



Research paper

Tyrosol and hydroxytyrosol derivatives as antitrypanosomal and antileishmanial agents



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ABSTRACT

Trypanosomiasis and leishmaniasis keep being a real challenge for health and development of African countries. Existing treatments have considerable side effects and increase resistance of the parasites. We have measured antitrypanosomal and antileishmanial activity of natural phenols, tyrosol (TYR) and hydroxytyrosol (HT) and several of their esters and metabolites. We found significant IC₅₀ values against *Trypanosoma brucei* for HT decanoate ester and HT dodecanoate ester (0.6 and 0.36 μM, respectively). This represents a large increase in activity with respect to HT (79 and 132 fold, respectively). Moreover, both compounds displayed a high selectivity index against MRC-5, a non-tumoral human cell line (118 and 106, respectively). Then, we synthesized a focused library of compounds to explore structure-activity. We found the ether and thiourea analogs of HT decanoate ester and HT dodecanoate ester also showed IC₅₀ values against *T. brucei* in the low micromolar range. In conclusion, the di-ortho phenolic ring and medium size alkyl chain are essential for activity whereas the nature of the chemical bond among them seems less important.

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1. Introduction

Infectious diseases caused by protozoan parasites affect millions of people around the world, especially in tropical and subtropical areas. Among them, leishmaniasis (caused by different species of *Leishmania*) is a major global health problem with around 12 million people infected and causing 1–2 million new cases every year. *Trypanosoma brucei*, transmitted by the tsetse fly, is responsible for sleeping sickness in humans and preclude the development of productive livestock and agricultural activity based on domesticated animals where it causes Nagana [1,2]. For these reasons, this parasite is considered to be one of the major root causes of hunger and poverty in sub-Saharan Africa. Current treatments for

both diseases present important drawbacks such as high toxicity, increased resistance and variability on their efficacy depending on the different strain of the parasite. Thus, the development of new antiparasitic therapies continues to be necessary.

Natural products have been tested for decades to find new antitrypanosomal and antileishmanial agents. Among them, several phenolics and polyphenols have shown relevant activity. For example, flavonoids such as 7,8-dihydroxyflavone, 3-hydroxyflavone and rhamnetin showed low micromolar IC₅₀ values for *T. brucei rhodesiense* and others such as luteolin and quercetin also displayed low micromolar IC₅₀s for *Leishmania donovani* [3]. In fact, quercetin glycosides present in the aqueous extract of *Kalanchoe pinnata* were active by oral administration in experimental cutaneous and visceral leishmaniasis infections produced *in vivo* [4,5].

Tyrosol (TYR, **1**) and hydroxytyrosol (HT, **2**) (Fig. 1) are natural phenolic antioxidants present in olives and olive oil. They have shown a diverse biological activity such as antibacterial [6,7], antiviral [8], anti-inflammatory [9–12], neuroprotective [13] and anticancer activity [14], inhibition of human LDL oxidation [15] and

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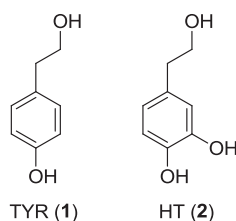


Fig. 1. Chemical structures of tyrosol (TYR, 1) and hydroxytyrosol (HT, 2).

prevention of platelet aggregation [16]. Recently, Moradi-Afrapoli et al. reported medium activity of TYR against *T. brucei rhodesiense* ($IC_{50} > 15 \mu M$) [17]. To the best of our knowledge, HT has not been examined for activity against *T. brucei*. Though, moderate antileishmanial activity was reported for HT against both, promastigotes of *Leishmania infantum*, *L. donovani*, and *Leishmania major*, and against *L. donovani* amastigotes that parasitize J774A.1 macrophages [18].

The preparation of chemically-modified natural products to improve their antiparasitic activity is still a very active source of potential new drugs. This is the case of chalcone [19] or caracasine acid derivatives [20], or the preparation of hybrids such as *Cinchona* alkaloids with bile acids [21] or caffeine-based chalcones [22]. Tyrosol and hydroxytyrosol derivatives have also been synthesized in order to improve the antioxidant and biological properties of the parent compounds. Hydroxytyrosol fatty acid esters increased the protection of proteins and lipids against oxidation caused by peroxyl radicals in a brain homogenate as an *ex vivo* model [23]. Similarly, hydroxytyrosol acetate was able to reduce the metabolic imbalance induced by a high-cholesterol diet in rats to a higher extent than HT [24]. Finally, HT alkyl ether derivatives, more stable under biological conditions than HT, have been reported to exert antiproliferative [25], neuroprotective [26,27], antiplatelet and anti-inflammatory effects [28] that are greater than those of HT.

Due to the improved biological activity found for several HT derivatives with respect to HT itself, we decided to investigate the antitrypanosomal and antileishmanial activity of a series of TYR and HT derivatives. We prepared a series of tyrosol and hydroxytyrosol fatty acid esters together with three metabolites of HT and TYR, tyrosol sulfate, tyrosol glucuronate and hydroxytyrosol glucuronate for a first *in vitro* screening. Later, we synthesized a focused library of compounds to explore structure-activity relevance varying the number of phenolic OH groups, the type of chemical bond between the phenolic ring and the alkyl chain, and the length of the alkyl chain.

2. Results and discussion

2.1. Chemistry

Tyrosol and hydroxytyrosol fatty acid esters (3–7 and 11–16, respectively) were synthesized by enzymatic acylation using Novozym 435 and the corresponding vinyl acyl donor in *t*-butyl methyl ether as reported previously [29–31] (Scheme 1). Tyrosol and hydroxytyrosol glucuronates 8 and 17, 18 were prepared by glycosylation of the acetyl protected TYR or HT derivatives (3 and 10, respectively). We used the acetyl protected trichloroacetimidate glucuronosyl derivative 19 as glycosyl donor and boron trifluoride etherate as the catalyst in the glycosylation step. Final acetyl deprotection yielded glucuronate derivatives 8 and 17, 18 as described previously [32]. TYR sulfate 9 was prepared following a similar strategy to the one used for the glucuronate derivative. Sulfation of tyrosol acetate 3 was carried out with $SO_3 \cdot NMe_3$ as

sulfating reagent, NEt_3 as base, and acetonitrile as solvent at $100^\circ C$ under microwave radiation. The reaction afforded the sulfated TYR derivative in good yield. Final acyl deprotection and reverse phase purification gave TYR sulfate 9.

We identified hydroxytyrosol decanoate ester 13 and hydroxytyrosol dodecanoate ester 14 as relevant hit compounds against *T. brucei* (see below, Table 2) after carrying out the first *in vitro* screening on *T. brucei* and *L. donovani*. We decided then to perform structure-activity studies on 13 and 14 by changing the phenolic hydroxyl groups, the type of chemical bond between the phenolic ring and the alkyl chain, and the length of the alkyl chain (Fig. 2) in order to improve its biological activity.

