Structure-Activity Relationship of α Mating Pheromone from the Fungal Pathogen Fusarium oxysporum*

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During sexual development ascomycete fungi produce two types of peptide pheromones termed α and β. The α pheromone from the budding yeast Saccharomyces cerevisiae, a 13-residue peptide that elicits cell cycle arrest and chemotropic growth, has served as paradigm for the interaction of small peptides with their cognate G protein-coupled receptors. However, no structural information is currently available for α pheromones from filamentous ascomycetes, which are significantly shorter and share almost no sequence similarity with the S. cerevisiae homolog. High resolution structure of synthetic α-pheromone from the plant pathogenic ascomycete Fusarium oxysporum revealed the presence of a central β-turn resembling that of its yeast counterpart. Disruption of the fold by d-alanine substitution of the conserved central Glyα-Glnβ residues or by random sequence scrambling demonstrated a crucial role for this structural determinant in chemotactant activity. Unexpectedly, the growth inhibitory effect of F. oxysporum α-pheromone was independent of the cognate G protein-coupled receptors Ste2 and of the central β-turn but instead required two conserved Trp1-Cys2 residues at the N terminus. These results indicate that, despite their reduced size, fungal α-pheromones contain discrete functional regions with a defined secondary structure that regulate diverse biological processes such as polarity reorientation and cell division.

Mating pheromone α from bakers’ yeast Saccharomyces cerevisiae has served as a model for studying the interaction of small peptides with G protein-coupled receptors (GPCRs)6 (1). Like most ascomycete fungi, S. cerevisiae cells of different mating types secrete small peptide pheromones (α and β) that function as sexual chemoattractants and are sensed by the cognate plasma membrane GPCRs Ste2 and Ste3, respectively (2). Ligand binding to the receptor elicits a range of cellular responses including Gαi cell cycle arrest, formation of a polarized cell projection (known as a shmoo), and chemotropic growth toward the pheromone of the opposite mating type (3-5).

The mature α-pheromone of S. cerevisiae is a 13-residue peptide with the sequence WHWLGKLKPGQPMY (6). The central residues Pro8-Gly9 were proposed to form a Type II β-turn necessary to orient the N- and C-terminal ends during the interaction with the Ste2 receptor and to adapt to the conformational changes when the receptor switches to an active state (1, 7, 8). Alanine replacement of these central residues, which is expected to destabilize the Type II β-turn, leads to a reduction in pheromone-receptor affinity (1, 9). Besides this central region, the residues close to the C terminus are important for physical interaction with Ste2, as replacement by alanines caused an up to 3000-fold decrease in receptor affinity and loss of growth arrest (1). Similarly, in the dimorphic human pathogen Candida albicans, α-pheromone tridecapeptides with d-alanine substitutions at C-terminal positions 10–12 largely lost the ability to induce pheromone-mediated processes such as mating and biofilm formation (10). On the other hand, the N terminus of S. cerevisiae α-factor plays a major role in receptor activation and downstream signaling events. Alanine scanning of this region resulted in modified peptides, which still bound strongly to Ste2, but failed to exert biological activity. Interestingly, these analogs function as antagonists of α-pheromone in shmoo formation, growth arrest, and gene induction assays (1).

The function of α pheromones in sexual development appears to be broadly conserved in ascomycetes and has been experimentally demonstrated for a number of species (11). Interestingly, α-pheromone peptides from filamentous ascomycetes share low to no sequence similarity with yeast α-pheromone, tend to be significantly shorter (typically 10 amino acids), and often carry conserved Trp1-Cys2 and Gly6-
Gln7 residues at their N terminus and central region, respectively (12, 13). The structure-function relationship for this type of α-pheromones has not been investigated so far. Here we present an extensive biophysical characterization and high resolution structure of synthetic α-H9251-pheromones of Fusarium oxysporum, a highly destructive ascomycete plant pathogen that attacks over a hundred different crop species and has also been reported as an emerging pathogen of humans (14). F. oxysporum was recently shown to encode a predicted α-H9251-pheromone peptide with chemoattractant activity (12). We show that, similar to its yeast counterpart, F. oxysporum α-pheromone adopts a β-turn structure in water that becomes more globular in the presence of intracellular-like cosolvents. Using di-alanine substitution as well as a scrambled derivative of the peptide, we demonstrate a crucial role for the central Gly6-Gln7 residues in α-pheromone bending and chemoattractant activity. We further show that α-pheromone inhibits hyphal growth of F. oxysporum and that the inhibitory function is independent of the plasma membrane GPCR Ste2 and of the central Gly6-Gln7 residues but instead requires the conserved Trp1-Cys2 residues at the N terminus.

