DELAMINOMYCINS, NOVEL EXTRACELLULAR MATRIX RECEPTOR ANTAGONIST

IV. STRUCTURE-ACTIVITY RELATIONSHIPS OF DELAMINOMYCINS AND DERIVATIVES

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Delaminomycins A, B, C and their derivatives were prepared and investigated biological activities of them. Among these compounds, spiro compounds (**A2**, **B2** and **C2**) showed stronger inhibitory activity than natural products (**A1**, **B1** and **C1**) on B16 melanoma cells adhesion assay and Con A-induced proliferation of murine splenic lymphocytes assay. In MLCR and antimicrobial assay, however, **A1**, **B1** and **C1** showed more potent inhibitory activity than spiro compounds (**A2**, **B2** and **C2**).

On the other hand, as to C-5′ substituents of pyrrolidine ring, the order of inhibitory activity was $R=OH>R=OCH_3>R=H$ on Con A-induced proliferation of murine splenic lymphocytes assay. In MLCR and antimicrobial assay, however, the order of inhibitory activities were $R=H>R=OCH_3>R=OH$.

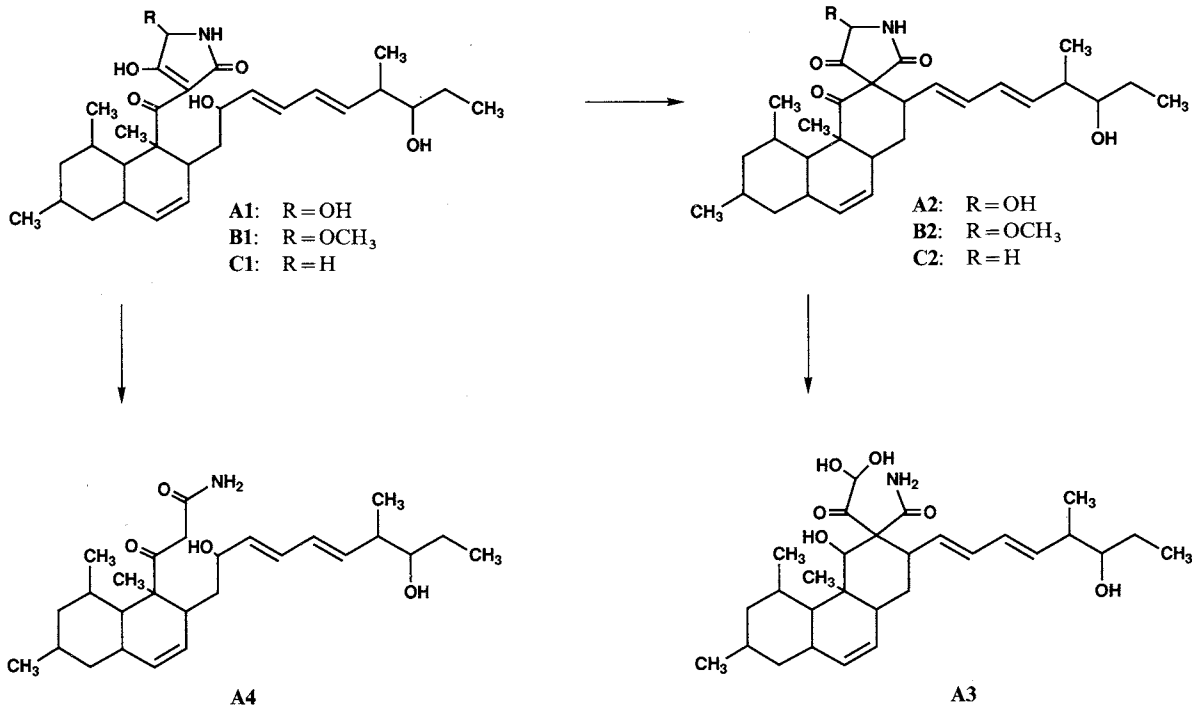
Inhibitory activities of **A4** which was lacked pyrrolidine ring were reduced on B16 melanoma cells adhesion assay and on cytotoxicity against tumor cells *in vitro* in comparison with those of **A1**.

In the course of screening for inhibitors of cell adhesion to fibronectin (FN), laminin (LM) and collagen type IV (CL), components of the extracellular matrix (ECM), we found new non-peptide antibiotics, delaminomycins, produced by *Streptomyces albulus* MJ202-72F3. In the preceding paper, we reported that delaminomycins suppressed immune responses *in vitro* and *in vivo* and exhibited antimicrobial effect on Gram-positive bacteria[1]. In this paper, we report the biological activities of delaminomycins and their derivatives and the structure-activity relationship of them.

