

New Benzophenone-Derived Bisphosphonium Salts as Leishmanicidal Leads Targeting Mitochondria through Inhibition of Respiratory Complex II

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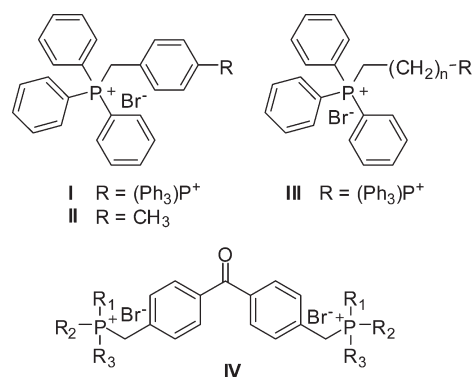
A set of benzophenone-derived bisphosphonium salts was synthesized and assayed for lethal activity on the human protozoan parasite *Leishmania*. A subset of them, mostly characterized by phosphonium substituents with an intermediate hydrophobicity, inhibited parasite proliferation at low micromolar range of concentrations. The best of this subset, 4,4'-bis((tri-*n*-pentylphosphonium)methyl)benzophenone dibromide, showed a very scarce toxicity on mammalian cells. This compound targets complex II of the respiratory chain of the parasite, based on (i) a dramatically swollen mitochondrion in treated parasites, (ii) fast decrease of cytoplasmic ATP, (iii) a decrease of the electrochemical mitochondrial potential, and (iv) inhibition of the oxygen consumption rate using succinate as substrate. Thus, this type of compounds represents a new lead in the development of leishmanicidal drugs.

Introduction

Leishmaniasis encompasses the different clinical manifestations caused by infection with the protozoan species belonging to the genus *Leishmania*. It ranges from the cutaneous form, in many cases self-healing, to the visceral leishmaniasis caused by species of the *donovani* complex, mortal if untreated. According to WHO estimations, 12 million people are infected worldwide, with 2 million new cases per year, from which 500 000 cases correspond to the visceral form (<http://www.who.int/leishmaniasis/burden/en/>). Nowadays, chemotherapy is the only effective way to fight the disease. Until recently, first-line drugs were constituted almost exclusively by pentavalent organic antimonials; aside from their intrinsic high toxicity, their efficacy is threatened by rampant resistance.¹ In fact, they are progressively being replaced by either liposomal formulations of amphotericin B, despite their high cost,² or miltefosine, the first successful oral drug against the visceral form of leishmaniasis.³ Nevertheless, the paucity of new leads in the pipeline is a worrisome scenario, raising concern about the near future. Thus, the search and development of new leads are an urgent priority.⁴

During the screening of in-house made libraries of aromatic and aliphatic dicationic derivatives against *Trypanosoma brucei*,^{5,6} the trypanocidal effect of the bisphosphonium derivative 4,4'-bis((tri-*n*-pentylphosphonium)methyl)benzophenone dibromide⁵ was discovered. This compound showed a good trypanocidal activity in vitro with a low DNA-binding affinity,⁷ in contrast to the high affinity of its guanidine counterpart, also trypanocidal. Previously, other authors reported for benzyltriphenylphosphonium derivatives cidal activities on animal infections by trypanosomatids such as *T. brucei*

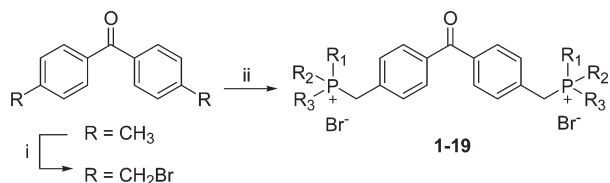
Chart 1. Known Triphenylphosphonium Compounds with Antischistosomal (**I**, **III**) or Antitrypanosomal Activity (**II**) and Newly Synthesized Benzophenone-Derived Bisphosphonium Salts (**IV**)



rhodesiense,^{8,9} *T. cruzi* (**II**, Chart 1),¹⁰ and *Leishmania mexicana*.¹¹ However, the literature about the pharmacological applications of phosphonium derivatives is relatively scarce, encompassing three major fields mainly. The first one consists of phosphonium compounds, such as *p*-xylylene bis(triphenylphosphonium)¹² or poly(methylene)bis(triphenylphosphonium) salt (**I** and **III**, Chart 1), which showed marked anticholinesterase and anticholinergic activities on *Schistosoma mansoni*.¹³ In fact, the phosphonium analogue of choline is metabolized and incorporated into phospholipids in mammalian cells without apparent toxicity.^{14,15}

A second group of phosphonium compounds is characterized by a strong amphipathic character endowed by the presence of at least one long alkyl chain substituent. They were extensively used as biocides, in either monomeric¹⁶ or polymeric form.^{17–19} They work as cationic detergents, disrupting the cell membrane. Some specificity for this process is achieved by profiting the higher anionic character of cell

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Scheme 1^a

^a Reagents and conditions: (i) NBS, benzoyl peroxide cat., CCl₄, 80 °C; (ii) R₁R₂R₃P, DMF or toluene, 110 °C.

membranes on prokaryotes and lower eukaryotes (reviewed in ref 20).

Finally, some phosphonium derivatives act as lipophilic ions with a delocalized charge and enhanced hydrophobicity. These characteristics afford their transfer across biological membranes despite their cationic nature, with a privileged accumulation in organelles with a strong electrochemical potential, such as mitochondria.^{21–23} Hence, the massive accumulation of these lipophilic cations into mitochondria may by itself lead to mitochondrial dysfunction, which in turn will trigger the apoptotic mitochondrial pathway (reviewed in ref 24). This is especially relevant in transformed cells, with a higher $\Delta\Psi_m$ ^a than their nontransformed counterparts.^{25,26} As far as *Leishmania* is concerned, the above-mentioned properties of phosphonium derivatives are particularly relevant if one considers the strong anionic character of the parasite plasma membrane²⁷ and the key role of mitochondria in the bioenergetics of this parasite. This could in turn represent a target for antileishmanial chemotherapy. To assess this hypothesis, we have synthesized and assayed a number of bisphosphonium benzophenone derivatives bearing different substituents on the phosphonium moieties (IV, Chart 1). As the DNA-binding properties of some of these compounds cannot fully account for their trypanocidal effects and likely for their leishmanicidal activities,⁷ we set out to study alternative mechanisms. In tune with the aforementioned properties of this class of compounds, their effects on plasma membrane integrity and mitochondrial functionality in *Leishmania* were studied in detail. By complementary bioenergetics and morphological approaches, we have defined the impairment of mitochondrial functionality as the basis for the leishmanicidal mechanism of bisphosphonium benzophenones, exemplified by 7, the best within this series. This compound severely impaired mitochondrial ATP synthesis through inhibition of the respiratory complex II (succinate dehydrogenase).

Results

Chemistry. The bisphosphonium salts 1–19 were synthesized by reaction of 4,4'-bis(bromomethyl)benzophenone with an excess of the corresponding phosphine in either anhydrous DMF or anhydrous toluene (Scheme 1). The bisphosphonium salts were isolated with satisfactory yields by crystallization from the reaction mixture. All the starting

phosphines were commercially available except triphenylphosphine, which was prepared as previously reported.⁵ The substitution pattern (R₁, R₂, and R₃) of compounds 1–19 is shown in Table 1.

Cytotoxicity against *Leishmania* Parasites. The reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by living cells constitutes a suitable parameter to assess the viability in an extensive range of cell lines, including *Leishmania*.²⁸ As such, the capability of bisphosphonium salts to inhibit MTT reduction by *Leishmania* parasites was used to assess the potential leishmanicidal activity of these compounds. The results were compiled in Table 1. From the initial set of 19 bisphosphonium compounds, 11 showed a LC₅₀ on *L. donovani* promastigotes at the low micromolar range. Under identical criteria, the same subset became active on *L. pifanoi* axenic amastigotes, except 19, with a LC₅₀ higher than 50 μ M, the cutoff established in the present study to select the active candidates (Table 1). Interestingly, amastigotes, the pathological form of the parasite in vertebrates, were more susceptible than promastigotes, with the sole exception of compound 14 and the aforementioned 19. The results underscore certain structural features modulating the leishmanicidal activity. For compounds with homoalkyl groups, the activity was restricted to those with a narrow range of substituents, from five- to eight-carbon length, suggesting an important role for hydrophobicity, tightly limited in values. Compound 7, with *n*-pentyl substituents, showed the highest potency. The combination of two alkyl groups plus an aromatic substituent was not enough to recover activity for methyl (10) or ethyl (11) substituents, but a better activity was achieved when two phenyl groups (13, 14) or cyclohexyl substituents were present (compound 12). The triphenylphosphonium (15) or diphenyltolyl (17, 18) compounds showed activities very close to the *n*-pentyl substituted compound. In particular, the diphenyl-*p*-tolyl analogue (18) displayed the second best activity of the whole series. Inclusion of a heterocycle was well tolerated for 2-pyridyl (16) but impaired activity by more than 1 log order for furanyl (19). As compound 7 turned out to be the most active molecule, on promastigotes and amastigotes, its toxicity on mammalian cells was assayed. The LC₅₀ of 7 on mouse peritoneal macrophages was 28.6 \pm 4.8 μ M. This represents a therapeutic index of 28, defined as the ratio between the respective LC₅₀ values for mammalian cells and for amastigotes, the pathological form in mammals. According to its highest leishmanicidal activity and scarce toxicity on mammalian cells, compound 7 was selected for the subsequent set of assays dealing with its mechanism of action.

