SYNTHESIS, CHARACTERIZATION AND *IN VITRO* BIOLOGICAL ACTIVITY PROFILE OF SOME DERIVATIVES OF C-9154 ANTIBIOTIC

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Abstract: Structural modification of the C-9154 antibiotic was carried out in an attempt to simultaneously improving its activity and lower its toxicity. An analogue of the C-9154 antibiotic and six derivatives of this analogue were synthesized. The approach was to significantly reduce the polarity of the analogue by structurally modifying it to get the derivatives in a bid to achieve increased permeability across cell membranes by conversion of the highly polar carboxylic group to an ester functional group. These compounds were synthesized by condensation of 2,4-dimethylaniline with maleic anhydride and then conversion of the terminal carboxylic acid functional group to an ester functional group using a thionyl chloride-mediated esterification process. The *in vitro* biological activity showed that the derivatives were more active than the analogue and significantly better than the standard drugs used for comparison. The compounds were fully characterized using Infrared, GC-MS and 1D and 2D NMR experiments.

Keywords: C-9154 antibiotics, bioactivity, permeability, fumaramidmycin, maleic anhydride, NMR

Introduction

After the 'fall of man', fighting disease has become a normal part of his existence. This he does by the use of compounds derived from nature. One such classes of compounds is called antibiotics. Vuillemin, a French bacteriologist, suggested using the word 'antiobiosis', meaning 'against life,' to describe the group of drugs that had detrimental effect against microorganisms (Aronson, 1997). Selman Waksman, the discoverer of streptomycin, later changed this term to antibiotic in 1942 (Radetsky, 1996). The term 'antibiotic' is used to describe any substance produced by a microorganism that is antagonistic to the growth of other microorganisms in high dilution (low concentration) (Waksman, 1947). With current advances in medicinal chemistry, antibiotics are now mostly semisynthetic modifications of various natural compounds (vonNussbaum *et al*., 2006). Some antibiotic compounds are still being isolated from living organisms like the aminoglycosides, whereas other antibiotics like the sulfonamides, the quinolones, and the oxazolidinones are produced solely by chemical synthesis (vonNussbaum *et al*., 2006). This implies that synthesis of antibiotic compounds plays an important and vital role in the fight against disease-causing organisms.

In light of emerging resistance to current antibiotic drugs, it has become imperative to synthesize new antibiotics to combat these resistances. 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 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.

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 specie was called *Streptomyces kurssanovii* and the isolated antibiotic referred to as fumaramidmycin (Maruyama *et al*., 1975). 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 (Hasegawa *et al*., 1975). The two new antibiotics were found from structural studies to be one and the same compound (Hasegawa *et al*., 1975; Suhara *et al*., 1975). This new antibiotic was found to inhibit the growths of various microorganism at concentrations between 10 - >100 µg/mL (Hasegawa *et al*., 1975). 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 (Maruyama *et al*., 1975). Its intraperitoneal LD_{50} value in mice was found to be between 75-100 mg/kg while its oral LD_{50} was found to be 1.25 - 2.5 g/kg (Maruyama *et al*., 1975). The structure of C-9154 (Fig. 1) was determined using elemental analysis procedures, IR and UV measurements and NMR and GC-MS experiments (Suhara *et al*., 1975; Jumina *et al*., 2001).

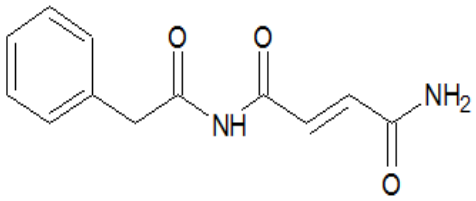


Fig. 1: C-9154 antibiotic

Analysis of the structure of C-9154 antibiotic showed that it was made up of two fragments, namely, phenyl acetic acid and fumaramide (Hasegawa *et al*., 1975; Suhara *et al*., 1975). Analogues of the C-9154 antibiotic have been previously synthesized (Suhara *et al*., 1975; Jumina *et al*., 2001; Jumina *et al*., 2002; Jumina *et al*., 2005). We have also previously reported the syntheses of some aniline derivatives (Bello *et al*., 2012a) and benzyl derivatives of C-9154 antibiotic (Bello *et al*., 2012b) and their biological activity profiles.

