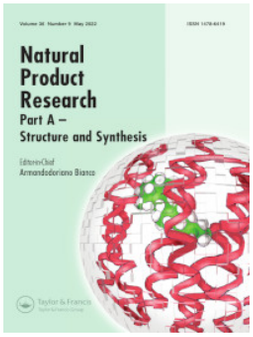

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Synthesis of a pyrrolidine derivative of a carvotacetone and monoterpenes for anti-methicillin-resistant *Staphylococcus aureus* and anti-cryptococcal properties

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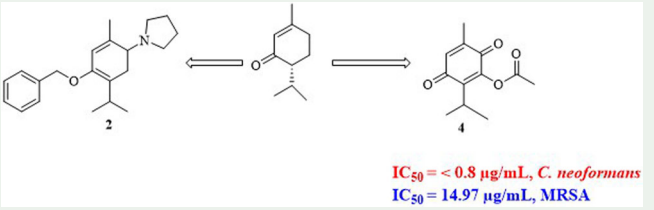
ABSTRACT

Monoterpene derivatives are of great biological relevance in the pharmaceutical industry. In the present study, pyrrolidine derivative of a carvotacetone, 3-*O*-benzylcarvotacetone (**1**), and selected monoterpenes (3-hydroxy-2-isopropyl-5-methyl-*p*-benzoquinone (**3**) and *cis*-piperitol (**5**)) were prepared to provide (*R*)-1-(4-(benzyloxy)-5-isopropyl-2-methylcyclohexa-1,3-dien-1-yl)-pyrrolidine (**2**), 2-isopropyl-5-methyl-3,6-dioxocyclohexa-1,4-dien-1-yl acetate (**4**), *cis*-3-hydroxypiperitone (**6**) and carvacrol (**7**). Structure of **2** was determined based on NMR and HRMS spectral data. Compound **4** exhibited activity against fungi *Cryptococcus neoformans* with an IC_{50} value of $< 0.8\,\mu g/mL$. In addition, this compound **4** had an IC_{50} value of $14.97\,\mu g/mL$ against methicillin resistant *Staphylococcus aureus* bacteria. Previous to the current study, both compound **6** and **7** had been reported to have anti-microbial and anti-fungal activities.



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1. Introduction

Scientists estimate that there are over 5 million fungal species worldwide with close to 300 species causing disease to humans (Perfect et al. 2010). Several species of fungi are known to cause invasive infections, which lead to death of about one million

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people every year worldwide (Brown et al. 2012; Krummenauer et al. 2019). One million cases of meningitis are reported annually worldwide with over half a million deaths estimated in endemic areas of sub-Saharan Africa (Mpoza et al. 2018; Park et al. 2009). The key treatment agents include polyenes, azoles and flucytosine. The emergence of drug resistance to the currently available anti-fungal drugs severely limits therapy because of the few treatment options available in the market (Perlin et al. 2017).

On the other hand, methicillin-resistant bacteria *Staphylococcus aureus* leads to bacteraemia, skin and soft tissue infections, osteomyelitis and endocarditis (Turner et al. 2019). The first medicament (1941) in the treatment of *S. aureus* infections involved use of penicillin, a *beta* lactam antibiotic on which resistance was immediately noted on introduction (North and Christie 1946). Emergence of resistant strains led to the development of methicillin in 1960s, a semisynthetic analogue of penicillin and in less than a decade, methicillin resistant strains of *S. aureus* (MRSA) were reported (Parker 1970). MRSA infections are majorly found in hospitals and communities, with several anti-microbials developed and others still under clinical trials such as daptomycin (Steenbergen et al. 2005), dalbavancin (Chen et al. 2007), linezolid (Brickner et al. 2008), ceftobiprole (Dauner et al. 2010), telavancin (Damodaran and Madhan 2011), ceftaroline (Laudano 2011), oritavancin (Zhanel et al. 2012) and tedizolid (Zhanel et al. 2015). Resistance of these pathogens to multiple antibiotics has activated the search and discovery of novel antibiotic agents with better activity and less side effects.

Plants contain thousands of useful secondary metabolites of medicinal value including essential oils composite of many volatile and odourless compounds (Boye et al. 2020). The essential oils constitute of terpenes which exist as hemiterpenes, monoterpenes or sesquiterpenes, some of which have been reported to be active against fungi, Gram-positive and Gram-negative bacteria (Bhatti et al. 2014; Loomis and Croteau 1980). For instance, carvacrol (**7**), an aromatic monoterpenoid has been reported to demonstrate anti-fungal activities against *Candida albicans* hyphae and *Cryptococcus neoformans* (Teixeira et al. 2020; Vale-Silva et al. 2010), anti-protozoal properties against *P. falciparum* chloroquine sensitive strain (Can Baser 2008), anti-oxidative properties (Prieto et al. 2007). In addition, carvacrol (**7**) has shown anti-microbial efficacy against several bacteria such as methicillin resistant *Staphylococcus aureus* and *E. coli* (Tabanca et al. 2001).

