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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

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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* epimastogotes and *L. infantum* promastogotes 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* (IC_{50}=6.8 nM), *T. cruzi* (IC_{50}=0.21 μM) and *L. infantum* promastigotes (IC_{50}=2.67 μM) and intracellular amastigotes (IC_{50}=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.

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1 INTRODUCTION

Trypanosomatid protozoan parasites of the genera *Trypanosoma* and *Leshmania* are the aetiological agents of serious diseases; human African trypanosomiasis (HAT or sleeping sickness), Chagas disease and the leishmaniases (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.[1] In Latin America, 5 - 8 million people are currently infected with *T. cruzi*,[2] and globally there are an estimated 1 million new infections with different *Leishmania* species each year.[3] 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.[4] 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.[5]

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.[6] In addition, increased drug resistance and high therapeutic failure rates have been reported recently in several foci.[7] Eflornithine, an alternative treatment, now given in combination with nifurtimox, is better tolerated, but difficult to administer.[8] 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.[9] These compounds were derived from conformationally constrained lipophilic spiro carbocyclic 2,6-diketopiperazine (2,6-DKP) scaffolds by introducing an acetohydroxamic acid moiety ($CH_2CONHOH$) into their imidic nitrogen atom. The hydroxamic acid unit (CONHOH) is indispensable for trypanocidal activity in this class of compound.[9] 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

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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.

2 METHODS AND MATERIALS

2.1 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 δ(ppm) with tetramethylsilane or solvent (DMSO-d_6) as internal standard. Splitting patterns are designated as s, singlet; d, doublet; dd, doublet of doublets; t, triplet; td, triplet of doublets; q, quartet; qd, quartet of doublets; m, multiplet; br, broad; v br, very broad; sym, symmetrical. The spectra were recorded at 293 K (20 °C) unless otherwise specified. Carbon multiplicities were established by DEPT experiments. 2D NMR experiments (HMQC 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_2O 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

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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.[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.[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.

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

Carboxylic acid **47** (1.33 g, 3.82 mmol) was treated with 1,1'-carbonyldiimidazol (743 mg, 4.58 mmol) in dry THF (75mL) as described for the preparation of **58** from **45**.[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^{-3} mmHg) in an Abderhalden apparatus gave **60** as a glass solid (1 g, 58%). This compound appears in the ^{1}H and ^{13}C NMR spectra as a mixture of *Z*/*E* conformers. ^{1}H NMR (600 MHz, $CDCl_3$, 273 K) δ 0.96 (t, 6H, *J*=3.6, 6.0 Hz, $CH(CH_3)CH_3$), 1.05-1.30 (br s, 1H, 1-H), 1.39-1.60 (complex m, 3H, 4'e, 9'e-H, $CHHCH(CH_3)_2$), 1.61-1.99 (complex m, 10H, 3', 5', 6', 7', 8', 10'e-H, $CHHCH(CH_3)_2$), 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_{AB}=15.0 Hz, $CH_2CONHOCH_2Ph$), 4.49-4.69 (q, AB, 0.93H, J_{AB}=16.8 Hz, $CH_2CONHOCH_2Ph$), 4.85, 4.89 (s+s, 2H, $CONHOCH_2Ph$), 7.28-7.43 (complex m, 5H, aromatic H), 8.61 (s, 0.38H, $CONHOCH_2Ph$), 9.03 (s, 0.33H, $CONHOCH_2Ph$); ^{13}C NMR (150 MHz, $CDCl_3$, 273 K) δ 21.2, 23.6 ($CH(CH_3)CH_3$), 24.5 ($CH(CH_3)_2$), 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 ($CH_2CONHOCH_2Ph$), 41.7 ($CH_2CH(CH_3)_2$), 51.6 (6-C), 60.7 (2, 2'-C), 78.2, 79.6 ($CONHOCH_2Ph$), 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 ($CONHOCH_2Ph$), 174.9, 175.9 (3, 5-C); ESI$^+$MS: m/z 454.5 $[M+H]^+$.

Compound **60** (856 mg, 1.89 mmol) was subjected to catalytic hydrogenation (H_2/Pd-C, 103 mg) in abs EtOH (90 mL) following the procedure previously described.[9] The hydrogenation product was chromatographed on silica gel column using 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^{-3} mmHg) in an Abderhalden apparatus gave **1d** as an off-white semifoamy solid (658 mg, 96%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.91 (d, 6H, *J*=6.0 Hz, $CH(CH_3)CH_3$), 1.37 (d, 1H, *J*=11.6 Hz, 9'e-H), 1.41-1.51 (m, 2H, 4'e-H, $CHHCH(CH_3)_2$), 1.53-