Chemistry

We have described the synthesis of 5′-Hydroxy-2-[(1*E*,3*E*)-6-hydroxy-5-methyl-1,3-octadienyl]-4a,5,7-trimethyl-1,2,3,4,4a,4b,5,6,7,8,8a,10a-dodecahydrophenanthrene-3-spiro-3′-pyrrolidine-2′,4,4′-trione (**A2**) from delaminomycin A (**A1**) in the preceding paper[2]. As shown in Fig. 1, **A1** was converted to form a spiro ring upon acid treatment into **A2**. Compounds 5′-Methoxy-2-[(1*E*,3*E*)-6-hydroxy-5-methyl-1,3-octadienyl]-4a,5,7-trimethyl-1,2,3,4,4a,4b,5,6,7,8,8a,10a-dodecahydrophenanthrene-3-spiro-3′-pyrrolidine-2′,4,4′-trione (**B2**) and 2-[(1*E*,3*E*)-6-hydroxy-5-methyl-1,3-octadienyl]-4a,5,7-trimethyl-1,2,3,4,4a,4b,5,6,7,8,8a,10a-dodecahydrophenanthrene-3-spiro-3′-pyrrolidine-2′,4,4′-trione (**C2**) were prepared from delaminomycins B (**B1**) and C (**C1**)[3] by the same method used for the synthesis of **A2**.

Fig. 1. Structures of delaminomycins and analogs.



Reduction of **A2** with $NaBH_4$ gave a tetrahydro derivative 4′,5′-Dihydroxy-2-[(1*E*,3*E*)-6-hydroxy-5-methyl-1,3-octadienyl]-4-hydroxy-4a,5,7-trimethyl-1,2,3,4,4a,4b,5,6,7,8,8a,10a-dodecahydrophenanthrene-3-spiro-3′-pyrrolidine-2′-one (**A3**) which was obtained as an epimeric mixture. An alkali hydrolysis of **A1** with NaOH gave 3-2-[(3*E*, 5*E*)-2,8-Dihydroxy-7-methyl-3,5-decadienyl]-1,6,8-trimethyl-1,2,4a,5,6,7,8,8a-octahydro-1-naphthyl]-3-oxopropionamide (**A4**).

Biological Activity

B16 Cell Adhesion Assay

B16 melanoma cell adhesion assays[1] were performed using the modified method reported by GRAF *et al*[4,5]. As shown in Table 1, **A1**, **A2** and **B2** at 2.8～6.5 μg/ml showed stronger inhibitory activity on adhesion of B16 melanoma to all ECM components tested than that of other delaminomycins. **A2**, **B2** and **C2** showed stronger inhibitory activity than **A1**, **B1** and **C1**, respectively. **C1** and **C2** showed no inhibition on FN at 100 μg/ml. Inhibitory activity of **A3** was slightly reduced reduced in compared with that of **A1**, and inhibitory activity of **A4** was further reduced than that of **A3**.

Cytotoxicity

Tumor cells were cultured in RPMI-1640 containing 10% FCS with test samples for 72 hours in 5% CO_2 and 95% air and cytotoxicity was determined by MTT assay[1]. As shown in Table 2, **A2** and **B2** showed stronger cytotoxicity than **A1** and **B1**, respectively, although **C1** showed stronger cytotoxicity than **C2** except for L1210. L1210 cells were rather resistant to delaminomycins and their analogs tested than other tumor cells.

Con A-induced Proliferation of Murine Splenic Lymphocytes.

Con A-induced proliferation assays were performed by the method described previously[1]. As shown in Table 3, all samples tested showed inhibitory activity against Con A-induced proliferation of murine splenic lymphocytes. **A2** and **B2** showed stronger inhibitory activity than **A1** and **B1**, respectively. **A2** showed the strongest activity and IC_{50} value was 0.78 μg/ml.

Mixed Lymphocytes Culture Reaction (MLCR)

MLCR assay was performed by the method described previously[1]. As shown in Table 4, all samples

Table 1. Inhibition of adhesion of B16 melanoma cells to ECM components by delaminomycins and analogs.

Compounds	IC_{50} (μg/ml)		
	Laminin	Fibronectin	Type IV collagen
A1	6.5	6.0	3.3
A2	4.4	3.7	4.4
A3	13.4	13.0	10.7
A4	36.9	50.0	47.9
B1	17.0	17.0	14.0
B2	3.3	2.8	4.8
C1	25.0	>100	14.5
C2	12.4	>100	22.3

Table 2. Cytotoxicity of delaminomycins and analogs on tumor cells in cultures.

