Toxin-Targeted Design for Anticancer Therapy. I: Synthesis and Biological Evaluation of New Thioimidate Heterobifunctional Reagents

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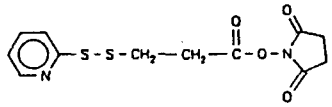
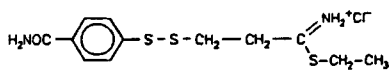
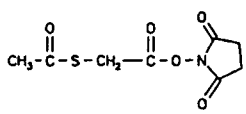
Abstract □ In an effort to obtain a more potent and specific immunotoxin for cancer therapy, we designed a series of heterobifunctional linkers characterized by a thioimidate group linked to a *S*-acetyl thiol (**4, 5**) or substituted aryldithio group (**6–10**). These ligands were synthesized by a Pinner-type process from the corresponding nitrile derivatives obtained by thiol–disulphide exchange reaction, reaction with substituted benzene-sulphenyl chloride, or other known procedures. To check the reagent of choice for immunoconjugate preparation, we studied thiol–disulphide exchange kinetics between the intermediate nitrile derivatives and cysteine. Among the tested aryldithio derivatives (**6–10**), we selected ethyl 3-(4-carboxamido-phenyldithio)propionthioimidate (CDPT, **9**) for further studies. By analyzing the rate of incorporation of the linkers **4**, **5**, and **9** in a model immunoglobulin G protein, we found similar results with CDPT **9** and ethyl *S*-acetyl 3-mercaptopropionthioimidate ester hydrochloride (AMPT, **5**) because both reagents showed a linear correlation between the number of introduced thiol groups and factors such as time and protein and reagent concentrations. Comparison of the two acetylthio-derivative ligands **4** and **5** showed that AMPT **5** was more stable toward deacetylation than ethyl *S*-acetyl 2-mercaptopropionthioimidate ester hydrochloride (AMAT, **4**). By comparing the kinetic and biological parameters of seven new thioimidate linkers, we found that two of these (CDPT and AMPT) could be superior ligands for protein–protein conjugation. They offer advantages over the commercially available compounds, such as minimal perturbation of the protein structure, controlled reactivity, and good stability.



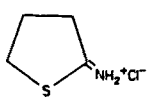
N–succinimidyl 3–(2–pyridyldithio)propionate (SPDP)



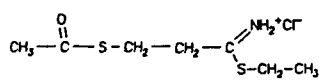
3–(4–carboxamidophenyldithio)–propionthioimidate (CDPT)



N–succinimidyl–acetylthioacetate (SATA)



2–iminothiolane (2–IT)



Ethyl S–acetyl–propionthioimidate (AMPT)

Conjugation of protein or synthetic polymers with drugs is a widely studied means of constructing hybrid conjugates with high activity and good specificity. Examples are immunotoxins (ITs) composed of a toxin and a monoclonal antibody (Mab) or chemoimmunoconjugates formed by a Mab coupled to a cytostatic drug.[1–8] The immunoconjugate concept is based on the idea that a therapeutic compound must be attached to the antibody in such a way that it can be delivered to the tumor without being released before reaching its target. The nature of the linkage is therefore important both for retention of activity and for the in vivo stability of the conjugate. Indeed, the objective of any conjugating procedure is to effect the attachment without altering the desired properties of the ligand and carrier molecules. For example, with immunoconjugation, it is necessary that after conjugation the antibody retains its specificity and the drug or toxin maintains its activity.

We have applied new coupling procedures to prepare an IT consisting of a whole ricin–antibody conjugate possessing strong and specific antitumor activity in nude mice grafted intraperitoneally with a human adenocarcinoma.[9–13] Generally, to link toxin and antibody, a heterobifunctional reagent such as *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP, **1**) was used.[14,15] By adding this reagent to both conjugating proteins and then removing the thiopyridyl group with dithiothreitol in only one of the two derivatized proteins, a disulfide-linked conjugate was formed via a disulfide exchange reaction. Alternatively, deprotected SPDP on one macromolecule can be reacted with an iodoacetyl group.

Instead of introducing thiol groups into proteins by acylation of the lysyl residues, as in the case of SPDP, amidination can be an alternative. In this way, the modified lysyl residues retain the cationic charges of the native molecule; this can be important for conformational stability. One of the reagents used was the 4-mercapto-butirrymidate ester which was found to spontaneously cyclize to form 2-iminothiolane (2-IT, **2**).[16–19]

2-IT has became a popular reagent to prepare immunoconjugates because it offers many advantages over other possible linkers. Nevertheless, 2-IT has a rigid cyclic structure that makes it difficult to vary its reactivity. For example, it is not possible to vary the distance between the two reactive groups (thioimidate group and thiol) or to change the reactivity of the thiol in the heterobifunctional linker. Moreover, relatively

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low yields are obtained using 2-IT, and this may be due to air oxidation during handling and storage of the free thiol group introduced into the protein. This could be avoided by shielding the sulfhydryl with a protective group.

Attempting to find a more suitable cross-linking reagent for attaching antibodies to proteins (such as a toxin or an enzyme), we prepared a series of acyclic spacers consisting of a thioimidate group linked through an ethylene hydrocarbon chain to a protected thiol. Protection of thiol group was realized following two routes: (1) by linking the thioimidate spacer to a readily cleavable substituted aryldithio group, and (2) by shielding the thiol with an acetyl group from which the free sulfhydryl group could be liberated by hydroxylamine, analogously to SATA 3, a widely used reagent for introducing an acetylthiogroup into proteins.[20,21] Alternatively, by varying the length of the ethylene hydrocarbon chain we were able to alter the stability and reactivity of the thiol-protected thioimidate linkers.

Results

Chemistry—The preparation as hydrochlorides of both *S*-acetyl **4** and **5** and substituted aryldithio thioimidates **6–10** is shown in Schemes I and II. A common method of synthesizing the thioimidate linkers **4–10** is to apply the Pinner synthesis[22] by bubbling dry hydrogen chloride through a solution of the corresponding nitriles and ethanthiol. The synthesis of *S*-acetyl thioimidate **4** and **5** began with the preparation of acetylthioacetonitrile **11** and the 3-acetylthiopropionitrile **12** through reaction of potassium thioacetate with chloroacetonitrile,[23] and reaction of acetyl chloride with 3-mercaptopropionitrile, respectively (Scheme I).

