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A liquid chromatography–thermospray ionisation–mass spectrometry guided isolation of a new sesquiterpene aryl ester from *Armillaria novae-zelandiae*

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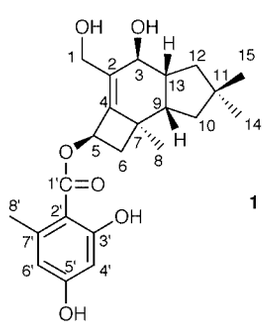
A liquid chromatography–thermospray ionisation–mass spectrometry guided fractionation of the mycelial extract of *Armillaria novae-zelandiae* (strain CBS 432.72) led to the isolation of a new protoilludane sesquiterpene aryl ester, 6′-chloro-10α-hydroxymelleolide (**9**). The antibacterial and antifungal activity of **9** was determined.

Introduction

The pathogenic basidiomycete *Armillaria* is the cause of widespread root disease in coniferous and deciduous trees.[1] *A. novae-zelandiae* Stevenson is a southern hemisphere species which is regarded as an important primary pathogen in New Zealand forest plantations and has also been reported as causing disease on ornamentals, garden plants and horticultural crops.[2] In Australia, it is considered as a secondary pathogen in forests stressed by such factors as drought and insect defoliation.[3]

Our previous liquid chromatography–thermospray ionisation–mass spectrometry (LC–TSI–MC) analysis of *Armillaria* cultures revealed a widespread distribution of protoilludane sesquiterpene aryl esters within the genus.[4] These metabolites are comprised structurally of two major types represented by armillyl orsellinate (**1**) and melleolide (**2**). Six sesquiterpene aryl



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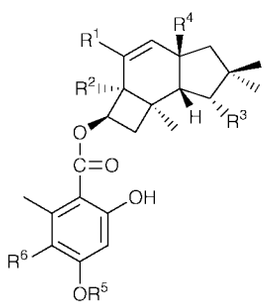
esters, melledonals B (**3**) and C (**4**), melleolides I (**5**) and J (**6**) and armellides A (**7**) and B (**8**), have been previously isolated from cultures of *A. novae-zelandiae*.[5] In this paper we report a LC–TSI–MC guided isolation and characterization of a new sesquiterpene aryl ester, 6′-chloro-10α-hydroxymelleolide (**9**), from cultures of *A. novae-zelandiae* CBS 432.72. Melledonal B (**3**), melledonal C (**4**), 10α-hydroxymelleolide (**10**), 13-hydroxy-4-methoxymelleolide (**11**) and dihydromelleolide (**12**) were also identified.



2 R^1=CHO, R^2=OH, R^3=R^4=R^5=R^6=H

3 R^1=CHO, R^2=R^3=R^4= OH, R^5=H, R^6=Cl

4 R^1=CHO, R^2=R^3=R^4=OH, R^5=CH_3, R^6=Cl

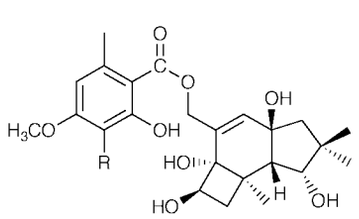
5 R^1=CH_2OH, R^2=R^3=OH, R^4=H, R^5=CH_3, R^6=Cl

6 R^1=CHO, R^2=OH, R^3=R^4=H, R^5=CH_3, R^6=Cl

10 R^1=CHO, R^2=OH, R^3=R^4=R^5=R^6=H

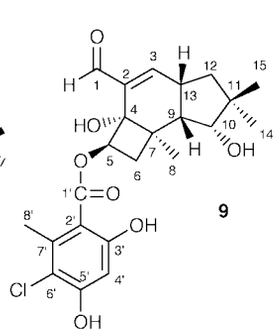
11 R^1=CHO, R^2=R^3=OCH_3, R^4=R^5=R^6=H

