Synthesis and Biological Evaluation of Fluorescent Leishmanicidal Analogues of Hexadecylphosphocholine (Miltefosine) as Probes of Antiparasite Mechanisms

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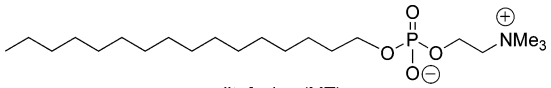
The leishmanicidal mechanism of miltefosine (hexadecylphosphocholine, MT) is not clearly understood. Valuable insights into its mode of action could be obtained by fluorescence techniques, given suitably emitting analogues. In this regard, the synthesis and biological characterization of two fully competent MT fluorescent analogues is reported here: *all*-(*E*)-13-phenyltrideca-6,8,10,12-tetraenylphosphocholine (PTE-MT) and *all*-(*E*)-13-phenyltrideca-8,10,12-trien-6-ynylphosphocholine (PTRI-MT). Both compounds show large absorption coefficients and a modest, but usable, fluorescence yield. Their activities were very similar to that of MT and were recognized by the MT uptake system of *Leishmania*. Their localization in living *L. donovani* promastigotes by confocal microscopy show a homogeneous intracellular distribution of the fluorescence. The concentration of PTRI-MT within the parasites (ca. 1.7 mM) showed a 100-fold enrichment relative to its external concentration. These results are consistent with a multiple target leishmanicidal mechanism for MT and validate the application of these analogues for pharmacokinetic and diagnostic studies concerning the chemotherapy of leishmaniasis.

Introduction

Leishmaniasis is a protozoal disease encompassing a complex of clinical manifestations caused by different species of human parasites of the genus *Leishmania*. The clinical forms of the disease range from some self-healing cutaneous leishmaniasis to the most severe visceral infection (kala-azar in India), fatal if untreated.[1] The disease is endemic in 88 countries, located mostly in tropical and subtropical regions, with a worldwide prevalence of 10−12 million, and 1.5 million new cases per year.[2,3] In addition, there is an important incidence of *Leishmania* co-infection in HIV[a] patients, due to the opportunistic character of the parasite. Nowadays, treatment of the disease relies exclusively on chemotherapy,[4] with organic pentavalent antimonials as a first line option. These drugs, which are administered parenterally in high doses requiring patient hospitalization, are cardiotoxic, show variable clinical response,[4,5] and are expensive. One alternative therapy is based on amphotericin B (AmB), an antifungal polyenic antibiotic that can be nephrotoxic. Liposome formulations of AmB show almost no side effects, but are unaffordable in the areas of high prevalence. Besides, the ever-increasing resistance against organic antimonials originated a major clinical concern, fostering the search for alternative chemotherapies.[5]

Miltefosine (hexadecylphosphocholine, MT) is a synthetic alkylphosphocholine initially developed as an antineoplastic agent, but also endowed with a potent leishmanicidal activity.[6,7] In recent years, MT has become the first successful oral treatment against human leishmaniasis in field-trials, with mild secondary effects,[8−11] and is currently licensed in India, Colombia, and Germany. On the other hand, the propensity of MT for selecting resistant strains of the parasite underlies the convenience of patient monitoring during treatment, as well as the introduction of a combined chemotherapy. In this regard, a simple test of MT-resistant parasites would be highly desirable.



miltefosine (MT)

The substantial knowledge on the clinical applicability of MT contrasts with the incomplete and often conflicting information on the drug's primary targets in *Leishmania*.[12,13] The antiparasite mechanism of MT has been associated to inhibition of key steps in the biosynthesis of ether-lipids and alkyl-anchored glycoproteins,[14] impaired fatty acid elongation and desaturation processes,[15] inverted PC:PE ratio,[16] disfunction of mitochondria,[17,18] or inhibition of choline transport,[19] among others. Regardless of the targets involved, MT causes an apoptosis-like death in both *Leishmania* stages.[20−22]

Up to now, the only high-resistant *Leishmania* phenotype to MT which has been described is characterized by the reduced intracellular drug accumulation.[23] This may result either from an increased efflux of the drug, carried out by overexpressed P-like glycoproteins,[23,24] or from its faulty inward translocation across the plasma membrane. In this last case, failure of drug internalization was related to a lack-of-function mutation of the

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[a] Abbreviations: ALP, alkyl-lysophospholipid; AmB, amphotericin B; BSA, bovine seroalbumin; DAPI, 4′,6-diamidino-2-phenylindole; DB99, 2,5-bis-(4-amidinophenyl)-3,4-dimethylfuran; DIBAL-H, diisobutylaluminium hydride; ET, edelfosine or 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; HIV, human immunodeficiency virus; LD_{50}, drug concentration required to inhibit 50% of parasite proliferation; LdMT, *Leishmania donovani* aminophospholipid translocase; MT, miltefosine or hexadecylphosphocholine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PTE-ET, *all*-(*E*)-1-*O*-(15′-phenylpentadeca-8′,10′,12′,14′-tetraenyl)-2-*O*-methyl-*rac*-glycero-3-phosphocholine; PTE-MT, *all*-(*E*)-13-phenyltrideca-6,8,10,12-tetraenylphosphocholine; PTRI-MT, *all*-(*E*)-13-phenyltrideca-8,10,12-trien-6-ynylphosphocholine; PTRI-OH, *all*-(*E*)-13-phenyltrideca-8,10,12-trien-6-ynylol; SDS, sodium dodecyl sulfate; THF, tetrahydrofuran; TMSOTf, trimethylsilyl trifluoromethanesulfonate.

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sole system involved in MT transbilayer transport in *Leishmania*, consisting of an ATP-dependent aminophospholipid translocase (LdMT) and its regulatory proteins.[25−27]

The identification of the subcellular MT distribution may provide important clues on the drug's parasiticidal mechanisms. By means of radiolabeled MT it was possible to quantitate the drug uptake by *Leishmania*.[25−27] However, this technique is not suitable for obtaining unambiguous information on the drug subcellular distribution, because of the difficulty of isolating intact large intracellular organelles. The lysis of this parasite was severely hampered by the high resistance of its plasma membrane, originating in a membrane-bound subpellicular network of microtubules which imposed harsh methods of disruption, with consequent damage of the more fragile organelles. Additionally, the small size, amphipatic properties, and relatively high water solubility of MT contribute to a fast exchange among different organelles during sample preparation, which may bias the original subcellular distribution.

An alternative way of determining MT distribution and intracellular target localization, with minimal manipulation and subcellular distortion, is by means of fluorescent analogues of the drug. The use of fluorescent ligands to assess the affinity and localization of receptors in cell biology is well documented.[28−30] When the cellular target can be isolated, the application of spectroscopic methods from solution photophysics may provide additional structural information with sub-nanometer resolution.[31,32] On the other hand, the large absorption coefficients of many fluorescent tags are useful in determining the concentration of the drug's analogue in whole cells or in specific organelles. The challenge is, of course, to develop a true fluorescent drug, in which the affinity and selectivity for the potential therapeutic target(s), as well as the pharmacodynamic properties of the parent drug, are preserved. Drugs of relatively high molecular mass (700−1000 Da) are likely to contain structural elements nonessential for the interaction with the target, thus providing convenient anchoring points for a fluorescent tag, as exemplified by the antitumoral drug paclitaxel (taxol).[33] However, the small size of MT, its amphipatic structure, and the paucity of structure−antiparasitic data make the goal of obtaining bioactive emitting analogues more demanding.

