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Reactive oxygen species altering the metabolite profile of the marine-derived fungus *Dichotomomyces cejpii* F31-1

Sharpkate Shaker[a], Ting-Ting Sun[a], Liang-Yue Wang[c], Wen-Zhe Ma[d], Dong-Lan Wu[a], Yong-Wei Guo[a], Jun Dong[e], Yan-Xiu Chen[a], Long-Ping Zhu[a], De-Po Yang[a], Hou-Jin Li[b] and Wen-Jian Lan[a]

[a]School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, China; [b]School of Chemistry, Sun Yat-sen University, Guangzhou, China; [c]School of Bioscience & Bioengineering, South China University of Technology, Guangzhou, China; [d]State Key Laboratory of Quality Research in Chinese Medicine, Macau Institute for Applied Research in Medicine and Health, Macau University of Science and Technology, Taipa, Macau, China; [e]School of Traditional Chinese Medicine, Guangdong Pharmaceutical University, Guangzhou, China

ABSTRACT

To investigate the influence of reactive oxygen species (ROS) on the secondary metabolites of the marine-derived fungus *Dichotomomyces cejpii* F31-1, hydrogen peroxide (H_2O_2) was added to the GPY culture medium. The HPLC chromatogram of the EtOAc extract of the culture broth was distinct from that of the H_2O_2 free GPY medium. Further study of the metabolites in the GPY medium with H_2O_2 resulted in the discovery of eight known compounds. Among them, (22*E*)-5α, 8α-epidioxyergosta-6, 22-dien-3β-ol (**2**) and ergosta-4,6,8(14),22-tetraene-3-one (**3**) were present in the highest concentration, while ergosterol and diketopiperazines are abundant in the H_2O_2 free medium. Additionally, a new compound, dichocetide D (**1**) containing a chlorine element and a known ergosterol (**10**) were isolated from the H_2O_2 free medium. (22*E*)-5α, 8α-epidioxyergosta-6, 22-dien-3β-ol (**2**) exhibited moderate cytotoxic activity against human prostate cancer cell line LNCaP-C4-2B.

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CONTACT De-Po Yanga lssydp@mail.sysu.edu.cn School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, 510006, China; Hou-Jin Lib ceslhj@mail.sysu.edu.cn School of Chemistry, Sun Yat-sen University, Guangzhou, 510275, China; Wen-Jian Lan lanwj@mail.sysu.edu.cn School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, 510006, China.

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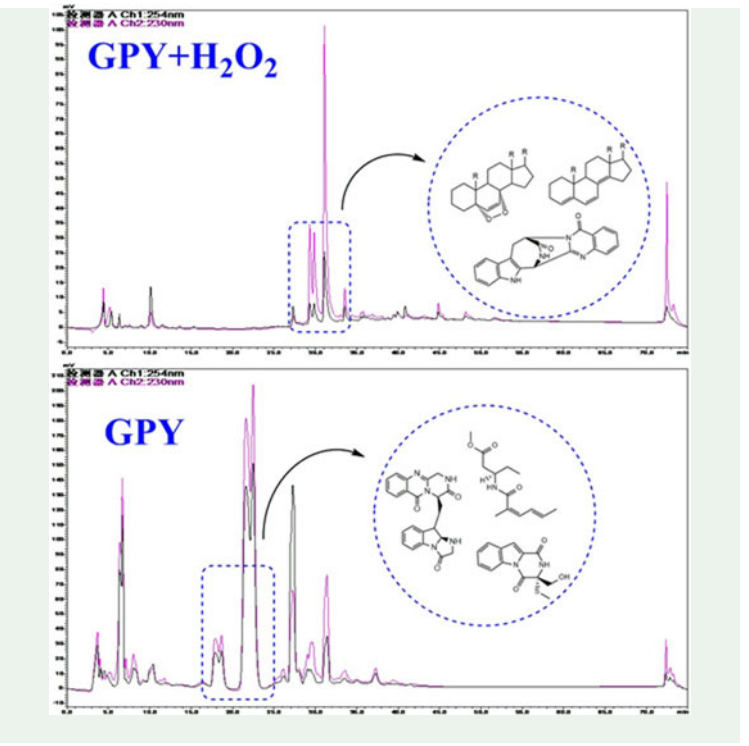


