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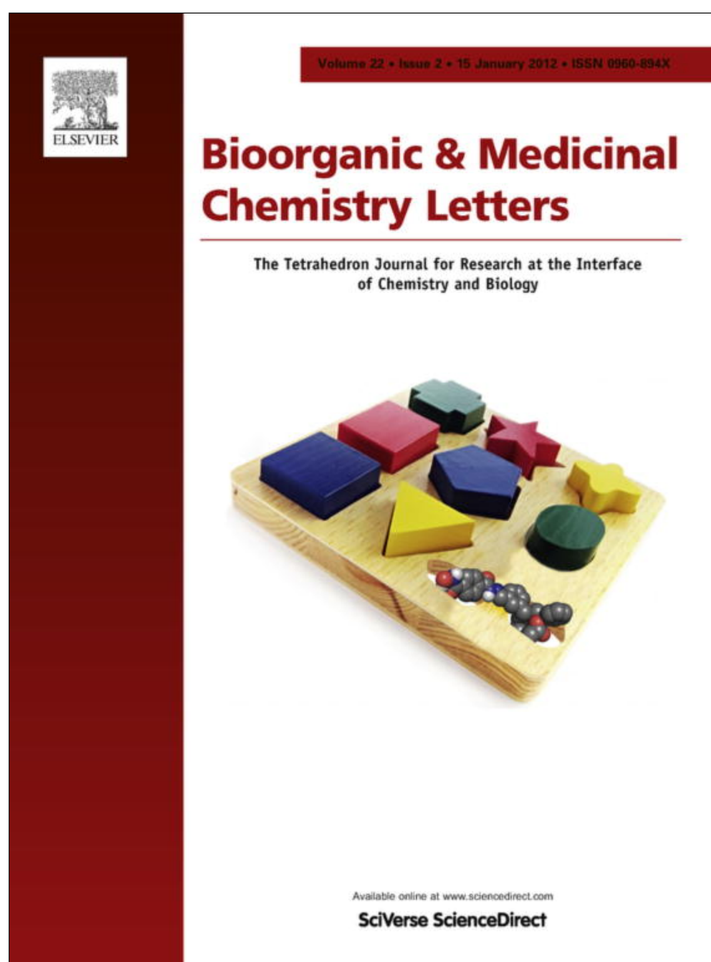

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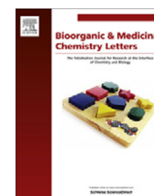

In vitro and in silico studies of polycondensed diazine systems as anti-parasitic agents

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ABSTRACT

Parasitic diseases caused by protozoarian agents are still relevant today more than ever. Recently, we synthesized several polycondensed diazine derivatives by means 1,3-dipolar cycloaddition reactions. A broad selection of these compounds were submitted to in vitro biological screening against *Plasmodium falciparum*, *Leishmania infantum*, *Trypanosoma* brucei, and *Trypanosoma cruzi*, resulting active at micromolar level. Induced Fit Docking/MM-GBSA studies were performed giving interesting indications about the probable mechanism of action of the most active compounds

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Parasitic diseases are the cause of significant mortality and morbidity worldwide. The WHO estimates that approximately 247 million people are infected with malaria annually, resulting in approximately 1 million deaths. In addition, an estimated 1 billion people are affected by parasitic infections, such as leishmaniasis, trypanosomiasis. The annual mortality associated with parasitic infections, excluding malaria, is an estimated 500,000 deaths.[1] The majority of the drugs used to treat these diseases is old and has several limitations, including high cost, poor efficacy, toxicity.[2] Moreover, the development of drug resistance makes actual the research of new molecules able to act as selective and effective anti-parasitic chemotherapeutic agents.

