PRODUCTION OF A NEW AMINOGLYCOSIDE ANTIBIOTIC BY A MUTANT OF *BACILLUS CIRCULANS*

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A new aminoglycoside antibiotic, S-11-A, was isolated from the fermentation broth of the 2-deoxystreptamine negative (DOS$^-$) mutant of *Bacillus circulans* S-11. The structure of S-11-A was elucidated as 1-deamino-1-hydroxyxylostasin, which contains an intermediate of DOS biosynthesis (S-11-P) and has resistance to some aminoglycoside-inactivating enzymes. This is the first finding of antibiotic production by a DOS$^-$ mutant without any supplementation of DOS or a DOS analog, and could be described as a novel method of getting a new aminoglycoside antibiotic.

Mutational biosynthesis has widely been used to produce new aminoglycoside antibiotics, since Shier, *et al.*[1] demonstrated the utility of this method. For this purpose, many 2-deoxystreptamine negative (DOS$^-$) mutants from various aminoglycoside-producing strains have been isolated, and addition experiments of many DOS analogs to culture broths have been carried out. Streptamine and 2-epistreptamine were the most convenient analogs for making new antibiotics such as hybrimycins[1] and mutamicins[2]. A DOS$^-$ mutant of *Bacillus circulans* was isolated by Claridge and co-workers[3], who showed that streptamine and streptidine could be incorporated to give butirosin-related new antibiotics. In spite of these reports, nothing has been reported about using DOS$^-$ mutants without the supplementation of DOS analogs for antibiotic production. We report here on a novel method of producing a new antibiotic, S-11-A, by one of the DOS$^-$ mutants of *B. circulans* in the absence of a DOS analog. This mutant strain S-11 also has the character of accumulating an intermediate of DOS biosynthesis (S-11-P, Fig. 2) in the culture broth[4,5]. The structure of S-11-A and the significance of S-11-A production using the DOS$^-$ mutant were also discussed.

Materials and Methods

Strain

Bacillus circulans S-11 is a DOS$^-$ mutant isolated from the xylostasin (XLN) producing strain Mot 3[6]. Isolation of this strain was described in the previous paper[4].

Fermentation

The fermentation procedure, using strain S-11 in seed medium S-4 and in fermentation medium F-5 in a 30-liter jar fermenter, was the same as that for preparing S-11-P[4].

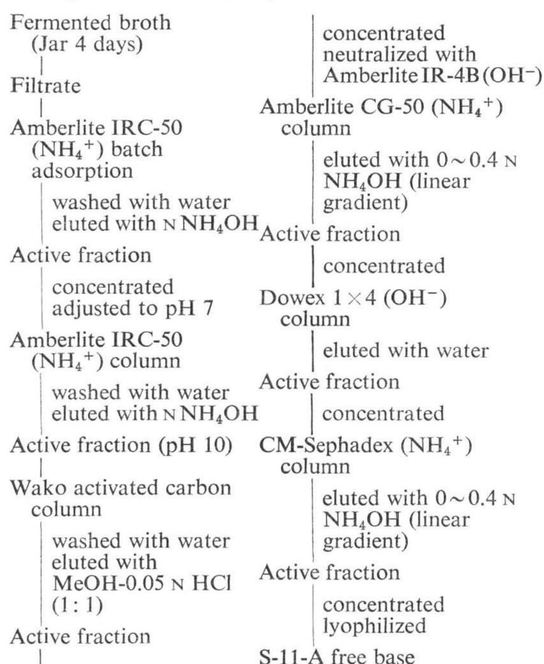
Isolation procedure of S-11-A

S-11-A was isolated from the culture broth by the procedure shown in Fig. 1.

Assay of S-11-A

Antibiotic activity of S-11-A was assayed using the paper disc diffusion method on an agar plate seeded with *Bacillus subtilis* PCI 219 as a test organism.

Fig. 1. Isolation and purification of S-11-A.



^{13}C NMR

^{13}C NMR spectra were taken with a Varian NV-14 spectrometer.

Chemicals

XLN and DOS were prepared in our laboratory, and ribostamycin (RBM) was purchased from Meiji Seika Kaisha, Ltd.

