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Phytochemistry 66 (2005) 1472–1475
PHYTOCHEMISTRY
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An anti-staphylococcal acylphloroglucinol from *Hypericum foliosum*

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Received 10 February 2005; received in revised form 5 April 2005
Available online 25 May 2005

Abstract

An investigation into the antibacterial properties of *Hypericum foliosum* Aiton. (Guttiferae) has led to the isolation of a new bioactive acylphloroglucinol natural product which by NMR spectroscopy and mass spectrometry was characterised as 1,3,5-trihydroxy-6-[2‴,3‴-epoxy-3‴-methyl-butyl]-2-[2″-methyl-butanoyl]-4-[3′-methyl-2″-butenyl]-benzene and is described here for the first time. This metabolite was evaluated against a panel of multidrug-resistant strains of *Staphylococcus aureus* and minimum inhibitory values ranged from 16 to 32 μg/ml.

Keywords: *Hypericum foliosum*; Acylphloroglucinol; Guttiferae; Antibacterial; MRSA; MDR; *Staphylococcus aureus*

1. Introduction

The genus *Hypericum* is a rich source of antibacterial metabolites of which hyperforin from *Hypericum perforatum* (St. John's Wort) is an exceptional example. Minimum inhibitory concentration (MIC) values for this natural product range from 0.1 to 1 μg/ml against penicillin-resistant *Staphylococcus aureus* (PRSA) and methicillin-resistant *S. aureus* (MRSA) strains (Schempp et al., 1999; Reichling et al., 2001). These results substantiate the use of St. John's Wort in several countries as a treatment for superficial burns and wounds that heal poorly (Reichling et al., 2001). Additionally, the possible use of this agent as an antibiotic is supported by the observation that no in vitro resistance has been observed at low concentrations and that even in strains with reduced susceptibility, no cross resistance with clinically used antibiotics could be detected (Hübner, 2003). Given the considerable need to find novel anti-staphylococcal compounds and the fact that surprisingly at present there are no single chemical entity plant-derived antibacterials used clinically, a molecule with hyperforin's activity may well become a development candidate.

Hyperforin is a member of the acylphloroglucinol group of natural products which are prolific within the Guttiferae family, although only a small number of species of *Hypericum* have been investigated for the antibacterial properties of these metabolites (Winkelmann et al., 2000, 2001; Gibbons, 2004). These natural products are based on an aromatic ring that in many cases has been reduced or has a keto-enol form. Many of these products are prenylated and/or farnesylated and possess simple acyl groups such as 2-methylpropanoyl which is found in hyperforin.

Our antibacterial studies on the *Hypericum* group started with an evaluation of 34 species and varieties collected from the national *Hypericum* collection at the Royal Botanic Gardens at Wakehurst Place, UK (Gibbons et al., 2002). This preliminary study revealed the potential of *Hypericum* metabolites as antibacterials

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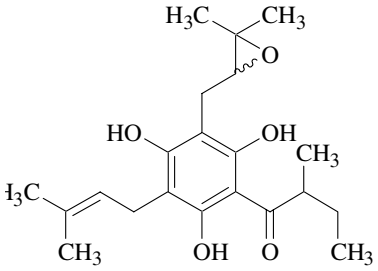
doi:10.1016/j.phytochem.2005.04.024

and prompted us to make several large scale collections, one of which was *Hypericum foliosum* Aiton, a species which is classified under section *Androsaemum* and is endemic to the Azores (Robson, 1977). There is little phytochemical data on this species although the essential oil has been characterised (Santos et al., 1999). This paper details the isolation and characterisation of the main anti-staphylococcal constituent of this species.



