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Biologically active *Phytophthora* mating hormone prepared by catalytic asymmetric total synthesis

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A *Phytophthora* mating hormone with an array of 1,5-stereogenic centers has been synthesized by using our recently developed methodology of catalytic enantioselective conjugate addition of Grignard reagents. We applied this methodology in a diastereand enantioselective iterative route and obtained two of the 16 possible stereoisomers of *Phytophthora* hormone α₁. These synthetic stereoisomers induced the formation of sexual spores (oospores) in A2 mating type strains of three heterothallic *Phytophthora* species, *P. infestans*, *P. capsici*, and *P. nicotianae* but not in A1 mating type strains. The response was concentration-dependent, and the oospores were viable. These results demonstrate that the biological activity of the synthetic hormone resembles that of the natural hormone α₁. Mating hormones are essential components in the sexual life cycle of a variety of organisms. For plant pathogens like *Phytophthora*, sexual reproduction is important as a source of genetic variation. Moreover, the thick-walled oospores are the most durable propagules that can survive harsh environmental conditions. Sexual reproduction can thus greatly affect disease epidemics. The availability of synthetic compounds mimicking the activity of *Phytophthora* mating hormone will be instrumental for further unravelling sexual reproduction in this important group of plant pathogens.

*Phytophthora* species are devastating plant pathogens that cause substantial yield losses in agricultural crops worldwide and destroy many indigenous plant species in natural vegetation (1). One of the most notorious species is *Phytophthora infestans*, the causal agent of late blight disease on potato and tomato and responsible for the Irish potato famine in the mid-19th century (2, 3). In the life cycle of *Phytophthora*, sexual reproduction plays an important role (2, 4). In 2005, Qi et al. (5) reported the purification of a *Phytophthora* mating hormone and revealed that the structure consists of an array of 1,5-stereogenic centers. Such structures can in principle be accessed by our recently developed powerful methodology of catalytic enantioselective conjugate addition of Grignard reagents (6–14) (Fig. 1). The challenging chemical structure of the mating hormone and its biological importance has prompted us to undertake the total synthesis of the target molecule and to test its biological activity.

Despite its fungal-like growth morphology (mycelium), *Phytophthora* is not a fungus. It belongs to the oomycetes, a diverse group in the stramenopile lineage that evolved entirely independently from fungi and includes both saprophytes and pathogens of plants, insects, fish, vertebrates, and microbes. Among the plant pathogenic oomycetes are nearly 80 *Phytophthora* species, a hundred or more *Pythium* species, and a variety of obligate biotrophs, including downy mildews and white rusts (1, 15). The closest relatives of oomycetes are brown algae and diatoms (16, 17). As most fungi, oomycetes have a vegetative and generative life cycle and propagate via spores. The thick-walled sexual spores, called oospores, are not only important as source of genetic variation; they are also crucial for surviving harsh environmental conditions. Sexual reproduction can either occur in single culture (homothallism) or requires mating of two strains that possess different sexual compatibility types (heterothallism). In heterothallic *Phytophthora* species, two mating types are known, A1 and A2 (18). Analysis of mating type inheritance in two species, *P. infestans* and *Phytophthora parasitica*, showed that A1 is governed by a heterozygous locus carrying one dominant and one recessive allele, whereas A2 is homozygous recessive (19, 20). In *P. infestans*, positioning of the mating type locus on a genetic linkage map (20, 21) and on a contig of genomic clones (22) showed that the locus is hemizygous and, in several strains, is linked to genetic abnormalities such as balanced lethality and translocations (19, 23, 24). Although the precise determinants of mating type in *Phytophthora* are still unknown, the mating type locus is thought to regulate either the synthesis of a mating hormone or the response to this hormone.

