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Microwave-assisted synthesis of antimicrobial dihydropyridines and tetrahydropyrimidin-2-ones: Novel compounds against aspergillosis☆

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Abstract—Ten 4-aryl-1,4-dihydropyridine and three 4-aryl-1,2,3,4-tetrahydropyrimidin-2-one derivatives have been synthesized and examined for their activity against pathogenic strains of *Aspergillus fumigatus* and *Candida albicans*. Although none of the three compounds belonging to pyrimidin-2-one series showed any activity against two pathogens, two of the compounds of the dihydropyridine series, that is, diethyl 4-(4-methoxyphenyl)-2,6-dimethyl-1,4-dihydropyridin-3,5-dicarboxylate and dimethyl 4-(4-methoxyphenyl)-2,6-dimethyl-1,4-dihydropyridin-3,5-dicarboxylate, exhibited significant activity against *A. fumigatus* in disc diffusion, microbroth dilution and percent spore germination inhibition assays. The most active diethyl dihydropyridine derivative exhibited a MIC value of 2.92 μg/disc in disc diffusion and 15.62 μg/ml in microbroth dilution assays. The MIC_{90} value of the most active compound by percent germination inhibition assay was found to be 15.62 μg/ml. The diethyl dicarboxylate derivative of dihydropyridine also exhibited appreciable activity against *C. albicans*. The in vitro toxicity of the most active diethyl dihydropyridine derivative was evaluated using haemolytic assay, in which the compound was found to be non-toxic to human erythrocytes even at a concentration of 625 μg/ml. The standard drug amphotericin B exhibited 100% lysis of erythrocytes at a concentration almost 16 times less than the safer concentration of the most active dihydropyridine derivative.

1. Introduction

Fungal infections are reported to cause lots of morbidity and mortality despite recent advances in antifungal chemotherapeutic regimen.[1] Immunocompromised individuals and patients with malignancies remain at higher risk of developing fatal mycosis, predominantly aspergillosis and candidiasis. *Aspergillus* and *Candida* species are the most frequent isolates from blood cultures in hospitals with large populations of immunocompromised patients.[2,3] Infections by these pathogenic fungal species are being recognized as emerging threat to public health.[4,5] It is, therefore, imperative to diagnose the mycotic infections at an early stage and treat them effectively. The drugs derived from natural sources or prepared synthetically are available for treating the fungal infections; however, none of these drugs is ideal.[6]

Amphotericin B, which is considered to be the drug of choice at present and remains effective therapeutic agent in severe conditions of invasive mycosis, has been found

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to be highly toxic and immunosuppressive.[7,8] The non-availability of an absorbable oral form of amphotericin B for long maintenance therapy in immunocompromised patients is another important drawback of this drug. Several synthetic drugs, such as ketoconazole, itraconazole and fluconazole, have been found to be very effective in treating the fungal diseases. Variconazole is a recent addition to the list of synthetic antifungals. However, all these triazole drugs also induce serious side effects in human beings.[9–11] Further, the development of resistance in fungi to most available drugs has been reported.[12–14] Therefore, there is an urgent need to identify new lead molecules from other classes of compounds to develop antifungal drugs with high therapeutic index.

Dihydropyridine derivatives, such as nifedipine, nitrendipine and nimodipine, have been found to be commercially useful molecules as calcium channel blockers.[15–17] A number of dihydropyridine calcium antagonists have been introduced as potential drugs for the treatment of congestive heart failure.[18,19] Further, cerebrocrast, a dihydropyridine derivative, has been introduced as a neuroprotective agent.[20] Together with calcium channel blocker and neuroprotective activity, a number of dihydropyridine derivatives have been found as vasodilators, antihypertensive, bronchodilators, antiatherosclerotic, hepatoprotective, antitumour, antimutagenic, geroprotective, antidiabetic and antiplatelet aggregation agents.[21–25] However, the potential of dihydropyridines as antifungal agents has not been studied. Therefore, the current study was undertaken to synthesize different dihydropyridine derivatives and investigate their antifungal potential using *Aspergilli* and *Candida* as model pathogens. Further, the interest to synthesize tetrahydropyrimidin-2-ones for their antifungal studies arises from their structural similarities to 1,4-dihydropyridines[26] and also because of interesting biological properties of several marine alkaloids[27–29] based upon dihydropyrimidine system, viz crambine, batzelladine (potent HIV gp-120-CD4 inhibitor), and ptilomycalin A. The derivatization of dihydropyrimidinones, specially at C-4, has led to the recognition of several lead compounds that show a very similar pharmacological profile to that of 1,4-dihydropyridine based drugs.[30–33]