Results

Chemotactant Activity of F. oxysporum α-Pheromone Requires the Central Gly6-Gln7 Residues—The synthetic F. oxysporum α-pheromone decapeptide WCTWRGQPCW was previously shown to elicit chemotropic growth in F. oxysporum germ tubes, exhibiting a bell-shaped dose-response curve (12). We performed alanine substitution of Gly6-Gln7, which are broadly conserved in α-pheromones of filamentous ascomycetes. The D-Ala6,7 analog showed a dramatically reduced chemotactic activity that was similar to that of the randomly scrambled peptide (Fig. 1, A and B). Both of these variants elicited a weak but significant response in the wild type but not in the ste2Δ strain (Fig. 1C), indicating that they are still able to bind Ste2, possibly with decreased receptor affinity. By contrast, alanine substitution of the N-terminal Trp1-Cys2 residues, which are also conserved across ascomycetes, did not significantly affect chemotactant activity (Fig. 1A). Thus, the central Gly6-Gln7, but not the N-terminal Trp1-Cys2 residues of α-pheromone, are strictly required for chemoattrac-
tant activity.

Growth Inhibitory Activity of F. oxysporum α-Pheromone Requires the N-terminal Trp1 and Cys2 but Not the Central Gly6-Gln7 Residues—In S. cerevisiae α-pheromone induces transient cell cycle arrest and growth inhibition (3–5). To test whether F. oxysporum α-pheromone exerts an analogous effect, we determined germ tube length as well as the number of hyphal compartments and nuclei in the absence or presence of synthetic α-pheromone. Germ tubes exposed to a gradient of 378 μM α-pheromone showed a significant decrease in the number of hyphal cell compartments and nuclei, resulting in significantly shorter hyphae compared with germ tubes exposed to trypsin-treated α-pheromone (negative control; p < 0.0001) (Fig. 2, A, C, and E). Interestingly, growth inhibition by α-pheromone was still active in the ste2Δ mutant (negative control; p = 0.0029) (Fig. 2, B and D). Similarly to the native

![A](image1.png)  ![B](image2.png)  ![C](image3.png)

**FIGURE 1.** The central Gly6 and Gln7 residues of α-pheromone are essential for chemoattractant activity. A and B, dose-response curves for directed growth of F. oxysporum germ tubes toward synthetic F. oxysporum α-pheromone (α-pher) (WCTWRGQPCW), its analogs (D-Ala1,2), and (D-Ala6,7) (A) or a randomly scrambled version (WRWPCCWGQT) (B). C, directed growth of F. oxysporum WT and ste2Δ germ tubes after 13 h exposure to a gradient of the indicated compounds (versus WT; *, p < 0.0001). For each measurement, five independent batches of cells (n = 100 cells per batch) were scored. Each bar represents the mean of two independent sets of measurements. Error bars show S.D.
α-pheromone, the D-Ala$^{6,7}$ analog induced a significant reduction in average cell/nuclear division and hyphal length, whereas the scrambled peptide and the D-Ala$^{1,2}$ analog lacked an inhibitory effect (versus α-pheromone, $p < 0.0001$) (Fig. 3, A–C). Thus, the N-terminal Trp$^1$-Cys$^2$ but not the central Gly$^6$-Gln$^7$ residues are required for the growth inhibitory activity of α-pheromone in a Ste2-independent manner.

