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Synthesis and Biological Evaluation of 2-alkylaminoethyl-1,1-bisphosphonic acids against *Trypanosoma cruzi* and *Toxoplasma gondii* targeting Farnesyl Diphosphate Synthase

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Abstract – The effect of a series of 2-alkylaminoethyl-1,1-bisphosphonic acids against proliferation of the clinically more relevant form of *Trypanosoma cruzi*, the etiologic agent of American trypanosomiasis (Chagas' disease), and against tachyzoites of *Toxoplasma gondii* has been studied. Most of these drugs exhibited an extremely potent inhibitory action against the intracellular form of *T. cruzi*, exhibiting IC_{50} values at the low micromolar level. This cellular activity was associated with a strong inhibition of the enzymatic activity of *T. cruzi* farnesyl diphosphate synthase (TcFPPS), which constitutes a valid target for Chagas' disease chemotherapy. Compound **17** was an effective agent against amastigotes exhibiting an IC_{50} value of 0.84 μM, while this compound showed an IC_{50} value of 0.49 μM against the target enzyme TcFPPS. Interestingly, compound **19** was very effective against both *T. cruzi* and *T. gondii* exhibiting IC_{50} values of 4.1 μM and 2.6 μM, respectively. In this case, **19** inhibited in at least two different enzymes of *T. cruzi* (TcFPPS and solanesyl diphosphate synthase (TcSPPS); 1.01 μM and 0.25 μM, respectively), while it inhibited TgFPPS in *T. gondii*. In general, this family of drugs was less effective against the activity of *T. cruzi* SPPS and against *T. gondii* growth *in vitro*. As bisphosphonate-containing compounds are FDA-approved drugs for the treatment of bone resorption disorders, their potential low toxicity makes them good candidates to control tropical diseases.

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‡ Both authors contributed equally to this work.

Introduction

The hemoflagellated protozoan *Trypanosoma cruzi* is the responsible agent of American trypanosomiasis (Chagas disease), which is the primary source of cardiac death where this disease is endemic[1]. It has been estimated that over 18 million people are infected and over 40 million individuals are at risk of infection by this parasite.[1,2] Of the infected individuals, most of them are in the chronic stage of the disease, in which 30 to 40% will develop serious, often lethal, cardiac and gastrointestinal tract lesions[3]. *T. cruzi* is transmitted to humans in the feces of its triatomine insect vector (such as *Triatoma infestans* or *Rhodnius prolixus*) and, as other kinetoplastid parasites, it has a complex life cycle, which includes obligatory stages in the mammalian host and in the vector. In the host *T. cruzi* has two forms: an intracellular dividing form (amastigote), and a non-dividing highly infective bloodstream form (trypomastigote) that can invade host cells. Two forms also occur in the vector: one replicative (epimastigote), and one nonreplicative (metacyclic trypomastigote).[4,5]

Chemotherapy of Chagas' disease, which is based on old and fairly non specific drugs empirically discovered, such as nifurtimox (**1**) and benznidazole (**2**), remains deficient.[6] This illness has also been found in non-endemic countries as a consequence of transfusion of contaminated blood from immigrants.[7] For this reason, it is very important to have an efficient agent to eradicate the bloodstream trypomastigotes from blood banks as well. Crystal Violet (**3**), the only drug employed for blood sterilization and discovered for that purpose some decades ago,[8] suffers from some disadvantages, since it was shown to be carcinogenic in *in vivo* assays (Figure 1).[9]
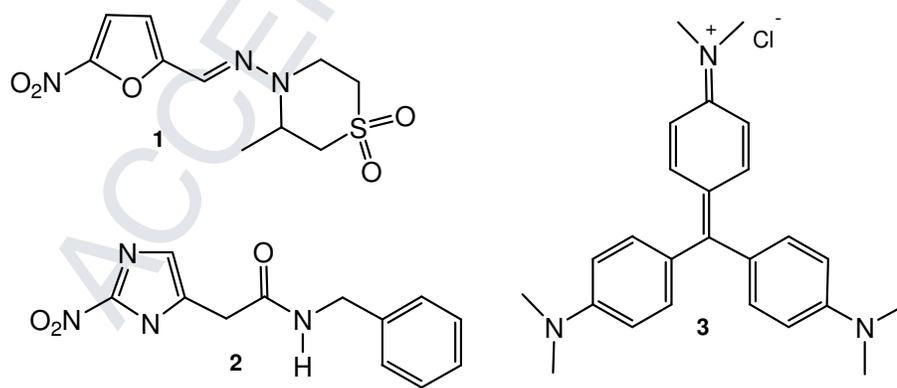
Figure 1. Current Drugs for the Treatment of Chagas' Disease and Blood Sterilization

Studies of *T. cruzi* biochemistry and physiology has led to recognize several crucial enzymes for parasite survival as potential targets for the design of new drugs.[10] One pathway that has been particularly useful for the identification of new targets against *T. cruzi* is the isoprenoid pathway. Enzymes studied so far that are involved in the synthesis of sterols[11] and farnesyl diphosphate,[12] and in protein prenylation[13] have been reported to be excellent drug targets against pathogenic parasites. A recent report about the presence of another important prenyltransferase in *T. cruzi*, a solanesyl diphosphate synthase (TcSPPS), which is involved in the synthesis of ubiquinone, suggested that it is another potential target for chemotherapy.[14] For example, farnesyl diphosphate synthase (FPPS) has been demonstrated to be the target of nitrogen-containing- and nitrogen free-bisphosphonates that have activity in vitro and in vivo against trypanosomatids and apicomplexan parasites.[6] The crystal structure of TcFPPS at 2 Å resolution has been recently published.[15] In addition, the fact that bisphosphonates can target multiple sites of isoprenoid biosynthesis reinforce the idea of testing different enzymes involved in this biosynthetic pathway.[16]