2. Results and discussion

2.1. Synthesis of 4-aryl-1,4-dihydropyridine derivatives 2a–2i, 3a and 3b

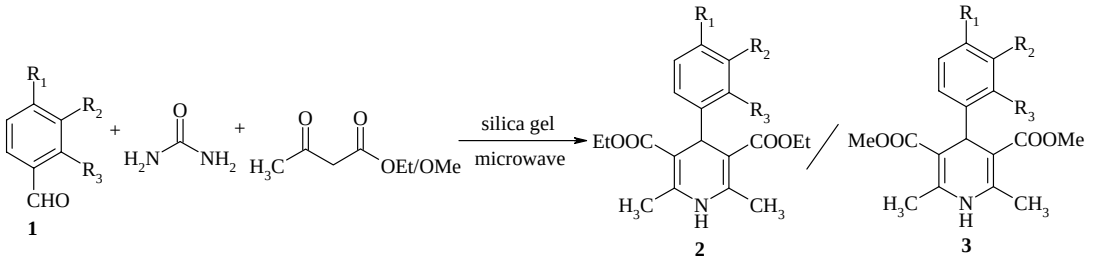
Diethyl 4-aryl-2,6-dimethyl-1,4-dihydropyridin-3,5-dicarboxylates **2a**–**2g** and dimethyl 4-aryl-2,6-dimethyl-1,4-dihydropyridin-3,5-dicarboxylates **3a** and **3b** were prepared by Biginelli cyclocondensation of ethyl acetoacetate/methyl acetoacetate, urea, and corresponding aromatic aldehydes **1a**–**1g** under microwave condition following the procedure of Yadav et al.[25] in 65–80% yield (Scheme 1). Epoxymethylation of one of the 4-aryl-1,4-dihydropyridines, that is, diethyl 2,6-dimethyl-4-(3-hydroxyphenyl)-1,4-dihydropyridin-3,5-dicarboxylate (**2d**), was carried out by refluxing the compound with epichlorohydrin in dry ethanol in the presence of fused potassium carbonate to afford diethyl 2,6-dimethyl-4-(3-epoxymethoxyphenyl)-1,4-dihydropyridin-3,5-dicarboxylate (**2h**) in 78% yield (Scheme 2). Treatment of compound **2h** with dilute hydrochloric acid in methanol led to the formation of diethyl 4-[3-(3-chloro-2-hydroxypropanoxy)phenyl]-1,4-dihydropyridin-3,5-dicarboxylate (**2i**) in 72% yield (Scheme 2). The structures of 4-aryl-1,4-dihydropyridine derivatives **2a**–**2i**, **3a** and **3b** were unambiguously established from the analysis of their spectral data (IR, ^{1}H-, ^{13}C NMR and mass). The structures of known compounds **2a**–**2g**, **3a** and **3b** were further confirmed by comparison of their melting points and/or spectral data with those reported in the literature.[34–39]

2.2. Synthesis of 4-aryl-1,2,3,4-tetrahydropyrimidin-2-one derivatives 5–7

Ethyl 4-(4-hydroxyphenyl)-6-methyl-1,2,3,4-tetrahydropyrimidin-2-one-5-carboxylate (**5**) was synthesized by Biginelli cyclocondensation reaction of ethyl acetoacetate, urea and 4-hydroxybenzaldehyde (**4**) in the presence of ferric chloride under microwave conditions in 85% yield following the modified literature procedure.[40] The compound **5** was acetylated to afford ethyl 4-(4-acetoxyphenyl)-6-methyl-1,2,3,4-tetrahydropyrimidin-2-one-5-carboxylate (**6**) in quantitative yield using acetic anhydride-pyridine and a catalytic amount of dimethylaminopyridine (DMAP) (Scheme 3). Further, epoxymethylation of tetrahydropyrimidin-2-one **5** was carried out by refluxing the compound with epichlorohydrin in dry ethanol in the presence of fused potassium carbonate to afford ethyl 4-(4-epoxymethoxy)phenyl-6-methyl-1,2,3,4-tetrahydropyrimidin-2-one-5-carboxylate (**7**) in 77% yield (Scheme 3). All the three hydroxyphenyl-, acetoxyphenyl- and epoxymethoxyphenyl-1,2,3,4-tetrahydropyrimidin-2-ones **5**–**7** were unambiguously characterized on the basis of their spectral data (IR, ^{1}H, ^{13}C NMR and mass). The structure of known tetrahydropyrimidine **5** was further confirmed by comparison of its melting point and spectral data with those reported in the literature.[40]

3. Antifungal activity

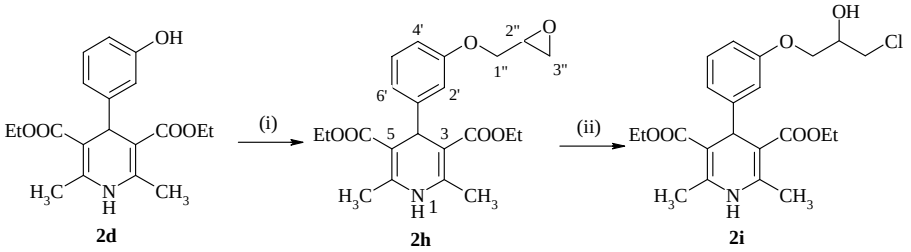
Ten 4-aryl-1,4-dihydropyridines **2a**–**2g**, **2i**, **3a** and **3b** and three 4-aryl-1,2,3,4-tetrahydropyrimidin-2-ones **5**–**7** have been examined for activity against pathogenic strains of *Aspergillus fumigatus* and *Candida albicans*. The anti-*Aspergillus* activity of all the 13 compounds has been evaluated by the disc diffusion (DDA), microbroth dilution (MDA) and percentage spore germination inhibition (PSGIA) assays;[41] the results are given in Table 1 (results of PSGIA are also depicted in Fig. 1). The anti-*Candida* activity of all the 13 compounds **2a**–**2g**, **2i**, **3a**, **3b**, and **5**–**7** has been evaluated by counting colony-forming units[42] (Fig. 2, inactive compounds have not been displayed in the figure). The anti-*Candida* activity of most active compound **2e**, identified on the basis of counting colony-forming units assay, was further evaluated by microbroth dilution assay (Fig. 3).