1. Introduction

Reactive oxygen species (ROS) comprising radical and non-radical oxygen, including superoxide anion (O^{2-}), hydroxyl radical (OH^{-}), and hydrogen peroxide (H_2O_2) are produced as natural byproducts of aerobic metabolism. ROS at low concentrations play indispensable roles in cell signaling and homeostasis. However, at higher levels, ROS can influence the progression of a variety of human disease (Devasagayam et al. 2004; Rajendran et al. 2014). When encountering environmental stress, such as drought, salinity, UV light or heat exposure, ROS concentrations can dramatically increase, resulting in damage to the cell membrane and changes in metabolism (Caverzan et al. 2016). Thus, to adapt to environmental changes, a counteracting oxidant response, referred to as the oxidative stress response, is triggered.

The synthesis of certain secondary metabolites is triggered by ROS accumulation, and the biosynthesis of mycotoxins can be influenced by the presence of reactive oxygen species (Reverberi et al. 2010). For example, oxidative stress triggers the production of citrinin in the fungus *Penicillium verrucosum* (Schmidt-Heydt et al. 2015). Oxidative stress also initiates aflatoxin B_1 biosynthesis in *Aspergillus flavus* (Grintzalis et al. 2014). ROS regulates the biosynthesis of lovastatin in *Aspergillus terreus* (Miranda et al. 2013, 2014).

To investigate the influence of oxidative stress on the secondary metabolites of the target fungus, ROS culture strategy was applied, in which one of the exogenous reactive oxygen species, H_2O_2, was added to the culture medium. Then the natural products produced by the fungus under the stress condition were studied. The fungus

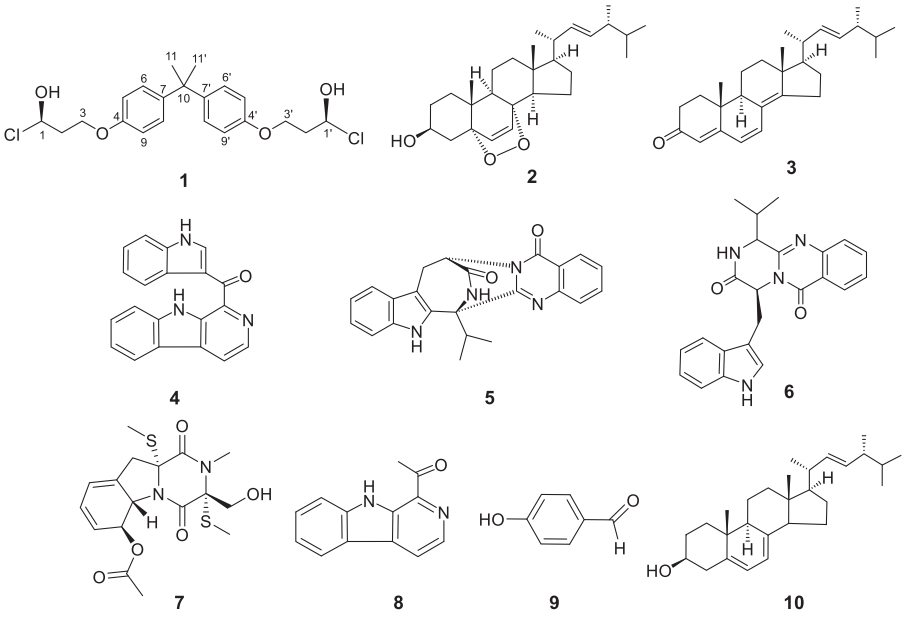


Figure 1. Chemical structures of compounds **1–10**.

