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Stabilized ^{111}In -Labeled sCCK8 Analogues for Targeting CCK2-Receptor Positive Tumors: Synthesis and Evaluation

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Radiolabeled cholecystokinin-8 (CCK8) peptide analogues can be used for peptide receptor radionuclide imaging and therapy for tumors expressing CCK2/gastrin receptors. Earlier findings indicated that sulfated CCK8 (sCCK8, Asp-Tyr(OSO₃H)-Met-Gly-Trp-Met-Asp-Phe-NH₂) may have better characteristics for peptide receptor radionuclide therapy (PRRT) than gastrin analogues. However, sCCK8 contains an easily hydrolyzable sulfated tyrosine residue and two methionine residues which are prone to oxidation. Here, we describe the synthesis of stabilized sCCK8 analogues, resistant to hydrolysis and oxidation. Hydrolytic stability was achieved by replacement of the Tyr(OSO₃H) moiety by a robust isosteric sulfonate, Phe(*p*-CH₂SO₃H). Replacement of methionine by norleucine (Nle) or homopropargylglycine (HPG) avoided undesired oxidation side-reactions. The phenylalanine analogue Phe(*p*-CH₂SO₃H) of L-tyrosine, synthesized by a modification of known synthetic routes, was incorporated in three peptides: sCCK8[Phe²(*p*-CH₂SO₃H),Met^{3,6}], sCCK8[Phe²(*p*-CH₂SO₃H),Nle^{3,6}], and sCCK8[Phe²(*p*-CH₂SO₃H),HPG^{3,6}]. All peptides were *N*-terminally conjugated with the macrocyclic chelator DOTA (1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid) and radiolabeled with In-111. In vitro binding assays on CCK2R-expressing HEK293 cells revealed that all three peptides showed specific binding and receptor-mediated internalization, with binding affinity values (IC₅₀) in the nanomolar range. In vitro oxidation studies demonstrated that peptides with Nle or HPG indeed were resistant to oxidation. In vivo targeting studies in mice with AR42J tumors showed that tumor uptake was highest for ^{111}In -DOTA-sCCK8 and ^{111}In -DOTA-sCCK8[Phe²(*p*-CH₂SO₃H),Nle^{3,6}] (4.78 ± 0.64 and 4.54 ± 1.15%ID/g, respectively, 2 h p.i.). The peptide with the methionine residues replaced by norleucine (^{111}In -DOTA-sCCK8[Phe²(*p*-CH₂SO₃H), Nle^{3,6}]) showed promising in vivo characteristics and will be further investigated for radionuclide imaging and therapy of CCK2R-expressing tumors.

INTRODUCTION

Cholecystokinin (CCK), a functional peptide hormone in the gastrointestinal tract and the brain, mediates a variety of hormonal and neuromodulatory functions mediated by two receptors, CCK1R and CCK2R (1). In addition, a splice variant of the CCK2R was identified, CCK2i4svR, which is expressed at relatively low levels in human colorectal cancer and in pancreatic cancer, but not in normal tissue (2). All three receptors belong to the superfamily of G-protein coupled receptors. Several tumor types, such as small cell lung cancers (SCLC) and medullary thyroid carcinomas (MTC), abundantly express CCK2/gastrin receptors (CCK2R) (3).

Since both gastrin and CCK8 display a high affinity for these receptors (3), radiolabeled analogues of these peptides may be considered for peptide receptor imaging and peptide receptor radionuclide therapy (PRRT). We have previously shown that sCCK8 (Asp-Tyr(OSO₃H)-Met-Gly-Trp-Met-Asp-Phe-NH₂) has good characteristics for PRRT compared to gastrin analogues (4). Sulfated CCK8 has affinity for both the CCK1 and CCK2 receptor, and shows better CCK2R

targeting characteristics as compared to nonsulfated CCK8 (nsCCK8). Behr et al. found that minigastrin analogues showed extremely high kidney uptake, whereas CCK8 exhibited low kidney uptake (5). The high kidney uptake was shown to be related to the pentaglutamate sequence of minigastrin, and was found to be reduced by coinjection of poly-Glu-containing peptides (6, 7). Unlike gastrin, sCCK8 includes a sulfated tyrosine (Tyr) residue that is easily hydrolyzable. Moreover, an Fmoc-Tyr(SO₃H)-OH building block is difficult to use in solid-phase peptide synthesis (SPPS), as it is acid labile (8). Another pitfall of sCCK8 is the presence of two methionines (Met) that are prone to oxidation to sulfoxides during radiolabeling and potentially also in vivo, leading to loss of receptor binding (9, 10). Therefore, we aimed to develop new and stabilized sCCK8 analogues, resistant to both hydrolysis and oxidation. To this end, we envisaged replacement of the Tyr sulfate moiety by a robust isosteric sulfonate. Since sulfonate isosteres of tyrosine sulfate are not commercially available, synthetic preparation of the phenylalanine analogue Phe(*p*-CH₂SO₃H) from L-tyrosine was required, to afford a Phe(*p*-CH₂SO₃H) building block that can be incorporated in peptide sequences under normal coupling conditions in SPPS without additional side chain protection (11). Second, to prevent oxidation, two sCCK8 analogues were designed with the Met residues replaced by norleucine (Nle) or homopropargylglycine (HPG) (12, 13). All peptides were *N*-terminally conjugated with the macrocyclic chelator DOTA (1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid) to allow radiola-

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beling. Finally, the three sCCK8 analogues were characterized both in vitro and in vivo and compared to DOTA-sCCK8.

EXPERIMENTAL PROCEDURES

Synthesis of Fmoc-Phe(p-CH₂SO₃Na)-OH. *General Methods.* Solvents were distilled from appropriate drying agents prior to use and stored under nitrogen. Chemicals were purchased from Sigma-Aldrich and used as received, unless stated otherwise. Reactions were carried out under inert atmosphere of dry nitrogen or argon. Standard syringe techniques were applied for the transfer of dry solvents and air- or moisture-sensitive reagents. Reactions were followed, and *R_F* values were obtained using thin-layer chromatography (TLC) on silica gel-coated plates (Merck 60 F254) with the indicated solvent mixture. Detection was performed with UV-light. NMR spectra were recorded on a Bruker DMX 300 (300 MHz) spectrometer in CDCl₃ solutions (unless otherwise reported). Chemical shifts are given in ppm with respect to tetramethylsilane (TMS) as internal standard. Coupling constants are reported as *J*-values in Hz. Mass was determined by LCQ measurement. Column or flash chromatography was carried out using ACROS silica gel (0.035–0.070 mm, and ca. 6 nm pore diameter).

