Immunomodulatory Constituents from an Ascomycete, *Microascus tardifaciens*

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Fractionation guided by the immunosuppressive activity of the defatted AcOEt extract of an Ascomycete, *Microascus tardifaciens*, afforded eight constituents, questin (emodin 8-*O*-methylether) (1), rubrocristin (2), 5,7-dihydroxy-4-methylphthalide (3), cladosporin (asperentin) (4), cladosporin 8-*O*-methylether (5), tardioxopiperazine A [*cyclo*-L-alanyl-5-isopentenyl-2-(1′,1′-dimethylallyl)-L-tryptophan] (6), tardioxopiperazine B [*cyclo*-L-alanyl-7-isopentenyl-2-(1′,1′-dimethylallyl)-L-tryptophan] (7), and asperflavin (8), among which 6 and 7 were new compounds. Compounds 1 and 2 showed considerably high immunosuppressive activity, 6 was moderate and, 3, 4, 5, 7 and 8 showed low activity.

Key words fungal metabolite; Ascomycete; *Microascus tardifaciens*; immunosuppressive activity; rubrocristin; tardioxopiperazine

Recently, several immunosuppressive 2-pyrones, multiforisins A—I from *Gelasinospora multiforis*,[1a,b] *G. heterospora*,[1b] and *G. longispora*,[1b] and macrophin from *Diplogelasinospora grovesii*,[1c] a macrocyclic sesterterpene, kobiin, and three 2-furanones, kobifuranones A—C from *G. kobi*,[1d] and a hexaketide, sordarial, from *G. heterospora*[1b] and *G. longispora*,[1b] have been isolated in our screening program on immunomodulatory components from fungi. Successively, it was found that the defatted AcOEt extract of an Ascomycete, *Microascus tardifaciens*, appreciably suppressed the proliferation (blastogenesis) of mouse splenic lymphocytes stimulated with mitogens, concanavalin A (Con A) and lipopolysaccharide (LPS). A solvent partition followed by repeated chromatographic fractionation of the extract guided by immunosuppressive activity afforded eight constituents, tentatively named MT-1 (**1**)—-8 (**8**). This report deals with the structures and immunosuppressive activities of these constituents recently isolated from *M. tardifaciens*.

Results and Discussion

The AcOEt extract of *M. tardifaciens* IFM4564[2] cultivated on sterilized rice was partitioned between *n*-hexane and water. The aqueous suspension (a defatted AcOEt extract) was further partitioned between AcOEt and water. After evaporation, *n*-hexane, AcOEt, and aqueous layers suppressed the Con A-induced proliferation of mouse splenic lymphocytes by 41, 99, and 74% at 50 μg/ml, and by 4, 64, and 11% at 10 μg/ml, respectively. Repeated chromatographic fractionation of the AcOEt layer guided by the immunosuppressive activity afforded eight constituents, MT-1—8 (**1**—**8**) [yields (%) from the AcOEt layer, **1**: 0.086, **2**: 0.029, **3**: 0.19, **4**: 0.77, **5**: 0.37, **6**: 0.13, **7**: 0.13, and **8**: 0.23]. Among them **1** and **2** showed considerably high immunosuppressive activity, **6** showed moderate activity, and **3**, **4**, **5**, **7**, and **8** were low.

MT-1 (**1**), yellow fine needles, was positive to the $FeCl_3$ reaction. The ^{1}H- and ^{13}C-NMR spectral data [in dimethyl sulfoxide-d_6 (DMSO-d_6)], including spin-decoupling ^{1}H-NMR, two dimensional ^{1}H–^{1}H shift correlation spectroscopy (COSY), and ^{1}H-detected heteronuclear correlation through multiple quantum coherence (HMQC) NMR data, suggested the presence of a methyl, a methoxyl, two pairs of *meta*-coupled aromatic protons, a hydrogen-bonded hydroxyl, four aromatic quaternary carbons, three aromatic quaternary carbons bearing oxygen, and two ketone carbonyls in **1** (Table 1). These structural units were connected by the aid of ^{1}H-detected heteronuclear multiple-bond correlation (HMBC) NMR data to construct a whole molecular structure (**1**), which was equal to the structure of questin [emodin (**9**) 8-*O*-methylether] isolated from *Penicillium frequentans*[3] and many other fungi.[4] MT-1 was identified with the authentic sample of questin (**1**) by direct comparison (Chart 1).

MT-2 (**2**), a fine red powder, $C_{16}H_{12}O_6$, was positive to the $FeCl_3$ reaction. The ^{1}H-NMR spectrum of **2** was quite similar to that of **1** except for the additional appearance of a signal due to a hydrogen-bonded OH at δ 13.07 (s) instead of the disappearance of that due to H-4, as well as a change of that due to H-2 from δ 7.13 (d) to δ 7.26 (s), indicating that MT-2 might be 4-hydroxyquestin (**2**). This was also supported by the ^{13}C-NMR spectral data (Table 1). 4-Hydroxyquestin, namely, 1,4,6-trihydroxy-8-methoxy-3-methylanthraquinone, has previously been isolated from *Aspergillus cristatus* and named rubrocristin.[5] The UV and electron impact (EI)-MS spectral data of MT-2 were similar to those of rubrocristin (**2**) and the ^{1}H-NMR spectral data of the 6-acetyl derivative of MT-2 was quite similar to that of rubrocristin-6-acetate (**10**) described in the literature (Table 1),[5] indicating that MT-2 is identical with **2** (Chart 1).

