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Biotin Derivatives Carrying Two Chelating DOTA Units. Synthesis, in Vitro Evaluation of Biotinidases Resistance, Avidin Binding, and Radiolabeling Tests

Alessandro Pratesi,[†] Francesca Bucelli,[†,¶] Ilaria Mori,[†,#] Marco Chinol,[§] Antonio Verdoliva,[‡] Giovanni Paganelli,[§] Vincenzo Rivieccio,[‡] Lucia Gariboldi,[§] and Mauro Ginanneschi*[,†]

[†]*Laboratory of Chemistry and Biology of Peptides and Proteins, Department of Organic Chemistry "Ugo Schiff", Via della Lastruccia 13, 50019 Sesto Fiorentino, Firenze, Italy,* [§]*European Institute of Oncology (IEO), Via Ripamonti 435, 20141 Milano, Italy, and* [‡]*Research and Development, Biochemistry Unit, Tecnogen S.p.A., Località La Fagianeria, 81013, Piana di Monte Verna, Caserta, Italy.* [¶]*Present address: Department of Public Health, Viale G. B. Morgagni 48, 50134 Firenze, Italy.* [#]*Present address: Novartis Vaccines and Diagnostics srl, Via Fiorentina 1, 53100 Siena, Italy.*

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The synthesis of four biotin derivatives carrying two DOTA moieties for each ligand (BisDOTA set) is reported, for increasing radiation/dose ratio and improving efficiency in the pretargeted avidin−biotin radioimmunotherapy. The biotin-containing scaffold of two BisDOTA was similar to the mono-DOTA derivative previously described. Then the scaffold was elongated by trifunctionalized spacers of different length and conjugated with one of the COOH groups of two DOTA. Two others were prepared starting from a on-resin lysine residue. The lysine α-NH_2 was bonded to biotin, and then spacers were appended to the ε-NH_2 and conjugated with two DOTA molecules. One compound contained a *p*-aminobenzoic acid spacer, which ensured higher head-to-tail distance and increased rigidity of the chain. These last two compounds had a very high ability to bond avidin and were labeled with ^{90}Y at high specific activity. All the compounds were resistant to the action of serum biotinidases.

Introduction

Tumor therapy is mostly implemented through the use of substances targeted at destroying cancer cells. This can be achieved with cytotoxic substances, which have to penetrate into the tumor cells in order to exert their full effect or by means of treatment of the tumor cells with radiation of sufficient energy to kill the cells. In both cases the main problem is to deliver the substance in a selective manner to the target cells to avoid possible damage to the surrounding healthy cells. The very high binding affinity of (+)-biotin toward the xenobiotic proteins avidin (Av) and streptavidin (Sav)[1] prompted researches in multidiscipcilinary fields to address the use of labeled biotin derivatives, in combination with Av or Sav and monoclonal antibodies (MoAbs), for delivering diagnostic or therapeutic radionuclides to cancer cells.[2] Actually, the so-called pretargeting protocols are widely used in the clinical practice[3−8] and, among them, the flexible three-steps method appeared especially useful for therapy of several type of tumors.[7,9−13] In designing new biotin conjugates for pretargeted radiotherapy of cancer, one must take in account that amide bonds between the valeric acid side arm of biotin and the spacer carrying the radiolabeled moiety could be degraded by the action of the serum biotinidases.[14] At the same time, molecular changes of the biotin side arm, carrying the radionuclide chelating unity, can affect the binding to Av or Sav, diminishing the affinity and causing loss of the radiating potency on cancer cells. The pivotal work of Wilbur's group[14,15] and of other researchers[16] has highlighted some structural characteristics of the biotin conjugates which hamper the action of biotinidase and allow retention of high affinity for Av and Sav pockets. Recently, J. Hainsworth et al. demonstrated that DOTA[a]-(α)-biotinamidolysine (Chart 1) is 93% streptavidin bound and that this molecule shows high stability in serum.[17] On the other hand, Pazy et al. showed that the Av pocket is much more sensible than Sav to structural changes on the biotin side chain, which caused the disordered conformation of a critical loop.[18] On the basis of these findings, these authors suggested an explanation of the fundamental role of streptavidin when administred in the three-step procedure.[13,14] Recently, we prepared a new biotin−mono-DOTA conjugate, r-BHD (Chart 2),[19] where the original carboxy group of the biotin was bonded to diaminohexane, then the amide CO reduced to CH_2 group, and finally, the *N*-hexylamine coupled to one of the four carboxymethyl chains of DOTA chelator. Preclinical evaluations (radiolabeling studies, binding to Av, and in vitro stability of the ^{90}Y labeled

*To whom correspondence should be addressed. Phone: +39 055 4573525. Fax: +39 055 4573531. E-mail: mauro.ginanneschi@unifi.it.

[a] Abbreviations: DOTA, 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-1,4,7,10-tetraacetic acid; DIPEA, diisopropylethylamine; DMF, dimethylformamide; DMSO-d_6, hexadeuteriodimethyl sulfoxide; DTPA, diethylenetriaminepentaacetic acid; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; FCC, fast column chromatography; Fmoc, 9*H*-fluoren-9-ylmethyloxycarbonyl; HABA, 4′-hydroxyazobenzene-2-carboxylic acid; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazole(4,5-*b*)pyridium-3-oxide hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; ITLC-SC, silica gel instant thin layer chromatography; MTT, methyltrityl; NMM, *N*-methylmorpholine; NMP, 1-methyl-2-pyrrolidinone; NMS, normal mouse serum; RCP, radiochemical purity; SPE, solid phase extraction; sulfo-NHS, *N*-hydroxysulfosuccinimide sodium salt; TBTU, 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium-3-oxide tetrafluoroborate; TFA, trifluoroacetic acid; THF, tetrahydrofurane; TIS, triisopropylsilane.

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complex) suggested that this bioconjugate is a good candidate for future applications in pretargeting protocols.[19] However, in the three-step pretargeting technique, the radioactivity is carried on the tumor by the radiolabeled biotin functionalized with the suitable chelating agent. Since only a small percentage really localizes on the tumoral lesion, it is important that the DOTA−biotin conjugate is labeled at high specific activity. The maximum allowed stoichiometry of a DOTA conjugated molecule is one metallic radionuclide per molecule, and this may limit the dose effectively delivered to the tumor. This prompted us to synthesize and test new biotin derivatives, carrying two DOTA groups per molecule (BisDOTA set). Substantially, BisDOTA are biotins modified through bifunctional spacers of different lengths and chemical structures, conjugated with two molecules of DOTA. Our aim was to label the BisDOTA set at higher specific activity than the mono-DOTA analogue, r-BHD. In fact, each BisDOTA can theoretically bind up to two radionuclide ions. In this way, the radiolabeled derivatives could deliver a higher radiation dose to the tumor and then improve the efficiency of targeted radionuclide therapy, provided the affinity for Av is retained at the highest level. We report herewith the synthesis of these new biotin derivatives featured by spacers chosen with the aim to have a suitable distance between the bulky DOTA chelating groups and, at the same time, between them and the biotine head (Schemes 1, 3, and 4). While two of the new derivatives (**5a** and **5b**) were prepared starting from the previously reported biotin *N*-hexylamino derivative, precursor of r-BHD (Schemes 1 and 3), the other compounds **12** and **16** were obtained by conjugation of biotin with the α-NH_2 of a Lys molecule via solid phase peptide synthesis (SPPS) strategy (Scheme 4). The avidin binding ability of BisDOTA derivatives was experimentally investigated by competitive binding studies with HABA/avidin complex, testing the thermal stability of Av/BisDOTA complexes by using CD spectroscopy. The Lys containing BisDOTA/avidin complexes showed an excellent stability when compared to the native (+)-biotin.

