Pyralomicins, Novel Antibiotics from *Microtetraspora spiralis*

III. Biosynthesis of Pyralomicin 1a

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The biosynthesis of pyralomicin 1a (**1**) was studied by feeding of ^{13}C and ^{15}N labeled compounds to the culture of *Microtetraspora spiralis* MI178-34F18. The result indicated that the benzopyranopyrrole unit of **1** was derived from two units of acetate, one unit of propionate and one unit of proline, and that the cyclitol unit of **1** was derived from glucose metabolites. And 4′-*O*-CH_3 was derived from the *S*-CH_3 group of methionine.

In the course of our screening program for novel antibiotics, we have isolated pyralomicins 1a～1d, 2a～2c[1]. Pyralomicin 1a (**1**) is the major component of pyralomicins, which has a unique structure as shown in Fig. 1. **1** has a benzopyranopyrrole as a chromophore, the nitrogen atom of which is alkylated by a cyclitol.

The benzopyranopyrrole structure was first reported as a skeleton structure of TAN-876A by FUNABASHI *et al.*[2]. This structure is biosynthetically interesting in relation to TAN-876B which is produced simultaneously (Fig. 2), but no biosynthetic study has been reported. We took a great interest in the origin of this unit to study.

The cyclohexene ring is closely related to valienamine, the partial structure of validamycins[3] or acarbose[4], except for the configuration of 1′-H. TOYOKUNI *et al.*[5] and DEGWERT *et al.*[6] demonstrated that the seven carbon skeleton of valienamine was constructed from C_2, C_2 and C_3 units derived from glycerol, glucose or other kinds of sugar, but not from C_4 and C_3 units which was shown in so called "*m*-C_7N" unit (a six membered carbocyclic ring bearing a carbon and a nitrogen substituent in a 1, 3 (*meta*) arrangement) of pactamycin[7], geldanamycin[8] and other ansamycin antibiotics[9].

In this paper, we report the biosynthetic origin of all the carbon and nitrogen atoms of **1**, and consider the biosynthetic pathway.

Materials and Methods

Strain and Seed Culture

An agar slant culture of *Microtetraspora spiralis* MI178-34F18 was inoculated into a 500-ml Erlenmeyer flask containing 110 ml of a seed medium consisting of galactose 2.0%, dextrin 2.0%, Bacto-soytone (Difco) 1.0%, corn steep liquor (Iwaki) 0.5%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2% and a drop of silicone oil (adjusted to pH 7.4 before sterilization). The inoculated medium was incubated at 30°C for 72 hours on a rotary shaker. This seed culture was stored at −20°C.

Labeled Compounds

^{13}C-labeled compounds were of 99% ^{13}C atom purity except for L-[1-^{13}C] proline (90% ^{13}C atom purity). Sodium [1-^{13}C] acetate, sodium [2-^{13}C] acetate, sodium [1,2-^{13}C] acetate, sodium [1-^{13}C] propionate, D-[U-^{13}C] glucose and L-[methyl-^{13}C] methionine were purchased from Aldrich Chemical Co., Milwaukee, U.S.A., and L-[1-^{13}C] proline from Shoko Co., Tokyo, Japan. ^{15}N-labeled proline was of 99% atom purity and purchased from ICON Co., N.Y., U.S.A.

Fig. 1. Structure of pyralomicin 1a (**1**).

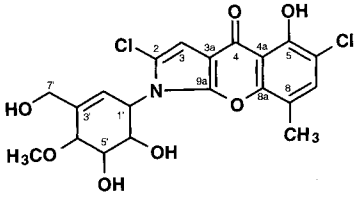
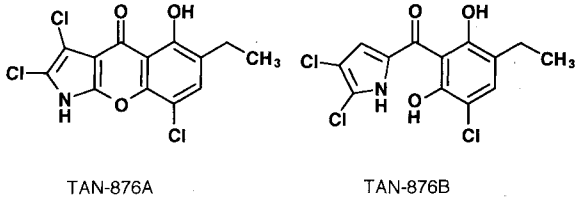


Fig. 2. Structures of TAN-876 A and B.



Incorporation of Stable Isotope-labeled Precursors into **1**

Two ml of the thawed seed culture was inoculated into a 500-ml Erlenmeyer flask containing 110ml of production medium consisting of starch 3.0%, Toast soya meal (Nisshin) 1.5%, corn steep liquor (Iwaki) 0.5%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, NaCl 0.3%, $CoCl_2 \cdot 6H_2O$ 0.001%, $CaCO_3 \cdot 6H_2O$ 0.3% and a small amount of silicone oil (adjusted to pH 7.2 before sterilization). This inoculated medium was incubated at 27°C on a rotary shaker (180 rpm).

