Electronic Supplementary Information

Interfacing phosphate catalytic reaction with microbial metabolism for production of azaphilone alkaloids

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Content

S1. General materials and methods

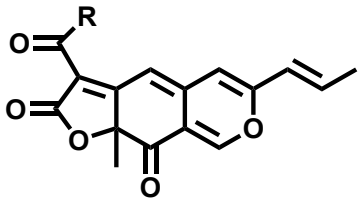
Acetonitrile (HPLC grade) was purchased from Avantor (US). Water was purified using a Mili-Q Ultra system (Millipore, US). Chloroform-*d* and methanol-d_4 were purchased from *Adamas-beta* (Shanghai, China). *p*-Aminobenzamide (ABA) was purchased from Macklin (Shanghai, China). 3-Morpholinopropanesulfonic acid (MOPS) was purchased from Aladdin (Shanghai, China). Other agents (chemical grade) were all purchased from Titan (Shanghai, China). Ultraviolet and visible light (UV-Vis) spectrum was recorded on a UV-2600 spectrometer (Shimadzu, Japan). Thin layer chromatography was performed on Merck TLC plates (silica gel 60 F254).

HPLC analysis: High performance liquid chromatography (HPLC) analysis was performed on Shimadzu LC-20AT system with a photodiode array (PDA) detector. An InertSustain C_{18} column, 4.6×250mm id., 5um (GL Sciences Inc., Japan), was used as analytical column. Gradient eluted with water (A)/acetonitrile (B) solvent system and detection wavelength at 500 nm was employed. The gradient eluting program was started from 10 % B, and changed to 25 % B within 2 min, then changed to 50 % B within 2 min, and changed to 80 % B within 4 min, then changed to 100% within 4min, then maintained 100 % B within 2 min, and changed to 10 % B within 1 min at a flow rate of 1.0 ml/min.

UPLC-MS Measurements: Ultra performance liquid chromatography-mass spectrometry (UPLC-MS) analysis was performed on a Water ACQUITY UPLC system. This system was equipped with a binary solvent delivery manager and a sample manager coupled with a Waters Q-TOF Premier Mass Spectrometer equipped with an electrospray interface (Waters Corporation, Milford, MA). An Acquity BEH C18 (100 mm×2.1 mm i.d., 1.7 μm, Waters, Milford, USA) analytical column was used and equipped with an ACQUITY UPLC BEH C18 (1.7 μm) Vanguard precolumn. The column was maintained at 50 ^{o}C and eluted with water (A)/acetonitrile (B) solvent system. The gradient eluting program was started from 10 % B, and changed to 50 % B within 5 min, then changed to 85 % B within 2 min, and changed to 100 % B within 2 min, then maintained 100 % B within 2 min, and changed to 10 % B within 0.5 min, and at last by equilibration at 10 % B for 2.5 min at a flow rate of 0.4 ml/min. The sample injection volume was 5 μL. High resolution mass spectrum (HRMS) was performed in positive ion model otherwise specified. The parameters were set as following: capillary voltage, 3 kV; sample cone, 35 V; source temperature, 100 ^{o}C; desolvation temperature, 350 ^{o}C; collision energy 4 eV; cone gas flow, 50 L/h, desolvation gas flow, 600 L/h. Data were collected in centroid mode from mass-to-charge ratio (m/z) 50 to 1000 at scan time of 0.25 s with an interval of 30 s. MS analysis was carried out via Masslynx 4.1 software (Waters MS Technologies, Manchester, UK).

NMR Spectroscopy: ^{1}HNMR and ^{13}CNMR spectra were obtained on a Avance Ⅲ 400 MHz spectrometer (Bruker, Germany) with TMS as the internal standard. All chemical shifts were given in ppm and coupling constants were given in Hz.

S2. Submerged culture for production of orange *Monascus* pigments (OMPs)



1 R= C_5H_{11}
2 R= C_7H_{15}

Strains, media and culture conditions: *Monascus ruber* (China Center of Industrial Culture Collection, CICC 5013) was used in this study. The strain was maintained on potato dextrose agar (PDA) medium (potato 200 g, glucose 20 g, and agar 15-20 g, per liter of tap water) at 4 °C. The seed culture medium consisted of glucose 20 g, $(NH_4)_2SO_4$ 4 g, peptone 10 g, KCl 0.5 g, KH_2PO_4 4 g and $FeSO_4{\cdot}7H_2O$ 0.01 g, and tap water 1 L. Inoculum culture was carried out in a 250 mL Erlenmeyer flask with 50 mL working volume at 30 °C and 200 rpm for 30 h.

The fermentation culture medium consisted of glucose 50 g, NH_4Cl 5 g, KH_2PO_4 2.4 g, K_2HPO_4 2.4 g, KCl 0.5 g, $MgSO_4{\cdot}7H_2O$ 8 g, $FeSO_4$ 0.01 g and $ZnSO_4$ 0.01 g in per liter of tap water. The initial pH of the fermentation culture medium was adjusted to 2.5 with 10 % (V/V) hydrochloric acid. Two milliliters of inoculums culture was added into 250 ml Erlenmeyer flask with 50 ml of working volume. The flasks were shaken at 30 °C, 200 rpm, and for 7 days.

Isolation and identification of OMPs: Extraction and isolation of OMPs followed a similar procedure in literature.[1] Briefly, after submerged culture of *M. ruber* for 7 days, 50 ml fermentation broth was mixed with equal volume of ethyl acetate, followed by 48 h of incubation on a reciprocal shaking water bath at 30 °C and 200 rpm. The mixture was moved into a 150 ml separating funnel and the ethyl acetate layer containing *Monascus* pigments was then separated. Ten grams of silica gel was added into the ethyl acetate phase for adsorption of the pigments while the ethyl acetate was further removed by evaporation. After that, 100 ml hexane was added to the pigment-silica gel complex and the mixture was incubated for 10 h at room temperature. The YMPs were extracted into the hexane phase while OMPs were resided in the pigment-silica gel complex.

One hundred milliliters of ethyl acetate was further added to the pigment-silica gel complex for dissolution of

the residual pigments. After filtration, crude OMPs as a solid was obtained by evaporation of the filtrate. The crude crystal OMPs was dissolved in 5 ml ethyl acetate, which was utilized for preparation of OMPs sample by further purification with silica gel column chromatography (Silica gel, 200-300 mesh, Haiyang, China; petroleum ether: ethyl acetate=85:15 as mobile phase).

The prepared OMPs were further purified by a preparative HPLC system (Shimadzu LC-6AD series equipped with an SPD-20 spectrophotometer using Shimadzu PRC-ODS EV0233 column). The method employed was isocratic elution (acetonitrile/water, 70:30, V/V) with a flow rate of 10 ml/min and detection wavelength at 470 nm. The sample injection volume was 1 ml. The purified rubropunctatin (1) and monascorubrin (2) were obtained, respectively.

Rubropunctatin (1). Orange needle solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.88 (s, 1H, CH), 6.89 (s, 1H, CH), 6.63 - 6.57 (m, 1H, CH), 6.16 (s, 1H, CH), 6.06 (d, *J* = 15.5 Hz, 1H, CH), 2.94 (m, 2H, CH_2), 1.96 (d, *J* = 7.0 Hz, 3H, CH_3), 1.71 (s, 3H, CH_3), 1.62 (m, 2H, CH_2), 1.32 (m, 4H, CH_2CH_2), 0.88 (t, *J* = 9.8 Hz, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 197.4 (C=O), 190.8 (C=O), 171.7 (C=O), 169.2 (*C*CH), 156.5 (*C*CH), 152.8 (CH), 141.7 (*C*CH), 136.4 (CH), 122.4 (*C*C), 116.3 (CH), 113.2 (CH), 109.6 (*C*CH), 104.1 (CH), 85.8 (CO), 41.6 (CH_2), 31.4 (CH_2), 28.3 (CH_3), 23.4 (CH_2), 22.6 (CH_2), 18.8 (CH_3), 14.0 (CH_3). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{21}H_{23}O_5$: 355.1545, found: 355.1564.

Monascorubrin (2). Orange needle solid. ^{1}HNMR(400MHz, $CDCl_3$) δ 7.86(s, 1H, CH), 6.88(s, 1H, CH), 6.63-6.51(m, 1H, CH), 6.14(s, 1H, CH), 6.04(d, *J*=14.3Hz, 1H, CH), 2.92(m, 2H, CH_2), 1.94(d, *J*=7.0Hz, 3H, CH_3), 1.70(s, 3H, CH_3), 1.59(m, 2H, CH_2), 1.26(m, 8H, $CH_2CH_2CH_2CH_2$), 0.86(t, *J*=7.1Hz, 3H, CH_3). ^{13}CNMR (100MHz, $CDCl_3$) δ197.6 (C=O), 191.0 (C=O), 171.8 (C=O), 169.4 (*C*CH), 156.6 (*C*CH), 153.0 (CH), 141.8 (*C*CH), 136.6 (CH), 122.6 (*C*C), 116.5 (CH), 113.4 (CH), 109.8 (*C*CH), 104.4 (CH), 86.0 (CO), 41.9 (CH_2), 31.9 (CH_2), 29.4 (CH_2), 28.5 (CH_3), 23.9 (CH_2), 22.8 (CH_2), 19.0 (CH_3), 14.3 (CH_3). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{23}H_{27}O_5$: 383.1858, found: 383.1863.

Standard curve used to quantify OMPs (1, 2): The OMPs (1, 2) in 70% ethanol aqueous solution (v/v; pH 2) was analysed by HPLC. OMPs (1, 2) concentration was calculated from the peak area of OMPs (1, 2) at *ca.* 13.1 and 14.0 min using the equation y=52.246x + 0.0518 (x=OMPs (1, 2) concentration; y= peak area/1000000).

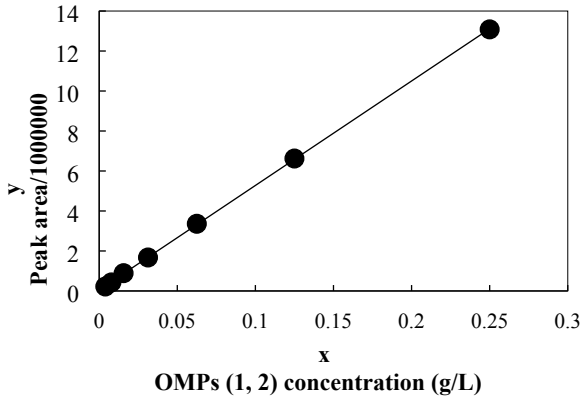
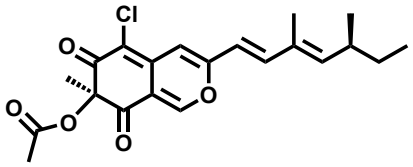


Figure S1. Standard curve used to quantify OMPs (1, 2)

S3. Submerged culture for production of sclerotiorin



sclerotiorin (3)

Fermentation of *P. sclerotiorum* FS-50: Marine-derived strain *P. sclerotiorum* FS-50 GDMCC 3.644 (GenBank accession number EU 807,940) was isolated from the sediment of South China Sea and stored in Guangdong Microbial Culture Collection Center (GDMCC). The strain was maintained on Potato Dextrose Agar (PDA) medium (potato 200 g, glucose 20 g, and agar 20 g, per liter of tap water) at 4 °C.

Liquid culture without stirring was carried out in Czapek liquid medium (glucose, 50g; $NaNO_3$, 2g; KH_2PO_4 1g; KCl 0.5g; $MgSO_4 \cdot 7H_2O$,0.5g; $FeSO_4 \cdot 7H_2O$,0.1g； tap water 1 L). The liquid culture of 2 L working volume was carried out at 26 °C without stirring for 24 days.

Isolation and identification of sclerotiorin (3): The mycelium was separated from the culture broth by filtration. And then, the dried mycelium was extracted twice with 1.0 L of ethyl acetate at room temperature for 24 hours. The mixture was suction-filtered. The extracts were combined and concentrated under reduced pressure. The residue was suspended in a petroleum (60-90 ^oC)/ethyl acetate mixture, subjected to a silica gel column (200-300 mesh; mobile phase: petroleum ether/ ethyl acetate=10/1; normal pressure). The fractions were collected and concentrated under reduced pressure to obtain the pure sclerotiorin (3).

^1H NMR (400 MHz, $CDCl_3$) δ 7.92 (s, 1H, CH), 7.05 (d, J = 15.7 Hz, 1H, CH), 6.63 (s, 1H, CH), 6.07 (d, J = 15.7 Hz, 1H, CH), 5.69 (d, J = 9.8 Hz, 1H, CH), 2.51 - 2.42 (m, 1H, CH), 2.16 (s, 3H, CH_3), 1.83 (d, J = 0.9 Hz, 3H, CH_3), 1.55 (s, 3H, CH_3), 1.42 (m, 1H, CH_2-H_a), 1.30 (m, 1H, CH_2-H_b), 1.00 (d, J = 6.6 Hz, 3H, CH_3), 0.85 (t, J = 7.4 Hz, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.7 (C=O), 185.9 (C=O), 170.0 (*C*OCH3), 158.0 (*C*CH), 152.6

(CH), 148.8 (CH), 142.8 (CH), 138.6 (*C*CH), 131.9 (*C*CH), 115.6 (CH), 114.5 (C-Cl), 110.8 (*C*CH), 106.3 (CH), 84.5 (CO), 35.1 (*C*HCH3), 30.0 (CH_2), 22.5 (CH_3), 20.2 (CH_3), 20.0 ($CO\mathit{C}H_3$), 12.3 (CH_3), 11.9 (CH_3). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{21}H_{24}ClO_5$: 391.1312, found: 391.1303.

