A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition GDCh Chemie

www.angewandte.org

Accepted Article

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To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201803329
Angew. Chem. 10.1002/ange.201803329

Link to VoR: http://dx.doi.org/10.1002/anie.201803329
http://dx.doi.org/10.1002/ange.201803329

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10.1002/anie.201803329

Identification of an Asexual Reproduction Inducer of Phytopathogenic and Toxigenic *Fusarium*

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10.1002/anie.201803329

Abstract

Asexual and sexual reproductions are the most important biological events in the lifecycle of phytopathogenic and toxigenic *Fusarium* and are responsible for disease epidemics. However, the signaling molecules which induce the asexual reproduction of *Fusarium* are unknown. Here we describe the structure elucidation including the absolute stereochemistry of Fusarium asexual reproduction inducer (FARI), a new sesquiterpene derivative, which was determined by spectroscopic analysis, total synthesis, and conidium–inducing assays of synthetic isomers. We have also uncovered the universality of FARI among *Fusarium* species. Moreover, the results of a mechanism of action study suggested that the Gpmk1 and LaeA signaling pathways are required for the conidial formation induced by FARI; conversely, the Mgv1 of mitogen-activated protein kinase is not involved in conidial formation. Furthermore, FARI exhibited conidium-inducing activity at an extremely low dose and high stereo-selection, which may suggest the presence of a stereospecific target.

The *Fusarium* genus is among the most important phytopathogenic and toxigenic filamentous fungus that causes destructive diseases in plants and contaminates food and feed with mycotoxins. *Fusarium* species can also directly cause human infections. The most notorious species, *F. graminearum*, is responsible for Fusarium head blight (FHB), which is a global problem in wheat, oat, barley, ear and stalk rot of maize and leads to severe yield and economic losses in these crops.[1-2] In addition to yield and quality losses, the fungus produces harmful mycotoxins in infected crops, food, and feed during the pre-harvest and post-harvest period.[3-4] These mycotoxins, such as deoxynivalenol, zearalenone, T-2 toxin, and fumonisin B1 are a severe threat to human and domestic animal health.[5-6] Direct *Fusarium* infection on humans causes various diseases such as sinusitis,[7] pneumonia,[8] and skin lesions[9] and is life-threatening in immunocompromised patients.[10-11] Asexual and sexual reproductions are key biological events in higher microorganisms. In nature, sexual and asexual reproductions are critical in the origin of outbreak strains for epidemic diseases, and the rapid spread of diseases is often through asexual reproduction by pathogenic fungi.[12] Therefore, discovery of asexual and sexual reproductive inducers and understanding the mechanism of asexual and sexual reproduction greatly contribute to the control of pathogenic fungi. For this purpose, we focused on the characterization of signaling molecules which induce the asexual or sexual reproduction of microorganisms for a long time. We previously characterized the mating hormones, α1 and α2, in *Phytophthora* oomycetes,[13-15] which morphologically resemble fungi but are evolutionarily distinct.

Asexual reproduction is the most important means of fungal reproduction. For plant pathogens like *Fusarium*, asexual reproduction can greatly affect epidemic diseases, while sexual reproduction is important as a source of genetic variation.

Studies have been performed to understand asexual reproduction of fungi for several decades. Results revealed that asexual spore production of *Aspergillus* spp. was affected by polyunsaturated fatty acids.[16] Also, reports indicated that conidiation of *Penicillium cyclopium* could be prematurely induced in a nutritionally sufficient medium by an endogenous diterpene and conidiation of *Trichoderma* was found to be influenced by the environment and endogenous biological rhythms.[17-18] Findings for the understanding of the regulation and signaling transduction on asexual reproduction also showed that the mitogen-activated protein kinase (MAPK),[19] velvet complex,[20-21] and AbaA[22] signaling pathways are involved in the asexual reproduction of *Fusarium* species. However, the signaling molecule that induces the asexual reproduction of *Fusarium* spp. is still unknown. Here, we report the isolation and characterization of a conidium-inducing signaling molecule, Fusarium asexual reproduction inducer (FARI (**1**), Figure 1A), from a *Fusarium* spp., and its generality in the genus *Fusarium* as well as a mechanism of action on conidium formation.