Results and Discussion

DOS^{-} mutants have been thought to produce no antibiotic without the supplementation of DOS or a DOS analog. However, *B. circulans* S-11 indicates that this is not the general character of DOS^{-} mutants. The strain S-11 produced an antibiotic activity (14 mm diameter of inhibition zone) in the absence of a DOS analog. Antibiotic activity was assayed against *B. subtilis* on a peptone agar plate using 6-mm diameter paper discs. This antibiotic showed a different Rf from that of XLN on the TLC-bioautogram (Table 2), and was designated as S-11-A. The DOS supplemented culture broth of strain S-11 (500 μg/ml) showed a strong antibiotic activity (25.5 mm diameter of inhibition zone), whose major component was XLN. This means the strain S-11 is a DOS^{-} mutant. Since the antibiotic S-11-A was simultaneously produced as a minor component even in the DOS supplemented culture, it was supposed that S-11-A might not contain DOS in the molecule. After the strain S-11 was cultivated with medium F-5 using a 30-liter jar fermenter at 28°C for 4 days, S-11-A was isolated from the culture broth by the procedure shown in Fig. 1.

Table 1. Physico-chemical properties of S-11-A.

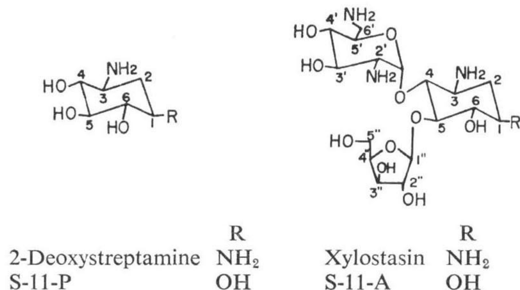
	Sulfate			N-Acetate		
Appearance	Crystalline powder			White powder		
Melting point	140~170°C (dec.)			>160°C		
Molecular formula	$C_{17}H_{33}N_3O_{11}\cdot\frac{3}{2}H_2SO_4\cdot 4H_2O$			$C_{17}H_{30}N_3O_{11}\cdot 3(CH_3CO)\cdot 2H_2O$		
Elemental analysis		Calcd.	Found.		Calcd.	Found
	C	30.27%	30.34%	C	44.72%	44.88%
	H	6.57	6.71	H	7.02	7.35
	N	6.23	6.08	N	6.80	6.81
	S	7.13	6.65			
Optical activity	$[\alpha]_D^{24.5}$ +38.2±0.8° (*c* 1, H_2O)			$[\alpha]_D^{26.0}$ +5.8±0.4° (*c* 1, H_2O)		
UV absorption	End absorption at 210 nm			End absorption at 210 nm		
Color reaction	Positive:	Ninhydrin, MOLISCH, Anthrone				
	Negative:	Fehling				
Acid hydrolysis	Xylose (1 N HCl, 60°C, 30 minutes)					
	S-11-P (6 N HCl, 100°C, 6 hours)					

Physico-chemical properties of S-11-A sulfate and N-acetate are summarized in Table 1. Acid hydrolysis of S-11-A afforded xylose (identified by paper chromatography) and S-11-P (identified by HPLC). This evidence strongly argues that the structure of S-11-A should be closely related to that of XLN, except the former contains S-11-P instead of DOS. ^{13}C NMR chemical shifts of S-11-A compared with XLN, RBM, S-11-P and DOS (Table 3) confirmed the above conjecture. The chemical shift of C-1 of S-11-A was quite different from that of XLN, and the C-2 and C-5 carbons were also different to a much lesser extent. These differences correspond to those of S-11-P and DOS. Small shifts of other carbons, especially C-3′ and C-5′, to higher field may be due to the effect of the slightly lower pD of S-11-A compared to XLN or RBM. From these results, the structure of S-11-A was elucidated as 1-deamino-1-hydroxyxylostasin (Fig. 2). According to the IUPAC-IUB tentative cyclitol nomenclature rules applied to the S-11-P moiety, S-11-A is named as 3-O-(β-D-xylofuranosyl)-4-O-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-(1 L)-1,3,5/2,4-5-aminocyclohexanetetrol. Although in the latter name the numbering about the S-11-P moiety was clockwise, C-1 was the same as indicated in Fig. 2.

Table 2. T.L.C. Comparison of S-11-A with xylostasin (XLN).

