Gibepyrone Biosynthesis in the Rice Pathogen Fusarium fujikuroi Is Facilitated by a Small Polyketide Synthase Gene Cluster*‡§

Received for publication, August 10, 2016, and in revised form, November 14, 2016. Published, JBC Papers in Press, November 17, 2016, DOI 10.1074/jbc.M116.753053

Slavica Janevskale, Birgit Arndt§, Eva-Maria Niehaus‡, Immo Burkhardt§, Sarah M. Rösler1, 2, Nelson L. Brock§, Hans-Ulrich Humpf§, Jeroen S. Dicksc#at§1, and Bettina Tudzynski¶1

From the ‡ Institut für Biologie und Biotechnologie der Pflanzen, Westfälische Wilhelms-Universität Münster, Schlossplatz 8, D-48143 Münster, the † Institut für Lebensmittelchemie, Westfälische Wilhelms-Universität Münster, Correnstrasse 45, D-48149 Münster, and the § Kekulé-Institut für Organische Chemie und Biochemie, Rheinische Friedrich Wilhelms-Universität Bonn, Gerhard-Domagk-Strasse 1, D-53121 Bonn, Germany

Edited by Gerald W. Hart

The 2H-pyran-2-one gibepyrones A and its oxidized derivatives gibepyrones B–F have been isolated from the rice pathogenic fungus Fusarium fujikuroi already more than 20 years ago. However, these products have not been linked to the respective biosynthetic gene clusters. Even though the biosynthesis has not yet been analyzed on a molecular level, feeding experiments with isotopically labeled precursors clearly supported a polyketide origin for the formal monoterpenoid gibepyrone A, whereas the terpenoid pathway could be excluded. Targeted gene deletion verified that the F. fujikuroi polyketide synthase PKS13, designated Gpy1, is responsible for gibepyrone A biosynthesis. Next to Gpy1, the ATP-binding cassette transporter Gpy2 is encoded by the gibepyrone gene cluster. Gpy2 was shown to have only a minor impact on the actual efflux of gibepyrone A out of the cell. Instead, we obtained evidence that Gpy2 is involved in gene regulation as it represses GPY1 gene expression. Thus, GPY1 was up-regulated and gibepyrone A production was enhanced both extra- and intracellularly in Δgpy mutants. Furthermore, expression of GPY genes is strictly repressed by members of the fungus-specific velvet complex, Vel1, Vel2, and Lae1, whereas Sge1, a major regulator of secondary metabolism in F. fujikuroi, affects gibepyrone biosynthesis in a positive manner. The gibepyrone A derivatives gibepyrones B and D were shown to be produced by cluster-independent P450 monoxygenases, probably to protect the fungus from the toxic product. In contrast, the formation of gibepyrones E and F from gibepyrone A is a spontaneous process and independent of enzymatic activity.

Fusarium fujikuroi is a member of the Fusarium (Gibberella) fujikuroi species complex that comprises some of the most detrimental fungal pathogens in agriculture (1, 2). It is the causative agent of the bakanane ("foolish seedling") disease of rice (3), which results in significant crop losses every year. The disease symptoms include the chlorotic hyperelongation of the internodes due to the fungus’ ability to produce and secrete gibberellic acids, a group of highly bioactive, growth-promoting plant hormones (4, 5). Indeed, their biosynthesis was the first to be elucidated on a molecular level among the secondary metabolites (SMs) produced by F. fujikuroi (6). Furthermore, the genes responsible for the biosynthesis of the pigments bikaverin and fusarubins (7, 8); the mycotoxins fusarins, fusaric acid, apicidin F, fumonisins, and beauvericin (9–14); and the terpenes (+)-eremophelene, (−)-α-acorenol, (−)-guaia-6,10(14)-diene, and (−)-koriaiol (15, 16) have been recently characterized. The sequencing of the genome of F. fujikuroi IMI58289 revealed the presence of altogether 48 SM key genes, encoding 18 polyketide synthases (PKSs), among those one type III PKS, 16 nonribosomal peptide synthetases (NRPSs), 12 terpen cyclases, and 2 dimethylallyltryptophan synthases (17). However, the majority of the corresponding products are still unknown.

Compounds with a 2H-pyran-2-one (α-pyrone) moiety (Fig. 1A) are highly abundant in animal, plant, and microbial systems. Microbial 2H-pyran-2-one metabolites exhibit a wide range of interesting properties, including antibiotic, antifungal, cytotoxic and phytotoxic effects. Some of them are of medical importance, displaying tumor- or HIV protease-inhibiting activities (18, 19). A diverse array of 2H-pyran-2-ones is produced by filamentous fungi, especially by species of the genera Alternaria, Aspergillus, Penicillium, Trichoderma, and Fusarium (18). Structurally simple metabolites from this group include triacetic acid lactone (Fig. 1B) isolated from Penicillium spp. (20, 21) and 6-pentyl-2H-pyran-2-one (Fig. 1C) produced by Trichoderma spp. (22, 23), the latter compound being toxic.

* This work was supported by the German Research Foundation (projects TU101/16-2 and HU730/9-3) and by the Collaborative Research Centre SFB 813 “Chemistry at Spin Centres.” The authors declare that they have no conflicts of interest with the contents of this article.

† This article contains supplemental Figs. S1–S20 and Tables S1–S3.

‡ To whom correspondence may be addressed. Tel.: 49-228-73-5797; E-mail: dickschat@uni-muenster.de.

§ To whom correspondence may be addressed. Tel.: 49-251-83-24801; Fax 49-251-83-21601; E-mail: tudzynski@uni-bonn.de.

© 2016 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
Gibepyrone Biosynthesis in *F. fujikuroi*

**FIGURE 1. Chemical structures of 2H-pyrane-2-ones.** Shown are structures of the parent compound 2H-pyran-2-one (A) and the fungal metabolites triacetic acid lactone (B); 6-pentyl-2H-pyran-2-one, its shorter and longer homologs, and derivatives with unsaturated side chain (C); gibepyrone A–F (D); fusalanipyron (E); nectriapyrione and pestaloxyprine (F).

to many fungal plant pathogens, such as *Botrytis cinerea* (24). Its shorter and longer homologs 6-propyl-2H-pyran-2-one and 6-heptyl-2H-pyran-2-one and the related unsaturated compounds (E)-6-(pent-1-en-1-yl)-2H-pyran-2-one and (E)-6-(hept-1-en-1-yl)-2H-pyran-2-one (Fig. 1C) are also known from *Trichoderma* spp. (25, 26). The 2H-pyran-2-ones isolated from species of the genus *Fusarium* include fusapyrone (27), acuminatopyrone (28), chlamydosporol (29), poaefusarin, and sporofusarin (30), compounds exhibiting mainly mycotoxic properties.

Metabolites from *F. fujikuroi* with a 2H-pyran-2-one structure, gibepyrone A and its formal oxidation products gibepyrones B–F (Fig. 1D), were first reported by Barrero et al. (31). Gibepyrone A was also recently detected in *F. fujikuroi* headspace extracts (32, 33). Gibepyrones A and B exhibit a moderate antimicrobial activity against Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and yeasts (*Saccharomyces cerevisiae* and *Candida albicans*) at a minimal inhibitory concentration of 100–200 μg/ml (31). Besides *F. fujikuroi*, gibepyrone A production was also reported for natural isolates of *Fusarium keratoplasticum* and *Fusarium petroliphilum*, two members of the *Fusarium solani* species complex (34). Gibepyrone D was found in cultures of endophytic isolates of *Fusarium oxysporum* and *Fusarium proliferatum* (35, 36), whereas gibepyrone F was detected in co-cultures of *Fusarium sp./Aspergillus clavatus* (37). Moreover, gibepyrone F was isolated from extracts of the South China Sea sponge *Jaspis stellifera* (38) which possibly originates also from a fungal symbiont, as described for other marine sponge-derived compounds (39, 40).

The (Z)-stereoisomer of gibepyrone A, fusalanipyrone (Fig. 1E), has been reported from *F. solani* and has antifungal and phytotoxic activities (41, 42). Based on its chemical structure, this C10 compound was first suggested to be of monoterprenoid origin, but subsequent feeding experiments with deuterated methionine provided evidence that fusalanipyrone could also represent a methylated polyketide product (41, 43). The phytotoxic methoxy derivatives nectriapyrione and pestaloxyprione (Fig. 1F) were first isolated from *Gyrostroma missouriensis* and *Pestalotiopsis oenotherae*, respectively (44–47). Due to incorporation of radioactive labeling from [2-14C]mevalonic acid, nectriapyrione was suggested to be a monoterprenoid, but this result is doubtful, because the “incorporation” of labeling into the widespread plasticizer *bis*-(2-ethylhexyl)phthalate, mistakenly identified as a natural product, was also reported in the same study (44). In an independent analysis, the incorporation of labeling from [1,2-13C2]acetate and [methyl-13C]methionine was described, providing evidence for a polyketide origin of this compound (48).

Although gibepyrone A–F are well known SMs of *F. fujikuroi* IMI58289 (31), the genes and enzymes involved in their biosynthesis have not been identified so far. Here we present feeding experiments with isotopically labeled precursors that validated the polyketide nature of the gibepyrones as well as the identification and molecular characterization of their small PKS gene cluster. Gibepyrone A biosynthesis is regulated by members of the fungus-specific *velvet* complex (Vel1, Vel2, and Lae1) and by Sge1, a major regulator of secondary metabolism in *F. fujikuroi*. Finally, the mechanisms for the formation of the oxidized gibepyrones B–F were elucidated, using a combination of chemical synthesis and gene knock-out experiments.

