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Aspochalamins A~D and Aspochalasin Z Produced by the Endosymbiotic Fungus *Aspergillus niveus* LU 9575

II. Structure Elucidation

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The structures of new cytochalasan fungal metabolites aspochalamins A~D have been elucidated by ESI-FTICR-MS, NMR spectroscopy, and chiral amino acid analysis. Aspochalamins A~D consist of different aspochalasin skeletons connected at position C-19 to the *N* terminus of the tripeptidic moiety amide anthranoyl-L-alanine-*E*-didehydrotryptamide. Furthermore, the structure of a new aspochalasin analog, aspochalasin Z, was derived from its molecular mass and NMR data as 10-isopropyl-14-methyl[11]-cytochalasa-6*Z*,13*E*,19*E*-triene-1,21-dione.

Five novel metabolites with retention times of 7.5 (**1**), 11.6 (**2**), 12.2 (**3**), 12.8 (**4**), and 13.8 (**5**) minutes in the HPLC-DAD screening[1)] were isolated from the mycelium extract of the endosymbiotic fungus *Aspergillus niveus* LU 9575. The taxonomy of the producing organism, fermentation, isolation, and biological activities of the metabolites are described in the preceding paper[2)]. Here, we present the structure elucidation of these compounds.

Results

With respect to its retention time, UV-Vis spectrum, and isolation characteristics compound **1** which had been isolated along with aspochalasin D differed clearly from metabolites **2**~**5**[2)]. An ESI-mass spectrum provided a molecular mass of 369 Da for **1** (*m/z* $[M+H]^+$ 370). The ^1H and ^{13}C NMR data (Table 1) accounted for the presence of five methyl, five methylene, five sp^3 methine and four sp^2 methine groups, as well as one sp^3 and four sp^2 quarternary carbons including two carbonyl carbons. A broad signal at δ_H 7.95 observed in the ^1H NMR spectrum was attributed to an amide group. Thus the molecular formula of **1** was deduced from its molecular mass and ^1H and ^{13}C NMR data as $C_{24}H_{35}NO_2$. Analysis of ^1H–^1H-COSY, HSQC, and HMBC spectra established **1** as 10-isopropyl-14-methyl[11]-cytochalasa-6*Z*,13*E*,19*E*-triene-1,21-dione[3)], a new member of the aspochalasin family designated as aspochalasin Z (Fig. 1). The (13*E*, 19*E*) configuration of the double bonds was concluded from the upfield chemical shift of C-25 (δ_C 16.7) and from the large vicinal coupling constant of $^3J_{19,20}$=16 Hz, respectively. Contrary to all known members of the aspochalasin family[4~11)], **1** lacks oxygen functionalities at positions C-17 to C-20 and can therefore be considered as the simplest aspochalasin analog discovered so far.

Metabolites **2**~**5** were analysed by high-resolution ESI-FTICR-MS, and molecular masses of 749 Da (**2**, **3**), 733 Da

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Table 1. ^{1}H and ^{13}C NMR data for aspochalasin Z (**1**) and aspochalamins A~D (**2**~**5**).

