward GRPR could be blocked by around 40% in the tumor and 60% in the pancreas at 60 min postinjection, indicating that a large part of the tracer uptake in the tumor and pancreas can be attributed to specific GRPR binding. Besides the tumor and pancreas, the highest accumulation of the radiotracer **[^{18}F]6b** was found in excretory organs such as the gallbladder, intestine, kidneys, and liver. Activity uptake in the gall bladder and intestine was much higher than that in the kidneys, suggesting a preponderance of hepatobiliary elimination versus renal excretion. In the intestine there was, however, no differentiation between GRPR binding naturally expressed in intestinal smooth muscle cells and intestinal content. Furthermore, the increasing uptake in bone over time indicated that ^{18}F-fluoride represents a major radio metabolite. In comparison to the bombesin analogue **[^{18}F]6b**, the radiotracer **[^{18}F]7b** was characterized by clearly superior tumor targeting. The absolute tumor accumulation was more than double reaching about 5% ID/g at 30 min p.i., and this uptake was persistent over four hours. Tracer uptake in the GRPR-rich pancreas amounted to almost 50% ID/g at 1 h postinjection. Uptake of compound **[^{18}F]7b** in the tumor and pancreas was highly specific since accumulation could be effectively blocked by coadministration of unlabeled bombesin (83% and 97% specificity, respectively). The biodistribution of **[^{18}F]7b** was also characterized by a fast blood clearance leading to favorable tumor-to-blood ratios of >30 at 60 min postinjection and later. In line with compound **[^{18}F]6b**, the bombesin analogue **[^{18}F]7b** was mainly excreted via hepatobiliary pathways resulting in bile-to-tumor ratios of >20. It is clear that at physiological pH, Arg and Ala(SO_3H) become positively and negatively

Table 3. Sequence of the Tested Bombesin Analoguess and Their Binding Affinity (K_i) Towards Human GRPR and Lipophilicities (log $D_{7.4}$)

peptide	linker	peptide sequence	IC_{50} (nM)	K_i (nM)	log $D_{7.4}$
1b	Ava	Gln-Trp-Ala-Val-Gly-His(3Me)-Sta-Leu-NH_2	97	28.0	n.d.[a]
2b	Arg-Ava	Gln-Trp-Ala-Val-Gly-His(3Me)-Sta-Leu-NH_2	0.8	0.2	−1.4
3b	Arg-βAla	Gln-Trp-Ala-Val-Gly-His(3Me)-Sta-Leu-NH_2	25	6.2	−1.0
4b	Arg-Ser	Gln-Trp-Ala-Val-Gly-His(3Me)-Sta-Leu-NH_2	69	6.4	n.d.[a]
5b	Arg-Ava	Gln-Trp-Ala-Val-Gly-His-FA01010-Leu-NH_2	6.0	1.5	−0.8
6b	Arg-Ava	Gln-Trp-Ala-Val-NMeGly-His-Sta-Leu-NH_2	2.7	0.7	−1.1
7b	Ala(SO_3H)-Ava	Gln-Trp-Ala-Val-NMeGly-His-Sta-Leu-NH_2	0.4	0.1	−1.1

[a] n.d.: not determined.

Table 4. Biodistribution of [^{18}F]6b and [^{18}F]7b in Nude Mice Bearing PC-3 Xenografts at Different Times p.i.[a]

