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Ushikulides A and B, Immunosuppressants Produced by a Strain of *Streptomyces* sp.

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Abstract Novel immunosuppressants, ushikulides A and B, were isolated from the culture broth of *Streptomyces* sp. IUK-102. Ushikulides A and B both have the same molecular formula, determined as $C_{40}H_{68}O_{10}$. The structures of both compounds were elucidated to be novel 22-membered macrolides. Both compounds showed immunosuppressive activity for murine splenocyte proliferation *in vitro*.

Keywords 22-membered macrolides, ushikulide, immunosuppressants

Cyclosporin A [1] and FK506 [2], extremely potent immunosuppressive agents, have revolutionized organ transplantation by blocking the activation of lymphocytes and are widely used in preventing rejection. Cyclosporin A and FK506 respectively bind to cyclophilin and FK506-binding protein (FKBP), so-called immunophilin [3]. Binding of each of these drug-protein complexes to a catalytic subunit of carcineurin causes inhibition of signal transduction in T cell activation [3]. However, these agents have some adverse side-effects such as nephrotoxicity and chronic rejection [4, 5]. Another potent immunosuppressive agent is desired to develop a safer medical treatment. During our screening for immunosuppressants from microbial products, novel immunosuppressants ushikulides A (**1**) and B (**2**) were found in the culture broth of *Streptomyces* sp. IUK-102 (Fig. 1). This paper describes the isolation, structure elucidation, and biological activities of these compounds.

The producing strain, *Streptomyces* sp. IUK-102, was isolated from a soil sample collected in Ibaraki prefecture,

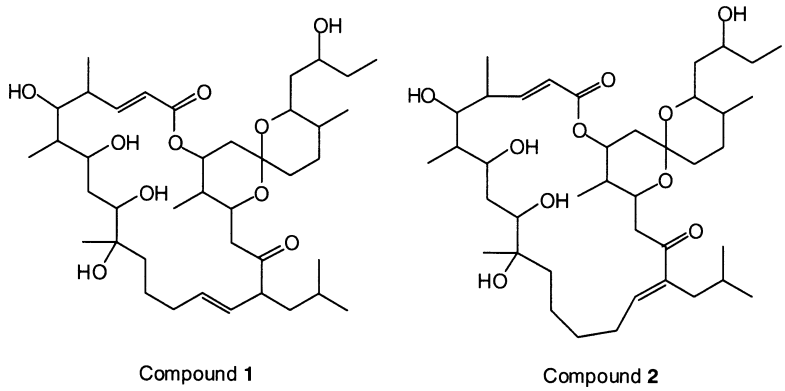

Fig. 1 Structures of ushikulides A (**1**) and B (**2**).

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Japan. This strain was incubated at 27°C for five days in 500-ml Erlenmeyer flasks, each containing 70 ml of medium composed of $(NH_4)_2SO_4$ 0.14%, KH_2PO_4 0.2%, $CaCl_2$ 0.03%, $MgSO_4 \cdot 7H_2O$ 0.03%, urea 0.03%, polypepton 0.5%, yeast extracts 0.1%, soybean meal 3%, glucose 1%, soluble starch 0.5%, $FeSO_4 \cdot 7H_2O$ 0.005%, $MnSO_4 \cdot 5H_2O$ 0.0016%, $ZnSO_4 \cdot 7H_2O$ 0.0014%, and $CoCl_2$ 0.002% (pH 7.0 before sterilization). After fermentation on a rotary shaker (230 rpm), four liters of the culture broth was centrifuged to separate mycelia and supernatant. The mycelia were extracted with acetone and the extract was evaporated to give an aqueous residue. This residue was then extracted with EtOAc and the soluble portion was concentrated *in vacuo* to obtain a brown residue (3.5 g). The residue was subjected to silica gel chromatography and eluted using $CHCl_3$ followed by a mixture of $CHCl_3$ and MeOH (90 : 10). Second elution with activity was evaporated and fractionated on a silica gel column using a mixture of $CHCl_3$ and MeOH (95 : 5). The fractions containing active substrates were combined and concentrated *in vacuo*. This active material was then subjected to reverse-phase HPLC (CAPCELL PAK C_{18} SG 120, Shiseido Co. Ltd., 20×250 mm) using a mixture of CH_3CN and H_2O (70 : 30) as the isocratic mobile phase at a flow rate of 5.0 ml/minute. Ushikulides A (**1**) and B (**2**) were eluted at 32.1 and 30.0 minutes, respectively. These fractions were collected and concentrated under reduced pressure. Finally, **1** (13 mg) and **2** (5 mg) were obtained as white powders.