	1[a)]		**2**[a)]		**3**[a)]		**4**[b,c)]		**5**[b)]	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	173.3 s	-	174.5 s	-	174.5 s	-	175.4 s	-	174.4 s	-
2	-	7.95	-	8.12	-	8.14	-	*	-	*
3	50.2 d	3.00	50.0 d	3.03	49.8 d	3.08	50.2 d	3.05	49.5 d	3.08
4	48.1 d	2.90	52.8 d	2.25	53.9 d	2.25	53.9 d	2.26	51.7 d	2.55
5	34.5 d	2.33	35.0 d	2.46	34.8 d	2.51	34.4 d	2.56	34.2 d	2.56
6	140.0 s	-	138.8 s	-	138.7 s	-	137.9 s	-	138.4 s	-
7	125.6 d[g)]	5.31	125.3 d	5.37	125.4 d	5.35	125.2 d	5.40	125.2 d	5.38
8	42.6 d	2.73	42.5 d	3.26	42.3 d	3.33	41.7 d	3.44	42.6 d	3.10
9	67.9 s	-	67.7 s	-	68.0 s	-	67.6 s	-	64.8 s	-
10	48.6 t	1.07	48.7 t	0.95	48.9 t	1.01	47.8 t	0.94	47.3 t	1.01
				1.01						1.09
11	13.3 q	1.16	12.9 q	1.12	12.9 q	1.15	12.4 q	1.09	12.4 q	1.14
12	19.6 q	1.71	19.4 q	1.71	19.4 q	1.72	18.9 q	1.68	18.7 q	1.68
13	125.4 d[g)]	5.97	124.3 d	6.08	125.5 d	6.19	124.2 d	6.12	122.5 d	6.35
14	136.6 s	-	136.0 s	-	135.9 s	-	137.1 s	-	135.2 s	-
15	40.7 t	1.90	36.7 t	1.99	32.7 t	1.83	39.5 t	2.05	40.9 t	2.03
				2.02		2.43		2.13		2.09
16	27.9 t	1.38	29.6 t	1.42	28.4 t	1.46	20.1 t	1.34	22.1 t	1.12
						1.56		1.51		1.59
17	27.1 t	1.50	78.5 d	3.75	70.9 d	3.50	34.2 t	1.69	19.0 t	1.23
		1.70						1.96		1.51
18	31.2 t	2.00	74.5 d	4.59	71.9 d	4.44	68.5 d	4.93	29.0 t	1.04
		2.05								1.72
19	141.8 d	6.59[d)]	51.3 d	3.35	51.1 d	3.38	51.5 d	3.49	45.8 d	4.10
20	130.5 d	7.22[d)]	42.7 t	1.93	43.0 t	1.91	41.5 t	2.09	41.6 t	2.24
				3.72		3.49		3.38		4.12
21	196.9 s	-	211.2 s	-	214.7 s	-	*	-	210.7	-
22	24.0 d	1.58	23.8 d	1.52	23.6 d	1.56	23.8 t	1.26	23.9 t	1.35
23[e)]	23.6 q	0.79	23.2 q	0.72	23.2 q	0.77	22.4 q	0.65	22.4 q	0.74
24[e)]	21.5 q	0.81	21.5 q	0.74	21.6 q	0.80	20.3 q	0.67	20.4 q	0.76
25	16.7 q	1.24	15.9 q	1.52	15.1 q	1.54	15.3 q	1.58	14.3 q	1.49

Table 1. (Continued).

	1[a)]		2[a)]		3[a)]		4[b,c)]		5[b)]	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
26			-	7.74	-	8.24	-	7.88	-	7.61
27			148.7 s	-	147.6 s	-	147.2 s	-	147.8 s	-
28			115.2 s	-	114.8 s	-	*	-	112.2 s	-
29			128.9 d	7.65	129.1 d	7.72	127.1 d	7.37	126.6 d	7.21
30			113.9 d	6.57	113.7 d	6.58	114.0 d	6.56	113.5 d	6.43
31			132.0 d	7.27	132.5 d	7.32	132.5 d	7.34	132.2 d	7.17
32			111.9 d	7.04	110.8 d	7.08	111.6 d	7.09	110.9 d	6.58
33			168.8 s	-	168.5 s	-	168.3 s	-	168.5 s	-
34			-	8.26	-	8.27	-	6.85	-	6.44
35			48.8 d	4.48	48.6 d	4.46	47.7 d	4.75	47.8 d	4.63
36			170.0 s	-	169.9 s	-	168.2 s	-	168.1 s	-
37			-	9.98	-	9.99	-	8.95	-	8.39
38[f)]			119.8 d	7.27	120.0 d	7.27	*	7.33	119.1 d	7.40
39[f)]			106.2 d	6.42	105.9 d	6.42	106.1 d	6.20	106.2 d	6.38
40			111.6 s	-	111.5 s	-	*	-	112.2 s	-
41			123.1 d	7.40	123.0 d	7.40	*	*	121.1 d	7.17
42			-	11.06	-	11.06	-	8.32	-	8.08
43			136.7 s	-	136.6 s	-	135.3 s	-	135.6 s	-
44			111.7 d	7.37	111.6 d	7.37	110.4 d	7.26	110.3 d	7.29
45			121.3 d	7.11	121.3 d	7.11	121.3 d	7.09	121.2 d	7.14
46			119.1 d	7.05	119.0 d	7.05	119.1 d	7.03	119.1 d	7.10
47			118.8 d	7.62	118.7 d	7.62	118.8 d	7.63	119.0 s	7.77
48			124.9 s	-	124.8 s	-	124.1 s	-	124.5 s	-
49			17.7 q	1.38	17.4 q	1.38	17.4 q	1.49	16.7 q	1.42

