ISOLATION OF AN INTERMEDIATE OF 2-DEOXYSTREPTAMINE BIOSYNTHESIS FROM A MUTANT OF *BACILLUS CIRCULANS*

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Eight 2-deoxystreptamine-negative (DOS$^-$) mutants were isolated from various strains of *Bacillus circulans*, normally producing butirosin, xylostasin and ribostamycin. These mutants were classified into two groups (converter and secretor) by the simple method of cosynthesis using agar plate culture. One of the cosynthetic pairs, strain S-11 and strain 236, was selected for further study From the fermentation broth of strain S-11, an intermediate of DOS biosynthesis, S-11-P, was isolated. This compound was converted to butirosin (BTN) effectively by strain 236. The structure of S-11-P was considered to be (1 L or 1 D)-1,3,5/2,4-5-aminocyclohexanetetrol, and the pathway of DOS biosynthesis is discussed here.

2-Deoxystreptamine (DOS) is a widely distributed component of many important aminoglycoside antibiotics, such as butirosin, xylostasin, ribostamycin, neomycin, kanamycin and gentamicin. However, the complete biosynthetic pathway has not been elucidated. RINEHART and STROSHANE[1] proposed two pathways of DOS biosynthesis, based on the results of ^{13}C-glucose and ^{13}C-glucosamine incorporation into neomycin. DAUM, *et al.*[2,3] examined the bioconversion of many synthesized cyclitols and aminocyclitols to gentamicins by a DOS$^-$ mutant of *Micromonospora purpurea* and supposed the pathway proposed by RINEHART, *et al.*, to be borne out. Recently, FURUMAI, *et al.*[4] reported that 2-deoxy-*scyllo*-inosose and 2-deoxy-*scyllo*-inosamine were converted to butirosin by butirosin-non-producing blocked mutants of *Bacillus circulans*. However, they used racemic compounds in their experiments, so it seems that the true pathway was still uncertain. Along with tracer experiments, isolation and analysis of a blocked mutant are very effective approaches to the study of a biosynthetic pathway. We report here the isolation of some kinds of DOS$^-$ mutants from butirosin (BTN), xylostasin (XLN), or ribostamycin (RBM) producing strains of *B. circulans*. Isolation of an aminocyclitol, S-11-P, which seems to be a precursor in DOS biosynthesis was performed with one of the mutants (secretor). The detection and assay of S-11-P were carried out by using another class of DOS$^-$ mutant (converter) having the ability to transform S-11-P to BTN.

Materials and Methods

Strains

DOS$^-$ mutants used in this experiment are listed in Table 1. Isolation procedures of the parent strains of each mutant were reported in the previous paper[5].

Cosynthesis by agar plate culture

Two mutant strains were streaked on a production agar medium in a Petri dish containing 1.0% glycerol, 2.0% Soytone (Difco), 0.3% $MgSO_4 \cdot 7H_2O$ and 1.5% agar, then incubated at 28°C for 5 days (Fig. 1). Agar pieces A and B were cut off with a cork borer and their antibiotic activities were assayed on an agar plate seeded with *Bacillus subtilis* PCI 219 as test organism. When the inhibition zone dia-

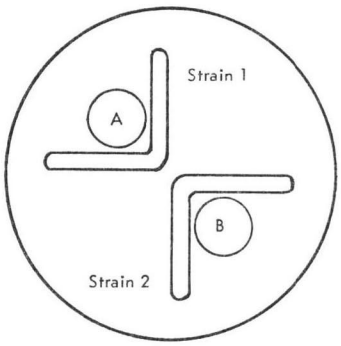
meter of agar piece A was larger than B, it was interpreted that strain 2 should be a secretor of a certain biosynthetic precursor which was converted to an antibiotic by strain 1, and *vice versa*. When both agar pieces A and B had little or no antibiotic activity, it was concluded that these two strains did not act as a cosynthetic pair.

Table 1. DOS⁻ mutants of *B. circulans*.

Strain	Parent	Product of DOS added culture
231	B15M	BTN
236	B15M	BTN
241	B15M	BTN
S-11	Mot 3	XLN
S-12	Mot 3	XLN
G-16	Mot 3	XLN
45110	451	RBM
45114	451	RBM

Fig. 1. Characterization of DOS⁻ mutants by a cosynthetic experiment on an agar plate.

The large circle represents the agar plate, and two DOS⁻ mutants, strains 1 and 2, were streaked as illustrated in the figure. Small circles A and B represent the agar pieces cut off after 5 days incubation at 28°C. See Materials and Methods for details.