F. graminearum strain PH-1 was used to isolate FARI. In a typical case, the supernatant (100 L) from the culture broths of *F. graminearum* was partitioned with EtOAc in batches. The active organic extracts were combined and chromatographed on a silica gel open column, followed by reversed-phase column chromatography. Further purification by repeated high performance liquid chromatography (HPLC) yielded 0.4 mg of pure FARI. Finally, a total of 0.8 mg of pure FARI was obtained from 201 L of supernatant culture broths. Conidium-inducing activity was evaluated in *F. graminearum* strain PH-1 as described in the Supporting Information (Figure S1) for all collected fractions from chromatography.

FARI has the molecular formula $C_{15}H_{26}O_2$, as determined by high-resolution ESI-MS measurement (found 261.1827, calculated for $C_{15}H_{26}O_2Na$ $[M + Na]^+$

261.1825). We analyzed the chemical structure of FARI by ^{1}H nuclear magnetic resonance (NMR) (Figure S2, Table 1), ^{13}C NMR (Figure S3, Table 1), and two-dimensional NMR spectroscopy (Figures S4-S6). The ^{1}H NMR spectrum of FARI show the presence of three methyl groups at δ_H 0.86 (3H, d, J = 6.8 Hz), 0.91 (3H, d, J = 6.8 Hz) and 2.10 (3H, s); four olefinic protons at δ_H 4.91 (1H, br s), 4.92 (1H, br s), 5.46 (1H, dd, J = 9.4, 15.8 Hz), 6.01 (1H, d, J = 15.8 Hz); one oxymethylene δ_H 3.58 (2H, t, J = 6.6 Hz); four methylenes at δ_H 1.51 (1H, m), 1.70 (2H, m), 1.78 (1H, m), 2.28 (2H, dd, J = 6.5, 9.0 Hz), and 2.42 (2H, m); and two methines at 1.61 (1H, m) and 1.78 (1H, m) (Table 1). The ^{13}C NMR spectrum of FARI revealed the presence of 15 carbon signals comprised of one ketone carbon (δ_C 212.2), three methyl groups (δ_C 19.7, 21.2 and 30.0), two double bonds (δ_C 114.4, 132.9, 134.9 and 147.2), an oxymethylene carbon (δ_C 62.7), four methylene groups (δ_C 27.6, 29.7, 32.6 and 42.6) as well as two methine groups (δ_C 33.6 and 51.0) (Table 1). A detailed analysis of a proton-proton correlation spectroscopy (^{1}H–^{1}H COSY) spectrum clarified the partial connectivity (in bold bonds in Figure S7). The heteronuclear multiple-bond connectivity (HMBC) spectrum connected the above partial structures to give a planar structure (Figure S7) as follows: H–1 to C–2; H–3 to C–1 and C–2; H–6 to C–8; H–7 to C–8 and C–12; H–9 to C–7 and C–8; H–10 to C–8; and H–12 to C–8 and C–9. The trans-geometry of the double bond at C6–C7 was determined from the coupling constant of H-6 and H-7, J_{6-7} = 15.8 Hz. The optical rotation of the natural FARI was $[\alpha]_D^{25}$ +7.0 (*c* 0.42, benzene).