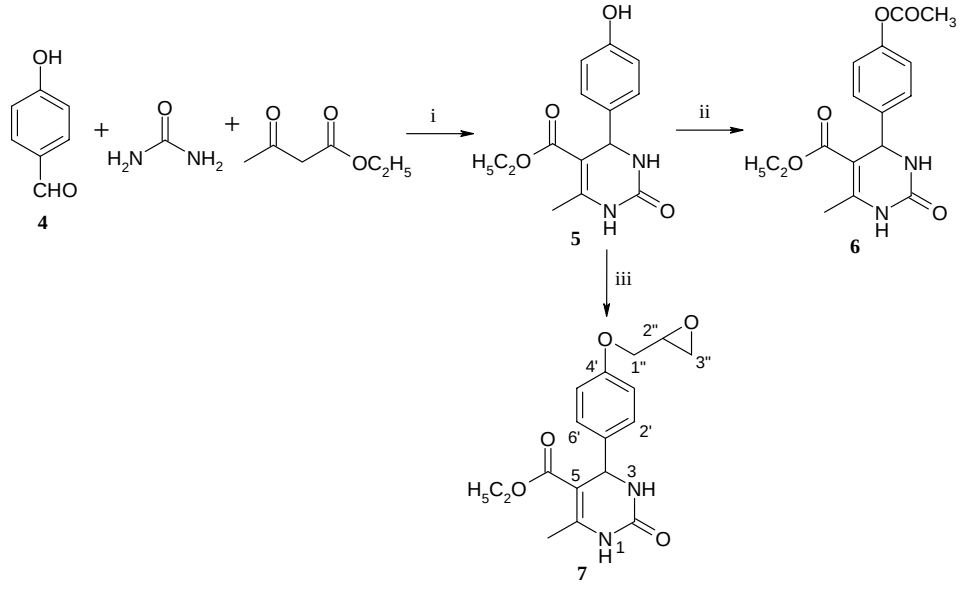
1 & 2	a	b	c	d	e	f	g
R_1	H	H	Br	H	OMe	OMe	OH
R_2	Cl	H	H	OH	H	OH	OMe
R_3	H	Br	H	H	H	H	H

3	a	b
R_1	OMe	H
R_2	H	OH
R_3	H	H

Scheme 1.



Scheme 2. Reagents and conditions: (i) epichlorohydrin, dry EtOH, fused K_2CO_3, reflux; (ii) MeOH, dilute HCl, stirring at 25–28 °C.



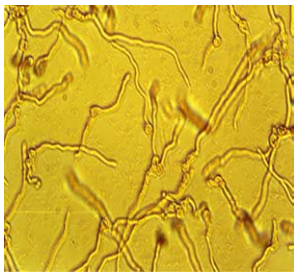
Scheme 3. Reagents and conditions: (i) microwave 1–2 min, $FeCl_3$, SiO_2; (ii) acetic anhydride, DMAP, 60 min, 25–30 °C; (iii) epichlorohydrin, dry EtOH, fused K_2CO_3, reflux.

3.0.1. Anti-*Aspergillus* activity. The results of anti-*Aspergillus* activity evaluation revealed that one of the dihydropyridines, that is, diethyl 4-(4-methoxyphenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (**2e**), is a potential inhibitor of the growth of *A. fumigatus*. The compound **2e** exhibited appreciable activity even at a concentration of 2.92 μg/disc in disc diffusion and 15.62 μg/ml in microbroth dilution assays (Table 1). The MIC_{90} value of the most active compound **2e** by percentage spore germination inhibition assay was found to be 15.62 μg/ml (Table 1 and Fig. 1). The next most active compound among dihydropyridines is dimethyl 4-(4-methoxyphenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (**3a**), which exhibited

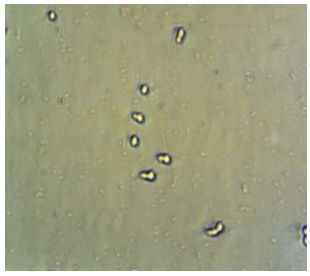
Table 1. Activity of dihydropyridine and tetrahydropyrimidin-2-one derivatives against *Aspergillus fumigatus* by disc diffusion (DDA), microbroth dilution (MDA) and percentage spore germination inhibition (PSGIA) assays

S. No.	Compound	DDA MIC (μg/disc)	MDA MIC (μg/ml)	PSGI MIC_{90} (μg/ml)
1.	Diethyl 4-(3-chlorophenyl)-2,6-dimethyl-1,4-dihydropyridin-3,5-dicarboxylate (**2a**)	—	—	—
2.	Diethyl 4-(2-bromophenyl)-2,6-dimethyl-1,4-dihydropyridin-3,5-dicarboxylate (**2b**)	—	—	—
3.	Diethyl 4-(4-bromophenyl)-2,6-dimethyl-1,4-dihydropyridin-3,5-dicarboxylate (**2c**)	187.5	500.0	500.0
4.	Diethyl 4-(3-hydroxyphenyl)-2,6-dimethyl-1,4-dihydropyridin-3,5-dicarboxylate (**2d**)	—	—	—
5.	Diethyl 4-(4-methoxyphenyl)-2,6-dimethyl-1,4-dihydropyridin-3,5-dicarboxylate (**2e**)	2.92	15.62	15.62
6.	Diethyl 4-(3-hydroxy-4-methoxyphenyl)-2,6-dimethyl-1,4-dihydropyridin-3,5- dicarboxylate (**2f**)	187.5	500.0	500.0
7.	Diethyl 4-(4-hydroxy-3-methoxyphenyl)-2,6-dimethyl-1,4-dihydropyridin-3,5- dicarboxylate (**2g**)	187.5	500.0	500.0
8.	Diethyl 4-[3-(3-chloro-2-hydroxypropoxy)phe-nyl]-2,6-dimethyl-1,4-dihydropyridin- 3,5-dicarboxylate (**2i**)	187.5	500.0	500.0
9.	Dimethyl 4-(4-methoxyphenyl)-2,6-dimethyl-1,4-dihydropyridin-3,5-dicarboxylate (**3a**)	11.68	31.25	31.25
10.	Dimethyl 4-(3-hydroxyphenyl)-2,6-dimethyl-1,4-dihydropyridin-3,5-dicarboxylate (**3b**)	187.5	500.0	500.0
11.	Ethyl 4-(4-hydroxyphenyl)-6-methyl-1,2,3,4-tetrahydropyrimidin-2-one-5- carboxylate (**5**)	—	—	—
12.	Ethyl 4-(4-acetoxyphenyl)-6-methyl-1,2,3,4-tetrahydropyrimidin-2-one-5- carboxylate (**6**)	—	—	—
13.	Ethyl 4-(4-epoxymethoxyphenyl)-6-methyl-1,2,3,4-tetrahydropyrimidin-2-one-5- carboxylate (**7**)	—	—	—
14[a]	Amphotericin B	0.73	1.95	1.95

