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THE FERMENTATION PROCESS AND PRODUCTION OF GENTAMICIN C

AN AMINOGLYCOSIDE ANTIBIOTIC COMPLEX *

by

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Aminoglycosides represent a group of antibiotics of major importance. Recently, an ever increasing number of these antimicrobial agents /e.g. streptomycin, neomycin, kanamycins, paromomycin, gentamicins, tobramycin, sisomicin, etc./ and their semisynthetic derivatives /e.g. amikacin, dibekacin, etc./ obtained by chemical modification have been introduced into human therapy. In addition, this type of antibiotics have found application in agriculture, too. Discoveries of new aminoglycosides either by conventional soil screening, by chemical modification of known aminoglycosides or by mutational biosynthesis /hybrimycins/ are needed to circumvent inherent toxicity problems and control the infections caused by cultures resistant to streptomycin, kanamycin, etc.

A detailed review by Price, Godfrey and Kawaguchi shows the structure-activity relationships of aminoglycosides containing 2-deoxystreptamine. The review of Takashi Nara gives also a good summary of recent advances in this field.

The sale of aminoglycosides over the world in 1977 is represented by the sum of 350 million $.

The gentamicin complex produced by various Micromonospora species was first described by Weinstein, Luedemann, Oden and Wagman in 1963. The main components of the gentamicin

complex are gentamicins C_1, C_2 and C_{1a} used widely in clinical practice. The gentamicin C complex is bactericidal and has greater antibacterial activity than streptomycin, neomycin or kanamycins. It is effective against many strains of Gram-negative bacteria including species of Escherichia, Enterobacter, Klebsiella, Salmonella, Serratia, Shigella, some Proteus and against Pseudomonas aeruginosa. Minimum inhibitory concentrations have been reported to range from 0.06 to 8 mcg per ml.

Among the Gram-positive organisms Staphylococcus aureus is highly sensitive to gentamicin with minimum inhibitory concentrations, being reported within the range of 0.125 to 1 mcg per ml. Other Gram-positive cocci are less sensitive, but Bacillus, Clostridium and Corynebacterium spp. may be inhibited by normal concentrations. Gentamicin sulfate is used to treat septicaemia and other severe systemic infections due to sensitive Gram-negative organisms. In the treatment of urinary-tract infections with gentamicin, alkalis should be given to raise the urinary pH above 7. The usual dose of gentamicin given intramuscularly is 100 to 800 mcg per kg body weight in every 8 hours. Gentamicin has been given intravenously in the same doses that are used intramuscularly. The course of treatment should generally be limited to 7 days. Gentamicin is poorly absorbed from the gastro-intestinal tract. If symptomy of ototoxicity occur, gentamicin should be withdrawn immediately. It

should be given with care and in reduced dosage to patients with impaired renal function.

Gentamicin C complex is being produced in Hungary since 1974. The producing strain is Micromonospora purpurea var. nigrescens nova varietas, isolated from soil in 1970, marked as X-148. This Actinomyces strain has been assigned accession number MNG 00112 in the Hungarian National Culture Collection. Table 1 below sets forth the comparison of strain X-148 with those of M. purpurea /NRRL-2953/ and M. purpurea var. violacea 55-5829 /accession number: Coll. Microb. Bulg., DKIILS, 55/3 III. 1971/.

Table 1

Characteristic	M. purpurea /MBL-2950/	Isolate /MBG 00112/	M. purpurea var. violacea 55-5809
Macro-morphology	No aerial mycelia, colonies are raised, spiral, abundant growth, waxy. Surface color: terracotta to brownish. Reverse: russet to deep brown. No soluble pigment.	No aerial mycelium, raised colonies but sink into the medium, waxy, slightly dry surface, very poor growth. Color: blackish deep purple. No soluble pigment.	No aerial mycelium, abundant growth, irregularly shaped, smooth colonies. Microscopically the surface is wrinkled, waxy, the radial ditches form strings which get smoothed in time. Colonies: violet.
Micro-morphology	Hyphae are long and branching, regular, not shaped, diameter: 0.5 µ. Spores are formed at the end of single sporophores, either round- or ellipsoidal-shaped, diameter: 1.0 µ.	Hyphae are long and extensively branching, diameter: 0.6-0.7 µ. Conidia are rare and round-shaped and smooth. Diameter: 1.0-1.3 µ, no sporophores.	On the young hyphae, unicellular spores are formed which are oval-shaped, diameter: 1.0 µ. On liquid medium hyphas are fine and completely basophyl in star like position.
Gelatin	Weak liquefaction	Liquefaction	Liquefaction
Sucrose	Inverted	Slightly inverted	Inverted
Starch	Hydrolyzed	Hydrolyzed	Hydrolyzed
Cellulose	Slightly decomposing	Slightly decomposing	
Glucose-aspa-ragine agar	Good growth, from bright peach to medium red orange color.	Very poor growth, blackish purple color.	
Tyrosine agar	Good growth, no soluble pigment.	A very slow growth only at 37°C, little tan color, soluble pigment.	Cream color, darker in the middle.

