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Yogesh Kumar
Department of Chemistry, Bhagini Nivedita College, University of Delhi, Kair, New Delhi, India

Vijay Bahadur
Department of Science, Alliance University - Central Campus, Chikkahadage Cross Chandapura-Anekal, Main Road, Bengaluru Karnataka, India

Anil Kumar Singh
Department of Chemistry, School of Physical Sciences, Mahatma Gandhi Central University, Motihari, Bihar, India

Poonam Mothsra
Department of Chemistry, Bhagini Nivedita College, University of Delhi, Kair, New Delhi, India

Correspondence Author;
Poonam Mothsra
Department of Chemistry, Bhagini Nivedita College, University of Delhi, Kair, New Delhi, India

Antifungal activity of 1, 2, 3-triazoles of 1-Benzyl-4-(phenoxymethyl) derivatives

Yogesh Kumar, Vijay Bahadur, Anil Kumar Singh and Poonam Mothsra

Abstract
In order to combat the alarming fungal infections, twenty-nine synthesised novel analogues of 1, 2, 3-triazole were further evaluated for their antifungal activity and cytotoxicity. The 1,2,3- triazole analogs were synthesized by using propyloxy benzene, phenyl or benzyl azide, commercially available copper sulphate, D-Glucose and N, N-diisopropylethylamine [DIPEA (2 mmol)], in THF: H_2O (2: 1) by various microwave assisted techniques. Characterisation of the structure was done using spectroscopic techniques 1H-NMR, 13C-NMR, MS and elemental analyses. All synthesized compound showed potent antifungal activity when compared to established drugs voriconazole (VCZ) and fluconazole (FCZ) taken as control. In vitro antifungal activities against five common human pathogenic fungi *viz. Candida albicans* MTCC 7253, *Candida parapsilosis* MTCC 7043, *Candida haemuloni* MTCC 8176, *Aspergillus niger* MTCC 9652, *Aspergillus flavus* MTCC 7133. The cytotoxicity of the compounds was also evaluated by using MCF-7 cell lines and IC_{80} was calculated. This work established the use of analogues of 1, 2, 3-triazole as potent antifungal compounds against the advent of resistant fungal strains.

Keywords: propyloxy benzene, phenyl azide, 1, 2, 3-triazole and antifungal activity

1. Introduction
1,2,3-Triazole derivatives have proved as an important class of heterocyclic compounds with several application invariably in all the field of sciences like chemical synthesis, material science and biology [1, 2]. The triazole scaffold has a wide range of therapeutic properties and is found quite commonly in the drugs. The chemotherapeutic properties of 1,2,3-triazoles have made them an important target moiety for the researchers. Some 1,2,3-triazoles are used as DNA cleaving agents and potassium channel activators [3]. In particular 1,2,3-triazole serves as an important pharmacophore which was found to be potent antimicrobial [4], analgesic [5], anti-inflammatory [5], anesthetic [6], antimalarial [7], antiviral [8], antiproliferating [9], anticonvulsant [10], antineoplastic [11] and anticancer [12] agents. Their synthesis and transformations are receiving great interest these days. Considering the pharmaceutical importance of 1,2,3-Triazole, our team decided to investigate the already synthesised novel 1,2,3-Triazole analogs for their antifungal activity as there is dire need to develop potent antifungal compound.

Fungal diseases have emerged as major causes of morbidity and mortality during the past few decades. The fungal infections have increased alarmingly in healthy and immune-compromised individuals. With evolutionary advent of resistant fungal strains, there is a dire need of novel antifungal drugs with remarkable antifungal potency and lower toxicity.

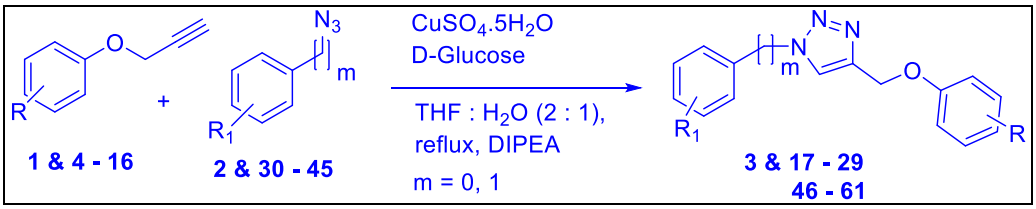
The most common antifungal drugs which have been used clinically are: azoles (e.g. fluconazole, voriconazole, and itraconazole) [13], polyene macrolides (e.g. amphotericin B) [14], allyamines (such as naftifine and terbinafine) [15] and echinocandins (e.g. caspofungin and micafungin) [16, 17] because of their broad spectrum, high efficiency and lower toxicity. But so far, azoles remain the stronghold for therapeutic procedures for severe fungal infections. The azoles are effective in severe *Candida albicans* and *Cryptococcus neoformans* infections but they are found to be ineffective against invasive Aspergillosis. These azoles act potently by inhibiting lanosterol 14a-demethylase (CYP51) in the process of biosynthesis of ergosterol through binding of the heterocyclic nitrogen atom (N-4 of triazoles) to the heme iron atom [18]. However, after so much of popularity azoles also lack in treating severe broad spectrum fungal infections because of their narrow spectrum, high risk

