Detection of VM55599 and Preparaherquamide from *Aspergillus japonicus* and *Penicillium fellutanum*: Biosynthetic Implications

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Received May 13, 2008

The secondary metabolites VM55599 (**4**) and preparaherquamide (**5**) have been identified by LC-MS^n analysis as natural metabolites in cultures of *Penicillium fellutanum*, whereas preparaherquamide has been identified only in cultures of *Aspergillus japonicus*. In accord with a previous proposal, the identification of both metabolites, which have a diastereomeric relationship, provides indirect support for a unified biosynthetic scheme.

The paraherquamides (**6**),[1] together with the asperparalines (**7**),[2] stephacidins,[3] brevianamides,[4] marcfortines,[5] notoamides,[6] sclerotamide,[7] and malbrancheamides,[8] are secondary metabolites derived from fungi that feature a common bicyclo[2.2.2]diazaoctane core. It has been postulated that this ring system is generated through an intramolecular Diels−Alder cycloaddition of the C_5 moiety across the α-carbons of the amino acid subunits, as depicted in Scheme 1.[9]

Members of this unique family of metabolites are all derived from tryptophan, isoprene units of mevalonate origin, and a cyclic amino acid residue consisting of either proline, *β*-methylproline (and derivatives), or pipecolic acid. In 1993, Everett and co-workers described the isolation of VM55599 (**4**), a minor metabolite from culture extracts of a *Penicillium* spp. (IMI332995) that also produces paraherquamide A (**6**), among other paraherquamides.[10] Taking into account the structural similarities between these co-occurring metabolites, these authors proposed that **4** might indeed be a biosynthetic precursor of paraherquamide A.[10] The relative stereochemistry of **4** was assigned by Everett and co-workers through extensive ^{1}H NMR NOE experiments, but the small quantity of this compound isolated precluded the determination of its absolute configuration.[10] Our laboratory has previously determined the absolute configuration of VM55599 produced by *Penicillium* spp. IMI332995 by an asymmetric, biomimetic total synthesis.[11] We further demonstrated that **4** is not a biosynthetic precursor to paraherquamide A through the synthesis of double ^{13}C-labeled, racemic VM55599 (**4**), for which the lack of incorporation into **6** in cultures of *P. fellutanum* cast doubt on the intermediacy of this species in paraherquamide biosynthesis.[12]

Asperparalines A (**7**) and C and several members of the paraherquamide family, including VM55599, have been identified as being biosynthesized from *β*-methylproline.[13] Previous studies from our laboratories have revealed that (*S*)-isoleucine (L-Ile) serves as the biosynthetic precursor of the *β*-methyl-*β*-hydroxyproline residue in paraherquamide A as well as the *β*-methylproline residue of **7**.[13] This mandates that the L-Ile side chain stereochemistry is retained at C-14 in paraherquamide A and that hydroxylation occurs with *net retention* at C-14. Accordingly, these results brought into question the capacity of VM55599 (**4**) to serve as a biosynthetic precursor to the paraherquamides. If, as one could reasonably speculate, L-Ile is a biosynthetic precursor not only to the paraherquamides but also to VM55599, the absolute stereochemistry of this compound must be that depicted in Scheme 2; we have rigorously confirmed the relative and absolute stereochemistry of **4** as mentioned above.[11] Thus, the absolute configuration of the bicyclo[2.2.2]diazaoctane core of VM55599 (**4**) is enantiomorphic to that of virtually all of the other members of the paraherquamide family.[14]