MT-4 (**4**), optically active pale yellow prisms, was positive to the $FeCl_3$ reaction. The ^{1}H- and ^{13}C-NMR spectral data (in DMSO-d_6) suggested the presence of a methyl, five aliphatic methylenes, three aliphatic methines bearing oxygen, two aromatic protons *meta*-coupled with each other, four aromatic quaternary carbons among which two bore oxygen, one ester carbonyl, and a hydrogen-bonded hydroxyl in **4** (Table 2). These structural units were connected on the basis of HMBC NMR data to build up a whole molecular structure (**4**) (without stereochemistry), which was equal to the structure of an antifungal metabolite, cladosporin (asperentin) isolated from *Cladosporium cladosporioides*[6a] and *Aspergillus flavus*.[6b] Comparison of the ^{1}H- and ^{13}C-NMR data, melting point, specific rotation ($[\alpha]_D$), EI-MS, UV, and circular dichroism (CD) spectral data of MT-4 with those of cladosporin described in the literature[6a,b] showed that MT-4 is

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Table 1. ^{1}H-NMR and ^{13}C-NMR Data for MT-1 (**1**) and MT-2 (**2**), and ^{1}H-NMR Data for 6-Acetyl MT-2 (**10**) and Rubrocristin-6-acetate[5)]

Position	**1** in DMSO-d_6		**2** in DMSO-d_6		**10** in $CDCl_3$	Rubrocristin-6-acetate[5)] in $CDCl_3$
	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{1}H-NMR
1		161.59 (s)		155.97 (s)		
HO-1	13.24 (s)		13.55 (s)		13.32 (s)[b)]	13.32 (s)[c)]
2	7.13 (d, 1.7)	124.06 (d)	7.26 (s)	129.35 (d)	7.17 (br s)	7.15 (m)
3		146.50 (s)		137.50 (s)		
CH_3-3	2.39 (3H, s)	21.26 (q)	2.25 (3H, s)	15.62 (q)	2.35 (3H, d, 0.9)	2.35 (3H, d, 1.0)
4	7.43 (d, 1.7)	118.99 (d)		155.41 (s)		
HO-4			13.07 (s)		13.22 (s)[b)]	13.22 (s)[c)]
4a		131.92 (s)[a)]		111.59 (s)		
5	7.21 (d, 2.3)	106.87 (d)	7.29 (d, 2.4)	106.57 (d)	7.78 (d, 2.3)	7.78 (d, 2.5)
6		164.40 (s)		164.46 (s)		
AcO-6					2.39 (3H, s)	2.39 (3H, s)
7	6.85 (d, 2.3)	104.87 (d)	6.86 (d, 2.4)	105.38 (d)	7.14 (d, 2.3)	7.15 (m)
8		163.34 (s)		163.48 (s)		
H_3CO-8	3.91 (3H, s)	56.21 (q)	3.90 (3H, s)	56.30 (q)	4.06 (3H, s)	4.07 (3H, s)
8a		112.50 (s)		112.85 (s)		
9		186.20 (s)		184.79 (s)		
9a		114.27 (s)		111.18 (s)		
10		182.20 (s)		186.92 (s)		
10a		136.69 (s)[a)]		136.49 (s)		

δ (ppm) from tetramethylsilane (TMS) as an internal standard [coupling constants (Hz) in parentheses]. *a*, *b*, *c*) may be interchangeable.

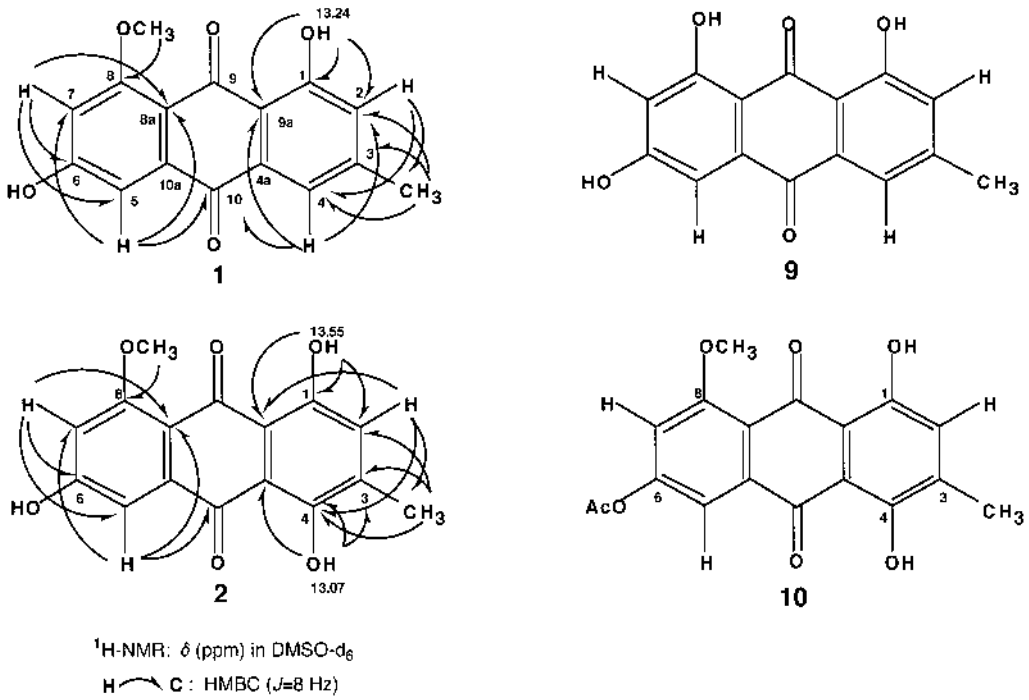

Chart 1

identical with cladosporin (**4**), including stereochemistry (Chart 2). The absolute configurations at positions 3, 10, and 14 in **4** were already determined to be (*R*), (*R*), and (*S*), respectively.[6c)]

MT-5 (**5**), an optically active pale yellow powder, was negative to the $FeCl_3$ reaction. The ^{1}H- and ^{13}C-NMR spectra of **5** were quite similar to those of **4**, except for the additional appearance of signals due to a methoxyl [δ_H 3.74 (3H, s), δ_C 55.54 (q)], instead of the disappearance of that due to a hydrogen-bonded OH-8 (Table 2), suggesting that **5** might be 8-*O*-methylether of **4**. On treatment with trimethylsilyldiazomethane, **4** afforded dimethylether (**11**), which was also obtained from **5** with the same treatment. Accordingly, MT-5 was deduced to be identical with cladosporin (asperentin) 8-*O*-methylether (**5**), which was already isolated from *Aspergillus flavus*.[6b)] It has been found that the signals of H-3 and C-3 in the ^{1}H- and ^{13}C-NMR spectra are present at δ_H 4.61 and δ_C 76.18, and the signs of $[\alpha]_D$ and $\Delta\varepsilon$ at *ca.* 300 nm in the CD spectrum are negative in the case of **4**, while the corresponding signals are present at δ_H 4.35 and δ_C 73.82 and the corresponding signs are positive in the case of **5** (see Table 2 and Experimental). These differences between the ^{1}H- and ^{13}C-NMR spectra, $[\alpha]_D$, and CD spectrum of **4** and those of **5** may be due to the difference between the con-

