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Anti-staphylococcal acylphloroglucinols from *Hypericum beanii*

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Abstract

As part of an ongoing project to investigate the anti-staphylococcal properties of the *Hypericum* genus, an acylphloroglucinol, 1,5-dihydroxy-2-(2′-methylpropionyl)-3-methoxy-6-methylbenzene (**1**), was isolated from the dichloromethane extract of the aerial parts of *H. beanii* (Guttiferae), together with a minor related acylphloroglucinol 1,5-dihydroxy-2-(2′-methylbutanoyl)-3-methoxy-6-methylbenzene (**2**) as a mixture in a 5:2 ratio. The known compounds 1,7-dihydroxyxanthone (**3**), stigmasterol, catechin and shikimic acid were also isolated from this plant. The structures of the compounds were characterized by extensive 1- and 2D NMR spectroscopy and mass spectrometry. The minimum inhibitory concentration (MIC) values the acylphloroglucinol mixture and (**3**) against a panel of multidrug-resistant strains of *Staphylococcus aureus* ranged from 16–32 μg/ml to 128–256 μg/ml, respectively.

Keywords: *Hypericum beanii*; Guttiferae; Acylphloroglucinol; Phloroglucinol; Xanthone; MRSA; *Staphylococcus aureus*; Antibacterial; MDR

1. Introduction

Multidrug-resistant *Staphylococcus aureus* (MRSA) infections, particularly those caused by methicillin-resistant *S. aureus*, have been a major threat to public health in hospitals and the community in the past decade. In the UK, the number of MRSA infections rose by nearly 5% between 2003 and 2004 (White, 2004). The current treatment of MRSA infections in the UK includes the glycopeptides vancomycin (Vancocin®) and teicloplanin (Targocid®), the oxazolidinone linezolid (Zyvox®), and a combination of the streptogramins, quinupristin and dalfopristin (Synercid®) (British National Formulary, 2006). Despite the new advances in antibiotic development, MRSA infections remain a considerable concern owing to the anticipated resistance to these new drugs. In 2002, MRSA strains fully resistant to vancomycin were isolated in the US (Morbidity Mortality Weekly Report, 2002). Resistance to linezolid has also been reported in some patients followed by prolonged antibiotic treatment in the US (Peeters and Sarria, 2005). Therefore, there is an urgent need to develop new classes of antibiotics to fight the problem of drug resistance.

The genus *Hypericum* (Guttiferae) is known to produce antibacterial metabolites, including the major antibacterial principal hyperforin from *Hypericum perforatum* (Schempp et al., 1999), hyperbrasilols from *Hypericum brasiliense* (Rocha et al., 1995, 1996) and drummondins from *Hypericum drummondii* (Jayasuriya et al., 1991). Hyperforin is an acylphloroglucinol, which consists of a phloroglucinol skeleton substituted with complex isoprene side-chains and a simple 2-methylpropanoyl group. Its antibacterial activity against penicillin-resistant *S. aureus* (PRSA) and MRSA is exceptional, with minimum inhibitory concentration (MIC) values ranging from 0.1 to 1 μg/ml (Schempp et al., 1999). These findings prompted us to investigate the antistaphylococcal activity of 34 *Hypericum* species collected from the National *Hypericum* collection at the Royal Botanic Gardens at Wakehurst Place, UK (Gibbons et al., 2002). The chloroform extract of *Hypericum beanii* was one of the most active species against *S. aureus* in the preliminary evaluation. This is the first report on the phytochemistry and anti-staphylococcal activity of this species.