**Peptide Oligomerization**—Small peptides in solution tend to be unstable and often oligomerize, giving rise to larger aggregates. Because the presence of two SH-free Cys residues in *F. oxysporum* α-pheromone suggested that it could form oligomeric structures through the formation of intermolecular disulfide bonds, we tested the presence of possible oligomers in solution by HPLC fractionation. In all cases only a single peptide species was detected, and dithiothreitol (DTT) treatment did not modify the HPLC behavior (Fig. 4A). We conclude that the α-pheromone peptide remained monomeric when newly prepared. These results also demonstrate that the Cys residues do not establish intermolecular or intramolecular disulfide bonds, which is in agreement with the nuclear magnetic resonance (NMR) data shown below. The finding that *F. oxysporum* α-pheromone and the scrambled peptide did not elute at identical retention times might imply that they adopt different conformations. The higher retention time of the scrambled peptide suggests that it is slightly more hydrophobic than the natural fungal pheromone.

**Secondary Structure: Effect of Salts and pH**—Fig. 4B shows the far-UV CD spectra of *F. oxysporum* α-pheromone and the three modified sequences in water and TFE mixtures. Due to
the high abundance of aromatic residues, which absorb in the same wavelength range, quantification of the secondary structure populations was meaningless to perform. However, in all cases CD data in water were compatible with an equilibrium of conformations with the presence of different contributions of β-turns. On the other hand, the use of TFE as a cosolvent stabilizes intramolecular hydrogen bonds in peptides and proteins and has been used to mimic low dielectric environments such as those with high concentrations of macromolecules and other cosolvents encountered in a cell (15). Moreover, in many cases H₂O/TFE mixtures were shown to stabilize pre-existing secondary structures in short linear peptides (16, 17). In contrast to helical peptides where 30% TFE typically increases the strength of CD minima at 208 and 222 nm, we found that the intensity of the spectral minima in α-pheromone was reduced. This change was less evident in the D-Ala₁,₂ and D-Ala₆,₇ peptides, where the band centered around 200 nm is smaller. Although these differences are difficult to interpret due to possible opposing spectral contributions from aromatic and amide groups, we attribute the result to the presence of the D-Ala residues, which produce a positive CD signal, whereas L-amino acids produce negative bands (18).

FIGURE 3. The N-terminal Trp¹ and Cys² residues of α-pheromone are essential for growth inhibitory activity. A and B, number of cellular compartments and nuclei (A), or length (B) of germ tubes was determined after 14 h of exposure to a gradient of 378 μM α-pheromone (α-pher), its analogs (D-Ala₁,₂), and (D-Ala₆,₇) (A) or a randomly scrambled version (*, p < 0.0001 versus the same concentration of α-pheromone). Length of germ tubes was measured using the ImageJ software. Mean values were calculated from 4 independent experiments, each with 50 germ tubes. Error bars show S.D. C, representative micrographs of F. oxysporum germlings whose nuclei are labeled with H1-ChFP, exposed to a gradient of the indicated peptides, and stained with CFW. Scale bar, 10 μm.
The CD spectra of *F. oxysporum* α-pheromone and the scrambled peptide did not change significantly upon the addition of up to 200 mM NaCl, CaCl₂, or MgCl₂ (data not shown). This indicates that the structure of the isolated peptide in aqueous solution is not affected by the presence of Cl⁻, Na⁺, Ca²⁺, or Mg²⁺ ions. Moreover, both the α-pheromone and the scrambled peptide showed a conformational transition corresponding to a pKₐ value of about 9.0, which can be attributed to the deprotonation of the N-terminal group, although contribution of Cys side-chain ionization cannot be discarded. As expected from the nature of the individual side chains of the amino acids that constitute the two peptides, no major changes were observed between pH 3.0 and 8.0. Thus, although the two peptides have different far-UV CD spectra and different conformational populations in equilibrium, the behavior of these conformations against pH follows an identical variation pattern.

