SACCHAROCIN, A NEW AMINOGLYCOSIDE ANTIBIOTIC

FERMENTATION, ISOLATION, CHARACTERIZATION AND STRUCTURAL STUDY

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A new aminoglycoside antibiotic, saccharocin has been isolated from the fermentation broth of *Saccharopolyspora* sp. AC-3440 (FERM P-6238) by column chromatography on a cation-exchange resin. Saccharocin is active against Gram-positive and Gram-negative bacteria. The structure was elucidated to be 4''-deamino-4''-hydroxyapramycin by ^{13}C NMR spectral analysis.

In the course of our screening program for new antibiotics, it has been found that an antimicrobial agent is produced by an actinomycetes strain AC-3440, identified as *Saccharopolyspora* sp. The organism was isolated from a soil sample collected at Ato Town, Yamaguchi Prefecture, Japan. This water-soluble basic antibiotic, named saccharocin, was purified from the broth filtrate by a cation-exchange resin process and chromatography. In this paper, the fermentation, isolation, characterization and structure of saccharocin is reported.

Fermentation and Isolation

Saccharopolyspora sp. AC-3440 was inoculated into 100 ml of a seed medium consisting of 1% dextrin, 1% glucose, 0.5% NZ-Amine type A, 0.3% yeast extract and 0.1% $CaCO_3$ (pH was adjusted to 7.0 before sterilization) in a 500-ml Erlenmeyer flask and incubated on a rotary shaker at 30°C for 72 hours.

Each 10 ml of the seed-cultured broth thus obtained, was inoculated into each 100 ml of a production medium composed of 0.2% glucose, 4% glycerin, 0.5% peptone, 0.2% starch, 0.5% soybean meal, 0.5% dry yeast, 0.5% NaCl and 0.2% $CaCO_3$ (pH was adjusted to 7.0 before sterilization) using one hundred 500-ml Erlenmeyer flasks. The fermentation was conducted at 30°C on a rotary shaker. The potency of the culture broth was estimated by a disc plate method against *Bacillus subtilis* PCI 219. After 96 hours incubation a maximum concentration (50 μg/ml as saccharocin) was obtained.

The fermentation broth (10 liters) containing saccharocin was centrifuged (4,000 rpm, 10 minutes) and the supernatant of the broth (9 liters) was absorbed onto a cation-exchange resin column of Amberlite IRC-50 (NH_4^+, 1 liter). The column was washed with water (2 liters) and the antibiotic was eluted with 2 M NH_4OH. The active eluate was concentrated to 50 ml *in vacuo*. The aqueous solution was adjusted to pH 7.0 and charged onto a column of Amberlite CG-50 (NH_4^+, 2×30 cm). After washing with deionized water the column was eluted with aqueous ammonia with a concentration gradient from water (1,000 ml) to 0.15 M (1,000 ml).

The eluate was monitored by silica gel TLC (E. Merck Art. 5735) which was developed with chloroform - methanol - 14% aqueous ammonia (1 : 2 : 1) and detected by ninhydrin color reaction. The

eluate containing saccharocin was concentrated and lyophilized to give a white powder (170 mg, 620 μg/mg).

The above crude powder was dissolved in water and charged onto a column of CM-Sephadex C-25 (NH_4^+, 2×25 cm), followed by elution with 0.05 M aqueous ammonia. By this chromatography, saccharocin was separated from other impurities. The eluate containing only saccharocin was lyophilized to afford a colorless solid (45 mg, 1,000 μg/mg).

Physico-chemical and Biological Properties

Saccharocin is a water-soluble basic white powder. It is stable at pH 2.0, 7.0 and 9.0 at 60°C for 30 minutes. Saccharocin gives positive color reactions to ninhydrin and $KMnO_4$ tests. Physico-chemical properties of saccharocin are listed in Tables 1 and 2. The IR and ^1H NMR spectra of saccharocin are shown in Figs. 1 and 2 respectively.

Saccharocin showed an activity against Gram-positive and Gram-negative bacteria including resistant strains of other aminoglycoside antibiotics, but it was slightly less active than apramycin[1~3] (Table 3). Antimicrobial activity of saccharocin was determined in a nutrient agar.

