Substituted 2-Iminothiolanes: Reagents for the Preparation of Disulfide Cross-Linked Conjugates with Increased Stability[1]

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Much attention has been focused recently on the stability of immunotoxin (antibody–toxin) conjugates linked by a disulfide bridge. Conflicting reports have appeared regarding the in vivo stability of such conjugates prepared with the two most commonly used cross-linking reagents, SPDP and 2-iminothiolane. We have developed (i) a series of reagents based on 2-iminothiolane substituted at the 4- and/or 5-positions (X2ITs) which, based on model studies with simple amines, should show enhanced disulfide stability when conjugated with antibodies or other proteins and (ii) a real-time method for monitoring the rate and extent of conjugation of these reagents with amino groups. Depending upon the substituent, the stability of model-activated disulfides relative to unsubstituted 2-iminothiolane was increased from 5- to 4000-fold as measured by glutathione-induced release of thionitrobenzoic acid. This family of cross-linking reagents should allow the construction of disulfide cross-linked toxin, drug, or enzyme conjugates with enhanced stability in vivo.

INTRODUCTION

Immunotoxins are a class of therapeutic agents typically composed of an antibody, capable of binding to specific cell-surface antigens on target cells, covalently cross-linked to a cytotoxic protein. For most immunotoxins prepared to date, the cytotoxic protein is ricin A chain (RTA),[2] which requires a reducible disulfide linkage with the targeting antibody for maximal expression of cytotoxic activity (*1*, *2*). However, while some studies have indicated that the disulfide linkages prepared with RTA or other toxins and two of the most commonly used cross-linking reagents, 2-iminothiolane (2IT) and N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), are stable in vivo (*3*, *4*), others (*5*, *6*) have suggested that such linkages may be unstable. In addition to decreasing the circulating levels of the antibody–toxin conjugate, such deconjugation would liberate free antibody which can then compete with the conjugate for binding to target cells.

Worrell et al. (*7*) and Thorpe et al. (*8*, *9*) have shown that hindering access to the disulfide bond linking the antibody and toxin by introducing a methyl group adjacent to the sulfur atom of reagents based upon SPDP increases the stability of the resultant conjugate both in vitro and in vivo. Moreover, alterations in spacer length have been found to alter the stability of conjugates in vitro (*10*). When combined with the experimental discrepancies noted above, these results suggest that the in vivo stability of immunotoxins may be influenced by the specific antibody–toxin pairing and can be enhanced by alterations in residues immediately adjacent to the disulfide bridge.

In an effort to more critically evaluate the requirements for improving disulfide bond stability in vivo, we have synthesized a series of sterically hindered reagents based on 2IT (*11*). 2IT offers several advantages over other possible linking reagents (Figure 1): (i) it reacts with primary amines to form stable amidinium derivatives, retaining the positive charge; (ii) inclusion of an aromatic disulfide such as 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in the reaction mixture both activates the newly exposed 2IT thiol and allows real-time spectrophotometric monitoring of the labeling reaction; and (iii) substitution on the 2-IT ring at the 4- and/or 5-positions can be used to introduce various groups into proximity with the disulfide bond, thus allowing alterations in the degree of steric hindrance. Here we have prepared a series of sterically hindered 2IT molecules (X2ITs, Table I) and have evaluated their reactivity with amino groups and the disulfide stability of model conjugates.

EXPERIMENTAL SECTION

Materials. 2IT and DTNB were obtained from Sigma. Anhydrous solvents, 10 M *n*-butyllithium in hexane, and all other reagents were from Aldrich Chemical Co., except as noted.

General Procedures. Melting points are uncorrected and were measured on a Fisher-Johns apparatus. ^{1}H NMR spectra were recorded at 60 MHz on a Varian EM-360 or at 400 MHz on a Bruker AM-400 spectrometer. Peaks are given as δ values relative to tetramethylsilane as internal standard. Coupling constants (J) are given in hertz. IR spectra were obtained either as KBr disks or as neat films on NaCl plates on a Perkin-Elmer Model 1330 spectrophotometer. The abbreviations used to describe the spectral peaks are v = very, s = strong, b = broad, sh = sharp, m = medium, w = weak. Wavelengths are given in inverse centimeters. Ultraviolet spectra were recorded on a Shimadzu Model 160 spectrophotometer. Flash chromatography was performed on silica gel 60 (Merck, 230–400 mesh) with apparatus supplied by J.T. Baker. Elemental analyses were performed by Desert Analytics,

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[1] A preliminary report of a portion of this work has appeared (Abstracts from the Fourth International Conference on Monoclonal Antibody Immunoconjugates for Cancer, San Diego, 1989).