These experimental observations led us to propose a unified biosynthesis of the paraherquamides and VM55599 (**4**), as shown in Scheme 2. In this proposal, the biosynthetic precursors of the paraherquamides and that of **4** would arise as diastereomeric products of the putative intramolecular Diels−Alder cycloaddition of a common azadiene through two of four possible diastereomeric transition states (**24** and **25**). The major pathway (via **24**) produces species **5** (named "pre-paraherquamide"), which is further processed in the respective organisms to produce the paraherquamides and asperparalines. In support of this hypothesis, we have synthesized double ^{13}C-labeled species **5** and have demonstrated that this compound incorporates into paraherquamide A (**6**) in cultures of *P. fellutanum*.[12] Despite the incorporation of **5** into the biosynthesis of paraherquamide A, this putative intermediate has heretofore not been detected as a secondary metabolite in either paraherquamide- or asperparaline-producing fungi. Herein, we demonstrate that (i) paraherquamides A (**6**) and B are produced in both *P. fellutanum* and *Aspergillus japonicus*; (ii) both VM55599 (**4**) and preparaherquamide (**5**) are natural metabolites of the paraherquamide-producing organism *P. fellutanum*; and (iii) preparaherquamide (**5**) is also observed as a natural metabolite from *A. japonicus* cultures. The observation of VM55599 (**4**) and preparaherquamide (**5**) provides additional, indirect support for the unified biogenesis outlined in Scheme 2.

Results and Discussion

P. fellutanum and *A. japonicus* JV-23 were cultured and first examined for viable production of paraherquamide A (**6**), asperparaline A (**7**), and their derivatives, respectively. Authentic paraherquamide A was first analyzed by LC-MS^n, and its MS, MS^2, MS^3, and MS^4 spectra were informative in identifying this alkaloid, establishing the successful application of LC-MS^n analysis in this study (Figure S1, Supporting Information). Next, paraherquamide A (**6**) in the *P. fellutanum* isolation was eluted at 14.47 min in the selected-ion monitoring (SIM) chromatograph and exhibited an ion at *m/z* 494.36 (calculated $[M + H]^+$: 494.26) by MS analysis (Figures 1C and 2A). Further MS^2, MS^3, and MS^4 analyses produced identical spectra to those of authentic paraherquamide A (Figures 2A and S1). Along with paraherquamide A, paraherqua-

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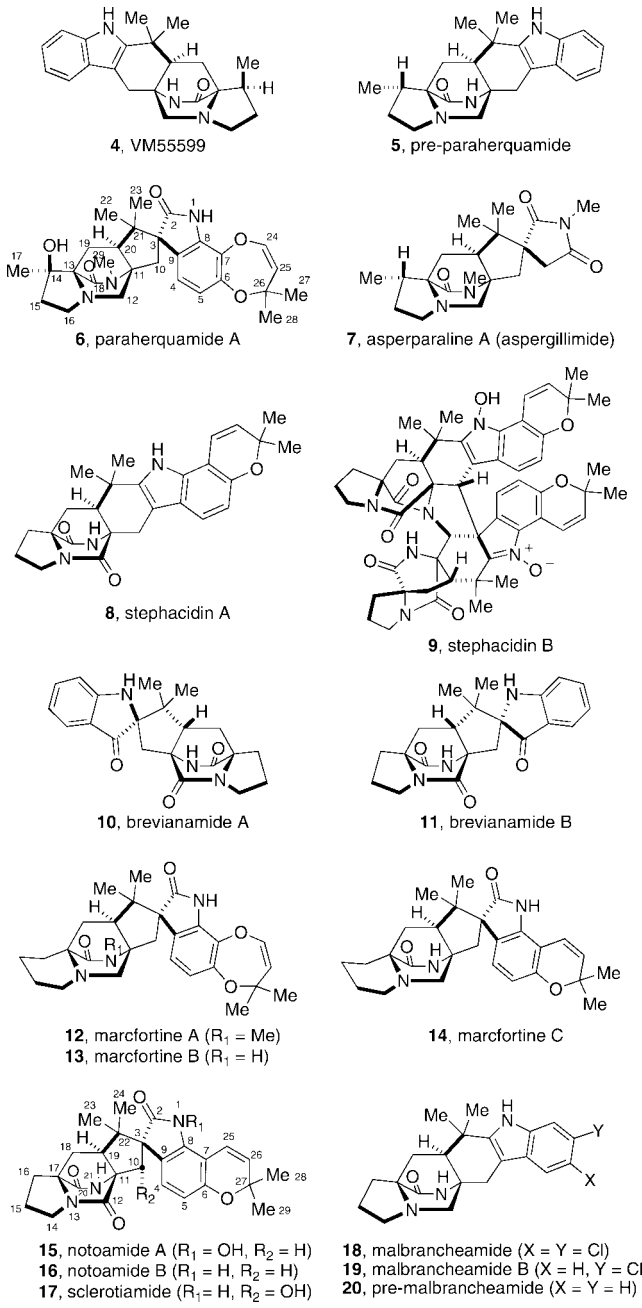
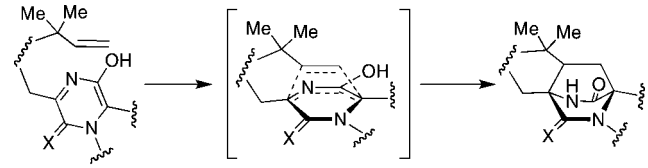
10.1021/np800292n CCC: $40.75
Published on Web 08/28/2008