Several azine or diazine heterocycles (pyridine, pyrazine, quinoxalines), as well as their ring-fused bioisosteric analogs, have shown marked activity in many biological systems. A large number of compounds incorporating these ring systems was found to posses antimicrobial or anti-parasitic activities.[3,4] Therefore we decided to study polycondensed diazine systems, incorporating the quinoxaline moiety, for their potential anti-parasitic activity. Recently, we have investigated the behaviour of quinoxalines as reactants for the one-pot building of complex ring systems through the well known 1,3-dipolar cycloaddition reactions (1,3-DCR) with nitrilimine dipoles, generated in situ from chloroarylhydrazones of type **2.**[5] By using this approach we successfully realized the one pot synthesis of the tetrahydrobis-1,2,4-triazolo[4,3-*a*:3′,4′-*c*]quinoxaline derivatives of type **4**. All possible biscycloadduct diastereoisomers (meso form RS, and racemic mixture RR and SS) were obtained, but the monocycloadduct **3** was never isolated[6] (Scheme 1).

On the contrary, when the dipolarophile component was non-symmetric (**5** or **7**), the reaction route was different leading also to the formation of the monocycloadducts **6bis** together with the biscycloadducts **6** or **8**, this last only as a RR/SS racemic mixture. However, the presence of the methyl group in position 2 on the quinoxaline **7** allowed also to isolate, from the reaction mixture, the spiro-cycloadduct of type **8bis**[7] (Scheme 2).

With the aim to explore further the influence of the dipolarophile asymmetry in the 1,3-DCR involving the pyrazine nucleus, 2-phenylpyrrole[2,3-*b*]pyrazine (**9**) was chosen as 2π component. The reaction with chloroarylhydrazones led to the tetrahydropyrrolo[2,3-*e*]bis[1,2,4]triazolo[3,4-*c*:4′,3′-*a*]pyrazines of type **10**. It was also possible to isolate, in good yields, as main component, the tricyclic monoadduct pyrrolo[2,3-*e*][1,2,4]triazolo[4,3-*a*]pyrazine **12** together with the monocycloadduct **13**[8] (Scheme 3).

A broad selection of these polycondensed azines presenting a wide pattern of substitutions was submitted to In vitro biological screening[9] against a panel of protozoarian agents: *Plasmodium falciparum* (chloroquine sensitive strain GHA), *Leishmania infantum, Trypanosoma brucei* and *Trypanosoma cruzi*. These test organisms were selected as primary screening model for the parasitic diseases malaria, leishmaniasis, sleeping sickness, and Chagas disease, respectively, so that a wide range of activity can be explored. Some of the tested compounds resulted active at micromolar level and, more significantly, structurally different compounds showed selectivity for