12 R^1=CH_2OH, R^2=OH, R^3=R^4=R^5=R^6=H



7 R=Cl

8 R=H



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The LC–TSI–MC analysis of a fraction resulting from the separation of a mycelial extract of *A. novae-zelandiae*, CBS 432.72 is shown in Fig. 1. The analysis was recorded in the positive ion mode with ammonium acetate added post column as an aqueous buffer. The mild ionization conditions of LC–TSI–MC leads to the formation of adduct ions, typically $[M + NH_4]^+$ with sesquiterpene aryl esters.[4] Selected ion monitoring at *m/z* 484, 498 and 468 revealed three components of

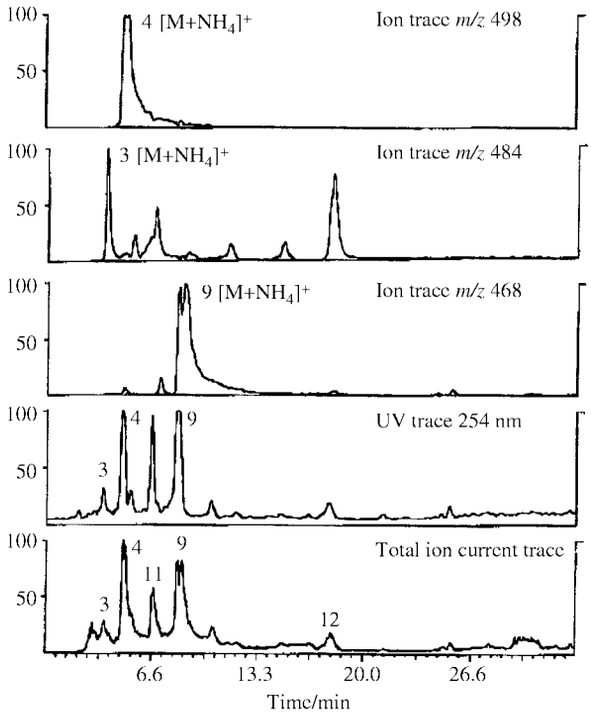


Fig. 1 LC–TSI–MS analysis of sub-fraction 3 of a chromatographic separation of the mycelial extract of *A. novae-zelandiae* (strain CBS 432.72). The UV trace at 254 nm and the total ion current trace are displayed in addition to selected ion traces corresponding to the ammonium adduct ions of **3**, **4** and **9**.

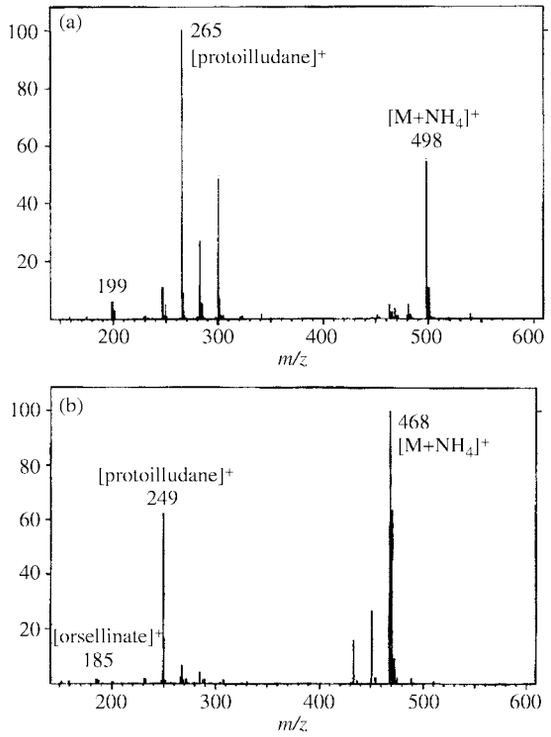


Fig. 2 LC–TSI–MS spectra of (a) **4** and (b) **9** recorded on line. For HPLC and TSI conditions, see Experimental section.

