Enhanced Stability *in Vitro* and *in Vivo* of Immunoconjugates Prepared with 5-Methyl-2-iminothiolane

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Substituted 2-iminothiolanes (X2ITs) are new heterobifunctional crosslinking agents designed for the preparation of disulfide-linked conjugates with enhanced resistance to reduction. Based upon 2-IT substituted at the 4 and/or 5 position, these reagents appear to function by sterically protecting the conjugate disulfide bond from attack by thiolate nucleophiles. Here, we have used the X2ITs to prepare and evaluate a series of immunoconjugates (antibody–cytotoxin conjugates) between the murine monoclonal antibody 791/T36, which recognizes a 72-kDa surface antigen present on many human tumor cells, and RTA_{30}, the naturally occurring 30-kDa glycoform of ricin A chain. The X2IT-linked conjugates were also compared to immunoconjugates prepared with *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) and 4-[(succinimidyloxy)carbonyl]-α-methyl-α-(2-pyridyldithio)toluene (SMPT), as well as with methyl- and dimethyl-substituted structural analogs of SPDP. *In vitro*, 791-(X2IT)–TNB model compounds exhibited a 6000-fold range of stabilities. In contrast, the corresponding 791-(X2IT)–RTA_{30} immunoconjugates were up to 20-fold more stable than conjugates made with unhindered linkages. These improvements resulted in immunoconjugates with prolonged serum half-lives in animals. Our data indicate that one of the crosslinking agents, 5-methyl-2-iminothiolane (M2IT), has optimal properties for the preparation of disulfide crosslinked immunoconjugates intended for therapeutic use in that (i) it is highly water soluble and reacts rapidly with protein amino groups at neutral pH, preserving the positive charge, (ii) it forms conjugates with RTA_{30} efficiently, and (iii) its conjugates exhibit enhanced disulfide bond stability *in vitro* and *in vivo*. The potential utility of M2IT and other X2ITs for the preparation of controlled release protein–drug conjugates is also discussed.

INTRODUCTION

Immunoconjugates (antibodies linked to cytotoxic proteins) represent a specialized class of protein–protein conjugates designed for therapeutic use (for a review, see refs 1 and 2). As such, they typically are prepared by covalently crosslinking an antibody molecule to a cytotoxin such as the A chain of ricin (RTA).[1] The antibody thus serves to target the action of the cytotoxic component to cells bearing the target antigen. Once internalized, the cytotoxin is released and then penetrates into the cytosol where it enzymically inactivates ribosomes, blocking protein synthesis and causing cell death. This approach for selective cellular elimination is currently being evaluated clinically for the treatment of autoimmune disorders and cancer (*2–8*).

Most of the RTA immunoconjugates prepared to date have utilized one of two crosslinking reagents, SPDP or 2IT, to generate a disulfide bond linking antibody to cytotoxin. That a reducible bond is required for maximal expression of cytotoxic activity has been demonstrated by numerous studies (*1, 2, 9*). However, many such conjugates are unstable in animals (*10, 11*), where cleavage of the disulfide bond regenerates free antibody and cytotoxin. For immunoconjugate therapy this deconjugation has two important consequences. First, it reduces the effective concentration of circulating immunoconjugate, and as a result, larger clinical doses may be required. Second, the released antibody may remain in circulation much longer than does conjugate, where it can compete for antigen binding sites on target cells. Thus, the disulfide bond linking antibodies to cytotoxin such as RTA must be sufficiently labile to facilitate intracellular cytotoxicity, but it must also be sufficiently stable to survive administration and delivery *in vivo*.

To address these issues, several new crosslinking reagents have been prepared and tested for immunoconjugate preparation, and the *in vitro* and *in vivo* properties of such conjugates have been studied. Each of these reagents contains one (*12, 13*) or two (*14*) methyl groups adjacent to the disulfide bond, and each has generated conjugates with enhanced stability (*12–15*) and improved efficacy (*15*) in animals. Thus, hindering access of reducing agents to the antibody–cytotoxin linkage results in immunoconjugates with improved *in vivo* stability and potency.

