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Structures and Biosynthesis of Aflastatins: Novel Inhibitors of Aflatoxin Production by *Aspergillus parasiticus*

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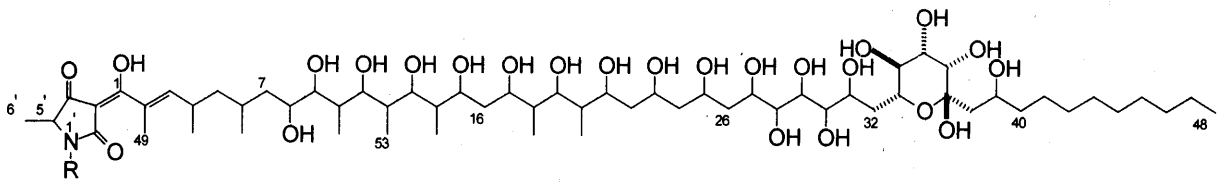
Two novel inhibitors of aflatoxin production by *Aspergillus parasiticus* were isolated from the mycelial extracts of *Streptomyces* sp. MRI142 and termed aflastatin A and B. The structures of aflastatin A (**1**) and B (**5**) were elucidated by NMR and chemical degradation experiments. These compounds have a novel skeleton of a tetramic acid derivative with a highly oxygenated long alkyl chain. The incorporation experiments using ^{13}C-labeled acetates, propionate, glucose and glycolate suggested that most of the C_2 and C_3 units involved in the alkyl chain moiety of aflastatin A were biosynthesized from acetic and propionic acids, but five C_2 units in the alkyl chain originated from glycolic acid.

Aflatoxin is a most potent environmental carcinogen. It is produced by some strains of *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. Since discovery of the potent carcinogenicity of aflatoxin in the 1960s and subsequent detection of the toxin in a wide variety of food commodities, control and management of this toxin have been issues of concern[1]. To protect foods and feeds from aflatoxin contamination, growth inhibitors of aflatoxigenic fungi are useful. However, effective fungicides are usually toxic to mammals to some extent and may provoke the emergence and pervasion of resistant strains as in the cases of many other drug-resistant microorganisms. On the other hand, it is known that the production of aflatoxin is not essential for the growth of its producing strain. Therefore, a specific inhibitor of aflatoxin biosynthesis may be a good candidate for a drug to depress aflatoxin contamination, which may not select the resistant strain and minimize the risk of pervasion of drug-resistant strains.

Until now, many substances have been screened for prevention of aflatoxin contamination in foods and feeds.[2] Among them, organo-phosphorous insecticides with choline esterase inhibitory activity, such as dichlorvos, were found to show unique inhibitory effects on aflatoxin production. They inhibit some esterases involved in the biosynthetic pathway of aflatoxin to result in the inhibition of aflatoxin biosynthesis.[3] Some inhibitors of pentaketide-derived melanin biosynthesis in fungi, such as tricyclazole and chlorobenthiazone, were recently shown to have an inhibitory activity against aflatoxin production in *A. flavus*. They are presumed to inhibit a reductase in the biosynthetic pathway of aflatoxin.[4,5] To obtain a new specific inhibitor of aflatoxin production, we have continued on screening of metabolites from microorganisms using a system of *A. parasiticus* and aflatoxin-sensitive bacteria. During the course of screening, *Streptomyces* sp. MRI142 was found to produce strong inhibitors, termed aflastatins, which inhibit aflatoxin production without essentially affecting the growth of its producer. We have already reported the isolation and preliminary elucidation of the structure and biosynthesis of the major component, aflastatin A.[6,7] This paper describes the detailed structural elucidation of aflastatin A and further investigation of its biosynthesis, and the isolation, structure and biological activity of aflastatin B.

Fig. 1. Structures of aflastatin A(**1**) and B(**5**).



Aflastatin A (**1**) R=CH_3
Aflastatin B (**5**) R=H

Fig. 2. COSY, relayed COSY and HMBC correlations observed in partial structure of **A**.

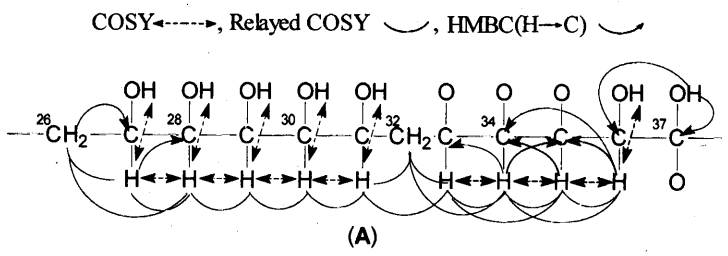
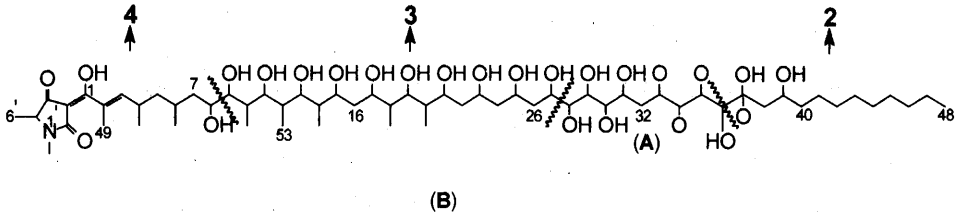


Fig. 3. Degradation experiments of **1**.



