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2-Alkylaminoethyl-1,1-Bisphosphonic Acids Are Potent Inhibitors of the Enzymatic Activity of *Trypanosoma cruzi* Squalene Synthase

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As part of our efforts aimed at searching for new antiparasitic agents, the effect of representative 2-alkylaminoethyl-1,1-bisphosphonic acids on *Trypanosoma cruzi* squalene synthase (*Tc*SQS) was investigated. These compounds had proven to be potent inhibitors of *T. cruzi*. This cellular activity had been associated with an inhibition of the enzymatic activity of *T. cruzi* farnesyl diphosphate synthase. 2-Alkylaminoethyl-1,1-bisphosphonic acids appear to have a dual action, since they also inhibit *Tc*SQS at the nanomolar range.

Inhibitors of squalene synthase (SQS) have great potential not only as cholesterol-lowering agents (23) but also as antiparasitic drugs (16, 37). In trypanosomatids, the enzyme catalyzes the first committed step in isoprenoid biosynthesis that leads to the formation of ergosterol instead of cholesterol such as occurs in mammals (37, 38). The reaction is a reductive coupling of two molecules of (*E*,*E*)-farnesyl pyrophosphate (compound 1 [FPP]) that takes place in two steps via the formation of a cyclopropane intermediate (compound 2) followed by a reductive rearrangement that gives rise to squalene (compound 3), as illustrated in Fig. 1. FPP is a branching point in isoprenoid biosynthesis. It can be either transformed into squalene or converted into other essential isoprenoids such as dolichol, coenzyme Q, or prenylated proteins (21).

Trypanosoma cruzi is the etiologic agent of American trypanosomiasis (Chagas' disease) (40) and exhibits a complex life cycle involving blood-sucking reduviid bugs and mammals (1). It multiplies in the insect gut as an epimastigote form and is spread as a nondividing metacyclic trypomastigote from the insect feces by contamination of intact mucosa or wounds produced by the blood-sucking activity of the vector. In the mammalian host, the parasite proliferates intracellularly as the amastigote form that is released into the bloodstream as a nondividing trypomastigote (1). Distribution of Chagas' disease could also take place via the placenta or by transfusion of infected blood (11, 14).

Bisphosphonic acids (compound 4) are metabolically stable pyrophosphate (compound 5) analogues in which a methylene group replaces the oxygen atom bridge between the two phosphorus atoms of the pyrophosphate unit. Substitution at the bridge has produced a large number of compounds (27). Bisphosphonates such as pamidronate (compound 6), alendronate (compound 7), risedronate (compound 8), and ibandronate (compound 9) are in clinical use for the treatment of different bone disorders (Fig. 2) (24, 25, 30). Bisphosphonic acids became relevant drugs after the calcification studies done close to 40 years ago (8, 9, 10).

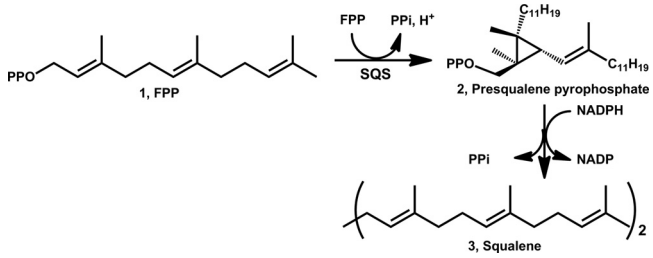

FIG 1 Squalene formation catalyzed by squalene synthase (SQS).

Besides their pharmacological properties with respect to bone, aminobisphosphonic acids had proven to be potent inhibitors of *T. cruzi* proliferation without toxicity to the host cells (20). Moreover, numerous bisphosphonic acids have been shown to be effective growth inhibitors of parasites other than *T. cruzi*, such as *T. brucei rhodesiense*, *Leishmania* spp., and apicomplexan parasites such as *Toxoplasma gondii* and *Plasmodium falciparum* (17, 22, 29, 32–36). As the acidocalcisomes are equivalent in composition to the bone mineral, gathering of bisphosphonic acids in these organelles facilitates their antiparasitic action (39). The mechanism of action of aminobisphosphonic acids has been narrowed down to protein prenylation (26). Farnesyl pyrophosphate synthase (FPPS) constitutes the main target of bisphosphonic acids (2, 6, 12, 13, 28). FPPS catalyzes the two mandatory biosynthetic steps to form farnesyl

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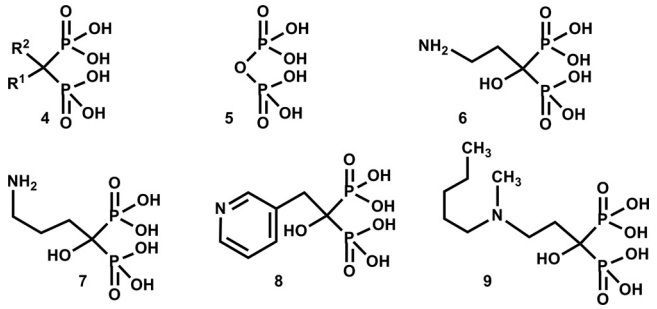

FIG 2 General formulas and chemical structures of representative FDA-approved bisphosphonic acids clinically employed for different bone disorders.