Table. 2 ^{1}H-NMR and ^{13}C-NMR Data for MT-4 (**4**), Cladosporin, MT-5 (**5**), and Cladosporin 8-*O*-Methylether

Position	**4** in DMSO-d_6		**4** in $CDCl_3$		Cladosporin[6c] in $CDCl_3$		**5** in DMSO-d_6		Cladosporin 8-*O*-Methylether[6b] in DMSO-d_6
	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR
1		169.24 (s)		170.14 (s)		169.5		161.18 (s)	
3	4.61 (m)	76.18 (d)	4.70 (m)	76.28 (d)	4.76	76.0	4.35 (m)	73.82 (d)	4.35
4	2.91 (2H, m)	37.25 (t)	2.82 (2H, m)	33.47 (t)	2.85	38.7	2.79 (2H, m)	34.41 (t)	2.80 (2H)
4a		142.23 (s)		141.64 (s)		141.2		143.94 (s)	
5	6.23 (d, 2.0)	106.93 (d)	6.31 (br s)	106.93 (d)	6.27	106.8	6.25 (d, 1.3)	106.49 (d)	6.34
6		163.40 (s)		164.15 (s)		163.9		163.58 (s)	
7	6.18 (d, 2.0)	100.89 (d)	6.18 (br s)	101.85 (d)	6.20	101.4	6.36 (d, 1.3)	98.60 (s)	6.22
8	11.08 (s, OH)	164.69 (s)	11.02 (br s)	163.64 (s)		164.4		162.84 (s)	
OCH_3-8							3.74 (3H, s)	55.54 (q)	3.75 (3H)
8a		100.09 (s)		101.06 (s)		100.4		104.37 (d)	
9	2.07 (ddd, 14.4, 10.4, 3.9)	32.61 (t)	2.01 (ddd, 14.1, 10.4, 3.7)	38.86 (t)	2.05 (2H)	33.5	2.00 (ddd, 14.3, 10.3, 3.9)	37.47 (t)	
	1.60 (m)		1.83 (m)				1.60 (m)		
10	3.96 (m)	65.75 (d)	4.14 (m)	67.83 (d)	4.00	66.2	3.94 (m)	66.26 (d)	*ca.* 3.9
11	1.29 (m), 1.60 (m)	29.77 (t)	1.36 (m), 1.68 (m)	30.59 (t)	1.33, 1.60	30.5	1.28 (m), 1.60 (m)	29.93 (t)	
12	1.60 (2H, m)	17.91 (t)	1.68 (2H, m)	18.04 (t)	1.60 (2H)	18.2	1.60 (2H, m)	17.99 (t)	
13	1.21 (m), 1.60 (m)	31.38 (t)	1.36 (m), 1.68 (m)	30.96 (t)	1.33, 1.60	31.1	1.21 (m), 1.60 (m)	31.42 (t)	
14	3.81 (m)	66.11 (d)	3.99 (m)	66.63 (d)	4.00	66.9	3.80 (1H, m)	65.77 (d)	*ca.* 3.6
15	1.09 (3H, d, 6.3)	19.83 (q)	1.22 (3H, d, 6.1)	19.00 (q)	1.18 (3H, d, 6.6)	19.2	1.08 (3H, d, 6.3)	19.83 (q)	1.08 (3H)

δ (ppm) from TMS as an internal standard [coupling constants (Hz) in parentheses].

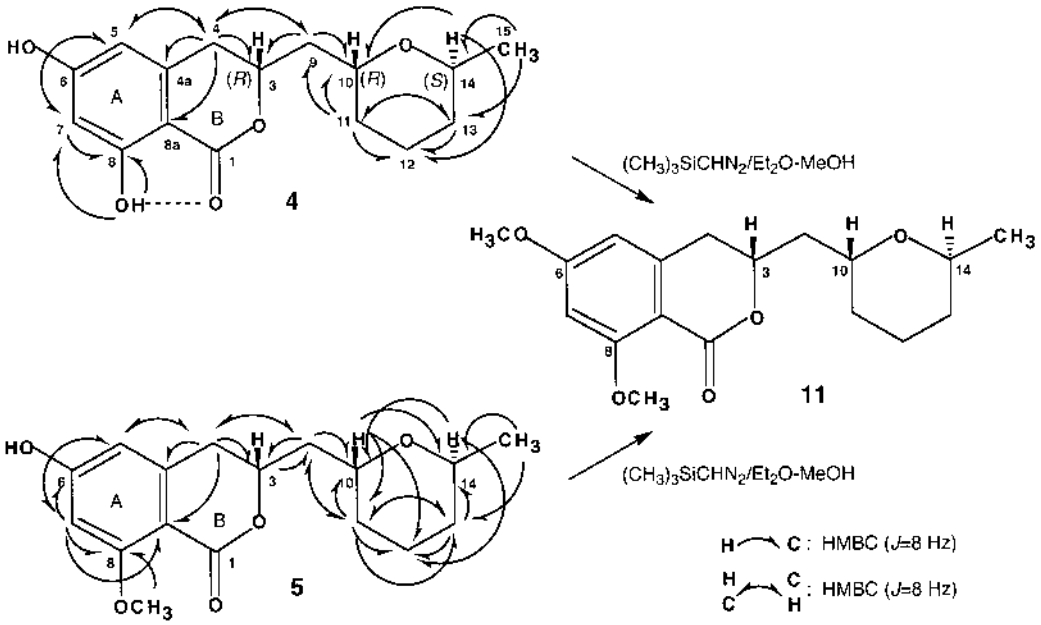

Chart 2

formation of the B ring strained by the presence of a hydrogen bond between HO-8 and CO-1 in **4** and that unstrained by the absence of such a hydrogen bond in **5**, as shown in Chart 2.

