Immunomodulatory Constituents from Three Ascomycetes, *Gelasinospora heterospora*, *G. multiforis*, and *G. longispora*

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Three new 2-pyrones (2*H*-pyran-2-ones) called multiforisins G (3), H (1), and I (4), and a known hexaketide sordarial (2) have been isolated from an Ascomycete *Gelasinospora heterospora*. Among them, 1, 2, and 3 have been proved to be the immunosuppressive components of the fungus. Compounds 1, 3, and 4 have also been isolated from *G.multiforis* together with multiforisin A (5), which was formerly isolated from this fungus as its main immunosuppressive feature, and 1—5 have also been isolated from *G. longispora*. The absolute stereostructure of 2, which was not previously certain, has finally been determined to be (3′*R*,4′*S*). It has been found that the multiforisins 1, 3, and 5 in which one of the two substituents at positions 3 and 5 is a hydroxymethyl group and the other is a formyl or an acetoxymethyl group, show high immunosuppressive activity; the immunosuppressive activity of 3 does not seem to be due to inhibition of interleukin 2 (IL-2) production.

Key words fungal metabolite; Ascomycete; *Gelasinospora*; immunosuppressive activity; multiforisin; sordarial

In our screening program on immunomodulatory components from fungi, several immunosuppressive 2-pyrones, multiforisins A—F[1a,b] and macrophin[1c] have been isolated from Ascomycetes, *Gelasinospora multiforis* CAILLEUX and *Diplogelasinospora grovesii* UDAGAWA *et* HORIE, respectively. Successively, it was found that the defatted AcOEt extracts of two Ascomycetes, *Gelasinospora heterospora* CAILLEUX, and *G. longispora* UDAGAWA, appreciably suppressed proliferation (blastogenesis) of mouse splenic lymphocytes stimulated with mitogens, concanavalin A (Con A) and lipopolysaccharide (LPS). Solvent partition followed by repeated chromatographic fractionation of the extract of *G. heterospora* guided by immunosuppressive activity afforded four compounds **1**—**4**, among which **1**, **2**, and **3** showed immunosuppressive activity. Compounds **1**, **3**, and **4** were also isolated at this time from *G. multiforis* together with multiforisin A (**5**); moreover, compounds **1**—**5** were also isolated from *G. longispora*. This report deals with the structures and immunosuppressive activity of these compounds isolated from the three fungi belonging to the genus *Gelasinospora*.

Results and Discussion

The AcOEt extract of *G. heterospora* 74-T-542-1[2] cultivated on sterilized rice was partitioned between *n*-hexane and H_2O to give an *n*-hexane layer and an aqueous suspension (the defatted AcOEt extract), which was further partitioned between AcOEt and H_2O to afford an AcOEt layer and an aqueous layer. After evaporation, the *n*-hexane, AcOEt, and aqueous layers suppressed the Con A-induced proliferation of mouse splenic lymphocytes by 39, 100, and 59% at 50 μg/ml, and by 4, 80, and 16% at 10 μg/ml, respectively. Repeated chromatographic fractionation of the AcOEt layer guided by the immunosuppressive activity afforded four constituents, compounds **1**—**4** [yields (%) from the AcOEt layer, **1**: 0.23, **2**: 0.13, **3**: 0.19, and **4**: 0.057]. Among them, **1**, **2**, and **3** showed immunosuppressive activity, while **4** did not show the activity.

Compound **3** was obtained as optically inactive colorless needles. The IR and UV spectra suggested the presence of a carbonyl group conjugated with C=C bonds and a hydroxyl group. The ^{1}H- and ^{13}C-NMR spectral data of **3** suggested that **3** might be a new homologue of the multiforisins,[1a,b] especially to be similar to multiforisin B (multiforisin A 10-acetate) (**6**), which had been isolated together with multiforisin A [5-formyl-3-hydroxymethyl-4-methoxy-6-((*E*)-1-propenyl)-2-pyrone] (**5**) from *Gelasinospora multiforis*. Comparison of the ^{1}H- and ^{13}C-NMR spectral data of **3** with those of **6** indicated that the formyl group at position 5 in **6** [δ 10.09 (s), 187.0 (d)] was replaced with a hydroxymethyl group [δ 4.52 (2H, s), 54.9 (t)] in **3** (see Table 1 and Chart 1). This estimation was also supported by the fact that the molecular formula of **3** was determined to be $C_{13}H_{16}O_6$, with the high-resolution electron-impact MS (HR-EI-MS) spectrum and the nuclear Overhauser effects (NOEs) being observed as 8% between H-7 (δ 6.43) and H_2-11 (δ 4.52) and as 5% between H-7 and H_3-9 (δ 1.96) in the differential NOE (DifNOE) experiment of **3**. Finally, reduction of **6** with $NaBH_4$ in EtOH afforded O^{11},11-dihydromultiforisin B which was identical with **3**. Accordingly, the structure of **3** was determined to be 3-acetoxymethyl-5-hydroxymethyl-4-methoxy-6-((*E*)-1-propenyl)-2-pyrone (see Chart 1).