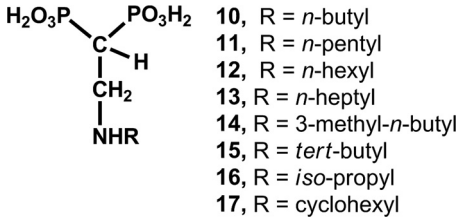

FIG 3 Representative members of 1-[(alkylamino)ethyl]-1,1-bisphosphonic acids.

pyrophosphate from dimethylallyl pyrophosphate. Inhibition of the enzymatic activity of FPPS blocks farnesyl pyrophosphate and geranylgeranyl pyrophosphate formation, which are required for the posttranslational prenylation of small GTP-binding proteins within osteoclasts (4).

Of special interest are 1,1-bisphosphonic acids derived from fatty acids, particularly the 2-alkylaminoethyl-1,1-bisphosphonic acid derivatives, which were shown to be potent growth inhibitors of the *T. cruzi* amastigote, which is the clinically more relevant form of the parasite, exhibiting 50% inhibitory concentrations (IC_{50}s) at the nanomolar range (29, 33). This class of bisphosphonic acids has proven to be more efficient than the parent drugs 1-hydroxy-, 1-amino-, and 1-alkyl-1,1-bisphosphonic acids as antiparasitic agents (33). Compound 12 arises as the main member of this class of bisphosphonic acids (14, 29, 32–36), with an IC_{50} of 0.84 μM (33). In initial studies, this cellular activity had been exclusively associated with the inhibition of the enzymatic activity of *T. cruzi* FPPS (*Tc*FPPS) (5), being a competitive inhibitor (35) with an IC_{50} of 0.49 μM (33). Compound 12 was also effective with respect to the enzymatic activity of *T. gondii* FPPS (IC_{50} = 0.14 μM) (33) and exhibited *in vitro* inhibitory action against tachyzoites of *T. gondii* (IC_{50} = 9.37 μM) (33) (Fig. 3).

It is worth pointing out that compound 12 also has exhibited modestly inhibitory action (IC_{50} = 1.35 μM) against an important prenyltransferase in *T. cruzi*, a solanesyl diphosphate synthase (*Tc*SPPS), which is involved in the synthesis of ubiquinone (7). This enzyme has been considered another potential target for chemotherapy (7).

Certain bisphosphonate derivatives have in addition been reported to be potent inhibitors of mammalian squalene synthase. Such is the case of the isoprenoid derivatives 18 to 24 (18, 19) and the closely structurally related compounds 25 to 27 (15) (Fig. 4). We therefore reasoned that, in a similar fashion, this could be the case with some of the compounds previously reported to be potent inhibitors of *T. cruzi* proliferation (compounds 10 to 17), which were straightforwardly prepared according to published procedures (33). Hence, here we tested a selection of bisphosphonic acids against recombinant *Tc*SQS.

Truncated soluble *T. cruzi* SQS enzyme was expressed and purified as previously described (31). Assessment of *Tc*SQS activity was based on measuring the conversion of [^{3}H]FPP to [^{3}H]squalene. Final assay concentrations were 50 mM morpholinepropanesulfonic acid–NaOH (pH 7.4), 20 mM $MgCl_2$, 5 mM CHAPS {3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 1% Tween 80, 10 mM dithiothreitol, 0.025 mg/ml bovine serum albumin, 0.25 mM NADPH, and 10 to 20 ng of purified recombinant *T. cruzi* SQS. The reaction was started with the addition of substrate ([^{3}H]farnesyl pyrophosphate; 0.1 nmol, 2.22 × 10^6 dpm), and the final volume of the reaction was 200 μl. After incubation at 37°C for 5 min, 40 μl of 10 M NaOH was added to stop the reaction, followed by 10 μl of a (100:1) mixture of 98% EtOH and squalene. The resulting mixtures were mixed vigorously by the use of a vortexing apparatus, and then 10-μl aliquots were applied to channels (2.5 by 10 cm) of a silica gel thin-layer chromatogram, and newly formed squalene was separated from the unreacted substrate by chromatography in toluene–EtOAc (9:1). The region of the squalene band was scraped and immersed in Hydrofluor liquid scintillation fluid and assayed for radioactivity. IC_{50}s were calculated from the hyperbolic plot of percent inhibition versus inhibitor concentration, using Sigma Plot (31).

