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Article

The Semisynthetic Landscape of Aphidicolin: Inspiration Towards Leishmanicidal Compounds

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Recentes estudos demonstraram que afidicolina, metabólito secundário do fungo endofítico *Nigrospora sphaerica*, apresenta alta atividade contra o protozoário *Leishmania major*. Apesar do promissor potencial leishmanicida, a afidicolina apresenta propriedades físico-químicas e perfil farmacocinético inadequado para terapêutica. Neste sentido, uma revisão da literatura apresenta as limitadas modificações para este terpeno e ainda traz informações sobre as propriedades moleculares dos compostos já descritos e sua correlação com compostos leishmanicidas. Essa revisão forneceu uma análise racional para o desenvolvimento do derivado oxima. Uma série de derivados da afidicolina bem como o análogo oxima foram sintetizados para avaliação preliminar dos requisitos estruturais para atividade leishmanicida da afidicolina e seus derivados semi-sintéticos. Oito compostos foram sintetizados e testados contra diferentes espécies de *Leishmania*. A avaliação preliminar demonstrou alta atividade leishmanicida da afidicolina enquanto que o derivado oxima apresenta moderada seletividade contra a espécie *L. braziliensis*, endêmica em diversos países da América do Sul. Nenhum dos compostos apresentou citotoxicidade contra células de mamíferos.

Recent studies have shown that aphidicolin, a secondary metabolite of the endophytic fungus *Nigrospora sphaerica*, has high activity against the protozoan *Leishmania major*. Despite its promising leishmanicidal potential, aphidicolin presents a therapeutically unsuitable physico-chemical and pharmacokinetic profile. In this sense, a review of the literature shows the limited types of modification for this terpene. In addition, it gives an idea about the molecular properties of the compounds produced and which were correlated to leishmanicidal derivatives. This analysis provided us a rationale for the development of an oxime derivative. We synthesized the oxime-aphidicolin and a series of derivatives for a preliminary evaluation of the structural requirements for the leishmanicidal activity of aphidicolin and its semisynthetic derivatives. Eight compounds have been synthesized and tested against different species of the *Leishmania* protozoa. The preliminary evaluation demonstrated high leishmanicidal activity for aphidicolin, while the oxime derivative shows moderate selectivity for the *L. braziliensis* species, which is commonly found in several South American countries. None of the compounds showed cytotoxicity against mammalian cells.

Keywords: diterpenes, aphidicolin, *Leishmania* spp., semisynthesis, molecular properties

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Introduction

The World Health Organization (WHO) has identified leishmaniasis as a major neglected disease worldwide and as an increasing public health problem.[1] Despite controversial classifications, there are four prevalent clinical forms of leishmaniasis that are particularly diverse: visceral leishmaniasis (VL) is usually fatal when untreated; muco-cutaneous leishmaniasis (MCL) causes mutilation; diffuse cutaneous leishmaniasis (DCL) is long-lasting due to a deficiency in the cellular-mediated immune response; and cutaneous leishmaniasis (CL) is considered a disabling disease.[2]

Recently, the WHO developed the concept of disability-adjusted life years (DALYs),[3] which tallies the years potentially lost due to premature mortality and the years of productivity lost due to disability. Leishmaniasis causes approximately 50,000 deaths annually and an estimated 2,357,000 DALYs, placing leishmaniasis ninth in a global analysis of infectious diseases.[1] In Brazil, CL is a major public health problem, with cases reported in every state and 25,000 new cases of CL reported each year.[1] Unlike other world regions in which the most prevalent species of *Leishmania* is *L. major*, *L. braziliensis* is the most common species in the Americas and is the primary cause of CL and MCL in Brazil.

The first-line drugs for treating cutaneous leishmaniasis are Pentostam® (sodium stibogluconate), Glucantime® (meglumine antimoniate) and Fungizone® (amphotericin B), which have problematic toxicities/side effects, teratogenicity, increasing resistance and efficacy issues.[4] Antimonials suffer from failure rates of approximately 60% after the first treatment course.[5] In rural endemic areas in Brazil, the use of sodium stibogluconate to treat CL caused by *L. braziliensis* has been associated with decreasing cure rates,[6] and CL caused by *L. braziliensis* has demonstrated a higher rate of therapeutic failure compared with CL caused by other *Leishmania* species.[7]

However, despite extensive efforts, no new drugs have been released on the market recently, although diversified approaches were used. One of the strategies used in drug discovery programs involves the use of natural products. The investigation of natural products has provided an important source of novel chemical compounds in recent years,[8,9] and structure-activity studies of active natural leads provide an alternative approach for developing new safe and selective leishmanicidal compounds against each *Leishmania* species.

Among natural products, diterpenes have shown high activity against different *Leishmania* species; clerodanes have shown activity against *L. donovani*, abietanes against *L. donovani*, cassanes against *L. amazonesis*, labdanes against *L. donovani*, *L. tropica*, *L. braziliensis*, and ribenols against *L. donovani*.[4,10,11] Besides terpene leads, semisynthetic derivatives have been widely screened against leishmaniasis[12] toward better activity and pharmacokinetic profile.

Aphidicolin (**1**) is a tetracyclic diterpene ($C_{20}H_{34}O_4$), first described by Brundret *et al.* in 1972, proven to be a potent inhibitor of *Herpes simplex* type I growth[13,14] and to prevent mitotic cell division by interfering with the activity of DNA polymerase-α.[15] Considered as a natural lead, **1** was applied in several drug discovery programs that resulted in the discovery of the semisynthetic water soluble aphidicolin glycinate, which achieved clinical trials in the 1980's as a potent antitumoral.[16] Later, *in vivo* studies revealed that **1** undergoes rapid metabolism, resulting in its inactivation and the loss of DNA polymerase-α inhibition.[17] The main metabolic process, constituting > 90% of the metabolic profile, produces 3-ketoaphidicolin (**7**), which exhibits approximately 10% of the activity of aphidicolin in inhibiting DNA polymerase-α (Figure 1).[17] Since then, **1** has been used as an important tool for studies of cell-cycle progression and DNA replication.[18-20]

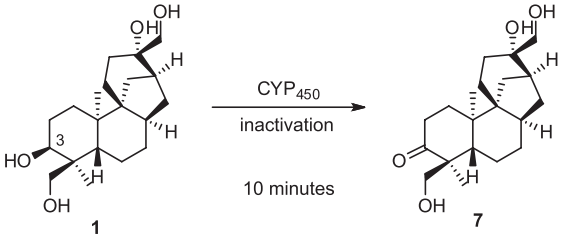


Figure 1. Aphidicolin metabolism to inactive metabolite.

Recently, **1** has been associated with antiparasitic activity. Aphidicolin **1** and semisynthetic derivatives have been screened against *Leishmania* spp. and **1** and analogues emerged as a leishmanicidal compounds, inhibiting the protozoa at a concentration of 0.19 μM.[21] These findings, together with the known pharmacodynamics and pharmacokinetics, make **1** an interesting lead compound for leishmaniasis medicinal chemistry research programs.

Despite the high leishmanicidal activity of **1** and derivatives, the search for new antiparasitic derivatives is not described in the literature. In fact, the semisynthesis of **1** can be considered underexplored toward lead optimization or SAR (structure-activity relationship) exploration. Therefore, this work analyzes the literature to describe the medicinal chemistry toolbox of **1**, trying to congregate the semisynthetic strategies with the physico-chemical properties of known derivatives. These data provided a rationale for the discovery of 3-oxime-aphidicolin (**8**) as an active leishmanicidal derivative of **1**, without the structural

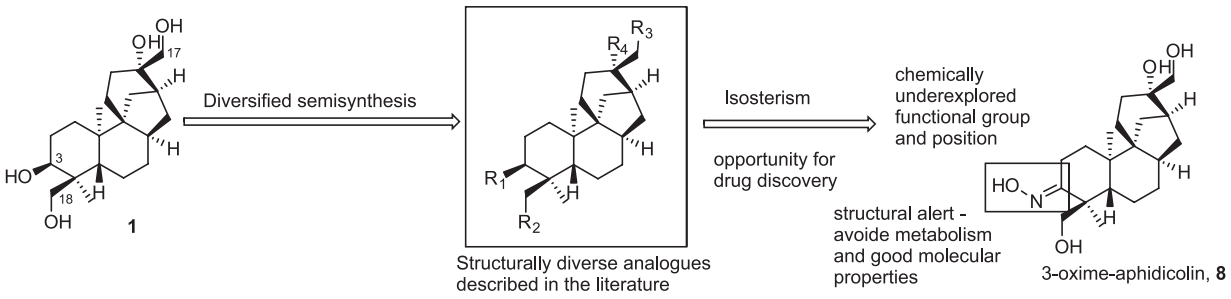


Figure 2. Rationale for the development of 3-oxime-aphidicolin.

pharmacokinetic alerts that make **1** fail during clinical trials (Figure 2). Also, the oxime group keeps the free hydroxyl, which is important to test the hypothesis that it is an important group for bioactivity and biological interactions. Finally, the molecular properties to druglikeness are still in good profile in the oxime.

