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Direct synthesis of C3-mono-functionalized oxindoles from N-unprotected 2-oxindole and their antileishmanial activity

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ABSTRACT

A novel approach for the synthesis of unprecedented C3-mono-functionalized indolin-2-ones is reported, starting from 2-oxindole and chalcones. The reactions proceed regioselectively under mild conditions, without di- and tri-alkylated side products. The new compounds have been evaluated in vitro for their antiproliferative effects against the protozoan *Leishmania infantum*. Interestingly, they appear able to kill *L. infantum* promastigotes and amastigotes, without significant cytotoxic effects.

1. Introduction

Leishmaniasis comprises a group of infectious diseases caused by several species of the genus *Leishmania* with three different clinical forms, visceral, cutaneous, and mucocutaneous. It is associated with significant rates of morbidity and mortality in many countries around the world and affects ca. 15 million people.[1]

The relevance of this parasitic disease is that the incidence of new cases is increasing daily and currently is emerging as a common and serious opportunist infection in human immunodeficiency virus (HIV)-infected patients and in organ transplant patients.[2]

Infection takes place when a sandfly vector inoculates *Leishmania* promastigotes (extracellular flagellated forms of the parasite) into the mammalian skin. Once in the host tissues, parasites are phagocytosed by mononuclear blood cells, especially macrophages, where they differentiate into amastigotes (obligate intracellular forms of the parasite). Amastigotes proliferate inside the macrophages before inducing the bursting of the host cell and being released into the bloodstream. This process occurs repeatedly, leading to tissue damage.[3]

To date, no effective vaccination is available. The choice of drugs (pentavalent antimonials, amphotericin B, liposomal amphotericin B, paromomycin, and miltefosine) has increased in the past decade, but current treatments are limited, have the potential to develop resistance, are expensive, and possess unacceptable toxicity.[4] Thus, there is a great necessity to develop new drugs that are efficacious, safe, and more accessible.

The indole ring is the most common heterocycle present in nature and several indole-based biologically active natural products and indole-derived drugs are known. For this reason, substituted indoles have been referred to as 'privileged structures' owing to their excellent binding ability for many receptors with high affinity.[5]

In particular, the 2-oxindole scaffold constitutes an important pharmacophoric moiety, encountered in natural products and pharmaceutical compounds, with a wide spectrum of biological effects, such as antitumor, phosphodiesterase inhibitor activity and tyrosine kinase inhibitor activity.[6–8]

Thus, the search of new methods for the preparation of oxindole-containing heterocycles, especially those substituted at the C-3 position, is of considerable interest.

The selective and direct monofunctionalization at C-3 of N-unsubstituted 2-oxindole by alkyl, alkenyl, aryl, or heteroaryl groups is a longstanding issue.[9] Indeed, the conventional methods (reactions with alkyl halides or Michael or Mannich reactions)

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suffer of some limitations, such as lack of regioselectivity and difficulty in stopping the reaction at the monosubstitution,[10,11] so that di- and tri- alkylated products are obtained, as a consequence of the close acidity (pK_a 18.5) of the protons at the C-3 and N-1 positions.[9]

Thereby, the reduction of 3-alkylidene- or 3-arylidene-oxindoles, prepared by condensation of oxindoles with aldehydes or ketones, is generally preferred, although this method suffers of limitations, such as the competitive aldol-type side reactions for aliphatic aldehydes.[12–14] Recently, this two-step procedure has been converted into a more useful one-pot reaction, starting from alcohol as aldehyde-precursor, in the presence of catalytic amount of iridium,[15] ruthenium complex,[16] or Nichel-Raney.[17] However, the process is expensive and requires relatively long reaction time.

In view of these observations, in the framework of our studies dealing with the design of poly functionalized N,O-heterocycles,[18–27] we propose a new, convenient and straightforward approach for the synthesis of unprecedented C3-mono-functionalized N-unprotected indolin-2-ones, aiming at the identification of novel small molecules with leishmanicidal effects.

Finally, we further expanded the scope of the reaction to the synthesis of spiro-oxindoles.

The cytotoxicity and the anti-proliferative effects against *Leishmania infantum*, the main causative agent of visceral leishmaniasis in Mediterranean region, of all synthesized compounds were evaluated and compared with those of amphotericin B.

2. Results and discussion

2.1. Chemistry

In our previous work,[28] we have described the AcOH-mediated Michael addition of 4-unsubstituted azol-5-ones to α,β-unsaturated carbonyl compounds. This procedure extended to oxindole **1** as pronucleophilic agent and chalcone **2** (R = Ph) as selected α,β-unsaturated carbonyl compound did not give the expected Michael adduct. Therefore, the development of an alternative method applicable to this substrate has been studied. In particular, in refluxing EtOH and in the presence of piperidine and 4 Å molecular sieves, the reaction of oxindole **1** and chalcones **2a–e** (R = Ph) yielded the Michael adducts **3a–e** (Scheme 1).

It was found that all chalcones **2a–e** can tolerate the reaction conditions with different yields (Scheme 1), in particular rising from those *m*-substituted up to be excellent for *p*-substituted. Conversely, the *o*-substituted chalcones did not react.

