Bioconjugate Chemistry

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pubs.acs.org/bc

Orthogonally Protected Artificial Amino Acid as Tripod Ligand for Automated Peptide Synthesis and Labeling with $[^{99m}Tc(OH_2)_3(CO)_3]^+$

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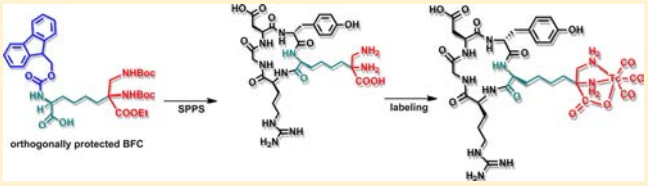
Supporting Information

ABSTRACT: 1,2-Diamino-propionic acid (Dap) is a very strong chelator for the $[^{99m}Tc(CO)_3]^+$ core, yielding small and hydrophilic complexes. We prepared the lysine based Dap derivative L-Lys(Dap) in which the ε-NH_2 group was replaced by the tripod through conjugation to its α-carbon. The synthetic strategy produced an orthogonally protected bifunctional chelator (BFC). The -NH_2 group of the α-amino acid portion is Fmoc- and the -NH_2 of Dap are Boc-protected. Fmoc-L-Lys(Dap(Boc)) was either conjugated to the N- and C-terminus of bombesin BBN(7−14) or integrated into the sequence using solid-phase peptide synthesis (SPPS). We also replaced the native lysine in a cyclic RGD peptide with L-Lys(Dap). For all peptides, quantitative labeling with the $[^{99m}Tc(CO)_3]^+$ core at a 10 μM concentration in PBS buffer (pH = 7.4) was achieved. For comparison, the rhenium homologues were prepared from $[Re(OH_2)_3(CO)_3]^+$ and Lys(Dap)-BBN(7−14) or cyclo-(RGDyK(Dap)), respectively. Determination of integrin receptor binding showed low to medium nanomolar affinities for various receptor subtypes. The IC_{50} of cyclo-(RGDyK(Dap[$Re(CO)_3$])) for $\alpha_v\beta_3$ is 7.1 nM as compared to 3.1 nM for nonligated RGD derivative. Biodistribution studies in M21 melanoma bearing nude mice showed reasonable $\alpha_v\beta_3$-integrin specific tumor uptake. Altogether, orthogonally protected L-Lys(Dap) represents a highly versatile building block for integration in any peptide sequence. Lys(Dap)-precursors allow high-yield ^{99m}Tc-labeling with $[^{99m}Tc(OH_2)_3(CO)_3]^+$, forming small and hydrophilic complexes, which in turn leads to peptide radiopharmaceuticals with excellent in vivo characteristics.

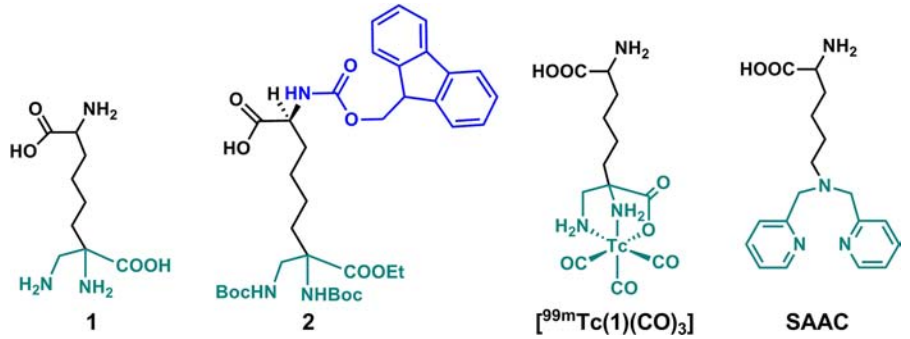


INTRODUCTION

Peptide-based targeting vectors as carriers for radionuclides are the focus of finding and developing new radiolabeled compounds for molecular imaging purposes. Since distinct peptide receptors are strongly overexpressed in many tumor cells; they represent attractive targets for radionuclide-based diagnosis and therapy in oncology.[1−7] Most peptide radiopharmaceuticals, which are either used in clinical studies or currently under preclinical evaluation, are structurally derived from naturally occurring peptide ligands. These have intrinsically evolved to be highly active ligands, but also, being mostly peptide hormones, to be rapidly degraded in vivo.[2] For the applicability of a radiolabeled peptide probe for in vivo imaging, however, high metabolic stability, suitable pharmacokinetics (e.g., fast clearance from the blood pool, low binding to plasma proteins or nontarget tissues, fast renal excretion) and high receptor affinity are crucial. Even if all these issues have been carefully addressed and an "optimized" small and metabolically stable peptide sequence with high affinity toward the target receptor is available, introduction of the radiolabel may represent a serious challenge on both receptor affinity and in vivo pharmacokinetics, depending on the radionuclide and labeling method chosen. For example, the use of radiometals requires the functionalization of the biomolecule with an usually bulky chelator for radiometal complexation. Even if this modification is well-tolerated in a given position of the peptide sequence and does not seriously challenge receptor affinity, it may still have substantial impact on the overall pharmacokinetics of the peptide radiopharmaceutical. This problem has been repeatedly observed for ^{99m}Tc-labeled peptides, and recent research has therefore been focused on the development of suitable bifunctional chelators (BFC) allowing ^{99m}Tc-labeling with high efficiency, leading to complexes with high stability. The influence of the type of chelator on labeling efficiencies and complex stabilities has recently been reviewed comprehensively several times.[8−12] However, in some cases, coordination chemistry and physicochemical characteristics such as lipophilicity of the complexes were not optimally compatible with the requirements for in vivo imaging applications.

Received: June 21, 2012
Revised: November 22, 2012
Published: December 14, 2012

Scheme 1. Basic Bifunctional, L-Lysine Based Chelators[a]



[a] 1,2-Diamino-propionic acid (Dap) as chelating unit conjugated to the ε-position (**1**),[26,27] the corresponding, orthogonally protected BFC (**2**) for automated SPPS, the LAT1 targeting radiopharmaceutical [^{99m}Tc(**1**)(CO)$_3$], and the bifunctional SAAC system.[16−18]

As a labeling precursor, the complex [^{99m}Tc(OH$_2$)$_3$(CO)$_3$]$^+$ has attracted much attention over the past years. The {^{99m}Tc(CO)$_3$}$^+$ core accepts a wide variety of different ligands due to the robust nature of the resulting complexes.[13−15] Among these BFCs, Valliant et al. introduced the L-lysine-based single amino acid chelator (SAAC) approach. In this concept, a BFC is obtained by modifying the ε-amino group with a heterocyclic tridentate, originally pyridine-based tripod.[16−18] Tether and ligand are altered while the entire ligand can be subjected to automated SPPS. The utility of this concept was demonstrated by the labeling of many peptides prepared via this approach.[11,19−24] A drawback of the concept is the lipophilicity of the tagged complex and its relatively large size. Lipophilicity can be reduced by ligand variations, however, but at the cost of molecular weight and label size.[25]

We have introduced a BFC (**1**) based on L-lysine in which the ε-amino group was replaced by 2,3-diamino propionic acid (Dap). Labeling of **1** with the {^{99m}Tc(CO)$_3$}$^+$ fragment resulted in [^{99m}Tc(**1**)(CO)$_3$] which was recognized and actively transported by the L-type amino acid transporter LAT1, demonstrating for the first time that small molecules can be labeled with ^{99m}Tc under retention of transporter and receptor affinity.[26] The α-amino group allows introduction of further functionalities for conjugating a wide variety of biomolecules with the small, hydrophilic, and strong Dap chelator.[27−29] Since [^{99m}Tc(**1**)(CO)$_3$] basically represents an "unnatural amino acid", the use of appropriately and orthogonally protected precursors should allow the substitution of structurally similar amino acids in a peptide sequence during peptide synthesis without strongly affecting receptor affinities.

