SAR of 4-Alkoxybenzoic Acid Inhibitors of the Trypanosome Alternative Oxidase

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ABSTRACT: The SAR of 4-hydroxybenzaldehyde inhibitors of the trypanosomal alternative oxidase (TAO), a critical enzyme for the respiration of bloodstream forms trypanosomes, was investigated. Replacing the aldehyde group with a methyl ester resulted in a 10-fold increase in TAO inhibition and activity against *T. brucei*. Remarkably, two analogues containing the 2-hydroxy-6-methyl scaffold (**9e** and **16e**) displayed single digit nanomolar TAO inhibition, which represents the most potent 4-alkoxybenzoic acid derivatives described to date. **9e** was 50-times more potent against TAO and 10-times more active against *T. brucei* compared to its benzaldehyde analogue **23**. The farnesyl derivative **16e** was as potent a TAO inhibitor as ascofuranone with IC_{50} = 3.1 nM. Similar to ascofuranone derivatives, the 2-hydroxy and 6-methyl groups seemed essential for low nanomolar TAO inhibition of acid derivatives, suggesting analogous binding interactions with the TAO active site.

The bloodstream forms (BSF) of African trypanosomes, which cause sleeping sickness in humans and animal trypanosomiasis (nagana) in livestock, rely exclusively on glucose metabolism for energy production. Lacking a functional oxidative phosphorylation pathway, BSF trypanosomes use the trypanosome alternative oxidase (TAO) as terminal oxidase to re-oxidize the NADPH that accumulates during glycolysis. TAO is a non-haem cyanide–insensitive membrane-bound di-iron protein that catalyzes the oxidation of ubiquinol and the four electron reduction of oxygen to water.[1] This enzyme, which is essential for the respiration of BSF trypanosomes[2] and is conserved among *T. brucei* subspecies,[3] has been validated as a drug target against trypanosomes.[4-7]

In recent years, different series of TAO inhibitors have been reported that are based on the structure of the natural isoprenoid antibiotic ascofuranone (Figure 1).[8-12] Kita's group reported the pharmacophore of ascofuranone[8] and, shortly after, the 3-D structures of TAO in the presence and absence of ascofuranone derivatives, allowing a better understanding of inhibitor binding modes.[13] However, to date ascofuranone is the only TAO inhibitor among the ascofuranone-based compounds that has shown in vivo curative activity in different mouse models of trypanosomiasis.[6, 14] The modest success of the ascofuranone-like inhibitors against *T. brucei* is attributed due to poor physicochemical (drug-like) properties of these compounds, as they are highly lipophilic and hardly soluble in water, resulting in poor pharmacokinetic properties. In these inhibitors, a high degree of lipophilicity is required not only for effective binding to TAO[8] (i.e. a hydrophobic tail is necessary, Figure 1) but probably also to retain the inhibitors in the mitochondrial membrane where TAO is located.[11] The requirement for these properties has hampered the search for inhibitors with drug-like properties.

Recently, we have developed different series of TAO inhibitors based on the 4-hydroxybenzoate and 4-alkoxybenzaldehyde chemotypes (Figure 1, **A** and **D**). These compounds displayed submicromolar to low nanomolar IC_{50} values against recombinant TAO (rTAO) and micromolar activities against *T. brucei*. The conjugation of these inhibitors with a mitochondrion-targeting lipophilic cation tail via a methylene linker yielded very potent trypanocides against wild-type and multidrug-resistant *T. brucei* strains.[15, 16] In particular, compound **A** ($R_1 = {}^{+}PPh_3$, $IC_{50\ (rTAO)}$ = 0.09 μM) was able to reduce the parasite load of mice infected with *T. b. brucei rhodesiense* by ip administration at 4×10 mg/kg.[15]

In spite of the promising initial in vivo results, the benzoate derivatives **A** present several drawbacks: 1) a metabolic liability dependent on the substituents of the aromatic head (i.e. limiting the options for SAR development); 2) less than optimum water solubility; 3) a reduced therapeutic window in vivo (e.g. dosage > 4×10 mg/kg/ip was not tolerated, unpublished data). In contrast, the 4-alkoxy inhibitors (e.g. **B**–**D**), which are devoid of an ester bond, are expected to be resistant to serum hydrolases. In fact, compound **1** (Figure 1, D) proved to be stable in mouse

serum although it appeared to bind serum proteins to high degree.[15] This behavior was attributed to the presence of the benzaldehyde group which may form Schiff's bases with the amino groups of the proteins.

