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A New Bifunctional Chelate, $BrMe_2HBED$: An Effective Conjugate for Radiometals and Antibodies

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A new bifunctional chelate, *N*-(2-hydroxy-3,5-dimethylbenzyl)-*N'*-(2-hydroxy-5-(bromoacetamido)benzyl)ethylenediamine-*N*,*N'*-diacetic acid ($BrMe_2HBED$), was designed and synthesized to bind trivalent cationic metals with monoclonal antibodies. The stability constants (log values) for indium complexed with a similar ligand, HBED, were increased over those of more commonly used ligands DTPA and EDTA. Predictably, the increased metal–ligand complex stability would expedite the in vivo clearance from nontarget regions and perhaps enhance the localization of the radiolabeled antibody (Ab). $BrMe_2HBED$ was conjugated with the Ab (24 h) and then rabiolabeled with indium-111 citrate (24 h). Additionally, the Ab was radiolabeled by using conventional methods (^{111}In–DTPA and ^{125}I–lactoperoxidase) and then compared by measuring the in vitro stability, in vitro immunoreactivity (IR), and in vivo distribution and clearance. A 10:1 $BrMe_2HBED$:Ab mole ratio resulted in good labeling efficiency with ^{111}In and more importantly a very high IR. In a hamster tumor model, ^{111}In–$BrMe_2HBED$-labeled monoclonal antibody (1A3) had high uptake in the tumor tissue and preferable blood clearance compared to either of the more conventional radiolabeled 1A3 monoclonal antibodies (^{111}In–DTPA or ^{125}I–lactoperoxidase).

Introduction

Bifunctional chelation techniques have been used to bind radiometals (e.g. ^{111}In, ^{68}Ga, ^{153}Gd) to proteins and antibodies (Ab) in high yields.[1-15] Although high radiolabeling yields and, in some cases, retained immunoreactivity were reported, these radiopharmaceuticals have been found to be less than adequate diagnostic agents. In many cases, the radiolabeled antibody, when evaluated in vivo, demonstrates less than the desired localization after a reasonable time, high nontarget uptake, slow blood clearance, and/or uncertain metabolism of the radiopharmaceutical (potentially masking Ab localization in liver and kidneys and also increasing an unnecessary radiation dose to the clearance organs).

Our goals have been to design a bifunctional chelate that is capable of rapid clearance of the chelated protein complex from the blood, that does not interfere with the Ab immunoreactivity, that has a very high metal stability constant, and whose rapid clearance from the blood and nontarget organs reduces the total radiation dose. Indium-111 has been the most widely utilized radionuclide; its 2.8-day physical half-life is sufficiently long to obtain high target to nontarget ratios, and its medium-energy γ-rays are reasonable for imaging with conventional nuclear medicine equipment. Diethylenetriaminepentaacetic acid (DTPA) anhydride has been utilized to bind In-111 to proteins and Abs;[16-20] however, in vivo, the radiolabeled proteins have slow clearance and some or most of the radioactivity remains in the liver or kidneys.[21,22] Many investigators have described this incomplete clearance from the liver (intact antibody) or kidneys (antibody fragments) when ^{111}In–DTPA-conjugated Ab was used.[23-26] Other approaches to radiolabeling antibodies that used functionalized EDTA or DTPA to covalently attach the chelate to the antibody have been utilized with similar results.[13-15,27-32]

Recent work on in vivo biodistribution of the radiometal complexes of HBED [*N*,*N'*-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N'*-diacetic acid] and its analogues (alkyl groups added to the aromatic rings), where rapid liver and blood clearance was observed,[33] led to the selection of a multidentate ligand, Me_4HBED, as the ligand to be derivatized for protein labeling (Figure 1). This ligand was modified by functionalizing a benzene ring with $-NHCOCH_2Br$, an effective protein linking group that binds via covalent bond formation through the alkylation of free amino groups in the protein by the active bromomethylene function.[31,34]

Experimental Section

Materials and Methods. 2,4-Dimethylphenol, *N*-acetylethylenediamine, 2-hydroxy-5-nitrobenzaldehyde, palladium on activated carbon (10%), borane–tetrahydrofuran complex (1.0 M solution in tetrahydrofuran), bis(trimethylsilyl)acetamide (BSA), trimethylsilyl bromoacetate,

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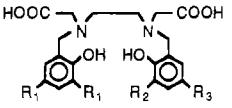


	R_1	R_2	R_3
HBED	H	H	H
Me_4HBED	CH_3	CH_3	CH_3
$BrMe_2$HBED	CH_3	H	$NHCOCH_2Br$

Figure 1. Structural formulas of the parent HBED ligand, the more lipophilic Me_4HBED, and the prototype bifunctional chelate $BrMe_2$HBED.

2,4,6-collidine, and bromoacetyl bromide were purchased from Aldrich Chemical Co., Inc., and were used without further purification. Benzene and methanol were dried with 4-Å molecular sieves, and tetrahydrofuran was refluxed with $LiAlH_4$ and redistilled. Thin-layer chromatography was performed on silica-60 F_{254} plates (EM Reagents). Sephadex G-50 and G-25 (Sigma Chemical Co.) was utilized for spin columns equilibrated with 0.01 M sodium citrate (Fisher Scientific Co.) (HPLC grade water from a Milli Q water system, Millipore Division, Waters Associates), pH adjusted to 8.0 with 1.0 N sodium hydroxide (Morton Thiokol, Alfa Products). Phosphate-buffered saline (PBS) consisted of 137 mM sodium chloride, 8.1 mM sodium phosphate, 1.46 mM potassium phosphate, and 2.7 mM potassium chloride, pH 7.3–7.4. Bovine immunoglobulin G (IgG) (Sigma Chemical Co.) was used to determine experimental conditions and initial radiolabeling efficacy. The Ab used in the animal studies was a murine monoclonal antibody (IgG_1, κ), designated 1A3, which binds to a lipid antigen found enriched in human colon cancer cells.[35-37] Hybridoma cells producing 1A3 were grown as ascites in pristane-primed BALB/c mice, and 1A3 monoclonal antibodies were purified by 40% $(NH_4)_2SO_4$ precipitation followed by Sephadex G-150 and DEAE Sephacryl chromatography. SDS-PAGE (7.5%) has shown these monoclonal Abs to be ca. 90% pure. The 1A3 was stored frozen (−80 °C) in PBS at ca. 7.5 mg/mL.

