Bisphosphonates Inhibit the Growth of *Trypanosoma brucei, Trypanosoma cruzi, Leishmania donovani, Toxoplasma gondii,* and *Plasmodium falciparum*: A Potential Route to Chemotherapy

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We have investigated the effects in vitro of a series of bisphosphonates on the proliferation of *Trypanosoma cruzi*, *Trypanosoma brucei rhodesiense*, *Leishmania donovani*, *Toxoplasma gondii*, and *Plasmodium falciparum*. The results show that nitrogen-containing bisphosphonates of the type used in bone resorption therapy have significant activity against parasites, with the aromatic species having in some cases nanomolar or low-micromolar IC_{50} activity values against parasite replication (e.g. *o*-risedronate, $IC_{50} = 220$ nM for *T. brucei rhodesiense*; risedronate, $IC_{50} = 490$ nM for *T. gondii*). In *T. cruzi*, the nitrogen-containing bisphosphonate risedronate is shown to inhibit sterol biosynthesis at a pre-squalene level, most likely by inhibiting farnesylpyrophosphate synthase. Bisphosphonates therefore appear to have potential in treating parasitic protozoan diseases.

Introduction

Parasitic protozoan diseases constitute the world's most widely spread human health problem. It is estimated that 3 billion individuals suffer from one or more parasitic infections, with the greatest causes of morbidity being attributed to the trypanosomatid and apicomplexan parasites.[1] The apicomplexan, *Plasmodium falciparum*, the causative agent of malaria, infects approximately 500 million individuals each year, resulting in 2−3 million deaths annually.[1,2] Of the trypanosomatid parasites, the combined burden of *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania* spp. results in an additional 20 million disease cases.[3−6] Since vaccinations against parasitic infections are still in development and control of environmental sources of transmission is ineffective and often impractical, the need for improved chemoprophylactic/chemotherapeutic approaches in the control of these pathogens is indisputable.

Investigations of the metabolic processes occurring in a range of parasitic protozoa have demonstrated the presence of a pathway derived from mevalonic acid, called "the mevalonate pathway". This pathway is responsible for the synthesis of a variety of sterols and polyisoprenoid compounds which are of vital importance to parasite survival.[7−10] The production of farnesylpyrophosphate (FPP) marks the branching point in the mevalonate pathway, with FPP being the primary precursor of farnesylated and geranylgeranylated proteins, as well as dolichol and sterol biosynthesis.

Recent studies have shown that nitrogen-containing bisphosphonates, such as the ones used to treat bone resorption diseases, are competitive inhibitors of farnesylpyrophosphate synthase (FPPS).[11−15] Bisphosphonates are nonhydrolyzable pyrophosphate (P−O−P) analogues in which the oxygen bridge has been replaced by a carbon (P−C−P). We have previously proposed that the potent nitrogen-containing bisphosphonates, such as pamidronate, alendronate, and risedronate, act as (aza)carbocation pyrophosphate intermediate analogues of the geranylpyrophosphate carbocation.[11]

In this study we explore the in vitro activity of a series of bisphosphonates against the parasitic protozoa *T. brucei*, *T. cruzi*, *L. donovani*, *T. gondii*, and *P. falciparum*, the causative agents of African sleeping sickness, Chagas' disease, visceral leishmaniasis, toxoplasmosis, and malaria, respectively.

Results and Discussion

We show in Table 1 the structures of the 19 bisphosphonates tested. **1** is alendronate (Merck's Fosamax), and **2** is risedronate (Procter and Gamble/Hoechst Marion Roussels' Actonel), while **5** is pamidronate (Novartis' Aredia). We tested these and other compounds (Table 1) against *T. brucei rhodesiense*, *T. cruzi*, *L. donovani*, *T. gondii*, and *P. falciparum* replication in vitro using the methods described in the Experimental Section. The experimental concentrations required to reduce parasite proliferation by 50%, the IC_{50} values, were extracted from experimental growth versus inhibitor concentration assays by fitting the rectangular hyperbolic function:

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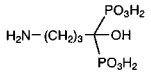
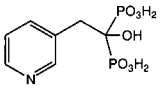
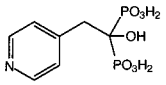
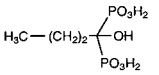
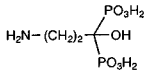
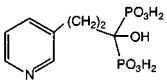
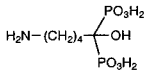
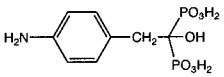
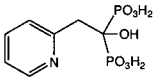
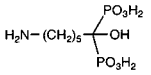
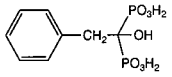
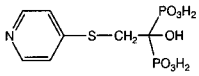
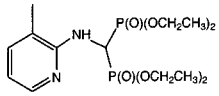
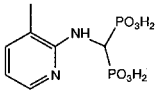
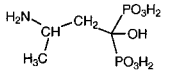
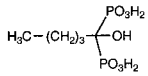
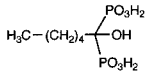
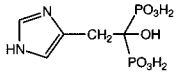
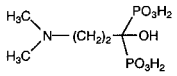
Table 1. Bisphosphonates Screened for Antiparasitic Activity