Decrease of Intracellular ATP Levels. The maintenance of an adequate intracellular ATP level is essential for cellular homeostasis. The *in vivo* luminescence of 3-Luc promastigotes affords a real-time monitorization of changes in the cytoplasmic ATP pool.²⁹ Accordingly, compound 7 caused a drop of the luminescence of the 3-Luc strain in a concentration-dependent manner, reaching the end-point 10–15 min after addition (Figure 1). Altogether, these results suggest a decrease of the cytoplasmic ATP pool caused by 7.

Maintenance of Plasma Membrane Integrity. The drop of intracellular ATP observed in the previous assay can be caused either by inhibition of mitochondrial ATP synthesis, essential in the bioenergetics of *Leishmania*, or by permeabilization of the parasite plasma membrane, leading to ATP release or its futile hydrolysis by ionic pumps, in an attempt

^a Abbreviations: $\Delta\Psi_m$, mitochondrial membrane potential; BSA, bovine serum albumin; DMNPE-luciferin, D-luciferin 1-(4,5-dimethoxy-2-nitrophenyl) ethyl ester; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HBSS-Glc, Hanks' balanced salt solution plus 10 mM D-glucose; LC₅₀, drug concentration required to inhibit 50% of parasite proliferation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; rhodamine 123, 2-(6-amino-3-imino-3*H*-xanthen-9-yl)benzoic acid methyl ester, chloride; SD, standard deviation; SDS, sodium dodecyl sulfate.

Table 1. In Vitro Antileishmanial Activity and $\Delta\Psi_m$ Variation Induced by Bisphosphonium Salts 1–19

compd	R ₁	R ₂	R ₃	LC ₅₀ (μM)		% ΔΨ _m ^c
				<i>L. donovani</i> promastigote ^a	<i>L. pifanoi</i> amastigote ^b	
miltefosine				12.50 ± 2.90	26.30 ± 3.40	75.00
1	Me	Me	Me	> 50	> 50	
2	Et	Et	Et	> 50	> 50	
3	<i>n</i> -Pr	<i>n</i> -Pr	<i>n</i> -Pr	> 50	> 50	
4	<i>i</i> -Pr	<i>i</i> -Pr	<i>i</i> -Pr	> 50	> 50	
5	<i>n</i> -Bu	<i>n</i> -Bu	<i>n</i> -Bu	> 50	> 50	
6	<i>i</i> -Bu	<i>i</i> -Bu	<i>i</i> -Bu	> 50	> 50	
7	<i>n</i> -C ₅ H ₁₁	<i>n</i> -C ₅ H ₁₁	<i>n</i> -C ₅ H ₁₁	1.09 ± 0.14	0.97 ± 0.01	48.00
8	<i>c</i> -C ₆ H ₁₁	<i>c</i> -C ₆ H ₁₁	<i>c</i> -C ₆ H ₁₁	3.30 ± 1.19	1.67 ± 0.06	42.00
9	<i>n</i> -C ₈ H ₁₇	<i>n</i> -C ₈ H ₁₇	<i>n</i> -C ₈ H ₁₇	5.66 ± 0.46	1.37 ± 0.05	10.80
10	Me	Me	Ph	> 50	> 50	
11	Et	Et	Ph	> 50	> 50	
12	<i>c</i> -C ₆ H ₁₁	<i>c</i> -C ₆ H ₁₁	Ph	2.10 ± 0.84	1.45 ± 0.17	83.12
13	Me	Ph	Ph	19.31 ± 0.64	1.64 ± 0.5	100.00
14	Et	Ph	Ph	6.90 ± 0.50	14.01 ± 1.20	100.00
15	Ph	Ph	Ph	2.73 ± 0.99	1.53 ± 0.03	67.25
16	Ph	Ph	2-pyridyl	3.03 ± 0.66	2.58 ± 0.30	100.00
17	Ph	Ph	<i>o</i> -tolyl	1.90 ± 0.01	1.51 ± 0.05	55.75
18	Ph	Ph	<i>p</i> -tolyl	1.20 ± 0.29	1.38 ± 0.02	53.25
19	2-furanyl	2-furanyl	2-furanyl	18.12 ± 1.20	> 50	

^a Promastigotes of *L. donovani* strain MHOM/SD/00/1S-2D. ^b Amastigotes of *L. pifanoi* strain MHOM/VE/60/Ltrod. ^c Percentage of rhodamine 123 accumulated in the promastigote mitochondrion after overnight incubation at the respective LC₅₀, relative to untreated cells.

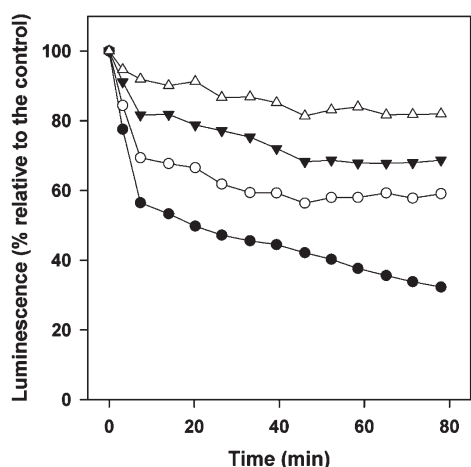


Figure 1. Variation of intracellular ATP level assessed by in vivo luminescence of 3-Luc promastigotes after addition of compound 7. Promastigotes (2×10^7 cells/mL) were preloaded with 25 μM DMNPE-luciferin. When the luminescence signal reached a plateau, luminescence was considered as 100% and compound 7 was added ($t = 0$). Variation in luminescence was normalized relative to the control untreated parasites. Compound 7 concentrations are as follows (μM): 0.6 (Δ), 1.0 (▼), 2.5 (○), 8.0 (●).

to recover the initial ionic gradients. The latter alternative was examined by monitoring the entrance of the vital dye SYTOX green. Even at 25 μM, i.e., a 25-fold higher concentration than its LC₅₀, compound 7 was unable to produce any significant increase of SYTOX green fluorescence, ruling out damage to the plasma membrane integrity (data not shown).

Morphological Alterations Induced by Compound 7. In a further step, the morphological damage inflicted to the

promastigotes by compound 7 was visualized by transmission electron microscopy. Electron micrographs showed an extremely swollen mitochondrion for parasites treated with 7 at 5 μM (Figure 2). This organelle appeared as a huge, electron translucent vacuole, occupying a large fraction of the intracellular space. In fact, mitochondrion was only identified by the presence of the kinetoplast, the mitochondrial network of concatenated DNA circles, characteristic of trypanosomatids. Furthermore, the plasma membrane appeared mostly intact, which confirmed the above results with SYTOX green.

Effect on Mitochondrial Functionality. The cumulative evidence of the results obtained by the two previous techniques prompted us to get a deeper insight into mitochondrial functionality. In the first step, we examined the variation of $\Delta\Psi_m$, essential to drive the mitochondrial ATP synthesis, assessed by rhodamine 123 accumulation in promastigotes, and monitored by cytofluorometry. The $\Delta\Psi_m$ -driven accumulation of rhodamine showed a consistent decreasing trend after incubation with growing concentrations of 7 (Figure 3). The same result was obtained when the incubation was carried out for 4 h in HBSS, conditions used for luminescence and cytotoxicity (data not shown). Rhodamine 123 accumulation also decreased around 50% for most of the remaining leishmanicidal benzophenone-derived bisphosphonium compounds at their respective LC₅₀ (Table 1), confirming mitochondrial damage inflicted by these drugs. Nevertheless, compounds 13, 14, and 16 did not follow the same trend (Table 1), suggesting alternative targets.

In a step forward, we set out to define the molecular target of 7 in the mitochondrial ATP synthesis. To afford free access of specific substrates and inhibitors to the different complexes of the respiratory chain, digitonin-permeabilized