N-tert-Butyloxycarbonyl-L-tyrosine Methyl Ester (1). 30.0 g (165.55 mmol) L-tyrosine was dissolved in 1.1 L MeOH and cooled on an ice bath. 15.65 mL (215.24 mmol, 1.3 equiv) SOCl₂ was added dropwise and the reaction mixture was refluxed at 75 °C overnight. The solvent was evaporated, and the crude product (38.2 g white solid) was used in the next step. ¹H NMR (D₂O) δ 3.25 (dq, 2H, Ar-CH₂-CH), 3.86 (s, 3H, -OCH₃), 4.39 (t, 1H, α-CH), 6.91–7.21 (dd, 4H, Ar-H).

Next, 38.2 g crude product was dissolved in 500 mL dioxane/H₂O (1/1). 34.65 g (412.5 mmol, 3 equiv) NaHCO₃ and 43.21 g (198 mmol, 1.2 equiv) Boc₂O were added and the mixture was stirred at RT for 2 h. Dioxane was removed in vacuo and EtOAc was added. The organic layer was washed with water and brine, dried on MgSO₄, filtered, and concentrated to yield an orange oil, that crystallized overnight, yielding 44.52 g (150.7 mmol, 91% over 2 steps) of white crystals. *R_F* (EtOAc/heptane 1/1) 0.49; mp 95 °C; ¹H NMR (CDCl₃) δ 1.42 (s, 9H, -C(CH₃)₃), 3.02 (dq, 2H, Ar-CH₂-CH), 3.71 (s, 3H, -OCH₃), 4.53 (m, 1H, α-CH), 4.98 (m, 1H, -NH), 5.50 (bs, 1H, -OH), 6.72–6.99 (dd, 4H, Ar-H).

N-tert-Butyloxycarbonyl-O-trifluoromethylsulfonyl-L-tyrosine Methyl Ester (2). (14, 15) 1.0 g (3.39 mmol) of compound **1** was dissolved in 40 mL CH₂Cl₂ and cooled on an ice bath. Then, 720 μL (4.23 mmol, 1.25 equiv) DIPEA and 1.45 g (4.23 mmol, 1.25 equiv) phenyltriflimide were added, before stirring the mixture at RT overnight. Solvent was removed in vacuo, and the remaining colorless oil was dissolved in 40 mL EtOAc. The solution was washed with H₂O, 0.1 M HCl, and brine, dried on MgSO₄, and concentrated. The crude product was purified by flash column chromatography (EtOAc/heptane 15/85). Yield: 1.095 g (2.56 mmol, 79%) white crystals (after drying in vacuo). *R_F* (EtOAc/heptane 1/1) 0.65; mp 48 °C (lit. 48–49 °C); ¹H NMR (CDCl₃) δ 1.41 (s, 9H, -C(CH₃)₃), 3.00–3.20 (dq, 2H, Ar-CH₂-CH), 3.71 (s, 3H, -OCH₃), 4.61 (m, 1H, α-CH), 5.00 (m, 1H, -NH), 7.22 (s, 4H, Ar-H). MS(ESI-TOF): 392.0 (M+H⁺), calcd 392 (M+H⁺).

N-tert-Butyloxycarbonyl-p-vinyl-L-phenylalanine Methyl Ester (3). 1.43 g (30.4 mmol, 1.2 equiv) of LiCl in a 250 mL flask was flame-dried in vacuo. 100 mL of dry DMF, 10.95 g (25.6 mmol) of compound **2**, and 8.23 mL (28.2 mmol, 1.1 equiv) tri-*n*-butyl vinyl tin were added. The mixture was stirred at RT for 10 min. (PPh₃)₂PdCl₂ (0.91 g, 3 mol %) was added, and the mixture was heated up to 90 °C and stirred overnight in sealed flask. Water was added and the product was extracted

with EtOAc/heptane (1/1, 3 × 20 mL). Organic layers were dried on MgSO₄ and concentrated in vacuo. The crude product was purified by flash column chromatography (CH₂Cl₂, EtOAc/heptane 1/1). Yield: 6.12 g (19.8 mmol, 68%) colorless oil. *R_F* (EtOAc/heptane 1/3) 0.42; ¹H NMR (CDCl₃) δ 1.42 (s, 9H, -C(CH₃)₃), 3.05–3.14 (m, 2H, Ar-CH₂-CH), 3.71 (s, 3H, -OCH₃), 4.58 (m, 1H, α-CH), 4.97 (m, 1H, -NH), 5.22 (d, *J* = 10.88, 1H, CH=CHH), 5.71 (d, *J* = 17.61, 1H, CH=CHH), 6.68 (dd, *J* = 17.61, 1H, CH=CH₂), 7.07 (d, *J* = 9.17, 2H, Ar-H), 7.33 (d, *J* = 8.06, 2H, Ar-H).

N-tert-Butyloxycarbonyl-(p-hydroxymethyl)-L-phenylalanine Methyl Ester (4). (11) Ozone was bubbled through a solution of the vinyl compound **3** (4.26 g, 13.96 mmol) in 60 mL MeOH at -60 °C until the solution turned blue. The mixture was stirred under N₂ for 10 min at -60 °C and 10.3 mL (139.6 mmol, 10 equiv) of Me₂S was added. The mixture was stirred for 1 h at -60 °C. Then, NaBH₄ (2.64 g, 69.8 mmol, 5 equiv) was added. The mixture was stirred at -60 °C for 10 min, then allowed to warm to 0 °C and stirred for an additional 45 min. Saturated NH₄Cl (60 mL) was added, and the product was extracted with EtOAc (2 × 100 mL). The organic layers were dried on MgSO₄, filtered, and concentrated to give a yellow-brown oil. The crude product was purified by flash column chromatography (EtOAc/heptane 2/3 → EtOAc 100%). Yield: 8.18 g (26.5 mmol, 50.4% over 2 steps, 74%) of white/yellow crystals. *R_F* (EtOAc/heptane 1/1) 0.31; mp 81 °C; ¹H NMR (CDCl₃) δ 1.42 (s, 9H, -C(CH₃)₃), 3.08 (dd, *J* = 10.36, 2H, Ar-CH₂-CH), 3.72 (s, 3H, -OCH₃), 4.57 (m, 1H, α-CH), 4.67 (d, *J* = 5.85, 2H, -CH₂OH), 4.97 (m, 1H, -NH), 7.11 (d, *J* = 8.02, 2H, Ar-H), 7.29 (d, *J* = 8.02, 2H, Ar-H). MS(ESI-TOF): 332.2 (M+Na⁺), calcd 332.16 (M+H⁺).

