Darlucins A and B, New Isocyanide Antibiotics from *Sphaerellopsis filum* (*Darluca filum*)†

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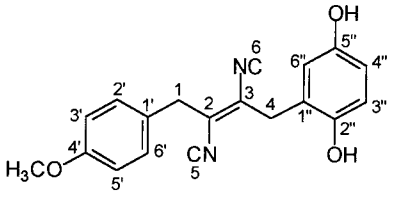
Two new xanthocillin type antibiotics, darlucin A (**1**) and B (**2**), were isolated from fermentations of *Sphaerellopsis filum* (*Darluca filum*). Their structures were established by spectroscopic methods. The darlucins are the first known compounds with a 1,2-diisocyanoalkene moiety. Both compounds exhibited antibacterial, antifungal and weak cytotoxic activities.

During our screening of mycophilic fungi growing on or in fruiting bodies of asco- and basidiomycetes, cultures of the widespread coelomycete *Sphaerellopsis filum* (Biv.-Bern. ex Fr.) Sutt. (*Darluca filum*) were found to produce antimicrobial metabolites. *S. filum*, the anamorph of *Eudarluca caricis* (Fr.) O. Eriks., is a destructive mycoparasite occurring world wide on rust fungi[1,2]. It is known from more than 360 hosts[3]. For some time, *S. filum* was considered to be useful for biological control of rust fungi, but no commercial product has been developed[4,5]. Toxins involved in the destruction of the host or other secondary metabolites from *S. filum* are not known. Therefore the antimicrobial active metabolites were isolated and elucidation of the structures revealed two new xanthocillin type metabolites. In this paper the production, isolation, biological activities and elucidation of the structures of darlucin A (**1**) and B (**2**) will be reported.

Formula 1



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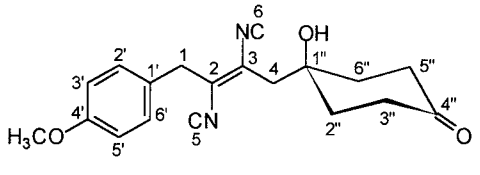
Formula 2



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Materials and Methods

General

Spectral data were recorded on the following instruments: ^1H and ^{13}C NMR, Bruker AM-400; MS, A.E.I. MS-50; FT-IR, Bruker IFS 48 and UV, Perkin-Elmer lambda 16. Optical rotations were recorded with a Perkin-Elmer 241 polarimeter. For TLC, aluminium foils coated with silica gel Merck 60 F_{254} were used. Preparative HPLC was conducted with Merck LiChrosorb Diol 7 μm; column size: 2.5 × 25 cm; flow rate 5 ml/minute.

Producing Organism

Sphaerellopsis filum, CBS 658.79, was cultivated and maintained on YMG agar composed of (g/liter): yeast extract 4, malt extract 10, glucose 4, and agar 15, pH 5.5. Freeze-dried cultures were made in skim milk from pycnidial cultures for long-term storage. After several subcultures, the strain lost the ability to sporulate. In this case, the strain was recultivated from lyophilized material.

Fermentation

Fermentations were carried out in a 20-liter fermentor (Biolafitte C-6) at 22°C with an aeration rate of 3.0 liters/minute and agitation (130 rpm). The fermentation medium was composed of (g/liter): maltose 20, glucose 10, peptone 2, yeast extract 1, KH_2PO_4 0.5, $MgSO_4 \cdot 7H_2O$ 1, $ZnSO_4 \cdot 7H_2O$ 0.002, $FeCl_3$ 0.01 and $CaCl_2 \cdot 2H_2O$ 0.074. The pH was adjusted to 5.5 prior to sterilization. As inoculum, a well grown culture in the same medium (250 ml) was used. Antifungal activity during fermentation was measured in the agar plate diffusion assay with *Nematospora coryli* as test organism.

† Parts of the results have been presented at the 33rd ICAAC, New Orleans, Louisiana, October 1993.

