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Activity of fluorine–containing analogues of WC-9 and structurally related analogues against two intracellular parasites: *Trypanosoma cruzi* and *Toxoplasma gondii*

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Abstract

Two obligate intracellular parasites, *Trypanosoma cruzi*, agent of Chagas disease, and *Toxoplasma gondii*, agent of toxoplasmosis, upregulate the mevalonate pathway of their host cells upon infection, suggesting that this host pathway could be a potential drug target. In this work we designed, synthesized and evaluated the effect of a number of compounds structurally related to **WC-9** (4-phenoxyphenoxyethyl thiocyanate), a known squalene synthase inhibitor, on *T. cruzi* and *T. gondii* growth in tissue culture cells. The fluorine-containing derivatives 3-(3-fluorophenoxy) and 3-(4-fluorophenoxy)phenoxyethyl thiocyanates exhibited EC_{50} values of 1.6 μM and 4.9 μM, respectively, against tachyzoites of *T. gondii*, while they showed similar potency as **WC-9** against intracellular *T. cruzi* (EC_{50} values 5.4 μM and 5.7 μM, respectively). In addition, 2-[3-(phenoxy)phenoxyethylthio]ethyl-1,1-bisphosphonate, which is a hybrid inhibitor containing 3-phenoxyphenoxy and bisphosphonate groups, has activity on *T. gondii* proliferation at sub micromolar levels (EC_{50} = 0.7 μM), suggesting combined inhibitory effect of the two functional groups.

Graphical Abstract

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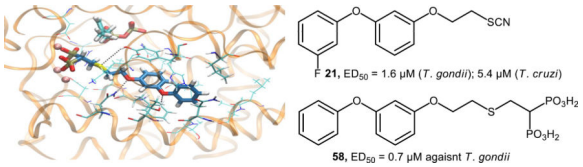
Supporting Information: Physical data and spectral information of the target molecules and the corresponding intermediates as well as copies of the ^{1}H NMR, ^{13}C NMR, and ^{19}F NMR spectra are included as supporting information.



Compound **58** interacting at the active site of *Tg*FPPS (IC_{50} = 0.076 μM) built by homology modeling.

1. Introduction

Obligate intracellular parasites depend on their host cell integrity to survive. They have evolved sophisticated strategies to manipulate their host and establish with them a close metabolic link in order to complete their development. A case in point is that of parasites like *Trypanosoma cruzi*, a trypanosomatid, and *Toxoplasma gondii*, an Apicomplexan parasite, which upon infection, upregulate host genes for the mevalonate pathway.[1–3] This up-regulation is probably in order to acquire cholesterol and other isoprenoids needed to accommodate an increasing intracellular parasite load and/or provide these lipids to be scavenged by the intracellular parasites.[3] *T. cruzi* is the agent of Chagas disease (American trypanosomiasis), the largest parasitic disease burden of the Americas.[4] Treatment of *T. cruzi* infection is with nifurtimox or benznidazole. Both these compounds are not FDA-approved drugs, and in the United States they are available only from CDC under investigational protocols. On the other hand, *T. gondii* is the agent of toxoplasmosis,[5] which affects a wide range of hosts, particularly humans and other warm-blooded animals.[6] Toxoplasmosis can be considered as one of the most prevalent parasitic diseases affecting almost one billion people worldwide.[7] This parasite can cause mortality among immune-compromised individuals such as AIDS patients and organ transplant recipients, as well as in congenitally infected children.[8] Toxoplasmosis may also lead to severe ocular disease in immune-competent patients.[9] The current chemotherapy for toxoplasmosis is also deficient as the available drugs may cause toxic side effects and they are not able to properly access the central nervous system. Another drawback of the present chemotherapy is its high cost.[10]

The up-regulation of the mevalonate pathway of the host by these intracellular parasites provides an additional potential drug target since its inhibition could affect the parasite and the host cell in which the parasite resides. *T. gondii* does not synthesize cholesterol and imports it from the host,[11] while it is also able to take up isoprenoids like farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) synthesized by the host. As other trypanosomatids, *T. cruzi* has a strict requirement for specific endogenous sterols for survival although it can take up cholesterol from its mammalian host.[12,13] Appropriate ergosterol biosynthesis inhibitors can induce parasitological cure in both acute and chronic experimental models of Chagas disease.[14] 4-Phenoxyphenoxyethyl thiocyanate (compound **1; WC-9**) is a potent inhibitor of the intracellular amastigote forms of *T. cruzi*.[15] **WC-9** is a non-competitive inhibitor of *T. cruzi* squalene synthase (*Tc*SQS) acting at low nanomolar concentration.[16] This enzyme catalyzes the first step in the sterol biosynthesis, which

consists in the reductive dimerization of two molecules of farnesyl diphosphate to yield squalene. Additional synthetic derivatives of **WC-9** (**2–6**) are shown in Figure 1.[15,17–21]

It is interesting to note that *T. gondii* lacks the mevalonate pathway and uses the essential 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway to make isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP).[22] As *T. gondii* does not synthesize cholesterol and imports it from the host,[11] it is reasonable to consider that inhibitors of the host SQS could eventually control *T. gondii* growth. Certainly, mevalonate pathway inhibitors modulate growth of different intracellular Apicomplexan parasites that are devoid of this pathway such as *Babesia divergens*,[23] *Plasmodium falciparum*,[23,24] *Cryptosporidium parvum*,[25] and *T. gondii*,[26] indicating that parasites lacking the mevalonate pathway are reliant on host precursors of isoprenoid biosynthesis. Interestingly, there is a synergistic effect when the mevalonate pathway of the host and the isoprenoid pathway of the parasite are targeted independently.[27] For example, zoledronic acid, a bisphosphonate that inhibits the *T. gondii* farnesyl diphosphate synthase (*Tg*FPPS), and atorvastatin, an statin that inhibits the host 3-hydroxymethyl-glutaryl CoA-reductase, exhibit a marked synergism in the inhibition of *T. gondii* growth. [27]

2. Rationale

WC-9 is one of the few examples of a pharmacologically important lead compound possessing a thiocyanate group covalently bound to its main skeleton.[28] At the present time, there is no crystal structure available for the complex **WC-9**–*Tc*SQS. However, an X-ray crystal structure of **WC-9** with human SQS has been recently reported (pdb code 3WCD).[29] On the other hand, the X-ray crystal structure of the complexes E5700–*Tc*SQS and ER-119884–*Tc*SQS are available.[29] The quinuclidine derivatives E5700 (**7**) and ER-119884 (**8**) are potent inhibitors of *T. cruzi* growth acting as *Tc*SQS inhibitors (Figure 2).[30,31] Both of these compounds are extremely potent inhibitors of the enzymatic activity of *Tc*SQS exhibiting IC_{50} values of 0.84 nM and 3.5 nM, respectively.[31]

Figure 3 shows a superimposition of the E5700–*Tc*SQS and ER-119884–*Tc*SQS complexes with the crystal structure of the complex **WC-9**–*human* SQS. A high degree of similarity is observed between the *T. cruzi* and *human* protein structures. Furthermore, the quinoclidine inhibitors occupy the same binding site as **WC-9**. Given that these inhibitors were found to be mixed-type (**7**) and non-competitive (**8**),[32] they provide further evidence that **WC-9** may in fact occupy the same binding site in *T. cruzi* SQS.

Interestingly, *Tc*SQS activity is also inhibited by bisphosphonates.[33] Bisphosphonates are the main modulators of farnesyl diphosphate synthase (FPPS), a key enzyme of isoprenoid biosynthesis.[34] Moreover, several X-ray crystal structures of *Tc*SQS are available with a number of bisphosphonates, such as BPH1344 (**9**) (pdb code 3WCG) (Figure 4).[29]

Figure 5 shows a superposition of the **WC-9**–hsSQS complex (*blue*, NewRibbons representation) with the BPH1344-*Tc*SQS complex (*red*), with the bisphosphonate deleted, showing the putative binding site that **WC-9** could occupy in *T. cruzi* SQS.[29] As can be observed, the two structures show a high degree of similarity, except for the alpha helix 284

– 294 in *T. cruzi* which acquires a loop organization in the corresponding human SQS structure.[29] The X-ray crystal structure of **WC-9** with dehydrosqualene synthase from *Staphylococcus aureus*, an enzyme very similar to SQS that catalyzes dehydrosqualene formation, is also available.[35]

At the present time, there is no a computer-assisted protocol to predict binding of **WC-9** analogues to *Tc*SQS. However, there are abundant structure activity relation (SAR) data available on *T. cruzi* and *T. gondii* cells that can be used to facilitate drug design.[17–21,36] In addition, there is strong evidence to state that the phenoxyethyl thiocyanate moiety of **WC-9** (Figure 1) is the pharmacophore of this family of molecules. A question that emerges, whose answer is still pending, is whether the optimum substitution pattern will be at the C-4′ or the C-3′ position. The availability of the Buchwald coupling reaction allowing us to access a variety of **WC-9** analogues encouraged us to go further in searching for better inhibitors against either *T. cruzi* or *T. gondii* cells.[37–40]

3. Results and Discussion

The introduction of a fluorine atom into the **WC-9** structure to give rise to compounds **2** and **3** was a significant structural change, which was quite beneficial for the inhibitory action.[19] Therefore, it seemed of interest to study the biological activity of the fluorine–containing analogues of the regioisomer of **WC-9**, compound **4**, envisioning compounds **11–13** as target molecules. Compound **10**[20] was treated with 2-fluoro, 3-fluoro, and 4-fluorophenol under typical Buchwald coupling reaction conditions to generate tetrahydropyranyl derivatives **11, 12**, and **13** in 53%, 33 and 71% yields, respectively. Each tetrahydropyranyl protecting group present in these compounds was cleaved by treatment with pyridinium *p*-toluenesulfonate producing the corresponding free alcohols **14, 15** and **16** in very good yields, which were tosylated to give **17, 18** and **19** in 66%, 73% and 84% yields, respectively. On treatment with potassium thiocyanate, in separate experiments, these compounds were converted into the target molecules **20, 21** and **22**, respectively, as illustrated in Scheme 1.

The fluoro derivatives of **WC-9** at C-4″ and C-3″, namely **2** and **3**, were more potent than our lead drug **WC-9**.[19] It seemed reasonable to also produce the fluorinated analogue at C-2″ (compound **27**), which had not been prepared before. Therefore, Buchwald coupling reaction of **23**[20] with 2-fluorophenol generated the tetrahydropyranyl derivative **24** in a low but reproducible yield, which on treatment with pyridinium 4-toluenesulfonate produced the free alcohol **25** in 64% yield. Tosylation of this compound to produce **26** followed by treatment with potassium thiocyanate resulted in the title compound **27** in 36% yield (Scheme 2).

An interesting structural variation was the replacement of the terminal phenyl group by a pyridyl group where the nitrogen atom occupied the 4″ position (compound **31**). We have previously synthesized and evaluated the corresponding 2-pyridyl[21] and 3-pyridyl[20] derivatives; then, the availability of **31** would complete the corresponding SAR analysis. A straightforward synthesis of **31** was accomplished starting form **23**,[20] which treated with 4-hydroxypyridine under typical coupling reaction generated **28**. On treatment with

pyridinium 4-toluenesulfonate, **28** was converted into alcohol **29**, which was further transformed into **30** by reaction with *N*-bromosuccinimide and triphenylphosphine[41] in 52% yield. On reaction with potassium thiocyanate, **30** was transformed into the title compound **31** in 36% yield (Scheme 2).

