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Original article

9- and 11-substituted 4-azapaullones are potent and selective inhibitors of African trypanosoma

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ABSTRACT

Trypanosomes from the "brucei" complex are pathogenic parasites endemic in sub-Saharan Africa and causative agents of severe diseases in humans and livestock. In order to identify new antitrypanosomal chemotypes against African trypanosomes, 4-azapaullones carrying α,β-unsaturated carbonyl chains in 9- or 11-position were synthesized employing a procedure with a Heck reaction as key step. Among the so prepared compounds, **5a** and **5e** proved to be potent antiparasitic agents with antitrypanosomal activity in the submicromolar range.

1. Introduction

Protozoan parasites from the genus *Trypanosoma* are responsible for zoonotic diseases affecting human and livestock. In Africa, *Trypanosoma brucei gambiense and Trypanosoma brucei rhodesiense* cause human sleeping sickness whereas *Trypanosoma brucei brucei*, *Trypanosoma vivax* and *Trypanosoma congolense* produce Nagana in cattle. These unicellular pathogens are transmitted by the bites of tsetse flies abundant in sub-Saharan Africa where more than 70 million people and 40 million domestic animals are at risk of trypanosomiasis [1].

The infection in humans has two major clinical presentations, acute (haemolymphatic form) and chronic (neurological form), that despite being highly disabling are fatal if left untreated. Interestingly, the proposal by MacLeod et al. that human pathogens from the "*brucei*" clade are host-range variants of the local *T. b. brucei* populations [2] has recently been supported by a thorough genetic survey of field isolates that demonstrate inter-subspecies genetic exchange [3,4] and the identification of single genes as determiner of human infectivity [5,6]. These findings also raise alarms about the genetic diversity available to *T. brucei* for counteracting natural or chemical (drugs) selection [3].

Among the animals affected by the disease are cattle, horses, sheep and goats that after severe infection show anemia, body weight loss, changes in food intake, which increases the susceptibility to secondary infections and decreases productivity [7]. Since the domestic animals are a major source of food and financial earnings of the rural population, Nagana accounts also for human malnutrition and impairment of social development. Affecting almost one third of the cattle population, Nagana is economically the most important African livestock disease [8].

The development of effective immunoprophylaxis against African trypanosomes remains unattainable due to sophisticated and successful evasion mechanism employed by the pathogens [9].

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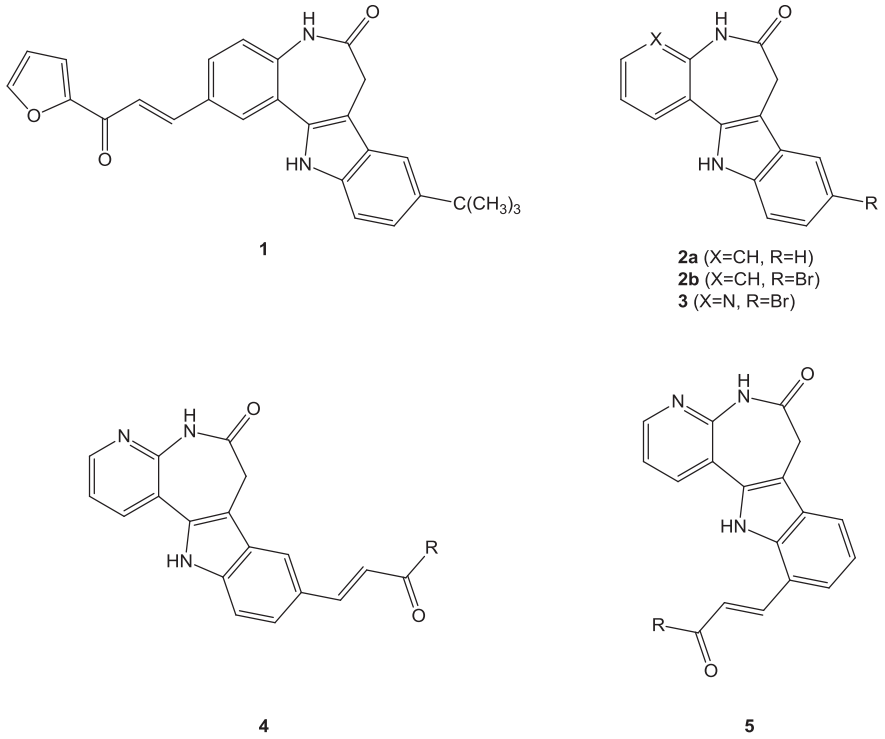


Chart 1. Paullones mentioned in the text.

Since more than six decades these diseases are treated using a limited number of drugs that suffer from poor safety and efficacy, and require an administration regime difficult to apply in humans. For instance, pentamidine and suramin are recommended for the treatment of the first stage of human African trypanosomiasis, whereas melarsoprol, efluornithine and, more recently, a combination of efluornithine/nifurtimox are drugs of choice when the parasites invade the central nervous system [10]. The lack of effectiveness of efluornithine and nifurtimox against *T. b. rhodesiense* and the increasing number of cases reporting drug resistance [11–14] exemplify how limited is the current chemotherapy. Nevertheless, an agreement between WHO and pharmaceutical companies (Sanofi and Bayer AG) make these drugs available to endemic countries free of charge [1].

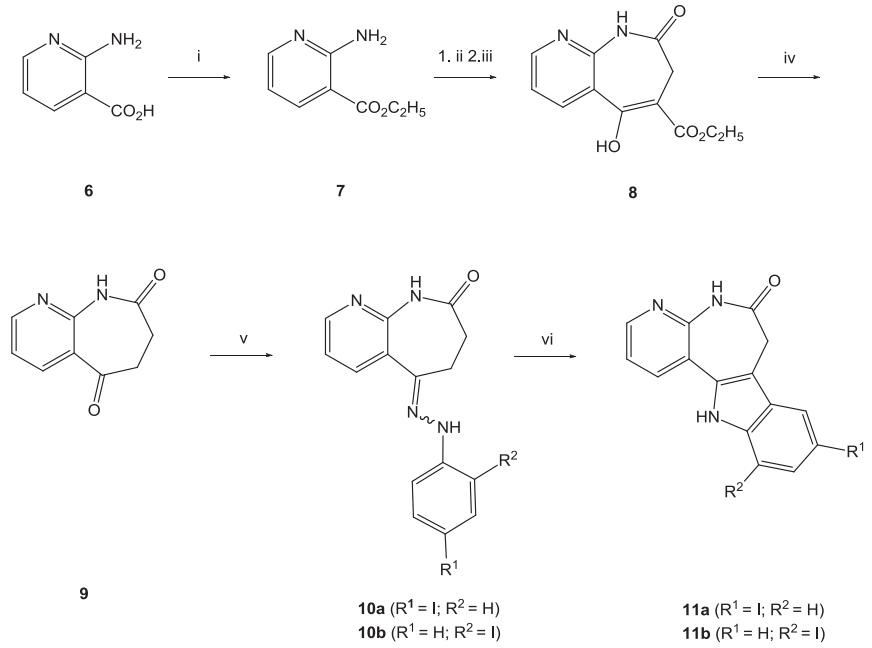
Nagana is treated with: the phenanthridinium ethidium chloride, the diamidine diminazene aceturate (DA), and isomethamidium chloride (ISM) [8,15]. Because of its mutagenic properties the use of ethidium for prophylaxis or therapy of infected animals is no longer recommended [16] and its effectiveness is furthermore hampered by widespread resistance of the parasites [17]. DA and ISM are cheap and easy to obtain, their private use is often not controlled by trained veterinary personnel. Thus, inadequate application regimes might have contributed to the numerous cases of resistance reported from 17 African countries between Mali and Mozambique [16,18].

