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Lipophilic conformationally constrained spiro carbocyclic 2,6-diketopiperazine-1-acetohydroxamic acid analogues as trypanocidal and leishmanicidal agents: an extended SAR study

Grigoris Zoidis¹, Andrew Tsotinis¹, Alexandra Tsatsaroni¹, Martin C. Taylor², John M. Kelly², Antonia Efstathiou³, Despina Smirlis³, George Fytas¹,*

¹School of Health Sciences, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, National and Kapodistrian University of Athens, Panepistimioupoli-Zografou, GR-15771 Athens, Greece
²Department of Pathogen Molecular Biology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK
³Laboratory of Molecular Parasitology, Department of Microbiology, Hellenic Pasteur Institute, 127 Vas. Sofias Ave, 11521 Athens, Greece

*To whom correspondence should be addressed. Phone: +302107224810. Fax: +30210 7274747. E-mail: gfytas@pharm.uoa.gr

Abstract
We have previously described a number of lipophilic conformationally constrained spiro carbocyclic 2,6-diketopiperazine (2,6-DKP)-1-acetohydroxamic acids as potent anti-trypanosomal agents. In this report, we extend the SAR analysis in this class of compounds with respect to in vitro growth inhibition of Trypanosoma and Leishmania parasites. Introduction of bulky hydrophobic substituents at the vicinal position of the basic nitrogen atom in the spiro carbocyclic 2,6-DKP ring system can provide analogues which are potently active against bloodstream-form T. brucei and exhibit significant activities towards T. cruzi epimastigotes and L. infantum promastigotes and intracellular amastigotes. In particular, compounds possessing a benzyl or 4-chlorobenzyl substituent were found to be the most active growth inhibitors, with activities in the low nanomolar and low micromolar ranges for T. brucei and L. infantum, respectively. The benzyl substituted (S)-enantiomer was the most potent derivative against T. brucei (IC₅₀=6.8 nM), T. cruzi (IC₅₀=0.21 μM) and L. infantum promastigotes (IC₅₀=2.67 μM) and intracellular amastigotes (IC₅₀=2.60 μM). Moreover, the (R)-chiral benzyl substituted derivative and its racemic counterpart displayed significant activities against L. donovani. Importantly, the active compounds show high selectivity in comparison with two mammalian cell lines.
1 INTRODUCTION

Trypanosomatid protozoan parasites of the genera *Trypanosoma* and *Leishmania* are the aetiological agents of serious diseases; human African trypanosomiasis (HAT or sleeping sickness), Chagas disease and the leishmaniasis (cutaneous, mucocutaneous and visceral). These three parasitic diseases severely affect human health, representing a huge social and economic burden. As recently as the turn of the century, there were estimated to be 0.3 million infections with *T. brucei* annually, although the numbers have since fallen significantly. In Latin America, 5 - 8 million people are currently infected with *T. cruzi* and globally there are an estimated 1 million new infections with different *Leishmania* species each year. Since there are no human vaccines, prevention and treatment of these devastating diseases relies on public health measures and chemotherapy. Unfortunately, the current drugs have many limitations.

Pentavalent antimony, the most widely prescribed treatment for visceral leishmaniasis, since its introduction several decades ago, has shortcomings which include serious side effects, the requirement for a prolonged course of treatment, and the emergence of drug resistance. Similarly, other visceral leishmaniasis drugs which have emerged over the past 10–15 years, also have drawbacks. With paromomycin, difficulties include administration (injectable, long treatment) and region-dependent efficacy. With miltefosine, the problems are high cost, long treatment and potential for teratogenicity. With the liposomal amphotericin B, high cost is also an issue, together with the need for hospitalization and potential teratogenicity.

For HAT, the first-line treatment for second-stage infections, since its introduction in 1949, has been the arsenic-based drug melarsoprol. However, it kills 5% those treated. In addition, increased drug resistance and high therapeutic failure rates have been reported recently in several foci. Efflorentine, an alternative treatment, now given in combination with nifurtimox, is better tolerated, but difficult to administer. Despite the urgency to develop new drugs for these neglected diseases, research and development has been limited by the lack of commercial interest.

We have previously reported on a series of acetohydroxamic acid analogues (Figure 1, compounds 1a-c, 1f, 1g, 2, 6a-c, 6f, 7a, 7b) which have potent anti-trypanosome activity. These compounds were derived from conformationally constrained lipophilic spiro carbocyclic 2,6-diketopiperazine (2,6-DKP) scaffolds by introducing an acetohydroxamic acid moiety (CH$_2$CONHOH) into their imidic nitrogen atom. The hydroxamic acid unit (CONHOH) is indispensable for trypanocidal activity in this class of compound. Thus, we assumed that these primary hydroxamic acids act by inhibiting a vital parasite metalloenzyme via the metal ion binding action of the hydroxamate group in the catalytic site. It was also found that the potency of these acetohydroxamic acid – based trypanocidal agents was affected by: (a) the structure of the spiro carbocyclic ring and, (b) the presence of alkyl substituents on either the basic nitrogen atom (N-methylation), or at its vicinal position (C-methylation or benzylation) in the spiro carbocyclic 2,6-DKP portion. Notably, attaching a benzyl group to the position adjacent to amine nitrogen (C-benzylation) significantly improved the potency against *T. brucei* and *T. cruzi* (Table 1, compounds 1f, 1g, 2, 6f) relative to the parent compounds (1a, 6a). This finding revealed that the stereoelectronic features and lipophilicity of the substituent at the above position play an important role in the potency of this class of hydroxamates. Subsequently, we
have shown that the introduction of a methyl substituent into the nitrogen atom of the hydroxamic unit (CONHOH) results in inactive compounds (Table 1, 1a, 2 vs 4, 5).\(^ {10}\)

The present work is an extension of our ongoing efforts to enrich the structure-activity relationships based on variation of the alkyl or benzyl substituent at the position adjacent to the amine nitrogen of the 2,6-DKP ring. Thus, we incorporated at this position an isobutyl (compounds 1d, 6d, 7d), a 2-(methylthio)ethyl (compound 1e) or a 4-chlorobenzyl group (compound 3). The trypanocidal properties of the newly synthesized compounds were assessed against cultured bloodstream-form *T. brucei*. In addition, compounds 1a-g, 2, 3, 6a-d, 6f, 7a, 7b and 7d were evaluated for their activity toward three different *Leishmania* sp., whilst their cytotoxicity against two established mammalian cell lines was investigated.

\section{METHODS AND MATERIALS}

\subsection{Chemistry}

Melting points were determined using a Büchi capillary apparatus and are uncorrected. \(^1\)H and \(^{13}\)C NMR spectra were obtained on Bruker MSL 400 (400 MHz \(^1\)H, 100 MHz \(^{13}\)C), Bruker AVANCE III 600 (600 MHz \(^1\)H, 150 MHz \(^{13}\)C), Varian 300 (75 MHz \(^{13}\)C), and Bruker AVANCE 200 (50 MHz \(^{13}\)C) spectrometers, using CDCl\(_3\) or DMSO-\(d_6\) as solvent. Chemical shifts are reported in \(\delta\) (ppm) with tetramethylsilane or solvent (DMSO-\(d_6\)) as internal standard. Splitting patterns are designated as \(\text{s}\), singlet; \(\text{d}\), doublet; \(\text{dd}\), doublet of doublets; \(\text{t}\), triplet; \(\text{td}\), triplet of doublets; \(\text{q}\), quartet; \(\text{qd}\), quartet of doublets; \(\text{m}\), multiplet; \(\text{br}\), broad; \(\text{v br}\), very broad; \(\text{sym}\), symmetrical. The spectra were recorded at 293 K (20 °C) unless otherwise specified. Carbon multiplicities were established by DEPT experiments. 2D NMR experiments (HMOC and COSY) were performed for the elucidation of the structures of the newly synthesized compounds. Low resolution mass spectra were recorded on either an API 2000 LC-MS/MS system, using positive electrospray ionization mode or Thermo Electron Corporation DSQ mass spectrometer in chemical ionization (CI) in positive ion mode with methane as CI reagent gas or in electron impact (EI). High resolution mass spectra (HRMS) were determined on a hybrid LTQ-Orbitrap Discovery mass spectrometer under electrospray ionization (ESI) in positive or negative ion mode. Optical rotations were measured on a Perkin Elmer 341 polarimeter at the sodium D line (589). Analytical thin-layer chromatography (TLC) was conducted on precoated Merck silica gel 60 F\(_{254}\) plates (layer thickness 0.2 mm) with the spots visualized by iodine vapors and/or UV light. Column chromatography purification was carried out on silica gel 60 (70-230 and 230-400 mesh ASTM). Elemental analyses (C, H, N) were performed by the Service Central de Microanalyse at CNRS (France) or Department of Microanalysis of NCSR “Democritos” (Greece), and were within ±0.4% of the calculated values. The purities of the tested compounds were determined by analytical HPLC and elemental analysis. The obtained results correspond to >95% purity. Analytical HPLC was performed on a Thermo Finnigan HPLC system (Thermo Finnigan, San Jose, USA) consisting of a SpectraSystem P4000 pump, a SpectraSystem 100 degasser, a SpectraSystem AS3000 autosampler, and a SpectraSystem UV2000 PDA detector, controlled by a SpectraSystem controller. ChromQuest 4.1 software was used for the management of the data. For the HPLC-DAD, a Supelco Analytical Discovery HS C18 (250 mm x 4.6 mm, 5.0 μm) column was used and the injection volume was 10 μL. The mobile phase consisted of H\(_2\)O and 1% acetic acid (solvent A) and acetonitrile (solvent B), and solvent gradient of A/B was 95/5 to 0/100. The analyses were performed at r.t. with a constant flow rate of 1 mL/min using a gradient elution of 0-50 min. The
commercial reagents were purchased from Alfa Aesar, Sigma-Aldrich and Merck, and were used without further purification except for the benzyl bromoacetate. This reagent was purified by distillation in vacuo prior to use. O-(4-Methoxybenzyl)hydroxylamine was synthesized according to the literature reported method.\textsuperscript{[11]} Organic solvents used were in the highest purity, and when necessary, were dried by the standard methods. Yields refer to chromatographically pure materials.