Each labeled precursor was dissolved in sterile water and added to the above mentioned producing culture twice equally, at the 48th and 52nd hour of cultivation. In the case of sodium [1-^{13}C] acetate, sodium [2-^{13}C] acetate, sodium [1,2-^{13}C] acetate, sodium [1-^{13}C] propionate, D-[U-^{13}C] glucose and L-[methyl-^{13}C] methionine, 240 mg (30 mg × 4 flasks × 2) of each labeled compound was added to 440 ml of the producing culture. And in the case of L-[1-^{13}C] proline and L-[^{15}N] proline, 80mg (10 mg × 4 flasks × 2) of each labeled compound was added. The cultures were incubated further and harvested at the 72nd hour of cultivation.

Isolation of **1**

Each harvested fermentation broth containing **1** (440 ml), which was fed with ^{13}C-labeled or ^{15}N-labeled precursors was filtered with the aid of celite. The filtrate was extracted with 200 ml of butyl acetate. The mycelial cake was extracted with 200 ml of methanol. This extract was concentrated under reduced pressure, and the resulting material was suspended in 50 ml of water. This suspension was extracted twice with 50 ml of butyl acetate. Both butyl acetate extracts from the filtrate and the mycelial cake were combined and evaporated to dryness. This crude material containing **1** was chromatographed on a silica gel column (Wako-gel C-300, 5 g) with a solvent of *n*-hexane - ethyl acetate (1 : 4). The fractions containing **1** were purified by preparative HPLC using Senshu-pak (ODS, H-5251, i.d. 20 × 250 mm), with a solvent of 55% MeOH. The fractions containing **1** were concentrated to give pale yellow powder. The production of **1** in each feeding experiment is summarized in Table 1.

Table 1. Dose of precursor and production of pyralomicin 1a.

Precursor fed (enrichment)	Dose of precursor	Production of pyralomicin 1a
Sodium [1-^{13}C] acetate (99%)	240mg	3.6mg
Sodium [2-^{13}C] acetate (99%)	240mg	3.8mg
Sodium [1,2-$^{13}C_2$] acetate (99%)	240mg	6.0mg
Sodium [1-^{13}C] propionate (99%)	240mg	5.0mg
L-[methyl-^{13}C] Methionine (99%)	240mg	5.2mg
D-[U-^{13}C] Glucose (99%)	240mg	2.7mg
L-[1-^{13}C] Proline (90%)	80mg	1.5mg
L-[^{15}N] Proline (99%)	80mg	1.4mg

NMR Analyses

^{13}C NMR spectra were measured in *N*,*N*-dimethylformamide (DMF)-d_7 on a JEOL JNM-A500 NMR spectrometer at 125 MHz. Enrichment ratios of carbons were determined from each signal intensity by comparison with the signal of unenriched position (C-4′-OCH_3 or C-8-CH_3) and with the corresponding signal of unenriched material recorded under the same conditions.

^{15}N NMR spectra were measured on a JEOL JNM-A500 NMR spectrometer using a 5 mm tunable probe, TH-5 at 50.55 MHz. Using dimethylsulfoxide (DMSO)-d_6 as a solvent, ^{15}N NMR spectra of ^{15}N enriched **1** (1.4 mg) derived from L-[^{15}N] proline and unenriched **1** (100 mg) were measured under the following conditions: pulse flip angle = 45°, data points = 32,768, spectral width = 25 kHz, gated decoupling (nondecoupling with NOE) and delay time between scans (PD) = 9 seconds, scan times = 7,000, DMF-d_7 signal as an external reference ($\delta_N = 103.2$).

^{1}H-^{15}N pulsed field gradients (PFG)-HMBC spectrum was measured by a JEOL JNM-A500 NMR spectrometer equiped with a JEOL NM-AFG field gradients unit and a 5 mm PFG probe.

Results and Discussion

As shown in Table 2, high levels of enrichment for carbons C-5, C-8a by [1-^{13}C] acetate, C-4a, C-6 by [2-^{13}C] acetate, and C-7 by [1-^{13}C] propionate were observed. A feeding experiment with [1,2-^{13}C] acetate revealed the ^{13}C-^{13}C coupling of C-4a − C-8, and C-5 − C-6. These results indicated the benzene ring was derived from two acetates and a propionate as shown in Fig. 3.

