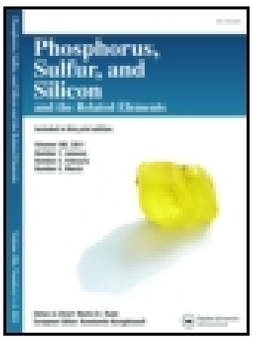

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Synthesis and biological evaluation of 2-substituted vinylgembisphosphonates against *Plasmodium falciparum* and *Trypanosoma brucei*

Siphamandla B. Simelane, Monisola I. Ikhile, Derek T. Ndinteh, and Xavier Y. Mbianda

Department of Applied Chemistry, University of Johannesburg, Doornfontein, South Africa

ABSTRACT

A series of 2-substituted vinylidene-1,1-bisphosphonate esters and their acids were synthesized and tested *in vitro* for activity against *Plasmodium falciparum* and *Trypanosoma brucei*. For each compound, % parasite viability in treated wells was calculated relative to untreated controls for both *P. falciparum* and *T. brucei*. Fifty percentage inhibitory concentration (IC_{50}) was also determined for the compounds. Chloroquine and pentamidine were used as positive control drug standards for activity against *P. falciparum* and *T. brucei*, respectively. The esters had better antiparasitic activity compared to their corresponding acids. Some of the compounds reduced % parasite viability to as low as 24.3% for *P. falciparum* and down to 0.602% for *T. brucei*. Tetraethyl-2-(o-tolyl)-ethene-1,1-bisphosphonate (**3b**) recorded the best IC_{50} against *T. brucei* which was 0.0345 μmol/mL.

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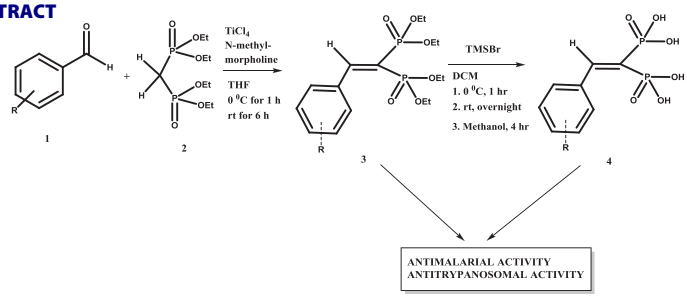
Substituted vinyl gem-bisphosphonic acids; antimalarial activity; antitrypasomal activity; drug resistance

GRAPHICAL ABSTRACT



Introduction

Malaria and Human African Trypanosomiasis (HAT) are diseases caused by protozoan parasites of the genera *Plasmodium* and *Trypanosoma*, respectively.[1,2] Malaria is responsible for about 1 million deaths each year whilst approximately half a million cases of HAT have been reported in Sub-Saharan Africa between 1990 and present.[3,4] Over the years, the deadly species causing malaria, *Plasmodium falciparum*, has gained resistance against first line drugs such as chloroquine.[5] Due to lack of vaccines and constant emergence of drug-resistant strains, it is necessary to develop improved drugs candidates.

Bisphosphonates (BPs) are synthetic organic compounds characterized by a P-C-P backbone structure. They are metabolically stable structural analogues of the inorganic pyrophosphate that has a P-O-P backbone.[6] BPs were discovered in the 19th century and their first synthesis was reported in 1865.[7] These compounds are also analogous to many units in molecules found in living organisms such as deoxyribonucleic acids (DNA), ribonucleic acids (RNA), adenosine triphosphate (ATP), and so on. Therefore, they have a great potential in pharmaceutical applications.[6] Initially, BPs were used as antiscaling and anticorrosive agents as well as complexing agents in the textile, fertilizer, and oil industries. Their potential for the treatment of calcium and bone associated disorders was discovered in the 1960s.[8] BPs are now clinically used to treat diseases such as arthritis, Paget's disease, and osteoporosis in women. Etidronate and elandronate are also clinically used in the prevention of osteoporosis.[7] They treat calcium associated disorders by binding to the mineral surfaces in bone and prevent the dissolution of calcium phosphate crystals thereby inhibiting bone resorption.[9]

