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Curvupallides, A New Class of Alkaloids from the Fungus *Curvularia pallescens*

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Abstract: Three alkaloids with a novel skeleton, named curvupallides A, B and C, are described from the phytopathogenic fungus *Curvularia pallescens*. They possess an unusual α,ß-unsaturated ene-amide γ-lactam. Curvupallides are produced - together with phytotoxic spirostaphylotrichines showing structural similarities with them - only under limitation of nitrogen in submerse culture. Their structure were determined using various NMR techniques and MSMS. They displayed no phytotoxic activity so their ecological function could not be determined.

Curvularia pallescens (teleomorph = *Pseudocochliobolus pallescens*) is a phytopathogenic fungus infecting food plants like maize (*Zea mays*), coriander (*Coriandrum sativum*), wheat (*Triticum vulgare*), or garlic (*Allium sativum*).[1] From its culture broth we identified triticone A and B, first isolated from *Drechslera tritici-repentis* (now *Pyrenophora tritici-repentis*),[2] as the phytotoxin. Tamm and coworkers isolated these toxins and similar compounds from the fungus *Staphylotrichum coccosporum*[3] and named this class of secondary metabolites spirostaphylotrichines. We recently reported on spirostaphylotrichines from *Curvularia pallescens* Palliser DSM 62482[4]. Beside curvulin and the known spirostaphylotrichines A, C and D (= triticone A and B), Q and R[5] we isolated the novel spirostaphylotrichines U and V. Furthermore, we found three compounds with a novel skeleton which we will discuss here in detail.

RESULTS AND DISCUSSION

Curvularia pallescens formed some alkaloids, which were identified as the spirostaphylotrichines A (**1**), C (**2**), D (**3**), Q (**4**) and R (**5**), and additionally the novel spirostaphylotrichines U (**7**) and V (**6**) only if grown under

the limitation of nitrogen. Besides these spirostaphylotrichines three compounds were found which could not belong to this class of alkaloids because of their ^{1}H and ^{13}C NMR spectra.