Compound **4** was obtained as optically inactive colorless needles. The ^{1}H- and ^{13}C-NMR data including spin-decoupling ^{1}H-NMR and two dimensional ^{1}H–^{1}H shift correlation spectroscopy (^{1}H–^{1}H COSY), ^{13}C–^{1}H COSY, and ^{1}H-detected heteronuclear multiple-bond correlation (HMBC) NMR experiments showed that **4** was also a new homologue of the multiforisins. Comparison of the ^{1}H- and ^{13}C-NMR spectral data of **4** with those of **3** indicated that the signal of AcO-10 in **3** [δ 2.08 (3H, s), 21.0 (1C, q), 170.9 (1C, s)] disappeared, and those of H_2-10, C-10, and C-3 were shifted to δ 4.60 (−0.46), 55.9 (−1.3), and 110.8 (+4.7), respectively. Thus, the structure of **4**, whose molecular formula was decided as $C_{11}H_{14}O_5$, by the HR-EI-MS spectrum, was deduced to be 3,5-bishydroxymethyl-4-methoxy-6-((*E*)-1-propenyl)-2-pyrone (see Chart 1). This was also supported by the fact that the NOEs were observed as 8% between H-7 (δ 6.42) and H_2-11 (δ 4.52) and as 4% between H-7 and H_3-9 (δ

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Table 1. ^{1}H-NMR and ^{13}C-NMR Data for Multiforisins B (**6**), G (**3**), H (**1**), I (**4**), and O^{11},11-Dihydromultiforisin B, and ^{1}H-NMR Data for Compound **7**, δ (ppm) from TMS as an Internal Standard in $CDCl_3$ [Coupling Constants (Hz) in Parentheses]

Position	**6**[1a)]		O^{11},11-Dihydromultiforisin B		**3**	
	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR
2		161.5 (s)		163.4 (s)		163.5 (s)
3		106.2 (s)		106.1 (s)		106.1 (s)
4		170.6 (s)		171.0 (s)		170.9 (s)
4-OMe	4.06 (3H, s)	63.1 (q)	4.06 (3H, s)	62.7 (q)	4.06 (3H, s)	62.7 (q)
5		108.8 (s)		111.4 (s)		111.4 (s)
6		164.4 (s)		158.0 (s)		158.0 (s)
7	7.34 (dq, 15.4, 1.5)	120.9 (d)	6.43 (dq, 15.6, 1.7)	119.3 (d)	6.43 (dq, 15.4, 1.7)	119.3 (d)
8	7.26 (dq, 15.4, 6.7)	145.7 (d)	6.88 (m)	138.3 (d)	6.88 (dq, 15.4, 6.8)	138.2 (d)
9	2.04 (3H, dd, 6.7, 1.5)	19.4 (q)	1.96 (3H, dd, 7.1, 1.7)	18.9 (q)	1.96 (3H, dd, 6.8, 1.7)	18.9 (q)
10	5.07 (2H, s)	56.4 (t)	5.06 (2H, s)	57.2 (t)	5.06 (2H, s)	57.2 (t)
10-OAc	2.10 (3H, s)	20.9 (q)	2.08 (3H, s)	21.0 (q)	2.08(3H, s)	21.0 (q)
		170.7 (s)		170.8 (s)		170.9 (s)
11	10.1 (s)	187.0 (d)	4.52 (2H, s)	55.0 (t)	4.52 (2H, s)	54.9 (t)
11-OAc						

Position	**1**		**4**		**7**
	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR
2		164.7 (s)		164.9 (s)	
3		110.8 (s)		110.8 (s)	
4		168.8 (s)		168.8 (s)	
4-OMe	4.07 (s)	63.2 (q)	4.12 (3H, s)	63.1 (q)	4.02 (3H, s)
5		107.9 (s)		111.8 (s)	
6		158.0 (s)		156.8 (s)	
7	6.39 (dq, 15.1, 1.7)	119.3 (d)	6.42 (dq, 15.3, 1.8)	119.3 (d)	6.39 (dq, 15.1, 1.5)
8	6.86 (dq, 15.1, 7.0)	138.2 (d)	6.84 (dq, 15.3, 7.0)	137.5 (d)	6.91 (dq, 15.1, 7.0)
9	1.97 (3H, dd, 7.0, 1.7)	18.9 (q)	1.96 (3H, dd, 7.0, 1.8)	18.9 (q)	1.97 (3H, dd, 7.0, 1.5)
10	4.59 (2H, s)	55.9 (t)	4.60 (2H, s)	55.9 (t)	5.05 (2H, s)
10-OAc					2.09 (3H, s)
11	4.97 (2H, s)	56.2 (t)	4.52 (2H, s)	55.1 (t)	4.97 (2H, s)
11-OAc	2.08 (3H, s)	20.9 (q)			2.09 (3H, s)
		170.8 (s)			

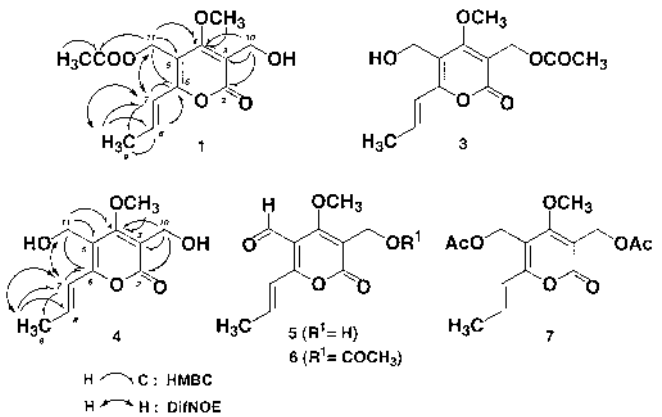

Chart 1

1.96) in the DifNOE experiment of **4**.

