Development and Biologic Evaluation of a Kit for Preformed Chelate Technetium-99m Radiolabeling of an Antibody Fab Fragment Using a Diamide Dimercaptide Chelating Agent

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A kit has been developed for ^{99m}Tc antibody radiolabeling via defined chemistry using an N_2S_2 diamide dimercaptide bifunctional chelating agent and the preformed chelate method. The process involved efficient transchelation of ^{99m}Tc from gluconate to 2,3,5,6-tetrafluorophenyl 4,5-bis-S-(1-ethoxyethyl) mercaptoacetamidopentanoate as an active ester ligand and subsequent conjugation to antibody lysine amine functional groups. The use of the ethoxyethyl group for sulfur protection allowed optimum yields of ^{99m}Tc N_2S_2 chelate formation with complete retention of the active ester. Subsequent addition of antibody Fab fragment gave ^{99m}Tc chelate conjugates indistinguishable from the stepwise in situ esterification and purification of the ^{99m}Tc N_2S_2 complex followed by conjugation as previously shown to give stable ^{99m}Tc antibody fragments with retained immunoreactivity and tumor-targeting properties.

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The development of monoclonal antibodies (Mabs) of high affinity and high specificity for tumors has generated widespread interest in radiolabeled Mabs for the detection and treatment of cancer. Although the feasibility for imaging was established with whole Mabs using longer-lived radionuclides such as ^{131}I and ^{111}In, improved imaging quality and convenience could be obtained by using ^{99m}Tc. Fragments of antibodies such as F(ab)'$_2$ (100,000 kD) and Fab (50,000 kD) clear more rapidly from the vascular system and provide useful target-to-background ratios within the 24-hr practical imaging window of the 6-hr half-life of ^{99m}Tc (*1*).

Among the several antibody radiolabeling methods reported in the literature, the majority involve direct labeling with ^{99m}Tc of the cysteine sulfhydryl groups generated using reducing mercaptans such as dithiothreitol or cysteamine and exchange of reduced, chelated ^{99m}Tc. Direct labeling involves metal binding to nitrogen, sulfur, and carboxylate protein donor atoms resulting in randomly distributed multiple binding sites of variable affinity (*2,3*). Despite the apparent simplicity of the direct labeling method, concerns of label stability, irreproducible generation of binding sites, and variable labeling yield of the radiolabeled product led us to the development of alternate methods of ^{99m}Tc antibody radiolabeling.

Antibodies have been radiolabeled with ^{99m}Tc via bifunctional chelating agents, but with suboptimal results. Bifunctional chelating agents, including diethylenetriaminepentaacetic acid (DTPA) (*4,5*) and bisthiosemicarbazones (*6*) have been studied for ^{99m}Tc labeling, but have been found to suffer from problems of: (a) nontarget in vivo uptake due to the requirement for several ligands per antibody; (b) adventitious binding of metal ^{99m}Tc to the protein; (c) formation and binding of colloids to the antibody (*7*); (d) control of oxidation state of technetium; and (e) low yields of specifically bound ^{99m}Tc (*5*).

We have applied a diamide dimercaptide ligand system (N_2S_2) to ^{99m}Tc labeling of antibody fragments. The N_2S_2 ligand system forms highly stable tetradentate complexes with technetium at the +5 oxidation state (*8*). Structure distribution studies of a large number of compounds containing the N_2S_2 donor group showed that high yields of ^{99m}Tc complexes of the predicted structure were obtained (*9–11*). The chemistry of ^{99m}Tc labeling of antibodies was controlled by preformation of the ^{99m}Tc N_2S_2 complex and then conjugation to the protein. Initial development thus involved formation of the carboxylate containing ^{99m}Tc N_2S_2 complex, esterification using phenol and carbodiimide, purification, and then conjugation with protein (*12*). However, the antibody labeling procedure required about 4 hr. As such it was unsuitable for routine use in clinical applications.

The development of a kit was based on minimization of synthetic steps. Thus, the ^{99m}Tc radiolabeling procedure was simplified by direct chelation of the ^{99m}Tc to the active ester form of the N_2S_2 chelating agent. This eliminated the

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stepwise in situ esterification and purification. The kit has been used for ^{99m}Tc labeling of anti-melanoma NR-ML-05 Fab antibody fragment. Clinical results have been reported elsewhere (*13,21*). This paper reports the synthesis of the N_2S_2 active ligand, active ester optimization of transchelation to the N_2S_2 active ester and conjugation to antibody fragment, in vitro characterization, and comparative in vivo biodistribution studies.

