Conjugates of 2,4-Dihydroxybenzoate and Salicylhydroxamate and Lipocations Display Potent Anti-parasite Effects by Efficiently Targeting the *Trypanosoma brucei* and *Trypanosoma congolense* Mitochondrion

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ABSTRACT

We investigated a chemical strategy to boost the trypanocidal activity of 2,4-dihydroxybenzoic acid (2,4-DHBA)- and salicylhydroxamic acid (SHAM)-based trypanocides with triphenylphosphonium and quinolinium lipophilic cations (LC). Three series of LC conjugates were synthesized that were active in the submicromolar (**5a**–**d** and **10d**–**f**) to low nanomolar (**6a**–**f**) range against wild-type and multi-drug resistant strains of African trypanosomes (*Trypanosoma brucei brucei* and *T. congolense*). This represented an improvement in trypanocidal potency of at least 200-fold, and up to >10,000-fold, compared with the non-LC coupled parent compounds 2,4-DHBA and SHAM. Selectivity over human cells was >500 and reached >23,000 for **6e**. Mechanistic studies showed that **6e** did not inhibit the cell cycle but affected parasite respiration in a dose-dependent manner. Inhibition of the trypanosome alternative oxidase (TAO) and the mitochondrial membrane potential was also studied for selected compounds. We conclude that effective mitochondrial targeting greatly potentiated the activity of these compound series.

INTRODUCTION

Human African trypanosomiasis (HAT) is a parasitic disease caused by two subspecies of trypanosomes, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, which are transmitted by the bite of infected tsetse flies.[1] Other species and subspecies of trypanosomes infect cattle and cause enormous economical loss in the tsetse belt in Africa.[2] Because the therapeutic options are limited and threatened by drug resistance,[3] and HAT is a fatal disease if left untreated, the search for new safe and effective trypanocidal drugs remains an important goal in tropical medicine.

In contrast to mammalian cells, which contain hundreds of mitochondria per cell, trypanosomes possess a single mitochondrion that is involved in vital cellular functions including maintenance and expression of genetic information, energy metabolism, RNA editing, Fe-S cluster biogenesis, etc.[4] Hence, this essential organelle represents a good chemotherapeutic target for the development of trypanocidal drugs.[5-7] Among the many validated mitochondrial targets of *T. brucei* (e.g. kDNA and topoisomerases, tRNA import, fatty acid biosynthesis),[8] the mitochondrial respiration of the parasite is a particularly attractive target.[9] In effect, during their life-cycle, trypanosomes adapt their energy metabolism to the availability of nutrients in their environment.[10] Hence, procyclic forms of *T. brucei* have a fully functional respiratory chain and synthesize ATP by oxidative phosphorylation in the mitochondrion. In contrast, bloodstream trypomastigotes of *T. brucei* (i.e. the form present in the mammalian host) rely exclusively on glycolysis for energy production as they have no oxidative phosphorylation, no cytochrome-mediated electron transport systems, and no tricarboxylic acid cycle.[4, 11] Clarkson et al have shown that respiration of *T. b. brucei* trypomastigotes is dependent on a plant-like alternative oxidase known as the trypanosome alternative oxidase (TAO), which is localized in the inner mitochondrial

membrane.[12] Because it is essential to the viability of bloodstream trypanosomes, and because it has no counterpart in the mammalian host, TAO is considered an excellent target for chemotherapy.[9, 13-15]

Earlier reports in the literature have shown that very simple chemical structures containing the 2,4-dihydroxybenzoic acid (2,4-DHBA, **1**) and benzhydroxamic acid (SHAM) scaffolds were trypanocidal in the low micromolar range against *T. brucei* (Chart 1).[16-21] Although these compounds did inhibit the respiration and growth of the parasite in a dose-dependent manner, an effect that was thought to be related to the inhibition of TAO, their trypanocidal activity proved disappointing - probably because the inhibitors did not effectively cross the inner mitochondrial membrane to reach their target.

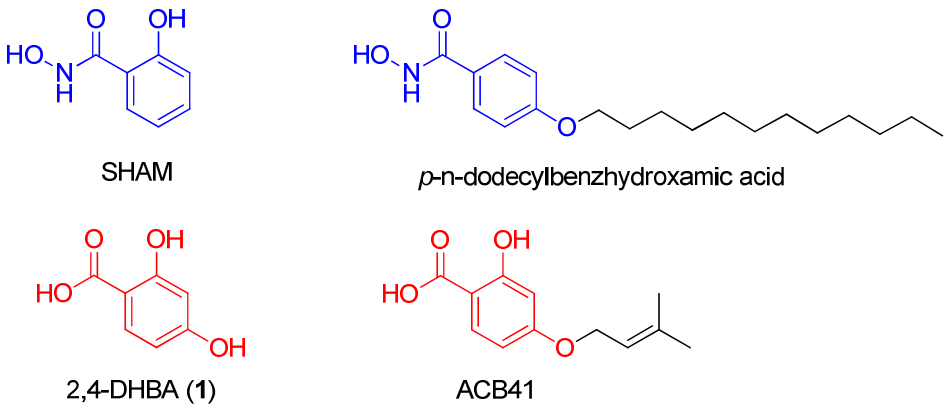


Chart 1. Examples of 2,4-dihydroxybenzoic acid and benzhydroxamic acid derivatives showing low micromolar activity against *T. brucei*.

In the present work, we investigated a strategy to enhance the antitrypanosomal potency of this class of compounds based on the conjugation of these trypanocides with a mitochondrion-targeting lipophilic cation (LC).[22] The triphenylphosphonium (TPP) cation is one of the most successful LC for mitochondria targeting,[23-25] and the use of

the "TPP strategy" to deliver trypanocidal drugs to the mitochondrion of trypanosomes has recently been demonstrated.[7, 26] Lipophilic cations can cross lipid bilayers by non-carrier mediated transport and accumulate specifically into mitochondria driven by the plasma and mitochondrial transmembrane potentials.[27-30] The strong accumulation of dications by the charged mitochondria allows the targeting of its various essential functions with relatively low extracellular drug concentrations.[6, 31, 32] In addition, LCs can cross the blood–brain barrier (BBB) and generate therapeutically effective concentrations in the brain,[24] which is particularly relevant for the treatment of late-stage sleeping sickness. Importantly, TPP-conjugates appear to be generally safe. For instance, the TPP-coupled antioxidant MitoQ was safely administered as a daily oral tablet to patients for a year in a controlled study with human volunteers.[33]

In this paper, we report the synthesis and characterization of three series of LC conjugates based on the 2,4-DHBA and SHAM scaffolds (Figure 1). Two different cationic groups were tested as mitochondrion targeting moieties: the bulky TPP cation and the flat heterocyclic 1-quinolinium cation. The position of conjugation of the lipophilic moiety via the benzoic acid group was motivated by the precedents in the literature showing that benzoate derivatives of related compounds, e.g. 3,4-dihydroxybenzoic acid, are better TAO inhibitors and have superior activity against trypanosomes than acid derivatives.[19, 21, 34] Linkers from 8 to 16 CH_2 units were chosen based on previous studies with esters of 3,4-dihydroxybenzoic acid[18, 21] showing that long methylene chains are preferred for higher activity against *T. brucei*. The compounds were evaluated in vitro against multiple African trypanosome species (*T. b. brucei*, *T. congolense*), including wild-type and multi-drug resistant strains. To assess whether these compounds do indeed target the parasite's mitochondrion, their effects on

the mitochondrial membrane potential, cell cycle, and parasite respiration were also evaluated.

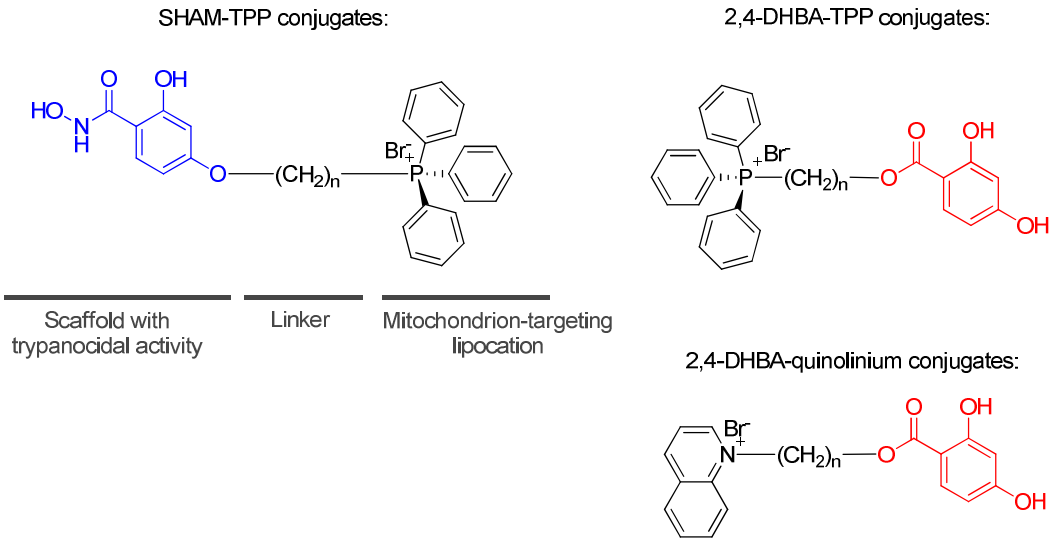


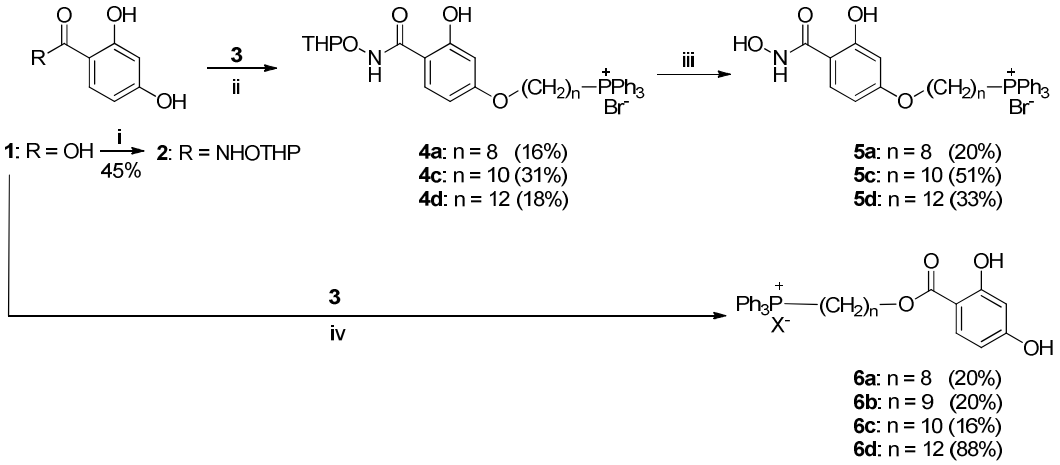
Figure 1. Design and general structure of the SHAM and 2,4-DHBA conjugates

RESULTS

Chemistry. The hydroxamic acid derivatives **5a**–**d** were synthesized in 3 steps starting from 2,4-dihydroxybenzoic acid (Scheme 1). Coupling of the THP-protected hydroxylamine[35] (THPO-NH_2) with 2,4-DHBA (**1**) using EDC/HOBt as coupling agents and microwave irradiation (MWI) yielded **2** which was isolated by silica chromatography (45%). Selective substitution of **2** with dibromoalkanes to get the 4-substituted bromoalkyl hydroxamic acid derivatives proved very tricky leading to very low yields of the desired product. Hence, we decided to use a convergent synthesis to prepare **4a**–**d**. The THP-protected hydroxamate **2** reacted under mildly basic conditions ($NaHCO_3$/CH_3CN/65 ºC/72h) with bromoalkyltriphenylphosphonium salts (**3a**–**d**) synthesized previously[36] to give **4a**–**d**. Addition of a catalytic amount of sodium iodide was useful to speed up this sluggish reaction. Removal of the THP group by acidolysis

using a catalytic amount of *p*-toluenesulfonic acid in methanol gave the target compounds **5a**–**d**. The 2,4-dihydroxybenzoate derivatives **6a–d** were obtained in one step by reacting 2,4-DHBA with **3** using a similar protocol as for the hydroxamate derivatives (but without NaI).