FARI significantly induced conidia formation (1200 ± 165 conidia) at 1 ng dose on *F. graminearum* strain PH-1 mycelia, and the number of total conidia increased in a dose-dependent manner at the range of 1 to 300 ng (Figure 1B) and peaked at 300

ng (Figure S8). Cross effect to other *Fusarium* species demonstrated that FARI induced the conidia formation on the other six tested species: *F. equiseti* and *F. verticillioides* (Figure 1B), and *F. proliferatum*, *F. avenaceum*, *F. culmorum*, and *F. oxysporum* (Figure S9). Moreover, the conidium-inducing activity of FARI on other fungi was examined. The results revealed that FARI was inactive on conidiation of *Botrytis cinerea* and *Penicillium digitatum* (Figure S10). Figure 1C shows a photomicrograph of conidia obtained in the conidium-inducing assay at a dose of 300 ng.

Since only trace amount of FARI was obtained in this study and the structure of FARI possesses two possible stereoisomers due to the chiral center at C5, the chemical synthesis of two enantiomers was necessary for the structure confirmation as well as the assignment of the absolute configuration at C5 (Figure 2, see the Supporting Information). Two enantiomers of FARI (**1**) were synthesized from commercially available chiral precursors, (*R*)- and (*S*)-limonene (**2**) (see the Supporting Information, Figures S11-S30). The synthesis of (*S*)-**1** from (*R*)-**2** is shown in Figure 2. First, site-selective hydrogenation of (*R*)-limonene (**2**) with Adams' catalyst (PtO_2) followed by Lemiux-Johnson oxidation (cat. OsO_4, $NaIO_4$) afforded (*R*)-keto aldehyde **3**.[23-25] The resulting aldehyde was then transformed into (*R*)-geminal dibromide **4** in two steps by Takeda's procedure.[26] The hydrazone prepared by treatment of (*R*)-**3** with hydrazine monohydrate was treated with copper (II) bromide and triethylamine in the presence of a molecular sieve (MS 4A) to give (*R*)-*gem*-dibromide **4** in 34% yield. Dehydrobromination of (*R*)-**4** with DBU provided (*S*)-vinyl bromide **5** in a good yield. Though the yield of **4** was relatively low, this route provided sufficient amount of **5** without epimerization at C5 for the synthesis of (*S*)-**1** with high optical purity. (*S*)-vinyl bromide **5** was coupled with alkenylborane

6[27] through the Suzuki-Miyaura coupling[28] to give (*S*)-**7** in a yield of 75%. Finally, removal of the TBS group with TBAF furnished (*S*)-**1** in a good yield. The (*R*)-enantiomer of **1** was also synthesized from (*S*)-limonene (**2**) in a similar manner to that of (*S*)-**1**. Optical purities of (*S*)- and (*R*)-**1** were determined by chiral HPLC to be 96.8 and 97.4% ee, respectively. The maximum wavelength of absorption (λ_{max}) of (*S*)-**1** was 231 nm and the molar absorption coefficients (ε) in methanol was 18217.

The synthetic stereoisomers did not show any difference from the natural compound as observed by ^{1}H and ^{13}C NMR data (Figures S31, S32, Tables S1, S2). Since assignment of the absolute configurations of natural FARI by spectroscopic methods is difficult, the conidium-inducing activities of the two synthetic isomers, (*S*)-**1** and (*R*)-**1**, were evaluated in comparison with the natural one. The (*S*)-**1** isomer induced significant conidia formation ($P < 0.001$) in *F. graminearum* at a dose of 1 ng, and the conidia increased in a dose-dependent manner, which is very similar to the results of the natural FARI (Figure 3). (*R*)-**1** isomer at a dose of 100 ng did not induce any remarkable conidia formation (Figure 3). Although a higher dose (300 ng) of (*R*)-**1** induced conidia formation, this activity might be derived from a small amount of the (*S*)-enantiomer (1.3%) containing in (*R*)-**1**, which may have arisen from the epimerization in the synthesis and/or from the starting material containing trace amounts of (*R*)-**2**. In addition, the optical rotation of natural FARI (**1**) [$[\alpha]_D^{25}$ +7.0 (*c* 0.42, benzene)] closely matched that of synthesized (*S*)-**1** [$[\alpha]_D^{24}$ +10.8 (*c* 1.05, benzene)] and opposite that of (*R*)-**1** [$[\alpha]_D^{23}$ -10.7 (*c* 1.07, benzene)]. These results strongly suggested that natural FARI has a *S* configuration at the C-5 position.