Recently, we described the synthesis and preliminary characterization of a new family of crosslinking reagents, termed X2ITs (*16*), which are based upon 2-iminothiolane (*17*). The X2ITs offer several advantages over other

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[1] Abbreviations: 2IT, 2-iminothiolane; 2-ME, 2-mercaptoethanol; 2TP, 2-thiopyridine; DTNB, dithionitrobenzoic acid; DTPO, 2,2′dithiobis(pyridine *N*-oxide); DTDP, 2,2′dithiodipyridine; GSH, reduced glutathione; HPSEC, high-performance size-exclusion chromatography; MSPDP, the methyl-SPDP analog *N*-succinimidyl 3-(2-pyridyldithio)butyrate; RTA, ricin toxin A chain; RTA_{30}, the 30-kDa glycoform of RTA; SAMBA, the dimethyl-SPDP structural analog *N*-hydroxysuccinimidyl 3-methyl-3-(acetylthio)butanoate; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; SMPT, 4-[(succinimidyloxy)carbonyl]-α-methyl-α-(2-pyridyldithio)toluene; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; TNB, thionitrobenzoic acid; TPO, 2-thiopyridine *N*-oxide; X2IT,

to form stable amidinium derivatives that retain the positive charge; (ii) inclusion of an aromatic disulfide (such as DTNB) in the reaction mixture both activates the newly exposed X2IT thiol and allows real-time spectrophotometric monitoring of the labeling reaction; and (iii) variation in the substituent at the 5-position (immediately adjacent to the linker thiol) alters the susceptibility of the resulting disulfide bond to reduction. For activated model compounds, these alterations in steric hindrance resulted in disulfide bonds that varied by over 4000-fold in their ability to be reduced by glutathione (*16*). The preparation and properties, both *in vitro* and *in vivo*, of RTA immunoconjugates prepared with the X2IT reagents are the subject of this report.

EXPERIMENTAL PROCEDURES

Materials. Solutions of DTNB (Sigma Chemical Co., St. Louis, MO) were prepared as described by Jocelyn (*18*). An E^{1mM}_{1cm} of 14.1 at 412 nm (*19*) was used to determine concentrations of the TNB anion. DTDP and DTPO were from Aldrich (Milwaukee, WI); the mM extinction coefficients (and wavelengths) used for 2TP (343 nm) and TPO (332 nm) were 7.06 and 4.16, respectively. Stock solutions of GSH (Sigma Chemical Co., St. Louis, MO) were prepared in PBS–EDTA (see below); prior to use, the concentration of free thiols was quantified by reaction with DTNB. DTT, 2-ME, Sephadex G25F, Phenyl-Sepharose (all from Sigma Chemical Co., St. Louis, MO), trisacryl GF-05LS, and Ultrogel AcA44 (both from IBF Biotechnics, France) were purchased as indicated. All other reagents were of analytical grade.

Crosslinking Reagents. 2IT, SMCC (both from Sigma Chemical Co., St. Louis, MO), and SPDP (Pierce Chemical, Rockford, IL) were obtained from the indicated sources. The X2IT crosslinking reagents, which have the structures shown in Table 1, were synthesized as described previously (*16*). Stock solutions were prepared in water; concentrations were determined spectrophotometrically by using appropriate extinction coefficients at 248 nm (*16*). In addition, three other crosslinkers were prepared for these studies. MSPDP and SMPT were synthesized according to Worrell et al. (*12*) and Thorpe et al. (*13*), respectively, with minor modifications. The structure of each linker was confirmed by ^{1}H NMR. *N*-Hydroxysuccinimidyl 3-methyl-3-(acetylthio)butanoate (SAMBA), a dimethyl-substituted structural analog of SPDP, was prepared as follows: 3-Methyl-3-(acetylthio)butanoic acid (ref 10, 2.17 g, 12.3 mmol) in CH_2Cl_2 (20 mL) was treated with *N*-hydroxysuccinimide (1.86 g, 16.2 mmol) and dicyclohexylcarbodiimide (3.34 g, 16.2 mmol) at room temperature for 66 h under N_2. The reaction mixture was filtered, concentrated *in vacuo*, and then subjected to flash chromatography on SiO_2 with elution in hexane/EtOAc (80/20, v/v, then 50/50). The desired ester was obtained as a pale yellow oil (2.78 g, 82% yield) that gave a white solid upon standing at room temperature: mp 63 °C; TLC (hexane/EtOAc, 80/20, v/v) R_f = 0.27; ^{1}H NMR (60 MHz, $CDCl_3$) 3.23 (s, 2H), 2.80 (s, 4H, NHS ester), 2.27 (s, 3H, SAc), 1.60 (s, 6H).