In all cases, any bi- or tri-substituted adducts were isolated, neither intramolecular ring closure products, although the adduct **3** seem to possess the appropriate functionalities for the synthesis of fused ring system.[29]

Piperidine was found the best choice, while the use of DBU provided an uninteresting mixture of di- and tri-alkylated derivatives.

The structure of new compounds **3a–e** was determined on the basis of analytical and spectroscopic data.

Interestingly, this reaction creates two new stereocenters, but the ^{1}H NMR spectrum in $CDCl_3$ of **3b**, selected as model compound, indicated the presence of only one diastereomer, and the relative configuration was assigned on the basis of NOE experiments. In particular, the irradiation of Ha (dd, 3.56 ppm, the upfield resonance of methylene proton) resulted in a signal enhancement for Hb (dd, 4.0 ppm, the downfield methylene proton), for Hc (ddd, 3.98 ppm) and for aromatic protons (d, 7.95 ppm, the orto protons of the benzoyl group; m, 7.00–7.06 ppm, oxindole protons) (Scheme 2). Furthermore, when Hd was irradiated (d, 3.80 ppm) a positive NOE effect was observed for Hb and for aromatic protons (d, 6.77 ppm, orto protons of the aryl substituent; m, 7.00–7.06 ppm, oxindole protons). These results support an anti relationship between Hd and Hc consistent with **3b′** structure (Scheme 2), having C3-R*, Cα-R* configuration.

However, after one hour both ^{1}H and ^{13}C NMR spectra of **3b′** in $CDCl_3$ appeared complicated by the presence of three sets of signals. In particular, three methyl groups at 2.27, 2.20 and 2.18 ppm (14:2:1 ratio) are clearly observed.

One- and two-dimensional NMR data revealed that the signals at 2.20 ppm (s, CH3), 3.50 ppm (dd, Ha), 3.82 ppm (d, Hd), 3.96 ppm (m, Hc) and 4.20 ppm (m, Hb) can be attributed to the diastereomer **3b‴** (Scheme 2). The positive NOE signals observed within Hd–Hc–Ha of **3b‴** fully support their *syn* relationship. The formation of **3b‴** can be rationalized by a keto–enol tautomerism causing racemization at C-3.

In d_6-DMSO/D_2O a faster equilibrium was observed as indicated by the presence of three singlets for three methyl groups at 2.05, 2.06 and 2.15 ppm respectively, with the relative ratio 4.5:1:4.5 (**3b‴**/**3b″**/**3b′**). One- and two-dimensional NMR data indicated that the signals at 2.15 ppm (s, CH3), 3.15 ppm (dd, Ha), 4.02 ppm (dd, Hb) and 5.10 (dd, Hc) can be assigned to the enol form **3b″** (Scheme 2).

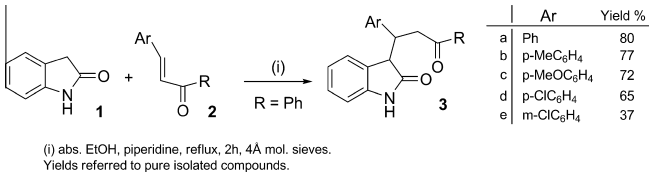
Moreover, a very fast equilibrium between the two keto-forms in $CDCl_3$ was observed for halogenated derivatives **3d** and **3e**, leading to a 3:2 and a 3:1 mixture respectively, within a few minutes (see ESM for NMR spectra).

Then, we turned our attention to testing the reactivity of benzalacetone **2′** (R = Me). Under similar reaction conditions, the reaction did not yield the expected Michael adduct.

Taking into account the experimental results, we suggest the mechanism summarized in Scheme 3. The 1,4-addition of piperidine to the α,β-unsaturated carbonyl compound[30] leads to the Mannich base **4** which could evolve according to two different pathways. For chalcone **2** (R = Ph), the intermediate **4** is intercepted by the oxindole **1**, undergoing a rapid proton exchange and a slow C-alkylation through SN_2, leading to the expected adduct **3**. This proposed mechanism could explain the unsuccessful reaction with the sterically hindered *o*-substituted chalcone and the absence of bis-adducts as final products, because of the steric hindrance of the chiral carbon at C-3.

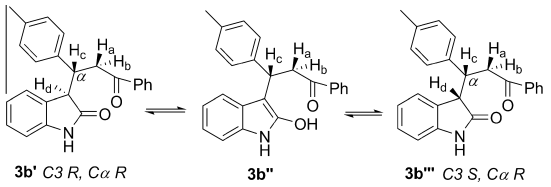
Conversely, for benzalacetone **2′** (R = Me), the intermediate **4** is preferentially intercepted by the piperidine and converted into the iminium ion **5**. The latter readily stabilizes, providing the dienamine **6**, after removal of the piperidinium ion **7**. Obviously, the electron-rich dienamine **6** is unable to be intercepted by the pronucleophile oxindole **1** (Scheme 3).