MATERIALS AND METHODS

General

Proton NMR spectra were obtained on a Varian Gemini-200 spectrometer with Me_4Si as an internal standard. Melting points were obtained on a Thomas-Hoover apparatus and are uncorrected. Elemental analyses were performed by Deseret Microanalytical Laboratories, Tucson, AZ. High-performance liquid chromatography (HPLC) was carried out on Beckman-Altex systems using 5 micron ODS (Beckman Ultrasphere) columns. Spectrophotometric detection was done on Beckman Model 153 fixed wavelength or 155-40 variable wavelength spectrophotometers. Antibody conjugate radiochemical purity was assessed by size-exclusion chromatography using Zorbax (DuPont de Nemours) HPLC with 0.2 *M* phosphate, pH 7.2, as mobile phase. Detection of monomeric and aggregate antibody forms separated from non-bound Tc-species was performed using a Beckman model 170 radiometric detector in series with a Beckman 331 UV detector. Separation of free- and protein-bound ^{99m}Tc was performed using silica gel impregnated glass fiber thin-layer chromatography (TLC) supplied by Gelman Sciences, Inc., Ann Arbor, MI as ITLC® SG. The silica gel strips were precut to a final dimension of 2 × 10 cm, activated, and stored according to the manufacturer's instructions. The TLC strips were developed with 12% w/v trichloroacetic acid (TCA) freshly prepared as elution solvent. In this TLC development solvent system, the ^{99m}Tc antimelanoma antibody Fab fragment remained at the origin and nonprotein bound ^{99m}Tc-labeled species migrated with the solvent front. The TLC strips were cut into two halves that were separately counted for final purity on a Packard MINAX1 500 series gamma counter.

Challenge Studies of (^{99m}Tc-$C_5N_2S_2$-NR-ML-05 Fab) Conjugate

The stability of the ^{99m}Tc binding to the antibody fragment was tested by incubation at 37°C in the presence of various challenging agents. They included human serum (diluted to 80%), 10 m*M* DTPA, 10 m*M* 2,3-bis(mercaptoacetamido)propanoate, 6 *M* urea, and 10 m*M* cysteine. Samples were removed at various times, analyzed by ITLC, and compared with that of the ^{99m}Tc-Fab conjugate prepared by the in situ active ester procedure (*12*).

Radiolabeled Cell Binding Assay

The immunoreactivity of ^{99m}Tc-$C_5N_2S_2$-NR-ML-05 Fab was assessed in an antigen-excess cell binding assay as previously described (*16*). Radioactivity bound to cells was determined at increasing FMX-metastatic (met) melanoma cell levels until maximum binding was shown (*17,18*). Correction was made for nonspecific binding by the addition of excess unlabeled antibody.

Biodistribution Studies of ^{99m}Tc-Labeled NR-ML-05 Fab

Time course biodistribution studies were conducted separately with "kit" and "in situ esterification" methods of labeled NR-ML-05 Fab in four groups of eight tumored nude mice at 4 and 20 hr postinjection. The standard protocol included intravenous administration via lateral tail vein of 10 μg of antibody fragment (50–100 μCi) in a 100-μl volume. The volume of injectate was determined by weighing the syringe before and after injection. At the designated times postinjection, mice were weighed, bled via the retro-orbital plexus, and killed by cervical dislocation. Thirteen organs and tissues were collected: blood (BL), tail (TA), tumor (TU), skin (SK), muscle (MU), bone (BO), lung (LU), liver (LI), spleen (SP), stomach (ST), neck (NE), kidneys (KI), and intestines (IN). Tissues were blotted when appropriate, weighed, and counted along with standards of the injected dose in a gamma scintillation counter with the counting window set from 80 to 200 keV.

RESULTS

The synthesis of 2,3,5,6-tetrafluorophenyl 4,5-bis-S-(1-ethoxyethyl)mercaptoacetamidopentanoate (*10*) prepared as described in the Appendix was purified by preparative reverse-phase TLC.

The chelation of active ester ligand with ^{99m}Tc was accomplished via exchange in aqueous isopropyl alcohol (IPA) organic solvent from intermediate ^{99m}Tc-gluconate by heating at 75°C for 15 min. This gives 80%–95% radiochemical yields of the ^{99m}Tc chelate ester (Fig. 1). Radiochemical yields were dependent upon the percent of

