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Probing Adamantane Arylhydroxamic Acids against *Trypanosoma brucei* and *Trypanosoma cruzi*

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Abstract: In this work, we present the synthesis and the anti-trypanosomal activity of the 2-(4-(adamant-1-yl)phenyl)-*N*-hydroxyarylamides, **1a**,**b** and the 2-(4-(adamant-1-yl)phenoxy)-*N*-hydroxyacetamide, **1c**. The 4-(adamant-1-yl)phenyl- and 4-(adamant-1-yl)phenoxy- moieties, which are endowed with promising drug-like properties, are functionalized at the side chain termini as hydroxamic acids. The phenoxy acetohydroxamic derivative, **1c**, shows the most interesting profile in terms of activity and toxicity against trypanosomes and merits further investigation.

Keywords: 4-(adamant-1-yl)phenyl; 4-(adamant-1-yl)phenoxy; arylhydroxamides; anti-trypanosomal activity

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1. Introduction

One-sixth of the global population, more than 1 billion, is infected with one or more neglected tropical diseases (NTDs) and an additional 2 billion are at risk [1]. Among the "most neglected" NTDs are the two types of trypanosomiasis: human African trypanosomiasis (HAT) and Chagas disease (CD) [2]. Public–private consortia bridge the gap between research and the current therapeutic regimens against these diseases [3,4]. Large-scale phenotypic screening has identified many drug candidates that are active against the kinetoplastid protozoa. However, validated targets against the parasitic diseases are fewer because of the complexities of pathogen biology and, in some cases, insufficiently robust genetic approaches [5]. The challenge for new classes of effective therapeutic agents involves investigation of target-based approaches of drug design [6].

The current drugs used against HAT and CD are suboptimal. The approved regimen presents many restrictions, such as serious adverse side effects, excessive toxicity, limited efficacy and increasing resistance [3,4]. The older therapeutical arsenal consists of pentamidine, eflornithine, suramin, melarsoprol and nifurtimox, while newer agents are benznidazole and the latter fexinidazole and acoziborole [5]. Over the last decade, the scientific community has made tremendous efforts toward NTD control and many more late-stage candidates will be generated in the pipeline, in due course [7–9].

We have been exploring the chemical space of structurally different adamantane derivatives and their biological role against trypanosomes [10–15]. Recently, we published the promising drug-like properties of 4-(adamant-1-yl)phenyl- and 4-(adamant-1-yl)phenoxy-moieties in various scaffolds with trypanocidal activity [16–18]. In the current work, we present the synthesis and the anti-trypanosomal activity of 2-(4-(adamant-1-yl)phenyl)-*N*-hydroxyarylamides **1a**,**b** and the 2-(4-(adamant-1-yl)phenoxy)-*N*-hydroxyacetamide **1c** (Figure 1). The new derivatives exhibit the 4-(adamant-1-yl)phenyl- and 4-(adamant-1-yl)phenoxy- cores, which are functionalized as hydroxamic acids. Hydroxamic acids exhibit

metal-chelating properties and act as inhibitors of metalloproteins with a wide spectrum of therapeutic roles [19]. Acetohydroxamic adamantane derivatives have shown significant activity against *T. brucei* [20] (Figure 1). We now explore the role of the phenyl or phenoxy substitution at the C1 position of the adamantane cage regarding the functional hydroxamic end of the side chain in the **1a**,**b** and **c** derivatives and their activity against trypanosomes.

