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Effect of Crude Drug Extracts on Trypacidin Production in *Aspergillus fumigatus*

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Abstract

We examined the production of fungal metabolites as biological responses to 120 crude drugs by culturing the filamentous fungus *Aspergillus fumigatus* CBS101355 with crude drugs and analyzing the culture extracts by HPLC. Nine crude drug extracts [Kyokatsu (Notopterygium), Kyonin (apricot kernel), Kujin (Sophora root), Goboshi (Burdock fruit), Goma (sesame), Shokyo (ginger), Shin'i (magnolia flower), Togashi (Benincasa seed), and Bukuryo (*Poria sclerotium*)] induced the production of trypacidin, which was not produced by culturing in potato dextrose broth without crude drugs.

Keywords

Aspergillus fumigatus, Crude Drug, Fungal Metabolites, Trypacidin

1. Introduction

Fungi produce various secondary metabolites, and of the metabolites isolated to date, some have proven useful, and others are harmful mycotoxins [1] [2] [3] [4]. These fungal metabolites are produced as a biological response to medium components, culture temperature, culture medium pH, or the addition of non-nutritional compounds such as epigenetic chemicals (5-azacytidine as DNA methyltransferase inhibitor, suberoylanilide hydroxamic acid, trichostatin A and sodium butyrate as histone deacetylase inhibitors, etc.) [5] [6] [7]. These findings led us to study fungal metabolite production as a biological response to Kampo medicines (traditional Japanese medicines) and their constituent crude drugs.

In our search for factors in Kampo medicines that affect the production of fungal metabolites, we found that Shimbu-to, a Kampo medicine used for gastrointestinal diseases and indigestion, promotes the production of emericellin and related compounds in *Aspergillus nidulans* [8]. We also reported that the Kampo medicine Shakuyaku-Kanzo-to induced the production of sterigmato-

cystin, a mycotoxin produced by *Emericella nidulans* IFM 60678; this inducing activity was enhanced only by peony extract [9].

Aspergillus fumigatus is a common, naturally occurring saprophytic fungus noted for producing a plethora of secondary metabolites (e.g., gliotoxin, helvolic acid, hexadehydroastechrome, trypacidin, endocrocin, neosartoricin, and fumagillin) [10].

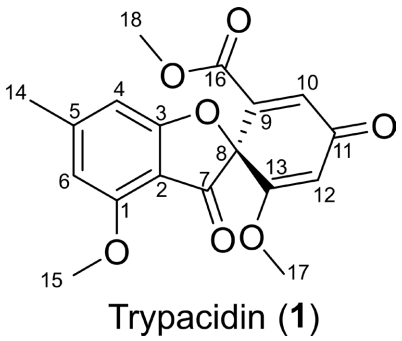
In this study, we examined the production of fungal metabolites as biological responses to 120 crude drugs by culturing *A. fumigatus* CBS101355 and crude drugs, followed by analysis of culture extracts by HPLC. The HPLC chromatograms of culture extracts for nine crude drugs [Kyokatsu (Notopterygium), Kyonin (apricot kernel), Kujin (Sophora root), Goboshi (Burdock fruit), Goma (sesame), Shokyo (ginger), Shin'i (magnolia flower), Togashi (Benincasa seed) and Bukuryo (*Poria sclerotium*)] exhibited a common new peak (**1**: tR = 13.8 mim) that was not detected in the control.

Compound **1** was isolated from a culture extract of *A. fumigatus* grown in medium supplemented with Goboshi (Burdock Fruit) extract, which induced production of the highest amount of **1**. Detailed analysis of NMR and MS data for **1** identified the compound as trypacidin (**1**), an anti-protozoal [11], cytotoxic [12] and antiphagocytic [13] substance produced by *A. fumigatus* (**Figure 1**). It is reported that the production of trypacidin and transcription level of trypacidin gene cluster were affected by cultural temperature [14]. These results indicate that nine crude drug extracts can induce the production of trypacidin in *A. fumigatus*.

2. Materials and Methods

2.1. Experimental Instruments

An LC-20 HPLC system equipped with a DAD detector (Shimadzu, Kyoto, Japan) was used for analytical HPLC. LC-MS was performed using an Agilent 1200 HPLC system and JMS-T100LP ESI-TOF-MS mass spectrometer (JEOL, Tokyo, Japan). Column chromatography was performed using a Sephadex LH-20 (GE Healthcare Japan, Toko, Japan). Preparative HPLC was performed using an LC-20AT pump and SPV-10AV UV detector (Shimadzu, Kyoto, Japan). NMR spectra were recorded on a JEOL ECAII 600 spectrometer (^{1}H: 600 MHz, ^{13}C: 150 MHz) (JEOL) using tetramethylsilane as an internal standard. Optical rotation



Trypacidin (**1**)

Figure 1. Structure of trypacidin (**1**).

was measured using a P-1020 photopolarimeter (JASCO, Tokyo, Japan). All fungi were fermented in a SANYO MIR-554 incubator (SANYO Electric Co., Ltd., Osaka, Japan). Centrifugation was performed using a FORCE 712-100V microcentrifuge (Select BioProducts Inc., New Zealand).

2.2. Fungal Strain

Aspergillus fumigatus CBS101355 was obtained from the Westerdijk Fungal Biodiversity Institute (CBS), Netherlands.

2.3. Screening for Crude Drugs that Affect Fungal Metabolite Expression

For the cultivation of *A. fumigatus* CBS101355, potato dextrose broth (PDB, Difco, BD, iNJ, Japan) was amended with 120 different crude drug extracts (see Supplementary Table 1) at a final concentration of 2.4 mg/mL. After sterilization, 2 mL of medium containing drug extract was poured into each well of a 24-well plate (TPP, Sweden), and all wells were inoculated with *A. fumigatus* CBS101355. The plate was then sealed with breathable film (Axygen BF-400-S, Corning, AZ, USA) and cultured at 25˚C for 1 week. After cultivation, each culture broth was lyophilized and extracted with 1.5 mL of methanol at room temperature for 1 day. The extract solution was filtered and dried under air flow.

