Design and Synthesis of Potent Antileishmanial Cycloalkylidene-Substituted Ether Phospholipid Derivatives

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Two series of novel ether phospholipids (EPs) have been synthesized. The first includes cyclodecylidene- or cyclopentadecylidene-substituted EPs carrying *N,N,N*-trimethylammonium or *N*-methylpiperidino or *N*-methylmorpholino head groups. The second series encompasses more rigid head groups in combination with cycloalkylidene moieties in the lipid portion. In addition, hydrogenated derivatives were obtained. All the new analogues, except **33**, were 1.5- to 62-fold more potent than miltefosine against the intracellular *L. infantum*, and the most active ones were also less cytotoxic against the human monocytic cell line THP1 and less hemolytic than miltefosine. The analogues that combine high potency with low cytotoxicity and hemolytic activity were **19**, **37**, **21 23**, **38**, **39**, and **40**. Cyclopentadecylpentylphosphocholine (**38**) possesses an IC_{50} of 0.7 μM against *L. infantum* amastigotes and is the least cytotoxic analogue, since it does not present toxicity against THP1 macrophages, even at a concentration that is 800-fold the antiparasitic IC_{50} value, and does not present significant hemolytic activity.

Introduction

Leishmaniasis, one of the most neglected tropical diseases, is a parasitic disease that is transmitted by the bite of the infected phlebotomine sandfly and constitutes a major public health problem especially in the tropical and subtropical regions of the world. It is currently endemic in 88 countries on five continents (Africa, Asia, Europe, and North and South America), and the population at risk reaches 350 million people. The parasite exists in two different forms: the flagellated one in the gut of the sandfly vector and the amastigote in the mammalian host that is the cause of the acute desease.

Visceral leishmaniasis (VL[a], or "kala-azar"), mucocutaneous leishmaniasis (MCL, ulceration of the skin and hyperdevelopment of the mucous membranes), and cutaneous and diffuse cutaneous leishmaniasis (CL and diffuse CL) are the distinguishable forms of the disease, which includes several clinical syndromes and, if untreated, can have devastating consequences. Establishment of the infection and progression of the disease are favored by the compromised immune system of patients, like those infected with HIV, and as a result, leishmania/HIV coinfection is now considered an extremely serious new disease with severe clinical, diagnostic, chemotherapeutic, epidemiological, and economic implications.[1]

Even though chemotherapy is currently the only way to treat the various forms of leishmaniasis, since no vaccine is yet available, the arsenal of drugs against the disease is still limited. Today, first line antileishmanial drugs include pentavalent antimonials (sodium stibogluconate and meglumine antimonate), pentamidine, and amphotericin B.[2]

The high resistance developed to pentavalent antimonials, high-dose regimens, and long treatment courses using parenteral administration, as well as, renal and cardiac toxicity, are major drawbacks. On the other hand, the high toxicity and declining efficacy of pentamidine have restricted its use. Amphotericin B and its lipid formulation proved to be very effective in the treatment of leishmaniasis, but the cost is still prohibitively high. Paromomycin and sitamaquine are two compounds currently undergoing clinical trials.[3,4]

The serious problems associated with the treatment of leishmania infections, such as development of drug resistance, the need for low cost drugs, and the limited funding available for parasitic diseases, have recently led many research groups to design and synthesize novel antileishmanial compounds as well as screen natural products for their antileishmanial activity. Most recent examples of synthetic compounds include nitrogen heterocycles, such as quinoline,[5,6] dihydropyridopyrimidine,[7] and pyrazolopyridine[8] derivatives as well as nitrogen-containing biphosphonates[9] and arylanthranilodinitriles.[10] Moreover, screening of natural products has identified several promising leads and has provided new scaffolds for chemical derivatization.[11–15]

In 1987, the "serendipitous" discovery of the antileishmanial activity of hexadecylphosphocholine (miltefosine, HePC),[16] an alkyllysophospholipid initially developed as an antitumor agent, constituted a major breakthrough in antileishmanial chemotherapy. This compound is effective against both visceral and cutaneous leishmaniasis, displays good bioavailability, and is currently registered as an oral drug for the treatment of the disease in India (in 2002) and Colombia (in 2005).[17] According to the latest clinical studies in Colombia, cure rates of 91−100% were obtained with a dose of 2.5 (mg/kg)/day for 28 days, with mild side effects (mostly gastrointestinal) in 35–60% of the patients.[18]

Despite its advantages, miltefosine has a long half-life (100–200 h) in humans and a low therapeutic ratio, characteristics that could encourage development of resistance, especially

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[a] Abbreviations: HePC, hexadecylphosphocholine; HIV, human immunodeficiency virus; VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; GPI, glycosylphosphatidylinositol; PI, propidium iodide.

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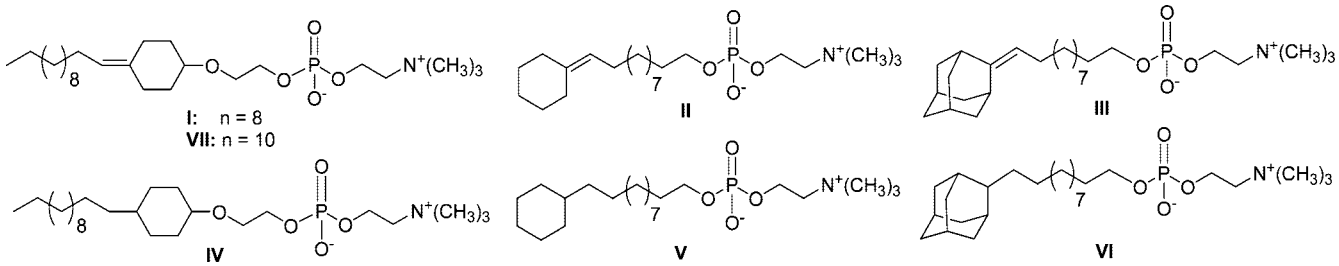


Figure 1. Structures of ether phospholipids **I**−**VII**.

in India where VL is an anthroponosis. Moreover, it is not suitable for pregnant women because it has been shown to cause teratogenesis in animals[19] and it did not give satisfactory results when administered to HIV-coinfected patients, since most of them relapsed.[20] The efficacy of miltefosine against other non-Indian VL and the efficacy against the wide range of CL syndromes in the New and the Old World are also major issues that have to be addressed.[21]

The mode of action of miltefosine is not yet fully understood. Several molecular targets in trypanosmatids have been suggested, including alkyl lipid metabolism and phospholipid biosynthesis[22] of the parasite, glycosylphosphatidylinositol (GPI) anchor biosynthesis, signal transduction[23] and induction of apoptosis,[24,25] and inhibition of the glycosome-located alkyl-specific acyl-CoA acyltransferase, an enzyme involved in lipid remodeling.[22] Recently, it has been demonstrated that miltefosine is inserted into the membrane by miscibility and interacts with sterols.[26] Miltefosine and the phospholipids of analogous structure edelfosine and perifosine as well as fluorescent phospholipid derivatives have been shown to be taken up across the plasma membrane by a specific protein-dependent translocation step that requires a miltefosine transporter and a protein factor, both recently identified and characterized. Inactivation of this inward-directed translocation activity causes resistance to miltefosine in vitro against both the promastigote and amastigote stages of the parasite and influences the parasite burden in the liver of infected mice.[27] Laboratory-induced resistance to miltefosine has also been shown to be related to two mutations on the aminophospholipid translocase.[28a] Recently, it has been reported that alkylphosphocholines can be recognized and actively transported out of the leishmania cell by an ABCG-like transporter, which belongs to the ATP-binding cassette transporters, suggesting that the parasite can employ multiple resistance mechanisms.[28b]

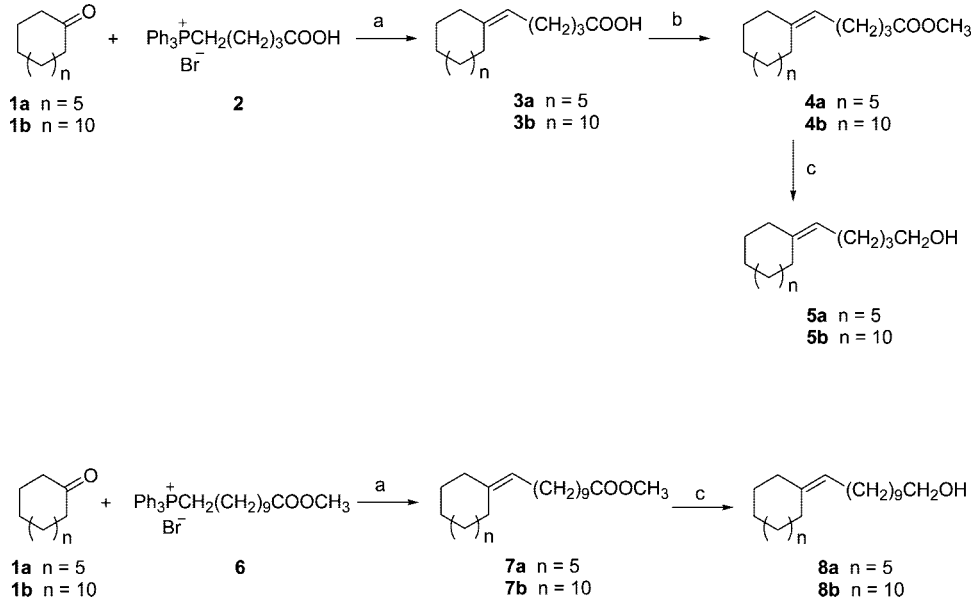
The current knowledge on the structure−antiparasitic actitvity relationships of miltefosine analogues is limited.[29,30] In addition, miltefosine is not the most active drug among compounds of the phospholipid structural family because some alkyllysophospholipid analogues that have been synthesized and tested for their antileishmanial activity have exhibited higher activity.[16,17,29,30] With the issues of resistance, side effects and production cost still requiring special attention and the mechanism of action still requiring elucidation, the field of design and synthesis of novel antileishmanial phospholipid derivatives is an ongoing challenge.