The synthesis of compounds 1a-c, 1f, 1g, 2, 4, 5, 6a-c, 6f, 7a, 7b, 9, 10, 13, 17, 18, 21, 25, 31, 32, 35, 39, 45, 46, 49, 52, 58, 66, 67, 74, 75, 77, 78, 80, 81, 83, 84 has been previously described in our published protocols.\textsuperscript{[9,10,12]} The experimental details, physical and chemical data of compounds 14, 15, 22, 23, 26, 28, 29, 36, 37, 40, 42, 43, 50, 51, 53, 55, 56, 59, 62-64, 68, 69, 71, 72, 76, 79, 82, 85-88 are given in the present work (Supporting information). The new compounds 1d, 1e, 3, 6d, 7d, 48, 60, 61, 65, 70, 73 (main manuscript), 11, 12, 16, 19, 20, 24, 27, 30, 33, 34, 38, 41, 44, 47, 54, 57, (Supporting Information) were synthesized as a part of this study.

\subsection*{2.1.1 (S)-N-Hydroxy-2-(2-methylpropyl)-3,5-dioxospiro[piperazine-2,2'-tricyclo[3.3.1.1\textsuperscript{3,7}] decane]-4-acetamide 1d}

Carboxylic acid 47 (1.33 g, 3.82 mmol) was treated with 1,1'-carbonyldimidazol (743 mg, 4.58 mmol) in dry THF (75mL) as described for the preparation of 58 from 45.\textsuperscript{[9]} Then, O-benzyl hydroxylamine hydrochloride (731 mg, 4.58 mmol) and triethylamine (509 mg, 5.04 mmol) were added, and the mixture was stirred at 28°C for 25 h under argon. The reaction was worked up following the same procedure described in 58, and the resulting viscous oil was chromatographed on silica gel column with AcOEt-n-hexane 1:1, as eluent, to afford the corresponding O-benzyl hydroxamate 60 as a white foamy solid, which strongly binds the aforementioned solvents. Removal of the entrapped solvents upon drying at 62-64°C under vacuum (10\textsuperscript{3} mmHg) in an Abderhalden apparatus gave 60 as a glass solid (1 g, 58%). This compound appears in the \textsuperscript{1}H and \textsuperscript{13}C NMR spectra as a mixture of Z/E conformers. \textsuperscript{1}H NMR (600 MHz, CDCl\textsubscript{3}, 273 K) \(\delta\) 0.96 (t, 6H, J=3.6, 6.0 Hz, CH(CH\textsubscript{2})\textsubscript{3}), 1.05-1.30 (br s, 1H, 1-H), 1.39-1.60 (complex m, 3H, 4'e, 9'e-H, CHHCH(\textsubscript{2}C\textsubscript{6})), 1.61-1.99 (complex m, 10H, 3', 5', 6', 7', 8', 10'e-H, CHHCH(\textsubscript{2}C\textsubscript{6})), 2.05-2.20 (complex m, 2H, 1', 4'a-H), 2.42 (~t, 1H, J=12.0, 12.6 Hz, 9'a-H), 2.87 (t, 1H, J=13.2, 13.8 Hz, 10'a-H), 3.65 (~br s, 1H, 6-H), 4.04-4.41 (q, AB, 1.2H, J=15.0 Hz, CH\textsubscript{2}CONHOCH\textsubscript{2}Ph), 4.49-4.69 (q, AB, 0.93H, J=16.8 Hz, CH\textsubscript{2}CONHOCH\textsubscript{2}Ph), 4.85, 4.89 (s=s, 2H, CONHOCH\textsubscript{2}Ph), 7.28-7.43 (complex m, 5H, aromatic H), 8.61 (s, 0.38H, CONHOCH\textsubscript{2}Ph), 9.03 (s, 0.33H, CONHOCH\textsubscript{2}Ph); \textsuperscript{13}C NMR (150 MHz, CDCl\textsubscript{3}, 273 K) \(\delta\) 21.2, 23.6 (CH\textsubscript{2}(\textsubscript{CH})\textsubscript{3}), 24.5 (CH\textsubscript{2}(\textsubscript{CH})\textsubscript{3}), 27.0, 27.3 (5', 7'-C), 31.1 (1'-C), 31.7 (4'-C), 32.7 (8'-C), 33.1 (9'-C), 33.9 (10'-C), 34.9 (3'-C), 37.9 (6'-C), 40.3, 40.4 (CH\textsubscript{2}CONHOCH\textsubscript{2}Ph), 41.7 (CH\textsubscript{2}(\textsubscript{CH})\textsubscript{3}), 51.6 (6-C), 60.7 (2, 2'-C), 78.2, 79.6 (CONHOCH\textsubscript{2}Ph), 128.6, 128.7, 128.9, 129.2, 129.4 (2, 3, 4, 5, 6-aromatic C), 134.2, 135.2 (1-aromatic C), 165.6, 170.8 (CONHOCH\textsubscript{2}Ph), 174.9, 175.9 (3, 5-C); ESI\textsuperscript{+}MS: m/z 454.5 [M+H]\textsuperscript{+}.

Compound 60 (856 mg, 1.89 mmol) was subjected to catalytic hydrogenation (H\textsubscript{2}/Pd-C, 103 mg) in abs EtOH (90 mL) following the procedure previously described.\textsuperscript{[9]} The hydrogenation product was chromatographed on silica gel column with AcOEt-n-hexane 1:1, as eluent, to afford the titled compound 1d as a white foamy solid, which strongly binds the eluting solvents. Removal of the entrapped solvents upon drying at 55°C under vacuum (10\textsuperscript{3} mmHg) in an Abderhalden apparatus gave 1d as an off-white semifoamy solid (658 mg, 96%): \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}) \(\delta\) 0.91 (d, 6H, J=6.0 Hz, CH(\textsubscript{3}CH)\textsubscript{2}), 1.37 (d, 1H, J=11.6 Hz, 9'e-H), 1.41-1.51 (m, 2H, 4'e-H, CHHCH(\textsubscript{2}C\textsubscript{6})), 1.53-

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1.98 (complex m, 10H, 3', 5', 6', 7', 8', 10'e-H, CHHCH(CH₃)₂), 2.07 (s, 1H, 1'-H), 2.14 (d, 1H, J=12.0 Hz, 4'a-H), 2.46 (d, 1H, J=11.2 Hz, 9'a-H), 2.69 (d, 1H, J=11.6 Hz, 1'H), 2.83 (d, 1H, J=10.8 Hz, 10'a-H), 3.42-3.54 (~td, 1H, J=2.6, 11.1 Hz, 6-H), 4.05-4.20 (q, AB, 1.4H, J₆₀=15.6 Hz, CH₂CONH₂OH, E-isomer), 4.33-4.50 (q, AB, 0.4H, J₆₀=16.8 Hz, CH₂CONH₂OH, Z-isomer), 8.82 (s, 0.57H, CONHOH, E-isomer), 9.24 (s, 0.14H, CONHOH, Z-isomer), 10.10 (s, 0.15H, CONHOH, Z-isomer), 10.49 (s, 0.53H, CONHOH, E-isomer); ¹³C NMR (75 MHz, DMSO-d₆) δ 21.2, 23.5 (CH₂(CH₃)CH₂), 24.1 (CH₂(CH₃)₂), 26.7, 27.0 (5', 7'-C), 30.3, 30.5 (1'-C, Z/E-isomers), 31.3 (4'-C), 32.2 (8'-C), 32.9 (9'-C), 33.4 (10'-C), 33.9, 34.0 (3'-C, Z/E-isomers), 37.8 (6'-C), 39.6 (CH₂CONHOH), 40.8, 40.9 (CH₂(CH₂CH₂)₂, Z/E-isomers), 51.1 (6-C), 59.7, 59.8 (2', 2'-C, Z/E-isomers), 164.2 (CONHOH, E-isomer), 169.6 (CONHOH, Z-isomer), 174.7, 174.8, 175.0, 175.1 (3, 5-C, Z/E-isomers); [α]$_D^{25}$ = -29.5 (c, 0.2, CHCl₃); HRMS (ESI): [M-H]$^-$ calc for C₁₅H₂₉N₂O₄: 362.2080, found, 362.2067. The hydrochloride salt (1d-HCl) was prepared by treating an ether solution of 1d with ethereal HCl under ice cooling. The white precipitate was collected by filtration, triturated with ether and dried in vacuo. Mp 200-203°C (dec). Anal. Calcd for C₁₅H₂₉N₂O₄: C, 57.06; H, 7.56; N, 10.51; Found: C, 56.72; H, 7.41; N, 10.82.

