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SCIENCE DIRECT
Bioorganic & Medicinal Chemistry
Bioorganic & Medicinal Chemistry 14 (2006) 2747–2752

Synthesis and antifungal activity of substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles

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Received 18 October 2005; revised 25 November 2005; accepted 29 November 2005
Available online 11 January 2006

Abstract—Series of substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles derivatives have been synthesized and examined for their activity against pathogenic strains of *Aspergillus fumigatus* (ITCC 4517), *Aspergillus flavus* (ITCC 5192) *Aspergillus niger* (ITCC 5405) and *Candida albicans* (ITCC No 4718). All synthesized compounds showed mild to moderate activity, except for 2-substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles **6a**–**d**. The most active 1-(4-chlorophenyl)-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indole **4c** exhibited a MIC value of 5.85 μg/disc against *A. fumigatus* and 11.71 μg/disc against *A. flavus* and *A. niger* in disc diffusion assay. Anti-*Aspergillus* activity of active compound **4c** by microbroth dilution assay was found to be 15.62 μg/ml in case of *A. fumigatus* and 31.25 μg/ml with *A. flavus* and *A. niger*. The MIC_{90} value of the most active compound by percent germination inhibition assay was found to be 15.62 μg/ml against *A. fumigatus*. The MIC_{90} values of substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles against *C. albicans* ranged from 15.62 to 250 μg/ml. The in vitro toxicity of the most active 1-(4-chlorophenyl)-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indole **4c** was evaluated using haemolytic assay, in which the compound was found to be non-toxic to human erythrocytes up to a concentration of 312.50 μg/ml. The standard drug amphotericin B exhibited 100% lysis at a concentration of 37.5 μg/ml.

1. Introduction

Invasive fungal infections, particularly in immunosuppressed patients, have continued to increase in incidence during the past 20 years and are now significant causes of morbidity and mortality.[1] This is particularly true in patients with haematological malignancies undergoing induction or consolidation chemotherapy (especially during the nadir of their granulocytopenia), in immunosuppressed organ transplant recipients and in patients with acquired immunodeficiency secondary to infection by human immunodeficiency viruses. These infections also occur in some iatrogenic or nosocomial clinical settings.[2,3] Autopsy data indicate that more than half of the patients who die with malignancies are infected with *Candida* spp., approximately one-third with *Aspergillus* spp., and increasing numbers with *Cryptococcus* spp. or other fungi such as *Fusarium* spp.[2,5]

Major factors which predispose patients to invasive fungal diseases include: prolonged neutropenia (chemotherapy induced); defective T-lymphocyte function (associated with organ transplantation and HIV infection); impaired macrophage function, particularly of pulmonary macrophages (associated with high doses and prolonged administration of corticosteroids); and barrier defects (associated with invasive medical procedures, vascular catheters, parenteral nutrition and haemodialysis and peritoneal dialysis) in compromised patients.[2–4,6–10]

Although invasive fungal diseases are now more frequent than during the first half of the century, they are still difficult to diagnose clinically. During the latter half of the century, particularly during the past two decades, a number of different classes of antifungal agents have been discovered.[2,3,6,10–13] Despite advances in antifungal therapies, many problems remain to be solved for

Keywords: 1,2,3,4-Tetrahydropyrazino[1,2-*a*]indoles; Benzotriazole; Antifungal agent; *Aspergillus*; *Candida*; Haemolytic activity.
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doi:10.1016/j.bmc.2005.11.054

