Thiol-Containing Cross-Linking Agent with Enhanced Steric Hindrance

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Ricin A chain immunotoxins disulfide cross-linked with conventional, sterically unhindered reagents have unsatisfactorily short circulating life times in vivo. (Acetylthio)succinic anhydride, a thiolating reagent with partial steric hindrance of the sulfur atom, does not remedy this situation. Sulfosuccinimidyl *N*-[3-(acetylthio)-3-methylbutyryl]-β-alaninate, a new cross-linker in which the carbon α to the sulfur is doubly methylated, creates disulfide bonds 2 orders of magnitude more resistant to reduction than unhindered disulfides. Nevertheless, this deactivated thiolating agent rapidly and reliably cross-links ricin A chain and antibodies to create immunotoxins with in vitro cytotoxicities comparable to those of 2-iminothiolane-coupled conjugates.

INTRODUCTION

Immunotoxins are protein conjugates in which a toxin is covalently attached to a monoclonal antibody (reviewed in refs 1–3). These chimeric molecules contain up to four functional regions. The antibody targets the drug in vivo to the desired cell population. The toxin is the effector portion responsible for cell death once bound to the cell surface (for surface-acting toxins) or internalized into the cytosol (for toxins which act on the protein-synthesis machinery). Most toxins are thought to contain a translocation domain which facilitates entry of the catalytic portion into the cytosol. Finally, there is a cleavable linking region, which must confer stability to the conjugate in vivo while in the circulation, but later allow the release of the toxin to enable its entry into the cytosol.

Often the toxin moiety is one of a variety of proteins capable of catalytically inactivating the protein-synthesis machinery of eukaryotic cells. These include holotoxins consisting of a catalytically active A fragment and binding B fragment [e.g. diphtheria toxin (*4*, *5*), *Pseudomonas* exotoxin A (*6*, *7*), ricin (*8*, *9*), and abrin (*10*, *11*)], hemitoxins consisting of only the enzymatic A fragment [e.g. the A chain of diphtheria toxin (*12*, *13*), ricin (*1*, *14*), or abrin (*15*, *16*)], and ribosome-inactivating proteins [e.g. gelonin (*17*, *18*), pokeweed antiviral protein (*19*, *20*), and saporin (*21*)]. In order to be effective in vivo, the toxin must remain attached to the antibody in the circulation. However, once the immunotoxin is inside the target cell, the release of the catalytic portion of the toxin is required in order to interact with its cytosolic target; A-chain-containing conjugates made with noncleavable thioether linkages are less than 1% as active as those containing easily reduced disulfide linkages (*14*, *22*). The type of linkage required between the toxin and antibody depends on the form of the toxin used.

Conjugates made with the standard heterobifunctional cross-linking reagents, *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP)[1] and 2-iminothiolane (2-IT), are labile in circulation (*23–26*, *18*). Premature cross-link cleavage reduces the amount of intact conjugate which can bind to target cells. In addition, the released antibody remains in the circulation longer than conjugate and can compete with the intact conjugate for target-cell binding (23, 24, 26–29). Finally, the slow, sustained release of ricin A chain may contribute to the increased toxicity of disulfide-linked conjugates compared to that of free ricin A chain, which is rapidly cleared by renal filtration (*23*, *30*). All of these factors probably have reduced immunotoxin efficacy in various in vivo models for solid-tumor therapy.

Thorpe et al. (*31*) and Worrell et al. (*32*) synthesized cross-linking reagents capable of yielding more stable disulfide bonds, on the basis of the finding that substitution of the α-carbon decreases the reactivity of the adjacent sulfur atom (*33*). One linker, 4-[(succinimidyloxy)-carbonyl]-α-methyl-α-(2-pyridyldithio)toluene (SMPT), sterically hindered the disulfide with a methyl group and a benzene ring attached to the carbon adjacent to the sulfur atom (*31*). The other linker, *N*-succinimidyl 3-(2-pyridyldithio)butyrate, substituted a methyl group on the α-carbon (*32*). Conjugates made with these new cross-linkers were more difficult to reduce chemically and had longer circulating half-lives than immunotoxins bearing

