Immunosuppressive constituents from an Ascomycete, *Sordaria gondaensis*

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Summary

Immunomodulatory activity-guided fractionation of the ethyl acetate extract of an Ascomycete, *Sordaria gondaensis*, afforded three dioxopiperazine-type constituents as the immunosuppressive features of this fungus. They were elucidated to be two known fungal metabolites, tryprostatin B and fumitremorgin B, and a new metabolite 13-oxofumitremorgin B by their physicochemical and spectral data and the chemical correlation. This is the first time that both tryprostatin B and fumitremorgin B have been isolated from this fungus.

Key words : *Sordaria gondaensis*, Ascomycete, immunosuppressant, 2,5-dioxopiperazine, 13-oxofumitremorgin B

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Introduction

In our recent search for immunomodulatory components from Ascomycetes, several novel immunosuppressants from *Gelasinospora multiforis*,^{1,2)} *G. kobei*,³⁾ *G. heterospora*,²⁾ *G. longispora*,²⁾ *Diplogelasinospora grovesii*,⁴⁾ and *Microascus tardifaciens*⁵⁾ were isolated. Continuously, it was found that the ethyl acetate (AcOEt) extract of *Sordaria gondaensis* IFM4583⁶⁾ suppressed significantly proliferation (blastogenesis) of mouse splenic lymphocytes stimulated with mitogens, concanavalin A (ConA) and lipopolysaccharide (LPS). Immunomodulatory activity-guided fractionation of the extract by solvent partition and chromatographic separation afforded three metabolites tentatively named SG-1 (1), -2 (2), and -3 (3) as the immunosuppressive features of this fungus. This report deals with the structure and immunosuppressive activity of these metabolites.

Materials and Methods

The general procedures for chemical and other experimental conditions, including those for the evaluation of immunomodulatory activity of samples against mouse splenic lymphocytes stimulated with Con A and LPS, were the same as described in our previous report [this method is based on the formation ratio of formazan from exogenous 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) in lymphocytes].²⁾ The thin layer chromatography (TLC) was carried out with Merck Kieselgel 60F₂₅₄ (ordinary phase) and/or RP18 F₂₅₄S (reverse phase) plates and an ultraviolet (UV)

detector at 254 and 360 nm. The infrared (IR) spectra, the UV spectra, the circular dichroism (CD) spectra, the electron impact mass spectra (EI-MS), the high-resolution EI-MS (HREI-MS), and the optical rotations were recorded with a JASCO FT-IR230, a Hitachi U-3200, a JASCO J-500, a Hitachi M-60, a JEOL JMS-HX110A, and a JASCO DIP-140 spectrometers, respectively. The ^1H - and ^{13}C -nuclear magnetic resonance (NMR) spectra were recorded in deuteriochloroform (CDCl_3) with a JEOL JNM-A400 or JNM-A500 spectrometer in δ (ppm) values from tetramethylsilane (TMS) as an internal standard at 400 and 100.4 MHz, or at 500 and 125.65 MHz, respectively.

Isolation of SG-1 (1), -2 (2), and -3 (3) Black or dark greenish brown colored mycelia of *Sordaria gondaensis* IFM4583⁶⁾ cultivated on sterilized rice media (200 g/flask $\times$ 50) at 25°C for 29 days were extracted with AcOEt (15 l) with shaking at room temperature for 5 hours two times to give an extract (after evaporation *in vacuo*, 32.3 g), which was then suspended in water (300 ml). The aqueous suspension was shaken with *n*-hexane (300 ml) two times to separate *n*-hexane-soluble portion (after evaporation *in vacuo*, 18.7 g), and then partitioned with AcOEt (300 ml) two times into AcOEt layer (after evaporation *in vacuo*, 11.3 g) and aqueous layer (after lyophilization *in vacuo*, 1.8 g). The AcOEt layer was subjected to silica gel column chromatography with CHCl_3 -MeOH to give six fractions I - VI. Fraction III (2.97 g) was then chromatographed on an octadecyl silica gel (ODS) column with MeOH and CHCl_3 to give MeOH-soluble fraction (Fraction III m) (1.25 g) and CHCl_3 -soluble fraction (Fraction III c) (111 mg). Fraction III m was further chromatographed on an ODS column with H_2O -MeOH (3:2, v/v), MeOH, and CHCl_3 to give six fractions III m1- III m6. Fraction III m3 afforded **1** (476 mg) directly after evaporation of the solvent. Fraction III m4 (80 mg) was further purified by preparative TLC on ordinary phase plates with CHCl_3 -acetone (4:1) to afford **2** (6.5 mg). Fraction III m5 (124 mg) was recrystallized with MeOH to afford **3** (32 mg).

