Antifungal potential of C-9154 derivatives against *Candida albicans* and *Aspergillus nigre*

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In view of the increasing resistance to current antifungal drugs and the sometimes adverse side effects associated with most, it has become imperative to search for new compounds with potent antifungal activity to combat the threat posed by pathogenic fungi. This has necessitated the syntheses of several analogues of C-9154 antibiotic and their ester derivatives. These compounds exhibited excellent in vitro biological activity against clinical isolates of *Candida albicans* and *Aspergillus nigre*. The compounds were synthesized by condensation of maleic anhydride with the required aryl amine and then conversion of the terminal carboxylic acid functional group to several ester functional groups using a thionyl chloride-mediated esterification reaction. This step was taken to increase their permeability across cell membranes thereby improving the activity of the derivatives over the analogues. Minimum bactericidal activity was observed for the compounds against *Candida albicans* and *Aspergillus nigre* at 2.5 - 10 µg/mL. They had no effect on *Trichophyton rubrum* however. The compounds were more active than the standard antifungal used for comparison. Hence they could complement or even replace current antifungal drugs that the fungi have developed resistance against.

Keywords Antifungal; C-9154 antibiotic; Biological activity; Candidosis; Aspergillosis

1. Introduction

Man has an extensive use of fungi because of their capacity to produce a large range of natural products with antimicrobial or other biological activities. Many species have been or are being used for the production of antibiotics, vitamins, anti-cancer and/or cholesterol-lowering drugs. One such important natural product is the penicillins, a structurally related group of β-lactam antibiotics that are synthesized from small peptides. [1] Another antibiotic produced by fungi include: cyclosporin, used as an immunosuppressant during transplant surgery, and fusidic acid which is used to help control infection from methicillin-resistant *Staphylococcus aureus*. [2] Other drugs produced by fungi include griseofulvin isolated from *Penicillium griseofulvum*, used to treat fungal infections, [3] and statins used to inhibit cholesterol synthesis. Examples of statins found in fungi include mevastatin from *Penicillium citrinum* and lovastatin from *Aspergillus terreus* and the oyster mushroom. [4]

Saccharomyces cerevisiae, also known as Baker's yeast is a single-celled fungus used to make bread and other wheat-based products, such as pizza dough and dumplings. Yeast species of the genus *Saccharomyces* are also used to ferment alcoholic beverages. [5] *Aspergillus oryzae* also known as 'Shoyu koji mold' is an essential ingredient in brewing Shoyu (soy sauce) and sake, and also used in the preparation of miso. [6] Several of these fungi are domesticated species that were bred or selected according to their capacity to ferment food without producing harmful mycotoxins which are produced by very closely related *Aspergilli*. [7]

Certain mushrooms are used as medicines in Traditional Chinese medicine. Notable medicinal mushrooms with a well-documented history of use include *Agaricus subrufescens*, [8, 9] *Ganoderma lucidum*, [10] and *Ophiocordyceps sinensis*. [11] Research has shown that these and other fungi produced compounds that have inhibitory biological effects against viruses [12, 13] and cancer cells. [14, 15]

Sadly though the story of fungi will not be complete without mentioning the adverse effect they can have on man and his continued existence.

Many fungi are parasites on plants, animals (including humans), and even other fungi. Some fungi can cause serious diseases in humans, several of which may be fatal if untreated. These include aspergilloses, candidoses and coccidioidomycosis. Immuno-deficient persons are particularly susceptible to disease by *Aspergillus*, *Candida* and *Cryptoccocus*, [16, 17, 18] Other fungi can attack the eyes, nails, hair, and the skin causing local infections such as ringworm, athlete's foot and onychomycosis. [19] Fungal spores also cause allergies, and can evoke allergic reactions. [20]

As has been previously mentioned many fungi produce biologically active compounds, several of which are toxic to animals or plants. These are called mycotoxins. Particularly relevant to humans are mycotoxins produced by molds which cause food spoilage. Some other mycotoxins include the aflatoxins, which are liver toxins and highly

carcinogenic metabolites produced by certain *Aspergillus* species often growing in or on grains and nuts consumed by humans.

Antifungal medications are pharmaceutical preparations used to treat mycoses such as athlete's foot, ringworm, candidiasis (thrush), serious systemic infections such as cryptococcal meningitis, and other fungal infections. Such drugs can be obtained by a doctor's prescription or purchased over-the-counter. Apart from side effects like liver damage or affecting estrogen levels, many antifungal medicines can cause allergic reactions in people. For example, the azole antifungals such as ketoconazole or itraconazole can be both substrates and inhibitors of the P-glycoprotein, [21] which (among other functions) excretes toxins and drugs into the intestines. Azole group of drugs is known to have caused anaphylaxis. [22]