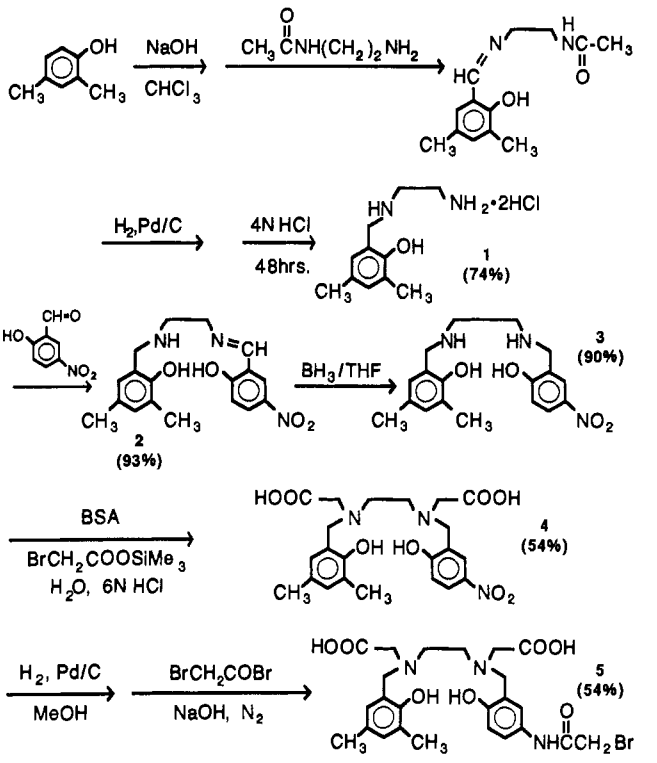
The radiolabeled Ab was evaluated by fast protein liquid chromatography (FPLC) on a Superose 12 column (Pharmacia/LKB) eluted with 0.05 M NaH_2PO_4/0.15 M NaCl (pH 7.2) at 0.5 mL/min. The column output is directly (flow thru) measured by a UV detector (280 nM) and for radioactivity [NaI (Tl), scintillation detector with a multichannel analyzer (Canberra)].

Indium-111 chloride (50 mCi/mL in sodium chloride, pH 1–2) was provided by Mallinckrodt, Inc. Ultrapure sodium acetate, indium acetate (99.99%), and indium chloride (99.999%) were purchased (Morton Thiokol, Aesar, and Aldrich Chemical Co., respectively). Diethylenetriaminepentaacetic acid (DTPA) anhydride was purchased (Sigma Chemical Co.) and diluted with chloroform (HPLC grade Fisher Scientific Co.). Glacial acetic acid was diluted to prepare indium solutions (Fisher Scientific Co.). Dehydrated ethanol was used in these experiments (USI Chemical Co.).

The proton NMR spectra were recorded with a Varian XL-200E NMR spectrometer operating at 200 MHz, and the chemical shifts are reported in ppm on the δ scale relative to tetramethylsilane. The mass spectra were obtained with a VG 70S analytical high-resolution double-focusing magnetic sector spectrometer, with attached VG 11/250J analytical data system. The C, H, and N elemental analyses were performed by Galbraith Laboratories, Inc.

Synthesis of $BrMe_2$HBED. The synthesis of $BrMe_2$HBED and intermediate compounds was carried out as shown in Scheme I and described below.

Scheme I



***N*-(2-Hydroxy-3,5-dimethylbenzyl)ethylenediamine (1).** 2-Hydroxy-3,5-dimethylbenzaldehyde (7.7 g, 0.051 mol), which was synthesized from 2,4-dimethylphenol by the Reimer–Tiemann reaction,[38] and 5.4 g (0.053 mol) of acetylethylenediamine were mixed and heated to 80 °C (bath) for about 30 min. About 120 mL of benzene was added and distilled azeotropically until the water produced was removed. The benzene was removed by distillation under reduced pressure, and a yellow crystalline residue was obtained. This Schiff base was dissolved in 120 mL of absolute ethanol, 1.0 g of Pd–C (10%) catalyst was added, and the mixture was hydrogenated under ~40 psi with vigorous shaking for 2 h. This reaction mixture was allowed to stand at room temperature for 16 h. The catalyst was filtered off, and the solvent was removed from the filtrate by vacuum evaporation. A nearly colorless product crystallized from the concentrated ethanol solution.

A 60-mL volume of a 4 N HCl solution was added to the above crude product, and the reaction mixture was heated to 110 °C (bath) for 48 h. After cooling, a large amount of crystalline material separated. This crude product was treated with charcoal and recrystallized from HCl–H_2O, washed with ethanol and ether, and air-dried. The total yield was 10.1 g, 74%, based on the starting aldehyde.

^{1}H NMR (in D_2O), (*p*-dioxane as internal standard at 3.58 ppm): 7.00 and 6.89 (s, 2 H, aromatic), 4.13 (s, 2 H, benzyl $-CH_2-$), 3.29 (s, 4 H, ethylene), 2.08 (s, 6 H, methyl).