The '—' in the table means no activity up to tested concentration, that is, 750 μg/disc by DDA and 1000 μg/ml by MDA & PSGIA.
[a] Amphotericin B has been used as standard drug.



Normal germinating Spores



Spores treated with 15.62 μg/ml of compound **2e**

Figure 1. Inhibition of spore germination by compound **2e**.

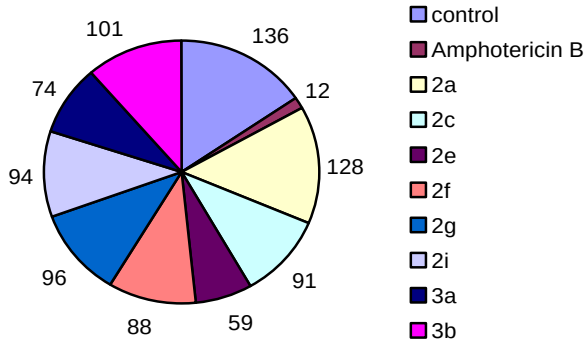


Figure 2. Inhibition of colonies of *Candida albicans* by different 1,4-dihydropyridines at a concentration of 31.25 μg/ml (inactive compounds **2b**, **2d** and **5**–**7** are not displayed in the figure).

activity at 11.68 μg/disc in DDA and 31.25 μg/ml, both in MDA and PSGIA. The only difference in the structure of compounds **2e** and **3a** is the presence of carbomethoxy group at C-3 and C-5 positions in compound **3a** instead of carboethoxy group at the same positions in compound **2e**; both these compounds have 4-methoxyphenyl substituents at C-4 position of dihydropyridine ring. The dihydropyridine derivatives, that is, **2c**, **2f**, **2g** and **2i** having carboethoxy groups at C-3 and C-5 positions, do not show any appreciable activity and the other three compounds of this series, **2a**, **2b** and **2d**, are completely inactive. In all these dihydropyridine derivatives having carboethoxy group at C-3 and C-5 positions, the C-4 aryl group is different than the 4-methoxyphenyl groups in active compounds **2e** and **3a**. This indicated that 4-methoxyphenyl group is one of the essential features for a dihydropyridine to exhibit inhibition of growth of *A. fumigatus*. Both compounds **2e** and **3a** have 4-methoxyphenyl substituent at C-4 position, but the activity of latter compound is less than the activity of the former indicating that C-3 and C-5-carboalkoxy groups also affect the antifungal activity of dihydropyridines, and carboethoxy groups at these positions are better than the carbomethoxy groups. It is interesting to note that compound **2f** is much less active than compound **2e**, although it has methoxy group at C-4 position in the phenyl ring of the compound together with carboethoxy groups at C-3 and C-5-positions. This may be because of the presence of a hydroxyl group at C-3 position in the C-4 phenyl moiety in compound **2f**. This again indicates the importance of substit-

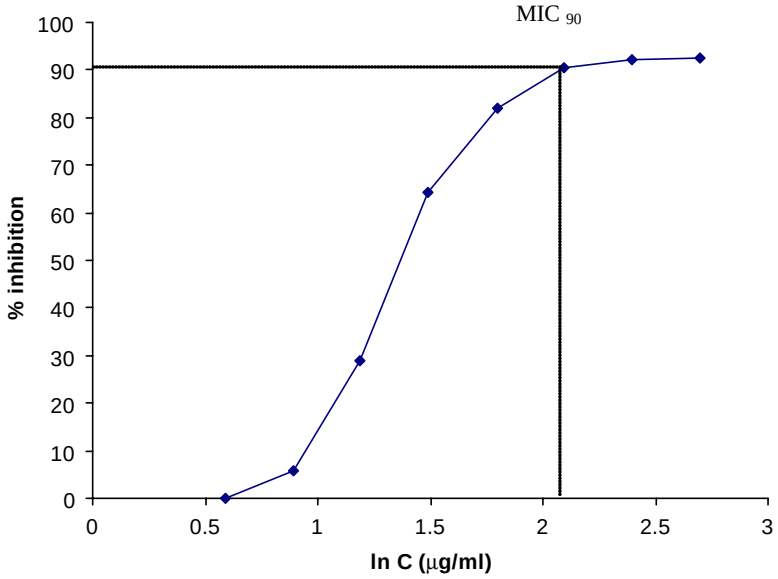


Figure 3. MIC_{90} of compound **2e** against *Candida albicans*.

uents present on the C-4 phenyl group of the dihydropyridines. On the basis of structure–activity relationship drawn above compound **3b** should not exhibit appreciable inhibition of *A. fumigatus* growth, which is also revealed by experiment. Finally to examine the importance of dihydropyridine ring, we investigated the activity of three tetrahydropyrimidin-2-one derivatives **5**–**7**, which have similar structures as the dihydropyrimidines under study; none of the compounds **5**–**7** showed inhibition of *A. fumigatus*.