Several bisphosphonates, which are potent inhibitors of bone resorption, are currently being used for the treatment of several bone disorders such as osteoporosis, Paget's disease, hypercalcemia, tumor bone metastases, etc.[17] Selective action on bone is based on the binding of the bisphosphonate moiety to the bone mineral.[17] The selective antiparasitic action of bisphosphonates was proposed to be due to the presence of acidocalcisomes, which, having an equivalent composition to the bone mineral, facilitate their accumulation.[18] Representative nitrogen-containing bisphosphonates such as pamidronate (**4**), alendronate (**5**), risedronate (**6**), and ibandronate (**7**) act by a mechanism that leads to osteoclast apoptosis (Figure 2).[19] The target of aminobisphosphonates has been limited to the isoprenoid pathway and more specifically, to protein prenylation.[20]
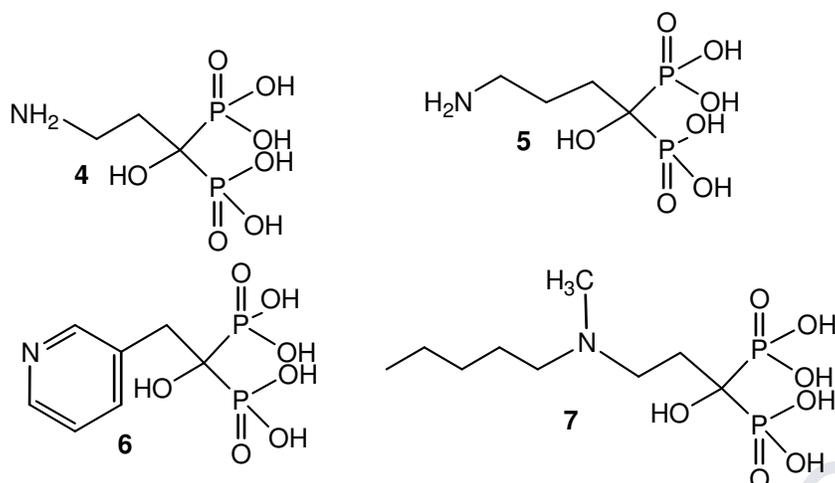
Figure 2. Chemical structure of representative FDA-approved bisphosphonates clinically employed for bone disorders.

Rationale

Nitrogen-containing bisphosphonates were originally found to be effective against *T. cruzi in vitro* and *in vivo* without toxicity to the host cells.[18] More recently, bisphosphonate derivatives were found to be effective growth inhibitors of pathogenic trypanosomatids other than *T. cruzi* (*T. brucei rhodesiense*, *Leishmania donovani*, and *L. mexicana*) and apicomplexan parasites (*Toxoplasma gondii* and *Plasmodium falciparum*).[21–27] *In vivo* assays of bisphosphonates have shown that risedronate can significantly increase survival of *T. cruzi*-infected mice.[28] The above results indicate that bisphosphonates are promising candidate drugs to treat pathogenic parasitic infections by *T. cruzi* and other protozoan parasites. In addition, bisphosphonates have the advantage that they are straightforwardly and inexpensively prepared and many compounds bearing the bisphosphonate moiety are FDA-approved drugs for the long-term treatment of several bone disorders. A low toxicity for bisphosphonate-containing drugs might be predictable.

Of special interest are bisphosphonates derived from fatty acids, which were shown to be potent growth inhibitors against the clinically more relevant form of *T. cruzi* exhibiting IC_{50} values at the low micromolar range.[23–25] Compound **8** arises as main member of this class of bisphosphonates with an IC_{50} value of 18 μM.[23] This cellular activity correlates quite properly with the inhibition of the enzymatic activity towards

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TcFPPS,[24] being a competitive inhibitor with an IC_{50} value of 1.94 μM and a K_i of 0.40 μM.[24] Compound **8** also inhibits the enzymatic activity of *T. brucei* FPPS,[24] and is active *in vitro* against *T. gondii*.[26] Besides, SAR studies indicated that the replacement of the hydroxyl group at C-1 of **8** by a hydrogen atom to yield **9** resulted in a less effective compound either as inhibitor of the enzymatic activity of TcFPPS or as inhibitor of *T. cruzi* growth.[24] The low efficacy observed for **9**, in which the hydroxyl group at C-1 is missing, may be attributable to the lack of ability to coordinate Mg^{2+} present at the active site of the target enzyme[29,30] or due to poor cell permeability. In contrast, the tridentate **8** has three coordination sites versus a bidentate structure that clearly justifies is effectiveness towards TcFPPS. The presence of the amino group would retain the ability to coordinate Mg^{2+} in a tridentate structure similar to compounds of the type **8**. On the other hand, 1-amino-1,1-bisphosphonate derivatives such as **10** were very potent against TcFPPS at the nanomolar range,[25] and also were very effective inhibitors of *T. cruzi* proliferation but to a lesser extent compared with 1-hydroxy-1,1-bisphosphonates.[23,25] Moreover, from the X-ray structure of the complex of risedronate with TcFPPS, it can be observed that residue Asp250 forms a hydrogen bond with the hydroxyl group present at the C-1 position of the molecule of risedronate. This observation justifies the poor biological action observed by bisphosphonates where either the hydroxyl group or the amino group is replaced by a hydrogen atom.[15] Bearing in mind the above results, it would seem of interest to carry out structural variations on compound **10** taken as lead drug. Therefore, compounds where the relative position of the nitrogen atom respect to the bisphosphonic unit is such as the compound of general formula **11** were envisioned. This family of compounds would retain the ability to coordinate Mg^{2+} in a tridentate structure. Since compounds **8** and **9** are nitrogen-free bisphosphonates, they could not act as carbocation transition state analogues of the substrate of FPPS as previously postulated.[31] In addition, nitrogen-containing bisphosphonates of the class **10** and **11** bear the nitrogen atom in such a position that their protonated form cannot act as an analogue of a transition state of the target enzyme (Figure 3).
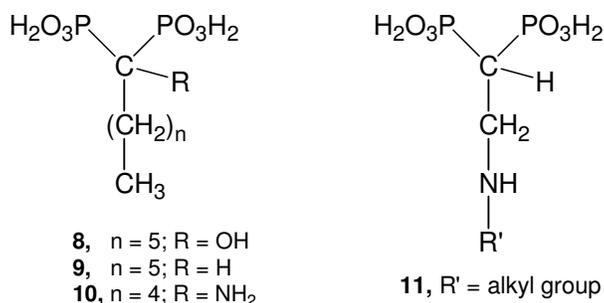
Figure 3. Representative bisphosphonic acids derived from fatty acids.