Scheme 1. Synthesis of Salicylhydroxamate and 2,4-Dihydroxybenzoate Derivatives 5a–d and 6a–d[a]

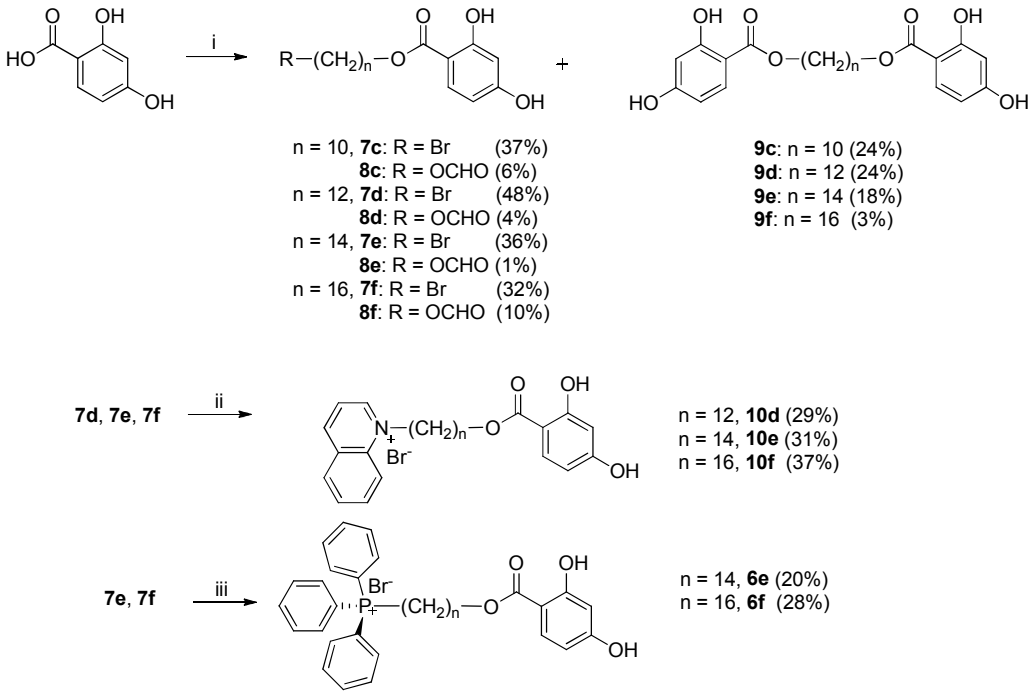


[a]Reagents and conditions: (i) $THPONH_2$, EDC, NMM, HOBt, DMF, MWI, 120 ºC, 30 min; (ii) Br-$(CH_2)_n$-$PPh_3^+Br^-$ (**3a–d**: n = 8, 10, 12), $NaHCO_3$, NaI, CH_3CN, 65 ºC, 3 days; (iii) TsOH (cat.), MeOH, rt; (iv) Br-$(CH_2)_n$-$PPh_3^+Br^-$ (**3a–d**: n = 8, 9, 10, 12), $NaHCO_3$, CH_3CN, 5 min at 120 ºC then 65 ºC, 3 days.

Alternatively, the quinolinium and phosphonium analogues were obtained by a route involving the synthesis of the bromoalkyl benzoate precursors **7c**–**f** (Scheme 2). As expected, the reaction of 1 equivalent of 2,4-DHBA with 1 equivalent of dibromoalkane in the presence of 1 equivalent of sodium bicarbonate led to a nearly 50/50 mixture of bromoalkyl benzoate **7c**–**f** and the dimeric compound **9c**–**f.** A minor formyl by-product (**8c**–**f**) was also isolated and characterized. This compound most probably results from

the formylation of **7c**–**f** in the presence of DMF as reaction solvent.[37] The reaction of **7d**–**f** in the presence of quinoline or triphenylphosphine in CH_3CN at 80 ºC yielded the quinolinium (**10d**–**f**) and phosphonium compounds (**6e**–**f**).

Scheme 2. Synthesis of 2,4-Dihydroxybenzoate Derivatives 10d–f and 6e–f[a]



[a]Reagents and conditions: (i) Br-$(CH_2)_n$-Br (n = 10, 12, 14, and 16; 1 equiv.), $NaHCO_3$, DMF, 65 ºC; (ii) Quinoline, CH_3CN, 80 ºC; (iii) PPh_3, CH_3CN, 80 ºC.

Biology

In vitro activity against *T. b. brucei* and *T. congolense* wild type and resistant strains. SHAM and 2,4-DHBA were active in the micromolar range against bloodstream trypomastigotes of *T. b. brucei* s427 (WT) (Table 1). The SHAM–TPP conjugates (**5a–d**) and the 2,4-DHBA-quinolinium conjugates (**10d–f**) displayed submicromolar EC_{50} values (0.1 to 0.4 μM) against this trypanosome strain. In contrast, the 2,4-DHBA–TPP derivatives (**6a**–**f**) were 10- to 66-times more active, with low to

mid-nanomolar EC_{50} values (0.0012 to 0.073 μM), i.e. in the same range as the reference drugs pentamidine and diminazene (Table 1). There was no absolute correlation as regards to the influence of the linker length on the activity against *T. brucei* but linkers with more than 8 methylene units seemed to be favored: 12 > 10 > 8 methylene units for **5a**–**d**, 10 > 14 > 12 ≈ 9 > 16 >> 8 for **6a**–**f**, and 14 ≈ 16 > 12 for **10d**–**f**, which is in agreement with previous reports.[18, 21] The synthetic intermediates **7d**, **7f**, and **8c**, lacking the TPP or 1-quinolinium cations, displayed micromolar range activities similar (**7d**) or approximately 2-fold lower than 2,4-DHBA (**7f**, **8c**) against *T. brucei*. This shows that the LC-carrier moiety greatly enhances the trypanocidal activity of the compounds whereas the linker does not seem to contribute favorably to the trypanocidal activity of the 2,4-DHBA scaffold.

Very little difference in activity was observed between WT and B48 cell lines, with resistance factors (RF) consistently close to 1 (Table 1). In general, the compounds' cytotoxicity against human cell lines was low (>200 μM), except for **6a**–**e** which displayed a cytostatic (as opposed to cytotoxic) effect in the low micromolar range. In most cases the selectivity indices (SI) were >500, and **6e** and **6f** reached SI>23,000.

[Table 1]

The compounds were generally less active against *T. congolense* strain IL3000 grown in culture (from 5- to 140-fold). However, with EC_{50} values for the best compounds (**6c**–**6f**) in the submicromolar range (Table 1), close to or better than that of the widely used[2] reference drug diminazene (EC_{50} = 0.15 μM), several compounds showed significant potential for use against this species.

Mode of action studies

Dynamics of trypanocidal action, mitochondrial membrane potential and DNA content. Propidium iodide assays were performed to monitor the effects of two representative 2,4-DHBA–LC conjugates (i.e. TPP and quinolinium derivatives with the same linker and high activity/selectivity profile) on *T. b. brucei* in real time. The effects of **6e** and **10e** on *T. brucei* s427 trypomastigotes was dose-dependent; at doses near their EC_{50} values the compounds increased rates of PI influx only marginally compared with untreated control cells, over the 6 hours of the experiments. For both compounds, at ~3-fold of their EC_{50} values (1.5 nM and 100 nM, respectively), killing of the trypanosomes was complete in approximately 4 h (Figure 2). These results show that there is no immediate disruption of the plasma membrane from the administration of these nanomolar concentrations of LC conjugates.

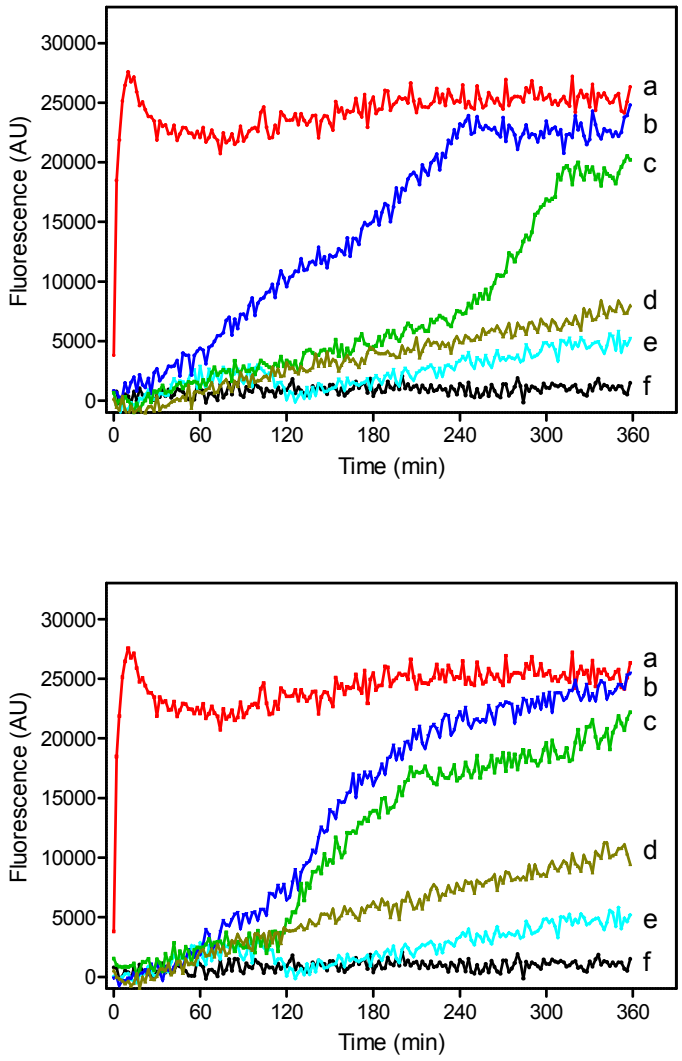


Figure 2. Viability assay for **6e** and **10e**. Top panel: cells were incubated with 10 μM digitonin (a) or with test compound **6e** at 6× (b), 4× (c) or 2× EC_{50} (d), or no test compound (e) in the presence of 9 μM propidium iodide. Background fluorescence was recorded for wells containing media only (f). Lower panel: parallel experiment with compound **10e**, at 3× (b), 2× (c) and 1× EC_{50} (d). An increase in fluorescence recorded as arbitrary units (A.U.) correlates with increased permeability to propidium iodide, reflecting membrane integrity.

If the lipophilic cations are, as designed, accumulating in the *T. brucei* mitochondrion, it is expected that this will impact on the mitochondrial membrane potential Ψ_m, as the

result of (1) the accumulation of cations in the mitochondrial matrix, and (2) disruption of mitochondrial functions involved in maintaining the ion gradients. Similar effects have been shown for various diamidines, choline-derived dications and bisphosphonium compounds.[5, 6, 31, 38] We thus determined Ψ_m by flow cytometry and found that **6e** indeed rapidly depolarized the mitochondrial membrane, as measured by the fluorescent probe TMRE. Figure 3 shows the percentage of cells in the population that accumulated >200 artificial units of TMRE fluorescence, which was set at 50% for the 0 time point of untreated cells; any increase in fluorescence such as induced by troglitazone signifies a hyperpolarization of the mitochondrial membrane and a decrease in fluorescence indicates depolarization. It is thus clear that **6e** rapidly decreases Ψ_m, although not as rapidly as the potassium ionophore valinomycin (Fig. 3). The reduced fluorescence is not simply the result of an increasing percentage of the cells dying, as can be seen from the narrow, monophasic peaks in the histograms of TMRE fluorescence for the individual determinations (Fig. S1), and thus represents a genuine collapse of Ψ_m that was highly reproducible and remarkably homogeneous throughout the cell population. The observed homogeneity is consistent with our expectations, in that these LC conjugates are believed to diffuse passively through the applicable membranes, and their rate of accumulation is therefore not subject to variable levels of expression of transport proteins as is often the case with less lipophilic drugs.

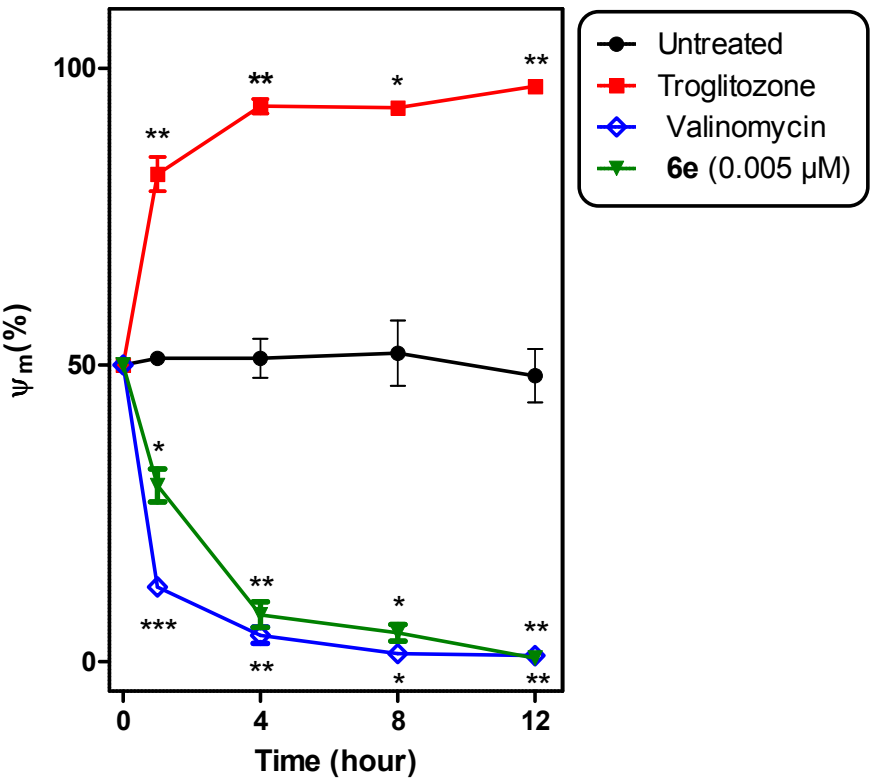


Figure 3. Mitochondrial membrane potential (%Ψ_m) of treated and untreated *T. b. brucei* s427 WT cells. Data points represent average and SEM of flow cytometric determinations of TMRE fluorescence and are expressed as the percentage of cells that exhibit >200 A.U. of fluorescence intensity in the analyzed populations. Valinomycin and Troglitazone were used as controls for hyperpolarization and depolarization, respectively. Statistically significant differences from untreated control populations were assessed using an unpaired Student's t-test; *, P<0.05; **, P<0.01; ***, P<0.001.