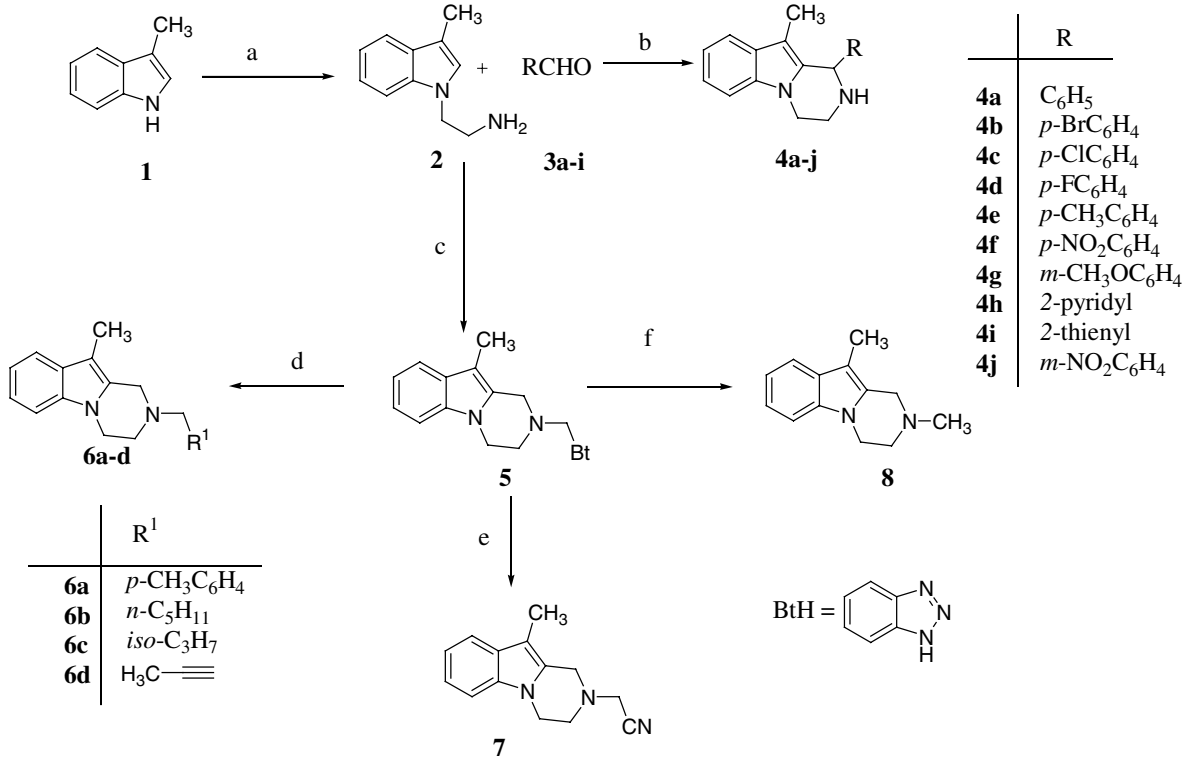
most antifungal drugs available. For example, the use of amphotericin B, known as the 'gold standard', is limited because of its infusion-related reactions and nephrotoxicity.[14,15] The use of azoles, such as fluconazole, ketoconazole and miconazole, has resulted in clinically resistant strains of *Candida* spp.[16,17] A 3.6–7.2% of vaginal isolates of *Candida albicans* from women with candidal vaginitis is resistant to fluconazole.[18] This situation highlights the need for advent of safe, novel and effective antifungal compounds.

Pyrazino[1,2-*a*]indoles have attracted a great deal of attention due to their therapeutic uses as serotonin antagonist,[19] thrombolytic,[20] cardiovascular diseases,[21] antidepressant, anxiolitics,[22] central nervous system depressants,[23] anticonvulsants,[24] antihistaminic,[25] protein kinase C inhibitors[26] and 5-HT_{2A},[27] 5-HT_{2C},[27,28] and selective imidazoline I_2 receptor ligands.[29] One report shows the activity of pyrazinoate towards resistant *Mycobacterium tuberculosis*.[30] Some triazino[5,6-*b*]-indoles are reported to have antifungal properties.[31] However, the potential of pyrazino[1,2-*a*]indoles as antifungal agents has not been studied. Increases in the incidence of fungal infections have prompted a search for new antifungal agents with broad antifungal activities and fungicidal actions, a low likelihood of resistance development and minimal toxicity. Therefore, the current study was undertaken to synthesize different substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indole **4**–**8** derivatives and investigate their antifungal potential using *Aspergilli* and *Candida* species as model pathogens.

