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Fluorine-containing aryloxyethyl thiocyanate derivatives are potent inhibitors of *Trypanosoma cruzi* and *Toxoplasma gondii* proliferation

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Abstract—As a part of our project aimed at developing new safe chemotherapeutic and chemoprophylactic agents against tropical diseases, fluorine-containing drugs structurally related to 4-phenoxyphenoxyethyl thiocyanate (**1**) were designed, synthesized, and evaluated as antiproliferative agents against *Trypanosoma cruzi*, the parasite responsible of American trypanosomiasis (Chagas' disease), and *Toxoplasma gondii*, the etiological agent of toxoplasmosis. This thiocyanate derivative had previously proven to be an effective agent against *T. cruzi* proliferation. Fluorine-containing thiocyanate derivatives **2** and **3** were threefold more potent than our lead drug **1** against intracellular *T. cruzi*. The biological evaluation against *T. gondii* was also very promising. The IC_{50} values corresponding to **2** and **3** were at the very low micromolar level against tachyzoites of *T. gondii*. Both of these drugs are interesting examples of effective antiparasitic agents that have outstanding potential not only as lead drugs but also to be used for further in vivo studies.

American trypanosomiasis (Chagas' disease) and toxoplasmosis are among the most prevalent parasitic diseases worldwide.[1] It has been estimated that around 18–20 million people are infected and over 40 million individuals are at risk of infection by the hemoflagellated protozoan *Trypanosoma cruzi*, the responsible agent of this disease.[2] As other kinetoplastid parasites, *T. cruzi* has a complex life cycle possessing four main morphological forms. It multiplies within the insect gut as an epimastigote form and is spread as a non-dividing metacyclic trypomastigote from the insect feces by contamination of intact mucosa or wounds produced by the blood-sucking activity of the vector. In the mammalian host, *T. cruzi* proliferates as the intracellular amastigote form, which is next released into the bloodstream as a non-dividing highly infective trypomastigote that can either invade other tissues or can infect the respective Chagas' disease vectors closing the cycle.[3–5] Transmission via the placenta or by blood transfusion is the responsible mechanism for the occurrence of Chagas' disease in developed countries where the disease is not endemic.[6,7]

Toxoplasmosis is caused by *Toxoplasma gondii*, a complex eukaryotic parasite that has adopted an essential intracellular survival strategy.[5,8] Most of *T. gondii* infections are asymptomatic. There are two asexual forms that can cause disease in humans. The tachyzoite form, which can invade all types of cells and divides rapidly leading to cell death, and the bradyzoite form that divides slowly and forms cysts in muscle and brain.[9]

The existing chemotherapy either for Chagas' disease or for toxoplasmosis remains deficient. The chemotherapy for Chagas' disease is based on two drugs empirically discovered, nifurtimox, now discontinued, and benznidazole. Although both of these compounds are able to cure at least 50% of recent infections as indicated by the disappearance of symptoms, and reduction of

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parasitemia and serology, they present severe side effects.[10,11] The standard treatment against *T. gondii* infections consists in the combination of pyrimethamine, which inhibits the enzymatic activity of dihydrofolate reductase, and sulfadiazine, whose target is dihydropteroate synthetase.[12] This therapy is frequently associated with toxic side effects.[13]

The knowledge of unique aspects of the biochemistry and physiology of *T. cruzi* has led to the finding of specific molecular targets for rational drug design,[14–19] among them, sterol biosynthesis arises as a valid target for Chagas' disease.[20] The parasitic pathway leads to ergosterol, while cholesterol is the final product in the mammalian host.[21] For example, triazole derivatives,[22–24] or azasterols[25,26] had shown to be effective antiparasitic agents against *T. cruzi* targeting ergosterol biosynthesis. In addition, we have demonstrated that aryloxyethyl thiocyanate derivatives are potent inhibitors of *T. cruzi* proliferation.[27–29] 4-Phenoxyphenoxyethyl thiocyanate also known as WC-9 (**1**) is a representative member of this family of compounds, which proved to be a potent agent against *T. cruzi* proliferation whose target is squalene synthase (SQS) (Fig. 1).[30]