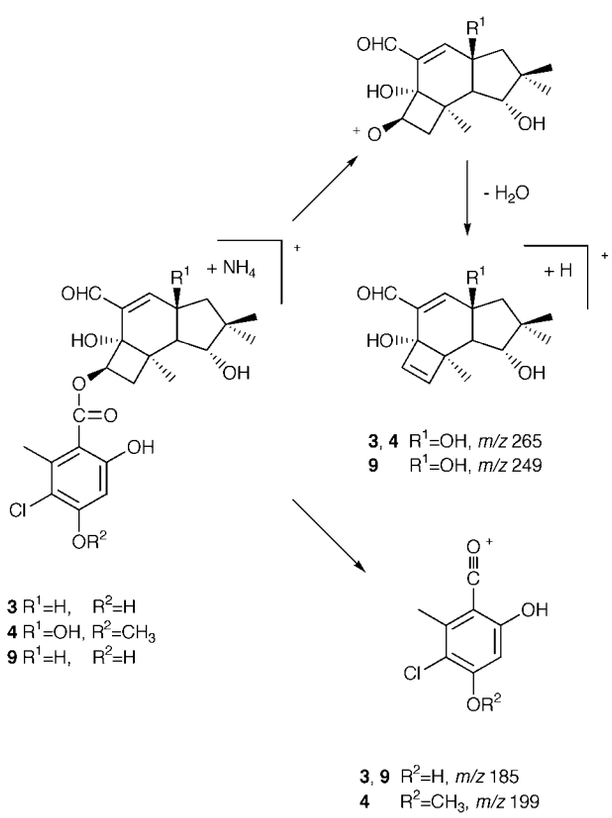
potential sesquiterpene aryl ester nature **3**, **4** and **9**. Taking these peaks as corresponding to ammoniated adduct ions led to molecular weight assignments of 466, 480 and 450 for **3**, **4** and **9** respectively. (These assignments are based on the more abundant ^{35}Cl isotope and the expected isotope pattern for a monochlorinated compound was observed.) Two non-chlorine containing sesquiterpene aryl esters **11** and **12** were also identified by this method.

The on-line TSI–MS spectra of **4** and **9** (Fig. 2) confirm this assignment with fragment ions indicative of a well-established fragmentation pathway for these compounds (Scheme 1). These fragments, due to cleavage of the aryl ester resulting in orsellinic and protoilludane fragments, account for the peaks at *m*/*z* 265 and 199 for compound **4** and at *m*/*z* 249 and 185 for compound **9**.

No previously isolated sesquiterpene aryl ester corresponded to a molecular weight of 450 amu. Component **3** (*M* 466) corresponded to melleolide I (**5**) or melledonal B while compound **4** (*M* 480) corresponded to melledonal C. These on-line spectroscopic data, together with chemotaxonomical consideration, suggested that compound **9** was an unknown sesquiterpene aryl ester. The separation of compounds **3**, **4** and **9** was subsequently achieved by medium pressure liquid chromatography (MPLC) on RP-8 using a MeOH–H_2O gradient. This isolation also yielded one further component **10**. These isolates were purified by repeated elution on Sephadex LH-20.

The direct chemical ionisation (DCI) and electron impact (EI) mass spectra of compound **9** confirmed a molecular weight of 450. The presence of the chlorinated orsellinate ester was indicated by a fragment peak at 185 in the EIMS. The presence of the orsellinate ester in compound **9** was further suggested from the IR spectrum which showed a band at 1645 cm^{-1}, corresponding to a chelated ester, and a hydroxy band at 3446 cm^{-1}.

The ^1H NMR spectrum (see Table 1) showed the presence of one aromatic proton (δ_H 6.31) and one aromatic methyl group at (δ_H 2.36). This, in conjunction with ^{13}C NMR resonances for six aromatic carbons and one ester carbonyl, confirmed



Scheme 1 Proposed fragmentation pathways for compounds **3**, **4** and **9**.

the presence of the chloroorsellinate ester. The position of the chlorine was assigned as 6′, *ortho* to the methyl and hydroxy group, by comparing its resonances with those of synthetic methyl 3-chloroorsellinate and methyl 5-chloroorsellinate.[6]

The presence of signals for 3 aliphatic methyl groups (δ_H 1.39, 1.02, 0.99) and an allylic aldehyde (δ_H 9.41) in the ^1H NMR of **9** suggested a protoilludane skeleton similar to melleolide (**2**). A vinylic proton was evident at δ_H 6.97 and

Table 1 ^{1}H NMR (CD_3OD) spectral data for 6′-chloro-10α-hydroxymelleolide (**9**)