Preparation of Linker-Modified Antibody. The murine IgG2b monoclonal antibody 791/T36 (791, M_r ca. 150 000) was produced in an Accusyst hollow fiber bioreactor (Endotronics, Minneapolis, MN) and purified as described (*20*). The purified antibody was derivatized with each crosslinker so as to incorporate an average of 1–1.5 linkers per mol of antibody. For modification with SPDP, MSPDP, SAMBA, or SMCC, 791 antibody at 2 mg/mL in reaction buffer (0.1 M $NaPO_4$, 0.1 M NaCl, pH 7.5) was first reacted with a 5- to 10-fold molar excess of crosslinker (previously dissolved in absolute ethanol). Following a 20-min incubation at 20 °C, excess reagent and reaction byproducts were removed by size-exclusion chromatography on a GF-05LS column equilibrated in reaction buffer at 4 °C. The number of crosslinkers introduced into the antibody was determined by spectrophotometric analysis following DTT-induced release of the 2TP leaving group (*21*). For some experiments, the 2TP leaving groups were replaced with TNB by mild reduction of the linker-modified antibody (0.1 mM DTT, 30 min, 25 °C), followed by reaction with 2 mM DTNB (30 min, 25 °C). The TNB-activated antibody was then purified by size-exclusion chromatography on a column of G5–05LS equilibrated in phosphate buffered saline containing 1 mM EDTA, pH 7.4 (PBS–EDTA) and stored at 4 °C.

The reaction of 2IT and the X2ITs with 791 antibody was monitored spectrophotometrically as follows (16): 791 antibody (3 mg/mL; 20 μM) and DTNB (2.5 mM) in reaction buffer were equilibrated at 25 °C in a 1-cm disposable cuvette and placed in a dual-beam spectrophotometer. An identical solution prepared without antibody was placed in the reference position. To initiate the reaction, X2IT (freshly dissolved in water) was rapidly added to each cuvette with mixing to a final concentration of 0.5 mM, and the absorbance at 412 nm was monitored. When the A_{412} reached a value of 0.28 (20 μM TNB, or 1 mol of TNB per mol of 791), the reaction mixture was rapidly desalted on a 1-cm × 20-cm column of Sephadex G25F equilibrated at 4 °C in PBS–EDTA. The excluded protein peak was pooled and stored at 4 °C. The number of linkers introduced per mole of antibody was determined spectrophotometrically as follows: The A_{280} of the linker-activated protein was first measured. Then, following reaction with 2 mM DTT, released TNB was quantified at 412 nm. The corrected protein A_{280} was calculated from the equation

$$A_{280}(\text{protein}) = A_{280}(\text{nonreduced}) - (0.33A_{412}(\text{reduced}))$$

and the concentration of 791 antibody was determined by using an E^{1mM}_{1cm} of 179 at 280 nm ($E^{1\ mg/mL}_{1cm}$ = 1.2). The linker/antibody ratio was then calculated from the molar values of TNB and protein.

Preparation and Purification of Immunoconjugates. Immunoconjugates containing SPDP-, SMPT-, and SMCC-activated 791 antibody and RTA_{30} (the naturally occurring 30-kDa glycoform of RTA) were prepared essentially as described (*16, 20*). Briefly, linker-activated antibody (1–2 mg/mL) in PBS–EDTA was reacted with a 5-fold molar excess of freshly reduced RTA_{30}. The disulfide exchange (SPDP, SMPT) or maleimide-based (SMCC) conjugation reactions proceeded for 16 h at 4 °C. For SAMBA-activated 791 antibody, the conjugation reaction proceeded differently. The free thiol on RTA_{30} (5 mg/mL in reaction buffer) was first activated by reaction with 2 mM DTNB, and the RTA_{30}–SS–TNB was purified by size-exclusion chromatography on Sephadex G25F. SAMBA-modified 791 antibody was then treated with 50 mM hydroxylamine (pH 7.5) for 30 min at 25 °C to remove the *S*-acetyl protecting group, and conjugation was initiated by the addition of RTA_{30}–SS–TNB (3-fold molar excess).

Immunoconjugates were separated from excess RTA_{30} and reaction byproducts by chromatography on a 1- × 50-cm column of Ultrogel AcA44 equilibrated at 4 °C in reaction buffer. The number of RTA_{30} molecules crosslinked to antibody was determined by densitometric

polyacrylamide gel electrophoresis in 5% gels under nonreducing conditions (*22*) and Coomassie blue staining. The monoconjugate species (1 RTA per 791 antibody) of selected immunoconjugates was purified by hydrophobic interaction chromatography on Phenyl-Sepharose (*20*), so as to remove residual free antibody and immunoconjugates containing multiple RTA_{30} moieties.