2.1.2  
(S)-N-Hydroxy-6-[(2-methylthio)ethyl]-3,5-dioxospiro[piperazine-2,2'-tricyclo[3.3.1.1^{3,7}]decane]-4-acetamide 1e

A mixture of benzyl ester 34 (850 mg, 1.86 mmol) and 10% Pd on charcoal (1275 mg) in EtOH-AcOEt 3:2 (70 mL) was hydrogenated as prepared for the preparation of 45 from 31[21] to give a white foamy solid (646 mg). The $^1$H NMR spectrum of the product material showed a mixture of the desired carboxylic acid 48 (purity ca. 91%, yield 86%) and some unchanged 34. The carboxylic acid 48 was regarded as sufficiently pure to be used in the next reaction without further purification [48]: $^1$H NMR (400 MHz, CDCl₃) δ 1.48-1.59 (q, 2H, J=12.4 Hz, 4'e, 9'e-H), 1.61-1.93 (complex m, 9H, CHHCH₃S, 3', 5', 6', 7', 8', 10'e-H), 2.11 (s, 4H, 1'-H, SCH₂), 2.18 (d, 1H, J=12.0 Hz, 9'a-H), 2.32-2.50 (m, 2H, CHHCH₂S, 4'a-H), 2.63-2.80 (m, 2H, CH₂S), 2.89 (d, 2H, J=12.0 Hz, 10'a-H), 3.86 (dd, 1H, J=3.4, 9.0 Hz, 6-H), 4.30-4.62 (q, AB, 2H, J₉₀=17.6 Hz, CH₂CO₂H), 5.46-6.65 (v br s, 2H, 1-H, CO₂H)]. This material (646 mg) was treated with 1,1'-carbonyldimidazol (311 mg, 1.92 mmol) in dry THF (32 mL) as described earlier for the preparation of 58 from 45[9]. Then, O-benzylhydroxylamine hydrochloride (306 mg, 1.92 mmol) and triethylamine (214 mg, 2.11 mmol) were added, and the mixture was stirred at 28°C for 25 h under argon. The reaction was worked-up in exactly the same way described in 58, and the resulting viscous oil residue was chromatographed over flash silica eluting first with Et₂O-n-hexane 1:1 and then AcOEt to afford the corresponding O-benzyl hydroxamate 61 as a colourless viscous oil, which binds the elution solvents. Removal of the entrapped solvents upon drying at 55°C under vacuum (10⁻³ mmHg) gave 61 as a glass solid (542 mg, 62% from 34). This compound appears in the $^1$H and $^{13}$C NMR spectra as a mixture of Z/E isomers. $^1$H NMR (600 MHz, CDCl₃, 283 K) δ 1.38-1.47 (br s, 1H, 1'-H), 1.51 (d, 1H, J=12.4 Hz, 4'e-H), 1.56 (d, 1H, J=11.6 Hz, 9'e-H), 1.64-1.91 (complex m, 9H, CHHCH₂S, 3', 5', 6', 7', 8', 10'e-H), 2.11 (s, 3H, SCH₂), 2.17 (~br s, 2H, 1', 9'a-H), 2.34-2.50 (m, 2H, CHHCH₂S, 4'a-H), 2.65-2.78 (m, 2H, CH₂S), 2.83-2.95 (br s, 1H, 10'a-H), 3.78-3.93 (br s, 1H, 6-H), 4.07-4.42 (q, AB, 1H, J₉₀=15.1 Hz, CH₂CONHOCH₂Ph), 4.54-4.73 (q, AB, 1H, J₉₀=16.8 Hz, CH₂CONHOCH₂Ph), 4.87, 4.91 (s, 2H, CONHOCH₂Ph), 7.34-7.41 (m, 5H, aromatic H), 8.19 (s, 0.44H, CONHOCH₂Ph), 8.61 (s, 0.42H, CONHOCH₂Ph); $^{13}$C NMR (150 MHz, CDCl₃, 283 K) δ 15.1 (SCH₂), 26.9, 27.2 (5', 7'-C), 30.4 (CH₂S), 30.8, 31.0 (1'-C), 31.1 (CH₂CH₂S), 31.4 (9'-C), 32.5 (8'-C), 33.1 (4'-C), 33.8 (10'-C), 35.0 (3'-C), 37.9 (6'-C), 40.2 (CH₂CONHOCH₂Ph), 51.7 (6-C), 60.9 (2,2'-C), 78.1,
Compound 61 (360 mg, 0.76 mmol) was subjected to catalytic hydrogenation (10% Pd-C, 550 mg) in EtOH-AcOEt 3:2 (35 mL) following the procedure previously described.[9] The hydrogenation material was chromatographed over flash silica eluting first with AcOEt-n-hexane 1:1 and then AcOEt to afford successively the unchanged compound 61 and title compound 1e as a white foamy solid, which strongly binds the elution solvents. Removal of the entrapped solvent upon drying at 62-64 °C under vacuum (10⁻³ mm Hg) in an Abderhalden apparatus gave 1e as an off-white solid (72 mg, 25%): mp 75-78 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 1.39 (d, 1H, J=11.7 Hz, 4'e-H), 1.46 (d, 1H, J=11.7 Hz, 9'e-H), 1.54-1.92 (complex m, 9H, 3', 5', 6', 7', 8', 10' e-H), 2.05 (s, 3H, SCH₃), 2.07 (s, 1H, 1'-H), 2.12-2.29 (m, 2H, 9'a-H, CHHCH₂S), 2.45 (d, 1H, J=10.8 Hz, 4'a-H), 2.67 (~t, 2H, J=7.3, 7.8 Hz, CH₂S), 2.82 (~d, 2H, J=11.4 Hz, 1, 10'a-H), 3.60-3.75 (sym quintet, 1H, 1', 9'a-H), 4.07-4.19 (q, AB, 1.36 H, Jₙ=15.6 Hz, CH₁CONHO, E-isomer), 4.21-4.31 (m, 0.38 H, CH₂CONHO, E-isomer), 4.36-4.53 (q, AB, 0.35 H, J=16.8 Hz, CH₂CONHO, Z-isomer), 8.84 (s, 0.7 H, CONHO, E-isomer), 9.26 (s, 0.2 H, CONHO, Z-isomer), 10.12 (s, 0.2 H, CONHO, Z-isomer), 10.52 (s, 0.7 H, CONHO, E-isomer); ¹³C NMR (100 MHz, DMSO-d₆) δ 14.4 (SCH₃), 26.6, 26.9 (5', 7'-C), 29.9 (CH₂S), 30.1, 30.2 (1'-C), 30.8, 30.9 (CH₂CH₂S), 31.1 (9'-C), 32.2 (8'-C), 32.8 (4'-C), 33.4 (10'-C), 34.0, 34.1 (3'-C), 37.8 (6'-C), 39.5, 39.9 (CH₂CONHO), 51.3, 51.4 (6-C), 59.9 (2, 2'-C), 164.1 (CONHO, E-isomer), 169.6 (CONHO, Z-isomer), 174.4, 174.6 (3, 5-C); HRMS (APCI⁺): [M+H⁺]⁺ calcd for C₁₇H₁₂N₂O₂S 382,1756, found 382,1791. The hydrochloride salt (1e-HCl) was prepared as described for 1d-HCl. Mp 139-143 °C (dec); [α]₂⁰Ring -22 (C, 0.1, DMSO). Anal Calcd for C₁₉H₁₉Cl₂N₂O₆S: C, 51.73; H, 6.75; N, 10.05. Found: C, 52.10; H, 6.98; N, 10.12.