Although a significant number of structural variations have been made on the structure of **WC-9**, only few of them correspond to substitutions at the A ring.[17] An interesting method to incorporate an acetyl group at the C-2′ is the photo-Fries rearrangement reaction.[42] Then, 4-phenoxyphenol (**32**) was acetylated to give **33** in excellent yield. **33** was irradiated at 254 nm to produce the corresponding photo-Fries rearranged product **34** in 39% yield. **34** was reacted with 2-bromoethyl tetrahydro-2*H*–pyran-2-yl ether *via* a Williamson etherification reaction to give **35** in 62% yield. This compound was deprotected by treatment with pyridinium 4-toluenesulfonate in methanol to generate free alcohol **36** (92% yield), which was treated with tosyl chloride in pyridine to give the expected tosylate **37** in 95% yield. This compound was further transformed into the thiocyanate derivative **38** by treatment with potassium thiocyanate in *N,N*-dimethylformamide at 80 ºC in 71% yield (Scheme 3).

The concept of a hybrid drug where one compound possesses a dual mode of action is fascinating.[43–45] For this reason, hybrid molecules such as **43** and **48** were envisioned with the phenoxy unit targeting SQS and the bisphosphonate moiety acting against FPPS.[34] The production of **42** was straightforward employing the already described azide **39**.[46] This compound was reduced to the corresponding amine **40** through a Staudinger reaction by treatment with triphenylphosphine in methylene chloride in 77% yield.[47] Michael-type addition of the resulting amine **40** with the Michael acceptor **41**[48] yielded **42** in 82% yield, which after hydrolysis by treatment with refluxing hydrochloric acid produced the title compound **43**. The hybrid compound **48** was straightforwardly prepared starting from the described tosylate **44**.[15] Upon treatment of **48** with potassium thioacetate in *N,N*-dimethylformamide at 90 ºC **45** was produced, which when treated with lithium aluminum hydride in anhydrous tetrahydrofuran yielded the corresponding mercaptan **46** in good yields. Michael addition reaction of this resulting compound with **41** followed by hydrolysis of the phosphonate esters by treatment with bromotrimethylsilane and digestion with methanol produced the title compound **48**. Following a quite similar approach, **31** was prepared from the already described tosylate **49**,[20] which was transformed into azide **50** by treatment with sodium azide in *N,N*.dimethylformamide. Under a Staudinger-type conditions **50** was converted into amine **51** that was reacted with **41** to produce the Michael adduct **52**. Hydrolysis of this compound by treatment with refluxing concentrated hydrochloric acid produced the title compound **53**. The synthesis of **58** was also straightforward. The known tosylate **54**[20] was reacted with potassium thioacetate *via* a S_N2 reaction in *N,N*-dimethylformamide at 80 ºC to give rise to **55** in 83% yield. Hydrolysis of the acetyl group by treatment with potassium carbonate followed by reduction with zinc in glacial acetic acid generated the corresponding mercaptan **56** in 75% yield. Michael addition of this mercaptan followed by hydrolysis by treatment with bromotrimethylsilane and methanol digestion produced the title compound **58** in 65% yield. Similarly, **63** was obtained from the already described tosylate **59**,[18] which was transformed into **60** by treatment with

sodium azide. This compound was transformed into **61** that was reacted with **41** to yield **62**. Treatment with refluxing concentrated hydrochloric acid resulted in the title compound **63** (Scheme 4).

As another structural variation, hydrophobic analogues of **WC-9** such as **68** and **73**, were also produced. Both of these compounds were easily prepared starting from β-naphthol (**64**) and α-naphthol (**69**), respectively. Therefore, each compound, in separate experiments, was treated with 2-bromoethyl tetrahydro-2*H*–pyran-2-yl ether under Williamson-type conditions to produce tetrahydropyranyl derivatives **65** and **70**, respectively, which were easily deprotected by treatment with pyridinium 4-toluenesulfonate to yield the corresponding free alcohols **66–71**, respectively. On treatment with excess of tosyl chloride, **66** and **71** were converted into tosylates **67** and **72**, respectively. On reaction with potassium thiocyanate in *N*,*N*-dimethylformamide, **67** and **72** were converted into the title molecules **68** and **73**, respectively as shown in Scheme 5.

Biological evaluation of these new compounds structurally related to **WC-9** was promising. All fluorine-containing derivatives of compound **4**, that is compounds **20–22**, were effective inhibitors of tachyzoites of *T. gondii* exhibiting EC_{50} values of 3.1 μM, 1.6 μM, and 4.9 μM, respectively, with **20** and **21** being slightly more potent than **4** (EC_{50} = 4.0 μM).[20] The fluorinated compounds were also effective growth inhibitors of the intracellular form of *T. cruzi* (amastigotes), which is the clinically more relevant replicative form of the parasite. In fact, all of these compounds bearing a fluorine atom bound to the C-2″, C-3″, and C-4″ positions exhibited relatively similar EC_{50} values (7.0 μM, 5.4 μM, and 5.7 μM, respectively) compared to **WC-9**, used as a positive control, under the same assay conditions.[20] The fluorine analogue of **WC-9** at C-2″, compound **27**, which is the regioisomer of **2** and **3**, was also a potent inhibitor of *T. gondii* tachyzoites growth having an EC_{50} value of 4.5 μM. Compound **27** was more potent than **2** (EC_{50} = 15.8 μM)[19] but exhibited quite similar efficacy as **3** (EC_{50} = 3.9 μM).[19] Quite unexpectedly, in spite of its regioisomers **2** and **3** being potent growth inhibitors of amastigotes of *T. cruzi*,[19] **27** was devoid of antiparasitic activity against these parasites. Pyridyl derivative **31** was free of antiparasitic activity against *T. cruzi*; however, **31** behaved as a growth inhibitor of *T. gondii* possessing an EC_{50} value of 7.9 μM. The introduction of an acetyl group at the C-2′ position giving rise to **38** resulted to be quite beneficial for the biological activity, as this compound showed a potent inhibitory action against *T. gondii*. In fact, its EC_{50} value was 2.4 μM. **38** was less effective against *T. cruzi* with an EC_{50} value of 11.3 μM. The hybrid molecules **53** and **58** were both devoid of activity against *T. cruzi* but they showed a strong inhibitory action against tachyzoites of *T. gondii*. The sulfur-containing analogue **58** showed a sub-micromolar activity against *T. gondii* having an EC_{50} value of 0.7 μM. In fact, the 3′-phenoxy substitution pattern found in **53** and **58** is more beneficial for biological activity against *T. gondii* than the 4′-phenoxy substitution present in **43** and **48**. Compounds **53** and **58** were also recognized as potent inhibitors of the target enzyme *Tg*FPPS exhibiting IC_{50} values of 0.040 μM and 0.076 μM, respectively. The simplified naphtyl derivatives **68** and **73** turned out to be potent inhibitors of *T. gondii* possessing EC_{50} values of 3.6 μM and 3.1 μM, respectively, but exhibited vanishing inhibitory action against *T. cruzi*. The results are presented in Table 1.

Given the high *in vitro* activity of hybrid derivatives of **WC-9**, we performed Molecular Dynamics-based computational studies to gain further insight on the interactions of this kind of compounds with the active site of FPPS. Taking **58** as a relevant example and as no crystal structure of *Tg*FPPS is available, we constructed that structure using homology modeling based on the crystal structure of *P. vivax* FPPS (pdb id 3mav), the enzyme showing the highest degree of similarity with *Tg*FPPS. We inserted **58**, three Mg^{2+} ions and isopentenyl pyrophosphate as a co-ligand, by alignment with the *Tc*FPPS alendronate complex structure (pdb id 1yhm). We carried out a 10 ns Molecular Dynamics simulation in explicit water, and performed a clustering analysis to obtain the most populated structure. Based on an implicit solvent energy decomposition calculation, we identified the key interactions present with the binding site. Tyr 303 forms a hydrogen bond with the sulfur at position 3 of **58**, Gln 204 forms a hydrogen bond with the first bridging oxygen and Thr 200 forms an H-bond with the second bridging oxygen. **π-π** interactions are present between Tyr 303 and ring A and between Phe 131 and ring B. Hydrophobic interactions take place between ring B and Leu 307 and Asn 196. The high degree of specificity of these interactions is an indication of why the *meta*-substituted **58** shows a much higher potency than the *para*-substituted **48** (Figure 6). Further investigation of the ability of different isoprenoids to rescue the growth inhibition could help to pin down the actual target of the bisphosphonate.

We cannot rule out that this inhibitory effect of **WC-9** analogs is in part due to inhibition of the host squalene synthase and of other host enzymes of the mevalonate pathway. In this regard, microarray experiments have shown that 3-HMG-CoA reductase, diphosphomevalonate decarboxylase, and farnesyl diphosphate synthase are induced 24 h after *T. gondii* infection of human foreskin fibroblasts (HFF).[1] Squalene epoxidase, the second enzyme in the cholesterol biosynthesis pathway and one of the rate-limiting enzymes is up-regulated 5-fold following infection of either HFF[1] or porcine kidney epithelial cells line PK13,[2] suggesting that induction of mevalonate biosynthetic enzymes is necessary to increase cellular levels of squalene that could be scavenged by the parasite.[1] Similarly, 3-HMG-CoA reductase, diphosphomevalonate decarboxylase and farnesyl diphosphate synthase are induced several fold 24–72 hours postinfection of *Macaca mulatta* LLcMK2 kidney cells with *T. cruzi*.[3]

It can be concluded that most target compounds exhibited potent action against *T. gondii* proliferation and to a less extent some of them were also growth inhibitors of *T. cruzi*. The drug-like character of our lead compound (for instance it follows all Lipinki rules[49]: no hydrogen bond donors, three hydrogen bond acceptors, molecular weight is 271.3 and logP is 4.20) and other closely related analogues offer good chances for optimizing their chemical structure. The availability of the crystal structure of **WC-9** with dehydrosqualene synthase from *S. aureus* together with crystallographic structure of this compound bound to human SQS and proper docking studies in homology models will allow the further production of more active **WC-9** analogues. A more detailed computational study is under way to further understand the interactions of current inhibitors and guide the development of new compounds, which will be published elsewhere.

Experimental Section

General methods

The glassware used in air- and/or moisture-sensitive reactions was flame dried, and the reactions were performed under a dry argon atmosphere. Unless otherwise noted, chemicals were commercially available and were used without further purification. Anhydrous *N*,*N*-dimethylformamide and anhydrous dimethyl sulfoxide were used as supplied from Aldrich. Nuclear magnetic resonance spectra were performed by using a Bruker AM-500 MHz apparatus. Chemical shifts are reported in parts per million δ relative to tetramethylsilane. ^{13}C NMR spectra were fully decoupled. High-resolution mass spectra were carried out by using a Bruker micrOTOF-Q II spectrometer, which is a hybrid quadrupole time of flight mass spectrometer with MS–MS capability. Melting points were determined by using a Fisher–Johns apparatus. Column chromatography was performed with E. Merck silica gel plates (Kieselgel 60, 230–400 mesh). Analytical thin-layer chromatography was performed by employing 0.2 mm coated commercial silica gel plates (E. Merck, DC-Aluminum sheets, Kieselgel 60 F254).