[2] The abbreviations used are as follows: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); PBS–EDTA, phosphate-buffered saline, pH 7.2, containing 0.5 mM EDTA, ethylenediaminetetraacetic acid; RTA, ricin toxin A chain; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; TNB, 5-mercapto-2-nitrobenzoic acid; 2IT, 2-iminothiolane; X2IT, 4- and/or 5-substituted 2-iminothi-

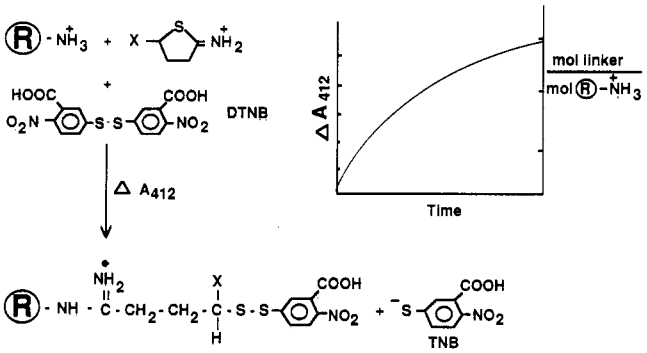
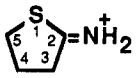
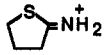
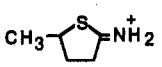
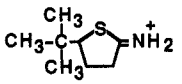
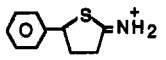
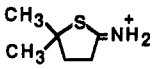
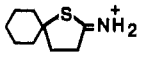
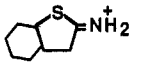


Figure 1. Chemistry of the X2ITs. Compounds containing a primary amine (R-NH_2) react with the X2ITs to create an amidinium linkage without neutralizing the positive charge. If the reaction is performed in the presence of DTNB, the newly exposed iminothiolane thiol cleaves DTNB in a disulfide-exchange reaction. This cleavage both activates the linker thiol (in preparation for conjugation) and releases free TNB, allowing real-time spectrophotometric monitoring of the rate and extent of reaction.

Table I. Structures of Substituted X2ITs



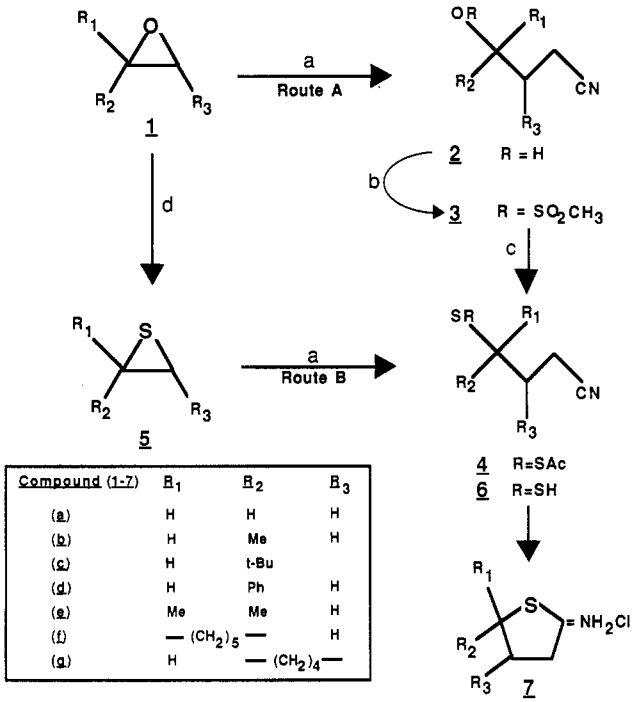
compd	substitution	no.[a]	structure
2IT	–	**7a**	
M2IT	5-methyl	**7b**	
TB2IT	5-*tert*-butyl	**7c**	
Ph2IT	5-phenyl	**7d**	
DM2IT	5,5-dimethyl	**7e**	
S2IT	5-spiro	**7f**	
R2IT	4,5-ring	**7g**	

[a] Refers to the structures shown in Scheme I. [b] The fused ring structures are shown here with the numbering system common to the iminothiolane family.

All synthetic reactions were stirred magnetically under an inert atmosphere; argon for reactions with *n*-butyllithium, otherwise nitrogen. The expression "dried" refers to passage of the organic layer through a pad of anhydrous sodium sulfate. Concentration of solutions in vacuo was performed under water aspiration with a Büchi R110 Rotavapor. Reactions were performed at room temperature unless otherwise noted. For each compound, the boldfaced designation in parentheses (e.g. **6b**) denotes the corresponding structure shown in Scheme I.