To confirm interspecies universality, quantitative analysis of FARI produced by *Fusarium* species was performed using the ultra high performance liquid chromatography-electrospray ionization tandem quadrupole mass spectrometry

(UHPLC-ESI-MS/MS) and FARI-d_2 ((*S*)-**1**-d_2) as the internal standard (IS) (See Supporting Information, Figures S33-S39 and Tables S3-S4). The tested species produced FARI at 0.10-0.32 μg L^{-1} (Table 2), which strongly supports the universality of FARI among *Fusarium* species. These results suggest that FARI is not only a conidium-inducing molecule of *F. graminearum* but also a universal regulator of conidia formation among *Fusarium* species.

To investigate the mechanism of action, the FgMgv1 and FgGpmk1 of MAPK, FgLaeA of velvet complex, and FgAbaA signaling pathways were examined to evaluate their effects on conidiation induced by FARI using corresponding mutants in carboxymethyl cellulose (CMC) liquid medium culture. Two concentrations, 200 and 600 ng mL^{-1}, that practically correspond to 100 and 300 ng doses on the solid medium assay, were used for all experiments. FARI significantly increased conidia formation on ΔFgMgv1 at tested concentrations, whereas it did not cause an increase in conidia number on ΔFgGpmk1 and ΔFgLaeA. These results indicated that the Mgv1 MAPK pathway did not play a crucial role, but the Gpmk1 MAPK and LaeA velvet pathways are involved in conidiation induced by FARI (Figure 4). As AbaA is known to be directly involved in the conidial formation, ΔFgAbaA did not make conidium regardless the existence of FARI.

In conclusion, to our knowledge, this is the first characterization of a conidium-inducing molecule in the pathogenic and toxigenic fungus, *Fusarium*. Isolation, structure elucidation, total synthesis of enantiomers, biological evaluation as well as an investigation of the mode of action were achieved. The absolute configuration of natural FARI was determined as 5*S* by comparing the optical rotation and conidium-inducing activities of the two synthetic stereoisomers with those of natural FARI. Chirality is certainly important in the conidium-inducing activity in

Fusarium, which strongly implies the existence of a stereospecific target at the beginning of the signal transduction cascade, leading to conidia formation. Despite being a genus of filamentous fungi, *Fusarium* is similar to phytopathogenic *Phytophthora* oomycetes, in which the configuration change for each methyl group in the chemical structure of synthetic mating hormones, α1 and α2, could have resulted in an almost complete loss of hormonal activities.[14-15] These results suggested that the mating hormones of *Phytophthora* target stereospecific receptors to initiate the oospore formation. Therefore, our findings also suggest that FARI is a possible hormone or hormone-like molecule which regulates the conidia formation in *Fusarium*.

A study of the mechanism of action showed that the Gpmk1 MAPK and velvet complex signaling pathways are involved in conidiation of *F. graminearum* induced by FARI. These results are consistent with previous reports by genetic methods in which conidium production was completely abolished in *AbaA* deletion mutant,[22] and the Gpmk1 pathway and LaeA pathway of the velvet complex were involved in conidiation.[19,21] The Mgv1 pathway is involved in the male fertilization and female fertilization, but not in asexual conidia formation.[29] These support the idea that FARI is an important inducer for conidia formation of *Fusarium* species.

Microorganisms may contain specific receptors for signaling molecules, which trigger asexual and sexual reproductions. Each endogenous reproductive signaling molecule targeting a specific receptor is possibly a natural occurrence in the microbial kingdom. Our findings imply that the formation of asexual spores in filamentous fungi may be regulated by hormones or hormone-like inducers, and the targets of such substances could be used for the discovery of specific novel antibiotics and fungicides against fungal pathogens for human and in agriculture, respectively.