Results and Discussion

Structural Elucidation of Aflastatin A

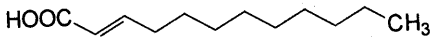
Aflastatin A (**1**) was isolated as a white powder after lyophilization. The pseudomolecular ions of $(M+Na)^+$ and $(M-H)^-$ were observed at *m/z* 1280 and 1256 in the positive and negative FAB-MS spectra of **1**, respectively. The HR-FABMS spectrum of **1** and elemental analysis strongly suggested that the molecular formula of **1** was $C_{62}H_{115}NO_{24}$, which was unambiguously confirmed after completing analysis of NMR spectra of **1** as later mentioned. The UV spectrum of **1** was very characteristic, which showed a shift from the absorption maxima at 299 and 247 nm in neutral and basic solutions to those at 314 and 237 nm in acidic solution, respectively. The IR and ^1H NMR spectra of **1** in DMSO-d_6 solution suggested that **1** contains a number of hydroxyl groups in its molecule. The NMR signals of the carbons involved in the chromophore moiety of **1** were not clearly observed because of signal broadening when measured with free acid of **1**, but they became detectable when the ^{13}C NMR spectrum was measured with diethylamine salt of **1**. By analyzing the DQF-COSY, DQF-relayed COSY, HMQC and HMBC spectra of **1**, which were measured using diethylamine salt of **1** in DMSO-d_6 solution, the presence of the partial structure (A) was clarified (Fig. 2). The structure A was highly oxygenated, and the presence of the seven hydroxyl groups in it was verified from the COSY and HMBC spectra of **1** as shown in Figure 2. Other small partial structures were also identified from the various spectra above mentioned, but it was difficult to determine the total structure of **1** by further NMR analysis with the intact molecule. Since the partial structure (A) having several diol moieties was obtained, oxidation of **1** with $NaIO_4$ was carried out to obtain fragment molecules in order to identify the remainder of

the structure (Fig. 3).

First, **1** was oxidized with $NaIO_4$, and followed by $NaBH_4$ reduction and acetylation. The crude products obtained were purified by Sephadex LH-20 column chromatography and reverse-phase HPLC to afford two main products, **2** and **3** (Fig. 4). The structure of a fragment was determined as **2** by analysis of its MS and NMR spectra. Another fragment had no characteristic absorption in its UV spectrum, and the molecular formula was determined as $C_{44}H_{70}O_{20}$ by its HR-FABMS spectrum. By analysis of COSY and HMQC spectra, ten partial structures, $AcOCH_2CH(CH_3)$–, $-CH(CH_3)CH(OAc)-\times 4$, $-CH_2CH(OAc)-\times 4$, and $-CH_2CH_2OAc$, which contained all the atoms involved in the fragment, were identified. To connect these partial structures, the HMBC spectrum was measured. In the spectrum, significant long-range couplings to establish the connections among them were observed as shown in Figure 5, which determined the structure of the fragment as **3**. Next, to obtain a fragment molecule containing the chromophoric moiety of **1**, **1** was oxidized with $NaIO_4$, and followed only by $NaBH_4$ reduction. In this case, after purification of the crude products by reverse-phase HPLC under basic conditions, the fragment **4**, whose UV spectrum showed the same absorption maxima as **1**, was obtained as a product. The HR-FABMS spectrum of **4** indicated that the molecular formula was $C_{17}H_{27}NO_4$. The UV spectra of **4** with a characteristic bathochromic shift in acidic solution and the chemical shifts of four carbon signals (δ_C 195.5, 175.8, 101.0, and 196.0 in MeOH-d_4 solution for C-1, -2′, -3′, and -4′, respectively) contained in **4** strongly suggested the presence of a tetramic acid skeleton.[8~11] This was confirmed by HMBC experiments, and further analysis of COSY, HMQC, HMBC, and NOE difference spectra led to determination of its structure as **4** (Fig. 6).

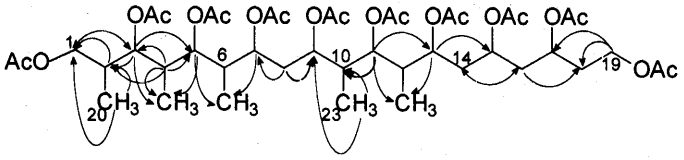
Since, all 62 carbon atoms of **1** were involved in structure A or fragment **2**, **3**, or **4**, its total carbon skeleton could be constructed with them. Based on the fact that **1** had no α, β-unsaturated carboxylic acid, acetoxymethyl, or hydroxymethyl groups in its molecule, the carbon skeleton of **1** was easily reconstructed as follows. The α, β-unsaturated carboxylic acid skeleton of **2** could be produced by cleavage of the hemiketal (ketone)-hydroxyl moiety in partial structure A and then reduction, acetylation, and β-elimination of the acetoxyl group. Thus, the connection between structure A and **2** was clarified. The acetoxymethyl groups at both ends of **3** indicated that two sets of vicinal diols were originally oriented in **1** across the group producing **3**. Fragment **4** should also originate by cleavage of a vicinal diol in **1**. Therefore, the connections between **3** and structure A and **3** and **4** were used to construct the carbon skeleton of **1**. The presence of a methylene group at C-26 of **1**, which was confirmed by the correlation between the methylene proton at C-26 and the methine carbon at

Fig. 4. Structure of fragment **2**.