Compound Number	Compound Name	Structure
1	4-amino-1-hydroxybutane-1,1-bisphosphonate *Alendronate*	
2	2-(3-pyridyl)-1-hydroxyethane-1,1-bisphosphonate; *Risedronate*	
3	2-(4-pyridyl)-1-hydroxyethane-1,1-bisphosphonate	
4	1-hydroxybutane-1,1-bisphosphonate	
5	3-amino-1-hydroxypropane-1,1-bisphosphonate; *Pamidronate*	
6	3-(3-pyridyl)-1-hydroxypropane-1,1-bisphosphonate	
7	5-amino-1-hydroxypentane-1,1-bisphosphonate	
8	2-(4-aminophenyl)-1-hydroxyethane-1,1-bisphosphonate	
9	2-(2-pyridyl)-1-hydroxyethane-1,1-bisphosphonate	
10	6-amino-1-hydroxyhexane-1,1-bisphosphonate *Neridronate*	
11	2-phenyl-1 hydroxyethane-1,1-bisphosphonate	
12	2-(4-pyridylthio)-1-hydroxyethane-1,1-bisphosphonate	
13	N-(2-(3-picolyl)) aminomethylene bisphosphonate tetraethyl ester	
14	N-(2-(3-picolyl)) aminomethylene bisphosphonate	
15	3-amino-1-hydroxybutane-1,1-bisphophonate	
16	1-hydroxypentane-1,1-bisphosphonate	
17	1-hydroxyhexane-1,1-bisphosphonate	
18	2-(4-imidazole)-1-hydroxyethane-1,1-bisphosphonate	
19	3-(N,N-dimethyl)-amino-1-hydroxypropane-1,1-bisphosphonate *Olpadronate*	

$$I = \frac{I_{max}C}{IC_{50} + C} \quad (1)$$

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. The regression analyses were performed with Sigma Plot 5.0 (SPSS Inc., Chicago, IL). By way of example, we show results for four assays in Figure 1: **2** in *T. gondii* tachyzoites (IC_{50} = 490 nM, Figure 1A), the *ortho*-isomer of risedronate (**9**) (2-(2-pyridyl)-1-hydroxyethane-1,1-bisphosphonate) in *T. gondii* tachyzoites (IC_{50} = 9.1 μM, Figure 1B), **9** in *T. brucei* bloodstream trypomastigotes (IC_{50} = 220 nM, Figure 1C), and 2-(4-imidazolyl)-1-hydroxyethane-1,1-bisphosphonate (**18**) in *T. cruzi* intracellular amastigotes (IC_{50} = 35 μM, Figure 1D).

In the case of axenically grown *T. brucei* trypomastigotes, our results indicate the lowest IC_{50} of any of the bisphosphonates tested in any organism studied here, with a value of 220 nM being found for **9**, Table 2 and Figure 1C. We also found some activity against *T. brucei* trypomastigotes with **2** ($IC_{50} \sim 8.6$ μM) and its higher homologue **6**, which has an additional methylene group in its side chain and showed an IC_{50} of ~1.7 μM. In addition, the aminomethylenebisphosphonate **14** showed very high activity, having an IC_{50} of 700 nM (Table 2). These results are all of interest since, on the basis of previous work, **2** has been found to have an extremely low IC_{50} for inhibiting FPPS, with values of 3.9 nM being reported for a recombinant human enzyme.[14] Moreover, **14** (Zeneca's compound **1** in ref 16) has been reported to have a 23 nM IC_{50} in FPPS inhibition