***N*-(2-Hydroxy-3,5-dimethylbenzyl)-*N'*-(2-hydroxy-5-nitrobenzylidene)ethylenediamine (2).** To 20 mL of absolute ethanol were added 0.80 g (0.020 mol) of sodium hydroxide and 2.67 g (0.010 mol) of *N*-(2-hydroxy-3,5-dimethylbenzyl)ethylenediamine dihydrochloride. This mixture was stirred at room temperature for 2 h, and the sodium chloride was removed by filtration. To the filtrate was added 1.67 g (0.01 mol) of 2-hydroxy-5-nitrobenzaldehyde, and the reaction mixture was stirred at 80 °C for 1 h. The bright yellow precipitate that formed was collected by filtration, washed with ethanol and ether, and then air-dried. The total yield was 3.1 g (93%).

^{1}H NMR (in DMSO-d_6) 8.4 (d, 1 H, aromatic, ortho to NO_2), 8.2 (s, 1 H, benzylidene $-CH{=}N$), 8.1 and 8.0 (d, d, 1 H, aromatic, ortho to NO_2), 6.8, 6.7, and 6.6 (d, 3 H, aromatic), 3.8 (s, 2 H, benzyl $-CH_2-$), 3.7 (t, 2 H, $-CH_2-N{=}$), 2.9 (t, $-NH-CH_2-$), 2.1 and 2.0 (6 H, two methyls). Anal. Calcd for $C_{18}H_{21}N_3O_4$: C, 63.0; H, 6.12; N, 12.24. Found: C, 62.48; H, 6.15; N, 12.35.

***N*-(2-Hydroxy-3,5-dimethylbenzyl)-*N'*-(2-hydroxy-5-nitrobenzyl)ethylenediamine (3).** A 2.7-g (0.0079-mol) sample of the above Schiff base (**2**) was suspended in 40 mL of dry THF in an ice-water bath. While the suspension was covered with dry N_2 gas, 40 mL of 1.0 M borane–tetrahydrofuran complex solution was added over a 20-min pe-

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riod. The reaction mixture was stirred at room temperature for another 20 min, and 12 mL of water was carefully added, followed by 6 mL of 6 M HCl. The THF and water were removed by distillation and vacuum distillation. To the pale yellow residue thus obtained was added 45 mL of water to form a clear solution. The pH of this solution was adjusted to about pH 8, and a large amount of yellow precipitate formed. This product was thoroughly washed with distilled water and vacuum-dried under P_2O_5 at room temperature for 48 h; 2.79 g of product as the dihydrate was obtained. Yield: ~90%.

^{1}H NMR (D_2O–NaOD): 8.0 (m, 2 H, aromatic, ortho to NO_2), 6.9, 6.8, and 6.5 (d, 3 H, aromatic), 3.7 and 3.6 (s, 4 H, two benzyl $-CH_2-$), 2.8 (s, 4 H, ethylene), 2.2 (s, 6 H, two methyls). Anal. Calcd for $C_{18}H_{23}N_3O_4 \cdot 2H_2O$: C, 56.4; H, 7.05; N, 10.97. Found: C, 56.09; H, 6.14; N, 10.61.

***N*-(2-Hydroxy-3,5-dimethylbenzyl)-*N'*-(2-hydroxy-5-nitrobenzyl)-ethylenediamine-*N,N'*-diacetic Acid (4).** To 0.70 g (0.0020 mol) of **3** under N_2 gas was added 10 mL of BSA, and the mixture was heated to 50–60 °C for ~1 h until it became a clear pale yellow solution. The excess BSA and byproduct were removed by vacuum distillation. To the yellow sticky residue were added 2.0 mL of dry benzene, 6.6 mL of trimethylsilyl bromoacetate, and 1.0 mL of 2,4,6-collidine. The reaction mixture was stirred at room temperature for 3 days. The excess unreacted reagents were removed by distillation under reduced pressure. To the residue was added 20 mL of 6 M HCl, and the mixture was allowed to stand at room temperature for 20 h. The pH was adjusted with 2.5 M NaOH to pH 11, and a clear light brown solution was obtained. The collidine was removed by ether extraction, and the aqueous phase was neutralized with 6 M HCl. The precipitate that formed at pH 8.6 was removed by filtration and discarded. A large amount of pale yellow precipitate was separated at a pH range 5.7–2.5, which was collected by filtration, washed with 0.010 M HCl, and dried under vacuum at room temperature for 20 h. Yield: ~0.4 g. Upon cooling of the filtrate, an additional 0.10 g was obtained. Total yield: ~0.50 g (54%).

^{1}H NMR (D_2O–NaOD): 8.1 (m, 2 H, aromatic, ortho to NO_2), 6.8 (d, 2 H, aromatic), 6.5 (d, 1 H, aromatic), 3.5 (s, 4 H, two benzyl $-CH_2-$), 3.1 (s, 4 H, two acetate $-CH_2-$), 2.6 (s, 4 H, ethylene), 2.1 (s, 6 H, two methyls). Anal. Calcd for $C_{22}H_{27}N_3O_8 \cdot {}^1/_2H_2O$: C, 56.2; H, 5.96; N, 8.94. Found: C, 56.54; H, 5.90; N, 8.62.

***N*-(2-Hydroxy-3,5-dimethylbenzyl)-*N'*-(2-hydroxy-5-(bromoacetamido)benzyl)ethylenediamine-*N,N'*-diacetic Acid (5).** A 92-mg, 0.2-mmol sample of compound **4** was suspended in 10 mL of methanol, and 58 mg of Pd–C (10%) and 0.16 mL of 2.5 M NaOH were added. This mixture was allowed to react with 1 atm of H_2 in an ice-water bath for 6–7 h. The reaction mixture was vented with an Ar blanket, and 0.25 mL of 2.5 M HCl was added. The catalyst was removed by filtration, and the filtrate was evaporated under reduced pressure to dryness, giving the amino compound.

^{1}H NMR (D_2O–DCl): 7.4–6.85 (m, 5 H, aromatic), 4.3 (s, 4 H, two benzyl $-CH_2-$), 4.0 (m, 4 H, two acetate $-CH_2-$), 3.7 (m, 4 H, ethylene), 2.1 (s, 6 H, two methyls). FAB MS: m/e 432, $(M + H)^+$.