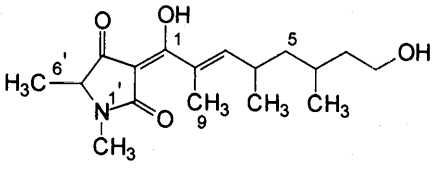
2

Fig. 5. HMBC correlation (H→C) in **3**.



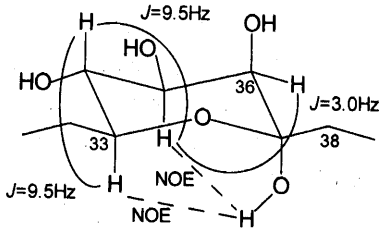
3

Fig. 6. Structure of fragment **4**.



4

Fig. 7. Relative stereochemistry of tetrahydropyran ring.



C-27 observed in a HMBC experiment, could determine the direction of linkage between **3** and structure A. From the above results, a large partial structure (B) containing all the carbon atoms of **1** was clarified (Fig. 3). This structure and molecular formula of **1** indicated that the only remaining problem was the position of an ether linkage. The formation of a tetrahydropyran ring by the ether linkage between C-33 and C-37 was revealed by the *J* values and NOEs around the ring protons as shown in Figure 7. Thus, the total structure of aflastatin A was determined as **1**. The assignments of protons and carbons in the NMR spectra of **1** are summarized in Table **1**.

Isolation, Structure and Biological Activity of Aflastatin B

Aflastatin B (**5**) was isolated as a minor component of aflastatins produced by *Streptomyces* sp. MRI142. The isolation procedure of **5** from a mycelial MeOH extract of the MRI142 strain was the same as that of **1**. At the final purification step with reverse-phase HPLC under basic conditions, the two compounds were separately eluted from the HPLC column.

The pseudo-molecular ion of $(M+Na)^+$ was observed at *m/z* 1266 in the FAB-MS spectrum of **5**. The HR-FABMS data revealed the molecular formula $C_{61}H_{113}NO_{24}$ which was one CH_2 less than that of **1**. The UV spectrum of **5** closely resembled to that of **1**, in which absorption maxima at 299 and 246 nm in neutral and basic solutions and at 314 and 237 nm in acidic solution were observed, indicating that **5** and **1** have a similar chromophore. The ^1H and ^{13}C NMR spectra of **5** were also almost the same as those of **1**, only one difference being observed in the signals of the *N*-methyl group. Namely, the signals of the *N*-methyl group of **1** (δ_H 2.68 and δ_C 26.3) were not observed in the NMR spectra of **5**. From these data, the structure of aflastatin B was determined as **5**, which is the *N*-demethyl derivative of **1**. The assignments of carbons in the ^{13}C NMR spectrum of **5** are summarized in Table 1, which were assigned based on the assignment of **1**.

The biological activity of **5** toward the aflatoxigenic fungus, *A. parasiticus* NRRL2999, was examined, and the results are summarized in Table 2. Aflastatin B (**5**) completely inhibited the aflatoxin production of the fungus at the concentration of 0.5 μg/ml in the medium, while the growth of the producer was not significantly affected at the same concentration. This was almost the same activity as **1**.[6]

Biosynthesis of Aflastatin A

Aflastatin A contains many hydroxyl groups in its alkyl side chain. If we assume that the alkyl chain is biosynthesized according to a usual polyketide pathway, several hydroxyl groups in **1** seem to attach to unexpected positions. Especially, the polyol skeleton present in the partial structure A is not usually observed in polyketide metabolites. This prompted us to investigate the biosynthesis of **1**. Firstly, feeding experiments using [1-^{13}C] and [2-^{13}C]acetate, and [1-^{13}C]-propionate were carried out. A labeled precursor was added, in one portion, to the culture of *Streptomyces* sp. MRI142 after the 48th hour of cultivation, around which time production of **1** started. The ^{13}C NMR spectrum of labeled **1** obtained was measured to determine the carbon enrichment. Table 3 shows the results of the feeding experiments. In the feeding experiment of [1-^{13}C]acetate, a clear ^{13}C incorporation was observed at each of the carbons, 15, 21, 23, 25, 31, 37, 39, 47 and 2′. Three of the five carbons, 41, 42, 43, 44, 45, were also clearly enriched, but it was not possible to determine which positions of the carbons were enriched because of signal overlapping. On the other hand, in the feeding experiment with [2-^{13}C]acetate no clear enriched peak was observed due to rapid turnover of the labeled acetate. In the feeding experiment of [1-^{13}C]propionate, a high level of ^{13}C incorporation into each of the carbons, 1, 3, 5, 9, 11, 13, 17 and 19 was observed. From the results of these incorporation experiments, it was indicated that most of the C_2 and C_3 units, which are involved in the portion of C-1 to C-48 and C-2′, 3′, originated from acetic and propionic acid according to an expected polyketide pathway. However, no enrichment was observed at the C_2 unit of C-7, 8, and two C_4 units of C-27, 28, 29, 30 and C-33, 34, 35, 36. As regards the origin of the C_2 unit of C-7, 8, a glycolic acid or a related molecule might be a most possible candidate as in the case with geldanamycin[12] or leucomycin.[13] On the other hand, two molecules of glycolic acid or a tetraose derivative might be incorporated into the two C_4 units. Secondly, a feeding experiment with [*U*-$^{13}C_6$]glucose was carried out to clarify the origin of these unusual groups. In this incorporation experiment, soluble starch was used as the carbon source for the cultivation in place of glucose to avoid dilution of the isotopically-labeled glucose. Since it was difficult to assign each ^{13}C-^{13}C coupling from the ^{13}C NMR spectrum of labeled **1** due to signals overlapping, ^{13}C-^{13}C COSY and *J*-resolved 2D NMR spectra were measured to analyze the couplings. The $^1J_{CC}$

