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Structures of Flagranones A, B and C, Cyclohexenoxide Antibiotics from the Nematode-trapping Fungus *Duddingtonia flagrans*

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Spectroscopic data define the structures of the flagranones A (**2**), B (**3**) and C (**4**) from the nematode-trapping fungus *Duddingtonia flagrans*. These antibiotics are structurally related to the farnesylated cyclohexenoxides of the oligosporon group recently isolated from the nematode-trapping fungus *Arthrobotrys oligospora*, and show similar antimicrobial activity.

The interactions between nematophagous fungi and their nematode prey have been extensively investigated,[1~3] and such fungi have potential for the biological control of nematode parasites of plants[4] and animals.[5] *Duddingtonia flagrans* (Duddington) Cooke, a predacious soil fungus which forms a mono-specific genus and employs adhesive net traps, has shown particular promise both *in vitro* and *in vivo* for the control of nematode infections of livestock.[6~9] Extracts from a cultured isolate of *D. flagrans* also showed larvacidal activity in a development assay employing *Haemonchus contortus* eggs.[10] The only secondary metabolites reported to date from predatory fungi are the antibiotics of the oligosporon group recently isolated from *Arthrobotrys oligospora*,[11,12] which like *D. flagrans* also forms adhesive net traps. Their novel structures are typified by that of oligosporon itself (**1**), and include various representatives reduced at the C-1 carbonyl or the C-4′,5′ olefin, hydroxylated at the 11′-methyl, or epoxidised at the C-10′,11′ olefin. We now describe the isolation and structural elucidation of the related metabolites flagranones A (**2**), B (**3**) and C (**4**) from cultures of *D. flagrans*, and the antimicrobial activity of flagranones A and B.

Experimental

General

NMR spectra were recorded for acid-free $CDCl_3$ solutions on a Varian Gemini-300 instrument at 300 MHz for ^1H and 75.43 MHz for ^{13}C. TMS and the ^{13}C resonance of $CDCl_3$ at 77.0 ppm were used as internal references. Signal assignments were based on direct spectrum analysis, APT and HMQC data, and comparison with literature data.[12] EIMS, HR-EIMS and ESMS were recorded on Fisons Instruments AutoSpec and Quattro II spectrometers. UV spectra were recorded on a Hewlett-Packard 845OA spectrophotometer.

Production and Isolation of the Flagranones

Wheat, oats, and barley grains (50 g each) were separately seeded with an inoculum of *D. flagrans* isolated from cattle faeces and incubated at 24°C for 4 weeks. The fermented grains were washed with water to remove spores, and portions of the remaining materials were extracted with EtOAc. Since TLC analysis of the three extracts showed the presence of similar components, which were not present in the uninoculated grains, the grains were combined and extracted by shaking for 1 hour with EtOAc (500 ml). Filtration, drying (Na_2SO_4) and evaporation of the solvent afforded a brown oil (1.06 g). This material showed no significant nematocidal activity in a larval development assay.[13] It inhibited the growth of *Bacillus subtilis* and *Streptomyces aureofaciens* but not of *Escherichia coli* or *Erwinia carotovora* incorporated into nutrient agar (Difco Laboratories, Detroit, USA), and inhibited the growing fronts of *Phytophthora cinnamomi* and to lesser extents of *Pythium ultimum* and *Rhizoctonia solani* on potato dextrose agar (Oxoid, Basingstoke, UK), in sensitivity disc assays.

Table 1. Physico-chemical properties of flagranones A, B and C.

	Flagranone A (**2**)	Flagranone B (**3**)	Flagranone C (**4**)
Appearance	Yellow oil	Yellow oil	Yellow oil
Molecular formula	$C_{26}H_{32}O_7$	$C_{18}H_{18}O_8$	$C_{16}H_{16}O_8$
ESMS *m/z*		363 (MH^+) 385 (MNa^+)	337 (MH^+) 359 (MNa^+)
HR-EIMS *m/z*	Found 456.2168 $C_{26}H_{32}O_7$ requires 456.2148	Found 302.0791 $C_{16}H_{14}O_6$ requires 302.0790	Found 234.0528 $C_{12}H_{10}O_5$ requires 234.0528
	Found 327.1257 $C_{19}H_{19}O_5$ requires 327.1232	Found 273.0763 $C_{15}H_{13}O_5$ requires 273.0763	Found 205.0501 $C_{11}H_9O_4$ requires 205.0501
	Found 69.0703 C_5H_9 requires 69.0704	Found 95.0496 C_6H_7O requires 95.0497	
UV λ_{max} (EtOH) nm	274_{sh} (32600), 286_{max} (34900)	266_{max} (18500)	220_{max} (18700), 302_{max} (6160), 316_{sh} (5710)