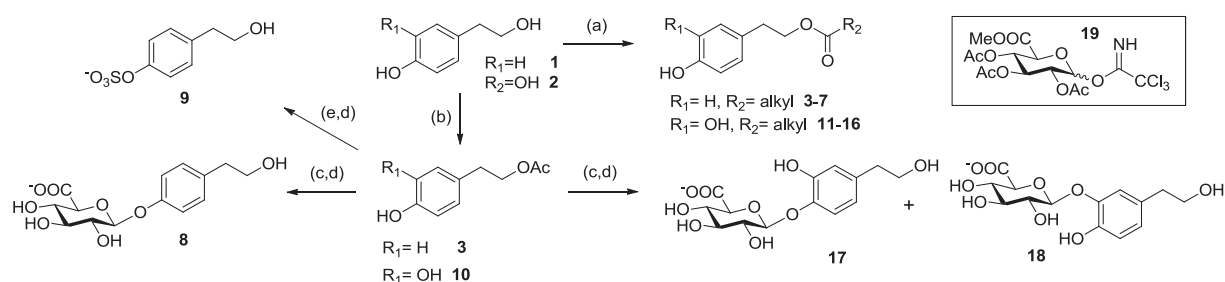
We synthesized a hydroxyl protected version of compound 14 by formation of the acetal derivative 22 and also a shorter version with a decanoate alkyl chain, compound 21 (Scheme 2). The cyclic acetal intermediate 20 was obtained by reaction of HT with CH_2Cl_2 in basic conditions and then acylated using the enzymatic conditions used previously for HT yielding the desired compounds. The ether analog of 14, compound 29, was prepared as reported previously [33] by benzyl protection of the phenolic groups, alkylation with 1-iodododecane under basic conditions and final hydrogenation. The HT ether decanoate derivative 28 was prepared following the same procedure. We also prepared the *p*-methoxybenzyl (PMB) protected HT derivative 24 trying to improve the yields of the route. However, deprotection with trifluoroacetic acid of the ether intermediate 27 was unsuccessful. The synthesis of the thioether analog of 14 was attempted next. Tosylation of derivatives 23 and 24 was followed by nucleophilic displacement with 1-dodecanethiol producing the protected thioether derivatives 32 and 33 with moderate yields. Neither hydrogenation nor reaction under strong acidic conditions yielded the final thioether deprotected products.

Dopamine hydrochloride was the starting material for the synthesis of the amide and thiourea analogs of 13 and 14 (compounds 37–40) (Scheme 3). Dopamine was also synthesized to compare its activity with HT. The thiourea derivatives were synthesized by reaction of the isothiocyanate intermediate 36 with the corresponding primary alkylamine to obtain the products in moderate yields. The amide analogs of 13 and 14 were prepared by reaction of dopamine hydrochloride with the corresponding fatty acids using HATU as the coupling reagent.

2.2. Biological evaluation

The *in vitro* antiparasitic activities of TYR, HT and their derivatives 3–18 were evaluated against *T. brucei brucei* and against axenic amastigotes of *L. donovani*. Those compounds exhibiting over 40% inhibition of axenic amastigotes at $20 \mu M$ were also evaluated on intracellular amastigotes of *L. donovani*. The cytotoxicity of these compounds was also evaluated against a human non-tumoral lung cell line (MRC-5). Suramin and amphotericin B were used as positive drug controls for *T. brucei* and *L. donovani*, respectively. Selectivity indices (SI) were calculated according to the formula: IC_{50} (MRC-5)/ IC_{50} parasite. The activity gain (AG) of each compound with respect to the reference compound (TYR or HT) was calculated according to the formulas: IC_{50} (TYR)/ IC_{50} compound and IC_{50} (HT)/ IC_{50} compound.

Table 1 presents the antiparasitic and cytotoxic data for TYR and its derivatives 3–9. IC_{50} values against *T. brucei* ranged from 10 to $62.7 \mu M$ and selectivity indices from 1.5 to 5. None of the TYR derivatives improved the activity of TYR itself as can be observed from the AG values. In the case of *L. donovani*, compounds 4 and 5 showed inhibition over 40% of axenic amastigotes at $20 \mu M$ concentration. These two derivatives were evaluated on intracellular amastigotes and showed IC_{50} values $> 10 \mu M$. Aissa et al. [34]



Scheme 1. Reagents and condition: (a) vinyl alkyl ester, Novozym 435, tBuOMe, 60 °C; (c) **19**, BF₃·OEt₂, CH₂Cl₂; (d) Na₂CO₃, MeOH, H₂O; (e) SO₃·NMe₃, TEA, CH₃CN, 100 °C, 20 min, MW.

Table 1
Cytotoxic, trypanocidal and leishmanicidal activities of tyrosol derivatives.

			Cytotoxicity MRC-5				<i>T. brucei</i>		<i>L. donovani</i>			
Compounds	R ₁	R ₂	IC ₅₀ (μM)	IC ₅₀ (μM)	SI	AG			Axenic amastigotes	Intracellular amastigotes		
									% Inhib. (20 μM)	IC ₅₀ (μM)	SI	AG
TYR (1)	H	H	>500	>10	>5	—			50	>10	5	—
3	COCH ₃	H	113.6 ± 11.3	79.4 ± 23.1	1.4	0.13			28	n.d.	—	—
4	CO(CH ₂) ₄ CH ₃	H	166.4 ± 0.6	59.1 ± 2.3	2.8	0.16			44	>10	16.6	1
5	CO(CH ₂) ₆ CH ₃	H	133.0 ± 9.8	56.8 ± 8.3	2.3	0.22			46	>10	13.3	1
6	CO(CH ₂) ₈ CH ₃	H	171.5 ± 15.7	81.4 ± 19.3	2.1	0.16			34	n.d.	—	—
7	CO(CH ₂) ₁₀ CH ₃	H	67.2 ± 0.2	16.2 ± 3.8	4.1	0.52			32	n.d.	—	—
8	H	GlcAc	110.2 ± 14.4	61.5 ± 23.8	1.8	0.17			36	n.d.	—	—
9	H	SO ₃ K ⁺	106.4 ± 17.2	41.6 ± 12.6	2.6	0.16			36	n.d.	—	—
Suramin	—	—	—	0.038 ± 0.003	—	—			—	—	—	—
Eflornitin	—	—	—	0.026 ± 0.002	—	—			—	—	—	—
Amphotericin B	—	—	—	—	—	—			—	0.10 ± 0.01	—	—

Experiments on *L. donovani* were carried out in axenic amastigotes and intracellular amastigotes.

SI = Selectivity Index (IC₅₀ MRC-5 / IC₅₀ parasite); AG = Activity gain (IC₅₀ tyrosol / IC₅₀ compound).

Table 2
Cytotoxic, trypanocidal and leishmanicidal activities of hydroxytyrosol derivatives.