Dichotomomyces cejpii F31-1 was isolated from the soft coral *Lobophytum crassum*. In our previous work, more than thirty compounds of chemical diversity were discovered from the marine-derived fungus *Dichotomomyces cejpii* F31-1 (Chen et al. 2017; Wu et al. 2018). The tremendous biosynthetic potential of this fungal strain inspired us to study it further. Using a small-scale 2 L culture, we found that the HPLC chromatograms of the EtOAc extracts of the culture broth obtained by using GPY medium supplemented with H_2O_2 and without H_2O_2 were distinct (Figure S6 and S7 in Supplementary material). Using a 50 L-scale culture, eight compounds, including (22*E*)-5α,8α-epidioxyergosta-6,22-dien-3β-ol (**2**) (Kwon et al. 2002), ergosta-4,6,8(14), 22-tetraene-3-one (**3**) (Schulte et al. 1968), pityriacitrin (**4**) (Mexia et al. 2015), sartorymensin (**5**) (Buttachon et al. 2012), fiscalin B (**6**) (Wong et al. 1993), 6-acetylbis-(methylthio)-gliotoxin (**7**) (Watts et al. 2010), 1-acetyl-8-hydroxy-β-carboline (**8**) (Zhou et al. 1998), 4-hydroxy-benzaldehyde (**9**) (Huang and Wang 2004) were obtained in the medium added with H_2O_2, and most of these compounds were not appeared in the previous research without H_2O_2. Additionally, a new compound, dichocetide D (**1**) and an ergosterol (**10**) (Goad and Akihisa 1997) were isolated as part of our previous investigation of the metabolites from H_2O_2 free medium. Other compounds isolated from the medium without H_2O_2 are the same with previously reported in our research (Chen et al. 2017; Wu et al. 2018). (Figure 1).

2. Results and discussion

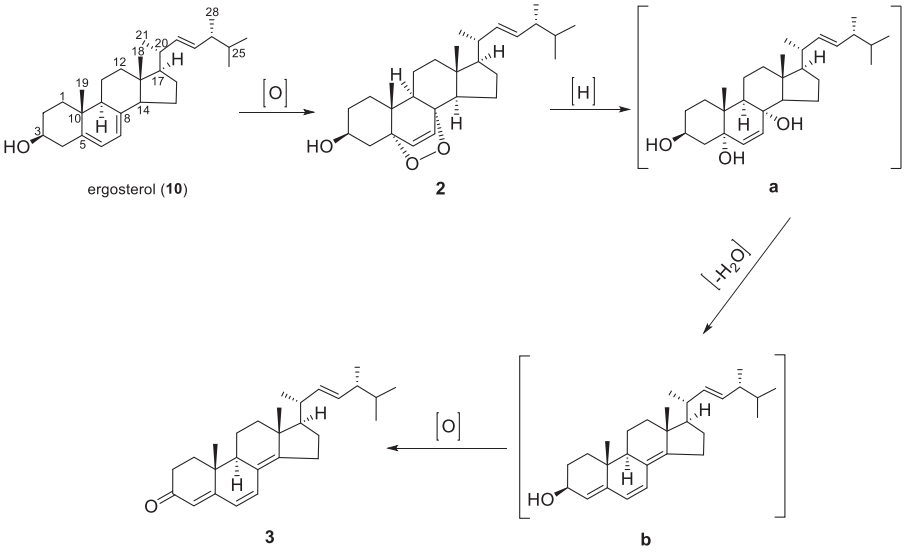
2.1. Structure determination

Dichocetide D (**1**) was acquired as a brown oil. It had the molecular formula of $C_{21}H_{26}Cl_2O_4$ with eight degrees of unsaturation, which was established on the basis