We have recently shown that treatment of *T. b. brucei* trypomastigotes with a different class of lipophilic cations, consisting of symmetrical bisphosphonium compounds, led to a rapid inhibition of the cell cycle by preventing initiation of S-phase; these compounds were shown to inhibit the mitochondrial F_oF_1 ATPase.[6] We thus investigated whether the LC conjugates might have a similar effect on the cell cycle. Figure S2 shows that **6e** did not exhibit a cell cycle-specific effect, as 0.005 µM of **6e**

had no effect on the percentage of cells in G1, S or G2 phase after as much as 24 h, while the lipophilic bisphosphonium compounds CD38 and AHI-9 significantly reduced the percentage of S-phase cells after 8 and 12 h of incubation, respectively.[6]

Effect on parasite respiration. In order to determine whether the antitrypanosomal activity displayed by these compounds might be related to the inhibition of parasite respiration, the susceptibility assays were repeated in the presence of 5 mM glycerol, which inhibits the *T. brucei* anaerobic ATP production pathway[39], which is essential when the aerobic respiration is disabled. During anaerobiosis or when TAO is inhibited, glycerol kinase (GK) becomes essential to BSF trypanosomes because it contributes to glycolysis via a thermodynamically unfavorable mechanism consisting in the catalysis of the transphosphorylation of ADP with a phosphoryl group from glycerol 3-phosphate (G3P), forming ATP (i.e. net production of 1 mole of ATP per glucose molecule consumed) and glycerol.[40-43] Hence, the co-administration of TAO inhibitors and glycerol is known to effectively kill the parasites[44] because the added glycerol competes with G3P as GK substrate, and thus inhibits anaerobic ATP production by mass action.[40]

Co-incubation with glycerol significantly ($P<0.05$) increased the trypanocidal activities of **6e**, **6f**, **10d**, **10f**, and SHAM, whereas it had no effect on the efficacy of control drugs pentamidine and diminazene (Table 2). This result indicates that the aerobic glycolytic pathway may be involved in the MOA of these test compounds.

As respiration of BSF trypanosomes is entirely dependent on TAO as the terminal oxidase, we next investigated whether the compounds were inhibitors of purified rTAO enzyme[45] in the ubiquinol oxidase assay. Unlike previously published inhibitor studies,[45] we used the physiological form of the enzyme, without its N-terminal 25

amino acid mitochondrial targeting sequence (MTS), which is cleaved off in the mitochondrion.[46] Two compounds (**6e**, **10e**) inhibited rTAO, with IC_{50} values of 1.46 and 1.36 μM, respectively. A further three compounds (**6f**, **10d**, **10f**), SHAM (IC_{50} = 5.93 μM), and 2,4-DHBA (IC_{50} = 120 μM) displayed IC_{50} values >5 μM.

Some of the compounds were further tested on a *T. b. brucei* line overexpressing TAO, as a further test for activity through inhibition of TAO, as it is not possible to delete the *TAO* gene, or even reduce its expression by RNAi. Of the compounds tested, only **6e** and SHAM were significantly less effective against this cell line than against the wild-type control, by 2.6 ($P=0.0001$) and 1.6-fold ($P<0.01$), respectively - a further indication of TAO involvement in the MOA of both compounds. However, the level of overexpression was really modest, as established by qPCR (Figure S3), owing to the already very high expression level of TAO in *T. brucei* trypomastigotes.

Compound **3c**, which lacks either a SHAM or a 2,4-DHBA group, but does have a mitochondrial targeting group (TPP), had no effect on rTAO activity at 10 μM, and displayed no differential effects against *T. b. brucei* in the presence of 5 mM glycerol, or against the AOX-OE line, indicating that indeed it was not an inhibitor of TAO. However, with an EC_{50} of just 1.8 ± 0.4 nM and a selectivity >3000 it might be worth investigating its mode of action separately.

Table 1. EC_{50} values (μM) against Wild Type and Resistant Strains of *T. b. brucei*, *T. congolense*, and Cytotoxicity against Human Cells ($CC_{50,}$ μM).

Cmpd	***T. brucei* WT**[a]	SI[b]	***T. brucei* B48**[c]	RF[d]	***T. congolense* WT**[e]	SI[f]	**Human cells**[g,h]
3c	0.0018 ± 0.0004	3339	0.0012 ± 0.0001	0.7	0.95 ± 0.05	6.3	6.01 ± 1.47[g]
5a	0.40 ± 0.14	>1000	nd[i]		27.2 (n=1)	>14.7	>400[g]
5c	0.20 ± 0.04	>2043	nd		nd		>400[g]
5d	0.14 ± 0.01	>2857	nd		46.4 (n=1)	8.6	>400[g]
6a	0.073 ± 0.003	106	0.068 ± 0.001	0.94	4.3 ± 1.6	1.8	7.73 ± 0.79[g]
6b	0.0059 ± 0.0025	789	0.0074 ± 0.0006	1.24	0.41 ± 0.13	11.5	4.68 ± 1.26[g]
6c	0.0013 ± 0.0010	1768	0.0011 ± 0.0001	0.86	0.18 ± 0.02	13.2	2.33 ± 0.53[g]
6d	0.0012 ± 0.0012	1334	0.0012 ± 0.0002	0.96	0.042 ± 0.003	5.5	1.65 ± 0.42[g]
6e	0.0015 ± 0.0003	23378	0.0012 ± 0.0001	0.78	0.061 ± 0.005	982	59.7± 6.4[h]
6f	0.009 ± 0.001	27714	0.008 ± 0.001	0.91	0.122 ± 0.006	2038	249 ± 66[h]
7d	14.5 ± 1.0	>28	14.9 ± 1.0	1.03	52.1 ± 3.7	>7.7	>400[g]
7f	45.7 ± 1.5	>9	49.0 ± 0.6	1.07	>100		>400[g]
8c	31.8 ± 0.9	>12	22.1 ± 4.1	0.69	>100		>400[g]
10d	0.33 ± 0.01	609	0.347 ± 0.002	1.05	7.0 ± 0.3	28.7	202 ± 7[h]
10e	0.10 ± 0.01	1657	0.125 ± 0.015	1.21	3.8 ± 0.2	45.3	172 ± 14[h]
10f	0.14 ± 0.01	2410	0.14 ± 0.01	1.01	3.0 ± 0.1	115	345 ± 24[h]
2,4-DHBA[j]	17.1 ± 1.0		nd		nd		nd
SHAM[k]	38.7 ± 4.8		nd		nd		nd
Pentamidine	0.0028 ± 0.0003		0.94 ± 0.03	98	nd		nd

Diminazene	0.065 ± 0.007	0.78 ± 0.04	0.151	
PAO[l]	0.0011 ± 0.00003			0.036 ± 0.004[f] 0.29 ± 0.02[g]

[a]Trypomastigotes of *T. b. brucei* s427 (n ≥ 4). [b]Selectivity index (SI) = CC_{50}/EC_{50} (*T.brucei.* WT). [c]*T. b. brucei* strain resistant to pentamidine, diminazene, and melaminophenyl arsenicals. [d]Resistance factor relative to WT. [e]Trypomastigotes of *T. congolense* IL3000 (n = 2). [f]Selectivity index (SI) = CC_{50}/EC_{50} (*T. congolense* WT). [g]Cytostatic activity on human embryonic kidney cells; no cytotoxic activity was observed up to 50 μM (n = 3). [h]Cytotoxicity on Human Foreskin Fibroblast (HFF) cells (n = 2). [i]Not determined. [j]2,4-Dihydroxybenzoic acid. [k]Salicylhydroxamic acid. [l]Phenylarsine oxide.

Table 2. EC_{50} values (μM) against *T. b. brucei* WT in the presence of glycerol (5 mM)

Cmpd	*T. b. brucei*[a]	RF[b]	t-test[c]
5d	0.158 ± 0.009	1.13	0.187
6a	0.084 ± 0.015	1.16	0.330
6b	0.005 ± 0.002	0.88	0.857
6c	0.002 ± 0.0001	1.26	0.829
6d	0.0012 ± 0.0003	1.01	0.996
6e	0.0008 ± 0.0001	0.55	0.116
6f	0.005 ± 0.0005	0.60	0.079
10d	0.23 ± 0.01	0.69	$2.76E^{-3}$
10e	0.078 ± 0.012	0.75	0.190
10f	0.089 ± 0.003	0.62	0.024
2,4-DHBA[d]	19 ± 1	1.11	0.261
SHAM[e]	7.0 ± 0.3	0.18	$1.36E^{-11}$
Pentamidine	0.004 ± 0.0006	1.32	0.135
Diminazene	0.063 ± 0.002	0.97	0.838

[a]Trypomastigotes of *T. b. brucei* (n = 3). [b]Resistance factor relative to WT without glycerol: RF = EC_{50} (in the presence of glycerol)/EC_{50} (without glycerol). [c]Unpaired Student's t-test compairing EC_{50} values against the WT strain in the presence and absence of 5 mM glycerol. [d]2,4-Dihydroxybenzoic acid. [e]Salicylhydroxamic acid.

The effect of **6e** on oxygen consumption by *T. b. brucei* bloodstream trypomastigotes was tested using a fluorescent oxygen reporter probe, and it was found to inhibit oxygen consumption of WT trypanosomes in a dose-dependent manner. The level of inhibition of **6e** corresponded with a similar effect as the TAO inhibitor SHAM when both were used at ~2×EC_{50} (Figure 4). These results clearly indicate an effect of **6e** on the respiration of *T. brucei* trypomastigotes.

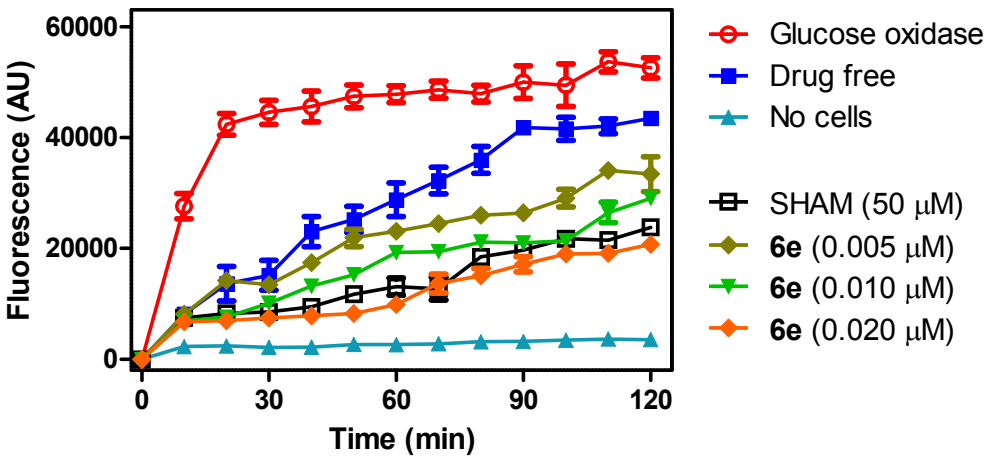


Figure 4. Oxygen consumption assay of **6e** on *T. b. brucei* s427, using the MitoXpress®-Xtra HS kit (Cambridge Bioscience), which generates a fluorescence signal inversely proportional to the oxygen concentration. Glucose oxidase was used to rapidly deplete the cell suspension of oxygen, generating a maximum (plateau) signal for reference, whereas wells without cells were used to establish a null/background fluorescence level. Trypanosomes near-depleted the medium of oxygen in approximately 90 min (drug free control), a rate that was dose-dependently reduced by **6e** and by SHAM. Symbols represent the average and SEM of 2 independent determinations.