Qi et al. (5) purified a compound from culture filtrate from *Phytophthora nicotianae* that can induce oospore formation in a *P. nicotianae* A2 mating type strain. They determined the structure of the purified compound and designated it mating hormone α₁ (MH-α₁). The structure of MH-α₁ is shown in Fig. 1A (1). Purification of 1.2 mg of MH-α₁ required >1,800 liters


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of culture filtrate suggesting that \textit{P. nicotianae} produces only minute amounts. The activity spectrum of MH-\(\alpha\)-1 was not limited to \textit{P. nicotianae}; in other heterothallic \textit{Phytophthora} species, MH-\(\alpha\)-1 also induced oospore formation. This is consistent with the phenomenon that an A1 strain of one \textit{Phytophthora} species can induce oospores in an A2 strain of another species, which points to conservation of mating hormones throughout the genus.

Although Qi et al. (5, 25, 26) reported the structural characterization of MH-\(\alpha\)-1, the relative and absolute configurations were not assigned, and the optical rotation was not determined. The four stereocenters in MH-\(\alpha\)-1 can lead to 16 possible stereoisomers. In the absence of stereochemical information for MH-\(\alpha\)-1, chemical synthesis requires a route that allows independent control of the configuration of each individual stereogenic center. Recently, we developed a highly enantioselective conjugate addition (CA) of Grignard reagents, in particular MeMgBr, to \(\alpha,\beta\)-unsaturated thioesters catalyzed by a copper bromide complex of Josiphos ligand (Fig. 1B) and applied this methodology in a diastereo- and enantioselective iterative route to deoxypropyionate chains (6–14).

The aim of the present study was to demonstrate the versatility of the catalytic asymmetric strategy in the stereoselective total synthesis of two stereoisomers of \textit{Phytophthora} mating hormone \(\alpha\)-1 and to demonstrate biological activity of the two stereoisomers. The construction of three \([C(3, 7, 15)]\) of four stereoisomers \(\{1\}\) was achieved in a catalytic enantioselective manner, using CA of MeMgBr to the corresponding thioesters. Biological assays showed that two different synthetically obtained stereoisomers can mimic the activity of MH-\(\alpha\)-1 and induce the formation of oospores. Our findings demonstrate that synthetic \textit{Phytophthora} mating hormone \(\alpha\)-1 can be readily obtained and can be exploited as a powerful tool to further investigate sexual reproduction in an important group of plant pathogens.

Results and Discussion

Catalytic Enantioselective Synthesis of Two Stereoisomers of Mating Hormone \(\alpha\)-1. Our retrosynthetic analysis for the stereoisomers of mating hormone \(\alpha\)-1 \((1)\) is illustrated in Fig. 2. Target molecule \(1\) may be derived from protected triol \(2\), in which the carbonyl group is masked as a dithiane. Compound \(2\) may be accessed via the assembly of two fragments \(3\) and \(4\) employing a dithiane coupling strategy (27–31). Both \(3\) and \(4\) are available from the \(\alpha,\beta\)-unsaturated thioesters \(5\) and \(6\) \((11–13)\), each with a different alcohol protecting group to allow selective deprotection in later stages of the synthesis.

In principle, any of the 16 putative stereoisomers can be prepared via this synthetic route by judicious selection of the configuration of the catalysts used to introduce each stereogenic center. Because the absolute stereochemistry of MH-\(\alpha\)-1 is unknown, we arbitrarily chose the absolute configuration of two stereoisomers of hormone \(\alpha\)-1 to be synthesized.

The synthesis of the dithiane fragment 3 was accomplished in six steps from the \(\alpha,\beta\)-unsaturated thioester 5 \((\text{Scheme } 1)\). Compound 5 itself was obtained in three steps from ethylene glycol on a multigram scale. Enantioselective CA of MeMgBr to 5 in the presence of 2 mol% of the chiral catalyst \((R, S)-7\) in \(\text{BuOMe} at \sim 78^\circ C\) provided 8 with 92\% enantiomeric excess (ee) and 75\% yield. The absolute configuration of the stereogenic center \(C(3)\) was assigned via subsequent derivatization to a known compound \((13)\). A two-step reduction of 8 with disobutylaluminum hydride (DIBALH) to alcohol 9, followed by silyl protection, provided protected alcohol 10. Selective deprotection of the tert-butyldimethylsilyl (TBS) group followed by iodoxybenzoic acid (IBX) oxidation of the corresponding alcohol 11 provided aldehyde 12. Treatment of 12 with propanedithiol at \sim 78^\circ C in the presence of BF\(_3\)-OEt\(_2\) resulted in the desired dithiane 3 \((88\% \text{ yield})\) [see supporting information (SI) Text, Remark 1].