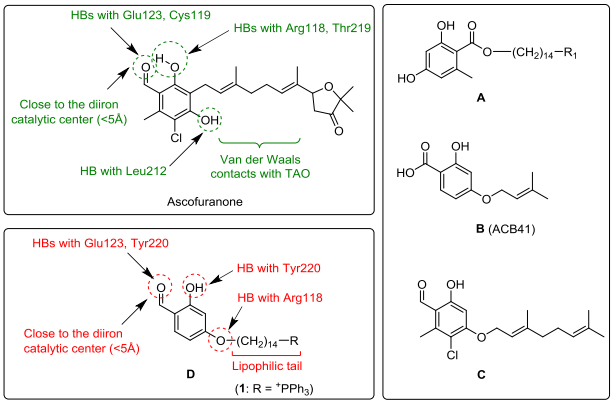


Figure 1. Structure of different TAO inhibitors chemotypes: ascofuranone, 4-hydroxybenzoates (**A**),[15, 16] 4-alkoxybenzoic acids (**B**),[17] and 4-alkoxybenzaldehydes (**C**, **D**).[12, 15] The main interactions of ascofuranone-like inhibitors with the TAO active site are shown in green color.[8, 13] The predicted main interactions of 4-alkoxybenzaldehyde **D** with the amino acids of the TAO active site[15] are highlighted in red color. HB = hydrogen bond.

In the current work, we further investigated the SAR of this promising TAO inhibitor scaffold by replacing the aldehyde group of **D** with a carboxylic acid (or ester), modulating the nature of the lipophilic tail (e.g. alkyl, geranyl, farnesyl) and of the substituent on the aromatic ring (Figure 2). In the designed compounds, the carboxylic acid function is expected to be in close proximity to the diiron center of TAO whereas the oxygen atom in 4-position is expected to engage in hydrogen bond (HB) interactions with polar amino acids from the TAO binding site (e.g. Arg118) similarly to **1** (Figure 1). The presence of a free acid group in the molecule should enhance the water solubility of the molecules.

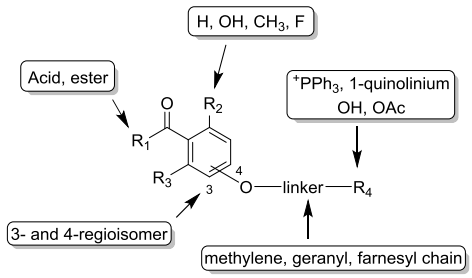
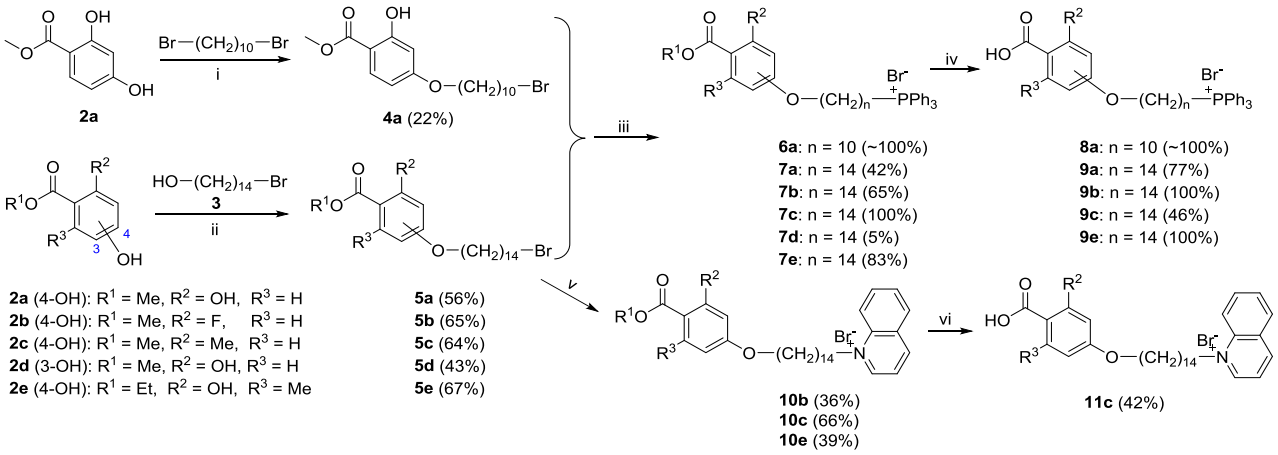


Figure 2. Structural modifications of 4-alkoxybenzoic acid derivatives studied in this work.