			Cytotoxicity MRC-5				<i>T. brucei</i>		<i>L. donovani</i>			
Compounds	R ₁	R ₂ , R ₃	IC ₅₀ (μM)	IC ₅₀ (μM)	SI	AG			Axenic amastigotes	Intracellular amastigotes		
									% Inhib. (20 μM)	IC ₅₀ (μM)	SI	AG
HT (2)	H	H, H	>50	47.5 ± 16.9	>1	—			0	n.d.	—	—
10	COCH ₃	H, H	>50	43.4 ± 14.0	>1	1			48	>10	—	—
11	CO(CH ₂) ₂ CH ₃	H, H	71.5 ± 15.5	9.4 ± 1.4	8	5			48	>10	7.1	—
12	CO(CH ₂) ₆ CH ₃	H, H	103.5 ± 15.2	9.6 ± 0.8	11	5			45	>10	10.3	—
13	CO(CH ₂) ₈ CH ₃	H, H	71.1 ± 6.9	0.6 ± 0.24	118	79			54	8.44 ± 1.6	8.4	—
14	CO(CH ₂) ₁₀ CH ₃	H, H	38.1 ± 2.4	0.36 ± 0.01	106	132			56	>10	—	—
15	CO(CH ₂) ₁₂ CH ₃	H, H	>50	2.29 ± 0.48	>22	21			53	>10	—	—
16	CO(CH ₂) ₁₄ CH ₃	H, H	>50	2.43 ± 0.33	>21	20			52	>10	—	—
17–18	H	GlcAc, H	118.8 ± 21.5	62.3 ± 16.8	2	0.8			31	n.d.	—	—

Experiments on *L. donovani* were carried out in axenic amastigotes and intracellular amastigotes.

SI = Selectivity Index (IC₅₀ MRC-5 / IC₅₀ parasite); AG = Activity gain (IC₅₀ hydroxytyrosol / IC₅₀ compound).

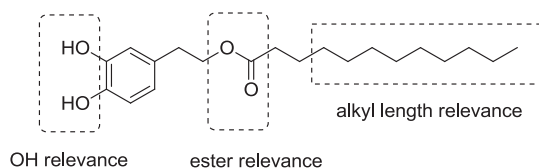
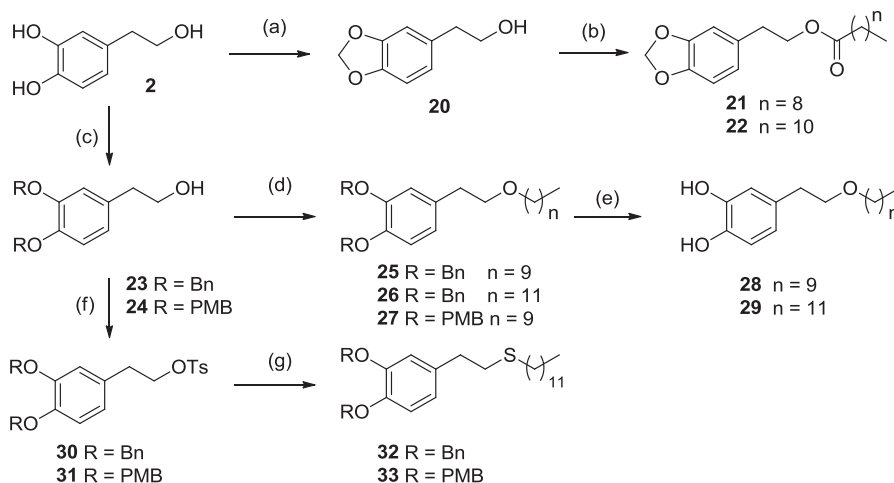


Fig. 2. Parts of compound **14** to focus structure-activity studies.

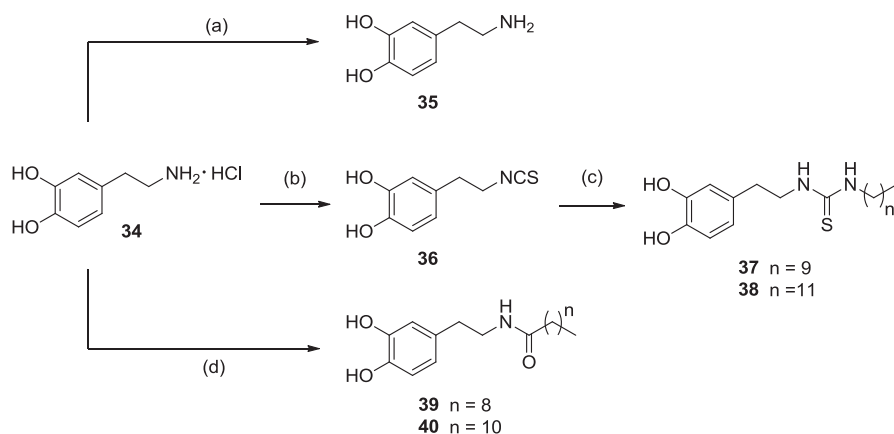
measured antileishmanial activity against *L. major* and *L. infantum* parasite species for tyrosol fatty acid esters from C2 to C18 chain length. They found the best IC₅₀ values for medium chain tyrosyl

derivatives **5–7** (e.g. tyrosol decanoate **6**: 65 and 132 μM, against *L. major* and *L. infantum*, respectively). The authors proposed this effect could be due to the surfactant activity reported for these medium chain tyrosyl fatty acid esters [35].

When HT and its derivatives **10–18** were evaluated against *T. brucei* (Table 2), compounds **13–16** showed IC₅₀s in the low micromolar range (0.36–2.43 μM). Remarkably, hydroxytyrosol decanoate **13** and hydroxytyrosol dodecanoate **14** displayed a high selectivity index (118 and 101, respectively) and a large increase in activity with respect to HT (AG values of 79 and 132, respectively). A length of the alkyl chain around 10 to 12 carbons seems to be the optimum on this series. When these compounds were examined



Scheme 2. Reagents and conditions: (a) CH_2Cl_2 , K_2CO_3 , DMF, Reflux; (b) vinyl alkyl ester, Novozym 435, tBuOMe ; (c) BrBn or PMBCl , K_2CO_3 , Acetone, Reflux; (d) $\text{I}(\text{CH}_2)_n\text{CH}_3$, KOH , DMSO, 50°C ; (e) H_2 , Pd, 4 atm, THF; (f) TsCl , TEM, DCM, 0°C ; (g) $\text{HS}(\text{CH}_2)_{11}\text{CH}_3$, K_2CO_3 , THF.



Scheme 3. Reagents and conditions: (a) KOH , MeOH ; (b) CS_2 , TEA, MeOH , THF; (c) $\text{H}_2\text{N}(\text{CH}_2)_n\text{CH}_3$, TEA, Pyridine; (d) $\text{CH}_3(\text{CH}_2)_n\text{COOH}$, HATU, TEA, THF.

against axenic amastigotes of *L. donovani*, three of them (compounds **11–13**) exhibited over 40% inhibition at $20\ \mu\text{M}$ concentration. When they were evaluated on intracellular amastigotes, the best IC_{50} value was displayed by hydroxytyrosol decanoate **13** ($8.44\ \mu\text{M}$). Kyriazis et al. [18] had previously reported IC_{50} data for hydroxytyrosol of $393\ \mu\text{M}$ against *L. donovani* and in the same range for *L. infantum* and *L. major*. We have observed that the addition of a fatty acid ester group to HT improves its activity with respect to HT against *L. donovani* although only to IC_{50} s in the low micromolar range.

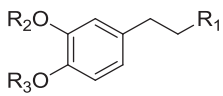
After performing this first *in vitro* screening on *T. brucei* and *L. donovani* for TYR, HT and their derivatives **3–18**, we decided to focus our efforts only on new compounds against *T. brucei*. We carried out a structural-activity study of analogs of hydroxytyrosol fatty acid esters **13** and **14** (Table 3) in order to probe the relevance of the phenolic hydroxyl groups, the type of chemical bond between the phenolic ring and the length of the alkyl chain.

