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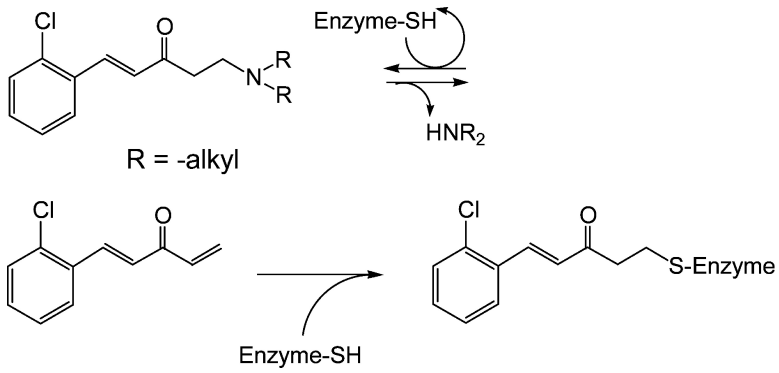

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Irreversible Inactivation of Trypanothione Reductase by Unsaturated Mannich Bases: A Divinyl Ketone as Key Intermediate

Brittany Lee,[†] Holger Bauer,[†,§] Johannes Melchers,[†,#] Thomas Ruppert,[#] Lauren Rattray,[‡] Vanessa Yardley,[‡] Elisabeth Davioud-Charvet,[*,†,§] and R. Luise Krauth-Siegel[*,†]

Biochemie-Zentrum der Universität Heidelberg, Im Neuenheimer Feld 504, 69120 Heidelberg, Germany, Zentrum für Molekulare Biologie der Universität Heidelberg, Im Neuenheimer Feld 282, 69120 Heidelberg, Germany, UMR 8525 CNRS-Université Lille II-Institut Pasteur de Lille, Institut de Biologie de Lille, 1 Rue du Professor Calmette, BP447 59021 Lille Cedex, France, and Department of Infections and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, U.K.

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Trypanothione reductase is a flavoenzyme unique to trypanosomatid parasites. Here we show that unsaturated Mannich bases irreversibly inactivate trypanothione reductase from *Trypanosoma cruzi,* the causative agent of Chagas' disease. The inhibitory potency of the compounds strongly increased upon storage of the DMSO stock solutions. HPLC, NMR, and mass spectrometry data of potential intermediates revealed a divinyl ketone as the active compound inactivating the enzyme. ESI- and MALDI-TOF mass spectrometry of trypanothione reductase modified by the Mannich base or the divinyl ketone showed specific alkylation of the active site Cys52 by a 5-(2′chlorophenyl)-3-oxo-4-pentenyl substituent. The reaction mechanism and the site of alkylation differ from those in *Plasmodium falciparum* thioredoxin reductase where the C-terminal redox active dithiol is modified. After deamination, unsaturated Mannich bases are highly reactive in polycondensation with trypanothione. Interaction of these compounds with both trypanothione and trypanothione reductase could account for their potent trypanocidal effect against *Trypanosoma brucei*.

Introduction

Trypanosomes and leishmanias are parasitic protozoa causing African sleeping sickness (*Trypanosoma brucei gambiense* and *Trypanosoma b. rhodesiense*), Chagas' disease (*Trypanosoma cruzi*), Nagana cattle disease (*Trypanosoma congolense* and *Trypanosoma b. brucei*), Espundia (*Leishmania brasiliensis*), Kala-azar (*L. donovani*), and Oriental sore (*L. tropica*). All of these parasites have a unique thiol metabolism based on the flavoenzyme trypanothione reductase (TR)[1], which maintains bis(glutathionyl)spermidine [trypanothione, $T(SH)_2$] and monoglutathionylspermidine in the reduced state:[2]

$$TS_2 + NADPH + H^+ \xrightarrow{TR} T(SH)_2 + NADP^+ \quad (1)$$

This trypanothione system replaces the glutathione/glutathione reductase (GR) couple and probably also the thioredoxin/thioredoxin reductase system occurring in the mammalian hosts:

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2\,GSH + NADP^+ \quad (2)$$

Although TR and GR are structurally and mechanistically closely related enzymes, they show mutually exclusive specificities toward their disulfide substrates.[3,4] TR has a negatively charged disulfide substrate binding site and binds cationic glutathionylspermidine conjugates.[5,6] In contrast, the active site of GR is positively charged and is specific for glutathione disulfide (GSSG) with an overall charge of −2. These differences in the charge distribution between TR and GR have been widely used to design compounds that govern the specific binding to the parasitic enzyme.[7] Both flavoenzymes have three residues that are directly involved in catalysis, the redox active dithiol/disulfide (Cys52−Cys57 in *T. cruzi* TR) and a His residue (His460′ in *T. cruzi* TR), which is provided by the second subunit of the homodimer.[8] The trypanothione disulfide binding site of TR is more hydrophobic and much wider than the GSSG binding site of GR and allows the accommodation of extremely large ligands. Several genetic studies have demonstrated that TR is essential for trypanosomes. Bloodstream *T. brucei* parasites with less than 10% of wild-type TR activity are more sensitive to oxidative stress and are unable to grow.[9] The absence of TR in the mammalian host, the structural differences to related host enzymes, and the essential role for parasite survival render TR a promising target for a rational drug design against African sleeping sickness, Chagas' disease, and the different forms of leishmaniasis.[10−15]

The structures of TR in complex with quinacrine, a competitive inhibitor, and of TR irreversibly inactivated by quinacrine mustard are the only three-dimensional structures of TR−ligand complexes solved so far.[16,17] In the latter structure, one quinacrine moiety is covalently bound to Cys52. Since more than 90% inactivation of

* To whom correspondence should be addressed. For E.D.-C.: phone, (49) 6221 54 41 88; fax, (49) 6221 54 55 86; e-mail: elisabeth.davioud@gmx.de. For R.L.K.-S.: phone, (49) 6221 54 41 87. fax, (49) 6221 54 55 86; e-mail: krauth-siegel@urz.uni.heidelberg.de.

[†] Biochemie-Zentrum der Universität Heidelberg.

[§] Delegate (E.D.C.) and Fellow (H.B.) of Centre National Recherche Scientifique, UMR 8525 CNRS-Université Lille II, France, in the frame of a German−French cooperation with Heidelberg University, Germany.

[#] Zentrum für Molekulare Biologie der Universität Heidelberg.

[‡] London School of Hygiene and Tropical Medicine.

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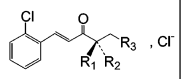
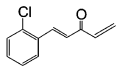
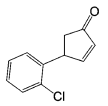
Table 1. Unsaturated Mannich Bases and Putative Reaction Intermediates Examined as Irreversible Inhibitors of *Trypanosoma cruzi* Trypanothione Reductase

structure	compnd	R_1	R_2	R_3	irreversible inhibition	$t_{1/2}$[a]
	**1**	H	H	$^+NH(Me)_2$	yes	8.6 min
	2	H	H		yes	7.5 min
	3	H	H		yes	2 min
	4	Me	Me	$^+NH(Me)_2$	no	-
	**5**				yes	< 45 s
	**6**				no	-

[a] The half time ($t_{1/2}$) of inactivation was determined at 100 μM inhibitor taken from a freshly prepared 4 mM stock solution in DMSO.

TR is needed to inhibit growth and to abolish the infectivity of trypanosomes in vitro,[9] purely competitive inhibitors must have nanomolar inhibitor constants or show slow binding behavior to be potential drug candidates.[18] Therefore, irreversible inhibitors might be more promising because substrate accumulation cannot overcome inhibition of the pathway. The first irreversible inhibitors shown to inactivate TR but not human GR were (2,2′:6′,2″-terpyridine)platinum(II) complexes,[19] which also proved to be cytotoxic in cultures of *T. cruzi, T. brucei*, and *L. donovani*.[20] Like the quinacrine mustard, these complexes probably bind to Cys52 in the active site.

