Lumpidin, a Novel Biomarker of Some Ochratoxin A Producing Penicillia

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The novel compound lumpidin (**1**) has been isolated as a major compound from an isolate of *Penicillium nordicum*. Compound **1** is a diketopiperazine with a unique ring system likely to be a condensate of one mole each of L-tryptophan, L-phenylalanine, and L-homo-proline. The fact that **1** has been detected from only three out of sixteen isolates of *P. nordicum* indicates that lumpidin-producing isolates might represent a separate and third ochratoxin A producing *Penicillium* species.

Keywords: *Ochratoxin A; Penicillium nordicum; diketopiperazin; biomarker; lumpidin*

INTRODUCTION

Penicillium nordicum is an important food-spoiling fungus and of major concern because it produces the mycotoxin ochratoxin A, a metabolite also produced by the closely related species *P. verrucosum* (*1*). Whereas *P. verrucosum* is frequently isolated from cereal products, *P. nordicum* is a contaminant of products with high levels of proteins and fats, such as meat and cheese, and is responsible for the frequent detection of ochratoxin A in such products (*2, 3*). Both species produce verrucolone and quinazoline metabolites (*1, 4*), however, only *P. nordicum* produces the quinazoline anacine, a metabolite also produced by *P. aurantiogriseum* (*5*).

In a large chemotaxonomic investigation of ochratoxin A producing penicillia, also including 24 isolates of *P. verrucosum*, anacine was found to be produced consistently by all 16 isolates of *P. nordicum* (*1*). The study revealed that two ochratoxin producing chemotypes seem to exist within *P. nordicum*. One group of isolates produced the benzodiazepine sclerotigenin (*6*) as a major secondary metabolite, whereas another small group of three isolates produced an unknown compound. This compound was named lumpidin because it was produced as a major compound from an isolate originally isolated from lumpfish roe.

This report describes the isolation and structural elucidation of lumpidin (**1**), which proved to be a novel diketopiperazine with a unique ring system.

MATERIALS AND METHODS

Fungal Material and Fermentation. The isolate of *Penicillium nordicum* (IBT 6573, ex lumpfish roe) was obtained from the IBT Culture Collection at BioCentrum-DTU, The Technical University of Denmark. The isolate was cultured as three-point mass inoculations on yeast extract sucrose agar (YES) (*7*) for 14 days in the dark. For preparation of the large extract IBT 6573 was cultivated on 200 Petri dishes of YES under similar conditions.

Extraction and Separations. The contents of the 200 Petri dishes were transferred to four large glass flasks and were extracted at still conditions for 16 h at room temperature with 2 L of EtOAc to give ca. 3.1 g of dried extract after evaporation. This extract was partitioned between 90% aqueous MeOH and hexane followed by 50% aqueous MeOH and dichloromethane to give three fractions: hexane, dichloromethane (D), and aqueous MeOH. Fraction D (ca 1.1 g) was further separated by vacuum liquid chromatography on a column (3 × 6.8 cm i.d.) packed with silica gel 60H (Merck). Nine fractions were obtained by eluting with mixtures of increasing polarity: from EtOAc/hexane (1:1) to 100% EtOAc followed by MeOH/EtOAc (5:95) to 100% MeOH. The fraction rich in lumpidin (**1**) was purified on a Merck Lichroprep RP-18 (310 × 25 mm i.d., 40−63 μm) column using H_2O-CH_3CN (50:50) as mobile phase at 4 mL/min flow rate, to give almost pure lumpidin. This fraction was subjected to HPLC on a Waters Prep Nova-Pak C18 cartridge (100 × 25 mm i.d., 6 μm) using H_2O-CH_3CN (70:30) as mobile phase at 20 mL/min flow rate, to give **1** (12 mg).

Apparatus. NMR spectra were recorded in 5-mm tubes at 600.13 MHz for ^{1}H and at 150.92 MHz for ^{13}C and at 300 K, in DMSO-d_6, on a Bruker DRX 600. The chemical shifts are given relative to DMSO (2.50 ppm) for ^{1}H and (39.5 ppm) for ^{13}C (Table 1). The double quantum filtered phase sensitive COSY experiment was performed using the Bruker standard program COSYDFPRTP (*8*), with 0.246-s acquisition time and 4K data points. F1 were zero filled to give a matrix of 4K × 1K points and was resolution enhanced in both dimensions by a shifted sinebell. The nuclear Overhauser effect spectroscopy was performed using the Bruker standard program NOESYPRTP (*9*), with a mixing time of 200 ms. The heteronuclear single quantum coherence spectroscopy (*10*) was performed using sensitivity improvement and echo/antiecho gradient selection. The heteronuclear multiple bond correlation spectra (*11*) were recorded using gradient selection Bruker standard pulse program INV4GSLPLRND with evolution delay of 60 ms. The spectra were assigned using the computer program Pronto (*12*), which allows the simultaneous display of different two-dimensional spectra and individual labeling of cross-peaks.