TLC on silica gel (Merck Kieselgel 60 F_{254}) in EtOAc-$CHCl_3$ (1 : 100) showed antimicrobial activity at Rf 0.37 and 0.60, and these spots could be visualised with UV light or iodine spray. Chromatography of this extract (300 mg) on silica gel (Merck Kieselgel 60, 40 g) in EtOAc-$CHCl_3$ (1 : 100) separated flagranone A (**2**) (38.7 mg) as a yellow oil from a mixture of flagranones B and C (50.3 mg). Re-chromatography of this mixture in EtOAc-$CHCl_3$ (4 : 100) gave flagranone B (**3**) (7.8 mg), and then in EtOAc-$CHCl_3$ (20 : 100) gave flagranone C (**4**) (0.7 mg). The physico-chemical properties of the flagranones are reported in Table 1, and their ^1H and ^{13}C NMR data in Tables 2 and 3.

Bioassay of the Flagranones

Antimicrobial assays[12] and nematode larval development assays[13] were carried out as described previously.

Results and Discussion

Structure of Flagranone A (**2**)

HR-EIMS established the molecular formula of flagranone A (**2**) as $C_{26}H_{32}O_7$ (Table 1), C_2O greater than that of oligosporon (**1**), $C_{24}H_{32}O_6$. Mass measurement of the base peak at *m/z* 69 defined its composition as $C_5H_9^+$, suggesting the presence of a terminal dimethylallyl unit in flagranone A.[12] ^1H and ^{13}C NMR spectroscopy extended this unit to a 4′,5′-dehydrofarnesyl chain, for which the chemical shift and multiplicity data closely matched that for oligosporon (**1**) from 2′-CH to 12′-CH_3 (Tables 2 and 3). One of the two acetoxy groups visible in the NMR spectra of flagranone A was attached to the 1′-position of this chain, however, shifting the 1′-H resonance downfield by 1.27 ppm relative to the corresponding proton in

Table 2. ^{1}H NMR data of oligosporon and flagranones A, B and C.[a]

Proton(s)	Oligosporon (**1**)	Flagranone A (**2**)	Flagranone B (**3**)	Flagranone C (**4**)
3-H	6.71, dt, 4.7, 1.3	6.53, t, 2.0	6.55, t, 2.0	6.56, t, 1.9
4-H	5.03, dm, 4.7			
6-H	3.29, d, 1.4	3.86, s	3.91, s	3.94, s
1'-H	5.17, d, 8.9	6.44, d, 9.7	6.31, d, 9.3	6.25, d, 8.9
1'-OAc		2.14, s[b]	2.15, s[b]	2.15, s[b]
2'-H	5.40, d, 8.9	5.23, d, 9.8	5.82, brd, 9.3	6.36, dq, 8.9, 1.5
3'-Me	1.89, d, 1.2	2.03, d, 1.6[c]	2.07, d, 1.1	1.96, d, 1.5
4'-H	6.16, d, 15.4	6.08, d, 15.3	7.07, d, 15.9	9.48, s
5'-H	6.54, dd, 15.3, 10.9	6.58, dd, 15.3, 10.9	6.28, dd, 15.9, 7.1	
6'-H	5.90, dm, 10.9	5.88, d, 11.0	9.61, d, 7.1	
7'-Me	1.82, d, 1.3	1.81, d, 1.6[c]		
8'-H_2	2.11, m	2.10, m		
9'-H_2	2.13, m	2.11, m		
10'-H	5.10, m	5.08, m		
11'-Me	1.62, d, 1.2	1.60, brs[d]		
12'-H_3	1.69, d, 1.1	1.68, brs[d]		
1"-H_a	4.76, dt, 14.1, 1.4	4.81, dd, 16.9, 1.9	4.81, dd, 17.3, 1.7	4.83, dd, 17.1, 1.7
1"-H_b	4.79, dt, 14.2, 1.2	4.97, dd, 16.9, 1.8	5.00, dd, 17.0, 1.6	5.00, dd, 17.1, 2.0
1"-OAc	2.09, s	2.05, s[b]	2.09, s[b]	2.11, s[b]

[a] Chemical shifts (δ) with TMS as internal reference, multiplicities and coupling constants (Hz) for solutions in $CDCl_3$ at 300 MHz.

[b,c,d] Assignments may be interchanged within groups.

oligosporon. Mass spectroscopic loss of both CH_3CO_2H and C_5H_9 from the molecular ion accounted for the strong fragment ion with composition $C_{19}H_{19}O_5$ at *m/z* 327 in EIMS.