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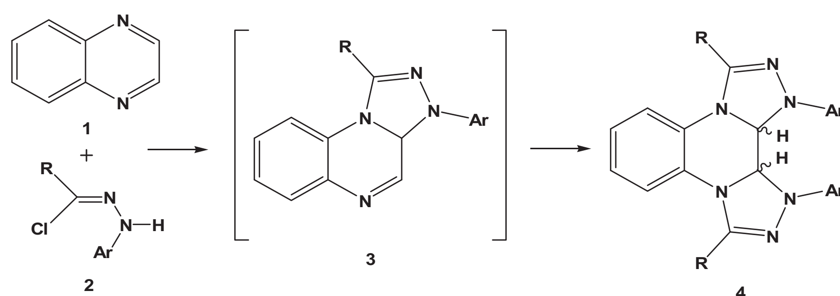
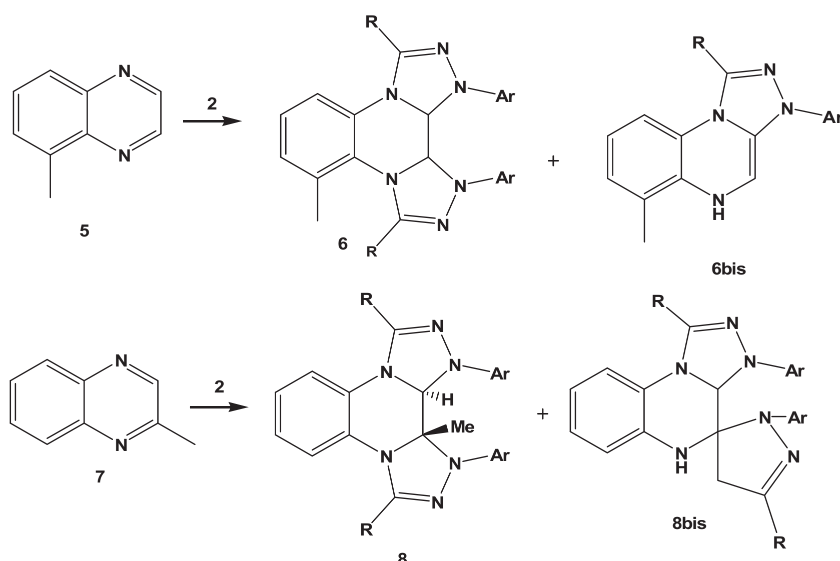
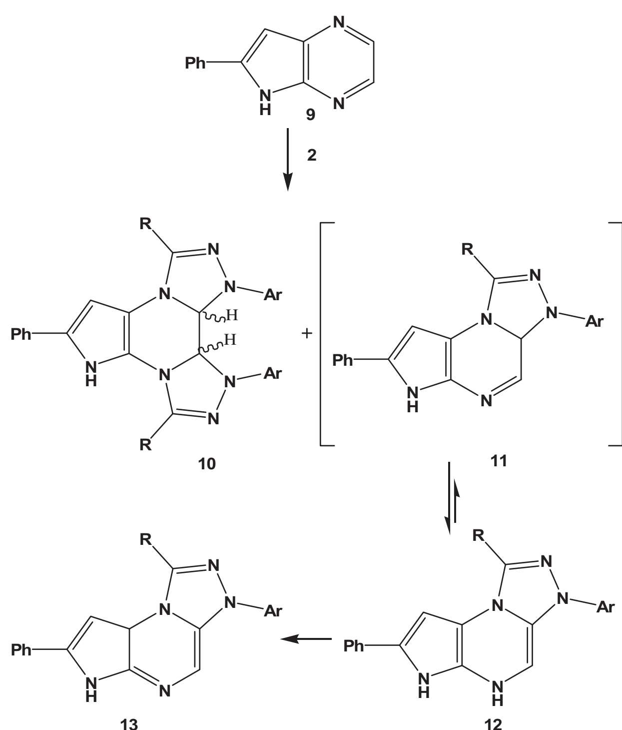


Scheme 1. 1,3-DCR with quinoxaline.



Scheme 2. 1, 3-DCR with nonsymmetric benzodiazines.



Scheme 3. 1,3-DCR with pyrrolopyrazine ring as dipolarophile.

different species of microorganisms (Table 1). Only derivatives **4b** and **8d** resulted aspecific, with IC_{50} in the range 2.1-12.6 μM against all the tested agents. Compound **4g** resulted active and very selective against *T. brucei* at IC_{50} = 2.06 μM in different condition of solubility, and against *T. cruzi* at IC_{50} <10 μM.

Other two compounds of the series of bis-triazoloquinoxaline showed selectivity against *T. cruzi* at IC_{50} <10 μM. Compound **8a** showed also activity against *P. falciparum* (IC_{50} = 11.62 μM). Bis-triazoloquinoxaline derivatives showed activity also against *T. brucei*: in fact compounds **4c, 4g** and **8d** demonstrated high level of inhibition capability with IC_{50} = 2.00 μM. But, whereas **8d** was active on all the four tested parasitic agents (IC_{50} in the range 2.06–10.08 μM), the only one revealed to be selective against *T. brucei* was compound **4c**.

Polycondensed azines were generally inactive against *Leishamania infantum*, but again compound **8d** was the only one of the tested series with IC_{50} <10 μM. Compound **8b**, a bis-triazoloquinoxaline derivative, resulted to be the most active and selective against *Plasmodium falciparum* (IC_{50} <1.9 μM), although also compounds **10a** and **10b**, belonging to the series of bis-triazolopyrrolopyrazine, exhibited inhibitory activity, but at higher concentration.

