DEFLECTINS, NEW ANTIMICROBIAL AZAPHILONES FROM *ASPERGILLUS DEFLECTUS**

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Five structurally related, angular azaphilones, the deflectins, were isolated from the mycelia of *Aspergillus deflectus*. The structures of all compounds have been established. Besides the inhibitory effects on the growth of bacteria and fungi, these compounds showed lytic activity towards bacteria and erythrocytes and cytotoxic activity towards cells of the ascitic form of Ehrlich carcinoma of mice.

The inhibitory effects of the deflectins could be reversed by the addition of serum or serum albumin.

Aspergillus deflectus, a member of the *A. ustus* group, was found to produce desferritriacetylfusigen when grown on a synthetic medium with a low iron content[2]. Since no other secondary metabolites have been reported so far from *A. deflectus*, this strain was the subject of further investigations.

On Raulin-Thom, Czapek-Dox or media based on various amounts of soybean meal and mannitol, no antibiotic activity could be detected in the culture filtrate or in mycelial extracts. However, when grown on a medium composed of yeast extract (4 g), malt extract (10 g) and glucose (20 g) (YMG-medium), mycelial extracts exhibited antibacterial activity. This paper deals with the isolation, the elucidation of structures and the biological activities of the antibiotics from *A. deflectus*.

Materials and Methods

Cultivation of Organisms

Bacteria (except *Actinomycetales*) were grown on nutrient broth (NB; Difco) or on a minimal medium composed of (g/liter): K_2HPO_4, 7 g; KH_2PO_4, 3 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; NaCl, 0.1 g; glucose (sterilized separately) 2 g.

Actinomycetales and fungi were grown on HA medium (yeast extract, 4 g; malt extract, 10 g; glucose, 4 g) or on a minimal medium composed of (g/liter): asparagine, 1.0 g; NaCl, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCO_3$, 0.2 g; glucose (sterilized separately) 10 g.

In some cases the minimal media were supplemented with a vitamin solution (5ml BME-Vitamins, Flow Laboratories, per liter).

For solid media 20 g Bacto-agar (Difco) were added to one liter of medium.

Fermentation of *A. deflectus*

A. deflectus was maintained on HA medium. The evaluation of the most suitable medium for the production of the deflectins was carried out in 500-ml Erlenmeyer flasks with one intrusion, containing 200 ml of medium. The flasks were incubated on a rotary shaker (120 rpm) at 27°C. The con-

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Parts of the results have been presented at the VIth International Fermentation Symposium, London, Canada, July 20～25, 1980, and at the 81st Annual Meeting of the American Society for Microbiology, Dallas, March 1～6, 1981.

ditions for the fermentors were: (a) 25 liters (type b 20, Braun Melsungen AG, Melsungen, FRG) with "intensor" system: 1000 rpm, 4 liters air/minute, 27°C; size of inoculum: 5%; (b) 200 liters (type b 200, AG für biologische Verfahrenstechnik, Giovanola Freres SA, Monthey, Switzerland) with "intensor" system: 1000 rpm, 40 liters air/minute, 27°C; size of inoculum: 12.5%. To prevent foaming, silicone antifoam (Merck) was added.

Assays

The disc-diffusion assay with *Bacillus subtilis* (ATCC 6051) as test organism in minimal agar was used for measuring the antibiotic content of the culture fluid and mycelial extracts. The effect of the deflectins on the macromolecular syntheses in bacteria and Ehrlich carcinoma ascitic cells (ECA cells) was tested as previously described by ANKE[3]. Mice bearing ECA were a gift from H. G. Probst, Tübingen.

Test for hemolytic activity: Hypotonic hemolysis (control) of bovine erythrocytes was determined by the hemiglobincyanide method, Merckotest 3317 (Merck)[4]. The effect of the deflectins on erythrocytes was tested in a similar way using an isotonic medium.

General Methods

Melting points were determined with a Büchi 510 apparatus and are uncorrected. TLC was performed on silica gel F-254 plates (Merck). IR spectra were determined on a Perkin-Elmer 297 spectrometer and UV spectra on a Zeiss DMR 21. Mass spectra were recorded on an AEI MS 9 apparatus (70 eV) and NMR spectra on Bruker WH 270 and WH 400, using tetramethylsilane as internal standard. The optical rotations were measured with a Perkin-Elmer 241. GLC was performed with a Packard 428 (50 m glass capillary column with SE 30; FID; temperatures: 300/180/300°C inject-column-detection). HPLC was carried out on a Waters system with detectors 450 and R 401.

