Identification of biosynthetic intermediates for the mating hormone α2 of the plant pathogen *Phytophthora*

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ABSTRACT

The heterothallic group of the plant pathogen *Phytophthora* can sexually reproduce between the cross-compatible mating types A1 and A2. The mating hormone α2, produced by A2 mating type and utilized to promote the sexual reproduction of the partner A1 type, is known to be biosynthesized from phytol. In this study, we identified two biosynthetic intermediates, 11- and 16-hydroxyphytols (**1** and **2**), for α2 by administering the synthetic intermediates to an A2 type strain to produce α2 and by administering phytol to A2 strains to detect the intermediates in the mycelia. The results suggest that α2 is biosynthesized by possibly two cytochrome P450 oxygenases via two hydroxyphytol intermediates (**1** and **2**) in A2 hyphae and secreted outside.

Keywords: plant pathogen, *Phytophthora*, mating hormone, sexual reproduction, biosynthesis

The genus *Phytophthora*, a group of filamentous fungus-like oomycetes, includes well-known plant pests, some of which are deleterious to a broad range of economically and ecologically important plant species (Erwin et al. 1996). Among them *P. infestans* is most notorious as the causal agent for the Irish Potato Famine during the mid-1840s, leading to mass starvation. Sexual reproduction is one of the most attractive biological events of *Phytophthora*, where the female and male organs fuse and are fertilized to produce sexual spore so-called oospores. The heterothallic (self-sterile) members require pairing of two compatibility types, mating types A1 and A2, for their sexual reproduction (Savage et al. 1968), and the hormonal factors termed α1 (secreted by A1) and α2 (secreted by A2) were predicted as the triggers for the *Phytophthora* sexual reproduction (Ko, 1978). Since the resulting oospores provide advantages such as a sustainable double-walled structure and the potential of accelerated evolution (Judelson et al. 2005), research on the mechanisms behind the *Phytophthora* sexual reproduction could be a key to controlling this agricultural pest. After long-term efforts, the mating hormones α1 (Qi, et al. 2005) and α2 (Ojika et al. 2011) were chemically identified (Fig. 1). The hormone α2 was found to be biosynthesized from phytol by A2 mating type and then converted to α1 by A1 mating type (Ojika et al. 2011). Since the biosynthesis of α2 is the starting point of the sexual reproduction of *Phytophthora*, the mechanism of α2 biosynthesis is worthy to be elucidated. Since α2 is regarded as 11,16-dihydroxyphytol, this hormone could be biosynthesized by cytochrome P450s via 11- or/and 16-hydroxyphytols (Fig. 2). The aim of the present study is to identify these plausible biosynthetic intermediates, which would

partially contribute to establish the molecular basis of the *Phytophthora* sexual reproduction system in the future.

(Figure 1)
(Figure 2)

Materials and methods

General

IR spectra were measured on a Jasco IR-4100 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on Jeol ECS400 spectrometer using the residual solvent peak at $\delta = 7.26$ (for ^{1}H) or $\delta = 77.0$ (for ^{13}C) as an internal standard. High resolution MS data were recorded on Jeol JMS-T100 and Agilent 6530 spectrometers. Column chromatography was performed with silica gel Wakogel-C200. Flush column chromatography was performed with Kanto Silica Gel 60 (spherical).

Phytophthora strains and cultivation.

P. nicotianae ATCC 38606 (A2 mating type) was purchased from the ATCC (YA, USA). *P. nicotianae* NBRC 33192 and *P. capsici* NBRC 8386 were obtained from the Biological Resource Centre, National Institute of Technology and Evaluation (NBRC, Chiba, Japan). A loopful of mycelia of a *Phytophthora* strain was inoculated onto a Petri plate (ϕ 9 cm) containing 20% V8 juice, 0.3% $CaCO_3$, and 2% agar, and precultured at 25 °C and 60% humidity for 7-9 days in the dark by using an Environmental Chamber KCL-2000 (EYELA, Tokyo, Japan). The mycelia on V8-agar medium were used for the next experiments.

LC-MS analysis of α hormones and α2 intermediates (1 and 2)

A standard solution (1-10 μL, 0.1-1 μM) of α1, α2, and synthetic hydroxyphytols (*mix*-**1** and *mix*-**2**) in 50-80% MeCN or a solution (1-10 μL) of *Phytophthora* culture extracts in an appropriate concentration and solvent was injected to a 1100 High-Performance Liquid Chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA) connected to an Agilent 6520 Accurate-Mass Q-TOF spectrometer. The HPLC conditions were as follows: column Poroshell 120 EC-C18 (ϕ 3.0 × 50 mm, Agilent Technologies); mobile phase (i) 75-100% (0-12.5 min) MeOH-10 mM ammonium formate or (ii) 60-100% (0-20 min) MeOH-10 mM ammonium formate; flow rate 0.2 mL min^{-1}. The MS conditions were as follows: ESI-

TOF, positive ion mode, gas temperature 300 °C, dry gas 7 L min^{-1}, nebulizer 35 psig, and capillary voltage 3500 V. The hormone α2 and intermediates (**1**, **2**) were detected on extracted ion chromatographs (EIC) by using $[M+Na]^+$ ion masses of 351.287, 335.292, and 335.292 at the retention times of 5.5/12.6, 10.7/18.1, and 11.0/18.5 min, respectively, in the conditions of (i)/(ii) mentioned above. The EIC peak areas were used for quantitative analysis.