Compound **1** was obtained as optically inactive colorless needles. The ^{1}H- and ^{13}C-NMR spectral data and the molecular formula $C_{13}H_{16}O_6$ suggested that **1** might be an isomer of **3** involved in the position of an acetyl group. Comparison of the ^{1}H- and ^{13}C-NMR spectral data of **1** with those of **4** showed the new appearance of the signal of an acetoxyl group [δ 2.08 (3H, s), 20.9 (1C, q), 170.8 (1C, s)], and those of H_2-11, C-11, C-5 were shifted to δ 4.97 (+0.45), 56.2 (+1.1), 107.9 (−2.9), respectively; this indicated that the structure of **1** was 5-acetoxymethyl-3-hydroxymethyl-4-methoxy-6-((*E*)-1-propenyl)-2-pyrone (Chart 1). This conclusion was supported by the fact that **1** gave on acetylation with acetic anhydride and pyridine 10-acetyl derivative of **1** (**7**) [δ 2.09 (3H, s, H_3COO-10), 5.05 (2H, s, H_2-10)], which was also obtained from a mixture of **1** and **3** under the same acetylation condition (Table 1). In the DifNOE experiment of **1**, the NOEs were observed as 9% between H-7 (δ 6.39) and H_2-11 (δ 4.97) and as 5% between H-7 and H_3-9 (δ 1.97).

Compound **2** was obtained as optically active pale yellow needles. The IR and UV spectra suggested the presence of hydroxyl groups, a hydrogen-bonded carbonyl group conjugated with benzene ring. The ^{1}H- and ^{13}C-NMR spectral data including spin-decoupling ^{1}H-NMR, ^{1}H–^{1}H COSY, ^{13}C–^{1}H COSY, and HMBC NMR spectra of **2** suggested that **2**, which gave the molecular ion at *m/z* 222 corresponding to $[C_{12}H_{14}O_4]^{\cdot+}$ in the EI-MS spectrum and the (+) optical rotation, might be identical with sordarial, a hexaketide from the Ascomycete *Sordaria macrospora*,[3)] including its stereochemistry (see Table 2, Chart 2, Experimental). Although the structure of sordarial was presented as 2-[(*E*)-*erythro*-3,4-di-

Table 2. ^{1}H-NMR and ^{13}C-NMR Data for Compound **2**, and ^{1}H-NMR Data for Sordarial, δ (ppm) from TMS as an Internal Standard [Coupling Constants (Hz) in Parentheses]

Position	**2**				Sordarial[3] ^{1}H-NMR (in CD_3OD)
	^{1}H-NMR (in CD_3OD)	^{13}C-NMR	^{1}H-NMR (in $CDCl_3$)	^{13}C-NMR	
1		118.8 (s)		117.33 (s)	
2		144.1 (s)		142.1 (s)	
3	7.03 (d, 7.8)	119.9 (d)	6.94 (d, 7.8)	118.9 (d)	7.04 (d, 7.7)
4	7.47 (t, 7.8)	138.1 (d)	7.46 (dd, 8.2, 7.8)	137.2 (d)	7.50 (dd, 8.2, 7.7)
5	6.83 (d, 7.8)	117.5 (d)	6.90 (d, 8.2)	117.30 (d)	6.84 (br d, 8.2)
6		163.7 (s)		162.8 (s)	
6-OH			11.86 (s)		
7	10.39 (s)	197.2 (d)	10.34 (s)	195.2 (d)	10.40 (s)
1′	7.25 (d, 15.6)	127.5 (d)	7.20 (d, 15.6)	126.9 (d)	7.26 (dd, 15.8)
2′	6.25 (dd, 15.6, 6.1)	137.7 (d)	6.17 (dd, 15.6, 6.1)	134.8 (d)	6.26 (dd, 15.8, 6.2)
3′	4.11 (dd, 6.1, 5.4)	77.5 (d)	4.34 (m)	75.8 (d)	4.10 (ddd, 6.2, 5.2, 1.5)
4′	3.77 (qd, 6.1, 5.4)	71.6 (d)	4.01 (m)	70.2 (d)	3.76 (qd, 6.4, 5.2)
5′	1.22 (3H, d, 6.1)	19.1 (q)	1.22 (3H, d, 6.4)	17.7 (q)	1.22 (3H, d, 6.4)

Table 3. ^{1}H-NMR Data for Compounds **8**—**11**, δ (ppm) from TMS as an Internal Standard in $CDCl_3$ [Coupling Constants (Hz) in Parentheses]

Position	**8**	**9**	**10**	**11**
3	6.92 (d, 8.3)	6.91 (d, 8.2)	6.94 (d, 8.3)	6.91 (d, 8.2)
4	7.45 (dd, 8.3, 7.7)	7.44 (dd, 8.2, 7.8)	7.47 (t, 8.3, 7.7)	7.45 (dd, 8.2, 7.8)
5	6.86 (d, 7.7)	6.82 (d, 7.8)	6.91 (d, 7.7)	6.86 (d, 7.8)
6-OH	11.86 (s)	11.86 (s)	11.86 (s)	11.86 (s)
7	10.08 (s)	10.21 (s)	10.20 (s)	10.24 (s)
1′	7.09 (d, 15.6)	7.13 (d, 15.6)	7.25 (d, 15.9)	7.20 (d, 15.7)
2′	6.05 (dd, 15.6, 5.9)	5.98 (dd, 15.6, 5.9)	6.15 (dd, 15.9, 7.5)	6.08 (dd, 15.7, 6.1)
3′	5.61 (m)	4.44 (br s)	5.59 (m)	4.49 (br s)
4′	4.13 (m)	5.31 (m)	4.07 (m)	5.31 (m)
5′	1.25 (3H, d, 6.3)	1.40 (3H, d, 6.6)	1.20 (3H, d, 6.4)	1.33 (3H, d, 6.6)

