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# Unsaturated Mannich Bases Active Against Multidrug-Resistant *Trypanosoma brucei brucei* Strains

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*A series of unsaturated Mannich bases possessing two electrophilic sites was recently identified as irreversible inhibitors of trypanothione reductase from* Trypanosoma cruzi. *New derivatives were synthesized by modifying the substitution pattern on the aromatic ring and by incorporating the melamine motif of melarsoprol. Their affinity to P2 transporter and their trypanocidal properties have been studied using three strains expressing various purine transporters. While the melamine derivatives showed some affinity to the P2 transporter, unsaturated Mannich bases without the melamine motif showed excellent potencies against pentamidine-resistant strains of* T. brucei brucei *suggesting alternative drug uptake routes. The Michael acceptor properties of the three most active compounds towards glutathione correlated with the observed trypanocidal activities.*

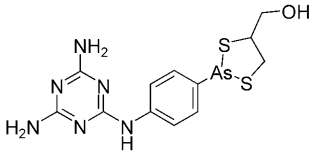
## Introduction

African trypanosomiasis, Chagas' disease, and the different forms of leishmaniases are human infectious diseases caused by kinetoplastid parasites. The homodimeric FAD-containing trypanothione reductase (TR, EC 1.8.1.12) is unique to these parasites and belongs to the family of NADPH-dependent oxidoreductases, including the human glutathione reductase (GR). TR is responsible for maintaining trypanothione (bis(glutathionyl)spermidine) in its dithiol form (Equation 1):

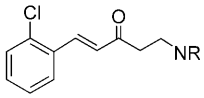
$$TS_2 + NADPH + H^+ \rightarrow T(SH)_2 + NADP^+ \quad (1)$$

The enzyme plays a crucial role in thiol redox metabolism and is known to be essential for all trypanosomatids studied so far.[1,2] TR/GR substrate recognition is governed by a difference in the charge of the active site, rendering these enzymes mutually exclusive towards their disulfide substrates. The active site of TR is negatively charged due to Glu 18, while an Arg residue at the cognate position 37 in GR gives a positively charged active site. Additionally, the active site of TR can accommodate larger ligands and is more hydrophobic due to Met 113 and Trp 21; the latter can also be involved in cation–$\pi$[3] and $\pi$–$\pi$ interactions.[1] Evidence that TR is a target for drug design includes a study showing that a *T. brucei* strain expressing less than 10% of normal TR levels was unable to infect mice, and also showed extreme sensitivity to oxidative stress in the absence of exogenous reducing agents.[2]

African sleeping sickness is invariably fatal if untreated. Current therapy of the late encephalitic stage with the melaminophenyl arsenical drug melarsoprol (mel, shown) has severe side effects with an overall mortality rate of 5%. Mel can act as a bisalkylating agent of dithiols including trypanothione and trypanothione reductase.[4] The drug is actively taken up into trypanosomes via the P2 adenosine transporter that recognizes melamine and the amidine motifs as well as 6-amino purines.[5] The mutation or loss of the P2 transporter correlates with resistance to mel and also some diamidines in laboratory studies, but the situation is more complex than originally thought with other transporters also capable of carrying mel[6] and diamidines.[7–12] The P2 transporter is one of several purine transporters in *T. brucei*, and is encoded by the *TbAT1* gene,[13] the loss




melarsoprol (arsobal®)




**1** NR = $N(Me)_2$
**2** NR = piperidine

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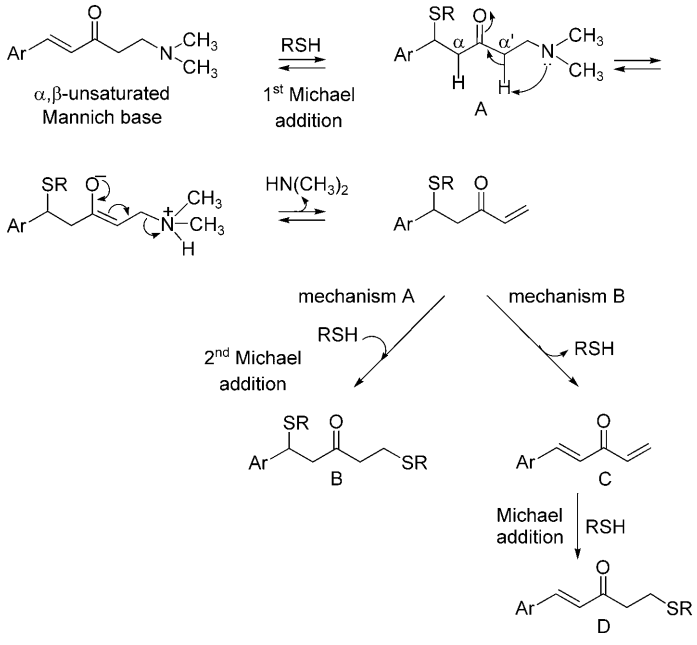
of which does not induce any notable negative impact on growth of trypanosomes.[14]

The P2 transporter was shown to recognize melamine- and benzamidine-based compounds,[5,9,15,16] which explains why these drugs enter via this transporter. Although loss of the transporter can correlate with resistance to drugs that enter via this route, it appears that other transporters also carry a number of melamine- and benzamide-based compounds since the *TbAT1* knockout lines frequently do not show an altered sensitivity to such compounds.[12] Thus, addition of melamine moieties to any cytotoxin has the potential to add specificity to the derivative through selective drug uptake. Apart from P2, two other transporters were also identified in pentamidine and possibly other melaminophenyl arsenicals uptake: the high affinity pentamidine transporter (HAPT1) and low affinity pentamidine transporter (LAPT1).[17,18] Diminished transport activity by P2 and HAPT1 were observed in the *T. brucei gambiense* subspecies that has been adapted to high levels of melaminophenyl arsenical resistance.[6]

With trypanothione reductase validated as a drug target in trypanosomes, our work aims at the design of inhibitors of this enzyme. A series of unsaturated Mannich bases illustrated by **1**—possessing two electrophilic sites–-was identified as potent and irreversible inhibitors of both *Plasmodium falciparum* thioredoxin reductase[19] and *Trypanosoma cruzi* trypanothione reductase[20] (Scheme 1, mechanisms A and B, respectively). Inactivation of TR was found to be more efficient than that of human GR, probably because the protonated amino group of the Mannich base is better recognized in the negatively charged TR active site. Incubation of the enzyme with the compounds in the presence of NADPH caused a covalent modification of Cys 52 in the active site (Scheme 1, mechanism B, adduct structure D). A divinylketone product of the parent compound (Scheme 1, mechanism B, intermediate C) is responsible for enzyme inactivation after base-catalyzed deamination and dethiolation reactions. In addition, unsaturated Mannich bases were revealed to be very reactive in polycondensation reactions with trypanothione after deamination (Scheme 1, mechanism A, adduct structure B). The specific reactivity of unsaturated Mannich bases could explain the potent trypanocidal effects of these compounds against *T. brucei*.[20] The identification of the α,β-unsaturated β-dialkylaminoketone as the minimal motif for mechanism-based inactivation of the trypanothione system, prompted us to design and synthesize new unsaturated Mannich bases.

Because the divinylketone is too reactive to be considered as a drug candidate, two strategies were followed to modulate the reactivity and bioavailability of the enone. In the first approach, a melamine group was introduced to facilitate drug uptake and to lower host drug cytotoxicity and metabolism (compounds **3a-c** and **4**). In the second approach, a related phenolic Mannich base was synthesized as a potential prodrug of quinone methide generated after biooxidation (compound **5**).[21]

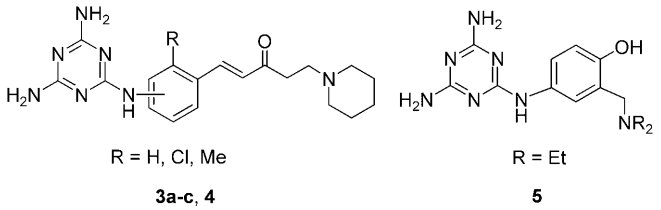



**Scheme 1.** Proposed reaction mechanisms for the modification of thiols (RSH) by an unsaturated Mannich base, such as compound **1** (Ar = 2-chlorophenyl).[20] Mechanism A: in the case of two reactive thiols, e. g. trypanothione in *T. cruzi*. Mechanism B: in the case of a single reactive protein thiol, for example, Cys 52 in *T. cruzi* trypanothione reductase (TR).

## Results

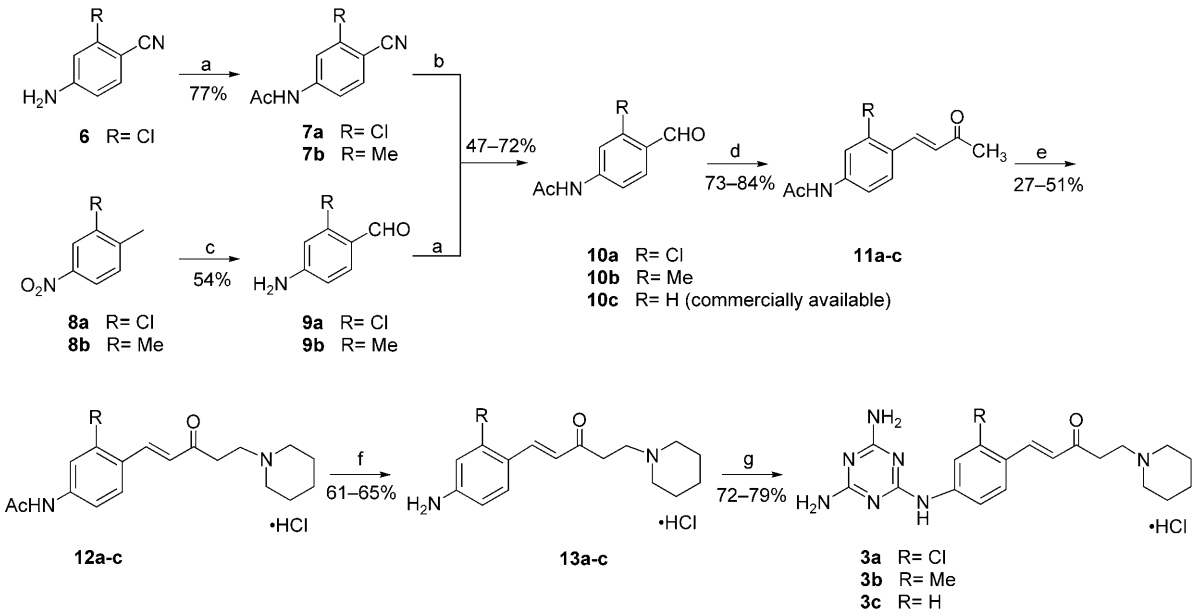
### Chemistry

Mel-derived Mannich base analogues were synthesized and investigated in this study (compounds **3**–**5**). Different functional groups were added to the aromatic ring in *ortho*, *meta*, and




*para* positions relative to the unsaturated ketone to vary the Michael acceptor properties of the molecule. The melamine derivatives were synthesized according to the procedure described in Schemes 2–4. The starting compounds **10a-b** in Scheme 2 were prepared via two different routes. In the first route, the cyano function of **7a–b** was reduced with Raney-Ni in formic acid after the protection of the amino group of derivative **6** as the acetamide with acetic anhydride. In the second route, reduction of the nitro group of compounds **8a-b** to the corresponding amine and oxidation of the methyl function to the aldehyde were achieved in one step by a Zinin reaction with $Na_2S{\cdot}9H_2O$ and sulfur in ethanol under basic conditions to yield compounds **9a-b**. The unsaturated ketones **11a-c** were obtained via a base-catalyzed Claisen–Schmidt condensation in acetone. An easy isolation of the product was established by