(p-Chloromethyl)-L-phenylalanine Methyl Ester (5). (16) Compound **4** (10.03 g) was dissolved in 350 mL of CH₂Cl₂ and SOCl₂ (25–30 equiv) was added dropwise, before refluxing overnight. After evaporation to dryness, the remaining solid was rinsed twice with Et₂O and dried. The product was used in the next step without further purification. Yield: 4.59 g (62%) of an off-white solid. *R_F* (CH₂Cl₂/MeOH/AcOH 9/1/0.1) 0.34; mp 178 °C (lit. 180 °C); ¹H NMR (MeOD) δ 3.15–3.32 (dq, 2H, Ar-CH₂-CH), 3.81 (s, 3H, -OCH₃), 4.33 (m, 1H, α-CH), 4.65 (s, 2H, -CH₂Cl), 7.28 (d, 2H, Ar-H), 7.43 (d, 2H, Ar-H); MS(ESI-TOF): 228.0 (M+H⁺), calcd 228.07 (M+H⁺).

(p-Sulfomethyl)-L-phenylalanine (6). (11, 16) Compound **5** (4.59 g, 20.2 mmol) was dissolved in 200 mL H₂O and 20.3 g (161.6 mmol, 8 equiv) Na₂SO₃ was added. The mixture was refluxed at 100 °C for 3 h. The solvent was removed in vacuo and the crude product was washed with EtOH, yielding 32.25 g of a white solid after drying in vacuo. The crude product was used in the next step. ¹H NMR (D₂O) δ 3.08–3.12 (dq, 2H, Ar-CH₂-CH), 3.97 (m, 1H, α-CH), 4.20 (s, 2H, -CH₂SO₃Na), 7.33 (d, 2H, Ar-H), 7.43 (d, 2H, H). MS(ESI-TOF): 280.3 (M+H⁺), calcd 280.3 (M+H⁺).

N-Fluorenyl-9-methoxycarbonyl-(p-sulfomethyl)-L-phenylalanine (7). (16) The sulfonate product **6** (5 mmol) was dissolved in 40 mL H₂O, cooled on an ice-bath and pH was set to 9.0 with 0.1 M NaOH. Next, 2.11 g (6.25 mmol, 1.25 equiv) Fmoc-OSu was dissolved in 50 mL dioxane and added to the mixture. The mixture was stirred on an ice-bath for 30 min, then at RT for 6 h while maintaining the pH between 8.5 and 9.0 with an automatic titrator filled with 0.1 M NaOH. Dioxane was evaporated and the excess Fmoc-OSu was extracted with diethyl ether. The aqueous solution was acidified to pH 5 with 1 M HCl and concentrated to dryness. The crude product was dissolved in methanol, the insoluble material was removed by filtration, and the methanol was evaporated. The residue was dissolved in water and was washed with ethyl acetate before concentration. This product was purified by column chroma-

tography (MeCN/H₂O 9/1), yielding 1.04 g of white solid (2.07 mmol, 41.4% over 2 steps). *R*_F (ACN/H₂O 9/1) 0.24; mp 185 °C (lit. 184 °C); ¹H NMR (MeOD) δ 2.93–3.18 (dq, 2H, Ar-CH₂-CH), 3.99 (m, 1H, α-CH), 4.20 (s, 2H, -CH₂SO₃Na), 7.15–7.41 (m, 8H, Ar-H (Fmoc)), 7.61 (d, 2H, Ar-H), 7.78 (d, 2H, Ar-H); MS(ESI-TOF): 480.1 (M–H⁺), calcd 480.11 (M–H⁺).

Synthesis of DOTA–Peptides. Peptides were synthesized on a peptide synthesizer (Peptide Synthesizer SP 4000-LAB, Labortec AG) by applying the Fmoc-strategy. In all coupling steps, DIPCDI and HOBt were used, except for coupling of Fmoc-Phe(*p*-CH₂SO₃H) and DOTA(*t*-Bu)₃, which was performed in the presence of HBTU and DIPEA. After completion of the synthesis of the peptide on the resin, the peptide was cleaved off by stirring in TFA/ethanedithiol (EDT)/triisopropyl silane (TIS)/H₂O (92.5/2.5/2.5/2.5) for 3–5 h. The mixture was filtered, the filtrate centrifuged with Et₂O. The precipitated peptide was washed with Et₂O and centrifuged several times. Formation of the correct product was corroborated with mass spectrometry (ESI-TOF).

The remaining *t*-Bu protecting groups of the chelator were removed by stirring the product in TFA in the presence of a few drops of concentrated HCl, leading to complete removal of all protecting groups within 1 h. Three peptides were synthesized: DOTA-sCCK8[Ph²(*p*-CH₂SO₃H)] (DOTA-D-Asp-Phe(CH₂SO₃H)-Met-Gly-Trp-Met-Asp-Phe-NH₂), DOTA-sCCK8[Ph²(*p*-CH₂SO₃H), Nle^{3,6}] (DOTA-D-Asp-Phe(CH₂SO₃H)-Nle-Gly-Trp-Nle-Asp-Phe-NH₂), and DOTA-sCCK8[Ph²(*p*-CH₂SO₃H), HPG^{3,6}] (DOTA-D-Asp-Phe(CH₂SO₃H)-HPG-Gly-Trp-HPG-Asp-Phe-NH₂).

Peptides were analyzed by analytical HPLC (Alltima C18 5 μm, 3.2 × 150 mm, Alltech, USA) with 5–75% ACN 0.1% TFA in H₂O 0.1% TFA as an eluent. DOTA-sCCK8[Ph²(*p*-CH₂SO₃H)] and DOTA-sCCK8[Ph²(*p*-CH₂SO₃H), Nle^{3,6}] were further purified by SP RP-HPLC (ReproSil 100 C18 250 mm × 10 mm, Dr. A. Maisch HPLC-GmbH, Germany), using ACN 0.1% TFA (5–75% over 30 min) in H₂O 0.1% TFA as an eluent. Peptide-containing fractions (detected by UV-light) were lyophilized and analyzed by HPLC and mass spectrometry. DOTA-sCCK8[Ph²(*p*-CH₂SO₃H)] = 1525.7 (M–H⁺), calcd = 1526.55. DOTA-sCCK8[Ph²(*p*-CH₂SO₃H), Nle^{3,6}] = 1489.8 (M–H⁺) calcd = 1490.64. DOTA-sCCK8[Ph²(*p*-CH₂SO₃H), HPG^{3,6}] = 1481.8 (M–H⁺) calcd = 1482.58.