We now report the syntheses of one analogue of the C-9154 antibiotic, and six of its derivatives. Four of these derivatives are novel and are being reported for the first time in this study. The *in vitro* biological activities of all the synthesized compounds are also being reported for the first time.

Materials and Methods

Infrared spectra were determined using a PerkinElmer Spectrum 100 series Universal ATR. 1D and 2D NMR experiments were carried out using a Bruker av400MHz NMR. The GCMS spectra were taken using an Agilent Technologies 6890 series GC coupled with an Agilent 5973 Mass Selective detector.

All chemicals and reagents unless otherwise stated were obtained from Merck Chemicals, Germany while all media were obtained from Oxoid, England.

Synthesis of C-9154 analogue

N-[(2,4-dimethylphenyl) amino] fumaramic acid was prepared according to reaction scheme 1 (Fig. 2). 2,4-dimethylaniline (1.0 g, 8.3 mmol) in toluene (5 mL) was transferred to a round bottom flask containing maleic anhydride (1.0 g, 10.2 mmol) in toluene (5 mL). The mixture was refluxed at 98°C with stirring for 3 h. The reaction mixture was allowed to cool to room temperature and filtered using a Buchner funnel. The residue was washed using ethyl acetate and dried to afford a dirty yellow solid labeled IA/35/1 (1.67 g, 92.3%). TLC was used to determine that the reaction had gone to completion. 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.

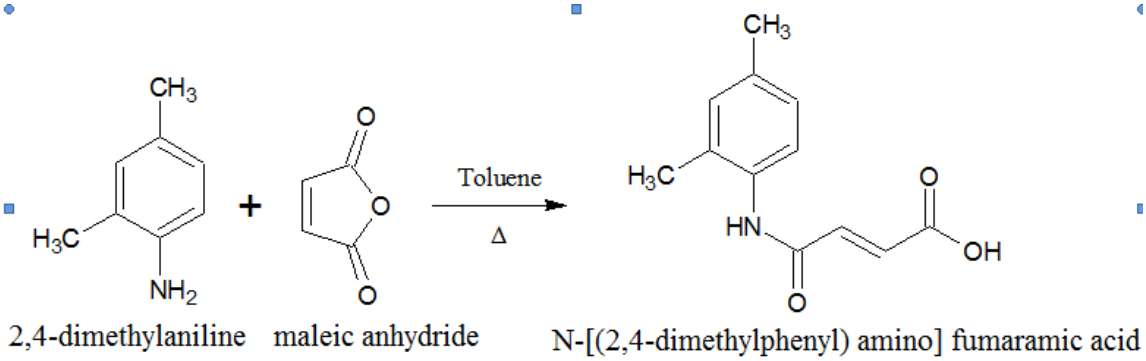
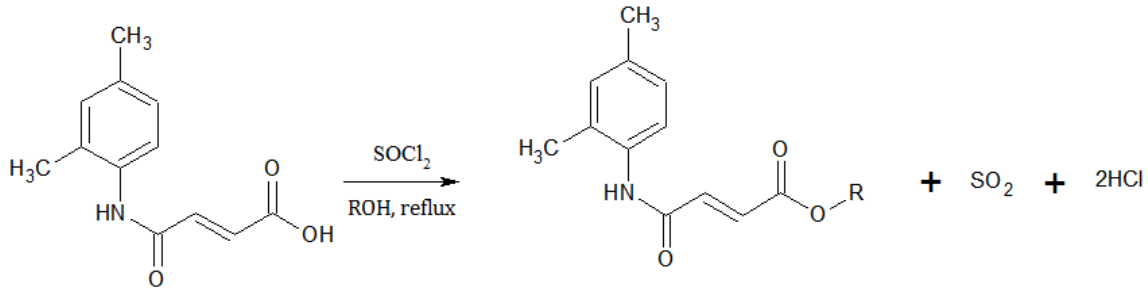


Fig. 2: Reaction scheme 1



Where: R = CH_3; CH_3CH_2; $CH_3CH_2CH_2$; $(CH_3)_2CH$; $CH_3CH_2CH_2CH_2$; $(CH_3CH_2)(CH_3)CH$

Fig. 3: Reaction scheme 2

Table 1: Synthesized C-9154 analogue and its derivatives

Sample Code	Type	Yield (mg)	Melting point (°C)	Physical State
IA/35/1	C-9154 Analogue	92.3%	125-127	yellow powdery solid
IA/43/1/B	Methyl ester	70, (0.64 mmol)	97	creamy crystalline solid
IA/44/1/B	Ethyl ester	140, (0.45 mmol)	70	creamy crystalline solid
IA/45/1/B	n-propyl ester	180, (0.69 mmol)	65	yellow crystalline solid
IA/46/1/B	Isopropyl ester	60, (0.75 mmol)	97	creamy crystalline solid
IA/47/1/B	n-butyl ester	160, (0.38 mmol)	41	yellow semi-solid
IA/48/1/B	2-butyl ester	10, (0.48 mmol)	76	creamy white solid