Results and Discussion

The medicinal chemistry toolbox of aphidicolin

The review of the literature reflects the scenario of the chemical exploration of a natural lead towards bioactive compounds. Based on the structure of **1**, the search for new naturally derived hits involves semisynthetic modification. In these cases, it is possible to observe that the hydroxyls of the terpene are the main target for classic organic synthesis or biocatalysis. The modification of **1** can be divided as following (Figure 3):

Type 1 - hydroxyl linear manipulation: take advantage of the hydroxyl reactivity for acylation (or similar reactions), and alkylation toward more lipophilic compounds;

Type 2 - heterocyclization (for protection or scaffold modification purposes): in general, it is used as a strategy to protect specific hydroxyls during chemical modification steps, although few examples of bioactive compounds were found;

Type 3 - ring cleavage: a strategy to create molecular diversity for both chemical reactivity and medicinal chemistry purposes. Simplified structures can be found;

Type 4 - oxidation state modification: primary and secondary hydroxyls are oxidized changing electronic and structural properties;

Type 5 - functional group introduction/modification: useful strategy to explore chemical reactivity of functional groups in the structure of **1** for more diversified structure pattern.

The literature fishing of chemical modification, via semisynthetic and biocatalytic reactions of **1**, reveals a chemically under-exploited natural lead, without any focused study toward the medicinal chemistry of this compound (Table 1 and Table 2). More specifically, Table 1 shows the types of transformation of **1** using conventional organic semisynthesis, which reflects a prevalence of acylation and similar reactions (e.g., tosylation) at the

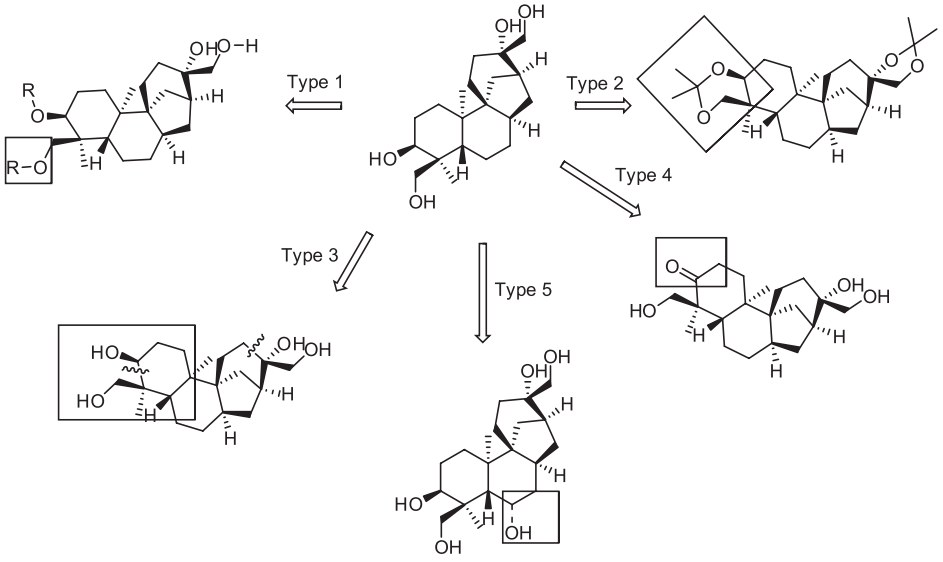


Figure 3. Main types of structural modifications of **1** using biocatalysis or conventional semisynthesis found in literature.

hydroxyls of **1** (24.4% of the occurrence), specially in the hydroxyl group at C-17, which was modified in all acylation reactions listed. Another common reaction involving **1** is oxidation. Several methods have been applied, from mild methods, such as Pfitzner-Moffat, which provides aldehydes at C-17 and C-18 positions, to more drastic methods as Jones oxidation, which gives the ketone at C-3 position in addition to the aldehyde groups. The treatment of **1** - hydroxyl at C-3 and C-18 previously protected - with periodic acid, gives the corresponding ketone at C-16 position. Common protective groups are usually introduced to the structure of 1, and are responsible for 15% of the chemical modification. Azide introduction and carbon-carbon cross coupling reactions, although explored, does not correspond to important modification strategies. Interestingly, simple alkylation and halogenation are not described in the literature, which demonstrated the lack of a comprehensive knowledge of the chemical reactivity of **1**. It is important to notice that substitution reactions involve mostly the electrophilic attack in **1**, as the cases of protection and acylation reactions. Reactions with 1 which involves a previous deprotonating step are not described in the literature, and we noticed that an intermediated susceptible to base needs to be introduced prior to the base attack.

Compared to conventional semisynthesis, biotransformation of **1** is much less studied, and our literature revision found only two works describing the

Table 1. Occurrence rates of reactions within the data set

Reaction type	No. of reactions	Total / %	Subtype	Position Carbon/ring	References
Acylation and related processes	23	24.20	Acetylation: 5	C-3, C-17, C-18	13, 19, 22-25
			Crotonation: 1	C-17	19
			Tosylation: 8	C-17	23, 26
			Succinic: 1	C-17	24
			Others: 8	C-3, C-17, C-16, C-18	13, 23
Oxidations	21	22.10	Pfitzner-Moffatt: 6	C-17, C-18	19, 23, 26
			Corey-Schmidt: 4	C-3	19, 23, 26
			Jones: 6	C-3, C-16, C-17	13, 19, 24, 26
			Catalytic: 1	C-3, C-17	26
			Periodic acid: 3	C-16	13, 24, 26
			Baeyer-Villiger: 1	C-16, C-17	13
Reductions	6	6.30	Catalytic: 3	C-18, C-2	24, 26
			Hydrides: 1	C-3	13, 26
			Wolff-Kishner: 2	C-16	13
Protections	15	15.70	Acetonide: 8	C-3, C-16, C-17, C-18	13, 19, 26
			TBDMSCl: 3	C-17, C-18	19, 23, 26
			MOM: 1	C-16	19
			MEM: 2	C-3, C-16, C-18	19
			Ethylenodioxy: 1	C-16, C-17	13
Deprotections	5	5.20	–	–	13, 19, 23, 26
C–C bond formation	6	6.30	Grignard: 3	C-16, C-3	19, 23, 26
			Organolithium: 3	C-17	23
Functional group interconversion	2	2.10	Alcohol to azide	C-17	23
			Alcohol to tioether	C-18	13
Dehydration	4	4.20	–	C-3, C-15, C-16	13, 19
Reductive elimination	5	5.20	–	C-17, C-18	13, 19, 26
Ring cleavage	4	4.20	–	D, A	13, 26
Heterocyclization	4	4.20	Epoxidation: 4	C-3, C-16, C-17, C-18	13, 23
Total	95	100			

TBDMSCl: *tert*-butyldimethylsilyl chloride; MOM: methoxy methyl ether; MEM: 2-methoxyehtoxy methyl ether.

Table 2. Biotransformation reactions involving aphidicolin

Reaction type	No. of reactions	Total / %	Position Carbon/ring	Microorganism	Classification	References
Acetylation	3	30	C-3, C-17, C-18	*Scopulariopsis constantini*	Fungi/Sordariomycetes	27
				Tolypocladium inflatlum	Ascineycetious fungus	28
Alkylation	1	10	C-3, C-18	*Tolypocladium inflatlum*	Ascineycetious fungus	28
Oxidation	4	40	C-3, C-18	*Trichothecium roseum*	Fungi/Ascomycetes	27
				Streptomyces griséus	Bacteria/Actinomycetes	27
				Chaetomium funiculum	Fungi/Sordariomycetes	27
Hydroxylation	1	10	C-6	*Streptomyces punipalus*	Bacteria/Actinomycetes	27
Epimerization	1	10	C-3	*Chaetomium funiculum*	Fungi/Sordariomycetes	27
Total	10					

use of microorganisms to make structural modifications of **1**. However, it is possible to see similarities between these strategies applied for the chemical transformations of **1**. Biocatalyzed by both fungi and bacteria, oxidation and acylation correspond to almost all of the reactions, while epimerization and alkylation seem to be exclusively observed in previously biotransformed derivatives (Table 2). Also, similarly to classical semisynthesis, the preferred positions for biotransformation are C-3 and C-18 (Figure S1), in which the hydroxyl groups are mainly acylated or oxidized. The epimerization at C-3 is an interesting modification that, together with alkylation, occurs exclusively in the biotransformation process.

