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Unusual antimicrobial compounds from *Aeollanthus buchnerianus*

J. E. Dellar, M. D. Cole[a] and P. G. Waterman*

Phytochemistry Research Laboratories, Department of Pharmaceutical Sciences, University of Strathclyde, 204 George Street, Glasgow G1 1XW (Scotland), Fax +44 141 552 6443, and [a]Forensic Science Unit, Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow G1 1XW (Scotland)
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Abstract. Using bioassay guided isolation, three novel 12 carbon polyoxygenated fatty acids and a novel abietane diterpene have been isolated from the the chloroform extract of aerial parts of *Aeollanthus buchnerianus* (Lamiaceae). Rigorous spectroscopic methods were used for compound identification. (*Z*,*Z*)-8ζ-acetoxy-5ζ-hydroxydodeca-2,6-dienoic acid and (*Z*,*Z*)-5ζ,8ζ-dihydroxydodeca-2,6-dienoic acid inhibited the spore germination of *Cladosporium cucumerinum* (both with Minimum Inhibitory Dose (MID) values of 1 μg) and *Aspergillus niger* (MID 5 and 25 μg respectively). Further, they also reduced the hyphal growth of *Pythium ultimum*. (*Z*)-5ζ-hydroxy-6ζ,7ζ,8ζ-triacetoxydodeca-2-dienoic acid exhibited short term inhibition of the growth of *Cladosporium cucumerinum*. The novel abietane diterpenoid, (*rel*)-14α-acetoxyabiet-7-en-18-oic acid inhibited the growth of the gram positive bacteria *Bacillus subtilis*, *Staphylococcus aureus* and *Streptomyces scabies* (MIC values 80, 20 and 20 μg ml^{-1} respectively).
Key words. *Aeollanthus buchnerianus*; Lamiaceae; dodecanoic acids; abietane diterpenes; antifungal and antibacterial bioactivity.

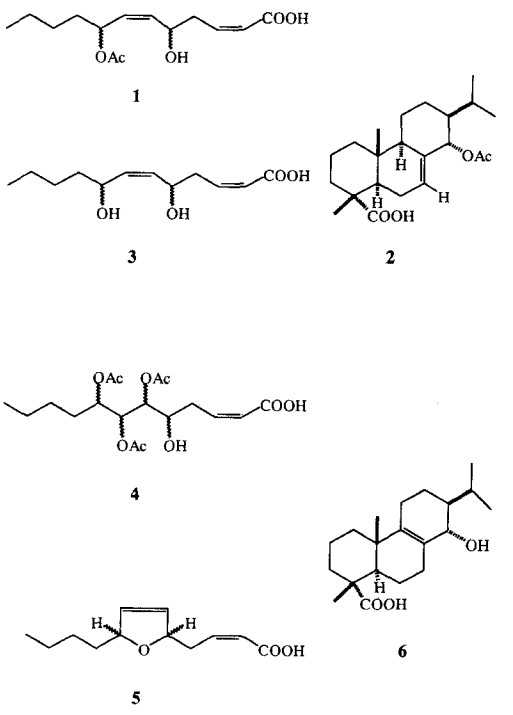
The genus *Aeollanthus* MART. ex SPRENG. (Lamiaceae) consists of over one hundred succulent and xerophytic species and occurs in southern Africa[1]. All species are aromatic due to the presence of low molecular weight terpenes. *A. buchnerianus* BRIQ. is a widespread species found in highland areas from Tanzania and Zaire southward[1]. It has been selected for study on the basis of an initial screening that showed that the chloroform extract had appreciable antifungal activity. In this paper we wish to report on the isolation of the compounds responsible for that activity, notably a group of unusual polyoxygenated (*Z*,*Z*)-dodeca-2,6-dienoic acid derivatives as well as an abietane diterpene. Some preliminary studies on the mechanism of action of the fatty acid derivatives are also reported.

Materials and methods

Plant material. The plant material was obtained from The Royal Botanic Gardens, Edinburgh, where a voucher specimen is deposited, and was propagated at Glasgow Botanic Garden.