3.0.2. Anti-*C. albicans* activity. The preliminary anti-*Candida* activity of ten 4-aryl-1,4-dihydropyridines **2a**–**2g**, **2i**, **3a** and **3b** and three 4-aryl-1,2,3,4-tetrahydropyridin-2-ones **5**–**7** have been evaluated by counting colony-forming units[42] (CFU/ml). The control experiment exhibited the growth of 136 colonies of *C. albicans*, out of which 124 colonies were inhibited by standard drug amphotericin B at a concentration of 31.25 μg/ml, that is, amphotericin B exhibited 91.2% inhibition (Fig. 2). As in the case of antifungal activity against *A. fumigatus*, compound **2e** exhibited maximum activity against *C. albicans* by inhibiting 77 colonies (56.6% inhibition) out of 136 colonies followed by compound **3a** (45.6%) at a concentration of 31.25 μg/ml (Fig. 2). This may again be due to the similar structures of compounds **2e** and **3a**, which differ only in the substitution at C-3 and C-5 positions of dihydropyridine ring. The anticandidal activity of compounds **2c**, **2f**, **2g** and **2i** is low and of the same order, that is, 33.1, 35.3, 29.4 and 30.9%, respectively (Fig. 2). Compounds **2a** and **3b** did not show any appreciable activity. The other two 4-aryl-1,4-dihydropyridine derivatives **2b** and **2d**, and three 4-aryl-1,2,3,4-tetrahydropyrimidin-2-ones **5**–**7** evaluated for their anti-*Candida* potential under this study were found inactive. These results again revealed that carboethoxy substituent at C-3 and C-5 positions and 4-methoxyphenyl substituent at C-4 position of phenyl ring in 4-aryl-1,4-dihydropyridine make the compound a better anticandidal agent. Among the two 4-methoxyphenyl-1,4-dihydropyridines **2e** and **3a**, one with carboethoxy substituent at C-3 and C-5 positions exhibited better therapeutic index than the other with carbomethoxy group at same positions. The anti-*Candida* activity of compound **2e** was further evaluated by microbroth dilution assay (Fig. 3). It was observed that there was more than 90% inhibition of growth of *C. albicans* cells in wells treated with 125.0 μg/ml of compound **2e** (Fig. 3). Thus, anti-*Candida* activity testing of 4-aryl-1,4-dihydropyridines and 4-aryl-1,2,3,4-tetrahydropyrimidin-2-ones by counting colony-forming units showed that compound **2e** is a potential anticandidal agent, which is further confirmed by 90% inhibition of growth of *C. albicans* cells by the compound in microbroth dilution assay.[43]

3.1. Cytotoxicity study on diethyl 4-(4-methoxyphenyl)-2,6-dimethyl-1,4-dihydropyridin-3,5-dicarboxylate (2e)

The in vitro cell cytotoxicity of 4-aryl-1,4-dihydropyridine **2e** was investigated using haemolytic assay[44] (Fig. 4). In a dose-dependent study, compound **2e** was

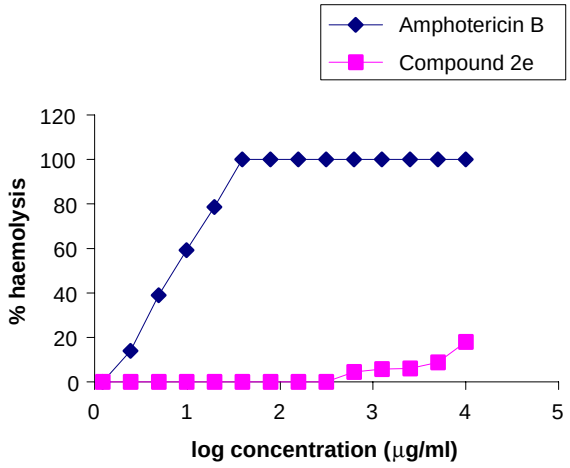


Figure 4. Cytotoxicity of compound **2e** against erythrocytes using haemolytic assay.

found to be non-toxic up to the concentration of 625.0 μg/ml and it lysed only 17.95% of human erythrocytes at the highest dose tested, that is, at a concentration of 10,000 μg/ml (Fig. 4). The standard drug amphotericin B lysed 100% erythrocytes at a concentration of 39.05 μg/ml. This result is further confirmed by the study of Cybulska et al.[45], who have reported that 1.70 μg/ml of amphotericin B caused 50% haemoglobin loss from erythrocytes.

4. Conclusion

Eleven 4-aryl-1,4-dihydropyridines, including the intermediate epoxide **2h** and three 4-aryl-1,2,3,4-tetrahydropyrimidine derivatives, have been synthesized out of which four compounds are novel and have not been synthesized earlier. The antifungal activity evaluation studies on 4-aryl-1,4-dihydropyridines **2a**–**2g**, **2i**, **3a** and **3b**, and 4-aryl-1,2,3,4-tetrahydropyrimidin-2-ones **5**–**7** have revealed that diethyl 4-(4-methoxyphenyl)-2,6-dimethyl-1,4-dihydropyridin-3,5-dicarboxylate **2e** is a potent antifungal agent. Although the potency of compound **2e** is less than one of the most active antifungal compounds amphotericin B, the toxicity of this active dihydropyridine derivative is about 16 times less than the toxicity of amphotericin B. This has revealed that the active dihydropyridine derivative is a much safer drug candidate and can be taken up for the development of a suitable antifungal drug through generation of a library of analogues of **2e** and for further studies.

