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ANTIMICROBIAL ABIETANE DITERPENOIDS FROM *PLECTRANTHUS ELEGANS*

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Key Word Index—*Plectranthus elegans*; Lamiaceae; abietane diterpenoids; antibacterial activity; antifugal activity.

Abstract—Two novel abietane diterpenoids have been isolated from the aerial material of *Plectranthus elegans* and identified as 11-hydroxy-12-oxo-7,9(11),13-abietatriene and 7α,11-dihydroxy-12-methoxy-8,11,13-abietatriene. Their structures were determined through rigorous use of spectroscopic methods. Both inhibited spore germination of the fungus *Cladosporium cucumerinum*, in direct bioautography, at a dose of 1 μg. The new diterpenes also inhibited the growth of Gram-positive bacteria, in the concentration range 10–40 μg ml^{-1} in broth dilution assay. No effect was observed against Gram-negative bacteria. The ecological implications of these findings are discussed.

INTRODUCTION

The Lamiaceae represents a valuable pool of plant species which contain biologically active molecules [1]. The genus *Plectranthus* consists of some 350 species, distributed from Africa, through to Asia and Australia [2]. Although some anecdotal evidence is available concerning the ethnobotanical uses of the genus [3], little is known of the phytochemistry of these plants [4, 5].

As a part of our continued phytochemical study of the family, the methanol, chloroform and hexane extracts of *Plectranthus elegans* Britten were screened for antimicrobial activity. We describe here the isolation, identification and characterization of two new diterpenes (**1, 2**) from the chloroform extract and report on the activity of these compounds against the test fungus *Cladosporium cucumerinum* and a number of Gram-positive and Gram-negative bacteria of economic importance.

RESULTS AND DISCUSSION

Preliminary testing of the crude chloroform-soluble extract of the aerial parts of *P. elegans* revealed considerable antimicrobial activity. Bioactivity-guided fractionation of the extract (see Experimental) gave two new active abietane diterpenes.

The more polar of the two diterpenes gave a weak $[M + H]^+$ ion *m/z* 301 by FAB-mass spectrometry indicating, by accurate mass measurement, an empirical formula $C_{20}H_{28}O_2$. The infrared spectrum indicated hydroxyl and C=O functional groups and the UV spectrum the occurrence of an extensive chromophore. The ^{13}C NMR spectrum (Table 1) revealed five methyls, four methylenes, four methines (two olefinic) and seven quaternary carbons, including four olefinic and one carbonyl. Major features of the ^1H NMR spectrum (Table 1) were the occurrence of two of the methyls and one methine in an isopropyl group, one of the olefinic protons as a sharp singlet, with the other a double doublet, and an O–H resonance at δ 7.47. The ^1H–^1H COSY spectrum showed the coupled olefinic proton to be part of a $=CH-CH_2-CH-$ spin system and the remaining three methylenes to be a single $-CH_2-CH_2-CH_2-$ spin system. Direct H–C couplings were established by means of an HC–COBIdec experiment [6] and long-range heteronuclear couplings through an HMBC experiment [7]. The most important correlations of the HMBC study are shown in Table 2 and required placement of the phenolic hydroxyl at C-11 and the carbonyl at C-12. These allowed the identification of the abietane nucleus and the unambiguous placement of all substituents, leading to the structure **1**.

Diterpene **2** gave an $[M + Na]^+$ *m/z* 355 in the FAB-mass spectrum, indicating an empirical formula $C_{21}H_{32}O_3$. The infrared spectrum still showed hydroxyl functionality but no ketone, which was confirmed by the less-complex UV spectrum. The NMR spectra (Table 1) revealed three double bonds but only a single olefinic proton, two hydroxyls, a methoxyl, but no ketone. An additional feature, not seen in **1**, was an oxymethine (δ_H 4.19, δ_C 78.8). Once again the HMBC spectrum provided

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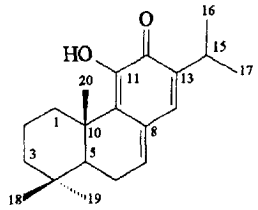

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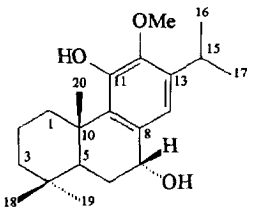

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critical information on connectivities (Table 2), allowing placement of hydroxyls at C-7 and C-11 and the methoxyl at C-12. The relative stereochemistry of the oxymethine proton at C-7 must be assigned as equatorial (β) in view of the absence of significant coupling with either H-6 protons.

