Aflastatin A, a Novel Inhibitor of Aflatoxin Production of *Aspergillus parasiticus*, from *Streptomyces*

Shohei Sakuda,*,† Makoto Ono,‡ Kazuo Furihata,†
Jiro Nakayama,† Akinori Suzuki,† and Akira Isogai§

Department of Applied Biological Chemistry
The University of Tokyo
Bunkyo-ku, Tokyo 113, Japan
Research Institute, Morinaga and Co., Ltd.
Tsurumi-ku, Yokohama 230, Japan
Graduate School of Biological Sciences
Nara Institute of Science and Technology
Ikoma, Nara 630-01, Japan

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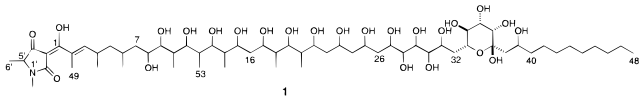
Aflatoxin, a mycotoxin produced by fungi, is one of the most potent environmental carcinogens, which causes worldwide concern about contamination of agricultural products. To protect foods from contamination, some fungicides are usually used to kill the producer. However, because they are toxic to mammals to some extent and easily produce a drug resistant strain, a safer drug having higher selectivity is desired. This prompted us to search for an inihibitor of aflatoxin biosynthesis using a system of *Aspergillus parasiticus* and an aflatoxin-sensitive bacteria.[1] During the course of the screening, *Streptomyces* sp. MRI142 was found to produce a strong inhibitor, named aflastatin A (**1**), which inhibits aflatoxin biosynthesis,



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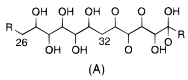
but does not inhibit the growth of its producer. This Communication describes the preliminary elucidation of the structure and biosynthesis of **1**.

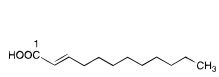
Aflastatin was isolated from a mycelial MeOH extract of the MRI142 strain as a white powder.[2] The molecular formula of **1** was determined as $C_{62}H_{115}NO_{24}$ from elemental analysis, and analysis of the HR-FABMS spectrum and NMR spectra. The UV spectrum of **1** was pH-sensitive, and the IR and ^{1}H NMR spectra of **1** in a DMSO-d_6 solution showed that **1** is polyhydroxylated. By analyzing the DQF-COSY, DQF-relayed COSY, HMQC, and HMBC spectra of **1**, the presence of a highly oxygenated partial structure (A) was clarified. Other small



(A)

partial structures were also identified from the spectra, but it was difficult to determine the total structure of **1** by further NMR analysis with the intact molecule. Since the structure A was obtained, oxidation of **1** with $NaIO_4$ was carried out to obtain fragment molecules to identify the remainder of the structure.

$NaIO_4$ oxidation of **1**, followed by $NaBH_4$ reduction and acetylation, afforded two main products, **2** and **3**.[3] The structure



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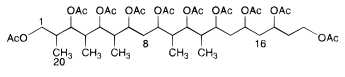

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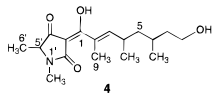
of a fragment was determined as **2** by analysis of its MS and NMR spectra. Another fragment had no characteristic absorption in its UV spectrum, and the molecular formula was determined as $C_{44}H_{70}O_{20}$ by its HR-FABMS spectrum. By analysis of COSY and HMQC spectra, 10 partial structures, $AcOCH_2CH(CH_3)-$, $-CH(CH_3)CH(OAc)-$ × 4, $-CH_2CH(OAc)-$ × 4, and $-CH_2CH_2OAc$, which contained all atoms involved in the fragment, were identified. The connections among these partial structures were determined by analyzing the HMBC spectrum, 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, the fragment, whose UV spectrum showed the same absorption maxima as **1**, was obtained as a product.[3] The HR-FABMS spectrum of **4**



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indicated that the molecular formula was $C_{17}H_{27}NO_4$. The UV spectrum of **4** and the chemical shifts of four carbon signals (δ_C 195.5, 175.8, 101.0, and 196.0 for C-1, -2′, -3′, and -4′, respectively) contained in **4** strongly suggested the presence of a tetramic acid skeleton,[4] which was confirmed by an HMBC experiment. By further analysis of COSY, HMQC, and NOESY spectra, its structure was determined as **4**.

Because all 62 carbon atoms of **1** were involved in structure A or fragment **2**, **3**, or **4**, its total structure could be constructed with them. Since **1** had no α,β-unsaturated carboxylic acid,

† The University of Tokyo.
‡ Morinaga and Co., Ltd.
§ Nara Institute of Science and Technology.