Synthesis of derivatives of C-9154 analogue

The synthesized analogue was then converted to its ester derivatives using methanol, ethanol, n-propanol, isopropanol, n-butanol and 2-butanol respectively by a thionyl chloride ($SOCl_2$) mediated esterification according to reaction scheme 2 (Fig. 3). Six portions of IA/35/1 (0.5 g, 2.1 mmol) 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 (10 mL), isopropanol (10 mL), n-butanol (10 mL), 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 each extracted using dichloromethane (2 x 25 mL) and combined. The combined dichloromethane fractions were individually dried using anhydrous sodium sulphate (Na_2SO_4) and concentrated to give dark yellow oils. These were chromatographed on separate 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 (Table 1). IR, 1D and 2D NMR and GC-MS were used to identify the compounds as the desired esters.

Biological screening

These compounds were then subjected to biological screening *in vitro* to ascertain their activity and the concentration at which this activity was exhibited.

This was carried out using Zones of inhibition measurements, Minimum Inhibitory Concentration measurements (MIC) and Minimum Bactericidal/Fungicidal Concentration measurements (MBC/MFC). Fourteen (14) microorganisms were selected for the screening to include both gram-positive and gram-negative bacteria and some fungi. These microorganisms are: Methicillin-Resistant *Staphylococcus aureus*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus subtilis*, and *Corynebacterium ulcerans* for gram positive bacteria, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella typhii*, *Shigella dysenteriae*, and *Klebsiella pneumonia* for gram negative, and *Candida albicans*, *Aspergillus nigre* and *Trichophyton rubrum* for fungi.

Zones of inhibition

The antimicrobial activity (Table 2) of the synthesized analogues and derivatives was determined using some pathogenic microorganisms obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria. All isolates were checked for purity and maintained in slants of blood agar. Diffusion method was the method used for screening the compounds (Foster and Woodruff, 1943).

The analogues (0.1 mg) and the derivatives (0.05 mg) were each weighed and dissolved in DMSO (10 mL) to obtain a concentration of 10 and 5 µg/mL, respectively. This was the initial concentration used to check the antimicrobial activities of the compounds. Mueller Hinton and Sabouraud agar were used as growth media for the bacteria and fungi, respectively. The media were prepared according to the manufacturer's instructions, sterilized at 121°C for 15 min and were poured into sterile Petri dishes. The plates were allowed to cool and solidify. Diffusion method was the method used for screening the compounds. The sterilized media were seeded with a standard inoculum (0.1 mL) 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 min. Using a standard cork-borer of 6 mm in diameter, a well was cut at the centre of each seeded plate. 0.1 mL of the compounds was then introduced into the well. The plates were then incubated at 37°C for 24 h for the bacteria and 30°C for 48 h for the fungi, 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 two standard drugs; Sparfloxacin (antibacterial) and Fluconazole (antifungal).

Minimum inhibitory concentration

The minimum inhibitory concentrations of the compounds (Table 3) were carried out using broth dilution method (Qaiyumi, 2007). Mueller Hinton and Sabouraud dextrose broth were prepared and 10 mL was dispensed into test-tubes and the broths were sterilized at 121°C for 15 min, the broths were allowed to cool. McFarland's turbidity scale number 0.5 was prepared to give turbid solution. Normal saline was prepared and the test microorganisms were inoculated and incubated at 37°C for 6 h. Dilution of the test microorganisms were 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. Two-fold 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, 0.1 mL of the standard inoculum of the test microorganisms in the normal saline were then inoculated into the different concentrations, and then incubated at 37°C for 24 h for the bacteria and 30°C for 48 h for the fungi, 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.