of toxicity and undesirable side effects. Moreover, the most serious issue is the emergence of high-level drug resistance [19] for several fungal infections. Because of this, therapeutic settings for fungal infections are now focused on several new triazoles and their derivatives. Some of the proven examples are voriconazole posaconazole, ravuconazole, which are currently in use in the late stages of clinical trials. In the present work, we have synthesized a series of azoles with substituted phenoxyalkyne and phenyl azides. These synthesized compounds have potential to emerge as potent antifungal agent with optimization taking in to account their excellent *in vitro* antifungal activity against most of the tested human pathogenic fungi.

2. Result and Discussions

2.1 Chemistry

The synthetic route [20] to the analogues of 1,2,3-triazole (3, 17-29, 46-61) involves the described propyloxy benzene [21], phenyl or benzyl azide [22], commercially available copper sulphate, D-Glucose and N, N-diisopropylethylamine [DIPEA (2 mmol)], in THF/H_2O (2: 1) as a solvent was irradiated in microwave at 70 ºC, 100 watt for 10 - 15 min. The completion of the reaction was monitored by TLC [ethyl acetate / petroleum ether (1: 3)]. The product was extracted using ethyl acetate (3 x 30 mL) which was further concentrated under reduced pressure and dried over Na_2SO_4. Column chromatography was used to purify and yield the desired products 3, 17–29 & 46–61. The structures of the compounds were confirmed by IR, mass, ^{1}H NMR and ^{13}C NMR spectroscopy (Supplementary Information available online) [20].



Scheme 1: Synthesis of 1,2,3-triazoles

2.2 Biology

2.2.1. Antifungal Activity

All the synthesized Triazoles compounds were experimented for their *in vitro* antifungal potency. Established drugs voriconazole (VCZ) and fluconazole (FCZ) were used as the positive control.

All Synthesized compounds were dissolved (Stock concentration 100µg/ml) in dimethyl sulfoxide (DMSO) and then stored at −20 °C. The compounds were serially diluted and the tested concentrations ranged from 100 to 0.047 µg/mL. For, five common human pathogenic fungi *viz. Candida albicans* MTCC 7253, *Candida parapsilosis* MTCC 7043, *Candida haemuloni* MTCC 8176, *Aspergillus niger* MTCC 9652, *Aspergillus flavus* MTCC 7133 were obtained from MTCC Chandigarh, India and revived in laboratory by culturing them on a tube containing 20 mL of Sabouraud Dextrose Agar (SDA) plus yeast extract at 35 °C for 48h. Known drugs voriconazole (VCZ) and fluconazole (FCZ) were used as control in same amount as that of synthesized triazole compounds to standardize the results and further to establish the relative comparison. *In vitro* minimal inhibitory concentrations (MICs) of the synthesized compounds were determined by the micro-broth dilution method suggested by the National Committee for Clinical Laboratory Standards (NCCLS 2002). For this, 96-well micro test plates (Nunc) were used MIC_{80} was calculated following an approximate 80 % reduction in growth was observed compared to the growth in a drug-free well. For antibacterial assays, 100 µl of all the serially diluted triazoles compounds as well as controls were incubated with 100 µl of all five revived fungal cultures (Cfu $4.5x10^5$) at 35 ºC. Growth MIC was determined at 72 h for *C. albican, C.parapsilosis* and *C. haemuloni* and 48h for *A. niger* and *A. flavus* sps. The results of antifungal activities and the cytotoxicity are summarized in Table 1. The data is represented as mean of the triplicates performed for each compound.