Unfortunately, the market volume for human and veterinary drugs against African trypanosomiasis is not attractive for international pharmacy companies [16]. However, the pharmacological weakness of the drugs currently available, the lack of back-up substitutes and the emergence of resistance urge on the development of new chemotherapeutics against African trypanosomes. To avoid the risk of cross-resistance with established drugs, new antitrypanosomal candidates should be unrelated to existing drugs by means of structure and potential mechanism of action. The study presented here was directed to the design of new antitrypanosomal compounds against African trypanosomes.

In preceding papers we reported upon the antileishmanial and antitrypanosomal activity of paullone derivatives substituted at the 2-position with side chains containing α,β-unsaturated carbonyl elements, e.g. the paullone–chalcone hybrid **1** [19,20]. However, these compounds showed some signs of toxicity when tested on human cell lines. Since the structures are based on the heterocyclic scaffold paullone (7,12-dihydroindolo[3,2-*d*][1]benzazepin-6(5*H*)-one, **2a**), which represents a well established class of mammalian protein kinase inhibitors [21–25], toxic effects of antitrypanosomal paullones may stem from undesired inhibition of host kinases. It has been shown that insertion of a nitrogen atom in position 4 of the paullone scaffold abrogates the kinase inhibitory potency in paullone congeners [26]. For example, 4-azakenpaullone **3** is more than two orders of magnitude less active as a GSK-3 inhibitor compared to its 4-carbaanalogue kenpaullone **2b** (Chart 1) [27]. We therefore designed new 4-azapaullones as potential antitrypanosomal compounds. To introduce an additional aspect of novelty, the side chains were attached to the 9-position (derivatives **4**) or to the 11-position (derivatives **5**), respectively.

2. Chemistry

For the preparation of the desired compounds **4** and **5**, an improved synthesis for the pivotal building block **9** was developed (Scheme 1) [27]. First, commercially available 2-aminopyridine-3-carboxylic acid (**6**) was esterified by means of iodoethane. The resulting ethyl ester **7** was acylated by ethyl succinyl chloride. The raw resulting amide was treated with potassium *tert*-butylate in a toluene/DMF mixture to yield the benzazepine derivative **8** by means of a Dieckmann condensation reaction. A dealk-oxycarbonylation of **8** in wet DMSO under neutral conditions furnished the cyclic ketone **9** which was reacted at 40 °C with either 4-iodo- or 2-iodophenylhydrazine in the presence of sulfuric acid, respectively, yielding the phenylhydrazones **10a** and **10b**. After purification, these phenylhydrazones were heated to 70 °C in the presence of sulfuric acid to produce the 4-azapaullones **11a** and **11b** by

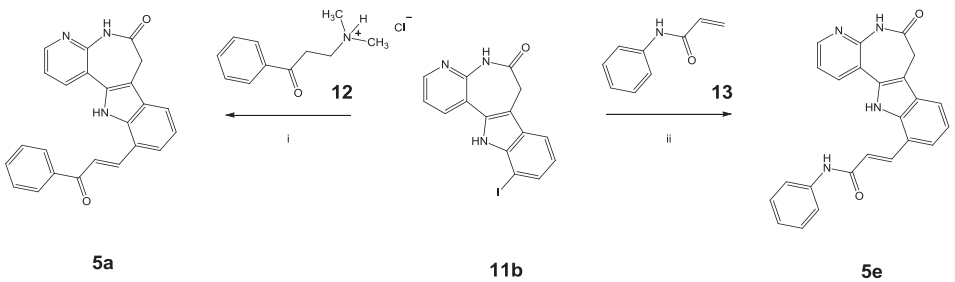


Scheme 1. Synthesis of 9- or 11-iodo-4-azapaullones. Reagents and conditions: (i) ICH_2CH_3, K_2CO_3, DMF, rt, 1 h, 81%; (ii) ethyl succinyl chloride, pyridine, THF, rt; (iii) KO-*tert*-Bu, toluene, DMF, N_2, 0 °C → rt, 33% from **7**; (iv) DMSO, H_2O, N_2, 150 °C, 5–7 h, 87%; (v) 2- or 4-iodophenylhydrazine, AcOH, H_2SO_4, rt → 40 °C, 67% and 89%, respectively; (vi) AcOH, H_2SO_4, 70 °C, 63% and 51%, respectively.

Fischer indole ring closure reactions. Starting from the iodoarenes **11a** or **11b**, respectively, the preparation of the title compounds was accomplished by Heck reactions as exemplified in Scheme 2. For the synthesis of the paullone–chalcone hybrid structure **5a, 11b** was reacted with *N*,*N*-dimethyl-3-oxo-3-phenylpropan-1-aminium chloride (**12**) as precursor of phenyl vinyl ketone in the presence of palladium acetate [28]. The product **5a** resulted in (*E*)-configuration as deduced from the coupling constant of the vinylic protons in the ^{1}H NMR spectrum. The low yield of this compound was caused by a cumbersome purification procedure during which most of the product was lost. A classical Heck reaction of **11b** with *N*-phenylacrylamide (**13**) yielded the paullone–cinnamide hybrid structure **5e** that also displayed (*E*)-configuration. The other test compounds **4** and **5** were synthesized by similar procedures.

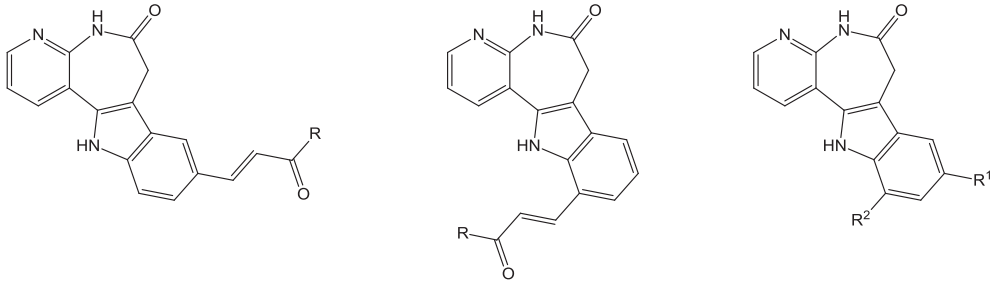
3. Biological evaluation and discussion

All new 4-azapaullones **4** and **5** were tested for growth inhibition of infective *T. b. brucei* cultivated *in vitro*. *T. b. brucei* appears as a suitable laboratory model for *in vitro* screening tests because it is not pathogenic for humans but expresses the full repertoire of genes controlling metabolic and other major cellular processes as the related pathogenic subspecies. The screening tests were performed at compound concentrations of 5 and 30 μM and cell viability was assessed by flow cytometry after 24 h incubation. For those compounds that at 5 μM reduced more than 90% of the cell population, EC_{50} values were determined. The results displayed in Table 1 show that indeed most of the new 4-azapaullones **4** and **5** are potent inhibitors of *T. b. brucei* parasites. In the series **4** substituted at the 9-position, the secondary cinnamides **4f** and **4g** showed higher antiparasitic activity than the tertiary amide **4e** or the chalcone-4-azapaullone hybrids **4a**–**4d**. This observation indicates that both an H-bond donor motif and an aromatic ring appear advantageous at the end of the side chain in the derivatives **4**. The results with the congeners **5** were more heterogeneous. While the chalcone-4-azapaullone hybrid **5a** showed a stronger antiparasitic activity compared to its 9-substituted analogue **4a**, *p*-substitution with a methoxy (**5b**) or a chloro substituent (**5c**) at the terminal phenyl ring led to derivatives that were significantly less potent compared to the analogues **4b** and **4c**, respectively. With an EC_{50} of 120 nM, the *N*-phenylcinnamide **5e** turned out to be the most potent antitrypanosomal compound in the series. The comparison with the modest activity displayed by the iodo-substituted 4-azapaullones **11a** and **11b** demonstrates that the α,β-unsaturated



Scheme 2. Synthesis of azapaullones **5a** and **5e** substituted in 11-position. The synthesis of azapaullones **4** substituted in 9-position was performed by similar methods. Reagents and conditions: (i) TEA, $Pd(OAc)_2$, DMF, 140 °C, 1 h, 6%; (ii) TEA, $Pd(OAc)_2$, DMF, microwaves, 100 °C, 40 min, 80%.

