A NEW AMINOGLYCOSIDE ANTIBIOTIC COMPLEX—THE SELDOMYCINS

II. ISOLATION, PHYSICOCHEMICAL AND CHROMATOGRAPHIC PROPERTIES

SEIJI SATO, SEIGO TAKASAWA, TOMOYASU SATO, MITSUYOSHI YAMAMOTO, RYO OKACHI, ISAO KAWAMOTO, TAKAO IIDA, ATSUKO MORIKAWA and TAKASHI NARA

Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., Ltd., 3-6-6 Asahimachi, Machidashi, Tokyo, Japan

(Received for publication August 12, 1976)

An antibiotic complex consisting of four components, seldomycin factors 1, 2, 3 and 5 was isolated from the fermentation broth of *Streptomyces hofunensis* sp. nov. by use of a cationic exchange resin. After silica gel column chromatography, the purified components were characterized as new aminoglycoside antibiotics by their physicochemical, chromatographic and antimicrobial properties.

Our previous paper[1] described the production of the seldomycin complex of new antibiotics by fermentation of *Streptomyces hofunensis* sp. nov. MK-88.

The seldomycin complex consists of 4 components, factors 1, 2, 3 and 5 (previously designated XK-88-1, XK-88-2, XK-88-3 and XK-88-5, respectively). The present report will describe their isolation and purification by use of cationic exchange resin and silica gel column chromatography and their characterization by silica gel thin-layer chromatography, physicochemical and antimicrobial properties.

Their activity against aminoglycoside-resistant organisms will be discussed in terms of the structures. The structure elucidation studies will be reported in subsequent papers[2,3].

Isolation and Purification

Seldomycin complex was isolated from the fermentation broth by an ion-exchange resin procedure. The whole broth was adjusted to pH 2.0 with 6 N H_2SO_4 and filtered. The filtrate was neutralized to pH 6.8 with 6 N NaOH and charged Amberlite IRC-50 cationic exchange resin in the ammonium cycle. The column of IRC-50 (NH_4^+ form) was washed with deionized water and then developed with 1 N NH_4OH. The active fractions were combined, concentrated *in vacuo*, adjusted to pH 4.5 with 6 N H_2SO_4 and passed through an active carbon column. The carbon column was eluted with water and 30% methanol. The active fraction was neutralized by passage through an anionic exchange resin, Dowex 44 (OH^-). The neutralized eluate was passed through Amberlite CG-50 cationic exchange resin in the ammonium cycle. The column was washed with deionized water and factor 1 was eluted with 0.15 N NH_4OH. A mixture of seldomycin factors 2, 3 and 5 was then eluted with 0.5 N NH_4OH. Each active fraction was concentrated *in vacuo* to dryness.

Seldomycin factor 1 was purified by a silica gel column and CG-50 (NH_4^+ cycle). The crude sample of seldomycin factor 1 was subjected to chromatography on a silica gel column developed with 10% ammonium acetate-methanol (1:1). The active fraction of seldomycin factor 1 was diluted with 4 volumes of deionized water and passed through a column of CG-50 (NH_4^+ form).

After washing with water, the column was eluted with 0.5 N NH_4OH. The active fraction was evaporated to dryness under reduced pressure to yield purified seldomycin factor 1 as free base.

The mixture of seldomycin factors 2, 3 and 5 was subjected to chromatography on silica gel column developed with a solvent system of *n*-butanol - ethanol - chloroform - conc. NH_4OH (4:5:2:5). The components were eluted separately in the order, factors 5, 2 and 3, and concentrated to dryness *in vacuo* yielding seldomycin factors 2, 3 and 5 as free bases. To obtain the sulfate salts of the seldomycins, concentrated solutions were adjusted to pH 4.5 with 6 N H_2SO_4 and methanol was added to precipitate the salts.

Physicochemical Properties

The physicochemical properties of seldomycin factors 1, 2, 3 and 5 sulfate are shown in Table 1. Seldomycin factors 1 and 3 are white amorphous solid bases, which are readily soluble in water, slightly soluble in methanol and insoluble in ethanol, acetone, and other organic solvents. Seldomycin factors 2 and 5 are similar in properties to factors 1 and 3, but

