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Design, Synthesis and Biological Evaluation of Sulfur-Containing 1,1-Bisphosphonic Acids as Antiparasitic Agents

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Abstract

As part of our efforts aimed at searching for new antiparasitic agents, 2-alkylmercaptoethyl-1,1-bisphosphonate derivatives were synthesized and evaluated against *Trypanosoma cruzi*, the etiologic agent of Chagas disease, and *Toxoplasma gondii*, the responsible agent for toxoplasmosis. Many of these sulfur-containing bisphosphonates were potent inhibitors against the intracellular form of *T. cruzi*, the clinically more relevant replicative form of this parasite, and tachyzoites of *T. gondii* targeting *T. cruzi* or *T. gondii* farnesyl diphosphate synthases (FPPSs), which constitute valid targets for the chemotherapy of these parasitic diseases. Interestingly, long chain length sulfur-containing bisphosphonates emerged as relevant antiparasitic agents. Taking compounds **37**, **38**, and **39** as representative members of this class of drugs, they exhibited ED_{50} values of 15.8 μM, 12.8 μM, and 22.4 μM, respectively, against amastigotes of *T. cruzi*. These cellular activities matched the inhibition of the enzymatic activity of the target enzyme (*Tc*FPPS) having IC_{50} values of 6.4 μM, 1.7 μM, and 0.097 μM, respectively. In addition, these compounds were potent anti-*Toxoplasma* agents. They had ED_{50} values of 2.6 μM, 1.2 μM, and 1.8 μM, respectively, against *T. gondii* tachyzoites, while they exhibited a very potent inhibitory action against the target enzyme (*Tg*FPPS) showing IC_{50} values of 0.024 μM, 0.025 μM, and 0.021 μM, respectively. Bisphosphonates bearing a sulfoxide unit at C-3 were also potent anti-*Toxoplasma* agents, particularly those bearing long aliphatic chains such as **43**–**45**, which were also potent antiproliferative drugs against tachyzoites of *T. gondii*. These compounds inhibited the enzymatic activity of the target enzyme (*Tg*FPPS) at the very low nanomolar range. These bisphosphonic acids have very good prospective not only as lead drugs but also as potential chemotherapeutic agents.

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Supporting Information: Copies of the ^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra Time-dependent ^{1}H and ^{31}P NMR spectra of tetraethyl esters and free bisphosphonic acids under oxidizing conditions are included as supporting information.

Introduction

The isosteric replacement of the oxygen atom bridge of inorganic pyrophosphate (**1**) with substituted methylene groups gives rise to a class of drugs known as bisphosphonates (**2**) [1], which became compounds of pharmacological importance since calcification studies carried out many decades ago [2–4]-Several bisphosphonates such as, pamidronate (**3**), alendronate (**4**) and risedronate (**5**) are in clinical use for the treatment and prevention of osteoclast-mediated bone resorption associated with various bone disorders (Figure 1) [5–8].

Besides their use in long-term treatment of different bone disorders, bisphosphonates exhibit a wide range of biological activities, such as antibacterial agents [9], anticancer agents [10–13], as selective inhibitors of acid sphingomyelinase [14], in stimulation of γδ T cells [15], and, particularly, as antiparasitic agents [16–20]. Some years ago, selected bisphosphonates, comprising the FDA-approved pamidronate (**3**) and alendronate (**4**), were found to be potent inhibitors of *T. cruzi* proliferation in *in vitro* and *in vivo* assays without toxicity to the host cells [21]. Based on the previous findings, other bisphosphonates were found to be potent antiproliferative agents against other trypanosomatids such as *T. brucei rhodesiense*, *Leishmania donovani*, and *L. mexicana*, and Apicomplexans such as *Toxoplasma gondii* and *Plasmodium falciparum* [17–20].

Bone mineral has a similar mineral composition than acidocalcisomes, which are acidic organelles of high-density with a high concentration of phosphorus present as pyrophosphate and polyphosphate, which is associated to calcium and other cations. Then, it is reasonable to anticipate that accumulation of bisphosphonates in these organelles facilitates their antiparasitic action [22,23] FPPS catalyzes the two committed biosynthetic steps to form farnesyl diphosphate from dimethylallyl and isopentenyl diphosphates [20,21].

Bisphosphonates derived from fatty acids are promising antiparasitic agents, in particular, 2-alkyl(amino)ethyl derivatives. These compounds exhibit cellular activity against intracellular *T. cruzi*, which is one of the clinically relevant forms of this parasite, having IC_{50} values at the low nanomolar level against the target enzyme [24,25]. In addition, 1-hydroxy-, 1-alkyl-, and 1-amino-bisphosphonates such as **6**–**9** have been mainly useful in SAR studies as antiparasitic agents [26–29]. For example, bisphosphonate **6** is a potent growth inhibitor against *T. cruzi* (amastigotes) [26] and also against *T. gondii* (tachyzoites) [29,30], while **7** is effective against *P. falciparum* [30]. Compounds **10** and **12** have cellular activity against *T. gondii,* the latter one being unusually effective against the target enzyme (IC_{50} = 93 nM) [30,31]. In addition, in contrast to what would be expected, α-fluoro-1,1-bisphosphonates are devoid of activity against *T. cruzi* cells and *Tc*FPPS regardless of the chain length [31]. However, these compounds behave as extremely potent inhibitors of the enzymatic activity of *T. gondii* FPPS [31]. Actually, **13** and **14** possess IC_{50} values of 35 nM and 60 nM, respectively, towards *Tg*FPPS, that is, they are even more effective than risedronate (IC_{50} = 74 nM) used as positive control (Figure 2) [31]. The high selectivity observed by these drugs towards *Tg*FPPS versus *Tc*FPPS is not surprising bearing in mind that the amino acid sequences of these enzymes have less than 50% identity [20].

T. cruzi and *T. gondii* are the etiologic agents of American trypanosomiasis (Chagas disease) and toxoplasmosis, respectively, two major parasitic diseases according to the World Health Organization [20,21]. Chemotherapy for this two parasitic diseases, based on empirically discovered drugs, is still a challenge [23,32–34]. *T. cruzi* has a complex life cycle involving blood-sucking Reduviid insects and mammals [35]. This parasite has four main morphological forms and the amastigote form is the more relevant replicative form of the parasite [35]. This blood-sucking activity is the main way of dissemination of Chagas disease, while infection via the placenta or by blood transfusion is the mechanism

responsible where this disease is not endemic [36]. The opportunistic parasite *T. gondii* is able to infect humans (basically all warm-blooded mammals) by contact with feces of infected cats, by eating undercooked meat or *via* the placenta from pregnant women [37,38]. Two asexual forms are able to affect humans: the tachyzoite form can invade cells and multiplies leading to host cell death, while the bradyzoite form proliferates slowly and forms cysts in muscle [39]. The main goal in toxoplasmosis is to develop a drug that is able to eliminate the cyst stage of the parasite to avoid recrudescence of the disease [20].

Rationale

In the last years, many efforts have been made to understand how bisphosphonic acids inhibit FPPS at the molecular level [40–42]. Recently, we were able to determine that *Tc*FPPS inhibitors **10** and **11** bind to the allylic site of the enzyme [43] with the phosphates group of the bisphosphonate moiety coordinating three Mg^{2+} atoms that bridge the compound to the enzyme in a similar way that was observed for the physiological substrates [44,45]. The nitrogen atom at the C-3 position is very important to maintain a high degree of biological activity.

Analyses of the 2-alkylaminoethyl-1,1-bisphosphonates–*Tc*FPPS complexes have indicated that methyl substitution at the *N*-linked carbon of the alkyl chain would be favorable for binding [43]. Then, **18** was envisioned for this purpose (Scheme 1). In addition, in order to study a potential synergistic effect, it was considered to add a hydroxyl group at C-1, present in many pharmacological important bisphosphonic acids, in the reference structure **11** to afford the 2-alkylaminoethyl-1-hydroxy-1,1-bisphosphonic acid **21**.

To assess the necessity of the amine group for inhibitory activity against *T. cruzi* or *T. gondii*, as well as their corresponding target enzymes *Tc*FPPS and *Tg*FPPS, we decided to replace it for a sulfide, sulfoxide, sulfone and methylalkylsulfonium group.

Results and Discussion

Preparation of the methyl analogue of the lead structure **10** (compound **18**) was conducted according to previously published procedures [24,25]. Briefly, the versatile Michael Acceptor **16** [46–48], which was straightforwardly obtained from commercially available tetraethyl methylenebis(phosphonate) (**15**), was reacted with 2-heptylamine in methylene chloride to afford the Michael adduct **17**. This compound was hydrolyzed by treatment with bromotrimethylsilane in methylene chloride followed by digestion with methanol [49] to afford the free bisphosphonic acid **18**. Additionally, the 1-[(*n*-alkylamino)ethyl]-1-hydroxy-1,1-bisphosphonic acid derivative **21** was readily prepared from *n*-heptylamine. Coupling reaction between this compound and benzyl bromoacetate in acetonitrile [50] afforded the expected benzyl *n*-alkylaminoacetate **19** in 84% yield, which was hydrogenated employing palladium on charcoal as catalyst to yield the free acid **20** in 67% yield, which was the substrate to form the title compound **21**. Then, on treatment with phosphorous acid and phosphorous trichloride employing benzenesulfonic acid as a solvent at 65 °C followed by hydrolysis, **20** was converted into **21** according to the widely employed method for the preparation of 1-hydroxy-1,1-bisphosphonic acids [51].