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[1] The abbreviations used are as follows: BSA, bovine serum albumin; DTDP, 4,4′-dithiodipyridine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSH, reduced glutatione; GSSTNB, 2-nitro-5-mercaptobenzoic acid disulfide of glutathione; GSSTP, 4-mercaptopyridine disulfide of glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HNSA, 4-hydroxy-3-nitrobenzenesulfonic acid; IAM, iodoacetamide; 2-IT, 2-iminothiolane; MEA, β-mercaptoethylamine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NaP_i, sodium phosphate buffer; NHS, *N*-hydroxysuccinimide; PSH, penicillamine; PSSTNB, 2-nitro-5-mercaptobenzoic acid disulfide of PSH; PSSTP, 4-mercaptopyridine disulfide of PSH; rRA, recombinant ricin A chain; rRA–TNB, 2-nitro-5-mercaptobenzoic acid disulfide of rRA; rRA–TP, 4-mercaptopyridine disulfide of rRA; SAMSA, (acetylthio)succinic anhydride; SMPT, 4-[(succinimidyloxy)carbonyl]-α-methyl-α-(2-pyridyldithio)-toluene; sNHS, *N*-hydroxysulfosuccinimide; sNHS-ATMBA, sulfosuccinimidyl *N*-[3-(acetylthio)-3-methylbutyryl]-β-alaninate; SPDP, succinimidyl 3-(2-pyridyldithio)propionate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TMBA, *N*-(3-mercapto-3-methylbutyryl)-β-alanine; TNB, 2-nitro-5-mercaptobenzoic acid;

the standard disulfide cross-links (*31*, *32*). Conjugates made with SMPT were as cytotoxic in vitro as conjugates synthesized with SPDP or 2-IT and had improved efficacy in vivo (*34*). Attempts to make an active-ester cross-linking reagent from 3-(2-pyridyldithio)isovaleric acid, a compound containing two methyl substituents on the carbon α to the disulfide, failed (*32*).

During an effort to make immunotoxins which would resist undesired disulfide cleavage in circulation, we found that conjugates cross-linked with (acetylthio)succinic anhydride (SAMSA), in which the α-carbon is substituted with either a carboxylate or a methyl carboxylate, are unstable in vivo. Therefore, we developed a cross-linking reagent, sulfosuccinimidyl *N*-[3-(acetylthio)-3-methylbutyryl]-β-alaninate (sNHS-ATMBA), in which the α-carbon is substituted with two methyl groups. The disulfide bond involving the sulfur adjacent to the tertiary carbon is 2 orders of magnitude more difficult to reduce than the analogous glutathione disulfide bond. Nevertheless, most conjugates made with this cross-linker have in vitro cytotoxic activity similar to that of analogous conjugates made with 2-IT.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals. (^{35}S)methionine (1086 Ci/mmol, cat. no. NEG-009A) and [1-^{14}C]iodoacetamide (24.1 mCi/mmol) were purchased from New England Nuclear (Boston, MA); 2-IT, SAMSA, *N*-hydroxysulfosuccinimide (sNHS), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and 4,4′-dithiodipyridine (DTDP) were from Pierce (Rockford, IL); β-mercaptoethylamine (MEA), iodoacetamide (IAM), *tert*-butyl-β-alanine hydrochloride, *N*-hydroxysuccinimide (NHS), dicyclohexyl carbodiimide, and carboxymethylated BSA were from Sigma (St. Louis, MO); chloroform ($CHCl_3$), methanol (MeOH), and methylene chloride (CH_2Cl_2) were from Burdick and Jackson (Muskegon, MI); triethylamine, dimethylacryloyl chloride, thiolacetic acid, trifluoroacetic acid (TFA), and D,L-penicillamine (α-amino-β-methyl-β-mercaptobutyric acid, PSH) were from Aldrich Chemical Co. (Milwaukee, WI); *N*,*N*-Dimethylformamide was from Fisher Scientific Co. (Fair Lawn, NJ); and activated Type 4A, 8–12 mesh molecular sieve was from J. T. Baker Chemical Co. (Phillipsburg, NJ). The 4-hydroxy-3-nitrobenzenesulfonic acid (HNSA) sodium salt was synthesized as previously described (*35*). Preparative chromatography was performed on a Chromatotron (Harrison Instruments, Palo Alto, CA).

Strains and Medium. Hybridoma cells were grown in HL-1 medium (Ventrex, Portland, ME) supplemented with Fungibact (Irvine Scientific, Santa Ana, CA) and 8 mM glutamine. When labeling the antibody metabolically by incorporation of (^{35}S)methionine, HL-1 medium deficient in methionine was used. For the in vitro kinetic studies, cells were grown in RPMI 1640 medium with and without methionine (Flow Laboratories, McLean, VA).

Mice. Female Balb/C nude (nu/nu) mice were obtained from Charles River Breeding Labs (Kingston, NY).

