DELAMINOMYCINS, NOVEL EXTRACELLULAR MATRIX RECEPTOR ANTAGONISTS

V. BIOSYNTHESIS

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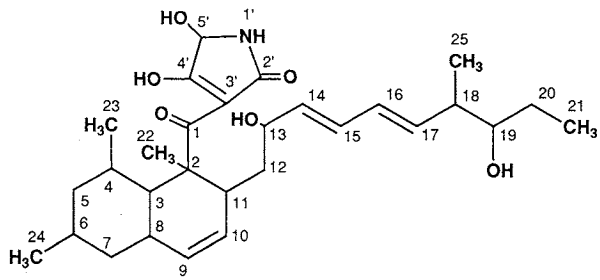
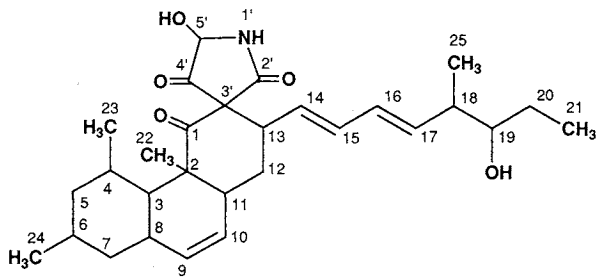
The biosynthesis of delaminomycin A, produced by *Streptomyces albulus* MJ202-72F3, was investigated by feeding ^{13}C-labeled compounds followed by ^{13}C NMR analyses. The results indicate that delaminomycin A is derived from six acetate units, five propionate units and one glycine unit.

In the course of screening for inhibitors of cell adhesion to fibronectin, laminin and collagen type IV, components of the extracellular matrix (ECM), we found new ECM antagonists, named delaminomycins, produced by *Streptomyces albulus* MJ202-72F3[1~4]. The major component was identified as delaminomycin A (**1**, Fig. 1). In this paper, we report incorporation experiments with single and multiple labeled ^{13}C

Fig. 1. Structures of delaminomycin A (**1**) and its derivative (**2**).



1
Delaminomycin A



2

precursors to determine the biosynthetic origin of the carbon atoms of **1**.

Materials and Methods

General Procedure

TLC was carried out on Silica gel 60 F_{254} plates of 0.2 mm thickness (Merck, Art. No. 5554) using $CHCl_3$ - MeOH - NH_4OH (40 : 10 : 1) as the developing solvent. Detection of **1** and **2** (Fig. 1) on the TLC plate was carried out by a color reaction with the vanillin-H_2SO_4 reagent (purple red) or fluorescence quenching under 254 nm UV light. The Rf values of **1** and **2** were 0 and 0.67, respectively.

HPLC analyses were carried out using a CAPCELL PAK $5C_{18}$ column (Shiseido, 4.6 × 250 mm) with a mobile phase of MeOH - CH_3CN - 25 mM NH_4OAc - 2-PrOH (30 : 30 : 35 : 5) at a flow rate of 1.0 ml/minute equipped with a Hitachi 655A-11 pump, a Sugai U-620 PSH column heater set at 35°C, a Hitachi L-4000 UV detector set at 235 nm and a Hitachi chromato-integrator. The retention time of **1** was 7.5 minutes.

Preparative HPLC was performed using a Gilson HPLC system controlled by NEC PC-9801 computer, Sugai U-620 PSH column heater set at 30°C, SIC chromatocorder-21 and a YMC-Pack SH-343 ODS column (20 × 250 mm).

^{13}C NMR spectra were measured in $CDCl_3$ on a Jeol JNM-A400 instrument operating at 100 MHz. Enrichment ratios were determined from each signal intensity by comparison with spectra of unenriched positions and with that of unenriched material recorded under the same conditions.

Labeled Compounds

^{13}C precursors contained >99 atom% ^{13}C at the labeled positions except for [1-^{13}C]acetate (98.6 atom% ^{13}C). Sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, sodium [1,2-$^{13}C_2$]acetate, sodium [1-^{13}C]propionate, DL-[1-^{13}C]alanine, [1,2-$^{13}C_2$]glycine and L-[*methyl*-^{13}C]methionine were purchased from Sigma Chemical Co., U.S.A.