DISCUSSION

The chemotherapy of HAT is still deficient despite recent efforts to discover new treatments effective for both stages of the illness.[47] Moreover, drugs against animal African trypanosomiasis (AAT, or nagana) are even more urgently needed than for the corresponding human condition.[2] As current drugs are becoming ineffective due to drug resistance, cross-resistance between existing drugs and new ones is one of the most important issues that must be tackled early in the search for new antitrypanosomal agents.[48]

In this work, two trypanocidal scaffolds (i.e. 2,4-dihydroxybenzoic acid and salicylhydroxamic acid) known to interact with mitochondrial targets[9, 12, 17] were conjugated with one of two mitochondrion-targeting lipophilic cations in order to boost their potency against trypanosomes. The activities against WT and multi-drug resistant *T. brucei* strains, and to a *T. congolense* strain, were studied in vitro. The first important result came from the low nanomolar range activities displayed by the 2,4-DHBA–TPP derivatives (**6a**–**f**), and the submicromolar activities of the 2,4-DHBA–quinolinium derivatives (**10d**–**f**), as compared with the micromolar EC_{50} values of the parent compound. In contrast, the SHAM–TPP derivatives displayed somewhat lower activities, even though this still represented an approximately 100-fold improvement in potency relative to SHAM. The superior antitrypanosomal activities observed with the TPP vs 1-quinolinium conjugates is consistent with earlier studies on diphenyl cationic trypanocides[28], and probably reflects the higher lipophilicity and charge dispersion around the phosphorus atom in the TPP cation, which is optimal for membrane permeation and accumulation in the mitochondrion. Secondly, the insignificant differences in susceptibility between the WT and the multi-drug resistant B48 cell lines

means that cross-resistance with existing first line HAT and AAT drugs, including pentamidine, diminazene, cymelarsan and melarsoprol, is highly unlikely to appear with these compounds, despite the diamidines, at least, also having mitochondrial targets.[5, 49] Indeed, some compounds (e.g. **6e**) were, if anything, slightly more effective against the *T. brucei* B48 resistant line compared to WT, although this did not reach statistical significance. The lack of cross-resistance of 2,4-DHBA and SHAM–LC conjugates with diamidines can be attributed to the fact that diamidine resistance in *T. brucei* is associated with the loss of specific cell surface transporters,[50, 51] whereas the lipophilic LC conjugates are likely to diffuse across biological membranes.

Also noteworthy is the submicromolar activity displayed by compounds **6b–6f** against *T. congolense*, the principal etiological agent of AAT. Their EC_{50} values are similar to the veterinary drug diminazene and their utility against AAT should be investigated further, as drugs against this condition are even more urgently needed than for HAT.[52]

Our preliminary study of the MOA of these compounds showed that, contrary to reported bisphosphonium salt derivatives that inhibit the mitochondrial F_oF_1 ATPase,[6] these compounds do not inhibit progression through the cell cycle. Since the compounds described here were designed as potential mitochondrion-targeted molecules, we studied their effect on parasite respiration and mitochondrial function, and investigated whether TAO might be involved in the observed antitrypanosomal activity. Compounds **6e-f**, **10d-f** and the control drug SHAM (inhibitor of the cyanide-insensitive respiration pathway) were significantly more active against *T. brucei* when co-administered with glycerol, indicating that the aerobic energy metabolic pathway may be a target of these compounds. Indeed, compound **6e** inhibited oxygen consumption of *T. brucei* WT in a similar dose-dependent manner as SHAM and rapidly depolarized the mitochondrial membrane. As TAO is essential for the respiration of bloodstream form trypanosomes

we investigated whether some of the compounds were inhibitors of rTAO. Five compounds were found to inhibit purified rTAO in the low micromolar range (i.e. similar to the reference compound SHAM), two of which were 2,4-DHBA–TPP conjugates (**6e**, **6f**), and three were 2,4-DHBA–quinolinium conjugates (**10d**, **10e**, **10f**). In contrast, 2,4-DHBA was > 20-times weaker inhibitor of rTAO (IC_{50} = 120 μM) and its trypanocidal activity was not potentiated when co-administered with glycerol. Since 2,4-DHBA is 2-fold more potent than SHAM against BSF trypanosomes in vitro, TAO is probably not its main trypanosomal target. Siedow et al. have shown that, in isolated mung bean mitochondria, free carboxylates have no effect on the alternative pathway but a single hydroxyl group in *para* position relative to a benzoate ester is sufficient to inhibit the cyanide-insensitive electron transfer pathway.[34] These results are consistent with our findings and show that the free carboxylate group in 2,4-DHBA may possibly be involved in the binding to TAO by coordinating the iron atoms in the active site. However, the presence of a lipophilic side chain (e.g. ACB41, K_i = 5 μM)[17] seems essential to enhance the interactions of the inhibitor with the TAO active site.

Interestingly, the C14 methylene linker (**6e**, **10e**), which seemed optimal for trypanocidal action also provided improved inhibitory activity against the pure recombinant TAO enzyme. However, the correlation between inhibition of purified rTAO and the trypanocidal effects of the reported LC conjugates is much complicated by the fact that the local concentration of the test compounds in functional, charged mitochondria, remains unknowable for the moment. As intended, this makes the apparent EC_{50} concentration in vitro much lower than the IC_{50} concentration against the isolated enzyme, exactly as reported for the inhibition of the *T. brucei* F_1F_o ATPase by lipophilic bisphosphonium compounds.[6] Although the evidence suggests that some of the LC conjugates have sufficiently low IC_{50} values to act principally through inhibition

of mitochondrial TAO, the possibility that (some of) the compounds also impact on other mitochondrial functions cannot be excluded.

CONCLUSION

In this work, several highly potent trypanocidal agents against *T. brucei* and *T. congolense* with very high selectivity indices (from >500 to >23,000 for **6e** and **6f**) and no cross resistance with existing trypanocidal drugs were synthesized. We showed that the linking of a lipophilic cation to the 2,4-DHBA or SHAM scaffold improved drastically the activity against *T. brucei* in vitro. The 2,4-DHBA scaffold gave the most potent compounds and the 14-methylene linker seemed optimal for trypanocidal action and TAO inhibition.

Compound **6e** in particular inhibited trypanosome growth with EC_{50} in the low nanomolar range (further enhanced in the presence of glycerol) with outstanding selectivity. Preliminary mechanistic studies indicated that its activity was not cell cycle-specific, in that it did not act on cells in a specific phase of the cell cycle, and that parasite respiration was a target of **6e**. Even though TAO was inhibited (in the low micromolar range) by some of the compounds reported here, more data will be needed to confirm the nature of the main target of these 2,4-DHBA–LC conjugates in whole cells. As benzoate derivatives may be susceptible to hydrolysis in vivo by serum hydrolases,[20] in vivo stability studies will have to be taken into account in the future development of these series of compounds. Further SAR studies with these series are in progress.

EXPERIMENTAL SECTION

Chemistry. Anhydrous solvents were purchased to Aldrich/Fluka in SureSeal™ bottles and used as received. Reactions heated under microwave irraditation were carried out in a Biotage Initiatior microwave oven reactor (frequency: 2045 GHz). Thin Layer chromatography (TLC) was performed on silica gel 60 F254 aluminum TLC plates (MERCK). Medium pressure silica chromatography was performed on a FlashMaster Personal system using FlashPack SI prepacked columns (2, 5, 10, 20, and 50 g). Melting points were measured with a Reichert-Jung Thermovar apparatus and are uncorrected. LC-MS spectra were recorded on a WATERS apparatus integrated with a HPLC separation module (2695), PDA detector (2996) and Micromass ZQ spectrometer. Three different cone voltages were used (20, 40 and 60 ēV) and detection was in positive or negative mode (ES^+ or ES^-). Analytical HPLC was performed with a SunFire C18-3.5 μm column (4.6 mm × 50 mm). Mobile phase A: CH_3CN + 0.08% formic acid and B: H_2O + 0.05% formic acid. UV detection was carried over 190 to 440 nm. ^1H NMR and ^{13}C NMR spectra were registered on a Bruker Avance-300, Varian Inova-300, Varian Inova-400, Varian-Mercury-400, and Varian-system-500 spectrometers. Chemical shifts of the ^1H NMR spectra were referenced to tetramethylsilane (δ 0) for $CDCl_3$ or the residual proton resonance of the deuterated solvents: DMSO-d_6 (δ 2.50), CD_3CN (δ 1.94), and CD_3OD (δ 3.31). Chemical shifts of the ^{13}C NMR spectra were referenced to $CDCl_3$ (δ 77.16), DMSO-d_6 (δ 39.52), CD_3CN (δ 1.32), and CD_3OD (δ 49.0). Coupling constants *J* are expressed in hertz (Hz). Accurate mass were measured with an Agilent Technologies Q-TOF 6520 spectrometer using electrospray ionization. All of the biologically tested compounds were ≥ 95% pure by HPLC.

2,4-Dihydroxy-*N*-((tetrahydro-2*H*-pyran-2-yl)oxy)benzamide (**2**). The synthesis was carried out in parallel in 4 microwave tubes. Each tube was charged with 2,4-dihydroxy

benzoic acid (150 mg, 1 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (287 mg, 1.5 mmol), HOBt (6.7 mg, 0.05 mmol), and O-(tetrahydro-2*H*-pyran-2-yl)hydroxylamine[35] (234 mg, 2 mmol). The tubes were purged with argon and anhydrous DMF (4 mL) and *N*-methylmorpholine (274 µL, 2.5 mmol) was added in each tube [Note 1: working at higher concentration leads to lower yields due to the formation of by-products]. The reaction mixture was heated 60 min at 120 ºC in the microwave oven reactor to give a clear yellow reaction mixture [Note 2: conventional heating, during approximately 12 h, can be used as well even though MWI heating give cleaner reaction mixtures]. The content of the 4 tubes was transferred to a round-bottomed flask and the solvent was removed under vacuum. The resulting yellow oil was partitioned between CH_2Cl_2 and water. The aqueous phase was acidified with 5% aqueous citric acid solution and extracted with CH_2Cl_2. The combined organic extracts were washed with brine, dried (Na_2SO_4) and evaporated. The crude product was purified by silica chromatography with CH_2Cl_2/EtOAc (100/0 → 85/15) to yield **2** as colorless foam (460 mg, 45%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.93 (s, 1H), 7.36 (d, J = 8.7 Hz, 1H), 6.40 (d, J = 2.4 Hz, 1H), 6.31 (dd, J = 2.4, 8.7 Hz, 1H), 5.00 (t, J = 3.1 Hz, 1H), 4.02 (ddd, J = 3.4; 8.6; 11.7 Hz, 1H), 3.61 (m, 1H), 1.82 (m, 3H), 1.58 (m, 3H). ^{13}C NMR (75 MHz, CD_3CN) δ 169.4, 164.1, 163.4, 129.1, 108.3, 105.9, 104.1, 103.1, 62.9, 28.8, 25.8, 19.3. HPLC (UV) = 95%. LRMS (ES^+) *m/z* = 254 $[M+H]^+$.

A) General procedure for the synthesis of 4a, 4c, and 4d. A mixture of **2** (100 mg, 0.4 mmol, 1 equiv.), sodium bicarbonate (0.48 mmol, 1.2 equiv), sodium iodide (0.08 mmol, 0.2 equiv.) and bromoalkyltriphenylphosphonium salt (**3a**, **3c**, and **3d**; 0.32 mmol, 0.8 equiv.) in anhydrous acetonitrile (5 mL) was stirred at 65 ºC under an argon atmosphere for 3 days. The white precipitate was filtered off and the filtrate was evaporated under vacuum. The crude residue was purified by silica chromatography (5g

SI prepacked column) using CH_2Cl_2/MeOH (100/0 → 90/10) as eluent to give **4a**, **4c**, and **4d** as colorless solids.

(8-(3-Hydroxy-4-(((tetrahydro-2*H*-pyran-2-yl)oxy)carbamoyl)phenoxy)octyl)triphenylphosphonium bromide (**4a**)**.** Starting from **3a** (173 mg, 0.492 mmol) and following the general procedure, we obtained **4a** as colorless solid (16%). HPLC (UV) > 95%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.38 (s, 1H), 7.94 (s, 1H), 7.80 (d, *J* = 8.7 Hz, 1H), 7.75 – 7.53 (m, 15H), 6.84 (s, 1H), 6.52 (dd, *J* = 1.9, 8.7 Hz, 1H), 4.90 (s, 1H), 4.0–3.78 (m, 3H), 3.59–3.29 (m, 3H), 1.83 – 1.41 (m, 4H), 1.50–1.10 (m, 14H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.5, 163.0, 158.1, 135.3 (d, *J* = 2.9 Hz), 133.7 (d, *J* = 10.0 Hz), 133.1 , 130.7 (d, *J* = 12.6 Hz), 118.2 (d, *J* = 85.9 Hz), 110.4, 109.4, 102.3, 100.7, 94.8, 68.9, 62.5, 30.8, 28.5, 28.2 (d, *J* = 12.2 Hz), 25.6, 25.2, 25.2 (d, *J* = 39.5 Hz), 22.7 (d, *J* = 49.6 Hz), 22.5, 19.9, 18.8. LRMS (ES^+) *m/z* 626 (M^+).