We found that the phenolic hydroxyl groups seem to be essential for activity against *T. brucei* since changing them to a methylene acetal (compounds **21** and **22**) or protecting them with benzyl or p-methoxybenzyl groups (compounds **25**, **27**, **32** and **33**) increased their IC_{50} values ($>10\ \mu\text{M}$) with respect to those of **13** and **14**. The type of bond linking the alkyl chain to the HT scaffold on these compounds (ester, ether or thioether) appears to be much

less important for their activity. When both phenolic hydroxyl groups and the medium size alkyl chain still remain as part of the structure of the compounds, the functional group connecting them seems to be less relevant. Actually, the hydroxytyrosol ether analogs **28** and **29**, and the thiourea analogs **37** and **38** showed similar IC_{50} values ($0.63\text{--}1.29\ \mu\text{M}$) to those of the ester derivatives **13** and **14**. In contrast, the amide derivatives **39** and **40** presented slightly worse antitrypanosomal activity (>10 and $8.74\ \mu\text{M}$, respectively) than the ester derivatives. However, none of the new compounds displayed better selectivity index than compounds **13** and **14** and their activity gain with respect to HT was lower too. Finally, the length of the alkyl chain seems optimum at 10–12 carbon atoms independently of the bond connecting it to the HT scaffold. Only a two-fold difference is observed for the ester and ether derivatives with decyl and dodecyl chains.

Hydroxytyrosol decanoate **13** and hydroxytyrosol dodecanoate **14** possess relevant surfactant properties, even better than medium chain tyrosyl fatty acid esters [35]. So, it could be argued that **13** and **14** are acting as nonspecific detergents disrupting the parasite's membrane. Another possibility is they could be inhibiting fatty acid biosynthesis, through inhibition of FabG, FabI or FabZ enzymes. The structural similarity of **13** and **14** with *trans*-2-hexadecenyl-(*N*-acetylcysteamine)-thioester, a FabI inhibitor [36,37], or with 2-, 5-, 6-, and 9-hexadecynoic acids (HDAs), inhibitors of FabG, FabI and

Table 3
Cytotoxic and trypanocidal activities of hydroxytyrosol derivatives **19–40**.

Compounds			Cytotoxicity MRC-5		<i>T. brucei</i>	
	R ₁	R ₂ , R ₃	IC ₅₀ (μM)	IC ₅₀ (μM)	SI	AG
21	OCO(CH ₂) ₈ CH ₃	–CH ₂ –	>50	>10 (n = 2)	>5	>5
22	OCO(CH ₂) ₁₀ CH ₃	–CH ₂ –	>50	>10 (n = 3)	>5	>5
25	O(CH ₂) ₉ CH ₃	Bn, Bn	>50	>10 (n = 3)	>5	>5
27	O(CH ₂) ₉ CH ₃	PMB, PMB	>50	>10 (n = 2)	>5	>5
28	O(CH ₂) ₉ CH ₃	H, H	>50	1.29 ± 0.21	>38	37
29	O(CH ₂) ₁₁ CH ₃	H, H	13.19 ± 2.90	0.63 ± 0.07	21	75
32	S(CH ₂) ₁₁ CH ₃	Bn, Bn	>50	>10 (n = 2)	>5	5
33	S(CH ₂) ₁₁ CH ₃	PMB, PMB	>50	>10 (n = 2)	>5	5
35	NH ₂	H, H	68.9 ± 15.11	>10 (n = 2)	7	5
36	NCS	H, H	21.13 ± 8.78	9.87 ± 1.02	2	5
37	NHSNH(CH ₂) ₈ CH ₃	H, H	24.8	0.74 ± 0.36	33	64
38	NHSNH(CH ₂) ₁₀ CH ₃	H, H	2.96 ± 1.20	0.77 ± 0.28	4	62
39	NHCO(CH ₂) ₈ CH ₃	H, H	>50	>10 (n = 4)	>5	5
40	NHCO(CH ₂) ₁₀ CH ₃	H, H	>50	8.74 ± 1.15	>5	5

SI = Selectivity Index (IC₅₀ MRC-5/IC₅₀ *T. brucei*); AG = Activity gain (IC₅₀ hydroxytyrosol/IC₅₀ compound).

FabZ of *P. falciparum* [38], could be pointing to these potential therapeutic targets. In both cases, the inhibitors possess long alkyl chains (13–16 atoms in length) together with one or two groups at the end of the compound capable of accepting or donating hydrogen bonds resembling the scaffolds of compounds **13** and **14**.

3. Conclusions

We have examined the trypanocidal and leishmanicidal activities of several tyrosol and hydroxytyrosol fatty acid esters together with three of TYR and HT metabolites. We found notable IC₅₀ values against *T. brucei* for HT decanoate ester **13** and HT dodecanoate ester **14** (0.6 and 0.36 μM, respectively) and against *L. donovani* for **14** (8.44 μM). We synthesized several analogs of **13** and **14** to investigate structure–activity relationship in *T. brucei*. We observed that the di-ortho phenolic ring and the medium size alkyl chain are essential for activity but the nature of the chemical bond among them is not so relevant.

4. Experimental section

4.1. General

All chemicals were used without further purification, unless otherwise noted. All reactions were monitored by TLC on precoated Silica-Gel 60 plates F254, and detected by heating with 5% sulfuric acid in ethanol or Mostain (500 ml of 10% H₂SO₄, 25 g of (NH₄)₆Mo₇O₂₄•4H₂O, 1 g Ce(SO₄)₄•4H₂O). Products were purified by flash chromatography with Silica gel 60 (200–400 mesh). Low resolution mass spectra were obtained on an ESI/ion trap mass spectrometer. High resolution mass spectra were obtained on an ESI/quadrupole mass spectrometer. NMR spectra were recorded on a 300, 400 or 500 MHz [300 or 400 MHz (1H), 75 or 100 (13C)] NMR spectrometers, at room temperature for solutions in CDCl₃, D₂O or CD₃OD. Chemical shifts are referred to the solvent signal. 2D experiments (COSY, TOCSY, ROESY, and HMQC) were done when necessary to assign the new compounds. Chemical shifts are in ppm. Data were processed using manufacturer software, raw data were multiplied by shifted exponential window function prior to Fourier transform, and the baseline was corrected using polynomial fitting.