2. Results and discussion

2.1. Chemistry

2-(3-Methyl-1*H*-indol-1-yl)ethylamine **2** was obtained by the reaction of 3-methylindole **1** with 2-chloroethylamine hydrochloride in CH_3CN in the presence of NaOH and tetrabutylammonium hydrogen sulfate (TBAHS).[32] Condensation of 2-(3-methyl-1*H*-indol-1-yl)ethylamine **2** with 1 equiv of benzotriazole (BtH) and 2 equiv of formaldehyde (37% aqueous solution) in MeOH/H_2O at 25 °C gave 2-(1*H*-1,2,3-benzotriazol-1-ylmethyl)-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indole **5** in 96% yield as Bt1 (benzotriazol-1-yl) isomers.[32] Bt group attached at an α-carbon to a nitrogen atom is easily replaced by nucleophiles. Treatment of **5** with 2 equiv of sodium borohydride in refluxing THF replaced the benzotriazolyl group with hydrogen to give 2,10-dimethyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indole **8** in 90% yield. The Bt group in **5** can be substituted by a cyano anion to afford 2-[10-methyl-3,4-dihydropyrazino[1,2-*a*]indol-2(1*H*)-yl]acetonitrile **7** in 96% yield. Nucleophilic substitution of **5** with *p*-tolyl, 1-propynl, *n*-pentyl and isopropylmagnesium bromides in dry THF furnished the 2-substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles **6a**–**d** in 90–96% yields. 1-substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles **4a**–**j** were obtained as a racemic mixture in high yields by the reaction of 2-(3-methyl-1*H*-indol-1-yl)ethylamine **2** with benzotriazole and aldehydes **3a**–**j** in the presence of catalytic amount of $AlCl_3$ in CH_2Cl_2 (Scheme 1).[32] The structures of all the synthesized



Scheme 1. Reagents and conditions: (a) $ClCH_2CH_2NH_2HCl$, NaOH, TBAHS, CH_3CN, reflux, 36 h; (b) BtH, DCM, catalytic $AlCl_3$, 25–28 °C, 1–6 h; (c) BtH, HCHO (2 equiv), 25–28 °C stirring; (d) R^1MgX, THF, reflux, 4–12 h; (e) NaCN, DMSO, 25 °C, 36 h; (f) $NaBH_4$, THF, reflux, 12 h.

compound were clearly supported by their spectral analyses.[32] The structures of new compounds are clearly supported by their ^{1}H, ^{13}C NMR spectra and microanalysis. The ^{1}H NMR spectra showed NC*H*-pyrazino singlet signal for **4a–j** at ~5.3 ppm and the presence of exchangeable NH pyrazino was confirmed by deuterium exchange.

2.2. Antifungal activity

Series of substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles **4a–j** and **5–8** have been examined for activity against pathogenic strains of *A. fumigatus*, *A. flavus*, *A. niger* and *C. albicans*. The anti-*Aspergillus* activity of all the synthesized compounds was evaluated by disc diffusion (DDA), microbroth dilution (MDA) and percentage spore germination inhibition (PSGI) assays;[33] the results are given in Table 1. The anti-*Candida* activity of all the synthesized compounds **4a–j** and **5–8** has been investigated by microbroth dilution assay[36] and the results are given in Table 1.