Using direct bioautography [8] **1** and **2**, each at doses of 1 μg, inhibited the germination of spores of *C. cucumerinum*. This activity persisted for 72 hr. The positive control, amphoteracin, inhibited the growth of the fungus with minimal inhibitory concentration (*MIC*) value < 0.5 μg, the activity of which persisted indefinitely. There was no inhibition of fungal growth in the solvent controls.

Both **1** and **2** inhibited the growth of the Gram-positive species of bacteria tested, while growth of the Gram-negative species was unaffected. The *MIC* values are presented in Table 3, the bacteriocidal concentrations in Table 4. Against *Bacillus subtilis*, compound **1** was more active than **2** as a bacteriostatic compound, although the response to the compounds was identical from the other Gram-positive species of bacteria. The former result is in accordance with the activity profile reported for abietane lactones from *Salvia* spp., where C-12 methyl ethers were less active than the ketones. In terms of bactericidal concentration (Table 4), no difference between the two compounds was observed in this study.

The activity of these two compounds against fungal and bacterial species suggests that these compounds may have a role in the chemical defence of *Plectranthus*. There are reports in the literature of chemical defence systems where a compound is active against more than one organism [10, 11]. In ecological terms this is advantageous to the plant, which only has to manufacture a few compounds to defend it against a wide range of potential pathogens.

EXPERIMENTAL

UV: MeOH. IR: thin film on KBr disks. NMR spectra run on a Bruker AMX-400 spectrometer. FAB-MS were run as a NOBA matrix. Petrol refers to the bp 40–60° fraction.

Table 1. ^{1}H and ^{13}C NMR chemical shift data for compounds **1** and **2**

	^{1}H		^{13}C	
C/H	**1**	**2**	**1**	**2**
1	1.55 *m* 3.02 *br d* (12.6)	1.32 *m* 3.00 *m*	37.0	36.5
2	1.56 *m* 1.67 *m*	1.40 *m* 1.56 *m*	19.4	19.5
3	1.25 *m* 1.46 *dt* (13.2, 1.6)	1.34 *m* 1.65 *m*	41.9	41.4
4			33.7	33.4
5	1.53 *m*	1.71 *br d* (12.0)	50.7	45.7
6	2.39 *ddd* (20.6, 6.8, 3.7) 2.57 *ddd* (20.6, 8.5, 6.9)	1.51 *m* 2.12 *br d* (14.0)	26.0	22.5
7	6.81 *d* (6.9, 3.6)	4.19 *br s* 5.09 (OH)	149.2	78.8
8			131.8	129.0
9			127.5	133.8
10			38.8	39.7
11	7.47 (OH)	5.60 (OH)	144.0	142.6
12			181.6	140.6
13			140.7	132.0
14	6.77 *s*	6.67 *s*	136.3	120.1
15	3.07 *hept* (6.8)	2.97 *hept* (6.8)	26.8	27.5
16	1.14 *d* (6.8)	1.20 *d* (6.8)	21.7*	22.4
17	1.14 *d* (6.8)	1.23 *d* (6.8)	22.0*	18.9
18	0.97 *s*	0.96 *s*	22.3	22.0
19	0.93 *s*	0.97 *s*	33.5	33.7
20	1.18 *s*	1.30 *s*	18.7	22.9
OMe	–	3.45 *s*		56.3

Spectra run in $CDCl_3$.