SG-1 (tryprostatin B⁷⁻⁹⁾ (**1**): pale yellow crystalline powder, mp. 101-103°C (lit.⁷⁻⁹⁾ 102-105°C). $[\alpha]_{\text{D}}^{19}$ -77° ($c=0.60$, CHCl_3) [lit.⁷⁻⁹⁾ -71.1° ($c=0.63$, CHCl_3)]. EI-MS m/z (%): 351 (M^+ , 15), 198 (100) [lit.⁷⁻⁹⁾ 351 (M^+ , 20), 198 (100)]. HREI-MS: m/z 351.1956 ($\text{C}_{21}\text{H}_{25}\text{N}_3\text{O}_2$ requires 351.1946). UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ): 224 (4.59), 273 (sh, 3.88), 283 (3.90), 290 (3.85) [lit.⁷⁻⁹⁾ 226 (4.38), 277 (3.94), 298 (sh, 3.86)]. CD (0.71 mM, MeOH) $\Delta\epsilon$ (nm): 0.03 (305), -0.15 (290), 0.27 (272), -0.04 (254), 6.55 (232), -10.04 (210) [lit.⁷⁻⁹⁾ 0.3 (303), 0.2 (292), 0.3 (272), 3.0 (232)]. ^1H - and ^{13}C -NMR: see Table 1.

SG-2 (13-oxofumitremorgin B) (**2**): pale yellow amorphous powder, $[\alpha]_{\text{D}}^{19} +56^\circ$ ($c=0.20$, CHCl_3). EI-MS m/z (%): 477 (M^+ , 12), 459 (12), 310 (32), 309 (100), 241 (53). HREI-MS: m/z 477.2246 ($\text{C}_{27}\text{H}_{31}\text{N}_3\text{O}_5$ requires 477.2263). UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm(log ϵ): 218 (sh, 4.42), 244 (sh, 4.13), 250 (4.16), 282 (4.06), 318 (sh, 3.89). CD (1.05 mM, EtOH) $\Delta\epsilon$ (nm): -0.36 (340), 3.23 (313), 1.12 (290), 1.93 (272), 1.03 (260), 1.70 (254), -4.25 (244), 0.67 (233), -2.79 (226), 10.07 (210), 11.80 (204). ^1H - and ^{13}C -NMR: see Table 2.

SG-3 (fumitremorgin B¹⁰⁻¹²⁾ (**3**): white crystalline powder, mp 210-211°C, $[\alpha]_{\text{D}}^{19} +31^\circ$ ($c=0.40$, CHCl_3), EI-MS m/z (%): 479 (M^+ , 87), 311 (100), 268 (71). CD (1.05 mM, EtOH) $\Delta\epsilon$ (nm): 1.32 (305), 1.40 (298), 1.20 (292), 2.75 (276), 2.13 (268), -0.91 (247) -0.77 (242), -1.92 (230), 0.71 (216), 0.27 (209). ^1H - and ^{13}C -NMR: see Table 2. This compound was identified with authentic sample of fumitremorgin B¹⁰⁻¹²⁾ in terms of mixed mp, TLC behavior on ordinary phase plates with CHCl_3 -acetone (5:1) and C_6H_6 -EtOH (10:1), ^1H - and ^{13}C -NMR (in CDCl_3), and CD spectra (in EtOH).