Table 1. Physicochemical properties of seldomycin sulfates

	Factor 1	Factor 2	Factor 3	Factor 5
Nature*	Basic, white powder	Basic, white powder	Basic, white powder	Basic, white powder
M. P.	200~240°C (dec.)	225~250°C (dec.)	215~240°C (dec.)	215~240°C (dec.)
$[\alpha]_D^{25}$	+77.9°(*c* 1.08, H_2O)	+74.7°(*c* 0.56, H_2O)	+87.3°(*c* 0.53, H_2O)	+93.9°(*c* 0.54, H_2O)
U. V.	End absorption	End absorption	End absorption	End absorption
Elem. Anal.	Found. Calcd.	Found. Calcd.	Found. Calcd.	Found. Calcd.
[%]				
C;	29.23 29.35	25.47 25.90	26.02 25.82	25.89 25.35
H;	6.35 6.48	6.75 6.52	6.72 6.39	6.50 6.62
N;	8.02 8.35	9.68 10.07	8.61 8.88	9.74 9.85
M. W.*	454	306	453	450
Formula*	$C_{17}H_{34}N_4O_{10}$	$C_{16}H_{26}N_4O_5$	$C_{17}H_{35}N_5O_9$	$C_{18}H_{38}N_6O_7$
Solubility				
Soluble	Water	Water	Water	Water
Slightly soluble	Methanol	Methanol, ethanol	Methanol	Methanol, ethanol
Insoluble	Ethanol, acetone, ether, $CHCl_3$, benzene	Acetone, ether, $CHCl_3$, benzene	Ethanol, acetone ether, $CHCl_3$ benzene	Acetone, ether, $CHCl_3$, benzene

*: Data of seldomycin free bases

Fig. 1. IR spectrum of seldomycin factor 1

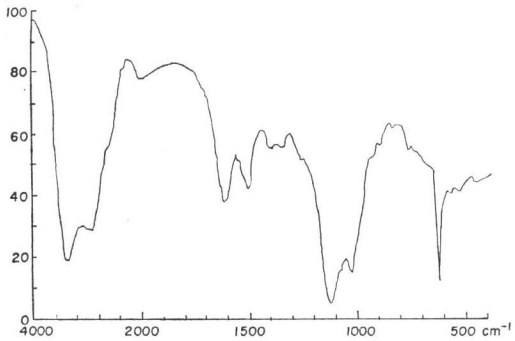

Fig. 2. IR spectrum of seldomycin factor 2

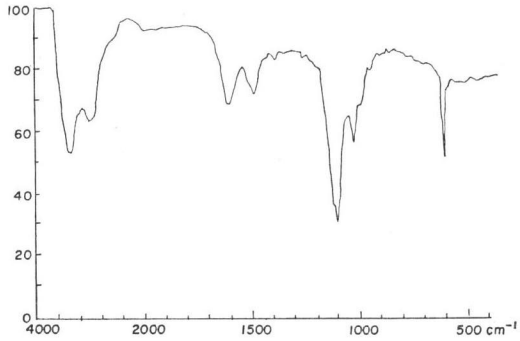

Fig. 3. IR spectrum of seldomycin factor 3

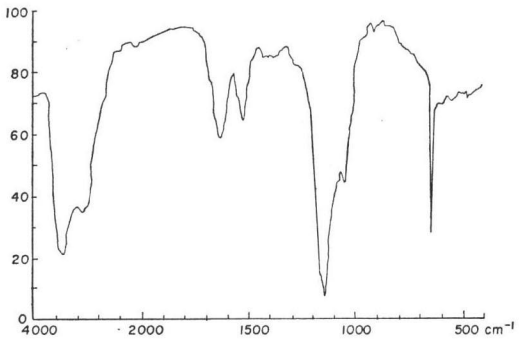

Fig. 4. IR spectrum of seldomycin factor 5

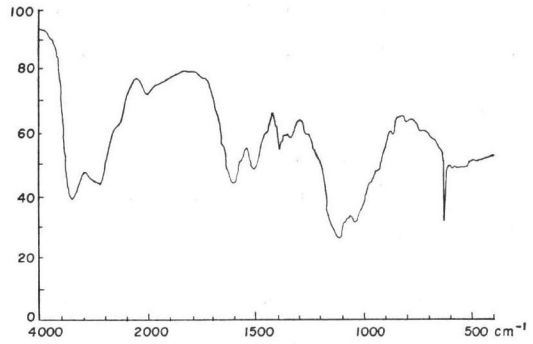

Table 2. Rf Values of seldomycins on TLC

Seldomycins	Rf		
	I	II	III
Factor 1	0.58	0.31	0.26
Factor 2	0.45	0.38	0.41
Factor 3	0.39	0.27	0.58
Factor 5	0.10	0.50	0.65

