Research Article

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Future Medicinal Chemistry

Two in one: bifonctional derivatives of trolox acting as antimalarial and antioxidant agents

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Background: The aim of the present work was to set-up compounds that are able to act simultaneously as antimalarial and antioxidants. Trolox, a known antioxidant was chosen as a core structure to ensure the antioxidant activity and contribute to antiplasmodial effect. **Results:** Ten compounds were prepared in one step and evaluated on chloroquino-sensitive (3D7) and chloroquino-resistant (FcB1) strains of *Plasmodium falciparum*. The most active compound (**3d**) shows antiplasmodial activity in the range of chloroquine against chloroquino-sensitive and chloroquino-resistant *P. falciparum* strain. The antioxidant activity of (**3d**) was conducted through four tests and was found to be more potent than trolox itself and L-ascorbic acid. **Conclusion:** Compound (**3d**) can be considered as an excellent lead molecule for further *in vivo* studies. This study paves the way for building large chemical libraries to be investigated in the field of malaria.

Graphical abstract:

First draft submitted: 13 April 2020; Accepted for publication: 24 July 2020; Published online: TBC

Keywords: antioxidants • antiplasmodial • chloroquine • malaria • trolox

Malaria kills around 0.5 million people a year. It is estimated that 90% of malaria cases are located in tropical and subtropical regions, peculiarly in sub-Saharan Africa [1]. Malaria is caused by a protozoal parasite of the genus *Plasmodium* which is transmitted by *Anopheles* mosquitoes [2]. Among *Plasmodium* genus, the major threat is *Plasmodium falciparum*, responsible for more than 95% of mortality due to malaria. In humans, after inoculation, the protozoa migrates to the hepatocytes where it multiplies and matures as a hepatic schizont which releases merozoites that enter the bloodstream and infect red blood cells [3–5]. Following the initial asexual replication in the liver, the parasite undergoes a new asexual multiplication schizogony in the erythrocytes (commonly named the blood stage parasites) that are responsible for symptoms of malaria.

It is admitted that infected red blood cells are under constant oxidative stress**,** however the relationship between the redox status of the parasite and the host is complex [6]. This stress is of different origins such as the effect of exogenous and endogenous reactive oxidant species (ROS) as well as reactive nitrogen species produced by the immune system of the host [7]. Moreover, ROS can also be produced by the mitochondrial electron transport and by various metabolic processes [8]. The consequence of the constant oxidative stress during infection of red blood cells is the alteration of the human defence system against such stress. Unfortunately, the major antimalaria drugs such as chloroquine and derivatives lack antioxidant activity to counterbalance the effect of oxidative stress. Chloroquine was even shown to exert oxidative potential, advocating hepatotoxicity [9], explaining the use of additive compounds as protectors against the chloroquine-induced oxidative stress [10].

Owing to the harmful role of the oxidative stress following the infection by *P. falciparum*, more effective antimalaria drugs should be active on *Plasmodium* spp. While possessing antioxidant potential.

Previously, we have demonstrated that **trolox analogs** are able to act as specific reversal agents for the ABCI4 transporter that confers resistance of *Leishmania* toward antimony treatments [11]. In continuing our program aimed

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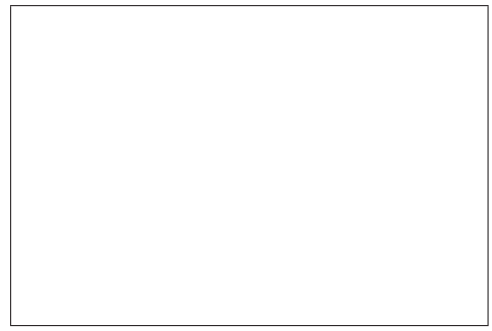


Figure 1. Structures of trolox and trolox derivatives.

at discovering efficient antiparasitic drug candidates, we targeted new chemical entities derived from trolox with the aim to design and obtain dual compounds having antimalarial and antioxidant activities.

Trolox (Figure 1) is the common name for 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble derivative of Vitamin E. Trolox was chosen because it possesses three key features, **a)** a pharmacophore, previously identified as important for reversing the resistance of parasitic strains toward antiparasitic drugs, **b)** high antioxidant activity, **c)** a carboxylic function that can be used for linking a variety of chemical entities.

In the present work, we targeted two types of compounds (Figure 1): **a)** trolox linked to chemical entities commonly found in antimalaria drugs, **b)** compounds having two trolox scaffolds linked through a linker.

