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WC-9 a Lead Drug with Great Prospects for American Trypanosomiasis and Toxoplasmosis

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Abstract: Trypanosomatids possess an unremitting requirement for distinctive endogenous sterols for their life cycle and cannot use the copious availability of cholesterol existing in their mammalian hosts. Exhaustion of endogenous sterols such as ergosterol or of its next biosynthetic product 24-ethylcholesta-5,7,22-trien-3β-ol brings forth an inhibition of proliferation on *Trypanosoma cruzi,* the etiologic agent of American trypanosomiasis or Chagas disease. These metabolites are crucial; consequently, the enzymes implicated in catalyzing their formation constitute interesting molecular targets for drug design. Selective inhibition of an enzyme associated to the ergosterol biosynthesis will produce *T. cruzi* cell arrest. Trypanosomatids, fungi, and yeasts have need of these endogenous sterols for cell viability and growth. In fact, some effective ergosterol biosynthesis inhibitors bearing suitable pharmacokinetic properties in mammals have become putative antiparasitic agents by inducing almost complete parasitological cure in both acute and chronic experimental Chagas disease. **WC-9** (compound **7**; 4-phenoxyphenoxyethyl thiocyanate) holds our attention bearing in mind that this compound exhibits ED_{50} values at the low nanomolar range against the clinically more relevant replicative form of *T. cruzi* (amastigotes). The cellular activity of **WC-9** is due to an exhaustion of endogenous sterols demonstrating a blockade of the biosynthetic pathway at a pre-squalene level.

Keywords: Antiparasitic Agents, Chagas disease, Squalene synthase, Toxoplasmosis, WC-9.

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The development of 4-phenoxyphenoxyethyl thiocyanate, known as **WC-9** (4-phenoxyphenoxyethyl thiocyanate; compound **7**) as an effective growth inhibitor of trypanosomatids and Apicomplexan parasites, is a quite exciting story. The juvenile hormones of insects are crucial metabolites, whose function is to conserve larval stages. They also have a key role in the maturation of the reproductive system in the female. The corresponding chemical structures of naturally occurring juvenile hormone of insects are illustrated in Fig. (**1**) as juvenile hormones 0, I, II, III, and 4-methyl juvenile hormone I, respectively [1]. The starting point of the development of **WC-9** was the fact that Chagas disease vectors such as *Rhodnius prolixus* or *Triatoma infestans* treated with juvenile hormone analogues were less vulnerable to be contaminated naturally with the responsible agent of Chagas disease or American trypanosomiasis, the hemoflagellated protozoan *Trypanosoma cruzi* than non treated vectors [2]. These findings led to consider the evaluation of juvenile hormone analogues against *T. cruzi* cells in order to seek if this level of protection was associated to a certain cellular activity. Certainly, some compounds structurally related to naturally occurring juvenile hormone and other ones whose chemical structures were envisioned taken the molecule of the insect growth regulator fenoxycarb (**6**; ethyl *N*-{2-[(4-phenoxyphenoxy)ethyl]}carbamate) as a model exhibited a modest but determined inhibitory action as inhibitors of *T. cruzi* proliferation [3]. Very interestingly, these former juvenile hormone analogues turned out to be cell growth inhibitors, whose chemical structures were further optimized to yield **WC-9** and additional closely related compounds [4-8]. Hence, this dual biological action resulted so relevant that encouraged us to carry out further studies to determine its precise mode of action and structure optimization.

As other trypanosomatids, *T. cruzi* cell has a complex life cycle, which involves blood-sucking interaction among Reduviid bugs and mammals [9]. The parasite proliferates in the insect gut as an epimastigote form, which is distributed as a non-dividing metacyclic trypomastigote along the insect feces. These contaminated excrements infect the mammalian host by contact with undamaged mucosa or through the lesions caused by the mentioned blood-sucking activity of the insect. Within the host, *T. cruzi* multiplies intracellularly in the amastigote form and is further liberated into the blood stream as a non-dividing trypomastigote, which invades other tissues [9]. Transmission of American trypanosomiasis could also take place *via* the placenta or by blood transfusion. Certainly, the appearance of Chagas disease in