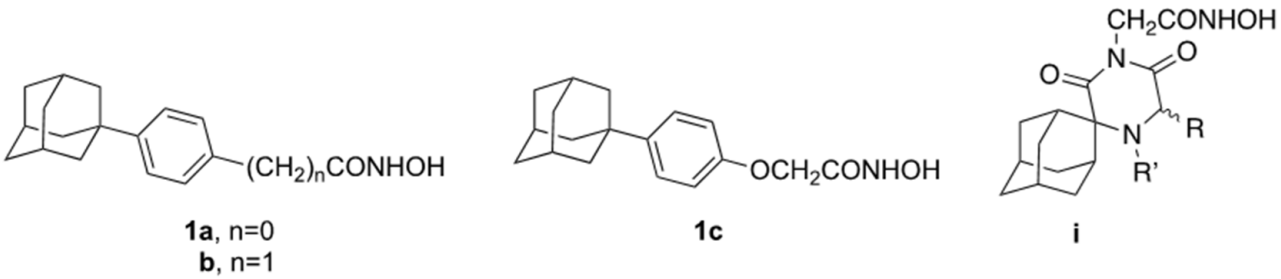
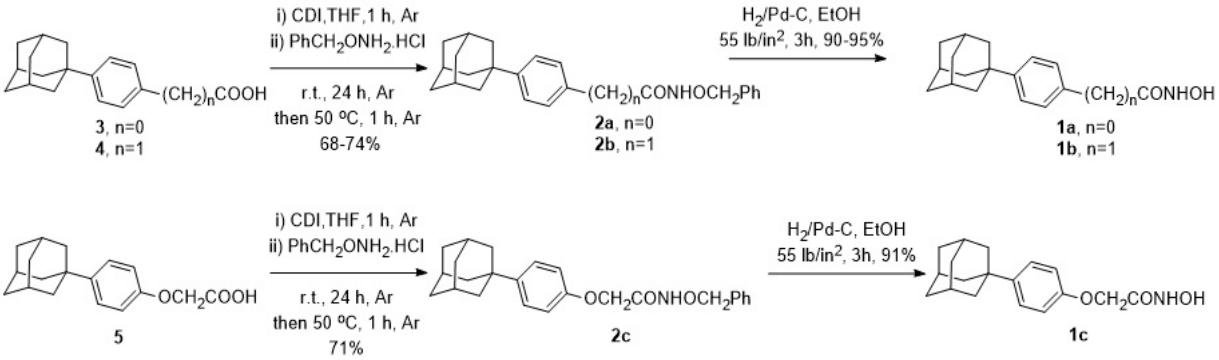


Figure 1. Adamantane hydroxamic derivatives with anti-trypanosomal activity.

2. Results and Discussion

2.1. Chemistry

The synthesis of the hydroxamic acids **1a–c** was accomplished by the reactions shown in Scheme 1. Carboxylic acids **3–5** were coupled with *O*-benzylhydroxylamine in the presence of 1,1′-carbonyldiimidazole (CDI) in THF to give the corresponding *O*-benzyl hydroxamates **2a–c**, in yields of 68–74%. Subsequent *O*-benzyl deprotection by hydrogenolysis in the presence of Pd/C in ethanol afforded the target hydroxamic acids, **1a–c**, in high yield (90–95%). The synthesis of the carboxylic acids **3** and **4** (Scheme 1) has been described in our previously published protocols [18,21], while the preparation of the 2-(4-(adamantan-1-yl)phenoxy)acetic acid **5** was reported by Lee et al. [22].



Scheme 1. Synthesis of the hydroxamic acid analogues **1a–c**.

2.2. Biological Evaluation

The new hydroxamic acid analogues **1a–c** were tested for their activity against the bloodstream form *Trypanosoma brucei* and *Trypanosoma cruzi* epimastigotes and the results are presented in Tables 1 and 2.

Table 1. Anti-*T. brucei* screening of derivatives **1a–c**.

Cmpd	*T. brucei* IC_{50} (μM) [a]	*T. brucei* IC_{90} (μM) [a]	L6 Cells IC_{50} (μM) [a]	S.I. L6/Tb IC_{50} [b]
1a	19.1 ± 0.3	26.4 ± 0.2	23.0 ± 0.5	1.2
1b	2.70 ± 0.18	4.14 ± 0.28	15.7 ± 1.3	5.8
1c	0.286 ± 0.007	0.631 ± 0.020	4.85 ± 0.50	17
Fexinidazole [c]	2.40 ± 0.18	4.51 ± 0.57		

[a] IC_{50} and IC_{90}; concentration that inhibits growth by 50% and 90%, respectively. [b] S.I.; selectivity index, the ratio of IC_{50} values obtained with the rat myoblast L6 cell line and *T. brucei*. [c] Fexinidazole was tested under the same conditions as for the reported derivatives.

