Table XIII. Percent Inhibition of ADP-Induced Platelet Aggregation Data for Compounds 1 and 58

expt	concn of compd[a]	% inhibn		
		PGE_1	1	58
1	32	94		
	10	56	82	
	3.2	26	38	
	1.0		29	
2	32	90		
	10	28	85	
	3.2	1	62	
	1.0		27	
3	32	72		
	10	37		68
	3.2	14		40
	1.0			27

[a] The concentration of PGE_1 is in ng/mL; the concentration of 1 and 58 is in μg/mL.

The platelet count for the guinea pig plasma was adjusted to 3.5 × 10^5 platelets/mm^3 and for the rat plasma to 5.0 × 10^5 platelets/mm^3 with autologous PPP.

An ADP concentration required to give slightly less than maximal aggregation was used to test compounds for inhibition of aggregation with the use of a Payton aggregation module coupled with an Omniscribe recorder. The concentrations of compounds in Table VIII represent those that gave approximately 50% inhibition (IC_{50}). The approximate IC_{50} of these compounds on ADP-induced platelet aggregation was obtained at $^1/_2$ log intervals of drug concentration. PGE_1 was used as an internal standard to adjust for individual variation in platelet sensitivity. Table XIII illustrates typical studies on different days for the first two compounds in Table VIII. No correction for platelet sensitivity was necessary as the IC_{50} of the standard PGE_1 was consistently between 10 and 32 ng/mL. Compounds that did not display approximately 50% inhibition at 10 μg/mL were considered inactive.

To test for inactivation of the drug by the blood, compounds active in the in vitro screen were incubated in whole blood (10 μg/mL) at room temperature for 0, 1, and 2 h. PRP was prepared from an aliquot of the blood and was tested for inhibition of platelet aggregation. Control aggregation studies were performed with blood incubated with solvent used to dissolve the drug.

Ex Vivo Studies. Compounds were dissolved in Emulphor/ethanol/Tyrodes and were administered gastrically to rats and guinea pigs via gavage. At 2 h after drug or placebo administration, blood samples were drawn from all animals as described previously. PRP was prepared and aggregation experiments were conducted as previously described. Activity was determined by demonstration of a p value of <0.05 (Student's t test) for inhibition of ADP-induced platelet aggregation in PRP with respect to placebo.

Acknowledgment. We thank the Physical and Analytical Chemistry Department of The Upjohn Company for elemental analyses, mass spectra, and IR spectroscopy.

Registry No. 1, 117688-40-1; 3, 4975-73-9; 4, 60006-28-2; 5, 60006-25-9; 6, 117688-41-2; 7, 117688-42-3; 8, 117688-43-4; 9, 117688-44-5; 10, 117688-45-6; 11, 117688-46-7; 12, 117688-47-8; 13, 117688-48-9; 14, 117688-49-0; 15, 117688-50-3; 16, 117688-51-4; 17, 107785-13-7; 18, 117688-52-5; 19, 29882-02-8; 20, 68897-42-7; 21, 117688-53-6; 22, 117688-54-7; 23, 117688-55-8; 24, 117688-56-9; 26, 117688-57-0; 28, 117688-58-1; 29, 117688-59-2; 30, 117688-60-5; 31, 117688-61-6; 32, 117688-62-7; 33, 117688-63-8; 34, 117688-64-9; 35, 117688-65-0; 40, 117688-66-1; 41, 117688-67-2; 42, 117688-68-3; 43, 117688-69-4; 44, 117688-70-7; 45, 117688-71-8; 46, 117688-72-9; 47, 117688-73-0; 48, 57257-21-3; 49, 123-61-5; 50, 104-49-4; 51, 91-97-4; 52, 3634-83-1; 53, 1014-98-8; 54, 77372-57-7; 55, 117688-74-1; 56, 117709-59-8; 57, 117688-75-2; 58, 117688-76-3; 59, 117688-77-4; 60, 117688-78-5; 61, 117688-79-6; 62, 117688-80-9; 63, 117688-81-0; 64, 117709-60-1; 65, 117688-82-1; 66, 117688-83-2; 67, 117688-84-3; 68, 117688-85-4; 69, 117688-86-5; 70, 117688-87-6; 71, 117688-88-7; 72, 117688-89-8; 73, 117688-90-1; 74, 4858-84-8; 75, 99191-71-6; 76, 117688-91-2; 77, 80-73-9; 78, 117688-92-3; DCC, 538-75-0; *i*-PrN=C=NPr-*i*, 693-13-0; $HN(C_6H_{11})_2$, 101-83-7; $HN(C_6H_5)_2$, 122-39-4; $HN(CH_3)_2$, 124-40-3; $OCN(CH_2)_5NCO$, 4538-42-5; $OCN(CH_2)_7NCO$, 18020-78-5; $OCN(CH_2)_8NCO$, 10124-86-4; $OCN(CH_2)_9NCO$, 7192-79-2; $OCN(CH_2)_{10}NCO$, 4538-39-0; $OCN(CH_2)_4NCO$, 4538-37-8; $ClCO(CH_2)_6COCl$, 10027-07-3; $ClCO(CH_2)_8COCl$, 111-19-3; $Cl(CO)_2Cl$, 79-37-8; $ClCOO(CH_2)_6OCOCl$, 2916-20-3; $I(CH_2)_{10}I$, 16355-92-3; BuI, 542-69-8; $MeNH(CH_2)_6NHMe$, 13093-04-4; $MeNH(CH_2)_2NHMe$, 110-70-3; *m*-$(MeNHCH_2)_2C_6H_4$, 23399-62-4; pyrrolidine, 123-75-1; piperidine, 110-89-4; thiomorpholine, 123-90-0; morpholine, 110-91-8; di(4-tolyl)carbodiimide, 726-42-1; hexamethylene diisocyanate, 822-06-0; piperazine, 110-85-0.

Synthesis of Novel Bifunctional Chelators and Their Use in Preparing Monoclonal Antibody Conjugates for Tumor Targeting

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Bifunctional derivatives of the chelating agents ethylenediaminetetraacetic acid and diethylenetriaminepentaacetic acid, in which a *p*-isothiocyanatobenzyl moiety is attached at the methylene carbon atom of one carboxymethyl arm, was synthesized by reductive alkylation of the relevant polyamine with (*p*-nitrophenyl)pyruvic acid followed by carboxymethylation, reduction of the nitro group, and reaction with thiophosgene. The resulting isothiocyanate derivatives reacted with monoclonal antibody B72.3 to give antibody–chelator conjugates containing 3 mol of chelator per mole of immunoglobulin, without significant loss of immunological activity. Such conjugates, labeled with the radioisotopic metal indium-111, selectively bound a human colorectal carcinoma implanted in nude mice when given intravenously. Uptake into normal tissues was comparable to or lower than that reported for analogous conjugates with known bifunctional chelators. It is concluded that substitution with a protein reactive group at this position in polyaminopolycarboxylate chelators does not alter the chelating properties of these molecules to a sufficient extent to adversely affect biodistribution and thus provides a general method for the synthesis of such chelators.