In the present work, MT fluorescent analogues were designed which maintain the phosphocholine head group, essential for preserving leishmanicidal activity *in vivo* (*vide infra*). Instead, nonpolar conjugated *all*-(*E*)-phenylpolyene groups were incorporated into the alkyl chain. Although these chromophores show modest emission yields and may photodegrade under intense illumination, they have large absorption coefficients in the near UV (340−370 nm) and tend to preserve overall lipid properties.[34−38] For the antitumoral ether-lipid edelfosine (ET), the phenylpolyene analogue *all*-(*E*)-1-*O*-(15′-phenylpentadeca-8′,10′,12′,14′-tetraenyl)-2-*O*-methyl-*rac*-glycero-3-phosphocholine (PTE-ET) displays the same membrane distribution and very similar proapoptotic activity in several tumor cell lines as the parent drug.[39,40]

Two emitting analogues of miltefosine are reported here, in which the original fully saturated C16 alkyl chain has been replaced by a shorter polymethylene (C5) connector linked to phenyltetraene (analogue PTE-MT) or phenyltrienyne (analogue PTRI-MT) groups. The resulting fluorescent analogues show potent leishmanicidal activity, compete with MT for intracellular uptake and reproduce the effect of the drug on MT-resistant and susceptible *Leishmania* strains. Similar antiparasite properties were also determined for the edelfosine analogue PTE-ET. Furthermore, the distribution of the analogues inside living parasites was obtained by standard methods of fluorescence confocal microscopy, as well as a reliable estimate of their intracellular concentration.

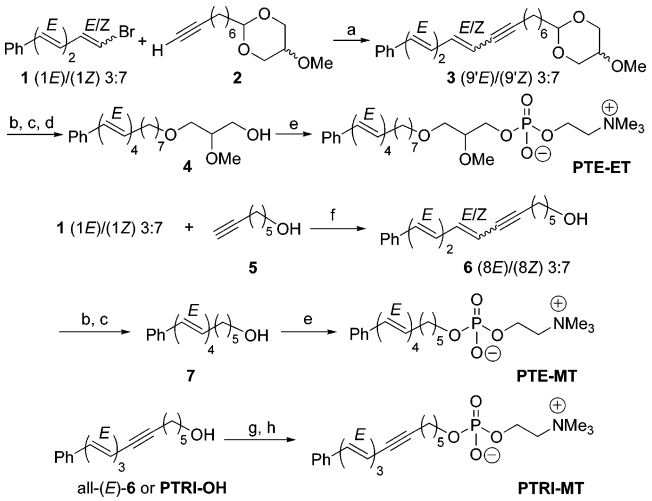
Chemistry. Molecular Design. Previous information on structure−activity relationships (SAR) of MT analogues is limited and only partly useful for the design of fluorescent alkylphosphocholines. MT was originally developed for antineoplastic applications, and therefore SAR studies are focused mainly on that specific property.[41−44] Since it its unknown whether the antitumor and antiparasite[12,45,46] effects of MT involve common mechanisms, only those SAR studies addressing antileishmanial effects of alkylphospholipids are considered here.

The effect of phospholipid alkyl chain length (C15 to C20), its branching, and other structural variables has been tested *in vitro* against *L. donovani* extracellular promastigotes and amastigotes in infected mouse peritoneal macrophages;[6] eight compounds, including MT (C16) and an alkyl phosphoethanolamine, were reported active at <6 μg mL^{-1}, but no clear relationship between antiparasite activity and chain length was evident. However, *in vivo* assays (mice infected with *L. donovani*) indicated that compounds with phosphocholine, but not with phosphoethanolammonium, head groups were active.[6] Twelve analogues of MT, including saturated C14−C22 alkylphosphocholines, *cis* or *trans* monounsaturated *N*,*N*,*N*-trialkyl-substituted alkylphosphoethanolammonium, and saturated analogues with terminal *N*-methylpyrrolidinium or *N*-methylpiperidinium polar groups, have been also tested *in vitro* for activity against promastigotes of three different *Leishmania* strains, isolated from patients with visceral leishmaniasis.[47] Under these conditions, it was found that higher cytotoxicity is associated to C16 or C18 polymethylene chain length. Interestingly, the presence of a *cis* or *trans* double bond in this chain does not change the activity significantly. When the analogues were assayed in infected mice, the C18 and, especially, the C16 (MT) phosphocholines were most active.[47] In another study, three series of phospholipid analogues of MT incorporating cycloalkylidene or aromatic rings in the alkyl group, and terminal *N*,*N*,*N*-trimethylamonium, *N*-methylpiperidinium, or *N*-methylmorpholinium polar groups, were tested *in vitro* on promastigotes of *L. donovani* or *L. infantum*.[48] It was concluded that introducing cycloalkylidene groups in alkylphosphocholines resulted in enhanced activity.[48] On the other hand, a miltefosine analogue with a terminal thiol group (16-mercaptohexadecylphosphocholine) has been synthesized, as an intermediate in the development of affinity chromatography methods. The leishmanicidal activity of this compound (*in vitro*) is identical to that of the parent drug.[49] Finally, a few analogues have been obtained incorporating reporter probes such as a tetrafluorophenylazido group[50] for photolabeling purposes, or a nitroxide paramagnetic group at position 12 of octadecylphosphocholine.[51] However, the antileishmanial activity of these compounds was not reported.

The above information suggests that desirable structural features for an emitting analogue of optimal activity are a lipophilic fluorescent group positioned at the end of a polymethylene chain, linked to a phosphocholine head group, and a total length close to that of a C16−C18 alkylphosphocholine. To maintain a small size for the emitting group, we selected the phenylpolyene chromophores mentioned above. The synthetic strategy for the preparation of MT analogues containing these specific structures is detailed below.

Synthesis. The edelfosine analogue PTE-ET was obtained as described elsewhere.[39] Briefly (Scheme 1), a Sonogashira−

Scheme 1[a]



[a] Reagents and conditions: (a) **2**/**1** mole ratio = 1.3, $Pd(PPh_3)_2Cl_2$ (7.5 mol %), CuI (25 mol %), Et_2NH, THF, Ar, room temp, 2 h, 90%; (b) Zn(Cu/Ag), MeOH/H_2O 1:1, room temp, 24 h, 95%; (c) I_2 (trace), hexane, Ar, 15 min reflux, 95%; (d) DIBAL-H, MePh, Ar, 0 °C, 99%; (e) 2-chloro-1,3,2-dioxaphospholane-2-oxide, Me_3N, MeCN, pressure tube, −78 °C, then room temp, 2 h, and 70 °C, 1 h, 36%; (f) **5**/**1** mol ratio = 1.3, conditions as in a, 80%: (g) 2-chloro-1,3,2-dioxaphospholane-2-oxide, Et_3N, benzene, Ar, room temp, 1 h, filter, solvent elimination, then THF−H_2O 1:1, Amberlite MB-3, filter, extract with MeOH; (h) TMSOTf, Me_3N, MeCN, pressure tube, −78 °C, then room temp, 2 h, 34% (g plus h).