As judged from the homogeneity of the ^{1}H, ^{13}C, ^{19}F and ^{31}P NMR spectra and HPLC analyses of the title compounds employing a Beckmann Ultrasphere ODS-2 column 5 μM, 250 × 10 mm eluting with acetonitrile–water (9:1) at 3.00 mL/min with a refractive index detector indicated a purity >97%.

Syntheses

3-(2-Fluorophenoxy)phenoxyethyl Tetrahydro-2*H*-pyran-2-yl Ether (11)—A mixture of compound **10** (900 mg, 2.6 mmol), 2-fluorophenol (580 mg, 5.2 mmol), copper(I) iodide (49.2 mg, 0.26 mmol), 2-picolinic acid (63.6 mg, 0.52 mmol) and potassium phosphate tribasic (1.1 g, 5.2 mmol) under anhydrous conditions was evacuated and back-filled with argon. This sequence was repeated twice. Then, dimethyl sulfoxide was added (3.0 mL) and the reaction mixture was stirred at 90 ºC for 4 days. The mixture was cooled to room temperature and was partitioned between methylene chloride (20 mL) and water (20 mL). The aqueous layer was extracted with methane chloride (2 × 20 mL) and the combined organic phases were washed with brine (5 × 50 mL), dried ($MgSO_4$) and the solvent was evaporated. The product was purified by column chromatography (silica gel) employing hexane–EtOAc (24:1) as eluent to produce 453 mg (53% yield) of pure compound **11** as a colorless oil.

3-(3-Fluorophenoxy)phenoxyethyl Tetrahydro-2*H*-pyran-2-yl Ether (12)—To a mixture of **10** (893 mg, 2.6 mmol), 3-fluorophenol (575 mg, 5.1 mmol), copper(I) iodide (48.9 mg, 0.26 mmol), 2-picolinic acid, (63.2 mg, 0.51 mmol), and potassium phosphate tribasic (1.092 g, 5.1 mmol) was added dimethyl sulfoxide was added (3.0 mL) as described for the preparation of **11**. The reaction mixture was stirred at 90 ºC for 14 days. After the usual work-up the product was purified by column chromatography (silica gel) employing hexane–EtOAc (49:1) as eluent to produce 280 mg (33% yield) of **12** as a colorless oil.

3-(4-Fluorophenoxy)phenoxyethyl Tetrahydro-2*H*-pyran-2-yl Ether (13)—A mixture of **10** (877 mg, 2.5 mmol), 4-fluorphenol (565 mg, 5.0 mmol), copper(I) iodide (48.0 mg, 0.25 mmol), 2-picolinic acid, (62.0 mg, 0.50 mmol), and potassium phosphate tribasic (1.072 g, 5.0 mmol) was treated with dimethyl sulfoxide (3.0 mL) as described for the preparation of **11**. The reaction mixture was stirred at 90 ºC for 4 days. The reaction was quenched as described for 11 and the product was purified by column chromatography (silica gel) employing hexane–EtOAc (24:1) as eluent to produce 594 mg (71% yield) of **13** as a colorless oil.

3-(2-Fluorophenoxy)phenoxyethanol (14)—A solution of **11** (426 mg, 1.3 mmol) in methanol (10 mL) was treated with pyridinium 4-toluenesulfonate (30 mg). The reaction mixture was stirred at room temperature overnight. Then, water (50 mL) was added and the mixture was extracted with methylene chloride (3 × 50 mL). The combined organic layers were washed with brine (3 × 50 mL), dried ($MgSO_4$), and the solvent was evaporated. The residue was purified by column chromatography eluting with hexane-EtOAc (85:15) to produce 297 mg (93% yield) of pure alcohol **14** as a colorless oil.

3-(3-Fluorophenoxy)phenoxyethanol (15)—A solution of **12** (269 mg, 0.81 mmol) in methanol (10 mL) was treated with pyridinium 4-toluenesulfonate (30 mg) as depicted for the preparation of **14**. The residue was purified by column chromatography eluting with hexane–EtOAc (83:17) to give 182 mg (91% yield) of alcohol **15** as a colorless oil.

3-(4-Fluorophenoxy)phenoxyethanol (16)—A solution of **13** (581 mg, 1.7 mmol) in methanol (10 mL) was treated with pyridinium 4-toluenesulfonate (30 mg) as described for the preparation of **14**. The crude product was purified by column chromatography (silica gel) eluting with hexane–EtOAc (41:9) to produce 399 mg (92% yield) of alcohol **16** as a colorless oil.

3-(2-Fluorophenoxy)phenoxyethyl 4-Toluenesulfonate (17)—A solution of alcohol **14** (253 mg, 0.95 mmol) in pyridine (3 mL) was treated with p-toluenesulfonyl chloride (546 mg, 2.9 mmol) and the mixture was stirred at room temperature for 4 h. Then, 5% HCl (50 mL) was added and the reaction mixture was stirred for an additional hour. The mixture was partitioned between methylene chloride (50 mL) and water (50 mL). The organic layer was washed with 5% HCl (3 × 50 mL) and water (3 × 50 mL). The organic phase was dried ($MgSO_4$) and the solvent was evaporated. The product was purified by column chromatography (silica gel) employing a mixture of hexane–EtOAc (9:1) as eluent to produce 226 mg of **17** (66% yield) as a colorless oil.

3-(3-Fluorophenoxy)phenoxyethyl 4-Toluenesulfonate (18)—A solution of alcohol **15** (175 mg, 0.70 mmol) in pyridine (3 mL) was treated with 4-toluenesulfonyl chloride (402 mg, 2.1 mmol) and the mixture was stirred at room temperature for 4 h. The reaction was quenched as depicted for the preparation of **17**. The product was purified by column chromatography (silica gel) employing a mixture of hexane–EtOAc (91:9) as eluent to produce 207 mg of **18** (73% yield) as a colorless oil.

3-(4-Fluorophenoxy)phenoxyethyl 4-Toluenesulfonate (19)—A solution of alcohol **16** (388 mg, 1.6 mmol) in pyridine (3.0 mL) was treated with 4-toluenesulfonyl chloride (894 mg, 4.7 mmol) as depicted for the preparation of **17**. The product was purified by column chromatography (silica gel) employing a mixture of hexane–EtOAc (91:9) as eluent to produce 226 mg of **19** (84% yield) as a colorless oil.

3-(2-Fluorophenoxy)phenoxyethyl Thiocyanate (20)—A solution of tosylate **17** (381 mg, 0.98 mmol) in anhydrous *N,N*-dimethylformamide (3.0 mL) was treated with potassium thiocyanate (460 mg, 4.7 mmol). The reaction mixture was heated at 100 ºC for 3 h. The mixture was allowed to cool to room temperature and water (20 mL) was added. The aqueous phase was extracted with methylene chloride (2 × 30 mL), and the combined organic layers were washed with brine (5 × 30 mL), water (2 × 30 mL), dried ($MgSO_4$), and the solvent was evaporated. The residue was purified by column chromatography (silica gel) eluting with hexane–EtOAc (23:2) to produce 226 mg (80% yield) of **20** as a colorless oil.

3-(3-Fluorophenoxy)phenoxyethyl Thiocyanate (21)—A solution of **18** (197 mg, 0.49 mmol) in anhydrous *N,N*-dimethylformamide (3.0 mL) was treated with potassium thiocyanate (237 mg, 2.4 mmol). The mixture was treated as described for the preparation of **20**. The residue was purified by HPLC eluting with methanol–H_2O (9:1) and employing a Bechman Ultrasphere 5 μM column (250 mm × 10 mm) to give rise to 107 mg (68% yield) of **21** as a colorless oil.

3-(4-Fluorophenoxy)phenoxyethyl Thiocyanate (22)—A solution of **19** (514 mg, 1.3 mmol) in anhydrous *N,N*-dimethylformamide (3.0 mL) was treated with potassium thiocyanate (647 mg, 6.7 mmol). The reaction mixture was treated as depicted for **20**. The residue was purified by column chromatography (silica gel) eluting with hexane–EtOAc (47:3) to produce 221 mg (58% yield) of **22** as a colorless oil.

4-(2-Fluorophenoxy)phenoxyethyl Tetrahydro-2*H*-pyran-2-yl Ether (24)—To a mixture of **23** (858 mg, 2.5 mmol), 2-fluorophenol (549.5 mg, 4.9 mmol), copper(I) iodide (46.0 mg, 0.24 mmol), 2-picolinic acid, (59.4 mg, 0.51 mmol), and potassium phosphate tribasic (1.026 g, 4.9 mmol) was added dimethyl sulfoxide was added (3.0 mL) as described for the preparation of **11**. The reaction mixture was stirred at 90 ºC for 12 days. After the usual work-up the product was purified by column chromatography (silica gel) employing hexane–EtOAc (19:1) as eluent to produce 295 mg (37% yield) of **24** as a colorless oil.

4-(2-Fluorophenoxy)phenoxyethanol (25)—A solution of **12** (280 mg, 0.84 mmol) in methanol (10 mL) was treated with pyridinium 4-toluenesulfonate (30 mg) as depicted for the preparation of **14**. The residue was purified by column chromatography eluting with hexane–EtOAc (9:1) to give 124 mg (62% yield) of alcohol **25** as a colorless oil.

4-(2-Fluorophenoxy)phenoxyethyl 4-Toluenesulfonate (26)—A solution of alcohol **25** (110 mg, 0.44 mmol) in pyridine (3.0 mL) was treated with 4-toluenesulfonyl chloride (250 mg, 1.3 mmol) and the mixture was stirred at room temperature for 24 h. The reaction was quenched as depicted for the preparation of **17**. The product was purified by column

chromatography (silica gel) employing a mixture of hexane–EtOAc (19:1) as eluent to produce 163 mg of **26** (92% yield) as a colorless oil.

4-(2-Fluorophenoxy)phenoxyethyl Thiocyanate (27)—A solution of **26** (151 mg, 0.38 mmol) in anhydrous *N*,*N*-dimethylformamide (3.0 mL) was treated with potassium thiocyanate (215.7 mg, 2.2 mmol). The reaction mixture was heated at 80 ºC for 6 h and was quenched as depicted for **20**. The residue was purified by column chromatography (silica gel) eluting with hexane–EtOAc (19:1) to produce 39.6 mg (36% yield) of **27** as a colorless oil.

4-(4-pyridyl)oxyphenoxyethyl Tetrahydro-2*H*-pyran-2-yl Ether (28)—To a mixture of **23** (2.145 g, 6.25 mmol), 4-hydroxypyridine (1.165 g, 12.3 mmol), copper(I) iodide (115.0 mg, 0.6 mmol), 2-picolinic acid, (149.2 mg, 1.28 mmol), and potassium phosphate tribasic (2.565 g, 12.3 mmol) was added dimethyl sulfoxide was added (10.0 mL) as described for the preparation of **11**. The reaction mixture was stirred at 90 ºC for 12 days. After the usual work-up the product was purified by column chromatography (silica gel) employing hexane–EtOAc (1:1) as eluent to produce 572 mg (29% yield) of **28** as a colorless oil.