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Acknowledgements

This work was supported by the National Natural Science Foundation of China [Grant No. 21661140001 and 21572204] (to J.Q.), a Grant-in-Aid for Scientific Research (B) (No. 16H04915) (to T.N.) and the JSPS A3 Foresight Program. All authors discussed the results and commented on the manuscript. The authors thank professors Su Zeng (Zhejiang University, China) and Zhujun Yao (Nanjing University, China) for valuable discussions. Dr. Julius Lopez (RIKEN, Japan) gave important comments to the manuscript. The authors acknowledge Dr. Kin-ichi Oyama (Chemical Instrumental Facility, Research Center for Material Science, Nagoya University) for measuring HRMS (EI) of deuterated compounds.

Conflict of interest

The authors declare no conflict of interest.

Keywords: asexual reproduction inducer · *Fusarium* · sesquiterpene · signal transduction · total synthesis

10.1002/anie.201803329

Figures and figure legends:

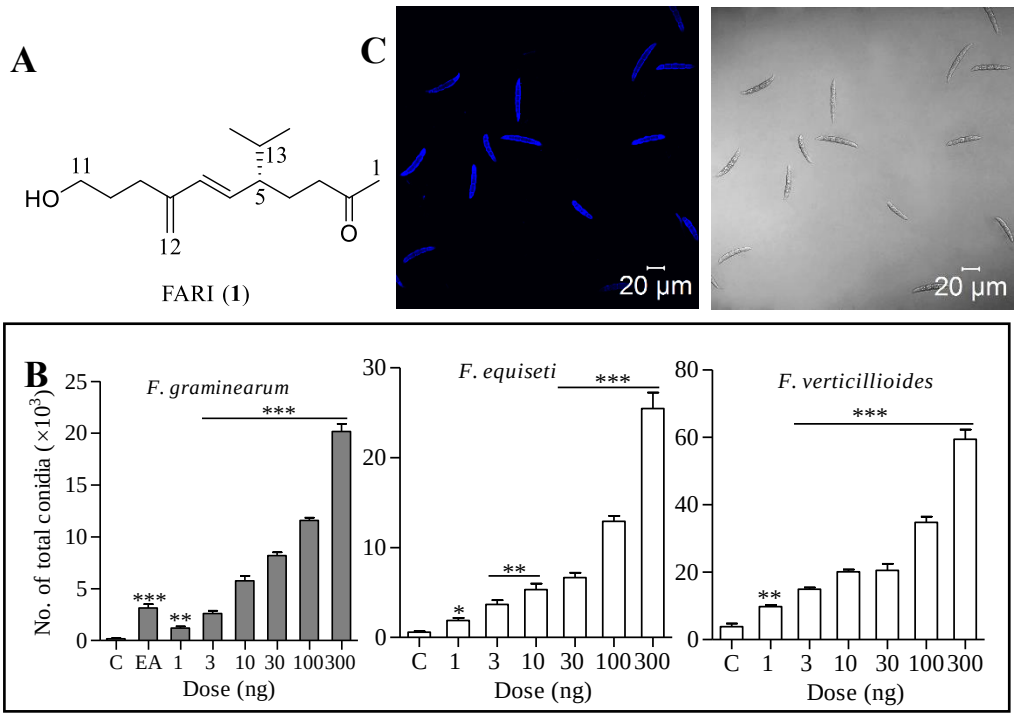

Figure 1. Chemical and biological character of natural FARI. A) The chemical structure of Fusarium asexual reproduction inducer (FARI, **1**) isolated from *Fusarium graminearum*. B) Dose-dependent increase of asexual spores (conidia) formation in *F. graminearum*, *F. equiseti* and *F. verticillioides* upon treatment with FARI. The total number of conidia (vertical axis) produced on a FARI-added well was counted. EA represents the EtOAc extract corresponding to 5 mL of culture broth; C is the negative control. Values are expressed in terms of means of six replicates ± SE. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 indicate significant difference from the corresponding negative control. The total number of conidia formed on the mycelia of *Fusarium* represented the conidium-inducing activity of test samples. C) Photomicrograph of conidia induced by 300 ng of FARI on *F. graminearum*. The left one is the micrograph of fluorescence image. The right one is the micrograph of light image. Fluorescence image of conidia stained with calcofluor white (CFW) were acquired