Table 1. ^{1}H and ^{13}C assignments of **1**, and ^{13}C assignment of **5**[a].

C-No.	δ_C of **1**	δ_H of **1**	δ_C of **5**	C-No.	δ_C of **1**	δ_H of **1**	δ_C of **5**
1	191.5		195.7	29-OH		4.12	
2	135.2		-[d]	30	72.5	3.45	72.5
3	139.3	5.45 br. d (10)	139.3	30-OH		4.17	
4	29.9	2.54	29.9	31	68.6	3.83	68.6
5	44.8	0.93 t (11), 1.34	44.8	31-OH		4.57	
6	26.1	1.90	26.3	32	35.8	2.06, 1.48	35.8
7	42.8	1.26	42.6	33	70.2	3.62	70.2
8	67.2	3.64	67.0	34	71.2	3.18 t (9.5)	71.2
9	75.0	6.26	75.0	35	70.7	3.56 dd (9.5, 3)	70.7
10	37.1	1.81	37.0	36	73.0	3.41	73.0
11	75.8	3.71	75.9	36-OH		4.45	
11-OH		5.20		37	98.4		98.6
12	38.1	1.62	38.1	37-OH		6.11	
13	78.9	3.67	78.9	38	41.6	1.82, 1.42	41.6
13-OH		5.26		39	67.5[e]	3.89	67.4
14	38.1	1.67	38.1	39-OH		4.75	
15	74.5	3.85	74.5	40	38.1[b]	1.30	38.1[f]
15-OH		4.79		41	24.9[c]	1.23	24.9[g]
16	34.9[b]	1.30, 1.60	34.8[f]	42	29.2[c]	1.23	29.2[g]
17	70.4	3.91	70.4	43	29.0[c]	1.23	29.1[g]
17-OH		4.71		44	29.0[c]	1.23	29.1[g]
18	41.6	1.65	41.6	45	28.7[c]	1.23	28.7[g]
19	76.2	3.46	76.2	46	31.3	1.23	31.3
19-OH		4.57		47	22.1	1.23	22.1
20	38.1	1.53	38.1	48	13.9	0.84 t (7)	14.0
21	73.5	3.81	73.5	49	13.3	1.67	13.2
21-OH		4.76		50	21.5	0.88 d (6.5)	21.4
22	41.6[b]	1.53	41.6[f]	51	20.8	0.85 d (7)	20.8
23	67.9	3.79	67.9	52	8.7	0.84 d (7)	8.7
23-OH		4.73		53	12.8	0.64 d (7)	12.8
24	44.5[b]	1.39, 1.55	44.5[f]	54	6.4	0.81 d (7)	6.5
25	68.6[e]	3.87	68.6	55	10.6	0.68 d (7)	10.5
25-OH		5.24		56	5.8	0.79 d (7)	5.8
26	41.0	1.35, 1.85	41.0	2'	173.4		-[d]
27	69.7	3.63	69.6	3'	98.1		-[d]
27-OH		4.66		4'	192.6		196.1
28	74.3	3.25	74.3	5'	59.4	3.20	54.3
28-OH		4.59		6'	15.9	1.11 d (7)	18.6
29	69.4	3.82	69.4	7'	26.3	2.68	

[a] Spectra were obtained in DMSO-d_6 on a JEOL GX-500. [b,c] May be interchanged. [d] Not detected because of signal broadening. [e] Assignments of these two carbons were interchanged in the previous report.[7] These were revised from the data of ^{13}C-^{13}C couplings obtained by the feeding experiment. [f,g] May be interchanged. Coupling constants in Hertz are given in parentheses.

Table 2. Effect of aflastatin B on aflatoxin production by *Aspergillus parasiticus*.

aflastatin B (μg/ml)	mycelium weight ± S.D.[a] (mg)	aflatoxin conc. ± S.D.[a] (total of B_1, G_1, B_2 and G_2, μg/flask)
None	34.0 ± 1.0	4.67 ± 1.33
0.03	32.7 ± 0.58	1.49 ± 0.34
0.12	32.3 ± 0.58	0.65 ± 0.57
0.5	31.7 ± 0.58	Tr.[b]

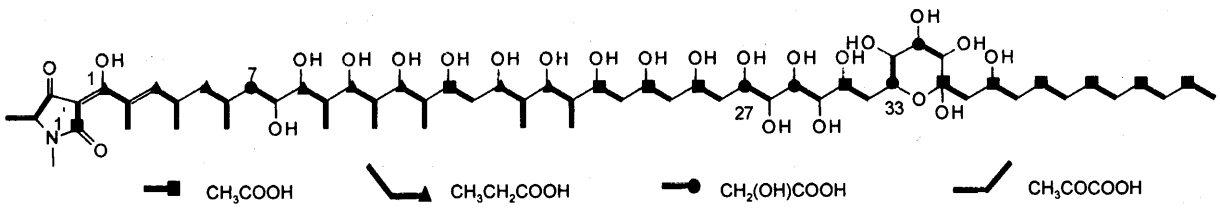
[a] Mean and standard deviation of triplicated experiments.
[b] Trace amount (<0.1 μg/ml) of aflatoxin was detected.