4-(4-pyridyl)oxyphenoxyethanol (29)—A solution of **28** (556 mg, 1.76 mmol) in methanol (10 mL) was treated with 4-toluenesulfonic acid (90 mg) as depicted for the preparation of **14**. The residue was purified by column chromatography eluting with EtOAc–MeOH (49:1) to give 240.1 mg (59% yield) of alcohol **29** as a colorless oil.

4-(4-pyridyl)oxyphenoxyethyl Bromide (30)—To a mixture of *N*-bromosuccinimide (187.0 mg, 1.05 mmol) and triphenylphosphine (275.4 mg, 1.05 mmol) in anhydrous methylene chloride (20 mL) cooled at 0 ºC was treated with alcohol **29** (220.3 mg, 0.95 mmol) and the reaction mixture was stirred at 0 ºC for 6 h. The solvent was evaporated and the residue was purified by column chromatography (silica gel) eluting with a mixture of CH_2Cl_2–MeOH (99:1) to give 154.2 mg (50% yield) of pure **30** as a colorless oil.

4-(4-pyridyl)oxyphenoxyethyl Thiocyanate (31)—A solution of **30** (130 mg, 0.44 mmol) in anhydrous *N*,*N*-dimethylformamide (3.0 mL) was treated with potassium thiocyanate (172.6 mg, 1.8 mmol). The reaction mixture was heated at 80 ºC for 6 h and was quenched as depicted for **20**. The residue was purified by column chromatography (silica gel) eluting with CH_2Cl_2–MeOH (19:1) to produce 40.8 mg (34% yield) of **31** as a colorless oil.

4-Phenoxyphenyl Acetate (33)—A solution of 4-phenoxyphenol (**32**; 2.08 g, 11.2 mmol) in pyridine (5 mL) was treated with acetic anhydride (3 mL) and the reaction mixture was stirred at room temperature for 16 h. Then, water (10 mL) was added and the mixture was stirred for 1 h. The mixture was extracted with methylene chloride (2 × 30 mL). The combined organic layers were washed with an aqueous 1 N solution of hydrochloric acid (2 × 30 mL), water (2 × 30 mL), dried ($MgSO_4$), and the solvent was evaporated. The product

was purified by column chromatography (silica gel) eluting with a mixture of hexane–EtOAc (19:1) to give 2.478 g (97% yield) of **33** as a colorless oil.

2-Acetoxy-4-phenoxyphenol (34)—A solution of acetate **33** (257.8 mg, 1.13 mmol) in cyclohexane (200 mL), previously degassed with dry nitrogen, in a septum stopped quartz tube was irradiated with germicide lamps (4 × 20 W) centered at 254 nm at room temperature for 12 h. The solvent was evaporated and the residue was purified by column chromatography (silica gel) eluting with a mixture of hexane–EtOAc to produce 100.5 mg (39% yield) of pure **34** as a white solid.

2-Acetoxy-4-phenoxyphenoxyethyl Tetrahydro-2*H*-pyran-2-yl Ether (35)—A solution of **34** (457.1 g, 2.0 mmol) in dimethyl sulfoxide (5.0 mL) was treated with potassium hydroxide (450 mg, 8.0 mmol). The mixture was stirred at room temperature for 5 min. Then, bromoethyl tetrahydropyranyl ether (418 mg, 2.0 mmol) was added, and the reaction mixture was stirred at room temperature overnight. The mixture was partitioned between water (35 mL) and methylene chloride (35 mL). The aqueous phase was extracted with methylene chloride (2 × 30 mL). The combined organic layers were washed with a saturated solution of sodium chloride (5 × 30 mL), dried ($MgSO_4$), and the solvent was evaporated. The product was purified by column chromatography (silica gel) eluting with hexane–EtOAc (19:1) to yield 442 mg (62% yield) of **35** as a colorless oil.

2-Acetoxy-4-phenoxyphenoxyethanol (36)—A solution of **35** (420 mg, 1.2 mmol) in methanol (10 mL) was treated with pyridinium 4-toluenesulfonate (30 mg) as depicted for the preparation of **14**. The residue was purified by column chromatography eluting with hexane–EtOAc (83:17) to give 301 mg (92% yield) of alcohol **36** as a colorless oil.

2-Acetoxy-4-phenoxyphenoxyethyl 4-Toluenesulfonate (37)—A solution of alcohol **36** (280.2 mg, 1.03 mmol) in pyridine (3 mL) was treated with 4-toluenesulfonyl chloride (383 mg, 2.0 mmol) and the mixture was stirred at room temperature for 6 h. The reaction was quenched as depicted for the preparation of **17**. The product was purified by column chromatography (silica gel) employing a mixture of hexane–EtOAc (9:1) as eluent to produce 417 mg of **37** (95% yield) as a colorless oil.

2-Acetoxy-4-phenoxyphenoxyethyl Thiocyanate (38)—A solution of **37** (348 mg, 0.82 mmol) in anhydrous *N*,*N*-dimethylformamide (3.0 mL) was treated with potassium thiocyanate (321.6 mg, 3.3 mmol). The reaction mixture was heated at 80 ºC for 6 h and was quenched as depicted for **20**. The residue was purified by column chromatography (silica gel) eluting with hexane–EtOAc (9:1) to produce 186.9 mg (71% yield) of **27** as a colorless oil.

4-Phenoxyphenoxyethyl amine (40)—A solution of azide **39** (1.020 g, 4.0 mmol) in tetrahydrofuran (20 mL) was treated with triphenylphosphine (2.308 g. 8.8 mmol). The reaction mixture was stirred at room temperature for 4 h. Then, water (2.0 mL) was added and the mixture was stirred for an additional hour. The solvent was evaporated and the residue was purified by column chromatography (silica gel) eluting with CH_2Cl_2–MeOH (19:1) to give 706 mg (77% yield) of **40** as a colorless oil.

Tetraethyl 1-[(4-phenoxyphenoxyethylamino)ethyl]-1,1-bisphosphonate (42)— To a solution of **41** (300 mg, 1.5 mmol) in anhydrous methylene chloride (10 mL) was added amine **40** (345.1mg, 1.5 mmol) under an argon atmosphere. The reaction mixture was stirred at room temperature overnight. The solvent was evaporated to produce 651 mg (82% yield) of **42**, which was used in next step without further purification.

1-[(4-Phenoxyphenoxyethylamino)ethyl]-1,1-bisphosphonic Acid (43)— Compound **42** (430.1 mg, 0.81 mmol) was treated with a concentrated aqueous solution of hydrochloric acid (3.0 mL). The resulting mixture was refluxed for 24 h. The solvent was evaporated and the residue was crystalized from H_2O–ethanol (1:1) to give 274.1 mg (81% yield) of **52** as a white solid.

S-(2-(4-phenoxyphenoxy)ethyl) ethanethioate (45)—A solution of tosylate **44** (1.90 g, 4.9 mmol) in anhydrous *N,N*-dimethylformamide (10 mL) under argon atmosphere was treated with potassium thioacetate (1.23 g, 10.8 mmol). The reaction mixture was stirred at 90 ºC for 3 h. The solvent was evaporated and the residue was partitioned between water (50 mL) and methylene chloride (50 mL). The aqueous phase was extracted with methylene chloride (2 × 50 mL). The combined organic layers were dried ($MgSO_4$), and the solvent was evaporated. The product was purified by column chromatography (silica gel) eluting with hexane–EtOAc (19:1) to produce 1.383 g (98% yield) of pure **45** as a colorless oil.

2-(4-phenoxyphenoxy)ethanethiol (46)—To a mixture of lithium aluminum hydride (238 mg, 63 mmol) in anhydrous tetrahydrofuran (20.0 mL) cooled at 0 ºC was added a solution of compound **45** (1.35 g, 4.7 mmol) in tetrahydrofuran (8.0 mL). The reaction mixture was allowed to reach room temperature and was stirred for 60 min. The reaction was quenched by addition of ethyl acetate (10 mL). The mixture was partitioned between an aqueous saturated solution of sodium potassium tartrate (50 mL) and methylene chloride (50 mL). The aqueous layer was extracted with methylene chloride (2 × 50 mL). The combined organic layers were dried ($MgSO_4$) and the solvent was evaporated. The residue was purified by column chromatography (silica gel) eluting with hexane–EtOAc (99:1) to give 1.064 g (92% yield) of **46** as a colorless oil.

Tetraethyl 1-[4-phenoxyphenoxyeth-1-ylthio)ethyl] 1,1-bisphosphonate (47)— To a solution of tetraethyl ethenylidenbisphosphonate (**41**; 300 mg, 1 mmol) in anhydrous dichloromethane (10 mL) was added triethylamine (139 μL, 101 mg, 1.0 mmol) and the corresponding mercaptan **24** (246 mg, 1.0 mmol). The reaction mixture was stirred at room temperature for 5h. Water (20 mL) was added, and the mixture was extracted with dichloromethane (3 × 10 mL). The combined organic layers were washed with brine (20 mL), dried ($MgSO_4$), and the solvent was evaporated to produce 336 mg (59% yield) of tetraethyl ester **47**. The product was used in the next step without further purification: colorless oil.

1-[4-Phenoxyphenoxyeth-1-ylthio)ethyl] 1,1-bisphosphonic acid (48)—A solution of the tetraethyl ester **47** (290 mg, 0.53 mmol) in anhydrous methylene chloride (10 mL) was treated with bromotrimethylsilane (10 equiv.) under an argon atmosphere. The

reaction mixture was stirred at room temperature for 48 h. Then, methanol (1.0 mL) was added and the solvent was evaporated. The residue was dissolved in methanol (10.0 mL) and the mixture was stirred at room temperature for 24 h. The solvent was evaporated and the residue redissolved and evaporated in methanol four times, to complete the hydrolysis of remaining trimethylsilyl bromide and to remove the recently formed hydrobromic acid. The residue was purified by column chromatography (Silica gel C18-reversed phase) eluting with a mixture of water–methanol (9:1) to produce 159 mg (69% yield) of **48** as an amorphous solid.

3-Phenoxyphenoxyethylazide (50)—A solution of tosylate **49** (999.6 mg, 2.60 mmol) in anhydrous *N,N*-dimethylformamide (5 mL) was treated with sodium azide (845.1 mg, 13.0 mmol). The reaction mixture was stirred at 80 ºC for 2 h. Then, the mixture was allowed to cool to room temperature and water (50 mL) was added. The mixture was extracted with methylene chloride (3 × 20 mL) and the combined organic layers were washed with an aqueous saturated solution of sodium chloride (5 × 20 mL), water (2 × 20 mL) and dried ($MgSO_4$). The solvent was evaporated and the product was purified by column chromatography (silica gel) using a hexane–EtOAc (97:3) as eluent to produce 590.1 mg (90% yield) of **50** as a colorless oil.

3-Phenoxyphenoxyethylamine (51)—A solution of 3-phenoxyphenoxyethyl azide (**50**, 545.3 mg, 2.1 mmol) in tetrahydrofuran (10 mL) was treated with triphenylphosphine (616.3 mg, 2.4 mmol). The reaction mixture was stirred 2 hours at room temperature. Then, water (20 mL) was added and the mixture was extracted with methylene chloride (3 × 20 mL). The combined organic phases were washed with water (2 × 20 mL), dried ($MgSO_4$), and the solvent was evaporated. The product was purified by column chromatography (silica gel) eluting with a mixture of CH_2Cl_2–methanol (49:1) as eluent to produce 431.1 mg (89.5 yield) of **51** as a colorless oil.

