Antibacterial constituents of *Neohyptis paniculata*

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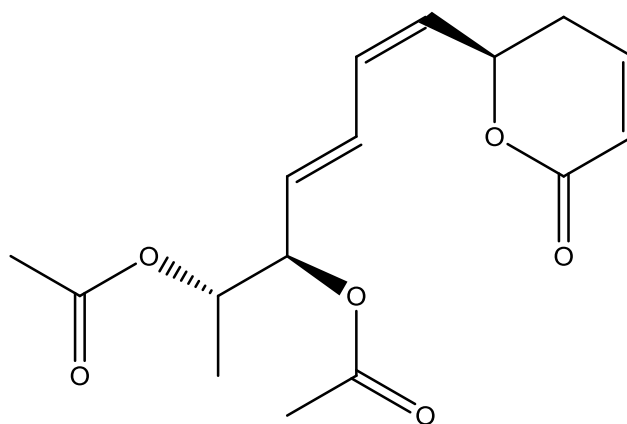
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Graphical Abstract

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Abstract

A new α-pyrone, 6*R*-[5*R*,6*S*-diacetyloxy-1*Z*,3*E*-heptadienyl]-5,6-dihydro-2H-pyran-2one (**1**), along with six known compounds including an α-pyrone, flavones and terpenes was isolated from the aerial parts of *Neohyptis paniculata*. The structure of **1** was established unambiguously by MS and a series of 1D and 2D-NMR spectroscopic analyses. The antibacterial activity of the compounds (**1-7**) was investigated against five strains of multi-drug resistant (MDR) and methicillin-resistant *Staphylococcus aureus* and minimum inhibitory concentrations (MICs) of these compounds were found to be in the range of 64-256 μg/mL.

1. Introduction

Neohyptis paniculata (Bak.) J. K. (Lamiaceae), a slender and erect perennial herb attaining a height of 1-3 ft., is a monotypic species that is characterized by a small and white corolla with purple dots. The plant grows well in savanna swamps of some African countries including Ghana, Guinea, Angola, Cameroon, Angola and Sierra-Leone [1]. This species has not been previously investigated for phytochemistry and bioactivity.

Infections caused by multidrug-resistant (MDR) and methicillin-resistant strains of *Staphylococcus aureus* (MRSA) are still problematic in the clinical environment and the need for new antibacterials is becoming increasingly urgent. As part of an on-going effort to characterize new compounds from species of Lamiaceae [2,3] with antibacterial activity against multidrug-resistant (MDR) strains of *Staphylococcus aureus*, we here report the isolation of a new compound (**1**) together with six known compounds including an α-pyrone, flavones and terpenes from the aerial parts of *N. paniculata* and the antibacterial activities of compounds **1**-**7** against five bacterial strains of MDR and methicillin-resistant *Staphylococcus aureus*.

2. Materials and Methods

2.1 General.

Optical rotations were measured on a Perkin Elmer Polarimeter 341. IR spectra were recorded as a dry film on a Perkin Elmer Spectrum 1000 FT-IR spectrometer. UV spectra were obtained on a Unicam UV 4-100 UV/vi spectrophotometer in MeOH. HREIMS were recorded on a Micromass Q-TOF Global Tandem Mass Spectrometer. NMR spectra (both 1D and 2D) were obtained on a Bruker AVANCE 500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C), using the residual solvent peaks as internal standard. Vacuum-liquid chromatography (VLC) was carried out

using Merck Si gel 60 H. Gel filtration was performed using Sephadex LH-20 (Sigma). TLC and PTLC were conducted on normal-phase Merck Si gel 60 PF_{254} and reverse-phase Merck Si gel RP-18 PF_{254} plates (20 cm X 20 cm). Spots on TLC and PTLC plates were visualised under UV light (254 and 366 nm) and spraying with 1% vanillin-H_2SO_4 followed by heating at 110°C for 5-10 min.

2.2.   Plant material

The aerial parts of *N. paniculata* were collected from Ghana in 2006. A herbarium specimen of this collection is maintained at the UCL School of Pharmacy, University of London, UK.

2.3.   Extraction and isolation

250 g of dried, ground plant material was sequentially extracted with hexane, $CHCl_3$ and methanol in a Soxhlet apparatus. This sequential extraction with solvents of increasing polarity allowed preliminary separation of the components based on the polarity of the metabolites. As both hexane and chloroform extracts showed antibacterial activity, these two extracts were analysed further for the isolation and purification of compounds.