Isolation of Darlucins A and B

After eight days of fermentation, the culture fluid (18 liters) was separated from the mycelia and the active components of the broth were extracted by adsorption onto Mitsubishi Diaion HP 21 resin (column size: 6.5 × 30 cm). The resin was washed with H_2O. Elution with two liters of acetone yielded a crude extract (1.34 g) which was applied onto a silica gel column (Merck 60; 60~200 μm diameter; 70 g). Upon elution with cyclohexane-EtOAc (1 : 1), 110 mg of a crude product were obtained. Final purification was achieved by preparative HPLC using a cyclohexane-EtOAc gradient: 20% EtOAc (75 minutes); 20~30% EtOAc (10 minutes); 30% EtOAc (10 minutes); 30~40% EtOAc (10 minutes); 40% EtOAc (35 minutes); 40~60% EtOAc (15 minutes). Darlucin B was eluted after 110 minutes, darlucin A after 150 minutes.

Tests for Biological Activities

Cytotoxic activity: HL60 cells (ATCC CCL 240) were grown in RPMI 1640 medium, HeLaS3 cells (ATCC CCL 2.2) in D-MEM medium and BHK21 cells (ATCC CCL 10) in G-MEM medium, all supplemented with 10% fetal calf serum. L1210 cells (ATCC CCL 219) were cultivated in Ham's F12 medium with 20% horse serum. All media contained 65 μg/ml penicillin G and 100 μg/ml streptomycin sulfate. The cultures were incubated in a humidified atmosphere containing 5% CO_2. Cytotoxicity was measured in microtiter plates with $3 \cdot 10^4 \sim 1 \cdot 10^5$ cells/ml. After 48 hours the cells were examined and counted under the microscope. In addition, effects on BHK and HeLa cells were determined according to the method of MIRABELLI *et al.*[6] with slight modifications[7].

Test for inhibition of respiration was carried out as described previously[8].

Phytotoxic activity was measured as described by ANKE *et al.*[9].

Darlucin A (1)

Colorless oil; Rf 0.48 (toluene-acetone 7:3); UV λ_{max}^{MeOH} nm (log ε) 225 (4.39), 283 (3.66); IR (KBr, Fig. 3) cm^{-1} 3285, 2107, 1610, 1513, 1455, 1353, 1303, 1252, 1200, 1178, 1033, 1003, 822, 772, 741; ^1H and ^{13}C NMR, Table 1; EI-MS (direct inlet, 180°C) *m/z* (relative intensity %) 320.1134 (49, M^+, calcd for $C_{19}H_{16}N_2O_3$ 320.1107), 319 (100, $C_{19}H_{15}N_2O_3$), 291 (46, $C_{18}H_{15}N_2O_3$), 121 (32, C_8H_9O).

Darlucin B (2)

Colorless oil; Rf 0.47 (toluene-acetone 7:3); $[\alpha]_D^{20}$ 0°; UV λ_{max}^{MeOH} nm (log ε) 226 (4.11), 275 (sh, 3.56); IR (KBr, Fig. 4) cm^{-1} 3440, 2930, 2111, 1712, 1612, 1513, 1251, 1179, 1120, 1032; ^1H and ^{13}C NMR, Table 2; EI-MS (direct inlet, 180°C) *m/z* (relative intensity %) 324.1464 (77, M^+, calcd for $C_{19}H_{20}N_2O_3$ 324.1474), 295 (23, $C_{18}H_{19}N_2O_2$), 267 (21, $C_{16}H_{15}N_2O_2$), 121 (100, C_8H_9O).