Tetraethyl 1-[(3-phenoxyphenoxyethylamino)ethyl]-1,1-bisphosphonate (52)—To a solution of **41** (451.0 mg, 1.5 mmol) in anhydrous methylene chloride (10 mL) was added amine **51** (350.8 mg, 1.5 mmol) under an argon atmosphere. The reaction mixture was stirred at room temperature overnight. The solvent was evaporated to give 770 mg (97% yield) of **52**, which was used in next step without further purification.

1-[(3-Phenoxyphenoxyethylamino)ethyl]-1,1-bisphosphonic Acid (53)—Compound **52** (411.2 mg, 0.78 mmol) was treated with a concentrated aqueous solution of hydrochloric acid (2 mL). The resulting mixture was refluxed for 24 h. The solvent was evaporated and the residue was crystalized from H_2O–ethanol (1:1) to give 114 mg (35% yield) of **53** as an amorphous solid.

***S*-[3-Phenoxy)phenoxyethyl] ethanethioate (55)—**A solution of **54** (660 mg, 1.7 mmol) in anhydrous *N,N*-dimethylformamide (3.0 mL) was treated with potassium thioacetate (392 mg, 3.4 mmol). The reaction mixture was stirred at 80 ºC for 3 h. The mixture was allowed to cool to room temperature and water (20 mL) was added. The aqueous phase was extracted with methylene chloride (2 × 30 mL) and the combined organic

layers were washed with brine (5 × 30 mL), water (2 × 30 mL), dried ($MgSO_4$), and the solvent evaporated. The residue was purified by column chromatography (silica gel) eluting with hexane–EtOAc (49:1) to give 411 mg (83% yield) of **55** as a colorless oil.

3-(Phenoxy)phenoxyethyl mercaptan (56)—To a solution of **55** (957 mg, 3.3 mmol) in methanol (10 mL) potassium carbonate (anhydrous powder) were added while stirring, followed by water (3.0 mL) to obtain complete solution. The reaction mixture was stirred at room temperature for 40 min and the solvent was evaporated. The residue was dissolved in glacial acetic acid, and zinc (1.8 g, 28 mmol) was added. The reaction mixture was refluxed for 1 h. The mixture was allowed to cool to room temperature and was partitioned between water (30 mL) and methylene chloride (30 mL). The organic phase was washed with water (2 × 30 mL), dried ($MgSO_4$), and the solvent was evaporated to yield 616 mg (75% yield) of **56** as a colorless oil, which was used in the next step without further purification.

Tetraethyl 2-[3-(Phenoxy)phenoxyethylthio]ethyl-1,1-bisphosphonate (57)—To a solution of tetraethyl ethenylidenbisphosphonate (**41**; 751 mg, 2.5 mmol) in anhydrous dichloromethane (10 mL) was added thiol **56** (616 mg, 2.5 mmol). The reaction mixture was stirred at room temperature for 1 day. Water (20 mL) was added, and the mixture was extracted with dichloromethane (3 × 10 mL). The combined organic layers were dried ($MgSO_4$), and the solvent was evaporated. The residue was purified by column chromatography (silica gel) eluting with CH_2Cl_2–methanol (49:1) to produce 1.1 g (81% yield) of **57** as a colorless oil.

2-[3-(Phenoxy)phenoxyethylthio]ethyl-1,1-bisphosphonic Acid (58)—A solution of **57** (1.1 g, 2.0 mmol) in anhydrous methylene chloride (10 mL) was treated with bromotrimethylsilane (3.1 g, 20.2 mmol) under an argon atmosphere. The reaction mixture was stirred at room temperature for 48 h. Then, methanol (1.0 mL) was added and the solvent was evaporated. The residue was dissolved in methanol (8 mL) and the mixture was stirred at room temperature for 24 h. The solvent was evaporated and the residue redissolved/evaporated in methanol four times. The residue was purified by column chromatography (reverse phase) eluting with water–methanol (1:1) to produce 568 mg (65%) of a **58** as a yellow pale solid after lyophilization.

Phenoxyethylazide (60)—A solution of **59** (1.1054 g, 3.78 mmol) in anhydrous *N*,*N*-dimethylformamide (5 mL) was added sodium azide (1.2290 g, 18.9 mmol). The reaction mixture was stirred at 80 ºC for 2 h. Then, the mixture was allowed to cool to room temperature and water (50 mL) was added. The aqueous phase was extracted with methylene chloride (2 × 20 mL) and the combined organic layers were washed with an aqueous saturated solution of sodium chloride (5 × 20 mL), water (2 × 20 mL) and dried ($MgSO_4$). The solvent was evaporated and the residue was purified by column chromatography (silica gel) eluting with hexane–EtOAc (97:3) to give 498.4 mg (81% yield) of **60** as a colorless oil.

Phenoxyethylamine (61)—A solution of azide **60** (463.6 mg, 2.84 mmol) in tetrahydrofuran (10 mL) was treated with triphenylphosphine (819.7 mg, 3.12 mmol) as depicted for the preparation of **40**. The residue was purified by column chromatography

(silica gel) eluting with a mixture of hexane–EtOAc (9:1) to afford 260.8 mg (67% yield) of **61** as a yellow pale oil.

Tetraethyl 1-[(Phenoxyethylamino)ethyl]-1,1-bisphosphonate (62)—To a solution of **41** (452.9 mg, 1.5 mmol) in anhydrous methylene chloride (10 mL) was added amine **61** (194.6 mg, 1.5 mmol) under an argon atmosphere. The reaction mixture was stirred at room temperature overnight. The solvent was evaporated to give 574.6 mg (88% yield) of **62**, which was used in next step without further purification.

1-[(Phenoxyethylamino)ethyl]-1,1-bisphosphonic Acid (63)—Compound **62** (547.6 mg, 1.25 mmol) was treated with a concentrated aqueous solution of hydrochloric acid (4.0 mL). The mixture was refluxed for 24 h. The solvent was evaporated and the residue was crystalized from H_2O–ethanol (1:1) to give 44.5 mg (11% yield) of **63** as a white solid.

2-(Naphthalen-2-yloxy)ethyl Tetrahydro-2*H*-pyran-2-yl Ether (65)—A solution of **64** (1.0121 g, 7.02 mmol) in dimethyl sulfoxide (10.0 mL) was treated with potassium hydroxide (877 mg, 15.6 mmol) and bromoethyl tetrahydropyranyl ether (1.7957 g, 8.6 mmol) as described for the preparation of **35**. The product was purified by column chromatography (silica gel) eluting with hexane–EtOAc (19:1) to produce 917.6 mg (48% yield) as a colorless oil.

2-(Naphthalen-2-yloxy)ethanol (66)—A solution of **65** (789.0 mg, 2.9 mmol) in methanol (10 mL) was treated with pyridinium 4-toluenesulfonate (30 mg) as depicted for the preparation of **14**. The residue was purified by column chromatography eluting with hexane–EtOAc (9:1) to give 463.5 mg (85% yield) of alcohol **66** as a colorless oil.

2-(Naphthalen-2-yloxy)ethyl 4-Toluenesulfonate (67)—A solution of alcohol **66** (450.3 mg, 2.45 mmol) in pyridine (5 mL) was treated with 4-toluenesulfonyl chloride (1.422 g, 7.5 mmol) and the mixture was stirred at room temperature for 4 h. The reaction was worked up as depicted for the preparation of **17**. The product was purified by column chromatography (silica gel) employing a mixture of hexane–EtOAc (91:9) as eluent to produce 696.3 mg of **67** (83% yield) as a colorless oil.

2-(Naphthalen-2-yloxy)ethyl Thiocyanate (68)—A solution of **67** (464.1 mg, 1.35 mmol) in anhydrous *N*,*N*-dimethylformamide (3.0 mL) was treated with potassium thiocyanate (531 mg, 5.5 mmol). The reaction mixture was treated as described for **20**. The residue was purified by column chromatography (silica gel) eluting with hexane–EtOAc (19:1) to produce 170.3 mg (55% yield) of **68** as a colorless oil.

2-(Naphthalen-1-yloxy)ethyl Tetrahydro-2*H*-pyran-2-yl Ether (70)—A solution of **69** (537.1 g, 3.82 mmol) in dimethyl sulfoxide (5.0 mL) was treated with potassium hydroxide (503 mg, 8.9 mmol) and bromoethyl tetrahydropyranyl ether (1.1157 g, 5.3 mmol) as described for the preparation of **35**. The product was purified by column chromatography (silica gel) eluting with hexane–EtOAc (19:1) to produce 675.7 mg (65% yield) as a colorless oil.

2-(Naphthalen-1-yloxy)ethanol (71)—A solution of **70** (562.8 mg, 2.07 mmol) in methanol (10 mL) was treated with pyridinium 4-toluenesulfonate (30 mg) as depicted for the preparation of **14**. The residue was purified by column chromatography eluting with hexane–EtOAc (9:1) to give 315.6 mg (81% yield) of alcohol **71** as a colorless oil.

2-(Naphthalen-1-yloxy)ethyl 4-Toluenesulfonate (72)—A solution of alcohol **71** (326.1 mg, 1.77 mmol) in pyridine (5 mL) was treated with 4-toluenesulfonyl chloride (1.027 g, 5.4 mmol) and the mixture was stirred at room temperature for 4 h. The reaction was worked up as depicted for the preparation of **17**. The product was purified by column chromatography (silica gel) employing a mixture of hexane–EtOAc (9:1) as eluent to produce 545.5 mg of **72** (90% yield) as a colorless oil.

2-(Naphthalen-1-yloxy)ethyl Thiocyanate (73)—A solution of **67** (445.4 mg, 1.29 mmol) in anhydrous *N,N*-dimethylformamide (3.0 mL) was treated with potassium thiocyanate (550 mg, 5.7 mmol). The reaction mixture was treated as described for **20**. The residue was purified by column chromatography (silica gel) eluting with hexane–EtOAc (19:1) to yield 171.6 mg (58% yield) of **73** as a colorless oil.

Drug Screening

***T. cruzi* amastigote assays—**These experiments were done as reported using tdTomato labeled trypomastigotes[50] with the modifications described by Recher et al., 2013.[51] EC_{50} values were determined by non-linear regression analysis using SigmaPlot.

***T. gondii* tachyzoites assays—**Experiments on *T. gondii* tachyzoites were carried out as described previously[52] using *T. gondii* tachyzoites expressing red fluorescent protein[53] with the modifications described by Recher et al., 2013.[51] Plates were read with covered lids, and both excitation (544 nm) and emission (590 nm) were read from the bottom.