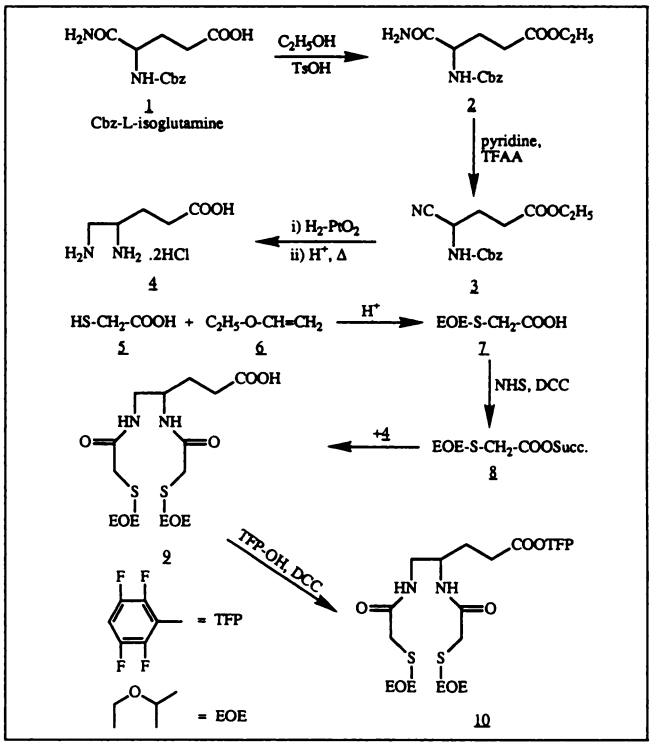

FIGURE 1. Synthesis of 2,3,5,6-tetrafluorophenyl-4,5-bis-(S-1-ethoxyethyl)mercaptoacetamidopentanoate (*10*).

TABLE 1
The Dependency of Isopropyl Alcohol in the Formation of ^{99m}Tc-2,3,5,6-tetrafluorophenyl 4,5-bis(thioacetamido)pentanoate

% Isopropanol	% Active ester formation by HPLC
0	55
7.7	76
13	84
19	88
24	92
32	94

TABLE 2
The Dependency of the Transchelation Reaction of ^{99m}Tc-Gluconate with 2,3,5,6-Tetrafluorophenyl 4,5-bis(1-ethoxyethyl mercaptoacetamido)pentanoate (N_2S_2 TFP) on pH

pH	^{99m}Tc-N_2S_2 TFP (%)
3.92	73
3.61	75
3.34	86
3.24	90
3.04	79
2.66	83

IPA used in the chelate exchange reaction (Table 1). The optimal concentration of IPA was found to be 30%. The selection of IPA was based on minimization of transesterification of the active ester due to its secondary alcohol nature. When ethanol was used as solvent, HPLC analysis indicated the formation of 3%–5% of the Tc-chelate ethyl ester via transesterification, which is unreactive in the antibody conjugation step.

The ethoxyethyl sulfur protecting group is lost during the chelation step (Fig. 2). This conclusion is based on obtaining the same product directly from the ethoxyethyl sulfur protected tetrafluorophenyl active ester chelate as from the procedure involving carboxylate dithiol chelation followed by esterification as reported earlier (*12*). Since the ethoxyethyl group on the sulfur is stable at pH 3, its loss probably involves technetium metal assistance. The radiochemical yields were dependent upon pH as shown in Table 2. Under the kit labeling conditions, negligible hydrolysis of the ester to the carboxylate form of the complex was observed. The analysis of the Tc-complex acid was determined by ^{99m}Tc radiolabeling of the acid form of the ligand under identical conditions and HPLC analysis. The cooling step of the reaction vial ensures lack of heat denaturation of the Fab fragment when added and minimizes hydrolysis during addition of the basic pH conjugation buffer.

The conjugation of the ^{99m}Tc ligand ester with antibody was carried out by increasing the pH of the labeled active ester and antibody fragment mixture to 10.0 and incubation at room temperature for 20 min. The conjugation reaction with the NR-ML-05 Fab afforded yields from 65% to 75%. Figure 3 illustrates the dependence of the degree of conjugation on the amount of antibody present. Although the conjugation yields also depend on pH (*12*), less than 10% change in conjugation yield was observed over the pH range 8.75–10.5.

In order to easily purify the ^{99m}Tc-N_2S_2 Fab conjugate and maintain pharmaceutical quality, a closed-system anion exchange column procedure was developed that allowed the radiolabeled antibody to pass through while retaining the ^{99m}Tc-impurities, yielding conjugates of 95% purity or greater.

In Vitro Analyses

The radiolabeled antibodies were routinely analyzed by HPLC using size-exclusion columns (Fig. 4), isoelectric focusing (IEF), and radiolabeled cell binding assay. The HPLC analysis showed ^{99m}Tc-labeled Fab fragments, aggregates, and small molecular weight forms such as ^{99m}Tc-N_2S_2 carboxylates. Retention times and peak shapes of the antibody conjugate prepared by the kit method were identical to those of the radiolabeled conjugate prepared by the in situ esterification and purification procedure reported earlier (*12*). Negligible loss of radioactivity was observed when the radiolabeled conjugate was subjected

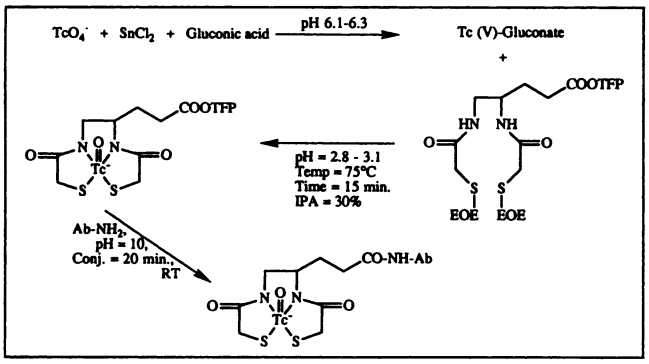

FIGURE 2. Technetium-99m kit labeling procedure of NR-ML-05 Fab.