The dehydrofarnesyl chain of flagranone A must be attached to a nucleus $C_9H_7O_5$, which thus carries two hydrogen atoms less than the 2-acetoxymethyl-4-hydroxy-5,6-epoxycyclohex-2-enone nucleus, $C_9H_9O_5$, of oligosporon (**1**). NMR data clearly establish the flagranone nucleus as the corresponding 2-acetoxymethyl-5,6-epoxycyclohex-2-en-1,4-dione depicted in structure (**2**) (Tables 2 and 3). Thus the nuclei of both metabolites contain an allylic acetoxymethyl group in which the methylene protons 1″-H_a and 1″-H_b are diastereotopic and coupled to the olefinic proton, although proton chemical shift differences indicate somewhat different environments in each compound. The ^{1}H and ^{13}C resonances of the 4-CHOH of oligosporon are replaced by the ^{13}C resonance of the 4-C=O of flagranone A, while H-3 and H-6 lose their couplings to H-4 and become a triplet and singlet, respectively. The olefinic 3-C and epoxide 5-C resonances of oligosporon retain their respective methine and quaternary multiplicity but move upfield by 6~8 ppm under the influence of the adjacent C=O in flagranone A, while the quaternary 2-C, now the α-carbon of an enedione rather than of an enone, moves 11.3 ppm downfield. The environment of the epoxide methine 6-C is relatively unaffected, as is its chemical shift. These ^{13}C shift differences are as expected for the change in oxidation level, and parallel those which are seen between the model cyclohexenoxide metabolites epiepoxydon (**5**)[14] and phyllostine (**6**)[15] which lack the acetate and dehydrofarnesyl substituents of flagranone A. They confirm that the position of attachment of the dehydrofarnesyl chain to the flagranone nucleus is the same as in oligosporon, and establish the structure (**2**) for flagranone A.

The all-*trans* olefinic stereochemistry of the flagranone A side chain follows directly from the ^{13}C chemical shifts

Table 3. ^{13}C NMR data of oligosporon and flagranones A and B.[a]

Carbon	Oligosporon (1)	Flagranone A (2)	Flagranone B (3)
1	192.4	190.9	190.3
2	131.9	143.2	143.7[b]
3	141.0	132.5	132.3
4	63.9	189.6	189.4
5	67.6	61.6	61.2
6	57.2	55.8	56.1
1'	67.5	64.3	64.6
2'	124.1	120.7	130.4
3'	140.6	141.5[b]	140.0[b]
3'-Me	13.3	13.4	13.4
4'	133.2	132.9	131.5
5'	127.0	127.7	154.5
6'	124.7	124.7	193.5
7'	141.3	142.5[b]	
7'-Me	17.0	16.9	
8'	40.1	40.1	
9'	26.5	26.5	
10'	123.7	123.7	
11'	131.9	131.8	
11'-Me	17.7	17.7	
12'	25.7	25.7	
1"	60.4	59.2	59.2
Ac-CO	170.5	169.8, 169.2	169.8, 169.1
Ac-Me	20.9	20.7, 20.6	20.7, 20.6

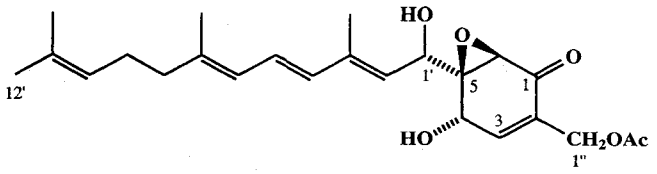
a Chemical shifts (δ) with $CDCl_3$ (77.0) as reference for solutions in $CDCl_3$ at 75.43 MHz.

b Assignments may be interchanged within groups.

of the 3′- and 7′-Me groups, and from the ^1H chemical shifts and mutual coupling constant of the olefinic protons 4′- and 5′-H, in comparison with the data for oligosporon itself (Tables 2 and 3) and literature data for model terpenes and carotenoids.[16]

Structures of Flagranones B (3) and C (4)

Flagranones B and C showed protonated and sodiated molecular ions in ESMS at *m/z* 363/385 and 337/359 (Table 1), but failed to give molecular ions under EIMS conditions. Their molecular formulae were deduced as $C_{18}H_{18}O_8$ and $C_{16}H_{16}O_8$, respectively, by mass measurement of fragment ions resulting from losses of CH_3CO_2H, CH_2CO, and CHO in EIMS. Comparison of their ^1H and ^{13}C NMR spectra (Tables 2 and 3) with that of flagranone A (**2**) confirmed the presence in all three metabolites of identical 2-acetoxymethyl-5,6-epoxycyclohex-2-en-1,4-dione nuclei, to which different side chains were attached at 5-C. In contrast to the $C_{17}H_{25}O_2$ dehydrofarnesyl chain of flagranone A, flagranones B and C must then carry $C_9H_{11}O_3$ and $C_7H_9O_3$ chains, which retain the 1′-acetoxy-3′-methyl-2′-ene substitution pattern of flagranone A although the 2′-H proton is shifted progressively to lower field, from 5.23 to 5.82 and 6.36 ppm (Table 2). Each of these shorter chains is terminated by an aldehyde function visible in ^1H NMR spectra as a low field doublet and singlet, respectively. Flagranone B (**3**) is clearly the