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1.98 (complex m, 10H, 3', 5', 6', 7', 8', 10'e-H, CH*H*C*H*(CH_3)$_2$), 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, J_{AB}=15.6 Hz, C*H$_2$*CONHOH, *E*-isomer), 4.33-4.50 (q, AB, 0.4H, J_{AB}=16.8 Hz, C*H$_2$*CONHOH, *Z*-isomer), 8.82 (s, 0.57H, CONHO*H*, *E*-isomer), 9.24 (s, 0.14H, CONHO*H*, *Z*-isomer), 10.10 (s, 0.15H, CON*H*OH, *Z*-isomer), 10.49 (s, 0.53H, CON*H*OH, *E*-isomer); ^{13}C NMR (75 MHz, DMSO-*d$_6$*) δ 21.2, 23.5 (CH(CH$_3$)CH$_3$), 24.1 (CH(CH_3)$_2$), 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 (*C*H$_2$CONHOH), 40.8, 40.9 (*C*H$_2$CH(CH_3)$_2$, *Z*/*E*-isomers), 51.1 (6-C), 59.7, 59.8 (2, 2'-C, *Z*/*E*-isomers), 164.2 (*C*ONHOH, *E*-isomer), 169.6 (*C*ONHOH, *Z*-isomer), 174.7, 174.8, 175.0, 175.1 (3, 5-C, *Z*/*E*-isomers); $[\alpha]^{21}_{589}$ -29.5 (c, 0.2, $CHCl_3$); HRMS (ESI): $[M-H]^-$ calc for $C_{19}H_{29}N_3O_4$, 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 $C_{19}H_{30}ClN_3O_4$: 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 described for the preparation of **45** from **31**[12] 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, $CDCl_3$) δ 1.48-1.59 (q, 2H, *J*=12.4 Hz, 4'e, 9'e-H), 1.61-1.93 (complex m, 9H, C*H*HCH$_2$S, 3', 5', 6', 7', 8', 10'e-H), 2.11 (s, 4H, 1'-H, SC*H$_3$*), 2.18 (d, 1H, *J*=12.0 Hz, 9'a-H), 2.32-2.50 (m, 2H, CH*H*CH$_2$S, 4'a-H), 2.63-2.80 (m, 2H, C*H$_2$*S), 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, J_{AB}=17.6 Hz, C*H$_2$*CO$_2$H), 5.46-6.65 (v br s, 2H, 1-H, CO$_2$*H*)]. This material (646 mg) was treated with 1,1'-carbonyldiimidazol (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 Et$_2$O-*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* conformers. ^{1}H NMR (600 MHz, $CDCl_3$, 283 K) δ 1.38-1.47 (br s, 1H, 1-H), 1.51 (d, 1H, *J*=12.4 Hz, 4'e), 1.56 (d, 1H, *J*=11.6 Hz, 9'e-H), 1.64-1.91 (complex m, 9H, C*H*HCH$_2$S, 3', 5', 6', 7', 8', 10'e-H), 2.11 (s, 3H, SC*H$_3$*), 2.17 (~br s, 2H, 1', 9'a-H), 2.34-2.50 (m, 2H, CH*H*CH$_2$S, 4'a-H), 2.65-2.78 (m, 2H, C*H$_2$*S), 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, J_{AB}=15.1 Hz, C*H$_2$*CONHOCH$_2$Ph), 4.54-4.73 (q, AB, 1H, J_{AB}=16.8 Hz, C*H$_2$*CONHOCH$_2$Ph), 4.87, 4.91 (s+s, 2H, CONHOC*H$_2$*Ph), 7.34-7.41 (m, 5H, aromatic H), 8.19 (s, 0.44H, CON*H*OCH$_2$Ph), 8.61 (s, 0.42H, CON*H*OCH$_2$Ph); ^{13}C NMR (150 MHz, $CDCl_3$, 283 K) δ 15.1 (S*C*H$_3$), 26.9, 27.2 (5', 7'-C), 30.4 (*C*H$_2$S), 30.8, 31.0 (1'-C), 31.1 (*C*H$_2$CH$_2$S), 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 (*C*H$_2$CONHOCH$_2$Ph), 51.7 (6-C), 60.9 (2,2'-C), 78.1,

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79.5 (CONHO*C*H_2Ph), 128.5, 128.8, 129.1, 129.2 (2, 3, 4, 5, 6-aromatic C), 134.1, 135.1 (1-aromatic C), 165.5, 170.7 (*C*ONHOCH_2Ph), 174.7, 174.8 (3, 5-C); ESI$^+$MS: m/z 472.2 $[M+H]^+$.