Table 2

Carbohydrate utilization

Carbohydrate	M. purpurea /NRRL-2953/	X-148 /MNG 00112/	M. purpurea var. violacea 55-5829
D-glucose	+	+	+
D-galactose	+	-	+
Sucrose	+	+	+
Maltose	0	+	+
Lactose	-	-	+
Raffinose	-	-	+
Sorbose	0	-	0
Cellobiose	0	+	0
Trehalose	0	[(+)]	0
Melibiose	0	-	0
Melecitose	0	[(+)]	0
Glycerol	0	-	-
Sorbitol	-	-	-
Starch, soluble	+	+	+
D-xylose	+	+	+
L-Arabinose	+	+	-
L-rhamnose	-	+	-
Galactitol	0	-	+
D-mannitol	-	-	-
Inositol	-	-	-
Salicin	0	-	0
α-methyl-D-glucoside	0	-	0
Fructose	-	0	-
Mannose	+	0	0
Cellulose	-	+	0
Dextrin	0	0	+

Based on the considerably differing characteristics the strain K-148 can be precisely distinguished from the following species or variants of Micromonospora strains producing gentamicins or gentamicin-type antibiotics:

Micromonospora purpurea NRRL-2953
Micromonospora purpurea var. violacea 55-5829
Micromonospora echinospora NRRL-2985
Micromonospora echinospora var. ferruginea NRRL-2995
Micromonospora echinospora var. pallida NRRL-2996
Micromonospora grisea NRRL-3800
Micromonospora rhodorangea NRRL-5326
Micromonospora inyoensis NRRL-3292

On the basis of the above, the new strain K-148 was designated as Micromonospora purpurea var. nigrescens nov. var. Description of the applied K-148 strain according to Waksman's system is given below:

Morphology:

Very slow growth. Colonies resemble to bacterial ones, with slightly dry surface and unbroken edges, as a rule, slightly raised from the nutrient agar but at the same time sink into it. Owing to non-diffusing pigment they have a blackish-purple color. Aerial mycelium is not formed. Microscopically, hyphae are long and extensively branched, diameter: 0.6-0.7 μ. Conidia (spores) rarely discernible on the hyphae, are round-shaped and smooth, diameter: 1.0-1.3 μ. No sporophores.

Biochemical, physical properties:

Nurtient agar: no growth

Glucose-asparagine agar: colonies are grown very poorly, surface is dry, slightly raised, stick into the agar, with unbroken edges, blackish-purple color.

Tyrosine agar: no growth at 26°C, very slow growth at 37°C, colonies are bright purple, slight tan color pigment diffusing into the agar.

Corn-meal agar: slow growth, similarly to that observed on glucose-asparagine agar.

Blood agar: color of colonies: grayish-pink, no haemolysis.

Löffler's serum-medium: colonies are meat-colored, strong proteolysis is observable.

Cellulose: poor growth.

NaCl-tolerance: 3.5 % /on glucose-yeast-extract agar/.

Gelatin: liquefaction.

I. Preservation of the gentamicin producing Micromonospora strain

1./ For the preservation of the strain bouillon agar slant pH 7.2, adjusted with 0.5 % peptone and 0.5 % NaCl is used. Slant cultures are incubated at 37°C for one day /in the case of inoculation from shake flask culture/ and 3-5 days /in the case of inoculation from a single colony/, respectively, then stored at 4° - 10°C. Under these conditions the producing capacity of the strain is generally preserved.

2./ The strain sporulated very poorly on the different media tested by us. Its producing capacity can be preserved, however, for a period of one year by means of lyophilizing a vegetative seed culture.

3./ At -15°C the strain can be preserved at least for 3 months. For this purpose the following medium is inoculated with agar slant culture:

0.3 % beef extract
0.1 % peptone
0.1 % sucrose
2.4 % starch, soluble
0.4 % $CaCO_3$

with tap water, pH 7.0 before sterilization. The medium is sterilized at 121°C for 20 min.

A 200 ml volume is shaken in a 500 ml Erlenmeyer flask for 2-3 days at 37°C, followed by addition of 3 % glycerol and freezing in mixture of acetone-CO_2.