An analysis of the reactions of **1** using classical semisynthesis and biotransformations shows differences in the type and positions modified. Although the amount of examples is higher for the classical semisynthesis, it is possible to see an expected tendency for more diversification in this approach compared to biotransformations. Supplementary Information shows the maps of transformation with the correlations between groups introduced for each two positions in the structure of **1**. The maps of transformations show the correlation of type and number of modifications for each two positions. These correlations are represented by balls, which indicate the functional groups in each position and the number of examples of this modification (Figures S7 and S8).

Analyzing the structures derived from classical semisynthesis (Figures S2-S6), it is confirmed the high diversification of groups introduced at C-17 (R_3) and C-18 (R_2), usually keeping the other hydroxyls unmodified. When multiple transformations appear, usually C-3 and C-18 are the most common sites (R_1 and R_2, respectively) reacted. On the other hand, the biotransformation derivatives tend to have C-3 (R_1) and C-18 (R_2) modified, although less diversified when compared to the classical semisynthesis. In both cases, R_4 is not so much explored, as expected for tertiary alcohols, due to steric hindrance. It is important to mention that this position is usually modified by oxidation reactions at C-16, dehydration and insertion of protective groups.

An interesting point to highlight regarding the chemical transformations of **1** is the limitation of solvents used for conventional semisynthesis. Indeed, methanol and pyridine are the most common solvents used for the reactions of aphidicolin, which could be responsible for the limitation in the synthetic strategies. However, despite the limited panel of reactions, several structural features were explored to evaluate the medicinal chemistry potential of this class of compounds, including manipulating physicochemical properties towards more bioavailable leads.

The chemical manipulation of **1** is usually correlated to the nucleophile characteristic of its hydroxyls, which leads to changes in the hydrogen bond type/number and other physicochemical properties. As **1** is well established in terms of pharmacokinetics, which is particularly correlated to lipophilicity (logP), size (e.g., expressed as molecular weight - MW), total polar surface area (TPSA) and the flexibility of the structure (number of rotatable bonds - RBC), we can assume that manipulations of the functional groups toward better bioavailable derivatives can be achieved considering both the structural alerts of metabolism and these properties.[29,30]

We calculated these mentioned parameters of **1** and all the derivatives (Tables S1 and S2) and analyzed the differences among the strategies used to modify the natural lead. Interestingly, **1** fits the parameters of rules of five (Ro5), although the experimental data showed that after absorption it is rapidly metabolized to 3-oxo-aphidicolin **7**. Comparing derivatives of **1** obtained from biotransformation and classic semisynthesis it is possible to see that in average they kept the same pattern of the

lead compound by fitting the parameters established for good bioavailability, although some major differences can be noticed (Table 3). Table 3 shows that both classic semisynthesis and biotransformation analogues present mean values of XLogP and molecular weight greater than **1**, while it is possible to observe reduction on TPSA and hydrogen bond donor. No significant alterations are observed for hydrogen bond acceptor potential (HBA) and rotatable bond. These findings shows that chemical transformations of 1 tend to produce more lipophilic and high MW compounds without markedly changing their flexibility. These characteristics correlate to the types of transformations listed in Tables 1 and 2, where it can be found that the hydroxyls are substituted by electrophiles, reducing the H-bond donor potential of the terpene, which consequently alters the hydrophobicity of the structure, and also due to its size, volume and TPSA. Chemically, the groups introduced are acyl groups (or similar - tosyl) with aromatic rings or small aliphatic chains, which explain the low increment in rotatable bonds and high lipophilicity.

Table 3. Comparative analysis of physicochemical and structural parameter differences between **1** and classical semisynthesis/biotransformation

Compound	Aphidicolin	Products of biotransformation	Products of semisynthesis
XLogP	1.12	2.63	3.27
HBD	4	2.83	1.77
HBA	4	4.75	3.8
MW	338.48	389	364.6
TPSA	80.9	79.57	63.06
RBC	2	2.5	2.28

XLogP: calculated lipophilicity; HBD: hydrogen bond donor potential; HBA: hydrogen bond acceptor potential; MW: molecular weight; TPSA: total polar surface area; RBC: rotatable bond count.

A brief comparison between biotransformation and classical semisynthesis shows that both strategies make more lipophilic compounds tend to increase MW and LogP. In addition, both strategies reduce hydrogen bond donor potential (HBD) and TPSA without changing HBA and RBC. Comparison to Lipinski's cutoff showed that all compounds, in average, lies into this druglikeness rule of bioavailable compounds. As the chemical manipulations tend to keep intact the basic structure of **1**, it was expected that logP and MW increase, considering that alkylation, oxidation and protection were the main type of modifications in the literature. However, HBD seems to be important for the transformations as it reduces far from the cutoff (HBD < 5) in both strategies. HBD reduction contributes to higher hydrophobicity, which is typical in the modification of **1**. On the other hand, alternative properties that also correlate with oral bioavailability are RBC (cutoff ≤ 10) and TPSA (≤ 140 Å^2). For RBC, which correlates to flexibility and escape from flat, the compounds derived from both strategies still lie into the flatland with low values of RBC. Meanwhile, TPSA sums the surface area of polar atoms in the structure and correlates well with human intestinal absorption. It is interesting to notice that classical semisynthesis reduces more the value of TPSA. Although these values respect the limits of Lipinski's rules and other parameters to good oral bioavailability, some recent data showed some properties alerts to toxicity, as TPSA < 75 and logP < 3.[31]

Extracting the leishmanicidal derivatives from the literature[21] and comparing the molecular properties to **1**, it is possible to see in all the compounds an increase in the value of XlogP and MW, following the same pattern as previously observed during semisynthesis analysis. However, the changes vary for each leishmanicidal derivative. The most important feature is that the most active compound showed high values for both TPSA and XLogP when compared to the others, including **1** (0.19 M). This seems to be an interesting profile to be explored in the search of new leishmanicidal derivatives of **1**. Also, structurally the most active compounds show free hydroxyl at C-3, an exception is seen for the acetal derivative (Figure 4).

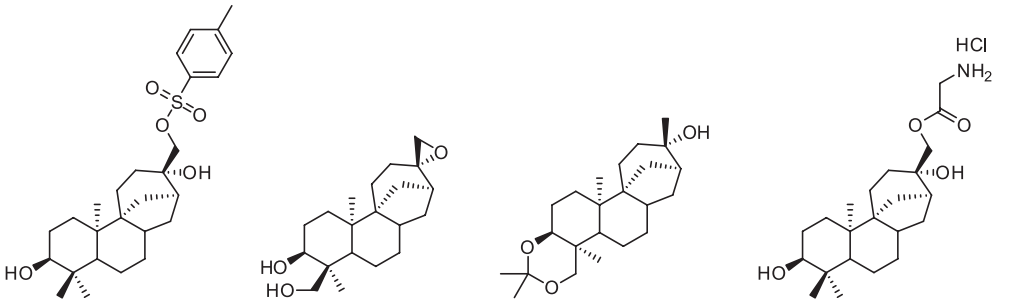
Considering the underpopulated chemical space of analogues of **1**, we designed a new leishmanicidal derivative guided by the alerts provided by the molecular properties and by the metabolic behavior of **1**. Therefore, we developed the oxime derivative **8**, maintaining the hydrogen bond donor at C-3 and introducing a functional group less susceptible to human CYP_{450}, without remarkable changes in the main descriptors described herein. Also, we expected that oxime has a higher TPSA changing slightly other molecular properties, which should correlates to a good biological activity.

Chemistry

Aphidicolin **1** and 3-deoxy-aphidicolin **2** (Figure 5a) were isolated from cultures of the endophytic fungus *Nigrospora sphaerica* as previously described.[32]

The 3D structure of **1** is shown in licorice view (Figure 5b). Previous work detailed the X-ray crystallography of an acetonide derivative of **1**,[2] which was used to confirm the structure of the natural compound.

We have obtained the direct crystallographic data of **1** for the first time, based on an X-ray single-crystal diffraction experiment. Two molecules were observed in an asymmetric unit (Figure 6a), which was packed in an orthorhombic unit cell containing 8 molecules



	L. major IC_{50} = 0.02 μM *L. donovani* IC_{50} = 0.02 μM	*L. major* IC_{50} = 0.15 μM *L. donovani* IC_{50} = 0.12 μM	*L. major* IC_{50} = 0.22 μM *L. donovani* IC_{50} = 0.32 μM	*L. major* IC_{50} = 0.28 μM *L. donovani* IC_{50} = 0.48 μM
TPSA (Å^2)	92.2	92.8	53	38.7
HBD	2	3	2	1
HBA	5	5	3	3
RBC	4	4	1	0
MW	476.67	379.53	320.47	362.55
XLogP	4.27	1.93	2.22	4.69

Figure 4. Semisynthetic derivatives of **1** with potent leishmanicidal activity.