5. Experimental

5.1. General

Melting points were determined on a sulfuric acid bath and are uncorrected. The IR spectra were recorded either on a Perkin-Elmer model 2000 FT-IR or RXI FT-IR spectrophotometer. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 300 spectrometer at 300 and 75.5 MHz, respectively, using TMS as internal standard. The chemical shift values are on δ scale and the coupling constant values (J) are in Hz. The HRMS were recorded on a JMS-AX 505W instrument at 70 eV in FAB (positive or negative ion mode) using bis-hydroxyethyldisulphide (HEDS) doped with sodium ions and 3-nitrobenzyl alcohol (NBA) as matrix. Microwave reactions were performed in a domestic microwave oven of 850 W and 1.2 Cft (33L, Infodisplay, Sharp Carosel). Analytical TLCs were performed on pre-coated Merck silica gel $60F_{254}$ plates; the spots were detected either by viewing under UV light or by charring with 10% alcoholic H_2SO_4.

5.2. Materials

Sabouraud's dextrose agar and Sabouraud's dextrose broth were purchased from Hi Media, Mumbai, India. Amphotericin B and DMSO were purchased from Sigma Chemical Company, USA.

5.3. Pathogens

Pathogenic strain of *A. fumigatus* was obtained from Microbiology Department of Vallabhbhai Patel Chest Institute, Delhi, India, and *C. albicans* was obtained from IARI, New Delhi, India. *A. fumigatus* and *C. albicans* were grown on Sabouraud's dextrose agar at 37 °C.

5.4. General method of synthesis of 4-aryl-1,4-dihydropyridine derivatives 2a–2g, 3a and 3b

A mixture of aromatic aldehyde (**1a**–**1g**, 4 mmol), ethyl acetoacetate/methyl acetoacetate (8 mmol) and urea (4 mmol) was thoroughly mixed with silica gel (100–200 mesh, 2 g). The mixture was taken in a beaker, placed in an alumina bath and subjected to microwave irradiation for 1.5–2 min at 850 W (Scheme 1).[25] After completion of the reaction as indicated by TLC, the reaction-mass was directly loaded on a silica gel column, and compounds **2a**–**2g**, **3a** and **3b** were eluted with 4-7% acetone in chloroform as colourless/pale yellow solids in 65–80% yields. The structures of **2a**–**2g**, **3a** and **3b** were unambiguously established on the basis of their spectral analysis (IR, ^{1}H-, ^{13}C and mass) and comparison of their melting points and/or spectral data with those reported in the literature.[34–39]

5.5. Synthesis of diethyl 4-[3-(3-chloro-2-hydroxypropoxy)phenyl]-2,6,dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (2i)

The title compound **2i** was synthesized in two steps starting from the refluxing of a mixture of compound **2d** (2 mmol) and epichlorohydrin (2.2 mmol) in dry ethanol (20 ml) in the presence of fused potassium carbonate (1 g) (Scheme 2). On completion of the reaction, potassium carbonate was filtered off and solvent was removed under reduced pressure. The crude product, thus obtained, was purified over silica gel column using petroleum ether–ethyl acetate as mobile phase to afford pure **2h** as a colourless solid (650 mg) in 78% yield. Mp. 105 °C; IR (KBr): 3336 (NH), 1693 (CO), 1489, 1300, 1271, 1202 and 1097 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.22 (6H, t, $J = 7.1$ Hz, 2× $COOCH_2CH_3$), 2.31 (6H, s, C-2 and C-6 CH_3), 2.72 (1H, dd, $J = 2.6$ and 4.8 Hz, C-3″H_α) and 2.90 (1H, t, $J = 4.6$ Hz, C-3″H_β), 3.33 (1H, m, C-2″H), 3.90 (1H, dd, $J = 5.6$ and 10.9 Hz, C-1″H_α), 4.07 (4 H, m, 2× $COOCH_2CH_3$), 4.15 (1H, dd, $J = 3.8$ and 11.0 Hz, C-1″H_β), 4.98 (1H, s, C-4H), 5.98 (1H, br s, NH), 6.66 (1H, dd, $J = 1.9$ and 7.9 Hz, C-6′H), 6.85 (1H, br s, C-2′H), 6.90 (1H, d, $J = 7.6$ Hz, C-4′H) and 7.11 (1H, t, $J = 7.7$ Hz, C-5′H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 14.68 (2× $COOCH_2CH_3$), 19.96 (2× CH_3), 39.95 (C-3″), 45.28 (C-2″), 50.61 (C-4), 60.12 (2× $COOCH_2CH_3$), 68.99 (C-1″), 104.30 (C-2 and C-6), 111.99, 115.28, 121.55, 129.04 (C-2′, C-4′, C-5′ and C-6′), 144.37 (C-3 and C-5), 149.85 (C-1′), 158.67 (C-3′) and 167.98 (2× CO); HRMS: calculated for $C_{22}H_{27}NO_6$ $[M]^+$ 401.1736, observed $[M]^+$ 401.1750. The epoxide **2h** (1.2 mmol) was dissolved in methanolic–HCl (15 ml) and stirred at 25–28 °C until complete conversion of the starting material into a slow moving product on TLC was seen. The