mides B−G have previously been isolated from *P. fellutanum* (previously named *P. charlesii*).[1] In this study, one compound at *m/z* 464.29 was detected in the fungal isolation and had a retention time of 14.95 min (Figures 1C and 2B). This compound was identified as paraherquamide B (calcd $[M + H]^+$: 464.25) by comparing its retention time and MS^2 spectrum to those of an authentic specimen (Figures 1 and S1).

In extracts from *A. japonicus* JV-23 cultures, asperparaline A (**7**) had a retention time of 7.58 min and exhibited an ion at *m/z* 360.29 (calcd $[M + H]^+$: 360.22) (Figures 1D and 2C). This metabolite was further analyzed by MS^2 analysis. In previous reports, *Aspergillus* species IMI 337664[2] and *A. sclerotiorum*[7] represented the first organisms outside of *Penicillium* spp. to produce paraherquamide congeners. In this study, we investigated paraherquamide production in *A. japonicus* JV-23. By comparing their retention times and MS^n spectra to those of authentic compounds, both paraherquamide A (14.45 min) and paraherquamide B (14.88 min) were identified in this *Aspergillus* spp. isolation, further indicating that both *Penicillium* spp. and *Aspergillus* spp. are able to produce these anthelmintic alkaloid metabolites (Figures 1 and S2). Moreover, this result strongly suggested one common

Scheme 1. Proposed Intramolecular Diels−Alder Construction of the Bicyclo[2.2.2]diazaoctane Core



biosynthetic pathway shared by both asperparalines and paraherquamides in this fungus.