Experimental chemistry

Materials & methods

NMR spectra were recorded on Bruker AC-400 instrument (400 MHz for ^{1}H and 100 MHz for ^{13}C). Tetramethylsilane (TMS) was used as an internal standard and chemical shifts are reported as δ values (p.p.m.) relative to TMS. Mass spectra were realized at the ICMG, FR 2607, Grenoble, France using a Fisons Trio 1000 instrument. Combustion analyses were performed at the Analytical Department of CNRS, Vernaison, France and all analyzed compounds have a purity of at least 98%. Melting points were measured by using a Büchi B540 melting point apparatus and are uncorrected. TLC was realized on Merck silica gel F-254 plates (0.25 mm, thickness) and flash chromatography was done on Merck silica gel 60, 200–400 mesh. Chemicals and reagents were obtained either from (Sigma-Aldrich, Saint-Quentain Fallavier, France) or (Acros, Gell, Belgium) companies and were used as obtained.

General procedure of the synthesis of compounds (2a-f) & (3a-d)

Trolox was dissolved in DMF under nitrogen atmosphere, amine (0.5 to 1 eq.) and triethylamine (3 eq.) was added successively. After stirring for 30 min at room temperature, BOP-Cl (1.3 eq.) was finally added to the reaction mixture and the solution was stirred overnight at room temperature. The mixture was mixed with cold water, extracted by ethyl acetate and washed with aqueous NaOH (1 M). The resulting organic phase was evaporated until dryness, then purified by column chromatography or by simple precipitation in diethyl ether. Crystallizations were attempted on compounds **2a** and **3b** and were unsuccessful. Therefore, no attempts were realized on the rest of the compounds.

N-(Phenyl)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamide (2a)

Purified by column chromatography (CH_2Cl_2/MeOH 99:1); Yield: 33%; white powder; mp: 169–171°C; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 7.17–6.62 (m, 5H, H-phenyl), 2.65 (m, 3H, H3, H3′, H4), 2.4 (m, 2H, H4′), 2.28 (s, 3H, CH_3), 2.21 (s, 3H, CH_3), 2.10 (m, 1H, H3′), 1.60 (s, 3H, CH_3); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 172.71 (CO), 147.43 (C-6), 146.04 (C-9), 144.17 (C arom), 138.59 (C arom), 129.9 (C arom), 122.01 (C-5), 121.7 (C-7), 119.26 (C-8), 111.3 (C arom), 109.74 (C arom), 106.35 (C arom), 78.65 (C-2), 29.6 (C-4), 24.48 (C-3), 20.63 (CH_3), 12.45 (CH_3), 12.23 (CH_3), 11.5 (CH_3); high resolution mass spectrometry (HRMS) calcd for $C_{20}H_{23}N_1O_3$: 325.1678; found 326.2200 $[M + H]^+$. Anal. Calcd for $C_{20}H_{23}NO_3$: C, 73.82; H, 7.12; N, 4.30. Found: C, 73.14; H, 7.01; N, 4.14.

6-Hydroxy-N-(3-hydroxyphenyl)-2,5,7,8-tetramethylchroman-2-carboxamide (2b)

Purified by precipitation in Et_2O; Yield: 66%; white powder; mp: 206–209°C; ^{1}H-NMR (CD3COCD3, 400 MHz) δ: 8.60 (bs, 1H, NH), 8.30 (bs, 1H, OH), 7.28 (s, 1H, H2′), 7.06 (t, *J* = 8.05 Hz, 1H, H5′), 6.96 (d, *J* = 8.02 Hz, 1H, H4′), 6.54 (bs, 1H, OH), 6.53 (d, *J* = 8.00 Hz, 1H, H6′), 2.62–2.33 (m, 3H, H-3, H-4, H-4′), 2.23 (s, 3H, CH_3), 2.15 (s, 3H, CH_3), 2.05 (s, 3H, CH_3), 1.88 (m, 1H, H-3′), 1.52 (s, 3H, CH_3); ^{13}C-NMR (CD3COCD3, 100 MHz) δ: 173.13 (CO), 158.72 (C-1′), 147.54 (C-6), 144.89 (C-9), 140.39 (C-3′), 130.41 (C-2′), 123.48 (C-5), 122.56 (C-7), 121.04 (C-8), 118.57 (C-10), 111.78 (C-4′), 111.55 (C-6′), 107.57 (C-5′), 79.05 (C-2), 30.51 (C-4), 24.54 (CH_3), 21.17 (CH_2), 12.84 (CH_3), 12.30 (CH_3), 11.87 (CH_3); HRMS calcd $C_{20}H_{23}NO_4$: 341.4009; found 342.5210 $[M + H]^+$. Anal. Calcd for $C_{20}H_{23}NO_4$: C, 70.36; H, 6.79; N, 4.10. Found: C, 70.12; H, 6.61; N, 4.00.