Preparation of fragment 4 began from \(\alpha,\beta\)-unsaturated thioester 6 \((13)\) \((\text{Scheme } 2)\). CA of MeMgBr to 6 catalyzed with 1 mol% of \((R, S)-7\) resulted in thioester 13 \((95\% \text{ yield and } 98\% \text{ ee})\). The absolute configuration of the stereogenic center \(C(15)\) was assigned in a similar manner as indicated for compound 8. DIBALH reduction of 13 to aldehyde 14, followed by a Wittig reaction provided ketone 15. Hydrogenation of 15 with Pd/C resulted in the saturated ketone 16 \((99\% \text{ yield})\).

We anticipated that the tertiary alcohol could be prepared through a catalytic asymmetric vinylogous Mukaiyama aldol condensation of the aliphatic ketone catalyzed by a Cu-complex of Tol-Binap (32). With \((R)-\text{Tol-Binap}, lactone 17 was obtained from ketone 16 in 81\% yield and 92.8\% dr. \((R)-\text{Tol-Binap leads to the } \(R\)-\text{-aldol product in accordance with the proposed stereocontrol by the } \(R\)-\text{Tol-Binap based catalyst (32). We tentatively assigned the } \(R\)-\text{-configuration to the newly formed stereogenic center C(11). After hydrogenation of the alken moiety in 17, lactone 18 was reduced with DIBALH to the corresponding lactol. A Wittig reaction on this lactol yielded the \(\alpha,\beta\)-unsaturated thioester 19 \((59\% \text{ yield})\). After protection of the tertiary alcohol with a triethylsilyl group, the third stereogenic center \(C(7)\) was formed through CA of MeMgBr to 20 with 2 mol% of the chiral catalyst \((S, R)-7\) in \(\text{BuOMe} at \sim 78^\circ C\) with de \(>90\%\). In analogy with the results of catalytic CA addition catalyzed by \((S, R)-7\), we assigned the \((R)-configuration to the
the ketone, using MeI in the presence of CaCO3 (30), furnished nearly complete recovery of excess of dithiane products. To improve the yield of the reaction, the ratio of with an equimolar ratio of undertaken, using a dithiane coupling strategy (Scheme 3) (30). With an equimolar ratio of

Scheme 2. Synthesis of iodide 4. Reagents: (a) MeMgBr and 1 mol % (R,S)-7 in 1BuOMe at −78°C. (b) DibalH in CH2Cl2 at −40°C. (c) PPh3=CH-COCH3 in THF at 66°C. (d) H2 and Pd/C in EtOAc at room temperature. (e) CH2=C=CH=CH2−COTMS(GeT), TBAT, 10 mol % Cu(OTf)2, and 10 mol % (R)-Tol-BINAP in THF at room temperature. (f) H2, Pd/C in EtOAc at room temperature. (g) DibalH in hexane at −78°C. (h) PPh3=CH−COSiEt in CH2Cl2 at 40°C. (i) TESOTf and 2,6-lutidine in CH2Cl2 at 0°C to room temperature. (j) MeMgBr and 2 mol % (S,R)-7 in BuOMe at −78°C. (k) DibalH in CH2Cl2 at −40°C. (l) PPh3, I2, and imidazole in CH2Cl2 at room temperature.