Hybridomas and Antibodies. The following mouse monoclonal antibodies and hybridomas were used in the study (*14*, *36*–*38*).

antibody	isotype	antigen	Accession No.
113F1	IgG_3, κ	37/60/93/200 kDa	HB 8490
2G3	IgG_1, κ	HMW protein	HB 8491
260F9	IgG_1, κ	55 kDa	HB 8488
317G5	IgG_1,	44-kDa glycoprotein	HB 8484
454A12	IgG_1, κ	transferrin receptor	IVI 10075
520C9	IgG_1, κ	210 kDa	HB 8696

Cell Lines. The human breast cancer cell line SK-Br-3 was a generous gift from Dr. J. Fogh (Sloan Kettering, New York, NY), and MCF-7 was obtained from E. G. & G. Mason Research Institute, Worcester, MA. The in vitro human breast cancer cell line MX-1 was a line adapted from the in vivo MX-1 tumor (*39*, *40*) by C. Vitt and A. Creasey (Department of Cell Biology, Cetus Corp.). The newborn human foreskin cell line HS27F (ATCC CRL1634) was used as a negative control because it bound none of the antibodies.

Chemical Analysis. ^{1}H NMR spectra were taken on a Varian FT-80A spectrometer. Chemical shifts of compounds in $CDCl_3$ are reported in ppm downfield from internal tetramethylsilane.

Synthesis of ATMBA. The *tert*-butyl ester of β-alanine hydrochloride (structure II, Figure 1) (1.8 g, 10 mmol) in 10 mL of CH_2Cl_2 was neutralized with 1 equiv of triethylamine (1.4 mL, 10 mmol). The precipitated triethylamine hydrochloride was filtered. The solution of β-alanine *tert*-butyl ester and 1.4 mL of triethylamine was placed in a dropping funnel and added dropwise to a nitrogen-flushed 100-mL three-neck round-bottom flask containing dimethylacryloyl chloride (structure I, Figure 1) (1.1 mL, 10 mmol) dissolved in 10 mL of CH_2Cl_2. The mixture was stirred at room temperature for 2 h. The reaction mixture was diluted to about 50 mL with CH_2Cl_2, washed with water (15 mL × 2) and brine (saturated aqueous sodium chloride), and dried over $MgSO_4$. The crude product was purified by Chromatotron chromatography on a 4-mm silica gel plate. The plate was eluted first with 0.5% MeOH in $CHCl_3$ to remove an impurity, then with 2% MeOH in $CHCl_3$ to elute the product *tert*-butyl ester of dimethylacryloyl-β-alanine (structure III, Figure 1). ^{1}H NMR: δ 6.25 (br s, 1 H), 5.5 (s, 1 H), 3.45 (q, 2 H), 2.4 (t, 2 H), 2.2 (s, 3 H), 1.8 (s, 3 H), 1.45 (s, 9 H). Yield = 1.5 g (66%).

To 1.2 g (5.4 mmol) of the *tert*-butyl ester of dimethylacryloyl-β-alanine in a 25-mL round-bottom flask was added 5 mL of freshly distilled thioloacetic acid. The reaction mixture was refluxed under nitrogen for 4 h. The solution was cooled and diluted with about 50 mL of ethyl ether. The ether solution was washed with 5% acetic acid, water, and brine and dried over $MgSO_4$. Evaporation of the ether gave a colorless oil which was not further purified.

The crude product (structure IV, Figure 1) was dissolved in 10 mL of TFA and stirred at room temperature for 1 h. The TFA was evaporated and the crude product was purified by Chromatotron chromatography on a 4 mm silica gel plate. Chromatography was started in $CHCl_3$ and the product eluted with 5% MeOH in $CHCl_3$. The product *N*-[3-(acetylthio)-3-methylbutyryl]-β-alanine (structure V, Figure 1) crystallized on evaporation of the solvent and was recrystallized from $CHCl_3$–hexane. ^{1}H NMR: δ 8.3 (br s, 1 H), 6.4 (br t, 1 H), 3.5 (q, 2 H), 2.75 (s, 2 H), 2.55 (t, 2 H), 2.25 (s, 3 H), 1.5 (s, 6 H). Yield = 0.7 g (52%).

HNSA or sNHS Esters. *N*-[3-(Acetylthio)-3-methylbutyryl]-β-alanine (617 mg, 2.5 mmol) was weighed into a 10-mL round-bottom flask. Sodium HNSA (602 mg, 2.5 mmol) or sNHS (542 mg, 2.5 mmol) was dissolved in about 3 mL of dimethylformamide and added to the flask, followed by 515 mg of dicyclohexylcarbodiimide, and the mixture was stirred at room temperature for 18 h. The mixture was filtered to remove dicyclohexylurea and added dropwise to 50 mL of ethyl ether with rapid stirring. The ether was stirred for about 0.5 h, then the precipitate was allowed to settle. The ether was decanted and the precipitate was washed with fresh ether four times. The solid product (structure VI, Figure 1) was collected by