Results and Discussion

The title compounds were straightforwardly prepared employing tetraethyl ethenylidenbisphosphonate (compound **13**) as a Michael acceptor,[32] which in turn was easily prepared from tetraethyl methylenebisphosphonate (compound **12**) in two steps according to a slightly modified Degenhart protocol.[33] Therefore, compound **13** was reacted with the corresponding amine in a Michael-type reaction to afford the respective Michael adduct. Once this synthetic intermediate was at hand, it was hydrolyzed by treatment with concentrated hydrochloric[34] acid to afford the title compounds, that is, the corresponding free bisphosphonic acids (**14–27**). The preparation of this new family of 2-alkylaminoethyl *gem*-bisphosphonates is illustrated in Scheme 1. Some of these compounds had previously been shown to be herbicidal agents.[35,36] Compounds **14–27** were evaluated as growth inhibitors against the amastigote form of *T. cruzi*, the clinically more relevant form of the parasite. **WC-9**, a well-known antiparasitic agent, was used as a positive control.[37] In addition, the correlation of the cellular activity with the action against its target enzyme (TcFPPS) as well as TcSPPS was studied. Besides, based on previous studies on structurally related bisphosphonates against the opportunistic pathogen *T. gondii*,[26,38] this series of new nitrogen-containing bisphosphonic acids was evaluated against *T. gondii* and its putative target enzyme TgFPPS.

Most of the title compounds were potent inhibitors of the enzymatic activity of TcFPPS. The efficacy of several compounds on this enzyme qualitatively correlated with their inhibitory action against growth of amastigotes of *T. cruzi*. Taken compound **17** as an example, this compound was a potent inhibitor of TcFPPS activity with an IC_{50} in the nanomolar range (0.49 μM). These data correlated completely with the efficacy of this

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compound as antiparasitic agent. In fact, compound **17** proved to be a potent inhibitor of the amastigote form of the parasite with an IC_{50} = 0.84 μM. Remarkably, **17** was > 10-fold more potent than **WC-9**,[37,39] a well-known antiparasitic agent employed as positive control, which in turn proved to be more effective than the currently drugs for the treatment of Chagas' disease such as nifurtimox and benznidazole under the same assays conditions. The cellular activity exhibited by **17** was > 20-fold more potent than **8** (IC_{50} = 18.1 μM),[23] taken as lead drug for the present study. A comparable degree of efficacy as inhibitors of TcFPPS activity was observed for compounds **14** and **18**. Surprisingly, in spite of being one order of magnitude more potent than **17** towards TcFPPS, compound **18** exhibited indistinguishable antiproliferative potency against amastigotes compared to **WC-9** and a notable less potency than that shown by **17**. These compounds have to cross two cell membranes to get to the target enzyme. Compound **18** is apparently less permeable than compound **17** despite its lower charge to mass ratio. For example, compound **14**, which was the most effective compound on TcFPPS (IC_{50} = 38 nM) was devoid of activity against the intracellular form of the parasite. These results were in agreement with our previous results with the isosteric analogue of **14**, compound **9**, in which the amino group was replaced by a methylene group and which exhibited an IC_{50} value against TcFPPS of 5.67 μM, and K_i = 0.47 μM and was completely devoid of activity against amastigotes. The above results indicated that the chain length was appropriate in the case of **14** but its permeability should be optimized. Compound **16** was the most potent compound against the intracellular form of *T. cruzi* (IC_{50} = 0.54 μM). This activity correlated quite well with the effect of this compound against the target enzyme (IC_{50} = 1.84 μM). Compound **19** was a very interesting example of a compound effective against growth of both *T. cruzi* and *T. gondii* showing IC_{50} values of 4.1 μM and 2.6 μM, respectively. **19** was very potent against both target enzymes TcFPPS and TcSPPS especially against the latter one with IC_{50} of 1.01 μM and 0.25 μM, respectively. The inhibitory action towards TcSPPS was not surprising since this compound has the longest side chain among the designed compounds; therefore, it was better recognized by TcSPPS due to substrate similarity. Moreover, **19** also exhibited potent inhibitory action against TgFPPS at the nanomolar range (IC_{50} = 87 nM). Compounds **17** and **18** were also good inhibitors towards *T. cruzi* SPPS showing IC_{50} values of 1.35 μM and 1.69 μM,

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respectively. However, a high selectivity against TcFPPS, except for **19**, was observed in all of the designed compounds. It is interesting to note the lack of trend in inhibition of TcFPPS between compounds **14**-**19**, where there is a logical stepwise variation in structure, but not in activity, and the lack of correlation between growth and enzyme inhibition in other cases (**14**, **18**, **21, 23, 24, 25**), suggesting in some cases (**16**) off-target effects.