MT-6 (**6**), an optically active pale yellow powder, $C_{24}H_{31}N_3O_2$, was positive to the Van Urk reaction,[7] suggesting the presence of an indole skeleton in **6**. The ^{1}H- and ^{13}C-NMR spectral data of **6** (in DMSO-d_6) showed that **6** is composed of seven partial structures, ***a***) 2,3,5-trisubstituted indole (C_8H_4N), ***b***) isopentenyl [$(CH_3)2C{=}CHCH_2$–], ***c***) 1,1-dimethylallyl [$CH_2{=}CHC(CH_3)_2$–], ***d***) $CH_3CH(NH)$–, ***e***) –$CH_2CH(NH)$–, ***f***) >CO, and ***g***) >CO. Then, the partial structures ***d***—***g*** were connected with the aid of HMBC NMR data to obtain an expanded partial structure, ***h***) 3-methyl-6-methylene-2,5-dioxopiperazine. Finally, the four partial structures ***a***, ***b***, ***c***, and ***h*** were united on the basis of detailed HMBC NMR data to construct a whole molecular structure (**6**) [*cyclo*-alanyl-5-isopentenyl-2-(1′,1′-dimethylallyl)tryptophan] (without stereochemistry). The constructed structure (**6**) was similar to the structure of echinulin [*cyclo*-L-alanyl-5,7-diisopentenyl-2-(1′,1′-dimethylallyl)-L-tryptophan] (**12**), a 2,5-dioxopiperazine isolated from *Aspergillus echinulatus* and other *Aspergillus glaucus* group fungi.[8a] This was also supported by the ^{1}H-NMR spectral data of **12** described in the literature.[8b] It is already known that **12** gave a similar molecular rotation ([ϕ]) in the optical rotatory dispersion (ORD) spectrum to those of *cyclo*-L-alanyl-2-(1′,1′-dimethylallyl)-L-tryptophan (**13**).[8c] MT-6 afforded a similar [ϕ] ([ϕ] (nm): −19000° (235), +18400° (212)) in the ORD spectrum to those of **12** (−15500° (238), +19950° (216)) and **13** (−27200° (230), +46250° (214)), as described in the literature,[8c] indicating that the stereostructure of MT-6 was de-

Table 3. ^{1}H-NMR and ^{13}C-NMR Data for MT-6 (**6**), MT-7 (**7**), and Echinulin

Position	**6**[a]		**7**[a]		Echinulin[b,8b]
	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR
NH-1	10.42 (s)			9.80 (s)	
2		141.46 (s)		141.34 (s)	
3		104.32 (s)		105.42 (s)	
3a		129.16 (s)		128.93 (s)	
4	7.19 (s)	116.92 (d)	7.27 (d, 7.8)	115.58 (d)	6.78 or 7.12
5		131.08 (s)	6.89 (dd, 7.8, 7.0)	118.81 (d)	
6	6.82 (d, 8.3)	121.31 (d)	6.81 (d, 7.0)	119.98 (d)	6.78 or 7.12
7	7.21 (d, 8.3)	110.70 (d)		123.75 (s)	
7a		133.39 (s)		133.59 (s)	
8	3.00 (dd, 14.5, 9.4)	31.12 (t)	3.07 (dd, 14.5, 9.4)	31.19 (t)	3.12—3.79
	3.30 (m)		3.35 (m)		
9	3.93 (m)	55.58 (d)	3.94 (m)	55.64 (d)	3.96—4.50
NH-10	7.44 (d, 3.0)		7.48 (d, 2.7)		8.04
11		167.84 (s)		167.90 (s)	
12	3.79 (m)	50.30 (d)	3.80 (m)	50.29 (d)	3.96—4.50
CH_3-12	1.29 (3H, d, 7.1)	20.72 (q)	1.27 (3H, d, 7.1)	20.69 (q)	1.51
NH-13	8.17 (d, 2.7)		8.16 (d, 2.4)		8.04
14		167.37 (s)		167.31 (t)	
15		39.92 (s)		39.92 (s)	
16	6.14 (dd, 17.1, 10.2)	146.54 (d)	6.24 (dd, 17.5, 10.6)	146.73 (d)	5.77—6.50
17	5.01 (dd, 10.2, 1.0)	110.98 (t)	5.05 (dd, 10.6, 1.2)	111.02 (t)	4.87—5.29
	5.04 (dd, 17.1, 1.0)		5.09 (dd, 17.5, 1.2)		
18	1.45 (3H, s)	27.97 (q)	1.52 (3H, s)	27.96 (q)	1.51
19	1.46 (3H, s)	27.97 (q)	1.53 (3H, s)	28.00 (q)	1.51
20	3.30 (2H, m)	34.17 (t)	3.60 (2H, d, 7.1)	28.89 (t)	3.12—3.79
21	5.31 (t, 7.3)	124.84 (d)	5.40 (m)	122.62 (d)	4.87—5.29
22		130.34 (s)		131.64 (s)	
23	1.68 (3H, s)	25.53 (q)	1.74 (3H, s)	25.56 (q)	1.73
24	1.69 (3H, s)	17.67 (q)	1.74 (3H, s)	17.72 (q)	1.73
25					3.12—3.79
26					4.87—5.29
27					
28					1.73
29					1.73

δ (ppm) from TMS as an internal standard [coupling constants (Hz) in parentheses]. *a*) in DMSO-d_6, *b*) in $CDCl_3$.