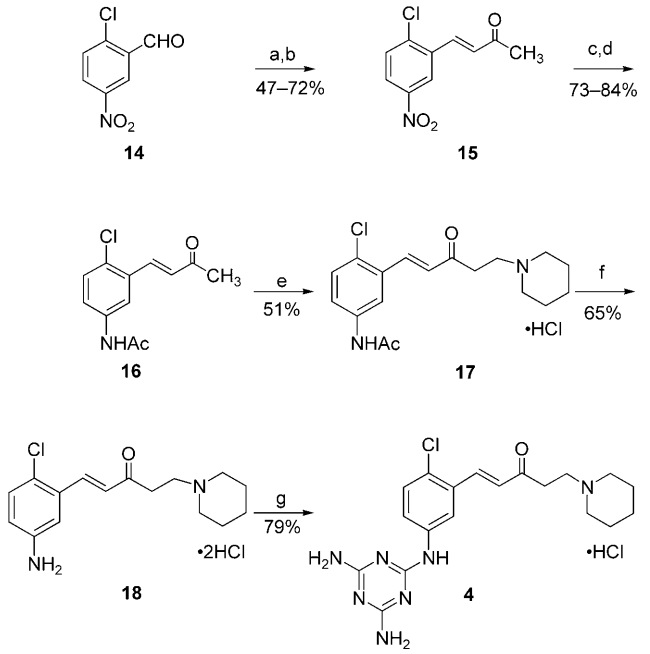
  




**Scheme 2.** Synthesis of the unsaturated Mannich base derivatives containing the melamine motif in the *para* position relative to the unsaturated ketone (compounds **3a-c**). *Reagents and conditions*: a) $Ac_2O$, TEA in toluene; b) Ra-Ni, formic acid; c) NaOH, $Na_2S{\cdot}9H_2O$, S in $H_2O$, EtOH, 3 h, reflux; d) 1 N NaOH, acetone, 1.5 h, RT; e) paraformaldehyde, piperidine hydrochloride, acid in EtOH, reflux, 1 d; f) 8 N HCl solution, reflux, 3 h; g) 2-chloro-4,6-diamino-1,3,5-triazine in MeOH, reflux, 2 h.

concentration of the reaction mixture resulting in the straightforward precipitation of the benzalacetone from the solution. To generate compounds **13a-c**, the Mannich reaction was performed under acidic conditions with paraformaldehyde and piperidine hydrochloride followed by deprotection of the acetamide under standard conditions. The desired Mannich base derivatives **3a-c** were synthesized via a nucleophilic substitution with 2-chloro-4,6-diamino-1,3,5-triazine. The melamine compounds showed a low solubility in most organic solvents, except DMSO and $H_2O$. Purification was achieved by recrystallization from methanol or ethanol.

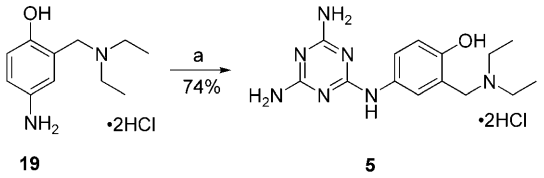
The presence of an amino group in the *para* position to the enone was expected to lower the Michael acceptor properties of the unsaturated Mannich base. For this reason, the synthesis of compound **18** containing the amino motif in the *meta* position and a chloride in the *ortho* position relative to the unsaturated ketone (Scheme 3) was designed to increase the activity. The unsaturated ketone **15** was obtained again via a Claisen–Schmidt reaction from compound **14** but in contrast to the general procedure, the reaction was performed in two steps under milder conditions. Firstly, the alcohol intermediate was formed using a catalytic amount of base at 0 °C. After isolation, the Claisen–Schmidt product **15** was obtained via acid-catalyzed dehydration. The nitro group of this compound was reduced with $SnCl_2$ under acidic conditions to form the amine, which was directly protected with acetic anhydride to give acetamide **16**. The resulting compound was then treated with paraformaldehyde and piperidine hydrochloride under acidic conditions to obtain the Mannich base **17**. The deprotection of the acetamide under standard conditions and the nucleophilic substitution of the aniline **18** with 2-chloro-4,6-diamino-1,3,5-triazine led to the desired Mannich base derivative **4**.




**Scheme 3.** Synthesis of the unsaturated Mannich base derivative **4** containing the melamine motif in *meta* to the unsaturated ketone. *Reagents and conditions*: a) acetone/water, 2 N NaOH, 30 min, 0 °C; b) MeOH/2.5 N $H_2SO_4$, 3 h , reflux; c) $SnCl_2$, concd HCl, EtOH, 4 h, 70 °C; d) $Ac_2O$, 24 h, RT; e) paraformaldehyde, piperidine hydrochloride, HCl, EtOH, 3 h, reflux; f) 8 N HCl, 3 h, reflux; g) 2-chloro-4,6-diamino-1,3,5-triazine, MeOH, 3 h, reflux.

The synthesis of the phenolic Mannich base derivative **5** is shown in Scheme 4. The compound was formed via a nucleophilic substitution reaction of the 2-chloro-4,6-diamino-1,3,5-triazine with the 4-amino-α-diethylamino-*ortho*-cresol dihydro-

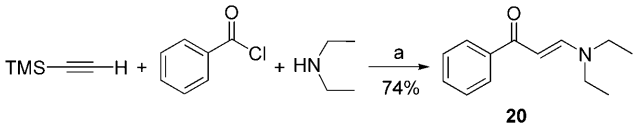
  




19 5

**Scheme 4.** Synthesis of the phenolic Mannich base derivative **5**. *Reagents and conditions*: a) 2-chloro-4,6-diamino-1,3,5-triazine in EtOH, reflux, 2 h.

chloride **19** in ethanol. The known *N,N*-dimethyl acrylophenone **20** was formed in a Sonogashira reaction according to the previously described procedure (Scheme 5).[22]




20

**Scheme 5.** Synthesis of the *N,N*-diethyl acrylophenone **20**. *Reagents and conditions*: a) $PdCl_2(PPh_3)_2$, CuI, TEA, dry THF.

### Glutathionylation of Mannich base derivatives

The Michael acceptor properties of compounds **1**, **2**, **13a** and **20** possessing one or two sites for thiol alkylation were evaluated by the rate of formation of the monoglutathionylated Mannich base adducts upon addition of one equivalent of glutathione (Scheme 1, adduct structure A). The reaction mechanism of unsaturated Mannich bases, such as **1** and **2**, towards glutathione was previously studied[19] and confirmed. The Michael addition of thiols to the enone group of unsaturated Mannich bases is much more rapid but also much more complex than with unsaturated ketones because it is reversible, that is, the reaction does not reach completion. Also, the retro-Michael addition (Scheme 1, A $\rightleftharpoons$ $\alpha,\beta$-unsaturated Mannich base) is more favored in the case of Mannich bases than for the corresponding ketone series because of the intramolecular deprotonation of the acidic $\alpha$ hydrogen. For this reason, addition of glutathione to unsaturated Mannich bases showed a linear rate up to 30% starting material consumption and reached an equilibrium at which point both dethiolation (retro-Michael addition) and deamination take place. Because of the complexity of these reactions we analyzed the linear phase of the Michael addition, that is, in short time periods upon addition of only one equivalent of glutathione. The results were expressed as the time-dependent [Mannich base]:[monoSG adduct] ratio given by the HPLC analysis and retention times ($t_R$). The monoSG adducts of compounds **1** (**1**, $t_R$ = 14.04 min; monoSG adduct of **1**, $t_R$ = 12.32/12.42 min), and **2** (**2**, $t_R$ = 14.54 min; monoSG adduct of **2**, $t_R$ = 11.99/12.15 min) appeared as a pair of two close peaks in the chromatogram revealing both diastereoisomers formed upon Michael addition of glutathione, while with **13a** (**13a**, $t_R$ = 13.63 min; monoSG adduct of **13a**, $t_R$ = 11.30 min), the monoSG adduct appeared as a single peak corresponding to the quinone imine structure (Figure 1).

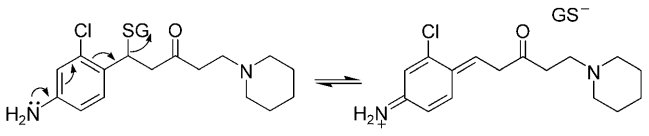



**Figure 1.** Structure of the quinone imine **13a**–SG adduct.

For compounds **1**, **2** and **13a**, the [Mannich base]:[monoSG adduct] ratio reached the value of 1, after 3 min, 4 min and 30 min, respectively (Figure 2a). When comparing compounds **2** and **13a**, these values are in agreement with the predicted

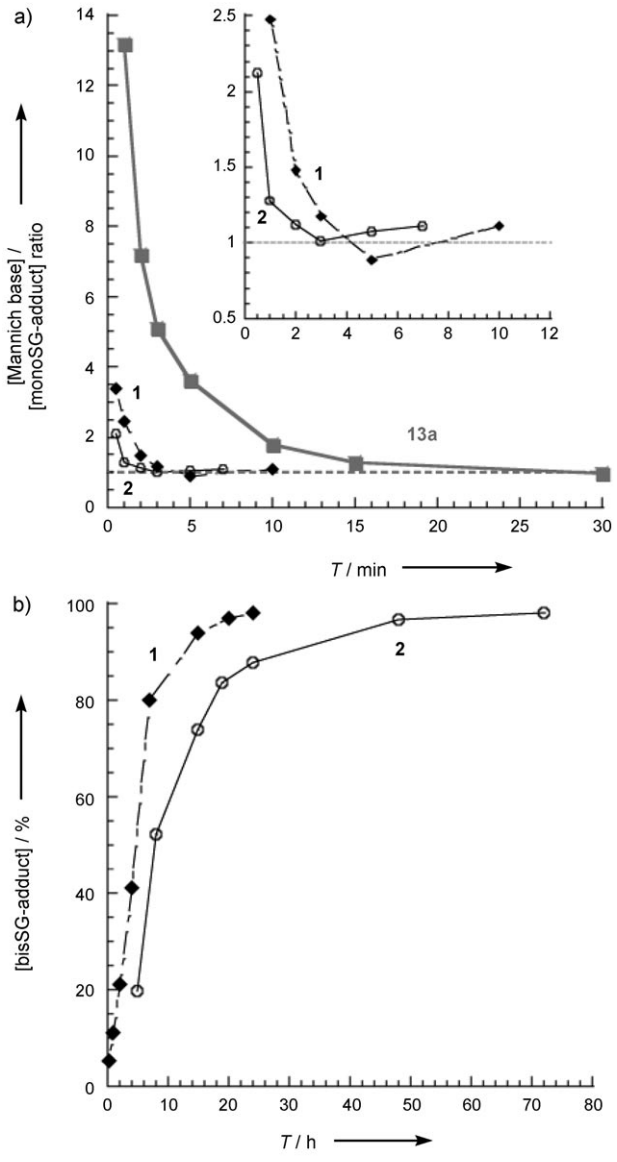



**Figure 2.** Glutathionylation rates of compounds **1**, **2**, and **13a** as Michael acceptors. a) Reaction of compound **1**, **2**, or **13a** in the presence of 1 equiv GSH at pH ~7. The time-dependent formation of the glutathione–Mannich base monoadduct is expressed as the [Mannich base]:[monoSG adduct] ratio versus time (min). The inset is a closer view of the primary plot. b) Reaction of compound **1** or **2** in the presence of 2 equiv GSH at pH ~7. The time-dependent formation of the glutathione–Mannich base bisadduct is expressed as the [bisSG adduct] (%) versus time (min).

reactivity of an unsaturated Mannich base possessing an electron-donating group on the phenyl ring in the *para* position relative to the enone; the amino group in **13a** causes the Michael addition to be ~10 times slower because of the increased electronic density in the double bond of the enone. In the case of the unsaturated Mannich base **20** ($t_R$ = 16.75 min), the Michael addition is so slow that the monoadduct was not observed even after 3 weeks. When comparing compounds **1** and **2**, both are almost as reactive in the first monoglutathionylation reaction; the reaction is so rapid that, at time 0, the [Mannich base]:[monoSG adduct] ratio cannot be estimated. Because the $pK_a$ value of the amine leaving group was shown to affect the rate of the base-dependent deamination reaction and consequently the rate of the enzyme inactivation,[19] the replacement of the piperidine in compound **2** by a dimethylamine in derivative **1** as the leaving group of the unsaturated Mannich base derivatives has, as expected, a significant influence on the formation of the bisglutathionylated Mannich bases in this experiment (Scheme 1, adduct structure B). In the case of compound **1** (bisSG adduct of **1**, $t_R$ = 14.34 min) the bisadduct formation is complete after 24 h, while for compound **2** (bisSG adduct of **2**, $t_R$ = 14.19 min) the formation is complete after 70 h (Figure 2b). The reaction curve did not reach 100% because a small amount of GSH oxidized to GSSG under the reaction conditions and did not participate further in the reaction.