solvent of the reaction was removed, ice-cold water (25 ml) was added and the product was extracted with chloroform (2 × 25 ml). Combined organic layer was dried over Na_2SO_4, solvent was removed and the crude product was purified over silica gel column using ethyl acetate petroleum ether as eluent to afford compound **2i** as a colourless solid (378 mg) in 72% yield. Mp. 141–142 °C; IR (KBr): 3400 (OH), 2979, 2366, 1669 (CO), 1492, 1373, 1215, 1125, 1047 and 747 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 1.25 (6H, t, J = 7.1 Hz, 2× $COOCH_2CH_3$), 2.31 (6H, s, C-2 and C-6 CH_3), 2.60 (1H, br s, C-2″ OH), 3.71 (2H, m, C-3″H), 4.10 (7H, m, 2× $COOCH_2CH_3$, C-1″H and C-2″H), 4.98 (1H, s, C-4H), 5.84 (1H, br s, NH), 6.67 (1H, dd, J = 1.9 and 7.9 Hz, C-6′H), 6.84 (1H, br s, C-2′H), 6.90 (1H, d, J = 7.6 Hz, C-4′H) and 7.11 (1H, t, J = 7.9 Hz, C-5′H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 14.66 (2× $COOCH_2CH_3$), 19.81 (2× CH_3), 40.02 (C-3″), 46.43 (C-4), 60.19 (2× $COOCH_2CH_3$), 68.86 and 70.34 (C-1″ and C-2″), 104.14 (C-2 and C-6), 112.16, 115.12, 121.64, 129.16 (C-2′, C-4′, C-5′ and C-6′), 144.64 (C-3 and C-5), 149.97 (C-1′), 158.45 (C-3′) and 168.16 (2× CO); HRMS: calculated for $C_{22}H_{28}NO_6Cl$ $[M]^+$ 437.1509, observed $[M]^+$ 437.1503.

5.6. Synthesis of ethyl 4-(4-acetoxyphenyl)-6-methyl-1,2,3,4-tetrahydropyrimidin-2-one-5- carboxylate (6)

A mixture of 4-hydroxybenzaldehyde (**4**, 5 mmol), ethyl acetoacetate (5.5 mmol), urea (15 mmol) and ferric chloride hexahydrate (5 mmol) was thoroughly mixed with silica gel (100–200 mesh, 2 g). The mixture was taken in a beaker, which was placed in an alumina bath and subjected to microwave irradiation of four shots of 15 s each at an interval of 1 min at 850 W (Scheme 3).[40] After completion of the reaction as indicated by TLC, the reaction-mass was directly loaded on a silica gel column and eluted with 1% methanol in chloroform to afford compound **5** as a yellow solid. For further purification, compound **5** was dissolved in methanol (50 ml), the solution passed through a pad of Celite (5 g) and the solvent was removed to afford **5** as a colourless solid (1.17 g, mp 140–141 °C; literature[40] mp 141–142 °C) in 85% yield. The structure of compound **5** was unambiguously established on the basis of its spectral analysis (IR, UV, ^1H-, ^{13}C and mass) and comparison of the data with those reported in the literature.[40] To a solution of compound **5** (1 mmol) in acetic anhydride (1.1 equiv) and pyridine (2 equiv) was added a catalytic amount of 4-*N*,*N*-dimethylaminopyridine and the reaction mixture was stirred at 25–28 °C for an hour when TLC examination showed complete conversion of the starting material compound into a product of higher R_f value. Ice-cold water (30 ml) was added to the reaction under vigorous stirring, and solid separated was filtered off and washed with water and petroleum ether to afford **6** as a white solid (280 mg) in 88% yield. Mp. 141–142 °C; IR (KBr): 3249, 3117, 2981, 1723 (CO of ethyl ester), 1704 (CO of acetate), 1651 (CO of amide), 1508, 1225, 1094, 785 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 1.17 (3H, t, J = 6.0 Hz, $COOCH_2CH_3$), 2.28 and 2.33(6H, 2s, 3H each, $OCOCH_3$ and C-6CH_3), 4.08 (2H, q, J = 6.0 Hz, $COOCH_2CH_3$), 5.40 (1H, d, J = 1.9 Hz, C-4H), 5.85 (1H, br s, N-1H), 7.02 (2H, d, J = 8.5 Hz, C-3′H and C-5′H), 7.32 (2H, d, J = 8.5 Hz, C-2′H and C-6′H) and 8.19 (1H, s, N-3H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 13.86 ($COOCH_2CH_3$), 18.39 and 20.79 ($OCOCH_3$ and C-6CH_3), 54.86 (C-4), 59.79 ($COOCH_2CH_3$), 101.03 (C-5), 121.46 (C-3′ and C-5′), 127.44 (C-2′ and C-6′), 140.99 (C-1′), 146.10 (C-6), 149.97 (C-4′), 153.01 (C-2), 165.24 ($COOCH_2CH_3$) and 169.01($OCOCH_3$); HRMS: calculated for $C_{16}H_{18}N_2O_5$ $[M]^+$ 318.1216, observed $[M]^+$ 318.1201.