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2. Results and discussion

Fractionation of the dichloromethane extract of the aerial parts of *H. beanii* led to the isolation of a mixture of a major compound (**1**) and a minor related compound (**2**) in a ratio of 5:2. HR ESI-TOF-MS of the mixture suggested molecular formulae of $C_{12}H_{17}O_4$ $[MH]^+$ (225.1129) (**1**) and $C_{13}H_{19}O_4$ $[MH]^+$ (239.1280) (**2**). Due to the similarity in polarity and size of these compounds, it was not possible to further purify (**1**) and (**2**). This problem was also experienced in isolating phloroglucinol derivatives from *Hypericum papuanum* by Winkelmann et al. (2000). The ^1H NMR spectrum (Table 1) showed the presence of one aromatic proton (δ_H 6.02, 1H), one methoxyl group (δ_H 3.86, 3H), one septet (δ_H 3.79, 1H), one deshielded methyl singlet (δ_H 1.97) and two overlapping methyl doublets (δ_H 1.14, 6H, $J = 7$ Hz). Six aromatic carbon signals were observed in the ^{13}C spectrum, indicating the presence of an aromatic ring. The three signals with chemical shifts of approximately 160 ppm implied that they were attached to an electron-withdrawing group, for example a hydroxyl group or a methoxyl group as they were deshielded. These carbon resonances were typical for a phloroglucinol (1,3,5-trihydroxylated benzene) (Gibbons et al., 2005). Assuming a phloroglucinol in the HMBC spectrum, the aromatic proton (H-4) coupled to four aromatic quaternary carbons (C-2, C-3, C-5 and C-6), two of which were oxygen-bearing (Fig. 1a). This proton was placed between the two oxygen-bearing carbons (C-3 and C-5). The methoxyl group was then placed next to the aromatic proton at C-3. This was confirmed by HMBC studies which showed correlations between the aromatic proton and the methoxyl group to an oxygen-bearing carbon (δ_C 162.1, C-3). A correlation between H-4 and the methoxyl in the NOESY spectrum also provided evidence that these groups were *ortho* to each other. The deshielded methyl group showed ^1H–^{13}C correlations in the HMBC spectrum with three aromatic carbons, one to which it was directly attached (C-6, δ_C 105.0) and two oxygen-bearing quaternary carbons (C-1, δ_C 166.1 and C-5, δ_C 162.2). This group was therefore placed at position 6 of the 1,3,5-trihydroxylated benzene.

The final substituent at position 2 included a methine septet which was coupled to the six-hydrogen methyl doublet in the COSY spectrum, indicating the presence of an isopropyl side chain. In the HMBC spectrum correlations were observed between the methyl doublets and the methine (^2J), methyl (^3J) and the carbonyl carbon (^3J, δ_C 211.4). This indicated that the isopropyl group was part of a 2′-methylpropionyl group. In the NOESY spectrum, cross-peaks were seen between the methoxyl group and the methyl groups of the isopropyl group. This correlation implied that the methoxyl group must be attached to a carbon next to the 2′-methylpropionyl-bearing carbon in the aromatic ring. Compound **1** was therefore identified as 1,5-dihydroxy-2-(2′-methylpropionyl)-3-methoxy-6-methylbenzene.

A minor compound **2** was isolated with compound **1**. The ^1H and ^{13}C data were almost identical with those of **1** with the exception of the side-chain at C-2 (Table 1). In the same ^1H spectrum, a methyl doublet (δ_H 1.16), a methine multiplet (δ_H 3.68, 0.4H), a methylene multiplet (δ_H 1.38 and δ1.81) and a methyl triplet (δ_H 0.92, 1.5H) were observed. In the COSY spectrum, the methyl triplet was coupled to the methylene which was coupled to the methine. The methine was also coupled to the methyl doublet. In the HMBC spectrum, this methyl doublet showed correlations to the methine (^2J), methylene (^3J) and a carbonyl (^3J) carbon (Fig. 1b). The methyl triplet exhibited ^1H–^{13}C correlations to the methylene (^2J) and methine (^3J). This confirmed the presence of the 2-methylbutanoyl side-chain in compound **2**. This side-chain was also found in the acylphloroglucinol isolated from *Hypericum foliosum* by our group (Gibbons et al., 2005). The NMR data for this side-chain showed a close agreement with those obtained by Gibbons et al. (2005). The molecular formula