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; ^{1}H NMR (400 MHz, DMSO-d_6) 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, C*H*HCH_2S), 2.05 (s, 3H, SCH_3), 2.07 (s, 1H, 1'-H), 2.12-2.29 (m, 2H, 9'a-H, CH*H*CH_2S), 2.45 (d, 1H, *J*=10.8 Hz, 4'a-H), 2.67 (~t, 2H, *J*=7.3, 7.8 Hz, CH_2S), 2.82 (~d, 2H, *J*=11.4 Hz, 1, 10'a-H), 3.60-3.75 (sym quintet, 1H, 6-H), 4.07-4.19 (q, AB, 1.36 H, J_{AB}=15.6 Hz, CH_2CONHOH, *E*-isomer), 4.21-4.31 (m, 0.38 H, CH_2CONHOH, *E*-isomer), 4.36-4.53 (q, AB, 0.35 H, *J*=16.8 Hz, CH_2CONHOH, *Z*-isomer), 8.84 (s, 0.7 H, CONHO*H*, *E*-isomer), 9.26 (s, 0.2 H, CONHO*H*, *Z*-isomer), 10.12 (s, 0.2 H, CON*H*OH, *Z*-isomer), 10.52 (s, 0.7 H, CON*H*OH, *E*-isomer); ^{13}C NMR (100 MHz, DMSO-d_6) δ 14.4 (S*C*H_3), 26.6, 26.9 (5', 7'-C), 29.9 (CH_2S), 30.1, 30.2 (1'-C), 30.8, 30.9 (*C*H_2CH_2S), 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 (*C*H_2CONHOH), 51.3, 51.4 (6-C), 59.9 (2, 2'-C), 164.1 (*C*ONHOH, *E*-isomer), 169.6 (*C*ONHOH, *Z*-isomer), 174.4, 174.6 (3, 5-C); HRMS (APCI$^+$): $[M+H]^+$ calcd for $C_{18}H_{27}N_3O_4S$ 382,1756, found 382,1791. The hydrochloride salt (**1e·HCl**) was prepared as described for **1d·HCl**. Mp 139-143 ^{o}C (dec); $[\alpha]_{589}^{26}$ -22 (C, 0.1, DMSO). Anal Calcd for $C_{18}H_{28}ClN_3O_4S$: 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 [3.3.1.1^{3,7}]decane]-4-acetamide 3

A mixture of 4-methoxybenzyl ester **38** (1.0 g, 1.86 mmol) and TFA (3 mL) in CH_2Cl_2 (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 **58** from **45**[9]. 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 ^{1}H and ^{13}C NMR spectra as a mixture of *E*/*Z* conformers. ^{1}H NMR (600 MHz, $CDCl_3$, 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, C*H*HC_6H_4Cl-4), 3.35 (~d, 1H, *J*=13.8 Hz, CH*H*C_6H_4Cl-4), 3.75-3.88 (m, 1H, 6-H), 3.78, 3.81 (s + s, 3H, OCH_3), 4.10-4.40 (q, AB, 1H, J_{AB}=15.3 Hz, CH_2CONHOC$H_2C_6H_4$OCH_3-4), 4.47-4.68 (q, AB, 1H, J_{AB}=16.8 Hz, CH_2CONHOC$H_2C_6H_4$OCH_3-4), 4.80, 4.83 (s + s, 2H, CONHOC$H_2C_6H_4$OCH_3-4), 6.84-6.95 (dd, 2H, *J*=8.4, 20.4 Hz, aromatic H), 7.14-7.36 (complex m, 6H, aromatic H), 8.53 (s, 0.4 H, CON*H*OC$H_2C_6H_4$OCH_3-4), 8.96 (s, 0.4 H, CON*H*OC$H_2C_6H_4$OCH_3); ^{13}C NMR (150 MHz, $CDCl_3$, 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 (*C*$H_2C_6H_4$Cl-4), 40.2, 40.3 (*C*H_2CONHOC$H_2C_6H_4$OCH_3-4), 54.1, 54.2 (6-C), 55.3, 55.4 (O*C*H_3),

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60.9 (2, 2'-C), 77.8, 79.3 (CONHO*C*$H_2C_6H_4OCH_3$-4), 113.8, 114.1, 126.2, 127.2, 128.8, 130.6, 130.7, 131.2, 132.8, 135.5, 159.9, 160.2 (aromatic C), 165.4, 170.6 (*C*ONHO$CH_2C_6H_4OCH_3$-4), 173.7, 174.4 (3, 5-C); CI^+ MS: m/z 552.2 ($[M+H]^+$, 1.5), 426. 2 ($[M – CH_2C_6H_4Cl\text{-}4]^+$, 8), 137.0 (30), 121.0 (100).

A mixture of compound **65** (451 mg, 0.82 mmol) and TFA (6 mL) in CH_2Cl_2 (23 mL) was stirred for 10 min. The solution was then treated dropwise with Et_3SiH (600 µL) *via* syringe, and the stirring was continued for 45 min at room temperature. In this time window, the colour 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 Na_2CO_3 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 (Na_2SO_4) 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 semifoamy solid (313 mg, 89%): ^1H NMR (400 MHz, DMSO-d_6) δ 1.30 (d, 2H, *J*=11.6 Hz, 4'e, 9'e-H), 1.48-1.73 (complex m, 5H, 4'a, 7', 8', 10'e-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, C*H*HC_6H_4Cl-4, 1, 10'a-H), 3.30 (dd, 1H, *J*=3.2, 14.0 Hz, CH*H*C_6H_4Cl-4), 3.72 (td, 1H, *J*=3.2, 10.9 Hz, 6-H), 4.16 (s, 1.4 H, C*H₂*CONHOH), *E*-isomer), 4.38-4.53 (q, AB, 0.43H, *J*=16.8 Hz, CH_2CONHOH, *Z*-isomer, 7.29-7.40 (m, 4H, aromatic H), 8.87 (s, 0.72 H, CONHO*H*, *E*-isomer), 9.28 (s, 0.2 H, CONHO*H*, *Z*-isomer), 10.16 (s, 0.2 H, CON*H*OH, *Z*-isomer), 10.55 (s, 0.7 H, CON*H*OH, *E*-isomer); ^{13}C NMR (50 MHz, DMSO-d_6) 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 ($CH_2C_6H_4$Cl-4), 37.7 (6'-C), 39.5 (*C*H_2CONHOH), 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 (*C*ONHOH, *E*-isomer), 169.5 (*C*ONHOH, *Z*-isomer), 173.5., 174.5 (3,5-C); HRMS (ESI): $[M + H]^+$, $[M + Na]^+$ calcd for $C_{22}H_{26}ClN_3O_4$ 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 $C_{22}H_{27}Cl_2N_3O_4$: 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]tridecane-4-aceta-mide 6d