Hagihara cross-coupling between 1-bromo-6-phenylhexa-1,3,5-triene (**1**) (3:7 *all*-(*E*)/(1*Z*,3*E*,5*E*) mixture) and *cis*- or *trans*-5-methoxy-2-oct-7′-ynyl-1,3-dioxane (**2**) (obtained by acetalization of non-8-ynal with *rac*-2-*O*-methylglycerol) yielded the corresponding coupling compound **3** with the same (*E*)/(*Z*) ratio. Partial reduction of the triple bond with activated zinc, followed by *Z*→*E* isomerization (iodine), gave the respective *all*-(*E*)-*cis* or *all*-(*E*)-*trans* tetraenes which, upon acetal cleavage/reduction with DIBAL-H, produced the *all*-(*E*) alcohol **4**. A final reaction of **4** with 2-chloro-1,3,2-dioxaphospholane-2-oxide and trimethylamine yielded the phenyltetraene edelfosine analogue PTE-ET in 17−28% overall yield. Miltefosine analogues *all*-(*E*)-13-phenyltrideca-6,8,10,12-tetraenylphosphocholine (PTE-MT) and *all*-(*E*)-13-phenyltrideca-8,10,12-trien-6-ynylphosphocholine (PTRI-MT) were obtained in a similar way, from the corresponding *all*-(*E*) alcohols **7** and **6**. Phenyltrienyne alcohol **6** was synthesized as (8*E*,10*E*,12*E*)/(8*Z*,10*E*,12*E*) 3:7 mixture by a similar cross-coupling reaction between bromotriene **1** and hept-6-yn-1-ol (**5**), following reported conditions.[52] Pure isomer *all*-(*E*)-**6** was easily separated from the (8*E*)/(8*Z*) mixture by selective precipitation with *n*-pentane from concentrated CH_2Cl_2 solutions. Subsequent partial reduction of **6** (pure *all*-(*E*) isomer or (8*E*)/(8*Z*) mixtures) with activated zinc, and isomerization with iodine of the resulting phenyltetraene, yielded the *all*-(*E*) alcohol **7**.[52]

Results

Spectral Properties and Leishmanicidal Activity of Fluorescent Miltefosine Analogues. The absorption and emission spectra of the phenyltetraene (PTE-MT) and phenyltrienyne (PTRI-MT) analogues differ very little from those of the isolated chromophores,[53] indicating the lack of electronic interaction between the fluorophore and the phosphocholine head group. Representative absorption and fluorescence spectra of PTRI-MT are shown in Figure 1. The absorption spectrum, recorded in the same Hanks buffer solution used to load the drug to parasites, shows an intense absorption band with maxima at 323, 338, and 355 nm, and absorption coefficients of 43000, 57700, and 47600 M^{-1} cm^{-1}, respectively; both analogues are nonfluorescent in this solution. The fluorescence spectrum shown in the same figure corresponds to PTRI-MT specifically bound to living promastigotes of *L. donovani*. The fluorescence, with an estimated quantum yield of 0.1−0.2,[53] appears bluish to the eye. The spectral properties of the PTE-MT analogue are very similar to those of PTRI-MT shown above, and also barely depart from those of PTE-ET, the corresponding edelfosine analogue described elsewhere.[39]

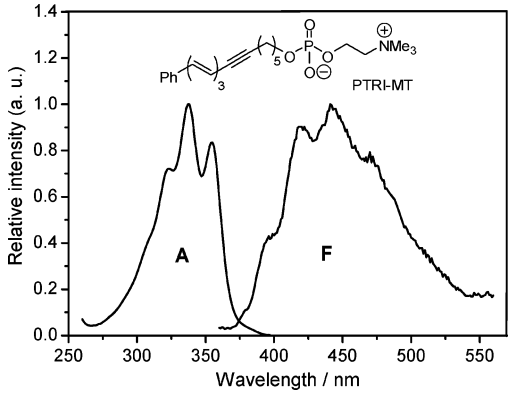
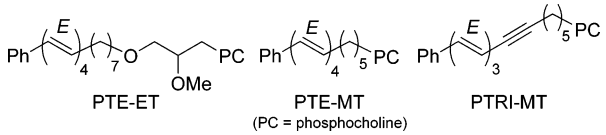


Figure 1. Absorption spectrum (A) of PTRI-MT in Hanks buffer solution, pH 7.2, and corrected fluorescence emission spectrum (F) of the analogue specifically bound to *Leishmania donovani* promastigotes resuspended in the same medium. PTRI-MT initial concentration 10 μM, 1.5 × 10^9 cells mL^{-1}, 26 °C, λ_{exc} = 335 nm.

The inhibition of parasite proliferation by the fluorescent analogues of MT was assayed with the promastigote form of *L. donovani* and the *L. pifanoi* amastigote, the pathological form of the parasite in vertebrates. Both PTE-MT and PTRI-MT analogues showed a potent concentration-dependent cytotoxic effect in the micromolar range (Table 1 and Figure 2). In the case of *L. pifanoi* amastigotes, the LD_{50} value of the analogues (drug concentration required to inhibit 50% of parasite proliferation) was the same as that of the parent drug MT, while their LD_{50} values against the promastigote form was 2-fold higher. Two sets of control experiments were also included in Table 1 and Figure 2. In the first one, the activity of the edelfosine analogue PTE-ET, which fully mimics the proapoptotic effect of ET in tumor cells,[39] was also assayed, since both ET and MT share the same membrane transporter in *Leishmania*.[25] In the second one, the effect of the MT analogues was tested on R-40 *L. donovani* promastigotes, a strain resistant to both MT and ET.[25] The data in Table 1 show a potent antiparasitic effect for the ET analogue PTE-ET, consistent with the sharing of a common transporter for the uptake of this ET analogue and for the MT analogues PTE-MT and PTRI-MT. Moreover, the emitting analogues, as well as the parent drug, failed to inhibit promastigote growth in the MT-resistant strain.

Intracellular Distribution of MT Analogues in *Leishmania* Parasites. Once the high leishmanicidal activity of the fluorescent analogues was established, these compounds were used to visualize their distribution inside the infectious organism. Images of parasites incorporating the emitting compounds PTE-ET, PTE-MT, and PTRI-MT were obtained by standard living-cell confocal microscopy, after careful elimination of the fraction not specifically bound to the parasite. The same uniform and homogeneous intracellular distribution (Figure 3) of the three analogues can be observed, in both wild-type strains of *L. donovani* promastigotes and of *L. pifanoi* amastigotes. In sharp contrast, MT-resistant *L. donovani* promastigotes, treated and observed under identical experimental conditions, appeared

Table 1. In Vitro Antileishmanial Activity[a] of Fluorescent Analogues of Miltefosine (MT) and Edelfosine (ET)



	$LD_{50} \pm SE$ (μM)[b]		
	L. donovani promastigotes		*L. pifanoi* amastigotes
compd	MT sensitive strain	MT resistant strain	MT sensitive strain
MT	12.5 ± 2.9	>60	26.3 ± 3.4
PTE-ET	9.0 ± 1.3	>60	22.5 ± 0.8
PTE-MT	28.6 ± 4.5	>60	26.0 ± 5.8
PTRI-MT	29.6 ± 2.6	>60	26.5 ± 1.8
PTRI-OH[c]	>150	>150	>150

[a] Expressed as mean $LD_{50} \pm SE$, $n = 3$, each experiment was repeated at least twice. [b] LD_{50}: drug concentration required to inhibit 50% parasite proliferation. [c] Alcohol *all*-(*E*)-**6**.