Based on recent reports,[31] we further expanded the scope of the reaction to dibenzalacetones **8** (Scheme 4), with the aim to

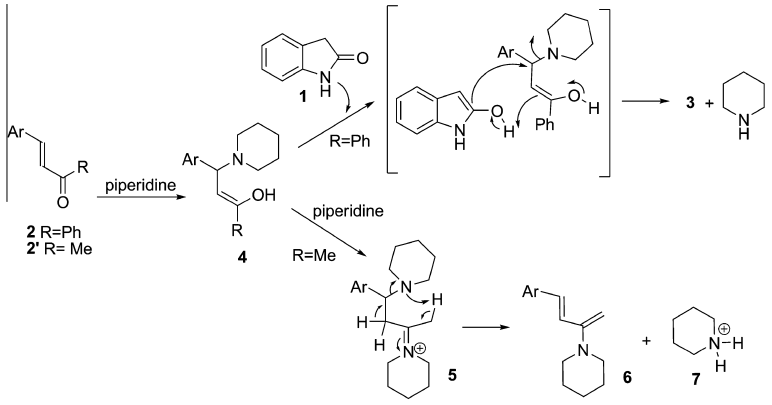


	Ar	Yield %
a	Ph	80
b	p-MeC$_6$H$_4$	77
c	p-MeOC$_6$H$_4$	72
d	p-ClC$_6$H$_4$	65
e	m-ClC$_6$H$_4$	37

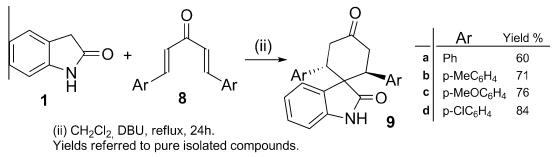
Scheme 1. Synthesis of compounds **3a–e** and yields.



Scheme 2. Keto–enol tautomerism of compounds **3**.



Scheme 3. Proposed mechanism.



Scheme 4. Synthesis of compounds **9a–d** and yields.

promote a double conjugate addition leading to spirocyclic compounds, which are intriguing combinations of multistereogenic cyclohexanone and oxindole motifs, with promising therapeutic perspectives.[32] Indeed, the spiro-oxindole is a privileged heterocyclic ring system, which constitutes the core of pharmaceutically relevant natural and unnatural products, exhibiting a wide spectrum of bioactivities such as antitumor, antimycobacterial, antimicrobial and antitubercular properties.[33]

Initially, the above described procedure has been applied to the reaction of oxindole **1** and dibenzalacetone **8**, but in refluxing EtOH and in the presence of piperidine and 4 Å molecular sieves, the reaction did not yield the expected spirans. In this case, DBU was found the best base-catalyst, in agreement with the literature,[34] confirming the role of piperidine as nucleophile in the reaction of chalcone **2**, not as base catalyst.

Our strategy is outlined in Scheme 4. Oxindole **1** reacted with dibenzalacetones **8a–d** in CH_2Cl_2 under reflux conditions, affording the spiro[cyclohexanone-oxindoles] **9a–d** in good yields (60–84%) and complete diastereoselectivity.

The proposed reaction mechanism consists in a double Michael addition of an enolate to the dibenzalacetone **8**, leading to **9** in a diastereoselective fashion.

The structure of compounds **9a–d** has been determined on the basis of analytical and spectroscopic data and by comparison with authentic samples.[32] According to literature data,[33] only the more stable trans isomer has been detected in the crude reaction mixture.

It is noteworthy that several advantages have been achieved with respect to previous methods,[32–36] such as quicker reaction time, mild conditions, and simple workup. Furthermore, the protection of the oxindole-NH[37] and the use of additives[32–36] are not required. However, the reaction proceeds with high diastereoselectivity.

2.2. Biology

All new synthesized compounds were evaluated in vitro for antileishmanial activity. The screening for leishmanicidal compounds was carried out with *Leishmania* promastigotes and axenic amastigotes.[38] All tests were performed in triplicate and amphotericin B was used as reference drug.[39]

Compounds **3a–e** and **9a–d** exhibit a good activity and toxicity profile with respect to the precursors oxindole **1** and chalcone **2** (Tables 1 and 2). In particular, oxindole **1** results inactive at all tested concentrations, while chalcone **2** show a good EC_{50}/24 h value (5.7 μg/mL for promastigotes; 5.1 μg/mL for amastigotes), but a significant toxicity (TC_{50}/24 h: 28.1 μg/mL; TC_{50}/48 h: 30.7 μg/mL).

After 24 h of incubation, **3a** and **9b** exhibit a EC_{50} value of 16.2 μg/mL and 6.1 μg/mL, respectively, with a quite high TC_{50} (71.6 μg/mL and 62.8 μg/mL, respectively) and a good TI (4.4 and 10.2, respectively).

After 48 h of incubation, **9b** and **3a** show high TI values for both promastigotes (18.7 and 10.6, respectively) and amastigotes (20.3 and 11.2, respectively).

Interestingly, **3e** presents a quite good therapeutic index TI for both promastigotes and amastigotes (6.1 and 6.3, respectively) after 24 h of incubation, suggesting that the presence of an electron-withdrawing group (Cl) at the *meta* position of the phenyl ring maintains the biological activity, reducing the toxicity. Conversely, the presence of an electron-donor group in **3b** and **3c** (Me, OMe) results in a decrease of antileishmanial activity.