During studies on screening for antibiotics that showed activity against bacteria resistant to various known antibiotics, a new antibiotic with a broad antibacterial spectrum was isolated from the whole agar culture of Streptomyces strain NR-7GGI. This Streptomyces species was called *Streptomyces kurssanovii* and the isolated antibiotic referred to as fumaramidmycin [23]. Another researcher working independently and slightly earlier than the previous researcher also found that a new species of *Streptomyces*, *Streptomyces ishigakiensis* produced a novel antibiotic which was named C-9154 [24]. The two new antibiotics were found from structural studies to be the same compound [24, 25]. This new antibiotic was found to inhibit the growths of various microorganisms at concentrations between 10–>100 μg/mL [24]. It was also shown to be active against certain strains that were resistant to ampicillin, cephalosporin, chloramphenicol, gentamicin, kanamycin, macrolides, neomycin, sulfonamides, streptomycin, and tetracyclines at concentrations between 3.12–>200 μg/mL [23]. Its intraperitoneal LD50 value in mice was found to be between 75–100 mg/kg while its oral LD50 was found to be 1.25–2.5 g/kg [23]. The structure of C-9154 was determined using elemental analysis procedures, IR and UV measurements, and NMR and GC-MS experiments [24, 25].

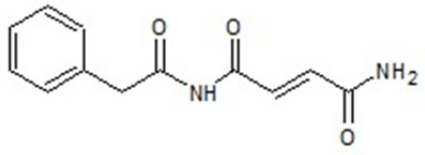


Fig. 1 C-9154 [24, 25]

Analogues of the C-9154 antibiotic have been previously reported [25 - 30]. The problem of resistance to antibiotics on the part of the microorganism, the adverse side effects associated with antibiotics in current use and the difficulty in obtaining these antibiotics in large (commercial) quantities from their natural sources implies that newer antibiotics have to be constantly sought for, to address these problems to give man the needed advantage in the ongoing battle between microbes and men. Synthesis of previously characterized antibiotics with structural modifications to imbue desirable qualities or remove undesirable ones provides a way to assist man in this great battle. We now report the syntheses of some analogues to the C-9154 antibiotic, their derivatives and their in vitro antifungal activity against clinical isolates of *Candida albicans*, *Aspergillus nigre* and *Trichophyton rubrum*.

2. Materials and Method

The melting points of all the synthesized compounds were determined using a micro melting point apparatus manufactured by Reichart, Austria (uncorrected). Infrared spectra were taken using a PerkinElmer Spectrum 100 series Universal ATR driven by Spectrum software 2006, by PerkinElmer Inc. 1D and 2D NMR experiments were carried out using a Bruker av400MHz NMR driven by ICON-NMR software for Topspin 2.1, by Bruker Biospin 2007 while the mass spectra were determined using an Agilent technologies 6890 series GC coupled with an Agilent 5973 Mass selective detector driven by Agilent Chemstation software.

All chemicals and reagents used for the syntheses were of standard quality and manufactured by Merck Chemicals, Germany while the media used for the biological activity studies were manufactured by Oxoid, England.

2.1. Synthesis of C-9154 Analogues.

2.1.1. *N-phenyl fumaramic acid (I)*

N-phenyl fumaramic acid was prepared by the addition reaction between aniline and maleic anhydride according to reaction scheme 1 (Figure 2). Aniline (1.0 g, 11.0 mmol) in toluene (5 mL) was transferred into a round bottom flask containing maleic anhydride (1.3 g, 13.3 mmol) in toluene (5 mL). The mixture was refluxed with stirring for 2hrs. TLC was used to determine that the reaction had gone to completion. The reaction was allowed to cool to room temperature

(25°C) and filtered using a Buchner funnel. The residue was washed using ethyl acetate and dried to afford a greyish solid which was recrystallized from methanol and labeled IA/01/1 (1.9 g, 90.0%). IR, 1D and 2D NMR and GC-MS were used to identify the compound as the desired N-phenyl fumaramic acid. Its melting point was determined to be 195-196°C.

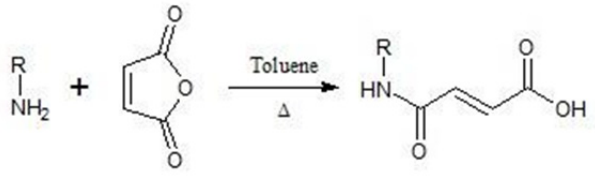


Fig. 2 Reaction Scheme 1

2.1.2. *N-[(3-hydroxyphenyl) amino] fumaramic acid (VIII)*

N-[(3-hydroxyphenyl) amino] fumaramic acid was prepared by the addition reaction between 3-aminophenol and maleic anhydride according to reaction scheme 1 (Figure 2). 3-aminophenol (1.0 g, 9.2 mmol) in toluene (5 mL) was transferred into a round bottom flask containing maleic anhydride (1.1 g, 11.2 mmol) in toluene (5 mL). The mixture was refluxed with stirring for 2hrs. TLC was used to determine that the reaction had gone to completion. The reaction was allowed to cool to room temperature (25°C) and filtered using a Buchner funnel. The residue was washed using ethyl acetate and dried to afford a yellow solid which was recrystallized from methanol and labeled IA/09/1 (1.7 g, 89.0%). IR, 1D and 2D NMR and GC-MS were used to identify the compound as the desired N-[3-hydroxyphenyl) amino] fumaramic acid. Its melting point was determined to be 190-191°C.