2.4. DAD-HPLC Analysis Conditions

The culture extracts were dissolved in 1 mL of 50% acetonitrile (CH_3CN) and centrifuged at 10,000 rpm for 5 min. The supernatant was analyzed by DAD-HPLC using a Mightysil RP-18 GPII column (3 × 250 mm, 5 μm, Kanto Chemicals, Tokyo, Japan), with the column oven temperature set to 40˚C. The column was eluted using water and CH_3CN as mobile phases, and the eluate was analyzed in gradient mode as follows: 0 min: 30% CH_3CN, 17 min: 100% CH_3CN, 35 min: 100% CH_3CN. The flow rate was 0.5 mL/min. The detection range of the DAD detector was 200 - 400 nm, and the chromatograms are shown at 200 nm.

2.5. LC-MS Analysis Conditions

Culture extracts were dissolved in 1 mL of methanol and centrifuged at 10,000 rpm for 5 min. The supernatant was analyzed by LC-MS using an Inertsil ODS-3 column (2.1 × 150 mm, 3 μm, GL Science, Tokyo, Japan), with the column oven temperature set to 40˚C. The mobile phases were 0.5% HCOOH (A) and CH_3CN with 0.5% HCOOH (B), and the column was eluted in gradient mode as follows: 0 min, 20% B; 17 min, 95% CH_3CN, and 30 min, 95% CH_3CN. The flow rate was 0.2 mL/min. The samples were analyzed in positive ESI mode.

2.6. Culture and Extraction of *Aspergillus fumigatus* on Medium Supplemented with Goboshi (Burdock Fruit) Extract

To 90 g of Goboshi (Burdock fruit) was added 900 mL of water, and the mixture was refluxed for 50 min. The extracted solution was filtered through double

gauze and lyophilized to obtain 9.3 g of Goboshi (Burdock fruit) extract.

Aspergillus fumigatus CBS101355 was inoculated into 1 L of PDB with 4.8 mg/mL Goboshi (Burdock fruit) extract, incubated at 25˚C for 1 week, and then lyophilized whole. The lyophilized product was extracted with 150 mL of methanol and filtered. The filtrate was evaporated *in vacuo* to obtain a methanol extract (2.5 g).

2.7. Isolation of Compound 1

The above methanol extract (2.5 g) was dissolved in 1 L of water and sequentially extracted with hexane, chloroform, ethyl acetate, and 1-butanol (each 1 L × 2) and evaporated *in vacuo*. The chloroform extract was chromatographed on a Sephadex LH-20 column eluted sequentially with hexane-chloroform (1:4; 200 mL), chloroform-acetone (3:2; 200 mL and 1:4; 200 mL), acetone (200 mL), and methanol (1 L) to obtain 10 fractions. Fraction 2 (4.2 mg) was purified by HPLC on an ODS column eluted with 45% acetonitrile to obtain **1** (2.4 mg) as a white amorphous powder.

3. Results

3.1. Crude Drugs that Affected Fungal Metabolite Expression

To study the effect of crude drug extracts on fungal metabolite expression, *A. fumigatus* was cultured in PDB supplemented with each of 120 crude drug extracts. As the results of examination for the amounts of crude drugs and cultural time, differences of secondary metabolites were effectively observed at concentration of 2.4 mg/mL crude drugs and 7 days cultural time. The 120 crude drug extracts examined in this study showed no antifungal activity against *A. fumigatus* at a concentration of 2.4 mg/mL (data not shown).

After 7 days of incubation, the culture extracts were analyzed by DAD-HPLC. A common new peak (**1**, t_R: 13.8 min) not observed in the control appeared in the HPLC chromatograms of PDB culture extracts with 9 of the 120 crude drug extracts (**Figure 2**). The 9 crude drugs were Kyokatsu (Notopterygium), kyonin (apricot kernel), Kujin (Sophora root), Goboshi (Burdock fruit), Goma (sesame), Shokyo (ginger), Shinni (magnolia flower), Togashi (Benincasa seed), and Bukuryo (*Poria sclerotium*).

Peak **1** (t_R = 13.8 min) showed a maximum UV spectrum at 287 nm by DAD-HPLC analysis. LC-MS analysis of the 9 culture extracts that exhibited common peak **1** confirmed a pseudo–molecular ion peak at *m/z* 345.11 (Supplementary **Figure 1**). The highest level of trypacidin was observed in the extract with Goboshi.

3.2. Identification of Compound 1

Aspergillus fumigatus was cultured in PDB supplemented with Goboshi extract, which produced the highest amount of **1** among the 9 herbal extracts.