a) In DMSO-d_6. b) In $CDCl_3$. c) Compound **4** was unstable under the experimental conditions so that a complete assignment was not available. d) $^3J_{19,20}$ = 16 Hz observed for **1**. e) Assignment of prochiral methyl groups is interchangeable. f) $^3J_{38,39}$ = 15 Hz observed for **2**-**5**. g) Signals may be interchangeable. *) Signal not observed or assignment not possible.

(**4**), and 717 Da (**5**) were determined. It was evident from the measurement that metabolites **2** and **3** were isomers sharing the same molecular formula, and that the mass difference between metabolites **2**/**3** and **4**, and between **4** and **5** corresponded to the loss of one oxygen. A mixture of compounds **2**~**5** containing an internal standard was used to determine the molecular formulae. Using restraints derived from ^1H and ^{13}C NMR data molecular formulae of $C_{44}H_{55}N_5O_6$ (**2**/**3** *m/z* $[M+H]^+$ 750.42475 found, 750.42251 calcd.), $C_{44}H_{55}N_5O_5$, (**4**, *m/z* $[M+H]^+$ 734.43087 found, 734.42759 calcd.), and $C_{44}H_{55}N_5O_4$ (**5**, *m/z* $[M+H]^+$ 718.43302 found, 718.43268 calcd.) were established.

^1H and ^{13}C NMR data of **2** (Table 1) comprised signals for six methyl, four methylene, nine sp^3 and thirteen sp^2 methine groups, as well as for one sp^3 and seven sp^2 quarternary carbons. Furthermore, the signals of four carbonyl carbons, one of them belonging to an isolated keto group ($\delta_{C\text{-}21}$ 211.2), were present. Five proton signals missing in the ^{13}C-HSQC spectrum were assigned to NH groups. The remaining two oxygen and two hydrogen atoms

Fig. 1. Structure of aspochalasin Z (**1**).

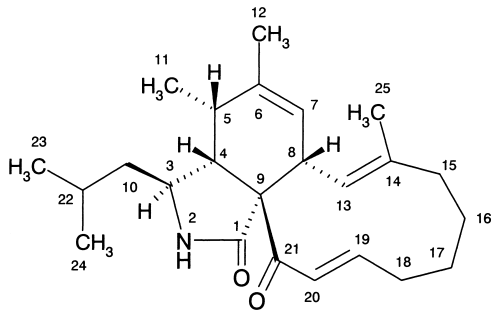
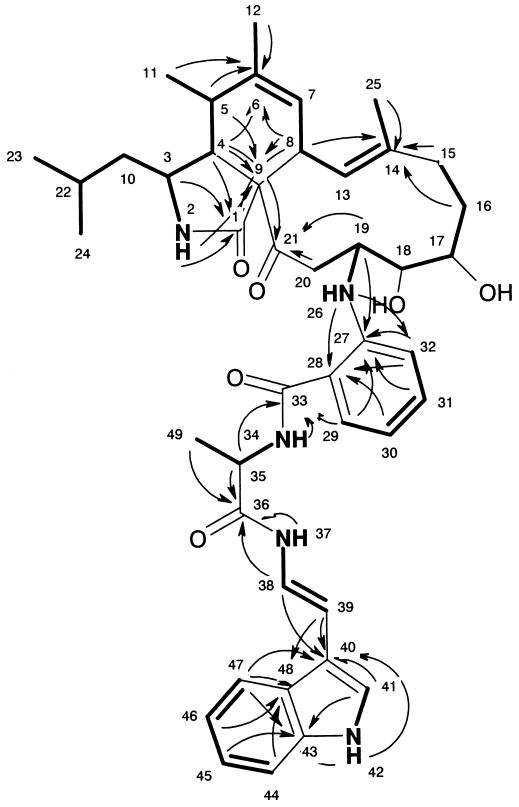


Fig. 2. Spin networks (bold lines) deduced from DQF-COSY and TOCSY spectra and selected HMBC connectivities (arrows) defining quarternary carbons and the linkage between the aspochalasin and tripeptidic moieties for aspochalamins A and B (**2**, **3**).



were attributed to two hydroxy groups bound to two of the sp^3 methine groups as indicated by their chemical shifts (δ_C 78.5 and δ_C 74.5).