Current efforts to use monoclonal antibodies as a delivery system for targeting drugs, toxins, or radioisotopes to tumor foci in vivo[1–4] have spurred interest in improved methods for preparing such immunoconjugates. The potential utility of radioisotope conjugates in both the detection and treatment of neoplastic disease has, in particular, led to increasing work on methods for linking ra-

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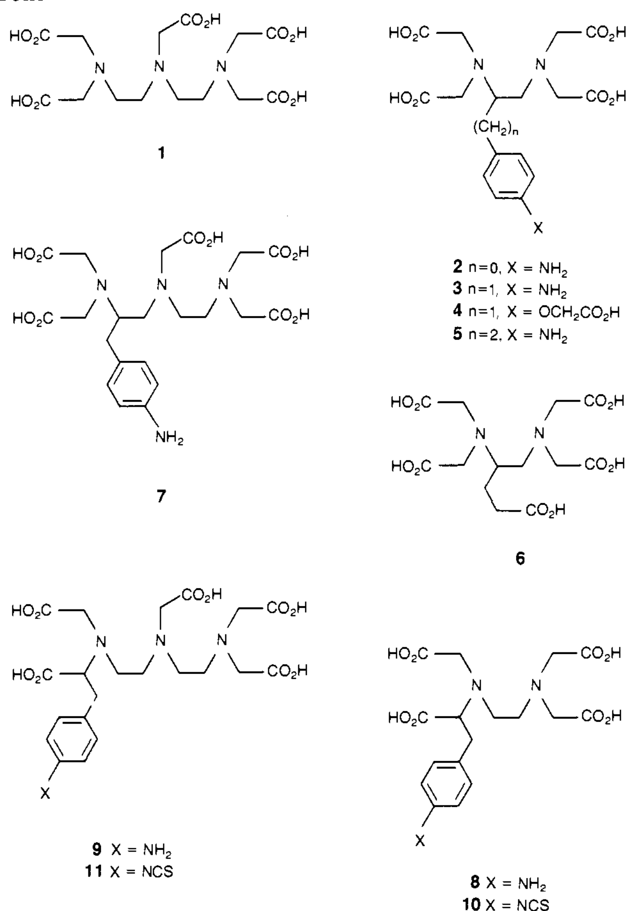
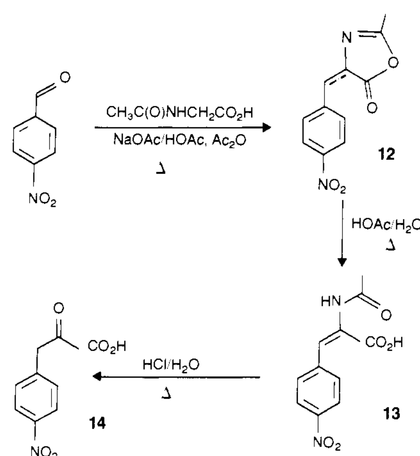
Scheme I



Scheme II



dioisotopic metal ions to immunoglobulins.[5-8] This is achieved by the use of "bifunctional" chelating agents,[8-10] namely, molecules possessing both an array of metal-binding groups (i.e., a chelating function) and an additional moiety through which the chelating portion of the molecule may be covalently linked to a protein (i.e., a protein-reactive function). Most work to date has employed diethylenetriaminepentaacetic acid (DTPA, **1**, Scheme I) as the bifunctional chelator, methods having been developed[11-16] that seek to use one of the five carboxymethyl groups in forming an amide bond to the antibody while the other four remain available for metal binding. However, as there is no intrinsic difference between the protein-reactive and chelating functions in DTPA, such procedures can lead to cross-linking and denaturation of the antibody,[14,15,17] with concomitant degradation of its ability to bind the target antigen. It is presently unclear to what extent the results obtained in vivo with DTPA-conjugated antibodies, particularly the high liver uptake seen both in animal models[18-20] and in man,[2,4,21-24] reflect such denaturation.

Bifunctional chelating agents that incorporate a unique protein-reactive site should avoid potential cross-linking problems and several such systems are currently known. The first was described in 1974 by Meares and co-workers,[25,26] who synthesized an EDTA derivative bearing a *p*-aminophenyl substituent (**2**, Scheme I) that may be coupled to proteins under mild conditions by conversion of the aromatic amine to a diazonium, isothiocyanate, or bromoacetamide moiety.[27] Benzyl (**3**, **4**, Scheme I),[28,29] phenethyl (**5**, Scheme I),[30] and 2-carboxyethyl (**6**, Scheme

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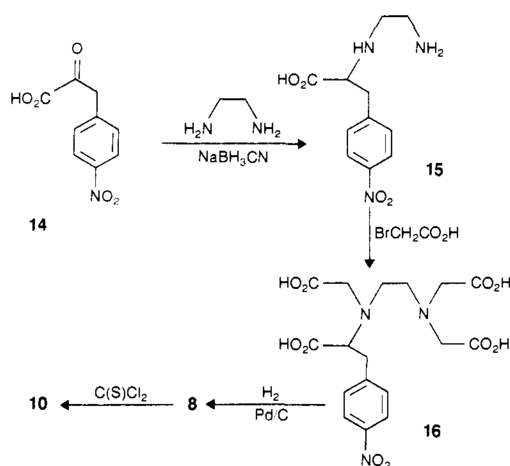
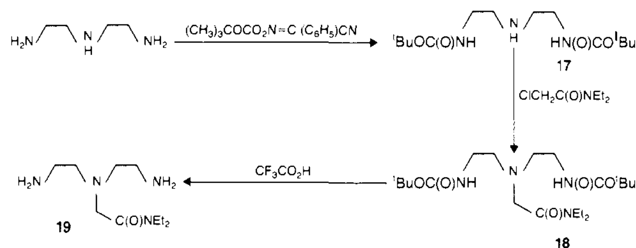
Scheme III



Scheme IV



I)[31] analogues have subsequently been described and, most recently, a DTPA analogue (**7**, Scheme I)[32,33] and a macrocyclic system[34] have been reported. A common feature of all of these structures is attachment of the protein-reactive function at a methylene carbon of the polyamine backbone. In seeking a more flexible, generic route for introducing a protein-reactive moiety into a wide variety of polyaminopolycarboxylate frameworks, we undertook the synthesis of agents in which the protein-reactive site is incorporated at a position common to, and accessible in, all such chelators, namely, on one of the carboxymethyl arms. This report describes the synthesis of the first such agents (**8**, **9**, Scheme I) and illustrates their use in the preparation of monoclonal antibody conjugates formed via the isothiocyanate derivatives (**10**, **11**, Scheme I). The ability of these conjugates to target a human colorectal carcinoma in vivo was evaluated in a nude mouse model and these data were compared with literature values for the same antibody conjugated to **1** and to isothiocyanate derivatives of **3** and **7**.

Chemistry. Inasmuch as the amine nitrogen atoms and carboxylate oxygen atoms of polyaminopolycarboxylate chelators are those groups involved in metal binding, there are only two positions within such molecules where derivatization with a protein-reactive substituent might be attempted with any prospect of leaving the chelating properties of the molecule unaffected. These are the methylene carbon chains of the polyamine backbone and the methylene carbon atoms of the carboxymethyl arms. We chose to target the latter, with the intent that any derivatization scheme that was developed should be applicable to the broadest possible spectrum of chelators, including those in which the methylene carbons of the polyamine backbone are not readily available for substitution, e.g., when these are part of a cyclic system.

The routes by which a protein-reactive moiety was introduced into the EDTA and DTPA frameworks are shown in Schemes II–V. The key step involved a reductive alkylation of the corresponding polyamine with (*p*-nitrophenyl)pyruvic acid (**14**, Scheme II). Borch et al.[35] had previously shown that a variety of pyruvic acids, including phenylpyruvic acid and (*p*-hydroxyphenyl)pyruvic acid, could be reductively aminated to the corresponding *dl* α-amino acids under mild conditions using ammonia and sodium cyanoborohydride. This step thus represented an extrapolation of their procedure from the use of ammonia to the use of a polyamine, the main issue being to avoid or minimize the formation of disubstituted species. Although no evidence was found to indicate formation of such species in the ethylenediamine sequence (Scheme III), mass spectra of crude reaction products from the diethylenetriamine reaction (Scheme V) clearly showed disubstituted materials to be present. These were separated from the desired product (**20**, Scheme V) by reverse-phase HPLC.