Table 1
Structures, antitrypanosomal Activity, trypanothione synthetase inhibition and toxicity of 4-Azapaullones



4 **5** **11**

	R	Viability[a] of bloodstream *T. b. brucei* @ conc. [%] ± 2SD; *n* or EC_{50} *T.b.b.* [μM][b]	Residual activity[c] of *Tb*TryS [%] ± 2SD @ 30 μM; *n*	Tox[d] [%] ± SD; *n*
4a	Ph-	54.0 ± 3.1 @ 5 μM; 2	61.9 ± 6.4; 4	7.6 ± 3.1 @ 26 μM; 3
4b	4-MeO-Ph-	44.5 ± 2.7 @ 5 μM; 2	77.6 ± 5.4; 4	20.3 ± 12.7 @ 12 μM; 3
4c	4-Cl-Ph-	38.4 ± 2.7 @ 5 μM; 2	79.8 ± 5.7; 4	4.73 ± 1.8 @ 48 μM; 3
4d	Furan-2-yl-	21.9 ± 0.001 @ 30 μM; 3	90.2 ± 3.8; 4	17.6 ± 6.9 @ 54 μM; 3
4e	Diethylamino-	22.1 ± 0.6 @ 30 μM; 2	91.5 ± 5.2; 4	<0 @ 59 μM; 3
4f	Phenylamino-	EC_{50} ~ 2.5 μM	68.1 ± 2.4; 4	<0 @ 58 μM; 4
4g	Benzylamino-	12.1 ± 4.4 @ 5 μM; 2	67.5 ± 4.2; 6	<0 @ 29 μM; 4
5a	Ph-	EC_{50} = 0.18 ± 0.02 μM	91.1 ± 7.2; 3	28.3 ± 7.9 @ 26 μM; 4
5b	4-MeO-Ph-	79.9 ± 10.5 @ 30 μM; 2	80.4 ± 3.1; 3	16.5 ± 11.7 @ 48 μM; 4
5c	4-Cl-Ph-	74.6 ± 7.3 @ 5 μM; 2	73.2 ± 7.8; 4	9.2 ± 6.6 @ 12 μM; 4
5d	Diethylamino-	81.6 ± 3.9 @ 30 μM; 2	102.0 ± 8.3; 4	<0 @ 56 μM; 4
5e	Phenylamino-	EC_{50} = 0.12 ± 0.04 μM	94.6 ± 2.7; 4	74.7 ± 4.4 @ 60 μM; 4
5f	Benzylamino-	EC_{50} = 0.45 ± 0.12 μM	81.8 ± 5.5; 4	4.16 ± 0.7 @ 24 μM; 2
11a	R^1 = I, R^2 = H	72.1 ± 7.6@ 30 μM; 2	96.7 ± 6.1; 4	12.3 ± 5.7 @ 10 μM; 4
11b	R^1 = H, R^2 = I	61.3 ± 18.5 @ 30 μM; 2	111.7 ± 8.1; 3	4.4 ± 4.1 @ 5 μM; 3

[a] Compounds were simultaneously tested @ 5 and 30 μM. Parasite viability was assessed by flow cytometry after 24 h incubation with test compounds. Nifurtimox 15 μM was used as 50% growth inhibition control drug.
[b] For the compounds reducing parasite viability for more than 90% @ 5 μM, EC_{50} values were determined. The EC_{50} is the compound concentration that inhibits parasite growth to 50% compared to untreated controls.
[c] The activity of recombinant *T. b. brucei* trypanothione synthetase (*Tb*TryS) was evaluated after incubation for 15 min without (control) or with test compounds by measuring the phosphate released from ATP with Biomol Green™ reagent. The values are expressed as % residual activity with respect to the control.
[d] Toxicity for mammalian cells measured with mouse macrophages using the xCELLigence system. Shown is the toxicity [%] at the highest concentration [μM] that was used depending on the solubility of the test compounds.

carbonyl motif plays an important role for antitrypanosomal activity in **4** and **5**. All compounds were also evaluated for non-specific toxicity using a xCELLigence assay with mouse macrophages as mammalian cell model. At the highest tested concentration, which depended on individual compound solubility, none of the derivatives (with the exception of **5e**) provoked a reduction in viability of more than 50%. Hence, EC_{50} values for these compounds were not calculated and compounds were rated to be of limited toxicity, at least in this test system. For instance, by comparing the toxicity parameters for **5a** it can be estimated that it presents selective inhibition towards parasites that is at least two-orders of magnitude higher than against macrophages. For an assessment of potential off-targets of the substituted 4-azapaullones, the derivatives showing highest antitrypanosomal activity (**5a** and **5e**) were tested on mammalian kinases that are typically inhibited by paullones. In the test panel also related kinases from the CMGC family were included (Table 2). Both **5a** and **5e** failed to inhibit all and most, respectively, protein kinases at the highest tested concentration (10 μM) with the only exception that compound **5e** exhibited an IC_{50} of 0.49 μM against the casein kinase 1 (CK1). Overall, this result corroborates our assumption that introduction of the nitrogen atom in 4-position of the paullone basic structure leads to loss of undesired inhibitory activity on a wide diversity of mammalian kinases.

Up to now, the biochemical mechanism underlying the antitrypanosomal activity of **4** and **5** remains speculative. Recently it was reported that N^5-substituted paullones inhibit the trypanothione synthetase (TryS) by competing with ATP-binding [29]. Being essential for maintaining the cellular redox status in *Kinetoplastidae* and absent in mammalian species, TryS was suggested as a druggable target for treatment of trypanosomiasis [30–32]. We therefore constructed a homology model of *T. b. brucei* TryS based on the published structure of the *Leishmania major* TryS (pdb file 2vpm) and the *T. b. brucei* TryS sequence (Q82MC2 from the Uniprot data base) (data not shown). All attempts to generate meaningful ligand–protein complexes by docking compounds **4** and **5** into the ATP binding site of the *T. b. brucei* TryS 3D-model failed. TryS have been shown to contain highly dynamic structural elements involved in substrate binding [33–35]. Thus, a structural

Table 2
Inhibition of Mammalian Kinases of the CMGC Family by 4-Azapaullones **5a** and **5e** [IC_{50}, μM].

	CDK1	CDK2/A	CDK5	CDK9/T	CK1	CLK1	DYRK1A	GSK-3
5a	>10	>10	>10	>10	>10	>10	>10	>10
5e	>10	>10	>10	>10	0.49	>10	>10	>10

model of TryS might not provide reliable information about conformational changes shaping the sites of ligand binding. Therefore, we decided to test all derivatives **4** and **5** for *in vitro* inhibition of recombinant *T. b. brucei* TryS (Table 1). Although some congeners (**4a**–**c**, **4f**–**g**, **5c**) showed a modest inhibition, the differences observed in potency against enzyme (e.g. 20–30% activity inhibition at 30 μM) and parasite growth ($EC_{50} \leq 5$ uM) suggests that other targets than TryS are responsible for the antitrypanosomal activity of the title compounds.