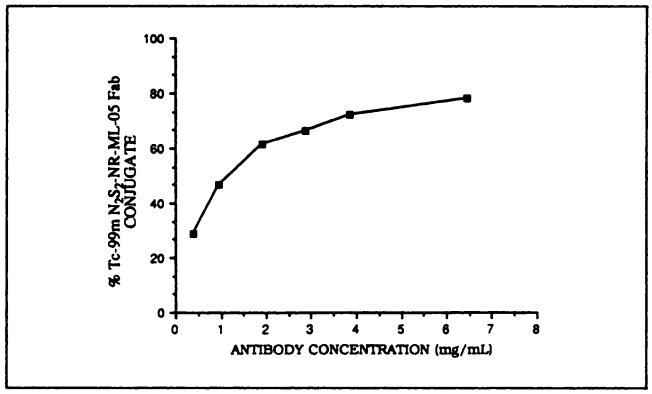

FIGURE 3. The dependency of reaction of the ^{99m}Tc-2,3,5,6-tetrafluorophenyl 4,5-bis(thioacetamido)pentanoate with NR-ML-05 Fab antibody fragment concentration.

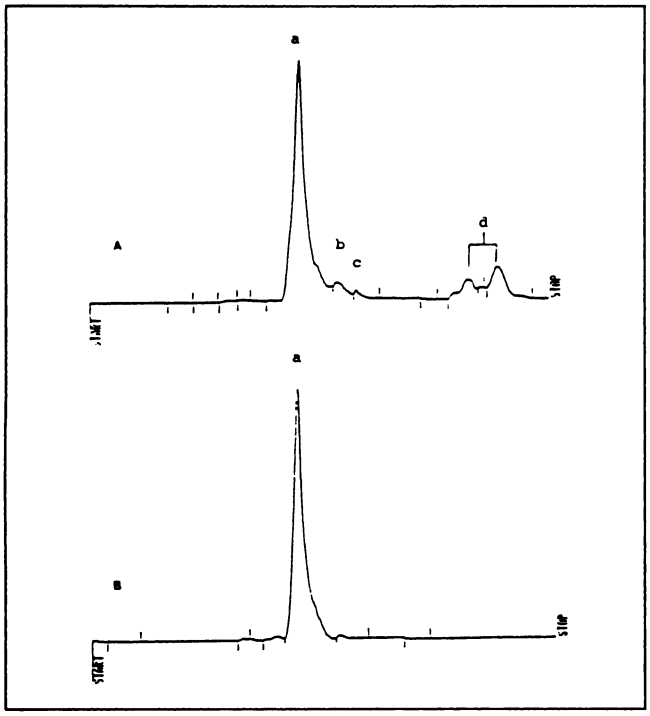

FIGURE 4. HPLC chromatograms of crude ^{99m}Tc conjugation product (radiometric trace in A) and purified ^{99m}Tc-N_2S_2-NR-ML-05 Fab (radiometric in trace B). The column used was Zorbax. Conditions: 0.2 *M* sodium phosphate (pH 7.0) at 1 ml/min. (a) Technetium-99m-N_2S_2-NR-ML-05 (monomeric form), (b) ^{99m}Tc-gluconate, (c) $^{99m}TcO_4^-$, and (d) ^{99m}Tc-N_2S_2 acids (epimeric forms).