(10-(3-Hydroxy-4-(((tetrahydro-2*H*-pyran-2-yl)oxy)carbamoyl)phenoxy)decyl)triphenylphosphonium bromide (**4c**). Starting from **3c** (190 mg, 0,395 mmol) and following the general procedure, we obtained **4c** as colorless solid (90 mg, 31%). HPLC (UV) > 95%; mp = 118 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.45 (s, 1H), 7.84 (d, *J* = 8.7 Hz, 1H), 7.83–7.59 (m, 16H), 6.90 (d, *J* = 2.2 Hz, 1H), 6.55 (dd, *J* = 8.7, 2.0 Hz, 1H), 4.96 (t, *J* = 3.0 Hz, 1H), 4.01 (t, *J* = 6.4 Hz, 2H), 3.91 (m, 1H), 3.59 (dd, *J* = 11.0, 5.1 Hz, 1H), 3.43 (m, 2H), 1.93 – 1.69 (m, 2H), 1.69–1.09 (m, 20H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.5, 163.0, 158.2, 135.3 (d, *J* = 3.4 Hz), 133.7 (d, *J* = 9.9 Hz), 133.0, 130.7 (d, *J* = 12.7 Hz), 118.3 (d, *J* = 85.8 Hz), 110.3, 109.4, 102.3, 100.6, 68.9, 62.4, 30.4 (d, *J* = 15.9 Hz), 28.9, 28.8, 28.7, 28.7, 28.5, 28.1, 25.3 (d, *J* = 15.6 Hz), 22.8 (d, *J* = 50.3 Hz), 22.7 (d, J = 5 Hz), 22.5, 18.8. LRMS (ES^+) *m/z* 654 (M^+).

(12-(3-Hydroxy-4-(((tetrahydro-2*H*-pyran-2-yl)oxy)carbamoyl)phenoxy)dodecyl)triphenylphosphonium bromide (**4d**). Starting from **3d** (160 mg, 0,322 mmol) and following the general procedure, we obtained **4d** as colorless solid (18%). HPLC (UV) > 95%; mp = 117 ºC. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.41 (s, 1H), 9.62 (brs, 1H), 7.81 (d, *J* = 8.7 Hz, 1H), 7.70 – 7.60 (m, 15H), 6.90 (d, *J* = 2.1 Hz, 1H), 6.52 (dd, *J* = 2.1, 8.7 Hz, 1H), 4.94 (t, *J* = 3.3 Hz, 1H), 4.00 (t, *J* = 6.5 Hz, 2H), 3.89 (ddd, *J* = 2.6, 9.4, 11.8 Hz, 1H), 3.59 – 3.53 (m, 1H), 3.46 (ddd, *J* = 5.7, 9.5, 12.8 Hz, 2H), 1.89–1.65 (m, 6H), 1.44–0.97 (m, 20H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.5, 163.0, 158.3, 135.3 (d, *J* = 3.1 Hz), 133.7 (d, *J* = 9.9 Hz), 133.0, 130.7 (d, *J* = 12.5 Hz), 118.3 (d, *J* = 86.0 Hz), 110.3, 109.3, 102.3, 100.5, 68.8, 62.4, 36.6, 30.5 (d, *J* = 15.7 Hz), 29.8, 29.1, 28.9, 28.9, 28.9, 28.7, 28.3, 28.1, 25.4 (d, *J* = 30.4 Hz), 22.83 (d, *J* = 50.0 Hz), 22.81, 22.78. LRMS (ES^+) *m/z* 682 (M^+).

B) General procedure for the synthesis of 5a, 5c, and 5d. To a stirred solution of **4a-d** (tipically 30–40 mg, 1 equiv.) in methanol (1 mL) was added p-toluenesulfonic acid (0.1 equiv.). The solution was stirred at room temperature until complete disappearance of the starting material as shown by TLC eluting with CH_2Cl_2/MeOH (9/1). The product was purified either by silica chromatography (**5a**, **5d**) eluting with CH_2Cl_2/MeOH (100/0 → 90/10) or via ether-mediated precipitation from the reaction mixture (**5c**).

(8-(3-Hydroxy-4-(hydroxycarbamoyl)phenoxy)octyl)triphenylphosphonium bromide **(5a)**. Starting from **4a** (25 mg, 0.046 mmol) and following the general procedure, we obtained **5a** as colorless hygroscopic solid (20%). HPLC (UV) > 95%; ^{1}H NMR (400 MHz, CD_3OD) δ 7.91 – 7.70 (m, 16H), 7.23 (d, *J* = 8.4 Hz, 2H), 6.48 (s, 1H), 4.09 (t, *J* = 6.2 Hz, 2H), 3.50 – 3.34 (m, 2H), 1.90 – 1.26 (m, 12H). ^{13}C NMR (101 MHz, CD_3OD) δ 163.5, 159.8, 136.3 (d, *J* = 3.0 Hz), 134.8 (d, *J* = 9.9 Hz), 133.4, 131.5 (d, *J* = 12.6 Hz), 129.8, 127.0, 120.0 (d, *J* = 86.4 Hz), 109.1, 100.9, 70.0, 31.3 (d, *J* =

16.4 Hz), 29.8, 29.5, 26.9, 23.4 (d, J = 5.1 Hz), 22.6 (d, J = 51.1 Hz), 21.3. LRMS (ES^+) *m/z* 542 (M^+). HRMS (ES^+) *m/z* 542.2469 (calculated for $C_{33}H_{37}NO_4P$: 542.2460).

(10-(3-Hydroxy-4-(hydroxycarbamoyl)phenoxy)decyl)triphenylphosphonium bromide **(5c)**. Starting from **4c** (23 mg, 0.031 mmol) and following the general procedure, the crude product was precipitated by addition of Et_2O and the tube was allowed to stand at 4 ºC overnight. The precipitate was triturated in Et_2O to give an off-white amorphous hygroscopic solid (10.4 mg, 51%). HPLC (UV) = 95%; ^1H NMR (300 MHz, CD_3OD) δ 8.07 – 7.65 (m, 16H), 6.58 – 6.42 (m, 2H), 4.10 (t, J = 6.17 Hz, 2H), 3.43 – 3.33 (m, 2H), 2.04 – 1.22 (m, 16H). ^{13}C NMR (75 MHz, CD_3OD) δ 166.9, 163.5, 159.8, 136.3, 134.8 (d, J = 10.1 Hz), 133.5, 131.5 (d, J = 12.8 Hz), 120.0 (d, J = 86.6 Hz), 112.4, 109.1, 100.9, 70.1, 31.5 (d, J = 16.1 Hz), 30.3, 30.2, 30.1, 29.9, 29.7, 27.1, 23.5 (d, J = 3.4 Hz), 22.7 (d, J = 51.8 Hz). HRMS (ES^+) *m/z* 570.2763 (calculated for $C_{35}H_{41}NO_4P$: 570.2773).

(12-(3-Hydroxy-4-(hydroxycarbamoyl)phenoxy)dodecyl)triphenylphosphonium bromide **(5d)**. Starting from **4d** (23 mg, 0.04 mmol) and following the general procedure, we obtained **5d** as colorless hygroscopic solid (33%). The compound is a mixture (approximately 3:1 ratio) of bromide and tosylate salts as shown by NMR. HPLC (UV) = 95%. ^1H NMR (500 MHz, $CDCl_3$) δ 7.79 (d, J = 8.4 Hz, 1H), 7.72 – 7.61 (m, 15H), 7.01 (d, J = 7.7 Hz, 1H), 6.87 (s, 1H, NH*OH*), 6.47 (d, J = 8.4 Hz, 1H), 4.04 – 3.92 (m, 2H), 3.44 – 3.33 (m, 2H), 2.24 (s, 1H, TsO^-), 1.81 – 1.62 (m, 2H), 1.26 – 1.00 (m, 18H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.7, 163.0, 158.5, 143.5 (TsO^-), 139.4 (TsO^-), 135.2, 133.7 (d, J = 10.2 Hz), 132.6, 130.7 (d, J = 12.4 Hz), 128.7 (TsO^-), 126.2 (TsO^-), 118.3 (d, J = 84.8 Hz), 109.3, 108.9, 100.7, 68.8, 30.3 (d, J = 15.3 Hz), 29.0, 28.6, 28.5, 28.4, 27.8, 25.3, 22.8, 22.8, 22.4, 21.5 (TsO^-). LRMS *m/z* 598 (M^+). HRMS (ES^+) *m/z* 598.3093 (calculated for $C_{37}H_{45}NO_4P$: 598.3086).

General procedure for the synthesis of 6a–6d. A Kimax tube was charged with a mixture of 2,4-dihydroxybenzoic acid (0.49 mmol, 1 equiv.), sodium bicarbonate (0.59 mmol, 1.2 equiv), and the bromoalkyltriphenylphosphonium salt (**3a**, **3b**, **3c**, and **3d;** 0.44 mmol, 0.9 equiv.) in anhydrous acetonitrile (4 mL). The tube was flushed with argon, stopped and heated at 120 ºC with stirring for 5 min. Then, the reaction mixture was stirred at 65 ºC for 3 days. The reaction mixture was filtered and the precipitate was rinsed with CH_3CN. The filtrate was evaporated under vacuum to give an oily residue. The crude residue was purified by silica chromatography (5g SI prepacked column) using CH_2Cl_2/MeOH (100/0 → 95/5) as eluent to give **6a-d** as hygroscopic colorless amorphous sticky solids. ^1H–^{13}C HMBC and NOESY experiments confirmed that the isolated isomers were the benzoate products and not the 4-alkyloxy-substituted benzoic acid isomers.

(8-((2,4-Dihydroxybenzoyl)oxy)octyl)triphenylphosphonium bromide **(6a)**. Starting from **3a** (200 mg, 0.37 mmol) and following the general procedure, we obtained **6a** as colorless foam (50 mg, 20%). HPLC (UV) > 95%. ^1H NMR (400 MHz, $CDCl_3$) δ 10.77 (s, 1H), 7.80 – 7.52 (m, 15H), 7.49 (d, *J* = 8.8 Hz, 1H), 6.75 (dd, *J* = 2.3, 8.8 Hz, 1H), 6.40 (d, *J* = 2.3 Hz, 1H), 4.14 (d, *J* = 6.3 Hz, 2H), 3.43 – 3.33 (m, 2H), 1.59 – 1.39 (m, 6H), 1.27 – 1.11 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.4, 164.8, 163.6, 135.3 (d, *J* = 3.4 Hz), 133.7 (d, *J* = 10.0 Hz), 130.7 (d, *J* = 12.5 Hz), 118.2 (d, *J* = 85.9 Hz), 109.2, 104.5, 103.4, 64.9, 30.5 (d, *J* = 16.2 Hz), 29.9, 28.95, 28.2, 25.9, 23.0 (d, *J* = 51.3 Hz), 22.7 (d, *J* = 4.9 Hz). LRMS (ES^+) *m/z* 527 (M^+). HRMS (ES^+) *m/z* 527.2349 (calculated for $C_{33}H_{36}O_4P$: 527.2351).

(9-((2,4-Dihydroxybenzoyl)oxy)nonyl)triphenylphosphonium bromide **(6b)**. Starting from **3b** (220 mg, 0.40 mmol) and following the general procedure, we obtained **6b** as colorless foam (55 mg, 20%). HPLC (UV) = 95%. ^1H NMR (400 MHz,

$CDCl_3$+CD_3OD) δ 7.79 – 7.73 (m, 3H), 7.68 – 7.58 (m, 12H), 7.57 (d, *J* = 8.8 Hz, 1H), 6.37 (dd, *J* = 2.3, 8.8 Hz, 1H), 6.27 (d, *J* = 2.3 Hz, 1H), 4.17 (t, *J* = 6.4 Hz, 2H), 3.33 – 3.18 (m, 2H), 1.65 – 1.59 (m, 6H), 1.33 – 1.14 (m, 8H). ^{13}C NMR (101 MHz, CD_3OD) δ 170.1, 164.0, 163.2, 135.3 (d, *J* = 3.0 Hz), 133.3 (d, *J* = 9.9 Hz), 131.4, 130.5 (d, *J* = 12.5 Hz), 117.8 (d, *J* = 86.2 Hz), 108.3, 104.5, 102.7, 64.8, 30.3 (d, *J* = 15.7 Hz), 28.9, 28.8, 28.7, 28.3, 25.8, 22.5 (d, *J* = 50.8 Hz), 22.4 (d, *J* = 4.4 Hz). LRMS (ES^+) *m/z* 541 (M^+). HRMS (ES^+) *m/z* 541.2498 (calculated for $C_{34}H_{38}O_4P$: 541.2508).

