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Chemotherapy of leishmaniasis part X: Synthesis and bioevaluation of novel terpenyl heterocycles ☆

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ABSTRACT

Some novel α and β ionone based chalcones and their dihydropyrazolidines/pyrazolidines have been synthesized and evaluated for their in vitro and in vivo antileishmanial activities against *Leishmania donovani*. Amongest all, one compound (**4d**) exhibited significant in vitro activity against intracellular amastigotes of *Leishmania donovani* with IC_{50} values of 7.49 μM and was found promising as compared to reference drug, miltefosine. On the basis of good Selectivity Index (S.I.), the compound was further tested for its in vivo response against *Leishmania donovani*/hamster model and has shown significant inhibition of parasite multiplication (81%). The present study has helped us in identifying a new lead that could be exploited as a potential antileishmanial agent.

Leishmaniasis is a family of parasitic diseases that affect about 12 million people in tropical and subtropical areas in the form of three clinical expressions: visceral leishmaniasis, which is fatal in the absence of treatment; muco-cutaneous leishmaniasis and cutaneous leishmaniasis, which is often self curing. Classical drugs such as antimonials (Pentostam and Glucantime) are toxic and drug resistance is increasing dangerously in the field.[1] A liposomal amphotericin B formulation (AmBisome) less toxic than amphotericin B deoxycholate is gradually becoming the first-line therapy, especially in immunocompromised patients, but this drug must be administered by a parenteral route.[2] Miltefosine (Impavido) was the first drug registered against visceral leishmaniasis in the last decade; however, its toxicity and the appearance of drug resistance justify the search for new chemical series in order to find an orally safe and active drug.[3] Currently, efforts are being made to search for new molecules from the natural sources and in this endeavor diarylheptanoids,[4] oxygenated abietanes,[5] diterpene quinones[6,7] are showing promise as new lead molecules. Rationally designed heterocyclic ionone like molecules[8] and some novel terpenyl 2,4-diamino pyrimidines[9] are showing promising antimicrobial and dihydrofolate reductase inhibitory activities. 2,4-diaminopyrimidines[10] and some de novo-designed molecules[11] are also giving further inputs in the leishmanial dihydrofolatereductase activity. In continuation of our studies on terpenylpyrimidines as novel antileishmanial agents,[12] we designed some novel dihydropyrazolidines/pyrazolidines, having added aryl substitution and evaluated for their in vitro and in vivo antileishmanial activity and the results are reported in this communication.

The synthesis of compounds (**4a–j**) and (**8a–e**) followed the general pathway outlined in Schemes 1 and 2. They were prepared in two steps. Firstly, the chalcones were obtained by direct condensation between the substituted aromatic aldehydes and β-ionone, α-ionone respectively, using phase transfer catalyzed conditions.[13] Secondly, reaction of different chalcones with phenyl hydrazine in refluxing ethanol leads to the formation of dihydropyrazolidine derivatives. Further, compounds (**8a–e**) were aromatized into compounds (**9a–e**) using Ag_2O in refluxing ethanol. The reaction of β-ionone with substituted benzaldehydes was very facile and furnished chalcones in good to excellent yield.

The reaction of phenyl hydrazine with chalcones (**3a–j**) and (**7a–e**) was not only facile but it was also regiospecific in manner. The reaction of chalcone **3a** with phenyl hydrazine in ethanol furnished dihydropyrazolidine **4a** in 40% yield as a crystalline solid melting at 138–140 °C. The structure was assigned on the basis of ^1H and ^{13}C NMR spectra.