II. Preservation and checking of the producing capacity

1./ The most successful way for preservation of the productivity is as follows:

The deep-freezed vegetative culture is plated onto bouillon-agar, pH 7.2. After incubation for 4 days at 37°C, inoculation onto agar slants of the same composition by means of individual colonies is carried out. After a further 4 days incubation at 37°C, the culture is inoculated into 500 ml Erlenmeyer flasks with 100 ml working volume, using three parallels, having the following composition:

3.0 % soya meal
1.5 % starch
0.4 % $CaCO_3$
2 mcg/ml $CoCl_2.6H_2O$

with tap water, pH 7.0-7.5 before sterilization. Sterilization is performed at 121°C for 20 min.

Incubation is at 37°C on a rotary shaker /320 r.p.m., 37 mm stroke/. From the 3-days-old cultures sterile samples are taken and kept at 4-10°C for a further 4 days. In the meantime, productivity of cultures are checked and, fom the correspondingly stored culture having the highest producing capacity, inoculation is performed onto bouillon slant, pH 7.2. After incubation at 37°C for one day seed cultures are inoculated again from the slant for freezing purposes using the method described under I/3.

2./ Checking of the production is carried out on the following medium:

In 500 ml Erlenmeyer flasks of 100 ml working wolume

3.0 % soya meal
1.5 % potato starch
0.4 % $CaCO_3$
2 mcg/ml $CoCl_2.6H_2O$

dissolved in tap water, pH 7.0-7.5, sterilization at 121°C for 20 min.

Media are inoculated with 1 ml of suspension made from agar slant or deep freezed vegetative culture, respectively, and shaken at 28°C on a rotary shaker /320 r.p.m., 37 mm stroke/.

Production, sterility and morphology are first checked at the age of 72 h and as a rule for a further three days.

3./ For a 72-hour-old culture, dense mycelium of thin slightly wavy hyphae is characteristic. In the 72th-96th hours slight sporulation starts, which gets increased by age. No considerable fragmentation is observable.

4./ pH of the culture from the initial 7.0 decreases by 0.5-1 unit, then is generally increased to 8.0-8.2 at the 144th h.

5./ Average production development:

Inoculated with agar slant culture:

72 h	200-300 mcg/ml
96 "	300-400 "
120 "	400-500 "
144 "	500-600 "

Inoculated with deep freezed vegetative culture:

72 h	200-250 mcg/ml
96 "	250-350 "
120 "	350-450 "
144 "	450-500 "

6./ Average component composition of the complex, determined from a gentamicin C complex, prepared from a 144 hours fermentation broth: /crude substance/

C_{1a}	19 %
C_2	45 %
C_1	36 %

III. Preparation of seed culture

1./ For preparation of seed culture deep freezed vegetative inoculum not older than three months is used. Composition of the medium:

0.3 % beef extract
0.1 % peptone
0.1 % sucrose
2.4 % starch, soluble
0.5 % yeast extract
0.4 % $CaCO_3$

tap water, pH 7.0.

200 ml medium is prepared in 500 ml Erlenmeyer flasks, sterilization at 121°C for 20 min.

2./ Three flasks containing 200 ml medium are inoculated with 1-1 ml seed and shaken for 48-72 h at 37°C. After microscopical checking 500 ml inoculum is used to inoculate 5 l medium.

3./ Microscopic characteristics of a 72-hour-old culture: colonies consist of thin, slightly wavy mycelia, not forming a continuous network. Slight spore formation, no fragmentation.

IV. Fermentation process

The culture thus obtained was used to inoculate 2000 liters of the sterilized medium in a 3 cubic meter fermentation tank having the following composition /grams/liter/

Potato starch	10 g
sucrose	5 "
yeast extract	5 ml /10 % solution/
casein hydrolyzate	20 " /20 % " /
peptone	2 g
corn steep liquor	1 " /50 % dry weight/
$CaCO_3$	4 "
palm oil	2 ml
tap water	

pH 7.0 before sterilization. The inoculated medium is aerated at a rate of 1/1 v/v and stirred 130 r.p.m. at 35°C for 50-60 h. The resulting culture is then used to inoculate the 30 cubic meter producing tank fermentor having 20 cubic meter of the following composition /grams/liter/

soya meal	30 g
potato starch	15 "
glycerol	15 ml
$CaCO_3$	5 g
$CoCl_2.6H_2O$	2 mg
palm oil	2 g
tap water	

pH 7.0 before sterilization. Sterilization is carried out while stirring at 121°C for 1 h. Fermentation is carried out in acid-fast steel tanks. Rate of stirring is 130 r.p.m. and aerated at 1/1 v/v.

Antibiotic production starts in the 35-40th h and highest value is reached by 100-110 h.

Total antibiotic activity of the broth referred to gentamicin-C standard is 850 U/ml / 1 U is equal to 1 mcg gentamicin standard; C_1: 32 %, C_2: 48 %, C_{1a}: 20 %/, determined on Staphylococcus epidermidis test organism by large plate agar diffusion method. The total amount of antibiotics produced is 1000-1050 mcg/ml from which 75 % is gentamicin-C. The composition of gentamicin-C fraction: C_1=36 %, C_2=45 %, C_{1a}=19 %.