Based on the ability of mercaptane derivatives to undergo 1,4-conjugate Michael-type addition reactions on a number of α,β-unsaturated carbonyl compounds [52–55], the synthetic precursors of the sulfur-containing 1,1-bisphosphonic acids (**22**–**30**) were successfully prepared *via* this 1,4-conjugated addition among commercial *n*-alkyl mercaptanes and the acceptor **16**, in the presence of triethylamine. Reaction yields ranged 68–94%. Hydrolysis of these tetraethyl intermediates by treatment with

bromotrimethylsilane in methylene chloride followed by digestion with methanol afforded the title compounds **31**–**39** in good yields (Scheme 2).

In order to obtain the sulfoxide derivatives of these compounds, it was first considered starting from the tetraethyl esters **22**–**30**. The controlled oxidation reaction of the corresponding thioethers is the most widely employed method of preparation of sulfoxides [56]. However, contrarily to what had been depicted in closely related compounds [57], in our hands all the attempts to oxidize any of the sulfides **22**–**30** by using sodium metaperiodate [58–60], hydrogen peroxide [61,62], or *m*-chloroperoxybenzoic acid [63] underwent a retro-Michael reaction. These results are in agreement with published data where alkylsulfides bonded at the β-position of aldehydes and ketones experienced a retro-Michael reaction when treated with an oxidizing agent affording the α,β-unsaturated carbonyl compounds and the corresponding alkylsulfanol [64–66].

Oxidation reaction on the free bisphosphonic acids **33**–**39** would not undergo retro-Michael addition. In order to test this hypothesis, the reaction of **34** with hydrogen peroxide was monitored by ^{1}H and ^{31}P NMR spectroscopy. On treatment with hydrogen peroxide (one equivalent) compound **34** was converted rapidly into sulfoxide **41**. No overoxidation was observed. Addition of a second equivalent of hydrogen peroxide gave rise to sulfone **47**. Therefore, sulfides **33**–**39** were transformed into sulfoxides **40**–**46** as illustrated in Scheme 2.

The methyl(octyl)sulfonium derivative **48** was succesfully prepared by treatment of the respective free bisphosphonic acid **37** bearing a sulfide moeity at C-3, with methyl iodide and silver tetrafluoroborate [67] in acetronitrile affording **48** in very good yield. Attempts to methylate **28** failed due to a retro-Michael type reaction occurred instead of formation of the expected sulfonium derivative as illustrated in Scheme 3.

Biological evaluation of the title compounds **18**, **21**, **31**–**48** resulted to be very interesting. Both nitrogen-containing bisphosphonic acids **18** and **21** were almost devoid of antiparasitic activity against the amastigote form of *T. cruzi* and also towards the target enzyme *Tc*FPPS. Compound **18** exhibited moderate potency against tachyzoites of *T. gondii* having an EC_{50} value of 11.4 μM. In addition, sulfur-containing bisphosphonic acids proved to be very potent antiparasitic agents. Certainly, alkylthioethyl derivatives with relatively long aliphatic chains were very effective against either *T. cruzi* or *T. gondii*, with ED_{50} values of 15.8 μM (**37**), 12.8 μM (**38**), and 22.4 μM (**39**) against amastigotes forms of the former. These activities are in accordance with the strong inhibition of *Tc*FPPS observed for these analogues (IC_{50} values of 6.4 μM, 1.7 μM, and 0.097μM, respectively). Besides their action against *T. cruzi,* these compounds were potent anti-*Toxoplasma* agents. In fact, they had ED_{50} values of 2.6 μM, 1.2 μM, and 1.8 μM, respectively against tachyzoites of *T. gondii*, while they exhibited a very potent inhibitory action against the target enzyme (*Tg*FPPS) showing IC_{50} values of 0.024 μM, 0.025 μM, and 0.021 μM, respectively. With the exception of **37**, all these compounds had equivalent potency to risedronate (ED_{50} = 2.4 μM against *T. gondii* and IC_{50} = 0.074 μM against *Tg*FPPS) used as positive control. Compound **36** maintained the anti-*Toxoplasma* activity exhibited by **37**–**39** (ED_{50} = 0.97 μM against tachyzoites of *T. gondii*; ED_{50} = 0.069 μM against *Tg*FPPS), but was devoid of anti-*T. cruzi* activity against either cells or *Tc*FPPS. On the other hand, short chain length derivatives **31**–**35** exhibited some antiparasitic activity but to a lesser extent than **36**–**39**.

Bisphosphonic acid derivatives bearing a sulfoxide moeity at the C-3 position were also potent anti-*Toxoplasma* agents, particularly those possessing long aliphatic chains such as **43**–**45**, which were potent antiproliferative drugs against tachyzoites of *T. gondii*. They had EC_{50} values of 5.0 μM, 3.0 μM, and 1.4 μM, respectively. **45** showed a similar efficacy

than risedronate under the same assay conditions. Above all, these compounds efficiently inhibited the enzymatic activity of the target enzyme (*Tg*FPPS) at the very low nanomolar range showing IC_{50} values of 0.009 μM, 0.016 μM, and 0.056 μM, respectively. Except sulfoxide **45**, which had moderate potency against intracellular *T. cruzi* (ED_{50} = 19.4 μM), the rest of lineal synthetic sulfoxides were devoid of biological activity against both *T. cruzi* cells and *Tc*FPPS. The lack of biological activity against *T. cruzi* was somewhat unusual taking into account the inhibitory action shown by lineal closely related bisphosphonic acids.[24–31] Sulfone **47** was just active against intracellular *T. cruzi*, while methylsulfonium **48** has proven to be an interesting antiparasitc agent exhibiting moderate antiproliferative action against both *T. cruzi* and *T. gondii* cells (ED_{50} = 32.0 μM and 7.0 μM, respectively, but behaved as a very potent drug against the target enzymes *Tc*FPPS and *Tg*FPPS (IC_{50} = 0.040 μM and 0.013 μM, respectively). Taking into account that the inhibitory activity does not always match with the ED_{50} values, it is necessary to consider that for cellular activity a compound has to cross the mammalian cell membrane, then, the parasite cell membrane, and finally has to reach the enzyme located in an organelle. At the concentrations used, no toxicity was associated to the title compounds. Assays were done with tissue culture cells infected with *T. cruzi* and toxicity on tissue culture cells could be seen easily if it happened (cells detach or show signs of necrosis). This was not observed and therefore the compounds have low or no toxicity against Vero cells. In addition, cytotoxicity studies (AlamarBlue™) of the most potent anti-*Toxoplasma* agents such as **36–39**, **45** against hTerT cells, used as hosts of *T. gondii* tachyzoites, indicated that these compounds were almost devoid of toxicity. In fact, all of them showed high selectivity. Biological data are shown in Table 1.

In summary, lineal sulfur-containing-1,1-bisphosphonic acids seem to be promising antiparasitic drugs that were able to inhibit efficiently the enzymatic activity of *T. gondii* farnesyl diphosphate synthase as well as *T. gondii* cells and to a lesser extent against TcFPPS and intracellular *T. cruzi* cells. This effect was more noticeable in compounds having a relative long aliphatic chain. Particularly, **38** and **39**, which contain twelve and thirteen atoms in their lineal aliphatic chains, including one sulfur atom at C-3, exhibited potent activity against the target enzymes *T. gondii* and *T. cruzi* FPPS. As a consequence of these enzymatic activities, these bisphosphonic acid derivatives had the ability to control *T. gondii* (tachyzoites) and *T. cruzi* (amastigotes) proliferation. Sulfoxide derivatives were more selective towards *Tg*FPPS and *T. gondii* cells as it was the case of **45**. Compound **43** was an extremely potent inhibitor towards *Tg*FPPS having an IC_{50} as low as 9 nM. Among the designed sulfur-containing 1,1-bisphosphonic acids, the methylsulfonium **48** was the most potent inhibitor of the enzymatic activity of the *T. cruzi* enzyme and also very efficient towards *Tg*FPPS, both at the low nanomolar range. In order to optimize this new family of bisphosphonic acids, structural modifications at the aliphatic chain including branching and conformational restriction tools will be considered as the next step. Work aimed at exploiting the potential value of these sulfur-containing bisphosphonic acids is currently being pursued in our laboratory.

Experimental Section

The glassware used in air and/or moisture sensitive reactions was flame-dried and carried out under a dry argon atmosphere. Unless otherwise noted, chemicals were commercially available and used without further purification. Solvents were distilled before use. Methylene chloride and acetonitrile were distilled from phosphorus pentoxide.

Nuclear magnetic resonance spectra were recorded using a Bruker AM-500 MHz spectrometer. Chemical shifts are reported in parts per million (δ) relative to tetramethylsilane. Coupling constants are reported in Hertz. ^{13}C NMR spectra were fully decoupled. Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet.

High-resolution mass spectra were obtained using a Bruker micrOTOF-Q II spectrometer, which is a hybrid quadrupole time of flight mass spectrometer with MS/MS capability.

Melting points were determined using a Fisher-Johns apparatus and are uncorrected. IR spectra were recorded using a Nicolet Magna 550 spectrometer.

Column chromatography was performed with E. Merck silica gel plates (Kieselgel 60, 230–400 mesh). Analytical thin layer chromatography was performed employing 0.2 mm coated commercial silica gel plates (E. Merck, DC-Aluminum sheets, Kieselgel 60 F_{254}).

As judged from the homogeneity of the ^{1}H, ^{13}C, ^{31}P NMR spectra of the title compounds **31**–**48** and HPLC analyses of the committed intermediates **22**–**30** employing a Beckmann Ultrasphere ODS-2 column 5 μM, 250 × 10 mm eluting with acetonitrile–water (1:1) at 3.00 mL/min with a refractive index detector indicated a purity >97%.