Ushikulide A (**1**) was obtained as a colorless powder with a specific rotation of −12.7° (*c* 0.5, MeOH, 25°C). The molecular formula of **1** was established as $C_{40}H_{68}O_{10}$ on the basis of HRFAB-MS (found 709.4910, calcd. 709.4883 for M+H). The IR spectrum (KBr) indicated the presence of hydroxyl and carbonyl groups at 3456 and 1719 cm^{-1}, respectively. From ^1H and ^{13}C NMR spectral data, compound **1** was shown to possess a ketone, an ester carbonyl, 4 sp^2 methines, 2 sp^3 quaternary carbons, 13 sp^3 methines including 7 oxymethines, 11 sp^3 methylenes, and 8 methyl carbons. Seven degrees of unsaturation were inferred from the molecular formula. The COSY and TOCSY spectra of **1** indicated four ^1H-^1H spin coupling systems (Fig. 2). Spin systems A and B were connected through a series of HMBC correlations from H-34 to C-9, C-10, and C-11, and from H_2-11 (δ_H 1.52/1.54) to C-10. Spin systems B and C were linked by HMBC correlations of H-16 and H-18 (δ_H 2.73) to C-17 (δ_C 212.5). The long range couplings from H-2 (δ_H 5.81) and H-21 (δ_H 5.33) to C-1 (δ_C 166.5) established the ester linkage of spin systems A and C, indicating the presence of a 22-membered lactone ring in the structure. The configurations of the two double bonds in the structure were demonstrated as (*E*) by coupling constant values of H-2 to H-3 (15.7 Hz), and H-14 to H-15 (15.0 Hz). HMBC correlations from H-19, H-22, H-24, and H-27 to C-23 (δ_C 98.9) indicated connectivity between spin systems C and D and the presence of 1,1′-dioxaspiro[5.5]undecane, which was fused onto the 22-membered lactone structure. Accordingly, the structure of **1** was determined to be that shown in Fig. 1. All the assigned proton and carbon signals are listed in Table 1.

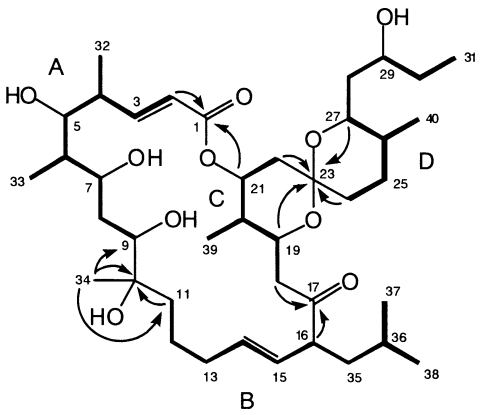

Fig. 2 2D-NMR correlations for **1**. Bold lines show TOCSY correlations, and arrows show HMBC correlations.

