# Verrucine F, a Quinazoline from *Penicillium verrucosum*

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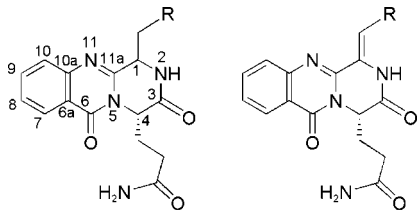
Verrucine F (**3**), a quinazoline similar to verrucines A (**1**) and B (**2**), contains one anthranilic acid residue, one L-glutamine residue, and one α,β-unsaturated phenylalanine residue, as determined by NMR, MS, and chemical methods. Compounds **1** and **3**, but not **2**, were produced by *Penicillium verrucosum* J255 and eight additional *P. verrucosum* strains. Verrucines were typically more concentrated in the inner (older) parts of the colony, and **3** peaked 4−8 days after **1**, but at 10-fold lower concentrations (∼200 μg/g). Verrucine F (**3**) formed spontaneously from **1** in buffered water solutions, probably by oxidation at C-1 followed by water elimination.

Mold spoilage of stored cereals is of major concern throughout the world. Growth of spoilage fungi may be detrimental to the smell, taste, or appearance of grains; more seriously, certain molds produce mycotoxins, rendering the grains unsuitable for use as food or feed. *Penicillium verrucosum* (Dierckx), of the family Trichocomaceae, is a common mold on stored cereals in temperate regions. This species produces several mycotoxins including ochratoxin A,[1] which has recently gained significant attention as a problem in food and feed. The European community has issued directives regarding the maximum concentrations of ochratoxin A allowed in cereals, wine, and coffee.[2,3] At present, we are investigating some aspects of the biosynthesis of ochratoxins A and B in *P. verrucosum*, including the search for key intermediates. In the course of this work, we have also come across a number of other secondary metabolites, including verrucines A (**1**) and B (**2**).[4] Larsen and co-workers, when first describing **1** and **2**, also indicated the existence of a series of oxygenated verrucine analogues, verrucines C, D, and E.[5] In the present study, we describe the isolation and characterization of a new related substance, with the proposed name verrucine F (**3**), as well as its occurrence in different strains of *P. verrucosum* and its formation from verrucine A.

Mycelia from 50 agar cultures of *P. verrucosum* were extracted with aqueous MeOH and were subsequently fractionated by SPE and preparative HPLC, which led to the isolation of **1** and **3**. Compound **1** was found to be partly isomerized to **2** when the solvent (acidic aqueous MeCN) was evaporated under reduced pressure. This epimerization has been observed in previous studies of **1** and the related compound anacine (**4**).[4,6] Verrucines A (**1**) and B (**2**) were identified by NMR and MS data, aided by comparison with literature data.[4]




**1** *S* at C-1, R = Ph
**2** *R* at C-1, R = Ph
**4** S at C-1, R = i-Pr




**3** R = Ph
**5** R = i-Pr

Verrucine F (**3**) yielded $^1$H NMR data similar to **1** and **2**, suggesting the presence of anthranilic acid and glutamine moieties.