On the other hand, it has been reported that sterol biosynthesis inhibitors such as azasterols act as selective inhibitors of *T. gondii* proliferation.[31,32] In particular, it was recently reported that two quinuclidine derivatives, SQS inhibitors, showed selective activity against tachyzoites of *T. gondii*.[33] As compound **1** is a potent inhibitor of *T. cruzi* proliferation, and bearing in mind that previous studies indicated that structural variation at the B ring had no influence on biological action,[27–29,34] it was thought that the introduction of a fluorine atom at the B ring would be of benefit for biological activity. The estimated log *P* values for the title compounds **2** and **3** were 4.71, while the corresponding one for **1** was 4.51. As the drugs must penetrate two cell membrane to reach TcSQS, it could be anticipated a better biological action. Then, fluorine-containing drugs were designed, synthesized, and biologically evaluated against the intracellular and the epimastigote forms of *T. cruzi* and the tachyzoite forms of *T. gondii*.

The introduction of a fluorine atom onto the B ring was carried out via a coupling reaction that involves an arylboronic acid and an appropriate phenol in the presence of pyridine and cupric acetate as illustrated in the Scheme.[35–37] The title compounds were synthesized starting from commercially available 4-benzyloxyphenol (**4**), which on reaction with 3-fluorophenylboronic acid in the presence of pyridine and cupric acetate was converted into compound **5** in 79% yield. The benzyl group of the resulting product was removed by catalytic hydrogenation to give phenol **7** in 90% yield, which after treatment with 2-bromoethyl tetrahydro-2*H*-pyran-2-yl ether in a suspension of potassium hydroxide in methyl sulfoxide[34] afforded **9** in 80% yield. Cleavage of the tetrahydropyranyl group of **9** by treatment with pyridinium *p*-toluenesulfonate gave alcohol **11**, which on reaction with tosyl chloride gave the corresponding tosylate **13** in 33% yield. The title compound **2** was obtained by nucleophilic displacement with potassium thiocyanate in *N*,*N*-dimethylformamide at 100 °C in 43% yield. Compound **3** was prepared following a similar synthetic approach as illustrated in Scheme 1.

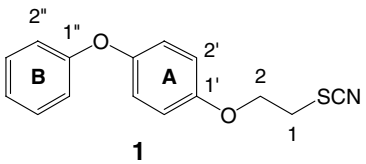

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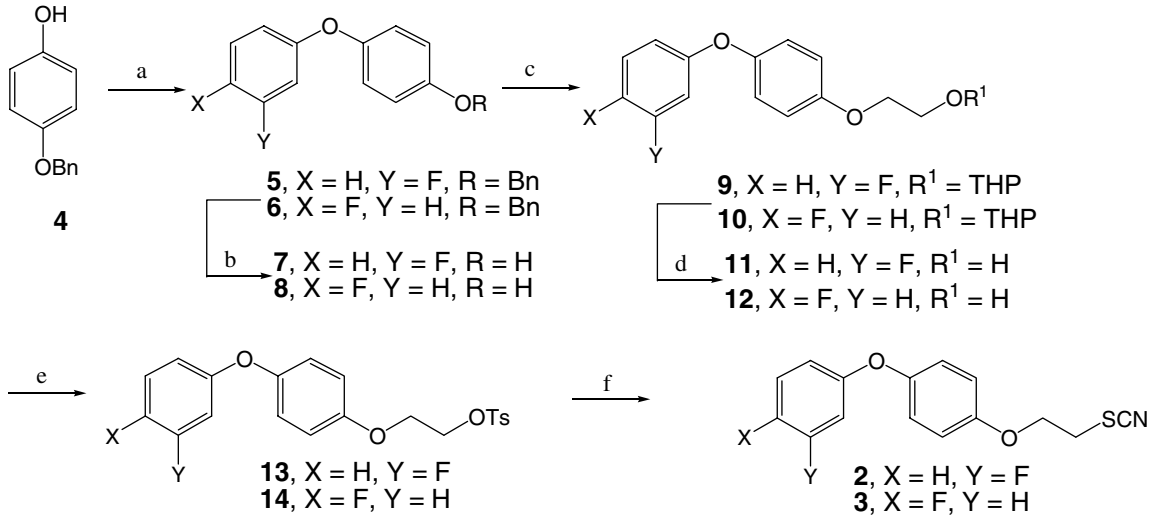
Figure 1. Chemical structure of 4-phenoxyphenylethyl thiocyanate.