2.2.2. Table 1- *In vitro* Cytotoxicity

Table 1: *In vitro* Cytotoxicity

					***Invitro* Minimum Inhibitory concentration of triazoles target compounds (MIC_{80}) µg/ml**					
Entry	**Compd.**	**R**	**R_1**	**m**	***C. alb.***	***C. hae***	***A. niger***	***A.Flavus***	***C. par.***	**IC_{50} (µg/ml)**
1	1 & 3	H	H	0	0.195	0.097	>50	>50	0.048	16.891
2	5 & 18	3-F	H	0	0.097	0.097	>50	>50	0.048	16.025
3	6 & 19	3-Cl	H	0	0.097	0.097	>50	>50	0.048	15.243
4	7 & 20	3-Br	H	0	0.781	0.781	>50	>50	0.781	12.254
5	8 & 21	4-F	H	0	0.195	0.0485	>50	>50	0.097	14.880
6	9 & 22	4-Cl	H	0	0.195	0.097	>50	>50	0.097	13.586
7	10 & 23	2,3,4,5,6-F	H	0	0.781	0.781	>50	>50	0.781	11.792
8	11 & 24	2-F	H	0	0.39	0.097	>50	>50	0.048	15.625
9	12 & 25	4-CHO	H	0	0.78	0.0485	>50	>50	0.195	12.254
10	13 & 26	2-$COCH_3$	H	0	0.39	0.097	>50	>50	0.39	12.5
11	14 & 27	3-$COCH_3$	H	0	0.39	0.097	>50	>50	0.39	11.574
12	15 & 28	4-$COCH_3$	H	0	0.39	0.097	>50	>50	0.197	12.254
13	16 & 29	4-NO_2	H	0	0.39	0.097	>50	>50	0.195	11.574

14	30 & 46	H	H	1	0.197	0.0485	>50	>50	0.047	10.964
15	31 & 47	H	4-NO_2	1	0.781	0.781	>50	>50	0.781	16.447
16	32 & 48	H	4-CH_3	0	0.39	0.097	>50	>50	0.097	11.574
17	33 & 49	H	3-CH_3	0	0.097	0.0485	>50	>50	0.097	12.019
18	34 & 50	H	2-CH_3	0	0.197	0.0485	>50	>50	0.047	16.447
19	35 & 51	H	4-OCH_3	0	0.39	0.097	>50	>50	0.39	11.160
20	36 & 52	H	3-OCH_3	0	0.197	0.097	>50	>50	0.39	10.416
21	37 & 53	H	2-OCH_3	0	0.197	0.097	>50	>50	0.39	11.160
22	38 & 54	H	4-F	0	0.197	0.0485	>50	>50	0.195	10.245
23	39 & 55	H	3-F	0	0.197	0.0485	>50	>50	0.195	16.447
24	40 & 56	H	2-F	0	0.197	0.0485	>50	>50	0.195	12.254
25	41 & 57	H	4-Cl	0	0.39	0.097	>50	>50	0.047	12.254
26	42 & 58	H	3-Cl	0	0.39	0.097	>50	>50	0.097	13.888
27	43 & 59	H	2-Cl	0	0.39	0.097	>50	>50	0.097	16.025
28	44 & 60	H	4-Br	0	0.781	0.781	>50	>50	0.781	10.245
29	45 & 61	H	3-Br	0	0.781	0.781	>50	>50	0.781	11.363
30	VCZ				0.197	0.39	0.197	0.781	0.195	12.254
31	FCZ				0.39	0.39	>50	>50	1.562	12.755

3. Experimental

3.1. General

A commercial microwave CEM-Discover-Coolmate in mono mode operating at frequency of 2.45 GHz with continuous irradiation power from 0 to 300W was applied to carry out microwave assisted synthesis of 1,2,3-triazoles in open and cooled vials. The chemicals & reagents were used as received from commercial sources. Analytical TLCs were performed on Merck silica gel 60_{F254} plates. Perkin-Elmer 2000 FT-IR spectrometer at Department of Chemistry, University of Delhi was used to record IR spectra. JEOL ECX-400P NMR at 400 MHz and 100 MHz was employed respectively for ^{1}H and ^{13}C NMR in $CDCl_3$ using TMS as internal standard at USIC, University of Delhi. Melting point of the compounds were determined using Buchi M-560 melting point apparatus and are uncorrected. The high-resolution mass spectra analysis was performed on JEOL JMS-SX-102A spectrometer at Institut fur Chemie und Biochemie, Freie Universitat Berlin, Takustr. 3, 14195, Berlin, Germany.

3.1.1. General procedure for the synthesis of compounds

A stoichiometric ratio of appropriate alkyne (amount 1 mmol), appropriate azide (amount 1.2 mmol), $CuSO_4.5H_2O$ (amount 0.2 equiv), D-glucose (amount 0.4 equiv) and N, N-diisopropylethylamine [DIPEA (2 mmol)] were taken with THF & water (1.5ml) in a ratio of 2:1 in a 10 mL glass vial. The glass vial was tightly sealed with an Teflon crimp top and was subjected to a small magnetic stirring bar along with irradiation in microwave. The irradiation continued for 10-15 minute at 70 ºC and 100 W irradiation power. The progress of the reaction was monitored through TLC [ethyl acetate / petroleum ether (1 : 3)]. The product was extracted after completion of the reaction with ethyl acetate (3 x 30 mL) which is further concentrated under pressure, dried over Na_2SO_4 and finally purified through column chromatography to yield the desired products 3, 18–29 & 46-61 (Scheme 1).

