Journal of Bioscience and Bioengineering
VOL. 119 No. 3, 289–296, 2015

ELSEVIER

www.elsevier.com/locate/jbiosc

Improved production of phleichrome from the phytopathogenic fungus *Cladosporium phlei* using synthetic inducers and photodynamic ROS production by phleichrome

Kum-Kang So,[1] Ik-Su Jo,[1] Min-Seon Chae,[1] Jung-Mi Kim,[2] Hea-Jong Chung,[1] Moon-Sik Yang,[1] Beom-Tae Kim,[1] Jin-Kug Kim,[3] Jong-Kyung Choi,[3] and Dae-Hyuk Kim[1,*]

Department of Molecular Biology, Department of Bioactive Material Sciences, Center for Fungal Pathogenesis, Chonbuk National University, Jeonju, Chonbuk 561-756, Republic of Korea,[1] Department of Bio-Environmental Chemistry, Wonkwang University, Iksan, Chonbuk 570-749, Republic of Korea,[2] and Jeonbuk Provincial Office, National Agricultural Products Quality Management Service, Jeonju, Chonbuk 561-202, Republic of Korea[3]

Received 28 May 2014; accepted 19 August 2014
Available online 16 September 2014

Two different diketopiperazines, *cyclo*-(L-Pro-L-Leu) and *cyclo*-(L-Pro-L-Phe), which were isolated from the culture filtrate of *Epichloe typhina* and found to be inducers of phleichrome production, were chemically synthesized and evaluated for use in the improved production of phleichrome from wild-type and UV-mutagenized strains (M0035) of *Cladosporium phlei*. When supplemented with PDA and V8 juice agar media, both inducers showed significant increases in the production of phleichrome. Phleichrome production was increased in a dose-dependent manner up to a concentration of maximum yield for both inducers. No further significant induction was observed by supplementing inducers over the concentration of maximum yield. Among the two inducers, *cyclo*-(L-Pro-L-Phe) showed better inducing capability than *cyclo*-(L-Pro-L-Leu). The maximum yield was observed from the M0035 strain grown on V8 juice media supplemented with 150 μM *cyclo*-(L-Pro-L-Phe), which was estimated to be 232.6 mg of phleichrome per gram of mycelia and 10.2 mg of secreted phleichrome per 20 agar-plugs. Interestingly, growth inhibition was observed on V8 juice agar media with 100, 150, and 200 μM *cyclo*-(L-Pro-L-Phe) but not on PDA with the same amount of inducer, which suggests that the inhibitory effect might be through the overproduction of phleichrome rather than the toxic effect of the inducer itself. Superoxide production by purified phleichrome was dramatically stimulated upon illumination, thus demonstrating photodynamic production of superoxide *in vitro* by phleichrome.

[**Key words:** *Cladosporium phlei*; Perylenequinone; Phleichrome; Diketopiperazine; Inducer]

The phytopathogenic fungus *Cladosporium phlei* (C. T. Gregory; de Vries) is a hypomycetous fungus that causes purple eyespot disease in timothy (*Phleum pratense*). The disease is characterized by circular purple (and later, brown) spots with white to grayish-fawn centers on host leaves, and has been shown to be the most common foliar disease affecting timothy (1). *C. phlei* produces a phytotoxic orange pigment, and the principal compound causing this phytotoxic effect was shown to be phleichrome (2).

Phleichrome belongs to a member of a group of fungal perylenequinones and has a structure of 1,12-bis-(2-hydroxypropyl)-2,6,7,11-tetramethoxy-4,9-dihydroxyperylene-3,10-quinone, a derivative of 4,9-dihydroxyperylene-3,10-quinone. Some compounds derived from 4,9-dihydroxy-3,10-perylenequinone are associated with photogenic diseases (e.g., hypericism, fagopyrism) and exhibit photodynamic properties (3). The compounds belonging to the latter are erythropines, fagopyrin, elsinochromes, cercosporin, phleichrome, and hypocrellin.