1-[(*n*-Hept-2-ylamino)ethyl] 1,1-bisphosphonic Acid (18)

A solution of compound **16** (300 mg, 1.0 mmol) in anhydrous methylene chloride (10 mL) was treated with the 2-heptylamine (115 mg, 1.1 mmol) under an argon atmosphere. The reaction mixture was stirred at room temperature overnight. The solvent was evaporated and the residue was purified by column chromatography (silica gel) employing hexane–EtOAc (17:3) as eluent to afford tetraethyl ester **17**. To a solution of this product in anhydrous methylene chloride (10 mL) was added dropwise trimethylsilyl bromide (12 equivalents) in an argon atmosphere. The reaction mixture was stirred at room temperature for 48 h. After cooling at 0 °C, anhydrous methanol (10 mL) was added, and the resulting mixture was allowed to reach room temperature. The solution was then concentrated under reduced pressure. The residue was dissolved in anhydrous methanol (10 mL) and subsequently concentrated under reduced pressure twice. The solvent was evaporated and the residue was crystallized from ethanol–water to afford pure **18** as a white solid: mp 165–166 °C; ^{1}H NMR (500.13 MHz, D_2O) δ 0.78 (t, J = 7.1 Hz, 3H, H-9), 1.21 (m, 4H, H-7, H-8), 1.22 (d, J = 6.6 Hz, 3H, CH_3 at C-4), 1.30 (m, 2H, H-6), 1.50 (m, 1H, H-5_a), 1.62 (m, 1H, H-5_b), 2.35 (tt, J = 21.4, 7.3 Hz, 1H, H-1), 3.25 (dt, J = 13.2, 6.6 Hz, 2H, H-2), 3.41 (m, 2H, H-4); ^{13}C NMR (125.77 MHz, D_2O) δ 13.2 (C-9), 15.5 (CH$_3$ at C-4), 21.7 (C-8), 24.0 (C-7), 30.6 (C-6), 32.7 (C-5), 36.1 (t, J = 120.8 Hz, C-1), 42.1 (C-2), 54.7 (C-4); ^{31}P NMR (D_2O) δ 16.01 m_{AB}. HRMS (ESI) calcd for $C_9H_{24}O_6NP_2$ $[M+H]^+$ 304.1079; found: 304.1062. Anal. Calcd. for ($C_9H_{23}O_6NP_2$.1.25H_2O): C, 33.18; H, 7.89; N, 4.30. Found C, 33.13; H, 7.27; N, 3.98.

1-[(*n*-Heptylamino)ethyl]-1-hydroxy-1,1-bisphosphonic Acid (21)

To a solution of heptylamine (1.00 g, 8.7 mmol) in acetonitrile (15 mL) cooled at 0 °C was added dropwise benzyl bromoacetate (1.99 g, 8.9 mmol). Then, triethylamine (2.4 mL, 17.3 mmol) and the reaction mixture was stirred overnight. The solvent was evaporated and the residue was purified by column chromatography (sílica gel) eluting with a mixture of hexane–EtOAc (19:1) to afford 1.919 g (84% yield) of pure **19** as a colorless oil. A solution of benzyl ester **19** (1.919 g, 7.2 mmol) in ethyl acetate (50 mL) in the presence of palladium on charcoal (50 mg) was treated with hydrogen at 3 atm in a Parr apparatus. The reaction mixture was shaken for 6 h and the mixture was filtered through a fritted glass funnel. The solvent was evaporated to give 847 mg (67% yield) of pure **20** as a white solid that was used in the next step without further purification: mp = 187–191 °C; ^{1}H NMR (500.13 MHz, CD_3OD) δ 0.91 (t, J = 7.0 Hz, 3H, H-10), 1.32 (m, 4H, H-8, H-9), 1.37 (m, 4H, H-6, H-7), 1.67 (p, J = 7.5 Hz, 2H, H-5), 2.97 (m, 2H, H-4); 3.46 (s, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 13.6 (C-10), 22.2 (C-9), 28.5 (C-8), 28.7 (C-7), 29.4 (C-6), 31.3 (C-5), 47.4 (C-4), 49.2 (C-2), 169.5 (C-1). Anal. Calcd. for ($C_9H_{19}O_2N$): C, 62.39; H, 11.05; N, 8.08. Found

C, 62.05; H, 10.62; N, 7.74. To a flame dried 100 mL three neck flask having an addition funnel and a reflux condenser through which was circulated water at 0 °C was added the carboxylic acid **20** (500 mg, 2.9 mmol), H_3PO_3 acid (273 mg, 2.9 mmol), and anhydrous benzenesulfonic acid (1.0 g, 6.3 mmol) under argon atmosphere. The reaction mixture was heated to 65 °C, then PCl_3 (500 μL, 5.8 mmol) was added dropwise with vigorous stirring. The reaction was stirred at 65 °C for 16 h. The reaction was allowed to cool to room temperature. Cold water (60 mL) was added and the reaction was stirred at 100 °C for 5 h. The reaction was cooled to room temperature and the p*H* was adjust to 4.3 with a 50% aqueous NaOH solution. Acetone (20 mL) was added, and the resulting mixture was cooled to 0 °C for 24 h. The product was filtrated and crystallized with water-ethanol. mp 155–159 °C; ^{1}H NMR (500.13 MHz, $CDCl_3$) δ 0.90 (t, *J* = 7.0 Hz, 3H, H-10), 1.18 (m, 6H, H-7, H-8, H-9), 1.24 (m, 2H, H-6), 1.60 (p, *J* = 7.4 Hz, 2H, H-5), 3.01 (t, *J* = 7.6 Hz, 2H, H-4), 3.39 (t, *J* = 11.7 Hz, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 13.3 (C-10), 21.8 (C-9), 25.3 (C-8), 25.5 (C-7), 27.8 (C-6), 30.7 (C-5), 48.3 (C-2), 49.9 (C-4), 70.3 (t, *J* = 137.7 Hz, C-1); ^{31}P NMR (D_2O) δ 15.31. HRMS (ESI) calcd for $C_9H_{24}O_7NP_{20}$ $[M+H]^+$ 320.1030; found: 320.1037. Anal. Calcd. for ($C_9H_{23}O_7NP_2.1.50H_2O$): C, 31.22; H, 7.57; N, 4.05. Found C, 31.53; H, 7.75; N, 4.36.

Synthesis of tetraethyl 2-[(alkylthio)ethyl] 1,1-bisphosphonates

General procedure—To a solution of tetraethyl ethenylidenbisphosphonate (**16**; 300 mg, 1 mmol) in anhydrous dichloromethane (10 mL) was added triethylamine (1 mmol) and the corresponding alkylmercaptane (1 mmol). The reaction mixture was stirred at room temperature for 1h. Water (20 mL) was added, and the mixture was extracted with dichloromethane (3 × 10 mL). The combined organic layers were washed with brine (20 mL), dried on sodium sulfate and the solvent was evaporated.

Tetraethyl 1-[(Ethylthio)ethyl] 1,1-bisphosphonate (22)

98% yield; colorless oil; ^{1}H NMR (500.13 MHz, $CDCl_3$) δ 1.29 (t, *J* = 7.3 Hz, 3H, H-5), 1.37 (t, *J* = 7.0 Hz, 12H, H-2′), 2.61 (q, *J* = 7.3 Hz, 2H, H-4), 2.62 (tt, *J* = 23.9, 5.9 Hz, 1H, H-1), 3.07 (dt, *J* = 16.3, 5.7 Hz, 2H, H-2), 4.23 (m, 8H, H-2′); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ 14.5 (C-5), 16.4 (d, *J* = 6.8 Hz, C-2′), 26.9 (C-4), 27.2 (t, *J* = 4.9 Hz, C-2), 39.1 (t, *J* = 131.6 Hz, C-1), 62.8 (dd, *J* = 18.6, 6.8 Hz, C-1′); ^{31}P NMR (202.46 MHz, $CDCl_3$) δ 21.74. HRMS (ESI) calcd for $C_{12}H_{28}O_6P_2S$ $[M+H]^+$ 363.1160; found: 363.1163.

Tetraethyl 1-[(*n*-Prop-1-ylthio)ethyl] 1,1-bisphosphonate (23)

95% yield; colorless oil; ^{1}H NMR (500.13 MHz, $CDCl_3$) δ 0.99 (t, *J* = 7.3 Hz, 3H, H-6), 1.35 (t, *J* = 7.1 Hz, 12H, H-2′), 1.63 (sext, 2H, H-5), 2.55 (t, *J* = 7.3 Hz, 2H, H-4), 2.59 (tt, *J* = 24.0, 5.9 Hz, 1H, H-1), 3.04 (dt, *J* = 16.3, 5.9 Hz, 2H, H-2), 4.20 (m, 8H, H-2′); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ 13.4 (C-6), 16.3 (d, *J* = 5.9 Hz, C-2′), 22.7 (C-5), 27.7 (t, *J* = 5.4 Hz, C-2), 35.1 (C-4), 39.1 (t, *J* = 131.1 Hz, C-1), 62.8 (dd, *J* = 18.6, 6.8 Hz, C-1′); ^{31}P NMR (202.46 MHz, $CDCl_3$) δ 21.75. HRMS (ESI) calcd for $C_{13}H_{30}O_6P_2S$ $[M+H]^+$ 377.1317; found: 377.1326.