The circular dichroism (CD) spectra were measured on a JASCO J-710 spectropolarimeter, and the UV spectra were measured on a Hewlett-Packard 8452A diode array spectrophotometer. The optical rotation was measured on a JASCO DIP 1000 digital polarimeter. Analytical HPLC conditions were

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Table 1. NMR Data for Lumpidin (1) (DMSO-d_6)

position	^{13}C	^{1}H: δ (mult, J = Hz)	HMBC to carbon #[a]	NOESY[b]
1	166.8			
2		8.42 (d, 2.0)	3, 4, 6	3 (m), 24a (w), 24b (m)
3	55.8	4.18 (ddd, 2.5, 4.0, 4.5)	1, 4, 24, 25	2 (m), 24a (s), 24b (s), 26/30 (m)
4	166.6			
5		7.37 (d, 2.0)	1, 3, 6, 7	6 (s), 7a (m), 21a(m)
6	50.7	3.07 (ddd, 2.0, 2.0, 9.5)	1, 4, 5, 7, 8	5 (s), 21a (m), 23 (m)
7a	41.7	2.22 (dd, 9.5, 14.5)	1, 6, 8, 9	5 (m), 23 (w)
7b		2.08 (dd, 2.0, 14.5)	1, 6, 8, 9	23 (m)
8	77.4			
9	137.1			
10	125.1	7.38 (d, 7.5)	8, 12, 14	
11	124.4	7.12 (t, 7.5)	9, 13	
12	129.6	7.32 (t, 7.5)	10, 14	
13	114.1	7.37 (d, 7.5)	9, 11	
14	137.6			
16	171.7			
17	66.3	3.10 (dd)	16	18a (w), 21b (m), 19b (m)
18a	24.8	1.82 (m)	20	Overlap 19a
18b		1.31 (m)	17, 19, 20	Overlap 19b
19a	23.0	1.83 (m)	17, 21	Overlap 18a
19b		1.30 (m)	17, 19, 21	Overlap 18b
20a	23.8	1.68 (m)	18, 19	21a (m), 21b (w)
20b		1.44 (m)	18, 19, 21	21a (w)
21a	49.3	2.48 (m)	17, 19	5 (m), 6 (m), 20a (m), 20b (w)
21b		2.05 (m)	17	17 (m), 19b (w), 20a (m)
23	84.4	4.25 (d, 0.5)	7, 8, 21	6 (m), 7a (w), 7b (m)
24a	39.1	3.11 (dd, 13.5, 4.0)	3, 4, 25, 26/30	2 (w), 3 (s), 26/30 (s)
24b		2.91 (dd, 13.5, 4.5)	3, 4, 25, 26/30	2 (w), 3 (s), 26/30 (s)
25	135.5			
26, 30	130.0	7.15 (7.5)	28	3 (m), 24a (s), 24b (s)
27, 29	128.2	7.29 (t)	25	
28	126.9	7.27 (t)	26/30	

[a] The HMBC data are reported as from ^{1}H to ^{13}C. [b] The magnitudes of the measured NOEs are divided into three categories: weak (w), medium (m), and strong (s).

similar to those given by Smedsgaard (*13*). Retention index (RI) of lumpidin was calculated according to Frisvad (*14*).

The FABMS, EIMS, and HRMS data were obtained using a JEOL JMS-HX/HX110A tandem mass spectrometer.

Lumpidin (1). Amorphous solid. $[\alpha]^{22}_{D} = -70.8°$ (*c* 0.01, EtOH). UV λ_{max} (EtOH) nm (log ϵ): 246 (4.99), 280 (sh, 4.33). CD (EtOH, *c* 0.07), λ nm ($\Delta\epsilon$): 217 (−22.81), 231 (+69.74), 253 (−6.34), 286 (−10.57). ^{1}H and ^{13}C NMR see Table 1. HREIMS *m*/*z* 460.2075 (−3.6 mmu calcd for $C_{26}H_{28}O_4N_4$). RI = 902.