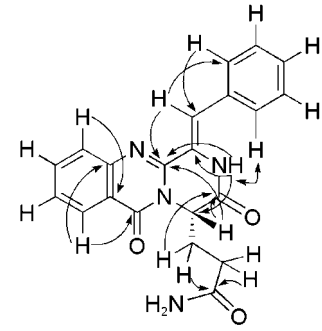



**Figure 1.** Relevant HMBC (single-headed arrows) and NOESY (double-headed arrow) correlations for verrucine F (**3**).

The presence of a glutamine side chain was indicated by MSMS data, which showed the sequential loss of $NH_3$ and CO from **3**, just as was observed for **1** and **2**.[4] The major difference in the $^1$H NMR data between **3** and both **1** and **2** was that the signals from the phenylalanine-derived methine and methylene groups of **1** and **2** were absent in **3**. Instead, a new olefinic proton singlet was present at $\delta$ 7.30 ($\delta_C$ 116.6), along with the signals from the phenyl group originating from phenylalanine. This indicated that **3** contained a double bond in the phenylalanine-derived part of the molecule. This was corroborated by cross-peaks in HMBC experiments, as indicated in Figure 1. The presence of an extra double bond in **3** was also supported by MS data, showing a $[M + H]^+$ ion at *m/z* 375 as compared to a $[M + H]^+$ ion at *m/z* 377 for both **1** and **2**. A similar unsaturated anacine analogue (**5**) was recently isolated from *P. aurantiogriseum*.[7] Analysis of **3** by HRFABMS yielded a $[M + H]^+$ ion at *m/z* 375.1489, in accordance with the molecular formula $C_{21}H_{18}N_4O_3$. NOESY experiments showed that the configuration of the double bond was *Z*, by a cross-peak between the phenyl ring CH signal at $\delta$ 7.67 and the amide proton signal at $\delta$ 10.59 (Figure 1). The corresponding *E*-isomer was not detected in any sample. The absolute configuration of the glutamine residue was determined by acidic hydrolysis followed by esterification with (*S*)-2-butanol, amide formation with perfluoropropanoic anhydride, and GC-MS analysis. Comparison with reference samples showed a 10:1 ratio between the derivatives of L- and D-glutamic acid in the sample, where the D-glutamic acid derivative was assumed to be formed by racemization during sample derivatization, as previously observed.[8] The structural difference between **1/2** and **3** was also reflected by the corresponding UV spectra. The UV spectrum of **3** in EtOH displayed peaks at 209, 230, 257, and 337 nm. Compounds **1** and **2** displayed UV peaks at similar wavelengths, but with overall lower log $\varepsilon$ values.[4] The UV result can be explained by the extended conjugated double-bond system in **3**,

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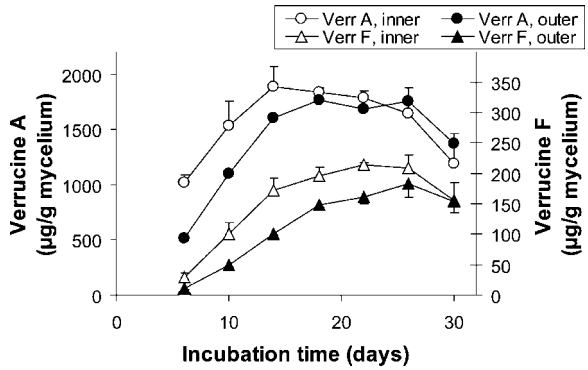
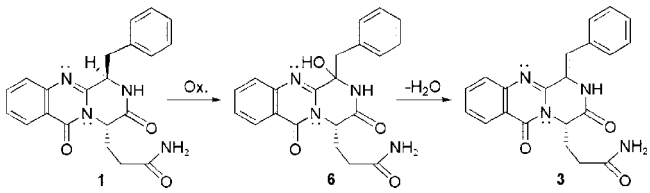



**Figure 2.** Verrucines A (**1**) and F (**3**) in inner and outer parts of colonies of *Penicillium verrucosum* J255 grown on yeast extract sucrose agar at 25 °C.

**Scheme 1**




but not in **1** or **2**. This difference was also apparent when analyzing samples containing **1** and **3** using LC-MS with simultaneous UV detection at 332 nm. Samples giving similar peak areas for **1** and **3** in the corresponding extracted ion chromatograms always yielded much larger peaks for **3** in the UV chromatogram.

