สารออกฤทธิ์ทางชีวภาพจากเชื้อราทะเล *Aspergillus terreus* CRIM 301

Bioactive Metabolites from the Marine-derived Fungus *Aspergillus terreus* CRIM 301

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บทคัดย่อ

การศึกษาสารจากเชื้อราทะเลสายพันธุ์ *Aspergillus terreus* CRIM 301 พบว่าผลิตสารที่มีรายงานแล้ว 12 ชนิด คือ neogeodin hydrate (1), asterric acid (2), geodin hydrate (3), methyl dichloroasterrate (4), methyl asterrate (5), butyrolactones I (6), II (7) and V (8), aspernolide A (9), questin (10), terrein (11) และ dimethoxydimethylphthalide (12) การพิสูจน์โครงสร้างทางเคมีของสาร 1-12 ใช้วิธีการวิเคราะห์ข้อมูลทางสเปคโตสโคปี และ สาร 1 ใช้การวิเคราะห์ผลึกเดี่ยวด้วยรังสีเอกซ์ จากการศึกษาพบว่า aspernolide A (9) มีฤทธิ์ต้านอนุมูลอิสระเมื่อทดสอบด้วยวิธี DPPH โดยมีค่า IC50 เท่ากับ 26.4 ไมโครโมล เมื่อเปรียบเทียบกับสารมาตรฐาน ascorbic acid (IC_{50} 21.2 ไมโครโมล) methyl asterrate (5) มีฤทธิ์ต้านอนุมูลอิสระซุปเปอร์ออกไซด์ โดยวิธีการ XXO (ยับยั้งเอนไซม์ xanthine oxidase) สารที่แยกได้ส่วนมากไม่มีฤทธิ์ความเป็นพิษต่อเซลล์ ยกเว้น terrein มีฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็งชนิดต่างๆ โดยมีค่า IC_{50} เท่ากับ 3.6, - 10.49 มคก/มล

คำสำคัญ: *เชื้อราทะเล ฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ความเป็นพิษต่อเซลล์*

Abstract

Chemical investigation of the marine-derived fungus *Aspergillus terreus* CRIM 301 led to the identification of twelve known compounds which were (1) neogeodin hydrate, (2) asterric acid, (3) geodin hydrate, (4) methyl dichloroasterrate, (5) methyl asterrate, (6) butyrolactones I, (7) II and (8) V, (9) aspernolide A, (10) questin, (11) terrein, and (12) dimethoxydimethylphthalide. Structure elucidation of 1-12 were achieved by analysis of spectroscopic data and by a single crystal X-ray crystallographic analysis for structure 1. Aspernolide A (9) scavenged DPPH free radicals with an IC_{50} value of 26.4 μM, comparable to a standard compound, ascorbic acid (IC_{50} 21.2 μM). Methyl asterrate (5) inhibited superoxide anion radical formation in the XXO assay. Most of the isolated compounds did not show cytotoxic activity, except terrein (11) that exhibited cytotoxicity with the IC_{50} ranges of 3.60-10.49 μg/mL.

Keywords: *marine-derived fungi, radical scavenging activity, cytotoxicity*

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1. Introduction

Marine organisms, as well as marine microbes, have proven to be rich sources of bioactive compounds, and marine-derived fungi in particular have been recognized as potential source of structurally novel and biologically active metabolites (Bugni and Ireland, 2003; Zhuang et al., 2012). Marine-derived fungi of the genus Aspergillus are particularly rich in bioactive metabolites, and recently we have isolated many new metabolites from this fungal genus (Antia et al., 2011; Ingavat et al., 2011; Sureram et al., 2012). We report herein the isolation and biological activities of metabolites from the marine-derived fungus *Aspergillus terreus* CRIM 301.

2. Objectives

2.1 To isolate and purify secondary metabolite from marine-derived fungus *Aspergillus terreus* CRIM 301.

2.2 To evaluate radical scavenging, antioxidant activities and cytotoxic activity of the isolated compounds.

3. Materials and Method

3.1 General experimental procedures

Melting points were measured on Buchi 535 Melting Point Apparatus and reported without correction. UV-Vis spectra were obtained using a Shimadzu UV-1700 PharmaSpec spectrophotometer. FT-IR data were recorded on a universal attenuated total reflectance (UATR) attachment on a Perkin-Elmer Spectrum One spectrometer. NMR spectra were acquired on a Bruker AVANCE 600 spectrometer (operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C). APCI-TOF MS spectra were recorded on a Bruker MicroTOF-LC spectrometer.