The ^1H NMR spectrum of **4a** displayed doublet of doublets at 2.87 ppm (J = 17, 7 Hz, 1H) & 3.85 ppm (J = 17, 12 Hz, 1H) for two geminal protons of the dihydropyrazolidine ring and doublet of

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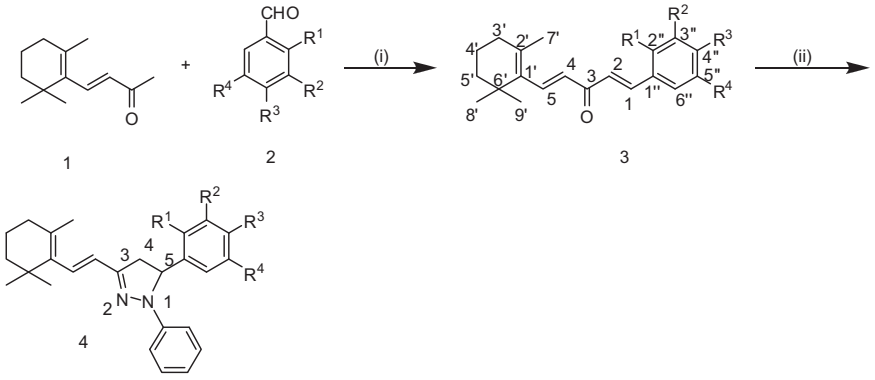


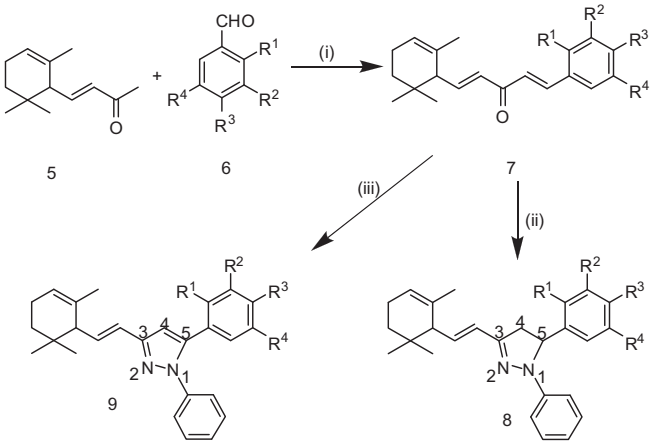
3, 4	R^1	R^2	R^3	R^4
a	NO_2	H	H	H
b	H	H	NO_2	H
c	H	NO_2	H	H
d	H	H	OCH_3	OCH_3
e	H	OCH_3	OCH_3	OCH_3
f	Cl	H	H	H
g	H	H	Cl	H
h	H	Cl	H	H
i	H	H	F	H
j	H	H	OBn	H

Scheme 1. Reagents and conditions: (i) Cetyl trimethyl ammonium bromide (CTABr), NaOH, H_2O, rt, 24 h; (ii) $PhNHNH_2$, EtOH, reflux, 8 h.

doublets at 5.69 (J = 12, 7 Hz, 1H) for H-5 proton (vicinal to the geminal protons) and it established the assigned structure **4a**. The compound **4d** was synthesized by the reaction of chalcone **3d** with phenyl hydrazine in ethanol (reflux, 8 h). The compound **4d**, Yellow crystalline solid which melted at 90–93 °C. The compound **4d** was characterized by IR, NMR and mass spectrum. The IR spectrum showed a C=N stretching band at 1597 cm^{-1}. The ^1H NMR spectrum of **4d** displayed three doublet of doublets at 2.85 ppm (J = 17, 7 Hz, 1H), 3.55 ppm (J = 17, 12 Hz, 1H) and 5.01 (J = 12, 7 Hz, 1H) for two geminal protons of dihydropyrazolidine ring and one for the proton vicinal to geminal protons respectively. Analysis of ^{13}C, DEPT-135 and mass spectra provided the final structural elucidation of compound **4d**. Cyclization in all the synthesized heterocyclic compounds took place near the aromatic ring rather than near the ionone ring. The presence of doublet near 2.25 (δ value) in ^1H NMR spectrum of compounds (**9a**–**e**) indicated that the cyclization took place near the aromatic ring. If cyclization had taken place near the ionone ring then a singlet would have been obtained in place of doublet in ^1H NMR spectrum of compounds (**9a**–**e**). Compounds (**4b**–**j**) and (**8a**–**e**) were synthesized in good yields under identical reaction conditions as used for the synthesis of compound **4a**. Few dihydropyrazolidines (**8a**–**e**) were aromatized using Ag_2O in refluxing ethanol and their structures were assigned by ^1H NMR and ^{13}C NMR spectra. We used the same reaction conditions, as used for compounds (**8a**–**e**), for aromatization of compounds (**4a**–**j**) but we didn't find any aromatized product.