Solvent (silica gel TLC; 20×20 cm)
I: 10% CH_3COONH_4 - methanol (1:1)
II: *n*-Butanol - ethanol - $CHCl_3$ - conc.NH_4OH (4:5:2:5)
III: $CHCl_3$- methanol - conc.NH_4OH - acetone (2:2:1:1)
Bioautographed on *B. subtilis* KY 4273

Table 3. Comparison with other aminoglycoside antibiotics

Antibiotics		Rf		
		I	II	IV
Seldomycin	factor 1	0.58	0.31	0.80
	factor 2	0.45	0.35	0.73
	factor 3	0.39	0.27	0.58
	factor 5	0.10	0.50	0.65
Gentamicin	A	0.39	0.38	0.66
	B	0.38	0.38	0.75
	C_{1a}	0.18	0.52	0.86
	C_1	0.17	0.59	0.87
	C_2	0.18	0.61	0.86
Kanamycin	A	0.75	0.25	0.77
	B	0.82	0.16	0.80
	C	0.84	0.17	0.84
Ribostamycin		0.72	0.18	0.75
Apramycin		0.35	0.15	0.85
Nebramycin	factor 4	0.45	0.16	0.78
	factor 5	0.65	0.14	0.79
Tobramycin		0.51	0.25	0.80
Neamine		0.71	—	0.40

Silica gel TLC (20×20 cm)
Solvent I; 10% CH_3COONH_4 - methanol (1:1)
II; *n*-Butanol - ethanol - $CHCl_3$ - conc. NH_4OH (4:5:2:5)
IV; $CHCl_3$ - methanol - 17%NH_4OH (2:1:1 upper layer)
Bioautographed on *B. subtilis* KY 4273.

slightly soluble in ethanol too. The ultraviolet absorption spectra exhibit no characteristic peaks. The infrared spectra of seldomycin factors 1, 2, 3 and 5 are given in Figs. 1, 2, 3 and 4, respectively. As a result of elemental analysis and molecular weight determination by the high resolution mass-spectrum method, the molecular formulas which are shown in Table 1, were calculated for these compounds. The antibiotics showed dextrorotation. All factors were positive in reactions such as ninhydrin, RYDON-SMITH, ELSON-MORGAN reagents, but negative in FEHLING, maltol and SAKAGUCHI reactions.

Seldomycin factors 1, 2, 3 and 5 showed Rf values given in Table 2 on thin-layer chromatography. The seldomycin mixture was found to contain several minor components such as neamine, paromamine and a few unidentified compounds. The components of seldomycin complex were designated factors 1 to 5 by the order of Rf values on silica gel TLC (solvent I).

These chromatographic, physicochemical and biological data including antibacterial spectra[1] clearly show that all the four factors belong to the class of basic and water-soluble antibiotics, probably new aminoglycosides. Subsequent comparative studies with many known aminoglycoside antibiotics, as shown in Table 3, eventually demonstrated that seldomycin factors 1, 2, 3 and 5 are new aminoglycosides. Seldomycin factor 2, after we isolated and found it new, has been recently reported by KAWAGUCHI and coworkers[4] as a degradation product of the new antibiotics 4′-deoxybutirosins. However factor 2 itself has never been isolated from a fermentation broth.

Activity of Seldomycin Factors 2 and 5 against Aminoglycoside-Resistant Organisms

The antibacterial activity of seldomycins *in vitro* and *in vivo* against Gram-negative bacteria was described in the previous paper[1]. Seldomycin factor 5 exhibited a broad spectrum of antibacterial activity against aminoglycoside-resistant *Escherichia coli* and lower minimal inhibitory concentrations than seldomycin factors 1, 2 and 3. The activity of seldomycin factors 2 and 5 was compared with reference aminoglycoside antibiotics against various strains of aminoglycoside-resistant *E. coli* and *Providencia* sp., whose mechanisms of resistance were known. As shown in Table 4, seldomycin factor 5 was inactive against *E. coli* KY8302

Table 4. *In vitro* antibacterial activities against aminoglycoside-resistant organisms

Test organisms	MIC mcg/ml (free base)						Inactivating enzymes
	GM-C	KM-A	NM-B	PM	SDM-5	SDM-2	
E. coli ATCC26	0.04	0.16	0.2	0.26	0.1	0.95	—
KY8302	0.09	>18	>17	>18	>50	>122	APH(3′)-I
KY8321	0.09	8.88	8.45	>18	0.75	61	APH(3′)-II
KY8327	0.75	4.44	0.53	2.23	0.1	1.9	ANT(2″)
KY8348	0.75	0.04	0.07	0.14	3.13	0.95	AAC(3)-1
KY8349	0.04	4.44	>17	>18	>50	>122	AAC(6′)
Providencia sp. 164	>5.5	1.13	>17	>18	50	>122	AAC(2′)