**Results**

*Chemical Synthesis of Gibepyrone A and Its Production by F. fujikuroi—*Analysis of liquid cultures of *F. fujikuroi* IMI58289 by HPLC-high resolution mass spectrometry (HRMS) showed the presence of an SM with the exact mass of gibepyrone A. The same compound was also detected via GS-MS in headspace extracts from *F. fujikuroi* agar plate cultures as reported previously (32, 33). For unambiguous identification of gibepyrone A, the compound was synthesized via a known procedure by treating tigloyl chloride (1) with triethyl amine in dichloromethane (31). Its isomer 6-(but-3-en-2-yl)-3-methyl-2H-pyran-2-one (2) was also obtained in this reaction (Fig. 2). The synthetic gibepyrone A was identical to the detected SM in *F. fujikuroi* with respect to the HPLC-MS and GC-MS retention times (9 and 28 min, respectively) and mass spectra (supplemental Fig. S1).
Feeding Experiments on the Biosynthesis of Gibepyrone A—To exclude a terpenoid origin for gibepyrone A, a feeding experiment with \([\text{methyl}-^2\text{H}_3]\)mevalonolactone was performed. No incorporation of labeling into gibepyrone A was detected (supplemental Fig. S2A), which contradicts a terpene biosynthetic pathway. Alternative feeding experiments were carried out to test for a polyketide origin of gibepyrone A. The formation of the carbon backbone from four acetate-derived C2 building blocks and two S-adenosyl-l-methionine (SAM)-derived C1 units seemed to be most likely, but the potential for formation from two propionate-derived C3 building blocks and two acetate-derived C2 building blocks, although atypical for fungi, was addressed as well. Additionally, the possibility of a biosynthetic pathway via an isoleucine-derived \((E)-2\)-methylbut-2-enoyl-CoA starter unit was evaluated. Feeding of \([\text{methyl}-^{13}\text{C}]\)methionine resulted in the incorporation of up to two C1 units with high rates (50%), as indicated by a 2-mass unit shift of the molecular ion of gibepyrone A, whereas no incorporation of labeling from \([^{2}\text{H}_10]\)propionate was observed (supplemental Fig. S2A). Up to four deuterium atoms were found in gibepyrone A in the feeding experiment with \([^{2}\text{H}_10]\)isoleucine but only with a low incorporation rate (1.3%; supplemental Fig. S2A). This finding is in agreement with a degradation of \([^{2}\text{H}_10]\)isoleucine to \([^{2}\text{H}_3]\)acetetyl-CoA and \([^{2}\text{H}_3]\)propiony-CoA along the isoleucine degradation pathway, followed by incorporation of \([^{2}\text{H}_3]\)acetetyl-CoA into gibepyrone A (supplemental Fig. S2B), but not with a hypothetical isoleucine-derived \((E)-2\)-methylbut-2-enoyl-CoA starter unit that would require incorporation of seven deuterium atoms. Indeed, feeding of \([2,^{13}\text{C}]\)acetate resulted in an incorporation of up to four units with a low incorporation rate (supplemental Fig. S2A). Taken together, these results confirmed that gibepyrone A is a tetraketide composed of four acetate building blocks and two SAM-derived methyl groups.

PKS13 Is Responsible for Gibepyrone A Biosynthesis—After demonstrating the polyketide origin of gibepyrone A by the feeding experiments described above, we confirmed the PKS origin also on a genetic and enzymatic level. Therefore, the \(F.\ fujikuroi\ Delta ppt1\) deletion mutant as well as the complemented strain \(PPT1^{+}\) were analyzed for gibepyrone A production. The phosphopantetheinyl transferase encoded by \(PPT1\) is essential for the activation of the major proportion of fungal PKSs (and NRPSs) through post-translational attachment of the phosphopantetheinyl prosthetic group (49). Analysis of culture fluids by HPLC-MS/MS revealed the production of gibepyrone A by the wild-type (WT) strain and \(PPT1^{+}\) but not by the \(Delta ppt1\) mutant (Fig. 3A), confirming the polyketide origin.

In the framework of an ongoing project to identify novel SMs produced by \(F.\ fujikuroi\), deletion strains for all 18 PKS-encoding genes have been generated. Targeted replacement of one of these genes (PKS13, \(F\text{FUJ}\_12020\)) with the hygromycin B resistance cassette (supplemental Fig. S3) resulted in total loss of gibepyrone A biosynthesis in three independent deletion mutants. In loco complementation of one deletion mutant with the full-length PKS13 gene resulted in full restoration of gibepyrone A formation (Fig. 3B and supplemental Fig. S4). Therefore, PKS13 is the key enzyme of gibepyrone biosynthesis, designated Gpy1.

The Gibepyrone A Biosynthetic Gene Cluster Includes a Transporter-encoding Gene—In addition to the key enzyme-encoding gene, SM gene clusters often comprise genes encoding pathway-specific transcription factors (TFs); several tailoring enzymes, such as methyltransferases, oxidoreductases, or P450 monooxygenases; and efflux transporters for the respective SM (50).

Upstream and downstream of \(GPY1\), several genes were identified that might belong to a putative gibepyrone biosynthetic gene cluster (Fig. 4A). As summarized in Table 1, a meth-
A methyltransferase-encoding gene (FFUJ_12019) was found upstream of GPY1, whereas two genes encoding an ATP-binding cassette (ABC) transporter (FFUJ_12021) and a fungus-specific Zn(II)$_2$Cys$_6$-type TF (FFUJ_12023) were detected downstream. The gene FFUJ_12022 does not harbor any domains or motifs that might confer a specific function. Moreover, a genomic alignment of different Fusarium spp. (F. proliferatum, Fusarium mangiferae, Fusarium verticillioides, and F. oxysporum) underlined that GPY1 and further adjacent genes are conserved in these species, whereas the gene of unknown function, FFUJ_12022, is not always clustered with GPY1 (supplemental Fig. S5). To assess the involvement of the other indicated genes in gibepyrone A biosynthesis, single deletion mutants were generated (supplemental Figs. S6–S8). Deletion of the methyltransferase- and TF-encoding genes FFUJ_12019 and FFUJ_12023, respectively, did not alter gibepyrone A yields, whereas

FIGURE 3. Gibepyrone A production depends on the presence of Ppt1 (A) and the SM biosynthetic key enzyme PKS13, designated Gpy1 (B). A, HPLC-MS/MS analysis of culture fluids of the WT, Δppt1, and PPT1C. The strains were grown in the presence of 6 mM glutamine for 7 days. The gibepyrone A peak was verified through comparison with the synthesized chemical standard. B, the WT, the Δgpy1 deletion mutant, and the complemented strain GPY1C were grown and analyzed as described above.

FIGURE 4. The gibepyrone biosynthetic gene cluster comprises a PKS-encoding gene and an ABC transporter-encoding gene, GPY1 and GPY2, respectively. A, schematic representation of GPY1 (FFUJ_12020), GPY2 (FFUJ_12021), and neighboring, non-cluster genes (gray). Arrows, direction of transcription; white bars, introns. B, HPLC-MS/MS analysis of culture fluids of the WT and indicated deletion mutants. The strains were grown in triplicate in the presence of 6 mM glutamine for 7 days with product formation of the WT being set to 100%. C, HPLC-MS/MS and Northern blot expressional analysis of the WT and the overexpression mutants of GPY1 and FFUJ_12023 (TF). The strains were grown and analyzed as described above for gibepyrone A quantification, whereas cell harvest was carried out after 3 days for expressional analysis. In the latter case, GPY1 and FFUJ_12023 were used as probes. Error bars, S.D.
deletion of the ABC transporter-encoding gene FFUJ_12021 resulted in a significantly enhanced level of extracellular gibepyrone A (Fig. 4B). Thus, adjacent to GPY1, a second gene was identified with impact on gibepyrone A production, designated GPY2.

Next, overexpression mutants were generated for GPY1 and the TF-encoding gene FFUJ_12023 (supplemental Fig. S9, A and B). Whereas the OE::GPY1 overexpression mutant showed significantly higher gibepyrone A production, overexpression of FFUJ_12023 did not have any impact (Fig. 4C), indicating that the encoded putative TF does not represent a pathway-specific TF for gibepyrone biosynthesis.

**The ABC Transporter Gpy2 Represses PKS Gene Expression**

In addition to the enhanced extracellular gibepyrone A levels upon deletion of the transporter-encoding gene GPY2 (Fig. 4B), significantly elevated intracellular gibepyrone A levels were also observed in the mutant compared with the WT (Fig. 5A). These increased product levels were in accordance with the up-regulated expression of the PKS-encoding gene GPY1 in Δgpy2 mutants (Fig. 5B). GPY1 gene expression and gibepyrone A formation were elevated throughout the whole cultivation period of the Δgpy2 mutant, as shown for days 1–3 in Fig. 5, C and D. The low producing WT phenotype could be restored through in loco complementation with the full-length GPY2 gene (supplemental Figs. S10 and S11).

To study the potential regulatory role of GPY2 in more detail, this ABC transporter-encoding gene was overexpressed in the WT and GPY1 overexpression background (supplemental Fig. S9C). Interestingly, the overexpression of GPY2 led to repression of GPY1 gene expression and gibepyrone A product formation in the WT (native GPY1 promoter) but not in the OE::GPY1 background (OE::GPY1/OE::GPY2) (Fig. 5, A and B). These data indicate that GPY1 regulation by Gpy2 occurs on the level of gene expression.

Whereas GPY2 was equally expressed in the WT and Δgpy1 background (supplemental Fig. S12A), the addition of 1 or 10 μg/ml synthetic gibepyrone A induced GPY2 expression both in the WT and Δgpy1 (supplemental Fig. S12B). Interestingly, the PKS-encoding gene GPY1 itself was also up-regulated upon the addition of gibepyrone A to the WT. In contrast, expression of the adjacent gene FFUJ_12019 was not affected (supplemental Fig. S12B). Taken together, these data indicate that gibepyrone A is able to induce its own production by an elevated GPY1 gene expression in a positive feedback loop (Fig. 5D and supplemental Fig. S12B). At the same time, expression of the ABC transporter-encoding gene GPY2 is also induced (supplemental Fig. S12B), causing a down-regulation of GPY1 expression in a negative feedback loop.

**Gibepyrone A Is Toxic to F. fujikuroi**—The highly sophisticated regulatory mechanism described above led to the assumption that gibepyrone A might have a biological activity not only against bacterial and yeast strains (31) but also against the producing fungus itself. A plate assay using synthetic gibepyrone A against the WT strain and the mutants Δgpy1 and Δgpy2 was performed. Indeed, the growth of all three strains was equally inhibited, as indicated by the reduced colony diameters to ~50 and 15% in the presence of 100 and 200 μg/ml gibepyrone A, respectively (supplemental Fig. S13).

