UV-Guided Isolation of Verrucines A and B, Novel Quinazolines from *Penicillium verrucosum* Structurally Related to Anacine from *Penicillium aurantiogriseum*

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Two novel quinazolines, verrucines A (**1**) and B (**2**), have been isolated as a major and a minor metabolite, respectively, of *Penicillium verrucosum*. Both are condensates of one mole each of anthranilic acid, phenylalanine, and glutamine. The structures were elucidated by spectroscopic methods, and the two compounds were found to be epimers. The spectroscopic data for the ostensible benzodiazepine anacine reported from *Penicillium aurantiogriseum*, composed of one mole each of anthranilic acid, leucine, and glutamine, appeared very similar to those of **1**. Therefore, a revised quinazoline structure for anacine is proposed.

Penicillium verrucosum is one of the most common fungi on stored cereals in temperate regions and is of major concern because it may produce the mycotoxins citrinin and ochratoxins A and B.[1] Verrucolone is another secondary metabolite reported from this species.[2] During the investigation of a large number of *P. verrucosum* isolates on solid media, we discovered that many of these contained another major metabolite (verrucine A) with a retention index and a UV spectrum very similar to those of anacine, a benzodiazepine metabolite first reported from *Penicillium aurantiogriseum*.[3] For some time we therefore believed this compound to be anacine. However, a closer investigation revealed the UV spectra of the two compounds to be slightly different. Moreover, the UV spectra of both were similar to that of fumiquinazoline F (Figure 1), a metabolite with an established quinazoline skeleton recently reported from *Penicillium thymicola*.[4] This was unexpected because the major chromophore of the quinazoline ring system has one additional unsaturation compared to that of the benzodiazepine ring system of anacine, which would therefore be expected to give rise to a significantly different UV spectrum. To investigate these inconsistencies, verrucine A (**1**) was isolated together with a minor, similar constituent, verrucine B (**2**), and the structures were determined.

The NMR spectra of **1** (DMSO-d_6) revealed the presence of 20 protons and 21 carbon atoms, while a mass spectrum showed a molecular weight of 376. Combined with the elemental analysis, the elemental composition $C_{21}H_{20}N_4O_3$ could thus be established. The ^{1}H NMR spectrum (Table 1) revealed the presence of an *ortho*-disubstituted benzene ring corresponding to an anthranilate moiety, together with a phenylalanyl moiety, and a glutaminyl moiety. A HETCOR experiment was consistent with the above analysis, allowing assignment of all protonated carbons. Comparison with the NMR data (Table 1) published[3] for anacine (**3**) revealed a close similarity when allowing for the different side chain at C-3. However, our data did not fit with such a benzodiazepine structure. In the H-H COSY spectrum of **1**, the two NH signals at δ 6.71 and 7.25 displayed a coupling. A strong correlation between the same two NH signals was also seen in the NOESY spectrum, and both signals showed additional correlations to the 16-CH_2 group (δ 2.2), consistent with the presence of a $-CH_2-CONH_2$ moiety, that is, with an open glutaminyl side chain, and not with a glutarimide structure such as that originally proposed for **3**.[3] This led us to propose the quinazoline structure **1** for verrucine A, also consistent with the UV data. This structure embraced the above open glutaminyl side chain as well as the phenylalanyl and anthranilate moieties. Furthermore, in the HMBC spectrum, correlations were observed between the NH at δ 8.61 and C-1, C-3, C-4, and C-14, compatible with the structure given as **1**, but not with a benzodiazepine structure similar to **3**.