The Mannich reaction is an important tool for C−C bond formation in organic chemistry and is widely used for the preparation of β-aminoketones and aldehydes. Mannich bases are versatile intermediates for the synthesis of Michael acceptors (via elimination of the amine moiety) and functionalized carbonyl compounds (via substitution of the amine moiety by nucleophiles).[21,22] There are numerous applications ranging from the synthesis of natural and pharmaceutical products to polymer chemistry.[23] Different Mannich bases are drugs or drug candidates, but few studies have aimed at the identification of the molecular targets and their mechanism of action. From a screening of 350 000 compounds of the Pfizer library, the unsaturated Mannich bases **1**−**3** (Table 1) were identified to be efficient mechanism-based inhibitors of *Plasmodium falciparum* thioredoxin reductase.[24] The fact that unsaturated Mannich bases readily react with dithiols prompted us to study these compounds for their ability to interact with trypanothione reductase as well as free trypanothione. The kinetic analysis revealed that stored solutions of the Mannich bases inactivated the enzyme to a greater extent and at a much faster rate than fresh solutions. Here, we report on kinetic and spectroscopic studies and HPLC, NMR, and mass spectrometric analyses of TR inactivated by unsaturated Mannich bases. We show that the divinyl ketone intermediate **5** is responsible for the irreversible inactivation of *T. cruzi* TR.

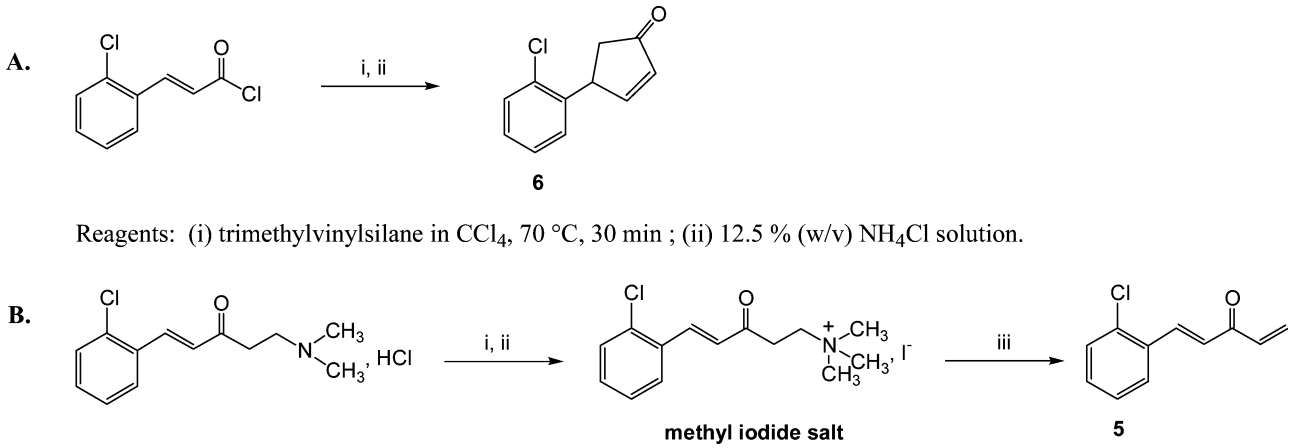
Results

Chemistry. The unsaturated Mannich bases **1**−**4** (Table 1) were prepared as described previously.[24] The cyclopentenone **6** was synthesized via the route of Kjeldsen et al. (Scheme 1A).[25] The mechanism is a Friedel−Crafts-like acylation using 2-chlorocinnamoyl chloride and trimethylvinyl silane in the presence of $AlCl_3$. The cyclopentenone **6** was obtained in 42% yield when the reaction was conducted at 70 °C. The reaction involves formation of the divinyl ketone **5**, which was described to be isolatable if the reaction is conducted at −10 °C. However, we were not able to separate the compound from the multiple reaction products. Thus, we prepared the divinyl ketone **5** in an overall yield of 67% by methylation of the unsaturated Mannich base **1** to the corresponding quaternary ammonium salt and subsequent thermal elimination (Scheme 1B).

Reduced *T. cruzi* TR Is Irreversibly Inactivated by Unsaturated Mannich Bases. The three unsaturated Mannich bases **1**−**3** (Table 1) were studied as inhibitors of *T. cruzi* TR. The enzyme was incubated with the compounds in the absence and presence of NADPH. After different time intervals, the remaining activity was determined in a standard assay. Incubation of 0.5 μM TR with 100 μM compound **1** in the presence of NADPH resulted in a time-dependent complete inactivation of the enzyme. The inhibitor concentration was then incrementally lowered. Compound **1** at 5 μM inhibited reduced TR to 30% within less than 5 min, but further inactivation was minimal at longer incubation times (Figure 1). No inactivation was observed in the absence of NADPH, indicating that prereduction of the enzyme was essential for inactivation. When these experiments were repeated using the DMSO stock solution of **1** that was kept for 2 days at 4 °C, inactivation of *T. cruzi* occurred at a faster rate and was much more complete (Figure 1). Compound **1** at 5 μM now inhibited TR within 5 min to 70%. Respective experiments with **2** and **3** showed that these compounds also act as irreversible inhibitors of reduced *T. cruzi* TR. Again, inactivation of the enzyme improved when stored stock solutions were used (data not shown).

At a concentration of 100 μM, fresh samples of **1**−**3** inactivated reduced TR with half times ($t_{1/2}$) of 8.6, 7.5, and 2 min, respectively (Table 1). In the proposed mechanism for the reaction of unsaturated Mannich bases with thiols,[24] both the elimination and addition reactions are pH-dependent, the optimum pH being linked to the p*K* value of the amine leaving group. The observed inactivation rates correlate with the predicted deamination rates for the R_3 group. Compound **3** has the best leaving group with a p*K* value of 8.8 and the shortest $t_{1/2}$, while compounds **2** and **1** have slower leaving groups (p*K* values 11.2 and 10.8, respectively) and longer half times for inactivation. The fourth unsaturated Mannich base **4** contained methyl groups as R_1 and R_2 substituents at the α-carbon, which prevent a base-dependent deamination. In accordance with the proposed reaction mechanism (Scheme 2), compound **4** did not inactivate TR even at a concentration of 200 μM.

Scheme 1. Synthesis of the Divinyl Ketone **5** and the Cyclopentenone **6**



To confirm the irreversible modification of the enzyme by the Mannich bases, TR was >99% inactivated by treatment with fresh or stored solutions of compound **1**. The low molecular mass reaction components were removed by repeated washing and centrifugation through Microcon YM-30 concentrators. The resulting enzyme solution did not show any activity even when treated with 2.6 mM DTE for 1 h.

Unsaturated Mannich Bases Are Converted into a Divinyl Ketone. HPLC analysis of a fresh solution of **1** showed a single peak with a retention time of 18.6 min. The peak gradually decreased with time, and a new peak appeared at 24.4 min that corresponded to the deamination product **5** [1-(2′-chlorophenyl)penta-1,4-dien-3-one or 1-(2′-chlorophenyl) divinyl ketone] (Table 1). Formation of the divinyl ketone was confirmed by ^{1}H NMR. A 4-day-old solution of compound **1** contained 14% of **5**. The spectrum showed the typical vinylic proton signals at 5.88, 6.35, and 6.74 ppm, identical to

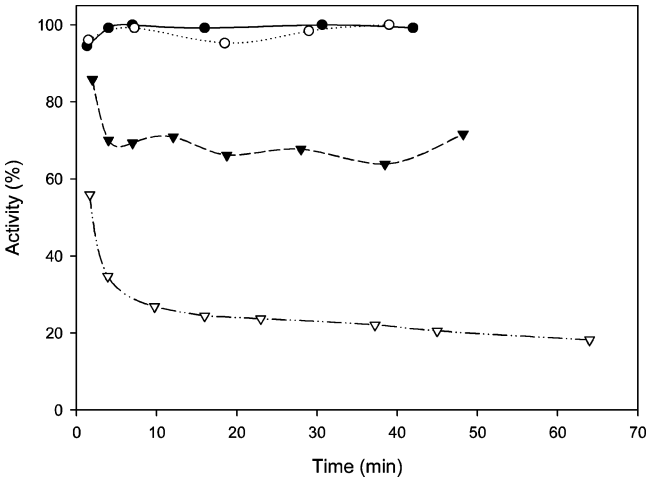
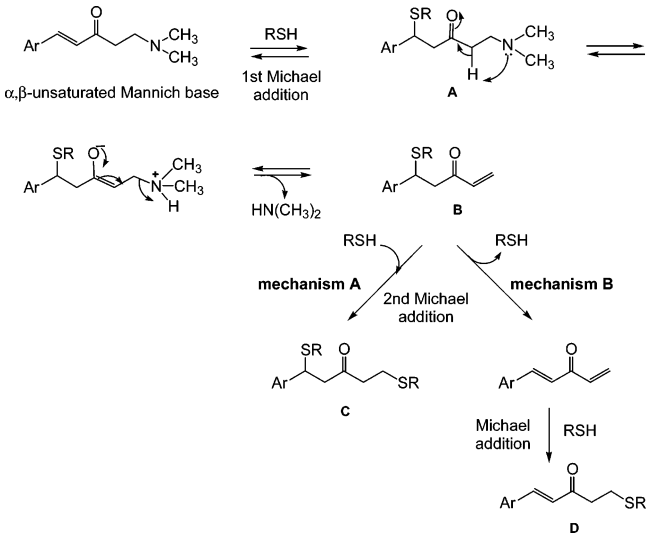


Figure 1. Inactivation of *T. cruzi* trypanothione reductase by the unsaturated Mannich base **1**. TR at 0.5 μM was incubated with 5 μM compound **1** and 200 μM NADPH at 25 °C for different times, 5 μL aliquots were removed, and the remaining activity was measured in a standard TR assay as described under Experimental Section. Compound **1** was taken from a fresh (▼) or a 2-day-old (▽) stock solution. Controls contained the enzyme and NADPH (●) and the enzyme and inhibitor (○). TR activity is given as the percentage of the activity measured for the controls at time zero.