2.1.3. *N-benzyl fumaramic acid (XV)*

N-benzyl fumaramic acid was prepared by the addition reaction between benzylamine and maleic anhydride according to reaction scheme 1 (Figure 2). Benzylamine (1.0 g, 9.3 mmol) in toluene (5 mL) was transferred into a round bottom flask containing maleic anhydride (1.1 g, 11.2 mmol) in toluene (5 mL). The mixture was refluxed with stirring for 3hrs. TLC was used to determine that the reaction had gone to completion. The reaction was allowed to cool to room temperature (25°C) and filtered using a Buchner funnel. The residue was washed using ethyl acetate and dried to afford a shiny white solid which was recrystallized from methanol and labeled IA/12/1 (1.76 g, 91.7%). IR, 1D and 2D NMR and GC-MS were used to identify the compound as the desired N-benzyl fumaramic acid. Its melting point was determined to be 125-127°C.

2.1.4. *N-[(2,4-dimethylphenyl) amino] fumaramic acid (XXII)*

N-[(2,4-dimethylphenyl) amino] fumaramic acid was prepared by the addition reaction between 2,4-dimethylaniline and maleic anhydride according to reaction scheme 1 (Figure 2). 2,4-dimethylaniline (1.0 g, 8.3 mmol) in toluene (5 mL) was transferred into a round bottom flask containing maleic anhydride (1.0 g, 10.2 mmol) in toluene (5 mL). The mixture was refluxed with stirring for 3hrs. TLC was used to determine that the reaction had gone to completion. The reaction was allowed to cool to room temperature (25°C) and filtered using a Buchner funnel. The residue was washed using ethyl acetate and dried to afford a dirty yellow powdery solid which was recrystallized from methanol and labeled IA/35/1 (1.67 g, 92.3%). IR, 1D and 2D NMR and GC-MS were used to identify the compound as the desired N-[(2,4-dimethylphenyl) amino] fumaramic acid. Its melting point was determined to be 125-127°C.

2.1.5. *N-[(4-nitrophenyl) amino] fumaramic acid (XXIX)*

N-[(4-nitrophenyl) amino] fumaramic acid was prepared by the addition reaction between 4-nitroaniline and maleic anhydride according to reaction scheme 1 (Figure 2). 4-nitroaniline (1.0 g, 7.2 mmol) in toluene (5 mL) was transferred into a round bottom flask containing maleic anhydride (0.85 g, 8.7 mmol) in toluene (5 mL). The mixture was refluxed with stirring for 1.5hrs. TLC was used to determine that the reaction had gone to completion. The reaction was allowed to cool to room temperature (25°C) and filtered using a Buchner funnel. The residue was washed using ethyl acetate and dried to afford a pale yellow solid which was recrystallized from methanol and labeled IA/49/1 (1.68 g, 98.2%). IR, 1D and 2D NMR and GC-MS were used to identify the compound as the desired N-[(4-nitrophenyl) amino] fumaramic acid. Its melting point was determined to be 197-198°C.

2.2. Synthesis of derivatives of C-9154 analogues

The synthesized acids were converted via the Fischer-Speier esterification procedure to their ester derivatives using methanol, ethanol, n-propanol, isopropanol, n-butanol and 2-butanol respectively using a thionyl chloride ($SOCl_2$) mediated esterification process, according to reaction scheme 2 (Figure 3).