Seven spin networks were deduced from TOCSY and DQF-COSY spectra as depicted in Fig. 2. The largest spin network extended from 2-H to 13-H and further included the isopropyl substituent at C-10 (22-H to 24-H_3) and the methyl group at C-14 (25-H_3). HMBC connectivities (Fig. 2) enabled the assignment of the quarternary sp^2 carbons C-6 and C-14, and served to define the vicinal positions of the central bridgehead carbon C-9, the amide carbonyl carbon C-1, and the keto carbon C-21. Observation of HMBC cross peaks to C-14 and C-21 from protons of the second spin system (15-H_2 to 20-H_2 and 26-H) established the aspochalasin moiety of the molecule as 19-amino-17,18-dihydroxy-10-isopropyl-14-methyl[11]cytochalasa-6*Z*,13*E*-diene-1,21-dione[3].

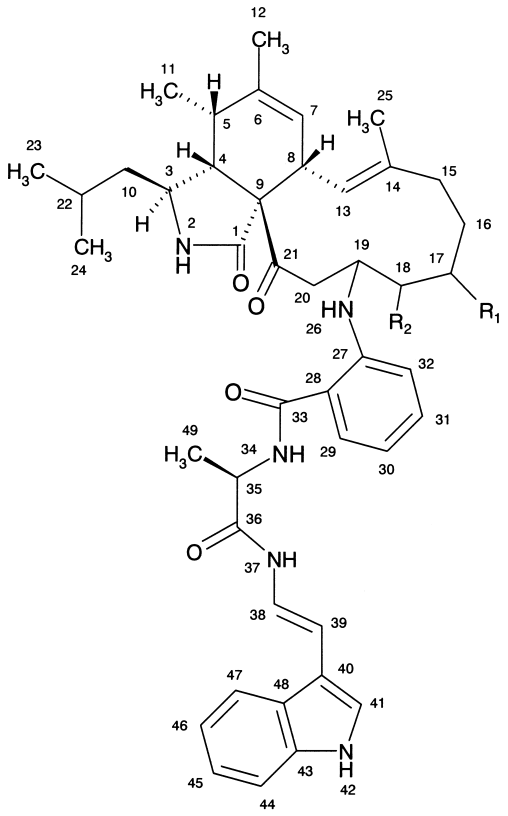
Starting from the indole nitrogen proton 42-H displaying the typical downfield shift (δ_H 11.06) the didehydrotryptamine moiety was secured fusing its three spin systems by HMBC connectivities. The (38*E*) configuration was deduced from the vicinal coupling constant of $^3J_{38,39}$=15 Hz. The amino acids anthranilic acid and alanine were derived from their spin systems and HMBC connectivities, and the tripeptidic sequence was determined from HMBC cross peaks of the amide protons (37-H to C-36 and 34-H to C-33). Finally, the connection between the aspochalasin and the tripeptidic part of the molecule became evident through HMBC cross peaks from 19-H to C-27 and from 26-H to C-28 and C-32.

Analysis of the NMR data of **3** (Table 1) revealed its planar structure to be identical to that of **2** (Fig. 3), while the planar structures of **4** and **5** (Fig. 3) were elucidated as the 17-deoxy and the 17,18-dideoxy analogs of **2** or **3**, respectively. Chiral amino acid analyses of **2**～**5** revealed the L configuration of the alanine moiety for each compound. The structures of **2**～**5** were thus elucidated to consist of an aspochalasin moiety varying among the metabolites in the presence and number of hydroxy groups and/or the stereochemistry and of the tripeptidic moiety anthranoyl-L-alanine-*E*-didehydrotryptamide. Emphasizing the amino group at position C-19 connecting the aspochalasin with the tripeptidic part metabolites **2**～**5** were named aspochalamins A～D.