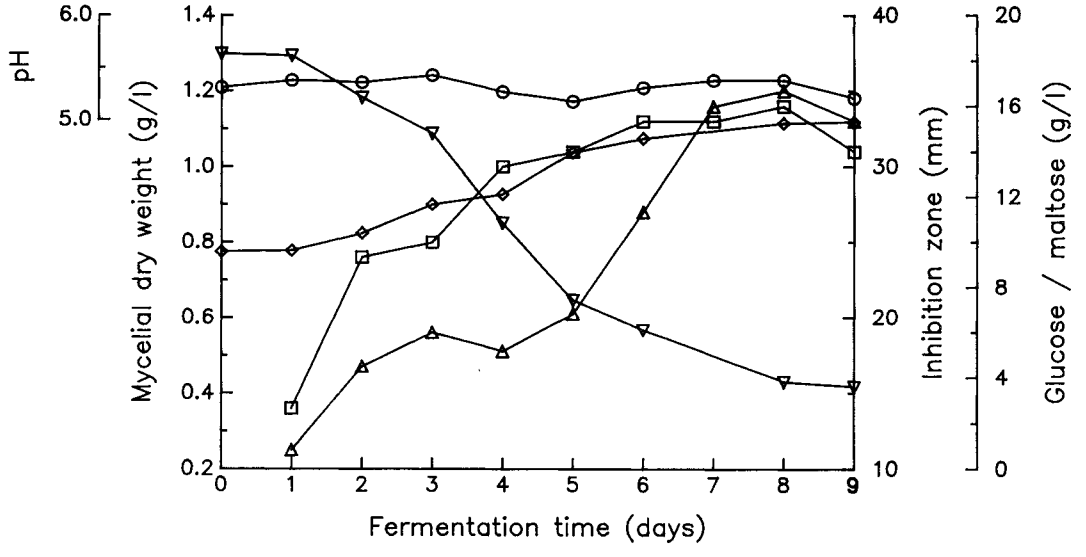
Results and Discussion

Production of Darlucins A and B

Despite being a parasitic organism, the fungus grew well on complex solid media. Growth and production of antimicrobial metabolites in submerged cultures occurred only when sporulating cultures from agar plates were used as inoculum. Formation of pycnidia, however, was sparse and not all fermentations yielded active metabolites. During a nine day fermentation in MGPY medium, the fungus consumed the maltose and left most of the glucose unused as shown in Fig. 1. The content

Fig. 1. Fermentation of *Sphaerellopsis filum* in 20 liters MGPY medium.

○ pH: △ mycelial dry weight (g/liter); ▽ maltose (g/liter); ◇ glucose (g/liter); □ inhibition zone (mm) caused by 10 μl of culture filtrate extract, corresponding to 1 ml culture broth.



of glucose actually increased due to the cleavage of maltose. Very little mycelium was formed. The antibiotic production paralleled the biomass formation. Fermentors were harvested when the biomass had reached its maximum. Mycelia containing no antibiotics were discarded. Following the isolation procedure described above, 7.6 mg of darlucin A (**1**) and 8.6 mg of darlucin B (**2**) were obtained from 18 liters of culture filtrate.

Structural Elucidation

The molecular formula of darlucin A (**1**) was determined as $C_{19}H_{16}N_2O_3$ by HR mass spectrometry. The ^1H NMR spectrum of **1** (Table 1) indicates the presence of two benzene rings which, according to their coupling patterns, are 1,4- and 1,2,4-substituted. In addition, singlets for two isolated methylene groups (δ_H 3.77 and 3.79), one methoxyl group (δ_H 3.79), and two exchangeable protons (δ_H 7.85 and 8.15) are observed. From the ^{13}C NMR data (Table 1) and the ^1H-^{13}C correlation given in Fig. 2 the presence of a 4-methoxybenzyl and a 2,5-dihydroxybenzyl residue can be discerned which accounts for 15 of the 19 carbon atoms of the molecule. The 4-methoxybenzyl residue is supported by a fragment ion at *m/z* 121 in the MS.

The IR spectrum (Fig. 3) of **1** exhibits an intense absorption at 2107 cm^{-1} which is characteristic for certain vinylisocyanides[10~12]. The presence of two isocyano groups is confirmed by the appearance of two ^{13}C NMR signals at δ_C 174.70 and 174.87. Their unusually large chemical shifts can only be explained by 1,2-attachment of the two isocyano groups to the olefinic double bond[10~13]. The signals of the olefinic carbons appear at δ_C 128.25 and 128.72 and are strongly broadened due to interaction with the nitrogens of the isocyano groups[12].