Photodynamic therapy (PDT) is an innovative and attractive method for the treatment of small and superficial tumors. PDT requires both a selective photosensitizer and a particular type of light. Briefly, when photosensitizers are exposed to a specific wavelength of light, they produce a reactive oxygen species (ROS) that kills nearby cancer cells. Photofrin II, hematoporphyrin derivatives, was the first approved PDT agent (approved in 1993) for the treatment of bladder cancer and has become the most commonly used and studied PDT sensitizer (4). However, many new compounds have been studied in an attempt to create more efficient treatments. Among the new candidates, hypocrellin isolated from the natural fungus sac of *Hypocrella bambusae* (Berk. et Broome) has gained considerable attention as a potential photosensitizer for PDT (5). Hypocrellin has several advantages over the current photodynamic therapeutic agent, Photofrin II, including a low aggregation tendency, high quantum yields of singlet oxygen ($^1O_2$), low toxicity, and rapid metabolism *in vivo* (6). However, large-scale and sustainable production of hypocrellin is hampered by the rarity of its natural source, *H. bambusae*, which is harvested in limited regions and seasons in China. Moreover, hypocrellin production from *H. bambusae* is largely dependent on factors that are difficult to control, such as weather, and thus limit production. Therefore, as an alternative, a chemical synthesis method involving total or semi-total synthesis from readily available starting materials has been introduced (7). In this method, calphostin D, a

* Corresponding author. Tel.: +82 63 270 3440; fax: +82 63 270 3345.
E-mail address: dhkim@jbnu.ac.kr (D.-H. Kim).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2014.08.011

stereoisomer of phleichrome, was used to prepare hypocrellin B (7). Moreover, a recent synthetic study confirmed the feasibility of transforming phleichrome into various potential PDT agents, including hypocrellin B (8). However, this alternative semi-total synthetic methodology for the preparation of the key precursor compound was the significant rate-limiting step. The chemical synthesis of phleichrome itself required more than 14 steps, starting from 3,5-dimethylbenzaldehyde. Thus, a method for the sustainable, large-scale production of the key precursor compound for the production of various derivatives will require the establishment of biological practices, including strain improvement and the optimization of the fermentation process. This appears to be the best option when compared to other means, and will facilitate the development of more efficient derivatives than hypocrellins.

In our previous study, we described the culture characteristics of *C. phlei*, the extraction of phleichrome, and the UV-mutagenesis of *C. phlei* to breed a fungal strain for the stable overproduction of phleichrome, which yielded 146 mg and 446 mg of phleichrome in the mycelia and the agar media of the bred strain, respectively, from 25 plates produced by pouring 40 ml of melted V8 juice agar (9,10). Although these studies demonstrated that it was possible to prepare phleichrome from biological sources, a further increase in phleichrome production is required for the sustainable conversion of phleichrome to hypocrellins and the development of other effective derivatives. A previous study using TLC bio-autography revealed that two diketopiperazines from *Epichloe typhina*, an endophytic fungus of the timothy plant, stimulated the production of phleichrome from *C. phlei* (11). Therefore, it demonstrated the possibility of stimulation of phleichrome production. However, the previous study used diketopiperazines, which were purified from a culture broth of *E. typhina*. In addition, since they tested the response of *C. phlei* to the inducers on TLC plates, no cultural practice has been conducted for the stimulation of phleichrome production. Also, no studies attempting the overproduction of phleichrome have been conducted.

Chemical synthesis to obtain a product has several advantages over the purification from natural resources in that it can be easy to manipulate the final product and easy to scale-up. Therefore, the current study describes the inducing capacity of chemically-synthesized inducers when supplemented into culture media. Cultural characteristics necessary for the sustainably improved production of phleichrome were analyzed using two different strains, a wild-type and a bred strain. In addition, photodynamic superoxide production by the purified phleichrome was demonstrated.

MATERIALS AND METHODS

Fungal strains, culture media, and growth conditions The *C. phlei* wild-type strain obtained from the American Type Culture Collection (ATCC 36193) and the bred strain M0035 obtained from the previous UV-mutagenesis study (10) were maintained on V8 juice agar plates under constant dark condition at 20°C, as previously described (9,10). The culture conditions and methods for the preparation of cultures on cellophane membrane overlaying agar plates have been previously described (9,10). Briefly, experimental cultures were initiated by placing uniform sized agar blocks, excised from the margins of actively growing colonies, at the center of the plates containing appropriate media with or without cellophane overlay. The media used for comparison were potato dextrose agar (PDA) (Difco, USA) and V8 juice agar [vegetable juice (Campbell Soup Co., USA) 20% v/v, $CaCO_3$ 0.3%, agar 2%].