We present in this work the synthesis of the bifunctional artificial amino acid **2**. BFC **2** contains orthogonally protected functional groups which enable its general use in automated SPPS. Exemplary incorporation into BBN(7−14) and an RGD peptide demonstrate the strength of this concept (Scheme 1). The resulting peptides were labeled with the {^{99m}Tc(CO)$_3$}$^+$ and {Re(CO)$_3$}$^+$ cores and evaluated in vitro and in vivo.

EXPERIMENTAL SECTION

General. All reagents and organic solvents were purchased from Aldrich, Acros, or ABCR in reagent grade or better and used without further purification. Column chromatography was accomplished on silica gel 60 (20−40 mesh). Electrospray ionization mass spectrometry (ESI-MS) spectra were recorded a Bruker HCT spectrometer and NMR spectra on a Bruker 400 or 500 MHz spectrometer in $CDCl_3$ solutions. All NMR chemical shifts are in ppm; coupling constants (J) are given in Hz. High-resolution electrospray ionization mass spectrometry was performed on a FinniganMAT 900 (Finnigan MAT95, San Jose, CA; USA) double-focusing magnetic sector mass spectrometer (geometry BE). Compounds **3**, **4**, **5**, and **6** were prepared according to literature. Characterization data (^{1}H and ^{13}C NMR, MS) were in agreement with literature procedures.[30,31]

For the preparation of the peptides **L1−L4** coordinated to the {Re(CO)$_3$}$^+$ moiety, we used (NEt$_4$)$_2$[ReBr$_3$(CO)$_3$] as starting material as described in the experimental procedures. Once dissolved in water, [ReBr$_3$(CO)$_3$]$^{2-}$ converts rapidly to [Re(OH$_2$)$_3$(CO)$_3$]$^+$ which then coordinates to the corresponding ligand.

*(S)-Benzyl 2-(benzyloxycarbonylamino)-6-(methylsulfonyloxy)hexanoate (**7**).* To a stirred solution of **6** (2g, 5.4 mmol) and H_3CSO_2Cl (0.466 mL, 6 mmol) in CH_2Cl_2 (10 mL) cooled to −78 °C, NEt_3 (0.797 mL, 6 mmol) was slowly added. The mixture was allowed to come to r.t., and stirring was continued for 3 h. The solution was diluted with 10 mL CH_2Cl_2, washed with H_2O, dried over $MgSO_4$, and evaporated. The crude product was purified by flash column chromatography (33% Et_2O in hexane) to give **7** as a pale yellow oil (2.3g, 5.12 mmol, 95%). R_f = 0.77 (SiO_2, TLC, Et_2O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.26 (s, 10H), 5.61 (d, 1H, J = 8 Hz), 5.02−5.13 (m, 4H), 4.34 (d, 1H, J = 5.2 Hz), 4.03 (t, 2H, J = 6.2 Hz), 2.81 (s, 3H), 1.32−1.78 (m, 6H). Anal. Calcd. for $C_{22}H_{27}NO_7S$: C, 58.78; H, 6.05; N, 3.12. Found: C, 58.39; H, 5.99; N, 3.04. MS-ESI: m/z 472.1 (calcd. 472.15) [(M + Na)$^+$].

*(7S)-8-Benzyl 1-ethyl 2-(tert-butoxycarbonylamino)-7-(benzyloxycarbonylamino)-2-cyan ooctanedioate (**8**).* Na° (0.046g, 2 mmol) was dissolved in abs. ethanol, and ethyl 2-(*tert*-butoxycarbonylamino)-2-cyanoacetate (0.46g, 2 mmol) was added; the mixture was stirred at 60 °C for 30 min. After cooling to r.t., ethanol was removed under vacuum, the residue dissolved in DMSO (5 mL), mesylate **7** (0.67g, 1.5 mmol) was added, and the resulting solution stirred at 70 °C for 10 h. DMSO was removed under high vacuum and the residue dissolved in EtOAc. The solution was washed with water, brine, dried over $MgSO_4$, and concentrated in vacuo. Purification on a silica gel column with EtOAc/hexane provided **8** (0.66g, 76%) as a colorless oil. R_f = 0.24 (SiO_2, TLC; hexane/EtOAc, 2/1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.26 (s, 10H), 5.41 (d, 1H, J = 7.6 Hz), 5.04−5.11 (m, 4H), 4.33 (s, 1H), 4.22 (t, 2H, J = 7 Hz), 1.58−1.96 (m, 4H), 1.38 (s, 9H), 1.18−1.31 (m, 7H).

dx.doi.org/10.1021/bc3003327 | Bioconjugate Chem. 2013, 24, 26−35

Anal. Calcd. for $C_{31}H_{39}N_3O_8$: C, 64.01; H, 6.76; N, 7.22. Found: C, 63.92; H, 6.68; N, 7.18. MS-ESI: m/z 582.4 (calcd. 582.27) $[(M + H)^+]$.

*(7S)-8-Benzyl 1-ethyl 2-(tert-butoxycarbonylamino)-7-(benzyloxycarbonylamino)-2-((tert-butoxycarbonylamino)-methyl)octanedioate (**9**).* Boc_2O (0.87g, 4 mmol) was added to a solution of **8** (1.20g, 2 mmol) in dry MeOH of 0 °C followed by $NiCl_2 \cdot 6H_2O$ (0.048g, 0.2 mmol). $NaBH_4$ (0.606g, 16 mmol) was then added in small portions over 30 min. The resulting mixture was allowed to warm to r.t. and left to stir for a further 2 h. The mixture was filtered through Celite and the filtrate evaporated to dryness in vacuo. The residue was taken up in EtOAc, washed with water, brine, dried over $MgSO_4$, and concentrated. Flash chromatography on silica gel column eluting with EtOAc/hexane provided **9** (0.96g, 70%) as a colorless oil. R_f = 0.4 (SiO_2, TLC; hexane/EtOAc, 2/1). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.24 (s, 10H), 5.42 (s, 1H), 5.35 (d, 1H, J = 7.5 Hz), 5.04−5.11 (m, 4H), 4.82 (s, 1H), 4.31 (s, 1H), 4.11 (d, 2H, J = 6.5 Hz), 3.60 (m, 2H), 1.57−1.99 (m, 4H), 1.41 (s, 18H), 1.06−1.20 (m, 7H). Anal. Calcd. for $C_{36}H_{51}N_3O_{10}$: C, 63.05; H, 7.50; N, 6.13. Found: C, 62.90; H, 7.23; N, 6.00. MS-ESI: m/z 708.5 (calcd. 708.35) $[(M + Na)^+]$.