Synthesis of stereoisomeric mixtures of 11-hydroxyphytols (*mix*-1)

10-Hydroxy-6,10,14-trimethylpentadecan-2-yl acetate (4) and 14-hydroxy-6,10,14-trimethylpentadecan-2-yl acetate (5). To a stirred solution of $RuCl_3$•xH_2O (5.2 mg, 25 μmol), $KBrO_3$ (250 mg, 1.5 mmol), and pyridine (4.0 μL, 50 μmol) in water (4 mL) was added a solution of **3** (Tori et al. 1985) (157 mg, 500 μmol) in MeCN (4 mL) at room temperature (McNeill et al. 2010). The mixture was stirred for 3 h at 60 °C. After cooling to room temperature, saturated Na_2SO_3 aq. solution was added carefully to the mixture. The aqueous layer was extracted with EtOAc, and the combined organic layer was washed with water and brine. The organic layer was dried with Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography with hexane-EtOAc (10:1) to give **4** (16.5 mg, 10%, diastereomeric mixture) and **5** (19.8 mg, 12%, diastereomeric mixture) as colorless oils, and the starting material **3** (75.8 mg, 48%) was recovered.

4: IR (film): ν_{max} = 3500(br), 1738 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 0.86 (d, *J* = 6.6 Hz, 3H), 0.88 (d, *J* = 6.6 Hz, 6H), 1.05-1.48 (m, 19H), 1.15 (s, 3H), 1.20 (d, *J* = 6.5 Hz, 3H), 1.55 (m, 1H), 2.03 (s, 3H), 4.89 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 19.9, 20.0, 21.4 (2C), 21.7, 22.6, 22.8/22.9 (total 1C), 27.0, 27.9, 32.6, 36.2, 36.7, 37.3, 37.6, 39.6, 42.2, 71.0, 72.8, 170.8; HR-DART-MS (*m*/*z*): $[M+H]^+$ calcd. for $C_{20}H_{41}O_3$ 329.3056, found 329.3058.

5: IR (film): ν_{max} = 3440(br), 1738 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 0.84-0.91 (m, 6H), 1.04-1.85 (m, 20H), 1.20 (s, 6H), 2.02 (s, 3H), 2.13-2.64 (m, 4H), 4.89 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 19.64, 19.69, 20.0, 21.3, 21.4, 21.8, 22.6, 22.7, 22.8, 24.4, 24.8, 26.8, 26.9, 28.0, 29.3, 32.6, 32.8, 36.2, 36.4, 36.8, 37.3, 37.4, 37.5, 37.6, 39.3, 41.5, 42.2, 42.3, 44.3, 70.8, 71.1, 72.6, 170.8 (The discrepancy between the number of signals and the carbon number of the compound reflects the presence of diastereomers); HR-DART-MS (*m*/*z*): $[M+H]^+$ calcd. for $C_{20}H_{41}O_3$ 329.3056, found 329.3046.

10-Hydroxy-6,10,14-trimethylpentadecan-2-one (6). To a stirred solution of **4** (1.84 g, 5.61 mmol) in MeOH (20 mL) was added K_2CO_3 (776 mg, 5.60 mmol). After stirring for 26 h, sat. NH_4Cl aq. was added to the mixture. The aqueous phase was extracted with EtOAc, and the

combined organic layer was washed with water and brine. The organic layer was dried with Na_2SO_4, and concentrated. The residue was dissolved in CH_2Cl_2 (40 mL), and the resulting solution was cooled to 0 °C. To the solution powdered MS4A (2 g) and PCC (1.32 g, 6.14 mmol) were added portionwise. After stirring for 90 min, the mixture was diluted with ether and filtered through a pad of silica gel. The filtrate was concentrated, and the residue was purified by column chromatography with hexane-EtOAc (5:1) to give **6** as a colorless oil (921 mg, 58%, diastereomeric mixture). IR (film): ν_{max} = 3490(br), 1714 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ = 0.86 (d, *J* = 6.9 Hz, 3H), 0.87 (d, *J* = 6.9 Hz, 6H), 1.03-1.67 (m, 19H), 1.14 (s, 3H), 2.13 (s, 3H), 2.39 (t, *J* = 7.3 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 19.2, 20.9, 21.0, 21.3, 22.3, 26.6, 27.6, 29.5, 32.3, 36.1, 37.1, 39.2, 41.7, 41.8, 43.6, 72.2, 208.9; HR-DART-MS (*m*/*z*): $[M+H]^+$ calcd. for $C_{18}H_{37}O_2$ 285.2794, found 285.2781.

Ethyl (*E*)-11-hydroxy-3,7,11,15-tetramethylhexadec-2-enoate (7). To a suspension of NaH (60% in oil, washed with hexane, 76 mg, 3.2 mmol) in THF (3 mL) was added triethyl phosphonoacetate (652 μL, 3.17 mmol) under Ar. After stirring for 1h, the mixture was cooled to 0 °C, and a solution of **6** in THF (1 mL) was added to the mixture. The mixture was stirred for 20 h at room temperature and quenched with sat. NH_4Cl aq. The aqueous phase was extracted with EtOAc, and the combined organic layer was washed with water and brine. The organic layer was dried with Na_2SO_4, and concentrated. The residue was purified by column chromatography with hexane-EtOAc (10:1) to give **7** as a colorless oil (61.3 mg, 85%, *E*:*Z*=4:1, diastereomeric mixture). A sample of pure (*E*)-isomer was partially obtained by the column chromatography. IR (film): ν_{max} = 3450(br), 1717, 1650 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ = 0.86 (d, *J* = 6.9 Hz, 3H), 0.87 (d, *J* = 6.9 Hz, 6H), 1.05-1.58 (m, 19H), 1.15 (s, 3H), 1.28 (t, *J* = 7.3 Hz, 3H), 2.11 (t, *J* = 7.8 Hz, 2H), 2.15 (d, *J* = 0.9 Hz, 3H), 4.14 (q, *J* = 7.3 Hz, 2H), 5.65 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 14.3, 18.7, 19.6, 21.3, 21.7, 22.6, 24.8, 27.0, 27.9, 32.6, 36.5, 37.5, 39.5, 41.2, 42.2, 59.4, 72.8, 115.5, 160.3, 166.9; HR-DART-MS (*m*/*z*): $[M+H]^+$ calcd. for $C_{22}H_{43}O_3$ 355.3212, found 355.3198.