2.1.3 (RS)-6-[[4-Chlorophenyl]methyl]-N-hydroxy-2,6-dioxospiro[piperazine-2,2′-tricyclo[3.3.1.1³,7]decane]-4-acetamide 3
A mixture of 4-methoxybenzyl ester 3B (1.0 g, 1.86 mmol) and TFA (3 mL) in CH₂Cl₂ (20 mL) was stirred for 90 min. The solution was evaporated to dryness under reduced pressure, and the residue was dissolved in dry THF (36 mL). Triethylamine (304 mg, 3 mmol) and 1,1′-carbonyldiimidazol (364 mg, 2.24 mmol) were added, and the mixture was stirred at 28 °C for 1h under argon. After this time, O-(4-methoxybenzyl)hydroxylamine (344 mg, 2.24 mmol) was added, and the mixture was stirred 24 h at 28 °C and 1 h at 55 °C under argon. The reaction was then worked up in the same way described for the preparation of 5B from 45. The resulting thick oil was chromatographed on silica gel column with AcOEt-n-hexane 1:2, as eluent, to afford the corresponding O-benzyl hydroxamate 65 as a white foamy solid, which strongly binds the elution solvents. Removal of the entrapped solvents as in 60 gave 65 as a glass solid (715 mg, 70%). This compound appears in the ¹H and ¹³C NMR spectra as a mixture of E/Z conformers. ¹H NMR (600 MHz, CDCl₃, 273K) δ 1.39-1.50 (m, 2H, 4'e, 9'e-H), 1.55-1.88 (complex m, 10H, 1, 3', 4'a, 5', 6', 7, 8', 10'-e-H), 1.99-2.17 (m, 2H, 1', 9'a-H), 2.81-3.0 (complex m, 2H, 10'a-H, CHHCH₂Cl-4), 3.35 (~d, 1H, J=13.8 Hz, CHHCH₂Cl-4), 3.75-3.88 (m, 1H, 6-H), 3.78, 3.81 (s + s, 3H, OCH₃), 4.10-4.40 (q, AB, 1H, J₉=15.3 Hz, CH₂CONHOCH₂C₆H₄OCH₃-4), 4.47-4.68 (q, AB, 1H, J₉=16.8 Hz, CH₂CONHOCH₂C₆H₄OCH₃-4), 4.80, 4.83 (s + s, 2H, CONHOCH₂C₆H₄OCH₃-4), 6.84-6.95 (dd, 2H, J₈=8.4, 20.4 Hz, aromatic H), 7.14-7.36 (complex m, 6H, aromatic H), 8.53 (s, 0.4 H, CONHOCH₂C₆H₄OCH₃-4), 8.96 (s, 0.4 H, CONHOCH₂C₆H₄OCH₃-4); ¹³C NMR (150 MHz, CDCl₃, 273 K) δ 26.9 (5', 7'-C), 30.6 (1'-C), 31.3, 31.4 (4'-C), 32.5 (9'-C), 33.2 (8'-C), 33.9 (10'-C), 34.8 (3'-C), 37.3 (6'-C), 37.9 (CH₂CH₂Cl-4), 40.2, 40.3 (CH₂CONHOCH₂C₆H₄OCH₃-4), 54.1, 54.2 (6-C), 55.3, 55.4 (OCH₃),
A mixture of compound 65 (451 mg, 0.82 mmol) and TFA (6 mL) in CH₂Cl₂ (23 mL) was stirred for 10 min. The solution was then treated dropwise with Et₃SiH (600 μL) via syringe, and the stirring was continued for 45 min at room temperature. In this time window, the color changed from ruby to pale yellow. The reaction mixture was evaporated to dryness under reduced pressure, and the residual material was quenched with water (15 mL) and ether (50 mL) under vigorous stirring. To this stirred two phase mixture was added solid Na₂CO₃ to pH=8-9. The organic layer was separated and the water phase was extracted with ether (3 x 40 mL). The combined organics were washed with brine (50 mL), dried (Na₂SO₄) and evaporated to dryness. The thick oily residue was chromatographed on silica gel column with AcOEt−n-hexane 2:1, as eluent, to afford the title compound 3 as a white foamy solid, which binds the elution solvents. Removal of the entrapped solvents as in 1d gave 3 as an off-white semi foamy solid (313 mg, 89%): ¹H NMR (400 MHz, DMSO-d₆) δ 1.30 (d, 2H, J=11.6 Hz, 4′e, 9′e-H), 1.48-1.73 (complex m, 5H, 5′-H), 1.76 (s, 1H, 5′-H), 1.82 (s, 1H, 3′-H), 2.04 (s, 1H, 1′-H), 2.18 (d, 1H, J=12.0 Hz, 9′a-H), 2.69-2.92 (complex m, 3 H, CHH₃C₆H₄Cl-4, 1, 10′a-H), 3.30 (dd, 1H, J=3.2, 14.0 Hz, CHH₃C₆H₄Cl-4), 3.72 (td, 1H, J=3.2, 10.9 Hz, 6-H), 4.16 (s, 1.4 H, CH₂CONOH), 4.38-4.53 (q, AB, 0.43H, J=16.8 Hz, CH₂CONOH), 7.29-7.40 (m, 4H, aromatic H), 8.87 (s, 0.72 H, CONOH, E-isomer), 9.28 (s, 0.2 H, CONOH, Z-isomer), 10.16 (s, 0.2 H, CONOH, Z-isomer), 10.55 (s, 0.7 H, CONOH, E-isomer), ¹³C NMR (50 MHz, DMSO-d₆) δ 26.5 (5′-C), 26.7 (7′-C), 29.9 (1′-C), 30.9 (4′-C), 32.0 (9′-C), 32.8 (8′-C), 33.3 (10′-C), 34.1 (3′-C), 36.5 (CH₂C₆H₄Cl-4), 37.7 (6′-C), 39.5 (CH₂CONOH), 54.1 (6-C), 60.0 (2, 2′-C), 127.9, 131.0 (2, 3, 5, 6-aromatic C), 130.8 (4-aromatic C), 137.8 (1-aromatic C), 164.1 (CONOH, E-isomer), 169.5 (CONOH, Z-isomer), 173.5., 174.5 (3,5-C); HRMS (ESI): [M + H]⁺, [M + Na]⁺ calcd for C₂₂H₂₆ClN₂O₄ 432.1690, 454.1510, found 432.1689, 454.1506. The hydrochloride salt was prepared as described for 1d·HCl. Mp 179-182 °C (dec). Anal. Calcd for C₂₂H₂₇Cl₂N₃O₄·HCl: C, 56.41; H, 5.81; N, 8.97; Found: C, 56.06; H, 6.12; N, 8.65.

2.1.4 (S)-N-Hydroxy-2-(2-methylpropyl)-3,5-dioxo-1,4-diazaspiro[5.7]tridecan-4-aceta-mide 6d

Carboxylic acid 54 (1.13 g, 5.5 mmol) was treated with 1, 1′-carbonyldimidazole (681 mg, 4.2 mmol) in dry THF-DMF 6:1 (70 mL) as described for the preparation of 58 from 45.⁹ Then O-benzyldihydroxyamine hydrochloride (670 mg, 4.2 mmol) and triethylamine (468 mg, 4.62 mmol) were added, and the mixture was stirred at 28 °C for 25 h under argon. The reaction was worked up in exactly the same way described in 58, and the resulting viscous oil was chromatographed on silica gel column with AcOEt-n-hexane 2:3, as eluent, to afford the corresponding O-benzyl hydroxamate 70 as a white foamy solid, which strongly binds the aforementioned solvents. Removal of the entrapped solvents as in 60 gave 70 as a white solid (1.07 g, 71.5%): mp 115-118 °C. This compound appears in the ¹H and ¹³C NMR spectra as a mixture of E/Z conformers. ¹H NMR (400 MHz, CDCl₃) δ 0.95 (t, 6H, J=12.6 Hz, CH(CH₃)₂CH₃), 1.10-1.27 (br s, 1H, 1-H), 1.31-2.08 (complex m, 16 H, 7, 8, 9, 10, 11, 12, 13-H, CH₂CH₂CH(CH₃)₂), 2.17-2.35 (m, 1H, 13-H), 3.62 (d, 1H, J=22.0 Hz, 2-H), 4.12-4.40 (br s, 1H, CH₂CONOCH₂Ph), 4.45-4.75 (br s, 1H, CH₂CONOCH₂Ph), 4.88 (s, 2H, CONOCH₂Ph), 7.37 (s, 5H, aromatic H), 8.12-8.45 (br s, 0.5H, CONOCH₂Ph), 8.57-8.88 (br s, CONOCH₂Ph), ¹³C NMR (50 MHz, CDCl₃) δ 20.8 (9-C), 21.2 (CH(CH₃)₂CH₃), 21.4 (11-C), 23.5 (CH₃CH₂CH₃), 24.5 (10-C, CH₂CH₂CH₃), 27.4 (8-C), 28.5 (7-C), 28.7 (12-C), 34.5 (13-C), 39.7 (CH₂CONOCH₂Ph), 40.5 (CH₂CH₃CH₂), 51.8 (2-C), 60.4

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Compound 7b (1.19 g, 2.8 mmol) was subjected to catalytic hydrogenation (10% Pd-C, 143 mg), in abs EtOH (126 mL) following the procedure previously described. The hydrogenation material was chromatographed on silica gel column eluting first with AcOEt-hexane 2:3 and then AcOEt to afford the title compound 6d as a white foam, which strongly binds the eluting solvent. Removal of the entrapped solvent as in 1e gave 6d as an off-white solid (850 mg, 90.5%): mp 129-132°C; 1H NMR (400 MHz, DMSO-δ) δ 0.84 (d, 3H, J=6.4 Hz, CH(CH3)2CH3), 0.88 (d, 3H, J=6.4 Hz, CH(CH3)2CH3), 1.30-1.96 (complex m, 16H, 7, 8, 9, 10, 11, 12, 13-H, CH2CH(CH3)2), 1.97-2.10 (q, 1H, J=9.2 Hz, 13-H), 2.42-2.48 (m, 1H, 1-H), 3.36-3.53 (m, 1H, 2-H), 4.0-4.13 (q, AB, 1.5H, Jab=15.6 Hz, CH2CONHOE, E-isomer), 4.30-4.42 (q, AB, 0.4H, Jab=16.8 Hz, CH2CONHOE, Z-isomer), 8.79 (s, 0.8H, CONHOE, E-isomer), 9.21 (s, 0.2H, CONHOE, Z-isomer), 10.08 (s, 0.2H, CONHOE, Z-isomer), 10.43 (s, 0.7H, CONHOE, E-isomer); 13C NMR (75 MHz, DMSO-δ) δ 20.4 (9-C), 21.0 (11-C), 21.1 23.5 (CH(CH3)2CH3), 24.0 (CH(CH3)2), 24.2 (10-C), 27.0 (8-C), 27.7, 27.8 (7-C), 28.3 (12-C), 33.4 (13-C), 39.2, 39.3 (CH2CONHOE), 39.6 (CH2CH(CH3)2), 51.1 (2-C), 59.5 (6-C), 164.0 (CONHOE, E-isomer), 169.5 (CONHOE, Z-isomer), 174.1, 176.9 (3, 5-C); [α]23[δB] -11 (c, 0.2, CHCl3); HRMS (ESI): [M-H] calcd for C12H20N2O4, 338.2080, found, 338.2069. The hydrochloride salt (6d-HCl) was prepared as described for 1d-HCl. Mp 212-214°C (dec). Anal. Calcd for C12H30ClN2O4: C, 54.32; H, 8.05; N, 11.18; Found: C, 53.98; H, 7.88; N, 10.89.