Fermentations carried out were batch fermentations, we have not any experience regarding semi-continuous fermentation. Economical requirements, of course, improve by raising the volume of the fermentors. We could attain economical production already in 30 cubic meter volume, though a 100 cubic meter volume would be economically more advantageous. Regarding automation, it is worth at least to automatize temperature, pH and addition of anti-foam agents.

The raw materials of the fermentation process listed above being agro-industrial products are practically locally available. As for sources of supply for the equipment: for fermentation of gentamicin no special requirements are needed, they might be purchased anywhere.

The anticipated losses due to contamination in case of trained work are around 5 %.

Isolation of crude gentamicin complex

To 1 cubic meter of the fermentation broth /850 mcg/ml/ produced by the above process, 5 N sulfuric acid was added at 20°C to adjust the pH to 2.0. The broth was filtered and the mycelial cake washed by 0.1 volume of tap water giving 920 l filtrate. To this filtrate, 2.7 kg oxalic acid was added, furthermore, the pH was adjusted to 7.5 by sodium hydroxyde solution. After 10 min heating at 80-90°C the broth was filtered and the mycelial cake washed with 50 l tap water. The 980 l /825 mcg/ml/ solution obtained was passed through a 3-column-resin-system at room temperature, each column /8x60 cm/ containing 3-3 l carboxyl-type cation exchange resin /Wofatit CP-300/ in Na form. Flow rate 50 l/h. During the absorption process the first column was practically saturated i.e. an effluent containing 800 mcg/ml activity was obtained. About 15 % of the total antibiotic activity was absorbed on the second column. The effluent of this column contained only 5 - 10 mcg/ml at the end of the absorption process. In case of continuous processing, this column was employed further for absorption of active broth. The first column was washed with 25 l distilled water then antibiotics were eluted with 4 N sulfuric acid at a flow rate of 0.5 l/h. The effluent fractions con-

taining more than 5000 mcg/ml were pooled, yielding 4500 ml solution of 136.000 mcg/ml activity.
/By batch process, from the second and third colums 3500 ml 40.000 mcg/ml and 2600 ml 15.000 mcg/ml eluates were obtained by a similar way./
The pH of the dark brown, acidic eluate obtained from the first column was adjusted to 4.5 by stirring with triethyl amine then methanol was given /about 2500 ml/ to the solution until the turbidity was just disappearing. To the solution activated charcoal was added and after 30 min stirring, it was filtered. The carbon was washed by distilled water resulting 6900 ml solution with 83.000 mcg/ml activity. This solution was pured while continuous stirring into 35 l methanol. The precipitated crude gentamicin was filtered, washed twice with methanol and dried in vacuo at 30-40°C. By this way 962 grams of antibiotic complex were obtained as sulfate salt, assaying at 536 mcg/mg, ash content: 2.0 %, rotation: $/\alpha/_D^{25}$ = +104° / 1.0 % in water/.
By batch process, the sulfuric acid eluates obtained from the second and third colums by a similar way yield a further 168 g of crude sulfate salt having 536 mcg/mg activity. Total yield: 1130 g crude gentamicin sulfate.

Isolation of components

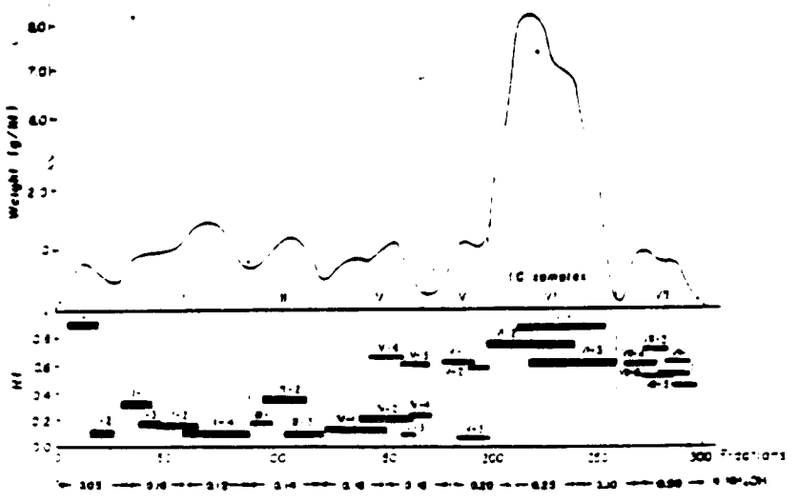
The crude gentamicin complex /500 g which corresponds to 670 g of crude sulfate salt above/ was chromatographed at first on Amberlite CG-50 Type-I resin developed with increasing concentration of aqueous ammonia. The pattern of chromatography is shown in Fig. 1. The antibiotic complex was separated into seven mixtures indicated on Table 3.

