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Bisphosphonates Derived from Fatty Acids are Potent Inhibitors of *Trypanosoma cruzi* Farnesyl Pyrophosphate Synthase

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Abstract—Studies on the mode of action of a series of bisphosphonates derived from fatty acids, which had previously proved to be potent inhibitors against *Trypanosoma cruzi* proliferation in in vitro assays, have been performed. Some of these drugs proved to be potent inhibitors against the intracellular form of the parasite, exhibiting IC_{50} values at the low micromolar level. As bisphosphonates are FDA clinically approved for treatment of bone resorption disorders, their potential innocuousness makes them good candidates to control tropical diseases.

American Trypanosomiasis (Chagas' disease) is an endemic disease widespread from southern United States to southern Argentina. It has been estimated that around 18 million people are infected with the etiological agent of this illness, the hemoflagellated protozoan *Trypanosoma cruzi*.[1] Chagas' disease is considered by the World Health Organization as one of the major parasitic diseases.[2] In rural areas, this disease is transmitted by reduviid bugs as a consequence of their blood-sucking activity.[3] As other kinetoplastid parasites, *T. cruzi* has a complex life cycle possessing three main morphological forms: the dividing non-infective epimastigotes, the non-dividing highly infective trypomastigotes, and the intracellular and clinically more relevant form, amastigotes.[4] This disease has an acute phase, which may take place nearly unnoticed, although rarely it can lead to fatal meningoencephalitis or acute myocarditis, predominantly in children; an indeterminate asymptomatic phase, which can continue for more than ten years or even for the entire life of the infected individual; finally, a chronic phase, associated with heart problems or enlargement of hollow viscera (esophagus and colon) that may lead to death. Chemotherapy for the treatment of Chagas' disease is still deficient.[4,5] It is based on two drugs empirically discovered, nifurtimox, now discontinued, and benznidazole. Although both of these compounds are able to cure at least 50% of recent infections as indicated by the disappearance of symptoms, and negativization of parasitemia and serology, they have important drawbacks such as selective drug sensitivity on different *T. cruzi* strains. These agents produce serious side effects including vomiting, anorexia, peripheral neuropathy, allergic dermopathy, and so on. Long-term treatment is an additional disadvantage.[6] Moreover, these compounds are not effective in the chronic stage of the disease. In addition, there are a number of uncertainties concerning gentian violet, the only drug available to prevent blood transmission of Chagas' disease, because it is carcinogenic in animals.[7] For the above reasons, there is a critical need to develop new drugs that are more effective and safer than those currently available.[7] The studies of unique aspects of the biochemistry and physiology of *T. cruzi* led to the recognition of specific molecular targets for drug design.[8–13] Among them, protein prenylation arises as a specially attractive target.[11,12]

Bisphosphonates are compounds structurally related to inorganic pyrophosphate in which a methylene group has replaced the oxygen bridge between the phosphor-

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ous atoms. Unlike pyrophosphate, geminal phosphonates present greater metabolic stability because they are not hydrolyzed by pyrophosphatases and are also stable to hydrolysis under acidic conditions. Compounds **1**–**3** (pamidronate, alendronate, and risedronate, respectively) and other bisphosphonates are effective inhibitors of bone resorption and are currently being used for the treatment of several bone disorders such as osteoporosis, Paget's disease, complications associated with bone metastases and multiple myeloma, hipercalcemia provoked by malignancy, bone inflammation associated with rheumatoid arthritis or periodontal disease (Fig. 1).[14–18]

Bisphosphonates were originally designed to mimic the chemical structure of pyrophosphate. In spite of having been used for more than 30 years, the target of these drugs, the isoprenoid pathway, has been elucidated only recently. Early work had postulated that these drugs were putative inhibitors of pyrophosphate-related metabolic pathways. In fact, protein prenylation occurs in pathogenic trypanosomes.[19] This process is responsible for the attachment of farnesyl and geranylgeranyl groups to the C-terminal cysteine residues of a number of proteins, such as the small GTPases such as Ras, Rac, Rab, and Rho, giving rise to farnesylated and geranylgeranylated proteins. These proteins are important signaling molecules involved in crucial cell processes for osteoclasts function.[20] The attached prenyl groups play an important role in anchoring proteins to membranes and also act in protein–protein interactions. Three enzymes have been identified in eukaryotic cells: protein farnesyl transferase (PFT) and protein geranylgeranyl transferases I and II (PGGT-I and II).[21] Selective inhibition of PFT impairs growth of human tumors due to farnesylation inhibition of oncogenic Ras.[22] This finding led to the development of many FPT inhibitors[22] as potential antitumor agents; some of them were potent inhibitors of *T. cruzi* and *T. brucei* proliferation.[19] The molecular target of nitrogen-containing bisphosphonates in osteoclasts,[23–25] plants,[26] and *Dictyostelium discoideum*[27] is farnesyl pyrophosphate synthase. This enzyme catalyzes the formation of the substrate for protein prenylation.[28] Bisphosphonates with the nitrogen atom at the C-3 position would act as carbocation transition state analogues of isoprenoid diphosphates for isoprenoid biosynthesis.[29,30]