11-Hydroxyphytol (*mix*-1). To a suspension of LAH (23 mg, 610 μmol) in ether (1 mL) was added a solution of **7** (30 mg, 85 μmol) in ether (1 mL) at 0 °C under Ar. After stirring for 90 min, water (23 μL), 15% NaOH aq. (23 μL), and water (23 μL) were added to the mixture. The resulting mixture was filtered through a pad of Celite, and the filtrate was concentrated. The residue was purified by flash column chromatography with hexane-EtOAc (3:1) to give *mix*-**1** as a colorless oil (23.6 mg, 89%, *E*:*Z*=4:1, diastereomer mixture). A sample of pure (*E*)-isomer was partially obtained by the flash column chromatography. IR (film): ν_{max} =

3348(br), 2932, 2867, 1464, 1377, 1307, 1247, 1154, 1083, 1016 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ = 0.86 (d, *J* = 6.9 Hz, 3H), 0.87 (d, *J* = 6.9 Hz, 6H), 1.03-1.48 (m, 15H), 1.15 (s, 3H), 1.55 (m, 1H), 1.66 (s, 3H), 1.99 (t, *J* = 7.3 Hz, 2H), 2.11 (t, *J* = 7.8 Hz, 2H), 2.15 (d, *J* = 0.9 Hz, 3H), 4.15 (d, *J* = 6.8 Hz, 2H), 5.40 (t, *J* = 6.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 16.2, 19.7, 21.3, 21.7, 22.6(x2), 25.0, 27.0, 27.9, 32.5, 36.4, 37.5, 39.5, 39.7, 42.1(x2), 59.4, 72.9, 123.2, 140.0; HR-DART-MS (*m*/*z*): $[M+H]^+$ calcd. for $C_{20}H_{41}O_2$ 313.3107, found 313.3100.

Synthesis of stereoisomeric mixtures of 16-hydroxyphytols (*mix*-2)

Ethyl (*E*)-14-acetoxy-2,6,10-trimethylpentadec-2-enoate (10). To a solution of **5** (1.96 g, 5.98 mmol) in pyridine (18 mL) was added $POCl_3$ (1.19 mL, 8.96 mmol) at –20 °C under Ar. After stirring for 16 h, sat. NH_4Cl aq. was added to the mixture, and the aqueous phase was extracted with EtOAc. The combined organic layer was washed with water and brine. The organic layer was dried with Na_2SO_4, and concentrated. The residue was purified by column chromatography with hexane-EtOAc (3:1) to give **8** as a colorless oil (1.37 g, 74%, *endo*:*exo*=7:2). This was employed in the next step without further purification. To a solution of **8** (150 mg, 483 mmol) in acetone/water (5:2, 3 mL) were added NMO (113 mg, 966 μmol) and a solution of 1% OsO_4 in *t*-BuOH (1.5 mL). After stirring for 50 h, the mixture was poured into water, and the aqueous phase was extracted with CH_2Cl_2. The combined organic layer was washed with water and brine. The organic layer was dried with Na_2SO_4, and concentrated. The residue was purified by column chromatography with hexane-EtOAc (1:1) to give **9** as a colorless oil (113 mg, 68%). This was employed in the next step without further purification. To a solution of **9** (75.8 mg, 220 μmol) in THF/water (4:1, 1 mL) was added $NaIO_4$ (141 mg, 661 μmol) at 0 °C under Ar. After stirring for 2 h, brine was added to the mixture, and the aqueous phase was extracted with EtOAc. The combined organic layer was washed with water and brine. The organic layer was dried with Na_2SO_4, and concentrated. The residue was dissolved in CH_2Cl_2 (1 mL), and (1-ethoxycarbonylethylidene)triphenylphosphorane (239 mg, 660 μmol) was added to the solution. After stirring for 14 h, the mixture was concentrated, and the residue was purified by flash column chromatography with hexane-EtOAc (20:1) to give **10** as a colorless oil (30.3 mg, 37%, diastereomeric mixture). IR (film): ν_{max} = 1738, 1713, 1652 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ = 0.83 (d, *J* = 6.4 Hz, 3H), 0.88 (d, *J* = 6.4 Hz, 3H), 1.02-1.58 (m, 16H), 1.20 (d, *J* = 6.4 Hz, 3H), 1.29 (t, *J* = 6.4 Hz, 3H), 1.83 (s, 3H), 2.02 (s, 3H), 2.16 (m, 2H), 4.18 (q,

J = 6.4 Hz, 2H), 4.89 (sex, J = 6.4 Hz, 1H), 6.74 (dt, J = 1.4, 7.3 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 14.2, 19.4, 19.5, 19.89, 19.93, 21.3, 22.8, 24.3, 29.7, 32.3, 32.6, 35.1, 36.2, 36.7, 37.1, 37.2, 60.0, 70.96, 70.99, 121.0, 149.6, 166.7, 170.7 (The discrepancy between the number of signals and the carbon number of the compound reflects the presence of diastereomers); HR-DART-MS (*m*/*z*): $[M+H]^+$ calcd. for $C_{22}H_{41}O_4$ 369.3005, found 369.3001.