Standard curve used to quantify sclerotiorin (3): The sclerotiorin (3) in 70 % ethanol aqueous solution (v/v; pH 2) was analyzed by HPLC. Sclerotiorin (3) concentration was calculated from the peak area of sclerotiorin (3) detected at *ca.* 13.8 min using the equation y=5.9075x + 0.0051 (x=sclerotiorin (3) concentration; y= peak area/1000000).

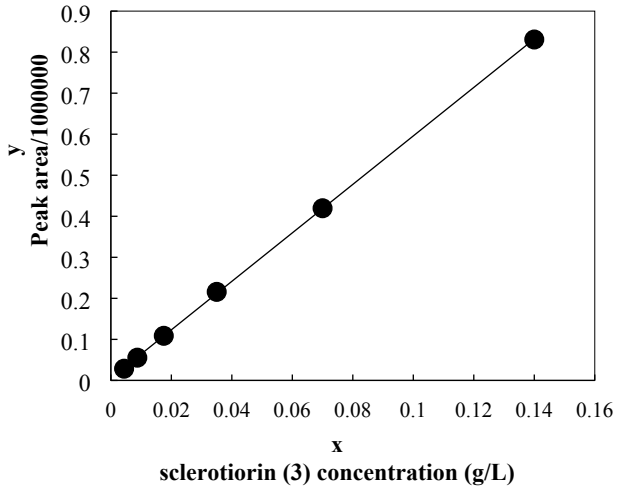
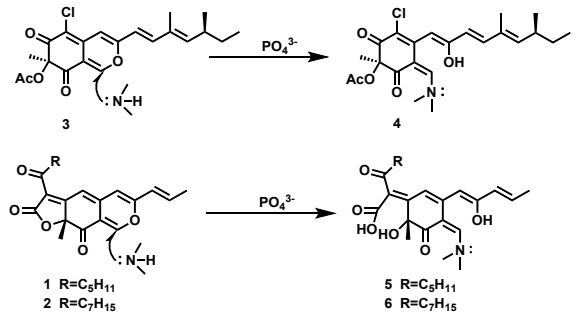


Figure S2. Standard curve used to quantify sclerotiorin (3)

S4. Chemical conversion of azaphilone into the corresponding enamine



To solutions of azaphilone compounds 1, 2 and 3 (2.6 mM) in phosphate aqueous solution (5 mL; 0.1 M, pH7), dimethylamine hydrochloride (26 mM) was added and the resulting mixtures were stirred for 72 hours at 30 ^oC with no pH variation, using the reactions in aqueous solution (5 mL; pH 7) as control. Upon completion, the

reaction mixtures were subjected to UPLC-MS analysis by a 15-fold dilution with ethanol aqueous solution (70%, V/V; pH 7).

The molecular formula of $C_{23}H_{31}NO_5Cl$ Na (*m/z* 458.1700) matched that of the sodium adduct of compound **4**. The peak at *m/z* 436.1880 was consistent with the $[M+H]^+$ ion of the desired compound **4** produced by compound **1** reaction with dimethylamine. Likewise, the molecular formula of $C_{23}H_{32}NO_6$ and $C_{25}H_{36}NO_6$ matched those of the $[M+H]^+$ ions of the desired compound **5** and **6**, respectively. The fragment ions of *m/z* 400.2110 and 428.2424 corresponded to the $[M-H_2O+H]^+$ ions of compounds **5** and **6**.

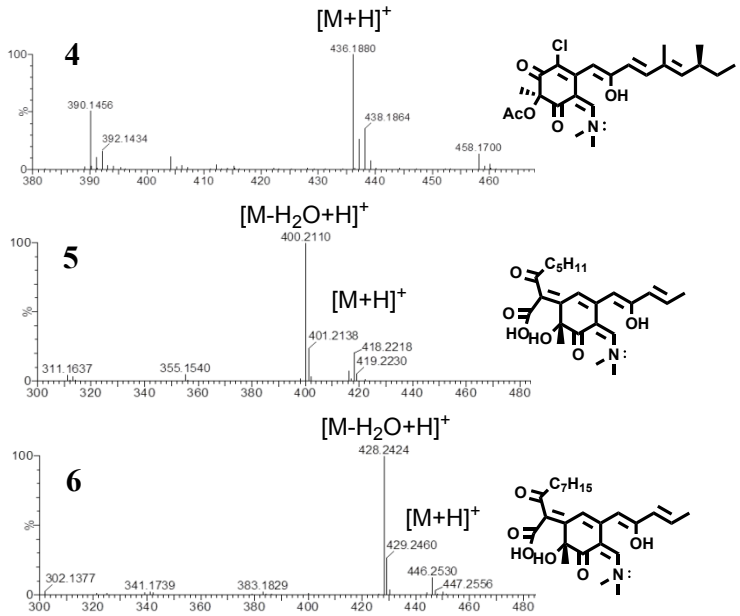
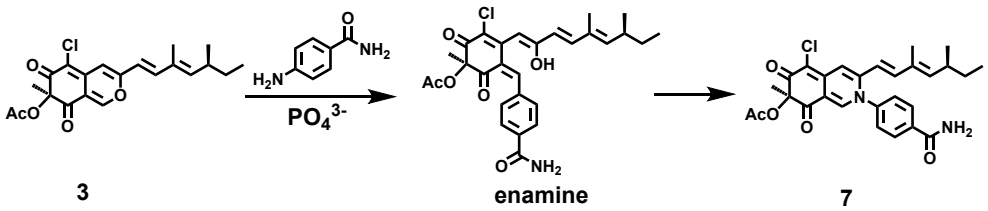


Figure S3. MS analysis of compounds **4, 5** and **6**

S5. Chemical conversion of sclerotiorin (3) into red sclerotiorin alkaloids

Preparation and characterization of sclerotiorin alkaloid (7):



A mixture of sclerotiorin (3) (2.6 mM) and ABA (5.2 mM) in ethanol/ phosphate aqueous solution (20 mL, volume ratio of ethanol to aqueous solution= 1/1; phosphate aqueous solution 0.1 M, pH 7) was stirred for 40 hours at 30 ^oC until sclerotiorin (3) was consumed completely (monitored by thin-layer chromatography (TLC), developing solvent: chloroform/ methanol/ water=56/ 10/ 0.9). The reaction mixture was concentrated by a rotary

evaporator.

After evaporation of solvent, the solid residue was redissolved by ethyl acetate/water (1/1, V/V; 50 mL). The mixture was transferred into a 150 mL separating funnel and an ethyl acetate layer was then separated. The ethyl acetate layer was concentrated in a rotary evaporator under vacuum to afford the red alkaloid (7). The purified compound **7** was further identified by MS and NMR analysis.

Red amorphous powder. ^{1}H NMR (400 MHz, CD_3Cl) δ 8.05 (d, *J* = 7.9 Hz, 2H, Ar-H), 7.80 (s, 1H, CH), 7.39 (d, *J* = 7.9 Hz, 2H, Ar-H), 7.13 (s, 1H, CH), 6.97 (d, *J* = 15.5 Hz, 1H, CH), 6.79 (s, 1H, NH), 6.04 (s, 1H, NH), 5.68 (d, *J* = 9.7 Hz, 1H, CH), 5.56 (d, *J* = 15.5 Hz, 1H, CH), 2.40 (m, 1H, *C*CH_2), 2.15 (s, 3H, CH_3), 1.58 (s, 3H, CH_3), 1.51 (s, 3H, CH_3), 1.40 (m, 1H, CH_2-H_a), 1.31 (dd, *J* = 14.3, 6.8 Hz 1H, CH_2-H_b), 0.97 (d, *J* = 6.6 Hz, 3H, CH_3), 0.84 (t, *J* = 7.4 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, CD_3Cl) δ 192.4 (C=O), 183.8 (C=O), 169.3 (*C*OCH_3), 166.4 (*C*ONH_2), 147.4 (*C*CH), 146.3 (CH), 143.0 (CH), 142.7 (CH), 142.0 (Ar-H), 139.7 (CH*C*), 133.9 (Ar-H), 130.8 (*C*CH), 128.6 (Ar-H), 128.6 (Ar-H), 125.8 (Ar-H), 125.8 (Ar-H), 114.9 (CH), 113.7 (C-Cl), 109.1 (*C*CH), 102.7 (CH), 83.9 (*C*CH_3), 34.0 (CH), 29.0 (CH_2), 22.2 (CH_3), 19.3 (CO*C*H_3), 19.1 (CH_3), 11.3 (CH_3), 10.9 (CH_3); UV/Vis (EtOH): λ_{max} (ε)= 495(4600), 395 nm (33000 $mol^{-1}dm^3cm^{-1}$); HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{28}H_{30}ClN_2O_5$: 509.1843, found: 509.1839.

Standard curve used to quantify sclerotiorin alkaloid (7): Sclerotiorin alkaloid (7) in 70 % ethanol aqueous solution (v/v; pH 2) was analysed by HPLC (detection wavelength 500 nm). Sclerotiorin alkaloid (7) concentration was calculated from the peak area of sclerotiorin alkaloid (7) at *ca.* 11.4 min using the equation y=11.385x + 0.0013 (x= sclerotiorin alkaloid (7) concentration; y= peak area/1000000).

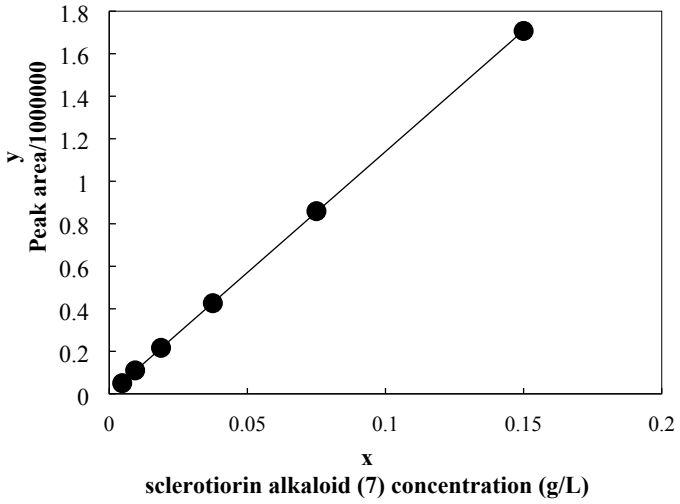


Figure S4. Standard curve used to quantify sclerotiorin alkaloid (7)

Effect of PO_4^{3-} concentration on amination reaction between sclerotiorin (3) and ABA: Mixtures of sclerotiorin (3) (2.6 mM) and ABA (5.2 mM) in a certain phosphate concentration (such as 0, 0.0125, 0.025, 0.05, 0.1, or 0.2 M) aqueous solution (5 mL; pH 5-9) were stirred for 72 hours at 30 °C with no pH variation. Upon completion, the reaction mixtures were subjected to HPLC analysis (detection wavelength 300 nm) by a 15-fold dilution with ethanol aqueous solution (70%, V/V; pH 7). The resulting mixture in phosphate aqueous solution (0.1 M, pH7; reaction time 72 hours) was analyzed by UPLC-MS. The $[M+H]^+$ ion of compound **7**, which had been identified by NMR analysis, was found at *m/z* 509.1839. The fragment ion at *m/z* 527.1940 matched the $[M+H]^+$ ion of the reaction intermediate sclerotiorin enamine.

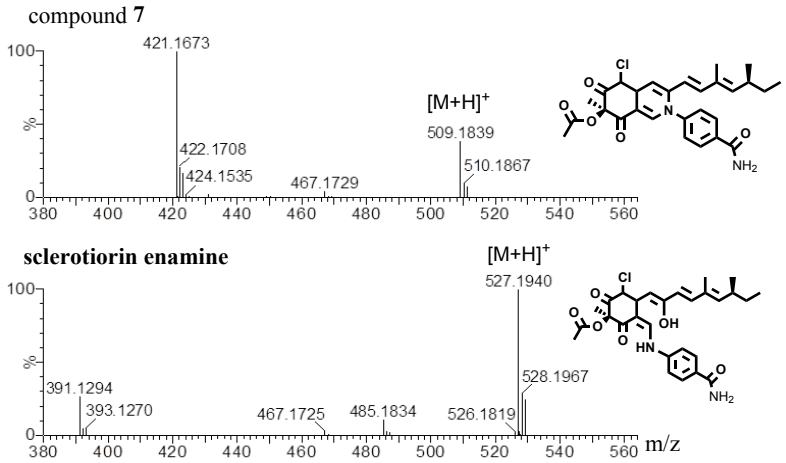


Figure S5. MS analysis of the reaction intermediate sclerotiorin enamine.