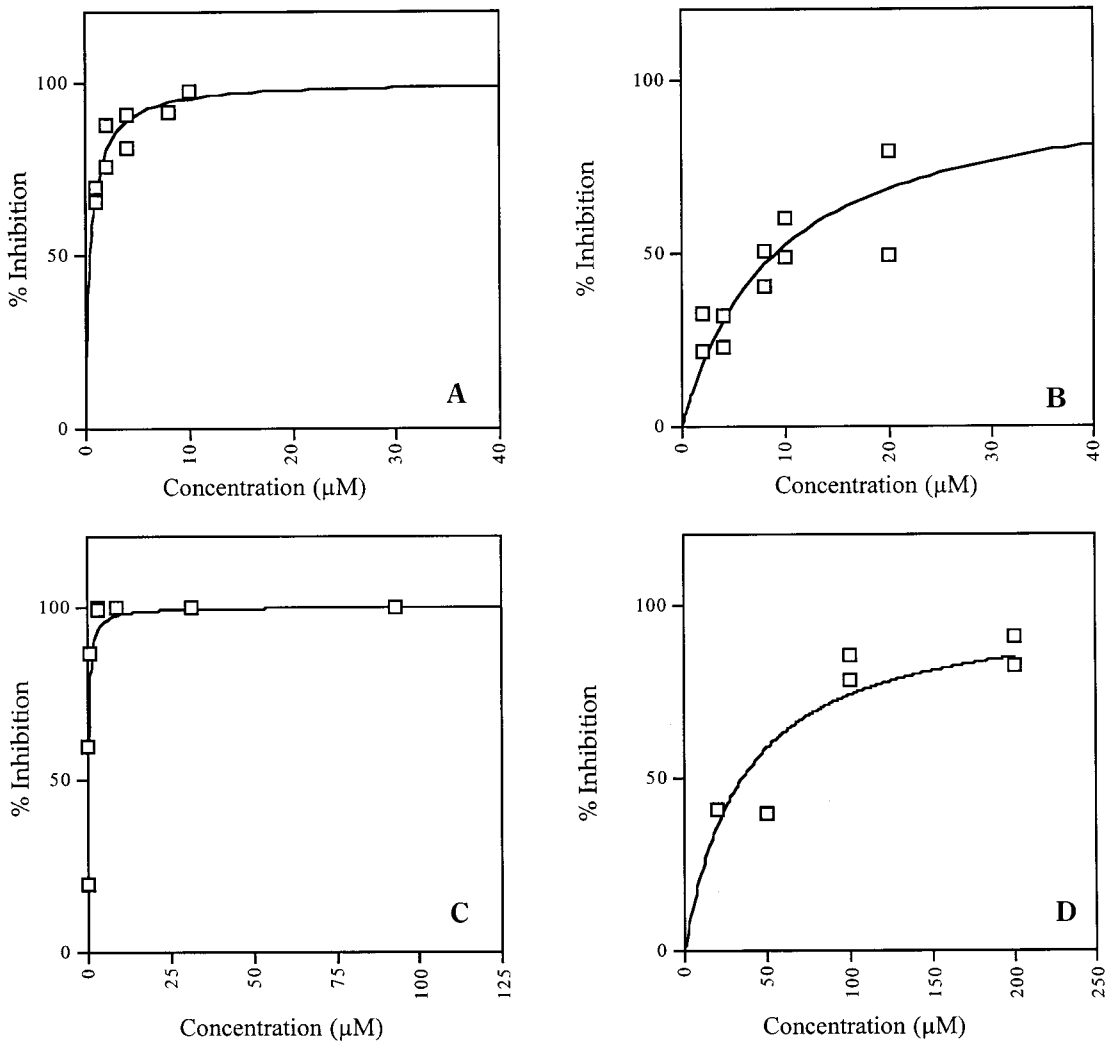


Figure 1. Four growth inhibition results. The percent (%) growth inhibition is plotted as a function of drug concentration. The experimental results are fit to a rectangular hyperbolic function (see text, eq 1). The drugs/organism tested are as follows: A, risedronate (**2**)/*T. gondii* tachyzoites; B, *o*-risedronate (**9**)/*T. gondii* tachyzoites; C, *o*-risedronate (**9**)/*T. brucei* trypomastigotes; D, 2-(4-imidazolyl)-1-hydroxyethane-1,1-bisphosphonate (**18**)/*T. cruzi* amastigotes.

Table 2. Growth Inhibition Results for Bisphosphonates against Five Parasitic Protozoa[a]

	IC_{50} (μM)				
compd	*T. brucei* trypomastigotes	*T. cruzi* amastigotes	*L. donovani* amastigotes	*T. gondii* tachyzoites	*P. falciparum* intraerythrocytic stages
1	>200	147 ± 31.2	82.5 ± 14.6	25 ± 6.3	>200
2	8.6 ± 2.7	123 ± 26.4	2.3 ± 0.3	0.49 ± 0.05	123 ± 22.5
3	>200	>200	>200	>200	>200
4	92 ± 13.4	>200	>200	9.1 ± 1.4	130 ± 37.6
5	177 ± 51.1	60 ± 11.4	t/100	35.6 ± 6.2	>200
6	1.7 ± 0.3	>200	>200	>200	>200
7	>200	>200	129 ± 18.3	>200	>200
8	40 ± 5.1	>200	>200	68.2 ± 13.2	>200
9	0.220 ± 0.09	105 ± 20.9	t/100	9.1 ± 1.2	>200
10	31.7 ± 8.7	>200	>200		>200
11	21.3 ± 7.6	>200	>200		7.7 ± 1.4
12	19.8 ± 7.2	>200	>200		158 ± 40.1
13	27.9 ± 13.1	>200	82.1 ± 9.0		>200
14	0.7 ± 0.3	147 ± 33.2	t/100		>200
15	7.8 ± 3.0	>200	>200		>200
16	99.8 ± 21.6	>200	>200		56.7 ± 7.1
17	62.4 ± 18.3	>200	>200		5.1 ± 1.6
18	8.6 ± 3.6	35 ± 8.3	t/+		>200
19	5.4 ± 0.9	>200	>200		>200