2.2.1. Anti-*Aspergillus* activity. The results of anti-*Aspergillus* activity evaluation revealed that one of the 1,2,3,4-tetrahydropyrazino[1,2-*a*]indole, that is, 1-(4-chlorophenyl)-10-methyl-1,2,3,4-tetrahydropyrazino-[1,2-*a*]indole (**4c**) is a potential inhibitor of the growth of *A. fumigatus*, *A. flavus* and *A. niger*. The compound **4c** exhibited appreciable activity even in the range of 5.85–11.71 μg/disc in disc diffusion and 15.62–31.25 μg/ml in microbroth dilution assays (Table 1). The MIC_{90} value of **4c** by percentage spore germination inhibition assay was found to be in the range of 15.62–31.25 μg/ml (Table 1). Significant activity was observed with compounds **4b** and **d** which exhibited MIC value in the range of 23.43–46.87 μg/disc in DDA and 62.50–125.05 μg/ml by MDA and PSGI assay, respectively. Compound **4h** show good activity against *A. fumigatus* with MIC 93.75 μg/disc and compound **4f** showed moderate activity against *A. flavus* and *A. niger* in the DDA assay. All compounds showed mild to moderate activity against the pathogenic strains used in the study, except for **6a–d**. However, pyrazino[1,2-*a*]indoles have not been investigated. We, for the first time, synthesized substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles for their antimycobial properties. The activity of the compound appeared to be associated with the 1 or 2 substitution on the 1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles. The presence of NH group with substitution on 1-position of the compound **4a–j** showed better activity than compound **5–8** with substituents present on 2-position. The compound **4b–d** showed potential activity due the presence of 4-fluorophenyl, 4-chlorophenyl and 4-bromophenyl at 1-position. Enhanced activity of l-substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles **4a–j** as compared to that of 2-substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles, **5–8**, was noticed due to the presence of hydrogen atom on the pyrazino nitrogen atom which might be important to exhibit inhibitory activity.

2.2.2. Anti-*Candidal* activity. The results of anti-*Candida* activity showed that all the synthesized compounds had variable activity against the pathogenic strains of *C. albicans* but no activity was found in compounds **6a–d**. The most active compound 1-(4-chlorophenyl)-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indole **4c** was a potential inhibitor of the growth of *C. albicans* with a MIC_{90} value of 15.62 μg/disc by microbroth dilution assay. The significant activity was found for compounds **4b** and **d**, which exhibited activity at 62.50 and 125.0 μg/ml, respectively, in microbroth dilution assay. The only difference in the structures of compounds **4b–d** is the presence of a halogen group at C-4 position of the 1-substituted phenyl group. However, 4-chlorophenyl derivative **4c** was found to be the most active compound among all the halogen

Table 1. In vitro antifungal activity against *Aspergilli* and *Candida albicans*

Compound	DDA MIC (μg/disc)			MDA MIC (μg/ml)			PSGI MIC_{90} (μg/ml)			MDA MIC_{90} (μg/ml)
	A. flavus	*A. fumigatus*	*A. niger*	*A. flavus*	*A. fumigatus*	*A. niger*	*A. flavus*	*A. fumigatus*	*A. niger*	*C. albicans*
4a	—	—	—	500.0	500.0	1000.0	500.0	500.0	500.0	125.0
4b	46.87	23.43	46.87	125.0	125.0	62.50	125.0	62.50	62.50	62.50
4c	11.71	5.85	11.71	31.25	15.62	31.25	31.25	15.62	15.62	15.62
4d	46.87	46.87	46.87	125.0	62.50	125.0	125.0	62.50	62.50	125.0
4e	—	—	—	500.0	500.0	1000.0	500.0	500.0	500.0	250.0
4f	187.5	187.5	—	500.0	250.0	500.0	500.0	250.0	250.0	250.0
4g	—	—	—	500.0	500.0	500.0	500.0	500.0	500.0	250.0
4h	187.5	93.75	187.5	500.0	250.0	500.0	250.0	125.0	250.0	125.0
4i	—	—	—	500.0	500.0	500.0	500.0	500.0	500.0	250.0
4j	—	—	—	500.0	500.0	500.0	500.0	250.0	500.0	250.0
5	—	—	—	500.0	500.0	500.0	500.0	500.0	250.0	250.0
6a	—	—	—	—	—	—	—	—	—	—
6b	—	—	—	—	—	—	—	—	—	—
6c	—	—	—	—	—	—	—	—	—	—
6d	—	—	—	—	—	—	—	—	—	—
7	—	187.5	—	500.0	250.0	500.0	500.0	250.0	250.0	250.0
8	—	—	—	500.0	500.0	500.0	500.0	250.0	500.0	—
AmpB	0.73	0.73	0.73	1.95	1.95	1.95	1.95	1.95	1.95	0.97

The '—' means no Activity.