(**1**)

2. Results and discussion

Antibacterial activity was concentrated in the hexane extract of the aerial parts of *H. foliosum* and the MIC was 64 μg/ml, against *S. aureus* SA-1199B. This strain possesses the NorA multidrug efflux transporter which is the major characterised drug efflux pump in *S. aureus* and confers resistance to certain fluoroquinolones and quaternary ammonium antiseptics (Marshall and Piddock, 1997). Preparative HPLC of vacuum liquid chromatography fraction 4 (eluted with 30% EtOAc in hexane), the most active fraction (MIC = 32 μg/ml), led to the isolation of compound **1** as a yellow oil.

HRCI-MS of **1** suggested a molecular formula of $C_{21}H_{30}O_5$ $[M]^+$ (362.2079). The ^1H NMR spectrum (Table 1) provided signals for two highly deshielded hydrogen bonded hydroxyl groups (δ_H 14.26, 14.25) and an additional broad signal at δ_H 6.31 attributable to a proton of another hydroxyl group. Further signals for an olefin (δ_H 5.28 t, 1H), two methine protons (δ_H 3.81, 3.74), three methylene groups, four methyl singlets, one methyl doublet and one methyl triplet completed the ^1H resonances for **1** . The olefinic triplet was reminiscent of an olefinic proton of a prenyl (dimethyl allyl) substituent (Nayar and Bhan, 1972) and this was supported by HMBC correlations between the protons of two methyl groups (δ_H 1.84, 1.79) and the carbon associated with this olefin. The olefinic resonance also coupled to the protons of one of the methylene groups (δ_H 3.40 d, J = 7.2 Hz) further confirming the presence of a prenyl moiety. In the HMBC spectrum, the protons of this methylene coupled to three aromatic carbons, one to which it was directly attached (δ_C 105.6) and to two oxygen-bearing quaternary carbons. Hydroxyl groups were attached to these carbon atoms and key correlations were seen by the protons of these groups to carbons in the aromatic ring (Fig. 1). The carbon spectrum was indicative of a 1,3,5-trihydroxylated benzene with six quaternary carbons, three of which were deshielded due to the presence of hydroxyl substituents (Table 1). This was supported by the presence of two hydrogen-bonded and one non-hydrogen bonded OH groups in the ^1H spectrum. With the prenyl group attached at C-4 of this substituted benzene and the lack of aromatic protons, positions 2 and 6 of the aromatic nucleus must be substituted with further moieties. This was proved by inspection of the HMBC spectrum which showed correlations between the protons of a methylene group (δ_H 2.86, 2.61) and an aromatic quaternary carbon (C-6). In the COSY spectrum, these methylene protons cou-

Table 1
^1H (500 MHz) and ^{13}C NMR (125 MHz) spectral data and ^1H–^{13}C long-range correlations of **1** recorded in $CDCl_3$

Position	^1H	^{13}C	^2J	^3J
1	–	153.9		
2	–	*105.6		
3	–	163.0		
4	–	*105.7		
5	–	160.0		
6	–	97.8		
1′	3.40 *d* (7.2)	21.9	C-2′, C-4	C-3, C-5, C-3′
2′	5.28 *t* (7.2)	122.1		
3′	–	136.5		
4′	1.79 *s*	26.1	C-3′	C-2′, C-5′
5′	1.84 *s*	18.1	C-3′	C-2′, C-4′
1″	–	210.7		
2″	3.74 *m*	46.4		
3″	1.43 *m*, 1.85 *m*	27.1		
4″	0.91 *t* (7.6)	12.1	C-3″	C-2″
5″	1.17 *d* (6.6)	17.0	C-2″	C-1″, C-3″
1‴	2.61 *dd* (16.7, 5.4)	26.2	C-6	
	2.86 *dd* (16.7, 5.0)			
2‴	3.81 *bs*	68.9		
3‴	–	78.3		
4‴	1.39 *s*	24.9	C-3‴	C-2‴, C-5‴
5‴	1.42 *s*	22.1	C-3‴	C-2‴, C-4‴
1/3 - OH	*14.26 / *14.25	–		C-2/C-4
5 - OH	6.31 *bs*	–	C-5	C-4, C-6

Resonances denoted * may be interchangeable.