The presence of **1**, **2**, and **3** in a number of *P. verrucosum* strains was tested by extracting mycelia from agar cultures with MeOH and analyzing the extracts by LC-MS. Compounds **1** and **3** were detected in all cultures, whereas **2** was not detected in any culture. For **1** the concentrations ranged from 0.3 to 4 mg/g agar plug, and for **3** from 5 to 100 $\mu$g/g agar plug. The production of **1** and **3** in *P. verrucosum* J255, a strain that also produces ochratoxin A and citrinin, was monitored by harvesting cultures at intervals over 30 days, followed by MeOH extraction and HPLC analysis. Verrucine A (**1**) attained maximum concentrations of ~1800 $\mu$g/g mycelium in both the inner and outer parts of the colony after 14−18 days (Figure 2). Initially, the concentration of **1** was approximately 2-fold greater in the inner (older) than the outer part of the colony, although after 18 days, concentrations in both parts were similar. Verrucine F (**3**) attained maximum concentrations of ~200 $\mu$g/g mycelium after 24−26 days and was typically more concentrated in the inner than the outer parts of the colony. The concentrations of both **1** and **3** decreased slightly after 26 days. This is consistent with a steady enzymatic or spontaneous formation of **3** from **1** or from **2** after epimerization. Enzymatic activity introducing a double bond in phenylalanine residues has previously been identified in other members of the *Penicillium* subgenus *Penicillium* section *Viridicata*, as part of the machinery forming the alkaloids cyclopenin and cyclopenol.[9] This enzymatic activity required the presence of $NAD^+$ and was optimum at pH 9.1.[10] Spontaneous formation of **3** could occur through oxidation of **1** or **2** at C-1, forming a hydroxylated analogue (**6**), a process that previously has been observed,[5,11,12] followed by elimination of $H_2O$ to form **3** (Scheme 1). A similar C-1 hydroxylated anacine analogue has also been reported.[7] As only the *Z*-isomer of **3** was observed, this could indicate involvement of an enzymatic process. However, if formation of **3** is spontaneous, via a C-1 oxidized intermediate, subsequent elimination of $H_2O$ would still be expected to lead mainly to the *Z*-isomer due to steric interactions between the phenyl ring and the quinazoline ring system in the *E*-isomer, as well as during the water elimination process.

To discriminate between these two modes of formation, verrucine A (**1**) was incubated with whole cell extracts of *P. verrucosum*, at pH 7 and 9, in the presence of $NAD^+$, as well as without cell extracts (pH between 4 and 9). LC-MS analyses showed that a fraction of **1** was transformed to **3** in the absence of cell extracts and that the addition of cell extracts did not enhance the formation. The formation of **3** was the highest at pH 4 and 9, which is consistent with acid- and base-catalyzed, respectively, water elimination from a C-1 hydroxylated verrucine A (Scheme 1); however, the conversion was also observed at neutral pH. Moreover, in all *P. verrucosum* samples containing **3**, as well as in all samples from incubation of **1**, a possible C-1 hydroxylated **1** was detected by LC-MS. This substance displayed a $[M + H]^+$ ion at *m/z* 393, from which water was lost to produce a daughter ion at *m/z* 375. These results indicate that the formation of **3** is not catalyzed by enzymes.

## Experimental Section

**General Experimental Procedures.** $^1H$ and $^{13}C$ NMR data were acquired on a Bruker DRX600 NMR spectrometer equipped with a 2.5 mm SEI microprobe ($^1H/^{13}C$). All NMR experiments were recorded at 30 °C in DMSO-$d_6$, and the chemical shifts were determined relative to the residual signal of the solvent ($\delta_C$ 39.5; $\delta_H$ 2.50). For structure elucidation, 1D $^1H$ NMR, COSY, NOESY (300 ms), DEPT-HSQC, and HMBC (65 ms) were applied, and the pulse sequences were used as provided by the manufacturer. HRFABMS was performed on a four-sector tandem mass spectrometer (Jeol SX/SX102A), equipped with a FAB ion-source (Xe), using glycerol as matrix and PEG as internal standard. LC-MS and LC-MSMS were performed on an Agilent 1100 HPLC system connected to a Bruker Esquire-LC ion-trap mass spectrometer (electrospray ionization, ESI) operated in the positive ion mode, and GC-MS on a HP5890/5970 GC-MS (Hewlett-Packard) using He as carrier gas. Preparative HPLC was run on a Gilson system. The mobile phase for LC-MS and HPLC comprised MeCN (HPLC isocratic grade) and deionized filtered water. SPE was performed with 10 g prepacked columns [Isolute C18 (EC), International Sorbent Technology, Hengoed, UK].

