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Accumulation of Narbonolide by the Addition of Sodium Arsenite†

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Addition of sodium arsenite to the fermentation medium of *Streptomyces venezuelae* MCRL–0376 caused an inhibition of antibiotic production and a simultaneous accumulation of narbonolide,[1~3] the aglycone of narbomycin. The accumulation of narbonolide was not observed in the absence of sodium arsenite. The inhibition of antibiotic production by sodium arsenite was not reversed by sodium acetate in contrast to erythromycin fermentation.[4,5]

As reported in the previous papers,[1~3] the authors investigated the production of narbonolide, the aglycone of narbomycin (Fig. 1), using *S. venezuelae* MCRL–0376 and found that this microorganism accumulated narbonolide in the presence of various organic acids.

Concerning the isolation of the aglycone part of macrolide antibiotics, Tardrew and Nyman[6] reported on erythromycin aglycone (erythronolide-B) employing a mutant strain derived from erythromycin-producing strain, *Streptomyces erythreus*.

We attempted the production of macrolide aglycone by using various metabolic inhibitors and found that *S. venezuelae* MCRL–0376 accumulated a large amount of narbonolide when the producer was cultured in the presence of sodium arsenite.

In this present paper, the identification and the accumulation of narbonolide observed by the addition of sodium arsenite are described.

MATERIALS AND METHODS

The strain used *Streptomyces venezuelae* MCRL–0376 which produce various macrolide antibiotics (picromycin as the main product, also narbomycin, methymycin, neomethymycin and 10-deoxymethymycin).

Basal culture medium used the following composition: glucose 10 g, glycerol 10 g, polypepton 10 g, meat extract 5 g, NaCl 5 g, $CaCl_2$ 2 g, yeast extract 1 g in one liter. Sodium salt of arsenic acid ($NaAsO_2$) was added to give a final concentration, as described in each experiments. All fermentations were carried out in 500 ml Erlenmeyer flasks on a rotatory-type shaker (about 170 rpm) at 27°C. Each 500 ml flask contained 100 ml of the medium.

During the fermentation, aliquots of culture broth were sampled, and the antibiotic activity and narbonolide accumulation were measured by the following procedures.

Antibiotic production was assayed by the cup plate method using *Bacillus subtilis* PCI–219 as a test organism and picromycin as a reference standard. The amount of antibiotics was expressed in terms of picromycin equivalent.

Narbonolide was detected by silica gel thin-layer chromatography (benzene: ethyl acetate=3: 2). The presence of narbonolide was observed at *Rf* 0.6 by spraying 40% H_2SO_4 followed by heating at 100°C for 10 min. The production of narbonolide in each fermentation broth was measured by the Hitachi MPF–2A Fluorescence Spectrophotometer as described in the previous report.[3]

RESULTS

1) *Identification of narbonolide produced by S. venezuelae MCRL–0376 cultured in the medium containing sodium arsenite*

S. venezuelae MCRL–0376 was cultured in the medium containing 3×10^{-4} M of sodium arsenite for 3 days. The filtrate of the culture was adjusted to pH 4.5 with 1 N HCl and the accumulated metabolites were extracted with ethyl acetate. The extract was chromatographed on a silica gel column and eluted with benzene: ethyl acetate (2: 1, v/v). This eluate was concentrated *in vacuo* and crystals appeared during the concentration. The crude crystals were separated from the liquid by

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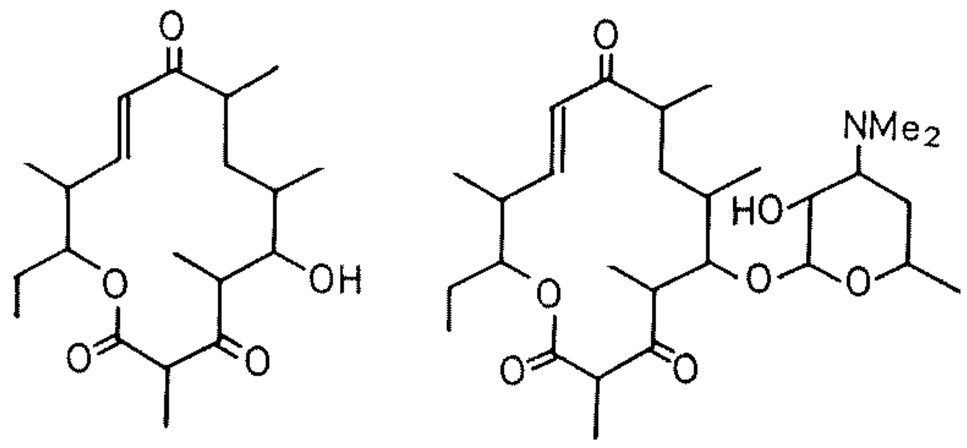