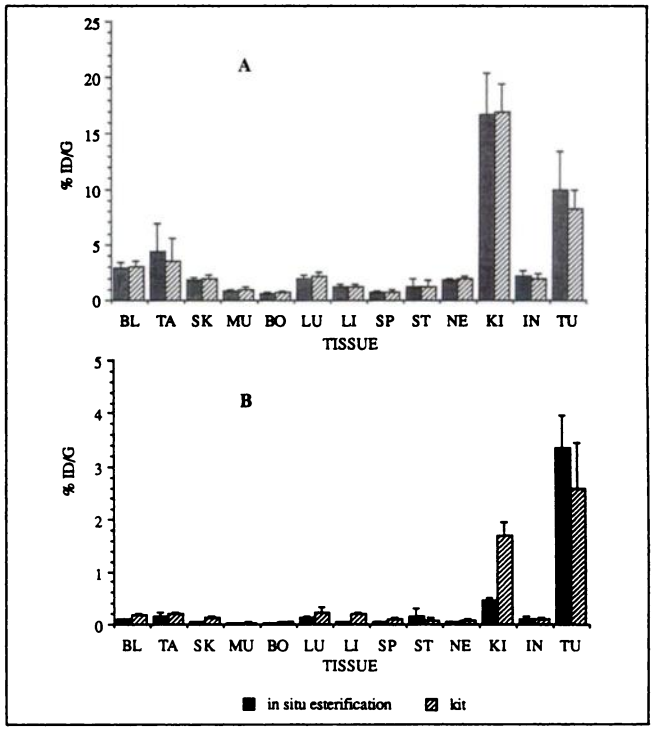

FIGURE 5. Biodistribution values in %ID/g of tissue with standard deviation of ^{99m}Tc-N_2S_2-NR-ML-05 Fab in mice bearing A375 m/m xenografts at (A) 4 hr and (B) 20 hr postinjection.

to challenge agents. In vitro incubation of Tc-labeled conjugate with human serum albumin and transferrin failed to exhibit any transchelation as assessed by HPLC under conditions that separated Fab fragment from these proteins. A maximum of ≤5% of activity of the purified preparation is ^{99m}Tc-$C_5N_2S_2$ acid forms. Of this impurity activity, 90% is readily excreted through the renal system and about 10% via the hepatobiliary route. Challenge experiments using 10 m*M* of DTPA, 10 m*M* of 2,3-bis(mercaptoacetamido)propanoate, and 6 *M* of urea as in the previous study (*12*) did confirm that a stable attachment of the radiolabeled conjugate was obtained by the preformed chelate technology. The stability of the Fab fragment preparations was evaluated by cell binding assay to measure immunoreactivity and HPLC for chemical form changes. The results as shown in Table 3 indicated that excellent stability is obtained for storage of up to 12 hr from the time of preparation.

TABLE 3
Stability of ^{99m}Tc-N_2S_2-NR-ML-05 Fab

Time* (hr)	Immunoreactivity†, %	Purity‡, % (TLC)
0	79.7	95.4
2	—	94.4
4	71.2	93.8
8	70.7	93.6
12	76	92.6

* Technetium-99m-N_2S_2-NR-ML-05 Fab post-labeled preparation stored at room temperature.

† Immunoreactivity of ^{99m}Tc-C_5-N_2S_2-NR-ML-05 Fab was assessed in an antigen excess cell binding assay by measuring radioactivity bound to FMX-met melanoma cells.

‡ The values represent the radiochemical purity of the conjugate by ITLC developed in 12% w/v TCA solution.

In Vivo Evaluation

The ^{99m}Tc-radiolabeled antibody fragment prepared by the "kit" method and the "in situ ester" procedure reported earlier (*12*) were evaluated in vivo for any difference between the two procedures. The antibody chosen for the in vivo evaluations by the kit method was the Fab fragment of NR-ML-05, an anti-melanoma antibody which reacts with the 250 kD proteoglycan antigen present on melanoma cells (*19*).

Biodistribution data is presented in Figure 5. Localization in normal tissues and tumor is equivalent at 4 hr and similar at 20 hr, with the exception of kidney which is higher for the kit preparation (1.71 ± 0.25 %ID/g) than for the in situ esterification preparation (0.45 ± 0.07 %ID/g). In a replicate kit labeling study (data not shown), less activity, 0.97 ± 0.10 %ID/g, was retained in the kidney at 20 hr. In any case, renal activity at 20 hr accounts for <0.5% of the injected dose, indicating that although there

may indeed be longer retention of kit metabolite in kidney, it accounts for a negligible fraction of the injected dose.

DISCUSSION

The results of these studies indicate that it is practical to radiolabel antibodies and/or their fragments with ^{99m}Tc by using defined chelation chemistry. The chemistry of the kit formulation involves forming a stable complex with ^{99m}Tc by exchange of technetium from an intermediate labile complex into a stable tetradentate N_2S_2 chelate and subsequently conjugating the complex by using an active-ester approach under mild conditions. Time required for the kit labeling procedure is about 60 min. High stability and complete retention of immunoreactivity have been demonstrated for the ^{99m}Tc-labeled antibody fragments by both in situ esterification and kit procedures. The challenge studies of antibody Fab fragment labeled with the C_5-N_2S_2 complex indicate that the label is stably bound. Equivalent biodistribution and tumor retention were also obtained for both procedures. The F(ab')$_2$ fragment of another anti-melanoma antibody, 9.2.27, an IgG_{2a} subclass antibody which reacts with the same antigen as that of NR-ML-05, was also radiolabeled by the kit method. No cleavage of F(ab')$_2$ to Fab' was seen, indicating that the mild reducing conditions present during the protein conjugation step do not cleave the disulfide bonds responsible for the integrity of the F(ab')$_2$. This has been problematic for other approaches that involved radiolabeling F(ab')$_2$ with reducing agents present such as stannous ion (*20*).