A significant improvement of antiprotozoal activity has been observed for compounds **9a–d** comparing with **3a–e**. Indeed, all spiro[cyclohexanone-oxindoles] **9a–d** are able to kill more than 74% of *L. infantum* promastigotes after 48 h of incubation, at all tested concentrations (data not shown). In particular, **9b**, although less active than commercial drug Am B, results the most interesting compound, with a TI more than two times higher than Am B, for both promastigotes and amastigotes.

3. Conclusions

In summary, an efficient approach for the synthesis of C3-mono-functionalized N-unprotected oxindoles has been achieved. The procedure offers several advantages, including mild conditions, high product yield, and simple workup, and provides straightforward access to important synthetic intermediates. Furthermore, spiro[cyclohexanone-oxindoles] have been synthesized in a more convenient fashion with respect to previous reports. The obtained compounds were evaluated in vitro for leishmanicidal activity against *L. infantum*. The results showed for both series **3** and **9** lack of cytotoxicity and significant dose-dependent antiproliferative activity. The overall activity profile demonstrated that the modification of the oxindole nucleus is a convenient way for the identification of new antiparasitic drugs. Further studies are currently in progress to explore the mechanism of action and more extensive details will be reported in future publications.

Table 1
Toxic concentration on RAW 264.7 cells, antileishmanial activity (vs promastigotes and axenic amastigotes) and therapeutic index of the tested compounds after 24 h of incubation

	RAW 264.7	Promastigotes			Amastigotes		
	TC_{50} ± SD (μg/mL)	EC_{50} ± SD (μg/mL)	EC_{95} ± SD (μg/mL)	TI (TC_{50}/EC_{50})	EC_{50} ± SD (μg/mL)	EC_{95} ± SD (μg/mL)	TI (TC_{50}/EC_{50})
3a	71.6 ± 4.5	16.2 ± 3.5	77.8 ± 6.7	4.4	15.3 ± 2.1	75.6 ± 4.2	4.7
3b	93.3 ± 6.4	62.0 ± 2.7	110.9 ± 4.6	1.5	60.1 ± 1.8	99.5 ± 3.4	1.6
3c	41 ± 2.3	29.7 ± 0.3	78.5 ± 1.2	1.3	24.2 ± 0.2	75.4 ± 0.9	1.7
3d	36.9 ± 3.3	19.7 ± 1.1	76.5 ± 1.3	1.8	18.5 ± 0.9	74 ± 1.1	2
3e	193.1 ± 12.4	31.6 ± 0.3	85.5 ± 0.5	6.1	30.5 ± 0.2	80.2 ± 0.3	6.3
9a	88.8 ± 7.3	29.8 ± 2.2	86.1 ± 2.6	2.9	28.5 ± 1.7	78.3 ± 1.5	3.1
9b	62.8 ± 3.5	6.1 ± 1.4	72.1 ± 0.3	10.2	5.9 ± 1.2	69.4 ± 0.2	10.6
9c	80.5 ± 6.4	23.9 ± 2.2	82.4 ± 3.7	3.3	21 ± 1.6	79.3 ± 2.5	3.8
9d	44 ± 3.5	23.4 ± 1.3	77.1 ± 1	1.8	21 ± 1.1	75.2 ± 0.7	2.1
1	125.8 ± 11.3	232.4 ± 19	447.4 ± 36	0.5	199 ± 11	430.5 ± 15.5	0.6
2	28.1 ± 2.3	5.7 ± 1.2	83.5 ± 1.3	4.9	5.1 ± 0.9	79.5 ± 1.1	5.5
AmB	19.6 ± 1.4	0.55 ± 0.08	3.9 ± 0.02	35.1	0.5 ± 0.05	3.9 ± 0.02	39.1

Table 2
Toxic concentration on RAW 264.7 cells, antileishmanial activity (vs promastigotes and axenic amastigotes) and therapeutic index of the tested compounds after 48 h of incubation

	RAW 264.7	Promastigotes			Amastigotes		
	TC_{50} ± SD (μg/mL)	EC_{50} ± SD (μg/mL)	EC_{95} ± SD (μg/mL)	TI (TC_{50}/EC_{50})	EC_{50} ± SD (μg/mL)	EC_{95} ± SD (μg/mL)	TI (TC_{50}/EC_{50})
3a	66.6 ± 5.4	6.3 ± 1.2	72.1 ± 0.3	10.6	5.9 ± 1.1	69.4 ± 0.2	11.2
3b	67.6 ± 4.1	51.6 ± 1	96 ± 1.6	1.3	48.4 ± 1	94.3 ± 1.1	1.3
3c	35.4 ± 4.2	14.2 ± 1.1	74 ± 0.4	2.5	13.1 ± 1.1	71.2 ± 0.3	2.7
3d	35 ± 5	11.5 ± 1.1	73.6 ± 0.4	3	10.1 ± 0.9	70.2 ± 0.2	3.5
3e	116.3 ± 9.9	13.2 ± 1.3	74 ± 0.7	8.8	12.5 ± 1.1	71.5 ± 0.5	9.3
9a	65.2 ± 7	6.9 ± 1.7	73.2 ± 0.9	9.4	5.7 ± 0.9	74.1 ± 0.7	11.4
9b	38.6 ± 4.7	2 ± 0.9	71.4 ± 0.7	18.7	1.9 ± 0.8	69.9 ± 0.5	20.3
9c	51 ± 5.9	16.2 ± 1.2	75.2 ± 0.2	3.1	15.1 ± 1	74.8 ± 0.4	3.4
9d	21.3 ± 1.9	5.1 ± 1.7	72.1 ± 0.8	4.2	4.9 ± 0.9	69.9 ± 0.7	4.4
1	154.5 ± 20.6	72.4 ± 6	146.4 ± 13.5	2.1	69.5 ± 4.1	135.5 ± 12.1	2.2
2	30.7 ± 3.4	1.5 ± 0.3	71.1 ± 0.1	20.1	1.3 ± 0.2	69.9 ± 0.7	23.7
AmB	3.1 ± 0.2	0.4 ± 0.07	3.9 ± 0.03	8.2	0.4 ± 0.07	3.4 ± 0.02	8.2