Chemical reaction of sclerotiorin (3) with primary amines at different pH: To solutions of sclerotiorin (3) (2.6 mM) in phosphate aqueous solution (5 mL; 0.1 M; pH3-7), ammonium chloride (AC), methylamine hydrochloride (MH), glycine (Gly), monosodium glutamate (MSG), glucosamine (GA) and *p*-aminobenzamide (ABA) (5.2 mM) were added, respectively. The resulting mixtures were stirred for 72 hours at 30 °C with no pH variation. Sclerotiorin (3) in phosphate aqueous solutions with different pH (pH 3-7) were used as blanks and the reactions of sclerotiorin (3) with the primary amines in an aqueous solution (pH 7, free of phosphate) were used as controls. Meanwhile, pH drift never exceeded 0.1 pH units. Upon completion, the reaction mixtures were subjected to UV analysis by a 15-fold dilution with ethanol aqueous solution (70%, V/V; pH 2) using UV-vis spectroscopy (detection at 520 nm). The results showed that the reactions between sclerotiorin and primary amines with less bulky group occurred easily.

A B

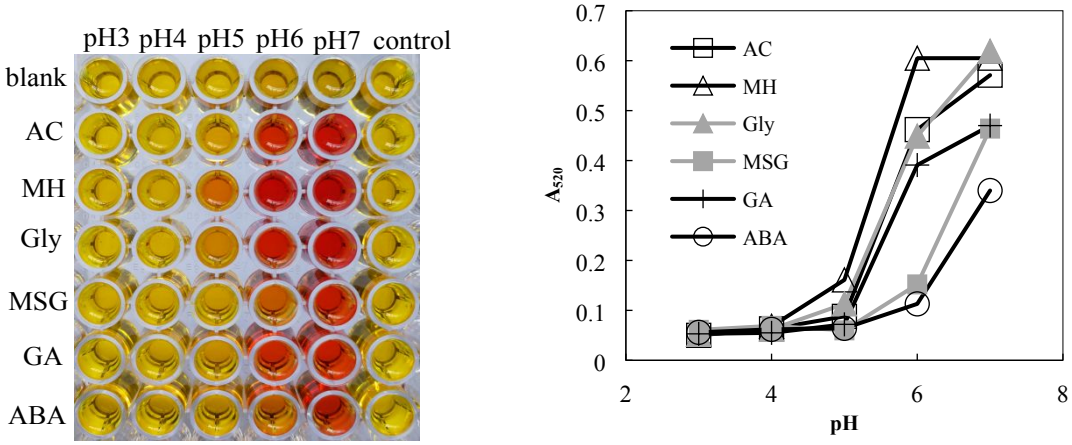
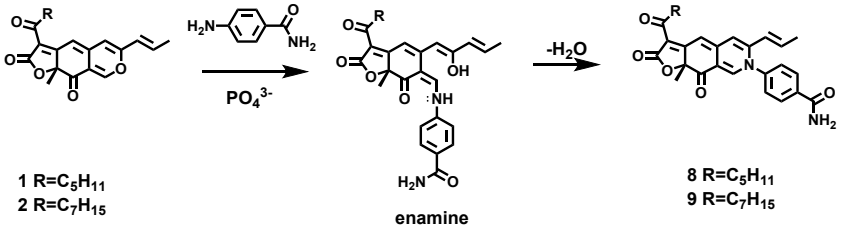


Figure S6. Amination of sclerotiorin (3) with primary amines in phosphate aqueous solutions with different pH (0.1 M; pH 3-7). (A) Photograph. (B) UV-vis analysis (detection at 520 nm). Sclerotiorin (3) in phosphate aqueous solutions with different pH (pH 3-7) were used as blanks and the reactions of sclerotiorin (3) with the primary amines in an aqueous solution (pH 7, free of phosphate) were used as controls.

S6. Chemical conversion of OMPs (1, 2) into red *Monascus* alkaloids (8, 9)



Preparation and characterization of *Monascus* alkaloid (8/9): A mixture of monascorubrin (2) (2.6 mM; preparation from fermentation broth as shown in S2) and ABA (5.2 mM) in ethanol/ phosphate aqueous solution (20 mL, volume ratio of ethanol to aqueous solution= 1/1; phosphate aqueous solution 0.2 M, pH 7) was stirred for 6 hours at 30 °C until monascorubrin (2) was consumed completely (monitored by thin-layer chromatography (TLC), developing solvent: chloroform/methanol/water=56/10/0.9). The reaction mixture was concentrated by a rotary evaporator. The approaches of purification and identification of the red alkaloid (9) were the same as those of sclerotiorin alkaloid (7).

Red amorphous powder. ^{1}H NMR (400 MHz, CD_3Cl) δ 8.19 (s, 1H, CH), 8.16 (d, J = 6.0 Hz, 2H, Ar-H), 7.56 (s, 1H, NH), 7.45 (d, J = 8.4 Hz, 2H, Ar-H), 6.80 (d, J = 13.6 Hz, 1H, CH), 6.49 (dd, J = 15.4, 6.9 Hz, 1H, CH), 5.87 (s, 1H, NH), 5.69 (d, J = 15.5 Hz, 1H, CH), 2.91 (m, 2H, CH_2), 1.83 (dd, J = 6.8, 1.4 Hz, 3H, CH_3), 1.64 (m, 5H, CH_3/ CH_2), 1.31 (m, 8H, $CH_2CH_2CH_2CH_2$), 0.89 (t, J = 11.8 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, CD_3Cl) δ 196.2 (C=O), 193.1 (C=O), 172.4 (C=O), 170.7 ($CONH_2$), 166.9 (*C*CH), 148.0 (*C*CH), 146.2 (CH), 141.5 (*C*CH),

140.7 (*C*C), 136.7 (Ar-H), 129.1 (Ar-H), 129.1 (Ar-H), 125.5 (Ar-H), 125.5 (Ar-H), 121.9 (Ar-H), 116.2 (CH), 114.9 (CH), 105.1 (*C*CH), 97.7 (CH), 84.9 (CO), 30.9 (CH_2), 30.6 (CH_2), 28.9 (CH_3), 28.7 (CH_2), 23.0 (CH_2), 21.6 (CH_2), 18.0 (CH_3), 13.0 (CH_3); UV/Vis (EtOH): λ_{max} (ε)= 515 (24000), 420 nm (19000 $mol^{-1}dm^3cm^{-1}$); HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{30}H_{32}N_2O_5$: 501.2390 , found: 501.2388.

The method for preparation of *Monascus* alkaloid (8) was the same as those of *Monascus* alkaloid (9). *Monascus* alkaloid (8) was identified by UPLC-MS analysis.

Red amorphous powder. UV/Vis (EtOH): λ_{max} (ε)= 515 (24000), 420 nm (19000 $mol^{-1}dm^3cm^{-1}$); HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{28}H_{29}N_2O_5$: 473.2076 , found: 473.2070.

Standard curve used to quantify *Monascus* alkaloids (8, 9): *Monascus* alkaloids (8, 9) in 70 % ethanol aqueous solution (v/v; pH 2) was analysed by HPLC (detection wavelength 500 nm). *Monascus* alkaloids (8, 9) concentrations were calculated from the peak area of *Monascus* alkaloids (8, 9) at *ca.* 11.0 and 12.0 min using the equation y=45.988x + 0.0591 (x= *Monascus* alkaloids (8, 9) concentration; y= peak area/1000000).

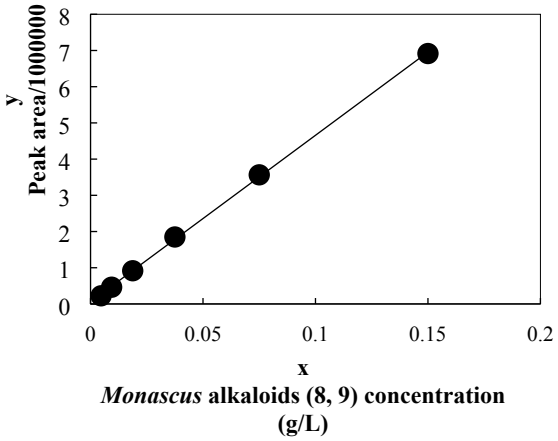


Figure S7. Standard curve used to quantify *Monascus* alkaloids (8, 9)

Amination reaction between OMPs (1, 2) and ABA catalyzed by PO_4^{3-}: A mixture of OMPs (1, 2) (2.6 mM) and ABA (5.2 mM) in a certain phosphate concentration (such as 0, 0.0125, 0.025, 0.05, 0.1, 0.2, or 0.4 M) aqueous solution (5 mL; pH 7) was stirred for 3 hours at 30 °C with no pH variation. Upon completion, the reaction mixtures were subjected to HPLC analysis by a 15-fold dilution with ethanol aqueous solution (70%, V/V; pH7).

The resulting mixture between OMPs (1, 2) and ABA in phosphate aqueous solution (0.2 M, pH7; reaction time 3 hours) was subjected to HPLC analysis (detection wavelength 500 nm) by a 15-fold dilution with ethanol aqueous solution (70%, V/V; pH7). The results showed that amination reaction between OMPs (1, 2) and ABA only occurred by PO_4^{3-} catalysis for the reaction time (Figure S8A). The resulting mixture was also subjected to

UPLC-MS analysis. The enamine was detected and identified by MS (Figure S8B). With the increase of PO_4^{3-} concentration, OMPs (1, 2) concentration reduced remarkably and the corresponding *Monascus* alkaloids (8, 9) concentration also increased (Figure S8 C&D).

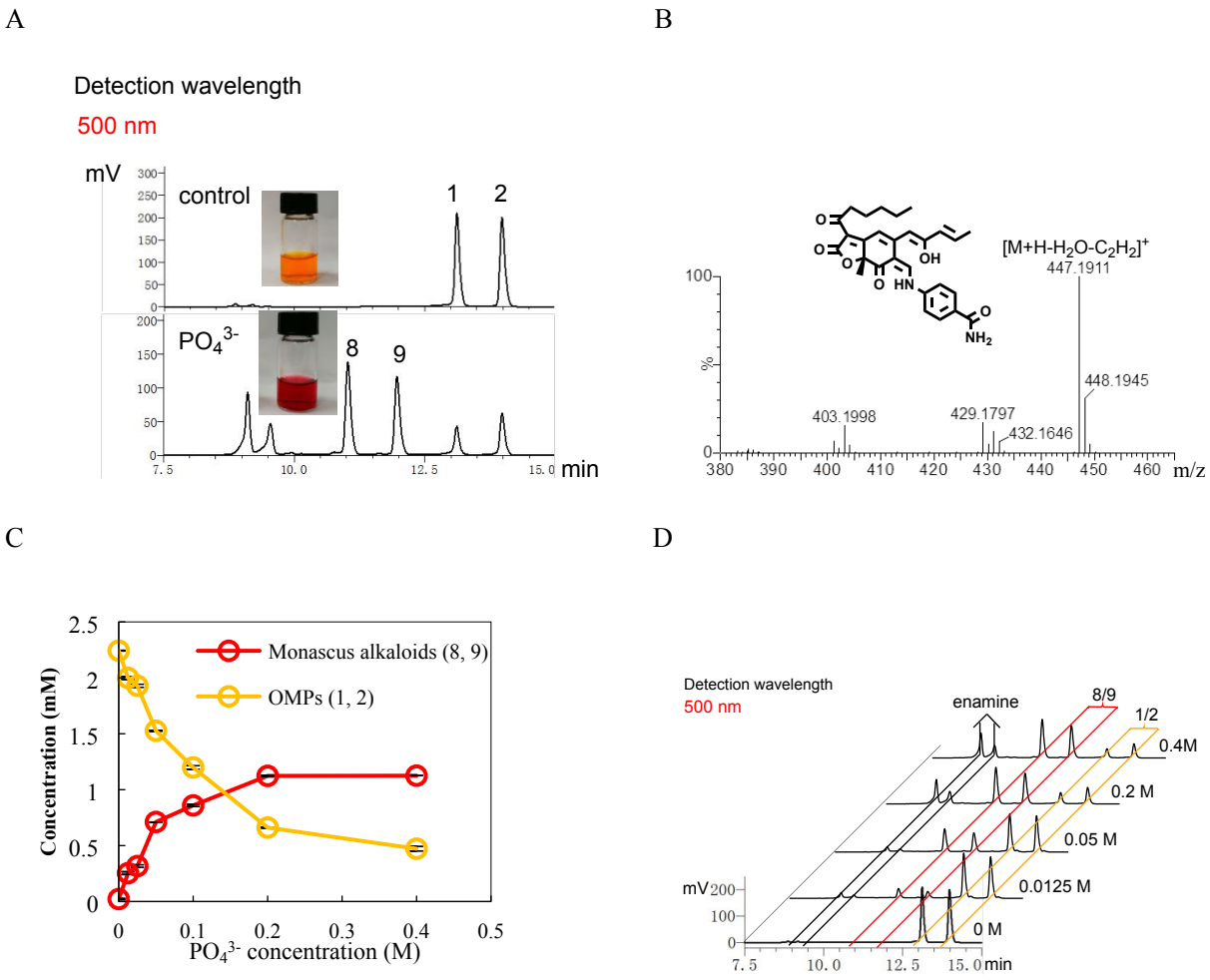


Figure S8. Chemical conversion of OMPs (1, 2**)** with *p*-aminobenzamide (ABA) into *Monascus* alkaloids (8, 9).