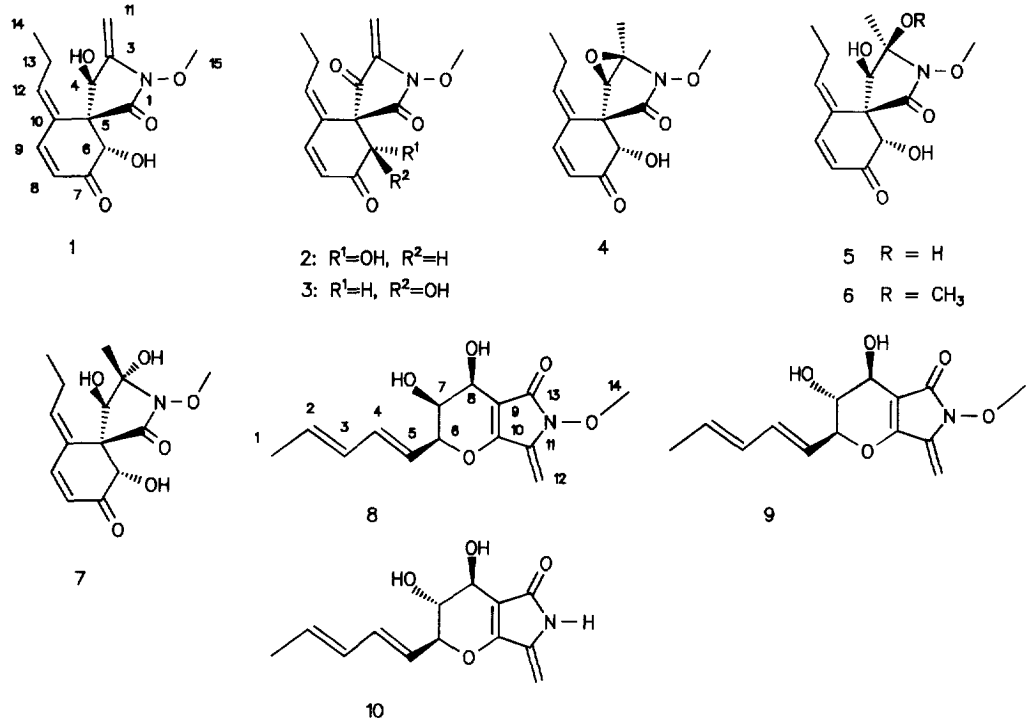


Figure 1: Metabolites of *Curvularia pallescens* DSM 62482

The most polar compound found in the extract of *Curvularia pallescens* was one of the main metabolites, its NMR resonances however were distinct from those of the spirostaphylotrichines. A fermentation in larger scale brought enough material for the structure elucidation of this compound and resulted also in the formation of two related minor metabolites. The main metabolite, named curvupallide C, displayed resonances of an all-trans pentadienyl moiety in the ^{1}H NMR spectrum. This side chain showed a coupling to a double douplet at low field (δ_H 4.61). $^{1}H\{^{1}H\}$ homonuclear shift-correlation spectroscopy (COSY-45) revealed the connectivities of the protons. The carbon backbone of this alkaloid could be elucidated by the use of the HMBC[6] NMR spectra. To discern between nitrogen or oxygen as the heteroatom in the five-membered ring the infrared spectrum proved to be useful. It displayed an absorption at 1687 cm^{-1} requiring a lactam and not a lactone as found in tetrodecamycin[7]. The problem whether a hydrogen is attached to the nitrogen or another heteroatom could be solved by mass spectroscopy. The EI^{+} spectrum showed a basepeak at m/z 110, a significant ion at m/z 231 and a small peak at m/z 249, the latter indicating a molecular weight of 249. Compound 10 is expected to undergo retro-Diels-Alder fragmentation[8] in the dihydropyran ring, leading to a fragmention at m/z 110 (Scheme 1). In the positive FAB mode m/z 250 was the basepeak of the spectrum. An MSMS experiment

Table 1: NMR data of the curvupallides A (**8**), B (**9**) and C (**10**) ($CDCl_3$)

	^{1}H NMR			^{13}C NMR		
	8	**9**	**10**	**8**	**9**	**10**
1	1.81 dd	1.70 dd	1.78 dd	18.5 +[a]	18.0 +	17.7 +
2	5.85 dq	5.86 dq	5.75 dq	133.0 +	132.4 +	131.8 +
3	6.14 ddq	6.18 ddq	6.04 ddq	130.6 +	130.2 +	130.1 +
4	6.47 dd	6.48 dd	6.31 dd	136.8 +	135.9 +	135.2 +
5	5.79 dd	5.81 dd	5.70 dd	123.3 +	123.5 +	123.7 +
6	4.75 d br	4.61 dd	4.61 dd	78.6 +	82.9 +	82.8 +
7	3.90 m	3.72 dd	3.81 dd	70.8 +	71.9 +	71.6 +
8	4.47 d	4.38 d	4.40 d	61.4 +	64.3 +	64.2 +
9	-	-	-	103.7 0	103.7 0	105.7 0
10	-	-	-	136.8 0	136.2 0	137.1 0
11	-	-	-	158.4 0	156.4 0	159.5 0
12	5.17 d	5.13 d	5.01 d	93.3 -	93.1 -	94.1 -
12'	5.07 d	5.04 d	4.82 d			
13	-	-	-	166.9 0	166.6 0	171.0 0
OMe	3.91 s	3.83 s	-	64.8 +	64.6 +	-

[a]amplitude of signals in DEPT-135 spectrum (CH_3 or CH = +; CH_2 = -; quat. C = 0)