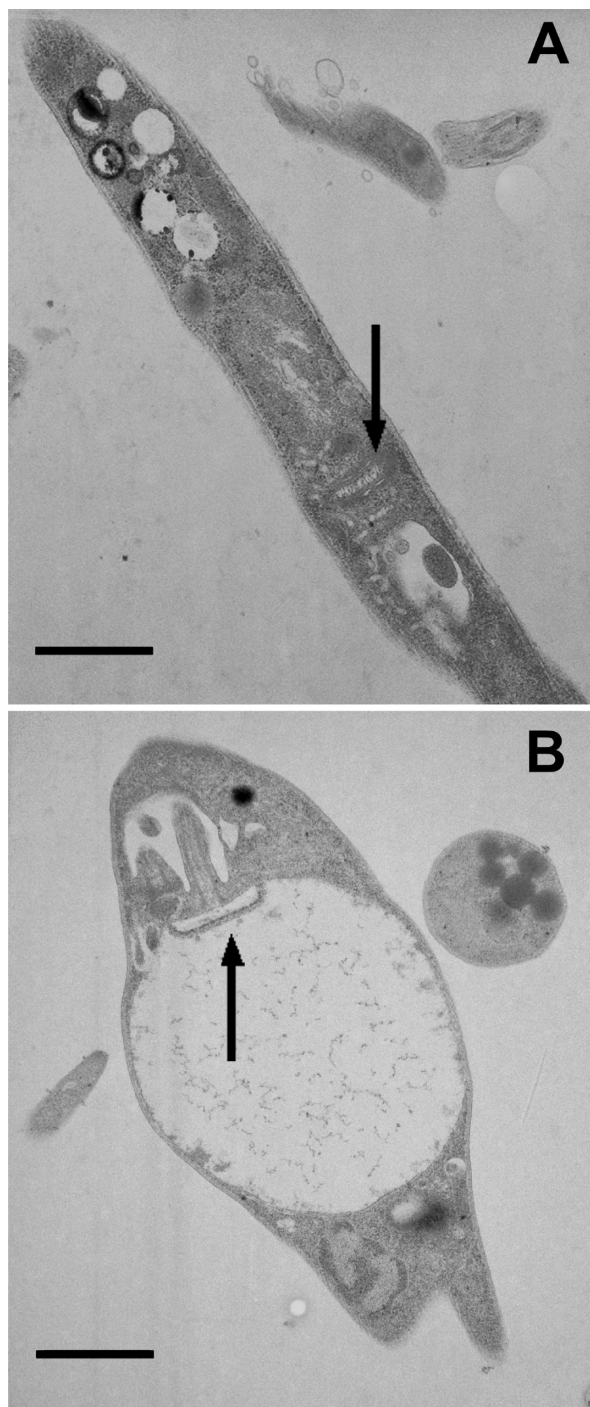


Figure 2. Electron microscopy of *L. donovani* promastigotes treated with compound 7: (A) control parasites; (B) parasites incubated for 4 h with 5 μM 7, a concentration that caused $\sim 80\%$ inhibition of proliferation. Mitochondrial kinetoplast is indicated by the arrow; bar, 1 μm .

parasites were used. The integrity of the inner mitochondrial membrane was preserved, as after ADP addition the respiration increased recovering state 3, in which ADP is no longer the limiting substrate for oxidative phosphorylation. At this state, oxygen consumption rate of digitonin-permeabilized parasites was $9.4 \text{ nmol} \times \text{min}^{-1} \times 10^{-8}$ cells (Figure 4B). The addition of 16 μM compound 7 to digitonized parasites leads to a full inhibition of the oxygen consumption rate using succinate as substrate (Figure 4B). Addition of 10 μM FCCP, a mitochondrial uncoupler, did

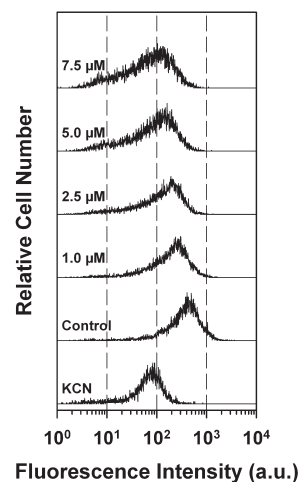


Figure 3. Variation of $\Delta\Psi_m$ of *L. donovani* promastigotes monitored by rhodamine 123 accumulation. Parasites treated with 7 overnight as well as control cells were loaded with 0.3 $\mu\text{g}/\text{mL}$ rhodamine 123, and fluorescence level was measured by cytofluorometry. Compound 7 concentration is shown at the side of each trace. Full depolarized parasites were obtained by incubation with 1 mM KCN.

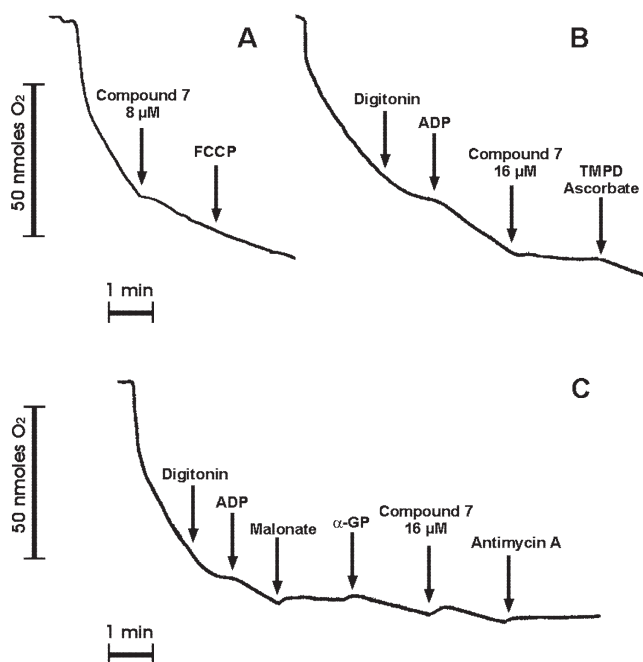


Figure 4. Variation of oxygen consumption rates of *L. donovani* promastigotes by 7. Bar scales for oxygen concentration (Y axis) and time (X axis) are included. Oxygen consumption rates were calculated from the corresponding slopes of the traces. Arrows stand for the addition of the indicated substrates and inhibitors at their respective final concentrations as stated: 10 μM FCCP, 60 μM digitonin, 100 μM ADP, 0.1 mM TMPD plus 1.7 mM ascorbate, 2 mM malonate, 6.7 mM α -glycerophosphate (α -GP), and 1 μM antimycin A: (A) intact parasites; (B, C) digitonin-permeabilized promastigotes.

not revert this inhibition, ruling out any specific inhibition of 7 on F_1F_0 -ATPase (Figure 4A). In contrast, addition of TMPD-ascorbate, an electron donor to cytochrome *c*,³⁰ restored the oxygen consumption rate at 50% of the initial value (Figure 4B). This excludes cytochrome *c* oxidase (complex IV) as the site of inhibition and circumscribes

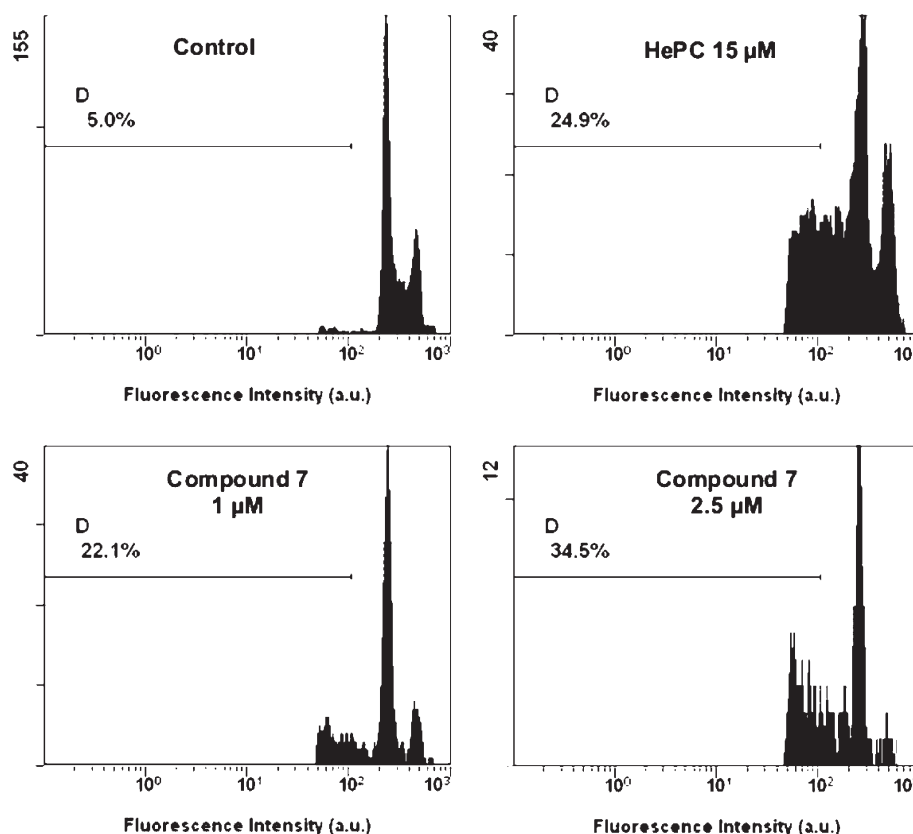
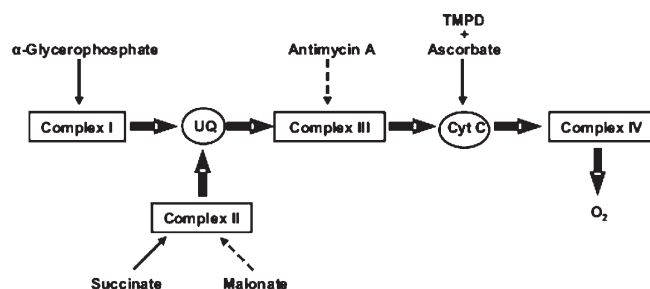


Figure 5. Assessment by cytofluorometry of apoptosis in *L. donovani* promastigotes treated with 7. The presence of a sub- G_1 peak, characteristic of the apoptotic cell population, was identified by nucleic acid staining with PI, and its percentage (D) is indicated in each histogram.

