ASHIMYCINS A AND B, NEW STREPTOMYCIN ANALOGUES

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Detailed analysis of the fermentation broth of *Streptomyces griseus* strain FT3-4 resulted in the identification of two new streptomycin analogues named ashimycins A and B. Their structures have been determined by NMR spectral analysis and chemical degradations.

During the attempts to improve the fermentation yield of streptomycin by *Streptomyces griseus* strain FT3-4, we noticed that the lower production yield of streptomycin was sometimes accompanied by the accumulation of new metabolites structurally related to streptomycin. We will report herein the fermentation, isolation, physico-chemical properties, structural elucidation and biological activities of these compounds named ashimycins A and B.

Fermentation

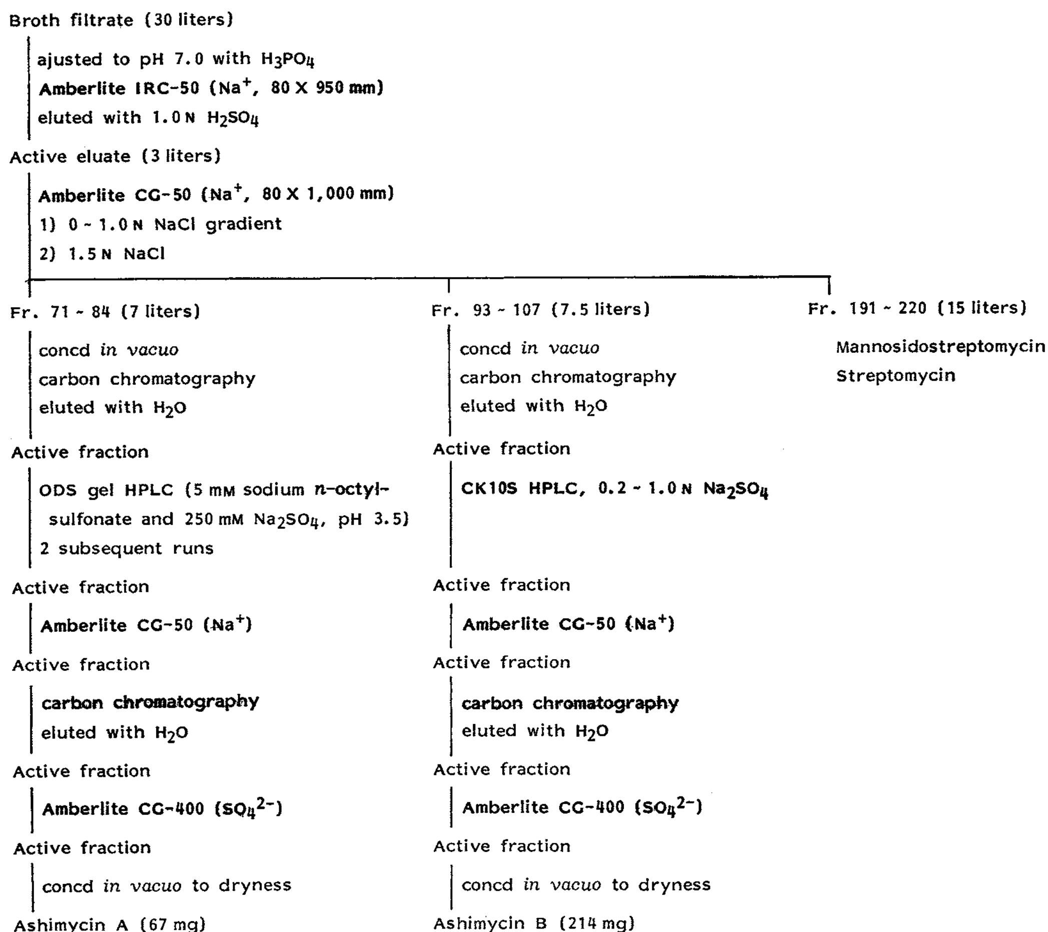
Ashimycins A and B have been isolated from the culture filtrate of *Streptomyces griseus* strain FT3-4 which is being used for commercial production of streptomycin at our company. A well-grown agar slant of the strain FT3-4 was used to inoculate 20 ml of a seed culture medium consisting of corn steep liquor 3.0%, yeast 0.25% and $CaCl_2 \cdot 2H_2O$ 0.1% (pH 7.5) in a 100-ml Erlenmeyer flask. The flask was shaken on a rotary shaker (220 rpm) at 28°C for 27 hours, and then 4 ml of the culture was transferred to 80 ml of the same medium in a 500-ml Erlenmeyer flask. After shaking at 28°C for 21 hours, 80 ml of the broth was used to inoculate 1,000 ml of the same medium in a 5-liter Erlenmeyer flask, and the fermentation was continued at 28°C for 30 hours. This seed culture was added to a 30-liter jar fermenter containing 20 liters of a production medium composed of soybean meal 5.0%, glucose 5.0%, mannose 0.5% and KH_2PO_4 0.1%, pH being adjusted to 7.6 before sterilization. The final fermentation was carried out at 28°C for 6 days with an agitation rate of 250 rpm and aeration of 8 liters per minute.