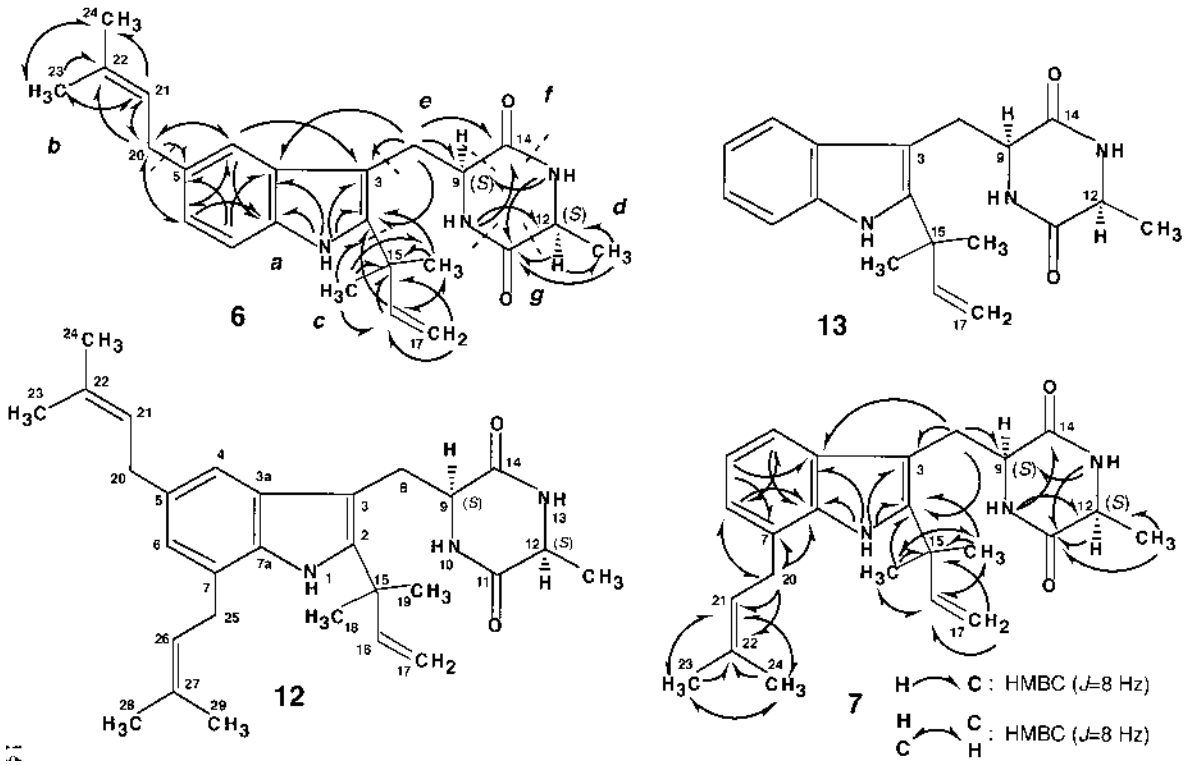

Chart 3

Table 4. ^{1}H-NMR and ^{13}C-NMR Data for MT-3 (**3**), MT-8 (**8**), 5,7-Dihydroxy-4-methylphthalide, and Asperflavin

	3		5,7-Dihydroxy-4-methylphthalide[9]		**8**		Asperflavin[9]
Position	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	Position	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR
1		173.36 (s)		1		204.02 (s)	
3	5.17 (2H, s)	70.16 (s)	5.2 (2H, s)	2	2.71, 2.79	52.49 (t)	2.78 (2H)
3a		150.16 (s)		3	(each d, 17.1)	71.24 (s)	
4		111.09 (s)		CH_3-3	1.34 (3H, s)	28.90 (q)	1.36 (3H)
CH_3-4	2.01 (3H, s)	10.27 (q)	2.05 (3H, s)	4	2.97 (2H, br s)	44.10 (t)	3.00 (2H)
5		164.29 (s)		4a		138.36 (s)	
6	6.34 (1H, s)	103.00 (d)	6.38 (1H, s)	5	6.51 (d, 2.2)	103.46 (d)	6.50
7		157.52 (s)		6		161.98 (s)	
7a		103.60 (s)		7	6.43 (d, 2.2)	98.88 (d)	6.43
				8		162.86 (s)	
				CH_3O-8	3.90 (3H, s)	56.29 (q)	3.92 (3H)
				8a		110.33 (s)	
				9		166.48 (s)	
				9a		110.25 (s)	
				10	6.75 (s)	117.68 (s)	6.75
				10a		143.69 (s)	

δ (ppm) from TMS as an internal standard in CD_3OD [coupling constants (Hz) in parentheses].

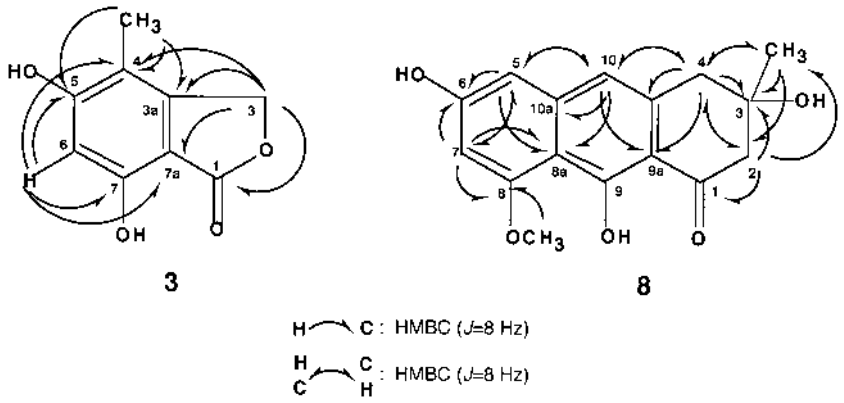

Chart 4

duced to be *cyclo*-L-alanyl-5-isopentenyl-2-(1′,1′-dimethylallyl)-L-tryptophan (**6**), as shown in Chart 3.