In parallel with anti-parasitic tests, cytotoxicity on host cells was also determined, using MRC-5 cells, because these tests represent a very important criterion for assessing the selectivity of the observed biological activities. The majority of diazine derivatives resulted non toxic at studied concentrations, although **4b**, **4c**, **4e**, **4g** showed toxicity at concentrations lower than 64 μM. However,

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Table 1
In vitro activity against parasitic agents and cytotoxicity on host cells (IC_{50} in μM)

Compds	R	Ar	Solubility*	*MRC-5*	*T. cruzi*	*L. infantum*	*T. brucei*	*P. falciparum*-GHA
4a	COMe	Ph	M	64.00	38.14	32.00	64.00	64.00
4b (meso)	COMe	Ph	M	29.63	12.60	32.46	8.06	8.48
4c	COPh	Ph	M	64.00	64.00	32.46	2.03	27.66
			NS	17.55			2.03	
4d	COOMe	Ph	M	64.00	64.00	64.00	64.00	12.26
4e (meso)	COOMe	Ph	M	33.07	42.47	64.00	34.56	25.58
4f	COOEt	$pCl-C_6H_4$	M	64.00	64.00	64.00	64.00	37.33
4g (meso)	COOEt	$pCl-C_6H_4$	M	48.50	8.75	32.00	2.06	18.61
			NS	12.44			2.01	
4h	H	Ph	M	64.00	64.00	64.00	64.00	29.76
6	COOMe	$pCl-C_6H_4$	M	64.00	64.00	64.00	64.00	42.57
6bis	COOMe	Ph	M	64.00	64.00	64.00	64.00	64.00
8a	COOMe	Ph	M	64.00	6.22	64.00	64.00	11.62
			M		4.29			
8b	COOEt	$pCl-C_6H_4$	M	64.00	64.00	64.00	64.00	0.50
			NS					1.90
8c	COMe	Ph	M	64.00	33.71	24.05	32.22	29.23
8d	COPh	Ph	M	64.00	10.08	8.00	2.06	8.23
8bis	COOMe	$pCl-C_6H_4$	M	64.00	64.00	64.00	64.00	64.00
10a	COOMe	Ph	M	64.00	64.00	32.46	64.00	6.87
10b	COMe	Ph	P	64.00	64.00	32.46	64.00	2.15
			NS					11.62
10c	COOEt	$pCl-C_6H_4$	M	64.00	40.32	64.00	32.23	26.91
13a	Ph	Ph	P	64.00	64.00	64.00	64.00	64.00
13b	COOMe	Ph	P	64.00	64.00	64.00	64.00	64.00

* M = moderate in 13% DMSO/water; P = poor in 100% DMSO; NS no solubility issue.

considering the Selectivity Index (ratio toxicity/activity) in the case of *T. brucei*, the more active compounds compound, **4c, 4g** and **8d**, resulted quite safe (SI range 2–32) as well as those, **4g** and **8a, 4d**, for which inhibitory capability towards *T. cruzi* was evidenced (SI range 6–16). Better selectivity was revealed by compounds active against *P. falciparum*, especially in the case of the most active one **8b**, which combines selectivity towards the protozoarian agent with safety (SI value reaching 128).

Once defined the inhibitory activity of these polycondensed diazines, the next step was to try to identify the molecular target related to each microorganism. Several proteins have been already identified as responsible of the metabolism of these parasites, therefore the research pointed towards new anti-parasitic inhibitors which have to be specific binders of parasite proteins. For instance, it is known that Plasmodium has a limited ability to synthesize amino acids, so that it needs to find them pre-formed in the intra-erythrocyte environment, degradating hemoglobin to get the required amino acids and energy, or it needs to synthesize nucleic acid by means dihydrofolate reductase (pfDHFR) which, on the contrary of the mammalian one, also works as timidilate synthase. The same concept can be extended to Trypanosomes and Leishmanias which, for instance, have to fight the oxidative stress by means specific enzyme as trypanothione reductase, the parasite analogous of the mammalian enzyme glutathione reductase, or use specific proteases as well. Therefore we carried out a mixed molecular modeling approach (InducedFit Docking (IFD)/MM-GBSA) in the attempt to identify the molecular target of our selective compounds.