*NMR Assignment*—It was previously reported that the tendency of linear peptides to form secondary structures can be confirmed by plotting ΔδHα, ΔδCα, and ΔδCβ as a function of peptide sequence (19). We thus obtained ¹H and ¹³C NMR chemical shifts for α-pheromone and the modified peptides (see “Experimental Procedures” for details). Because aromatic amino acid rings cause major distortions in the chemical shift values due to the ring current effects (20), the large proportion of tryptophan residues (3/10 in the wild type, scrambled, and D-Ala₁,₂ peptides) and 2/10 in D-Ala⁶⁻⁷ peptides) made it difficult to extract conformational tendencies and to quantify populations from Δδ data. Thus, calculation of the three-dimensional structures on the basis of the NOE data was man-
NMR chemical shifts can be used to monitor the oxidation state of cysteine side chains, because the difference in chemical shifts between oxidized and reduced forms of $^{13}$C is large (10–13 ppm) in comparison with the effects on the aromatic side chains. The measured $^{13}$C chemical shifts of Cys$_2$, Cys$_9$, and Cys$_{5,6}$ were between 27.7 and 28.9 ppm (Fig. 5A), in agreement with values reported for reduced Cys. The expected value for the $^{13}$C nuclei in the oxidized state should be close to 41.1 ppm (21). These data are in line with those obtained by HPLC, confirming the reduced state of all cysteines (Cys$_2$ and Cys$_9$ in α-pheromone and D-Ala$_{6,7}$; Cys$_{5,6}$ in the scrambled peptide; Cys$_9$ in D-Ala$_{1,2}$) and the absence of inter- or intradisulfide bonds under the conditions used in this study.

X-P Trans/Cis Conformational Equilibrium in Solution—In all cases two different sets of signals were present in the NMR spectra, corresponding to the X-P trans/cis bond conformational equilibrium (Fig. 5B). The chemical shift difference between Pro $^{13}$C$_{β}$ and $^{13}$C$_{γ}$ carbons (22) ($δ Cβ–δ Cγ$ ≈ 4.5 ppm) and the characteristic sequential NOEs observed between the H$_α$ proton of X and the H$_β$ and H$_δ$ protons of P confirmed the trans rotamer as the major species. However, the populations varied between the different peptides. In the α-pheromone and the D-Ala$_{1,2}$ analog in H$_2$O, the Gln$_7$–Pro$_8$ trans/cis relation was 7/1, corresponding to the expected trans/cis ratio for flexible linear peptides (23). By contrast, in the scrambled sequence and the D-Ala$_{6,7}$ analog, the populations around Trp$_3$–Pro$_4$ and D-Ala$_7$–Pro$_8$ trans/cis bond were much more similar than in the wild type peptide (1.5–2/1). A similar effect was observed in other linear peptides when a Pro residue was preceded by an aromatic amino acid (23), as is the case for the scrambled sequence. Also, in the native state of proteins there is a marked trend for an aromatic amino acid to precede a cis proline.

In all four peptides tested, the equilibria were not significantly affected in the presence of 30% TFE.

Three-dimensional Structure in Solution—The three-dimensional structures of α-pheromone and its derived peptides (trans-X-P major form) in water and TFE mixtures were calculated based on the NMR data (Fig. 6). Table 1 shows the main structural statistics of the calculations. In H$_2$O, α-pheromone adopted a $\beta$-turn structure comprising residues $^4$WRGQ$^7$ (Fig. 6A). The turn is very well determined with a r.m.s.d. of 0.35 Å for the backbone atoms. In contrast, the N and C termini ($^1$WCT and $^8$PCW), particularly the side chains, were more flexible and disordered and failed to adopt defined conformations. No side-chain interactions were found to stabilize the turn. In H$_2$O/TFE (Fig. 6B), the NOE data of α-pheromone are compatible with a main globular conformation. This preferred fold was a well packed $\beta$-hairpin-like structure composed by two strands, $^1$WCT and $^8$PCW, linked through a $\beta$-turn formed by $^4$WRGQ$. The backbone and all side chains were very well determined (Table 1), and all the φ and ψ angles were located within the permitted region of the Ramachandran plot. The structure is stabilized by a medium distance hydrogen bond between the backbone NH of Gln$_7$ and the O of Trp$_4$. Interestingly, π–π interactions between the aromatic rings of Trp$_3$ and Trp$_8$ also appeared to play an important role in peptide stabilization. Regarding electrostatics, the positive Arg$_5$...
The central Gly⁶ and Gln⁷ residues are crucial for defined β-turn structure in solution. Solution structure of the preferred conformation of α-pheromone and related peptides in H₂O (A, C, E, and G) and H₂O/TFE mixture (B, D, F, and H) calculated by NMR. The superposition of the backbone of the best 20 structures in each family is represented in gray. Side chains of the energetically best structure in solution are in different colors depending on the sequence position; 1, blue; 2, red; 3, yellow; 4, cyan; 5, magenta; 6, gray; 7, violet; 8, orange; 9, green; 10, brown. Peptide termini are indicated by N and C. Residues are labeled with the one letter code and sequence number.