3.2 Fungal material, extraction, and isolation

The marine-derived fungus *Aspergillus terreus* CRIM 301 was isolated from marine sediment collected from Surin Islands, Phang-Nga province, Thailand. The fungus *A. terreus* CRIM 301 was identified based on morphological characteristics, and it was deposited at Chulabhorn Research Institute (CRI), Bangkok, Thailand.

A. terreus was cultured in potato dextrose broth (constituted seawater instead of distilled H_2O) under static conditions for 28 days. Fungal cells and broth (5 L) were separated by filtration, and the filtrate was extracted three times with an equal volume of EtOAc to obtain a crude extract (3.2 g). Fungal cells were extracted sequentially with MeOH and CH_2Cl_2, yielding a crude extract (2.7 g). The extract was subjected to Sephadex LH-20 column chromatography (CC) (3x85 cm), eluted with MeOH, to yield 14 fractions (A1-A14). Fractions A4 contained terrein (11) (1.43 g). Fraction A5 was further purified by Sephadex LH-20 CC (1.5x115 cm) using MeOH as eluent, giving 7 fractions (B1-B7). Fraction B4 was subjected to silica gel CC, eluted with a mixture of EtOAc:CH_2Cl_2 (9:1), to furnish butyrolactone V (8) (33.8 mg) and aspernolide A (9) (26.4 mg). Fraction A6 was re-crystallized from MeOH to afford asterric acid (2) (18.7 mg). Fraction A7 was further purified by Sephadex LH-20 CC (1.5x115 cm), eluted with MeOH, to yield 6 fractions (C1- C6). Fraction C1 contained geodin hydrate (3) (32.0 mg), while a fraction C6 contained a diphenyl ether (1) (30.7 mg). Fraction A8 was purified by preparative TLC, developed with a mixture of EtOAc:CH_2Cl_2 (2:8), to furnish butyrolactone II (7) (10.5 mg). Fraction A10 was re-crystallized from acetone to yield questin (10) (108.5 mg). A crude cell extract (2.7 g) was separated by Sephadex LH-20 CC (3x85 cm), eluted with MeOH, yielding 13 fractions (D1-D13). Fraction D3 was re-crystallized from MeOH to afford terrein (11) (228.5 mg). Fraction D4 was further purified by preparative TLC, developed with a mixture of EtOAc:hexane (1:9), to furnish dimethoxydimethylphthalide (12) (6.1 mg). Fraction D5 was further purified by preparative TLC, developed with a mixture of EtOAc:hexane (2:8), yielding methyl dichloroasterrate (4) (7.5 mg) and methyl asterrate (5) (18.9 mg). Fraction D6 was further purified by preparative TLC, developed with a mixture of EtOAc:CH_2Cl_2 (1: 9), to furnish butyrolactone I (6) (47.0 mg).

3.3 X-ray crystallography

Crystals of 1 were obtained by slow vapor diffusion of MeOH–acetone solution (1:1) in CH_2Cl_2 as $C_{17}H_{14}Cl_2O_8$ in the triclinic space group *P*-1 (no. 2). X-ray diffraction experiment was performed at 298(2) K using a Bruker X8 APEX2 Kappa CCD area-detector diffractometer with MoKα radiation (λ = 0.71073 Å). The structure was solved by direct methods and refined with full-matrix least squares on F^2 using SHELXL-97 (Sheldrick, G.M., University of Gottingen, Gottingen, Germany, 1997). The final $R_1(F^2)$ = 0.041 and $wR(F^2)$ = 0.113 for 2,980 data with $F^2 > 2\sigma(F^2)$. Data have been deposited with the Cambridge Crystallographic Data Centre (CCDC 838232). Copies

of these data can be obtained, free of charge, on application to the CCDC via www.ccdc.cam.ac.uk/conts/retrieving.html (or 12 Union Road, Cambridge CB2 1EZ, UK, fax: +44 1223 336033, e-mail: deposit@ccdc.cam.ac.uk).

3.4 Bioassays

3.4.1 Radical scavenging and antioxidant activities

Scavenging DPPH free radicals, XXO assay, inhibition of TPA-induced superoxide anion radical formation, and ORAC assay were carried out using the method described by Gerhauser and co-workers (2003). In the XXO assay, inhibition of superoxide anion radical formation was measured only when the tested compounds did not inhibit xanthine oxidase. In the ORAC assay, an antioxidant potential of the test compounds (1 μM) was compared with that of 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), a water soluble vitamin E analog. Results were expressed as ORAC units, where 1 ORAC unit equals the net protection of β-phycoerythrin produced by 1 μM of Trolox. Scavenging capacities >1 ORAC unit were considered as positive.