Structural Elucidation

The structural elucidation of saccharocin was performed by comparing the ^1H NMR and ^{13}C NMR spectra to those of apramycin[4~6], which is an antibiotic structurally very similar to saccharocin.

Table 1. Physico-chemical properties of saccharocin.

Appearance	Basic white powder	
Mp	188~190°C	
$[\alpha]_D^{24}$	+163.5° (*c* 1.0, H_2O)	
UV $\lambda_{max}^{H_2O}$ nm	End absorption	
Elemental analysis	$C_{21}H_{40}N_4O_{12}\cdot 2H_2O$	
(%)	Found	Calcd.
C	43.35	43.74
H	7.46	7.69
N	9.74	9.72
IR ν_{max}^{KBr} cm^{-1}	3350, 2900, 1585, 1460, 1380, 1340, 1140, 1090, 990	

Table 2. Rf values of aminoglycoside antibiotics.

Antibiotics	A	B
Saccharocin	0.32	0.26
Apramycin	0.32	0.22
Oxyapramycin	0.31	0.16

Solvent systems; A: $CHCl_3$ - MeOH - 28% NH_4OH, (2:3:2). B: $CHCl_3$ - MeOH - 14% NH_4OH, (1:2:1).

Silica gel TLC; TLC plastic sheets silica gel 60-F_{254} pre-coated (E. Merck Art. 5735).

Fig. 1. IR spectrum of saccharocin.

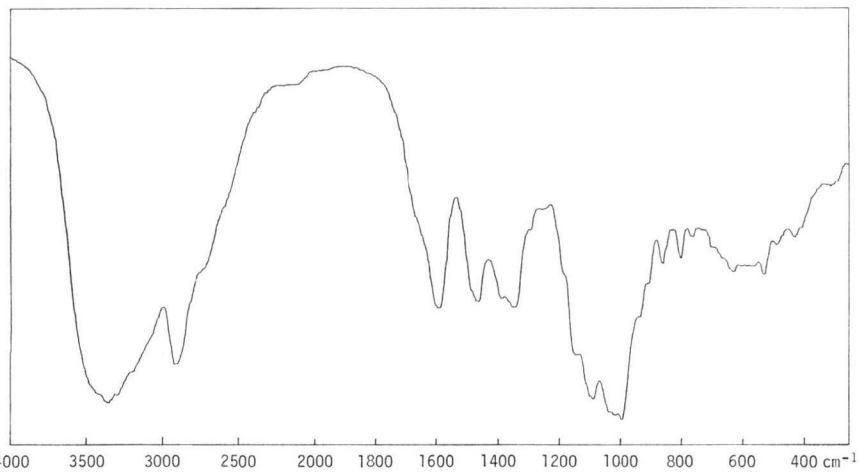


Fig. 2. ^{1}H NMR spectrum of saccharocin.