The culture extract was purified by various chromatographic methods to

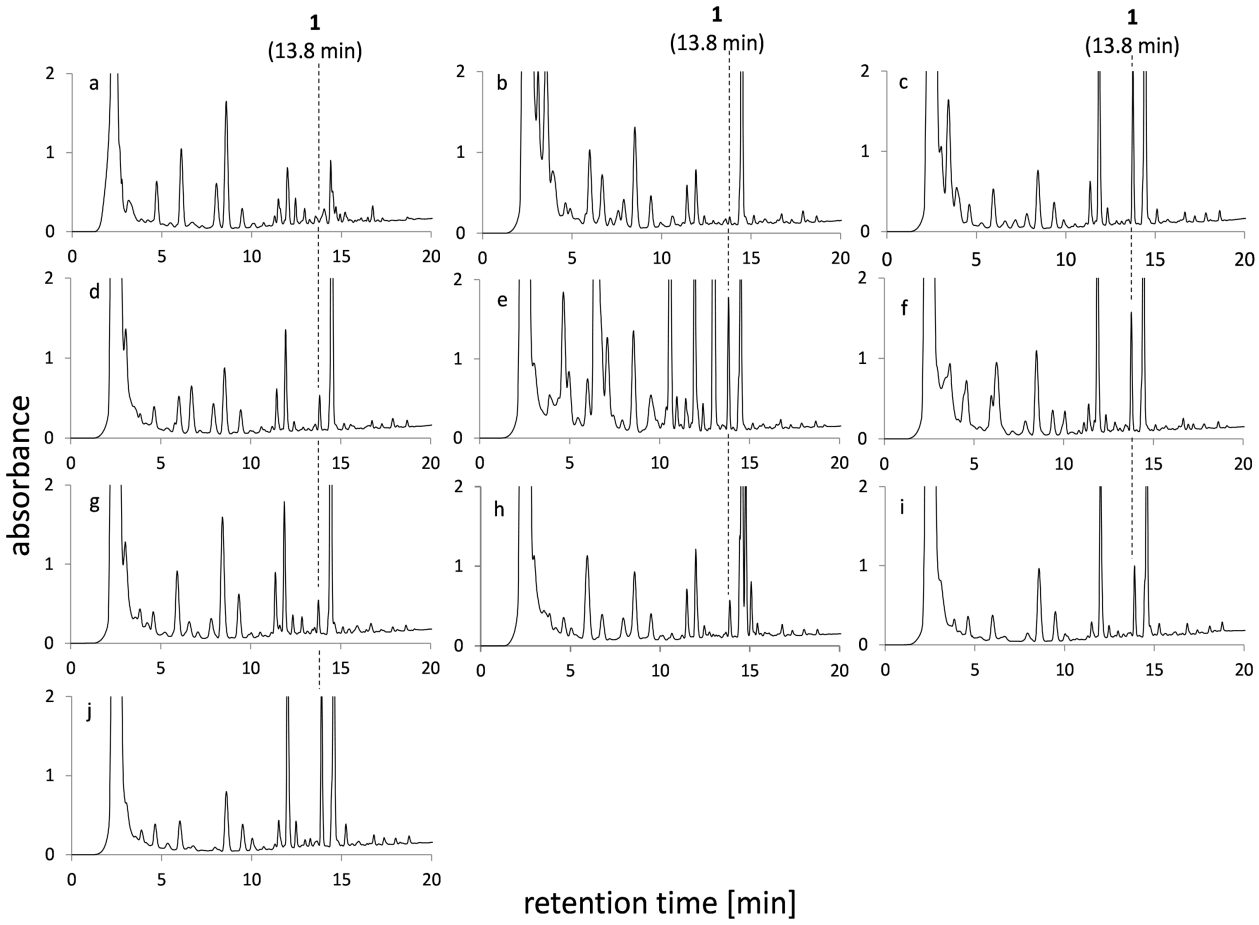


Figure 2. HPLC chromatograms of extracts of *A. fumigatus* cultured on PDB supplemented with crude drug extracts. Chromatograms were recorded at 200 nm. a: culture extract without crude drug; b: culture extract with Kyokatsu (Notopterygium); c: Kyonin (apricot kernel); d: Kujin (Sophora root); e: Goboshi (Burdock fruit); f: Goma (sesame); g: Shokyo (ginger); h: Shin'i (magnolia flower); i: Togashi (Benincasa seed); j: Bukuryo (*Poria sclerotium*).

obtain **1** (2.4 mg) as a white amorphous powder. The structure of **1** was determined as (-)-trypacidin ($[\alpha]_D^{20} = -68.1$, $c = 0.05$, MeOH) by comparison of MS and NMR spectral data and optical rotation in reference to previous data [11], [15] and detailed analyses of 2D-NMR data in DMSO-d_6 (**Table 1** and Supple. **Figures S1-S7**).

3.3. Factors in Crude Drug Extracts that Induce Trypacidin Production

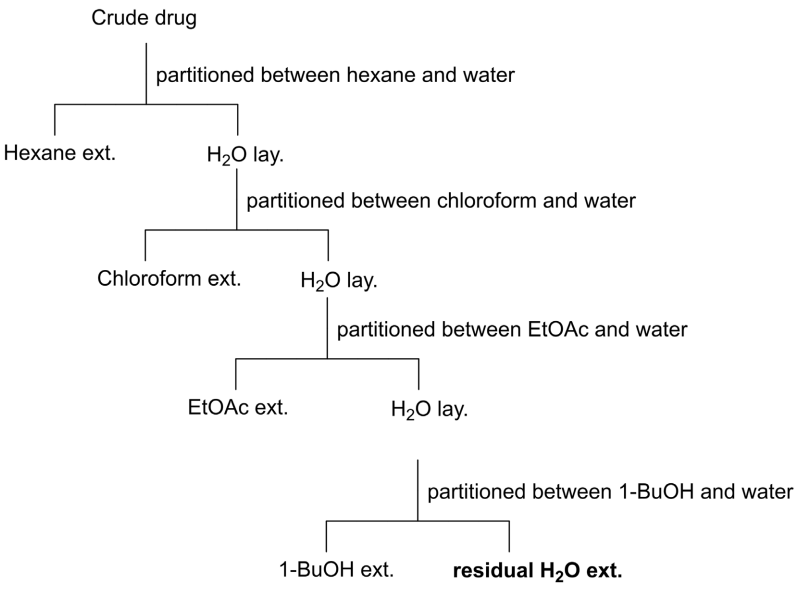
Aspergillus fumigatus produced trypacidin (**1**) in the presence of the 9 crude drug extracts. These results suggested that these crude drug extracts contain inducers of trypacidin (**1**) production in *A. fumigatus*. To identify the trypacidin inducers in the 9 crude drug extracts, each of the extracts was sequentially partitioned into liquid solutions using chloroform, ethyl acetate, and 1-butanol. Then, *A. fumigatus* was cultured in PDB supplemented with these partitioned extracts.

DAD-HPLC analysis confirmed the production of trypacidin only when the

aqueous fraction of all 9 herbal extracts was added, and no trypacidin production was observed when other extract fractions were added to the culture (Figure 3).

Table 1. NMR data of trypacidin (**1**).