3.4.2 Inhibition of aromatase (CYP19)

Aromatase inhibitory assay was performed according to the method reported by Stresser (Stresser et al., 2000). The reference compound, ketoconazole, typically exhibits the IC_{50} value of 2.4 μM.

3.4.3 Cytotoxicity

Cytotoxic activity for adhesive cell lines including HuCCA-1, A549, and HepG2 cancer cell lines was evaluated with the MTT assay (Carmichael et al., 1987). Cytotoxic activity of non-adhesive cells, MOLT-3 cell lines, was assessed using the XTT assay (Doyle and Griffiths, 1997). Etoposide and doxorubicin were used as the reference drugs.

4. Results and Discussion

Twelve known compounds including neogeodin hydrate (1), asterric acid (2), geodin hydrate (2,4-dichloroasterric acid) (3), methyl dichloroasterrate (4), methyl asterrate (5), butyrolactones I (6), II (7) and V (8), aspernolide A (9), questin (10), terrein (11), and dimethoxydimethylphthalide (12), were isolated from the marine-derived fungus *Aspergillus terreus* CRIM 301. The structure of neogeodin hydrate (1) (Figure 1) was elucidated by analysis of spectroscopic data, and confirmed by a single crystal X-ray crystallographic analysis (Jongrungruangchok et al., 2013). Spectroscopic data of known compounds (2-12) were identical to those reported in the literature (Curtis et al., 1964; Parvaktar et al., 2009; Ohashi et al., 1992; Natori and Nishikawa, 1962; Hargreaves et al., 2002; Rao et al., 2000; Lin et al., 2009; Fujimoto et al., 1999; Kolb et al., 1990; Bradamante et al., 2002).

Compound 1, namely neogeodin hydrate, was isolated as colorless crystals. The molecular formula for 1, $C_{17}H_{14}Cl_2O_8$, was indicated by APCITOF MS. The presence of the two chlorine atoms in neogeodin hydrate (1) was evident from the MS spectrum, showing the chlorine isotope pattern of $[M-H]^-$, $[M+2-H]^-$, and $[M+4-H]^-$ with the ratio

of 10:6:1. Careful analysis of these spectroscopic data revealed that the spectral data of compound 1 shared a great deal of similarity with those of geodin hydrate (3) (Figure 1), suggesting that 1 was a diaryl ether derivative of 3. The molecular formula $C_{17}H_{14}Cl_2O_8$ obtained from the MS spectrum implied that the two aromatic rings in 1 should be linked, similar to that of geodin hydrate (3), through an ether bond. Fortunately, crystals of neogeodin hydrate (1) were obtained and subjected to a single crystal X-ray crystallographic analysis. ORTEP plot of 1 is in Figure 2, and this X-ray analysis conclusively confirmed the structure of neogeodin hydrate (1).

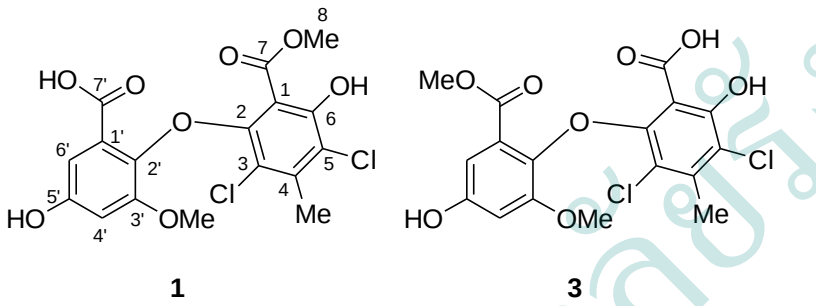

Figure. 1 Structures of neogeodin hydrate (1) and geodin hydrate (3)