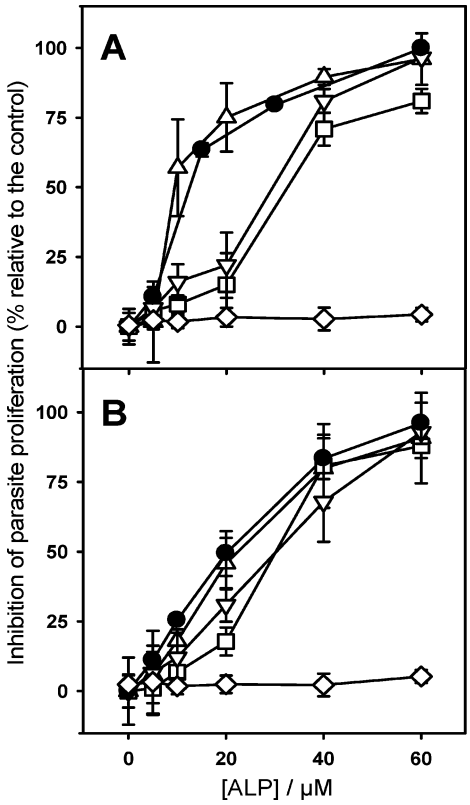


Figure 2. Inhibition of *Leishmania* proliferation by fluorescent alkyllysophospholipid (ALP) analogues of miltefosine and edelfosine, expressed as percentage relative to untreated parasites. Proliferation was determined as a function of ALP concentration in cell cultures as described in the Experimental Section. (A) *L. donovani* promastigotes. (B) *L. pifanoi* amastigotes. Each point represents the mean ± SE (n = 3) obtained from at least two independent experiments. ●, MT; △, PTE-ET; ▽, PTE-MT; □, PTRI-MT; ◇, alcohol *all*-(*E*)-**6**.

barely stained. The PTE-MT analogue bound to parasites underwent faster photobleaching than PTRI-MT in the confocal microscope. Since both compounds display similar activity (Table 1) and distribution patterns within *Leishmania* (Figure 3), PTRI-MT was selected for the rest of the experiments reported here.

The absence of a privileged accumulation of PTRI-MT inside subcellular organelles, as observed in Figure 3, was confirmed by sequential labeling of the parasite with MitoTracker Red, which stains preferentially the mitochondrion, one of the reported targets of MT in *Leishmania*,[18,22,54] and then with the PTRI-MT analogue. The fluorescence patterns of the parasite-bound forms of the two dyes differed substantially, as shown in Figure 4; whereas PTRI-MT blue fluorescence appears homogenously distributed throughout the inside of the parasite, the pattern for MitoTracker Red emission is clearly restricted to the areas of the mitochondrion included in the observed section.

Metabolic Stability of the Fluorescent Analogues of Miltefosine. The interpretation of the fluorescence images recorded after the incorporation of the MT analogues into *Leishmania* parasites may be misleading if fluorescent degradation products were also present within the parasite. The metabolic stability of these analogues, as well as that of PTE-ET, was investigated by allowing the parasite to incorporate a sublethal amount of each fluorescent compound; after 4 h of incubation the possible degradation products were extracted and separated by TLC. As shown in Figure 5, fluorescent degradation products were not detected, since all fluorescent spots are coincident with those of the corresponding initial analogue. As a further test, compounds **7** and *all*-(*E*)-**6**, the precursor alcohols for PTE-MT and PTRI-MT, respectively, which are the most likely fluorescent degradation products from parasite esterases, were run in the same plate. Figure 5 shows that none of these products can be detected in the corresponding lane. Hence, it can be concluded that the fluorescent analogues are metabolically stable in *L. donovani* promastigotes and *L. pifanoi* amastigotes, ensuring that the fluorescence images presented above correspond to molecules of the original emitting analogue.

Specific Uptake and Intracellular Amount of PTRI-MT in *Leishmania* Promastigotes. The competition between PTRI-MT and the parent drug MT for the uptake by *Leishmania* parasites can be easily probed by recording the changes in fluorescence intensity of the cells. As shown in Figure 6, the incorporation of the emitting analogue to *L. donovani* promastigotes was inhibited by increasing concentrations of MT, in a dose-dependent manner, decreasing the fluorescent staining of the parasites. The time frame was reduced to 1 h in order to avoid PTRI-MT reaching the steady state.

In addition, the actual concentration of the emitting analogues internalized by the parasite can be determined from their characteristic absorption spectrum. Although absorption measurements are not as sensitive as fluorescence detection, the large absorption coefficients of these compounds compensate for the lower sensitivity, with the advantage that absorption spectra are usually less affected than emission yield by the chromophore environment. To determine the internal concentration in *L. donovani* promastigotes, the uptake of PTRI-MT (15 μM) was

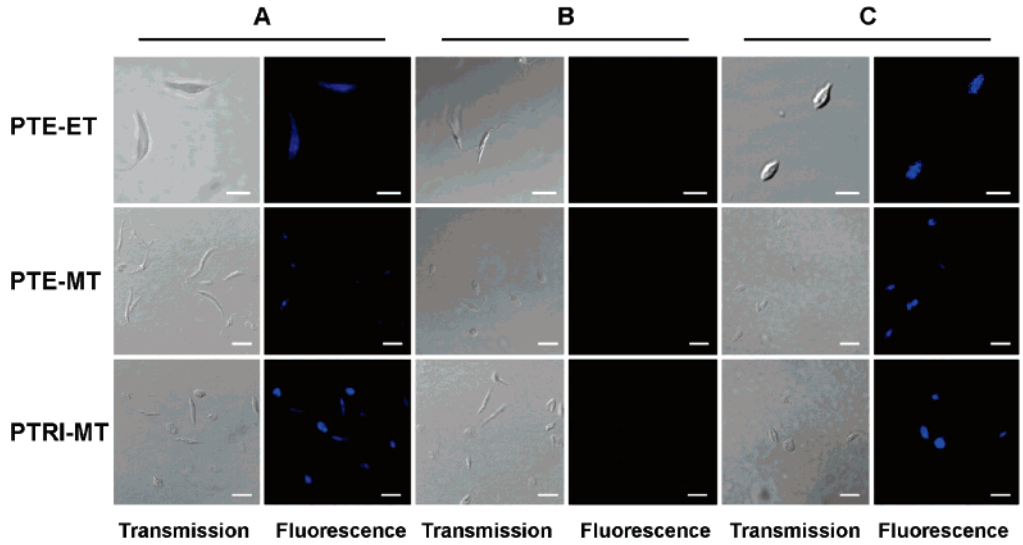


Figure 3. Images of the specific binding of fluorescent analogues of edelfosine (PTE-ET) and miltefosine (PTE-MT, PTRI-MT) to *Leishmania* parasites, using live-cell confocal microscopy. (A) *L. donovani* promastigotes, wild type, incubated with each fluorescent analogue (7.5 μM, 4 h, 26 °C) in Hanks medium. (B) *L. donovani* promastigotes, MT-resistant strain, incubated with the same fluorescent analogues (7.5 μM, 4 h, 26 °C) and recorded with identical laser power and gain settings. (C) *L. pifanoi* amastigotes incubated with each fluorescent analogue (7.5 μM, 4 h, 32 °C) in Hanks medium. Microscope settings: $\lambda_{exc} = 351$ nm, $\lambda_{em} = 460$ nm; bar = 8 μm. Additional details are given in the Experimental Section.