N-(3,4-dimethoxyphenethyl)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamide (2c)

Purified by precipitation in Et_2O; Yield: 60%; white powder; mp: 118–120°C; ^{1}H-NMR (CD3COCD3, 400 MHz) δ: 6.83 (bs, 1H, NH), 6.74 (m, 3H, H4′′, H5′′), 6.71 (s, 1H, OH), 6.50 (dd, *J* = *8 Hz, J* = *1.79 Hz*, 1H, H5′′), 6.48 (s, 3H, OCH_3), 3.74 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 3.37 (m, 2H, N-CH_2), 2.59–2.25 (m, 3H, H-3, H-4, H-4′), 2.46 (m, 2H, CH_2-Ph), 2.12 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 1.98 (s, 3H, CH_3), 1.74 (m, 1H, H-3′), 1.39 (s, 3H, CH_3); ^{13}C-NMR (CD3COCD3, 100 MHz) δ: 174.35 (CO), 150.44 (C' arom), 148.97 (C' arom), 147.15 (C-6), 145.09 (C-9), 132.64 (C' arom), 123.20 (C-5), 122.32 (C-7), 121.56 (C' arom), 120.73 (C-8), 118.48 (C-10), 113.40 (C' arom), 112.92 (C' arom), 78.81 (C-2), 56.19 (OCH_3), 56.02 (OCH_3), 40.95 (CH_2-Ph), 35.96 (CH_2-N), 30.56 (C-4), 25.11 (CH_3), 21.20 (C-3), 12.84 (CH_3), 12.15 (CH_3), 11.90 (CH_3), HRMS calcd $C_{24}H_{31}NO_5$: 413.5066; found 414.6903 $[M + H]^+$. Anal. Calcd for $C_{24}H_{31}NO_5$: C, 69.71; H, 7.56; N, 3.39. Found: C, 69.49; H, 7.53; N, 3.28.

6-Hydroxy-2,5,7,8-tetramethyl-N-(quinolin-2-yl)chroman-2-carboxamide (2d)

Purified by precipitation in Et_2O; Yield: 66%; white powder; mp: 213–2015°C; ^{1}H-NMR (DMSO-d6, 400 MHz) δ: 10.84 (bs, 1H, NH), 8.82 (d, *J* = 2.45 Hz, 1H, H5"), 8.62 (d, *J* = 7.38 Hz, 1H, H7"), 8.37 (d, *J* = 7.99 Hz, 1H, H2"), 7.59 (m, 3H, H3", H4", H6"), 3.31 (m, 1H, H2), 2.40 (s, 3H, CH_3), 2.08 (s, 3H, CH_3), 1.95 (s, 3H, CH_3), 1.56 (m, 1H, H3), 1.20–1.00 (m, 1H, H3′); ^{13}C-NMR (DMSO-d6, 100 MHz) δ: 172.32 (CO), 148.75 (C-6), 146.25 (C-9), 143.44 (CH arom), 137.65 (CH arom), 136.66 (CH arom), 133.47 (CH arom), 127.69 (CH arom), 127.02 (CH arom), 123.04 (CH arom), 122.37 (C-5), 122.04 (C-7), 121.47 (C-8), 116.90 (C-10), 115.21 (CH arom), 78.04 (C-2), 47.49 (CH_2), 33.34 (CH_2), 24.92 (CH_3), 12.78 (CH_3), 11.87 (CH_3), 11.78 (CH_3); HRMS calcd $C_{23}H_{24}N_2O_3$: 376.4483; found 377.56 $[M + H]^+$. Anal. Calcd for $C_{23}H_{24}N_2O_3$: C, 73.38; H, 6.43; N, 7.44. Found: C, 73.32; H, 6.39; N, 7.36.

N-(5-aminonaphthalen-1-yl)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamide (2e)