Isolation and Purification

The procedures for isolating a crude streptomycin complex and purifying the individual components are schematically shown in Fig. 1. Active substances were traced by the maltol reaction after separation by analytical HPLC (column: CK10S, Mitsubishi Chemical Industries Limited, 7.0×250 mm; solvent: linear gradient from 0.2 to 1.0 M Na_2SO_4, flow rate: 1.0 ml/minute). The broth filtrate contained the ashimycins in addition to mannosidostreptomycin and streptomycin as shown in Fig. 2.

The broth filtrate was adsorbed on an Amberlite IRC-50 column (Na^+, 4.8 liters), and elution with

Fig. 1. Isolation scheme for ashimycins A and B.



1.0 N H_2SO_4 gave an active eluate (3 liters) which was concentrated to give a crude powder (269 g). Separation of the individual antibiotics was achieved by Amberlite CG-50 column chromatography (Na^+, $80 \times 1,000$ mm) developed with a 0~1.0 N NaCl gradient (72 liters). The eluate was cut into 0.5-liter fractions. Ashimycin A was eluted in fractions 71~84 and ashimycin B in fractions 93~107. Mannosidostreptomycin and streptomycin were eluted by 1.5 N NaCl.

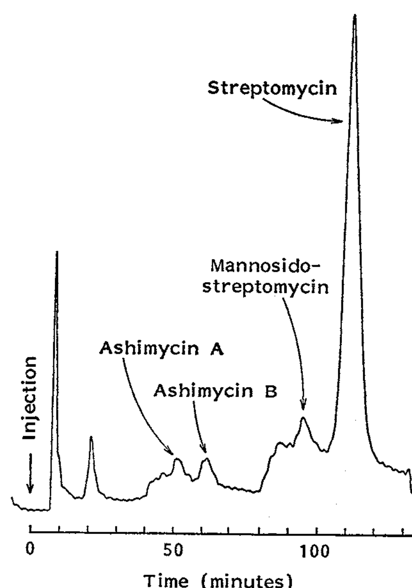
Ashimycin A was further purified by carbon column chromatography (Wako, activated carbon, 40×360 mm) eluted with water followed by liquid chromatography on YMC-ODS (Shimakyu, Co. 50×500 mm, equilibrated with an aqueous solution containing 5 mM of sodium *n*-octylsulfonate and 250 mM of sodium sulfate adjusted to pH 3.5) developed with the same solution. The purification procedure by liquid chromatography was repeated twice. The concentrated fraction containing ashimycin A was adsorbed on a column of Amberlite CG-50 (Na^+, 60×360 mm) followed by elution with a gradient from 0~1.0 N NaCl. The active fraction was concentrated and was subjected to carbon column chromatography (10×64 mm) developed with water. The concentrated active solution of the eluate was adsorbed on a column of Ambrelite CG-400 (SO_4^{2-}, 12×88 mm) and developed with water to yield a colorless hygroscopic powder of ashimycin A sulfate (67 mg).