4-Mercaptopentanenitrile (6b). To dry THF (100 mL) cooled to −75 °C was added 10.0 M *n*-butyllithium (15.0 mL), followed carefully dropwise with a solution of CH_3CN (7.84 mL, 0.15 mol) in THF (20 mL). After 15 min 2-methylthiirane (11.75 mL, 0.15 mol) was added dropwise. The white suspension stirred a further 5 min at −75 °C and then the cooling bath was removed. An exothermic reaction ensued to give a pale yellow homogeneous solution. After 2 h the reaction was quenched with 1/1 concentrated

Scheme I.[a] Synthetic Scheme for the Preparation of X2ITs



Compound (1-7)	R_1	R_2	R_3
(a)	H	H	H
(b)	H	Me	H
(c)	H	t-Bu	
(d)	H	Ph	H
(e)	Me	Me	H
(f)	— $(CH_2)_5$ —		H
(g)	H	— $(CH_2)_4$ —	

[a] Details can be found in Experimental Procedures. Reagents: (a) $LiCH_2CN$/THF; (b) $ClSO_2CH_3$/benzene/Et_3N; (c) CsSAc/DMF; (d) KSCN/EtOH/H_2O or 3-methylbenzothiazole-2-thione/TFA/CH_2Cl_2; (e) HCl(g)/MeOH. The products of the reactions are indicated by underlined number (stage of synthesis), letter (R groups) combinations. For example, 7a refers to the final X2IT hydrochloride where $R_1 = R_2 = R_3 = H$ (2IT).

The combined organic layers were dried and concentrated in vacuo to give a crude amber liquid (10.1 g, 59%). Distillation in vacuo gave **6b** as a colorless liquid (6.1 g, 35%): ^1H NMR (60 MHz, $CDCl_3$) 2.72–3.28 (m, 1 H, CH), 2.53 (t, J = 7, 2-CH_2), 1.62–2.18 (m, 2 H, 3-CH_2), 1.48 (d, 1 H, J = 7, exchanges with D_2O, SH), 1.40 (d, 3 H, J = 6, 5-CH_3); IR (film) 2550 (SH), 2245 (CN).

4-Mercapto-4-methylpentanenitrile (6e). To a 20 mL of dry THF cooled at −75 °C was added 10.9 M *n*-BuLi (3.0 mL), followed dropwise by a solution of CH_3CN (1.60 mL, 30 mmol) in THF (5 mL). After addition was complete the mixture, containing a white precipitate of $LiCH_2CN$, was stirred for 10 min. Then 2,2-dimethylthiirane (*19*) (2.7 g, 30 mmol) was added dropwise. The cooling bath was removed and an exothermic reaction ensued to give a pale yellow homogeneous solution. After 1.5 h the reaction was quenched by the addition of 5 mL of 1/1 concentrated HCl/H_2O. The mixture was extracted (3 × 10 mL) with EtOAc. The combined organic layers were dried and concentrated in vacuo to give a pale yellow liquid (3.66 g, 92%) with a strong thiol stench, which was used without further purification: ^1H NMR (60 MHz, $CDCl_3$) 2.88 (t, 2 H, J = 7, 2-CH_2), 2.05 (t, 2 H, J = 7, 3-CH_2), 1.53 (s, 6 H, 2 CH_3); IR (film) 2250 (w), 2190 (m).

4-Hydroxy-4-phenylbutanenitrile (2d) (*13*). Sodium amide (10.61 g, 0.271 mol) was transferred to a tared 500-mL three-neck round-bottom flask in a glove bag under Ar. An addition funnel and thermometer were added, then 150 mL of dry THF was cannulated in. The suspension was cooled to −30 °C on a CCl_4/dry ice bath and then CH_3CN (13.5 mL, 0.259 mol) was added dropwise, while the temperature was kept <−20 °C. This was followed by addition of styrene oxide (26.0 mL, 0.228 mol) over 30 min. The mixture was allowed to warm to 0 °C over 80 min,

was then recooled to −35 °C and quenched with saturated aqueous NH_4Cl (35 mL). The mixture was diluted with EtOAc (200 mL), and the layers were separated. The aqueous layer was extracted (2 × 50 mL) with EtOAc. The combined organic layers were dried and concentrated in vacuo. TLC indicated two major products. Repeated flash chromatography on silica (hexanes/EtOAc) gave **2d** as a pale yellow oil (3.48 g, 10%): TLC (70/30 hexanes/EtOAc) R_f = 0.52; ^{1}H NMR (60 MHz, $CDCl_3$) 7.30 (s, 5 H, ar), 4.73 (m, 1 H, benzylic), 2.16–2.80 (m, 2 H), 1.70–2.16 (m, 2 H); IR (film) 3420 (vs, b, OH), 2240 (CN).

