METABOLITES OF GENTAMICIN-PRODUCING *MICROMONOSPORA* SPECIES

I. ISOLATION AND IDENTIFICATION OF METABOLITES*

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From the cultural broth of a *Micromonospora* species 25 aminocyclitol antibiotics were isolated by repeated ion-exchange chromatographic processes. The main components of the metabolites were identified as gentamicin C_1, C_2, and C_{1a}. The other previously reported gentamicin type antibiotics and some other degradation products including gentamicin A, B, B_1, X_2, sisomicin, garamine, gentamines *etc.*, were identified by chemical, PMR, and mass spectroscopic studies. Besides these, seven new gentamicin type antibiotics were isolated and characterized.

Gentamicins and related antibiotics are basic, water-soluble substances of the aminoglycoside family produced by various *Micromonospora* species.[1] The main components of the gentamicin complex are gentamicin C_1, C_2, and C_{1a} used widely in clinical practice. Their structures were elucidated in the period 1969~1975[2~5]. Some other gentamicin related antibiotics, *e.g.* gentamicins A, A_1, A_2, A_3, A_4, B, B_1, C_{2a}, C_{2b}, and X_2, produced by gentamicin C-producing *Micromonospora purpurea* and *Micromonospora echinospora* species in a minor amount were also identified recently[6~11]. Some other antibiotics structurally related to gentamicins, *e.g.* antibiotics G-418[12], JI-20A, JI-20B[13], sisomicin[14], verdamicin[15], antibiotics G-52[16], 66-40B, and 66-40D[17] are produced by other *Micromonospora* species.

During the period 1972~1976 we isolated from the cultural broth of a new gentamicin C-producing *Micromonospora purpurea* var. *nigrescens* strain[18], numerous metabolites including the previously identified gentamicin antibiotics and other related metabolites together with several new substances. The separation of the metabolites was made by repeated ion-exchange chromatography. The identification of the separated components was based on chemical degradation, PMR spectral analysis, mass spectrometry and direct chemical and chromatographic comparisons.

In the present paper we describe the isolation and some properties of 25 metabolites and discuss briefly the identification of the components published previously in the literature**.

Materials and Methods

Before analysis all samples were dried for 24 hours over P_2O_5 and KOH at 80°C in N_2 atmosphere. Melting points, determined on a Kofler hot stage apparatus, are uncorrected. All optical rotation values were measured in 1% solutions of water on an OPTON LEP IIA polarimeter. N-Acetyl derivatives were obtained by suspending one part of the free base in a mixture of 5 parts of acetic anhydride and 50 parts of methanol. After 24 hours at room temperature the solvent was evaporated

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** The structures of the new metabolites were determined by combination of the chemical and spectroscopic methods. Details of these studies will be published in this Journal.

under reduced pressure and the N-acetates precipitated with diethyl ether. The permethylation of the N-acetyl derivatives for mass spectrometry was made by the modified HAKAMORI method[19].

Mass spectra were taken on a Varian MAT SM-1 instrument. Mass spectral data given in the text consist of the mass numbers of characteristic ions, the ion giving rise to the base peak is printed in italics. PMR spectra were recorded on Varian A-60 D and Jeol C-60 HL spectrometers, respectively. Chemical shifts are given in δ values for solutions in D_2O, using DSS as internal standard.

Paper chromatography was performed on Macherey-Nagel 214 paper (PC). The solvent system for paper chromatography of free bases consisted of methyl ethyl ketone - *tert*-butyl alcohol - methanol - 6.5 N ammonium hydroxide 16: 3: 1: 6 (System A). 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 50×8 (Na^+) plates[20] 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 alcohol (System C). All components were detected with ninhydrin spray. The bioautographic detection was made with *Bacillus subtilis* ATCC 6633 as a test organism.

Column chromatography:

All chromatographic fractions were analysed by TLC using system B and tested for biological activity in serial dilution method against *B. subtilis*. The fractions with similar composition were pooled and evaporated under reduced pressure at a temperature of 60°C to a syrup and finally dehydrated with a mixture of benzene - ethanol 1: 1.

(a) Ion-exchange chromatography:

Carboxyl- or sulphonyl-type ion-exchange resins (Amberlite CG-50 Type I or II, Amberlite XE-69, Dowex 50-×8) or dextran gels (CM-Sephadex C-25, SP-Sephadex C-25) were used for the separation of components. All ion-exchange materials were used in the ammonium form. The freshly regenerated resins were washed with ion-free water to pH 9. The aqueous solutions of antibiotic bases were charged to the columns after decolorization with activated carbon and washed with ion-free water. The elutions were made with 0.05~0.60 N ammonium hydroxide solutions by stepwise gradient elution technique. Flow rate was 50 ml/cm²/hour.

(b) Ion-exclusion chromatography:

The chromatography was carried out on the strongly basic ion-exchange resins (Dowex 1-X2, 200~400 mesh, Amberlite CG-400 Type II, Amberlite IRA-401S) or dextran gels (QAE-Sephadex A-25) in the hydroxyl form, using ion- and CO_2-free water as eluent. The free bases were charged to a column in 30~50% aqueous solutions. Flow rate was 20 ml/cm²/hour.