*(2S)-2-Amino-7-(tert-butoxycarbonylamino)-7-((tert-butoxycarbonylamino)methyl)-8-ethoxy-8-oxooctanoic acid (**10**).* Thirty milligrams of 10% Pd/C was added to an anhydrous EtOH (15 mL) solution of **9** (0.27g, 0.4 mmol). H_2 was then bubbled through the solution for 2 days at r.t. The solution was filtered with Celite to remove the catalyst and the solvent removed by rotary evaporation. The residue was purified by C_{18} RP chromatography (1:1 CH_3OH/H_2O) to give **10** (0.16 g, 88%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): 7.94 (s, 1H), 5.73 (s, 1H), 5.15 (s, 1H), 4.12 (d, 1H, J = 6 Hz), 3.57 (m, 3H), 1.41 (s, 18H), 1.18−1.82 (m, 11H). MS-ESI: m/z 484.4 (calcd. 484.27) $[(M + Na)^+]$. HRMS (ESI): Calcd for $[M+H]^+$, 462.2815; Found, 462.2804.

*(2S)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-7-(tert-butoxycarbonylamino)-7-((tert-butoxycarbonylamino)-methyl)-8-ethoxy-8-oxooctanoic acid (**2**).* Compound **10** (0.6g, 1.3 mmol) was dissolved in dioxane (4 mL) and a 5% aqueous solution of Na_2CO_3 (8 mL). The reaction mixture was immersed in an ice bath and a solution of FmocCl (0.336g, 1.3 mmol) in dioxane(4 mL) was added. The solution was allowed to warm to r.t. and stirred for 3 h. The solvent was evaporated under high vacuum and the resulting material was purified on a silica gel column eluting with $CHCl_2$/MeOH. Compound **2** was received as a white solid (0.77g, 87%). R_f =0.3 (SiO_2, TLC; CH_2Cl_2/MeOH, 30:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.26−7.77 (m, 8H), 5.53 (s, 1H), 4.89 (s, 1H), 4.19−4.50 (m, 5H), 3.68 (m, 2H), 1.41 (s, 18H), 1.25−2.10 (m, 11H). MS-ESI: m/z 682.5 (calcd. 682.34) $[(M-H)^-]$. HRMS (ESI): Calcd for $[M+Na]^+$, 706.3315; Found 706.3314.

Synthesis of BBN Analogues (L1−L3). Peptides were prepared either on an automated peptide synthesizer or by manual synthesis using Fmoc chemistry. The BBN analogues prepared and characterized were as follows: NH_2-Lys(Dap)-Gln-Trp-Ala-Val-Gly-His-Leu-Met-$CONH_2$ (N-terminal, **L1**), NH_2-Gln-Trp-Ala-Val-Lys(Dap)-Gly-His-Leu-Met-$CONH_2$ (integrated, **L2**), and NH_2-Gln-Trp-Ala-Val-Gly-His-Leu-Met-Lys(Dap)-$CONH_2$ (C-terminal, **L3**). Peptide synthesis used traditional Fmoc chemistry with HBTU or HATU activation of the carboxylate groups on the reactant for coupling with the N-terminal amino group on the growing peptide anchored via the C-terminus to the resin. Rink amide MBHA resin and Fmoc-protected amino acids with appropriate side-chain protection were used for the synthesis. The final products were cleaved from the resin by a standard procedure using a cocktail containing thioanisol, water, phenol, and trifluoroacetic acid in a ratio of 5:5:5:85 and precipitated into methyl *t*-butyl ether. At this point, the carboxylate group in the Dap unit was still esterified. Coordination to the $\{Re(CO)_3\}^+$ or to the $\{^{99m}Tc(CO)_3\}^+$ moiety hydrolyzes this group concomitantly. Crude peptides were purified by preparative HPLC (gradient from 0% to 100% solvent B over the course of 60 min). ESI-MS was used to determine the molecular constitution of the conjugates. ESI-MS for **L1**, **L2**, **L3**: m/z 1183.7 $[(M + H)^+]$.

*Synthesis of RGD-Peptide Cyclo-(Arg-Gly-Asp-D-Tyr-Lys-(Dap)) (**L4**).* The peptide was synthesized by solid-phase peptide synthesis (SPPS) on 2-chlorotrityl chloride resin using Fmoc-strategy. The resin was loaded with Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Dap)-OH, Fmoc-D-Tyr(tBu)-OH, and Fmoc-Asp(OtBu) were sequentially coupled with HBTU (4 equiv), HOBt (4 equiv), and DIEA (10 equiv) in DMF. Cleavage of protected linear peptide **11** from the resin was performed without affecting the side-chain protecting groups by using mildly acidic conditions (0.8% TFA in DCM or HOAc/TFA/DCM = 1:1:8). Head-to-tail cyclization to afford compound **12** was carried out in DMF with HBTU (4 equiv), HOBt (4 equiv), and DIEA (10 equiv). Finally, the ethyl ester was hydrolyzed with LiOH in a mixture of MeOH and H_2O and the remaining side-chain protecting groups were removed by treatment with trifluoroacetic acid/water/triisopropylsilane (TIPS) (95:2.5:2.5) at room temperature for 1.5 h to yield **L4**. The reaction mixture was concentrated and precipitated with ice-cold ether and purified by preparative HPLC (gradient from 5% to 25% solvent B over the course of 70 min). ESI-MS: m/z 707.3 $[(M + H)^+]$, 354.1 $[(M + 2H)^{2+}]$.

*Synthesis of [Re(L4)(CO)$_3$] (**13**), [Re(L1)(CO)$_3$] (**14**), [Re-(L2)(CO)$_3$] (**15**), and [Re(L3)(CO)$_3$] (**16**).* An excess of $(NEt_4)_2[ReBr_3(CO)_3]$ was added to 6 mg of an aqueous solution of **L1**, **L2**, **L3**, or **L4**, respectively. The solution was heated at 70 °C for 6−20 h (longer times do not reduce the yields) with stirring. Quality control of the reaction mixture was done by RP-HPLC. The desired product was purified by HPLC, lyophilized, and characterized by ESI-MS. ESI-MS for **14**, **15**, **16**: m/z 1425.6 $[(M + H)^+]$, 713.3 $[(M + 2H)^{2+}]$, **13**: m/z 977.3 $[(M + H)^+]$.

Determination of Binding Affinity and Specificity Using Solubilized Integrins. The inhibitory activity and selectivity of **L4** and $[Re(\mathbf{L4})(CO)_3]$ were determined using an ELISA assay based on previously reported methods with some optimizations.[32,33]

Human integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were purchased from Millipore, $\alpha_{IIb}\beta_3$ from Enzyme Research Laboratories, and $\alpha_5\beta_1$ and $\alpha_v\beta_3$ from R&D Systems. Vitronectin was purchased from Millipore, fibronectin from Sigma, fibrinogen from Calbiochem, and LAP protein from R&D Systems.