Purified by column chromatography (EtOAc/c-Hex 4:6); Yield: 28%; white powder; mp: 175–178°C; ^{1}H-NMR (MeOD, 400 MHz) δ: 7.83 (d, *J* = *8.53 Hz*, 1H, H6"), 7.67 (d, *J* = *7.1 Hz*, 1H, H2"), 7.37 (m, 1H, H7"), 7.03 (m, 2H, H3"), 6.75 (d, *J* = *7.37 Hz*, 1H, H4"), 6.41 (d, *J* = *8.43 Hz*, 1H, H8"), 2.53 (m, 3H, H2, H3, H3′), 2.29 (s, 6H, CH_3), 2.23 (s, 6H, CH_3), 2.08 (s, 6H, CH_3), 1.93 (m, 2H, H2′), 1.70 (s, 6H, CH_3); ^{13}C-NMR (MeOD, 100 MHz) δ: 175.73 (CO), 147.82 (C arom), 145.96 (C-6), 145.55 (C-9), 133.0 (C arom), 130.18 (C arom), 128.33 (C arom), 125.82 (C-5), 125.46 (C-7), 124.85 (C arom), 123.2 (C arom), 123.14 (C-8), 122.42 (C-10), 121.21 (C arom), 119.34 (Carom), 111.28 (C arom), 110.99 (C arom), 80.29 (C-2), 31.15 (C-4), 25.85 (C-3), 22.0 (CH_3), 13.07 (CH_3), 12.45 (CH_3), 12.06 (CH_3); HRMS calcd for $C_{24}H_{26}N_2O_3$: 390.4748; found 391.2111 $[M + H]^+$. Anal. Calcd for $C_{24}H_{26}N_2O_3$: C, 73.82; H, 6.71; N, 7.17. Found: C, 73.77; H, 6.66; N, 7.08.

N-(2-[1H-indol-3-yl]ethyl)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamide (2f)

Purified by column chromatography (EtOAc/c-Hex 4:6); Yield: 46%; white powder; mp: 185–187°C; ^{1}H-NMR (CD3COCD3, 400 MHz) δ: 9.99 (bs, 1H, NH), 7.56 (d, *J* = 7.83 Hz, 1H, H7′), 7.36 (d, *J* = 8.07 Hz, 1H, H4′), 7.06 (m, 2H, H5′, H6′), 6.62 (s, 1H, H2′), 3.48 (bs, 1H, CH_2-NH), 2.91 (s, 1H, CH_2-CH_2-NH), 2.89–2.27 (m, 3H, H3, H4, H4′), 2.11 (s, 3H, CH_3), 2.05 (s, 3H, CH_3), 1.75 (m, 1H, H3′), 1.4 (s, 3H, CH_3); ^{13}C-NMR (DMSO-d6, 100 MHz) δ: 174.96 (CO), 147.74 (C-6), 145.73 (C-9), 138.45 (CH arom), 129.03 (CH arom), 124.02 (C-5), 123.86 (C-7), 123.01 (C-8), 122.81 (CH arom), 121.42 (C-10), 113.59 (CH arom), 112.80 (CH

arom), 79.37 (C-2), 40.70 (CH_2), 26.84 (CH_2), 25.50 (CH_3), 13.42 (CH_3), 12.67 (CH_3), 12.48 (CH_3); HRMS calcd $C_{24}H_{28}N_2O_3$: 378.4641; found 379.6050 $[M + H]^+$. Anal. Calcd for $C_{24}H_{28}N_2O_3$: C, 73.44; H, 7.19; N, 7.14. Found: C, 73.28; H, 7.12; N, 7.06.

N,N′-(1,4-phenylene)bis(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamide) (3a)

Purified by column chromatography (EtOAc/c-Hex 1:1); Yield: 3%; oil; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 8.17 (bs, 2H, NH), 7.26 (d, *J* = 8.59 Hz, 2H, H arom), 6.70 (d, *J* = 8.59 Hz, 2H, Harom), 2.64 (m, 2H, H2), 2.26 (s, 6H, CH_3), 2.21 (s, 6H, CH_3), 2.10 (s, 6H, CH_3), 2.06–1.96 (m, 6H, H2′, H3, H3), 1.60 (s, 6H, CH_3); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 173.78 (CO), 147.35 (C-6), 145.63 (C-9), 144.87 (C arom), 130.40 (C arom), 123.4 (C-5), 123.06 (C arom), 122.37 (C-7), 120.66 (C-8), 119.63 (C-10), 116.97 (C arom), 79.99 (C-2), 31.06 (C-4), 25.83 (C-3), 22.04 (CH_3), 13.85 (CH_3), 13.63 (CH_3), 12.91 (CH_3); HRMS calcd $C_{34}H_{40}N_2O_6$: 572.6912; found 573.90 $[M + H]^+$. Anal. Calcd for $C_{34}H_{40}N_2O_6$: C, 71.31; H, 7.04; N, 4.89. Found: C, 71.06; H, 6.98; N, 4.82.