Proton(s)	δ (multiplicity, J/Hz)	^{1}H–^{1}H COSY
H-1	9.41 (s)	
H-3	6.97 (d, 2.9)	H-13
H-5	5.69 (t, 8.6)	H-6α, 6β
H-6α	2.08 (br t, 11.05, 3.8)	H-5, H-6β
H-6β	1.64 (br t, 10.4, 9.3)	H-5, H-6α
H-8	1.39 (s)	
H-9	2.46 (dd, 9.6, 3.8)	H-13, H-10β
H-10β	3.58 (d, 3.7)	
H-12α	1.54 (dd, 13.1, 6.2)	
H-12β	2.08 (br t, 11.05, 3.8)	H-12α, H-13
H-13	3.11 (m)	H-3, H-9, H-12α, H-12β
H-14	1.02 (s)	
H-15	0.99 (s)	
H-4′	6.31 (s)	
8′	2.36 (s)	

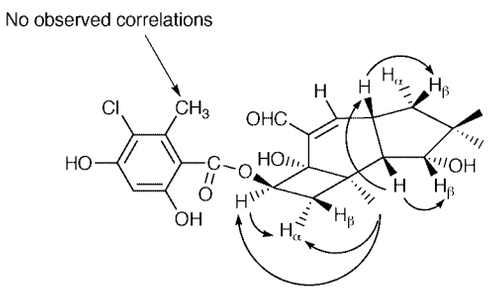


Fig. 3 Observed ^{1}H–^{1}H NOESY correlations of 6′-chloro-10α-hydroxymelleolide (**9**).

corresponded to H-3. The appearance of this signal as a doublet with a coupling constant of 2.9 Hz, indicated protonation on the neighbouring C-13 position. A doublet at δ_H 3.58 was characteristic of H-10 in the presence of C-10 hydroxylation. The coupling constant of 3.7 Hz corresponded to *syn*-coupling and indicates that H-9 and H-10 are in a *syn*-relationship. The triplet at δ_H 5.69 was assigned to H-5 which together with the diastereotopic H-6α and H-6β forms an ABX system. The resonances for H-6α and H-6β appeared at δ_H 2.08 and 1.64 and were detected from the ^{1}H–^{1}H COSY coupling with H-5. Again, use of ^{1}H–^{1}H COSY showed coupling between H-3 (δ_H 6.97) and a multiplet at δ_H 3.11, facilitating assignment of the latter as H-13. This multiplet also showed coupling to signals at δ_H 1.54 and 2.08 leading to their assignment as H-12β and H-12α, respectively, with the latter signal overlapping with that of H-6α to give a broad triplet. Coupling of H-13 (δ_H 3.11) to the signal at δ_H 2.46 helped to assign this as H-9, which was confirmed by its coupling to H-10 at δ_H 3.58. This led to an assignment of the sesquiterpenoid portion as identical to 10α-hydroxymelleolide (**10**),[7] although the latter was recorded in deuteriochloroform, whereas **9** was run in deuteriomethanol due to problems with its solubility in deuteriochloroform. The ^{13}C NMR spectrum also resembled that of 10α-hydroxymelleolide with a carbonyl carbon (δ_C 196.25), two olefinic carbons (δ_C 137.07 and 158.25) and C-10 appearing at a deshielded resonance of δ_C 82.21, due to hydroxylation at this position.

The relative stereochemistry of the ring system was determined by ^{1}H–^{1}H NOESY measurements. Firstly, the correlation between CH_3-8 (δ_H 1.39), assumed as α, and H-5 (δ_H 5.69) indicated the *syn*-relationship of this proton and the *anti*-position of the aryl ester relative to CH_3-8. This signal was also correlated to that at δ_H 2.08, confirming this as the assignment of the 6α proton and thus δ_H 1.64, as the 6β proton. Cross-peaks were also observed between H-9β (δ_H 2.46) and H-10β (δ_H 3.58) and also H-9β with H-13β (δ_H 3.11). These measurements defined the expected *cis–trans–cis* geometry of the ring system as shown in Fig. 3.[7] H-13 (δ_H 3.11) was also correlated to the signal at (δ_H 2.08), allowing assignment of the H-12β proton. The 6′ position of the chlorine was confirmed in the NOESY studies by the absence of a cross-peak between CH_3-8′ (δ_H 2.36) and the aromatic proton H-4′ (δ_H 6.31). Compound **9** was thus characterized as 6′-chloro-10α-hydroxymelleolide (**9**). Compounds **3**, **4** and **10** were obtained as white crystalline powders. Analysis of their spectroscopic data led to their identification as the previously known melledonal B (**3**), melledonal C (**4**) and 10α-hydroxymelleolide (**10**), respectively. [Note, our published[7] structure for 10α-hydroxymelleolide (**10**) has a *trans* cyclobutane–cyclohexene ring junction, but the present study and other work by Arnone *et al.*[5] suggest that a *cis* cyclobutane–cyclohexene ring junction is correct.]