4.2. Synthesis

4.2.1. Preparation of 2-(benzo[d][1,3]dioxol-5-yl)ethyl decanoate (**21**)

To a solution of **20** [39] (45 mg, 0.271 mmol) in *tert*-butyl methyl ether (3 ml), vinyl decanoate (0.061 ml, 0.271 mmol) and *Candida antarctica* lipase (Novozym 435, 200 mg) were added. The reaction was stirred by an orbital shaker for 24 h at 55 °C and then the enzyme was separated and washed. The solvent was concentrated and purified by silica-gel flash column chromatography using a mixture of hexane/ethyl acetate, 9:1 (v/v). Evaporation of the solvents yielded 18.6 mg of the pure product as a white solid (Yield = 22%; R_f = 0.67 (Hexane/ethyl acetate, 4:1)). ¹H NMR (300 MHz, CDCl₃) δ = 6.77–6.47 (m, 3H, CH_{arom}), 5.86 (s, 2H, OCH₂O), 4.16 (t, J = 7.0 Hz, 2H, CH₂O), 2.77 (t, J = 6.9 Hz, 2H, CH₂CH₂O), 2.21 (t, J = 7.5 Hz, 2H, CH₂COO), 1.51 (d, J = 6.2 Hz, 2H, CH₂CH₂COO), 1.19 (s, 12H, CH₂), 0.81 (t, J = 5.4 Hz, 3H, CH₃); ¹³C NMR (75 MHz, CDCl₃) δ = 174.0 (COO), 147.9 (C³_{ipso}O), 146.4 (C⁴_{ipso}O), 131.9 (C¹_{ipso}), 122.0 (C⁶_{arom}), 109.6 (C²_{arom}), 108.5 (C⁵_{arom}), 101.1 (OCH₂O), 65.1 (C₁), 35.1 (C₂), 34.6 (CH₂COO), 32.1 (CH₂), 29.7 (CH₂), 29.5 (CH₂), 29.4 (CH₂), 25.2 (CH₂), 22.9 (CH₂), 14.4 (CH₃); ESI-HRMS [M + Na] calcd for C₁₉H₂₈O₄ 343.1885, found 343.1875.

4.2.2. Preparation of 2-(benzo[d][1,3]dioxol-5-yl)ethyl dodecanoate (**22**)

Following the same procedure used to prepare compound **21**, with vinyl dodecanoate (0.075 ml, 0.289 mmol) as the acylating agent, afforded 71.5 mg as a white solid (Yield = 71%; R_f = 0.65 (Hexane/ethyl acetate, 4:1)). ¹H NMR (300 MHz, CDCl₃) δ = 6.72–6.53 (m, 3H, CH_{arom}), 5.86 (s, 2H, OCH₂O), 4.16 (t, J = 7.0 Hz, 2H, CH₂O), 2.77 (t, J = 7.0 Hz, 2H, CH₂CH₂O), 2.21 (t, J = 7.5 Hz, 2H, CH₂COO), 1.58–1.47 (m, 2H, CH₂CH₂COO), 1.19 (s, 16H, CH₂), 0.81 (t, J = 6.6 Hz, 3H, CH₃); ¹³C NMR (75 MHz, CDCl₃) δ = 174.0 (COO), 147.9 (C³_{ipso}O), 146.4 (C⁴_{ipso}O), 131.9 (C¹_{ipso}), 122.0 (C⁶_{arom}), 109.6 (C²_{arom}), 108.5 (C⁵_{arom}), 101.1 (OCH₂O), 65.1 (C₁), 35.1 (C₂), 34.6 (CH₂COO), 32.2 (CH₂), 29.9 (CH₂), 29.7 (CH₂), 29.6 (CH₂), 29.5 (CH₂), 29.4 (CH₂), 25.2 (CH₂), 22.9 (CH₂), 14.4 (CH₃); ESI-HRMS [M + Na] calcd for C₂₁H₃₂O₄ 371.2198, found 371.2204.

4.2.3. Preparation of 2-(3,4-bis(benzyloxy)phenyl)ethan-1-ol (**23**)

Benzyl bromide (1.62 ml, 13.62 mmol) was added to a mechanically stirred degassed suspension of hydroxytyrosol (1 g, 6.49 mmol) and potassium carbonate (3.59 g, 25.9 mmol) in dry

acetone (20 ml) and the resulting mixture was refluxed for 12 h. The reaction was then filtered through a celite © pad and purified by silica-gel flash chromatography column (hexane/ethyl acetate, 3:1 (v/v) as solvents) obtaining 1.68 g of the desired product as a white solid (Yield = 77%; R_f = 0.16 (hexane: ethyl acetate, 3:1)). The spectroscopic data coincide with the previous report [33].

4.2.4. Preparation of 2-(3,4-bis(4-methoxybenzyloxy)phenyl)ethan-1-ol (**24**)

Following the same procedure used to prepare compound **23**, with hydroxytyrosol (175 mg, 1.135 mmol), potassium carbonate (377 mg, 2.72 mmol) and 4-methoxybenzyl chloride (0.339 ml, 2.497 mmol) in DMF (2 ml), afforded 249.5 mg of the pure product as a white solid (yield = 56%; R_f = 0.35 (Hexane/ethyl acetate, 2:1)). Solvent purification mixture: Hexane/ethyl acetate, 4:1 (v/v). ^1H NMR (500 MHz, CDCl_3) δ = 7.23 (dd, J = 8.7, 2.0 Hz, 4H, H_{PMB}), 6.76 (dd, J = 8.5, 1.8 Hz 5H, H_{PMB} and $\text{H}'2$), 6.70 (d, J = 1.9 Hz, 1H, $\text{H}'5$), 6.61 (dd, J = 8.1, 2.0 Hz, 2H, $\text{H}'6$), 4.94 (s, 2H, $\text{CH}_2^{\text{PMB}}\text{O}$), 4.93 (s, 2H $\text{CH}_2^{\text{PMB}}\text{O}$), 3.69 (s, 3H, CH_3O), 3.69 (s, 3H, CH_3O), 3.66 (t, J = 6.4 Hz, 2H, CH_2OH), 2.64 (t, J = 6.5 Hz, 2H, $\text{CH}_2\text{CH}_2\text{OH}$); ^{13}C NMR (75 MHz, CDCl_3) δ = 159.3 (C_{ipso}), 149.1 (C_{ipso}), 147.9 (C_{ipso}), 131.8 (C_{ipso}), 129.5 (C_{ipso}), 129.4 (C_{ipso}), 129.1 ($\text{C}_{\text{arom}}^{\text{PMB}}$), 129.0 ($\text{C}_{\text{arom}}^{\text{PMB}}$), 121.9 (C_{arom}^6), 116.5 (C_{arom}^2), 115.8 (C_{arom}^5), 113.8 ($\text{C}_{\text{arom}}^{\text{PMB}}$), 71.4 ($\text{C}_{\text{arom}}^{\text{PMB}}\text{H}_2\text{O}$), 71.2 ($\text{C}_{\text{arom}}^{\text{PMB}}\text{H}_2\text{O}$), 63.7 (CH_2OH), 55.3 (CH_3O), 38.7 ($\text{CH}_2\text{CH}_2\text{OH}$); ESI-HRMS [$\text{M} + \text{Na}$] calcd for $\text{C}_{24}\text{H}_{26}\text{O}_5$ 417.1678, found 417.1658.