Narbonolide Narbomycin

FIG. 1.

TABLE I. PHYSICOCHEMICAL PROPERTIES OF ISOLATED AND AUTHENTIC NARBONOLIDE

		Isolated	Authentic
mp		125~126°C	125~126°C
M.W. (Mass)		352	352
UV λ_{max}^{EtOH} nm (ε)		228.5 (8200)	228.5 (8200)
IR ν_{max}^{Nujol} cm^{-1}		3440 (OH)	3440 (OH)
		1723 (C=O)	1723 (C=O)
		1680 (C=O)	1680 (C=O)
		1620 (C=C)	1620 (C=C)
Rf	A[a]	0.77	0.77
	B[b]	0.50	0.50
	C[c]	0.60	0.60

a) Silica gel plate, *n*-hexane: ethyl acetate (1: 1, v/v).
b) Silica gel plate, benzene: acetone (9: 1, v/v).
c) Silica gel plate, benzene: Ethyl acetate (3: 2, v/v).

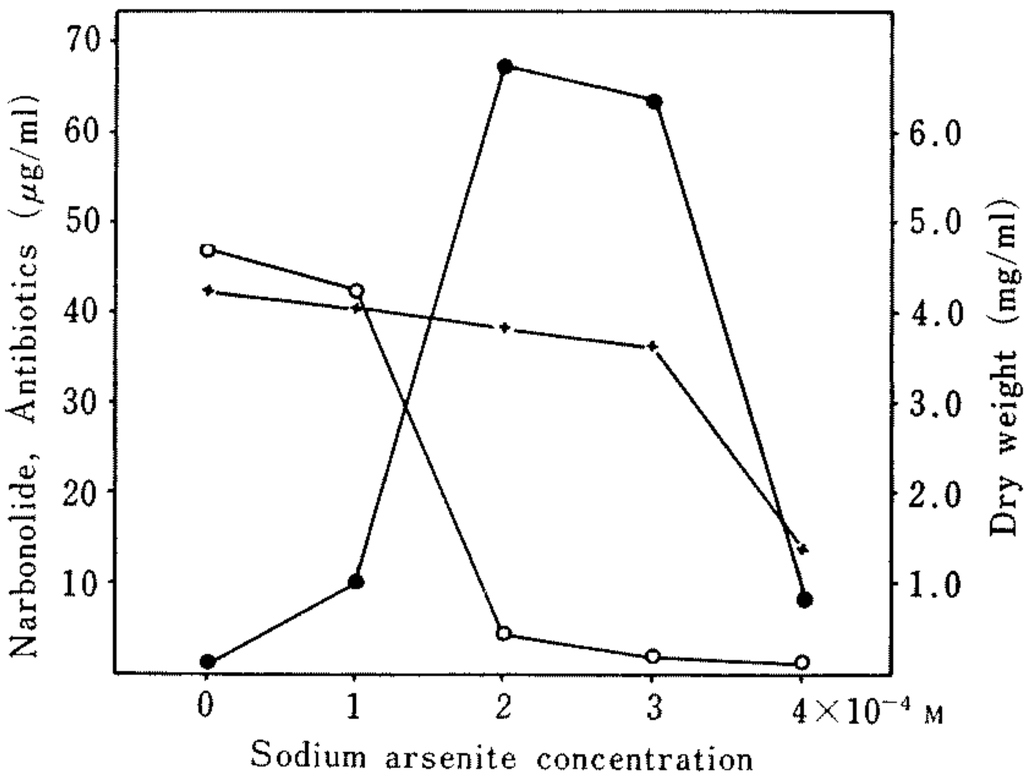

FIG. 2. Effect of Concentration of Sodium Arsenite on Antibiotic Production, Narbonolide Accumulation and Dry Weight of Cell.