Solvent system	Rf	
	S-11-A	XLN
i-PrOH - 28% NH_4OH (1:1)	0.32	0.32
$CHCl_3$-MeOH-17% NH_4OH (2:1:1 upper phase)	0.53	0.46
$CHCl_3$-MeOH-28% NH_4OH-H_2O (1:4:2:1)	0.35	0.28

Fig. 2. Structures of S-11-A, XLN, DOS and S-11-P.



	R
2-Deoxystreptamine	NH_2
S-11-P	OH

	R
Xylostasin	NH_2
S-11-A	OH

Table 3. ^{13}C-Chemical shifts (δ) of S-11-A compared with xylostasin (XLN), ribostamycin (RBM), S-11-P and 2-deoxystreptamine (DOS).

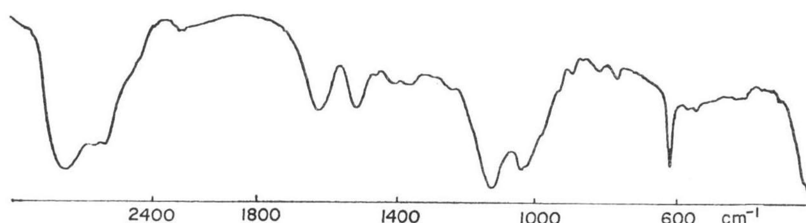
	δ (ppm)			Δδ
	S-11-A pD 4.7	XLN pD 6.6	RBM pD 7.2	δ(S-11-A) −δ(XLN)
C-1	70.0	51.2	51.2	18.8
C-2	32.7	31.7	31.2	1.0
C-3	49.0	49.7	49.6	−0.7
C-4	77.2	78.6	78.6	−1.4
C-5	85.7	86.6	86.1	−0.9
C-6	76.3	74.1	74.0	2.2
C-1′	95.8	95.8	96.1	0
C-2′	54.3	54.8	54.8	−0.5
C-3′	68.9	69.8	69.9	−0.9
C-4′	71.4	71.9	71.9	−0.5
C-5′	68.9	69.8	69.6	−0.9
C-6′	40.9	41.2	41.2	−0.3
C-1″	112.6	112.7	111.1	−0.1
C-2″	81.6	81.5	76.0	0.1
C-3″	75.3	75.2	69.9	0.1
C-4″	83.7	83.5	83.2	0.2
C-5″	61.4	61.4	61.8	0

	δ (ppm)		Δδ
	S-11-P pD 6.5	DOS pD 6.6	δ(S-11-P) −δ (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

For the usual mutational biosynthesis, the DOS analog has to be prepared chemically, but in this example, S-11-P could be thought of as a DOS analog, and was simultaneously produced by the strain itself along with S-11-A, so it was unnecessary to synthesize and supplement the DOS analog. If S-11-P is supplemented to the culture of one of the converter group of DOS^- mutants such as strain 236[4], S-11-

Fig. 3. IR spectra of S-11-A sulfate and S-11-A N-acetate (KBr).

S-11-A sulfate (KBr)



S-11-A N-acetate (KBr)

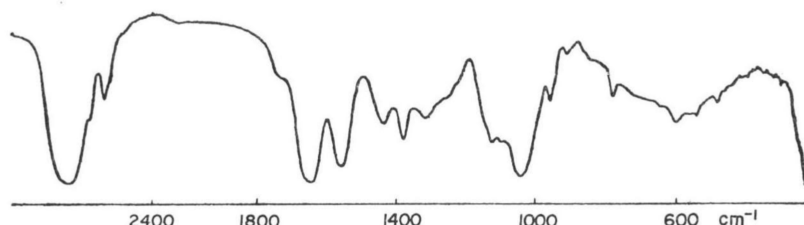
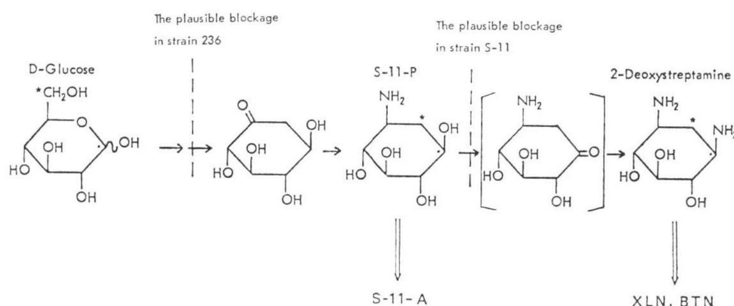


Fig. 4. Biosynthetic pathways of S-11-A, XLN and butirosin.