DOTA-sCCK8 was prepared as described before (17).

Radiolabeling. The DOTA-conjugated peptides were radio-labeled with ¹¹¹InCl₃ (Covidien, Petten, The Netherlands) in 0.25 M ammonium acetate buffer, pH 5.0, for 30 min at 95 °C. Labeling efficiency and radiochemical purity were checked by HPLC (1100 series LC system, Agilent Technologies, Palo Alto, CA, USA, RP-C18 column 5 μm, 4.6 × 250 mm) with a gradient of H₂O containing 0.1% TFA and ACN containing 0.1% TFA, and ITLC on ITLC-SG strips (Pall Life Sciences, Milford, MA, USA) with 0.1 M NH₄OAc/0.1 M EDTA (1/1 v/v) (*R*_F labeled peptide = 0, *R*_F unbound ¹¹¹In = 1), as well as MeOH/3.5% NH₃ (1/1 v/v) (*R*_F colloid = 0, *R*_F unbound ¹¹¹In or labeled peptide >0.8) as eluents. The radiochemical purities of peptides used in the studies described here were always above 95%. Maximum specific activity was 5.5 GBq/μmol.

Determination of Partition Coefficient (log P). To determine the lipophilicity of the radiolabeled peptides, approximately 60 000 dpm ¹¹¹In-DOTA-peptide was diluted in 3 mL PBS. An equal volume of 1-octanol was added to obtain a binary system. The system was vortexed for 10 s and mixed more gently for another 4 min. The two layers were separated by centrifugation (3000 g, 5 min). Three samples of 250 μL were taken of both layers and the radioactivity was determined in a 3" well-type gamma-counter (Wallac 1480-Wizard 3, Perkin-Elmer, Boston,

MA, USA). The log P value was calculated with the following formula:

$$\text{Log } P_{\text{octanol/water}} = \log(\text{cpm}_{\text{octanol}}/\text{cpm}_{\text{water}})$$

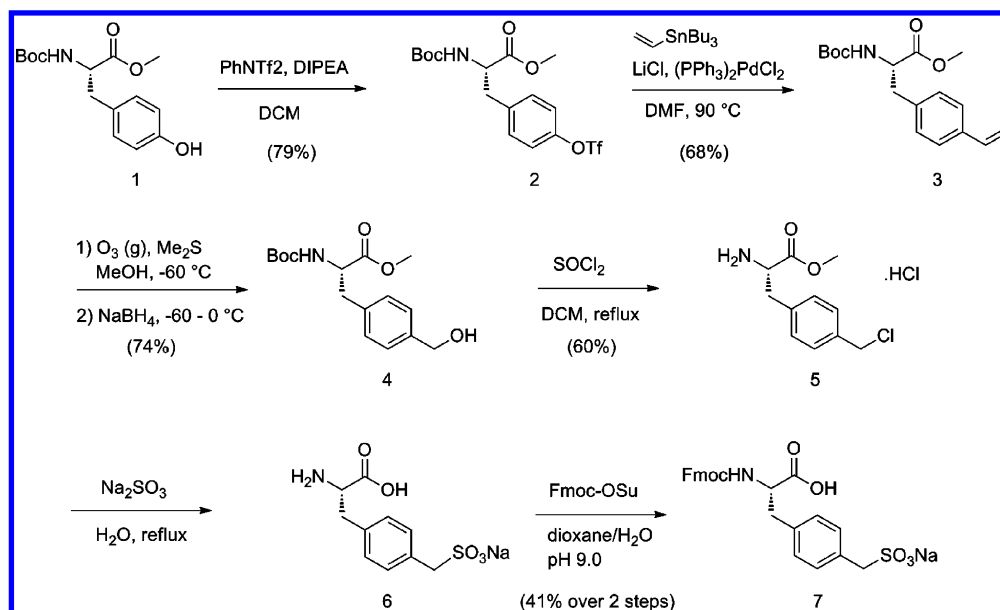
Oxidation Experiments. To study the susceptibility of the peptide analogues to oxidation, the ¹¹¹In-labeled peptides were incubated with oxidation buffer (H₂O₂/acetic acid/NH₄OAc 1/3/36) for 10 min at RT. Before and after incubation, the peptides were analyzed using RP-HPLC.

Stability Experiments. To study the stability of the peptide analogues, the ¹¹¹In-labeled peptides were incubated in human serum for 4 h at 37 °C. The samples were centrifuged at 3000 g for 5 min. The plasma was removed and mixed with acetonitrile. Before and after incubation, the peptides were analyzed using RP-HPLC.

Internalization Experiments. The internalization of the radiolabeled DOTA–peptides was investigated using HEK293-CCK2R cells (obtained from M. Hellmich, Univ. of Texas Medical Branch, Galveston, USA). Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) and supplemented with 10% fetal calf serum (FCS) and G418 (400 μg/mL). Cells were grown in a humidified atmosphere with 5% CO₂ at 37 °C. Cells were grown to confluency in 6-well plates. Radiolabeled peptide was added at a concentration of 4.4 × 10^{–2} nM (12 000 dpm/well). Cells were incubated for 0.5–2 h at either 0 or 37 °C, medium was removed, and cells were washed with ice-cold binding buffer (DMEM supplemented with 0.5% w/v bovine serum albumin (BSA)). To remove receptor-bound radiolabeled peptide, cells were incubated with acid buffer (0.1 M acetic acid, 154 mM NaCl, pH 2.0) for 10 min at 0 °C. After washing the cells twice with ice-cold PBS, the internalized fraction was determined by counting the cells in a gamma counter. The receptor-bound activity was also determined by counting the acid wash fractions.