Microorganism

Streptomyces albulus MJ202-72F3 was used to produce **1**.

Fermentation

Strain MJ202-72F3 on an agar slant was inoculated into a 500-ml Erlenmeyer flask containing 110 ml of seed medium and cultured at 30°C for 3 days on a rotary shaker (180 rpm). The seed medium consisted of glucose 1.5%, yeast extract (Nippon-Seiyaku) 0.25%, Casamino acids (Difco) 0.25%, $CaCO_3$ 0.4%; the pH of the medium was not adjusted. Two ml of this seed culture was inoculated into 100 ml of production medium in a 500-ml Erlenmeyer flask and cultured at 28°C for 6 or 7 days on a rotary shaker (180 rpm). The production medium consisted of glucose 3.0% yeast extract 0.5%, Casamino acids 0.5%, $NaNO_3$ 0.2%, KCl 0.2%, $CaCO_3$ 0.4%; the pH of the medium was not adjusted.

After 3 days fermentation, at a suitable stage in delaminomycin production, cultures received the first addition of ^{13}C-labeled precursor. In the case of [1-^{13}C, 2-^{13}C, 1,2-$^{13}C_2$]acetate and [1-^{13}C]propionate, precursors were added in doses of 0.05% (w/v) at 72 and 120 hours after inoculation. DL-[1-^{13}C]alanine, [1,2-$^{13}C_2$]glycine and L-[*methyl*-^{13}C]methionine were added in doses of 0.025% (w/v) at 72, 96, 120 and 144 hours after inoculation, and the cultures were incubated further for 24 hours.

Preparation of ^{13}C-Labeled **2**

Conversion of **1** to **2**: Each fermentation broth (500 ml) containing **1** labeled with a particular ^{13}C-labeled precursor was centrifuged and the mycelium was extracted with 200 ml of acetone. The extract was concentrated under reduced pressure to give an aqueous solution. The solution was extracted twice with 30 ml of *n*-BuOH. The organic layer was concentrated under reduced pressure. The crude material containing **1** was treated with 8 ml of 1 N HCl - acetone (1 : 3, v/v) at room temperature for 17 hours. Under the treatment, **1** was converted to **2**, showing a main spot at Rf 0.67 with the vanillin-H_2SO_4 reagent[2].

Isolation of **2**: The reaction mixture containing **2** was concentrated under reduced pressure to give an aqueous solution. The solution was extracted with EtOAc. The organic layer was washed with saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was dissolved

Table 1. Production of **1** in cultures and the yields of **2**.

Expt.	Additives	Production of **1** (μg/ml)[a]	Amounts of **2** (mg)	Total yield of **2** (%)
I	None	88[b]	13.6	30.9
	Sodium [2-^{13}C]acetate	70[b]	9.4	26.9
	Sodium [1,2-$^{13}C_2$]acetate	62[b]	6.8	21.9
II	None	179	—	—
	L-[*Methyl*-^{13}C]methionine	65	5.6	17.2
III	None	155	—	—
	Sodium [1-^{13}C]acetate	146	15.2	20.8
	DL-[1-^{13}C]Alanine	270	28.0	20.7
	[1,2-$^{13}C_2$]Glycine	259	26.6	20.5
IV	Sodium [1-^{13}C]propionate	220	23.0	20.9

[a] Determined by HPLC analysis.
[b] A shaker was out of order at 3rd day for 15 hours.

Table 2. ^{13}C NMR analysis of **2** enriched by incorporation of isotopic precursors.