Blocking and binding steps were always performed with TS buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$) containing 1% BSA (TSB-buffer). Washing steps after the incubation time were done with PBST buffer (10 mM Na_2HPO_4, pH 7.5, 150 mM NaCl, and 0.01% Tween 20).

For the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, the binding was visualized using a mouse antihuman integrin α-V monoclonal antibody for α_v subunit (MAB 1978 purchased from Chemicon); for $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ were used antibodies from BD Biosciences (mouse

antihuman CD49e for $\alpha_5\beta_1$ and mouse antihuman CD41b for $\alpha_{IIb}\beta_3$) and Sigma (antimouse IgG-peroxidase). Peroxidase development was performed using the substrate solution 3,3,5,5′-tetramethylethylenediamine (TMB from Seramun Diagnostic GmbH) and 3 M H_2SO_4 to stop the reaction.

The absorbance (450, 492 nm) was recorded with a POLARstar Galaxy plate reader (BMG Labtechnologies). Every concentration was analyzed in duplicate and the resulting inhibition curves were analyzed using *OriginPro 7.5G* software. Each plate also contained either Tirofiban[34] or Cilengitide[35] as reference compounds.

$\alpha_v\beta_3$ Assay. Flat-bottom 96-well ELISA plates (from Brand) were coated with 100 μL of vitronectin (2 μg/mL) overnight at 4 °C in 15 mM of Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6 (carbonate buffer).

After removal of the coating solution, the wells were blocked for 1 h at r.t. with 150 μL/well of TSB. Plates were then washed three times with 200 μL/well of PBST.

Next, 4.0 μg/mL of soluble integrin $\alpha_v\beta_3$ and a serial dilution of integrin inhibitors and the control Cilengitide were incubated in the coated wells for 2 h at r.t.

After washing three times, the plate was treated with 100 μL/well of primary antibody (MAB1978 diluted 1:500 in TSB) and the secondary antibody (antimouse IgG-peroxidase) at 2.0 μg/mL for 1 h at r.t. After three washing steps, integrin binding was visualized with TMB. The oxidation was allowed to proceed for 5 min, and the product absorbance was measured at 450 nm.

$\alpha_v\beta_6$ Assay. For this assay, ELISA plates were coated with 100 μL of LAP protein (0.4 μg/mL) overnight at 4 °C in coating TS buffer. Blocking and washing steps were performed as described for $\alpha_v\beta_3$. Next, 0.5 μg/mL of soluble integrin $\alpha_v\beta_6$ and a serial dilution of integrin inhibitors and the control Cilengitide were incubated in the coated wells for 1 h at r.t. All subsequent steps were performed as described for $\alpha_v\beta_3$.

$\alpha_v\beta_5$ Assay. ELISA plates were coated with 100 μL of vitronectin (5 μg/mL) overnight at 4 °C in coating TS buffer. Blocking and washing steps were performed as described for $\alpha_v\beta_3$. Next, 3.0 μg/mL of soluble integrin $\alpha_v\beta_5$ and a serial dilution of integrin inhibitors and the control Cilengitide were incubated in the coated wells for 1 h at r.t. After washing three times, the plate was incubated with 100 μL/well of primary antibody (MAB1978) diluted 1:500 in TSB and secondary antibody (antimouse IgG-peroxidase) at 1.0 μg/mL for 1 h at r.t. All subsequent steps were performed as described for $\alpha_v\beta_3$.

$\alpha_5\beta_1$ Assay. Flat-bottom 96-well ELISA plates were coated overnight at 4 °C with 100 μL/well of 0.50 μg/mL of fibronectin in carbonate buffer (see $\alpha_v\beta_3$). Blocking and washing steps were performed as described. Next, 1.0 μg/mL of soluble integrin $\alpha_5\beta_1$ and a serial dilution of integrin inhibitors and the control Cilengitide were incubated in the coated wells for 1 h at r.t. After washing three times, the plate was treated with 100 μL/well of primary antibody (CD49e) at 1.0 μg/mL (1:500 dilution) and secondary antibody (antimouse IgG-peroxidase) at 2.0 μg/mL (1:385 dilution) for 1 h at r.t. All subsequent steps were performed as described for $\alpha_v\beta_3$.

$\alpha_{IIb}\beta_3$ Assay. ELISA plates were coated overnight at 4 °C with 100 μL/well of 10.0 μg/mL of fibrinogen in carbonate buffer. After washing and blocking, 2.5 μg/mL of soluble integrin $\alpha_{IIb}\beta_3$ and a serial dilution of integrin inhibitors and the control molecules Cilengitide and Tirofiban were incubated in the coated wells for 1 h at r.t. After three washing steps, wells were treated with 100 μL/well of primary antibody (CD41b) at 2.0 μg/mL (1:250 dilution) and secondary antibody (antimouse IgG-peroxidase) at 1.0 μg/mL (1:770 dilution) for 1 h at r.t. All subsequent steps were performed as described for $\alpha_v\beta_3$.

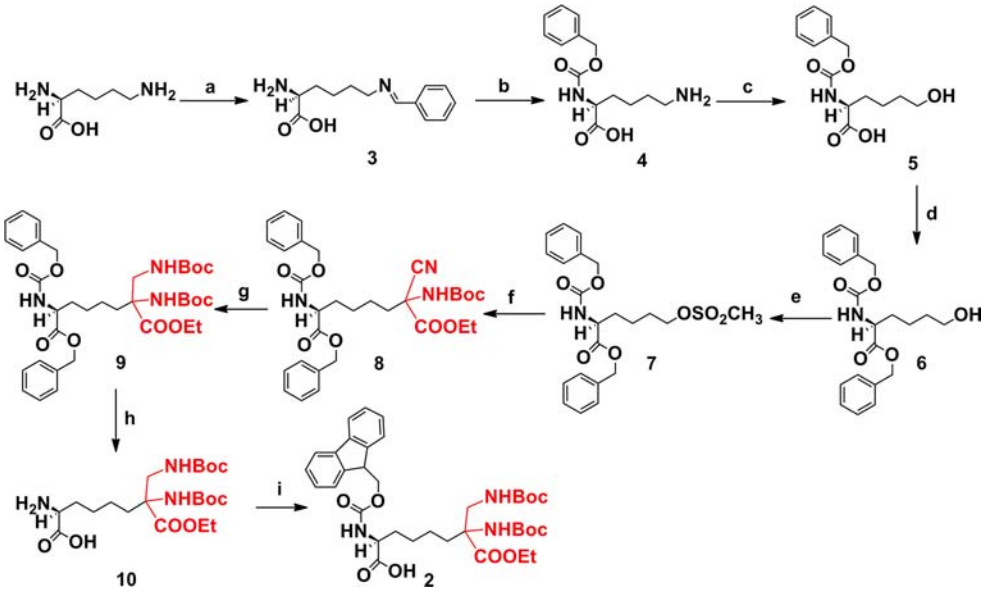
Determination of Binding Affinity Using M21 Cells. The in vitro integrin binding affinities were assessed via a cellular displacement assay using ^{125}I-echistatin (Perkin-Elmer, Rodgau, Germany; specific activity: 2200 Ci/mmol) as an integrin specific radioligand on M21 human melanoma cells. One day prior to the experiment, M21 cells were harvested using trypsin/EDTA (0.05% and 0.02%) in PBS (Biochrom), centrifuged, and resuspended in culture medium. The cells were transferred into 24-well plates (approximately 200 000 cells per well in 1 mL) and placed in the incubator overnight. The plates were used when confluence reached approximately 80%. Before the experiment, the culture medium was removed and binding buffer (20 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.4, 150 mM NaCl, 2 mM $CaCl_2{\cdot}2H_2O$, 1 mM $MgCl_2{\cdot}6H_2O$, 1 mM $MnCl_2{\cdot}4H_2O$, 0.1% (m/m) BSA) was added. The cells were incubated with ^{125}I-echistatin (120 000–140 000 cpm/well) and increasing concentrations of the RGD ligands (10^{-11}–10^{-4} mol/L), both dissolved in binding buffer. The total incubation volume was adjusted to 500 μL by addition of binding buffer. After the cells were incubated for 2 h at r.t., the supernatant was removed and the cells were washed twice with cold PBS. The cells were lysed with 1 M sodium hydroxide solution and transferred to vials. Quantification of bound radioactivity was performed using a gamma counter. The experiments were performed in duplicate and repeated three times. The best-fit IC_{50} (inhibitory concentration of 50%) values were calculated by fitting the data by nonlinear regression using GraphPad *Prism* (GraphPad Software, Inc.).