NMR data position	1		Ref. data	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	158.3		158.3	
2	108.3		108.3	
3	174.4		174.3	
4	105.5	6.55 (brs)	105.5	6.55 (d, 2.1)
5	152.1		152.2	
6	105.3	6.37 (brs)	105.3	6.37 (d, 2.1)
7	190.4		190.0	
8	89.5		84.0	
9	138.2		138.2	
10	137.1	7.11 (d, 2.0)	137.1	7.10 (d, 1.2)
11	185.7		185.7	
12	104.0	5.77 (brs)	103.9	5.77 (d, 1.2)
13	169.4		169.4	
14	23.2	2.44 (s)	23.2	2.44 (s)
15	56.1	3.95 (s)	56.1	3.95 (s)
16	163.5		163.5	
17	56.7	3.66 (s)	56.7	3.66 (s)
18	52.8	3.69 (s)	52.8	3.69 (s)



(a)

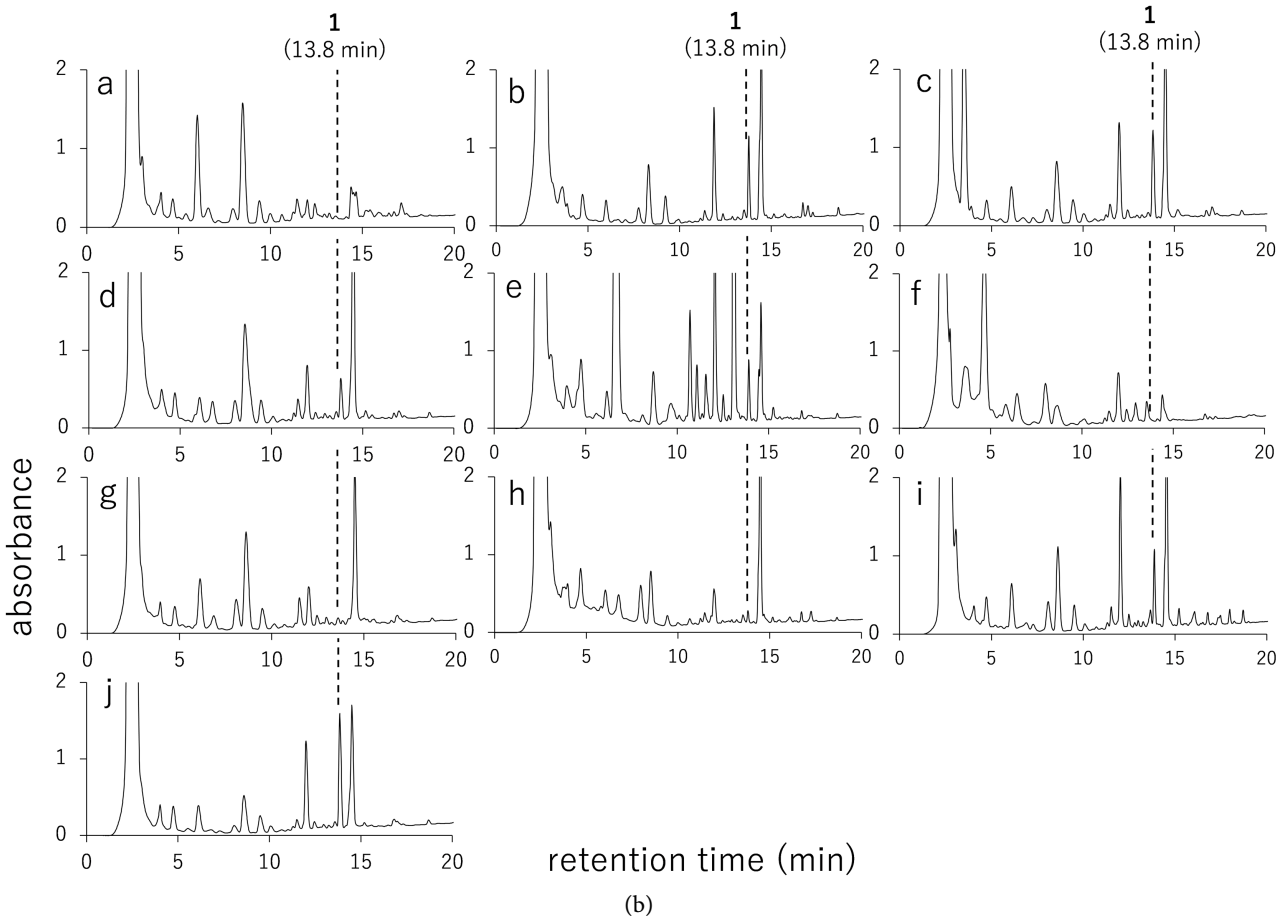


Figure 3. Separation procedure for crude drug extracts and HPLC chromatograms of *Aspergillus fumigatus* culture extracts supplemented with water fractions of crude drug extracts. (a) Separation procedure; (b) HPLC chromatograms. Chromatograms were recorded at 200 nm. a: culture extract without crude drugs, b: culture extract with Kyokatsu (Notopterygium), c: Kyonin (apricot kernel), d: Kujin (Sophora root), e: Goboshi (Burdock fruit), f: Goma (sesame), g: Shokyo (ginger), h: Shin'i (magnolia flower), i: Togashi (Benincasa seed), j: Bukuryo (*Poria sclerotium*).

4. Discussion

In the present study, *A. fumigatus* was cultured in medium supplemented with 120 crude drug extracts derived from components of Kampo medicine in order to explore the factors that influence the production of secondary metabolites in *A. fumigatus*.

Our analyses confirmed that 9 crude drug extracts [Kyokatsu (Notopterygium), Kyonin (apricot kernel), Kujin (Sophora root), Goboshi (Burdock fruit), Goma (sesame), Shokyo (ginger), Shin'i (magnolia flower), Togashi (Benincasa seed), and Bukuryo (*Poria sclerotium*)] commonly induced *A. fumigatus* to produce trypacidin (**1**). Furthermore, only the aqueous fraction of the 9 crude drug extracts induced the production of trypacidin. These results indicate that the factor that induces the production of **1** is a water-soluble substance.

Since the aqueous fraction contained glucose, fructose, and sucrose as major components, the ability of these sugars to induce the production of trypacidin by *A. fumigatus* was examined. None of these sugars induced the production of

trypacidin (data not shown).

Trypacidin was first isolated from *A. fumigatus* and reported as an anti-protozoal agent [11] that is also cytotoxic to human A549 lung cells [12]. These biological activities are thought to play a role in predator avoidance, as trypacidin is present in the conidia of *A. fumigatus*. However, trypacidin is also a clinically important chemical because its bioactivity is thought to play a role in the pathogenesis of pulmonary aspergillosis caused by *A. fumigatus* [13].