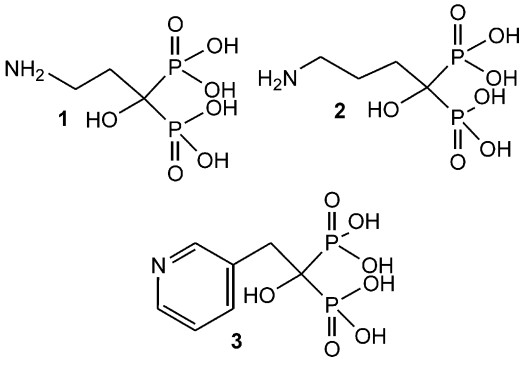


Figure 1. Chemical structures of representative member of bisphosphonates currently employed for the treatment of bone disorders.

Nitrogen-containing bisphosphonates such as compounds **1**–**3** inhibit *T. cruzi* proliferation in vitro and in vivo without toxicity to the host cells.[31] For example, pamidronate (**1**) and alendronate (**2**) are able to impair *T. cruzi* growth (amastigotes) both with IC_{50} values close to 65 μM. Pamidronate is also able to arrest parasitemia in a murine model of an acute *T. cruzi* infection.[31] In addition, it was found that several bisphosphonates are potent inhibitors of several pathogenic trypanosomatids (*T. cruzi*, *T. brucei rhodesiense*, and *Leishmania donovani*) and apicomplexan parasites (*Toxoplasma gondii* and *Plasmodium falciparum*).[32,33] Risedronate possesses IC_{50} values of 0.22 μM for *T. brucei rhodesiense*, and 0.49 μM for *T. gondii*.[33] Bisphosphonates derived from fatty acids, in which no nitrogen atom is present in their chemical structure, were shown to be potent inhibitors of *T. cruzi* proliferation possessing IC_{50} values at the low micromolar level.[34] Taking into account that bisphosphonates derivatives are FDA-approved drugs for long-term treatment of bone disorders; it might be anticipated low toxicity for new compounds bearing the bisphosphonate moiety. Bearing in mind that the pharmacophore corresponded to the *gem*-phosphonate unit, and on the basis of the potent inhibitory action exhibited by bisphosphonates derived from fatty acids, it was decided to validate the molecular target of these new bisphosphonates as well as to study the influence of the hydroxyl group at the C-1 position on their biological activity. For that reason, a new set of bisphosphonate derivatives lacking the hydroxyl group was designed, prepared and evaluated against *T. cruzi* growth. In addition, both 1-hydroxy-1,1-bisphosphonates and 1,1-bisphosphonates were evaluated as inhibitors of *T. cruzi* farnesyl pyrophosphate synthase (TcFPPS).

Compounds **4**–**10** were prepared as previously described (Fig. 2).[34] 1-Alkyl-1,1-bisphosphonates were synthesized following a slightly modified published procedure employing tetraethyl ethenylidenbisphosphonate (compound **13**) as a Michael acceptor,[35] which was straightforwardly prepared from methylenebisphosphonate (compound **11**) in two steps according to the Degenhart protocol.[36–38] The corresponding tetraalkyl bisphosphonic ester were converted into the free bisphosphonic acids by treatment with concentrated hydrochloric acid.[39] Therefore, the appropriate Grignard reagent prepared from the corresponding alkyl halide was added slowly to a solution of the Michael acceptor **13** to afford the desired tetraethyl alkyl bisphosphonate in moderate yields.[40] Methyllithium was used to incorporate an extra carbon instead of methyl magnesium iodide, which resulted not to be effective in this transformation.