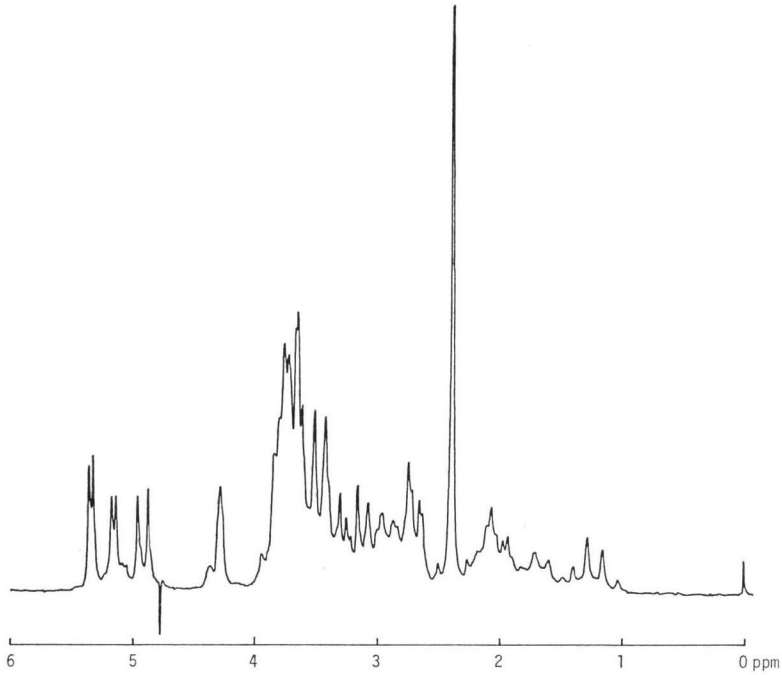


Table 3. Antimicrobial activity of saccharocin, apramycin and oxyapramycin.

Test organism	Saccharocin	Apramycin	Oxyapramycin
Staphylococcus aureus ATCC 6538 P	12.5	3.1	12.5
S. aureus MS27	12.5	1.6	12.5
S. aureus O119	6.3	1.6	6.3
S. epidermidis sp-al-1	6.3	1.6	6.3
Streptococcus pyogenes N.Y.5	25	12.5	50
Bacillus subtilis ATCC 6633	0.8	0.4	1.6
Escherichia coil NIHJ-JC 2	6.3	3.1	6.3
E. coil W3630	1.6	1.6	3.1
E. coil W3630 RGN14	3.1	3.1	6.3
Citrobacter freundii GN346	6.3	3.1	6.3
Klebsiella pneumoniae ATCC 10031	3.1	3.1	6.3
Salmonella enteritidis Gaertner	6.3	3.1	6.3
Shigella sonnei E33	6.3	6.3	12.5
Proteus morganii 0239	6.3	3.1	6.3
P. rettgeri ACR	3.1	1.6	6.3
Enterobacter aerogenes 0655	6.3	3.1	6.3
E. cloacae GN336	6.3	3.1	6.3
Serratia marcescens	6.3	3.1	6.3
Pseudomonas aeruginosa IAMI1095	25	25	50
P. aeruginosa ML4561	25	12.5	25
P. aeruginosa ML4561 Rms 166	12.5	6.3	12.5
P. aeruginosa ML4561 Rms 164–1	25	12.5	50
P. aeruginosa ML4561 RP4	12.5	6.3	12.5
P. aeruginosa 1946	>100	>100	>100
P. aeruginosa 2512	50	25	50
P. putida 1842	25	12.5	25
P. maltophilia 1850	>100	>100	>100

The protonated molecular ion at m/z 541 of desorption chemical ionization mass spectrum[7,8] and elemental analysis for saccharocin agreed with the molecular formula of $C_{21}H_{40}N_4O_{12}$. The ^1H NMR spectrum (Fig. 2) of saccharocin revealed the presence of three anomeric protons, a doublet at 5.34 ppm (1H, J = 3.5 Hz), a doublet at 5.16 ppm (1H, J = 3.5 Hz) and a doublet at 4.92 ppm (1H, J = 8.5 Hz), and one *N*-methyl group as a singlet at 2.38 ppm (3H). The characteristic chemical shifts and coupling patterns of the four protons between 2.3 and 1.0 ppm indicated the presence of a 2-deoxystreptamine moiety.

The ^{13}C NMR carbon chemical shifts of saccharocin are shown in Table 4 together with those of apramycin, and the data are very similar.

The signal at 53.3 ppm in the ^{13}C NMR spectrum (pD 11) of apramycin, which has been assigned to the 4″ carbon, has been replaced by a resonance in the 70~75 ppm region in the spectrum of saccharocin. Such a change is consistent with the replacement of NH_2 with OH.

When characteristic signals of the β-aprosamine[4] portion (C-1~C-8′) is subtracted from the ^{13}C NMR spectrum of saccharocin, the remaining six resonances accord well with the spectrum of the α-D-glucopyranose.

Consequently, the structure deduced for saccharocin is 4″-deamino-4″-hydroxyapramycin as shown in Fig. 3.