$[^{99m}Tc(OH_2)_3(CO)_3]^+$ Labeling Studies. The precursor $[^{99m}Tc(OH_2)_3(CO)_3]^+$ was prepared using an IsoLink Kit with 1.0–2.5 GBq of $Na^{99m}TcO_4$ in 1–2 mL saline.[36] After neutralization to pH = 7 with HCl, quantitative labeling was achieved by mixing an aliquot of the $[^{99m}Tc(OH_2)_3(CO)_3]^+$ precursor with a 0.1 mM stock solution of **L1**–**L3** at 90 °C for 30 min and **L4** at 70 °C for 25 min. Alternatively, labeling can be performed in a microwave oven. After one minute at 130 °C, one single product was received in yields better than 98%. Quality control (radiochemical yield and purity determination) of the product was determined by RP-HPLC. The identity was assessed by comparing HPLC retention times with the corresponding rhenium compounds (coinjection). Lipophilicity of $[^{99m}Tc(\mathbf{L4})(CO)_3]$ was determined as reported previously.[37]

In Vivo Biodistribution Study. To establish tumor growth, 1.5×10^7 M21 melanoma cells were injected subcutaneously into the shoulder of female CD1 nu/nu mice (Charles River, Sulzfeld, Germany).[38] At the time of the experiment (approximately six weeks after cell inoculation), tumor masses ranged from 32 to 250 mg. For the biodistribution study, 740 kBq of $[^{99m}Tc(\mathbf{L4})(CO)_3]$ in 100 μL of PBS were injected intravenously into the tail vein of the M21 tumor bearing nude mice (n = 5). To demonstrate $\alpha_v\beta_3$ integrin specificity of tumor accumulation, mice (n = 3) were coinjected with 590 μg Cilengitide per mouse (23.5 mg/kg body weight). At 60 min post injection (p.i.), mice were sacrificed, and the organs of interest were dissected, weighed, and counted in a Gamma counter (Wallach, Turku). Data are given in percent of injected dose per gram tissue [%ID/g] and represent means ± standard deviation.

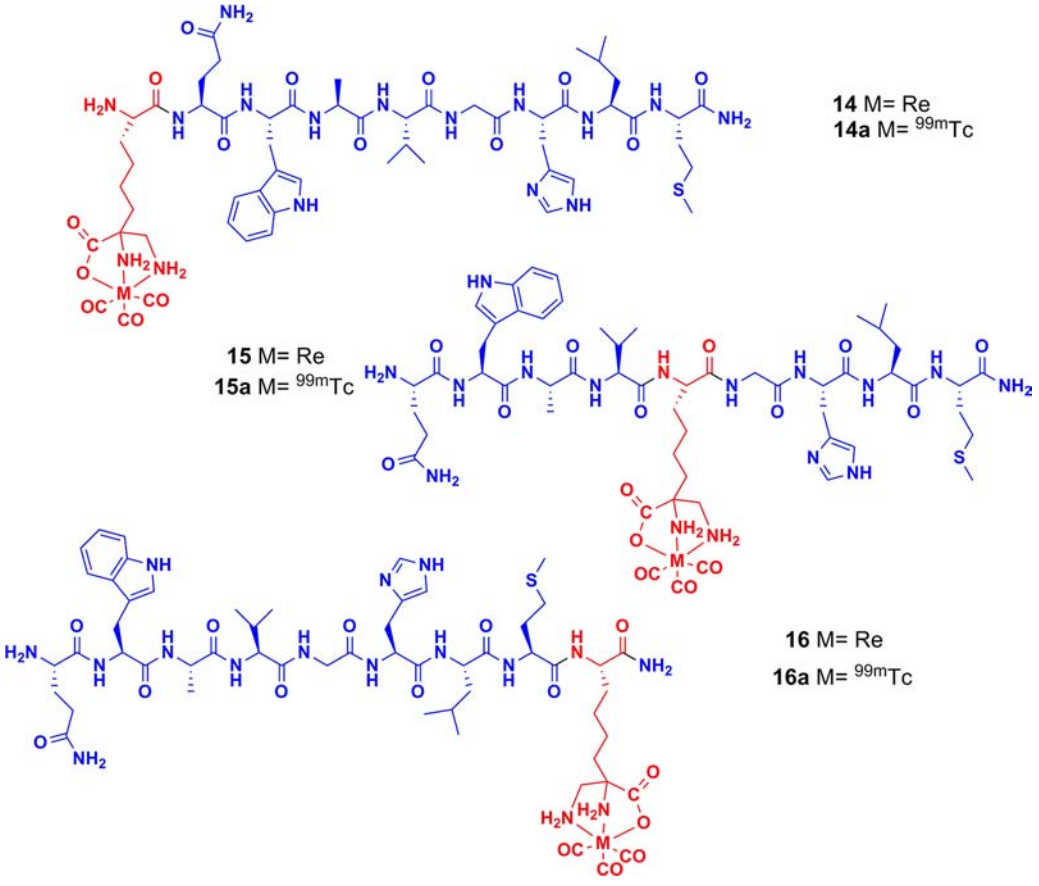
dx.doi.org/10.1021/bc3003327 | *Bioconjugate Chem.* 2013, 24, 26–35

Scheme 2. Synthesis of the Orthogonally Protected BFC 2[a]



[a](a) LiOH, 4 °C, benzaldehyde; (b) EtOH/1 M NaOH, benzyl chloroformate, −20 °C; (c) 4 M NaOH, pH = 9.5, sodium nitroprusside; (d) $PhCH_2Br$, Et_3N; (e) $MeSO_2Cl$, CH_2Cl_2, Et_3N; (f) ethyl 2-(*tert*-butoxycarbonylamino)-2-cyanoacetate, Na[OEt], DMSO, 70 °C; (g) Boc_2O, $Na[BH_4]$, $NiCl_2{\cdot}6H_2O$, MeOH; (h) Pt/C, H_2, EtOH; (i) FmocCl, H_2O/dioxane, Na_2CO_3, r.t.