Determination of antifungal activity. Aerial parts were lyophilised (151 g) and then extracted for 72 h at room temperature, first with chloroform and then with methanol. The two extracts were concentrated under reduced pressure using a rotary film evaporator at 40 °C to give 7 g and 5.3 g of extract, respectively. Bioautography[2] of the two extracts, using *Cladosporium cucumerinum* IMI 299104 as the test fungus on silica gel TLC plates, revealed the presence of appreciable levels of antifungal activity in the chloroform extract.

Isolation of antifungal compounds. Bioautography on silica gel using chloroform-ethyl acetate (3:2) as the



Scheme.

* Corresponding author.

developing solvent revealed four antifungal bands at Rf 0.27, 0.37, 0.49 and 0.60. Preparative TLC on silica gel using chloroform-ethyl acetate (9:1) lead to the isolation of compounds corresponding to the Rf values of 0.49 (**1**, 25.4 mg) and 0.60 (**2**, 42.1 mg). Similar preparative TLC but using chloroform-ethyl acetate (1:1) gave **3** (26.6 mg), which corresponded to Rf 0.37. Column chromatography (silica gel, 10 g; solvent chloroform) yielded the final compound, **4**, (210 mg).

Identification of isolated compounds

*(Z,Z)-8ξ-Acetoxy-5ξ-hydroxydodeca-2,6-dienoic acid (**1**).* An oil, giving a yellow-brown spot when sprayed with 1% vanillin in conc. sulphuric acid and heated to 100 °C. $[\alpha]_D + 9.1°$ (c. 1.27, MeOH) IR γ_{max} (KBr) 3470, 1738 broad, 1380, 1245, 1050, 817, 760 cm^{-1}. NMR – see table 1. FABMS *m/z* 275 $(M - H_2O + Na)^+$.

*(rel)-14α-Acetoxyabiet-7-en-18-oic acid (**2**).* An oil, giving a purple spot when sprayed with 1% vanillin in conc. sulphuric acid and heated to 100 °C. $[\alpha]_D - 77.0°$ (c. 2.7, MeOH) IR γ_{max} (KBr) 1735, 1696, 1238 cm^{-1}. NMR – see table 2. FABMS *m/z* 385 $(M + Na)^+$.

*(Z,Z)-5ξ,8ξ-Dihydroxydodeca-2,6-dienoic acid (**3**).* An oil, giving a violet-brown spot when sprayed with 1% vanillin in conc. sulphuric acid and heated to 100 °C. $[\alpha]_D + 5.6°$ (c. 0.53, MeOH). IR γ_{max} (KBr) 3445, 1721, 1383, 1250, 1046, 1024, 818, 757 cm^{-1}. NMR – see table 1. FABMS *m/z* 233 $(M - H_2O + Na)^+$, 211 $(M - H_2O + H)^+$.

*(Z)-5ξ-Hydroxy-6ξ,7ξ,8ξ-triacetoxydodeca-2-enoic acid (**4**).* An oil, giving a grey spot when sprayed with 1% vanillin in conc. sulphuric acid and heated to 100 °C. $[\alpha]_D + 9.8°$ (c. 1.43, MeOH). IR γ_{max} (KBr) 3455, 1746 broad, 1374, 1222, 1045, 816, 757 cm^{-1}. NMR – see table 1. FABMS *m/z* 393 $(M - H_2O + Na)^+$.

Determination of Minimum Inhibitory Dose (MID) and Minimum Inhibitory Concentration (MIC). The MID required to prevent fungal spore germination was determined using direct bioautography with *C. cucumerinum* and *Aspergillus niger* IMI 17454 as test organisms. The test against *C. cucumerinum* was carried out as previously described[2]. To test against *A. niger*, spores from an actively growing colony were suspended in sterile saline (20 ml) and 100 µl aliquots added to 10 ml of molten agar. This seeded agar medium was sprayed over a TLC plate (silica gel) that had been previously impregnated with a range of concentrations of the test compound and with suitable controls, so that the plate was covered with a thin uniform layer of agar. The plates were then incubated at 25 °C for 48 hours.