Determination of IC₅₀ Values. The 50% inhibitory concentration (IC₅₀) of the peptides for binding the CCK2R was determined on HEK293-CCK2R cells in a competitive binding assay. Cells were grown to confluency in 6-well plates. Cells were washed with binding buffer and incubated at RT for 10 min in binding buffer. Subsequently, unlabeled peptide was added in the range 0.1–100 nM together with a trace amount of ¹¹¹In-DOTA-sCCK8[Ph²(*p*-CH₂SO₃H), HPG^{3,6}] (12 000 dpm). After incubation at RT for 1 h, binding buffer was removed and cells were washed twice with binding buffer. Cell-associated radioactivity was determined. The IC₅₀ was defined as the peptide concentration at which 50% of binding without competitor was reached. IC₅₀ values (including standard deviations) were calculated using *GraphPad Prism* software (version 4.00 for Windows, GraphPad Software, San Diego, CA, USA).

Biodistribution Studies. The in vivo tumor targeting potential of the ¹¹¹In-DOTA-peptides was investigated in female athymic BALB/c mice. Subcutaneous tumors were induced by inoculation with CCK2R expressing AR42J cells (rat tumor of the exocrine pancreas). Cells were cultured in DMEM with 4500 mg/L D-glucose (Gibco, Invitrogen, Breda, The Netherlands), supplemented with 10% FCS and 1% penicillin/streptomycin. Mice were inoculated s.c. with 4 × 10⁶ AR42J cells (200 μL) in the left flank. When tumors had reached a weight of 0.2 g, mice were divided in groups of 5 mice each and 370 kBq ¹¹¹In-DOTA-peptide (0.1 μg) was injected intravenously. Specificity was studied in groups (*n* = 5) which received a 1000-fold molar excess of unlabeled sCCK8. Mice were killed 2 h postinjection (p.i.), a blood sample was drawn, and tissues of interest were dissected, weighed, and counted in a gamma-counter along with a standard of the injected activity to allow calculation of the injected dose per gram tissue (% ID/g). Animal experiments

Scheme 1. Synthesis Route of Fmoc-Phe(*p*-CH₂SO₃H)-OH

were approved by the local animal welfare committee and carried out according to national regulations.

Statistical Analysis. All mean values are expressed as mean \pm standard deviation (SD). Statistical analysis was performed using one-way analysis of variance using *GraphPad InStat* software (version 3.10, Graphpad software). The level of significance was set at $p < 0.05$.

RESULTS

Synthesis of Phe(*p*-CH₂SO₃H) and Peptides. Since sulfonate building blocks are not commercially available, Fmoc-Phe(*p*-CH₂SO₃H)-OH was synthesized starting from L-tyrosine, by modification of known routes (Scheme 1). First, the amino group and the acid functionality were protected by a Boc- and a methyl group, respectively. The hydroxy group of **1** was activated by triflation using phenyltriflimide and DIPEA, giving the triflate **2** in 79% yield (14). The next step, a Stille vinylation (14, 18), was performed under strictly dry conditions and required careful workup and rapid purification to obtain the vinylated product **3**. Ozonolysis and reduction of the vinyl compound was performed immediately to obtain the hydroxymethylated compound **4** (15, 18). The chloromethyl derivative **5**, obtained from alcohol **4** by treatment with thionyl chloride, was converted into the sulfonate **6** by refluxing in water in the presence of a large excess of Na₂SO₃ (9). NMR spectra showed that the methyl group was concomitantly removed in this reaction, thereby obviating a separate deprotection step. The final step in the synthesis of the sulfonate building block was Fmoc-protection of the amine under basic conditions (16). The overall yield of the synthesis starting from L-tyrosine was 9% (19).

Having the phenylalanine sulfonate building block **7** at hand, we investigated the synthesis of sCCK8 analogues by solid-phase peptide synthesis. Initial application of standard coupling conditions (HOBt, DIPCDI) to Fmoc-Phe(*p*-CH₂SO₃H)-OH failed to afford incorporation of Phe(*p*-CH₂SO₃H)-OH in the peptide. Fortunately, switching of reagents to HBTU and DIPEA led to the desired octapeptide uneventfully. To obtain other more stable sCCK8 peptide analogues not susceptible to oxidation, methionine residues were replaced by either norleucine (Nle) or homopropargylglycine (HPG) residues. After incorporation of the final amino acid, the macrocyclic chelator DOTA was coupled on the resin as tri-*tert*-butyl protected derivative. The

latter coupling reaction was found to proceed optimally under the action of HBTU and DIPEA. After cleavage from the resin with TFA/H₂O (92.5/2.5, v/v) in the presence of ethanedithiol (EDT) and triisopropyl silane (TIS), with simultaneous protective group removal, the peptides were purified by RP-HPLC. Molecular structures of the peptides are depicted in Figure 1.

Partition Coefficient. For ¹¹¹In-DOTA-sCCK8[Phe²(*p*-CH₂SO₃H)] and ¹¹¹In-DOTA-sCCK8[Phe²(*p*-CH₂SO₃H),Nle^{3,6}], the log P values were -2.97 ± 0.02 and -2.93 ± 0.13 , respectively. The log P value for ¹¹¹In-DOTA-sCCK8[Phe²(*p*-CH₂SO₃H),HPG^{3,6}] was -2.86 ± 0.14 . These values were comparable to that of the lead compound, ¹¹¹In-DOTA-sCCK8: -2.91 ± 0.20 . All compounds were relatively hydrophilic, and the molecular changes did not affect the hydrophilicity of the peptide significantly.

Oxidation Experiments. After 10 min incubation in oxidation buffer (containing hydrogen peroxide and acetic acid), about 66% of the ¹¹¹In-labeled DOTA-sCCK8[Phe²(*p*-CH₂SO₃H),Met^{3,6}] was oxidized, as judged by RP-HPLC. Both ¹¹¹In-DOTA-sCCK8[Phe²(*p*-CH₂SO₃H),Nle^{3,6}] and ¹¹¹In-DOTA-sCCK8[Phe²(*p*-CH₂SO₃H),HPG^{3,6}] did not show any oxidation.

Stability Experiments. In vivo, the peptide analogues should remain intact long enough to target the gastrin/CCK2 receptors. After 4 h incubation in human serum at 37 °C, peptides remained intact. Of the ¹¹¹In-labeled DOTA-sCCK8[Phe²(*p*-CH₂SO₃H),Met^{3,6}] 2.3% was oxidized after incubation in the serum.