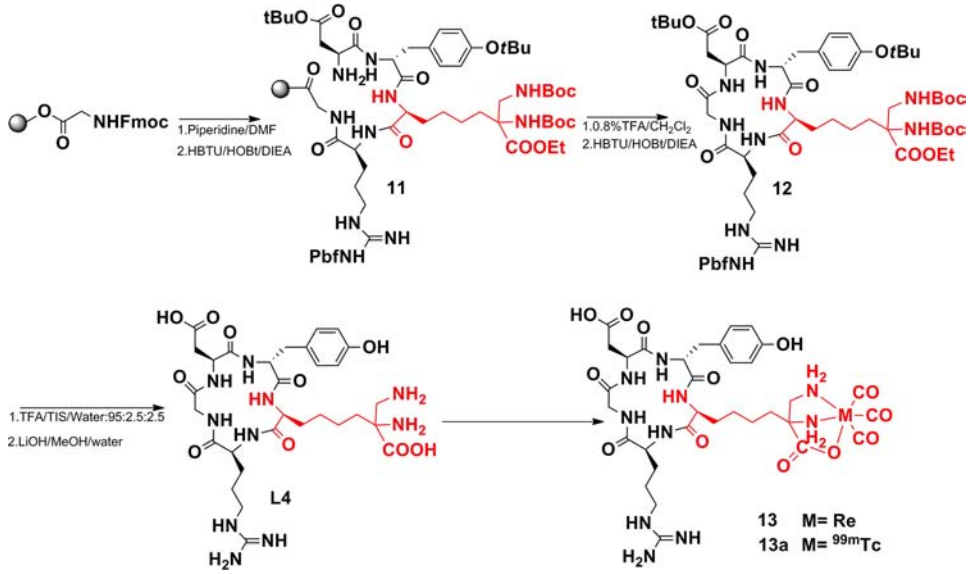
Scheme 3. Structures of the Different BBN(7-14) Derivatives of L1−L3 with the L-Lys(DAP) Amino Acid Conjugated to the N-Terminus (Top), Integrated into the Sequence (Middle), and Bound to the C-Terminus (Bottom)[a]



[a]Before coordinating **L1−L3** to the $\{Re(CO)_3\}^+$ or the $\{^{99m}Tc(CO)_3\}^+$ moiety, respectively, the carboxylate group in the Dap chelator is protected by an ethyl ester. Hydrolysis occurs concertedly with coordination.

dx.doi.org/10.1021/bc3003327 | *Bioconjugate Chem.* 2013, 24, 26−35

Scheme 4. Synthesis of RGD Peptide Analogue cyclo-(Arg-Gly-Asp-D-Tyr-Lys(DAP)) and Its Re (13) and ^{99m}Tc (13a) Complexes: $[Re(OH_2)_3(CO)_3]^+/H_2O$, 70°C; $[^{99m}Tc(OH_2)_3(CO)_3]^+$/PBS, 90 °C



RESULTS AND DISCUSSION

Synthesis. The synthesis of Fmoc-Lys(Dap)-OH **2** followed an orthogonal protection strategy starting from L-lysine as shown in Scheme 2. In the first step, the ε-amino group was masked by formation of the benzylidene imine **3**. Benzyloxycarbonyl was then introduced for protecting the α-amino group. Hydrolytic in situ cleavage of the ε-imine gives the *N*-α-benzyloxycarbonyl-L-lysine **4**. In a key transformation, the ε-amino group of **4** was converted to a hydroxyl group to yield Cbz-protected L-α-aminoadipic acid **5**. The carboxylic acid moiety was protected to give the benzyl ester **6**, and the ε-hydroxyl function was mesylated to yield **7**. Nucleophilic displacement of the mesylate by the sodium salt of ethyl acetamido-cyanoacetate was difficult but proceeded in reasonable yield when carried out in DMSO.[39] The resulting product **8** was catalytically reduced to Boc protected **9** in the presence of $Na[BH_4]$. The removal of carboxy-benzyl (Cbz) and the benzyl (Bn) protecting groups by catalytic hydrogenation gave the desired compound **10**. The final step was Fmoc-protection of the α-amine group under basic conditions to yield the final product, the orthogonally protected bifunctional chelator **2**. The overall yield of the synthesis starting from L-lysine is about 10% (Scheme 2).

Although the synthesis of the orthogonally protected bifunctional chelator **2** requires nine steps when starting from L-Lys, the individual steps are fast and provide good yields. In addition, reagents are cheap and the procedure is feasible on a large scale without difficulties. Building block **2** offers numerous possibilities for derivatization and conjugation. Since it is an α-amino acid, it can be subjected to SPPS with essentially any peptide sequence (vide infra). Coupling to other targeting molecules via either the $-NH_2$ or the $-COOH$ group (or both) is possible without affecting the coordinating properties of the second function. Deprotection of the ligand yields a very strong chelator not only for the $[^{99m}Tc(CO)_3]^+$ moiety, but potentially also for other metals. In addition, the ligand $-NH_2$ groups can be extended by further chelating functions, yielding ligands with a denticity higher than three.

Peptide Conjugation. To assess the utility of **2**, three different peptide sequences, each containing nine residues but with a variable L-Lys(Dap) position, were selected for the synthesis of BBN analogues. In those sequences, the single amino acid chelate L-Lys(Dap) was introduced at the N- or C-terminus or near the center of the peptide. The protected amino acids were assembled sequentially on the Rink amide resin using a standard HBTU coupling protocol. Peptides were cleaved from the resin with simultaneous Boc protecting group removal using a cocktail consisting of 85% TFA, 5% thioanisole, 5% phenol, and 5% water. It is important to exclude oxygen during the cleavage reaction in order to avoid oxidation of the methionine containing peptides. Following precipitation using cold methyl *t*-butyl ether and centrifugation, the peptide–chelator conjugates were purified by RP-HPLC (SI). Peptides were obtained in overall yields of 45–50% based on the initial resin loading of 0.69 mmol g^{-1}.

The synthetic strategy for preparation of the cyclic RGD peptide is shown in Scheme 4. Essentially, the synthesis involved three key steps: (1) attachment to the solid support via the α-carboxyl of Fmoc-Gly-OH, (2) linear chain formation, and (3) head-to-tail cyclization in solution through amide bond formation between the α-carboxyl group of Gly and the α-amino group of Asp. Purification of the crude products with preparative RP HPLC yielded the target compound **L4** in 20% overall yield, with >95% purity as determined by analytical HPLC.