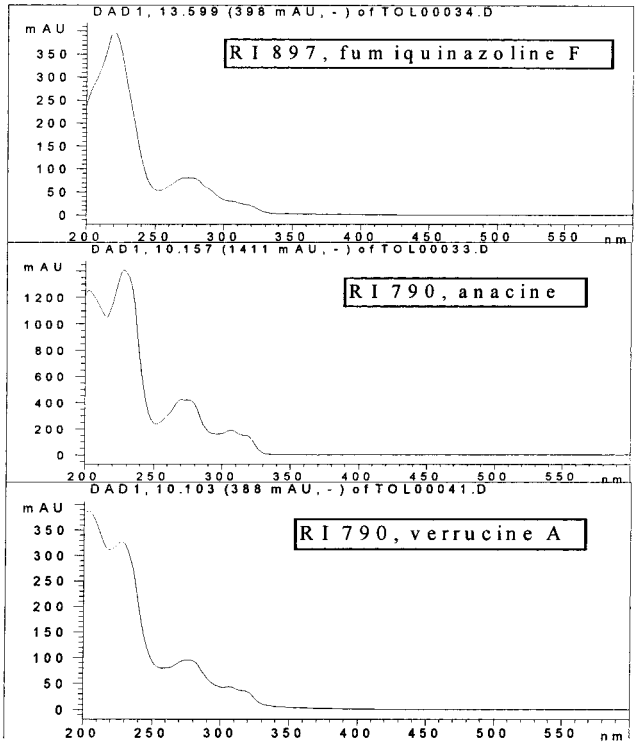

Figure 1. UV spectra (200−600 nm) of the fungal metabolites fumiquinazoline F (from *Penicillium thymicola*), anacine (**3**, from *P. aurantiogriseum*) and verrucine A (**1**, from *P. verrucosum*). Retention indices (RI) were calculated according to Frisvad and Thrane.[11]

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Table 1. NMR Data for Verrucines and Analogues

	verrucine A (**1**)[a]		verrucine B (**2**)[a]		anacine (**3**)[a,b]		*cyclo*-anthranilylleucine dipeptide (**5**)[a,b]
position	^{1}H: δ (*J*)	^{13}C	^{1}H: δ (*J*)	13C	1H: δ (*J*)	^{13}C	^{1}H: δ (*J*)
1		166.2		167.3		166.6	
2(NH)	8.61 (br 4)		8.50 (s)		8.87 (br d)		8.43 (d)
3	4.76 (obsc)	57.0	5.18 (t, 5.0)	55.4	4.39 (m)	53.8	3.60 (m)
4		151.0		151.0		152.0	
6		146.9		146.4		147.0	
7	7.66 (br 8)	126.7	7.72 (br 8)	127.1	7.63 (dd)	126.7	7.09 (d)
8	7.85 (8; 7; 1.5)	134.9	7.87 (8; 7; 1.5)	134.9	7.81 (dt)	134.7	7.51 (t)
9	7.54 (8; 7; 1)	126.8	7.55 (8; 7; 1)	126.5	7.52 (dt)	126.7	7.21 (t)
10	8.14 (8; 1.5)	126.3	8.13 (8; 1.5)	126.4	8.13 (dd)	126.2	7.75 (d)
11		119.8		119.8		119.7	
12		160.1		159.9		160.1	
13							
14	4.76 (obsc)	54.8	4.95 (t, 4.4)	53.7	4.86 (m)	54.9	
15	1.34; 1.86 (m's)	28.8	2.15; 2.18 (m's)	26.5	1.96−2.14 (m)	29.3	
16	2.20; 2.23 (m's)	32.2	2.18 (m)	31.1	2.25−2.40 (m)	32.2	
17($CONH_2$)	7.25; 6.71(br s's)	172.8	7.23; 6.70 (br s's)	172.7	7.31; 6.74 (br)	172.8	10.36 (s)
18	3.28 (13.5; 7.5); 3.25 (13.5; 5)	42.8	3.55(14.5; 5); 3.32 (obsc)	40.2	1.69−1.79 (m)	47.2	1.55 (m)
19		136.3		136.9	1.89 (sept)	24.0	1.79 (m)
20, 24	7.27 (br d, 7)	129.8	7.38 (7)	130.0	0.97 (dd)	23.0	0.85 (d)
21, 23	7.21 (br t, 7)	128.5	7.21 (br t, 7)	128.0	0.97 (dd)	21.4	0.77 (d)
22	7.18 (br t, 7)	127.0	7.17 (br t, 7)	127.1			

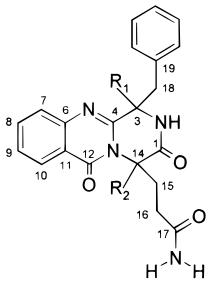
[a] DMSO-d_6 (δ 2.50; 39.5). [b] Data from Boyes-Korkis et al.[3]