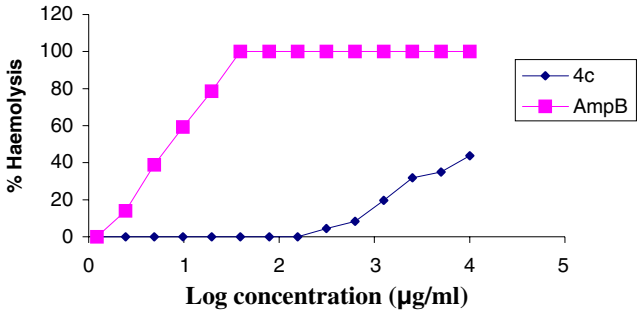


Figure 1. The cytotoxicity of 1-(4-chlorophenyl)-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indole (**4c**) analysed by haemolytic assay.

derivatives **4b–d**. Mild to moderate activity was shown by the 2-substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles **5–8** against the *C. albicans* strain, except for compounds **6a–d**. (10-Methyl-3,4-dihydro-1*H*-pyrazino[1,2-*a*]indol-2-yl)-acetonitrile **7** and 2-benzotriazol-1-ylmethyl-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indole **5** showed moderate activity. Activity data of all the compounds listed in Table 1 revealed that the presence of a hydrogen atom on the pyrazino nitrogen atom showed potent antifungal activity. Presence of alkyl, alkynyl and *p*-tolyl groups in compounds **6a–d** at 2-position of compounds does not cause any significant effect on the antifungal activity.

2.2.3. Cytotoxicity study of 1-(4-chlorophenyl)-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indole 4c. The in vitro cell cytotoxicity of 1-(4-chlorophenyl)-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indole **4c** was investigated using haemolytic assay.[35] In a dose-dependent study, compound **4c** was found to be non-toxic up to a concentration of 312.50 µg/ml and lysed only 4.5% of human erythrocytes (Fig. 1). The standard drug amphotericin B (AmpB) exhibited 100% lysis at a concentration of 37.5 µg/ml. However, it was found to be much less toxic than the standard drug amphotericin B up to the tested concentration, that is, 10,000.0 µg/ml and lysed only 43.75% of human erythrocytes.

3. Conclusion

Series of substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles has been synthesized and the antifungal activity evaluation studies on 1-substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles **4a–j** and 2-substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles **5–8** have revealed that 1-(4-chlorophenyl)-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indole **4c** is a potent antifungal agent. Although the potency of compound **4c** was less than that of the standard antifungal compound amphotericin B, the toxicity of this active pyrazino[1,2-*a*]indole derivative was much less than that of the standard drug amphotericin B. This shows that pyrazino[1,2-*a*]indole derivatives are safe drug candidates and are taken up for the development of safer antifungal drugs through generation of a library of analogues of **4c** and for further studies.

4. Experimental

4.1. General

All reagents used were of AR grade. THF was distilled from sodium/benzophenone prior to use. Melting points were determined with a Thomas Hoover melting point apparatus and are uncorrected. ^{1}H (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Bruker 300 NMR spectrometer in $CDCl_3$ (with TMS for ^{1}H and chloroform-*d* for ^{13}C as internal references) unless otherwise stated. Mass spectrum was recorded on a Hybrid Quadrupole-TOF LC\MS\MS mass spectrometer (Q. Star XL). Infrared spectra (ν_{max}) were recorded on a Perkin Elmer FTIR spectrophotometer as thin films on KBr plates (for oils) or KBr discs (for solids). Column chromatography was performed on silica gel (230–400 mesh). Microanalyses were obtained with an Elemental Analysensysteme GmbH VarioEL V3.00 element analyser. The reactions were monitored by thin-layer chromatography (TLC) using aluminium sheets with silica gel 60 F_{254} (Merck). All the reactions were carried out under nitrogen atmosphere.