The amino compound from the above catalytic hydrogenation was placed in a small flask with a combination pH microelectrode and protected with argon gas in an ice-water bath. It was dissolved in 1.0 mL of degassed water and several drops of 2.5 M NaOH until the pH of the clear solution became 7.5–8.0. Bromoacetyl bromide was added in 10-μL portions. Whenever the pH of the reaction solution dropped to below 6.0, 1.0 M NaOH was added dropwise until the pH of the reaction solution became 7.5–8.0. About 6–8 portions of 10 μL of bromoacetyl bromide were added, and the solution was checked for the amino group until negative to fluorescamine.[39] The reaction was complete within 30–40 min, and 2.5 M HCl was then added until the pH of the aqueous phase became 2. The product precipitated as a pale brown solid that adhered to the wall of the flask. The aqueous phase, which contained sodium chloride, sodium bromide, and bromoacetic acid, was removed with a pipette. The product was washed with ether and 0.01 M HCl repeatedly to remove the excess bromoacetyl bromide, bromoacetic acid, and sodium salts. The product was vacuum-dried at 1 mmHg, 0 °C, for 1 h. FAB MS showed m/e 552 and 554, $(M + H)^+$ with the characteristic isotopic pattern for one Br atom. No hydrolyzed $HOCH_2CONH-C_6H_4-$ species peaks, which should have m/e 489 for $(M + H)^+$, were found. The total yield was 60 mg, which is ~50%, based on the nitro compound **4**.

Conjugation of Ab with $BrMe_2$HBED. The ligand was dissolved in ethanol (1.0 mg/mL) immediately prior to use. An appropriate volume of the solution was removed and roto-evaporated to leave a thin film on a 10-mL pear-shaped flask. The antibody in 0.01 M sodium citrate (pH 8) was quickly added to the flask with the ligand, and the solution was mixed gently for about 1 min and then transferred to a plastic tube. The ligand and antibody concentrations were determined so as to obtain the desired mole ratio; generally, 500 μg of Ab was used in a 10:1 (L:Ab) mole ratio. The antibody was incubated with $BrMe_2$HBED at room temperature for 24 h. The unbound $BrMe_2$HBED was removed from the $BrMe_2$HBED–Ab complex by gel chromatography separation on a 1-mL Sephadex G-50 spin column in 0.01 M sodium citrate (pH 8.0). The spin column technique[40] allows large molecular weight (>30000) compounds to pass through the gel bed during a 7-min centrifugation at 1000g (GLC-2B Sorvall centrifuge with HL4 swinging-bucket rotor). A 5–10% loss of mass can occur from the column procedure. A spin column prewashed with serum albumin eliminates the nonspecific binding on the column; instead, the recovery volume was determined and the protein concentration was measured by a colorometric assay (Bio-Rad protein assay).

DTPA conjugation of antibody was also carried out with the use of DTPA cyclic anhydride. The ligand solution was prepared by diluting 1 mg in 2 mL of chloroform, from which an aliquot (appropriate volume to achieve the desired mole ratio) was removed and evaporated to dryness with nitrogen. The antibody was added to the tube with the ligand film, and the solution was mixed gently. The L–Ab solution was transferred to a plastic tube and incubated at room temperature for 1 h; after the incubation, DTPA–Ab was separated on a Sephadex G-50 1-mL spin column. The conjugated antibody was then mixed with ^{111}In in citrate buffer (0.1 M, pH 8) or acetate buffer (0.1 M, pH ~5.5). The labeling was incubated at room temperature for 1 h and then purified on a 1-mL spin column. The ^{111}In-labeled antibody was collected with the column eluent. If radiolabeling was in low efficiency (<90%), subsequent purifications on additional spin columns were carried out.

Conjugation efficiency was determined by titration of the conjugated Ab with indium.[15,31,34,41-43] A small amount of ^{111}In was added as a tracer for the carrier indium. Radiolabeling was carried out as previously described with several aliquots of purified (no free ligand) conjugated Ab and various amounts of indium. The Ab samples were eluted from 1-mL Sephadex G-50 spin columns, and the radiolabeling efficiency was determined.

DTPA–Ab (10:1 in moles) was titrated with 20, 8, 4, 2, 1, and 0 excess moles of indium compared to moles of Ab. Indium acetate (1 mg/0.1 mL in 10% acetic acid, pH 1–2) diluted (1:10) with 0.4 M acetate buffer (pH 5) adjusted to a final pH of 4–5 with 1 N NaOH as necessary and doped with 7–10 μCi of indium-111 acetate (1 μL of $^{111}InCl_3$ in 76 μL of 0.4 M acetate buffer, pH 5) was utilized. Various volumes of the indium solution were diluted with 0.4 M acetate buffer (pH 5) to maintain a constant incubation volume and the desired moles of indium.

$BrMe_2$HBED–Ab (10:1 in moles) was titrated with 20, 8, 2, 1, 0.5, 0.2, and 0 excess moles of indium. The indium citrate solution (1 mg/mL of 0.1 M citrate buffer, pH 8) was mixed with radioactive indium-111 citrate (~10 μCi; 1 μL of ^{111}In-Cl_3 in 100 μL of 0.1 M citrate buffer, pH 8). Aliquots of the indium-111-enriched indium citrate were mixed with the purified conjugated Ab, maintaining a constant volume by the addition of the appropriate volume of 0.1 M citrate buffer, and then incubated at room temperature for 24 h. The In–$BrMe_2$HBED–Ab solutions were eluted on 1-mL spin columns, and the incorporation of indium was determined by measuring the effective radiolabeling.