In this context, we recently reported[30,31] the synthesis of a series of ring-substituted ether phospholipids, incorporating 4-alkylidenecyclohexyloxylethyl, cyclohexylidene, or adamantylidene groups in the lipid portion and polar head groups other than choline in order to evaluate how these structural changes affect the antileishmanial activity of the compounds. Our results indicated that introduction of cycloalkane rings in alkylphosphocholines provides compounds with enhanced activity, especially against the strain *L. infantum*, which causes the AIDS associated coinfection in Europe. In particular, the presence of a 2-(4-alkylidenecyclohexyloxy)ethyl moiety renders the resulting compounds more active against *L. infantum* promastigotes while the presence of *ω*-cyclohexylidenealkyl or *ω*-adamantylidenealkyl groups results in enhanced activity against *L. donovani* promastigotes. The most active analogues against both *L. donovani* and *L. infantum* promastigotes were 2-(4-dodecylidenecyclohexyloxy)ethylphosphocholine (**I**), 11-cyclohexylideneundecylphosphocholine (**II**), 11-adamantylideneundecylphosphocholine (**III**), their hydrogenated analogues (**IV**−**VI**), and 2-(4-tetradecylidenecyclohexyloxy)ethylphosphocholine (**VII**), which were all more potent than miltefosine (Figure 1). Moreover, preliminary evaluation of the cytotoxicity of the most active ether phospholipids on the human monocytic cell line THP1 revealed that the new compounds 11-cyclohexylideneundecylphosphocholine and 11-adamantylideneundecylphosphocholine were not only more potent antileishmanial agents but also less cytotoxic than miltefosine.[31]

Our lead discovery of the new antileishmanial ring-substituted ether phospholipid derivatives urged more in-depth assessments of the structural requirements for enhanced antileishmanial activity and minimal cytotoxicity for this class of compounds. Thus, we have designed and synthesized two series of novel compounds. In the first series we increased the ring size of the cycloalkane functionality in the lipid portion by incorporating cyclodecylidene or cyclopentadecylidene groups linked to the phosphate polar headgroup by an oligomethylene bridge of 5 or 11 carbons, as previously reported.[30] In addition, we have varied the polar headgroup, using *N,N,N*-trimethylammonium, *N*-methylpiperidino, or *N*-methylmorpholino moieties. In the second series, in order to explore the influence of the flexibility of the headgroup on the activity of the compounds, we incorporated more rigid head groups, namely, the *trans*-2-hydroxy-*N,N,N*-trimethylcyclopentanamino or the *trans*-2-hydroxy-*N,N,N*-trimethylcyclohexanamino group, the 3-(2-hydroxyethylidene)-8,8-dimethyl-8-azoniabicyclo[3.2.1]octane group and (4-hydroxy-but-2-ynyl)trimethylammonium group in combination with cyclopentadecylidene-, cyclohexylidene-, and adamantylidene-substituted lipid portions. Finally, we synthesized hydrogenated analogues of the first series in order to explore the effect of the double bond on the activity and the toxicity of the compounds.

The antileishmanial activity of the new analogues was evaluated in vitro against the promastigote forms of *L. infantum* and *L. donovani* and against the intracellular amastigote form of *L. infantum*. The cytotoxicity of the new compounds was assessed on the human monocytic cell line THP1. Moreover, since hemolysis is a problem associated with alkylphosphocho-

Scheme 1[a]



[a] Reagents and conditions: (a) $[(CH_3)_3Si]_2NK$, THF; (b) MeOH, H^+, 40 °C; (c) $LiAlH_4$, THF.

lines, preventing their use in injectable form, we studied the hemolytic properties of the new compounds on human red blood cells.

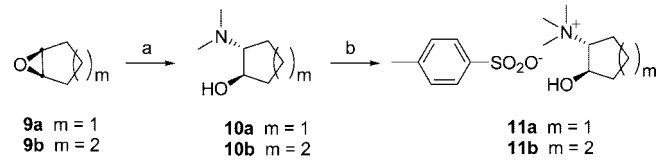
Chemistry

The synthetic strategy followed for the preparation of the ether phospholipids **19–36** is depicted in Schemes 5 and 6 and involves phosphorylation of the appropriate alcohols using $POCl_3$ and subsequent attachment of the desired head groups. For the synthesis of the required starting alcohols **5a**,**b** (Scheme 1) a Wittig reaction between the cyclodecanone (**1a**) or cyclopentadecanone (**1b**) and 4-carboxybutyl triphenylphosphonium bromide (**2**) was employed. The resulting unsaturated acids **3a**,**b** were transformed to the corresponding methyl esters **4a**,**b** which, after treatment with $LiAlH_4$ in THF, produced the alcohols **5a**,**b** in high yields (82%). Alcohols **8a**,**b** were obtained in a similar manner (Scheme 1); however, in this case 10-methoxycarbonyldecyltriphenylphosphonium bromide (**6**)[30] was employed in the Wittig reaction, and as a result, the corresponding unsaturated esters **7a**,**b** were obtained directly. Reduction of esters **7a**,**b** afforded the desired alcohols **8a**,**b** in almost quantitative yields (91–100%). Alcohols **8c** and **8d** were prepared as previously described.[30]

The required head groups were obtained after quaternization of *N*-methylpiperidine or *N*-methylmorpholine to *N*-(2-hydroxyethyl)-*N*-methylpiperidinium bromide or *N*-(2-hydroxyethyl)-*N*-methylmorpholinium bromide, respectively, which was effected upon treatment with 1-bromoethanol, as previously described.[30] The synthesis of the sterically constrained *trans*-2-hydroxy-*N*,*N*,*N*-trimethylcyclohexanamino and *trans*-2-hydroxy-*N*,*N*,*N*-trimethylcyclopentanamino head groups is depicted in Scheme 2 and involves aminolysis of the epoxide ring of **9a** or **9b**, with aqueous dimethylamine to provide the corresponding *trans*-amino alcohols **10a** and **10b**.[33] N-Methylation of **10a,b** by *p*-toluenesulfonate yields the respective quaternized amines **11a** and **11b**, isolated as the *p*-toluenesulfonate salts after recrystallization from acetone.

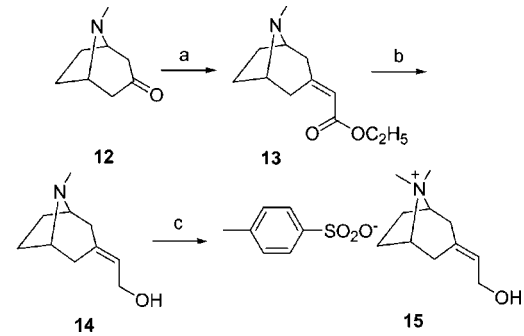
In addition, we prepared the bicyclic headgroup **15** by performing a Wadsworth−Emmons reaction between tropinone (**12**) and triethyl phosphonoacetate followed by reduction of the

Scheme 2[a]



[a] Reagents and conditions: (a) $(CH_3)_2NH$, 40% in H_2O; (b) methyl *p*-toluenesulfonate, Et_2O, room temp.

Scheme 3[a]



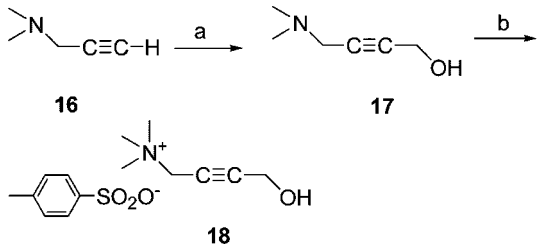
[a] Reagents and conditions: (a) $[(CH_3)_3Si]_2NK$, $(C_2H_5O)_2P(O)CH_2C(O)C_2H_5$, THF, 80 °C; (b) $LiAlH_4$, THF; (c) methyl *p*-toluenesulfonate, Et2O, room temp.

derived ester to the allylic alcohol **14** and methylation of the amino group to the corresponding *p*-toluenesulfonate salt **15** (Scheme 3).

The variety of sterically constrained head groups that we used for our structure–activity relationship studies was further enriched with compound **18**, which bears a carbon-carbon triple bond. The synthesis of this compound is shown in Scheme 4 and involves hydroxymethylation of the terminal acetylenic carbon of 3-(dimethylamino)-1-propyne (**16**) followed by quaternization to the *p*-toluenesulfonate salt.

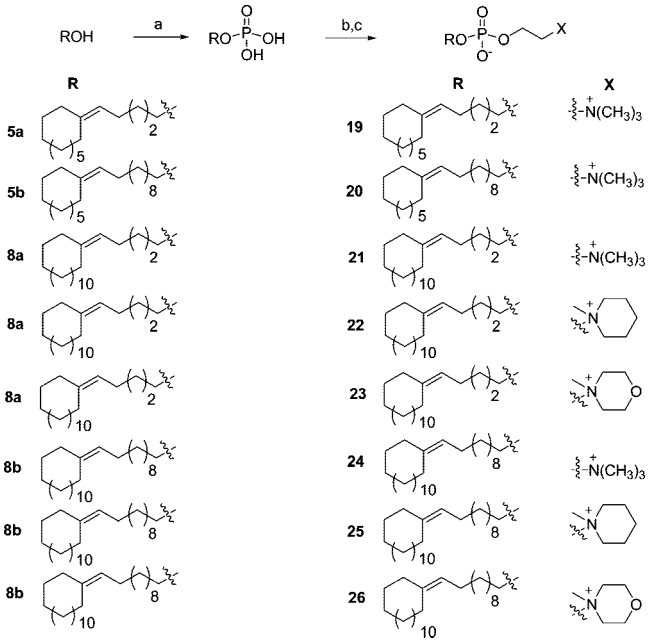
The synthesis of the desired ether phospholipids was affected via two methods. Method A (Scheme 5), which we have previously employed to synthesize other phospholipid derivatives,[30,34] involves phosphorylation of the alcohols with phos-

Scheme 4[a]



[a] Reagents and conditions: (a) *n*-BuLi, HCHO, THF; (b) methyl *p*-toluenesulfonate, Et_2O, room temp.