**Organisms and Cultivation.** The following strains of *Penicillium verrucosum* were grown as single-point cultures on yeast extract sucrose agar[13] at 25 °C: J255 (Culture Collection, Dept of Microbiology, Swedish University of Agricultural Sciences, Uppsala), OTA11 (National Food Administration, Uppsala, Sweden), BFE489, BFE490, BFE491, BFE492, BFE493, BFE495, and BFE505 (Max Rubner Institute, Institute of Nutrition and Food, Institute for Safety and Quality of Fruits and Vegetables, Karlsruhe, Germany).

**Isolation of Verrucines A (1), B (2), and F (3).** Mycelia from 50 cultures of *P. verrucosum* J255 (14 days) were ground in liquid nitrogen and subsequently extracted with 1 L of aqueous 40% MeOH for 10 min in an ultrasonic bath, followed by 1 h with stirring at room temperature. The extract was filtered through a textile cloth, then sequentially through two different filter papers (3 and 00H, respectively, Munktell, Sweden), and subsequently diluted with $H_2O$ (3 L). The resulting solution (4 L) was loaded onto five 10 g SPE columns. Each SPE column was washed with 50 mL of $H_2O$ and eluted with 50 mL of aqueous 95% MeCN. The combined 95% MeCN extract was dried under vacuum, redissolved in 4 mL of aqueous 40% MeCN, and injected onto a preparative C-18 column (20 × 100 mm with a 20 × 30 mm guard column, 5 $\mu$m, Dr. A. Maisch High Performance LC GmbH, Germany). The column was eluted with a gradient of MeCN in $H_2O$ (with 0.1% TFA), running from 40% to 70% MeCN in 10 min, followed by 70% for 10 min, at 10 mL/min. The eluate was monitored at 332 nm, and fractions were collected in deep-well plates (2 mL). Selected fractions were analyzed by ESI-MS, and the fractions containing putative verrucines, as guided by *m/z* values, were pooled and dried under reduced pressure. The pooled fractions were fractionated further on the same preparative HPLC column (40% aqueous MeCN with 0.1% TFA, at 10 mL/min). The fractions containing **3** were dried under reduced pressure, yielding 1 mg of pure substance. Verrucine A (**1**) was isolated in parallel, but during evaporation of the solvent (40% to 70% MeCN gradient with 0.1% TFA) under reduced pressure, **1** was partly isomerized to **2**. Pure **1** and **2** were obtained by

preparative HPLC on the same column using aqueous 40% MeCN without TFA.

**Verrucine F (3):** white powder; UV $\lambda_{max}$ (EtOH) nm (log $\varepsilon$) 209 (4.52), 230 (4.35), 257 (4.03), 337 (4.27); $^1$H NMR (DMSO-$d_6$, 600 MHz) $\delta$ 10.59 (1H, s, NH), 8.17 (1H, d, $J$ = 8 Hz, H-7), 7.88 (1H, t, $J$ = 8 Hz, H-9), 7.77 (1H, d, $J$ = 8 Hz, H-10), 7.67 (2H, d, $J$ = 8 Hz, Ph-2/6), 7.56 (1H, t, $J$ = 8 Hz, H-8), 7.46 (2H, t, $J$ = 8 Hz, Ph-3/5), 7.36 (1H, t, $J$ = 8 Hz, Ph-4), 7.30 (1H, s, C*H*-Ph), 7.25 (1H, s) and 6.70 (1H, s, $CONH_2$), 5.25 (1H, t, $J$ = 7 Hz, H-4), 2.19 (2H, m, $CH_2$-$CONH_2$), 2.13 (1H, m) and 2.07 (1H, m, $CH_2$-$CH_2$-$CONH_2$); $^{13}$C NMR (DMSO-$d_6$, 150 MHz) $\delta$ 172.2 ($CONH_2$), 165.7 (CO, C-3), 160.1 (CO, C-6), 146.9 (C, C-10a), 146.6 (C, C-11a), 134.6 (CH, C-9), 133.4 (C, Ph-1), 129.0 (CH, Ph-2/6), 128.1 (CH, Ph-3/5), 127.8 (CH, Ph-4), 126.8 (CH, C-8), 126.6 (CH, C-10), 126.6 (C, C-1), 125.9 (CH, C-7), 119.6 (C, C-6a), 116.6 (CH, *C*H-Ph), 54.6 (CH, C-4), 30.2 ($CH_2$, $CH_2$-$CONH_2$), 28.2 ($CH_2$, $CH_2$-$CH_2$-$CONH_2$); HRFABMS *m*/*z* 375.1489 [M + H]$^+$ (calcd for $C_{21}H_{19}N_4O_3$ 375.1452).