On the basis of these results, structure **1** can be assigned to darlucin A. The (*E*)-configuration at the olefinic double bond is given arbitrarily and has to be confirmed

Fig. 2. ^1H-^{13}C correlation for **1** by COLOC experiment.

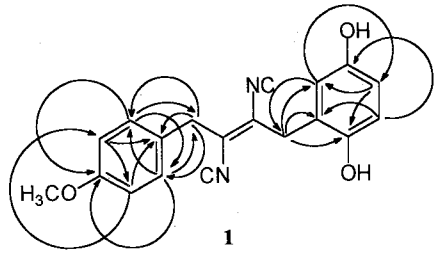


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Table 1. ^1H (400 MHz) and ^{13}C (100.6 MHz) NMR data of darlucin A (**1**) in acetone-d_6.[a]

Proton	δ (ppm)		J (Hz)	Carbon	δ (ppm)		J (Hz)
1-H_2	3.79[b]	s		C-1	37.44	tt	133/4
				C-2	128.25[c]	m	
				C-3	128.72[c]	m	
4-H_2	3.77[b]	s		C-4	33.15	td	133/5
				C-5	174.70[d]	s	
				C-6	174.87[d]	s	
				C-1′	127.76	m	
2′/6′-H	7.24[f]	'd'	8.5	C-2′/6′	130.66	ddt	160/7/5
3′/5′-H	6.92[f]	'd'	8.5	C-3′/5′	115.03	dd	160/5
				C-4′	160.25	m	
				C-1″	122.42	m	
				C-2″	149.20	m	
3″-H	6.75	d	8.8	C-3″	116.52	d	158
4″-H	6.64	dd	8.8/3.0	C-4″	115.99	dd	159/5
				C-5″	151.22	m	
6″-H	6.72	d	3.0	C-6″	118.06	dm	156
4′-OCH_3	3.79	s		4′-OCH_3	55.48	q	144
2″-OH	7.85[e,g]	s, br					
5″-OH	8.15[e,g]	s, br					

[a] Assignments of carbons have been confirmed by 2D NMR experiments.
[b~e] Assignments may be interchanged.
[f] AA′BB′ system.
[g] Signal disappears after addition of D_2O.

Fig. 3. IR spectrum of darlucin A (**1**) in KBr (100 μg/33 mg).

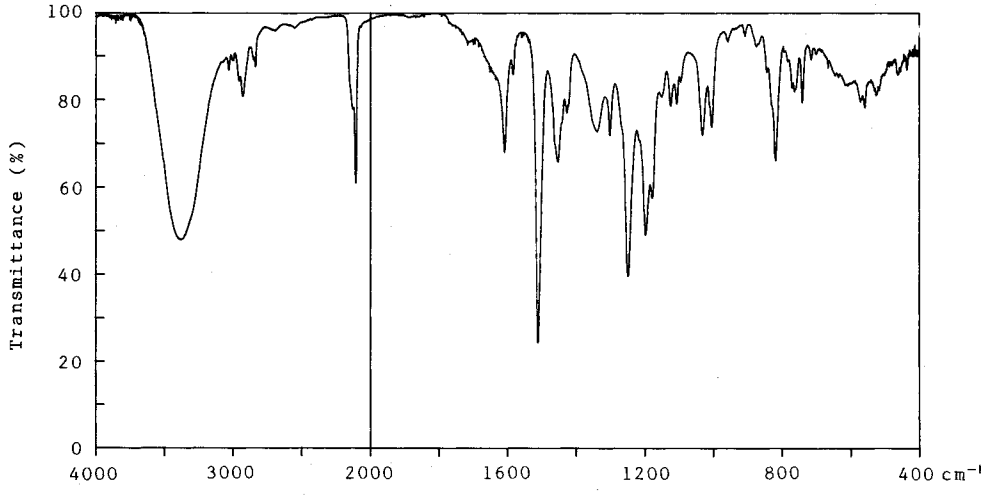


Fig. 4. IR spectrum of darlucin B (2) in KBr (100 μg/33 mg).