4.2. 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.

4.3. Pathogens

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

4.4. General method of synthesis for the 1-substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles 4a–j

To a mixture of 2-(3-methyl-1*H*-indol-1-yl) ethylamine (**2**, 0.5 g, 2.9 mmol), an aldehyde (**3a–j**) (2.9 mmol), benzotriazole (0.342 g, 2.9 mmol), in the presence of catalytic amount of Lewis acid ($AlCl_3$) in dichloromethane (20 ml) was stirred at 25 °C for 0.5–8.0 h (completion of the reaction was monitored by TLC).[32] The reaction mixture was quenched with water and extracted with ethyl acetate (3× 100 ml). The combined extract was washed with 1 N NaOH, brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give solid compounds **4a–j** in 70–90% yields. For microanalysis, compounds were recrystallized from ethyl acetate/hexanes. The structures of **4a–f** and **h**,**i** 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.[32]

4.4.1. 1-(3-Methoxyphenyl)-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indole 4g. Colourless needless; mp 108–111 °C; Found: C, 77.94; H, 6.98; N, 9.75. $C_{19}H_{20}N_2O$ requires C, 78.05; H, 6.89; N, 9.58; ν_{max} (KBr) 3311, 3048, 3041, 2960, 2921, 2870, 753, 677 cm^{-1}; δ_H (300 MHz, $CDCl_3$) 7.52–6.50 (8H, m, Ph), 5.34 (1H, s), 4.08–3.91 (2H, m), 3.85 (3H, s, OCH_3), 3.21–3.12 (2H, m), 2.42 (1H, s, NH); 2.39 (3H, s, CH_3); δ_C (75 MHz, $CDCl_3$) 8.6, 40.2, 42.4, 56.2, 58.5, 106.2, 108.7, 118.5, 120.0, 121.2, 125.2, 129.0, 129.2, 130.2, 132.5, 135.4, 141.2, 143.5, 147.5; LCMS *m/z* 291 (100%, M−1), 293.4 (20%, M+1), 292.2 (8%, M).

4.4.2. 10-Methyl-1-(3-nitrophenyl)-1,2,3,4-tetrahydropyrazino[1,2-*a*]indole 4j. (Yellow crystals from EtOAc/hexanes); mp 121–123 °C; Found: C, 70.31; H, 5.56; N, 13.45. $C_{18}H_{17}N_3O_2$ requires C, 70.34; H, 5.58; N, 13.67; ν_{max} (KBr) 3321, 3050, 3042, 2955, 2922, 2876, 1520, 1360, 750, 680 cm^{-1}. δ_H (300 MHz, $CDCl_3$) 7.61–6.93 (8H, m, Ph); 5.30 (1H, s), 4.18–3.94 (2H, m), 3.25–3.12 (2H, m), 2.65 (3H, s, CH_3), 1.72 (1H, s, NH); δ_C (75 MHz, $CDCl_3$) 8.6, 40.8, 42.5, 56.5, 109.2, 111.5, 118.0, 118.9, 120.0, 127.5, 128.5, 129.5, 132.5, 132.8, 139.5, 137.0, 138.5, 142.5; LCMS *m/z* 307 (80%, M), 308 (20%, M+1), 185.2 (14%, M−122).