Rhenium Complexes. The complexes were synthesized as references for the corresponding ^{99m}Tc-labeled peptides. Aqueous solutions of peptides (**L1–L4**) were reacted with $[Re(OH_2)_3(CO)_3]^+$ in H_2O under N_2 at 70 °C for 6–20 h to give the Re compounds in quantitative yields (Schemes 3 and 4). The conjugates were purified by RP-HPLC and lyophilized. ESI-MS gave the correct $[m/z]^+$ (SI). No release of the $\{Re^I(CO)_3\}$ moiety from the peptides was observed, demonstrating the stability of the complex.

^{99m}Tc-Labeling Studies. $[^{99m}Tc(OH_2)_3(CO)_3]^+$ was prepared from $Na^{99m}TcO_4$ according to literature methods.[36] Aqueous ^{99m}Tc solutions in the concentration range of 10^{-6} to 10^{-9} M were adjusted to pH = 7 with 1 M HCl and labeling

dx.doi.org/10.1021/bc3003327 | *Bioconjugate Chem.* 2013, 24, 26–35

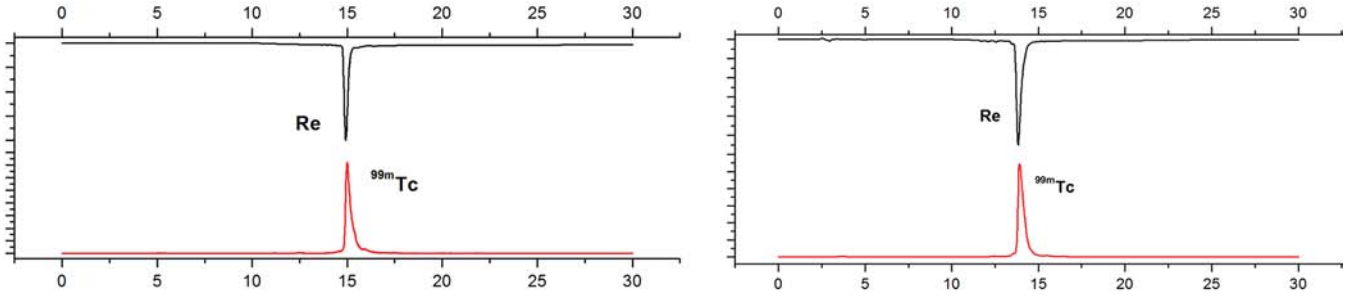


Figure 1. HPLC traces of $[M(\mathbf{L1})(CO)_3]$ (**14**, M = Re and **14a** M = ^{99m}Tc) conjugates (left) and of $[M(\mathbf{L4})(CO)_3]$ (**13** M = Re and **13a** M = ^{99m}Tc) (right).

Table 1. Competitive Binding Affinities of L4 and $[Re(\mathbf{L4})(CO)_3]$ to Different Solubilized Integrins Determined via ELISA[a]

	$\alpha_v\beta_6$[c]	$\alpha_v\beta_1$[d]	$\alpha_v\beta_5$[e]	$\alpha_{IIb}\beta_3$[f]	$\alpha_v\beta_3$[g]
reference peptide[b]	30 (3.5)	6.1 (0.5)	41 (1.2)	1.5 (0.3)	0.2 (0.02)
L4	254 (4.1)	41 (1.9)	30 (3.1)	440 (8.3)	3.4 (0.05)
$[Re(\mathbf{L4})(CO)_3]$	540 (3.9)	42 (1.4)	72 (1.2)	432 (5.1)	7.1 (1.2)

[a]Data represent IC_{50} values [nM] (mean of 4–6 separate experiments (SD)). [b]Data for the respective reference peptides are cited from the literature. [c]Reference peptide RTDlinear IC_{50} = 30 nM.[43] [d]Reference peptide Cilengitide IC_{50} = 15 nM.[44] [e]Reference peptide Cilengitide IC_{50} = 50 nM.[45] [f]Reference peptide Tirofiban IC_{50} = 0.6 nM.[44] [g]Reference peptide Cilengitide IC_{50} = 0.5 nM[35,46]

efficiencies determined by reacting these stock solutions with peptide concentration from 10^{-3} to 10^{-5} M. Over this range, **L1** and **L4** could be labeled with $[^{99m}Tc(OH_2)_3(CO)_3]^+$ at 90 °C after 30 min in yields better than 98%. Even at a concentration of 10^{-6} M, the peptides could be labeled in 85% yield after 70 min, underlining the efficacy of the Dap chelator. In the presence of 0.1 M cysteine or histidine at 37 °C, no transmetalation was found after 24 h, further confirming the chemical robustness of the Dap complex.[27] It should be emphasized at this point that the labeling yield did not depend on the absolute amount of ^{99m}Tc activity, an observation in agreement with the pseudo first-order labeling kinetics in Dap. Typical specific activities as achieved in our labeling studies were on the order of 1.1 TBq/μmol. Dap is, thus, suitable for labeling biomolecules to very high specific activities. It is important to note that specific activities are calculated from the crude radiolabeling solution and that, due to the very low amounts of Dap-conjugate needed for efficient $[^{99m}Tc(OH_2)_3(CO)_3]^+$ complexation, an HPLC separation of ^{99m}Tc-labeled product from unreacted precursor is not necessary. The identity of the ^{99m}Tc-labeled conjugates was confirmed by HPLC coinjection with the corresponding rhenium complexes (Figure 1). Due to the close structural similarity between Re and Tc homologues, retention times should be close to identical.

In Vitro Evaluation. For an exemplary evaluation of the effect of Lys-by-Lys(Dap) substitution on receptor binding affinity of cyclic RGD analogues, we investigated the binding affinity of $[Re(\mathbf{L4})(CO)_3]$ to different clinically relevant integrin receptor subtypes.[40,41]

The integrins with the highest documented relevance for imaging applications contain the α_v subunit, especially the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ subtype. The former is known to be overexpressed on many tumor types and tumor neovasculature, but is also expressed at lower levels in noncancerous tissues.[42] Table 1 summarizes the binding affinities (IC_{50} in nM) of $[Re(\mathbf{L4})(CO)_3]$ as well as of the uncomplexed labeling precursor **L4** to these and three other integrin receptor subtypes.