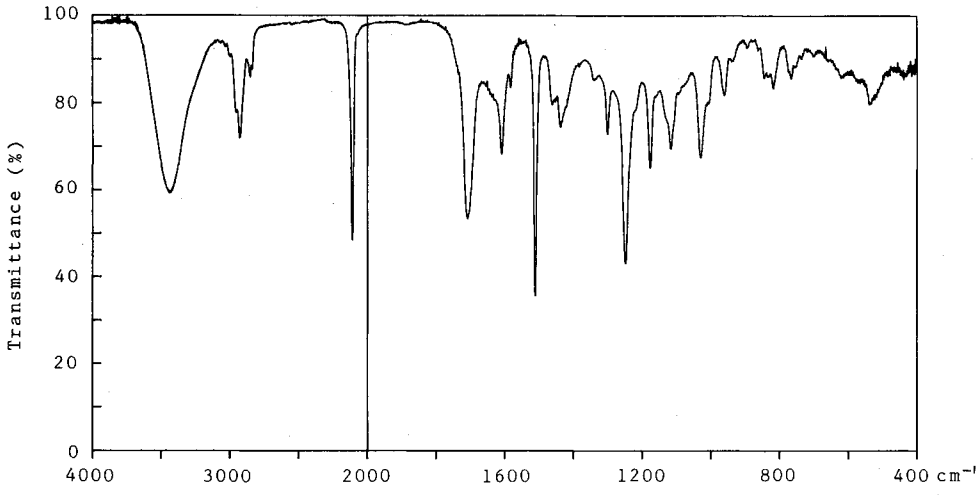


Table 2. ^1H (400 MHz, in $CDCl_3$) and ^{13}C (100.6 MHz, in acetone-d_6) NMR data of darlucin B (2).

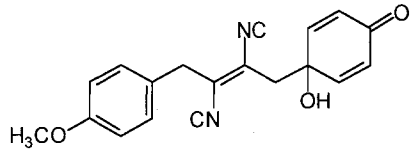
Proton	δ (ppm)		J (Hz)	Carbon	δ (ppm)
1-H_2	3.73	s		C-1	37.65
				C-2	[a]
				C-3	[a]
4-H_2	2.71	s		C-4	44.98[b]
				C-5	175.06[c]
				C-6	175.36[c]
				C-1′	127.72
2′/6′-H	7.19[d]	'd'	8.8	C-2′/6′	130.66
3′/5′-H	6.88[d]	'd'	8.8	C-3′/5′	115.03
				C-4′	160.20
				C-1″	72.11
2″/6″-H_{ax}	1.92	ddd	13.8/13.6/5.0	C-2″/6″	37.24[b]
2″/6″-H_{eq}	2.07	ddm	13.8/6.3		
3″/5″-H_{ax}	2.70	ddd	15.0/13.6/6.3	C-3″/5″	37.15
3″/5″-H_{eq}	2.28	ddm	15.0/5.0		
				C-4″	209.72
4′-OCH_3	3.80	s		4′-OCH_3	55.49

[a] Signal invisible.
[b] Doublet due to ^3J-coupling with the axial OH-group ($J_{C4,OH}$ = 2.4 Hz; $J_{C2'',OH}$ = 5.2 Hz).
[c] Assignments may be interchanged.
[d] AA′BB′ system.

by further investigations.

A comparison of the NMR data (Table 2) of darlucin B, $C_{19}H_{20}N_2O_3$, with those of **1** reveals the presence of an identical 2-(4-methoxybenzyl)-1,2-diisocyanovinyl unit in both compounds. The vicinal isocyano groups give rise to an IR band at 2111 cm^{-1} (Fig. 4). The corresponding ^{13}C NMR signals appear at δ_C 175.06 and 175.36 whereas the olefinic resonances are invisible due to strong line broadening. The remaining $C_7H_{11}O_2$ fragment of **2** consists of two identical CH_2CH_2 units (δ_C 37.15, 37.24) linked to a carbonyl (δ_C 209.72) and a tertiary carbinol group (δ_C 72.11). This indicates the presence of a 1-hydroxy-4-oxo-1-cyclohexyl residue in

Formula 3



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accordance with the close agreement of its NMR data with those of 4-hydroxy-4-methyl-cyclohexanone[14,15]. Connecting both partial structures by means of the remaining CH_2 group (δ_C 44.98) leads to structure **2** for darlucin B. It is in correspondence with the lack of optical activity of this antibiotic.