The strong anti-bacterial activities of the aerial extracts of *Foeniculum vulgare* against *Staphylococcus saprophyticus* and *Escherichia coli* were attributed to the presence of several monoterpenoid metabolites such as *cis*-3-hydroxypiperitone (**6**), a monoterpenoid carvotacetone (Zellagui et al. 2011). On the other hand, nitrogenous derivatives including pyrrolidine, morpholine, piperazine and piperidine derivatives are also constituents of secondary metabolites and synthetic bioactive molecules and is deemed as an important pharmacophore in the drug industry (Bellina and Rossi 2006; Enyedy et al. 2001).

In continuation of our previous report on synthesis of 3-*O*-benzyl-carvotacetone and 3-hydroxy-2-isopropyl-5-methyl-*p*-benzoquinone (Masila et al. 2020), in this paper, we describe a strategy to synthesise (*R*)-1-(4-(benzyloxy)-5-isopropyl-2-methylcyclohexa-1,3-dien-1-yl)-pyrrolidine (**2**), 2-isopropyl-5-methyl-3,6-dioxocyclohexa-1,4-dien-1-yl

acetate (**4**), *cis*-3-hydroxypiperitone (**6**) and carvacrol (**7**) that could be of anti-microbial potential. Full spectroscopic characterization and HRMS of the synthesized nitrogenous derivative (**2**) is discussed together with the anti-cryptococcal and anti-MRSA activities of 2-isopropyl-5-methyl-3,6-dioxocyclohexa-1,4-dien-1-yl acetate (**4**).

2. Results and discussion

2.1. Synthesised compounds

The synthesis began from 3-*O*-benzyl-carvotacetone (**1**) as synthesized in Masila et al. (2020). Compound **1** was subjected to *N*-alkylation in presence of pyrrolidine to provide (*R*)-1-(4-(benzyloxy)-5-isopropyl-2-methylcyclohexa-1,3-dien-1-yl)-pyrrolidine (**2**) in 38% (w/w) yield. Success of this reaction was confirmed from both NMR (Figure S2-S7) and HRMS (Figure S8) spectroscopic data. Compound **2** had *m/z* 312.2249 [M + H] corresponding to a molecular formula of $C_{21}H_{29}NO$. From the ^{13}C-NMR, the carbonyl signal at δ 198.1 of the 3-*O*-benzyloxycarvotacetone had disappeared and additional methylene carbons were observed at δ 62.5 (C-11/14) and δ 29.4 (C-12/13) as envisioned in the DEPT spectrum, a clear indication that the pyrrolidine moiety had been incorporated in the compound leading to the formation of an enamine. The deshielded methylene carbon at δ 62.5 confirmed presence of a heteroatom (N). In the ^{1}H-NMR spectrum, the deshielded methylene protons appeared at δ 4.71 (4H, H-11/14) and the other set resonated at δ 1.28 (4H, H-12/13). The presence of a high field deshielded olefinic proton resonating at δ 8.13 (H-3) with strong HMBC correlations with carbon one and two (H-3 $\rightarrow$ C-1, C-2) confirmed formation of an enamine.

Acylation of 3-hydroxy-2-isopropyl-5-methyl-*p*-benzoquinone (**3**) in acetic anhydride (Ranu et al. 2003), provided **4** in 60% (w/w) yield. In addition to the peaks observed in compound **3** as described by Masila et al. (2020), a carbonyl peak of the acyl group (C-1′) resonated at δ 168.1 and the methyl (C-2′) carbon at δ 20.4 ppm with its protons observed at δ 2.37 (H-2′, *s*, 3H) ppm integrating for three protons appearing as a singlet (Figure S10 and S11).The observed values were in agreement with the literature values as reported by Joseph-Nathan and Burgueno-Tapia (2000) (Table S9).

Riley oxidation of *cis*-piperitol (**5**) in the presence of SeO_2 and 1,4-dioxane was also carried out to obtain *cis*-3-hydroxypiperitone (**6**) in 40% (w/w) yield as a reddish-brown oil. The ^{13}C NMR and ^{1}H NMR spectrum (Figure S13 and S14) had a close similarity to compound **5** (Masila et al. 2020). The major difference was presence of an oxy-methine carbon resonating at δ 69.1 (C-4) with an oxy-methine proton observed as a multiplet at δ 4.38 ppm (H-4). The observed and literature values were in agreement as reported by Zellagui et al. (2011) (Table S12). *Cis*-3-hydroxypiperitone has previously been obtained through biotransformation of (-)-(R-)-α-phellandrene (İşcan et al. 2012) and previously isolated as a secondary metabolite from the aerial parts of *Foenicium vulgare* (Zellagui et al. 2011).