**C-Methylation Mechanism in the Biosynthesis of Gibepyrone A**—As underlined by feeding experiments with isotopically labeled precursors, gibepyrone A consists of a polyketide backbone that is modified by two methylation steps (supplemental Fig. S2A). Because deletion of the methyltransferase-encoding gene FFUJ_12019 did not have an impact on gibepyrone A production (Fig. 4B), the C-methyltransferase domain within the highly reducing PKS Gpy1 might solely be responsible for attaching both methyl groups to the nascent polyketide backbone. To verify this hypothesis, an amino acid substitution was introduced into the catalytic center of the C-methyltransferase domain of Gpy1. Glycine at position 1443 was exchanged for valine (G1443V) because corresponding mutations in the *F. verticillioides* fumonisin synthase (Fum1) and *Phoma* sp. C2932 squalestatin tetraketide synthase (SQTKS) were described as sterically inhibiting SAM substrate binding (51, 52). An amino acid alignment of the C-methyltransferase domains of Gpy1, Fum1, and SQTKS underlined the conserved position of this active site glycine (supplemental Fig. S14A). The Δgpy1 strain was complemented with this mutated GPY1 variant, yielding independent transformants of GPY1*G1443V*. Although expression of GPY1*G1443V* was similar to GPY1*Δ*, re-introduction of this point-mutated gene copy could not restore gibepyrone A production (supplemental Fig. S14, B and C), suggesting that the PKS alone is able to condense the methylated polyketide gibepyrone A. Single-methylated or completely

### Table 1

| Gene name (accession number) | Length | Length | Domains and motifs | Predicted function |
|-----------------------------|--------|--------|--------------------|-------------------|
| FFUJ_12019                  | bp     | 1798   | IPR029063 S-adenosyl-l-methionine-dependent methyltransferase | Methyltransferase; probably does not belong to the cluster |
| GPY1 (FFUJ_12020)          |        | 8438   | IPR020841 PKS, β-ketoacyl synthase domain | PKS |
|                            |        |        | IPR020801 PKS, acyltransferase domain | |
|                            |        |        | IPR020807 PKS, dehydratase domain | |
|                            |        |        | IPR020863 S-adenosyl-l-methionine-dependent methyltransferase | |
|                            |        |        | IPR020843 PKS, enoylreductase domain | |
| GPY2 (FFUJ_12021)          | 5007   | 1432   | IPR031227 ABC transporter type 1, transmembrane domain | ABC transporter |
| FFUJ_12022                  | 1132   | 244    | IPR003593 AAA + ATPase domain | Unknown function; probably does not belong to the cluster |
| FFUJ_12023                  | 2362   | 725    | IPR001138 Zn (2)-C6 fungus-type DNA-binding domain | Zn(II)Cys6-type transcription factor; probably does not belong to the cluster |

*a* Amino acids.
non-methylated gibepyrone A derivatives could not be identified in \textit{GPY1}\textsuperscript{G1443V} mutants by HPLC-HRMS analysis (supplementary Fig. S15).

**Production of Gibepyrone A Derivatives**—The initial report on gibepyrone A by Barrero \textit{et al}. (31) also mentioned the presence of a series of oxygenated derivatives, gibepyrones B–F, in \textit{F. fujikuroi} (Fig. 1D). All of these compounds were assumed to have a common biosynthetic origin. In fact, gibepyrones B–D are characterized through oxidations at the methyl group C8, whereas gibepyrone E carries a 6,7-epoxide function in the side chain. Furthermore, gibepyrone F was hypothesized to be generated through oxidative cleavage of the C6=C7 double bond (31). No genes encoding oxidizing proteins were identified in \textit{GPY1} (Table 1), suggesting that independent enzymes encoded outside of the gene cluster might facilitate these reactions.

The oxidation of a single carbon atom (\textit{e.g.} the conversion of gibepyrone A (methyl group) to gibepyrone D (acid group) via gibepyrone B (hydroxyl group) and gibepyrone C (aldehyde group)) is a typical reaction sequence catalyzed by P450 monooxygenases. To test this hypothesis, WT cultures were compared with those of the \textit{Δcpr} mutant. CPR encodes the major NADPH-cytochrome P450 reductase in \textit{F. fujikuroi} that is associated with P450 monooxygenases, being essential for the electron transfer from NADPH onto the prosthetic heme group of the P450s (53). Thus, in the \textit{Δcpr} mutant, the majority of all P450s is inhibited, which has been first demonstrated for the P450s encoded by the gibberellic acid gene cluster (53). To further examine the involvement of CPR in the biosynthesis of the oxidized gibepyrones, a chemical synthesis of gibepyrones B, C, and E was performed (Fig. 2). Gibepyrone E was synthesized via epoxidation of gibepyrone A using \textit{meta}-chlorobenzoic acid, whereas 2 was used as starting material for the synthesis of 6-(2-hydroxybut-3-en-2-yl)-3-methyl-2\textit{H}-pyran-2-one (3) using modified Riley conditions (54). Compound 3 was converted into gibepyrone B via acidic rearrangement of the tertiary allylic hydroxy function using methanesulfonic acid or to gibepyrone C via oxidative rearrangement using pyridinium chlorochromate (Fig. 2).

The synthetic compounds were used as standards to search for oxidized gibepyrones in the \textit{F. fujikuroi} WT and mutant strains. Gibepyrones B and D were identified by HPLC-HRMS
Gibepyrone Biosynthesis in F. fujikuroi

FIGURE 6. Production of gibepyrone A derivatives, gibepyrones B–E. HPLC-HRMS analysis of culture fluids of the WT and the Δcpr mutant. The strains were grown in the presence of 6 mM glutamine for 7 days, and the supernatant was analyzed without further processing. Shown are extracted ion chromatograms for the calculated masses of gibepyrones A–E for the two strains as well as for the synthesized chemical standards. The gibepyrone E peak cannot be found in the cultures. However, in the presence of water, both the cultures and the standard show an identical double peak (Gibepyrone E derivatives).

analysis in WT cultures and in increased amounts in the OE::GPY1 strain, but not in the Δgpy1 mutant, linking the production of these metabolites to the gibepyrone PKS (Fig. 6 and supplemental Fig. S16). In contrast, gibepyrone C was detected in neither WT cultures nor the OE::GPY1 mutant (supplemental Fig. S16). Comparison of WT and Δcpr cultures revealed that gibepyrones B and D were missing in the mutant, whereas an accumulation of gibepyrone A was observed (Fig. 6). These data are in full agreement with the conversion of gibepyrone A to gibepyrones B and D by one or multiple CPR-dependent P450 monooxygenases encoded outside of the gibepyrone biosynthetic gene cluster.

The HPLC-HRMS analysis of an aged synthetic gibepyrone E standard showed a slow compound degradation in aqueous solution. Furthermore, gibepyrone E could not be detected in culture fluids, whereas two of its degradation products were present in cultures of the WT and the Δcpr mutant (Fig. 6). Presumably, these two compounds represent diastereomeric diols that are formed from gibepyrone E by hydrolysis of its epoxide function in the presence of water (supplemental Fig. S17A). Because the two compounds were also found in the Δcpr mutant, the conversion of gibepyrone A into gibepyrone E must be independent of the P450 monooxygenase(s) involved in the formation of the other oxidized gibepyrones. Instead, gibepyrone E formation and its hydrolysis were also observed in an aqueous solution of synthetic gibepyrone A (supplemental Fig. S17B), indicating its autoxidation in the presence of molecular oxygen.

Gibepyrone F was only detected in headspace extracts of the F. fujikuroi WT via GC-MS and not in liquid cultures via HPLC-HRMS. Similar to gibepyrone E, this compound was also formed spontaneously from synthetic gibepyrone A (supplemental Fig. S17C) and was also detectable in headspace extracts from the Δcpr mutant strain (supplemental Fig. S18), indicating its formation independent of enzymatic activity.

Gibepyrone A Production by F. solani—The reported structure of fusalanipyrone from F. solani DSM 62416 (41) represents the (Z)-stereoisomer of gibepyrone A. However, genome sequencing of the strain F. solani 77-13-4 (55) revealed that this organism encodes a PKS with the same domain organization and 84% amino acid identity compared with Gpy1 (17) (accession number NECHADRAFT_123282; supplemental Fig. S5). Although a homolog of the ABC transporter-encoding gene GPY2 cannot be found adjacent to the F. solani GPY1 homolog, the methyltransferase-encoding border gene (a homolog of FFUJ_12019) is conserved in F. solani, strongly suggesting that NECHADRAFT_123282 is the true Gpy1 homolog (supplemental Fig. S5). This bioinformatic analysis underlined that F. solani may also produce gibepyrone A, whereas the production of the stereoisomer fusalanipyrone by Gpy1 would be difficult to understand. Comparison of the NMR data reported for fusalanipyrone (41) with the NMR data of synthetic gibepyrone A (see “Experimental Procedures”) showed the identity of these compounds. Their identity was additionally verified by comparison of liquid culture extracts from F. solani DSM 62416 and F. fujikuroi IMI58289 with the synthetic gibepyrone A standard by GC-MS (supplemental Fig. S19). Therefore, the structure of fusalanipyrone must be revised to that of gibepyrone A.

Regulation of Gibepyrone A Biosynthesis—F. fujikuroi SM biosynthesis was shown to be differentially regulated by global regulators, such as members of the fungus-specific velvet complex (Ve1, Vel2, and Lae1) (56) as well as Sge1, a recently char-
characterized master regulator of several SM gene clusters in this fungus (57). To study their impact on gibepyrone A production, the yields were compared between the WT and the regulatory mutants. Gibepyrone A levels were elevated in deletion mutants of the velvet complex, $\Delta vel1$, $\Delta vel2$, and $\Delta lae1$, but down-regulated in $\Delta sge1$ (Fig. 7A). Analysis of $GPY1$ and $GPY2$ expression by quantitative RT-PCR (qRT-PCR) verified these results (Fig. 7B). Besides $GPY1$ and $GPY2$, no further adjacent genes were up-regulated in the $\Delta vel1$ mutant (Fig. 7C), underlining that these two genes represent a co-regulated small biosynthetic gene cluster.

**Discussion**

Before this study, the biosynthetic origin of gibepyrone A and its oxidized derivatives was unknown. Therefore, this study focused on the biosynthetic pathway of gibepyrone A–F, the elucidation of their biosynthetic gene cluster, and the regulation of these genes in *F. fujikuroi*.