Two different methods were used for the synthesis of the substituted aryldithiopropionitriles **13–19**, precursors of the aryldithio thioimidates **6–10** (Scheme II): (A) the reaction of thiol-disulfide exchange between symmetrical diaryl disulfide and 3-mercaptopropionitrile; and (B) the reaction of substituted benzenesulphenyl chloride with 3-mercaptopropionitrile. The thiol–disulfide exchange reaction is of general use.[24–27] In our case, two important factors were considered in applying it. One is the presence, on the phenyl ring of the diaryl disulfide, of sufficiently strong electron-withdrawing groups to allow an appropriate choice of a nearly neutral (as for **20** and **21**) or a fairly alkaline (as for **22**) aqueous solution. A second important factor was the solubility of the reacting products; for example, we failed to prepare the thioimidate esters from sodium salt of 3-(4-sulphonylphenyldithio) propionitrile **15** and of 3-(4-nitro-2-sulphonylphenyldithio) propionitrile **14** because of the insolubility of these intermediates in ethanthiol and in other solvents compatible with the synthesis.

When the thiol–disulfide exchange reaction was difficult to accomplish because of the modest electronegativity of the aryl substituents and/or the insufficient solubility of the reagent, the chlorinolysis method (method B) was followed (Scheme II).[26–28] The benzenesulphenyl chlorides **27–29** obtained were used as crude products for the preparation of the 3-(4-carboxamidophenyldithio)-propionitrile **17** and the 4-sulfonamido analogue **18** from equimolar amount of 3-mercaptopropionitrile in dry acetic acid. This procedure resulted in only moderate yields of the required mixed disulfides, but it had the advantage of avoiding appreciable -S-S- bond scission that usually occurs at neutral or alkaline pH.[29]

The symmetrical diaryl disulfide intermediates were obtained following established procedures (see *Experimental Section*).[26–29] All the thioimidate esters **4–10** synthesized required storage under very anhydrous conditions and were stocked in tightly stoppered flasks at −4 °C under argon. Under these conditions, they remained crystalline and were stable for several months.

To compare the reactivity of the differently substituted aryldithio groups with the 2-pyridyldithio residue of SPDP, we synthesized the 3-(2-pyridyldithio)propionitrile **30** by thiol–disulfide exchange between 3-mercaptopropionitrile and bis 2-pyridyl disulfide.[18]

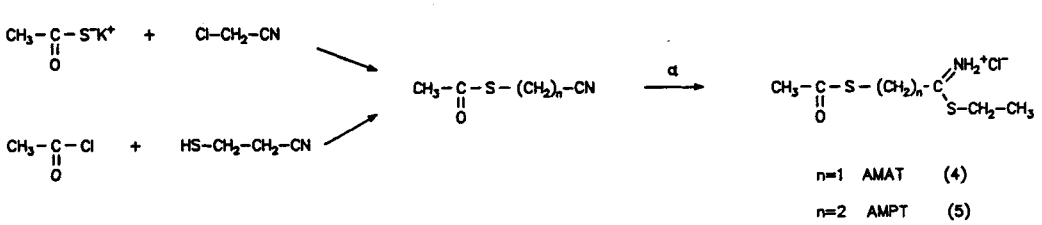
Kinetic Studies—In the series of derivatives with arylthio protective residues, we made preliminary[16,18] studies on disulfide bond reactivity towards thiolated reagents such as cysteine to select the most suitable cross-linker for immunoconjugate preparation via thiol–disulfide exchange. Disulfide reactivity towards cysteine in sodium phosphate buffer at pH 6.5 was measured spectrophotometrically on the intermediate nitrile derivative rather than on the final thioimidate ester because this group easily undergoes hydrolysis, which could complicate the calculation (Table I).[30,31]

The reaction of thiols with aryldithio derivatives involves two consecutive steps[32–36]: (A) Ar-S-S-B + X-SH ⇌ Ar-SH + X-S-S-B and (B) X-S-S-B + X-SH ⇌ X-S-S-X + B-SH. In the initial step (A), the aryldithio derivative Ar-S-S-B (aryldithiopropionitrile **13–19**) gives, in the presence of the thiolated reagent X-SH (cysteine), the mixed disulfide X-S-S-B and the chromophoric thiophenol Ar-SH. In the second step (B), the excess of X-SH converts X-S-S-B to the symmetrical dialkyl disulfide X-S-S-X.

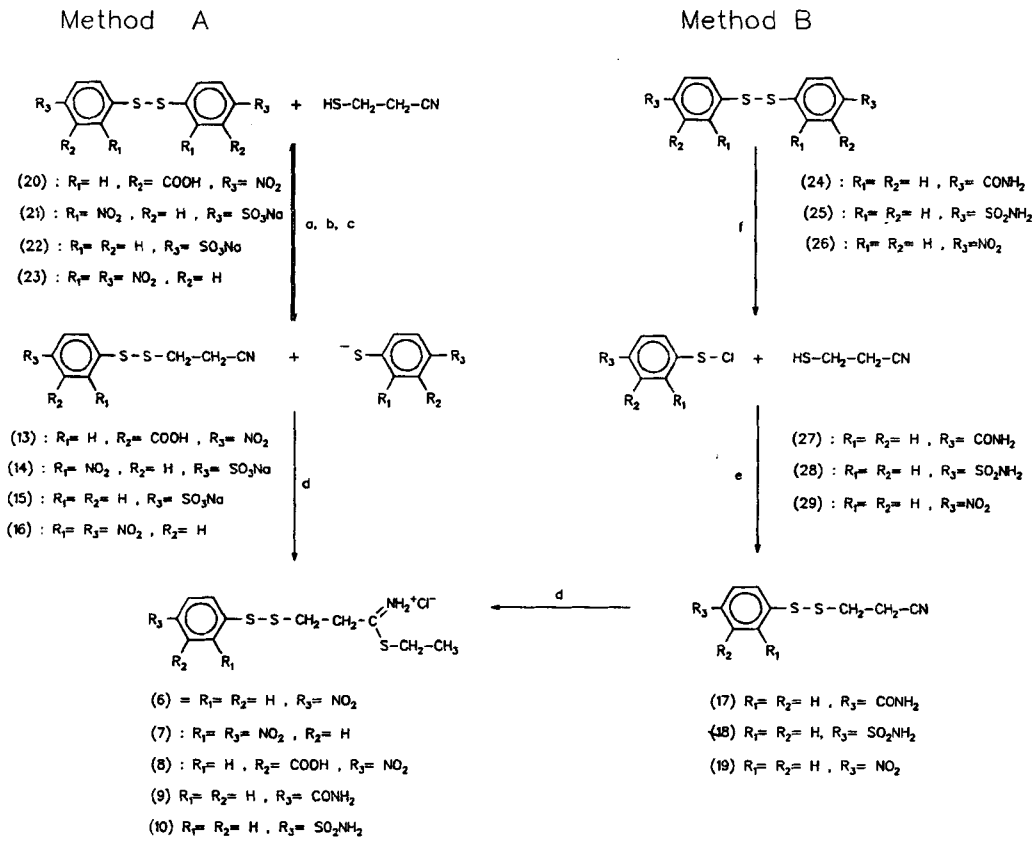
For simplicity, we calculated the kinetic constants relative to the initial phase (A) when no more than 20% of the disulfide Ar-S-S-B had reacted and the inverse reaction (X-S-S-B → Ar-S-S-B) could still be neglected. Under these conditions, the equilibrium shown in B can be ignored.