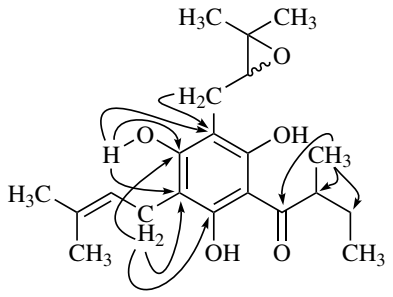


Fig. 1. Key HMBC correlations for compound **1**.

pled to a broad singlet of an oxymethine group with resonances attributable to an epoxide moiety (Asakawa et al., 1991). Two methyl singlets exhibited ^{3}J correlations to the carbon associated with this oxymethine and a ^{2}J correlation to an additional oxygen bearing portion of the epoxide to which the methyl groups were directly attached. This revealed the second substituent to be an epoxidised prenyl group and resonances for this portion are in close agreement to those found in the literature for epoxyprenyl groups which are directly attached to an aromatic ring and *ortho* to an hydroxyl (Asakawa et al., 1991).

Resonances for the final substituent included a methyl triplet which was coupled to a methylene group in the COSY spectrum. This methylene moiety further coupled to a methine proton which was coupled to by a methyl doublet. This methyl doublet exhibited correlations in the HMBC spectrum to the methine (^{2}J), methylene (^{3}J) and a ketonic carbon (^{3}J). These resonances completed the final substituent and identified it as a 2-methylbutanoyl moiety which is a side chain found in acylphloroglucinol natural products from *Hypericum papuanum* (Winkelmann et al., 2000). The point of attachment of this 2-methylbutanoyl moiety must be at C-2 of the aromatic ring allowing hydrogen-bonded deshielding of the hydroxyl groups at C-1 and C-3 via interaction with the carbonyl of the 2-methylbutanoyl group. Compound **1** is therefore assigned as the new acylphloroglucinol 1,3,5-trihydroxy-6-[2‴,3‴-epoxy-3‴-methyl-butyl]-2-[2″-methylbutanoyl]-4-[3′-methyl-2″-butenyl]-benzene and is described here for the first time. Acylphloroglucinols that are structurally related to **1** include adhumulone which possesses a central 1,3,5-trioxygenated ring system, two prenyl and one 2-methylbutanoyl substituents (Hermans-Lokkerbol and Verpoorte, 1994). Being the major component of the active fraction, compound **1** was tested for its ability to inhibit the growth of three effluxing strains and one standard strain of *S. aureus* and minimum inhibitory concentrations are given in Table 2.

Compound **1** was slightly more active against strains which possess efflux mechanisms of resistance (MIC = 16 μg/ml) when compared to a standard *S. aureus* ATCC 25923. XU212, in addition to possessing the TetK efflux transporter which confers resistance to tetracycline, is also a methicillin-resistant *S. aureus* (MRSA). SA-1199B possesses the NorA MDR efflux transporter which is resistant to certain fluoroquinolones and antiseptics and **1** was marginally more active than the fluoroquinolone norfloxacin. In the UK, the number of citations in death certificates that mention MRSA has dramatically risen during the 1990s and is a major clinical burden (Crowcroft and Catchpole, 2002). Given the dearth of novel classes of anti-staphylococcal agents, the occurrence of vancomycin resistance in MRSA (Appelbaum and Bozdogan, 2004) and the appearance of resistance to linezolid (Tsiodras et al., 2001), one of the newest anti-MRSA agents, further investigation of the acylphloroglucinol class as anti-staphylococcal leads is appropriate.

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded on a Bruker AVANCE 500 MHz spectrometer. Chemical shift values (δ) were reported in parts per million (ppm) relative to appropriate internal solvent standard and coupling constants (J values) are given in Hertz. Mass spectra 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.