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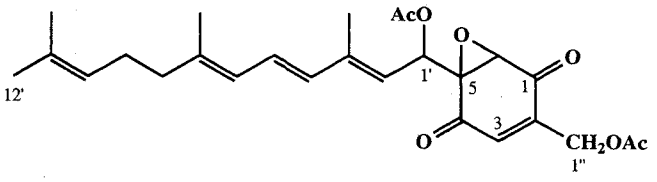

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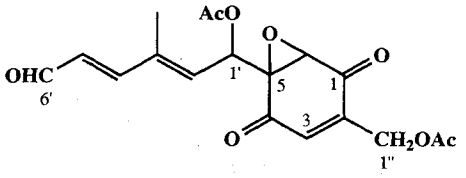

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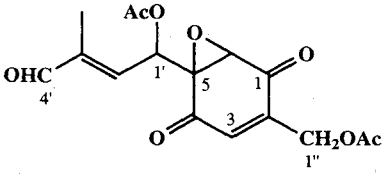

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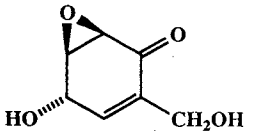

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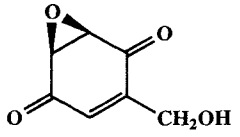

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$\alpha,\beta,\gamma,\delta$-unsaturated aldehyde vinylogue of the α,β-unsaturated aldehyde flagranone C (**4**). The base peak with composition C_6H_7O at *m/z* 95 in the EIMS of flagranone B (**3**) arises by cleavage of the 1′,2′-bond and retention of the charge on the dienal segment.

Both flagranones B (**3**) and C (**4**) retain where possible the *trans* olefinic stereochemistry of the flagranone A side chain, as shown by the ^{13}C chemical shifts of the 3′-Me groups, and by the large mutual coupling constant of the olefinic protons 4′- and 5′-H of flagranone B.

Biological Activity of the Flagranones

Flagranones A and B were tested for antimicrobial activity against a range of bacteria and fungi. The MIC value for flagranone A (**2**) against the Gram-positive bacteria *B. subtilis* and *S. aureofaciens* was 25 μg/ml, for flagranone B (**4**) 50 and 100 μg/ml respectively. Only flagranone B inhibited growth of the Gram-negative bacterium *E. carotovora*, and that was incomplete at 100 μg/ml. Antifungal activity was also limited, with an MIC value for flagranone B of 100 μg/ml against *P. cinnamomi*, and incomplete inhibition of *P. ultimum* and *R. solani* by flagranones B and A respectively. The amount of flagranone C available was insufficient for quantitive bioassay.

Conclusion

Flagranone A (**2**) from *D. flagrans* and the oligosporon metabolites from *A. oligospora*, exemplified by oligosporon itself (**1**), probably share a common biosynthetic pathway, with carbon skeletons formed by alkylation of a polyketide-derived nucleus[17~19] with a terpenoid-derived farnesyl unit. Flagranones B (**3**) and C (**4**) represent oxidative cleavage products of the side chain of flagranone A at the 6′,7′- and 4′,5′-olefinic bonds, respectively. The enedione nucleus of the flagranones is at a higher oxidation level than the hydroxyenone and enediol nuclei seen to date in the oligosporons[11,12]. The 1′-*O*-acetate side chain substituent is also characteristic of the former group, although the nuclear 2-acetoxymethyl functionality is common to both groups. The flagranones and oligosporons share all-*trans* olefinic stereochemistry in the side chains, and probably also correspond in absolute configuration at the respective stereogenic centres. While the absolute configuration of the substituted 7-oxabicyclo[4.1.0]hept-3-ene nucleus of the oligosporons has been established by circular dichroic spectroscopy,[12] interpretation of chiroptical spectra of the flagranones is complicated by the presence of overlapping chromophores and associated Cotton effects in the 220~350 nm region.

The flagranones display moderate antimicrobial activity, primarily against Gram-positive bacteria, and lack strong nematocidal action. This bioactivity spectrum parallels that of the more active of the oligosporons.[12] The occurrence of such structurally and biogenetically related metabolites as the oligosporons and the flagranones in two different genera of adhesive net-forming nematophagous fungi is of taxonomic interest. It is notable that recent phylogenetic

analysis based upon ribosomal DNA sequences indicates that *A. oligospora* and *D. flagrans*, and other predacious fungi which trap nematodes with various adhesive structures, are monophyletic and have evolved *via* a single lineage from a common ancestor.[20,21] The oligosporons and the flagranones may share a common role in the biology of these predacious Deuteromycetes.

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