Table 3. Chromatographic separation of crude gentamicin

Fractions	Antibiotic mixtures	Main component(s)	Weight (g)
6~ 30	I	Compound I-1	24.2
31 ~ 91	II	Gentamicin A, antibiotic G-418, compound II-2	49.8
92~123	III	garamine, gentamicin A_1	17.1
124~175	IV	gentamicin B, B_1	18.6
176~198	V	sisomicin	17.3
199~260	VI	gentamicin C_1, C_2, C_{1a}	345.5
261~298	VII	gentamine C_2	16.2

Fig. 1. Chromatographic separation of gentamicin components. Weights and Rf values of components. *(TLC, system B)

Resin: Amberlite CG-50 I (100 ~ 200 mesh)
Column: 12 × 120 cm
Sample: 500 g crude gentamicin
Elution: 30 ~ 30 liters of 0.05, 0.10, 0.12, 0.14, 0.16, 0.18, 0.20, 0.25, 0.30 and 0.50 N ammonium hydroxide
Fractions: 1,000 ml

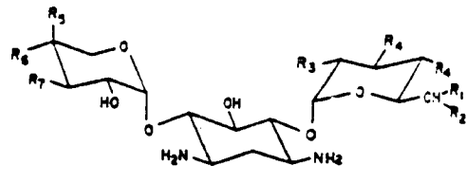


Chromatographic analyisis by systems A,B and C of the I~VII mixtures indicate their complex nature. There are more or less overlaping between mixtures and each of them contains 4~12 components. The bioautographic detection showed that all of the components, except components I-1 and III-2, have bioactivity against B. subtilis. Application of the stepwise gradient elution method resulted in a good resulution of substances having different structural features but similar polarity. The proper combination of various ion-exchange chromatographic procedures enabled the separation of 35 pure components of the gentamicin complex.

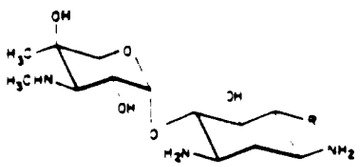
Table 4. Physical and chemical properties of gentamicin minor components

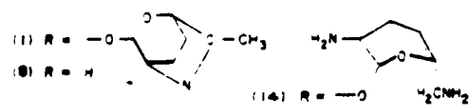
Structure	Compound number	Identical	Formula	MW	$[\alpha]_D^{20}$ (1% water)	PC Rf rel. B_1 System A	TLC Rf System B	TLC Rf System C
1	I-1	New compound	$C_{20}H_{40}N_4O_7$	444	+144°	1.60	0.93	0.16
2	I-2	Gentamicin A_2	$C_{17}H_{36}N_6O_{11}$	455	+138°	0.25	0.08	0.47
3	II-1	G-418	$C_{20}H_{40}N_4O_{10}$	496	+168°	0.73	0.27	0.60
4	II-2	New compound	$C_{19}H_{38}N_4O_{10}$	482	+140°	0.52	0.15	0.73
5	II-3	Gentamicin X_2	$C_{19}H_{38}N_4O_{10}$	482	+158°	0.56	0.16	0.66
6	II-4	Gentamicin A	$C_{18}H_{36}N_4O_{10}$	468	+146°	0.36	0.08	0.84
7	III-1	New compound	$C_{19}H_{38}N_4O_{10}$	482	+156°	0.46	0.18	0.67
8	III-2	Garamine	$C_{18}H_{37}N_5O_6$	321	+137°	0.91	0.31	0.74
9	III-3	Gentamicin A_1	$C_{18}H_{36}N_4O_{10}$	468	+167°	0.37	0.09	0.82
10	IV-1	Gentamicin B	$C_{19}H_{38}N_4O_{10}$	482	+155°	0.41	0.15	0.79
11	IV-2	Gentamicin B_1	$C_{20}H_{40}N_4O_{10}$	496	+163°	1.00	0.23	0.62
12	IV-3	Л-20А	$C_{19}H_{39}N_5O_6$	481	—	0.39	0.14	0.76
13	IV-4	Л-20В	$C_{20}H_{41}N_5O_6$	495	+150°	0.94	0.25	0.46
14	V-1	Sisomicin	$C_{19}H_{37}N_5O_7$	447	+188°	0.86	0.60	0.24
15	V-2	New compound	$C_{19}H_{39}N_5O_7$	449	+156°	0.75	0.56	0.19
16	V-3	Gentamicin A_3	$C_{19}H_{38}N_4O_{10}$	468	−166°	0.29	0.06	0.84
17	VII-1	New compound	$C_{20}H_{41}N_5O_7$	463	−142°	1.13	0.69	0.13
18	VII-2	Gentamine C_1	$C_{14}H_{30}N_4O_4$	318	−88°	1.35	0.73	0.19
19	VII-3	New compound	$C_{19}H_{39}N_5O_7$	449	−148°	0.92	0.55	0.17
20	VII-4	Gentamine C_2	$C_{19}H_{38}N_4O_4$	304	−92°	1.12	0.59	0.27
21	VII-5	New compound	$C_{19}H_{37}N_5O_7$	435	−144°	0.71	0.48	0.20
22	VII-6	Gentamine C_{1a}	$C_{19}H_{38}N_4O_4$	290	−97°	0.82	0.42	0.28