Scheme 5[a]

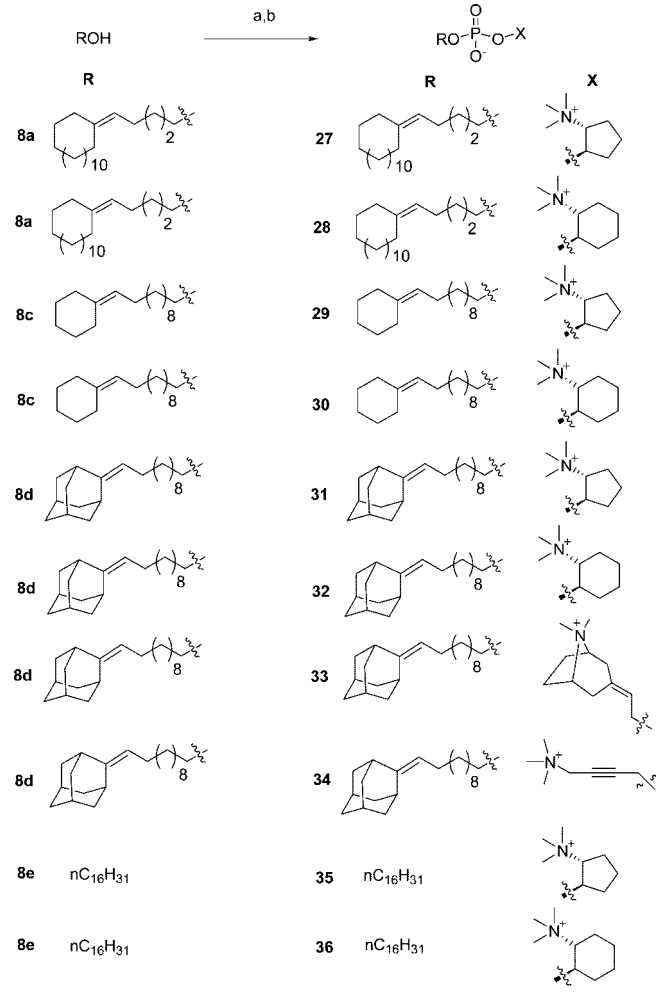


[a] Reagents and conditions: (a) (1) $P(O)Cl_3$, Et_3N, THF; (2) H_2O, 2-propanol; (b) pyridine, 40 °C; (c) pyridine, MSNT, $HOCH_2CH_2N^+R^1R^2R^3$, 40 °C.

phorus oxychloride in the presence of triethylamine in THF, followed by hydrolysis and treatment with pyridine in order to form the corresponding pyridinium salts. These were in turn coupled to the appropriate choline, *N*-methylpiperidino or *N*-methylmorpholino head groups in the presence of 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazolide (MSNT) as a condensing agent. This method was successfully used for the synthesis of compounds **19**–**26**, but it did not prove to be efficient in the synthesis of compounds **27**–**36** substituted by the constrained head groups, probably because of steric hindrance. As a result, the more direct one-pot phosphorylation method B (Scheme 6) was employed in which the appropriate alcohols **8a**−**d** were treated with $POCl_3$, and to the dichlorophosphate ester formed in situ was added a pyridine/chloroform solution of the *p*-toluenesulfonate salt of the corresponding quaternized amino alcohol (**11a**, **11b**, **15**, and **18**) to afford after hydrolysis the desired phospholipids **27**–**36**.

The lipid portion of phospholipids **27**–**32** was selected on the basis of the highest activity and minimum toxicity exhibited by the novel ether lipids **19**−**26** and our previous findings on analogous lead compounds.[30] Thus, from 5-(cyclopentadecylidene)pentanol (**5b**) we prepared compounds **27** and **28** and from 11-cyclohexylideneundecanol **(8c)** and 11-adamantylideneundecanol **(8d)** (prepared as described previously[30]) we obtained compounds **29**–**34**. In addition, we have synthesized

Scheme 6[a]



[a] Reagents and conditions. Method B: (a) (1) $P(O)Cl_3$, Et_3N, THF; (2) **11a**, **11b**, **15**, or **18**, pyridine,$CHCl_3$; (b) H_2O, 2-propanol.

the miltefosine analogues **35** and **36**, incorporating the sterically hindered *trans*-2-hydroxy-*N*,*N*,*N*-trimethylcyclopentanamino and *trans*-2-hydroxy-*N*,*N*,*N*-trimethylcyclohexanamino head groups.

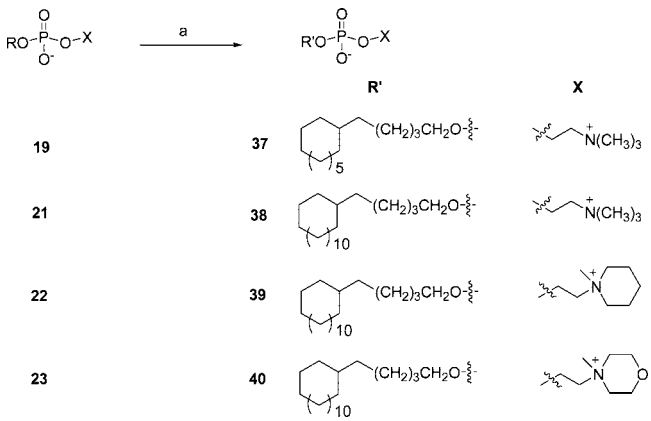
Finally, in order to investigate the effect of the double bond present in the lipid portion of our compounds, we synthesized the saturated analogues of selected ether phospholipids (Scheme 7). Thus, catalytic hydrogenation of compounds **19**, **21**, **22**, and **23** using 10% Pd/C in methanol provided compounds **37**, **38**, **39**, and **40**, respectively.

Biological Evaluation

Two in vitro models were employed for the evaluation of antileishmanial activity, as described in the Experimental Section. Thus, the new analogues **19**–**40** (Schemes 5−7) were evaluated in vitro against promastigote cultures of *Leishmania donovani* MON 703 and *Leishmania infantum* MON 235 (Table 1). Furthermore, the antiparasitic activity of derivatives **19**–**40** was assessed using the intracellular amastigote form of *Leishmania infantum* MON 235 in infected human monocytic THP-1 cells (Table 1).

The adverse effects of the new derivatives were evaluated in two systems. In the first system we assessed the apoptosis effect of the compounds against the THP-1 host cell line, and in the second system we evaluated the compounds' toxic effect on

Scheme 7[a]



[a] Reagents and conditions: (a) H_2, 10% Pd/C, MeOH, 1 atm.

Table 1. In Vitro Antileishmanial Activity[a] against the Promastigote Forms of *L. infantum* and *L. donovani* and the Intracellular Amastigote Form of *L. infantum*

	potency promastigotes IC_{50} (μM)		intracellular amastigote form *L. infantum*
compd	*L. infantum* MON 235	*L. donovani* MON 703	MON 235 IC_{50} (μM)
19	76.8	<3.2	3.3 ± 0.6
20	2.9 ± 0.3	1 ± 0.24	0.11 ± 0.02
21	2.2 ± 0.7	2.3 ± 0.7	3.8 ± 0.8
22	1.7 ± 0.5	2 ± 0.9	4.1 ± 0.9
23	1.8 ± 0.5	1.94 ± 0.5	4.4 ± 0.5
24	100	68.2	0.75 ± 0.04
25	84.5	65.9	3.6 ± 0.3
26	50.2	41.5	0.9 ± 0.1
27	<3.1	<3.1	0.2 ± 0.02
28	>100	6 ± 2.1	1.6 ± 0.4
29	>100	8.6 ± 1.4	1.1 ± 0.2
30	>100	36.6/16.1	1.1 ± 0.22
31	38 ± 2.5	3.9 ± 0.8	0.30 ± 0.2
32	>100	16.6	0.8 ± 0.1
33	>100	41.6	9.1 ± 0.5
34	44.3/95.9	41.1 />100	5.2 ± 1
35	>100	54.3/>100	3.3 ± 1.2
36	>100	71.9/>100	1.7 ± 0.5
37	21.7	<3.13	2.2 ± 0.2
38	1.4 ± 0.2	0.9 ± 0.1	0.7 ± 0.4
39	2.8 ± 0.3	1 ± 0.1	1 ± 0.4
40	1.9 ± 0.3	1 ± 0.2	0.4 ± 0.1
miltefosine	23.9 ± 4.2	8.7 ± 0.7	6.7 ± 1

[a] Results are expressed as the mean ± SEM of three independent experiments.

human red blood cells (Table 2). All experiments were performed in three independent experiments.

Results and Discussion

The first screening of the new analogues **19–40** was performed on the promastigote form of the parasite and provided a general comparative evaluation of the compounds against the two leishmania species employed in this study. In general, all analogues showed increased activity against the promastigote form of *L. donovani* than against *L. infantum* (Table 1). More specifically, in the first series the 5-cyclodecylidenepentylcholine-substituted ether phospholipid **19** was marginally active against *L. infantum* (IC_{50} =76.8 μM) while it possessed good activity against *L. donovani* (IC_{50} < 3.2 μM). Introduction of an 11-carbon spacer between the phosphate group and the 10-membered ring, compound **20**, led to an increased activity against both promastigote strains with IC_{50} values of 2.9 μM against *L. infantum* and 1 μM against *L. donovani*. In the

Table 2. In Vitro Cytotoxicity[a] in the Human Monocytic Cell Line THP1 and Hemolytic Activity in Human Erythrocytes

compd	cytotoxicity IC_{50} (μM)	cytotoxicity >50 (μM)	hemolysis HC_{50} (μM)	hemolysis at 100 μM (%)	hemolysis at 500 μM (%)
19	>50	349.1	>100	2.1	41.8
20	37.9 ± 2		>100	25.5	43.0
21	>50	153.6	>100	38.0	57.4
22	>50	272.2	68.4 ± 2.8	71.6	100.0
23	>50	372.3	>100	45.4	88.3
24	37.7 ± 2		100	17.7	100.0
25	>50	72.61	>100	36.0	51.4
26	36.2 ± 3.1		>100	16.9	100.0
27	>50	75.4	44.3 ± 1.9	80.5	100.0
28	>50	88.54	76.7 ± 2.9	60.7	100.0
29	30.7 ± 3.2		76.3 ± 4.7	66.4	100.0
30	28.7 ± 3.2		59.8 ± 2	81.6	100.0
31	>50	74.95	>100	30.8	100.0
32	39.7 ± 0.3		66.6 ± 5.3	66.8	100.0
33	>50	147.3	61.3 ± 3.6	82.3	100.0
34	>50	75.04	~100		100.0
35	34.1 ± 0.4		43.1 ± 3.6	100.0	100.0
36	21.3 ± 3.2		>100	20.4	55.4
37	>50	363.3	>100	1.7	100.0
38	>50	>500	>100	8.7	100.0
39	>50	332.8	>100	28.7	80.8
40	>50	243.8	>100	34.8	81.1
miltefosine	28.6 ± 2.5		38.3 ± 2.8	96.1	100.0

[a] Results are expressed as the mean ± SEM of three independent experiments.

cyclopentadecylidene series, analogues **21–26**, a more detailed SAR was performed, varying not only the oligomethylene bridge but the headgroup as well. The 11-cyclopentadecylideneundecyl derivatives **24–26** show moderate to very low activity against both promastigote strains with IC_{50} values ranging from 41.5 to 100 μM, with the choline substituted analogue **24** being the least active and the *N*-methylmorpholino derivative **26** the most potent of the three. Replacement of the undecyl chain by a pentyl group, compounds **21–23**, results in high activity against both promastigote leishmania strains, which is not influenced by the nature of the headgroup (IC_{50} values in the range of 2 μM, Table 1).