4. Experimental

4.1. General

Melting points were determined on a Kofler melting apparatus and are uncorrected. IR spectra were recorded in Nujol with a Nicolet Impact 410D spectrometer. ^{1}H and ^{13}C NMR spectra were obtained on a Varian 500 MHz spectrometer. The chemical shifts (δ) and coupling constants (J) are expressed in ppm and hertz respectively. Microanalyses and mass spectrometry analyses were carried out on a Carlo Erba EA 1102 and on a 3200 QTRAP (Applied Biosystem SCIEX) respectively. All solvents and reagents were obtained from commercial sources and purified before use if necessary. Merck Kieselgel 60F_{254} plates were used for TLC, and Merck Silica gel 60 (0.063–0.100 mm) for column chromatography.

4.1.1. Typical procedure for the preparation of compounds 3a–e

To a stirred solution of **1** (0.5 g, 7.5 mmol) in abs EtOH (20 mL) chalcone **2a**–**e** (7.5 mmol) and piperidine (7.5 mmol) were added. The mixture was stirred and heated at reflux for 2 h. After cooling, the reaction mixture was evaporated, affording compounds **3a**–**e**. For compound **3a**, the resulting residue was washed with cool H_2O (20 mL) and the aqueous suspension was then extracted with Et_2O (3 × 30 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. The residue was recrystallized from MeOH leading to **3a** as white crystals.

4.1.1.1. (C3-*R,Cα-*R**)-3-(1,3-diphenylpropyl)indolin-2-one (3a).** White solid; yield: 80%; mp: 150–152 °C; IR (Nujol): 1704, 1682; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.58 (dd, J = 6.6, 16.3 Hz, 1H), 3.81 (d, J = 7.1 Hz, 1H), 3.96–3.99 (m, 1H), 3.99–4.04 (m, 1H), 6.70 (t, J = 8.4 Hz, 2H Ar), 6.87 (t, J = 7.5 Hz, 1H Ar), 7.14–7.21 (m, 6H, Ar), 7.44 (t, J = 7.0 Hz, 2H Ar), 7.55 (t, J = 7.0 Hz, 1H Ar), 7.94 (d, J = 7.0 Hz, 2H Ar); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 41.9, 42.7, 49.7, 109.4, 122.0, 125.4, 127.1, 127.9, 128.0, 128.1, 128.2, 128.3, 128.5, 128.6, 133.1, 137.0, 141.0, 178.2, 198.3; ESI-MS (m/z) = 342.14 $[M+1]^+$; Anal. Calcd for $C_{23}H_{19}NO_2$: C, 80.95; H, 5.68; N, 4.20.

4.1.1.2. (C3-*R,Cα-*R**)-3-(3-oxo-3-phenyl-1-*p*-tolylpropyl)indolin-2-one (3b).** White solid; yield: 77%; mp: 158–160 °C; IR (Nujol): 1702, 1677; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.28 (s, 3H), 3.56 (dd, J = 6.1, 15.5 Hz, 1H), 3.80 (d, J = 6.6 Hz, 1H), 3.98 (ddd, J = 6.1, 6.6, 7.0 Hz, 1H), 4.0 (dd, J = 7.0, 15.5 Hz 1H), 6.73 (d, J = 8.0 Hz, 1H Ar), 6.77 (d, J = 7.1 Hz, 1H Ar), 6.89 (t, J = 7.5 Hz, 1H Ar), 7.0–7.06 (m, 4H Ar), 7.16 (t, J = 8.0 Hz, 1H Ar), 7.45 (t, J = 8.4 Hz, 2H Ar), 7.54 (t, J = 8.4 Hz, 1H Ar), 7.95 (d, J = 8.4 Hz, 2H Ar); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 21.0, 42.0, 42.4, 49.8, 109.4, 121.9, 125.4, 128.1, 128.2, 128.6, 129.0, 133.0, 136.6, 137.0, 137.9, 141.4, 178.1, 198.2; ESI-MS (m/z) = 356.14 $[M+1]^+$; Anal. Calcd for $C_{24}H_{21}NO_2$: C, 81.15; H, 5.99; N, 3.98.