We started identifying into the Protein Data Bank (PDB)[10] the 3D structures relative to most common, and crucial for the parasites metabolism, enzymes: pfDHFR, plasmepsin, dihydroorotate dehydrogenase (DODH) for *Plasmodium*; trypanothione reductase (TR) and DHFR timidilate synthase (tcDHFR-TS), cruzain for *Trypanosoma cruzi*; pteridine reductase 1 (PTR1) for *Trypanosoma brucei*; and trypanothione reductase (TR) for *Leishmania*. More than one hundred structures were retrieved, but they were filtered according to the presence of co-crystallized inhibitors. Moreover a preliminary docking calculation by means Glide XP[11] was carried out with the aim to identify which protein-inhibitor complex could be better reproduced. Calculation of crystallized-docked superimposition root mean square deviation (RMSD) gives an estimation of the good quality of docking runs, and allows to select the more suitable complex to be used in further analysis. The lower value of RMSD for each protein-inhibitor complex guided the selection of the structure to be used for the IFD analysis of polycondensed diazine compounds; also in the case of the presence of a single protein-inhibitor complex RMSD values are suitable to select the structure. A list of the filtered complexes is reported in Table 2.

Thus, IFD calculation on identified complexes was carried out first. IFD is a mixed Molecular Dynamics/Docking protocol,[12] developed by Schrödinger Inc. (http://www.schrodinger.com/). This approach combines, in an iterative fashion, the ligand docking techniques with those for modeling receptor conformational changes. We used the same calculation settings set in our recent work.[13] IFD calculation were followed by Prime/MM-GBSA for the estimation of $\Delta G_{binding}$. The MM-GBSA approach employs molecular mechanics, the generalized Born model and the solvent accessibility method to elicit free energies from structural information circumventing the computational complexity of free energy simulations wherein the net free energy is treated as a sum of a comprehensive set of individual energy components, each with a physical basis.[14,15]

Table 2
Filtered PDB complexes and RMSD values (expressed in Å) for the superimposition of crystallized-docked ligands

		LIGAND	PDB	RMSD
Plasmodium	Plasmepsin	RS370	1LF2	1.3078
	pfDHFR	Pyrimethamine	2BL9	0.2973
	DODH	Genz667348	3O8A	0.2215
T. brucei	PTR1	Pemetrexed	2X9G	1.8340
L .infantum	TR	Antimonium	2W0H	2.4228
T. cruzi	TR	Spermidine	1BZL	0.8565
	tcDHFR-TS	Methotrexate	3CL9	0.7310
	Cruzain	Zya	1AIM	0.6806

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We carried out simulation only on most active compounds (IC_{50} <10 μM), and $\Delta G_{binding}$ were also calculated for co-crystallized ligands as reference compounds, in order to compare the binding capability of our polycondensed diazine derivatives with that of known inhibitors interacting with the proposed target. A first consideration can be done for *Plasmodium* DODH, for *T. brucei* PTR1 and *L. infantum* TR: no valuable results were obtained for these targets. Therefore anti-malarial activity of the studied polycondensed diazines seems not dependent from inhibition of DODH; nor tripanosomal inhibition found for **4b, 4c, 4g** can be related to reductase interaction. Moreover, it is unlikely that the only compound active against *L. infantum* (**8d**) interacts with TR.