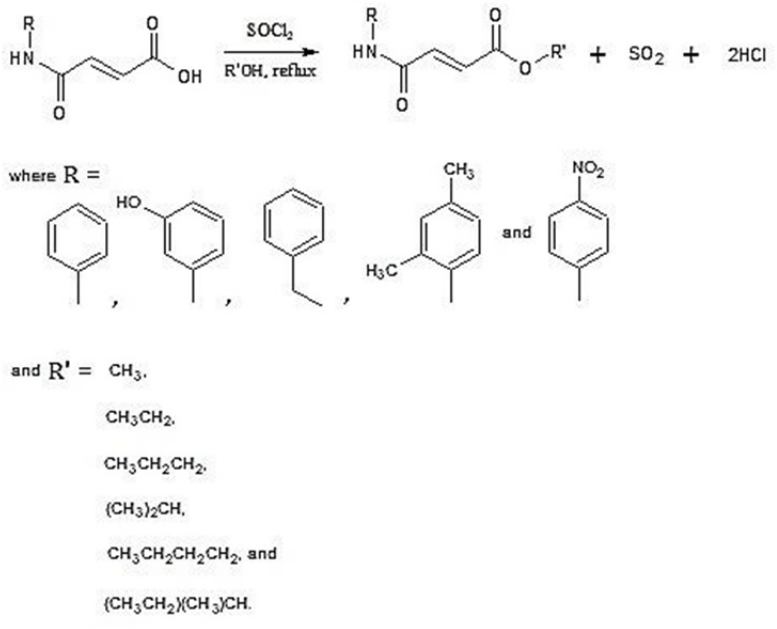


Fig. 3 Reaction Scheme 2

Six portions of the desired acid (I (0.5 g, 2.6 mmol), VIII (0.5 g, 2.4mmol), XV (0.5 g, 2.4 mmol), XXII (0.5 g, 2.1mmol) or XXXIV (0.5 g, 2.3mmol)) were individually transferred to six round-bottomed flasks in ice baths. Thionyl chloride (2 mL) was added in drops with constant stirring. The excess thionyl chloride was removed using a rotary evaporator. Methanol (10 mL), ethanol (10 mL), n-propanol (10mL), isopropanol (10mL), n-butanol (10mL), or 2-butanol (10 mL) was added to each flask and the mixtures refluxed. At the end of the reactions as determined by TLC, saturated sodium carbonate (Na_2CO_3) solution was added to each flask until the solutions just turned alkaline as indicated by litmus paper. Water (20 mL) was added to each flask and the mixtures were individually transferred to different separatory funnels. The mixtures in the different separatory funnels were extracted using dichloromethane (2 × 25 mL). The combined dichloromethane fractions were then individually dried using anhydrous sodium sulphate (Na_2SO_4) and concentrated to oils. These were chromatographed on silica gel columns and eluted using ethyl acetate: hexane (3 : 7), to give the desired esters which crystallized on standing. All the esters were obtained as crystalline solids except the 2-butyl ester of I which was obtained as a golden brown oil and the methyl and butyl esters of XV which were obtained as colourless oils. (Tables 1-5). IR, 1D and 2D NMR, and GC-MS were used to identify the compounds as the desired esters.

2.3. Biological Screening.

The synthesized analogues and their derivatives were tested for activity against *Candida albicans, Aspergillus nigre* and *Trichophyton rubrum*, using Zone of Inhibition measurements, Minimum Inhibitory Concentration (MIC) measurements and Minimum Fungicidal Concentration (MFC) measurements.

2.3.1. Zones of Inhibition.

The antimicrobial activity (Tables 6-10) of the synthesized analogues and derivatives was determined using clinical isolates of *Candida albicans, Aspergillus nigre* and *Trichophyton rubrum,* obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria. The isolates were checked for purity and maintained in slants of blood agar. The analogues (0.1 mg) and the derivatives (0.05 mg) were each weighed and dissolved in DMSO (10mL) to obtain concentrations of 10 µg/mL and 5 µg/mL respectively. This was the initial concentration used to check the antimicrobial activities of the compounds. Sabouraud agar was the growth media used for the fungi. The medium was prepared according to manufacturer's instructions, sterilized at 121°C for 15 minutes

and poured into sterile Petri dishes. The plates were allowed to cool and solidify. Diffusion method was used for screening the compounds. The sterilized medium was seeded with a standard inoculum (0.1mL) of the test microorganisms. This was spread evenly over the surface of the plate by using a sterile swab. The plates were dried at 37°C for 30 minutes. Using a standard cork-borer of 6mm in diameter, a well was cut at the centre of each seeded plate. 0.1mL of the compounds was then introduced into the well. The plates were then incubated at 30°C for 48 hrs, after which the plates were observed for zones of inhibition of growth. The zones were measured using a pair of dividers and a ruler and the result recorded in millimeters. The activity of the compounds was compared against a standard antifungal drug, Fluconazole.

2.3.2. Minimum Inhibitory Concentration.

The minimum inhibitory concentrations (Tables 6-10) of the compounds were carried out using broth dilution method. Sabouraud dextrose broth was prepared and 10mL was dispensed into test tubes, sterilized at 121°C for 15minutes and allowed to cool. McFarland's turbidity scale number 0.5 was prepared to give a turbid solution. Normal saline was prepared and the test microorganisms were inoculated and incubated at 37°C for 6 hrs. Dilution of the test microorganisms was done continuously in the normal saline until the turbidity matched that of the McFarland's scale by visual comparison. At that point the test microbe was at a concentration of about 1.5 x 10^8 CFU/mL. Twofold serial dilutions of the compounds in the broth were made to obtain the different concentrations of the compounds in the broth. Having obtained the different concentrations, 0.1mL of the standard inoculum of the test microorganisms in the normal saline was then inoculated into the different concentrations, and then incubated at 30°C for 48 hrs, after which each test tube was observed for turbidity (growth). The MIC was the test tube with the lowest concentration of the compounds which showed no turbidity.

2.3.3. Minimum Fungicidal Concentration.

MFC (Tables 6-10) was carried out to check whether test microorganisms were killed or only their growths were inhibited. Sabouraud dextrose agar was prepared, sterilized, and poured into sterile Petri dishes. These were allowed to cool and solidify. The content of the MIC in the serial dilution was then sub-cultured onto the prepared media. These were then incubated at 30°C for 48 hrs after which each plate was observed for colony growth. The MFC was the plate with lowest concentration of the compounds without colony growth.