The scrambled peptide (trans Trp-Pro major form) failed to adopt a completely folded structure either in H₂O or in TFE/H₂O mixtures (Fig. 6, C and D). In water, the structure was...
mainly random with some tendency to form a β-turn centered at C5–C6. Compared with the wild type peptide, the conformational ensemble of the scrambled peptide in water was less ordered, the r.m.s.d. values were higher, and the hydrophobic side chains projected toward the solvent to a higher degree. In H2O/TFE, the central turn was stabilized, and the structural convergence was slightly better. Similar to the wild type peptide, the structure of the D-Ala1,2 analogue in H2O showed a turn centered at Trp4–Gln7 and was more compact in TFE even though it lacked the tryptophan at position Trp1 (Fig. 6, E and F). In this case the structure was only stabilized by the hydrogen bond of the turn between the backbone atoms of Trp4 and Gln7. By contrast, the D-Ala6,7 analog showed a strikingly different behavior. This sequence could not bend and behaved as a linear unfolded peptide without any secondary structure in either solvent (Fig. 6, G and H). The tendency of this analog to adopt a linear conformation is in accordance with previous studies reporting that substitution of two adjacent L-amino acids of a linear peptide by the corresponding D-isomers resulted in a reduced structure and increased water accessibility and flexibility (24).

In summary, the central β-turn, although populated to varying degrees, was detected in all tested peptides except the D-Ala6,7 analog, where the two consecutive D-Ala residues at positions 6 and 7 prevented bending. In general, all peptides were more ordered in TFE mixtures, suggesting that the cosolvent stabilizes the preformed conformations. Importantly, these results indicate that F. oxysporum α-pheromone could adopt different structures, more extended or flexible in the diluted conditions that favor induced fit recognition such as the extracellular environment.

**Discussion**

Elucidation of three-dimensional structures has been widely used to study structure-function relationships in small biologically active peptides (1, 25, 26). NMR analysis of S. cerevisiae α-pheromone suggested that the residues close to the N terminus are important for receptor activation, whereas those at the C terminus are implicated in receptor binding, and those in the center are required for orienting the signaling and binding domains of the pheromone (1). Moreover, the central region encompassing residues 7KPGQ10 was shown to form a transient Type II β-turn, which is required for activation of Ste2 (1). These results revealed that even a relatively short peptide contains regions associated with distinct biological functions. So far, however, the lack of a high atomic resolution structure has prevented atomic modeling of the pheromone-receptor interaction.

In this work we performed structure-function analysis of the mating pheromone α from the fungal pathogen F. oxysporum. Such information is crucial to understand how this small peptide interacts with and activates its cognate GPCR Ste2, which was recently shown to play a key role in chemotropic growth of F. oxysporum toward both α-pheromone and the host plant tomato (12, 27). Similar to most α-pheromones from filamentous ascomycetes (13), the F. oxysporum peptide is significantly shorter than its yeast counterpart. In addition, F. oxysporum α-pheromone contains two cysteines that could potentially be involved in inter- or intramolecular disulfide bonds, whereas the S. cerevisiae α-factor has two prolines, one of which was shown to be involved in the central β-turn structure (1).