In the second series, compounds **27–34**, we opted to use the 5-cyclopentadecylidenepentyl, 11-cyclohexylideneundecyl, and 11-adamantylideneundecyl groups as lipid portions on the basis of the in vitro results of the present report and our previous work.[30,31] In addition, two new straight-chain derivatives bearing a hexadecyl group, as miltefosine, were synthesized in order to get an insight on the effect on activity of cyclic moieties in the lipid portion and the headgroup of alkylphosphocholines. All but three compounds (**27**, **31**, **34**) were inactive against the promastigote form of *L. infantum*, IC_{50} values greater than 100 μM, while the activities against the promastigote *L. donovani* were moderate to satisfactory, IC_{50} values between 3.1 and 71.9 μM.

The intracellular amastigote form is the more relevant parasitic stage for compound biological evaluation, and we were very pleased to find that all new analogues, except compound **33**, were more potent than miltefosine (IC_{50} = 6.7 μM) against the intracellular amastigote *L. infantum* (Table 1). A more detailed SAR is now described. In the first series, compounds **19–26**, the 11-cyclodecylideneundecyltrimethylammonium derivative **20** is the most potent with an IC_{50} value of 0.1 μM. The activity decreases by 30-fold for the 5-cyclodecylidenepentyltrimethylammonium ether phospholipid **19** (IC_{50} = 3.3 μM) in which the distance between the cyclodecylidene moiety and the phosphate group is shorter. The 5-cyclopentadecylidenepentyl-substituted ether phospholipids **21–23** possess similar

activity with IC_{50} values of 3.8, 4.1, and 4.4 μM, respectively. Replacement of the pentyl group by an undecyl in the cyclopentadecylidene series resulted in a marked increase of activity against the intracellular amastigotes of *L. infantum*, which was influenced by the headgroup. The trimethylammonium derivative **24** and the *N*-methylmorpholino analogue **26** possessed similar activity (IC_{50} = 0.8 and 0.9 μM, respectively) while, the *N*-methylpiperidino-substituted ether phospholipid **25** was the least potent of the three (IC_{50} = 3.6 μM).

The second series, compounds **27**–**36**, comprises ether phospholipids bearing constrained head groups and more specifically *trans*-2-hydroxy-*N*,*N*,*N*-trimethylcyclohexanamino or *trans*-2-hydroxy-*N*,*N*,*N*-trimethylcyclopentanamino groups. Within the *trans*-2-hydroxy-*N*,*N*,*N*-trimethylcyclopentanamino-containing ether phospholipids **27**, **29**, **31**, and **35** the most active are the 5-cyclopentadecylidenepentyl-substituted analogue **27** and the 11-adamantylidene-substituted derivative **31** (IC_{50} = 0.2 μM and 0.3 μM, respectively). The activity against *L. infantum* amastigotes was decreased for the 11-cyclohexylideneundecyl analogue **29** (IC_{50} = 1.1 μM), the least potent being the straight-chain derivative **35** (IC_{50} = 3.3 μM). The order of activity against the intracellular amastigote *L. infantum* is slightly different in the case of the *trans*-2-hydroxy-*N*,*N*,*N*-trimethylcyclohexanamino headgroup, analogues **28**, **30**, **32**, and **36**. The most potent is compound **32** (IC_{50} = 0.8 μM), which contains an 11-adamantylideneundecyl moiety followed by the 11-cyclohexylideneundecyl analogue **30** (IC_{50} = 1.1 μM) and the 5-cyclopentadecylidenepentyl-substituted ether phospholipid **28** (IC_{50} = 1.6 μM) and the straight chain analogue **36** (IC_{50} = 1.7 μM). Since the 11-adamantylideneundecyl derivatives **31** and **32** exhibited high activity, two additional adamantylidene compounds were synthesized, **33** and **34**, which contained a tropane-derived headgroup and an alkynyl headgroup, respectively. Unfortunately, both **33** and **34** were less active against the amastigote *L. infantum* than the other two adamantylidene derivatives **31** and **32**; moreover, they were the least active in this series with IC_{50} values of 9.1 and 5.2 μM, respectively.

Since human monocytic THP-1 cells infected with the appropriate *Leishmania* species are used for the evaluation of the leishmanicidal activity of compounds against the intracellular amastigote stages of the parasite, we assessed the cytotoxicity of all new analogues against THP-1 cells (Table 2), and the majority of them was found to be less cytotoxic than miltefosine (IC_{50} = 28.6 μM).

More specifically, in the first series the presence of the undecyl group confers toxicity to the ether phospholipids **20**, **24**, **25**, and **26** with IC_{50} values of 37.9, 37.7, 72.6, and 36.2 μM, respectively. Conversely, the pentyl-substituted derivatives **19**, **21**, **22**, and **23** are not cytotoxic with IC_{50} values 349.1, 153.6, 272.2, and 372.3 μM, respectively. The 5-cyclodecylidenepentylcholine-substituted **19** is 2-fold less cytotoxic than the 5-cyclopentadecylidenepentylcholine derivative **21**. Within the 5-cyclopentadecylidenepentyl derivatives **21**–**23** the headgroup influences toxicity, which decreases in the order of choline-substituted **21**, *N*-methylpiperidino-substituted **22**, and *N*-methylmorpholino-substituted derivative **23**, which is the least cytotoxic of this series.

The analogues of the second series **29**, **30**, **32**, **35**, and **36** exhibit toxicity comparable to miltefosine with IC_{50} values of 30.7, 28.7, 39.7, 34.1, and 21.3 μM, respectively. Conversely, derivatives **27**, **28**, **31**, **33**, and **34** are less cytotoxic than miltefosine with IC_{50} values of 75.4, 88.54, 74.95, 147.3, and 75.04 μM, respectively. With respect to the lipid portion SAR, the 5-cyclopentadecylidenepentyl- and 11-adamantylideneundecyl-substituted analogues are less cytotoxic than the 11-cyclohexylideneundecyl- or hexadecyl-substituted ether phospholipids. With respect to headgroup SAR, the *trans*-2-hydroxy-*N*,*N*,*N*-trimethylcyclopentanamino headgroup is preferred over the *trans*-2-hydroxy-*N*,*N*,*N*-trimethylcyclohexanamino headgroup with respect to toxicity against THP1 macrophages. Furthermore, the more constrained tropane-derived headgroup renders the corresponding ether phospholipid **33** the least cytotoxic of the second series (IC_{50} value of 147.3 μM).

Since hemolysis is a problem associated with alkylphosphocholines, which prevents their use in injectable form, it was considered important to study the hemolytic properties of the new compounds. Thus, all the new analogues were evaluated for their hemolytic activity against red blood cells (Table 2). All the new compounds exhibit lower hemolytic activity than miltefosine (HC_{50} = 38.3 μM). Specifically, from the first series, analogues **19**–**21** and **23**–**26** possess HC_{50} of >100 μM while compound **22** has HC_{50} of 68.4 μM.

In the second series, compounds **27**–**36**, the 5-cyclopentadecylidenepentyl-substituted derivatives **27** and **28** possess HC_{50} values of 44.3 and 76.7 μM, respectively, and the 11-cyclohexylideneundecyl-substituted ether phospholipids **29** and **30** possess HC_{50} values of 76.3 and 59.8 μM, respectively. Among the 11-adamantylideneundecyl derivatives **31**–**34**, analogue **31** bearing the *trans*-2-hydroxy-*N*,*N*,*N*-trimethylcyclopentanamino headgroup possess HC_{50} of >100 μM and analogue **34** bearing the alkynyl headgroup exhibits HC_{50} of ~100 μM, while **32** and **33** are more hemolytic with HC_{50} values of 66.6 and 61.3 μM, respectively. Concerning the straight-chain analogues **35** and **36**, the nature of the headgroup influences the hemolytic activity to a great extent with the *trans*-2-hydroxy-*N*,*N*,*N*-trimethylcyclopentanamino group rendering **35** hemolytic (HC_{50} = 43.1 μM) and the *trans*-2-hydroxy-*N*,*N*,*N*-trimethylcyclohexanamino group rendering **36** nonhemolytic (HC_{50} > 100 μM). By comparision of the percent hemolysis at 100 and 500 μM (Table 2) of compounds **19**–**21** and **23**–**26**, there are differences that depend on both the lipid portion and the headgroup. It seems that choline is the headgroup of choice followed by the *N*-methylmorpholino and finally the *N*-methylpiperidino head groups. However, there is no clear trend as to which combination of structural features is the optimum.

The influence of the double bond on antileishmanial activity was explored by comparing ether phospholipids **19** and **21**−**23**, which are the least cytotoxic against THP1 monocytes and possess hemolytic activity higher than 100 μM and better antileishmanial activity against the intracellular amastigotes than miltefosine, with their saturated congeners **37**–**40**, which were obtained by catalytic hydrogenation. We were delighted to find out that antileishmanial activity was improved and toxicity was reduced. More specifically, the absence of the double bond in the cyclodecadecylidene-substituted choline derivative increased antileishmanial activity against the amastigotes *L. infantum* by 1.5-fold (IC_{50} = 2.2 μM) while slightly reducing cytotoxicity against THP-1 monocytes (IC_{50} = 363.3 μM) and hemolysis of human erythrocytes (HC_{50} > 100 μM, hemolysis of 1.7% at 100 μM). The absence of a double bond in the cyclodecapentylidene-substituted ether phospholipids was more dramatic especially for the choline derivative **38** whose antileismanial activity against intracellular *L. infantum* increased 5.5-fold (IC_{50} = 0.7 μM), being 9-fold more active than HePC; moreover, **38** is noncytotoxic (IC_{50} > 500 μM), has hemolytic activity (HC_{50} >100 μM), and undergoes hemolysis (8.7% at 100 μM). The same trend was observed for the *N*-methylpiperidino-substituted derivatve **39** whose antileismanial activity against intracellular