Chart 1. Carboxylate Function α to the Biotinamide

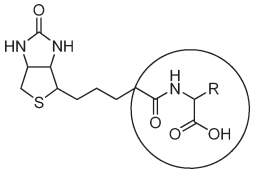
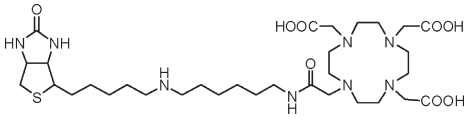
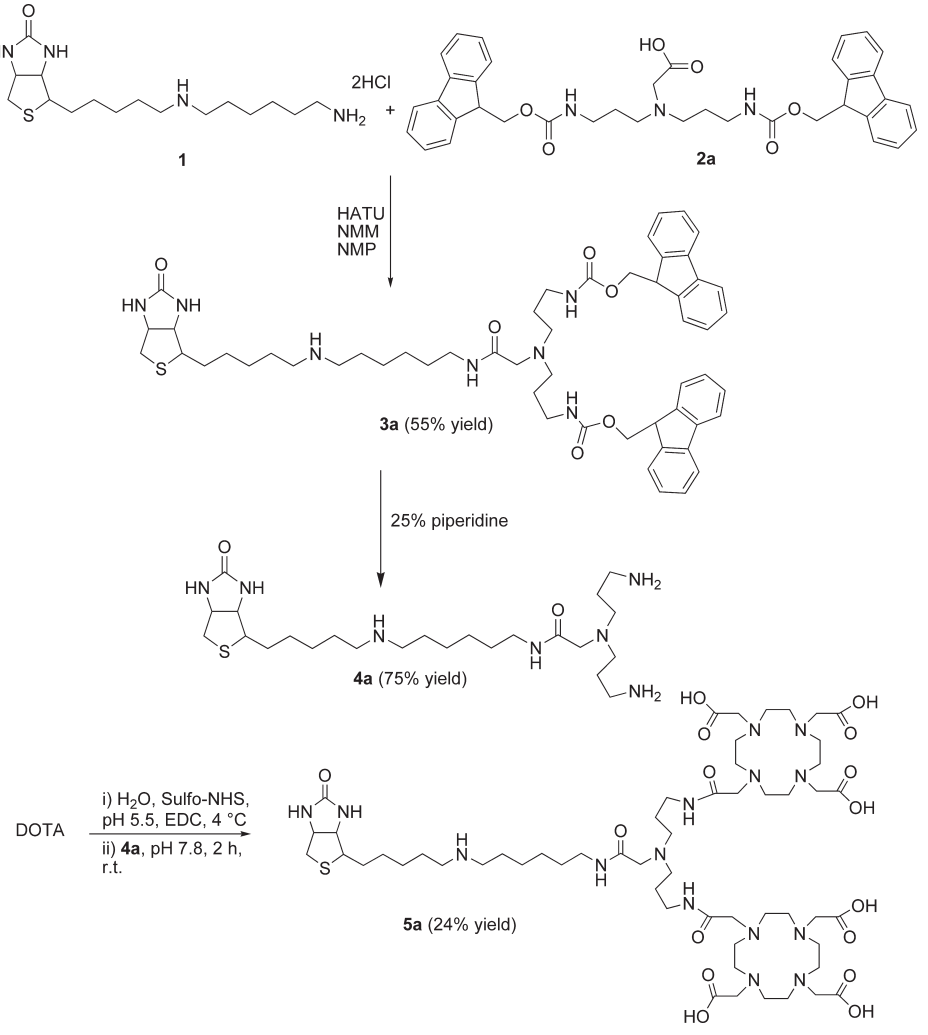


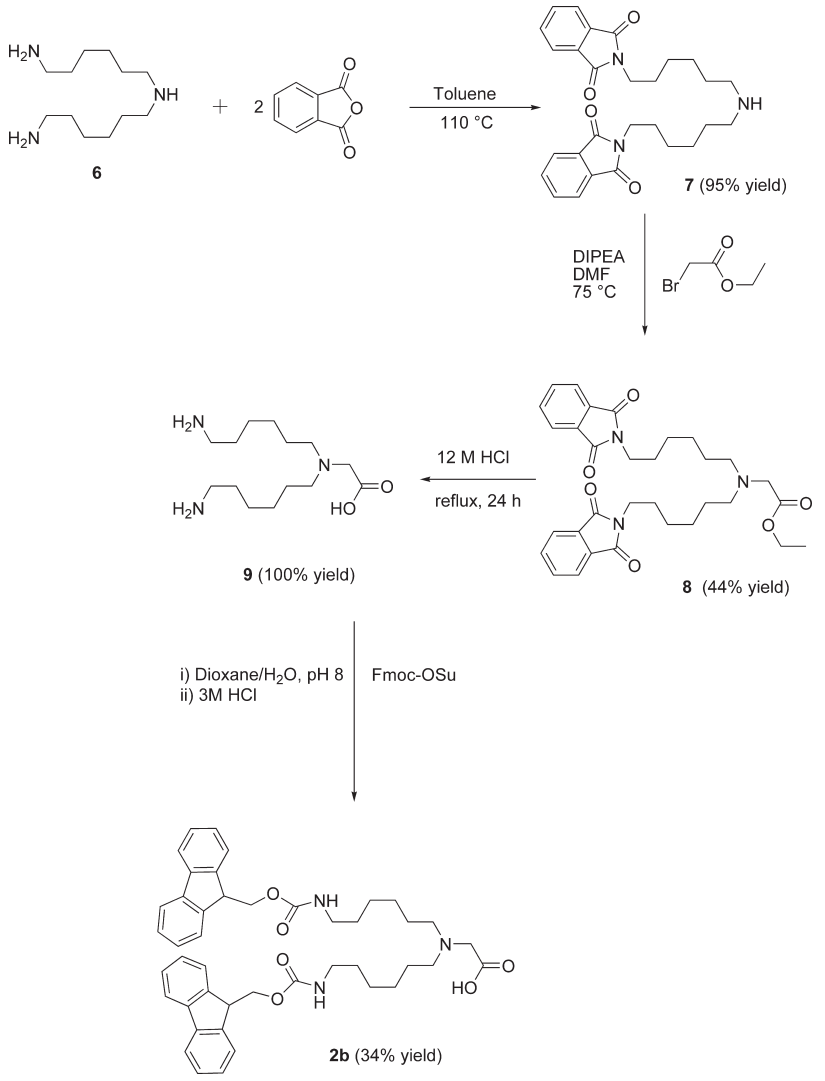
Chart 2. Structure of r-BHD



Scheme 1. Synthesis of BisDOTA-C_3



Scheme 2. Synthesis of the Fmoc-Protected Spacer C_6 (**2b**)