It may be speculated that the Michael acceptor motif present in **4** and **5** could react with vital thiols in the parasite cells. This mode of action has recently been proven for the antitrypanosomal sesquiterpene lactone cynaropicrin which leads to depletion of trypanothione and glutathione in the parasite by formation of covalent adducts [36]. On the other hand, based on the inhibition of mammalian CK1 by **5e** and the finding that the isoform 2 of the homologue protein from *Trypanosoma brucei* has been shown to be indispensable for the infective form of the parasite [37] it is tempting to speculate that this compound exerts its trypanosomal toxicity by inhibiting this molecular target. Future studies will address the mechanism of action of the most potent paullones described in this work.

4. Conclusion

The design and synthesis of 4-azapaullones carrying α,β-unsaturated carbonyl chains in 9- or 11-position led to compounds **4** and **5** showing inhibitory activity towards the infective stage of *T. b. brucei*. The most potent compounds **5a** and **5e** inhibited the parasites in submicromolar concentrations. A low degree of toxicity for these compounds can be assumed since mammalian cells (mouse macrophages) were only slightly affected in significant higher concentrations on the one hand and a selection of mammalian kinases was not inhibited by **5a** and **5e**, respectively, on the other hand. The exception was compound **5e** that has been identified here as a selective inhibitor of mammalian CK1. The mode of action of these antitrypanosomal agents is still unknown. The trypanothione synthetase of *T. b. brucei* was eliminated as possible intracellular target by docking experiments and by inhibition tests with recombinant enzyme. Since **5a** and **5e** offer many options for molecular variations they appear as appropriate starting points for a further development of drugs against African trypanosomes.

5. Experimental protocols

5.1. Synthetic chemistry

5.1.1. General

Melting points (mp) were determined on an electric variable heater (Barnstead Electrothermal IA 9100) and were not corrected. IR-spectra were recorded as KBr discs on a Thermo Nicolet FT-IR 200. ^{1}H NMR-spectra and ^{13}C NMR-spectra were recorded on the following instruments: Bruker Avance DRX-400 and Bruker Avance II-600; solvent DMSO-d_6 if not stated otherwise; internal standard tetramethylsilane; signals in ppm (δ scale). Elemental analyses were determined on a CE Instruments FlashEA® 1112 Elemental Analyser (Thermo Quest). Mass spectra were recorded on a double-focused sector field mass spectrometer Finnigan-MAT 90. Accurate measurements where conducted according to the peakmatch method using perfluorokerosene (PFK) as an internal mass reference; (EI)-MS: ionization energy 70 eV. TLC: Polygram® Sil G/UV254, Macherey–Nagel, 40 × 80 mm, visualization by UV-illumination (254 nm). Column chromatography: silica gel 60 (Merck), column width: 2 cm, column height 10 cm unless stated otherwise. Purity was determined by HPLC using the following devices and settings [38]: Elite LaChrom (Merck/Hitachi), Pump L-2130, autosampler L-2200, Diode Array Detector L-2450, organizer box L-2000; column: Merck LiChroCART 125-4, LiChrosphere 100, RP 18, 5 μm; flow rate 1.000 mL/min, isocratic, volume of injection: 10 μL; detection (DAD) at 254 and 280 nm; AUC-%-method.; time of detection 15 min, net retention time (t_N), dead time (t_m) related to DMSO. All compounds employed in biological tests were used in >90% purity. Microwave accelerated reactions were carried out in sealed 10 mL microwave glass tubes using a mono mode microwave device (CEM discover, Kamp-Lintfort, Germany). For reactions in a parallel synthesis reactor a carousel 12 place reaction station was used (Radley Discovery Technologies, Shire Hill, Saffron Walden, Essex, UK). *Mannich* bases [39] and *N*-substituted acrylamides [40,41] employed as reagents in Heck reactions were synthesized according to published standard methods. Other starting materials were purchased from commercial suppliers (Acros Organics, Geel, Belgium), Sigma Aldrich (St. Louis, MO, USA), Merck KGaA (Darmstadt, Germany) and were used without further purification. Synthesis procedures and characterization of 2-iodophenylhydrazine as well as test compounds **4a**–**g, 5b**–**d** and **5f** are described in the supporting information.

5.1.2. 2-Aminopyridine-3-carboxylic acid ethyl ester (**7**)

To a solution of 2-aminopyridine-3-carboxylic acid (**6**, 2.00 g, 14.0 mmol) in DMF (56 mL) was added potassium carbonate (2.50 g, 28.0 mmol). After stirring the suspension for 30 min at room temperature, a solution of iodoethane (2.20 g, 14.0 mmol, 1.10 mL) in DMF (14 mL) was added dropwise. After stirring for 1 h the mixture was poured on ice water (300 mL). The precipitated crystals were collected by filtration. The filtrate was extracted with ethyl acetate (4 × 75 mL). The combined ethyl acetate layers were dried (Na_2SO_4) and evaporated. To the remaining residue diethyl ether (100 mL) was added and the resulting solution was washed with aqueous 30% lithium chloride solution (4 × 25 mL). After drying with Na_2SO_4, the organic solvent was evaporated. The residue was combined with the precipitate that was collected earlier and the material was crystallized from ethanol to yield 1.90 g (80.8%) colorless crystals. mp: 93–94 °C (Lit.: 94–96 °C [42]); IR (KBr): 3428 (NH_2), 1695 (C=O), 1625 (NH_2), 1245, 770 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.22 (*dd*, 1H, $^3J_{H,H}$ = 4.8 Hz, $^4J_{H,H}$ = 1.9 Hz), 8.15 (*dd*, 1H, $^3J_{H,H}$ = 7.8 Hz, $^4J_{H,H}$ = 1.9 Hz), 6.63 (*dd*, 1H, $^3J_{H,H}$ = 7.8 Hz, $^3J_{H,H}$ = 4.8 Hz), 4.35 (*q*, 2H, $^3J_{H,H}$ = 7.1 Hz), 1.39 (*t*, 3H, $^3J_{H,H}$ = 7.1 Hz); ^{13}C NMR (150.9 MHz, $CDCl_3$): δ = 167.0, 159.4, 106.5 (quat C); 153.5, 140.1, 112.7 (tert C); 60.9 (sec C); 14.3 (prim. C); $C_8H_{10}N_2O_2$ (166.18); calcd. C 57.82, H 6.07, N 16.86; found C 57.68, H 6.22, N 17.20.

5.1.3. 2-[(4-Ethoxy-1,4-dioxobutyl)amino]pyridine-3-carboxylic acid ethyl ester

To a solution of 2-aminopyridine-3-carboxylic acid ethyl ester (**7**, 4.00 g, 24.00 mmol) in THF (80 mL) were successively dropwise added pyridine (2.00 g, 24.8 mmol, 2.00 mL) and succinic acid ethyl ester chloride (7.20 g, 44.0 mmol, 6.00 mL). After stirring the mixture for 2 h at room temperature, water (160 mL) was added and the resulting mixture was extracted with ethyl acetate (3 × 120 mL). The combined organic layers were dried (Na_2SO_4) and evaporated. The remaining viscous oil (4.96 g) was used for the following synthesis step without further purification. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 10.94 (*s*, 1H), 8.57 (*d*, 1H, $^3J_{H,H}$ = 4.4 Hz), 8.33 (*d*, 1H, $^3J_{H,H}$ = 7.8 Hz), 7.09 (*dd*, 1H, $^3J_{H,H}$ = 7.4 Hz, $^3J_{H,H}$ = 5.0 Hz), 4.40 (*q*, 2H, $^3J_{H,H}$ = 7.1 Hz), 4.16 (*q*, 2H, $^3J_{H,H}$ = 6.9 Hz), 2.98 (*t*, 2H, $^3J_{H,H}$ = 6.8 Hz), 2.75 (*t*, 2H, $^3J_{H,H}$ = 6.8 Hz), 1.41 (*t*, 3H, $^3J_{H,H}$ = 7.2 Hz), 1.26 (*t*, 3H, $^3J_{H,H}$ = 7.1 Hz).