To date, 13 gene clusters involved in the biosynthesis of trypacidin have been reported [13] [16]. Lind also reported that these biosynthetic gene clusters are regulated by the global regulators LaeA and BrlA [17].

Based on the results of the present study, we hypothesized that the increase in trypacidin production induced by the 9 crude drug extracts is due to chemical components in the extracts that affect either the trypacidin biosynthesis genes or the regulator genes.

To our knowledge, there have been no reports of chemicals that control the production of trypacidin, although some studies have described the effects of physical factors such as temperature and light or modification of gene clusters using molecular biological techniques. Identifying the substances in the aqueous fraction that affect the production of trypacidin may aid in the development of methods to control the production of trypacidin by *A. fumigatus*.

5. Conclusion

In this study, we found that the aqueous fractions of 9 crude drug extracts induce the production of trypacidin in *A. fumigatus*. The identification of trypacidin producing substances in these aqueous fractions is expected to facilitate the development of methods to inhibit trypacidin production in *A. fumigatus* in the future.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplementary Tables and Figures

Table S1. Crude drugs used in this study.

Japanese name	English name	Japanese name	English name	Japanese name	English name
Ireisen	Clematis Root	Saishin	Asiasarum Root	Chimpi	Citrus Unshiu Peel
Inchinko	Artemisia Capillaris Flower	Sahuran	Saffron	Tenma	Gastrodia Tuber
Ukon	Turmeric	Sankirai	Smilax Rhizome	Tenmondo	Asparagus Root
Uyaku	Lindera Root	Sanshishi	Gardenia Fruit	Togashi	Benincasa Seed
Engosaku	Corydalis Tuber	Sanshichininjin	Panax notoginseng root	Toki	Japanese Angelica Root
Ougi	Astragalus Root	Sansyuyu	Cornus Fruit	Tojin	Codonopsis Root
Ogon	Scutellaria Root	Sansyo	Japanese Zanthoxylum Peel	Tonin	Peach Kernel
Obaku	Phellodendron Bark	Sansonin	Jujube Seed	Dokukatsu	Aralia Rhizome
Ohi	Cherry Bark	Sanyaku	Dioscorea Rhizome	Tocyu	Eucommia Bark
Oren	Coptis Rhizome	Sanryo	Sparganium Rhizome	Nikujuyo	Cistanche Herb
Onji	Polygala Root	Jio	Rehmannia Root	Ninjin	Ginseng
Gaiyo	Artemisia Leaf	Shigoka	Eleutherococcus Senticosus Rhizome	Baimo	Fritillaria Bulb
Kasyu	Polygonum Root	Jigoppi	Lycium Bark	Hakusempi	Dictamnus root bark
Gajutsu	Zedoary	Shitsuritsu	Tribulus Fruit	Bakumondo	Ophiopogon Root
Kakkon	Pueraria Root	Syakuyaku	Peony Root	Hakka	Mentha Herb
Karokon	Trichosanthes Root	Syazenshi	Plantago Seed	Hange	Pinellia Tuber
Kankyo	Processed Ginger	Jukujio	Processed Rehmannia Root	Byakusi	Angelica Dahurica Root
Kanzo	Glycyrrhiza	Syukusya	Amomum Seed	Byakujutsu	Atractylodes Rhizome
Kikyo	Platycodon Root	Syokyo	Ginger	Biwayo	Loquat Leaf
Kikuka	Chrysanthemum Flower	Syoma	Cimicifuga Rhizome	Binroji	Areca
Kijitsu	Immature Orange	Shin'i	Magnolia Flower	Bukuryo	Poria Sclerotium
Kyokatsu	Notopterygium	Jingyo	Large gentian root	Boi	Sinomenium Stem and Rhizome
Kyonin	Apricot Kernel	Sekisyaku	Red Peony Root	Bokon	Imperata Rhizome
Kujin	Sophora Root	Senkyu	Cnidium Rhizome	Bohu	Saposhnikovia Root and Rhizome
Kumazasa	Sasa keaf	Kiboshininjin	Raw dried Panax ginseng	Hobushi	Processed Aconite Root
Keigai	Schizonepeta Spike	Sojutsu	Atractylodes Lancea Rhizome	Bokusoku	Quercus Bark
Keiketto	Spatholobus suberectus	Souhakuhi	Mulberry Bark	Hokotsushi	Psoralea seed
Keihi	Cinnamon Bark	Soyo	Morus leaf	Botampi	Moutan Bark
Genjin	Scrophularia root	Zokudan	Dipsacus asperoides root	Mao	Ephedra Herb
Koka	Safflower	Soyo	Perilla Herb	Mashinin	Hemp Fruit
Gokahi	Eleutherococcus	Daio	Rhubarb	Mankeishi	Shrub chaste tree fruit
Kojin	Red Ginseng	Taiso	Jujube	Mokkou	Saussurea Root
Kobushi	Cyperus Rhizome	Takusya	Alisma Tuber	Motsuyaku	Myrrh
Koboku	Magnolia Bark	Tanjin	Salvia Miltiorrhiza Root	Yakuchi	Bitter Cardamon
Goshitsu	Achyranthes Root	Chikujo	Bamboo culm	Yakumoso	Leonurus Herb
Gosyuyu	Euodia Fruit	Chikusetsuninjin	Panax Japonicus Rhizome	Yokuinin	Coix Seed
Goboshi	Burdock Fruit	Chimo	Anemarrhena Rhizome	Ryutan	Japanese Gentian
Goma	Sesame	Cyoji	Clove	Ryokyo	Alpinia Officinarum Rhizome
Gomishi	Schisandra Fruit	Cyotoko	Uncaria Hook	Reishi	Ganoderma
Saiko	Bupleurum Root	Cyorei	Polyporus Sclerotium	rengyo	Forsythia Fruit