Ethyl 14-acetoxy-2,6,10-trimethylpentadecanoate (11). To a solution of **10** (47.1 mg, 128 μmol) in EtOAc (1 ml) was added 10% Pd/C (11 mg) under Ar. After replacing the argon in the flask with hydrogen, the mixture was stirred for 2 h. The mixture was filtered through a pad of Celite, and the filtrate was concentrated. The residue was purified by column chromatography with hexane-EtOAc (10:1) to give **11** as a colorless oil (46.0 mg, 97%, diastereomeric mixture). IR (film): ν_{max} = 1738 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 0.81 (d, J = 6.9 Hz, 6H), 0.99-1.66 (m, 20H), 1.10 (d, J = 6.9 Hz, 3H), 1.17 (d, J = 6.4 Hz, 3H), 1.22 (t, J = 6.9 Hz, 3H), 1.99 (s, 3H), 2.24-2.42 (m, 1H), 4.09 (q, J = 6.9 Hz, 2H), 4.86 (sex, J = 6.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 14.2, 17.0, 17.1, 19.55, 19.57, 19.86, 19.91, 21.3, 22.8, 24.4, 24.57, 24.60, 25.2, 26.5, 32.58, 32.62, 34.06, 34.08, 34.3, 36.2, 36.5, 36.72, 36.74, 36.8, 37.3, 39.5, 59.9, 60.0, 70.94, 70.96, 170.6, 173.7, 176.8 (The discrepancy between the number of signals and the carbon number of the compound reflects the presence of diastereomers); HR-DART-MS (*m*/*z*): $[M+H]^+$ calcd. for $C_{22}H_{43}O_4$ 371.3161, found 371.3154.

Ethyl 2,6,10-trimethyl-14-oxopentadecanoate (12). To a solution of **11** (46.0 mg, 124 μmol) in EtOH (1 mL) was added K_2CO_3 (8.6 mg, 62 μmol). After stirring for 24 h at 40 °C, an additional K_2CO_3 (8.6 mg, 62 μmol) was added to the mixture. After stirring for 4 h, the mixture was allowed to cool to room temperature, and the mixture was poured into sat. NH_4Cl aq. The aqueous phase was extracted with EtOAc. The combined organic layer was washed with water and brine. The organic layer was dried with Na_2SO_4, and concentrated. The residue was dissolved in CH_2Cl_2 (3 mL), and cooled to 0 °C. To the solution powdered MS4A (58 mg) and PCC (39.0 mg, 181 μmol) were added portionwise. After stirring for 3 h, the mixture was diluted with ether, and filtered through a pad of silica gel. The filtrate was concentrated, and the residue was purified by column chromatography with hexane-EtOAc (10:1) to give **12** as a colorless oil (36.6 mg, 91%, diastereomeric mixture). IR (film): ν_{max} = 1734, 1720 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 0.82 (d, J = 6.9 Hz, 3H), 0.84 (d, J = 6.4 Hz, 3H), 1.00-1.68 (m, 18H), 1.12 (d, J = 7.3 Hz, 3H), 1.23 (t, J = 6.9 Hz, 3H), 2.11 (s, 3H), 2.25-2.42 (m, 3H), 4.11 (q, J = 6.9 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 14.2, 17.0, 17.1, 19.5, 19.6, 21.4,

22.8, 24.4, 24.59, 24.63, 25.3, 26.6, 29.8, 32.60, 32.63, 34.08, 34.10, 34.4, 36.4, 36.6, 36.8, 37.2, 37.3, 39.5, 44.1, 60.0, 60.1, 173.8, 176.9, 209.2 (The discrepancy between the number of signals and the carbon number of the compound reflects the presence of diastereomers); HR-DART-MS (*m*/*z*): $[M+H]^+$ calcd. for $C_{20}H_{39}O_3$ 327.2899, found 327.2902.

Diethyl (*E*)-3,7,11,15-tetramethylhexadec-2-enedioate (13). To a solution of triethyl phosphonoacetate (74.5 μL, 373 μmol) in THF (1 mL) was added a solution of *n*-BuLi in hexane (1.6 M, 175 μL, 280 μmol) at 0 °C under Ar. After stirring for 15 min at room temperature, a solution of **12** (15.2 mg, 46.6 μmol) in THF (1 mL) was added to the mixture at 0 °C. The mixture was stirred for 43 h and poured into sat. NH_4Cl aq. The aqueous phase was extracted with EtOAc, and the combined organic layer was washed with water and brine. The organic layer was dried with $MgSO_4$, and concentrated. The residue was purified by flash column chromatography with hexane-EtOAc (30:1) to give an isomeric mixture containing **13** as a colorless oil (5.4 mg, 29%, *E*:*Z*=5:2). The starting material **12** (8.7 mg, 57%, diastereomeric mixture) was recovered. A sample of pure *E* isomer (**13**) was obtained by the flash column chromatography. IR (film): ν_{max} = 1736, 1718, 1649 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ = 0.83 (d, *J* = 6.4 Hz, 3H), 0.85 (d, *J* = 6.0 Hz, 3H), 1.01-1.68 (m, 19H), 1.13 (d, *J* = 6.9 Hz, 3H), 1.25 (t, *J* = 6.9 Hz, 3H), 1.28 (t, *J* = 7.2 Hz, 3H), 2.11 (m, 1H), 2.15 (d, *J* =1.0 Hz, 3H), 2.41 (m, 1H), 4.13 (m, 4H), 5.66 (d, *J* = 1.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 14.26, 14.32, 17.0, 17.1, 18.7, 19.6, 24.4, 24.6, 24.8, 25.1, 32.6, 34.12, 34.13, 36.5, 36.8, 36.8, 37.26, 37.31, 37.32, 39.6, 41.2, 59.4, 60.0, 115.4, 160.3, 166.9, 176.9 (The discrepancy between the number of signals and the carbon number of the compound reflects the presence of diastereomers); HR-DART-MS (*m*/*z*): $[M+H]^+$ calcd. for $C_{24}H_{45}O_4$ 397.3318, found 397.3315.