Scheme 2. Proposed Reaction Mechanisms for the Modification of Protein Thiols (RSH) by an Unsaturated Mannich Base Such as **1** (Ar = 2-Chlorophenyl) (A) in the Case of Two Vicinal Reactive Protein Thiols, e.g., Cys535 and Cys540 in *P. falciparum* Thioredoxin Reductase (TrxR)[24] and (B) in the Case of a Single Reactive Thiol, e.g., Cys52 in *T. cruzi* Trypanothione Reductase (TR)



the synthesized divinyl ketone **5**. ESI-MS of a fresh sample of the unsaturated Mannich base **1** revealed a single mass peak at 238 *m/z* corresponding to the theoretical mass of the compound. Upon storage in DMSO, three new peaks with *m/z* values of 157, 192, and 430 appeared that represent the dechlorinated divinyl ketone, the divinyl ketone, and an artificial adduct between compounds **1** and **5**, respectively (data not shown).

The absorption spectra of fresh compound **1** and the divinyl ketone **5** show maxima at 287 and 305 nm, respectively. After 20 days, λ_{max} of compound **1** had shifted to 296 nm (Figure 2A). In comparison, the λ_{max} of pure divinyl ketone **5** remained constant (Figure 2B). The overall slow absorption decrease reflects degradation of the divinyl ketone. The spectra of compounds **2** and **3** showed a comparable shift of λ_{max} with time (not shown). In contrast, the spectrum of **4**, which does not contain acidic hydrogens in positions R_1 and R_2, was

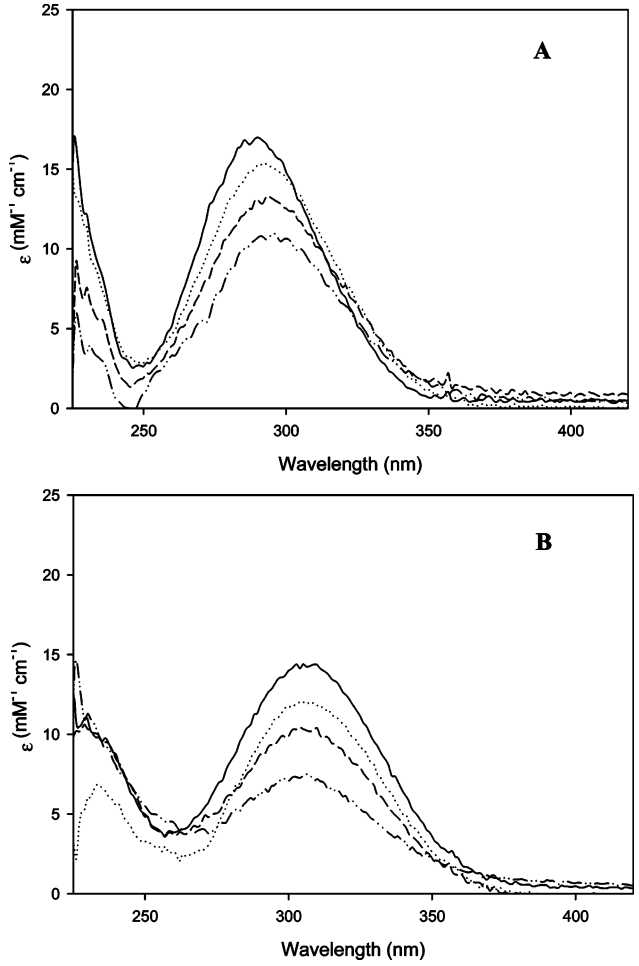


Figure 2. Changes of the absorption spectra of the unsaturated Mannich base **1** and divinyl ketone **5** upon storage in DMSO. The 10 mM stock solutions of (A) compound **1** and (B) the divinyl ketone **5** were prepared in DMSO and kept at 25 °C. After different times, 1 μL aliquots were removed and diluted in 1 mL of water. The spectra show a fresh (—) dilution and dilutions prepared after 24 h (···), 6 days (- - -), and 20 days (—··) of storage.

stable. Formation of the cyclopentenone **6**, another putative degradation product of compound **1**, was ruled out by the different spectroscopic studies.

Reaction of α,β-Unsaturated Ketones with Glutathione. Because **5** proved to be a potent irreversible inhibitor of *T. cruzi* TR (see below), we investigated the reactivity of the divinyl ketone and related α,β-unsaturated compounds with glutathione (GSH). When equal concentrations of 0.1 mM **5** and GSH were allowed to react at neutral pH, the yellow color of the divinyl ketone faded within less than a minute. HPLC analysis after 30 min revealed a single new peak ($t_R \approx 19.1$ min) that represents the glutathione monoadduct. ^{1}H NMR clearly showed the loss of two vinylic protons from the terminal double bond. At neutral pH, a second molecule of GSH does not readily add to the remaining conjugated double bond. However, incubation of **5** with 2 equiv of GSH at pH ~9.5 and at 60 °C for 3 h resulted in a mixture of the monoadduct and the diastereomeric forms of the bis-adduct, which eluted after 16.4 and 16.5 min from the HPLC column.

The 4-(2′-chlorophenyl)-2-cyclopentenone **6**, which may be formed from **5**,[25−27] reacts with GSH at neutral pH much more slowly than the divinyl ketone. After 1 h of incubation of 0.1 mM **6** and 0.1 mM GSH at 60 °C and pH 7, about 50% of the cyclopentenone had disappeared, yielding the two diastereomeric monoadducts that eluted from the HPLC column after 18.3 and 18.5 min. Even at pH 9.5 we did not observe significantly more addition products. The reactivity of **6** is rather low toward the nucleophilic attack by thiols as previously observed for other simple α,β-unsaturated ketones.[28] A conjugation-stabilized double bond such as that in 2-chlorobenzylidene acetone, an intermediate in the synthesis of **1**, was almost unreactive with GSH (data not shown). In contrast, addition of a thiol to the conjugated double bond of an unsaturated Mannich base like **1** or, even more pronounced, to the terminal double bond of divinyl ketone is favored because of the electron-withdrawing group opposite the conjugated double bond.[29]

Reaction of the Unsaturated Mannich Base 1 with Trypanothione. Reaction of equimolar concentrations (0.1 mM) of **1** with trypanothione at physiological pH yielded a product that eluted from the HPLC column after 20.5 min. Most probably, the compound is the monoadduct as it is observed in the reaction with GSH. After overnight incubation at 25 °C, compound **1** and the monoadduct were no longer detectable, but a pattern of polymeric products appeared in the HPLC chromatogram. On the basis of mass spectrometry, we conclude that these products represent oligomers and polymers made up of trypanothione and deaminated compound **1**. As shown before, reaction between equimolar concentrations of the Mannich base and glutathione under the same conditions resulted in the formation of 8.5% bis-adduct, 37.5% monoadduct, and 30.1% free compound **1**.[24] These data clearly show that trypanothione reacts much more quickly with **1** than does glutathione and, in contrast to the monothiol, leads to the formation of polymeric conjugates, as described for the reaction of other dithiols with unsaturated Mannich bases.[23]

The Divinyl Ketone 5 Is a More Potent Inhibitor of TR Than the Parent Unsaturated Mannich Bases. Based on the serendipitous finding that inactivation of TR improved when the Mannich bases were stored in DMSO, two possible degradation intermediates, the divinyl ketone **5** and the cyclopentenone **6**, were synthesized and studied for their ability to inactivate TR. A 100 μM solution of **6** did not cause any inhibition of reduced TR, while 5 μM **5** completely inhibited reduced TR (Figure 3).