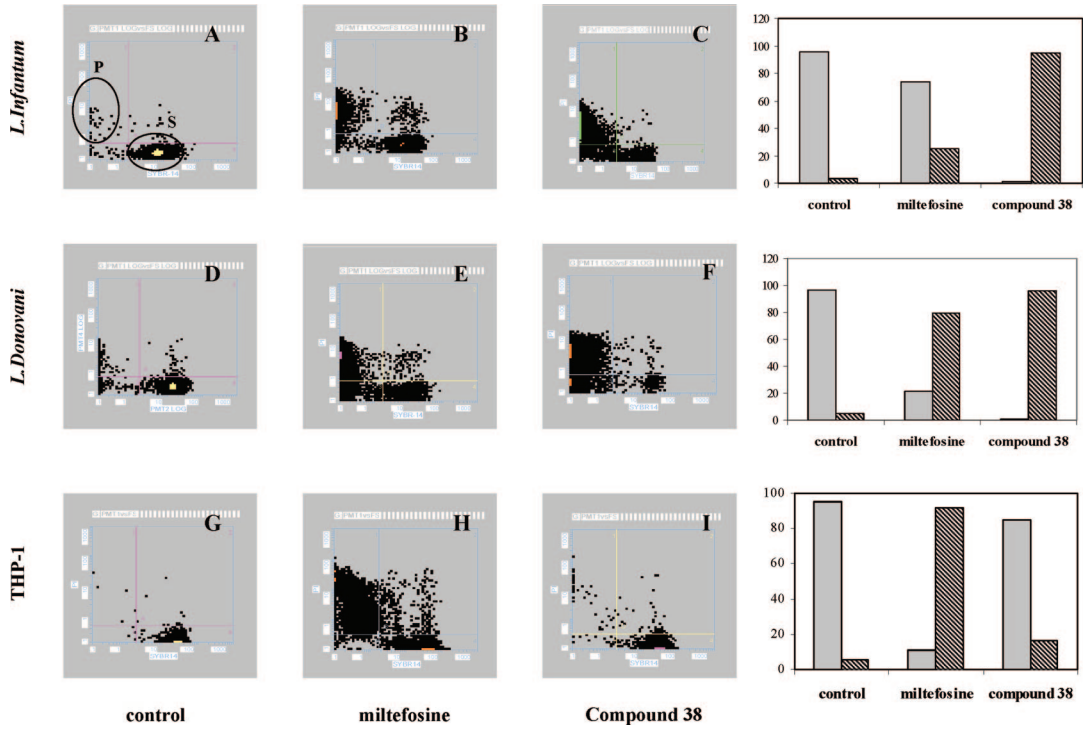


Figure 2. Comparative assessment of the effect of miltefosine (20 μM) and compound **38** (20 μM) on *L. infantum* (B, C) and *L. donovani* (E, F) promastigotes and after treatment of THP-1 cells with 50 μM miltefosine (H) or 50 μM compound **38** (I). Cells were double-stained with PI and SYBR-14, and dead/alive ratios were sorted by flow cytometry. Population stained with SYBR14 is sorted in the area S, and PI stained population is sorted in the area P. Quantification of dead/alive cells, after measuring 10 000 events per sample is depicted in the histograms. Gray bars represent the SYBR 14 stained alive cells, and slashed bars represent the PI stained dead cells.

L. infantum increased 4-fold (IC_{50} = 1. μM) (6.5-fold more active than HePC) and its cytotoxicity (IC_{50} = 332.8 μM) and hemolytic activity decreased (HC_{50} > 100 μM, hemolysis of 28.7% at 100 μM). Finally, for the *N*-methylmorpholino-substituted derivative **40**, hydrogenation of the double bond led to an increased antiparasitic activity against intracellular *L. infantum* (10.6-fold, IC_{50} = 0.41 μM), rendering **40** 16-fold more active than HePC. However, its cytotoxicity increased slightly (IC_{50} = 243.8 μM) while its hemolytic activity decreased (HC_{50} > 100 μM, hemolysis of 34.8% at 100 μM).

It has been previously observed that both promastigote and amastigote stages of *L. donovani, L. major*, *L. tropica*, *L. aethiopica*, *L.mexicana*, and *L. panamensis* vary in their in vitro sensitivity. In all assays *L. donovani* was the most sensitive species.[35] The difference in activity of the various ether phospholipids of this study against *L. infantum* and *L. donovani* promastigotes may be due to dissimilarities in both membrane sterol[36,37] and lipid content[38] of the two *Leishmania* species. Miltefosine and the new ether phospholipids of the present study, except analogues **21**–**23**, were more active against the intracellular *L. infantum* than the promastigote form of the parasite. Although the in vitro test systems for amastigotes and promastigotes are not directly comparable, alkylphosphocholines appear to be more active against the intracellular amastigotes, and this could be partially explained by their ability to activate macrophages.[39]

There has been increasing numbers of patients with *L. infantum* and HIV coinfection, especially in Mediterranean countries during the past decade,[40] with clinical, diagnostic, chemotherapeutic, epidemiological, and economic implications. The coexistence of these diseases produces cumulative deficiency of the immune response because *Leishmania* parasites and HIV destroy the same cells, leading to an exponential increase of disease severity and consequences. These immunocompromised patients generally have a poor response to antimonials, and failure or relapse rates of 25% within 1−36 months are commonly reported.[40b] Thus, VL is considered a major contributor to a fatal outcome in coinfected patients.[41] All new analogues, except compound **33**, were 1.5- to 62-fold more potent than miltefosine against the intracellular amastigote *L. infantum*, and the most active ones were also less cytotoxic and hemolytic than HePC. These data, taken together, may indicate that ring-substituted ether phospholipids may be useful in the treatment of *Leishmania*/HIV coinfection. In addition, the length of the alkyl chain of the most active ether phospholipids of the present study varies from 5 to 11 carbon atoms, and this could be advantageous for their metabolic clearance. Furthermore, all new analogues are less hemolytic than miltefosine, which indicates that some of the new compounds could be administered intravenously, and thus, they may show reduced gastrointestinal toxicity and higher plasma concentrations in vivo.

Compound **38** is the least cytotoxic analogue of the present study, while its antileishmanial activity is in the submicromolar range, being 9-fold more active than miltefosine. A comparative flow cytometric assessment of the cytotoxicity of **38** and miltefosine on THP-1 cells, as well as their effect against leishmania promastigotes, is shown in Figure 2. We observe a notable difference in the ability of compound **38** (20 μM) to kill *L. infantum* (Figure 2C) and *L. donovani* promastigotes (Figure 2F) compared to miltefosine (20 μM) (parts B and E of Figure 2, respectively). Moreover, the comparison of cytotoxicity of HePC and **38** is striking and worth mentioning. Miltefosine at 50 μM is very cytotoxic to THP1 macrophages (Figure 2H) while, after treatment with compound **38** (50 μM) (Figure 2I), the THP1 monocytes are

similar to the control (untreated THP1 monocytes) (control in Figure 2G).

Conclusions

To date, there is no vaccine against leishmaniasis and chemotherapy is the main weapon in our arsenal. There is an urgent need for better drugs against *Leishmania*. Teratogenicity, potential of resistance, and low therapeutic window pose limitations on miltefosine, and it is desirable to optimize its structure with regard to activity and/or overcoming these drawbacks. We have elucidated some of the structural features required for antileishmanial activity of alkylphosphocholines and shown that the hemolytic activity and cytotoxicity of this class of compounds can be substantially decreased by introducing cycloalkanes into the lipid portion while activity is increased. The series of 22 new ring-substituted ether phospholipids reported in the present study allows us to further refine the pharmacophoric elements of this class of antileishmanial compounds. All new analogues, except compound **33**, are 1.5- to 62-fold more potent than miltefosine against the intracellular amastigote *L. infantum*. The presence of constrained head groups is beneficial to activity; however, it results in increased toxicity and hemolytic activity in comparison to the choline-substituted congeners. The analogues that combine high potency and low cytotoxicity and hemolytic activity are 5-cyclodecylidenepentylphosphocholine (**19**) and its hydrogenated derivative **37**, 5-cyclopentadecylidenepentylphosphocholine (**21**) and its *N*-methylmorpholino congener **23**, and the hydrogenated derivatives **38**−**40**. All the hydrogenated derivatives **37**–**40** are not cytotoxic, being more potent than their unsaturated counterparts. 5-Cyclopentadecylpentylphosphocholine (**38**) is the least cytotoxic analogue (toxicity against THP1 macrophages of >500 μM), possessing very low hemolytic activity (HC_{50} > 100 μM) and high potency against *L. infantum* amastigotes IC_{50} = 0.7 μM. These findings make compound **38** an interesting and suitable candidate for further studies in vivo as well as against trypanosmatidae.

Experimental Section

Chemistry. All reactions were carried out under scrupulously dry conditions. NMR spectra of all new compounds were recorded in $CDCl_3$, unless stated otherwise, using a Bruker AC 300 spectrometer operating at 300 MHz for ^{1}H, 75.43 MHz for ^{13}C, and 121.44 MHz for ^{31}P. ^{1}H NMR spectra are reported as δ with $CHCl_3$ resonance at 7.24 ppm used as the chemical shift resonance. ^{13}C NMR shifts are expressed in units relative to $CDCl_3$ at 77.00 ppm, while ^{31}P NMR spectra are reported as δ relative to 85% H_3PO_4 used as an external standard. Silica gel plates (Merck F_{254}) were used for thin-layer chromatography. Chromatographic purification was performed with silica gel (200–400 mesh). Analyses of the elements were carried out by the microanalytical section of the Institute of Organic and Pharmaceutical Chemistry of the National Hellenic Research Foundation.

General Procedure for the Preparation of Ether Phospholipids. Method A. To a solution of phosphorus oxychloride (0.09 mL, 1 mmol) in dry THF (5 mL) was added at 0 °C dry triethylamine (0.25 mL, 1.8 mmol) and subsequently a solution of the corresponding alcohol (1 mmol) in dry THF (7 mL). The mixture was stirred at ambient temperature for 2 h, and then H_2O (5 mL) was added and stirring was continued for 1 h. The aqueous layer was extracted first with EtOAc and then with CH_2Cl_2. The combined organic extracts were dried over anhydrous Na_2SO_4 and the solvent was evaporated in vacuo to afford the corresponding phosphoric acid derivative, which was transformed to the pyridinium salt by the addition of 5 mL of anhydrous pyridine and stirring for 12 h at 40 °C. After the mixture was cooled, the solvent was evaporated in vacuo. To a solution of the above salt (1 mmol) in pyridine (7 mL) was added dropwise, with cooling, 1-(mesitylen-2-sulfonyl)-3-nitro-1*H*-1,2,4-triazole (MSNT) (593 mg, 2 mmol) followed by the addition of choline chloride (205 mg, 1.5 mmol) or *N*-(2-hydroxyethyl)-*N*-methylpiperidinium bromide (448 mg, 1.5 mmol) or *N*-(2-hydroxyethyl)-*N*-methylmorpholinium bromide (452 mg, 1.5 mmol), and the mixture was stirred at 40 °C for 48 h. After cooling, the mixture was hydrolyzed by the addition of 2-propanol/H_2O, 7:2, and stirred for 0.5 h at ambient temperature. The solvents were evaporated in vacuo and the residue was subjected to flash column chromatography (CH_2Cl_2/MeOH, 60:50, and then CH_2Cl_2/MeOH/25% NH_4OH, 60:50:5) to afford the desired ether phospholipids.