Our initial attempts had focused on a reaction sequence analogous to that shown in Scheme III but employing (*o*-nitrophenyl)pyruvic acid as this material is commercially available. However, although the reductive alkylation and subsequent carboxymethylation steps both proceeded smoothly and pure samples of the *o*-nitrobenzyl analogue of **16** could be obtained, upon reduction of the nitro group the preponderant product obtained was the six-membered lactam (**23**, Scheme VI). To avoid such problems, we therefore synthesized (*p*-nitrophenyl)pyruvic acid by acid hydrolysis of the azalactone obtained from the reaction of *p*-nitrobenzaldehyde with acetylglycine (Scheme II).

Intramolecular condensation leading to the formation of a lactam also proved to be a problem in the carboxymethylation of **15**. Although this condensation did not occur during the reductive alkylation step that gave **15**, the conditions for carboxymethylation (elevated temperature at a basic pH) always led to some lactam formation, with subsequent carboxymethylation such that the lactam was isolated as the bis(carboxymethyl) derivative (**24**, Scheme VI). The secondary nitrogen atom of diethylenetriamine was first protected with a diethylacetamide moiety (Scheme IV), which was eventually hydrolyzed to form the fifth carboxymethyl arm after the alkylation and reduction steps had been completed (Scheme V) in order to circumvent similar difficulties in the DTPA sequence.

Reduction of the nitrobenzyl substituted chelators (**16**, **21**) and subsequent transformation into the ultimate protein-reactive forms used in these studies (**10**, **11**) were carried out by using literature methods.[25,26] Typical overall yields of **10** and **11** based on (*p*-nitrophenyl)pyruvic acid were 9% and 7%, respectively.

Immunochemistry. B72.3 is a murine monoclonal antibody developed by Schlom and co-workers[36] that recognizes a high molecular weight mucin antigen, termed

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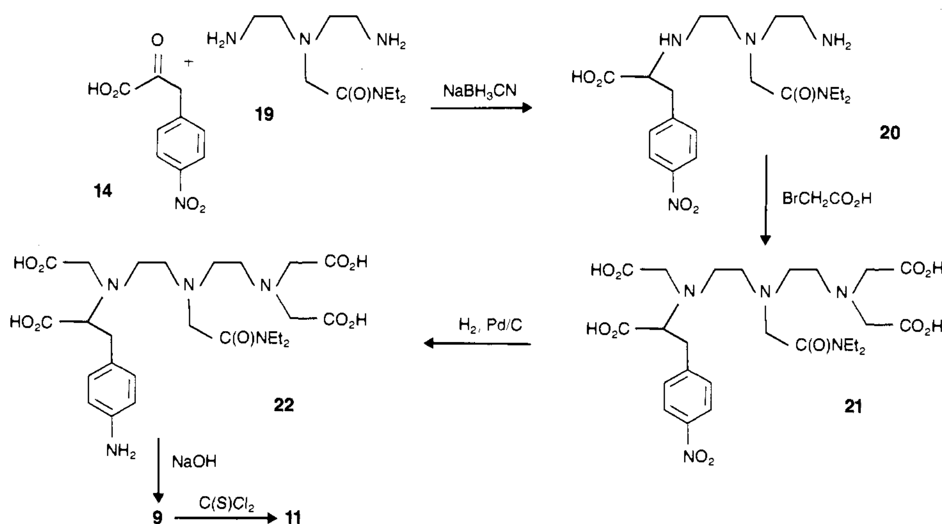
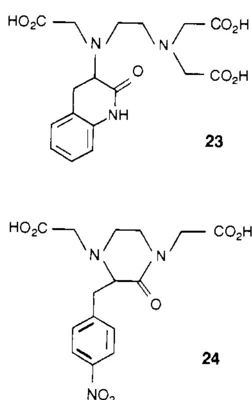
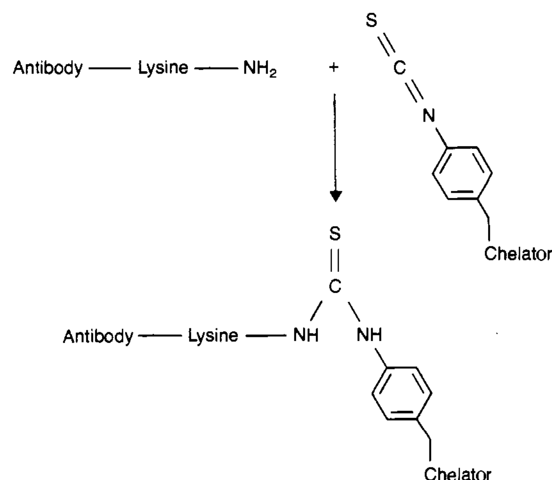
Scheme V



Scheme VI



Scheme VII



TAG-72,[37] which is expressed by a wide variety of human adenocarcinomas, including those of the colon, lung, breast, and ovaries.[38] This antibody has been extensively studied as a means of targeting radioisotopes to such tumors both in animal models[32,33,39-43] and in man.[44,45] Because of the ready availability of literature data with which to compare the performance of new antibody conjugates, we chose the B72.3 system as a model to test the utility of compounds **10** and **11**.

Conjugates were obtained by reacting the antibody, at a concentration of 10 mg/mL in 0.1 M phosphate/0.1 M bicarbonate buffer, pH 8.5, for 3 h at 37 °C with the isothiocyanate derivative of the chelator, leading to the formation of a thiourea linkage between the chelating agent and the ϵ-amino group of lysine residues present in the protein (Scheme VII). The resulting conjugates were purified by serial dialysis, the last dialysis step being carried out against the 0.05 M citrate buffer, pH 6.0, that was used in the subsequent radiolabeling step. Conjugates thus obtained were evaluated by a radiocobalt binding assay previously described by Meares et al.,[27] in order to determine the average number of chelating groups introduced into each immunoglobulin molecule, and by an enzyme linked immunosorbent assay (ELISA) procedure, to test whether the ability of the antibody to bind its antigen had been affected by the conjugation procedure.

Every monoclonal antibody is unique in its susceptibility to loss of immunological activity on chemical derivatization and, as a result, for each antibody there is an optimum set of reaction conditions that lead to conjugates having the maximum level of substitution consistent with retention of full immunological activity. These optimum conditions can only be established empirically, through a series of trial conjugations performed at various charging stoichiometries defined in terms of the number of moles of chelator introduced into the reaction mixture per mole of immunoglobulin present. For B72.3, the highest substitution level that could be achieved without adversely affecting the ELISA titration curve was found to be approximately 3 chelators per antibody molecule, this substitution level being achieved at a charging stoichiometry of 5 mol of chelator per mol of immunoglobulin under the conditions