Table 2. Anti-*T. cruzi* screening of derivatives **1a–c**.

Cmpd	*T. cruzi* IC_{50} (μM) [a]	*T. cruzi* IC_{90} (μM) [a]	L6 Cells IC_{50} (μM) [a]	S.I. L6/Tc IC_{50} [b]
1a	8.81 ± 0.33	17.8 ± 0.4	23.0 ± 0.5	2.6
1b	2.25 ± 0.06	4.25 ± 0.08	15.7 ± 1.3	7.0
1c	0.432 ± 0.037	0.737 ± 0.083	4.85 ± 0.50	11
Benznidazole [d]	5.39 ± 0.19	9.74 ± 0.88		

[a] IC_{50} and IC_{90}; concentration that inhibits growth by 50% and 90%, respectively. [b] S.I.; selectivity index, the ratio of IC_{50} values obtained with the rat myoblast L6 line and *T. cruzi*. [d] Benznidazole was tested under the same conditions as for the reported derivatives.

We found that the acetohydroxamide **1b** was more potent than the benzohydroxamide **1a**, which had little activity against trypanosomes. Of the three derivatives tested, the phenoxy acetamide **1c** was the most active. Insertion of the oxygen atom of the phenoxy group enhanced activity against *T. brucei* more than ninefold compared with **1b** (IC_{50} of **1c** versus IC_{50} of **1b**, Table 1). For comparison purposes, the phenoxy acetamide **1c** was 12-fold more potent against *T. cruzi* epimastigotes and more than eight times more potent against the *T. brucei* bloodstream forms than benznidazole and fexinidazole, two heterocyclic nitro-drugs that are used to treat *T. cruzi* and *T. brucei,* respectively. The activity of both compounds against *T. cruzi* followed the same pattern. These results have prompted us to further investigate these structure-activity relationships and pursue in the future the synthesis of related structures.

3. Materials and Methods

3.1. Chemistry

All chemicals and solvents were obtained from commercial suppliers and used without further purification. Concentrated refers to the removal of solvents with a rotary evaporator at normal water aspirator pressure, followed by further evacuation on a high-vacuum line. Reactions were monitored by thin-layer chromatography. Thin-layer chromatography was performed using E. Merck precoated silica gel 60 F_{254} plates. Developed TLC plates were visualized with UV light (254 nm) and iodine. The chromatographic purification of the products was carried out using Silica gel 60 (40–63 μm, 230–400 mesh ASTM, Silica flash). Melting points were determined on a Büchi 530 apparatus and are uncorrected.

^{1}H-NMR and ^{13}C-NMR spectra (Supplementary Materials) were taken in $CDCl_3$ or DMSO-d_6 at 298 K (25 °C) and recorded on a Bruker Ultrashiel™ Plus Avance III 600 spectrometer (Bruker, Billerica, Massachusetts, U.S.) and a Bruker DRX400 spectrometer.The measured chemical shifts are reported in δ (ppm), and the residual solvent signal was used as the internal calibration standard ($CDCl_3$): ^{1}H = 7.26 ppm, ^{13}C = 77.0 ppm; (DMSO-d_6): ^{1}H = 2.50 ppm; and ^{13}C = 39.52 ppm. Splitting patterns are designated as follows: s, singlet; br. s, broad singlet; d, doublet; t, triplet; q, quartet; multiplet; and complex m, complex multiplet. Coupling constants (*J*) are expressed in units of Hertz (Hz). IR spectra were recorded by using the attenuated total reflection (ATR) method on a FTIR Bruker Tensor 27.

Microanalyses were carried out by the NCSR Demokritos, Greece, and the results obtained had a maximum deviation of ±0.4% from the theoretical value.

The ^{1}H and ^{13}C NMR spectra for the hydroxamic acid derivatives described in this report (compounds **1a–c**), are consistent with a *Z*/*E* conformation of these molecules in solution. The detected double set of characteristic peaks in the ^{1}H and ^{13}C NMR spectra confirm the presence of the two carbonyl rotamers. The assignment of the *Z* or *E* rotamers was based on literature data concerning *Z*/*E* geometry studies in simple hydroxamate structures (Figure 2) [23–27].