The configurations at C-3 and C-14 were determined by hydrolysis of **1**, which required more severe conditions than usual.[4] When analyzing the hydrolysis mixture using Marfey's reagent [N_α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide][5] and HPLC comparison with references, we found a ca. 3:2 proportion of both L,D-phenylalanine and of L,D-glutamine, showing that significant epimerization had taken place during the hydrolysis, but strongly suggesting that **1** incorporates L-phenylalanine and L-glutamine as shown. Epimerization under acidic conditions has also been reported for anacine.[3] Spectroscopic proof for the syn orientation of the two side chains was obtained by recording the ^{1}H NMR spectrum in DMSO-d_6−C_6H_6-d_6 (3:4). In this solvent, the H-3 and H-14 signals were well separated (δ 5.15 and 4.98, respectively), but no NOE was seen between them in the NOESY spectrum. However, a significant interaction could be seen between (one of) the benzylic protons (18-CH_2) at δ 3.50 and the C-15 proton at δ 1.77, implying a boatlike conformation of the piperazine ring with the two large substituents in a *syn*-1,4-diaxial disposition. It has been shown that such a conformation is energetically preferred in pyrazinoquinazolines similar to those discussed here.[6]

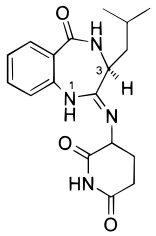
MS and NMR data (Table 1) indicated the minor metabolite verrucine B (**2**) to be an isomer of **1**. The ^{13}C NMR spectrum was assigned by analogy to that of **1**, and the NOESY spectrum allowed assignment of the ^{1}H NMR signals. The main differences when comparing the spectra of **1** and **2** were seen for H-3/H-18 and for H-14/H-15, signifying that **2** must be an epimer of **1**, that is, with an *anti*-disposition of the two substituents of the piperazine rings. Consistent with this, a NOESY correlation was observed between H-3 (δ 5.18) and one of the protons at C-15 (δ 2.18). The absolute configuration was not determined due to the epimerization experienced with **1**.

HPLC analysis of an extract of another isolate of *P. verrucosum* (IBT 11622) also showed the presence of **1** and **2**; however, for this isolate **2** was the major metabolite. This finding demonstrates that both metabolites must be natural products despite the observed epimerizations. Boyes-Korkis et al. also observed epimerization of anacine; however, they also concluded that the two epimers they detected by HPLC of their native extracts were natural products.[3]

In view of the spectral similarities of anacine with **1**, the quinazoline structure **4** seems more likely than the originally proposed structure **3**. Apparently, the benzodiazepine structure was assigned[3] because of the presence of an ion *m/z* 130 in the mass spectrum similar to the one found in auranthine and attributed to the indolic fragment C_8H_4NO. However, structure **3** ($[M + H]^+$ *m/z* 343) cannot accommodate two prominent ions at *m/z* 326 and 298, which were explained as loss of ammonia and successive loss of carbon monoxide, events that would fit well with structure **4**. The NMR data also point to the latter. In fact, a comparison with an ^{1}H NMR spectrum of the model compound cyclo-anthranilyl-leucine dipeptide[3] (**5**) shows poor overall similarity (Table 1). On the other hand, a very good similarity is found when comparing the NMR data of **3** and **4** with literature data for glyantrypine[7] and other analogous quinazolines.[8,9]



1 R_1, R_2 = β-H,β-H
2 R_1, R_2 = α-H,β-H or β-H,α-H



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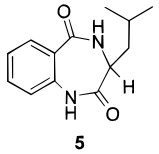

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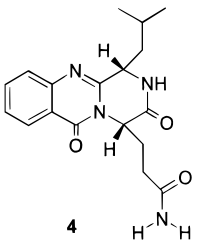

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Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Varian FT-NMR spectrometer at 500 and 125 MHz, for ^{1}H and ^{13}C, respectively. ESMS data were obtained using a Varian Trio 2000 mass spectrometer. The circular dichroism (CD) spectra were measured on a JASCO J-710 spectropolarimeter, and the UV spectra, on a Hewlett−Packard 8452A diode array spectrophotometer. Analytical HPLC conditions were similar to those given by Smedsgaard,[10] and retention indices (RI) of fungal metabolites were calculated according to Frisvad and Thrane.[11]

Fungal Material and Fermentation. The isolates of *P. verrucosum* (IBT: 11622, origin unknown; 14249, ex peanut) were obtained from the Culture Collection at the Department of Biotechnology (IBT), The Technical University of Denmark. IBT 14249 was cultured for 14 days in the dark on 200 SYES agar plates[12] as three-point mass inoculations. IBT 11622 was cultivated on 1 SYES agar plate.