Disulfide Bond Stability Assay. The susceptibility of 791–RTA_{30} immunoconjugates to reduction *in vitro* was evaluated in a high-performance size-exclusion chromatographic assay (HPSEC) which quantifies physical dissociation of the antibody–RTA_{30} conjugate[2] (*23*). Immunoconjugates (0.23 mg/mL in PBS–EDTA) were incubated at 37 °C for 30 min with increasing concentrations of reduced glutathione (0–10 mM). Upon completion, free thiols were quenched by the addition of excess iodoacetic acid (pH 7.5; final concentration, 50 mM), and aliquots were chromatographed on a BioSil TSK-250 column (BioRad Labs, Richmond, CA) equilibrated at 25 °C in 50 mM $NaPO_4$, 100 mM Na_2SO_4, pH 6.8. The flow rate was 1.0 mL/min, the column effluent was monitored at 280 nm, and the amount of RTA_{30} released was quantified by area integration. By comparison to samples incubated with 50 mM 2-ME (which resulted in 100% deconjugation), plots were constructed correlating percent RTA_{30} release with the concentration of glutathione in the incubation mixture. That concentration of glutathione which released 50% of the conjugated RTA_{30} was termed the RC_{50}.

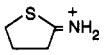
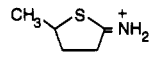
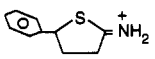
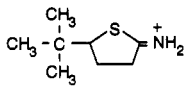
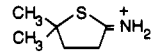
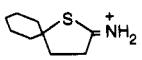
Cytotoxicity Assay. The cytotoxicities of 791–RTA_{30} immunoconjugates were determined using the 791T/M osteosarcoma cell line, which expresses the antigen recognized by 791 antibody (*20*). Cells (4×10^5/mL) were incubated in a humidified 5% CO_2 incubator with increasing concentrations of immunoconjugates at 37 °C. After 42 h, ^{3}H-thymidine (1 μCi/well) was added, and incubation was continued for an additional 18 h. Upon completion, cell-associated radioactivity was determined by liquid scintillation counting. The IC_{50} was calculated as the concentration of immunoconjugate necessary to inhibit incorporation of radioactivity by 50% relative to untreated controls. These IC_{50} values were corrected for the number of RTA_{30} molecules conjugated to antibody for each preparation by multiplying the conjugate IC_{50} by the RTA/Ab ratio. These normalized values compensate for slight variations in the number of cytotoxins per antibody in the different preparations and are expressed in terms of pM RTA_{30}.

Pharmacokinetic Studies. Pharmacokinetic studies of selected immunoconjugates were performed in 5-week-old male Sprague–Dawley rats (Simonsen Laboratories, Gilroy, CA) weighing an average of 149 g (range 122–175 g) at the initiation of dosing. All animals were delivered healthy to the XOMA animal care facility, where they were acclimated for at least 5 days prior to dosing, and were housed using standard NIH guidelines for husbandry procedures.

Only purified monoconjugates were used in these studies. Each monoconjugate was radiolabeled with ^{125}I by the Iodogen method (*24*) to a specific activity of 0.3–2 mCi/mg and was injected intravenously (33–50 μg/kg) into 42 rats per study (three rats per timepoint). At selected timepoints (0.05, 0.25, 0.5, 0.75, 2, 4, 8, 12, 18, 24, 36, 48, 72, and 96 h), blood samples were collected via the orbital sinus, and serum aliquots were counted in an LKB γ counter. Serum samples from each timepoint were also analyzed by SDS–PAGE and autoradiography to determine the fraction of intact monoconjugate prior to pharmacokinetic analysis (*13*). Pharmacokinetic parameters were determined from a two compartmental analysis using the program PCNONLIN (Statistical Consultants, Inc., Lexington, KY).

[2] Carroll, S. F., Goff, D., Reardan, D., and Trown, P. W. (1989) Abstracts from the fourth international conference on monoclonal antibody immunoconjugates for cancer, San Diego, CA, p 161.