(A) The reaction occurs in phosphate aqueous solution (0.2 M; pH 7), using the reaction in aqueous solution (pH 7) as control. The reaction time was 3 hours with no pH variation. The intermediate enamines were detected at 8.9 min and 9.4 min.

(B) MS analysis of the enamine. $[M+H]^+$ ion with *m/z* 447.1911 was postulated as the fragment of the intermediate enamine resulted from the dissociation of the intermediate enamine by the loss of a water molecular and C_2H_2 of the alkene branch chains at high energy.

(C) The influence of PO_4^{3-} concentration on PO_4^{3-} catalytic activity in phosphate aqueous solutions (pH 7).

(D) HPLC analysis (detection wavelength 500 nm) of the effect of PO_4^{3-} concentration on PO_4^{3-} catalytic activity in phosphate aqueous solutions (pH 7).

S7. Interfacing biocompatible amination with microbial metabolism

Interfacing of biocompatible reaction with *Penicillium* metabolism: Cell suspension culture followed a similar procedure in literature.[2] *P. sclerotiorum* FS50 was grown on PDA plate in the dark at 26 °C for 4 days. Sterile water was poured on a well-grown PDA plate and the spores on the plate surface were gently scraped off with a coating bar. Then, the spore-containing sterile water was aspirated into a sterile beaker to obtain a spore solution. Submerged culture was carried out in Czapek liquid medium (glucose 20 g, $NaNO_3$ 3g, KH_2PO_4 1 g, KCl 8 g, $MgSO_4•7H_2O$ 0.5 g, $FeSO_4•7H_2O$ 0.1 g, $CuSO_4•5H_2O$ 0.05 g, per liter of tap water with natural pH). Initial pH was adjusted to approximately 7. Spore solution (OD_{600}=2) with volume of 2 mL was added into a 250 mL Erlenmeyer flask with 50 mL working volume. Submerged culture was carried out by incubation of the flasks at 26 °C and stirring at 110 rpm for 4 days.

After incubation for 4 days, mycelia were collected by filtration and rinsed with equal volume of water (pH=7, 50 mL) for 3 times to scour off the residual nutrients. Approximate 7 g/L DCW was added into 100 ml Erlenmeyer flask containing 25 mL of the liquid medium (KCl 8 g, $MgSO_4•7H_2O$ 0.5 g, $FeSO_4•7H_2O$ 0.1 g, $CuSO_4•5H_2O$ 0.05 g, per liter of tap water with pH 7). The biocompatibility of phosphate catalytic reaction interfacing with metabolism was performed in the liquid medium with KH_2PO_4 (6.8 g/L) and ABA (1 g/L). The liquid medium with ABA (1 g/L) was utilized as control. The initial pH of the fermentation culture medium was adjusted to 7 with sodium hydroxide (3 M). Cell suspension culture was carried out by incubation of the flasks at 26 °C and stirring at 110 rpm for 11 days. All the above operations were conducted under non-sterile condition in triplicate.

After cell suspension culture, the total fermentation broth was subjected to filtration. The filtrate was utilized for analysis of residual glucose (standard 3, 5-dinitrosalicylic acid method, DNS) and final pH value. The residue (wet mycelia) was incubated in 50 mL ethanol aqueous solution (ethanol: water=7:3, water was adjusted to pH=2 by addition of hydrochloric acid (1 M)) for more than 1 h. After extraction, the extract was analysed by HPLC (detection wavelength 500 nm). And the wet mycelia were further utilized to estimate dry cell weight (DCW).

A B

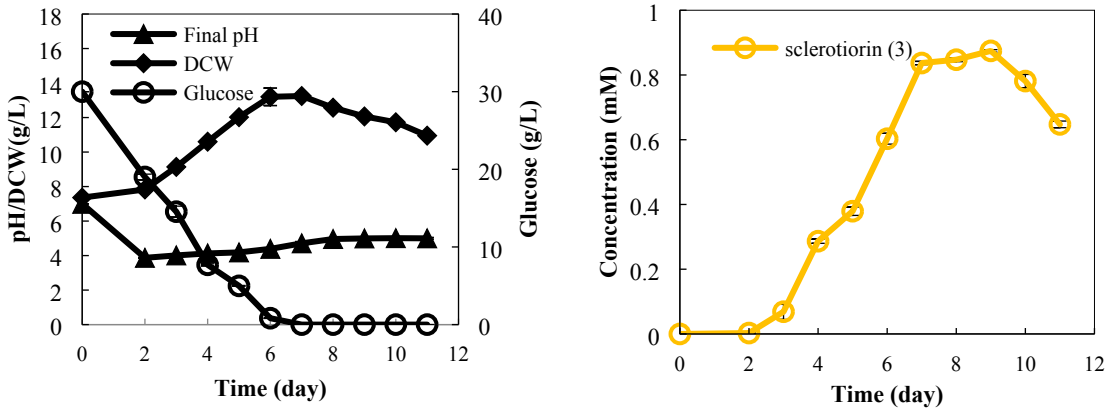


Figure S9. The time course of cell suspension culture of *P. sclerotirum* metabolism in the presence of ABA. A) Basic parameters of microbial metabolism, such as cell growth, glucose consumption and pH change; B) Production of sclerotiorin (3) during microbial metabolism. The culture condition was 30 g/L glucose, 1 g/L ABA, intial pH 7.

Combining biocompatible amination with *Monascus* metabolism: Cell suspension culture followed a similar procedure in literature.[3] The method of preparing inoculums culture was by reference to Section S2. The fermentation culture medium consisted of glucose 50 g, MSG 5 g, KH_2PO_4 2.4 g, K_2HPO_4 2.4 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 8 g, $FeSO_4$ 0.01 g, $ZnSO_4$ 0.01 g and Triton X-100 50 g in per liter of tap water. The initial pH of the fermentation culture medium was adjusted to 2.5 with 10 % (V/V) hydrochloric acid. Two milliliters of inoculums culture was added into 250 mL Erlenmeyer flask with 50 mL of working volume. The flasks were shaken at 30 °C, 200 rpm, and for 5 days.

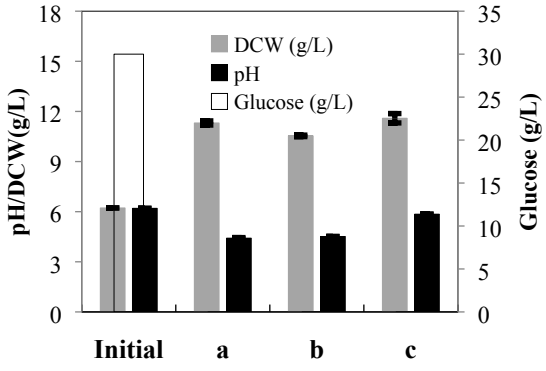
After incubation for 5 days, wet mycelia in the culture medium were collected by filtration and rinsed with water (equal to the volume of fermentation broth, pH 7) for 3 times to scour of the nonionic surfactants and the residual nutrients. Approximate 6 g/L DCW was added into a 100 mL Erlenmeyer flask containing 25 mL of culture medium (KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 8 g, $FeSO_4$ 0.01 g, $ZnSO_4$ 0.01 g, per liter of tap water with pH 7) for cell suspension culture. The conditions for cell suspension culture were shown as Figure S10A. Then, cell suspension culture was carried out by incubation of the flasks at 30 °C and 200 rpm for 3 days.

After cell suspension culture, the whole culture medium was subjected to filtration. The filtrate was utilized for analysis of residual glucose (DNS) and final pH value. The residue was utilized for determination of DCW. Besides, the whole culture medium (25 mL), adjusted to pH=2 by hydrochloric acid, followed by addition of 58 mL ethanol, was incubated in the ethanol aqueous solution (ethanol: water=7:3) for more than 1 h. The extract was analyzed by HPLC (detection wavelength 500 nm). All the above operations were conducted under non-sterile condition in triplicate.

A

Entry	Glucose 30 g/L	ABA 1 g/L	Phosphate 0.2 M, pH=7
a	+	-	-
b	+	+	-
c	+	+	+

B



C

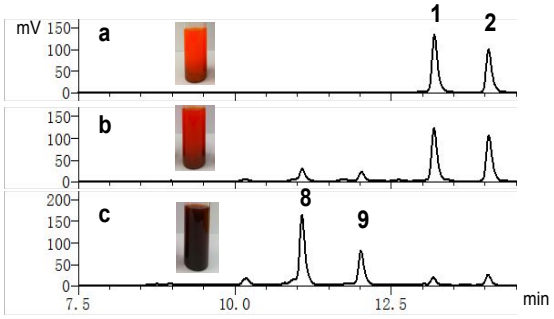


Figure S10. Combining biocompatible amination with *Monascus* metabolism.

A) Table of conditions for cell suspension culture.

B) Biocompatibility of the amination reaction. Cell suspension culture under different culture conditions was carried out by incubation of the flasks at 30 °C and 200 rpm for 3 days. Microbial growth (increase of DCW), glucose consumption (from the initial 30 g/L to zero) and final pH variants from the initial 7 was observed.

C) *Monascus* alkaloids (8, 9) production in different fermentation broths. *Monascus* alkaloids (8, 9) concentrations were illustrated by HPLC chromatograph. The concentration of *Monascus* alkaloids (8, 9) increased dramatically (from 0.14 mM (entry b) to 1.56 mM (entry c)).

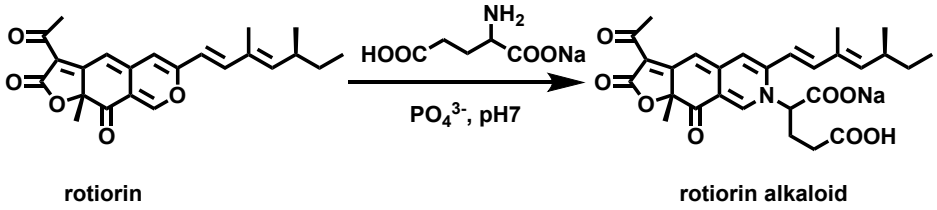
S8. Chemical conversion of rotiorin into the corresponding alkaloid by phosphate catalysis

Fermentation, isolation and identification of rotiorin: *P. sclerotiorum* FS-50 GDMCC 3.644 was incubated in Raulin-Thom medium (glucose, 50g; ammonium tartrate 2.6 g, tartaric acid 2.6 g, $(NH_4)_2HPO_4$ 0.4g, K_2CO_3 0.4g, $MgCO_3$ 0.26 g, $(NH_4)_2SO_4$ 0.17g, $ZnSO_4$ 0.05g, $FeSO_4$ 0.05g; tap water 1 L). The liquid culture of 2 L working volume was carried out at 26 °C without stirring for 20 days. After fermentation, extraction and isolation of rotiorin followed a similar procedure to those of sclerotiorin (Section S3). Then, rotiorin was identified by UPLC-MS analysis. The $[M+H]^+$ ion of rotiorin was found at *m/z* 381.16844 (Figure S11 B). And the characteristic absorbance wavelength in acetonitrile was 490 nm. The data was consistent with the literature results.[4] Herein, pure

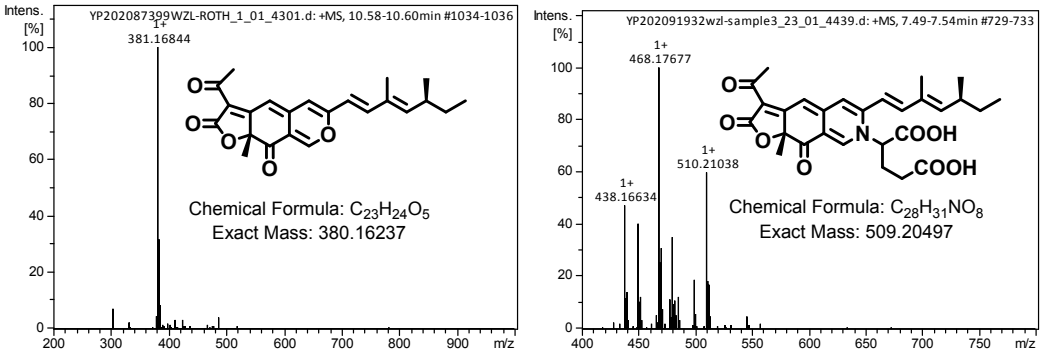
rotiorin was difficult to obtain owing to limited production as well as the instability of rotiorin.

Phosphate catalyzed the reaction between rotiorin and monosodium glutamate: A mixture of crude rotiorin (2.6 mM) and monosodium glutamate (5.2 mM) in phosphate aqueous solution (5 mL; 0.2 M, pH 7) was stirred for 6 hours at 30 °C with no pH variation, using the reaction in aqueous solution (pH 7) as control. Upon completion, the reaction mixtures were subjected to HPLC analysis (detection wavelength 550 nm) by a 15-fold dilution with ethanol aqueous solution (70%, V/V; pH2). The results showed that amination reaction between rotiorin and monosodium glutamate only occurred by PO_4^{3-} catalysis for the reaction time (Figure S11C). The product (rotiorin alkaloid) was identified by UPLC-MS analysis. The $[M+H]^+$ ion of the rotiorin alkaloid was found at *m/z* 510.21038 (Figure S11 B).

