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Synthesis of 16-mercaptohexadecylphosphocholine, a miltefosine analog with leishmanicidal activity

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Abstract—The alkylphosphocholine miltefosine (*n*-hexadecylphosphocholine, MT) has been introduced recently as a very effective drug for the oral treatment of human leishmaniasis. However, the parasiticidal mechanism of MT at a molecular level is far from being understood. Here we report the synthesis and biological characterization of 16-mercaptohexadecylphosphocholine, a thiol analog of MT which was designed to facilitate the search of MT interacting targets within the parasite by a variety of analytical methods. This analog presents the same leishmanicidal effect as the parent drug against *Leishmania donovani* promastigotes and *Leishmania pifanoi* axenic amastigotes, and has been used to develop an affinity chromatography method to attempt the isolation of putative *Leishmania* proteins that bind to the phosphocholine part of the molecule.
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Miltefosine (*n*-hexadecylphosphocholine, MT) is a synthetic alkyl phospholipid with a major application in the treatment of human leishmaniasis, a group of diseases caused by the infection with *Leishmania* parasites.[1,2] MT is the first effective oral drug against both visceral[3] and cutaneous[4,5] forms of the infection, preserving its activity against antimonial-resistant parasites[1,6] or on immunodepressed patients,[7] without the severe side effects common to most of the first-line leishmanicidal drugs.[8] In contrast, the information available on the origin of the efficient parasiticidal mechanism of MT and on the metabolism and the subcellular interactions of the drug in *Leishmania* is scarce.[9–11] Interestingly, it has been known recently that MT induces apoptosis-like death in *Leishmania donovani*,[12,13] although the primary targets of the drug remain to be determined. In fact, the sole mechanism of *Leishmania* resistance to MT so far described consists in a faulty uptake of the drug,[14] associated to loss-of-function of a plasma membrane translocator.[15]

The current knowledge on the structure–antiparasite activity relationship of MT analogs is also limited.[1,16] It has been shown that an alkyl chain length of 16 or 18 methylene groups yields the highest in vitro activity, while in vivo assays indicated that the C_{16} analog is the most active.[17,18] MT analogs with the lipophilic groups cyclohexylideneundecyl, adamantylideneundecyl, dodecylidenecyclohexyloxyethyl or tetradecylidene-cyclohexyloxyethyl showed more in vitro leishmanicidal activity than MT, and among them only the two latter compounds demonstrated higher cytotoxicity in vivo than the parent drug. Analogs with shorter chains such as phenoxyhexadecyl or 2-naphthyloxyethyl groups were devoid of activity.[19] Regarding the polar head group, it has been found that compounds with the terminal trimethylammonium group, as in phosphocholine, present higher in vitro activity than the corresponding analogs with ammonium, *N*-methylpiperidinium, or *N*-methylmorpholinium terminal groups.[17–19] In addition, a few phosphocholine derivatives have been

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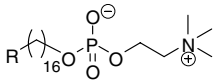
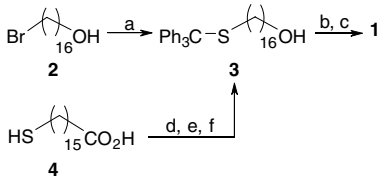
synthesized which contain a specific reporter group (probe). That is the case of MT analogs bearing the tetrafluorophenylazido photolabeling group[20] or nitroxide paramagnetic group.[21] These compounds were produced to investigate different aspects of the biological effects of alkylphosphocholines, although the leishmanicidal activity remains to be determined. Finally, a fluorescent miltefosine analog has been obtained recently, in which the potent anti-*Leishmania* activity of the parent drug has been preserved.[22]

The availability of SH-substituted analogs of MT with antiparasite properties would be of great utility in the search of possible mechanisms of activity of the parent drug. First, these analogs would make it possible to develop isolation techniques of putative MT receptors and targets in *Leishmania* parasites based on affinity chromatography methods.[23] In addition, self-assembled monolayers of miltefosine may be obtained by covalent bonding of the SH-substituted analog to a gold surface.[24] This derivatized gold substrate may be used as a specific sensor of *Leishmania* proteins that bind to the phosphocholine part of the molecule. Finally, the presence of the reactive SH-group in the MT analog would allow both the covalent attachment to the drug of a variety of probes (as biotin) and the reversible linkage of carrier molecules.[25] With these possible applications in mind, we wanted to report the synthesis and preliminary study of the in vitro leishmanicidal activity of 16-mercaptohexadecylphosphocholine (**1**, MT-SH), a MT analog substituted with the moderately lipophilic mercapto group at the end of the molecule alkyl chain.