The kit has been found to be useful in extensive clinical trials. The low degree of retention of ^{99m}Tc radioactivity in the liver, spleen, and bone marrow resulted in high detection rates in these organ systems (*13,21*).

Rhenium-186 and ^{188}Re have attractive properties for radioimmunotherapy. Thus, virtually identical structural chemistry of the N_2S_2 Tc and Re complexes (*22*) provide a basis for an imaging (^{99m}Tc) and therapeutic (^{186}Re, ^{188}Re) matched pair. The preformed chelate approach allows adjustment in chelation conditions to compensate for the differences in rates and redox chemistries of rhenium and technetium. Thus, similar kits have been developed for use with rhenium. Our studies of ^{186}Re and ^{188}Re indicate that the same N_2S_2-diamide dithiolate technology is applicable to rhenium labeling of proteins and gives biodistribution properties that are comparable to that of the ^{99m}Tc-N_2S_2 antibody fragment conjugates (*23*).

APPENDIX

Synthesis of 2,3,5,6-Tetrafluorophenyl 4,5-bis-(S-1-ethoxyethyl mercaptoacetamido)pentanoate (Fig 1)

Carbobenzyloxy-L-isoglutamine (*1*, Cbz-L-Isoglutamine) was prepared according to the literature procedure of Straka and Zaoral (*14*).

N-Cbz-L-isoglutamine Ethyl Ester (2)

A stirred suspension of Cbz-L-isoglutamine (*1*) (28 g, 100 mmol) and p-toluenesulfonic acid monohydrate (1.9 g, 10 mmol) in 560 ml of absolute ethanol was gently refluxed for 12–14 hr until TLC (1:5:94 HOAC/H_2O/CH_3CN) indicated that esterification was complete. The reaction mixture was concentrated in vacuo and recrystallized from ethyl acetate/hexane to give 21.8 g (71%) of a white solid: mp 144–145°. H^1 NMR ($CDCl_3$): δ 1.23 (t,3H), 1.95 (m,1H), 2.02 (m,1H), 2.46 (m,2H), 4.21 (q,2H), 4.24 (m,1H), 5.09 (s,2H), 5.85 (br,2H,$CONH_2$), 6.47 (br,1H,NH), 7.33 (s,5H,C_6H_5).

N-Cbz-γ-cyano-γ-aminobutyric Acid Ethyl Ester (3)

To a stirred suspension of Cbz-L-isoglutamine ethyl ester (*2*) (15.42 g, 50 mmol) and pyridine (8.4 ml, 105 mmol) in 360 ml of anhydrous THF at 0°C was added dropwise a solution of trifluoroacetic anhydride (7.7 ml, 55 mmol) in 40 ml of THF at such a rate as to maintain a temperature of 0–5°C (*15*). The reaction mixture was allowed to stir at 0°C for 1–2 hr or until reaction was complete as evidenced by TLC (5% H_2O/94% CH_3CN/1% HOAC; $Cu(OAc)_2$ stain). The reaction mixture was concentrated in vacuo to a clear oil. The oil was taken up in ethyl acetate, washed twice with dilute aqueous HCl, water, brine, and dried (Na_2SO_4). The mixture was filtered and concentrated in vacuo to a clear oil. Recrystallization from cold ethanol/water gave 11.90 g (82%) of white needles: mp 61–62°C. H^1 NMR ($CDCl_3$): δ 1.23 (t,3H), 2.19 (m,2H), 2.49 (m,2H), 4.13 (q,2H), 4.35 (m,1H), 5.12 (s,2H), 5.92 (br,1H,NH), 7.34 (s,5H,C_6H_5).

4,5-Diaminopentanoic Acid Dihydrogen Chloride (4)

A 500-ml Parr Shaker bottle was charged with 3.00 g of N-Cbz-γ-cyano-γ-amino-butyric acid ethyl ester (*3*), 500 mg of PtO_2 catalyst (Aldrich), 80 ml of EtOH, and 80 ml of 6N HCl. The mixture was shaken for 16 hr under 50–60 psi H_2 pressure and then filtered and concentrated. The resulting oily residue was dissolved in 150 ml of 6N HCl and heated at 70° for 4 hr. After concentrating under vacuum, 100 ml of EtOH was added to the resulting syrup. The mixture was allowed to stand in the refrigerator, and the resulting solid was collected by filtration to yield 2 g of white powder. H^1 NMR (DMSO-d_6): δ 1.88 (t,2H), 2.43 (t,2H), 3.10 (d,2H), 3.46 (m,1H), 9.07 (br,6H,NH_3^+). Anal. Calcd. for $C_5H_{12}N_2O_2$.2HCl: C, 29.28, H, 6.88, N, 13.65, Cl, 34.51; Found: C, 29.35, H, 7.05, N, 13.63, Cl, 34.62.