N,N′-(propane-1,3-diyl)bis(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamide) (3b)

Purified by column chromatography (EtOAc/c-Hex 4:6); Yield: 13%; white powder; mp: 151–153°C; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 6.78 (bs, 1H, NH), 3.00 (m, 4H,CH_2-N), 2.45 (m, 3H, H2, H3, H3′), 2.21 (s, 3H, CH_3), 2.18 (s, 3H, CH_3), 2.09 (s, 3H, CH_3), 2.01 (s, 3H, CH_3), 1.85 (m, 1H, H2′), 1.80 (m, 2H, CH_2-CH_2-N), 1.52 (s, 3H, CH_3), 1.50 (s, 3H, CH_3); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 176.36 (CO), 147.13 (C-6), 145.94 (C-9), 123.73 (C-5), 123.12 (C-7), 120.63 (C-8), 119.46 (C-10), 79.83 (C-2), 37.19 (C-4), 31.5 (CH_2-CH_2-N), 31.16 (CH_2-CH_2-N), 26.20 (C-3), 22.15 (CH_3), 13.83 (CH_3), 12.89 (CH_3), 12.80 (CH_3); HRMS calcd $C_{31}H_{42}N_2O_6$: 538.6750; found 539.3212 $[M + H]^+$. Anal. Calcd for $C_{31}H_{42}N_2O_6$: 69.12; H, 7.86; N, 5.20. Found: C, 68.98; H, 7.83; N, 5.11.

N,N′-([1r,4r]-cyclohexane-1,4-diyl)bis(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamide) (3c)

Purified by column chromatography (EtOAc/c-Hex 1:1); Yield: 25%; white powder; mp: 216–219°C; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 7.49 (bs, 1H, NH), 6.90 (bs, 2H, OH), 3.38 (m, 2H, H1", H4"), 3.33 (bs, 4H, 2 CH_2), 2.48 (bs, 4H, 2 CH_2), 2.25 (m, 3H, H2, H3, H3′), 2.05 (s, 3H, CH_3), 2.04 (s, 3H, CH_3), 1.96 (s, 3H, CH_3), 1.71 (m, 1H, H2′), 1.32 (s, 3H, CH_3); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 174.52 (CO), 147.82 (C-6), 145.85 (C-9), 124.71 (C-5), 123.07 (C-7), 122.34 (C-8), 119.15 (C-10), 79.04 (C-2), 48.71 (CH_2), 32.25 (C-4), 31.45 (CH_2), 25.78 (CH_3), 22.04 (C-3), 14.77 (CH_3), 13.99 (CH_3), 13.80 (CH_3); HRMS calcd $C_{34}H_{46}N_2O_6$: 578.7388; found 579.2738 $[M + H]^+$. Anal. Calcd for $C_{34}H_{46}N_2O_6$: 70.56; H, 8.01; N, 4.84. Found: C, 70.56; H, 7.95; N, 4.77.

Piperazine-1,4-diylbis([6-hydroxy-2,5,7,8-tetramethylchroman-2-yl]methanone) (3d)

Purified by precipitation in Et_2O; Yield: 60%; white powder; mp: 227–229°C; ^{1}H-NMR (DMSO-d6, 400 MHz) δ: 7.50 (s, 1H, NH), 5.74 (s, 1H, NH), 5.56 (bs, 1H, OH), 5.54 (bs, 1H, OH), 2.45 (m, 2H, H2′), 2.03 (bs, 6H, CH_3), 1.94 (bs, 3H, CH_3), 1.58 (s, 15H, CH_3), 1.14 (m, 14H, CH_2); ^{13}C-NMR (DMSO-d6, 100 MHz) δ: 172.66 (CO), 157.17 (C-6), 153.98 (C-9), 124.94 (CH arom), 124.90 (CH arom), 122.71 (CH arom), 119.05 (CH arom), 80.19 (CH_2), 49.51 (CH_2), 35.37 (CH_3), 33.25 (N-CH_2), 27.34 (C-3), 26.48 (CH_3), 22.49 (N-CH_2), 14.76 (CH_3), 13.79 (CH_3); HRMS calcd $C_{32}H_{42}N_2O_6$: 550.6857; found 551.3500 $[M + H]^+$. Anal. Calcd for $C_{32}H_{42}N_2O_6$: 69.79; H, 7.69; N, 5.09. Found: C, 69.71; H, 7.66; N, 4.98.

Antiplasmodial activity

Blood stages (FcB1-Columbia, FcM29 and F32) of *P. falciparum* were cultured in human erythrocytes maintained in RPMI 1640 supplemented with 5% human serum. The cultures were synchronized every 48 h by 5% D-sorbitol lysis (Merck, Darmstadt, Germany) in order to discard old stage parasite. *In vitro* antimalarial activity testing was performed by following [^{3}H]-hypoxanthine (Perkin-Elmer, France) incorporation [12]. The IC_{50} values were graphically determined from inhibition versus concentration curves as described elsewhere [13] each drug concentration being tested independently three-times in triplicate, the final IC_{50} was the mean of the three obtained values.