No.	δ ^{13}C (ppm)	[1-^{13}C]-Acetate	[2-^{13}C]-Acetate	[1,2-$^{13}C_2$]-Acetate	L-[*Methyl*-^{13}C]-methionine	[1-^{13}C]-Propionate
1	212.2	2.36*	1.47	1.94 (N.C.[b])[a]	0.77	9.19**
2	54.3	1.09	2.93*	2.09 (33.2)	1.22	0.96
3	43.6	2.99*	1.87	2.19 (34.4)	0.97	8.14**
4	35.5	0.84	3.05*	2.15 (34.3)	0.91	0.66
5	47.2	3.16*	2.30	2.75 (33.6)	0.91	8.58**
6	33.1	1.06	3.76*	2.11 (32.8)	0.92	0.81
7	42.3	7.46**	1.45	1.87 (33.6)	0.96	0.96
8	40.4	0.89	5.00**	1.56 (33.6)	0.90	0.70
9	130.6	7.06**	1.19	1.09 (48.0)	0.94	0.93
10	126.8	0.88	6.35**	1.35 (48.0)	0.88	0.74
11	46.1	7.35**	1.18	1.47 (32.0)	1.01	0.93
12	32.0	0.99	6.94**	1.84 (32.2)	1.00	0.83
13	44.1	5.43**	0.88	1.21 (44.3)	0.84	1.03
14	128.8	0.95	5.85**	1.41 (44.3)	1.00	0.84
15	133.8	6.06**	0.99	1.24 (54.9)	0.86	0.91
16	129.9	0.68	5.15**	1.32 (54.9)	0.78	0.68
17	137.4	1.96*	1.24	1.59 (43.5)	0.70	8.05**
18	41.9	0.89	3.18*	2.30 (41.2)	0.92	0.74
19	76.6	3.33*	1.42	1.61 (38.1)	O.L.[d]	4.97**
20	26.4	0.72	3.53*	3.04 (38.2)	0.78	0.77
21	10.5	1.06	3.73*	3.09 (35.1)	0.82	0.93
22	17.1	1.08	3.93*	2.97 (37.3)	0.97	1.00
23	18.9	1.02	3.20*	2.22 (34.3)	0.95	0.87
24	22.1	1.11	3.31*	2.50 (35.1)	1.02	0.96
25	14.1	1.19	3.23*	2.39 (35.8)	1.10	0.99
2′	168.5	2.86*	0.66	0.95 (47.3)	0.48	0.79
3′	72.4	1.22	5.21**	1.83 (47.3)	1.31	1.21
4′	205.5	0.60	0.60	1.11 (N.C.)	0.60	1.00
5′	79.5	1.00[c]	1.00[c]	1.00[c]	1.00[c]	1.00[c]

[a] *J*-values are in parentheses (Hz).
[b] Not coupled.
[c] Relative enrichments were normalized to peak intensities for the C-5′ signal.
[d] Overlapped with solvent peak.
* Low level of enrichment was observed.
** High level of enrichment was observed.

in acetonitrile and applied to a reverse phase HPLC column (YMC-pack ODS, 20 × 250 mm) and eluted with 80% acetonitrile. Fractions containing **2** were collected and concentrated under reduced pressure, loaded onto a Sephadex LH-20 column (65 ml) and eluted with acetonitrile. The fractions containing **2** were concentrated to give a white powder.

Results and Discussion

As described in our previous papers[1,2], the major component of delaminomycins in the fermentation broth and mycelium was **1**; however, the ^{13}C NMR spectrum of **1** was very complicated due to the presence of possible equilibrium structures. Thus, we converted **1** to **2** in order to clarify the mode of incorporation of ^{13}C-labeled precursors by ^{13}C NMR spectroscopic analyses.

As shown in Table 1, feeding of DL-[1-^{13}C]alanine, [1,2-$^{13}C_2$]glycine and sodium [1-^{13}C]propionate enhanced the production of **1**, but feeding of L-[*methyl*-^{13}C]methionine inhibited growth of the producing strain and reduced the production of delaminomycins including **1** in cultures. Throughout these incorporation experiments, total yields of ^{13}C-labeled **2** after purification were between 17 to 31%.

