DUOCARMYCINS, NEW ANTITUMOR ANTIBIOTICS PRODUCED BY *STREPTOMYCES*; PRODUCING ORGANISMS AND IMPROVED PRODUCTION

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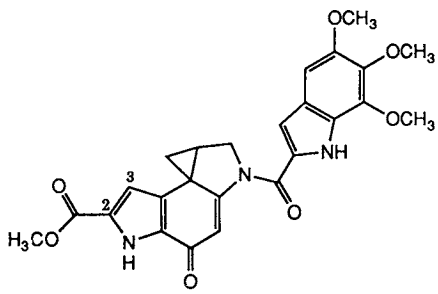
Six duocarmycins have been discovered during our search for new antitumor antibiotics and they showed extremely potent cytotoxic activity with IC_{50} values of 10^{-12} M ~ 10^{-9} M on HeLa S3 cell. Three different producing strains isolated from soils were taxonomically assigned as *Streptomyces*. Duocarmycin A was unstable in culture broth, so improved culture conditions were designed to produce a high titer of duocarmycins B1, B2, C1 and C2 which are halogenated seco-compounds of duocarmycin A. Duocarmycin SA, one of the most potent cytotoxic agents yet discovered, was shown to be more stable in culture media than duocarmycin A, despite the structural similarity on their spirocyclopropylhexadienone moiety. In contrast to the duocarmycin A fermentation, no halogenated seco-compounds of duocarmycin SA were detected in culture broth supplemented with Br^- or Cl^-. All duocarmycins could be produced using one producing strain with improved media and culture conditions.

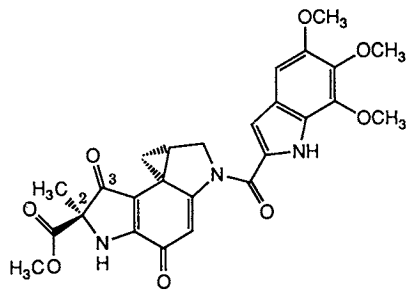
In the course of screening for new antitumor agents, we have isolated new potent antitumor antibiotics duocarmycins A (DC88-A)[1], B1, B2[2], C1 (DC89-A1)[3], C2 and SA (DC113)[4] produced by *Streptomyces*. OHBA *et al.*[5] have reported pyrindamycins A and B which are identical to duocarmycins C2 and C1, respectively. The structures of duocarmycins were revealed by spectroscopic and chemical analysis[6] (Fig. 1). Duocarmycins A and SA have a spirocyclopropylhexadienone moiety which is known to be the DNA alkylating group of "left hand segment" of CC-1065[7~9]. Recent studies have indicated that duocarmycin A can alkylate DNA in mechanisms similar to those of CC-1065[10,11]. The structural similarities suggest that duocarmycin SA also binds to DNA and alkylated DNA by a common mechanism. Duocarmycins B1, B2, C1, C2 possess five or six-membered ring which were generated *via* attacking of bromide or chloride ion at the different carbon position on cyclopropane ring of duocarmycin A. We have succeeded in purification of duocarmycin A, however it seemed difficult to prepare duocarmycin A from culture broth in a large quantity because of its chemical instability: Duocarmycin A is unstable in aqueous solution and protic solvent, which will be published in a separate paper[12]. Therefore we studied improved conditions for large scale production and purification of the halogenated seco-compounds, duocarmycins B1, B2, C1, C2 which are more stable than duocarmycin A. On the other hand duocarmycin SA has been shown to be more stable than duocarmycin A, so we investigated the improved production and purification of duocarmycin SA.

We report here the taxonomic comparison of the three producing strains, increased production through manipulation of the culture conditions which could support production of all duocarmycins using any one of the producing strains.