Chemical synthesis of inducers *N*-Boc-L-Phe-L-proline methyl ester (**2a**) and *cyclo*-(L-Phe-L-Pro) (**3a**): *N*,*N*-diisopropylethylamine (DIEA) (1.31 ml, 7.54 mmol), *N*-Boc-L-phenylalanine (0.85 g, 3.0 mmol), and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU) (1.72 g, 4.52 mmol) were added to a solution of L-proline methyl ester hydrochloride (0.63 g, 3.77 mmol) in dimethylformamide (DMF) as a solvent. The reaction mixture was stirred at room temperature overnight. The DMF was removed *in vacuo* and the residue was diluted in ethyl acetate (EtOAc), washed with $NaHCO_3$ and brine, dried over anhydrous $MgSO_4$, and filtered. The solvent was evaporated *in vacuo*. The resulting crude product was purified by column chromatography using silica gel (ethyl acetate/hexane, 7:3, v/v), yielding a linear dipeptide, *N*-Boc-L-Phe-L-proline methyl ester, **2a** in 99% of yield. *N*-Boc-L-Phe-L-proline methyl ester (0.12 g, 0.30 mmol) was treated with CF_3CO_2H (1.2 ml) in CH_2Cl_2 (1 ml) at room temperature to give a quantitative yield of L-Phe-L-proline methyl ester. The trifluoroacetate salt of the dipeptide methyl ester (0.10 g, 0.28 mmol) was dissolved in 5% aqueous $NaHCO_3$ (0.5 ml) and stirred for 4 h at room temperature. The reaction mixture was extracted with methylene chloride (50 m × 3), and the combined extracts were dried over anhydrous $MgSO_4$, and evaporated to yield a crude product, which was purified by column chromatography using silica gel (CH_2Cl_2/MeOH, 95:5, v/v), yielding the corresponding cyclized dipeptide, *cyclo*-(L-Phe-L-Pro) (**3a**), at an 87% yield.

N-Boc-L-Leu-L-proline methyl ester (**2b**) and *cyclo*-(L-Leu-L-Pro) (**3b**): Compounds **2b** and **3b** were synthesized by the same procedure described above for the preparation of compounds **2a** and **3a**. The isolated yields of those compounds were 95 and 85%, respectively.

Melting points were recorded on an Electrothermal melting point apparatus and uncorrected. LC/MS spectra were recorded on an Agilent 6410B Triple Quad, and the solvent system for elution was a co-solvent containing equal volumes of water and acetonitrile containing 0.1% formic acid, and the procedure was performed in an isocratic manner. ^1H and ^{13}C NMR spectra were recorded on a Jeol 400 MHz spectrometer (Tokyo, Japan).

Spectroscopic data Methyl 1-(2-(*tert*-butoxycarbonylamino)-3-phenylpropanoyl)pyrrolidine-2-carboxylate (*N*-Boc-L-Phe-L-Pro) (**2a**): pale yellow oil; ^1H NMR ($CDCl_3$, 400 MHz): δ 7.22 (m, 5H), 5.27 (d, 1H, J = 8.8 Hz), 4.60 (m, 1H), 4.45 (m, 1H, H-5), 3.69 (s, 3H), 3.55 (m, 1H), 3.15 (m, 1H), 3.05 (m, 1H), 2.86 (m, 2H), 2.11 (m, 1H), 1.88 (m, 3H), 1.33 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 172.11, 170.47, 154.99, 136.24, 129.53, 128.14, 126.55, 79.37, 58.73, 53.08, 51.98, 46.63, 38.94, 28.83, 28.14, 24.69; ESIMS (m/z): $(M + H)^+$ 377.3.