The ^{13}C NMR spectra of **2** derived from [1-^{13}C]acetate, [2-^{13}C]acetate, [1-^{13}C]propionate and [1,2-$^{13}C_2$]glycine are shown in Fig. 2. The numbering depicted in the signals indicates the enriched carbon signals. The enriched ratio and ^{13}C-^{13}C coupling constants of ^{13}C-labeled **2** are listed in Table 2. Enrichment ratios were calculated from the relative intensity of C-5′ as 1.0. As shown in Table 2, enrichment of carbons C-7, 9, 11, 13, 15, 2′ by [1-^{13}C]acetate and C-8, 10, 12, 14, 16, 3′ by [2-^{13}C]acetate were observed. ^{13}C-^{13}C coupling constants of [1,2-$^{13}C_2$]acetate-labeled **2** were equal in couples of carbons. These results indicate the incorporation of acetate into a pentaketide chain (C-7 to 16) and a single acetate unit (C-2′ and C-3′). As enriched signals were not observed in C-4′ and C-5′, these carbons are thought to be derived from another precursor(s). The low level of incorporation of [2-^{13}C]acetate into all methyl carbons (C-21, 22, 23, 24 and 25) and carbons adjacent to these methyl groups (C-2, 4, 6, 18 and 20) suggest the metabolism of [2-^{13}C]acetate to propionate with consequent label dilution before incorporation at these sites.

To confirm this hypothesis, incorporation experiments using L-[*methyl*-^{13}C]methionine and [1-^{13}C]propionate were performed. As shown in Table 2, no enriched signals were observed in any

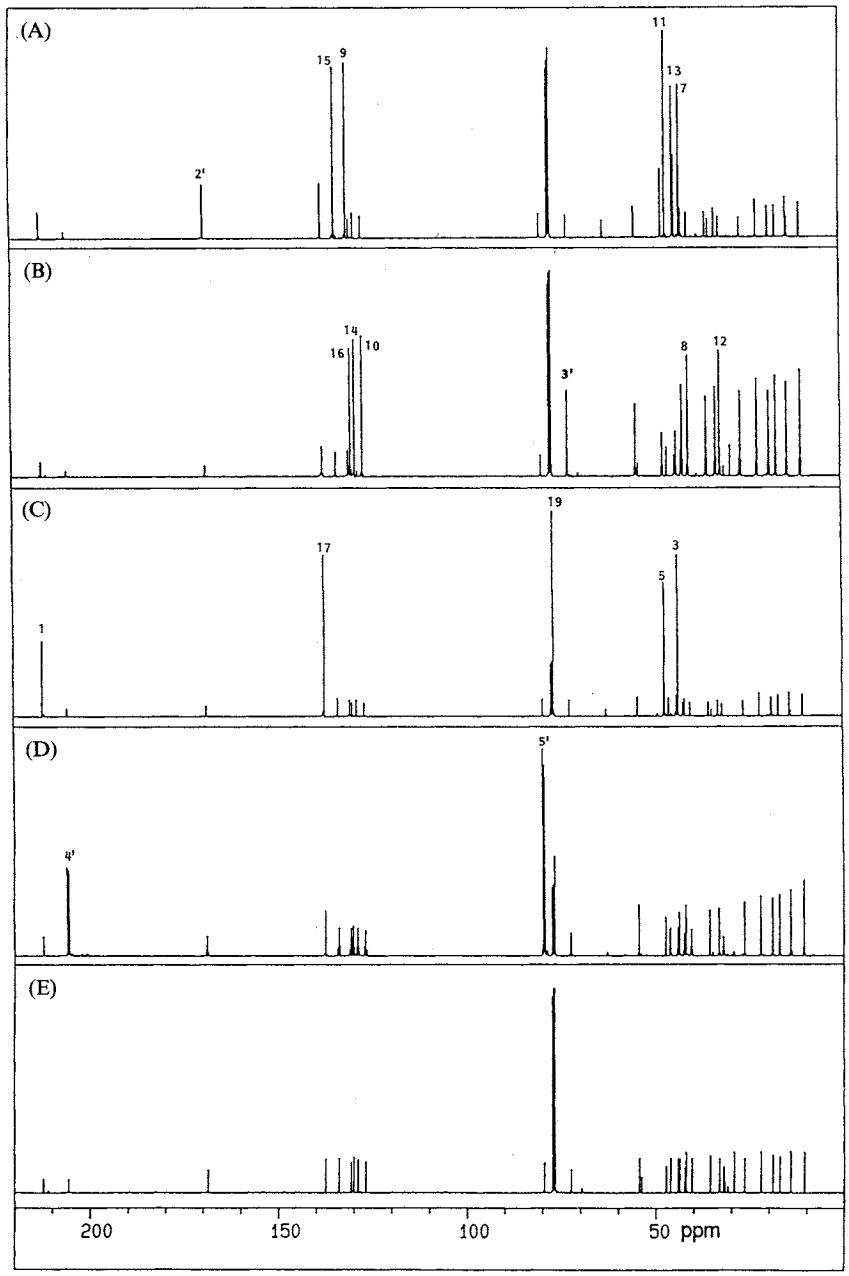
Table 3. ^{13}C NMR analysis of **2** enriched with stable isotope precursors.