**Determination of Absolute Configuration of Verrucine F (3).** Verrucine F (20 $\mu$g) was hydrolyzed in 1 mL of 6 M HCl in a sealed test tube at 110 °C for 18 h. The sample was dried under reduced pressure and was treated with 200 $\mu$L of (*S*)-2-butanol/acetyl chloride (10:1) in a closed vial at 100 °C for 45 min. After drying under a stream of nitrogen, the sample was reacted with 200 $\mu$L of perfluoropropionic anhydride in a closed test tube at 100 °C for 40 min. The reagent was removed with a stream of nitrogen and the sample dissolved in EtOAc (150 $\mu$L) and analyzed by GC-MS (HP-5MS, 30 m × 0.25 mm, 0.25 $\mu$m, Agilent Technologies) with the oven held at 150 °C for 5 min and then raised to 200 °C at 5°/min. The temperature of both the injector and the transfer line to the mass spectrometer was 240 °C. As references, samples of D- and L-Glu were derivatized and analyzed using the same protocol (D-Glu, 18.83 min; L-Glu, 18.96 min).

**Identification and Quantification of 3 in Cultures.** *P. verrucosum* strains were grown for 14 days, after which two agar plugs (5 mm diameter) from the inner and outer parts of the colony were removed from each culture, pooled, and extracted with MeOH (1 mL) by vortexing. The mixtures were held at room temperature for 1 h, then were vortexed again, after which the plugs were discarded. The extracts were centrifuged (13 000 rpm, 10 min), and the supernatant was transferred to HPLC vials for LC-MS analysis. LC-MS analysis was performed on a C-18 column (2.1 × 125 mm, 3 $\mu$m, Dr. A. Maisch High Performance LC GmbH) eluted with a gradient of MeCN in $H_2O$ (0.1% TFA), running from 20% to 70% MeCN in 18 min, from 70% to 100% in 1 min, maintained at 100% for 5 min, and re-equilibrated at 20% for 8 min, at 0.2 mL/min and with UV detection at 332 nm. MS data were used for supporting the identification of the compounds, whereas UV data were used for quantification. As references for identification and quantification, samples containing known amounts of **1**, **2**, and **3** were used. For quantification of **1** and **3** during growth on YES agar, *P. verrucosum* J255 cultures were frozen at −20 °C 6 days after inoculation and then every 4 days for a total of 30 days. After thawing, mycelia were divided at half the radius into inner and outer portions, and excess agar was scraped from the reverse with a spatula. Each portion was weighed and extracted into 10 times volume MeOH by vortexing, followed by sonication for 10 min, and vortexing again. Extracts were centrifuged in preparation for HPLC analysis. Triplicate samples were analyzed at each time point, and analyses were performed on a C-18 column (4.6 × 150 mm, 5 $\mu$m, Zorbax SB 300, Agilent Technologies), eluted with a gradient of MeCN in 2% acetic acid: held initially at 20% MeCN for 5 min, then from 20% to 70% MeCN in 20 min, from 70% to 100% in 3 min, maintained at 100% for 6 min, and re-equilibrated at 20% for 4 min, at 0.8 mL/min. Verrucines A (**1**) and F (**3**) were quantified by UV detection as previously described.