Even though BPs are popular for the treatment of these calcium disorders, recent studies have demonstrated that they have a huge potential for other pharmaceutical applications. Several studies have reported that BPs also have anticancer, antimalarial, and antibacterial properties.[10–12] They also activate γT cells of the immune system which help the body fight infections as well as cancer.[6,13] BPs can be conjugated with other molecules (drugs and imaging agents) to target bone tissue as delivery systems because they have

CONTACT Derek T. Ndinteh dndinteh@uj.ac.za Department of Applied Chemistry, University of Johannesburg, Doornfontein 2028, South Africa.
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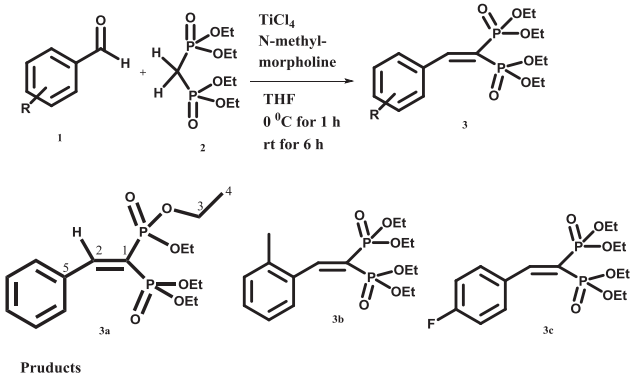
high affinity for hydroxyapatite, the main component of bone mineral.[14] Many studies have revealed that BPs are potent inhibitors of protozoan parasites and also have anti-tumor properties.[11] Martin et al. showed that BPs can also inhibit the growth of *Trypanosoma brucei* and *P. falciparum* which cause HAT and malaria, respectively.[15] The challenge with BPs currently available for clinical applications is their strong affinity for bone mineral which makes them unsuitable when targeting other tissues.[10] Vinyl gem-BPs, unlike most of the clinical BPs, do not have the hydroxyl group in the α-position, which reduces affinity toward bone mineral. To the best of our knowledge there are no studies in literature that have reported the biological evaluation of substituted vinyl gem-BPs without reducing the double bond. In this paper, we report the synthesis and biological evaluation of vinyl *gem*-BPs esters and their acids against *T. brucei* and *P. falciparum*.



Scheme 1. Synthesis of vinyl bisphosphonate esters.

Results and discussion

Synthesis

Substituted vinyl *gem*-BPs were successfully synthesized by allowing to react aldehydes and tetraethyl methylenebisphosphonate **2** in the presence of titanium tetrachloride ($TiCl_4$) and *N*-methylmorpholine as shown in Scheme 1.[16,17] Nuclear magnetic resonance (NMR) results were in agreement with those reported in literature.[18]

^{1}H NMR spectra (Figure 1) of the products showed two distinct triplets for C**H$_3$** protons (accounting for 6 protons each) at about 1.2 and 1.4 ppm, whereas their signal appears as one triplet in **2**. This indicates that there is no rotation in the molecule, which confirms the presence of the double bond. Two separate signals were also observed for the OC**H$_2$** protons at about 3.80 and 4.30 ppm. The presence of the double bond was also confirmed by the appearance of a doublet of doublets (accounting for one proton) corresponding to the vinylic proton at about 8.25 ppm. The *cis*- and *trans*-coupling constants, $^2J_{PH}$, were 29.1 and 47.7 Hz, respectively.

The ^{31}P NMR spectrum (Figure 2) showed two doublets at 12 and 17.5 ppm with a coupling constant $2J_{pp}$ around 50 Hz. The two phosphorus atoms appeared at different chemical shifts because there is no free rotation around the double bond, thus the two phosphorus atoms are in different magnetic environments.

Our first attempt to hydrolyze vinyl *gem*-BPs **3** was by refluxing with hydrochloric acid, as it is a fast and simple method. ^{31}P NMR analysis showed major peaks as singlets at 1.4, −1.5, and −1.8 ppm which are not consistent with the expected bisphosphonic acid **4**. These peaks correspond to phosphate groups which indicate that the vinyl BP was destroyed under these conditions.