Compound **8a** and **8d** showed a good binding capability both for Cruzain and tcTR if compared to reference compounds, with $\Delta G_{binding}$ up to 6 Kcal/mol more negative than that calculated for Zya and spermidine, respectively. But, no valuable $\Delta G_{binding}$ could be calculated for compound **4g**, the other derivative which showed to be good inhibitor of *T. cruzi* strains. When docked to Cruzain, compound **8a** forms a H-bond interaction with Gly166 by means the triazole nitrogen; the benzofused portion of quinoxaline inserts in the hydrophobic pocket created by Leu67, Ala133, and Leu157, as shown in Figure 1a. Other hydrophobic interactions stabilize the binding: the ester methyl interact with Trp26 and Ala156, respectively, but also the methyl bound to quinoxaline contributes to the interactions by means Leu67. When we consider tcTR-**8a** docking mode (Fig. 1b), no H-bonds formation was evidenced and the number of hydrophobic interactions is lower than in the case of Cruzain-**8a** complex. Therefore although **8a** can bind to both proteins, more stable could be considered the complex with tcTR.

When docked to Cruzain, compound **8d** also forms a H-bond with Gly166, this time by means the carbonyl oxygen, which involves that the benzofused moiety interacts only with Leu67 (Fig. 2a). One of the phenyl bound to carbonyl strongly interacts with the hydrophobic pocket created by Trp26, Met68, Ala133, Leu157, as far as the phenyl bound to triazole nitrogen interacts with Ala156. When we consider docking of the same compound **8d** to tcTR (Fig. 2b), no H-bonds were identified, but the higher calculated binding capability, with respect to **8a,** is due to more tight hydrophobic interactions which are established with phenyl rings of substituents (Leu63, Phe367, Cys58, Leu334, Pro336, Val59, Val54).

In the case of compound **8b**, the most active and selective against *P. falciparum* (SI = 128) in our series, excellent results were found only when docking to pfDHFR was considered, with binding

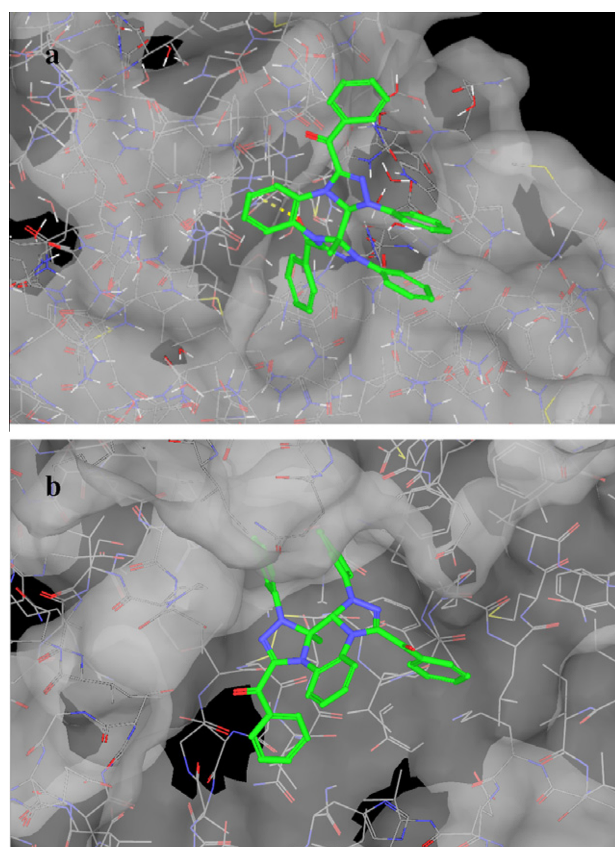

Figure 2. Compound **8d** docked to Cruzain (a) and to tcTR (b).