Compounds **14** and **17** resulted to be moderately effective against *T. gondii* with IC_{50} values of 6.33 µM and 9.37 µM, respectively. The mode of action of these compounds would also be by affecting protein prenylation at the level of TgFPPS (IC_{50} values of 0.30 µM and 0.14 µM, respectively). Atovaquone was used as a positive control against *T. gondii* growth ($IC_{50} = 0.29$ µM). Recent work[40] has indicated that TgFPPS is a bifunctional enzyme, catalyzing the formation of both farnesyl diphosphate (FPP) and geranyl geranyl diphosphate (GGPP). In agreement with that work, we found that the correlation between inhibition of TgFPPS and TcSPPS (a longer chain prenyl synthase) by 12 of the bisphosphonates assayed in this work was high ($R^2 = 0.69$; Figure 4), while there was no correlation between of TgFPPS and TcFPPS inhibition, or between TcFPPS and TcSPPS inhibition (data not shown).

It can be concluded that the isosteric replacement of the methylene group at the C-3 position of 1-alkyl-1,1-bisphosphonates such as compound **9** to give compounds of general formula **11** was of the great effectiveness against both *T. cruzi* and the target enzyme TcFPPS. Most of the designed compounds were even much more potent than the *gem*-bisphosphonic acid **8**, taken as a reference structure, against both *T. cruzi* and the target enzyme, *T. cruzi* farnesyl diphosphate synthase. Most of these compounds proved to be inhibitors of the enzymatic activity of TcSPPS but to a lesser extent than TcFPPS as previously discussed. Moreover, some of these 1-[2-(alkylamino)ethyl] bisphosphonic acids or 1-(3-azaalkyl)-1,1 bisphosphonic acids were shown to be moderately effective anti-*T. gondii* agents indicating the broad scope that this type of compounds has. Work aimed at optimizing the chemical structure of 1-(3-azaalkyl)-1,1 bisphosphonic acids such as compound **17** and other closely related analogues is currently being pursued in our laboratory.
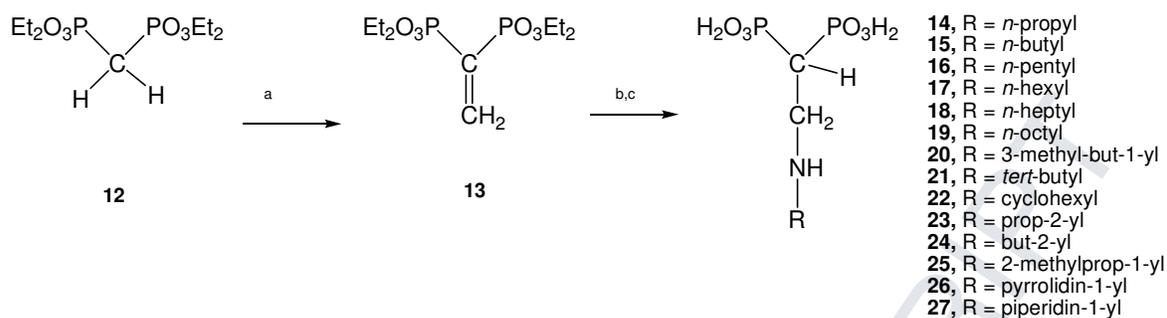
Scheme 1. Reagents and conditions: (a) references 40–42; (b) RNH$_2$, CH$_2$Cl$_2$ (anh.), rt, 30 min; (c) (conc.) HCl, reflux, 24 h, 94% for **14**, 92% for **15**, 98% for **16**, 85% for **17**, 91% for **18**, 78% for **19**, 88% for **20**, 71% for **21**, 66% for **22**, 79% for **23**, 80% for **24**, 68% for **25**, 77% for **26**, 84% for **27**.

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Table 1

Comp.	IC$_{50}$ (μM)	IC$_{50}$ (μM)	IC$_{50}$ (μM)	IC$_{50}$ (μM)	IC$_{50}$ (μM)
	T. cruzi amastigotes	*T. gondii* tachyzoites	*Tc*FPPS	*Tc*SPPS	*Tg*FPPS
14	> 50	6.33	0.038 ± 0.008	11.01 ± 1.87	0.298 ± 0.035
15	4.8	48.67	2.28 ± 0.34	8.96 ± 2.18	0.206 ± 0.010
16	0.54	>50	1.84 ± 0.25	16.79 ± 4.37	0.080 ± 0.023
17	0.84	9.37	0.49 ± 0.12	1.35 ± 0.22	0.137 ± 0.087
18	10.0	>50	0.058 ± 0.009	1.69 ± 0.39	0.095 ± 0.024
19	4.1	2.60	1.014 ± 0.457	0.252 ± 0.050	0.087 ± 0.038
20	10.0	36.94	0.42 ± 0.18	147.1 ± 77.2	0.275 ± 0.041
21	10.0	42.84	1.21 ± 0.13	59.8 ± 13.9	0.286 ± 0.059
22	36.3% at 10 μM	57.3	0.013 ± 0.003	77.4 ± 4.09	0.337 ± 0.138
23	> 50	69.81	1.399 ± 0.496	0.761 ± 0.471	0.253 ± 0.069
24	> 50	> 100	0.944 ± 0.211	1.980 ± 0.359	0.112 ± 0.004
25	> 50	74.47	0.515 ± 0.104	3.493 ± 2.678	0.181 ± 0.050
26	14	13.30	0.760 ± 0.316	8.276 ± 6.474	0.361 ± 0.203
27	6.1	70.60	0.287 ± 0.021	10.034 ± 2.307	0.165 ± 0.045
WC-9	12.0				