**Biosynthesis of the Polyketide Gibepyrone A**—In this work, we provide evidence for gibepyrone A biosynthesis by *F. fujikuroi* PKS13 (FFUJ_12020), designated Gpy1. Feeding experiments with isotopically labeled precursors and analysis of the $\Delta ppt1$ mutant ruled out a terpenoid origin for this compound. Although a polyketide biosynthetic pathway was suggested for the structurally related compounds fusalanipyrene and necryptapyrene (43, 48), unambiguous proof on a molecular level was lacking for this group of metabolites. By deleting $GPY1$, the responsible key gene of gibepyrone biosynthesis was identified, encoding a highly reducing PKS. A proposed biosynthetic pathway to gibepyrone A by Gpy1 uses an acetyl-CoA starter unit, three malonyl-CoA extender units, and two SAM-dependent methylations to establish the gibepyrone A carbon backbone, followed by product release upon intramolecular cyclization (Fig. 8). Introduction of a loss-of-function mutation into the $C$-methyltransferase domain of Gpy1 and the non-effective deletion of a methyltransferase encoding gene adjacent to $GPY1$ (FFUJ_12019) verified that both methylation steps are catalyzed by the PKS itself. It is unclear whether PKS biosynthesis in the $Gpy^{C144S}$ mutant is blocked or results in an unidentified shunt product that may be unstable or quickly metabolized. The latter was suggested for *Phoma* sp. C2932 SQTKS (52), whereas for other systems, including the lovastatin PKS, the formation of defined non-methylated shunt products was shown by *in vitro* experiments in the absence of SAM (58).
The data are in agreement with those reported recently for fusarubin and bikaverin pigment biosynthesis.
Gibepyrone Biosynthesis in F. fujikuroi

these cases, it was highly anticipated that the SMs are toxic to the producing fungi. Such SM-specific transporters conferring self-protection are major facilitator superfamily transporters as encoded by the *Aspergillus fumigatus* glio toxin cluster (Glia) (62), the *Fusarium graminearum* trichothecene cluster (Tri12) (63), and the *Fusarium* spp. fusaric acid cluster (Feb11) (11, 64). Deletion of *gla* and *Feb11* resulted in a reduced intra- and extracellular accumulation of glio toxin and fusaric acid, respectively, most likely due to the detoxification of these compounds via derivatization, whereas cluster gene expression was not affected (11, 62, 64). Similarly, deletion of *Tri12* led to diminished trichothecene levels via a yet unknown mechanism (63).

Far fewer SM gene clusters harbor ABC transporter-encoding genes. Examples are the *Fusarium verticillioides* fumonisins cluster (Fun19) (65), the *Leptosphaeria maculans* sirodesmin cluster (SirA) (66), and the *F. fujikuroi* beauvericin cluster (14). Upon disruption of *Fum19*, little impact was detected on extracellular fumonisins contents, and the authors assumed that other transporters are able to compensate for *Fum19* (65). To the best of our knowledge, only two reports on enhanced extracellular SM contents upon deletion of a transporter-encoding gene have been described before, a phenotype that would be comparable with the one observed for *F. fujikuroi* *Dpy2*. Thus, the *L. maculans* sirodesmin NRPS-encoding gene *sirp* was up-regulated in *ΔSir* ABC transporter mutants. Unfortunately, the authors did not quantify intracellular sirodesmin levels, but they did find enhanced extracellular sirodesmin in *ΔSir* mutants (66). Additionally, an up-regulation of NRPS gene expression and SM product formation was observed upon deletion of *F. fujikuroi* *BEA3*, encoding the beauvericin ABC transporter that we analyzed recently (14).

It is tempting to hypothesize that this regulatory role of SM transporters is specific to ABC transporters; however, further research is needed to prove this. A transporter that has been implicated in having a sensing function in addition to its transport function is the *S. cerevisiae* general amino permease Gap1 (67). The authors were able to introduce C-terminal truncations that only affected the sensing function and not the transport efficiency of Gap1, establishing that the signaling pathway is mediated via protein kinase A (67). A potential signal transduction pathway that might be responsible for *Gpy1* repression via the ABC transporter Gpy2 as well as its biological function remain to be elucidated.

*Global Regulatory Mechanisms Controlling Gibepyrone A Biosynthesis*—Because no cluster-specific TF is encoded by the small gene cluster, we evaluated the impact of global regulators on gibepyrone A biosynthesis. Thus, members of the fungus-specific *velvet* complex, Vel1, Vel2, and Lae1, represent repressors of gibepyrone A biosynthesis, whereas Sge1 acts as positive regulator.

In addition to the pigment bikaverin, gibepyrone A represents the second SM that is influenced in a negative manner by members of the *velvet* complex in *F. fujikuroi*. In the case of bikaverin, however, only Vel1 and Vel2 act as repressors, whereas Lae1 does not (56). The mode of action of the *velvet* complex is still not fully understood. Whereas homologs of the *velvet* domain proteins Vel1 and Vel2 have been described as having DNA-binding properties (68), the putative histone methyltransferase Lae1 has been implicated to act on the highest hierarchical level of chromatin remodeling (69). Furthermore, the complex may vary in its conformation when regulating different aspects of differentiation and secondary metabolism (70). Notably, this is the first example in which all three members of the *velvet* complex seem to work together to repress gibepyrone A biosynthesis in *F. fujikuroi*.

Sge1 was shown to be a major positive regulator of secondary metabolism in *F. fujikuroi* (57), which also held true for gibepyrone A biosynthesis. Also for Sge1, the mode of action is not fully resolved. Although the yeast Sge1-homologs in *C. albicans* and *Histoplasma capsulatum* have been described to bind the promoters of their target genes (71, 72), no common DNA-binding motif has been identified in filamentous fungi so far. In fact, Sge1 in *F. fujikuroi* was suggested to act on a high hierarchical level, also regulating genes encoding histone-modifying enzymes on a transcriptional level (57).

In conclusion, this work provides a comprehensive overview of the biosynthesis and regulation of gibepyrone A and its derivatives in *F. fujikuroi*, applying feeding experiments, chemical synthesis of gibepyrone standards, gene knock-out, and overexpression strategies as well as regulatory mutants. As summarized in Fig. 9, the PKS Gpy1 is responsible for the production of gibepyrone A, whereas one or several cluster-independent P450 monoxygenases account for the production of gibepyrone B and D. Furthermore, gibepyrone E and F are gained in the absence of enzymatic activity. We found evidence that the ABC transporter Gpy2 represses expression of the PKS-encoding gene *GPY1* via a yet unknown mechanism. Finally, gibepyrone biosynthesis underlies global regulatory mechanisms, as verified for Sge1 and the *velvet* complex that affect gibepyrone A biosynthesis in a positive and negative manner, respectively.

**Experimental Procedures**

**Fungal Strains, Media, and Growth Conditions**

The WT strain *F. fujikuroi* IMI58289 (Commonwealth Mycological Institute, Kew, UK) was used as a parental strain for the generation of deletion and overexpression mutants. Concerning the analysis of gene expression as well as product formation, the following strains were applied additionally: *Δppt1, PPT1* (49), *Δcpr* (53), *Δvel1, Δvel2, Δlae1* (56), *Δsge1* (57), and *F. solani* DSM 62416 (41).

For all experiments in liquid culture, the fungal strains were preincubated for 3 days in 300-ml Erlenmeyer flasks containing 100 ml of Darken medium (73) on a rotary shaker at 180 rpm and 28 °C in the dark. 500 μl of this preculture were transferred into 100 ml of synthetic ICI medium (Imperial Chemical Industries Ltd., London, UK) (74) supplemented with 6 mM glutamine as the sole nitrogen source. This main culture was incubated under the indicated conditions for an additional 1–7 days. Feeding experiments in liquid culture were performed after the addition of 0, 1, or 10 μg/ml synthetic gibepyrone A to a 2-day-old 30-ml ICI culture. After 2 h of induction, the mycelium was harvested and used for the isolation of RNA. For protoplasting *F. fujikuroi*, 500 μl of the preculture were transferred into 100 ml of ICI medium with 10 g/liter fructose instead of glucose as well as 0.5 g/liter (NH₄)₂SO₄ as a nitrogen source and
shaken for no longer than 16 h. The general maintenance of the fungal strains was carried out on solidified complete medium (CM) (75), whereas, before DNA isolation, the strains were incubated on CM covered with cellophane for 2–4 days at 28 °C in the dark. Hyphal growth of fungal strains was assessed on solidified CM supplemented with 0–200 μg/ml synthetic gibepyrone A. Plates were incubated for 4 days at 28 °C in the dark. The solvent (ethanol absolute) was added as a control, not resulting in an inhibition of fungal growth (data not shown).

Plasmid Constructions

The cloning of deletion, complementation, and overexpression constructs was accomplished with the use of yeast recombinational cloning (76, 77). For targeted gene deletion, ~0.6–1-kb large upstream (5′) and downstream (3′) regions of the gene of interest were amplified with the primer pairs 5F/5R and 3F/3R, respectively (supplemental Table S1). The hygromycin resistance cassette hphR, consisting of the hygromycin B phosphotransferase gene hph and the strong trpC promoter from Aspergillus nidulans, was amplified with hph_F/hph_R from the template pCSN44 (78). S. cerevisiae FY834 (79) was transformed with the obtained fragments as well as with the EcoRI/XhoI-restricted vector pRS426, yielding the complementation vector pGPY1C (supplemental Fig. S4A). For introducing the G1443V mutation into the Gpy1 C-methyltransferase domain (51, 52), a modified in loco complementation vector was cloned, pGPY1G1443V. Using overlapping primers that harbor the nucleotide exchange, GPY1 was amplified in four overlapping fragments, two of which were based on the novel primer combinations 12020_G1443V_F and 12020_G1443V_R (supplemental Table S2). Furthermore, for in loco complementation of the ABC transporter-encoding gene GPY2 (FFUJ_12021), the following gene-specific fragments were amplified: 12021_5F/12021_c_R1, 12021_c_F2/12021_c_R2, and 12021_3F/12021_3R (supplemental Table S2). S. cerevisiae FY834 was transformed with the obtained fragments, Tgluc and natR, as well as with the EcoRI/XhoI-restricted vector pRS426,
Gibepyrone Biosynthesis in F. fujikuroi

yielding the complementation vector pGPY2C (supplemental Fig. S10A). The overexpression of GPY1 was achieved through fusion to the strong oliC promoter from A. nidulans. Thus, GPY1 was again amplified in three overlapping fragments using a proofreading polymerase, in this case with 12020_OE_F/12020_c_R1, 12020_c_F2/12020_c_R2, and 12020_c_F3/12020_OE_R (supplemental Table S2). The latter primer combination included ~300 bp of the GPY1 terminator sequence. S. cerevisiae was transformed with these fragments as well as with the NcoI/SacII-restricted plasmid pNDN-OGG (77), resulting in pOE::GPY1 (supplemental Fig. S9A). Similarly, for overexpressing FFUJ_12023 via PoliC, the gene of interest was amplified with the primer pair 12023_OE_F/12023_OE_R (supplemental Table S2). The NcoI/NotI-restricted plasmid pNAH-OGG (77) served as the plasmid backbone, yielding pOE::12023 (supplemental Fig. S9B), and for overexpression of GPY2 in the WT and OE::GPY1 overexpression backgrounds, the first 1.4 kb of GPY2 were amplified with the primer pair 12021_OE_F/12021_OE_R (supplemental Table S2). The fragment was fused to the NcoI/SacII-restricted plasmid pNAH-GGT harboring the strong ghaA promoter from F. fujikuroi (11), resulting in pOE::GPY2 (supplemental Fig. S9C). The correct assembly of all complementation and overexpression vectors was verified by sequencing with primers listed in supplemental Table S2.