Antibiotic activity	Converter	Secretor
A > B	Strain 1	Strain 2
A < B	Strain 2	Strain 1

Cosynthesis by liquid culture

Seed medium (S-4) containing 2.0% soluble starch, 1.0% tryptone (Difco), 1.0% yeast extract (Difco), and 0.3% $MgSO_4 \cdot 7H_2O$, (pH 7) was inoculated with a loopful of cells grown on a soybean agar slant and incubated at 28°C for 24 hours on a reciprocal shaker. Each 0.2 ml of the seed culture prepared by two mutant strains to be tested was simultaneously inoculated into 10 ml of fermentation medium (F-5) containing 3.0% corn starch, 3.0% oat meal, 2.0% Polypepton, 0.3% $MgSO_4 \cdot 7H_2O$, and 1.0% $CaCO_3$. Antibiotic activity after 5 days culture at 28°C on a reciprocal shaker was assayed, and the product was purified by column chromatography using Amberlite IRC-50 (NH_4^+) and identified by TLC-bioautography.

Assay of an intermediate of DOS biosynthesis (S-11-P)

For the assay of S-11-P, strain 236 was used. The assay procedure was essentially the same as in the case of cosynthesis by liquid culture. A 0.4-ml amount of seed culture of strain 236 was transferred into 10 ml of the production medium (F-5) and incubated for 24 hours on a reciprocal shaker. An S-11-P containing sample was supplemented to this culture broth and, after 4 days incubation, antibiotic activity was assayed with *B. subtilis* as test organism. A blank culture was made without the addition of S-11-P.

Fermentation and isolation of S-11-P

A seed culture (medium S-4) of strain S-11 was used to inoculate to 20 liters of medium F-5 in a 30-liter jar fermenter. After 4 days fermentation at 28°C, the culture filtrate was adsorbed on Amberlite IRC-50 (NH_4^+). The material eluted with 1 N aqueous ammonia was again adsorbed on the column of the same resin. The S-11-P fraction eluted from the column with 1 N ammonia was concentrated under reduced pressure. The concentrate was purified by repeated chromatography using an Amberlite CG-50 (NH_4^+) column with linear gradient elution by ammonia (0~0.4 N), followed by passage through a column of Dowex 1×4 (OH^-). The purified fraction containing S-11-P thus obtained was lyophilized and used for structure elucidation.

Results

Cosynthetic experiments on agar plates were performed by cultivating simultaneously 2 mutant

Table 2. Summary of cosynthetic experiments and the classification of mutants.

		Secretor				
		231	45114	S-11	S-12	G-16
Converter	236	18*, BTN** (15)	23, BTN (17)	17, BTN (0)	19, BTN (17)	18, BTN (13)
	241	21 (14)	23 (16)	18 (14)	21 (18)	18 (14)
	45110	20, RBM (17)	20, RBM (0)	18, RBM (0)	18, RBM (16)	18, RBM (15)

* Figure indicates the inhibition zone diameter (mm) of an agar piece (10 mm dia.) from agar plate culture, figure in parenthesis indicates the inhibition zone diameter of the counterpart of the agar piece.

** BTN or RBM indicates the product of cosynthesis by liquid culture. Products of cosynthesis with converter strain 241 were not identified.

strains selected from 8 DOS$^-$ mutants. The pairs for cosynthesis were reconfirmed by mixed cultures in liquid medium and the products were characterized by TLC-bioautography. The data obtained on all combinations (56 pairs) are summarized in Table 2. The eight mutants were divided into two groups. One is the converter group consisting of strains 236, 241 and 45110 and the other is the secretor group of strains 231, S-11, S-12, G-16 and 45114. Among the cosynthetic pairs listed in Table 2, we selected strain S-11 (secretor) and strain 236 (converter) for further study to clarify the pathway of DOS biosynthesis. The culture

Fig. 2. Time course of S-11-P production by *B. circulans* S-11.

S-11-P titer was expressed as arbitrary unit which obtained by BTN activity produced by strain 236. At the indicated times the sample was withdrawn from the fermentation of strain S-11 in a jar fermenter. The sample was centrifuged and S-11-P titer was assayed as described in Materials and Methods. The titer of S-11-P 70 units at 4 days culture roughly corresponds to 420 μg/ml of pure S-11-P base.