All compounds were routinely characterized by using ^{1}H and ^{13}C NMR spectroscopy at 500 MHz and 125 MHz, respectively (Bruker AM-500 apparatus). Elemental analysis data for all new compounds were satisfactory.[38]

The title compounds **2** and **3** were potent growth inhibitors of the intracellular form of *T. cruzi*. Certainly, these drugs exhibited IC_{50} values of 4.3 and 3.7 μM, respectively, being fourfold more potent than our lead drug **WC-9**, used as a positive control, under the same assay conditions. The same behavior was observed against the epimastigote form of *T. cruzi* where drugs **2** and **3** were more potent than **1** but to a lesser extent than against amastigotes. The tetrahydropyranyl precursors of the title drugs, that is, compounds **9** and **10**, were also potent inhibitors of *T. cruzi* (amastigotes) proliferation showing IC_{50} values of 19.0 and 14.4 μM, respectively. This fact was in agreement with our previous results in other closely related compounds, where the biological activity of drugs bearing the thiocyanate group correlated quite well with the activity exhibited by their natural tetrahydropyranyl ether precursors when bonded to the same aromatic skeleton.[28,29] The efficacy decreased proportionally in all cases. In addition, fluorine-containing drugs were also potent inhibitors of *T. gondii* (tachyzoites) possessing IC_{50} values at the low micromolar level. Compounds **1** and **3** proved to be the more effective growth inhibitors of *T. gondii* exhibiting IC_{50} values of 2.80 and 3.99 μM, respectively. **WC-9**, a well-known antiparasitic agent, and atovaquone were used as positive controls for assays on *T. cruzi* and *T. gondii*, respectively. The results are shown in Table 1.

Biological assays on epimastigotes were performed as previously described.[34,39] Experiments on the intracellular form of the parasite were conducted on *T. cruzi*-infected L_6E_9 myoblasts (Y strain) as described before.[40–42] Experiments on *T. gondii* tachyzoites were carried out as previously published.[43,44]

It can be concluded that fluorine-containing thiocyanate derivatives were potent inhibitors of *T. cruzi* proliferation exhibiting an efficacy superior than that presented by **WC-9** our lead drug. These compounds offer excellent prospective as antiparasitic agents because they also exhibit potent inhibitory action on *T. gondii*.



Scheme 1. Reagents and conditions: (a) 3-FPhB(OH)$_2$ or 4-FPhB(OH)$_2$, Cu(OAc)$_2$, CH_2Cl_2, py, rt, 5 days, 79% for **5**, 35% for **6**; (b) H_2/Pd (C), rt, overnight, 90% for **7**, 92% for **8**; (c) KOH, DMSO, $BrCH_2CH_2OTHP$, rt, 16 h, 80% for 9, 85% for 10; (d) PPTS, MeOH, rt, 14 h, 95% for **11**, 78% for **12**; (e) ClTs, py, rt, 8 h, 33% for **13**, 55% for **14**; (f) KSCN, DMF, 100 °C, 5 h, 43% for **2**, 48% for **3**.