Fig. 2. Structures of compounds



	R_1	R_2	R_3	R_4	R_5	R_6	R_7
2	H	OH	NH_2	OH	H	OH	OH
3	CH_3	OH	NH_2	OH	OH	CH_3	$NHCH_3$
4	CH_3	OH	NH_2	OH	H	OH	$NHCH_3$
5	H	OH	NH_2	OH	OH	CH_3	$NHCH_3$
6	H	OH	NH_2	OH	H	OH	$NHCH_3$
7	CH_3	OH	NH_2	OH	OH	H	$NHCH_3$
9	H	OH	NH_2	OH	OH	H	$NHCH_3$
10	H	NH_2	OH	OH	OH	CH_3	$NHCH_3$
11	CH_3	NH_2	OH	OH	OH	CH_3	$NHCH_3$
12	H	NH_2	NH_2	OH	OH	CH_3	$NHCH_3$
13	CH_3	NH_2	NH_2	OH	OH	CH_3	$NHCH_3$
15	CH_3	NH_2	NH_2	H	OH	CH_3	NH_2
16	H	NH_2	OH	OH	OH	H	$NHCH_3$
17	CH_3	$NHCH_3$	NH_2	H	OH	H	$NHCH_3$
19	CH_3	NH_2	NH_2	H	OH	H	$NHCH_3$
21	H	NH_2	NH_2	H	OH	H	$NHCH_3$

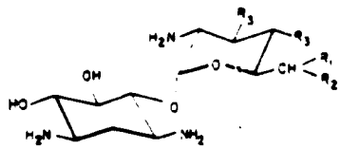




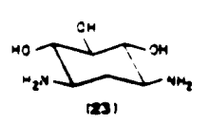
(1) R = —O—C(=O)—CH_3

(8) R = H

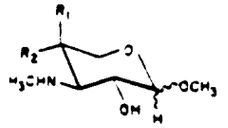
(14) R = —O—CH_2CNH_2



	R_1	R_2	R_3
(18)	CH_3	$NHCH_3$	H
(20)	CH_3	NH_2	H
(22)	H	NH_2	H
(25)	H	OH	OH
(26)	CH_3	OH	OH
(29)	H	NH_2	OH
(30)	CH_3	NH_2	OH



(23)



	R_1	R_2
(24)	OH	CH_3
(27)	H	OH
(28)	OH	H

Mixture I: The relatively apolar compound I-1 was separated from compound I-2 /gentamicin A_2/ on QAE-Sephadex column with water as eluent. Compound I-1 was eluted firstand it was purified by repeated ion-exclusion chromatography of Dowex 1-X2 resin. Gentamicin A_2 was isolated from the later fractions by chromatography on CM-Sephadex eluted with 0.05, 0.07 and 0.10 N ammonium hydroxide, successively. Crude gentamicin A_2 was purified by precipitation with ether from methanolic solution.

Mixture II: We separated the main components of this complicated mixture as follows: The first step was a repeated chromatography on Amberlite CG-50 Type-II resin with gradient elution by 0.08~0.12 N ammonium hydroxide. Compound II-1 /antibiotic G-418/ was isolated from the first enriched fractions by ion-exclusion chromatography on Dowex 1-X2 resin. The following active fractions were pooled and separated again by chromatography on Amberlite XE-69 resin. The elution was carried out by 0.08, 0.10 and 0.12 N ammonium hydroxide, successively. The final /0.12 N/ fractions of this chromatography contained compound II-4 /gentamicin A/ crystallized from ethanol-water. Fractions containing both compounds II-2 and II-3 /gentamicin X_2/ were pooled and rechromatographed on Amberlite CG-400 Type-II resin using ion-free water as eluent. Gentamicin X_2 was eluted first followed by

compound II-2. The final purification of components were achieved on Amberlite CG-50 Type-I resin columns by elution with 0.1 N ammonium hydroxide.