4.1.1.3. (C3-*R,Cα-*R**)-3-(1-(4-methoxyphenyl)-3-oxo-3-phenylpropyl)indolin-2-one (3c).** white solid; yield: 72%; mp: 145–146 °C; IR (Nujol): 1703, 1678; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.57 (dd, J = 6.5, 16.2 Hz, 1H), 3.75 (s, 3H), 3.78 (dd, J = 6.9 Hz, 1H), 3.94–3.97 (m, 2H), 6.73 (d, J = 8.8 Hz, 2H Ar), 6.80 (d, J = 7.3 Hz, 1H Ar), 6.90 (t, J = 7.2 Hz, 1H Ar), 7.06 (d, J = 8.8 Hz, 2H Ar), 7.16 (t, J = 7.2 Hz, 1H Ar), 7.43–7.46 (m, 3H Ar), 7.55 (t, J = 7.3 Hz, 1H Ar),

7.95 (d, J = 7.3 Hz, 2H Ar); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 42.0, 42.1, 50.0, 55.1, 109.4, 113.6, 122.0, 125.4, 128.0, 128.1, 128.2, 128.6, 129.5, 132.9, 133.1, 137.0, 178.3, 198.2; ESI-MS (m/z) = 372.16 $[M+1]^+$; Anal. Calcd for $C_{24}H_{21}NO_3$: C, 77.69; H, 5.75; N, 3.71.

4.1.1.4. 3-(1-(4-Chlorophenyl)-3-oxo-3-phenylpropyl)indolin-2-one (3d). Inseparable 3:2 mixture of (C3-*R**,Cα-*R**) and (C3-*S**,Cα-*R**) white solid; yield: 65%; mp: 160–162 °C; IR (Nujol): 1704, 1675; (C3-*R**,Cα-*R**) isomer ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.60 (m, 1H), 3.83 (d, J = 0.4 Hz, 1H), 4.05 (dd, J = 7.0, 14.5 Hz, 1H), 4.19–4.23 (m, 1H), 6.64 (d, J = 8.0 Hz, 1H Ar), 6.87 (s, 1H Ar), 7.02 (t, J = 7.9 Hz, 1H Ar), 7.04–7.18 (m, 5H Ar), 7.44–7.46 (m, 3H Ar), 7.47–7.57 (m, 1H Ar), 8.03 (d, J = 7.1 Hz, 1H Ar); (C3-*R**,Cα-*R**) isomer ^{13}C NMR (125 MHz, $CDCl_3$): δ = 41.6, 42.0, 49.8, 109.3, 122.4, 124.5, 127.9, 128.0, 128.2, 128.6, 129.7, 133.2, 133.3, 137.0, 140.8, 178.0, 198.5; (C3-*S**,Cα-*R**) isomer ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.58 (dd, J = 7.4, 7.3 Hz, 1H), 3.79 (d, J = 7.0 Hz, 1H), 4.19–4.23 (m, 2H), 6.75 (d, J = 7.5 Hz, 1H Ar), 6.85 (s, 1H Ar), 6.93 (t, J = 7.3 Hz, 1H Ar), 7.04–7.18 (m, 5H Ar), 7.33 (d, J = 7.5 Hz, 1H Ar), 7.44–7.46 (m, 2H Ar), 7.47–7.57 (m, 1H Ar), 7.93 (d, J = 7.0 Hz, 1H Ar); (C3-*S**,Cα-*R**) isomer ^{13}C NMR (125 MHz, $CDCl_3$): δ = 39.7, 41.3, 49.8, 109.6, 122.1, 125.2, 127.4, 128.0, 128.4, 128.7, 129.9, 132.5, 137.0, 138.0, 140.8, 177.8, 198.0; ESI-MS (m/z) = 376.16 $[M+1]^+$; Anal. Calcd for $C_{23}H_{18}ClNO_2$: C, 73.55; H, 4.88; Cl, 9.47; N, 3.74.

4.1.1.5. 3-(1-(3-Chlorophenyl)-3-oxo-3-phenylpropyl)indolin-2-one (3e). Inseparable 3:1 mixture of (C3-*R**,Cα-*R**) and (C3-*S**,Cα-*R**); white solid; yield: 37%; mp: 145–146 °C; IR (Nujol): 1704, 1675; (C3-*R**,Cα-*R**) isomer ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.54–3.58 (m, 1H), 3.85 (d, J = 0.2 Hz, 1H), 3.94–3.98 (m, 1H), 4.15–4.17 (m, 1H), 6.67 (d, J = 7.5 Hz, 1H Ar), 7.01–7.19 (m, 5H Ar), 7.29 (d, J = 7.1 Hz, 1H Ar), 7.41–7.48 (m, 4H Ar), 7.53–7.58 (m, 1H Ar), 8.02 (d, J = 7.5 Hz, 1H Ar), 8.03 (br s, NH); (C3-*R**,Cα-*R**) isomer ^{13}C NMR (125 MHz, $CDCl_3$): δ = 41.5, 42.3, 49.9, 109.6, 122.4, 124.6, 126.6, 127.1, 127.7, 128.1, 128.2, 128.4, 128.6, 129.5, 133.2, 133.9, 136.9, 141.1, 141.8, 178.5, 197.6; (C3-*S**,Cα-*R**) isomer ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.54–3.58 (m, 1H), 3.80 (d, J = 0.4 Hz, 1H), 4.15–4.17 (m, 2H), 6.68 (d, J = 7.0 Hz, 1H Ar), 6.91 (t, J = 7.5 Hz, 1H Ar), 7.01–7.19 (m, 6H Ar), 7.41–7.48 (m, 4H Ar), 7.93 (d, J = 7.5 Hz, 1H Ar), 8.42 (br s, NH); (C3-*S**,Cα-*R**) isomer ^{13}C NMR (125 MHz, $CDCl_3$): δ = 39.5, 41.6, 49.8, 109.8, 122.1, 125.1, 126.9, 127.3, 127.6, 128.0, 128.4, 128.5, 129.3, 129.5, 133.2, 134.1, 136.8, 141.6, 143.2, 178.3, 198.4; ESI-MS (m/z) = 376.18 $[M+1]^+$; Anal. Calcd for $C_{23}H_{18}ClNO_2$: C, 73.56; H, 4.89; Cl, 9.44; N, 3.76.