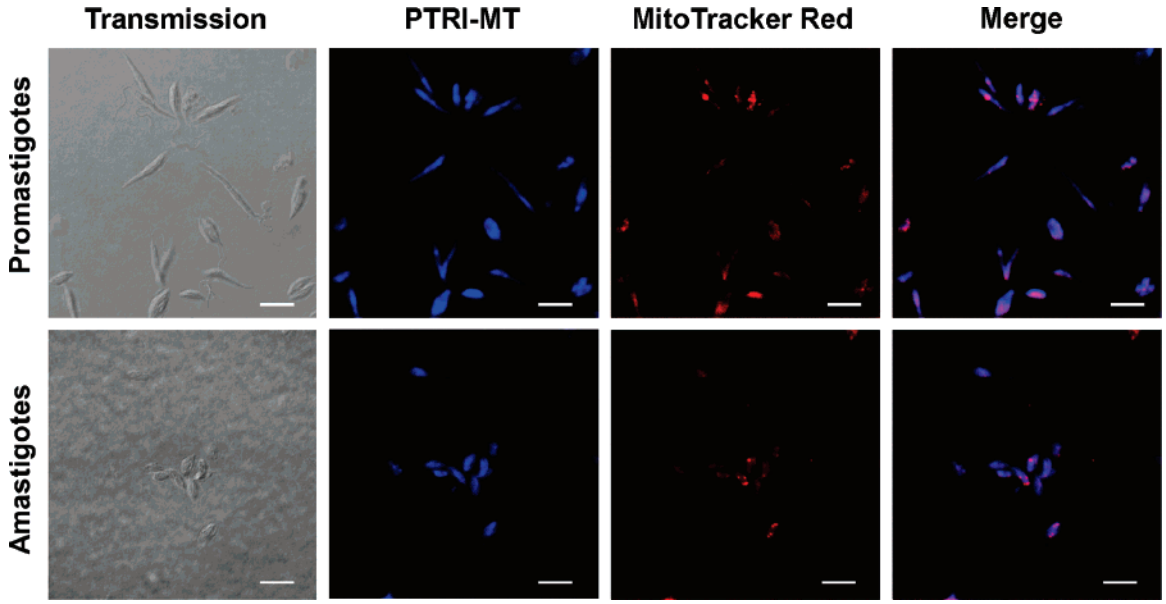


Figure 4. Visualization by living-cell confocal microscopy of *Leishmania* parasites double-labeled with the miltefosine analogue PTRI-MT and MitoTracker Red. *L. donovani* promastigotes (top row) and *L. pifanoi* amastigotes (lower row) were incubated successively with 0.1 μM MitoTracker Red (15 min) and 7.5 μM PTRI-MT (4 h). In the overlay images preferential labeling of mitochodria by MitoTracker Red (purple pixels) can be distinguished clearly from the uniform distribution of the bound PTRI-MT (blue pixels). Microscope settings: PTRI-MT, $\lambda_{exc} = 351$ nm, $\lambda_{em} = 460$ nm; MitoTracker Red, $\lambda_{exc} = 543$ nm, $\lambda_{em} = 599$ nm. Bar = 8 μm.

allowed to proceed for 2 h, to ensure a steady level of intracellular accumulation. Afterward, the analogue was extracted and assayed by HPLC (see Experimental Section). Under these conditions, the total amount of PTRI-MT incorporated into MT-sensitive promastigotes was 7.6 ± 0.7 nmol mg^{-1} of protein, a value to be compared with that previously described[27] of 5.0 ± 0.7 nmol mg^{-1} of protein at saturation, determined from the uptake of radiolabeled MT (initial external concentration 2.5 μM). Using a value of 4.3 × 10^{-6} L mg^{-1} of protein for the intracellular volume of *L. donovani* promastigotes,[55] the intracellular concentration of PTRI-MT in promastigotes would be ca. 1.7 mM. When the external PTRI-MT concentration was reduced to 7.5 μM, a similar 50% reduction was determined in the intracellular concentration (0.7 mM).

Discussion

The literature on leishmanicidal fluorescent drugs is scarce. The well-known nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI) and 2,5-bis-(4-amidinophenyl)-3,4-dimethylfuran (DB99), two fluorescent analogues of pentamidine, a cationic diamidine used as a second-line alternative in the treatment of leishmaniasis, have been used before to study drug accumulation in *Leishmania*.[56,57] Both compounds were shown to concentrate in the mitochondrion of pentamidine-sensitive strains of the parasite. In the present work, we wanted to develop functional emitting analogues of the first-line anti-*Leishmania* drug MT with similar purposes. However, the use of labeled lipids to study living-cell processes, as, e.g., metabolism, subcellular distribution or partition into membrane domains, usually requires

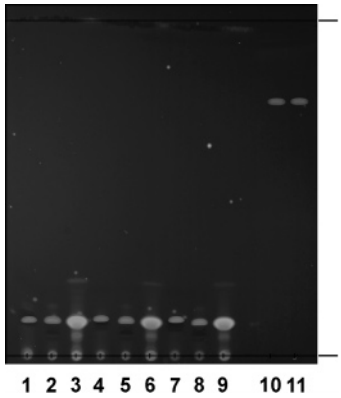


Figure 5. Metabolic stability of fluorescent analogues of miltefosine (MT) and edelfosine (ET) incorporated into *Leishmania* promastigotes and amastigotes. Both forms of *Leishmania* parasites were allowed to incorporate the analogues (7.5 μM) for 2 h, stripped of nonspecifically bound compound, and further incubated for 4 h. The analogues were finally extracted from the parasites with $CHCl_3$−MeOH 2:1 and run into TLC plates. Original samples of the analogues: lanes (1) PTE-ET, (2) PTE-MT, and (3) PTRI-MT. Analogues extracted from *L. donovani* promastigotes: lanes (4) PTE-ET, (5) PTE-MT, and (6) PTRI-MT. Analogues extracted from *L. pifanoi* amastigotes: lanes (7) PTE-ET, (8) PTE-MT, and (9) PTRI-MT. Alcohols **7** and *all*-(*E*)-**6**: lanes (10) and (11), respectively.