2.1.5 (S)-N-Hydroxy-2-(2-methylpropyl)-3,5-dioxospiro-1,4-diazaspiro[5.6]dodecane-4-acetamide 7a

Carboxylic acid 57 (1.52 g, 4.9 mmol) was treated with 1,1'-carbonyldiimidazol (957 mg, 5.9 mmol) in dry THF-DMF 6:1 (100 mL) as described for the preparation of 58 from 45.[9] Then, O-benzylhydroxylamine hydrochloride (942 mg, 5.9 mmol) and triethylamine (657 mg, 6.5 mmol) were added, and the mixture was stirred at 28°C for 25 h under argon. The reaction was worked up in exactly the same way described in 58, and the resulting thick oil was chromatographed on silica gel column with AcOEt-hexane 2:3, as eluent, to afford the corresponding O-benzyl hydroxamate 7a as a white foam, which strongly binds the aforementioned solvents. Removal of the entrapped solvents as in 60 gave 7a as a white solid (1.54 g, 76%): 129-131°C. This compound appears in the 1H and 13C NMR spectra as a mixture of E/Z conformers. 1H NMR (400 MHz, CDCl3) δ 0.93 (d, 3H, J=6.0 Hz, CH(CH3)2), 0.96 (d, 3H, J=6.3 Hz, CH(CH3)2), 1.13-1.37 (br s, 1H, 1-H), 1.38-2.03 (complex m, 14H, 7, 8, 9, 10, 11, 12-H, CH2CH(CH3)2), 2.27 (~t, 1H, J=9.9, 12.0 Hz, 12-H), 3.60 (d, 1H, J=8.0 Hz, 2-H), 4.09-4.39 (br s, 1H, CH2CONHOCH2Ph), 4.45-4.69 (br s, 1H, CH2CONHOCH2Ph), 4.87 (s, 2H, CONHOCH2Ph), 7.37 (s, 5H, aromatic H), 8.24-8.44 (br s, 0.34H, CONHOCH2Ph), 8.75-8.93 (s, 0.37H, CONHOCH2Ph); 13C NMR (50 MHz, CDCl3) δ 21.2 (CH2CH3), 21.8 (8-C), 22.7 (11-C), 23.5 (CH2CH3), 24.5 (CH(CH3)2), 29.4, 29.6 (9, 10-C), 33.6 (7-C), 38.8 (12-C), 39.0 (CH2CONHOCH2Ph), 40.3 (CH2CH(CH3)2), 51.8 (2-C), 61.2 (6-C), 78.5, 79.6 (CONHOCH2Ph), 128.8, 129.4 (2, 3, 4, 5, 6-aromatic C), 134.9 (1-aromatic C), 165.7, 170.8 (CONHOCH2Ph), 174.3, 177.6 (3, 5-C); 13C MS: m/z 416.2 ([M+H]+, 5), 359.1 ([M+H]+, 5), 265.1 ([M+H]+, 5), 264.1 (59), 91.0 (100).
Compound 73 (1.62 g, 3.9 mmol) was subjected to catalytic hydrogenation (10% Pd-C, 194 mg), in abs EtOH (175 mL) following the procedure previously described.\(^9\) The hydrogenation material was chromatographed on silica gel column with AcOEt-n-hexane 3:1, as eluent, to afford the title compound 7d as a white foamy solid, which strongly binds the aforementioned solvents. Removal of the entrapped solvent as in 1e gave 7d as an off-white crystalline solid (1.18 g, 93%): mp 152-155°C; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 0.88 (d, 3H, J=6.4 Hz, CH(CH\(_3\))CH\(_3\)), 0.92 (d, 3H, J=6.8 Hz, CH(CH\(_3\))CH\(_3\)), 1.33-1.80 (complex m, 12H, 7H, 8, 9, 10, 11, 12-H, CH\(_2\)CH(CH\(_3\))\(_2\)), 1.83-2.0 (m, 2H, CH\(_2\)CH\(_3\)), 2.02-2.16 (m, 1H, 12-H), 2.59 (d, 1H, J=12.4 Hz, 1-H), 3.41-3.56 (m, 1H, 1-H), 4.06-4.19 (q, AB, 1.5H, J\(_{ab}=15.6\) Hz, CH\(_2\)CONHOH, E-isomer), 4.35-4.51 (q, AB, 0.4H, J\(_{ab}=16.8\) Hz, CH\(_2\)CONHOH, Z-isomer), 8.84 (s, 0.7H, CONHOH, E-isomer), 9.25 (s, 0.2H, CONHOH, Z-isomer), 10.13 (s, 0.2H, CONHOH, Z-isomer), 10.48(s, 0.7H, CONHOH, E-isomer); \(^{13}\)C NMR (75 MHz, DMSO-\(d_6\)) \(\delta\) 21.1 (CH(CH\(_3\))CH\(_3\)), 21.3 (8-C), 22.3 (11-C), 23.5 (CH(CH\(_3\))CH\(_3\)), 24.0 (CH(CH\(_3\))\(_2\)), 29.1, 29.3 (9, 10-C), 33.1 (7-C), 37.7 (12-C), 39.4 (CH\(_2\)CONHOH), 39.5 (CH\(_2\)CH(CH\(_3\))\(_2\)), 51.1 (2-C), 60.2, 60.3 (6-C), 164.1 (CONHOH, E-isomer), 169.5 (CONHOH, Z-isomer), 174.1, 177.4 (3, 5-C); [\(\alpha\)]\(_{23}^{20}\) \(\delta\) 21 (c, 0.2, CHCl\(_3\)); HRMS (ESI): [M-H] calcd for C\(_{36}\)H\(_{28}\)N\(_3\)O\(_4\), 524.1923, found, 524.1911. The hydrochloride salt (7d-HCl) was prepared as described for 1d-HCl. Mp 211-213°C (dec). Anal. Calcd for C\(_{36}\)H\(_{28}\)Cl\(_2\)N\(_3\)O\(_4\): C, 53.10; H, 7.80; N, 11.61; Found: C, 53.39; H, 7.65; N, 11.32.

2.2 Biological evaluation

2.2.1 Trypanocidal assays

Bloodstream form T. brucei (strain 221) were cultured in modified Iscove’s medium, as outlined previously.\(^{13}\) Assays were performed in 96-well microtitre plates and the compound concentrations which inhibited growth by 50% (IC\(_{50}\)) and 90% (IC\(_{90}\)) were determined. Parasites were first diluted to 2.5 \(\times\) 10\(^5\) mL\(^{-1}\), compounds were added at range of concentrations, and the plates incubated at 37°C. Resazurin was added after 48 h (12.5 \(\mu\)g/ml), the plates incubated for a further 16 h, and then read in a Spectramax plate reader (excitation 555 nm/emission 585 nm). Results were analysed using GraphPad Prism. Each drug concentration was tested in triplicate.

T. cruzi epimastigotes (strain CL Brener) were cultured as described.\(^{14}\) Trypanocidal activity was determined in microtitre plates as outlined above, with the following modifications. Experiments were initiated by seeding the parasites at 2.5 \(\times\) 10\(^5\) mL\(^{-1}\), and after the addition of test compounds, cultured at 28°C for 4 days. Resazurin was added, the plates were incubated for a further 2 days, and then assessed as above.

2.2.2 Leishmanicidal assays and cytotoxicity assays on murine macrophages J774.1. cells

L. donovani (strain LG13, MHOM/ET/0000/HUSSEN), L. infantum (MHOM/GR/2002/GH12) and L. amazonensis (MPRO/BR/72/M1845) promastigotes were cultured at 26°C in RPMI 1640 (RPMI) medium supplemented with 10% heat-inactivated fetal bovine serum (fully supplemented RPMI), 10 mM HEPES and antibiotics (penicillin/ streptomycin) as previously described.\(^{15}\) The murine macrophage J774.1 cell line (American Type Culture Collection, Manassas, VA) was cultured as well as described above, in fully supplemented RPMI, and incubated at 37°C in 5% CO\(_2\). The inhibitory activity and the cytotoxicity of the compounds were determined with the use of an MTT-based assay, the Alamar blue, as previously described.\(^{16}\) More specifically, 2.5 \(\times\) 10\(^6\) parasites/ml and 2 \(\times\) 10\(^5\) macrophages/ml were seeded into 96-well plates (in total volume 200\(\mu\)l) and they were incubated for 72 hours with different concentrations of the compounds at 26°C and 37°C (in 5% CO\(_2\))
respectively. 20μl of alamar blue were added upon the 72 hour incubation and 24 hours later the colorimetric readings were performed (wavelength: 550 nm, reference wavelength: 620 nm). The 50% maximal inhibitory concentration (IC$_{50}$) was calculated using a nonlinear regression curve fit.[17] For evaluating the inhibitory activity of compounds against intracellular amastigotes, J774.1 macrophages were seeded into 96-well flat bottom plates at a density of 2 × 10$^5$ cells/mL and a total volume of 200μl, and were left to adhere overnight at 37 °C in 5% CO$_2$. Then, the macrophages were infected with stationary phase 4×10$^6$ promastigotes/mL, at a ratio of 10 parasites per 1 macrophage, and incubated for a further 24 h at 37 °C in 5% CO$_2$ as previously described.[16] Upon the incubation, the medium was removed, wells were thoroughly washed with PBS to remove free parasites and 200μl of fresh medium containing the different concentrations of the compounds was added. The plate was incubated for further 72 hours at 37 °C in 5% CO$_2$ and 20μl of alamar blue was added as described above. All experiments were performed at least three independent times in triplicate.