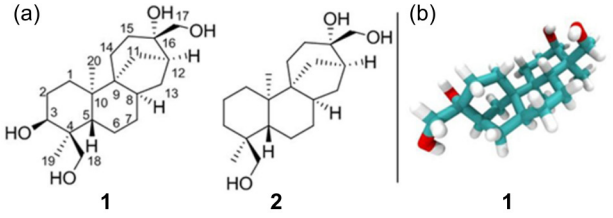


Figure 5. (a) Natural products aphidicolin **1** and 3-deoxy-aphidicolin **2** structures. (b) Aphidicolin **1** is shown in licorice view.

(4 asymmetric units) with a = 11.7093(1), b = 11.7636(2), c = 25.5233(5) Å. Additional crystallographic data and the data collection details, structural solution and refinement are summarized in the Supplementary Information data (Table S3). The molecule has a hydrophobic 'body' composed of three 6-carbon and one 5-carbon linked rings and is bound to two methyl and two hydroxymethyl groups (Figure 6b). No solvent molecules or highly disordered atoms were observed in the unit cell. The molecules interact via hydrogen bonding between the hydroxyl groups of the molecules from different asymmetric units, generating planes perpendicular to the c-axis throughout the crystal. All bond distances and angles, fractional coordinates,

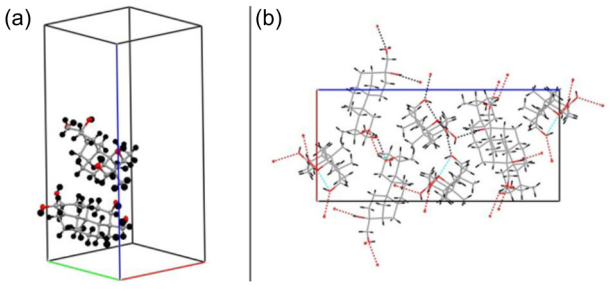


Figure 6. (a) Asymmetric unit with two aphidicolin molecules. (b) Hydrogen bond network between aphidicolin molecules shown along a plane.

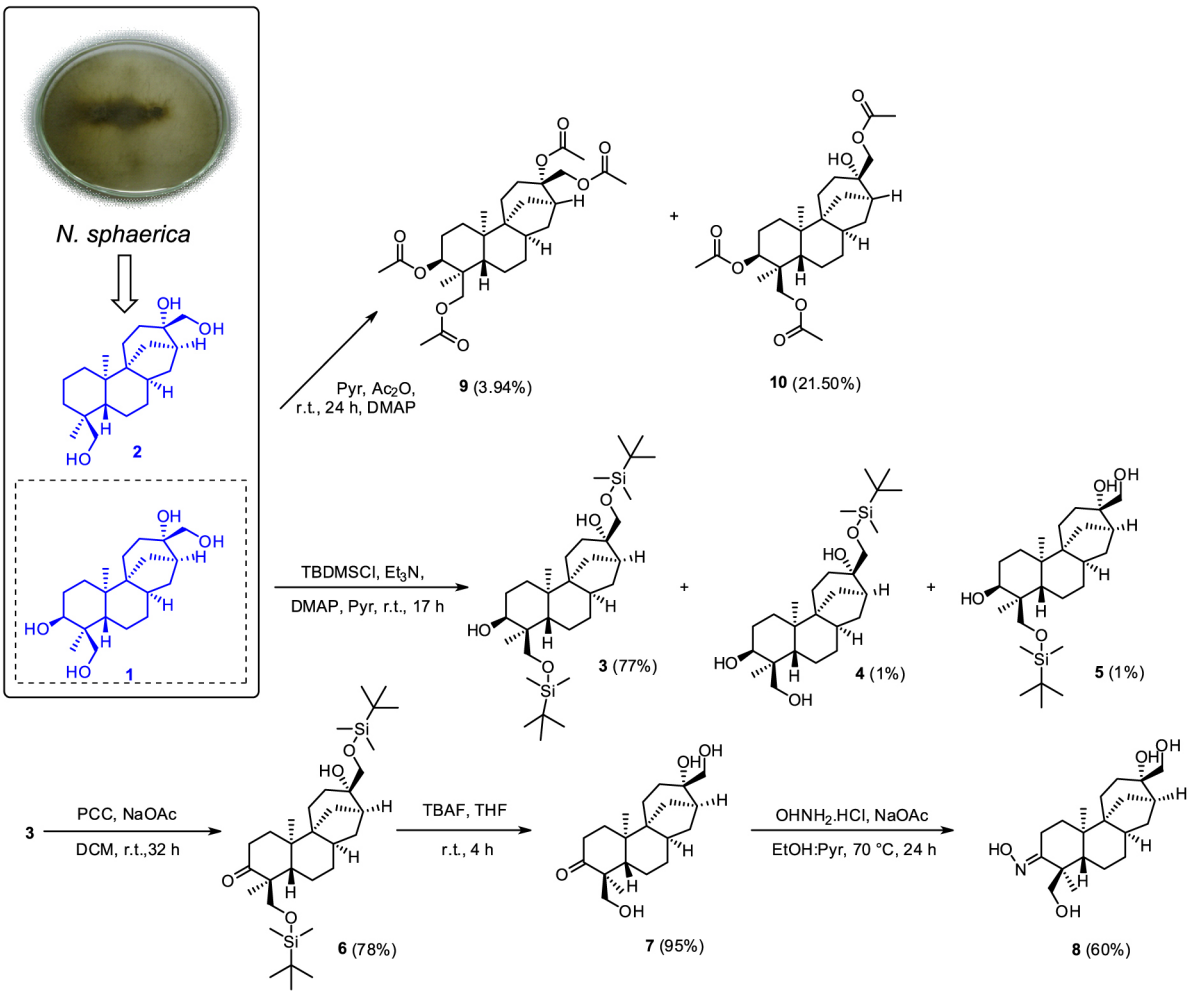
equivalent isotropic displacement parameters and other relevant information can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif under deposit number CCDC 915899.

3-Deoxy-aphidicolin **2** was identified based on the ^{1}H nuclear magnetic resonance (^{1}H NMR) (500 MHz, $CDCl_3$) spectrum that presented two singlets at δ 0.99 and 0.78, which refer to methyl hydrogens, and four signals related to carbinolic hydrogens between δ 3.05-3.47. The absence of a deshielding signal corresponding to H-3 suggests that there is no hydroxyl group at C-3 when comparing with the spectral data of **1**. All spectroscopic data of **2** were comparable to those previously described.[33]

The modifications of **1** were planned based on the metabolism stability and the H-bonding donor potential on C-3 position. The sites of metabolism on **1** were evaluated by consensus scoring on the basis of the softwares MetaPrint-2D-react[34] and SmartCyp,[35] which revealed that atoms C-3 (NOR = 0.747), C-17 (NOR = 0.251) and C-18 (NOR = 0.730) are more susceptible to CYP_{450} oxidation. One potential candidate obtained from this study was the derivative **8**, due to the great stability on C-3 position (C-3 NOR = 0.000), and which maintains the hydroxyl group at C-3 atom indispensable for leishmanicidal activity.

Thus, the analogues **3**-**10** (Figure S9) were synthesized from **1** using a series of reactions involving protection-deprotection methodologies, oxidation, oximation and acylation. The modifications were planned in order to investigate the activity of isosteres at C-3 position and also the importance of the free hydroxyl groups of **1** for the leishmanicidal activity (Scheme 1).

The protection of the primary hydroxyl groups with *tert*-butyldimethylsilyl chloride (TBDMSCl) (2.2 eq.)



Scheme 1. Semisynthesis of derivatives (**3**-**10**) from **1**.

catalysed by dimethylaminopyridine (DMAP) produced the derivatives **3**, **4** and **5** with yields ranging from 1 to 77%.