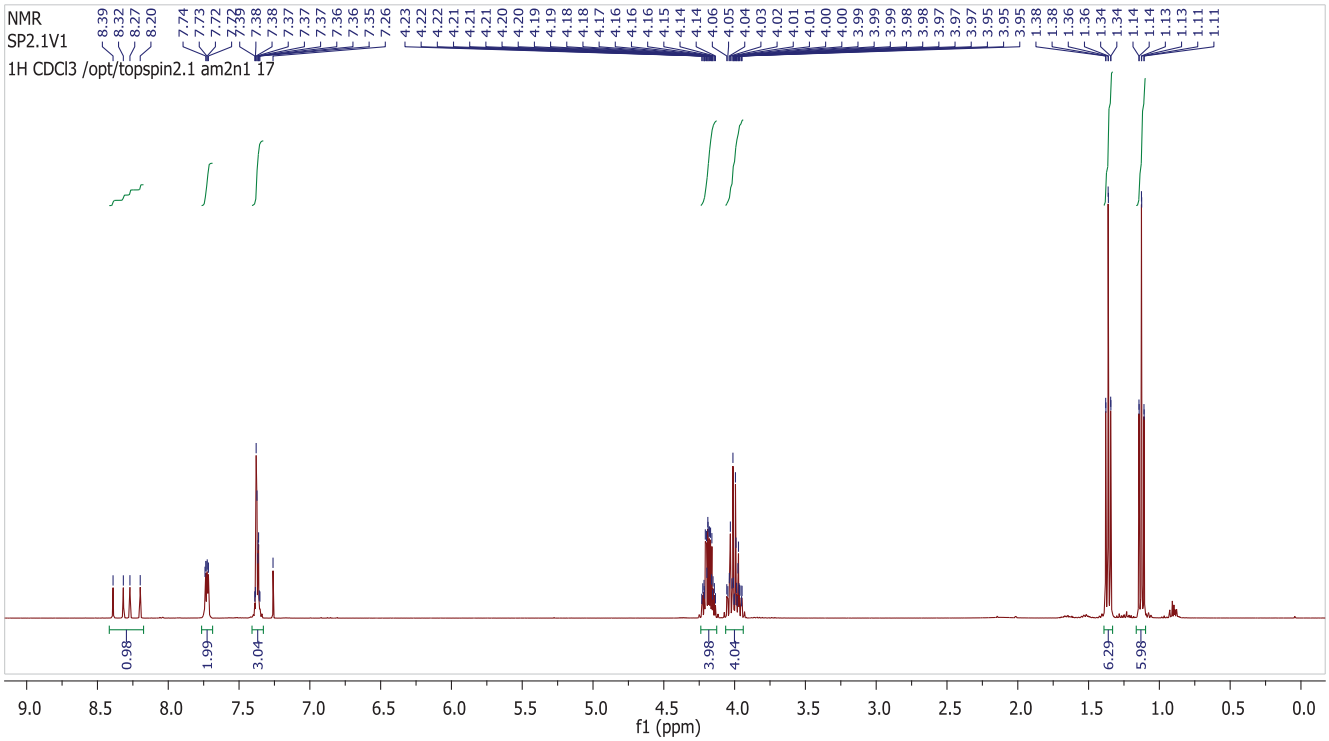


Figure 1. ^{1}H NMR spectrum of tetraethyl 2-phenyl-ethene-1,1-PB **3a**.