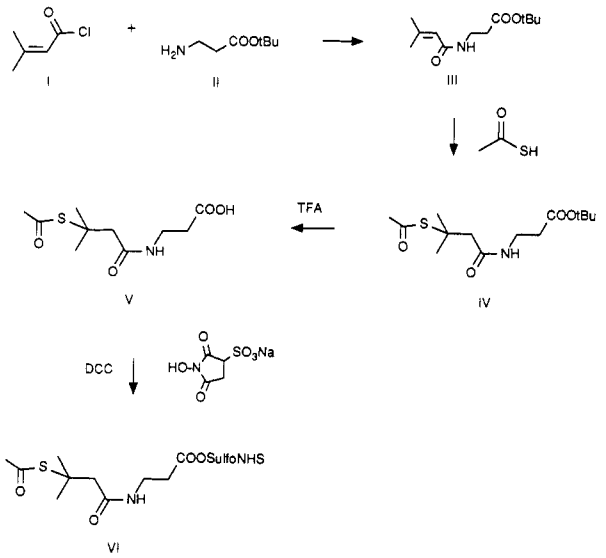

Figure 1. Synthesis of sNHS-ATMBA. The structures are (I) dimethylacryloyl chloride; (II) *tert*-butyl ester of β-alanine; (III) *tert*-butyl ester of (dimethylacryloyl)-β-alanine; (IV) *tert*-butyl ester of *N*-[3-(acetylthio)-3-methylbutyryl]-β-alanine; (V) *N*-[3-(acetylthio)-3-methylbutyryl]-β-alanine; and (VI) sulfosuccinimidyl ester of *N*-[3-(acetylthio)-3-methylbutyryl]-β-alanine.

NHS Ester. *N*-[3-(Acetylthio)-3-methylbutyryl]-β-alanine (494 mg, 2 mmol) was weighed into a 25-mL round-bottom flask. NHS (230 mg, 2 mmol), in 10 mL of CH_2Cl_2, was added followed by 412 mg of dicyclohexylcarbodiimide. The reaction was stirred at room temperature for 18 h. The precipitated dicyclohexylurea was filtered off and the solvent evaporated to give a white powder. The product was recrystallized from ethanol. ^1H NMR: δ 6.3 (br s, 1 H), 3.6 (q, 2 H), 2.85 (s, 4 H), 2.8 (t, 2 H), 2.75 (s, 2 H), 2.25 (s, 3 H), 1.5 (s, 6 H). Yield = 350 mg (50%).

Characterization of Stock Concentrations of Linker. Stock 10 mM solutions of the HNSA ester of the linker were freshly prepared prior to use by dissolving ca. 2 mg in 500 μL of water. The concentration of active ester was determined in buffer (either 100 mM NaP_i, 1 mM EDTA, pH 7.6, or 100 mM HEPES, 0.2 M NaCl, 1 mM EDTA, pH 7.6) as described by Aldwin and Nitecki (*35*). Briefly, the free HNSA dianion concentration was measured at 406 nm with a molar extinction coefficient of 4.6×10^3 M^{-1} cm^{-1}. The initial concentration of the free HNSA dianion was determined. Following the addition of 5 N NaOH to a final concentration of 240 mM the total concentration of HNSA was determined. The concentration of ester was calculated from the difference in the initial and final values.

A stock (25 mM) solution of the NHS ester of the linker was prepared by dissolving 3 mg in acetonitrile and storing at −20 °C. The amount of the anion of NHS was measured at 259 nm with an extinction coefficient of 8600 M^{-1} cm^{-1} (*41*, *42*). To determine the concentration of ester, the stock solution was diluted into 0.25 M Tris-Cl, pH 8.0, and the absorbance at 259 nm was monitored with time. The ester concentration was determined from the difference between the final and initial (extrapolated) absorbances.

Stock (10 mM) solutions of the sNHS ester of the linker were made in dry dimethylformamide and stored at −70 °C. Following dilution of the stock in 100 mM NaP_i, pH 6.0, the initial absorbance at 269 nm was measured. Hydroxylamine was added to a final concentration of 5 mM, and the measurement was repeated 1 min later. The aminolysis of the ester was complete in 10 s. Ester concentrations, using a molar extinction coefficient at 269 nm of 6100 M^{-1} cm^{-1} at pH 6.0.

Kinetic Studies. Hydrolysis rates were studied spectrophotometrically in the indicated buffers as a function of time at 25 °C. Reactions were carried out for 15 min and monitored at 15-s intervals. Hydrolysis was followed at 406 nm for the HNSA ester and at 269 nm for the sNHS ester. The total ester was determined as described above.