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using a Zeiss LSM 780 confocal microscope. Conidia were obtained after filtration of hyphae and agar. Scale bar, 20 μm.

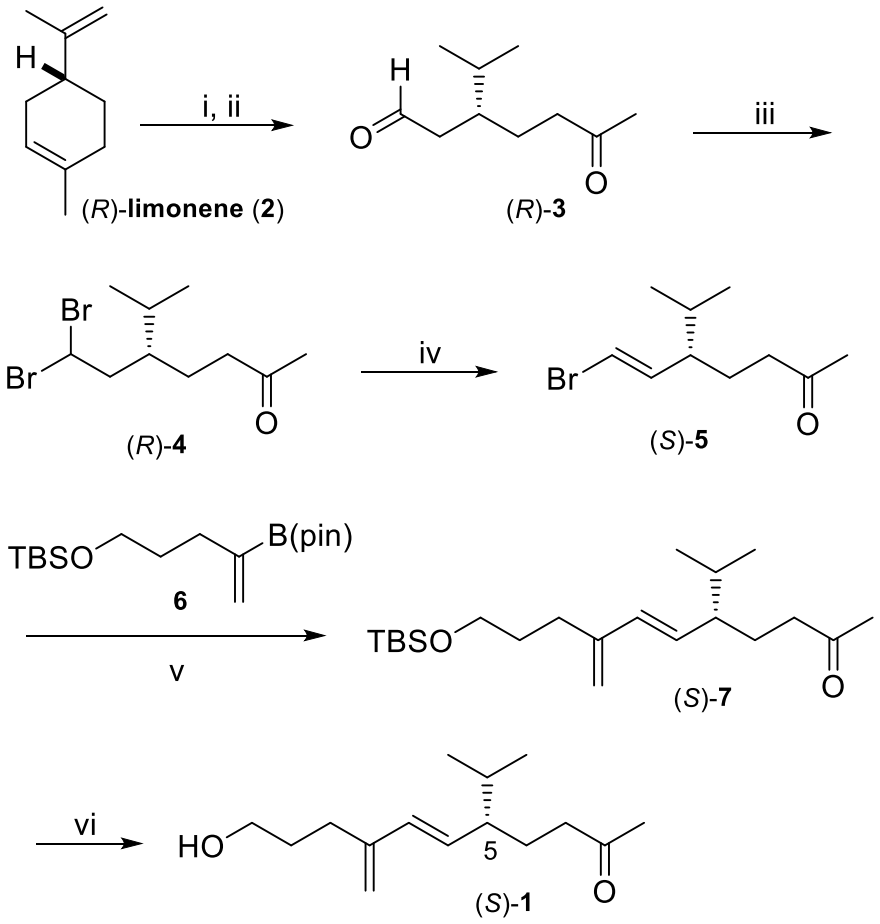

Figure 2. Synthesis of (*S*)-**1**. Reagents and conditions: (i) H_2, PtO_2, EtOH; (ii) OsO_4, $NaIO_4$, pyridine, THF-H_2O; (iii) $(H_2N)_2 \cdot H_2O$, CH_2Cl_2, rt, then Et_3N, $CuBr_2$, MS4A, 0 °C to rt, 20 min (34%); (iv) DBU, DMF, 50 °C to reflux, 5 h (67%); (v) $Pd(PPh_3)_4$, Cs_2CO_3, DMF-H_2O, 50 °C, 4 h (75%); (vi) TBAF, THF, rt, 80 min (67%). Abbreviation: MS4A, molecular sieve 4A; TBS, *tert*-butyldimethylsilyl; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DMF, *N,N*-dimethylformamide.