2.2.3 Cytotoxicity assays on rat skeletal myoblast L6 cells
Cytotoxicity against L6 cells was assessed using microtitre plates. Briefly, cells were seeded in triplicate at 1 × 10$^4$ mL$^{-1}$ in growth medium containing different compound concentrations. The plates were incubated for 6 days at 37 °C and resazurin then added to each well. After a further 8 h incubation, the fluorescence was determined using a Spectramax plate reader.

3 RESULTS AND DISCUSSION
3.1 Chemistry
The synthetic routes for the preparation of the target compounds (1a-g, 2-5, 6a-d, 6f, 7a, 7b and 7d) is illustrated in Scheme 1. 2,6-Diketopiperazine-1-acetic acids 45-57 and 83-85 were the pivotal intermediates in preparing the acetoxyhydroxamic acid analogues 1a-g, 2, 4, 5, 6a-d, 6f, 7a, 7b and 7d. Thus, coupling of these carboxylic acids with O-benzylhydroxylamine or O-benzyl-N-methylhydroxylamine in the presence of 1,1'-carbonyldiimidazol (CDI) in THF or THF-DMF mixtures afforded the corresponding O-benzyl hydroxamates 58-64, 66-73 and 86-88 in yields 58-95%. Subsequent benzyl deprotection by hydrogenation over 10% Pd-C gave the respective hydroxamic acids 1a-g, 2, 4, 5, 6a-d, 6f, 7a, 7b and 7d. It is of note that this reductive deprotection provided 1a-d, 1f, 1g, 2, 4, 5, 6a-d, 6f, 7a, 7b and 7d in high yields (80-96%), whilst it was low yielding (<10%) for 1e. This is presumably due to catalyst poisoning by the methionine sulfide group. A better yield of 1e (25%) was achieved when the catalyst amount was 1.5-fold the weight of its corresponding O-benzyl hydroxamate precursor 61.

The preparation of the chloro substituted hydroxamic acid 3 needed an alternative synthetic process as shown in Scheme 1. 4-Methoxybenzyl ester 38 was treated with trifluoroacetic acid (TFA) to give the respective carboxylic acid (not shown), which was coupled with O-(4-methoxybenzyl)hydroxylamine in the presence of CDI to form the O-(4-methoxybenzyl) hydroxamate 65 (70% from 38). Removal of the 4-methoxybenzyl protecting group of 65 was effected by treatment with trifluoroacetic acid in the presence of triethylsilane to give the desired compound 3 in 89% yield. The requisite 4-methoxybenzyl ester 38 was synthesized from 24 as described for the preparation of the benzyl esters 31-37 and 39-44, except that 4-methoxybenzyl bromoacetate was used in place of benzyl bromoacetate. It is noteworthy that the 4-methoxybenzyl bromoacetate used was prepared by employing a new facile and efficient experimental protocol involving the
treatment of 4-methoxybenzyl alcohol with bromoacetyl chloride in the presence of NaHCO$_3$ in dry CH$_2$Cl$_2$ (Supporting Information).

The $^1$H and $^{13}$C NMR spectra for all acetohydroxamic acid analogues (compounds 1a-g, 2-5, 6a-d, 6f, 7a, 7b and 7d) are consistent with a Z/E conformational behavior of these molecules in solution. The NMR spectral data of compounds 1a-c, 1f, 1g, 2, 6a-c, 6f, 7a and 7b have already been reported.$^{[9]}$

However, the assignment of the Z and E conformers given in this report was erroneous. Our next, more detailed conformational study,$^{[10]}$ has reversed these assignments, i.e. the original data referring to the E isomer correspond to the Z isomer structure and vice versa.

3.2 Biological evaluation

Compounds 1a-g, 2-5, 6a-d, 6f, 7a, 7b and 7d were tested against bloodstream form $T$. brucei in vitro. Analogues 1a, 1b and 1f were also tested against $T$. cruzi epimastigotes. The results are shown in Table 1 and expressed as IC$_{50}$ and IC$_{90}$ values. The in vitro anti-leishmanial activity of compounds 1a-g, 2, 3, 6a-d, 6f, 7a, 7b and 7d was evaluated against promastigote and intracellular amastigote forms of $L$. infantum GH12, $L$. donovani LG13 and $L$. amazonensis LV78. The IC$_{50}$ values are given in Table 2. The cytotoxicities of the hydroxamic acid derivatives against mammalian cells were determined using the rat skeletal myoblast L6 and murine macrophage J174.1 cell lines.

Inspection of Table 1 shows that compounds 1a-g, 2, 3, 6a-d, 6f, 7a, 7b and 7d exhibited potent activity against $T$. brucei as either free bases or hydrochloride salts (IC$_{50}$ = 6.6-1870 nM and 9.2-1150 nM respectively). In particular, compounds 1f, 1g, 2, 3, 6d and 6f were the most potent against African trypanosomes, with IC$_{50}$ values in the low nanomolar range (6.8-72 nM), while hydroxamates 1a, 1b and 1f were found to be significantly active against $T$. cruzi epimastigotes. Structure-activity relationship studies show that the substitution pattern of the spiro carbocyclic 2,6-DKP scaffold is an essential factor in the trypanocidal potency of these acetohydroxamic acid-based analogues.

Methylation on the basic nitrogen of the 2,6-DKP ring of the adamantane parent compound 1a resulted in 2.1-fold less activity against $T$. brucei (compare 7b vs 7a). However, analogous methylation on the cyclooctane and cycloheptane parent molecules 6a and 7a provided a 1.9 and 6.6-fold increase in the potency of their N-methyl analogues 6b and 7b, respectively (Table 1). These results indicate that N-methyl substitution on the 2,6-DKP ring has a favorable effect on the trypanocidal activity only in the cases of the cyclooctane or cycloheptane-containing acetohydroxamic acid analogues in this series of compounds.

Table 1.

We next investigated the effect of alkyl or arylmethyl substitution at the vicinal position of the basic nitrogen atom within the spiro carbocyclic 2,6-DKP framework, using methyl, isobutyl, 2-(methylthio)ethyl, benzyl and 4-chlorobenzyl substituents. As shown in Table 1, introduction of a methyl, isobutyl or 2-(methylthio)ethyl substituent to the adamantane parent 1a, leading to the (S)-enantiomer of the corresponding C-alkylated analogues 1c, 1d and 1e, reduced potency against $T$. brucei. Compounds 1c, 1d and 1e, in the free base form, were 1.5, 3.1 and 1.9-fold less potent than 1a. The lowest activity relative to 1a resulted from the introduction of an isobutyl group (1d). However, similar C-methylation or isobutylolation of the cyclooctane parent molecule 6a appeared to
have a beneficial effect on the trypanocidal potency; the resulting (S)-methyl and (S)-isobutyl substituted analogues 6c and 6d had 2.4 and 4.2-fold better activities than 6a, respectively. It is interesting that a significantly improved activity (19-fold) was observed with the cycloheptane-containing (S)-isobutyl substituted congener 7d relative to the parent compound 7a. Therefore, the presence of a methyl or isobutyl group at the vicinal position of the amine nitrogen atom in the 2,6-DKP ring seems to favor trypanocidal potency toward T. brucei in the context of the spiro cyclooctane or cycloheptane 2,6-diketopiperazine-1-acetohydroxamic acids.

Attachment of a bulky hydrophobic substituent, such as a benzyl or 4-chlorobenzyl group, to the methylene carbon in the 2,6-DKP ring of the parent molecules 1a and 6a led to the most potent analogues (1f, 1g, 2, 3 and 6f) in this series against bloodstream-form T. brucei, with activity in low nanomolar range (IC\textsubscript{50} = 6.8-32 nM, Table 1). Of the adamantane-based compounds possessing a benzyl substituent, the S-enantiomer (1f, IC\textsubscript{50} = 6.8 nM) was more potent than the R-enantiomer counterpart (1g, IC\textsubscript{50} = 9.1 nM), or racemate (2, IC\textsubscript{50} = 17 nM). Their activities in the free base form were found to be 5.3-13 times higher than the unsubstituted parent 1a and 8-42 times higher than their C-methyl and C-isobutyl substituted congeners 1c and 1d. Incorporation of a 4-chlorobenzyl substituent at the same position of the parent 1a, leading to the racemate p-chlorobenzyl substituted derivative 3, caused an increase in activity (2.8-fold), albeit to a lesser extent than the respective benzylic substitution (racemate 2 versus 3). A similar potency profile was observed for the cyclooctane C-benzyl substituted analogue 6f (S-enantiomer). This compound was significantly more potent (10-fold) than the parent 6a, and had 4.3 and 2.5 more trypanocidal activity than the (S)-enantiomers of the C-methyl and C-isobutyl counterparts 6c and 6d, respectively (Table 1). The higher increase in potency of the C-benzylated analogues 1f, 1g, 2, 3 and 6f is ascribed to the strongly favorable stereoelectronic and lipophilic effects exerted by the benzylic substituent in the binding site.