The antibacterial MIC value was determined using the broth dilution method[3], the test organisms being *Bacillus subtilis* NCTC 8326 (4×10^{13} cfu/ml), *Staphylococcus aureus* NCTC 6751 (9×10^{13} cfu/ml), *Pseudomonas aeruginosa* NCTC 6750 (9×10^{13} cfu/ml), *Erwinea carotovora* IMI 347390 (4×10^9 cfu/ml), *Pseudomonas syringae* IMI 349170 (4×10^9 cfu/ml) and *Streptomyces scabies* IMI 349428 (3×10^8 cfu/ml). The first three species were incubated at 37 °C, whilst the others were incubated at 25 °C, all in microtitre plates. Test compounds were dissolved in methanol and 10 ml aliquots added to each well, giving final concentrations of test compounds in the range of 0.01–100 µg ml^{-1}. Chloramphemicol was used as the positive control. The results were recorded after 24 hours. To determine whether the observed antibacterial activities were due to bacterioci-

Table 1. ^1H and ^{13}C NMR chemical shift data for compounds **1**, **3** and **4** (coupling constants, *J* Hz, in parentheses).

C/H	^1H			^{13}C		
	1	**3**	**4**	**1**	**3**	**4**
1				163.7	164.0	162.5
2	6.04 dd (9.8, 2.6)	6.03 ddd (9.8, 2.5, 1.2)	6.03 ddd (9.8, 2.7, 0.9)	121.8	121.6	121.7
3	6.87 ddd (9.8, 5.9, 2.6)	6.88 ddd (9.8, 6.4, 2.5)	6.87 ddd (9.8, 6.2, 2.4)	144.4	145.0	144.2
4	2.45 ddt (18.4, 11.6, 2.6)	2.32–2.47 m	2.53 ddt (18.3, 11.8, 2.6)	30.2	30.1	25.4
	2.28 dt (18.4, 5.3)		2.30 dddd (18.3, 6.0, 4.0, 0.8)			
5	5.38 m	5.32 ddd (12.1, 7.4, 4.7)	4.53 ddd (11.8, 5.8, 4.1)	74.2	73.9	75.4
6	5.72 dd (11.1, 8.2)	5.61 dd (11.2, 7.6)	5.35 t (4.4)	130.3	127.6	70.8
7	5.53 dd (11.1, 10.2)	5.66 dd (11.2, 8.0)	5.33 t (4.4)	131.9	138.1	70.9
8	5.42 m	4.41 dt (7.1, 7.0)	5.02 dt (5.8, 6.3)	69.6	67.9	71.9
9	1.64–1.73 m	1.58–1.64 m	1.54–1.59 m	34.5	36.9	30.5
	1.47–1.56 m	1.38–1.44 m				
10	1.20–1.36 m	1.20–1.36 m	1.24–1.33 m	22.6	22.8	22.5
11	1.20–1.36 m	1.20–1.36 m	1.24–1.33 m	27.4	27.6	27.2
12	0.89 t (6.4)	0.89 t (6.9)	0.88 t (6.8)	14.1	14.2	14.0
6-OAc			2.10 s			20.9
						170.9
7-OAc			2.14 s			21.1
						169.8
8-OAc	2.02 s		2.07 s	21.3		20.8
				170.3		170.6

All spectra run in $CDCl_3$. ^1H at 400 MHz, ^{13}C at 100.56 MHz.

dal or bacteriostatic effects, loops were taken from the test solutions with no visible bacterial growth and sub-cultured onto fresh nutrient agar and incubated overnight. Where bacterial growth resumed, the inhibition observed was recorded as bacteriostatic whilst where growth did not resume, the activity was recorded as bacteriocidal.