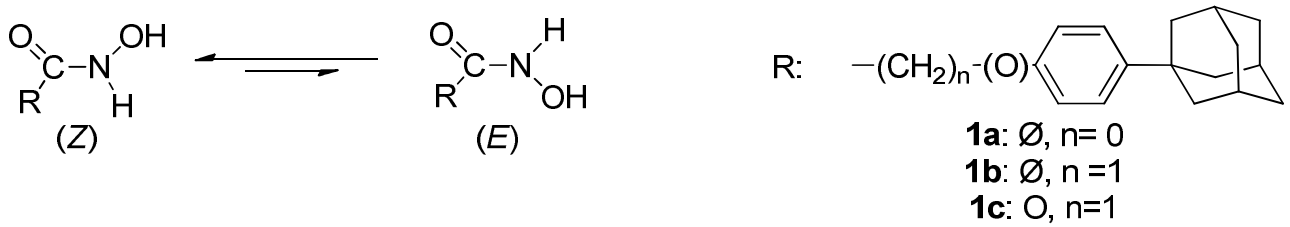


Figure 2. Tautomers of hydroxamic acids **1a–c.**

3.2. Synthesis

3.2.1. 4-(Adamant-1-yl)-*N*-(benzyloxy)benzamide **2a**

To a solution of the carboxylic acid **3** [18] (400 mg, 1.56 mmol) in dry THF (20 mL) was added 1,1′-carbonyldiimidazole (304 mg, 1.87 mmol), and the mixture was stirred at ambient temperature for 1 h under argon. Then, *O*-benzylhydroxylamine hydrochloride (299 mg, 1.87 mmol) and triethylamine (284 mg, 2.81 mmol) were successively added, and the stirring was continued at room temperature for 24 h and then at 50 °C for 1 h under argon. After removal of the solvent *in vacuo*, water was added, and the mixture was extracted with ethyl acetate (×3). The combined extracts were washed with brine (×2), water (×2), dried over Na_2SO_4 and evaporated in vacuo. The residue was purified by flash column chromatography, using as eluent a mixture of DCM:MeOH, 95:5 to give *O*-benzyl hydroxamate **2a** as a white solid. Yield 420 mg (74%). M.p.: 137–138 °C.

Mixture of two rotamers ***E*** and ***Z***. *E*/*Z* ≈ 1.8.

^{1}H-NMR (400 MHz, $CDCl_3$), δ (ppm): (Rotamer ***E***): 1.63–1.73 (~q, 6H, 4,6,10-H), 1.79 (s, 6H,2,8,9-H), 2.01 (br. s, 3H, 3,5,7-H), 4.94 (s, 2H, OCH_2), 7.26–7.30 (complex m, 9H, H_{ar}), 8.58 (s, 1 H, NH); ^{13}C-NMR (50 MHz, $CDCl_3$), δ (ppm): 27.81 (3,5,7-C), 33.62 (1-C), 35.66 (4,6,10-C), 41.94 (2,8,9-C), 77.79 (OCH_2), 124.26 (3,5-C_{ar}), 126.85, 127.04, 127.24, 127.39, 127.69 (2′,3′,4′,5′,6′-C_{ar}), 127.64 (2,6-C_{ar}), 127.86 (1-C_{ar}), 128.33 (1′-C_{ar}), 150.62 (4-C_{ar}), 158.68 (C=O).