Compounds	IC_{50} (μg/ml)			
	L1210	$P388D_1$	EL4	B16
A1	>100	14.0	17.9	21.1
A2	3.7	10.0	4.1	8.3
A3	17.4	N.D.	N.D.	18.0
A4	>100	N.D.	N.D.	52.2
B1	30.0	13.3	11.1	>100
B2	8.0	12.1	6.8	17.9
C1	42.0	3.1	1.9	7.4
C2	3.6	22.0	13.3	16.9

Table 3. Inhibitory effect of delaminomycins and analogs on Con A induced proliferation of splenic lymphocytes.

	Compounds					
	A1	**A2**	**B1**	**B2**	**C1**	**C2**
IC_{50} (μg/ml)	17.5	0.78	4.0	0.85	1.0	1.6

Table 4. Inhibitory effect of delaminomycins and analogs on MLCR.

	Compounds					
	A1	**A2**	**B1**	**B2**	**C1**	**C2**
IC_{50} (μg/ml)	8.6	13.9	1.1	8.6	0.5	37.9

Table 5. Antimicrobial activity of delaminomycins and analogs.

Microorganisms	MIC (μg/ml)					
	A1	**A2**	**B1**	**B2**	**C1**	**C2**
Staphylococcus aureus FDA209P	12.5	12.5	6.25	50	3.12	100
S. aureus Smith	25	12.5	6.25	>100	3.12	>100
S. aureus MS9610	25	12.5	6.25	>100	3.12	>100
S. aureus No. 5 (MRSA)	25	12.5	6.25	>100	3.12	>100
S. aureus No. 17 (MRSA)	25	12.5	6.25	>100	3.12	>100
Micrococcus luteus FDA16	6.25	6.25	3.12	12.5	1.56	>100
M. luteus IFO3333	6.25	6.25	3.12	12.5	1.56	100
M. luteus PCI1001	100	12.5	6.25	>100	1.56	>100
Bacillus anthracis	3.12	6.25	1.56	25	<0.78	100
B. subtilis NRRL B-558	6.25	6.25	6.25	>100	3.12	>100
B. subtilis PCI219	6.25	12.5	6.25	>100	3.12	>100
B. cereus ATCC 10702	6.25	6.25	3.12	50	1.56	>100
Corynebacterium bovis 1810	3.12	12.5	6.25	100	3.12	>100

tested showed inhibitory activity against MLCR. Among them **A1**, **B1** and **C1** showed stronger inhibitory activity than **A2**, **B2** and **C2**, respectively. **C2** showed the lowest activity (37.9 μg/ml in IC_{50}) and **C1** showed the strongest inhibitory activity against MLCR, and IC_{50} value was 0.5 μg/ml.

Antimicrobial Activity

Antimicrobial activity of drugs was examined by the serial agar dilution method using Mueller-Hinton agar (Difco) for antibacterial tests with incubation at 37°C for 18 hours and a nutrient agar containing 1% glucose for antifungal tests with incubation at 27°C for 42 hours. Minimum inhibitory concentration (MIC) value is expressed as the minimum concentration which inhibits growth of the microorganisms.

As shown in Table 5, **A1** at 3.12~100 μg/ml, **A2** at 6.25~12.5 μg/ml, **B1** at 1.56~6.25 μg/ml and **C1** at 0.78~3.12 μg/ml showed antibacterial activity only against Gram-positive bacteria. None of samples tested showed antibacterial activity Gram-negative bacteria or fungi at 100 μg/ml (data not shown). **C1** showed the strongest antibacterial activity. Antimicrobial activities of **B2** and **C2** were reduced in compared with those of **B1** and **C1**, respectively. **C2** only showed the activity at more than 100 μg/ml.

Discussion

The structure-activity relationship of delaminomycins and their derivatives on biological activities was studied. In B16 cell adhesion assay, delaminomycins **A1**, **A2** and **B2** showed stronger inhibitory activity to all ECM components tested. The inhibitory activity of **A4** was reduced on cell adhesion in compared with that of **A1**. These facts indicated that pyrrolidine moiety can be thought to play an important role in inhibiting cell adhesion to ECM components. **A2**, **B2** and **C2** showed stronger inhibitory activities than

A1, **B1** and **C1**, respectively. Therefore, spiro compounds (**A2**, **B2** and **C2**) are more potent than natural products (**A1**, **B1** and **C1**) on inhibition of B16 adhesion to ECM components.