Investigation of reduction of radial hyphal growth in response to test compounds. Batches of molten malt extract agar (10 ml) were mixed with test compounds (previously dissolved in 1 µl methanol) to give concentrations of 5, 10, 25, 50 and 100 µg ml^{-1}. Methanol only and agar only controls were also performed. The agar was then allowed to solidify in a 9 cm petri dish and each plate inoculated with a 6 mm fungal plug taken from an actively growing colony of *Pythium ultimum* IMI 30827 or *Thanatephorus cucumeris* IMI 303152. Plates were incubated for 48–72 h at 25 °C and radial growth measured by taking the average diameter (four measurements).

Investigation of the effect of compounds on hyphal morphology. Test compounds were dissolved in methanol and added in 1 µl aliquots to 1 ml portions of molten malt extract agar, to give final concentrations of 10, 25, 50 and 100 µg ml^{-1}. Suitable controls were also prepared. Each agar sample was applied to a sterilised slide as an even layer and placed in a petri dish on a damp filter paper. A 6 mm plug taken from actively growing cultures of either *P. ultimum* or *T. cucumeris* was placed in the centre of the slide and dishes were covered and incubated for 18–48 h. Hyphae were examined for morphological changes, abnormal growth patterns or lysis of hyphal tips using light microscopy and were compared with negative controls. Evan's Blue stain (0.05% in distilled water) was applied as an indicator of cell membrane dysfunction[4].

Results

Identification of compounds 1–4. Compounds **1**, **3** and **4** showed similar spectral features. The FTIR of each indicated the presence of hydroxyl and carbonyl functional groups. The ^{13}C NMR spectrum of **3** (table 1) revealed the presence of twelve carbons (1 × CH3, 4 × CH2, 2 × CH(O), 4 × =CH, 1 × C = O). The 2D COSY-45 spectrum allowed a complete assignment of all ^2J and ^3J homonuclear ^1H-^1H couplings which suggested the structure **3**. The direct H-C couplings were established by the HC-COBIdec technique[5] and long range heteronuclear couplings by the inverse-detected HMBC pulse sequence[5].

The FAB-MS failed to give a pseudomolecular ion but a major fragment was observed at m/z 233, which can be attributed to $[M - H_2O + Na]^+$. On this basis **3** was assigned the structure (*Z*,*Z*)-5ζ,8ζ-dihydroxydodeca-2,6-dienoic acid. The stereochemistry of the two double bonds is based on the ^1H-^1H-coupling constants of the olefinic protons (under 12 Hz in each case). The alternative dihydrofuranoid structure **5** could be discounted because of the lability of **3** with EIMS and the absence of any ^3J heteronuclear interactions between oxymethine protons and carbons in the HMBC spectrum.

Table 2. NMR chemical shift data and ^2J and ^3J H-C coupling constants for compound **2**.

C/H	^1H	^{13}C	HMBC	
			^2J	^3J
1	ca. 1.50, 1.83	38.8		
2	ca. 1.59	18.1		
3	1.70–1.78	37.1		
4		46.4		
5	1.90 m	44.5		
6	1.75–2.00	25.1*		
7	5.80 d (5.8)	127.7		44.5(C5), 76.2(C14)
8		136.1		
9	ca. 2.00	48.7		
10		34.9		
11		24.9*		
12	1.72–1.80, 1.34–1.45	23.7		
13	ca. 1.00	48.5		
14	5.52 s	76.2		23.7(C12), 48.7(C-9), 127.7(C7), 170.7(Ac)
15	ca. 1.44	29.0		
16	0.89 d (6.8)	21.1**	29.0(C15)	48.5(C13), 21.0/1(C17)
17	0.89 d (6.8)	21.0**	29.0(C15)	48.5(C13), 21.0/1(C16)
18		184.9		
19	1.27 s	17.3	46.4(C4)	37.1(C3), 44.5(C5), 184.9(C18)
20	0.82 s	15.4	34.9(C10)	38.8(C1), 44.5(C5), 48.7(C9)
Ac	2.02 s	170.7/21.9		

Spectra run in $CDCl_3$. ^1H at 400 MHz, ^{13}C at 100.56 MHz.
*signals interchangeable.
**signals interchangeable.