The unsubstituted parent compounds 1a, 6a, 7a and their substituted derivatives 1b, 1c, 1e, 6b, 6c, 6f and 7b had a marginal activity toward all the Leishmania sp. tested. In contrast, compounds bearing an isobutyl substituent at the vicinal position of the nitrogen atom of the respective spiro carbocyclic 2,6-DPK residue (1d, 6d, 7d), or compounds possessing a benzylic or 4-chlorobenzyl substituent at the same position of the adamantane-based spiro 2,6-DPK core (1f, 1g, 2, 3), exhibited significant activities against L. infantum promastigotes and intracellular amastigotes, in either free base form or hydrochloride salt, with IC\textsubscript{50} values at micromolar to low micromolar levels. Their anti-promastigote and anti-amastigote activities in the free base form were in the range of 2.67-17.7 μM and 2.23-7.85 μM, respectively. Similar ranges of activities against the two forms of this parasite were detected for the corresponding hydrochloride salts (2.86-13.2 μM and 2.04-8.90 μM, respectively).
Moreover, compounds 1g and 2 exhibited micromolar or low micromolar activities against L. donovani (Table 2). None of the tested compounds showed any significant activity against L. amazonensis promastigotes.

Relative to the parent structures 1a, 6a and 7a, the C-isobutyl analogues 1d, 6d and 7d, (S-enantiomers) were active against the L. infantum promastigotes (1d, IC50 = 7.23 μM, 6d, IC50 = 6.16 μM, and 7d, IC50 = 17.7 μM). However, they gave better potencies (3.2, 1.8 and 2.3-fold, respectively) toward the intracellular amastigotes, indicating that this form of L. infantum is more sensitive to these compounds. In both cases, the cycloheptane-containing C-isobutyl analogue 7d was less potent than the adamantane and cyclooctane congeners 1d and 6d, respectively (Table 2). No significant activity against the two other Leishmania sp. was observed with the isobutyl analogues 1d, 6d and 7d. As it is evident from Table 2, theadamantane-based C-benzyl or 4-chlorobenzyl substituted analogues 1f, 1g, 2 and 3 were the most efficient growth inhibitors of both forms of L. infantum, possessing activities in the low micromolar range (promastigotes form, IC50=2.67-4.85 μM, and amastigote form, IC50=2.43-4.40 μM). These analogues in the free base form were 1.5-2.7-fold more effective than the isobutyl counterpart 1d against promastigotes, while their efficacy toward intracellular amastigotes was comparable to that of 1d. Among the C-benzylated compounds (1f, 1g, 2), the S-enantiomer 1f was the most potent and selective against L. infantum exhibiting almost equal anti-promastigote and anti-amastigote potencies. It was 1.4 and 1.7-fold more active than the corresponding R-enantiomer 1g against promastigotes and intracellular amastigotes, respectively. A similar result against the promastigote form of L. infantum was observed when comparing 1f to the racemic compound 2. Somewhat surprisingly, the S-enantiomer 1f was almost equipotent to the racemic mixture 2 (1f, IC50=2.60±1.37 μM; 2, IC50=2.74±1.59 μM) toward intracellular amastigotes, when these compounds were tested as free bases. However, a difference in activity was detected between their respective hydrochlorides, with 1f.HCl (S-enantiomer) being more potent (2.3-fold) than 2.HCl (racemic), as expected. Noticeably, enhanced activities were observed against promastigotes of L. donovani in the cases of the R-enantiomer 1g and racemate 2, when compared to the corresponding S-enantiomer 1f. They retained low micromolar anti-promastigote potencies, although the racemic mixture 2 was slightly less active. R-enantiomer 1g also exhibited a noteworthy activity toward the intracellular amastigotes in the free base form (IC50 = 15.0 μM). These findings indicate that the R-stereochemistry is preferred over the S with respect to L. donovani potency. Introduction of a chloride atom at the benzene ring 4-position of the benzylc substituent in the racemic compound 2 slightly reduced the potency against L. infantum promastigotes, as shown from the IC50 values of compounds 2 and 3 (Table 2). On the other hand, this chloro-substitution did not significantly affect the activity against the intracellular amastigotes, when the analogues 2 and 3 were screened as free bases, while a marked difference in potency was detected between the corresponding hydrochloride salts (2.HCl and 3.HCl). In the case of the latter, the hydrochloride salt of the chloro-substituted compound 3 was 3.9 times more potent than the hydrochloride salt of its unsubstituted counterpart 2.

On the basis of these anti-leishmanial data, the presence of a branched alkyl chain, such as isobutyl group, at the vicinal position of the nitrogen atom of the 2,6-DKP ring of compounds 1d, 6d and 7d, or a benzyl substituent at the same position of the adamantane-based compounds 1f, 1g, 2 and 3, is responsible for their enhanced potency. This points to the important influence of these substituents in determining anti-leishmanial activity, possibly due to lipophilic and/or stereo electronic effects.
The observed differences in activity (anti-trypanosomal or anti-leishmanial) between the free base and the corresponding hydrochloride salt forms (Table 1 and 2) might be due to their differential cell permeability, resulting from differences in solubility at the pH of the culture media.

It is noteworthy that the active compounds, show very low cytotoxicity against mammalian cells (rat skeletal myoblast L6 and murine macrophage J 774.1 cell lines). In the case of the myoblast L6 cells, the selectivity indices varied from 63 (1e) to more >3600 (1a). With respect to the murine macrophage cells, the IC\textsubscript{50} values were found to be >200 μM, with the exception of compound 1f (IC\textsubscript{50}=29.2 μM). These results indicate significant selectivity of the compounds, up to two orders of magnitude greater than has been reported previously for DKP-based compounds with activity against T. brucei.\textsuperscript{[20]} If similar properties can be established in vivo, this would represent a significant advantage over current trypanocidal drugs, where toxic side effects are one of the major drawbacks. In this study, despite the lower antileishmanial activity of antimonials- and thus similar activity to the most active hydroxamic acid derivatives, in comparison to amphotericin B\textsuperscript{[15,21]} (i.e. 135 times lower for Glucantime over amphotericin B\textsuperscript{[21]}), we selected not to use antimonials as reference drugs, as parasites may have inherent or even develop spontaneous drug resistance during in vivo or cell culture passages. We thus validated the phenotypic screen by using the highly active amphotericin B. Our results show that the inhibitory activity of Amphotericin B (IC\textsubscript{50} = 0.09 μM and 0.10 μM in L. infantum and L. donovani promastigotes respectively and 0.18 μM and 0.20 μM L. infantum and L. donovani in intracellular amastigotes respectively) is in the anticipated range.\textsuperscript{[22]}

### 4 CONCLUSION

The present work has extended the structure-activity relationships of spiro carbocyclic 2,6-DKP-1-acetohydroxamic acids in determining in vitro growth inhibition of trypanosomal and leishmanial parasites. Our studies demonstrate that the anti-parasitic activity of this class of compounds is greatly dependent upon the alkyl or the arylalkyl substitution on either the basic nitrogen atom (N-methylation) or at its vicinal position (C-alkylation or arylmethylation) in the spiro carbocyclic 2,6-DKP skeleton. N-methylation had a positive influence on the potency against blood-stream form T. brucei, only in the cases of cyclooctane or cycloheptane-containing analogues (Table 1, compounds 6b, 7b). C-alkylation or isobutylation (C-alkylation) reduced potency for the adamantane-based (S)-methyl and (S)-isobutyl substituted analogues 1c and 1d, respectively. In contrast, enhanced activity against T. brucei was observed for the cyclooctane and cycloheptane (S)-methyl or (S)-isobutyl counterparts (6c, 6d, 7d), although the isobutylation produced a more pronounced effect with respect to the methylation. In contrast, C-arylmethylation, such as C-benzylation or 4-chlorobenzylation, yielded (S) or (R)-chiral or racemic analogues (1f, 1g, 2, 3), which were the most potent against blood-stream-form T. brucei, with low nanomolar IC\textsubscript{50} values (6.8-32 nM) as shown in Table 1. Of note, compounds 1a, 1b and 1f displayed significant activity against T. cruzi epimastigotes. The (S)-chiral benzyl substituted compound 1f was the most active derivative against T. brucei and T. cruzi, with IC\textsubscript{50} values of 6.8 nM and 210 nM, respectively. In the case of T. cruzi, this value is 5-10 fold lower than the current front line drugs, benznidazole and nifutimox.\textsuperscript{[23]}

With respect to the anti-leishmanial activity of the tested compounds, only the C-isobutylation or C-arylmethylation (C-benzylation or 4-chlorobenzylation) produced a significant anti-parasitic toxicity against the L. infantum promastigotes and amastigotes (Table 2, compounds 1d, 1f, 1g, 2, 3, 6d). However, this was not the case for the cyclooctane C-benzylated analogue 6f. Among the C-benzylated compounds 1f, 1g and 2, the S-enantiomer 1f was the most potent and selective against
L. infantum. However, the R-enantiomer 1g and the racemate 2 displayed satisfactory micromolar activities against L. donovani, as compared to the corresponding S-enantiomer. These results are indicative of the enhanced potency of the R-enantiomer against L. donovani. The addition of a chlorine atom at the benzene ring C4-position of the benzylic substituent in the racemic compound 2 has a little effect on the potency toward L. infantum (Table 2, 3 vs 2).