*5.1.4. 5-Hydroxy-8-oxo-8,9-dihydro-7H-pyrido[2,3-b]azepine-6-carboxylic acid ethyl ester (**8**)*

The raw 2-[(4-ethoxy-1,4-dioxobutyl)amino]pyridine-3-carboxylic acid ethyl ester (4.96 g) prepared as described above was dissolved in a mixture of toluene (53 mL) und DMF (8 mL). This solution was added dropwise to a precooled solution of potassium *tert*-butoxide (10.0 g, 89.0 mmol) in toluene (27 mL) at 0 °C under nitrogen. The mixture was allowed to warm up to room temperature and was stirred for further 4 h under nitrogen. After addition of glacial acetic acid (5.3 mL) and water (53 mL) a precipitate formed which was filtered and washed with petrol ether. The material was crystallized from ethanol to yield a colorless solid (1.90 g, 33.4% over two steps from **7**); mp: 207–208 °C; IR (KBr): 3204 (NH), 1690 (C=O), 1403, 1193, 764 cm^{-1}; ^1H NMR (600 MHz, DMSO-d_6): δ = 12.47 (*s*, 1H), 10.74 (*s*, 2H), 8.58 (*dd*, 1H, $^3J_{H,H}$ = 4.7 Hz, $^4J_{H,H}$ = 1.9 Hz), 8.23 (*dd*, 1H, $^3J_{H,H}$ = 7.9 Hz, $^4J_{H,H}$ = 1.9 Hz), 7.34 (*dd*, 1H, $^3J_{H,H}$ = 7.9 Hz, $^3J_{H,H}$ = 4.7 Hz), 4.31 (*q*, 2H, $^3J_{H,H}$ = 7.1 Hz), 3.03 (*s*, 1H), 1.31 (*t*, 3H, $^3J_{H,H}$ = 7.1 Hz); ^{13}C NMR (150.9 MHz, DMSO-d_6): δ = 171.7, 170.2, 164.3, 149.2, 119.8, 96.1 (quat C); 151.5, 137.7, 119.3 (tert C); 61.5, 30.9 (sec C); 14.0 (prim. C); $C_{12}H_{12}N_2O_4$ (248.23); calcd. C 58.06, H 4.87, N 11.29; found C 57.99, H 4.77, N 11.30.

*5.1.5. 6,7,8,9-Tetrahydro-5H-pyrido[2,3-b]azepine-5,8-dione (**9**)*

A mixture of 5-hydroxy-8-oxo-8,9-dihydro-7*H*-pyrido[2,3-*b*]azepine-6-carboxylic acid ester (**8**, 678 mg, 2.70 mmol), DMSO (48.6 mL) and water (5.4 mL) was heated for 5–7 h to 150 °C under nitrogen. After cooling to room temperature, water (54 mL) was added. Upon standing at room temperature a precipitate formed which was filtrated and crystallized from ethanol to yield a beige solid (412 mg, 86.6%); mp: 218–221 °C; IR (KBr): 3433 (NH), 2976 (CH aliphat.), 2917 (CH aliphat.), 1674 (C=O), 1592 (CONH), 767 cm^{-1}; ^1H NMR (600 MHz, DMSO-d_6): δ = 10.40 (*s*, 1H), 8.56 (*dd*, 1H, $^3J_{H,H}$ = 4.6 Hz, $^4J_{H,H}$ = 1.9 Hz), 8.26 (*dd*, 1H, $^3J_{H,H}$ = 7.8 Hz, $^4J_{H,H}$ = 1.9 Hz), 7.26 (*dd*, 1H, $^3J_{H,H}$ = 7.8 Hz, $^3J_{H,H}$ = 4.6 Hz), 3.08–2.85 (*m*, 2H), 2.85–2.63 (*m*, 2H); ^{13}C NMR (150.9 MHz, DMSO-d_6): δ = 197.2, 172.8, 150.78, 120.9 (quat. C); 153.1, 139.8, 119.2 (tert C); 37.1, 29.3 (sec C); $C_9H_8N_2O_2$ (176.17); calcd. C 61.36, H 4.58, N 15.90; found C 60.70, H 4.43, N 15.87.

*5.1.6. 5-[2-(2-Iodophenyl)hydrazono]-6,7-dihydro-5H-pyrido[2,3-b]azepine-8(9H)-one (**10b**)*

6,7,8,9-Tetrahydro-5*H*-pyrido[2,3-*b*]azepine-5,8-dione (**9**, 200 mg, 1.13 mmol), 2-iodophenylhydrazin hydrochloride (460 mg, 1.70 mmol) and sodium acetate (139 mg, 1.70 mmol) were stirred in glacial acetic acid (10 mL) under nitrogen. After stirring for 5 h, concentrated sulfuric acid (0.4 mL) was added and stirring at room temperature was continued for 2 h. Subsequently the mixture was stirred for 2 h at 40 °C. The reaction was terminated by addition of 5% aqueous sodium acetate solution (40 mL). The resulting precipitate was filtered, washed with water and crystallized from ethanol to yield a colorless solid (295 mg, 66.6%); mp: 253–254 °C; IR (KBr): 3433, 3048 (NH), 2893, 1688 (C=O), 1589, 1452, 741 cm^{-1}; ^1H NMR (600 MHz, DMSO-d_6): δ = 10.12 (s, 1H), 8.39 (*dd*, 1H, $^3J_{H,H}$ = 4.7 Hz, $^4J_{H,H}$ = 1.9 Hz), 8.11 (*dd*, 1H, $^3J_{H,H}$ = 7.7 Hz, $^4J_{H,H}$ = 1.8 Hz), 7.94 (*s*, 1H), 7.75 (*dd*, 1H, $^3J_{H,H}$ = 7.8 Hz, $^4J_{H,H}$ = 1.4 Hz), 7.43 (*dd*, 1H, $^3J_{H,H}$ = 8.2 Hz, $^4J_{H,H}$ = 1.5 Hz), 7.36–7.28 (*m*, 1H), 7.23 (*dd*, 1H, $^3J_{H,H}$ = 7.7 Hz, $^3J_{H,H}$ = 4.7 Hz), 6.70 (*ddd*, 1H, $^3J_{H,H}$ = 7.8 Hz, $^3J_{H,H}$ = 7.2 Hz, $^4J_{H,H}$ = 1.6 Hz), 3.10 (*dd*, 2H, $^3J_{H,H}$ = 7.4 Hz, $^3J_{H,H}$ = 5.6 Hz), 2.68–2.61 (*m*, 2H); ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 172.4, 148.9, 145.2, 144.7, 125.2, 84.1 (quat C); 148.5, 138.6, 138.4, 129.2, 122.3, 119.9, 114.7 (tert C); 30.9, 28.0 (sec C); $C_{15}H_{13}IN_4O$ (392.19); HPLC: 99.3% at 254 nm, 99.5% at 280 nm, t_N = 13.1 min, t_M = 1.09 min (ACN/H_2O; 40:60).