Tetraethyl 1-[(*n*-But-1-ylthio)ethyl] 1,1-bisphosphonate (24)

88% yield; colorless oil; ^{1}H NMR (500.13 MHz, $CDCl_3$) δ 0.91 (t, *J* = 7.4 Hz, 3H, C-7), 1.35 (t, *J* = 7.1 Hz, 12H, H-2′), 1.40 (sext, *J* = 7.4 Hz, 2H, H-6), 1.58 (p, *J* = 7.4 Hz, 2H, H-5), 2.57 (t, *J* = 7.5 Hz, 2H, H-4), 2.59 (tt, *J* = 24.0, 6.0 Hz, 1H, H-1), 3.04 (dt, *J* = 16.3, 5.8 Hz, 2H, H-2), 4.20 (m, 8H, H-2′); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ 13.6 (C-7), 16.4 (d, *J* = 6.6 Hz, C-2′), 21.9 (C-6), 27.7 (t, *J* = 4.9 Hz, C-2), 31.5 (C-5), 32.8 (C-4), 39.1 (t, *J* = 131.6 Hz, C-1), 62.8 (dd, *J* = 18.6, 6.8 Hz, C-1′); ^{31}P NMR (202.46 MHz, $CDCl_3$) δ 21.8. HRMS (ESI) calcd for $C_{14}H_{32}O_6P_2S$ $[M+H]^+$ 391.1473; found: 391.1476.

Tetraethyl 1-[(*n*-Pent-1-ylthio)ethyl] 1,1-bisphosphonate (25)

72% yield; colorless oil; ^{1}H NMR (500.13 MHz, $CDCl_3$) δ 0.90 (t, *J* = 7.1 Hz, 3H, H-8), 1.32 (m, 4H, H-6, H-7), 1.35 (t, *J* = 7.1 Hz, 12H, H-2′), 1.60 (p, *J* = 7.4 Hz, 2H, H-5), 2.56 (t, *J* = 7.4 Hz, 2H, H-4), 2.59 (tt, *J* = 23.7, 5.6 Hz, 1H, H-1), 3.04 (dt, *J* = 16.3, 5.9 Hz, 2H, H-2), 4.20 (m, 8H, H-2′); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ 13.9 (C-8), 16.4 (d, *J* = 5.9 Hz, C-2′), 22.3 (C-7), 27.7 (t, *J* = 5.0 Hz, C-2), 29.1 (C-6), 31.0 (C-5), 33.1 (C-4), 39.1 (t, *J* = 131.2 Hz, C-1), 62.8 (dd, *J* = 18.7, 6.8 Hz, C-1′); ^{31}P NMR (202.46 MHz, $CDCl_3$) δ 21.77. HRMS (ESI) calcd for $C_{15}H_{34}O_6P_2S$ $[M+Na]^+$ 427.1449; found: 427.1457.

Tetraethyl 1-[(*n*-Hex-1-ylthio)ethyl] 1,1-bisphosphonate (26)

94% yield; colorless oil; ^{1}H NMR (500.13 MHz, $CDCl_3$) δ 0.88 (t, *J* = 7.0 Hz, 3H, H-9), 1.29 (m, 6H, H-6, H-7, H-8), 1.35 (t, *J* = 7.0 Hz, 12H, H-2′), 1.59 (p, *J* = 7.4 Hz, 2H, H-5), 2.56 (t, *J* = 7.4 Hz, 2H, H-4), 2.59 (tt, *J* = 24.0, 6.0 Hz, 1H, H-1), 3.04 (dt, *J* = 16.3, 5.8 Hz, 2H, H-2), 4.20 (m, 8H, H-2′); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ 14.0 (C-9), 16.4 (d, *J* = 5.9 Hz, C-2′), 22.5 (C-8), 27.8 (t, *J* = 4.9 Hz, C-2), 28.5 (C-7), 29.4 (C-6), 31.4 (C-5), 33.2 (C-4), 39.1 (t, *J* = 131.4 Hz, C-1), 62.8 (dd, *J* = 18.8, 6.7 Hz, C-1′); ^{31}P NMR (202.46 MHz, $CDCl_3$) δ 21.77. HRMS (ESI) calcd for $C_{16}H_{37}O_6P_2S$ $[M+H]^+$ 419.1786; found 419.1792.

Tetraethyl 1-[(*n*-Hept-1-ylthio)ethyl] 1,1-bisphosphonate (27)

87% yield; colorless oil; ^{1}H NMR (500.13 MHz, $CDCl_3$) δ 0.88 (t, *J* = 7.0 Hz, 3H, H-10), 1.29 (m, 8H, H-6, H-7, H-8, H-9), 1.35 (t, *J* = 7.1 Hz, 12H, H-2′), 1.59 (p, *J* = 7.4 Hz, 2H, H-5), 2.56 (t, *J* = 7.5 Hz, 2H, H-4), 2.59 (tt, *J* = 24.2, 6.0 Hz, 1H, H-1), 3.04 (dt, *J* = 16.2, 5.6 Hz, 2H, H-2), 4.21 (m, 8H, H-2′); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ 14.1 (C-10), 16.4 (d, *J* = 5.9 Hz, C-2′), 22.6 (C-9), 27.8 (t, *J* = 4.9 Hz, C-2), 28.8 (C-8), 28.9 (C-7), 29.4 (C-6), 31.7 (C-5), 33.2 (C-4), 39.1 (t, *J* = 133.0 Hz, C-1), 62.8 (dd, *J* = 19.4, 6.3 Hz, C-1′); ^{31}P NMR (202.46 MHz, $CDCl_3$) δ 21.77. HRMS (ESI) calcd for $C_{17}H_{39}O_6P_2S$ $[M+H]^+$ 433.1943; found 433.1952.

Tetraethyl 1-[(*n*-Oct-1-ylthio)ethyl] 1,1-bisphosphonate (284)

83% yield; colorless oil; ^{1}H NMR (500.13 MHz, $CDCl_3$) δ 0.88 (t, *J* = 7.0 Hz, 3H, H-11), 1.28 (m, 10H, H-6, H-7, H-8, H-9, H-10), 1.35 (t, *J* = 7.1 Hz, 12H, H-2′), 1.59 (p, *J* = 7.4 Hz, 2H, H-5), 2.56 (t, *J* = 7.5 Hz, 2H, H-4), 2.59 (tt, *J* = 24.0, 6.0 Hz, 1H, H-1), 3.04 (dt, *J* = 16.2, 5.9 Hz, 2H, H-2), 4.21 (m, 8H, H-2′); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ 14.1 (C-11), 16.4 (d, *J* = 5.9 Hz, C-2′), 22.6 (C-10), 27.8 (t, *J* = 4.9 Hz, C-2), 28.9 (C-9), 29.2 (C-7, C-8), 29.4 (C-6), 31.8 (C-5), 33.2 (C-4), 39.1 (t, *J* = 130.1 Hz, C-1), 62.8 (dd, *J* = 19.0, 6.7 Hz, C-1′); ^{31}P NMR (202.46 MHz, $CDCl_3$) δ 21.77. HRMS (ESI) calcd. for $C_{18}H_{41}O_6P_2S$ $[M+H]^+$ 447.2099; found 447.2107.

Tetraethyl 1-[(*n*-Non-1-ylthio)ethyl] 1,1-bisphosphonate (29)

90% yield; colorless oil; ^{1}H NMR (500.13 MHz, $CDCl_3$) δ 0.89 (t, *J* = 7.0 Hz, 3H, H-12), 1.26 (m, 12H, -C*H*$_2$-), 1.35 (t, *J* = 7.1 Hz, 12H, H-2′), 1.59 (p, *J* = 7.4 Hz, 2H, H-5), 2.55 (t, *J* = 7.4 Hz, 2H, H-4), 2.59 (tt, *J* = 23.9, 5.8 Hz, 1H, H-1), 3.04 (dt, *J* = 16.3, 5.8 Hz, 2H, H-2), 4.20 (m, 8H, H-2′); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ 14.1 (C-12), 16.4 (d, *J* = 5.9 Hz, C-2′), 22.7 (C-11), 27.8 (t, *J* = 4.9 Hz, C-2), 28.9 (C-10), 29.2 (C-8, C-9), 29.4 (C-7), 29.5 (C-6), 31.8 (C-5), 33.2 (C-4), 39.1 (t, *J* = 131.1 Hz, C-1), 62.8 (dd, *J* = 18.6, 6.8 Hz, C-1′); ^{31}P NMR (202.46 MHz, $CDCl_3$) δ 21.77. HRMS (ESI) calcd for $C_{19}H_{43}O_6P_2S$ $[M+H]^+$ 461.2256; found 461.2256.

Tetraethyl 1-[(*n*-Dec-1-ylthio)ethyl] 1,1-bisphosphonate (30)

68% yield; colorless oil; ^{1}H NMR (500.13 MHz, $CDCl_3$) δ 0.88 (t, *J* = 7.0 Hz, 3H, H-13), 1.26 (m, 14H, -C*H*$_2$-), 1.35 (t, *J* = 7.1 Hz, 12H, H-2′), 1.59 (p, *J* = 7.5 Hz, 2H, H-5), 2.56 (t,

J = 7.3 Hz, 2H, H-4), 2.59 (tt, *J* = 23.9, 5.8 Hz, 1H, H-1), 3.04 (dt, *J* = 16.3, 5.8 Hz, 2H, H-2), 4.21 (m, 8H, H-2′); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ 14.1 (C-13), 16.4 (d, *J* = 5.9 Hz, C-2′), 22.7 (C-12), 27.8 (t, *J* = 4.9 Hz, C-2), 28.9 (C-11), 29.2 (C-10), 29.3 C-9), 29.4 (C-8), 29.53 (C-7), 29.54 (C-6), 31.9 (C-5), 33.2 (C-4), 39.1 (t, *J* = 131.1 Hz, C-1), 62.8 (dd, *J* = 18.6, 6.8 Hz, C-1′); ^{31}P NMR (202.46 MHz, $CDCl_3$) δ 21.78. HRMS (ESI) calcd for $C_{20}H_{45}O_6P_2S$ $[M+H]^+$ 475.2412; found 475.2412.