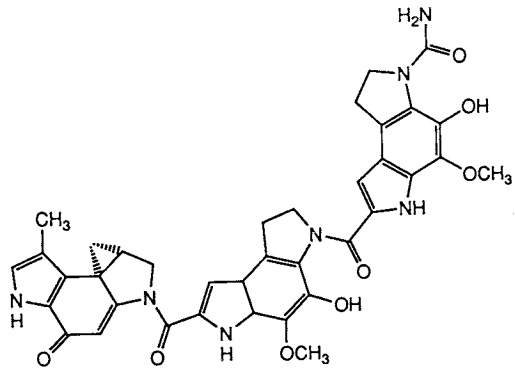
Fig. 1. Structures of duocarmycins and CC-1065.



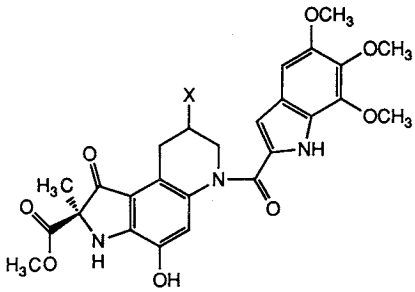
Duocarmycin SA



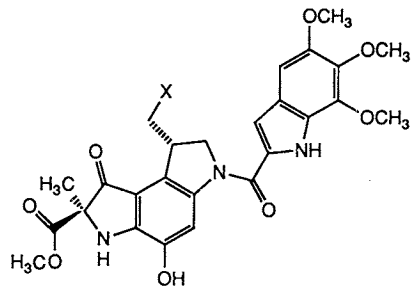
Duocarmycin A



CC-1065



Duocarmycin B1 X=Br
Duocarmycin C1 X=Cl



Duocarmycin B2 X=Br
Duocarmycin C2 X=Cl

Materials and Methods

Taxonomic Studies

Growth characteristics and carbohydrate utilization were determined by the method of the International Streptomyces Project (ISP)[13]. Color codes were assigned to the substrate and aerial mass pigments according to the Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago). The spores and mycelia of the strains were observed by scanning electron microscopy (scanning microscope model S-570, Hitachi Co., Ltd.).

Analysis of diaminopimelic acid was performed on the hydrolysate of cultures grown in a medium (glucose 10 g, starch 10 g, beef extract 3 g, yeast extract 5 g, $CaCO_3$ 2 g per liter of water, pH 7.0) for 48 hours at 28°C.

Producing Strains

Three strains producing duocarmycins were isolated from soils collected in Japan and taxonomically assigned to *Streptomyces*. The following high producing strains were used for fermentation: Strain DO-89 No. 3-88 for production of duocarmycins A, B1, B2, C1, C2, strain DO-113 No. N-133 for duocarmycin SA. For the investigation on co-production of duocarmycins, strain DO-88 No. 1 was used.

Culture Conditions

A seed medium (glucose 5 g, soluble starch 25 g, yeast extract 1 g, Peptone-A 10 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCO_3$ 1 g per liter of water, pH 7.2) was inoculated with a loopful of vegetative mycelium on agar slant, and was incubated at 28°C for 48 hours, and then was transferred into a fermentation medium. The effect of carbon, nitrogen sources and other ingredient in the fermentation medium was investigated in 250 ml shake-flasks. The details of fermentation media and culture conditions were described in results and legends of tables and figures.

Culture growth was evaluated by centrifuging untreated fermentation broth in 10-ml test tubes at $1,200 \times g$ for 10 minutes. The packed cell solids were recorded as % of total broth volume (packed cell volume: PCV).

HPLC Analysis of Duocarmycins

The mycelium obtained by centrifugation of 5 ml of fermentation broth at $1,200 \times g$ for 10 minutes was mixed with 2 ml of propanol and stirred for 30 minutes. After centrifugation at $1,200 \times g$ for 10 minutes, 10 μl of the supernatant was subjected to HPLC analysis. Duocarmycins eluted from the column were analyzed with a multi-channel photodiode array detector (Shimadzu SPD-M6A PACII).