Ushikulide B (**2**) was obtained as a colorless powder with a specific rotation of −46.7° (*c* 0.5, MeOH, 25°C). The molecular formula of **2** was determined to be $C_{40}H_{68}O_{10}$ by HRFAB-MS (found 707.4728, calcd. 707.4734 for M–H), which was the same as that of **1**. The IR spectrum (KBr) indicated the presence of hydroxyl and carbonyl groups at 3437 and 1718 cm^{-1}, respectively. Comparison of the ^1H NMR spectra of **2** and **1** revealed that the double bond proton signals at δ_H 5.60 and δ_H 5.44 present in **1** were missing in **2**. Instead, a proton signal at δ_H 6.97 was identified in **2**. ^{13}C NMR spectrum of **2** showed four carbon signals at δ_C 152.2, 149.4, 143.5, and 122.7 attributed to olefinic carbons. The significant difference observed between the NMR spectra resulted from a change in the olefin position. Besides this obvious difference, the 1D NMR spectra of these compounds resembled each other. Additionally, TOCSY and HMBC correlations of **2** (Fig. 3a) suggested that the basal structure of this compound was similar to that of **1**, however, more information was still required to establish the structure of **2**. Final structure confirmation was accomplished by a ROESY spectrum (Fig. 3b). The configuration of a double bond composed of C-15 and C-16 was shown to be (*E*) from ROESY correlation between H-15 and H-18 (δ_H 2.31). Correlation of H-27 with H-19 revealed a spiroketal

Table 1 NMR spectral data of ushikulides A (**1**) and B (**2**) in CD_3OD

No.	Ushikulide A (**1**)		Ushikulide B (**2**)	
	δ_H (*J* Hz)	δ_C	δ_H (*J* Hz)	δ_C
1		166.5		166.5
2	5.81 d (15.7)	122.7	5.80 d (15.8)	122.7
3	6.76 dd (15.7, 10.0)	152.2	6.75 dd (15.8, 10.0)	152.2
4	2.46 m	42.9	2.45 m	43.0
5	3.62 br.d (9.9)	81.3	3.59 dd (9.9, 1.7)	80.6
6	1.56 m	38.6	1.52 m	39.7
7	4.10 br.s	76.0	4.03 m	76.1
8	1.69 m	36.4	1.52 m	40.0
	1.73 m		1.69 m	
9	3.17 br.d (10.8)	75.0	3.28 (CD_3OD overlapped)	75.6
10		75.5		75.4
11	1.52 m	39.8	1.42 m	39.9
	1.54 m		1.57 m	
12	1.43 m	24.1	1.29 m	23.6
	1.52 m		1.47 m	
13	2.04 m	34.6	1.57 m	30.4
	2.23 m		1.63 m	
14	5.60 ddd (15.0, 9.4, 4.2)	136.7	2.37 m	29.8
			2.44 m	
15	5.44 dd (15.0, 9.0)	129.6	6.97 dd (8.7, 5.1)	149.4
16	3.07 ddd (8.6, 8.6, 6.4)	57.8		143.5
17		212.5		203.5
18	2.73 dd (17.4, 8.0)	42.0	2.31 dd (13.3, 4.8)	39.2
	2.78 dd (17.8, 6.4)		3.60 dd (13.3, 10.9)	
19	4.33 br.t (6.3)	67.2	4.19 m	70.2
20	2.05 m	34.7	1.85 m	34.7
21	5.33 ddd (11.1, 5.4, 5.4)	71.3	5.21 ddd (10.6, 4.9, 4.9)	71.4
22	1.68 m	36.4	1.72 m	36.3
	1.72 m			
23		98.9		99.4
24	1.43 m	30.6	1.47 m	30.5
	1.65 m		1.67 m	
25	1.40 m	27.5	1.42 m	27.6
	2.19 m		2.14 m	
26	1.48 m	32.1	1.59 m	32.1
27	4.02 m	69.1	4.09 m	69.3
28	1.28 m	41.7	1.28 m	41.4
	1.63 m		1.62 m	
29	3.73 br.s	70.6	3.78 m	70.4
30	1.47 m	31.9	1.49 m	32.2
	1.53 m		1.54 m	
31	0.97 t (7.4)	10.2	1.00 t (7.4)	10.3
32	1.14 d (6.4)	18.0	1.13 d (6.6)	18.0
33	0.85 d (6.9)	4.2	0.85 d (6.9)	4.9
34	1.06 s	22.7	1.08 s	23.2
35	1.41 m	41.3	2.22 d (7.1)	35.3
	1.53 m			
36	1.53 m	26.7	1.63 m	29.3
37	0.91 d (5.9)	23.2	0.86 d (5.4)	23.0
38	0.87 d (6.2)	22.4	0.82 d (6.6)	22.9
39	0.79 d (6.9)	6.2	0.92 d (6.9)	6.1
40	0.94 d (6.9)	11.5	0.95 d (6.9)	11.5