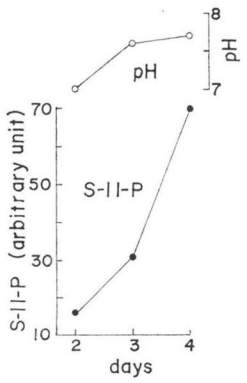
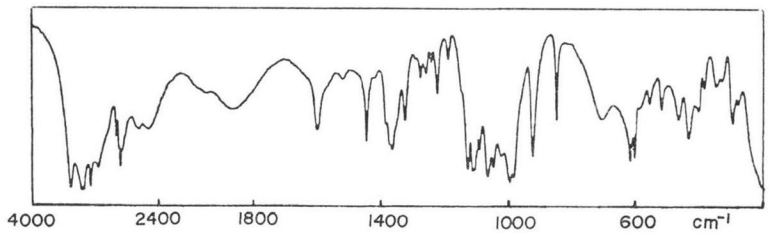


Table 3. Physico-chemical properties of S-11-P sulfate.

Optical activity	$[\alpha]_D^{24.5°}+3.6\pm0.5°$ (c 1, H_2O)
Elemental analysis	$C_6H_{13}NO_4\cdot\frac{1}{2}H_2SO_4\cdot H_2O$
Calcd.	C31.30, H7.00, N6.08, S6.96%
Found	C31.53, H7.14, N5.99, S7.08%
Mass spectrum	m/e 164 (MH^+)
PPC (BuOH - Pyridine - H_2O - AcOH, 6: 4: 3: 1)	Rf 0.11
Color reaction	Positive: $AgNO_3$, ninhydrin

Fig. 3. IR spectrum of S-11-P free base (KBr).



filtrate of strain S-11 had the same effect as DOS for producing butirosin when it was added to a culture of strain 236 and fermented for several days. This DOS-like substance in the culture filtrate of strain S-11 was designated as S-11-P. The amount of S-11-P increased gradually up to 4 days in jar fermentation (Fig. 2). As S-11-P was a basic, water soluble substance, it was isolated and purified as described in the Materials and Methods.

Physico-chemical properties of S-11-P sulfate are summarized in Table 3. From these data, S-11-P was supposed to be a DOS-like substance, with one amino group replaced by a hydroxyl group.

^{13}C NMR spectra of DOS (**1**) and S-11-P in D_2O clearly show that the structure of S-11-P corresponds to **2** or its enantiomer (**3**) (Fig. 4, Table 4). By comparison with chemically synthesized samples[6], S-11-P was identified as **2**.

Table 4. ^{13}C-Chemical shifts (δ) of S-11-P and 2-deoxystreptamine.

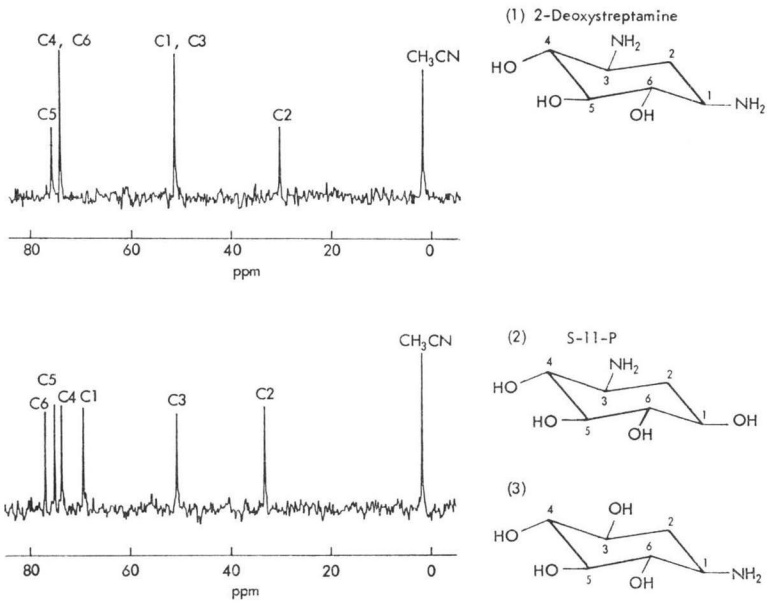
	δ (ppm)		$\Delta\delta$
	S-11-P pD 6.5	DOS pD 6.6	δ(S-11-P) $-\delta$(DOS)
C-1	69.5*	51.1	18.4
C-2	33.2	30.2	3.0
C-3	50.7*	51.1	−0.4
C-4	73.8*	74.1	−0.3
C-5	75.2	75.7	−0.5
C-6	77.1*	74.1	3.0

* C-1 and C-3, C-4 and C-6 may be reversed.

According to the IUPAC-IUB tentative cyclitol nomenclature rules[7], S-11-P is named as (1L)-1,3,5/2,4-5-amino-cyclohexanetetrol. In relation to DOS and by the same numbering system as that of DOS (**1**), S-11-P (**2**) is named as 1-deamino-2-deoxy-1-hydroxystreptamine.