The spectroscopic data of the synthetic stereoisomers 1 and 1′ of the hormone α1 (high-resolution mass spectrometry, 1H, 13C NMR, and IR spectroscopy) were identical to those reported for the natural product (33) (see Materials and Methods). (C) Graphical display of change in colony shape induced by the two stereoisomers, 1 and 1′. The y axis shows the ratio a/b in which “a” and “b” are the diameters of the colony measured along the lines marked by “a” and “b” in A, respectively. The graph shows the result of one experiment. The experiment was repeated three times with the same outcome. (D and E) Morphology of hyphae visualized by light microscopy. Strain NL88133 was grown in the absence (D) or presence (E) of stereoisomer 1 (1,600 ng). The arrow points to one of the many bulbed protrusions observed on the hyphae in the presence of the synthetic hormone.

Scheme 3. Synthesis of and stereoisomers. Reagents: (a) BuLi in 10% HMPA in THF at −78°C. (b) MeI and CaCO3 in MeCN, H2O, and THF (4/1/1) at 50°C. (c) TBAF in THF at room temperature.
Table 1. Oospore formation in Phytophthora A2 mating type strains induced by synthetic hormone is concentration-dependent

<table>
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<th>Phytophthora species</th>
<th>A2 strain</th>
<th>Days*</th>
<th>0</th>
<th>200</th>
<th>400</th>
<th>800</th>
<th>1,600</th>
<th>2,000</th>
<th>4,000</th>
<th>8,000</th>
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<tr>
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<td>CN505502B</td>
<td>7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>nd</td>
<td>nd</td>
</tr>
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<td></td>
<td></td>
<td>28</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>nd</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>P. capsici</td>
<td>LT3241</td>
<td>7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>nd</td>
<td>+</td>
<td>+++</td>
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<td>–</td>
<td>+</td>
<td>+</td>
<td>nd</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>P. nicotianae</td>
<td>PS82</td>
<td>7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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</table>

*Number of days after addition of synthetic hormone.
†The amount shown is in nanograms per 10 μl of solution. The approximate number of oospores per 2 cm²: –, none; 1, 1–10; 1+, 10–50; 1++, 50–100; 1+++ >100; nd, not determined. The oospore inducing activity of stereoisomer 1 and 1’ was in the same range, but 1 seemed to be slightly more active.
The P. capsici oospores induced by synthetic hormone readily germinated and produced one or more germination tubes (Fig. 4C and Fig. S6). Multiple germ tubes are common for P. capsici oospores and are produced by more than half of the oospores resulting from a mating between A1 and A2 P. capsici strains (34). P. capsici is a species that easily gives rise to sexual progeny and has the potential to become an oomycete model species for genetic analysis (35). P. infestans oospores are difficult to germinate under in vitro conditions and the percentage of oospores that give rise to viable progeny is relatively low (1, 35).

Taken together, we conclude that the synthetic stereoisomers 1 and 1′ both have biological activity. Only A2 mating type strains and not A1 strains respond to the presence of the synthetic stereoisomers and in that respect 1 and 1′ have the same activity spectrum as the natural MH-1 (5). With respect to sensitivity of the different species, however, the synthetic stereoisomers differ from MH-1. Qi et al. (5) reported that P. nicotianae, the species from which they purified MH-1, was the most sensitive of the four tested species, and, in contrast to our observations, they found that P. infestans produced far fewer oospores than P. nicotianae. Theoretically, this difference could be due to the origin of the strains, which differed from the ones we used. Many Phytophthora species are notorious for their phenotypic variability, and this depends highly on culture and storage conditions. The two P. infestans A2 strains tested by us showed a similar change in growth behavior but one of the two produced less oospores in response to the same amounts of synthetic hormone. Still, in our hands, even the least sensitive of the two tested P. infestans A2 strains was more sensitive than P. nicotianae. An alternative explanation is a slight difference in the structure of the synthetic hormone and the natural MH-1 that cannot be detected by the standard spectroscopic analyses but influences the biological activity in a species-specific manner. The apparent conservation of mating hormones throughout the Phytophthora genus does not exclude the existence of species-specific variants of these hormones. In this respect, it is worth testing the biological activity of synthetic versions of the 14 remaining stereoisomers of hormone 1.