Second-order rate constants for thiol–disulfide exchange reactions with DTNB or DTDP, releasing 2-nitro-5-mercaptobenzoic acid (TNB) [molar extinction coefficient at 412 nm of 13 600 M^{-1} cm^{-1} (*43*)] or 4-mercaptopyridine (TP) [molar extinction coefficient at 324 nm of 19 800 M^{-1} cm^{-1} (44)], were determined by monitoring the reaction spectrophotometrically at 5-s intervals for 10 min at 23 °C in 100 mM NaP_i, pH 7.0 or 8.0. When one reagent was in great excess, the reaction exhibited first-order kinetics with respect to the limiting reagent, and the pseudo-first-order rate constant was calculated from the initial linear part of the graph of ln [unreacted reagent] vs time. The second-order rate constant was calculated by dividing the pseudo-first-order constant by the concentration of the excess species. For fast reactions, equal concentration of the two reagents were added, and the second-order rate constant was calculated from a plot of $(1/[A]) - (1/[A_0])$ vs time. Finally, when the concentrations of the two reagents were not equal but neither was in great excess, a plot of $(1/([A_0] - [B_0])) \times \ln (([A] \times [B_0])/([A_0] \times [B]))$ vs time yielded the second-order rate constant.

The reactivity of the TMBA thiol was examined following derivatization of antibody, deacetylation with hydroxylamine, and desalting.

Synthesis of Immunoconjugates with 2-Iminothiolane. Recombinant ricin A (rRA) chain produced in *Escherichia coli* (*45*) and 2-IT conjugates were prepared by the Cetus Process and Product Development group using previously published methods (*46*, *47*).

Synthesis of Radioactive Immunoconjugates with SAMSA. For the preparation of metabolically ^{35}S-labeled 260F9 antibody [(^{35}S)-260F9], hybridoma cells were grown in HL-1 medium at 37 °C under 10% CO_2 for 2 days. Cells were harvested, washed, and resuspended in HL-1 medium deficient in methionine to a final cell density of 1×10^6 viable cells per mL. (^{35}S)methionine was added to a final specific activity of 50 μCi/mL (50 nM), and the culture was incubated an additional 24 h at 37 °C under 10% CO_2. The radiolabeled antibody was concentrated from the supernatant by chromatography over a Bio-Gel HPHT hydroxyapatite HPLC column (100 mm × 7.8 mm, Bio-Rad Laboratories, Richmond, CA) using a sodium phosphate gradient. The antibody was further purified by chromatography over a BioGel TSK-phenyl-5-PW HPLC column (75 mm × 7.5 mm, Bio-Rad, Richmond, CA) with a simultaneously descending gradient of ammonium sulfate (1.0–0 M) and ascending gradient of propylene glycol (0–30%) in phosphate buffer (100 mM NaP_i, pH 8.0). The antibody was at least 95% pure based on SDS–PAGE and autoradiography.

A stock solution (5 mM) of SAMSA was prepared in acetonitrile. (^{35}S)-260F9 (640 μg/mL, 1.1×10^5 dpm/μg) was derivatized with a 15-fold excess of SAMSA in 10 mM HEPES, 0.2 M NaCl, 1 mM EDTA, pH 7.6, for 16 h to generate approximately 1.8 linkers per antibody, and the acetyl group was removed by treatment with 50 mM hydroxylamine at 23 °C for 1 h. Following activation of the thiol by reaction with 1 mM DTNB at 23 °C for 1 h, the preparation was dialyzed against 100 mM NaP_i, pH 8.0.

1 mM dithiothreitol (DTT) and desalted over a PD-10 column (Pharmacia, Piscataway, NJ) in 100 mM NaP_i, pH 8.0. For conjugation, a 2.5 molar excess of rRA thiols over antibody thiols was reacted at 23 °C for 72 h under nitrogen. Conjugate containing one rRA per antibody (1-mer) was purified as described below.

Synthesis of Immunoconjugates with TMBA. Antibody (10 mg/mL) was derivatized in 100 mM HEPES, 200 mM NaCl, 0.1 mM EDTA, pH 7.6, with 3.3-fold excess of sNHS-ATMBA (or 8.5-fold excess of HNSA-ATMBA) for 16 h at 23 °C, resulting in 1.8–2 thiols per antibody. The linker thiol was deprotected by treatment of the modified antibody with 50 mM hydroxylamine at pH 8.0 for 1 h and the preparation desalted over a PD-10 column in 100 mM NaP_i, pH 8.0. rRA was freshly reduced with 1 mM DTT and desalted over a PD-10 column, the thiol was activated by treatment with 1 mM DTNB, and the unreacted products were removed by dialysis. Conjugation proceeded by mixing 1.5 mol of activated rRA–TNB disulfides per titratable antibody thiol at 4 °C for 16 h. Unreacted thiols were blocked by addition of 40 mM IAM for 1 h at 23 °C, and the conjugate was purified as described by Ferris et al. (*47*).