Radiolabeling was achieved by mixing small aliquots (~20 μL) of indium-111 chloride with 100 μL of 0.1 M sodium citrate (final pH 7–8). Indium-111 citrate was added to the $BrMe_2$HBED–Ab recovered from the spin column. The labeling solution was incubated for 1–24 h at room temperature before a final purification was carried out with an additional 1-mL Sephadex G-50 spin column. ^{111}In–$BrMe_2$HBED–Ab passes through the column while unbound indium-111 remains within the gel bed. Control separations of indium-111 citrate, Ab, and conjugated Ab were carried out, and the amounts of recovered protein and radioactivity were determined. (Indium-111 citrate does not elute, while the Ab and conjugated Ab do elute, recovery ca. 90%.) Radiolabeling, then, was measured as the amount of radiolabeled material eluted through the spin column compared to the total amount applied to the column.

Iodinated 1A3 was prepared by using a conventional lactoperoxidase technique.[44] ^{125}I–1A3 was separated from unbound ^{125}I by use of a Sephadex G-25 spin column.[31,40] With the use of this method, the specific

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activity of labeled 1A3 after dilution with unlabeled 1A3 was 0.97 μCi/μg with 95% of the radioactivity recovered in a precipitable protein fraction (by TCA precipitation).[45]

In vitro immunoreactivity assay (IRA) was developed so the immunoreactivity of each radiolabeled Ab preparation could be tested prior to in vivo use. The direct binding assay uses GW39 human colon carcinoma cell suspensions as the target, and conditions of antigen excess as described elsewhere.[46] Immunoreactivity values were determined by using regression analysis and linear extrapolation of the data to binding at infinite antigen excess.[46]

Animal biodistribution experiments were carried out in 3–6-week-old immunocompetent male Golden Syrian hamsters with GW39 human colon carcinoma tumors.[47] A 0.5-mL volume of 50% (v/v) tumor cell suspensions was injected in the right-thigh musculature of the hamsters and allowed to grow for 2–10 days before radiolabeled 1A3 was injected. For 24 h prior to radiolabeled-1A3 injection, hamsters received potassium iodide in their drinking water to block thyroid uptake of any unbound ^{125}I. Hamsters were coinjected intracardially with 25 μg of ^{111}In–1A3, $BrMe_2HBED$ or DTPA conjugated, and 25 μg of ^{125}I–1A3. Control hamsters received 25 μg of ^{111}In- or ^{125}I-labeled 1A3 only. Tumors were harvested 1, 3, or 5 days after the radiolabeled 1A3 injection. Samples of blood, skin, muscle, liver, and intestine were taken as well as the entire tumor, bladder, heart, lungs, stomach, kidneys, spleen, and thyroid. All organs were rinsed in phosphate-buffered saline, blotted, weighed, and then counted in an automated γ scintillation NaI(Tl) well-type counter (Beckman Gamma 8000).

Results

The bifunctional ligand (**5** in Scheme I) is a sensitive compound that slowly deteriorates at room temperature. It was stored at 0 °C and was packed in dry ice when transported from Texas A&M to Washington University for antibody-labeling experiments. The instability is probably related to the fact that it has amido nitrogen and phenolic groups in para positions and thus may be prone to slow air oxidation. Fortunately, it is insoluble in water and in aqueous acid solution, and this was the basis for its separation and purification. Treatment of the solid with dilute HCl, as described in the Experimental Section, removed NaCl, NaBr, and any sodium bromoacetate that may have been formed by hydrolysis of excess bromoacetyl bromide. Extraction with ether removed excess bromoacetyl bromide, as well as any bromoacetic acid that may have been present. NMR measurements in D_2O were not carried out because of its insolubility in acid and the fact that it gradually loses the bromine by hydrolysis in basic solution, in which it is soluble. However, mass spectral measurements (FAB) provided the necessary characterization and criteria of purity. The pH of acylation was controlled at 7.5–8.0 to direct the reaction to the amino group, which is unprotonated in that pH range, and to prevent the possibility of O-acylation. The intense double spectral peak, at m/e for $(M + H)^+$ of 552 and 554, showing the isotopic ratio of Br, and complete absence of a peak at m/e for $(M + H)^+$ of 489 proved that the molecule had been acylated at the expected position and that hydrolysis of the bromine to form the $-NHCOCH_2OH$ group did not occur. It also demonstrated that no alkylation, which would produce the group $-NHCH_2COOH$, with the same mass, had taken place. The presence of only a single bromine in the bifunctional ligand also showed that additional acylation of the phenolic groups does not occur under the reaction conditions employed. It should also be noted that the acylation reaction, under the conditions described in the Experimental Section, is a virtually instantaneous reaction, but it was carried out stepwise over a 0.5-h period to maintain the pH control needed to prevent side reactions such as bromide hydrolysis and O-acylation.

The amount of $BrMe_2HBED$ conjugated to the Ab was varied from a 1:1 to 100:1 (L:Ab) mole ratio, and the radiolabeling incubation time allowed for the conjugation to occur was varied from 1 to 24 h. The increased incorporation of radioactivity, without regard for ligand concentration, after a 24-h conjugation and a subsequent incubation for 24 h with radioactivity gave the overall highest radiolabeling yields (Tables I and II). Additionally, the effect of added ligand (DTPA or $BrMe_2HBED$) concentration on immunoreactivity of radiolabeled 1A3 was examined. The immunoreactivity (% IR) was dramatically reduced as the concentration of added DTPA anhydride was increased, while in contrast there were only minimal differences observed in % IR with the $BrMe_2HBED$-conjugated 1A3 (Figure 2). In these cases, the $BrMe_2HBED$-conjugated 1A3 had greater retention of immunoreactivity than DTPA-conjugated 1A3 and values comparable or better than iodinated 1A3 as determined by IRA (Table III). The optimal conditions for radiolabeling Ab with $BrMe_2HBED$ and indium-111, then, are to conjugate $BrMe_2HBED$ and Ab at room temperature for 24 h (10:1 L:Ab mole ratio) and, after a purification on a spin column, radiolabel with Indium-111 citrate (pH ~8) for 24 h at room temperature. These conditions provided a radiolabeled antibody (83.4 ± 22.1%) with 82.8 ± 8.5% retained immunoreactivity (determined by IRA) (Tables I–III). The conjugated efficiencies of the bifunctional chelates were determined with a 10:1 L:Ab ratio. Knowing the