**Structural Characteristics of F. oxysporum α-Pheromone—** Despite the difference in length, S. cerevisiae and F. oxysporum α-pheromones share a number of common features. Both are cationic peptides with a pI around 8. Both have, at the center of the ordered β-turn, a charged residue (Arg in F. oxysporum and Lys in S. cerevisiae) whose side chains point toward the solvent, suggesting that these residues may act as a molecular antenna, playing a key role in regulating potential intermolecular interactions. An interesting feature that differentiates *F. oxysporum* α-pheromone from its ortholog in *S. cerevisiae* is the presence of two cysteines, both of which were found to be reduced in *vitro*. Although it is currently unknown whether this result reflects the biological context of the pheromone-receptor interaction, the finding that the cysteines are not involved in the formation of intra- or interdisulfide bonds together with the high reactivity and known biological functions of the thiol groups opens the intriguing hypothesis that *F. oxysporum* α-pheromone may have a previously unreported function in redox regulated processes, whereas *S. cerevisiae* pheromone, which lacks the cysteines, would not. Cysteines can easily function as nucleophiles and thus could form covalent adducts with different molecules such as lipids or ADP, a hypothesis that might be of interest for future investigations.
Three-dimensional Structure of α-Pheromone Reveals the Presence of a Central β-Turn Essential for Chemoattractant Activity—The three-dimensional structure of F. oxysporum α-pheromone resembles that of the longer α-factor of S. cerevisiae. Both peptides contain a central β-turn with a cationic amino acid residue, Lys in S. cerevisiae and Arg in F. oxysporum. A low resolution model for S. cerevisiae α-factor bound to Ste2 was previously proposed based on biochemical and biophysical data (28). The model places α-factor bent around the Pro-Gly center of the peptide, with the Lys side chain facing away from the transmembrane domains and interacting with a binding pocket formed by the extracellular loops of the receptor. A similar model could be proposed for F. oxysporum α-pheromone in the interaction with its cognate receptor. Although the structure of the peptide in the complex could be modified with respect to the free form (i.e. X-Pro cis/trans equilibrium) by an interacting induced fit, the presence of a preferred conformation in solution should be energetically favorable for the process.

Previous studies revealed that α-pheromone elicits a robust chemotropic response in germ tubes of F. oxysporum, which is dependent on the cognate GPCR Ste2 (12, 29). Here we found that chemoattraction requires the Gly⁶ and Gln⁷, but not the Trp¹ and Cys² residues of α-pheromone. This is in line with the structural role of Gly⁶ and Gln⁷ in the maintenance of the three-dimensional structure and strongly suggests that activation of Ste2-mediated chemotropic growth depends on the secondary β-turn structure of α-pheromone rather than on its amino acid composition or pl. The idea is further supported by the finding that the N-terminal Trp¹ and Cys² residues which play no substantial role in the structure of α-pheromone are not required for chemoattraction. Indeed, alanine substitutions at the N terminus of α-pheromone in S. cerevisiae and C. albicans led to increased pheromone activity, suggesting that this region could have an inhibitory function role in receptor-mediated signaling (1, 10).

Besides chemoattraction, α-pheromone triggers a cell cycle and growth arrest in S. cerevisiae and C. albicans (30, 31). Here we found that hyphae exposed to α-pheromone contained fewer cell compartments and nuclei, indicating that similar to S. cerevisiae and C. albicans (5, 32), α-pheromone inhibits cell division in F. oxysporum. In both S. cerevisiae and C. albicans α-pheromone inhibits cell cycle progression via Ste2-mediated activation of a dedicated signaling cascade (33–36). Unexpectedly, pheromone-mediated growth inhibition of F. oxysporum germ tubes does not require the cognate receptor Ste2. These results suggest the presence of additional, currently unknown α-pheromone ligands or receptors in this species, with differential roles in regulation of cell growth and chemotropism. Interestingly, the growth inhibitory activity was abolished in the D-Ala¹⁹² and the scrambled analogs, both of which lack a tryptophan residue at the N or the C terminus, respectively. Aromatic residues, particularly tryptophan, have been reported to undergo specific interactions with lipid moieties resulting in anchoring to the membrane (37). The presence of the tryptophan residues Trp¹ and Trp¹⁰ at the two termini suggests that they could act as a structural clamp during a potential membrane interaction linked to the growth inhibitory activity of the α-pheromone.