No.	δ ^{13}C (ppm)	Relative enrichments	
		[1,2-$^{13}C_2$]Glycine	[1-^{13}C]Alanine
1	212.2	1.00[a]	1.00[a]
2	54.3	1.08	1.19
3	43.6	0.95	1.41
4	35.5	0.91	1.21
5	47.2	1.07	1.36
6	33.1	1.04	1.29
7	42.3	0.67	1.35
8	40.4	0.57	1.30
9	130.6	0.66	1.28
10	126.8	0.60	1.16
11	46.1	0.59	1.42
12	32.0	0.55	1.30
13	44.1	0.61	1.02
14	128.8	0.62	1.34
15	133.8	0.60	1.16
16	129.9	0.61	1.11
17	137.4	0.97	0.91
18	41.9	0.91	1.07
19	76.6	1.04	O.L.[b]
20	26.4	1.17	0.92
21	10.5	1.37	1.30
22	17.1	1.25	1.57
23	18.9	1.13	1.45
24	22.1	1.07	1.41
25	14.1	1.17	1.64
2′	168.5	0.63	0.71
3′	72.4	0.73	1.61
4′	205.5	4.76**	0.82
5′	79.5	5.13**	1.36

[a] Relative enrichments were normalized to peak intensities for the C-1 signal of **2**.
[b] Overlapped with solvent peak.
** High level of enrichment was observed.

Fig. 2. ^{13}C NMR spectra of **2** derived from labeled precursors in $CDCl_3$.

(A) [1-^{13}C]acetate, (B) [2-^{13}C]acetate, (C) [1-^{13}C]propionate, (D) [1,2-$^{13}C_2$]glycine, (E) natural abundance.



methyl carbon (C-21, 22, 23, 24 and 25). This indicates that these atoms are not derived from S-adenosylmethionine. As shown in Table 2 and Fig. 2, enriched signals were observed in C-1, 3, 5, 17 and 19. This indicates the incorporation of five propionate units into **2**. These results support the hypothesis described above.

Fig. 3. Signals for C-4′ and C-5′ in 100 MHz ^{13}C NMR spectrum of **2** labeled by [1,2-$^{13}C_2$]glycine. $J_{4',5'}=42.8$ Hz.