S. venezuelae was grown in the basal medium containing the indicated quantity of sodium arsenite for 70 hr.
○—○, antibiotics; ●—●, narbonolide; ×—×, dry weight of cell.

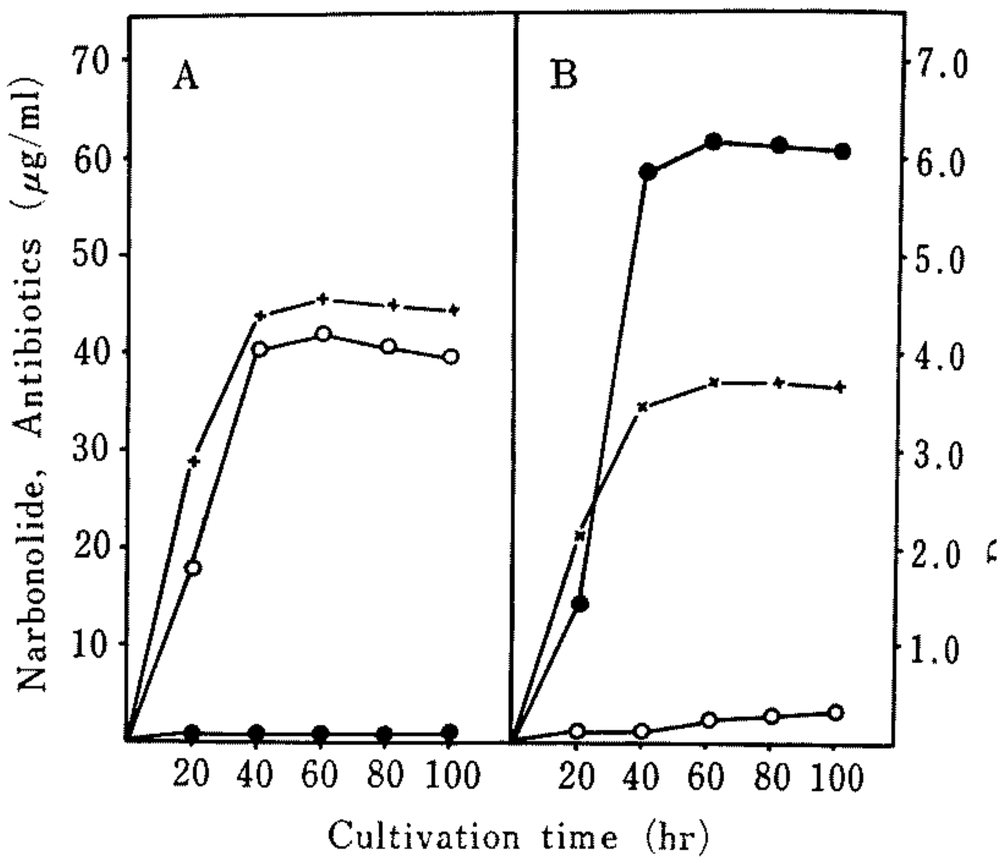

FIG. 3. The Course of Antibiotic Production, Narbonolide Accumulation and Dry Weight of Cell in Culture of *S. venezuelae* Grown in Absence (A) and Presence (B) of 3×10^{-4} M Sodium Arsenite.

○—○, antibiotics; ●—●, narbonolide; ×—×, dry weight.