Scheme 2. Respiratory Chain in *Leishmania* with the Specific Substrates and Inhibitors Used in This Study^a



^aSites of electron feeding or inhibition are indicated by solid or dotted line, respectively. UQ, ubiquinone; Cyt C, cytochrome *c*.

the potential target upstream of cytochrome *c* (Scheme 2). The oxygen consumption rate inhibited by 7 was partially (33%) reinstated upon addition of 6.7 mM α -glycerophosphate (data not shown), a substrate of complex I.³⁰ Consequently, this result ruled out complexes I and III as targets for 7 and supports complex II as the more likely target for this drug. To verify this hypothesis, the succinate-dependent respiration was inhibited with malonate at the level of complex II, and oxygen consumption was allowed to proceed with α -glycerophosphate as the sole substrate through complex I. Under these conditions, i.e., without a functional complex II, compound 7 did not exert any inhibitory effect on the α -glycerophosphate-dependent respiration (Figure 4C), pointing at complex II as the main target within oxidative phosphorylation.

Leishmania Killing Involves an Apoptotic Process. Since the mitochondrion plays a pivotal role in the onset of apoptosis in *Leishmania*, the induction of apoptosis by compound 7 was investigated.

Overnight incubation of promastigotes with different concentrations of compound 7 led to an increase in the sub- G_1 peak region, accounting for fragmented chromatin and hence for apoptosis (Figure 5). The percentage of promastigotes with a PI fluorescence in the peak region typical of apoptotic cells was 22% at the LC₅₀, while in parasites treated with miltefosine, a typical apoptosis inducer in *Leishmania*,³¹ the percentage was 25% at 15 μ M, its LC₅₀ on promastigotes (Figure 5).

Discussion

The choice of phosphonium substituents with moderate hydrophobicity led to compounds with an intermediate lipophilicity capable of traversing the plasma membrane and accumulating in organelles driven by electrical potentials.²⁴ According to this hydrophobicity criteria for the substituents, from the set of compounds tested, those with leishmanicidal activity belong to this group. The substituents are medium length alkyl chains (five to eight carbons), aryl groups, or a combination of both. Nevertheless, the literature regarding these compounds is mostly based on the presence of a single phosphonium group in the molecule. Actually, the uptake of dications may be impaired by the double charge present in the molecule. In fact, the desolvation process (i.e., energy required to remove solvating water molecules) is the main energy demand to move a cation from the aqueous medium to

the hydrophobic lipid membrane.²¹ In practical terms, the bisphosphonium cations require a higher threshold hydrophobicity than monophosphonium compounds to traverse the membrane²¹ which for the set of compounds assayed was provided by the benzophenone scaffold connecting the two phosphonium moieties. As the entrance of the vital dye SYTOX green into parasites is nil, even at concentrations higher than those required for full lethality, plasma membrane permeabilization was ruled out as a major cause of parasite killing. Thereby, the lethal activity must involve intracellular targets and thus uptake of compound **7** and other active compounds by the parasite. Accordingly, the balance of hydrophobicity with charge, required for the translocation across the membrane, is finely tuned. In fact, changes in the overall polarity of the whole molecule, achieved with a single incorporation of a heterocycle with slightly higher polarity than the phenyl substituent, impaired (2-pyridinyl, **16**) or almost abrogated (2-furanyl, **19**) leishmanicidal activity. The same happened when hydrophobicity was decreased for short alkyl substituents (**1–6**, **10**, **11**). This exquisite modulation by subtle changes in the hydrophobicity of phosphonium substituents has been employed to optimize the mitochondrial uptake of MitoQ, consisting of an antioxidant ubiquinone coupled to a phosphonium group.^{31,32}

The cellular uptake and accumulation for phosphonium compounds are driven by electrical potential;²² hence, the plasma membrane potential and in a further step the higher $\Delta\Psi_m$ of the mitochondrion will lead to a massive accumulation of these compounds in this organelle.^{23,24,33} Although in this work we did not attempt to define the intracellular distribution of compound **7**, from the experimental results it was concluded beyond reasonable doubt that mitochondria constitute an essential target for that compound. This is demonstrated by morphological data, obtained from micrographs where the mitochondrion of *Leishmania* appeared dramatically swollen in parasites treated with **7**. This result was validated from a functional perspective; **7** provokes a prominent decrease of cytoplasmic ATP level, as assessed in real time by the rapid decay of luminescence of living 3-Luc parasites.²⁹ Once the leakage of intracellular ATP or its enhanced hydrolysis associated with membrane permeabilization was discarded as the reason for these effects, impairment of ATP synthesis, essentially of mitochondrial origin in *Leishmania*, came out as the most feasible target for compound **7**. In fact, this compound causes the decrease of $\Delta\Psi_m$, which drives mitochondrial ATP synthesis, a trend also observed for most of the compounds for which leishmanicidal effect is associated with inhibition of rhodamine 123 accumulation.

Finally, the mitochondrial dysfunction created by **7** was unequivocally pinpointed to the inhibition of complex II, according to the systematic dissection of the functionality of the whole set of components involved in the oxidative phosphorylation process.

According to experimental data provided by other authors, we surmise two major alternatives to account for the inhibition of complex II: either by an unspecific effect due to a general mitochondrial dysfunction or by a specific effect of **7** on complex II.

Under the hypothesis of an unspecific origin for the inhibition of complex II by **7**, this would likely be due to the perturbation of the biophysical parameters of the inner mitochondrial membrane after massive $\Delta\Psi_m$ -driven insertion of **7** as a lipophilic phosphonium compound. In fact, the

specific tumoricidal activity of phosphonium compounds was based on their larger accumulation in mitochondria, due to the significant higher $\Delta\Psi_m$ in transformed versus nontransformed cells.^{24,34–36} For *Leishmania*, the $\Delta\Psi_m$ values reported are similar to those for mammalian cells (-150 mV in *L. donovani* promastigotes³⁷ vs -140 to -180 mV in mammalian cells³⁸). Although plasma membrane potential is higher in *Leishmania* (about -100 to -110 mV)^{39–41} than in many typical eukaryotic resting cells (-30 to -60 mV),⁴² the only predicted effect will be a faster transfer of bisphosphonium compounds from the external medium into the intracellular space. Nevertheless, the final mitochondrial accumulation of **7**, driven solely by $\Delta\Psi_m$, will be similar in both types of cells. Additional facts to discard an unspecific effect are provided by the integrity of the inner mitochondrial membrane throughout the time span of the experiment, as well as preservation of the functionality of the rest of the components within the respiratory chain. This is evidenced by the unaltered oxygen consumption rate obtained in the presence of **7**, when the system was artificially fed with substrates outside complex II, i.e., α -glycerophosphate and TMPD-ascorbate.

Hence, a specific inhibition of compound **7** on the mitochondrial complex II is the most feasible scenario to account for its leishmanicidal activity. In cancer cells, other lipophilic cations such as rhodamine 123 or AA-1 (thiopyrilium) work by specific inhibition, in this case the F_1F_0 -ATPase.²⁵ Druggability of complex II in *Leishmania* has been reported for other compounds^{43,44} also leading to apoptosis of the parasite,⁴³ as happens with **7**.

The uniqueness of the complex II of *Leishmania* as target for **7** may be disputed on the basis of the following. (i) Its dicationic character will presumably afford binding to either nuclear or kinetoplast DNA, as described for its guanidine homologue, but this possibility was ruled out on the grounds of the poor affinity of **7** for DNA.⁷ (ii) Compound **7** is active on the bloodstream forms of the African trypanosome *T. b. rhodesiense*.⁵ It is well established that the bioenergetic requirements of this form of parasite are fulfilled by glycolysis rather than through oxidative phosphorylation,⁴⁵ as *Leishmania* does. Nevertheless, for this trypanosomal form the maintenance of a rudimentary mitochondrion with a residual $\Delta\Psi_m$ is essential, although sustained by the electrogenic activity of mitochondrial ATPase⁴⁶ without the course of cytochrome-containing complexes. Altogether these data suggest the existence of additional targets aside from complex II, at least for other trypanosomatids.

Conclusions

Although the potential of phosphonium compounds as leishmanicidal drugs has been previously described for African and American trypanosomes and very succinctly mentioned for *L. braziliensis* and *L. mexicana*, this work demonstrates the feasibility of benzophenone-derived bisphosphonium salts as new leishmanicidal lead compounds. A fine modulation of the activity and specificity was achieved by the adequate selection of substituent based on their hydrophobicity. Two additional facts stressed this assertion. First is the higher susceptibility of amastigotes, as the pathological form of the parasite in vertebrates, over promastigote, and second, compound **7** acts over complex II of the mitochondrial respiratory chain. The importance of this target for *Leishmania* is highlighted by the total dependence of this parasite on mitochondrial ATP synthesis to fulfill its

bioenergetic requirements. Furthermore, in quantitative terms, complex II is the main gate for the metabolic feeding of the respiratory chain. All these items make highly attractive the exploitation of bisphosphonium salts as new leishmanicidal leads. Further progress in SAR studies is currently ongoing.