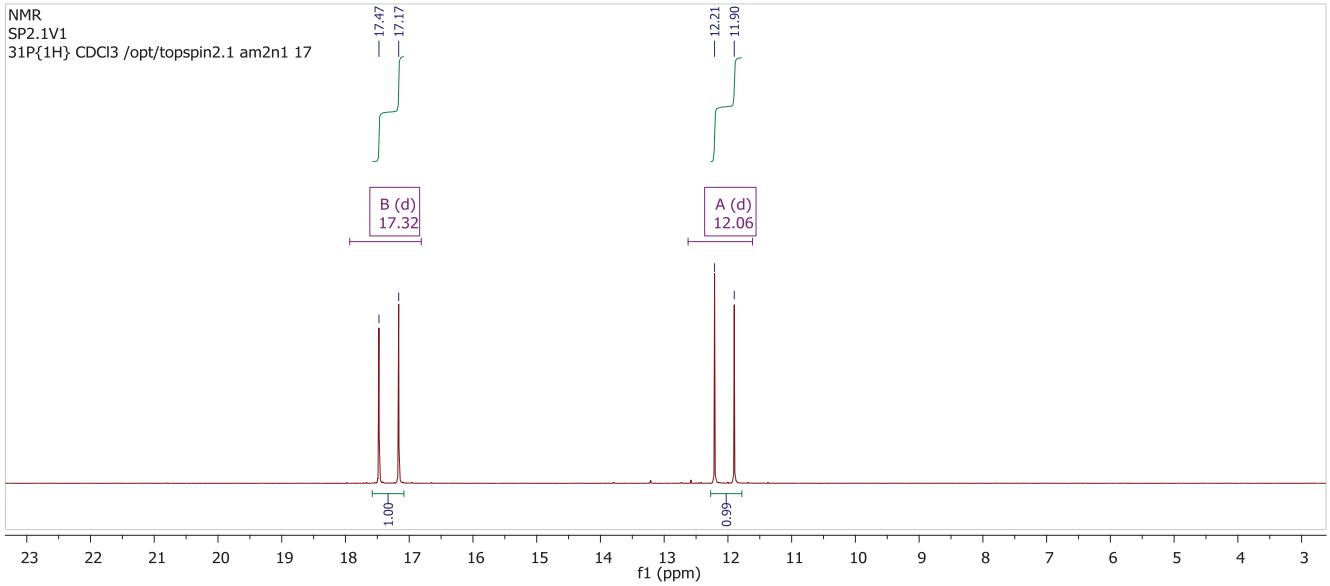
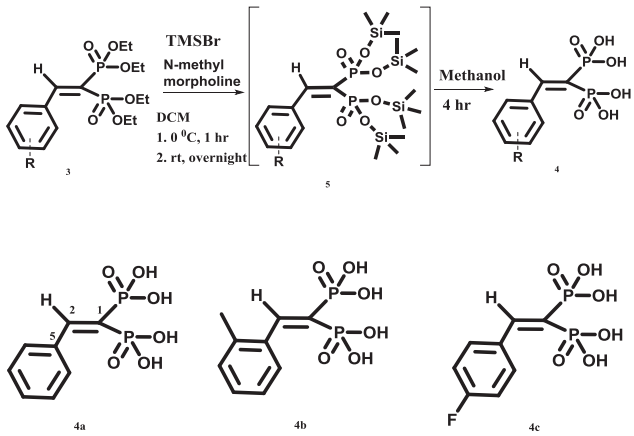


Figure 2. ^{31}P NMR spectrum of tetraethyl 2-phenylethene-1,1-BP **3a**.



Scheme 2. TMSBr assisted dealkylation of vinyl *gem*-BPs **3**.

Table 1. Biological evaluation of compounds **3** and **4**.

	% Parasite and cell viability		
	Antimalarial activity	Antitrypanosomal activity	Cytotoxicity
3a	24.3	0.602	47.5
3b[a]	34.2	0.711	73.7
3c	27.0	0.895	16.0
4a	119.9	100.4	91.8
4b	117.5	104.8	83.1
4c	113.9	91.8	79.9

[a]IC_{50} calculated for compounds with high potency and low cytotoxicity.
IC_{50} for compound **2** – 0.0345 µmol/mL.IC_{50} for Pentamidine – 0.009612 uM.