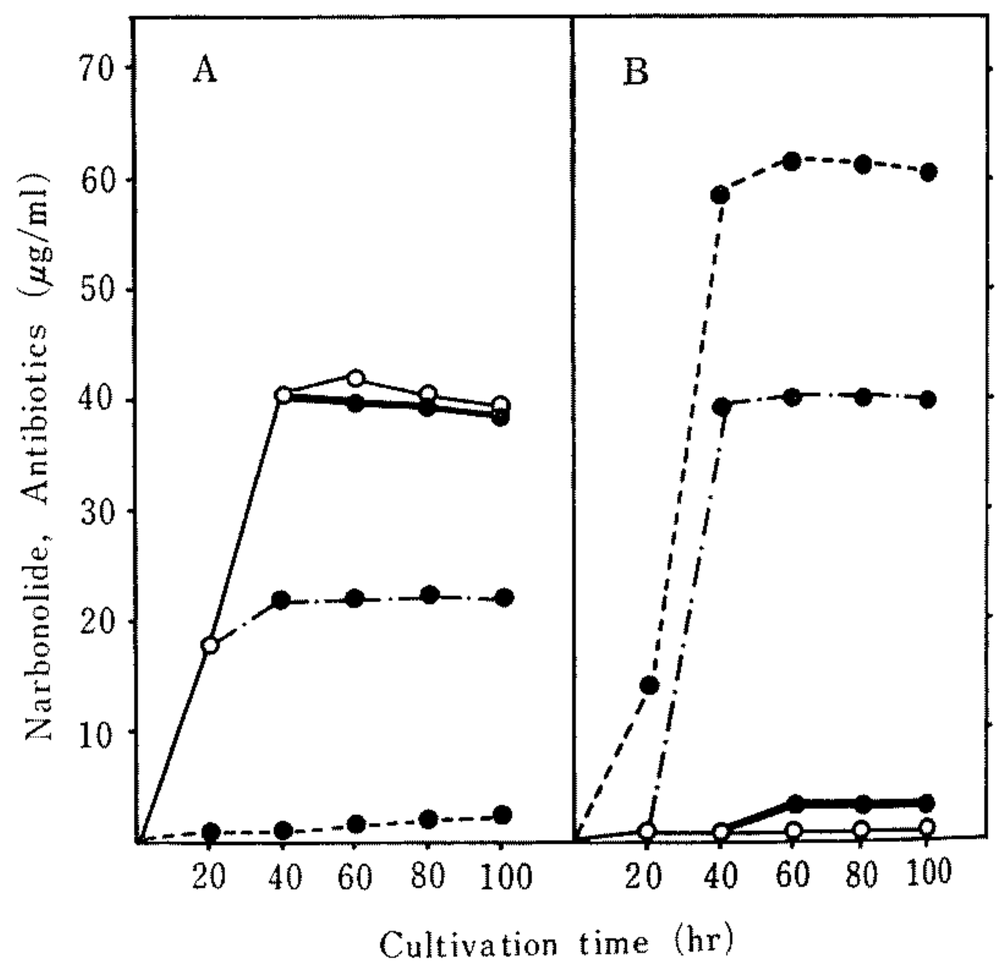

FIG. 4. Effect of Sodium Arsenite on Antibiotic Production (A) and Narbonolide Accumulation (B).

S. venezuelae was grown in the basal medium added with sodium arsenite (3×10^{-4} M) at zero hr, 20 hr and 40 hr. ○—○, no addition; ●---●, zero hr; ●-·-●, 20 hr; ●—●, 40 hr.

filtration and were recrystallized from ethyl acetate-*n*-hexane to afford colorless needles.

The crystals were compared with an authentic narbonolide[1] which was isolated from the culture harvested in the medium containing sodium acetate. The physicochemical pro-

perties of the isolated crystals were identical with those of the authentic narbonolide as shown in Table I.

2) Effects of sodium arsenite on narbonolide accumulation, antibiotic production and cell growth

S. venezuelae MCRL–0376 was grown in the medium containing varied concentrations of sodium arsenite. After 70 hr of cultivation, the production of narbonolide and antibiotics was measured and the dry weight of the cells in each culture were determined.

As summarized in Fig. 2, a considerable accumulation of narbonolide was observed in the fermentation broth containing sodium arsenite, and the optimum concentration of arsenite on narbonolide accumulation was 2 to 3×10^{-4} M.