Duocarmycins A, B1, B2, C1, C2: YMC ODS-AM-312 (6.0 × 150 mm, Yamamura Chemical) column was run with 0.05 M phosphate buffer (pH 4.0) - MeOH (2 : 3) at a flow rate of 1 ml/minute. The amount of duocarmycins was estimated from its absorbance at 254 nm. Rt at 33°C of duocarmycins: A, 11.7 minutes; B1, 17.3 minutes; B2, 41.8 minutes; C1, 14.6 minutes; C2, 35.0 minutes.

Duocarmycin SA: YMC ODS-AM-303 (4.6 × 250 mm, Yamamura Chemical) column, was run with 0.05 M phosphate buffer (pH 4.0) - MeOH (1 : 1) at a flow rate of 0.7 ml/minute. Duocarmycin SA from the column was detected with a UV monitor at 350 nm. Rt of duocarmycin SA was 9.4 minutes at 30°C.

Results and Discussion

Comparison of Producing Organisms

The duocarmycins were discovered during our search for new antitumor antibiotics from actinomycetes. Duocarmycin A was isolated from a culture of *Streptomyces* sp. DO-88 (FERM BP 1002) and duocarmycin C1 was produced by *Streptomyces* sp. DO-89 (FERM BP 988). Two strains were isolated in 1981 from soils collected at Mishima, the foot of Mt. Fuji (DO-88), and at Mt. Rokko in Hyogo, Japan (DO-89), respectively. Duocarmycin SA was found later from a culture of *Streptomyces* sp. DO-113 (FERM BP 2222) which was isolated in 1986 from a soil collected at Rokkakudo temple in Kyoto.

Whole cell analysis indicated the presence of LL-diaminopimelic acid in three strains. Based on morphology and the above whole cell analysis, three duocarmycins producing strains were identified as *Streptomyces* spp.[14~18]. The vegetative mycelium of the organisms grew abundantly on complex and synthetic media. No soluble pigment was produced by these three strains. All three strains formed spiral spore chains and the spore surface of the strains were smooth (Fig. 2).

Strain DO-113 decomposed cellulose, whereas other strains DO-88 and DO-89 could not. While DO-113 could not utilize both D-fructose and sucrose, DO-88 and DO-89 could utilize these sugars. Thus, strain DO-113 showed different physiological characteristics from the other strains DO-88 and DO-89 which seems to be similar taxonomically (Table 1).

Fig. 2. Scanning electron micrographs of strain DO-88 (A), DO-89 (B), DO-113 (C).