Table 3. ^{13}C Abundances and ^{13}C-^{13}C couplings in **1** obtained from feeding experiments with ^{13}C-labelled precursors.

C-No.	Relative abundance[a] in **1** biosynthesized from:				$^{1}J_{cc}$[b] (Hz)	^{13}C-^{13}C COSY[b] relation
	[1-^{13}C]propionate	[1-^{13}C] acetate	[2-^{13}C] acetate	[1-^{13}C] glycolate		
1	10.9	2.2	0.6	1.0		
2	1.1	0.9	*f*	1.2		
3	5.7	1.5	*f*	0.9		
4	1.0	1.0	1.0	1.0		
5	8.5	1.3	1.0	0.9		
6	1.2	0.8	*g*	1.0		
7	1.3	0.8	0.6	1.4	37.2	8
8	1.3	1.0	0.7	1.0	37.2	7
9	9.1	1.4	0.8	1.0		
10	1.3	0.9	1.2	0.8		
11	9.8	1.5	0.9	1.0		
12	*c*	*d*	*g*	1.0		
13	10.0	1.4	0.8	0.9		
14	*c*	*d*	*g*	1.0		
15	1.8	3.0	0.7	1.0	40.1	16
16	*c*	*d*	*g*	1.1	40.1	15
17	9.3	1.2	0.9	1.2		
18	*c*	*d*	*g*	*h*		
19	10.3	1.3	0.9	1.1		
20	*c*	*d*	*g*	1.0		
21	1.3	2.7	0.7	1.0	*i*	22
22	*c*	*d*	*g*	*h*	46.5	21
23	1.5	3.1	0.8	1.1	36.0	24
24	*c*	*d*	*g*	1.1	36.0	23
25	1.3	3.1	*g*	*h*	35.8	26
26	*c*	0.9	1.9	1.2	35.8	25
27	1.6	1.0	0.7	1.3	46.7	28
28	1.7	1.1	0.8	1.0	46.5	27
29	1.5	0.9	0.7	1.4	46.4	30
30	1.6	0.9	0.9	1.0	46.4	29
31	1.3	3.1	*g*	*h*	39.7	32
32	1.4	0.8	1.8	1.0	39.9	31
33	*c*	1.0	1.0	1.3	42.2	34
34	1.8	0.9	0.8	1.0	42.2	33
35	1.6	0.8	0.8	1.4	38.4	36
36	1.7	0.9	0.8	1.1	38.4	35
37	1.6	2.8	0.8	1.1	46.7	38
38	*c*	*d*	*g*	*h*	46.5	37
39	1.9	3.3	1.0	1.0	39.1	40
40	*c*	*d*	*g*	1.0	39.3	39
41	*c*	*e*	*g*	1.0	33.6	42
42	*c*	*e*	*g*	1.1	33.6	41
43	*c*	*e*	*g*	*h*	*i*	44
44	*c*	*e*	*g*	*h*	*i*	43
45	*c*	*e*	*g*	1.1	35.2	46
46	1.4	0.8	1.6	1.0	35.2	45
47	1.6	3.1	0.8	1.1	34.1	48
48	1.3	0.8	1.7	1.0	34.2	47
49	1.2	0.9	0.9	1.0		
50	1.2	0.9	0.9	1.0		
51	1.2	1.1	1.1	1.1		
52	1.2	1.1	1.1	0.9		
53	1.2	1.1	1.1	1.1		
54	1.3	1.1	1.1	1.0		
55	1.3	1.2	1.2	1.1		
56	1.4	1.2	1.2	1.1		
2'	1.8	3.6	0.8	0.9		
3'	1.2	0.8	1.3	0.8		
4'	2.3	1.4	1.2	1.1	36.9	5'
5'	1.0	0.9	0.3	1.1	*i*	4', 6'
6'	1.1	0.9	0.5	0.8	37.0	5'
7'	1.5	1.0	*g*	1.1		

[a] Peak height ratio of ^{13}C enriched to natural abundance. [b] In **1** from [*U*-$^{13}C_6$]glucose. [c,d] Can not be determined due to signal overlappings or ambiguous assignments. No enrichment was observed. [e] Can not be determined due to ambiguous assignments, but three of them were clearly enriched. [f] Not detected. [g] Can not be determined due to signal overlappings or ambiguous assignments. [h] Can not be determined due to signal overlappings or ambiguous assignments. No enrichment was observed. [i] Can not be determined due to signal overlappings.