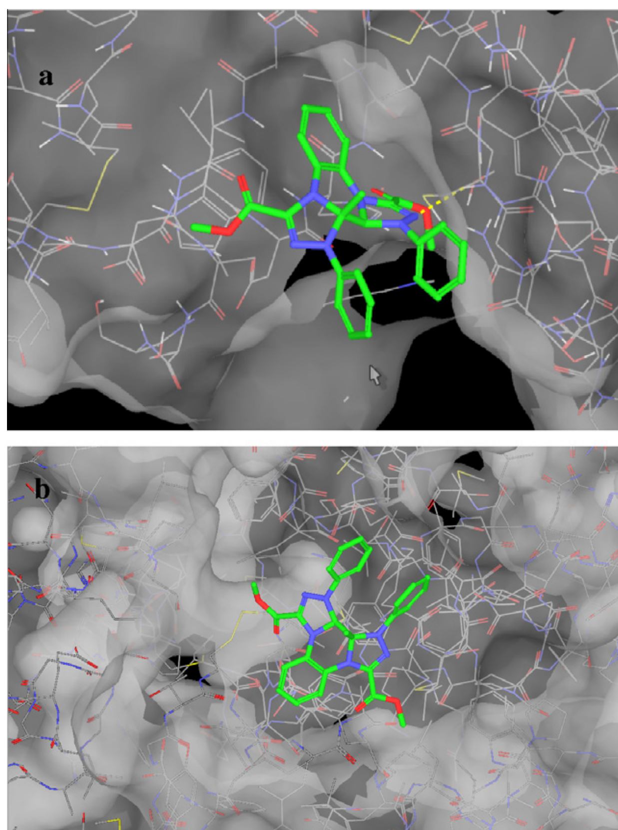

Figure 1. Compound **8a** docked to Cruzain (a) and to tcTR (b).

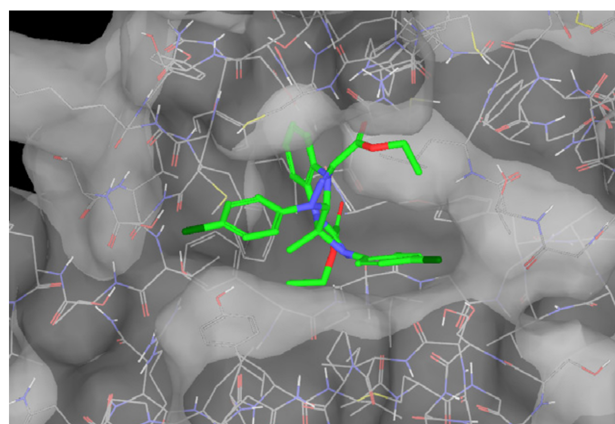

Figure 3. Compound **8b** docked to pfDHFR.

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capability comparable to that of the reference compound pyrimethamine, paralleling thus the results of in vitro screenings (Fig. 3). The ethyl chain inserts into the hydrophobic pocket created by Met54, Phe57, Ile121, and Leu128. The docking mode was strengthened also by the other hydrophobic interactions of *p*-chloro-phenyl rings with Tyr125, for one of them, and Pro122 and Leu45 for the other one, together with the interactions between the benzofused ring of quinoxaline with Leu45 and Trp47.

In summary, in this work we presented a series of polycondensed diazine compounds which revealed interesting in vitro activity against some parasites, generally joined to low level of toxicity. By means in silico studies we tried to identify their molecular mechanism of action. For some of the more active compounds it was possible define the most likely molecular target, so that the subsequent optimization of these series can be undertaken.