This observation and the UV, NMR, and ESI-MS data strongly support the interpretation that the divinyl ketone is the active intermediate responsible for the irreversible inactivation of *T. cruzi* TR by the Mannich bases studied here. The proposed reaction mechanism starts with the reversible addition of a protein thiol (Cys52) to the double bond of the α,β-unsaturated ketone **1** as it has been suggested for the inactivation of thioredoxin reductase by unsaturated Mannich bases.[24] Because Cys57 does not react as second thiol, the subsequent deamination is followed by the (protein) base-catalyzed generation of the divinyl ketone. Finally, the nucleophilic attack of Cys52 at the terminal double bond of the divinyl ketone leads to the irreversible inhibition of the enzyme (Scheme 2, mechanism B).

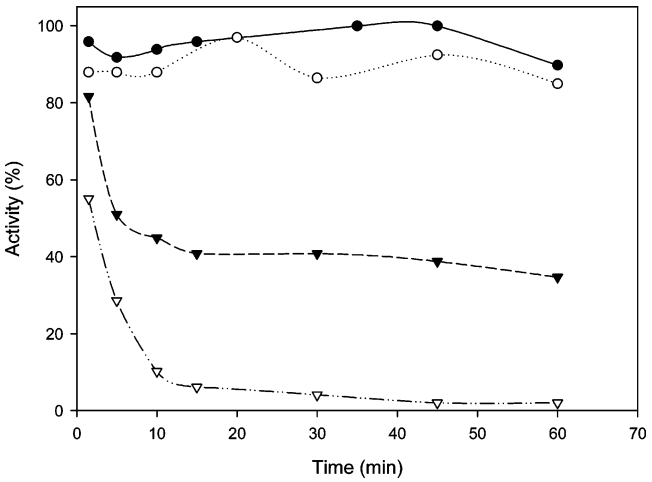


Figure 3. Inactivation of *T. cruzi* trypanothione reductase by **5**. Four reaction mixtures in TR assay buffer were incubated at 25 °C. The two experimental samples contained 0.5 μM TR, 200 μM NADPH, and 2.5 μM (▼) or 5 μM (▽) **5**. The control reactions contained (●) the enzyme and NADPH and (○) the enzyme and inhibitor but no NADPH. At different times, 5 μL aliquots were removed and the remaining activity was determined in a standard TR assay. The activity is expressed as a percentage of the maximum activity of the controls.

Mass Spectrometric Analyses of TR Inactivated by the Unsaturated Mannich Base 1 and the Divinyl Ketone 5. TR inactivated by **1** or **5** was subjected to mass spectrometry. ESI-MS analysis of TR reacted with **5** yielded a protein species of 53 807 Da. The mass shift of 192 Da when compared with the unmodified protein corresponds to one 5-(2′chlorophenyl)-3-oxo-4-pentenyl group introduced in the protein (Figure 4). The small peak at 54 004 Da probably reflects additional modification of Cys468 (see below). When TR was inactivated by a fresh sample of **1**, the ESI spectra showed two main peaks at 53 664 and 53 854 Da (not shown). Both masses were 48 Da higher than the masses of free TR and the protein treated with **5** (Figure 4). The reason for this is not clear, but again, the mass difference between the two peaks was 190 Da, in agreement with an identical modification of TR by **1** and **5**.

To identify the site of modification, TR inactivated by **1** or **5** was treated with iodoacetamide to alkylate the remaining free cysteine residues and digested by trypsin, and the peptides were analyzed by MALDI-TOF mass spectrometry. Trypanothione reductase possesses seven cysteine residues at positions 52, 57, 175, 220, 374, 443, and 468. Cys52 and Cys57 form the redox active dithiol/disulfide and occur in a single tryptic peptide that is detected in its reduced form as a protonated ion with a mass of 3041 Da (Figure 5A). Treatment of reduced TR with 10 μM **5** and subsequent alkylation of the remaining free cysteine residues caused a mass increase of this peptide by 249 Da, which corresponds to the addition of a molecule **5** (192 Da) to one cysteine and an acetamide group (57 Da) to the second cysteine (Figure 5B). A corresponding result was obtained when the enzyme was treated with 100 μM compound **1** (Figure 5D). However, 10 μM **1** was not sufficient to completely modify TR, and a second peptide species with 3155 Da occurs (Figure 5C). The mass increase of 114 Da when compared to the free reduced peptide corresponds to the introduction of two acetamide groups. Peptide sequencing by MALDI-TOF-TOF MS confirmed that **5** and **1** cause a modification of Cys52, whereas Cys57 was alkylated by iodoacetamide (not shown).

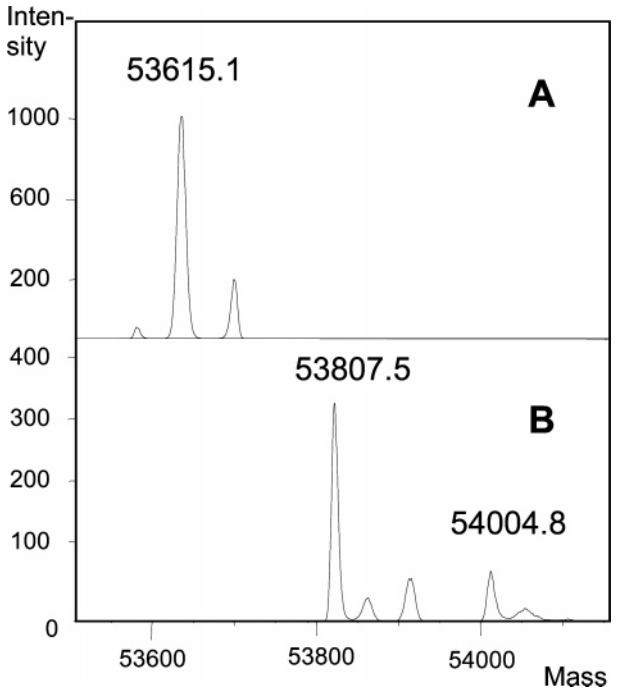


Figure 4. ESI-MS analysis of TR modified by **5**. TR was inactivated by the divinyl ketone **5** as described under Experimental Section. (A) Free enzyme. The mass of 53 615.1 Da corresponds to the theoretical mass of the polypeptide lacking the two N-terminal methionine residues with one oxygen molecule bound to the protein. (B) TR inactivated by **5**. The mass shift of 192 Da when compared to that of the free protein shows incorporation of one molecule of **5**. The small peak at 54 004.8 Da could represent the addition of two molecules of **5**. The other small peaks are probably oxidation products of TR.

As shown by MALDI-TOF analysis, reaction of reduced TR with compound **5** also caused some modification of Cys468. In the case of **1,** all cysteine residues, except Cys57, were partially modified. A quantitative estimation of these reactions was not possible. The N_2 laser used for detection works at 337 nm, which leads to a much higher ionization efficiency of a peptide modified by **1** or **5** in comparison to the respective peptide alkylated by iodoacetamide. Since the ESI-MS spectra did not show peaks at higher masses, reactions with other cysteine residues are negligible.

TR Inactivated by 1 Shows Charge-Transfer Absorption. Two-electron-reduced TR is characterized by a long-wavelength absorption around 530 nm that is caused by the interaction of the thiolate anion of Cys57 with the flavin ring.[3,4,8] The charge-transfer absorption is higher in the NADPH-reduced enzyme than in TR reduced by DTE, as observed in related flavoenzymes.[30,31] TR inactivated by **1** also shows a charge-transfer band (Figure 6). This is in accordance with the covalent modification of Cys52, the active site residue that is responsible for the thiol−disulfide interchange with trypanothione disulfide during catalysis. The lower intensity of the absorption around 530 nm may reflect some steric hindrance of the interaction between Cys57 and the flavin ring. Such an observation has been made in lipoamide dehydrogenase where, in contrast to glutathione reductase and trypanothione