### Enzymatic assay

The capability of compounds **2**, **13a** and **3a** to inactivate thiol redox enzymes was studied in assays using the two flavoenzymes *P. falciparum* TrxR and *T. cruzi* TR. The enzymes were pre-incubated for 5 min with NADPH and the inhibitor or DMSO (control). The residual activity was measured under steady-state conditions in the presence of 100 μM NADPH and the disulfide (3 mM DTNB or 100 μM $TS_2$), and an aliquot of the pre-incubated enzyme solution. Inhibition of the DTNB reduction rate by 45% was observed after incubation of *P. falciparum* TrxR with compound **2** (50 μM), **13a** (100 μM), and **3a** (400 μM), respectively. Thus, replacement of the hydrogen by an electron-donating amino group *para* to the enone decreases the Michael acceptor properties of the conjugated system resulting in a significant loss of activity. The increased electron density of the double bond led to a decrease in thiol attack at this site. In contrast, incubation of TR with 200 μM of compounds **2** and **13a**, and 100 μM of compound **3a** for 5 min resulted in 58%, 47% and 52% inactivation, respectively. Thus, despite the weaker Michael acceptor properties, compound **3a** was the most efficient TR inhibitor because the melamine motif, a cyclic amidine group, improved the binding of compound **3a** to TR, probably through specific ionic or cation–π interactions[3] with the acidic and hydrophobic residues of the $TS_2$ binding site. In contrast, compound **20**, introduced in the study to evaluate the requirement of one or two electrophilic centers for disulfide reductase inhibition, had no effect on TR, even at a concentration of 400 μM.

### Antiparasitic activities and toxicity in the primary screening assays

The biological activities of the Mannich bases **2–5**, **12a-c**, **13a-c**, **17**–**18**, and **20** against the intracellular amastigote stages of *T. cruzi* and *L. donovani* and the bloodstream form of *T. brucei brucei*, are given in table S1 (Supporting Information). The cytotoxicity of the compounds was determined in assays using the mammalian MRC-5 cell line. The $IC_{50}$ values represent the drug concentration that inhibits the growth of the parasites or the human cells by 50%. Compounds with $IC_{50}$ values below 5 μM towards *T. cruzi* and below 1 μM in the case of *T. brucei* can be considered as trypanocidal lead molecules.

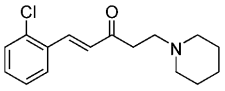
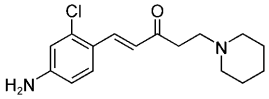
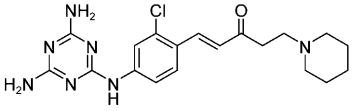
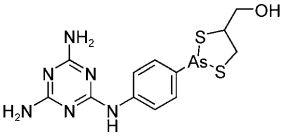
The newly synthesized derivatives did not display significant activities against *Leishmania* species or *T. cruzi*. The phenolic Mannich base **5** was completely inactive towards all parasites, suggesting that oxidative metabolism to the reactive quinone imine did not occur to a significant extent in *T. brucei*. The most potent compounds against *T. brucei* were the unsaturated Mannich bases possessing two electrophilic centers (compounds **2**–**4**, **12a-c**, **13a-c**,**17**–**18**) rather than one electrophilic center (compound **20**). The introduction of the melamine motif *meta* to the enone group (compounds **18**, **4**) significantly lowered the $IC_{50}$ values against the human cell line suggesting high cytotoxicity; this was not observed when the amino or the melamino group was *para* to the enone group (compounds **3a**, **3c**, **13a-c**). The melamine motif *para* position to the enone did not result in any improvement in the trypanocidal potencies of the final compounds **3a-c** when compared with the parent unsaturated Mannich bases (**2** and **13a-c**). The converse was observed for compounds **12a** and **17** bearing an acetamide group *para* and *meta* to the enone, respectively; the introduction of a substituent at the *meta* position led to an increase in activity against *T. brucei* with a twofold lower toxicity against mammalian MRC-5 cells, and good activity against *L. infantum* was observed for the first time.

### P2 transporter studies in *T. brucei brucei*

To determine whether compounds **2**, **3a** and **13a** are taken up by *T. brucei brucei* (strain 427) via the P2 transporter two approaches were followed. Firstly, the $IC_{50}$ values against several *T. brucei brucei* lines were determined. The cell lines were the wild type (strain 427), a *TbAT1*$^{-/-}$ knockout (KO) cell line[14] and the B48 strain derived from the AT1 KO line by selection with pentamidine.[6] The compounds exhibited potent trypanocidal activity against all three strains (Table 1). The specific $IC_{50}$ values against the wild-type cells ranged from 0.2 to 0.3 μM. Against the *TbAT1*$^{-/-}$ knockout line, comparable $IC_{50}$ values were observed. In the case of the B48 cell line, the $IC_{50}$ values for compounds **2** and **3a** were even lower than for the wild-type cells. Thus, neither the *TbAT1*$^{-/-}$ knockout cell line nor the B48 cell line displayed any resistance to the Mannich bases. This strongly suggests that the P2 and/or HAPT1 transporters are not the main routes of uptake of these compounds.

**Table 1.** Effects of Mannich base derivatives on the growth of *T. brucei brucei* strains lacking the P2 transporter.

| Cpnd | structure | *T. brucei brucei* strains $IC_{50}$ values (μM) wild type | P2-KO | P2-KO[R][a] |
|---|---|---|---|---|
| **2** |  | 0.19 ± 0.07 | 0.22 ± 0.04 | 0.07 ± 0.04 |
| **13a** |  | 0.20 ± 0.04 | 0.11 ± 0.07 | 0.08 ± 0.11 |
| **3a** |  | 0.29 ± 0.05 | 0.34 ± 0.09 | 0.38 ± 0.12 |
| mel[b] |  | 0.007 | 0.018 | 0.110[c] |

[a] P2-KO[R] selected for pentamidine resistance. [b] mel = melarsoprol. [c] Data from reference [6].

### Uptake assay

Compounds **2**, **3a** and **13a**, showing the highest activity against the *T. brucei brucei* parasites (table S1, Supporting Information), were studied for their ability to inhibit the adenosine uptake by the P2 aminopurine transporter of *T. brucei*. The [$^3$H]adenosine uptake assay contained 1 mM inosine to saturate the P1 transporters,[5] leaving P2 as the major route of entry for substrates with affinity for purine transporters. As shown in Figure 3, increasing concentrations of compounds **2** and **13a** up to 100 μM did not completely block [$^3$H]adenosine uptake, suggesting that the compounds do not have a high affinity for the P2 transporter. In contrast, the melamine derivative **3a** was able to compete with the adenosine uptake with an $IC_{50}$ value (i.e. concentration whereby 50% of the transporters are occupied with the compound) of 4.0 ± 0.4 μM. The ability of compound **3a** to inhibit the uptake of [$^3$H]adenosine correlates with the presence of the melamine group attached to the chlorobenzyl ring.

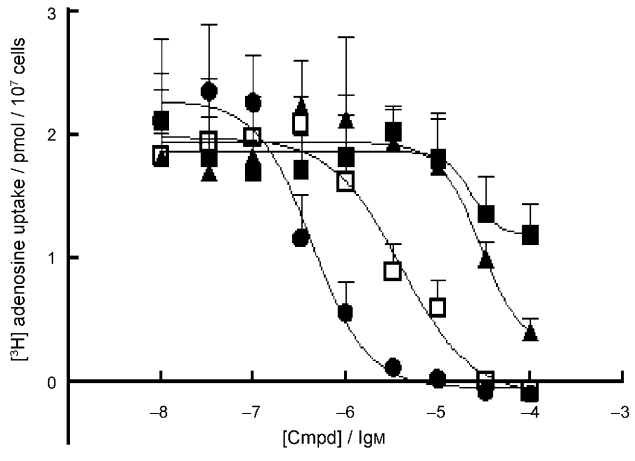



**Figure 3.** [$^3$H]Adenosine uptake of *T. brucei brucei* (wild type) in the presence of increasing concentrations of compounds **2** (■), **3a** (□), **13a** (▲), and pentamidine (●). The cells were incubated for 30 s in the presence of inosine (1 mM) and the compound of interest. Tritium incorporation was measured and the values given are the mean of raw data with standard deviations obtained in duplicate from three independent experiments.

The Mannich base **3a** and the diamidine pentamidine both competed with adenosine for P2 uptake. Since unsaturated Mannich bases are able to alkylate proteins, we investigated the reversibility of the impaired adenosine uptake caused by compound **3a**. Reversible impairment of adenosine uptake would indicate that both compounds are competitive substrates for the transporter, while irreversible impairment would indicate that compound **3a** alkylates the transporter. Wild-type trypanosomes were pretreated with compound **3a** or pentamidine for 1 and 20 min, respectively, followed by washing, and then subjected to an [$^3$H]adenosine uptake assay. The [$^3$H]adenosine uptake by cells pretreated with compound **3a** became saturated, achieving a maximum value of 8 ± 2 pmoles per $10^7$ cells after ~2 min (Figure 4a). There was no significant difference between cells pretreated for 1 or 20 min with compound **3a** or pentamidine (Figure 4b) and untreated cells (Figure 4c). Thus, compound **3a** does not covalently modify the P2 transporter.