Synthesis of 2-(alkylthio)ethyl-1,1-bisphosphonic acids (31–39)

General procedure—A solution of the corresponding tetraethyl 2-[(alkylthio)ethyl] 1,1-biphosphonate (1 mmol) in anhydrous methylene chloride (10 mL) was treated with trimethylsilyl bromide (10 equiv.) under an argon atmosphere. The reaction mixture was stirred at room temperature for 48 h. Then, methanol (1.0 mL) was added and the solvent was evaporated. The residue was dissolved in methanol (8 mL) and the mixture was stirred at room temperature for 24 h. The solvent was evaporated and the residue redissolved/evaporated in methanol four times, to complete the hydrolysis of remaining trimethylsilyl bromide and eliminate the hydrobromic acid created. The residue was purified by column chromatography on reverse phase with a mixture of water–methanol as eluent and the pure compound was obtained after lyophilization. Yields are reported relatively to compound **16**.

1-[(Ethylthio)ethyl]-1,1-bisphosphonic Acid (31)

10% yield; Amorphous solid; ^{1}H NMR (500.13 MHz, D_2O) δ 1.17 (t, *J* = 7.4 Hz, 3H, H-5), 2.32 (tt, *J* = 22.3, 6.6 Hz, 1H, H-1), 2.55 (q, *J* = 7.4 Hz, 2H, H-4), 2.95 (dt, *J* = 15.6, 6.9 Hz, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 13.7 (C-5), 25.9 (C-4), 26.8 (t, *J* = 3.9 Hz, C-2), 39.3 (t, *J* = 120.3 Hz, C-1), ^{31}P NMR (202.46 MHz, $CDCl_3$) δ 19.54. HRMS (ESI) calcd for $C_4H_{12}O_6P_2S$ $[M+Na]^+$ 272.9728; found 272.9728.

1-[(*n*-Propylthio)ethyl]-1,1-bisphosphonic Acid (32)

50% yield; Amorphous solid; ^{1}H NMR (500.13 MHz, D_2O) δ 0.87 (t, *J* = 7.3 Hz, 3H, H-6), 1.53 (sxt, *J* = 7.3 Hz, 2H, H-5), 2.42 (tt, *J* = 22.8, 6.5 Hz, 1H, H-1), 2.51 (t, *J* = 7.3 Hz, 2H, H-4), 2.94 (dt, *J* = 15.9, 6.5 Hz, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 12.7 (C-6), 22.0 (C-5), 27.1 (t, *J* = 4.5 Hz, C-2), 34.1 (C-4), 39.3 (t, *J* = 122.6 Hz, C-1); ^{31}P NMR (202.46 MHz, D_2O) δ 20.12. HRMS (ESI) calcd for $C_5H_{15}O_6P_2S$ $[M+H]^+$ 265.0065; found 265.0062.

1-[(*n*-Butylthio)ethyl]-1,1-bisphosphonic Acid (33)

Amorphous solid; ^{1}H NMR (500.13 MHz, D_2O) δ 0.81 (t, *J* = 7.4 Hz, 3H, H-7), 1.31 (sxt, *J* = 7.4 Hz, 2H, H-6), 1.51 (p, *J* = 7.4 Hz, 2H, H-5), 2.39 (tt, *J* = 22.6, 6.6 Hz, 1H, H-1), 2.55 (t, *J* = 7.4 Hz, 2H, H-4), 2.94 (dt, *J* = 15.8, 6.4 Hz, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 12.8 (C-7), 21.3 (C-6), 27.2 (t, *J* = 4.3 Hz, C-2), 30.6 (C-5), 31.7 (C-4), 39.3 (t, *J* = 121.0 Hz, C-1); ^{31}P NMR (202.46 MHz, D_2O) δ 19.97. HRMS. calcd for $C_6H_{16}O_6P_2S$ $[M+Na]^+$: 301.0041; found 301.0039.

1-[(*n*-Pentylthio)ethyl]-1,1-bisphosphonic Acid (34)

44% yield; Amorphous solid; ^{1}H NMR (500.13 MHz, D_2O) δ 0.79 (t, *J* = 7.3 Hz, 3H, H-8), 1.25 (m, 4H, -C*H*$_2$-), 1.53 (p, *J* = 7.3 Hz, 2H, H-5), 2.42 (tt, *J* = 22.8, 6.3 Hz, 1H, H-1), 2.54 (t, *J* = 7.6 Hz, 2H, H-4), 2.94 (dt, *J* = 15.8, 6.6 Hz, 2H, H-2); ^{13}C NMR (125.77 MHz, DMSO) δ 13.2 (C-8), 21.6 (C-7), 27.1 (t, *J* = 4.9 Hz, C-2), 28.2 (C-6), 30.3 (C-5), 32.0 (C-4), 39.3 (t, *J* = 121.8 Hz, C-1); ^{31}P NMR (202.46 MHz, D_2O) δ 20.12. HRMS (ESI) calcd for $C_7H_{18}O_6P_2SNa$ $[M+Na]^+$ 315.0197; found 315.0192.

1-[(*n*-Hexylthio)ethyl]-1,1-bisphosphonic Acid (35)

Purificartion by column chromatography (C-18 silica gel) eluting with methanol–water (9:1) afforded 129 mg of **35** (41% yield) as an amorphous solid: ^{1}H NMR (500.13 MHz, D_2O) δ 0.78 (t, *J* = 7.0 Hz, 3H, H-9), 1.21 (m, 4H, -C*H*$_2$-), 1.30 (p, *J* = 7.1 Hz, 2H, H-6), 1.53 (p, *J* = 7.4 Hz, 2H, H-5), 2.41 (tt, *J* = 22.8, 6.5 Hz, 1H, H-1), 2.54 (t, *J* = 7.4 Hz, 2H, H-4), 2.94 (dt, *J* = 15.8, 6.6 Hz, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 13.3 (C9), 21.9 (C-8), 27.3 (t, *J* = 4.1 Hz, C-2), 27.7 (C-7), 28.5 (C-6), 30.6 (C-5), 32.0 (C-4), 39.3 (t, *J* = 121.7 Hz, C-1); ^{31}P NMR (202.46 MHz, D_2O) δ 20.06. HRMS (ESI) calcd for $C_8H_{21}O_6P_2S$ $[M+H]^+$ 307.0534; found 307.0522.

1-[(*n*-Heptylthio)ethyl]-1,1-bisphosphonic Acid (36)

Purificartion by column chromatography (C-18 silica gel) eluting with methanol–water (7:3) afforded 83 mg of pure **36** as an amorphous solid: ^{1}H NMR (500.13 MHz, D_2O) δ 0.78 (t, *J* = 7.0 Hz, 3H, H-10), 1.20 (m, 6H, -C*H*$_2$-), 1.30 (p, *J* = 7.0 Hz, 2H, H-6), 1.54 (p, *J* = 7.4 Hz, 2H, H-5), 2.38 (m, 1H, H-1), 2.54 (t, *J* = 7.4 Hz, 2H, H-4), 2.94 (dt, *J* = 15.8, 6.5 Hz, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 13.4 (C-10), 22.0 (C-9), 27.2 (t, *J* = 3.9 Hz, C-2), 28.0 (C-7, C-8), 28.5 (C-6), 31.0 (C-5), 32.1 (C-4), 39.3 (t, *J* = 122.3 Hz, C-1); ^{31}P NMR (202.46 MHz, D_2O) δ 19.97. HRMS (ESI) calcd for $C_9H_{22}O_6P_2SNa$ $[M+Na]^+$ 343.0510; found 343.0591.

1-[(*n*-Octylthio)ethyl]-1,1-bisphosphonic Acid (37)

Purificartion by column chromatography (C-18 silica gel) eluting with methanol–water (7:3) afforded 65 mg of pure **37** as a syrup: ^{1}H NMR (500.13 MHz, D_2O) δ 0.78 (t, *J* = 7.0 Hz, 3H, H-11), 1.22 (m, 8H, -C*H*$_2$-), 1.31 (p, *J* = 6.9 Hz, 2H, H-6), 1.53 (p, *J* = 7.5 Hz, 2H, H-5), 2.36 (tt, *J* = 22.5, 6.6 Hz, 1H, H-1), 2.48 (2.55 (t, *J* = 7.4 Hz, 2H, H-4), 2.94 (dt, *J* = 15.7, 6.6 Hz, 2H, H-2); ^{13}C NMR (125.77 MHz, DMSO) δ 13.4 (C-11), 22.0 (C-10), 27.3 (t, *J* = 3.9 Hz, C-2), 28.0 (C-9), 28.25 (C-8). 28.32 (C-7), 28.5 (C-6), 31.1 (C-5), 32.1 (C-4), 39.4 (t, *J* = 119.4 Hz, C-1); ^{31}P MR (202.46 MHz, D_2O) δ 19.80. HRMS (ESI) calcd for $C_{10}H_{24}O_6P_2SNa$ $[M+Na]^+$ 357.0667; found 357.0671.

1-[(*n*-Nonylthio)ethyl]-1,1-bisphosphonic Acid (38)

The product was purified by column chromatography (C-18 silica gel) eluting with methanol–water (1:1) to afford 66 mg (19% yield) of pure compound **38** as an amorphous solid: ^{1}H NMR (500.13 MHz, CD_3OD) δ 0.89 (t, *J* = 7.0 Hz, 3H, H-12), 1.30 (m, 10H, -C*H*$_2$-), 1.40 (p, *J* = 7.2 Hz, 2H, H-6), 1.60 (p, *J* = 7.4 Hz, 2H, H-5), 2.44 (tt, *J* = 23.2, 6.0 Hz, 1H, H-1), 2.58 (t, *J* = 7.4 Hz, 2H, H-4), 3.03 (dt, *J* = 16.2, 6.2 Hz, 2H, H-2); ^{13}C NMR (125.77 MHz, CD_3OD) δ 14.4 (C-12), 23.7 (C-11), 28.3 (t, *J* = 4.4 Hz, C-2), 29.9 (C-10), 30.4 (C-9), 30.4 (C-8), 30.6 (C-7), 30.7 (C-6), 33.1 (C-5), 33.6 (C-4), 41.5 (t, *J* = 125.7 Hz, C-1); ^{31}P NMR (202.46 MHz, D_2O) δ 20.48. HRMS (ESI) calcd for $C_{11}H_{27}O_6P_2S$ $[M+H]^+$ 349.1004; found 349.1010.