tissue	0.5 h	1.0 h	1.0 h[b]	2.0 h	4.0 h
	3-CN-4-[^{18}F]Fluoro-Bz-Arg-Ava--Gln-Trp-Ala-Val-NMeGly-His-Sta-Leu-NH_2 (**[^{18}F]6b**)				
blood	0.93 ± 0.28	0.27 ± 0.04	0.32 ± 0.02	0.15 ± 0.03	0.21 ± 0.16
kidneys	2.92 ± 0.44	1.35 ± 0.11	1.46 ± 0.13	0.87 ± 0.18	1.10 ± 0.74
liver	3.26 ± 0.60	1.23 ± 0.17	1.35 ± 0.24	0.61 ± 0.06	0.72 ± 0.39
spleen	0.56 ± 0.10	0.28 ± 0.06	0.21 ± 0.04	0.17 ± 0.03	0.19 ± 0.09
lung	1.13 ± 0.27	0.48 ± 0.16	0.69 ± 0.19	0.28 ± 0.04	0.29 ± 0.14
muscle	0.29 ± 0.03	0.15 ± 0.05	0.20 ± 0.08	0.07 ± 0.02	0.07 ± 0.02
gallbladder	82.06 ± 19.10	81.38 ± 52.78	132.49 ± 73.06	64.13 ± 34.51	58.81 ± 20.33
bone	4.36 ± 0.34	4.64 ± 0.42	4.04 ± 0.35	7.80 ± 0.89	7.98 ± 1.58
intestine	12.90 ± 1.53	6.76 ± 5.84	9.66 ± 2.06	8.92 ± 1.83	11.04 ± 0.10
pancreas	2.57 ± 0.03	1.30 ± 0.59	0.51 ± 0.10	1.26 ± 0.09	1.54 ± 0.47
adrenals	0.86 ± 0.11	0.65 ± 0.40	0.70 ± 0.39	0.26 ± 0.07	0.44 ± 0.04
tumor	2.36 ± 0.47	1.80 ± 1.56	1.11 ± 0.30	1.28 ± 0.27	1.61 ± 0.23
T/blood	2.59 ± 0.24	6.82 ± 6.19	3.46 ± 0.97	8.55 ± 2.29	11.37 ± 7.23
	3-CN-4-[^{18}F]Fluoro-Bz-Ala(SO_3H)-Ava--Gln-Trp-Ala-Val-NMeGly-His-Sta-Leu-NH_2 (**[^{18}F]7b**)				
blood	0.52 ± 0.13	0.16 ± 0.01	0.48 ± 0.21	0.10 ± 0.01	0.09 ± 0.02
kidneys	3.00 ± 2.20	0.66 ± 0.05	1.16 ± 0.48	0.42 ± 0.04	0.36 ± 0.12
liver	1.37 ± 0.18	0.50 ± 0.13	1.76 ± 0.56	0.37 ± 0.08	0.34 ± 0.12
spleen	0.63 ± 0.23	0.30 ± 0.02	1.32 ± 0.49	0.31 ± 0.12	0.28 ± 0.35
lung	0.51 ± 0.18	0.28 ± 0.03	23.04 ± 6.99	0.20 ± 0.03	0.11 ± 0.02
muscle	0.10 ± 0.01	0.05 ± 0.00	0.14 ± 0.05	0.06 ± 0.02	0.04 ± 0.02
gallbladder	230.12 ± 88.04	102.63 ± 74.25	137.40 ± 106.91	66.80 ± 54.48	137.22 ± 109.07
bone	0.84 ± 0.08	1.52 ± 0.22	1.61 ± 0.35	1.60 ± 0.12	1.75 ± 0.23
intestine	11.54 ± 2.91	11.75 ± 2.86	11.81 ± 5.52	9.88 ± 2.76	12.14 ± 2.38
pancreas	41.95 ± 5.37	49.01 ± 8.07	1.63 ± 0.65	34.49 ± 4.96	30.70 ± 5.14
adrenals	4.49 ± 1.27	3.45 ± 0.24	0.97 ± 0.10	2.31 ± 0.73	1.17 ± 0.19
tumor	4.67 ± 0.04	4.88 ± 0.36	0.84 ± 0.26	5.40 ± 0.58	5.16 ± 1.32
T/blood	9.50 ± 2.81	30.59 ± 1.05	1.80 ± 0.29	51.77 ± 2.52	55.03 ± 0.56

[a] Results are expressed as % ID/g ± SD (n = 3). [b] Blockade study: animals received 100 μg of bombesin coinjected with the radiolabeled compound.

charged, respectively. Although bearing the same binding peptide sequence and having the same lipophilicity, the charge of the linker obviously plays a major role in *in vivo* pharmacokinetics. Similar results have been reported for different bombesin analogues labeled with ^{99m}Tc, ^{64}Cu, and ^{68}Ga (*43*−*46*) suggesting that a negatively charged linker has a profound effect on the biodistribution profile of bombesin analogues. Furthermore, significant *in vivo* defluorination of compound **[^{18}F]7b** was not evident from the % ID/g values in the bone.