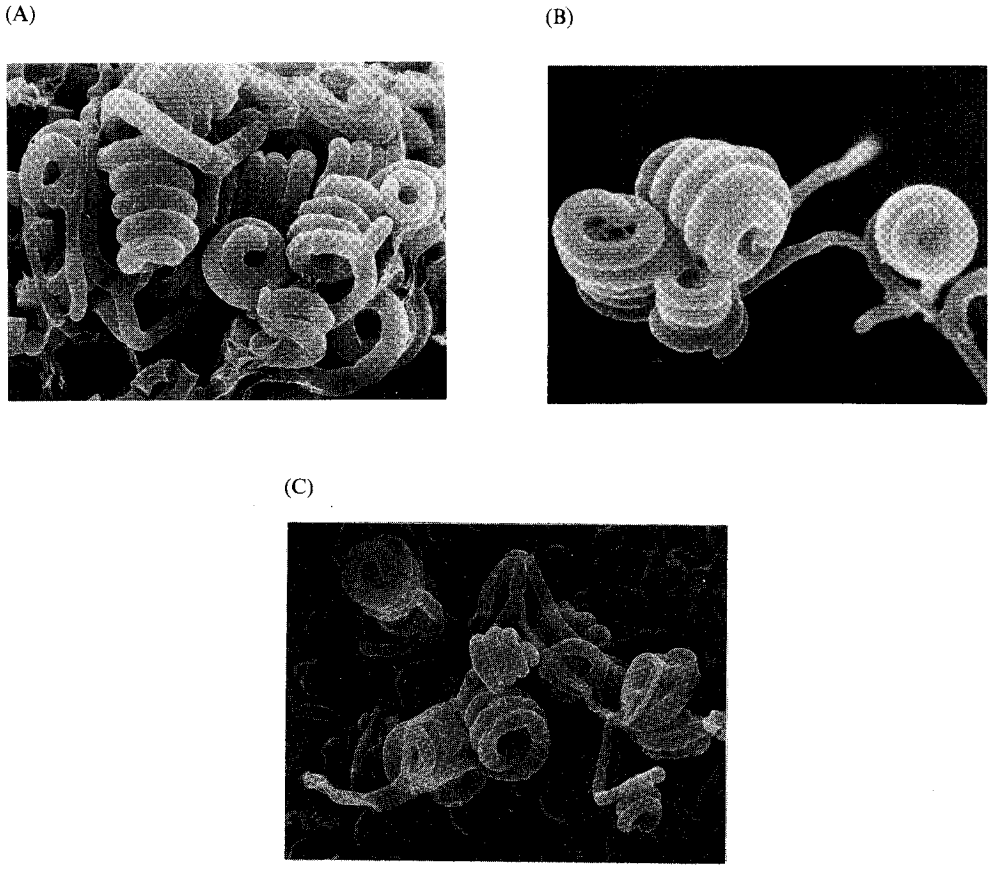


Table 1. Comparison of duocarmycin producing strains.

	Strain				Strain		
	DO-88	DO-89	DO-113		DO-88	DO-89	DO-113
Spore chain	*Spira*	*Spira*	*Spira*	Hydrolysis of starch	+	+	+
Spore surface	Smooth	Smooth	Smooth	Utilization of carbon sources:			
Aerial mass color	Gy, W	Gy	Gy,Y	L-Arabinose	+	+	+
Melanoid pigment:				D-Xylose	+	+	+
Tyrosine agar	−	−	−	D-Glucose	+	+	+
Peptone-yeast extract-iron agar	−	−	−	D-Fructose	+	+	−
				D-Mannitol	+	+	+
Soluble pigment	−	−	−	Sucrose	+	+	−
Liquefaction of gelatin	−	−	−	*m*-Inositol	+	+	+
Peptonization of milk	+	+	+	Raffinose	+	+	+
Decomposition of cellulose	−	−	+	L-Rhamnose	+	+	+

Gy: Gray, W: white, Y: yellow.

Fermentation

Duocarmycin A and Its Halogenated Seco-compounds, B1, B2, C1, C2

As previously reported, duocarmycin A was isolated from culture broth of *Streptomyces* sp. DO-88[1] and duocarmycins B1, B2, C1, C2 were isolated from culture broth of *Streptomyces* sp. DO-89[2,3]. Early conclusions from the experiments for optimization of titers were as follows: Duocarmycins B1 and B2 were produced in the presence of KBr and duocarmycins C1 and C2 were produced in the presence of KCl. Thus, we have considered that duocarmycin A is biosynthesized and then duocarmycins B1, B2, C1, C2 are generated in the same mode of chemical reaction of duocarmycin A with halogen ions in the fermentation broth.

Both strain DO-88 and strain DO-89 could produce duocarmycins A, B1, B2, C1, C2 according to the manipulation of media. Titers of duocarmycins A and C1 was less than 1 μg/ml by using the original media and culture conditions. Duocarmycin A is unstable in culture media whereas halogenated seco-compounds are more stable. Thus we focused fermentation studies on the optimization of media and culture condition for the halogenated seco-compounds. A high producing strain DO-89 No. 3-88 was obtained by monospore isolation and used in the following studies.

The maximum titer depended heavily on nitrogen sources. Ebios (Asahi Breweries, Limited) and dry yeast are good nitrogen sources, and the mixture of Ebios (2.5%) and dry yeast (1.5%) supported the high titers of C1 (2.1 μg/ml) and C2 (7.6 μg/ml) (Table 2).