Extraction and Separation. Agar plates with IBT 14249 were extracted repeatedly with EtOAc (2 L) to give a crude extract (1.8 g) after evaporation of the solvent. This extract was subjected to vacuum−liquid chromatography on silica (heptane, heptane−EtOAc, EtOAc−EtOH, EtOH as eluents) to give four fractions. The fraction containing verrucines A (**1**) and B (**2**) was purified on a Merck Lichroprep RP_{18} (25 × 310 mm, 40−63 μm) column using H_2O−CH_3CN (50:50) as mobile phase at 6 mL/min flow rate, to give a fraction rich in **1** and another rich in **2**. The two fractions were each subjected to HPLC on a Waters Prep Nova-Pak C_{18} cartridge (25 mm × 100 mm, 6 μm) using H_2O−CH_3CN (70:30) as mobile phase at 20 mL/min flow rate, to give **1** (32 mg) and **2** (6 mg). IBT 11622 was extracted according to Smedsgaard.[10]

Hydrolysis of Verrucine A (1). Hydrolysis of **1** (0.35 mg) was performed with 6N HCl (250 μL) for 1 h at 155 °C. The products were treated with N_α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide according to the directions given by Marfey.[5] Using the same solvent system, the resulting diastereomers were analyzed by HPLC and compared to Marfey derivatives prepared from L-phenylalanine (t_R = 20.03 min) and D,L-phenylalanine (t_R = 20.10 min and t_R = 22.89 min), together with L-glutamic acid (t_R = 11.72 min) and D,L-glutamic acid (t_R = 11.72 min and t_R = 13.40 min).

Verrucine A (1): amorphous solid; $[\alpha]^{22}_D$ +37° (*c* 0.1, EtOH); UV λ_{max} (EtOH) nm (log ϵ) 227sh (3.65), 272 (3.17), 278 (3.17), 306 (2.82), 317 (2.68); CD (EtOH, *c* 0.006), $\Delta\epsilon$ (λ nm) 210 (−22.80), 227 (+10.69), 252 (−1.90), 273 (−3.26), 309 (+3.98), 320 (+1.45); ^{1}H and ^{13}C NMR, see Table 1. The following HMBC correlations were observed: from C-1 to H-2, H-3, H-14, and CH_2-15; from C-3 to H-2 and H-18; from C-4 to H-2, H-3, H-14, and H-18; from C-6 to H-7, H-8, H-9, and H-10; from C-7 to H-8 and H-9; from C-8 to H-7, H-9, and H-10; from C-9 to H-7, H-8, and H-10; from C-10 to H-8 and H-9; from C-11 to H-7, H-8, and H-9; from C-12 to H-7, H-9, H-10, and H-14; from C-14 to H-2, CH_2-15, and H-16; from C-15 to H-14 and H-16; from C-16 to H-14, CH_2-15, and H-17 (6.71 ppm); from C-17 to CH_2-15, H-16, and H-17 (7.25 ppm); from C-18 to H-3, H-20, and H-24; from C-19 to H-3, H-18, H-20, H-21, H-23, and H-24; from C-20 and C-24 to H-21, H-22, and H-23; from C-21 and C-23 to H-20, H-22, and H-24; from C-22 to H-20, H-21, H-23, and H-24; elemental analysis: found C 66.83; H 5.51, N 15.03; calcd for $C_{21}H_{20}O_3N_4$, C 67.01, H 5.36, N 14.88%; ESMS $[M + H]^+$ at *m/z* 377; RI = 790.

Verrucine B (2): amorphous solid; $[\alpha]^{22}_D$ +124° (*c* 0.08, EtOH); UV λ_{max} (EtOH) nm (log ϵ) 227sh (3.56), 272 (2.90), 278 (2.87), 306 (2.69), 317 (2.65); CD (EtOH, *c* 0.008), $\Delta\epsilon$ (λ nm) 205 (+11.56), 231 (+11.17), 278 (+1.51), 287 (0.94), 303 (+0.82), 318 (+0.50); ^{1}H and ^{13}C NMR, see Table 1; ESMS $[M + H]^+$ at *m/z* 377; RI = 880.

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