The presence of sodium arsenite showed remakable inhibition of antibiotic production. The production of antibiotics was inhibited more than 95% of control at the above concentration of sodium arsenite, whereas the cell growth was decreased by only 15% of control. More than 4×10^{-4} M concentration showed inhibition of narbonolide production and cell growth.

In the culture without sodium arsenite, the strain MCRL–0376 produced about 40 μg/ml of antibiotics and the cell growth reached almost maximum (4.6 mg/ml) at 40 hr of fermentation, while narbonolide accumulation was not observed (Fig. 3). On the other hand, when sodium arsenite was added to the culture at a concentration of 3×10^{-4} M (at zero time), the cell growth reached the maximum (3.8 mg/ml) at 40 hr and antibiotic production was almost completely inhibited. Narbonolide was produced in the period of active cell growth and attained the maximum (60 μg/ml) at 40 hr of fermentation. Afterwards, the accumulation remained unchanged. When sodium arsenite was added at different times of fermentation, marked effects of sodium arsenite on the antibiotic and narbonolide production were observed at the earlier time of fermentation. Namely, the antibiotic production was inhibited almost completely at zero time-addition, but the addition of sodium arsenite did not show any effect at 40 hr of fermentation. Meanwhile, the highest narbonolide accumulation was shown at zero time-addition and the accumulation was very small (about 3 to 4 μg/ml) at 40 hr addition (Fig. 4).

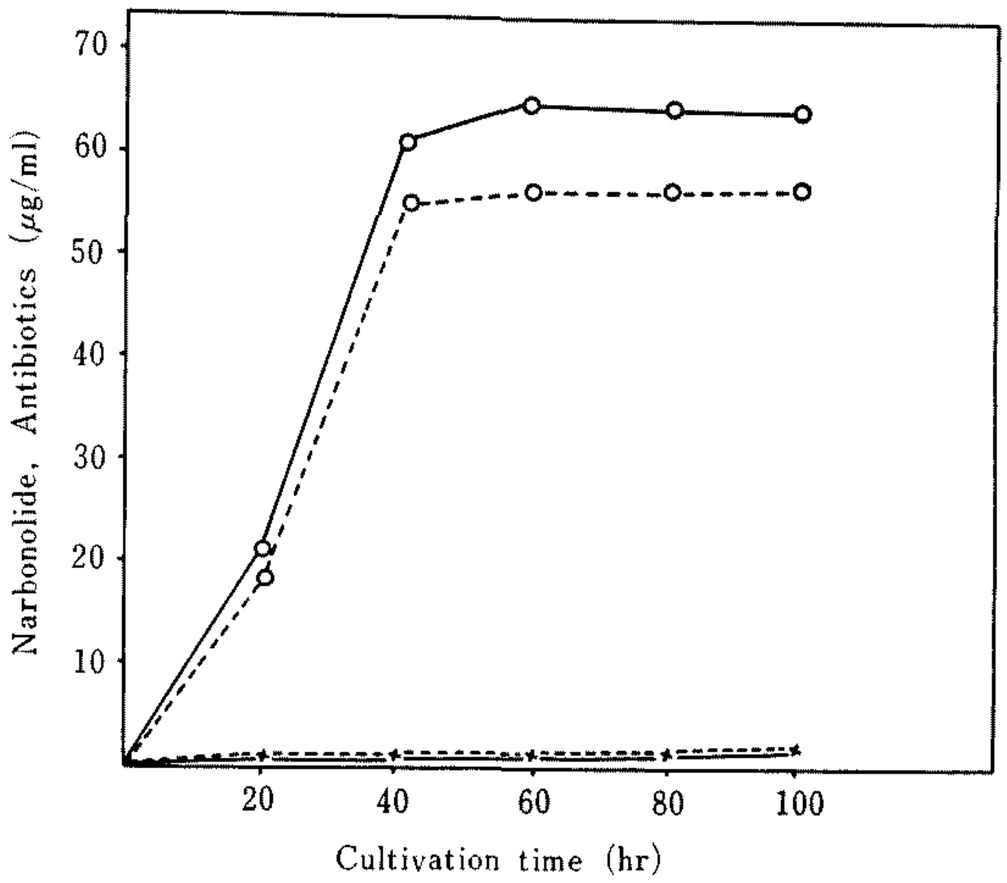

FIG. 5. Effect of Sodium Arsenite and Sodium Acetate on Antibiotic Production and Narbonolide Accumulation.