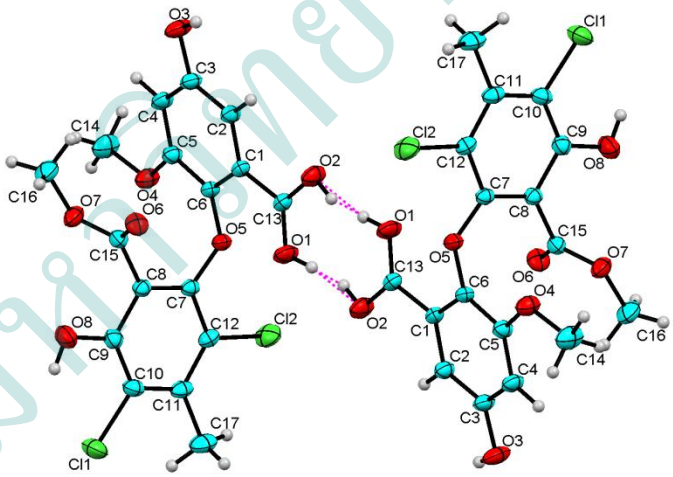

Figure. 2 ORTEP plot of 1 (30% probability level) showing a centrosymmetric dimer stabilized by intermolecular O–H···O hydrogen bonds through disordered carboxylic acid groups.

The isolated fungal metabolites were evaluated for their radical scavenging, antioxidant, aromatase inhibitory, and cytotoxic activities. Aspernolide A (9) exhibited potent DPPH radical scavenging activity with an IC_{50} value of 26.4 μM, comparable to a standard compound, ascorbic acid (IC_{50} 21.2 μM), while methyl asterrate (5), butyrolactones II (7) and V (8) showed the activity with IC_{50} values of 56.7, 42.2, and 33.8 μM, respectively (Table 1). Methyl asterrate (5) inhibited

superoxide anion radical formation in the xanthine/xanthine oxidase (XXO) assay with an IC_{50} value of 50.4 μM, while neogeodin hydrate (1) and asterric acid (2) exhibited respective IC_{50} values of 179.1 and 449.2 μM, which were much less active than 1. Compound 5 and terrein (11) inhibited xanthine oxidase (IXO) with respective IC_{50} values of 1.7 and 143.4 μM (Table 1). The isolated compounds did not suppress superoxide anion generation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in differentiated HL-60 human promyelocytic leukemia cells (Table 1). Among the metabolites tested, butyrolactones I (6) and II (7) exhibited ORAC antioxidant activity with 3.4-4.4 ORAC units (Table 1). It should be noted that the isolated compounds did not inhibit aromatase enzyme. Most of the isolated compounds showed only weak cytotoxic activity (IC_{50} ranges of 11.87-47.50 μg/mL) or were inactive towards the cell lines tested, except terrein (11) that exhibited cytotoxicity with the IC_{50} ranges of 3.60-10.49 μg/mL (Table2)

Table 1 Radical scavenging and antioxidant activities of compounds 1-12

Compound	Radical scavenging and antioxidant activities (IC_{50}, μM)				
	DPPH[a]	XXO[b]	IXO[c]	HL-60[b]	ORAC[d]
1	>250	179.1±13.1	>500	>100	1.7 ±0.6
2	>250	449.2±36.3	>500	>100	2.1±0.2
3	>250	>500	>500	>100	0.9±0.1
4	ND	ND	ND	ND	ND
5	56.7±0.9	50.4±2.6	1.7±0.1	>100	1.7±0.1
6	>250	>500	>500	>100	3.4±0.1
7	42.2±0.3	>500	>500	>100	4.4±0.2
8	33.8±0.4	>500	>500	>100	2.0±0.3
9	26.4±1.7	>500	>500	>100	2.1±0.4
10	>250	>500	>500	>100	2.2±0.4
11	>250	>500	143.4±15.0	>100	0.1±0.03
12	ND	ND	ND	ND	ND

DPPH = Scavenging 2,2-diphenyl-1-picrylhydrazyl free radicals; XXO = Inhibition of superoxide anion radical formation by xanthine/xanthine oxidase; IXO = Inhibition of xanthine oxidase; HL-60 = Inhibition of 12-*O*-tetradecanoylphorbol-13-acetate-induced superoxide anion radical generation in differentiated HL-60 cells; and ND = Not determined.

[a] Ascorbic acid was used as the reference compound (IC_{50} value 21.2 μM).

[b] Superoxide dismutase was used as a positive control (scavenging 100% of the radicals).

[c] Allopurinol is the reference inhibitor (IC_{50} value 3.0 μM).

[d] Results were expressed as ORAC units, where one ORAC unit equals the net protection of β-phycoerythrin produced by 1 μM of Trolox.