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Table I. Biodistribution of Indium-111-Labeled B72.3–Chelator Conjugates in Nude Mice Bearing LS174T Tumors

	percentage of injected dose of indium-111 per g of tissue[a]					
	DTPA amides		EDTA isothiocyanates		DTPA isothiocyanates	
tissue	1[b]	1[c]	3[b]	10[d]	7[b]	11[d]
blood	10.67 (0.03)	11.23 (5.51)	19.82 (0.62)	12.1 (2.8)	18.37 (0.28)	8.9 (1.4)
tumor (Ag +)	18.97 (0.09)	42.22 (15.14)	20.82 (0.05)	21.1 (4.6)	25.94 (1.01)	19.5 (2.3)
tumor (Ag −)		6.23 (0.88)		5.2 (1.3)		4.1 (0.3)
liver	17.20 (0.09)	19.39 (10.97)	14.26 (0.15)	6.1 (1.1)	9.09 (0.07)	6.9 (3.3)
spleen	9.14 (0.04)	12.12 (7.25)	11.68 (0.15)	3.9 (0.7)	5.03 (0.09)	4.0 (1.2)
kidney	17.14 (0.08)	12.64 (1.66)	16.91 (0.18)	5.9 (1.5)	8.99 (0.08)	2.3 (0.1)
lungs	6.30 (0.03)	6.15 (2.52)	9.19 (0.53)	5.5 (1.3)	9.94 (0.12)	4.1 (0.4)

[a] All data are for sacrifice 48 h after intravenous administration. [b] Data are from ref 33; values shown are mean (±S.E.M) for $n = 3$ or 4. [c] Data are from ref 43; values shown are mean (±S.D.) for $n = 5$. [d] This work; values shown are mean (±S.D.) for $n = 5$.

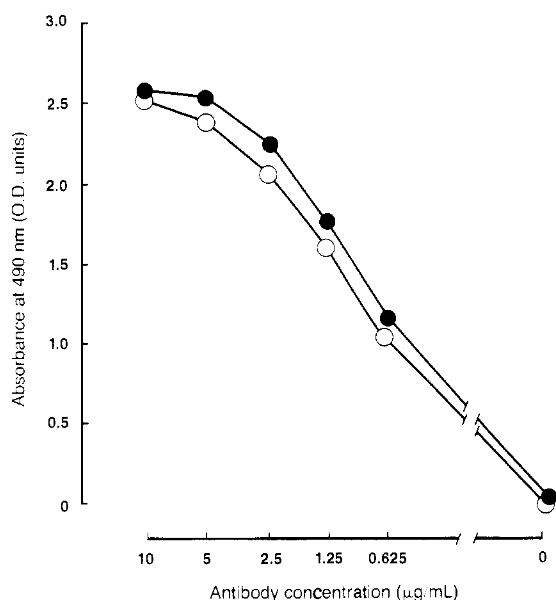

Figure 1. ELISA plots for underivatized B72.3 (—●—) and for a conjugate with **11** containing an average of 3.1 DTPA groups per antibody (—○—).

employed in this work. A typical ELISA titration curve for a conjugate having this level of substitution is compared with that for the underivatized antibody in Figure 1.

Biology. B72.3 conjugates of **10** and **11** were labeled with the γ-emitting radiometal indium-111 and their biodistributions were determined in nude mice bearing xenografts of the human colorectal carcinoma line, LS174T, which expresses high levels of TAG-72 when grown as a solid tumor. This model has been described in detail elsewhere.[33,40,42,43] Animals were inoculated on one flank with the antigen-positive tumor line, LS174T, and on the opposing flank with a human melanoma line, A375, which does not express the TAG-72 antigen and therefore serves as a control for nonspecific accumulation of immunoglobulin in tumor xenografts. Biodistribution data obtained 48 h after an intravenous injection of 2 μg of the indium-111-labeled conjugates appear in Table I.

The B72.3 conjugates of **10** and **11** were comparable in their overall performance, each achieving a 4–5-fold higher accumulation in the antigen-positive xenograft than in the antigen-negative tumor. In both cases, the xenograft was the only tissue that showed active accumulation of the label relative to the level found in the bloodstream. There were no significant differences in whole body retention of radioactivity between the two groups, those animals given the conjugate of **10** retaining 63 (±6)% of the injected dose at sacrifice while those given the conjugate of **11** had 67 (±4)% of the dose still present. Mean LS174T tumor weights in the two groups were equivalent, that in the group given the conjugate of **10** being 0.33 (±0.19) g and that in the group receiving the conjugate of **11** being 0.36 (±0.20) g. This is of significance in that levels of tumor activity achieved in models of this type are known to be dependent on tumor size.[46]

Discussion. Currently, the major limitation on use of indium-111-labeled antibodies for tumor detection via external photoscanning techniques is the high background caused by uptake of these agents into nontarget organs and, in particular, into the liver. Reticuloendothelial scavenging of denatured protein and/or redistribution of the indium-111 label to liver parenchyma via transferrin following loss of the label from the chelator–antibody conjugate are the two mechanisms most often invoked to explain elevated liver activity. The relative importance of these mechanisms is presently unclear, although it seems likely that both play a role.

When the distributions obtained with **10** and **11** are compared with those achieved by analogous conjugates with previously known chelators (Table I), it is apparent that uptake into nontarget tissues is markedly higher for conjugates of **1** than for those of **10**, **11**, and the isothiocyanate derivative of **7**. This is consistent with the decreased potential for antibody denaturation anticipated for the isothiocyanate derivatives compared to the amide conjugates of **1**, which were prepared by reaction of B72.3 with the bicyclic dianhydride of **1**—an agent possessing intrinsic cross-linking properties. Less readily explained is the apparently substantial difference seen between normal tissue uptake from the conjugate of **10** and that from the corresponding isothiocyanate derivative of **3**. Differential denaturation seems unlikely here and, inasmuch as the chelating moiety is identical in each case, the thermodynamic stability of the indium chelate label should be the same in both conjugates. However, others have observed that the presence of a bulky benzyl substituent within the chelator framework can cause apparent changes in the kinetic stability of the resulting indium complexes that are reflected in the rates at which indium is lost on exposure to serum.[47] It is possible that subtle differences of this type, dependent upon the position within the EDTA framework at which the bulky substituent is attached, could be responsible for the observed differences in liver, spleen, kidney, and, to a lesser extent, lung activity seen in the biodistribution patterns of these two bifunctional EDTA derivatives.

Although the mechanisms underlying normal tissue uptake from conjugates of this type clearly require further study, it is evident that compounds **10** and **11** do give

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antibody–chelator conjugates that display immunologically mediated tumor targeting in vivo and exhibit nonspecific uptake of the metal label into normal tissues that is comparable to, or lower than, that seen with any previously known bifunctional chelator. We conclude that incorporating protein reactive sites into the carboxymethyl arms of polyaminopolycarboxylate chelators does not affect the chelating properties of the parent molecule to a sufficient extent to adversely impact biodistribution and thus provides a viable strategy for converting any such chelator into a bifunctional derivative.

Experimental Section

Chelator Synthesis. General Procedures. Melting points were determined in open capillary tubes on an Electrothermal Engineering Ltd. melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on EM 360 or GE QE-300 spectrometers, chemical shifts being reported in parts per million relative to tetramethylsilane (TMS) or 3-(trimethylsilyl)-1-propanesulfonic acid (TSP) as an internal standard. All coupling constants are reported in hertz. ^{13}C NMR spectra were obtained on the GE QE-300 spectrometer, chemical shifts being given in parts per million relative to either TMS, acetonitrile, or dioxane. Mass spectra were obtained by the fast atom bombardment technique on a Kratos MS-50 mass spectrometer. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. Analytical HPLC characterization was carried out on a Waters Delta Prep 3000 system, using a Waters μ-Bondpak C-18 column (0.39 × 30 cm). Unless otherwise specified, all starting materials and other chemicals were purchased from Aldrich Chemical Co., Milwaukee, WI, and were used without further purification.