4-Phenyl-4-(thioacetyl)butanenitrile (4d). Hydroxy nitrile **2d** (3.48 g, 21.6 mmol) in benzene (20 mL) with Et_3N (3.13 mL, 22.5 mmol) was cooled to 0 °C and treated dropwise with methanesulfonyl chloride (2.54 g, 22.2 mmol) in benzene (15 mL). The ice bath was removed and stirring continued for 30 min. The Et_3NHCl was filtered off and the filtrate concentrated in vacuo to give cyanomesylate **3d**: ^{1}H NMR (60 MHz, $CDCl_3$) 7.37 (s, 5 H, ar), 5.58 (m, 1 H, benzylic), 2.72 (s, 3 H, SO_2CH_3), 2.03–2.67 (m, 4 H). Crude **3d** was dissolved in dry DMF (5 mL) and treated with a solution of CsSAc (22 mmol, prepared according to the method of Kellog and Strijtveen, ref 15) in DMF (10 mL). After overnight stirring the mixture was diluted with Et_2O (150 mL) and rinsed (5 × 20 mL) with H_2O. The organic layer was dried and concentrated in vacuo to give a brown liquid which was purified by flash chromatography (hexanes/EtOAc). Thioacetate **4d** was obtained as an orange oily liquid (3.37 g, 72%): TLC (90/10 hexanes/EtOAc) R_f = 0.21; ^{1}H NMR (60 MHz, $CDCl_3$) 7.27 (s, 5 H, ar), 4.58 (m, 1 H, benzylic), 2.30 (s, 3 H, SAc), 2.10–2.30 (m, 4 H); IR (film) 2250 (CN), 1688 (C=O).

2-(Cyanomethyl)-1-mercaptocyclohexane (6g). To dry THF (30 mL) at −75 °C was added 10.0 M *n*-BuLi (3.59 mL), followed dropwise by CH_3CN (1.88 mL, 35.9 mmol). Ten minutes after addition was complete, cyclohexene sulfide (*17*) (3.90 g, 34.2 mmol) in THF (10 mL) was added dropwise. When addition was complete the cooling bath was removed. After 2 h the mixture was recooled to 0 °C and quenched by addition of a mixture of concentrated HCl (6 mL) and H_2O (15 mL). The layers were separated, and the aqueous layer was extracted (2 × 25 mL) with EtOAc. The combined organic layers were dried and concentrated in vacuo, followed by vacuum distillation to give **6g** as a colorless liquid (2.26 g, 43%): bp 85–89 °C (0.3 mm); ^{1}H NMR (60 MHz, $CDCl_3$) 2.68 (d, 2 H, CH_2-CN), 2.32 (s, 1 H), 1.00–2.30 (m, 10 H); IR (film) 2250 (CN).

1-(2-Cyanoethyl)-1-mercaptocyclohexane (6f). To a solution of 10.0 M *n*-BuLi (0.67 mL) in THF (10 mL) at −75 °C was added CH_3CN (0.35 mL, 0.67 mmol) dropwise. After 10 min, 7-thiaspiro[5.2]octane [0.86 g, 0.67 mmol, prepared from 7-oxaspiro[5.2]octane (*20*) according to the method of ref 16] in 2 mL of dry THF was added. The cooling bath was removed and after 2 h the reaction was quenched with a mixture of concentrated HCl (1.5 mL) and H_2O (3.5 mL). The layers were separated, and the aqueous layer was extracted twice with EtOAc (25 mL). The combined organic layers were dried and concentrated in vacuo to give crude **6f** as a foul-smelling oil (0.48 g), which was used without further purification.

5,5-Dimethyl-4-mercaptohexanenitrile (6c). To a solution of 10.0 M *n*-BuLi (5.6 mL) in dry THF (50 mL) at −75 °C was added CH_3CN (2.9 mL, 56 mmol) dropwise. After 10 min, a solution of 2-*tert*-butylthiirane (*16*) (5.94 g, 51 mmol) in dry THF (5 mL) was added dropwise. After 10 min the cooling bath was removed and the mixture stirred for 2 h, whereupon it was recooled to 0 °C The mixture was diluted with CH_2Cl_2, and the layers were separated. The organic layer was rinsed four times with H_2O, dried, and concentrated in vacuo to give a pale yellow liquid (4.96 g). Kugelrohr distillation [90–100 °C (0.6 mm)] gave 2.94 g (34%) of colorless **6c**: ^{1}H NMR (60 MHz, $CDCl_3$) 3.70 (dd, 1 H, J = 6, 10, CH), 2.36–3.36 (m, 3 H), 1.70–2.36 (m, 2 H), 1.03 (s, 9 H, *t*-Bu); IR (film) 3200–3300 (s, b), 2960 (vs, b), 2250 and 2210 (both sh, m, CN), 1620, 1475, 1375.

General Procedure for the Synthesis of Substituted 2-Iminothiolanes (7b–g). The appropriate 4-mercaptonitrile (**6**) or 4-(thioacetyl)nitrile (**4**) was dissolved in dry MeOH (ca. 10 mL/g) and bubbled vigorously with HCl(g) for 3–5 min. The mixture was stirred overnight or until IR spectroscopy showed complete loss of the nitrile absorbance at 2200 cm^{-1}. The solvent was removed in vacuo. The residue was slurried in EtOAc and then warm EtOH was added until a solution was obtained. The mixture was cooled to −20 °C. If crystallization did not occur within several hours, Et_2O was added. Further cooling gave the following hydrochlorides.