Table 2: Zones of inhibition (mm) of the analogue and derivatives

	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	Sparfloxacin (20µg/mL)	fluconazole (50µg/mL)
MRSA	24	30	30	31	29	30	31	0	22	0
S. aureus	26	29	31	30	30	30	30	0	27	0
S. pyogenes	0	27	30	30	27	29	30	0	24	0
B. subtilis	33	34	32	31	32	31	34	0	30	0
C. ulcerans	20	30	31	30	27	30	0	0	0	0
E. coli	24	24	22	24	27	26	27	0	27	0
P. mirabilis	0	27	27	26	26	25	24	0	22	0
P. aeruginosa	0	0	0	0	0	0	0	0	20	0
S. typhii	27	27	26	25	24	26	24	0	21	0
S. dysenteriae	30	24	25	25	24	22	24	0	27	0
K. pneumoniae	29	27	25	26	25	24	26	0	25	0
C. albicans	24	21	20	20	21	20	22	0	0	24
A. nigre	20	0	0	0	0	0	0	0	0	0
T. rubrum	0	0	0	0	0	0	0	0	0	20

Table 3: Minimum inhibitory concentration (above) and minimum bactericidal/fungicidal concentration (below) of the analogue and derivatives (µg/mL)[a]

	IA/35/1	IA/43/1/B	IA/44/1/B	IA/45/1/B	IA/46/1/B	IA/47/1/B	IA/48/1/B	DMSO	Sparfloxacin	Fluconazole
MRSA	2.5	0.625	0.625	0.625	1.25	0.625	0.625	ND	10	ND
	10	2.5	2.5	2.5	5	5	2.5			
S. aureus	2.5	1.25	0.625	0.625	0.625	0.625	0.625	ND	10	ND
	5	5	2.5	5	2.5	2.5	5			
S. pyogenes	ND	1.25	0.625	0.625	1.25	1.25	0.625	ND	10	ND
	ND	5	5	2.5	5	5	5			
B. subtilis	1.25	0.625	0.625	0.625	0.625	0.625	0.625	ND	5	ND
	5	2.5	2.5	2.5	2.5	2.5	2.5			
C. ulcerans	2.5	0.625	0.625	0.625	1.25	0.625	ND	ND	ND	ND
	10	5	2.5	5	5	5	ND			
E. coli	2.5	1.25	1.25	1.25	1.25	1.25	1.25	ND	10	ND
	10	5	5	5	5	5	5			
P. mirabilis	ND	1.25	1.25	1.25	1.25	1.25	1.25	ND	10	ND
	ND	5	5	5	5	5	5			
P. aeruginosa	ND	ND	ND	ND	ND	ND	ND	ND	10	ND
	ND	ND	ND	ND	ND	ND	ND			
S. typhii	2.5	1.25	1.25	1.25	1.25	1.25	1.25	ND	10	ND
	5	5	5	5	5	5	5			
S. dysenteriae	1.25	1.25	1.25	1.25	1.25	1.25	1.25	ND	5	ND
	5	5	5	5	5	5	5			
K. pneumoniae	2.5	1.25	1.25	1.25	1.25	1.25	1.25	ND	5	ND
	5	5	5	5	5	5	5			
C. albicans	2.5	1.25	1.25	1.25	1.25	1.25	1.25	ND	ND	25
	10	5	5	5	5	5	5			
A. nigre	2.5	ND	ND	ND	ND	ND	ND	ND	ND	ND
	10	ND	ND	ND	ND	ND	ND			
T. rubrum	ND	ND	ND	ND	ND	ND	ND	ND	ND	25
	ND	ND	ND	ND	ND	ND	ND			

[a] Upper values are MIC and lower values are MBC or MFC as the case may be. **ND**=Not Determined

Minimum bactericidal/fungicidal concentration

MBC (Table 3) was carried out to check whether the test microorganisms were killed or only their growths were inhibited using the method described by Qaiyumi, 2007 with some modifications. Briefly, Mueller Hinton and Sabouraud dextrose agar were prepared, sterilized and poured into sterile Petri dishes. These were allowed to cool and solidify. The content of the MIC in the serial dilution were then sub-cultured onto the prepared media. These were then incubated at 37°C for 24 h for the bacteria and 30°C for 48 h for the fungi after which each plate was observed for colony growth. The MBC/MFC was the plate with lowest concentration of the compounds without colony growth.

Results and Discussion

Seven compounds were synthesized and fully characterized using 1D and 2D NMR experiments, infrared spectrophotometry and gas chromatography-mass spectrometry.