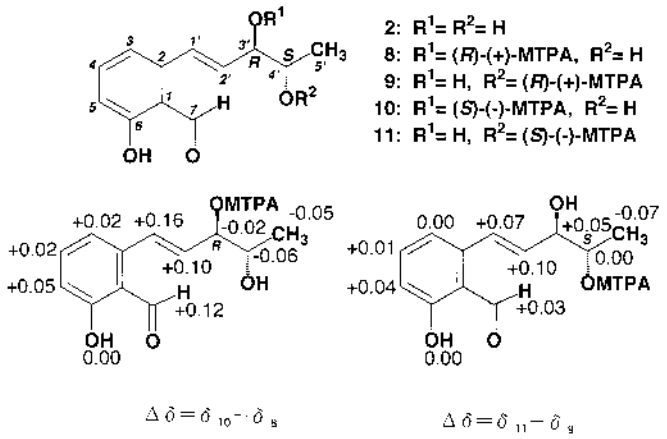

Chart 2

hydroxy-1-pentenyl]-6-hydroxybenzaldehyde by Bouillant *et al*,[3] the absolute configurations at positions 3′ and 4′ have not yet been decided between (3′*R*,4′*S*) or (3′*S*,4′*R*). To determine the absolute configurations at positions 3′ and 4′ in sordarial, a modification of Mosher's method[4] was applied to **2**, and the two (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetates of **2** [(*R*)-MTPA esters **8** and **9**] and the two (*S*)-(−)-MTPA esters of **2** [(*S*)-MTPA esters **10** and **11**] were prepared from **2**. Comparison of the ^{1}H-NMR spectral data of **8** and **9** with those of **2** showed that the signals of H-3′ and -4′ were shifted to δ 5.61 (+1.27) and 4.13 (+0.12) in the spectrum of **8**, and shifted to δ 4.44 (+0.10) and 5.31 (+1.30) in that of **9**, indicating that **8** and **9** were the 3′-(*R*)- and 4′-(*R*)-MTPA esters of **2**, respectively. Comparison of the ^{1}H-NMR spectral data of **10** and **11** with those of **2** showed that the signals of H-3′ and -4′ were shifted to δ 5.59 (+1.25) and 4.07 (+0.06) in the spectrum of **10**, and shifted to δ 4.49 (+0.15) and 5.31 (+1.30) in that of **11**, indicating that **10** and **11** were the 3′-(*S*)- and 4′-(*S*)-MTPA esters of **2**, respectively (see Table 3). The $\Delta\delta$ values ($\delta_{(S)\text{-MTPA}} - \delta_{(R)\text{-MTPA}}$) between **8** and **10**, and those between **9** and **11** were calculated as in Chart 2, indicating that the absolute configurations at positions 3′ and 4′ in **2** were (*R*) and (*S*), respectively. Accordingly, the structure of sordarial was determined as 2-[(*E*)-(3*R*,4*S*)-3,4-dihydroxy-1-pentenyl]-6-hydroxybenzaldehyde (**2**), as shown in Chart 2. To our knowledge, this is the first time that sordarial (**2**) was isolated as an immunosuppressive factor from a natural source.

Six immunosuppressive 2-pyrones called multiforisins A (**5**)—F had previously been isolated from the AcOEt layer, which was obtained from the partition between AcOEt and H_2O of the AcOEt extract of *Gelasinospora multiforis* IFM4498.[1a,b] This time, further search for 2-pyrone components was made from the AcOEt layer obtained from this newly cultivated fungus. The result was that three new 2-pyrones, which were identical with **1**, **3**, and **4** from *G. heterospora*, were isolated together with **5** from *G. multiforis* [yields (%) from the AcOEt layer, **1**: 0.25, **3**: 0.20, **4**: 1.13, and **5**: 0.05]. We propose to call these **1**, **3**, and **4** multi-

Table 4. Suppressive Effects of Compounds **1**—**7**, and Azathioprine, Cyclosporin A, and FK506 on Con A- and LPS-Induced Proliferations of Mouse Splenic Lymphocytes [IC_{50} (μg/ml)]

Compound	Con A-induced	LPS-induced	Compound	Con A-induced	LPS-induced
1	1.8	0.9	**2**	6.5	5.1
3	0.9	1.2	Azathioprine	2.7	2.7
4	>25	19	Cyclosporin A	0.04	0.07
5[1a,b)]	0.6	0.6	FK506 (tacrolimus)	1.5×10^{-5}	1.6×10^{-3}
6[1a,b)]	24	22			
7	4.7	4.5			

The IC_{50} value of each sample was calculated from the correlation curve between the sample concentration (horizontal axis) and the cell proliferation (vertical axis). The curve of each sample was drawn with 6 points, each of which represented the mean of 3 experiments on each correlation between 6 different concentrations and cell proliferations.