Cytotoxicity

The toxicity of compound **3d** was estimated using MCF-7 cells (human breast carcinoma). These cell lines were cultured in the same conditions as *P. falciparum*, where the 5% human serum was replaced with 10% fetal calf serum. After the addition of the compound **3d** at 100 µM concentration, cell growth was estimated by [^{3}H]-hypoxanthine incorporation following a 48-h incubation and was compared with a control sample (untreated with **3d**). The mean of the corresponding wells was referred to as 100%) [14].

In vivo toxicity

The most active derivative **3d** was evaluated for *in vivo* toxicity. Oral acute toxicity of **3d** was investigated at a dose of 10, 50 and 100 mg/kg. Animals were observed for gross body changes such as loss of appetite, hair erection, lacrimation, convulsions, salivation, diarrhea, mortality and other signs of overt toxicity. The observation was made twice daily for two weeks after the administration.

Handling of the animals was assured according to UE guidelines for use and maintenance of experimental animals.

Antioxidant activity

Alkaline phosphatase assay

In this assay, we used the catalytic activity of the enzyme alkaline phosphatase (ALP) (2 mU/ml in glycine buffer) in the presence of compound **3d**, trolox or L-ascorbic acid. A compound is considered as antioxidant if it is able to preserve the catalytic activity of the enzyme in the presence of AAPH-generated peroxyl radicals. The enzymatic activity of ALP was observed by following the enzyme-induced dephosphorylation of MUP (20 µM) to the fluorescent 4-methyllumbelliferone using a continuous spectrofluorimetric assay [15]. The ability of the tested compound to protect the activity of ALP was calculated according to the following equation: % ALP protection = $([ha_{sample}\text{-}ha_{ox}]/[ha_{non\text{-}ox}\text{-}ha_{ox}]) \times 100$ where: ha_{sample} = the hydrolytic activity of oxidized samples; ha_{ox} = oxidized controls, $ha_{non\text{-}ox}$ = non-oxidized controls.

The EC_{50} is the compound concentration which induces 50% protection of ALP enzymatic activity from a peroxyl radical-induced oxidation after 90 min.

Oxygen radical absorbance capacity assay

This assay relies on the same conditions as the ALP assay. In oxygen radical absorbance capacity (ORAC) assay, ALP was replaced by a fluorescein solution (6×10^{-8}M). The fluorescence of oxidized samples ($fluo_{sample}$) and oxidized controls ($fluo_{FLox}$) were read at λ_{Ex} 485 ± 20 nm and λ_{Em} 528 ± 20 nm. The fluorescence of non-oxidized controls ($fluo_{FLnon\text{-}ox}$) was calculated as following: % remaining fluorescein = $([Fluo_{sample}\text{-}Fluo_{flox}]/[Fluo_{non\text{-}ox} - Fluo_{flox}])$ x 100.

The results are expressed as EC_{50} that represents the sample concentration which protects 50% of fluorescein from oxidation induced by peroxyl radicals after 90 min [16,17].

ABTS assay

The antioxidant activity of was assessed by spectrophotometry in microplates by measuring the decolorization of the $ABTS^{\bullet-}$ radical [18]. The activity was expressed as ER_{50}, which corresponds to the ratio of compound concentration over the radical concentration inducing a decrease in absorbance by 50% after 90 min.

DPPH assay

This assay is similar to ABTS assay where the $ABTS^{\bullet-}$ radical was replaced by DPPH● radical. The activity is represented by ER_{50} that was determined with a dose–response curve and represents the ratio of compound concentration to DPPH● concentration producing a 50% decrease in DPPH● after 90 min of incubation.

Statistical analysis

Data are the means ± SD from duplicate samples of at least three independent experiments. Differences between the mean values were analyzed by Student's *t*-test, and the results were considered significant when $p < 0.05$.

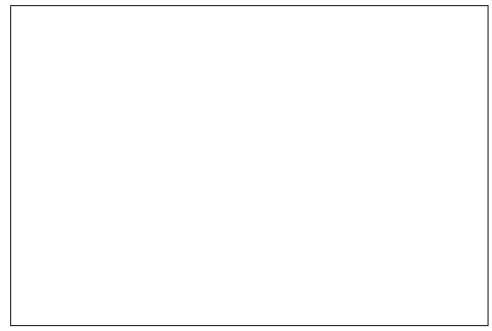


Figure 2. One step synthesis of trolox derivatives.