Table 2. Long-range C–H correlations for compounds **1** and **2**, determined by HMBC experiment

H	^2J	^3J
1		
3.02 (H-1)	19.1 (C-2), 38.8 (C-10)	
2.57/2.39 (H-6)	50.7 (C-5), 149.2 (C-7)	38.8 (C-10), 131.8 (C-8)
6.81 (H-7)	26.0 (C-6)	50.7 (C-7)
7.47 (OH-11)	144.0 (C-11)	127.5 (C-9), 181.6 (C-12)
6.77 (H-14)		127.5 (C-9), 149.2 (C-7), 181.6 (C-12)
3.07 (H-15)	21.7 22.0 (C-16/17), 140.7 (C-13)	136.3 (C-14), 181.6 (C-12)
1.14 (H_3-16, H_3-17)	26.8 (C-15)	21.7/22.0 (C-16/17), 140.7 (C-13)
0.97 (H_3-18)	33.7 (C-4)	33.5 (C-19), 41.5 (C-3), 50.7 (C-5)
0.93 (H_3-19)	33.7 (C-4)	22.3 (C-18), 41.5 (C-3), 50.7 (C-5)
1.18 (H_3-20)	38.8 (C-10)	37.0. (C-1), 50.7 (C-5), 127.5 (C-9)
2		
4.19 (H-7)		45.7 (C-5)
5.60 (OH-11)	142.6 (C-11)	133.8 (C-9), 140.6 (C-12)
3.45 (12-OMe)		140.6 (C-12)
6.67 (H-14)		27.5 (C-15), 78.8 (C-7), 133.8 (C-9), 140.6 (C-12)
2.97 (H-15)	18.9/22.3 (C-16/17), 132.0 (C-13)	120.1 (C-14), 140.6 (C-12)
1.20/1.23 (H_3-16, H_3-17)	27.5 (C-15)	18.9/22.3 (C-16/17), 132.0 (C-13)
0.96 (H_3-18)	33.4 (C-4)	33.7 (C-19), 41.4 (C-3), 45.7 (C-5)
0.97 (H_3-19)	33.4 (C-4)	22.0 (C-18), 41.4 (C-3), 45.7 (C-5)
1.30 (H_3-20)	39.7 (C-10)	36.7 (C-1), 45.7 (C-5), 133.8 (C-9)

Table 3. *MIC* values of compounds **1**, **2** and chloramphenicol against test bacteria

Organism	Density (cfu ml^{-1})	*MIC* value (μg ml^{-1})		
		1	**2**	Chloramphenicol
Erwinia carotovora	4×10^9	> 100	> 100	1
Pseudomonas aeruginosa	9×10^{13}	> 100	> 100	40
Pseudomonas syringae	4×10^{13}	> 100	> 100	1
Bacillus subtilis	4×10^{13}	40	60	1
Staphylococcus aureus	9×10^{13}	10	10	1
Streptomyces scabies	3×10^8	40	40	5

Table 4. Bacteriocidal concentration of compounds **1** and **2** against Gram-positive test bacteria

Organism	Bacteriocidal concentration (μg ml^{-1})	
	1	**2**
Bacillus subtilis	> 100	> 100
Staphylococcus aureus	40	40
Streptomyces scabies	40	40

Plant material. Plant material was collected from the Living Collection Division, Royal Botanic Garden, Edinburgh (Accession Number Burtt, B.L. 004525), where a voucher specimen is deposited, and was cultivated at Glasgow Botanic Gardens.

Isolation of the diterpenes. Lyophilized and milled aerial material of *P. elegans* (78.2 g) was extracted for 72 hr at room temp. in $CHCl_3$ at 100 mg ml^{-1}, yielding 1.65 g oil. The components of the oil were sepd using VLC [12], eluting with petrol–EtOAc mixtures of increasing polarity, in 50 ml frs. The column frs were monitored using TLC (silica gel, petrol—EtOAc 3:2) and visualized with acidified vanillin reagent. The biological activity of fractions was assessed by direct bioautography [8], the active frs being correlated with the TLC data. Antifungal activity was detected in VLC frs 3–7 which eluted from the VLC column in 97–90% petrol in EtOAc. The active zones gave an R_f value of 0.67 when developed with petrol–EtOAc (3:2) and gave a brown colour reaction with the vanillin reagent. The active fractions were pooled and the components separated by CC (silica gel, 230–400 mesh, 25 × 3 cm i.d.), eluting isocratically with $CHCl_3$. The active compounds were finally isolated using multiple pass prep. TLC in petrol–EtOAc (95:5) on silica gel, finally yielding **1** (24.3 mg) and **2** (37.6 mg).