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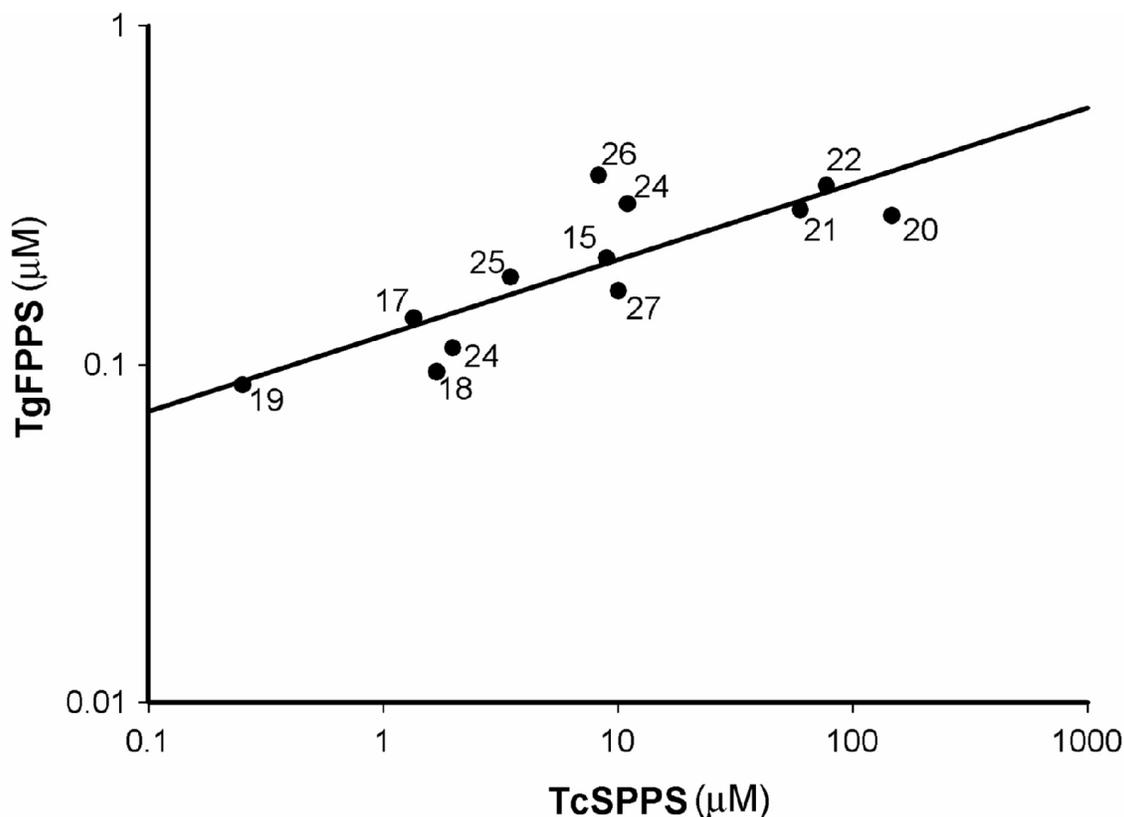
Figure 4. Prenyl synthase inhibition correlation. Correlation between TgFPPS and TcSPPS IC_{50} values, for the bisphosphonates indicated with numbers.

Experimental Section.

General. The glassware used in air- and/or moisture-sensitive reactions was flame-dried and reactions were carried out under dry argon. Unless otherwise noted, chemicals were commercially available and used without further purification. Solvents were distilled before use. Dichloromethane was distilled from phosphorus pentoxide. Nuclear magnetic resonance spectra were recorded with a Bruker AM-500 MHz spectrometer. The ^{1}H NMR spectra are referenced with respect to the residual $CHCl_3$ proton of the solvent $CDCl_3$ at $\delta = 7.26$ ppm. Coupling constants are reported in Hz. ^{13}C NMR spectra were fully decoupled and are referenced to the middle peak of the solvent $CDCl_3$ at $\delta = 77.0$ ppm. ^{31}P NMR spectra are referenced with respect to the peak of 85% H_3PO_4 as external reference. Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quadruplet; dd, double doublet, etc. Melting points were determined with a Fisher–Johns apparatus and are uncorrected. IR spectra were recorded with a Nicolet Magna 550 spectrometer. Analytical TLC was performed on commercial 0.2 mm aluminum-coated

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silica gel plates (F254) and visualized by 254 nm UV or immersion in an aqueous solution of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.04 M), $Ce(SO_4)_2$ (0.003 M) in concentrated H_2SO_4 (10%). Elemental analyses were conducted by Atlantic Microlab Inc., Norcross, Georgia. The results were within ±0.4% of the theoretical values.

Synthesis of 1-[2-alkylaminoethyl]-1,1-bisphosphonic acids

General Procedure. A solution of compound **13** (10 mmol) in anhydrous methylene chloride (10 mL) was treated with the corresponding amine (10 mmol) under an argon atmosphere. The reaction mixture was stirred at room temperature for 30 min. The solvent was evaporated and the residue 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 crystallized from ethanol.

1-[(Prop-1-ylamino)ethyl] 1,1-bisphosphonic Acid (14). 94% Yield; mp 195 ℃; ^{1}H NMR (D$_2$O) δ 0.86 (t, J = 7.4 Hz, 3H, H-6); 1.60 (sex, J = 7.2 Hz, 2H, H-5); 2.43 (tt, J = 21.3, 7.0 Hz, 2 H, H-1), 2.95 (t, J = 7.1 Hz, 2H, H-4), 3.33 (dt, J = 14.0, 7.1 Hz, 2H, H-2); ^{13}C NMR (D$_2$O) δ 10.04 (C-6), 19.07 (C-5), 35.98 (t, J = 121.7 Hz, C-1), 44.58 (C-2), 49.17 (C-4); ^{31}P NMR (D$_2$O) δ 16.14. Anal. ($C_5H_{15}NO_6P_2$) Calcd. C 24.30, H 6.12, N 5.67. Found C 24.01, H 6.12, N 5.70.