RESULTS AND DISCUSSION

Characterization of Lumpidin (1). The molecular formula $C_{26}H_{28}O_4N_4$ of lumpidin (**1**) was obtained from the HREIMS spectrum and indicated a compound with 16 degrees of unsaturation. The UV spectrum of **1** was very similar to that of verrucofortine (compound **2**, Figure 1) (*15*) suggesting the compound to be a diketopiperazine derived from tryptophan and phenylalanine. The presence of a phenylalanine residue in **1** was supported by the ^{1}H NMR and ^{13}C NMR data (Table 1) which gave several partial structures of **1** including a monosubstituted phenyl ring represented by the three aromatic hydrogens at δ 7.15 (2 H, m), 7.27 (1 H, m), and 7.29 (2 H, m). The presence of a tryptophan moiety was supported by the presence of an *ortho*-disubstituted benzene ring represented by the four aromatic hydrogens at 7.12, 7.32, 7.37, and 7.38 ppm. Furthermore, the three signals at 166.6, 166.8 ,and 171.7 ppm in the ^{13}C NMR spectrum indicated the presence of three amide bonds in **1**. The ^{1}H−^{1}H COSY spectrum of **1** showed the presence of two CH_2−CH−NH fragments and one $(CH_2)_4$−CH− fragment. Finally, the ^{1}H NMR and ^{13}C NMR data showed the presence of a methine proton at δ 4.25 attached to a carbon resonating at 84.4 ppm.

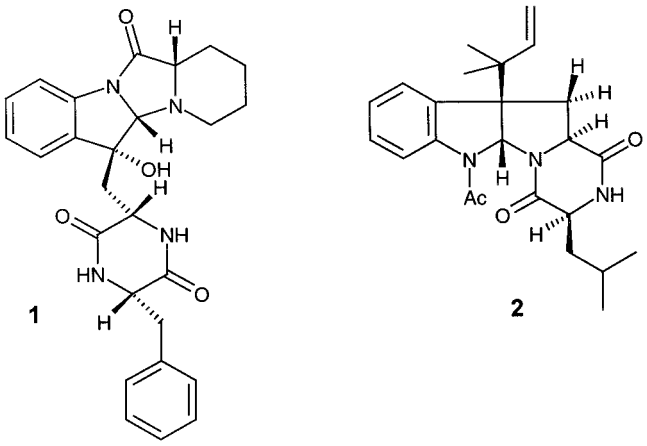

Figure 1. Proposed structure of lumpidin (**1**) produced by *Penicillium nordicum* (IBT 6573) and structure of the known compound verrucofortine (**2**) (*15*).

The partial structures described above could be linked together using the information available from the HMBC spectrum (Table 1). The most important 2- and 3-bond long-range correlations are shown in Figure 2. The diketopiperazine ring can be seen to be derived from a combination of phenylalanine and tryptophan moieties as correlations were observed between the α-proton H-3 at δ 4.18 and the carbons C-1, C-4, C-24, and C-25, and between the α-proton H-6 at δ 3.07 and the carbons at C-1, C-4, C-7, and C-8 (Table 1). Also, the NH protons at 7.37 and 8.42 ppm both correlate with the carbons at C-3 and C-6, supporting the diketopiperazine structure. The tryptophan part of the molecule can be seen to be further combined with one moiety of homo-proline because no NH functionality and double bond can be

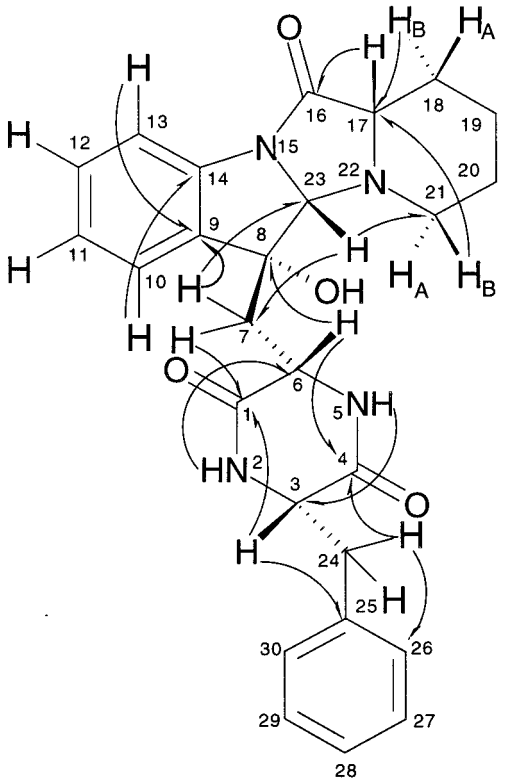

Figure 2. Important 2- and 3-bond long-range HMBC correlations observed for lumpidin (**1**).