Synthesis of Radioactive Immunoconjugate with TMBA. (^{35}S)-260F9 (850 μg, 1 mg/mL) in 10 mM HEPES, 200 mM NaCl, 1 mM EDTA, pH 7.6 was derivatized with 15-fold excess of HNSA-ATMBA at 23 °C for 18 h. The preparation was deacetylated and conjugated as described above. The conjugate mixture was chromatographed over a Bio-Gel TSK-phenyl-5-PW HPLC column (75 mm × 7.5 mm) and eluted at 1 mL/min with a simultaneously descending gradient of sodium chloride (1.5–0 M) and ascending gradient of propylene glycol (0–30%) in phosphate buffer (100 mM, pH 6.8). Fractions containing predominantly intact 1-mer, as determined by estimated molecular weight from SDS nonreducing PAGE and autoradiography, were pooled. Contaminating free rRA was removed by chromatography at 1 mL/min over a Zorbax Bio Series GF-250 HPLC column (25 cm × 9.4 cm, Du Pont, Wilmington, DE) equilibrated with 100 mM sodium phosphate, pH 6.8, 250 μg/mL human serum albumin (Travenol, Laboratores, Inc, Glendale, CA). The final conjugate had a specific activity of 7.5×10^5 dpm.

Detection of Cysteine Thiols following Deacetylation of 260F9–ATMBA. Monoclonal antibody 260F9 (33 mg/mL) was derivatized in 100 mM HEPES, 0.2 M NaCl, 1 mM EDTA, pH 7.6, with 6.7 molar excess of sNHS-ATMBA, deacetylated with 50 mM hydroxylamine, pH 8, and desalted over a PD10 column. DTNB analysis indicated 1.9 thiols per antibody. To 1 mg of underivatized antibody (260F9 in 397 μL), 1 mg of derivatized antibody prior to deacetylation (260F9–ATMBA, in 344 μl), or 1 mg of derivatized, deacetylated antibody (260F9–TMBA, in 166 μL) was added a 5 molar excess of [1-^{14}C]-IAM (24.1 mCi/mmol, 16 μL) at 23 °C for 16 h. Unincorporated label was removed by desalting over a PD10 column equilibrated with 20 mM *N*-ethylmorpholine, pH 8.0. In addition, [1-^{14}C]IAM was reacted with a 12-fold molar excess of cysteine and deacetylated TMBA. For acid hydrolysis, each sample was dried under vacuum, resuspended in 300 μL of 6 N HCl, sealed in glass capillary tubes under vacuum, and incubated at 100 °C for 16 h. Samples were chromatographed on a microcrystalline cellulose Baker-flex plate (J.T. Baker, Phillipsburg, NJ) in *n*-butanol–pyridine–glacial acetic acid–water (90:60:18:72), dried, and autoradiographed at −70 °C.

In Vitro MTT Assay. The in vitro activity of conon the ability of mitochondrial dehydrogenase enzymes to cleave the tetrazolium ring of the salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the violet crystal formazan (*48*). Assays were performed by the Cetus Assay Development group, using a procedure developed by C. Vitt of Cetus. Briefly, wells of a 96-well tissue culture plate were filled with 50 μL of MEM Eagles medium (Irvine Scientific, Santa Ana, CA) containing 10% fetal calf serum (Flow Laboratories, McLean, VA) and 1% penicillin–streptomycin (Irvine Scientific, Santa Ana, CA). Dilutions of the test conjugate were added in 25 μL followed by the addition of 100 μL of cell suspension (at 1×10^5 cells/mL). Following incubation at 37 °C (6% CO_2 for 72 h) 75 μg of MTT was added, and the plates were incubated for an additional 4–6 h at 37 °C under 5% CO_2. The liquid was removed by aspiration, 150 μL of 3% SDS, 0.04 N HCl–2-propanol was added, and the plates were incubated for 30–60 min to allow color development. The plates were read at 570 nm in a Titertek Multiscan plate reader.

Kinetics of in Vitro Cytotoxicity. OVCAR3 cells were trypsinized, counted, and seeded in 96-well plates at a cell density of 6.7×10^4 viable cells/mL followed by the addition of 10 nM conjugate. At the indicated times, the cells were washed, incubated for 45 min with methionine-deficient RPMI 1640 medium supplemented with 8 μCi/mL (^{35}S)methionine, washed, and harvested onto filter paper. Incorporated radioactivity was precipitated with TCA and counted.