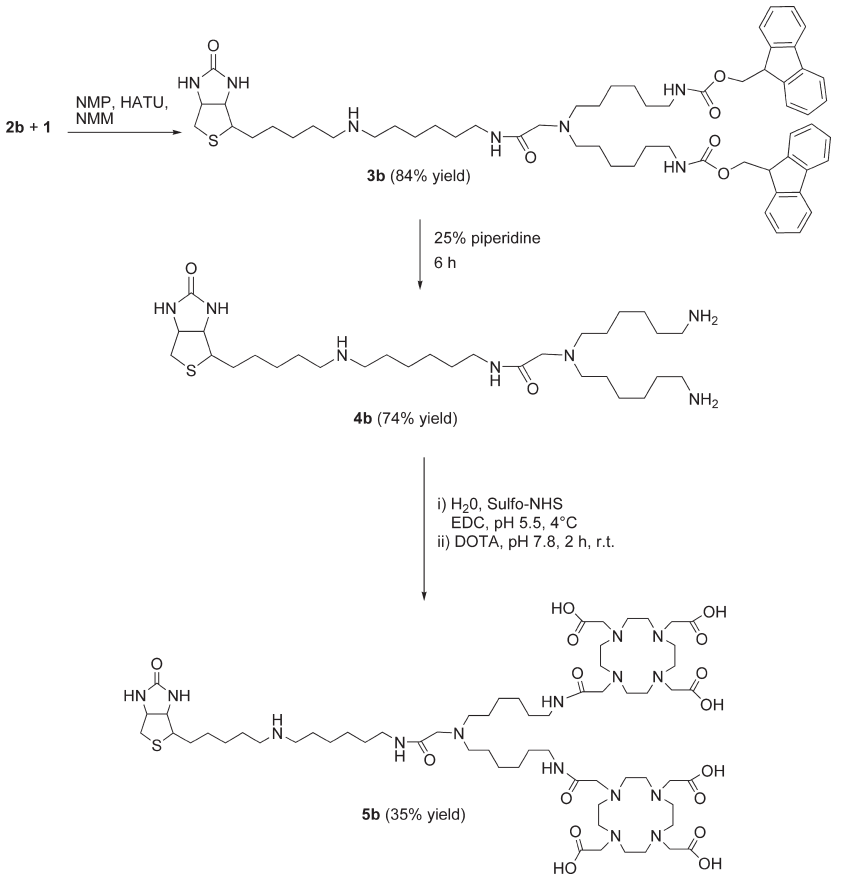


Moreover, biotinidase stability assay was also performed on these compounds, giving for all BisDOTA the expected good results.

Results and Discussion

Synthesis. BisDOTA derivative **5a** was prepared starting from the *N*-hexylaminobiotinyl amine **1** (Scheme 1) that was insensitive to the action of biotinidases.[19] The spacer **2a** was then introduced on the amino side arm for appending the two DOTA moieties. Notably, the spacer lengths were chosen in order to avoid the two chelating groups hanging too far away from the avidin pocket. The first spacer, **2a**, is commercially available as hydrogen sulfate salt. All the reaction steps were carried out in solution. Because of the low solubility of **1** in the commonly used organic solvents, the coupling with **2a** was carried out in NMP. The carboxy group of the spacer **2a** was activated by the uronium (aminium) salt before the addition of the amino component.[20] Moreover, the coupling requires basic conditions, and the use of the tertiary base NMM avoids reaction with the **2a** active ester. However, only 1 mol equiv of NMM was used for leaving most of the secondary NH protected as the hydrochloric acid salt. In the Fmoc deprotection of compound **3a**, piperidine should be accurately washed away because in the next step this base can compete in the coupling with the activated DOTA. This latter was activated following the method reported for the synthesis of r-BHD.[19] In order to minimize the risk of reaction on the secondary amine group, the coupling was performed at pH 7.8, and the presence of the free NH group in the final product **5a** was detected by NMR spectrum and confirmed by the Feigl spot test.[19] The synthesis of the BisDOTA homologue, **5b**, required the preparation of the new C_6-chain spacer, **2b**, that was obtained starting from the bis-(6-aminohexyl)amine **6** and following the method reported by Bambino et al. for the preparation of **2b**,[21] by means of an appropriate protection/deprotection sequence (Scheme 2). In the ESI spectrum of **2b**, the peak corresponding to m/z 1435.1 is a Fmoc-mediated cluster due to the experimental ionization and disappears in the MS−MS spectrum carried out with soft ionization conditions for avoiding fragmentation of the molecular peak. Ongoing from this step, the reaction pathway for the preparation of BisDOTA-C_6 followed the same procedure as for BisDOTA-C_3 (**5a**), affording the expected compound **5b** in higher yields than the corresponding C_3-derivative (Scheme 3). In compounds **5a**,**b**, the DOTA chelating groups are bonded to the biotinylated moiety by means of one of the four acetic acid side chains. We demonstrated in our preceding paper[19] that