N-Hydroxysuccinimidyl S-(1-ethoxyethyl)mercaptoacetate (8)

Mercaptoacetic acid (*5*) was converted to the S-(1-ethoxyethyl) derivative (*7*) in the following manner: A solution of mercaptoacetic acid (*5*) (17.4 ml, 250 mmol) in 125 ml of dichloromethane containing p-toluenesulfonic acid (0.24 g, 1.26 mmol) was cooled to −18 to −25°C with stirring. Ethyl vinyl ether (*6*) (23.9 ml, 250 mmol) in 125 ml of dichloromethane was added dropwise to the cold solution over a period of 90 min. The stirring was continued for an additional 30 min with the temperature maintained in the −18 to −25°C range. Then 200 ml of phosphate buffer (pH = 7) was added and the reaction mixture was allowed to warm with stirring for 10 to 15 min. The mixture was then poured into a flask containing 900 ml of ethyl acetate and 200 ml of water. Layers were separated, and the aqueous portion extracted twice with ethyl acetate. The organic layers were combined, washed with brine, and dried ($MgSO_4$). Removal of the solvent left 31.4 g of S-(1-ethoxyethyl)mercaptoacetic acid (*8*) as a colorless oil

(77% yield). H^1 NMR ($CDCl_3$): δ 1.15 (t, J= 7.0 Hz, 3H), 1.52 (d, J = 6.4 Hz, 3H), 3.36 (s, 2H), 3.60 (m, 2H), 4.84 (q, J = 6.4 Hz, 1H), 11.65 (s, 1H). The material was used without further purification for conversion of carboxylic acid to succinimidate ester.

A solution of S-(1-ethoxyethyl)mercaptoacetic acid (*7*) (5.76 g, 35.1 mmol), and N-hydroxysuccinimide (4.85 g, 42.1 mmol) was prepared in 100 ml of anhydrous THF. To this was added a solution of 1,3-dicyclohexylcarbodiimide (8.70 g, 42.1 mmol) in 65 ml of anhydrous THF. The mixture was stirred at room temperature for 2 hr or until TLC analysis indicated complete formation of the succinimidyl ester. The mixture was then filtered, and the filtrate was concentrated to a viscous residue. The residue was dissolved in ethyl acetate, washed with water, brine, and dried ($MgSO_4$). Removal of the solvent left the crude succinimidyl ester as an oil, which was further purified by flash chromatography on silica gel using ethyl acetate-hexanes as the column eluent to give 5.1 g of N-hydroxysuccinimidyl S-(1-ethoxyethyl)mercaptoacetate (*8*) as a colorless oil (56% yield). H^1 NMR ($CDCl_3$): δ 12.1 (t, J = 7.0 Hz, 3H), 1.58 (d, J = 6.4 Hz, 3H), 2.83 (s, 4H), 3.60 (m, 4H), 4.88 (q, J = 6.4 Hz, 1H).

4,5-bis-(S-1-ethoxyethylmercaptoacetamido) pentanoic acid (9)

To a stirring suspension of 4,5-diaminopentanoic acid dihydrochloride (*4*) (1.64 g, 8.0 mmol) in 32 ml of anhydrous dimethylformamide containing triethylamine (6.7 ml, 48.0 mmol) was added N-hydroxysuccinimidyl S-(1-ethoxyethyl)mercaptoacetate (*8*) (4.60 g, 17.6 mmol) dissolved in 12 ml of anhydrous dimethylformamide. The reaction mixture was stirred at room temperature for 90 min or until TLC analysis indicated complete formation of 4,5-bis-(S-1-ethoxyethylmercaptoacetamido)pentanoic acid. Then the reaction mixture was filtered, and the filtrate was concentrated to a viscous oil. The oil was dissolved in ethyl acetate and washed with successive portions of water, until no N-hydroxysuccinimide was evident in the organic phase by TLC. The organic phase was washed with brine and dried ($MgSO_4$). Removal of solvent afforded 2.0 g of 4,5-bis-[S-1-ethoxyethyl) mercaptoacetamido]pentanoic acid (*9*) as a viscous oil which solidified upon trituration with ether (59% yield). H^1 NMR ($CDCl_3$): δ 1.18 (t, J = 7.2 Hz, 6H), 1.53 (d, J = 6.6 Hz, 6H), 1.88 (m, 2H), 2.45 (t, J = 6.8 Hz, 2H), 3.30 (s, 4H), 3.55 (m, 6H), 4.10 (m, 1H), 4.77 (q, J = 6.6 Hz, 2H), 7.33 (m, 2H), 9.44 (br, 1H). Anal. Calcd. for $C_{17}H_{32}N_2O_6S_2$: C, 48.10, H, 7.59, N, 6.59, S, 15.07: Found: C, 48.06, H, 7.77, N, 6.56, S, 15.09.