Diprotection is always preferred over selective monoprotection involving primary hydroxyls of **1**, even when stoichiometric quantities of the reactants are used. While the diprotected derivative **3** was obtained in good yields (77%), the monoprotected derivatives **4** and **5** were obtained in low yields (about 1% each). The structure of **3** was suggested based on the ^{1}H NMR and ^{13}C NMR spectra, in comparison with the NMR data of **1**. The ^{1}H NMR spectra of **3** exhibited singlet signals at δ_{H} 0.90 (s, 9H), 0.89 (s, 9H), 0.08 (s, 3H), 0.07 (s, 3H) and 0.05 (s, 6H), which were not observed in the NMR spectra of **1**. In addition, the ^{13}C NMR signals at δ_{C} –5.8, –5.4, –5.3, 15.1, 17.6, 17.9, 24.9, 25.6, 25.9 and 26.5 are indicated to refer to the methyl groups in the structure of the *tert*-butylsilyl protecting group. The electrospray ionization mass spectrometry (ESI-MS) analysis $[M + Na]^{+}$ 589.4064 confirmed the hypothesis for the structure. The other ^{1}H signals exhibited great similarity to those of **1**. The structure of the monoprotected derivative **4** was suggested by ^{1}H NMR (500 MHz, CD_3OD) with a singlet at δ 0.89 (9H), which is related to the methyl hydrogens on the protecting group. A singlet was observed at δ 0.05 (6H), which refers to the methyl hydrogens attached to the silicon atom at C-18 position. Compound **5** exhibited a singlet at the δ 0.90 (9H) position in the ^{1}H NMR spectrum, which indicated methyl hydrogens on the protective group. Two singlet signals appeared at δ 0.08 and 0.06, which refer to methyl groups attached to the silicon atom. We proposed that the reaction occurred at the C-18 position since there are two different signals for the methyl groups bonded to the silicon atom and the same methyl group appears as a singlet in the NMR spectra for derivative **4**. This may occur due to an intramolecular interaction between the hydroxyl groups at the C-3 and C-18 positions, which may cause a conformational restriction at the protective group at C-18, affecting the chemical environment for the methyl groups. Based on these data, we suggested that derivative **5** is the monoprotected compound at C-18 position.

The classical Jones oxidation of **3** to produced ketone **6** was carried out in moderate yields. The structure of **6** was suggested by the presence of the C=O stretching mode at 1696 cm^{-1} in the infrared spectrum and by the signal at δ_C 218.0 in the ^{13}C NMR spectrum, which typically results from a carbonyl group. The structure of **6** was confirmed by both the absence of a signal at δ_H 3.61 in the ^1H NMR spectrum, which refers to carbinolic hydrogen at the C-3 position, and by the ESI-MS analysis of the adduct at $[M + Na]^+$ 587.3888.

The deprotection of **6** with tetrabutylammonium fluoride (TBAF) in THF produced ketone **7**, as confirmed by the formation of the ion $[M + H]^+$ 337.2373 in the mass spectra. The presence of the carbonyl group at the C-3 position influenced the ^1H NMR spectra, deshielding the border methylene hydrogens at the C-18 position, from δ 3.45 in the aphidicolin spectra to δ 3.71, which also confirmed the proposed 3-oxo-aphidicolin structure. The C=O stretch at 1690 cm^{-1} in the infrared spectra confirmed the structure of the ketone. The treatment of **7** with hydroxylamine chloride and sodium acetate yielded **8** in a 60% yield, which was confirmed by the presence of a δ_H 8.47 signal in the ^1H NMR spectra (C=N–OH) and a δ 162.4 signal in the ^{13}C NMR (C=N–OH). The ESI-MS $[M + H]^+$ 352.2482 was consistent with the proposed structure.

To obtain additional lipophilic compounds to be tested against the *Leishmania* species, we planned the acylation of the hydroxyl groups of **1** with acetic anhydride, catalysed by DMAP in pyridine. The steric restriction did not appear to interfere with the course of the reaction; therefore, the tri- and tetra-acetylated compounds could be isolated in modest yields. Compound **9** was identified as a tetra-acetylated derivative, as confirmed by the δ 171.2, 170.8, 170.5 and 170.1 signals in the ^{13}C NMR spectrum and the ion peak $[M + Na]^+$ 529.2822 in the mass spectrum. The two-dimensional heteronuclear multiple-bond correlation spectroscopy (HMBC) experiment exhibits that the hydrogen at δ 4.84 (H-3) showed a correlation to the carbons C-1, C-5 and C-21 (δ 170.1). Also, it shows that the hydrogen atoms at δ 3.76 and 4.00 (H-18) are correlated to C-3 (δ 73.2), C19 (δ 17.1) and C22 (δ 171.2). The hydrogens at δ 4.53 and 4.37 (H-17) are assigned to carbons C-12 (δ 39.7), C-15 (δ 26.1), C-16 (δ 84.8) and C-23 (δ 170.8). All these observations confirmed that the hydroxyl groups reacted with acetic anhydride to form the tetra-acetylated derivative **9**.

Compound **10** presented a molecular ion peak at *m/z* $[M + Na]^+$ 487.2658 in the mass spectrum, which is equivalent with the exact mass of the tri-acetylated derivative. The structure was confirmed by the two-dimensional HMBC experiment, which showed that the hydrogen atom at δ 4.81 (H-3) is correlated to the carboxyl signals at δ 170.1 (C-21), and also with the carbon at δ 26.8 (C-1) and 38.0 (C-5). Also, the hydrogens at δ 3.70 and 4.00 (H-18) showed to be correlated to carbons C-19 (δ 17.0), C-22 (δ 171.2) and C-3 (δ 73.3). The hydrogen at δ 3.95 (H-17) showed correlation to carbons at C-12 (δ 41.3), C-23 (δ 171.2) and C-16 (δ 73.2). These data provide strong evidence for the formation of **10**.

Biological evaluation

The natural products and their semisynthetic derivatives were screened for *in vitro* leishmanicidal activity against the promastigote stage of *L. major* and *L. braziliensis* (Table 4), which are of epidemiological relevance worldwide. This study is the first report of the *in vitro* activity of aphidicolin derivatives against *L. braziliensis*, which is the most common cause of tegumentary leishmaniasis disease in South America. Two compounds, **1** and **8**, demonstrated promising activity, with no toxicity to mammalian macrophages. The acetylated derivatives **9** and **10** did not exhibit activity against any of the tested species, which suggests that the free hydroxyl groups are important to the *Leishmania* target. The protected derivatives **3**, **4** and **5**, and compound **6** presented no activity, which corroborates previous findings that demonstrate the importance of the free hydroxyl groups at the C-17 and C-18 positions for anti-leishmanicidal activity.[9] Slight activity was observed with 3-oxo-aphidicolin **7** against *L. braziliensis*, but not against any other species, which is in agreement with the literature and supports the theory that, as with the oxidation on C-3, modifying the ring A reduces the leishmanicidal activity.[9] According to earlier studies, 3-oxo-aphidicolin **7** is inactive on mammalian cells because it is a product of the bio-inactivation metabolism in rat livers.[5] Therefore, this compound could be considered a safe antiprotozoal starting point for further studies, as when modified to the oxime **8**, it generated a very active compound. Since compounds **1** and **8** demonstrated higher potential for the inhibition of *Leishmania* growth, they were selected for cytotoxic activity studies in mammalian cells.

Table 4. IC_{50} values of compounds **1**, **2**, **7** and **8** against different promastigote *Leishmania* spp.

Compound	*L. braziliensis* (average ± SD)	*L. major* (average ± SD)
1	0.37 M ± 0.11	0.17 M ± 0.05
2	2.28 M ± 0.33	0.95 M ± 0.16
7	2.7 M ± 1.17	Inactive
8	0.85 M ± 0.23	Inactive
G-418[a]	5.7×10^{-6} M	3.3×10^{-6} M

[a]Positive control. SD: standard deviation.

The cytotoxicity assays demonstrated that the most active compounds, **1** and **8**, had no effect on the growth of normal cells (Figure 7). Because **1** and **8** demonstrated the IC_{50} values on promastigote cells and exhibited no cytotoxic effects on mammalian cells, they were tested against amastigotes, the stage of the parasite adapted to living in vertebrate host cells.

These compounds exhibited potent activity against the amastigotes (Table 5), the evolutionary form responsible for the pathological development. Compound **1** reduced the infection index of the *Leishmania* amastigotes by 90% at concentrations of 0.1 M, and compound **8** reduced the infection index by approximately 70% at 0.5 M (Figure 8).