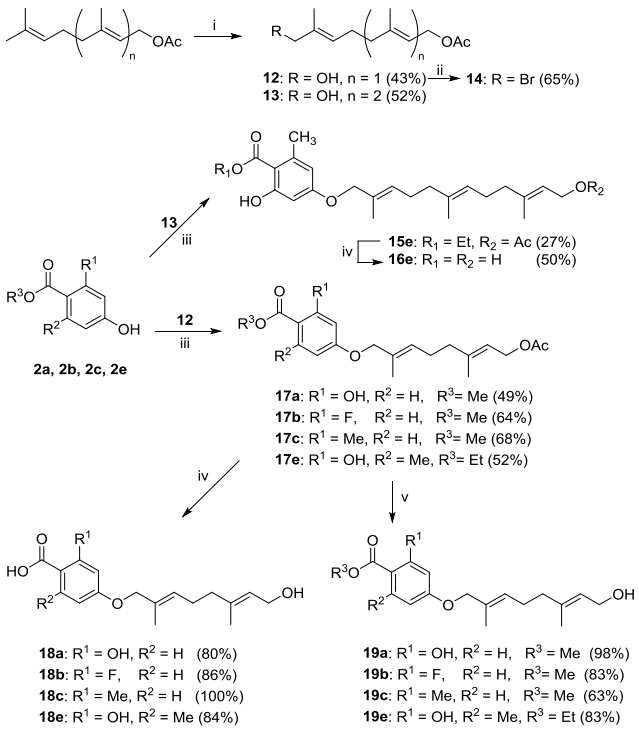
Results and discussion. The inhibitors with a lipocation alkyl tail (**6a**, **7a-e**, **10b**, **10c**, and **10e**) were synthesized in two steps starting from the corresponding 4-hydroxybenzoates **2a-e** (Scheme 1). Alkylation of **2a** with 1,10-dibromodecane and $NaHCO_3$ gave the bromoalkoxy compound **4a** with low yield (22%). Better yields of **5a-e** (43–67%) were obtained using the Mitsunobu protocol[18] with 14-bromotetradecan-1-ol.[19] Nucleophilic substitution of these bromo derivatives with triphenylphosphine or quinoline yielded the phosphonium (**6a**, **7a-e**) and quinolinium salts (**10b**, **10c**, **10e**). Smooth hydrolysis of the ester function with potassium carbonate[20] in MeOH/H_2O at 50 ºC yielded the carboxylic acids **8a** and **9a-e** with excellent yields. For the quinolinium salts, the hydrolysis was performed with 48% aqueous HBr to yield the acid derivatives **11b-e** (Scheme 1). The TAO inhibitors with a farnesyl (**15e**) or geranyl (**17a-c**, **17e**) tail were synthesized from **2a-e** by the Mitsunobu reaction using alcohols **12** and **13** (Scheme 2). Treatment of **15e**, **17a-c** and **17e** with KOH/MeOH at 60 ºC led to complete hydrolysis of the acetate and benzoate groups to give high yields of the carboxylic acids **16e**, **18a-c**, and **18e**. Selective hydrolysis of the acetate group of **17a-e** was performed with potassium carbonate to yield **19a-e**, respectively.

Scheme 1. Synthesis of TAO inhibitors with lipocation alkyl tail[a]



[a]Reagents and conditions. (i) $NaHCO_3$, CH_3CN, 65 ºC; (ii) DIAD, Ph_3P, THF, 0 ºC to rt; (iii) Ph_3P, CH_3CN, 80 ºC; (iv) K_2CO_3 (3 eq.), MeOH/H_2O (5/1) or EtOH/H_2O (5/1, for **7e**), 50 ºC; (v) Quinoline, CH_3CN, 80 ºC; (vi) HBr 48%, H_2O, 60 ºC.