Table 1. Reaction of Substituted 2-Iminothiolanes with 791 Antibody[a]

linker	substitution	structure	reaction rate[a] $k \times 10^5$
2IT	(none)		5.0
M2IT	5-methyl		4.0
Ph2IT	5-phenyl		8.8
TB2IT	5-*tert*-butyl		7.4
DM2IT	5-dimethyl		4.6
S2IT	5-spiro		4.6
R2IT	4,5-ring		5.6

[a] Rates of reaction of X2ITs (0.5 mM) with 791 antibody (20 μM) as monitored by coupling the reaction with 2.5 mM DTNB in 0.1 M NaPO4, 0.1 M NaCl, pH 7.5, and monitoring the change in absorbance at 412 nm. First-order rate constants were determined from the linear slopes of plots for log [X2IT] against time.

RESULTS

Reaction of X2ITs with Proteins. The structures of the X2IT crosslinking reagents are summarized in Table 1. Prior studies had shown that the reactivity of the X2ITs with the amino group of glycine was relatively unaffected by the X2IT ring substituent (*16*). We therefore examined the reactivity of the X2ITs with protein amino groups, in preparation for conjugate production. Each X2IT (0.5 mM) was incubated at pH 7.5 with the murine IgG2b monoclonal antibody 791 (20 μM) in the presence of DTNB (2.5 mM), and changes in the absorbance at 412 nm were recorded. Following reaction of the X2ITs with the protein amino groups, DTNB undergoes disulfide exchange with the newly exposed X2IT thiol to yield a free TNB group (monitored at 412 nm) and an activated 791–(X2IT)–SS–TNB molecule. This coupling of the reactions between protein modification and TNB production simplifies the analysis of the rate and extent of the reaction. As was found for the reaction with glycine (*16*), reaction rates for the X2ITs with 791 antibody followed first-order kinetics and varied less than 2-fold for the entire series of crosslinkers (Table 1). Similar reaction conditions were therefore employed for the preparation of protein conjugates utilizing each X2IT linker.

Stability of Model Disulfides. The relative stability of X2IT model protein disulfides was assessed by measuring the rates of release of the TNB leaving group from 791–(X2IT)–SS–TNB molecules following incubation with 200 μM reduced glutathione (GSH). For comparison, two additional control analogs were examined. 791–(SPDP)–SS–TNB was prepared by derivatizing 791 antibody with the heterobifunctional crosslinking reagent SPDP and replacing the 2TP leaving group with TNB. A similar

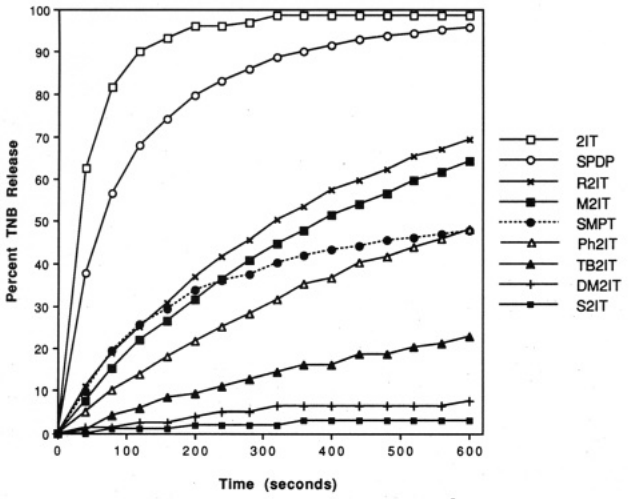


Figure 1. Glutathione-induced release of TNB from 791–TNB analogs. Samples of activated conjugates (791–(X)–SS–TNB, 10 μM) in PBS–EDTA were placed in a cuvette at 25 °C, and at T = 0 °C, reduced glutathione was added to a final concentration of 200 μM. The release of TNB was monitored spectrophotometrically at 412 nm for 500 s, and 2-ME was then added to a final concentration of 200 mM to determine maximal release of TNB. Results were normalized by quantifying percent maximal release, as determined by dividing the absorbance at any timepoint by that obtained with 200 mM 2-ME and then multiplying the product by 100.