5-Methyl-2-iminothiolane hydrochloride (M2IT·HCl, 7b): yield 6.67 g (83%); mp 114.5–115.5 °C (EtOAc/EtOH); ^{1}H NMR (400 MHz, DMSO-d_6) 7.46 (t, 1 H, J = 50.7, NH), 4.13–4.22 (m, 1 H, 5-H), 3.31–3.39 (m, 1 H), 3.19–3.28 (m, 1 H), 2.39–2.47 (m, 1 H), 1.88–1.97 (m, 1 H), 1.45 (d, 3 H, J = 6.7, 5-CH_3); IR (KBr) 3410, 2810 (vs, vb), 1615 (s, C=N), 1535, 1450, 1415, 1240 1025, 990, 885, 690. Anal. Calcd for $C_5H_{10}ClNS$: C, 39.60; H, 6.65; N, 9.24; S, 21.14. Found: C, 39.63; H, 6.62; N, 9.02; S, 21.34.

5-(1,1-Dimethylethyl)-2-iminothiolane hydrochloride (TB2IT·HCl, 7c): yield 1.22 g (41%); mp 224–226 °C; ^{1}H NMR (400 MHz, DMSO-d_6) 7.43 and 7.40 (2 t, J = 50.7, major t centered at 7.40, combined integral 1.7 H, NH_2^+), 4.12 (dd, 1 H, J = 5.2, 10.9, H-5), 3.17–3.33 (m, 2 H, 3-CH_2), 2.28–2.32 (m, 1 H, H-4), 1.96–2.06 (m, 1 H, H-4), 1.01 (s, 9 H, *t*-Bu); IR (KBr) 2800–3000 (vs, vb), 1630 (s, C=N), 1535, 1475, 1410, 1375, 1260, 1185, 1000, 895, 695. Anal. Calcd for $C_8H_{16}ClNS$: C, 49.60; H, 8.32; N, 7.23; S, 16.55. Found: C, 49.52; H, 8.37; N, 7.37; S, 16.34.

5-Phenyl-2-iminothiolane hydrochloride (Ph-2IT·HCl, 7d): yield 1.22 g (44%); mp 172 °C dec; ^{1}H NMR (400 MHz, DMSO-d_6) 7.52 (d, 2 H, J = 6.9), 7.36–7.45 (m, 3 H), 5.35 (dd, 1 H, J = 5.5, 10.4, 5-H), 3.30–3.44 (m, 2 H, 3-CH_2), 2.64–2.71 (m, 1 H), 2.39–2.48 (m, 1 H); IR (KBr) 3360, 2870 (vs, vb), 1615 (s, br, C=N), 1515, 1495, 1450, 1405, 1325, 1020, 985, 830, 770, 760, 700, 675. Anal. Calcd for $C_{10}H_{12}ClNS$: C, 56.20; H, 5.66; N, 6.55; S, 15.08. Found: C, 55.94; H, 5.54; N, 6.60; S, 15.05.

5,5-Dimethyl-2-iminothiolane hydrochloride (DM2IT·HCl, 7e): yield 3.0 g (65%); mp 156–159 °C; ^{1}H NMR (400 MHz, DMSO-d_6) 7.43 (t, 1 H, J = 51.3, NH), 3.43 (t, 2 H, J = 7.1, 3-CH_2), 2.18 (t, 2 H, J = 7.1, 4-CH_2), 1.59 (s, 6 H, 2 CH_3); IR (KBr) 2900 (vs, vb), 1610 (s, b, C=N), 1510, 1325, 1240, 1215, 1120, 990, 875, 845, 690. Anal. Calcd for $C_6H_{12}ClNS$: C, 43.50; H, 7.30; N, 8.45; S, 19.35. Found: C, 43.08; H, 7.45; N, 8.63; S, 18.88.

8-Imino-7-thiaspiro[5.4]decane hydrochloride (S2IT·HCl, 7f): yield 0.17 g (35%); mp 130–135 °C; ^{1}H NMR (400 MHz, DMSO-d_6) 7.37 (t, J = 50.8) and 7.34 (t, J = 50.7) (the major t is at 7.34, the combined integral of both 1.8 H, NH_2^+, 3.39 (t, 2 H, J = 7.1, 3-CH_2), 2.20 (t, 2 H, J = 7.1, 4-CH_2), 2.00 (m, 2 H), 1.69–1.79 (m, 4 H), 1.58 (m, 1 H), 1.33–1.38 (m, 3 H); IR (KBr) 2500–3500 (vs, vb), 1610 (s, C=N), 1525, 1445, 1405, 1130, 1100, 1020, 990, 875, 690.