By general procedures,[32–38] the progress of the disulfide reductive scission for each aryldithiopropionitrile derivative (process A) was followed at the absorption wavelength of the thiophenol released and the related rate constants k ($L \cdot mol^{-1} s^{-1}$) were calculated. The rate constants calculated for disulfide exchange with twofold molar excess of cysteine are given in Table I and compared, under the same conditions, to the rate constant obtained for the 3-(2-pyridyldithio)propionitrile **30** that is related to SPDP.

Among the compounds tested, the 4-sulphonamido phenyldithio derivative **18** showed the most favorable reactivity to thiol–disulfide exchange. 3-(2,4-Dinitrophenyldithio)propionitrile **16** and the sodium salt of 2-(2-nitro-4-sulphonylphenyldithio)propionitrile **14** were found to be too reactive for kinetic measurements as demonstrated by the rapid release of thiophenol observed when the corresponding thioimidate esters were dissolved in buffer at pH 7.4. The 3-(4-nitrophenyldithio)propionitrile **19**, although less reactive



Scheme I—Synthesis of thioimidate reagents. Key: (a) dry HCl, EtSH.



Scheme II—Synthesis of thioimidate reagents. Key: (**a**) from **20** or **21**, pH = 7.4 aqueous solution; (**b**) from **22**, pH = 9 aqueous solution; (**c**) from **23**, DMF; (**d**) dry HCl, EtSH; (**e**) CH_3COOH, reflux; (**f**) Cl_2, dry CH_2Cl_2.

than the corresponding dinitro derivative, showed the highest measurable rate constant.

3-(4-Sulphonamidophenyldithio)propionitrile **18** gave a rate constant comparable to that found by 3-(2-pyridyldithio)propionitrile **30**. Thus, presumably the corresponding thioimidate **10** might have the same reactivity of the widely used heterobifunctional cross-linker SPDP. Unfortunately, **10** was too insoluble in water, and this has precluded further investigations.

On the other hand, the 3-(4-carboxamidophenyldithio)propionitrile **17** was less reactive towards cysteine than the sulphonamido derivative **18**, whereas the corresponding thioimidate derivative **9** was much more soluble than **10**. Consequently, we chose **9** as the most convenient linker for further testing of protein aminolysis reactivity.

Reaction of Thioimidate Ester Ligands with Immunoglobulin—To evaluate the reactivity of the thioimidate linkers towards the ϵ-amino groups of a protein, bovine immunoglobulin G (IgG) was used as a model. Incorporation of the linkers into the protein was checked at pH 5.1, 6.05, 7.4, and 9.05 at 20 °C and at two different IgG concentrations (40 and 60 μM).

At first we tested the *S*-acetylthioimidate derivatives **4** (AMAT) and **5** (AMPT; Figures 1 and 2). At pH 7.4, these two reagents prolonging the reaction time increased the degree of substitution on the protein in proportion to the molar excess of ligand used and to the protein concentration. After 3 h of reaction, a fall in the degree of substitution was observed with AMAT **4**, whereas, using the AMPT linker, the degree of protein derivatization was generally efficient with both thioacetylated reagents. At acidic pH (5.1 and 6.05), an appreciable derivatization of the bovine IgG, strictly related to linker concentration and reaction time, was shown by AMPT **5** (Figure 1).

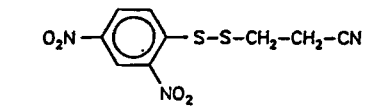
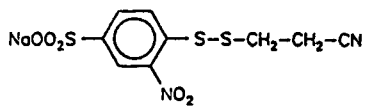
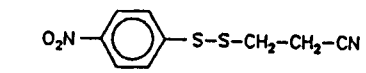
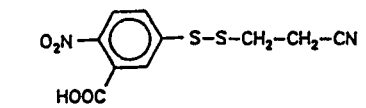
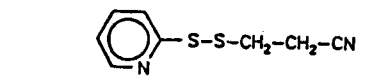
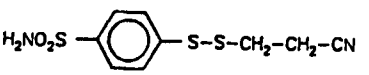
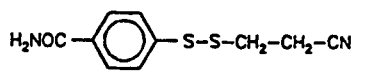
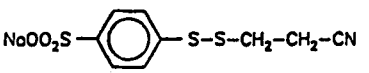
The in vitro stability of the AMPT or AMAT derivatized bovine IgG stocked at 4 °C was evaluated by monitoring spontaneous deacetylation at different time intervals (Figure 3). A striking difference was observed in the stability of the two linkers AMAT **4** and AMPT **5**. Even at low substitution degree (2–3 acetylthio groups introduced/protein molecule), the AMAT-modified IgG spontaneously deacetylated. In contrast, no deacetylation was observed till 60–80 days after the ligand introduction when 1–4 acetylthio groups were introduced per IgG molecule with AMPT. Deacetylation was only observed when derivatization efficiency was high (6–9 acetylthio groups introduced/IgG molecule).

Thus, the lower efficiency in protein derivatization shown by AMPT at the longer time intervals could be related to its spontaneous deacetylation with consequent oxidative and cross-linking side-reactions.

Among the aryldithio derivatives, we evaluated the reactivity of ethyl 3-(4-carboxamidophenyldithio)propionthioimidate (**9**, CDPT) towards bovine IgG. As with AMPT (Figure 1), the degree of substitution increased with reaction time both at pH 7.4 and 9.05. This result indirectly confirmed that no thiol displacement occurred even at alkaline pH. To compare our acyclic thioimidate derivatives with known commercially available linkers we derivatized bovine IgG, under identical conditions, with the cyclic thioimidate 2-iminothiolane (2-IT). The results (Figure 2) show that the cyclic thioimidate ester 2-IT was about equally efficient as our acyclic disulfide thioimidate derivatives in introducing thiol groups into the protein over short reaction times.

