ALBAFLAVENONE, A SESQUITERPENE KETONE WITH A ZIZAENE SKELETON PRODUCED BY A STREPTOMYCETE WITH A NEW ROPE MORPHOLOGY

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A novel antibiotic α,β-unsaturated sesquiterpene ketone, albaflavenone with a zizaene skeleton was isolated from a morphologically novel, highly odorous *Streptomyces* species which was identified with the species group *S. albidoflavus*, cluster 1. The new compound, partly responsible for the odour, was assigned the structure of 2*R*',6,7,7-tetramethyl-1*S*',8*R*'-tricyclo-[6.2.1.$0^{1,5}$]undec-5-en-4-one based on spectroscopic studies including 2D NMR (COSY, HETCOR, ROESY, NOE-difference) experiments.

The majority of known terpenoids are produced by eukaryotic organisms. However, a variety of actinomycetes, most notably members of the genus *Streptomyces*, produce sesquiterpenes which have been implicated in the production of earthy odours in water[1,2], and in soil[3]. These were identified as geosmin, methylisoborneol in combination with the heterocyclic 2-isopropyl-3-methoxy-pyrazine[4]. Other odorous sesquiterpenes have been isolated from broths of streptomycetes and include cadin-4-ene-1-ol[5], selina-4(14),7(11)-diene-9-ol(1)[6] and mucidone[7]. The antibiotic, arenamycin, is one of the few sesquiterpenes produced by streptomycetes reported to have biological activity[8]. This metabolite, also known as pentalenolactone, is biosynthesized *via* the enzymatic cyclization of farnesyl pyrophosphate to pentalenene.[9]

We wish to report the isolation of an unusually odoriferous actinomycete which produces a novel sesquiterpene, albaflavenone, identified as 2,6,7,7-tetramethyltricyclo[6.2.1.$0^{1,5}$]undec-5-en-4-one. The characteristics of this strain together with the isolation and identification of the novel metabolite and its antibiotic activity are described.

Materials and Methods

NMR spectra were recorded on a Bruker AMX 600 spectrometer and IR on a Perkin Elmer 1760X FT-IR spectrophotometer. UV spectra originate from a Hewlet Packard 8452A Diode Array instrument and MS on a JEOL JMS-HX/HX 110A tandem mass spectrometer. A Hewlett Packard P 5890 gas chromatograph coupled to a VG 12-250 mass spectrometer was used for GC-MS investigations. HPLC was monitored with a Hewlett Packard 5880 HPLC detection system.

Isolation and Taxonomy of Producing Strain

Starch casein medium[10] and the membrane filter method[11] were used for isolation from seeds. The strain, DSM 5415 was characterized and identified using the methods of WILLIAMS *et al.*[12]. A 16*S* rRNA *Streptomyces* genus probe as defined by STACKEBRANDT *et al.*[13] was used to confirm generic status of DSM 5415 using the method of STACKEBRANDT *et al.*[13]. Whole cell hydrolysates were analysed by TLC[14], and chemotaxonomic analysis was carried out by the German Collection of Microorganisms and Cell Cultures (DSM) and included analysis of cell wall amino acids, menaquinone type and its abundance in the cytoplasmic membrane and whole cell fatty acids.

Fermentation

Two ml of a spore suspension of DSM 5415 were inoculated into 500-ml baffled Erlenmeyer flasks containing 100 ml of the medium which consisted of (g/liter): soy tone 40.0, potato flour 100.0, Na_2HPO_4 12, BAN 120 L (Novo Nordisk A/S) 0.2 ml, pluronic (antifoam) 0.2 ml, pH 7.0. The flasks were incubated at 30°C for 5 days at 280 rpm.

Isolation of Albaflavenone

The whole broth (2.5 liters) was extracted with 3 × 400 ml redistilled EtOAc at pH 6.6. The extract was dried over $MgSO_4$ (30 minutes) and after filtration, concentrated *in vacuo*. The dried residue (2.6 g) was extracted with 5 × 10 ml *n*-heptane - EtOAc (1 : 1), yielding a yellow oil (139 mg), which was subjected to initial purification on a LiChroprep Lobar Si 60 column of 40 ~ 63 μm (Merck) with EtOAc - *n*-heptane 2 : 3. The effluent was monitored at 260 nm, and each component investigated by GC-MS and by analytical HPLC (RP 18, 5 μm column, acetonitrile - water 80 : 20) monitored with a photo diode array detector. A fraction (3.2 mg displaying a signal at *m/z* 218) was further purified by preparative HPLC, using a LiChrosorb Hibar RP 18 column (25 × 1 cm, 7 μm) with acetonitrile - water (85 : 15) to give 1.1 mg pure albaflavenone.