Internalization Experiments. Internalization of the peptides by HEK293-CCK2R cells was investigated in vitro. After 2 h incubation at 37 °C, $35.2 \pm 2.1\%$ of the added ¹¹¹In-DOTA-sCCK8 was internalized by the cells, most of it during the first 30 min (Figure 2). Receptor bound fraction after 2 h was $11.6 \pm 0.7\%$. Of both ¹¹¹In-DOTA-sCCK8[Phe²(*p*-CH₂SO₃H)] and ¹¹¹In-DOTA-sCCK8[Phe²(*p*-CH₂SO₃H),HPG^{3,6}], about 13% of the added radiolabeled compound was internalized by the CCK2R after 2 h at 37 °C, while 10–12% was still receptor-bound. The internalized fraction of ¹¹¹In-DOTA-sCCK8[Phe²(*p*-CH₂SO₃H),Nle^{3,6}] after 2 h was $26.8 \pm 1.4\%$ with a receptor-bound fraction of $9.4 \pm 0.6\%$ (Figure 2). Internalization kinetics of ¹¹¹In-DOTA-sCCK8 and ¹¹¹In-DOTA-sCCK8[Phe²(*p*-CH₂SO₃H),Nle^{3,6}] showed to be similar, although the percentage of internalized fraction was about 7% lower for the synthetic peptide ($P < 0.05$). Similar data were found using AR42J cells (data not shown).

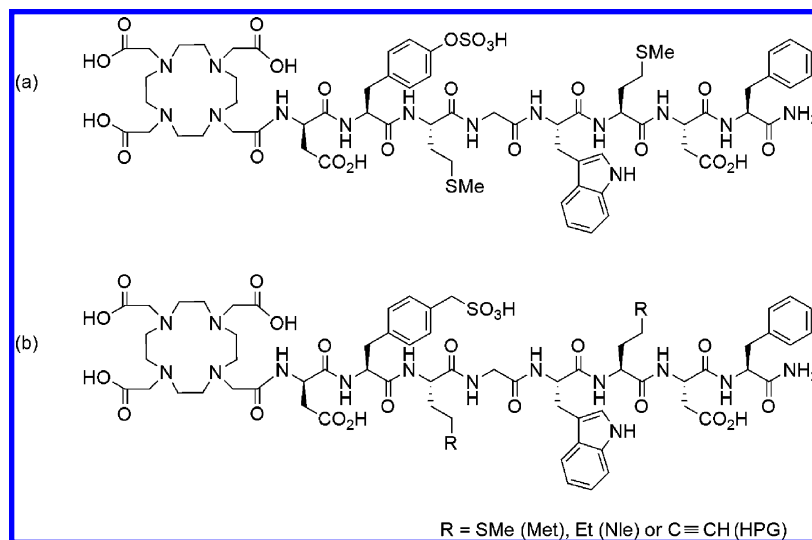


Figure 1. Molecular structures of (a) DOTA-sCCK8 and (b) synthetic DOTA-peptides.

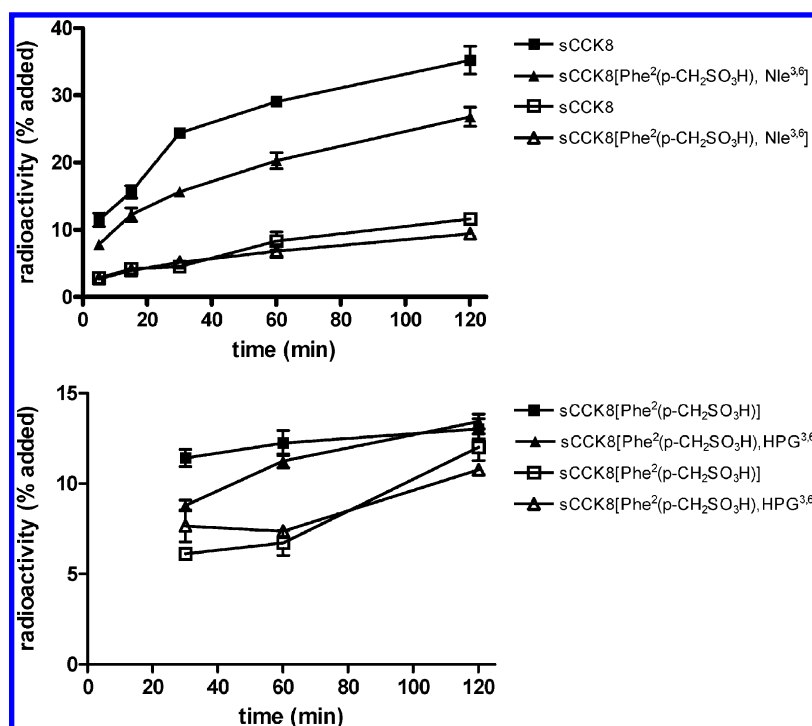


Figure 2. Internalization and receptor bound fractions in HEK293-CCK2R cells. Closed symbols indicate internalized fraction and open symbols indicate receptor-bound fraction. Values are expressed as percentage of the total amount added.

Determination of IC_{50} Values. The apparent IC_{50} values for binding CCK2R were determined for all peptides, using HEK293-CCK2R cells in a solid-phase competitive binding assay (Figure 3). All peptides showed an IC_{50} in the low nanomolar range, although the apparent IC_{50} values of the three synthesized peptides were approximately 10-fold higher than that of the lead compound. The apparent IC_{50} value for sCCK8 was 1.16 ± 0.06 nM, for DOTA-sCCK8[Phe 2 (*p*-CH $_2$ SO $_3$ H)] 27.2 ± 1.44 nM, for DOTA-sCCK8[Phe 2 (*p*-CH $_2$ SO $_3$ H), Nle 3,6] 11.6 ± 1.06 nM, and for DOTA-sCCK8[Phe 2 (*p*-CH $_2$ SO $_3$ H), HPG 3,6] 13.2 ± 1.12 nM.

Biodistribution. The potential of the radiolabeled DOTA-peptides for in vivo targeting of CCK2R expressing tumors was investigated in athymic mice with AR42J tumors. The biodistribution 2 h p.i. for each of the four ^{111}In -labeled peptides is summarized in Figure 4.