[a] Pentamidine IC_{50} = 35 nM (*T. brucei*); chloroquine IC_{50} = 3 nM (*P. falciparum*); pentostam IC_{50} = 4.0 μg/mL (*L. donovani*); benznidazole IC_{50} = 3.5 μM (*T. cruzi*); t/100 = toxic to cells, no parasites presents; t/+ = toxic to cells, parasites present.

in a daffodil chromoplast assay, which strongly suggests (but does not prove) that FPPS is a major target for the bisphosphonate inhibitors in *T. brucei* trypomastigotes.

For *T. cruzi* inhibition, we used three assay systems. In the first, we used γ-irradiated myoblasts to assay for amastigote proliferation. In the second, we used Vero cells to assay for both amastigote proliferation and host cell toxicity induced by the bisphosphonates. And in the third, we used epimastigotes to investigate sterol biosynthesis inhibition induced by **2** alone and in combination with a sterol biosynthesis inhibitor (SBI), ketoconazole.

In the case of *T. cruzi* amastigote proliferation in irradiated myoblasts (which do not undergo cell division), **1**, **2**, and **5** have some activity, as does **9**, but the IC_{50} values are typically on the order of 100 μM, considerably larger than found in the *T. brucei* trypomastigote system. Nevertheless, as noted previously, **5** decreases the extent and delays the onset of parasitemia in an in vivo murine model of acute Chagas' disease.[17] Of greater interest is the observation that the imidazolium compound **18**, an isomer of zoledronate,[18] has the lowest IC_{50} of any compound tested against *T. cruzi* amastigote replication, with an IC_{50} = 35 μM. We believe that this correlates with the observation that **18** has a very low inhibitory concentration for FPPS (IC_{50} = 1 nM) in the daffodil chromoplast assay[19] and readily docks into the FPPS active site (data not shown). The higher IC_{50} values found with the *T. cruzi* amastigotes are at least in part a reflection of the increased permeability barriers in this intracellular parasite, and similar differences between in vitro enzyme inhibition IC_{50} values and corresponding cell growth inhibition IC_{50} values have previously been reported in bone resorption work.[14,20,21]

Thus, in both *T. brucei* and *T. cruzi*, some of the most effective inhibitors of parasite growth have been shown in other systems to have extremely low IC_{50} values in FPP synthesis inhibition: **14** in *T. brucei* trypomastigotes correlating with a 23 nM IC_{50} in the plant assay and **18** in *T. cruzi* amastigotes correlating with a 1 nM IC_{50} in the same assay. These results strongly suggest that FPPS is a major target for the nitrogen-containing bisphosphonates in these protozoa.

Since sterol biosynthesis should also be affected by FPPS inhibition, we investigated the effects of one of the more potent bisphosphonates, **2**, on sterol biosynthesis in *T. cruzi*, together with the effects of ketoconazole, a known 14α-demethylase SBI. We show in Figure 2 the effects of **2** and ketoconazole on *T. cruzi* epimastigote proliferation as a function of time. There is clearly a substantial decrease in epimastigote proliferation in the presence of 100 μM **2** or 0.3 μM ketoconazole. The drugs were added at a cell density of 1×10^7 epimastigotes/mL (time = 50 h); 72 h after the addition of the drugs (time = 152 h) a marked reduction in the growth rate was observed, probably due to the reduction of endogenous sterol and isoprenoid content. The sterol analyses were carried out 120 h after the addition of the drugs (time = 168 h). In Table 3 it can be seen that **2** alone causes a marked reduction in the level of endogenous sterols, which drop to less than one-third of total sterols. Treatment with ketoconazole leads, as expected, to the disappearance of the parasite's 4,14-desmethyl sterols and a concomitant accumulation of di- and trimethylated sterols, particularly 24-methylenedihydrolanosterol, which becomes the most abundant sterol in the cells. When the two drugs are used in combination, Table 3, there is again a large increase in the relative proportion of the exogenous sterol, cholesterol, showing that the blockage of endogenous sterol synthesis induced by **2** is at a pre-lanosterol level. Since there is also no accumulation of squalene detected (data not shown), these results suggest that **2** inhibition must be at the pre-squalene level, consistent with the idea that FPPS is a principal target.