Scheme 2. Synthesis of TAO inhibitors with geranyl and farnesyl tail[a]



[a]Reagents and conditions. (i) 1) SeO_2, ^tBuOOH, salicylic acid (cat.), CH_2Cl_2, 30 min, rt; 2) geranyl acetate or farnesyl acetate, 45 h, rt; (ii) CBr_4, Ph_3P, CH_2Cl_2; (iii) DIAD, Ph_3P, THF, 0 ºC to rt; (iv) 1) 1M KOH, MeOH, 60 ºC, 2) 1M HCl; (v) K_2CO_3, MeOH, rt (for **17a**) or K_2CO_3, EtOH, 35 ºC (for **17e**).

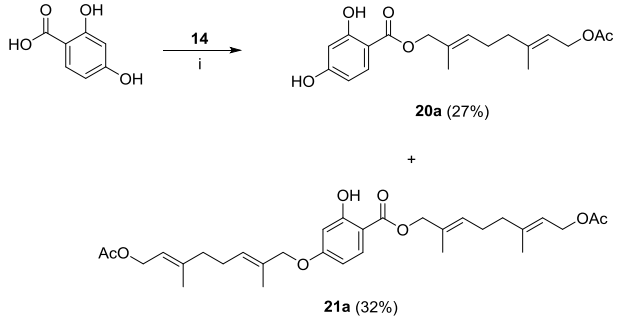
The compounds were assayed as inhibitors of the ubiquinol oxidase activity of purified ΔMTS-TAO as described.[15]

Cationic TAO inhibitors: The replacement of the aldehyde group of **23** by a methyl ester (**7a**) led to a 15-fold increase in TAO inhibition potency resulting in a 7-fold improvement in activity against *T. brucei* (Table 1).In this methyl ester series, replacement of the 2-OH group by a fluorine (**7b**) or methyl substituent (**7c**) resulted in 50- and >300-fold decreased inhibition compared with **7a**, respectively (2-OH > 2-F>> 2-CH_3).These data confirmed the need of a hydrogen bond-forming substituent at this position for tight binding to TAO. As noted earlier with the 4-hydroxybenzoate series,[15, 16] a methylene linker of less than C14 was detrimental to TAO inhibition (compare **6a**/**7a**). Switching the 4-alkoxy tail (**7a**) to the 3-position (**7d**) of the aromatic ring hardly influenced TAO inhibition (0.015 and 0.012 μM, respectively), which correlated with similar trypanocidal activities for both compounds (0.018 and 0.028 μM, respectively).

Interestingly, TAO inhibition was limited by the size of the ester substituent at C1: an ethyl ester substituent was detrimental with a 60-fold drop in inhibitor potency compared to the free acid (compare **7e/9e**, **15e/16e**). The same trend was observed for the geranyl series (Table 2) with higher IC_{50} values for the ethyl ester **19e** (>5 μM) compared to the free acid **18e** (2.4 μM). Intriguingly, replacement of the methyl ester (**7a** and **7b**) or aldehyde group (**23**) at C1 by a carboxylic acid led to a drastic loss of inhibition (IC_{50} >5 μM for **9a** and **9b**). In contrast, the 2-hydroxy-6-methyl acid analogue **9e** inhibited TAO in the low nanomolar range (IC_{50} = 4.2 nM) indicating that the presence of the 6-methyl substituent in **9e** is playing a key role in the binding of inhibitors with an acid functional group, similar to the ascofuranone-based inhibitors.[8] The triphenylphosphonium cation was more favorable than the 1-quinolinium cation for upper inhibition of TAO (compare **7e**/**10e**, **7b**/**10b**). In this series of quinolinium salts, an aldehyde group at C1 (**24**, IC_{50} = 1.23 μM) was superior to an ester or acid group (IC_{50} > 5 μM).

Non-cationic inhibitors with methylene, geranyl, and farnesyl tail: the acid derivative **15e** with a farnesyl lipophilic tail was the most potent inhibitor of these series (IC_{50} = 3.1 nM), superior to the geranyl counterpart **18e** and the rest of geranyl acid derivatives (**18a**–**c**) which were single digit micromolar inhibitors (Table 2). Remarkably, one extra isoprenyl unit (**15e**) was responsible for an increase of inhibition by 3 orders of magnitude within this series. In contrast, the benzoate derivatives (**15e**, **17a**, **17c**, **19b**, **19e**) were much less potent TAO inhibitors (IC_{50} > 5 μM), with the exception of **5a** and **19c** (IC_{50} ≈ 2 μM). These inhibition values are in the same range as previously reported TAO inhibitors bearing a 4-alkoxy isoprenoid chain such as **B** (ACB41, K_i = 5μM)[17] or **C** (IC_{50} = 1 μM).[12, 17] We decided to study whether a geranyl tail would have the same (weak) effect on the inhibitory potency of the benzoate series derived from scaffold A. The benzoate derivative **20a** with the geranyl tail at C1 was synthesized by reaction of 1 equivalent of 2,4-dihydroxybenzoic acid and 1 equivalent of **14** in the presence of potassium carbonate in anhydrous acetone at 60 ºC. The product of dialkylation **21a** was also isolated by silica chromatography (Scheme 3).