Preliminary studies of the activity against *L. donovani* promastigotes were performed with natural leads **1** and **2**, demonstrating IC_{50} values of 0.54 and 1.93 M, respectively. These results will lead to further investigations of mammalian amastigote cells when the resistance of *L. donovani* responsible for visceral leishmaniasis becomes a worldwide problem.[10]

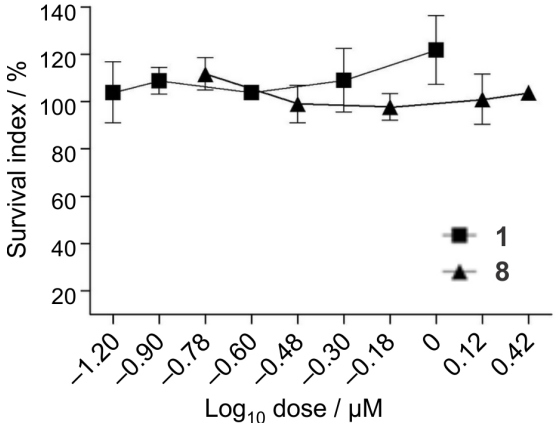


Figure 7. Cytotoxicity assay of compounds **1** and **8** on mammal macrophages.

Table 5. Physicochemical properties and biological evaluation of aphidicolin and derivatives

Compound	XLogP	HBD	HBA
1	1.12	4	4
2	2.29	3	3
7	1.42	3	4
8	1.48	4	5

Molecular property analysis of **1**, **2**, **7** and **8**

In comparison to **1**, the bioactive compounds found in this work have similar molecular properties, although bigger variations are seen for TPSA. The natural derivative 3-deoxy-aphidicolin **2** is the only example of reduced MW with leishmanicidal activity. However, low values of TPSA seem to affect the biological activity as discussed previously, although XLogP seems to be important for activity against *L. major*. Compound **7** has low activity against *Leishmania spp*. tested in this work although the molecular properties are similar to **8**. This could be due to the same structural features that prevent inhibitory activity against DNA polymerase. Interestingly, **8** shows high activity against *L. braziliensis* which correlates with the increased XLogP and high value of TPSA, as observed for previously described leishmanicidal compounds. Also, the oxime function at C-3 in compound **8** keeps the HBD character of **1** (hydroxyl group), which in addition to other structural features discussed earlier makes it a better lead for further drug discovery studies. The main structural alert for **1** is the oxidation at C-3 that reduces the bioavailability and produces an inactive metabolite. For **8**, this type of metabolism reaction is prevented due to functional group

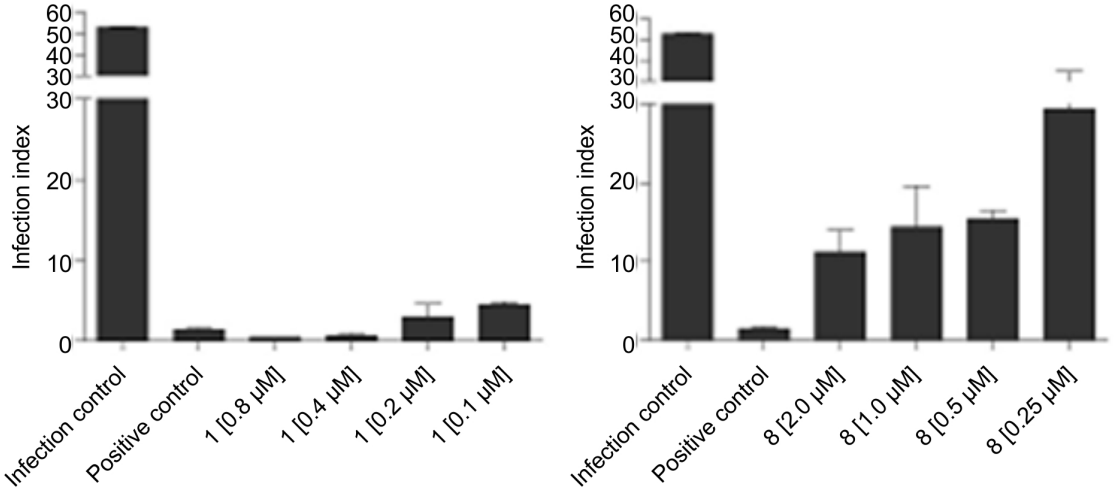


Figure 8. Biological evaluation of compounds **1** (left) and **8** (right) against amastigotes of *Leishmania braziliensis*. Negative control: non-treated infected macrophages. Positive control: infected macrophages treated with geneticin [10 μg mL^{-1}].

modification to the oxime, which also increases the value of TPSA, reducing the toxicology potential, as experimentally tested in this work. Also, TPSA lies within the safety values. Besides, the oxime structure allows further modifications toward more lipophilic compounds, which makes it a good lead against *Leishmania braziliensis*.

Conclusions

Lead discovery and chemical optimization have played important roles in the discovery of novel therapeutic agents from nature. Considering that the total synthesis of **1** has been published from different methods and mechanisms, but there are few semisynthetic derivatives described in the last decades, this is the first time that a rational analysis of the medicinal chemistry of **1** was presented. As the review of the literature provided us little information about the medicinal chemistry of **1** in neglected diseases, we congregate the molecular property analyses for druglikeness to develop **8**, to prove the hypothesis that structural alerts together with molecular properties cutoffs can generate a rationale for the development of bioactive compounds. The biological results with **8** reaffirm that modifications to the A ring are not well tolerated, although the preliminary evidences indicate that the presence of a hydrogen bond donor appears to be more relevant for the maintenance of the leishmanicidal profile and gave an idea about the influence of logP and TPSA in the leishmanicidal activity. In addition, this is the first work discussing the semisynthesis of **1**, which proved to be an efficient strategy and an open opportunity in hit-to-lead programs.

Experimental

General

All chemicals and reagents were purchased from Sigma-Aldrich; the solvents were obtained from Vetec and Synth (Brazil). High-resolution mass spectra (HRMS) were obtained using an electrospray ionization time-of-flight mass spectrometer (ESI-TOF). The NMR data were recorded with a Bruker DPX-500 and DRX-300 instrument by using $CDCl_3$ and CD_3OD as a solvent and tetramethylsilane (TMS) as an internal standard. Infrared (IR) spectra were measured with a Perkin-Elmer Spectrum RX IFTIR system.

Thin-layer chromatography was performed on silica gel 60 plates and visualized with a 0.25% solution of vanillin and sulphuric acid in ethanol followed by brief heating on hot plate. Flash chromatography was performed using silica gel 60 (230-400 mesh, Merck). All reagents were obtained from commercial suppliers unless otherwise stated.

The literature review was made using Scifinder (CAS), using the structure of **1** as starting material in the "Search reaction" option. Also, in Scifinder we applied the following filter for results retrieving: aphidicolin + semisynthesis or aphidicolin + medicinal chemistry. The physicochemical properties for the aphidicolin and set of derivatives were calculated using the program Vortex (Dotmatics, UK).

Synthesis

General method for hydroxyl protection of **1** (**3**-**5**)

Compound **1** and TBDMSCl were added to a solution of triethylamine and DMAP in dry pyridine at room temperature. The mixture was stirred for 19 h at room temperature under atmospheric N_2 and then poured into a saturated $NaHCO_3$ solution (15 mL). The mixture was extracted with ethyl acetate and the organic layer was dried using anhydrous sodium sulfate. The extract was then evaporated in vacuum, and the residue was purified by classical column chromatography (silica gel, EtOAc/hexane 2:3) to afford compounds **3**, **4** and **5** as white solids. All structures were confirmed by NMR and mass spectra as described below.

Compound **3**: 436.5 mg, 77%; m.p. 115-117 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.62 (bs, 1H), 3.53 (d, 1H, *J* 10.0 Hz), 3.43 (d, 1H, *J* 10.0 Hz), 3.32 (m, 2H), 2.41 (d, 1H, *J* 12.0 Hz), 2.15 (m, 2H), 2.10 (m, 1H), 2.09 (m, 1H), 1.87 (m, 1H), 1.86 (m, 1H), 1.85 (m, 1H), 1.79 (m, 1H), 1.69 (m, 1H), 1.65 (m, 1H), 1.58 (m, 1H), 1.56 (m, 1H), 1.33 (m, 2H), 1.24 (m, 1H), 0.84 (m, 1H), 0.69 (s, 3H), 0.96 (s, 3H), 0.90 (s, 9H), 0.89 (s, 9H), 0.08 (s, 3H), 0.07 (s, 3H), 0.05 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 76.1, 73.8, 71.6, 68.3, 49.1, 41.4, 40.7, 40.1, 39.8, 33.5, 32.8, 31.3, 28.1, 26.9, 26.5, 25.9, 25.6, 24.9, 23.0, 18.3, 17.9 17.6, 15.1, –5.3, –5.4, –5.8; IR (ATR) ν_{max}/cm^{-1} 3496, 2956, 2926, 2856, 1696, 1465, 1257, 1088, 1060, 853, 783, 668; ESI-MS (*m/z*) calcd. for $C_{32}H_{60}NaO_4Si_2$ $(M + Na)^+$: 589.4079; found: 589.4054.