*5.1.7. 11-Iodo-7,12-dihydropyrido[2′,3′:2,3]azepino[4,5-b]indol-6(5H)-one (**11b**)*

To a mixture of 5-[2-(2-iodophenyl)hydrazono]-6,7-dihydro-5*H*-pyrido[2,3-*b*]azepin-8(9*H*)-one (**10b**, 230 mg, 0.580 mmol) and glacial acetic acid (10 mL) was added concentrated sulfuric acid (0.7 mL). After stirring for 1 h at 70 °C under nitrogen the mixture was cooled to room temperature. A 5% aqueous solution of sodium acetate (40 mL) was added. A precipitate formed which was filtered off, washed with water and crystallized from ethanol to yield a colorless solid (65.0 mg, 51.2%); dec. starting at 330 °C; IR (KBr): 3421, 3192 (NH), 1667 (C=O) cm^{-1}; ^1H NMR (600 MHz, DMSO-d_6): δ = 11.40 (*s*, 1H), 10.36 (*s*, 1H), 8.45 (*dd*, 1H, $^3J_{H,H}$ = 4.7 Hz, $^4J_{H,H}$ = 1.8 Hz), 8.35 (*dd*, 1H, $^3J_{H,H}$ = 7.8 Hz, $^4J_{H,H}$ = 1.8 Hz), 7.75 (*d*, 1H, $^3J_{H,H}$ = 7.7 Hz), 7.62 (*dd*, 1H, $^3J_{H,H}$ = 7.4 Hz, $^5J_{H,H}$ = 0.9 Hz), 7.34 (*dd*, 1H, $^3J_{H,H}$ = 7.8 Hz, $^3J_{H,H}$ = 4.7 Hz), 6.92 (*dd*, 1H, $^3J_{H,H}$ = 7.9 Hz, $^3J_{H,H}$ = 7.5 Hz), 3.59 (*s*, 2H); ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 171.5, 147.6, 139.8, 131.5, 126.9, 117.3, 109.6, 76.8 (quat C); 147.7, 137.2, 131.9, 121.3, 118.9, 118.3 (tert C); 31.9 (sec C); $C_{15}H_{10}IN_3O$ (375.16); MS (EI): *m/z* = 375 ($[M]^{+\bullet}$, 100), 346 ($[M\text{-}CHO]^+$, 51), 219 (25); HRMS (EI): *m/z* ($[M]^{+\bullet}$) calcd. 374.98631, found 374.98555; HPLC: 98.1% at 254 nm, 97.8% at 280 nm, t_N = 6.7 min, t_M = 1.09 min (ACN/H_2O; 40:60).

*5.1.8. (E)-11-(3-Oxo-3-phenylprop-1-en-1-yl)-7,12-dihydropyrido[2′,3′:2,3]azepino-[4,5-b]indol-6(5H)-on (**5a**)*

11-Iodo-7,12-dihydropyrido[2′,3′:2,3]azepino[4,5-*b*]indol-6(5*H*)-one (**11b**, 200 mg, 0.530 mmol), *N,N*-dimethyl-3-oxo-3-phenylpropan-1-aminium chloride (**12**, 126 mg, 0.590 mmol), triethylamine (1 mL), $Pd(OAc)_2$ (7 mg) and DMF (5 mL) were stirred for 1 h at 140 °C in a carousel parallel synthesis reactor station. After filtration of the reaction mixture through a charcoal frit ethyl acetate (220 mL) was added. A precipitate formed, which was successively filtered off and washed with n-hexane (320 mL). In the combined washing solutions a precipitate formed, which was filtered off and crystallized from ethanol to yield a brown solid (12 mg, 6.0%) dec. starting at 319 °C; IR (KBr): 3246 (NH), 2909 (CH aliphat.), 1672 (C=O), 1645 (C=O), 1326, 1218 cm^{-1}; ^1H NMR (600 MHz, DMSO-d_6): δ = 12.09 (*s*, 1H), 10.41 (*s*, 1H), 8.58 (*d*, 1H, $^3J_{H,H}$ = 15.4 Hz), 8.46 (*dd*, 1H, $^3J_{H,H}$ = 4.7 Hz, $^4J_{H,H}$ = 1.8 Hz), 8.37 (*dd*, 1H, $^3J_{H,H}$ = 7.8 Hz, $^4J_{H,H}$ = 1.8 Hz), 8.25–8.20 (*m*, 2H), 8.08 (*d*, 1H, $^3J_{H,H}$ = 15.3 Hz), 8.04–7.98 (*m*, 1H), 7.86 (*d*, 1H, $^3J_{H,H}$ = 7.8 Hz), 7.72–7.67 (*m*, 1H), 7.60 (*dd*, 2H, $^3J_{H,H}$ = 8.1 Hz, $^3J_{H,H}$ = 7.2 Hz), 7.38 (*dd*, 1H, $^3J_{H,H}$ = 7.8 Hz, $^3J_{H,H}$ = 4.7 Hz), 7.22 (*t*, 1H, $^3J_{H,H}$ = 7.7 Hz), 3.66 (*s*, 2H); ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 188.9, 171.3, 147.6, 137.7, 137.2, 131.3, 127.5, 188.8, 117.4, 108.4 (quat C); 147.7, 139.3, 136.3, 133.0, 128.7 (2C), 128.4 (2C), 121.5, 121.0, 120.9, 119.7, 119.0 (tert C); 31.7 (sec C); $C_{24}H_{17}N_3O_2$ (379.41); MS (EI): *m/z* = 379 ($[M]^{+\bullet}$, 100), 362 (55), 302 ($[C_{18}H_{12}N_3O_2]^+$, 39), 274 ($[C_{17}H_{12}N_3O]^+$, 70), 105 (59); HRMS (EI): *m/z* ($[M]^{+\bullet}$) calcd. 379.13153, found 379.13143; HPLC: 91.1% at 254 nm, 95.6% at 280 nm, t_N = 5.6 min, t_M = 1.09 min (ACN/H_2O; 50:50).

*5.1.9. (E)-3-(6-oxo-5,6,7,12-tetrahydropyrido[2′,3′:2,3]azepino[4,5-b]indol-11-yl)-N-phenylacrylamide (**5e**)*

A mixture of 11-iodo-7,12-dihydropyrido[2′,3′:2,3]azepino[4,5-*b*]indol-6(5*H*)-one (**11b**, 200 mg, 0.530 mmol), *N*-phenylacrylamide (**13**, 87 mg, 0.59 mmol), triethylamine (101 mg, 1.00 mmol, 0.14 mL), $Pd(OAc)_2$ (7 mg, 0.03 mmol), and DMF (3 mL) were heated for 40 min to 100 °C (power: 100 W; pressure 150 PSI) in a microwave device in a sealed microwave reaction vessel. After filtration of the mixture through a charcoal frit, ethyl acetate (220 mL) was added to the filtrate. A solid precipitated, which was filtered off and washed with n-hexane (320 mL). Further material precipitated from the washing liquid, which was collected by filtration and combined with the material collected previously. Crystallization of