## Discussion

Unsaturated Mannich bases can react spontaneously with thiols, such as GSH present in cells at millimolar concentrations, to form the monoadduct initially, through a reversible Michael addition. During this process a base-dependent deamination allows the second electrophilic center to be exposed and the second Michael addition to occur. The bisadducts are then expected to be extruded out of the cells by ATP-dependent glutathione conjugate transporters. As shown here, the presence of an electron-donating group on the phenyl ring

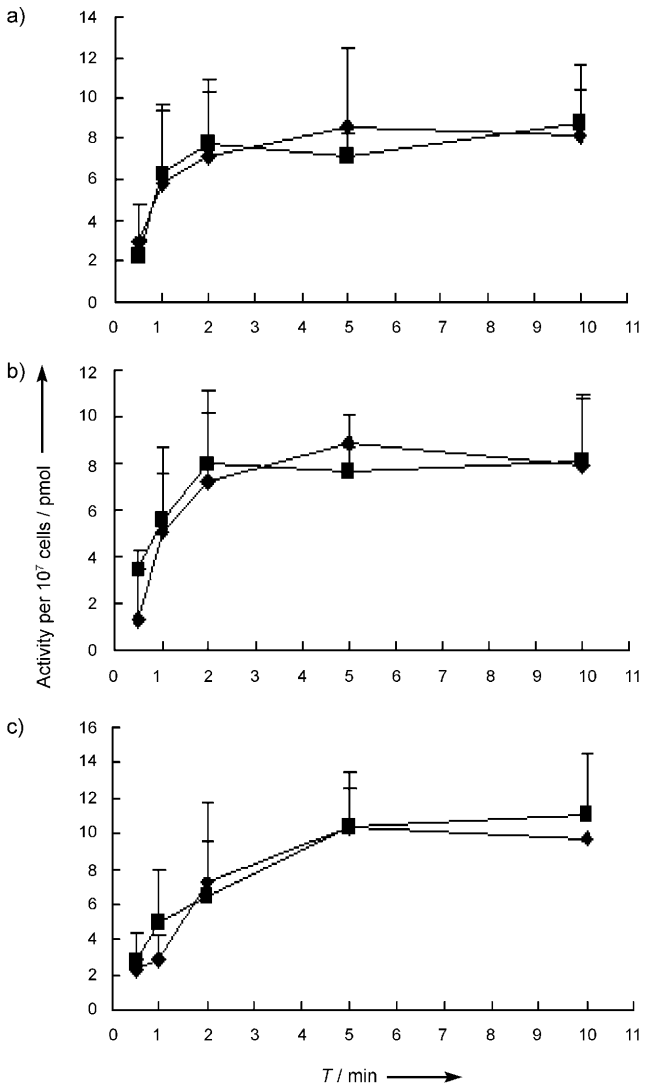



**Figure 4.** [$^3$H]Adenosine uptake by *T. brucei brucei* pretreated with different drugs. Wild-type cells were pre-incubated in the presence of inosine (1 mM) for 1 min (◆) or 20 min (■) with a) 1.5 μM **3a**, b) 2 nM pentamidine, or c) without inhibitor. Uptake activities are expressed as $^3$[H]adenosine incorporation followed over 10 min as described in the Experimental Section. Tritium incorporation was measured and the values represent the mean of raw data with standard deviations obtained in duplicate from three independent experiments.

*para* to the enone led to a delay of the first Michael addition. The Michael addition to compound **13a**, with a -$NH_2$ group on the phenyl ring *para* to the enone, was tenfold slower than that of compound **2** because of the increased electronic density at the double bond of the enone. This is a desired parameter, as rapid clearance of the drug by glutathionylation is avoided, allowing the drug to reach the parasite where the more reactive dithiols will shift the equilibrium of Michael addition and base-dependent deamination reactions to completion. Notably, the reaction between an unsaturated Mannich base (**1** or **2**) and trypanothione was observed to generate polymers.[20] Once formed in the parasites these polymers are not expected to be easily extruded, potentially causing the specific toxicity towards *T. brucei* parasites.

The unsaturated Mannich base **2** was previously shown to be active against *T. brucei rhodesiense* and *L. donovani*, exhibiting $IC_{50}$ values of 0.8 μM and 13.0 μM, respectively.[20] Therefore, compound **2** was selected as a scaffold for the synthesis of novel derivatives to increase the specificity and possibly the potency of the unsaturated Mannich base. Two such compounds were **13a**, with a primary amino group attached to the chlorobenzyl ring at position 5, and **3a** which was modified by a melamine group at the same position.

Mutant cell types derived from *T. brucei brucei* (strain 427) were included in the in vitro toxicity assay. These were the *TbAT1*$^{-/-}$ knockout line and the KO-B48 line that was derived from the knockout by selection of high level pentamidine resistance, which led to loss of HAPT1 activity.[6,14] Increasing resistance towards the diamidines and melaminophenylarsenicals has been documented for these cell types. For example, the resistance factors exhibited by the *TbAT1*$^{-/-}$ and KO-B48 towards pentamidine compared to the wild-type cells were 2.4 and 130, respectively.[6,14] The unsaturated Mannich bases exerted similar trypanocidal activity against all three cell lines including the wild type. This indicates that these compounds continue to enter trypanosomes regardless of the presence of the TbAT1 or HAPT1 transporters. The P2 transporter apparently plays a role in the uptake of compound **3a**, whereas the derivatives that lack the melamine moiety (compounds **2** and **13a**) do not inhibit the transporter. Compound **3a** appears to be a reversible competitive substrate for the P2 transporter and not an irreversible inhibitor since activity returned once the compound had been removed. Thus, compound **3a** did not form covalent thioether bond(s) with the P2 transporter. This result is significant as it indicates that the unsaturated Mannich base does not interact via Michael addition with carrier proteins at the cell surface. Compound **3a** exhibited similar potency against all three cell types tested (wild type, *TbAT1*$^{-/-}$ and KO-B48) indicating that other route(s) of uptake exist, as has been reported for some melamine-based nitroheterocycles and monobenzamidine compounds.[23,24] In these studies Stewart and co-workers showed that the compounds have affinity for P2 but continued to exhibit potent trypanocidal activity against the *TbAT1*$^{-/-}$ mutants.[23,24] These results are encouraging in view of overcoming the problem of drug resistance and cross resistance in the field. Various alternative routes of uptake have been suggested for the auxotrophic parasites, including the folate/biopterin transporters[25] and amino acid transporters,[26] which should also be optimized in drug delivery.

## Conclusions

Our studies were aimed at the identification of trypanocidal Mannich base derivatives capable of inducing selective toxicity through selective uptake of compounds into *T. brucei* using the P2 aminopurine transporter and other carriers capable of recognizing melamine-bearing moieties. The compounds synthesized were investigated for their ability to antagonize uptake of radiolabeled adenosine through the P2 transporter. The result of the binding studies indicated that the com-

pounds without a melamine motif were potent trypanocidal agents that appeared to be taken up effectively into cells despite the absence of P2 and HAPT1 carriers. This suggests that the main route of entry for the compounds was not the aminopurine transporters. Accordingly, other transporters or passive diffusion could be involved in drug uptake. They also do not bind irreversibly with nitrogen-rich molecules (e.g. proteins) in spite of potential Michael reactions occurring. However, the presence of the melamine moiety on compound **3a** did confer some affinity for the P2 transporter, albeit to a lower extent when compared to pentamidine, and weaker activity against the parasites. It was not possible to directly correlate the affinity of the P2 transporter and the trypanocidal activity because there were no remarkable changes of in vitro activities against *T. brucei* compared to the activity against the P2 transporter deficient *T. brucei* knockout line.

Interestingly, compounds **2** and **13a** showed poor affinity for the P2 transporter but marked in vitro activity against *T. brucei*, especially against the pentamidine-resistant strain. They also showed approximately the same trypanocidal activity against *T. brucei* KO. Resistance resulting from simple loss of the P2 transporter should not be an issue when other routes of drug uptake exist for these compounds. Some of the analogues synthesized with aromatic substitution variations showed good trypanocidal activity in intact parasites with a two- to tenfold lower toxicity against mammalian MRC-5 cells (table S1, Supporting Information). Altogether, these novel compounds appear to be promising candidates for trypanosomal chemotherapy and overcoming drug resistance. Further work is required to obtain better selectivity. We adopted the premise that formation of the divinylketone is a necessary part of the mechanism of action of these compounds (Scheme 1). With this in mind, the *N,N*-diethyl acrylophenone **20** containing only one electrophilic site was predicted to have a very low activity against the parasites compared to other derivatives where a divinylketone intermediate (two electrophilic sites) can be formed. Due to the fact that the phenolic Mannich base **5** showed very low activity against the intact parasites, we concluded that no highly expressed oxidative metabolism might transform the phenolic Mannich base to a reactive quinone imine in *T. brucei*.

## Experimental Section

### Chemistry

Melting points were determined on a Büchi melting point apparatus and were not corrected. $^1$H (300 MHz) and $^{13}$C (75 MHz) NMR spectra were recorded on a Bruker DRX-300 spectrometer; chemical shifts were expressed in ppm relative to TMS; the protons are indicated as $H_{vin}$ (H linked to the double bond), $H_{pip}$ (H linked to the piperidine moiety) and $H_{Ar}$ (H linked to the aryl group) in the $^1$H spectra; multiplicity is indicated as s (singlet), d (doublet), t (triplet), and m (multiplet); C indicates a quaternary carbon in $^{13}$C spectra. Elemental analyses were carried out at the Mikroanalytisches Laboratorium der Chemischen Fakultät der Universität Heidelberg. EI-MS, ESI-MS, FAB and MALDI-TOF were recorded using the facilities at the Institut für Organische Chemie der Universität Heidelberg. Analytical TLC was carried out on pre-coated Sil G-25 $UV_{254}$ plates from Macherey & Nagel. Flash chromatography was performed using silica gel G60 (230–400 mesh) from Macherey & Nagel. The unsaturated Mannich bases, 1-(2-chlorophenyl)-5-(dimethylamino)pent-1-en-3-one hydrochloride (**1**) and 1-(2-chlorophenyl)-5-(*N*-piperidino)pent-1-en-3-one hydrochloride (**2**) were synthesized as previously described.[19] (*E*)-3-Diethylamino-1-phenylpropenone (**20**) was formed in a Pd-catalyzed one-pot reaction with TMSA, phenylcarboxylic acid chloride and diethylamine according to described procedure.[22] 4-Amino-2-chloro-benzaldehyde (**9a**) was synthesized according to described procedure.[27,28] *N*-(3-Chloro-4-cyano-phenyl)acetamide (**7a**), 4-amino-2-methyl-benzaldehyde (**9b**), *N*-(3-chloro-4-formyl-phenyl)acetamide (**10a**), and *N*-[4-((*E*)-3-oxo-but-1-enyl)phenyl]acetamide (**11c**) are known compounds but were produced according to new routes. The purity of new compounds **3**–**5**, **12**–**19** was controlled by melting points and elemental analyses that agreed with the calculated values within 0.4%.

***N*-(3-Chloro-4-cyano-phenyl)acetamide 7a**: $Ac_2O$ (6.0 mL, 58 mmol) and dry TEA (18.5 mL, 133 mmol) were added dropwise to a suspension of 4-amino-2-chloro-benzonitrile (8.1 g, 53 mmol) in dry toluene (360 mL). The mixture was refluxed for 24 h. The precipitate was filtered, washed with AcOH and water, and dried in vacuo to obtain compound **7a** as a solid (7.9 g, 77%): $^1$H NMR (250 MHz, [$D_6$]DMSO) $\delta = 10.53$ (s, 1H, CON*H*), 8.02 (s, 1H, $H_{Ar}$), 7.87 (d, $J = 8.45$ Hz, 1H, $H_{Ar}$), 7.58 (d, $J = 8.07$ Hz, 1H, $H_{Ar}$), 2.11 ppm (s, 3H, C$H_3$CONH).

**4-Amino-2-methyl-benzaldehyde 9b**: A refluxing solution of NaOH (27.0 g, 324 mmol), $Na_2S{\cdot}9H_2O$ (80 g, 468 mmol) and sulfur (15 g, 468 mmol) in distilled water (500 mL) was treated slowly with 1,2-dimethyl-4-nitro-benzene (54.04 g, 360 mmol) in EtOH (300 mL). The solution was refluxed for 3 h and the EtOH was evaporated. The mixture was extracted with chloroform, and the organic phase was dried ($MgSO_4$), filtered and evaporated in vacuo to obtain **9b** as a solid (26 g, 54%): $^1$H NMR (300 MHz, $CDCl_3$) $\delta =$ 10.00 (s, 1H, C*H*O), 7.63 (d, $J = 8.31$ Hz, 1H, $H_{Ar}$), 6.56 (d, $J = 8.30$ Hz, 1H, $H_{Ar}$), 6.46 (s, 1H, $H_{Ar}$), 2.59 ppm (s, 3H, C$H_3$).