Results and Discussion

Production and Isolation of the Deflectins

Aspergillus deflectus grows well on the above mentioned YMG medium. After 3~4 days the mycelium becomes deep red and shortly thereafter the production of antibiotically active metabolites is observed. During longer incubation periods, antibiotic activity can be detected in the culture filtrate due to hyphal breakdown. However, more than 90% of the antibiotics were extracted from the mycelium. The mycelia were separated from the culture broth, which was discarded. The antibiotics were extracted from the mycelia with acetone. After evaporation of the solvent, the aqueous residue was extracted with chloroform. The chloroform extract was concentrated and applied onto a silica gel column. The column was eluted with chloroform followed by chloroform - methanol (increasing amounts of methanol). The fractions containing the antibiotics were detected using an agar diffusion assay with *Bacillus subtilis* as test organism and combined. Upon concentration yellow crystals were obtained, which were washed and recrystallized from methanol.

Elucidation of Structures

Although this nicely crystalline material seemed to be homogeneous on TLC, NMR and mass spectra indicated that it consisted of two or more structurally related components. HPLC on reversed-phase silica gel (RP-18) with methanol - triethylammonium formate buffer (8: 2, pH 6.0) as eluent revealed that four major and several minor components were present (Fig. 1). Five of these, **1a~c** and **2a, b** could be obtained in a pure state by reversed-phase chromatography on a preparative scale and recrystallization from methanol. From the physical data given in Table 1, two groups can be formed, deflectins A, **1a~c**, and deflectins B, **2a, b**. The relative amounts of the different deflectins in the crude cell extract can easily be determined by analytical HPLC with selective detection at 339 nm (Fig.

Fig. 1. Analytical HPLC of deflectins on RP-18.

A. 8 μg purified mixture of deflectins. B. 90 μg cell extract with detection at 339 nm. C. 3 mg cell extract with differential refractometer detection.

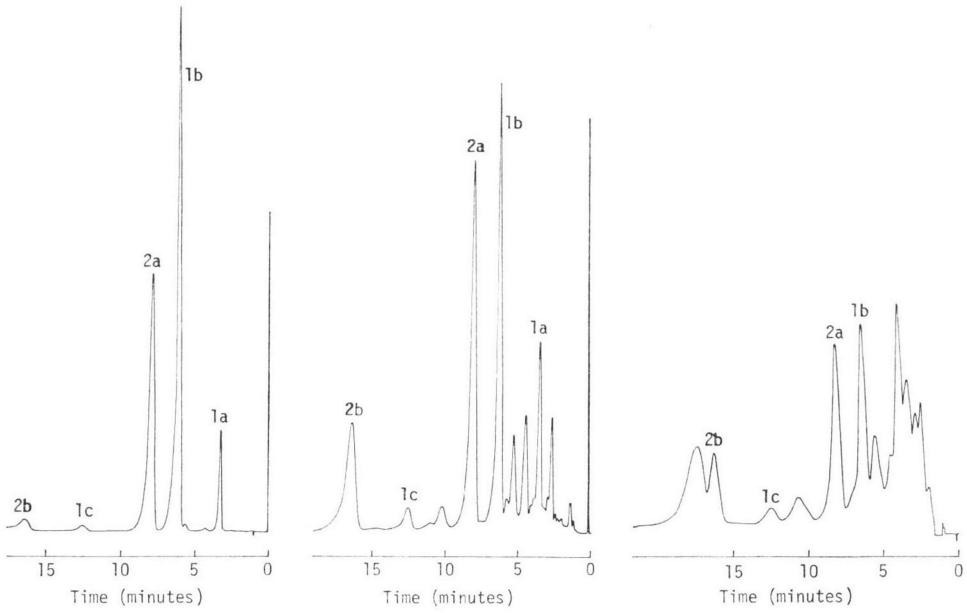
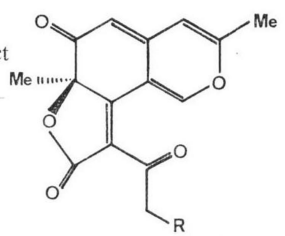


Table 1. Physical data of deflectins **1a~c** and **2a,b**.