Carboxylic acid **54** (1.13 g, 3.5 mmol) was treated with 1, 1'-carbonyldiimidazol (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*-benzylhydroxylamine 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 ^{13}C NMR spectra as a mixture of *E*/*Z* conformers. ^1H NMR (400 MHz, $CDCl_3$) δ 0.95 (t, 6H, *J*=12.6 Hz, CH(C*H₃*)C*H₃*), 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, C*H₂*C*H*$(CH_3)_2$), 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, CH_2CONHOCH_2Ph), 4.45-4.75 (br s, 1H, C*H₂*CONHOCH_2Ph), 4.88 (s, 2H, CONHOC*H₂*Ph), 7.37 (s, 5H, aromatic H), 8.12-8.45 (br s, 0.5H, CON*H*OCH_2Ph), 8.57-8.88 (br s, CON*H*OCH_2Ph); ^{13}C NMR (50 MHz, $CDCl_3$) δ 20.8 (9-C), 21.2 (CH(*C*H_3)CH_3), 21.4 (11-C), 23.5 (CH(CH_3)*C*H_3), 24.5 (10-C, *C*H$(CH_3)_2$), 27.4 (8-C), 28.5 (7-C), 28.7 (12-C), 34.5 (13-C), 39.7 (*C*H_2CONHOCH_2Ph), 40.5 (*C*H_2CH$(CH_3)_2$), 51.8 (2-C), 60.4

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(6-C), 78.4, 79.5 (CONHOCH_2Ph), 128.7, 129.3 (2, 3, 4, 5, 6-aromatic C), 135.0 (1-aromatic C), 165.6, 170.7 (*C*ONHOCH_2Ph), 174.3, 177.2 (3, 5-C); CI^+ MS: m/z 430.2 ($[M+H]^+$, 34), 429.3 ($[M]^+$, 13), 373.2 ($[M+H-CH_2CH(CH_3)_2]^+$, 18), 322.1 ($[M-OCH_2Ph]^+$, 18), 307.2 ($[M-NHOCH_2Ph]^+$, 22), 306.1 (49), 279.2 ($[M-CONHOCH_2Ph]^+$, 100), 91.0 (90).

Compound **70** (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.[9] The hydrogenation material was chromatographed on silica gel column eluting first with AcOEt-*n*-hexane 1:1 and then AcOEt to afford the title compound **6d** as a white foamy solid, 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-d_6) δ 0.84 (d, 3H, *J*=6.4 Hz, CH(CH_3)CH$_3$), 0.88 (d, 3H, *J*=6.4 Hz, CH(CH$_3$)CH_3), 1.30-1.96 (complex m, 16H, 7, 8, 9, 10, 11, 12, 13-H, CH_2CH(CH$_3$)$_2$), 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, J_{AB}=15.6 Hz, CH_2CONHOH, *E*-isomer), 4.30-4.42 (q, AB, 0.4H, J_{AB}=16.8 Hz, CH_2CONHOH, *Z*-isomer), 8.79 (s, 0.8H, CONHO*H*, *E*-isomer), 9.21 (s, 0.2H, CONHO*H*, *Z*-isomer), 10.08 (s, 0.2H, CON*H*OH, *Z*-isomer), 10.43 (s, 0.7H, CON*H*OH, *E*-isomer); ^{13}C NMR (75 MHz, DMSO-d_6) δ 20.4 (9-C), 21.0 (11-C), 21.1 23.5 (CH(*C*H$_3$)*C*H$_3$), 24.0 (*C*H(CH$_3$)$_2$), 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 (*C*H$_2$CONHOH), 39.6 (*C*H$_2$CH(CH$_3$)$_2$), 51.1 (2-C), 59.5 (6-C), 164.0 (*C*ONHOH, *E*-isomer), 169.5 (*C*ONHOH, *Z*-isomer), 174.1, 176.9 (3, 5-C); $[\alpha]^{23}_{589}$ -11 (c, 0.2, CHCl$_3$); HRMS (ESI): $[M-H]^-$ calcd for $C_{17}H_{29}N_3O_4$, 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 $C_{17}H_{30}ClN_3O_4$: 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 7d