***N*-(3-Chloro-4-formyl-phenyl)acetamide 10a**: 4-Amino-2-chlorobenzaldehyde **9a** (14.8 g, 95 mmol) was dissolved in $Ac_2O$ (250 mL) and stirred overnight at RT. The orange-red suspension was poured into ice water and the precipitate was filtered and dried in vacuo to afford **10a** as white-yellow cristals (13.4 g, 72%): mp: 150–151 °C; $^1$H NMR (300 MHz, [$D_6$]DMSO) $\delta = 10.51$ (s, 1H, C*H*O), 10.20 (s, 1H, CON*H*), 7.97 (s, 1H, $H_{Ar}$), 7.83 (d, $J = 8.62$ Hz, 1H, $H_{Ar}$), 7.57 (d, $J = 8.52$ Hz, 1H, $H_{Ar}$), 2.11 ppm (s, 3H, C$H_3$CONH); $^{13}$C NMR (75 MHz, [$D_6$]DMSO) $\delta = 188.31$ (*C*H), 169.49 (C), 145.40 (C), 137.32 (C), 130.66 (*C*H), 126.72 (C), 119.05 (C), 117.40 (*C*H), 24.26 ppm (*C*$H_3$); MS (EI) *m/z*: 196.94 (M+): Anal. calcd for $C_9H_8ClNO_2$: C 54.70, H 4.08, N 7.09, Cl 17.94, found: C 54.78, H 4.23, N 6.99, Cl 18.17%.

**(*E*)-4-(2-Chloro-5-nitro-phenyl)but-3-en-2-one 15**: A solution of compound **14** (10.49 g, 57 mmol) in acetone (400 mL) and water (200 mL) was cooled to 0 °C and treated with aq NaOH (8 mL, 2 N) until the colorless solution turned orange. The reaction mixture was immediately neutralized with aq $H_2SO_4$ (2.5 N) and the organic solvent was concentrated in vacuo. The precipitate was isolated, redissolved in MeOH/2.5 N aq $H_2SO_4$ (1:1) and heated to reflux for 3 h. The reaction mixture was diluted with water and the precipitate was filtered and dried in vacuo to afford **15** as a yellow-white solid (10.8 g, 85%): mp: 110–112 °C; $^1$H NMR (300 MHz, $CDCl_3$) $\delta =$ 8.52 (s, 1H, $H_{Ar}$), 8.19 (d, $J = 8.73$ Hz, 1H, $H_{Ar}$), 7.90 (d, $J = 16.22$ Hz,

1H, $H_{vin}$), 7.64 (d, $J=8.79$ Hz, 1H, $H_{Ar}$), 6.84 (d, $J=16.23$ Hz, 1H, $H_{vin}$), 2.46 ppm (s, 3H, $CH_3$); $^{13}C$ NMR (75 MHz, $CDCl_3$) $\delta=197.24$, 146.83, 141.31, 136.40, 134.35, 131.44, 131.25, 125.15, 122.48, 28.17 ppm; MS (EI) *m/z*: 225.02 (M+); Anal. calcd for $C_{10}H_8ClNO_3 \cdot 0.4H_2O$: C 51.59, H 3.81, N 6.02, Cl 15.23, found: C 51.88, H 3.62, N 6.00, Cl 14.80%.

***N*-[4-Chloro-3-((*E*)-3-oxo-but-1-enyl)phenyl]-acetamide 16**: A solution of compound **15** (4.6 g, 20 mmol) in EtOH (65 g) was treated with $SnCl_2$ (17.27 g, 91 mmol) in concd HCl (35.5 g) and the reaction was stirred for 4 h at 70 °C. After cooling to RT, the solid was filtered and the filtrate was basified with NaOH solution. The resulting suspension was extracted with DCM, the combined organic layers were dried ($MgSO_4$), filtered, and the solvent was removed in vacuo. The residue was dissolved in $Ac_2O$ and left overnight. The reaction mixture was poured into ice water and the precipitate was filtered and dried in vacuo to obtain compound **16** as a yellow solid (4.8 g, 72%): mp: 155–158 °C; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta=$ 8.52 (s, 1H, $H_{Ar}$), 8.19 (d, $J=8.73$ Hz, 1H, $H_{Ar}$), 7.90 (d, $J=16.22$ Hz, 1H, $H_{vin}$), 7.64 (d, $J=8.79$ Hz, 1H, $H_{Ar}$), 6.84 (d, $J=16.23$ Hz, 1H, $H_{vin}$), 2.46 ppm (s, 3H, $CH_3$); $^{13}C$ NMR (75 MHz, $CDCl_3$) $\delta=197.24$, 146.83, 141.31, 136.40, 134.35, 131.44, 131.25, 125.15, 122.48, 28.17 ppm; MS (FAB) *m/z*: 238.0 (M+); Anal. calcd for $C_{12}H_{12}ClNO_2 \cdot 0.6$ HCl: C 55.53, H 4.89, N 5.40, Cl 21.85, found: C 55.40, H 4.68, N 5.42, Cl 17.20%.

**Claisen–Schmidt reaction (general procedure 1)**: A solution of aq NaOH (5%) and acetone (1:25) was stirred for 20 min at RT. The aldehyde was added and the reaction mixture was stirred for a further 3 h. The reaction solution was triturated with water causing the α,β-unsaturated ketone to precipitate. The solid was filtered, washed with water and dried in vacuo.

***N*-[3-Chloro-4-((*E*)-3-oxo-but-1-enyl)phenyl]-acetamide 11a**: Compound **10a** (3 g, 15 mmol) was used as the starting material and treated according to the general procedure 1 to give compound **11a** as a light yellow solid (2.6 g, 73%): mp: 185–187 °C; $^1H$ NMR (300 MHz, [D$_6$]DMSO) $\delta=10.32$ (s, 1H, CONH), 7.92 (s, 1H, $H_{Ar}$), 7.88 (d, $J=8.71$ Hz, 1H, $H_{Ar}$), 7.74 (d, $J=16.21$ Hz, 1H, $H_{vin}$), 7.49 (d, $J=$ 8.69 Hz, 1H, $H_{Ar}$), 6.82 (d, $J=16.19$ Hz, 1H, $H_{vin}$), 2.33 (s, 3H, $CH_3$CONH), 2.08 ppm (s, 3H, $CH_3$CO); $^{13}C$ NMR (75 MHz, [D$_6$]DMSO) $\delta=197.51$ (C), 168.96 (C), 142.09, 136.95, 134.42, 128.54, 127.75, 126.07, 119.00, 117.78 (CH), 27.81 ($CH_3$), 24.10 ppm ($CH_3$); MS (EI) *m/z*: 237.1 (M+); Anal. calcd for $C_{12}H_{12}ClNO_2$: C 60.64, H 5.09, N 5.89, Cl 14.92, found: C 60.59, H 5.19, N 5.82, Cl 14.92%.

***N*-[3-Methyl-4-((*E*)-3-oxo-but-1-enyl)phenyl]-acetamide 11b**: *N*-(4-Formyl-3-methyl-phenyl)acetamide **10b** (3 g, 17 mmol) was used as the starting material and treated according to the general procedure 1 to give compound **11b** as a light yellow solid (3.1 g, 84%): mp: 143–145 °C; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta=10.07$ (s, 1H, CONH), 7.72 (d, $J=16.30$ Hz, 1H, $H_{vin}$), 7.66 (m, 1H, $H_{Ar}$), 7.49 (s, 1H, $H_{Ar}$), 7.46 (m, 1H, $H_{Ar}$), 6.65 (d, $J=16.11$ Hz, 1H, $H_{vin}$), 2.39 (s, 3H, $CH_3$), 2.33 (s, 3H, $CH_3$), 2.05 ppm (s, 3H, $CH_3$); $^{13}C$ NMR (75 MHz, $CDCl_3$) $\delta=197.86$ (C), 168.56 (C), 141.04 (C), 139.70 (CH), 138.61 (C), 127.51 (C), 127.24 (CH), 126.38 (CH), 120.38 (CH), 116.84 (CH), 27.46 ($CH_3$), 24.06 ($CH_3$), 19.53 ppm ($CH_3$); MS (FAB) *m/z*: 218.2 (M+); Anal. calcd for $C_{13}H_{15}NO_2$: C 71.87, H 6.96, N 6.45, found: C 71.89, H 7.02, N 6.49%.

***N*-[4-((*E*)-3-Oxo-but-1-enyl)phenyl]acetamide 11c**: 4-Acetamidobenzaldehyde (5 g, 31 mmol) was used as the starting material and treated according to the general procedure 1 to give compound **11c** as light yellow solid (6.06 g, 81%).

**Mannich reaction (general procedure 2)**: The suspension of piperidine hydrochloride (1.1–1.3 equiv) and paraformaldehyde (1.25–1.3 equiv) in dry EtOH was refluxed for 1.5–2 h. The reaction mixture was treated with aq HCl (25%) until a colorless solution was obtained (~1 drop). The reaction mixture was treated with the α,β-unsaturated ketone (1 equiv) and heated at reflux overnight. The solvent was removed in vacuo and the residue was washed with acetone and recrystallized from pure EtOH or EtOH/water (95:5).

***N*-[3-Chloro-4-((*E*)-3-oxo-5-piperidin-1-yl-pent-1-enyl)phenyl]acetamide 12a**: Compound **11a** (2.5 g, 11 mmol) was used as the starting material and treated according to the general procedure 2 to give compound **12a** as a yellow solid (1.1 g, 31%): mp: 208–210 °C; $^1H$ NMR (300 MHz, $CD_3OD$) $\delta=8.03$ (d, $J=16.09$ Hz, 1H, $H_{vin}$), 7.90 (s, 1H, $H_{Ar}$), 7.82 (d, $J=8.71$ Hz, 1H, $H_{Ar}$), 7.51 (d, $J=$ 8.56 Hz, 1H, $H_{Ar}$), 6.92 (d, $J=16.06$ Hz, 1H, $H_{vin}$), 3.66–3.52 (m, 2H, $CH_2$), 3.52–3.40 (m, 2H, $CH_2$), 3.39–3.26 (m, 2H, $H_{pip}$), 3.10–2.90 (m, 2H, $H_{pip}$), 2.18 (s, 3H, $CH_3$CO), 2.08–1.68 (m, 5H, $H_{pip}$), 1.68–1.41 ppm (m, 1H, $H_{pip}$); $^{13}C$ NMR (75 MHz, $CD_3OD$) $\delta=197.84$ (C), 172.34 (C), 143.28 (C), 139.94 (CH), 137.07 (C), 129.53 (CH), 128.44 (C), 126.99 (CH), 121.41 (CH), 119.57 (CH), 54.82 ($CH_2$), 53.12 ($CH_2$), 36.17 ($CH_2$), 24.25 ($CH_2$), 24.17 ($CH_3$), 22.61 ppm ($CH_2$); MS (FAB) *m/z*: 335.3 (M+); Anal. calcd for $C_{18}H_{23}ClN_2O_2 \cdot$ 1HCl: C 58.23, H 6.51, N 7.54, Cl 19.10, found: C 58.11, H 6.56, N 7.49, Cl 19.34%.