Cytotoxicity for Vero cells—The cytotoxicity was tested using the Alamar Blue™ assay as described by Recher et al., 2013.[51]

Computational Methods

Model building—The structure of *Tg*FPPS was built using homology modeling using SwissModel,[54] based on the structure of *P. vivax* FPPS (pdb id 3mav), a protein showing the highest degree of similarity with *Tg*FPPS. The constructed *Tg*FPPS structure was aligned with the *Tc*FPPS alendronate complex (pdb id 1yhm),[55] the structure of **58** was built on that of alendronate, Mg^{2+} ions and the co-ligand isopentenyl pyrophosphate were added. Charges for **58** and isopentenyl pyrophosphate were obtained using the RESP method at the Hartree Fock 6–31G* level, and the compounds were parameterized using the Generalized Amber Force Field. The complex was built using the *tleap* module in AmberTools 15.[56] The Amber FF14SB Force Field was used to parameterize the protein structure, the charge was neutralized by the addition of 7 Na^+ ions and the complex was solvated with a box of TIP3P waters extending 10 Å beyond the complex.

Molecular Dynamics (MD) Simulations—Simulations were run using the *sander* module in AmberTools 15. The Mg and phosphate units of **58** were restrained during all simulations to their original coordinates using a 100 Kcal/mol harmonic potential. The complex was minimized for 1000 steps of steepest descent followed by 1000 steps of conjugate gradient. It was then heated from 0 K to 300 K for 20 ps using Langevin dynamics followed by an 80 ps optimization using the NPT ensemble. The production run consisted of a 10 ns molecular dynamics simulation using the NVT ensemble.

Energy calculations—a calculation of binding energy was obtained using the MMPBSA.py module in AmberTools 15. The Generalized Born Surface Area model was used with igb = 5 and the corresponding mbondi2 radii, and a salt concentration o f 0.1 M. 100 frames at 100 ps intervals were taken from the MD simulation. Pairwise energy decomposition was carried out with the ligand decomposed fragment-wise using an in-house modified version of MMPBSA.py.

Clustering analysis—A clustering analysis was performed with the *cpptraj* module in AmberTools 15 on the MD simulation stripped of solvent using the dbscan algorithm, with an epsilon of 0.75 Å for the residues at 4 Å of the ligand. The MD snapshot corresponding to the most populated cluster was minimized for 1000 steps of steepest descent followed by 1000 steps of conjugate gradient keeping the explicit waters for the minimization to assemble Figure 6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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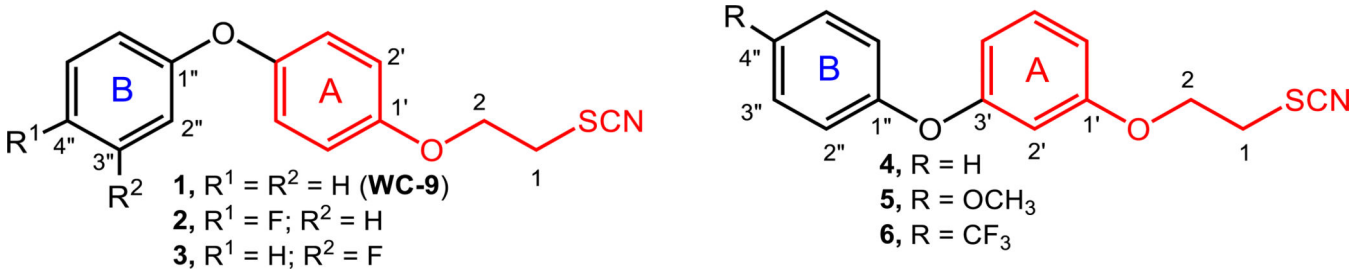


Figure 1.

Chemical structure of **WC-9** and other closely related inhibitors of *T. cruzi* proliferation

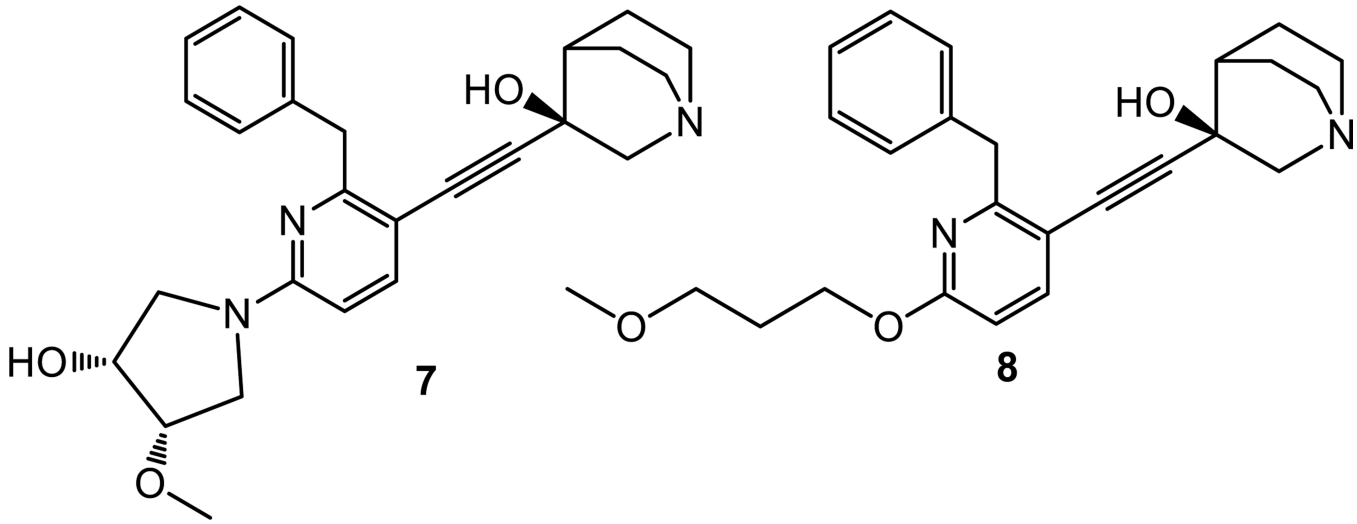


Figure 2.
Chemical structures of quinuclidine derivatives E5700 (**7**) and ER-119884 (**8**).

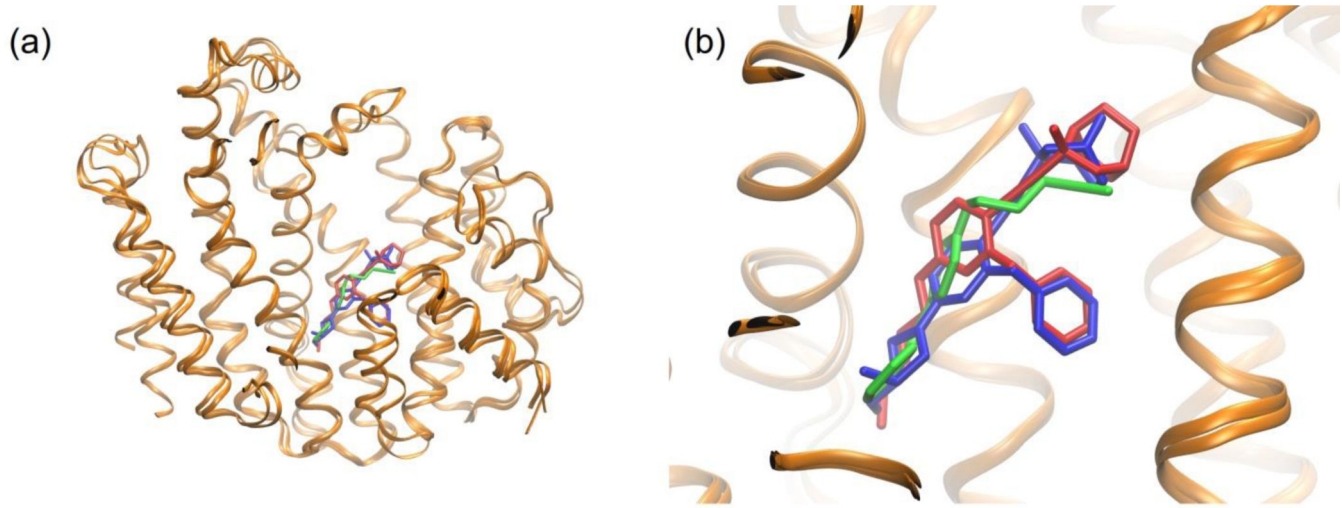


Figure 3.

(a) Superposition of the crystal structures of human SQS with **WC-9** and *T. cruzi* SQS with **7** and **8**. A high degree of similarity is observed between the protein chains. (b) Expansion of the structures showing that the quinuclidine derivatives **7** (red) and **8** (blue) occupy the same site S2 (homoallylic site) as **WC-9** (green). The mechanisms of action of these compounds (**7** mixed-type and **8** non-competitive) provide further evidence that **WC-9** may indeed bind to the S2 site in *T. cruzi* SQS.

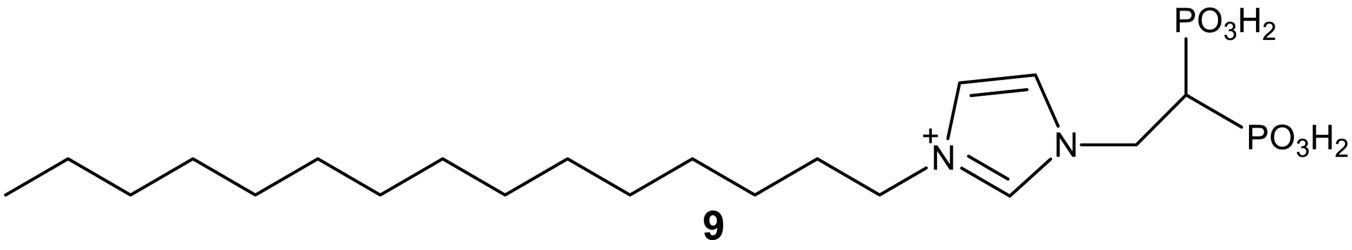


Figure 4.
Chemical structure of bisphosphonate known as BPH1344 (**9**).

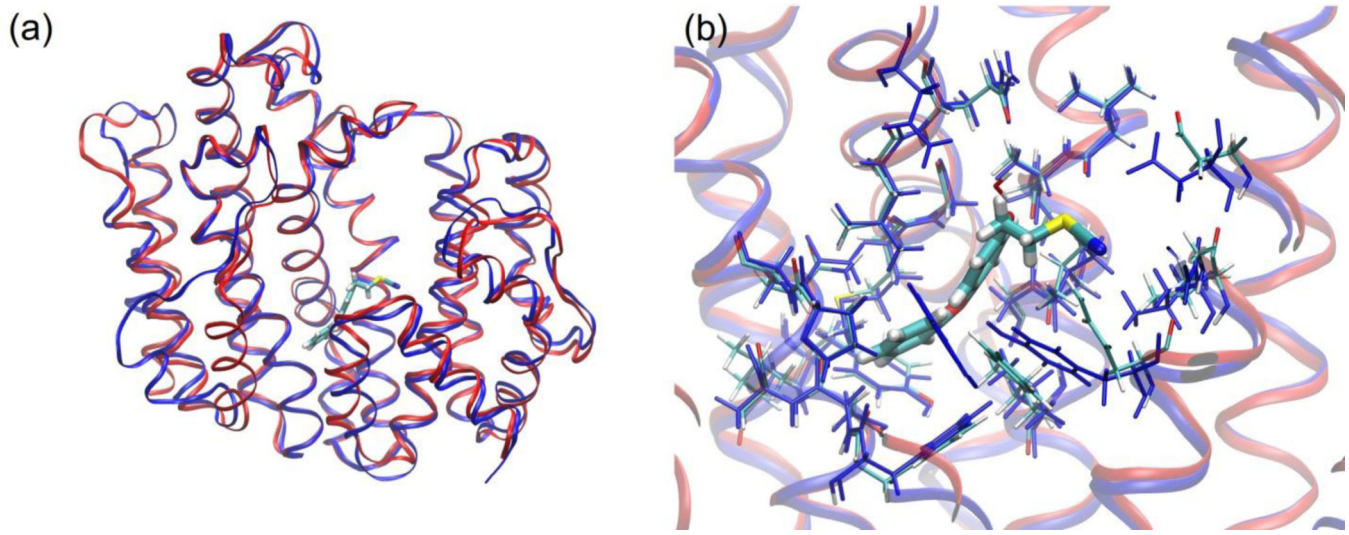


Figure 5.