The hexane extract (900 mg) was subjected to gel filtration over Sephadex LH20 (27 g) using a mobile phase of hexane, $CHCl_3$ and MeOH in a ratio of 2:5:1 to obtain a total of 8 fractions (50 mL each fraction). Solid phase extraction (SPE) (Si 60G; mobile phase 100% hexane to 100% EtOAc) on fraction 2 (200 mg) was carried out using hexane and EtOAc in a mixture of increasing polarity collecting 50 mL of each eluent. Preparative TLC (Si gel 60 PF_{254}; 100% hexane) on the SPE sub-fraction eluted with 100% hexane afforded **2** (2.2 mg) and **3** (3.1 mg) while compounds **1** (2.5 mg) and **4** (10.6 mg) were isolated from the SPE sub-fraction eluted with 50% EtOAc in hexane followed by preparative-TLC (Merck Si gel RP-18 PF_{254}; MeOH: H_2O:

AcOH= 59:40:1). Further, preparative TLC (Si gel 60 PF$_{254}$; 15% EtOAc in toluene plus 3-4 drops of AcOH) on the SPE sub-fraction eluted with 10-20% EtOAc hexane led to the isolation of **5** (4.1 mg) and **7** (6.3 mg).

The CHCl$_3$ extract (5.5 g) was fractionated by VLC over Si gel 60H using hexane-EtOAc and EtOAc-MeOH mixtures of increasing polarity. The eluates were combined together on the basis of TLC analysis. VLC fractions eluted with 20-25% EtOAc in petroleum ether were further subjected to preparative-TLC to yield **5** (14.5 mg) and **6** (16.6 mg). Gel filtration over Sephadex LH20 (hexane: CHCl$_3$: MeOH = 2:5:1) on the VLC fractions eluting with 40-50% EtOAc in hexane gave 15.4 mg of **7**.

*2.4.   6R-[5R,6S-diacetyloxy-1Z,3E-heptadienyl]-5,6-dihydro-2H-pyran-2one (**1**)*

White gum. $[\alpha]_D^{20}$ −30.1 (CHCl$_3$; c 4.55). UV λ_{max}^{MeOH} nm (logε): 229 (4.26). IR (solution in CHCl$_3$) ν cm^{-1}: 1736, 1360, 1225, 1070, 1025, 955, 800; ^{1}H NMR, ^{13}C NMR and HMBC, see Table 2. HRMS *m/z* [M+Na] peak at *m/z* 331.1157 (Calcd. 331.1163).

2.5.   Bacterial strains

A standard *S. aureus* strain ATCC 25923 and a clinical isolate (XU212), which possesses the TetK efflux pump and is also an MRSA strain, were obtained from E. Udo [4]. Strain RN4220 which has the MsrA macrolide efflux pump was provided by J. Cove [5]. EMRSA-15 [6] was obtained from Dr Paul Stapleton, UCL. Strain SA1199B which over-expresses the NorA MDR efflux pump was the gift of Professor Glenn Kaatz [7]. Norfloxacin was obtained from the Sigma Chemical Co.

2.6.   Antibacterial assay

Overnight cultures of each strain were made up in 0.9% saline to an inoculum density of 5 x 10^5 cfu/mL by comparison with a MacFarland standard. Tetracycline and oxacillin were dissolved directly in MHB, whereas norfloxacin and erythromycin

were dissolved in DMSO and then diluted in MHB to give a starting concentration of 512 µg/mL. Using Nunc 96-well microtitre plates, 125 µL of MHB were dispensed into wells 1-11. 125µL of the test compound or the appropriate antibiotic were dispensed into well 1 and serially diluted across the plate leaving well 11 empty for the growth control. The final volume was dispensed into well 12, which being free of MHB or inoculum served as the sterile control. Finally, the bacterial inoculum (125 µL) was added to wells 1-11 and the plate was incubated at 37ºC for 18 hours. A DMSO control (3.125%) was also included. All MICs were determined in duplicate. The MIC was determined as the lowest concentration at which no growth was observed. A methanolic solution (5 mg/mL) of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Lancaster) was used to detect bacterial growth by a colour change from yellow to blue [8].

3. *Results and discussion*

The HREIMS of **1** showed an [M+Na] peak at *m/z* 331.1157 (calc. 331.1163) and thereby, established its molecular formula as $C_{16}H_{20}O_6$. The ^{13}C and DEPT135 NMR experiments revealed the presence of 16 carbons; three methyls (15.2, 21.3 and 21.4), one methylene (29.9), three carbonyls (164.0, 170.3 and 170.6) and nine methines, three of which were oxygenated. The ^1H NMR spectrum (500 MHz, CDCl$_3$, Table 1) of **1** showed two acetoxy methyl groups which resonated at 2.06 (3H, s) and 2.09 (3H, s) and a further 3H signal at 1.22 (3H, d, *J*= 6.5 Hz) which was attributable to a terminal secondary methyl group. The spectrum also exhibited two olefinic protons of a dihydropyrone skeleton [9] at 6.09 (dq, *J* = 10.0, 1.0 Hz) and 6.92 (ddd, *J* = 10.0, 5.5, 3.0 Hz), two *cis* olefinic protons (5.64, dd, *J* = 10.5, 9.0 Hz; 6.18, dd, *J* = 11.5, 10.5 Hz) and two *trans* olefinic protons (6.52, dd, *J* = 15.0, 11.5 Hz; 5.77, dd, *J* = 15.0, 7.0 Hz), one methylene (2.44, m) and three oxy-methines (5.08, 5.37 and 5.45).