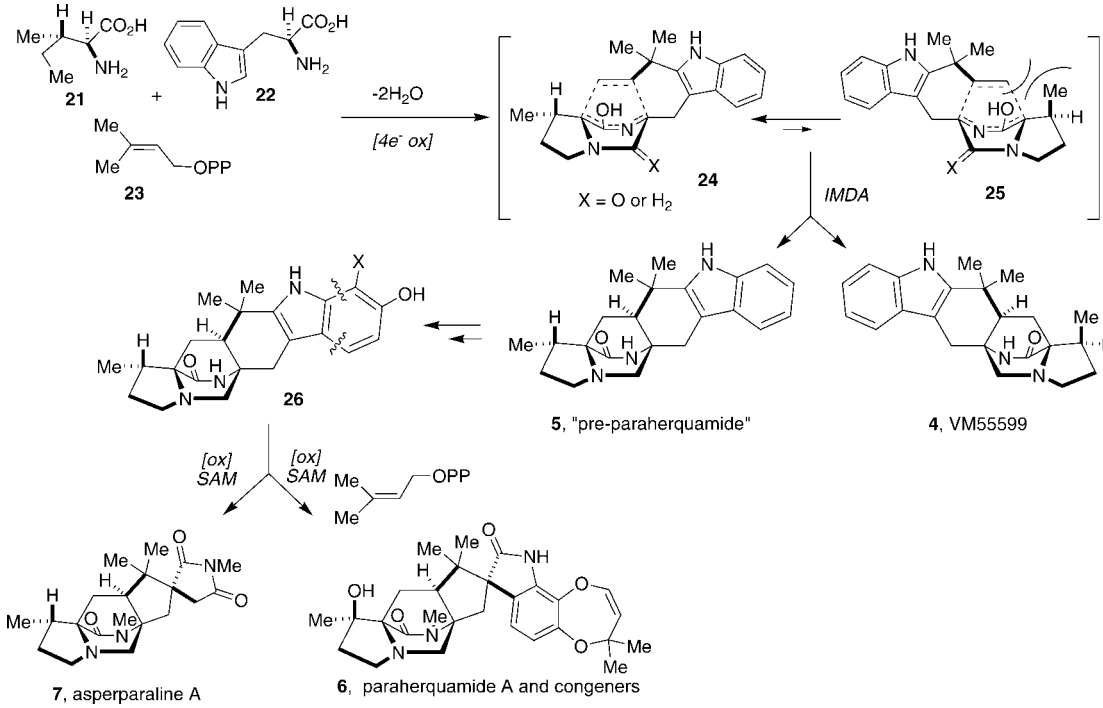
The *A. japonicus* JV-23 strain produced more paraherquamide B than paraherquamide A (**6**) in PDB medium under the growth conditions given. *A. japonicus* JV-23 is the first reported *Aspergillus* spp. to produce paraherquamide A itself, to the best of our knowledge, although Everett and co-workers isolated the paraherquamide congeners VM54159, SB203105, and SB200437 from *Aspergillus* strain IMI 337664.[2c] In extracts from *P. fellutanum*, two metabolites with *m/z* of 350 were separated and identified by LC-MS/MS analysis (Figure 3). The first peak had a retention time of 12.15 min, while the second metabolite was eluted at 12.64 min. These peaks were initially proposed to be VM55599 (**4**) and preparaherquamide (**5**), considering their theoretical molecular weights ($C_{22}H_{27}N_3O$, 349.22) and the previous isolation of **4** as the minor metabolite from *Penicillium* spp. IMI337664.[10]

To further identify these metabolites, synthetic and authentic (racemic) samples of **4** and **5** were used to secure standard MS and MS^2 fragmentation data (Figure S3). Interestingly, these two compounds, which are diastereomers, exhibited different fragmentation patterns in their MS^2 spectra. The ratio of the peak at *m/z* 322.26 to the peak at *m/z* 305.25 in the VM55599 MS^2 spectrum was larger than 1.0, while this ratio was significantly smaller than 1.0 in the preparaherquamide (**5**) MS^2 spectrum with the fragment at *m/z* 305.24 as the most intense peak, which likely serves as the distinctive feature of the MS^2 spectra of these two compounds (Figure S3). CO was lost from the peptide bond of parent ions to produce the first fragment at *m/z* 322.26, which was further fragmented to give a signal at *m/z* 305.25 by losing NH_3 (Figures 4 and S3). The same fragmentation pathway was observed for **5**. The fragmentation discrepancy observed in the MS^2 spectra of **4** and **5** was apparently affected by the single relative stereochemical difference at C-14 (paraherquamide numbering) between these two compounds. Comparing their MS^2 spectra with those of authentic compounds, VM55599 (**4**) (t_R = 12.15 min) and preparaherquamide (**5**) (t_R = 12.64 min) were identified in the extract from *P. fellutanum* cultures (Figures 3A−C and 4A, B). Preparaherquamide was thus observed as one natural metabolite, further strongly validating the putative pathway in Scheme 2.