MT-7 (**7**), an optically active pale yellow powder, $C_{24}H_{31}N_3O_2$, was positive to the Van Urk reaction. The ^{1}H- and ^{13}C-NMR spectral data of **7** indicated that signals due to the isopentenyl group at position 5 and the aromatic CH at position 7 in **6** were substituted with those of an aromatic CH and an isopentenyl group in **7**, respectively (Table 3), suggesting that MT-7 might be *cyclo*-alanyl-7-isopentenyl-2-(1′,1′-dimethylallyl)tryptophan (**7**). The fact that MT-7 gave a similar [ϕ] ([ϕ] (nm): −28200° (232), +27500° (215)) to that of **6** indicated that the stereostructure of **7** was deduced to be *cyclo*-L-alanyl-7-isopentenyl-2-(1′,1′-dimethylallyl)-L-tryptophan (**7**) (Chart 3). To our knowledge, this is the first time that *cyclo*-L-alanyl-5-isopentenyl-2-(1′,1′-dimethylallyl)-L-tryptophan (**6**) and *cyclo*-L-alanyl-7-isopentenyl-2-(1′,1′-dimethylallyl)-L-tryptophan (**7**) have been isolated from a natural source. We propose to call **6** and **7** tardioxopiperazines A and B, respectively.

MT-3 (**3**), a white powder, was positive to the $FeCl_3$ reaction. The ^{1}H- and ^{13}C-NMR data suggested the presence of a methyl, an aliphatic methylene bearing oxygen, an aromatic CH, five aromatic quaternary carbons, and an ester carbonyl. These structural units were united by the aid of HMBC NMR data to construct a whole molecular structure (**3**), being compatible with the molecular ion *m/z* 180 in the EI-MS spectrum. The whole molecular structure (**3**) was equal to the structure of 5,7-dihydroxy-4-methylphthalide isolated from *Aspergillus flavus*.[9] Comparison of the ^{1}H-NMR data, melting point, and UV spectrum of MT-3 with those of 5,7-dihydroxy-4-methylphthalide described in the literature[9] showed that MT-3 was deduced to be identical with 5,7-dihydroxy-4-methylphthalide (**3**) (Chart 4).

MT-8 (**8**) was optically active and positive to the $FeCl_3$ reaction. The ^{1}H- and ^{13}C-NMR data showed the presence of a *tert*-methyl, a methoxyl, two aliphatic methylenes, three aromatic protons among which two were *meta*-coupled with each other, an aliphatic quaternary carbon bearing oxygen, seven aromatic quaternary carbons among which three bore oxygen, and a ketone carbonyl in **8**. A whole molecular structure (**8**) was constructed for MT-8 from these structural units on the basis of HMBC NMR data. The whole molecular structure (**8**), supported by the molecular ion *m/z* 288 in the EI-MS spectrum, was equal to the structure of asperflavin isolated together with **3** from *Aspergillus flavus*.[9] However, the appearance and specific rotation of MT-8 (greenish amorphous, +89°) were considerably different from those of asperflavin described in the literature (citrine prism, +4°),[9]

Table 5. Immunosuppressive Effects of MT-1 (**1**)—MT-8 (**8**), MT-4 Dimethylether (**11**), and Some Related Compounds on the Con A-Induced and LPS-Induced Proliferation of Mouse Splenic Lymphocytes

Compound	IC_{50} (μg/ml) Con A-induced	LPS-induced
1	0.3	0.3
2	0.8	0.1
3	17	3.0
4	13	7.5
5	2.3	13
6	4.5	0.7
7	>25	15
8	16	3.6
11	23	12
Emodin (**9**)	0.2	0.2
Azathioprine	2.7	2.7
Cyclosporin A	0.04	0.07

suggesting that MT-8 (**8**) may be an optically active state of partially racemized asperflavin.

The immunosuppressive activities (IC_{50} values) of **1**—**9** and **11** were calculated against Con A- (T-cells) and LPS-induced (B-cells) proliferation of mouse splenic lymphocytes, as shown in Table 5. Two anthraquinones, **1** and **2**, which were isolated as main immunosuppressive features from *M. tardifaciens*, considerably suppressed the proliferation of both T- and B-cells, as well as emodin (**9**). The immunosuppressive activity of **9** has already been demonstrated on human mononuclear cells.[10] To our knowledge, this is the first time that **1** and its 4-hydroxy derivative **2**, and **4** and its 8-*O*-methylether (**5**) have been isolated as immunosuppressive features from a natural source. Tardioxopiperazine A [*cyclo*-L-alanyl-5-isopentenyl-2-(1′,1′-dimethylallyl)-L-tryptophan] (**6**) moderately suppressed the proliferation of both T- and B-cells, but tardioxopiperazine B [*cyclo*-L-alanyl-7-isopentenyl-2-(1′,1′-dimethylallyl)-L-tryptophan] (**7**) suppressed them only slightly. The immunosuppressive activities of **3**—**5**, **8**, and **11** were low in comparison with the immunosuppressive activities of two known immunosuppressants, cyclosporin A (IC_{50} against Con A- and LPS-induced proliferations: 0.04 and 0.07 μg/ml) and azathioprine (2.7 and 2.7 μg/ml).

Experimental

The general procedures for chemical experiments and other experimental conditions, including those for the evaluation of suppressive activity (IC_{50} values) of samples against the proliferation of mouse splenic lymphocytes stimulated with Con A and LPS, were the same as those described in our previous report (this method is based on the incorporation ratio of exogenous [^{3}H]thymidine into lymphocytes or the formation ratio of formazan from exogenous 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) in lymphocytes).[1b] Chemical shifts are expressed in δ (ppm) values from tetramethylsilane (TMS) as an internal standard.