Table 2. Relative Stabilities of TNB-Activated 791 Antibody Analogs

analog	TNB release rate[a] ($k \times 10^4$)	stability increase relative to 2IT[b]	stability increase relative to SPDP[c]
791–(2IT)–SS–TNB	235	1.0	0.4
791–(R2IT)–SS–TNB	21.7	10.8	4.7
791–(M2IT)–SS–TNB	18.2	12.9	5.6
791–(Ph2IT)–SS–TNB	11.5	20.4	8.9
791–(TB2IT)–SS–TNB	4.5	52.2	22.7
791–(DM2IT)–SS–TNB	0.039	6030	2620
791–(S2IT)–SS–TNB	0.036	6530	2830
791–(SPDP)–SS–TNB	102	2.3	1.0
791–(SMPT)–SS–TNB	14.7	16.0	6.9

[a] Reaction mixtures contained 791–X–SS–TNB (20 μM) and reduced glutathione (40–103 mM) and were incubated at 25 °C and monitored at 412 nm. Plots of log [791–X–SS–TNB] vs time were linear for all analogs except 791–(SMPT)–SS–TNB. Pseudo-first-order reaction rates were calculated by computerized nonlinear curve fitting (GraFit, version 2.0, Erithacus Software Ltd., Staines, U.K.). [b] Relative increase in disulfide stability compared to the 2IT analog. [c] Relative increase in disulfide stability compared to the SPDP analog.

procedure was used to prepare 791–(SMPT)–SS–TNB, which incorporates the methyl-hindered crosslinking reagent developed by Thorpe et al. (*13*). Figure 1 indicates that the X2IT reagents create model protein disulfides which vary greatly in their susceptibility to reduction by GSH. However, each of the substituted X2ITs produced linkages that were significantly more stable than those produced by SPDP or 2IT. At appropriate concentrations of reductant, pseudo-first-order rate constants for TNB release were calculated (Table 2). Relative to 2IT, the most stable linkages (DM2IT and S2IT) were more than 6000-fold more resistant to reduction by GSH. The order from least to most stable was as follows: 2IT < R2IT < M2IT < Ph2IT < TB2IT < DM2IT < S2IT. For the most stable analogs (those made with DM2IT and S2IT), prolonged incubation (30–60 min) with 200 mM 2-ME was required for complete release of TNB. In this assay,

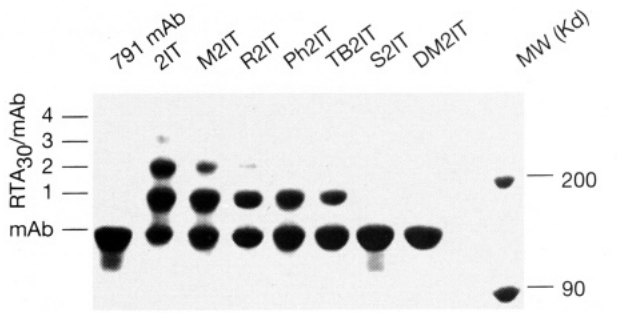


Figure 2. Conjugate formation by 791–TNB analogs. Each 791–(X2IT)–SS–TNB analog (1 TNB per 791 antibody) was incubated with a 5-fold molar excess of freshly reduced RTA_{30} in PBS–EDTA. The final concentration for both 791–(X2IT)–TNB and RTA_{30} was 1.6 mg/mL. After 16 h at 4 °C, aliquots (10 μg) were analyzed by SDS–PAGE in a 5% gel under nonreducing conditions. Upon completion, the gel was stained with Coomassie blue.

Preparation of RTA_{30} Immunoconjugates. Antibody–RTA immunoconjugates are typically prepared by performing a disulfide-exchange reaction between the free –SH group of RTA and an activated linker disulfide present on the antibody. Because this exchange reaction (like the stability assay described above) is essentially a reductive cleavage of the activated linker–SS–TNB bond, variations might be expected in the efficiency with which the linker-activated antibody is converted to immunoconjugate. Activated 791–(X2IT)–SS–TNB antibodies (1.0–1.3 linkers/Ab) were therefore individually reacted with a 5-fold molar excess of RTA_{30} for 16 h at 4 °C and then aliquots were analyzed by SDS–PAGE. The results (Figure 2) suggest an inverse correlation between disulfide bond stability and the efficiency of conjugation, as determined by either the disappearance of the free antibody band or by the appearance of higher molecular weight conjugate bands. Utilizing densitometry to quantify the conversion of antibody into immunoconjugate, we found that the efficiency of conjugation followed the order 2IT > M2IT > R2IT = Ph2IT > TB2IT. Under identical conditions, SMPT-activated antibody was converted to immunoconjugate roughly as efficiently as was TB2IT-activated antibody (data not shown).