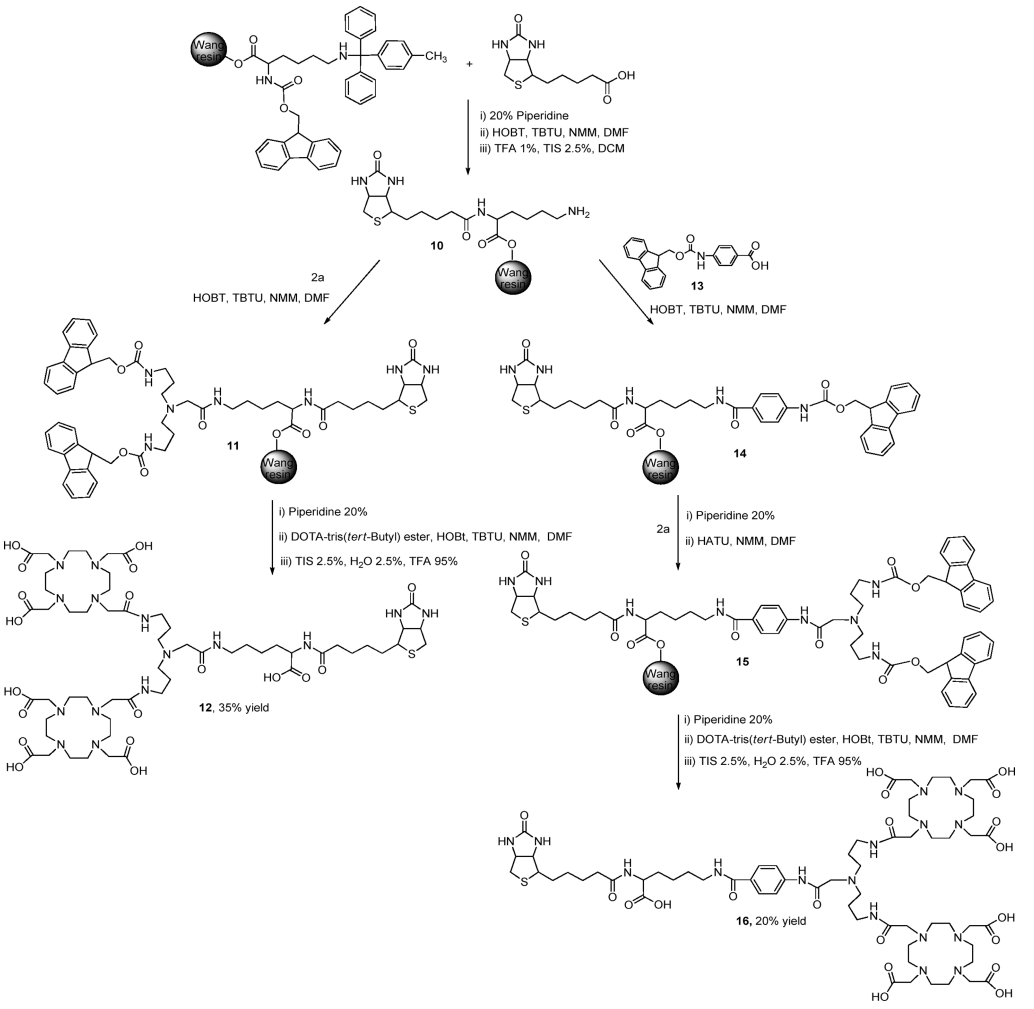
Scheme 3. Synthesis of BisDOTA-C_6



the use of one of the DOTA carboxy groups for the link to the biotinylated moiety did not significantly affected the ability of the azamacrocycle units to chelate the radioactive ions we used in the labeling. As depicted in Schemes 1 and 3, the coupling of DOTA to give compounds **5a** and **5b** was best achieved in aqueous solution by activating the free tetraacetic acid molecule with the water-soluble EDC/sulfo-NHS system. Taking into account the necessity of activating mainly one of the carboxylic acid groups, the solution pH was 5.5, as previously described.[19] This procedure, although giving low yields of the pure BisDOTA-C_3 and BisDOTA-C_6 (25−35%), is easy to scale up and is cheap. Another critical point of both of these synthetic routes is the preparation of the starting amine **1** by reduction of a suspension of the corresponding amide in THF with $BH_3 \cdot THF$ reagent. This reaction required a time-expensive workup of the crude product, and the final yield is not high.[19] The finds of Hainsworth et al. on the good affinity of biotin−lysine derivatives carrying one DOTA group for Sav[17] suggested us to bond biotin directly with the α-amino group of lysine and test the affinity of the so obtained BisDOTA derivatives to Av and, at the same time, their stability toward the biotinidases action. Concerning this latter point, we considered that the presence of two bulky groups like the DOTA moieties, very close each other, should be a barrier against the enzymatic hydrolysis despite the presence of an amide group near the biotin head. On this basis, we were prompted to prepare α-biotinamidolysine derivatives by coupling the biotin with L-lysine suitably protected on the ε-amino group. The introduction of the bifunctional spacer and the conjugation of two DOTA groups completed the synthetic design. Aiming to ensure a fast approach to the final derivatives, we decided to exploit the SPPS, which is routinely used in our laboratory, because this technique can rapidly afford the products and allow an easy scale-up of the synthesis. Moreover, in this technique, at the end of each step, the byproduct can be removed from the solid phase by simple washings.

The preparation of the biotinylated lysine derivatives BisDOTA-Lys-C_3 (**12**) and BisDOTA-Lys-(pAB)-C_3 (**16**) is outlined in Scheme 4. The orthogonally protected (Fmoc/MTT) lysine was purchased already bonded to Wang resin. After deprotection of the α-amino group by piperidine, only one coupling step with the activated (+)-biotin was necessary for the conjugation. Removal of the MTT group and reaction with the Fmoc-protected spacer **2a**, easy to hand in SPPS, afforded the protected diamine **11**. In another synthetic route, designed to move the bulk of the radiolabeled DOTA moiety further away from the Av pocket, we prepared a longer spacer holding an aromatic, rigid, structure. In this scheme, the on-resin biotinamidolysine was smoothly coupled with *N*-protected *p*-aminobenzoic acid (pAB) which, after deprotection, was, in turn, coupled with **2a** giving the bis-amine **15**. The poor nucleophilicity of the amino group of pAB complicated this coupling step and lowered the yields. Different activating agents, especially designed for peptide synthesis, were used in this task. In our conditions, the HATU/NMM method gave the best results. Because the Kaiser test for establishing the level of coupling of the NH_2 group of pAB gave unreliable results, the progress of the reaction was controlled by a series of microcleavages. Finally, after cleavage of the Fmoc protecting groups of **11** and **15**, 4-fold molar excess of tris(*tert*-butyl)

Scheme 4. Synthetic Route for BisDOTA-Lys-C_3 (**12**) and BisDOTA-Lys-(pAB)-C_3 (**16**)



ester of DOTA, well soluble in DMF and activated as reported in Scheme 4, was added giving the required BisDOTA. The on-resin final compounds were cleaved with a solution of 95% TFA, 2.5% H_2O, and 2.5% TIS. Notably, the very high TFA concentration was also able to hydrolyze the *tert*-butyl ester functions at room temperature (4−5 h), affording the expected BisDOTA **12** and **16** as the free carboxylic acids.

Testing Av Binding Ability and Thermal Stability of Av/BisDOTA Complexes. The avidin binding activity of BisDOTA derivatives was determined by HABA assay in comparison with (+)-biotin.[22] The assay utilizes the observation that HABA shows dramatic spectral changes when it binds to avidin. Free HABA has an absorption peak at 348 nm, while the HABA/avidin complex has strong absorption at 500 nm. Since the affinity between HABA and avidin is relatively weak ($K_d = 5.8 \times 10^{-6}$ M) compared to the affinity between (+)-biotin and avidin ($K_d = 1 \times 10^{-15}$ M), (+)-biotin can easily replace HABA from the HABA/avidin complex, resulting in a decrease of absorption at 500 nm. To estimate the *apparent* avidin binding affinity of biotin and BisDOTA derivatives, several competitive binding studies have been performed adding (+)-biotin and corresponding BisDOTA derivatives to the HABA/avidin sample and measuring any change in absorbance. As shown in Figure 1, all BisDOTA compounds were able to inhibit the HABA/avidin complex in a dose dependent manner.

Nevertheless, because of the differences in their spacer arms, BisDOTA-Lys-C_3 (**12**) and BisDOTA-Lys-(pAB)-C_3 (**16**) molecules showed an avidin binding capacity stronger than BisDOTA-C_3 and very similar to the native (+)-biotin. For sake of clarity, the relevant HABA displacement abilities are also compared in Table 1, comprising BisDOTA-C_6 that is not reported in Figure 1.

In strict agreement with these results, the BisDOTA-C_3 interaction with avidin stabilizes the complex, upon heat denaturation, only partially if compared to wild type avidin alone (Figure 2). In fact, the melting curve of avidin/BisDOTA-C_3 complex shows an inflection point at about 90 °C, which is higher than that of the avidin alone (about 80 °C). Indeed, the melting curves of avidin/BisDOTA-Lys-C_3 and avidin/BisDOTA-Lys-(pAB)-C_3 complexes show no valuable inflection point in the temperature range 25−95 °C, similar to avidin/(+)-biotin complex.

Results of the Evaluation of the Maximum SA. Three biotin derivatives, namely, **5a**, **12**, and **16**, were radiolabeled with ^{90}Y at high SA with high RCP. In particular, RCP above 99% was obtained with a SA of 2.6, 5.2, and 10.6 MBq/nmol. A further increase in SA (51.2, 212, 530 MBq/nmol) showed a decrease in RCP but still higher than 95%. The possibility of increasing considerably the specific activity maintaining high RCPs may represent a great advantage in the clinical application of the avidin−biotin system. A new targeted locoregional therapy, based on avidin and ^{90}Y labeled

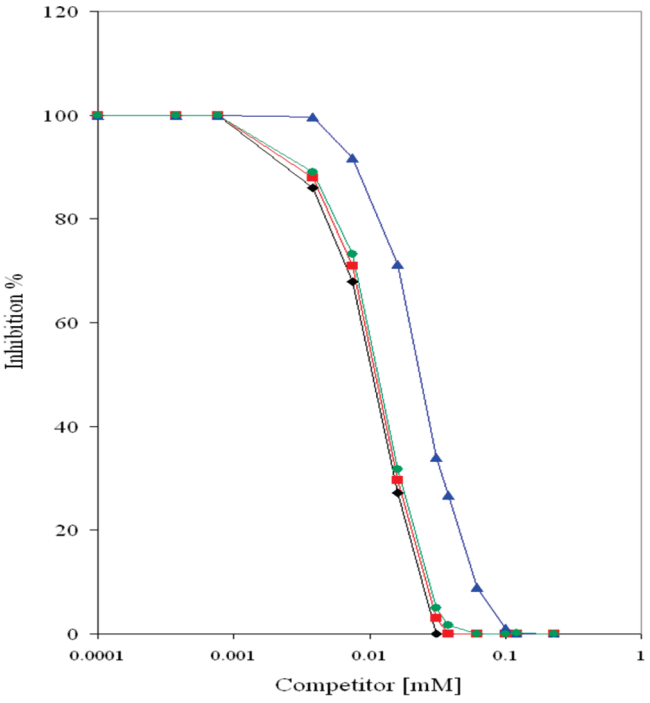


Figure 1. Inhibition of the HABA/avidin complex by (+)-biotin (◆, black line), BisDOTA-Lys-C_3 (■, red line), BisDOTA-Lys-(pAB)-C_3 (●, green line), and BisDOTA-C_3 (▲, blue line). Data are representative of three different experiments.