Assayed by agar dilution assay at pH 8.0

GM-C; gentamicin C, KM-A; kanamycin A, NM-B; neomycin B, PM; paromomycin, SDM-5; seldomycin factor 5, SDM-2; seldomycin factor 2, APH(3′)-I and APH(3′)-II; aminoglycoside-3′-phosphotransferase I and II, ANT(2″); aminoglycoside-2″-nucleotidyltransferase, AAC(3)-1; aminoglycoside-3-acetyltransferase-1, AAC(6′); aminoglycoside-6′-acetyltransferase, AAC(2′); aminoglycoside-2′-acetyltransferase,

producing neomycin-kanamycin phosphotransferase[5,6] (aminoglycoside-3′-phosphotransferase I; APH(3′)-I), but was active against *E. coli* KY8321 possessing neomycin-kanamycin phosphotransferase II[7,8] (aminoglycoside-3′-phosphotransferase II; APH(3′)-II). This pattern of the activity of seldomycin factor 5 is quite similar to the properties of 4′-deoxybutirosin and 4′-deoxykanamycin, as reported by KAWAGUCHI *et al.*[8] and UMEZAWA *et al.*[7]. respectively.

A clinical isolate *E. coli* KY8327 is a gentamicin-resistant strain having 2″-nucleotidyltransferase (aminoglycoside-2″-nucleotidyltransferase; ANT(2″))[9], and *E. coli* KY8321 has not only neomycin-kanamycin phosphotransferase II, but also gentamicin nucleotidyltransferase[10]. Seldomycin factors 2 and 5 were active against the both strains, KY8321 and KY8327. The reason for this is that factor 5 does not have a 2″-OH group in its structure and factor 2 is

devoid of the second amino sugar, as shown by the following structure studies[2]. *E. coli* KY8349 and *Providencia* sp. 164 showed cross-resistance against seldomycin factors 2 and 5. *E. coli* KY8349 is a clinical isolate possessing kanamycin-6′-NH_2 acetylating enzyme[11] (aminoglycoside-6′-acetyltransferase; AAC(6′)), and *Providencia* sp. 164 is a gentamicin-resistant strain possessing 2′-NH_2 acetylating enzyme which was initially reported by BENVENISTE and DAVIES.[12,13] This suggests that seldomycin factors 2 and 5 have 2′-and 6′-amino groups in their structure.

E. coli KY8348 is resistant specifically to gentamicin and sisomicin possessing an enzyme capable of acetylating the 3-NH_2 group of deoxystreptamine in those antibiotics[14]. It is interesting that seldomycin factor 5 is inactive against this strain, but factor 2 is active against this strain. This suggests that the pentosamine moiety in factor 5 determines the susceptibility of this antibiotic to gentamicin-acetyltransferase 1 (aminoglycoside-3-acetyltransferase 1; AAC(3)-1). As to the specificity of gentamicin-acetyltransferase 1, it is suggestive that gentamicin X is susceptible to this enzyme, but gentamicin A is not inactivated by this enzyme[15], and that structural difference of gentamicin X from gentamicin A is the presence of 4″-methyl in the garosamine moiety of gentamicin X and its absence in gentamicin A, and 4″-OH of gentamicin X (axial) is an epimer of that of gentamicin A (equatorial).

Acknowledgement

The authors are grateful to Drs. A. C. SINCLAIR, J. B. MCALPINE and his associates in Abbott Laboratories, North Chicago, Ill., U.S.A. for their kind advice and encouragement. They are also thankful to Dr. J. DAVIES, University of Wisconsin, Madison, Wis., U.S.A. through Abbott Laboratories for the gift of *Providencia* sp. 164.