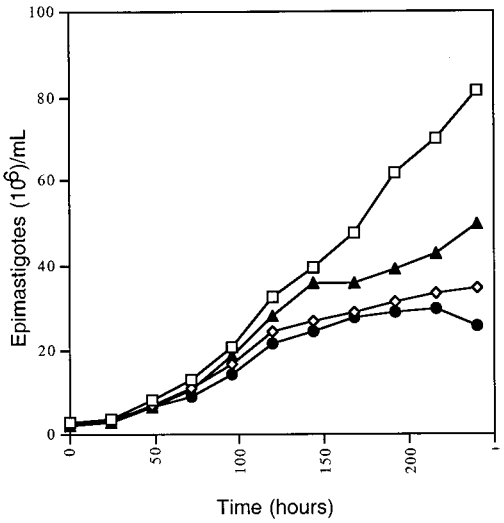


Figure 2. Effects of risedronate (**2**) and ketoconazole on *T. cruzi* epimastigote proliferation: □, control; ◇, risedronate (100 μM); ▲, ketoconazole (0.3 μM); ●, risedronate (100 μM) plus ketoconazole (0.3 μM).

These results are clearly of interest since they represent the first demonstration of the effects of bisphosphonates on sterol biosynthesis in *T. cruzi*, and similar effects are seen with *L. mexicana* as well (data not shown). However, in both cases, the decrease in endogenous sterol production alone caused by **2** is insufficient to stop parasite growth.[22] On the basis of the correlations with FPPS inhibition activity of **2**, **9**, and **14** (in other systems), we therefore expect a significant contribution to parasite growth inhibition due to FPPS inhibition, resulting in decreases in protein prenylation and dolichol and ubiquinone formation, as well as the decreased sterol production.

We have also investigated the effects of several bisphosphonates on *T. cruzi* amastigote proliferation inside Vero cells, to assess the general toxicity of such compounds toward host cells. Vero cells, in contrast to irradiated myoblasts, proliferate under our experimental conditions. Results are shown in Table 4. As can be seen, in this system **2** and its higher homologue **6** at 150 μM are very potent inhibitors of replication of the intracellular amastigote forms of *T. cruzi* and have no effect on the host cells. The picolylaminomethylenebisphosphonate ethyl ester **13** was also significantly active, while compounds **11** and **12** were inactive against both amastigotes and host cells. All other compounds (including **1**, **2**, and **14**) were toxic toward the host cells at the same concentration, leading to detachment and lysis. Toxicity was clear at 24 h and cells were dead at 48 h. Further work with lower drug

Table 3. Effects of Risedronate and Ketoconazole on the Free Sterol Composition of *T. cruzi* Epimastigotes (EP stock)

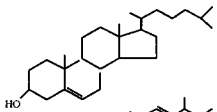
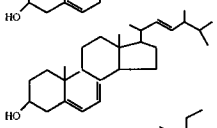
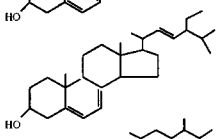
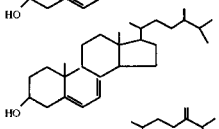
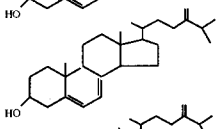
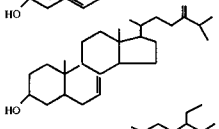
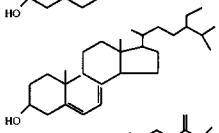
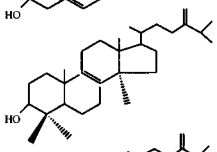
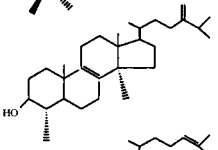
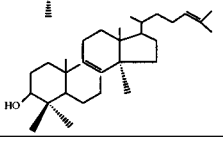
Sterol	Structure	Control	Risedronate (100μM)	Ketoconazole (0.3μM)	Risedronate (100μM) + Ketoconazole (0.3μM)
Exogenous: Cholesterol		44.1	67.3	28.3	71.5
Endogenous: 24-methyl-5,7,22-cholesta-triene-3-ol (Ergosterol)		22.5	13.5	2.2	<1
24-ethyl-5,7,22-cholesta-triene-3-ol		12.3	11.7	<1	<1
Ergosta-5,7-diene-3-ol		8.3	<1	<1	<1
Ergosta-5,7,24(24')-diene-3-ol		8.0	<1	<1	<1
Ergosta-7,24(24')-diene-3-ol		4.8	<1	<1	<1
24-ethyl-5,7- cholesta-diene-3-ol		<1	7.5	2.9	<1
Endogenous, 14-methyl: 24-methylene-dihydrolanosterol		<1	<1	39.6	28.5
4,14-dimethyl-ergosta-8,24,(24[1])- diene-3-ol (Obtusifoliol)		<1	<1	17.0	<1
Lanosterol		<1	<1	10.0	<1