Analysis of Radioactive Conjugates in Vitro. Six female Balb/C nude (nu/nu) mice were injected with 0.1 mL of (^{35}S)-260F9–SAMSA–rRA (6 μg, 5.4×10^5 dpm) in 100 mM NaP_i, pH 7, 200 μg/mL carboxymethylated BSA. At 0.25, 1.25, 2.25, 4.25, 8.25, and 12.33 h, mice were bled retroorbitally with heparinized capillaries and sacrificed. Alternatively, eight mice were injected with 0.1 mL of (^{35}S)-260F9–TMBA–rRA (3.6 μg); four mice were bled and sacrificed at both 5 and 24 h. Samples of blood were counted, and plasma was electrophoresed on a 5–10% gradient polyacrylamide SDS gel, dried, and autoradiographed at −70 °C using an intensifying screen.

RESULTS

Analysis of (^{35}S)-260F9–SAMSA–rRA in Vitro. Thorpe et al. (*31*) and Worrell et al. (*32*) have reported that cross-linkers with a methyl substituent on the carbon α to the thiol stabilize conjugates in vivo. We investigated the commercially available reagent SAMSA, which is branched at the carbon α to the sulfur (Figure 2). Due to reagent asymmetry, the substituent following derivatization can either be a carboxymethyl or a carboxylate group (Figure 2). Despite the substitution, SAMSA conjugates were unstable in vivo (Figure 3), as has been observed with 2-IT (*16*, *23–25*) and SPDP (11, 16, 26) conjugates. Because this substitution at the α-carbon failed to stabilize the conjugate in vivo, we investigated a new cross-linking reagent in which the α-carbon is substituted with two methyl groups.

Synthesis of ATMBA. Initially, penicillamine was chosen for the linker backbone (Figure 2). The 4-nitrophenyl ester of *N*-carbobenzoxy-*S*-benzylpenicillamine has been used in peptide synthesis (*49*). Preparation of several esters (NHS, HNSA, 2,4-dinitrophenol, and 4-nitrophenol) of *N*,*S*-diacetylpenicillamine was attempted. Only 4-nitrophenol yielded an ester product (data not shown). Unfortunately, this reagent, 4-nitrophenyl *N*,*S*-diacetylpenicillaminate, was not sufficiently water soluble to be useful as a protein cross-linker.

The inability to esterify the penicillaminecarboxylate

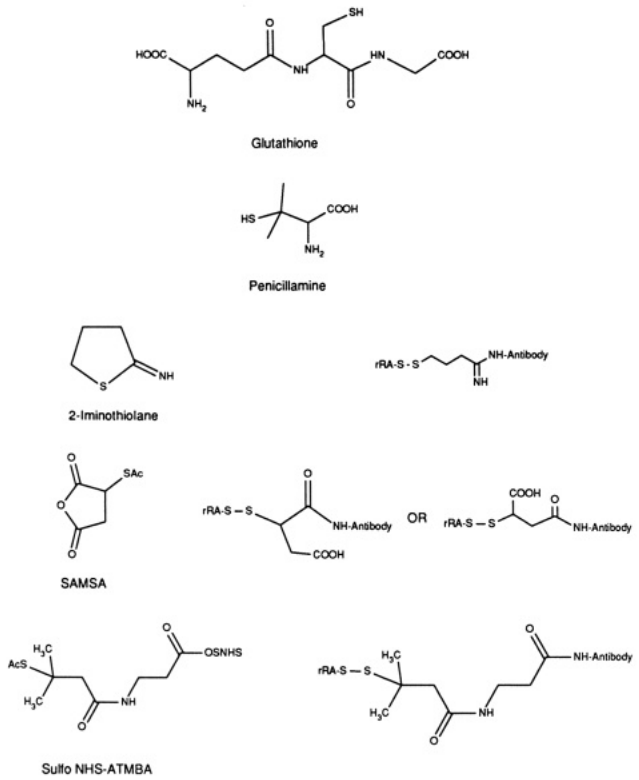

Figure 2. Chemical structures of thiol-containing compounds and cross-linkers. The thiol-containing compounds glutathione and penicillamine were used to compare the reactivity of sulfur atoms adjacent to a primary and tertiary carbon, respectively (Table I). The structures of the cross-linkers used in this study (2-IT, SAMSA, and TMBA) both before (left) and after (right) reacting with proteins are shown.