Table 1
^1H (500 MHz) and ^{13}C NMR (125 MHz) spectral data and ^1H–^{13}C long-range correlations of **1** and **2** recorded in CD_3OD

1					**2**			
Position	^1H	^{13}C	^2J	^3J	^1H	^{13}C	^2J	^3J
1	–	166.1			–	166.1		
2	–	105.0			–	105.0		
3	–	162.1			–	162.1		
4	6.02	91.2	C3, C5	C2, C6	6.02	91.2	C3, C5	C2, C6
5	–	162.2			–	162.2		
6	–	105.0			–	105.0		
7	1.97	7.41	C6	C1, C5	1.97	7.41	C6	C1, C5
1′	–	211.4			–	211.2		
2′	3.79 *m*	40.5			3.68 *m*	47.3		
3′	1.14 *d* (7)	19.8	C2′	C1′, C4′	1.38 *m*, 1.81 *m*	28.3		
4′	1.14 *d* (7)	19.8	C2′	C1′, C3′	0.92 *t* (7.5)	12.4	C3′	C2′
5′	–	–	–	–	1.17 *d* (6)	17.2	C2′	C1′, C3′
3-OCH_3	3.86	55.9		C3	3.86	55.9		C3

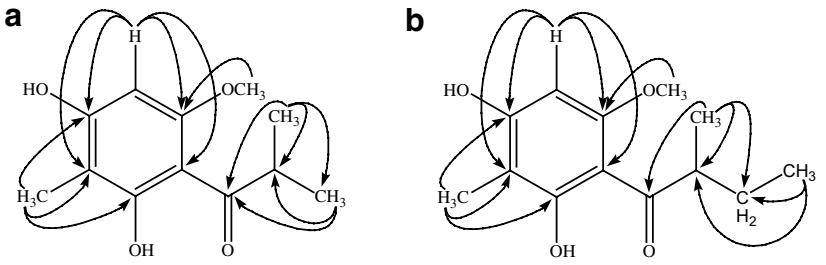
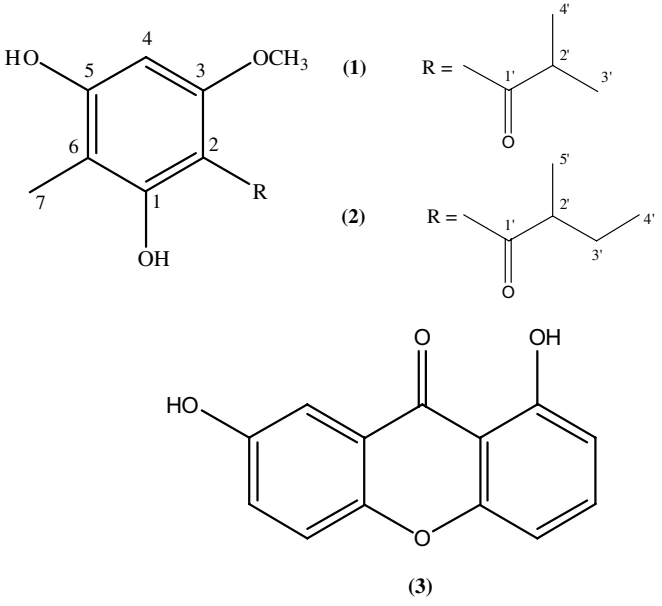


Fig. 1. Key HMBC correlations for **1** (a) and **2** (b).

of **2** differed to that of **1** by an addition of a CH_2 as suggested by mass spectrometry. Compound **2** was therefore identified as 1,5-dihydroxy-2-(2′-methylbutanoyl)-3-methoxy-6-methylbenzene and is described here for the first time.