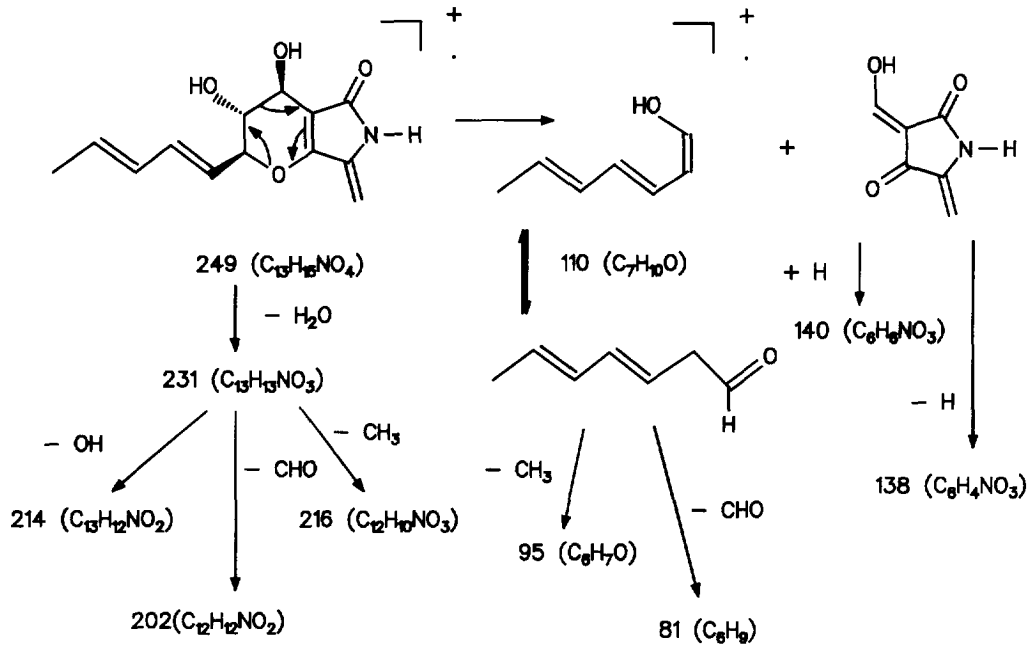
J (Hz): **8**: 1,2=7;1,3=1;2,3=15;3,4=10;4,5=15;5,6=7;6,7=1;7,8=2;12,12'=2. **9**, **10**: 1,2=7;1,3=1;2,3=15;3,4=10;4,5=15;5,6=8;6,7=7;7,8=6;12,12'=1.

showed the main daughter ions of m/z 250 to be m/z 232 and m/z 140 corresponding to an elimination of water and the RDA product from the $(M+H)^+$ ion. In the negative FAB mode an ion at m/z 248 of middle intensity was detected. This produced daughterions at m/z 230 and m/z 138 representing the same fragmentation pathway for the $(M-H)^-$ ion. These data clearly established a molecular weight of 249 for compound **10** thus showing the substituent attached to the nitrogen to be hydrogen. Furthermore a high resolution EI^+ measurement of the ion m/z 249 yielded an elemental composition of $C_{13}H_{15}NO_4$.

Taking all these informations together the structure **10** for curvupallide C results. The relative configuration of the substituents at the dihydropyran ring could be deduced as all-trans according to the coupling constants of $J_{6H,7H}$ = 7 Hz and $J_{7H,8H}$ = 6 Hz in the ^{1}H NMR spectrum[9].

The two minor compounds displayed very similar resonances in the NMR spectra but both showed an

additional resonance of a methoxy moiety. One of these metabolites, named curvupallide B, displayed in the ^{1}H NMR spectrum resonances which were - despite the additional methoxy signal - almost identical to the ones of curvupallide C. With these informations in hand curvupallide B was identified as compound **9**. The most obvious differences between curvupallide B (**9**) and the remaining metabolite, named curvupallide A, were in the coupling constants of 7-H (Table 1). This proton displayed in curvupallide A only small couplings to 6-H and 8-H pointing to compounds which are epimeric at 7-H. The identification of **8** as curvupallide A was



Scheme 1: Fragmentation of curvupallide C **10** in EI-MS

corroborated by the ^{13}C NMR data showing a shielding of C-6 (δ_C 78.6 in **8** compared to δ_C 82.9 in **9**) and C-8 (δ_C 61.4 in **8** compared to δ_C 64.3 in **9**) caused by the axial position of the hydroxy group at C-7.

The absolute configuration of the curvupallides were adopted according to the one of spirostaphylotrichin A **1** which was determined by Sandmeier and Tamm using Nakanishi's benzoate method[10].

DISCUSSION

Curvupallides A - C displayed no effects against *Bacillus cereus* DSM 318, *Staphylococcus aureus* ATCC 13709, *Escherichia coli* ATCC 9637, *Salmonella gallinarum* ATCC 9184, *Mycobacterium smegmatis* ATCC 607, *Candida albicans* ATCC 10231, *C. tropicalis* DSM 1346, *Rhodotorula glutinis* DSM 70398, *Aspergillus niger* (spores) DSM 737, *A. flavus* (spores) BD 27 and *Mucor rouxii* (spores) DSM 1691 in concentrations

below 100µg/ml. The metabolites in the same concentration were not toxic for the brine shrimp *Artemia salina* and did not effect cress seedlings. These negative results gave no hints of the biological or ecological function of these alkaloids. In contrast to the curvupallides the ecological role of the spirostaphylotrichines is more obvious. They are phytotoxins or derivatives thereof and formed under nitrogen starvation of the fungus which makes sense because under this circumstances the host plant is attacked and nitrogen is available to the fungus.