IR spectra were recorded on a Nicolet 360 FT-IR spectrophotometer and UV spectra on a Thermo Electron Corporation Helios spectrophotometer.

3.2. Plant material

H. foliosum was collected from the Royal Botanic Garden at Wakehurst Place in Surrey in May 2003, which forms part of the National *Hypericum* Collection (Accession No. 1984-5158). A voucher specimen has been deposited at the Centre for Pharmacognosy and Phytotherapy.

3.3. Extraction and isolation

Four hundred and fifty one grams of air-dried and powdered aerial parts were extracted in a Soxhlet apparatus using sequential extraction by hexane (3 l),

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

Strain (resistance mechanism)	1	Norfloxacin	Erythromycin	Tetracycline
ATCC 25923	32	2	0.25	0.25
SA-1199B (NorA)	16	32	0.25	0.25
RN4220 (MsrA)	16	2	128	0.25
XU212 (TetK, *mecA*)	16	16	>256	128

All MICs were determined in duplicate.

chloroform (3 l) and finally methanol (3 l). The hexane extract (10.52 g) was subjected to vacuum liquid chromatography (VLC) on silica gel (15 g) eluting with hexane containing 10% increments of ethyl acetate to yield 12 fractions. The fraction eluted with 30% ethyl acetate was further purified by multiple preparative reverse-phase HPLC (four times on two coupled 40×100 mm 6 μm Nova-Pak HR C_{18} columns) using a gradient system from 100% water to 100% acetonitrile both containing 0.1% AcOH. This was performed by holding at 100% water for 2 min and linearly increasing to 100% acetonitrile at 15 min and maintaining this composition until 20 min. The flow rate was 50 ml/min and compound **1** (56.6 mg) had a retention time of 14.6 min.

3.4. Antibacterial assay

S. aureus strain ATCC 25923 was the generous gift of E. Udo (Kuwait University, Kuwait). *S. aureus* RN4220 containing plasmid pUL5054, which carries the gene encoding the MsrA macrolide efflux protein, was provided by J. Cove (Ross et al., 1989). Strain XU212, which possesses the TetK tetracycline efflux protein, was provided by E. Udo (Gibbons and Udo, 2000). SA-1199B, which overexpresses the *norA* gene encoding the NorA MDR efflux protein was provided by G. Kaatz (Kaatz et al., 1993). All *S. aureus* strains were cultured on nutrient agar and incubated for 24 h at 37 °C prior to MIC determination. Bacterial inocula equivalent to the 0.5 McFarland turbidity standard were prepared in normal saline and diluted to give a final inoculum density of 5×10^5 cfu/ml. The inoculum (125 μl) was added to all wells and the microtitre plate was incubated at 37 °C for 18 h. The MIC was recorded as the lowest concentration at which no bacterial growth was observed as previously described (Gibbons and Udo, 2000).

3.5. 1,3,5-Trihydroxy-6-[2‴,3‴-epoxy-3‴-methyl-butyl]-2-[2″-methyl-butanoyl]-4-[3′-methyl-2′-butenyl]-benzene (*1*)

Pale yellow oil; $[\alpha]_D^{21}$ $+80^\circ$ (*c* 0.075, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε): 241 (3.47), 279 (3.50) nm; IR ν_{max} (thin film) cm^{-1}: 3852, 3170, 2974, 2924, 1716, 1635, 1540, 1123, 1050; ^1H NMR and ^{13}C NMR ($CDCl_3$): see Table 1; HRCI-MS (*m/z*): 362.2079 $[M]^+$ (calc. for $C_{21}H_{30}O_5$, 362.2093).

Acknowledgements

We thank the Engineering and Physical Sciences Research Council (Grant No. GR/R47646/01) and the Royal Society (RSRG 24268) for funding.

References

Appelbaum, P.C., Bozdogan, B., 2004. Vancomycin resistance in *Staphylococcus aureus*. Clinics in Laboratory Medicine 24, 381–402.