***N*-[3-Methyl-4-((*E*)-3-oxo-5-piperidin-1-yl-pent-1-enyl)phenyl]acetamide 12b**: Compound **11b** (3 g, 14 mmol) was used as the starting material and treated according to the general procedure 2 to give compound **12b** as a yellow solid (2.21 g, 51%): mp: 199–201 °C; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta=7.97$ (d, $J=15.8$ Hz, 1H, $H_{vin}$), 7.69 (d, $J=8.45$ Hz, 1H, $H_{Ar}$), 7.51 (d, $J=7.41$ Hz, 1H, $H_{Ar}$), 7.49 (s, 1H, $H_{Ar}$), 6.80 (d, $J=16.09$ Hz, 1H, $H_{vin}$), 3.47 (t, 2H, $CH_2$), 3.16 (t, 2H, $CH_2$), 2.46 (s, 3H, $CH_3$CONH), 2.17 (s, 3H, $CH_3$-Ar), 2.00–1.50 ppm (m, 10H, $H_{pip}$); $^{13}C$ NMR (75 MHz, $CDCl_3$) $\delta=198.65$ (C), 172.52 (C), 142.69 (C), 142.66 (CH), 141.37 (C), 130.14 (C), 128.95 (CH), 126.13 (CH), 122.97 (CH), 119.31 (CH), 55.18 ($CH_2$), 53.55 ($CH_2$), 46.15 ($CH_2$), 24.69 ($CH_2$), 24.54 ($CH_3$), 24.16 ($CH_2$), 23.52 ($CH_2$), 23.06 ($CH_2$), 20.46 ppm ($CH_3$). MS (FAB) *m/z*: 315.2 (M+); Anal calcd for $C_{19}H_{26}N_2O_2 \cdot$ 1.2HCl $\cdot$ 0.2$H_2O$: C 63.08, H 7.69, N 7.74, Cl 11.76, found: C 62.83, H 7.94, N 8.09, Cl 11.36%.

***N*-[4-((*E*)-3-Oxo-5-piperidin-1-yl-pent-1-enyl)phenyl]-acetamide 12c**: *N*-[4-((*E*)-3-Oxo-but-1-enyl)phenyl]acetamide **11c** (2 g, 9.84 mmol) was used as the starting material and treated according to the general procedure 2 to give compound **12c** as a yellow solid (1.3 g, 45%): mp: 221–222 °C; $^1H$ NMR (300 MHz, $CD_3OD$) $\delta=$ 7.72 (d, $J=16.22$ Hz, 1H, $H_{vin}$), 7.66 (s, 4H, $H_{Ar}$), 6.83 (d, $J=16.23$ Hz, 1H, $H_{vin}$), 3.70–3.42 (m, 4H, $CH_2$), 3.22–2.84 (m, 3H, $H_{pip}$), 2.17 (s, 3H, $CH_3$CO), 2.05–1.45 ppm (m, 7H, $H_{pip}$); $^{13}C$ NMR (75 MHz, $CD_3OD$) $\delta=198.48$ (C), 172.25 (C), 145.50 (CH), 142.56 (C), 131.13 (C), 130.69 (CH), 125.13 (CH), 121.14 (CH), 54.79 ($CH_2$), 53.13 ($CH_2$), 45.76 ($CH_2$), 24.25 ($CH_2$), 24.15 ($CH_3$), 22.63 ppm ($CH_2$); MS (ESI) *m/z*: 301.3 (M+); Anal. Calcd for $C_{18}H_{24}N_2O_2 \cdot$ 1HCl: C 64.18, H 7.48, N 8.32, Cl 10.52, found: C 64.01, H 7.49, N 8.25, Cl 10.70%.

***N*-[4-Chloro-3-((*E*)-3-oxo-5-piperidin-1-yl-pent-1-enyl)phenyl]-acetamide 17**: Compound **16** (2.5 g, 11 mmol) was used as the starting material and treated according to the general procedure to give compound **17** as a yellow-white solid (2.1 g, 60%): mp: 203–207 °C; $^1H$ NMR (300 MHz, $CD_3OD$) $\delta=8.17$ (s, 1H, $H_{Ar}$), 8.04 (d, $J=16.16$ Hz, 1H, $H_{vin}$), 7.56 (d, $J=8.76$ Hz, 1H, $H_{Ar}$), 7.42 (d, $J=$ 8.72 Hz, 1H, $H_{Ar}$), 6.89 (d, $J=16.12$ Hz, 1H, $H_{vin}$), 3.73–3.22 (m, 5H, $CH_2$, $H_{pip}$), 3.04 (t, $J=11.35$ Hz, 2H, $H_{pip}$), 2.05–1.38 ppm (m, 7H, $H_{pip}$); $^{13}C$ NMR (75 MHz, $CD_3OD$) $\delta=197.38$ (C), 171.86 (C), 140.12

(CH), 139.58 (C), 133.73 (C), 131.49 (CH), 130.82 (C), 128.82 (CH), 124.34 (CH), 119.58 (CH), 54.76 ($CH_2$), 52.98 ($CH_2$), 45.73 ($CH_2$), 24.27 ($CH_2$), 23.96 ($CH_3$), 22.61 ppm ($CH_2$); MS (FAB) *m/z*: 335.1 (M+); Anal. Calcd for $C_{18}H_{23}ClN_2O_2 \cdot 1HCl$: C 58.23, H 6.51, N 7.54, Cl 19.10, found: C 57.87, H 6.57, N 7.47, Cl 19.10%.

**Deprotection (general procedure 3)**: The acetamide (1 equiv) was dissolved in an excess of aq HCl (8 N) and the reaction mixture was heated at reflux for 3 h. After removal of the solvent, the residue was recrystallized from EtOH/diethylether.

**(*E*)-1-(4-Amino-2-chloro-phenyl)-5-piperidin-1-yl-pent-1-en-3-one 13a**: Compound **12a** (395 mg, 1.18 mmol) was used as the starting material and treated according to the general procedure 3 to give compound **13a** as a brown solid (329 mg, 95%): mp: 174–175 °C; $^1$H NMR (300 MHz, $CD_3OD$) $\delta = 8.04$ (d, $J = 15.27$ Hz, 1H, $H_{vin}$), 7.99 (d, $J = 7.82$, 1H, $H_{Ar}$), 7.47 (s, 1H, $H_{Ar}$), 7.34 (d, $J = 8.55$ Hz, 1H, $H_{Ar}$), 7.01 (d, $J = 16.13$ Hz, 1H, $H_{vin}$), 3.67–3.34 (m, 5H, $CH_2$, $H_{pip}$), 3.13–2.92 (m, 2H, $H_{pip}$), 2.06–1.45 ppm (m, 7H, $H_{pip}$); $^{13}$C NMR (75 MHz, $CD_3OD$) $\delta = 197.24$ (C), 138.79 (CH), 138.50 (C), 137.44 (C), 131.93 (C), 130.68 (CH), 128.69 (CH), 123.90 (CH), 121.91 (CH); 54.72 ($CH_2$); 52.99 ($CH_2$); 36.31 ($CH_2$); 24.42 ($CH_2$); 22.59 ppm ($CH_2$); MS (FAB) *m/z*: 293.2 (M+); Anal. calcd for $C_{16}H_{21}ClN_2O \cdot 1.8HCl \cdot 0.3H_2O$: C 52.82, H 6.48, N 7.70, Cl 27.28, found: C 52.54, H 6.34, N 7.55, Cl 27.52%.

**(*E*)-1-(4-Amino-2-methyl-phenyl)-5-piperidin-1-yl-pent-1-en-3-one 13b**: Compound **12b** (2 g, 6.36 mmol) was used as the starting material and treated according to the general procedure 3. After cooling with an ice bath, the red solution was diluted with water, made alkaline with concd NaOH solution and extracted with DCM. The combined organic layers were dried ($MgSO_4$), filtered and the organic solvent was removed in vacuo to afford **13b** as an orange solid (1.06 g, 63%): mp: 177–179 °C; $^1$H NMR (300 MHz, $CD_3OD$) $\delta = 8.00$ (d, $J = 16.12$ Hz, 1H, $H_{vin}$), 7.88 (d, $J = 9.05$ Hz, 1H, $H_{Ar}$), 7.34 (s, 1H, $H_{Ar}$), 7.33 (d, $J = 6.70$, 1H, $H_{vin}$), 6.91 (d, $J = 16.08$ Hz, 1H, $H_{vin}$), 3.68–3.34 (m, 5H, $CH_2$, $H_{pip}$), 3.05 (t, $J = 12.18$ Hz, 2H, $H_{pip}$), 2.55 (s, 3H, $CH_3$-Ar), 2.02–1.43 ppm (m, 7H, $H_{pip}$); $^{13}$C NMR (75 MHz, $CD_3OD$) $\delta = 197.62$ (C), 141.88 (C), 140.66 (CH), 135.40 (C), 133.69 (C), 129.53 (CH), 128.93 (CH), 126.14 (CH), 122.13 (CH), 54.69 ($CH_2$), 53.09 ($CH_2$), 36.07 ($CH_2$), 24.2 5 ($CH_2$), 22.63 ($CH_2$), 19.82 ppm ($CH_3$); MS (FAB) *m/z*: 273.3 (M+); Anal. calcd for $C_{17}H_{24}N_2O \cdot 2.1HCl$: C 58.83, H 7.70, N 8.07, Cl 19.41, found: C 58.51, H 7.74, N 8.03, Cl 19.56%.

**(*E*)-1-(4-Amino-phenyl)-5-piperidin-1-yl-pent-1-en-3-one 13c**: Compound **12c** (2 g, 6.66 mmol) was used as the starting material and treated according to the general procedure 3 to give compound **13c** as a red-brown solid (1.6 g, 94%): mp: 112–114 °C; $^1$H NMR (300 MHz, $CDCl_3$) $\delta = 7.51$ (d, $J = 16.04$, 1H, $H_{vin}$), 7.39 (d, $J = 8.23$ Hz, 2H, $H_{Ar}$), 6.67 (d, $J = 8.21$, 2H, $H_{Ar}$), 6.58 (d, $J = 16.08$ Hz, 1H, $H_{vin}$), 4.03 (br s, 2H, $NH_2$-Ar), 2.93–2.79 (m, 2H, $CH_2$), 2.79–2.62 (m, 2H, $CH_2$), 2.51–2.30 (m, 4H, $H_{pip}$), 1.70–1.34 ppm (m, 6H, $H_{pip}$); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta = 199.58$ (C), 149.04 (C), 143.32 (C), 130.24 (CH), 124.57 (CH), 122.45 (CH), 114.87 (CH), 54.52 ($CH_2$), 54.18 ($CH_2$), 38.15 ($CH_2$), 25.98 ($CH_2$), 24.30 ppm ($CH_2$); MS (FAB) *m/z*: 259.3 (M+); Anal. calcd for $C_{16}H_{22}N_2O \cdot 1.9HCl \cdot 2.2H_2O$: C 55.02, H 7.59, N 8.02, Cl 19.29, found: C 55.38, H 7.70, N 8.02, Cl 18.98%.

**(*E*)-1-(5-Amino-2-chloro-phenyl)-5-piperidin-1-yl-pent-1-en-3-one 18**: Compound **17** (1.5 g, 4.49 mmol) was used as the starting material and treated according to the general procedure 3 to give compound **18** as an orange solid (1 g, 79%): mp: 187–189 °C; $^1$H NMR (300 MHz, $CD_3OD$) $\delta = 8.03$ (d, $J = 16.15$ Hz, 1H, $H_{vin}$), 7.91 (s, 1H, $H_{Ar}$), 7.70 (d, $J = 8.54$ Hz, 1H, $H_{Ar}$), 7.49 (d, $J = 8.59$ Hz, 1H, $H_{Ar}$), 7.07 (d, $J = 16.15$ Hz, 1H, $H_{vin}$), 3.72–3.33 (m, 6H, $CH_2$, $H_{pip}$), 3.04 (t, $J = 11.77$ Hz, 2H, $H_{pip}$), 2.10–1.42 ppm (m, 6H, $H_{pip}$); $^{13}$C NMR (75 MHz, $CD_3OD$) $\delta = 197.16$ (C), 138.23 (CH), 136.24 (C), 135.64 (C), 132.98 (CH), 132.12 (C), 130.47 (CH), 127.06 (CH), 123.53 (CH), 54.74 ($CH_2$), 54.42 ($CH_2$), 52.84 ($CH_2$), 24.24 ($CH_2$), 22.58 ppm ($CH_2$); MS (FAB) *m/z*: 293.1 (M+); Anal. calcd for $C_{16}H_{21}ClN_2O \cdot 1.9HCl \cdot 0.2H_2O$: C 52.55, H 6.42, N 7.66, Cl 28.12, found: C 52.48, H 6.47, N 7.45, Cl 28.09%.