3. Results and Discussion

A total of thirty-five compounds were synthesized and fully characterized using 1D and 2D NMR experiments, infrared spectrophotometry, and gas chromatography-mass spectrometry. Antifungal activity was carried out using three pathogenic fungi. The results are presented below.

Table 1 Synthesized N-phenyl fumaramates

Sample Code	Type	Yield (mg)	Melting point (°C)	Description
IA/24/1/B **(II)**	Methyl ester	160, (0.78mmol)	74	Bright yellow crystalline solid
IA/21/1/B **(III)**	Ethyl ester	100, (0.46mmol)	63	Light brown crystalline solid
IA/22/1/B **(IV)**	n-propyl ester	170, (0.73mmol)	55	Dark brown crystalline solid
IA/23/1/B **(V)**	Isopropyl ester	105, (0.45mmol)	70	Dark brown crystalline solid
IA/19/1/B **(VI)**	n-butyl ester	200, (0.81mmol)	42	Yellow waxy solid
IA/20/1/B **(VII)**	2-butyl ester	150, (0.61mmol)	not determined	Golden brown oil

Table 2 Synthesized N-[(3-hydroxyphenyl) amino] fumaramates

Sample Code	Type	Yield (mg)	Melting point (°C)	Description
IA/37/1/B **(IX)**	Methyl ester	175, (0.79mmol)	119	Light brown shiny crystalline solid
IA/38/1/B **(X)**	Ethyl ester	75, (0.32mmol)	115	Dark brown crystalline solid
IA/39/1/B **(XI)**	n-propyl ester	40, (0.16mmol)	176	Dark brown crystalline solid
IA/40/1/B **(XII)**	Isopropyl ester	80, (0.32mmol)	154	Light brown shiny crystalline solid
IA/41/1/B **(XIII)**	n-butyl ester	105, (0.40mmol)	110	Brown crystalline solid
IA/42/1/B **(XIV)**	2-butyl ester	60, (0.23mmol)	102	Yellowish brown crystalline solid

Table 3 Synthesized N-benzyl fumaramates

Sample Code	Type	Yield (mg)	Melting point (°C)	Description
IA/27/1/B **(XVI)**	Methyl ester	140, (0.64mmol)	not determined	Colourless oil
IA/28/1/B **(XVII)**	Ethyl ester	105, (0.45mmol)	79	Light yellow crystalline solid
IA/29/1/B **(XVIII)**	n-propyl ester	170, (0.69mmol)	40	White crystalline solid
IA/30/1/B **(XIX)**	Isopropyl ester	185, (0.75mmol)	67	White crystalline solid
IA/31/1/B **(XX)**	n-butyl ester	100, (0.38mmol)	not determined	Colourless oil
IA/32/1/B **(XXI)**	2-butyl ester	125, (0.48mmol)	not determined	Colourless oil

Table 4 Synthesized N-[(2,4-dimethylphenyl) amino] fumaramates

Sample Code	Type	Yield (mg)	Melting point (°C)	Description
IA/43/1/B **(XXIII)**	Methyl ester	70, (0.64mmol)	97	Shiny creamy crystalline solid
IA/44/1/B **(XXIV)**	Ethyl ester	140, (0.45mmol)	70	Creamy crystalline solid
IA/45/1/B **(XXV)**	n-propyl ester	180, (0.69mmol)	65	Light yellow crystalline solid
IA/46/1/B **(XXVI)**	Isopropyl ester	60, (0.75mmol)	97	Creamy crystalline solid
IA/47/1/B **(XXVII)**	n-butyl ester	160, (0.38mmol)	41	Yellow semi-solid
IA/48/1/B **(XXVIII)**	2-butyl ester	10, (0.48mmol)	76	Creamy solid

Table 5 Synthesized N-[(4-nitrophenyl) amino] fumaramates

Sample Code	Type	Yield (mg)	Melting point (°C)	Description
IA/51/1/B **(XXX)**	Methyl ester	70, (0.28mmol)	109	Yellowish white crystalline solid
IA/52/1/B **(XXXI)**	Ethyl ester	70, (0.27mmol)	105	Dark yellow crystalline solid
IA/53/1/B **(XXXII)**	n-propyl ester	20, (0.07mmol)	99	Dark yellow crystalline solid
IA/54/1/B **(XXXIII)**	Isopropyl ester	10, (0.04mmol)	102	Dark yellow crystalline solid
IA/55/1/B **(XXXIV)**	n-butyl ester	60, (0.21mmol)	92	Dark yellow crystalline solid
IA/48/1/B **(XXXV)**	2-butyl ester	10, (0.03mmol)	91	Dark yellow crystalline solid

Table 6 Zone of Inhibition (mm), (top), Minimum Inhibitory Concentration (μg/mL), (middle) and Minimum Fungicidal Concentration (μg/mL), (bottom) of Series 1

