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New alkenyl derivative from *Piper malacophyllum* and analogues: Antiparasitic activity against *Trypanosoma cruzi* and *Leishmania infantum*

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Abstract

Alkylphenols isolated from *Piper malacophyllum* (Piperaceae), gibbilimbols A and B, showed interesting activity against the parasites *Trypanosoma cruzi* and *Leishmania infantum*. In continuation to our previous work, a new natural product from the essential oil of the leaves of *P. malacophyllum* was isolated, the 5-[(3*E*)-oct-3-en-1-il]-1,3-benzodioxole, and also a new set of five compounds was prepared. The antiparasitic activity of the natural product was evaluated in vitro against these parasites, indicating potential against the promastigote/trypomastigote/amastigote forms (IC_{50} 32-83 µM) of the parasites and low toxicity (CC_{50} >200 µM) to mammalian cells. The results obtained to the synthetic compounds indicated that the new derivatives maintained the promising antiparasitic activity, but the cytotoxicity was considerably lowered The amine derivative LINS03011 displayed the most potent IC_{50} values (13.3 and 16.7 µM) against amastigotes of *T. cruzi* and *L. infantum*, respectively, indicating comparable activity to the phenolic prototype LINS03003, with 3-fold decreased (CC_{50} 73.5 µM) cytotoxicity, leading the selectivity index (SI) towards the parasites up to 24.5. In counterpart, LINS03011 has not shown membrane disruptor activity in Sytox Green model. In summary, this new set showed the hydroxyl is not essential for the antiparasitic activity, and its substitution could decrease the toxicity to mammalian cells.

American trypanosomiasis (Chagas' disease) and visceral leishmaniasis are among the most important parasitic infections known as neglected tropical diseases.[1] They mainly affect developing and poor countries, especially in Latin America, Middle East and Asia, and are caused by the protozoans of the genus *Trypanosoma* and *Leishmania*, respectively.[2-5] Although these diseases affect millions of people worldwide,[1] only few drugs are available for their treatment (amphotericin B, benznidazole, miltefosine, nifurtimox, pentamidine and antimonials), which are poorly effective and considerably toxic. Despite the urgent necessity for new drugs to treat these conditions, low investments are directed to this aim, giving their "neglected" status.[6,7]

Considering this background, the search for prototypes to drug discovery against neglected diseases is an important research field, and natural products can be considered good starting point. In this respect, two alkylphenol derivatives, gibbilimbols A and B, isolated from the essential oil from the leaves of *Piper malacophyllum*, showed promising activity against both of the parasites responsible for Chagas' disease and visceral leishmaniasis, *Trypanosoma cruzi* and *Leishmania* (L.) *infantum,* respectively.[8,9] This previous study[8] revealed that gibbilimbol B was

capable of affecting the permeability of the plasma membrane of *L.* (L.) *infantum* in a similar way than amphotericin B, suggesting that this action could be result from the similarity to the membrane phospholipids, being the hydroxyl group the “polar head” and the alkyl chain the “lipophilic tail”. Moreover, gibbilimbol B is more potent than the A isomer, also suggesting the functionalization closer to the aromatic ring would improve the activity.

In a previous report,[9] we presented a set of eight gibbilimbol analogues to evaluate the role of functionalization and molecular modifications in the “lipophilic tail”. This set included substitution close to aromatic ring and lower homologues with six-atom length instead of ten. These analogues were tested against trypomastigote and amastigote forms of *T. cruzi* and promastigote and amastigote forms of *L. (L.) infantum*, in addition to the evaluation of cytotoxicity to mammalian cells. The data resulted in preliminary structure-activity relationship (SAR) information. LINS03003 (Figure 1) was the most promising molecule, exhibiting IC_{50} values from 28.6 to 1.8 μM against these parasites. However considerable cytotoxicity to mammalian cells (CC_{50} 23.5 μM) was verified. Other molecules of the series showed more potent IC_{50} values than the natural prototypes, but they showed higher cytotoxicity than both gibbilimbols A and B indeed.