S-11-P was converted to BTNs A and B by strain 236, and the conversion rate was nearly equal to that of DOS. Chemically synthesized S-11-P[6] was also converted to BTNs A and B by strain 236.

Fig. 4. ^{13}C NMR spectra of DOS and S-11-P in D_2O, and the structures of DOS (**1**), S-11-P (**2**), and the enantiomer (**3**) of S-11-P.



Discussion

We reported the isolation of xylostasin- and ribostamycin-producing mutants from the butirosin-

producing strain of *B. circulans* B15M, by the cylinder culture and double indicator method[5]. Along with the isolation of these mutants, many antibiotic-non-producing mutants were simultaneously isolated. These mutants were further examined for antibiotic production using DOS-containing cultures. Eight DOS$^-$ mutants were isolated in this way. By a simple experiment on agar plate culture, the detection of cosynthetic pairs and the characterization of each mutant were carried out. The mutants were divided into two classes, secretor and converter. Cosynthetic behavior of the mutants in liquid medium and their secretor-converter characters were confirmed by identifying the product.

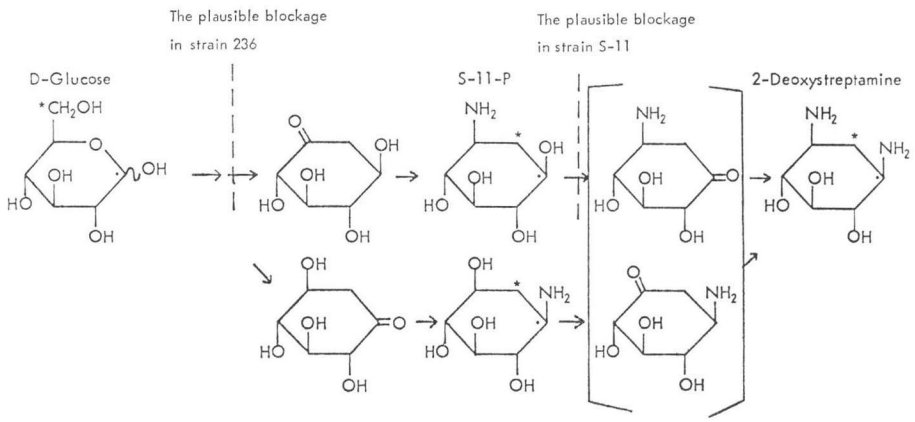
Based on the above observation, we selected secretor strain S-11, and isolated S-11-P from the fermentation broth of this strain. S-11-P is a very important aminocyclitol, which was isolated from a DOS$^-$ mutant for the first time. S-11-P was converted to DOS and further to BTN or RBM, depending on converter strains. These facts give the direct evidence concerning the DOS biosynthetic pathway.

S-11-P corresponds to one of the intermediates of DOS biosynthesis postulated by RINEHART and STROSHANE[1]. DAUM, *et al.*, found that by the supplementation of DL-2,4/3,5-tetrahydroxycyclohexanone in the culture medium, a DOS$^-$ mutant of *Micromonospora purpurea* produced gentamicins[3], and by the addition of *scyllo*-inosamine-2, it produced 2-hydroxygentamicins[2]. FURUMAI, *et al.*[4], found that DL-1,3,5/2,4-5-amino-cyclohexanetetrol, as well as DL-2,4/3,5-tetrahydroxycyclohexanone, were incorporated into BTNs by the mutants of *B. circulans*. From these data and from our results, we conclude that the upper line of Fig. 5 including S-11-P could be the true pathway of DOS biosynthesis, common to *Streptomyces, Micromonospora* and *Bacillus*.

Although the blockage in strain S-11 has still not been established, the dehydrogenation step seems to be a plausible one as illustrated in Fig. 5, because S-11-P was accumulated in the culture broth of this strain. Another possibility is that the blockage could be at the transamination step, if the dehydrogenation step of S-11-P is reversible and the equilibrium is favored to S-11-P as in the case of *myo*-inositol 2-dehydrogenase[8]. On the other hand, the blockage in strain 236 is supposed to be at a nearer step to glucose, because by the addition of *scyllo*-inosose to a culture of this strain, it produced a 2-hydroxybutirosin-like antibiotic.

Fig. 5. The pathway of DOS biosynthesis which we suppose to be the true one (upper line), and the alternative pathway including the enantiomers of each intermediate (lower line).

The structures in the parenthesis are intermediates proposed by RINEHART and STROSHANE, but no experimental evidence has been obtained.



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