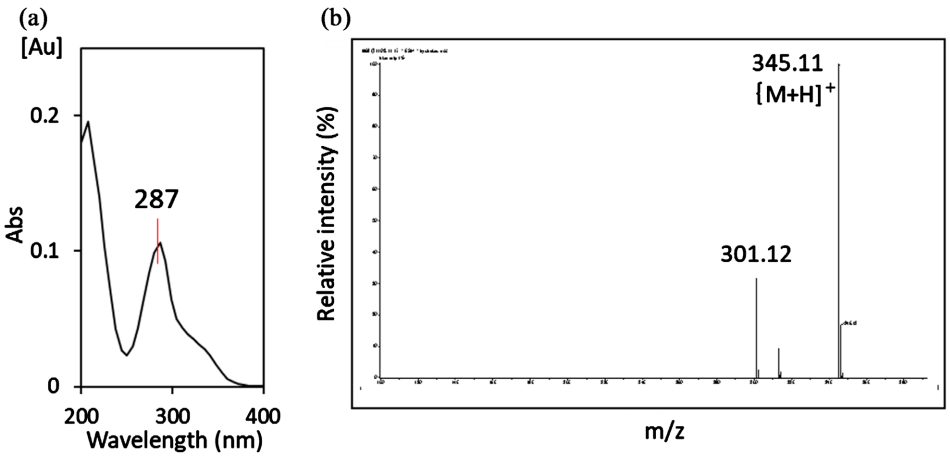

Figure S1. UV and MS spectra of compound **1**. (a) UV spectrum of **1** from DAD-HPLC analysis, (b) MS spectrum.

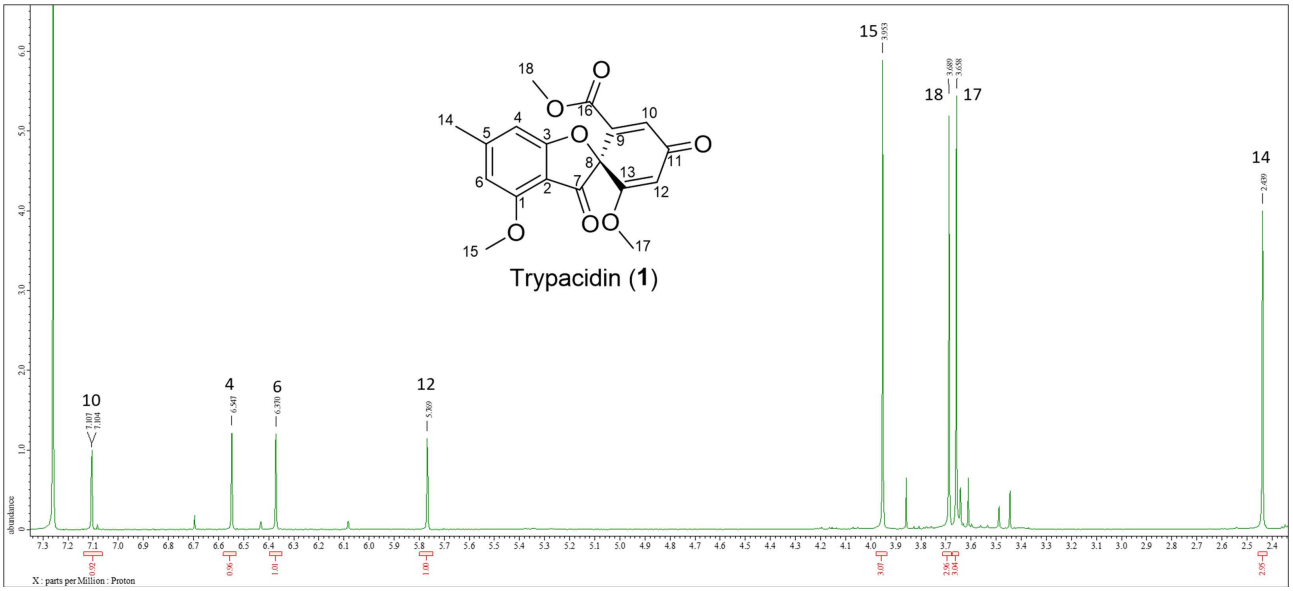

Figure S2. ^{1}H-NMR spectrum of **1** in $CDCl_3$.

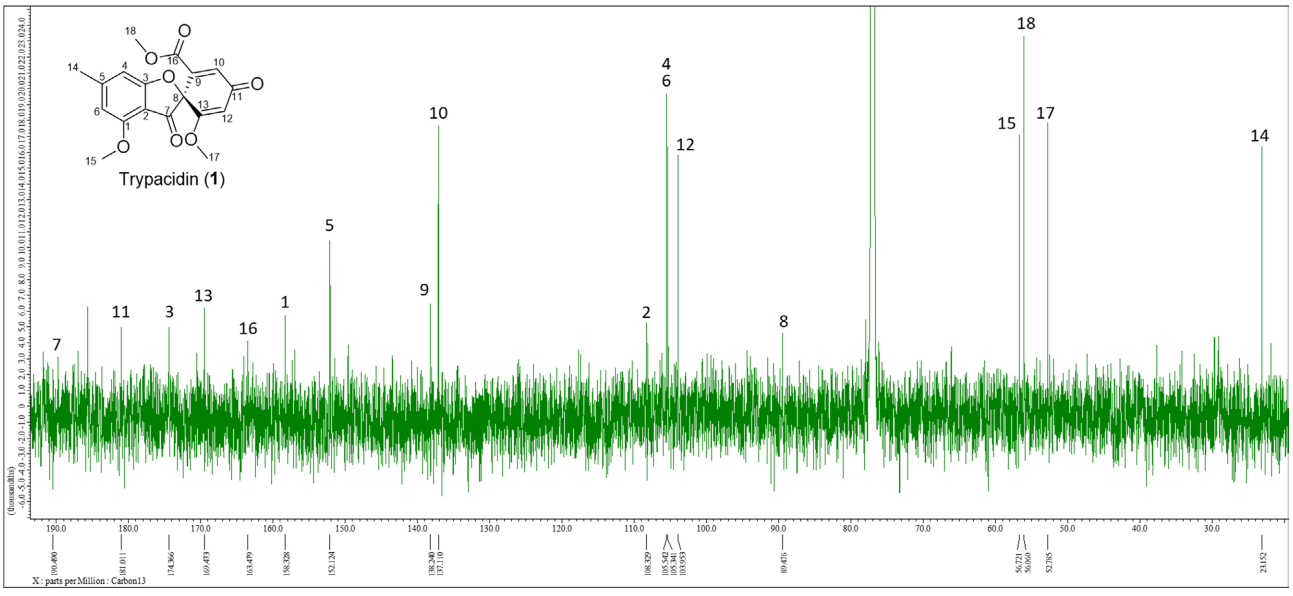

Figure S3. ^{13}C-NMR spectrum of **1** in $CDCl_3$.