Miltefosine (MT): R = H
1 (MT-SH): R = SH

Thiol **1** was synthesized from bromoalcohol **2** in three steps (Scheme 1): (1) reaction of **2** with trityl sulfide, yielding the thiol-protected alcohol **3** with 90% yield;[26] (2) introduction of the phosphocholine group in **3** by reaction with 2-chloro-2-oxo-1,3,2-dioxaphospholane and trimethylamine;[27] and (3) trityl deprotection with triethylsilane/trifluoroacetic acid.[28] Thiol **1** was thus isolated in pure form as a white powder in amounts in the range of hundreds of milligrams and with fair overall yield (36%).[29]



Scheme 1. Synthesis of thiol **1**. Reagents and conditions: (a) Ph_3CSH, K_2CO_3, MeOH, Ar, 90%; (b) 2-chloro-2-oxo-1,3,2-dioxaphospholane, Me_3N, MeCN, Ar, pressure tube, −78 °C, then rt, 2 h, and 70 °C, 4 h, 43%; (c) triethylsilane, TFA/CH_2Cl_2 1:1, Ar, rt, 1 h, 83%; (d) Ph_3CCl, DMF, Ar, rt, 48 h, 60%; (e) Cl_2SO, MeOH, Ar, 0 °C, then rt, 1 h, 98%; (f) DIBAL-H, MePh, Ar, rt, 1 h, 85%.

Alcohol **3** could be also obtained, albeit with lower overall yield (50%), from the carboxylic acid **4**, after trityl protection of the terminal mercapto group and esterification/reduction of the carboxylic acid group,[30,31] following methods previously used for the incorporation of protected mercapto groups to lipid molecules.[32]

Thiol **1** is stable in solid form, provided it is stored at low temperature under an inert atmosphere, and the freshly prepared samples are free of disulfide derivatives, as determined by Ellman titration[33] and ^{1}H NMR spectroscopy. The MT-SH methanol solution is stable for weeks at room temperature and in the presence of air. However, **1** is slowly oxidized to disulfide in DMSO solution, as other thiols.[34]

The leishmanicidal activity of MT-SH was assayed in vitro following standard protocols.[35,36] In this way, the inhibition of *L. donovani* promastigote proliferation by MT-SH (Fig. 1B) and, more important, of *Leishmania pifanoi* axenic amastigotes (Fig. 1D)—the form responsible for the pathology in vertebrates—was found to be identical to that of the parent drug (Figs. 1A and C). The data of Fig. 1 also show that both, the original drug and its mercapto analog, produce a reduced effect on the respective MT-resistant strains. Furthermore, the concentration-dependent leishmanicidal activity was the same for both compounds, regardless of the parasite stage and the MT-resistance of the isolate assayed. In fact, the difference between the respective LD_{50} values (Table 1) has no statistical significance. Note also that the similarity of the axenic amastigotes with those obtained from infected macrophages has been demonstrated by functional, morphological, antigenic, and metabolic criteria.[37]

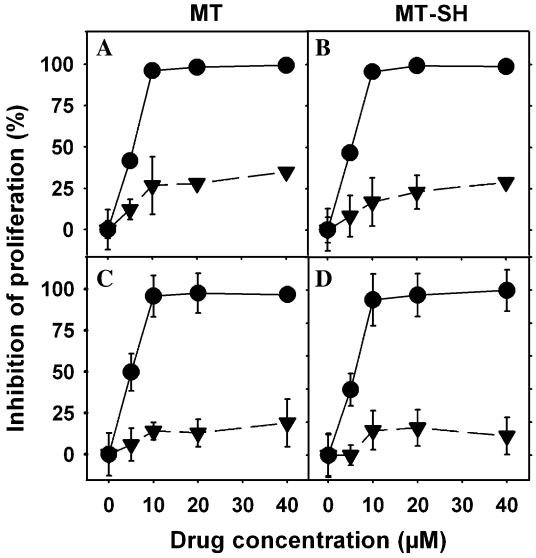


Figure 1. Leishmanicidal activity of miltefosine (MT) (A and C) and 16-mercaptohexadecylphosphocholine (MT-SH) (B and D), expressed as the percentage of proliferation inhibition relative to that of parasites grown in the absence of drug. Both compounds were tested on *Leishmania donovani* promastigotes (A and B) and on *Leishmania pifanoi* axenic amastigotes (C and D). (●) MT-susceptible parasites; (▼) MT-resistant strains. For experimental conditions, see Ref. 36.