In summary, the data obtained from this study show that introduction of branched alkyl (e.g. isobutyl) or benzylic substituents at the vicinal position of the amine nitrogen of the 2,6-DKP skeleton tends, in general, to generate analogues with greatly enhanced activity toward trypanosomal and leishmanial parasites. We surmise that this could be due to the strong lipophilic and/or stereoelectronic influence of these bulky substituents in the target binding site. Importantly, the most potent compounds were found not to have significant toxicity against mammalian cells, indicating a high selectivity over the parasites. The results suggest that these acetohydroxamic acid derivatives can be considered promising structures for further investigation in developing new more efficient and safe agents for the treatment of trypanosomiasis or leishmaniasis. Efforts in this direction, including modifications on either the spirocarbocyclic 2,6-DKP residue or the acetohydroxamate acid pharmacophoric moiety, while retaining the carboxyhydroxamic acid unit (CONHOH), are currently underway.

Figure and Scheme legends

Figure 1. Structures of lipophilic spiro carbocyclic 2,6-diketopiperazine-1-acetohydroxamic acid derivatives 1a-g, 2-5, 6a-d, 6f, 7a, 7b and 7d.

Scheme 1. Reagents and conditions: (a) NaCN, appropriate α-amino acid alkyl ester hydrochloride, DMSO/H₂O 29:1 (v/v), rt, 48 h; (b) (i) H₂SO₄, 97% for 17, 18, 25, rt, 24 h or H₂SO₄ 97%, CH₂Cl₂, rt, 24 h for 19, 20, 26-30 or 48 h for 21-24; (ii) ice and then aq NH₂ 26% to pH 7-8; (c) (i) (Me₂Si)₂NK (1 equiv), THF, 0-5°C, then rt, 1 h, argon; (ii) BrCH₂CO₂CH₂Ph or BrCH₂CO₂CH₂C₆H₄OCH₃-4 only for 38, DMF, rt, 48 h, argon, 78-95% for 31-37, 38-42, 44, 62% for 43 from 8; (d) H₂/Pd-C 10%, EtOH for 45, 46, 52, 53, 56, 83-85, 1a-d, 1f, 1g, 2, 4, 5, 6a-d, 6f, 7a, 7b, 7d or EtOH-AcOEt 3:2 (v/v) for 47-51, 54, 55, 57, 1e, 50 psi, rt, 3 h, >99% for 45-47, 49-57, 83-85, 86% for 48, 80-96% for 1a-d, 1f, 1g, 2, 4, 5, 6a-d, 6f, 7a, 7b, 7d, 25% for 1e; (e) (i) CDI, THF for 58-64, 66, 67, 69, 71, 86-88 or THF/DMF 3:4 for 68 or THF/DMF 6:1 for 70, 73 or THF/DMF 4:1 for 72, 28°C, 1 h, argon; (ii) PhCH₂ONH₂·HCl, Et₃N or PhCH₂ONHCH₂ for 66, 67, 28°C, 25 h, argon, for 59-63, 69-71, 73 or 28°C, 24 h, then 45°C, 1 h, argon for 58, 64, 66-68, 72, 86-88, 58-95% for 58-60, 62-64, 66-73, 86-88, 62% for 61 from 34 (f) (i) CF₃CO₂H, CH₂Cl₂, rt, 90 min; (ii) CDI, Et₃N, THF, 28°C, 1 h; (iii) 4-CH₂OC₆H₄ONH₂, 28°C, 24 h, then 55°C, 1 h, 70% from 38; (g) CF₃CO₂H, CH₂Cl₂, rt, 10 min, then Et₃SiH, rt, 45 min, 89% from 65; (h) as (c) (i), then CF₃CO₂H (1 equiv), >99%; (i) (i) aq CH₂O 37%, MeOH/THF 1:1 for 77 or MeOH/THF 1:3 for 78, 79, rt, 3 h, then NaCNBH₃, rt, 4 h, at pH 6-7 (maintained by adding AcOH); (ii) 1N NaOH and Na₂CO₃ to pH 8, 80-92%; (j) NaH, DMF, rt, 1 h, argon and then as (c) (ii) using BrCH₂CO₂CH₂Ph, 83-92%.
REFERENCES


SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.
Table 1. Activity of acetohydroxamic acid analogues 1a-g, 2-5, 6a-d, 6f, 7a, 7b and 7d (Figure 1) tested against cultured bloodstream-form *T. brucei* (pH=7.4) and *T. cruzi* epimastigotes, and cytotoxicity of the most active compounds against cultured rat skeletal myoblast L6 cells (Supporting information).

<table>
<thead>
<tr>
<th>Cpds</th>
<th>Activity</th>
<th>Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>T. brucei</em></td>
<td><em>T. cruzi</em></td>
</tr>
<tr>
<td></td>
<td>IC\textsubscript{50} (nM)\textsuperscript{a,b,c}</td>
<td>IC\textsubscript{90} (nM)\textsuperscript{a,b,c}</td>
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<tr>
<td>1a</td>
<td>90±16 (79±6)\textsuperscript{f}</td>
<td>155±7 (148±8)\textsuperscript{f}</td>
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<tr>
<td>1b</td>
<td>193±28 (340±28)</td>
<td>328±28 (622±84)</td>
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<tr>
<td>1c</td>
<td>134±33 (405±98)</td>
<td>276±12 (909±209)</td>
</tr>
<tr>
<td>1d</td>
<td>283±27 (253±35)</td>
<td>462±99 (355±17)</td>
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<tr>
<td>1e</td>
<td>168±42 (163±11)</td>
<td>251±14 (248±6)</td>
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<td>1f</td>
<td>6.8±1.4 (42±5)</td>
<td>11.5±2 (80±22)</td>
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<td>1g</td>
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<tr>
<td>2</td>
<td>17±1 (18±1)</td>
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<td>3</td>
<td>32±4 (31±3)</td>
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<td>246×10\textsuperscript{3} (106×10\textsuperscript{3})</td>
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<td>673±56 (717±113)</td>
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<tr>
<td>7d</td>
<td>99±4 (97±9)</td>
<td>148±8 (135±15)</td>
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\textsuperscript{a}Concentrations required to inhibit growth of *T. brucei* and *T. cruzi* by 50% and 90%, respectively.

\textsuperscript{b}For the active compounds 1a-g, 2, 3, 6a-d, 6f, 7a, 7b and 7d, IC\textsubscript{50} and IC\textsubscript{90} data are the mean of triplicate experiments ± SEM.

\textsuperscript{c}IC\textsubscript{50} and IC\textsubscript{90} for the respective hydrochloride are shown in brackets.
Cytotoxicity was determined by establishing the concentration required to inhibit growth of cultured L6 cells by 50% (IC$_{50}$) (Supporting Information). Data are the mean of triplicate experiments ± SEM.

Selectivity indices were calculated as the ratio of the IC$_{50}$ for L6 cells and IC$_{50}$ for T. brucei.

Table 2. In vitro inhibitory activity of acetohydroxamic acid analogues 1a-g, 2, 3, 6a-d, 6f, 7a, 7b and 7d (Figure 1) against Leishmania sp. promastigotes and intracellular amastigotes, and their cytotoxicity against murine macrophages J 774.1.

<table>
<thead>
<tr>
<th>Cpds</th>
<th>L. infantum GH12 IC$_{50}$ (μM)$^{abc}$</th>
<th>L. donovani Lg13 IC$_{50}$ (μM)$^{abc}$</th>
<th>Macrophages J774.1. IC$_{50}$ (μM)$^d$</th>
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<td>1a</td>
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<td>-</td>
<td>ni</td>
</tr>
<tr>
<td>1b</td>
<td>ni</td>
<td>-</td>
<td>ni</td>
</tr>
<tr>
<td>1c</td>
<td>ni</td>
<td>-</td>
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<tr>
<td>1d</td>
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<td>(7.32±1.78)$^c$</td>
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<tr>
<td>1f</td>
<td>2.67±0.70</td>
<td>(2.86±0.98)</td>
<td>2.60±1.37</td>
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<td>(2.95±0.86)</td>
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<tr>
<td>6d</td>
<td>6.16±1.29</td>
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<td>Amphotericin B</td>
<td>0.09 ± 0.005</td>
<td>0.18 ± 0.02</td>
<td>0.10 ± 0.01</td>
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$^a$Concentrations required to inhibit growth of Leishmania sp promastigotes and intracellular amastigotes by 50%.  $^b$IC$_{50}$ data are the means of triplicate experiments ± SEM.  $^c$IC$_{50}$ data for the respective hydrochloride are shown in brackets.  $^d$Cytotoxicity against the murine macrophage cell-line J 774.1.  $^e$ni - no inhibition at 10 μM.
a: R=R_{1}=R_{2}=H
b: R=R_{1}=H, R_{2}=CH_{3}
c: R=CH_{3}, R_{1}=R_{2}=H, (S)-enantiomer
d: R=CH_{2}CH(CH_{3})_{2}, R_{1}=R_{2}=H, (S)-enantiomer
e: R=CH_{2}CH_{2}SCH_{3}, R_{1}=R_{2}=H, (S)-enantiomer
f: R=CH_{2}C_{6}H_{5}, R_{1}=R_{2}=H, (S)-enantiomer
g: R=CH_{2}C_{6}H_{5}, R_{1}=R_{2}=H, (R)-enantiomer
2: R=CH_{2}C_{6}H_{5}, R_{1}=R_{2}=H, racemic
3: R=CH_{2}C_{6}H_{4}Cl-4, R_{1}=R_{2}=H, racemic
4: R=R_{2}=H, R_{1}=CH_{3}
5: R=CH_{2}C_{6}H_{5}, R_{1}=CH_{3}, R_{2}=H, racemic

n=7: 6a-d, 6f
n=6: 7a, 7b, 7d