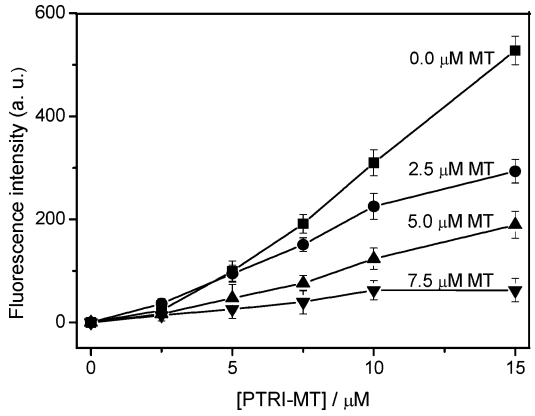


Figure 6. Inhibition of the uptake of the fluorescent analogue PTRI-MT by miltefosine (MT) in *L. donovani* promastigotes. Parasites were allowed to incorporate PTRI-MT at increasing concentration of MT for 1 h. The uptake was estimated by the fluorescence intensity associated to parasites at λ_{exc} = 351 nm and λ_{em} = 460 nm, after removal of the nonspecifically bound analogue. MT concentration is shown on its respective trace.

additional controls and cautious data interpretation, in comparison with labeled hydrophilic drugs. As noted above, this is a consequence of the changes in the lipid physical and chemical properties, which are likely to result from the introduction of the fluorescent tag.[58] An additional source of uncertainty in *in vivo* experiments is the impaired recognition of the labeled drug by the different biological systems involved in cellular uptake, intracellular traffic, metabolism, and target binding.[59,60]

In the present work, the suitability of inserting conjugated phenylpolyene fluorophores in the aliphatic chain of MT to produce functional analogues of the drug was evaluated. This approach was used successfully before for the antineoplastic alkyl-ether phospholipid ET,[39,61] which also presents leishmanicidal activity[62] and shares a common membrane transporter with MT in *Leishmania*.[25] The results presented above show that PTE-MT and PTRI-MT are fully functional analogues of MT, being also recognized by the miltefosine LdMT transporter, as evidenced by the low uptake by MT-resistant promastigote strains.[25] Additional resistance traits were not present in this strain, as indicated by the rescue of MT sensitivity through episomal expression of a functional LdMT.[25]

The blue emission of the analogue PTE-MT is adequate for mechanistic studies in cell suspensions using standard plate-reader or spectrofluorimetric techniques. However, once incorporated into the parasite, the PTE group underwent irreversible photodegradation on the confocal microscope, thereby hampering the recording of high-resolution images with long exposure times. As a result, a new analogue was obtained, PTRI-MT, in which the phenylpolyunsaturated system contains a triple bond. Photochemical studies in model compounds[53] indicated a higher photostability of this chromophore, which was also observed in the corresponding analogue. The antiparasitic activity of both PTE-MT and PTRI-MT analogues is very similar, despite the decreased molecular flexibility of the last one due to the triple-bond. This was not totally unexpected, in view of the SAR data discussed above.[48]

Interestingly, the impairment of antiparasite activity due to the presence of the fluorophore in the MT alkyl chain was lower in the case of *Leishmania sp.* amastigote, the pathological form of the parasite in vertebrates, than in promastigotes. The reason for this difference is unknown. Both stages differ substantially in overall metabolism and protein expression patterns,[63,64] the latter possibly including some MT targets. Alternatively, the regulation of the lipid transporter, or of other efflux systems, may vary depending on the stage of the parasite. More work is needed to substantiate any of these hypotheses.

The previously reported high metabolic stability of MT,[27] was also preserved in the fluorescent analogues. Degradation byproducts could not be detected after extensive intracellular incubation of the incorporated analogues, regardless of the form of the parasite used. This gives additional support to extrapolating the recorded fluorescence pattern in the parasite to that of the parent drug.

An important observation of this study is the rather homogeneous distribution of the emitting analogues inside the promastigote form, even though some of the MT targets previously described have been localized inside membrane-bound organelles such as mitochondria[17,18] or glycosome[14] and, therefore, are devoid of direct physical communication with the cytoplasm. As shown here, the cytotoxic effect of the analogues takes place without any apparent privileged accumulation in any cell organelles. This was further substantiated by the different images observed in double-labeling experiments with PTRI-MT and MitoTracker Red stains. Unlike the case of PTRI-MT, the intracellular fluorescence pattern of MitoTracker Red was patchy and restricted to the mitochondrial areas included in the confocal section. Accordingly, the perturbation of mitochondrial functionality by the parent drug MT does not result from a preferential accumulation of the drug inside this organelle.

The absorption spectral parameters allowed a reliable estimation of the intracellular concentration of the analogues. In this way it could be shown that PTRI-MT internal concentration in promastigote forms of *L. donovani* after 2 h uptake was 1.7 mM, that is, a factor of ∼100 higher than the external initial concentration (15 μM). Both the uptake kinetics and the internal concentration values of the analogue were similar to those previously reported for radiolabeled MT.[27] These values may appear surprising, although comparable data were also found for other leishmanicidal drugs. Assuming the same intracellular volume and amount of protein in promastigotes regardless of the strain and *Leishmania* species, the intracellular concentration of Sb^{3+} into *L. tarentolae* promastigotes was ca. 2 mM,[65] while that of AmB in *L. donovani* promastigotes was 0.4 mM.[66] For

pentamidine, another leishmanicidal drug, the reported values are not as large (0.053 mM),[67] which reflect the lower accumulation factor (5−6). In the case of furamidine, a pentamidine analogue, the intracellular concentration in African trypanosomes reached 12.2 mM.[68] It should be noted that for AmB and pentamidine a large fraction of the drug incorporated to *Leishmania* parasites was found associated with membrane ergosterol or kinetoplast DNA, respectively.[57] Hence, the drug local concentration might be much higher than the average intracellular concentration.

The high intracellular concentration of PTRI-MT and MT enables the interaction with receptors with modest binding affinity for the drug. In other words, a multitarget mode of antiparasite action of these compounds would be quite feasible. If true, this may represent a major pharmacological advantage of MT, because a single inactivating mutation of one of its therapeutic targets may not lead necessarily to full loss of drug's potency. In fact, until now MT-resistance has been solely produced by the faulty accumulation of the drug inside promastigotes.[23] On the other hand, the amphipatic nature of MT will drive a substantial fraction of the drug to partition into intracellular membranes, likely affecting their physicochemical properties, as well as the activity of enzymatic and transport systems modulated by these properties; indeed, changes in phospholipid composition have been reported in *Leishmania*,[15,16] as well as in *Trypanosoma cruzi* epimastigotes,[69] after MT treatment.

Conclusions

Two blue-emitting analogues of miltefosine (MT), PTE-MT and PTRI-MT, have been successfully synthesized and characterized. The respective fluorophores were lipophilic phenyltetraene and phenyltrienyne conjugated groups, linked through a pentamethylene chain to a phosphocholine polar head. Both analogues displayed the same cytotoxic activity as the parent drug against amastigotes of *L. pifanoi*, and a lower but still potent activity against promastigotes of *L. donovani*. In addition, it is shown here that (i) PTE-MT and PTRI-MT compete with the parent drug for the intracellular incorporation to *L. donovani* promastigotes at the low micromolar range, and (ii) the analogues are not internalized by MT-resistant parasites, characterized by a defective MT inward translocator.[24] These results were interpreted as due to the presence of a common inward translocator for the original drug and its analogues in the parasite plasma membrane. The metabolic stability and emitting properties of these molecules were appropriate for visualizing their localization within living parasites by confocal microscopy. The first images of this kind revealed a uniform intracellular distribution of the analogues in *L. donovani* promastigotes, which paralleled that of the parent drug. If so, these images ruled out a preferential accumulation of MT inside organelles, e.g., glycosomes or mitochondria, containing described MT targets.[17,18] The absorption properties of the analogues provided a direct method to estimate the intracellular concentration of these compounds, amounting to ca. 1.7 mM for PTRI-MT in *L. donovani* promastigotes, similar to that of the parent drug MT. This high concentration would permit substantial binding of MT to low-affinity receptors. All these observations are consistent with a multitarget leishmanicidal mechanism of MT. Further insight into the details of the anti-*Leishmania* activity of MT and similar drugs at a molecular level, as well as of its pharmacokinetic properties, are expected to be now more accessible using the emitting analogues described here.