5-Oxo-2-methyl-4-(4′-nitrobenzylidene)-4,5-dihydrooxazole (12). A solution of *N*-acetylglycine (7.0 g, 60 mmol) in HOAc (20 mL) and acetic anhydride (21 mL) containing sodium acetate (14.4 g, 176 mmol) and 4-nitrobenzaldehyde (9.7 g, 64 mmol) was stirred at 100 °C for 2 h. After the mixture was cooled to 10 °C, H_2O (100 mL) was added with vigorous stirring and 12.9 g (93%) of 12 was collected by filtration. Recrystallization from HOAc gave pure 12 in the form of orange crystals, mp 184–185 °C (lit.[48] mp 185–186 °C).

α-Acetamido-4-nitrocinnamic Acid (13). A solution of 12 (7.9 g, 34 mmol) in HOAc (200 mL) was heated to 100 °C. H_2O (5 mL) was added and the mixture stirred at 100 °C for a further 15 min. Upon allowing the solution to cool slowly to room temperature, 7.2 g (85%) of 13 separated as yellow crystals, mp 232–235 °C (lit.[48] mp 234–235 °C).

(4-Nitrophenyl)pyruvic Acid (14). A suspension of 13 (7.2 g, 29 mmol) in 3 M HCl (50 mL) was stirred at reflux for 7 h. After the mixture was cooled to 0 °C, the product was collected by filtration, washed with cold H_2O, and dried under vacuum, yielding 5.4 g (89%) of 14 as an orange powder, mp 192–194 °C (lit.[49] 194 °C).

***N*-(2-Aminoethyl)-3-(4-nitrophenyl)alanine Dihydrochloride (15).** To a solution of 14 (15.0 g, 71.7 mmol) in MeOH (500 mL) was added a solution of ethylenediamine dihydrochloride (11.6 g, 87 mmol) in H_2O (100 mL), and the pH of the resulting mixture was brought to 6.0 by using 7 M NaOH. Sodium cyanoborohydride (7.86 g, 125 mmol) was added to the reaction mixture and the pH readjusted to 6.0 by using 6 M HCl. The mixture was stirred at room temperature for 3 days and then concentrated HCl (30 mL) was carefully added. After stirring the solution for a further 30 min, it was concentrated under vacuum until an orange precipitate formed. The latter was extracted into EtOAc and the aqueous layer evaporated to dryness under vacuum. The resulting residue was chromatographed on Dowex 50X-2-200 (H^+ form), eluting initially with H_2O (700 mL) and then with 5% (v/v) NH_4OH. UV-absorbing fractions that were positive to Ninhydrin were combined and evaporated to dryness under vacuum and then the residue was dissolved in EtOH. Addition of concentrated HCl to this solution caused a dense white precipitate to form, which was collected by filtration, washed with EtOH and Et_2O, and air-dried to give 6.56 g (23%) of 15 as a white solid: ^{1}H NMR (D_2O, TSP standard) δ 3.31–3.55 (m, 6 H), 4.20–4.28 (m, 1 H), 7.48–7.57 (m, 2 H), 8.19–8.28 (m, 2 H); ^{13}C NMR (D_2O, dioxane standard) δ 34.00, 34.68, 42.92, 60.62, 123.03, 129.52, 140.68, 146.26, 169.12; mass spectrum, m/e 254 $(M + H)^+$. Anal. ($C_{11}H_{17}N_3O_4Cl_2 \cdot H_2O$) C, H, N, Cl.

***N*-(Carboxymethyl)-*N*-(2-(bis(carboxymethyl)amino)ethyl)-3-(4-nitrophenyl)alanine (16).** 15 (3.00 g, 9.20 mmol) was added to a solution of bromoacetic acid (4.51 g, 32.5 mmol) in H_2O (25 mL) and the mixture heated to 45 °C. The pH of the reaction mixture was brought to 10 by using 7 M NaOH, and it was then stirred at 45 °C for 24 h, the pH being maintained at 10 by periodic addition of 7 M NaOH. Subsequently, the reaction mixture was cooled to room temperature and chromatographed on Bio-Rad AG1-X4 (formate form, bed volume 250 mL), eluting successively with 1 L each of H_2O, 3.5 M formic acid, and 6.0 M formic acid. Fractions were evaluated by HPLC (20% MeOH/0.01 M triethylamine–acetic acid as mobile phase) and those containing the product, which eluted in the 6 M formic acid, were combined and evaporated to dryness, affording 1.78 g (45%) of 16 as a yellow gum: ^{1}H NMR (DMSO, TMS standard) δ 3.00–3.25 (m, 4 H), 3.30–3.50 (m, 3 H), 3.70 (d, 1 H, J = 18.0), 3.90 (t, 1 H, J = 7.5), 4.10–4.25 (m, 4 H), 7.60 (d, 2 H, J = 9.0), 8.15 (d, 2 H, J = 9.0); ^{13}C NMR (D_2O, dioxane standard) δ 35.47, 50.76, 52.90, 56.19, 59.93, 69.54, 124.43, 130.95, 146.80, 149.53, 179.70, 180.51, 180.87; mass spectrum, m/e 428 $(M + H)^+$. Anal. ($C_{17}H_{21}N_3O_{10} \cdot H_2O$) C, H, N.

***N*-(Carboxymethyl)-*N*-(2-(bis(carboxymethyl)amino)ethyl)-3-(4-aminophenyl)alanine (8).** A solution of 16 (0.91 g, 2.13 mmol) in H_2O (100 mL) and formic acid (15 mL) was hydrogenated at room temperature and 35 psi over 10% palladium on carbon (0.10 g) for 2 h. The catalyst was removed by filtration through Celite and the filtrate evaporated to dryness. The resulting residue was dissolved in 4 M HCl (50 mL) and evaporated to dryness under vacuum to give 0.87 g (95%) of 8 in the form of the trihydrochloride salt: ^{1}H NMR (D_2O, TSP standard) δ 2.95–4.30 (m, 13 H), 7.30–7.60 (m, 4 H); ^{13}C NMR (DMSO, TMS standard) δ 34.96, 48.50, 51.65, 53.75, 54.74, 65.08, 123.50, 130.13, 130.61, 138.21, 167.83, 172.37, 173.67; mass spectrum, m/e 398 $(M + H)^+$. Anal. ($C_{17}H_{26}N_3O_8Cl_3 \cdot H_2O$) C, H, N, Cl.

***N*-(Carboxymethyl)-*N*-(2-(bis(carboxymethyl)amino)ethyl)-3-(4-isothiocyanatophenyl)alanine Dihydrochloride (10).** To a solution of 8 (0.68 g, 1.34 mmol) in 3 M HCl (12 mL) was added 1.70 mL of an 85% solution of thiophosgene in CCl_4 (v/v), and the resulting mixture was stirred at room temperature for 6 h. At the end of this time, the aqueous layer was negative to fluorescamine and a sticky orange solid had formed as a clump in this layer. Addition of Et_2O and trituration produced a white precipitate, which was filtered off, washed with Et_2O, and dried under vacuum to give 0.38 g (55%) of 10: ^{1}H NMR (DMSO, TMS standard) δ 2.65–3.05 (m, 6 H), 3.25–3.55 (m, 6 H), 3.70 (t, 1 H, J = 7.5), 7.33 (s, 4 H); ^{13}C NMR (DMSO, TMS standard) δ 34.96, 49.79, 52.04, 52.34, 54.30, 64.79, 125.57, 127.84, 130.59, 132.82, 138.62, 170.99, 172.72, 173.09; mass spectrum, m/e 440 $(M + H)^+$. Anal. ($C_{18}H_{23}N_3O_8SCl_2 \cdot H_2O$) C, H, N, Cl.