In summary our results establish that F. oxysporum α-pheromone adopts a defined secondary structure and, despite its shorter size, contains discrete regions involved in different biological processes such as polarity reorientation and cell cycle control. We consider it likely that these findings apply to α-pheromones from other ascomycetes. The signaling functions of fungal sex pheromones might thus be more complex than previously anticipated.

Experimental Procedures

Fungal Strains and Culture Conditions—F. oxysporum f. sp. lycopersici strain 4287 (race 2) was used in all experiments. Generation and molecular characterization of the F. oxysporum FoH1-ChFP strain constitutively expressing ChFP fused to histone 1 and the ste2Δ mutant was described previously (12, 38). Fungal strains were stored as microconidial suspensions with 30% glycerol at −80 °C. Strain culture and microconidia production were performed as previously described (39).

Quantification of Fungal Chemotropism—To measure the directed growth of F. oxysporum germ tubes toward gradients of synthetic peptides a chemotropic plate assay was used. Plate preparation, chemoattractant application, and scoring were performed as described (12). For each concentration of test compound 5, independent batches of cells (n = 100 cells per batch) were scored. Each set of measurements was repeated in two independent experiments. Statistical analysis was conducted using t test. Directed growth was quantified with an Olympus binocular microscope (Olympus Iberia, Barcelona, Spain) (200× magnification).

For the synthesis of the scrambled peptide, 10 different scrambled α-pheromone sequences were generated using the free Scrambleware software (Mimotopes, Victoria, Australia). Among these, a sequence that did not carry the conserved N-terminal Trp¹-Cys² and the central Gly⁶-Gln⁷ dipeptide residues was selected for synthesis.

Synthetic F. oxysporum α-pheromone (WCTWRGQPCW), a scrambled version thereof (WRWPCCWGQT), and the di-alanine substituted analogues D-Ala¹⁹² and D-Ala⁶⁷ were obtained from GenScript (Piscataway, NJ). Lyophilized peptides were dissolved in 50% (v/v) methanol and assayed at the indicated concentrations.

Concentrations of synthetic α-pheromone and its analogs varying from 8 μM to 4 mM were chosen to cover the complete range of directional responses toward the chosen peptides. A significant chemotropic response was typically observed at a concentration of α-pheromone of 95 μM, which is higher than in some reports published in S. cerevisiae (40, 41), albeit similar or lower to those described in Candida spp. to elicit pheromone response phenotypes (10, 35, 36, 42). The discrepancy in pheromone concentration may be due to the different experimental setup of the chemotropism assays used in Fusarium and Saccharomyces (in our study pheromone gradients were established over mm rather than μm distances), differences between biological systems (multicellular versus unicellular organism), pheromone adsorption to the agar surface, or different temperature conditions.
Quantification of Germ Tube Length, Cell Compartments, and Nuclei—Freshly obtained microconidia of the *F. oxysporum* FoH1-CHFP strain and of the ste2Δ mutant were embedded in 5 ml of water-agarose (WA; 2%, w/v) (Pronadisa, Madrid, Spain) at a final concentration of 2.5 × 10⁶ conidia ml⁻¹ and poured into a standard Petri dish. A central scoring line was drawn on the bottom of the plate, and a parallel well was cut into the WA layer at 5 mm distance from the scoring line and filled with 40 µl of either 378 µM or 756 µM concentrations of the tested peptide. As a negative control, α-pheromone was treated with 1 mg ml⁻¹ trypsin (Sigma) overnight at 37 °C and incubated for 20 min at 100 °C to remove protease activity. Plates were maintained at 28 °C in the dark for 14 h. A 1 × 1-cm square of WA was then transferred from the plate to a microscope glass slide, and the position of the scoring line and the chemoattractant well was marked with two parallel lines on the bottom of the glass slide. To visualize septa, a 10-µl drop of 0.005% (w/v) calcifluor white (CFW) (Sigma) was added on the WA, a coverslip was applied on top, and samples were incubated for 5 min in the dark. Staining of nuclei in the ste2Δ mutant was accomplished by supplementing the CFW solution with propidium iodide (12.5 µg ml⁻¹) (Sigma) and RNase A (20 µg ml⁻¹) (Sigma). Observation of red fluorescent nuclei and CFW-stained septa was carried out using a Zeiss Axio Imager M2 microscope (Zeiss, Barcelona, Spain) equipped with an Evolve Photometrics EM512 digital camera (Photometrics Technology, Tucson, AZ). Examination using epifluorescence microscopy was performed on a Zeiss M2 microscope (Zeiss, Barcelona, Spain) equipped with an Axiovision 4.8 software (Zeiss). The SPARKY software (48). The 13C resonances were identified on the basis of the correlations between the protons and the bound carbon atoms present in the 13C,1H HSQC spectra.