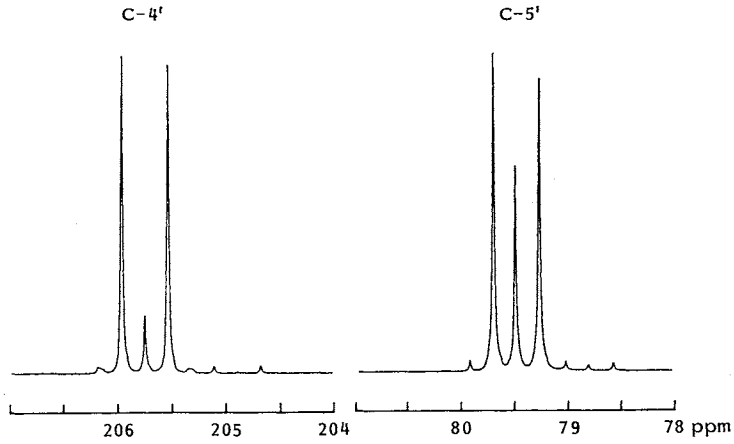


Fig. 4. Labeling patterns in delaminomycin A derived from ^{13}C-labeled precursors.

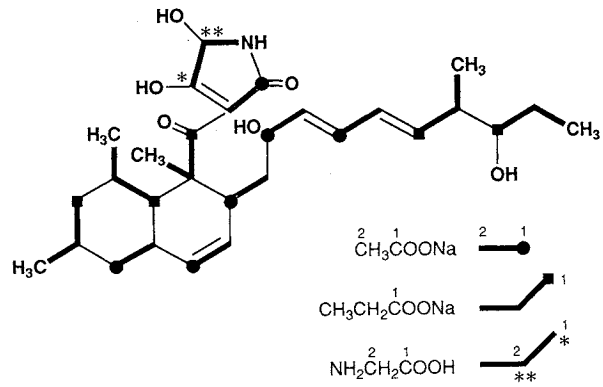
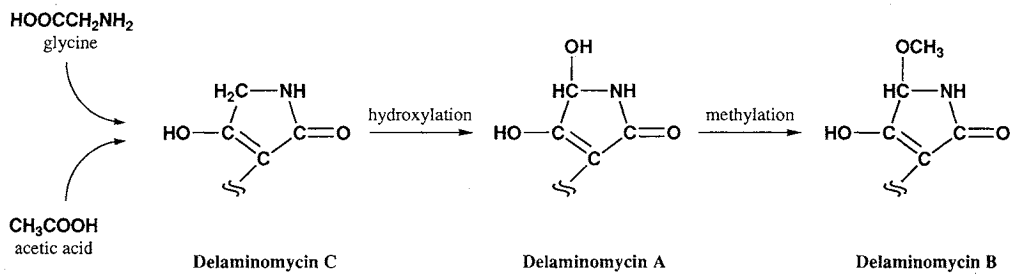


Fig. 5. Proposed biosynthetic scheme for delaminomycins A, B and C.



The incorporation data for DL-[1-^{13}C]alanine and [1,2-$^{13}C_2$]glycine into **2** are shown in Table 3. Enrichment ratios were calculated from the relative intensity of C-1 as 1.0. With [1,2-$^{13}C_2$]glycine, enriched signals were observed at C-4′ and 5′ with a coupling constant of $J_{4',5'}=42.8$ Hz as shown in Figs. 2 and 3. The results indicate the intact incorporation of glycine into the pyrrolidine moiety. No

incorporation was observed into **2** from DL-[1-^{13}C]alanine.

The results obtained from the above feeding experiments with ^{13}C-labeled precursors demonstrate that one mole of delaminomycin A is biosynthesized from six moles of acetate, five moles of propionate and one mole of glycine. Hence, the origin of the all carbon atoms of delaminomycin A has been established and can be summarized as shown in Fig. 4.

In the feeding experiment with [1,2-$^{13}C_2$]glycine, labeled glycine was highly incorporated into the delaminomycin molecule. This result suggests that intact glycine is incorporated into delaminomycin C. Thus, it appears that delaminomycin C is biosynthesized first and is a precursor for delaminomycins A and B. Therefore, the biosynthetic pathway to delaminomycins can be considered to be as shown in Fig. 5. First, delaminomycin C is biosynthesized by the incorporation of glycine into pyrrolidine moiety. Second, delaminomycin C is hydroxylated enzymatically to delaminomycin A. Third, delaminomycin A is methylated enzymatically to delaminomycin B.

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