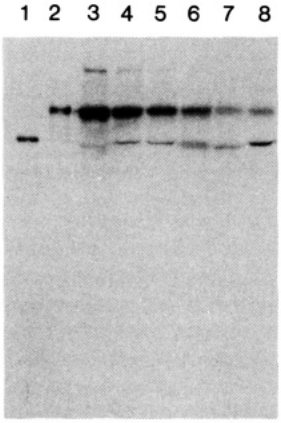

Figure 3. In vivo lability of 260F9–SAMSA–rRA conjugate. Six mice were injected intravenously with 6 μg of (^{35}S)-260F9–SAMSA–rRA and sacrificed at 0.25 (lane 3), 1.25 (lane 4), 2.25 (lane 5), 4.25 (lane 6), 8.25 (lane 7) and 12.3 h (lane 8). Plasma was isolated and electrophoresed on a 5–10% gradient SDS polyacrylamide gel. The gel was dried and autoradiographed. Lane 1 is (^{35}S)-260F9 and lane 2 is (^{35}S)-260F9–SAMSA–rRA injectate.

doubly branched α-carbon. To avoid the problem, the linker backbone was elongated before the introduction of the sulfur, as outlined in Figure 1. On the basis of the approach of Worrell et al. (*32*), dimethylacryloyl chloride was used as the starting material but the linker backbone was extended by reacting with β-alanine *tert*-butyl ester. Thioloacetic acid was added to the double bond, and the *tert*-butyl blocking group was removed. The carboxy-

Table I. Reactivity of Various Nucleophiles with Activated Thiols[a]

activated thiol	k_2, M^{-1} s^{-1} GSH	MEA	PSH	DTT
DTNB	2200 (5)	nd[b]	2100 (6)	nd
DTDP	1900 (3)	nd	1700 (4)	nd
GSSTNB	200 (2)	770 (1)	140 (3)	nd
GSSTP	360 (1)	890 (1)	210 (1)	nd
PSSTNB	1 (1)	3.2 (2)	0.73 (2)	1.7 (1)
PSSTP	1.6 (2)	1.8 (2)	nd	1.4 (1)
rRA-TNB	nd	nd	160 (1)	nd

activated thiol	k_2, M^{-1} s^{-1} hydroxylamine	260F9–TMBA	520C9–TMBA
diacetyl-PSH	0.34 (1)	nd	nd
DTNB	nd	88 (8)	nd
sNHS-ATMBA	0.018 (6)	nd	nd
rRA-TNB	nd	nd	7 (1)
rRA-TP	nd	nd	3.6 (1)

[a] The indicated two reagents were mixed and the release of the chromophore, TNB or TP, was followed spectrophotometrically at 23 °C as described in the Experimental Procedures. The concentration of the nucleophiles and the activated thiols ranged between 1×10^{-3} and 2×10^{-5} M. The rate of deacetylation of diacetyl-PSH (7×10^{-5} M) and sNHS-ATMBA (5×10^{-5} M) with hydroxylamine (50 mM) was monitored by measuring the exposed thiols by the inclusion of DTNB (1×10^{-4} M). The second-order rate constants (k_2) were determined during the initial part of the reaction. The number of determinations is indicated in parentheses. (rRA–TNB, rRA–2-nitro-5-mercaptobenzoate, rRA–TP, rRA–4-mercaptopyridine; 260F9–TMBA and 520C9–TMBA, derivatized antibody in which the thiol of the linker has been deblocked.) [b] Nd, not determined.

alcohols (HNSA, NHS, and sNHS). All three esters were evaluated for coupling efficiency.

Reactivity of Blocked Thiol. Penicillamine (Figure 2) was used to model the thiol reactivity of TMBA (Table I). The reactivity of the penicillamine (PSH) thiol with DTNB, DTDP, GSSTNB (2-nitro-5-mercaptobenzoic acid disulfide of glutathione), and GSSTP (4-mercaptopyridine disulfide of glutathione) was similar to those of reduced glutathione (GSH) and MEA (Table I). The reactivity of the penicillamine thiol with GSSTNB (k_2 = 140 M^{-1} s^{-1}) was similar to that with activated rRA, in which the thiol of rRA was activated with TNB (k_2 = 160 M^{-1} s^{-1}). In contrast, the 2-nitro-5-mercaptobenzoic acid (PSSTNB) and 4-mercaptopyridine (PSSTP) disulfides of penicillamine were at least 2 orders of magnitude less reactive than the corresponding GSH derivatives toward reduction by GSH or MEA (Table I). Rabenstein and Theriault (*50*) have reported similar findings in which the penicillamine thiol acts as an efficient nucleophile while disulfide bonds involving the penicillamine sulfur resist cleavage.

Forms of Ester. Efficiency of protein modification with ATMBA is dependent upon competing aminolysis and hydrolysis reactions. As the hydrolysis rate is buffer dependent (Table II), more efficient derivatization could be achieved by selecting buffers which minimize this rate. It was found that HEPES buffer resulted in both a lower hydrolysis rate and a higher derivatization efficiency when compared to phosphate buffer (Tables II and III). This buffer effect on both hydrolysis rate and derivatization efficiency was greater for the HNSA ester than the sNHS ester (Tables II and III).

HNSA is a water-soluble and spectrophotometrically monitorable active-ester leaving group (*35*). Aldwin and Nitecki (*35*) found that the degree of derivatization assessed

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