Alkaloids bearing the carbon skeleton of the curvupallides are novel. A substructure search for the unusual α,ß-unsaturated ene-amide γ-lactam moiety revealed no other natural product reported in the literature. Only a biliverdin derivative was reported to bear this type of lactam.[11] The oxygen analogue is found in the antibiotic protoanemonin[12] and as a substructure in a multitude of bioactive compounds (e. g. tetrodecamycin or tetronomycin[13]).

EXPERIMENTAL

Curvularia pallescens DSM 62482 was grown in 60 1-liter Erlenmeyer flasks filled with 200 ml of the medium containing glucose (10 g), ammonium chloride (0.1 g), dipotassium hydrogenphoshate (0.5 g), potassium chloride (1 g), magnesium sulfate heptahydrate (0.2 g), calcium chloride dihydrate (0.1 g) in 1 litre of deionized water. After 7 days the culture broth was filtered and extracted three times with ethyl acetate. After drying with sodium sulfate the solvent was evaporated and the crude extract was separated on a Si-60 column with a n-hexane/ethyl acetate gradient (changing from 19:1 to 0:1). When necessary the collected fractions were purified further by preparative TLC.

Mass spectra were recorded on a tandem high-resolution mass spectrometer of $E_1B_1E_2B_2$ configuration (JMS-HX/HX110A, JEOL Tokyo) at 10 kV accelerating voltage. Resolution of both MS was set to 1:1000 except for high resolution measurements, where the first MS was set to a resolution of 1:10000. EI spectra were recorded at 70 eV. In this case, the sample (solution in methanol) was introduced into the ion source via a heated direct inlet probe (JEOL MS-DP11). The probe was heated from 25 to 200°C with a rate of 2°C/min. For $FAB^{+/-}$ measurements thioglycerol served as the matrix. The JEOL FAB gun was operated at 6kV with xenon as the FAB gas. Collision-induced fragmentation took place in the third field free region. Helium served as the collision gas at a pressure sufficient to reduce the precursor ion signal to 30% of the original value. The collision cell was operated at a potential of 3 kV in the positive and 2 kV in the negative FAB mode. FAB-MS/MS spectra (linked scans of MS2 at constant B/E ratio) were recorded at 300 Hz filtering with a JEOL DA 7000 data system in profile mode.

The ^1H and ^{13}C NMR spectra were obtained at 400 and 75.5 Mhz, respectively. Deuterochloroform was the solvent and tetramethylsilane the internal standard. IR spectra were measured on potassium bromide, UV spectra in methanol. R_f values were determined with Si-60 HPTLC plates with the mobile phase ethyl acetate.

From 12 liter of fermentation broth curvupallides A (**8**, 7mg), B (**9**, 30mg) and C (**10**, 90mg) were isolated beside curvulin (3mg), spirostaphylotrichine A (**1**, 17mg), C, and D (**2** and **3**, 79mg), Q (**4**, 51mg), and V (**6**,

6mg).

Curvupallide A (**8**): R_f 0.48, oil, UV (λ_{max}): 230, 262 nm. IR: 3388, 1720, 1628, 1456 cm^{-1}. MS (m/z): 279.1097 (279.1107 calc. f. $C_{14}H_{17}NO_5$)(%), 238(), 196(5), 170(4), 167(5), 164(5), 149(14), 110(100), 95(41).

Curvupallide B (**9**): R_f 0.43, oil, IR: 3373, 1710, 1634, 1456, 732 cm^{-1}. MS (m/z): 279.1106 (279.1107 calc. f. $C_{14}H_{17}NO_5$)(6%), 238(3), 230(4), 196(4), 167(5), 154(37), 110(100), 95(45).

	589nm	578nm	546nm	
$[\alpha]^{27}$ =	-92.7°	-93.7°	-86.2°	(c=1.00, methanol)

Curvupallide C (**10**): R_f 0.11, instable, m. p. 168-170°C (decomp.), IR: 3287, 1687, 1639 cm^{-1}. MS (m/z): 249.1005 (249.1001 calc. f. $C_{13}H_{15}NO_4$) (1), 231 (9), 214 (4), 202 (3), 190 (4), 188 (3), 164 (3), 140 (21), 138 (4), 124 (3), 110 (100), 95 (74), 81 (30), 67 (12).

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