Table I. Effect of Incubation Time on Radiolabeling IgG after 24-h Conjugation with $BrMe_2HBED$ (10:1)

time, h	% radiolabeled	time, h	% radiolabeled
0.25	25.6 ± 2.1	3.5	53.7 ± 0.2
1.0	24.2 ± 2.7	6.0	63.1 ± 3.9
2.5	44.4 ± 0.4	18.0	73.8 ± 2.2
3.0	50.0 ± 3.7	24.0	85.7 ± 5.6

Table II. Effect of $BrMe_2HBED$ Concentration and Conjugation Time on Radiolabeling IgG (When Radiolabeled for 24 h)

L:Ab	conjugation time, h	% radiolabeled	L:Ab	conjugation time, h	% radiolabeled
2:1	1	18.1 ± 4.2	100:1	1	15.2 ± 8.1
	3	57.1 ± 1.1		3	65.0 ± 7.8
	24	75.5 ± 5.6		24	76.4 ± 3.0
10:1	1	14.5 ± 3.8			
	3	65.1 ± 11.9			
	24	85.7 ± 5.6			

Table III. Effect of Various Radiolabeling Techniques on IRA Values with 1A3

radioisotope	ligand	experiment no.	% IR
^{111}In	$BrMe_2HBED$	1	81
		2	92
		4	69
^{111}In	DTPA	3	61
		4	43
^{125}I	LP	1	76
		2	82
		3	89
		4	36

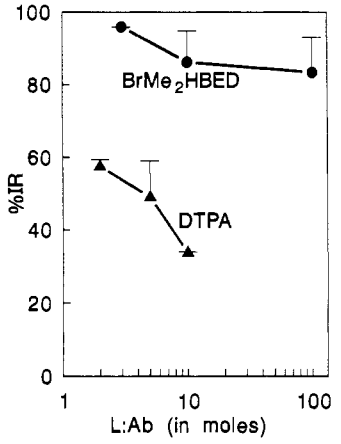

Figure 2. Effect of added ligand concentration (relative to Ab) on immunoreactivity (% IR).

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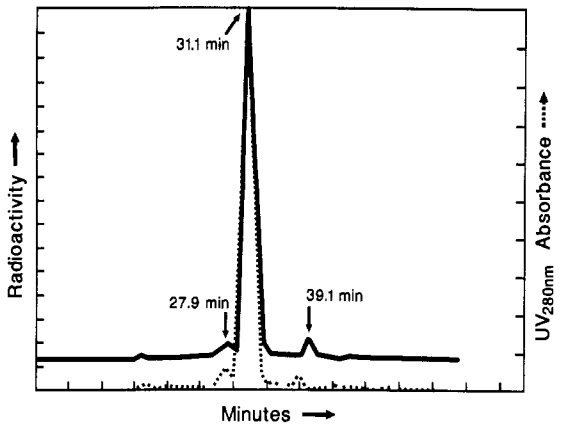

Figure 3. FPLC of ^{111}In-BrMe$_2$HBED-1A3 (—) and unlabeled 1A3 (···) on a Superose 12 column eluted with 0.05 M NaH_2PO_4/0.15 M NaCl (pH 7.2) at 0.5 mL/min.

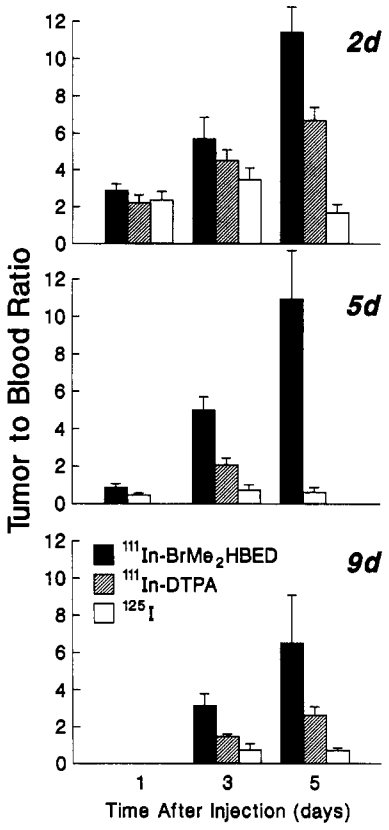

Figure 4. Tumor to blood ratios obtained 1, 3, or 5 days after injection of radiolabeled Ab in male Golden Syrian hamsters implanted with GW39 colorectal carcinoma 2 days (top), 5 days (middle), and 9 days (bottom) prior. Each animal was coinjected with ^{111}In-1A3 and ^{125}I-1A3 (50 μg/hamster).

number of moles of indium added, and assuming that the percent of indium-111 bound reflects the total distribution of indium and that M-L binding should be 1:1, the amount of bound ligand can readily be determined for the titration curves. A 3.46-mol amount of DTPA and 0.8 mol of BrMe$_2$HBED were attached per mole of Ab. Even though less BrMe$_2$HBED and DTPA is attached to Ab (per mole), the IR is much higher with BrMe$_2$HBED-1A3 when equivalent amounts of L are attached (i.e. 10:1 BrMe$_2$HBED is equivalent to 2:1 DTPA) (Figure 2).

^{111}In-BrMe$_2$HBED-1A3 had an elution profile on FPLC identical with that of unlabeled 1A3 (Figure 3). This shows that the labeling has not altered the molecular weight profile. At 10:1 DTPA:Ab ratios similar FPLC profiles are obtained, suggesting negligible cross-linking at this DTPA anhydride to Ab ratio.