16-Hydroxyphytol (*mix*-2). To a solution of **13** (1.8 mg, 4.6 μmol) in CH_2Cl_2 (1 mL) was added a solution of DIBAL in hexane (1.0 M, 24 μL, 24 μmol) at –78 °C under Ar. After stirring for 4.5 h, an additional solution of DIBAL in hexane (1.0 M, 12 μL, 12 μmol) was added to the mixture. After stirring for 90 min at 0 °C, the mixture was poured into sat. Rochelle salt aq. The aqueous phase was extracted with CH_2Cl_2, and the combined organic layer was washed with water and brine. The organic layer was dried with $MgSO_4$, and concentrated. The residue was purified by flash column chromatography with hexane-EtOAc (3:1) to give *mix*-**2** as a colorless oil (1.0 mg, 70%, diastereomer mixture). IR (film): ν_{max} = 3320(br), 2926, 2856, 1463, 1377, 1159, 1010 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ = 0.85 (d, *J* = 6.9 Hz, 6H), 0.92 (d, *J* = 6.9 Hz, 3H), 1.02-1.47 (m, 18H), 1.54 (br, 2H), 1.61 (m, 1H),

1.67 (s, 3H), 1.99 (t, J =7.3 Hz, 2H), 3.42 (dd, J = 6.6, 10.5 Hz, 1H), 3.51 (dd, J = 6.0, 10.5 Hz, 1H), 4.15 (d, J = 7.0 Hz, 2H), 5.41 (br.t, J = 7.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 16.2, 16.55, 16.64, 19.7, 19.8, 24.3, 24.41, 24.44, 25.1, 32.66, 32.70, 32.74, 33.4, 33.5, 35.8, 36.6, 37.2, 37.31, 37.32, 37.4, 37.32, 39.8, 59.4, 68.4, 68.5, 123.1, 140.3 (The discrepancy between the number of signals and the carbon number of the compound reflects the presence of diastereomers); HR-ESI-MS (m/z): $[M+H]^+$ calcd. for $C_{20}H_{41}O_2$ 313.3101, found 313.3098.

Quantitative analysis of intermediates (1, 2) in *Phytophthora* cultures

Two *Phytophthora* A2 mating type strains, were used for evaluating the productivity of **1** and **2**. A whole agar plate (9 cm diameter) of precultured *P. nicotianae* NBRC 33192 was soaked in 0.1% V8 juice medium (200 mL) supplemented with an ethanol solution of phytol (200 μg/200 μL) in a 500-mL Erlenmeyer flask, and incubated at 27 °C, 80 rpm for 1-3 d in a BioShaker BR-23FP (TAITEC, Koshigaya, Saitama, Japan). In the case of *P. capsici* NBRC 8386, incubation was carried out in ultrapure water for 2-4 days. The supernatant was separated from mycelia and agars by decantation, and extracted with EtOAc (100 mL, 2 times). The mycelia and agar were shaken in MeCN (100 mL) at room temperature, 100 rpm for 1 h and then removed by decantation. The EtOAc and MeCN extracts were concentrated to yield supernatant and mycelial extracts, respectively. These were dissolved in MeOH (2 mL), and a portion (10 μL, equiv. to 1 mL-culture broth) was used for LC/MS analysis with the mobile phase (i).

Quantitative analysis of intermediates (1, 2) in a large-scale culture of *P. capsici*

Seventeen pieces (1 cm square each) of precultured *P. capsici* NBRC 8386 mycelia on V8-agar medium were inoculated into 20% V8 juice medium (1.7 L) supplemented with an ethanol solution of phytol (1.7 mg/1.7 mL) in a 3-L Erlenmeyer flask. A pair of cultures (3.4 L in total) were incubated at 27 °C, 80 rpm for 6 d in a shaking incubator TB-98RS (Takasaki Scientific Instruments, Saitama, Japan). The cultures were filtered by suction to separate into supernatant and mycelia. The mycelia (wet weight 55 g in total) were stored at –30 °C until use. A supernatant was extracted with EtOAc (1 L, 0.5 L), and the extracts were combined and washed with water (150 mL). The organic layer was concentrated and combined with another batch of culture, yielding the supernatant extract (311 mg) from 3.4 L cultures. The mycelia were homogenized in MeOH (400 mL) by a blender and filtered by suction. The mycelial cake was washed with MeOH (100 mL, twice), and combined filtrates were

concentrated. The residue was dissolved in 90% MeOH (200 mL) and extracted with hexane (200 mL, twice). The hexane layers were combined and concentrated to give mycelial hexane extract (0.81 g). The supernatant extract was dissolved in MeCN (3.4 mL) and a portion (10 μL) was diluted with 75% MeCN (990 μL). A portion (2.5 μL, equiv. to 25 μL-culture broth) of the resulting solution was injected to the LC/MS apparatus. On the other hand, the mycelial hexane extract was dissolved in MeOH (3.4 mL) and a portion (0.1 mL) was passed through a Sep-Pak C18 cartridge (100 mg) with MeOH (1 mL). A portion (2.5 μL, equiv. to 250 μL-culture broth) of the resulting solution was injected to the LC/MS apparatus. The mobile phase (ii) was used for LC.

Conversion of synthetic intermediates (*mix*-1, *mix*-2) to α2 by *P. nicotianae*

Two pieces (1 cm square each) of precultured *P. nicotianae* ATCC 38606 mycelia on V8-agar medium were inoculated into 100 mL ultrapure water supplemented with an ethanol solution of *mix*-**1** (0.5 mg/0.1 mL) or *mix*-**2** (0.1 mg/0.1 mL) in 200-mL Erlenmeyer flask, and incubated at 27 °C, 80 rpm for 24 h in a BioShaker BR-23FP. To the culture was added MeCN (25 mL) and the mixture was shaken at 25 °C, 120 rpm for 30 min. The mixture was filtered and the filtrate was concentrated to dryness and dissolved in 75% MeCN (1 mL). A portion (1 μL, equiv. to 0.1 mL-culture broth) was used for LC/MS analysis with the mobile phase (i).