Repeated chromatography on the DCM extract yielded compound **3** as a yellow oil. HR ESI-MS of **3** suggested a molecular formula of $C_{13}H_8O_4$ $[M+H]^+$ (229.0506). The ^{13}C NMR spectrum displayed 13 signals in the aromatic region, including a signal for carbonyl carbon. The DEPT-135 spectrum showed 6 positive signals, indicating that 7 quaternary carbons were present and that methylene groups were absent. These signals were characteristic for a xanthone. The ^1H NMR spectrum revealed 3 aromatic protons in the ABD system with resonances at δ7.45 (*d*, $J_{ortho} = 9.0$ Hz, H-5), δ7.33 (*dd*, $J = 3.0$, 9.0 Hz, H-6) and δ7.54 (*d*, $J_{meta} = 3.0$ Hz, H-8) and 3 aromatic protons in an ABC system with resonances at δ 6.75 (*dd*, $J = 0.5$, 7.8 Hz, H-2), δ 7.64 (*t*, $J = 8.3$ Hz, H-3) and δ 6.97 (*dd*, $J = 1.0$, 8.5 Hz, H-4). Compound **3** was identified as 1,7-dihydroxyxanthone. The NMR data for this compound were in close agreement with that in the literature (Lin et al., 1996).



Fractionation of the hexane fraction led to the isolation of stigmasterol. Fractionation of the acetone fraction led to the isolation of catechin and shikimic acid. The structures of these compounds were elucidated from 1D and 2D NMR experiments and their molecular formula confirmed by HR ESI-MS. The data for these compounds are in agreement with that published (Forgo and Kover, 2004; Foo et al., 1996; Hall, 1964). These metabolites were inactive against *S. aureus*.

The mixture of compounds **1** and **2** was active against the tested *S. aureus* strains with MIC values of 16–32 μg/ml (Table 2). It was more active against multi-drug resistant strains XU212 and RN4220 than the standard *S. aureus* strain ATCC 25923. XU212, which possesses the TetK efflux transporter, and is resistant to both tetracycline and methicillin. RN4220 carries the MsrA macrolide efflux protein and is resistant to erythromycin. The activity of the mixture against SA-1199B was comparable to that of norfloxacin, the control antibiotic. SA-1199B possesses the NorA efflux protein which confers resistance to certain fluoroquinolones and quaternary ammonium antiseptics. Compound **3** exhibited weak anti-staphylococcal activity against all the tested strains with MIC values of 128–256 μg/ml. It had a similar activity against RN4220 as erythromycin. However, it was less active than the control antibiotics against other strains. Both compounds **1** and **2** are small molecules with simple structures and moderate anti-staphylococcal activity which could be enhanced. Derivatives of these compounds could be readily synthesized to investigate the structure–activity relationship of the acylphloroglucinol class of compounds.

Table 2
MICs of **1** and **2**, **3** and standard antibiotics in μg/ml

Strain (MDR efflux protein)	**1** and **2**	**3**	Norfloxacin	Tetracycline	Erythromycin
ATCC 25923	32	256	1	–	–
SA-1199B (NorA)	32	256	32	–	–
XU212 (TetK)	32	256	–	128	–
RN4220 (MsrA)	16	128	–	–	128

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded on a Bruker AVANCE 500 MHz spectrometer. Chemical shifts values (δ) were reported in parts per million (ppm) relative to the appropriate internal solvent standard and coupling constants (J values) were given in Hertz. IR spectra were recorded on a Nicolet 360 FT-IR spectrophotometer and UV spectra on a Thermo Electron Corporation Helios spectrophotometer. Accurate mass spectrum of mixture **1** and **2** was obtained using a micrOTOF spectrometer. Mass spectra of all other compounds were recorded on a Finnigan MAT 95 high resolution, double focusing, magnetic sector mass spectrometer. Accurate mass measurement was achieved using voltage scanning of the accelerating voltage. This was nominally 5 kV and an internal reference of heptacosa was used. Resolution was set between 5000 and 10,000.