Table 1. Displacement of HABA from Avidin Complex by Biotin and BisDOTA Derivatives (31 μM, Av/Ligand = 1:4)

compd	HABA/Av inhibition, %
(+)-biotin	100
BisDOTA-C_3	66
BisDOTA-C_6	64
BisDOTA-Lys-C_3	95
BisDOTA-Lys-(pAB)-C_3	97

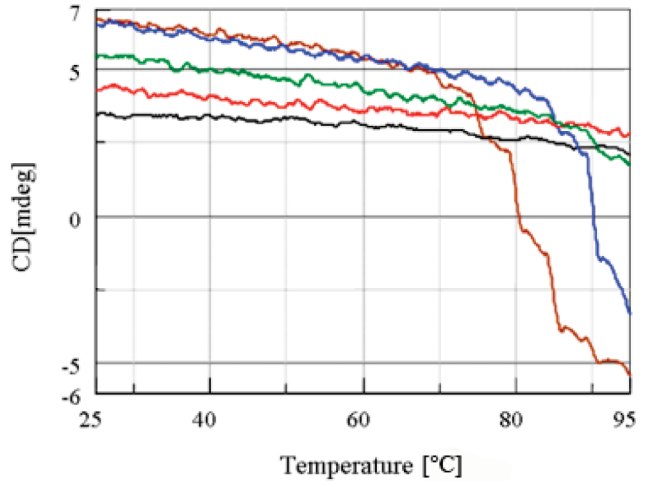


Figure 2. Melting curves of avidin (brown line), avidin/BisDOTA-C_3 complex (blue line), avidin/BisDOTA-Lys-C_3 complex (red line), Avidin/BisDOTA-Lys-(pAB)-C_3 complex (green line), and avidin/(+)-biotin complex (black line).

r-BHD, has shown great potential in the treatment of breast cancer reducing the duration of radiotherapy.[23] In this approach, r-BHD has been labeled at a specific activity of 2.6 MBq/nmol. Using the herein described BisDOTA derivatives radiolabeled at high SA may lead to a higher uptake by the targeted tumor area administering lower ^{90}Y activity, thus reducing the radiation burden to nontarget organs.

Stability Assay for Biotinidases. Biocytin is the natural substrate of the biotinidases, a hydrolytic enzyme family normally present in human and mouse sera, involved in the assimilation process of (+)-biotin (vitamin H) from diet. Activity of biotinidases in freshly collected NMS was assessed by incubating commercial biocytin with NMS, at 37 °C, and verifying the presence of (+)-biotin as product of the reaction. As shown in Figure 3 (panels A and B, profiles 2 and 3), after 2 h of incubation, biocytin (retention time 12 min; $[M + H]^+ = 373.5$ Da) was effectively hydrolyzed to produce a new species, at about $t_R = 12.5$ min, with a molecular weight $[M + H]^+ = 245.1$ Da, corresponding to (+)-biotin molecular weight (profile 3). Analysis of BisDOTA-Lys-(pAB)-C_3 (**16**) incubated with NMS for 2 h at 37 °C (profile 4) shows that no peak, with a retention time of about 12.5 min and corresponding to (+)-biotin, appeared, thus underlining the resistance of **16** to biotinidases activity. In strict agreement with these results, the peak of BisDOTA-Lys-(pAB)-C_3 was fully recovered after the incubation step. Similar results were obtained with BisDOTA-C_3, -C_6, and BisDOTA-Lys-C_3 derivatives (data not shown).

Conclusions

In summary, this study describes the syntheses and characterizations of four new biotin derivatives resistant to the biotinidases action and able to carry two radioisotopes for ligand unit. Radiolabeling experiments with ^{90}Y confirmed the expected achievable high specific activity. Experimental data on the thermal stability of avidin/ligand complexes suggested, at least for the derivatives containing a lysine spacer, a very high affinity for the protein and pointed out the different behavior between the spacers containing or not the lysine chain. Nevertheless, further studies in vitro and in vivo with the labeled BisDOTA will be necessary before considering these compounds good candidates for improving RIT protocols.

Experimental Procedures

General. DOTA and tris(*tert*-butyl)-DOTA were purchased from Macrocyclics (Richardson, TX) while standard (+)-biotin (B4639) and biocytin (B4261) were purchased from Sigma-Aldrich. *N*,*N*-Bis[3-(Fmoc-amino)propyl]glycin hydrogen sulfate potassium salt (**2a**) was from Fluka (Sigma-Aldrich, Switzerland). Fmoc-Lys(MTT)-Wang resin (100−200 mesh) was from Novabiochem AG (Laufelfingen, Switzerland), 6-Cl HOBt from Luxembourg (Israel), HATU from PerSeptive Biosystem, and TBTU from Chem Impex International (Wood Dale, IL). Peptide-synthesis grade DMF was from Scharlau (Barcelona, Spain). HPLC-grade MeCN was purchased from Carlo Erba (Italy). Dry solvents were distilled immediately before use: THF over sodium/benzophenone. All other chemicals were commercial compounds and were used as received. ^{90}Y chloride in hydrochloric acid (0.04 M) was purchased from QSA Global, Braunschweig, Germany. HABA was from Sigma-Aldrich (Switzerland) and avidin from Tecnogen (Italy). ^{1}H NMR spectra were recorded on a Varian Gemini 200 MHz or Varian Inova 400 MHz spectrometer in DMSO-d_6, D_2O, or $CDCl_3$. Chemical shifts are reported in ppm (δ) downfield from tetramethylsilane. CD spectra were acquired on Jasco J-715 spectropolarimeter equipped with a thermostatic water bath. Electrospray ionization mass spectra (ESI-MS) were acquired on LCQ-Advantage ESI ion trap spectrometer (Thermo Finnigan) for positive and negative ions detection. Fast atom