Scheme 3. Synthesis of 4-hydroxybenzoate inhibitor 20a.



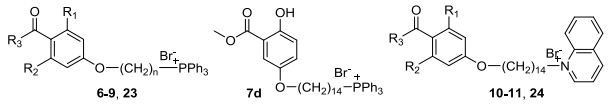
[a]Reagents and conditions. (i) K_2CO_3, acetone, 60 ºC, 2 h.

Compound **20a** inhibited TAO in the low nanomolar range (IC_{50} = 9.1 nM), with a potency similar to that of its 14-bromotetradecane benzoate analogue (10.8 nM).[15] This results indicates that the geranyl tail is a valid biososteric replacement of the methylene tail for the 4-hydroxybenzoate inhibitor series (A) but it is less effective for the 4-alkoxybenzoic acid derivatives. Not surprisingly, the disubstituted derivative **21a** with the 4-OH position blocked by the 4-alkoxygeranyl group inhibited TAO poorly (> 5 μM). The 4-hydroxybenzoate inhibitors are expected to bind TAO with the 4-OH group close to the diiron catalytic center, which is not possible with this disubstituted compound.

The trypanocidal activity of the new compounds was assessed against wild-type and multidrug-resistant strains of *T. b. brucei*.

In the cationic series, the triphenylphosphonium conjugates (**7b**, **7c**, **7e**) were 6- to 48-times more potent than the quinolinium salts (**10b**, **10c**, **10e**; Table 1) in agreement with previous results.[15, 16] However, the benzoates quinolinium salts **10c** and **10e** were 5- to 10-fold more potent than the benzaldehyde hit **1** (EC_{50} = 1.75 µM). Replacing the aldehyde group (**1**, R_3 = H) by a methyl ester function (**7a**, R_3 = OMe) resulted in a 7-fold increase in trypanocidal activity against *T. b. brucei*. In this benzoate series, replacement of the 2-OH with a 2-Me (**7c**) had no influence on the activity whereas a 2-F (**7b**) or (2-OH, 6-Me) substituents (**7e**) gave slightly less potent compounds (2.5-fold). The influence on trypanocidal activity of a free acid group in R_3 depended on the nature of the substituents R_1 and R_2; the benzoate derivatives **7b** (R_2 = F, R_3 = H) and **7e** (R_2 = Me, R_3 = OH) were 1.4- to 4-times less active than the free acids **9b** and **9e**, respectively. In contrast, the benzoates **7a** (R_1 = OH, R_2 = H), **7c** and **10c** (R_1= Me, R_2 = H) were 5-, 10-, and 66-times more active than the free acids **9a**, **9c** and **11c**, respectively.

Table 1. Biological evaluation of 4-alkoxybenzoate-lipocation conjugates against rTAO (IC_{50}, µM), *T. b. brucei* (EC_{50}, µM), and human cells (CC_{50}, µM).

