Dawenol, a New Polyene Metabolite from the Myxobacterium *Stigmatella aurantiaca*[*]

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A group of new polyene metabolites was detected in several crude extracts of the myxobacterium *Stigmatella aurantiaca*. The main compound dawenol (**1)** was isolated from cultures of *S. aurantiaca*, strain DW4/3-1. Its n-eicosaheptaene structure was elucidated by spectroscopic methods.

Key words: Myxobacteria, *Stigmatella aurantiaca*, Polyene Metabolite Dawenol

Introduction

Myxobacteria have been demonstrated to be a rich source of novel natural products with unique structures and biological activities [1]. In continuation of our biological and chemical screening for new secondary metabolites, crude extracts of a collection of *Stigmatella aurantiaca* strains were examined by HPLC/DAD. Six out of fifty strains investigated were found to contain a group of new polyene metabolites showing a structured band UV at 362 nm characteristic for six or seven conjugated double bonds. Due to the instability of these compounds we concentrated our efforts on the characterization of the main component which was later named dawenol (**1**). For the production and isolation of the polyene metabolites *S. aurantiaca*, strain DW4/3-1 [2] was selected, which in addition produces the electron-transport inhibitor myxothiazol [3] and of which the molecular biology of secondary metabolite biosynthesis and its regulation is under investigation. The biosynthetic gene cluster of myxothiazol from strain DW4/3-1 has recently been cloned and characterized [4], and the genome was scanned for the presence of further gene loci that encode both polyketide synthases (PKS) and none-ribosomal peptide synthetases (NRPS) genes [5]. In this article we report on the production, isolation, structure elucidation, physico-chemical and biological properties of **1**.

Results and Discussion

Fermentations batches of strain DW3/3-1 were harvested 70 h after inoculation and the polyene metabolites were isolated from the culture broth as described in the experimental section. The physico-chemical properties of dawenol are summarized in Table 1. The UV spectrum of **1** showed a maximum at 362 nm with three shoulders at 328, 344 and 383 nm consistent with six conjugated double bonds substituted with several alkyl groups. The elemental composition of **1** was established by HR DCI/MS to be $C_{28}H_{42}O_3$ which implied eight double bond equivalents. NMR spectroscopic data of dawenol (**1**) are given in Table 2. From the ^{13}C NMR spectrum seven C,C double bonds with signals in the range of 124 to 140 ppm and an *O*-acetyl group at 21.4 and 172.4 were identified, accounting for the eight double bond equivalents calculated. In the ^1H NMR spectrum three doublets for methyl groups (C-1, C-21, C-23) bound to aliphatic carbon atoms, four singulets and one doublet for methyl groups (C-22, C-24, C-25, C-26 and C-20) bond to olefinic carbon atoms were observed. The remaining 18 carbon atoms form the polyunsaturated backbone of dawenol. From the ^1H, ^1H COSY

Table 1. Physico-chemical properties of dawenol (**1**).

Appearance	Yellow oil
Molecular weight	426
Molecular formula	$C_{28}H_{42}O_3$
DCI-MS *m/z*	444 $(M+NH_4)^+$, 427 $(M+H)^+$, 232, 204, 162, 127, 110.
HR DCI-MS $(M+H)^+$	
Calcd. for $C_{28}H_{43}O_3$	427.3212
Found	427.3220
UV λ_{max} (nm) in MeOH	328 (sh), 344 (sh), 362, 383 (sh)

[*] Article No. 95 on antibiotics from gliding bacteria. Article No. 94: F. Sasse, T. Leibold, B. Kunze, G. Höfle, and H. Reichenbach: Cyrmenins, new β-methoxyacrylate inhibitors of the electron transport. Production, isolation, physico-chemical and biological properties. J. Antibiotics, submitted.

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Table 2. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of dawenol (**1**)in CD_3OD.