1-[(*n*-But-1-ylamino)ethyl] 1,1-bisphosphonic Acid (15). 92% Yield; mp 214 ℃; IR (BrK, cm^{-1}) 3101, 2314, 1273, 1180, 956; ^{1}H NMR (D$_2$O) δ 0.83 (t, J = 7.4 Hz, 3H, H-7), 1.30 (sex, J = 7.5, 2H, H-6), 1.58 (pent, J = 7.5 Hz, 2H, H-5), 2.42 (tt, J = 21.7, 7.0 Hz, 1H, H-1), 3.01 (t, J = 7.4 Hz, 2H, H-4), 3.35 (dt, J = 14.3, 7.3 Hz, 2H, H-2); ^{13}C NMR (D$_2$O) δ 12.6 (C-7), 20.0 (C-6), 27.5 (C-5), 36.1 (t, J = 120.3 Hz, C-1), 44.7 (C-2), 47.4 (C-4); ^{31}P NMR (D$_2$O) δ 15.99. Anal. ($C_6H_{17}NO_6P_2$) Calcd. C 27.59, H 6.56, N 5.36. Found C 27.94, H 6.72, N 5.57.

1-[(*n*-Pent-1-ylamino)ethyl] 1,1-bisphosphonic Acid (16). 98% Yield; mp 204 ºC; IR (BrK, cm^{-1}) 3101, 2310, 1273, 1180, 960; ^{1}H NMR (D$_2$O) δ 0.78 (t, *J* = 6.9 Hz, 3H, H-8), 1.26 (m, 4H, H-6, H-7), 1.60 (q, *J* = 7.3 Hz, 2H, H-4), 1.59 (q, *J* = 7.4 Hz, 2H, H-4), 2.41 (tt, *J* = 21.4, 7.3 Hz, 1H, H-1), 3.01 (t, *J* = 7.3 Hz, 2H, H-4), 3.35 (dt, *J* = 14.3, 7.2 Hz, 2H, H-2); ^{13}C NMR (D$_2$O) δ 13.0 (C-8), 21.4 (C-7), 25.1 (C-6), 27.7 (C-5), 36.1 (t, *J* = 121.2 Hz, C-1), 44.7 (C-2), 47.6 (C-4) ; ^{31}P NMR (D$_2$O) δ 15.99. Anal. (C$_7$H$_{19}$NO$_6$P$_2$·½H$_2$O) Calcd. C 29.59, H 7.09, N 4.93. Found C 29.78, H 6.86, N 4.93.

1-[(*n*-Hex-1-ylamino)ethyl] 1,1-bisphosphonic Acid (17). 85% Yield; mp 212 ºC; IR (BrK, cm^{-1}) 3105, 2307, 1712, 1273, 1176, 956; ^{1}H NMR (D$_2$O) δ 0.76 (t, *J* = 6.6 Hz, 3H, H-9), 1.21 (m, 4H, H-7, H-8), 1.28 (m, 2H, H-6), 1.59 (pent, *J* = 7.4 Hz, 2H, H-5), 2.42 (tt, *J* = 21.5, 7.2 Hz, 1H, H-1), 3.00 (t, *J* = 7.4 Hz, 2H, H-4), 3.34 (dt, *J* = 14.3, 7.3 Hz, 2H, H-2); ^{13}C NMR (D$_2$O) δ 13.1 (C-9), 21.6 (C-8), 25.2 (C-7),* 25.4 (C-6),* 30.3 (C-5), 36.1 (t, *J* = 120.7 Hz, C-1), 44.7 (C-2), 47.7 (C-4) ; ^{31}P NMR (D$_2$O) δ 16.02. Anal. (C$_8$H$_{21}$NO$_6$P$_2$) Calcd. C 33.22, H 7.32, N 4.84. Found C 33.39, H 7.13, N 4.95.

1-[(*n*-Hept-1-ylamino)ethyl] 1,1-bisphosphonic Acid (18). 91% Yield; mp 207 ºC; IR (BrK, cm^{-1}) 3094, 2310, 1269, 1180, 1003; ^{1}H NMR (D$_2$O) δ 0.76 (t, *J* = 6.0 Hz, 3H, H-9), 1.18-1.24 (m, 8H, H-8 H-7 H-6 H-5), 1.59 (q, *J* = 7.1 Hz, 2H, H-4), 2.43 (tt, *J* = 21.4, *J* = 7.0 Hz, 1H, H-1), 3.00 (t, *J* = 6.9 Hz, 2H, H-3), 3.34 (dt, *J* = 13.9, 7.1 Hz, 2H, H-2); ^{13}C NMR (D$_2$O) δ 13.3 (C-9), 21.8 (C-8), 25.4 (C-7),∗ 25.5 (C-6),* 27.8 (C-4), 30.7 (C-5), 36.0 (t, *J* = 120.3 Hz, C-1), 44.7 (C-2), 47.7 (C-3) ; ^{31}P NMR (D$_2$O) δ •15.61. Anal. (C$_9$H$_{13}$NO$_6$P$_2$) Calcd. C 35.65, H 7.65, N 4.62. Found C 35.38, H 7.59, N 4.80.

1-[(*n*-Oct-1-ylamino)ethyl] 1,1-bisphosphonic Acid (19) 78% Yield; mp 202 ºC; ^{1}H NMR (D$_2$O) δ 0.70 (t, *J* = 6.9 Hz, 3H, H-11), 1.13 (m, 10H, H-6, H-7, H-8, H-9, H-10), 1.36 (pent, *J* = 7.4 Hz, 2H, H-5), 1.73 (tt, *J* = 21.0, 6.9 Hz, 1H, H-1), 2.48 (t, *J* = 7.6 Hz, 2H, H-4), 2.84 (dt, *J* = 14.3, 6.2 Hz, 2H, H-2);. ^{13}C NMR (D$_2$O) δ 13.3 (C-11), 21.9 (C-10), 26.5 (C-9), 28.1 (C-8), 28.3 (C-7), 28.5 (C-6), 31.1 (C-5), 40.6 (t, *J* = 116.7 Hz, C-

1), 48.1 (C-2), 48.3 (C-4); ^{31}P NMR (D$_2$O) δ 17.99. Anal. (C$_{10}$H$_{25}$NO$_6$P$_2$) Calcd. C 37.86, H 7.94, N 4.41. Found C 37.69, H 7.85, N 4.40.