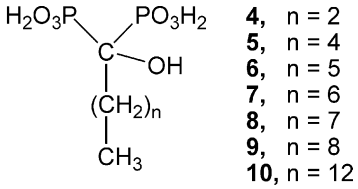
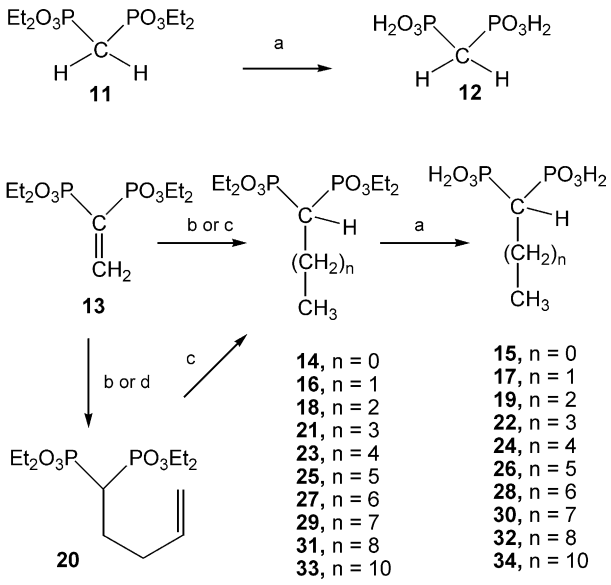


Figure 2. Chemical structures of bisphosphonates derived from fatty acids.

The acidic hydrolyses were achieved in good yields in all cases (70–90%). The preparation of this new family of *gem*-bisphosphonates is illustrated in Scheme 1.

1-Hydroxy-1,1-bisphosphonates derived from fatty acids (compounds **4**–**10**) were potent and competitive inhibitors[30] of TcFPPS activity. The efficacy of each drug on this enzyme (Table 1) correlated quite well with the inhibitory action that had previously exhibited against *T. cruzi* (amastigotes) growth.[34] Taken compound **5** as an example, this compound was a potent inhibitor of TcFPPS activity with an IC_{50} value of 1.94 μM and a K_i of 0.40 μM. These data correlated thoroughly with the effectiveness of this drug as antiparasitic agent. Compound **5** proved to be a potent inhibitor of the clinically more relevant form of the parasite with an $IC_{50} = 18$ μM.[34] Compound **5** lacks the nitrogen atom of conventional bisphosphonates, therefore the validation of TcFPPS as the actual target of these type of biphosphonates is extremely surprising. In this case, it is not possible to hypothesize a carbocation transition state



Scheme 1. Reagents and conditions: (a) HCl (concd), reflux, 12 h, 74% for **12**, 70% for **15**, 76% for **19**, 71% for **22**, 80% for **24**, 81% for **26**, 98% for **28**, 75% for **30**, 72% for **32**, 79% for **34**; (b) MeLi, THF, 0 °C, 2 h, 40% for **16**, $ClCH_2(CH_2)_nCH_3$, Mg, THF, 0 °C, 85% for **18**, 18% for **23**, 17% for **25**, 30% for **27**, 40% for **29**, 45% for **31**, 42% for **33**; (c) H_2, Pd/C, 3 atm, 2 h, 95% for **14**; (d) $CH_2{=}CHCH_2Cl$, Mg, THF, 0 °C, 2 h 31% for **20**, (ii) H_2, Pd/C, 3 atm, 2 h 85% for **21**.

Table 1. Effect of non-nitrogen-containing bisphosphonates on *Trypanosoma cruzi* farnesyl pyrophosphate synthase activity for compounds **4**–**8**, **17**, **19**, **22**, **24**, **26**, and **28**

Compd	IC_{50} (μM)[a]	K_i (μM)[a]	Compd	IC_{50} (μM)[a]	K_i (μM)[a]
4	42.83	5.04	**17**	>100	nt
5	1.94	0.40	**19**	150.36	16.99
6	2.37	0.24	**22**	5.71	0.79
7	9.36	0.98	**24**	5.67	0.47
8	8.45	0.59	**26**	4.54	0.54
			28	19.73	1.88
			30	4.25	0.31

[a]Values are means of three experiments, (nt, not tested). IC_{50} and K_i were calculated as described.[30]

analogue of TcFPPS at the enzyme active site as it had been postulated for nitrogen-containing bisphosphonates.[29,30] The same degree of efficiency as inhibitors of TcFPPS activity was observed for compounds **6**–**8**. Both of these drugs were formerly shown to be potent inhibitors against *T. cruzi* proliferation.[34] The results are shown in Table 1.