Results & discussion

Chemistry

The investigated compounds were prepared in one step according to Figure 2 by coupling of trolox to various primary amines, primary diamines or 1,4-piperazine in the presence of a bis(2-oxo-3-oxazolidinyl)phosphonic chloride (BOP-Cl) as the coupling agent and trimethylamine in DMF as the solvent. When diamines were used, the amount of product formed from reaction of only one amine functionality was comparatively low. In the latter case, a simple purification by chromatography allowed the isolation of the target compound. Table 1 summarizes all compounds obtained.

Biological assays

Antiplasmodial activities

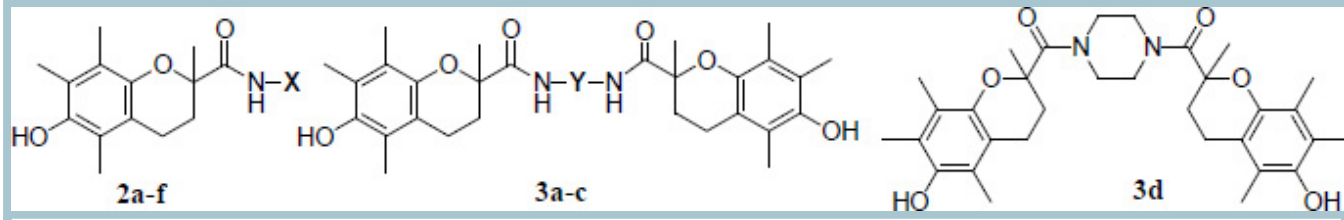
Overall, 10 compounds were obtained and tested *in vitro* for antiplasmodial activity (Table 1). Prior to antiparasitic assays, the cytotoxicity of the synthesized compounds was assessed at 100 μM concentration against the human breast cancer cell line MCF7 as described [19,20] and showed neglectable toxicity as the cell viability remained over 90%.

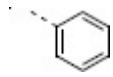
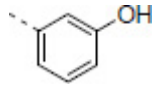
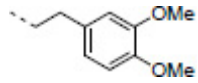
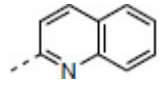
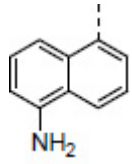
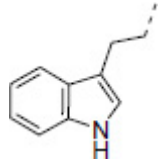
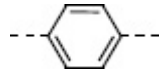
The antiparasitic activity was evaluated *in vitro* using **chloroquino-sensitive (3D7) and chloroquino-resistant (FcB1) strains** of *P. falciparum*. The results are expressed as the concentration giving 50% of parasite growth inhibition (IC_{50}) and compared with chloroquine, used as the reference drug (Table 1). Although, the number of investigated compounds is low, some structure-activity relationships can be highlighted. Among compounds having a phenyl ring linked to trolox, the substitution at the phenyl group seems to be determinant (**2a** vs **2b**). Among compounds bearing one trolox unit, the most active are those having two fused rings: quinoline (**2d**), amino-naphthyl (**2e**) and indolyl (**2f**). It is interesting to note this feature (two fused rings) and especially the quinoline motif is present in several antimalarial drugs, including chloroquine. Moreover, it can be highlighted that the replacement of the quinoline ring (in which the nitrogen is embedded in the ring) by amino-naphtyl (where the nitrogen is exocyclic) does not affect activity. Among compounds bearing two trolox units (**3a–d**), compounds **3c** and **3d** showed the highest inhibition activities with an IC_{50} in the nanomolar range. Regarding the structure-activity relationship, it can be noticed the positive effect of the rigidity of the linker, notably by comparing the activities of **3c**/**3d** versus **3b**. Moreover, the linker should be non-aromatic (compounds **3c/3d** vs **3a**). Finally, it is interesting to note that trolox derivatives either highly or moderately active show roughly similar activity profiles on both *P. falciparum* strains.

Antioxidant activity

The antioxidant activity of the most active compound **3d** was conducted through four tests (**ALP, ORAC, ABTS and DPPH test**) [15]. The ALP test is a fluorimetric test to assess the antioxidant capacity of a compound to protect proteins from loss of activity caused by reactive oxygen species (ROS) [21]. The ORAC test is commonly used to measure a compound capacity to scavenge free-radicals. It is based on the fluorescence degradation of fluorescein [22,23]. The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical test allows the determination of the concentration of free radicals. It is based on the neutralization of a radical-cation arising from the oxidation of ABTS● [18].