**Nucleophilic aromatic substitution (general procedure 4)**: A stirred suspension of the Mannich base derivative (1–1.6 equiv) and 2-chloro-4,6-diamino-1,3,5-triazine (1 equiv) in MeOH was refluxed for 2–3 h. The precipitate was filtered, washed with cold MeOH and dried in vacuo.

**(*E*)-1-[2-Chloro-4-(4,6-diamino-[1,3,5]triazin-2-ylamino)phenyl]-5-piperidin-1-yl-pent-1-en-3-one 3a**: Compound **13a** (329 mg, 1.12 mmol) was used as the starting material and treated according to the general procedure 4 to give compound **3a** as an orange solid (108 mg, 28%): mp: dec. > 120 °C; $^1$H NMR (300 MHz, $D_2O$) $\delta =$ 7.66 (d, $J = 16.35$ Hz, 1H, $H_{vin}$), 7.61 (s, 1H, $H_{Ar}$), 7.48 (d, $J = 8.74$ Hz, 1H, $H_{Ar}$), 7.15 (d, $J = 8.35$ Hz, 1H, $H_{Ar}$), 6.65 (d, $J = 16.12$ Hz, 1H, $H_{vin}$), 3.60–3.45 (m, 2H, $CH_2$), 3.45–3.33 (m, 2H, $CH_2$), 3.27–3.18 (m, 2H, $H_{pip}$), 2.96 (t, $J = 11.51$ Hz, 2H, $H_{pip}$), 2.04–1.33 ppm (m, 6H, $H_{pip}$); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta = 199.93$ (C), 161.50 (C), 159.13 (C), 158.18 (C), 141.55 (C), 140.27 (CH), 136.75 (C), 128.91 (CH), 127.30 (C), 126.00 (CH), 121.40 (CH), 119.75 (CH), 54.61 ($CH_2$), 52.55 ($CH_2$), 35.60 ($CH_2$), 23.81 ($CH_2$), 22.04 ppm ($CH_2$); MS (MALDI-TOF) *m/z*: 402.4 (M+); Anal. calcd for $C_{19}H_{24}N_7ClO \cdot 2HCl \cdot 0.7H_2O$: C 46.82, H 5.67, N 20.12, Cl 21.82, found: C 46.80, H 5.72, N 18.89, Cl 21.94%.

**(*E*)-1-[4-(4,6-Diamino-[1,3,5]triazin-2-ylamino)-2-methyl-phenyl]-5-piperidin-1-yl-pent-1-en-3-one 3b**: Compound **13b** (142 mg, 0.411 mmol) was used as the starting material and treated according to the general procedure 4 to give compound **3b** as a yellow solid (107 mg, 82%): mp: dec. > 200 °C; $^1$H NMR (300 MHz, $D_2O$) $\delta =$ 7.48 (d, $J = 16.00$ Hz, 1H, $H_{vin}$), 7.23 (d, $J = 8.57$ Hz, 1H, $H_{Ar}$), 7.01 (s, 1H, $H_{Ar}$), 7.00 (d, $J = 8.58$ Hz, 1H, $H_{Ar}$), 6.46 (d, $J = 16.02$ Hz, 1H, $H_{vin}$), 3.52–3.40 (m, 2H, $CH_2$), 3.35–3.22 (m, 2H, $CH_2$), 3.22–3.06 (m, 2H, $H_{pip}$), 2.98–2.80 (t, $J = 12.23$ Hz, 2H, $H_{pip}$), 1.96–1.81 (m, 2H, $H_{pip}$), 1.81–1.57 (m, 3H, $H_{pip}$), 1.52–1.32 ppm (m, 1H, $H_{pip}$); $^{13}$C NMR (75 MHz, $D_2O$) $\delta = 200.16$ (C), 160.25 (C), 160.18 (C), 142.33 (CH), 141.07 (C), 140.56 (C), 128.30 (C), 127.94 (CH), 124.51 (CH), 122.19 (CH), 118.64 (CH), 54.62 ($CH_2$), 52.69 ($CH_2$), 35.34 ($CH_2$), 23.82 ($CH_2$), 22.07 ($CH_2$), 20.17 ppm ($CH_3$); MS (MALDI-TOF) *m/z*: 382.4 (M+); Anal. calcd for $C_{20}H_{27}N_7O \cdot 0.9HCl \cdot 0.7H_2O$: C 56.27, H 6.92, N 22.97, Cl 7.47, found: C 56.06, H 6.76, N 22.63, Cl 7.54%.

**(*E*)-1-[4-(4,6-Diamino-[1,3,5]triazin-2-ylamino)phenyl]-5-piperidin-1-yl-pent-1-en-3-one 3c**: Compound **13c** (500 mg, 1.93 mmol) was used as the starting material and treated according to the general procedure 4 to give compound **3c** as an orange-brown solid (562 mg, 79%): mp: dec. > 180 °C; $^1$H NMR (300 MHz, $D_2O$) $\delta =$ 7.52 (d, $J = 16.27$ Hz, 1H, $H_{vin}$), 7.45 (s, 4H, $H_{Ar}$), 6.66 (d, $J = 16.26$ Hz, 1H, $H_{vin}$), 3.59–3.43 (m, 2H, $CH_2$), 3.43–3.31 (m, 2H, $CH_2$), 3.28–3.17 (m, 2H, $H_{pip}$), 2.93 (t, $J = 12.22$ Hz, 2H, $H_{pip}$), 2.02–1.83 (m, 2H, $H_{pip}$), 1.83–1.58 (m, 3H, $H_{pip}$), 1.54–1.32 ppm (m, 1H, $H_{pip}$); $^{13}$C NMR (75 MHz, $D_2O$) $\delta = 200.42$ (C), 161.23 (C), 158.54 (C), 145.87 (CH), 140.54 (C), 130.69 (C), 130.50 (CH), 124.80 (CH), 122.06 (CH), 54.55 ($CH_2$), 52.64 ($CH_2$), 34.83 ($CH_2$), 23.76 ($CH_2$), 22.03 ppm ($CH_2$); MS (MALDI-TOF) *m/z*: 368.2 (M+); Anal. calcd for

$C_{19}H_{25}N_7O{\cdot}2HCl{\cdot}0.8H_2O$: C 50.18, H 6.34, N 21.56, Cl 15.59, found: C 50.18, H 6.33, N 21.22, Cl 15.88%.

**(*E*)-1-[2-Chloro-5-(4,6-diamino-[1,3,5]triazin-2-ylamino)phenyl]-5-piperidin-1-yl-pent-1-en-3-one 4**: Compound **18** (100 mg, 0.342 mmol) was used as the starting material and treated according to the general procedure 4 to give compound **4** as a yellow solid (79 mg, 57%): mp: dec. $>252\,^{\circ}C$; $^1H$ NMR (300 MHz, $D_2O$) $\delta=$ 7.75 (d, $J=16.26$ Hz, 2H, $H_{vin}$), 7.66 (s, 1H, $H_{Ar}$), 7.41 (d, $J=7.55$ Hz, 2H, $H_{Ar}$), 7.28 (d, $J=7.80$ Hz, 1H, $H_{Ar}$), 6.53 (d, $J=16.41$ Hz, 1H, $H_{vin}$), 3.71–3.55 (m, 2H, $CH_2$), 3.55–3.44 (m, 2H, $CH_2$), 3.44–3.31 (m, 2H, $H_{pip}$), 3.06 (m, 2H, $H_{pip}$), 2.15–1.93 (m, 2H, $H_{pip}$), 1.93–1.40 ppm (m, 4H, $H_{pip}$); $^{13}C$ NMR (75 MHz, $D_2O$) $\delta=200.26$ (C), 161.27 (C), 161.41 (C), 140.66 (CH), 137.50 (C), 132.40 (C), 131.35 (CH), 131.05 (C), 128.04 (CH), 124.99 (CH), 119.33 (CH), 54.65 ($CH_2$), 52.48 ($CH_2$), 35.06 ($CH_2$), 23.83 ($CH_2$), 22.06 ppm ($CH_2$); MS (MALDI-TOF) *m/z*: 402.0 (M+); Anal. calcd for $C_{19}H_{24}ClN_7O\cdot 2HCl\cdot 1.4H_2O$: C 45.66, H 5.77, N 19.62, Cl 21.28, found: C 45.54, H 5.39, N 21.82, Cl 21.21%.

**4-(4,6-Diamino-[1,3,5]triazin-2-ylamino)-2-diethylaminomethyl-phenol 5**: A stirred suspension of 2-chloro-4,6-diamino-1,3,5-triazine (500 mg, 3.44 mmol) and 4-amino-α-diethylamino-*ortho*-cresol dihydrochloride **19** (1.1 g, 4.12 mmol) in EtOH (7 mL) was refluxed for 2 h. The precipitate was filtered, washed with cold EtOH and dried to afford **5** as a white hydrochloride salt (771 mg, 74%): mp: 250–252 °C; $^1H$ NMR (300 MHz, [$D_6$]DMSO) $\delta=10.43$ (s, 1H, OH), 10.10 (br s, 1H, NH), 8.53–7.59 (m, 4H, $H_{triazine}$), 7.86 (s, 1H, $H_{Ar}$), 7.36 (d, $J=8.76$ Hz, 1H, $H_{Ar}$), 7.03 (d, $J=8.76$ Hz, 1H, $H_{vin}$), 4.19 (d, $J=4.79$ Hz, 2H, Ar-$CH_2$), 3.15–3.01 (m, 4H, N($CH_2CH_3$)$_2$), 1.27 ppm (t, $J=7.17$ Hz, 6H, N($CH_2CH_3$)$_2$); $^{13}C$ NMR (75 MHz, [$D_6$]DMSO) $\delta=$ 158.38 (C), 156.34 (C), 153.64 (C), 146.94 (C), 129.14 (C), 126.80 (CH), 125.21 (CH), 116.24 (CH), 115.83 (CH), 49.38 ($CH_2$), 46.09 ($CH_2$), 8.48 ppm ($CH_3$); MS (FAB) *m/z*: 304.3 (M+); Anal. calcd for $C_{14}H_{21}N_7O\cdot 2HCl\cdot H_2O$: C 42.65, H 6.39, N 24.87, Cl 17.98, found: C 42.91, H 6.16, N 26.23, Cl 17.53%.

**Time-dependent formation of glutathione–Mannich base monoadducts**: GSH (20 mM in $H_2O$, 50 μL) and inhibitor (20 mM in DMSO, 50 μL) were added to a solution of aq $NH_4HCO_3$ (25 μM, 250 μL) and $H_20$ (650 μL) and incubated at RT (pH ~7). An aliquot was injected into an HPLC apparatus to determine the [Mannich base]:[monoSG adduct] ratio versus time (min) at various time intervals. The HPLC analysis was performed on a Hitachi Merck L-4000 equipped with a UV detector set at 254 nm. HPLC retention times were obtained using the following conditions: 100% eluent A (0.05% TFA in $H_2O$) for 5 min, a gradient up to 100% B (0.05% TFA in $H_2O/CH_3CN$ (1:4)) within 10 min, 100% B for 5 min, then again a gradient up to 100% A within 5 min at a flow rate of 1 mL min$^{-1}$.