Conclusions

Total synthesis of two stereoisomers of Phytophthora mating hormone 1 has been completed in a catalytic enantioselective manner with a longest linear sequence of 15 steps with an overall 8.1% yield. Key steps in the synthesis include the CA of MeMgBr to create three of the stereochemical centers, Mukaiyama aldol condensation to generate the chiral tertiary alcohol, and a dithiane coupling. Via this synthetic route, the 16 putative stereoisomers of the hormone can be accessed. The relative and absolute configuration of the natural MH-1 cannot be detected by the standard spectroscopic analyses but influences the biological activity in a species-specific manner. The apparent conservation of mating hormones throughout the Phytophthora genus does not exclude the existence of species-specific variants of these hormones. In this respect, it is worth testing the biological activity of synthetic versions of the 14 remaining stereoisomers of hormone 1.

Materials and Methods

Chemical Synthesis of Two Diastereomers of Phytotrophina Mating Hormone 1. Details for the synthesis of two stereoisomers 1 and 1′ are provided in SI Appendix.

Phytophthora Strains. The activity of the stereoisomers 1 and 1′ was tested on three Phytophthora species. The strains used were P. infestans NL80029 (A1 mating type), NL88133 (A2) and CN5055028 (A2), P. capsici LT3112 (A1), LT3241 (A2) and LT3145 (A2), and P. nicotianae P0270797 (A1) and P582 (A2). P. capsici strains and P. nicotianae P0270797 were kindly provided by K. Lamour (University of Tennessee) and the Netherlands Plant Protection Service in Wageningen, respectively. Strains were cultured on rye sucrose agar or V8 agar according to standard procedures (1).

Bioassays. Stock solutions of synthetic mating hormone were prepared by dissolving the synthesized stereoisomers 1 and 1′ in ethyl acetate (EA) to a final concentration of 160 µg/ml. Dilutions were made in EA and ranged from 1,600 ng/µl to 1.25 ng/µl. Stock solutions and dilutions were stored at -20°C and kept on ice during handling.

The experimental setup of the bioassay is shown in Fig. S1. We prepared Petri dishes (diameter (Ø) = 9 cm) containing 10% clarified-V8 agar with two wells in the agar. A mycelium plug (Ø = 7 mm) was cut from a fresh Phytophthora culture and placed exactly in the middle of the Petri dish. The culture was then incubated at 20°C in the dark. After 3 (P. capsici and P. nicotianae) or 7 days (P. infestans), 10 µl of the diluted solution of stereoisomer 1 or 1′ was added to each of the two wells. Before continuing the incubation at 20°C, the Petri dishes were placed for a few minutes with an open lid in a sterile hood to allow the EA to evaporate. The following days, growth was monitored by measuring the diameters of the colony along the lines marked by “a” and “b” (Fig. S1). After 7 and 28 days, the total number of oospores that were produced within a 2-cm² area around the well was counted under an inverted microscope (Zeiss; Axiosvert 100). Because the solvent evaporates and the synthetic hormone 1 probably diffuses in the agar, we present the data in relation to the absolute amounts of the stereoisomers 1 and 1′ in nanograms instead of a concentration in nanograms per microliter.

Assessment of Oospore Viability. To assess the viability of oospores, we used 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) staining (Sigma; catalog no. M5655) (1, 36). One volume of 0.1% MTT solution
[in 0.1 M phosphate buffer (pH 5.8)] was mixed with an equal volume of oospore suspension. After incubation for 2 days at 36–37°C, staining of the oospores was examined. Pink and red colored oospores were considered to be viable, and unstained or black oospores to be nonviable. As controls for the viability staining we collected oospores from a normal cross of which the majority is viable. Part of this oospore suspension was autoclaved (30 min, 120°C) to kill the oospores for the nonviable control staining.


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