The following fraction from the initial chromatography (2.8 mg) contained a component with M^+ at *m/z* 220, believed to represent the alcohol corresponding to albaflavenone. Further purification of this fraction gave 400 μg of an impure compound which is still under investigation.

Tests for Antibiotic Activity

The *in vitro* activity of albaflavenone was tested against *Bacillus subtilis* ATCC 6633 which was grown in 10 ml tubes each containing 1 ml of a medium which consisted of (g/liter): Trypticase (BBL) 20.0, yeast extract (Difco) 5.0, $FeCl_2 \cdot 4H_2O$ 0.006, $MnCl_2 \cdot 4H_2O$ 0.001, $MgSO_4 \cdot 7H_2O$ 0.015, pH 7.3. Different concentrations of pure albaflavenone were added to the tubes. The tubes were incubated at 30°C with shaking (300 rpm) for 20 hours. The lowest concentration that inhibited growth of *B. subtilis* was recorded as the MIC.

Results

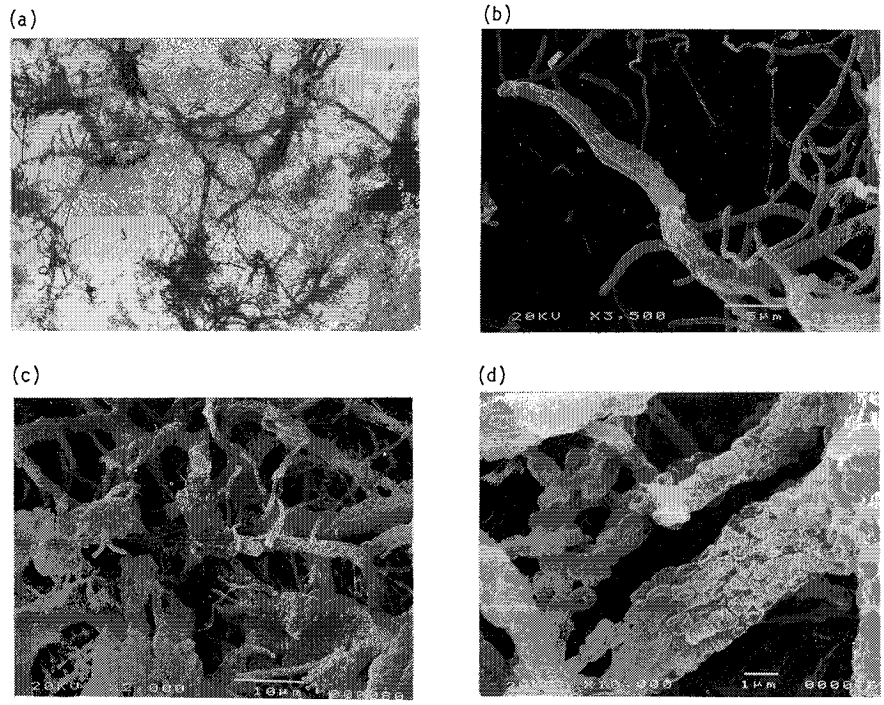
Taxonomy of producer

The producing strain DSM 5415 was isolated from corn seed. The generic identity was confirmed as *Streptomyces* by the presence of LL-DAP and glycine in the cell wall and a positive signal was obtained following hybridization of RNA with the ^{32}P-labelled 16*S* rRNA genus probe. The predominant

menaquinones were MK-9(H_4, H_6, H_9) typical for the *Streptomyces* genus[15]. However, an atypical feature was noted in the quantitative distribution of MK-9(H_6), as this is usually the most abundant meaquininone but in DSM 5415 MK-9(H_4) gave the highest peak. This difference was not regarded as sufficient to affect the generic identification as the relative abundance of the menaquinones has been shown to vary with stages in the growth cycle[15]. The fatty acid profile showed major amounts of the *iso*- and *anteiso*-fatty acids characteristic of the genus *Streptomyces* with major amounts of the fatty acid methyl esters with chain length 16:0, *iso*-16:0, *anteiso*-15:0, *anteiso*-17:0, *iso*-14:0, *iso*-15:0, in order of abundance. DSM 5415 produced a stable mycelium but the long chains of spores were aggregated into ropes observed by scanning electron microscopy (SEM) (Figs. 1b, c, d); these ropes were clearly visible under the light microscope (Fig. 1a). The substrate mycelium and non-sporulating aerial mycelium also formed ropes (Fig. 1b). Phenotypically DSM 5415 was identified with the species-group *Streptomyces albidoflavus*, cluster 1, with an identification score for the Willcox probability of 0.988, taxonomic distance (δ) of 0.417 and SE of δ 2.105. The spore mass was powdery, yellow to cream on all media used for identification tests. An unusual, strong odour was produced both on solid and in liquid media. DSM 5415 matched to the group *S. albidoflavus* as defined by WILLIAMS *et al.*[15] in vol. 4 of BERGEY's manual, the diagnostic features for which are given by WILLIAMS *et al.*[15]. This positive identification showed that the strain shared the features most diagnostic for the group including spore chain *Rectiflexibles*, smooth spore surface, no production of a diffusible pigment, melanin negative, antibiosis against fungi only (those given in Ref. 15), positive scores for lipolyisis, elastin, arbutin and xanthine degradation, resistance to penicillin,