## Biology

**In vitro antiparasitic bioassays for primary inhibitor screening** (table S1, Supporting Information): The *Leishmania infantum* MHOM/MA (BE)/67 strain used was maintained in the Golden Hamster and spleen amastigotes were collected for preparing infection inocula. Primary peritoneal mouse macrophages were used as host cells and were collected 2 d after peritoneal stimulation with a 2% potato starch suspension. Assays were performed in 96-well microtiter plates, with each well containing 10 μL of the compound dilutions together with 190 μL of macrophage/parasite inoculum ($3\times10^5$ cells $\pm3\times10^6$ parasites/well//RPMI-1640 + 5% inactivated Fetal Calf Serum (FCSi). After 5 d incubation, parasite burdens (mean number of amastigotes/macrophage) were microscopically assessed after Giemsa staining. The results are expressed as the % reduction in parasite burden compared to untreated control wells and an $IC_{50}$ value (50% inhibitory concentration) was calculated. In the primary evaluation, the compounds were tested at five concentrations (64, 16, 4, 1 and 0.25 μM or μg mL$^{-1}$). Pentostam® ($IC_{50}=6.8\pm0.9$ μM) and miltefosin ($IC_{50}=5.2\pm0.8$ μM) were included as reference drugs. A compound was classified as inactive when the $IC_{50}$ value is higher than 16 μg mL$^{-1}$ or μM. When the $IC_{50}$ value was lower than 1 μg mL$^{-1}$ or μM, the compound was classified as active and was further evaluated in a secondary screening, which involves the *L. donovani* MHOM/ET/67 L82 and *L. infantum* strains over an extended dose range (twofold compound dilutions).

The *Trypanosoma brucei brucei* squib 427 strain (suramin sensitive) was used. The strain was maintained in Hirumi (HMI-9) medium, supplemented with 10% FCSi. Assays were performed in 96-well microtiter plates, with each well containing 10 μL of the compound dilutions together with 190 μL of the parasite suspension ($7\times10^4$ parasites mL$^{-1}$). After 3 d incubation, parasite growth was assessed fluorometrically after addition of resazurin. After 24 h at 37 °C, fluorescence was measured ($\lambda_{ex}$ 550 nm, $\lambda_{em}$ 590 nm). The results are expressed as the % reduction in parasite growth/viability compared to control wells and an $IC_{50}$ value (50% inhibitory concentration) was calculated. Compounds were tested at five concentrations (64, 16, 4, 1 and 0.25 μM or mg mL$^{-1}$). Suramin was included as the reference drug ($IC_{50}=0.12\pm0.07$ μM). When the $IC_{50}$ value was lower than 1 μg mL$^{-1}$ or μM, the compound was classified as active and was further evaluated in a secondary screening, which involved an extended dose range (twofold compound dilutions), additional reference drugs (suramin, pentamidine, melarsoprol) and species (*T. b. rhodesiense* or *T. b. gambiense*).

*Trypanosoma cruzi*, tulahuen CL2, β galactosidase strain (nifurtimox sensitive) was used. The strain was maintained on MRC-$5_{SV2}$ (human lung fibroblast) cells in MEM medium, supplemented with 200 mM L-glutamine, 16.5 mM $NaHC0_3$, and 5% FCSi. All cultures and assays were conducted at 37 °C under an atmosphere of 5% $C0_2$. Assays were performed in sterile 96-well microtiter plates, with each well containing 10 μL of the compound dilutions together with 190 μL of MRC-5 cell/parasite inoculum ($2\times10^4$ cells mL$^{-1}$ $\pm2\times10^5$ parasites mL$^{-1}$). Parasite growth was compared to untreated infected controls (100% growth) and uninfected controls (0% growth) after 7 d incubation. Parasite burdens were assessed after adding the substrate CPRG (chlorophenol red β-D-galactopyranoside): 50 μL well$^{-1}$ of a stock solution containing CPRG (15.2 mg) and Nonidet (250 μL) in PBS (100 mL). The change in color was measured spectrophotometrically at 540 nm after 4 h incubation at 37 °C. The results are expressed as % reduction in parasite burdens compared to control wells and $IC_{50}$ values were calculated. Compounds were tested at five concentrations (64, 16, 4, 1 and 0.25 μM or mg mL$^{-1}$). Nifurtimox ($IC_{50}=0.845\pm0.2$ μM) was included as a reference drug. When the $IC_{50}$ value was lower than 1 μg mL$^{-1}$ or μM, the compound was classified as active on the condition that it also demonstrated selective action (absence of cytotoxicity).

**Evaluation of the cytotoxicity against human cell lines**: Human lung MRC-$5_{SV2}$ cells were cultured in Earl's MEM medium supplemented with 5% FCSi. Assays were performed in 96-well microtiter plates, with each well containing ~$10^4$ cells per well. After 3 d of incubation, cell viability was assessed fluorometrically; fluorescence was measured after addition of resazurin ($\lambda_{ex}$ 550 nm, $\lambda_{em}$ 590 nm). The results are expressed as % reduction in cell growth/viability

compared to untreated control wells and an $IC_{50}$ value was determined. Compounds were tested at five concentrations (64, 16, 4, 1 and 0.25 μM or mg mL$^{-1}$). When the $IC_{50}$ value was lower than 4 μg mL$^{-1}$ or μM, the compound was classified as toxic. Cytotoxic reference compounds included vinblastine or paclitaxel ($IC_{50}$ $<0.01$ μM), but these were rarely included because of associated health hazards. Alternatives are, for example, niclosamide and invermectin.

**Toxicity assay for P2 transporter studies in *T. brucei brucei***: Different cell types of the bloodstream *T. brucei brucei* (strain 427, wild type, TbAT1$^{-/-}$[14] and KO-B48[6] were cultivated in HMI-9 medium (BioSera Ltd., UK)[29] supplemented with 2 mM β-mercaptoethanol (Sigma–Aldrich, UK) and 10% FCSi (BioSera Ltd., UK) at 37 °C in a humidified 5% $CO_2$ atmosphere. Trypanotoxicity was determined using an adapted version of the Alamar Blue assay by Räz and co-workers.[30] Cells (100 μL of $1\times10^4$ cells ml$^{-1}$) were added to wells of 96-well plates containing doubling dilutions of each test compound (100 μL) and incubated for 48 h. Alamar Blue reagent (20 μL, 0.49 mM in PBS, pH 7.4; Sigma–Aldrich, UK) was added to each well. After 24 h, fluorescence was measured using a LS 55 luminescence spectrophotometer (PerkinElmer Life and Analytical Sciences, USA) set at excitation and emission wavelengths of 530 nm and 590 nm, respectively. Data was analyzed and $IC_{50}$ values determined with Prism 5.0 (GraphPad Software, USA) software. Experiments were performed in duplicate on three independent occasions.

**Uptake assay**: The uptake of [$^3$H]adenosine (Moravek Biochemicals Inc., USA) was studied using the rapid oil-stop method.[8] Bloodstream forms of *T. brucei brucei* (strain 427, wild type) were isolated from rat blood (female Winstar rat, Harlan UK) at peak parasitemia using a diethylaminoethyl cellulose, DE52 (Whatman, Maidstone, UK) anion exchange column. Cells were washed in assay buffer (33 mM Hepes, 98 mM NaCl, 4.6 mM KCl, 0.3 mM $CaCl_2$, 0.07 mM $MgSO_4$, 5.8 mM $NaH_2PO_4$, 0.3 mM $MgCl_2$, 23 mM $NaHCO_3$ and 14 mM glucose, pH 7.3) and resuspended to $\sim1\times10^8$ cells mL$^{-1}$ at RT. Aliquots of cells (100 μL) were added to microcentrifuge tubes containing equal volumes of assay buffer with [$^3$H]adenosine (100 nM), inosine (2 mM, Sigma–Aldrich, UK) and various test substrates at twice the required final concentration, layered over di-*n*-butyl phthalate (90 μL, BDH Labs, UK). Transport was terminated after 30 s by rapid centrifugation (13 000 g, 1 min, Heraeus Instruments, Germany) of cells through the oil layer and immediate flash freezing. Cell pellets were collected into respective scintillation vials and lysed with 2% *w/v* SDS (200 μL, ICN Biomedicals Inc., USA). Optiphase HiSafe 2 scintillation liquid (3 mL, PerkinElmer Life and Analytical Sciences, USA) was added to each sample and left overnight prior to ascertaining the counts min$^{-1}$ of radioactivity (Liquid Scintillation & Luminescence Counter, PerkinElmer Life and Analytical Sciences, USA). Total radioactivity present within the interstitial space was determined by performing an uptake assay on ice, followed by immediate centrifugation through oil and flash freezing. The background radioactivity was subsequently subtracted from the final count of all samples. Data analysis was performed using the Prism 5.0 (GraphPad Software, USA) software. All experiments were performed in duplicate on three independent occasions.

**Transporter binding assay**: Trypanosomes were pretreated with test substrates prior to the [$^3$H]adenosine uptake assay to assess the possibility of interaction with the P2 transporter via Michael addition. Briefly, bloodstream forms of *T. brucei brucei* (strain 427, wild type) from a female Wistar rat were isolated as mentioned above and incubated with various test substrates at five times their known $IC_{50}$ values for different time periods (1 min and 20 min) at RT. Cells were washed twice in assay buffer to remove test substrates before being subjected to [$^3$H]adenosine uptake as detailed above over a period of time (30 s to 10 min). Experiment was performed in triplicate on two independent occasions.

**Comparative inhibitor studies of TR, GR, and TrxR by the Mannich base derivatives 2, 3a, 13a and 20**: The activities of *T. cruzi* TR, *P. falciparum* and human GR, and *P. falciparum* TrxR were measured at 25 °C in 1 mL cuvets containing 40 mM HEPES and 1 mM EDTA (pH 7.5), 47 mM potassium phosphate buffer (pH 6.9), 200 mM KCl and 1 mM EDTA and 100 mM phosphate buffer (pH 7.4), 2 mM EDTA, respectively. Initial rates were calculated from NADPH oxidation measured at 340 nm using the physiological substrates and at 412 nm using DTNB as a substrate. 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 GSSG with GR, and 1 mM GSSG and 26 μM *P. falciparum* $TrxS_2$ or 3 mM DTNB in the case of TrxR. $IC_{50}$ values were determined in duplicate in the presence of four to six inhibitor concentrations (0–200 μM). The reactions were started by adding 5 μL enzyme solution (9 mU or 1.63 pmol *T. cruzi* TR; 10 mU or 1.14 pmol human GR; 9.65 mU or 7.7 pmol *P. falciparum* TrxR). The final content of DMSO in the cuvette was 1%.

**$IC_{50}$ values from time-dependent inactivation assays**: The activity of *T. cruzi* TR, *P. falciparum* GR, human GR, and *P. falciparum* TrxR after pre-incubation with inhibitor and NADPH for 5 min was determined by monitoring disulfide reduction. 5 μL of the enzyme solution containing 16.7 nmol *T. cruzi* TR, 54.2 pmol *P. falciparum* GR, 45 pmol human GR, and 771 pmol *P. falciparum* TrxR, respectively, were allowed to react with 160 μM NADPH and 0–400 μM **2**, **3a**, **13a** and **20** in a final volume of 50 μL or 200 μL 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).

Data sets of primary screening assays for compounds **2**, **3–5**, **12a-c**, **13a-c**, **17**–**18**, and **20** are available as Supporting Information.

Animal use was approved by the Ethical Committee of the University of Antwerp.

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