Methyl 1-(2-(*tert*-butoxycarbonylamino)-4-methyl-pentanoyl)pyrrolidine-2-carboxylate (*N*-Boc-L-Leu-L-Pro) (**2b**): pale yellow oil; ^1H NMR ($CDCl_3$, 400 MHz): δ 5.08 (d, 1H, J = 9.2 Hz), 4.44 (m, 2H), 3.73 (m, 2H), 3.68 (s, 3H), 3.57 (m, 2H), 2.17 (m, 1H), 1.96 (m, 3H), 1.72 (m, 1H), 1.38 (s, 9H), 0.95 (d, 3H, J = 6.8 Hz), 0.91 (d, 3H, J = 6.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 172.42, 171.75, 155.61, 79.41, 58.59, 52.11, 50.20, 46.63, 41.87, 28.88, 28.26, 24.83, 24.46, 23.30, 21.73; ESIMS (m/z): $(M + H)^+$ 343.3.

3-Benzyl-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione (cyclo-(L-Phe-L-Pro)) (**3a**): mp: 129°C; ^1H NMR ($CDCl_3$, 400 MHz): δ 7.28 (t, 2H, J = 7.8 Hz), 7.21 (t, 1H, J = 7.3 Hz), 7.15 (d, 2H, J = 6.8 Hz), 5.64 (s, 1H, −NH), 4.20 (dd, 1H, J = 2.9, 10.7 Hz), 4.00 (t, 1H, J = 7.3 Hz), 3.53 (m, 3H), 2.72 (dd, 1H, J = 10.5, 14.6 Hz), 2.25 (m, 1H), 1.90 (m, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 169.33, 165.01, 135.92, 129.21, 129.08, 127.49, 59.09, 56.15, 45.40, 36.75, 28.31, 22.50; ESIMS (m/z): $(M + H)^+$ 245.2.

3-Isobutyl-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione (cyclo-(L-Leu-L-Pro)) (**3b**): mp: 155°C; ^1H NMR ($CDCl_3$, 400 MHz): δ 6.48 (s, 1H), 4.07 (t, 1H, J = 7.8 Hz), 3.96 (dd, 1H, J = 3.4, 9.2 Hz), 3.52 (m, 2H), 2.29 (m, 1H), 2.15–1.90 (m, 3H), 1.85 (m, 1H), 1.75 (m, 1H), 1.48 (m, 1H), 0.95 (d, 3H, J = 6.8 Hz), 0.91 (d, 3H, J = 6.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.35, 166.20, 58.91, 53.36, 45.42, 38.49, 28.01, 24.55, 23.20, 22.68, 21.20; ESIMS (m/z): $(M + H)^+$ 211.2.

Induction experiments by supplementation of synthetic inducers Each inducer was dissolved in methanol to prepare a 100 mM stock solution, and the inducers were supplemented to the culture media at an appropriate concentration before pouring. Four different final concentrations (50, 100, 150, and 200 μM) of inducers were selected and two different representative fungal culture media, PDA and V8 juice agar plates, were used to examine the induction capability of the synthesized inducers.

An agar block (0.5 cm in diameter) containing actively growing young hyphae was inoculated on cellophane layered on top of the appropriate agar medium, where it was cultured under constant dark conditions at 20°C until use. The mycelia were then harvested by scraping from the detached cellophane overlaying media, which was used to determine the yield of phleichrome. In order to determine the yield of secreted phleichrome, 20 uniform-size (0.5-cm-diameter) agar plugs of media were obtained from positions 2 cm away from the center of the plate (10).

To examine the culture characteristics of the strains, the hyphal growth rate was compared to the growth rate of the wild-type strain. The fungal growth rate was assessed via measurements of the diameter of each colony at 90° angles, until no further expansion was observed (9). To evaluate the fungal biomass, mycelial pads were stripped from colonies that had been grown on cellophane overlaying media, weighed to determine the fresh weight, and dried further for two days at 80°C to determine the dry weight (9). The experimental design included five replicates and the experiment was repeated a minimum of three times.

Quantification of phleichrome To determine phleichrome content, phleichrome was extracted from mycelia and agar media using EtOAc, as described previously (10). To rapidly determine if phleichrome was present, the crude extract was analyzed using thin-layer chromatography (TLC) on silica gel with a resolving solution (CH_2Cl_2/MeOH = 19:1, v/v) and purified phleichrome as a control, as described previously (10). In addition, the absorbance of the EtOAc extract was measured with a spectrophotometer at a wavelength of 474 nm by using a model DU530 spectrophotometer (Beckman, USA) (11). The concentration of phleichrome was calculated by reference to a regression line that was established with a known amount of purified phleichrome as a standard using a spectrophotometer at 474 nm.