We then turned our attention to TMSBr assisted hydrolysis developed by McKenna with modification as shown in Scheme 2.[19,20] ^{31}P NMR analysis showed that the vinyl BPs were converted to the silyl ester intermediate **5**. The ^{31}P NMR spectra of **5** showed two doublets at 7.5 and 3.1 ppm with $^{2}J_{PP}$ of about 69 Hz.

The silyl esters **5** were then stirred with methanol to give the substituted vinyl *gem*bisphosphonic acids **4**. ^{31}P NMR spectrum of the bisphosphonic acid **4a** showed two doublets at about 14 and 9 ppm with a coupling constant of about 56 Hz.

Purification of the product **4** to remove the morpholinium salt required multiple recrystallizations which resulted in poor yield. The reaction was therefore repeated without adding *N*-methylmorpholine (Scheme 2). After complete silylation and methanolysis, the solvent was removed under reduced pressure and the product washed with acetone.

Results of ^{1}H NMR analysis were consistent with the substituted vinyl *gem*-bisphosphonic acids **4**. The spectra showed the absence of ethoxy groups, which confirmed complete dealkylation of the vinyl BPs. The NMR signal of H-2 appeared at 8 ppm as a doublet of doublets with $^{3}J_{PH}$ (cis) of 29 Hz and $^{3}J_{PH}$ (trans) of 49 Hz. These values are similar to those observed for the vinyl BP esters which confirmed that the double bond was not destroyed during dealkylation.

Antiplasmodial activity

Table 1 shows that the activity of vinyl BP esters against *P. falciparum* (strain 3D7) is significantly higher than that of the corresponding acids. Compounds **3a–c** reduced percentage parasite viability to 24.3%, 34.2%, and 27.0%, respectively. On the wells treated with the vinyl bisphosphonic acids (compounds **4a–c**) percentage parasite viability remained above 100% indicating that they were actually growing. The activity of compounds **3a** and **3c** could be caused by the general cytotoxicity of the compounds. As can be seen in Table 1 they reduced percentage cell viability to 47.5% and 16.0%, respectively. Compound **3c** had better antiplasmodial activity and lower cytotoxicity compared to the other esters. Saito et al. reported that BPs interfere with protein isoprenylation by targeting farnesyl pyrophosphate synthase (FPPS) thereby killing the parasite.[21] Hwan et al. suggested that geranyl pyrophosphate synthase could be another target for antimalarial drugs.[10] One structural difference between **3b** and the other two esters is the electron donating methyl substituent at the phenyl ring. The poor activity of the acids could be due to poor lipophilicity hence

they cannot easily diffuse into the cells. This phenomenon has also been observed in other studies.[10]

Antitrypanosomal activity

The activity of the esters against *T. brucei* was also found to be higher compared to the acids, as shown in Table 1. However, in this case, all the esters reduced viability of *T. brucei* to about 0%, which is very high compared to the activity against *P. falciparum*. Many studies have reported that the BPs kill the parasite by inhibiting *T. brucei* FPPS enzyme.[22,23]. The lower activity of the esters against *P. falciparum* relative to *T. brucei* could be attributed to poor uptake of the BPs into the parasite.[15] Fifty percentage inhibitory concentration (IC_{50}) was calculated for compound **3b** because it had good antitrypanosomal activity and lower cytotoxicity. It was found to be 0.0345 µmol/mL, which is comparable to that of the standard drug pentamidine.

It was also noted in Table 1 that the vinyl *gem*-BPs with electron-withdrawing substituents at the phenyl ring (**3a** and **3c)** have higher cytotoxicity and better antiparasitic activity compared to the one with electron donating substituents **3b**.

Conclusions

The results presented show that the BPs described have a potential in treating diseases caused by protozoan parasites. The results also showed that esters have better potency than the corresponding acids. This suggests that lipophilicity of the BPs plays a role in the efficiency of the compounds. One of the most effective compounds (**3b)** against *T. brucei* showed minimal cytotoxicity against human cells while reducing parasite viability to almost 0%.