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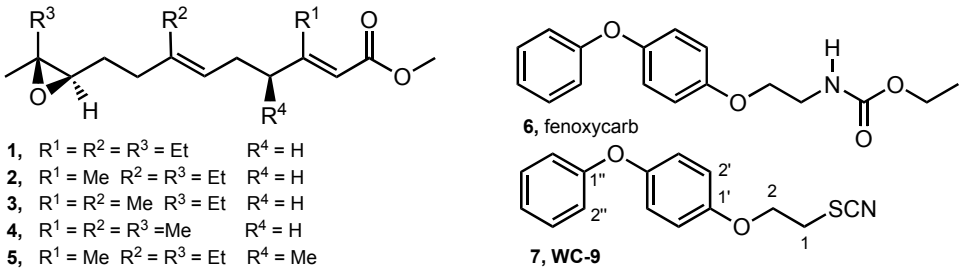


Fig. (1). Chemical structures of naturally occurring juvenile hormones of insects, the insect growth regulator fenoxycarb (**6**), and **WC-9**.

countries where it is not endemic should be attributed mainly to transfusion of contaminated blood [10, 11].

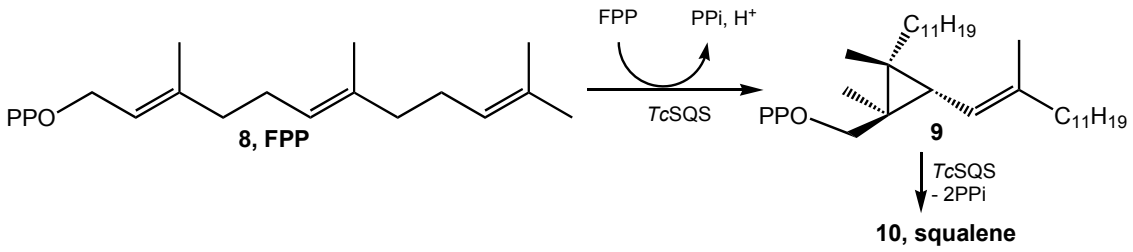
WC-9 proved to be an extremely efficient inhibitor of intracellular *T. cruzi* proliferation, which as previously mentioned, is the more clinically relevant dividing form of the parasite. This cellular activity exhibited ED_{50} values at the low nanomolar range. This compound behaved still more effective than nifurtimox, one of the drugs currently employed to treat this sickness under the same assays conditions [7]. Naturally, the first question that arose was what would be the target of **WC-9**. Our pioneering studies on the mode of action of these drugs had indicated that there was tantalizing evidence to believe that this compound would block ergosterol biosynthetic pathway based on the fact that **WC-9** inhibited steroidogenesis in Leydig tumor cells [12, 13]. Later, biological studies on epimastigotes of *T. cruzi* treated with **WC-9** indicated that the growth inhibition observed was associated with an increment of concentration of low molecular weight compounds from mevalonate to squalene in this biosynthetic pathway [14] suggesting that the target enzyme should be located at a pre-squalene level.

Squalene synthase (SQS) is a crucial enzyme in sterol biosynthesis catalyzing a reductive dimerization reaction between two molecules of farnesyl pyrophosphate (FPP) to give rise to squalene, which is an obligated intermediate to biosynthesize the required endogenous sterols. This enzyme is the molecular target of **WC-9**. This mode of action was confirmed employing wild type enzyme acquired from highly purified glycosomes and mitochondrial membrane vesicles of the epimastigote forms of *T. cruzi* [15]. Definitely, **WC-9** is an extremely potent inhibitor of the enzymatic activity of both glycosomal and mitochondrial *T.cruzi* SQS having IC_{50} values of 88 nM and 129 nM, respectively [16]. Furthermore, analysis of the corresponding dose-response curves regarding the activity of **WC-9** towards the target enzyme *Tc*SQS are in agreement with a non-competitive or allosteric inhibition, that is, $K_i = IC_{50}$. It is worth mentioning that the K_i values are two to three orders of magnitude lower that the K_m respective the substrates [16].