1-[(*n*-Decylthio)ethyl]-1,1-bisphosphonic Acid (39)

Amorphous solid; ^{1}H NMR (125.77 MHz, DMSO-d_6) δ 0.84 (t, *J* = 7.0 Hz, 3H, H-13), 1.23 (m, 12H, -C*H*$_2$-), 1.30 (p, *J* = 7.5 Hz, 2H, H-6), 1.49 (p, *J* = 7.3 Hz, 2H, H-5), 2.12 (tt, *J* = 22.5, 6.0 Hz, 1H, H-1), (t, *J* = 6.9 Hz, 2H, H-4), 2.85 (dt, *J* = 15.6, 6.1 Hz, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 13.9 (C-13), 22.7 (C-12), 27.5 (t, *J* = 3.9 Hz, C-2), 29.1 (C-10, C-11), 29.3 (C-8, C-9), 29.6 (C-7), 30.0 (C-6), 32.1 (C-5), 32.6 (C-4), 39.3 (t, *J* = 127.2 Hz, C-1); ^{31}P NMR (202.46 MHz, D_2O) δ 20.28. HRMS (ESI) calcd for $C_{12}H_{29}O_6P_2S$ $[M+H]^+$ 363.1160; found 363.1161.

Synthesis of 2-(alkylsulfinyl)ethyl-1,1-bisphosphonic acids (40–46)

General procedure—To a solution of the corresponding 2-(alkylthio)ethyl-1,1-biphosphonic acid (1 mmol) in deuterated water (2 mL) was added 30% hydrogen peroxide dropwise (1 mmol) and the mixture is stirred at room temperature. The reaction was monitored by proton NMR until the reaction was complete. The reaction mixture was freezed and lyophilized. The product was purified by column chromatography (reverse phase C-18 silica gel) eluting with water. Purity was determined not only by homogeneity (>95%) of the NMR data, but also by HPLC analysis eluting with a mixture of water methanol (4:1) employing a reversed phase column (250 × 10 mm).

1-[(*n*-Butylsulfinyl)ethyl]-1,1-bisphosphonic Acid (40)

Amorphous solid; ^{1}H NMR (500.13 MHz, D_2O) δ 0.85 (t, *J* = 7.4 Hz, 3H, H-7), 1.40 (m, 2H, H-6), 1.66 (p, *J* = 7.6 Hz, 2H, H-5), 2.59 (m, 1H, H-1), 2.79 (m, 1H, H-4$_a$), 2.93 (m, 1H, H-4$_b$), 3.18 (m, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 12.8 (C-7), 21.2 (C-6), 23.9 (C-5), 33.3 (t, *J* = 123.2 Hz, C-1), 48.4 (t, *J* = 3.9 Hz, C-2), 51.2 (C-4); ^{31}P NMR (202.46 MHz, D_2O) δ 17.81 m_{AB}. HRMS (ESI) calcd for $C_6H_{16}O_7P_2SNa$ $[M+Na]^+$ 316.9990; found 316.9985.

1-[(*n*-Pentylsulfinyl)ethyl]-1,1-bisphosphonic Acid (41)

Amorphous solid p; ^{1}H NMR (500.13 MHz, D_2O) δ 0.78 (t, *J* = 7.3 Hz, 3H, H-8), 1.24 (sxt, *J* = 7.2 Hz, 2H, H-7), 1.34 (m, 2H, H-6), 1.66 (m, 2H, H-5), 2.59 (m, 1H, H-1), 2.76 (ddd, *J* = 13.4, 8.1, 5.6 Hz, 1H, H-4$_a$), 2.90 (ddd, *J* = 13.4, 8.5, 7.6 Hz, 1H, H-4$_b$), 3.16 (m, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 13.0 (C-8), 21.45 (C-7), 21.48 (C-5), 30.0 (C-6), 33.1 (t, *J* = 124.7 Hz, C-1), 48.2 (t, *J* = 3.8 Hz, C-2), 51.5 (C-4); ^{31}P NMR (202.46 MHz, D_2O) δ 18.02 m_{AB}. HRMS (ESI) calcd for $C_7H_{18}O_7P_2SNa$ $[M+Na]^+$ 331.0146; found 331.0140.

1-[(*n*-Hexylsulfinyl)ethyl]-1,1-bisphosphonic Acid (42)

Amorphous solid; ^{1}H NMR (500.13 MHz, D_2O) δ 0.76 (t, *J* = 7.1 Hz, 3H, H-9), 1.21 (m, 4H, H-8, H-7), 1.37 (m, 2H, H-6), 1.65 (m, 2H, H-5), 2.61 (m, 1H, H-1), 2.78 (m, 1H, H-4$_a$), 2.91 (m, 1H, H-4$_b$), 3.16 (m, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 13.2 (C-9), 21.7 (C-8), 21.8 (C-5), 27.4 (C-6), 30.4 (C-7), 33.2 (t, *J* = 124.1 Hz, C-1), 48.3 (t, *J* = 3.7 Hz, C-2), 51.6 (C-4); ^{31}P NMR (202.46 MHz, D_2O) δ 17.99 m_{AB}. HRMS (ESI) calcd for $C_8H_{20}O_7P_2SNa$ $[M+Na]^+$ 345.0303; found 345.0291.

1-[(*n*-Heptylsulfinyl)ethyl]-1,1-bisphosphonic Acid (43)

Amorphous solid; ^{1}H NMR (500.13 MHz, D_2O) δ 0.79 (t, *J* = 6.9 Hz, 3H, H-10), 1.17 (m, 4H, -C*H*$_2$-), 1.24 (p, *J* = 7.0 Hz, 2H, H-7), 1.35 (m, 2H, H-6), 1.65 (m, 2H, H-5), 2.61 (m, 1H, H-1), 2.76 (ddd, *J* = 13.6, 8.1, 5.8 Hz, 1H, H-4$_a$), 2.89 (ddd, *J* = 13.5, 8.4, 7.7 Hz, 1H, H-4$_b$), 3.15 (m, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 13.3 (C-10), 21.79 (C-9), 21.84 (C-5), 27.6 (C-6), 27.8 (C-7), 30.7 (C-8), 33.1 (t, *J* = 124.6 Hz, C-1), 48.2 (t, *J* = 3.9 Hz, C-2), 51.6 (C-4); ^{31}P NMR (202.46 MHz, D_2O) δ 18.03 m_{AB}. HRMS (ESI) calcd for $C_9H_{22}O_7P_2SNa$ $[M+Na]^+$ 359.0459; found 359.0441.

1-[(*n*-Octylsulfinyl)ethyl]-1,1-bisphosphonic Acid (44)

Amorphous solid; ^{1}H NMR (500.13 MHz, D_2O) δ 0.79 (t, *J* = 7.0 Hz, 3H, H-11), 1.22 (m, 6H, -C*H*$_2$-), 1.29 (p, *J* = 7.0 Hz, 2H, H-7), 1.41 (m, 2H, H-6), 1.70 (m, 2H, H-5), 2.34 (ddt, *J* = 21.0, 9.0, 5.9 Hz, 1H, H-1), 2.77 (ddd, *J* = 13.4, 8.5, 5.9 Hz, 1H, H-4$_a$), 2.94 (ddd, *J* = 13.3, 9.0, 7.4 Hz, 1H, H-4$_b$), 3.15 (m, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 13.5 (C-11), 22.1 (C-10), 22.2 (C-5), 28.0 (C-6), 28.4 (C-7), 28.5 (C-8), 31.3 (C-9), 33.1 (t, *J* =

124.8 Hz, C-1), 48.3 (C-2), 51.7 (C-4); ^{31}P NMR (202.46 MHz, D_2O) δ 16.36 (d, *J* = 99.4 Hz). HRMS (ESI) calcd for $C_{10}H_{24}O_7P_2SNa$ $[M+Na]^+$ 373.0616; found 373.0595.

1-[(*n*-Nonylsulfinyl)ethyl]-1,1-bisphosphonic Acid (45)

Amorphous solid; ^{1}H NMR (500.13 MHz, D_2O) δ 0.77 (t, *J* = 7.0 Hz, 3H, H-12), 1.19 (m, 8H, -C*H*$_2$-), 1.27 (p, *J* = 6.8 Hz, 2H, H-7), 1.38 (m, 2H, H-6), 1.96 (m, 2H, H-5), 2.42 (m, 1H, H-1), 2.81 (ddd, *J* = 13.4, 8.5, 5.8 Hz, 1H, Hs4$_a$), 2.92 (ddd, *J* = 13.3, 9.0, 7.4 Hz, 1H, H-4$_b$), 3.15 (m, 2H, H-2); ^{31}P NMR (202.46 MHz, D_2O) δ 16.99 m_{AB}. HRMS (ESI) Calcd. for $C_{11}H_{27}O_7P_2S$ $[M+H]^+$ Calcd 365.0953. Found 365.0936.