No immunoconjugates were detected in reaction mixtures containing DM2IT- or S2IT-modified antibody, suggesting that RTA_{30} (like GSH and 2-ME) could not easily displace the TNB leaving group from these two linkers disubstituted at the 5 position (immediately adjacent to the disulfide bond). Similarly, little or no conjugation was detected with DM2IT- or S2IT-modified antibody even following prolonged incubation with RTA_{30} for several months at 4 °C or after increasing the incubation temperature to 25 or 37 °C. Antibody activated by reaction with a dimethyl-substituted analog of SPDP (synthesized according to Worrell et al. (*12*)) was also incapable of making conjugates when incubated with an excess of RTA_{30} (data not shown).

Conjugation Efficiency Is Influenced by the Leaving Group. Because the reactions of RTA_{30} with DM2IT–TNB- or S2IT–TNB-activated antibodies were not productive, the effect of alternate leaving groups on conjugation efficiency was investigated. Initially, 791–(SPDP)–SS–2TP was studied, together with SPDP linkages activated by two additional diaryl disulfides. As before, the 2TP leaving group of SPDP was first removed by mild reduction, and the newly exposed linker thiol was then

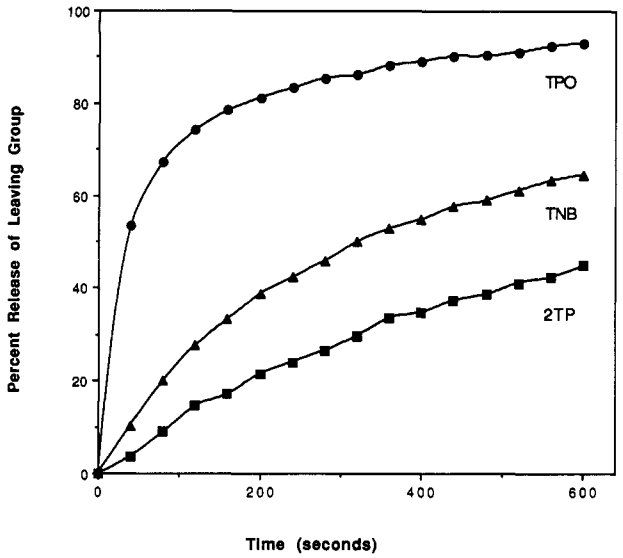


Figure 3. Glutathione-induced release of leaving groups from activated 791 antibody. Aliquots of 791–(SPDP)–SS–2TP were converted to the corresponding 791–(SPDP)–SS–TNB and 791–(SPDP)–SS–TPO analogs by mild reduction and subsequent reaction with the corresponding diaryl disulfide (DTNB and DTPO, respectively). The activated 791 antibodies were isolated by size-exclusion chromatography, and glutathione-induced release of the leaving groups was monitored as described in the legend for Figure 1. The final concentration of GSH in these assays was 40 μM.

spectively. These compounds were then evaluated for their susceptibility to reduction by GSH. As shown in Figure 3, the TPO derivative was most easily reduced, followed by TNB and then 2TP. On the basis of first-order rate constants, the release of TPO was 8-fold faster than TNB and 15-fold faster than 2TP. When these activated antibodies were reacted with RTA_{30}, conjugation efficiency was also highest for the TPO analog, followed again by TNB and then 2TP (data not shown). Essentially identical results were obtained with the M2IT-activated 791 antibody (791 reacted with M2IT in the presence of DTDP, DTNB, or DTPO); i.e., the TPO derivative was most easily reduced and was most efficiently conjugated with RTA_{30} (data not shown). In fact, the conjugation efficiency of 791–(M2IT)–SS–TPO exceeded 95%, even when only a 3-fold molar excess of RTA_{30} was used for conjugation. As before, however, no immunoconjugates were detected following reaction of 791–(DM2IT)–SS–TPO or 791–(S2IT)–SS–TPO with RTA_{30} under any of the reaction conditions tested.