The analogue was synthesized by reaction between the required N-phenyl amine (3-amino phenol) and maleic anhydride to get the desired fumaramic acid. This fumaramic acid (analogue) was then converted to its methyl, ethyl, n-propyl, isopropyl, n-butyl, and 2-butyl esters using a thionyl chloride - mediated esterification procedure. The yields from the reactions were excellent (92%) in the analogue and poor to moderate (1.5 – 30%) in the derivatives. This could be attributed to unwanted side reactions that were occurring during the esterification step. Attempts to mitigate this unwanted side reactions were unsuccessful. These compounds were then subjected to biological screening *in vitro*. The results and associated data are presented below.

IA/35/1 (N-[(2,4-dimethylphenyl) amino] fumaramic acid)

The compound was obtained as a dirty yellow powdery solid which melted at 125-127ºC. It had the following spectra data.

^{13}C-NMR (400MHz, $CDCl_3$ and DMSO-d_6). 170.3 (C10), 169.7 (C7), 141.5 (C1), 137.2 (C4), 136.4 (C2), 139.1 (C9), 137.9 (C8), 136.2 (C3), 131.8 (C5), 130.0 (C6), 25.7 (C11), 22.8 (C12).

^{1}H-NMR (400MHz, $CDCl_3$ and DMSO-d_6) δ 2.16 (3H, s, H-12), 2.23 (3H, s, H-11), 6.24 (1H, d, J=12.85 Hz, H-9), 6.62 (1H, d, J=12.85 Hz, H-8), 6.93 (1H, d, J=8.60 Hz, H-5), 6.97 (1H, s, H-3), 7.22 (1H, d, J=7.96 Hz, H-6), 10.38 (1H, s, 1-NH).

EI-MS: m/z 201 {[M - 17]$^+$, 100%}.

IR_{vmax} (neat) cm^{-1}: 3276.31 (N-H), 3058.06 (O-H), 2922.49 (C-H), 1707.10, 1633.36 (C=O).

IA/43/1/B (Methyl N-[(2,4-dimethylphenyl) amino] fumaramate).

The compound was obtained as a shiny creamy crystalline solid which melted at 97 °C. It had the following spectra data.

^{13}C-NMR (400MHz, $CDCl_3$). 166.9 (C7), 162.1 (C10), 139.2 (C9), 135.2 (C1), 133.1 (C4), 131.2 (C3), 130.3 (C2), 127.0 (C5), 125.1 (C8), 123.6 (C6), 52.6 (C13), 20.9 (C11), 18.0 (C12).

^{1}H-NMR (400MHz, $CDCl_3$) δ 2.27 (3H, s, H-12), 2.28 (3H, s, H-11), 6.17 (1H, d, J=13.05 Hz, H-8), 6.43 (1H, d, J=13.09 Hz, H-9), 6.98-6.99 (2H, H-3 and H-5), 7.69 (1H, d, J=8.80 Hz, H-6), 9.391 (1H, s, 1-NH).

EI-MS: m/z 149 {[M + H - 85]$^+$, 100%}.

IR_{vmax} (neat) cm^{-1}: 3215.08 (N-H), 3002.12 (C-H), 1731.24, 1667.08 (C=O).

IA/44/1/B (Ethyl N-[(2,4-dimethylphenyl) amino] fumaramate)

The compound was obtained as a creamy crystalline solid which melted at 70°C. It had the following spectra data.

^{13}C-NMR (400MHz, $CDCl_3$). 166.5 (C7), 162.0 (C10), 140.0 (C9), 135.1 (C1), 133.0 (C4), 131.2 (C3), 129.9 (C2), 127.1 (C5), 125.2 (C8), 123.3 (C6), 61.9 (C13), 20.9 (C11), 18.1 (C12), 14.0 (C14).
^{1}H-NMR (400MHz, $CDCl_3$) δ 1.33 (3H, t, J=7.18 Hz, H-14), 2.29 (3H, s, H-12), 2.30 (3H, s, H-11), 4.27 (2H, q, J=7.15 Hz, H-13), 6.20 (1H, d, J=13.29 Hz, H-8), 6.46 (1H, d, J=13.29 Hz, H-9), 7.01 (2H, s, H-3 and H-5), 7.77 (1H, d, J=8.68 Hz, H-6), 10.21 (1H, s, 1-NH).
EI-MS: m/z 247 {$[M]^+$, 50%}.
IR_{vmax} (neat) cm^{-1} : 3241.02 (N-H), 2985.12 (C-H), 1723.62, ~1600.00 (C=O).