In cytotoxicity against tumor cells, **A2**, **B2** and **C2** showed stronger cytotoxicity than **A1**, **B1** and **C1**, respectively. Since cytotoxicity of **A4** against tumor cells was reduced in compared with that of **A1**, pyrrolidine moiety should play important role for cytotoxicity as well as for inhibition of cell adhesion to ECM components.

In Con A-induced proliferation assay, the order of inhibitory effect was **A2**$\geq$**B2**$\geq$**C1**$\geq$**C2**>**B1**>**A1**. This result suggests two structure-activity relationships on the assay. Firstly, spiro form plays major role in inhibition on Con A-induced proliferation assay, and secondly, substitution of C-5′ in pyrrolidine ring plays minor role in inhibition on the assay. The order of potency for inhibition on the assay was R=OH>R=OCH_3>R=H.

In MLCR assay, natural products (**A1**, **B1** and **C1**) exhibited more potent suppressive activity than spiro compounds (**A2**, **B2** and **C2**). In addition, the inhibitory activity on MLCR was affected by replacements at C-5′ position of pyrrolidine ring. The order of inhibitory effect on MLCR was R=H>R=OCH_3>R=OH. Therefore, C-5′ position of pyrrolidine ring has importance on the structure-activity relationship as well as spiro ring form.

In antimicrobial assay, the order of potency against Gram-positive bacteria was **C1**>**B1**>**A2**>**A1**$\gg$**B2**$\geq$**C2**. Thus, natural products (**A1**, **B1** and **C1**) is more potent than spiro ring compounds (**A2**, **B2** and **C2**). Moreover, C-5′ position of pyrrolidine ring is important. The order of antimicrobial activity against Gram-positive bacteria on the substitution of C-5′ was R=H>R=OCH_3>R=OH.

In conclusion, pyrrolidine ring of delaminomycins and analogs is important for biological activities, and replacements at C-5′ position of pyrrolidine ring also affect biological activities. Furthermore, the change of original form to spiro ring form affects significantly on biological activities.

Experimental

General

NMR spectra were recorded on a JEOL JNM-GX400 NMR spectrometer and mass spectra were measured using a JEOL JMS-SX102 spectrometer. UV spectra were recorded on a Hitachi 228A spectrometer and IR spectra on a Hitachi 260-10 spectrometer.

Acid hydrolysis of A1 (Preparation of A2)

To 3.0 g of **A1** was added 48 ml of 1N HCl-acetone (1:3, v/v), and the mixture was stirred at room temperature for 17 hours. The reaction mixture was concentrated and extracted with EtOAc. The organic layer was washed with saturated NaCl solution and dried with anhydrous Na_2SO_4 and concentrated to dryness. The residue was subjected to centrifugal partition chromatography (Sanki Engineering) previously equilibrated with the lower layer of *n*-hexane-EtOAc-CH_3CN (7:2:3) at 25°C, 900 rpm. Chromatography was first performed with 1,000 ml of the mobile phase of the upper layer of *n*-hexane-EtOAc-CH_3CN (7:2:3) in the ascending mode and **A2** was remained in the immobile phase, and was eluted with the lower layer of *n*-hexane-EtOAc-CH_3CN (7:2:3) in descending mode and dried up under reduced pressure. The resulting residue contained **A2** was applied to a preparative HPLC (YMC-Pack SH-343, ODS, 20×250 mm) and eluted with a linear gradient from 70% MeOH to MeOH. The fractions containing **A2** were combined and concentrated to give 1.04 g of a colorless powder in 35.9% yield. UV λ_{max}^{MeOH} ($E_{1cm}^{1\%}$); 231 nm (614). IR ν_{max}^{KBr}; 3350, 2950, 2900, 1790, 1700, 1680, 1450, 1250, 1080 and 990 cm^{-1}. FAB-MS *m/z*; 482 $(M-H)^-$. Molecular formula; $C_{29}H_{41}NO_5$. TLC; Rf=0.67 ($CHCl_3$-MeOH-NH_4OH=40:10:1, Art. 5554 Kieselgel 60F_{254}, Merck)

Reduction of A2 with $NaBH_4$ (Preparation of A3)