Table 2 MIC values for antibacterial activities of armillyl orsellinate (**1**), melleolide (**2**), melledonal C (**4**), 6′-chloro-10α-hydroxymelleolide (**9**) and 10α-hydroxymelleolide (**10**) as estimated by bioautographic and agar dilution assays

	Bacillus subtilis		*Escherichia coli*	
Compound	TLC (μg)	Dilution (μg mL^{-1})	TLC (μg)	Dilution (μg mL^{-1})
1	0.5	—[a]	1.0	250.0
2	0.5	—	1.0	—
4	0.5	20.0	1.0	—
9	0.5	10.0	1.0	20.0
10	0.5	—	1.0	20.0
Ampicillin	0.001	NT[b]	NT	NT

[a] Inactive at concentrations tested. [b] Not tested.

Table 3 MIC values for antifungal activities of armillyl orsellinate (**1**), melleolide (**2**), melledonal C (**4**), 6′-chloro-10α-hydroxymelleolide (**9**) and 10α-hydroxymelleolide (**10**) as estimated by bioautographic and agar dilution assays

	Cladiosporium cucumerinum		*Candida albicans*	
Compound	TLC assay (μg)	Dilution assay (μg mL^{-1})	TLC assay (μg)	Dilution assay (μg mL^{-1})
1	—[a]	—	1.0	—
2	1.0	—	1.0	—
4	—	NT[b]	3.0	—
9	1.0	—	1.0	—
10	1.0	20.0	1.0	—
Amphotericin B	1.0	10.0	1.0	10.0
Nystatin	1.0	10.0	1.0	1.0

[a] Inactive at concentrations tested. [b] Not tested.

The antimicrobial activities of **4**, **9**, **10** and also armillyl orsellinate (**1**) and melleolide (**2**) are outlined in Tables 2 and 3. While all of the compounds showed antibacterial activity on TLC plate only 6′-chloro-10α-hydroxymelleolide (**9**) was active in dilution assays against the Gram-positive bacterium *Bacillus subtilis* and the Gram-negative bacterium *Escherichia coli.* Melledonal C (**4**) showed activity in the dilution assay against *B. subtilis* while 10α-hydroxymelleolide (**10**) was active against *E. coli*. The precise mode of antibacterial action is unknown. Melleolide (**2**), 6′-chloro-10α-hydroxymelleolide (**9**) and 10α-hydroxymelleolide (**10**) were active on TLC against the plant pathogen *Cladiosporium cucumerinum*, while only 10α-hydroxymelleolide (**10**) showed activity in the dilution assay. All of the compounds tested were active against the yeast, *Candida albicans* on TLC plate, however this activity was not found in dilution assays. This discrepancy between TLC and dilution assays is due to the difference in procedure between the two assays. The sample to be tested is added directly to the liquefied medium in the dilution assay, whereas in the overlay

bioautography assay, it makes contact with the microorganism only after diffusion into the medium. Armillyl orsellinate (**1**) and melleolide (**2**) were previously reported as being among the most active sesquiterpene aryl ester metabolites.[8] These results establish a similar level of activity for the isolates melledonal C (**4**), 6′-chloro-10α-hydroxymelleolide (**9**) and 10α-hydroxymelleolide (**10**). The NMR characterization of compounds **3**, **4** and **9** confirmed the initial molecular weight and structural assignments by on-line LC–TSI–MC analysis. In this study the sensitivity and selectivity of LC–TSI–MC analysis was useful for the targeted isolation of a new member of a structurally closely related class of fungal metabolites.