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-*n*-hexane 2:3, as eluent, to afford the corresponding *O*-benzyl hydroxamate **73** as a white foamy solid, which strongly binds the aforementioned solvents. Removal of the entrapped solvents as in **60** gave **73** as a white solid (1.54 g, 76%): 129-131°C. This compound appears in the ^1H and ^{13}C NMR spectra as a mixture of *E*/*Z* conformers. ^1H NMR (400 MHz, CDCl$_3$) δ 0.93 (d, 3H, *J*=6.0 Hz, CH(CH_3)CH$_3$), 0.96 (d, 3H, *J*=6.3 Hz, CH(CH$_3$)CH_3), 1.13-1.37 (br s, 1H, 1-H), 1.38-2.03 (complex m, 14H, 7, 8, 9, 10, 11, 12-H, CH_2C*H*(CH$_3$)$_2$), 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, CH_2CONHOCH$_2$Ph), 4.45-4.69 (br s, 1H, CH_2CONHOCH$_2$Ph), 4.87 (s, 2H, CONHOCH_2Ph), 7.37 (s, 5H, aromatic H), 8.24-8.44 (br s, 0.34H, CON*H*OCH$_2$Ph), 8.75-8.93 (s, 0.37H, CON*H*OCH$_2$Ph); ^{13}C NMR (50 MHz, CDCl$_3$) δ 21.2 (CH(*C*H$_3$)CH$_3$), 21.8 (8-C), 22.7 (11-C), 23.5 (CH(CH$_3$)*C*H$_3$), 24.5 (*C*H(CH$_3$)$_2$), 29.4, 29.6 (9, 10-C), 33.6 (7-C), 38.8 (12-C), 39.8 (*C*H$_2$CONHOCH$_2$Ph), 40.3 (*C*H$_2$CH(CH$_3$)$_2$), 51.8 (2-C), 61.2 (6-C), 78.5, 79.6 (CONHO*C*H$_2$Ph), 128.8, 129.4 (2, 3, 4, 5, 6-aromatic C), 134.9 (1-aromatic C), 165.7, 170.8 (*C*ONHOCH$_2$Ph), 174.3, 177.6 (3, 5-C); CI^+ MS: m/z 416.2 ($[M+H]^+$, 5), 359.1 ($[M+H-CH_2CH(CH_3)_2]^+$, 5), 265.1 ($[M-CONHOCH_2Ph]^+$, 18), 264.1 (59), 91.0 (100).

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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) δ 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, 7, 8, 9, 10, 11, 12-H, CH_2CH(CH$_3$)$_2$), 1.83-2.0 (m, 2H, C*H*(CH$_3$)$_2$, 7-H), 2.02-2.16 (m, 1H, 12-H), 2.59 (d, 1H, J=12.4 Hz, 1-H), 3.41-3.56 (m, 1H, 2-H), 4.06-4.19 (q, AB, 1.5H, J_{AB}=15.6 Hz, CH_2CONHOH, *E*-isomer), 4.35-4.51 (q, AB, 0.4H, J_{AB}=16.8 Hz, CH_2CONHOH, *Z*-isomer), 8.84 (s, 0.7H, CONHO*H*, *E*-isomer), 9.25 (s, 0.2H, CONHO*H*, *Z*-isomer), 10.13 (s, 0.2H, CON*H*OH, *Z*-isomer), 10.48(s, 0.7H, CON*H*OH, *E*-isomer); ^{13}C NMR (75 MHz, DMSO-d_6) δ 21.1 (CH(*C*H$_3$)CH$_3$), 21.3 (8-C), 22.3 (11-C), 23.5 (CH(CH$_3$)*C*H$_3$), 24.0 (*C*H(CH$_3$)$_2$), 29.1, 29.3 (9, 10-C), 33.1 (7-C), 37.7 (12-C), 39.4 (*C*H$_2$CONHOH), 39.5 (*C*H$_2$CH(CH$_3$)$_2$), 51.1 (2-C), 60.2, 60.3 (6-C), 164.1 (*C*ONHOH, *E*-isomer), 169.5 (*C*ONHOH, *Z*-isomer), 174.1, 177.4 (3, 5-C); $[\alpha]^{23}_{589}$ -21 (c, 0.2, CHCl$_3$); HRMS (ESI): [M-H]$^-$ calcd for $C_{16}H_{27}N_3O_4$, 324.1923, found, 324.1911. The hydrochloride salt (**7d·HCl**) was prepared as described for **1d·HCl**. Mp 211-213°C (dec). Anal. Calcd for $C_{16}H_{28}ClN_3O_4$: 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 2.5×10^4 mL^{-1}, compounds were added at range of concentrations, and the plates incubated at 37 °C. Resazurin was added after 48 h (12.5 μg/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 epimastogotes (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 2.5×10^5 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 ^{o}C in RPMI 1640 (RPMI) medium supplemented with 10% heat-inactivated fetal bovine serum (fully supplmented 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 ^{o}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, 2.5X10^6 parasites/ml and 2X10^5 macrophages/ml were seeded into 96-well plates (in total volume 200μl) and they were incubated for 72 hours with different concentrations of the compounds at 26 ^{o}C and 37 ^{o}C (in 5% CO$_2$)