Fig. 1. Novel rope-forming morphology of DSM 5415 growing on oatmeal agar.

(a) Light micrograph showing clear roping of aerial spore chains, unfixed, growing on a glass coverslip (×300). (b) Aerial mycelium ropes observed by SEM. (c) Rope formation by aggregation of spore chains (SEM). (d) Chains of ropes comprising rope structure (SEM).



7% NaCl, phenol 0.1%, potassium tellurite 0.001%, thallous acetate 0.001% and utilization of mannitol. Negative scores for diagnostic characters were obtained for presence of soluble pigments and substrate mycelial pigments other than yellow-brown, ability to degrade licithin, pectin, hippurate, resistance to neomycin, growth at 45°C, utilization of L-hydroxyproline, L-rhamnose, raffinose, dextran and xylitol. The sole atypical character was the inability to produce H_2S.

Structure Elucidation of Albaflavenone

The colourless solid ketone, named albaflavenone, gave a parent ion in the EI-HR-MS at *m/z* 218.1664 (Calcd for $C_{15}H_{22}O$ 218.1671). All fifteen carbon atoms were resolved in the ^{13}C NMR spectrum (Table 1). DEPT experiments established the presence of 4 methyl, 4 methylene, 2 methine, and 2 saturated quaternary carbon atoms and three sp^2 hybridized carbon atoms indicating a sesquiterpenoid structure. The proton spin systems were analysed using homonuclear decoupling and COSY experiments. A CH-correlation experiment using inverse detection (HMQC sequence[16]) served to connect the carbon and proton resonances as shown in Table 1. According to the ^{13}C NMR data the three sp^2-hybridized carbon atoms participate in an α,β-unsaturated ketone moiety. This was substantiated by the UV high intensity band (λ_{max}^{EtOH} 261 nm, ε 7,000) compared to findings for $\Delta^{8,14)}$-ergostenol-15-one acetate[17] (λ_{max}^{EtOH} 259 nm, ε 13,300) and two strong IR vibrations (ν^{KBr} 1704 and 1619 cm^{-1} for C=O and C=C, respectively). The remaining three degrees of unsaturation demand the presence of a tricyclic system.

The ^1H NMR chemical shift of the allylic 13-CH_3 group (2.09 ppm) predicts this group to reside at the β-position of the α,β-unsaturated carbonyl system. This was substantiated by CH-correlation with inverse detection (HMQC sequence[16]), optimized for long range couplings, revealing the 8-CH–7-C(14-CH_3, 15-CH_3)–6-C(13-CH_3)=C-5 sequence. The position of the methyl bearing carbons of this sequence was supported by the observation of a 2% enhancement of the geminal methyl groups at C-7 by irradiation at the position of the signal originating from 13-CH_3. The spin system 11-CH_2–8-CH–9-CH_2–10-CH_2

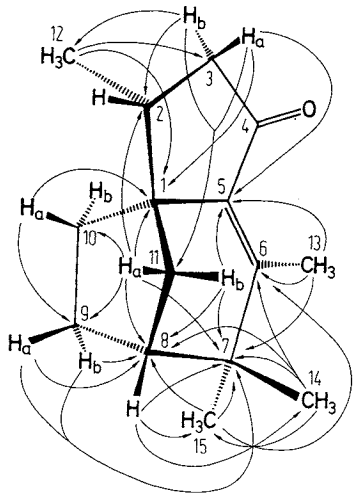
Table 1. NMR data of albaflavenone.