The rhenium reference peptide $[Re(\mathbf{L4})(CO)_3]$ and the uncomplexed labeling precursor **L4** display similar IC_{50} values for all integrin subtypes investigated. However, in the case of the $\alpha_v\beta_6$, $\alpha_v\beta_5$, and $\alpha_v\beta_3$ integrins, complexation with the $[Re(CO)_3]^+$ fragment leads to a loss in binding affinity by a factor of 2. Nevertheless, binding affinity of **L4** integrates well into a series of different RGD-based precursors for ^{99m}Tc-labeling such as Pz1-RGD, HYNIC-RGD, Cys-RGD, and L2-cRGD with IC_{50} values of 3, 6, 6.6, and 11.8 nM, respectively, in a comparable assay.[47] The $\alpha_v\beta_3$-integrin affinity of $[Re(\mathbf{L4})(CO)_3]$ is comparable to that of other clinically used radiolabeled RGD-analogues such as $[^{18}F]$Galacto-RGD. $[^{18}F]$-Galacto-RGD shows an affinity of 5 nM to the immobilized $\alpha_v\beta_3$ receptor, but significantly higher $\alpha_v\beta_3$ selectivity (IC_{50} ($\alpha_v\beta_5$) = 1000 nM, IC_{50} ($\alpha_{IIb}\beta_3$) = 6000 nM) than the peptides investigated in this study.[48]

The results obtained in the binding study using M21 cells and $[^{125}I]$echistatin as the radioligand show the same tendency: $Re(CO)_3$-complexation of **L4** leads to a loss in binding affinity (IC_{50} (**L4**) = 242 nM, IC_{50} ($[Re(\mathbf{L4})(CO)_3]$) = 866 nM), albeit somewhat more pronounced than observed when using immobilized integrin. The affinity determined for $[^{18}F]$Galacto-RGD in the same assay is 319 nM.[49]

In Vivo Biodistribution Study. The biodistribution data obtained for $[^{99m}Tc(\mathbf{L4})(CO)_3]$ in M21 melanoma bearing nude mice are summarized in Table 2.

$[^{99m}Tc(\mathbf{L4})(CO)_3]$ shows rapid clearance from the circulation and no particular predominance of renal vs hepatobiliary clearance or vice versa, but modest accumulation in all excretion organs. This represents a major advantage of this new Lys(Dap)-coupled RGD analogue over previous derivatives using other BFCs for $[^{99m}Tc(CO)_3]^+$ complexation.[50] For example, $[^{99m}Tc(CO)_3]$Pz1-cRGD, in which pyrazolyl functionalities serves for $[^{99m}Tc(CO)_3]^+$ complexation, shows 5-fold higher hepatic and intestinal accumulation. This may be due to the significantly reduced lipophilicity of $[^{99m}Tc(\mathbf{L4})(CO)_3]$ as compared to $[^{99m}Tc(CO)_3]$Pz1-cRGD (log P = −1.82 vs −0.92). Surprisingly, the accumulation of $[^{99m}Tc(\mathbf{L4})(CO)_3]$ in the excretion organs is as low as or even lower than that of

dx.doi.org/10.1021/bc3003327 | Bioconjugate Chem. 2013, 24, 26−35

Table 2. Biodistribution of [^{99m}Tc(L4)(CO)$_3$] in M21 Melanoma Bearing Nude Mice 60 min p.i.[a]

organ	control	blocking study	tumor/organ ratio
blood	0.37 ± 0.02	0.31 ± 0.25	3.8 ± 0.5
heart	0.34 ± 0.02	0.20 ± 0.04	4.2 ± 0.5
lung	0.78 ± 0.13	0.41 ± 0.06	1.8 ± 0.4
spleen	0.63 ± 0.13	0.22 ± 0.04	2.3 ± 0.5
pancreas	0.24 ± 0.01	0.10 ± 0.01	5.9 ± 0.7
liver	2.10 ± 0.50	1.42 ± 0.27	0.7 ± 0.2
stomach	1.02 ± 0.15	0.35 ± 0.23	1.4 ± 0.3
intestines	1.89 ± 0.45	1.31 ± 0.77	0.8 ± 0.2
kidney	2.02 ± 0.29	1.22 ± 0.22	0.7 ± 0.1
muscle	0.18 ± 0.01	0.07 ± 0.02	7.7 ± 1.0
tumor	1.41 ± 0.16	0.25 ± 0.14	

[a]Control animals (n = 5) were injected with tracer only, for the blocking study (n = 3), the tracer and 590 μg Cilengitide per mouse (23.5 mg/kg body weight) were coinjected. Data are given as %ID/g and are means ± SD.

[^{99m}Tc]EDDA/HYNIC-cRGD, which shows an even lower log P of −3.57.[47] This emphasizes once more the observation that, especially in the case of ^{99m}Tc-labeled peptide radiopharmaceuticals, the labeling method itself rather than the physical parameter "hydrophilicity" governs excretion pathways from the circulation.

Absolute tumor accumulation of [^{99m}Tc(**L4**)(CO)$_3$] is lower than that observed for [^{99m}Tc(CO)$_3$]Pz1-cRGD or [^{99m}Tc]-EDDA/HYNIC-cRGD in the same tumor model at 1 h p.i. (2.5% and 2.7% ID/g, respectively). However, as demonstrated by the blocking experiment (coinjection of an excess of unlabeled Cilengitide), tumor uptake is almost exclusively integrin-mediated, which highlights the integrin targeting efficiency of [^{99m}Tc(**L4**)(CO)$_3$].

Due to its comparably low accumulation in nontarget tissues, [^{99m}Tc(**L4**)(CO)$_3$] shows reasonable tumor-to-background ratios (Table 2), which approximate or even exceed those previously achieved with ^{99m}Tc-labeled monomeric RGD peptides with higher tumor accumulation such as [^{99m}Tc]-EDDA/HYNIC-cRGD (t/blood: 2.8, t/liver: 1.0, t/intestines: 1.3, t/kidney: 0.7, t/muscle: 3.6). These features make [^{99m}Tc(**L4**)(CO)$_3$] a promising candidate for future evaluation in small animal SPECT-imaging of integrin expression.

CONCLUSION

Orthogonally protected L-Lys(Dap) represents an artificial, single amino acid chelate which can be implemented into solid-phase peptide syntheses. Being an orthogonally protected amino acid, incorporation into any desired position in the peptide sequence is possible and thus enables facile preparation of libraries of peptides including a single amino acid chelate at any position. We have demonstrated this concept by conjugating L-Lys(Dap) to three different positions in BBN(7−14). In addition, we have replaced the original lysine in the c-RGDyK sequence. As shown in a receptor binding study, both introduction of the Lys(Dap)-moiety as well as complex formation with [Re(CO)$_3$]$^+$ do not seriously affect binding affinity of the peptide to different integrin subtypes, in particular, to the $\alpha_v\beta_3$ receptor. Labeling of L-Lys(Dap)-functionalized peptides with the [^{99m}Tc(CO)$_3$]$^+$ core usually proceeds quantitatively at around 10 μM concentrations, leading to ^{99m}Tc-labeled peptide radiopharmaceuticals with high specific activities. Overall, the investigated Dap-based tripod ligand is one of the smallest and most efficient tridentate ligands for [^{99m}Tc(CO)$_3$]$^+$ complexation described so far, and is suited for labeling not only peptides, but also other biomolecules or pharmacophores. As HYNIC, it has the potential to become a standard in ^{99m}Tc-based molecular imaging, but unlike for HYNIC-based ^{99m}Tc complexes, the authenticity of the conjugates is well-defined.

ASSOCIATED CONTENT

Supporting Information

Additional analytical data such as ^{13}C NMR, HPLC traces, and mass spectra and general synthetic procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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