1-[(*n*-Decylsulfinyl)ethyl]-1,1-bisphosphonic Acid (46)

Amorphous solid; ^{1}H NMR (500.13 MHz, D_2O) δ 0.82 (t, *J* = 7.0 Hz, 3H, H-13), 1.24 (m, 10H, -C*H*$_2$-), 1.33 (p, *J* = 7.0 Hz, 2H, H-7), 1.44 (m, 2H, H-6), 1.74 (m, 2H, H-5), 2.38 (m, 1H, H-1), 2.81 (ddd, *J* = 13.6, 8.5, 5.5 Hz, 1H, H-4$_a$), 2.98 (ddd, *J* = 13.2, 8.9, 7.4 Hz, 1H, H-4$_b$), 3.20 (m, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 13.8 (C-13), 22.6 (C-12), 22.7 (C-5), 28.7 (C-6, C-7), 29.4 (C-10), 29.6 (C-8), 29.7 (C-9), 31.9 (C-11), 33.0 (t, *J* = 128.1 Hz, C-1), 48.4 (C-2), 51.9 (C-4); ^{31}P NMR (202.46 MHz, D_2O) δ 14.15. HRMS (ESI) Calcd. for $C_{12}H_{28}O_7P_2SNa$ $[M+Na]^+$ Calcd 401.0929. Found 401.0904.

1-[(*n*-Pentylsulfonyl)ethyl]-1,1-biphosphonic Acid (47)

Amorphous solid; ^{1}H NMR (500.13 MHz, D_2O) δ 0.82 (t, *J* = 7.2 Hz, 3H, H-8) 1.28 (m, 2H, H-7), 1.36 (m, 2H, H-6), 1.78 (p, *J* = 7.7 Hz, 2H, H-5) 2.63 (tt, *J* = 22.8, 5.1 Hz, 2H, H-1), 3.27 (m, 2H, H-4), 3.59 (dt, *J* = 23.8, 5.2 Hz, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 12.9 (C-8), 21.3 (C-7), 21.4 (C-6), 29.8 (C-5), 33.2 (t, *J* = 122.9 Hz, C-1), 48.3 (t, *J* = 3.7 Hz, C-2), 51.3 (C-4); ^{31}P NMR (202.46 MHz, D_2O) δ 17.49. HRMS (ESI) Calcd. for $C_7H_{18}O_8P_2SNa$ $[M+Na]^+$ Calcd 347.0095. Found 347.0100.

(2,2-Diphosphonoethyl)(methyl)(octyl)sulfonium Tetrafluoroborate (48)

To a mixture of compound **37** (295 mg, 0.88 mmol), iodomethane (0.6 mL) in acetonitrile (20 mL) was added silver tetrafluoroborate (150 mg, 0.88 mmol) under argon atmosphere. The reaction mixture was stirred at room temperature for 2 h. The solvent was evaporated and the product was purified by column chromatography (C-18 silica gel) eluting with methanol to afford 131 mg (36% yield) of pure **48** as a white solid: mp = 96–97 °C; ^{1}H NMR (500.13 MHz, D_2O) δ 0.78 (t, *J* = 6.6 Hz, 3H, H-11), 1.20 (m, 8H, -C*H*$_2$-), 1.28 (p, *J* = 6.8 Hz, 2H, H-7), 1.41 (p, *J* = 7.2 Hz, 2H, H-6), 1.76 (m, 2H, H-5), 2.51 (m, 1H, H-1), 2.86 (s, 3H, S(+)C*H*$_3$), 3.19 (ddd, *J* = 12.3, 8.9, 6.3 Hz, 1H, H-4$_a$), 3.35 (ddd, *J* = 12.3, 9.1, 7.0 Hz, 1H, H-4$_b$), 3.50 (m, 1H, H-2$_a$), 3.59 (m, 1H, H-2$_b$); ^{13}C NMR (125.77 MHz, D_2O) δ 13.3 (C-11), 21.93 (C-10), 23.12 (C-5), 23.17 (S(+)*C*H$_3$), 27.5 (C-6), 27.9 (C-7), 28.0 (C-8), 30.9 (C-9), 35.6 (t, *J* = 118.5 Hz, C-1), 40.8 (t, *J* = 3.2 Hz, C-2), 42.7 (C-4); ^{31}P NMR (202.46 MHz, D_2O) δ 14.59. HRMS (ESI) calcd for $C_{11}H_{27}O_6P_2S$ $[M]^+$ 349.1004; found 349.1008.

Drug Screening

***T. cruzi* amastigote assays—**Gamma-irradiated (2,000 Rads) Vero cells (3.4×10^4 cells/well) were seeded in 96 well plates (black, clear bottom plates from Greiner Bio-One) in 100 μL RPMI media (Sigma) with 10% FBS. Plates were incubated overnight at 35 °C and 7% CO_2. After overnight incubation, Vero cells were challenged with 3.4×10^5 trypomastigotes/well (CL strain overexpressing a tdTomato red fluorescent protein) in 50 μL volume and incubated for 5 h at 35 °C and 7% CO_2. After infection, cells were washed once with Hanks solution (150 μL/well) to eliminate any extracellular parasites and compounds were added in serial dilutions in RPMI media in 150 μL volumes. Each dilution

was tested in quadruplicate. Each plate also contained controls with host cells and no parasites (for background check), and controls with parasites and no drugs (positive control). Drugs were tested on *T. cruzi* at 1.56 μM, 3.125 μM, 6.25 μM, 12.5 μM, 25 μM. For each set of experiments, benznidazole was also used as a positive control 0.39 μM, 0.78 μM, 1.56 μM, 3.125 μM, and 6.25 μM. After drug addition, plates were incubated at 35 °C and 7% CO_2. At day 3 post-infection, plates were assayed for fluorescence [69] IC_{50} values were determined by non-linear regression analysis using SigmaPlot. There was no evident cytotoxicity on the host cells (visual assay) with any of the drugs tested at concentrations as high as 25 μM.

***T. gondii* tachyzoites assays—**Experiments on *T. gondii* tachyzoites were carried out as described previously [70] using *T.gondii* tachyzoites expressing red fluorescent protein [71]. Cells were routinely maintained in hTerT cells grown in High Glucose Dulbecco's modified Eagle's medium (DMEM-HG) supplemented with 1% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, at 37 °C in a humid 5% CO_2 atmosphere. Confluent monolayers grown in 96-well black plates with optical bottoms (black, clear bottom plates from Greiner Bio-One) 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/well in the same media. The plates were incubated at 37 °C and read daily in a Molecular Devices fluorescence 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 [72]. For the calculation of the EC_{50}, the percent of growth inhibition was plotted as a function of drug concentration by fitting the values to the function: $I = I_{max}\ C/(EC_{50} + C)$, where I is the percent inhibition, I_{max} = 100% inhibition, C is the concentration of the inhibitor, and EC_{50} is the concentration for 50% growth inhibition. There was no evident cytotoxicity on the host cells with any of the drugs tested (visual assay).

***Tc*FPPS and *Tg*FPPS Assays and Product Analysis—**Drugs were tested on the enzymes first at 1 and 20 μM (*T. cruzi*) or 1 and 10 μM (*T. gondii*). If no activity was detected at 20 or 10 μM, respectively, then they were not further tested. For *Tc*FPPS [73–75] 100 μL of assay buffer (10 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 2 mM dithiothreitol, 4.7 μM [4-^{14}C]IPP (10 μCi/μmol)), and 55 μM DMAPP were 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 6.0 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. For *Tg*FPPS the reaction conditions were the same except that 1 mM $MgCl_2$ was used.

General method for measuring cytotoxicity or proliferation using AlamarBlue™ by spectrophotometry

Confluent monolayers of hTERT cells were seeded in 96 well plates (black, clear bottom from Greiner Bio-One Cat#655090) in 150 μL DMEM high glucose no phenol red (Gibco Cat# 21063) with 10% Cosmic Calf Serum. Plates were incubated overnight at 35 °C and 7% CO_2. After overnight incubation, wells were washed once with Hanks (150 μL/well) to eliminate any detached host cells, and drug compounds were added in serial dilutions in DMEM media in 150 μL volumes. Each dilution was tested in quadruplicate. Each plate also contained controls with host cells and no drug added. Plates containing drug dilutions were incubated at 35°C and 7% CO_2 for 3–4 days. After 3–4 days, Alamar Blue indicator (AbD serotec cat# BUF012B) was aseptically added in an amount equal to 10% of the

culture volume. Cultures were incubated at 35°C for 6 hours. After incubation, absorbance was measured at 570 and 600 nm. To calculate the percent difference in reduction (of Alamar Blue) between treated and control cells the following formula was used:

$$\text{Percentage difference between treated and control cells} = \frac{(\varepsilon_{OX})^{\lambda 2} A^{\lambda 1} - (\varepsilon_{OX})^{\lambda 1} A^{\lambda 2}}{(\varepsilon_{OX})^{\lambda 2} A^{0,\lambda 1} - (\varepsilon_{OX})^{\lambda 1} A^{0,\lambda 2}} \times 100$$

Where:

ε_{OX} = molar extinction coefficient of Alamar Blue TM oxidized form (BLUE)

A = absorbance of test wells

A^0 =absorbance of positive growth control well (cells plus Alamar BlueTM but no test agent)

λ1 = 570nm

λ2 = 600nm

Wavelength (λ)	ε_{OX}
570nm	80,586
600 nm	117,216

The Percentage difference obtained is then subtracted from 100 to obtain the percent of growth inhibition in the test well compared to that of the control.