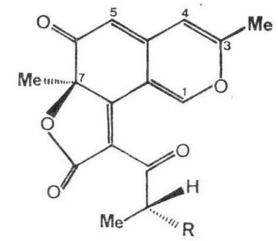
Compound	Molecular formula	Mol. wt.	M.p. °C	$[\alpha]_D^{21}$ (c 5, EtOAc)	Rel. amount cell extract (%)
1a	$C_{21}H_{24}O_5$	356	161	+431°	7
1b	$C_{23}H_{28}O_5$	384	152	+405°	34
1c	$C_{25}H_{32}O_5$	412	141	—[a)]	3
2a	$C_{24}H_{30}O_5$	398	122	+397°	39
2b	$C_{26}H_{34}O_5$	426	111	+346°	17

[a)]: Sufficient amounts of the pure product were not available.

Fig. 2. Structures of the deflectins.



1a R=*n*-C_6H_{13}
1b R=*n*-C_8H_{17}
1c R=*n*-$C_{10}H_{21}$



2a R=*n*-C_8H_{17}
2b R=*n*-$C_{10}H_{21}$

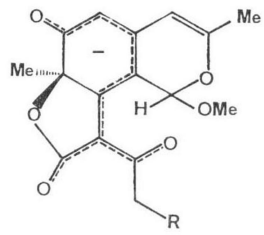
1B). Detection with a differential refractometer (Fig. 1C) shows, in addition to deflectins, several other compounds which are presently under investigation.

The mass spectra of all deflectins show a very strong base peak at m/z=230.0578 ($C_{13}H_{10}O_4$) which represents a common building block, containing the chromophoric system with UV maxima at 209 (7900), 219 (7200), 261 (11500), 340 (10700), 395 (sh 205), 530 nm (sh 110) for **1a~c** and 208 (8600), 218 (7800), 261 (12700), 338 (11600), 395 (sh 220), 430 nm (sh 130) for **2a** and **2b** in ethanol. In the IR spectrum (KBr) strong bands are observed at 1760, 1680, and 1615 cm^{-1} with small differences in the finger print region for deflectins A and B. From these data and the proton NMR spectra an azaphilone structure can be deduced for **1** and **2**[5,6].

Whereas the signals for H-4 and H-9 (6.08 and 2.20 ppm, J=0.5 Hz), for H-5 (5.28 ppm) and the methyl group at C-7 (1.68 ppm) are identical in **1** and **2**, H-1 is found at 8.76 ppm in **1** and 8.71 ppm in **2** with the characteristic coupling of J=1 Hz to H-5. The aliphatic side chain carboxylic acids

can be split off by fusion with sodium hydroxide and identified as their methyl esters by GLC and MS. Oxidation of **2a** and **2b** with potassium permanganate allowed the isolation of optically pure α-branched carboxylic acids with (*S*) configuration. The absolute configuration at C-7 of the deflectins as (*R*) follows from the comparison of optical rotation with related azaphilones[7,8]. Like other azaphilones the deflectins react rapidly with ammonia exchanging oxygen-2 for NH[5]. The transient deep red colour observed in this reaction, can be obtained also by addition of bases like NaOH, NaOMe or triethylamine to a solution of the deflectins in alcohol. From the spectroscopic data (λ_{max}: 210 (10800), 265 (11900), 300 (5700), 367 (10600), 530 nm (9100); no lactone carbonyl band; H-1=5.57 ppm) the anionic structure **3** (Fig. 3) can be deduced for the red compounds and on addition of acid **1** and **2** are regenerated without loss of optical purity. In the titration with NaOH in MeOH/H_2O the deflectins behave as weak acids with pKa=7.1. We believe that the biological activity of **1** and **2** is connected to the apparent high reactivity of C-1 towards nucleophiles.

Fig. 3. Anionic structure.



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Biological Activity

Little is known on the biological activity of azaphilones of the rotiorin and isorotiorin type; whereas citrinin, a related compound, has been investigated quite extensively[9,10]. Deflectins A (**1b**) and B (**2a**), the two major components, were used for the evaluation of the biological activities of the deflectins. Both compounds showed antibacterial and weak antifungal activity with minimum inhibitory concentrations (MIC) ranging from less than 1 μg/ml to 150 μg/ml, depending on the medium. In synthetic media the MIC's were 20~100 fold lower than in complex media. The antibacterial spectra are given in Table 2. *Bacillus brevis* and *B. subtilis* were the most sensitive organisms. Deflectin B was slightly

Table 2. Spectra of antibacterial activity of deflectins A and B.
Minimum inhibitory concentrations (MIC) in the serial dilution test; size of inoculum: 10^6 cells or spores per ml. I: minimal medium; II: minimal medium+vitamins; III: minimal medium+serum (50 μl/ml); IV: complex medium.