^{1}H-NMR (400 MHz, $CDCl_3$), δ (ppm): (Rotamer ***Z***): 1.63–1.73 (~q, 6H, 4,6,10-H), 1.75 (s, 6H,2,8,9-H), 1.84 (br. s, 3H, 3,5,7-H), 4.96 (s, 2H, OCH_2), 7.26–7.30 (complex m, 9H, H_{ar}), 8.58 (s, 1 H, NH); ^{13}C-NMR (50 MHz, $CDCl_3$), δ (ppm): 27.99 (3,5,7-C), 33.62 (1-C), 35.66 (4,6,10-C), 40.18 (2,8,9-C), 74.61 (OCH_2), 124.26 (3,5-C_{ar}), 126.85, 127.04, 127.24, 127.39, 127.69 (2′,3′,4′,5′,6′-C_{ar}), 127.64 (2,6-C_{ar}), 127.86 (1-C_{ar}), 128.21 (1′-C_{ar}), 149.94 (4-C_{ar}), 158.68 (C=O).

Anal Calcd. for $C_{24}H_{27}NO_2$ (%): C, 79.74; H, 7.53; N, 3.87; found (%) C, 79.62; H, 7.47; N, 3.77.

3.2.2. 2-(4-(Adamantan-1-yl)phenyl)-*N*-(benzyloxy)acetamide **2b**

Prepared from the carboxylic acid **4** [21] in a similar manner to benzamide **2a**. Yield 68%. M.p.: 81–83 °C.

Mixture of two rotamers ***E*** and ***Z***. *E*/*Z* ≈ 1.5.

^{1}H-NMR (600 MHz, $CDCl_3$), δ (ppm): (Rotamer ***E***): 1.73–1.79 (~d, 6H, 4,6,10-H), 1.89 (br. s, 6H,2,8,9-H), 2.09 (s, 3H, 3,5,7-H), 3.76 (s, 2H, CH_2CO), 4.97 (s, 2H, OCH_2Ph), 7.29–7.41 (complex m, 9H, H_{ar}), 7.53 (s, 1 H, NH); ^{13}C-NMR (150 MHz, $CDCl_3$), δ (ppm): 29.10 (3,5,7-C), 36.20 (1-C), 36.94 (4,6,10-C), 40.97 (CH_2CO), 43.33 (2,8,9-C), 79.27 (OCH_2Ph), 125.37

(3,5-C_{ar}), 128.75, 128.86, 128.96, 129.38, 129.45 (2′,3′,4′,5′,6′-C_{ar}), 130.19 (2,6-C_{ar}), 133.67 (1′-C_{ar}), 135.23 (1-C_{ar}), 152.36 (4-C_{ar}), 159.60 (C=O).

^{1}H-NMR (600 MHz, $CDCl_3$), δ (ppm): (Rotamer ***Z***): 1.73–1.79 (~d, 6H, 4,6,10-H), 1.89 (br. s, 6H,2,8,9-H), 2.09 (s, 3H, 3,5,7-H), 3.47 (s, 2H, $C\underline{H_2}CO$), 4.99 (s, 2H, $OC\underline{H_2}Ph$), 7.29–7.41 (complex m, 9H, H_{ar}), 7.81 (s, 1 H, NH); ^{13}C-NMR (150 MHz, $CDCl_3$), δ (ppm): 29.10 (3,5,7-C), 36.20 (1-C), 36.94 (4,6,10-C), 40.97 ($\underline{C}H_2CO$), 43.33 (2,8,9-C), 78.95 (OCH_2Ph), 125.37 (3,5-C_{ar}), 128.75, 128.86, 128.96, 129.38, 129.45 (2′,3′,4′,5′,6′-C_{ar}), 130.19 (2,6-C_{ar}), 133.67 (1′-C_{ar}), 135.27(1-C_{ar}), 150.76 (4-C_{ar}), 159.67 (C=O).

Anal Calcd. for $C_{25}H_{29}NO_2$ (%): C, 79.96; H, 7.78; N, 3.73; found (%) C, 80.10; H, 7.96; N, 3.77.

3.2.3. 2-(4-(Adamant-1-yl)phenoxy)-*N*-(benzyloxy)acetamide **2c**

Prepared from the carboxylic acid **5** [22] in a similar manner to benzamide **2a**. Yield 71%. M.p.: 104–105 °C.