The unambiguous assignment of all carbons and protons of **1** was achieved by a series of 2D experiments including HMQC, HMBC, COSY and NOESY. In the HMBC experiment, the proton at 6.92 (H-4) showed ^3J connectivity to a carbonyl at 164.0 (C-2), and to an oxygenated methine carbon at 74.0 (C-6; δ_H 5.37 from HMQC). In the COSY experiment, H-4 revealed an expected interaction with H-3 (δ_H 6.09; δ_C 121.9) and H_2-5 (2.44; δ_C 29.9 from HMQC). These chemical shifts and their correlations supported the presence of 5,6-dihydro-2H-pyran-2-one as a part of the molecule. The olefinic proton at 5.64 (H-1'; δ_C 128.8 from HMQC) coupled to H-6 and H-2' (δ_H 6.18; δ_C 131.4) in the COSY experiment and showed ^3J HMBC connectivity to a methylene carbon at 29.9 (C-5) and to an olefinic carbon at 128.4 (C-3'; δ_H 6.52). The latter proton (H-3') coupled to H-2' and H-4' (δ_H 5.77; δ_C 130.6) in the COSY experiment and exhibited a ^3J HMBC correlation to C-1' and to an oxygenated methine 74.8 (C-5'). The remaining oxy-methine proton at 5.08 (H-6') revealed a COSY interaction with H-5' and to a methyl doublet (J = 6.5 Hz) at 1.22 (H_3-7'; δ_C15.2 from HMQC). The remaining methyls at 2.06 and 2.09 ppm showed ^2J HMBC connectivity to their respective acetyl carbonyl carbons at 170.3 and 170.6. The ^3J HMBC correlation by H-5' and H-6' to the carbonyls at 170.6 and 170.3 confirmed their connectivity *via* C-5' and C-6', respectively. Accordingly, compound **1** was therefore identified as 6ζ-[5ζ,6ζ-diacetyloxy-1Z,3E-heptadienyl]-5,6-dihydro-2H-pyran-2one (**1**, Fig 1; named neohyptolide) which is a new compound. Because of paucity of the compound, it was not possible to carry out further study to confirm the absolute stereochemistry of the compound. However, *R*-configuration was proposed at C-5 by comparing its ^{1}H NMR data to other structurally related hyptolides [9, 10]. Again, in view of the similarity of the coupling constants of the side chain protons at C-5' and C-6' of **1** to hyptolides reported from *Hyptis oblongifolia* [9] and

pectinolides from *Hyptis pectinata* [11], the configurations of these chiral centers of **1** were assumed to be the same as in those compounds, i.e., *R*-configuration at C-5' and *S*-configuration at C-6'. So taking these configurations into account, compound 1 was considered to be 6*R*-[5*R*,6*S*-diacetyloxy-1*Z*,3*E*-heptadienyl]-5,6-dihydro-2H-pyran-2one.

By direct comparison of spectral data to those published in the literature, compounds **2-7** were identified as α-himachalene (**2**) [12], 4-deacetoxy-10-epi-olguine (**3**) [9], isoneocembrene-A (**4**) [13], β-caryophyllene oxide (**5**) [14], 3,5-dihydroxy-7,4'-dimethoxyflavone (**6**) [15], 5-hydroxy-3,7,4'-trimethoxyflavone (**7**) [15].

All compounds (**1-7**) were assessed for anti-staphylococcal activity in a minimum inhibitory concentration (MIC) assay but displayed only weak to moderate activity (Table 2). Among the compounds, the comparatively better anti-staphylococcal activity was exhibited by compound **1**. α–Pyrones with structural similarity to **1** have been reported to exhibit antibacterial properties including those against the clinical isolates of *Staphylococcal aureus* [16]. The cytotoxic potential of pectinolides was also reported with a number of cultured cell lines [16]. It is suggested that the presence of an alpha-beta unsaturated ketone, which is part of the lactone moiety may make this compound a substrate for biological nucleophiles and it is likely that this compound is also cytotoxic to mammalian cells as is seen in the case of the pectinolides [16].