On prolonging reaction time (up to 5 h), 2-IT showed a fall in IgG derivatization efficiency in contrast to the more stable AMPT and CDPT. Probably the introduction of unprotected thiol groups by 2-IT made the thiolated protein more sensitive

Table I—Aryldithio Group Reactivity[a]

Aryldithiopropionitrile	Compound	k, $L \cdot mol^{-1} \cdot s^{-1}$
	16	ND[b]
	14	ND
	19	410 ± 10
	13	330 ± 10
	30	116 ± 5
	18	99 ± 5
	17	51 ± 2
	15	28 ± 2

[a] Calculation of kinetic constants of thiol–disulfide exchange is shown in the *Appendix*. [b] ND, Not determined.

to air oxidation and/or aggregation than after derivatization with AMPT or CDPT.

Discussion

With a new bifunctional linker with the favorable properties shown by the 2-IT reagent, we designed a series of thioimidate derivatives provided with thioacetyl (as **4** and **5**) or aryldithio groups (as **6–10**). Thioimidates are known to be useful reagents for protein amidination by reaction with the ϵ-amino groups of lysine.[39,40] Like the corresponding imido esters,[41,42] the thioimidates undergo nucleophilic addition from suitable amino groups of proteins, forming a tetrahedral intermediate[33] that undergoes elimination of a thiol leading to the desired amidinated protein.

Selection of the bifunctional cross-linker most suitable for the preparation of disulfide-linked conjugates was accomplished by studying the thiol–disulfide exchange reaction involving intermediate nitrile derivatives **13–19** and a model thiolated reagent such as cysteine. By considering the kinetic constants of the thiol–disulfide exchange of eight differently substituted dithioaryl derivatives **13–19**,[32] a direct relationship between the k and the electron-withdrawing properties of the substituents was apparent (Table I).[43,44]

The acetylthioimidate derivatives **4** and **5** were designed as alternative ligands to SATA, a reagent used to incorporate sulfhydryl groups into proteins or to prepare immunotoxins.[9,20,21] SATA offers several advantages over SPDP in thiolation of proteins because it eliminates the need for a strong reducing agent such as dithiothreitol to deprotect thiol during protein conjugation. Instead, SATA deprotection is accomplished with hydroxylamine, which does not interfere with the protein coupling reaction. Moreover, SATA shows high stability in introducing thiol groups into Mabs in comparison with SPDP and some other disulfide linkers.[45,46]

To check the ability of the thioimidate linkers **4**, **5**, and **9** to derivatize Mabs or proteins as well as the stability of the thioacetylated proteins, **4**, **5**, and **9** were reacted with bovine IgG. At different pH and concentration (Figures 1 and 2), we found similar results with CDPT **9** and AMPT **5**. At alkaline

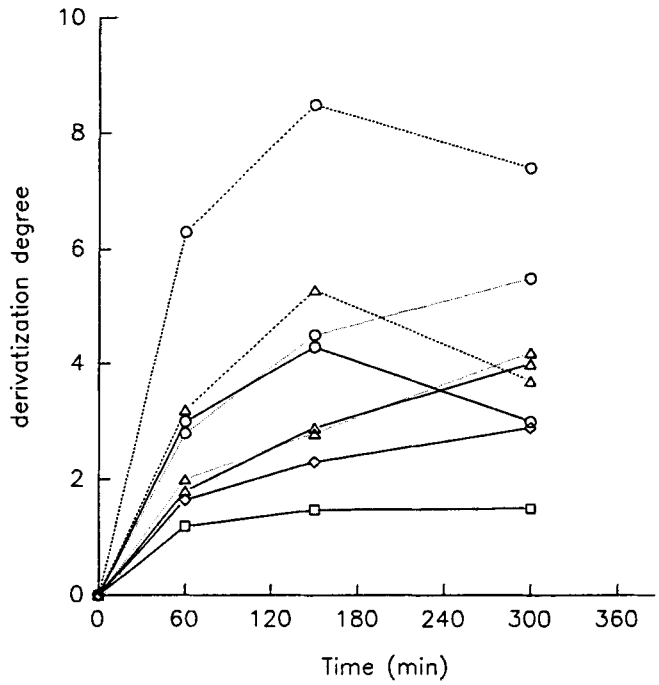


Figure 1—Bovine IgG derivatization degree at various pH. Key: AMPT (solid line); AMAT (dotted line); CDPT (short dashed); pH 9.50 (circle); pH 7.40 (triangle); pH 6.05 (diamond); pH 5.11 (square).

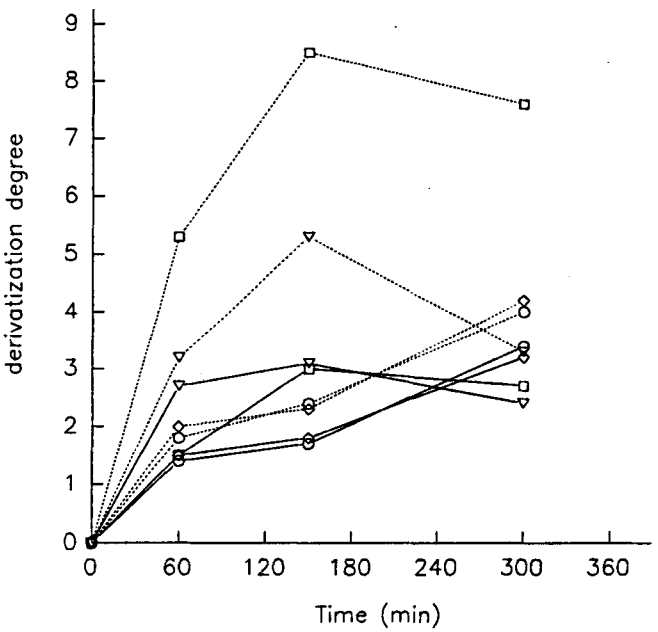


Figure 2—Bovine IgG derivatization degree (60 μM) with various linkers [AMPT (circle), AMAT (triangle), 2-IT (square), CDPT (diamond)] at different molar excesses [10× (solid line), 30× (dotted line)].