(1) The assay method for screening and biological activity of aflastatin will be published in detail elsewhere.

(2) After removing the solvent of the mycelium extract, the residue was triturated with *n*-BuOH, and the *n*-BuOH layer was concentrated. The residual oil was suspended in tetrahydrofuran, and an active amorphous precipitate was obtained. After this precipitate was washed with a solution of $CHCl_3$/MeOH (2:1), the residue was finally purified by reversed-phase HPLC under basic conditions to afford **1** in the yield of 12 mg/100 mL. **1**: HR-FABMS (positive, glycerol matrix) *m*/*z* 1280.7749 $(M + Na)^+$ (calcd for $C_{62}H_{115}O_{24}NNa$, 1280.7707); FABMS (negative, glycerol matrix) *m*/*z* 1256 $(M - H)^-$; Anal. Calcd for $C_{62}H_{115}O_{24}N{\cdot}7H_2O$: C, 53.78; H, 9.39; O, 35.82; N, 1.04. Found: C, 53.96; H, 8.95; O, 35.77; N, 1.08; IR ν_{max} (KBr) (cm^{-1}) 3380, 1600, 1450, 1060; UV λ_{max} (nm) (ϵ) (MeOH−H_2O, 1:1) 299 (6200), 247 (11 000); (MeOH−0.01 N NaOH, 1:1) 299 (6200), 247 (11 000); (MeOH−0.01 N HCl, 1:1) 314 (7300), 237 (7900); $[\alpha]^{19}_D$ −2.6° (*c* 0.545, DMSO).

(3) **2** : EIMS *m*/*z* 198 $(M)^+$; ^{1}H NMR 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); ^{13}C NMR 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). **3**: HR-FABMS (positive, NBA matrix) *m*/*z* 941.4405 $(M + Na)^+$ (calcd for $C_{44}H_{70}O_{20}Na$, 941.4358); $[\alpha]^{20}_D$ +10.7° (*c* 1.319, MeOH); ^{1}H NMR 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); ^{13}C NMR 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). **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 (5500), 245 (9400); (MeOH−0.01 N NaOH, 1:1) 299 (5500), 245 (9200); (MeOH−0.01 N HCl, 1:1) 316 (7300), 234 (7300); $[\alpha]^{20}_D$ +56.6° (*c* 1.381, MeOH); ^{1}H NMR 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); ^{13}C NMR 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-1,4,5,9,10, H-4 to C-2,3,10, H-5 to C-3,4,6,7,10,-11, H-7 to C-5,6,8,11, H-8 to C-7, H-9 to C-1,2,3, H-10 to C-3,4,5, H-11 to C-5,6,7, H-5′ to C-2′,4′,6′, H-6′ to C-4′,5′, H-7′ to C-2′,5′.

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acetoxymethyl, or hydroxymethyl groups in its molecule, these functional groups in the three fragments had to be produced during the chemical degradation reactions described above. 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**. **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 correlation between the methylene proton at C-26 and the methine carbon at C-27 in an HMBC experiment supported the linkage between **3** and structure A.

From the above results and molecular formula of **1**, the only remaining problem was the position of an ether linkage. The *J* values around C-33 to C-37 ($J_{33,34} = J_{34,35} = 9.5$ Hz, $J_{35,36} = 3$ Hz) strongly suggested that a tetrahydropyran ring was formed by the ether linkage betweeen C-33 and C-37, and it was present in the chair form. This was further confirmed by NOEs between the proton of the hydroxyl group at C-37 and H-33, and this proton and H-35. 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.

To elucidate the biosynthesis of **1**, feeding experiments with [1-^{13}C]- and [2-^{13}C]acetate and [1-^{13}C]propionate were performed. The ^{13}C-labeling pattern of **1** deduced from the experiments 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, but 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. A glycolic acid or a related molecule might be incorporated into these parts as in the case with geldanamycin[5] or luecomycin biosynthesis.[6] The origin of these unusual groups is under investigation.

Aflastatin inhibited aflatoxin production in *A. parasiticus* at the concentration of 0.5 μg/mL completely, but did not inhibit its growth at the same concentration. Since no accumulation of any biosynthetic intermediates of aflatoxin was detected in the broth of *A. parasiticus* when **1** was added to the culture, it may not be an inhibitor of an enzyme involved in the biosynthetic pathway of aflatoxin. It is known that a protein phosphatase inhibitor, tautomycetin, regulates the secondary metabolite production of the fungus *Penicillium urticae*.[7] Thus, inhibitory activity of **1** toward some protein phosphatases was examined. In a preliminary study, type 2A protein phosphatase was found to be inhibited by **1**, but only weakly. Studies to elucidate the stereochemistry and a mode of action of **1** are now in progress.