IA/45/1/B (n-propyl N-[(2,4-dimethylphenyl) amino] fumaramate)

The compound was obtained as a light yellow crystalline solid which melted at 65 °C. It had the following spectra data.
^{13}C-NMR (400MHz, $CDCl_3$). 166.6 (C7), 162.0 (C10), 140.0 (C9), 135.0 (C1), 133.1 (C4), 131.2 (C3), 129.9 (C2), 127.1 (C5), 125.1 (C8), 123.3 (C6), 67.4 (C13), 21.8 (C11), 20.9 (C12), 18.1 (C14), 10.3 (C15).
^{1}H-NMR (400MHz, $CDCl_3$) δ 0.97 (3H, t, J=7.46 Hz, H-15), 1.71 (2H, m, H-14), 2.29 (3H, s, H-12), 2.30 (3H, s, H-11), 4.17 (2H, t, J=6.72 Hz, H-13), 6.21 (1H, d, J=13.25 Hz, H-8), 6.46 (1H, d, J=13.33" Hz, H-9), 7.01 (2H, s, H-3 and H-5), 7.77 (1H, d, J=8.64 Hz, H-6), 10.24 (1H, s, 1-NH).0
EI-MS: m/z 261 {$[M]^+$, 50%}.
IR_{vmax} (neat) cm^{-1}: 3219.94 (N-H), 2973.55 (C-H), 1720.66, 1672.34 (C=O).

IA/46/1/B (Isopropyl N-[(2,4-dimethylphenyl) amino] fumaramate)

The compound was obtained as a creamy crystalline solid which melted at 97 °C. It had the following spectra data.
^{13}C-NMR (400MHz, $CDCl_3$). 166.0 (C7), 162.0 (C10), 139.9 (C9), 135.0 (C1), 133.1 (C4), 131.2 (C3), 129.9 (C2), 127.1 (C5), 125.7 (C8), 123.3 (C6), 69.8 (C13), 21.7 (C14 and C15), 20.9 (C11), 18.1 (C12).
^{1}H-NMR (400MHz, $CDCl_3$) δ 1.30 (6H, s, H-14 and H-15), 2.29 (3H, s, H-12), 2.30 (3H, s, H-11), 5.11 (1H, m, H-13), 6.17 (1H, d, J=13.29 Hz, H-8), 6.44 (1H, d, J=13.37 Hz, H-9), 7.01 (2H, s, H-3 and H-5), 7.77 (1H, d, J=8.68 Hz, H-6), 10.31 (1H, s, 1-NH).
EI-MS: m/z 261 {$[M]^+$, 50%}.
IR_{vmax} (neat) cm^{-1} : 3324.75 (N-H), 2984.96 (C-H), 1706.07, ~1600.00 (C=O).

IA/47/1/B (n-butyl N-[(2,4-dimethylphenyl) amino] fumaramate)

The compound was obtained as a yellow semi-solid which melted at 47 °C. It had the following spectra data.
^{13}C-NMR (400MHz, $CDCl_3$). 166.0 (C7), 162.1 (C10), 139.3 (C9), 135.0 (C1), 133.0 (C4), 131.2 (C3), 130.1 (C2), 127.0 (C5), 125.4 (C8), 123.5 (C6), 65.7 (C13), 30.4 (C-14), 20.9 (C11), 19.1 (C15), 18.2 (C-12), 13.6 (C-16).
^{1}H-NMR (400MHz, $CDCl_3$) δ 0.93 (3H, t, J=7.38 Hz, H-16), 1.39 (2H, m, H-15), 1.64 (2H, m, H-14), 2.28 (6H, s, H-11 and H-12), 4.19 (2H, t, J=6.66 Hz, H-13), 6.17 (1H, d, J=13.13 Hz, H-8), 6.42 (1H, d, J=13.13 Hz, H-9), 6.98 (1H, s, H-6), 6.99 (1H, s, H-5), 7.72 (1H, d, J=8.72 Hz, H-3), 10.09 (1H, s, 1-NH).
EI-MS: m/z 275 {$[M]^+$, 50%}.
IR_{vmax} (neat) cm^{-1} : 3322.20 (N-H), 2962.12 (C-H), ~1700.00, 1676.14 (C=O).