Experimental

Synthesis

Starting materials were purchased from Sigma Aldrich or Merck Chemicals unless specified otherwise. Solvents were dried using standard procedures in organic chemistry. NMR spectra were recorded with a Bruker Avance 400 MHz instrument operating at 400 MHz (^{1}H), 100 MHz (^{13}C), and 160 MHz (^{31}P) as well as with a Bruker 500 instrument operating at 500 MHz (^{1}H), 126 MHz (^{13}C) and 200 MHz (^{31}P). Mass spectrometry analysis was obtained using the Bruker Compact Q-TOF mass spectrometer. Reactions were monitored by thin layer chromatography on silica gel 60 (F254) plates. The Supplemental Materials contains sample ^{1}H, ^{13}C, and ^{31}P NMR spectra of products 4 (Figures S1–S9).

General procedure for synthesis of vinyl BP esters: Benzaldehyde (10 mmol) was dissolved in dry THF (80 mL) and $TiCl_4$ (3 mL) in toluene (20 mL) was added drop wise. The mixture was stirred for 5 min at 0 °C. A solution of tetraethyl methylenebisphosphonate (12 mmol) and *N*-methylmorpholine (24 mmol) in dry THF (20 mL) was added drop wise. The mixture was stirred for 1 h at 0 °C, and then stirred for 3 h at room temperature. The reaction was quenched with water (50 mL) and extracted three times with ethyl acetate (100 mL). The organic phase was dried with anhydrous sodium sulfate and purified by column chromatography (acetone:EtOAc, 1:2) to give the product.

Tetraethyl-2-(phenyl)-ethene-1,1-bisphosphonate ***(3a)***: Pale yellow oil (56%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.16$ (t, $J = 6.9$ Hz, 6H, CH_3), 1.39 (t, $J = 7.3$ Hz 6H, CH_3), 4.00 − 4.06 (m, 4H, CH_2), 4.17 − 4.25 (m, 4H, CH_2), 7.30 − 7.41 (m, 3H, arom-H), 7.50 − 7.76 (m, 2H, arom-H), 8.32 (dd, $J = 47.7$ Hz, 29.1 Hz, 1H, CH). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 15.1$ (d, $J = 7.0$ Hz, CH_3); 16.1 (d, $J = 6.0$ Hz, CH_3); 61.7 (d, $J = 6.3$ Hz, CH_2), 61.8 (d, $J = 5.3$ Hz, CH_2), 119.3 (t, $J = 169.3$ Hz, C1); 127.1 (s, C-arom.); 129.5 (s, C-arom.); 129.8 (s, C-arom.); 133.9 (dd, $J = 22.6$ Hz, 8.6 Hz, C_{ipso}); 159.5 (s, C2). ^{31}P NMR (160 MHz, $CDCl_3$): $\delta = 12.0$ (d, $J = 49.6$ Hz), 17.3 (d, $J = 49.6$).

Tetraethyl-2-(o-tolyl)-ethene-1,1-bisphosphonate ***(3b):*** Pale yellow oil (80%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.10$ (t, $J = 7.1$ Hz, 6H, CH_3), 1.40 (t, $J = 7.1$ Hz, 6H, CH_3), 2.30 (s, 3H, CH_3), 3.83 − 4.00 (m, 4H, CH_2), 4.16 − 4.23 (m, 4H, CH_2), 7.17 − 7.30 (m, 3H, arom-H), 7.58 − 7.70 (m, 1H, arom-H), 8.35 (dd, $J = 47.5$ Hz, 28.0 Hz, 1H, CH). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 15.1$ (d, $J = 7.0$ Hz, CH_3); 15.5 (d, $J = 3.0$ Hz, CH_3); 19.1 (s, CH_3); 61.6 (d, $J = 6.0$ Hz, CH_2); 61.8 (d, $J = 6.0$ Hz, CH_2); 124.7 (t, $J = 170.4$ Hz, C1); 124.4 (s, C-arom.); 128.2 (s, C-arom.); 129.0 (s, C-arom.); 134.2 (dd, $J = 20.5$ Hz, 8.3 Hz, C_{ipso}); 135.0(s, C-arom.); 160.5 (s, C2). ^{31}P NMR (160 MHz, $CDCl_3$): $\delta = 11.8$ (d, $J = 52.8$ Hz), 16.5 (d, $J = 52.8$ Hz).