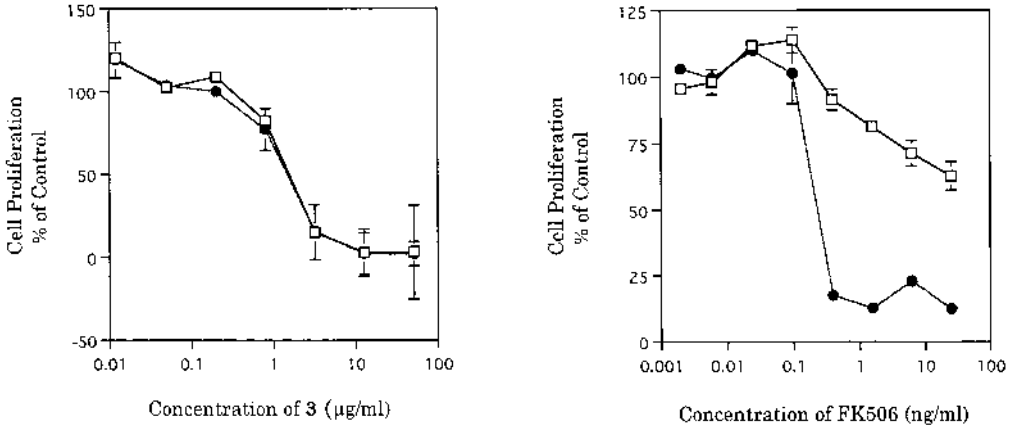

Fig. 1. Effects of Multiforisin G (**3**) and FK506 (a Positive Control) on the Proliferation of Mouse Splenic Lymphocytes Stimulated with Ionomycin and PMA in Cultivation with and without Exogenous IL-2

—□—, proliferation with exogenous IL-2; —●—, proliferation without exogenous IL-2. Each point represents the mean±S.E. of 3 experiments.

forisins H, G, and I, respectively. Differing from the previously isolated multiforisins A (**5**)—F,[1a,b)] these newly isolated multiforisins G (**3**), H (**1**), and I (**4**) have a hydroxymethyl group at position 5 (see Chart 1).

Recently, it has been found that the defatted AcOEt extract of an Ascomycete *Gelasinospora longispora* IFM4617[2)] also suppressed the Con A-induced proliferation of mouse splenic lymphocytes by 85 and 47% at 50 and 10 μg/ml, respectively. The defatted extract was partitioned between AcOEt and H_2O to afford an AcOEt layer and an aqueous layer. The AcOEt layer was repeatedly chromatographed to also give **1**—**5** [yields (%) from the AcOEt layer, **1**: 1.46, **2**: 1.09, **3**: 1.30, **4**: 14.7, and **5**: 0.26].

The immunosuppressive activities (IC_{50} values) of **1**—**4**, and **7** were calculated against Con A- (T-cells) and LPS-induced proliferations of mouse splenic lymphocytes (B-cells) as shown in Table 4; those of **5**, **6**[1a,b)] and three known immunosuppressants as positive controls, azathioprine, cyclosporin A, and FK506 (tacrolimus)[5)] are also shown. These data indicated that multiforisins such as **1**, **3**, and **5** in which one of the two substituents at positions 3 and 5 is a hydroxymethyl group and the other is a formyl or an acetoxymethyl group show a high immunosuppressive activity, and **7** with the acetoxymethyl groups at positions 3 and 5 slightly lowers the activity. But **4**, in which the hydroxymethyl groups are present at positions 3 and 5 or **6** which has an acetoxymethyl group at position 3 and a formyl group at position 5, largely lowers the activity. The IC_{50} values of, **1**, **3**, **4**, **5**, and **7** were found to be 5, 5, >50, 1, and 5 μg/ml against human leukemic HL-60 cells respectively and that of **5** was found to be 10 μg/ml against human KB cells.[1a)] Thus, **1** and **3** display immunosuppressive activity at a slightly lower concentration than that at which **1** and **3** show cytotoxicity against HL-60 cells; **5** displays immunosuppressive activity at a little lower and considerably lower concentrations than that at which **5** shows cytotoxicity against HL-60 and KB cells, respectively; and **7** displays immunosuppressive activity at a similar concentration to that at which **7** shows cytotoxicity against HL-60 cells. Generally speaking, the multiforisins display immunosuppressive activity at a slightly lower concentration than that at which they show cytotoxicity. Therefore, it also seems necessary to study the immunosuppressive activity of the multiforisins from the aspect of cytotoxicity.

The effect of exogenous interleukin 2 (IL-2) on the immunosuppressive activity of **3** was investigated using the method by Dumont *et al*.[6)] Compound **3** suppressed the proliferation of mouse splenic lymphocytes (T-cells) stimulated with ionomycin and phorbol 12-myristate 13-acetate (PMA) on cultivations both with and without exogenous IL-2. On the other hand, the positive control FK506,[5)] which is known to inhibit IL-2 production and IL-2 receptor expression in splenic T-cells,[5c,6)] strongly suppressed the proliferation of T-cells in the cultivation without exogenous IL-2, but only slightly suppressed it in the cultivation with exogenous IL-2, as shown in Fig. 1. Therefore, the immunosuppressive activity of **3** was not due to inhibition of IL-2 production.

Experimental

The general procedures for the chemical experiments and other experimental conditions, including those for evaluation of suppressive activity (IC_{50} values) of samples against proliferation of mouse splenic lymphocytes stimulated with Con A and LPS, were the same as described in our previous report (this method is based on the incorporation ratio of exogenous [^{3}H]-thymidine into lymphocytes).[7] Chemical shifts were expressed in δ (ppm) values from tetramethylsilane (TMS) as an internal standard.