Table 4. Effects of Bisphosphonates at 150 μM on the Proliferation of *T. cruzi* Amastigotes in Vero Cells

compd	% infected Vero cells	% inhibition of amastigote proliferation	Vero cell appearence
none	32 ± 4	0	normal
1			detached, lysed
2	0	100	normal
3			detached, lysed
5			detached, lysed
6	6 ± 2	81	normal
9			detached, lysed
11	30 ± 3	6	normal
12	33 ± 4	0	normal
13	9 ± 2	69	normal
14			detached, lysed
18			detached, lysed

levels is underway. Similarly, in the case of *L. donovani* intracellular amastigotes, **2** was once again found to have the lowest IC_{50} of any of the compounds investigated, with an IC_{50} = 2.3 μM, Table 2.

In addition to investigating the effects of bisphosphonates on these trypanosomatid parasites, we also investigated their effects on the proliferation of the apicomplexan parasites, *T. gondii* and *P. falciparum*. In the case of *T. gondii* intracellular tachyzoites, we found the lowest IC_{50} with **2**: IC_{50} = 490 nM, Table 2. In addition, significant activity was observed with **9**, IC_{50} = 9.1 μM, and the simple hydroxybutane bisphosphonate **4**, IC_{50} = 9.1 μM. The significant activity of **2** and its isomer **9** is not unexpected, based on the results found with these two nitrogen-containing bisphosphonates in the trypanosomatid parasites, Table 2. More surprising is the observation that **4** also has significant activity, even though it lacks the (aza)carbocation center found in the (typically) more potent nitrogen-containing bisphosphonates. This apparently anomalous effect is even more pronounced in the case of *P. falciparum* intraerythrocytic stages, where as shown in Table 2 the two most effective inhibitors of proliferation are the unsubstituted aromatic/aliphatic bisphosphonates **11** (IC_{50} = 7.7 μM) and **17** (IC_{50} = 5.1 μM), which have benzyl and pentyl substituents, respectively. In the case of the intraerythrocytic stages of *P. falciparum*, it seems

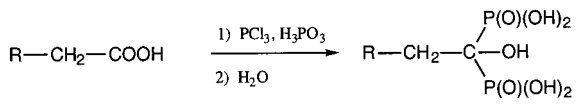
possible that drug delivery across the erythrocyte membrane may be less effective with the polar side chains found in the nitrogen-containing bisphosphonates. Further work with a homologous series of alkylbisphosphonates is underway.

Conclusions

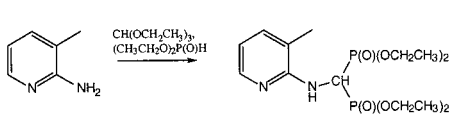
The results we have obtained above are of interest for a number of reasons. They represent the first detailed screening of a library of bisphosphonates against the major trypanosomatid and apicomplexan parasites: *T. brucei*, *T. cruzi*, *L. donovani*, *T. gondii*, and *P. falciparum*. The results show that nanomolar to low-micromolar IC_{50} values are found in all systems. In addition, our results indicate that risedronate, **2**, one of the most powerful nitrogen-containing bisphosphonates, inhibits sterol biosynthesis in *T. cruzi* at a presqualene level, most likely at the level of FPPS, based on similar activity profiles found in plants, mammals, and *D. discoideum*. Furthermore, on the basis of results with *T. cruzi* amastigotes in cultured Vero cells, risedronate, **2**, appears least cytotoxic to host cells but is highly effective against *T. cruzi* amastigote proliferation. Also, with *L. donovani*, risedronate, **2**, again appears to have a very low IC_{50} and little toxicity to the host cells. For the apicomplexan parasites *T. gondii* and *P. falciparum*, our results show that the non-nitrogen-containing bisphosphonates (**4**, **11**, **17**) have surprisingly high activity (~5−10 μM). In the case of *P. falciparum* these bisphosphonates, which contain simple aryl/alkyl groups, displayed a 10−20-fold lower IC_{50} than the nitrogen-containing bisphosphonates, indicating either a difference in uptake into the parasite cell and/or a different site of action. Since none of the compounds tested above were obtained as the product of any rational (parasite-directed) drug design program, but nonetheless exhibit IC_{50} values in the nanomolar to low-micromolar range, these results appear very promising from the perspective of designing more effective bisphosphonate chemotherapeutic agents based on compounds specifically active against parasite enzymes.

Experimental Section

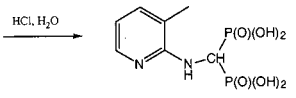
Synthetic Aspects. We prepared the 19 bisphosphonates shown in Table 1 using the Merck procedure for the 1-hydroxy-1,1-bisphosphonates (**1**−**12**, **15**−**19**):[23]



The aminomethylenebisphosphonates (**13**, **14**) were prepared by suitable modification of the procedures of Soloducho et al.:[24]



13



14

as described in detail below.