Method B. To a solution of phosphorus oxychloride (0.09 mL, 1 mmol) in dry THF (5 mL) was added at 0 °C dry triethylamine (0.25 mL, 1.8 mmol) and subsequently a solution of the corresponding alcohol (1 mmol) in dry THF (18 mL). The mixture was stirred at ambient temperature for 2 h. Subsequently, choline *p*-toluenesulfonate (358 mg, 1.3 mmol) or toluene-4-sulfonate(2-hydroxycyclopentyl)trimethylammonium (**11a**) (410 mg, 1.3 mmol) or toluene-4-sulfonate(2-hydroxycyclohexyl)trimethylammonium (**11b**) (428 mg, 1.3 mmol) or toluene-4-sulfonate[3-(2-hydroxyethylidene)-8,8-dimethyl-8-azoniabicyclo[3.2.1]octane] (**15**) (459 mg, 1.3 mmol) or toluene-4-sulfonate(4-hydroxybut-2-ynyl)trimethylammonium (**18**) (388 mg, 1.3 mmol), dry pyridine (12 mL), and dry $CHCl_3$ (10 mL) were added and the reaction mixture was stirred at ambient temperature for 48 h. Hydrolysis and purification, according to method A, afforded the desired ether phospholipids.

{2-[(5-Cyclodecylidenepentyloxy)hydroxyphosphinyloxy]ethyl}-*N,N,N*-trimethylammonium Inner Salt (19). Method A described above using 5-(cyclodecylidene)pentanol (**5a**) (224.3 mg, 1 mmol) and choline chloride (205 mg, 1.5 mmol) led to the formation of compound **19** as a yellowish, gummy solid (67 mg, 17%). ^{1}H NMR (δ) 5.08 (t, J = 6.71 Hz, 1H), 4.22 (bs, 2H), 3.75 (bs, 4H), 3.32 (s, 9H), 2.16–1.91 (m, 6H), 1.60–1.20 (m, 18 H); ^{31}P NMR (δ) −1.04; ^{13}C NMR (δ) 138.5, 126.0, 66.2, 66.1, 65.6, 65.5, 59.2, 54.2, 35.6, 35.2, 30.9, 30.8, 27.8, 27.5, 26.5, 26.2, 26, 25.9, 25.8, 25.7, 24.7, 24.5, 23.2, 20.8, 20.5. Anal. ($C_{20}H_{40}NO_4P$) C, H,N.

{2-[(5-Cyclodecylideneundecyloxy)hydroxyphosphinyloxy]ethyl}-*N,N,N*-trimethylammonium Inner Salt (20). Following method A described above using 11-(cyclodecylidene)undecanol (**8a**) and choline chloride, compound **20** was obtained as a yellowish, gummy solid (246 mg, 52%). ^{1}H NMR (δ) 5.13 (t, J = 6.71 Hz, 1H), 4.25 (bs, 2H), 3.76 (bs, 4H), 3.38 (s, 9H), 2.14–1.94 (m, 4H), 1.65–1.22 (m, 32H); ^{31}P NMR (δ) −1.22; ^{13}C NMR (δ) 137.9, 126.6, 66.1, 65.8, 59.2, 54.3, 35.1, 31.9, 31.0, 30.8, 29.9, 29.6, 29.4, 28.2, 28.1, 26.0, 25.7, 24.8, 24.7, 24.5, 23.1, 22.3. Anal. ($C_{26}H_{52}NO_4P$) C, H, N.

{2-[(5-Cyclopentadecylidenepentyloxy)hydroxyphosphinyloxy]ethyl}-*N,N,N*-trimethylammonium Inner Salt (21). Following method A described above using 5-(cyclopentadecylidene)pentanol (**5b**) and choline chloride, compound **21** was afforded as a yellowish, gummy solid (152 mg, 33%). ^{1}H NMR (δ) 5.03 (t, J = 6.71 Hz, 1H), 4.19 (bs, 2H), 3.73 (bs, 4H), 3.26 (s, 9H), 1.93 (bs, 6H), 1.55–1.28 (m, 28H); ^{31}P NMR (δ) −1.07; ^{13}C NMR (δ) 140.3, 124.6, 66.2, 66.1, 65.7, 65.6, 59.2, 54.2, 37.6, 30.7, 29.9, 27.8, 27.7, 27.6, 27.4, 27.1, 26.7, 26.6, 26.5, 26.4. Anal. ($C_{25}H_{50}NO_4P.3H_2O$) C, H, N.

1-{2-[(5-Cyclopentadecylidenepentyloxy)hydroxyphosphinyloxy]ethyl}-1-methylpiperidinium Inner Salt (22). Following method A described above using 5-(cyclopentadecylidene)pentanol (**5b**) and *N*-(2-hydroxyethyl)-*N*-methylpiperidinium bromide, compound **22** was formed as a yellowish, gummy solid (214 mg, 43%). ^{1}H NMR (δ) 5.04 (t, J = 6.71 Hz, 1H), 4.27 (bs, 2H), 3,81–3,53 (m, 8H), 3.30 (s, 3H), 1.95–1.50 (m, 14H), 1.30–1.22 (m, 26H); ^{31}P NMR (δ) −0.50; ^{13}C NMR (δ) 140.1, 124.6, 65.4, 65.3, 63.3, 61.9, 61.7, 61.6, 61.5, 61.4, 61.3, 61.0, 58.7, 58.5, 58.4, 55, 48.5, 37.5, 29.9, 27.7, 27.5, 27.3, 27.1, 26.7, 26.6, 26.4, 20.8, 20.1. Anal. ($C_{28}H_{54}NO_4P$) C, H, N.

4-{2-[(5-Cyclopentadecylidenepentyloxy)hydroxyphosphinyloxy]ethyl}-4-methylmorpholinium Inner Salt (23). Following method A described above using 5-(cyclopentadecylidene)pentanol (**5b**) and

N-(2-hydroxyethyl)-*N*-methylmorpholinium bromide, compound **23** was formed as a yellowish, gummy solid (121 mg, 24%). ^{1}H NMR (δ) 5.04 (t, J = 6.71 Hz, 1H), 4.28 (bs, 2H), 3.99–3.71 (m, 12 H), 3.45 (s, 3H), 1.94 (bs, 6H), 1.56–1.29 (m, 28H); ^{31}P NMR (δ) −0.57; ^{13}C NMR (δ) 138.5, 126, 66.2, 66.1, 65.6, 59.2, 54.2, 35.6, 35.2, 30.9, 30.8, 27.9, 27.5, 26.5, 26.2, 26.0, 25.9, 25.8, 25.7, 24.7, 24.5, 23.2, 20.8, 20.5. Anal. ($C_{27}H_{52}NO_5P\cdot 2.5H_2O$) C,H,N.

{2-[(11-Cyclopentadecylideneundecyloxy)hydroxyphosphinyloxy]ethyl}-*N,N,N*-trimethylammonium Inner Salt (24). Following method A described above using 11-(cyclopentadecylidene) undecanol (**8b**) and choline bromide, compound **24** was afforded as a yellowish, gummy solid (281 mg, 52%). ^{1}H NMR (δ) 5.04 (t, J = 6.71 Hz, 1H), 4.17 (bs, 2H), 3.71 (bs, 4H), 3.31 (s, 9H), 1.89 (bs, 6H), 1.55–1.28 (m, 40H); ^{31}P NMR (δ) −0.69; ^{13}C NMR (δ) 139.7, 125.2, 66.1, 66.0, 65.5, 59.1. 54.1, 37.5, 31.1, 31.0, 30.0, 29.9, 29.7, 29.5, 29.4, 27.8, 27.5, 27.4, 27.1, 26.8, 26.7, 26.4, 25.9, 25.7. Anal. ($C_{31}H_{62}NO_4P\cdot 3H_2O$) C, H, N.

1-{2-[(11-Cyclopentadecylideneundecyloxy)hydroxyphosphinyloxy]ethyl}-1-methylpiperidinium Inner Salt (25). Following method A described above using 11-(cyclopentadecylidene)undecanol (**8b**) and *N*-(2-hydroxyethyl)-*N*-methylpiperidinium bromide, compound **25** was obtained as a yellowish, gummy solid (291 mg, 50%). ^{1}H NMR (δ) 4.99 (t, J = 6.71 Hz, 1H), 4.17 (bs, 2H), 3,76–3,50 (m, 8H), 3.23 (s, 3H), 1.95–1.50 (m, 14H), 1.30–1.22 (m, 38H); ^{31}P NMR (δ) −0.47; ^{13}C NMR (δ) 139.6, 125.2, 65.4, 63.3, 61.7, 58.5, 49.6, 48.5, 37.4, 31.0, 30.9, 30.1, 29.9, 29.6, 29.4, 29.3, 27.7, 27.4, 27.3, 27.1, 26.7, 26.6, 26.4, 26.3, 25.8, 20.8, 20.1. Anal. ($C_{34}H_{66}NO_4P\cdot 2H_2O$) C, H, N.

4-{2-[(11-Cyclopentadecylideneundecyloxy)hydroxyphosphinyloxy]ethyl}-4-methylmorpholinium Inner Salt (26). Following method A described above using 11-(cyclopentadecylidene)undecanol (**8b**) and *N*-(2-hydroxyethyl)-*N*-methylmorpholinium bromide afforded compound **26** as a yellowish, gummy solid (215 mg, 37%). ^{1}H NMR (δ) 5.05 (t, J = 6.71 Hz, 1H), 4.24 (bs, 2H), 3.96–3.70 (m, 12 H), 3.44 (s, 3H), 1.94 (bs, 6H), 1.53–1.19 (m, 40H); ^{31}P NMR (δ) −0.63; ^{13}C NMR (δ) 139.7, 125.2, 65.6, 65.0, 64.2, 60.7, 58.5, 48.1, 37.5, 31.1, 31.0, 30.2, 30.1, 30.0, 29.9, 29.8, 29.6, 29.5, 29.4, 29.3, 29.0, 27.8, 27.5, 27.4, 27.1, 26.8, 26.7, 26.4, 25.9. Anal. ($C_{33}H_{64}NO_5P$) C, H, N.

***trans*-{2-[(5-Cyclopentadecylidenepentyloxy)hydroxyphosphinyloxy]}-*N,N,N*-trimethylcyclopentaneammonium Inner Salt (27).** Following method B described above using 5-(cyclopentadecylidene)pentanol (**5b**) and *trans*-(2-hydroxycyclopentyl)trimethylammonium toluene-4-sulfonate (**11a**), compound **27** was obtained as a white, gummy solid (11 mg, 22%). ^{1}H NMR (δ) 5.05 (t, J = 6.71 Hz, 1H), 4.85 (bs, 1H), 3.93–3.70 (m, 3H), 3.73 (s, 9H), 2.15–2.09 (m, 2 H), 1.96 (bs, 6H), 1.75–1.71 (m, 4 H), 1.57–1.30 (m, 28H); ^{31}P NMR (δ) −1.60; ^{13}C NMR (δ) 139.4, 124.7, 80.1, 80.0, 74.6, 74.5, 65.8, 65.3, 65.0, 52.1, 50.2, 37.5, 37.1, 35.5, 32.4, 30.9, 30.6, 29.9, 29.5, 29.3, 29.2, 28.6, 28.2, 27.8, 27.7, 27.6, 27.4, 27.3, 27.2, 26.9, 26.8, 26.7, 26.6, 26.5, 26.0, 25.8, 25.6, 24.6, 20.3. Anal. ($C_{28}H_{54}NO_4P$) C, H, N.