When the total amount of radiolabeled 1A3 (^{111}In-BrMe$_2$HBED and ^{125}I) was varied (10–100 μg) and coinjected

Table IV. Biodistribution of Various Amounts of Radiolabeled 1A3 in Hamsters with 5-Day-Old GW39 Tumors (Experiment 1): % ID/g ($\bar{x}$ ± SD; n = 6) 3 Days after Injection

	tot. amount of 1A3 injected, μg		
	10	50[a]	100
	^{111}In-BrMe$_2$HBED-1A3		
blood	0.76 ± 0.11	0.60 ± 0.07	0.66 ± 0.07
tumor	3.45 ± 0.81	3.01 ± 0.53	3.04 ± 0.33
muscle	0.34 ± 0.06	0.31 ± 0.03	0.28 ± 0.05
liver	1.86 ± 0.43	1.58 ± 0.35	1.80 ± 0.29
kidney	4.52 ± 1.02	3.93 ± 0.46	3.80 ± 0.44
	^{125}I-1A3		
blood	3.17 ± 0.41	2.84 ± 0.28	3.04 ± 0.32
tumor	2.05 ± 0.37	2.04 ± 0.66	1.99 ± 0.39
muscle	0.18 ± 0.02	0.15 ± 0.02	0.16 ± 0.01
liver	0.40 ± 0.06	0.45 ± 0.07	0.47 ± 0.07
kidney	0.10 ± 0.07	0.18 ± 0.03	0.12 ± 0.05

[a] n = 5.

Table V. Biodistribution of Radiolabeled 1A3 in Hamsters with 5-Day-Old GW39 Tumors (Experiment 1): % ID/g ($\bar{x}$ ± SD; n = 4)

	time after injection, days		
	1	3[a]	5
	^{111}In-BrMe$_2$HBED-1A3		
blood	2.79 ± 0.32	0.60 ± 0.07	0.21 ± 0.01
tumor	2.37 ± 0.52	3.01 ± 0.53	2.29 ± 0.55
muscle	0.26 ± 0.05	0.31 ± 0.03	0.31 ± 0.01
liver	1.73 ± 0.18	1.55 ± 0.35	2.12 ± 0.27
kidney	3.14 ± 0.43	3.93 ± 0.46	3.89 ± 0.45
	^{125}I-1A3		
blood	4.94 ± 0.27	2.84 ± 0.28	1.71 ± 0.14
tumor	2.25 ± 0.58	2.04 ± 0.66	1.04 ± 0.46
muscle	0.17 ± 0.04	0.15 ± 0.02	0.11 ± 0.01
liver	0.92 ± 0.09	0.45 ± 0.06	0.27 ± 0.03
kidney	0.32 ± 0.10	0.18 ± 0.03	0.07 ± 0.03

[a] n = 5.

Table VI. Biodistribution of ^{125}I-1A3 and ^{111}In-BrMe$_2$HBED-1A3 Coinjected in Hamsters with GW39 Tumors (Experiment 2): % ID/g ($\bar{x}$ ± SD; n = 4)

	tumor age at injection, days; time after injection, days			
	2; 0.9	2; 3	2; 5	5; 3
	^{111}In-BrMe$_2$HBED-1A3			
blood	2.65 ± 0.07	1.08 ± 0.40	0.43 ± 0.07	1.01 ± 0.21
tumor	7.57 ± 0.97	5.60 ± 0.68	4.80 ± 0.28	3.88 ± 0.82
muscle	0.36 ± 0.08	0.39 ± 0.07	0.32 ± 0.04	0.41 ± 0.09
liver	1.78 ± 0.10	1.69 ± 0.59	1.74 ± 0.31	1.41 ± 0.22
kidney	3.17 ± 0.31	3.82 ± 0.60	4.08 ± 0.33	3.72 ± 0.51
	^{125}I-1A3			
blood	4.44 ± 0.15	2.58 ± 1.14	2.01 ± 0.24	3.20 ± 0.46
tumor	11.59 ± 1.95	6.82 ± 1.06	3.35 ± 0.86	1.71 ± 0.54
muscle	0.21 ± 0.08	0.17 ± 0.02	0.12 ± 0.01	0.22 ± 0.05
liver	1.38 ± 0.19	0.61 ± 0.06	0.39 ± 0.08	0.78 ± 0.17
kidney	0.10 ± 0.08	0.51 ± 0.12	0.29 ± 0.06	0.52 ± 0.11

in hamsters with GW39 colorectal tumors 5 days after tumor implantation, very little differences in biodistribution at 3 days was observed (Table IV). The antibody concentration was adjusted to 50 μg total for each hamster in all subsequent experiments. The biodistribution of ^{111}In-BrMe$_2$HBED-1A3 did alter between 1 and 5 days after administration (Table V). Greater tumor uptake was observed with ^{111}In-BrMe$_2$HBED-1A3 than with ^{125}I-1A3, and additionally, blood clearance of indium-111 radioactivity was more rapid, yielding enhanced tumor to blood ratio values (Figure 4). It has become apparent, in additional experiments, that the time after tumor cell implant is critical in this animal model; so we evaluated the radiolabeled Ab in hamsters 2, 5, and 9 days after implant. In the rapidly growing tumors (2 days after implant), high uptake of both ^{111}In-BrMe$_2$HBED-1A3 and ^{125}I-1A3 was observed (Table VI). In order to directly