^{1}H-NMR (400 MHz, $CDCl_3$), *δ* (ppm): 1.62–1.70 (~q, 6H, 4,6,10-H), 1.77 (br. s, 6H,2,8,9-H), 1.99 (s, 3H, 3,5,7-H), 4.45 (s, 2H, $OC\underline{H_2}CO$), 4.85 (s, 2H, $OC\underline{H_2}Ph$), 6.68–6.70 (d, 2H, $J \approx 8.2$ Hz, 2,6-H_{ar}) 7.17–7.22 (m, 7H, H_{ar}), 7.49 (s, 1 H, NH).

Anal Calcd. for $C_{25}H_{29}NO_3$ (%): C, 76.70; H, 7.47; N, 3.58; found (%) C, 76.63; H, 7.41; N, 3.78.

3.2.4. 4-(Adamant-1-yl)-*N*-hydroxybenzamide **1a**

O-benzyl hydroxamate **2a** (350 mg, 0.97 mmol) in absolute EtOH (30 mL) was hydrogenated (Pd/C 10%, 35 mg) for 3 h at room temperature and 55 psi pressure. The catalyst was filtered off, washed with EtOH (×3), and the combined filtrates were evaporated in vacuo. The residue was purified by flash column chromatography, using as eluent a mixture of *n*-hexanes/AcOEt, 50:50, increasing to 20:80 to afford the title compound **1a** as a white crystalline solid. Yield 250 mg (95%). M.p.: 144–145 °C; IR, *v*(NH): 3415–3362 cm^{-1}, *v*(C=O): 3201 cm^{-1}. *v*(C=O): 1650 cm^{-1}.

Mixture of two rotamers ***E*** and ***Z***. *Z* > *E*.

^{1}H-NMR (400 MHz, $CDCl_3$), *δ* (ppm): (Rotamer ***Z***): 1.63–1.72 (m, 6H, 4,6,10-H), 1.75–1.77 (~d, 6H,2,8,9-H), 1.98 (s, 3H, 3,5,7-H), 5.99 (br. s, 1H, OH), 7.16–7.36 (m, 4H, H_{ar}) 7.58 (s, 1 H, NH); ^{13}C-NMR (50 MHz, $CDCl_3$), *δ* (ppm): 27.82 (3,5,7-C), 33.63 (1-C), 35.67 (4,6,10-C), 41.96 (2,8,9-C), 125.98 (3,5-C_{ar}), 127.52 (2,6-C_{ar}), 139.98 (1-C_{ar}), 154.23 (4-C_{ar}), 168.43 (C=O).

^{1}H-NMR (400 MHz, $CDCl_3$), *δ* (ppm): (Rotamer ***E***): 1.63–1.72 (m, 6H, 4,6,10-H), 1.79–1.82 (~d, 6H,2,8,9-H), 2.01 (s, 3H, 3,5,7-H), 6.16 (br. s, 1H, OH), 7.16–7.36 (m, 4H, H_{ar}), 7.57 (s, 1H, NH); ^{13}C-NMR (50 MHz, $CDCl_3$), *δ* (ppm): 30.74 (3,5,7-C), 33.63 (1-C), 35.67 (4,6,10-C), 45.75 (2,8,9-C), 126.31 (3,5-C_{ar}), 126.57 (2,6-C_{ar}), 139.98 (1-C_{ar}), 154.71 (4-C_{ar}), 168.69 (C=O).

Anal Calcd. for $C_{17}H_{21}NO_2$ (%): C, 75.25; H, 7.80; N, 5.16; found (%) C, 75.43; H, 7.84; N, 5.36.

3.2.5. 2-(4-(Adamantan-1-yl)phenyl)-*N*-hydroxyacetamide **1b**

Prepared from *O*-benzyl hydroxamate **2b** in a similar manner to *N*-hydroxybenzamide **1a**. Yield 90%. M.p.: 93–95 °C; IR, *v*(NH): 3400 cm^{-1}, *v*(OH): 3273 cm^{-1}, *v*(C=O): 1650 cm^{-1}.