Compound **4**: 4.9 mg, 1.1%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.68 (s, 1H), 3.45 (t, 2H, *J* 10.0 Hz), 3.37 (d, 1H, *J* 11.0 Hz), 3.32 (d, 1H, *J* 11.0 Hz), 2.41 (dd, 1H, *J* 10.1 Hz), 2.08 (m, 1H), 2.00 (m, 3H), 1.86 (m, 2H), 1.65 (m, 3H), 1.59 (m, 3H), 1.47 (m, 2H), 1.25 (s, 6H), 1.12 (s, 1H), 0.98 (s, 3H), 0.89 (s, 9H), 0.69 (s, 3H), 0.05 (s, 6H); ESI-MS (*m/z*) calcd. for $C_{26}H_{48}NaO_4Si^+$ $(M + Na)^+$: 475.3214; found: 475.3220.

Compound **5**: 5.8 mg, 1.3%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.62 (br s, 1H), 3.55 (d, 1H, *J* 11.6 Hz), 3.44 (d, 1H, *J* 12.0 Hz), 3.35 (d, 1H, *J* 12.1 Hz), 3.32 (d, 1H,

J 10.7 Hz), 2.42 (m, 1H), 2.16 (m, 2H), 2.10 (m, 2H), 2.00 (m, 4H), 1.91 (m, 3H), 1.79 (m, 3H), 1.73 (m, 3H), 1.59 (m, 7H), 0.96 (s, 3H), 0.90 (s, 9H), 0.69 (s, 3H), 0.08 (s, 3H), 0.06 (s, 6H); HRMS-ESI (*m/z*) calculated for $C_{26}H_{48}NaO_4Si^+$ $(M + Na)^+$: 475.3214; found: 475.3213.

(4*S*,4a*S*,6a*R*,8*S*,9*S*,11a*R*,11b*R*)-4,9-*bis*(((*tert*-butyldimethylsilyl)oxy)methyl)-9-hydroxy-4,11b-dimethyldodecahydro-8,11a-methanocyclohepta[*a*]naphthalen-3(2*H*)-one (**6**)

Pyridinium chlorochromate (PCC) (276 mg, 1.26 mmol) was added to a stirred solution of sodium acetate (86 mg, 1.03 mmol) and **3** (330 mg, 0.61 mmol) in dry dichloromethane. The mixture was stirred at room temperature for 32 h and protected from moisture with an attached $CaCl_2$ dry tube. The mixture was filtered through a Buchner filter and eluted with diethyl ether. The residue was filtered once again through a florisil pad (4 g) and sodium sulfate (1 g) and eluted with diethyl ether. The residue was then purified via classical column chromatography (silica gel, EtOAc/hexane 2:3) to produce compound **6** as a white solid (268.8 mg, 78%). M.p. 115-117 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.55 (d, 1H, *J* 8.5 Hz), 3.40 (d, 1H, *J* 10.0 Hz), 3.27 (d, 1H, *J* 9.5 Hz), 3.18 (d, 1H, *J* 9.0 Hz), 2.64 (m, 1H), 2.21 (m, 2H), 2.07 (m, 2H), 1.99 (d, 1H, *J* 11.0 Hz), 1.89 (m, 3H), 1.64 (m, 1H), 1.43 (m, 1H), 1.37 (s, 2H), 1.24 (m, 2H), 1.19 (m, 1H), 0.90 (s, 3H), 0.84 (s, 9H), 0.82 (s, 3H), 0.79 (s, 9H), 0.00 (s, 6H), –0.05 (s, 3H), –0.07 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 218.0, 77.6 77.3, 77.1, 74.1, 70.1, 68.4, 53.0, 48.5, 41.6, 40.7, 39.3, 38.6, 37.0, 33.3, 32.2, 31.6, 30.9, 30.7, 30.0, 28.3, 26.7, 26.3, 26.1, 25.9, 25.1, 23.0, 18.7, 18.5, 18.4, 15.0, 14.4, 1.3, –5.0, –5.0, –5.30, –5.35. IR (ATR) ν_{max}/cm^{-1} 3496, 2956, 2926, 2856, 1696, 1465, 1257, 1088, 1060, 853, 783, 668; HRMS-ESI (*m/z*) calcd. for $C_{32}H_{60}NaO_4Si_2$ $(M + Na)^+$: 587.3922; found: 587.3888.

Synthesis of (4*S*,4a*S*,6a*R*,8*S*,9*S*,11a*R*,11b*R*)-9-hydroxy-4,9-*bis*(hydroxymethyl)-4,11b-dimethyldodecahydro-8,11a-methanocyclohepta[*a*]naphthalen-3(2*H*)-one (**7**)

Compound **6** (0.45 mmol, 257 mg) in dry THF (20 mL) and tetrabutyl ammonium fluoride (3.30 mmol, 862 mg) were added to a round-bottomed flask. The mixture was stirred for 4 h, poured into water and extracted with ethyl acetate. The organic layer was then dried with anhydrous sodium sulphate and evaporated under vacuum. The residue was purified via classical column chromatography (silica gel, EtOAc) to afford the pure compound **7** as a white solid (143.8 mg, 95%). M.p. 136-138 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 3.71 (d, 1H, *J* 10.0 Hz), 3.48 (d, 1H, *J* 10.0 Hz), 3.37 (t, 2H, *J* 10.0 Hz), 2.70 (m, 1H), 2.35 (m, 2H), 2.22 (m, 1H), 2.17 (s, 2H), 1.98 (m, 2H), 1.74 (m, 5H), 1.71 (m, 4H), 1.57 (m, 2H), 1.41 (m, 3H), 1.32 (m, 2H), 1.24 (m, 1H), 1.17 (s, 3H), 1.00 (s, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 213.7, 73.0, 66.1, 53.5, 52.2, 50.5, 48.9, 48.8, 46.3, 45.6, 44.7, 44.3, 41.0, 38.6, 32.9, 31.5, 29.4, 26.8, 24.4, 22.3; IR (KBr) ν_{max}/cm^{-1} 3393, 2946, 1690, 1037; HRMS-ESI (*m/z*) calcd. for $C_{10}H_{32}O_4$ $(M + H)^+$: 337.2379; found: 337.2373.

Synthesis of (4*R*,4a*S*,6a*R*,8*S*,9*S*,11a*R*,11b*R*,*E*)-9-hydroxy-4,9-*bis*(hydroxymethyl)-4,11b-dimethyldodecahydro-8,11a-methanocyclohepta[*a*]naphthalen-3(2*H*)-one oxime (**8**)

Compound **7** (62 mg, 0.180 mmol) and hydroxylamine chloride (32 mg, 0.472 mmol) were added to a stirred solution of sodium acetate (36 mg, 0.43 mmol) and triethylamine (2 mL, 14.3 mmol) in pyridine and ethanol (7:3 v/v). The mixture was stirred for 24 hours, in batches at 70 °C under a N_2 atmosphere. The mixture was evaporated under vacuum, and the obtained residue was washed with water and extracted with ethyl acetate. The residue was purified via classical column chromatography (silica gel, EtOAc) to yield compound **8** as a white solid (3.9 mg, 60%). M.p. 210 °C; ^{1}H NMR (500 MHz, DMSO) δ 8.47 (s, 1H), 5.07 (s, 1H), 4.28 (m, 2H), 3.83 (m, 1H), 3.18 (m, 2H), 3.13 (d, 1H), 2.64 (d, 1H), 2.18 (d, 2H), 2.15 (d, 2H), 2.02 (t, 1H), 1.84 (d, 2H), 1.33 (s, 3H), 1.29 (m, 1H), 1.18 (m, 2H), 0.93 (m, 2H), 0.83 (m, 2H), 0.68 (s, 3H); ^{13}C NMR (125 MHz, DMSO) δ 162.4, 73.4, 69.6, 67.7, 49.0, 48.3, 44.4, 41.1, 32.9, 31.2, 30.5, 28.0, 30.6, 28.0, 26.5, 25.2, 24.6, 20.9, 20.6, 18.3, 14.5; IR (KBr) ν_{max}/cm^{-1} 3420, 2923, 1653, 1090, 1027; HRMS-ESI (*m/z*) calcd. for $C_{20}H_{33}NO_4$ $(M + H)^+$: 352.2488; found: 352.2482.

General method for acylation of **1** (**9**-**10**)

Acetic anhydride (2.5 mmol, 0.236 mL) and dimethylaminopyridine (DMAP) (0.02 mmol, 3.0 mg) were added to a solution of **1** (0.5 mmol, 169.0 mg) in dry pyridine (10 mL). The reaction mixture was maintained under a N_2 atmosphere for 24 h, and then poured into cool water. The residue was filtered and dried under vacuum, resulting in a mixture of products **9** and **10**. The residue was then purified via classical column chromatography to produce a 3.9% yield of compound **9** (silica gel, EtOAc/hexane 3:7) and 21.5% yield of compound **10** (silica gel, EtOAc/hexane 1:1) as white solids. All structures were confirmed by NMR and mass spectra as discussed below.