IA/48/1/B (2-butyl N-[(2,4-dimethylphenyl) amino] fumaramate)

The compound was obtained as a creamy white solid which melted at 76°C. It had the following spectra data.
^{13}C-NMR (400MHz, $CDCl_3$). 166.2 (C7), 162.0 (C10), 140.0 (C9), 135.0 (C1), 133.2 (C4), 131.2 (C3), 130.0 (C2), 127.1 (C5), 125.6 (C8), 123.3 (C6), 74.4 (C13), 28.8 (C11)21.0 (C12), 19.4 (C14), 18.2 (C-16), 9.7 (C-15).
^{1}H-NMR (400MHz, $CDCl_3$) δ 0.92 (3H, t, J=7.48 Hz, H-15), 1.28 (3H, d, J=6.36 Hz, H-14), 1.62 (1H, m, H-13), 2.29 (3H, s, H-12), 2.30 (3H, s, H-11), 4.96 (1H, q, J=6.30 Hz, H-13), 6.19 (1H, d, J=13.41 Hz, H-8), 6.45 (1H, d, J=13.41 Hz, H-9), 7.01 (2H, s, H-3 and H-5), 7.78 (1H, d, J=8.72 Hz, H-6), 10.37 (1H, s, 1-NH).
EI-MS: m/z 275 {$[M]^+$, 50%}.
IR_{vmax} (neat) cm^{-1} : 3321.41 (N-H), 2978.18 (C-H), 1705.46, ~1600.00 (C=O).
Four of these derivatives; **IA/45/1/B, IA/46/1/B, IA/47/1/B, and IA/48/1/B** are novel and are being reported for the first time in this study.
The results (Table 3) show that the synthesized antibiotics had remarkable activity in the range 0.625 - 10 µg/mL against the microorganisms for which they were active against.
The derivatives showed higher activity than the analogue. This is most likely 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 (Strand, 1983; Eckert *et al*., 1988; Alberts *et al*., 1989). The fumaramates have been shown to be more active than the fumaric acids (Jumina *et al.*, 2001; Jumina *et al.*, 2002; Jumina *et al.*, 2005). In fact, A fumaric acid ester is currently under investigation for treatment of multiple sclerosis (Moharregh-Khiabani *et al.*, 2009). The activity of derivatives of C-9154 antibiotic has been shown to be due to the aryl moiety and the enedione moiety (Jha *et al.*, 2010) but the polarity of the derivatives also plays a key part in enhancing activity as has been shown when the ester derivatives were found to be more active than the acid analogues because the more polar carboxylic group impedes passage through the cell membranes of cancer cells which are lipophilic and selectively allow the passage of lipophilic compounds (Jha *et al.*, 2010). All the synthesized compounds showed better activity than the standard drugs used for comparison. The antibacterial standard drug, sparfloxacin, was up to four-fold less active than most of the synthesized derivatives while the antifungal standard drug, fluconazole, was up to ten-fold less active than the synthesized analogue and derivatives for the microorganism against which they were active. All the synthesized compounds except (IA/48/1/B) were able to inhibit the growth of *C. ulcerans* whereas the standard antibacterial could not. While the standard antifungal and the derivatives could not inhibit the growth of *A. nigre*, the analogue (IA/35/1) was able to inhibit its growth but could not inhibit the growths of *S. pyogenes* and *P. mirabilis*. All the synthesized compounds could not inhibit the growths of *P. aeruginosa* and *T. rubrum*. These results have far-reaching consequences for these types of compounds with respect to what they can be used for as the have been shown to be well tolerated in mice whereby doses up to and including 300 mg/kg did not cause any mortality (Jha *et al.*, 2010).

Conclusion

The results show that the synthesized compounds have a clear advantage over the tested standard drugs, and this has opened up the possibility of their application in the treatment of various ailments for which the sensitive microorganisms are responsible for.
The possible applications of these compounds are endless and with further studies could compliment or even replace some of the antibiotic drugs currently in the market since they are less toxic and well tolerated. Other applications for these new antibiotics can be in the veterinary and agricultural fields where they could be useful in combatting some of the diseases that plague both animals and plants.

Some *in vivo* work is on-going to establish the activity of the synthesized compounds as anti-cancer, anti-HIV, antimalarial, antitrypanosomiasis, anti-tuberculosis etc.

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