Experimental Section

Chemistry. All dry solvents were purchased from Aldrich or Fluka in Sure/Seal bottles. All reactions requiring anhydrous conditions or an inert atmosphere were performed under a positive pressure of N₂. All reactions were monitored by HPLC–MS. ¹H and ¹³C NMR spectra were recorded on a Bruker Advance 300 or Varian Inova 400 spectrometer. Chemical shifts of the ¹H NMR spectra were internally referenced to the residual proton resonance of the deuterated solvents: CDCl₃ (7.24 ppm) and DMSO (δ 2.5 ppm). *J* values are given in Hz. Chemical shifts of the ¹³C NMR spectra were referenced to the carbon resonance of the deuterated solvents: CDCl₃ (77.23 ppm) and DMSO (δ 39.51 ppm). Melting points were determined in open capillary tubes with a SMP3-Stuart Scientific apparatus and are uncorrected. The solvent used for crystallization is indicated between parentheses. The purity of the compounds was ≥95% as assessed by combustion analysis or HPLC. Elemental analysis was performed on a Heraeus CHN-O rapid analyzer. Analytical results were within ±0.4% of the theoretical values unless otherwise noted. Analytical HPLC–MS was run with a Waters Sunfire C18 3.5 μm (4.6 mm × 50 mm) column on a Waters 2695 separation module coupled with a Waters Micromass ZQ spectrometer using electrospray ionization (ES⁺). The following HPLC conditions were used: column temperature = 30 °C, gradient time = 5 min, H₂O/CH₃CN (90:10 → 0:100) (HCO₂H 0.1%), flow rate = 1 mL/min, UV detection with diode array (λ = 190–400 nm). HRMS spectra were recorded on a QTOF 6520 Agilent Technologies mass spectrometer.

General Procedure for the Synthesis of the Bisphosphonium Salts 1–19. A Kimax tube was charged with 4,4'-bis(bromo-methyl)benzophenone⁵ (100 mg, 0.272 mmol) and flushed with argon. Anhydrous DMF (3 mL) was added followed by the phosphine (1.1 mmol, 4 equiv). The tube was flushed with argon, stopped, and the reaction mixture was stirred at 110 °C for 20 h. Different workup procedures were used depending on whether the product precipitated from the reaction mixture or not. For workup I, the mixture was allowed to cool to room temperature and the precipitated product was collected by filtration, rinsed successively with toluene and Et₂O, and dried under vacuum. For workup II, the reaction mixture was transferred to a flask. Then toluene (10 mL) was added to precipitate the product. The flask was stored in the refrigerator overnight. The supernatant was removed and the precipitate rinsed with toluene. Et₂O (10 mL) was added, and the precipitate was triturated with a spatula. The solid was collected, rinsed with Et₂O, and dried under vacuum.

The synthesis of compounds **7**⁵ and **15**^{47,48} was reported before. We report here for the first time the detailed ¹H and ¹³C NMR spectroscopic data for **15**.

4,4'-Bis((trimethylphosphonio)methyl)benzophenone Dibromide (1). The reaction was carried out following the general procedure with trimethylphosphine (1 M in toluene). The product was obtained as a colorless solid (82%) following workup I: mp 208–213 °C with previous softening (DMF/toluene). ¹H NMR (300 MHz, DMSO) δ 7.81 (d, *J* = 7.8, 4H, ArH), 7.52 (dd, *J* = 8.2, 2.3, 4H, ArH), 3.97 (d, *J* = 17.5, 4H, PhCH₂P), 1.86 (d, *J* = 14.7, 18H, CH₃). ¹³C NMR (75 MHz, DMSO) δ 195.21 (CO), 136.59 (d, *J* = 3.7, C_q), 135.01 (d, *J* = 9.1, C_q), 130.77 (d, *J* = 3.2, CH), 130.49 (d, *J* = 5.2, CH), 29.84 (d, *J* = 48.2, CH₂), 7.43 (d, *J* = 54.0, CH₃). LRMS (ES⁺) *m/z* 359.11 [(M – H)⁺], 180.23 [M²⁺, 100%]. ESI-HRMS *m/z* 359.1696 (M – H)⁺ (C₂₁H₂₉OP₂ requires 359.1688). HPLC ≥95% pure.

4,4'-Bis((triethylphosphonio)methyl)benzophenone Dibromide (2). The reaction was carried out following the general procedure using dry toluene instead of DMF and using triethylphosphine (1 M in THF). The yellow precipitate was collected by filtration and rinsed with toluene. Recrystallization from hot DMF afforded **2** as a yellowish solid (58%): mp 284–288 °C with previous softening (DMF). ¹H NMR (300 MHz, DMSO) δ 7.79 (d, *J* = 8.0, 4H, ArH), 7.58 (dd, *J* = 8.0, 2.0, 4H, ArH), 4.02 (d, *J* = 16.1, 4H, PhCH₂P), 2.26 (dq, *J* = 15.2, 7.6, 12H, CH₂-CH₃), 1.14 (dt, *J* = 18.3, 7.6, 18H, CH₃). ¹³C NMR (75 MHz, DMSO) δ 195.05 (CO), 136.54 (d, *J* = 3.4, C_q), 134.85 (d, *J* = 8.5, C_q), 130.76 (d, *J* = 6.7, CH), 130.68 (d, *J* = 3.9, CH), 24.92 (d, *J* = 44.1, PhCH₂P), 11.13 (d, *J* = 48.1, CH₃), 5.58 (d, *J* = 5.4, CH₃). LRMS (ES⁺) *m/z* 443.25 [(M – H)⁺], 222.32 [M²⁺, 100%]. ESI-HRMS *m/z* 443.2634 (M – H)⁺ (C₂₇H₄₁OP₂ requires 443.2627). HPLC ≥95% pure.

4,4'-Bis((tripropylphosphonio)methyl)benzophenone Dibromide (3). The reaction was carried out following the general procedure using dry toluene instead of DMF and using tri-*n*-propylphosphine. The mixture was allowed to cool to room temperature whereupon a yellow sticky residue was precipitated on the walls of the tube. The supernatant was discarded and the precipitate dissolved in a little DMF. Then **3** was obtained as a yellowish solid (61%) following workup I: mp 90–95 °C with previous softening (DMF/toluene). ¹H NMR (300 MHz, DMSO) δ 7.80 (d, *J* = 8.1, 4H, ArH), 7.57 (dd, *J* = 8.1, 1.9, 4H, ArH), 4.01 (d, *J* = 16.0, 4H, PhCH₂P), 2.24–2.17 (m, 12H, PCH₂CH₂), 1.53 (dp, *J* = 14.9, 7.3, 12H, CH₂CH₃), 1.01 (dt, *J* = 7.3, 1.2, 18H, CH₃). ¹³C NMR (75 MHz, DMSO) δ 194.64 (CO), 136.60 (d, *J* = 3.4, C_q), 134.92 (d, *J* = 8.6, C_q), 130.74 (d, *J* = 2.8, CH), 130.64 (d, *J* = 5.0, CH), 25.93 (d, *J* = 43.9, PhCH₂P), 20.01 (d, *J* = 46.2, PCH₂), 15.64 (d, *J* = 16.7, CH₃), 15.06 (d, *J* = 4.3, CH₃). LRMS (ES⁺) *m/z* 527.33 [(M – H)⁺], 264.35 [M²⁺, 100%]. Anal. Calcd (C₃₃H₅₄Br₂OP₂·2H₂O): C, 54.70; H, 8.07. Found: C, 54.56; H, 7.90.

4,4'-Bis((triisopropylphosphonio)methyl)benzophenone Dibromide (4). The reaction was carried out following the general procedure using dry toluene instead of DMF and using triisopropylphosphine (technical grade 90%). Compound **4** was obtained as a yellowish solid (59%) following workup I and recrystallization from DMF: mp 277–278 °C (DMF). ¹H NMR (300 MHz, DMSO) δ 7.78 (d, *J* = 8.1, 4H, ArH), 7.61 (dd, *J* = 8.1, 1.7, 4H, ArH), 4.20 (d, *J* = 14.9, 4H, CH₂P), 3.05–2.78 (m, 6H, CH), 1.31 (dd, *J* = 15.9, 7.2, 36H, CH₃). ¹³C NMR (75 MHz, DMSO) δ 194.78 (CO), 136.43 (d, *J* = 2.8, C_q), 135.55 (d, *J* = 8.0, C_q), 131.07 (d, *J* = 4.6, CH), 130.75 (CH), 22.04 (d, *J* = 40.4), 20.50 (d, *J* = 41.0), 16.63 (d, *J* = 3.2, CH₃). LRMS (ES⁺) *m/z* 527.27 [(M – H)⁺]. Anal. Calcd (C₃₃H₅₄Br₂OP₂·¹/₃ H₂O·¹/₃ C₃H₇NO): C, 56.80; H, 7.99, Br, 22.23. Found: C, 56.64; H, 8.38; Br, 22.08.