Ashimycin B was purified by carbon chromatography (40×360 mm) eluted with water followed by liquid chromatography on CK10S (10 μm, 50×500 mm) with a 0.2~1.0 N Na_2SO_4 gradient system. The concentrated solution was adsorbed on a column of Amberlite CG-50 (Na^+, 60×360 mm) and eluted with a gradient from 0~1.0 N NaCl. The active fraction was concentrated and was subjected to carbon column chromatography (10×64 mm) developed with water. The concentrated active solution of the eluate was adsorbed on a column of Amberlite CG-400 (SO_4^{2-}, 12×88 mm) and developed with water to yield a colorless powder of ashimycin B sulfate (214 mg).

Physico-chemical Properties

Physico-chemical properties of ashimycins A and B are listed in Table 1. Both compounds were soluble in water and insoluble in methanol and chloroform. The molecular formula of ashimycins A and B were determined by secondary ion mass spectrometry (SI-MS) and elemental analysis. The IR absorption at 1660~1630 cm^{-1} (Figs. 3 and 4) suggested that these antibiotics contained guanidino groups. Ashimycin A was positive to Sakaguchi, maltol and Elson-Morgan reactions, while ashimycin B was negative to Elson-Morgan reaction.

Fig. 2. HPLC analysis of the fermentation broth of *Streptomyces griseus* FT3-4.



Column: CK10S (7.0×250 mm), mobile phase; 0.2~1.0 M Na_2SO_4, pH 3.5 (gradient), detection; maltol reaction.

Structures of Ashimycins A and B

In the 400 MHz ^1H NMR spectrum of ashimycin A in D_2O (Fig. 5), three anomeric proton signals were observed at δ 5.30 ($J_{1',2'}$=1.4 Hz, 1'-H), 5.74 ($J_{1'',2''}$=3.5 Hz, 1''-H) and 5.36 (s, 1'''-H). The first two were ascribed to the anomeric protons of streptose and *N*-methylglucosamine, respectively. The last singlet signal at δ_H 5.36 was assigned to the anomeric proton

Table 1. Physico-chemical properties of ashimycins A and B.

	Ashimycin A	Ashimycin B
Appearance	White amorphous powder	White amorphous powder
MP(°C)	150	205
Optical rotation $[\alpha]_D^{23}$	−37.0° (*c* 1.0, H_2O)	−85.3° (*c* 0.04, H_2O)
SI-MS (*m/z*, (M+H))	758	640
Molecular formula	$C_{27}H_{47}N_7O_{18}\cdot 1\frac{1}{2}H_2SO_4\cdot 2H_2O$	$C_{23}H_{41}N_7O_{14}\cdot H_2SO_4\cdot H_2O$
Elemental analysis	C H N	C H N
Calcd:	34.47, 5.79, 10.42	36.56, 6.00, 12.98
Found:	34.10, 5.68, 10.15	36.39, 6.04, 13.31
IR ν_{max}^{KBr} cm^{-1}	3400, 1660, 1630, 1400, 1100, 600	3340, 1660, 1630, 1400, 1100, 600
Color reaction		
Positive:	Sakaguchi, maltol, Elson-Morgan	Sakaguchi, maltol
Negative:		Elson-Morgan

Fig. 3. IR spectrum (KBr) of ashimycin A.