Far-UV spectra were recorded on a Jasco J-715 spectropolarimeter (Easton, MD) in 0.1-cm optical path quartz cells. The results are expressed as mean residue weight ellipticities in units of degree × cm² × dmol⁻¹. The mean residue mass employed was 132.2 Da, calculated from their respective amino acid sequences. Peptides were dissolved in 50 mM sodium phosphate (with or without 30% TFE) (pH 7.0) at a concentration of 80 µM. Ellipticity at 222 nm was also recorded as a function of different pH values (from 3.0 to 10.5) and salt concentrations (NaCl, CaCl₂, or MgCl₂) in the range of 0–200 mM (45).

NMR—NMR samples were prepared at a concentration of 0.1–0.5 mM concentrations of peptide in H₂O and H₂O/TFE (7/3 v/v) (TFE D₃ Eurisotop, France) and pH 5.0. Spectra were recorded at 5 °C and 25 °C on a Bruker spectrometer equipped with a cryo-probe and operated at 800 MHz for the proton.

All peptides exhibited limited solubility in H₂O and were generally more soluble in H₂O/TFE. In some cases this made the evaluation of weak NOE signals and the complete assignment of signals from the *cis* isomer in the equilibrium more difficult.

Phase-sensitive two-dimensional correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY), and nuclear Overhauser enhancement spectroscopy (NOESY) spectra were recorded by standard techniques using the time-proportional phase incrementation mode. The water signal was suppressed by either presaturation or by using a 3–9–19 pulse sequence. TOCSY spectra were obtained by using a DIPSI2 pulse sequence with a 60-ms mixing time and with a z filter spin-lock sequence. The NOESY mixing time was 150 ms. 13C,1H heteronuclear single quantum coherence (HSQC) spectra were recorded at 13C natural abundance. Data were processed with the standard TOPSPIN program (Bruker Biospin, Karlsruhe, Germany). The two-dimensional data matrices were multiplied by a square-sine-bell window function with the corresponding shift optimized for every spectrum and zero-filled to 2 × 1 K complex matrices before Fourier transformation. Baseline correction was applied in both dimensions. The 13C δ-values were indirectly referenced by using the IUPAC-IUB recommended H1/13C chemical shift ratio (46).

Assignments of the 1H spectra in both solvents were obtained following the sequential assignment protocols (47) with the help of the SPARKY software (48). The 13C resonances were identified on the basis of the correlations between the protons and the bound carbon atoms present in the 13C,1H HSQC spectra.

Structure calculations of the major trans X-P bond conformers were performed with CYANA 2.1 program (49). NOE integrated cross-peaks were translated into distance restraints, and the Φ and Ψ dihedral angle restraints were obtained using TALOS + websolver (50). Typically 200 structures were calculated using a standard protocol. The lists of distance constraints were checked with the corresponding NOESY spectra, and ambiguous constraints were relaxed or removed in order to generate a final list used as input for a standard simulated annealing CYANA 2.1 calculation. The 20 conformers with the lowest target function values were selected. The structural ensembles were visualized and examined using MOLMOL (51) and PyMOL (52).
Fusarium Oxysporum α-Pheromone Structure Activity

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