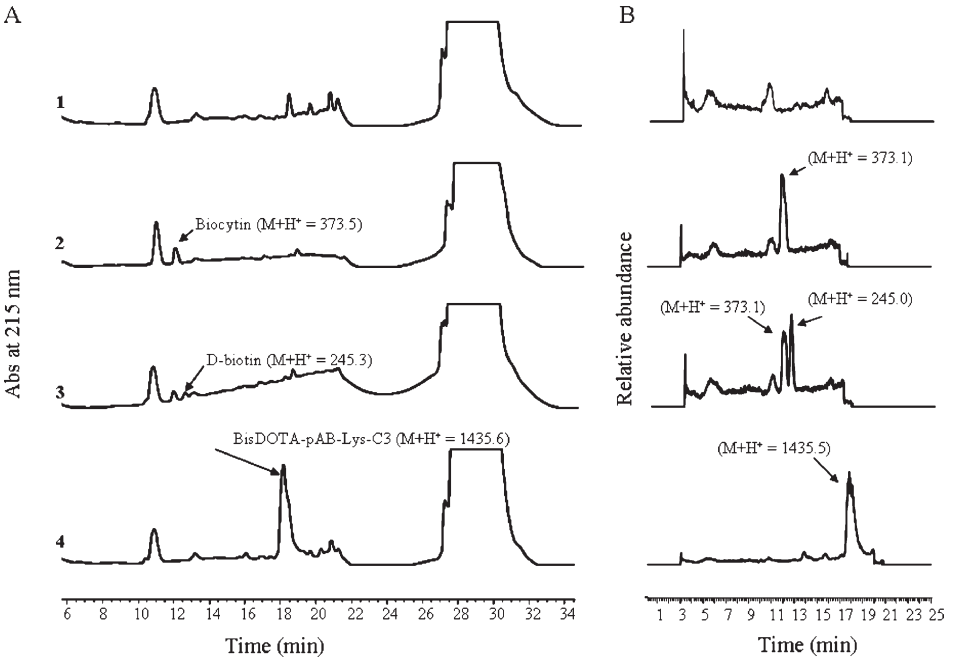


Figure 3. LC−MS analyses of BisDOTA derivatives resistance to biotinidase activity: (A) UV chromatograms from C_2−C_{18} RP-HPLC column; (B) total ionic current (TIC) from ESI source; (1) NMS; (2) biocytin in NMS at $t = 0$ min.; (3) biocytin in NMS incubated for 2 h at 37 °C; (4) BisDOTA-Lys-(pAB)-C_3 in NMS incubated for 2 h at 37 °C. In panel A arrows indicate the identified species, with their theoretical molecular weight as protonated species reported in parentheses and expressed in daltons. In panel B arrows indicate the corresponding molecular weights experimentally assigned and expressed in daltons.

bombardment mass spectrum (FAB-MS) was registered on a Finnigan-MAT TSQ70 triple stage quadrupole apparatus equipped with an Ion Tech (Teddington, U.K.) atom gun with xenon as bombarding gas and on a VG 70-250 FAB-MS spectrometer (Micromass). The purity of the final compounds (>95%) was checked by HPLC and, in some cases, also by combustion analysis, performed on a Perkin-Elmer 240 C elemental analyzer.

Chromatography. TLC was carried out on SiO_2 (Merck, 60 Å F_{254}), and spots were located with UV light (254 and 366 nm), methanolic ninhydrin, Fluram (Fluka; fluorescamine, 4-phenylspyro[furan-2(3*H*),1′-isobenzofuran]-3,3′-dione) in acetone, Cl_2/O-tolidine [*O*-tolidine (75 mg)], AcOH (3 mL), and KI (21 mg) in water (47 mL). SPE was performed on RP-LiChroprep resins, and FCC purifications were performed on silica gel 60, 40−63 μm, as detailed in the text. Semipreparative HPLC was carried out by a Supelco C_{18} 180 Å (250 mm × 10 mm, 5 μm, metals < 10 ppm) colum at 28 °C using a Beckman Gold Noveau instrument (detector diode array). Analytical HPLC was run on 5 μm Phenomenex Jupiter columns C_{18} 300 Å (250 mm × 4.6 mm). The solvent systems used for gradients were A (0.1% TFA in H_2O) and B (0.1% TFA in CH_3CN). The flow rates were 1 mL/min for analytical HPLC and 4 mL/min for semipreparative HPLC, with the linear gradients indicated when necessary. LC−ESI MS analyses were performed by a Phenomenex Aqua C_{18} column (5 μm, 150 mm × 2.0 mm) (flow rate, 200 μL/min) or a Vydac 218MS51 C_{18} mass spectometry column (5 μm, 250 mm × 1.0 mm) (flow rate, 300 μL/min) on a Thermo Finnigan Surveyor HPLC system coupled to the ESI-MS, using the solvent systems H_2O (A), MeCN (B), 1% TFA in H_2O (C). Silica gel ITLC paper strips for the labeling procedure were from ITLC-SG, Gelman Science, Ann Arbor, MI. The radiochromatographic profile was determined by Cyclone (Packard BioScience, Meriden, CT). For determination of the serum stability, the apparatus was a C_2−C_{18} RP-HPLC column (2.1 mm × 100 mm, GE Healthcare) connected to a LC−ESI-MS system (LCQ Duo, Thermo-Finnigan). The solvent systems used were A (0.08% TFA in H_2O) and B (0.08% TFA in CH_3CN).

Synthesis of BisDOTA-C_3 (5a). Bis[(9*H*-fluoren-9-yl)methyl]-3,3′-[2-oxo-2-[[6-[[5-[(3a*S*,4*S*,6a*R*)-hexahydro-2-oxo-1*H*-thieno-[3,4-*d*]imidazol-4yl]pentyl]amino]hexyl]amino]ethylazanodiyl] Bis-(propane-1,3-diyl) Dicarbamate (3a). The compound was obtained by adding the activated (+)-biotin (2 mol equiv) in two steps to a solution of **2a** in NMP and stirring for 5 h at room temperature. The product was purified by SPE (LiChroprep RP_8, 40−63 μm; 170 mm × 20 mm; eluent, $CH_3CN/H_2O/HCl$ = 50:50:0.1, 1 mL/min), and the yellow-orange oil was checked by TLC inspection ($CH_3CN/H_2O/HCl$ = 50:50:0.1). Analytical HPLC (30−100% of B in 20 min): t_R = 15.4 min. ^{1}H NMR (298 K), δ (DMSO-d_6, 200 MHz): 9.63 (br s, 1H), 8.64 (br s, 3H), 7.87 (d, 4H), 7.65 (d, 4H), 7.43−7.27 (m, 8H), 6.41 (d, 2H), 4.31−4.14 (m, 8H), 3.85 (s, 2H), 3.14−3.03 (m, 11H), 2.82−2.76 (m, 4H), 2.6 (d, 2H), 1.76−1.25 (m, 20H). ESI-MS (positive ions): m/z calcd $[M + H]^+$ 944.5; found 944.5 $[M + H]^+$.

2-[Bis(3-aminopropyl)amino]-*N*-[6-[[5-[(3a*S*,4*S*,6a*R*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl]pentyl]amino]hexyl]-acetamide (4a). The compound was obtained after evaporation of DMF solution, dissolved in MeOH, and precipitated by ethyl ether. The solid, dissolved in H_2O and lyophilized, was then purified by SPE (LiChroprep RP_{18}, 25−40 μm, 170 mm × 20 mm; eluent, H_2O/100%−MeOH 4−100%). The pure compound was collected from the aqueous fractions as a colorless solid. Analytical HPLC (5−15% of B in 30 min): t_R = 18.2 min. ^{1}H NMR (298 K), δ ($CDCl_3$, 200 MHz): 8.12 (br s, 2H), 6.45 (s, 2H), 4.35−4.25 (m, 1H), 4.15−4.05 (m, 1H), 3.59−2.95 (m, 7H), 2.95−2.70 (m, 12H), 2.50 (d, 2H), 1.80−1.20 (m, 20 H). ESI-MS (positive ions): m/z calcd $[M + H]^+$ 499.4; found 500.4 $[M + H]^+$.