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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 $4X10^6$ 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 acetohydroxamic 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[18, 19] used was prepared by employing a new facile and efficient experimental protocol involving the

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treatment of 4-methoxybenzyl alcohol with bromoacetyl chloride in the presence of $NaHCO_3$ in dry CH_2Cl_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 J 174.1 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 isobutylation of the cyclooctane parent molecule **6a** appeared to

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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_{50} = 6.8-32 nM, Table 1). Of the adamantane-based compounds possessing a benzyl substituent, the *S*-enantiomer (**1f**, IC_{50} = 6.8 nM) was more potent than the *R*-enantiomer counterpart (**1g**, IC_{50} = 9.1 nM), or racemate (**2**, IC_{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-menthyl 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** vs **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 adamantane-containing free bases **1a, 1b** and **1f** were significantly active against cultured *T. cruzi* epimastigotes, with micromolar to submicromolar IC_{50} values (Table 1). It is noteworthy, that *N*-methylation in analogue **1b** leads to an opposite effect to that in *T. brucei*. Similarly, *C*-benzylation on **1a**, giving compound **1f**, led to a much more potency against *T. cruzi* (26-fold).[9]

The results of the *in vitro* assay for the anti-leishmanial activity of compounds **1a**-**g**, **2**, **3**, **6a**-**d**, **6f**, **7a**, **7b** and **7d** are presented in Table 2. These compounds were first screened against the promastigote form of each of the three *Leishmania* sp. at 10 µM. IC_{50} values were further determined for those displaying 100% parasite growth inhibition at this concentration.

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-chlorobenzylic 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_{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,

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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**, IC_{50}= 7.23 μM, **6d**, IC_{50}= 6.16 μM, and **7d**, IC_{50}= 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, the adamantane-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, IC_{50}=2.67-4.85 μM, and amastigote form, IC_{50}=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**, IC_{50}=2.60±1.37 μM; **2**, IC_{50}=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 (IC_{50}= 15.0 μM). These findings indicate that the *R*-stereochemistry is preferred over the *S* with respect to *L. donovani* potency. Introduction of a chlorine atom at the benzene ring 4-position of the benzylic substituent in the racemic compound **2** slightly reduced the potency against *L. infantum* promastigotes, as shown from the IC_{50} 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 stereoelectronic effects.

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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_{50} values were found to be >200 μM, with the exception of compound **1f** (IC_{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.*[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[15,21] (i.e. 135 times lower for Glucantime over amphotericin B[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_{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.[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*-methylation 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 bloodstream-form *T. brucei,* with low nanomolar IC_{50} values (6.8-32 nM) as shown in Table1. 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_{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.[23]
With respect to the anti-leishmanial activity of the tested compounds, only the *C*-isobutylation or *C*-arylmethylation (*C*-benzylation or 4-chlorobenzyaltion) 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

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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 carbohydroxamic 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/H_2O 29:1 (v/v), rt, 48 h; **(b) (i)** H_2SO_4 97% for **17**, **18**, **25**, rt, 24h or H_2SO_4 97%, CH_2Cl_2, rt, 24 h for **19**, **20**, **26-30** or 48 h for **21-24**; **(ii)** ice and then aq NH_3 26% to pH 7-8; **(c) (i)** $(Me_3Si)_2NK$ (1 equiv), THF, 0-5°C, then rt, 1 h, argon; **(ii)** $BrCH_2CO_2CH_2Ph$ or $BrCH_2CO_2CH_2C_6H_4OCH_3$-4 only for **38**, DMF, rt, 48 h, argon, 78-95% for **31-37**, **38-42**, **44**, 62% for **43** from **8**; **(d)** H_2/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)** $PhCH_2ONH_2 \cdot HCl$, Et_3N or $PhCH_2ONHCH_3$ 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)** CF_3CO_2H, CH_2Cl_2, rt, 90 min; **(ii)** CDI, Et_3N, THF, 28°C, 1 h; **(iii)** 4-$CH_3OC_6H_4CH_2ONH_2$, 28°C, 24 h, then 55°C, 1 h, 70% from **38**; **(g)** CF_3CO_2H, CH_2Cl_2, rt, 10 min, then Et_3SiH, rt, 45 min, 89% from **65**; **(h)** as **(c) (i)**, then CF_3CO_2H (1 equiv), >99%; **(i) (i)** aq CH_2O 37%, MeOH/THF 1:1 for **77** or MeOH/THF 1:3 for **78**, **79**, rt, 3 h, then $NaCNBH_3$, rt, 4 h, at pH 6-7 (maintained by adding AcOH); **(ii)** 1N NaOH and Na_2CO_3 to pH 8, 80-92%; **(j)** NaH, DMF, rt, 1 h, argon and then as **(c) (ii)** using $BrCH_2CO_2CH_2Ph$, 83-92%.