Fungal Transformations and Analysis of Transformants

Protoplast transformation of F. fujikuroi was carried out as described previously (81). About 10⁶ protoplasts were transformed with the amplified replacement cassettes to generate deletion mutants. Amplification was achieved with the primer combination SF/3R (supplemental Table S1), whereas the circular deletion vectors served as DNA templates for in loco complementation of Δgpy1 T5 with pGPY1C (supplemental Fig. S4A) or pGPY1G1443V, 30 µg of the vectors were linearized with Apal before transformation. Similarly, for in loco complementation of Δgpy2 T9 with pGPY2C (supplemental Fig. S10A), 30 µg of the vector was linearized with Asel. Furthermore, 15 µg of the overexpression vectors pOE::GPY1 (supplemental Fig. S9A), pOE::12023 (supplemental Fig. S9B), and pOE::GPY2 (supplemental Fig. S9C) were introduced in a circular manner. Selection of transformants was achieved under the use of 100 µg/ml hygromycin B (Calbiochem) or 100 µg/ml nourseothricin (Werner-Bioagents, Jena, Germany), depending on the resistance marker.

Homologous integration of the resistance cassettes and absence of the WT genes were verified by Southern blotting analyses as well as diagnostic PCR. In the latter case, the following primer combinations were applied: 5diag/trpC_T (5’), 3diag/trpC_P2 (3’), and WT_F/WT_R (untransformed nuclei). Diagnostic PCRs for three independent transformants of Δ12019, Δgpy1, Δgpy2, and Δ12023, respectively, can be found in supplemental Figs. S3 and S6–S8. Concerning the in loco complementation, transformants unable to grow on hygromycin B (deletion phenotype) but able to grow on nourseothricin (complementation phenotype) were additionally analyzed for the homologous integration of the complementation constructs: 5diag/c_diag (5’), 3diag/nat1_R1 (3’), and 5diag/trpC_T (untransformed nuclei). Diagnostic PCRs for three independent transformants of GPY1G1443V showed the same signals (data not shown). Diagnostic PCRs for two independent transformants of GPY2C are shown in supplemental Fig. S10C. Finally, the presence of the overexpression vectors was verified using PoliC_Seq_F2/12020_OE_diag for two independent transformants of OE::GPY1 (supplemental Fig. S9A), PoliC_Seq_F2/12023_WT_R for three independent transformants of OE::12023 (supplemental Fig. S9B), and GS_Prom_M/OE_12021_diag for three independent transformants of OE::GPY2 and one transformant of OE::GYPY1/OE::GYPY2 (supplemental Fig. S9C).

Standard Molecular Methods

For DNA isolation from F. fujikuroi, lyophilized mycelium was ground in the presence of liquid nitrogen and extracted following the protocol of Cenis (82). Generated deletion mutants were analyzed via Southern blotting (83) regarding ectopically integrated replacement cassettes. Thus, their genomic DNA was digested with an appropriate restriction enzyme (Fermentas, St. Leon-Rot, Germany), whereupon it was separated in 1% (w/v) agarose gel and transferred onto a nylon membrane (Nytran™ SPC, Whatman, Sanford, ME) by downward alkali blotting (84). Hybridization was performed with 32P-labeled probes that were generated with the random oligomer-primer method (85). Amplified 5’ or 3’ sequences served as templates for probe generation (supplemental Table S1). Southern blotting analyses of Δgpy1 and Δgpy2 can be found in supplemental Figs. S3 and S7, respectively. Plasmid DNA from S. cerevisiae as well as Escherichia coli Top10 F’ (Invitrogen, Darmstadt, Germany) was isolated with the NucleoSpin® plasmid kit (Machery-Nagel, Düren, Germany). Concerning DNA amplification by PCR, BioTherm™ DNA polymerase (GeneCraft, Lüdinghausen, Germany) was chosen for standard applications, especially diagnostic PCR, and handled according to the manufacturer’s instructions. For the purpose of amplifying long fragments, TaKaRa LA Taq® DNA polymerase (Takara Bio, Saint-Germain-en-Laye, France) was used, whereas Phusion® high-fidelity DNA polymerase (Finnzymes, Vantaa, Finland) was applied in case proofreading was essential.

RNA isolation was carried out with the use of TRI Reagent™ (Sigma-Aldrich, Steinheim, Germany). For expression analyses by Northern blotting (86), 20 µg of total RNA were separated in a 1% (w/v) denaturing agarose gel (85). Upon transfer of the separated RNA onto a nylon membrane (Nytran™ SPC, Whatman), it was hybridized with 32P-labeled probes, as indicated above (85). ~1-kb fragments of GPY1 and FFUJ_12023 served as templates for probe generation and were amplified with primer pairs 12020_WT_F/12020_WT_R and 12023_WT_F/12023_WT_R, respectively (supplemental Table S1). Before expression analyses by qRT-PCR, 1 µg of total RNA was treated with DNase I (Fermentas) and transcribed into cDNA using oligo(dT) primers and SuperScript® II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Subsequently, qRT-PCR was performed with iQ SYBR Green Supermix (Bio-Rad, München, Germany) and cDNA as template using an iCycler iQ real-time PCR system (Bio-Rad). The annealing temperature was 60°C, and the
Quantitative Analysis of Gibepyrone A and Its Derivatives by HPLC-MS/MS and HPLC-HRMS

For quantifying gibepyrone A in the supernatant as sensitively as possible, a QTRAP 6500 MS system (SCIEX, Darmstadt, Germany) was used. Seven-day-old cultures were filtered using 0.45-μm membrane filters (BGB Analytik, Schlossböckelheim, Germany). Metabolite extraction from the mycelium was performed with a modified method described by Niehaus et al. (12). Briefly, 0.1 g of harvested, washed, and lyophilized mycelium was mixed thoroughly with 1.5 ml of ethyl acetate/MeOH/dichloromethane (3:2:1, v/v/v) for 2 h. 0.75 ml of the extract was evaporated to dryness and taken up in 0.75 ml of 15% (v/v) MeOH.

For relative quantification of gibepyrone A via HPLC-MS, methylparaben (MePa) was used as an internal standard, and chromatographic separation was carried out on a 150 × 2.1-mm inner diameter, 5-μm, Eclipse XDB-C18, column (Agilent Technologies) with MeOH + 1% formic acid (FA) as solvent A and H2O + 1% FA as solvent B. The column was tempered to 40 °C. After isocratic running for 3 min at 15% A, the gradient rose to 100% A in 10 min. With the concentration of solvent A, the flow rate also rose from 400 to 450 μl. After holding these conditions for 2 min, the column was equilibrated for 3 min. A divert valve was used to discard polar substances from the medium in the first 3 min of the run. For MS/MS analysis with electrospray ionization (ESI), the following parameters were used. The curtain gas (nitrogen) was set to 30 p.s.i., the nebulizer gas (zero grade air) was set to 35 p.s.i., and the drying gas (zero-grade air) was set to 40 p.s.i. The ion spray voltage was set to +5500 V in the positive and to −4500 V in the negative mode. Nitrogen was also used as the collision gas in medium mode. Gibepyrone A was analyzed with a declustering potential of 56 V, an entrance potential of 10 V, and a cell exit potential of 56 V, whereasthe CE and the CXP varied for each transition. Quantifier MePa analysis, negative polarity was used. The declustering potential was set to −50 V, whereas the CE and the CXP varied for each transition. Quantifier MePa (M − H)−: m/z 151.0/92.0 (CE −23 V, CXP −11 V), qualifier MePa: m/z 151.0/121.0 (CE −28 V, CXP −11 V).

For the identification of gibepyrone A derivatives, HPLC-HRMS measurements were carried out on an HPLC system (Accela LC with Accela pump 60057-60010 and Accela autosampler 60057-60020, Thermo Scientific, Dreieich, Germany) coupled to a Fourier transform mass spectrometer with a heated ESI source (LTQ Orbitrap XL, Thermo Scientific). The HPLC column was a 150 × 2.00-mm inner diameter, 5-μm, Gemini C18 with a 4 × 2-mm Gemini NX C18 guard column (Phenomenex, Aschaffenburg, Germany). The MS parameters were set as described elsewhere (17) with a change in capillary temperature from 275 to 300 °C. 1% FA in MeOH (v/v, solvent A) and 1% FA in H2O (v/v, solvent B) were used as solvents with a flow rate of 250 μl/min. The gradient started with 10% A, holding this condition for 5 min. Until 15 min, the gradient rose to 100% A. Then the column was equilibrated again with 10% A for 10 min. The injection volume was set to 10 μl. For HPLC-HRMS measurements, standard substances were dissolved in ethyl acetate and diluted with MeOH (1:10, v/v). A 10 μg/ml solution (in MeOH/H2O 1:10, v/v) was applied for the measurements. For analyzing the non-enzymatic conversion of gibepyrone A into gibepyrone E, 20 μg of gibepyrone A standard was incubated in 1 ml of H2O at 28 °C and 180 rpm for 7 days.

Calculated m/z ratios of the different gibepyrone derivatives (Fig. 1D) are as follows: m/z gibepyrone A (M + H)+ 165.0910, m/z gibepyrone B (M + H)+ 181.0859, m/z gibepyrone C (M + H)+ 178.630, m/z gibepyrone D (M + H)+ 195.0652, m/z gibepyrone E (M + H)+ 181.0859, m/z gibepyrone F (M + H)+ 153.0546. To differentiate between gibepyrone B and E, data-dependent fragmentation (most intense ion from mass list) of gibepyrone derivatives was applied. Therefore, higher energy collisional dissociation (HCD) with 50% normalized collision energy was used.