(R2IT·HCl, 7g): yield 3.13 (59%); mp 254–255 °C dec; ^{1}H NMR (400 MHz, DMSO-d_6) 7.34 (t, 1 H, J = 50.7), 3.57 (td, 1 H, J = 11.5, 3.4, H-9), 3.24 (dd, 1 H, J = 17.0, 5.7, H-3$_{eq}$), 2.91 (dd, 1 H, J = 17.0, 13.1, H-3$_{ax}$), 2.22 (m, 1 H), 1.94–2.03 (m, 2 H), 1.75–1.84 (m, 2 H), 1.45–1.54 (m, 1 H), 1.28–1.39 (m, 3 H); IR (KBr) 2840 (vs, vb), 1624 (s, C=N), 1528, 998, 895, 880, 694. Anal. Calcd for $C_8H_{14}ClNS$: C, 50.12; H, 7.36; N, 7.31; S, 16.72. Found: C, 50.02; H, 7.29; N, 7.21; S, 16.79.

Preparation of Thiol-Activated Model Compounds. TNB-activated X2ITs were prepared by aminolysis in the presence of the aromatic disulfide DTNB. Each X2IT (final concentration 0.5 mM) was added to a solution of 1.0 mM DTNB in 30 mM NH_4HCO_3, pH 8.0. After 1 h at 25 °C, the reactions were terminated, and solvent was removed, by lyophilization. The activated model compounds (NH_2-X2IT-TNB) were resuspended in 0.5 mL of 25 mM sodium citrate, pH 3.2, and applied to separate 1-mL columns of S-Sepharose Fast Flow (Pharmacia) equilibrated in the same buffer. Each column was washed with citrate buffer until the absorbance at 412 nm approached zero, and the model disulfides were then eluted with PBS–EDTA.

Preparation of IND1-M2IT-RTA. A mixture of the murine IgG2a IND1 antibody (20 μM, 3 mg/mL, 2 mL), DTNB (5.0 mM), and M2IT (3.4 mM) in PBS–EDTA was incubated at 25 °C until the absorbance of the reaction mixture at 412 nm reached 0.54. Excess reagents and reaction byproducts were then removed by desalting on a 1 cm × 20 cm column Sephadex G25F equilibrated at 4 °C in PBS–EDTA. The thiolated IND1–M2IT-TNB antibody so prepared (6.4 μM, 5 mL) was mixed with a 5-fold molar excess of RTA, and the mixture was incubated at 4 °C for 16 h. The IND1–M2IT–RTA immunotoxin was subsequently purified by size-exclusion chromatography on a 1 cm × 32 cm column of AcA44 equilibrated in PBS at 4 °C.

RESULTS

Synthesis and Characterization of Substituted 2-Iminothiolanes. The X2IT·HCl analogues (Table I) were prepared by cyclization of γ-mercapto or γ-thioacetyl nitriles in methanolic HCl (Scheme I). Both the parent unsubstituted 2IT (**7a**) and M2IT (**7b**) have been previously prepared in this way (*11*, *12*). The nitriles were prepared either by ring opening of the appropriate epoxide with sodium or lithium acetonitrile (*13*, *14*), followed by mesylation of the γ-hydroxy nitrile and displacement with cesium thioacetate (*15*) (route A), or by ring opening of a thiirane with lithium acetonitrile (route B). The thiiranes were prepared from the corresponding epoxides either with potassium thiocyanate in EtOH (or MeOH)/H_2O (*16*) or with 3-methylbenzothiazole-2-thione (*17*). Route B was useful when nucleophilic substitution of a mesylate would be difficult [TB2IT (**7c**) and DM2IT (**7e**)]. Similarly, the cis-fused bicyclic R2IT (*cis*-**7g**) could not be prepared by substitution of mesylate **3g** or the corresponding triflate with cesium thioacetate, but the trans-fused (*trans*-**7g**) compound was readily prepared via cyclohexene sulfide (route B). The reaction of lithioacetonitrile with 2-phenylthiirane proceeded poorly, so in this case Ph2IT (**7d**) was prepared via route A. The preparation of nonracemic 5-substituted X2ITs was not investigated, but should be feasible from commercially available chiral epoxides such as (*R*)-(+)-styrene oxide and (*S*)-(−)-propylene oxide or the derived thiiranes (*16*, *18*).