PET Study. The excellent tumor targeting of compound **[^{18}F]7b** encouraged us to test the capability of **[^{18}F]7b** to visualize PC3 tumors *in vivo* by PET imaging. Two nude mice bearing subcutaneous PC-3 tumor xenografts next to the right shoulder were scanned from 60 to 105 min p.i. in a dedicated small-animal PET system both under baseline and blockade conditions (Figure 3). The tumor was clearly imaged under baseline conditions, whereas no tumor visualization was possible under blocking conditions. As expected from the *ex vivo*

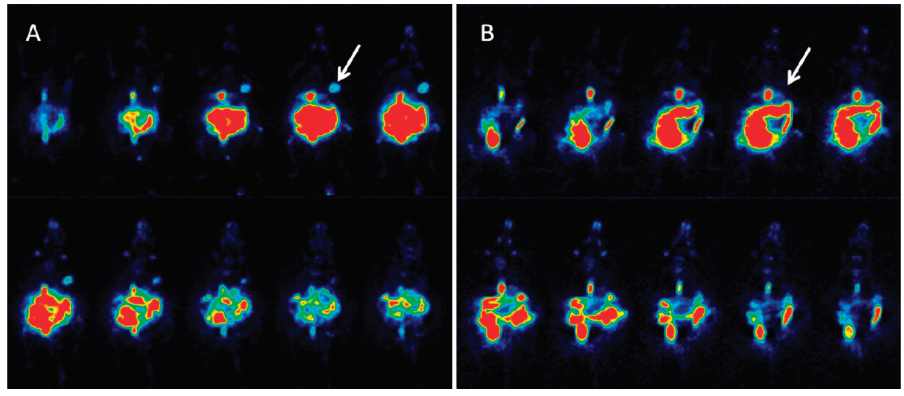

Figure 3. Series of horizontal whole body slices (ventral to dorsal) of two nude mice bearing subcutaneous PC-3 tumor xenografts next to the right shoulder (see arrows), which were injected with **[^{18}F]7b** and scanned from 60 to 105 min p.i., both under baseline (left) and blockade conditions (right). Image data were normalized to the injected dose per body weight.

biodistribution study, highest activity concentrations in the *in vivo* PET data were identified in the gall bladder and the intestine. The pancreas could not be visualized by PET due to the proximity to the bowel. Postmortem tissue sampling of these mice after PET scanning (107 min p.i.) confirmed the data which were obtained in the biodistribution study (Table 4). The absolute radioactivity uptake values in the GRPR-rich tissues were 5% ID/g for the tumor and 30% ID/g for the pancreas. The specificity of uptake amounted to 85% and 95% for the tumor and pancreas, respectively.

CONCLUSIONS

The direct ^{18}F-fluorination protocol developed previously in our lab was successfully applied to new bombesin peptide analogues. Amino acids such as histidine, tryptophan, and arginine, and non-natural amino acids such as statine and cysteine sulfonic acid in the peptide sequence did not require any protection group during radiosynthesis. *In vitro* binding studies of the synthesized bombesin analogues revealed nanomolar binding affinities toward the human GRPR. The most promising peptide sequence in this study was Gln-Trp-Ala-Val-NMeGly-His-Sta-Leu-NH_2. Two bombesin analogues containing this optimal peptide sequence, one positively (Arg-Ava) and another one negatively (Ala(SO_3H)-Ava) charged, were radiolabeled with ^{18}F and evaluated in biodistribution studies in nude mice bearing PC-3 xenografts. The comparative *in vivo* analysis of **[^{18}F]6b** and **[^{18}F]7b** showed superior characteristics for **[^{18}F]7b** with respect to (1) the total tracer accumulation in tumors and the GRPR-rich pancreas, (2) the specificity of uptake in target tissues, and (3) the target to nontarget ratios. The positive *in vivo* results of **[^{18}F]7b** suggest that this compound represents a promising PET tracer candidate for the visualization of GRPR-positive tumors in humans.

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