In addition, *cis*-piperitol (**5**) synthesised from piperitone (Masila et al. 2020) was successively subjected to Riley oxidation to yield carvacrol (**7**), 33% (w/w) yield. The ^{13}C NMR (Figure S17) spectrum had a total of 10 signals just like the starting material (**5**) (Masila et al. 2020) with one signal at δ 24.1 integrating for two methyl carbons (C-9/C-10) and a methine carbon for the isopropyl unit resonating at δ 33.7 (C-8) ppm. An

additional methyl carbon attached to an aromatic ring was observed at δ 15.4 (C-7) ppm. However, instead of two olefinic carbons, the compound seemed to have aromatized with three sp^2 hybridized carbons appearing at δ 113.0 (C-6), 118.8 (C-4) and 130.9 (C-3). An oxygenated sp^2 hybridized quaternary carbon appeared at δ 153.6 (C-1) with other two quaternary sp^2 hybridized carbons appearing at δ 120.9 (C-2) and 148.5 (C-5) as supported by DEPT spectrum (Figure S18). The isopropyl unit was notably observed, the methine proton appeared at δ 2.86 (*m*, 1H, H-8) and the methyl protons at δ 1.26 (*d*, $J = 5$ *Hz*, 6H, H-9/10). The methyl protons at position C-7 attached to the aromatic ring appeared at δ 2.26, observed as a sharp singlet. In addition, three aromatic protons were observed at δ 6.70 (*s*, 1H, H-6), δ 6.78 (*d*, 1H, H-4) and δ 7.08 (*d*, 1H, H-3) (Figure S16). These observed values were in agreement with the literature values (Tang et al. 2011) (Table S15).

2.2. Anti-microbial activities

Compound **4**, an acylated benzoquinone monoterpenoid, exhibited anti-bacterial activity against MRSA (ATCC **33591**) (IC_{50} value of 14.97 μg/mL) with the benzoquinone (**3**) showing no activity towards the same pathogen (IC_{50} value > 20 μg/mL). The standard drug cefotaxime, meropenem and methicillin had IC_{50} values of 10.50, 2.63 and 17.57 μg/mL (Figure S19). In addition, compound **4** exhibited anti-fungal activity towards *C. neoformans* (ATCC8 **90113**) with an IC_{50} value of < 0.80 μg/mL while the standard drug amphotericin B had an IC_{50} value of 0.53 μg/mL (Figure S20). Furthermore, compound **4** had an $IC_{50} > 20$ μg/mL against the yeast *Candida albicans* (ATCC **90028**) and *Aspergillus fumigatus* (ATCC **204305**), and the bacteria *E. coli* (ATCC **35218**), *Pseudomonas aeruginosa* (ATCC **27853**) and VRE.

3. Experimental

3.1. Instrumentation and reagents

All reagents and other chemicals were purchased from Loba Chemie, Merck and Santa Cruz and used without further purification. Reactions requiring anhydrous conditions were performed under nitrogen. The reactions were done in glassware that were dried in an oven (110 °C) for at least 2 hours and allowed to cool under nitrogen. Solvents for column chromatography were distilled in the laboratory in glass apparatus. Chromatographic separation was done on silica gel (60-120 mesh). Thin layer chromatography was performed on Merck silica gel (60 F_{254}) TLC plates and visualised under 254 nm UV lamp.

^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) were recorded on an Agilent DD2-500 NMR spectrometer (Santa Clara, CA, USA) with tetramethylsilane as an internal standard. The high-resolution mass spectrum was acquired via LCMS (Agilent).