1,7-Bis(*tert*-butoxycarbonyl)diethylenetriamine (17). A solution of diethylenetriamine (15.0 g, 145.39 mmol) and triethylamine (44.14 g, 436.20 mmol) in THF (250 mL) was cooled in an ice bath and stirred as a solution of 2-[[(*tert*-butoxycarbonyl)oxy]imino]-2-phenylacetonitrile (71.61 g, 290.78 mmol) in THF (1 L) was added over 50 min. After being stirred for 2 h in the ice bath and then for a further 1 h at room temperature, the THF was removed by evaporation under vacuum to give a golden oil, which was dissolved in CH_2Cl_2 (500 mL) and extracted with 5% NaOH (w/v) (1600 mL). The organic layer was dried over anhydrous Na_2SO_4 and then evaporated to dryness under vacuum, and the residue was chromatographed by HPLC, using a PrepPak-500/silica column and isocratic elution with 3% MeOH:97% CH_2Cl_2. Fractions containing the desired product were identified by TLC on silica plates developed in 10% MeOH/90% CH_2Cl_2 (R_f = 0.5) and these were combined and evaporated to dryness under vacuum to give 22.03 g (50%) of 17 in the form of a white solid: ^{1}H NMR ($CDCl_3$, TMS standard) δ 1.45 (s, 18 H), 1.65 (br s, 1 H), 2.70–2.75 (m, 4 H), 3.15–3.25 (m, 4 H), 5.10 (br s, 2 H); ^{13}C NMR ($CDCl_3$, TMS standard) δ 28.19,

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40.21, 48.74, 79.15, 156.13; mass spectrum, m/e 304 (M + H)$^+$.

1,7-Bis(*tert*-butoxycarbonyl)-4-(*N*,*N*-diethylacetamido)diethylenetriamine (18). A solution of 17 (21.5 g, 70.86 mmol), 2-chloro-*N*,*N*-diethylacetamide (10.77 g, 72 mmol), and triethylamine (7.29 g, 72.04 mmol) in EtOH (200 mL) was refluxed for 4 days. The solvent was removed by evaporation under vacuum to give a pale yellow residue, which, on addition of CH_2Cl_2 and trituration, gave a white precipitate that was filtered off. The filtrate was extracted with 5% Na_2CO_3 (w/v) (600 mL) and then dried over anhydrous Na_2SO_4 and chromatographed by HPLC, using a PrepPak-500/Silica column and isocratic elution with 5% MeOH/95% CH_2Cl_2. Fractions containing the desired product were identified by TLC on silica plates developed in 10% MeOH/90% CH_2Cl_2 (R_f = 0.6) and these were combined and evaporated to dryness under vacuum to give 25.63 g (87%) of 18 in the form of a yellow oil: ^{1}H NMR ($CDCl_3$, TMS standard) δ 1.10–1.20 (m, 6 H), 1.45 (s, 18 H), 2.65–2.75 (m, 4 H), 3.10–3.45 (m, 10 H), 5.50 (br s, 2 H); ^{13}C NMR ($CDCl_3$, TMS standard) δ 12.4, 13.8, 28.1, 38.2, 39.7, 40.7, 54.0, 55.6, 78.3, 155.9, 169.3; mass spectrum, m/e 417 (M + H)$^+$.

4-(*N*,*N*-Diethylacetamido)diethylenetriamine Trihydrochloride (19). Trifluoroacetic acid (100 mL) was added to 18 (25.0 g, 60.02 mmol), and the mixture was stirred for 2 h, after which time gas evolution had ceased. Evaporation to dryness under vacuum yielded a yellow oil, which was dissolved in 4 M HCl (200 mL), and this solution again evaporated to dryness under vacuum, to give 18.42 g (94%) of 19 as the trihydrochloride salt in the form of a white solid: ^{1}H NMR (D_2O, TSP standard) δ 1.05–1.25 (m, 6 H), 3.25–3.75 (m, 12 H), 4.40 (s, 2 H); ^{13}C NMR (D_2O, acetonitrile standard) δ 12.22, 13.21, 35.11, 41.57, 42.18, 53.05, 55.71, 164.83; mass spectrum, m/e 217 (M + H)$^+$.

***N*-(2-((2′-Aminoethyl)(*N*″,*N*″-diethylacetamido)amino)ethyl)-3-(4-nitrophenyl)alanine Trihydrochloride (20).** A solution of 19 (9.0 g, 27.63 mmol) in H_2O (25 mL) was added to a solution of 14 (5.90 g, 28.21 mmol) in MeOH (200 mL). The pH was adjusted to 6 by using 7 M NaOH; then solid sodium cyanoborohydride (2.61 g, 41.53 mmol) was added, the pH readjusted back to 6 by using 6 M HCl, and the reaction mixture stirred at room temperature for 4 days. Concentrated HCl (30 mL) was then added and, after gas evolution had ceased, the resulting solution was evaporated to dryness under vacuum to give an orange residue. This was suspended in H_2O (250 mL) and extracted with EtOAc (600 mL). The aqueous layer was then evaporated to dryness under vacuum to give a pale yellow residue, which was chromatographed by HPLC using a Waters PrepPak-500/C_{18} column and isocratic elution with 25% MeOH/75% 0.01 M triethylammonium acetate buffer. Product-containing fractions were identified by TLC on silica plates developed in 20% concentrated NH_4OH/80% EtOH (R_f = 0.7) and these were combined and evaporated to dryness under vacuum to yield an orange residue. Buffer salts were removed from this residue by HPLC using a PrepPak-500/C_{18} column and isocratic elution with 25% MeOH/75% H_2O. Product-containing fractions were again identified by TLC and were combined and evaporated to dryness under vacuum. The resulting residue was dissolved in 4 M HCl (100 mL) and this solution evaporated to dryness under vacuum to yield 3.14 g (22%) of 20 in the form of a white solid: ^{1}H NMR (D_2O, TSP standard) δ 1.00–1.20 (m, 6 H), 2.95–3.35 (m, 14 H), 3.70 (s, 2 H), 4.25 (t, 1 H, J = 6.3), 7.55 (d, 2 H, J = 9.0), 8.25 (d, 2 H, J = 9.0); ^{13}C NMR (D_2O, acetonitrile standard) δ 12.48, 13.39, 35.68, 36.86, 41.78, 42.17, 45.18, 52.86, 52.94, 55.04, 61.71, 124.63, 131.19, 142.48, 147.80, 169.79, 171.00; mass spectrum, m/e 410 (M + H)$^+$. Anal. ($C_{19}H_{34}N_5O_5Cl_3{\cdot}H_2O$) C, H, N.