In continuation to our work exploiting the potential from natural and synthetic compounds, the present work reports a new compound from the essential oil from leaves of *P. malacophyllum*, 5-[(3*E*)-oct-3-en-1-il]-1,3-benzodioxole (**1**, Figure 1). The structure of this compound was determined by detailed analysis of NMR and MS spectral data (see Supplementary Information) and despite the novel proposed structure, this compound are closely related to gibbilimbol B. Considering this similarity, we decided to investigate the activity of this new natural product against *T. cruzi* and *L. (L.) infantum*, as well as its cytotoxicity against NCTC mammalian cells.

The present report aims to deal with the cytotoxicity issue. All the compounds of the previous set (and also gibbilimbols) present a phenol group, which is responsible for toxic effects in several molecules,[10-14] and sometimes can be classified as pan-assay interference compounds (PAINS)[15] due to its possible interference as redox cycler. However, this phenolic hydroxyl would be part of the pharmacophore when considering the membrane disruption hypothesis observed previously.[8,9] Considering this, and the results from compound **1**, a new set of compounds (**2**-**6**, Figure 1) were designed and synthesized with a new site of modifications, the 4-position substituent on the aromatic ring, as an attempt to investigate the importance of the hydroxyl in the biological activity. The proposed molecular modifications included the methylation of the hydroxyl and its removal, represented by the methoxybenzene and benzene groups, respectively.

The compounds were synthesized in a similar fashion than the previous reported set (Figures 2 and 3, see Supplementary Information).[9] Compounds **2** and **3** were prepared from the corresponding benzaldehydes and 1-octylamine by mixing them together in equimolar amount under ultrasonic conditions. The reaction led to moderate yields for both compounds. Compound **4** was then prepared using compound **2** as starting material, which was converted to the corresponding amine by reacting with $NaBH_4$, with good yield.

The amide derivative **5** was synthesized from aminolysis of benzoyl chloride with 1-octylamine, in presence of triethylamine (TEA). The product was then purified by recrystalization in water:ethanol mixture, also with good yield. Finally, compound **6** was obtained through aldol condensation of anisaldehyde and 2-nonanone in basic conditions, with adequate yields.

The antiprotozoal activity of the new set of analogues **1–6** was evaluated *in vitro* against trypomastigote/amastigote forms of *T. cruzi* as well as promastigote/amastigote forms of *L. (L.) infantum* using the method from Varela et al.,[9] except by use of bone marrow-derived mouse macrophages differentiated using cytokine M-CFS obtained from L929 cell-conditioned medium. Benznidazole and miltefosine were used as positive control against *T. cruzi* and *L. (L.) infantum*, respectively. Cytotoxicity was determined in mammalian NCTC clone 929 cells as previously described.[9]

The natural product **1** has shown interesting activity against all parasite forms, with IC_{50} values ranging from 32 to 82 µM. It must be stressed this compound was more active against the amastigote forms of the parasites, the most relevant form in the diseases. This activity profile is very similar to the gibbilimbol B, however the substitution of the phenol for the benzodioxole showed to improve the activity. In addition, the cytotoxicity was quite low for this compound (>200 µM). Considering the higher activity of the benzodioxole **1** in comparison to the phenolic gibbilimbols, it is suggested the phenolic hydroxyl is not essential to the activity, and thus it is an important site for structural exploitation to obtain analogues with improved activity as well as reduced cytotoxicity.

Among the five new analogues synthetized, only the compound **4** showed significant activity against the trypomastigote form of *T. cruzi*, with IC_{50} value of 21 µM. The remaining compounds showed poor activity against this form of the parasite (>100 µM). However, three compounds showed considerable activity against the amastigote form of *T. cruzi*, being the compound **4** the most active (IC_{50} 13 µM). As observed in the previous results from our group,[9] the amine group in the “tail” moiety

leads to promising activity, especially against the amastigote form. Molecules **2** and **6** also showed interesting activity, but were less active against the trypomastigotes than compound **4**. According to Drugs for Neglected Disease initiative (DNDi) the intracellular amastigotes are the most important target to drug discovery studies, but candidates that eliminate the trypomastigotes are of great interest, due to recrudescence of the parasite.[16] In our study, compound **4** fulfilled these requirements, also showing a selectivity towards *T. cruzi* with a SI value of 5.5 (considering the intracellular amastigotes).