	IA/01/1 (10μg/mL)	**IA/24/1/B (5μg/mL)**	**IA/21/1/B (5μg/mL)**	**IA/22/1/B (5μg/mL)**	**IA/23/1/B (5μg/mL)**	**IA/19/1/B (5μg/mL)**	**IA/20/1/B (5μg/mL)**	**DMSO**	**fluconazole (50μg/mL)**
C. albicans	22 2.5 10	22 1.25 5	24 1.25 5	27 1.25 2.5	30 0.625 2.5	24 1.25 5	21 1.25 5	0 ND	24 25
A. nigre	21 2.5 10	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND	0 ND
T. rubrum	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND	20 25

Table 7 Zone of Inhibition (mm), (top), Minimum Inhibitory Concentration (μg/mL), (middle) and Minimum Fungicidal Concentration (μg/mL), (bottom) of Series 2

	IA/09/1 (10μg/mL)	**IA/37/1/B (5μg/mL)**	**IA/38/1/B (5μg/mL)**	**IA/39/1/B (5μg/mL)**	**IA/40/1/B (5μg/mL)**	**IA/41/1/B (5μg/mL)**	**IA/42/1/B (5μg/mL)**	**DMSO**	**fluconazole (50μg/mL)**
C. albicans	20 2.5 10	22 1.25 5	21 1.25 5	19 2.5 5	20 1.25 5	21 1.25 5	20 1.25 5	0 ND	24 25
A. nigre	20 2.5 10	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND	0 ND
T. rubrum	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND	20 25

Table 8 Zone of Inhibition (mm), (top), Minimum Inhibitory Concentration (μg/mL), (middle) and Minimum Fungicidal Concentration (μg/mL), (bottom) of Series 3

	IA/12/1 (10μg/mL)	**IA/27/1/B (5μg/mL)**	**IA/28/1/B (5μg/mL)**	**IA/29/1/B (5μg/mL)**	**IA/30/1/B (5μg/mL)**	**IA/31/1/B (5μg/mL)**	**IA/32/1/B (5μg/mL)**	**DMSO**	**fluconazole (50μg/mL)**
C. albicans	26 2.5 5	22 1.25 5	23 1.25 5	20 1.25 5	20 1.25 5	22 1.25 5	21 1.25 5	0 ND	24 25
A. nigre	21 2.5 10	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND	0 ND
T. rubrum	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND	20 25

Table 9 Zone of Inhibition (mm), (top), Minimum Inhibitory Concentration (μg/mL), (middle) and Minimum Fungicidal Concentration (μg/mL), (bottom) of Series 4

	IA/35/1 (10μg/mL)	**IA/43/1/B (5μg/mL)**	**IA/44/1/B (5μg/mL)**	**IA/45/1/B (5μg/mL)**	**IA/46/1/B (5μg/mL)**	**IA/47/1/B (5μg/mL)**	**IA/48/1/B (5μg/mL)**	**DMSO**	**fluconazole (50μg/mL)**
C. albicans	24 2.5 10	21 1.25 5	20 1.25 5	20 1.25 5	21 1.25 5	20 1.25 5	22 1.25 5	0 ND	24 25
A. nigre	20 2.5 10	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND	0 ND
T. rubrum	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND	20 25

Table 10 Zone of Inhibition (mm), (top), Minimum Inhibitory Concentration (μg/mL), (middle) and Minimum Fungicidal Concentration (μg/mL), (bottom) of Series 5

	IA/49/1 (10μg/mL)	**IA/51/1/B (5μg/mL)**	**IA/52/1/B (5μg/mL)**	**IA/53/1/B (5μg/mL)**	**IA/54/1/B (5μg/mL)**	**IA/55/1/B (5μg/mL)**	**IA/56/1/B (5μg/mL)**	**DMSO**	**fluconazole (50μg/mL)**
C. albicans	20 2.5 10	22 1.25 5	20 1.25 5	21 1.25 5	22 1.25 5	20 1.25 5	21 1.25 5	0 ND	24 25
A. nigre	21 2.5 10	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND	0 25
T. rubrum	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND ND	0 ND	20 25

IA/01/1 (N-phenylamino fumaramic acid) (I).

The compound was obtained as a grey powdery solid which melted at 195-196°C. It had the following spectra data.
^{13}C-NMR (400MHz, $CDCl_3$ and DMSO-d_6). 165.1 (C10), 163.7 (C7), 136.6 (C1), 133.7 (C9), 132.7 (C8), 128.4 (C3 and C5), 125.0 (C4), 120.3 (C2 and C6).
^{1}H-NMR (400MHz, $CDCl_3$ and DMSO-d_6): δ 6.23 (1H, d, J=12.69 Hz, H-9), 6.55 (1H, d, J=12.73 Hz, H-8), 7.09 (1H, t, J=7.40 Hz, H-4), 7.27 (2H, t, J=7.88 Hz, H-3 and H-5), 7.57 (2H, d, J=7.84 Hz, H-2 and H-6), 7.68 (1H, s, 1-NH), 10.79 (1H, s, 10-OH).
EI-MS: m/z 173 {$[m - H]^+$, 100%}.
IR_{vmax} (neat) cm^{-1} : 3271.36 (N-H), 3071.75 (O-H), 2883.57 (C-H), 1694.60, 1618.99 (C=O).