the combined materials from ethanol yielded a gray-brown solid (168 mg, 80.4%); decomposition starting at 289 °C; IR (KBr): 3435 (NH), 2908 (CH aliphat.), 1652 (C=O), 1624 (C=O), 1442, 1359 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 12.03 (*s*, 1H), 10.40 (*s*, 1H), 10.25 (*s*, 1H), 8.46 (*dd*, 1H, $^3J_{H,H}$ = 4.7 Hz, $^4J_{H,H}$ = 1.8 Hz), 8.42–8.32 (*m*, 2H), 7.83–7.72 (*m*, 3H), 7.58 (*d*, 1H, $^3J_{H,H}$ = 7.4 Hz), 7.44–7.29 (*m*, 3H), 7.21 (*t*, 1H, $^3J_{H,H}$ = 7.7 Hz), 7.13–7.04 (*m*, 1H), 6.99 (*d*, 1H, $^3J_{H,H}$ = 15.5 Hz), 3.65 (*s*, 2H); ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 171.4, 163.8, 147.7, 139.4, 136.7, 131.3, 127.5, 119.1, 117.5, 108.4 (quat C); 147.6, 136.3, 135.6, 128.8 (2C), 123.3, 122.3, 119.9, 119.8, 119.4, 119.2 (2C), 119.1 (tert C); 31.8 (sec C); $C_{24}H_{18}N_4O_2$ (394.43); MS (EI): *m/z* = 394 ($[M]^{+\bullet}$, 52), 302 ($[C_{18}H_{12}N_3O_2]^+$, 100), 274 ($[C_{17}H_{12}N_3O]^+$, 99), 259 (56); HRMS (EI): *m/z* ($[M]^{+\bullet}$) calcd. 394.14243, found 394.14233; HPLC: 98.1% at 254 nm, 98.9% at 280 nm, t_N = 8.5 min, t_M = 1.09 min (ACN/H_2O; 40:60).

5.2. Biological assays

5.2.1. Expression, purification and activity assay of T. b. brucei trypanothione synthetase (TbTryS)

5.2.1.1. Expression and purification of recombinant TbTryS. *Tb*TryS was produced in recombinant form with an N-terminal His-tag using *Escherichia coli* strain BL21(DE3) transformed with the construct pET-15b *Tb*TryS [43] (a kind gift of Dr. Alan Fairlamb, Dundee University, Scotland; Gen Bank™ accession number CAC87573.1). Starter cultures of freshly transformed cells were prepared in LB medium containing 100 μg/mL ampicillin and grown overnight under aerobic conditions at 37 °C and 180 rpm. Five mL of the overnight culture was inoculated into a 2 L Erlenmeyer flask containing 500 mL of Terrific Broth medium supplemented with 10 g/L glucose and 100 μg/mL ampicillin. Culture growth was initiated by incubation at 37 °C and 180 rpm and extended until an optical density at 600 nm between 0.8 and 1.0 was achieved. The flask was then chilled at 4 °C for 15 min. After addition of 0.5 mM isopropyl 1-thio-β-D-galactopyranoside bacterial growth was resumed at 25 °C and 180 rpm for further 5 h. Cells were harvested by centrifugation at 4000× g for 15 min at 4 °C (centrifuge Sorvall RC-6, Thermo Fisher Scientific) and resuspended in 50 mM NaH_2PO_4, pH 7.2, 300 mM NaCl (buffer A) and 10 mM imidazol at a ratio of 1 g wet weight pellet per 5 mL buffer. Cell lysis was achieved by gently shaking with lysozyme (30 mg % w/v) for 1 h at 4 °C followed by sonication on ice (4 pulses during 30 s interspersed with pauses of 60 s) at an amplitude of 40% using a Digital Sonifier 450 (Branson). The lysate was centrifuged twice at 20,000× g for 20 min at 4 °C (centrifuge Sorvall RC-6, Thermo Fisher Scientific) to remove debris and the supernatant containing soluble protein was cleared by filtration (0.7 μM filter) and then loaded onto a 1 mL HisTrap Fast Flow column (GE Healthcare) pre-equilibrated in buffer A. The column was washed with buffer A added with 25 mM imidazol and the recombinant protein eluted in an isocratic fashion with 10 mL of buffer B (buffer A with 500 mM imidazol). This chromatography was performed at 4 °C and at a flow rate of 1 mL/min using a peristaltic pump (TRIS Teledyne ISCO). Fractions containing homogenous and active protein (tested by a kinetic assay in saturating conditions [33]) were pooled and concentrated with a 30 kDa cut-off Amicon filter (Millipore). Recombinant TryS was subjected to a second purification step by size exclusion chromatography (SEC) in a Superdex™ 200 10/300 GL (GE healthcare) run on reaction buffer (see below "*TryS activity assay*") added of 150 mM NaCl. Elution fractions displaying homogenous and active enzyme were pooled (see below). The final purification step consisted of an anion exchange chromatography using a MonoQ HR 5/5 column (GE Healthcare). Prior to injection into the MonoQ column, the protein samples were desalted on a PD-10 column (Sephadex G25 matrix; GE Healthcare) equilibrated with reaction buffer. TryS recovered from the PD-10 column was applied to the MonoQ column pre-equilibrated with reaction buffer. The column was washed with reaction buffer until absorbance 280 nm approaches the baseline and bound proteins eluted with a linear gradient from 0 to 500 mM NaCl in reaction buffer. The last two chromatographies were performed at room temperature using an Äkta-FPLC device (GE Healthcare).

Protein purity was assessed after each purification step by SDS-PAGE 12% gel under reducing conditions. Protein concentration was determined by the Bicinconinic Acid assay with bovine serum albumin as standard and enzyme activity was determined by the end-point assay detailed below (see "*TryS activity assay*"). The protocol described above yielded ~1–1.5 mg recombinant protein per liter of culture medium with a purity ≥95%. The protein was stored at − 20 °C in reaction buffer added of 40% (v/v) glycerol (Carlo Erba Reagents SA) without significant loss of activity for at least 6 months.

5.2.1.2. TryS activity assay. The end-point assay is based on the determination of inorganic phosphate released from ATP during TryS catalysis, by its complexation (phosphomolybdate) to the cationic triphenylmethane dye, malachite green (BIOMOL GREEN™ Enzo® Life Sciencies). The green complex presents an absorption maximum at 650 nm. The reaction buffer consisted of 100 mM HEPES-K, 0.5 mM EDTA, 10 mM $MgSO_4$ and 5 mM DTT, pH 7.4. Master Mix (MM) solutions containing substrates at concentrations adjusted according to their intracellular levels in *T. brucei* [44,45] and the kinetic parameters reported in the literature for *Tb*TryS [30,43] were prepared. Thus, spermidine concentration was fixed to 2 mM and glutathione concentration was adjusted to 50 μM. Due to interference with the assay, ATP concentration was adjusted to 150 μM.

Assays were conducted at room temperature, using 96-well plates (Corning Incorporated, costar® 3590) and a total reaction volume of 50 μL: 40 μL of MM, 5 μL of test compound dissolved in DMSO (J.T. Baker) and 5 μL enzyme solution (*Tb*TryS: 230 nM, ~0.070 μmol/min mg). A reaction control was prepared by adding 5 μL DMSO. An inhibitory control was performed for the enzyme by adding 5 μL of a N^5-substituted paullone at its respective IC_{50} concentration of 30 μM [46]. Blanks lacking enzyme were prepared for each tested compound and controls. Compounds, controls and blanks were tested in quadruplicates.

The reaction was initiated by addition of enzyme (or reaction buffer, in the case of blanks) and stopped after 15 min by adding 200 μL BIOMOL GREEN™ reagent. The colorimetric signal was allowed to develop for 20 min and then absorbance at 650 nm was measured with a MultiScan EX plate reader (Thermo SCIENTIFIC). The results are expressed as residual activity (%) and are calculated as follow:

$$[(A_{650\,\mathrm{nm\,corr\,compound}}/A_{650\,\mathrm{nm\,corr\,reaction\,control}}) \times 100],$$

where

$$A_{650\,\mathrm{nm\,corr\,compound}} = A_{650\,\mathrm{nm\,compound}} - A_{650\,\mathrm{nm\,compound\,blank}};$$

$$A_{650\,\mathrm{nm\,corr\,reaction\,control}} = A_{650\,\mathrm{nm\,reaction\,control}} - A_{650\,\mathrm{nm\,reaction\,control\,blank}};$$

A_{650} nm corr compound is the corrected absorbance measured for a given compound, and A_{650} nm corr reaction control is the corrected absorbance measured for the reaction control. The error is expressed as 2 S.D., estimated as $2\sigma^{n-1}$.