Mixture of two rotamers ***E*** and ***Z***. $Z/E \approx 9.2$

^{1}H-NMR (600 MHz, DMSO-d_6), *δ* (ppm): (Rotamer ***Z***): 1.71–10.76 (~q, 6H, 4,6,10-H), 1.85 (s, 6H,2,8,9-H), 2.05 (s, 3H, 3,5,7-H), 3.43 (s, 2H, $C\underline{H_2}CO$), 6.11 (s, 1H, OH), 7.16–7.17 (d, 2H, $J \approx 8.2$ Hz, 3,5-H_{ar}), 7.25–7.27 (d, 2H, $J \approx 8.2$ Hz, 2,6-H_{ar}), 8.19 (s, 1H, NH); ^{13}C-NMR (150 MHz, DMSO-d_6), *δ* (ppm): 28.26 (3,5,7-C), 35.38 (1-C), 36.15 (4,6,10-C), 41.30 ($\underline{C}H_2CO$), 42.62 (2,8,9-C), 124.26 (3,5-C_{ar}), 128.90 (2,6-C_{ar}), 132.99 (1-C_{ar}), 148.64 (4-C_{ar}), 167.10 (C=O).

^{1}H-NMR (600 MHz, DMSO-d_6), *δ* (ppm): (Rotamer ***E***): 1.71–1.76 (~q, 6H, 4,6,10-H), 1.80–1.85 (s, 6H,2,8,9-H), 2.05 (s, 3H, 3,5,7-H), 3.72 (s, 2H, $C\underline{H_2}CO$), 6.11 (s, 1H, OH), 7.19–7.20 (d, 2H, $J \approx 8.2$ Hz, 3,5-H_{ar}), 7.29–7.30 (d, 2H, $J \approx 8.2$ Hz, 2,6-H_{ar}), 7.37 (s, 1H, NH); ^{13}C-NMR (150 MHz, DMSO-d_6), *δ* (ppm): 28.26 (3,5,7-C), 35.38 (1-C), 36.15 (4,6,10-C), 42.55

($\underline{C}H_2CO$), 42.62 (2,8,9-C), 124.36 (3,5-C_{ar}), 128.55 (2,6-C_{ar}), 132.99 (1-C_{ar}), 148.64 (4-C_{ar}), 173.00 (C=O).

Anal Calcd. for $C_{18}H_{23}NO_2$ (%): C, 75.76; H, 8.12; N, 4.91; found (%) C, 75.68; H, 7.93; N, 4.87.

3.2.6. 2-(4-(Adamantan-1-yl)phenoxy)-*N*-hydroxyacetamide **1c**

Prepared from *O*-benzyl hydroxamate **2c** in a similar manner to *N*-hydroxybenzamide **1a**. Yield 90%. M.p.: 104–105 °C; IR, v(NH): 3396 cm^{-1}, v(OH): 3150 cm^{-1}, v(C=O): 1655 cm^{-1}.

Mixture of two rotamers $\underline{\boldsymbol{E}}$ and $\underline{\boldsymbol{Z}}$. $E/Z \approx 5.3$.

^{1}H-NMR (600 MHz, DMSO-d_6), δ (ppm): (Rotamer ***Z***): 1.69–1.74 (~q, 6H, 4,6,10-H), 1.82 (s, 6H,2,8,9-H), 2.03 (s, 3H, 3,5,7-H), 4.39 (s, 2H, O$\underline{C}H_2$CO), 6.17 (s, 1H, OH), 6.88–6.99 (d, 2H, $J \approx 8$ Hz, 2,6-H_{ar}), 7.24–7.2 (d, 2H, $J \approx 8$ Hz, 3,5-H_{ar}), 8.27 (s, 1 H, NH); ^{13}C-NMR (150 MHz, DMSO-d_6), δ (ppm): 28.43 (3,5,7-C), 35.14 (1-C), 36.25 (4,6,10-C), 42.87 (2,8,9-C), 65.94 (OCH_2), 114.22 (2,6-C_{ar}), 125.53 (3,5-C_{ar}), 143.73 (4-C_{ar}), 155.67 (1-C_{ar}), 164.50 (C=O).