To a solution of **A2** (15 mg) in MeOH (1 ml) was added $NaBH_4$ (7 mg), and the mixture was stirred at room temperature for 28 hours. The crude product was subjected to a preparative HPLC (YMC-Pack SH-343, ODS) and eluted with a linear gradient from 80% MeOH to MeOH. The fractions containing **A3** were combined and concentrated under reduced pressure. The residue was loaded onto a Sephadex LH-20 column and eluted with MeOH. The fractions containing **A3** were combined and concentrated to

give 8.6 mg of a colorless powder. UV λ_{max}^{MeOH} ($E_{1cm}^{1\%}$); 236 nm (728). IR ν_{max}^{KBr}; 3380, 2950, 2900, 1670, 1450, 1375, 1290~1240, 1100~1040, 995, 970, 750 and 710 cm^{-1}. FAB-MS *m/z*; 488 $(M+H)^+$, 486 $(M-H)^-$. Molecular formula; $C_{29}H_{45}NO_5$. TLC; Rf=0.44 ($CHCl_3$ - MeOH - NH_4OH=40 : 10 : 1, Art. 5554 Kieselgel 60F_{254}, Merck).

Alkali Hydrolysis of **A1** (Preparation of **A4**)

To a solution of **A1** (38 mg) in MeOH (3 ml) was added 2 N NaOH (1 ml), and the mixture was stirred at room temperature for 8 days. The crude product was concentrated and loaded onto a Diaion HP-20 column (15 ml). Eluate with MeOH was concentrated under reduced pressure and subjected to preparative HPLC (YMC-Pack, ODS) and eluted with a linear gradient from 80% MeOH to MeOH. The fractions containing **A4** were combined and concentrated under reduced pressure. The residue was loaded onto a Sephadex LH-20 column and eluted with MeOH. The fractions containing **A4** were combined and concentrated under reduced pressure to give 9.0 mg of a colorless powder. UV λ_{max}^{MeOH} ($E_{1cm}^{1\%}$); 234 nm (400). IR ν_{max}^{KBr}; 3420, 2910, 1680, 1460 and 1000 cm^{-1}. FAB-MS *m/z*; 468 $(M+Na)^+$, 444 $(M-H)^-$. Molecular formula; $C_{27}H_{43}NO_4$. TLC; Rf=0.65 ($CHCl_3$ - MeOH - NH_4OH=40 : 10 : 1, Art. 5554 Kieselgel 60 F_{254}, Merck)

Preparation of **B2**

To 100 mg of **B1** was added 4 ml of 1 N HCl - acetone (1 : 3, v/v), and the mixture was stirred at room temperature for 18 hours. The crude product was subjected to a preparative HPLC (YMC-Pack, silica gel) using the same gradient system of that of **A2**. The fractions containing **B2** were concentrated and injected to a preparative HPLC (YMC-Pack, ODS) and eluted with a linear gradient from 80% MeOH to MeOH. The fractions containing **B2** were combined and concentrated under reduced pressure. The residue was loaded onto a Sephadex LH-20 column and eluted with MeOH. The fractions containing **B2** were combined and concentrated to give 34.6 mg of a colorless powder. UV λ_{max}^{MeOH} ($E_{1cm}^{1\%}$); 232 nm (588). IR ν_{max}^{KBr}; 3400, 2950, 2900, 1780, 1700, 1680, 1450, 1380, 1260, 1080 and 1000 cm^{-1}. FAB-MS *m/z*; 520 $(M+Na)^+$, 496 $(M-H)^-$. Molecular formula; $C_{30}H_{43}NO_5$. TLC; Rf=0.78 ($CHCl_3$ - MeOH - NH_4OH=40 : 10 : 1, Art. 5554 Kieselgel 60 F_{254}, Merck)

Preparation of **C2**

To a solution of **C1** (22 mg) in MeOH (2 ml) was added 1 N HCl (0.5 ml), and the mixture was stirred at room temperature for 17 hours. The crude product was subjected to a preparative HPLC (YMC, ODS) and eluted with a linear gradient from 80% MeOH to MeOH. The fractions containing **C2** were concentrated and loaded onto a Sephadex LH-20 column and eluted with MeOH. The fractions containing **C2** were combined and concentrated to give 9.8 mg of a colorless powder. UV λ_{max}^{MeOH} ($E_{1cm}^{1\%}$); 230 nm (708). IR ν_{max}^{KBr}; 3390, 2940, 2900, 1780, 1690, 1450, 1370, 1265 and 950 cm^{-1}. FAB-MS *m/z*; 468 $(M+H)^+$, 466 $(M-H)^-$. Molecular formula; $C_{29}H_{41}NO_4$. TLC; Rf=0.80 ($CHCl_3$ - MeOH - NH_4OH=40 : 10 : 1, Art. 5554 Kieselgel 60 F_{254}, Merck)

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