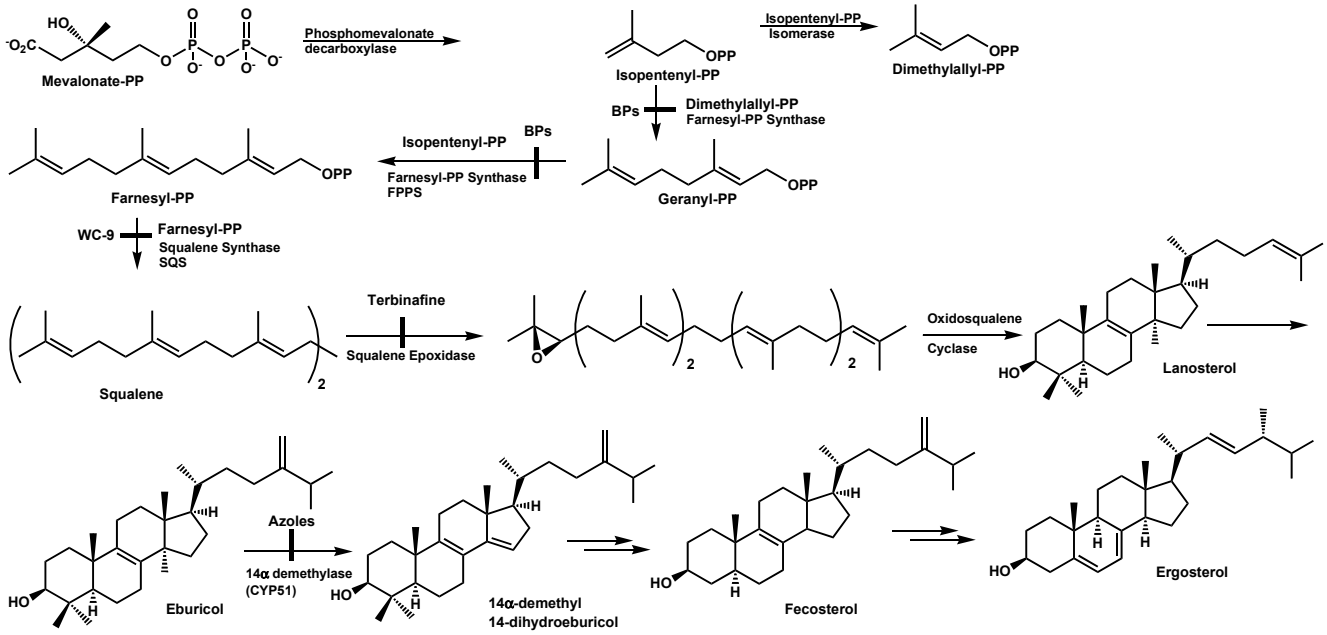
The nanomolar activity of **WC-9** towards *Tc*SQS can be attributed to the presence of the electrophilic center of the thiocyanate group bonded to the carbon-1 position of the hydrocarbonated skeleton of the molecule. The carbon-nitrogen triple bond would emulate the carbocationic transition state of the first step of the reaction that leads to the cyclopropylcarbinyl presqualene-diphosphate **9** as depicted for other inhibitors of the enzymatic activity of this enzyme [17, 18]. The intermediate **9** gives rise to squalene as illustrated in Scheme **1**. The same comprehensive mechanism of action might support the potent inhibitory action exhibited by arylquinuclidines against both mammalian and *T. cruzi* SQS [19, 20].

Ergosterol biosynthesis in trypanosomatids, and, particularly in *T. cruzi*, is a relevant molecular target for drug design and development [21−23]. Sterol biosynthesis in trypanosomatids is unlike that the corresponding one in mammalian hosts. The former one leads to ergosterol rather than cholesterol, the central sterol that arises in humans, in the latter case. Exhaustion of endogenous sterols such as ergosterol or 24-ethylcholesta-5,7,22-trien-3β-ol brings out growth arrest of *T. cruzi* as these metabolites cannot be replaced by the abundant supply of cholesterol present in the host (Scheme **2**) [22, 23]. In fact, there are important and recent reviews describing relevant sterol biosynthesis inhibitors that could be used as potential antiparasitic agents [24-27].

The point for consideration is the optimization of the chemical structure of **WC-9**. This compound is an allosteric



Scheme (1). Reductive dimerization of farnesyl pyrophosphate to yield squalene.