1-[(3-Methyl-but-1-ylamino)ethyl] 1,1-bisphosphonic Acid (20). 88% Yield; mp 231 ºC; ^{1}H NMR (D$_2$O) δ 0.83 (d, J = 6.4 Hz, 6 H, H-6), 1.49 (dd, J = 15.1, 7.1 Hz, 2 H, H-4), 1.57 (hept, J = 6.6 Hz, H, H-5), 2.40 (tt, J = 21.5, 7.3 Hz, 1 H, H-1), 3.04 (t, J = 7.7 Hz, 2 H, H-3), 3.35 (dt, J = 14.3, 7.2 Hz, 2 H, H-2); ^{13}C NMR (D$_2$O) δ 21.29 (C-6), 25.03 (C-5), 34.20 (C-4), 36.18 (t, J = 120.3 Hz, C-1), 44.85 (C-2), 46.09 (C-3); ^{31}P NMR (D$_2$O) δ 15.87. Anal. (C$_7$H$_{19}$NO$_6$P$_2$)) Calcd. C 30.55, H 6.96, N 5.09. Found C 30.79, H 6.96, N 5.08.

1-[(*tert*-Butylamino)ethyl] 1,1-bisphosphonic Acid (21). 71% Yield; mp 238 ºC; ^{1}H NMR (D$_2$O) δ 1.28 (s, 9 H, C(CH_3)$_3$), 2.31 (tt, J = 21.4, 7.3 Hz, 1 H, H-1), 3.31 (dt, J = 14.2, 7.3 Hz, 2 H, H-2); ^{13}C NMR (D$_2$O) δ 24.98 C(CH$_3$)$_3$), 36.34 (t, J = 120.8 Hz, C-1), 38.86 (C-2), 57.05 (C-3); ^{31}P NMR (D$_2$O) δ 16.04. Anal. (C$_6$H$_{17}$NO$_6$P$_2$) Calcd. C 27.59, H 6.56, N 5.36. Found C 27.21, H 6.55, N 5.14.

1-[(Cyclohexylamino)ethyl] 1,1-bisphosphonic Acid (22). 66% Yield; mp 227 ºC; ^{1}H NMR (D$_2$O) δ 2.38 (tt, J = 21.5, 7.3 Hz, 3.07 (m, 1 H, H-), 3.35 (dt, J = 14.3, 7.2 Hz, 2 H, H-); ^{13}C NMR (D$_2$O) δ 23.64, 24.42, 28.92, 36.01 (t, J = 121.7 Hz, C-1), 41.76, 57.09; ^{31}P NMR (D$_2$O) δ 16.12. Anal. (C$_8$H$_{19}$NO$_6$P$_2$) Calcd. C 33.46, H 6.67, N 4.88. Found C 33.38, H 6.64, N 5.01.

1-[(Prop-2-ylamino)ethyl] 1,1-bisphosphonic Acid (23) 79% Yield; mp 227–228 ºC; ^{1}H NMR (D$_2$O) δ 1.23 (d, J = 6.6 Hz, 6H, H-5), 2.37 (tt, J = 21.5, 7.2 Hz, 1H, H-1), 3.33 (dt, J = 14.2, 7.1 Hz, 2H, H-2), 3.37 (sept, J = 6.5 Hz, 1H, H-4); ^{13}C NMR (D$_2$O) δ 18.3 (C-5), 36.1 (t, J = 121.7 Hz, C-1), 42.0 (C-2), 50.8 (C-4); ^{31}P NMR (D$_2$O) δ 16.04. Anal. (C$_5$H$_{15}$NO$_6$P$_2$)) Calcd. C 24.30, H 6.12, N 5.67. Found C 24.50, H 6.27, N 5.74.

1-[(But-2-ylamino)ethyl] 1,1-bisphosphonic Acid (24) 80% Yield; mp 197 ºC; ^{1}H NMR (D$_2$O) δ 0.83 (dist. t, J = 7.4 Hz, 3H, H-6), 1.16 (d, J = 6.6 Hz, 3H, C*H$_3$* at C-4), 1.49 (m, 1H, H-5$_a$), 1.60 (m, 1H, H-5$_b$), 2.38 (tt, J = 21.7, 7.3 Hz, 1H, H-1), 3.15 (m, 1H, H-4), 3.26 (m, 1H, H-2$_a$) 3.37 (m, 1H, H-2$_b$); ^{13}C NMR (D$_2$O) δ 8.7 (C-6), 15.1 (*CH$_3$* at C-4), 35.8 (t, J = 122.1 Hz, C-1), 25.9 (C-5), 41.9 (C-2), 56.0 (C-4); ^{31}P NMR (D$_2$O) δ 16.14 (d, J = 7.4 Hz, P$_a$), 16.14 (d, J = 7.4 Hz, P$_b$). Anal. (C$_6$H$_{17}$NO$_6$P$_2$)) Calcd. C 27.60, H 6.56, N 5.36. Found C 27.81, H 6.65, N 5.44.