Oxidation of SG-3 (3) A solution of **3** (2 mg) in pyridine (20 μl) was added to a complex

prepared with CrO_3 (5 mg) and pyridine (30 μ l) under ice-cooling, then the reaction mixture was stirred under ice-cooling for 1 hour and at room temperature for 4 hours. After addition of 1 drop of MeOH, the mixture was diluted with water and extracted with Et_2O . The Et_2O layer was treated as usual to give a product mixture, which was purified by a preparative TLC on an ordinary phase plate with CHCl_3 -acetone (5:1) to give a product tentatively named Oxo-SG-3 (0.5 mg) as amorphous powder. This product was identified with **2** in terms of TLC behavior on an ordinary phase plate with CHCl_3 -acetone (5:1), ^1H -NMR (in CDCl_3) (see Table 2) and CD spectra (in EtOH).

Results and Discussion

The AcOEt extract of *S. gondaensis* IFM4583⁶⁾ cultivated on sterilized rice suppressed by 99, 91, 77, and 55% of the proliferation of mouse splenic lymphocytes stimulated with Con A (T cells) at 50, 10, 2.0, and 0.4 μ g/ml, respectively. After separating *n*-hexane-soluble (fatty) portion, the extract was partitioned with AcOEt- H_2O . The AcOEt layer suppressed by 50% of the Con A-induced proliferation of the lymphocytes at less than 10 μ g/ml, while both the fatty portion and the aqueous layer suppressed it by 50% at more than 25 μ g/ml. Immunosuppressive activity-guided fractionation with repeated chromatography of the AcOEt layer afforded SG-1 (**1**), SG-2 (**2**), and SG-3 (**3**) as the immunosuppressive features of this fungus [yield (%) from the AcOEt layer, **1**: 4.2, **2**: 0.058, and **3**: 0.28].

SG-1 (**1**) was obtained as pale yellow crystalline powder, $\text{C}_{21}\text{H}_{25}\text{N}_3\text{O}_2$, and was optically active. The ^1H - and ^{13}C -NMR spectral data including the spin-decoupling ^1H -NMR, the two-dimensional ^1H - ^1H shift correlation spectroscopy (COSY) NMR, ^1H -detected heteronuclear correlation through multiple quantum coherence (HMQC) NMR, and ^1H -detected heteronuclear multiple-bond correlation (HMBC) NMR (see Table 1) suggested that the structure of **1** quite resembled that of a cytotoxic dioxopiperazine, tryprostatin B,⁷⁻⁹⁾ isolated from *Aspergillus fumigatus* (see Chart 1). Comparison of the physicochemical and spectral data of SG-1 with those of tryprostatin B described in the literature⁷⁻⁹⁾ showed that SG-1 was identical with tryprostatin B (**1**) (see Table 1 and Materials and Methods).

SG-3 (**3**) was obtained as white crystalline powder, and was optically active. The physicochemical and spectral data including the ^1H - and ^{13}C -NMR data of **3** were very similar to those of a tremorgenic dioxopiperazine, fumitremorgin B,¹⁰⁻¹²⁾ isolated from *Aspergillus fumigatus* (see Table 2). SG-3 was identified with the authentic sample of fumitremorgin B (**3**) by direct comparison (see Chart 1). This is the first time that both tryprostatin B and fumitremorgin B have been isolated from *S. gondaensis*.

SG-2 (**2**) was obtained as pale yellow amorphous powder, $\text{C}_{27}\text{H}_{31}\text{N}_3\text{O}_5$, and was optically active. Comparison of the ^1H - and ^{13}C -NMR spectra of **2** with those of **3** exhibited that all of the signals in the ^1H - and ^{13}C -NMR spectra of **2** were quite similar to those of **3** except for the disappearance of both signals of H-13 and OH-13 and the down-field shift of the signal of C-13 to δ_c 182.13 (+113.17), indicating that **2** might be a new compound having the structure of 13-oxo derivative of **3**. On oxidation of **3** with CrO_3 -pyridine complex, the hydroxyl group at position 13 in **3** was oxidized to give a 13-oxo derivative of **3**, which was identified with **2** by direct comparison (see Materials and Methods). Thus, the structure of SG-2 was finally elucidated to be 13-oxofumitremorgin B (**2**).