**Incubation of 1 with Cell Extracts.** Mycelia of *P. verrucosum* J255 were ground using a magnetic mill cooled with liquid nitrogen (Spex 6700). Ground material (1.0 g) was thawed and suspended in Tris-HCl buffer (2.0 mL, 50 mM, pH 7.0 or 9.0), and the resulting cell suspension used for incubation of **1**. To 2 mL HPLC vials containing 200 $\mu$g of dried **1** (duplicate samples) were added cell suspension (200 $\mu$L), $H_2O$ (200 $\mu$L), and $NAD^+$ (18 mM, 50 $\mu$L). Samples without **1** and samples with buffer instead of cell suspension were included as controls. All samples were stirred at 30 °C, and after 1 and 6 h, 150 $\mu$L was withdrawn and stored at −18 °C. Samples were subsequently analyzed by LC-MS [same column as above, eluted with aqueous 40% MeCN (0.1% TFA) at 0.1 mL/min and with UV detection at 210 nm]. Prior to LC-MS analysis, the samples were thawed and centrifuged (13 000 rpm, 5 min) and the supernatants transferred to HPLC vials. All samples without cell suspension were also analyzed after 22 days. In addition, samples of **1** were treated at pH 4.1 (100 mM acetate buffer), pH 6.4 (100 mM bicarbonate buffer), and pH 7.6 (100 mM phosphate buffer), also at 30 °C, and were repeatedly analyzed by LC-MS over a period of 6 weeks.

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## References and Notes

(1) Frisvad, J. C.; Lund, F.; Elmholt, E. *J. Appl. Microbiol.* **2005**, *98*, 684–692.
(2) Commission Regulation (EC) No. 123/2005 of January 26, 2005, amending regulation (EC) No. 466/2001 as regards ochratoxin A. *Off. J. Eur. Union*, **2005**, *L 25*, 3−5.
(3) Commission Recommendation of August 17, 2006, on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2, and HT-2, and fumonisins in products intended for animal feeding. *Off. J. Eur. Union*, **2006**, *L 229*, 7−9.
(4) Larsen, T. O.; Franzyk, H.; Jensen, S. R. *J. Nat. Prod.* **1999**, *62*, 1578–1580.
(5) Larsen, T. O.; Svendsen, A.; Smedsgaard, J. *Appl. Environ. Microbiol.* **2001**, *67*, 3630–3635.
(6) Boyes-Korkis, J. M.; Guerney, K. A.; Penn, J.; Mantle, P. G.; Bilton, J. N.; Sheppard, R. N. *J. Nat. Prod.* **1993**, *56*, 1707–1717.
(7) Hong Xin, Z.; Fang, Y.; Du, L.; Zhu, T.; Duan, L.; Chen, J.; Gu, Q.-Q.; Zhu, W.-M. *J. Nat. Prod.* **2007**, *70*, 853–855.
(8) Broberg, A.; Menkis, A.; Vasiliauskas, R. *J. Nat. Prod.* **2006**, *69*, 97–102.
(9) Framm, J.; Nover, L.; El Azzouny, A.; Richter, H.; Winter, K.; Werner, S.; Luckner, M. *Eur. J. Biochem.* **1973**, *37*, 78–85.
(10) Aboutabl, S. *Pharmazie* **1974**, *29*, 73.
(11) Wang, H.; Sim, M. M. *J. Nat. Prod.* **2001**, *64*, 1497–1501.
(12) Larsen, T. O. Personal communication.
(13) Hocking, A. D.; Pitt, J. I.; Samson, R. A.; Thrane, U. *Advances in Food Mycology*; Springer: New York, 2006; p 356.

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