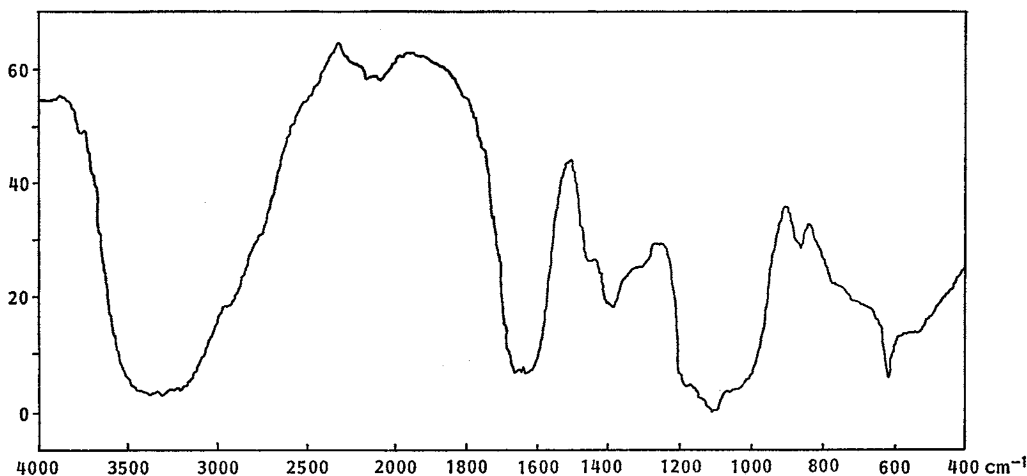
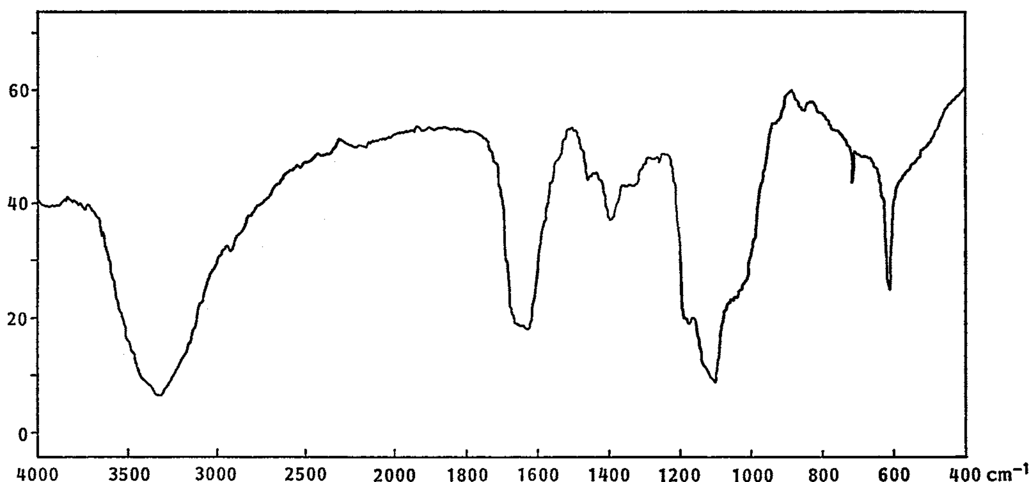
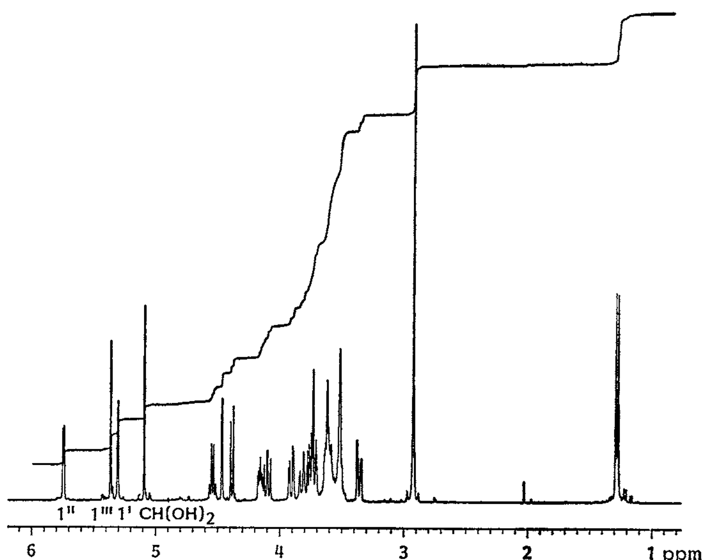


Fig. 4. IR spectrum (KBr) of ashimycin B.