Asakawa, Y., Kondo, K., Takikawa, N.K., Tori, M., Hashimoto, T., Ogawa, S., 1991. Prenyl bibenzyls from the liverwort *Radula kojana*. Phytochemistry 30, 219–234.

Crowcroft, N.S., Catchpole, M., 2002. Mortality from methicillin-resistant *Staphylococcus aureus* in England and Wales: analysis of death certificates. British Medical Journal 325, 1390–1391.

Gibbons, S., Udo, E.E., 2000. The effect of reserpine, a modulator of multidrug efflux pumps, on the in vitro activity of tetracycline against clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) possessing the tet(K) determinant. Phytotherapy Research 14, 139–140.

Gibbons, S., Ohlendorf, B., Johnsen, I., 2002. The genus *Hypericum* – a valuable resourceof anti-Staphylococcal leads. Fitoterapia 73, 300–304.

Gibbons, S., 2004. Anti-staphylococcal plant natural products. Natural Product Reports 21, 263–277.

Hermans-Lokkerbol, A.C.J., Verpoorte, R., 1994. Preparative separation and isolation of three α bitter acids from hop, *Humulus lupulus* L., by centrifugal partition chromatography. Journal of Chromatography A 664, 45–53.

Hübner, A.T., 2003. Treatment with *Hypericum perforatum* L. does not trigger decreased resistance in *Staphylococcus aureus* against antibiotics and Hyperforin. Phytomedicine 10, 206–208.

Kaatz, G.W., Seo, S.M., Ruble, C.A., 1993. Efflux-mediated fluoroquinolone resistance in *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 37, 1086–1094.

Marshall, N.J., Piddock, L.J.V., 1997. Antibacterial efflux systems. Microbiologia 13, 285–300.

Nayar, M.N.S., Bhan, M.K., 1972. Coumarins and other constituents of *Hesperethusa crenulata*. Phytochemistry 11, 3331–3333.

Reichling, J., Weseler, A., Saller, R., 2001. A current review of the antimicrobial activity of *Hypericum perforatum* L. Pharmacopsychiatry 34 (Suppl. 1), S116–S118.

Robson, N.K.B., 1977. Studies in the genus *Hypericum* L. (Guttiferae) I. Infrageneric classification. Bulletin of the British Museum (Natural History) Botany 5, 118–119.

Ross, J.I., Farrell, A.M., Eady, E.A., Cove, J.H., Cunliffe, W.J., 1989. Characterisation and molecular cloning of the novel macrolide-streptogramin B resistance determinant from *Staphylococcus epidermidis*. Journal of Antimicrobial Chemotherapy 24, 851–862.

Santos, P.A.G., Figueiredo, A.C., Barroso, J.G., Pedro, L.G., Scheffer, J.J.C., 1999. Composition of the essential oil of *Hypericum foliosum* Aiton from five Azorean Islands. Flavour and Fragrance Journal 14, 283–286.

Schempp, C.M., Pelz, K., Wittmer, A., Schopf, E., Simon, J.C., 1999. Antibacterial activity of hyperforin from St. John's Wort, against multiresistant *Staphylococcus aureus* and Gram-positive bacteria. Lancet 353, 2129.

Tsiodras, S., Gold, H.S., Sakoulas, G., Eliopoulos, G.M., Wennersten, C., Venkataraman, L., Moellering, R.C., Ferraro, M.J., 2001. Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. Lancet 358, 207–208.

Winkelmann, K., Heilmann, J., Zerbe, O., Rali, T., Sticher, O., 2000. New phloroglucinol derivatives from *Hypericum papuanum*. Journal of Natural Products 63, 104–108.

Winkelmann, K., Heilmann, J., Zerbe, O., Rali, T., Sticher, O., 2001. New prenylated bi- and tricyclic phloroglucinol derivatives from *Hypericum papuanum*. Journal of Natural Products 64, 701–706.