Fig. 8. Biosynthetic origin of **1**.



values deduced from the spectra are listed in Table 3. ^{13}C-^{13}C coupling was observed between a carbon pair in the C_2 unit of C-7, 8 as well as between each carbon pair in all the C_2 units that derived from acetic acid. The coupling was also observed between each carbon pair in the C_2 units of C-27, 28, C-29, 30, C-33, 34 and C-35, 36, but no coupling between C-28 and C-29 or between C-34 and 35 was observed. This indicated that a tetraose derivative such as erythrose, whose whole carbon skeleton directly originated from the glucose carbon skeleton, was not intactly incorporated into two C_4 units. Other two ^{13}C-^{13}C couplings were also observed between C-4′ and C-5′, and C-5′ and C-6′, indicating that the C_3 unit of C-4′, 5′, 6′ originated from the glucose molecule. Thus, it was easily presumed that the origin of the C_3 unit should be alanine biosynthesized *via* pyruvate.

The results obtained suggested that a glycolic acid or its derivative might be incorporated into the C_2 unit of C-7, 8 and the C_4 units of C-27～30 and C-33～36. Finally, a feeding experiment using [1-^{13}C]glycolate was performed. In this case, a high level of ^{13}C incorporation was not observed, but enrichment of C-7, 27, 29, 33 and 35 was clearly determined (Table 3). From the above information, we may illustrate the biosynthetic origin of aflastatin A as summarized in Figure 8. The origin of all carbon atoms of **1** except for the C_1 unit at C-7′ has been clarified. Since aflastatin B, *N*-demethyl derivative of **1**, was obtained as a minor component, *N*-methylation of aflastatin B might be the final step in the biosynthetic pathway of aflastatin A. The biosynthetic origin of the two tetraol moieties of **1** is very unique. To our knowledge, this is the first case of a tetraol skeleton, which is biosynthesized from two molecules of glycolic acid.

The structures of aflastatins are tetramic acid derivatives with a highly oxygenated long alkyl chain. A saturated hydrocarbon skeleton forms the end part of their alkyl chain. Until now, a large number of natural products containing a tetramic acid skeleton have been isolated,[8～11] but the structures of aflastatins are very unique among them. Very recently, the structure of blasticidin A was determined, and it was shown that blasticidin A and aflastatin A have a similar structure and biological activity.[14] They may make up a new small group of antibiotics produced by *Streptomyces*. Work to investigate the stereochemistry and mode of action of aflastatins is now in progress.

Experimental

Isolation of Aflastatins

Culture method of *Streptomyces* sp. MRI142 for production of aflastatin A (**1**) and its isolation procedure have been reported previously.[6] As aflastatin B (**5**) was isolated as a minor component of aflastatins produced by strain MRI142, its isolation procedure was the same as that of **1**. Aflastatin B was precipitated from a tetrahydrofuran solution along with **1** as crude aflastatins. After the precipitate was washed with a solution of chloroform-methanol (2:1), the residue was purified by using HPLC on a C_{18} column (Capcell Pak C_{18} AG120, 15 mm × 250 mm, Shiseido Co., Tokyo). Elution was carried out with methanol-0.5% diethylamine in water (62:38) and a flow rate of 7.0 ml/minute at room temperature. Aflastatin B was eluted earlier than **1** from the HPLC column. The retention times of **1** and **5** were 18.8 and 16.1 minutes, respectively. Because of poor solubility of aflastatins in the mobile phase, this HPLC was repeated to obtain crude **5**, in which a small amount of **1** was contaminated. Rechromatography of the crude **5** under the same conditions, followed by lyophilization, gave 5.7 mg of **5** from 4.3 liters of culture broth. This yield was much smaller than that of **1** (500 mg from the same culture broth).

1: HR-FABMS (positive, glycerol matrix) *m/z* 1280.7749 $(M+Na)^+$ (Calcd for $C_{62}H_{115}NO_{24}Na$, 1280.7707); FAB-MS (negative, glycerol matrix) *m/z* 1256 $(M-H)^-$; *Anal* Calcd for $C_{62}H_{115}NO_{24}\cdot 7H_2O$:

C, 53.78; H, 9.39; N, 1.04, Found: C, 53.96; H, 8.95; N, 1.08; IR ν_{max} (KBr) cm^{-1} 3380, 1600, 1450, 1060; UV λ_{max} nm (ε), (MeOH-H_2O, 1:1): 299 (6,200), 247 (11,000); (MeOH-0.01 N NaOH, 1:1): 299 (6,200), 247 (11,000); (MeOH-0.01 N HCl, 1:1): 314 (7,300), 237 (7,900); $[\alpha]_D^{19}$ −2.6° (*c* 0.545, DMSO).

5: HR-FABMS (positive, glycerol matrix) *m/z* 1266.7477 $(M+Na)^+$ (Calcd for $C_{61}H_{113}NO_{24}Na$, 1266.7550); IR ν_{max} (KBr) cm^{-1} 3320, 1600, 1450, 1070; UV λ_{max} nm (ε), (MeOH-H_2O, 1:1): 299 (6,300), 246 (10,600); (MeOH-0.01 N NaOH, 1:1): 299 (6,300), 246 (10,600); (MeOH-0.01 N HCl, 1:1): 314 (7,300), 237 (8,000); $[\alpha]_D^{23}$ −0.6° (*c* 0.165, DMSO).