Experimental

The melting points (uncorrected) were determined on a Kofler hot-stage apparatus. UV spectra were recorded on a Pye Unicam SP8-400 spectrophotometer and IR spectra were recorded as KBr pellets on a Mattson Galaxy series FTIR 3000 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Varian VXR 200 instrument at 200 and 50.3 MHz, respectively. Chemical shift are in ppm downfield from TMS as internal reference. The DEPT sequence was used to distinguish the methylene carbon signals from those due to methine, methyl, and quaternary carbons. All 2D NMR spectra were recorded on a Bruker-500 FT spectrometer and on a Varian Unity 500 MHz spectrometer. EIMS data were recorded on a VG micromass 707 OH spectrometer and on a VG 12-250 quadrupole. DCIMS data were recorded on a Finnigan-MAT TSQ-700 instrument with NH_3 as the CI gas. Sephadex LH-20 and silica gel 60 were used for column chromatographic separations. MPLC separation was achieved on a column (id 2.6 × 46 cm) packed with LiChroprep RP-8 (15–25 μm) phase. For LC–TSI–MC analyses a Waters 600MS solvent delivery system, on-line UV Water 490MS multiwavelength detector, and Waters 590MS pump for post-column addition of buffer formed the HPLC configuration. Separations were on a Hypersil ODS 5 μ (250 × 4.66 mm) column equipped with a Nova-Pak Guard pre-column using a linear gradient of MeOH–H_2O from 75 : 25 to 90 : 10 in 30 minutes (1 mL min^{-1}). An aqueous solution of NH_4OAc was added post-column (0.5 M, 0.2 mL min^{-1}) to help ionization. LC–MS interfacing was achieved with a Thermospray 2 (Finnigan MAT) interface used with the following conditions: source temperature 200 °C, vaporizer 95 °C, aerosol 200–220 °C (beginning–end of gradient). The electron multiplier voltage was 1800 V, dynode 15 kV and the filament and discharge mode were off. Full-scan spectra (140–600 amu) were obtained in the positive-ion mode (scan time 1.2 s). MS detection was achieved on a Finnigan-MAT TSQ-700 triple stage quadrupole instrument. Samples were analysed by dissolving 30 μg in 1 mL of MeOH. The injection volume was 10 μL.

Fungal material

Armillaria novae-zelandiae, CBS 432.72 was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, and stock cultures were maintained on 5% malt agar slants at 4 °C. The strain was then grown on DIFCO potato dextrose broth in 12 × 1 L ROUX flasks (each containing 200 mL) in the dark for 35 days at 24 °C.

Extraction and isolation

The culture was harvested by filtration and the mycelium macerated in MeOH (2 × 300 mL) for 48 h at room temperature, evaporated to dryness and then partitioned into $CHCl_3$–MeOH–H_2O (65 : 35 : 20) (2 × 1500 mL). Evaporation of the combined $CHCl_3$ layers yielded the crude mycelial extract. Gel filtration of 4.73 g of this extract on Sephadex LH-20 (MeOH) yielded five fractions. Fraction II (4.034 g) was chromatographed on silica gel ($CHCl_3$–MeOH, 100 : 1–10 : 1) yielding five sub-fractions. All fractions were subjected to LC–TSI–MC analyses and sub-fraction 3 (0.433 g) was targeted for further isolation work. MPLC separation of this fraction on LiChroprep RP-8 (MeOH–H_20, 30 : 70) at a flow rate of 10 mL min^{-1} gave four compounds which were further purified by repeated elution on Sephadex LH-20 to yield **3** (4.4 mg) melledonal B, **4** (8.0 mg) melledonal C, **9** (30.0 mg) 6′-chloro-10α-hydroxymelleolide and **10** (11.0 mg) 10α-hydroxymelleolide. The ^{1}H NMR, ^{13}C NMR and MS data for **3**, **4** and **10** were in agreement with published values.[7]

6′-Chloro-10α-hydroxymelleolide (9)