Gibepyrone B (m/z 181.0859, (M + H)+, retention time (RT) 11.7 min) @HCD 50%: 109.0648 ((M + H)+-C2H4O2, 100%), 107.0855 ((M + H)+-C3H6O2, 61%), 137.0599 ((M + H)+-C4H8O, 41%), 141.0862 ((M + H)+, 29%), 135.0806 ((M + H)+-CH2O2, 25%), 105.0689 ((M + H)+-C2H3O, 17%), 117.0699 ((M + H)+-CH3O, 10%), 115.0543 ((M + H)+-CH2O, 6%), 163.0756 ((M + H)+-C2H3O, 10%), 111.0805 ((M + H)+-C3H4O2, 3%), 107.0491 ((M + H)+-C4H6O2, 1%), 125.0961 ((M + H)+-C2O2, 2%), 151.0756 ((M + H)+-CH2O, 2%).

Gibepyrone E 1 (presumably diol 1) (m/z 181.0859, (M + H)+, RT 8.4 min) @HCD 50%: 109.0284 ((M + H)+-C2H4O2, 100%), 109.0755 ((M + H)+-C3H6O2, 70%), 111.0804 ((M + H)+-C4H8O2, 30%), 138.0677 ((M + H)+-C2H3O2, 12%), 109.0647 ((M + H)+-CH2O2, 6%), 121.0649 ((M + H)+-C3H4O2, 1%), 181.0863 ((M + H)+-CHO, 4%), 152.0834 ((M + H)+-CHO, 4%), 135.0806 ((M + H)+-CH2O2, 3%), 111.0440 ((M + H)+-C2H2O, 2%).

Gibepyrone E 2 (presumably diol 2) (m/z 181.0859, (M + H)+, RT 9.1 min) @HCD 50%: 109.0284 ((M + H)+-C2H4O2, 100%), 139.0755 ((M + H)+-C3H6O2, 72%), 111.0804 ((M + H)+-C4H8O2, 30%), 138.0677 ((M + H)+-C2H3O2, 12%), 121.0649 ((M + H)+-C2H3O2, 1%), 181.0863 ((M + H)+, 4%), 152.0834 ((M + H)+-CHO4, 1%), 109.0647 ((M + H)+-CHO3, 4%), 135.0806 ((M + H)+-CH2O2, 3%), 111.0440 ((M + H)+-C2H2O, 2%).
Gibepyrone Biosynthesis in F. fujikuroi

((M + H)⁺ - C₆H₅O, 100%), 139.0750 ((M + H)⁺ - C₅H₃O, 67%), 119.0801 ((M + H)⁺ - C₄H₂O, 28%), 109.0647 ((M + H)⁺ - C₃H₂O, 16%), 138.0672 ((M + H)⁺ - C₂H₂O, 11%), 152.0829 ((M + H)⁺ - CHO, 9%), 135.0806 ((M + H)⁺ - CH₂O, 8%), 181.0863 ((M + H)⁺, 8%), 121.0649 ((M + H)⁺ - C₂H₅O, 6%).

General Synthetic and Analytical Methods—Chemicals were purchased from Acros Organics (Geel, Belgium) or Sigma-Aldrich and used without purification. Commercially available isotopically labeled compounds were purchased from Euriso-Top (Saarbrücken, Germany) or Sigma-Aldrich. Thin layer chromatography was performed with 0.2-mm precoated plastic sheets Polygram® Sil G/UV254 (Machery-Nagel). Normal phase column chromatography was carried out using Merck silica gel 60 (70–200 mesh). Reversed phase column chromatography was carried out using Merck Lichroprep RP-18 silica gel (40–63 μm).

1H NMR and 13C NMR spectra were recorded on Bruker AV I (300 MHz), AV I (400 MHz), and AV III HD Prodigy (500 MHz) spectrometers and were referenced against CDCl₃ (δ = 7.26 ppm), C₆D₆ (δ = 7.16 ppm), and CDCl₂ (δ = 5.32 ppm) for 1H NMR and CDCl₃ (δ = 77.01 ppm), C₆D₆ (δ = 128.06 ppm), CDCl₂ (δ = 53.84 ppm) for 13C NMR.

Analytical data (UV–vis; 70 eV; m/z (%) = 100, 136 (64), 121 (77), 109 (62), 93 (22), 91 (15), 82 (7), 77 (11), 55 (14), 53 (40).

High resolution electron ionization mass spectrometry (HREIMS) calculated for C₁₀H₁₂O₂⁺: m/z 164.0823; found: 164.0823.

1H NMR (400 MHz, CD₂Cl₂): δ = 7.12 (dq, J₃,H₇ = 6.9 Hz, J₄,H₆ = 1.2 Hz, 1H, CH), 6.54 (dq, J₅,H₆ = 6.9 Hz, 1H, CH), 6.06 (dq, J₆,H₅ = 6.9 Hz, 1H, CH), 2.04 (s, 3H, CH₃), 1.85 (d, J₇,H₈ = 1.1 Hz, 3H, CH₃), 1.83 (d, J₈,H₉ = 6.9 Hz, 3H, CH₃) ppm.

13C NMR (100 MHz, CD₂Cl₂): δ = 163.4 (C₆), 160.1 (C₅), 140.2 (CH), 128.0 (CH), 127.5 (C₄), 123.4 (C₃), 100.9 (CH), 167.7 (CH), 14.3 (CH₃), 12.2 (CH₃) ppm.

IR (ATR): ν = 3040 (w), 2978 (w), 2920 (w), 2856 (w), 1697 (s), 1645 (s), 1449 (m), 1381 (w), 1210 (w), 1130 (m), 1111 (m), 1050 (s), 1016 (m), 992 (m), 958 (m), 848 (w), 801 (s), 740 (s), 696 (m), 557 (w), 529 (m), 471 (w) cm⁻¹.

UV–vis (MeOH): λmax (lg ε) = 331 (1.602), 240 (1.642) nm. Electro spray ionization (ESI)-MS (70 eV): m/z (%) = 164 (100), 136 (64), 121 (77), 109 (62), 93 (22), 91 (15), 82 (7), 77 (11), 55 (14), 53 (40).

High resolution electron ionization mass spectrometry (HREIMS) calculated for C₁₀H₁₂O₂⁺: m/z 274.16; found: 274.16.

Pyrone—(Cyclohexene/EnOAc) 2.1: Rf = 0.65. GC (HP-5): I = 1240. 1H NMR (400 MHz, CDCl₃): δ = 6.25 (dq, J₃,H₇ = 6.6 Hz, J₄,H₆ = 1.2 Hz, 1H, CH), 5.65 (dd, J₅,H₆ = 17.3 Hz, J₆,H₅ = 10.0 Hz, 1H, CH), 4.91 (dd, J₇,H₈ = 1.2 Hz, J₈,H₉ = 17.4 Hz, J₉,H₇ = 1.4 Hz, 1H, CH), 4.90 (dd, J₉,H₈ = 1.3 Hz, J₈,H₇ = 10.2, J₇,H₆ = 1.4 Hz, 1H, CH), 2.85 (dq, J₈,H₉ = 7.0 Hz, J₇,H₈ = 7.0 Hz, 1H, CH), 1.84 (d, J₆,H₅ = 1.2 Hz, 3H, CH₃), 1.02 (d, J₅,H₄ = 7.0 Hz, 3H, CH₃) ppm.

13C NMR (100 MHz, CDCl₃): δ = 165.1 (C₆), 162.9 (C₅), 138.7 (CH), 138.5 (CH), 123.0 (C₄), 115.8 (CH₂), 100.9 (CH), 41.8 (CH₂), 17.6 (CH₂), 16.7 (CH₃) ppm.

IR (ATR): ν = 3084 (w), 2973 (w), 2937 (w), 2924 (w), 2886 (w), 1706 (versus), 1642 (s), 1580 (s), 1453 (w), 1434 (w), 1417 (w), 1380 (m), 1360 (m), 1228 (w), 1216 (w), 1108 (s), 1075 (w), 1042 (m), 995 (m), 920 (m), 817 (m), 732 (m), 691 (w), 673 (w), 543 (w), 531 (w).

UV–vis (dichloromethane): λmax (lg ε) = 297 (2.657), 202 (3.737) nm. EI-MS (70 eV): m/z (%) = 164 (45), 136 (18), 121 (16), 109 (100), 93 (5), 91 (5), 81 (12), 53 (51), 39 (5).

HREIMS calculated for C₁₀H₁₂O₂⁺: m/z 164.0823; found: 164.0826.

Gibepyrone A—A solution of triethyl amine (8.96 g, 88.6 mmol, 1.05 eq) in dichloromethane (10 ml) was added dropwise over 15 min to a solution of tigloyl chloride (10.0 g, 84.3 mmol, 1.0 eq) in dichloromethane (80 ml) under argon atmosphere at room temperature. The solution changed colors immediately from colorless to deep red and was stirred for 18 h at room temperature. The solvent was removed in vacuo, and the solid residue was taken up in EtO. The insoluble material was removed by filtration, and the filtrate was concentrated in vacuo, giving a red oil, which was subjected to column chromatography on reversed phase silica gel (H₂O/MeOH, 3:1). The product–yielding fractions were collected, concentrated to an aqueous suspension, and extracted with dichloromethane (3×).

The combined organic layers were dried over MgSO₄, and the solvent was evaporated, yielding gibepyrone A (1.26 g, 7.67 mmol, 18%) as a colorless highly viscous oil and its isomer 5-(but-3-en-2-yl)-3-methyl-2H-pyran-2-one (2, 1.18 g, 7.22 mmol, 17%) as a yellow oil.

TLC (pentane/ EtO₅, 5:1): Rf = 0.29. GC (HP-5): I = 1523. 1H NMR (400 MHz, CD₂Cl₂): δ = 7.12 (dq, J₃,H₇ = 6.9 Hz, J₄,H₆ = 1.2 Hz, 1H, CH), 6.54 (dq, J₅,H₆ = 6.9 Hz, 1H, CH), 6.06 (dq, J₆,H₅ = 6.9 Hz, 1H, CH), 2.04 (s, 3H, CH₃), 1.85 (d, J₇,H₈ = 1.1 Hz, 3H, CH₃), 1.83 (d, J₈,H₉ = 6.9 Hz, 3H, CH₃) ppm.