Scheme (2). Brief overview of the ergosterol biosynthesis in trypanosomatids. Bars indicate inhibitors.

inhibitor of the enzymatic activity of *Tc*SQS acting at the low nanomolar range [7, 16], but the binding site at the target enzyme is still unknown. At the present time, crystal structures of *Tc*SQS are not available. In spite of that, the X-ray crystallographic structure of the complex **WC-9**-dehydrosqualene synthase (CrtM) from *Staphylococcus aureus* has been accounted not long ago by other authors [28]. CrtM catalyzes dehydrosqualene formation, a biosynthetic precursor of staphyloxanthin. Based on this X-ray structure, it has been assumed that our compound **WC-9** might behave likewise into the equivalent hydrophobic S2 pocket in the target enzyme *Tc*SQS as observed in CrtM keeping identical polar interactions with the thiocyanate group [28], which is the polar portion of the molecule. Furthermore, the crystal structure of **WC-9** bound to human SQS has been recently accomplished by other group as well [29]. On the contrary, all the efforts to do so with *Tc*SQS, to be precise, the X-ray structure of the complex **WC-9**−*Tc*SQS, were unsuccessful [29]. Notwithstanding, the above crystallographic information is very relevant because superimposition of **WC-9** bound to *h*SQS and CrtM, respectively [28, 29], would probably provide binding affinity predictions of the complexes *Tc*SQS−**WC-9**s by *in silico* design and extended molecular dynamics. Human SQS and *Tc*SQS share less than 50% amino acid identity and experience the same fold [29].

Based on the chemical structure of **WC-9**, we have established a rigorous structure activity relationship (SAR) bringing about that the phenoxyethyl thiocyanate unit should be considered as the pharmacophore [8, 14, 30-32]. Certainly, modifications in the A and B rings have been made, the more relevant findings result that the fluorine-containing analogues like **11** and **12** are four-fold more effective than our reference molecule of **WC-9** against *T. cruzi* multiplying intracellularly (Fig. **2**) [30]. It is important to notice that the substitution pattern of the aryloxy group (B ring) at the C-4′ position as occurs in **WC-9** is not necessarily required for keeping a potent inhibitory action against *T. cruzi* cells. In fact, compounds **13** and **14**, where the aryloxy moiety is bonded at the C-3′ position, exhibit a similar level of efficacy than **WC-9** as growth inhibitors of *T. cruzi* (amastigotes) [31, 32]. All in all, structural variations have been made all through the chemical structure of **WC-9**. The more relevant ones can be summarized as illustrated in the compound of general formula **15** (Fig. **2**). Therefore, in order to study the ability of the oxygen bridge, which bonds the A and B rings (X in compound **15**) on the biological activity, this atom was replaced by a sulfur atom, a nitrogen atom, a methylene group, and a methyleneoxy group concluding that the original oxygen atom was the best selection for keeping a high level of cellular activity [6, 8, 14]. A similar study was conducted at the oxygen atom at the C-1′ position (Y in compound **15**) by replacement this atom by a sulfur atom, an amino group, a methylene group, etc., inferring that this oxygen atom is crucial for inhibitory action [6, 8, 14]. Usually, the substitution at the B ring maintains the same level of efficacy than **WC-9** [6, 8, 14]. Moreover, the chlorine derivative of **16**, where the terminal phenyl group is missing, exhibited promising antiparasitic activity against epimatigotes of *T. cruzi* (ED_{50} = 1.0 μM), but was devoid of activity against intracellular *T. cruzi* [8]. The bromine derivative of **16** was also free of inhibitory action against amastigotes of *T. cruzi* [32].

On the other hand, toxoplasmosis is a major parasitic disease whose etiologic agent is the opportunistic Apicomplexan parasite *Toxoplasma gondii* [33]. *T. gondii* is an obligate intracellular parasite extended worldwide from the very hot and humid in tropical weather to mild one. This parasite has the feline as its ideal host and is able to infect all warm-blooded mammals including humans [34]. In order to