of a new sugar moiety. ^{13}C NMR spectral comparison of ashimycin A and streptomycin (Table 2) suggests that the new antibiotic is structurally very similar to streptomycin except for the presence of a new sugar[1,2]. The functionalities of the carbon atoms in the new sugar are as follows; 1 × anomeric carbon, 1 × COOH, 1 × C-O, 2 × CH-O and 1 × CH_2-O. The partial structures of the new sugar from C-3‴ to C-5‴ (Fig. 6) were determined by proton spin decoupling experiments. The ^{13}C chemical shifts of C-4‴ (δ 83.3) and C-5‴ (δ 62.0) are very similar to those of C-4′ and C-5′ of 9-β-D-xylofuranosyl adenine (δ_C 83.0 and 60.0 respectively)[3], suggesting that C-4‴ must be attached to C-1‴ *via* an ethereal oxygen. The downfield shift of C-4‴ is reasonably explained in terms of an alkylation shift. Since C-1‴ and C-3‴ were not coupled to each other, they must be separated by a quaternary oxycarbon (δ 82.0) and the carboxyl group is therefore linked to C-2‴. Furthermore, the presence of a carboxyl group in ashimycin A was revealed by positive coloration of a hydroxamic acid derivative[4]. The configurations of C-1‴ and C-2‴ remain to be established.

Fig. 5. 400 MHz ^{1}H NMR spectrum of ashimycin A.



The position of the new sugar within the molecule was determined as follows. Compared with streptomycin, C-4″ is shifted downfield by 8.5 ppm, and C-3″ and C-5″ are shifted upfield by 1.8 and 1.9 ppm, respectively, in ashimycin A. Thus, the C-4″ position of the streptomycin moiety is glycosylated by the new sugar in ashimycin A. Accordingly, the structure of ashimycin A was determined as shown in Fig. 7. We propose to name the new sugar as ashimose. A sugar with the same planar structure had been prepared by oxidation of methyl β-D-glucopyranoside with oxygen in alkaline, aqueous solution[5].

In the 200 MHz ^{1}H NMR spectrum of ashimycin B in D_2O (Fig. 8), two anomeric proton signals were observed at δ 5.35 ($J_{1',2'}=1.5$ Hz, 1′-H) and 5.70 ($J_{1'',2''}=3.4$ Hz, 1″-H). The ^{13}C NMR spectrum (Table 2) suggests that ashimycin B is structurally very similar to streptomycin except for the presence of two new signals due to carbonyl (δ_C 171.5) and methylene (δ_C 58.9) carbons, assignable to a glycolic acid moiety (δ_C C-1, 177.0 and C-2, 60.5)[6]. The downfield shifts of NCH_3 by 8.2 ppm and of C-2″ (δ_C 66.4)

Table 2. ^{13}C NMR shifts of ashimycins A and B[a].

Carbon	Ashimycin A	Ashimycin B	Streptomycin
1	59.6	59.7	59.7
2	72.1	71.7	71.5
3	59.3	59.3	59.0
4	76.6	78.7	78.9
5	74.3	74.0	74.2
6	72.3	72.4	72.4
C=NH (1)	159.1	159.1	159.1
C=NH (3)	158.3	158.5	158.6
1′	107.3	106.4	106.7
2′	81.6	84.1	85.3
3′	83.4	83.2	83.1
4′	78.8	78.0	78.3
5′	12.3	13.7	13.4
CHO (3′)	89.9	90.5	90.5
1″	92.6	95.9	95.2
2″	62.0	66.4	62.3
3″	68.6	68.4	70.4
4″	78.6	71.0	70.1
5″	71.8	73.5	73.7
6″	60.2	61.3	61.2
NCH_3 (2″)	32.7	41.3	33.1
C=O		171.5	
CH_2OH		58.9	
1‴	106.7		
2‴	82.0		
3‴	74.3		
4‴	83.3		
5‴	62.0		
COOH	177.2		

From internal dioxane in ppm. pD 5.5.

[a] For the assignments of streptomycin and related substances, see refs 1 and 2.

Fig. 6. Partial structures of ashimycin A.