For assessing the activity of compounds against the amastigote stage of the parasite, mouse macrophage cell line (J-774A.1) infected with promastigotes expressing luciferase firefly reporter gene was used. Cells were seeded in a 96-well plate (4×10^4 cell/100 μL/well) in RPMI-1640 containing 10% foetal calf serum and the plates were incubated at 37 °C in a CO_2 incubator. After 24 h, the medium was replaced with fresh medium containing stationary phase promastigotes (4×10^5/100 μL/well). Promastigotes invade the macrophage and are transformed into amastigotes. The test compounds were added at two fold dilutions up to 7 points in complete medium starting from 40 μM conc. after replacing the previous medium and the plates were incubated at 37 °C in a CO_2 incubator for 72 h. At the end of the incubation, the supernatants were removed and 50 μL PBS was added in each well and mixed with an equal volume of Steady Glo reagent. After gentle shaking for 1–2 min, the reading was taken in a luminometer.[14–16] The values are expressed as relative luminescence units (RLU). IC_{50} of antileishmanial activity was calculated by nonlinear regression analysis of the concentration response curve using the four parameter Hill equations.

The cell viability was determined using the MTT assay.[17] Exponentially growing cells (KB Cell line) (1×10^5 cells /100 μl/well) were incubated with test compounds for 72 h. The test compounds were added at three fold dilutions up to 7 points in complete medium starting from 400 μM concentration, and were incubated at 37 °C in a humidified mixture of CO_2 and 95% air in an incubator. Podophyllotoxin was used as a reference drug and control wells containing dimethyl sulfoxide (DMSO) without compounds were also included in the experiment. Stock solutions of compounds were initially dissolved in DMSO and further diluted with fresh complete medium. After incubation, 25 μml of MTT reagent (5 mg/ml) in PBS medium, followed by syringe filtration was added to each well and incubated at 37 °C for 2 h. At the end of the incubation period, the supernatant were removed and 150 μL of pure DMSO was added to each well. After 15 min. of shaking the



7, 8 and 9	R^1	R^2	R^3	R^4
A	NO_2	H	H	H
B	H	H	NO_2	H
C	H	H	F	H
D	H	H	OCH_3	OCH_3
E	H	OCH_3	OCH_3	OCH_3

Scheme 2. Reagents and conditions: (i) Cetyl trimethyl ammonium bromide (CTABr), NaOH, H_2O, rt, 24 h; (ii) $PhNHNH_2$, EtOH, reflux, 8 h; (iii) $PhNHNH_2$, Ag_2O, EtOH, reflux, 18 h.

readings were recorded as absorbance at 544 nm on a micro plate reader. The cytotoxic effect was expressed as 50% lethal dose, that is, as the concentration of a compound which provoked a 50% reduction in cell viability compared to cell in culture medium alone. IC_{50} values were estimated as described by Huber and Koella.[18] The selectivity index (SI) for each compound was calculated as ratio between, cytotoxicity (CC_{50}) and activity (IC_{50}) against Leishmania amastigotes.