of the high resolution electrospray ionization mass spectroscopy (HR-ESI-MS) at m/z 411.11342 $[M-H]^-$ (calcd. 411.11354). The relative abundance ratio [M: (M + 2): (M + 4) = 9: 6: 1] verified the presence of two chlorine atoms. Further analysis of the 1 D NMR data and HSQC spectrum indicated the highly symmetric structure of this compound. The 1 D NMR displayed 26 proton and 21 carbon signals, comprising four pairs of symmetric aromatic sp^2 methine groups [δ_C 114.0, δ_H 6.82 (d, $J = 8.4$); δ_C 128.0, δ_H 7.14 (d, $J = 8.8$)], two pairs of symmetric sp^2 quaternary carbons (δ_C 144.0, 156.2), two pairs of symmetric methylene groups [δ_C 46.1, δ_H 3.74 (ddt, $J = 5.1$, 10.0, 13.6); δ_C 68.5, δ_H 4.07 (m)], symmetric sp^3 methines [δ_C 70.0, δ_H 4.20 (ddd, $J = 5.0$, 5.1, 10.0)], and symmetric methyls (δ_C 31.1, δ_H 1.64, s) attached to the sp^3 quaternary carbon (δ_C 41.9). The aromatic sp^2 methine groups as well as COSY correlation between δ_H 6.82 (d, $J = 8.4$) and δ_H 7.14 (d, $J = 8.8$) implied the existence of para-substituted benzene moities. The HMBC correlations from H_3-11 to C-10, C-7 indicated the symmetric methyls attached to one quaternary carbon as a bridge between para-substituted phenyl-rings. The 1-chlorobutan-1-ol moiety and the ether bond were determined from the careful analysis of the characteristic down-field shift of C-3 (δ_C 68.5) and the H-1/H_2-2, H_2-2/H_2-3 cross-peaks in the COSY spectrum, as well as the HMBC correlations from H_2-3 to C-4, C-1. Besides, the hydroxyl and chlorine at C-1 validated by the characteristic down-field shift of C-1 (δ_C 70.0), which were in accordance with the molecular formula $C_{21}H_{26}Cl_2O_4$. Finally, the planar structure of dichocetide D (**1**) was established as shown in Figure 1. To determine the stereochemistry of dichocetide D (**1**), the optical rotation was conducted. It was found that the experimental OR value is zero. Therefore, dichocetide D (**1**) was subjected to the chiral-phase HPLC, however, dichocetide D (**1**) could not be resolved by chiral-phase HPLC to afford a pair of enantiomers, and it was determined as a mesomer.

2.2. Interconversion among ergosterol (10) and compounds 2–3

By careful analysis of the amounts and chemical structures of the compounds obtained from two different kinds of media, it was found that (in decreasing order of abundance) diketopiperazines, indole alkaloids and polyketides (including polyketide alkaloids) are privileged in the H_2O_2 free GPY medium, while in the GPY medium with H_2O_2, steroids, indole alkaloids as well as a little amount of a diketopiperazine are dominant (Figure S6 and S7 in Supplementary material). The result is consistent with the literature report that the metabolite of *Aspergillus niger* treated with H_2O_2 was a steroid analogue (Lv et al. 2015). Probably the production of the steroids (22*E*)-5α, 8α-epidioxyergosta-6,22-dien-3β-ol (**2**) and ergosta-4,6,8(14),22-tetraene-3-one (**3**) was associated with the addition of H_2O_2. In the chemical structure point of view, (22*E*)-5α, 8α-epidioxyergosta-6,22-dien-3β-ol (**2**) was the oxidation product of ergosterol (**10**). The cleavage of the peroxide bond furnished the intermedia with a two adjacent hydroxyl groups. The dehydration of two hydroxyl groups resulted in the formation of the intermedia **b**. Finally, further oxidation of the hydroxyl group at C-3 position of **b** generated ergosta-4,6,8(14),22-tetraene-3-one (**3**) (Scheme 1). For this transformation, the formation of the peroxide bridge in compound **2** was supposed to be related to H_2O_2.



Scheme 1. Interconversion among ergosterol (**10**) and compounds **2–3**.

Among eight compounds (**2**-**9**) from the medium with H_2O_2, the sterol (22*E*)-5α, 8α-epidioxyergosta-6,22-dien-3β-ol (**2**) has the highest content 1.01mg/L, and the yield of ergosta-4,6,8(14),22-tetraene-3-one (**3**) came second with 0.82 mg/L. Interestingly, ergosterol (**10**) was not found from the medium with H_2O_2, however, the content of ergosterol (**10**) was higher than 1.00mg/L in the H_2O_2 free medium. Obviously, H_2O_2 induces the increase and oxidation formation of compound **2**. Oxidative-stress of the fungus activates the related reductase, resulting in the breakage of the peroxide bridge and further production of **3**.