Similar activity profile was observed when the compounds were tested against *L. infantum* parasite. The amine compound **4** was again the most active against both forms of the parasite, promastigotes and amastigotes, being more active against the promastigotes. On the other hand, the prototype LINS03003 was more active against amastigote form of *L. infantum* than against the promastigotes. Compounds **2** and **3** were also active against both forms of *L. infantum*, showing IC_{50} values in the range of 28-40 μM. The amide derivative **5** lacks considerable activity against both parasites, as previously observed to the phenolic analogues, although it showed no considerable cytotoxicity indeed.

Maybe the most important finding in this work is the low cytotoxicity of this new set of gibbilimbol analogues. Practically all the compounds showed decreased toxicity to mammalian cells than the previous set from our group.[9] With exception of compound **4**, which exhibited a CC_{50} value of 74 μM, the compounds showed CC_{50} >200 μM. Although compound **4** was considerably cytotoxic, the selectivity indexes towards the parasites were over than 3.6, and up to 25 considering the *L. infantum* promastigotes. Perhaps this low cytotoxicity can be attributed to the absence of phenolic hydroxyl in this new set, corroborating with the hypothesis of the toxicity of this group. Moreover, the compounds suggest the hydroxyl is not essential to the biological activity albeit it increases the activity, especially against *T. cruzi* trypomastigotes. As example, when comparing the methoxyl compound **6** to its hydroxyl analogue LINS03001,[9] the activity against *T. cruzi* amastigote is almost the same, but the cytotoxicity is quite low when the methoxy group is present. The same behavior can be observed for compound **4**, which exhibited CC_{50} 3-fold increased than the hydroxyl molecule LINS03003. On the other hand, the benzodioxole **1** suggests that additional substitution in the 3-position of the aromatic ring may improve the activity keeping the cytotoxicity low, and in future works we should prepare synthetic 3-substituted derivatives.

Considering the gibbilimbols could act through membrane disruption,[8] the compound **4** was studied using the fluorescent probe Sytox Green in late growth-phase (non-stationary) of promastigotes of *L. infantum*[8,17,18] (Figure 4). Sytox Green (Molecular Probes) is a high-affinity nucleic acid stain that penetrates cells with compromised plasma membranes and enhances its fluorescence by more than 500-fold upon nucleic acid binding. The non-ionic surfactant Triton X-100 (Tx100) was used as the positive control, resulting in the maximal intensity of fluorescence. Fluorescence was

measured in a microplate reader with excitation and emission wavelengths of 485 and 520 nm, respectively. Gibbilimbol B showed to promote the penetration of the dye, suggesting the disruption of the plasma membrane permeability of *Leishmania*.[8] However, compound **4** showed no increased fluorescence inside the cells at the EC_{50} value, suggesting other mechanism than gibbilimbol against *Leishmania*, which caused disruption of the plasma membrane. At elevated concentration (EC_{90}) compound **4** slightly increased the dye fluorescence; possibly the hydroxyl of gibbilimbol B plays an important role in this action, acting as the "polar head" to guide its membrane insertion. Since compound **4** do not present the hydroxyl group, this action in the membrane was reduced. Further studies must be done to confirm this statement.

Natural products are always reported as inspiring molecules to achieve novel antiparasitic derivatives. Several works are reported reporting semi-synthetic molecules to evaluate the role of substituents in the activity. Morais et al. reported the promising activity of natural and semi-synthetic masticadienoic acid derivatives against *T. cruzi* and *L. infantum*, achieving good IC_{50} values for both parasites.[19] Aphidicolin was also used to prepare some semi-synthetic derivatives to assess the antileishmania activity in several species.[20] However, natural products are frequently very complex structures which difficult the synthesis of cheap bioactive compounds, an important requirement for future drugs to neglected diseases. The compounds presented herein are much simpler structures in comparison to these cited examples, allowing the easy design of several synthetic analogues to improve the activity profile.