IA/24/1/B (Methyl N-phenylamino fumaramate) (II).

The compound was obtained as a bright yellow crystalline solid which melted at 74°C. It had the following spectra data.
^{13}C-NMR (400MHz, $CDCl_3$). 167.4 (C7), 161.5 (C10), 137.8 (C1), 140.5 (C9), 124.9 (C8), 129.0 (C3 and C5), 124.6 (C4), 120.1 (C2 and C6), 52.8 (C11).
^{1}H-NMR (400MHz, $CDCl_3$) δ 3.83 (3H, s, H-11), 6.21 (1H, d, J=13.33 Hz, H-8), 6.42 (1H, d, J=13.25 Hz, H-9), 7.11 (1.0H, t, J=7.38 Hz, 4-H), 7.32 (2H, t, J=7.60 Hz, H-3 and H-5), 7.64 (2H, d, J=7.88 Hz, H-2 and H-6), 10.82 (1H, s, 1-NH).
EI-MS: m/z 205 {$[M]^+$, 50%}.
IR_{vmax} (neat) cm^{-1} : 3250.97 (N-H), 2953.75 (C-H), 1730.94, 1666.54 (C=O).

IA/21/1/B (Ethyl N-phenylamino fumaramate) (III).

The compound was obtained as a light brown crystalline solid which melted at 63°C. It had the following spectra data.
^{13}C-NMR (400MHz, $CDCl_3$). 166.7 (C7), 162.0 (C10), 137.9 (C1), 138.9 (C9), 126.0 (C8), 129.0 (C3 and C5), 124.6 (C4), 120.1 (C2 and C6), 61.9 (C11), 14.0 (C12).
^{1}H-NMR (400MHz, $CDCl_3$) δ 1.30 (3H, t, J=7.20 Hz, H-12), 4.26 (2H, q, J=7.14 Hz, H-11), 6.16 (1H, d, J=13.09 Hz, H-8), 6.39 (1H, d, J=13.09 Hz, H-9), 7.10 (1H, t, J=7.36 Hz, H-4), 7.31 (2H, t, J=7.84 Hz, H-3 and H-5), 7.65 (2H, d, J=7.96 Hz, H-2 and H-6), 10.7 (1H, s, 1-NH).
EI-MS: m/z 219 {$[M]^+$, 50%}.
IR_{vmax} (neat) cm^{-1} : 3258.58 (N-H), 2926.30 (C-H), 1716.89, 1667.67 (C=O).

IA/22/1/B (n-propyl N-phenylamino fumaramate) (IV).

The compound was obtained as a dark brown crystalline solid which melted at 55°C. It had the following spectra data.
^{13}C-NMR (400MHz, $CDCl_3$). 166.7 (C7), 162.5 (C10), 137.9 (C1), 137.2 (C9), 126.6 (C8), 128.9 (C3 and C5), 124.5 (C4), 120.2 (C2 and C6), 67.3 (C11), 21.7 (C12) 10.3 (C13).
^{1}H-NMR (400MHz, $CDCl_3$) δ 0.83 (3H, t, J=7.42 Hz, H-13), 1.57 (2H, m, H-12), 4.04 (2H, t, J=6.74 Hz), 6.04 (1H, d, J=12.69 Hz, H-8), 6.31 (1H, d, J=12.69 Hz, H-9), 7.00 (1H, t, J=7.42 Hz, H-4), 7.21 (2H, d, J=7.64 Hz, H-3 and H-5), 7.56 (2H, d, J=7.76 Hz, H-2 and H-6), 10.3 (1H, s, 1-NH).

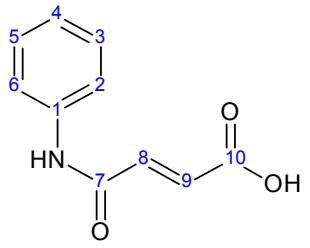
EI-MS: m/z 233 {[M]$^+$, 50%}.
IR$_{vmax}$ (neat) cm^{-1} : 3259.72 (N-H), 2878.49 (C-H), 171946, 1662.54 (C=O).

IA/23/1/B (Isopropyl N-phenylamino fumaramate) (V).

The compound was obtained as a dark brown crystalline solid which melted at 70°C. It had the following spectra data.
^{13}C-NMR (400MHz, $CDCl_3$). 166.4 (C7), 161.6 (C10), 137.9 (C1), 140.3 (C9), 125.8 (C8), 129.0 (C3 and C5), 124.5 (C4), 120.1 (C2 and C6), 70.0 (C11), 21.7 (C12 and C13).
^{1}H-NMR (400MHz, $CDCl_3$) δ 1.32 (6H, d, J=6.28 Hz, H-12 and H-13), 5.15 (1H, m, H-11), 6.18 (1H, d, J=13.36 Hz, H-8), 6.41 (1H, d, J=13.45 Hz, H-9), 7.12 (1H, t, J=7.38 Hz, H-4), 7.34 (2.1H, d, J=15.81 Hz, H-3 and H-5), 7.67 (1.9H, d, J=7.84 Hz, H-2 and H-6), 11.0 (1H, s, 1-NH).
EI-MS: m/z 233 {[M]$^+$, 50%}.
IR$_{vmax}$ (neat) cm^{-1} : 3238.13 (N-H), 2936.22 (C-H), ~1700.00, 1660.48 (C=O).