ND = Not determined

Table 2 Cytotoxic activity of compounds 1-12 against four cancer cell lines

Compound	Cytotoxic activity (IC50, µg/mL); mean (±s.d.), n=3			
	HuCCA-1	A549	MOLT-3	HEPG2
1	>50	>50	>50	>50
2	>50	>50	>50	>50
3	>50	>50	>50	>50
4	46.5 ± 0.70	35.4 ± 2.62	27.93 ± 0.62	41.50 ± ±6.36
5	>50	>50	23.40 ± 3.37	45.0 ± 4.3
6	>50	>50	34.38 ± 5.92	>50
7	>50	>50	47.50 ± 2.09	>50
8	>50	>50	>50	>50
9	>50	>50	35.90 ± 2.47	>50
10	>50	>50	11.87 ± 2.47	45.0 ± 6.30
11	3.60 ± 0.20	10.49 ± 0.22	4.34 ± 0.22	5.67 ± 0.29
12	ND	ND	ND	ND
Doxorubicin Etopioside	0.40 ±0.282	0.35 ± 0.00	ND	ND
	ND	ND	0.025 ± 0.007	0.35 ± 0.13

HuCCA-1 = human lung cholangiocarcinoma cancer cells; A549 = human lung carcinoma cell line; MOLT-3 = T-lymphoblast (acute lymphoblastic leukemia); HepG2 = human hepatocellular liver carcinoma cell line

ND = Not determined

Although only known compounds were obtained from the present work, some of the known compounds isolated, i.e. butyrolactone I (6) and terrein (11), were important bioactive agents with promising therapeutic applications. It should be noted that terrein (11) was isolated on a gram scale (1.43 g) from the fungus *A. terreus* CRIM 301. Terrein (11) enhanced osteoblast biocompatibility on titanium surface (Lee et al., 2010), and reduced pulpal inflammation in human dental pulp cells (Lee et al., 2008). Terrein (11) had a strong antiproliferative effect on human skin equivalents and might be used to treat hyperproliferative skin diseases such as *psoriasis vulgaris* (Kim et al., 2007); further study revealed that it inhibited extracellular signal-regulated protein kinase and decreased the expressions of cyclin B1 and Cdc2 complex (Kim et al., 2008). Terrein (11) induced apoptosis in Hela human cervical carcinoma cells through p53 and ERK regulation (Porameesanaporn et al., 2013); moreover, terrein promoted apoptotic cell death on both fibroblasts and pulmonary tumor cell lines (Demasi et al., 2010). Terrein (11) was a potent melanogenesis inhibitor (Park et al., 2004), which decreased melanogenesis through ubiquitin-dependent proteasomal degradation as well as via decreased expression of its mRNA (Park et al., 2009). Terrein (11) had an additive effect with KI-063 (a tyrosinase inhibitor) and might be used for

skin whitening (Kim et al., 2008). Butyrolactone I (6) is a potent inhibitor of cyclin-dependent kinase (Kitagawa et al., 1994) with potential application for the treatment of cancer (Suzuki et al., 1999). Recently, butyrolactone I (6) has a positive impact on animal breeding programs (De Bem et al., 1999; Quetglas et al., 1999; Ferreira et al., 2009). Butyrolactones I (6) and II (7) and aspernolide A (9) also exhibited lipoxygenase inhibitory and DPPH radical-scavenging activities (Sugiyama et al., 2010). Although chemical syntheses of butyrolactone I (6) and terrein (11) have been established (Braña et al., 2004; Lee et al., 2005) the present work suggests that the marine-derived fungus *A. terreus* CRIM 301 could be an alternative source of these bioactive agents, particularly a gram scale production of terrein (11) by this fungus.

5. Conclusion

Twelve known compounds including neogeodin hydrate (1), asterric acid (2), geodin hydrate (2,4-dichloroasterric acid) (3), methyl dichloroasterrate (4), methyl asterrate (5), butyrolactones I (6), II (7) and V (8), aspernolide A (9), questin (10), terrein (11), and dimethoxydimethylphthalide (12), were isolated from the marine-derived fungus *A. terreus* CRIM 301. These compounds have been proved to be with lots of bioactivities. Some of the known compounds isolated were important bioactive agents with promising therapeutic applications.

6. Acknowledgements

We are grateful acknowledgement the grant of Rangsit University, Assoc. Prof. Dr. S. Wiyakrutta for the identification of the fungus, S. Sitthimonchai for determining radical scavenging and aromatase inhibitory activities; and P. Intachote, S. Sengsai, and B. Saimanee for cytotoxicity testing, Thai Government Stimulus Package 2 (TKK2555), and the National Research University Project (FW657B). We are also thankful the Thailand Research Fund and the Center of Excellence on Environmental Health and Toxicology, Science & Technology Postgraduate Education and Research Development Office (PERDO), Ministry of Education for their support.

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