4,4'-Bis((tributylphosphonio)methyl)benzophenone Dibromide (5). The reaction was carried out following the general procedure with tri-*n*-butylphosphine. Compound **5** was obtained as a beige solid (87%) following workup II: mp 154.2–157.7 °C with previous softening (Et₂O). ¹H NMR (300 MHz, DMSO) δ 7.79 (d, *J* = 7.9, 4H, ArH), 7.57 (d, *J* = 7.9, 4H, ArH), 4.01 (d, *J* = 16.0, 4H, PhCH₂P), 2.19 (br m, 12H, PCH₂CH₂), 1.41 (br m, 24H, CH₂CH₃), 0.90 (t, *J* = 6.8, 18H, CH₃). ¹³C NMR (75 MHz, DMSO) δ 195.12 (CO), 136.64 (d, *J* = 3.4, C_q), 134.98 (d, *J* = 8.4, C_q), 130.67 (CH), 130.61 (CH), 25.90 (d, *J* = 43.8, PhCH₂P), 23.70 (d, *J* = 15.9, CH₂), 22.92 (d, *J* = 4.5, CH₂), 17.79 (d, *J* = 46.8, PCH₂), 13.60 (CH₃). LRMS (ES⁺) *m/z* 611.35 [(M – H)⁺], 306.38 [M²⁺, 100%]. ESI-HRMS *m/z* 611.4507 (M – H)⁺ (C₃₉H₆₅OP₂ requires 611.4505). HPLC ≥95% pure.

4,4'-Bis((triisobutylphosphonio)methyl)benzophenone Dibromide (6). The reaction was carried out following the general procedure with triisobutylphosphine. Compound **6** was obtained as an off-white solid (56%) following workup II: mp 79–85 °C (DMF/toluene). ¹H NMR (300 MHz, DMSO) δ 7.80 (d, *J* = 8.0, 4H, ArH), 7.60 (dd, *J* = 8.0, 1.8, 4H, ArH), 4.09 (d, *J* = 15.6, 4H,

PhCH₂P), 2.26 (dd, *J* = 13.1, 6.1, 12H, PCH₂CH), 2.12 (m, *J* = 6.1, 6H, CH), 1.02 (d, *J* = 6.6, 36H, CH₃). ¹³C NMR (75 MHz, DMSO) δ 195.13 (CO), 136.63 (d, *J* = 3.3, C_q), 134.91 (d, *J* = 8.7, C_q), 131.00 (d, *J* = 4.8, CH), 130.64 (d, *J* = 2.3, CH), 28.00 (d, *J* = 4.3, CH₂P), 27.62 (d, *J* = 42.8, CH₂P), 24.88 (d, *J* = 8.6, CH₃), 23.03 (d, *J* = 4.6, CH₂). LRMS (ES⁺) *m/z* 611.37 [(M - H)⁺], 306.33 [M²⁺, 100%]. ESI-HRMS *m/z* 611.4514 (M - H)⁺ (C₃₀H₆₅OP₂ requires 611.4505). HPLC 92% pure.

4,4'-Bis((tricyclohexylphosphonio)methyl)benzophenone Dibromide (8). The reaction was carried out following the general procedure with tricyclohexylphosphine. The mixture was concentrated to ~1 mL, resulting in a yellowish oil that was diluted with toluene (10 mL). Addition of Et₂O (10 mL) caused precipitation of an oily residue. The flask was stored in the refrigerator overnight. The supernatant was removed, and the oily precipitate was rinsed with toluene. Et₂O (10 mL) was added to the oily precipitate which was triturated with a spatula to afford **8** as a colorless solid (75%): mp 151–156 °C with previous softening (DMF/toluene/Et₂O). ¹H NMR (300 MHz, DMSO) δ 7.79 (d, *J* = 7.7, 4H, ArH), 7.57 (d, *J* = 7.7, 4H, ArH), 4.17 (d, *J* = 14.7, 4H, PhCH₂P), 2.58 (m, 6H, PCH), 2.15–1.14 (m, 60H, CH₂). ¹³C NMR (75 MHz, DMSO) δ 195.09 (CO), 136.64 (C_q), 135.79 (d, *J* = 7.9, C_q), 131.03 (d, *J* = 4.5, CH), 130.72 (CH), 29.89 (d, *J* = 39.4, PCH₂), 26.41 (CH₂), 26.24 (CH₂), 25.31 (CH₂), 21.80 (d, *J* = 39.9, PCH). LRMS (ES⁺) *m/z* 767.44 [(M - H)⁺], 383.99 [M²⁺, 100%]. ESI-HRMS *m/z* 767.5451 (M - H)⁺ (C₅₁H₇₇OP₂ requires 767.5444). HPLC ≥95% pure.

4,4'-Bis((trioctylphosphonio)methyl)benzophenone Dibromide (9). The reaction was carried out following the general procedure with tri-*n*-octylphosphine (technical grade 90%). Compound **9** was isolated as a yellowish hygroscopic solid (33%) following the same workup as described for **8**. ¹H NMR (300 MHz, CDCl₃) δ 7.57 (m, 4H, ArH), 7.45 (d, *J* = 7.6, 4H, ArH), 4.66 (d, *J* = 16.1, 4H, PhCH₂P), 2.31 (br m, 12H, PCH₂CH₂), 1.7–1.1 (m, 72H, (CH₂)₆), 0.79 (t, *J* = 5.5, 18H, CH₃). ¹³C NMR (75 MHz, CDCl₃) δ 194.53, 136.43, 134.37 (d, *J* = 9.1), 130.72, 130.64, 31.77, 30.99 (d, *J* = 14.8), 29.05 (d, *J* = 6.6), 22.70, 22.02 (d, *J* = 4.7), 19.71 (d, *J* = 24.1), 18.94, 14.18. LRMS (ES⁺) *m/z* 947.86 [(M - H)⁺], 473.96 [M²⁺, 100%]. ESI-HRMS *m/z* 473.4093 (M - H)²⁺ (C₆₃H₁₁₂OP₂ requires 473.4094). HPLC ≥95% pure.

4,4'-Bis((dimethylphenylphosphonio)methyl)benzophenone Dibromide (10). The reaction was carried out following the general procedure with dimethylphenylphosphine. Compound **10** was obtained as an off-white solid (79%) following workup II: mp 95–100 °C (dec). ¹H NMR (300 MHz, DMSO) δ 8.05–7.89 (m, 4H, ArH), 7.82 (dd, *J* = 7.6, 5.7, 2H, ArH), 7.76–7.61 (m, 8H, ArH), 7.31 (dd, *J* = 8.3, 2.4, 4H, ArH), 4.25 (d, *J* = 17.0, 4H, PhCH₂P), 2.25 (d, *J* = 14.5, 12H, CH₃). ¹³C NMR (75 MHz, DMSO) δ 195.15 (CO), 136.56 (d, *J* = 3.9, C_q), 134.62 (d, *J* = 2.6, C_q), 134.51 (d, *J* = 9.1), 132.31 (d, *J* = 10.0, CH), 130.58 (m, CH), 129.79 (d, *J* = 12.4, CH), 120.75 (d, *J* = 84.2, C_q), 30.83 (d, *J* = 46.5, CH₃), 6.42 (d, *J* = 54.5, CH₃). LRMS (ES⁺) *m/z* 483.13 [(M - H)⁺], 242.23 [M²⁺, 100%]. ESI-HRMS *m/z* 483.2000 (M - H)⁺ (C₃₁H₃₃OP₂ requires 483.2001). HPLC 94% pure.

4,4'-Bis((diethylphenylphosphonio)methyl)benzophenone Dibromide (11). The reaction was carried out following the general procedure with diethylphenylphosphine. Compound **11** was obtained as an off-white solid (60%) following workup II: mp 69–76 °C (DMF/toluene). ¹H NMR (300 MHz, DMSO) δ 8.04–7.78 (m, 6H, ArH), 7.72 (dt, *J* = 7.4, 3.7, 4H, ArH), 7.64 (d, *J* = 8.0, 4H, ArH), 7.29 (dd, *J* = 8.2, 2.1, 4H, ArH), 4.37 (d, *J* = 16.0, 4H, PhCH₂P), 2.81–2.59 (m, 8H, CH₂CH₃), 1.16 (dt, *J* = 19.2, 7.5, 12H, CH₃). ¹³C NMR (75 MHz, DMSO) δ 195.01 (CO), 136.51 (d, *J* = 3.6, C_q), 134.88 (d, *J* = 2.6, C_q), 134.27 (d, *J* = 8.5), 132.99 (d, *J* = 8.7), 130.60 (d, *J* = 5.6, CH), 130.52 (CH), 130.13 (d, *J* = 11.7, CH), 117.22 (d, *J* = 79.4, C_q), 26.74 (d, *J* = 44.7, CH₂P), 11.55 (d, *J* = 49.3, CH₂P), 5.76 (d, *J* = 5.0, CH₃). LRMS (ES⁺) *m/z* 539.2 [(M - H)⁺], 270.29 [M²⁺, 100%]. ESI-HRMS *m/z* 539.2635 (M - H)⁺ (C₃₅H₄₁OP₂ requires 539.2627). HPLC 92% pure.

4,4'-Bis((dicyclohexylphenylphosphonio)methyl)benzophenone Dibromide (12). The reaction was carried out following the general procedure with dicyclohexylphenylphosphine. Workup II followed by recrystallization from EtOH/Et₂O afforded **12** as a yellowish solid (33%): mp > 300 °C (dec). ¹H NMR (400 MHz, CDCl₃) δ 8.10 (t, *J* = 8.7, 4H, ArH), 7.67 (m, 6H, ArH), 7.12 (brs, 8H, ArH), 5.33 (d, *J* = 15.2, 4H, PhCH₂P), 3.09 (m, 4H, PCH), 2.25–1.00 (m, 40H, CH₂). ¹³C NMR (101 MHz, CDCl₃) δ 194.27 (CO), 135.70 (d, *J* = 3.2), 135.12 (d, *J* = 8.7), 134.32 (d, *J* = 2.3), 134.18 (d, *J* = 7.6), 130.83 (d, *J* = 4.8), 130.24, 130.10 (d, *J* = 5.9), 115.35 (d, *J* = 73.7, C_q), 31.28 (d, *J* = 42.3, PCH₂), 26.73, 26.55, 25.74, 24.82 (d, *J* = 40.7, PCH₂). LRMS (ES⁺) *m/z* 755.36 [(M - H)⁺], 378.21 [M²⁺, 100%]. ESI-HRMS *m/z* 755.4508 (M - H)⁺ (C₅₁H₆₅OP₂ requires 755.4505). HPLC ≥ 95% pure.