2,2′,2″-[10-[2,9-Dioxo-22-[(3a*S*,4*S*,6a*R*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl]-7-[3-[2-[4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane-*N*-1-yl]acetamide]propyl]-3,7,10,17-tetraazadocosyl]-1,4,7,10-tetraazacyclododecane-*N*′,*N*″,*N*‴-1,4,7-triyl]triacetic Acid (5a). After the coupling of **4a** with DOTA, the aqueous solution was evaporated to dryness and the crude compound was purified by SPE as for **4a**. Crude **5a** was collected from the methanolic solution and further purified by semipreparative RP-HPLC, affording the pure compound as a colorless

solid. ^{1}H NMR (298 K), δ (D_2O, 400 MHz): 4.51−4.48 (m, 1H), 4.32−4.29 (m, 1H), 3.92 (br s, 2H), 3.72−2.65 (m, 65H), 1.81−1.23 (m, 20H). ESI-MS (positive ions): m/z calcd $[M + H]^+$ 1272.73, $[M + 2H]^{2+}$ 636.86 (100%); found 1272.71 $[M + H]^+$, 637.2 $[M + 2H]^{2+}$. Anal. ($C_{56}H_{101}N_{15}O_{16}S \cdot 6TFA \cdot 5H_2O$) C, H, N.

Synthesis of the C_6-Chain Spacer (2b). 2,2′-(Iminodi-6,1-hexanediyl)bis-1*H*-isoindole-1,3(2*H*)-dione (7). The compound was collected as an orange oil and used in the next step without further purification. ^{1}H NMR (298 K), δ ($CDCl_3$, 200 MHz): 7.81−7.77 (m, 4H), 7.68−7.64 (m, 4H), 3.62 (t, 4H), 2.57 (t, 4H), 2.20 (s, 1H), 1.75−1.55 (m, 4H), 1.5−1.4 (m, 8H), 1.35−1.2 (m, 4H). ESI-MS (positive ions): m/z calcd $[M + H]^+$ 476.3; found 476.5 $[M + H]^+$.

***N*,*N*-Bis[6-(1,3-dihydro-1,3-dioxo-2*H*-isoindole-2-yl)hexyl]-glycine Ethyl Ester (8).** The compound was collected from evaporation of DMF and purified through FCC (eluent, AcOEt/petroleum ether = 1:1), affording **8** as an yellow-orange oil. Analytical HPLC (30−80% of B in 20 min), t_R = 15.6 min. ^{1}H NMR (298 K), δ ($CDCl_3$, 200 MHz): 7.81−7.75 (m, 4H), 7.70−7.64 (m, 4H), 4.10 (q, 2H), 3.62 (t, 4H), 3.24 (s, 2H), 2.49 (t, 4H), 1.66−1.56 (m, 4H), 1.44−1.26 (m, 15H). ESI-MS (positive ions): m/z calcd $[M + H]^+$ 562.3; found 562.4 $[M + H]^+$.

***N*,*N*-Bis(6-aminohexyl)glycine (9).** Analytical HPLC (20−60% of B in 20 min): t_R = 6.47 min. ^{1}H NMR (298 K), δ (DMSO-d_6, 200 MHz): 10.22 (br s, 1H), 8.18 (br s, 4H), 4.06 (s, 2H), 3.13−3.08 (m, 4H), 2.8−2.55 (m, 4H), 1.58−1.55 (m, 8H), 1.4−1.15 (m, 8H). ESI-MS (positive ions): m/z calcd $[M + H]^+$ 274.3; found 274.3 $[M + H]^+$.

***N*,*N*-Bis[[[6-(9*H*-fluoren-9-ylmethoxy)carbonyl]amino]hexyl]-glycine (2b).** The compound was extracted from the reaction solution previously acidified with 3 M HCl. The crude product was purified by FCC (eluent, $CHCl_3$/MeOH = 3:1) to provide compound **2b**. Analytical HPLC (30−100% of B in 20 min): t_R = 19.67 min. ^{1}H NMR (298 K), δ ($CDCl_3$, 400 MHz): 7.71 (d, 4H), 7.56 (d, 4H), 7.38−7.22 (m, 8H), 4.33−4.29 (m, 2H), 4.16 (d, 4H), 3.60−3.44 (m, 2H) 3.3−2.95 (m, 8H), 1.72−1.20 (m, 16H). ESI-MS (positive ions): m/z calcd $[M + H]^+$ 718.4, $[2M + H]^+$ 1434.8; found 718.4 $[M + H]^+$, 1435.1 $[2M + H]^+$.

Synthesis of BisDOTA-C_6 (5b). *N*,*N*-Bis[(9*H*-fluoren-9-yl)methyl]3,3′-[2-oxo-2-[[6-[[5-[(3a*S*,4*S*,6a*R*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl]pentyl]amino]hexyl]amino]ethylazanodiyl]bis(hexan-1,6-diyl)dicarbamate (3b). After evaporation of NMP under reduced pressure, the oily residue was treated with H_2O and then purified by SPE (LiChroprep RP_8, 40−63 μm, 170 mm × 20 mm; eluent, H_2O/CH_3CN = 2:3, 1 mL/min), affording compound **3b**. Analytical HPLC as for **2b**: t_R = 16.57 min. ^{1}H NMR (298 K), δ (DMSO-d_6, 200 MHz): 7.84−7.20; Fmoc. ESI-MS (positive ions): m/z calcd $[M + H]^+$ 1028.6; found 1028.5 $[M + H]^+$.

2-[Bis(6-aminohexyl)amino]-*N*-[6-[[5-[(3a*S*,4*S*,6a*R*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl]pentyl]amino]hexyl]acetamide (4b). The crude compound was collected and purified as reported for **4a**. However, from the SPE eluate the pure product was found in the methanolic fraction as a colorless solid. Analytical HPLC (5−30% of B in 30 min): t_R = 18.48 min. ^{1}H NMR δ (DMSO-d_6, 200 MHz): Fmoc signals disappeared. ESI-MS (positive ions): m/z calcd $[M + H]^+$ 584.5; found 584.7 $[M + H]^+$.

2,2′,2″-[10-[16-Carboxy-2,9-dioxo-22-[(3a*S*,4*S*,6a*R*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl]-7-[6-[2-[4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane-*N*-1-yl]acetamide]-hexyl]-3,7,10,17-tetraazadocosyl]-1,4,7,10-tetraazacyclododecane-*N*′,*N*″,*N*‴-1,4,7-triyl]triacetic Acid (5b). The workup was carried out as reported for compound **5a**. Analytical HPLC as for **4b**: t_R = 19.8 min. ^{1}H NMR (298 K), δ (D_2O, 400 MHz): 4.55−4.50 (m, 1H), 4.33−4.30 (m, 1H), 3.98 (br s, 2H), 3.89−2.64 (m, 65H), 1.94−1.14 (m, 32H). ESI-MS (positive ions): m/z calcd $[M + H]^+$ 1356.82, $[M + 2H]^{2+}$ 678.91; found 1356.7 $[M + H]^+$, 679.2 $[M + 2H]^{2+}$ (100%). Anal. ($C_{62}H_{113}N_{15}O_{16}S \cdot 9HCl \cdot 8H_2O$) C, H, N.