Disulfide Bond Stability and Cytotoxicity of 791–RTA_{30} Immunoconjugates *in Vitro*. The *in vitro* stabilities of 791–RTA_{30} immunoconjugates were analyzed directly by monitoring thiol-dependent release of RTA_{30}. In addition to the conjugates described above, three additional immunoconjugates were also prepared, purified, and tested. 791–(MSPDP)–SS–RTA_{30} (which incorporates a methyl-substituted analog of SPDP) and the thioether-linked conjugate 791–(SMCC)–CS–RTA_{30} (which is not reducible) were prepared by standard reactions with linker-modified antibodies. The third conjugate was prepared in a effort to evaluate an immunoconjugate disubstituted at the carbon atom adjacent to the disulfide bond and, since conjugations with DM2IT- and S2IT-linked antibodies were unsuccessful, required alternate chemistries. An analog of SPDP was therefore prepared (SAMBA) which incorporated two methyl groups adjacent to the linker thiol and an *S*-acetyl protecting group instead of the usual 2TP moiety. Following reaction of the

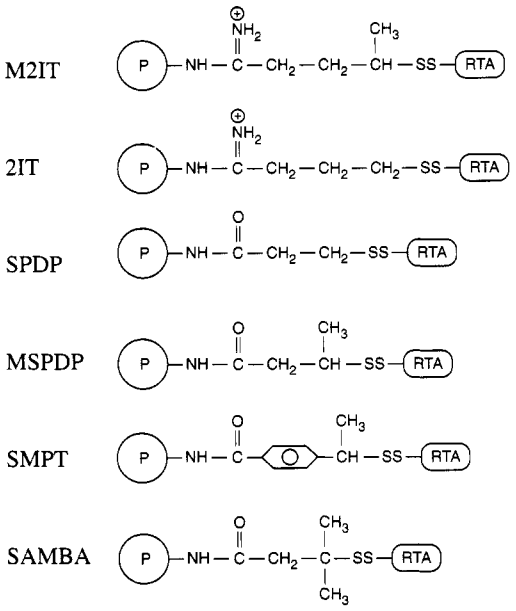


Figure 4. Linkage structures formed by the different crosslinking agents. For the X2ITs, only M2IT is shown as an example.

Table 3. Stability and Cytotoxicity of 791–RTA_{30} Immunotoxins *in Vitro*

immunotoxin	RC_{50}[a]	stability increase relative to 2IT[b]	stability increase relative to SPDP[c]	IC_{50} (pM RTA_{30})
791–(2IT)–SS–RTA_{30}	1.9	1.0	0.6	70.6
791–(R2IT)–SS–RTA_{30}	11.8	6.2	3.7	65.8
791–(M2IT)–SS–RTA_{30}	26.2	13.8	8.3	69.0
791–(Ph2IT)–SS–RTA_{30}	16.5	8.7	5.2	94.6
791–(TB2IT)–SS–RTA_{30}	9.5	5.0	3.0	93.6
791–(SPDP)–SS–RTA_{30}	3.2	1.7	1.0	89.1
791–(MSPDP)–SS–RTA_{30}	11.8	6.2	3.7	72.2
791–(SAMBA)–SS–RTA_{30}	32.9	17.3	10.4	89.6
791–(SMPT)–SS–RTA_{30}	5.7	3.0	1.8	76.1
791–(SMCC)–CS–RTA_{30}	nd[e]			9949.5

[a] The concentration of reduced glutathione, in mM, that releases 50% of the RTA_{30} from the immunotoxin. [b] Obtained by dividing the RC_{50} for each conjugate by 1.9, the value for 791–(2IT)–SS–RTA_{30}. [c] Obtained by dividing the RC_{50} value for each conjugate by 3.2, the value for 791–(SPDP)–SS–RTA_{30}. [d] The concentration of immunotoxin that inhibited protein synthesis in 791T/M cells by 50%. The data are expressed in terms of RTA_{30} equivalents. The RTA/Ab ratios varied between 1.1 and 1.5. [e] Not determined. The amount of RTA_{30} released by reducing agents did not exceed 10%.

SAMBA NHS ester with antibody amino groups, a free –SH group was exposed on the linker by treatment with hydroxylamine. Thiol-activated RTA_{30}–SS–TNB was then added, and conjugation occurred via disulfide exchange, thus producing 791–(SAMBA)–SS–RTA_{30}. The linkage structures of these and other immunoconjugates are shown in Figure 4 (note that the linkage made by SAMBA is identical to that which would have been made by dimethyl-SPDP).

Following preparation and purification, the stability of the disulfide bond linking antibody and RTA_{30} to reduction with GSH *in vitro* was then examined. The calculated RC_{50} values (the concentration of GSH causing 50% release of RTA_{30}) for all immunoconjugates are shown in Table 3. On the basis of these analyses, the dimethyl-substituted SAMBA conjugate was the most stable, followed closely by conjugates made with M2IT and Ph2IT. However, unlike the 791–TNB protein-leaving group compounds (which exhibited stabilities over an 6000-fold range), the

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