IA/19/1/B (n-butyl N-phenylamino fumaramate) (VI).

The compound was obtained as a yellow waxy solid which melted at 42°C. It had the following spectra data.
^{13}C-NMR (400MHz, $CDCl_3$). 166.6 (C7), 162.8 (C10), 136.2 (C1), 138.0 (C9), 126.8 (C8), 128.8 (C3 and C5), 124.5 (C4), 120.2 (C2 and C6), 65.4 (C11), 30.3 (C12), 19.0 (C13), 13.6 (C14).
^{1}H-NMR (400MHz, $CDCl_3$) δ 0.84 (3H, t, J=7.60 Hz, H-14), 1.31 (2H, m, H-13), 1.56 (2H, m, H-12), 4.12 (2H, t, J=6.78 Hz, H-11), 6.07 (1H, d, J=12.69 Hz, H-8), 6.38 (1H, d, J=12.53 Hz, H-9), 7.04 (1H, t, J=7.40 Hz, H-4), 7.24 (2H, t, J=7.64 Hz, H-3 and H-5), 7.63 (2H, d, J=8.20 Hz, H-2 and H-6), 10.2 (1H, s, 1-NH).
EI-MS: m/z 247 {[M]$^+$, 75%}.
IR$_{vmax}$ (neat) cm^{-1} : 3298.98 (N-H), 2957.43 (C-H), ~1700.00, 1659.63 (C=O).



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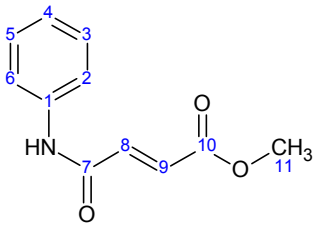

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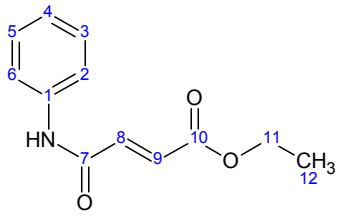

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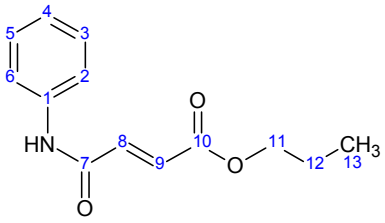

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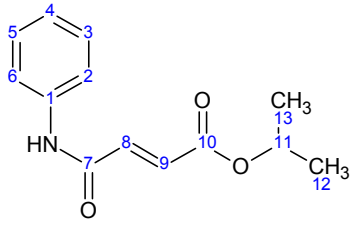

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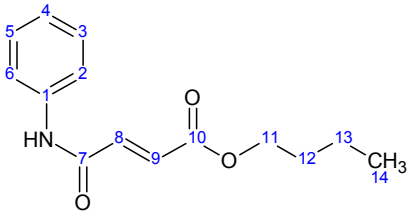
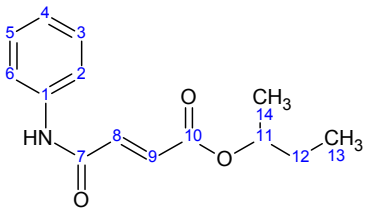


VI



VII

Fig. 4 Spectral data and structures for series 1 is provided. Data for the other series can be made available on request.

The results show that the synthesized compounds showed remarkable activity in the range 0.625–2.5 μg/mL. The derivatives showed higher activity than the analogue as expected. This is largely due to the reduction in polarity when the highly polar carboxylic functional group was converted to the less polar ester functional group. This has been shown to increase cell membrane permeability [31-33].

All the synthesized compounds showed better activity against *Candida albicans* than fluconazole. The antifungal standard drug was found to be up to 10-20fold less active than all the synthesized compounds and even up to 40fold less active than IA/32/1/B. The results show that the compounds have the potential to compliment or even replace current antibiotics used especially against drug-resistant *Candida albicans* and infections by the microorganism acquired from hospital environment after further studies.

All the derivatives and the standard antifungal used for comparison could not inhibit the growth of *Aspergillus nigre*, whereas the analogues could at 2.5 μg/mL. This is particularly noteworthy as it could be used to combat *Aspergillus nigre*, which produces ochratoxin A, a potential carcinogen and a neurotoxin. [34, 35].

All the synthesized compounds could however not inhibit the growth of *Trichophyton rubrum* at the 5 μg/mL which was probably too low for the fungi which is quite resilient.

3. Conclusion

The synthesized compounds were shown to be quite potent when compared to the standard antifungal drug, Fluconazole. The very low concentration at which this activity was exhibited implies that fungi, especially those which have become resistant to standard drugs can now be possibly addressed if the compounds are found after further studies to be non-toxic.

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