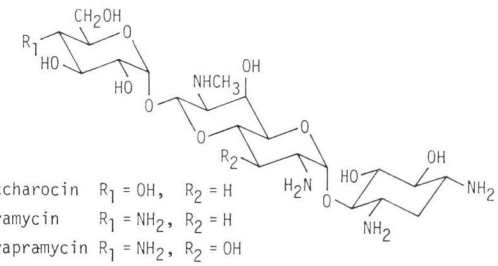
Table 4. ^{13}C NMR chemical shifts.

Carbon	Saccharocin		Apramycin*		Treha-lose*
	pD 11	pD 1	pD 11	pD 1	
C-1	51.1	50.7	51.1	50.7	
2	36.8	29.1	36.8	29.1	
3	50.2	49.5	50.2	49.5	
4	87.9	78.9	87.9	78.6	
5	76.8	75.9	76.8	75.9	
6	78.5	73.2	78.5	73.2	
1′	101.6	96.0	101.6	96.0	
2′	49.7	48.9	49.7	48.9	
3′	32.9	27.8	32.9	27.8	
4′	67.9	66.8	67.9	66.8	
5′	71.0	70.5	71.0	70.4	
6′	66.4	63.7	66.2	63.5	
7′	62.4	60.3	62.3	60.3	
8′	96.5	93.6	96.5	93.6	
1″	95.5	95.5	95.7	95.3	94.0
2″	71.8	71.2	72.0	71.2	72.9
3″	74.3	73.8	74.3	70.0	73.4
4″	70.9	70.2	53.3	53.2	70.6
5″	73.9	73.2	73.6	68.9	71.9
6″	61.7	61.4	61.8	61.3	61.4
N-Me	33.2	31.4	33.0	31.3	

^{13}C NMR spectra were recorded in D_2O (pD 11 and pD 1) with dioxane (67.4 ppm) as internal standard.

* Values obtained in our laboratory are in excellent agreement with the published ones[4].

Fig. 3. Structures of saccharocin, apramycin and oxyapramycin.



Saccharocin R_1 = OH, R_2 = H
Apramycin R_1 = NH_2, R_2 = H
Oxyapramycin R_1 = NH_2, R_2 = OH

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References

1) Stark, W. M.; M. M. Hoehn & N G. Knox: Nebramycin, a new broad spectrum antibiotic complex. I. Detection and biosynthesis. Antimicrob. Agents Chemother. -1967: 314~323, 1968
2) Higgens, C. E. & R. E. Kaster: Nebramycin, a new broad spectrum antibiotic complex. II. Description of *Streptomyces tenebrarius*. Antimicrob. Agents Chemother. -1967: 324~331, 1968

3) THOMPSON, R. G. & E. A. PRESTI: Nebramycin, a new broad spectrum antibiotic complex. III. Isolation and chemical-physical properties. Antimicrob. Agents Chemother. -1967: 332~340, 1968
4) WENKERT, E. & E. W. HAGAMAN: Carbon-13 nuclear magnetic resonance spectroscopy of naturally occurring substances. XXIX. Apramycin — an application of amine protonation parameters. J. Org. Chem. 41: 701~703, 1976
5) O'CONNOR, S.; L. K. T. LAM, N. D. JONES & M. O. CHANEY: Apramycin, a unique aminocyclitol antibiotic. J. Org. Chem. 41: 2087~2092, 1976
6) KOCH, K. F.; K. E. MERKEL, S. C. O'CONNOR, J. I. OCCOLOWITZ, J. W. PASCHAL & D. E. DORMAN: Structures of some of the minor aminoglycoside factors of the nebramycin fermentation. J. Org. Chem. 43: 1430~1434, 1978
7) HUNT, D. F.; J. SHABANOWITZ, F. K. BOTZ & D. E. BRENT: Chemical ionization mass spectrometry of salts and thermally labile organics with field desorption emitters as solids probes. Anal. Chem. 49: 1160~1163, 1977
8) TAKADA, N.; K. I. HARADA, M. SUZUKI, A. TATEMATSU & T. KUBODERA: Application of emitter chemical ionization mass spectrometry to structural characterization of aminoglycoside antibiotics -2. Org. Mass Spectrum. 17: 247~252, 1982