S. venezuelae was grown in the basal medium containing the indicated quantity of sodium arsenite and sodium acetate.
Antibiotic production: ×---×, 3×10^{-4} M sodium arsenite; ×—×, 3×10^{-4} M sodium arsenite and 0.04 M sodium acetate. Narbonolide accumulation: ○---○, 3×10^{-4} M sodium arsenite; ○—○, 3×10^{-4} M sodium arsenite and 0.04 M sodium acetate.

Since the inhibition of erythromycin production by sodium arsenite was reversed by sodium acetate, the effect of sodium acetate on the antibiotic production of *S. venezuelae* MCRL–0376 and the narbonolide accumulation were examined in the culture added with 3×10^{-4} M sodium arsenite. As shown in Fig. 5, narbonolide accumulation was not affected by the addition of sodium acetate and reached the maximum at 40 hr, yielding about 10 to 15% higher than that in the control which used only sodium arsenite. On the other hand, the production of antibiotics was repressed as well in the culture with addition of sodium arsenite and reversion of antibiotic production by the addition of sodium acetate was not observed in contrast to erythromycin fermentation.[4,5]

DISCUSSION

In the studies of erythromycin fermentation, it has been reported by Musílek and Ševčík[7] that the addition of sodium arsenite caused an accumulation of pyruvic acid and acetaldehyde with a simultaneous inhibition of the antibiotic production. However, the data given by our experiments on the effect of sodium arsenite in the culture of *S. venezuelae* MCRL–0376 showed a distinctly different result from those of erythromycin fermentation. Namely, sodium arsenite caused a large accumulation of narbonolide and a simultaneous inhibition of antibiotic production.

Other investigators[4,5,8] reported that the inhibition of erythromycin production by sodium arsenite was removed to some extent by the presence of sodium acetate, sodium propionate and sodium formate. This suggests that these organic acids are closely connected with the biosynthesis of erythromycin and sodium arsenite may inhibit a series of organic acid metabolism catalyzed by lipoic acid.

However, as to *S. venezuelae* MCRL–0376, the inhibition of antibiotic production by sodium arsenite was not reduced by sodium acetate, and sodium acetate increased narbonolide accumulation to some extent. In the culture of the strain MCRL–0376, sodium arsenite appeared to affect sugar residue (desosamine) formation rather than aglycone formation.

The accumulation of narbonolide by *S. venezuelae* MCRL–0376 would be a specific phenomenon, because the addition of sodium arsenite was not successful in detecting the aglycone part in the culture of 14–membered macrolide producing strains such as *Streptomyces narbonensis* ISP–5016 (narbomycin-producing strain) and *Streptomyces erythreus* NRRL–0338 (erythromycin-producing strain)

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REFERENCES

1) T. Hori, I. Maezawa, N. Nagahama and M. Suzuki, *Chem. Commun.*, **1971**, 304.
2) T. Hori, I. Maezawa, N. Nagahama and M. Suzuki, 14th Symposium on the Chemistry of Natural Products, Fukuoka City, Japan, 1970, p. 85.
3) I. Maezawa, T. Hori, and M. Suzuki, *Agr. Biol. Chem.*, **38**, 91 (1974).
4) V. Musílek and V. Ševčík, *Folia Microbiol.*, **4**, 316 (1959).
5) Z. Vaněk and J. Majer, "Antibiotics," Vol. II, ed. by D. Gottlieb and P. W. Show, Springer-Verlag, Berlin, 1967, p. 154.
6) P. L. Tardrew and M. A. Nyman, U. S. Patent, 3127315 (1965). [*C.A.*, **60**, 16469 (1965)].
7) V. Musílek and V. Ševčík, *Naturwissenschaften*, **45**, 215 (1958).
8) V. Ševčík and V. Musílek, *Antibiotiki*, **6**, 9 (1961).