In summary, the results presented herein demonstrated that this new set of gibbilimbol analogues also showed considerable activity but with improved selectivity towards the parasites, possibly due to the absence of the toxicophoric phenolic group. These molecules present important information to guide the design of molecules with improved activity, and in fact a new set of derivatives based on the amine derivatives **4** and LINS03003, in addition to the characteristics of compound **1** are in progress by our group to investigate the role of other substituents in these molecules.

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Figures captions

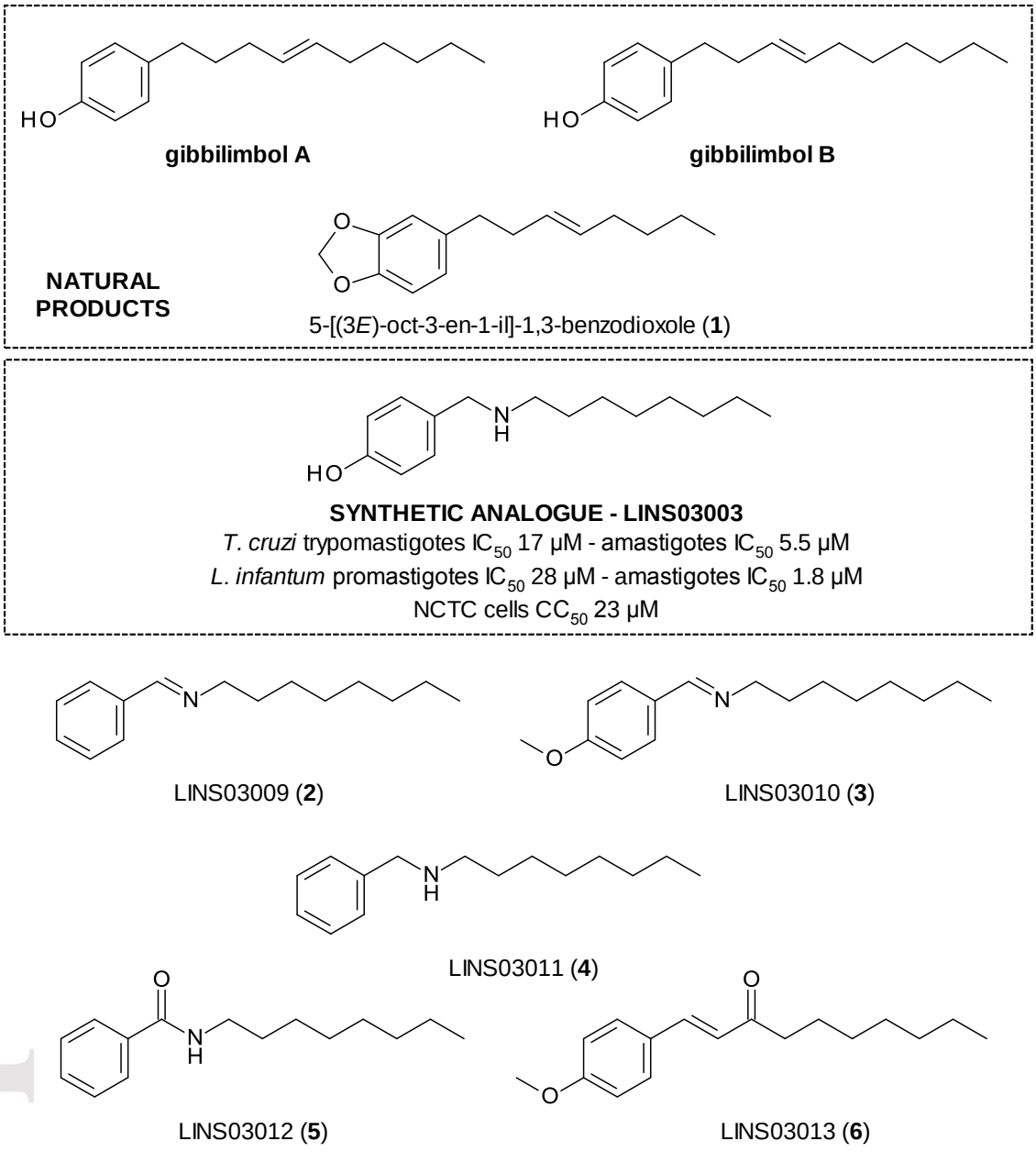

Figure 1. Natural products, lead compounds and the new set of LINS03 molecules.

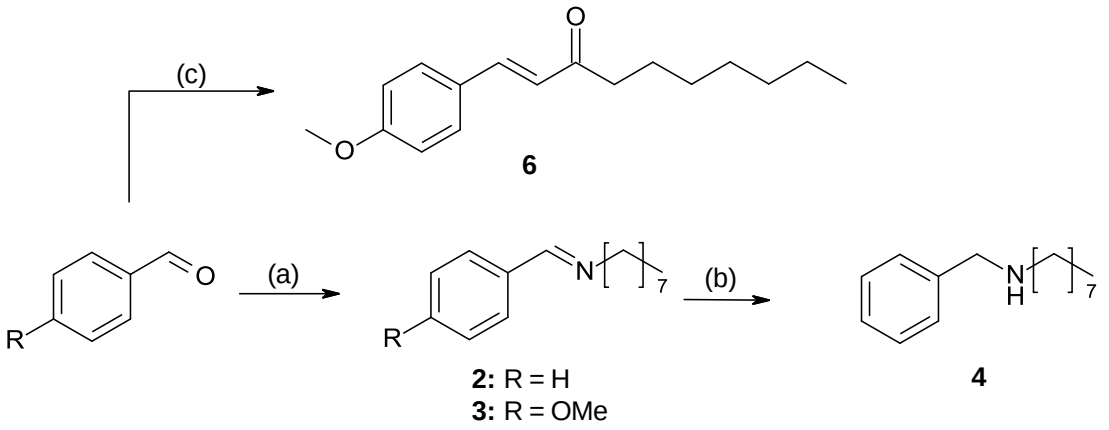

Figure 2. Synthesis of LINS03 compounds **2**-**4** and **6**. Reagents and conditions: (a) octylamine, THF, 1 h,))); (b) $NaBH_4$, MeOH, 4 h; (c) 2-nonanone, NaOH 40%, EtOH, 12 h.

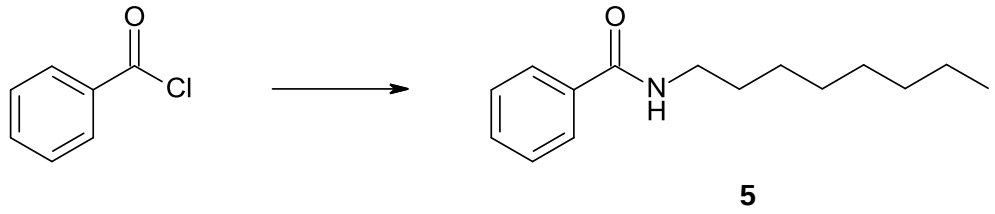

Figure 3. Synthesis of compound **5**. Reagents and conditions: octylamine, TEA, CH_2Cl_2, 60 °C, 6 h.