This Figure shows the binding site (amino acids within 4 Angstroms of the ligand) of **WC-9** in the human SQS structure (amino acids shown in Licorice Representation, *blue*) (a), with the putative site it would occupy in *T. cruzi* SQS (amino acids in Licorice Representation with the Name color scheme) (b). A high degree of similarity is observed between the two binding sites. In terms of sequence, all amino acids in the binding sites are the same, except for Ser 256 in the *T. cruzi* vs. Cys 254 in the human enzyme.

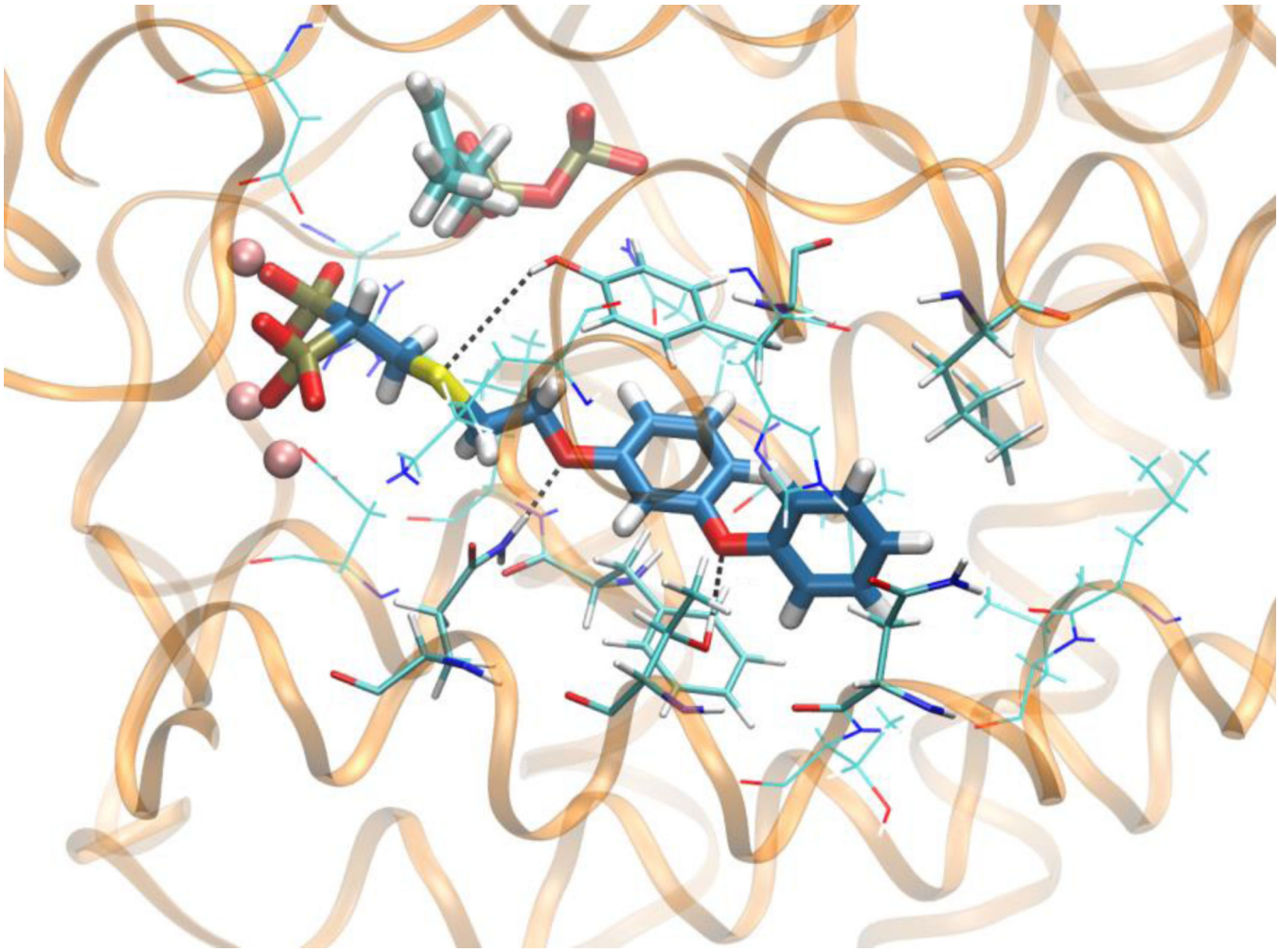
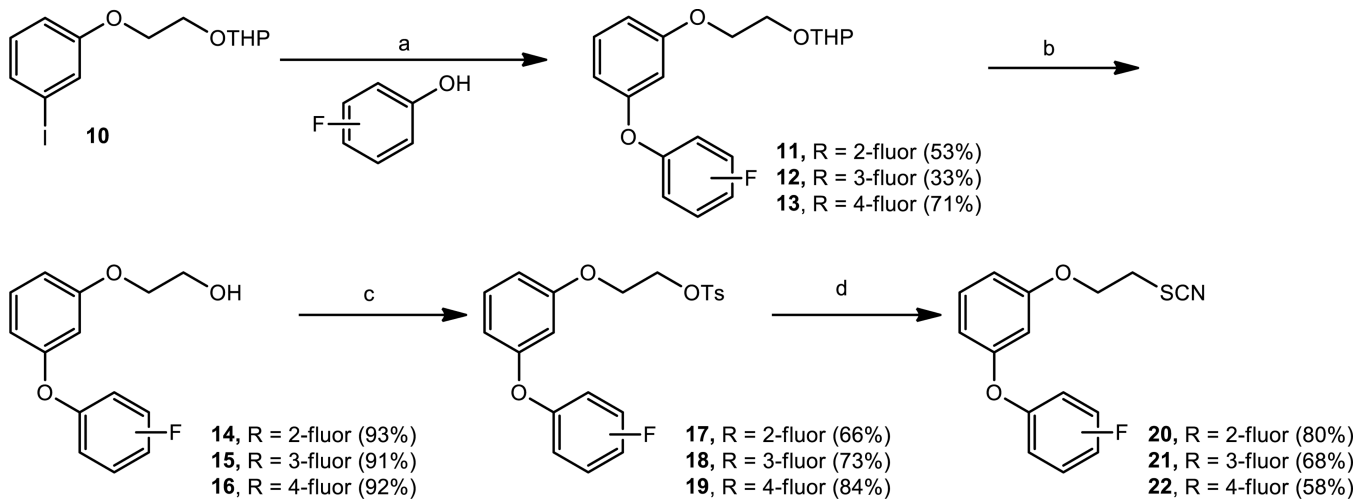


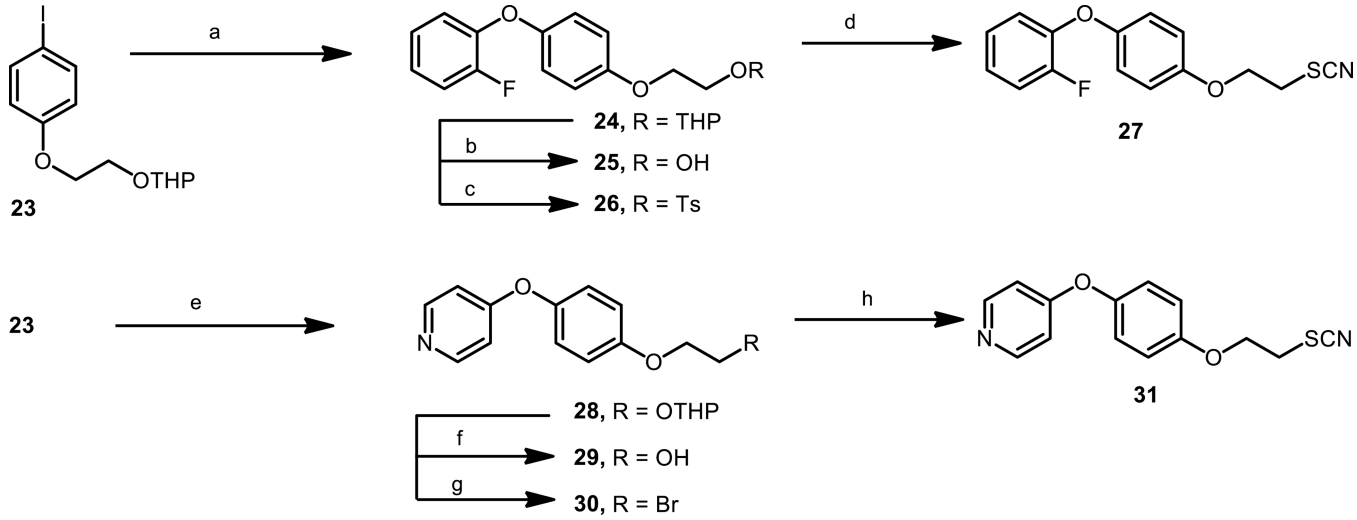
Figure 6.

Interaction of **58** with *Tg*FPPS. The structure was formed by homology modeling from *P. vivax* FPPS, and **58** (blue), three Mg ions (pink) and isopentenyl pyrophosphate (cyan) were inserted by alignment with the *Tc*FPPS alendronate crystal structure (pdb id 1yhm). Residues at 4 Å from the ligand are shown in thick Line representation, with those contributing significantly to the binding energy in thin Licorice representation.



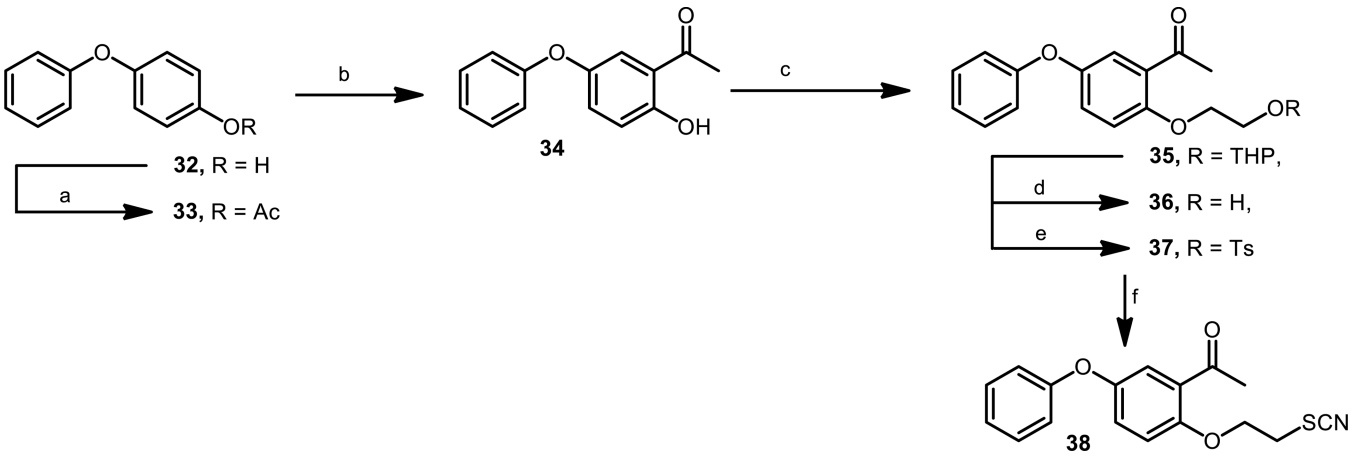
Scheme 1.