2.3. Cytotoxic bioassay

All of the compounds were screened for their cytotoxic activities against human prostate cancer cells LNCaP-C4-2B, murine melanoma cells B 16 and human breast cancer cells MDA-MB 231. Among them, (22*E*)-5α, 8α-epidioxyergosta-6, 22-dien-3β-ol (**2**) exhibited moderate cytotoxic activity against the LNCaP-C4-2B cell line with an IC_{50} value of 35.53 μM and weak cytotoxic activity against B 16 cell line with an IC_{50} value of 78.77 μM respectively. Fiscalin B (**6**) showed weak cytotoxic activity against the LNCaP-C4-2B cancer cell line with an IC_{50} value of 60.91 μM.

3. Experimental section

3.1. Fungal material, culture, extraction, and isolation

The marine fungus *Dichotomomyces cejpii* F31-1 was obtained from the inner tissue of the soft coral *Lobophytum crassum* collected from Hainan Sanya National Coral Reef Reserve, P. R. China. This fungal strain was conserved in 15% (v/v) glycerol aqueous solution at $-80\,^{\circ}$C. A voucher specimen was deposited in the School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, P. R. China. By BLAST

database screening, the ITS rDNA of the strain F31-1 showed a 100% match to the model strain *Dichotomomyces cejpii* NRRL 26980 (Accession No. GenBank EF669956). The fungal strain was identified as *Dichotomomyces cejpii*. The marine fungus *Dichotomomyces cejpii* F31-1 was cultured in the medium which contained 20 g/L glucose, 5 g/L peptone, 2 g/L yeast extract, 2 g/L Trp, 2 g/L Phe, 30 g/L sea salt, 1 mL/L H_2O_2 (30%) and 1 L H_2O at pH 7.5. Fungal mycelia were cut and transferred aseptically to 1 L Erlenmeyer flasks, each adding 600 mL of sterilized liquid medium. The flasks were incubated at 25 °C for 60 days. Fifty liters of liquid culture were filtered through cheesecloth to separate the culture broth and the mycelia. The culture broth was successively extracted three times with EtOAc (30 L) and then was concentrated by low-temperature rotary evaporation to give a crude extract (18 g).

The extract from the GPY medium with H_2O_2 was chromatographed on a silica gel column (diameter: 8 cm, length: 80 cm, silica gel: 180 g) with a gradient of petroleum ether-EtOAc (10:0–0:10, v/v) followed by EtOAc-MeOH (10:0–0:10, v/v) to afford 13 fractions (Fr. 1–Fr. 13). Fr. 3 and Fr. 4 were subjected to a Sephadex LH-20 column and eluted with CH_2Cl_2-MeOH (1:1, v/v) to give four sub-fractions (Fr. 3-1–Fr. 3-4 and Fr. 4-1–Fr. 4-4) respectively. Then compounds **2** (50.3 mg), **3** (40.8 mg) were obtained from Fr. 4-2, which is chromatographed on silica gel column using a step gradient elution with $CHCl_3$-EtOAc (10:0–0:10, v/v). Fr. 4-3 was further purified with a preparative RP HPLC (MeCN-H_2O, 35:65, v/v) to acquire **7** (8.3 mg), **8** (2.3 mg). Fr. 9 was purified by silica gel column using a step gradient elution with petroleum ether-EtOAc (10:0–0:10, v/v) to get 10 subfractions (Fr. 9.1–Fr. 9.10) after gathering the similar fractions as monitored by TLC analyses. Compounds **5** (2.0 mg) and **6** (2.5 mg) were obtained from Fr. 9.4 with a preparative RP HPLC with MeOH-H_2O (38:62, v/v). Fr. 9.7 was separated via Sephadex LH-20 (MeOH) to give **4** (2.0 mg), **9** (8.0 mg). The extracts from the H_2O_2 free GPY medium also were chromatographed on a silica gel column (diameter: 8 cm, length: 80 cm, silica gel: 300 g) with a gradient of petroleum ether-EtOAc (10:0–0:10, v/v) followed by EtOAc-MeOH (10:0–0:10, v/v) to afford 9 fractions (Fr. 1–Fr. 9). Fr. 2 was purified by silica gel column using a step gradient elution with petroleum ether-EtOAc (10:0–0:10, v/v) to get 7 subfractions (Fr. 2-1–Fr.2-7) after gathering the similar fractions as monitored by TLC analyses. Fr. 2-3 was separated via Sephadex LH-20 (MeOH) to give compound **1** (7.2 mg). Fr. 4 was loaded on a silica gel column using a step gradient elution with petroleum ether-EtOAc (10:0–0:10, v/v) to get 9 subfractions (Fr. 4-1–Fr.4-9) after gathering the similar fractions as monitored by TLC analyses. Fr. 4-3 was subjected to ODS (octadecylsilyl) column using a step gradient elution with MeOH-H_2O (6:4–10: 0, v/v) to give compound **10** (48.2 mg).