4.1.2. Detection of compound 3b″

Five milligrams of compound **3b** was dissolved in 0.7 mL of d_6-DMSO. The ^{1}H NMR spectra were recorded at different times (1 min, 10 min). One drop of D_2O was then added and the relative ^{1}H NMR spectrum was registered (see ESM).

4.1.3. Typical procedure for the preparation of compounds 9a–d

To a stirred solution of **1** (0.5 g, 7.5 mmol) in CH_2Cl_2 (20 mL) dibenzalacetone **8a–d** (7.5 mmol) and DBU (7.5 mmol) were added. The mixture was stirred and heated at reflux for 24 h. After cooling, the reaction mixture was evaporated. A 10% solution of HCl and 5 mL of MeOH were added to the residue and the product was then extracted with Et_2O (3 × 30 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure, leading to compounds **9a–d**.

4.1.3.1. (2S,6S)-2,6-Diphenylspiro[cyclohexane-1,3′-indoline]-2′,4-dione (9a). Lit.[32]

4.1.3.2. (2S,6S)-2,6-Dip-tolylspiro[cyclohexane-1,3′-indoline]-2′,4-dione (9b). Lit.[32]

4.1.3.3. (2S,6S)-2,6-Bis(4-methoxyphenyl)spiro[cyclohexane-1,3′-indoline]-2′,4-dione (9c). Lit.[32]

4.1.3.4. (2S,6S)-2,6-Bis(4-chlorophenyl)spiro[cyclohexane-1,3′-indoline]-2′,4-dione (9d). Lit.[32]

4.2. Biological materials and methods

4.2.1. Activity assay against promastigotes and axenic amastigotes of *L. infantum*

The in vitro efficacies of the synthesized compounds were performed on promastigotes and on axenic amastigotes of *L. infantum* (zymodeme MON-1). Promastigotes were cultured at 26 °C in Schneider's Drosophila Medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) at pH 7.2. Parasites were harvested from the medium, in the logarithmic phase; were counted in a Neubauer's chamber and adjusted to a concentration of 5 × 10^6 parasites/mL^{-1}, for the drug assay.[40–43]

Axenic amastigotes were grown at 37 °C with 5% CO_2 in a cell free medium called MAA/20 (medium for axenically grown amastigotes).[44] MAA/20 consisted of modified medium 199 (Gibco) with Hank's balanced salt solution supplemented with 0.5% tryptocasein (Oxoid), 15 mM d8-glucose (Sigma), 5 mM glutamine (Gibco), 4 mM $NaHCO_3$ (Sigma), 0.023 mM bovine hemin (Fluka), and 25 mM HEPES to a final pH of 6.5 and supplemented with 20% heat inactivated fetal calf serum FBS (Gibco). The growth of parasites was determined by Image stream multispectral imaging flow cytometer.

Tested compounds, solubilized in DMSO (the highest concentration used was 1% v/v, not hazardous to the parasites), were added to the above parasite culture for screening, using a 96-well microtitre plate, in a concentration range of 100–10 μg/mL. Promastigote or axenic amastigote cultures were distributed in 96-well flat-bottom microplates (195 μL/well); 5 μL of each concentration of products were added to the culture in a range from 10 to 100 μg/mL, with a final volume of 200 μL/well and incubated at 26 °C or 37 °C with 5% CO_2, respectively for promastigotes and axenic amastigotes, for 24/48 h.

After 24/48 h of incubation at specific temperatures (26 °C for promastigotes and 37 °C with 5% CO_2 for axenic amastigotes), parasite's survival was evaluated. The number of surviving promastigotes was counted in Neubauer's chamber and compared to the controls (parasites without the drugs).

The viability of axenic amastigotes was evaluated by ImageStream Multispectral Imaging Cytometer (Ideas v4.0, Amnis Corp., Seattle, WA) using the IDEAS data analysis software (Inspire v4.0, Amnis Corp.).

Axenic amastigotes treated and untreated (control) were stained with molecular probe TO-PRO-3 (Invitrogen) for nucleic acid labeling (absorbance at 642 nm and emission at 661 nm), maintained for 10 min at room temperature before analyses.