Table 1. Leishmanicidal (as in Fig. 1) activity of miltefosine (MT) and 16-mercaptohexadecylphosphocholine (MT-HS) in vitro

Parasite	LD_{50}[a] (μM)	
	MT	MT-SH
L. donovani promastigote	5.3 (±0.5)	5.1 (±0.1)
L. donovani promastigote (MT-resistant)	>50	>50
L. pifanoi axenic amastigote	5.0 (±0.2)	5.5 (±0.1)
L. pifanoi axenic amastigote (MT-resistant)	>50	>50

[a] LD_{50}: drug concentration required to inhibit 50% parasite proliferation; mean values of three experiments; standard deviation given between parentheses.

The use of axenic amastigotes, which proliferate in the absence of host cells, removes interfering effects due to the uptake and transit of MT or its analog MT-SH through the macrophage, in order to reach the intracellular amastigote.

As noted above, the reactivity of the thiol group and the potent antiparasite effect of **1** provide the basis for several applications to investigate the MT leishmanicidal mechanism, as well as that of drug's resistances that would likely emerge in treated patients. For instance, we have prepared an immobilized form of MT by the facile reaction of **1** with iodoacetyl-modified agarose.[38] This material is being used as an affinity column to capture putative MT target proteins from *Leishmania* parasite lysates.

Acknowledgments

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26. Compound **3**: colorless powder, 90% from **2**; TLC, R_f (hexane/Et_2O, 1:1): 0.75; ^1H NMR (300 MHz, $CDCl_3$): δ 1.02–1.48 (m, 24H, H-3 to H-14), 1.37 (qn, J = 7.2 Hz, 2H, H-15), 1.56 (qn, J = 7.3 Hz, 2H, H-2), 2.13 (t, J = 7.2 Hz, 2H, H-16), 3.64 (t, J = 7.4 Hz, 2H, CH_2OH), 7.19 (t, J = 7.1 Hz, 3H, H_p-Ph), 7.27 (t, J = 7.3 Hz, 6H, H_m-Ph), 7.41 (d, J = 7.5 Hz, 6H, H_o-Ph); ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.7 (C-3), 28.4–29.6 (C-4 to C-15), 32.0 (C-16), 32.8 (C-2), 63.5 (C-1), 66.8 (CPh_3), 126.9 (C_p), 128.2 (C_m), 130.02 (C_o), 145.5 (C_i); FT IR (KBr) ν_{max}: 3435, 2918, 2850, 1631, 1591, 1483, 1465, 1445, 743, 701 cm^{-1}; MS ES^+, *m/z*: 539.3 $[M+Na]^+$, 243.1 $[CPh_3]^+$; Anal. Calcd for $C_{29}H_{42}O_2S$ (502.33): C, 81.22; H, 9.22; S, 6.38. Found: C, 81.25; H, 9.38; S, 6.15.
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29. Compound **1** (MT-SH): purified by column chromatography ($CHCl_3$/MeOH, 9:1, v/v and then $CHCl_3$/MeOH/H_2O, 65:25:5) and successive precipitation from $CHCl_3$/acetone and $CHCl_3/Et_2O$; white powder, 32% overall yield from **2**; mp 229–231 °C. R_f = 0.38 ($CHCl_3$/MeOH/H_2O, 65:25:5); ^1H NMR (300 MHz, DMSO-d_6): δ 1.11–1.38 (m, 24H, H-3 to H-14), 1.45 (m, 2H, H-2), 1.51 (q, J = 7.0 Hz, 2H, H-15), 2.22 (t, J = 7.6 Hz, 1H, S*H*), 2.46 (m, J = 7.2 Hz, 2H, H-16), 3.13 (br s, 9H, $N(CH_3)_3$), 3.48 (br s, 2H, CH_2N), 3.57 (q, J = 6.5 Hz, 2H, H-1), 3.99 (br s, 2H, OCH_2CH_2N); ^{13}C NMR (75 MHz, DMSO-d_6): δ 23.7 (C-16), 25.5–29.1 (C-3 to C-14), 30.6 (d, J = 7.3 Hz, C-2),