Table 1. Effect of fluorine-containing drugs against *Trypanosoma cruzi* and *Toxoplasma gondii* for compounds **1**–**3**, **9**, and **10**

Compound	IC_{50}[a] (μg/ml)	IC_{50}[a] (μM)	IC_{50} (μM)
	T. cruzi epimastigotes	*T. cruzi* amastigotes	*T. gondii* tachyzoites
1 (WC-9)	2.6	12.0	2.80
2	1.74	4.3	15.8
3	1.95	3.7	3.85
9	>20.0	19.0	3.99
10	>20.0	14.4	6.16
Atovaquone			0.29

[a] Values are means of three experiments.

Work aimed at optimizing the chemical structure of compounds such as **2** and **3** as well as to establish a more complete structure–activity relationship is currently being pursued in our laboratory.

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38. Selected data for title compounds **2** and **3**.
4-(3-Fluorophenoxy)phenoxyethyl thiocyanate **(2)**: yellow pale oil; R_f 0.5 (hexane/EtOAc, 7:3); IR (film, cm^{-1}) 3075, 2927, 2872, 2158, 1607, 1509, 1271, 1122, 1080, 968, 772; ^1H NMR (CDCl3) δ 3.34 (t, J = 5.8 Hz, 2H, H-1), 4.31 (t, J = 5.8 Hz, 2H, H-2), 6.64 (dt, J = 10.4, 2.3 Hz, 1H, H-2′), 6.74 (m, 2H, H-5″, H-6″), 6.93 (d, J = 9.0 Hz, 2H, H-2′), 7.05 (d, J = 8.9 Hz, 2H, H-3′), 7.24 (dt, J = 8.2, 6.8 Hz, 1H, H-4″); ^{13}C NMR (CDCl3) δ 33.26 (C-1), 66.34 (C-2), 105.03 (d, J = 24.6 Hz, C-2″), 109.31 (d, J = 21.2 Hz, C-4″), 111.70 (S*C*N), 112.98 (d, J = 3.4 Hz, C-6″), 115.93 (C-2′), 121.29 (C-3′), 130.39 (d, J = 10.2 Hz, C-5″), 150.17 (C-4′), 154.45 (C-1′), 159.69 (d, J = 11.0 Hz, C-1″), 163.47 (d, J = 246.7 Hz, C-3″); MS (*m*/*z*, relative intensity) 289 (M^+, 96), 203 (100), 147 (27), 95 (63), 86 (67); Anal. Calcd for $C_{15}H_{12}FNO_2S$: C, 62.28; H, 4.15; N, 5.19; S, 11.07. Found: C, 62.71; H, 4.41; N, 4.77; S, 10.78.
4-(4-Fluorophenoxy)phenoxyethyl thiocyanate (**3**): yellow pale oil: R_f 0.48 (hexane/EtOAc, 7:3); IR (film, cm^{-1}) 2930, 2169, 1515, 1219, 1041, 842; ^1H NMR ($CDCl_3$)δ 3.33 (t, J = 5.8 Hz, 2H, H-1), 4.30 (t, J = 5.8 Hz, 2H, H-2), 6.90 (d, J = 9.2 Hz, 2H, H-3′), 6.92 (m, 2H, H-3″), 6.95 (d, J = 9.2 Hz, 2H, H-2′), 7.00 (m, 2H, H-2″); ^{13}C NMR ($CDCl_3$)δ 33.28 (C-1), 66.40 (C-2), 111.71 (S*C*N), 115.99 (d, J = 18.7 Hz, C-3″), 116.24 (C-2′), 119.41 (d, J = 8.5 Hz, C-2″), 120.14 (C-3′), 151.69 (C-4′), 153.86 (C-1′), 158.45 (d, J = 241.6 Hz, C-4″); MS (*m*/*z*, relative intensity) 289 (M^+, 100), 203 (99), 147 (24), 95 (41), 86 (42); Anal. Calcd for $C_{15}H_{12}FNO_2S$: C, 62.28; H, 4.15; N, 5.19; S, 11.07. Found: C, 62.42; H, 4.30; N, 4.81; S, 10.93.
39. *Trypanosoma cruzi* epimastigotes (Y strain) were grown in a LIT medium containing 5% NCS and 1% P/S. Five-day-old culture was centrifuged and resuspended in fresh medium to get a $2–3 \times 10^6$ cell/ml suspension. Parasites were then placed in sterile screw-cap tubes (2 ml/tube). Two tubes were filled with medium for blank. Each drug was tested at four different concentrations (1, 2.5, 5, 10, and 20 μg/ml), each one in triplicate. Drugs stock solutions were prepared in absolute ethanol and then were diluted in a LIT medium. A control without drug was done for each group tested. The concentrations of cells were determined by measuring the absorbance of the culture medium containing parasites at 600 nm against a blank with culture medium alone. To calculate percent inhibition, the following formula was used: percent inhibition = $100 - (\Delta A_d \times 100)/\Delta A_c$, where ΔA_c and ΔA_d are the differences in the absorbance of control cultures and drug-treated cultures, respectively, at the beginning and at day 5.