Tumor uptake of ^{111}In -DOTA-sCCK8 and ^{111}In -DOTA-sCCK8[Phe 2 (*p*-CH $_2$ SO $_3$ H), Nle 3,6] were comparable (4.78 ± 0.64 and $4.54 \pm 1.15\%$ ID/g, respectively). The radioactivity concentration of these peptides in the tumor was the highest of all tissues studied ($P < 0.001$). For both other compounds, the tumor uptake was significantly lower ($2.18 \pm 0.18\%$ ID/g for ^{111}In -DOTA-sCCK8[Phe 2 (*p*-CH $_2$ SO $_3$ H)], $P < 0.001$) and $1.92 \pm 0.31\%$ ID/g for ^{111}In -DOTA-sCCK8[Phe 2 (*p*-CH $_2$ SO $_3$ H), HPG 3,6], $P < 0.001$), and pancreatic concentration for these peptides was at the same level as the concentration in the tumor. Tumor uptake of all compounds could be blocked by coinjection of an excess of unlabeled sCCK8, indicating receptor-mediated uptake. For all compounds tested, the uptake in the normal tissues, such as the blood, lungs, muscle, spleen, and small intestine was low. Specific uptake was also found in the pancreas and, to a lesser extent, in the stomach. Most likely, this is due to relatively high CCK2R

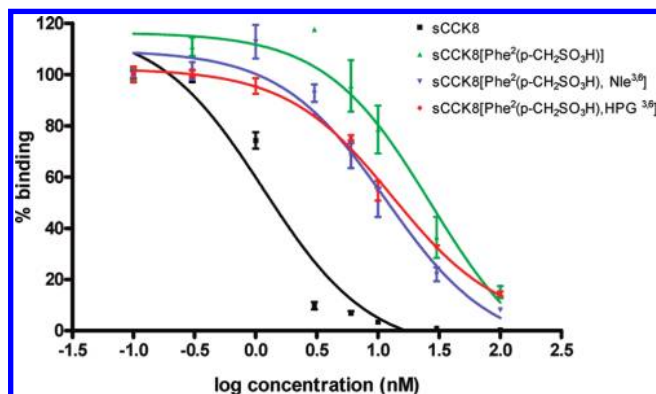


Figure 3. Competition binding analyses (IC_{50}) of four peptides using HEK293-CCK2R cells.

expression in murine pancreatic tissue en stomach. Kidney uptake of all peptides was low (<1.5% ID/g).

DISCUSSION

In earlier studies, we as well as others have shown that radiolabeled CCK8 peptide analogues show promise for peptide receptor radionuclide targeting of small cell lung cancers and medullary thyroid carcinomas (4, 5, 8, 12). In particular, sCCK8 was found to possess better CCK-receptor targeting behavior (4). An important drawback of the peptides used to date, however, is the intrinsic hydrolytic instability of the tyrosine sulfate ester. Moreover, since CCK8 contains two methionine residues, radiolabeling inevitably leads to varying amounts of thioether oxidation, which negates the binding affinity of the peptide to CCKR. Having these drawbacks in mind, we aimed for sCCK8 peptide analogues with improved stability as compared to the lead compound sCCK8. This study describes

the characterization of three DOTA-conjugated analogues of the sCCK8 peptide.

A promising method to avoid the instability of the sulfate ester is the replacement of the sulfated tyrosine by a phenylalanine sulfonate isostere, featuring a covalent C–S bond that is fully resistant to water. However, lack of commercial availability of such an unnatural amino acid necessitated a suitable synthetic preparation.

Synthesis started with a tyrosine triflate derivative (2), which is readily available from the corresponding protected tyrosine 1 by reaction with phenyl triflimide. For the synthesis of the hydroxymethylphenylalanines, we investigated different routes: the palladium-catalyzed coupling of the tyrosine triflate with *tert*-butyl acrylate (Heck reaction) and the coupling of the tyrosine triflate to vinyltributylstannane in the presence of metallic palladium and lithium chloride (Stille reaction), leading to styrene derivative 3. The latter reaction was found to give the best results, which is similar to the published data (14, 18). Both Tilley et al. and the group of Larsen described this reaction with slightly different tyrosine derivatives and they could obtain the allylated compounds in good yields (65–82%) after purification (14, 18). We found that careful workup and immediate ozonolysis and reduction were essential to obtain the hydroxymethylated product 4 in acceptable yields. We also attempted to follow the four-step synthesis route described by Miranda et al. (16), but the chloromethylation of phenylalanine, the first step in this route, also failed to succeed in our hands, as reported earlier (19). In addition, a third attempt involved synthesis of the hydroxymethylphenylalanine directly by a Stille reaction with (tributylstannyl)methanol (20). Several palladium complexes and solvents were investigated, but the desired compound could not be produced.

The chloromethyl derivative 5 was obtained from the alcohol 4 by treatment with thionyl chloride. We found that during this