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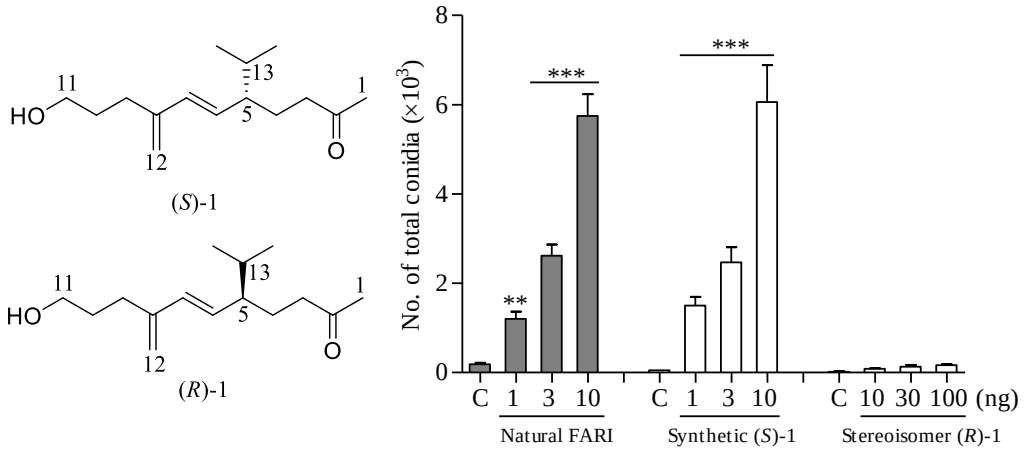

Figure 3. Biological properties of the synthetic FARI stereoisomers. Conidia formation in *F. graminearum* induced by two synthesized isomers (*S*)-**1** and (*R*)-**1** at doses of 1, 3, and 10 ng well^{-1}, and 10, 30, 100 ng well^{-1}, respectively, in comparison with natural FARI at same doses of (*S*)-**1**. C, negative control. Values are expressed as means of six replicates ± SE. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant difference from the corresponding negative control.

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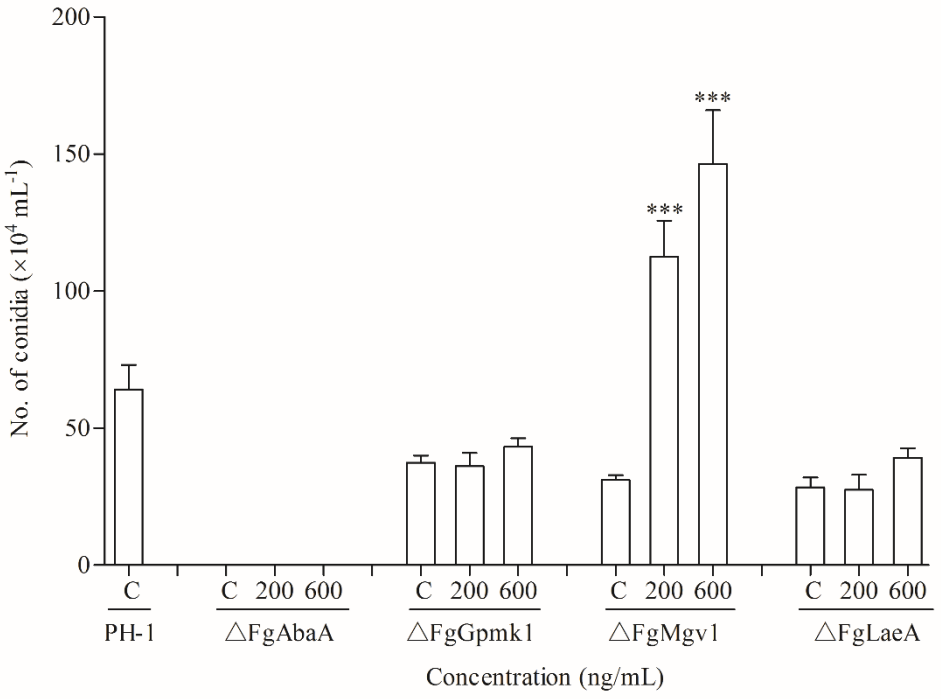