(10-((2,4-Dihydroxybenzoyl)oxy)decyl)triphenylphosphonium bromide **(6c)**. Starting from **3c** (235 mg, 0.42 mmol) and following the general procedure, we obtained **6c** as colorless foam (47 mg, 16%). HPLC (UV) > 95%. ^{1}H NMR (400 MHz, CD_3OD) δ 7.90 – 7.81 (m, 6H), 7.78 – 7.70 (m, 9H), 7.65 (d, *J* = 8.7 Hz, 1H), 6.33 (dd, *J* = 2.3, 8.7 Hz, 1H), 6.26 (d, *J* = 2.3 Hz, 1H), 4.28 (t, *J* = 6.4 Hz, 2H), 3.41 (tdd, *J* = 2.3, 5.3, 7.90, 2H), 1.73 (dd, *J* = 6.4, 8.1 Hz, 2H), 1.67 – 1.61 (m, 2H), 1.57 – 1.51 (m, 2H), 1.40 (m, 2H), 1.33 – 1.27 (m, 8H). ^{13}C NMR (101 MHz, CD_3OD) δ 171.4, 165.6, 165.0, 136.2 (d, *J* = 3.0 Hz), 134.8 (d, *J* = 10.0 Hz), 132.6, 131.5 (d, *J* = 12.6 Hz), 120.0 (d, *J* = 86.1 Hz), 109.1, 105.6, 103.5, 65.9, 31.5 (d, *J* = 16.2 Hz), 30.3, 30.2, 30.15, 29.8, 29.6, 27.0, 23.5 (d, *J* = 4.5 Hz), 22.7 (d, *J* = 50.7 Hz). LRMS (ES^+) *m/z* 555 (M^+). HRMS (ES^+) *m/z* 555.2685 (calculated for $C_{35}H_{40}O_4P$: 555.2664).

(12-((2,4-Dihydroxybenzoyl)oxy)dodecyl)triphenylphosphonium bromide **(6d)**. Starting from **3d** (240 mg, 0.41 mmol) and following the general procedure, we obtained **6d** as colorless oily-sticky solid (238 mg, 88%). HPLC (UV) > 95%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.80 (s, 1H), 9.60 (s, 1H), 7.74 – 7.58 (m, 15H), 7.51 (d, *J* = 8.8 Hz, 1H), 6.73 (dd, *J* = 2.3, 8.8 Hz, 1H), 6.40 (d, *J* = 2.3 Hz, 1H), 4.20 (t, *J* = 6.0 Hz, 2H), 3.56 – 3.43 (m, 2H), 1.65 (p, *J* = 6.5 Hz, 2H), 1.59 – 1.41 (m, 2H), 1.41 – 0.97 (m, 16H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5, 164.8, 163.6, 135.2 (d, *J* = 3 Hz), 133.7 (d,

J = 10 Hz), 131.2, 130.6 (d, *J* = 12.5 Hz), 118.3 (d, *J* = 85.9 Hz), 109.1, 104.5, 103.3, 64.7, 30.7 (d, J = 15.7 Hz), 29.4, 29.25, 29.18, 28.83, 28.82, 28.26, 28.21, 25.6, 22.8 (d, *J* = 50 Hz), 22.77, 22.72. LRMS (ES^+) *m/z* 583.4 (M^+). HRMS (ES^+) *m/z* 583.2986 (calculated for $C_{37}H_{44}O_4P$: 583.2977).

(14-((2,4-Dihydroxybenzoyl)oxy)tetradecyl)triphenylphosphonium bromide **(6e)**. A solution of **7e** (24.6 mg, 0.057 mmol) and triphenylphosphine (17.3 mg, 0.08 mmol) in anhydrous acetonitrile (1 mL) was heated at 80 ºC for 68 h in a Kimax tube under argon atmosphere. The crude product was precipitated by addition of Et_2O and the tube was allowed to stand at 4 ºC overnight. The solid obtained was purified by successive precipitations from MeOH/Et_2O and CH_2Cl_2/EtOAc. The pure product was obtained as colorless oily hygroscopic solid (7.5 mg, 20%). HPLC (UV) > 95%. ^{1}H NMR (300 MHz, CD_3OD) δ 7.89 – 7.74 (m, 15H), 7.66 (d, *J* = 8.8 Hz, 1H), 6.32 (dd, *J* = 2.6, 8.8 Hz, 1H), 6.27 (d, *J* = 2.6 Hz, 1H), 4.30 (t, *J* = 6.5 Hz, 2H), 3.45 – 3.20 (m, 2H), 1.82 – 1.06 (m, 24H). ^{13}C NMR (75 MHz, CD_3OD) δ 171.4, 166.0, 165.0, 136.3 (d, *J* = 3.1 Hz), 134.8 (d, *J* = 9.9 Hz), 132.6, 131.5 (d, *J* = 12.6 Hz), 120.0 (d, *J* = 86.1 Hz), 109.2, 105.5, 103.6, 65.9, 31.6 (d, *J* = 16.0 Hz), 30.59, 30.57, 30.48, 30.3, 30.2, 29.8, 29.7, 27.0, 23.5 (d, *J* = 4.1 Hz), 22.7 (d, *J* = 50.1 Hz). LRMS (ES^+) *m/z* 611 $[M]^+$. HRMS (ES^+) *m/z* 611.3290 (calculated for $C_{39}H_{48}O_4P$: 611.3290).

(16-((2,4-Dihydroxybenzoyl)oxy)hexadecyl)triphenylphosphonium bromide (**6f**). A solution of **7f** (20 mg, 0.044 mmol) and triphenylphosphine (11.5 mg, 0.044 mmol) in anhydrous acetonitrile (1 mL) was heated at 80 ºC for 68 h in a Kimax tube under argon atmosphere. The crude product was precipitated by addition of Et_2O and the tube was allowed to stand at 4 ºC overnight. The solid obtained was purified by successive precipitations from MeOH/Et_2O and CH_2Cl_2/EtOAc. The pure product was obtained as colorless oily hygroscopic solid (9 mg, 28%). HPLC (UV) > 95%. ^{1}H NMR

(300 MHz, CD_3OD) δ 7.90 – 7.75 (m, 15H), 7.66 (dd, *J* = 4.1, 8.6 Hz, 1H), 6.33 (dd, *J* = 2.5, 8.6 Hz, 1H), 6.27 (d, *J* = 2.5 Hz, 1H), 4.30 (t, *J* = 6.3 Hz, 2H), 3.49 – 3.27 (m, 2H), 1.80 – 1.12 (m, 28H). ^{13}C NMR (75 MHz, CD_3OD) δ 171.4, 165.8, 165.0, 136.3 (d, *J* = 3.1 Hz), 134.8 (d, *J* = 10.0 Hz), 132.6, 131.5 (d, *J* = 12.5 Hz), 120.0 (d, *J* = 86.5 Hz), 109.1, 105.7, 103.5, 65.9, 31.6 (d, *J* = 16.6 Hz), 30.6, 30.53, 30.49, 30.3, 30.2, 29.9, 29.7, 27.0, 23.5 (d, *J* = 4.2 Hz), 22.7 (d, *J* = 50.4 Hz). LRMS (ES^+) *m/z* 639.5 $[M]^+$. HRMS (ES^+) *m/z* 639.3615 (calculated for $C_{41}H_{52}O_4P$: 639.3603).

General procedure for the synthesis of 7c–7f. A Kimax tube was charged with an equimolar quantity of 2,4-dihydroxybenzoic acid (142 mg, 0.9 mmol), $NaHCO_3$ (78 mg, 0.9 mmol), the dibromoalkane (0.9 mmol) and anhydrous DMF (10 mL). The tube was stopped and the reaction mixture was stirred at 65 ºC under argon atmosphere from 24 to 48 h. The solvent was evaporated to dryness under vacuum to give a crude solid containing the three mains products **7**, **8**, and **9**. Purification by silica chromatography (10 g SI prepacked column) using hexane/EtOAc (100/0 → 50/50) as eluent allowed the isolation of **7** (major product, > 30%), **8** (< 30%), and **9** (< 10%).

10-Bromodecyl 2,4-dihydroxybenzoate (**7c**). Starting from 1,10-dibromodecane (276 mg, 0.92 mmol) and following the general procedure, **7c** was isolated by silica chromatography using hexane/EtOAc (98/2) as eluent. Off-white solid (127 mg, 37%). HPLC (UV) = 92%; mp = 41.3 ºC. ^1H NMR (300 MHz, $CDCl_3$) δ 11.06 (s, 1H), 7.73 (d, *J* = 8.4 Hz, 1H), 6.43 – 6.33 (m, 2H), 6.08 – 5.59 (brs, 1H), 4.30 (t, *J* = 6.6 Hz, 2H), 3.40 (t, *J* = 6.6 Hz, 2H), 1.92 – 1.20 (m, 16H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 163.8, 162.1, 132.0, 107.9, 106.2, 103.3, 65.3, 34.2, 33.0, 29.5, 29.5, 29.3, 28.9, 28.7, 28.3, 26.1. LRMS (ES^+) *m/z* 373, 375 $[M+H]^+$. HRMS (ES^+) *m/z* 372.0925 (calculated for $C_{17}H_{25}BrO_4$: 372.0936).

12-Bromododecyl 2,4-dihydroxybenzoate (**7d**). Starting from 1,12-dibromododecane (142 mg, 0.92 mmol) and following the general procedure, **7d** was isolated by silica chromatography using hexane/EtOAc (97/3) as eluent. Colorless solid (176 mg, 48%). HPLC (UV) > 95%; mp = 48–51 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.00 (s, 1H), 7.67 (d, *J* = 8.4 Hz, 1H), 6.33 (d, *J* = 2.3 Hz, 1H), 6.30 (dd, *J* = 2.3, 8.4 Hz, 1H), 5.68 (brs, 1H), 4.23 (t, *J* = 6.5 Hz, 2H), 3.33 (t, *J* = 6.7 Hz, 2H), 1.86 – 1.61 (m, 6H), 1.44 – 1.12 (m, 14H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 163.8, 162.0, 132.0, 107.9, 106.3, 103.3, 65.4, 34.2, 33.0, 29.6, 29.55, 29.35, 28.9, 28.7, 28.3, 26.1. LRMS (ES^+) *m/z* 401, 403 $[M+H]^+$. HRMS (ES^+) *m/z* 400.1266 (calculated for $C_{19}H_{29}BrO_4$: 400.1249).

14-Bromotetradecyl 2,4-dihydroxybenzoate (**7e**). Starting from 1,14-dibromotetradecane (285 mg, 0.8 mmol) and following the general procedure, **7e** was isolated by silica chromatography using hexane/EtOAc (98/2) as eluent. Colorless solid (123 mg, 36%). HPLC (UV) > 95%; mp = 72–73.8 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.99 (s, 1H), 7.67 (d, *J* = 8.4 Hz, 1H), 6.34 – 6.31 (m, 1H), 6.29 (d, *J* = 2.6 Hz, 1H), 5.59 (brs, 1H), 4.23 (t, *J* = 6.4 Hz, 2H), 3.33 (t, *J* = 6.6 Hz, 2H), 1.90-1.55 (m, 6H), 1.52 – 1.01 (m, 18H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 163.8, 162.0, 132.0, 107.9, 106.3, 103.3, 65.4, 34.2, 33.0, 29.73, 29.68, 29.63, 29.57, 29.4, 28.9, 28.8, 28.4, 28.3, 26.1. LRMS (ES^+) *m/z* 429, 431 $[M+H]^+$. HRMS (ES^+) *m/z* 428.1577 (calculated for $C_{21}H_{33}BrO_4$: 428.1562).

16-Bromohexadecyl 2,4-dihydroxybenzoate (**7f**). Starting from 1,16-dibromohexadecane (93 mg, 0.24 mmol) and following the general procedure, **7f** was isolated by silica chromatography (5g SI cartridge) using hexane/EtOAc (98/2) as eluent. Colorless amorphous solid (35 mg, 32%). HPLC (UV) > 95%; mp = 58–60 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.98 (s, 1H), 7.67 (d, *J* = 8.4 Hz, 1H), 6.33 (d, *J* = 2.5

Hz, 1H), 6.30 (dd, *J* = 2.5, 8.4 Hz, 1H), 5.43 (brs, 1H), 4.23 (t, *J* = 6.5 Hz, 2H), 3.34 (t, *J* = 6.7 Hz, 2H), 1.78 (p, *J* = 6.9 Hz, 4H), 1.67 (p, *J* = 6.8 Hz, 4H), 1.46 – 1.23 (m, 20H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 163.9, 161.9, 132.0, 107.8, 106.4, 103.3, 65.4, 34.2, 33.0, 29.9, 29.8, 29.70, 29.69, 29.65, 29.6, 29.4, 28.9, 28.8, 28.3, 26.1. LRMS (ES^+) *m/z* 457, 459 $[M+H]^+$. HRMS (ES^-) *m/z* 456.1868 (calculated for $C_{23}H_{37}BrO_4$: 456.1875).

10-(Formyloxy)decyl 2,4-dihydroxybenzoate (**8c**). **8c** was isolated by silica chromatography using hexane/EtOAc (96/4) as eluent. Off-white amorphous solid (20 mg, 6%). HPLC (UV) > 95%; mp = 62–68 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.96 (s, 1H), 7.99 (s, 1H), 7.66 (d, *J* = 8.5 Hz, 1H), 6.33 (d, *J* = 2.4 Hz, 1H), 6.30 (dd, *J* = 2.4, 8.5 Hz, 1H), 4.23 (t, *J* = 6.5 Hz, 2H), 4.10 (t, *J* = 6.6 Hz, 2H), 1.72 – 1.09 (m, 16H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 163.8, 162.3, 161.6, 132.0, 107.9, 106.2, 103.3, 65.3, 64.4, 29.8, 29.5, 29.28, 29.26, 28.7, 28.6, 26.1, 25.9. LRMS (ES^+) *m/z* 339 $[M+H]^+$. HRMS (ES^+) *m/z* 338.1740 (calculated for $C_{18}H_{26}O_6$: 338.1729).