The X2IT·HCl compounds (**7a–g**) were characterized

Table II. Properties of Substituted 2-Iminothiolanes

linker	λ_{max}	ε, 1 mM, 248 nm	reaction rates (25 °C); k, s^{-1} hydrolysis[a] (×10^{-5})	reaction rates (25 °C); k, s^{-1} glycine[b] (×10^{-3})
2IT	247	8.72	1.4	5.3
R2IT	250	9.07	1.7	3.4
M2IT	247	9.27	0.7	4.8
Ph2IT	246	9.00	0.2	5.2
TB2IT	248	11.7	0.8	4.4
DM2IT	247	10.8	0.9	3.5
S2IT	248	10.7	0.7	3.0

[a] The X2ITs (100 μM) were incubated at 25 °C in PBS–EDTA and changes in the absorbance at 248 nm were monitored. Plots of log [X2IT] vs time were linear, and first-order rate constants were calculated from the slopes. [b] The rates of reaction of the X2ITs (100 μM) with glycine (160 mM) in PBS–EDTA were examined by coupling the reaction with 0.5 mM DTNB and monitoring changes at 412 nm. First-order rate constants were determined from the linear slopes of log [X2IT] vs time plots.

analysis. The recrystallized X2ITs are easily handled and stable for at least 6 months when stored dry at 4 °C. The compounds are readily soluble in H_2O or polar solvents such as DMSO. The ^{1}H NMR spectra of the X2ITs are straightforward, showing signals for the 4-CH_2 from 1.9 to 2.7 ppm, for the 3-CH_2 from 2.9 to 3.4 ppm, and for the 5-CH (when present) from 3.5 to 5.4 ppm. The trans ring fusion of R2IT (**7g**) was assigned on the basis of the assumed anti opening of cyclohexene sulfide with lithioacetonitrile and by the 11.5 Hz coupling observed between the bridgehead protons.

The UV spectra of the X2ITs have a maximum at 246–250 nm with mM extinction coefficients of ca. 10 (Table II). The UV absorbance of the X2ITs is lost upon ring opening and this provides a convenient method of monitoring hydrolysis or aminolysis rates. The rates of aqueous hydrolysis of the X2ITs (**7a–g**) in PBS could therefore be followed by (i) the loss of absorbance at 248 nm or (ii) coupling the reaction with DTNB. As shown in Table II, aqueous hydrolysis of the X2ITs was relatively slow, followed first-order kinetics, and showed little dependence upon ring substitution. The rates shown are for the direct optical assay; similar values were obtained when the released thiol was trapped with DTNB and the reaction monitored at 412 nm (data not shown). The rates of X2IT hydrolysis were thus unaffected by the presence of DTNB.

Reactivity with Glycine. Reaction of the X2ITs with amino groups was analyzed by incubating each crosslinker with glycine at pH 7.2. In order to better mimic the reaction with proteins, these reactions were performed in the presence of DTNB, thus allowing both real-time monitoring of the reaction, as well as activation of the newly exposed iminothiolane thiol. Each X2IT was therefore incubated with glycine and DTNB, and changes in the absorbance at 412 nm were monitored. Under these conditions, the reaction rates were first-order and were again unaffected by the ring substituent (Table II). Similar rates were also obtained when the reactions were monitored optically at 248 nm in the absence of DTNB (data not shown). Under the experimental conditions employed, the rates of reaction of the X2ITs with glycine were typically 5000-fold greater than the rates of aqueous hydrolysis.

Preparation and Stability of Model Disulfides. In order to assess the effect of the various ring substituents on subsequent disulfide bond stability, TNB-activated model conjugates were prepared by reacting each X2IT with ammonium bicarbonate in the presence of DTNB. Aminolysis with ammonium bicarbonate was chosen for

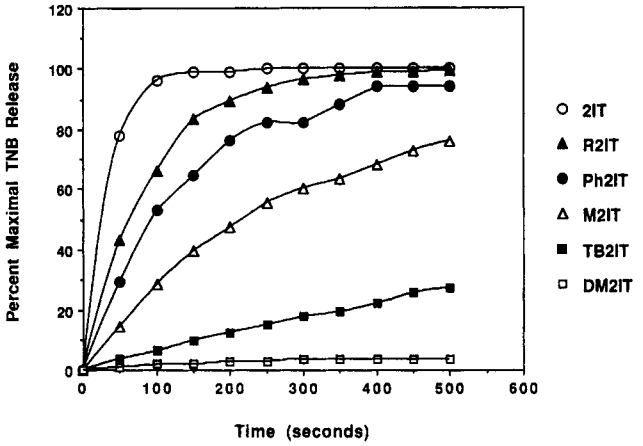


Figure 2. Glutathione-induced release of TNB from NH_2-X2IT-TNB model conjugates. Samples of the activated model conjugates (20 μM) in PBS–EDTA were placed in a cuvette thermostated to 25 °C and, at $T = 0$, reduced glutathione was added to a final concentration of 200 μM. The release of TNB was monitored optically at 412 nm for 500 s, and 2-mercaptoethanol was then added to a final concentration of 200 mM to determine maximal release of TNB. The plots for the different linkers were normalized by determining percent maximal TNB release, as calculated by dividing the amount of TNB released at any timepoint by the amount released with 2-mercaptoethanol.