Results and Discussion

Synthesis of hydroxyphytols (*mix*-1 and *mix*-2)

The starting material **3**, prepared from phytol according to the methods reported previously (Tori et al. 1985), was subjected to ruthenium-catalyzed C–H oxygenation (McNeill et al. 2010) to give tertiary alcohols **4** and **5** in 10% and 12%, respectively (Scheme 1). The position of hydroxylation in **4** was determined by two-dimensional NMR analysis and that in **5** was readily clarified by the resonances at δ 3.42 (dd) and 3.51 (dd). For preparing 11-hydroxyphytol (*mix*-**1**), the acetyl group of **4** was removed and the resulting alcohol was oxidized to ketone **6**. Two-carbon elongation of **6** by Horner-Wadsworth-Emmons (HWE) reaction gave unsaturated ester **7**, which was converted to *mix*-**1** by hydride reduction. For preparing 16-hydroxyphytol (*mix*-**2**), dehydration of **5** was performed to give a mixture of *endo* and *exo*-alkene (**8**) in the ratio of 7:2. The mixture was treated with osmium tetroxide and the desired 14,15-diol **9** was chromatographically purified. The oxidative cleavage of **9**

followed by Wittig reaction afforded unsaturated ester **10**, which was then converted to saturated ester **11** by hydrogenation. The acetyl group of **11** was removed and the resulting alcohol was oxidized to ketone **12**. Two-carbon elongation of **12** by HWE reaction gave unsaturated ester **13**, which was finally converted to *mix*-**2** by hydride reduction. Although both *mix*-**1** and *mix*-**2** are the epimeric mixtures at the positions 11 and 15, respectively, the stereoisomers were not distinguished each other by ^{1}H and ^{13}C NMR and reversed-phase HPLC.

(Scheme 1)

Identification of α2 biosynthetic intermediates in *Phytophthora* cultures.

Two *Phytophthora* A2 mating type strains, *P. nicotianae* NBRC 33192 and *P. capsici* NBRC 8386, were used for examining their productivity of **1** and **2** from phytol. The strains were incubated in the presence of phytol (1 mg/L) for 1-4 d. The supernatant and mycelia were separately extracted and then analyzed by liquid chromatography-electrospray ionization time-of-flight mass spectrometry (LC-ESI-TOF MS). The mating hormone α2 was detected in both strains in the concentrations of 2.1 μg/L (1 day) and 5.0 μg/L (4 days), respectively (Fig. 3a). Among the biosynthetic intermediates, only 11-hydroxyphytol (**1**) was detected in both strains in the concentrations of 0.11 μg/L (1 day) and 0.25 μg/L (4 days), respectively (Fig. 3a).

To confirm the production of the α2 intermediates (**1** and **2**) in A2 mating type, *P. capsici* NBRC 8386 was again cultured in a large scale (1.7 L/flak). This time the nutritious 20% V8 juice medium was used to promote the growth of mycelia, in which the less polar intermediates might be richer than in supernatant. The culture period was set for 6 days, when the α2 production reaches the plateau (Tomura et al. 2017). The supernatants were separated from mycelia and extracted with EtOAc. The mycelia were extracted with MeOH and the extract was partitioned between hexane and aqueous MeOH. Non-polar extracts from the supernatant and mycelia were analyzed by LC-MS, demonstrating that both intermediate **1** (2.4 μg/L) and **2** (3.7 μg/L) were detected in mycelia. It is worthy to note that the intermediates were detected only in mycelia and the mating hormones were in supernatant. Without the external addition of phytol, the production of α2 was not observed, and lower production of **1** (0.15 μg/L), **2** (0.28 μg/L), and α1 (12 μg/L) was observed, which were generated from natural phytol in V8 vegetable juice (data not shown). These results confirmed

the intermediacy of **1** and **2** and suggested that the mating hormones are biosynthesized inside the hyphal cells via the intermediates **1** and **2** before being secreted into the medium. Since the intermediates lodge in A2 cells and, after further oxygenation, α2 is secreted outside, this enzymatic reaction could be executed inside the cells. On the other hand, α1 could be biosynthesized on the surface of A1 cells by capturing free α2 in the medium. Although α1 had been regarded to be produced by A1 mating type, we have recently found that there were many A2 strains that produce α1; *P. capsici* NBRC 8386 produced both hormones as mentioned in this paper and *P. cryptogea* NBRC 32326 surprisingly produces only α1 in a high yield (Tomura et al. 2017). Although this appears inconsistent with the mating types, the generally used definition of the *Phytophthora* mating type is based on the responsiveness (oospore formation) to the counter mating type rather than hormone productivity. The two exceptional strains described above actually produce oospores when co-cultured with an A1 mating type strain. *P. capsici* NBRC 8386 and other several A2 type strains possibly possesses not only α2 biosynthetic enzymes but also an α1 biosynthetic enzyme, and the secreted free α2 is readily converted to α1 on the cell surface of A2 type like A1. The identification of these biosynthetic enzymes is under progress.

(Figure 3)

Conversion of synthetic intermediates to α2 by *Phytophthora*

Further evidence for the intermediacy of **1** and **2** for α2 biosynthesis was obtained by the conversion of the synthetic intermediates *mix*-**1** and *mix*-**2** to α2. The synthetic intermediates were added to cultures of *P. nicotianae* ATCC 38606 strain (A2 mating type). After incubating for 24 h in water, the amount of α2 in the cultures was analyzed by LC-MS. The results demonstrated that both synthetic compounds were converted to α2 (Fig. 4); 5 mg/L of *mix*-**1** was converted to 0.35 mg/L of α2 (6.6% yield), 1 mg/L of *mix*-**2** was to 0.041 mg/L of α2 (3.9% yield). The yields are based on the weight including the unnatural stereoisomer but, if the enzymatic reaction was stereospecific, the yields could be 13.2% and 7.8%, respectively. These results clearly support the intermediacy of both hydroxyphytols **1** and **2**.