The immunosuppressive activities (IC_{50} values) of **1-3** were calculated against Con A- (T-cells) and LPS-induced (B-cells), and mitogen-free proliferations of mouse splenic lymphocytes, as shown in

Table 1. ^1H -NMR and ^{13}C -NMR data for SG-1 (1) and Typrostatin B (1)⁷⁻⁹, δ (ppm) from TMS in CDCl_3 (coupling constants in parentheses)

Position	SG-1		Typrostatin B ⁷⁻⁹	
	^1H -NMR	^{13}C -NMR	^1H -NMR	^{13}C -NMR
1-NH	8.14-(brs)		8.03 (brs)	
2		136.44 (s)		136.43 (s)
3		104.51 (s)		104.61 (s)
3a		127.93 (s)		127.97 (s)
4	7.47 (d, 7.7)	117.67 (d)	7.47 (d, 7.7)	117.74 (d)
5	7.09 (t, 7.7)	119.81 (d) ^{a)}	7.09 (t, 7.7)	119.91 (d)
6	7.15 (t, 7.7)	121.76 (d)	7.16 (t, 7.7)	121.87 (d)
7	7.30 (d, 7.7)	110.75 (d)	7.31 (d, 7.7)	110.79 (d)
7a		135.31 (s) ^{b)}		135.44 (s)
8	2.96 (dd, 15.1, 11.2), 3.68 (m)	25.58 (t)	2.96 (dd, 15.0, 11.0), 3.68 (dd, 15.0, 3.5)	25.61 (t)
9	4.37 (br dd, 11.2, 2.8)	54.55 (d)	4.37 (br dd, 11.0, 3.5)	54.57 (d)
10-NH	5.67 (brs)		5.64 (brs)	
11		169.31 (s)		169.35 (s)
12	4.05 (brt, 7.6)	59.20 (d)	4.06 (br dd, 8.0, 7.5)	59.27 (d)
13	2.02 (m), 2.32 (m)	28.30 (t)	1.97-2.08 (m), 2.33 (m)	28.34 (t)
14	1.90 (m), 2.02 (m)	22.57 (t)	1.85-1.95 (m), 1.97-2.08 (m)	22.64 (t)
15	3.62 (2H, m)	45.36 (t)	3.68 (overlapped with H-8) 3.59 (ddd, 12.0, 8.5, 3.0)	45.41 (t)
17		165.76 (s)		165.80 (s)
18	3.46 (2H, m)	25.07 (t)	3.44 (dd, 17.0, 6.5), 3.49 (dd, 17.0, 7.0)	25.12 (t)
19	5.30 (brt, 7.2)	119.74 (d) ^{a)}	5.31 (br dd, 7.0, 6.5)	119.70 (d)
20		135.41 (s) ^{b)}		135.48 (s)
21	1.77 (3H, s)	25.68 (q)	1.79 (3H, s)	25.74 (q)
22	1.74 (3H, s)	17.92 (q)	1.75 (3H, s)	17.98 (q)

a),b) : interchangeable

Chart 1

Table 2. ^1H -NMR and ^{13}C -NMR data for SG-2 (**2**), SG-3 (**3**) and Oxo-SG-3 (**2**), δ (ppm) from TMS in CDCl_3 (coupling constants in parentheses)