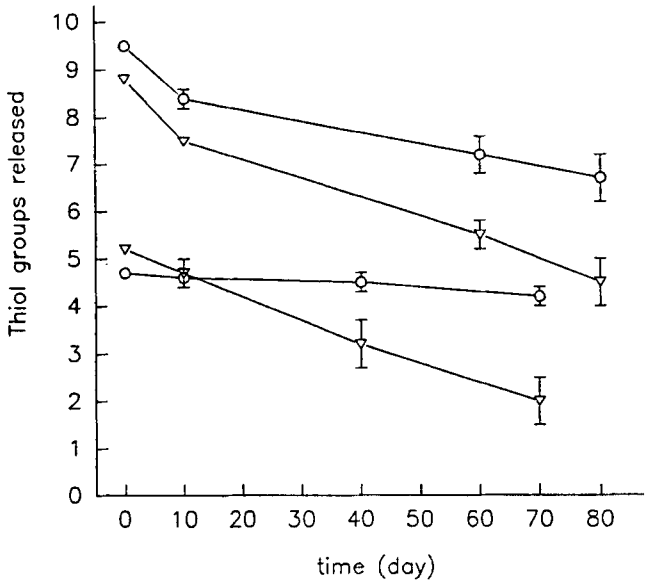


Figure 3—In vitro stability of AMPT (circle) and AMAT (triangle); spontaneous deacetylation at 4 °C with time.

pH, the SATA-related linkers AMPT **5** and AMAT **4** began to deacetylate, thus causing a lower derivatization yield in comparison with CDPT **9**. Generally AMAT was found to be more reactive but also more sensitive to deacetylation than AMPT. Probably a shorter chain length could induce minor stability of the linker as previously shown.[45]

AMPT **5** and CDPT **9** appeared to be as reactive as the cyclic thioimidate 2-iminothiolane **2**. These reagents, which present the reactivity of the thioimidate ester in one part of the molecule and that of SPDP or SATA on the other side, could potentially be more useful than the traditional linkers in coupling used to prepare ITs. For examples CDPT, the thioimidate derivative related to SPDP, was able to derivatize proteins at acidic pH, whereas SPDP became very unstable due to protonation of the pyridyl group.

AMPT could offer some advantage over 2-IT to introduce thiol groups into proteins because during the coupling procedure, the sulfydryl group remains protected from air oxidation, which usually gives rise to heterogeneous products. In addition it appeared to be more stable than SATA to spontaneous deacetylation. Consequently, it could be very useful as an alternative linker to SATA in thiolating proteins or in preparing thioether or disulfide-linker ITs by reaction of an AMPT-derivatized protein with an iodoacetylated or a CDPT-derivatized one.

In conclusion, in the present work, we prepared two new cross-linking reagents (CDPT and AMPT) that in addition to the advantage of producing minimal perturbation of the protein structure (as is generally true for thioimidate reagents) offered the major benefits of controlled reactivity and good stability during protein coupling.

Experimental Section

Melting points were determined with a Reichert Kofler apparatus and are uncorrected. ^{1}H NMR spectra were recorded on Jeol EX-400, GX/270, or JNM-PMX 60 spectrometers, with $SiMe_4$ as internal standard. Fast-atom bombardment (FAB)[1] mass spectra were obtained with a VG Analytical 70-70 EQ-HF spectrometer. IR spectra were recorded on a Perkin-Elmer 781. Microanalyses for C, H, N, O, S, and Cl were within +0.4% of theoretical values and were performed on a elemental analyzer 1106 (Carlo Erba Strumentazione).

The reactions were checked on F_{254} silica gel precoated sheets (Merck); after development, the sheets were exposed to iodine vapor and viewed under UV light. Purification was done by column flash chromatography on 230–400 silica gel (Merck). The progress of thiol–disulfide exchange reactions was followed with a Beckman DU-70 spectrometer.

Bovine IgG and protein A-sepharose CL-4B were purchased from Sigma (St. Louis, MO). 2-IT was obtained from Pierce Europe (3260 BA Oud-Beijerland, The Netherlands).

Compound Preparation—3-Mercaptopropionitrile was obtained by following the procedure of Traut et al.[16] The bis 2,4-dinitrophenyl disulfide **23** and bis 4-nitrophenyl disulfide **20** were prepared according to the method of Marston et al.[27] 4,4′-Dithiodibenzamide **24** and 4,4′-dithiodibenzensulfonamide **25** were obtained according to the procedure of Aiello and Pappalardo.[48] Sodium 4,4′-dithiodibenzensulfonate **22** and sodium 3,3′-dinitro-4,4′-dithiodibenzensulfonate **21** were respectively prepared as reported by Smith et al.[49] and Pollak and Deutscher.[28] 4-Carbamoylbenzensulfenyl chloride **27**, 4-sulfonamidobenzensulfenyl chloride **28**, and 4-nitrobenzensulfenyl chloride **29** were obtained from chlorinolysis of the corresponding symmetrical disulfides in anhydrous dichloroethane according to Behforouz and Kerwood.[50] The sulfenyl chlorides were directly used as crude products. All the other reagents were purchased commercially. Diethyl ether was distilled from lithium aluminum hydride, and dry dichloroethane and tetrahydrofuran (THF) were respectively obtained by distillation from calcium hydride and sodium.

Thioimidate Linkers Synthesis: Preparation of Intermediate Nitriles—*Acetylthioacetonitrile (11)*—Chloroacetonitrile was reacted with potassium thioacetate in diethyl ether to yield 75% of **11** according to the method of Bohme and Dick.[23] The product was purified by vacuum distillation (bp 95–96 °C at 14 mmHg); IR (liquid film): 2250 (CN), 1700 (C=0), and 1360 (CH_3) cm^{-1}; ^{1}H NMR (CCl_4): 3.63 (s, 2H, CH_2) and 2.4 (s, 3H, CH_3) ppm.

3-Acetylthiopropionitrile (12)—This compound was prepared in a manner analogous to the one described by Benary[51] for the preparation of acetylthioacetic acid. Distilled 3-mercaptopropionitrile (4.5 g, 52 mmol) was added in a dropwise manner to acetyl chloride (4.5 g, 58 mmol) at 0 °C under an Ar atmosphere. The reaction mixture was then refluxed for 1 h and was submitted to fractional distillation under reduced pressure to give **12** as a colorless oil in a yield of 3 g (45%), bp 195–200 °C at 15 mmHg; IR (liquid film): 2250 (CN), 1700 (C=0), and 1360 (CH_3) cm^{-1}; ^{1}H NMR (CCl_4): 3.15 (t, 2H, CH_2-S-C=0), 2.68 (t, 2H, CH_2-CN), and 2.4 (s, 3H, CH_3) ppm.