No	δ_C (ppm)	δ_H (ppm), mult, J(Hz)	^{1}H,^{1}H-COSY
1	14.0	1.19, d, J = 6.4	2-H
2	72.8	5.31, m	1-H, 3-H
3	40.7	2.05, m	2-H, 4-H, 21-H
4	80.8	3.70, d, J = 9.6	3-H
5	135.5	–	–
6	133.7	5.26, dbr J = 9.0	7-H, 22-H_3
7	33.1	3.5, m	6-H, 8-H, 23-H_3
8	138.9	5.45, dbr J = 8.7	7-H, 24-H_3
9	134.1	–	–
10	139.5	6.31, d, J = 15.1	11-H
11	124.5*	6.57, dd, J = 11.0, 15.1	10-H, 12-H
12	133.4	6.19, d, J = 11.0	11-H, 25-H_3
13	136.2	–	–
14	138.5	6.34, d, J = 15.1	15-H
15	125.1*	6.60, dd, J = 11.1, 15.1	14-H, 16-H
16	132.2	6.12, d, J = 11.1	15-H, 26-H_3
17	135.7	–	–
18	135.1	5.93, d, J =11.9	19-H, 20-H_3
19	125.5	5.50, m	18-H, 20-H_3
20	15.4	1.90, d, J = 7.4	18-H, 19-H
21	10.8	0.79, d, J = 7.2	H-3
22	11.2	1.69, s	6-H
23	21.8	1.08, d, J = 6.6	7-H
24	12.7**	1.89, s	8-H
25	12.8**	1.98, s	12-H
26	17.4	2.02, s	16-H
2-OCOCH_3	21.4	2.05, s	–
2-O$COCH_3$	172.4	–	–

*, ** : Interchangeable.

spectrum a 2,4-diol partial structure C-1 to C-4 was deduced. The acetyl group was placed at 2-OH according to the chemical shift of δ 5.31 for 2-H. As there is no further direct coupling to 4-H a methyl substituted double bond is attached in 5,6 position. 6-H is coupled to an aliphatic 7-H which is flanked by another methyl-substituted double bond in 8,9 position. The latter is part of the conjugated hexaene which is in good agreement with the UV band at 362 nm, and is terminated by a methyl group at C-20. According to the vicinal coupling of the remaining olefinic protons the remaining methyl groups C-25 and C-26 have to be placed at C-13 and C-17. The overall substitution pattern derived from spectral data is in full accordance with the supposed polyketide biosynthesis of dawenol.

The configuration of the Δ^{10} , Δ^{14} and Δ^{18} double bonds in dawenol was established to be *E*, *E* and *Z* on the basis of the vicinal proton coupling constants $J_{11-H,12-H}$ = 15.1, $J_{14-H,15-H}$ =15.1 and $J_{18-H,19-H}$ = 12.0 Hz. The configuration of the remaining double bonds Δ^5, Δ^8, Δ^{12} and Δ^{16} was determined by a ROESY experiment. Strong cross peaks for 4-H/6-H, 8-H/10-H, 11-H/25-H_3, and 15-H/26-H indicate an *E* configuration of all these double bonds.

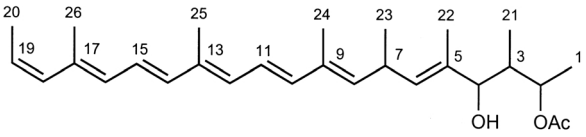

Fig. 1. Structure of dawenol (**1**).

From the above, the structure of dawenol (**1**) was elucidated unambiguously as acetic acid 2-(5*E*,8*E*, 10*E*,12*E*,14*E*,16*E*,18*Z*)-4-hydroxy-3,5,7,9,13,17-hexamethyl-eicosa-5,8,10,12,14,16,18-heptaenyl ester (Fig. 1). Though care was taken to purify dawenol (**1**) as fast as possible while avoiding unnecessary exposure to chromatographic media, light, heat, air, and pH changes, only small amounts of **1** could be isolated from the culture extracts. Attempts to determine the absolute stereochemistry of the two chiral centers in **1** were precluded by chemical instability.

The antibiotic activity of dawenol (**1**) was determined using the agar diffusion assay with paper discs. The compound (5 μg/disc) showed no inhibitory activity against various bacteria (*Staphylococcus aureus*, *Micrococcus luteus*, *Brevibacterium ammoniagenes*, *Escherichia coli*, *Pseudomonas fluorescens*), yeasts (*Saccharomyces cerevisiae*, *Candida albicans*, *Rhodotorula glutinis)* or filamentous fungi (*Mucor hiemalis*, *Botrytis cinerea*). The cytotoxicity of dawenol was determined by an MTT assay [6] with the mouse fibroblast cell line L929 and no inhibition was observed up to a concentration of 3 μg/ml.