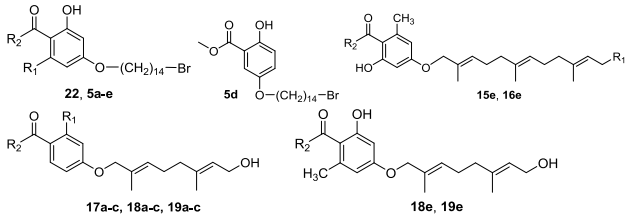


Cmpd	n	R_1	R_2	R_3	rTAO[a]	*T. b. brucei* WT[b]	HEK cells[c]	SI[d]
1[e]	14	OH	H	H	0.22 ± 0.01	0.133 ± 0.003	>200	>1500
6a	10	OH	H	OMe	>5	0.002 ± 0.001	0.44 ± 0.02	184
7a	14	OH	H	OMe	0.015 ± 0.003	0.018 ± 0.003	ND[f]	
7b	14	F	H	OMe	0.74 ± 0.04	0.05 ± 0.01	0.22 ± 0.04	4.5
7c	14	Me	H	OMe	>5	0.018 ± 0.004	0.35 ± 0.01	19
7e	14	OH	Me	OEt	0.250 ± 0.011	0.05 ± 0.01	0.63 ± 0.01	13
7d	14	OH	H	OMe	0.012 ± 0.008	0.028 ± 0.02	0.64 ± 0.03	10
8a	10	OH	H	OH	>5	0.73 ± 0.01	8.2 ± 0.1	11
9a	14	OH	H	OH	>5	0.086 ± 0.005	19.6 ± 1.3	227
9b	14	F	H	OH	>5	0.037 ± 0.001	8.35 ± 0.53	225
9c	14	Me	H	OH	>5	0.19 ± 0.01	4.24 ± 0.10	22
9e	14	OH	Me	OH	0.0042 ± 0.0002	0.013 ± 0.003	0.83 ± 0.06	64
10b	14	F	H	OMe	>5	2.4 ± 0.1	3.36 ± 0.18	1.4
10c	14	Me	H	OMe	>5	0.16 ± 0.01	0.30 ± 0.04	1.9
10e	14	OH	Me	OEt	>5	0.29 ± 0.05	0.61 ± 0.001	2.1
11c	14	Me	H	OH	>5	10.6 ± 0.5	24.4 ± 2.8	2.3
23[h]	14	OH	H	H	1.23	1.75 ± 0.01	>200	>114
AF[g]					0.002 ± 0.0004			
SHAM[h]					5.93 ± 0.13	62.9 ± 0.6		
Pent[i]						0.0042 ± 0.0009		
PAO[j]							1.1 ± 0.15	

[a]Purified recombinant trypanosome alternative oxidase from *T. b. brucei* (n = 3). [b]Trypomastigotes of *T. b. brucei* s427. [c]Cytotoxicity on human endothelial kidney cells. [d]Selectivity index = CC_{50}/EC_{50} (*T. brucei* WT). [e]Taken from reference [15]. [f]Not determined. [g]Ascofuranone. [h]Salicylhydroxamic acid. [i]Pentamidine. [j]Phenylarsine oxide.

The non-cationic benzoate inhibitors with a geranyl or farnesyl tail holding an OAc group (**15e**, **17a**, **17c**) displayed low micromolar activity against *T. brucei* (< 20 µM). In contrast, the inhibitors with a free acid group (**18a**–**c**, **18e**, **16e**) were all inactive at the highest dose tested (100 µM), possibly due to poor uptake of these negatively charged carboxylate compounds (pK_a < 5) into the trypanosomes' mitochondria. The benzoate derivatives with a 14-bromotetradecane chain (**5a**–**c**) were inactive except **5d** (8.1 µM) and **5e** (8.9 µM) which displayed IC_{50} < 10 µM.

Table 2. Biological evaluation of 4-alkoxybenzoates with alkyl, geranyl and farnesyl tail against rTAO (IC_{50}, µM), *T. b. brucei* (EC_{50}, µM), and human cells (CC_{50}, µM).



Cmpd	R_1	R_2	rTAO[a]	*T. b. brucei* WT[b]	HEK cells[c]	SI[d]
22[c]	H	H	0.073 ± 0.011	17.6 ± 0.5		
5a	H	OMe	2.3 ± 0.2	NE/100	ND	
5e	Me	OEt	>5	8.9 ± 1.2	29.6 ± 5.6	3.3
5d			>5	8.1 ± 1.0	28.6 ± 0.4	3.5
15e	OAc	OEt	>5	6.6 ± 1.3	20.8 ± 4.8	3.1
16e	OH	OH	0.0031±0.0004	NE/100	ND	
17a	OH	OMe	>5	19.3 ± 1.3	31.8 ± 4.2	1.7
17c	Me	OMe	>5	17.0 ± 0.5	45.1 ± 1.2	2.7
18a	OH	OH	2.1 ± 0.4	NE/100	ND	
18b	F	OH	1.1 ± 0.1	NE/100	ND	
18c	Me	OH	0.92 ± 0.15	NE/100	ND	
19a	OH	OMe	ND	ND	ND	
19b	F	OMe	>5	NE/100	>100	
19c	Me	OMe	1.69 ± 0.38	NE/100	>100	
19e		OEt	>5	ND	ND	
18e		OH	2.4 ± 0.5	NE/100	ND	

Footnotes and control drugs: see Table 1.