Table III. Relative Stability of TNB-Activated NH_2-Iminothiolanes

	TNB release rate (25 °C),[a] k ($\times 10^{-4}$), s^{-1}	stability increase relative to 2IT[b]
NH_2-2IT-TNB	318	1.0
NH_2-R2IT-TNB	113	2.8
NH_2-Ph2IT-TNB	70.0	4.5
NH_2-M2IT-TNB	32.5	9.8
NH_2-TB2IT-TNB	3.22	99
NH_2-DM2IT-TNB	0.076	4184

[a] Reactions contained 20 μM NH_2-X2IT-TNB in PBS–EDTA at 25 °C and were initiated by the addition of reduced glutathione as described in the legend to Figure 2. The concentration of added glutathione was varied between 50 μM and 200 mM in order to achieve linear plots of log [NH_2-X2IT-TNB] vs time, and first order rate constants were then determined. [b] Calculated by dividing the rate constant for TNB release for each model conjugate by that obtained for NH_2-2IT-TNB.

be removed by lyophilization, thereby minimizing ionic effects. Conditions were chosen such that each linker was quantitatively converted to the corresponding activated model conjugate (NH_2-X2IT-TNB). Following lyophilization, the model conjugates were purified by cation-exchange chromatography to remove unreacted DTNB and TNB. The relative stabilities of these mixed disulfides were then examined by reacting the model conjugates with reduced glutathione. The release of TNB was monitored at 412 nm, providing a direct measure of glutathione-induced disulfide bond cleavage.

A comparative rate plot for the activated X2IT model conjugates (20 μM) incubated with 200 μM glutathione is shown in Figure 2. By varying the concentration of glutathione (0.05 to 250 mM) for the different compounds, first-order rate constants for TNB release were determined (Table III). The results indicate that the relative stability of the disulfide bonds formed by the X2ITs vary by a factor of roughly 4000, with DM2IT being the most stable, followed by TB2IT and M2IT.

Preparation of Protein–Protein Conjugates. To confirm the utility of the X2IT reagents for the preparation of antibody–toxin conjugates, one of the linkers (M2IT) in the presence of DTNB. The reaction was monitored optically at 412 nm and was terminated when the absorbance indicated 1.9 activated thiols/mol of protein. Following purification, an aliquot was removed and treated with 0.1 mM DTT; the activated IND1–M2IT-TNB antibody contained 2.0 TNB/mol. Subsequent conjugation with RTA gave IND1–M2IT–RTA in high yield.

DISCUSSION

The X2ITs described here represent a new family of cross-linking reagents that should prove useful in the preparation of stabilized protein conjugates linked by a disulfide bridge. Like 2IT (*11*), the X2ITs are highly water soluble, they react with amino groups to produce a stable amidinium linkage (thereby preserving the positive charge), and the reaction rate and extent can be monitored in real time by including an aromatic disulfide in the reaction mixture. In addition, the relative stability of the conjugate disulfides can be controlled by appropriate substitution on the 2IT ring, particularly at the 5-position. With the model conjugates prepared here, disulfide bond stability was increased from 6- to 4000-fold relative to unsubstituted 2IT and was well-correlated with the degree of steric hindrance (see below). Importantly, these increases were achieved without adversely affecting either the rate of reaction with glycine or the aqueous lability of the linker.

In designing cross-linking reagents for the preparation of disulfide-linked immunoconjugates, several features critical to actual therapeutic use must be evaluated (*1*). Some considerations (and consequences) include (i) the effect of linker derivatization on protein function (reduced binding or enzymatic activity, altered charge, etc.), (ii) variations in subsequent conjugation efficiency (lower efficiency necessitates higher linker/protein ratios and reagent needs), and (iii) relative stability in vivo (rapid deconjugation reduces the effective serum concentration and liberates competitive ligand). Whereas some of these concerns are empirical (i.e., the effect of linker derivatization), the remainder can be readily controlled by appropriate linker chemistry and selection.

Each of the above concerns can be minimized by the use of a linking reagent that allows both efficient conjugation with thiol-containing compounds, as well as the ability to control disulfide bond stability. Thus, the absolute number of linkers/molecule can be minimized, reducing the probability that protein function will be affected. Preliminary results suggest that the efficiency of conjugation (i.e., the efficiency with which the aromatic leaving groups are replaced by the protein thiol) for the X2ITs ranges between 60 and 100%, depending upon the diaryl disulfide present in the reaction mixture (unpublished data). These results, together with the preservation of positive charge and the controlled bond stability, support the use of the X2ITs in the preparation of human therapeutics.

Mechanistically, the observed increases in the stability of model X2IT disulfides conferred by α-alkylation can be rationalized by assuming that the incoming thiolate nucleophile (glutathione in this case) must attack the sulfur atom derived from the X2IT (see Figure 1). It has been postulated that this thiol-exchange process is a nucleophilic substitution with the attacking thiolate approaching along the extension of the S–S bond (22). According to this model, the σ^*(S–S) orbital interacts with the approaching nucleophile. Molecular modeling indicates that α-methyl groups do provide significant shielding of the adjacent S and that this shielding increases with

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