Figure 4. Mechanism of action of FARI. Conidium-inducing activity of FARI on mutants (ΔFgAbaA, ΔFgGpmk1, ΔFgMgv1, and ΔFgLaeA) in comparison with *F. graminearum* strain PH-1. Conidia were counted after incubation of the wide type and mutants in CMC liquid culture medium for five days at 25 ºC, 180 rpm. The concentrations of 200 and 600 ng mL^{-1} which practically correspond to the doses of 100 and 300 ng on solid medium assay, respectively, were used in all experiments. C is the negative control. Values are expressed in terms of means of six replicates ± SE. ***P < 0.001 indicates significant difference from the corresponding negative control.

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Tables

Table 1. The ^{1}H and ^{13}C NMR data for natural FARI (in CD_3OD, ppm)[a]

Carbon No.[b]	δ_H	δ_C
1	2.10 s	30.0
2	-	212.2
3	2.42 m	42.6
4	1.78 m 1.51 m	27.6
5	1.78 m	51.0
6	5.46 dd (9.4,15.8)	132.9
7	6.01 d (15.8)	134.9
8	-	147.2
9	2.28 dd (6.5, 9.0)	29.7
10	1.70 m	32.6
11	3.58 t (6.6)	62.7
12	4.91 br s 4.92 br s	114.4
13	1.61 m	33.6
14	0.86 d (6.8)	19.7
15	0.91 d (6.8)	21.2

[a] 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR, coupling constants (J in Hz) are in parentheses.

[b] Numbering of FARI (see Fig. 1A)

10.1002/anie.201803329

Table 2. Interspecies universality of FARI in *Fusarium* species (μg L^{-1}, n=3).

Species	Strain[b]	FARI[a]
F. proliferatum	CGMCC3.4759	0.18 ± 0.04
F. avenaceum	CGMCC3.6813	0.11 ± 0.01
F. equiseti	CGMCC3.6911	0.30 ± 0.05
F. culmorum	CGMCC3.4595	0.32 ± 0.10
F. oxysporum	CGMCC3.6787	0.15 ± 0.01
F. verticillioides	CGMCC3.7995	0.10 ± 0.02

[a] The values were determined by UHPLC-ESI-MS/MS analysis of FARI containing fractions which were obtained from the cartridge separation of EtOAc layer of filtered culture broths using a synthetic FARI-d_2 as the internal standard. The experiments were repeated three times and the data were presented as means ± SEM.

[b] The strains were purchased from the China General Microbiological Culture Collection Center (CGMCC, Beijing, China).

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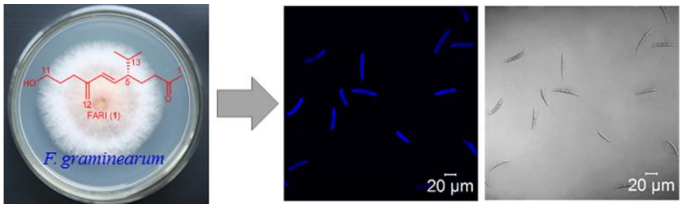

Fusarium asexual reproduction inducer (FARI), a new sesquiterpene derivative, induced conidial formation on notorious *Fusarium graminearum* at an extremely low dose. The universality of FARI among *Fusarium* species was uncovered. Furthermore, mechanism of action study revealed that Gpmk1 and LaeA signaling pathways are required for the conidial formation induced by FARI.