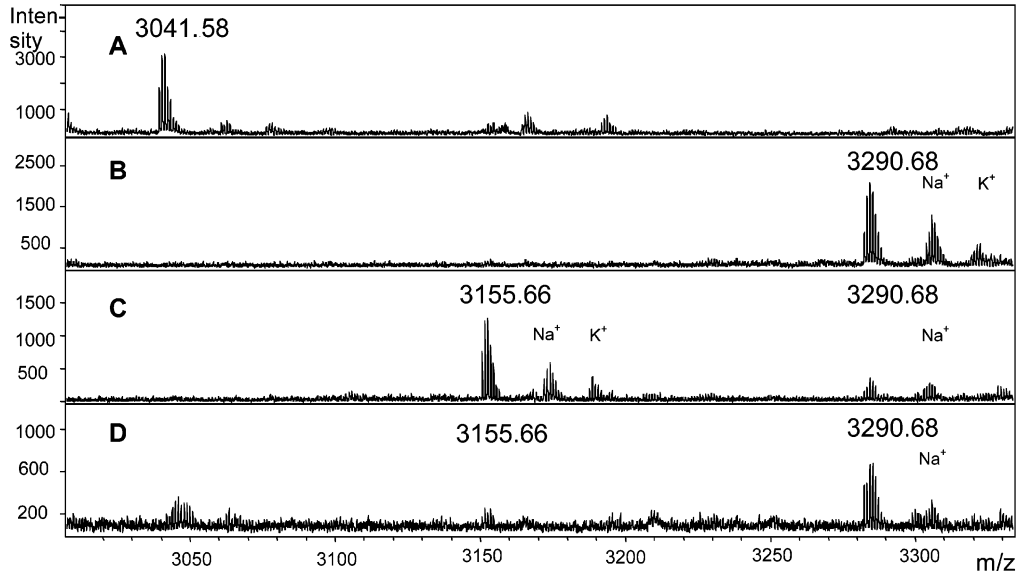


Figure 5. MALDI-TOF analysis of the active site peptide of TR inactivated by **5** or **1**: (A) unmodified, reduced tryptic peptide with Cys52 and Cys57 as free SH groups; (B) peptide obtained from TR treated with 10 μM **5**, (C) 10 μM **1**, and (D) 100 μM **1** and subsequently with iodoacetamide as described under Experimental Section. The mass shift of 249 Da (from 3041 to 3290 Da) is consistent with the modification of one cysteine by iodoacetamide (57 Da) and one by the divinyl ketone **5** (192 Da). The mass of 3155.66 Da (C) corresponds to the peptide with both cysteines alkylated by iodoacetamide. Thus under these conditions, TR is only partially modified by **1**. The additional peaks (Na^+ and K^+) are sodium and potassium adducts of the respective preceding peak. Reaction of TR with **1** or **5** yields peptide species with identical molecular masses, which shows that the unsaturated Mannich base **1** is converted into divinyl ketone **5** prior to reacting with the enzyme (see Scheme 1).

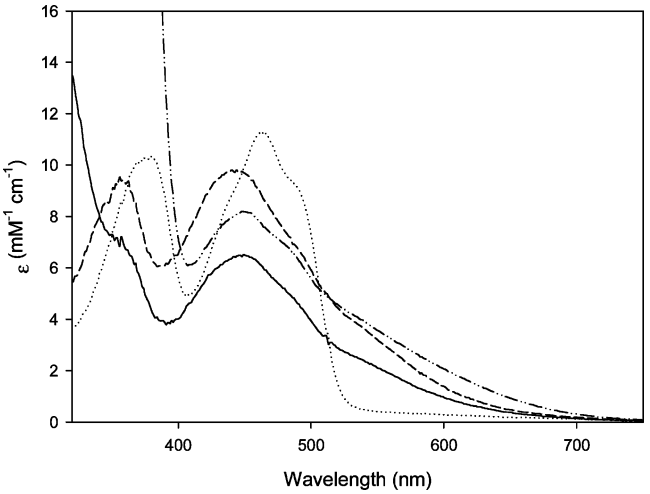


Figure 6. Visible absorption spectrum of trypanothione reductase inactivated by the unsaturated Mannich base **1**. The spectra were recorded in TR assay buffer (40 mM Hepes, 1 mM EDTA, pH 7.5). TR was completely inactivated using a freshly prepared 10 mM solution of **1** in the presence of NADPH. After removal of the non-protein components, the spectrum of inactivated TR (−) was recorded. The spectra of oxidized TR (···), the enzyme reduced by excess DTE (- - -), and NADPH (−··) are depicted for comparison.

reductase, alkylation of the distal cysteine causes the loss of the charge-transfer interaction between the proximal cysteine and the flavin ring.[32] The spectroscopic data fully agree with the mass spectrometric results. The unsaturated Mannich base **1** inactivates TR by covalently modifying Cys52, leaving Cys57 free for interaction with the isoalloxazine ring.

Interaction of the Divinyl Ketone 5 with Related FAD Disulfide Oxidoreductases. Inhibition of human glutathione reductase (hGR) and *P. falciparum* thioredoxin reductase (PfTrxR) by **5** was followed in two different assay systems. In the first system, the activity was directly measured in the presence of different concentrations of **5**. In the second system, the enzymes were preincubated with NADPH and the inhibitor for 5 min, an aliquot was removed, and the remaining activity was determined in a standard assay. In both cases, the concentration of the disulfide substrate in the assay was 5 times that of the respective K_m value. The inhibitor concentrations causing 50% inhibition are given in Table 2. The much lower IC_{50} value of TR for **5** in comparison to GR in the direct assay probably reflects a higher affinity of the parasite enzyme toward the divinyl ketone. The high GSSG concentration may compete with the inhibitor for binding because lowering the GSSG concentration lowered the IC_{50} value to the same extent. When the enzymes were preincubated with **5** in the presence of NADPH, the IC_{50} values were comparable, which indicates that the conversion of the E−I complex into the covalently modified enzyme species occurs at similar rates. Taken together, all three

Table 2. Inactivation of Different Disulfide Reductases by the Divinyl Ketone **5**[a]

	IC_{50} (μM)					
	T. cruzi TR preincubation time (min)		human GR preincubation time (min)		*P. falciparum* TrxR preincubation time (min)	
substrate	0	5	0	5	0	5
100 μM TS_2	8.0	2.0				
500 μM GSSG			190.0[b]	5.0		
34 μM $TrxS_2$[c]					3.7	0.4

[a] IC_{50} values were determined under steady-state conditions (0 min) and after 5 min of preincubation. The substrate concentration in the assays was selected to be 5 times the K_m value of the physiological disulfide substrate of the respective enzyme. [b] In the presence of 50 μM GSSG, the IC_{50} was 18.5 μM. [c] Recombinant *Drosophila melanogaster* thioredoxin-2 ($TrxS_2$) was used as the substrate of *P. falciparum* TrxR.[50]

Table 3. In Vitro Antiparasitic Activities

	ED_{50} (μM)				
compd	*L. donovani*[a]	*T. brucei rhodesiense*[a]	*T. cruzi*[a]	*P. falciparum*[a]	cytotoxicity[a]
1	41.6	1.8	8.7	27.0	14.2
2	13.0	0.8	11.8	16.2	7.3
5	68.0[b]	1.9	12.5	46.7	1230

[a] The standard drugs pentostam, benznidazole, and pentamidine served as positive controls for *L. donovani*, *T. cruzi*, and *T. brucei*, respectively, and displayed ED_{50} values of 28.9−32.0 μM, 4.2−7.3 μM, and 0.1−2 nM. Chloroquine showed an ED_{50} value of 5.8−7.7 nM against the chloroquine-sensitive *P. falciparum* 3D7 strain. Podophyllotoxin exhibited an ED_{50} value of 0.07 μM against the human KB cell line. [b] Toxicity was observed toward macrophages at ≥156 μM.

FAD disulfide oxidoreductases are irreversibly inactivated by **5**. In both assay systems, the plasmodial thioredoxin reductase had the higher reactivity.

Antiparasitic Activities. The activities (ED_{50} values) of the Mannich bases **1** and **2** and of the divinyl ketone **5** against the intracellular amastigote stages of *T. cruzi* and *L. donovani*, the bloodstream form of *T. b. rhodesiense,* and intraerythrocytic *P. falciparum* are given in Table 3. The cytotoxicity of the compounds was determined in assays using the KB cell line. Compounds having ED_{50} values below 5 μM toward *T. cruzi* or *L. donovani* and ≤1 μM in the case of *T. b. rhodesiense* can be considered as trypanocidal lead molecules. Compounds displaying ED_{50} values in the low-nanomolar range might be considered as antimalarial lead compounds.

1, **2**, and **5** displayed only modest activity against all intracellular parasites and the human cell line but showed a significant effect against the extracellular *T. b. rhodesiense*. In the case of intracellular parasites, reaction of the compounds with glutathione in the host cells may lower the effective drug concentration reaching the parasite compartment. The more pronounced cytotoxicity against *T. b. rhodesiense* may be due to reaction with both trypanothione reductase and trypanothione as outlined here.