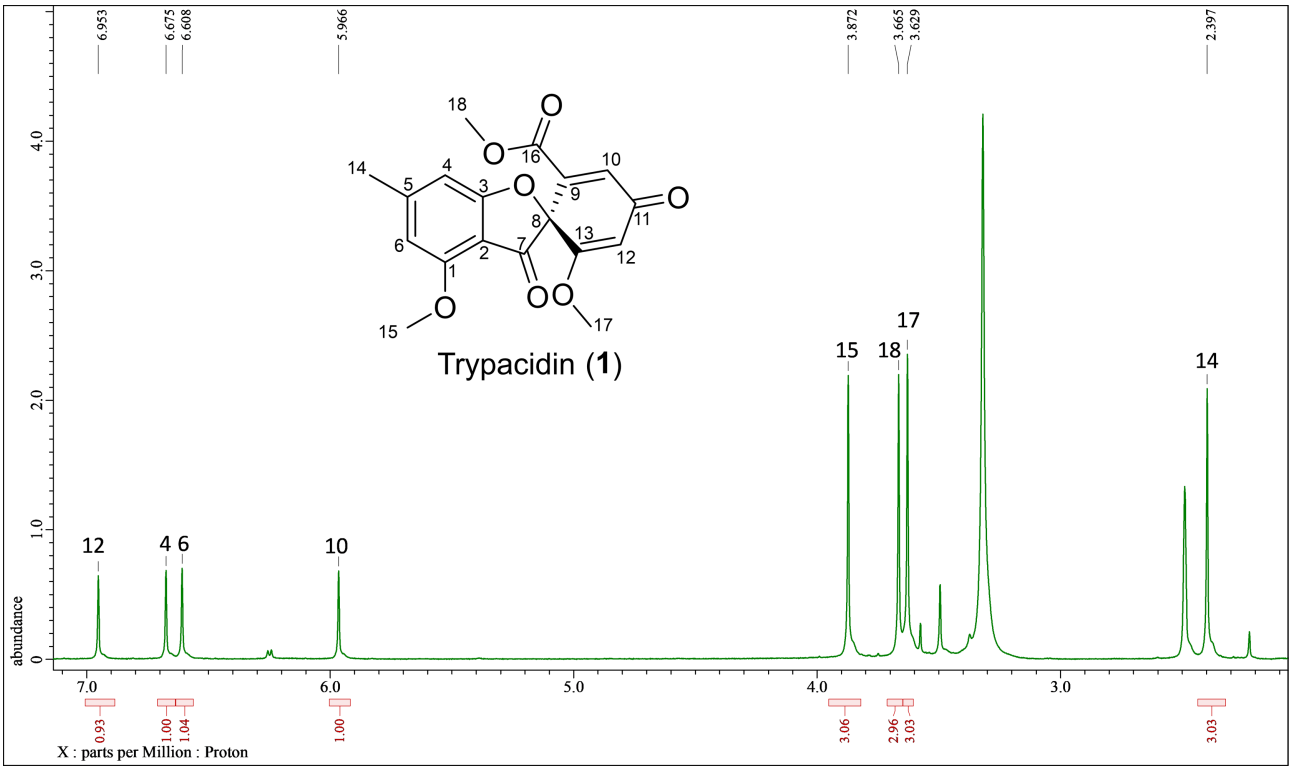

Figure S4. [1]H-NMR spectrum of **1** in DMSO-d_6.

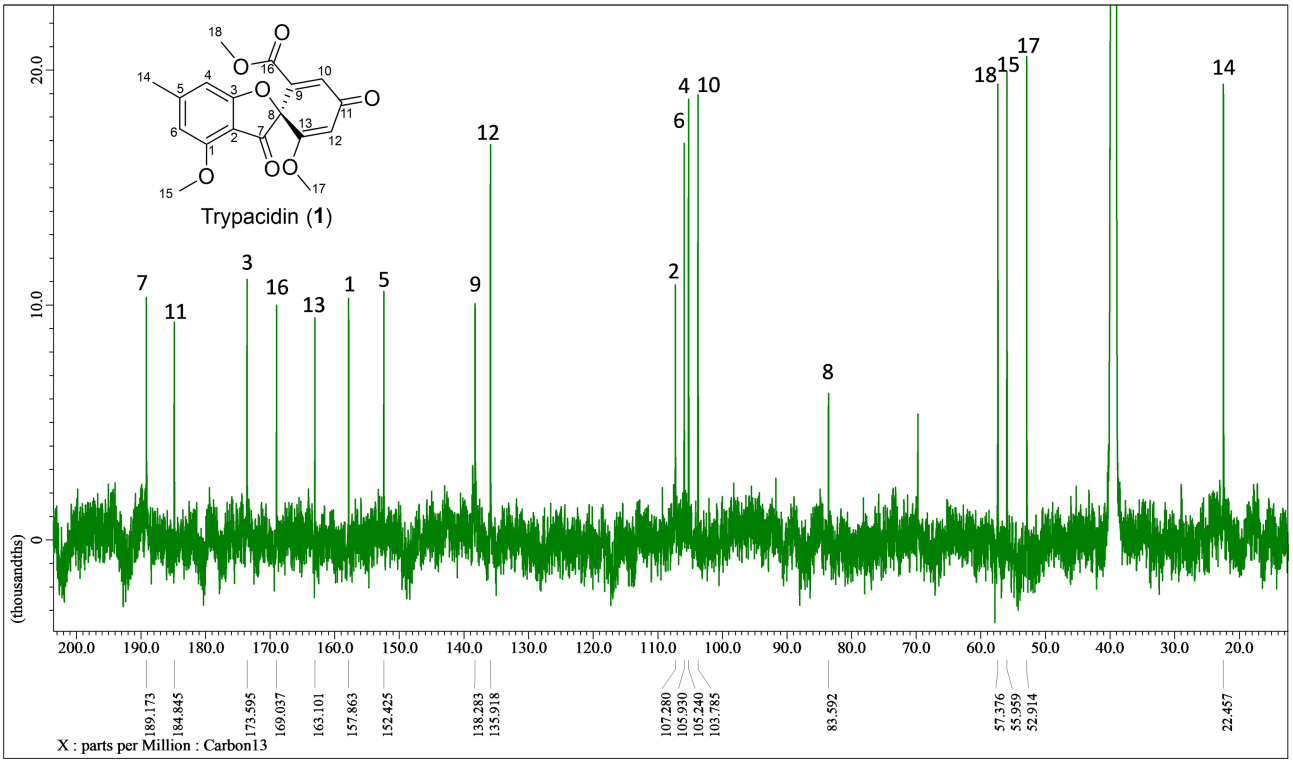

Figure S5. [13]C-NMR spectrum of **1** in DMSO-d_6.