Tetraethyl-2-(p-fluorophenyl)-ethene-1,1-bisphosphonate ***(3c)***: Clear oil (60%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.25$ (dd, $J = 47.6$ Hz, 29.1 Hz, 1H, –CH=); 7.83 (m, 2H, arom-H); 7.09 (t, $J = 8.6$ Hz, 2H, arom-H); 4.21 (m, 4H, CH_2); 4.06 (m, 4H, CH_2); 1.39 (t, $J = 6.9$ Hz, 6H, CH_3); 1.20 (t, $J = 6.9$ Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 163.4$ (d, $^{1}J_{CF} = 266.9$ Hz, C-F); 159.2 (s, –CH=); 133.6 (t, $J = 8.0$ Hz, C_{ipso}); 136.4 (d, $^{3}J_{CF} = 8.0$ Hz, C-arom); 120.1 (t, $J = 167.5$ Hz, =C<); 114.4 (d, $^{2}J_{CF} = 21.0$ Hz, C-arom); 61.8 (d, $J = 6.0$ Hz, CH_2); 61.9 (d, $J = 7.0$ Hz, CH_2); 15.6 (d, $J = 7.1$ Hz, CH_3); 15.4 (d, $J = 7.1$ Hz, CH_3). ^{31}P NMR (160 MHz, $CDCl_3$): $\delta = 17.3$ (d, $J = 48.0$ Hz); 12.5 (d, $J = 48.0$ Hz).

General procedure for synthesis of vinyl bisphosphonic acids: Bromotrimethylsilane (1.47 g, 9.6 mmol) was added to a solution of tetraethyl 2-(phenyl)-ethene-1,1-bisphosphonate **3a** (0.452 g, 1.20 mmol) in dichloromethane (5 mL) at 0 °C and stirred for 1 h. The reaction mixture was then allowed to warm up to room temperature and stirred overnight. Methanol (5 mL) was added and stirring was continued for 4 h. The solvents were removed under reduced pressure and the solid product was washed with acetone and dried under vacuum.

2-(Phenyl)-ethene-1,1-bisphosphonic acid ***(4a)***: Off-white solid, 0.317 g (90%): ^{1}H NMR (400 MHz, D_2O): $\delta = 7.86$ (dd, $J = 46.9$ Hz, 29.1 Hz, 1H, CH), 7.35 (d, $J = 4.4$ Hz, 2H, arom-H), 7.16 (s, 3H, arom-H). ^{13}C NMR (126 MHz, D_2O): $\delta = 157.0$ (s, C2), 134.9 (dd, $J = 21.6$ Hz, 9.0 Hz, C_{ipso}), 130.2

(s, C-arom.), 129.5 (s, C-arom.), 128.2 (s, C-arom.), 125.7 (dd, $J = 161.5$ Hz, 158.5 Hz, C1). ^{31}P NMR (162 MHz, D_2O): $\delta = 15.0$ (d, $J = 53.7$ Hz), 9.8 (d, $J = 53.7$ Hz). HRMS (ESI^+): $m/z = 265.0030$ (calcd. $[M + H^+]$ for $C_8H_{10}O_6P_2 = 264.9953$).

2-(o-Tolyl)-ethene-1,1-bisphosphonic acid ***(4b)***: White crystals (83%). ^{1}H NMR (500 MHz, D_2O): $\delta = 8.08$ (dd, $J = 46.7$ Hz, 28.0 Hz, 1H, CH); 7.32 (d, $J = 7.6$ Hz, 1H, arom-H); 7.20 (t, $J = 7.4$ Hz, 1H, arom-H); 7.16 – 7.08 (m, 2H, arom-H); 2.13 (s, 3H, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 161.5$ (s, C2); 151.5 (s, C-arom.); 140.0 (dd, $J = 22.5$ Hz, 9.1 Hz, C_{ipso}); 139.3 (s, C-arom.); 132.8 (s, C-arom.), 129.3 (dd, $J = 168.7$ Hz, 164.0 Hz, C1), 128.4 (s, C-arom), 128.2 (s, C-arom), 20.1 (s, CH_3). ^{31}P NMR (202 MHz, D_2O): $\delta = 14.0$ (d, $J = 56.3$ Hz), 9.9 (d, $J = 56.3$ Hz). HRMS (ESI^+): $m/z = 279.0183$ (calcd. $[M + H^+]$ for $C_9H_{12}O_6P_2 = 279.0109$).