A



B



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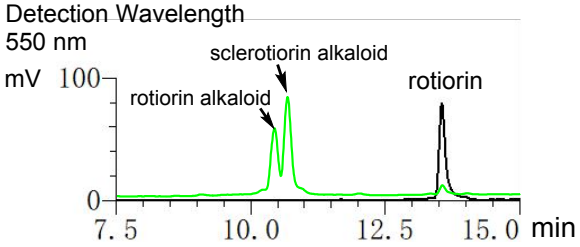

Figure S11. Chemical conversion of rotiorin with monosodium glutamate into rotiorin alkaloid.

(A) Schematic illustration of conversion of rotiorin into rotiorin alkaloid by PO_4^{3-} catalysis. (B) MS analysis of rotiorin and corresponding alkaloid. (C) The reaction occurs in phosphate aqueous solution (0.2 M; pH 7), using the

reaction in aqueous solution (pH 7) as control. The reaction time was 6 hours with no pH variation. Black line and green one were the HPLC analysis of the reaction mixtures in the absence and presence of phosphate. 550 nm was chosen as the detection wavelength for rotiorin to avoid the interference of sclerotiorin.

S9. References

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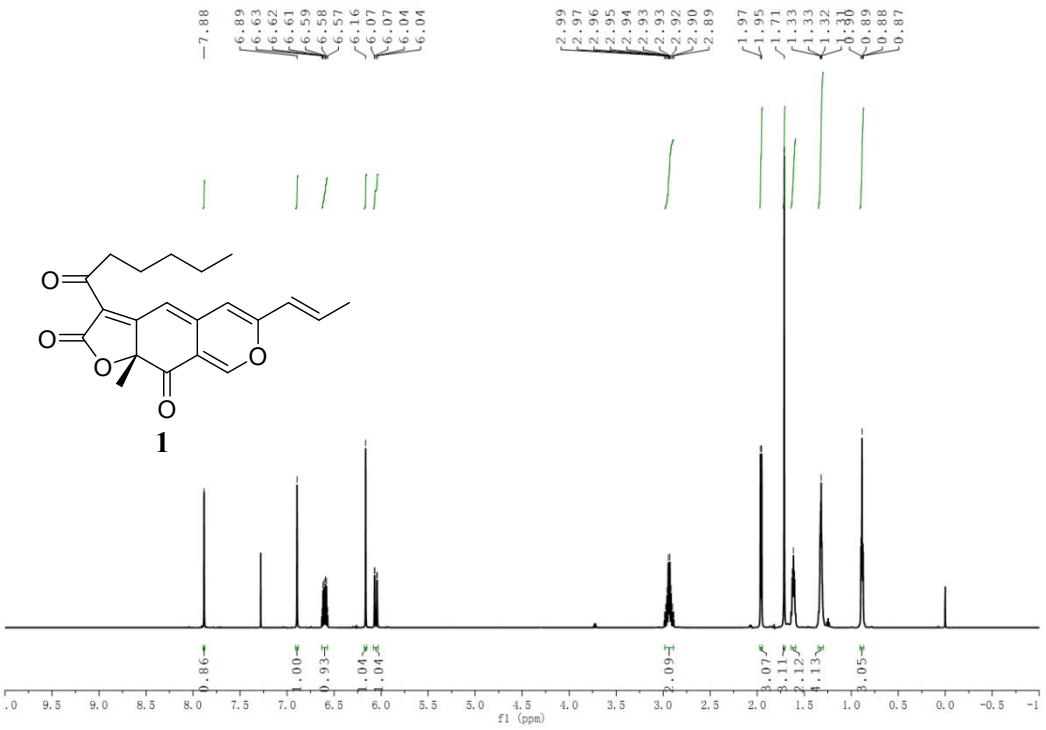
3 Y. Huang, L. Liu, G. Zheng, X. Zhang, Z. Wang, *World J. Microb. Biot.* 2019, **35** (1), 13.

4 S. Kanokmedhakul, K. Kanokmedhakul, P. Nasomjai, S. Louangsysouphanh, K. Soytong, M. Isobe, P. Kongsaeree, S. Prabpai, A. Suksamrarn, *J. Nat. Prod.* 2006, **69**(6), 891-895.

S10. ^{1}H and $^{13}CNMR$ spectra



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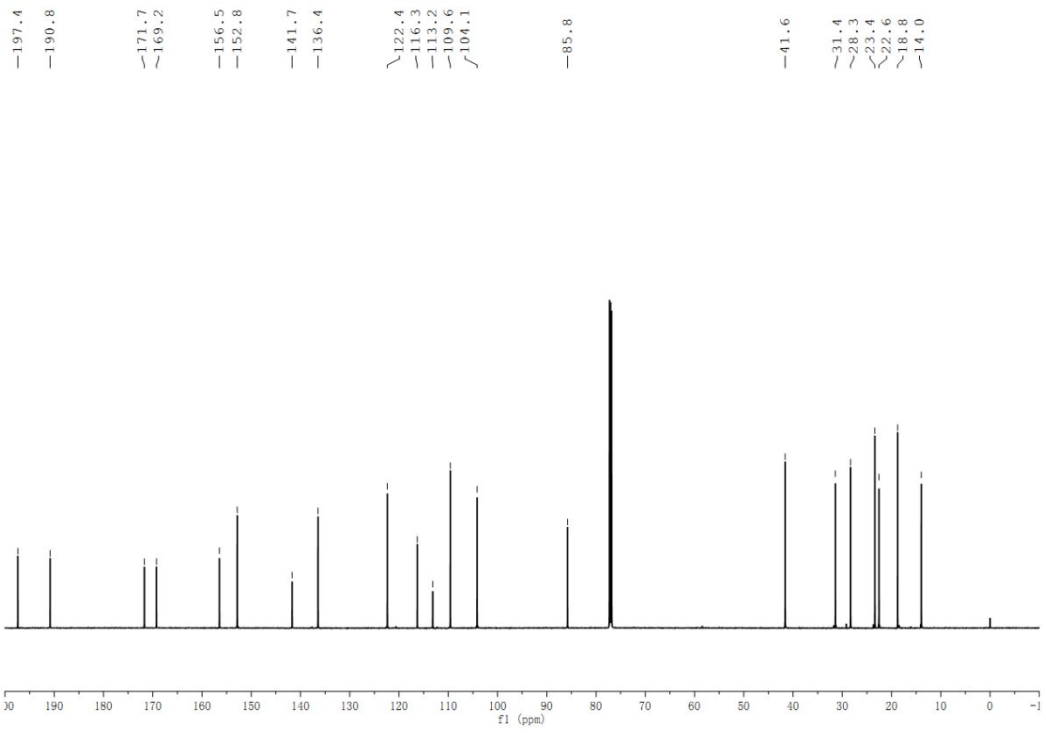