Isolation of MT-1(1)—-8(8) from *M. tardifaciens* *M. tardifaciens* IFM4564[2] was cultivated on sterilized rice (200 g/flask×300) at 25 °C for 27 d. The brown colored moldy rice was extracted with AcOEt (60.0 l) with shaking at room temperature for 6 h two times to give an AcOEt solution (120.0 l), which gave, after evaporation *in vacuo*, an AcOEt extract (37.8 g). The AcOEt extract was partitioned with *n*-hexane–H_2O (1 : 1, v/v) (2.0 l) into an *n*-hexane layer (after evaporation *in vacuo*, 29.1 g) and an aqueous suspension, which was then partitioned with AcOEt (1.0 l) into an AcOEt layer (7.0 g) and an aqueous layer (0.14 g). The *n*-hexane, AcOEt, and aqueous layers suppressed the Con A-induced proliferation of mouse splenic lymphocytes by 41, 99, and 74% at 50 μg/ml, and by 4, 64, 11% at 10 μg/ml, respectively. The AcOEt layer was subjected to chromatography on a silica gel column with *n*-hexane–acetone (4 : 1), (2 : 1), (1 : 1), and acetone to give four fractions: I (2.9 g), II (0.93 g), III (2.0 g), and IV (1.1 g). Fractions I, II, III, and IV suppressed the Con A-induced proliferation of the lymphocytes by 28, 72, 64, and 49% at 10 μg/ml, respectively. Fraction II was further chromatographed on a silica gel column with C_6H_6–AcOEt and on a Sephadex LH-20 (Pharmacia) column with MeOH to give three fractions, IIa—IIc. Fraction III was also further chromatographed in the similar way to give four fractions, IIIa—IIId. A mixture of fractions IIb and IIIc (67 mg) was then chromatographed on a silica gel column with $CHCl_3$–MeOH and on an octadecyl silica gel (ODS) column with CH_3CN–H_2O to afford **1** (6 mg) and **2** (2 mg). Fraction IIa (276 mg) was also chromatographed on a silica gel column with $CHCl_3$–MeOH two times, on a Sephadex LH-20 column with MeOH, and successively on a high performance liquid chromatographic (HPLC) ODS column with CH_3CN–H_2O (1 : 1) at a flow rate of 8.0 ml/min to afford **4** (54 mg) and **3** (13 mg). Fraction IIIb (960 mg) was chromatographed on an ODS column with MeOH–H_2O to give three fractions, IVa—IVc. Fraction IVb (91 mg) was chromatographed on preparative TLC plates with *n*-hexane–AcOEt (1 : 5) and on an HPLC ODS column with CH_3CN–H_2O (3 : 7) at a flow rate of 8.0 ml/min to afford **5** (14 mg) and **8** (16 mg). Fraction IVc (296 mg) was further chromatographed on a silica gel column with $CHCl_3$–MeOH, on an ODS column with CH_3CN–H_2O, and successively on an HPLC ODS column with CH_3CN–H_2O (1 : 1) to give **6** (9 mg), **7** (9 mg), and **5** (12 mg).

MT-1 (**1**) (Questin): Fine yellow needles from EtOH, mp 297—299 °C (lit.[3] bright-yellow needles, mp 301—303 °C). EI-MS *m/z* (%): 284 (65, M^+), 267 (15), 213 (28), 198 (25), 182 (36), 77 (100). IR ν_{max}^{KBr} cm^{-1}: 3262 (O—H), 1680, 1631 (C=O), 1592, 1473 (C=C), 1437, 1352, 1270, 1218 (C—O). UV λ_{max}^{EtOH} nm (log ε): 224 (4.60), 248 (4.13), 286 (4.41), 425 (3.99). This compound was identical with an authentic sample of questin (**1**) in terms of mixed mp, ^{1}H-NMR spectra, and TLC behavior [Kieselgel 60 F_{254}S (Merck), *n*-hexane–acetone (1 : 1); RP18 F_{254}S (Merck), CH_3CN–H_2O (3 : 1)]

MT-2 (**2**) (Rubrocristin): Fine red powder, mp 280—282 °C. EI-MS *m/z* (%): 300 (100, M^+), 282 (28), 253 (36), 213 (30), 197 (31), 183 (38) [lit.[5] 300 (100, M^+), 282 (55), 254 (25), 217 (15)], high-resolution EI-MS (HR-EI-MS) *m/z*: 300.0642 ($C_{16}H_{12}O_6$ requires 300.0634). UV λ_{max}^{EtOH} nm (log ε): 231 (5.01), 253 (4.55), 280 (4.77), 306 (sh, 4.50), 481 (4.51), 495 (sh, 4.49), 515 (sh, 4.35), 530 (4.19) [lit.[5] 230 (4.49), 254 (4.08), 280 (4.25), 300 (sh, 4.06), 480 (4.00), 495 (3.99), 512 (3.87), 528 (3.74)].

MT-3 (**3**) (5,7-Dihydroxy-4-methylphthalide): Fine white powder, mp 203—205 °C (lit.[9] 200—203 °C). EI-MS *m/z* (%): 180 (55, M^+), 151 (100), 77 (30), 69 (76). UV λ_{max}^{MeOH} nm (log ε): 216 (4.41), 259 (4.05), 297 (3.68) (lit.[9] 227 (4.20), 260 (3.97), 297 (3.61)).

MT-4 (**4**) [Cladosporin (Asperentin)]: Pale yellow prism, mp 191.5—192 °C (lit.[6a] 188.5—189 °C), $[\alpha]_D^{22}$ −23.3° (*c*=0.1, EtOH) [lit.[6a] −24.8° (*c*=0.96, EtOH)]. EI-MS *m/z* (%): 292 (13, M^+), 194 (14), 179 (56), 151 (100). UV λ_{max}^{MeOH} nm (log ε): 216 (4.31), 230 (sh, 4.03), 268 (4.10), 301 (3.73) [lit.[6a] 217 (4.32), 230 (infl, 4.08), 270 (4.11), 303 (3.78)]. CD (1.69 mM, MeOH) $\Delta\varepsilon$ (nm): −0.70 (301), +2.40 (270), −0.42 (249), +2.47 (235) [lit.[6b] −0.86 (302), +2.45 (268), −0.58 (247), +2.30 (234)].

MT-5 (**5**) [Cladosporin 8-*O*-Methylether (Asperentin 8-*O*-Methylether)]: Pale yellow powder, mp 236.5—237 °C [lit.[6b] mp 225 and 235 °C (double mp)], $[\alpha]_D^{19}$ +71° (*c*=0.33, $CHCl_3$) [lit.[6b] $[\alpha]_D^{20}$ +72° (*c*=0.1, $CHCl_3$)]. EI-MS *m/z* (%): 306 (3, M^+), 288 (9), 208 (19), 193 (34), 165 (28), 81 (100). UV λ_{max}^{MeOH} nm (log ε): 214 (4.33), 226 (sh, 4.23), 265 (4.10), 298 (3.82) [lit.[6b] 227 (4.27), 266 (4.15), 298 (3.89)] CD (1.47 mM, MeOH) $\Delta\varepsilon$ (nm): +1.34 (297), +1.12 (288), +4.18 (270), −0.78 (251), +4.01 (233) [lit.[6b] +1.51 (296), +4.70 (269), −0.91 (251), +4.39 (232)].