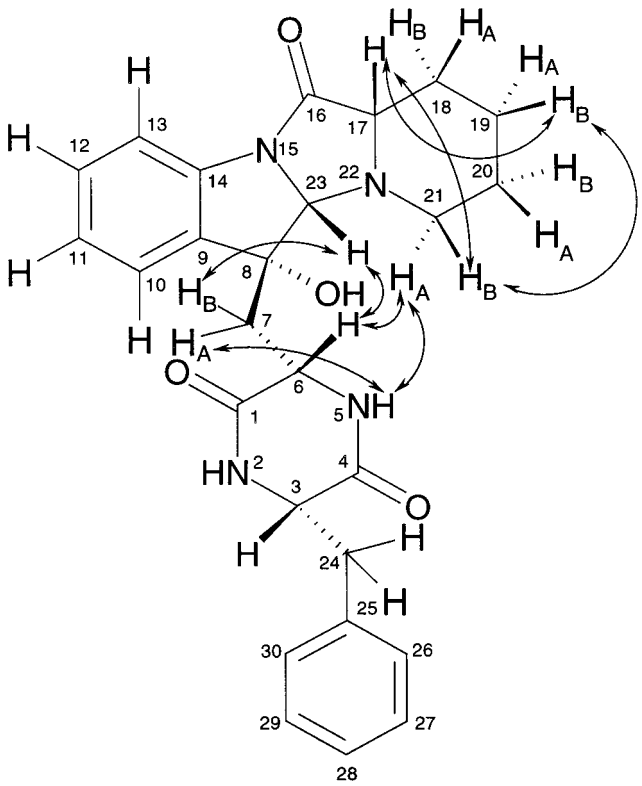

Figure 3. Important NOE correlations observed in lumpidin (**1**).

seen in the indoline nucleus. The HMBC correlations observed between H-23 and C-21, and between the two H-21 protons to C-17 (Figure 2), proved the proposed four ring partial structure of **1**, thus establishing at which positions the linkages had occurred. Homo-proline is linked with the tryptophan moiety of the compound in a manner similar to that in which glycine and alanine are linked with tryptophan in other fungal metabolites such as fumiquinazolines and fiscalins (*16*, *17*). These metabolites also carry a hydroxy group at the same position in the molecule as the quarternary carbon C-8 at 77.4 ppm. The position of C-8 in **1** was established by its HMBC correlation to the protons H-6, H-7, H-10, and H-23 (Table 1).

At present, the absolute configuration of lumpidin, having five stereocenters, has not been established. However, lumpidin is likely to be biosynthesized from L-phenylalanine and L-tryptophan giving *S* configurations at both position 3 and position 6 in lumpidin, as seen for several other diketopiperazines such as verrucofortine (*15*).

Assuming that the configuration at position 6 is *S*, the relative stereochemistry at positions 8 and 23 can be established based on a number of observed NOE correlations (Table 1; Figure 3). The NH-5 can be seen to correlate to H-7_A and H-21_A, H-6 correlates to H-21_A and H-23, and H-7_B correlates to H-23, which taken together establish that the configuration at position 8 is *R* and at position 23 is *S*.

Finally, the three axial protons H-17, H-19_B, and H-21_B correlate with each other, showing that these three protons are in the same plane of the homo-proline ring. The coupling constants of the protons at positions 18−21 could not be measured accurately; however, when large geminal couplings (>10 Hz) are omitted, H-17 could be seen to have a medium coupling (8−10 Hz) to H-18_B and a small coupling (3−5 Hz) to H-18_A. Similarly, H-18_B had a large coupling to H-19_B and a small coupling to H-19_A; H-19_B had a large coupling to H-20_B and a small coupling to H-20_A; and, finally, H-20_B had a large coupling to H-21_B and a small coupling to H-21_A; altogether strongly indicating that the homo-proline ring is in a chair conformation, in agreement with the above-mentioned NOE correlations. A chair conformation of the homo-proline ring could be the result of incorporation of both D- and L-homo-proline into lumpidin, but the present data do not indicate the configuration at position 17. Because L-homo-proline is the naturally occurring form, the *S* configuration is the most likely one, as suggested here.

Chemotaxonomic Significance. At present lumpidin is the only known diketopiperazine produced by ochratoxin A producing penicillia. The fact that lumpidin was found to be produced by only 3 out of 16 isolates of *P. nordicum*, and never by isolates that also produce sclerotigenin (*1*), indicates that lumpidin-producing isolates might represent a separate species. Ongoing research, including molecular biological investigations, is in progress in our laboratory to establish whether lumpidin-producing isolates should be considered as a separate, and thereby third, ochratoxin-producing species in the series *Verrucosa* in the genus *Penicillium*.

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