33.4 (C-15), 52.9, 53.0, 53.1 ($N(CH_3)_3$), 58.1 (d, $J = 4.8$ Hz, CH_2N), 63.8 (d, $J = 6.0$ Hz, C-1), 65.5 (br s, OCH_2CH_2N); FT IR (KBr) ν_{max}: 3028, 2917, 2849, 2551, 1483, 1471, 1243, 1079, 1060, 968 cm^{-1}; ESI^+ MS, *m/z*: 440.4 $[M+H]^+$, 879.5 $[2M+H]^+$, 1318.9 $[3M+H]^+$; HR MS (L-SIMS) calcd for $(C_{21}H_{46}NO_4PS)H^+$: 440.2963; found: 440.2965. Anal. Calcd for $C_{21}H_{46}NO_4PS{\cdot}H_2O$ (457.65): C, 55.11; H, 10.57; N, 3.06; S, 7.29. Found: C, 55.07; H, 10.18; N, 3.22; S, 6.91.

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36. *Growth conditions.* Promastigotes of *L. donovani* (MHOM/SD/00/1S-2D) were cultured at 26 °C in RPMI-1640 medium (Gibco), supplemented with 10% heat-inactivated fetal calf serum (HIFCS), gentamicin, penicillin, and 2 mM glutamine. Resistant strains were kindly provided by Prof. S. L. Croft (London School of Tropical Medicine and Hygiene). *L. pifanoi* axenic amastigotes (MHOM/VE/60Ltrod) were grown at 32 °C in M199 medium (Gibco-BRL 31100), supplemented with 20% HIFCS, 5% trypticase, and 50 μg/mL hemin, pH 7.2. Its resistant strains were obtained from parasites grown under a stepwise increase of MT concentration, and cultured as the parental strain, except for the addition of 40 μg/mL MT to the growth medium. *Antileishmanial activity determination in vitro.* Parasites harvested at a late growth exponential phase were resuspended in the respective growth medium (4×10^6 parasites/mL) and transferred to a 96-microwell plate (50 μL/well); an equal volume of MT solution in growth medium at twice the final concentration was added to each well. Parasites were allowed to proliferate for 72 h at 27 °C (promastigotes) or for 120 h at 32 °C (axenic amastigotes). Then, parasites were removed from the plate and washed twice with 1 mL Hanks medium (136 mM NaCl, 4.2 mM Na_2HPO_4, 4.4 mM KH_2PO_4, 5.4 mM KCl, and 4.1 mM $NaHCO_3$, pH 7.2), supplemented with 20 mM D-glucose. *Assay of inhibition of parasite proliferation.* Parasites were resuspended in 100 μL of 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution in Hanks medium. MTT reduction was carried out at 26 °C or 32 °C for promastigotes or amastigotes, respectively, for 2 h. The resulting formazan was solubilized by addition of an equal volume of 10% w/v sodium dodecylsulfate, and incubated overnight at 37 °C; absorbance was measured at 595 nm in a 450 Bio-Rad Microplate Reader. All assays were performed in triplicate and the experiments were repeated at least twice. LD_{50} (drug concentration required to inhibit 50% parasite proliferation) was calculated using SigmaPlot software.
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38. *Linking of MT-SH to agarose.* A solution of thiol **1** (2 μmol) in a mixture of methanol (0.2 mL) and pH 8.50 buffer (50 mM Tris, 5 mM EDTA) (0.8 mL) was made to react with iodoacetyl-modified agarose (SulfoLink Coupling Gel, Pierce, Rockford, IL) (1 mL). The coupling reaction was completed after 45 min at room temperature (first 15 min with stirring), as determined by the negative result of Ellman test for free SH-groups. After washing with 1 M NaCl, any remaining iodoacetyl groups in the agarose were blocked by cysteine (50 mM, conditions as above).