Table VII. Comparison of Biodistribution of Radiolabeled 1A3 in Hamsters with GW39 Tumors (Experiment 3): % ID/g ($\bar{x}$ ± SD; n = 5)

	tumor age at injection, days; time after injection, days			
	2; 0.9	2; 3	2; 5	5; 3[a]
	^{111}In–DTPA–1A3			
blood	4.12 ± 0.31	1.52 ± 0.15	0.78 ± 0.08	1.92 ± 0.54
tumor	9.01 ± 1.76	6.82 ± 0.86	5.18 ± 0.41	3.83 ± 0.44
muscle	0.29 ± 0.48	0.36 ± 0.05	0.39 ± 0.11	0.34 ± 0.08
liver	1.67 ± 0.21	1.42 ± 0.12	1.20 ± 0.11	1.52 ± 0.39
kidney	3.87 ± 0.51	3.97 ± 0.23	3.90 ± 0.42	4.28 ± 0.52
	^{125}I–1A3			
blood	5.61 ± 0.43	2.55 ± 0.28	1.67 ± 0.17	3.38 ± 0.93
tumor	13.14 ± 2.89	8.77 ± 1.48	4.42 ± 1.04	2.35 ± 0.42
muscle	0.20 ± 0.05	0.18 ± 0.03	0.21 ± 0.17	0.16 ± 0.05
liver	1.69 ± 0.28	0.77 ± 0.17	0.41 ± 0.03	0.82 ± 0.20
kidney	1.03 ± 0.16	0.48 ± 0.06	0.32 ± 0.06	0.55 ± 0.15

[a] n = 4.

Table VIII. Biodistribution of Radiolabeled 1A3 in Hamsters with 9-Day-Old GW39 Tumors (Experiment 4): % ID/g ($\bar{x}$ ± SD; n = 4)

	time after injection, days	
	3	5
	^{111}In–BrMe$_2$HBED–1A3	
blood	1.23 ± 0.27	0.45 ± 0.05[a]
tumor	3.73 ± 0.14	2.86 ± 1.03[a]
muscle	0.73 ± 0.16	0.78 ± 0.14[a]
liver	3.46 ± 0.56	2.44 ± 2.21[a]
kidney	10.06 ± 0.74	10.01 ± 2.64[a]
	^{111}In–DTPA–1A3	
blood	1.58 ± 0.10	0.98 ± 0.06
tumor	1.74 ± 1.01	2.58 ± 0.62
muscle	0.35 ± 1.04	0.29 ± 0.05
liver	1.37 ± 0.17	1.36 ± 0.13
kidney	6.04 ± 0.42	5.52 ± 0.03
	^{125}I–1A3	
blood	2.23 ± 0.27	1.26 ± 0.16[a]
tumor	1.54 ± 0.64	0.90 ± 0.23[a]
muscle	0.14 ± 0.01	0.15 ± 0.03[a]
liver	0.80 ± 0.12	0.50 ± 0.08[a]
kidney	0.54 ± 0.08	0.37 ± 0.13[a]

[a] n = 3.

compare each preparation of radiolabeled Ab, a repetetive time point was evaluated (5-day-old tumor after 3 days) for which similar distribution results were observed (see Table V). Also, the uptake of ^{111}In–DTPA–1A3 in 2-day-old tumors was high (Table VII), but clearance of blood radioactivity was not as rapid as with BrMe$_2$HBED-conjugated Ab (Figure 4). When ^{111}In–BrMe$_2$HBED–1A3 or ^{111}In–DTPA–1A3 was coinjected with ^{125}I–1A3 in older tumors (9 days after implant), the tumor uptake was somewhat lower (Table VIII) but the blood clearance pattern, as observed in the other animal distribution experiments, was similar (Figure 4).

Discussion

The new bifunctional chelate BrMe$_2$HBED has been developed as a prototype to attach radiometals to proteins and antibodies. Methods of conjugating the ligand to antibodies as well as radiolabeling have been optimized so that very high in vitro immunoreactivity was obtained with this ligand. This type of ligand has several advantages over the derivatized DTPA and EDTA ligands commonly used for antibody labeling; these are the higher stability constant of HBED-type ligands and the potential for other substitutions on the aromatic ring to prepare ligands that are more hydrophilic or hydrophobic than the initial prototype ligands studied herein. The HBED derivative with its aromatic groups will be less hydrophilic than the DTPA derivatives. The FPLC elution profile of the radiolabeled Ab is very similar (Figure 3) to that of the unlabeled Ab, and the retention of the immunoreactivity is much greater for BrMe$_2$HBED-conjugated Ab than for DTPA-conjugated Ab (Figure 2 and Table III). Compared to DTPA-conjugated Ab, significantly less BrMe$_2$HBED is attached per mole of Ab, although high labeling efficiencies are obtained (even when 2–5 mCi of indium-111 is utilized). When the DTPA anhydride:Ab ratio is reduced to 2:1, the labeling yield is significantly reduced. So, even though a relatively low concentration of BrMe$_2$HBED is attached, a useful radiolabeled antibody can be prepared.

One of the goals of this work was to prepare bifunctional chelates that would rapidly clear radioactivity from the liver or kidneys. This prototype HBED ligand does not accomplish this (although more rapid blood clearance is observed). This could be due to several reasons: (1) metabolism of the carboxyl groups on HBED; (2) trapping of the catabolic fragment in liver cells; (3) release of indium from the complex.

The exact liver "trapping" mechanism for ^{111}In–BrMe$_2$HBED–Ab needs to be evaluated. ^{14}C-labeled DTPA experiments suggests DTPA liver entrapment occurs after catabolism,[48] while, for derivatized EDTA, Deshpande et al. have suggested that the residual catabolic fragment cannot clear from the liver cell.[49] Reduction of ligand catabolism might be achieved by further modifying the protein; we are pursuing this by preparing derivatives of *N,N,N′,N′*-tetrakis(2-hydroxy-3,5-dimethylbenzyl)ethylenediamine and by adding more lipophilic substituents to the benzene rings in the HBED derivatives to enhance liver clearance.

In conclusion we have prepared a new bifunctional ligand that has potential to chelate radiometals to proteins and antibodies while retaining high immunoreactivity or biological integrity. Higher target to blood ratios are obtained with BrMe$_2$HBED than with conventionally labeled antibodies, although radioactivity is observed in the clearance organs after several days. This new type of bifunctional ligand is a prototype for a series of bifunctional ligands based upon both the HBED and PLED structures that are currently under investigation.

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