***trans*-{2-[(5-Cyclopentadecylidenepentyloxy)hydroxyphosphinyloxy]}-*N,N,N*-trimethylcyclohexaneammonium Inner Salt (28).** Following method B described above using 5-(cyclopentadecylidene)pentanol (**5b**) and *trans*-(2-hydroxy-cyclohexyl)trimethylammonium toluene-4-sulfonate (**11b**), compound **28** was afforded as a white, gummy solid (117 mg, 23%). ^{1}H NMR (δ) 5.05 (t, J = 6.71 Hz, 1H), 4.38 (bs, 1H), 3.83–3.76 (m, 3H), 3.31 (s, 9H), 2.39–2.36 (bs, 1H), 2.19 (bs, 1H), 1.95–1.88 (m, 7H), 1.68–1.51 (m, 3 H), 1.27 (bs, 31H); ^{31}P NMR (δ) −2.26; ^{13}C NMR (δ) 138.8, 125.0, 73.9, 73.8, 65.5, 65.4, 53.3, 37.5, 37.1, 35.5, 33.9, 30.8, 30.7, 30.5, 30.4, 29.9, 29.5, 29.3, 29.1, 28.5, 28.1, 28.0, 27.8, 27.7, 27.5, 27.4, 27.1, 26.9, 26.6, 26.5, 26.4, 26.0, 25.9, 25.7, 25.5, 24.6, 23.4. Anal. ($C_{29}H_{56}NO_4P$) C, H, N.

***trans*-{2-[(11-Cyclohexylideneundecyloxy)hydroxyphosphinyloxy]}-*N,N,N*-trimethylcyclopentaneammonium Inner Salt (29).** Following method B described above using 11-(cyclohexylidene) undecanol (**8c**) and *trans*-(2-hydroxycyclopentyl)trimethylammonium toluene-4-sulfonate (**11a**) gave compound **29** as a white, gummy solid (128 mg, 28%). ^{1}H NMR (δ) 5.01 (t, J = 6.71 Hz, 1H), 4.86 (bs, 1H), 3.97–3.65 (m, 3H), 3,36 (s, 9H), 2.09–1.90 (m, 8H), 1.73 (bs, 4 H), 1.48 (bs, 8H), 1.21 (bs, 14H); ^{31}P NMR (δ) −1.09; ^{13}C NMR (δ) 139.3, 121.4, 79.8, 79.7, 74.3, 74.2, 65.3, 65.2, 52.0, 37.1, 32.2, 31.0, 30.9, 30.2, 29.6, 29.5, 28.6, 27.8, 27.1, 27.0, 26.9, 25.9, 24.6, 19.8. Anal. ($C_{25}H_{48}NO_4P\cdot 2H_2O$) C, H, N

***trans*-{2-[(11-Cyclohexylideneundecyloxy)hydroxyphosphinyloxy]}-*N,N,N*-trimethylcyclohexaneammonium Inner Salt (30).** Following method B described above using 11-(cyclohexylidene) undecanol (**8c**) and *trans*-(2-hydroxycyclohexyl)trimethylammonium toluene-4-sulfonate (**11b**) gave compound **30** as a white, gummy solid (90 mg, 19%). ^{1}H NMR (δ) 5.01 (t, J = 6.71 Hz, 1H), 4.41 (bs, 1H), 3.83–3.74 (bs, 3H), 3.36 (s, 9H), 2.36 (bs, 1H), 2.22 (bs, 1H), 2.08–1.83 (m, 7H), 1.64–1.21 (m, 27H); ^{31}P NMR (δ) −2.10; ^{13}C NMR (δ) 139.3, 121.4, 73.7, 73.6, 53.3, 50.0, 37.1, 34.0, 30.9, 30.8, 30.2, 29.6, 29.5, 29.4, 29.3, 29.1, 29.0, 28.8, 28.6, 28.5, 27.8, 27.1, 27.0, 26.9, 25.9, 24.9, 23.5. Anal. ($C_{26}H_{50}NO_4P$) C, H, N.

***trans*-{2-[[11-(Adamantylidene)undecyloxy]hydroxyphosphinyloxy]}-*N,N,N*-trimethylcyclopentaneammonium Inner Salt (31).** Following method B described above using 11-(adamantylidene) undecanol (**8d**) and *trans*-(2-hydroxycyclopentyl)trimethylammonium toluene-4-sulfonate (**11a**), compound **31** was obtained as a white, gummy solid (90 mg, 18%). ^{1}H NMR (δ) 4.99 (t, J = 6.71 Hz, 1H), 4.88 (bs, 1H), 3.95–3.72 (m, 3H), 3,35 (s, 9H), 2.78 (s, 1H), 2.28 (s, 1H), 2.14 (bs, 2 H), 1.91–1.53 (m, 18H), 1.23 (bs, 16H); ^{31}P NMR (δ) −1.16; ^{13}C NMR (δ) 147.3, 116.3, 81.1, 80.0, 79.9, 74.4, 74.3, 71.0, 65.4, 65.3, 52.2, 45.9, 40.5, 39.9, 38.9, 37.3, 32.3, 32.0, 31.0, 30.9, 30.4, 29.7, 29.6, 29.3, 28.7, 26.5, 25.9, 24.7, 19.9. Anal. ($C_{29}H_{52}NO_4P$) C, H, N.

***trans*-{2-[[11-(Adamantylidene)undecyloxy]hydroxyphosphinyloxy]}-*N,N,N*-trimethylcyclohexaneammonium Inner Salt (32).** Following method B described above using 11-(adamantylidene) undecanol (**8d**) and *trans*-(2-hydroxycyclohexyl)trimethylammonium toluene-4-sulfonate (**11b**) led to the formation of compound **32** as a white, gummy solid (172 mg, 33%). ^{1}H NMR (δ) 4.98 (t, J = 6.71 Hz, 1H), 4.41 (m, 2H), 3.80–3.78 (bs, 2H), 3.33 (s, 9H), 2.76 (s, 1H), 2.33–2.27 (m, 3H), 1.81–1.41 (m, 20H), 1.23 (bs, 16H); ^{31}P NMR (δ) −2.67; ^{13}C NMR (δ) 147.3, 116.3, 74.0, 73.9, 65.4, 65.3, 53.4, 40.5, 40.4, 39.9, 39.8, 39.7, 39.6, 38.9, 37.3, 37.2, 33.9, 32.0, 31.0, 30.9, 30.4, 29.7, 29.6, 29.5, 29.3, 29.2, 29.1, 29.0, 28.9, 28.8, 27.7, 28.6, 28.5, 28.4, 26.6, 26.5, 26.4, 25.9, 25.7, 24.9, 23.5. Anal. ($C_{30}H_{54}NO_4P$) C, H, N.

8,8-Dimethyl 3-{2-[11-[adamantylidene)undecyloxy]hydroxyphosphinyloxy]ethylidene}-8-azoniabicyclo[3.2.1]octane Inner Salt (33). Following method B described above using 11-(adamantylidene)undecanol (**8d**) and 3-(2-hydroxyethylidene)-8,8-dimethyl-8-azoniabicyclo[3.2.1]octane, *p*-toluenesulfonate (**15**) led to the formation of compound **33** as a white, gummy solid (55 mg, 10%). ^{1}H NMR (δ) 5.74 (bs, 1H), 4.98 (t, J = 7.32 Hz, 1H), 4.32–4.27 (m, 3H), 4.08 (bs, 1H) 3.76–3.74 (bs, 2H), 3.44–3.07 (m, 7H), 2.76 (bs, 3H), 2.36–2.25 (m, 4H), 1.94–1.54 (m, 16H), 1.23–1.18 (m, 16H); ^{31}P NMR (δ) −0.41; ^{13}C NMR (δ) 147.3, 129.6,127.8, 116.3, 74.0, 69.4, 69.0, 65.4, 65.3, 60.3, 50.9, 43.9, 40.5, 39.8, 38.9, 37.3, 36.7, 32.0, 31.0, 30.9, 30.5, 30.3, 29.7, 29.6, 29.5, 29.2, 28.6, 26.5, 25.9. Anal. ($C_{32}H_{54}NO_4P$) C, H, N.

{4-[(11-Adamantylideneundecyloxy)hydroxyphosphinyloxy]-but-2-ynyl}-*N,N,N*-trimethylammonium Inner Salt (34). Following method B described above using 11-(adamantylidene)-undecanol (**8d**) and toluene-4-sulfonate(4-hydroxybut-2-ynyl) trimethylammonium (**18**) gave compound **34** as a gummy solid (291 mg, 50%). ^{1}H NMR (δ) 4.98 (t, J = 7.32 Hz, 1H), 4.49 (bs, 2H), 4.47 (bs, 2H), 3.74–3.72 (m, 2H), 3.36 (s, 9H), 2.76 (bs, 1H), 2.26 (bs, 1H), 1.89–1.54 (m, 16H), 1.23–1.20 (m, 14H); ^{31}P NMR (δ) −0.50; ^{13}C NMR (δ) 147.2, 116.3, 89.4, 76.5, 65.5, 64.9, 56.1, 52.6, 40.5, 39.8, 39.0, 38.9, 37.3, 32.0, 31.9, 30.9, 29.7, 29.6, 29.5, 29.2, 28.9, 28.6, 28.5, 28.4, 28.3, 26.4, 25.9. Anal. ($C_{28}H_{48}NO_4P\cdot H_2O$) C, H, N.

***trans*-{2-[Hexadecyloxy]hydroxyphosphinyloxy]}-*N,N,N*-trimethylcyclopentaneammonium Inner Salt (35).** Following method B described above using hexadecanol (**8e**) and *trans*-(2-hydroxy-cyclopentyl)trimethylammonium toluene-4-sulfonate (**11a**) afforded

compound **35** as a gummy solid (204 mg, 40%). ^{1}H NMR (δ) 4.80 (bs, 1H), 3.88–3.62 (m, 3H), 3.24 (s, 9H), 2.09–2.03 (m, 2H), 1.73–1.66 (m, 4H), 1.53–1.49 (m, 2H), 1.19–1.17 (m, 26H), 0.79 (t, J = 7.0 Hz, 3H); ^{31}P NMR (δ) −1.07; ^{13}C NMR (δ) 79.94, 79.9, 74.3, 74.2, 65.4, 65.3, 51.9, 32.2, 31.9, 31.8, 30.9, 30.8, 30.2, 29.6, 29.5, 29.4, 29.3, 25.8, 24.5, 22.6, 19.8, 14.0. Anal. ($C_{24}H_{50}NO_4P$) C, H, N.