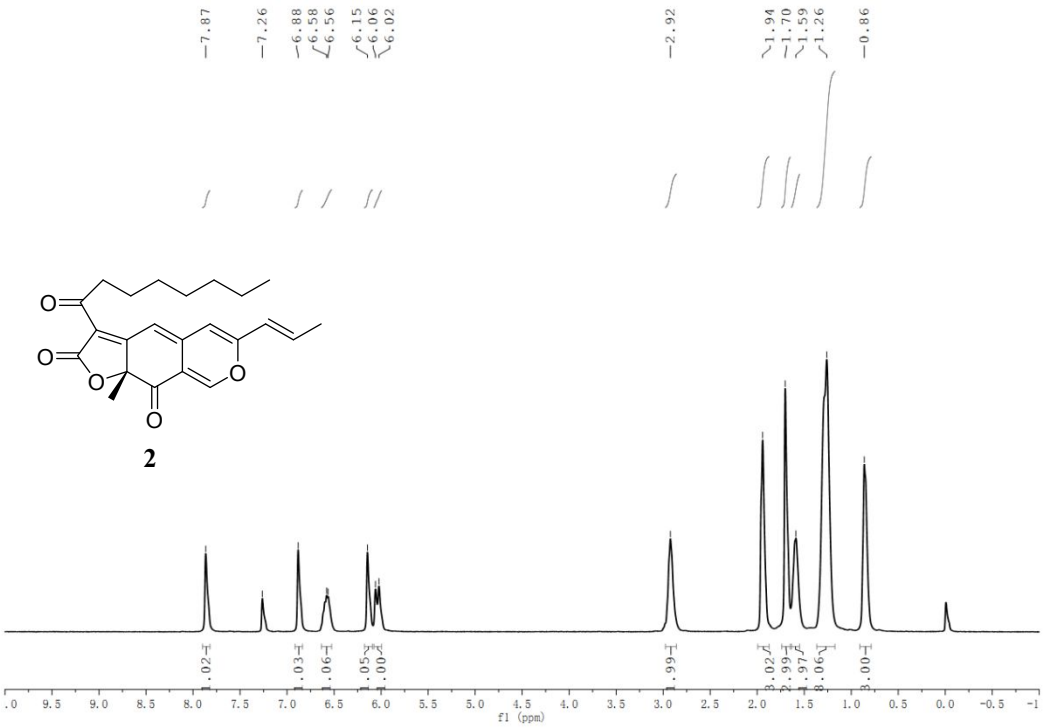
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1.02
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3.00
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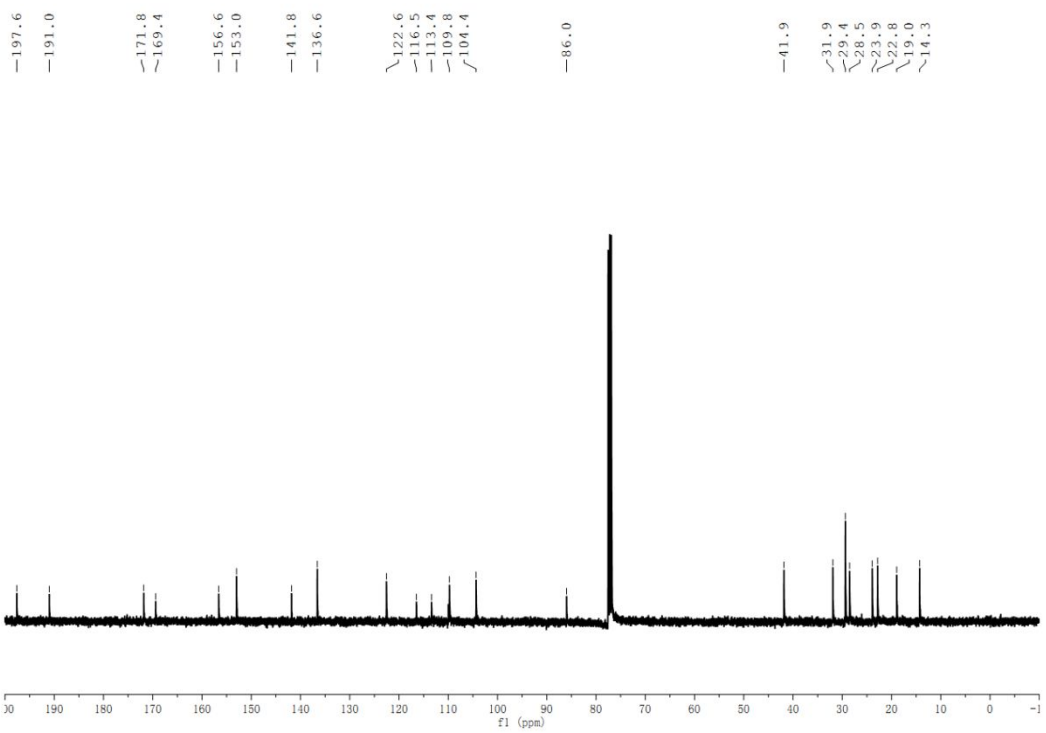
2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153.0
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28.5
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22.8
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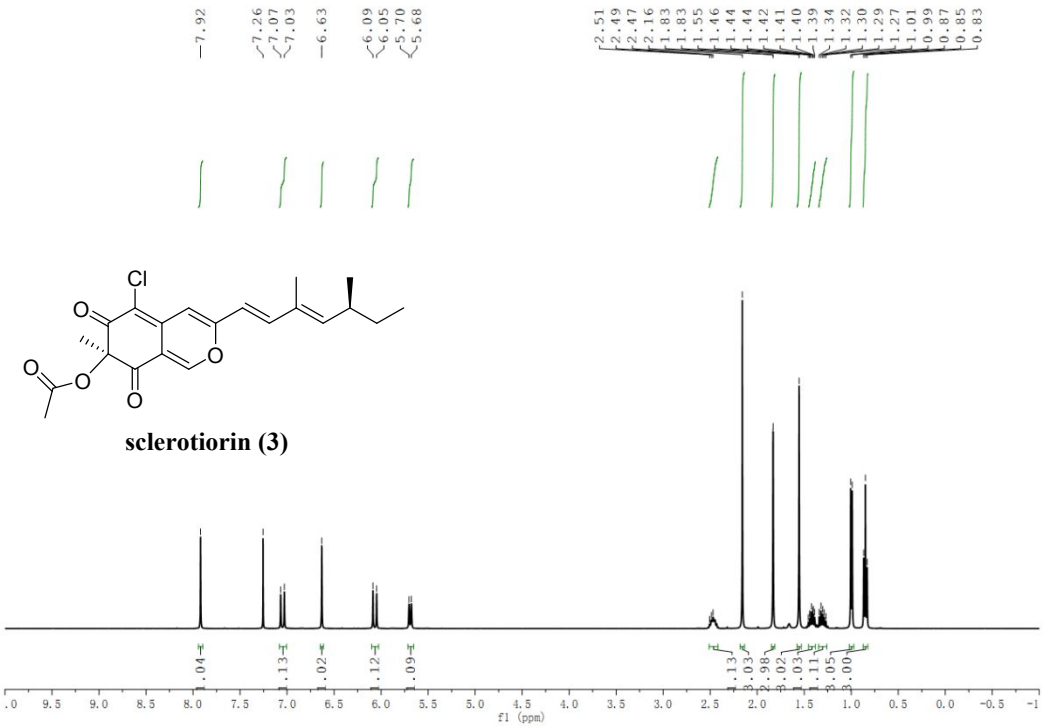
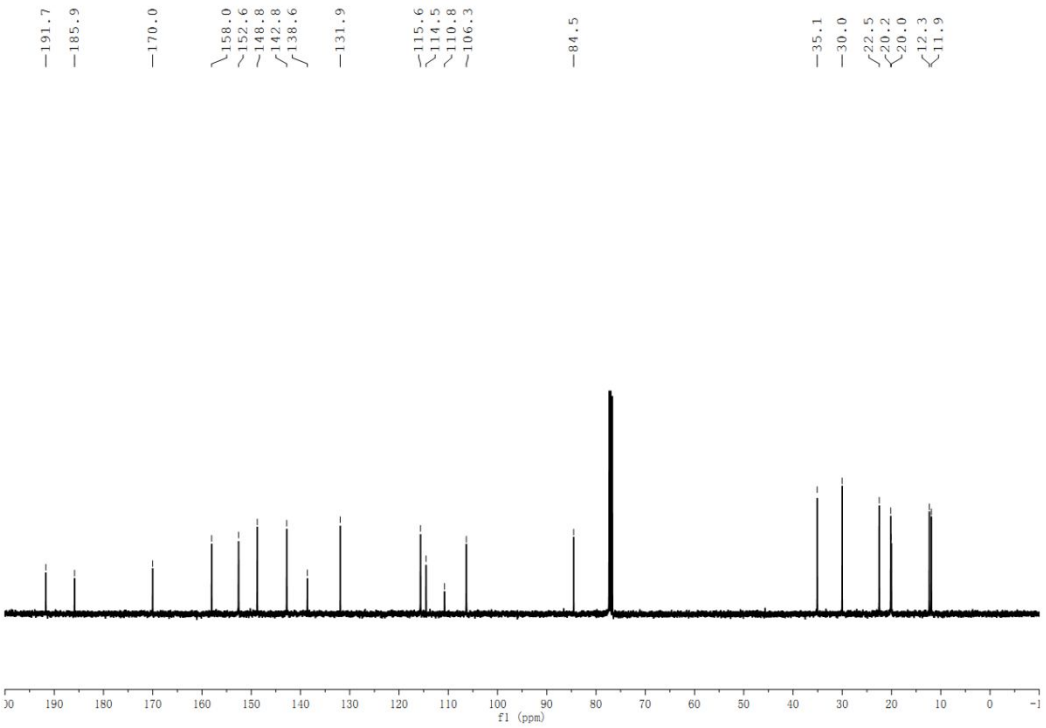
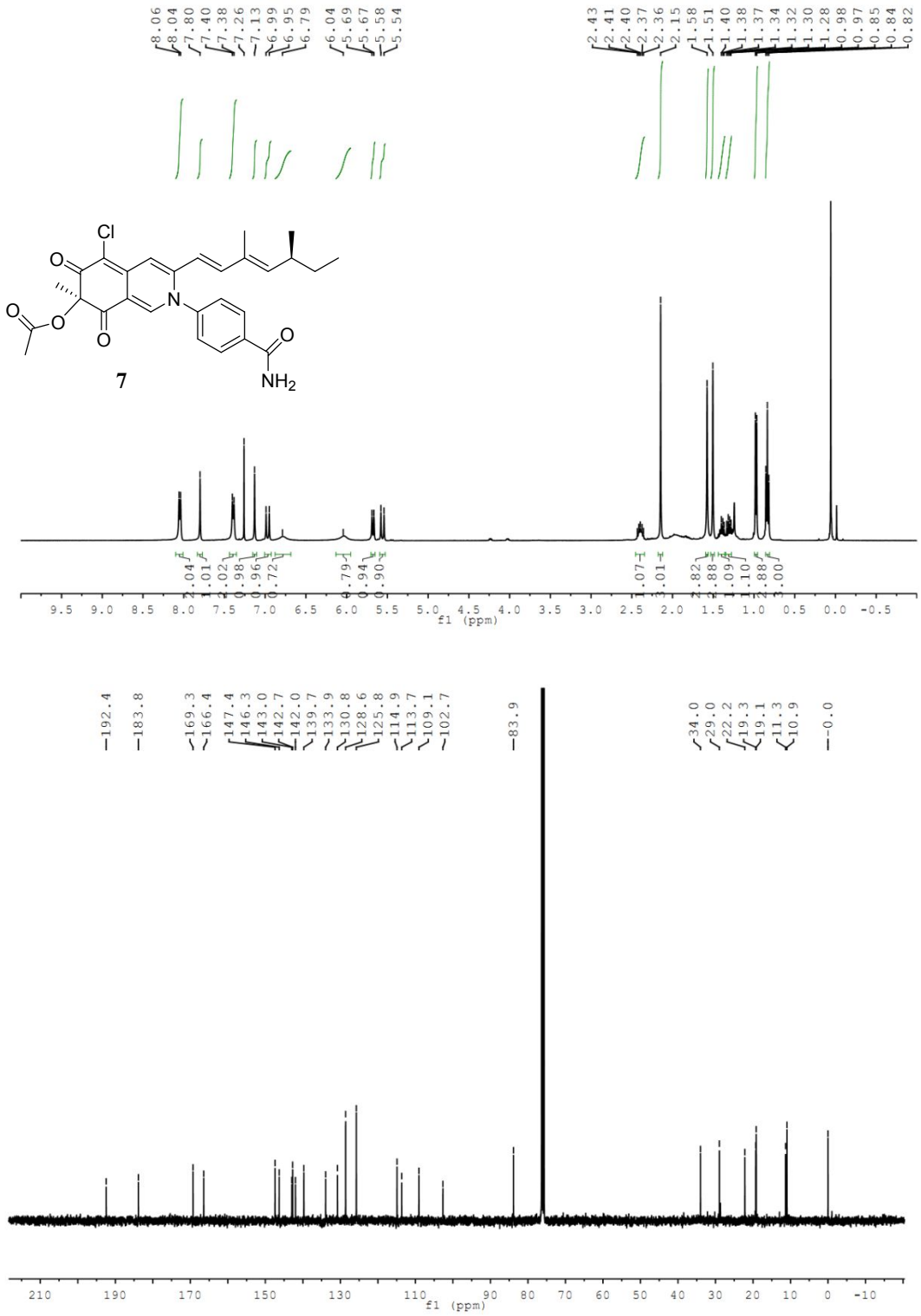
f1 (ppm)



sclerotiorin (3)

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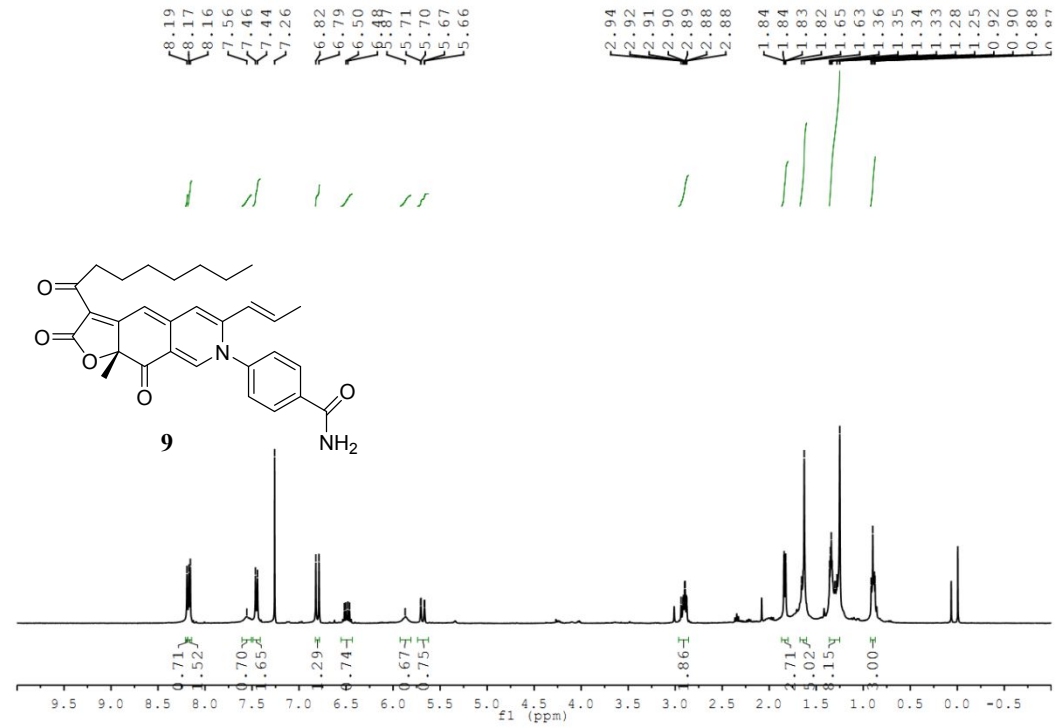
f1 (ppm)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1.63
1.36
1.35
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1.33
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0.88
O
O
O
O
N
O
NH2
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1.65
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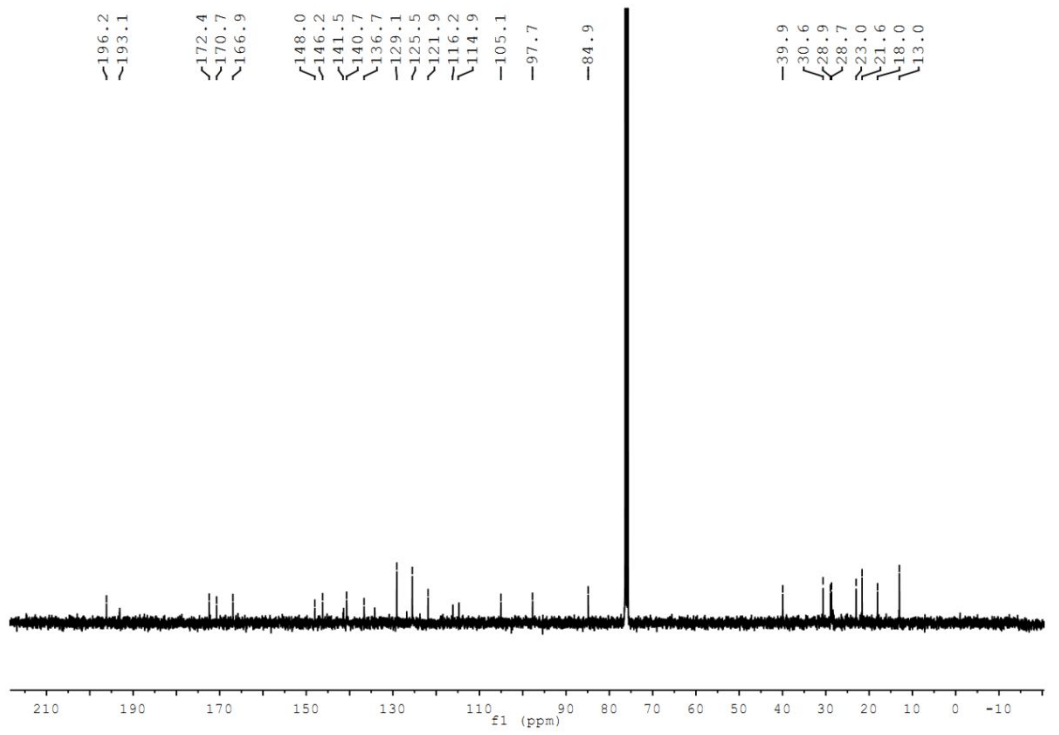
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

9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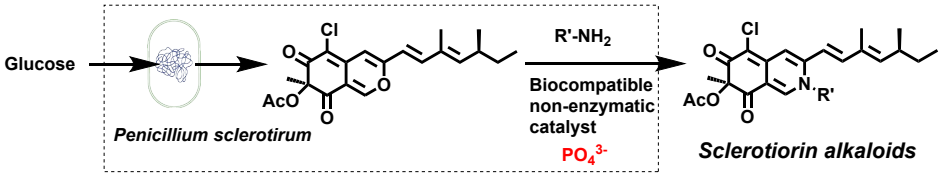
114.9
105.1
97.7
84.9
39.9
30.6
28.9
28.7
23.0
21.6
18.0
13.0
210 190 170 150 130 110 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

Table of contents entry



Synopsis: Exploring PO_4^{3-} as a Brønsted acid catalyst, the biocompatible amination reaction was successfully interfaced with *Penicillium* sp. metabolism to produce sclerotiorin alkaloids.