On the other hand, bisphosphonates without the hydroxyl group at the C-1 position were also potent inhibitors of TcFPPS but to a slightly lesser extent than drugs **5**, **6**, and **8**, as it was the case of **24** and **26**. Nevertheless, such inhibition of the enzymatic activity did not correlate with the cellular activity observed by this family of drugs. Certainly, compound **26** was moderately effective against the intracellular form of the parasite with an IC_{50} value close to 70 μM. Similar results were observed with the hydroxy-containing bisphosphonates,[34] these compounds were devoid of activity against the epimastigote forms of the parasite (Table 2). Moreover, all the intermediate tetraethyl bisphosphonate esters exhibited marginal activity against the intracellular form of *T. cruzi* (Table 3). This lack of biological activity may be attributable to the inability of phosphonic esters to coordinate Mg^{2+}, present at the active site of the enzyme, in a bidentate manner or due to poor cell permeability. In contrast to the above behaviour, the tridentate 1-hydroxy-1,1-bisphosphonate derivatives have three coordination

Table 2. Effect of non-nitrogen-containing bisphosphonates against *Trypanosoma cruzi* (epimastigotes) for compounds **4**–**9**, and **16**–**19**, and **21**–**32**

Compd	IC_{50} (μM)[a,b]	Compd	IC_{50} (μM)[a]	Compd	IC_{50} (μM)[a]
4	>70	**16**	>70	**17**	>100
5	>70	**18**	>70	**19**	>100
6	>70	**21**	>70	**22**	>70
7	>70	**23**	>50	**24**	>70
8	>70	**25**	>50	**26**	>70
9	>70	**27**	>50	**28**	>70
		29	>50	**30**	>70
		31	>50	**32**	>70

[a]Values are means of three experiments.
[b]Data taken from ref 34.

Table 3. Effect of non-nitrogen-containing bisphosphonates against *Trypanosoma cruzi* (amastigotes) for compounds **4**–**9**, and **16**–**19**, and **21**–**32**

Compd	IC_{50} (μM)[a,b]	Compd	IC_{50} (μM)[a]	Compd	IC_{50} (μM)[a]
4	21.4	**16**	nt	**17**	>100 (21%)[a]
5	>70 (34%)[c]	**18**	>70 (27%)[c]	**19**	>90 (28%)
6	18.1	**21**	>50	**22**	>70
7	>70 (41%)[c]	**23**	nt	**24**	nt
8	65.8	**25**	nt	**26**	>70 (47%)[c]
10	>70 (10%)[c]	**27**	nt	**28**	>70 (49%)[c]
		29	>50	**30**	22.36

[a]Values are means of three experiments.
[b]Data taken from ref 34.
[c]Maximum inhibition values obtained at the indicated concentrations (ca. 100.0 or 70.0 μM) are given in parentheses (nt, not tested).

Table 4. Effect of non-nitrogen-containing bisphosphonates against *Trypanosoma brucei* farnesyl pyrophosphate synthase activity for compounds **4–8**

Compd	IC_{50} (μM)[a]
4	> 100
5	3.12
6	0.66
7	3.57
8	4.54

[a]Values are means of three experiments.

sites that clearly justify their effectiveness against *T. cruzi* growth.

1-Hydroxy-1,1-bisphosphonates were also able to block farnesyl pyrophosphate synthase from *T. brucei* (TbFPPS), the etiological agent for sleeping sickness, another important parasitic disease targeting the central nervous system.[41] Compound **6** was a very potent inhibitor of this enzyme at the nanomolar level. Bearing in mind that TbFPPS was more susceptible to compound **6** than TcFPPS, it might be anticipated that *gem*-phosphonates derived from fatty acids have potential utility to control other parasitic diseases like sleeping sickness. Drugs **5**, **7**, and **8** were also potent inhibitors of TbFPPS activity. The results are shown in Table 4. A gene encoding the farnesyl pyrophosphate synthase of *T. brucei* was recently cloned and sequenced.[42] The activity of the enzymes (TcFPPS or TbFPPS) was determined by a radiometric assay based on that described before.[30,42–44]

It can be concluded that non-nitrogen-containing bisphosphonates derived from fatty acids were potent inhibitors of TcFPPS and TbFPPS, some of them were even more potent than representative nitrogen-containing bisphosphonates.[29,30] The presence of a hydroxyl group at C-1 position was very significant for biological activity because it would provide an extra site of coordination with Mg^{2+} ion. The blockade of a phosphonic acid as tetraethyl phosphonic ester voids its ability to coordinate with Mg^{2+} resulting in a loss of efficacy both to inhibit TcFPPS activity and to impair *T. cruzi* growth. These results are very encouraging to improve drug design because it would allow the use of homology modelling of TcFPPS and TbFPPS taking into account that the X-ray structure of the avian FPPS has been solved.[45]

Work aimed at exploiting the potential biological activity of different bisphosphonates as well as to establish a rigorous structure–activity relationship is currently being pursued in our laboratory.