Discussion

The unsaturated Mannich base **1** was first described as an inhibitor of *Plasmodium falciparum* thioredoxin reductase,[24] an FAD disulfide oxidoreductase mechanistically and structurally related to trypanothione reductase.[8] As shown here, unsaturated Mannich bases are also potent irreversible inhibitors of *T. cruzi* trypanothione reductase, but the mode of inactivation is strikingly different. *P. falciparum* TrxR possesses (in addition to the dithiol/disulfide near the flavin cofactor that is present in all FAD disulfide oxidoreductases) a C-terminal redox center composed of Cys535 and Cys540.[33,34] One of these cysteines attacks the double bond of compound **1** to generate the first reversible adduct (Scheme 2, species **A**). Following elimination of the *N,N*-dialkylamino moiety and formation of a new highly reactive Michael acceptor (species **B**), base catalysis by the enzyme enables the inhibitor to bind irreversibly.[24] The final reaction product was proposed to be a macrocyclic adduct composed of the inhibitor, Cys535, and Cys540 (mechanism B, species **C**).

The reaction of **1** with GR and TR is different. The enzymes have the redox active dithiol/disulfide (Cys52−Cys57 in *T. cruzi* TR, Cys58−Cys63 in human GR) adjacent to the flavin cofactor, but they lack a second thiol redox pair.[8,34] The disulfide substrates (TS_2 and GSSG, respectively) have direct access to the central redox center. Despite the structural and mechanistic similarities between TR and GR, the unsaturated Mannich base **1** is a poor inhibitor of GR. As shown recently, only 10% inhibition occurs with 1 mM inhibitor after 5 min of preincubation of the reduced enzyme.[24] The inhibitory potency toward TR is much higher, resulting in almost 40% inactivation within 5 min at 5 μM compound **1**. These differences may be attributed to the structural characteristics of the disulfide substrate binding sites of the enzymes. TR has a high affinity for positively charged ligands that interact with its negatively charged trypanothione disulfide binding site.[5] In addition, the active site of TR, characterized by a hydrophobic wall formed by Try21 and Met113, is much wider than that of GR and can accommodate very bulky ligands. Thus, the initial binding of **1** to the parasitic enzyme versus the human enzyme should be favored.

Reaction of reduced TR with the unsaturated Mannich base **1** or the divinyl ketone **5** leads to an inactive enzyme species with long-wavelength absorbance around 530 nm. This charge-transfer absorbance is derived from the interaction of the thiolate of Cys57 with the isoalloxazine ring of FAD and demonstrates that this residue is not modified. In contrast, Cys52, which during catalysis directly forms a mixed disulfide with TS_2, is easily alkylated. Because of the lack of a second reacting thiol in TR, Mannich bases such as **1** must obviously first form the divinyl ketone to allow irreversible modification of a single cysteine residue. The intermediate dethiolation step that generates **5** may be catalyzed by a basic residue in the active site such as His460′. The attack of Cys52 at the terminal double bond of **5** then leads to the irreversible product **D** (Scheme 2, mechanism B). In addition, **5** is slowly formed from the Mannich bases in solution. Inactivation of TR by **1** or **5** causes a mass shift of 192 Da. Although the mass analysis cannot discriminate between two potential addition products (**B** and **D** in Scheme 2) compound **B** can be ruled out as an end product. Addition of a thiol to the double bond of the Mannich base and the subsequent deamination step (first three reactions in Scheme 2) are freely reversible[28] as shown by the reaction of stoichiometric concentrations of glutathione and **1**. The reaction yielded a mixture of free Mannich base, species **A** and **C**, but compound **B** was not detectable.[24] In addition, the spontaneous reaction of glutathione with **5** results in formation of **D**, and again, species **B** is not observed.

The cytostatic effect of **1** against *T. b. rhodesiense* trypomastigotes in vitro prompted us to investigate also the reaction of the compound with trypanothione, the main nonprotein thiol in trypanosomatids. As described in the literature, α,β-unsaturated Mannich bases readily react with dithiols forming poly(β-ketothioethers). This polycondensation reaction between unsaturated Mannich bases and dithiols has been extensively applied in the field of polymer chemistry.[23] Trypanothione [bis(glutathionyl)spermidine, $T(SH)_2$] differs from GSH not only by being a dithiol but also because of the higher reactivity of its SH groups. This is mainly attributed to

the lower thiol pK_a value of 7.4 versus ~9.2 in GSH.[35,36] In the spontaneous reaction between the unsaturated Mannich bases and trypanothione, the secondary amino group in the spermidine bridge should facilitate deamination of the Mannich bases. The first reaction between one of the two thiol functions and the α,β-unsaturated Mannich base is a reversible Michael addition of the thiol to the double bond of compound **1**. A prerequisite for the polymerization is the subsequent elimination of the *N,N*-dimethylamino moiety, a process that, as we show here, proceeds under physiological pH conditions. This base-dependent reaction accounts for the rapid and complete reaction of **1** with trypanothione in comparison to glutathione. In the reaction with $T(SH)_2$, two potential products may be formed resulting from intra- or intermolecular reactions, namely, a macrocyclic adduct and open-chain polymers of different lengths. The mass spectrometry data did not provide evidence for a macrocyclic adduct but showed a pattern of polymers. A macrocyclic adduct was also not detectable when reacting **1** with dithiothreitol (data not shown). This finding is in agreement with the entropic factor favoring the formation of open chains between the low M_r dithiol and compound **1**.

The antitrypanosomal activity of the unsaturated Mannich base **1** and related derivatives against the extracellular *T. b. rhodesiense* in culture might at least partially be due to its high reactivity toward TR and trypanothione. The comparably poor cytostatic effect of the compounds against intracellular trypanosomatids and against human KB cells may be explained by reaction with glutathione present in millimolar concentrations in the cytosol of the mammalian (host) cells.

Our studies indicate that the spontaneous reaction between trypanothione and the unsaturated Mannich base **1** leads to the formation of polymers. Indeed, various polymers were shown to be toxic against different cancer cell lines since they are not easily extruded from cells, as are glutathione conjugates, probably because of transport inhibition mechanisms.[37,38] Optimization of unsaturated Mannich bases by generating prodrugs to improve the uptake into the parasites is a promising strategy for the development of new antitrypanosomal and antileishmanial drugs.

Experimental Section

Chemistry. General Methods. All chemicals were purchased from Acros and Aldrich and were used without further purification. Thin-layer chromatography (TLC) was carried out on Whatman flexible PE/SIL G/UV-254 silica gel plates. The components were detected by their absorption at 254 nm, followed by staining with 5% phosphomolybdic acid in ethanol and heating or by 0.1% 2,4-dinitrophenylhydrazine in MeOH/H_2O (90:10). Silica gel 60z (Merck 230−400 mesh) was used for chromatography. Analytical HPLC was run on a Nucleosil 100-5 C18 column (Machery & Nagel) using a Merck-Hitachi L-6200 instrument with an L-4000 UV detector set at 254 nm. The compounds were dissolved in a mixture of 50% (v/v) ethanol and 0.05% TFA in H_2O and applied onto the column. The components were separated at a flow rate of 1.0 mL/min using the following conditions: 100% solvent A (0.05% TFA in H_2O) for 5 min, followed by a gradient from 100% solvent A to 100% solvent B (0.05% TFA in 80% (v/v) acetonitrile) over 15 min and 100% B for another 15 min. The retention times (t_R) are given in min. ^{1}H NMR (300 MHz), ^{13}C NMR (75 MHz), and elemental analyses were conducted at the Organisch Chemisches Institut der Universität Heidelberg. ESI-MS of the free ligands were carried out by Drs. Jens Pfannstiel and Johannes Lechner at the Biochemie-Zentrum der Universität Heidelberg.

The unsaturated Mannich bases, 1-(2-chlorophenyl)-5-(dimethylamino)pent-1-en-3-one hydrochloride (**1**), 1-(2-chlorophenyl)-5-(*N*-piperidino)pent-1-en-3-one hydrochloride (**2**), 1-(2-chlorophenyl)-5-(*N*-morpholino)pent-1-en-3-one hydrochloride (**3**), 1-(2-chlorophenyl)-4,4-dimethyl-5-(dimethylamino)pent-1-en-3-one hydrochloride (**4**), were synthesized as previously described.[24] Elemental analyses were in agreement with the calculated values (see Supporting Information).