3-(2,4-Dinitrophenyldithio)propionitrile (16)—2,4-Dinitrophenyl disulfide **23** (4 g, 10 mmol) was dissolved in deaerated dimethylformamide (DMF, 200 mL) by heating and stirring. 3-Mercaptopropionitrile (0.437 g, 5 mmol) in 5 mL of DMF was then added in a dropwise manner to the solution at room temperature under an Ar atmosphere and the reaction mixture was stirred for 20 min. Then, 300 mL of water were added, and the resulting yellow precipitate was isolated by precipitation and washed with CH_2Cl_2 (3 × 25 mL). The aqueous filtrate was also extracted with CH_2Cl_2 (3 × 100 mL). The organic extracts were pooled, dried (Na_2SO_4), and evaporated under reduced pressure. The solid residue was purified by flash chromatography (CH_2Cl_2:petroleum ether, 50:50 and 60:40), giving 712 mg of **16** as light yellow needles in 50% yield; mp 103–105 °C; IR (KBr): 2260 (CN), 1600, 1530 (NO_2), 1350 (NO_2), 840 and 740 cm^{-1}; ^{1}H NMR ($CDCl_3$): 9.2 (s, 1H, Ar-H), 8.7 (s, 2H, Ar-H) and 3.05 (m, 4H, CH_2-CH_2) ppm.

5-(2-Cyanoethyldithio)-2-nitrobenzoic acid (13)—2,2′-Dinitro-5,5′-dithiodibenzoic acid **20** (3.96 g, 10 mmol) was dissolved in 80 mL of deaerated sodium phosphate buffer at pH 7.4 under an Ar atmosphere. Distilled 3-mercaptopropionitrile (435 mg, 5 mmol) was then added in a dropwise manner over a period of 30 min. After stirring for 30 min at room temperature, the reaction mixture was acidified to pH 3 with 2 N HCl and extracted with ethyl acetate (3 × 25 mL). The organic extracts were dried (Na_2SO_4) and concentrated under reduced pressure. Purification was obtained by flash chromatography (CH_2Cl_2:CH_3OH:CH_3COOH, 98:2:2) to give 568 mg of **13** as a viscous oil in 40% yield; IR (KBr): 3500–2800 (OH), 2250 (CN), 1660 (C=0), 1600, 1580, 1530 (NO_2), 1370 (NO_2), and 840 cm^{-1}; ^{1}H NMR (CD_3COCD_3): 10.3 (s, 1H, COOH), 7.9 (s, 3H, Ar-H), and 3.06 (m, 4H, CH_2CH_2) ppm.

Sodium 4-(2-cyanoethyldithio)-3-nitrobenzensulfonate (14)—3-Mercaptopropionitrile (261 mg, 3 mmol) was added in a dropwise manner to a solution of sodium 3,3′-dithio-4,4′-dithiodibenzensulfonate (3 g, 6 mmol) in deaerated sodium phosphate buffer at pH 7.4 under an Ar atmosphere. The reaction mixture was stirred for 30 min at room temperature. The solution was then evaporated to dryness

under reduced pressure, and the solid residue was purified by flash chromatography (CH_2Cl_2:CH_3OH, 90:10). The pure product **14** was obtained as yellow crystals in a yield of 410 mg (40%); IR (KBr): 2250 (CN), 1590, 1520 (NO_2), 1335 (NO_2), 1230, 1200 (SO_2), and 885 and 650 (S-O) cm^{-1}; ^1H NMR (CD_3OD): 8.9 (s, 1H, Ar-H), 8.6 (s, 2H, Ar-H), and 3.06 (m, 4H, CH_2CH_2) ppm.

Sodium 4-(2-cyanoethyldithio)benzensulfonate (15)—The title compound was prepared and purified in a manner analogous to that of **14** with the exception that the reaction was carried out in sodium borate buffer at pH 9. The pure product **15** was obtained as a white powder in a 40% yield; IR (KBr): 2250 (CN), 1580, 1230, 1180 (SO_2), 1050 (SO_2), 820, 760, and 660 (S-O) cm^{-1}; ^1H NMR (DMSO-d_6): 7.7 (d, 4H, Ar-H) and 3.0 (m, 4H, CH_2CH_2) ppm.

3-(4-Nitrophenyldithio)propionitrile (19)—4-Nitrobenzensulfenyl chloride (3 g, 16 mmol) was suspended in 60-mL of glacial acetic acid and heated to reflux. Distilled 3-mercaptopropionitrile (1.4 g, 16 mmol) in 3 mL of glacial acetic acid was then added to the resulting solution. The reaction mixture was stirred at 80–90 °C for 1.5 h, and, after cooling to room temperature, the precipitate formed was removed by filtration. Iced water (100 mL) was then added to the filtrate, and the resulting precipitate was collected and washed with water. The crude product was purified by flash chromatography (light petroleum:CH_2Cl_2, 50:50) to give light crystals of **19** in a yield of 80%; mp 83–84 °C; IR (KBr): 2250 (CN), 1600, 1585, 1510 (NO_2), 1340 (NO_2), 850, 840, and 740 cm^{-1}; ^1H NMR ($CDCl_3$): 8.2 (d, 2H, Ar-H), 7.7 (d, 2H, Ar), and 2.9 (m, 4H, CH_2CH_2) ppm.

4-(2-Cyanoethyldithio)benzensulfonamide (18)—4-Sulfonamidobenzensulfenyl chloride (3 g, 12 mmol) was dissolved in hot glacial acetic acid (70 mL) and distilled 3-mercaptopropionitrile (1.04 g, 12 mmol) in 3 mL of glacial acetic acid was added. The reaction mixture was stirred at 80–90 °C for 2 h, and, after cooling to room temperature, the resulting precipitate was eliminated by filtration and the filtrate was diluted with water. The aqueous solution was extracted with ethyl acetate (3 × 50 mL), and the organic extracts were dried (Na_2SO_4) and evaporated under reduced pressure. The solid residue was purified by flash chromatography (CH_2Cl_2:CH_3OH, 99:1) to give **18** as colorless crystals in a yield of 1.5 g (46%); mp 75 °C; IR (KBr): 3350 (NH), 3250 (NH), 2250 (CN), 1580, 1330 (SO_2), 1160 (SO_2), and 810 cm^{-1}; ^1H NMR (CD_3OD): 7.9 (d, 4H, Ar-H) and 3.03 (m, 4H, CH_2CH_2) ppm.