11-*hydroxy*-12-*oxo*-7,9(11),13-*abietatriene* (**1**). A yellow oil $[\alpha]_D$ + 25.9° (MeOH; *c* 1.54) UV_{max} nm: 279, 310,

399. IR_{max} cm^{-1}. 3319, 1658, 1612, 1565, 1362. ^1H NMR ^{13}C NMR: see Table 1. FAB-MS: *m/z* 301 $[M + H]^+$.

7α,11-dihydroxy-12-methoxy-8,11,13-abietatriene (**2**). An orange oil $[\alpha]_D$ + 4.1° (MeOH; *c* 0.57) UV_{max} nm: 279, 313. IR_{max} cm^{-1}: 3340, 1610, 1564, 1461, 1362, 756. ^1H NMR, ^{13}C NMR: see Table 1. FAB-MS: *m/z* 355 $[M + Na]^+$.

Fungal cultures. *C. cucumerinum* IMI 299104 was obtained from the International Mycological Institute, Kew, U.K., and cultured on MEA agar at 25°.

Bacterial cultures. Cultures of *Erwinia carotovora* IMI 347390, *Pseudomonas syringae* IMI 349170 and *Streptomyces scabies* IMI 349428 were maintained on NA at 25°, while cultures of *Pseudomonas aeruginosa* NCTC 6750. *B. subtilis* NCTC 8326 and *Staphylococcus aureus* NCTC 6751 were maintained on NA at 37°.

Antifungal assay. This was performed by direct bioautography, based on a method previously described [8]. A stock soln, in which the conidiospores of *C. cucumerinum* were suspended, was prepared using 7 g KH_2PO_4, 3 g $NaH_2PO_4 \cdot 2H_2O$, 4 g KNO_3, 1 g $MgSO_4 \cdot 7H_2O$ and 1 g NaCl per litre of tap water. A 60 ml aliquot of this soln was added to 10 ml 30% aq. glucose soln, to which 4 drops Tween 80 had been added. Following autoclaving at 120° for 20 min, the conidiospores from a 3-day-old slope culture of *C. cucumerinum* were suspended in the medium. The TLC chromatograms on which the assays were to be performed were run in duplicate, either to separate the components of active fractions, or with pure compounds to determine the *MIC* value. Both positive (amphoteracin) and negative controls (solvent alone) were also prepd. One chromatogram was thoroughly air dried, and sprayed with the conidial suspension. The fungal culture was incubated in a humid chamber at 25° for up to 72 hr, until growth and inhibition zones could clearly be observed. The other TLC chromatogram was visualized with acidified vanillin reagent, and the R_f data and colour reactions used to monitor the column fractions.

Antibacterial assay. Determination of MIC values. The antibacterial activity of the compounds was determined using a broth dilution assay in a 96-well microtitre plate. Test compounds were dissolved in MeOH to give final concns of 0.01–100 $\mu g\,ml^{-1}$ in the test well. To 10 μl of the soln of test compound was added 290 μl double-strength nutrient broth. Bacterial suspensions taken from 18–48 hr old nutrient agar slopes were resuspended in 20 ml double-strength nutrient broth and 50 μl suspension CFU density given in Table 3) added to each well. Each test was carried out in triplicate. Chloramphenicol, in the same concn range as the test compounds was employed as the positive control, as was a broth only, and MeOH only, negative control. Microtitre plates were incubated at 25° for 18–36 hr. The *MIC* value was scored as the lowest concn at which no growth occurred, following viable counting.

Determination of bacteriocidal concentrations. To determine whether the observed inhibition was a bacteriocidal and bacteriostatic effect, loops of the suspension in which no growth was observed were plated out in 20 ml nutrient agar in a 9.1 cm Petri dish. The plates were incubated overnight at 25° or 37° as appropriate. Where growth was observed the activity was depend merely bacteriostatic, where no growth was observed, the activity was scored as bacteriocidal.

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