2-(p-Fluorophenyl)-ethene-1,1-bisphosphonic acid ***(4c)***: Light brown crystals (95%). ^{1}H NMR (500 MHz, D_2O): $\delta = 7.93$ (dd, $J = 46.1$ Hz, 28.9 Hz, 1H, CH), 7.59 (m, $J = 7.8$ Hz, 6.0 Hz, 2H, arom-H), 7.05 (t, $J = 8.8$ Hz, 2H, arom-H). ^{13}C NMR (125 MHz, D_2O) $\delta = 163.5$ (d, $^1J_{CF} = 248.4$ Hz, C-F), 155.2 (s, –CH=), 132.2 (d, $^3J_{CF} = 8.7$ Hz, C-arom), 131.3 (m, C_{ipso}), 126.1 (m, =C<), 115.2 (d, $^2J_{CF} = 22.0$ Hz, C-arom). ^{31}P NMR (200 MHz, D_2O): $\delta = 15.1$ (d, $J = 53.4$ Hz), 9.33 (d, $J = 53.4$ Hz). HRMS (ESI^+): $m/z = 283.9933$ (calcd. $[M + H^+]$ for $C_8H_9O_6P_2 = 283.9858$).

Biological evaluation

Antimalarial activity: To determine antimalarial potency, compounds were added to parasite cultures (*P. falciparum* strain 3D7) in 96-well plates and incubated for 48 h in a 37 °C CO_2 incubator. Twenty microliter of culture was removed from each well and mixed with 125 µL of a mixture of Malstat solution and NBT/PES solution in a fresh 96-well plate. These solutions measure the activity of the parasite lactate dehydrogenase (pLDH) enzyme in the cultures. A purple product is formed when pLDH is present, and this product can be quantified in a 96-well plate reader by absorbance at 620 nm (Abs_{620}). The Abs_{620} reading in each well is thus an indication of the pLDH activity in that well and also the number of parasites in that well. Compounds were tested in a range extending from 100 to 0.006 uM (4 fold-dilutions). For each compound concentration, % parasite viability – the pLDH activity in compound-treated wells relative to untreated controls – was calculated. Compounds were tested in duplicate wells, and a standard deviation (SD) was derived. For each compound, percentage viability was then plotted against log (compound concentration) and the **IC_{50}** (50% inhibitory concentration) was obtained from the resulting dose-response curve by non-linear regression. For comparative purposes, chloroquine was used as a positive control.

Antitrypanosomal activity: To determine the antitrypanosomal potency of test compounds, serial dilutions of the compounds were added to *in vitro* cultures of *T. brucei* in 96-well plates and incubated for 48 h. The numbers of parasites surviving exposure to the individual compound concentrations were determined by adding a resazurin-based reagent. For each compound concentration, % parasite viability – the resorufin fluorescence in compound-treated wells relative to untreated controls – was calculated. Compounds were tested in duplicate wells, and a standard deviation (SD) was derived. For each compound, percentage viability was then plotted against log (compound concentration) and the **IC_{50}** (50% inhibitory concentration) was obtained from the resulting dose-response curve by non-linear regression. Pentamidine was used as a positive control.

Cytotoxicity: Cytotoxicity of the compounds was determined by incubating HeLa (human cervix adenocarcinoma) cells with compounds at a concentration of 20 uM for 48 h. Percentage cell viability was determined using the resazurin-based reagent and reading resorufin fluorescence in a multi-wall plate reader. Compounds were tested in duplicates and the standard deviation was calculated. Emetine was used as a positive control standard.

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