The in vivo leishmanicidal activity was determined in golden hamsters (*Mesocricetus auratus*) infected with MHOM/IN/80/Dd8 strain of *Leishmania donovani* obtained through the courtesy of P.C.C. Garnham, Imperial College, London (UK). The method of Beveridge et al[19] as modified by Bhatnagar et al[20] and Gupta et al[21] was used for in vivo evaluation. Golden hamsters (Inbred strain) of either sex weighing 40–45 g were infected intracardiacally with 1×10^7 amastigotes per animal. The infection is well adapted to the hamster model and establishes itself in 15–20 days. Meanwhile, hamsters gain weight (85–95 g) and can be subjected to repeated spleen biopsies. Pre-treatment spleen biopsy in all the animals was carried out to assess the degree of infection. The animals with +1 infection (5–15 amastigotes/100 spleen cell nuclei) were included in the chemotherapeutic trials. The infected animals were randomized into several groups on the basis of their parasitic burdens. Five to six animals were used for each test sample. Drug treatment by intraperitoneal (i.p.) route was initiated after 2 days of biopsy and continued for 5 consecutive days. Post-treatment biopsies were done on day 7 of the last drug administration and amastigote counts are assessed by Giemsa staining. Intensity of infection in both, treated and untreated animals, and also the initial count in treated animals was compared and the efficacy was expressed in terms of percentage inhibition (PI) using the following formula:

$$PI = 100 - [ANAT \times 100/(INAT \times TIUC)]$$

Table 1
Antileishmanial activity of dihydropyrazolidines/pyrazolidines against *L. donovani*

Compd. No.	In vitro antiamastigote activity IC_{50} (μM)	CC_{50} (μM)	Selectivity index (S.I.) CC_{50}/IC_{50}	In vivo activity (% inhibiton) (50 mg/Kg × 5 days, i.p. dose)
4a	>20	—	—	—
4b	>40	—	—	—
4c	16.95	279.49	16.48	7.13 ± 8.95
4d	7.49	220.66	29.46	80.80 ± 11.91
4e	>20	—	—	—
4f	>20	—	—	—
4g	>20	—	—	—
4h	>40	—	—	—
4i	>40	—	—	—
4j	>40	—	—	—
8a	>20	—	—	—
8b	>40	—	—	—
8c	>40	—	—	—
8d	>20	—	—	—
8e	>20	—	—	—
9a	>40	—	—	—
9b	>40	—	—	—
9c	>40	—	—	—
9d	>20	—	—	—
9e	>40	—	—	—
Miltefosine	12.50	3.23	0.26	95.28 ± 2.49

Where PI is Percent Inhibition of amastigotes multiplication, ANAT is Actual Number of Amastigotes in Treated animals, INAT is Initial Number of Amastigotes in Treated animals and TIUC is Time Increase of parasites in Untreated Control animals.

The leishmanicidal activity of aryl substituted dihydropyrazolidines/pyrazolidines (**4a–j**), (**8a–e**) and (**9a–e**) was evaluated against *L. donovani* intracellular amastigotes and results have been presented in Table 1. The dihydropyrazolidines (**4a–j**) prepared from β ionone showed marginal to good in vitro antileishmanial activity. Among all the compounds, **4d** has shown promising antiamastigote activity with IC_{50} of 7.5 μM and selectivity index of 29.5. Rest of the compounds have shown marginal activity. The in vitro antileishmanial response of this compound was better than the reference drug, miltefosine (IC_{50} = 12.5 μM, S.I. = 0.26). We found very little correlation between type of substitution on the aromatic ring and the in vitro biological activity. The dihydropyrazolidines (**8a–e**) prepared from α ionone and their aromatized compounds (**9a–e**) showed only marginal in vitro activity.

The compound **4d** was also selected for in vivo efficacy evaluation against *L. donovani*/hamster model at the intraperitoneal (i.p.) dose of 50 mg kg^{-1} × 5 days. The compound exhibited significant in vivo response (81% inhibition in parasite multiplication).

In summary, synthesis and biological evaluation of these terpenyl heterocycles led us to discover compound **4d** as good antileishmanial agent which is more active than miltefosine in vitro. Selectivity index of compound **4d** is 113.3 fold higher than miltefosine. These investigations revealed that these terpenyl heterocycles can serve as prototype for development of more efficacious antileishmanial agents.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.10.110.

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