1-(2′-Chlorophenyl)penta-1,4-dien-3-one (5). The hydrochloride of **1** (580 mg, 2.1 mmol) was dissolved in 10 mL of water, and 2.5 N NaOH (10 mL) was added. The free base was extracted with ether, dried with $MgSO_4$, and used without further purification. It was dissolved in toluene (25 mL). Methyl iodide (900 mg, 6.3 mmol) was added, and the mixture was stirred overnight at room temperature. After addition of another 300 mg of methyl iodide, the reaction mixture was heated for 1.5 h at 50 °C and then refluxed for 45 min to release compound **5** by thermal elimination. The salt precipitate was removed by filtration, the solvent was evaporated, and the crude material was chromatographed on silica gel with petroleum ether/acetone (8:1), resulting in a yellow oil (271 mg, 1.4 mmol, 67% yield) that was stored under nitrogen at 4 °C until further use. ^{1}H NMR ($CDCl_3$) δ 5.88 (d, 1H, J = 10.6 Hz), 6.35 (d, 1H, J = 17.4 Hz), 6.74 (dd, 1H, J = 10.6 and 17.4 Hz), 6.93 (d, 1H, J = 16.1 Hz), 7.14−7.65 (m, 4H), 8.04 (d, 1H, J = 16.1 Hz). ^{13}C NMR ($CDCl_3$) δ 189.44, 139.61, 135.34, 134.88, 132.91, 131.23, 130.23, 128.97, 127.61, 127.10, 126.84. ESI-MS (*m/z*) calcd, 192.67; found, 192.72. Anal. ($C_{11}H_9ClO$) C, H. HPLC (Nucleosil C-18) t_R = 24.4 min.

4-(2′-Chlorophenyl)-2-cyclopentenone (6). To a stirred solution of aluminum chloride (490 mg, 3.7 mmol) in 7 mL of CCl_4 was added a solution of 2-chlorocinnamoyl chloride (700 mg, 3.5 mmol) and trimethylvinyl silane (420 mg, 4.2 mmol) in 4 mL of CCl_4. The mixture was stirred at ~70 °C for 30 min, and then hydrolysis was started by adding 20 mL of NH_4-Cl solution (12.5% w/v). The aqueous phase was extracted with CH_2Cl_2 (2 × 25 mL). The organic phase was washed with water and a diluted $NaHCO_3$ solution and dried over $MgSO_4$. The crude material was chromatographed on silica gel using CH_2-Cl_2 to give compound **6** (280 mg, 42% yield) as a faintly yellow oil. ^{1}H NMR ($CDCl_3$) δ 2.20 (dd, 1 H, J = 2.6 and 19.1 Hz), 2.97 (dd, 1 H, J = 7.0 and 19.1 Hz), 4.63 (m, 1 H), 6.37 (dd, 1 H, J = 2.2 and 5.6 Hz), 6.98−7.41 (m, 4 H), 7.69 (dd, 1H, J = 2.6 and 5.7). ^{13}C NMR ($CDCl_3$) δ 165.12, 139.18, 134.92, 133.70, 129.80, 128.42, 127.55, 127.35, 43.22, 42.62. Anal. ($C_{11}H_8ClO$) C, H. HPLC (Nucleosil C-18) t_R = 23.9 min.

Stability Studies of the Unsaturated Mannich Bases. Fresh solutions of 20 mM compounds **1** and **2** in DMSO were prepared and kept in the dark at 20 °C. After different times (10 min to 4 days) aliquots were removed and diluted with a 1:1 (v/v) mixture of ethanol and solvent A (0.05% TFA in H_2O) to give a final concentration of 1 mM. Chloroquine (0.2 mM in solvent A with 50% v/v EtOH) served as an internal reference. Equal volumes of the chloroquine solution and the diluted Mannich bases were mixed, and 20 μL of the mixture was injected into the HPLC system. The analytical HPLC was performed on a Hitachi L 6200A system equipped with a Macherey & Nagel C18 Nucleosil column (4 mm × 300 mm, 100 μm, 300 Å) and detection at 254 nm. The reaction products were separated at a flow rate of 1 mL/min under the following conditions: 100% solvent A for 5 min, a linear gradient from 100% solvent A to 100% solvent B (0.05% TFA, 20% H_2O, 80% CH_3CN) over 15 min, and then 100% solvent B for 10 min. The retention times were 16.4 min for the internal reference (chloroquine), 18.6 min for **1**, 20.1 min for **2**, 24.4 min for **5**, and 23.9 min for **6**. The percentage of the starting materials converted with time was calculated from the peak areas using chloroquine as the internal reference.

The DMSO solution of compound **1** was also analyzed by ^{1}H NMR and ESI-MS after different times of storage (10 min to 36 days by NMR; 10 min to 66 days by ESI-MS).

Glutathionylation of 5. The monoglutathionylation product of compound **5**, 1-(2′-chlorophenyl)-5-glutathionyl-pent-1-en-3-one), was prepared by mixing a 100 mM solution of **5** (8.6 mg in 450 μL of ethanol) with an equal volume of 100 mM GSH in 0.1 N NaOH. The suspension was allowed to react at room temperature in an ultrasound bath. The diglutathionylation product of **5**, (1-(2′-chlorophenyl)-1,5-bis-glutathionyl-pentan-3-one), was obtained by reacting a 100 mM solution of **5** with one volume of 200 mM GSH in 0.4 N NaOH (pH ∼9.5) at 60 °C for 3 h. Both glutathionylation products were analyzed by HPLC (Nucleosil C-18), ^{1}H NMR, and mass spectrometry (ESI-MS). HPLC yielded a t_R value of 19.1 min for the monoadduct and t_R values of 16.3 and 16.4 min (diastereomers) for the diadduct.

Glutathionylation of 6. A 100 mM solution of 4-(2′-chlorophenyl)-2-cyclopentenone in EtOH was mixed with an equal volume of 100 mM GSH in 0.1 N NaOH. The reaction mixture was incubated in the ultrasound bath and analyzed by HPLC (Nucleosil C-18). The t_R value of the conjugate was 18.3 min (diastereomers).

Trypanothionylation of 1. A sample of 1 M $NaBH_4$ in 0.1 M NaOH (10 μL) was added to 500 μM TS_2 in 100 mM sodium phosphate buffer, pH 7.4 (1 mL), under anaerobic conditions. After complete hydrolysis of borohydride (∼15 min), 50 μL of a 10 mM solution of compound **1** in DMSO was added, and the reaction was analyzed by HPLC. The reaction mixture was diluted 1:5 with solvent A, and 10 μL of the resulting solution was co-injected with 10 μL of 100 mM chloroquine as a reference. The HPLC conditions were as described above. The t_R value of the monoadduct was 20.5 min.

Enzymes and Biochemical Materials. Recombinant *T. cruzi* TR was purified following published procedures.[39] Trypanothione disulfide (TS_2) was purchased from Bachem, Switzerland. Stock solutions (5−10 mM) of the unsaturated Mannich bases and potential intermediates (Table 1) were made in DMSO and, when not immediately used, stored at 4°C.

Trypanothione Reductase Standard Assay. TR activity was measured at 25 °C in TR assay buffer (40 mM Hepes, 1 mM EDTA, pH 7.5) as described.[40] The standard assay (1 mL) contained 100 μM NADPH and 5−10 mU *T. cruzi* TR. The reaction was started by adding 110 μM trypanothione disulfide (TS_2), and the absorption decrease due to NADPH consumption was monitored at 340 nm in a Hitachi U 150-20 spectrophotometer. V_{max} was calculated using a K_m of 18 μM for TS_2 under these assay conditions.

Irreversible Inhibition of Trypanothione Reductase. To monitor the time-dependent inactivation, 0.5 μM TR was incubated at 25 °C in the presence and absence of 200 μM NADPH with various concentrations of inhibitor in TR assay buffer (total volume of 100−200 μL). At different time intervals, aliquots of 5 μL were removed and the remaining activity was measured in a standard TR assay. Owing to the dilution, reversible inhibition is not recorded. As a control, TR was incubated with NADPH and the respective amount of DMSO. Under these conditions, DMSO does not affect enzyme activity. A second control contained enzyme and inhibitor but no NADPH. To confirm the irreversible inhibition of TR by compound **1**, an enzyme sample with <5% remaining activity was prepared. The low molecular mass components were removed by centrifugation in a Microcon YM-30 centrifugal filter (Sorvall Centrifuge RC-5C, 4 °C). After repeated rinses with TR assay buffer, the enzyme solution was recovered and its activity measured in a standard TR assay. In addition, the sample was treated with 2.6 mM dithioerythritol for 1 h, and again, the enzyme activity was measured.

Mass Spectrometric Analyses of TR Inactivated by the Unsaturated Mannich Base 1 or the Divinyl Ketone 5. ESI-MS of the protein was measured on a QTOF mass spectrometer (Applied Biosystems). MALDI-TOF MS was carried out using an Ultraflex instrument (Bruker).