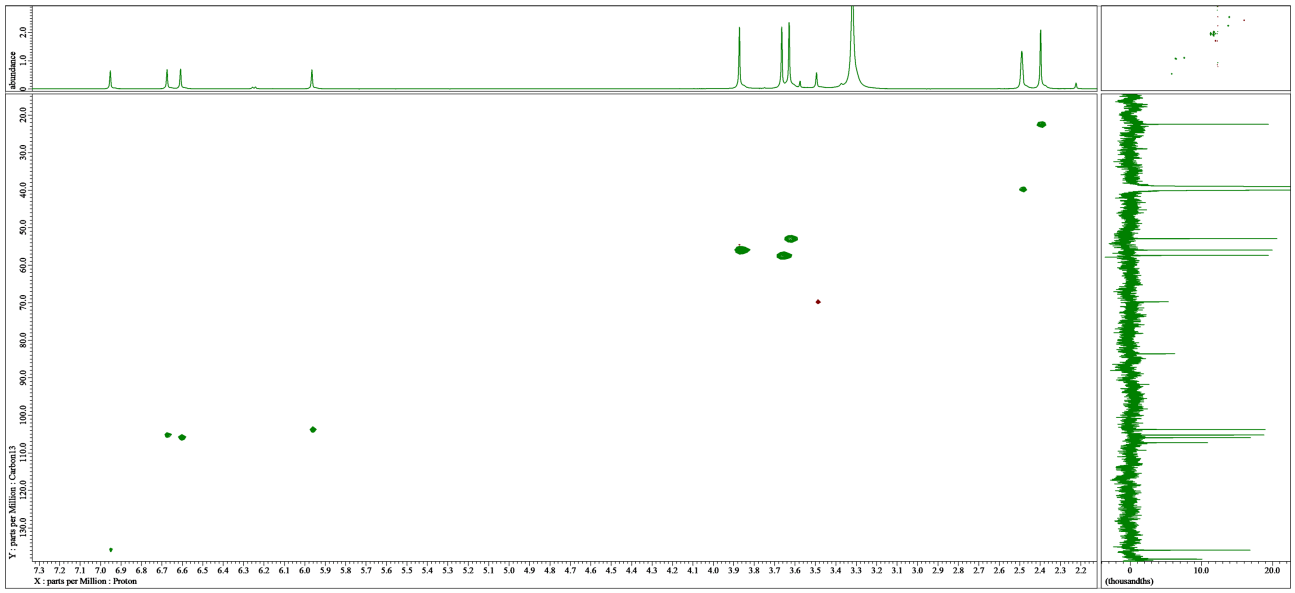

Figure S6. HSQC spectrum of **1** in DMSO-d_6.

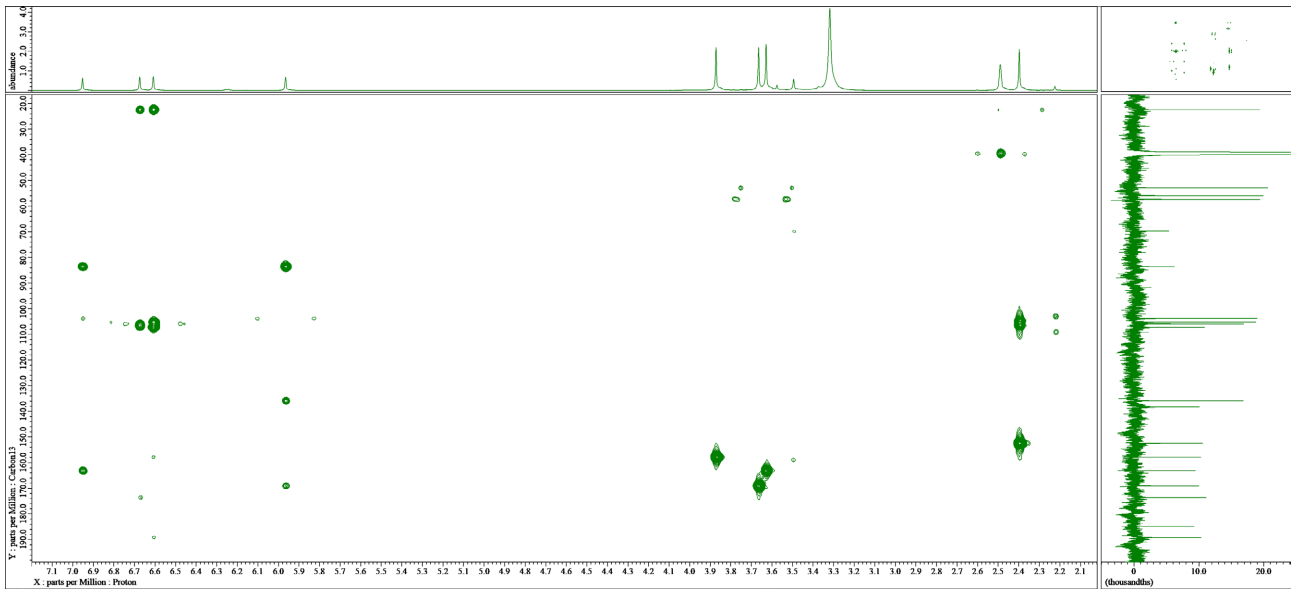

Figure S7. HMBC spectrum of **1** in DMSO-d_6.