References

1) NARA, T.; M. YAMAMOTO, S. TAKASAWA, S. SATO, T. SATO, I. KAWAMOTO, R. OKACHI, I. TAKAHASHI & A. MORIKAWA: A new aminoglycoside antibiotic complex—The seldomycins. I. Taxonomy, fermentation and antibacterial properties. J. Antibiotics 30: 17~24, 1977
2) EGAN, R. S.; A. C. SINCLAIR, R. L. DEVAULT, J. B. MCALPINE, S. L. MUELLER, P. C. GOODLEY, R. S. STANASZEK, M. CIROVIC, R. J. MAURITZ, L. A. MITSCHER, K. SHIRAHATA, S. SATO & T. IIDA: A new aminoglycoside antibiotic complex—The seldomycins. III. The structure of seldomycin factors 1 and 2. J. Antibiotics 30: 31~38, 1977
3) MCALPINE, J. B.; A. C. SINCLAIR, R. S. EGAN, R. L. DEVAULT, R. S. STANASZEK, M. CIROVIC, S. L. MUELLER, P. C. GOODLEY, R. J. MAURITZ, N. E. WIDEBURG, L. A. MITSCHER, K. SHIRAHATA, H. MATSUSHIMA, S. SATO & T. IIDA: A new aminoglycoside antibiotic complex—The seldomycins. IV. The structure of seldomycin factor 5. J. Antibiotics 30: 39~49, 1977
4) KONISHI, M.; K. NUMATA, K. SHIMODA, H. TSUKIURA & H. KAWAGUCHI: Aminoglycoside antibiotics. VI. Structure determination of 4′-deoxybutirosins (Bu-1975 C_1 and C_2). J. Antibiotics 27: 471~483, 1974
5) YAGISAWA, M.; H. YAMAMOTO, H. NAGANAWA, S. KONDO, T. TAKEUCHI & H. UNEZAWA: New enzyme in *Escherichia coli* carrying R-factor phosphorylating 3′-hydroxy of butirosin A, kanamycin, neamine and ribostamycin. J. Antibiotics 25: 748~750, 1972
6) UMEZAWA, H.; H. YAMAMOTO, M. YAGISAWA, S. KONDO, T. TAKEUCHI & A. CHABBERT: Kanamycin phosphotransferase I: Mechanism of cross resistance between kanamycin and lividomycin. J. Antibiotics 26: 406~411, 1973
7) UMEZAWA, S.; Y. NISHIMURA, Y. HATA, T. TSUCHIYA, M. YAGISAWA & H. UMEZAWA: Synthesis of 4′-deoxykanamycin and its resistance to kanamycin phosphotransferase II. J. Antibiotics 27: 722~725, 1974
8) KAWAGUCHI, H.; K. TOMITA, T. HOSHIYA, T. MIYAKI, K. FUJISAWA, M. KIMEDA, K. NUMATA, M. KONISHI, H. TSUKIURA, M. HATORI & H. KOSHIYAMA: The 4′-deoxybutirosin (Bu-1975 C_1 and

C_2), new aminoglycoside antibiotics of bacterial origin. J. Antibiotics 27: 460~470, 1974

9) BENVENISTE, R. & J. DAVIES; R factor mediated gentamicin resistance: A new enzyme which modified aminoglycoside antibiotics. FEBS Letters 14: 293~296, 1971

10) OKACHI, R.; I. KAWAMOTO, S. TAKASAWA, M. YAMAMOTO, S. SATO, T. SATO & T. NARA: A new antibiotic XK-62-2 (Sagamicin). I. Isolation, physicochemical and antibacterial properties. J. Antibiotics 27: 793~800, 1974

11) BENVENISTE, R. & J. DAVIES; Enzyme acetylation of aminoglycoside antibiotics by *Escherichia coli* carrying an R-factor. Biochem. 10: 1787~1796, 1972

12) BENVENISTE, R. & J. DAVIES; Aminoglycoside antibiotic inactivating enzymes in *Actinomycetes* similar to those present in clinical isolates of antibiotic-resistant bacteria. Proc. Natl. Acad. Sci. 70: 2276~2280, 1973

13) BENVENISTE, R. & J. DAVIES; Mechanism of antibiotic resistance in bacteria. Ann. Rev. Biochem. 42: 471~506, 1973

14) BRZEZINSKA, M.; R. BENVENISTE, J. DAVIES, P. J. L. DANIELS & J. WEINSTEIN: Gentamicin resistance in strains of *Pseudomonas aeruginosa* mediated by enzymatic N-acetylation of the deoxystreptamine moiety. Biochemistry 11: 761~776, 1972

15) PRICE, K. E.; J. C. GODFREY & H. KAWAGUCHI: Effect of structural modifications on the biological properties of amino glycoside antibiotics containing 2-deoxystreptamine. in "Advances in Applied Microbiology" Ed., by D. PERLMAN, Vol. 18, pp. 191~307, Academic Press (New York, San Francisco and London), 1974