Experimental Section

Chemistry. All reactions were carried out under an argon atmosphere. Reagents were used as obtained commercially. Solvents were purified by standard techniques and deoxygenated thoroughly by argon bubbling prior to use. Yields refer to the isolated pure compound. Analytical thin layer chromatography (TLC) was carried out on precoated silica gel plates, Merck 60F254, 0.25 mm. Compounds were visualized with UV light and phosphomolybdic acid solution followed by heating. Flash column chromatography was performed on silica gel Merck 60, 230−400 mesh, 0.040−0.063 mm. High performance liquid chromatography (HPLC) analyses were carried out with an Agilent 1100 chromatograph equipped with a 5-μm C18 reverse phase column (4.6 × 150 mm) and a diode array detector, using as eluent MeOH−H_2O 9:1 v/v plus 10 mM H_3PO_4 at a flow rate of 1.2 mL min^{-1}, and detection at 346, 354, and 362 nm. For quantitative HPLC analysis of PTRI-MT incorporation in *L. donovani* promastigotes, calibration plots with a 30 μM MeOH solution of phospholipid were used. Lipid concentration was determined by phosphate assay subsequent to total digestion.[70] ^{1}H NMR spectra were recorded on an INOVA-300 spectrometer. Chemical shifts are reported in parts per million (ppm), using as internal reference the proton signal of the trace of undeuterated solvent: δ 7.26 in $CDCl_3$. Abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), m (complex multiplet). (*E*)/(*Z*) ratios were calculated from ^{1}H NMR relative integrals of characteristic signals. IR spectra (in cm^{-1}) were recorded on Perkin-Elmer 681 and FT-Spectrum One spectrometers. Low-resolution mass spectra were recorded by electrospray ionization in the positive mode (ESI^+) in a Hewlett-Packard 1100 spectrometer. High-resolution mass spectra were determined in an AutoSpec Micromass (Waters) apparatus in the L-SIMS mode using Cs^+ (30 kV) in *m*-NBA matrix, with PEG as internal standard. UV−vis absorption spectra were registered on Varian CARY-3E or Perkin-Elmer Lambda-2 spectrophotometers. Steady-state corrected fluorescence spectra were recorded in a SLM 8000D spectrofluorimeter. Alcohol **4** and PTE-ET were synthesized as described elsewhere.[39] Alcohols **6** and **7** were obtained by cross-coupling between bromopolyene **1** and alkyne **5**, following reported conditions.[52]

***all*-(*E*)-13-Phenyltrideca-6,8,10,12-tetraenylphosphocholine (PTE-MT).** Trimethylamine (ca. 2 mL) was condensed into a solution of alcohol **7** (35 mg, 0.13 mmol) in acetonitrile (5 mL) in a pressure tube at −78 °C under argon. 2-Chloro-1,3,2-dioxaphospholane-2-oxide (0.12 mL, 1.3 mmol) was then added to the cooled solution, and the reactor was closed and left 2 h at room temperature and 1 h at 70 °C. After cooling back, the reactor was opened, the solvent and excess trimethylamine were vacuum-evaporated, and the residual solid was dissolved in THF−H_2O 9:1 v/v (10 mL). Amberlite (MB-3 or TMD-8) was then added, the mixture was stirred 10 min and filtered, and the separated solid was washed with MeOH (3 × 10 mL). The filtrate and washings were collected, the solvent was vacuum-evaporated, and the residual solid was purified by column chromatography on silica gel, first with $CHCl_3$−MeOH 9:1 and then with $CHCl_3$−MeOH−H_2O 65:25:5. The isolated solid was finally precipitated from a $CHCl_3$ solution with *n*-pentane. Yellowish waxy solid. Yield 20 mg, 36%. R_f = 0.2 (MeOH−H_2O 9:1). R_t (HPLC): 2.38 min ^{1}H NMR (300 MHz, $CDCl_3$, 30 °C): δ 7.38 (d, J = 15.8 Hz, 2 H, H_o), 7.29 (m, 2 H, H_m), 7.19 (m, 1 H, H_p), 6.82 (m, 1 H, H-12), 6.52 (d, J = 15.8 Hz, 1 H, H-13), 6.35 (m, 2 H, H-10, H-11), 6.23 (m, 2 H, H-8, H-9), 6.10 (m, 1 H, H-7), 5.72 (m, 1 H, H-6), 4.30−3.70 (m, 6 H, CH_2-CH_2N, CH_2N, H-1), 3.30 (s, 9 H, N(CH_3)$_3$), 2.11 (m, 2 H, H-5), 1.67 (m, 2 H, H-2), 1.40 (m, 4 H, H-3, H-4). ESI^+ MS: 434.3 [M + H^+]. HR MS (L-SIMS): calcd for $C_{24}H_{37}NO_4P$ (M + H^+) 434.2460, observed 434.2461. Phosphorus analysis: calcd for $C_{24}H_{36}NO_4P$ 7.13, observed 7.39. UV/vis (MeOH), λ_{max} (ϵ, M^{-1} cm^{-1}): 325 (50000), 340 (71000), 359 (58700) nm; (DMSO) λ_{max} (ϵ, M^{-1} cm^{-1}): 333 (37400), 349 (52000), 368 (42400) nm.

***all*-(*E*)-13-Phenyltrideca-8,10,12-trien-6-ynylphosphocholine (PTRI-MT).** 2-Chloro-1,3,2-dioxaphospholane-2-oxide (0.6 mL, 6.53 mmol) was added to a solution of alcohol *all*-(*E*)-**6** (90