2,3,5,6-Tetrafluorophenyl 4,5-bis(S-1-ethoxyethyl mercaptoacetamido)pentanoate (10)

To a solution of 4,5-bis-[S-(1-ethoxyethyl)mercaptoacetamido] pentanoic acid (*9*) (1.50 g, 3.53 mmol) and 2,3,5,6-tetrafluorophenol (0.88 g, 5.3 mmol) in 16 ml of anhydrous tetrahydrofuran was added 1,3-dicyclohexylcarbodiimide (0.95 g, 4.6 mmol) with rapid stirring. The mixture was stirred at room temperature for 18–24 hr or until TLC analysis indicated complete conversion to the ester. Then the mixture was filtered, and the filtrate was concentrated to give a solid. The solid was dissolved in a minimal amount of ethyl acetate and allowed to stand at 5°C for 2 hr. The solution was then filtered to remove any precipitated dicyclohexylurea, and the filtrate was concentrated to afford solid 2,3,5,6-tetrafluorophenyl 4,5-bis-(S-1-ethoxyethylmercaptoacetamido)pentanoate. The solid was washed with ether to remove any remaining 2,3,5,6-tetrafluorophenol. After drying in vacuo, 1.64 g of 2,3,5,6-tetrafluorophenyl 4,5,-bis-(S-1-ethoxyethylmercaptoacetamido)pentanoate was obtained (81% yield). H^1 NMR ($CDCL_3$): δ 1.22 (t, J = 7.2 Hz, 6H), 1.56 (d, J = 6.6 Hz, 6H), 2.06 (m, 2H), 2.83 (t, J = 8 Hz, 2H), 3.33 (s, 4H), 3.60 (m, 6H), 4.15 (m, 1H), 4.75 (q, J = 6.6 Hz, 2H), 7.22 (m, 3H). MS (CI): 573 (M + 1). Anal. Calcd. for $C_{23}H_{32}N_2O_6S_2F_4$: C, 48.24, H, 5.63, N, 4.89, S, 11.20; Found: C, 48.46, H, 5.74, N, 5.09, S, 10.87.

Preparation of ^{99m}Tc-2,3,5,6-tetrafluorophenyl 4,5-bis(thioacetamido)pentanoate NR-ML-05 Fab

Preparation of ^{99m}Tc radiolabeled antibody conjugate by the kit procedure involved five sequential steps:

1. Preparation of acidified ligand active ester solution.
2. Preparation of intermediate ^{99m}Tc-gluconate complex.
3. Preparation of ^{99m}Tc ligand active ester chelate.
4. Conjugation of ^{99m}Tc ester chelate to antibody fragment.
5. Purification of radiolabeled antibody fragment on anion exchange chromatographic column.

Acidified ligand active ester solution was prepared by adding 0.16 ml of 0.2 *M* hydrochloric acid-glacial acetic acid (14:2 ratio) to 0.6 ml of 2,3,5,6-tetrafluorophenyl 4,5-bis-(S-1-ethoxyethylmercaptoacetamido)pentanoate (*10*) (0.3 mg, 0.0005 mole freshly dissolved in 0.9 ml isopropyl alcohol). Then 0.5 ml of this solution was added to 1.1 ml of ^{99m}Tc-gluconate (the ^{99m}Tc-gluconate was prepared from 0.12 mg $SnCl_2.2H_2O$, 5.0 mg of sodium gluconate at pH 6.1–6.3 and 100 mCi of [^{99m}Tc]pertechnetate). The reaction mixture was then heated at 75°C for 15 min followed by cooling on ice. Then 0.1 ml of 1.0 *M* carbonate buffer, pH 10, was added and followed immediately by 10 mg of NR-ML-05 Fab antibody fragment [in 0.9 ml of a 1.25:1 mixture of phosphate-buffered saline (6 m*M* phosphate in 150 m*M* NaCl, pH 7.2) and 1.0 *M* carbonate buffer, pH 10] for conjugation. After incubation for 20 min at room temperature, the ^{99m}Tc-$C_5N_2S_2$-NR-ML-05 Fab conjugate was purified by loading onto a 5-ml QAE-Sephadex® (Pharmacia) column (equilibrated in 37 m*M* phosphate buffer, pH 6.8) and elution with 5 ml of 75 m*M* sodium chloride solution.

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