4.2.5. Preparation of 1,2-bis((4, 4'-methoxybenzyl)oxy)-4-(2-(decyloxy)ethyl)benzene (**27**)

Compound **24** (50 mg, 0.127 mmol) and potassium hydroxide (49.8 mg, 0.887 mmol) were added to DMSO (2 ml) in a round-bottomed flask. 1-Iododecane (0.081 ml, 0.380 mmol) was then added and the reaction was heated to 50 °C and stirred overnight. The reaction mixture was diluted with hydrochloric acid (5%, 25 ml) and extracted with CH_2Cl_2 (3 $\times$ 25 ml). The combined organic layers were then dried with MgSO_4 , filtered and concentrated. The crude was purified by silica-gel flash column chromatography eluting with hexane/ethyl acetate, 3:1 (v/v) obtaining 40.5 mg of the pure product as a white solid (Yield = 60%; R_f = 0.8 (hexane/ethyl acetate, 2:1)). ^1H NMR (300 MHz, CDCl_3) δ = 7.27 (dd, J = 8.5, 4.7 Hz, 4H, H_{PMB}), 6.84–6.73 (m, 6H, H_{PMB} , $\text{H}'2$ and $\text{H}'5$), 6.68–6.61 (m, 1H, $\text{H}'6$), 4.97 (s, 2H, $\text{CH}_2^{\text{PMB}}\text{O}$), 4.95 (s, 2H, $\text{CH}_2^{\text{PMB}}\text{O}$), 3.73 (s, 3H, CH_3O), 3.72 (s, 3H, CH_3O), 3.48 (t, J = 7.3 Hz, 2H, CH_2O), 3.33 (t, J = 6.7 Hz, 2H, CH_2O), 2.70 (t, J = 7.2 Hz, 2H, $\text{CH}_2\text{CH}_2\text{O}$), 1.49 (dd, J = 13.5, 6.8 Hz, 2H, $\text{CH}_2\text{C}_{\text{ipso}}$), 1.18 (s, 16H, CH_2), 0.80 (t, J = 6.6 Hz, 3H, CH_3CH_2); ^{13}C NMR (126 MHz, CDCl_3) δ = 159.3 (C_{ipso}), 159.2 (C_{ipso}), 149.0 (C_{ipso}), 147.6 (C_{ipso}), 132.6 (C_{ipso}), 129.6 (C_{ipso}), 129.5 (C_{ipso}), 129.1 ($\text{C}_{\text{arom}}^{\text{PMB}}$), 129.0 ($\text{C}_{\text{arom}}^{\text{PMB}}$), 121.7 (C_{arom}^6), 116.4 (C_{arom}^2), 115.7 (C_{arom}^5), 113.8 ($\text{C}_{\text{arom}}^{\text{PMB}}$), 113.8 ($\text{C}_{\text{arom}}^{\text{PMB}}$), 71.9 (CH_2O), 71.4 (CH_2O), 71.2 (CH_2O), 71.1 (CH_2O), 55.3 (CH_3O), 35.9 (CH_2), 31.9 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 29.6 (CH_2), 29.5 (CH_2), 29.3 (CH_2), 26.2 (CH_2), 22.7 (CH_2), 14.1 (CH_3CH_2); ESI-HRMS [$\text{M} + \text{Na}$] calcd for $\text{C}_{34}\text{H}_{46}\text{O}_5$ 557.3243, found 557.3237.

4.2.6. Preparation of 3,4-bis(benzyloxy)phenethyl 4-methylbenzenesulfonate (**30**)

Tosyl chloride (0.855 g, 4.49 mmol) was dissolved in CH_2Cl_2 (1.5 ml) in a 10 ml flask under argon atmosphere. Compound **23** (1 g, 2.99 mmol) and triethylamine (1.250 ml, 8.97 mmol) were dissolved in CH_2Cl_2 (1.5 ml) in a second 10 ml flask under argon atmosphere and then cooled to 0 °C. The tosyl chloride solution was added at a rate of 0.75 ml/h (time = 2 h) whilst stirring and the reaction was then left to react for another 21 h at room temperature. The reaction mixture was then washed with water (3 $\times$ 20 ml)

and the organic phases were combined, concentrated and purified by silica gel column eluting with hexane/ethyl acetate, 3:1 (v/v) to give a yellow oil (Yield = 98%). ^1H NMR (300 MHz, CDCl_3) δ = 7.71 (d, J = 8.2 Hz, 2H, H^{Ts}), 7.48 (d, J = 7.0 Hz, 4H, H^{Bn}), 7.39 (dd, J = 16.2, 8.5 Hz, 6H, H^{Bn}), 7.30 (d, J = 8.1 Hz, 2H, H^{Ts}), 6.87 (d, J = 8.1 Hz, 1H, H^{HT}), 6.75 (s, 1H, $\text{H}'2$), 6.67 (d, J = 8.1 Hz, 1H, H^{HT}), 5.14 (d, J = 15.3 Hz, 4H, $\text{CH}_2\text{O}^{\text{Bn}}$), 4.19 (t, J = 7.0 Hz, 2H, CH_2O), 2.89 (t, J = 7.9 Hz, 2H, $\text{CH}_2\text{CH}_2\text{O}$), 2.44 (s, 3H, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ = 149.2 (C_{ipso}), 148.2 (C_{ipso}), 144.9 (C_{ipso}), 137.6 (C_{ipso}), 137.4 (C_{ipso}), 133.3 (C_{ipso}), 130.0 (C_{ipso}), 129.8 (C_{arom}), 128.7 (C_{arom}), 128.1 (C_{arom}), 127.6 (C_{arom}), 127.5 (C_{arom}), 122.1 (C_{arom}), 116.1 (C_{arom}), 115.5 (C_{arom}), 71.6 (CH_2O), 71.6 (CH_2O), 71.0 (CH_2O), 35.1 ($\text{CH}_2\text{CH}_2\text{O}$), 21.9 (CH_3); ESI-HRMS [$\text{M} + \text{Na}$] calcd for $\text{C}_{29}\text{H}_{28}\text{O}_5\text{S}$ 511.1555, found 511.1561.

4.2.7. Preparation of 3,4-bis((4-methoxybenzyl)oxy)phenethyl 4-methylbenzenesulfonate (**31**)

Following the same procedure used to prepare compound **30**, with compound **24** (200 mg, 0.507 mmol) as starting material, tosyl chloride (145 mg, 0.761 mmol) and triethylamine (0.212 ml, 1.521 mmol) in 3 ml of CH_2Cl_2 , afforded 184 mg of pure product as a yellow oil (Yield = 66%, R_f = 0.46 (hexane/ethyl acetate/Acetone, 4:2:1)). ^1H NMR (300 MHz, CDCl_3) δ = 7.52 (d, J = 8.2 Hz, 2H, H^{Ts}), 7.20 (d, J = 7.3 Hz, 4H, H^{PMB}), 7.10 (d, J = 8.0 Hz, 2H, H^{Ts}), 6.74 (dd, J = 8.6, 2.9 Hz, 4H, H^{PMB}), 6.68 (d, J = 8.2 Hz, 1H, $\text{H}'5$), 6.57 (s, 1H, $\text{H}'2$), 6.48 (d, J = 8.1 Hz, 1H, $\text{H}'6$), 4.88 (s, 2H, $\text{CH}_2\text{O}^{\text{PMB}}$), 4.84 (s, 2H, $\text{CH}_2\text{O}^{\text{PMB}}$), 4.01 (t, J = 6.9 Hz, 2H, CH_2O), 3.64 (s, 3H, OCH_3), 3.63 (s, 3H, OCH_3), 2.69 (t, J = 6.9 Hz, 2H, $\text{CH}_2\text{CH}_2\text{O}$), 2.24 (s, 3H, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ = 159.6 (C_{ipso}), 159.6 (C_{ipso}), 149.4 (C_{ipso}), 148.3 (C_{ipso}), 145.0 (C_{ipso}), 133.2 (C_{ipso}), 130.1 (C_{ipso}), 129.8 (C_{ipso}), 129.7 (C_{ipso}), 129.6 (C_{arom}), 129.4 (C_{arom}), 129.3 (C_{arom}), 128.1 (C_{arom}), 122.1 (C_{arom}), 116.4 (C_{arom}), 115.8 (C_{arom}), 114.1 (C_{arom}), 71.5 (CH_2O), 71.4 (CH_2O), 71.1 (CH_2O), 55.5 (CH_3O), 35.1 (CH_3O), 21.9 (CH_3); ESI-HRMS [$\text{M} + \text{Na}$] calcd for $\text{C}_{31}\text{H}_{32}\text{O}_7\text{S}$ 571.1766, found 571.1771.