4,4'-Bis((diphenylmethylphosphonio)methyl)benzophenone Dibromide (13). The reaction was carried out following the general procedure with methylphenylphosphine. Compound **13** was obtained as an off-white solid (71%) following workup II: mp 110–113 °C (dec). ¹H NMR (300 MHz, DMSO) δ 7.97 (dd, *J* = 12.8, 7.3, 8H, ArH), 7.86 (dd, *J* = 8.0, 6.2, 4H, ArH), 7.74 (td, *J* = 7.6, 3.4, 8H, ArH), 7.61 (d, *J* = 7.9, 4H, ArH), 7.38–7.26 (m, 4H, ArH), 4.82 (d, *J* = 16.9, 4H, PhCH₂P), 2.63 (d, *J* = 14.1, 6H, CH₃). ¹³C NMR (75 MHz, DMSO) δ 195.03 (CO), 136.60 (d, *J* = 3.8), 135.02 (d, *J* = 2.5), 133.85 (d, *J* = 8.3), 133.06 (d, *J* = 10.1, CH), 130.93 (d, *J* = 5.3), 130.43 (d, *J* = 3.0), 130.16 (d, *J* = 12.4, CH), 120.07 (d, *J* = 84.6, C_q), 29.05 (d, *J* = 47.4, PCH₂), 4.86 (d, *J* = 55.6, CH₃). LRMS (ES⁺) *m/z* 607.13 [(M - H)⁺]. Anal. Calcd (C₄₁H₃₈Br₂OP₂·2C₃H₇NO): C, 62.79; H, 5.39, Br, 18.99. Found: C, 62.70; H, 5.83; Br, 18.74. HPLC ≥95% pure.

4,4'-Bis((diphenylethylphosphonio)methyl)benzophenone Dibromide (14). The reaction was carried out following the general procedure with ethyldiphenylphosphine. Compound **14** was obtained as an off-white solid (67%) following workup II: mp 118–123 °C with previous softening (DMF/toluene). ¹H NMR (300 MHz, DMSO) δ 8.05–7.65 (m, 20H, ArH), 7.54 (d, *J* = 7.8, 4H, ArH), 7.17 (m, 4H, ArH), 4.90 (d, *J* = 16.2, 4H, PhCH₂P), 3.02 (m, 4H, PCH₂CH₃), 1.14 (td, *J* = 7.4, 21, 6H, CH₃). ¹³C NMR (75 MHz, DMSO) δ 195.01 (CO), 136.56 (d, *J* = 3.7), 135.20 (d, *J* = 2.1), 133.77 (d, *J* = 9.5, CH), 130.76 (d, *J* = 5.2), 130.34 (CH), 130.18 (CH), 117.91 (d, *J* = 82.7, C_q), 27.11 (d, *J* = 45.7, PCH₂), 12.64 (d, *J* = 50.6, PCH₂), 5.97 (d, *J* = 4.7, CH₃). LRMS (ES⁺) *m/z* 635.18 [(M - H)⁺], 318.26 [M²⁺, 100%]. ESI-HRMS *m/z* 635.2628 (M - H)⁺ (C₄₃H₄₁OP₂ requires 635.2627). HPLC 92% pure.

4,4'-Bis((triphenylphosphonio)methyl)benzophenone Dibromide (15).^{47,48} The reaction was carried out following the general procedure with triphenylphosphine. Compound **15** was obtained as an off-white solid (66%) following workup II: mp > 200 °C (dec). ¹H NMR (300 MHz, DMSO) δ 8.30–7.61 (m, 30H, ArH), 7.52 (d, *J* = 7.8, 4H, ArH), 7.14 (d, *J* = 7.7, 4H, ArH), 5.33 (d, *J* = 16.1, 4H, CH₂). ¹³C NMR (75 MHz, DMSO) δ 195.08 (CO), 136.81 (d, *J* = 3.7, C_q), 135.60, 134.44 (d, *J* = 10.0), 133.47 (d, *J* = 8.4), 131.33 (d, *J* = 4.9), 130.56 (d, *J* = 12.5), 130.27 (d, *J* = 2.7), 117.93 (d, *J* = 85.9, C_q), 28.43 (d, *J* = 46.1, CH₂). LRMS (ES⁺) *m/z* 731.21 [(M - H)⁺], 366.3 [M²⁺, 100%]. HPLC ≥95% pure.

4,4'-Bis((diphenyl-2-pyridylphosphonio)methyl)benzophenone Dibromide (16). The reaction was carried out following the general procedure with diphenyl-2-pyridylphosphine. Compound **16** was obtained as a beige solid (69%) following workup II: mp 175–178 °C (dec). ¹H NMR (300 MHz, DMSO) δ 9.03 (d, *J* = 4.5, 2H, ArH, 6-H_{pyr}), 8.21 (dt, *J* = 7.8, 6.1, 2H, ArH), 8.13–7.98 (m, 2H, ArH), 7.92 (dd, *J* = 14.6, 6.1, 6H, ArH), 7.83–7.64 (m, 16H, ArH), 7.46 (d, *J* = 7.9, 4H, ArH), 7.20–7.04 (m, 4H, ArH), 5.29 (d, *J* = 16.4, 4H, CH₂). ¹³C NMR (75 MHz, DMSO) δ 195.01 (CO), 152.44 (d, *J* = 19.5), 144.04 (d, *J* = 116.4, C_q), 138.84 (d, *J* = 9.9), 136.65 (d, *J* = 3.7), 135.61 (d, *J* = 2.7), 134.62 (d, *J* = 9.8, CH), 133.46 (d, *J* = 8.4), 132.17 (d, *J* = 23.8), 131.30 (d, *J* = 5.5), 130.43 (d, *J* = 12.5, CH), 130.12 (d, *J* = 2.7), 128.99 (d, *J* = 3.2), 117.26 (d, *J* = 84.9, C_q), 27.93 (d, *J* = 46.4,

CH_2). LRMS (ES^+) m/z 733.12 [(M - H)⁺], 366.89 [M^{2+} , 100%]. Anal. Calcd ($\text{C}_{49}\text{H}_{40}\text{Br}_2\text{N}_2\text{O}_2$): C, 65.79; H, 4.51, N, 3.13. Found: C, 65.98; H, 4.80; N, 3.20.

4,4'-Bis((diphenyl-*o*-tolylphosphonio)methyl)benzophenone Dibromide (17). The reaction was carried out following the general procedure with diphenyl(*o*-tolyl)phosphine. Compound **17** was obtained as an off-white solid (55%) following workup II: mp 255.9–262.7 °C with previous softening (DMF/toluene). ¹H NMR (300 MHz, CDCl_3) δ 8.17 (br, 2H, ArH), 7.9–7.45 (m, 26H, ArH), 7.22 (m, 8H, ArH), 5.60 (d, $J = 15.0$, 4H, CH_2), 1.99 (s, 6H, CH_3). ¹³C NMR (75 MHz, CDCl_3) δ 195.58 (CO), 143.50 (d, $J = 8.4$), 137.01 (d, $J = 3.7$), 136.81 (d, $J = 10.9$), 136.0 (d, $J = 2.2$), 135.45 (d, $J = 2.4$), 134.53 (d, $J = 9.8$), 133.82 (d, $J = 11.0$), 133.19 (d, $J = 8.2$), 132.14 (d, $J = 5.4$), 130.75 (d, $J = 12.6$), 130.53 (d, $J = 2.4$), 128.65, 117.93 (d, $J = 84.7$, C_q), 116.55 (d, $J = 83.2$, C_q), 32.24 (d, $J = 47.6$, CH_2), 23.29 (d, $J = 4.4$, CH_3). LRMS (ES^+) m/z 759.35 [(M - H)⁺], 380.1 [M^{2+} , 100%]. ESI-HRMS m/z 759.2936 (M - H)⁺ ($\text{C}_{53}\text{H}_{45}\text{OP}_2$ requires 759.2940). HPLC 92% pure.