Basal UF1 medium was designed as follows; maltose 50 g, Ebios 25 g, dry yeast 15 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g and $CaCO_3$ 5 g in 1 liter of water, pH 5.0. Table 3 shows the production of duocarmycins in UF1 and UF1 with 1% KBr (UF1-B medium) and UF1 with 1% KCl (UF1-C medium).

We examined the addition of high porous resins to adsorb duocarmycins and found that accumulation of duocarmycins C1 and C2 was significantly increased without inhibition of cell growth. Among the resins tested, Amberlite XAD-7 was the most effective one to accumulate duocarmycins C1 and C2 (Table 4). The same fermentation method with KBr instead of KCl supported an effective production of duocarmycins B1 and B2 (Table 4). From further experiments on ingredients of fermentation medium in the presence of Amberlite XAD-7 resin, UF2-B medium with fructose (1%) and maltose (4%) was selected for large scale fermentation of duocarmycins B1 and B2. UF2-B medium; maltose 4%, fructose 1%, Ebios 2.5%, dry yeast 1.5%, KBr 1.0%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.5%, pH 5.0. Amberlite XAD-7 (6.6%) was added at 48 hours after inoculation. Fig. 3 shows the time course of duocarmycins

Table 2. Effect of nitrogen sources on production of duocarmycins C1 and C2.

N sources	Duocarmycins (μg/ml) at 120 hours	
	C1	C2
Soybean meal (4%)	<0.1	0.6
Yeast extract (4%)	<0.1	0.3
Pharmamedia (4%)	<0.1	<0.1
Dry yeast (4%)	0.2	0.7
Ebios (4%)	1.0	5.5
Ebios (2.5%)+dry yeast (1.5%)	2.1	7.6

Basal fermentation medium (g/liter): Maltose 50, KCl 10, KH_2PO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, $CaCO_3$ 5, pH 4.0.

Table 3. Selective production of duocarmycin A and halogenated seco-compounds with supplement of Br^- or Cl^- in medium.

Medium	Duocarmycins (μg/ml)				
	A	B1	B2	C1	C2
UF1	1.0	<0.05	<0.05	<0.05	<0.05
UF1-B	1.0	7.2	26.3	<0.05	<0.05
UF1-C	1.0	<0.05	<0.05	4.9	17.1

Strain DO-89 No. 3-88 grown in seed medium was inoculated into UF1, UF1-B and UF1-C media and cultured for 120 hours at 28°C. Duocarmycins were analyzed by HPLC.

Table 4. Effect of high porous resin on production of duocarmycins B1, B2, C1, and C2.

(A)

High porous resin	Duocarmycins (μg/ml)		pH	PCV (%)
	C1	C2		
None	2.3	12.3	5.4	48
Diaion HP-20	9.1	31.8	5.4	45
Amberlite XAD-2	12.4	39.4	5.6	48
Amberlite XAD-7	16.7	89.8	5.5	48

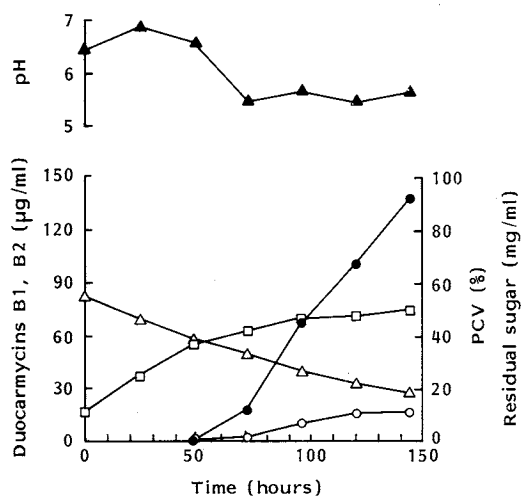
(B)

High porous resin	Duocarmycins (μg/ml)		pH	PCV (%)
	B1	B2		
None	7.2	26.3	6.0	48
Amberlite XAD-7	19.1	104.1	6.1	48

Medium UF1-C was used for experiment (A) and medium UF1-B was used for experiment (B). High porous resin (6.6%) was added at 48 hours after inoculation in all experiments. Cell growth (PCV), pH and titer of duocarmycins were determined after 120 hours of fermentation at 28°C.