In extracts from *A. japonicus* JV-23 cultures, only one metabolite at *m/z* 350 was identified by LC-MS/MS analysis (Figure 3D). When compared to the MS and MS^2 spectra of authentic standards, the metabolite with the retention time of 12.62 min was validated as preparaherquamide (Figure 4C). This represents the first identification of this putative precursor in an asperparaline-producing organism. Preparaherquamide (**5**) has been proposed by this laboratory as the key, common biosynthetic precursor to both the paraherquamides and the asperparalines.[9,13c] The identification of this substance in the paraherquamide- and asperparaline-producing *A. japonicus* JV-23 strain further supports the unified biogenetic hypothesis detailed in Scheme 2. Curiously, VM55599 was not detected as a natural metabolite from the *A. japonicus* JV-23 cultures.

Numerous alkaloids that display a wide spectrum of biological activities have been isolated from various fungi. An important and growing family of prenylated indole alkaloids is constituted by a common bicyclo[2.2.2]diazaoctane core derived mainly from tryptophan, proline, substituted proline derivatives, and isoprene units.

Scheme 2. Proposed Unified Biogenesis of Paraherquamides and Asperparalines



This family includes the paraherquamides,[1] asperparalines,[2] stephacidins,[3] brevianamides,[4] marcfortines,[5] notoamides,[6] sclerotiamide,[7] and the malbrancheamides.[8] Among these alkaloids, malbrancheamide is further distinguished by the presence of two chlorine atoms in the indole aromatic nucleus. Premalbrancheamide, which lacks the C-14 methyl group found in preparaherquamide, was recently isolated and identified as the precursor in malbrancheamide B biosynthesis.[15] By LC-MS/MS analysis, this natural metabolite exhibited a similar fragmentation pattern to VM55599 (**4**) and preparaherquamide (**5**). Similar to the results we obtained in precursor feeding experiments with **5**, synthetic doubly ^{13}C-labeled premalbrancheamide was also successfully incorporated into malbrancheamide B in *Malbranchea aurantiaca*.[12,15] The identification of both preparaherquamide and premalbracheamide as natural, trace metabolites suggests that structurally related common precursors may be involved in the biosynthesis of other subgroups of alkaloids within this family and that the structural diversity of these alkaloids are likely introduced by downstream tailoring enzymes following the construction of the bicyclo[2.2.2]diazaoctane core.

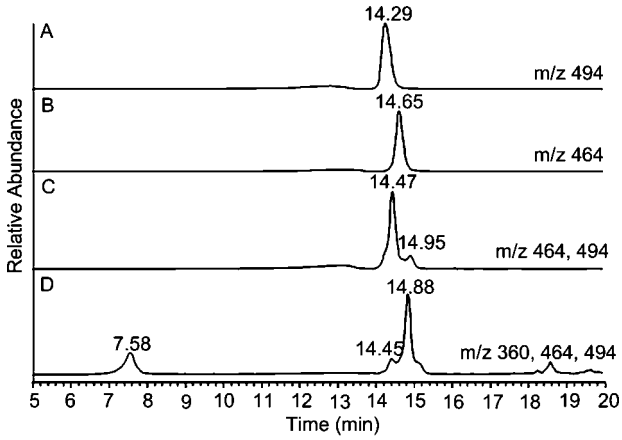


Figure 1. Selected-ion monitoring (SIM) chromatographs corresponding to authentic paraherquamide A (**6**) (*m/z* 494) (A), authentic paraherquamide B (*m/z* 464) (B), isolation from *P. fellutanum* culture (*m/z* 464 and 494) (C), and isolation from *A. japonicus* JV-23 (*m/z* 360, 464, and 494) (D).