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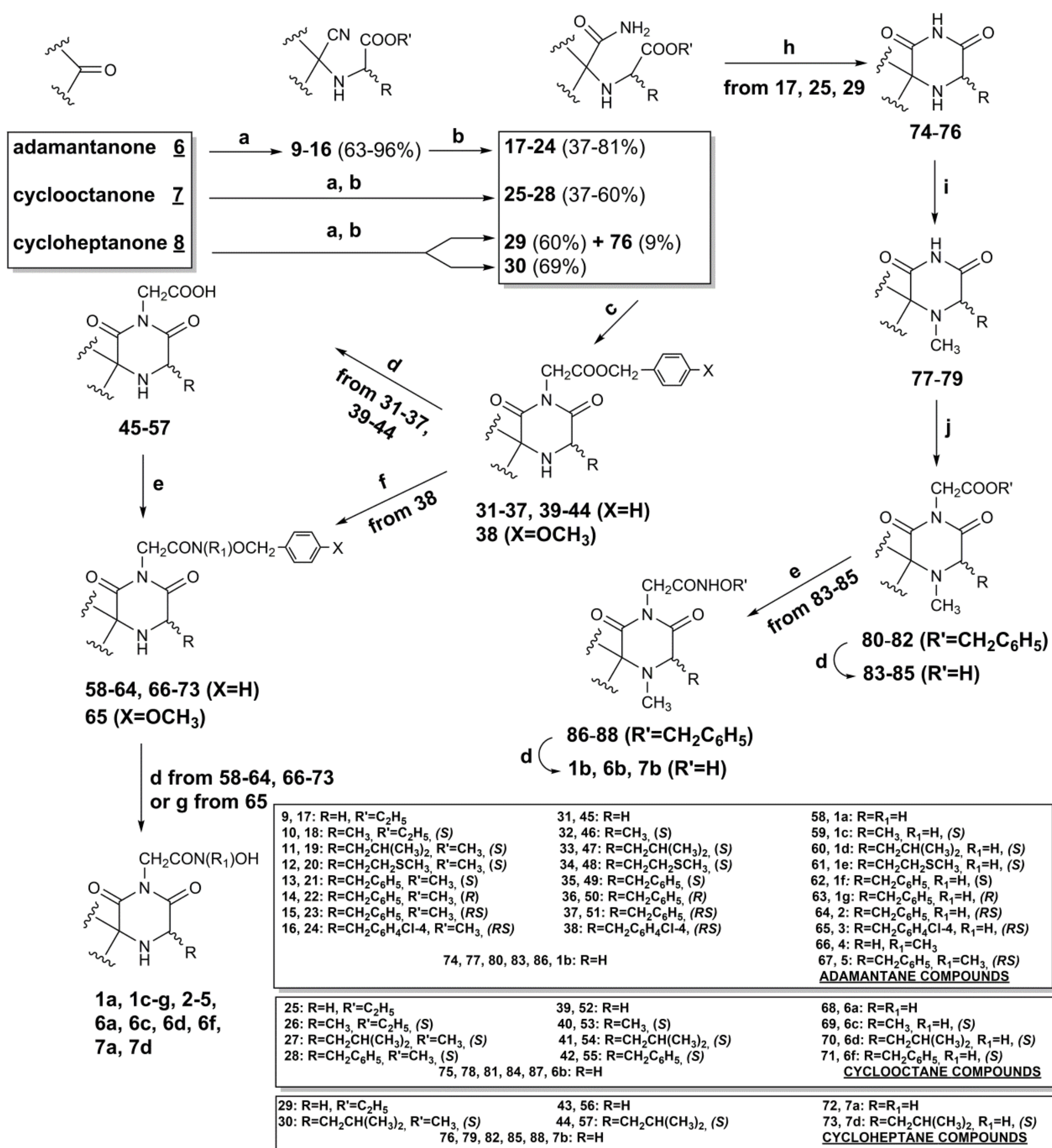

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Table 1. Activity of acetohydroxamic acid analogues **1a-g**, **2-5**, **6a-d**, **6f**, **7a**, **7b** and **7d** (Figure 1) tested against cultured bloodstream-form *T. brucei* (pH=7.4) and *T. cruzi* epimastigotes, and cytotoxicity of the most active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