Position	SG-2		SG-3		Oxo-SG-3
	^1H -NMR	^{13}C -NMR	^1H -NMR	^{13}C -NMR	^1H -NMR
2		147.59 (s)		131.14 (s)	
3	6.07 (d, 9.6)	48.60 (d)	5.97 (d, 10.0)	49.02 (d)	6.08 (d, 9.8)
5		165.70 (s)		166.22 (s)	
6	4.72 (t, 8.3)	60.00 (d)	4.45 (dd, 9.6, 7.1)	58.74 (d)	4.76 (t, 8.3)
7	2.15 (m), 2.33 (m)	28.60 (t)	1.94 (m), 2.47 (m)	28.93 (t)	2.10 (m), 2.38 (m)
8	1.88 (m), 2.04 (m)	23.11 (t)	2.08 (2H, m)	22.59 (t)	1.92 (2H, m)
9	3.55 (2H, m)	45.54 (t)	3.63 (2H, dd, 9.0, 4.6)	45.24 (t)	3.53 (m), 3.62 (m)
11		172.66 (s)		170.45 (s)	
12		81.66 (s)		82.94 (s)	
12-OH			4.05 (brs)		
13		182.13 (s)	5.77 (d, 2.7)	68.96 (d)	
13-OH			4.71 (brd, 2.7)		
14		108.21 (s)		104.38 (s)	
15		118.54 (s)		120.51 (s)	
16	8.13 (d, 8.7)	123.02 (d)	7.85 (d, 8.7)	121.34 (d)	8.20 (d, 8.7)
17	6.80 (dd, 8.7, 2.0)	111.68 (d)	6.80 (dd, 8.7, 2.2)	109.29 (d)	6.96 (dd, 8.8, 2.1)
18		157.74 (s)		156.19 (s)	
18-OCH ₃	3.82 (3H, s)	55.64 (q)	3.84 (3H, s)	55.72 (q)	3.86 (3H, s)
19	6.70 (d, 2.0)	94.90 (d)	6.69 (d, 2.2)	93.85 (d)	6.72 (d, 2.0)
20		139.01 (s)		137.88 (s)	
21	4.48 (dd, 16.1, 4.3), 4.55 (dd, 16.1, 6.7)	42.67 (t)	4.54 (2H, brd, 5.6)	41.77 (t)	4.53 (dd, 16.6, 5.4), 4.60 (dd, 16.6, 6.8)
22	4.99 (brt, 5.5)	118.54 (d)	5.03 (brt, 5.6)	120.27 (d)	5.02 (brt, 5.7)
23		136.33 (s)		134.58 (s)	
24	1.84 (3H, s)	18.33 (q)	1.85 (3H, s)	18.18 (q)	1.87 (3H, s)
25	1.71 (3H, s)	25.56 (q)	1.70 (3H, s)	25.68 (q)	1.74 (3H, s)
26	4.77 (d, 9.6)	122.36 (d)	4.70 (d, 10.0)	122.96 (d)	4.83 (d, 10.1)
27		137.99 (s)		135.20 (s)	
28	1.95 (3H, s)	18.76 (q)	1.99 (3H, s)	18.35 (q)	1.97 (3H, s)
29	1.63 (3H, s)	25.56 (q)	1.63 (3H, s)	25.51 (q)	1.66 (3H,s)

Table 3. The IC_{50} values of three related dioxopiperazines, tardioxopiperazines A (**4**) and B (**5**) from *Microascus tardifaciens*,⁵⁾ and echinuline (**6**) from *Aspergillus echinulatus* and other *Aspergillus glaucus* group fungi¹³⁻¹⁵⁾ (see Chart 1), and some positive controls against Con A- and LPS-induced, and mitogen-free proliferations of the lymphocytes are also shown in Table 3. Among these six dioxopiperazines, **1-4** show moderate immunosuppressive activity, while **5** and **6** show slight one. The fact that the activity of **2** is a little higher than that of **3** suggests that substitution of a hydroxyl group at position 13 in **3** by a carbonyl group slightly increases the activity. It has been already known that **1** and **3** possess inhibitory activity against the cell cycle progression of mouse tsFT210 cells in the M phase.⁷⁻⁹⁾ The immunosuppressive activity of **1**, **2**, and **3** seem to be connected with their cytotoxic property.