Example calculation: Percent difference between treatment and control cells = 62%. This would indicate that the amount of reduction in the test well is 62% of that in the control well, or put another way, that growth in the test well is inhibited by 38% when compared to that of the control. As this assay was performed using non-irradiated hTERT cells, cell confluency was also checked in control wells during the 4 days of the assay to evaluate cell death as a consequence of overgrowth. At day 4, there was a minimum amount of cells detached in control wells. Different concentrations of DMSO were tested as positive control of toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Sulfur-containing bisphosphonates were active against amastigotes of *T. cruzi*
- These compounds were also active against tachyzoites of *T. gondii*
- This effect was associated with farnesyl pyrophosphate synthase (FPPS) blockage
- Some compounds inhibited the TgFPPS with IC_{50} values at the low nanomolar range

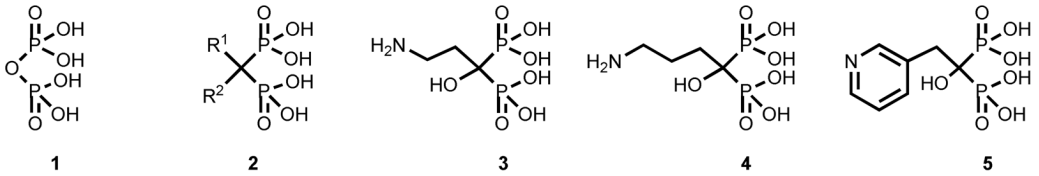


Figure 1.
General formula and chemical structure of representative FDA-approved bisphosphonates clinically employed for the treatment of bone disorders.

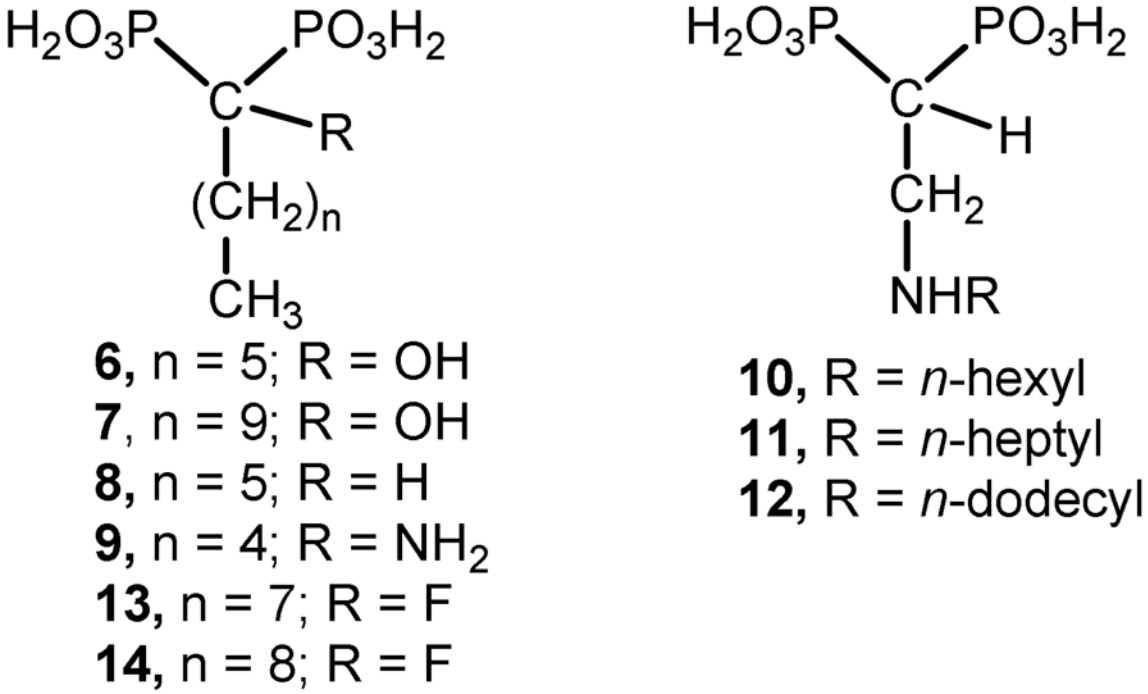
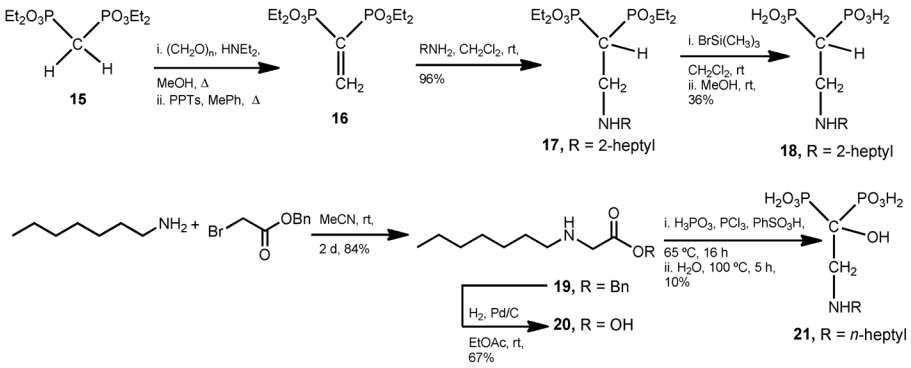
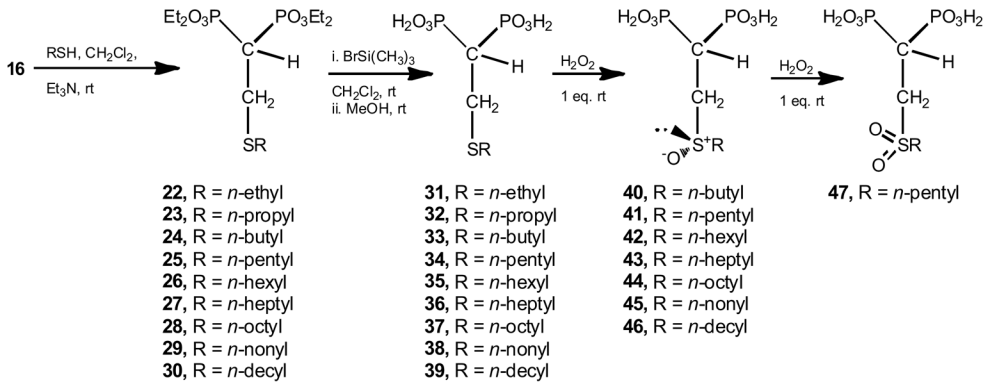


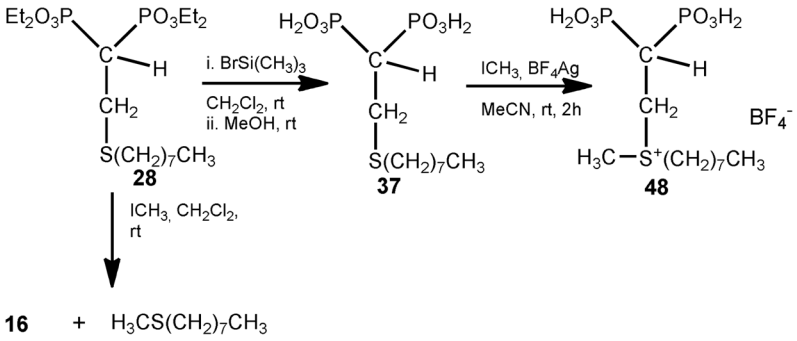
Figure 2.
Chemical structure of representative members of bisphosphonic acids derived from fatty acids.



Scheme 1.
Synthetic approach for the preparation of modified alkylaminoethyl bisphosphonates.



Scheme 2.
Synthetic approach to access to sulfur-containing bisphosphonates.



Scheme 3.
Method of preparation of the methyl sulfonium derivative **48**.

Table

Biological activity of sulfur containing 1,1-bisphosphonic acids against *Tc*FPPS, *Tg*FPPS, *T. cruzi* (amastigotes), and tachyzoites of *T. gondii.*

Compound	TcFPPS IC_{50} (μM)	ED_{50} *T. cruzi* amastigotes	TgFPPS IC_{50} (μM)	ED_{50} *T. gondii* (μM)	Citotoxicity hTerT cells (μM)*
18	> 10	> 20	< 1.0	11.4	
21	> 10	> 20	> 1.0	> 10	
31	0.627 ± 0.433	> 20	1.834 ± 0.145	> 10	
32	0.925 ± 0.639	> 20	0.066 ± 0.027	> 10	
33	0.789 ± 0.531	> 20	0.048 ± 0.016	> 10	
34	> 10	> 20	0.110 ± 0.037	> 10	
35	> 10	> 20	0.307 ± 0.202	6.5	
36	> 10	> 20	0.069 ± 0.046	0.97	> 200
37	6.386 ± 0.847	15.8	0.024 ± 0.016	2.6	> 1000
38	1.70 ± 0.186	12.8	0.025 ± 0.015	1.2	≥ 500
39	0.097 ± 1.494	> 20	0.021 ± 0.014	1.8	> 200
40	> 10	> 20	> 10	> 10	
41	> 10	> 20	> 10	> 10	
42	> 10	> 20	> 10	> 10	
43	> 10	> 20	0.009 ± 0.004	5.0	
44	> 10	> 20	0.016 ± 0.002	3.0	
45	> 10	19.4	0.056 ± 0.028	1.4	> 1000
46	> 10	> 20	> 10	7.6	
47	> 10	> 20	> 10	> 10	
48	0.040 ± 0.016	> 20	0.013 ± 0.006	7.0	
Benznidazole	ND[a]	1.7 ± 1.03	ND[a]	ND[a]	
Risedronate	0.027 ± 0.01 [68]	55.0 ± 5.0 [68]	0.074 ± 0.017 [30]	2.4 ± 0.7 [29]	

[a] ND = not determined

* Values are maximal concentrations at which no toxicity was observed. DMSO controls showed toxicity at a concentration of ≥ 0.25% under similar conditions.