Fig. 3. Time course of duocarmycins B1, B2 fermentation in a 2,000-liter tank fermenter.

pH (▲), PCV (□), residual sugar (△), titers of duocarmycin B1 (○), and duocarmycin B2 (●).



Fermentation medium UF2-B medium (1,000 liters) in 2,000-liter tank fermenter were inoculated with 100 liters of seed culture and incubated for 144 hours at 28°C with agitation at an impeller speed of 120 rpm and aeration of 400 liters/minute. The pH of culture broth was controlled at 5.5 using 6 N H_2SO_4 during fermentation. Amberlite XAD-7 (6.6%) was added to culture medium at 48 hours after inoculation.

B1, B2 production in 2,000-liter tank fermenter under the optimum conditions. The pH of culture broth was controlled at 5.5 with 6 N H_2SO_4 during fermentation. Cell growth continued until 144 hours. Production of duocarmycins B1 and B2 started at about 72 hours and 17 μg/ml of duocarmycin B1 and 138 μg/ml of duocarmycin B2 were accumulated. 10 g of duocarmycin B1 and 50 g of duocarmycin B2 were obtained from 1,000-liter culture broth using the isolation procedure described previously[2]. These culture conditions were also applicable for production of duocarmycins C1 and C2.

Duocarmycin A has been available by the chemical conversion from B2, that is, treatment of duocarmycin B2 with 1,5-diazabicyclo[5.4.0]undecene-5 (DBU) in acetonitrile gave duocarmycin A quantitatively[2].

Duocarmycin SA

Titer of duocarmycin SA was less than 0.1 μg/ml by using the original medium and culture conditions[4]. A high producing strain DO-113 No. N-133 was obtained by monospore isolation and used in the following studies. We manipulated the ingredients of media, and the fermentation titers of duocarmycin SA were increased to about ten times.

Ingredients that aided production of duocarmycin SA include soluble starch, Ebios and trace metals (Tables 5 and 6). The effective compounds of the metal mixture were $CoSO_4$ (1 μg/ml) and $NiSO_4$ (1 μg/ml). Among amino acids tested, supplement of tryptophan (1 mg/ml) increased the titer of duocarmycin SA (elicited 30%, data not shown). From these results we designed the improved fermentation medium UF3: soluble starch 50 g, Ebios 25 g, tryptophan 1 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CoSO_4$ 1 mg, $NiSO_4$

Table 5. Effect of carbon and nitrogen sources on production of duocarmycin SA.

C or N sources	Duocarmycin SA at 96 hours (μg/ml)
With dry yeast (1.4%) as N sources:	
Soluble starch	0.32
Glycerol	0.14
Dextrin	0.14
Sucrose	<0.1
Maltose	<0.1
With soluble starch (5%) as C sources:	
Ebios	0.32
Dry yeast	<0.1
Soybean meal	<0.1
Yeast extract	<0.1

Basal fermentation medium (g/liter): KH_2PO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, $CuSO_4$ 0.001, $NiSO_4 \cdot 7H_2O$ 0.0005, $CrK(SO_4)_2 \cdot 12H_2O$ 0.001, $CaCO_3$ 5, pH 7.0.

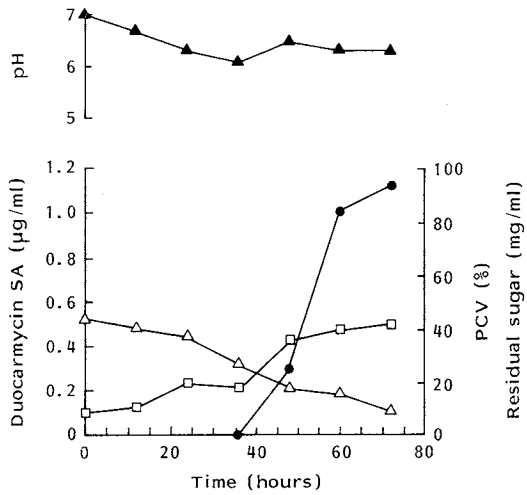
Table 6. Effect of trace metals on production of duocarmycin SA.