Preparation of Fragment **2** and **3**

A solution of $NaIO_4$ (340 mg) in water (8 ml) was mixed with a solution of aflastatin A (250 mg) in methanol (40 ml). The solution was stirred for 5 hours and left for 19 hours at room temperature in the dark. After decomposing excess $NaIO_4$ by adding a small amount of ethylenegylcol, $NaBH_4$ (1.0 g in 6 ml of 0.1 M NaOH) was added to the solution in an ice bath, which was stirred in an ice bath for 1 hour and then for 1 hour at room temperature. The reaction solution was neutralized with 3 N HCl and concentrated. The residue obtained was extracted with methanol and the extract was concentrated. A mixture of acetic anhydride (2 ml), pyridine (8 ml) and 4-dimethylaminopyridine (10 mg) was added to the residue, and the reaction mixture was stirred for 24 hours at room temperature, then was poured into water (140 ml). After adjusting pH to 2.0 with conc. HCl, the solution was extracted with ethyl acetate (50 ml × 3). The ethyl acetate extract was washed with 5% $NaHCO_3$ and concentrated to give a crude product (246 mg), which was chromatographed on a Sephadex LH-20 column (26 mm × 70 cm) packed in and eluted with methanol. A crude fragment **2** (50 mg) was eluted from the column at the elution volume from 246 ml to 308 ml. After evaporating the methanol, the obtained residue was purified with preparative HPLC on a C_{18} column (Capcell Pak C_{18} AG120, 15 mm × 250 mm, Shiseido Co., Tokyo). Elution was carried out with a linear gradient of methanol-0.1% diethylamine in water, starting with 35:65 to 60:40 for 40 minutes, then held 5 minutes, and a flow rate of 5.0 ml/minute at room temperature. The peak at the retention time of 42.2 minutes afforded fragment **2** (8.0 mg). **2**: EI-MS *m/z* 198 $(M)^+$; δ_H (CD_3OD, 500 MHz) 6.86 (dt, *J*=16, 7 Hz, H-3), 5.78 (dt, *J*=16, 1.5 Hz, H-2), 2.19 (H-4), 1.45 (H-5), 1.3 (H-6~11), 0.89 (t, *J*=7 Hz, H-12); δ_C (CD_3OD, 125 MHz) 171.5 (C-1), 149.7 (C-3), 124.0 (C-2), 33.1 (C-4), 33.1 (C-10), 30.7, 30.5, 30.4 and 30.3 (C-6, 7, 8, 9), 29.4 (C-5), 23.7 (C-11), 14.5 (C-12).

To obtain fragment **3**, a degradation experiment of **1** (250 mg) was carried out again with the same procedure by which **2** was obtained. A crude fragment **3** (119 mg) was eluted from the Sephadex LH-20 column (26 mm × 70 cm, MeOH) at the elution volume from 154 ml to 209 ml. Fragment **3** was purified with HPLC on the same column that **2** was purified. Elution was carried out with the linear gradient of acetonitrile-water, starting with 60:40 to 100:0 for 40 minutes, then held 15 minutes, and a flow rate of 8.0 ml/minute at room temperature. The peak at the retention time of 11.5 minutes afforded fragment **3** (28.2 mg). **3**: HR-FABMS (positive, NBA matrix) *m/z* 941.4405 $(M+Na)^+$ (Calcd for $C_{44}H_{70}O_{20}Na$, 941.4358); $[\alpha]_D^{20}$ +10.7° (*c* 1.319, MeOH); δ_H (CD_3OD, 500 MHz) 4.93 (dd, *J*=3, 8 Hz, H-3), 4.92 (H-7), 4.90 (H-9), 4.89 (H-15), 4.88 (H-17), 4.87 (H-13), 4.82 (H-5), 4.81 (H-11), 4.07 (H-19), 3.94 (dd, *J*=7, 11 Hz, H-1a), 3.83 (dd, *J*=6, 11 Hz, H-1b), 2.20 (H-4), 2.18 (H-2), 2.12 (H-10), 2.06 (H-6), 2.06 (H-12), 1.93 (H-18a), 1.91 (H-8a), 1.9 (H-16), 1.82 (H-18b), 1.78 (H-8b), 0.96 (d, *J*=7 Hz, H-20), 0.96 (d, *J*=7 Hz, H-21), 0.95 (d, *J*=7 Hz, H-22), 0.91 (d, *J*=7 Hz, H-24), 0.90 (d, *J*=7 Hz, H-23), 2.10, 2.07, 2.05, 2.04, 2.03, 2.03, 2.02, 2.02, 2.01 and 1.99 (Ac); δ_C (CD_3OD, 125 MHz) 76.5 (C-5), 75.9 (C-11), 75.2 (C-3), 75.0 (C-7), 73.6 (C-13), 73.2 (C-9), 70.1 (C-15), 69.8 (C-17), 67.9 (C-1), 61.7 (C-19), 40.0 (C-10), 39.6 (C-16), 39.3 (C-12), 39.1 (C-4), 39.1 (C-6), 38.2 (C-14), 35.3 (C-2), 34.2 (C-18), 32.8 (C-8), 14.6 (C-21), 11.4 (C-20), 11.4 (C-23), 9.6 (C-22), 9.0 (C-24), 21 and 172 (Ac).