12-(Formyloxy)dodecyl 2,4-dihydroxybenzoate (**8d**). **8d** was isolated by silica chromatography using hexane/EtOAc (90/10) as eluent. Colorless amorphous solid (14.6 mg, 4%). HPLC (UV) > 95%; mp = 68–70 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.99 (s, 1H), 8.00 (s, 1H), 7.66 (d, *J* = 8.4 Hz, 1H), 6.34 (d, *J* = 2.4 Hz, 1H), 6.31 (dd, *J* = 2.4, 8.4 Hz, 1H), 6.15 (brs, 1H), 4.23 (t, *J* = 6.6 Hz, 2H), 4.10 (t, *J* = 6.6 Hz, 2H), 1.73 – 1.56 (m, 6H), 1.22 (m, 14H). LRMS (ES^+) *m/z* 367 $[M+H]^+$. HRMS (ES^+) *m/z* 366.2050 (calculated for $C_{20}H_{30}O_6$: 366.2042).

14-(Formyloxy)tetradecyl 2,4-dihydroxybenzoate (**8e**). **8e** was isolated by silica chromatography using hexane/EtOAc (96/4) as eluent. Colorless solid (3 mg, 1%). HPLC (UV) = 93%; mp = 70–71 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.96 (s, 1H), 8.00

(s, 1H), 7.66 (d, *J* = 8.0 Hz, 1H), 6.38 – 6.22 (m, 2H), 5.66 (br, 1H), 4.24 (d, *J* = 6.6 Hz, 2H), 4.11 (d, *J* = 6.8 Hz, 2H), 1.74 – 1.52 (m, 6H), 1.33 – 1.22 (m, 18H). LRMS (ES^+) *m/z* 395 $[M+H]^+$. HRMS (ES^+) *m/z* 394.2370 (calculated for $C_{22}H_{34}O_6$: 394.2355).

16-(Formyloxy)hexadecyl 2,4-dihydroxybenzoate (**8f**). **8f** was isolated by silica chromatography using hexane/EtOAc (96/4) as eluent. Colorless solid (11 mg, 10%). HPLC (UV) = 92%; mp = 76–77 ºC. ^1H NMR (300 MHz, $CDCl_3$) δ 10.96 (s, 1H), 7.99 (s, 1H), 7.67 (d, *J* = 8.5 Hz, 1H), 6.33 (d, *J* = 2.4 Hz, 1H), 6.29 (dd, *J* = 2.4, 8.5 Hz, 1H), 5.36 (brs, 1H), 4.23 (t, *J* = 6.5 Hz, 2H), 4.10 (t, *J* = 6.7 Hz, 2H), 1.89 – 0.97 (m, 28H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.4, 164.2, 162.2, 161.7, 132.2, 108.0, 106.6, 103.5, 65.6, 64.6, 30.1, 30.0 (m, overlapping peaks), 29.91, 29.87, 29.60, 29.55, 29.0, 28.9, 26.4, 26.2. LRMS (ES^+) *m/z* 423 $[M+H]^+$. HRMS (ES^+) *m/z* 422.2687 (calculated for $C_{24}H_{38}O_6$: 422.2668).

Decane-1,10-diyl bis(2,4-dihydroxybenzoate) (**9c**). **9c** was isolated by silica chromatography using hexane/EtOAc (70/30) as eluent. Off-white solid (83 mg, 24%). HPLC (UV) > 95%; mp = 118.6–120.1 ºC. ^1H NMR (300 MHz, DMSO-d_6) δ 10.92 – 10.18 (br, 4H), 7.63 (d, *J* = 8.7 Hz, 2H), 6.37 (dd, *J* = 8.7, 2.4 Hz, 2H), 6.29 (d, *J* = 2.4 Hz, 2H), 4.25 (t, *J* = 6.4 Hz, 4H), 1.68 (p, *J* = 6.5 Hz, 4H), 1.30 (m, 12H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 169.3, 164.2, 162.8, 131.4, 108.3, 104.0, 102.4, 64.6, 28.8, 28.5, 28.0, 25.4. LRMS (ES^+) *m/z* 447 $[M+H]^+$. HRMS (ES^+) *m/z* 446.1936 (calculated for $C_{24}H_{30}O_8$: 446.1941).

Dodecane-1,12-diyl bis(2,4-dihydroxybenzoate) (**9d**). **9d** was isolated by silica chromatography using hexane/EtOAc (50/50) as eluent. Colorless amorphous solid (90 mg, 24%). HPLC (UV) > 95%; mp = 96–97 ºC. ^1H NMR (300 MHz, $CDCl_3$) δ 10.91 (s, 2H), 9.32 (s, 2H), 7.61 (d, *J* = 8.6 Hz, 2H), 6.35 (d, *J* = 2.4 Hz, 2H), 6.32 (dd, *J* = 2.4,

8.6 Hz, 2H), 4.21 (t, *J* = 6.5 Hz, 4H), 1.68 (t, *J* = 7.1 Hz, 4H), 1.35 – 1.20 (m, 16H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.3, 164.1, 163.8, 131.5, 108.3, 104.9, 103.1, 65.0, 29.7, 29.5, 29.3, 28.7, 26.0. LRMS (ES^+) *m/z* 475 $[M+H]^+$. HRMS (ES^+) *m/z* 474.2263 (calculated for $C_{26}H_{34}O_8$: 474.2254).

Tetradecane-1,14-diyl bis(2,4-dihydroxybenzoate) (**9e**). **9e** was isolated by silica chromatography using hexane/EtOAc (70/30) as eluent. Colorless solid (61 mg, 18%). HPLC (UV) > 95%, mp = 132.7–134.8 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.97 (brs, 2H), 7.62 (dd, *J* = 2.0, 7.3 Hz, 2H), 6.33 – 6.25 (m, 4H), 4.21 (t, *J* = 6.5 Hz, 4H), 1.67 (p, *J* = 6.5 Hz, 4H), 1.40 – 1.15 (m, 20H). ^{13}C NMR (75 MHz, $CDCl_3$ δ 170.2, 163.8, 163.3, 131.6, 108.2, 104.9, 102.6, 65.1, 29.6, 29.52, 29.47, 29.2, 28.6, 26.0. LRMS (ES^+) *m/z* 503 $[M+H]^+$.

Hexadecane-1,16-diyl bis(2,4-dihydroxybenzoate) (**9f**). **9f** was isolated by silica chromatography using hexane/EtOAc (70/30) as eluent. Colorless amorphous solid (3 mg, 3%). HPLC (UV) = 87%; mp = 106–110 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.99 (s, 2H), 7.70 – 7.57 (m, 2H), 6.29 (m, 4H), 4.21 (t, *J* = 6.5 Hz, 4H), 1.81 – 1.56 (m, 4H), 1.55 – 0.99 (m, 20H), 0.77 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.3, 163.8, 163.3, 131.7, 108.3, 105.0, 102.6, 65.1, 32.6, 30.7, 30.1, 29.7, 29.7, 29.6, 29.5, 29.3, 28.7, 26.0, 25.8. LRMS (ES^+) *m/z* 531 $[M+H]^+$. HRMS (ES^+) *m/z* 530.2887 (calculated for $C_{30}H_{42}O_8$: 530.2880).

Synthesis of the target compounds 10d–f.

1-(12-((2,4-Dihydroxybenzoyl)oxy)dodecyl)quinolin-1-ium bromide (**10d**). A solution of **7d** (48 mg, 0.12 mmol) and quinoline (16 μL, 0.15 mmol) in anhydrous acetonitrile (1 mL) was heated at 80 ºC for 39 h in a Kimax tube. The precipitate was collected and washed with cold CH_3CN to give a light-brown solid (18.3 mg, 29%).

HPLC (UV) > 95%, mp = 151–153 ºC. ^{1}H NMR (300 MHz, CD_3OD) δ 9.42 (d, *J* = 6.6 Hz, 1H), 9.21 (d, *J* = 7.3 Hz, 1H), 8.56 (d, *J* = 8.3 Hz, 1H), 8.44 (d, *J* = 7.8 Hz, 1H), 8.35 – 8.24 (m, 1H), 8.07 (d, *J* = 7.5 Hz, 2H), 7.66 (d, *J* = 8.6 Hz, 1H), 6.42 – 6.18 (m, 2H), 5.1 (m, 2H), 4.58 (m, 2H), 4.29 (m, 2H), 2.18 – 1.96 (m, 2H), 1.89 – 1.64 (m, 2H), 1.64 – 1.12 (m, 16H). ^{13}C NMR (75 MHz, CD_3OD) δ 171.4, 165.7, 165.0, 150.3, 149.0, 137.3, 132.6, 132.2, 131.8, 131.4, 123.0, 119.7, 109.1, 103.5, 65.9, 59.4, 31.1, 30.4, 30.1, 29.6, 27.5, 27.0. LRMS (ES^+) *m/z* 450 $[M]^+$. HRMS (ES^+) *m/z* 450.2642 (calculated for $C_{28}H_{36}O_4N$: 450.2644).

1-(14-((2,4-Dihydroxybenzoyl)oxy)tetradecyl)quinolin-1-ium bromide (**10e**). A solution of **7e** (30 mg, 0.07 mmol) and quinoline (9.5 μL, 0.08 mmol) in anhydrous acetonitrile (1 mL) was heated at 80 ºC for 6 days in a Kimax tube. The precipitate was collected and triturated in Et_2O to give an off-white solid (12 mg, 31%). HPLC (UV) > 95%; mp = 143–146 ºC. ^{1}H NMR (300 MHz, CD_3OD) δ 9.42 (dd, *J* = 1.5, 5.9 Hz, 1H), 9.21 (d, *J* = 8.4 Hz, 1H), 8.56 (d, *J* = 9.1 Hz, 1H), 8.44 (dd, *J* = 1.6, 8.4 Hz, 1H), 8.30 (td, *J* = 3.7, 7.3, 7.6 Hz, 1H), 8.16 – 7.99 (m, 2H), 7.66 (d, *J* = 8.8 Hz, 1H), 6.33 (dd, *J* = 2.4, 8.7 Hz, 1H), 6.27 (d, *J* = 2.4 Hz, 1H), 5.09 (t, *J* = 7.7 Hz, 2H), 4.57 (brs, 2H), 4.30 (t, *J* = 6.4 Hz, 2H), 2.20 – 1.99 (m, 2H), 1.83 – 1.67 (m, 2H), 1.60 – 1.18 (m, 20H). ^{13}C NMR (75 MHz, CD_3OD) δ 171.4, 165.7, 165.0, 150.3, 149.0, 139.4, 137.3, 132.6, 132.2, 131.8, 131.4, 123.0, 119.7, 109.1, 105.7, 103.5, 66.0, 59.4, 31.1, 30.62, 30.58, 30.52, 30.49, 30.47, 30.2, 30.1, 29.7, 27.5, 27.0. LRMS (ES^+) *m/z* 478 $[M]^+$. HRMS (ES^+) *m/z* 478.2965 (calculated for $C_{30}H_{40}O_4N$: 478.2957).

1-(16-((2,4-Dihydroxybenzoyl)oxy)hexadecyl)quinolin-1-ium bromide (**10f**). A solution of **7f** (11 mg, 0.024 mmol) and quinoline (3 μL, 0.03 mmol) in anhydrous acetonitrile (0.5 mL) was heated at 80 ºC for 3 days in a Kimax tube. The precipitate was collected and triturated in Et_2O to give a reddish solid which was recrystallized in

MeOH. Light-brown solid (5.2 mg, 37%). HPLC (UV) > 95%, mp = 138–139 ºC. ^{1}H NMR (400 MHz, CD_3OD) δ 9.42 (dd, *J* = 1.4, 5.8 Hz, 1H), 9.21 (dt, *J* = 1.1, 8.4 Hz, 1H), 8.56 (d, *J* = 9.1 Hz, 1H), 8.44 (dd, *J* = 1.4, 8.3 Hz, 1H), 8.30 (ddd, *J* = 1.5, 7.0, 8.9 Hz, 1H), 8.12 – 8.03 (m, 2H), 7.66 (d, *J* = 8.8 Hz, 1H), 6.33 (dd, *J* = 2.4, 8.8 Hz, 1H), 6.27 (d, *J* = 2.4 Hz, 1H), 5.11 – 5.04 (m, 2H), 4.58 (brs, 2H), 4.30 (t, *J* = 6.54 Hz, 2H), 2.15 – 2.05 (m, 2H), 1.80 – 1.71 (m, 2H), 1.53 – 1.27 (m, 24H). ^{13}C NMR (101 MHz, CD_3OD) δ 171.4, 165.6, 165.0, 150.3, 149.0, 139.4, 137.3, 132.6, 132.2, 131.8, 131.5, 123.1, 119.7, 109.1, 105.7, 103.5, 66.0, 59.4, 31.1, 30.7, 30.61, 30.56, 30.53, 30.50, 30.48, 30.2, 30.2, 29.7, 27.5, 27.0. LRMS (ES^+) *m/z* 506 $[M]^+$. HRMS (ES^+) *m/z* 506.3285 (calculated for $C_{32}H_{44}O_4N$: 506.3270).