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40. ***Gem*-bisphosphonates. General procedure**: A solution of alkyl halide (10 mmol) in tetrahydrofuran (15 mL) was treated with freshly activated metallic magnesium and iodine under argon atmosphere. It was necessary to heat the reaction mixture when the alkyl halide presented more than five carbons in its chemical structure. Once the Grignard reagent was formed, the dark solution was cooled at 0 °C. Then, a solution of **13** in tetrahydrofuran was added slowly. The reaction mixture was stirred at 0 °C for 1 h, and then quenched with an aqueous saturated solution of ammonium chloride (10 mL). The mixture was extracted with CH_2Cl_2 (3×15 mL). The combined organic layers were washed with brine (2×20 mL), dried ($MgSO_4$), and the solvent was evaporated. The residue was purified by column chromatography eluting with mixtures of hexane–EtOAc. Each purified tetraethyl phosphonic ester was treated with concentrated hydrochloric acid (10 mL) and the mixture was refluxed for 12 h. The product was purified by reversed-phase column chromatography eluting with a mixture of methanol–water. Finally the product was crystallized from ethanol. Selected spectroscopic data for compounds **19** and **26**. Compound **19**: mp 181–183 °C; IR (KBr, cm^{-1}) 3393, 2972, 2887, 2266, 1468, 1105, 1040, 932, 762, 721; ^1H NMR (D_2O, 500.13 MHz) δ 0.68 (t, $J=7.3$ Hz, 3H, H-4) 1.23–1.42 (m, 2H, H-3) 1.48–1.76 (m, 2H, H-2), 2.08 (tt, $J=23.6$, 5.8 Hz, 1H, H-1); ^{13}C NMR (D_2O, 125.3 MHz) δ 13.78 (C-4) 22.78 (t, $J=6.8$ Hz, C-2) 27.82 (t, $J=4.7$ Hz, C-3) 37.82 (t, $J=126.8$ Hz, C-1); ^{31}P NMR (D_2O, 202.45 MHz) δ 22.84; MS (*m*/*z*, relative intensity) 217 (M^+, 1), 191 (3), 169 (2), 155 (3), 139 (4), 125 (9) 111 (18), 97 (28), 85 (25), 83 (24), 71 (46), 69 (44), 57 (100), 43 (86). Anal. calcd for ($C_4H_{12}O_6P_2$) C 22.03, H 5.55; found C 22.02, H 5.65. Compound **26**: mp 163–165 °C; IR (KBr, cm^{-1}) 3485, 2930, 2858, 2363, 2338, 1665, 1467, 1159, 930, 712; ^1H NMR (D_2O, 500.13 MHz) δ 0.68 (t, $J=6.4$ Hz, 3H, H-7), 1.06–1.18 (m, 6H, H-4, H-5, H-6), 1.30–1.44 (m, 2H, H-3), 1.56–1.86 (m, 2H, H-2), 2.11 (tt, $J=23.6$, 6.0 Hz, 1H, H-1); ^{13}C NMR (D_2O, 125.3 MHz) δ 14.17 (C-7), 22.74 (C-6), 25.86 (t, $J=4.7$ Hz, C-3) 29.03 (C-4) 29.51 (t, $J=7.2$ Hz, C-2), 31.57 (C-5) 38.34 (t, $J=124.61$ Hz, C-1); ^{31}P NMR (D_2O, 202.45 MHz) 23.03; MS (*m*/*z*, relative intensity) 260 (M^+, 1) 207 (4), 193 (.76) 176 (1) 123 (1), 109 (2), 97 (3), 83 (4), 69 (8), 57 (12), 55 (15), 44 (100). Anal. calcd for ($C_7H_{18}O_6P_2$) C 32.32, H, 6.97; found C 32.17, H. 7.01.
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43. **FPPS assay and product analysis**. Briefly, 100 μL of assay buffer [10 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 2 mM dithiothreitol, 47 μM [4-^{14}C]IPP (10 μCi/μmol)], and 55 μm DMAPP or GPP was prewarmed to 37 °C. The assay was initiated by the addition of recombinant protein (10–20 ng). The assay was allowed to proceed for 30 min at 37 °C and was quenched by the addition of 6 M HCl (10 μL). The reactions were made alkaline with 6 M NaOH (15 μL), diluted in water (0.7 mL), and extracted with 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.
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