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9. For full details of the biological assays used in this publication see: Cos, P.; Vlietinck, A. J.; Vanden Berghe, D.; Maes, L. *J. EthnoPharm.* **2006**, 106, 290. Stock solutions of test compounds were prepared in sterile distilled water and DMSO. Drug solutions were serially diluted with culture medium and introduced to parasite cultures on plates comprising 96-wells. The concentration causing 50% inhibition (IC_{50}) was obtained from the drug concentration–response curve and the results were expressed as the mean determined from several independent experiments. *In vitro model for Leishmania*: the visceral species *Leishmania donovani* or *Leishmania infantum* are best suited for drug screening. The use of promastigotes must be discouraged, because of lower level of validation. To prepare the inoculum for infection, amastigotes are harvested from the spleen of infected donor hamsters. Murine peritoneal macrophages are generally used as host cell and are obtained after intraperitoneal stimulation with 2% starch in water. The peritoneal cells are harvested about 24–48 h later and plated in 96-well microplates at about 10^4 cells/well. After adding 10^5 amastigotes per well and 5 days of incubation, parasite burdens are microscopically assessed after Giemsa staining. *In vitro model for African trypanosomes*: because of the non-pathogenicity for man, a drug sensitive *Trypanosoma brucei* strain is preferred for primary screening purposes. The bloodstream (trypomastigote) forms are axenically grown in Hirumi-9 (HMI) medium at 37 °C under an atmosphere of 5% CO_2. Assays are performed in 96-well tissue culture plates, each well containing about 10^4 parasites. After 4 days incubation, parasite growth is assessed by adding Alamar Blue^{TM} or resazurin for fluorimetric reading (excitation 530 nm; emission 590 nm) after 4 h at 37 °C. *In vitro model for Chagas disease*: according to existing bio-safety guidelines, *Trypanosoma cruzi* is a biohazard class-3 pathogen and all laboratory work should be carried out under BSL-3 containment. Particularly strict safety precautions must be adopted (working in a LAF safety cabinet, wearing gloves, a safety mask and a laboratory safety gown). Extreme care has to be taken with needles to prevent puncture accidents. The Tulahuen strain of *Trypanosoma cruzi* is nifurtimox-sensitive and can be maintained on MRC-5 cells. Assays are performed in 96-well tissue culture plates, each well containing the compound dilutions together with 3×10^3 MRC-5 cells and 3×10^4 parasites. Recently, a strain transfected with β-galactosidase (Lac Z) gene has been developed enabling colorimetric reading after addition of chlorophenolred β-D-galacto-pyranoside as substrate. *In vitro model for malaria*: of the four species that infect humans, only *Plasmodium falciparum* can be cultured in vitro. Several strains can be used and it is advised to include drug-sensitive as well as drug-resistant strains in the primary screening panel. Drug-sensitive (chloroquine) strains GHA were employed. The strains are maintained in continuous log phase growth in RPMI-1640 medium supplemented with 10% human serum and 4% human erythrocytes. The human serum can also be replaced by lipid-rich bovine serum albumin (AlbuMAXII). All cultures are conducted at 37 °C under micro-aerophilic (4% CO_2, 3% O_2 and 93% N_2) atmosphere. Assays are performed in 96-well tissue culture plates, each well containing the compound dilutions together with the parasite inoculum (1% parasitaemia, 2% haematocrit). After 72 h of incubation at 37 °C, plates are stored at 20 °C until further processing. After thawing, 20 μl of haemolysed parasite suspension from each well is transferred into another plate together with 100 μl Malstat™ reagent and 10 μl of a 1/1 mixture of phenazine ethosulphate (PES, 2 mg/ml) and NBT (Nitro Blue Tetrazolium Grade III, 0.1 mg/ml). The plates are kept in the dark for 2 h and change in colour is measured spectrophotometrically at 655 nm. Alternatives to the Malstat assay for quantification of parasite growth is [^{3}H]-hypoxanthine incorporation assay (addition of 0.2 μCi and reading with liquid scintillation counter after 24 h), the use of the DNA fluorochrome Picogreen® or simple microscopic reading of Giemsa-stained smears. *Cytotoxicity assays*: MRC-5 cells are cultured in MEM medium, supplemented with 20 mM L-glutamine, 16.5 mM $NaHCO_3$ and 5% fetal calf serum. Assays are performed at 37 °C and 5% CO_2 in 96-well tissue culture plates with confluent monolayers. After 4–7 days incubation, cell proliferation and viability is assessed after addition of Alamar Blue^{TM} or resazurin. After 4 h at 37 °C, fluorescence is measured (550 nm excitation, 590 nm emission).
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