S-11-A is produced using S-11-P by the unsupplemented culture and XLN is produced by the DOS supplemented culture of the strain S-11. Strain 236 could produce butirosin by the addition of S-11-P or DOS to the culture medium. The structure in the paranthesis is not known.



P is transformed to DOS and incorporated into butirosin (Fig. 4). Thus, S-11-A could not be produced by the usual mutational biosynthetic method, unless the special DOS^- mutant accumulating S-11-P was used. We propose the biosynthetic pathway of S-11-A shown in Fig. 4. This is a novel method of getting a new aminoglycoside antibiotic by a mutant of a known aminoglycoside-producing strain. According to this method, the production of 1-deamino-1-hydroxyneomycin could be expected by the S-11-P accumulating DOS^- mutant of a neomycin producer. 1-Deamino-1-hydroxykanamycin production could also be expected by the same type of the mutant derived from a kanamycin producer.

The *in vitro* antibiotic activity of S-11-A was weaker than XLN or RBM about 8 fold (Table 4). This suggests that the C-1 amino group of DOS-containing antibiotics plays an important role for the biological activity. It is generally recognized in the aminoglycosides that the substitution of an amino group by a hydroxyl group reduces both the antibiotic activity and toxicity[7]. For example, neomycin B is more toxic than paromomycin I and kanamycin B has the same tendency by comparison with

Table 4. *In vitro* antibacterial activities of S-11-A sulfate compared with xylostasin sulfate (XLN) and ribostamycin sulfate (RBM) in agar dilution test.

	Resistance mechanism	MIC (μg/ml)		
		S-11-A	XLN	RBM
Escherichia coli NIHJ JC-2		25	3.13	3.13
W677/R5HL	AAC(6′)	>100	>100	>100
W677/JR88	AAC(3)-I	50	6.25	6.25
W677/JR225	AAC(3)-II	25	100	>100
W677/JR214	ANT(2″)	>100	>100	>100
W677/JR35	APH(3′)-I	>100	>100	>100
80750	3′P	100	>100	>100
80750*		50	3.13	6.25
Klebsiella pneumoniae Shionogi		3.13	0.39	0.39
ATCC 27736		12.5	1.56	3.13
Serratia marcescens ATCC 13880		>100	6.25	25
Proteus rettgeri Ret-29		25	1.56	1.56
Proteus vulgaris ATCC 6390		25	3.13	1.56
Salmonella typhimurium ATCC 1334		50	6.25	12.5
Staphylococcus aureus FDA 209P JC-1		50	3.13	1.56

Abbreviations of resistance mechanism: AAC, aminoglycoside acetyltransferase. ANT, aminoglycoside nucleotidyltransferase. APH, aminoglycoside phosphotransferase. 3′P, a kind of 3′-O-phosphotransferase, attacking XLN, RBM and KM, but not well characterized.

* The resistance lost strain of *E. coli* 80750.

kanamycin A. Therefore, S-11-A can be expected to have weaker toxicity than XLN. The important finding from Table 4 is that S-11-A is active against *Escherichia coli* W677/JR225 having the aminoglycoside-acetylating enzyme, AAC(3)-II. This enzyme acetylates the C-3 amino group of many DOS-containing compounds, including XLN, but the substitution of an amino group by a hydroxyl group on S-11-A is not at the attacking site of this enzyme. Butirosin A, a compound acylated with 4-amino-2-hydroxybutanoic acid at the C-1 amino group of XLN, is resistant to this enzyme. However, S-11-A has no such acyl group. Conversion of an amino group to a hydroxyl group at the C-1 position seems to be a new mode of acquiring resistance to AAC(3)-II. S-11-A is also resistant to some kind of 3′-phosphorylating enzyme of the strain *E. coli* 80750. These findings may serve as important suggestions to the study of structure-activity relationships and the modification of aminoglycoside antibiotics.

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