All the ^{13}C signals belonging to the cyclitol unit showed the ^{13}C-^{13}C splittings in the feeding experiment with D-[U-^{13}C] glucose. The coupling constants showed the coupling of C-1′ − C-2′, C-1′ − C-6′, C-3′ − C-7′ and C-4′ − C-5′. Only C-1′ coupled to two carbons, and others coupled to one carbon. The result showed that C-3′ − C-7′ and C-4′ − C-5′ units were derived from C_2 metabolites, and C-2′ − C-1′ − C-6′ unit was derived from C_3 metabolite of glucose. This combination was the same as that of valienamine, the component of validamycins[3] or acarbose[4], but not as that of the *m*-C_7N unit of pactamycin[7], geldanamycin[8] and other ansamycin antibiotics[9] which showed $C_4 + C_3$ patterns in labeling experiments using D-[U-^{13}C] glucose. TOYOKUNI *et al.*[5] and DEGWERT *et al.*[6] demonstrated that the seven carbon skeleton of valienamine was constructed *via* a pentose phosphate pathway. So, the cyclitol unit of **1** was expected to be synthesized in the same pathway.

L-[methyl-^{13}C] Methionine enriched the methoxy carbon of the 4′-position (Table 2). This was consistent

Table 2. ^{13}C NMR analyses of pyralomicin 1a by incorporation of isotopic precursors.

Position	δ (ppm)	[1-^{13}C]-Acetate	[2-^{13}C]-Acetate	[1,2-$^{13}C_2$]-Acetate	[1-^{13}C]-Propionate	D-[U-^{13}C]-Glucose	L-[methyl-^{13}C]-Methionine	L-[1-^{13}C]-Proline
2	119.8	1.55	1.45	1.27**(80.4)	1.38	1.08	1.28	-
3	99.7	0.96	1.78	1.47**(80.4)	1.05	1.03	1.01	1.06
3a	105.2	1.15	0.97	1.16	1.21	0.99	0.94	-
4	177.5	0.99	0.77	0.86	0.95	0.63	0.85	33.30*
4a	110.1	-	13.08*	15.01*,**(69.1)	0.86	0.98	1.16	0.55
5	155.5	1.82[c],*	0.99	7.99*,**(77.9)	0.52	0.67	0.67	0.70
6	114.2	-	11.83*	9.99*,**(77.9)	0.52	0.82	1.02	0.82
7	135.6	0.78	1.30	1.98	48.75*	0.82	0.85	0.91
8	117.8	-	1.29	0.91	0.43	0.75	0.93	0.85
8-CH_3	14.8	1.32	1.30	1.82	0.96	1.00[b]	1.00[b]	0.96
8a	151.4	10.34*	1.83	9.72*,**(69.1)	0.88	0.83	0.94	-
9a	150.2	-	1.21	0.87	0.80	0.67	0.78	-
1'	60.7	0.75	0.98	0.87	0.97	0.98**(36.4, 41.5)	0.86	0.78
2'	119.4	0.87	0.99	0.92	0.95	1.67**(41.5)	0.84	0.87
3'	143.5	0.81	0.77	0.83	0.99	0.93**(46.5)	0.74	0.80
4'	82.8	1.02	1.04	0.96	1.03	1.80**(41.5)	0.84	1.08
4'-OCH_3	59.4	1.00[a]	1.00[a]	1.00[a]	1.00[a]	1.03	65.25*	1.00[a]
5'	76.8	0.91	0.92	0.90	0.98	1.62**(41.5)	0.91	0.97
6'	73.0	1.06	1.07	0.98	1.08	1.64**(36.4)	0.99	1.02
7'	61.8	0.98	1.00	0.85	1.04	1.60**(46.5)	0.94	1.08

J-values are in parentheses (Hz).

[a] Relative enrichments were normalized to peak intensities for the C-4'-OCH_3 signal.

[b] Relative enrichments were normalized to peak intensities for the C-8-CH_3 signal.

[c] This signal was broad as compared with the other signals.