(Figure 4)

Supplementary material

Supplementary material is available at Bioscience, Biotechnology, and Biochemistry online.

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Data availability

The data underlying this article are available in the article and in its online supplementary material.

Author contribution

M.O. designed the experiments. S.A. and Y.I. cultured the microorganism and analyzed the biosynthetic intermediates. R.O., R.K., and A.Y. synthesized the biosynthetic intermediates. T.S. supported LC/MS analysis.

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Disclosure statement

The authors declare no conflicts of interest.

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Figure captions

Figure 1. The *Phytophthora* mating hormones

Figure 2. Biosynthetic route for the *Phytophthora* mating hormones with plausible biosynthetic intermediates

Figure 3. Production of plausible α2 intermediates (1, 2) in *Phytophthora* strains

(a) The production of α2 (square) and intermediates (**1**: circle; **2**: triangle) by *P. nicotianae* NBRC 33192 (0.1% V8 medium) and *P. capsici* NBRC 8386 (water medium) in a small scale (100 mL). The values are the sum of the production in supernatant and mycelia. (b) The production of mating hormones (α1, α2) and intermediates (**1**, **2**) by *P. capsici* NBRC 8386 in 20% V8 juice medium in a large scale (1.7 L, 6 days). The content in mycelia and supernatant is separately indicated.

Figure 4. Conversion of synthetic intermediates *mix*-1 and *mix*-2 to α2 by *Phytophthora*

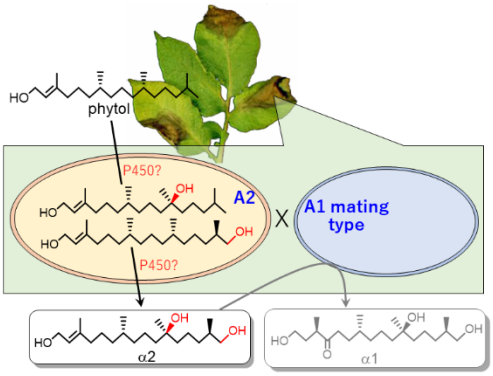
Extracted ion chromatographs (EIC) of α2 (*m/z* 351.28, red lines) of the extract of *P. nicotianae* ATCC 38606 cultured in the presence of *mix*-**1** (a) or *mix*-**2** (b) (*m/z* 335.29, black lines).

Scheme 1. Synthesis of 11-hydroxyphytol (*mix*-1) and 16-hydroxyphytol (*mix*-2).

Reagents and conditions: (a) $RuCl_3$•xH_2O, $KBrO_3$, pyridine, MeCN, H_2O, 60 °C (10% for **4**, 12% for **5**, and 48% recovered **3**); (b) 1) K_2CO_3, MeOH; 2) PCC, MS4A, CH_2Cl_2 (58% in 2 steps); (c) triethyl phosphonoacetate, NaH, THF, 0 °C to r.t. (85%); (d) LAH, ether, 0 °C to r.t. (89%); (e) $POCl_3$, pyridine, –20 °C (74%); (f) OsO_4, NMO, acetone, *t*-BuOH, H_2O, then separation (68%); (g) 1) $NaIO_4$, THF, H_2O; 2) (1-ethoxycarbonylethylidene)triphenylphosphorane, CH_2Cl_2 (37% in 2 steps); (h) H_2, Pd/C, EtOAc (97%); (i) 1) K_2CO_3, EtOH, 40 °C; 2) PCC, MS4A, CH_2Cl_2 (93% in 2 steps); (j) triethyl phosphonoacetate, *n*-BuLi, THF, 0 °C (29% and 57% recovered **12**); (k) DIBAL, CH_2Cl_2, –78 to 0 °C (70%).

Caption for Graphical Abstract

The mating hormone α2 of *Phytophthora* (A2 type) is biosynthesized from phytol via 11- and 16-hydroxyphytols, promoting the sexual reproduction of the partner A1 type.