***trans*-{2-[(Hexadecyloxy)hydroxyphosphinyloxy]}-*N,N,N*-trimethylcyclohexaneammonium Inner Salt (36).** Method B described above using hexadecanol (**8e**) and *trans*-(2-hydroxycyclohexyl)trimethylammonium toluene-4-sulfonate (**11b**) afforded compound **36** as a gummy solid (286 mg, 50%). ^{1}H NMR (δ) 4.39–4.37 (m, 1H), 3.89–3.75 (m, 3H), 3.24 (s, 9H), 2.65–2.51 (m, 4H), 2.43–2.39 (m, 1H), 2.19–2.15 (m, 1H), 1.86–1.21 (m, 30H), 0.83 (t, J = 6.7 Hz, 3H); ^{31}P NMR (δ) −1.88; ^{13}C NMR (δ) 73.8, 73.7, 65.7, 65.6, 53.4, 33.9, 31.9, 30.7, 29.6, 29.3, 25.7, 24.9, 23.2, 22.6, 14.0. Anal. ($C_{25}H_{52}NO_4P\cdot H_2O$) C, H, N.

{2-[(5-Cyclodecylpentyloxy)hydroxyphosphinyloxy]ethyl}-*N,N,N*-trimethylammonium Inner Salt (37). To a solution of compound **19** (20 mg, 0.05 mmol) in MeOH (1 mL) was added a catalytic amount of Pd/C (10% w/w). The suspension was stirred under 1 atm of hydrogen gas for 24 h. The reaction mixture was filtered through Celite and the filtrate was concentrated in vacuo to afford compound **37** as a yellowish, gummy solid (20 mg, 100%). ^{1}H NMR (δ) 4.08 (bs, 2H), 3.68 (bs, 2H), 3.46 (bs, 2H), 3.05 (s, 9H), 1.45–1.0 (m, 27 H); ^{31}P NMR (δ) −0.53; ^{13}C NMR (δ) 66.1, 58.9, 53.9, 36.9, 35.4, 30.5, 30.4, 30.3, 27.1, 25.9, 25.4, 25.3, 24.7, 23.5. Anal. ($C_{20}H_{42}NO_4P$) C, H, N.

{2-[(5-Cyclopentadecylpentyloxy)hydroxyphosphinyloxy]ethyl}-*N,N,N*-trimethylammonium Inner Salt (38). 38 was prepared following the procedure described for compound **37**, starting from compound **21** (139 mg, 0.3 mmol) and giving a yellowish, gummy solid (130 mg, 93%). ^{1}H NMR (δ) 4.20 (bs, 2H), 3.72 (bs, 4H), 3.29 (s, 9H), 1.53–1.22 (m, 37H); ^{31}P NMR (δ) −1.00; ^{13}C NMR (δ) 66.1, 65.8, 59.2, 54.2, 50.0, 44.9, 36.6, 35.3, 32.4, 31.0, 27.7, 27.2, 27.0, 26.8, 26.6, 26.5, 26.3, 24.6. Anal. ($C_{25}H_{52}NO_4P$) C, H, N.

4-{2-[(5-Cyclopentadecylpentyloxy)hydroxyphosphinyloxy]-ethyl}-1-methylpiperidinium Inner Salt (39). 39 was prepared following the procedure described for compound **37**, starting from compound **22** (195 mg, 0.39 mmol) and giving a yellowish, gummy solid (190 mg, 97%). ^{1}H NMR (δ) 4.18 (bs, 2H), 3.76–3.46 (m, 8H), 3.24 (s, 3H), 1.77 (bs, 4H), 1.59–1.47 (bs, 4H), 1.21–1.15 (m, 35H); ^{31}P NMR (δ) −0.53; ^{13}C NMR (δ) 65.5, 65.4, 63.2, 61.7, 58.5, 48.6, 36.4, 35.2, 32.4, 31.1, 31, 27.6, 27, 26.9, 26.7, 26.6, 26.4, 26.3, 24.6, 20.8, 20.1. Anal. ($C_{28}H_{56}NO_4P$) C, H, N.

4-{2-[(5-Cyclopentadecylpentyloxy)hydroxyphosphinyloxy]-ethyl}-4-methylmorpholinium Inner Salt (40). 40 was prepared following the procedure described for compound **37**, starting from compound **23** (110 mg, 0.22 mmol) and giving a yellowish, gummy solid (110 mg, 100%). ^{1}H NMR (δ) 4.30 (bs, 2H), 4.03–3.74 (m, 12 H), 3.48 (s, 3H), 1.56–1.29 (m, 37H); ^{31}P NMR (δ) −1.07; ^{13}C NMR (δ) 66.1, 65, 60.7, 58.6, 48.3, 45, 36.6, 35.3, 32.4, 31, 27.7, 27.2, 26.9, 26.8, 26.7, 26.6, 26.5, 26.4, 25.9, 24.6. Anal. ($C_{27}H_{54}NO_5P$) C, H, N.

Evaluation of in Vitro Antiparasitic Activity. Cell Culture and Parasite Strains. The human monocytic cell line THP-1 was used as a host cell for the leishmania parasites. Infection rate of this cell line ranged between 35% and 45% and was significantly lower than the infection rate achieved with mouse peritoneal macrophages (85–95%). However, the use of THP-1 as the parasite host cell for the evaluation of the compound antileishamnial activity against the intracellular parasite proved to be highly reproducible and convenient for the large number of compounds to be tested in three independent experiments. THP-1 cells are maintained in culture in RPMI 1640 supplemented with 10% FCS, L. glutamine, and antibiotics at 37 °C/5% CO_2. Promastigotes of *Leishmania infantum* MHOM/TN/80/IPT1/LEM 235 and *Leishmania donovani* MHOM/IN/80/DD8/LEM 703 were grown in RPMI 1640 supplemented with 10% FCS, L. glutamine, and antibiotics at 26 °C.

Determination of in Vitro Antileishmanial Activity in Promastigote Cultures. All compounds were dissolved in DMSO/ethanol, 50/50 v/v, to a final concentration of 65 mM, and linear 5-fold dilutions ranging from 100 to 0.8 μM were done in the culture medium. An amount of 200 μL of promastigote culture at 2×10^6 cells/mL was incubated with 200 μL of the appropriate compound concentration in a 24-well tissue culture plate (CELLSTAR, Greiner) at 26 °C. After 72 h, cultures were washed in PBS, were resuspended in 300 μL of Hepes buffered solution, and were stained with propidium iodide (PI) and SYBR-14 using the LIVE/DEAD viability kit (Molecular Probes) according to the manufacturer recommendations. Cell samples were analyzed by flow cytometry as described below. Calculation of IC_{50} values was done according to Hills et al.[42,43]

Determination of in Vitro Antileishmanial Activity against the Intracellular *L. infantum*. THP-1 cells were differentiated with 1 mM retinoic acid (Sigma) for 3 days at 37 °C and 5% CO_2. Infection of THP-1 cells with promastigotes was achieved by mixing the THP-1 and the parasite cultures at a ratio of 1:4 and incubation at 37 °C and 5% CO_2. The next day cells were washed by centrifugation at 400g for 10 min, the pellet was resuspended in RPMI medium, and the suspension was overlaid with an equal volume of Histopaque 1077 (Invitrogen). The free promastigotes were removed by centrifugation at 1000g for 20 min. The cell layer was washed with PBS twice and resuspended in RPMI at 4×10^5 cells/mL. Infection rate was assessed microscopically by Giemsa staining of the infected THP-1 suspension. An amoutn of 200 μL of infected THP-1 was plated in a 24-well tissue culture plate (Cellstar, Greiner) and mixed with an equal volume of the appropriate compound concentration. Incubation proceeded for 72 h at 37 °C and 5% CO_2, and the percentage of infected cells was monitored microscopically after Giemsa staining. The IC_{50} was calculated as described for the promastigotes above.

Assessment of Hemolytic Potential. EDTA-preserved peripheral blood from healthy volunteers was centrifuged in order to remove serum, and red blood cells were washed thrice in PBS. After the final wash, cells were distributed in 96-well microplates (100 μL/well) and an equal volume of the appropriate compound concentration was added. The tested compounds were diluted in PBS in concentrations ranging from 100 to 6.25 μM. Incubation proceeded at 37 °C for 1 h, and red blood cells were then centrifuged at 800g for 10 min. Absorbance of the supernatants were measured at 550 nm with reference filter at 625 nm. The percentage of hemolytic activity of each drug at different concentrations was estimated using $[(A - A_0)/(A_{max} - A_0)] \times 100$, where A_0 is the background hemolysis obtained by incubation with PBS and A_{max} is the 100% hemolysis achieved after incubation in H_2O.

Assessement of Cytotoxicity in THP-1 Monocytic Cells. As a quantitative measurement of the cell damage after incubation with different concentrations of drugs, dual staining with SYBR-14 and PI (Molecular Probes, The Neatherlands) was used. THP-1 cell cultures were incubated at 1×10^6 cells/mL with different concentrations of the compounds ranging from 50 to 1.56 μM. After an incubation period of 72 h approximately 4×10^6 cells were stained with PI and SYBR-14 as described for the promastigote staining. The ratio alive/dead cells was assessed by flow cytometry as described below.

Flow Cytometric Analysis. Cell samples were analyzed on an Epics Elite model flow cytometer (Coulter, Miami, FL). Excitation of both dyes was done at 488 nm. Differential monitoring of the dyes was achieved by reading the green fluorescence of SYBR-14 at 545 nm and the red fluorescence of PI at 645 nm. At least 10 000 cells were analyzed per sample, and each staining experiment was repeated thrice. Data analysis was performed on fluorescence intensities that excluded cell autofluorescence and cell debris.

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Supporting Information Available: Elemental analysis data for compounds **5a**,**b**, **8a**,**b**, **11a**,**b**, **15**, **18–40** and experimental procedures and spectroscopic data for compounds **3a**,**b**, **4a**,**b**, **5a**,**b**, **7a**,**b**, **8a**,**b**, **10a**,**b**, **11a**,**b**, **13**−**15**, **17**, and **18**. This material is available free of charge via the Internet at http://pubs.acs.org.

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