Mixture III: Crude compound III-1 was isolated from the first few fractions of repeated Amberlite CG-50 Type-II chromatography of this mixture with 0.12~0.14 N ammonium hydroxide and was purified by ion-exclusion chromatography on Dowex 1-X2 resin. The pooled and evaporated fractions, free from compound III-1 were chrometographed again on CM-Sephadex column eluted with 0.10, 0.12 and 0.14 N ammonium hydroxide, successively. The early fractions enriched in compound III-2 /garamine/ were subjected to ion-exclusion chromatography to obtain pure garamine. The final fractions containing mainly compound III-3 /gentamicin A_1/ were pooled and purified by repeated chromatography on CM-Sephadex.

Mixture IV: The fore run of the ion-exclusion chromatography of this mixture on Dowex 1-X2 resin contained apolar components /compounds IV-5 and IV-6/ and compounds IV-3 /antibiotic JI-20A/ and IV-4 /antibiotic JI-20B/. These fractions were pooled and separated by Amberlite CG-50 Type-II resin chromatography using 0.12, 0.14 and 0.16 N ammonium hydroxide as eluent. The fractions containing antibiotics JI-20A and JI-20B

were pooled and the mixture was separated by preparative TLC using system B as eluent. The main fractions of the ion-exclusion chromatography of mixture IV, containing IV-1 /gentamicin B/ and IV-2 /gentamicin B_1/ were chromatographed on CM-Sephades column with 0.12 and 0.15 N ammonium hydroxide as eluent and gentamicins B and B_1 were separated.

Mixture V: This mixture was chromatographed on SP-Sephadex column. The successive elution with 0.12, 0.14, 0.16 and 0.18 N ammonium hydroxide gave compounds V-3 /gentamicin A_3/, V-1 /sisomicin/, and V-2 in order. Fractions containing each component were pooled and purified by repeated chromatography on Amberlite CG-50 Type-II columns using 0.15 N ammonium hydroxide as eluent.

Mixture VI: By repeated chromatography of mixture VI /gentamicin C complex/ on Amberlite CG-50 Type-II resin, we isolated pure gentamicin C_2 from the early fractions. The subsequent fractions were pooled and chromatographed on CM-Sephadex column with 0.15 and 0.18 N ammonium hydroxide as eluent. The 0.15 N eluate contains gentamicins C_{1a} and C_2, while the 0.18 N eluate contains practically pure gentamicin C_1. Subsequently to the gentamicin C_{1a}~C_2 mixture, which was separated by silica gel chromatography, a small amount of a new component was eluted /compound VI-4/. It is very similar in all

respects /molecular formula, PMR and mass spectra, bioactivity/ to gentamicin C_2, except the optical rotation /$[\alpha]$+124°/. We suppose that this material may be identical to gentamicin C_{2a}.

The isolation of mixture VI for purpose of industrial production of gentamicin C sulfate:

Eluate fractions 199-260 /62 l/ were pooled and evaporated in vacuo at 85°C until 30 % concentration /dry weight/. To this concentrated solution charcoal was added at room temperature, the mixture was stirred for 30 min, then filtered. The carbon cake was washed with water. The pH of the stirred, cooled filtrates was adjusted to 4.5 with 5 N sulfuric acid. The solution was decolorized again by carbon, stirred for 30 min, filtered and washed thoroughly with water. The filtrates thus obtained were poured while stirring into 10 volumes of puriss methanol. After a few hours standing at 5°C, the gentamicin C sulfate complex was filtered and washed with methanol. It was subsequently dried in vacuo at 50°C until constant weight. The yield of gentamicin sulfate corresponds to about 468 g of practically pure, anhydrous product, assay 640 mcg/mg. Ash content: about 0.1 %, volatiles: 2.8 %. Rotation: $/\alpha/_D^{25}$ = +112° /1.0 % in water/. Components: C_1=34-36 %, C_2=45-49 %, C_{1a}=17-19 %. Over all yield calculated on gentamicin C of broth is 67.5%.

Mixture VII: This rather complicated mixture containing at least 12 components was chromatographed first on Amberlite XE-69 resin with 0.16, 0.20, 0.30, 0.40 and 0.60 N ammonium hydroxide, successively. The first few fractions contained compound VII-4 /gentamine C_2/ which was purified by crystallization from ethanol-water. The following fractions were divided into three parts. Each submixture was chromatographed on CM-Sephadex columns and the separated components were purified by ion-exclusion chromatography on QAE-Sephadex or Dowex 1 x 2 columns. From the first submixture /0.20 0.30 N/ compounds VII-5 and VII-6 /gentamine C_{1a}/ were isolated. Chromatography of the second /0.40 N/ and third /0.60 N/ submixtures resulted in compounds VII-2 /gentamine C_1/ and VII-3, and compound VII-1, respectively.