13C NMR (75 MHz, CDCl₃): δ = 163.4 (C₆), 160.1 (C₅), 140.2 (CH), 128.0 (CH), 127.5 (C₄), 123.4 (C₃), 100.9 (CH), 167.7 (CH), 14.3 (CH₃), 12.2 (CH₃) ppm.

IR (ATR): ν = 3458 (w, br), 3099 (w), 3052 (w),
Gibepyrone Biosynthesis in F. fujikuroi

6-(2-Hydroxybut-3-en-2-yl)-3-methyl-2H-pyran-2-one (3)—t-BuOOH (70% in H2O, 1.96 g, 15.3 mmol, 2.50 eq) was added to a suspension of SeO2 (372 mg, 3.36 mmol, 0.55 eq) in dichloromethane. The reaction was cooled to 0°C and diluted with H2O and argon at room temperature, and the resulting solution was heated under reflux for 6 days. The reaction was cooled to 0°C and diluted with Et2O and dichloromethane. The layers were separated, and the H2O layer was extracted with dichloromethane (3 × 5 ml). The combined organic layers were dried with MgSO4 and the solvent was removed in vacuo. The crude product was subjected to column chromatography (Flosril, petroleum ether/EtOAc, 5:1 to 2:1), yielding gibepyrone 5 (380 mg, 2.11 mmol, 35%).

TLC (cyclohexane/EtOAc, 2:1) Rf = 0.19. GC (HP-5): I = 1471. 1H NMR (400 MHz, CDCl3): δ = 6.30 (dq, J1,2H = 6.7 Hz, J2,3H = 1.2 Hz, 1H, CH), 5.99 (dd, J1,2H = 10.6 Hz, J2,3H = 17.2 Hz, 1H, CH), 5.92 (d, J2,3H = 6.7 Hz, 1H, CH), 5.34 (dd, J2,3H = 17.2 Hz, J2,4H = 1.1 Hz, 1H, CH), 4.98 (dd, J2,3H = 10.6 Hz, J2,4H = 1.1 Hz, 1H, CH), 1.79 (d, J2,3H = 1.2 Hz, 3H, CH3). 13C NMR (100 MHz, CDCl3): δ = 165.7 (Cq), 162.9 (Cq), 141.5 (CH), 139.3 (Cq), 123.2 (Cq), 114.0 (CH), 100.0 (Cq), 73.3 (Cq), 26.7 (CH2), 16.6 (CH3). IR (ATR): ν = 3520 (m, br), 3049 (w), 2981 (w), 2927 (w), 1692 (s), 1638 (s), 1577 (s), 1452 (w), 1410 (w), 1382 (w), 1363 (m), 1338 (m), 1199 (m), 1107 (s), 1046 (s), 994 (s), 928 (s), 827 (s), 761 (s), 538 (m) cm−1. UV-visible (dichloromethane): λmax (lg e) = 298 (3.756), 201 (3.606) nm. EI-MS (70 eV): m/z (%) = 178 (90), 163 (7), 150 (100), 149 (19), 135 (44), 133 (11), 122 (50), 121 (20), 109 (58), 108 (21), 107 (55), 96 (63), 91 (20), 82 (25), 81 (16), 79 (38), 77 (27), 69 (16), 68 (42), 53 (91), 52 (14), 51 (16), 43 (19), 41 (14), 39 (23). HREIMS calculated for C10H12O3: 178.0625, found: 178.0621.

GC-MS Analysis and Feeding Experiments

For analysis of emitted volatile compounds, the fungal strains were inoculated on solid CM using a small piece of preculture grown on the same medium and grown for 7 days at 28°C in the dark, before collection of the volatiles on charcoal filter traps by use of a closed loop stripping apparatus, followed by analysis of headspace extracts by GC-MS, as described previously (32).

For the comparative analysis of gibepyrone A production by F. fujikuroi IMI58289 and F. solani DSM 62416, both strains were cultivated in liquid ICI medium (6 mM glutamine) in the medium used for the isolation of fusalanipyrone (41) for 7 days at 28°C and 180 rpm. After filtration through a cotton cloth and centrifugation, 45 ml of the respective culture supernatant was extracted with EtOAc (2 × 5 ml). The combined organic layers were dried over MgSO4 and subjected directly to GC-MS analysis.

Gibepyrone B—A mixture of tetrahydrofurand (3 ml) and H2O (0.7 ml) was degassed by an argon stream for 60 min. Hydroxy- pyrone 3 (110 mg, 0.67 mmol, 1.00 eq) and methanesulfonic acid (118 mg, 1.22 mmol, 2 eq) were added subsequently under argon atmosphere at room temperature, and the resulting mixture was stirred at room temperature for 25 days in the dark. The reaction mixture was diluted with H2O and extracted with EtOAc (3 × 3). The combined organic layers were dried with MgSO4, the solvent was removed in vacuo, and the crude product was subjected to column chromatography on silica gel (cyclohexane/EtOAc, 1:2), yielding gibepyrone B (35 mg, 0.19 mmol, 29%) as a pale yellow viscous oil.

TLC (cyclohexane/EtOAc, 2:1) Rf = 0.11. GC (HP-5): I = 1817. 1H NMR (400 MHz, CDCl3): δ = 7.04 (dq, J1,2H = 6.9 Hz, J2,3H = 1.2 Hz, 1H, CH), 6.46 (dq, J1,2H = 6.5 Hz, J2,3H = 1.1 Hz, 1H, CH), 6.05 (d, J1,2H = 6.9 Hz, 1H, CH), 4.27 (d, J1,2H = 6.5 Hz, 2H, CH2), 1.96 (br s, 3H, CH3), 1.78 (br s, 3H, CH3). 13C NMR (125 MHz, CD2Cl2): 163.3 (Cq), 159.2 (Cq), 140.0 (CH), 131.7 (CH), 127.9 (Cq), 124.5 (Cq), 102.1 (CH2), 59.8 (CH2), 16.7 (CH2). IR (ATR): ν = 3472 (br m), 3044 (w), 2863 (w), 1669 (vs), 1642 (vs), 1615 (s), 1552 (vs), 1454 (m), 1382 (m), 1226 (m), 1203 (w), 1069 (s), 1069 (s), 908 (w), 812 (s), 726 (s), 594 (8m), 556 (m), 532 (m), 489 (m) cm−1. UV-visible (dichloromethane): λmax (lg e) = 324 (3.900), 235 (3.911), 213 (3.981) nm. EI-MS (70 eV): m/z (%) = 180 (20), 151 (100), 123 (5), 109 (19), 107 (5), 105 (6), 95 (8), 91 (9), 81 (6), 79 (6), 77 (6), 53 (24), 43 (10), 41 (6), 39 (6). HREIMS calculated for C10H12O3: 180.0781, found: 180.0781.
Gibepyrone Biosynthesis in F. fujikuroi

The mutant strain F. fujikuroi Δvel1 (56) was used for all feeding experiments and was precultured on CM solid medium. For the feeding experiments, [methyl-13C]methionine (2 mM), [1H10]isoleucine (2 mM), [2-13C]acetate (2 mM), [2H5]pro- 
pionate (3 mM), or [6,6,6-2H3]malvalonolactone (2 mM) (88) was added to CM solid medium after autoclaving. The agar plates were inoculated with about 0.25 cm² of the preculture, incubated for 4 days at 28°C, and used for closed loop stripping apparatus/GC-MS headspace analysis. Incorporation rates were calculated from integrated peak areas using ion trace chromatograms of the respective molecular ion for each isotopomer.

Author Contributions—S. I., B. A., I. B., H.-U. H., J. S. D., and B. T. conceived and designed the experiments; S. J., B. A., E.-M. N., I. B., S. M. R., and N. L. B. performed the experiments; S. J., B. A., E.-M. N., I. B., S. M. R., N. L. B., H.-U. H., J. S. D., and B. T. analyzed the data; H.-U. H., J. S. D., and B. T. contributed materials; and S. J., B. A., J. S. D., and B. T. wrote the paper.