5.7. Synthesis of ethyl 4-(4-epoxymethoxyphenyl)-6-methyl-1,2,3,4-tetrahydropyrimidin-2-one-5- carboxylate (7)

A mixture of compound **5** (2 mmol) and epichlorohydrin (2.2 mmol) in dry ethanol (20 ml) was refluxed in the presence of fused potassium carbonate (2 mmol). On completion of the reaction, potassium carbonate was filtered off, solvent was removed under reduced pressure and the crude product, thus obtained, was purified over silica gel column using $CHCl_3$ as mobile phase to afford pure **7** as a colourless solid (511 mg) in 77% yield. Mp. 196–198 °C; IR (KBr): 3246 (NH), 3119, 2927, 1721 (CO), 1648 (CO), 1513, 1457, 1235, 1099, 1033, 765 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 1.16 (3H, t, J = 6.0 Hz, $COOCH_2CH_3$), 2.34 (3H, s, C-6CH_3), 2.74 (1H, br s, C-3″H_α), 2.89 (1H, t, J = 6.03 Hz, C-3″H_β), 3.33 (1H, br s, C-2″H), 3.94 (1H, dd, J = 5.4 and 10.8 Hz, C-1″H_α), 4.07 (2H, q, J = 6.0 Hz, $COOCH_2CH_3$), 4.20 (1H, dd, J = 1.5 and 10.8 Hz, C-1″H_β), 5.35 (1H, br s, C-4H), 5.41 (1H, br s, N-1H), 6.85 (2H, d, J = 6.8 Hz, C-3′H and C-5′H), 7.24 (2H, d, J = 6.8 Hz, C-2′H and C-6′H) and 7.28 (1H, br s, N-3H); ^{13}C NMR (75.5 MHz, $CDCl_3$ + few drops of DMSO-d_6): δ 14.15 ($COOCH_2CH_3$), 18.17 (C-6CH_3), 44.17 (C-3″), 49.96 (C-2″), 54.25 (C-4), 59.39 ($COOCH_2CH_3$), 68.82 (C-1″), 100.36 (C-5), 114.35 (C-3′ and C-5′), 127.79 (C-2′ and C-6′), 137.71 (C-1′), 147.53 (C-6), 152.85 (C-4′), 157.69 (C-2) and 165.79 ($COOCH_2CH_3$); HRMS: calculated for $C_{17}H_{20}N_2O_5$ $[M]^+$ 332.1372, observed $[M]^+$ 332.1372.

6. Antifungal activity assay

The anti-*A. fumigatus* activity of all the compounds was studied by disc diffusion, microbroth dilution and percentage spore germination inhibition assays.[41] The activity against *C. albicans* was carried out by the method of Riffel et al.[42] and Iijima et al.[43]

7. Anti-*Aspergillus* activity assay

7.1. Disc diffusion

The disc diffusion assay was performed in radiation sterilized petri plates of 10.0 cm diameter (Tarsons) as described.[41] Different concentrations in the range of 750–1.46 μg of the test compounds were impregnated on the sterilized discs (5.0 mm in diameter) of Whatman filter paper No 1. The discs were placed on the surface of

the agar plates already inoculated with *A. fumigatus* spores. The plates were incubated at 37 °C and examined at 24, 48 and 96 h for zone of inhibition, if any, around the discs. The concentration, which developed the zone of inhibition of at least 6.0 mm diameter, was considered as minimum inhibitory concentration (MIC).

7.2. Microbroth dilution

The test was performed in 96-well culture plates (Nunc, Nunclon). Various concentrations of synthetic compounds in the range of 1000–7.81 μg/ml were prepared in the wells by twofold dilution method. Assay was performed as per standard method described earlier.[41]

7.3. Percentage spore germination inhibition

Various concentrations of the test compounds in 90.0 μl of culture medium were prepared in 96-well flat-bottomed micro-culture plates (Nunc, Nunclon) by double dilution method. The wells were prepared in triplicates for each concentration. Each well was then inoculated with 10.0 μl of spore suspension containing 100 ± 5 spores. The plates were incubated at 37 °C for 8 h and then examined for spore germination with an inverted microscope (Nikon, Diphot). The number of germinated and non-germinated spores was counted. The percentage spore germination inhibition was calculated.[41] All the tests were repeated at least three times. The lowest concentration of the compound, which resulted in >90% inhibition of germination of spores in the wells was considered as MIC_{90}.

8. Anti-candidal assays

8.1. Colony forming units

Cells of *C. albicans* were harvested from stock culture suspension and diluted at 10^1 to 10^6 in Sabouraud's dextrose broth. *Candida* cells in 100.0 μl of suspension were treated with 31.25 μg/ml of compounds. A volume of 100.0 μl of compound treated cells was laid on Sabouraud's dextrose agar plates and incubated for 24 h at 37 °C. The colonies developed in the plates were counted macroscopically[42] and results were expressed as CFU/ml.

8.2. Percentage growth inhibition of *C. albicans*

Candida albicans cells in the exponential phase of growth were suspended in Sabouraud's dextrose medium at a density of 1.6×10^3 cells/ml and a volume of 100.0 μl of the suspension was inoculated into each well of 96-well microtitre plate with 100.0 μl of test compound solution. After incubation at 37 °C for 12–16 h, the OD was measured at A_{650} nm of the suspension to assess the inhibition of cell growth due to treatment with compounds.[43]

9. Haemolytic assay

The toxicity of dihydropyridine derivatives having antifungal potential was investigated by using haemolytic assay.[44] A slight modification was employed to determine the haemolytic effect of antifungal dihydropyridine derivative **2e**. Human erythrocytes collected from apparently healthy volunteers were washed thrice with PBS and 2.0% (v/v) suspension of erythrocytes was prepared in phosphate buffer saline at pH 7.2. Half millilitre of (2.0%) human erythrocyte suspension in 16 duplicate sets of tubes was treated with compound **2e** at a concentration of 1.22 μg/ml for 1 h at 37 °C. After incubation, tubes were centrifuged at 5000 rpm for 10 min. The supernatant was collected and the OD was measured at A_{415} nm using a spectrophotometer (UV–Vis Spect Lambda Bio 20 Perkin-Elmer). Results were expressed as % haemolysis by the compound. Only buffer of pH 7.2 was used for background lysis in negative control sets, whereas in positive controls, lysis buffer was used for completely lysing the erythrocytes.

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