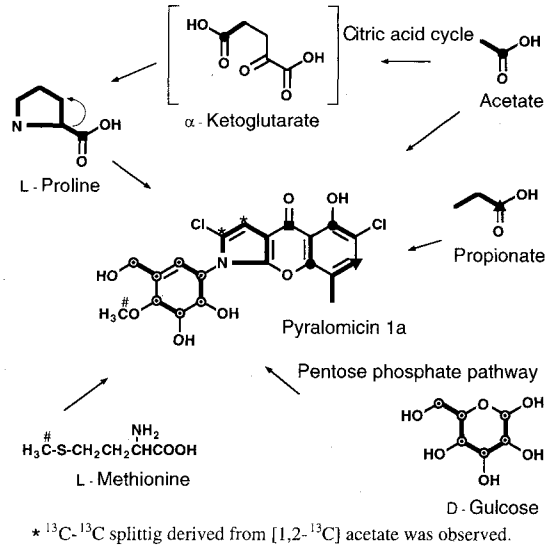
*High level of enrichment was observed. ** ^{13}C-^{13}C coupling was observed.

with the fact that *O*-methyl group of various metabolites were derived from the methyl group of methionine.

As described above, the carbon sources of the benzene unit and the cyclitol unit were determined. The origin of the 3-carbonyl pyrrole unit remained to be investigated. A feeding experiment with [1,2-^{13}C] acetate revealed the ^{13}C-^{13}C coupling between C-2 and C-3, but this incorporation was at a low level. Feeding D-[U-^{13}C] glucose did not yield a product whose ^{13}C NMR spectrum showed any splitting pattern. This implies that acetate is not a direct precursor, but a building unit of the intermediate for the 3-carbonyl pyrrole unit of **1**.

FUNABASHI *et al.*[2] reported two closely related antibiotics, TAN-876A and B, both of which were produced by an actinomycete strain (Fig. 2). Both compounds have a benzene ring and a pyrrole ring linked through a carbonyl carbon. Interestingly, in TAN-876A the cabonyl carbon is attached to 3-position carbon of the pyrrole ring, while in TAN-876B the carbonyl carbon is attached to 2-position of the pyrrole ring. Judging from their similarities especially from the position of the chlorine atoms and the ethyl group, it is possible that TAN-876A and TAN-876B are biosynthesized from a common intermediate, and in the biosynthetic pathway, the carbonyl group could have migrated intramolecularly

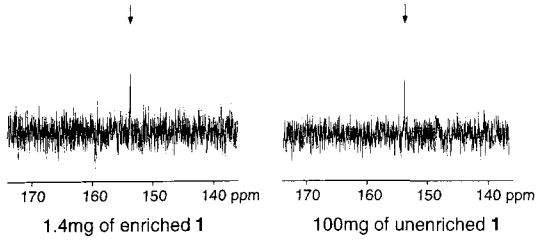
Fig. 3. Biosynthesis of pyralomicin 1a.



★ ^{13}C-^{13}C splittig derived from [1,2-^{13}C] acetate was observed.

from the 2-/3-position to the 3-/2-position of the pyrrole ring. **1** has a similar structure as TAN-876A, so an intermediate which has a 2-carbonyl pyrrole as TAN-876B is probable, and a 2-carbonyl pyrrole could be derived from proline. Since an acetate is incorporated into the 4- and 5-position of proline *via* the citric acid

Fig. 4. ^{15}N NMR spectra of pyralomicin 1a (**1**) enriched by the supplementation of L-[^{15}N]proline and unenriched **1**. (in DMSO-d_6).



cycle[10], this hypothesis could explain the low level incorporation of [1,2-^{13}C] acetate into C-2 and C-3 of **1**.

The result of the feeding experiment with L-[1-^{13}C] proline showed enrichment of C-4 (Table 2). The feeding experiment with L-[^{15}N] proline gave 1.4 mg of enriched **1** which showed ^{15}N signal at δ 153.5 in DMSO-d_6. As shown in Fig. 4, both ^{15}N signals of the enriched **1** (1.4 mg) and unenriched **1** (100 mg) were observed at δ 153.5, and their S/N ratios were 2.59 : 1 and 3.30 : 1, respectively. From the S/N ratios and the weight ratio of the samples, the ^{15}N enrichment ratio was calculated as 56 : 1. The ^{15}N signal at δ 153.5 of the enriched **1** was coupled to 3-H and 2′-H in the ^{1}H-^{15}N PFG HMBC spectra. The enrichment of C-2 and C-3 with [1,2-^{13}C]-acetate was consistent with the proline incorporation into the pyrrole unit, since acetate was incorporated specifically into γ- and δ-carbons of α-ketogultaric acid (precursor of proline) *via* the citric acid cycle[10]. These results suggested that the 3-carbonyl pyrrole unit of **1** was derived from L-proline (Fig. 3).

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