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42. Gamma-irradiated L_6E_9 myoblasts (1×10^7 cells/plate) in DMEM containing 20% FCS were plated in 12-well tissue culture plates and incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. After 24 h, wells were washed once and fresh media were added containing 4.17×10^6 trypomastigotes or amastigotes/well in DMEM. One well was left without parasites for control. After 2 h of incubation at 37 °C in a 7% CO_2 atmosphere, cultures were washed twice with Hanks' solution, and culture medium was replaced to remove extracellular parasites. At this time, 0.5–1 μCi of [5,6-^{3}H]uracil/well (specific activity, 40–50 Ci/mmol) and drug solutions in water were added. Two wells were left for infection control. Cultures were incubated for 72 h. Incorporation of the [5,6-^{3}H]uracil into trichloroacetic acid (TCA)-precipitable material was measured at day 3. The supernatants from the monolayers were transferred to glass tubes, the cells were dissolved with 1.3 ml of 1% sodium dodecyl sulfate containing 100 μg of cold uracil per ml, and the suspension was transferred to the glass tubes. The wells were rinsed with 3 ml of 5% TCA (ice-cold) which was combined with the previous suspension. The samples were maintained in ice for 15 min and collected on glass fiber filters (Whatman GF/B) by using a sampling manifold (Millipore, Bedford, MA). After filtering, the tubes were rinsed twice with 4 ml of 5% TCA and the filters were rinsed twice with TCA and once with 95% ethanol. After drying the filters, they were placed in scintillation vials containing 4–5 ml of scintillation cocktail Ecolume. Vials were vortexed for 20 s and counted. The percent inhibition was calculated by employing the following formula: percent inhibition = $100 - (\Delta C_d \times 100)/\Delta C_c$, where ΔC_c and ΔC_d are the differences in the count per minute of control cultures and drug-treated cultures, respectively.
43. *Toxoplasma gondii* tachyzoites of the m2m3 clone expressing yellow fluorescence protein[44] were routinely maintained in vitro in human foreskin fibroblast monolayers (HFF) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, at 37 °C in a humid 5% CO_2 atmosphere. Confluent HFF monolayers grown in 96-well black plates with optical bottoms (Falcon/Becton–Dickinson, Franklin Lakes, NJ) were used and drugs dissolved in the same medium and serially diluted in the plates. Freshly isolated tachyzoites were filtered through a 3 μm filter and passed through a 22 gauge needle, before use. The cultures were inoculated with 10^4 tachyzoites/ml in the same media. The plates were incubated at 37 °C and read daily in a Molecular Devices fluorescent plate reader. To preserve sterility the plates were read with covered lids, and both excitation (510 nm) and emission (540 nm) were read from the bottom.[44] For the calculation of the IC_{50}, the percent of growth inhibition was plotted as a function of drug concentration by fitting the values to the function: $I = I_{max}\, C/(IC_{50} + C)$, where I is the percent inhibition, I_{max} = 100% inhibition, C is the concentration of the inhibitor, and IC_{50} is the concentration for 50% growth inhibition.
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