mg, 0.34 mmol) and triethylamine (5 mL) in benzene (10 mL) at room temperature under argon and with stirring. After 1 h in these conditions, the mixture was filtered, the solvent was vacuum-eliminated, and the residue was dissolved in THF−H_2O 1:1 (10 mL). Amberlite (MB-3 or TMD-8) was added to the solution, and the mixture was stirred for 10 min and filtered, the separated solid was washed with MeOH (3 × 10 mL), the filtrate and washings were collected, and the solvent was vacuum-evaporated. The residual solid was dissolved in acetonitrile (10 mL), the solution was placed in a pressure tube under argon, trimethylsilyl triflate (0.2 mL, 1.1 mmol) was then added at room temperature, and the mixture was stirred for 30 min. After cooling to −78 °C, trimethylamine (ca. 2 mL) was condensed and the reactor was closed. After stirring for 2 h at room temperature, the reactor was opened and the solvent and excess trimethylamine were vacuum-evaporated. The residual solid was purified by column chromatography on silica gel, first with $CHCl_3$−MeOH 9:1 and then with $CHCl_3$−MeOH−H_2O 65:25:5. The isolated solid was finally precipitated from a $CHCl_3$ solution with acetone. Yellowish waxy solid, mp 187−190 °C. Yield 50 mg, 34%. R_f = 0.2 (MeOH−H_2O 9:1). t_R (HPLC): 1.97 min ^{1}H NMR (300 MHz, $CDCl_3$, 30 °C): δ 7.38 (m, 2 H, H_o), 7.29 (m, 2 H, H_m), 7.21 (m, 1 H, H_p), 6.78 (m, 1 H, H-12), 6.58 (m, 1 H, H-9), 6.57 (d, J = 15.8 Hz, 1 H, H-13), 6.37 (m, 2 H, H-10, H-11), 5.62 (m, 1 H, H-8), 4.26 (m, 2 H, CH_2CH$_2$N), 3.81 (m, 2 H, H-1), 3.74 (m, 2 H, CH_2N) 3.31 (s, 9 H, N(CH_3)$_3$), 2.34 (m, 2 H, H-5), 1.58 (m, 6 H, H-2 to H-4). ESI$^+$ MS: 432.2 [M + H$^+$]. HR MS (L-SIMS): calcd for $C_{24}H_{35}NO_4P$ (M + H$^+$) 432.2304, observed 432.2294. Phosphorus analysis: calcd for $C_{24}H_{34}NO_4P$ 7.18, observed 7.18. UV/vis (MeOH) λ_{max} (ϵ, M^{-1} cm^{-1}): 321 (43200), 335 (58000), 353 (52000) nm; (DMSO) λ_{max} (ϵ, M^{-1} cm^{-1}): 329 (43400), 345 (62100), 363 (51300) nm.

Growth Conditions. *L. donovani* promastigotes, line MHOM/ET/67/L82, and its MT resistant strain MHOM/ET/67/L82R40 were kindly provided by Prof. S. L. Croft (London School of Hygiene and Tropical Medicine) and were grown at 26 °C in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, gentamycin, penicillin, and 2 mM glutamine (complete growth medium).[12] Its resistant strain was grown as the susceptible parental strain, except for the addition of 40 μM MT into the growth medium. The axenic line of *L. pifanoi* amastigotes MHOM/VE/60/Ltrod was cultured at 32 °C, as described.[71]

Leishmanicidal Activity. *Leishmania* parasites were harvested at a late exponential phase of growth, washed twice, and resuspended at 2 × 10^6 parasites mL^{-1} in growth medium in the presence of the corresponding concentration of MT, or their fluorescent analogues, in a 96-microwell plate. They were allowed to proliferate in the presence of the ALP at 26 °C for 72 h for promastigotes, or at 32 °C for 120 h for amastigotes.[72] Then, parasites were collected by centrifugation, washed with Hanks medium (136 mM NaCl, 4.2 mM Na_2HPO_4, 4.4 mM KH_2PO_4, 5.4 mM KCl, 4.1 mM $NaHCO_3$, pH 7.2) supplemented with 10 mM D-glucose (Hanks+Glc), and resuspended into 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution at 0.5 mg mL^{-1}. MTT reduction was carried out for 2 h at 26 °C or 32 °C for promastigotes or amastigotes, respectively. The resulting formazan was solubilized by addition of an equal volume of 10% w/v SDS, and incubated overnight at 37 °C; absorbance values were then measured at 595 nm in a 450 Bio-Rad Microplate Reader.[49] All assays were performed in triplicate and the experiments were repeated twice at least. The inhibition caused by each fluorescent analogue was expressed as percentage, relative to the untreated parasites. LD_{50} and its standard error were calculated using SigmaPlot 9.0 software.

Promastigote Uptake of PTRI-MT. *L. donovani* promastigotes from resistant and susceptible strains were harvested at a late exponential phase of growth, washed twice in Hanks+Glc, resuspended at a final density of 1.5 × 10^7 parasites mL^{-1} in the same medium with the respective concentration of PTRI-MT, and incubated at 26 °C for 2 h. Then, parasites were harvested by centrifugation and washed twice with 10 mL of fatty acid free bovine serum albumin (BSA) (10 mg mL^{-1}) in Hanks medium, and the resulting pellet was extracted with $CHCl_3$−MeOH 2:1 v/v (3 × 0.5 mL). The solvent was evaporated, the residue was redissolved in 1 mL of MeOH and filtered (Whatman filter, 0.2 μm), and the PTRI-MT incorporated into the parasite was determined by HPLC as mentioned above. Competition experiments with MT were carried out as above, except that the volume was reduced to 1 mL, and uptake was stopped after 1 h, before reaching a steady state. The parasites were harvested, resuspended in 100 μL of the same medium, and transferred into a 96-microwell microplate. Under these conditions, the detergent effect associated to the fluorescent analogue plus MT was always <5%, as assessed by the entrance of the fluorescent vital dye SYTOX green.[18] Fluorescence intensity was measured in a Polarstar Galaxy microplate reader equipped with 340 and 460 nm filters for excitation and emission wavelengths, respectively.

Fluorescence Confocal Microscopy. Parasites were resuspended in growth medium containing each fluorescent analogue at a final concentration of 7.5 μM and incubated for 4 h at 26 °C. Excess analogue not specifically bound was eliminated by washing the parasites three times with Hanks + 10 mg mL^{-1} BSA (fatty acid free). The parasites were resuspended in 25 μL of the same medium and transferred into a fluorescence black 15-well slide (Cell-Line, Portsmouth, NH). In double-labeling experiments, parasites were loaded with 0.1 μM MitoTracker Red CMXRos (Molecular Probes, Leiden, Holland) for 15 min at 26 °C, before the incubation with the fluorescent analogue. Confocal fluorescence images were obtained on a Leica TCS-SP2-AOBS-UV ultraspectral confocal microscope (Leica Microsystems, Heidelberg, Germany), with excitation at 351 nm and emission at 460 nm for the fluorescent analogues, and with excitation at 543 nm and emission at 599 nm for MitoTracker Red.

Metabolic Stability of the Fluorescent Analogues. Uptake of the analogues at 7.5 μM by *L. donovani* promastigotes or *L. pifanoi* amastigotes was carried out according to the standard procedure described above, except that the volume of the parasite suspension was raised to 10 mL (total number of parasites 8 × 10^7) for promastigotes, or 30 mL of the same density for amastigotes, and the incorporation was stopped after 2 h. Afterward, the nonspecifically incorporated dye was removed from the parasites by three washes with 10 mL of BSA 10 mg mL^{-1} in Hanks medium at 4 °C. The parasites were resuspended in Hanks+Glc and incubated at their corresponding temperature for additional 4 h. The fluorescent analogues and their possible degradation products were extracted from the cell pellet with $CHCl_3$−MeOH 2:1 v/v, the solvents were evaporated under nitrogen, and the residue was solubilized with 20 μL of $CHCl_3$−MeOH−H_2O 65:25:5 v/v/v and loaded onto a TLC silica gel plate, which was run with the same solvent. The corresponding alcohols *all*-(*E*)-**6** and **7** were run as controls. Spots were identified under UV illumination and photographed.

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