^{1}H-NMR (600 MHz, DMSO-d_6), δ (ppm): (Rotamer ***E***): 1.69–1.74 (~q, 6H, 4,6,10-H), 1.82 (s, 6H,2,8,9-H), 2.08 (s, 3H, 3,5,7-H), 4.43 (s, 2H, O$\underline{C}H_2$CO), 6.81 (s, 1H, OH), 6.88–6.99 (d, 2H, $J \approx 8$ Hz, 2,6-H_{ar}), 7.24–7.26 (d, 2H, $J \approx 8$ Hz, 3,5-H_{ar}), 7.46 (s, 1 H, NH); ^{13}C-NMR (150 MHz, DMSO-d_6), δ (ppm): 30.62 (3,5,7-C), 35.14 (1-C), 36.25 (4,6,10-C), 42.87 (2,8,9-C), 66.85 (OCH_2), 114.22 (2,6-C_{ar}), 125.53 (3,5-C_{ar}), 143.73 (4-C_{ar}), 162.64 (1-C_{ar}), 170.53 (C=O).

3.3. Biological Assays

3.3.1. Cytotoxic Activity against Rat Skeletal Myoblast L6 Cells

Cytotoxicity against mammalian cells was assessed using microtitre plates. Briefly, L6 cells (a rat skeletal muscle line) were seeded at 1×10^4 mL^{-1} in 200 µL of RPMI-1640 growth medium containing seven different compound concentrations in a range previously established to encompass both the IC_{50} and IC_{90} values. The plates were incubated for 6 days at 37 °C and 20 µL Alamar Blue (Biosource Ltd, Wilton, North Yorkshire England.) was then added to each well. After an additional 8 h incubation, the fluorescence was determined using a Gemini fluorescent plate reader (Molecular Devices). Inhibition of growth was calculated by comparison with control values and IC_{50} and IC_{90} values were determined in triplicate using linear regression analysis.

3.3.2. *Trypanosoma brucei* Culturing and Drug Testing

Bloodstream form *T. brucei* (strain 427) were cultured at 37 °C in modified Iscove's medium. Trypanocidal activity was assessed by growing parasites in microtiter plates in the presence of various drug concentrations. Parasites were seeded at 0.25×10^{-5} mL^{-1} in 200 µL of growth medium containing seven different compound concentrations in a range previously established to encompass both the IC_{50} and IC_{90} values. The plates were incubated for 48 h at 37 °C and 20 µL Alamar Blue was then added to each well. After an additional overnight incubation, the fluorescence was determined using a Gemini fluorescent plate reader (Molecular Devices).

3.3.3. *Trypanosoma cruzi* Culturing and Drug Testing

T. cruzi epimastigotes (strain CL Brener) were cultured at 28 °C in supplemented RPMI-1640 medium. Trypanocidal activity was assessed by growing parasites in microtiter plates in the presence of various drug concentrations. Parasites were seeded at 2.5×10^{-5} mL^{-1} in 200 µL of growth medium containing 7 different compound concentrations in a range previously established to encompass both the IC_{50} and IC_{90} values. The plates were incubated for 4 days at 28 °C and 20 µL Alamar Blue was then added to each well. After an additional 3 days incubation, the fluorescence was determined using a Gemini fluorescent plate reader (Molecular Devices).

Supplementary Materials: The following material is available online. Figure S1: ^{1}H-NMR spectrum of compound **2a**; Figure S2: ^{13}C-NMR spectrum of compound **2a**; Figure S3: ^{1}H-NMR spectrum of compound **2b**; Figure S4: ^{13}C-NMR spectrum of compound **2b**; Figure S5: ^{1}H-NMR spectrum of compound **2c**; Figure S6: ^{1}H-NMR spectrum of compound **1a**; Figure S7: ^{13}C-NMR spectrum of compound **1a**; Figure S8: IR Spectrum of compound **1a**; Figure S9: ^{1}H-NMR spectrum of compound **1b**; Figure S10: ^{13}C-NMR spectrum of compound **1b**; Figure S11: IR Spectrum of compound **1b**; Figure S12: ^{1}H-NMR spectrum of compound **1c**; Figure S13: ^{13}C-NMR spectrum of compound **1c**; **Figure S14**: IR Spectrum of compound **1c**.

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