Position	δ_C	δ_H	J	ROE
1	51.8 s			
2	33.3 d	2.20	6.8, 13.2, 7.3	3-Ha, 3-Hb
3	47.4 t	a 2.40	7.3, 17.3	2-H, 3-Hb
		b 2.02	13.2, 17.3	2-H, 3-Ha, 12-H
4	207.4 s			
5	138.8 s			
6	153.0 s			
7	42.7 s			
8	46.2 d	1.91	6.8, 5.4	9-Hb, 11-Ha, 14-H, 15-H
9	24.4 t	a 1.75	3.5, 6.8, 11.4, 13.8	14-H, 8-H, 10-Hb
		b 1.79	9.1, 6.3, 1.8, 13.8	8-H, 10-Ha, 11-Ha
10	29.6 t	a 1.61	6.3, 11.4, 11.3	11-Ha, 9-Ha, 10-Hb
		b 1.39	2.3, 9.1, 11.3	10-Ha, 9-Hb
11	37.0 t	a 1.70	5.4, 10.9	9-Ha, 10-Ha, 11-Hb
		b 1.56	1.8, 10.9, 2.3	11-Ha, 14-H
12	14.2 q	1.07	6.8	2-H, 3-Ha, 3-Hb
13	13.0 q	2.09		15-H, 14-H
14	28.3 q	1.15		11-Hb, 8-H, 13-H, 15-H
15	24.5 q	1.12		14-H, 13-H, 8-H, 9-Hb

Spectra were measured in $CDCl_3$ solution at 600 MHz for protons and at 150.9 MHz for carbon. Chemical shifts are expressed in ppm relative to internal TMS and J in Hz.

lacks couplings between 8-H and 9-Hb and 11-Hb. Molecular models demonstrate the C-8–H bond to be orthogonal to both the C-9–Hb and the C-11–Hb bonds. C-11–Hb is in a "W" relationship to C-10–Hb and C-9–Hb giving rise to long range couplings. The presence of the sequence 12-CH_3–2-CH–3-CH_2–C-4 was deduced from the data obtained by the inverse detection techniques mentioned above. Further support for these assignments was obtained from a ROESY experiment (Table 1). In addition these data clarified most of the relative stereochemistries since cross peaks between the signals at 1.75~1.61~1.70 ppm define the *cis* relationship of protons 9-Ha, 10-Ha, and 11-Ha. Similarly cross peaks between the resonances at 1.15 and 1.56 and 1.91 ppm, respectively, demonstrate the *cis* relationship between 14-CH_3, 11-Hb and 8-H. Cross peaks between resonances at 1.12 and 1.79 and 1.91 shows 15-CH_3 to be spatially close to 9-Hb and 8-H. No cross peaks appeared between the resonances of 12-H~10-H and 2-H~11-H. However, NOE difference experiments demonstrated enhancement between 2-H and 11-Hb and between 12-H and 10-Ha. Inspection of models disclose that the enhancements observed are only consistent with the proposed structure provided the relative configuration is 1*S*',2*R*',8*R*'. Based on these deductions we assign the structure of albaflavenone as 2*R*',6,7,7-tetramethyl-1*S*',8*R*'-tricyclo-[6.2.1.$0^{1,5}$]undec-5-en-4-one analogous to the saturated sesquiterpene skeleton zizaane (2,6,7,7-tetramethyl-[6.2.1.$0^{1,5}$]undecane). The skeleton of albaflavenone has previously been obtained synthetically from khusimol[18]. The structure of albaflavenone in Fig. 2 is not intended to depict the absolute stereochemistry, which is at present unknown. Albaflavenone exhibits $[\alpha]_D^{20}$ 120° (EtOH, *c* 0.024) and CD $\Delta\varepsilon_{256}$ −2.15 and $\Delta\varepsilon_{350}$ 0.87 M^{-1} cm^{-1} (EtOH, 1.436 10^{-4} M).

Fig. 2. Structure of 2,6,7,7-tetramethyltricyclo-[6.2.1.$0^{1,5}$] undec-5-en-4-one.



: The results of the HMQC correlations are shown by arrows.

GC-MS investigations of the crude EtOAc extract revealed the presence of geosmin identified by comparison with computerized library data. The characteristic odour of the broth was due to a mixture of geosmin and albaflavenone. The powerful odour of pure albaflavenone is best described as earthy-camphor-like.

Biological Activity

Pure albaflavenone was active against *Bacillus subtilis*. In the serial dilution assay the MIC for albaflavenone was determined as 8~10 μg/ml. Further studies of the antimicrobial activity are in progress.

Discussion

The strain DSM 5415 was characterised by a novel morphology, which has not previously been described for a streptomycete. Long ropes of mycelium and spore chains were formed on a range of media. A further isolate, DSM 6012 was found with the same morphology. Studies of this strain are in progress. Strain DSM 5415 had an atypical menaquinone composition. Major amounts of MK9(H4) were detected. Broth cultures of DSM 5415 had a strong and characteristic odour originating from the sesquiterpenes

geosmin and albaflavenone. The latter is an unusual, odorous, volatile metabolite with antibacterial activity.

Acknowledgments

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