Compound **1** had very similar spectral characteristics to **3**, with the addition of signals attributable to an acetoxyl substituent. Placement of the acetoxyl at C8 rather than C5 was established by COSY-45, which identified the H8 oxymethine proton and then HMBC which showed that proton to couple with C9, C10 and the acetoxyl carbonyl. Thus compound **1** must be (*Z*,*Z*)-8ζ-acetoxy-5ζ-hydroxydodeca-2,6-dienoic acid.

The ^{13}C NMR spectrum of **4** again revealed a dodecanoic acid, in this case with a single double bond and four oxymethine carbons, three of which were acetylated. From the COSY-45 and HMBC spectra it was obvious that the double bond at C6/C7 was no longer present and that these two carbons were now oxygenated, leading to a 5,6,7,8-tetraoxygenated fatty acid. Placement of the acetoxyls at C6, C7 and C8 was confirmed first by identification of the oxymethine protons at these positions (COSY-45) and then by the long-range heteronuclear coupling of these protons to the acetoxyl carbonyls. On this basis compound **4** must be (*Z*)-5ζ-hydroxy-6ζ,7ζ,8ζ-triacetoxydodeca-2-dienoic acid.

The final compound was revealed as *m*/*z* 362 by FABMS, which solved for $C_{22}H_{34}O_4$. The ^1H and ^{13}C NMR spectra (table 2) showed the presence of an acetoxyl substituent leaving a 20-carbon (diterpene) skeleton. Resonances attributable to an isopropyl side chain suggested a compound of the abietane class, with one double bond (trisubstituted), an oxymethine and a carboxylic acid. Tracking of ^2J and ^3J heteronuclear coupling from the HMBC spectrum (table 2) placed the carboxylic acid at C18/C19 and allowed identification of all carbons except C2, C6, C11 and C12, with placement of the olefinic bond at C7/C8 and the oxymethine at C14. The carboxylic acid could be assigned as C18 (equatorial) by comparison of the shift value for the geminal methyl with published data for similar compounds[6].

The stereochemistry of the acetoxyl at C14 must be axial because of the strong nuclear Overhauser interaction between H7 and H14 (fig. 1). As the methyls of the isopropyl system also showed an nOe with H14 that substituent must be equatorial. This establishes **2** as (*rel*)-14α-acetoxy-abiet-7-en-18-oic acid, on the assumption that chirality at C5 (αH), C9 (αH), C10 (βMe) and C13 αH) are those of a normal abietane diterpene. This appears to be novel and closely related to suaveolic acid (**6**), which has been isolated from *Hyptis suaveolens* (also Lamiaceae)[7], but differs in the position of the double bond.

Minimum inhibitory dose (antifungal) of 1–4. All four compounds were effective at preventing spore germination of *Cladosporium cucumerinum* (table 3), the two dodecadienoic acids (**1** and **3**) being active down to 1 µg with this activity persisting for more than 88 h. Both **1** and **3** still exhibited strong activity (5 µg) after 11 days

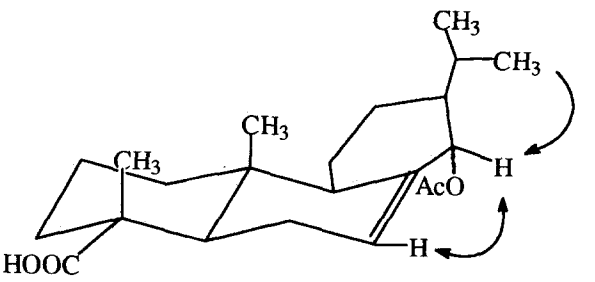


Figure 1. Nuclear Overhauser interactions in **2**.

(264 h). These two compounds were also active, although less so, against *Aspergillus niger*. In this experiment **2** or **4** were inactive and **1** appeared marginally more active than **3**. Once again the antifungal activity of **1** and **3** persisted.

Antibacterial activity of isolated compounds. Only the diterpene (**2**) displayed antibacterial activity, and this was limited to gram-positive organisms. The MIC values against six different bacteria are shown in table 4.