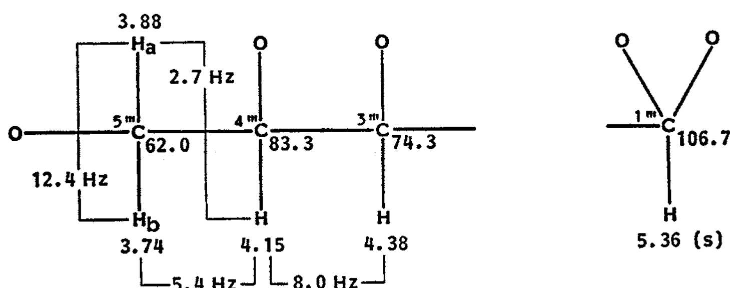


Fig. 7. Structures of ashimycins A and B.

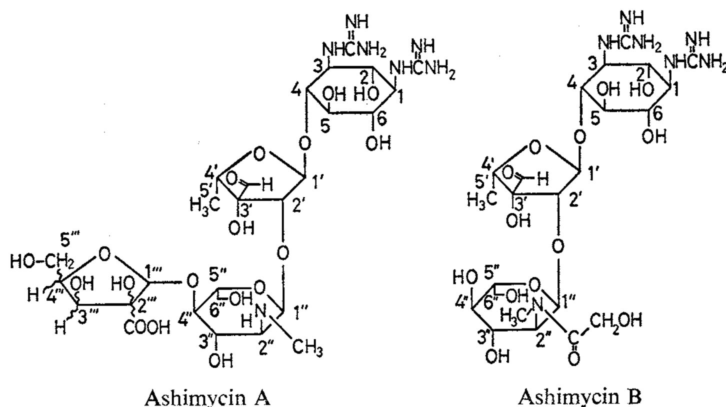
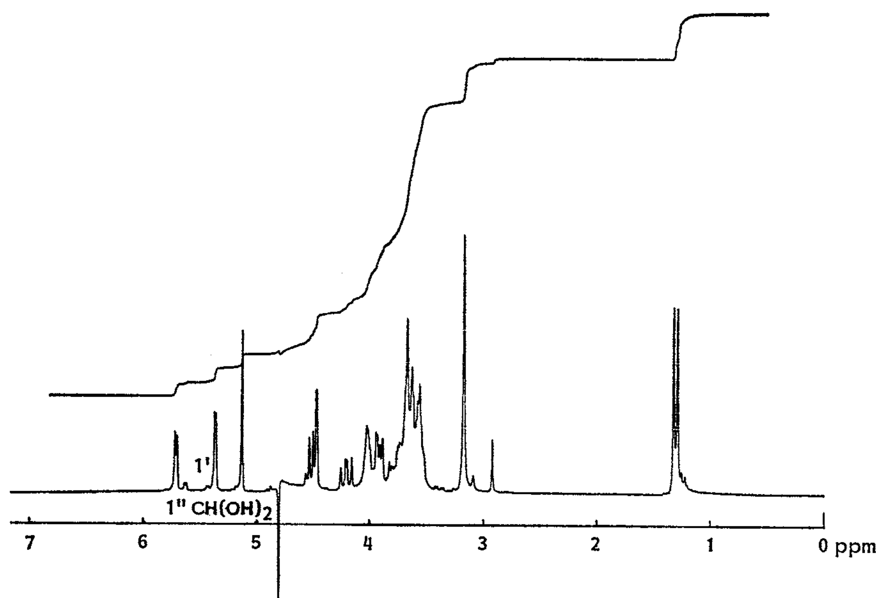


Fig. 8. 200 MHz ^{1}H NMR spectrum of ashimycin B.



by 4.1 ppm in the ^{13}C NMR data of ashimycin B suggest that the *N*-methyl group is acylated l glycolic acid in ashimycin B. In agreement with this conclusion, long range selective proton decour ing (LSPD) of the *N*-methyl proton collapsed the carbonyl carbon signal (δ_C 171.5) to a broa

Fig. 9. 200 MHz ^{1}H NMR spectrum of methyl *N*-glycolyl-*N*-methylglucosaminide peracetate.