Isolation of Compounds 1—4 from *G. heterospora* *G. heterospora* 74-T-542-1[2] was cultivated on sterilized rice (200 g/flask×150) at 25 °C for 24 d. The dark gray or black colored moldy rice was extracted with AcOEt (40.0 l) with shaking at room temperature for 6 h two times to give an AcOEt solution (80.0 l), which gave, after evaporation *in vacuo*, an AcOEt extract (170 g). A portion of the extract (50 g) was partitioned with *n*-hexane–H_2O (1 : 1, v/v) (2.0 l) into *n*-hexane layer (after evaporation *in vacuo*, 15.5 g) and an aqueous suspension, which was then partitioned with AcOEt (1.0 l) into AcOEt layer (15.0 g) and aqueous layer (3.6 g). The *n*-hexane, AcOEt, and aqueous layers suppressed the Con A-induced proliferation of mouse splenic lymphocytes by 39, 100, and 59% at 50 μg/ml, and by 4, 80, and 16% at 10 μg/ml, respectively. A portion of the AcOEt layer (4.0 g) was subjected to chromatography on a silica gel column with *n*-hexane–AcOEt–MeOH (50 : 50 : 0), (25 : 25 : 1), (4.5 : 4.5 : 1), (0.5 : 0.5 : 1), and MeOH to give five fractions I (0.62 g), II (0.87 g), III (1.13 g), IV (0.65 g), and V (0.07 g), respectively. Fraction II, which inhibited the Con A-induced proliferation of the lymphocytes by 50% at 6.9 μg/ml, was separated into a $CHCl_3$-soluble portion (6.72 g) and a $CHCl_3$-insoluble portion which showed no immunosuppressive activity (0.18 g). The $CHCl_3$-soluble portion was chromatographed on a silica gel column with *n*-hexane–AcOEt (1 : 3), on an octadecyl silica gel (ODS) column with H_2O–MeOH (1 : 1), and then on an ODS HPLC column with H_2O–CH_3CN (5 : 1) at a flow rate of 8.0 ml/min to afford **1** (9.3 mg), **3** (7.5 mg), and **2** (5.1 mg). Fraction III, which inhibited the Con A-induced proliferation of the lymphocytes by 50% at 7.4 μg/ml, was further chromatographed on a silica gel column with *n*-hexane–AcOEt, on an HPLC ODS column with H_2O–CH_3CN (7 : 3), and on an HPLC ODS column with H_2O–MeOH (3 : 2) at 8.0 ml/min to afford **4** (2.3 mg).

Isolation of Compounds 1, 3, 4 and 5 from *G. multiforis* *G. multiforis* IFM4498[2] was cultivated on sterilized rice (200 g/flask×100) at 25 °C for 28 d. The dark violet-brown colored moldy rice was extracted with AcOEt (30.0 l) two times in the manner described for the extraction of *G. heterospora* to give an AcOEt extract (200 g), which was then partitioned with AcOEt–H_2O (1 : 1) (1.0 l) into AcOEt layer (after evaporation *in vacuo*, 140 g) and aqueous layer (57 g). A portion of the AcOEt layer (10 g) was subjected to chromatography on a silica gel column with *n*-hexane–AcOEt (2 : 1), (1 : 1), and acetone to give three fractions I (0.53 g), II (2.45 g), and III (6.41 g), respectively. Fraction II was further chromatographed on a silica gel column with *n*-hexane–AcOEt (1 : 1), on an ODS column with H_2O–MeOH (2 : 3), and on a silica gel column with *n*-hexane–AcOEt (1 : 2) and (1 : 3) to give **3** (20 mg), **1** (25 mg), and **5** (5 mg). Fraction III was further chromatographed on a silica gel column with *n*-hexane–AcOEt followed by recrystallization from acetone to afford **4** (113 mg).

Isolation of Compounds 1—5 from *G. longispora* *G. longispora* IFM4617[2] was cultivated on sterilized rice (150 g/flask×6) at 25 °C for 17 d. The brownish light gray colored moldy rice studded with large black perithecia of this fungus was extracted with AcOEt (1.1 l) two times in the way described for the extraction of *G. heterospora* to give an AcOEt extract (4.32 g). The AcOEt extract was partitioned with *n*-hexane–H_2O (1 : 1) (60 ml) into *n*-hexane layer (after evaporation *in vacuo*, 1.34 g) and an aqueous suspension (after evaporation *in vacuo*, defatted AcOEt extract). The defatted AcOEt extract was then partitioned with AcOEt–H_2O (1 : 1) (60 ml) into AcOEt layer (1.92 g) and aqueous layer (0.93 g). The AcOEt layer was subjected to chromatography on a silica gel column with *n*-hexane–AcOEt (2 : 1), (1 : 1), (1 : 2), (1 : 5), AcOEt, and MeOH to give five fractions I (95 mg), II (136 mg), III (315 mg), IV (801 mg), and V (142 mg), respectively. Fraction III was further chromatographed on a silica gel column with $CHCl_3$–MeOH to give four fractions IIIa—d. Fraction IIIb (183 mg) which was eluted with $CHCl_3$–MeOH (50 : 1) and (20 : 1) was chromatographed on an HPLC ODS column with H_2O–CH_3CN (4 : 1) at 8.0 ml/min to give **3** (25 mg), **1** (28 mg), and **5** (5 mg). Fraction IIIc (92 mg) which was eluted with $CHCl_3$–MeOH (20 : 1) was chromatographed on an HPLC ODS column with H_2O–CH_3CN (4 : 1) to give **2** (21 mg). Fraction IV was recrystallized from acetone to give **4** (283 mg).

Compound **1** (Multiforisin H): Colorless needles from aqueous CH_3CN, mp 118—120 °C. Electron impact-MS (EI-MS) *m/z* (%): 268 (11, M^+), 223 (7), 179 (3), 167 (11), 149 (100). high-resolution EI-MS (HR-EI-MS) *m/z*: 268.0974 ($C_{13}H_{16}O_6$ requires 268.0947). IR ν_{max}^{KBr} cm^{-1}: 3390 (O–H), 1736, 1674 (C=O), 1610, 1543 (C=C), 1387, 1236 (C–O). UV λ_{max}^{MeOH} nm (log ε): 227 (4.48), 321 (3.99).