MT-6 (**6**) [Tardioxopiperazine A (*cyclo*-L-Alanyl-5-isopentenyl-2-(1′,1′-dimethylallyl)-L-tryptophan)]: Pale yellow amorphous, $[\alpha]_D^{22}$ −30° (*c*=0.20, $CHCl_3$), EI-MS *m/z* (%): 393 (15, M^+), 267 (100), 251 (25). HR-EI-MS *m/z*: 393.2396 ($C_{24}H_{31}N_3O_2$ requires 393.2416). UV λ_{max}^{MeOH} nm (log ε): 229 (4.46), 281 (sh, 3.85), 287 (3.86), 296 (sh, 3.79). ORD (*c*=0.03, MeOH) [ϕ](nm): +1150° (305), −19000° (235), +18400° (212).

MT-7 (**7**) [Tardioxopiperazine B (*cyclo*-L-Alanyl-7-isopentenyl-2-(1′,1′-dimethylallyl)-L-tryptophan)]: Pale yellow amorphous, $[\alpha]_D^{22}$ −16° (*c*=0.20, $CHCl_3$), EI-MS *m/z* (%): 393 (20, M^+), 267 (100), 251 (26). HR-EI-MS *m/z*: 393.2397 ($C_{24}H_{31}N_3O_2$ requires 393.2416). UV λ_{max}^{MeOH} nm (log ε): 226 (4.40), 273 (sh, 3.79), 280 (3.79), 291 (sh, 3.70). ORD (*c*=0.03, MeOH) [ϕ] (nm): +720° (299), −28200° (232), +27500° (215).

MT-8 (**8**) (Asperflavin): Greenish amorphous (lit.[9] citrine prisms, mp 225—230 °C) , $[\alpha]_D^{22}$ +89° (*c*=0.20, MeOH) [lit.[9] +4° (*c*=0.3, MeOH)], EI-MS *m/z* (%): 288 (23, M^+), 270 (14), 255 (16). 241 (25), 230 (100). UV λ_{max}^{MeOH} nm (log ε): 232 (4.23), 270 (4.56), 318 (3.68), 332 (3.57), 389 (4.02)

[lit.[9] 230 (4.13), 269 (4.51), 317 (3.64), 335 (3.40), 392 (3.91)]. CD (0.35 mM, MeOH) $\Delta\varepsilon$ (nm): +0.06 (394), −0.15 (322), +0.17 (280), −0.14 (261), +0.02 (242).

Formation of 6-Acetyl MT-2 (10) Acetic anhydride (2 drops) was added to a solution of **2** (1.5 mg) in pyridine (2 drops) to prepare a reaction mixture, which was allowed to stand at room temperature for 3.5 h. After the addition of a small volume of ice-water, the reaction mixture was extracted with AcOEt. The AcOEt layer was washed with water and evaporated *in vacuo* to give a crude product (1.4 mg). This product was purified by preparative thin layer chromatography [plate: silica gel $60F_{254}$ (Merck), developing solvent: *n*-hexane–AcOEt (1 : 1), extraction solvent: $CHCl_3$] to afford an orange amorphous powder (0.8 mg), which was identical with rubrocristin-6-acetate (**10**) in comparison of the ^{1}H-NMR spectrum (in $CDCl_3$) with the spectrum of rubrocristin-6-acetate described in the literature (see Table 1).[5]

Formation of MT-4 Dimethylether (11) A solution of 10% trimethylsilyldiazomethane in *n*-hexane (Nacalai) (100 μl) was added to a solution of **4** (5.0 mg) in a mixture of Et_2O (100 μl) and MeOH (100 μl) to prepare a reaction mixture, which was then stirred at room temperature for 17 h. Evaporation of the reaction mixture *in vacuo* afforded MT-4 dimethylether (**11**) (5.3 mg), pale yellow amorphous, $[\alpha]_D^{19}$ +79° (c=0.10, $CHCl_3$), ^{1}H-NMR δ (ppm) from TMS in DMSO-d_6: 1.08 (3H, d, J=6.3 Hz, CH_3-15), 1.21 (1H, m, C$\underline{H}_a$H$_b$-13), 1.29 (1H, m, C$\underline{H}_a$H$_b$-11), 1.62 (5H, m, C$\underline{H}_a$H$_b$-9, CH$_a\underline{H}_b$-11, CH_2-12, CH$_a\underline{H}_b$-13), 2.03 (1H, m, CH$_a\underline{H}_b$-9), 2.88 (2H, m, CH_2-4), 3.81 (3H, s, CH_3O-8 or -6), 3.82 (1H, m, CH-14), 3.84 (3H, s, CH_3O-6 or -8), 3.94 (1H, m, CH-10), 4.39 (1H, m, CH-3), 6.49 (1H, d, J=2.1 Hz, CH-5), 6.54 (1H, d, J=2.1 Hz, CH-7). CD (1.56 mM, MeOH) $\Delta\varepsilon$ (nm): +1.53 (297), +6.35 (269), −1.64 (250), +4.28 (232).

Formation of Compound 11 from MT-5 A solution of 10% trimethylsilyldiazomethane in *n*-hexane (Nacalai) (500 μl) was added to a solution of **5** (5.0 mg) in a mixture of Et_2O (200 μl) and MeOH (300 μl) to prepare a reaction mixture, which was then stirred at room temperature for 40 min. Evaporation of the reaction mixture *in vacuo* afforded a product (4.2 mg), pale yellow amorphous, which was identical with **11** in terms of ^{1}H-NMR spectrum, $[\alpha]_D$, and CD spectrum.

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