Inhibition of fungal radial growth by 1. Regression analysis of the results obtained from this assay were used to determine ID_{50} values (the concentration at which fungal growth was inhibited by 50% in comparison with a methanol control) at 24 hours. For **1** the ID_{50} value against *Pythium ultimum* was 178 µg ml^{-1} and against *Thanatephorus cucumeris* it was 248.9 µg ml^{-1}. Insufficient **3** was available to run this test.

The effect of 1 and 3 on fungal morphology. The controls all allowed lush, organised and linear hyphal growth with no occurrence of curling or shrinkage of hyphae at any stage of the experiment. At a concentration of 100 µg ml^{-1} compound **3** almost completely inhibited the growth of *Pythium ultimum*, causing hyphal curling and shrinkage. To a lesser extent the same effects were observed in the dose range of 10–50 µg ml^{-1}. Abnormal hyphae failed to take up Evan's Blue stain, indicating that the cell membranes were still able to function normally.

Somewhat surprisingly compound **1** showed little effect on *P. ultimum* growth, even at the highest concentra-

Table 3. Minimum inhibitory dose (µg) of compounds **1–4** against *Cladosporium cucumerinum* and *Aspergillus niger*.

Time (h)	Compound				
	1	**2**	**3**	**4**	Amphoteracin
C. cucumerinum					
40	1	5	1	25	<0.5
88	1	25	1	25	<0.5
264	5	>100	5	>100	<0.5
A. niger					
40	5	>100	10	>100	<0.5
88	10	>100	25	>100	<0.5
264	10	>100	25	>100	<0.5

Table 4. Minimum inhibitory concentration (MIC) of **2** against several bacteria.

Organism	MIC ($\mu g\ ml^{-1}$)	Bacteriocidal concentration ($\mu g\ ml^{-1}$)	MIC Chloramphenicol ($\mu g\ ml^{-1}$)
Erwinia carotovora	>100	na	1
Pseudomonas aeruginosa	>100	na	40
Pseudomonas syringae	>100	na	1
Bacillus subtilis	80	80	1
Staphylococcus aureus	20	60	1
Streptomyces scabies	20	40	5

na = not applicable.

tion. Neither compound was active against *Thanatephorus cucumeris* in this test.

Discussion

Direct bioautography revealed that *Aeollanthus buchnerianus* extracts contained compounds with significant antimicrobial activity. Bioassay guided separation has led to the isolation and identification of four novel compounds, three oxygenated dodecanoic acid derivatives which were responsible for the antifungal activity and an abietane diterpene which exhibited limited antibacterial activity.

Most interest lies in the three fatty acids. Of these the di-oxygenated dodecadienoic acids (**1** and **3**) proved to be far more active that the tetraoxygenated dodecenoic acid (**4**). The present investigation failed to reveal the mode of action of the fatty acids but showed they did not disrupt fungal membranes or inhibit the release or activity or extracellular enzymes.

Although 12-carbon polyoxygenated fatty acids are rare in higher plants a number of 18-carbon compounds with similar structures are known. (*Z*,*Z*)-8-hydroxyoctadeca-9,12-dienoic acid, from the basidiomycete *Laetisaria arvalis*, has also been found to inhibit mycelial growth in *P. ultimum*[8]. (*E*)-9,12,13-trihydroxyoctadec-10-enoic acid is produced in the tubers of *Colocasia antiquorum* (Araceae) in response to infection with the black rot fungus *Ceratocystis fimbriata* and is able to inhibit spore germination at 50 p.p.m.[9]. Other 18-carbon oxygenated fatty acids have been implicated in the resistance of some rice varieties to the rice blast fungus *Pyricularia oryzae*, by inhibiting spore germination and germ tube elongation at concentrations of 10–50 p.p.m.[10].

There is now evidence for the occurrence of a wide range of preformed anti-fungal compounds in plants[11], but oxygenated fatty acids of the type found here are unusual. An investigation of structure activity relationships of fatty acids against *P. oryzae* indicated that conjugation of the carboxylic acid with a double bond enhanced activity and that a chain length of 10–13 carbons was optimal[12]. The most active compounds in this study (**1** and **3**) both conform to those requirements.

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