Table 1. Structures and antiplasmodial activity of trolox derivatives.



Compound	X	Y	IC_{50} μM (FcB1)†	IC_{50} μM (3D7)‡
2a			10.7	12.1
2b			4	4.1
2c			27	17.5
2d			3.5	2.5
2e			3.7	2.5
2f			4.2	2.8
3a			55	33
3b			4.6	6.2
3c			**0.4**	**0.41**
3d			**0.3**	**0.25**
Chloroquine			0.15	0.025

†Resistant strain toward chloroquine.
‡Sensitive strain toward chloroquine.
The dashed bonds indicate the position of the linkage.

Finally, we realized the well-known DPPH• test which is based on the ability of the stable 2,2-diphenyl-1-picrylhydrazyl free radical to react with hydrogen donors.

Following the four tests highlighted above, we compared the antioxidant activity of the lead compound **3d** to trolox and L-ascorbic acid, used as antioxidant references. As shown in Table 2, trolox derived compound **3d** is

Table 2. Antioxidant activity of derivative 3d, trolox and L-ascorbic acid.

Compound	ALP (EC_{50}, μM)†	ORAC† (EC_{50}, μM)†	ABTS (ER_{50})‡	DPPH (ER_{50})‡
3d	6.46 ± 0.8	11.7 ± 0.95	0.25 ± 0.04	0.13 ± 0.01
Trolox	14.1 ± 0.72	18.2 ± 0.76	0.28 ± 0.04	0.18 ± 0.02
L-ascorbic acid	30.9 ± 0.67	57.5 ± 0.93	0.30 ± 0.03	0.28 ± 0.08

†EC_{50} is the concentration that produce 50% of antioxidant activity.
‡ER_{50} is the ratio of antioxidant concentration to ABTS or DPPH• concentration producing a 50% decrease in ABTS or DPPH at steady state (compounds with low ER_{50} values are more potent).
ALP: Alkaline phosphatase; ORAC: Oxygen radical absorbance capacity.

more potent than trolox and L-ascorbic, in particular when ALP and ORAC tests were used. The higher antioxidant activity of **3d** is an added value to its potential development as antimalarial drug.

Conclusion

In the field of drug discovery, the multitarget drugs is an emerging strategy, meaning one drug, multiple actions. Our work falls in this strategy, dealing with the development of compounds with dual actions, antiplasmodial and antioxidant activities. Herein, we have shown that trolox derivatives may offer promising antimalarial drug candidates together with high antioxidant potential. The lead compound (**3d**) characterized by the linkage of two trolox molecules to a 1,4-piperazine motif shows the highest antiplasmodial and antioxidant activities. This drug candidate can be synthesized in multigram scale in one step starting from commercially and affordable compounds. In order to pave the way for further development, the compound (**3d**) was assessed for *in vivo* toxicity. We performed toxicity studies on mice. After two weeks of treatment compound **3d** was shown to be well tolerated by animals as there were no signs of acute toxicity. However, it is worth mentioning that further preclinical development need prior formulation and drug delivery studies as compound **3d** is poorly soluble in aqueous mediums (CLogP = 6.78). Moreover, further toxicology studies and in particular toxicokinetic studies will be needed before going further for preclinical studies.

Future perspective

Malaria is caused by a parasite of the genus *Plasmodium*. It is a worldwide problem that need the identification of new class of antimalarials. It has been demonstrated that the *P. falciparum*-infected red blood cell is under oxidative stress and that the human defence system is not fully effective against it. Hence, bifunctional drugs with antiplasmodial activity and possessing antioxidant potential will continue be the golden approach for development of molecules to effectively fight malaria which continue to be a public health issue, especially in low-income countries.

Summary points

- Compounds derived from trolox were conceived as bifunctional agents with antiplasmodial and antioxidant activities to target malaria.
- The antiparasitic activity was evaluated *in vitro* using chloroquino-sensitive (3D7) and chloroquino-resistant (FcB1) strains of *Plasmodium falciparum*.
- The antioxidant activity of representative compounds shown in Table 1 was conducted through four tests (alkaline phosphatase, oxygen radical absorbance capacity, ABTS and DPPH test).
- Compounds **3c** and **3d** showed the highest activities comparable with those of chloroquine.
- Trolox derivative **3d** showed antioxidant activity higher than trolox and L-ascorbic acid.
- The one-step synthesis and displayed activity make the trolox derivative **3d** promising drug candidate for the treatment of malaria.

Acknowledgments

The authors D Cressend, E Nicolle and A Boumendjel dedicated this manuscript to the memory of P-A CARRUPT from the University of Geneva.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

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