Referee: 2 Comments to the Author

This paper entitled "Interfacing phosphate catalytic reaction with microbial metabolism for production of azaphilone alkaloids" is a very nice addition to the synthetic strategies now being developed within Green Chemistry principles. The authors show a very smart strategy to couple bio- and chemosynthesis using a very cheap and homogeneous catalyst, H2PO3(2-). However, some clarification is to be done to give a certain perspective of the importance of their findings.

Thanks for your good suggestion!

The typos and language grammar have been corrected according to your suggestion.

Apart from some minor corrections in the attached pdf regarding typos and language grammar, I am more concerned abotu the importance of the products being synthetised and the concentration in which they are obtained. On the first hand, the authors should provide further insights, even market prices of similar products, indicating the commercial importance of some of their products (as this journal has to do with engineering): prospective applications are just suggested, but very slightly.

Thanks for your good suggestion!

Commercial food additive Red *Monascus* pigments® is the similar product of sclerotiorin alkaloid (7) that the price in Chinese market is 1000 RMB/Kg. Sclerotiorin alkaloid (7) is regarded as a potential alternative owing to no mycotoxin accompanying production.

The corresponding content was added in the end of the 2nd paragraph of Page 4 in the present manuscript.

On the other, the concentrations are very low (in the range of 500-600 mg/L). Thus, how these compounds can be purified from the reaction media? Some discussion is needed in this sense.

Thanks for your suggestion!

In the food industry, the pigment concentration is characterized as AU value performed on UV-vis spectrophotometer without purification operation. For example, the content of red pigments in Red *Monascus* pigments® measured by AU_{500} (AU value under detection wavelength 500 nm). For scientific investigation, the experimental data were presented in terms of solutions of a specified molarity in the present work.

COMMUNICATION

Interfacing phosphate catalytic reaction with microbial metabolism for production of azaphilone alkaloids

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Synthetic biology is becoming a conventional technique to maximize production of interesting small molecule chemicals. However, it remains difficult to find the corresponding genes encoding enzymes for most of new natural products or non-natural products biosynthesis. Many strategies, such as designing of non-natural enzyme or artificial enzyme, have been tried to cross the boundary of microbial metabolism. Herein, PO_4^{3-} as a Brønsted acid catalyst is explored to catalyze the chemical reaction between azaphilones (a subgroup of fungi polyketides) and amines under mild condition. We further demonstrate that the mild chemical reaction is biocompatible and can be directly interfaced with microbial metabolism of azaphilones. The combination of chemo- and biosynthesis into a one-pot process in a simple way exhibits many advantages, such as diversifying natural azaphilone alkaloids, eliminating the possible product inhibition or degradation, and acting as a novel strategy for sustainable chemistry.

Nature has evolved highly efficient biosystems by combination of enzymatic and non-enzymatic reactions for multistep syntheses. The corresponding natural product is an attractive source for potential drug discovery.[1] With the vast development of molecular biology, synthetic biology is becoming a conventional technique to modulate and construct pathways within microorganisms to maximize production of interesting small molecule products.[2,3] However, the chemical reaction catalyzed by natural enzymes encoded by heterologous genes depends upon many factors, such as the availability of gene sequences to encode enzymes, substrate promiscuity, and cofactor requirements.[4] Furthermore, the catalytic repertoire of natural enzymes is limited comparing to the vast chemical reactions. Thus, protein engineering, designing of non-natural enzyme via modification of the amino acid series involving in a natural enzyme, has been developed to catalyze new chemical reaction beyond the natural repertoire.[5,6] This is a potential strategy to expand the reaction space of genetically encoded biocatalyst.

Alternatively, biocompatible chemical reaction, i.e., non-enzymatic chemical reactions under mild condition that have no influence on microbial metabolism and, vice versa, microbial metabolisms that also involve no disturbance on the chemical reaction, is the complementary to enzymatic reaction due to the non-enzymatic chemical reaction exhibiting the advantages of broad reactivity, substrate flexibility, and product diversity.[7] However, the mismatch between the optimal condition of non-enzymatic chemical reaction and enzymatic reaction in living organisms is one of the major challenges.[8] Artificial enzyme, the design of non-natural catalytic centers with macromolecular and supramolecular systems by mimicking natural enzyme, can also catalyze abiological reaction.[9] Despite most of artificial enzymes exhibit low catalytic activity, limited substrate range, and moderate selectivity, the similar characters between artificial enzyme and natural enzyme provide the possibility for combining chemo- and biosynthesis in a one-pot process. On the other hand, there are many successful examples of the integration of mild chemical reaction with microbial metabolism or enzymatic catalysis by elegant selection of metal-, organo-, photo-, and electrocatalyst.[10-14]

Azaphilone, possessing a highly oxygenated pyranoquinone bicyclic core and a quaternary carbon center, is a structurally variable family of fungal metabolites.[15] The biosynthetic pathway involves the formation of a key intermediate via a non-reducing polyketide synthase (NR-PKS), and the further construction of a diverse library of azaphilones from the intermediates by divergent enzymatic reactions in various filamentous fungi (Scheme 1A).[16] The corresponding azaphilone alkaloids have been exploited as an attractive source for potential drugs discovery due to their bioactivities, such as anti-inflammatory and cytotoxicity against cancer cell lines.[17] Due to their brilliant color, azaphilone alkaloids are also deve-

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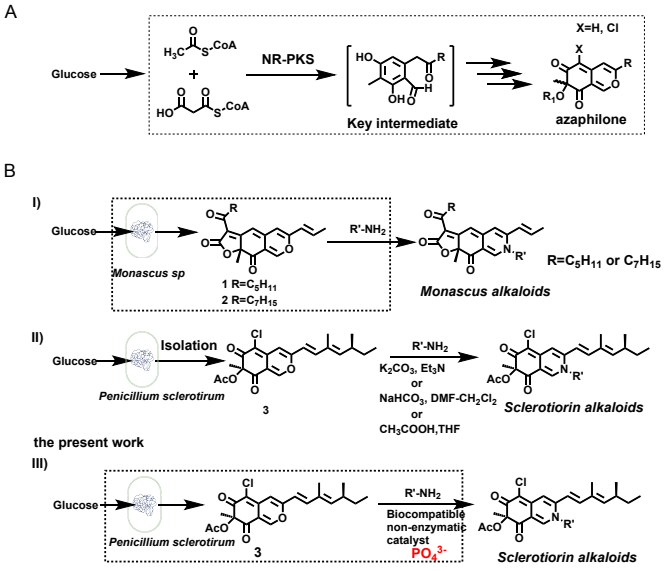


Scheme 1 Schematic illustration of the production of azaphilone alkaloids. A) The diversity of azaphilones in biosynthetic process. B) Chemical conversion of azaphilones into the corresponding azaphilone alkaloids. I) Fermentation of *Monascus* alkaloids involving a spontaneous chemical reaction between the microbial metabolites OMPs (**1**/**2**) and primary amines (denoted within the dotted box). II) Two-step process for production of sclerotiorin alkaloids via biosynthesis of sclerotiorin (**3**) and chemical modification of the isolated sclerotiorin (**3**) under harsh conditions. III) By exploring PO_4^{3-} as a novel catalyst, interfacing a biocompatible chemical reaction with microbial metabolism for direct production of sclerotiorin alkaloids.

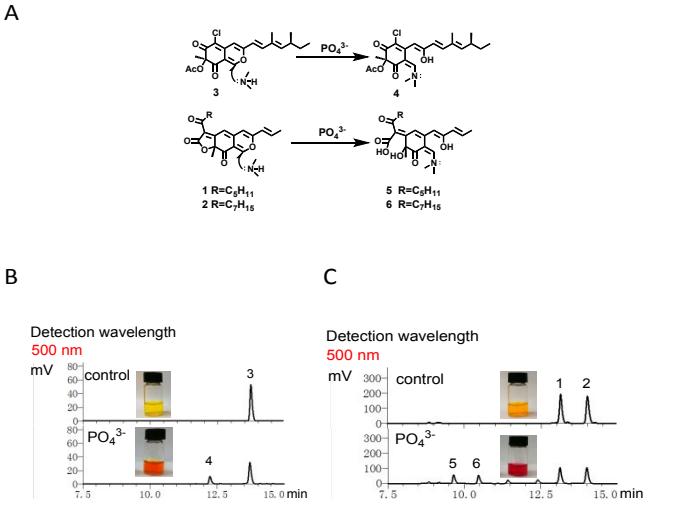


Fig.1 Chemical modification of azaphilones into the corresponding enamines by PO_4^{3-} catalysis. A) Scheme for formation of enamines between azaphilones and dimethylamine hydrochloride using PO_4^{3-} as a novel catalyst. B) The reaction between sclerotiorin (**3**) (2.6 mM) and dimethylamine hydrochloride (26 mM) in aqueous solution (pH 7) catalyzed by PO_4^{3-} (0.1 M). C) The reaction between OMPs (**1**, **2**) (2.6 mM) and dimethylamine hydrochloride (26 mM) in aqueous solution (pH 7) catalyzed by PO_4^{3-} (0.1 M). The aqueous solution (pH 7) in the absence of PO_4^{3-} was utilized as control. The reaction time was 72 hours with no pH variation and the basic condition was detailed in section S4. The reaction reagents sclerotiorin (**3**), OMPs (**1**, **2**) were identified by NMR and MS (Section S2 and S3) while the corresponding products **4**, **5**, and **6** were identified by UPLC-MS (Fig. S3) while no further identified by NMR due to the limited source for preparation of sample.

loped as natural food colorants. [18] *Monascus* alkaloids (red *Monascus* pigments, RMPs), one of the most famous exemplary food colorant, have been applied for more than 1000 years in China.[19] Herein, orange *Monascus* pigments (OMPs, **1** & **2**, formation of five-membered lactone via Knoevenagel cyclization) and sclerotiorin (**3**, possessing the electrondrawing group Cl in the bicyclic core) were considered as the exemplary azaphilones. Amination reaction between the microbial metabolite OMPs (**1**/**2**) and primary amines to form RMPs occurs spontaneously during microbial fermentation (Scheme 1B). It is reported that the production of RMPs can be improved by microbial fermentation in some non-aqueous media via intensifying the amination reaction. [20,21] Sclerotiorin (**3**) can be converted into sclerotiorin alkaloid by microbial transformation (*Beauveria bassiana*).[22] Alternatively, sclerotiorin (**3**) can also be chemically modified into sclerotiorin alkaloids under harsh conditions, such as organic solvent (DMF-CH_2Cl_2, Et_3N or THF), alkaline catalysts (K_2CO_3) or acetic catalyst (acetic acid) (Scheme 1B).[23-25] With regards to the benefits of interfacing non-enzymatic reaction with microbial metabolism, the aim of the present work is managed to explore PO_4^{3-} as a Brønsted acid catalyst to interface the mild chemical conversion of azaphilones into the corresponding alkaloids with microbial metabolism (Scheme 1B).

Herein, our study began by checking the catalytic activity of PO_4^{3-} on formation of enamine between azaphilone and dimethylamine (Fig. 1A). In the control (pH=7; absence of PO_4^{3-}), no chemical reaction occurred between sclerotiorin (**3**) and dimethylamine where the solution remained orange, no pH drifted, and only sclerotiorin (**3**) was detected by HPLC (upper row of Fig. 1B). However, the corresponding phosphate aqueous solution (pH=7) changed from orange to red-orange. HPLC analysis further confirmed the formation of enamine (**4**) (down row of Fig. 1B). The results indicated that PO_4^{3-} catalyzed the formation of enamine under mild condition. A similar result was also observed between OMPs (**1**, **2**) and dimethylamine to produce the corresponding enamines (**5**, **6**) (Fig. 1C).