Paper chromatography was performed on Macherey-Nagel 214 paper/PC/. The solvent system for paper chromatography of free bases consisted of methyl ethyl katonetert-butyl alcohol-methanol-6.5N ammonium hydroxide 16:3:1:6/SystemA/. Thin layer chromatography/TLC/was performed on Silica gel HF plates using the lower phase of chloroform-methanol-25% ammonium hydroxide 12/19/20 system as eluent/System B/. Ion-exchange TLC was performed on precoated Fixion 50 x 8/Na^+/ plates at 50°C, using 0.5 M Na_2HPO_4 solution/adjusted to pH 6.5/containing 2.5 M sodium chloride and 5% tert-butyl alochol/System C/. All components were detected with ninhydrin spray. The bioautographic detection was made with Bacillus subtilis ATCC 6633 as a test organism.

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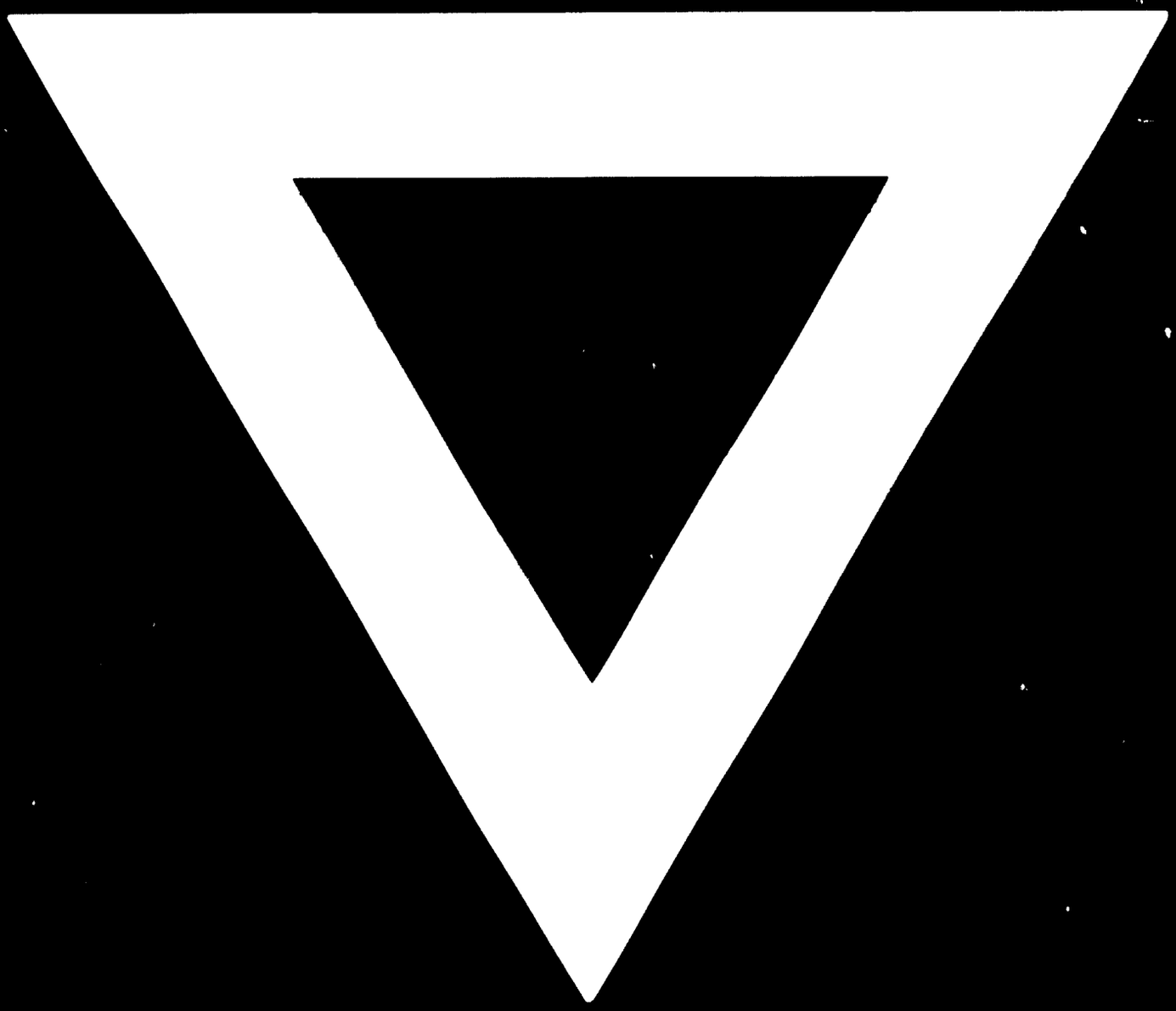

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