3.2. Synthesis of compound 2, 4, 6 and 7

The compounds were synthesised as represented in Figure 1

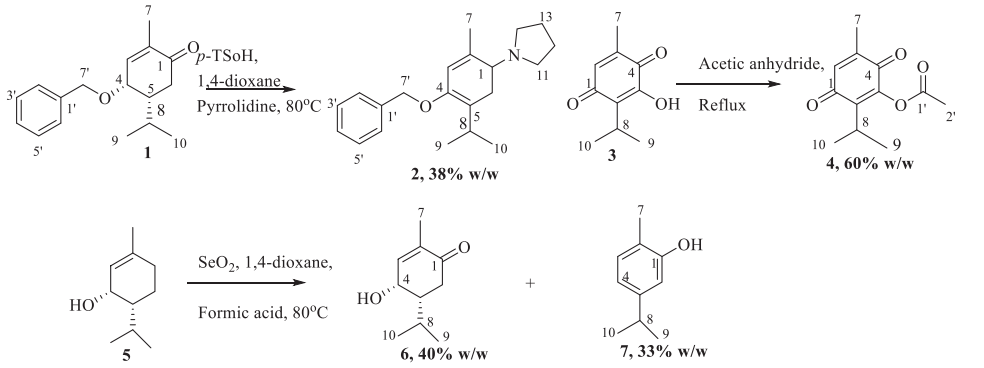

Figure 1. Synthetic route to compounds **2**, **4**, **6** and **7**.

3.2.1. N-alkylation of 3-O-benzyl-carvotacetone (1)

Pyrrolidine (0.1 mL) was added to a solution of 3-*O*-benzyl-carvotacetone **1**, (160 mg, 6.2×10^{-4} mmol) in toluene (10 mL) in a 50 mL round bottomed flask. A catalytic amount of *p*-toluene sulfonic acid (16 mg, 8.41×10^{-5} mmol) was added to the reaction mixture with constant stirring and the mixture refluxed at 80 °C for 72 hours. Formic acid (5 mL) was then added and mixture refluxed for six hours then neutralized with NaOH after cooling. Work up was done by concentrating the mixture on a rotary evaporator. The residue was diluted with water (5 mL) and then extracted with CH_2Cl_2 (20 mL twice). The organic phase was dried (Na_2SO_4), filtered and concentrated under reduced pressure and purified on column chromatography by eluting with 100% dichloromethane to afford a reddish brown oil of **2** (60 mg, 38% (w/w) yield).

3.2.2. Acylation of 3-Hydroxy-2-isopropyl-5-methyl-p-benzoquinone (3)

A mixture of 3-hydroxy-2-isopropyl-5-methyl-*p*-benzoquinone **3**, (10 mg, 5.5×10^{-5} mmol) and acetic anhydride (approximately 0.1 mL) in a 50 mL round bottomed flask was heated at 80 °C for 3 hours. The acetic anhydride was evaporated and the product extracted with diethyl ether (5 mL) then purified by column chromatography on silica gel with 40% dichloromethane in *n*-hexane to provide **4** (6 mg, 60% (w/w) yield).

3.2.3. Riley oxidation of cis-piperitol (5)

Cis-piperitol **5**, (4 g, 0.026 mol) was dissolved in a mixture of formic acid (2 mL) in dioxane (50 mL) in a 100 mL flask. Selenium dioxide (2.5 g, 0.023 mol) was added to the reaction flask and refluxed at 80 °C with constant stirring for three days. The mixture was then concentrated under reduced pressure. The work up was accomplished by adding water (30 mL) and extraction done with dichloromethane (2×200 mL). The combined extract was dried over Na_2SO_4, filtered and then concentrated on a rotary evaporator. The residue was purified on column chromatography with 80% dichloromethane in *n*-hexane to obtain reddish brown oil of **7** (1 g, 33% (w/w) yield). In addition, using the same procedure, *cis*-piperitol, **5**, (200 mg, 1.31×10^{-3} mmol) was also

subjected to Riley oxidation and purification carried out with dichloromethane on the column chromatography to yield **6** (80 mg, 40% (w/w) yield).

3.3. Spectroscopic data

(*R*)-1-(4-(Benzyloxy)-5-isopropyl-2-methylcyclohexa-1,3-dien-1-yl)-pyrrolidine (**2**): Reddish brown oil. ^{1}H and ^{13}C NMR ($CDCl_3$, 500 and 125 MHz): See Table S1, Figure S2-S7: *m/z* 312.2249 [M + H] See Figure S8.

3.4. Anti-microbial assay

All the organisms in the antimicrobial assays were obtained from the American Type Culture Collection (Manassas, VA) and included yeasts *C. albicans* (ATCC **90028**), the fungi *C. neoformans* (ATCC **90113**) and *A. fumigatus* (ATCC **204305**), and the bacteria MRSA (ATCC **33591**), *E. coli* (ATCC **35218**), *P. aeruginosa* (ATCC **27853**) and VRE. Drug control cefotaxime, meropenem and methicillin for bacteria and amphotericin B for yeasts and fungi were included in each assay. Susceptibility testing was performed at the National Centre for Natural Products Research, University of Mississippi, as described (Samoylenko et al. 2009).

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Disclosure statement

Authors declare no conflict of interest.

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