***N*-(Carboxymethyl)-*N*-(2-((2′-(bis(carboxymethyl)amino)ethyl)(*N*′,*N*′-diethylacetamido)amino)ethyl)-3-(4-nitrophenyl)alanine Trihydrochloride (21).** 20 (3.00 g, 5.78 mmol) was added as a solid to a solution of bromoacetic acid (3.61 g, 25.98 mmol) in H_2O (25 mL), and the resulting solution was heated to 45 °C and the pH adjusted to 12 by using 7 M NaOH. The reaction mixture was then stirred at 45 °C for 24 h, the pH being maintained at 12 by periodic addition of 7 M NaOH. After cooling to room temperature, the solution was then applied to a Bio-Rad AG1-X4 (formate form) anion exchange column (bed volume = 250 mL, 300 mequiv), the column being eluted successively with H_2O (1250 mL) and 1.2 M formic acid (1 L). The desired product eluted in the formic acid, product-containing fractions being identified by TLC on silica plates developed in 20% concentrated NH_4OH/80% EtOH (R_f = 0.6). These fractions were combined and evaporated to dryness to give a yellow oil, which was redissolved in 4 M HCl (200 mL) and reevaporated to dryness to give 2.21 g (55%) of 21 in the form of a white solid: ^{1}H NMR (D_2O, TSP standard) δ 1.05–1.20 (m, 6 H), 3.20–3.50 (m, 14 H), 3.60–3.95 (m, 7 H), 4.25–4.45 (m, 2 H), 7.50 (d, 2 H, J = 9.0), 8.20 (d, 2 H, J = 9.0); ^{13}C NMR (D_2O, acetonitrile standard) δ 12.50, 13.47, 35.55, 41.67, 42.27, 49.73, 49.94, 52.02, 52.95, 54.67, 54.93, 55.66, 66.67, 124.32, 130.80, 146.75, 146.98, 164.27, 173.37, 174.98, 176.40; mass spectrum, m/e 584 (M + H)$^+$. Anal. ($C_{25}H_{40}N_5O_{11}Cl_3{\cdot}H_2O$) C, H, N.

***N*-(Carboxymethyl)-*N*-(2-((2′-(bis(carboxymethyl)amino)ethyl)(*N*′,*N*′-diethylacetamido)amino)ethyl)-3-(4-aminophenyl)alanine Tetrahydrochloride (22).** A solution of 21 (2.12 g, 3.06 mmol) in H_2O (300 mL) and formic acid (30 mL) was hydrogenated at room temperature and 35 psi of H_2 over 10% palladium on carbon (0.21 g) for 2 h. The catalyst was then removed by filtration through Celite and the filtrate was evaporated to dryness under vacuum. The resulting residue was dissolved in 4 M HCl (200 mL) and again evaporated to dryness to give 2.01 g (94%) of 22 in the form of a white solid: ^{1}H NMR (D_2O, TSP standard) δ 1.05–1.25 (m, 6 H), 3.00–3.60 (m, 14 H), 3.65–3.95 (m, 7 H), 4.40–4.60 (m, 2 H), 7.30–7.50 (m, 4 H); ^{13}C NMR (D_2O, acetonitrile standard) δ 12.43, 13.44, 35.04, 41.71, 42.26, 49.58, 50.55, 52.36, 52.90, 54.80, 55.14, 55.73, 67.08, 123.72, 128.94, 131.24, 139.15, 164.48, 171.94, 174.81, 176.10; mass spectrum, m/e 554 (M + H)$^+$. Anal. ($C_{25}H_{43}N_5O_9Cl_4{\cdot}H_2O$) C, H, N.

***N*-(Carboxymethyl)-*N*-(2-((2′-(bis(carboxymethyl)amino)ethyl)(carboxymethyl)amino)ethyl)-2-(4-aminophenyl)alanine Tetrahydrochloride (9).** A solution of 22 (1.90 g, 2.72 mmol) in 7 M NaOH was refluxed for 10 h and then cooled to room temperature and chromatographed on Bio-Rad AG1-X4 (formate form) anion exchange resin (bed volume = 350 mL, 430 mequiv). The column was eluted successively with H_2O (2 L), 0.2 M formic acid (1.5 L), and 1.0 M HCl (1.5 L). The desired product eluted in the HCl fractions and was identified by TLC on silica plates developed in 20% concentrated NH_4OH/80% EtOH (R_f = 0.6). Product-containing fractions were combined and evaporated to dryness under vacuum, and the resulting residue redissolved in 4 M HCl (200 mL) and again evaporated to dryness to yield 1.43 g (82%) of 9 in the form of a white solid: ^{1}H NMR (D_2O, TSP standard) δ 3.05–3.55 (m, 10 H), 3.60–4.05 (m, 9 H), 7.35–7.50 (m, 4 H); ^{13}C NMR (D_2O, acetonitrile standard) δ 34.57, 50.45, 50.81, 51.50, 52.23, 53.48, 54.25, 55.76, 66.96, 123.92, 129.11, 131.34, 138.93, 169.93, 171.63, 174.35, 175.51; mass spectrum, m/e 499 (M + H)$^+$.

***N*-(Carboxymethyl)-*N*-(2-((2′-(bis(carboxymethyl)amino)ethyl)(carboxymethyl)amino)ethyl)-3-(4-isothiocyanatophenyl)alanine Trihydrochloride (11).** An 85% solution to thiophosgene in CCl_4 (v/v) (0.2 mL, 2.23 mmol) was added to a solution of 9 (0.10 g, 0.16 mmol) in 3 M HCl (4 mL). The resulting mixture was stirred at room temperature for 6 h and then evaporated to dryness under vacuum to give 0.093 g (92%) of 11 in the form of a tan solid: ^{1}H NMR (DMSO-d_6, TMS standard) δ 2.85–3.50 (m, 11 H), 3.55–3.75 (m, 5 H), 3.80 (t, 1 H, J = 7.5), 4.20–4.45 (m, 2 H), 7.35 (s, 4 H); ^{13}C NMR (DMSO-d_6, dioxane standard) δ 34.83, 48.29, 48.82, 51.06, 51.57, 52.31, 53.26, 54.31, 64.80, 125.74, 128.05, 130.69, 132.93, 138.35, 167.85, 171.53, 172.49, 173.62; mass spectrum, m/e 541 (M + H)$^+$.

Preparation of Antibody–Chelator Conjugates. General Procedures. Monoclonal antibody B72.3 was produced in tissue culture in an airlift fermenter and was purified from the culture medium by affinity chromatography on protein A Sepharose CL-4B (Repligen Corp., Cambridge, MA),[50] followed by exhaustive dialysis against 0.1 M phosphate-buffered normal saline, pH 7 (PBS). Prior to the coupling procedure, the antibody was buffer exchanged by dialysis overnight at 2–8 °C against 0.1 M KH_2PO_4/0.1 M $NaHCO_3$, pH 8.5. Antibody concentrations were determined by the Bradford dye binding assay (Bio-Rad Laboratories, Richmond, CA), which was performed according to the manufacturer's directions. All buffer salts were reagent grade

(50) Grunert, F.; AbuHarfeil, N.; Schwarz K.; von Kleist, S. *Int. J. Cancer* 1985, *36*, 357.

and buffers were prepared by using water from a MILLI-Q system (Millipore Corp., Bedford, MA) purified to a resistivity of 18 megaohm. Antibody solutions were concentrated where necessary by ultrafiltration, using an ultrafiltration cell equipped with a membrane having a nominal molecular weight cut-off of 10000 (Amicon Corp., Danvers, MA).

Conjugation Procedure. The method used was based on that described by Meares et al.[27] (Scheme VII). The antibody, in 0.1 M KH_2PO_4/0.1 M $NaHCO_3$, pH 8.5, was adjusted to a concentration of 10 mg/mL. A 5-fold molar excess of either **10** or **11** was dissolved in a minimum volume of the same buffer and added to the antibody solution, which was then incubated at 37 °C for 3 h. Subsequently, the conjugate solution was dialyzed for 24–48 h at 2–8 °C against a 0.1 M solution of DTPA in 0.05 M citrate buffer, pH 6, and then for a further 72 h against multiple changes of 0.05 M citrate buffer, pH 6. If necessary, the protein concentration was readjusted to 10 mg/mL and then the conjugate solution was aliquoted into acid-washed glass vials and stored at 2–8 °C until needed. Conjugations were typically performed on a scale of from 5 to 25 mg of antibody.