***N*-(2-(3-Picolyl))aminomethylenebisphosphonate Tetraethyl Ester (13) and *N*-(2-(3-Picolyl))aminomethylenebisphosphonate (14).** *N*-(2-(3-Picolyl))aminomethylenebisphosphonate **(14)** and its tetraethyl ester **13** were prepared by heating 0.05 mol of 2-amino-3-picoline to reflux with 0.10 mol of diethyl phosphite and 0.05 mol of triethyl orthoformate at 100 °C for 12 h.[24] The alcohol formed was distilled off and the remaining product was purified on a silica gel column (EtOAc/acetone, 1:1) yielding compound **13**. This compound was then hydrolyzed in 20 mL of 6 N HCl at 95 °C for 3 h yielding compound **14** as a crystalline product. The purity of all 19 samples prepared (Table 1) was verified by microchemical analysis (H/C/N/P) and via ^{13}C, ^{31}P, and ^{1}H NMR spectroscopy, the ^{1}H NMR experiments being performed in triplicate using an internal maleic acid quantitation standard. Absolute compound purity as determined from these experiments was 98.6%, on average. (See Supporting Information for analytical and spectroscopic data for target compounds.)

T. brucei. *T. brucei rhodesiense* (strain STIB900) bloodstream form trypomastigotes were maintained in HMI-18 medium supplemented with 20% HIFCS at 37 °C in a 5% CO_2−air mixture. *T. brucei rhodesiense* tests were performed in HMI-18 medium as above. Compounds were tested in triplicate. Parasites were diluted to 2×10^5/mL and added in equal volumes to the test compounds in 96-well, flat bottom Microtest III tissue culture plates (Becton Dickinson and Co., NJ) with pentamidine isethionate (Aventis, Frankfort) as the positive control, set up in parallel. Plates were maintained for 3 days at 37 °C in a 5% CO_2−air mixture. Compound activity was determined by the use of an Alamar Blue fluorometric assay[25] on day 3.

T. cruzi. γ-Irradiated L_6E_9 myoblasts (1×10^6 cells/well) in DMEM medium containing 20% fetal calf serum were plated in 12-well tissue culture plates (Corning Glass Works, Corning, NY) and incubated at 35 °C in a 7% CO_2 atmosphere for 24 h.[17] Cell monolayers were challenged with 5×10^7 trypomastigotes/well. After 2 h of incubation at 35 °C in a 7% CO_2 atmosphere, cultures were washed twice with Hanks solution and the culture medium was replaced, so that remaining extracellular trypomastigotes were removed. At this time, 1.0 μCi of [5,6-^{3}H]uracil/well (specific activity, 40−50 Ci/mmol; NEN Research Products, Boston, MA) and different concentrations of drugs were added, and cultures were incubated for an additional 72-h period. Incorporation of the [^{3}H]uracil into trichloroacetic acid (TCA)-precipitable material was measured at this time. The supernatants from the monolayers were removed by aspiration, the cells were washed twice with Hanks solution and dissolved with 1 mL of 1% sodium dodecyl sulfate containing 100 μg of cold uracil/mL. The contents of each well were then pipetted to dissolve the cell layer and then transferred to tubes. 3.0 mL of 5% TCA (vol/vol) solution was added to each tube. The resulting precipitates were maintained for 15 min on ice and collected on glass fiber filters (Whatman GF/B) using a sampling manifold (Millipore, Bedford, MA). The filters were washed twice with 3 mL of 5% TCA and once with 95% ethanol, dried and placed in 5 mL of scintillation cocktail (BudgetSolve, Research Products International, Mount Prospect, IL). Radioactivity was measured with a Packard (Model Tri-Carb 2100 TR) liquid scintillation system. To investigate the effects of antimicrobial agents on the replication of amastigotes, the drugs were added after the *T. cruzi* challenge. Compounds were tested in triplicate. The percent inhibition of [^{3}H]uracil incorporation (parasite growth) was calculated with the following formula: $(A - B/A) \times 100$, where A is the mean counts per minute of infected control-treated myoblasts and B is the mean counts per minute of infected drug-treated myoblasts. Subtraction of the minor labeling of the uninfected controls (typically around 300−400 cpm after 72-h incubation) yielded the incorporation that could be ascribed to the intracellular parasites (typically around 25 000 cpm after 70-h incubation).