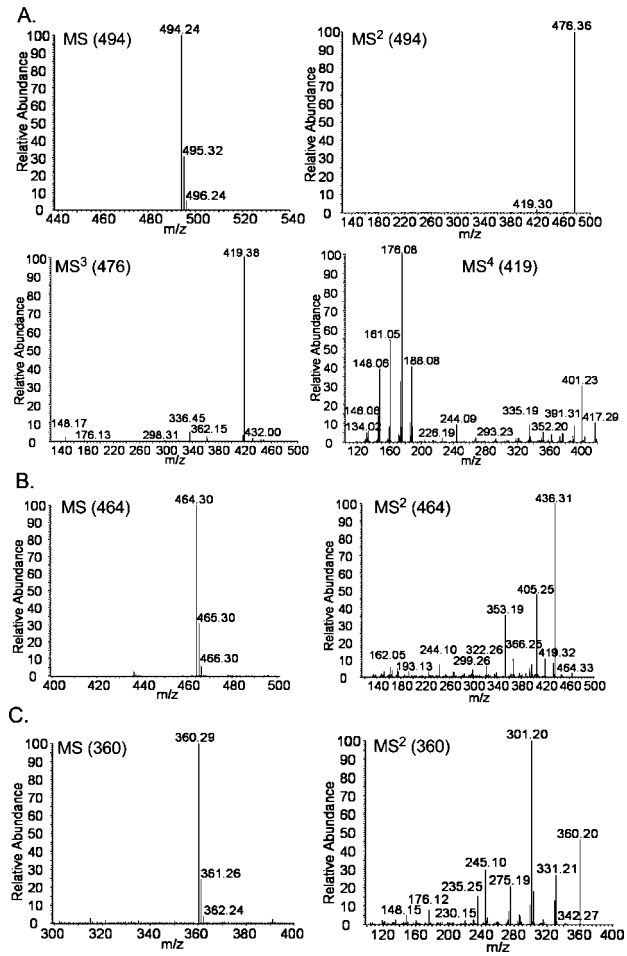


Figure 2. MS^n spectra of paraherquamide A (**6**) (A) and paraherquamide B (B) from the isolation of *P. fellutanum* cultures, and asperparaline A (**7**) (C) from the extracts of *A. japonicus* JV-23. The integral *m/z* values of ions for each MS^n analysis are included in the corresponding graphs.

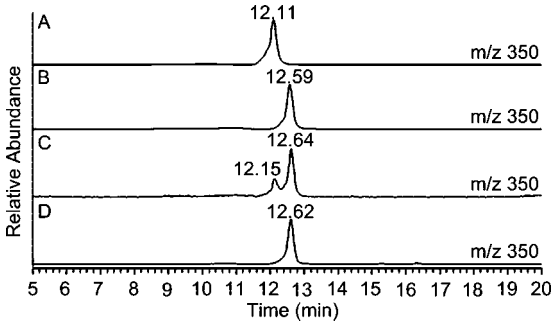


Figure 3. Selected-ion monitoring (SIM) chromatographs corresponding to the LC-MS^n analysis of authentic VM55599 (**4**) (*m*/*z* 350) (A), authentic preparaherquamide (**5**) (*m*/*z* 350) (B), isolation from *P. fellutanum* cultures (*m*/*z* 350) (C), and isolation from *A. japonicus* JV-23 (*m*/*z* 350) (D).