Experimental Section

General

NMR spectra were recorded with a Bruker ARX 400 spectrometer (^{1}H: 400.1 MHz, ^{13}C: 100.6 MHz) using the solvent signal as internal standard. DCI-MS spectra were recorded with a Finnigan spectrometer MAT 95 (resolution $M/\Delta M$ = 1000; high-resolution data from peak matching, $M/\Delta M$ = 10 000). UV spectra were measured with a Shimadzu UV/Vis scanning spectrometer UV-2102. Analytical HPLC was performed using a Hewlett Packard series 1100 instrument (chromatographic conditions: column ET 125 × 2 mm and precolumn, Nucleosil 120-5-C18; solvent: 0.2% acetic acid (A)/acetonitrile (B) gradient, 50% B – 5 min to 95% at 20 min; flow rate 0.5 ml/min; detection 200 – 400 nm).

Producing organism and fermentation

S. aurantiaca DW4/3-1 was initially grown in tryptone liquid medium containing (g/l): Tryptone 10, $MgSO_4 \cdot 7H_2O$ 2, pH 7.2). Batch cultures of 100 ml or of 400 ml in 250 ml or 1,000 ml Erlenmeyer flasks, respectively, were incubated at 30 °C on a gyratory shaker at 160 rpm for 3 – 4 days. Stock cultures were stored in a deep freezer at −80 °C or in liquid nitrogen. Fermentations were carried out in media based on technical substrates *e. g.* Probion (single cell protein prepared from *Methylomonas clarae*; Hoechst AG Frankfurt) or zein; 5 l of a culture strain of DW4/3-1 grown as described above for 4 days in zein liquid medium containing (g/liter): Zein 8, peptone from casein tryptically digested 1, $MgSO_4 \cdot 7H_2O$ 1, HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) 50 mM, pH 7.2) were inoculated in a 150-l bioreactor (Bioengineering, Wald, Switzerland) containing 85 liters of the same medium (modification: HEPES 10 mM) to which 1% (v/v) of the adsorber resin Amberlite XAD-16 (Rohm & Haas, Frankfurt, Germany) had been added before sterilisation. In order to reduce foam formation an emulsion of dodecanol/methanol (50:50 v/v) was continuously added during fermentation with a pump rate of 0.11 g/h. The bioreactor was kept at 30 °C with aeration of 7.4 l air per min and agitation at 110 – 180 rpm with a flat-blade turbine stirrer. The pH of the culture drifted to about 6.5 within the first 20 h of fermentation. After adjustment to 7.0 by titration with 1N KOH at 28 h the pH rose to 7.6 after 62 h. It was kept constant at this value by titration with 30% acetic acid until the end of the fermentation. The pO_2 in the culture was recorded continuously with a polarographic oxygen electrode. Within the first 25 h it fell from about 90% to about 10% saturation. At this level it was regulated till the end of the fermentation by continuously increasing the stirring rate.

Isolation

The adsorber resin was separated from the culture by passing the content of the bioreactor through a process filter of 210 μm mesh size. The cell mass was subsequently collected from the filtrate by centrifugation. Both, the cell mass (0.89 kg) and the Amberlite XAD 16 adsorber resin (1.24 kg) from 90 l of culture broth of DW4/3-1 were eluted with 8 l of methanol and 7 l of acetone. The combined eluates were concentrated *in vacuo* until an oil/water mixture was formed. This mixture was diluted with water and extracted three times with ethyl acetate. The combined organic layers were dried with sodium sulfate and concentrated *in vacuo* to yield 48.2 g of a dark oily residue which was partitioned between methanol and heptane to remove highly lipophilic by-products. Concentration of the methanol layer yielded 29.8 g of a brown oil which was chromatographed on Sephadex LH-20 (100 i.d. × 1000 mm; eluent methanol, flow rate 35 ml/min, UV detection at 366 nm). According to HPLC analysis, the fractions containing the dawenol complex were combined, concentrated *in vacuo* (residue 8.1 g) and chromatographed on a preparative RP18-HPLC column (YMC ODS-AQ, 100 i.d. × 400 mm, linear gradient of methanol/water 60:40 to 100% methanol, flow rate 200 ml/min, UV detection at 366 nm) to yield 1.32 g of a crude product. Further separation and final purification was achieved by chromatography in small batches on a Sephadex LH-20 column (20 mm i.d. × 700 mm, eluent dichloromethane/methanol 8:2, flow rate 1.3 ml/min, UV detection at 366 nm) yielding approx. 20 mg of dawenol (**1**). Due to degradation especially during the last purification step this represented only a minor part of the total content of approx. 240 mg of **1** in the culture extract.

Biological assays

Antimicrobial activity was determined in the agar diffusion assay using paper discs. Test for cytotoxicity against L929 cells (mouse, connective tissue, ATCC CCL 1) was determined as reported [6].

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