Conventional flow cytometric methods do not provide direct morphologic evidence of cell death. Imagestream system, with its technology can provide the statistical power offered by flow cytometry, coupled with the critical assessment capabilities associated with microscopic analysis.[45]

Briefly, images acquired on the Imagestream imaging cytometer (at least 10,000 events) were collected for each sample. Labeled parasites were excited using a 642 nm laser. Brightfield, side

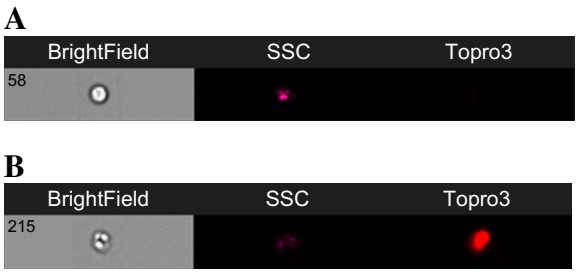


Figure 1. ImageStream multispectra analysis of axenic amastigotes. Before acquisition, amastigotes were labeled with ToPro-3, allowing differentiation of viable (A) from non-viable parasites (B).

scatter, fluorescent parasite image were acquired at 40× magnification (Fig. 1).

Post-acquisition data analysis was performed using IDEAS image analysis software package, analyzing the morphological characteristics, size, and vitality (Fig. 2).[46]

Antileishmanial activity of the compounds were estimated by their EC_{50} and EC_{95} values (dosage causing 50% and 95% parasites death, respectively) at 24 and 48 h in comparison to identical cultures without the compounds.

4.2.2. Cytotoxicity assay

The cytotoxicity effect of tested compounds was assayed on RAW 264.7 cells grown in RPMI 1640 medium, supplemented with 1 mmol L-1 L-glutamine, 10% (v/v) fetal bovine serum (FBS), Penicillin/Streptomycin. The 1×10^5 cells/well were cultivated on microplates and incubated at 37 °C overnight in a humidified 5% CO_2 atmosphere to ensure the adherence of the cells. After 24 h of incubation, the compounds were added to the cell culture at the respective concentration already assayed for *L. infantum* (10, 50 and 100 μg/mL) and re-incubated at 37 °C for 24 h and 48 h. After incubation, 20 μL aliquots of (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) MTT (5 mg/mL), were added to each well and incubated for 4 h at 37 °C, followed by low centrifugation at 400 rpm for 10 min. Then, the 200 μL of supernatant culture medium were carefully aspirated and the reaction was interrupted with 100 μL of DMSO to dissolve the formation of crystals. The culture plate was placed on a microplate reader and the absorbance was measured at 540 nm.[38,40,41] The amount of color produced is directly proportional to the number of viable cell. All assays were performed in triplicate. Cell viability rate was calculated as the percentage of MTT absorption as follows: %survival = (mean experimental absorbance/mean control absorbance) × 100.

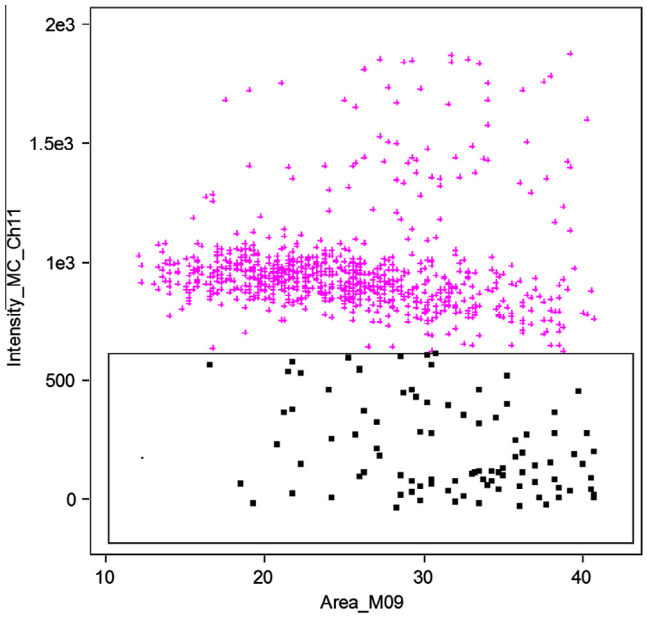


Figure 2. Bivariate plotting of 'Brightfield Area' versus 'Intensity To-Pro-3' performed using IDEAS software, from data obtained using the ImageStream cytometer. Area M 09 = Brightfield Area; Intensity MC Ch11 = Intensity To-Pro-3. Pink and black spots represent non-viable and viable amastigote distributions, respectively.

Toxicity of the compounds were assessed by their half maximal toxic concentration (TC_{50}) (dosage causing 50% macrophage death) values at 24 and 48 h in comparison to identical cultures without the compound.

An Excel add-in ED50V10 was used for calculating EC_{50}, EC_{95} and TC_{50}. Therapeutic index (TI) (TC_{50}/EC_{50}) was computed in order to find compounds with large therapeutic window.

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Supplementary data

Supplementary data (^1H and ^{13}C NMR spectra of new compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.12.039.

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