Compound **2** (Sordarial): Pale yellow needles from aqueous CH_3CN, mp 90.5—92.0 °C (lit.[3] yellow powder). $[\alpha]_D^{20}$ +22° (c=0.37, MeOH) [lit.[3] +18.2° (c=2.03, MeOH)]. EI-MS *m/z* (%): 222 (9, M^+), 204 (4), 178 (36), 160 (73), 147 (100). IR ν_{max}^{KBr} cm^{-1}: 3401 (O–H), 1637 (CHO), 1560, 1450 (C=C), 1313, 1240 (C–O). UV λ_{max}^{MeOH} nm (log ε): 226 (4.21), 272 (3.84), 343 (3.55) [lit.[3] 230 (3.66), 277 (3.80), 351 (3.49)].

Compound **3** (Multiforisin G): Colorless needles from aqueous CH_3CN, mp 125—128 °C. EI-MS *m/z* (%): 268 (14, M^+), 225 (77), 181 (14), 165 (6), 149 (17), 69 (100). HR-EI-MS *m/z*: 268.0956 ($C_{13}H_{16}O_6$ requires 268.0946). IR ν_{max}^{KBr} cm^{-1}: 3465 (O–H), 1714, 1700 (C=O), 1651, 1558 (C=C), 1369, 1265 (C–O). UV λ_{max}^{MeOH} nm (log ε): 226 (4.40), 325 (3.98). This compound was identical with O^{11},11-dihydromultiforisin B derived from **6** in terms of the ^{1}H- and ^{13}C-NMR spectra ($CDCl_3$), and the TLC behavior on a silica gel plate with *n*-hexane–AcOEt (1 : 4).

Compound **4** (Multiforisin I): Colorless needles from acetone, mp 154.5—156 °C. EI-MS *m/z* (%): 226 (65, M^+), 209 (32), 193 (28), 181 (21), 69 (100). HR-EI-MS *m/z*: 226.0838 ($C_{11}H_{14}O_5$ requires 226.0841). IR ν_{max}^{KBr} cm^{-1}: 3309 (O–H), 1711 (C=O), 1649, 1608, 1539 (C=C), 1385, 1254 (C–O). UV λ_{max}^{MeOH} nm (log ε): 229 (3.71), 327(3.25).

Compound **5** (Multiforisin A): Pale yellow needles from *n*-hexane–AcOEt, mp 81.5—82.5 °C. This compound was identical with authentic multiforisin A (**5**)[1a,b] in terms of the ^{1}H- and ^{13}C-NMR spectra ($CDCl_3$) and the TLC behavior on a silica gel plate with *n*-hexane–AcOEt (1 : 4).

Formation of O^{11},11-Dihydromultiforisin B A solution of **6**[1a,b] (27 mg) in EtOH (100 μl) was added to a solution of $NaBH_4$ (2 mg) in EtOH (200 μl) to prepare a reaction mixture, which was stirred at room temperature for 1 min. After dilution with water, the reaction mixture was extracted with AcOEt. After treatment as usual, the AcOEt solution was evaporated *in vacuo* to give a residue (13 mg), which was subjected to preparative TLC on a silica gel plate with *n*-hexane–AcOEt (1 : 3) to afford O^{11},11-dihydromultiforisin B, colorless needles from aqueous CH_3CN. Mp 125—128 °C. EI-MS *m/z* (%): 268 (14, M^+), 225 (77). ^{1}H-NMR and ^{13}C-NMR ($CDCl_3$): 4.52 (2H, s), 54.9 (1C, t).

Preparation of 3,5-Bisacetoxymethyl-4-methoxy-6-((*E*)-1-propenyl)-2-pyrone (7) Ac_2O (120 μl) was added to a solution of **1** (3.9 mg) in pyridine (120 μl), and the reaction mixture was allowed to stand at room temperature for 1 h, and treated as usual to give **7** (4.5 mg), a pale yellow oil, ^{1}H-NMR ($CDCl_3$): 2.09 (6H, s), 4.97 (2H, s), 5.05 (2H, s). Compound **7** was also obtained from acetylation of a mixture of **1** (5.0 mg) and **3** (5.0 mg) with Ac_2O (300 μl) and pyridine (300 μl) at room temperature for 1 h.

3′-(*R*)- and 4′-(*R*)-MTPA Esters of 2 (8, 9) Compound **2** (5.3 mg), (*R*)-MTPA acid (14.1 mg), and dicyclohexylcarbodiimide (DCC) (15.5 mg) were dissolved in a mixture of pyridine (25 μl) and CH_2Cl_2 (1.0 ml) to prepare a solution under ice-cooling. The solution was allowed to stand under ice-cooling for 1 h and at room temperature for 1.7 h. The reaction mixture was purified by chromatography on a silica gel column with *n*-hexane, *n*-hexane–AcOEt (3 : 1), (2 : 1), (1 : 1), and (1 : 2), and on a preparative TLC silica gel plate with *n*-hexane–AcOEt (3 : 2) to give 4′-(*R*)-MTPA ester of **2** (**9**) (2.2 mg), pale yellow oil, and 3′-(*R*)-MTPA ester of **2** (**8**) (2.0 mg), pale yellow oil.