To further verify the catalytic effect of PO_4^{3-}, the secondary amine dimethylamine (Fig. 1A) was replaced by the primary amine *p*-aminobenzamide (ABA) to produce sclerotiorin alkaloid (**7**) (Fig. 2A). Initially, the effect of PO_4^{3-} concentration on the catalytic activity was evaluated (Fig. 2B). During the whole progress, pH maintained at 7. The corresponding enamine was detectable by HPLC analysis (sclerotiorin enamine with a preferential detection wavelength 300 nm, and the corresponding MS data shown in Fig. S5). Obviously, there was a strong positive correlation between the enamine concentration and PO_4^{3-} concentration. No sclerotiorin alkaloid (**7**) as well as the enamine was observed in the absence of PO_4^{3-}. With the increase of PO_4^{3-} concentration, sclerotiorin (**3**) concentration reduced and sclerotiorin alkaloid (**7**) as well as the enamine concentration increased. This result confirmed the catalytic effect of PO_4^{3-} on the formation of sclerotiorin alkaloid (**7**). Subsequently, the effect of pH on the catalytic activity of PO_4^{3-} (0.1 M) was also evaluated (Fig. 2C). Sclerotiorin alkaloid (**7**) was accumulated markedly at pH

above 5 and maintained a relatively high concentration at pH 7~8. However, the relative low concentrations of both sclerotiorin (**3**) and the corresponding alkaloid (**7**) were observed at pH 9, which might be caused by sclerotiorin (**3**) instability under alkalic condition. Correspondingly, enamine was also observed at pH above 6. This result clearly indicated that the catalytic activity of PO_4^{3-} was strongly dependent on pH value. Furthermore, the conversion of sclerotiorin (**3**) into the enamine catalyzed by PO_4^{3-} was reversed, which was confirmed by the adjustment of pH from 7 to 2 with hydrochloric acid (1 M) (Fig. 2D). This result further confirmed that low pH is unfavorable for PO_4^{3-} catalysis (Fig. 2C). Finally, a time course of the conversion of sclerotiorin (**3**) into sclerotiorin alkaloid (**7**) was carried out. Sclerotiorin (**3**) conversion 44 % and sclerotiorin alkaloid (**7**) yield 1 % were achieved at the 12th hour while conversion 100 % and yield 70 % were obtained at the 120th hour (Fig. 2E). The phenomenon might be attributed to the enamine accumulation during the phosphate catalytic reaction.

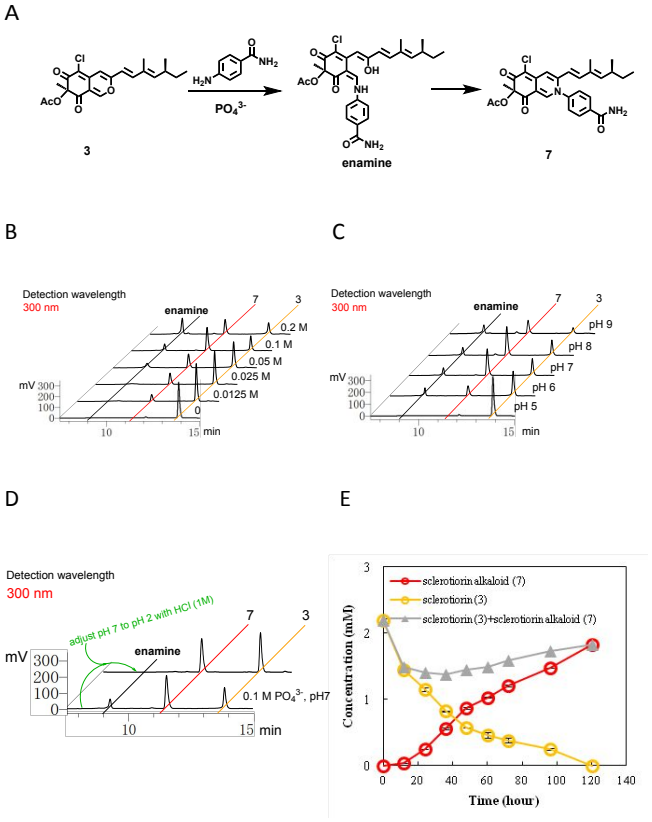
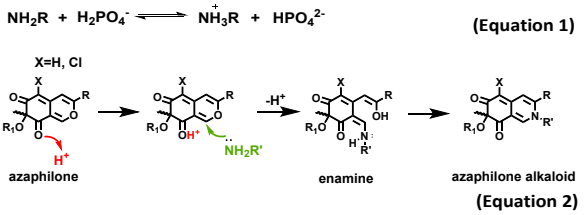


Fig.2 Conversion of sclerotiorin (**3**) into the sclerotiorin alkaloid (**7**). A) Schematic illustration of conversion of sclerotiorin (**3**) into sclerotiorin alkaloid (**7**) by PO_4^{3-} catalysis. B) The effect of PO_4^{3-} concentration on PO_4^{3-} catalytic activity in aqueous solutions (pH 7). Enamine was detected at 9.2 min by HPLC analysis (detection wavelength 300 nm). C) Evaluation of pH effect on PO_4^{3-} catalytic activity. The reaction was carried out in phosphate aqueous solutions (0.1 M; pH 5-9). D) Reverse conversion between enamine and sclerotiorin (**3**) by HPLC analysis with detectable wavelength 300 nm. The resulting mixture of sclerotiorin (**3**) and ABA in phosphate aqueous solutions (0.1 M, pH 7; 72 hours) was adjusted to pH 2 with hydrochloric acid (1 M). E) Time course of the PO_4^{3-} catalytic reaction in phosphate aqueous solutions (0.1 M; pH 7). The identification of sclerotiorin alkaloid (**7**) by NMR and MS and the identification of enamine by MS were detailed in Section S5. A mixture of sclerotiorin (**3**) (2.6 mM) and ABA (5.2 mM) was reacted for 72 hours with no pH variation. The basic experimental condition was described in section S5. Conversion (mol %) was determined as the ratio of sclerotiorin (**3**) concentration to the initial one while yield (mol %) was determined as the ratio of the sclerotiorin alkaloid (**7**) concentration to the initial sclerotiorin (**3**) concentration (2.6 mM) at a certain point of time.

The chemical modification of azaphilone involves two steps, i.e., nucleophilic addition of primary amine to azaphilone produces an enamine which further dehydrates into the azaphilone alkaloid.[26] The present work confirmed that PO_4^{3-} catalyzed the nucleophilic adduct of secondary amines to azaphilones to form the corresponding enamines (Fig. 1). It was further confirmed that the catalytic effect of PO_4^{3-} was strongly depended on PO_4^{3-} concentration and pH of reaction medium (Fig. 2B &C). Based on those results, a possible acid-base catalytic mechanism by using phosphate as a Brønsted acid catalyst is proposed (Scheme 2). Under neutral to weakly alkaline condition, NH_2R and $H_2PO_4^-$ are the major components of phosphate and primary amine in aqueous solution, respectively (Equation 1). $H_2PO_4^-$ acting as Brønsted acid catalyst provides a proton (H^+) (red) to activate azaphilone. At the same time, NH_2R with a lone pair of electrons attacks the activated azaphilone smoothly (green). Then, an intramole-cular proton (H^+) transfer leads to formation of enamine (Equation 2). In addition, plausible hydrogen-bond between the enamine and phosphate ion also facilitates the formation of enamine.[27] This assumption is consistent with the fact that phosphate catalytic activity depends on pH value (Fig. 2C).

To further check the above assumption, the primary amine ABA (Fig. 2A) was expanded to other primary amines. It was observed that the chemical reaction of sclerotiorin with other primary amines was also catalyzed by PO_4^{3-} and the obvious catalytic activity also exhibited under near-neutral condition (Fig. S6). The expansion of sclerotiorin (Fig. 2A) to other azaphilones is limited by the microbial resources, the optimization of microbial culture conditions as well as the downstream separation and identification of the secondary metabolites. Benefits from our previous works,[28] reaction between the classic OMPs (**1**, **2**) (Scheme 1) and the primary amine ABA was further checked (Section S6). It was also observed that the accumulation of *Monascus* alkaloids (**8**, **9**) was enhanced with the increase of PO_4^{3-} concentration (Fig. S8). Furthermore, rotiorin, a minor azaphilone component of culture of *P. sclerotirum*, was also isolated (Section S8). It fur-

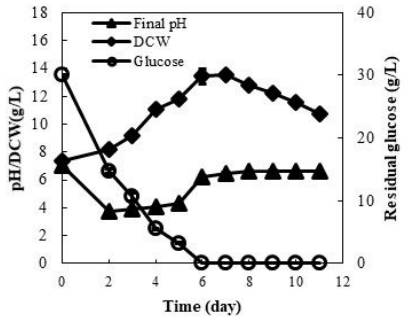


Scheme 2 Proposed mechanism for phosphate as a Brønsted acid catalyst. Crooked arrows denotes electron transfer. Under neutral or weakly alkaline condition, the primary amine exists as NH_2R. Otherwise, existing as NH_3R^+ (the acid forms of NH_2R) under acidic condition, which is unfavorable for the nucleophilic addition to azaphilone. At the same time, the major components of phosphate in an aqueous solution are $H_2PO_4^-$ under weak acidic or neutral condition. Thus, both NH_2R and $H_2PO_4^-$ are only presented under neutral or weakly alkaline condition (Equation 1).

ther confirmed that phosphate catalyzed the reaction of the azaphilone rotiorin with monosodium glutamate (Fig. S11). Thus, phosphate as a Brønsted acid catalyst actually provides a novel strategy for conversion of azaphilones (a subgroup of polyketides) into the corresponding alkaloids under a very mild condition rather than the reported harsh conditions (Scheme 1B & Fig. 2).

Chemical modification of sclerotiorin (**3**) into sclerotiorin alkaloid (**7**) catalyzed by PO_4^{3-} (Fig. 2A) is a mild chemical reaction, which is further pushed to interface with microbial metabolism. In our previous work, suspension culture of *Monascus* sp. confirms that the non-natural primary amine ABA is non-involved in nitrogen microbial metabolism.[20] Hence, cell suspension culture of *P. sclerotirum* in the presence of ABA as well as phosphate was designed to interface the mild chemical reaction (Fig. 2A) with microbial metabolism (Fig. 3). During the first 5 days, fast consumption of glucose, decrease of pH and then maintaining pH approximately 4, and increase of dried cell weight were observed (Fig. 3A). At the same time, sclerotiorin (**3**) concentration increased sharply (Fig. 3B), while no sclerotiorin alkaloid (**7**) was detectable. This result was consistent with that the low pH was unfavorable for chemical reaction (Fig. 2C). From the 6th to 8th day, glucose was consumed completely, and pH climbed up to about 6.6 (Fig. 3A). In the meanwhile, sclerotiorin alkaloid (**7**) was detectable

A



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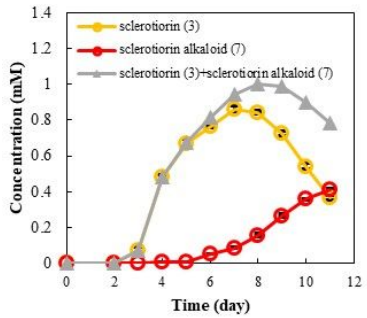

Fig. 3 The time course of cell suspension culture of *P. sclerotirum* to produce sclerotiorin alkaloid (**7**). A) Basic parameters of microbial metabolism, such as cell growth, glucose consumption and pH change. B) Production of sclerotiorin (**3**) and sclerotiorin alkaloid (**7**) during microbial metabolism. The culture condition was 30 g/L glucose, 1 g/L ABA, 6.8 g/L KH_2PO_4, initial pH 7. The basic experimental procedure was detailed in Section S7.

owing to the favorable chemical reaction (approximately neutral pH) (Fig. 2C). Furthermore, the total concentration including sclerotiorin (**3**) and sclerotiorin alkaloid (**7**) increased (Fig. 3B). The result confirmed that biosynthesis of sclerotiorin (**3**) and chemical modification of sclerotiorin (**3**) into its alkaloid (**7**) were fulfilled at the same time, i.e. the mild chemical reaction was successfully merged with microbial metabolism. After the 8th day, no glucose consumption and pH maintaining approximately 6.6, the concentration of sclerotiorin alkaloid (**7**) increased and that of sclerotiorin (**3**) decreased sharply (Fig. 3B). Using cell suspension culture in the presence of ABA while free of phosphate as control, sclerotiorin (**3**) was accumulated while no sclerotiorin alkaloid (**7**) was detectable (Fig. S9). The result clearly showed that the production of sclerotiorin alkaloid (**7**) during cell suspension culture was attributed to the PO_4^{3-} catalysis effect on the chemical reaction (Fig. 2A). Similarly, *Monascus* alkaloids were also observed during the cell suspension of *Monascus sp* (Fig. S10). Compared to the design of artificial enzyme or non-natural enzyme,[5,6,9] interfacing chemical reaction with microbial metabolism by application of PO_4^{3-} as Brønsted catalyst provides a simple strategy to modify a family of azaphilones into the corresponding azaphilone alkaloids (Scheme 1). Furthermore, commercial food additive Red *Monascus* pigments® is the similar product of sclerotiorin alkaloid (7) that the price in Chinese market is 1000 RMB/Kg. Sclerotiorin alkaloid (7) arises as a promising alternative owing to no mycotoxin accompanying production.[18,22]

Conclusions

In summary, phosphate catalyzing a class of chemical reactions between azaphilones and amines is first reported in the present work. The chemical reactions are biocompatible and can be interfaced with microbial metabolism, which exhibits many potential advantages. Firstly, biocompatible reaction expands the diversity of natural azaphilone alkaloids. Secondly, interfacing the biocompatible reaction with microbial metabolism also eliminates the possible product inhibition or degradation. Thirdly, combining chemo- and biosynthesis into a one-pot process, which circumvents the two-step process operating under harsh conditions, provides a novel strategy for sustainable chemistry.

Conflicts of interest

There are no conflicts of interest to declare.

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