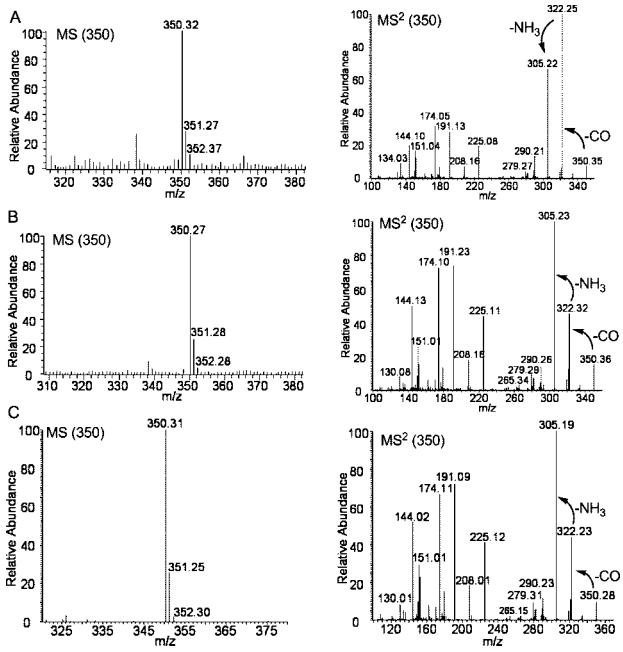


Figure 4. MS and MS^2 spectra of VM55599 (**4**) (A) and preparaherquamide (**5**) from the isolation from *P. fellutanum* culture (B), and preparaherquamide (**5**) from the extracts of *A. japonicus* JV-23 cultures (C). The integral *m*/*z* values of ions for each analysis are included in the corresponding graphs.

LC-MS/MS analysis has several important advantages with respect to sensitivity and selectivity in the detection of trace natural metabolites. In natural products identification, NMR techniques are widely used to directly provide structural information when sufficient amounts of purified substances are available. However, in the case of biosynthetic intermediates, there is often a paucity of material that is thus insufficient for NMR structural studies. This is a manifestation of biosynthetic intermediates being largely consumed by downstream tailoring enzymes, and thus these substrates do not accumulate. LC-MS/MS is an effective and powerful alternative in these cases and has been successfully used to identify many natural products in crude extracts by comparison with the respective reference compounds.[16] Herein, we were able to successfully deploy LC-MS/MS analysis to identify the presence of paraherquamide A (**6**), paraherquamide B, asperparaline A (**7**), VM55599 (**4**), and preparaherquamide (**5**) in crude fungal extracts using this technique.

In conclusion, we have demonstrated for the first time that preparaherquamide (**5**) is a natural secondary metabolite of the paraherquamide-producing organism *P. fellutanum* and the paraherquamide- and asperparaline-producing organism *A. japonicus* JV-23. This report constitutes the first confirmatory evidence for the natural existence of preparaherquamide, albeit at low concentration levels. This provides additional support for the unified biogenesis we have proffered.[9,11−13] VM55599 (**4**) is also produced by *P. fellutanum* and is consistent with the initial identification of this substance from the related paraherquamide-producing organism *Penicillium* spp. IMI332995 described by Everett and co-workers.[10] As the identification of new paraherquamide-producing fungi are discovered in various environments around the world, we speculate here that VM55599 can be expected to be detected as a co-metabolite. It should be further noted that Miller and co-workers recently described the detection of VM55599 as a metabolite in several strains of *Penicillium paneum* on the basis of mass spectrometric data.[17] It is entirely possible that these workers might have instead detected preparaherquamide (or both), which has the same mass as VM55599. As in the previously established case of *P. fellutanum*, we conclude that VM55599 is a shunt (dead-end) metabolite, as it possesses the incorrect absolute (and relative) stereochemistry to be processed further to a paraherquamide-like structure. Likewise, we speculate that in *A. japonicus* the major pathway metabolite **5** is largely consumed by the downstream biosynthetic machinery responsible for the substantial oxidative elaboration of **5** into the asperparalines and paraherquamides. We are currently pursuing the synthesis and labeling of several plausible metabolites downstream of preparaherquamide to gain insight into the sequence of events following the construction of this early pathway metabolite in both paraherquamide- and asperparaline-producing fungi. Efforts to clone the biosynthetic gene clusters for the biosynthesis of these structurally unique and biologically important natural products are also currently under investigation in our laboratories.