The darlucins constitute new members of the xanthocillin group of isonitrils[16,17]. To our knowledge, simple 1,2-diisocyanoalkenes have not been previously described, the only known compounds of the general type being 1,2-diisocyanobenzenes[18]. Another remarkable property of the darlucins is the presence of 2,5-dihydroxybenzene or 4-hydroxycyclohexanone rings in **1** or **2**, respectively. These compounds appear to be derived from a common quinol intermediate **3** *via* (4-hydroxyphenyl)pyruvic acid→homogentisic acid rearrangement[19] or reduction of the conjugated double bonds, respectively. Related isocyanides with partially reduced aromatic rings have been isolated from cultures of *Leptosphaeria* sp.[12] and *Mycoleptodiscus terrestris*[20].

Biological Properties

The antibacterial spectra of darlucins A and B are shown in Table 3. Gram-negative and positive organisms are equally sensitive, the MICs ranging from 2.5 to 20 μg/ml in nutrient broth. The antifungal activity was slightly less (Table 4) with MICs for most strains between

Table 3. Antibacterial spectrum of darlucin A (**1**) and darlucin B (**2**) in the serial dilution assay. (Size of inoculum: 1×10^5).

Organism	MIC (μg/ml)	
	1	**2**
Acinetobacter calcoaceticus	2.5	2.5
Bacillus brevis	2.5	2.5
B. subtilis	2.5	2.5
Escherichia coli K12	5	5
Micrococcus luteus	2.5	2.5
Mycobacterium phlei	5	5
Salmonella typhimurium TA 98	5	5
Streptomyces spec. ATCC 23836	5	20

Table 4. Antifungal activity of darlucin A (**1**) and darlucin B (**2**) in the serial dilution assay. (Size of inoculum: 1×10^5 cells or spores/ml).

Organism	MIC (μg/ml)	
	1	**2**
Yeasts:		
Nadsonia fulvescens	5	5
Nematospora coryli	10	10
Saccharomyces cerevisiae S 288 c	50	50
S. cerevisiae is 1	10	10
Filamentous fungi:		
Fusarium oxysporum	>50	>50
Mucor miehei	2.5	5
Paecilomyces variotii	10	10
Penicillium notatum	10	10

Table 5. Cytotoxic activities (inhibition of proliferation (IC_{50}) and total lysis) of darlucin A (**1**) and darlucin B (**2**).

Cell line	IC_{50} (μg/ml)		Lysis (μg/ml)	
	1	**2**	**1**	**2**
BHK21	25	100	100	>100
HeLaS3	10	100	25	>100
L1210	10	25	25	100
HL60	50	50	>100	>100

2.5 and 50 μg/ml. Interestingly, **1** and **2** showed only weak cytotoxic activity (Table 5). Proliferation of the cells was reduced to 50% between 10 and 100 μg/ml.

Darlucin A was not phytotoxic at 600 μg/ml. Both compounds had no effect on the respiration of *Penicillium notatum* up to 50 μg/ml.

Compounds with an isonitril moiety have been isolated from many different fungi. The first compound, xanthocillin X was isolated in 1948 from *Penicillium notatum*[21] and its structure was elucidated in 1957 by HAGEDORN *et al.*[22]. Ascomycetes, mainly Eurotiales and their anamorphs produce compounds of this type. Various biological activities like antibacterial, antifungal, cytotoxic, antitumor, anthelmintic, antiviral and enzyme inhibiting properties have been reported. For comprehensive reviews see ref.[16,17].

Acknowledgments

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