For the ESI-MS analysis, 5 μM TR in 100 μL of assay buffer was incubated with 0.3 mM NADPH and 50 μM divinyl ketone **5** or 50 or 100 μM compound **1**. After 1 h at room temperature, 15 mM DTT (Fluka) was added and the solution was again incubated for 30 min. Iodoacetamide was added to a final concentration of 45 mM to alkylate residual free cysteine residues. Total mass determination was done on an HPLC system (Agilent) on-line-coupled to the ESI-QTOF instrument. The protein solution was loaded on a 50 Poros R1 trapping column. After 1.5 min of washing (0.1% TFA, 0.4 mL/min), the proteins were eluted to the electrospray ion source with 80% acetonitrile, 0.1% TFA, 20 μL/min. The QTOF MS was calibrated with apomyoglobin (Sigma).

To identify the site of modification, 50 μL of 0.5 μM TR was treated with 10 or 100 μM compound **1** or 10 μM **5**, as described above. The modified protein was digested with trypsin (sequencing grade, Promega) at a TR to a protease ratio of 10:1 (w/w) for 4 h at 37°C. The peptides were desalted on reversed-phase material (μC18 ZipTip, Millipore) and eluted with a saturated solution of α-cyanohydroxycinnamic acid (Bruker) in acetonitrile, 0.1% TFA (1:1 v/v) representing the MALDI matrix.

Spectroscopic Studies. The absorption spectra of oxidized, reduced, and inactivated TR were recorded between 200 and 800 nm in a Beckman DU 7200 at 25°C. In a 100 μL microcuvette, absorption of 22.6 μM oxidized TR (99 μL) was measured, 2 mM DTE was added, and after complete reduction, another spectrum was collected. The concentration of oxidized TR is given by its absorption at 462 nm (ϵ = 11.3 $mM^{-1}\,cm^{-1}$). Excess NADPH was added, and the concentration of the reduced enzyme was calculated taking into account dilution of the solution. From these data the ϵ values for the reduced enzyme were estimated.[3] The absorption spectrum of 19.8 μM TR reduced by 500 μM NADPH was taken 2 min after mixing in a 1 mL cuvette. The spectrum of TR irreversibly inactivated by **1** was recorded in a total volume of 1 mL containing 15.07 μM TR, 850 μM NADPH, and 430 μM compound **1**. After removal of the nonprotein components as described above, another spectrum of inactivated TR was recorded and the enzyme concentration was determined by Micro BCA-Assay (Protein Assay Reagent Kit, Pierce). The spectra were normalized by setting the absorption at 770−800 nm to zero.

For compounds **1**−**6**, 10 mM stock solutions were prepared in DMSO and stored at room temperature. At 0 h, 24 h, 6 days, 20 days, and 40 days, a 1 μL aliquot was removed and, after dilution to a final concentration of 10 μM in water, the absorption spectrum was recorded. The spectrum of a solution containing 1 μL of DMSO in 1 mL of H_2O was subtracted. The spectra were normalized by setting the absorption at 750−800 nm to zero.

Comparative Inactivation of TR, GR, and TrxR by the Divinyl Ketone 5. The activities of *T. cruzi* TR, human GR, and *P. falciparum* TrxR were followed in a total volume of 1 mL of 100 mM phosphate buffer, pH 7.0, 100 mM potassium phosphate buffer, pH 7.0, 200 mM KCl, and 100 mM phosphate buffer, pH 7.4, and 2 mM EDTA, respectively. Initial rates were calculated from NADPH oxidation measured at 340 nm and 25 °C. The stock solutions of the inhibitors were made in DMSO. All assays of a series contained the same amount of DMSO.

IC_{50} Values from Direct Assays. The standard assay mixtures contained 100 μM NADPH and 100 μM TS_2 in the case of TR, 500 μM or 50 μM GSSG with GR, and 34 μM *Drosophila melanogaster* $TrxS_2$ in the case of TrxR. IC_{50} values were determined in duplicate in the presence of six to eight inhibitor concentrations ranging from 0 to 100 μM. The reactions were started by adding 5 μL of enzyme solution (9 mU or 1.63 pmol of *T. cruzi* TR, 8 mU or 0.86 pmol of human GR, 9.65 mU or 7.7 pmol of *P. falciparum* TrxR). The final content of DMSO in the cuvette was 1%.

IC_{50} Values from Time-Dependent Inactivations. The activities of *T. cruzi* TR, human GR, and *P. falciparum* TrxR after preincubation with inhibitor and NADPH for 5 min were determined by monitoring disulfide reduction. 5 μL of enzyme solution containing 20.42 pmol of *T. cruzi* TR, 17.2 pmol of human GR, or 30.8 pmol of *P. falciparum* TrxR was allowed

to react with 160 μM NADPH and 0−20 μM divinyl ketone **5** in a final volume of 50 μL of buffer for 5 min at 25 °C. All reaction mixtures contained 2% DMSO. 5 μL of each reaction mixture was removed, and the residual activity was measured in the respective standard assay (see above).

Parasite Cultures. *L. donovani* (strain MHOM/ET/67/HU3) was maintained routinely in special pathogen-free (SPF) female Golden hamsters (Charles Rivers Ltd., U.K.) by passage every 12 weeks. *T. cruzi* (strain MHOM/CL/00/Tulahuan-LacZ)[41] trypomastigotes were derived from L6 fibroblasts in RPMI 1640 medium (Sigma, U.K.) with 10% heat-inactivated fetal calf serum (HIFCS) (Harlan Sera-Lab., U.K.) at 37 °C and 5% CO_2/air mixture. *T. brucei rhodesiense* (STIB900) bloodstream trypomastigotes were maintained in HMI-18 medium[42] supplemented with 20% HIFCS at 37 °C in a 5% CO_2/air mixture. The chloroquine-sensitive 3D7 clone of the NF54 isolate[43] of *Plasmodium falciparum* was obtained from MR4 (Malaria Research and Reference Reagent Resource Center, Manassas, VA). *P. falciparum* in vitro culture was carried out using standard protocols[44] with modifications. Briefly, parasites were maintained in tissue culture flasks in human A Rh+ erythrocytes at 5% hematocrit in RPMI 1640 supplemented with 25 mM HEPES, 24 mM $NaHCO_3$, 0.2% (w/v) glucose, 0.03% L-glutamine, 150 μM hypoxanthine, and 0.5% Albumax II (Invitrogen) in a 5% CO_2/air mixture at 37 °C, and the medium was changed daily.

In Vitro Antiparasitic Bioassays. Growth inhibition of *L. donovani*, *T. cruzi*, and *T. b. rhodesiense* by potential drugs was followed using published protocols.[45] Drug activity against *L. donovani* was evaluated by counting the percentage of macrophages infected compared to untreated controls, with sodium stibogluconate ($NaSb^V$) (Glaxo-Wellcome, U.K.) as a positive reference.[46] Activity against *T. cruzi* was evaluated colorimetrically[41] with benznidazole (Bayer, Germany) as the control drug. The activity of the compounds against *T. b. rhodesiense* was determined in a tetrazolium salt colorimetric assay[47] after 3 days of treatment with pentamidine isethionate (Sigma, U.K.) as the reference. Drug susceptibility of *P. falciparum* was studied using a modified method[48] of the protocol described previously.[49] All assays included chloroquine diphosphate (Sigma, U.K.) as a standard and control wells with untreated infected and uninfected erythrocytes. ED_{50} values were derived by sigmoidal regression analysis (Microsoft *xl*fit).

Evaluation of the Cytotoxicity. Cytotoxicity on KB cells (human oral pharyngeal carcinoma) was evaluated using the Alamar Blue assay as described.[48] The positive control drug was podophyllotoxin (Sigma). ED_{50} values were calculated compared to blanks and untreated controls.

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Appendix

Abbreviations. CTC, charge-transfer complex; DMSO, dimethyl sulfoxide; DTE, dithioerythritol; ESI-MS, electrospray ionization mass spectrometry; E_{ox}, enzyme with oxidized flavin and redox active disulfide; EH_2, two-electron-reduced enzyme; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; QTOF MS, quadrupole time-of-flight mass spectrometry; TFA, trifluoroacetic acid; t_R, retention time; TrxR, thioredoxin reductase; $TrxS_2$, oxidized thioredoxin; TS_2, trypanothione disulfide; $T(SH)_2$, trypanothione; TR, trypanothione reductase.

Supporting Information Available: Results from elemental analysis of compounds **1**−**6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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