Biological evaluation of 2-(alkylamino)ethyl-1,1-bisphosphonic acids indicated that these compounds are potent inhibitors of the enzymatic activity of *T. cruzi* SQS. Particularly, compounds 11 to 13 arose as the most efficient examples of this type of compound. Interestingly, compound 11 exhibited an IC_{50} of 5.0 nM against *Tc*SQS and had previously shown a potent action as a growth inhibitor of amastigotes of *T. cruzi* with an IC_{50} of 0.54 μM (33). However, compound 11 exhibited only a moderate inhibitory action toward *Tc*FPPS (IC_{50} = 1.84 μM) (33). Compound 12 was another example of bisphosphonate that had previously exhibited potent activity against intracellular amastigotes (IC_{50} = 0.84 μM) (33). Indeed, this cellular activity had been attributed to inhibition of the enzymatic activity of *Tc*FPPS (IC_{50} = 0.49 μM) (33), and yet we now show that it is also an effective inhibitor of *Tc*SQS, with an IC_{50} of 21 nM. Compound 13 was also a potent inhibitor of *Tc*SQS (IC_{50} = 12 nM), while its cellular activity against *T. cruzi* amastigotes was moderate (IC_{50} = 10.0 μM) (33). Thus, with the exception of compounds 15 and 16, all the tested compounds were potent inhibitors of *Tc*SQS, with IC_{50}s in the low nanomolar range (Table 1). These results suggest that the primary target for some of these compounds may be *Tc*SQS instead of *Tc*FPPS as initially considered. This would imply that the interruption of carbon flow toward sterol intermediates is a major mechanism of action within the parasite. Work aimed at designing optimized bisphosphonate molecules targeting either *Tc*SQS or *Tc*FPPS is currently being pursued in our laboratory.

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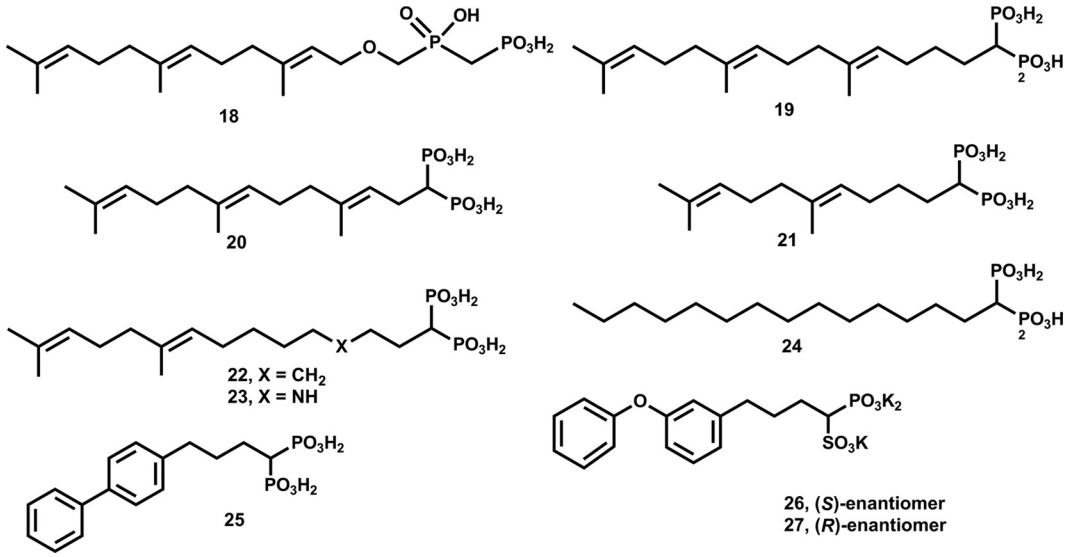
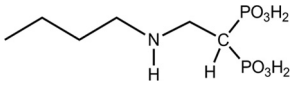
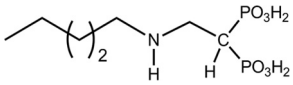
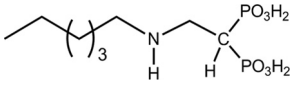
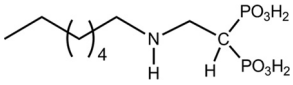
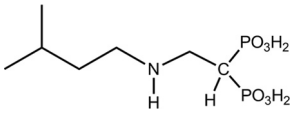
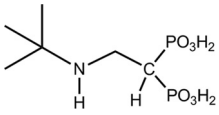
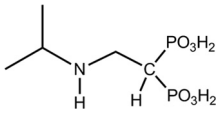
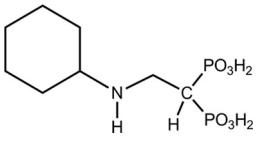


FIG 4 Chemical structures of selected inhibitors of SQS activity bearing a bisphosphonate moiety.

TABLE 1 Structures and IC_{50}s of antiparasitic compounds

Compound	Structure	IC_{50} (nM)		
		*Tc*SQS	*Tc*FPPS[a]	Amastigotes[a]
10		39.0 ± 8.1	2.28 ± 0.34	4.8
11		5.0 ± 2.1	1.84 ± 0.25	0.54
12		21.4 ± 3.3	0.490 ± 0.12	0.84
13		11.9 ± 0.5	0.058 ± 0.009	10
14		22.0 ± 5.4	>50	0.94
15		>1,000	1.21 ± 0.13	10
16		>1,000	69.8	1.39
17		30.0 ± 6.0	57.3	>10

[a] Data taken from reference 33.

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