4.2.8. Preparation of (3,4-bis(benzyloxy)phenethyl)(dodecyl)sulfane (**32**)

To a mechanically stirred two socket flask under argon atmosphere was potassium carbonate (141 mg, 1.025 mmol) suspended in acetonitrile (5 ml) and heated to reflux. In a second and third flask under argon atmosphere was dodecane-1-thiol (0.054 ml, 0.225 mmol) dissolved in acetonitrile (1 ml) and compound **30** (100 mg, 0.205 mmol) dissolved in acetonitrile (2 ml), respectively. The second and third flask solutions were simultaneously added to the refluxing two socket flask and left to react for 48 h. The reaction product was then filtered, concentrated and purified by silica-gel flash chromatography column using a mixture of hexane/ethyl acetate, 9:1 (v/v) as solvents to obtain 50 mg of the desired product as a yellow oil (Yield = 47%, R_f = 0.83 (Hexane: ethyl acetate, 4:1)). ^1H NMR (300 MHz, CDCl_3) δ = 7.37 (dd, J = 8.0, 2.4 Hz, 4H, H^{Bn}), 7.26 (dtd, J = 8.6, 6.7, 1.8 Hz, 6H, H^{Bn}), 6.79 (d, J = 8.2 Hz, 1H, $\text{H}'5$), 6.73 (d, J = 1.9 Hz, 1H, $\text{H}'2$), 6.64 (dd, J = 8.1, 1.9 Hz, 1H, $\text{H}'6$), 5.07 (s, 2H, CH_2O), 5.05 (s, 2H, CH_2O), 2.74–2.64 (m, 2H, CH_2S), 2.64–2.55 (m, 2H, CH_2S), 2.46–2.34 (m, 2H, $\text{C}_{\text{ipso}}\text{CH}_2\text{CH}_2$), 1.52–1.45 (m, 2H, $\text{CH}_2\text{CH}_2\text{S}$), 1.18 (s, 18H, CH_2), 0.80 (t, J = 6.6 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ = 149.3 (C_{ipso}), 147.8 (C_{ipso}), 137.7 (C_{ipso}), 137.6 (C_{ipso}), 134.5 (C_{ipso}), 128.7 (C^{Bn}), 128.0 (C^{Bn}), 128.0 (C^{Bn}), 127.6 (C^{Bn}), 127.6 (C^{Bn}), 121.6 (C_{arom}^6), 116.0 (C_{arom}^2), 115.5 (C_{arom}^5), 71.7 (OCH_2), 71.7 (OCH_2), 36.2 (SCH_2), 34.0 (SCH_2), 32.6 (CH_2), 32.2 (CH_2), 29.9 (CH_2), 29.9 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 29.5 (CH_2), 29.2 (CH_2), 23.0 (CH_2), 14.4 (CH_3). ESI-HRMS [$\text{M} + \text{H}$] calcd for $\text{C}_{34}\text{H}_{46}\text{O}_2\text{S}$ 519.3297, found 519.3295.

4.2.9. Preparation of (3,4-bis((4-methoxybenzyl)oxy)phenethyl)(dodecyl)sulfane (**33**)

Following the same procedure used to prepare compound **32**, with compound **31** (100 mg, 0.182 mmol), potassium carbonate (126 mg, 0.911 mmol) and dodecane-1-thiol (0.048 ml, 0.2 mmol), afforded 97 mg of pure product as a yellow oil (Yield = 92%, R_f = 0.3 (Hexane: ethyl acetate, 6:1)). ^1H NMR (300 MHz, CDCl_3) δ 7.26 (dd, J = 8.4, 4.6 Hz, 4H, H^{PMB}), 6.86–6.75 (m, 5H, H^{PMB} and $\text{H}'5$), 6.72 (d, J = 1.5 Hz, 1H, $\text{H}'2$), 6.63 (d, J = 8.1 Hz, 1H, $\text{H}'6$), 4.97 (s, 2H, CH_2O), 4.95 (s, 2H, CH_2O), 3.72 (s, 3H, CH_3O), 3.72 (s, 3H, CH_3O), 2.75–2.65 (m, 2H, CH_2S), 2.65–2.56 (m, 2H, CH_2S), 2.42 (t, J = 7.4 Hz, 2H, $\text{C}_{\text{ipso}}\text{CH}_2\text{CH}_2$), 1.57–1.42 (m, 2H, $\text{CH}_2\text{CH}_2\text{S}$), 1.18 (s, 18H, CH_2), 0.80 (t, J = 6.5 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ = 159.6 (C_{ipso}), 159.5 (C_{ipso}), 149.3 (C_{ipso}), 147.9 (C_{ipso}), 134.5 (C_{ipso}), 129.8 (C_{ipso}), 129.7 (C_{ipso}), 129.4 (C_{arom}), 129.3 (C_{arom}), 121.6 (C_{arom}), 116.3 (C_{arom}), 115.9 (C_{arom}), 114.1 (C_{arom}), 71.6 (CH_2O), 71.6 (CH_2O), 55.5 (CH_3O), 36.2 (SCH_2), 34.1 (SCH_2), 32.6 (CH_2), 32.2 (CH_2), 30.0 (CH_2), 29.9 (CH_2), 29.9 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 29.6 (CH_2), 29.2 (CH_2), 23.0 (CH_2), 14.4 (CH_3); ESI-HRMS [$\text{M} + \text{H}$] calcd for $\text{C}_{36}\text{H}_{50}\text{O}_4\text{S}$ 579.3508, found 579.3486.

4.2.10. Preparation of 4-(2-isothiocyanatoethyl)benzene-1,2-diol (**36**)

Triethylamine (1.617 ml, 11.60 mmol) was added to a stirring suspension of dopamine hydrochloride **34** (2 g, 10.55 mmol) in THF (26 ml). Methanol (20.80 ml) was slowly added to dissolve the dopamine hydrochloride, forming a clear solution. Carbon disulfide (3.17 ml, 52.7 mmol) was added to the mixture which was stirred for 1 h at 28 °C under argon atmosphere. The yellowish reaction mixture was cooled to 0 °C and hydrogen peroxide (0.942 ml, 30% in water) was added drop wise (30%). The solution was immediately acidified with concentrated hydrochloric acid and then concentrated *in vacuo*. The resulting mixture was filtered and rinsed with water. The filtrate was extracted with ethyl acetate and the combined organic layers were dried over MgSO_4 , filtered, and concentrated affording 740 mg of crude product as an oil (Yield = 36%). The spectroscopic data coincide with the previous report [40,41].