4-(2-Cyanoethyldithio)benzamide (17)—The same procedure followed for the preparation of **18** was used. The crude product was purified by flash chromatography (diethyl ether:ethyl acetate, 99.5:0.5). White light crystals of **17** were obtained in a yield of 40%; mp 135 °C; IR (KBr): 3330 (NH), 3100 (NH), 2240 (CN), 1660 (C=0), 1610 (NH), 1580, 1400, and 840 cm^{-1}; ^1H NMR (CD_3COCD_3): 8.2 (d, 2H, Ar-H), 7.7 (d, 2H, Ar-H), and 3.1 (m, 4H, CH_2CH_2) ppm.

3-(2-Pyridyldithio)propionitrile (30)—The title compound was obtained from 2,2′-dithiopyridine and 3-mercaptopropionitrile in deaerated methanol according to the procedure of King et al.[18] for the preparation of 3-(4-pyridyldithio)propionitrile. The pure product was obtained from a silica gel column by flash chromatography (light petroleum:ethyl acetate, 90:10). Compound **30** was obtained as a colorless oil in a yield of 40%; IR (liquid film): 2250 (CN) cm^{-1}; ^1H NMR ($CDCl_3$): 8.4 (d, 1H, Py-H), 7.6 (d, 2H, Py-H), 7.1 (m, 1H, Py-H), and 2.93 (m, 4H, CH_2CH_2) ppm.

General Procedure for Thioimidate Ester Hydrochlorides Preparation—The thioimidate ester hydrochlorides **4–10** were obtained from the corresponding nitriles **11–13** and **16–19** according to the Pinner synthesis.[22] Hydrogen chloride gas (1.38 g, 38.3 mmol), dried by passing through concentrated sulfuric acid in two washing bottles, was bubbled through ice-cold ethanthiol (3.25 mL, 43.5 mmol). The purified nitrile (8.7 mmol) was quickly added to the cold solution under stirring, and the flask was tightly stoppered and left overnight at 0 °C. The solid nitriles **13**, **16**, and **19** were dissolved in dry CH_2Cl_2 prior to being added to iced ethanthiol, whereas the nitriles **17** and **18** were previously dissolved in dry THF. Anhydrous cold diethyl ether (7 mL) was then added to the reaction mixture, and the flask was left at 0 °C until a white crystalline precipitate was formed. The supernatant was decanted, and the precipitate was washed three times with dry cold diethyl ether under an Ar atmosphere under reduced pressure at room temperature.

Ethyl S-acetyl 2-mercaptoacetothioimidate Ester Hydrochloride (AMAT, 4)—Yield 90%; mp 115–116 °C; IR (KBr): 3300–2300 (NH), 1700 (C=0), 1620 (CN), and 1360 (CH_3C=0) cm^{-1}; ^1H NMR ($CDCl_3$): 4.4 (s, 2H, -CH_2-S-C=0), 3.5 (q, 2H, -S-$\overline{CH_2}$-CH_3), 2.43 (s, 3H, CH_3-C=0), and 1.43 (t, 3H, -S-CH_2-$\overline{CH_3}$) ppm.

Anal.—Calc. for $C_6H_{12}ClNOS_2$: C, 33.71%; H, 5.66%; N, 6.55%; 0, 7.48%; S, 30.00%; Cl, 16.59%. Found: C, 34.16%; H, 5.71%; N, 6.49%; 0, 7.38%; S, 29.89%; Cl, 16.82%.

Ethyl S-acetyl 3-mercaptopropionthioimidate Ester Hydrochloride (AMPT, 5)—Yield 88%; mp 64–66 °C; IR (KBr): 3300–2400 (NH), 1690 (C=0), 1620 (CN), and 1360 (CH_3C=0) cm^{-1}; ^1H NMR ($CDCl_3$): 3.48 (q, 2H, -S-$\overline{CH_2}$-CH_3), 3.26 (m, 4H, -CH_2CH_2-), 2.38 (s, 3H, CH_3C=0), and 1.45 (t, 3H, -S-$CH_2\overline{CH_3}$) ppm.

Anal.—Calc. for $C_7H_{14}ClNOS_2$: C, 36.91%; H, 6.19%; N, 6.15%; O, 7.02%; S, 28.15%; Cl, 15.56%. Found: C, 36.79%; H, 6.11%; N, 6.42%; 0, 7.35%; S, 28.91%; Cl, 15.81%.

Ethyl 3-(2,4-dinitrophenyldithio)propionthioimidate Ester Hydrochloride (7)—Yield 80%; mp 120–125 °C; IR (KBr): 3100–2500 (NH), 1620 (CN), 1590, 1530 (NO_2), 1350 (NO_2), 840, and 740 cm^{-1}; Mass (FAB+): 348 (M^+ + 1).

Anal.—Calc. for $C_{11}H_{14}ClN_3O_4S_3$: C, 34.41%; H, 3.68%; N, 10.95%; O, 16.67%; S, 25.06%; Cl, 9.23%. Found: C, 34.40%; H, 3.60%; N, 10.88%; O, 16.01%; S, 24.67%; Cl, 10.56%.

Ethyl 3-(3-carboxy-4-nitrophenyldithio)propionthioimidate Ester Hydrochloride (8)—Yield 80%; mp 117–120 °C; IR (KBr): 3300–2500 (NH and OH), 1730 (C=0), 1610 (CN), 1570, 1530 (NO_2), 1370 (NO_2), 870, and 830 cm^{-1}; ^1H NMR ((CD_3)$_2$SO): 7.8 (s, 1H, Ar-H), 8.1 (d, 2H, Ar-H), 3.7 (t, 2H, -S-S-$\overline{CH_2}$-CH_2-), 3.42 (q, 2H, -S-$\overline{CH_2}$-CH_3), 3.3 (t, 2H, -S-S-CH_2-$\overline{CH_2}$-), and 1.4 (t, 3H, -S-CH_2-$\overline{CH_3}$) ppm; Mass (FAB+): 347 (M^+ + 1).

Anal.—Calc. for $C_{12}H_{15}ClN_2O_4S_3$: C, 37.64%; H, 3.95%; N, 7.32%; O, 16.71%; S, 25.12%; Cl, 9.26%. Found: C, 37.17%; H, 3.99%; N, 7.36%; O, 16.32%; S, 25.14%; Cl, 10.19%.