Biology

Test Organisms and culture media. Three strains of *Trypanosoma brucei* (bloodstream trypomastigotes only) were used in this study: (1) Wild type strain *Trypanosoma brucei brucei* Lister 427 (427-WT)[53]; (2) A multi-drug resistant strain, B48 which was created from 427-WT after deletion of the TbAT1 drug transporter[54] followed by adaptation to increasing concentrations to pentamidine;[55] (3) A 427-WT-derived clone, *Tb*AOX, generated by transfecting the wild-type cells with the vector pHD1336[56] containing the TAO gene, exactly as described for the expression of TbAT1.[57]

All *T. b. brucei* strains were used only as bloodstream trypomastigotes, and cultured in standard HMI-9 medium, supplemented with 10 % heat inactivated Fetal Bovine Serum (FBS), 14 μL β-mercaptoethanol, and 3.0 g sodium hydrogen carbonate per litre of medium (pH7.4). Parasites were cultured in vented flasks at 37 ºC in a 5% CO_2

atmosphere and were passage every 3 days. Bloodstream forms of the *T. congolense* savannah-type strain IL3000 were cultured exactly as described by Coustou et al[58] and were kindly provided by Theo Baltz (Université Victor Segalen Bordeaux 2, Bordeaux, France).

Drug susceptibility assays. The drug susceptibilities of bloodstream form trypanosomes *T. b. brucei* s427 and B48 were determined using the resazurin (Alamar blue) assay following a previously described resazurin-based method[59, 60] with slight modifications. The assays were performed in 96-well plates with of 2×10^4 cells/well for *T. brucei* and 5×10^4 cells/well for *T. congolense*. Trypanosomes and test drugs were incubated for a period of 48 hours followed by the addition of 20 μL of Alamar Blue solution (125 mg/L resazurin sodium salt (Sigma-Aldrich) in phosphate buffered saline (PBS), followed by further 24 hours incubation. Four trypanocides were used as positive controls including: pentamidine, diminazene aceturate, salicylic hydroxamic acid (SHAM), and phenylarsine oxide (PAO) (all from Sigma-Aldrich). Fluorescence was measured using a FLUOstar Optima (BMG Labtech, Durham, NC, USA) at wavelengths of 544 nm for excitation, 590 for emission. EC_{50} values were calculated by non-linear regression using an equation for a sigmoidal dose-response curve with variable slope using Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Drug sensitivity using Propidium Iodide (PI) assay. This procedure was used to monitor how monitor the effects of the test compounds act on trypanosomes in real time.[61] Trypanosomes become fluorescent when the plasma membrane is breached and PI enters the cell and binds to nucleic acids.[62] In this method, 100 μL of HMI-9 was added to each well of a 96-well plate and 100 μL from various compounds at different

concentrations, also in HMI-9, was added to the first column; wells receiving only media and served as drug free controls. To each well was added 100 μL of HMI-9 containing 2×10^6 trypanosomes and 18 μM of PI. Wells containing the same final concentration of PI (9 μM) in HMI-9 but no cells served to record background fluorescence. The plates were incubated in a FLUOstar OPTIMA fluorimeter (BMG Labtech) at 37 ºC with 5% CO_2 atmosphere, and the fluorescence was recorded at 544 nm excitation and 620 nm emission for 6 hours.

Cytotoxicity assay using Human Embryonic Kidney (HEK)/ Human Foreskin Fibroblast (HFF) 293-T cells. Toxicity of drugs to mammalian cells was carried out in mammalian cell lines according to a method previously described,[63] with slight modifications. Briefly, HEK or HFF cells were grown in a culture containing 500 mL Dulbecco's Modified Eagle's Medium (DMEM) (Sigma), 50 mL New-born Calf Serum (NBCS) (Gibco), 5 mL Penicillin/Streptomycin (Gibco) and 5 mL L-Glutamax (200 mM, Gibco). Mammalian cells were incubated at 37 ºC/5% CO_2 and were passaged when they reached 80-85% confluence in vented flasks. For the assay, cells were suspended at a density of 3×10^5 cells/mL, of which 100 μL was added to each well of a 96-well plate. The plate was incubated at 37 ºC + 5% CO_2 for 24 hours to allow cell adhesion. Serial drug dilutions were prepared in a separate sterile plate and 100 μL was transferred to the wells containing the cells; PAO was used as positive control. The plate was then incubated at 37 ºC/5% CO_2 for an additional period of 30 h followed by the addition of 10 μL of resazurin solution (125 mg/L in PBS) and a final incubation at 37 ºC/5% CO_2 for 24 hours. The plate was read in a FLUOstar OPTIMA fluorimeter at wavelengths 530 nm for excitation and 590 nm for emission. The data were analysed

using GraphPad Prism 5.0 to determine EC_{50} values. The selectivity index was calculated as EC_{50} (HEK)/EC_{50} (Trypanosoma).

Oxygen Consumption assays. The Oxygen Consumption Rate Assay Kit (Cayman chemicals, Ann Arbor MI, USA) designed to measure extracellular oxygen consumption in cells was adapted to trypanosomes following manufacturer protocol with substantial modifications. Cells were seeded in a sterile 96-well flat bottom tissue culture plate at a seeding density of 8×10^4 cells/well (i.e. 5×10^5 cells/mL) in 150 μL of HMI-9 and test compounds were added in 10 μL to the appropriate wells, immediately prior to measurement; three blank wells received culture medium only. Glucose Oxidase Stock Solution (10 μL, provided by the kit), was added to a control well to deplete all oxygen from the medium, and 10 μL of SHAM was added to another control well (50 μM final concentration). Finally, 10 μL of the probe solution was added to every well except the blank wells; all reagents used had been freshly prepared. Using a repeating pipette, every well was gently overlaid with 100 μL of mineral oil pre-warmed to 37 ^oC. The plate was immediately read kinetically for 120 minutes using a fluorimeter set at 37 ^oC and at a wavelength of 380 nm for excitation and 650 nm for emission. Gain was adjusted so that the fluorescent signal of probe in 21% O_2 (air saturated) buffer was equal to 20% of the maximum measureable signal.

Mitochondrial membrane potential (Ψ_m) determination using flow cytometry. Fluorescence Activated Cell Sorting technology (FACS) was employed in the determination of the change in mitochondrial membrane potential (MMP) due to exposure of trypanosomes to the test compounds by using tetramethylrhodamine ethyl ester (TMRE).[64] The cell density was adjusted to 1×10^6 cells/mL with and without test compounds for the start of the experiment. 1 mL of sample was transferred at each time point into a microfuge tube and centrifuged at 4500 rpm for 10 min at 4 °C. The pellet

was re-suspended in 1 mL PBS containing 200 nM of TMRE, followed by incubated at 37 °C for 30 min. The suspension was placed on ice for at least 30 minutes before analysis by a Becton Dickinson FACS Calibur using a FL2-heigth detector and CellQuest and FlowJo software.[31] Valinomycin (100 nM) and troglitazone (10 μM) were employed as negative (mitochondrial membrane depolarisation) and positive (mitochondrial membrane hyperpolarisation) controls respectively (Denninger *et al*., 2007).[64] Mitochondrial membrane potential was determined at 0, 1, 4, 8 and 12 h.

Cell cycle assay (DNA content assay) using flow cytometry. Fluorescence Activated Cell Sorting Technology (FACS) was also used to study the effects of test compounds on DNA content in *Trypanosoma brucei brucei* s427 WT. Cell density was adjusted to 1×10^7 cells/mL with and without test compounds for the duration of the experiment. 1 mL of sample was transferred at each time point into microfuge tubes and centrifuged at 1620 × *g* for 10 min at 4 °C, washed once in PBS containing 5 mM of EDTA and re-suspended and fixed in 1 mL of 70% methanol and 30% PBS/EDTA. The tube with the cells was left at 4 °C overnight in the dark, and the samples were subsequently washed once with 1 mL PBS/EDTA, re-suspended in 1 mL PBS/EDTA containing 10 μg/mL propidium iodide and incubated at 37 °C for 45 minutes. RNase A (10 μg/mL) was added before the samples were analysed by a Becton Dickinson FACSCalibur using the FL2-Area detector and CellQuest software. The data obtained were analysed using flowJo software (Flowjo LLC, Ashland, OR, USA).

Production of recombinant TAO. Recombinant TAO was produced essentially as described in the haem-deficient *E. coli* strain FN102/pTAO in which TAO is the only oxidase activity, providing functional complementation of an otherwise lethal

phenotype,[45] with small modifications. Specifically, the first 75 nucleotides of the open reading frame were omitted in order to produce the physiologically active TAO protein without the 25 amino acid mitochondrial targeting sequence (MTS). Briefly, the recombinant protein, containing an N-terminal 6-His tag and Small ubiquitin Modifier Protein (SUMO) sequence, produced in *E. coli*, was purified using nickel column chromatography. The 6×-HIS/SUMO tag was cleaved off after purification using Ubiquitin-like-specific protease 1, ULP-1, yielding the purified ΔMTS-TAO. The details of this improved method for obtaining rTAO will be reported in a separate paper, in preparation. The compounds were evaluated as inhibitors of rTAO activity using the ubiquinol oxidase assay exactly as described.[38] In this assay, ubiquinol oxidase activity is measured by recording the absorbance change of 150 μM ubiquinol-1 at 278 nm in the presence of rTAO in Tris-HCl (pH 7.4), in the presence or absence of test compound at various concentrations at 25 ºC.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Histograms of TMRE fluorescence in populations of *T. b. brucei* trypomastigotes incubated with **6e** (Figure S1). Effect of **6e** on the cell cycle (Figure S2). Expression of TAO in *T. b. brucei* trypomastigotes (Figure S3). Molecular formula strings (CSV)

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ABBREVIATIONS USED

AAT, animal African trypanosomiasis; BSF trypanosome, bloodstream form trypanosome; 2,4-DHBA, 2,4-dihydroxybenzoic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HAT, human African trypanosomiasis; HFF cells, human foreskin fibroblast cells; LC, lipophilic cation; Ψ_m, mitochondrial membrane potential; PAO, phenylarsine oxide; SHAM, salicylhydroxamic acid; TAO, trypanosome alternative oxidase; TMRE, tetramethylrhodamine ethyl ester; TPP, triphenylphosphonium; MWI, microwave irradiation; RF, resistance factor; SI, selectivity index.

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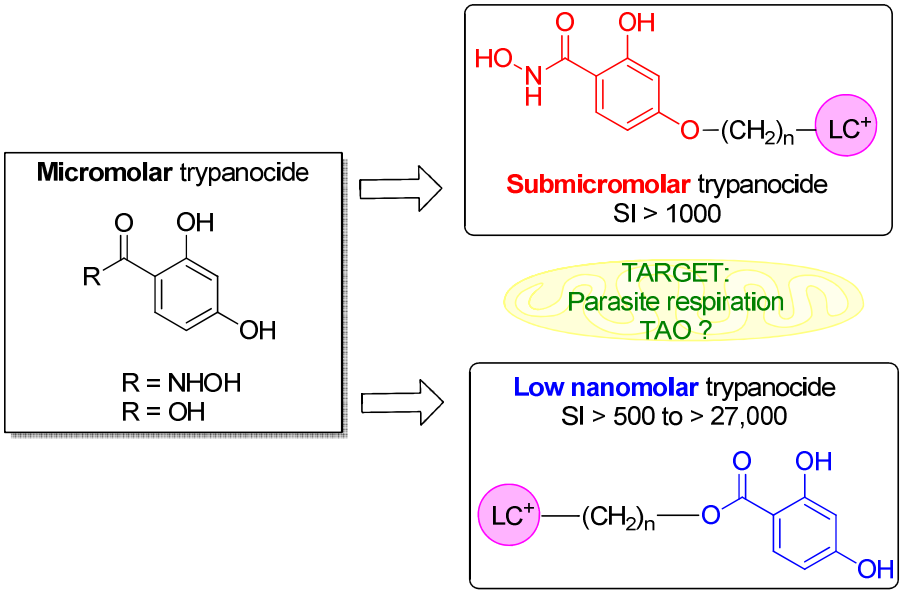
TOC graphic


Micromolar trypanocide
R = NHOH
R = OH
Submicromolar trypanocide
SI > 1000
TARGET:
Parasite respiration
TAO ?
Low nanomolar trypanocide
SI > 500 to > 27,000