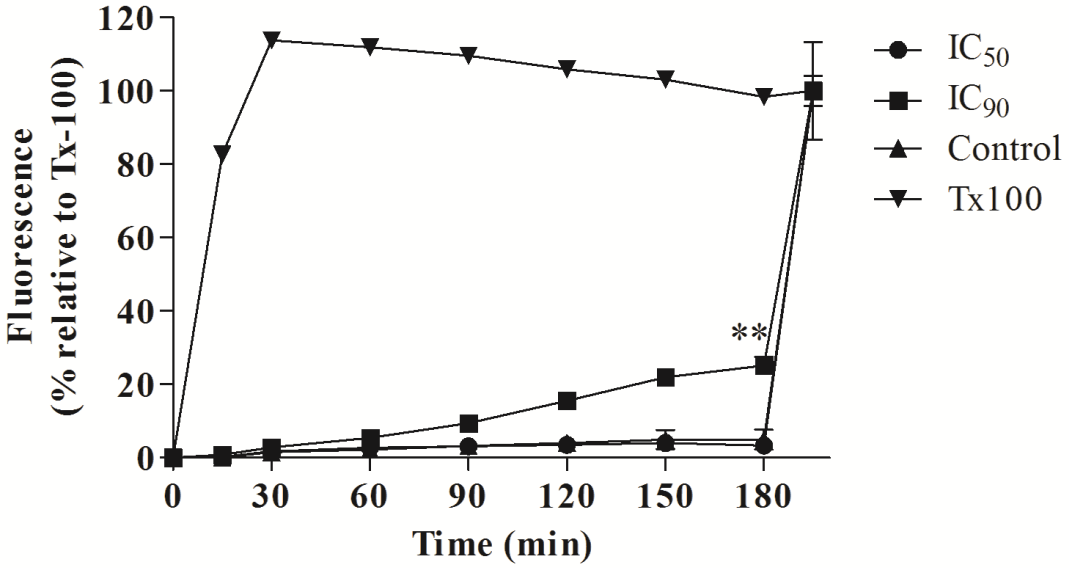

Figure 4. Effect of compound **4** on the membrane of *Leishmania* promastigotes using the fluorescent probe Sytox Green. Promastigotes of the control group were treated with 0.5% MeOH.

Tables

Table 1. Antiparasitic activities of the natural product **1**, and of the synthetic derivatives **2**–**6** against trypomastigote/amastigote forms of *T. cruzi* and promastigote/amastigote forms of *L. (L.) infantum*.

Compounds	IC_{50} µM (±SEM)				CC_{50} µM (±SEM)	SI[a]			
	T. cruzi **trypomastigotes**	***T. cruzi*** **amastigotes**	***L. (L.) infantum*** **promastigotes**	***L. (L.)infantum*** **amastigotes**	**NCTC cells**	**TCT**	**TCA**	**LIP**	**LIA**
1	67.3±2.6	32.6±1.5	82.5±5.2	51.6±4.6	>200	>2.9	>6.1	>2.4	>3.9
2	>100	50.0±18.3	28.3±1.8	40.4±15.8	>200	-	>4.0	>7.0	>4.9
3	>100	>100	27.8±1.3	34.9±2.8	>200	-	-	>7.2	>5.7
4	20.7±0.9	13.3±1.4	3.0±0.06	16.7±3.5	73.5±1.9	3.6	5.5	24.5	4.4
5	>100	>100	>100	>100	>200	-	-	-	-
6	>100	26.2±6.1	>100	>100	>200	-	>7.6	-	-
gibbilimbol A[b]	102.5	NA	NA	135.7	224.6	2.2	-	-	1.7
gibbilimbol B[b]	75.3	NA	100.4	94.9	254.1	3.4	-	2.5	2.7
miltefosine	ND	ND	16.7[b]	7.4±0.9	241.4[b]	-	-	14.5	32.6
benznidazole	16.4±2.1	5.2±0.3	ND	ND	>200	16.0	51.9	-	-

IC_{50}: 50% inhibitory concentration determined after 48 h and 72 h treatment for extracellular and intracellular forms, respectively

NA: not active

ND: not determined

CC_{50}: 50% cytotoxic concentration determined after 48 h treatment

[a]SI: selectivity index calculated from CC_{50}/IC_{50} (TCT: *T. cruzi* trypomastigotes, TCA: *T. cruzi* amastigotes, LIP: *L. (L.) infantum* promastigotes, LIA: *L. (L.) infantum* amastigotes)

[b]Available from Varela et al.[9]