3.2 Biology

3.2.1. Antifungal Activity

All the synthesized compounds were dissolved (Stock concentration 100µg/ml) in dimethyl sulfoxide (DMSO) and then stored at −20 °C. The compounds were serially diluted and the tested concentrations ranged from 100 to 0.047 µg/mL. For their *in vitro* antifungal potency, five common human pathogenic fungi *viz. Candida albicans* MTCC 7253, *Candida parapsilosis* MTCC 7043, *Candida haemuloni* MTCC 8176, *Aspergillus niger* MTCC 9652, *Aspergillus flavus* MTCC 7133 were obtained from MTCC Chandigarh, India and revived in laboratory by culturing them on a tube containing 20 mL of Sabouraud Dextrose Agar (SDA) plus yeast extract at 35 °C for 48h. Established drugs voriconazole (VCZ) and fluconazole (FCZ) were procured from their respective manufacturers and were used as the positive control at same concentrations as of triazoles compounds. *In vitro* minimal inhibitory concentrations (MICs) of the synthesized compounds were determined by the micro-broth dilution method suggested by the National Committee for Clinical Laboratory Standards (NCCLS 2002). For this, 96-well micro test plates (Nunc) were used MIC_{80} was calculated following an approximate 80 % reduction in growth was observed compared to the growth in a drug-free well. For antibacterial assays, 100 µl of all the serially diluted triazoles compounds as well as controls were incubated with 100 µl of all five revived fungal cultures (Cfu $4.5x10^5$) at 35 ºC. Growth MIC was determined at 72 h for *C. albican*, *C. parapsilosis* and *C. haemuloni* and 48h for *A. niger* and *A. flavus* sps. The results of antifungal activities and the cytotoxicity are summarized in Table 1. The data is represented as mean of the triplicates performed for each compound.

3.2.2. *In vitro* Cytotoxicity

MTT colorimetric assay was used to screen the compounds for their cytotoxicity for which MCF-7 cells were maintained in RPMI-1640 medium supplemented with heat inactivated FCS (10% v/v) and 100 U/ml of streptomycin. Further, humidified 5% CO_2 atmosphere at 37 °C was maintained to culture the cells. 100µl of MCF-7 cells were incubated for 16 h for adherence after seeding cells on to 96-well plates (Nunc Maxi Sorp) (2×10^5 cells/well). After 16h, media was aspirated from the wells and the cells were washed once with RPMI-1640 without FCS.

The proven antifungal drugs voriconazole (VCZ) and fluconazole (FCZ) (as control) & products 3, 17–29 & 46–61 were dissolved in a final concentration of 100, 25 and 6.25 µg ml^{-1} of DMSO and 75 µl of each compound was added to the separate wells. As mentioned earlier, Similar conditions of 37 °C in a humidified 5% CO_2 atmosphere was maintained for incubating plates for 4h. Media comprising of synthesised compounds were replaced with

normal RPMI-1640 with 200 µl and further incubated under similar conditions for next 48 h. Media was again replaced and incubated for 2h with 200 µl of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, 0.5 mg/ml of RPMI-1640). 100 µl iso-propanol containing 0.06 M HCl and 0.5% SDS were suspended with formazan crystals after their formation. Colour intensity was evaluated spectrophotometrically using ELISA plate reader (Biotek, ELx800) at 540 nm of each aliquots drawn from well. Cells without MTT were considered blank however untreated cells with 100% viability are refered as controls. The relative cell viability (%) in comparison to control cells was calculated through [abs] sample/ [abs] control × 100.
IC_{50} was designed when 50% of the cells were found to be dead.

Conclusion
Many of the synthesized 1,2,3-Triazole compounds have performed better on several fungal cultures than the proven antifungal drugs voriconazole (VCZ) and fluconazole (FCZ) as indicated in Table-1. Almost all synthesized 1,2,3-triazole (3, 17-29, 46-61) have shown potential activity against fungal culture *C.parapsilosis*. Compounds (1 & 3), (5 &18), (6 &19) have shown significantly improved results amongst all analogs of synthesized 1,2,3-Triazoles on all fungal cultures *C.albican*, *C.parapsilosis* and *C. haemuloni* and 48h for *A. niger* and *A. flavus* sps. However, no structure activity relation can be established from the result.

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Supplementary data
Supplementary data associated with this article can be found, in the online version, at doi: 10.5281/zenodo.5792095.

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