Synthesis of BisDOTA-Lys-C_3 (12) and BisDOTA-Lys-(pAB)-C_3 (16). The products were synthesized using the classical SPPS method, on Fmoc-Lys(MTT)-Wang resin using the manual synthesizer PLS 4 × 4. Each coupling step was checked by performing a Kaiser test on microcleavages.[24] Briefly, the biotin was coupled to the deprotected lysine according to the TBTU/HOBt/NMM activation method, using a 2-fold molar excess of activated biotin. The removal of MTT group was performed with a solution of 1% TFA in DCM, containing 5% TIS as scavenger. The spacers **2a** and **13** were introduced by adding 2-fold molecular excess of the activated reagents, while the DOTA amount was 4 mol equiv. The intermediate compounds were checked by microcleavages and HPLC−MS spectra of the isolated compounds. After the DOTA coupling, the cleavage solution was concentrated under nitrogen fluxing, Et_2O was added, and the suspension was centrifuged and lyophilized. The crude products were purified by RP-HPLC (5−30% of B in 30 min). The yields were calculated on the basis of the resin loading.

2,2′,2″-[10-[16-Carboxy-2,9-dioxo-22-[(3a*S*,4*S*,6a*R*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl]-7-[3-[2-[4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-*N*-1-yl]acetamido]propyl]-3,7,10,17-tetraazadocosyl]-1,4,7,10-tetraazacyclododecane-*N*′,*N*″,*N*‴-1,4,7-triyl]triacetic Acid (12). Analytical HPLC was performed as for **5b**: t_R = 11.1 min. ^{1}H NMR (298 K), δ (D_2O, 400 MHz): 4.51−4.48 (m, 1H), 4.32−4.29 (m, 1H) 4.23−4.19 (m, 1H), 3,93−2.68 (m, 63H), 2.21 (t, 2H), 1.84−1.29 (m, 16H). ESI-MS (positive ions): m/z calcd $[M + H]^+$ 1316.68, $[M + 2H]^{2+}$ 658.84; found 1316.5 $[M + H]^+$, 659.2 $[M + 2H]^{2+}$ (100%). Anal. ($C_{56}H_{97}N_{15}O_{19}S \cdot 6H_2O \cdot 6TFA$) C, H, N.

2,2′,2″-[10-[2-[3-[[3-[2-[4,7,10-Tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-*N*-1-yl]acetamido]propyl][2-[4-[5-carboxy-5-[5-(2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido]-pentylcarbamoyl]phenylamino]-2-oxoethyl]amino]propylamino]-2-oxoethhyl]-1,4,7,10-tetraazacyclododecane-*N*′,*N*″,*N*‴-1,4,7-triyl]triacetic Acid (16). Analytical HPLC conditions were as for **12**: t_R = 12.97 min. ^{1}H NMR (298 K), δ (D_2O, 400 MHz): 7.70−7.52 (dd, 4H), 4.44−4.41 (m, 1H), 4.26−4.23 (m, 1H), 4.20−4.18 (m, 1H), 3.70−3.06 (m, 60H), 2.83−2,80 (m, 2H), 2.62−2.58 (m, 1H), 2.15−2.10 (m, 2H), 1.91−1.12 (m, 16H). ESI MS (positive ions): m/z calcd $[M + H]^+$ 1435.72, $[M + 2H]^{2+}$ 718.86; found 1436.6 $[M + H]^+$, 718.9 $[M + 2H]^{2+}$ (100%). Anal. ($C_{63}H_{102}N_{16}O_{20}S \cdot 7H_2O \cdot 8TFA$) C, H, N.

HABA Assay. Preliminary experiments were performed by varying the concentration of HABA (from 3 mM to 0.3 μM) and avidin (15.1, 7.6, and 3.8 μM) to calculate their optimal concentrations in the competitive binding studies. In the final experiments, carried out in 1 mL sample cuvettes, various concentrations of competitors [(+)-biotin and BisDOTA derivatives] were added to the HABA/Av complex, obtained by mixing avidin and HABA to a final concentration of 7.6 and 0.25 μM, respectively. Changes in absorbance were measured at 500 nm. All experiments were in triplicate, and the % inhibition was calculated as {[(absorbance with competitor) − (absorbance without HABA)/(Av complex)]/[(absorbance without competitor) − (absorbance without HABA)/(Av complex)]} × 100.

CD Assay on Avidin/Bis-DOTA Complexes Stability. Each spectrum (195−260 nm) was obtained from averaging three scans and subtracting the contribution from the buffer solution. Other experimental settings were as follows: 20 nm × min scan speed, 2.0 nm bandwidth, 0.2 nm resolution, 50 mdeg sensitivity, and 4 s response. Melting curves of wild type avidin, with or without 4 equiv of D-biotin and its BisDOTA derivatives, were recorded by following the decrease of dichroic signal at 225 nm, keeping the temperature in the range 25−95 °C. Instrument settings were as follows: bandwidth 1 nm, response 0.5 s, data pitch 1 °C, temperature slope 10 °C/min. At every temperature increment of 5 °C, a far-UV CD spectrum was collected in the experimental conditions reported above.

General Radiolabeling Procedure. Radiolabeling general procedure was performed using the three derivatives BisDOTA-C_3 (**5a**), BisDOTA-Lys-C_3 (**12**), and BisDOTA-Lys-(pAB)-C_3 (**16**) at a concentration of 3 mg/mL in saline. Equal volumes of 1.0 M sodium acetate buffer (pH 5.0) and of the radionuclide chloride solution were used. In each experiment, biotin solution was added to the buffer and transferred into the radionuclide supplier vial. The mixture was then mixed and heated at 95 °C for 30 min. RCP was assayed in triplicate for each radiolabeled sample by ITLC. An aliquot (usually 0.05 mL) of the radiolabeling solution was mixed with 0.2 mL of an avidin−DTPA solution (0.4 mM Av and 2.5 mM DTPA, final pH 6.0) and kept at room temperature for 5 min. Subsequently, an amount of 5 μL of the radioactive mixture was spotted on a silica gel ITLC paper strip and then developed in saline solution. In this chromatographic system, Av-radio-biotin complex remains at the origin whereas free radiometal, bound to DTPA, migrates to the solvent front. The radiochromatographic profile was determined by an autoradiographic system using high performance storage phosphor screen and RCP consequently calculated. Labeling yields greater than 97% were routinely achieved.

High Specific Activity (SA) Radiolabeling. To determine the achievable SA (expressed as MBq/nmol), the three derivatives **5a**, **12**, and **16** were radiolabeled with ^{90}Y at the following increasing of SA: 2.6, 5.2, 10.6, 51.2, 212, 530 MBq/nmol. RCP was determined in each sample as previously described.

Serum Stability. Normal mouse serum, freshly collected just before each experiment, was used as the source of biotinidase activity. Each experiment was carried out by mixing 50 μL of NMS with 10−25 μL of samples (BisDOTA derivatives or Biocytin). The volume was adjusted to 500 μL with reaction buffer (100 mM sodium acetate, pH 5.5), and each sample was incubated at 37 °C in water bath up to 2 h. To stop the reaction, an amount of 50 μL of each sample was diluted 10-fold with 0.08% TFA (v/v) in water, either before and after incubation, and 40 μL of this solution was applied to the HPLC columns. The chromatographic separations were carried out at a flow rate of 0.2 mL/min and by applying a gradient from 2% to 60% of B in 20 min. Main settings of ESI source were the following: spray voltage, 4.2 kV; capillary temperature, 300 °C; capillary voltage, 6 V.

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Supporting Information Available: Synthesis and purification of BisDOTA-C_3, spacer-C_6, BisDOTA-C_6, BisDOTA-Lys-C_3, and BisDOTA-Lys-(pAB)-C_3; HPLC, analytical, and ESI-MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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