(3*S*,4*S*,4a*S*,6a*R*,8*S*,9*S*,11a*R*,11b*R*)-4,9-*bis*(acetoxymethyl)-4,11b-dimethyltetradecahydro-8,11a-methanocyclohepta[*a*]naphthalene-3,9-diyl diacetate (**9**): ^{1}H NMR (500 MHz, CD_3OD) δ 4.84 (bs, 1H), 4.53 (d, 1H, *J* 12.0 Hz), 4.37 (d, 1H, *J* 12.0 Hz), 4.01 (d, 1H, *J* 10.0 Hz), 3.76 (d, 1H, *J* 10.0 Hz), 2.80 (bs, 1H), 2.61 (s, 3H), 2.06 (s,

3H), 2.04 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.96 (m, 1H), 1.86 (m, 4H), 1.85 (m, 2H), 1.82 (m, 1H), 1.78 (m, 1H), 1.72 (m, 1H), 1.70 (m, 1H), 1.69 (m, 1H), 1.52 (m, 2H), 1.50 (m, 1H), 1.40 (m, 1H), 1.39 (m, 1H), 1.31 (m, 1H), 1.01 (s, 3H), 0.99 (s, 3H), 0.81 (m, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 171.2, 170.8, 170.5, 170.1, 84.8, 73.2, 70.7, 64.4, 48.4, 39.7, 39.6, 39.4, 38.1, 32.8, 30.7, 26.9, 26.3, 26.1, 24.3, 23.3, 22.2, 21.1, 20.8, 17.1, 14.7; HRMS-ESI (*m/z*) calcd. for $C_{28}H_{42}NaO_8$ $(M + Na)^+$: 529.2772; found: 529.2822.

Synthesis of ((3*S*,4*S*,4a*S*,6a*R*,8*S*,9*S*,11a*R*,11b*R*)-3-acetoxy-9-hydroxy-4,11b dimethyltetradecahydro-8,11a-methanocyclohepta[*a*]naphthalene-4,9 diyl)*bis*(methylene) diacetate (**10**): ^{1}H NMR (500 MHz, CD_3OD) δ 4.81 (bs, 1H), 4.00 (d, 1H, *J* 10.0 Hz), 3.95 (m, 2H), 3.75 (d, 1H, *J* 10.0 Hz), 2.09 (m, 5H), 1.97 (m, 8 H), 1.83 (m, 6H), 1.78 (m, 2H), 1.72 (m, 1H), 1.68 (m, 1H), 1.66 (m, 1H), 1.49 (m, 1H), 1.38 (m, 1H), 1.35 (m, 1H), 1.28 (m, 1H), 1.25 (m, 3H), 1.22 (m, 1H), 0.98 (s, 6H), 0.97 (m, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 171.2, 171.2, 170.1, 73.3, 73.2, 70.7, 69.7, 48.7, 41.3, 39.7, 39.4, 39.4, 38.0, 32.3, 31.2, 28.3, 26.8, 26.5, 24.2, 23.3, 22.6, 20.8, 20.7, 20.7, 17.0, 14.8; HRMS-ESI (*m/z*) calcd. for $C_{26}H_{40}NaO_7$ $(M + Na)^+$: 487.2666; found: 487.2658.

X-ray diffraction analysis

The crystal was mounted on the goniometer of a Nonius Kappa CCD diffractometer (95 mm CCD camera on κ-goniostat) with graphite monochromated Mo K_α ($\lambda = 0.71073$ Å) radiation. The X-ray diffraction intensities (ϕ scans and ω scans with κ offsets) were measured with the COLLECT program[36] and the reflections were integrated and scaled with the HKL Denzo-Scalepack software package.[37] All reflections were used to calculate the final unit cell parameters and no absorption correction was applied. The structure was solved using direct methods implemented in SHELXS-97.[38] The models were refined with anisotropic thermal parameters by full-matrix least-squares procedures on F^2 using SHELXL-97.[38] All the hydrogen atoms were stereochemically placed and refined with the riding model and the conformations of the methyl groups were refined by treating them as rigid bodies which were allowed to rotate around the corresponding C–CH_3 bond. The programs Mercury[39] and VMD[40] were used to prepare the molecular graphics for publication.

Leishmanicidal assay

Animals

We used 6-to-8-week-old BALB/c female mice, which were bred and maintained under standard conditions at the animal house of the School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil. All animals were handled in strict accordance with good animal practice, as defined by the relevant national and local animal welfare bodies of the University of São Paulo.

Leishmania cultivation

The *Leishmania* strain used in this study was *L. braziliensis* H3227 (MHOM/BR/94/H-3227), which was kindly provided by Prof Aldina Barral (Professor of Immunology, Centro de Pesquisas Gonçalo Moniz, FIOCruz-Bahia, Brazil). *L. braziliensis* was maintained *in vitro* in M199 medium and *in vivo* by serial passage in hamsters (*Mesocricetus auratus*) at the laboratory of Molecular Parasitology, Department of Molecular and Cellular Biology of Pathogen Bioagents, Faculty of Medicine, University of São Paulo, Brazil.

Anti-promastigote assay

The anti-promastigote assay was carried out according to the method of Dutta *et al.*.[41] We diluted 0.02-10 μM of the aphidicolin derivatives in Schneider's medium (supplemented with 10% betal bovine serum (FBS) and 2% human urine) in 96-well microplates. A total of 4×10^4 parasites were seeded *per* well (2×10^5 parasites *per* mL), and the plates were incubated at 26 °C for 72 h. Subsequently, 100 μg of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dissolved in 10 μL of phosphate buffered saline (PBS) were added *per* well, and the plates were incubated at 37 °C for 4 h. Following incubation, the plates were centrifuged at 3000 × g for 5 min, the supernatant was removed, and precipitated formazan was dissolved in 100 μL of dimethyl sulfoxide (DMSO). The absorbance was measured at 492 nm, and the data for 2 assays performed in triplicate were analyzed by using Graph Pad Prism 4.0 software.

Determination of cytotoxicity against mammalian cells

Cytotoxicity was determined by using the MTT assay, as described above. Briefly, BALB/c mice were injected with 5% thioglycolate medium. After 72 h, murine peritoneal cells were harvested in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco Invitrogen Corporation, New York, USA), pH 7.6. A total of 1×10^5 cells were plated on coverslips in 96-well plates and allowed to adhere for 12 h at 37 °C in an atmosphere of 5% CO_2. Macrophages were incubated with 0.032-12.5 μM of the aphidicolin derivatives for 48 h at 37 °C in 5% CO_2. Control cells were incubated in DMSO without drugs. Analysis of the mean of 2 assays performed in triplicate was carried out with Graph Pad Prism 5.0 software.

Determination of the *in vitro* infection index

Peritoneal macrophages from BALB/c mice were obtained as described above. A suspension of 5×10^5 cells in RPMI 1640 medium was distributed on glass coverslips (13 mm diameter) in 24-well plates and incubated for 1 h at room temperature. Non-adherent cells were removed by rinsing the cover slips with RPMI 1640. Adherent cells were incubated in RPMI 1640 (supplemented with 10% FBS [Gibco, Brazil], 100 UI *per* mL penicillin, and 0.1 mg *per* mL streptomycin) and infected with 10 *L. braziliensis* promastigotes *per* macrophage for 6 h at 37 °**C** in 5% CO_2. The aphidicolin derivatives to be tested were subsequently added at a concentration of 6 μM. After a 48 h incubation at 37 °C, the coverslips were washed with PBS, stained by using the Panótico Rápido LB kit (Laborclin, Paraná, Brazil), dried, mounted on glass slides with Tissue-Tek® mounting medium (Sakura Finetek Europe B.V., Alphen aan den Rijn, Holland), and examined microscopically. The number of infected macrophages and the average number of parasites per macrophage were determined for 200 cells. The results were expressed as the infection index, which is the percentage of infected macrophages multiplied by the average number of amastigotes *per* macrophage. The negative controls were non-treated infected macrophages. The positive controls were infected macrophages treated with Geneticin [10 μg *per* mL]. Analysis of the mean of 2 assays performed in duplicate was carried out with Graph Pad Prism 5.0 software.

Supplementary Information

Supplementary information (Figures S1-S37 and Tables S1-S2) is available free of charge at http://jbcs.sbq.org.br as PDF file.

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