Organism	MIC (μg/ml)							
	Deflectin A				Deflectin B			
	I	II	III	IV	I	II	III	IV
Arthrobacter citreus	5	—[a]	—	>100	1	—	—	75
Bacillus brevis	ng[b]	ng	ng	10	ng	ng	ng	5
B. subtilis ATCC 6633	ng	0.5	30	10	ng	0.5	30	5
B. subtilis ATCC 6051	0.5	5	>30	100	0.2	5	>30	100
Escherichia coli K12	>50	—	—	>100	>50	—	—	>100
Nocardia brasiliensis	1	5	>30	100	1	10	>30	100
Proteus vulgaris	1	—	—	50	1	—	—	20
Pseudomonas fluorescens	>50	—	—	>100	>50	—	—	>100
Staphylococcus aureus	—	5	>30	>100	—	5	>30	>100
Streptococcus faecalis	3	—	>30	70	1	—	30	30
Streptomyces viridochromogenes	ng	5	>30	50	ng	5	>30	50

[a] not tested; [b] no growth

Table 3. Effect of deflectin B on the incorporation of thymidine, uracil, leucine, and *N*-acetylglucosamine into trichloroacetic acid-precipitable material of cells of *Bacillus subtilis* ATCC 6051 in minimal medium.

Deflectin B (μg/ml)	Serum (μl/ml)	Incorporation*			
		Thymidine	Uracil	Leucine	*N*-Acetylglucosamine
0.2	—	26	21	22	12
1.0	—	12	12	16	6
5.0	—	4	7	7	3
5.0	10	92	98	100	102

* % of the control without antibiotic

Table 4. Effect of deflectin A on the incorporation of thymidine, uridine, and leucine into trichloroacetic acid-precipitable material in cells of the ascitic form of Ehrlich carcinoma.

Deflectin A (μg/ml)	Serum (μl/ml)	Incorporation*		
		Thymidine	Uridine	Leucine
10	0	48	37	34
10	10	88	80	81
20	0	6	1	1
20	10	57	36	32
20	20	99	96	89

* % of the control without antibiotic

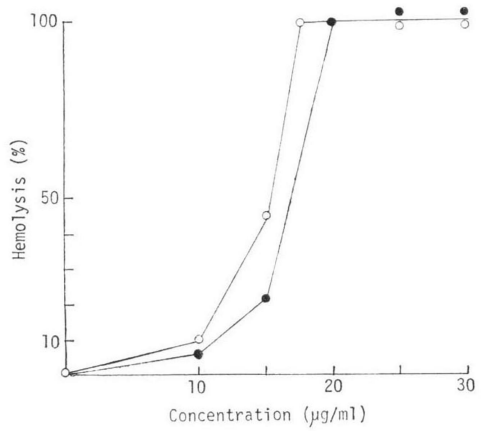
more active than deflectin A. The antibacterial effects of both antibiotics could be reversed by the addition of serum or serum albumin, as shown in Tables 2 and 3. Similar effects have been reported for citrinin[11]. The MIC's in minimal medium supplemented with 50 μl per ml of serum were comparable to those in complex media. The growth of fungi on minimal media containing agar was inhibited at 100 μg of deflectin A or B per disc in the agar diffusion assay.

In exponentially growing cells of *B. subtilis*, the effects of deflectins A and B on the macromolecular syntheses were measured. The inhibitory effects of the two compounds were almost identical. Table 3 shows, that the incorporation of thymidine, uracil, leucine, and *N*-acetylglucosamine was inhibited by deflectin B to the same extent. The same was true for deflectin A (data not shown). At the highest concentration (5 μg/ml) lysis of the cells was observed.

In cells of the ascitic form of Ehrlich carcinoma, the incorporation of thymidine, uridine, and leucine was almost simultaneously inhibited by deflectins A and B. As shown in Table 4, 10 μl of serum are necessary to compensate the inhibitory effect of 10 μg deflectin A. With chicken embryo fibroblasts, both normal cells and those transformed by Rous sarcoma virus, no effect on the uptake or incorporation of thymidine, uridine, and leucine was observed at concentrations up to 50 μg/ml (J. KUPKA, personal communication).

Deflectins A and B exhibit some hemolytic activity as shown in Fig. 4. At 20 μg/ml of deflectin A and 17 μg/ml of deflectin B complete hemolysis of bovine erythrocytes could be observed. This suggests that in sensitive cells one of the primary targets of the deflectins might be the cytoplasmic membranes.

Fig. 4. Hemolytic activity of deflectins A and B.
● deflectin A; ○ deflectin B



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