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Table 1

^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz) and HMBC data of **1** in CDCl$_3$

Position	^{1}H	^{13}C	HMBC	
			^2J	^3J
2	-	164.0	-	-
3	6.09, dq, J = 10.0, 1.0 Hz	121.9	C-2, C-4	C-5
4	6.92, ddd, J = 10.0, 5.5, 3.0 Hz	144.8	C-3	C-2, C-6
5	2.44, m	29.9	C-4	C-3, C-1'
6	5.37, ddt, J = 10.0, 5.0, 1.0 Hz	74.0	-	C-4, C-2'
1'	5.64, dd, J = 10.5, 9.0 Hz	128.8	C-2'	C-5, C-3'
2'	6.18, dd, J = 11.5, 10.5 Hz	131.4	C-1', C-3'	C-6, C-4'
3'	6.52, dd, J = 15.0, 11.5 Hz	128.4	C-2', C-4'	C-1', C-5'
4'	5.77, dd, J = 15.0, 7.0 Hz	130.6	C-3', -5'	C-4', -6'
5'	5.45, ddd, , J= 7.0, 3.5, 1.0 Hz	74.8	C-6'	C-3', -7'
6'	5.08, dq, J = 6.5, 3.5 Hz	70.7	C-5'	C-4'
7'	1.22, d, J = 6.5 Hz	15.2	C-6'	C-5'
C<u>H</u>$_3$CO-5'	2.09, s	21.3	CO-5'	-
C<u>H</u>$_3$CO-6'	2.06, s	21.4	CO-6'	-
CH$_3$<u>C</u>O-5'	-	170.6	-	-
CH$_3$<u>C</u>O-6'	-	170.3	-	-

Table 2

Minimum Inhibitory Concentrations (μg/mL) of compounds **1-7** against clinical isolates of multi-drug resistant (MDR) and methicillin-resistant strains of *Staphylococcus aureus*.

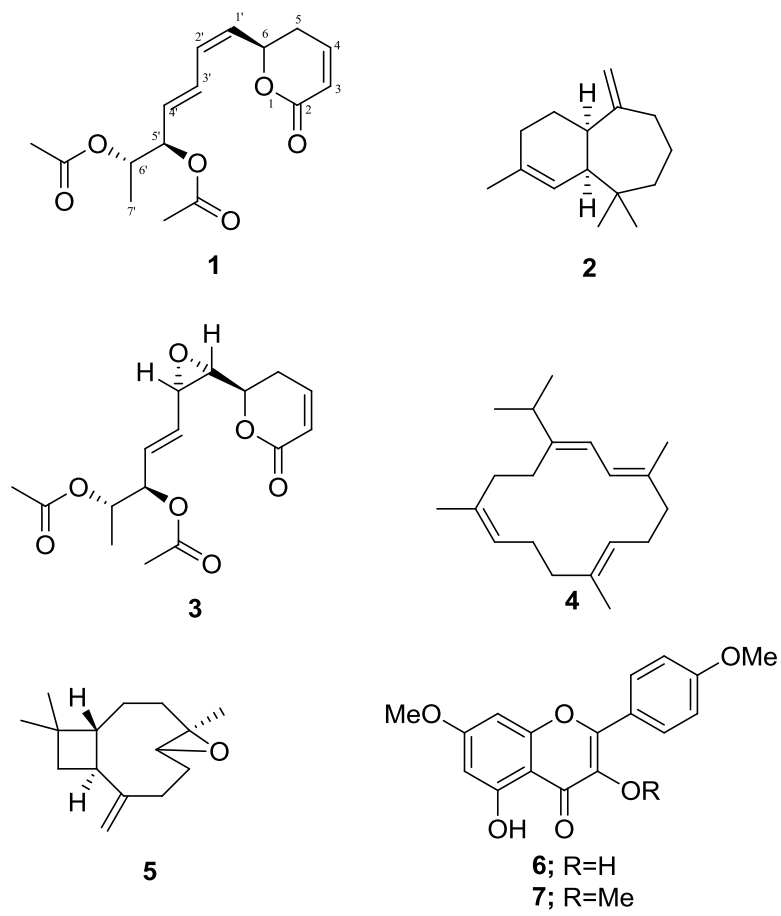| Compounds	SA1199B	RN4220	EMRSA-15	XU-212	ATCC25923
1	64	64	64	64	128
2	64	64	128	64	128
3	64	128	64	128	128
4	64	128	128	64	128
5	256	256	256	128	128
6	256	128	128	128	128
7	256	256	256	128	256
Norfloxacin	32	2	1	16	1

Fig 1. Structures of compound **1-7**