4.5. General method for the synthesis of 2-substituted–10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles 5–8

The 2-substituted-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indoles **5–8** were synthesized by the literature procedure[32] and the structures of **5–8** were unambiguously established on the basis of their spectral analysis (IR, ^1H, ^{13}C and mass) and comparison of their melting points and/or spectral data with those reported in the literature.[32]

4.5.1. 2-Hexyl-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indole 6b. Colourless oil; ν_{max} (KBr) 3324, 3053, 3046, 2952, 2910, 2865, 759, 680 cm^{-1}; δ_H (300 MHz, $CDCl_3$) 7.41 (1H, d, $J = 7.2$ Hz), 7.13 (1H, d, $J = 7.4$ Hz), 7.07–6.98 (2H, m), 3.95 (2H, t, $J = 5.3$ Hz), 3.65 (2H, s), 2.83 (2H, t, $J = 5.3$ Hz), 2.48 (2H, t, $J = 7.4$ Hz), 2.12 (3H, s, CH_3), 1.52–1.18 (8H, m), 0.82 (3H, t, $J = 6.6$ Hz, Me); δ_C (75 MHz, $CDCl_3$) 8.0, 14.2, 22.5, 28.0, 31.0, 31.5, 42.0, 46.5, 53.2, 54.5, 105.2, 108.1, 118.2, 119.2, 120.7, 128.9, 131.2, 136.2; TOF-MS ES *m/z*: 271.41 (M+1).

4.5.2. 2-Isobutyl-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indole 6c. Colourless oil; ν_{max} (KBr) 3324, 3059, 3034, 2951, 2920, 2846, 757, 689 cm^{-1}; δ_H (300 MHz, $CDCl_3$) 7.39 (1H, d, $J = 7.1$ Hz), 7.16 (1H, d, $J = 7.6$ Hz), 7.09–7.01 (2H, m), 4.10 (2H, t, $J = 5.3$ Hz), 3.78 (2H, s), 2.92 (2H, t, $J = 6.2$ Hz), 2.52 (2H, d, $J = 7.8$ Hz), 2.14 (3H, s, CH_3), 2.04–2.01 (1H, m), 1.21 (6H, d, $J = 6.9$ Hz); δ_C (75 MHz, $CDCl_3$) 8.0, 22.0 × 2, 30.1, 46.5, 53.2, 54.2, 65.5, 105.6, 109.2, 118.7, 119.5, 122.1, 128.7, 130.9, 136.1; TOF-MS ES *m/z*: 243.4 (M+1).

5. Antifungal activity assay

The anti-*Aspergillus* activity of all the compounds was studied by disc diffusion, microbroth dilution and percentage spore germination inhibition assays.[33] The activity against *C. albicans* was carried out by the methods of Iijima et al.[36] and Chhillar et al.[34]

6. Anti-*Aspergillus* activity assay

6.1. Disc diffusion

The disc diffusion assay was performed in radiation-sterilized petri plates of 10.0 cm diameter (Tarsons) as described.[33] 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). 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.

6.2. 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 the standard method described earlier.[33]

6.3. Percentage spore germination inhibition

Different concentrations of the test compounds in 90.0 μl of culture medium were prepared in 96-well flat bottomed microculture plates (Nunc, Nunclon) by double dilution method. The wells were prepared in triplicate 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 16 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.[33] 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}.

7. Anti-*Candidal* activity assay

The activity against *C. albicans* was carried out by the methods of Iijima et al.[36] and Chhillar et al.[34] *C. 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 a 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.

8. Haemolytic assay

The toxicity of pyrazino indole derivatives having an antifungal potential was investigated by using haemolytic assay.[35] A slight modification[37] was employed to determine the haemolytic effect of antifungal 1-(4-chlorophenyl)-10-methyl-1,2,3,4-tetrahydropyrazino[1,2-*a*]indole **4c** derivative. Human erythrocytes collected from apparently healthy volunteers were washed thrice with PBS and 2.0% (v/v) suspension of erythrocytes was prepared in phosphate-buffered saline at pH 7.2. Half millilitre of (2.0%) human erythrocyte suspension in 16 duplicate sets of tubes was treated with compound **4c** 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 percentage haemolysis by the compound. Only a 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.

Acknowledgments

We gratefully acknowledge the financial support from the Department of Science and Technology, New Delhi. R.K. thanks Jean and Ashit Ganguly Trust for JRF and J.S. is thankful to CSIR for JRF.

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