3′-(*S*)- and 4′-(*S*)-MTPA Esters of 2 (10, 11) Compound **2** (5.2 mg), (*S*)-MTPA acid (15.3 mg), and DCC (15.0 mg) were dissolved in a mixture of pyridine (25 μl) and CH_2Cl_2 (1.0 ml) to prepare a solution under ice-cooling. The solution was allowed to stand under ice-cooling for 1 h and at room temperature for 1.7 h. The reaction mixture was treated in the same way as described for the preparation of **8** and **9** to give 4′-(*S*)-MTPA ester of **2** (**11**) (3.3 mg), pale yellow oil, and 3′-(*S*)-MTPA ester of **2** (**10**) (2.1 mg), pale yellow oil.

Evaluation of Effects of Samples on Addition of Exogenous IL-2 To 87.0 ml of RPMI-1640 medium (Gibco), 10.0 ml of fetal bovine serum (FBS) (Gibco), 2.0 ml of penicillin–streptomycin (5000 IU/ml–5000 μg/ml) (Flow Laboratories), and 1.0 ml of 3.0% glutamine (Nissui)/RPMI-1640 medium were added to prepare an FBS/RPMI-medium. A suspension of the splenic lymphocytes from three male BALB/c mice (7—11 weeks old, Nippon SLC) was prepared with the FBS/RPMI medium at a concentration of 4.0×10^6 cells/ml in a manner similar to that in our previous report.[7] PMA (Wako) (1.0 mg) was dissolved in dimethyl sulfoxide (DMSO) to prepare a solution at 10 μg/ml, which was further diluted with the FBS/RPMI-medium to prepare a PMA solution at 100 ng/ml. Ionomycin (free acid) (Calbiochem) (1.0 mg) was dissolved in DMSO to prepare a solution at

167 μg/ml, which was further diluted with the FBS/RPMI-medium to prepare an ionomycin solution at 1.67 μg/ml. Recombinant human IL-2 (genzyme) [5.0 mg (20000 units)] was dissolved in 10 mM acetic acid to prepare a solution at 1.0 mg/ml (4000 units/ml), which was further diluted with the FBS/RPMI-medium to prepare an IL-2 solution at 100 μg/ml (400 units/ml). A sample was dissolved in 1.0% EtOH in the FBS/RPMI-medium to prepare a sample solution, 50 μl of which was incubated with 50 μl of the cell suspension, 20 μl of the PMA solution, 30 μl of the ionomycin solution, and 50 μl of the IL-2 solution in a U-bottom 96-well microtiter plate (Inter Med, Nunclon) at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 48 h. Then, 20 μl of the MTT/PBS(−) solution, which was prepared in advance by dissolution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Sigma) in phosphate buffered saline without $Ca^{2+}Mg^{2+}$ [PBS(−)] (Nacalai tesque) at 5.0 mg/ml, was added to the culture, and the culture was further incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 4 h. Then, the culture was centrifuged at 1000 rpm for 5 min at room temperature. After removal of the supernatant by an aspirator, 100 μl of DMSO was added to the precipitated cells to extract formazan, and the DMSO solution was vibrated to complete extraction with a microplate mixer (Iwaki, model MPX-96) for a few min. The absorbance of each DMSO solution was measured at 570 (a test wavelength) and 630 nm (a reference wavelength) with a microplate reader (Bio Rad, model 550).[8] The effect of each sample on presence and absence of the exogenous IL-2 was evaluated in triplicate, and expressed as a percentage of the absorbance of formazan, which was proportional to the cell proliferation,[8] formed from MTT in the incubation with the sample and that with FK506 (positive control) to that without the sample (control).[6]

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References and Notes

1) *a*) Fujimoto H., Satoh Y., Nakayama M., Takayama T., Yamazaki M., *Chem. Pharm. Bull*., **43**, 547—552 (1995); *b*) *Idem*, The 37th Symposium on the Chemistry of Natural Products Symposium Papers, Tokushima,1995, pp. 625—630; *c*) Fujimoto H., Nagano J., Yamaguchi K., Yamazaki M., *Chem. Pharm. Bull*., **46**, 423—429 (1998).
2) These strains, *G. heterospora* 74-T-542-1, *G. multiforis* IFM4498, and *G. longispora* IFM4617, were deposited earlier at Research Institute for Chemobiodynamics, Chiba University (present name: Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University). Now, these voucher specimens are also on deposit in our laboratory.
3) Bouillant M.L., Bernillon J., Favre-Bonvin J., Salin N., *Z. Naturforsch*., **44c**, 719—723 (1989).
4) Kusumi T., *Yuki Gosei Kagaku Kyokai Shi*., **51**, 462—470 (1993).
5) *a*) Tanaka H., Kuroda A., Marusawa H., Hatanaka H., Kino T., Goto T., Hashimoto M., *J. Am. Chem. Soc*., **109**, 5031—5033 (1987); *b*) Kino T., Hatanaka H., Hashimoto M., Nishiyama M., Goto T., Okuhara M., Kohsaka M., Aoki H., Imanaka H., *J. Antibiotics*, **40**, 1249—1255 (1987); *c*) Kino T., Hatanaka H., Miyata S., Inamura N., Nishiyama M., Yajima T., Goto T., Okuhara M., Kohsaka M., Aoki H., Ochiai T., *ibid*., **40**, 1256—1265 (1987).
6) Dumont F. J., Staruch M. J., Koprak S. L., Melino M. R., Sigal N. H., *J. Immunology*, **144**, 251—258 (1990).
7) Fujimoto H., Nakayama Y., Yamazaki M., *Chem. Pharm. Bull*., **41**, 654—658 (1993).
8) Mosmann T., *J. Immunological Methods*, **65**, 55—63 (1983).