References
1. Nirenberg, H. I., and O’Donnell, K. (1998) New Fusarium species and combinations within the Gibberella fujikuroi species complex. Mycologia 90, 434–458
2. Leslie, J. F., and Summerell, B. A. (2006) Fusarium laboratory workshops: a recent history. Mycotoxin Res. 22, 73–74
3. Sun, S., and Snyder, W. C. (1981) The bakanae disease of the rice plant. In Fusarium: Diseases, Biology and Taxonomy (Nelson, P. E., Tousson, T. A., and Cook, R. J., eds) pp. 104–113, Pennsylvania State University Press, University Park, PA.
4. Tuzdylski, B. (2005) Gibberellin biosynthesis in fungi: genes, enzymes, evolution, and impact on biotechnology. Appl. Microbiol. Biotechnol. 66, 597–611
5. Bömeke, C., and Tuzdylski, B. (2009) Diversity, regulation, and evolution of the gibberellin biosynthetic pathway in fungi compared to plants and bacteria. Phytochemistry 70, 1876–1893
6. Tuzdylski, B., and Höltel, K. (1998) Gibberellin biosynthetic pathway in Gibberella fujikuroi: evidence for a gene cluster. Fungal Genet. Biol. 25, 157–170
7. Wiemann, P., Willmann, A., Straeten, M., Kleigrewe, K., Beyer, M., Humphf, H. U., and Tuzdylski, B. (2009) Biosynthesis of the red pigment bikaverin in Fusarium fujikuroi: genes, their function and regulation. Mol. Microbiol. 72, 931–946
8. Studt, L., Wiemann, P., Kleigrewe, K., Humphf, H. U., and Tuzdylski, B. (2012) Biosynthesis of fusarins accounts for pigmentation of Fusarium fujikuroi perithecia. Appl. Environ. Microbiol. 78, 4468–4480
9. Niehaus, E. M., Kleigrewe, K., Wiemann, P., Studt, L., Sieber, C. M. K., Connolly, L. R., Freitag, M., Göldner, U., Tuzdylski, B., and Humphf, H. U. (2013) Genetic manipulation of the Fusarium fujikuroi fusarin gene cluster yields insight into the complex regulation and fusarin biosynthetic pathway. Chem. Biol. 20, 1055–1066
10. Niehaus, E. M., von Bargen, K. W., Espino, J. J., Pfannmüller, A., Humphf, H. U., and Tuzdylski, B. (2014) Characterization of the fusaric acid gene cluster in Fusarium fujikuroi. Appl. Microbiol. Biotechnol. 98, 1749–1762
11. Studt, L., Janewska, S., Niehaus, E. M., Burkhardt, I., Arndt, B., Sieber, C. M., Humphf, H. U., Dickschat, J. S., and Tuzdylski, B. (2016) Two separate key enzymes and two pathway-specific transcription factors are involved in fusaric acid biosynthesis in Fusarium fujikuroi. Environ. Microbiol. 18, 936–956
12. Niehaus, E. M., Janewska, S., von Bargen, K. W., Sieber, C. M. K., Harrer, H., Humphf, H. U., and Tuzdylski, B. (2014) Apicidin F: characterization and genetic manipulation of a new secondary metabolite gene cluster in the rice pathogen Fusarium fujikuroi. PLoS One 9, e103336
33. Dicketsch, J. S. (2014) Capturing volatile natural products by mass spectrometry. Nat. Prod. Rep. 31, 838–861
34. Short, D. P. G., O’Donnell, K., Thrane, U., Nielsen, K. F., Zhang, N., Juba, J. H., and Geiser, D. M. (2013) Phylogenetic relationships among members of the Fusarium solani species complex in human infections and the descriptions of F. keratoplasticum sp. nov. and F. pertolutophilum stat. nov. Fungal Genet. Biol. 53, 59–70
35. Wang, Q. X., Li, S. F., Zhao, F., Dai, H. Q., Bao, L., Ding, R., Gao, H., Zhang, L. X., Wen, H. A., and Liu, H. W. (2011) Chemical constituents from endophytic fungus Fusarium oxysporum. Fitoterapia 82, 777–781
36. Liu, D., Li, X., Li, C., and Wang, B. (2013) Sesterterpenes and 2H-pyran-2-ones (=α-pyrones) from the mangrove-derived endophytic fungus Fusarium proliferatum MA-84. Helv. Chim. Acta 96, 437–444
37. Bertrand, S., Azzollini, A., Schumpp, O., Bohni, N., Schrenzel, J., Monod, M., Gindro, K., and Wolﬀender, J. (2014) Multi-well fungal co-culture for de novo metabolite-induction in time-series studies based on untargeted metabolomics. Mol. Biosyst. 10, 2289–2298
38. Tang, S., Xu, R., Lin, W., and Duan, H. (2012) Jaspiferin A and B: two new phytotoxins produced by the phytopathogenic Fusarium oxysporum sp. nov. J. Nat. Prod. 75, 154–164
39. Abrell, L. M., Cheng, X., and Crews, P. (1994) New nectriapyrones by salt water culture of a fungus separated from an Indo-Pacific sponge. Tetrahedron Lett. 35, 6911–6914
40. Hentschel, U., Piel, J., Degnan, S. M., and Taylor, M. W. (2012) Genomic insights into the marine sponge microbiome. Nat. Rev. Microbiol. 10, 641–654
41. Abraham, W., and Arfmann, H. (1988) Fusalanipyrene, a monoterpenoid metabolite from Fusarium solani. Phytochemistry 27, 3310–3311
42. Shimizu, B., Saito, F., Miyagawa, H., Watanabe, K., Ueno, T., Sakata, K., and Ogawa, K. (2005) Phytotoxic components produced by pathogenic Fusarium against morning glory. Z. Naturforsch. C 60, 862–866
43. Abraham, W., Knoch, I., and Witte, L. (1990) Biosynthesis of the terpenoid polyketide fusalanipyrene. Phytochemistry 29, 2877–2878
44. Nair, M. S. R., and Carey, S. T. (1975) Metabolites of phycocyanomycetes II: nectriapyrene, an antibiotic monoterpenoid. Tetrahedron Lett. 16, 1655–1658
45. Venkatasingh, P., and van Dyke, C. G. (1991) Phytotoxins produced by Pestalotia obovata, a pathogen of evening primrose. Phytochemistry 30, 1471–1474
46. Lee, J. C., Yang, X., Schwartz, M., Strobel, G., and Clardy, J. (1995) The relationship between an endangered North American tree and an endophytic fungus. Chem. Biol. 2, 721–727
47. Türkkan, M., Andolfi, A., Zonno, M. C., Erper, I., Perrone, C., Cimmino, A., Vurro, M., and Evidente, A. (2011) Phytotoxins produced by Pestalotiopsis guepinii, the causal agent of hazelnut twig blight. Phytopathol. Mediterr. 50, 154–158
48. Avent, A. G., Hanson, J. R., and Truneh, A. (1992) The biosynthesis of nectriapyrene and vermopyrone. Phytochemistry 31, 3447–3449
49. Wiemann, P., Brown, D. W., Kleigrew, K., Bok, J. W., Keller, N. P., Humpf, H. U., and Tudzynski, B. (2010) FvE1 and FvA1, components of a velvet-like complex in Fusarium fujikuroi, affect differentiation, secondary metabolism and virulence. Mol. Microbiol. 77, 972–994
50. Michielse, C. B., Studt, L., Janovsky, S., Sieber, C. M. K., Arndt, B., Espino, J. J., Humpf, H. U., Güldener, U., and Tudzynski, B. (2015) The global regulator FgSg1 is required for expression of secondary metabolite gene clusters but not for pathogenicity in Fusarium fujikuroi. Environ. Microbiol. 17, 2690–2708
51. Yu, F., Zhu, X., and Du, L. (2005) Developing a genetic system for functional manipulations of Gibberella fujikuroi: Contribution of supernumerary chromosomes to gene expansion. PLoS Gen. 5, e1000618
52. Wiemann, P., Brown, D. W., Kleigrew, K., Bok, J. W., Keller, N. P., Humpf, H. U., and Tudzynski, B. (2010) FvE1 and FvA1, components of a velvet-like complex in Fusarium fujikuroi, affect differentiation, secondary metabolism and virulence. Mol. Microbiol. 77, 972–994
53. Proctor, R. H., Brown, D. W., Plattner, R. D., and Desjardins, A. E. (2003) Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in Gibberella moniliformis. Fungal Genet. Biol. 38, 237–249
54. Gardiner, D. M., Jarvis, R. S., and Howlett, B. J. (2005) The ABC transporter gene in the sirodesmin biosynthetic gene cluster of Leptosphaeria maculans is not essential for sirodesmin production but facilitates self-protection. Fungal Genet. Biol. 42, 257–263
55. Donatou, M. C. V., Holsbeeks, I., Lagatie, O., Van Zeebroeck, G., Crauwels, M., Winderickx, J., and Thevelein, J. M. (2003) The Gap1 general amino acid permease acts as an amino acid sensor for activation of protein kinase A targets in the yeast. Saccharomyces cerevisiae. Mol. Microbiol. 50, 911–929
56. Ahmed, Y. L., Gerke, J., Park, H. S., Bayram, O., Neumann, P., Ni, M., Dickmans, A., Kim, S. C., Yu, J. H., Braus, G. H., and Ficner, R. (2013) The velvet family of fungal regulators contains a DNA-binding domain structurally similar to NF-κB. PLoS Biol. 11, e1001750
57. Bok, J. W., Noordermeer, D., Kale, S. P., and Keller, N. P. (2006) Secondary metabolic gene cluster silencing in. Aspergillus nidulans. Mol. Microbiol. 61, 1636–1645
**Gibepyrone Biosynthesis in F. fujikuroi**

70. Bayram, O., and Braus, G. H. (2012) Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. *FEMS Microbiol. Rev.* **36**, 1–24

71. Nguyen, V. Q., and Sil, A. (2008) Temperature-induced switch to the pathogenic yeast form of *Histoplasma capsulatum* requires Ryp1, a conserved transcriptional regulator. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 4880–4885

72. Lohse, M. B., Zordan, R. E., Cain, C. W., and Johnson, A. D. (2010) Distinct class of DNA-binding domains is exemplified by a master regulator of phenotypic switching in *Candida albicans*. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 14105–14110

73. Darken, M. A., Jensen, A. L., and Shu, P. (1959) Production of gibberellic acid by fermentation. *Appl. Microbiol.* **7**, 301–303

74. Geissman, T. A., Verbiscar, A. J., Phinney, B. O., and Cragg, G. (1966) Studies on the biosynthesis of gibberellins from (−)-kaurenoic acid in cultures of *Gibberella fujikuroi*. *Phytochemistry* **5**, 933–947

75. Pontecorvo, G., Roper, J. A., Hemmons, L. M., Macdonald, K. D., and Button, A. W. J. (1953) The genetics of *Aspergillus nidulans*. *Adv. Genet.* **5**, 141–238

76. Colot, H. V., Park, G., Turner, G. E., Ringelberg, C., Crew, C. M., Litvinkova, L., Weiss, R. L., Borkovich, K. A., and Dunlap, J. C. (2006) A high-throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 10352–10357

77. Schumacher, J. (2012) Tools for *Botrytis cinerea*: new expression vectors make the gray mold fungus more accessible to cell biology approaches. *Fungal Genet. Biol.* **49**, 483–497

78. Staben, C., Jensen, B., Singer, M., Pollock, J., Schechtmann, M., Kinsey, I., and Selker, E. (1989) Use of a bacterial hygromycin B resistance gene as a dominant selectable marker in *Neurospora crassa* transformation. *Fungal Genet. Newslett.* **36**, 79–81

79. Winston, F., Dollard, C., and Ricupero-Hovasse, S. L. (1995) Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. *Yeast* **11**, 53–55

80. Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H., and Hieter, P. (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110**, 119–122

81. Tuzdyski, B., Homann, V., Feng, B., and Marzluf, G. A. (1999) Isolation, characterization and disruption of the areA nitrogen regulatory gene of *Gibberella fujikuroi*. *Mol. Gen. Genet.* **261**, 106–114

82. Cenis, J. L. (1992) Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Res.* **20**, 2380

83. Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**, 503–517

84. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) *Current Protocols in Molecular Biology*, Wiley, New York

85. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

86. Church, G. M., and Gilbert, W. (1984) Genomic sequencing. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1991–1995

87. Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45

88. Dickschat, J. S., Citron, C. A., Brock, N. L., Riclea, R., and Kuhz, H. (2011) Synthesis of deuterated mevalonolactone isotopomers. *Eur. J. Org. Chem.* **2011**, 3339–3346