Reagents and conditions: a) 5% CuI, 10% picolinic acid, (2-fluoro-, 3-fluoro-, 4-fluorophenol), K_3PO_4, DMSO, 80 ºC, 24 h; b) PPTs, CH_3OH, rt, 16 h; c) ClTs, py, 0 ºC, 6 h; d) KSCN, DMF, 100 ºC, 6h.



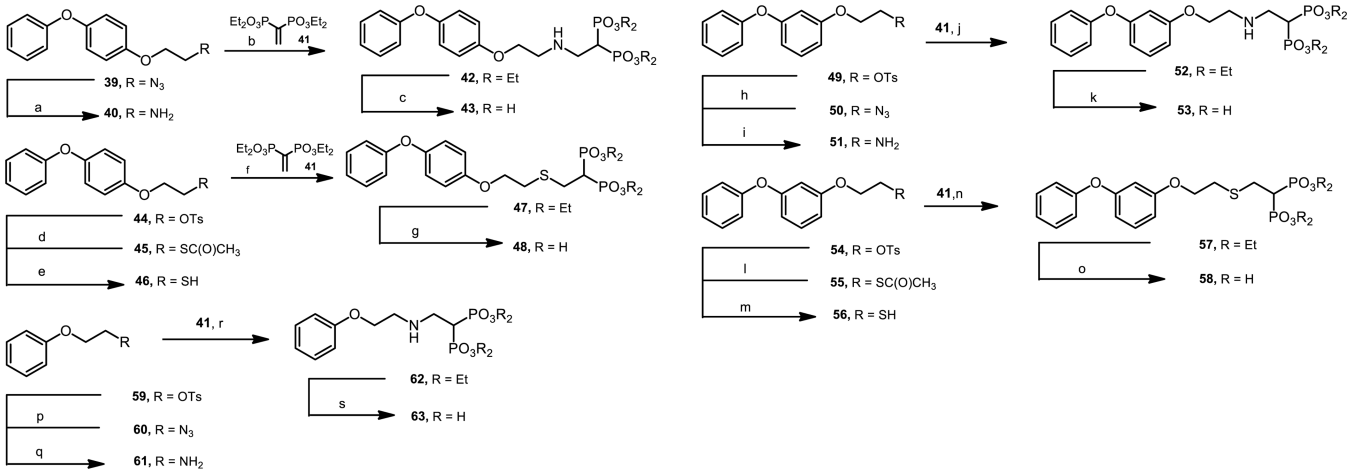
Scheme 2.

Reagents and conditions: a) 5% CuI, 10% picolinic acid, 2-fluorophenol, K_3PO_4, DMSO, 80 ºC, 24 h, 37%; b) PPTs, CH_3OH, rt, 24h, 62%; c) ClTs, py, rt, 24h, 92%; d) KSCN, DMF, 80 ºC, 6h, 36%; e) CuI, 10% picolinic acid, 4-hydroxypyridine, K_3PO_4, DMSO, 80 ºC, 24 h, 29%; f) PPTs, CH_3OH, rt, 24h, 59%; g) NBS, Ph_3P, 0ºC, 6h, 50%; h) KSCN, DMF, 80 ºC, 6h, 34%



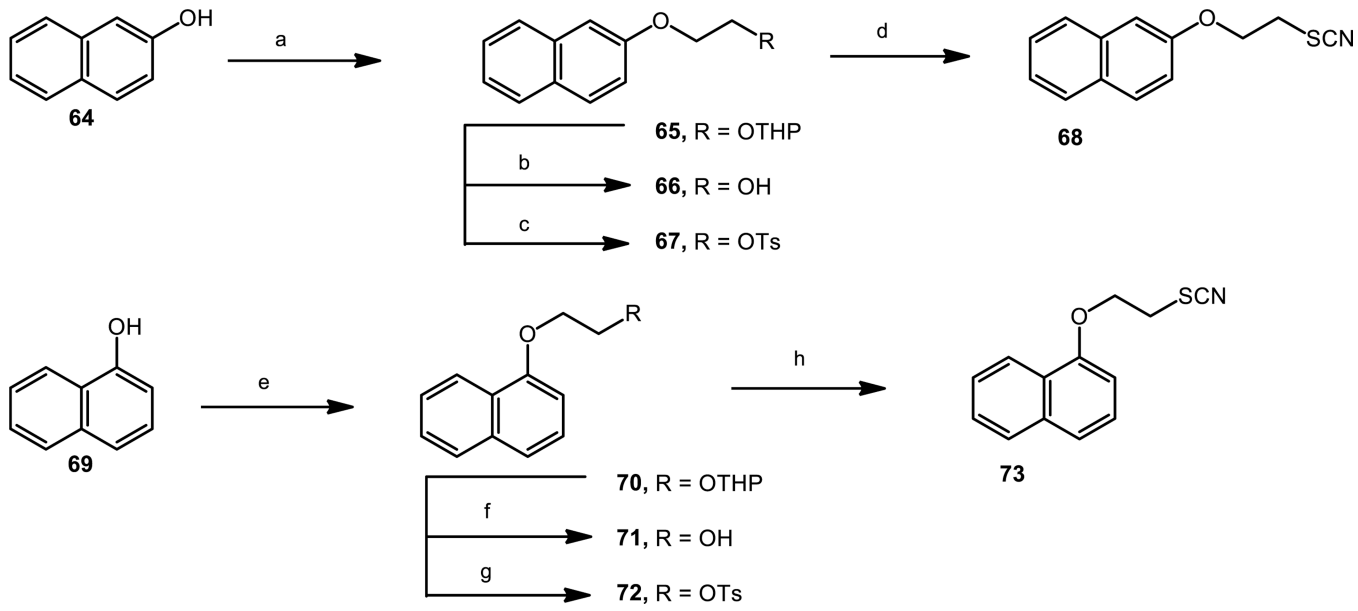
Scheme 3.

Reagents and conditions: a) Ac_2O, py, rt 16 h, 97%; b) hν, C_6H_{12}, rt, 12h, 39%; c) KOH, DMSO, $BrCH_2CH_2OTHP$, rt, 12h, 62%; d) PPTs, CH_3OH, rt, 16h, 92%; e) ClTs, py, rt, 6h, 95%; f) KSCN, DMF, 80 ºC, 6h, 71%.



Scheme 4.

Reagents and conditions: a) PPh_3, CH_2Cl_2, rt, 4h, 77%; b) **41**, CH_2Cl_2, NEt_3, rt, 4h, 82%; c) HCl (c), reflux, 7h, 81%; d) $CH_3C(O)SK$, DMF, 2h, 90 ºC, 83%; e) $LiAlH_4$, THF, rt, 3h, 92%; f) **41**, CH_2Cl_2, NEt_3, rt, 4h, 82%; g) i. $BrSi(CH_3)_3$, CH_2Cl_2, rt, 24h, ii. CH_3OH, rt, 12h, 69%; h) NaN_3, DMF, 80 ºC, 2h, 90%; i) PPh_3, THF, rt, 2h, 90%; j) **41**, CH_2Cl_2, NEt_3, rt, 24h, 97%; k) HCl (c), reflux, 7h, 35%; l) $CH_3C(O)SK$, DMF, 2h, 80 ºC, 83%; m) i. K_2CO_3, CH_3OH/H_2O, 1h, rt, ii. Zn/AcOH, 3h, 110 ºC, 75%; n) **41**, CH_2Cl_2, NEt_3, rt, 16h, 75%; o) i. $BrSi(CH_3)_3$, CH_2Cl_2, rt, 24h, ii. CH_3OH, rt, 12h, 65%; p) NaN_3, DMF, 80 ºC, 2h, 81%; q) PPh_3, THF, rt, 2h, 67%; r) **41**, CH_2Cl_2, NEt_3, rt, 24h, 90%; s)) HCl (c), reflux, 7h, 81%.



Scheme 5.

Reagents and conditions: a) KOH, DMSO, $BrCH_2CH_2OTHP$, rt, 5 days, 48%; b) PPTs, CH_3OH, rt, 24h, 85%; c) ClTs, py, rt, 0 ºC, 83%; d) KSCN, DMF, 80 ºC, 6h, 55%; f) KOH, DMSO, $BrCH_2CH_2OTHP$, rt, 5 days, 65%; g) PPTs, CH_3OH, rt, 24h, 81%; e) ClTs, py, rt, 0 ºC, 90%; f) KSCN, DMF, 80 ºC, 6h, 58%.

Table 1

Biological activity of **WC-9** analogues against *T. gondii* (tachyzoites), *T. cruzi* (amastigotes), and Vero cells.

Compound	*T. gondii* growth EC_{50} (μM)	*Tg*FPPS IC_{50} (μM)	*T. cruzi* growth EC_{50} (μM)	*Tc*FPPS IC_{50} (μM)	*Hh*FPPS IC_{50} (μM)	Cytotoxicity EC_{50} (μM)
20	3.12 ± 0.37	ND	7.01 ± 0.51	ND	ND	>60
21	1.63 ± 0.36	ND	5.38 ± 0.82	ND	ND	>50
22	4.92 ± 1.94	ND	5.69 ± 0.47	ND	ND	>55
27	4.54 ± 0.57	ND	>10	ND	ND	>55
31	7.86 ± 0.78	ND	>10	ND	ND	>200
38	2.42 ± 0.81	ND	11.3 ± 2.5	ND	ND	75.7 ± 7.3
43	5.90 ± 0.07	0.094 ± 0.020	>10	ND	>10	>100
48	> 10.0	0.362 ± 0.002	>20	0.8 ± 0.12	>15	>200
53	2.40 ± 0.70	0.040 ± 0.002	>10	>10	>10	>50
58	0.67 ± 0.23	0.076 ± 0.019	>10	ND	>10	>100
63	> 10	0.180 ± 0.015	>10	0.32 ± 0.04	>10.0	ND
68	3.62 ± 0.38	ND	>10	ND	ND	ND
73	3.13 ± 0.58	ND	>10	ND	ND	7.5
WC-9	4.8 ± 0.41[20]	ND	5.0 ± 1.1[20]	ND	ND	82.6 ± 7.3
Benznidazole	ND	ND	1.52 ± 0.10	ND	ND	ND

Values are means ± SD of results from at least three independent experiments, n = 3. ND, not determined