Discussion

The aspochalasin family[3~11] is a subgroup of the cytochalasans characterised by a 2-methyl-propyl group at the C-3 position of the perhydroisoindol-1-moiety and an 11-membered carbocyclic or 12-membered macrocyclic lactone ring connecting the C-8 and C-9 positions. With the

Fig. 3. Structures of aspochalamins A (**2**), B (**3**), C (**4**), and D (**5**).



Aspochalamin A (**2**): R^1=R^2=OH
Aspochalamin B (**3**): R^1=R^2=OH
Aspochalamin C (**4**): R^1=H, R^2=OH
Aspochalamin D (**5**): R^1=R^2=H

exception of the C-16 substitution in phomacins A~C[10], variations among aspochalasins occur only at positions C-17 to C-20. The substitution pattern of a 17,18-diol moiety as in aspochalamins A (**2**) and B (**3**) is already known from the 17-epimers aspochalasins C and D[5,13], and from aspochalasin I[10]. The relative configurations of these aspochalasin analogs were determined by X-ray[12] and NOE and coupling constant analyses[10,13]. Aspochalamin C (**4**) resembles TMC-169[8] and aspochalamin J[10] in possessing a single hydroxy group at position C-18, while the absence of oxygen functionalities at positions C-17 to C-20 links aspochalamin D (**5**) with aspochalasin Z (**1**).

It is generally assumed that the stereochemistry of the cyclohexene and isoindole moieties is the same in all cytochalasans[14]. Therefore, we depicted the stereochemistry of **1**~**5** as shown in Figs. 1 and 3. However, apart from the determination of the absolute configuration of C-35 (L-alanine moiety), stereochemical studies of **1**~**5** have not been conducted yet. Of particular interest will be the stereochemistry at positions C-17 to C-19 in aspochalamins A~D.

What sets aspochalamins apart from aspochalasins and other cytochalasan compounds is the tripeptidic substitution. Tripeptidic substances containing a didehydrotryptamide functionality have been reported as fungal metabolites[15~17]. Penidiamide[17], which is anthranoyl-glycine-*E*-didehydrotryptamide, is closely related to the tripeptidic moiety of the aspochalamins. The aspochalamins constitute a unique combination of structural elements known from fungal metabolites.

Experimental

NMR Spectroscopy

NMR spectra of aspochalasin Z (**1**) were recorded on a Varian Inova 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C) in DMSO-d_6 at 308 K.

NMR experiments on aspochalamins A~D (**2**~**5**) were carried out on a Bruker AMX2-600 spectrometer at 600 MHz (^{1}H) and 150 MHz (^{13}C) in DMSO-d_6 at 305 K (**2**, **3**) and in $CDCl_3$ at 298 K (**4**, **5**). Chemical shifts are given in ppm relative to the solvent signal at δ_H 2.50 and δ_C 39.5 (DMSO-d_6) or relative from TMS as an internal standard.

Mass Spectrometry

ESI-mass spectra of **1** were acquired on a Finnigan LQC mass spectrometer.

High resolution electrospray ionisation Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS)[18] of aspochalamins A~D (**2**~**5**) was performed on a passively shielded 4.7 Tesla Apex^{TM} II ESI/MALDI-FTICR mass spectrometer (Bruker Daltonik, Bremen, Germany). The mass spectrometry software XMASS version 5.0.10 (Bruker Daltonik) running on a Silicon Graphics O2 Workstation was used for data acquisition and processing. In general 512k data points were acquired. An internal four point calibration was performed. Mass calculation was done by using the standard elemental mass compilation of AUDI & WAPSTRA[19]. Direct infusion experiments with ESI (Analytica of Branford) were performed in the positive mode with a grounded capillary sprayer needle mounted 60° off-axis. No supporting nebulizer gas was used applying a flow rate of 1 μl/minute.

Chiral Amino Acid Analysis

Samples (approx. 50 nmol) were hydrolysed in 6 N HCl at 110°C for 24 hours. The free amino acids were derivatised to their *N*-trifluoroacetylated ethyl esters[20] and analysed on a GC-mass spectrometer (MSD 6890/5973, Agilent, Waldbronn, Germany) using a fused silica capillary coated with Lipodex E - PS 255 (30 : 70).

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