Vero Cell Culture of *T. cruzi* Amastigotes. Amastigotes were cultured in Vero cells maintained in minimal essential medium supplemented with 2% fetal calf serum in a humidi-

fied 95% air−5% CO_2 atmosphere at 37 °C. Cells were infected with 10 tissue culture-derived trypomastigotes/cell for 2 h and then washed three times with phosphate-buffered saline (PBS) to remove nonadherent parasites. Fresh medium with or without drugs was added and the cells were incubated for 96 h with a change of medium at 48 h. Quantitation of the number of infected cells and the number of parasites per cell were determined by use of light microscopy. Statistical analysis of the results was carried out as described previously.[26]

Sterol Analyses. Sterols were extracted from *T. cruzi* epimastigotes cultured in LIT medium in the presence or absence of drugs for 120 h.[27] Drugs were added at a cell density of 1×10^7 epimastigotes/mL. Sterols were separated from polar lipids by silicic acid column chromatography and analyzed by quantitative capillary gas−liquid chromatography and mass spectrometry. Results are expressed as mass percent.

L. donovani. *L. donovani* (strain MHOM/ET/67/L82) was maintained in pathogen-free (SPF) female Golden hamsters (Wright's strain, Charles River Ltd.) by passage every 6−8 weeks.[28] For in vitro assays, *L. donovani* peritoneal macrophages were harvested from female CD1 mice (Charles River Ltd., Margate U.K.) by peritoneal lavage 24 h after induction by soluble starch (Merck Ltd., Leicester, U.K.). After two washes in medium the exudate cells were dispensed into 16-well Lab-tek tissue culture slides (Nunc Inc., IL) at 4×10^4/well in a volume of 200 mL of RPMI-1640 medium (Sigma-Aldrich Co. Ltd., Dorset, U.K.) plus 10% HIFCS. After 24 h, macrophages were infected at a ratio of 10:1 (4×10^5/well) with *L. donovani* amastigotes freshly isolated from hamster spleen. Infected macrophages were then maintained in the presence of drug in a 3-fold dilution series, with quadruplicate cultures at each concentration, for 5 days. After these periods of drug exposure slides were fixed by methanol and Giemsa stained. Drug activity was determined by counting the percentage of macrophages cleared of amastigote in treated cultures in comparison with untreated cultures. Sodium stibogluconate (GlaxoSmithKline) was used as a control.

T. gondii. For the proliferation assays with *T. gondii*, human foreskin fibroblast monolayers were grown in DMEM medium containing 10% fetal calf serum,[29] plated in 12-well tissue culture plates and incubated at 37 °C in a 5% CO_2 atmosphere for 3 days. Cell monolayers were challenged with 6×10^4 tachyzoites/well. After 4 h of incubation at 37 °C in a 5% CO_2 atmosphere, cultures were washed twice with Hanks solution and the culture medium replaced so that remaining extracellular tachyzoites were removed. Different concentrations of drugs were then added and the plates incubated for 48 h at 37 °C (assay performed in triplicate). At this time, 1.0 μCi of [5,6-^{3}H]uracil/well (specific activity, 40−50 Ci/mmol; New England Nuclear Research Products, Boston, MA) was added, and cultures were incubated for 4 h. Incorporation of [^{3}H]uracil into TCA-precipitable material was measured at this time. The cells were dissolved with 1 mL of 1% sodium dodecyl sulfate containing 100 μg unlabeled uracil/mL. The contents of each well were then pipetted to dissolve the cell layer and transferred to tubes. 3.0 mL of 5% TCA (vol/vol) was added to each tube. The resulting precipitates were maintained at 4 °C for 15 min and collected on glass fiber filters (Whatman GF/B) using a sampling manifold (Millipore, Bedford, MA). The filters were washed twice with 3 mL of 5% TCA and once with 95% ethanol, dried and placed in 5 mL of scintillation cocktail (BudgetSolve, Research Products International, Mount Prospect, IL). Radioactivity was measured with a Packard Tri-Carb 2100 TR liquid scintillation system.

P. falciparum. *P. falciparum* (strain 3D7) were maintained in human A^+ erythrocytes in RPMI1640 medium supplemented with Albumax II at 37 °C in a 5% CO_2−air mixture. *P. falciparum* intraerythrocytic cultures were set up as above, with 1% ring stage parasitemia, 2.5% hematocrit, in triplicate in 100 mL of medium in 96 well, flat-bottomed Microtest III tissue culture plates. Drugs were added in 3-fold dilution series and cultures incubated for a total of 48 h at 37 °C in a 5% CO_2−air mixture. After 24 h, [^{3}H]hypoxanthine (0.2 mCi) was added to each well. At the end of the assay, plates were rapidly freeze−thawed (3×), harvested onto a Unifilter 96-well plate (Canberra Packard, Meriden, CT), dried, sealed with 50 mL of MicroScint40 and read on a Packard Topcount. Drugs were tested against chloroquine-sensitive *P. falciparum* 3D7.

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Supporting Information Available: Analytical data for target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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