Little difference in activity was observed between WT and the multidrug resistant cell line B48 with resistance factors (RF) close to 1 (Tables S1)), indicating they do not utilise the known drug transporters TbAT1 and HAPT.[21, 22] In contrast, several inhibitors (**5d**, **5e**, **8a**, **9c**, **9e**, **10b, 20a**) were more effective against the *T. brucei* cell line from which all three aquaporins were deleted (AQP1-3 triple KO[23]). These trypanosomes are particularly sensitive to TAO inhibitors because they cannot dispose of the glycerol produced in large quantity under anaerobic conditions (i.e. when TAO is inhibited). Accumulation of glycerol is toxic to the cells as a result of the inhibition of the glycerol kinase and depletion of ATP production.[14, 24] The same compounds were also significantly more effective against *T. b. brucei* WT (RF < 1) when co-incubated with 5 mM glycerol, which inhibits the anaerobic ATP production pathway (Tables S1). Analogous results were obtained with the benzaldehyde inhibitors **1**, **22**, and **23**,[15] supporting the view that TAO is highly likely to be the main target of **5d**, **5e**, **8a**, **9c**, **9e**, **10b**, and **20a**. In contrast, compounds **7b**, **7c**, **7e**, **9a**, and **9b** appeared to be less dependent on TAO inhibition as shown by the RF > 2 against the AQP1-3 knockout line and against *T. b. brucei* WT+glycerol indicating that these compounds probably have

multi-target activity. Cytotoxicity against HEK cells was low (> 20 µM) for the non-cationic inhibitors with a geranyl (**17a**, **17c**, **20a**), farnesyl (**15e**) and methylene (**5d**, **5e**) tail, and the cationic inhibitors with a free acid group (**8a**, **9a**–**c**) (CC_{50} > 4 µM, SI from 11 to 227). In contrast, the inhibitor **9e** was more cytotoxic (CC_{50} = 0.83 µM) although this still yielded selectivity indexes of 64 and 230 towards *T. brucei* WT and AQP1-3 knockout strains, respectively. Other inhibitors with a lipocation tail displayed variable cytotoxicity against human cells (CC_{50} < 1 µM for **6a**, **7a**–**d**; CC_{50} > 20 µM for **11c**).

In summary, cationic 4-alkoxy benzoic acid derivatives are more potent TAO inhibitors than their benzaldehyde counterparts. The 2,4-dihydroxy-6-methyl carboxylic acid scaffold, in particular, gave the most potent TAO inhibitors (**9e**, **16e**). Most of the 4-alkoxybenzoate-lipocation conjugates were also effective nanomolar range trypanocides. However, higher toxicity against human cells was observed resulting in lower selectivity indexes compared with previous series.

ASSOCIATED CONTENT

Supporting Information

Synthesis of compounds **4**–**21**. Biology experimental protocols. Table S1. The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

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ABBREVIATIONS

BSF trypanosome, bloodstream form trypanosome; DIAD, diisopropylazodicarboxylate; MTS, mitochondrion-targeting sequence; HB, hydrogen bond; RF, resistance factor; TAO, trypanosome alternative oxidase

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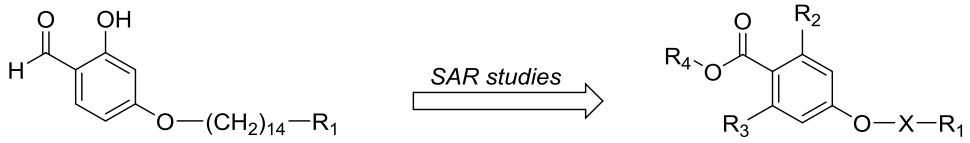

	R_1	IC_{50} (μM) rTAO	IC_{50} (μM) *T. b. brucei*
23	$^{+}PPh_3$	0.22	0.13
24	1-quinolinium	1.23	1.75

	X	R_1	R_2	R_3	R_4	IC_{50} (μM) rTAO	IC_{50} (μM) *T. b. brucei*
7a	$(CH_2)_{14}$	$^{+}PPh_3$	OH	H	OMe	0.015	0.018
9e	$(CH_2)_{14}$	$^{+}PPh_3$	OH	CH_3	OH	0.004	0.013
15e	farnesyl	OH	OH	CH_3	OH	0.003	> 100