Compounds	Duocarmycin SA at 96 hours (μg/ml)
None	0.26
$ZnSO_4$ (100 μg/ml)	0.36
$MnSO_4$ (100 μg/ml)	0.29
$CuSO_4$ (1 μg/ml)	0.31
$CoSO_4$ (1 μg/ml)	0.63
$NiSO_4$ (1 μg/ml)	0.69

Basal fermentation medium (g/liter): Soluble starch 50, Ebios 15, KH_2PO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, $CaCO_3$ 5, pH 7.0.

Fig. 4. Time course of duocarmycin SA fermentation in a 2,000-liter tank fermenter.

pH (▲), PCV (□), residual sugar (△), and titer of duocarmycin SA (●).



Fermentation medium (1,000 liters) in 2,000-liter tank fermenter were inoculated with 50 liters of seed culture and incubated for 72 hours at 26°C with agitation at an impeller speed of 120 rpm and aeration of 400 liters/minute.

1 mg, $CaCO_3$ 5 g in 1 liter of water, pH 7.0. Fig. 4 shows the time course of duocarmycin SA production in 2,000-liter tank fermenter under the optimum conditions. Cell growth continued until 72 hours. Duocarmycin SA production started at about 40 hours and 1.1 μg/ml of duocarmycin SA was accumulated during 80 hours of fermentation. Using the isolation procedure described previously 100 mg of duocarmycin SA was isolated from 1,000 liters fermentation broth[4].

As described in the previous section, halogenated seco-compounds of duocarmycin A could be produced by opening at the cyclopropane ring by bromide or chloride ion in culture media. In contrast, in the case of duocarmycin SA, no halogenated seco-compounds was detected in the fermentation broth with supplement of KBr or KCl. Thus the difference in reactivity seems to depend on the different electronic characteristics of each terminal five-membered ring structure.

Co-production of Duocarmycins A and SA

Although duocarmycins SA and A shows different reactivity with the halogen ions, they have the common structure except the different substitution at C-2 and C-3 position (Fig. 1). Thus, it seemed to be possible that duocarmycins A and SA are synthesized *via* common biosynthetic pathway.

We investigated whether both compounds can be co-produced by three different duocarmycins producing strains. In this experiment strain DO-88 No. 1 and DO-89 No. 3-88 were cultured in UF2-C fermentation medium (30 ml) for 95 hours and 116 hours, respectively. In both cases, Amberlite XAD-7

(2 ml) was added at 48 hours after inoculation. DO-113 No. N-133 was grown in UF-3 fermentation media for 90 hours.

Each mycelium obtained by centrifugation of 30 ml of culture broth at $1,200 \times g$ for 10 minutes was combined with 30 ml of propanol and stirred for 90 minutes in cold room (4°C). After centrifugation, the supernatant was applied to Diaion HP-20 resin (2 ml). After washing the eluate with ethyl acetate (6 ml) was concentrated and redissolved in 1 ml of chloroform.

The HPLC analysis of the chloroform solution revealed that all these three strains could produce duocarmycins A and SA. The peaks of duocarmycins A and SA were identified by their Rt on HPLC and UV spectrums recorded with a multi-channel photodiode array detector. The quantities of both compounds of each 30 ml fermentation broth are as follows: DO-88 No. 1, duocarmycins A 23.0 μg and SA 1.0 μg; DO-89 No. 3-88, duocarmycins A 2.7 μg and SA 0.1 μg; DO-113 No. N-133, duocarmycins A 1.5 μg and SA 1.0 μg.

The biosynthetic relationship between duocarmycins A and SA is interesting and remains to be studied.

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