1-[(2-Methylprop-1-ylamino)ethyl] 1,1-bisphosphonic Acid (25) 68% Yield; mp 220–222 ºC; ^{1}H NMR (D$_2$O) δ 0.89 (d, J = 6.9 Hz, 6H, H-6), 1.90 (n, J = 6.8 Hz, 1H, H-5), 2.42 (tt, J = 21.3, 7.3 Hz, 1H, H-1), 2.84 (d, J = 7.0 Hz, 1H, H-4), 3.34 (dt, J = 14.0, 7.4 Hz, 2H, H-2); ^{13}C NMR (D$_2$O) δ 18.9 (C-6), 25.5 (C-5), 35.9 (t, J = 121.2 Hz, C-1), 45.1 (C-2), 54.6 (C-4); ^{31}P NMR (D$_2$O) δ 16.36. Anal. (C$_6$H$_{17}$NO$_6$P$_2$) Calcd. C 27.60, H 6.56, N 5.36. Found C 27.31, H 6.50, N 5.20.

1-[(2-Pyrrolidin-1-ylamino)ethyl] 1,1-bisphosphonic Acid (26) 77% Yield; mp 235–236 ºC; ^{1}H NMR (D$_2$O) δ 1.95 (m, 2H, H-), 2.07 (m, 2H, H-), 2.53 (tt, J = 21.6, 7.7 Hz, 1H, H-1), 3.05 (2H, H-), 3.48 (ddd, J = 14.8, 12.6, 7.8 Hz, 2H, H-), 3.65 (p, J = 5.6 Hz, 2H, H-); ^{13}C NMR (D$_2$O) δ 22.8 (C-5), 36.1 (t, J = 121.0 Hz, C-1), 52.8 (C-2), 54.4 (C-4); ^{31}P NMR (D$_2$O) δ 15.51. Anal. (C$_6$H$_{15}$NO$_6$P$_2$•2H$_2$O) Calcd. C 24.42, H 6.49, N 4.75. Found C 24.70, H 6.47, N 4.66.

1-[(2-Piperidin-1-ylamino)ethyl] 1,1-bisphosphonic Acid (27) 84% Yield; mp 226–228 ºC; ^{1}H NMR (D$_2$O) δ 1.38 (m, 1H, H-), 1.58 (m, 2H, H-), 1.69 (m, 1H, H-), 1.85 (m, 2H, H-), 2.61 (tt, J = 21.4, 7.7 Hz, 1H, H-1), 2.86 (t, J = 11.1 Hz, 2H, H-), 3.31 (m, 2H, H-), 3.49 (t, J = 11.1 Hz, 2H, H-); ^{13}C NMR (D$_2$O) δ 21.3 (C-5), 23.4 (C-5), 34.0 (t, J = 121.0 Hz, C-1), 53.4 (C-2), 54.3 (C-4); ^{31}P NMR (D$_2$O) δ 15.60. Anal. (C$_7$H$_{17}$NO$_6$P$_2$) Calcd. C 30.78, H 6.27, N 5.13. Found C 30.33, H 6.30, N 4.99.

Drug Screening

Experiments on the intracellular form of the parasite were conducted on *T. cruzi*-infected L_6E_9 myoblasts (Y strain) as described before.[41,42] Gamma-irradiated L_6E_9 myoblasts (1×10^7 cells/plate) in DMEM medium 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 was added containing 4.17 x 10^6 trypomastigotes or amastigotes/well in DMEM medium. 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 Hank's 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 seconds and counted. The percent inhibition was calculated by employing the following formula: Percent inhibition = $100 – (\Delta C_d \text{ x } 100)/\Delta C_c$, where ΔC_c and ΔC_d are the differences in the counts per minute of control cultures and drug-treated cultures, respectively.

Experiments on *T. gondii* tachyzoites were carried out as previously published.[43] Briefly, *T.gondii* tachyzoites of the m2m3 clone expressing yellow fluorescence protein[43] 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.

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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.[41] 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.

TcFPPS and TgFPPS Assays and Product Analysis.

For TcFPPS[31,44,45] 100 µL of assay buffer (10 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 2 mM dithiothreitol, 100 µM [4-^{14}C]IPP (10 µCi/µmol)), and 100 µM DMAPP were prewarmed to 37 ºC. The assay was initiated by the addition of recombinant protein (10–20 ng). The assay was allowed to proceed for 30 min at 37 ºC and was quenched by the addition of 6 M HCl (10 µL). The reactions were made alkaline with 6.0 M NaOH (15 µL), diluted in water (0.7 mL), and extracted with of hexane (1 mL). The hexane solution was washed with water and transferred to a scintillation vial for counting. One unit of enzyme activity was defined as the activity required to incorporate 1 nmol of [4-^{14}C]IPP into [14-^{14}C]FPP in 1 min. For TgFPPS the assay conditions were as described above except that the buffer contained 1 mM $MgCl_2$.

TcSPPS assay

The activity of the enzyme was determined by a radiometric assay based on that described before.[14,46] Briefly, 100 µl of assay buffer (100 mM Tris-HCl buffer, pH 7.4, 1 mM $MgCl_2$, 1% (v/v) Triton X-100, 100 µM [4-^{14}C]IPP (10 µCi/ µmol)), and 50 µM GGPP was prewarmed to 37 °C. The assay was initiated by the addition of 10-20 ng of recombinant protein. The assay was allowed proceed for 30 min at 37 °C and was

quenched by chilling quickly in an ice bath. The reaction products were extracted with 1 ml of 1-butanol saturated with water. The organic layer was washed with water saturated with NaCl, and transferred to a scintillation vial with 4 ml of scintillation solution Ecolume for counting. One unit of enzyme activity was defined as the activity required to incorporate 1 nmol of [4-^{14}C]IPP into [4-^{14}C]FPP in 1 min.

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Synthesis and biological evaluation of 2-alkylaminoethyl-1,1-bisphosphonic acids against *Trypanosoma cruzi* and *Toxoplasma gondii* targeting farnesyl diphosphate synthase

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17, IC_{50} = 0.84 µM (against *T. cruzi*)
IC_{50} = 0.49 µM (against TcFPPS*)*