4,4'-Bis((diphenyl-*p*-tolylphosphonio)methyl)benzophenone Dibromide (18). The reaction was carried out following the general procedure with diphenyl(*p*-tolyl)phosphine. Compound **18** was obtained as an off-white solid (50%) following workup II: mp 296.9–298.5 °C (DMF/toluene). ¹H NMR (300 MHz, CDCl_3) δ 8.0–7.0 (m, 36H, ArH), 5.73 (br, 4H, CH_2), 2.44 (s, 6H, CH_3). ¹³C NMR (75 MHz, CDCl_3) δ 194.92 (CO), 146.47 (d, $J = 2.5$), 135.0 (br), 134.86, 134.68 (d, $J = 10.2$, CH), 133.13 (d, $J = 8.5$), 132.29 (d, $J = 5.0$), 131.23 (d, $J = 13.0$), 130.36 (d, $J = 12.5$, CH), 118.29 (d, $J = 86.0$, C_q), 114.08 (d, $J = 88.4$, C_q), 31.24 (d, $J = 46.1$, CH_2), 22.22 (CH_3). LRMS (ES^+) m/z 759.21 [(M - H)⁺], 380.24 [M^{2+} , 100%]. ESI-HRMS m/z 759.2929 (M - H)⁺ ($\text{C}_{53}\text{H}_{45}\text{OP}_2$ requires 759.2940). HPLC $\geq 95\%$ pure.

4,4'-Bis((tri-2-furanylphosphonio)methyl)benzophenone Dibromide (19). The reaction was carried out following the general procedure with tri-2-furylphosphine. Compound **19** was obtained as a beige solid (76%) following workup II: mp 214.5–223.7 °C with previous softening (DMF/toluene). ¹H NMR (300 MHz, CDCl_3) δ 8.03 (br m, 14H, ArH), 7.43 (m, 6H, ArH), 6.78 (br s, 6H, ArH, 4-*H*_{furanil}), 5.55 (br m, 4H, CH_2). ¹³C NMR (75 MHz, CDCl_3) δ 195.21 (CO), 154.06 (d, $J = 8.6$, CH), 137.14 (d, $J = 4.3$, C_q), 133.33 (d, $J = 22.0$, CH), 131.86 (d, $J = 6.3$, CH), 131.28 (d, $J = 9.9$, C_q), 131.05 (d, $J = 11.9$, CH), 130.1 (d, $J = 125.2$, C_q), 114.26 (d, $J = 10.0$, CH), 32.54 (d, $J = 53.4$, CH_2). LRMS (ES^+) m/z 671.08 [(M - H)⁺], 336.0 [M^{2+} , 100%]. ESI-HRMS m/z 671.1379 (M - H)⁺ ($\text{C}_{39}\text{H}_{29}\text{O}_7\text{P}_2$ requires 671.1383). HPLC $\geq 95\%$ pure.

Cultured Cells. Promastigotes from the *Leishmania donovani* strain MHOM/SD/00/IS-2D, its derived isogenic strain, 3-Luc, which expresses a C-terminal mutated form of luciferase (cytoplasmic luciferase), and *L. pifanoi* axenic amastigotes (MHOM/VE/60/Ltrod) were grown as described.^{29,49}

Peritoneal macrophages from BALB/c mice were obtained by previous elicitation of the mouse by a single intraperitoneal injection of 1 mL of 3% sodium thioglycolate, 4 days prior to macrophage harvesting by peritoneal lavage.

Unless otherwise stated, reagents were purchased from Sigma-España (Madrid, Spain). Propidium iodide, SYTOX Green, rhodamine 123 [2-(6-amino-3-imino-3*H*-xanthen-9-yl)benzoic acid methyl ester, chloride], and DMNPE-luciferin [d-luciferin 1-(4, 5-dimethoxy-2-nitrophenyl) ethyl ester] were purchased from Invitrogen (Barcelona, Spain).

Fluorescence and luminescence measurements were recorded in a Polarstar Galaxy microplate reader (BMG Labortechnologies, Offenburg, Germany) fitted with the corresponding optical setting, unless otherwise stated.

Cytotoxic Activity Measurements. Promastigotes and amastigotes were harvested at the late exponential phase, washed twice in Hanks' buffer, supplemented with 10 mM D-glucose (pH 7.2) (HBSS-Glc) at 4 °C, and resuspended in the same buffer at 2×10^7 cells/mL. These standard conditions were maintained

for the rest of the experiments unless otherwise stated. Aliquots (120 μL) of this cellular suspension were incubated with the drugs for 4 h, at either 25 or 32 °C for promastigotes or amastigotes, respectively. Afterward, 20 μL aliquots of this suspension were taken, added to 1 mL of HBSS-Glc, and centrifuged to remove unbound reagent. Washed parasites were resuspended in 100 μL of their respective growth medium devoid of phenol red, transferred into a new 96-well microplate, and allowed to proliferate (48 h, 25 °C for promastigotes; 96 h, 32 °C for amastigotes). Finally, an amount of 100 μL of MTT solution (1 mg/mL) in HBSS-Glc was added and substrate reduction allowed to proceed for 2 h at 25 or 32 °C for promastigotes or amastigotes, respectively. Precipitated formazan was solubilized by addition of 100 μL of 10% (w/v) SDS solution and read in a 450 Bio-Rad ELISA microplate reader equipped with a 600 nm filter.²⁹ To measure the short-term effect, an amount of 100 μL (1.6×10^6 promastigotes) of the remaining parasites was washed with 1 mL of HBSS-Glc, resuspended, and assayed for MTT reduction as above.

The induction of apoptosis by **7** was monitored by analysis of the sub- G_1 peak in parasites incubated overnight with different concentrations of **7**. After the incubation, parasites were stained with 50 $\mu\text{g/mL}$ propidium iodide (PI) and individual cellular fluorescence was measured in a Beckman Coulter FC500 MPL cytofluorometer. As positive control for apoptosis induction, parasites were treated in identical conditions with 15 μM miltefosine (hexadecylphosphocholine).³¹

BALB/c peritoneal macrophages were resuspended in their culture medium at a final density of 10^6 cells/mL, plated in a 96-well culture microplate (100 μL /well), and incubated with compound **7** for 24 h at 37 °C. Cytotoxicity was assessed using the colorimetric MTT reduction assay and expressed as the percentage of the value obtained for control cells.

Changes in Intracellular ATP Levels. The in vivo variation of intracellular ATP levels in *L. donovani* promastigotes was measured in 3-Luc parasites expressing a cytoplasmic form of *Photinus pyralis* luciferase.²⁹ Briefly, the substrate for luciferase, DMNPE-luciferin, was prepared as a 5 mM stock solution in DMSO. It was added to the 3-Luc promastigote suspension (2×10^7 cells/mL) in HBSS-Glc at a final concentration of 25 μM , and aliquots of this suspension were immediately distributed into a 96-well microplate (100 μL /well). Compound **7** was added once the luminescence reached a plateau. This point was considered as time zero and its luminescence taken as 100%. Changes in luminescence were recorded for 80 min using a luminescence optics setting. The measurements were averaged every 4 s. In vitro inhibition of luciferase (EC 1.13.12.7) by the compound was tested as described²⁹ using a commercial purified enzyme (Roche Applied Sciences, Madrid, Spain).

Plasma Membrane Permeabilization. It was assessed by the entrance of the vital dye SYTOX green (MW 600), according to the procedure described previously.⁵⁰ Briefly, compound **7** was added to *L. donovani* promastigotes in HBSS-Glc (2×10^7 promastigotes/mL) preincubated with 1 μM SYTOX green. Entrance of the dye into the intracellular compartment is only afforded through injured membranes, with lesions large enough to allow dye passage. The increase of fluorescence, due to binding of the dye to intracellular nucleic acids, was monitored using 485 and 520 nm filters for excitation and emission wavelengths, respectively. Full permeation was considered as that achieved by 0.1% Triton X-100 (TX-100).

Electron Microscopy. Promastigotes were incubated with compound **7** according to the standard procedure, washed twice in PBS, and fixed in 5% (w/v) glutaraldehyde in the same medium. Afterward, parasites were included with 2.5% (w/v) OsO_4 for 1 h, gradually dehydrated in ethanol (30%, 50%, 70%, 90%, and 100% (v/v); 30 min each) and propylene oxide (1 h), embedded in Epon 812 resin, and observed in a Jeol-1230 electron microscope as described.⁵⁰

Effects on Mitochondrial Activity. Variation of the mitochondrial membrane potential ($\Delta\Psi_m$) of promastigotes was measured

by rhodamine 123 accumulation.³⁹ To this end, promastigotes were incubated in their growth medium with the leishmanicidal benzophenone-derived bisphosphonium compounds at their respective LC₅₀ overnight, and dye incorporation was measured in a Beckman Coulter FC500 MPL cytofluorometer (excitation and emission wavelengths of 488 and 525 nm, respectively). Parasites either untreated or fully depolarized by incubation with 1 mM KCN were used as controls. Following the same protocol, a more extensive range of concentrations was used to study the effect of compound **7** on rhodamine 123 accumulation.

Oxygen consumption rates were measured in a Clark oxygen electrode (Hansatech, KingsLynn, U.K.) at 25 °C, using 1 mL of a promastigote suspension (10⁸ cells/mL) in respiration buffer supplemented with 5 mM succinate and 1 mg/mL fatty acid free BSA as described.³⁷ Cells were permeabilized with 60 μM digitonin, which allows a selective permeation of the plasma membrane but not of the inner mitochondrial membrane.⁵¹ Afterward, 100 μM ADP was added to restore state 3 of respiration, and once a steady rate was reached, compound **7** was added, followed by a selective set of substrates and inhibitors specific for the different complexes of the respiratory chain, at concentrations stated in the corresponding figures.

Statistical Analysis. Data represent the mean of triplicate samples ± SD. LC₅₀ values were calculated from the sigmoidal inhibition curves using the SigmaPlot 11.0 software.

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