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Table 3. Immunosuppressive effects of SG-1 (**1**) ~-3 (**3**), and some related compounds on the Con A- induced, LPS-induced and mitogen-free proliferations of mouse splenic lymphocytes

Compound	IC ₅₀ (μg/ml)		
	Con A-Induced	LPS-Induced	Mitogen-Free
1	12	3.0	6.0
2	2.3	0.4	1.5
3	7.0	0.8	2.9
Tradioxopiperazine A (4)	4.5	0.7	0.4
Tradioxopiperazine B (5)	>25	15	0.8
Echinuline (6)	>25	8.0	11.8
Azathiopurine	2.7	2.7	not tested
Cyclosporin A	0.04	0.07	0.05
FK506 (Tacrolimus)	0.015×10 ⁻³	1.6×10 ⁻³	2.1×10 ⁻³

References and Notes

- 1) Fujimoto, H., Satoh, Y., Nakayama, M., Takayama, T., Yamazaki, M. : Chem. Pharm. Bull., **43**, 547-552 (1995).
- 2) Fujimoto, H., Sumino, M., Nagano, J., Natori, H., Okuyama, E., Yamazaki, M. : Chem. Pharm. Bull., **47**, 71-76 (1999).
- 3) Fujimoto, H., Satoh, Y., Yamazaki, M. : Chem. Pharm. Bull., **46**, 211-216 (1998).
- 4) Fujimoto, H., Nagano, J., Yamaguchi, K., Yamazaki, M. : Chem. Pharm. Bull., **46**, 423-429 (1998).
- 5) Fujimoto, H., Fujimaki, T., Okuyama, E., Yamazaki, M. : Chem. Pharm. Bull., **47**, 1426-1432 (1999).
- 6) This strain was deposited earlier at Research Institute for Chemobiodynamics, Chiba University (present name: Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University). Now, the voucher specimen is also on deposit in our laboratory.
- 7) Cui, C. B., Kakeya, H., Okada, G., Onose, R., Ubukata, M., Takahashi, I., Isono, K., Osada, H. : J. Antibiot., **48**, 1382-1384 (1995).
- 8) Cui, C. B., Kakeya, H., Okada, G., Onose, R., Osada, H. : J. Antibiot., **49**, 527-533 (1996).
- 9) Cui, C. B., Kakeya, H., Osada, H. : J. Antibiot., **49**, 534-540 (1996).
- 10) Yamazaki, M., Fujimoto, H., Akiyama, T., Sankawa, U., Iitaka, Y. : Tetrahedron Lett., **1975**, 27-28.
- 11) Yamazaki, M., Fujimoto, H., Kawasaki, T. : Chem. Pharm. Bull., **28**, 245-254 (1980).
- 12) Yamazaki, M., Suzuki, K., Fujimoto, H., Akiyama, T., Sankawa, U., Iitaka, Y. : Chem. Pharm. Bull., **28**, 861-865 (1980).
- 13) Quilico, A., Panizzi, L. : Chem. Ber., **76**, 348-358 (1943).
- 14) Nakashima, R., Slater, G. P. : Tetrahedron Lett., **1971**, 2649-2650.
- 15) Fujimoto, H. : Annual Report of Research Institute for Chemobiodynamics, Chiba University, **5**, 9-43 (1977).

子囊菌 *Sordaria gondaensis* の免疫抑制活性成分

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免疫調節活性を指標とした子囊菌*Sordaria gondaensis*の酢酸エチル抽出エキスの分画において, 3種のジオキソピペラジン系成分が, 本菌の免疫抑制活性本体として単離された。それらは, 2種の既知菌類代謝産物, トリプロスタチンBとフミトレモルジンB, 並びに1種の新代謝産物, 13-オキソフミトレモルジンB, であることを, それらの物性とスペクトルデータおよび化学的関連付けから明らかにした。トリプロスタチンBとフミトレモルジンBが本菌から単離されたのは, 今回が初めてである。

キーワード: 子囊菌, 免疫抑制物質, 2, 5-ジオキソピペラジン, 13-オキソフミトレモルジンB