3.2. Plant material

The aerial parts of *H. beanii* were collected from the Royal Botanic Garden at Wakehurst Place in Surrey in August 2003 (Accession No. 1988-8790). A voucher specimen was deposited in the herbarium at the Centre for Pharmacognosy and Phytotherapy at the University of London School of Pharmacy.

3.3. Extraction and isolation

Air-dried, powdered aerial parts of *H. beanii* (650 g) were extracted exhaustively in a Soxhlet apparatus with solvents (3 l) of increasing polarity (hexane, dichloromethane, acetone and methanol). LH-20 Sephadex chromatography of the dichloromethane extract (5.7 g) eluted with dichloromethane yielded five different fractions by combining fractions showing similar TLC profile and one fraction with a final methanol wash. The fraction eluted with methanol was subjected to reverse phase solid phase extraction (SPE; Phenomenex Strata silica, 10 g/60 ml giga tubes) using a step gradient system with 10% increments from 100% water to 100% methanol, yielding 11 fractions. The fraction eluted with 100% methanol was further separated by preparative thin-layer chromatography (pTLC) using toluene–ethylacetate–acetic acid (TEA 80:18:2), yielding a mixture of compounds **1** and **2** (1.7 mg) and **3** (1.6 mg). The mixture of **1** and **2** and compound **3** had R_f values of 0.70 and 0.75, respectively.

3.4. Bacterial strains

S. aureus standard strain ATCC 25923 and tetracycline-resistant strain XU212 which possesses the TetK tetracycline efflux protein were provided by Gibbons and Udo (2000). Strain SA-1199B which overexpresses the norA gene encoding the NorA MDR efflux pump was provided by Kaatz et al. (1993). Strain RN4220 which possess the MsrA macrolide efflux protein was provided by Ross et al. (1989).

3.5. Minimum inhibitory concentration (MIC) assay

All strains were cultured on nutrient agar (Oxoid) and incubated for 24 h at 37 °C prior to MIC determination. Control antibiotics norflorxacin, tetracycline and erythromycin were obtained from Sigma Chemical Co. Mueller-Hinton broth (MHB; Oxoid) was adjusted to contain 20 and 10 mg/l of Ca^{2+} and Mg^{2+}, respectively. An inoculum density of 5×10^5 cfu of each *S. aureus* strain was prepared in normal saline (9 g/l) by comparison with a 0.5 MacFarland turbidity standard. The inoculum (125 µl) was added to all wells and the microtitre plate was incubated at 37 °C for 18 h. For MIC determination, 20 µl of a 5 mg/ml methanolic solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added to each of the wells and incubated for 20 min. Bacterial growth was indicated by a colour change from yellow to dark blue. The MIC was recorded as the lowest concentration at which no growth was observed (Gibbons and Udo, 2000).

3.6. 1,5-Dihydroxy-2-(2′-methylpropionyl)-3-methoxy-6-methylbenzene (*1*)

Pale yellow oil; UV (MeOH) λ_{max} (log ε): 293 (4.0), 206 (4.3) nm; IR ν_{max} (thin film) cm^{-1}: 3650, 1655, 1560, 1543, 1458, 1026; ^1H NMR and ^{13}C NMR (MeOD): see Table 1; HR ESI-TOF-MS (m/z): 225.1129 $[MH]^+$ (calc. for $C_{12}H_{17}O_4$, 226.1200).

3.7. 1,5-Dihydroxy-2-(2′-methylbutanoyl)-3-methoxy-6-methylbenzene (*2*)

Pale yellow oil; UV (MeOH) λ_{max} (log ε): 293 (4.0), 206 (4.3) nm; IR ν_{max} (thin film) cm^{-1}: 3650, 1655, 1560, 1543, 1458, 1026; ^1H NMR and ^{13}C NMR (MeOD): see Table 2; HR ESI-TOF-MS (m/z): 239.1280 $[MH]^+$ (calc. for $C_{13}H_{19}O_4$, 240.1356).

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