Experimental Section

Chemicals and Strains. Ethyl acetate and methanol were HPLC grade from Sigma Aldrich (St. Louis, MO), while acetonitrile used in LC-MS^n analysis was LC-MS grade from J. T. Baker (Phillipsburg, NJ). A MilliQ H_2O purification system (Millipore Ltd., Bedford, MA) generated water for LC-MS^n analysis. Trifluoroacetic acid (99%, reagent plus) and formic acid (>98%, ACS reagent) were also purchased from Sigma Aldrich. Authentic paraherquamide A (**6**),[18] VM55599 (**4**),[11,12] and preparaherquamide (**5**)[12] were synthesized following previously published procedures. Paraherquamide B (as the unnatural enantiomer) was obtained by total synthesis.[18b] *P. fellutanum* (ATCC20841) was purchased from American Type Culture Collection (Manassas, VA), while *A. japonicus* JV-23 was provided by Dr. Hideo Hayashi of Osaka Prefecture University.

Cultures of *P. fellutanum* and *A. japonicus* JV-23. *P. fellutanum* and *A. japonicus* JV-23 were initially grown in two different solid media (20 g of malt extract, 20 g of glucose, 1 g of peptone, and 20 g of agar in 1 L of deionized water) and (20 g of potato-dextrose broth and 20 g of agar in 1 L of deionized water), respectively, at 25 °C in the dark for 2 weeks. Fungal mycelia and spores were then transferred into 300 mL of sterile corn steep liquor medium (22 g of corn steep liquor and 40 g of glucose per liter of deionized water) for *P. fellutanum* or 300 mL of sterile potato-dextrose broth (PDB) (24 g of potato-dextrose broth per liter of deionized water) for *A. japonicus* JV-23, in 2 L Erlenmeyer flasks. Both fungal strains were then grown at 25 °C in the dark for 4 weeks.

Sample Extraction. The cultures with fungal mycelium were adjusted to pH 10−12 by 10 M KOH. The cultures were then extracted with an equal volume of ethyl acetate twice. The combined organic layer from each culture was washed with water, dried over anhydrous magnesium sulfate, and evaporated to dryness. The residues were redissolved in methanol prior to LC-MS^n analysis.

LC-MS^n Analysis. LC-MS^n analysis was performed using a ThermoFinnigan LTQ linear ion-trap instrument equipped with electrospray source and Surveyor HPLC system at room temperature. Separations were carried out with a Waters XBridge C_{18} (3.5 μm, 2.1 × 150 mm) column at a flow rate of 210 μL/min with solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). Solvent B was kept at 15% in solvent A for 2.5 min and then was gradually

increased to 40% over 12.5 min and 80% over 2 min and then maintained at 80% for 6 min to elute fungal metabolites. The column was further re-equilibrated with 15% solvent B for 25 min. For mass spectrometry, the capillary temperature was set to 275 °C with the source voltage at 3.6 kV, the source current at 3.5 μA, the capillary voltage at 30 V, and the tube lens at 119 V. Sheath gas flow was set to 28 psi, and auxiliary gas flow was 5 arbitrary units. The normalized collision energy for ion fragmentation was 30%. The injection volume was 5−10 μL, and spectra were recorded in the positive-ion mode. Selected-ion monitoring (SIM) chromatographs were obtained at the selected *m*/*z* values.

Acknowledgment. We gratefully acknowledge financial support from the National Institutes of Health (CA70375) and an Eli Lilly & Company predoctoral fellowship (to Y.D.). We are indebted to Professor Hideo Hayashi for generously providing us with a culture of *Aspergillus japonicus* JV-23 and authentic specimens of asperparaline. Mass spectra (CSU) were obtained on instruments supported by the NIH Shared Instrumentation Grant GM49631.

Supporting Information Available: MS^n spectra of authentic paraherquamide A (**6**) and authentic paraherquamide B; MS^n spectra of two metabolites at 14.45 and 14.88 min from the extract from *A. japonicus* JV-23; MS and MS^2 spectra of authentic VM55599 (**4**) and authentic preparaherquamide (**5**). This material is available free of charge via the Internet at http://pubs.acs.org.

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NP800292N