5.2.2. Proliferation assay for infective T. b. brucei

The biological activity of the compounds was evaluated in the infective form of *T. brucei brucei* strain 427, cell line 449 (encoding one copy of the tet-represor protein: Pleo [47]) transfected with a construct encoding a redox biosensor (Sardi, Comini unpublished). These parasites were cultivated aerobically in HMI-9 medium [48] supplemented with 10% (v/v) fetal bovine serum (FBS) (PAA), 10 U/mL penicillin (Gibco®), 10 μg/mL streptomycin (Gibco®), 0.2 μg/mL phleomycin (Gibco®) and 5 μg/mL hygromicyn (Invitrogen), inside a humidified incubator (Thermo SCIENTIFIC) with controlled temperature (37 °C) and CO_2 (5%).

Stock solutions at 3–24 mM in DMSO were prepared for each compound according to their solubility. All compounds were subjected to a primary screening at 5 and 30 μM.

For compounds showing more than 90% parasite growth inhibition at 5 μM, EC_{50} values were determined. To determine EC_{50}, two-fold serial dilutions in DMSO were prepared from the compound's stock solution.

Trypanosomes were grown to mid-exponential phase (~2 million cells/mL), counted with a Neubauer chamber (Precicolor HBG, Germany) and harvested by centrifugation at 2000 *g* for 10 min at room temperature. The cell pellet was resuspended at a density of 5×10^5 cells/mL in fresh culture medium. Two μL from the compound solutions or DMSO (proliferation control) was added onto each well of a 96-well culture plate (Corning Incorporated, costar® 3599). Immediately after, 200 μL of the cell suspension was plated into each well and the culture plate was incubated at 37 °C with 5% CO_2. After 24 h, 100 μL from each well was transferred to individual 1.1 mL tubes (T100, Biotube™ System), diluted with 200 μL sterile phosphate buffered saline, pH 7.0, with 1% (w/v) glucose and added of 2 μL propidium iodide (exclusion dye) at 200 mg/mL, homogenized by quick vortexing and analyzed using a CyAn™ ADP (DakoCytomation) flow cytometer. Flow cytometry analysis was performed with a 488 nm solid-state laser as the excitation light source. The positive events (cells) were gated by forward-scatter (FSC) and side-scatter (SSC) parameters whereas the fluorescence of propidium iodide was collected at $\lambda_{em} = 613/30$ nm to distinguish dead cells. The signals were detected with logarithmic amplifiers. All measurements were made at constant flow and acquisition time (60 s per sample). The data were analyzed using the Summit (Dako) and Flow-Jo (Tree star Inc.) software. A positive control included Nifurtimox (Lampit® from Bayer) tested at its EC_{50} (15 μM). All conditions were tested by triplicate.

The relative percentage of viable parasites at 5 or 30 uM is expressed as follow:

$$\%\,\text{Viability} = [(\text{number of parasites for compound X at concentration Y})/(\text{number of parasites in the proliferation control})] \times 100.$$

The EC_{50} values were obtained from dose response curves fitted to a sigmoidal Boltzmann equation (errors calculated using errors' propagation) or extrapolated from non-linear fitting plots. The error is expressed as 2 S.D., estimated as $2\sigma^{n-1}$.

5.2.3. Assay for inhibition of mammalian protein kinases

5.2.3.1. Protein kinase assay buffers. Buffer A: 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 25 mM Tris–HCl pH 7.5, 50 μg heparin/mL.

Buffer B: 50 mM $MgCl_2$, 90 mM NaCl, 30 mM Tris–HCl pH 7.4.

5.2.3.2. Kinase preparations and assays. Kinase activities for each enzyme were assayed in buffer A or B, with their corresponding substrates, in the presence of 15 μM ATP in a final volume of 30 μL. After 30 min incubation at 30 °C, the reaction was stopped by harvesting, using a FilterMate harvester (Packard), onto P81 phosphocellulose papers (GE Healthcare) which were washed in 1% phosphoric acid. Scintillation fluid was added and the radioactivity measured in a Packard counter. Blank values were subtracted and activities calculated as pmoles of phosphate incorporated during the 30 min incubation. The activities were expressed in % of the maximal activity, i.e. in the absence of inhibitors. Controls were performed with appropriate dilutions of DMSO.

CDK1/cyclin B (M phase starfish oocytes, native), *CDK2/cyclin E* (human, recombinant, from A. Echalier), *CDK5/p25* (human, recombinant, expressed in *E. coli*) were assayed in Buffer A (supplemented extemporaneously with 0.15 mg BSA/mL, except for CDK2) with 25 μg of histone H1. *CDK9/cyclin T* (human, recombinant, expressed in insect cells) was assayed as described for CDK1/cyclin B, but using 8.07 μg of CDK7/9-Tide (YSPTSPSYSPTSPSYSPTSPSKKKK) [49].

DYRK1A (human, recombinant, expressed in *E. coli* as a GST fusion protein), and *CLK1* (mouse, recombinant, expressed in *E. coli* as a GST fusion protein) were assayed as described for CDK1/cyclin B with 1 μg of RS peptide (GRSRSRSRSRSR) as a substrate [50].

GSK-3α/β (porcine brain, native, affinity purified on axin 1-sepharose beads [51]) was assayed as described for CDK1/cyclin B, but using a GSK-3 specific substrate (GS-1: YRRAAVPPSPSLSRHSSPHQpSEDEEE where pS stands for phosphorylated serine).

Casein kinase 1 (CK1δ/ε) (porcine brain, native, purified by affinity chromatography on axin 2-sepharose beads [52]) was assayed with 0.67 μg of CKS peptide (RRKHAAIGpSAYSITA), a CK1 specific substrate.

5.2.4. Toxicity assay on murine macrophages by xCELLingence device

Toxicity assays were performed with the xCELLigence System (Roche Applied Systems) using mouse bone marrow-derived macrophages generated as described previously [53]. After seeding 7.5×10^4 macrophages in 75 μL medium (Dulbecco's Modified Eagle Medium (Gibco), supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, penicillin and streptomycin) to each well of an E-plate VIEW 96 (Roche Applied Science) for 24 h, cells were treated with 125 μL medium containing the different test compounds at the highest concentration depending on the respective solubility. Impedance measurements were performed on the Real-Time Cell Analyzer SP instrument (Roche Applied Systems) every 30 min. All experiments were run for 24 h. Cell indices obtained were analyzed using the Real-Time Cell Analyzer Software 1.2 (Roche Applied Science). The percentage of toxicity was calculated as follows:

$$\%\,\text{Toxicity} = 100 - (\text{cell index test compound}/\text{cell index medium}) \times 100$$

5.3. In-silico analysis

5.3.1. Homology modeling

Homology Modeling was performed using MOE [54] and the homology modeling module. The available X-ray structure for *L. major* TryS (pdb 2vpm) was used as template structure and the *T. b. brucei*

TryS sequence from UniProt data base with the reference number: Q82MC2 was used as modeling sequence. For model creation standard settings together with the Amber99 force field were used.

5.3.2. Molecular docking

For docking GOLD 5.2 was used with standard settings and ChemPLP for pose creation and scoring [55,56]. For each molecule, 50 poses were created.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.06.020.

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