^1H and ^{13}C NMR were observed at 500 and 125 MHz, respectively.

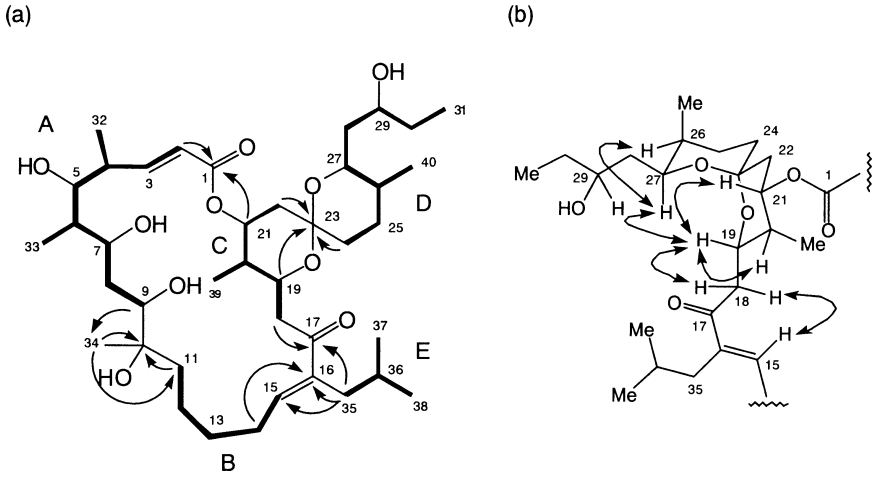

Fig. 3 2D-NMR correlations for **2**: (a) TOCSY (bold lines) and HMBC (arrows) correlations, (b) key ROESY correlations for spiroketal moiety.

subunit connecting spin systems C and D. Moreover, additional ROESY cross peaks were observed as shown in Fig. 3b. The configuration of the spiroketal in **2** was quite similar to that in cytovaricin [6], therefore, the structure of **2** was determined as that in Fig. 1. The complete assignments of proton and carbon signals in **1** and **2** revealed the considerable difference of proton chemical shifts of H_2-18 between these compounds (Table 1). This suggested that the positional change of the olefin near the ketone group caused a conformation of 22-membered lactone ring to be changed. As a result, H_2-18 at δ_H 2.31 and 3.60 in **2** were placed in a shielding area of the ketone group and a deshielding area of the ketone group, respectively.

The immunosuppressive activities of these compounds were estimated by concanavalin A (ConA) stimulated lymphocyte blastogenesis [7]. Both compounds inhibited murine splenic lymphocyte proliferation with IC_{50} values of 70 nM, comparable to that of cyclosporin A. Compounds **1** and **2** did not show significant cytotoxicity for KB cells at a concentration of 10 μM.

Ushikulides A (**1**) and B (**2**) are related to phthoramycin [8], kaimonolide A [9], and the aglycone of cytovaricin [6, 10], which are thought to be members of the oligomycin family from the point of view of biosynthesis. Oligomycin F [11] and 41-demethylhomooligomycin B [12], which have a 26-membered lactone ring in their structures, are known to possess immunosuppressive activities. As far as we know, this is the first report describing a 22-membered macrolide with a spiroketal moiety that shows immunosuppressive activity.

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