Preparation of Fragment **4**

A solution of $NaIO_4$ (340 mg) in water (8 ml) was mixed with a solution of aflastatin A (250 mg) in methanol (40 ml). The solution was stirred for 5 hours and left for 15 hours at room temperature in the dark. After decomposing excess $NaIO_4$ by adding a small amount of ethyleneglycol, $NaBH_4$ (670 mg in 6 ml of 0.1 M NaOH) was added to the solution in an ice bath, which was stirred in an ice bath for 1 hour and then for 1 hour at room temperature. The pH of the reaction solution was adjusted to 6.0 with 2 N HCl in an ice bath and concentrated. The obtained residue was extracted with methanol and the extract was concentrated, which was chromatographed on a Sephadex LH-20 column (26 mm × 70 cm, MeOH). A crude fragment **4** (149 mg) was eluted from the column at the elution volume from

176 ml to 242 ml. After evaporating the methanol, the obtained residue was purified with preparative HPLC on the same column that **2** was purified. Elution was carried out with a linear gradient of methanol - 0.1% diethylamine in water, starting with 30 : 70 to 50 : 50 for 20 minutes, and a flow rate of 5.0 ml/minute at room temperature. The peak at the retention time of 19.1 minutes afforded fragment **4** (30.0 mg). **4**: HR-FABMS (positive, glycerol matrix) m/z 310.2019 $(M+H)^+$ (Calcd for $C_{17}H_{28}O_4N$, 310.2026); UV λ_{max} nm (ε), (MeOH - H_2O, 1 : 1): 299 (5,500), 247 (9,400); (MeOH - 0.01 N NaOH, 1 : 1): 299 (5,500), 247 (9,200); (MeOH - 0.01 N HCl, 1 : 1): 314 (7,300), 237 (7,300); $[\alpha]_D^{20}$ + 56.6° (c 1.381, MeOH); δ_H (CD_3OD, 500 MHz) 5.70 (br.d, J=9.9 Hz, H-3), 3.56 (H-8), 3.47 (q, J=6.8 Hz, H-5'), 2.85 (H-7'), 2.65 (H-4), 1.83 (d, J=1.4 Hz, H-9), 1.67 (H-6), 1.48 (H-7a), 1.34 (H-7b), 1.34 (H-5a), 1.26 (d, J=6.8 Hz, H-6'), 1.12 (H-5b), 1.00 (d, J=6.7 Hz, H-10), 0.90 (d, J=6.7 Hz, H-11); δ_C (CD_3OD, 125 MHz) 196.0 (C-4'), 195.5 (C-1), 175.8 (C-2'), 143.3 (C-3), 136.9 (C-2), 101.0 (C-3'), 61.8 (C-5'), 60.9 (C-8), 46.2 (C-5), 41.7 (C-7), 31.7 (C-4), 28.3 (C-6), 26.9 (C-7'), 21.1 (C-10), 20.4 (C-11), 16.3 (C-6'), 13.5 (C-9); HMBC correlations ($^nJ_{CH}$ = 8 Hz): H-3 to C-1, 4, 5, 9 and 10, H-4 to C-2, 3 and 10, H-5 to C-3, 4, 6, 7, 10 and 11, H-7 to C-5, 6, 8 and 11, H-8 to C-7, H-9 to C-1, 2, and 3, H-10 to C-3, 4 and 5, H-11 to C-5, 6 and 7, H-5' to C-2', 4' and 6', H-6' to C-4' and 5', H-7' to C-2' and 5'.

Evaluation of the Effect of Aflastatin B

Broth dilution method was employed to evaluate the effect of aflastatin B on the growth and the aflatoxin production of *Aspergillus parasiticus* according to the procedure previously reported.[6]

Administration of Labeled Compounds

Sodium [1-^{13}C]acetate (99 atom% ^{13}C), sodium [2-^{13}C]acetate (99 atom% ^{13}C) and sodium [1-^{13}C]propionate (99 atom% ^{13}C) were purchased from Aldrich Chemical Co. [U-$^{13}C_6$]glucose (99 atom% ^{13}C) was purchased from Isotech Inc., USA. Calcium[1-^{13}C]glycolate was prepared from [1-^{13}C]bromoacetic acid (99 atom% ^{13}C, Aldrich). Each labeled compound, except for calcium[1-^{13}C]glycolate, was dissolved in water at a concentration of 60 mg/ml. Calcium[1-^{13}C]-glycolate was dissolved in water at a concentration of 30 mg/ml because of its restricted solubility in water. The solutions of all of the compounds were autoclaved before administration. Portions (60 mg for sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, sodium [1-^{13}C]propionate and [U-$^{13}C_6$]glucose, and 120 mg for calcium[1-^{13}C]-glycolate) of each labeled compound were added to each 500 ml Erlenmeyer flask containing the medium (glucose 3%, soy bean meal 1.5%, K_2HPO_4 0.1%, $CaCO_3$ 0.4%, pH 7.2) at the 48th hour of cultivation. In the case of the experiment with [U-$^{13}C_6$]glucose, glucose in the medium was replaced with 3% soluble starch. After further cultivation for 120 hours and work up, 17.3 mg, 11.6 mg, 17.1 mg, 37.6 mg, and 11.9 mg of **1** were obtained from 8 × 60 ml broth in the experiments with sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, sodium [1-^{13}C]-propionate and [U-$^{13}C_6$]glucose, and calcium[1-^{13}C]-glycolate, respectively.

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