3.2. Characterization of dichocetide D (1)

Dichocetide D (**1**) isolated as a brown oil; $[\alpha] = 0$ (c 0.10, MeOH). UV (MeOH) λ_{max} nm (log ε): 201 (4.61), 227 (4.20), 276 (3.55), 284 (3.51); IR (KBr) ν_{max} 3375, 2963, 2929, 2872, 1705, 1607, 1583, 1508, 1458, 1295, 1244, 1182, 1107, 1084, 828, 750 cm^{-1}. HRESIMS (-) *m/z* 411.11342 $[M-H]^-$ (calcd for $C_{21}H_{28}Cl_2O_4$, 411.11354, Δ −0.29 ppm).

^{1}H NMR: (400 MHz, $CDCL_3$) δ: 7.14 (H × 4, d, J = 8.8 Hz, 5/5′/9/9′), 6.82 (H × 4, d, J = 8.4 Hz, 6/6′/8/8′) , 4.20 (H × 2, ddd, J = 5.0, 5.1, 10.0), 4.07 (H_2×2, m, 3/3′), 3.74 (H_2×2, (ddt, J = 5.1, 10.0, 13.6 Hz, 2/2′), 1.64 (H, s, 11/11′)

^{13}C NMR: (100 MHz, $CDCL_3$) δ: 156.2 (C-4/4′), 144.0 (C-7/7′), 128.0 (CH-6/6′/8/8′), 114.1 (CH-5/5′/9/9′), 70.0 (CH_2-1/1′), 68.5 (CH_2-3/3′), 46.1 (CH_2-2/2′), 41.9 (C-10), 31.1 (CH_3-11/11′)

3.3. Cytotoxicity assay

All the compounds were evaluated cytotoxic activity against human prostate cancer cells LNCaP-C4-2B, murine melanoma cells B 16 and human breast cancer cells MDA-MB 231 by the colorimetric MTT assay. Briefly, LNCaP-C4-2B, B 16 and MDA-MB 231 cells were cultured in 96-well plates at a density of 5×10^3 per well and incubated at 37 °C for 24 h, respectively. Then, various concentrations (0.125-100 μM) of the compounds were added to the wells. After 48 h culture, 20 microliters MTT (Genview, Houston, TX, USA) solution (5 mg·mL^{-1}) was added into each well. These cells were cultured at 37 °C for another 4 h. In the end, the supernatant was discarded and the sediment was dissolved by dimethyl sulfoxide (DMSO) (100 microliters per well). The absorbance was read at 490 nm, and the data was calculated by SPSS software.

4. Conclusion

In conclusion, one new compound (**1**) and a known ergosterol were isolated from the culture broth of the H_2O_2 free medium, and eight known compounds were obtained from the culture broth of the H_2O_2 added medium. among eight compounds obtained from H_2O_2 added medium, six of them were not discovered from the fungus *Dichotomomyces cejpii* F31-1 in our previous research. In addition, this study showed that the ROS culture strategy alters the metabolite profile of the marine-derived fungus *Dichotomomyces cejpii* F31-1, inducing the fungus to produce high content and bioactive steroids. However, it is unclear whether the metabolite profile of dominant steroids is specific for the fungus *Dichotomomyces cejpii* F31-1 or it is common for the fungi. To elucidate this issue, the ROS culture strategy will be applied to a variety of fungi in the near future.

Disclosure statement

No potential conflict of interest was reported by the authors.

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