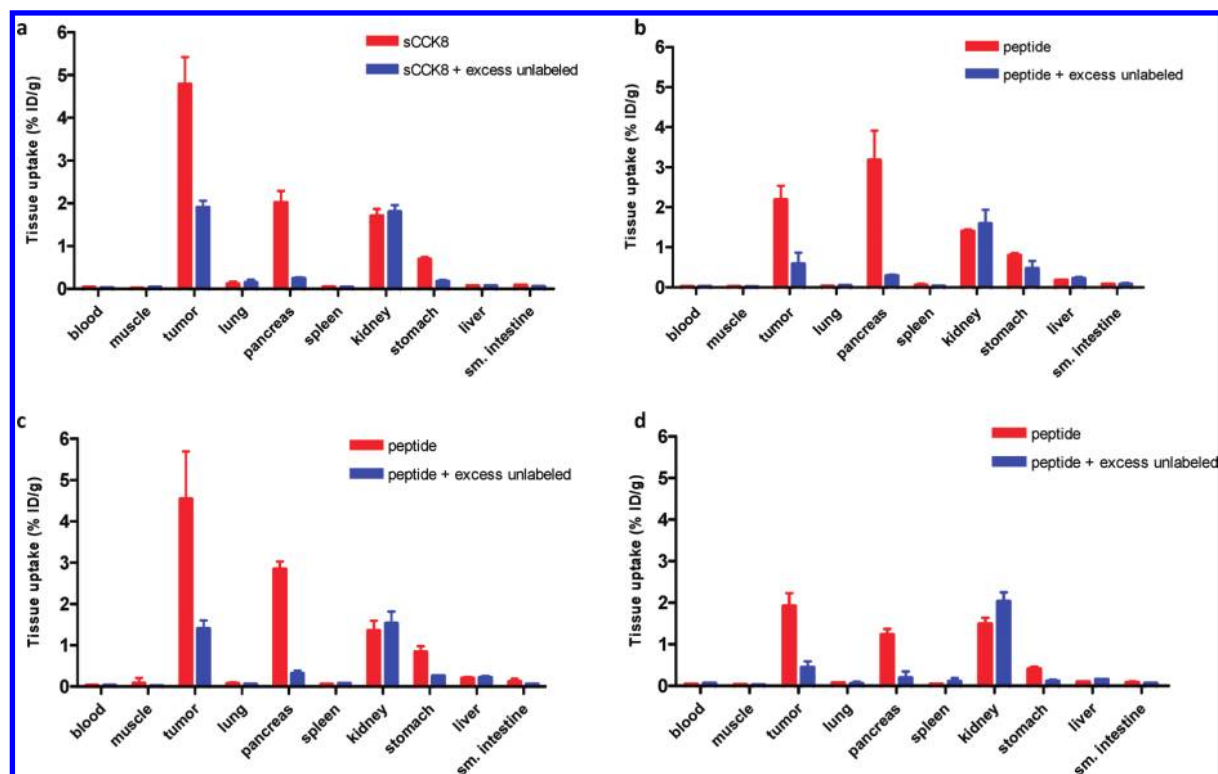


Figure 4. Biodistribution of (a) DOTA-sCCK8, (b) DOTA-sCCK8[Phe²(p-CH₂SO₃H)], (c) DOTA-sCCK8[Phe²(p-CH₂SO₃H), Nle^{3,6}], and (d) DOTA-sCCK8[Phe²(p-CH₂SO₃H), HPG^{3,6}] in athymic mice bearing CCK2R expressing tumors in the left flank. Values are expressed as percentage of the injected dose per gram tissue ($n = 5$ mice/group). Blocking was performed by coinjection of a 1000-fold molar excess of unlabeled sCCK8. Mice were dissected at 2 h p.i.

reaction the Boc-protective group was conveniently removed as well, probably due to the formation of HCl in this reaction. The next step, conversion to the sulfonate **6**, was also accompanied by an unintended but useful side reaction, i.e., hydrolysis of the methyl ester, thereby saving another deprotection step. On the basis of the whole sequence, it can be concluded that a practical, multigram-scale procedure has been developed in only 8 steps starting from L-tyrosine, leading to the final Fmoc-protected sulfonate building block **7** in an acceptable yield (9%).

The Fmoc-Phe(*p*-CH₂SO₃H)-OH building block was smoothly incorporated in the peptide sequence on solid phase, but required replacement of the typical DIPCDI/HOBt mixture with HBTU and DIPEA as coupling reagents. Three peptides were synthesized with the sulfonate building block, differing in the presence of Met, Nle, or HPG residues on both positions 3 and 6. Finally, all peptides were N-terminally conjugated with the macrocyclic chelator DOTA, also requiring HBTU/DIPEA for activation.

Receptor binding studies and internalization studies were performed on HEK293 cells stably expressing the CCK2 receptor. Substituting the tyrosine sulfate by its phenylalanine isostere resulted in sCCK8 analogues with apparent affinities for the CCK2 receptor in the low nanomolar range. When the Met residues were replaced by Nle residues, a more stable peptide was obtained with comparable tumor uptake in CCK2R positive tumors. Reubi et al. also found that replacement of methionine by norleucine in a DTPA-conjugated nonsulfated CCK8-peptide does not affect the binding affinity for the CCK2 receptor, but increases the plasma stability of the compound (12). In contrast, Mather et al. found that substitution of the methionine residue with norleucine in gastrin peptide analogues lowered both in vitro binding affinity and tumor uptake in vivo. As Mather synthesized gastrin analogues and not CCK8 analogues, these peptides may be affected differently by substitution of methionine by norleucine. This difference may also be due to the two additional histidines on the N-terminus (9). The group of Aloj synthesized and evaluated a series of cyclic sCCK8 peptide analogues, in which the tyrosine sulfate and both methionines were preserved. They found that cyclization of the peptides caused a 1000-fold decrease in affinity for both the CCK1 and CCK2 receptor (21, 22).

Homopropargylglycine has not earlier been applied in tumor-targeting peptides, but has been generally shown to be a suitable and stable substitute for methionine (13). We found that replacement of methionine by homopropargylglycine caused reduction of CCK2R affinity resulting in reduced uptake of the radiolabeled peptide in the AR42J tumor.

The CCK2 receptor belongs to the family of G-protein-coupled receptors. When gastrin or CCK8 binds to the receptor, the complex is internalized, after which the receptor can be recycled or metabolized. After binding and internalization of ^{111}In -labeled peptides, the radiometal is retained intracellularly in the lysosomes. The peptides in this study all showed time-dependent internalization. About 27% of ^{111}In -DOTA-sCCK8[Phe²(*p*-CH₂SO₃H), Nle^{3,6}] was internalized after 2 h, where ^{111}In -DOTA-sCCK8 showed 35% internalization ($P < 0.05$).

The peptides in this study showed very low kidney retention (1.5% ID/g, 2 h p.i.) and display also low uptake in the main peripheral tissue. This is in contrast with ^{111}In -labeled minigastrin analogues, which display a very high kidney uptake, 40–60% ID/g (5). Derivatives of minigastrin, lacking the pentaglutamate sequence, showed a comparably low kidney uptake compared to CCK8 analogues (23). Low kidney and peripheral tissue retention are prerequisites for receptor-targeted imaging and therapeutic agents. The observed specific uptake in the pancreas can be explained by the fact that in rodents the

pancreas expresses the CCK-receptor, whereas in human pancreatic tissue, the CCK2 receptor is not expressed. Therefore, in a clinical setting, pancreatic uptake of radiolabeled sCCK8 analogues is not expected.

In summary, DOTA-sCCK8[Phe²(*p*-CH₂SO₃H), Nle^{3,6}] is a peptide with receptor affinity and tumor uptake comparable to sCCK8, but with an increased stability and therefore is a promising peptide for use in PRRT.

CONCLUSION

A successful synthetic route was developed to a tyrosine isostere that is not susceptible to hydrolysis of the sulfate ester linkage and therefore is a valuable building block for the preparation of medicinally relevant peptides. A series of radiolabeled sCCK8 analogues with increased stability over the natural peptide was successfully synthesized by solid-phase peptide synthesis. The peptide with methionine residues replaced by norleucine (DOTA-sCCK8[Phe²(*p*-CH₂SO₃H), Nle^{3,6}]) showed promising characteristics for CCK2R targeting and will be further investigated for its potency in imaging and therapy.

Supporting Information Available: NMR-spectra, HPLC-spectra, biodistribution studies. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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