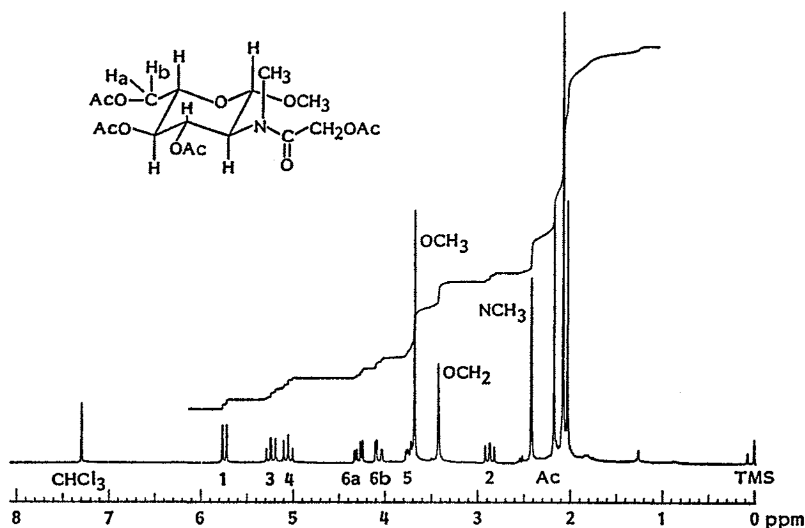


Table 3. Antibacterial activities of ashimycins A and B.

Test organisms	Ashimycin A	Ashimycin B	Streptomycin
Staphylococcus aureus Smith I	50	12.5	1.56
S. aureus FDA 209P	25	25	1.56
S. aureus Apo-1	50	50	3.13
S. epidermidis 109	50	100	100
Micrococcus flavus FDA 16	100	25	1.56
Bacillus anthracis	50	12.5	0.78
B. subtilis PCI 219	25	6.25	0.39
Corynebacterium bovis 1810	100	25	1.56
Escherichia coli K-12 R-5	50	100	100
E. coli NIHJ JC-2	50	25	3.13
Klebsiella pneumoniae	200	25	1.56
Proteus vulgaris OX19	50	12.5	1.56
P. rettgeri GN311	25	12.5	0.78

MIC (μg/ml) in Mueller-Hinton agar.

doublet signal (J=4.9 Hz). Furthermore, the *N*-methyl proton (δ_H 3.16) of ashimycin B is shifted downfield by 0.26 ppm as compared with the corresponding proton of streptomycin (δ_H 2.90).

In order to confirm the structure of ashimycin B, it was subjected to acid catalyzed hydrolysis. Ashimycin B was dissolved in 1 N H_2SO_4 and allowed to stand at room temperature for 18 hours. After removal of precipitated streptidine sulfate by filtration, the filtrate was treated with concentrated HCl for 2 hours at 100°C. The residue was passed through a column of Dowex 50 (H^+) and then an *N*-methylglucosamine derivative was eluted with 4 N NH_4OH. Treatment of the dried hydrolysate with HCl - methanol solution, followed by acetylation with acetic anhydride in the presence of anhydrous sodium acetate gave a crude powder of methyl *N*-glycolyl-*N*-methylglucosaminide peracetate. This material was purified by silica gel chromatography eluting with $CHCl_3$ - CH_3OH (100 : 1). Physico-chemical properties of the peracetate were as follows: $[\alpha]_D^{23}$ −22.8° (c 0.07, $CHCl_3$); SI-MS m/z 434 $(M+H)^+$; the 200 MHz ^{1}H NMR spectrum in $CDCl_3$ is shown in Fig. 9.

Anal Calcd for $C_{18}H_{27}NO_{11} \cdot H_2O$: C 47.89, H 6.47, N 3.10.
Found: C 48.36, H 6.11, N 3.03.

Thus the glycol unit is attached to NCH_3 by amide bond. Consequently the structure of ashimycin B was established as shown in Fig. 7.

Antibacterial Properties

The MICs of ashimycins A and B assayed by the agar dilution method are given in Table 3. They showed similar activities against *Staphylococcus aureus* Apo-1. Ashimycin A showed higher activities than ashimycin B except for *Staphylococcus epidermidis* 109 and *Escherichia coli* K-12 R-5.

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