Graphical Abstract

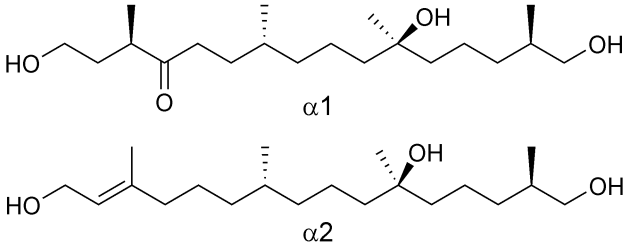


Figure 1

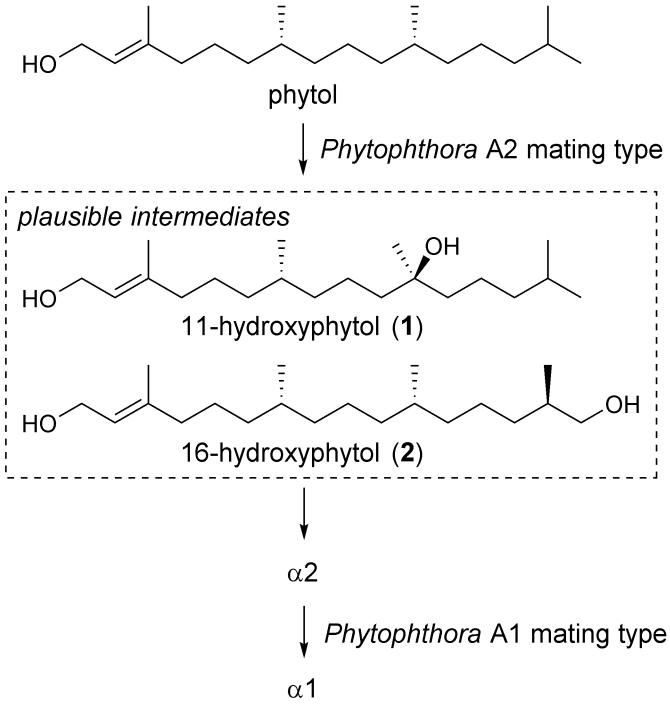
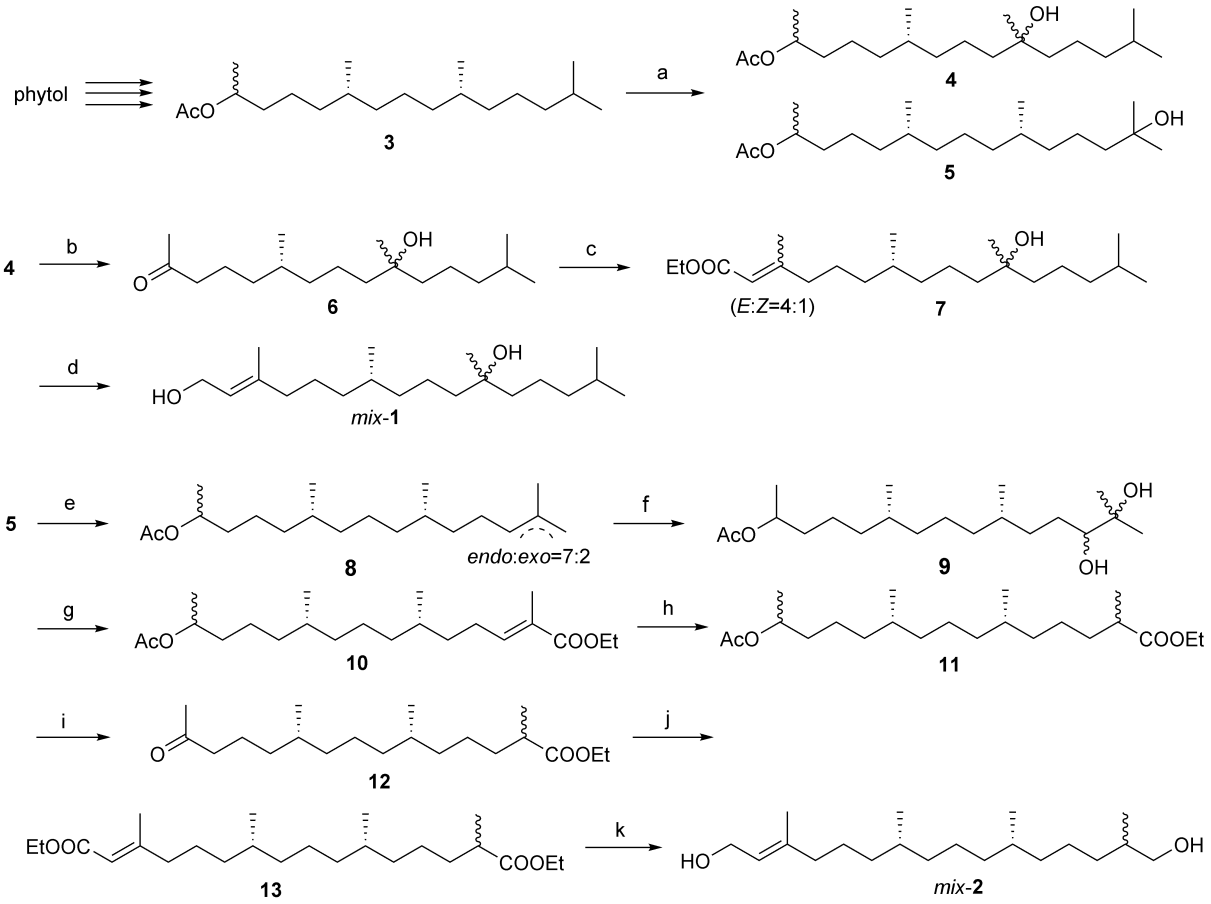


Figure 2



Scheme 1

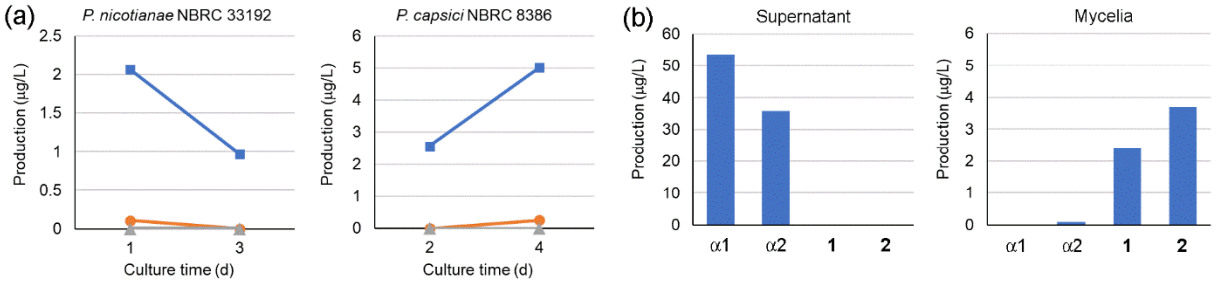


Figure 3

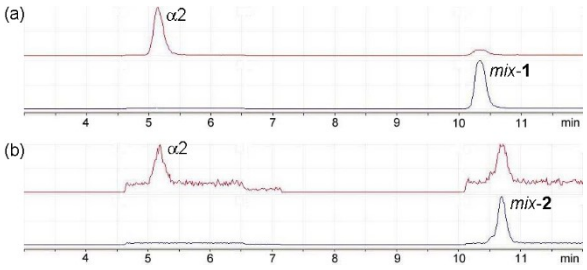


Figure 4