White crystalline solid; mp 123–125 °C; UV (MeOH) λ_{max} (log ε) 213 (4.43), 265 (4.13) and 306 (3.70) nm; IR (KBr) ν_{max} 3400, 1687 and 1643 cm^{-1}; ^{13}C NMR (CD_3OD, 50.3 MHz) δ 19.70 (CH_3, C-8), 21.51 (CH3, C-8′), 24.10 (CH_3, C-14), 28.60 (CH_3, C-15), 33.81 (CH_2, C-6), 37.2 (C, C-7), 37.36 (CH, C-13), 43.71 (C, C-11), 44.31 (CH_2, C-12), 47.71 (CH, C-9), 75.25 (C, C-4), 76.25 (CH, C-5), 82.21 (CH, C-10), 102.61 (CH, C-4′), 108.50 (C, C-2′), 115.40 (C, C-6′), 137.10 (C, C-2), 139.74 (C, C-7′), 158.20 (CH, C-3), 159.62 (C, C-5′), 162.01 (C, C-3′), 170.92 (C, C-1′) and 196.2 (CH, C-1); EIMS *m*/*z* (%) 450 $[M]^{+\bullet}$ (5), 432 $[M - H_2O]^+$ (10), 406 $[M - CO_2]^+$ (17), 249 $[M - 201]^+$ (4), 185/187 (100/33) and 150 (82); DCIMS *m*/*z* (%) 468 $[M + NH_4]^+$, 451 $[M + H]^+$, 433 $[M + NH_4 - H_2O]^+$, 249 (64) and 185 [assumes ^{35}Cl; isotope peaks for ^{37}Cl-containing fragments also observed]; for ^{1}H NMR data, see Table 1.

Antibacterial assays

Compounds **1**, **2**, **3**, **4** and **9** were subjected to overlay bioautography and agar dilution assays according to previously reported procedures.[9] Luria–Bertani agar inoculated with either *B. subtilis* (ATCC 6633) or *E. coli* was the medium for both assays. In the overlay bioautography assay 10.0, 5.0, 1.0 and 0.5 μg of each compound were deposited on glass backed silica gel plates, which were developed with a $CHCl_3$–MeOH (10 : 1) solvent system. After inoculation and incubation at 30 °C for 24 h, activity was visualised by spraying the plates with an aqueous solution of methylthiazoyltetrazolium chloride (2.5 mg mL^{-1}). For agar dilution assays geometric dilution of each compound were prepared at 2–10^{-2} μg mL^{-1}. Aliquots of these stock solutions were added to inoculated Luria–Bertani medium to give final concentrations of 0.1, 1.0, 10.0 and 20.0 μg mL^{-1}. This assay was carried out in duplicate in 96 well microtiter plates. After incubation at 30 °C for 24 h, activity was estimated visually. Ampicillin was used as a positive control and MeOH was used as a negative control.

Antifungal assays

The antifungal activites of compounds **1**, **2**, **3**, **4** and **9** against *C. albicans* were determined in an identical manner as described above for *B. subtilis* and *E. coli* with the qualification that the medium is an inoculum of yeasts (approx 10^5 cells mL^{-1}) in molten Malt Agar MA (Oxoid). Activity against *C. cucumerinum* was determined by direct bioautographic methods previously reported.[10] Quantities (10.0, 5.0, 1.0 and 0.5 μg) of each compound were deposited on aluminium backed silica gel plates which were developed with a $CHCl_3$–MeOH (10 : 1) solvent system. The chromatograms were then sprayed with a spore suspension of *C. cucumerinum* in a nutritive medium and incubated at room temperature for 2–3 days in a moist atmosphere. Activity was estimated by the presence of clear inhibition zones. For the agar dilution assay against *C. cucumerinum* identical concentrations of compounds **1**, **2**, **3**, **4** and **9**, as before, were added to Sabouraud agar medium. The medium was placed in Petri dishes and a spore suspension of *C. cucumerinum* in distilled water was spread over the agar. Incubation was

at 30 °C for 24 h at which point activity was estimated visually. Amphotericin B and Nystatin were used as positive controls and MeOH was used as a negative control in these assays.

Acknowledgements

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