Ethyl 3-(4-nitrophenyldithio)propionthioimidate Ester Hydrochloride (6)—Yield 83%; mp 113–116 °C; IR (KBr): 3200–2500 (NH), 1600, 1630 (CN), 1580, 1510 (NO_2), 1340 (NO_2), 850, 840, and 740 cm^{-1}; ^1H NMR ((CD_3)$_2$SO): 8.25 (d, 2H, Ar-H), 7.7 (d, 2H, Ar-H), 3.7 (t, 2H, -S-S-$\overline{CH_2}$-CH_2-), 3.4 (q, 2H, -S-$\overline{CH_2}$-CH_3), 3.3 (t, 2H, -S-S-CH_2-$\overline{CH_2}$-), and 1.39 (t, 3H, -S-CH_2-$\overline{CH_3}$) ppm; Mass (FAB+): 303 (M^+ + 1).

Anal.—Calc. for $C_{11}H_{15}ClN_2O_2S_3$: C, 38.98%; H, 4.46%; N, 8.26%; O, 9.44%; S, 28.38%; Cl, 10.46%. Found: C, 38.85%; H, 4.32%; N, 8.12%; 0, 9.25%; S, 28.12%; Cl, 10.05%.

Ethyl 3-(4-sulfonamidophenyldithio)propionthioimidate Ester Hydrochloride (10)—Yield 70%; mp 135–165 °C; IR (KBr): 3500–2200 (sulfonamidic NH_2 and imidic NH), 1620 (CN), 1580, 1330 (SO_2), 1160 (SO_2), and 810 cm^{-1}; ^1H NMR (CD_3OD): 7.87 (q, 4H), 3.4–3.1 (m, 6H), 1.4 (t, 3H) ppm.

Anal.—Calc. for $C_{11}H_{17}ClN_2O_2S_4$: C, 35.42%; H, 4.59%; N, 7.85%; O, 8.67%; S, 34.52%; Cl, 10.15%. Found: C, 35.38%; H, 4.66%; N, 7.51%; 0, 8.58%; S, 34.39%; Cl, 9.80%.

Ethyl 3-(4-carboxamidophenyldithio)propionthioimidate Ester Hydrochloride (CDPT, 9)—Yield 65%; mp 110–115 °C; IR (KBr): 3500–2200 (-NH_2 and =NH_2^+), 1650 (C=0), 1620 (CN), 1590, and 840 cm^{-1}; ^1H NMR (CD_3OD): 7.85 (q, 4H), 3.4–3.15 (m, 6H), 1.47 (t, 3H) ppm; Mass (FAB+): 301 (M^+ + 1).

Determination of Aryldithio Group Reactivity—Stock solution (0.25 mM) of the synthesized aryldithiopropionitriles **13–19** were prepared in EtOH (95%) and diluted to 0.028 M with a sodium phosphate buffer (0.0056 M Na_2HPO_4, 0.014 M $NaH_2PO_4 \cdot H_2O$, 0.2 M NaCl, 0.03 M EDTA bisodic salt, pH 6.5) that was well deaerated and flushed with Ar. To 900 μL of each freshly prepared aqueous disulfide solution in a photometer cell were quickly added 100 μL of different concentrations of freshly prepared solutions of cysteine chloride monohydrate (0.5 and 1 mM) in the same buffer. For each aryldithiopropionitrile, the progress of the thiol–disulfide exchange reaction was spectrophotometrically monitored by registering the curve absorbance–time relative to the displacement of the substituted thiophenol (which is in balance with its ionized form) in the presence of double and fourth molar excess of cysteine.

Reaction of Bovine IgG with the Thioimidate Ester Ligands AMAT (4), AMPT (5), and CDPT (9): General Procedure—The buffers used were sodium borate (100 mM, pH 9.05), sodium phosphate (100 mM, pH 7.04 and pH 6.05), and sodium acetate (100 mM, pH 5.10) (all buffers contained 100 mM NaCl and 1 mM EDTA and all were deaerated and flushed with Ar). Bovine IgG (Sigma) was dissolved in the buffer to concentrations of 40 and 60 μM ($E^{1\%}_{1\ cm}$ at 280 nm was 14.0) and mixed at 20 °C with different molar excesses of the thioimidate reagent previously dissolved in absolute ethanol or anhydrous DMF. Samples were drawn from the stirred reaction mixture at different time intervals, and the excess of ligand was

separated by gel filtration onto a Bio-Gel P6DG column pre-equilibrated in PBS-EDTA (100 mM, pH 7.4, 100 mM NaCl, 1 mM EDTA) at 20 °C.

Determination of Ligand:IgG Molar Substitution Ratio—The aryldithio groups linked to the IgG were spectrophotometrically evaluated by incubating protein samples (1 mL) with 2-mercaptoethanol in PBS-EDTA (50 μL, 11 mM) and NaOH (40 μL, 1.0 mM) to a final pH value of 8.8–9.4. After 20 min of incubation at room temperature, the absorbance of the thiolate anion released was measured at 313 nm. The molar absorbtivity value for the 4-carboxamidophenylthiolate anion was 15200 ± 300 at 313 nm under these conditions.

The thioacetylated groups linked to the IgG were calculated with the deacetylating reactive hydroxylamine according to a procedure described elsewhere.[20]

Appendix

Determination of Aryldithio Group Reactivity and Calculation of Kinetic Constants of Thiol–Disulfide Exchange—The kinetic constants were calculated from the UV registered curve absorbance–time relative to the displacement of the substituted thiophenol with the following second-order equation (see *Experimental Section* for details):

$$dx/dt = k(a - x)(b - x); k = \frac{1}{t(b - a)} \cdot \ln\frac{(b - x)a}{(a - x)b} \quad \text{(A1)}$$

In eq A1, a is the aryldithiopropionitrile initial molar concentration, b is the cysteine initial molar concentration, and x is the thiophenol molar concentration at time t (s). The value of x is calculated by eq A2:

$$dx/dt = k(A - A_o/A_{inf} - A_o)a \quad \text{(A2)}$$

In eq A2, A is the absorbance at time t (s), A_o is the initial absorbance of aryldithiopropionitrile, and A_{inf} is the final absorbance at 100% of reaction with cysteine.

For each couple A/t relative to the registered kinetic curves, the values of x and the kinetic constant k ($L \cdot mol^{-1} \cdot s^{-1}$) were calculated.

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