Table 1. ^{1}H and ^{13}C Assignments of **1**[a]

C no.	δ_C	δ_H	C no.	δ_C	δ_H
1	191.5		29-OH		4.12
2	135.2		30	72.5	3.45
3	139.3	5.45, br d (10)	30-OH		4.17
4	29.9	2.54	31	68.6	3.83
5	44.8	0.93, t (11), 1.34	31-OH		4.12
6	26.1	1.90	32	35.8	2.06, 1.48
7	42.8	1.26	33	70.2	3.62
8	67.2	3.64	34	71.2	3.18, t (9.5)
9	75.0	3.26	35	70.7	3.56, dd (9.5, 3)
10	37.1	1.81	36	73.0	3.41
11	75.8	3.71	36-OH		4.45
11-OH		5.20	37	98.4	
12	38.1	1.62	37-OH		6.11
13	78.9	3.67	38	41.6	1.82, 1.42
13-OH		5.26	39	68.6	3.87
14	38.1	1.67	39-OH		4.75
15	74.5	3.85	40	38.1[b]	1.30
15-OH		4.79	41	24.9[c]	1.23
16	44.5[b]	1.39, 1.55	42	28.7[c]	1.23
17	70.4	3.91	43	29.0[c]	1.23
17-OH		4.71	44	29.0[c]	1.23
18	41.6	1.65	45	29.2[c]	1.23
19	76.2	3.46	46	31.3	1.23
19-OH		4.57	47	22.1	1.23
20	38.1	1.53	48	13.9	0.84, t (7)
21	73.5	3.81	49	13.3	1.67
21-OH		4.76	50	21.5	0.88, d (6.5)
22	41.6[b]	1.53	51	20.8	0.85, d (7)
23	67.9	3.79	52	8.7	0.84, d (7)
23-OH		4.73	53	12.8	0.64, d (7)
24	34.9[b]	1.30, 1.60	54	6.4	0.81, d (7)
25	67.5	3.89	55	10.6	0.68, d (7)
25-OH		5.24	56	5.8	0.79, d (7)
26	41.0	1.35, 1.85	2′	173.4	
27	69.7	3.63	3′	98.1	
27-OH		4.66	4′	192.6	
28	74.3	3.25	5′	59.4	3.20
28-OH		4.59	6′	15.9	1.11, d (7)
29	69.4	3.82	7′	26.3	2.68

[a] Spectra were obtained in DMSO-d_6 on a JEOL GX-500. Coupling constants in hertz are given in parentheses. [b,c] May be interchanged.

Supporting Information Available: Table of the summary of the feeding experiments, HMBC spectrum of **1**, partial HMBC spectrum of **1**, partial DQF-relayed COSY spectrum of **1**, partial COSY spectrum of **1**, summary of COSY, relayed COSY, and HMBC correlations observed in partial structure A, HMBC spectrum of **3**, and summary of HMBC correlations in **3** (8 pages). See any current masthead page for ordering and Internet access instructions.

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(4) A large number of naural products containing a tetramic acid skeleton have been isolated. Recent examples: (a) Phillips, N. J; Goodwin, J. T.; Fraiman, A.; Cole, R. J.; Lynn, D. G. *J. Am. Chem. Soc.* **1989**, *111*, 8223−8231. (b) Hayakawa, Y.; Kanamaru, N.; Morisaki, N.; Furihata, K.; Seto, H. *J. Antibiot.* **1991**, *44*, 288−292. (c) Matsunaga, S.; Fusetani, N.; Kato, Y.; Hirota, H. *J. Am. Chem. Soc.* **1991**, *113*, 9690−9692. (d) Kanazawa, S.; Fusetani. N.; Matsunaga, S. *Tetrahedron Lett.* **1993**, *34*, 1065−1068.

(5) Haber, A.; Johnson, R. D.; Rinehart,Jr., K. L. *J. Am. Chem. Soc.* **1977**, *99*, 3541−3544.

(6) Omura, S.; Tsuzaki, K.; Nakagawa, A.; Lukacs, G. *J. Antibiot.* **1983**, *36*, 611−613.

(7) Sakuda, S.; Miki, K.; Kitaoka, S.; Reugjitchachawaly, M.; Yamada, Y. *Biosci., Biotechnol., Biochem.* **1995**, *59*, 133−134.