4.2.11. Preparation of 1-(3,4-dihydroxyphenethyl)-3-decylthiourea (**37**)

Compound **36** (355.7 mg, 1.822 mmol) and decan-1-amine (0.364 ml, 1.820 mmol) were dissolved in pyridine (10 ml) to give a yellow solution. The reaction was stirred for 3 h and triethylamine (0.254 ml, 1.822 mmol) was then added. The reaction was stirred for 1 h and then concentrated, diluted with 20 ml of hydrochloric acid (5%) and extracted with ethyl acetate (3 $\times$ 20 ml). The combined organic phases were concentrated and purified via silica-gel flash column chromatography while eluting with hexane/ethyl acetate, 2:1 (v/v) to give 338 mg of the pure product as a yellow solid (Yield = 53% R_f = 0.2 (hexane/ethyl acetate, 2:1)). ^1H NMR (300 MHz, CDCl_3) δ = 6.72 (d, J = 8.1 Hz, 2H, C^2_{arom} and C^5_{arom}), 6.56 (d, J = 7.9 Hz, 1H, C^6_{arom}), 3.64 (t, J = 7.1 Hz, 2H, CH_2N), 3.39 (m, 2H, CH_2N), 2.74 (t, J = 7.1 Hz, 2H, $\text{CH}_2\text{C}_{\text{ipso}}$), 1.55 (t, 2H, $\text{CH}_2\text{CH}_2\text{N}$), 1.31 (s, 16H, CH_2), 0.91 (t, J = 6.7 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ = 202.0 (NCN), 145.1 (C^4_{arom}), 143.6 (C^3_{arom}), 130.8 (C^1_{arom}), 120.0 (C^6_{arom}), 115.8 (C^2_{arom} or C^5_{arom}), 115.3 (C^2_{arom} or C^5_{arom}), 34.6 (CH_2), 32.0 (CH_2), 29.6 (CH_2), 29.3 (CH_2), 26.9 (CH_2), 22.6 (CH_2), 13.4 (CH_3); ESI-HRMS [$\text{M} + \text{H}$] calcd for $\text{C}_{19}\text{H}_{32}\text{N}_2\text{O}_2\text{S}$ 353.2252, found 353.2263.

4.2.12. Preparation of 1-(3,4-dihydroxyphenethyl)-3-dodecylthiourea (**38**)

Following the same procedure used to prepare compound **37**, from dodecan-1-amine (0.42 ml, 1.822 mmol), afforded 392.7 mg of the pure product as a yellow solid (Yield = 57% R_f = 0.5 (hexane/

ethyl acetate, 1:1)). ^1H NMR (300 MHz, CDCl_3) δ = 6.68 (d, J = 8.0 Hz, 2H, C^2_{arom} and C^5_{arom}), 6.53 (dd, J = 8.0, 2.0 Hz, 1H, C^6_{arom}), 3.61 (s, 2H, CH_2N), 3.30 (dt, J = 3.2, 1.6 Hz, 2H, CH_2N), 2.71 (t, J = 7.2 Hz, 2H, $\text{CH}_2\text{C}_{\text{ipso}}$), 1.56–1.46 (m, 2H, $\text{CH}_2\text{CH}_2\text{N}$), 1.29 (s, 18H, CH_2), 0.89 (t, J = 6.2 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ = 196.0 (NHCNH), 146.3 (C^4_{arom}), 144.5 (C^3_{arom}), 131.9 (C^1_{arom}), 121.1 (C^6_{arom}), 116.9 (C^2_{arom} or C^5_{arom}), 116.4 (C^2_{arom} or C^5_{arom}), 35.7 (CH_2), 35.7 (CH_2), 33.1 (CH_2), 30.7 (CH_2), 30.4 (CH_2), 28.0 (CH_2), 23.7 (CH_2), 14.4 (CH_3); ESI-HRMS [$\text{M} + \text{H}$] calcd for $\text{C}_{21}\text{H}_{36}\text{N}_2\text{O}_2\text{S}$ 381.2576, found 381.2563.

4.3. Biological assays

4.3.1. *In vitro* antitrypanosomal activity

Bloodstream forms (BSF) of *T. brucei* brucei 'single marker' S427 (S16) were grown at 37 °C, 5% CO_2 in HMI-9 medium supplemented with 10% (heat-inactivated fetal bovine serum, hiFBS). Drug susceptibility assay was performed as described in Ref. [42]. Briefly, parasites (1×10^4 BSF per ml) were incubated in 96-well plates with increasing concentration of drugs/compounds for 72 h at 37 °C, 5% CO_2 in culture medium. Cell proliferation was determined using the alamarBlue[®] assay [43]. Fluorescence was recorded with an Infinite[®] F200 microplate reader (Tecan Austria GmbH, Austria) equipped with 550 and 590 nm filters for excitation and emission wavelengths, respectively.

4.3.2. *In vitro* antileishmanial activity

The experiments of drug susceptibility on *L. donovani* were carried out in axenic amastigotes and intracellular amastigotes. Axenic *L. donovani* MHOM/ET/67/HU3 amastigote parasites were grown in Schneider medium supplemented with 20% hiFBS, pH 5.4 at 37 °C and 5% CO_2 . 10^6 axenic amastigotes/ml in a 96-well plate were incubated with increasing concentrations of compounds for 72 h at 37 °C, followed by a resazurin-based assay, as described [44]. Additionally, we use intracellular amastigotes of a *L. donovani* (MHOM/ET/67/HU3) line with luciferase gene integrated into the parasite genome [45]. The susceptibility of intracellular *L. donovani* amastigotes to synthesized compounds was determined using the Luciferase Assay System Kit (Promega, Madison, Wis.) as previously described [44]. Briefly, macrophage-differentiated-THP-1 cells were plated at a density of 3×10^4 macrophages/well in 96-well white polystyrene microplates and were infected at a macrophage/parasite ratio of 1:10 with late-stage promastigotes. Infected cell cultures were incubated at different compound concentrations in RPMI 1640 medium plus 10% hiFBS at 37 °C with 5% CO_2 for 72 h and then lysed. Luminescence intensity was measured as indicative of the intracellular parasite growth, according to the instructions of the supplier.

4.3.3. Cytotoxicity assay

Human myelomonocytic cell line THP-1 were grown at 37 °C and 5% CO_2 in RPMI-1640 supplemented with 10% hiFBS, 2 mM glutamate, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. 3×10^4 cells/well in 96-well plates were differentiated to macrophages with 20 ng/ml of PMA treatment for 48 h followed by 24 h of culture in fresh medium [44]. MRC-5 cells, a SV-40 transformed human fetal lung fibroblast cell line, were maintained at 37 °C and 5% CO_2 in DMEM supplemented with 10% hiFBS, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cells were harvested by treatment with 0.05% (w/v) trypsin plus 0.48 mM EDTA for 5 min, diluted to 4×10^4 cell/ml in 96-well plates and incubated at 37 °C and 5% CO_2 before toxicity assay [44]. Cellular toxicity of all compounds was determined using the colorimetric MTT-based assay after incubation at 37 °C for 72 h in the presence of increasing concentrations of compounds [44].

The results are expressed as EC₅₀ values, as the concentration of compound that reduce cell growth by 50% versus untreated control cells.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejmech.2016.04.047>.

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