Determination of Chelator Content. The average number of moles of chelator incorporated per mole of antibody was determined by using a radiocobalt binding assay that has been previously described.[27] Briefly, aliquots containing a known amount of antibody–chelator conjugate were incubated with a series of $CoCl_3$ solutions labeled with $^{57}CoCl_3$ (NEN-du Pont, Billerica, MA) to varying, known specific activities. Following an EDTA chase to complex that fraction of ^{57}Co not taken up by the antibody-bound chelator, protein-bound radioactivity was separated from unbound radiocobalt by TLC on silica gel plates (Redi-Plate, Fisher Scientific, Pittsburgh, PA) developed in a 1:1 (v:v) mixture of MeOH and 10% NH_4OAc. The analogous assay used to determine indium-111 incorporation[27] was also performed on silica gel impregnated fiberglass strips (Gelman Sciences, Ann Arbor, MI) developed in normal saline, as these could be developed much more rapidly and avoided the radiation hazard associated with scraping the finely powdered silica gel from glass plates.[51] In both cases, antibody-bound activity remained at the origin while unbound activity migrated close to the solvent front.

Evaluation of Immunological Activity. The impact of chelator conjugation on the ability of the antibody to bind antigen was assessed by an ELISA procedure.[52,53] For these purposes we employed bovine submaxillary mucin (BSM, Cooper Biomedical, Malvern, PA), an inexpensive and readily available mucin with which B72.3 is strongly cross-reactive.

Plate coating procedure: 96-well microtiter plates (Immulon 2, Dynatech Laboratories, Arlington, VA) were coated with BSM by incubating in each well a solution of the antigen (1.0 μg) in 10 mM Tris, pH 7.4 (100 μL). The wells were covered and incubated overnight at room temperature and then were emptied and washed twice with deionized water. The wells were then overcoated by placing in each well 100 μL of a solution of bovine serum albumin (Sigma Chemical Co., St. Louis, MO, 0.1% w/v in PBS, pH 7.4) and incubating at room temperature for 2 h. The overcoated plates were stored at 2–8 °C until needed, with an aliquot of the overcoating solution (100 μL) in each well. Immediately before use, the plates were emptied and washed five times with PBS, pH 7.4.

Assay procedure: parallel ELISA assays were performed on (a) the antibody–chelator conjugate and (b) the native (underivatized) antibody from which that conjugate had been prepared. Both assays were run on the same microtiter plate, employing successive 2-fold dilutions of solutions adjusted to an initial protein concentration of 10 μg/mL, dilutions being made in a solution of bovine serum albumin (1.0% w/v) and Tween 20 (0.1% v/v, Sigma) in PBS, pH 7.4. To each well was first added an aliquot (50 μL) of this dilution buffer, followed by an aliquot (50 μL) of the antibody solution. The plate was covered and incubated at 37 °C for 1 h and then emptied and washed five times with PBS, pH 7.4. An aliquot (100 μL) of a solution of goat anti mouse antibody conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD, 0.06 μg/mL in 1.0% bovine serum albumin/0.1% Tween 20/PBS, pH 7.4) was added to each well. The plate was again covered and incubated at 37 °C for 1 h and then was emptied and washed five times with PBS, pH 7.4. An aliquot (100 μL) of *o*-phenylenediamine solution, prepared according to the manufacturer's directions from a preformulated kit (Abbott Laboratories), was added to each well. The plate was incubated and protected from light for 15 min at room temperature; then 0.5 M H_2SO_4 (100 μL) was added to each well to quench the enzymatic reaction. Both the enzyme conjugate solution and the *o*-phenylenediamine solution are unstable and these were prepared no more than 30 min and 10 min before use, respectively. The color generated in each well was read at 490 nm by using a microtiter plate reader (Minireader II, Dynatech). Duplicate wells were run at each concentration for each antibody preparation.

Antibody titration curves were prepared by plotting the mean optical density at 490 nm against antibody concentration and the curve for the conjugate was compared to that for the underivatized antibody. A semiquantitative estimate of the immunoreactivity retained after coupling could be obtained by expressing the absorbance of the conjugate as a percentage of the absorbance of the native antibody at 50% titration.

Nude Mouse/Human Tumor Model Studies. Labeling of Antibody–Chelator Conjugates with Indium-111. Carrier-free indium-111 chloride (1.0 mCi, Atomic Energy of Canada, Ltd., Kanata, Ontario) was added to an aliquot of the antibody–chelator conjugate (100 μL) at a concentration of 10 mg/mL in 0.05 M citrate buffer, pH 6. After a 30-min incubation at room temperature, the radiochemical yield of indium-111-labeled B72.3 was determined by incubating an aliquot (50 μL) of the solution with 0.05 M DTPA, pH 6 (25 μL), for 10 min and then diluting this solution 50-fold with normal saline and spotting 3 μL of the resulting solution onto a TLC plate. TLC analysis,[27] using either silica gel plates or the silica gel impregnated fiberglass strips, typically showed that ≥95% of the applied ^{111}In activity remained bound at the origin. For animal studies, the stock ^{111}In-B72.3 solution at approximately 1 mCi/mg and 10 mg/mL was diluted with normal saline to a final concentration of 20 μg/mL.

Biodistribution Studies. Female athymic nude mice (nu/nu, BALB/c background, Charles River Biotechnology Services, Inc., Wilmington, MA) were injected subcutaneously with 5×10^6 A375 human melanoma cells (American Type Culture Collection, Rockville, MD, CRL 1619) in the left rear flank. Fourteen days later, 1.25×10^8 LS174T human colorectal carcinoma cells (ATCC, CL 188) were similarly injected into the right flank. After a further 7–14 days, the solid tumors that developed had reached a size of from 100 to 500 mg. Animals were then randomized into treatment groups and injected iv via the tail vein with 2.0 μg of indium-111-labeled antibody in normal saline (100 μL). At 48 h post-injection, all animals were sacrificed by cervical dislocation and the tumors plus all internal organs were removed, weighed, and counted in a gamma counter (LKB Model 1272 Clinigamma, Pharmacia LKB Biotechnology Inc., Gaithersburg, MD) with windows set for 0–300 keV. Weighed aliquots of blood muscle and skin were counted, as was the residual carcass. The tail was counted separately to check for extravasation at the injection site. A 100-μL aliquot of the injectate was counted at the same time as the tissues and the radioactivity measured in each tissue was then expressed as a percentage of this injected dose per gram of tissue.

Registry No. **8**, 117499-10-2; **9**, 117499-11-3; **10**, 117499-12-4; **11**, 117526-32-6; **12**, 117499-13-5; **13**, 70973-01-2; 14, 38335-24-9; **15**, 117499-14-6; **16**, 117499-15-7; **17**, 117499-16-8; **18**, 117499-17-9; **19**, 117499-18-0; **20**, 117499-19-1; **21**, 117499-20-4; **22**, 117499-21-5; *N*-acetylglycine, 543-24-8; 4-nitrobenzaldehyde, 555-16-8; ethylenediamine dihydrochloride, 333-18-6; bromoacetic acid, 79-08-3; thiophosgene, 463-71-8; diethylenetriamine, 111-40-0; 2-[[(*tert*-butoxycarbonyl)oxy]imino]-2-phenylacetonitrile, 58632-95-4; indium-111, 15750-15-9; 2-chloro-*N*,*N*-diethylacetamide, 2315-36-8.

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