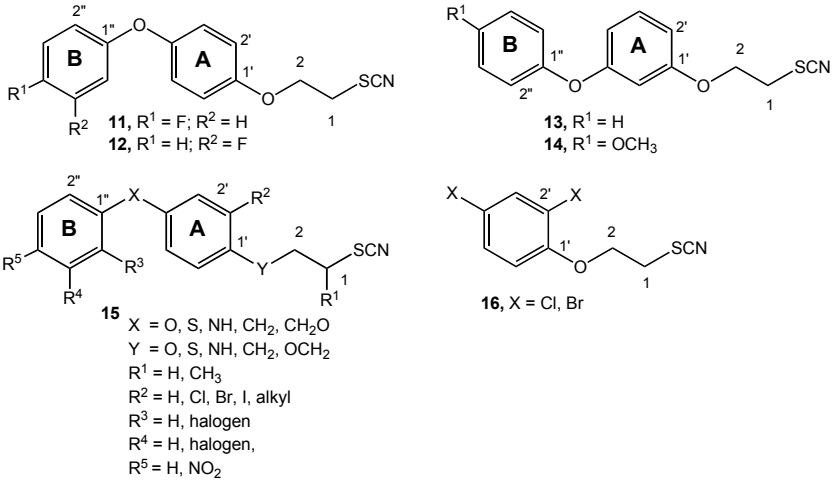
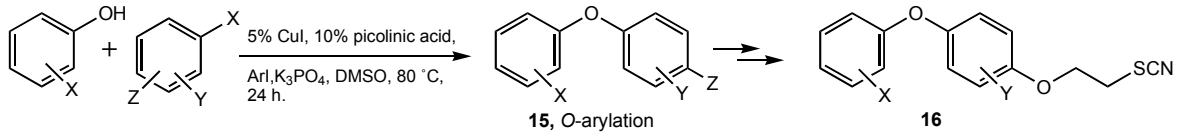


Fig. (2). Chemical structures of relevant **WC-9** analogues.



Scheme (3). Buchwald coupling reaction to prepare highly functionalized diaryl ethers, which leads to **WC-9** analogues.

circumvent the fact that the mevalonate pathway is absent in *T. gondii*, the parasite takes advantage of a prokaryotic-type 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway to biosynthesize IPP and DMAPP instead, which locates in the apicoplast and is crucial [35]. As *T. gondii* does not biosynthesize the essential cholesterol, this metabolite should be taken from the host suggesting that inhibitors of the enzymatic activity of human SQS could putatively inhibit *T. gondii* proliferation [36]. In fact, it has been reported that inhibitors of the mevalonate pathway can control proliferation of different Apicomplexan parasites such as *Plasmodium falciparum* [37, 38], *Babesia divergens* [37], *Cryptosporidium parvum* [39], and *T. gondii* [40], suggesting that in the case of these classes of parasites, where the so-called mevalonate pathway is missing, are needed of host biosynthesis of metabolites of the isoprenoid pathway.

It is of paramount importance to remark that it is possible to inhibit the mevalonate pathway of the host with and the DOXP pathway synergistically or FPP and GGPP of *T. gondii* [38]. In fact, the used of two FDA-approved and extensively used compounds, such as zoledronic acid and atorvastatin exhibit a marked synergistic effect towards *T. gondii* proliferation [41]. In this context, **WC-9** and their structurally similar analogues are potent growth inhibitors of tachyzoites of *T. gondii* acting at low micromolar concentrations. Actually, **WC-9** shows an ED_{50} value of 4.8 μM against *T. gondii* cells, while it exhibits an ED_{50} value of 5.0 μM against intracellular *T. cruzi* [30-32].

Fortunately, the development of the Buchwald coupling reaction has a dramatic effect in the scope of **WC-9** family of compounds, which has proven to be a strong approach to access asymmetric substituted diaryl ethers as illustrated in Scheme **3** [42-44]. With the aid of this method, it will be possible to synthesize diverse **WC-9** analogues bearing different substituents bonded either at the A or B rings of formula **16**. In fact, this is a very reliable and robust protocol compared to the coupling reaction of very expensive phenylboronic acids with phenols [45, 46].

In summary, **WC-9** targeting *Tc*SQS has an immense outlook. At the present time, it is known that its parasitic activity against *T. cruzi* cells is due to a strong non competitive inhibition of the enzymatic activity of *Tc*SQS [16]. The site of the allosteric binding is still unknown. Once its precise mode of action were at hand, it will be possible the design of inhibitors that better fit their molecular target. For that reason, phenoxyethyl thiocyanate derivatives are promising lead structures not only as potential antiparasitic agents, but also as cholesterol-lowering molecules to be used in humans.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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