Cpds	Activity				Cytotoxicity	
	T. brucei		*T. cruzi*		L6 cells	
	IC_{50} (nM)[a,b,c]	IC_{90} (nM)[a,b,c]	IC_{50} (nM)[a,b]	IC_{90} (nM)[a,b]	IC_{50} (nM)[d]	SI[e]
1a	90±16 (79±6)[c]	155±7 (148±8)[c]	5510±680	11110±1190	>325000	>3600
1b	193±28 (340±28)	328±28 (622±84)	3620±310	5990±160	40400±5600	210
1c	134±33 (405±98)	276±12 (909±209)	-	-	-	-
1d	283±27 (253±35)	462±99 (355±17)	-	-	32000±2000	110
1e	168±42 (163±11)	251±14 (248±6)	-	-	10500±500	63
1f	6.8±1.4 (42±5)	11.5±2 (80±22)	210±40	360±10	10400±900	1500
1g	9.1±0.2 (9.2±0.5)	14±1 (14±1)	-	-	11600±1500	1300
2	17±1 (18±1)	26±3 (24±1)	-	-	23700±400	1400
3	32±4 (31±3)	43±3 (40±2)	-	-	21000±1000	660
4	246×10^3 (106×10^3)	523×10^3 (198×10^3)	-	-	-	-
5	37×10^3 (35×10^3)	47×10^3 (45×10^3)	-	-	-	-
6a	300±25 (266±19)	635±14 (495±28)	-	-	-	-
6b	158±34 (162±15)	300±34 (327±9)	-	-	-	-
6c	125±30 (134±13)	270±41 (259±25)	-	-	-	-
6d	72±2 (73±3)	92±9 (111±4)	-	-	204000±47000	2830
6f	29±3 (25±2)	39±1 (35±1)	-	-	93800±18800	3200
7a	1870±80 (1150±130)	2530±290 (1710±40)	-	-	-	-
7b	285±9 (311±7)	673±56 (717±113)	-	-	-	-
7d	99±4 (97±9)	148±8 (135±15)	-	-	191000±2000	1930

[a]Concentrations required to inhibit growth of *T. brucei* and *T. cruzi* by 50% and 90%, respectively.
[b]For the active compounds **1a-g**, **2**, **3**, **6a-d**, **6f**, **7a**, **7b** and **7d**, IC_{50} and IC_{90} data are the mean of triplicate experiments ± SEM.
[c]IC_{50} and IC_{90} for the respective hydrochloride are shown in brackets.

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[d]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. [e]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.

Cpds	***L. infantum* GH12** IC_{50} (μM)[a,b,c]		***L. donovani* Lg13** IC_{50} (μM)[a,b,c]		**Macrophages J774.1.** IC_{50} (μM)[d]
	promastigotes	**intracellular amastigotes**	**promastigotes**	**intracellular amastigotes**	
1a	ni	-	ni	-	>200
1b	ni	-	ni	-	>200
1c	ni	-	ni	-	>200
1d	7.23±0.25 (7.32±1.78)[c]	2.23±1.26 (2.76±1.11)		-	>200
1e	ni	-	ni	-	>200
1f	2.67±0.70 (2.86±0.98)	2.60±1.37 (3.51±1.20)	ni	-	29.3 (32)
1g	3.70±0.23 (2.95±0.86)	4.40±0.69 (4.70±0.43)	8.35±0.86 (6.51±0.32)	15±1.57 (18±1.34)	>200
2	3.80±0.46 (4.05±0.28)	2.74±1.59 (7.90±0.78)	9.73±0.33 (ni)	-	>200
3	4.85±1.22 (4.26±1.39)	2.43±1.18 (2.04±0.77)	ni	-	>200
6a	ni	-	ni	-	>200
6b	ni	-	ni	-	>200
6c	ni	-	ni	-	>200
6d	6.16±1.29 (5.96±0.73)	3.50±1.44 (3.20±1.59)	ni	-	>200
6f	ni	-	ni	-	>200
7a	ni	-	ni	-	>200
7b	ni	-	ni	-	>200
7d	17.7±1.96 (13.2±0.35)	7.85±1.56 (8.90±0.46)	ni	-	>200
Amphotericin B	0.09 ± 0.005	0.18 ± 0.02	0.10 ± 0.01	0.20 ± 0.02	-

[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.

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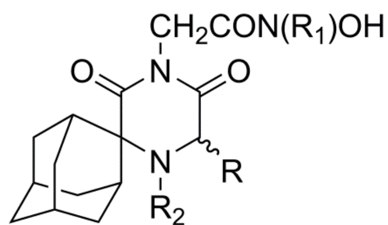

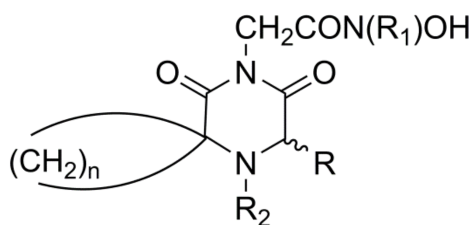

1a-g, 2-5

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

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: $R=CH_2CH(CH_3)_2$, $R_1=R_2=H$, (*S*)-enantiomer
e: $R=CH_2CH_2SCH_3$, $R_1=R_2=H$, (*S*)-enantiomer
f: $R=CH_2C_6H_5$, $R_1=R_2=H$, (*S*)-enantiomer
g: $R=CH_2C_6H_5$, $R_1=R_2=H$, (*R*)-enantiomer
2: $R=CH_2C_6H_5$, $R_1=R_2=H$, racemic
3: $R=CH_2C_6H_4Cl$-4, $R_1=R_2=H$, racemic
4: $R=R_2=H$, $R_1=CH_3$
5: $R=CH_2C_6H_5$, $R_1=CH_3$, $R_2=H$, racemic

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