Methanolysis was carried out by refluxing the anhydrous bases with hydrogen chloride-saturated methanol. Acidic hydrolysis was made at 100°C with 6 N hydrochloric acid. Separation of the degradation products, if not stated otherwise, was made by ion-exchange chromatography on Amberlite CG-50 resin.

6-Amino-6-deoxy-D-glucose and 2-deoxystreptamine were isolated from acidic hydrolysate of kanamycin A. Paromamine and neamine were isolated by methanolysis of paromomycin and neomycin, respectively. Methyl garosaminide and gentamines were obtained by methanolysis of gentamicin C complex and gentamicin C components, respectively. Garamine was prepared from sisomicin by published method.[14]

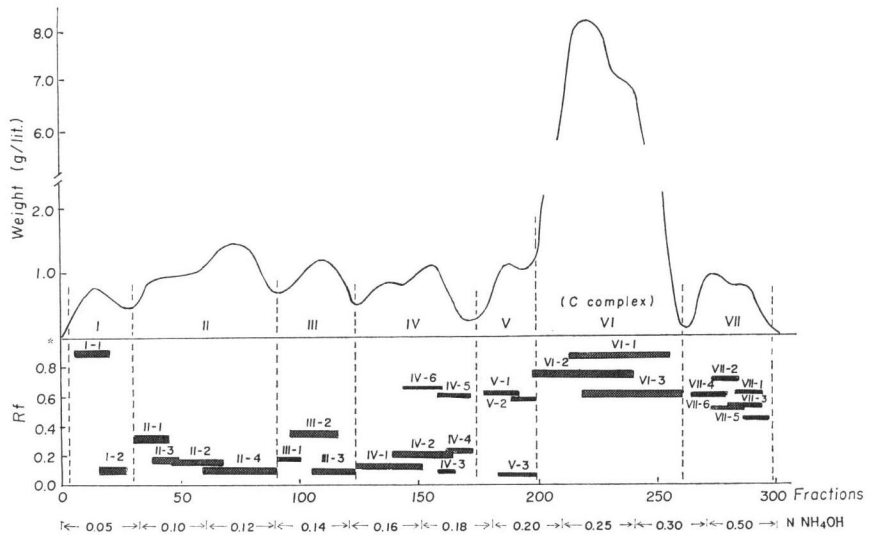
Results and Discussion

The extensive chromatographic analysis of the crude gentamicin obtained by ion-exchange process from the cultural filtrate of *Micromonospora purpurea* var. *nigrescens* MNG-122 strain[18] indicated the presence of about 50 basic, water-soluble components. The main components identified by chemical and spectroscopic methods and by direct comparisons as gentamicin C_1, C_2, and C_{1a}, represent 65~75% of all materials isolated. Some other components designated compounds I-1, II-1, II-2, II-4,

Table 1. 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



III-2, III-3, IV-1, IV-2, V-1, and VII-4 are present in 1~5% amount. Other minor components are present in an almost negligible qauntity.

During our investigation we isolated 35 homogeneous components of this mixture, out of which 14 were found identical with the previously described antibiotics of different *Micromonospora* species. Four further compounds proved to be identical with gentamine C_1, C_2, C_{1a}, and garamine, isolated formerly by chemical degradation of gentamicin C components and sisomicin. Furthermore, on investigating the remaining components we found 7 new compounds of gentamicin type. These components are characterized by some physical and chemical properties (Table 2). Their structures are depicted on Fig. 2. The details of their structural characterization will be published in the subsequent communications. The amounts of further substances isolated in pure form so far are insufficient for detailed chemical characterization.

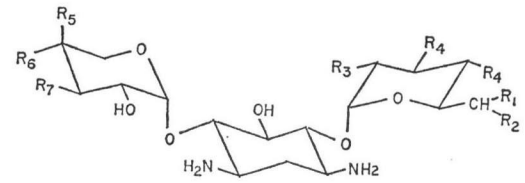
Isolation of Components

The crude gentamicin complex was chromatographed at first on Amberlite CG-50 Type-I resin developed with increasing concentration of aqueous ammonia[21]. The pattern of chromatography is shown in Fig. 1. The antibiotic complex was separated into seven mixtures indicated on Table 1. Chromatographic analysis by system A, B and C of the I ~ VII mixtures indicated their complex nature. There are more or less overlapings 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 resolution 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.

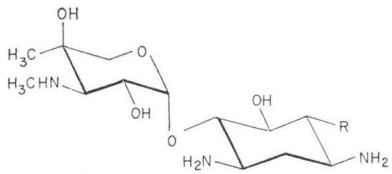
Mixture I: The relatively apolar compound I-1 was separated from compound I-2 (gentamicin A_2) on QAE-Sephadex column with water as eluent. Compound I-1 was eluted first and it was purified by repeated ion-exclusion chromatography of Dowex 1-X2 resin. Gentamicin A_2 was isolated from the later fractions 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.

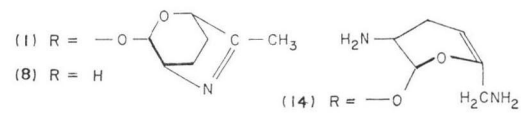
Mixture II: We separated the main components of this complicated mixture as follows: The first step was a repeated chromatography on Amberlite CG-50 Type-II resin with gradient elution by 0.08 ~ 0.12 N ammonium hydroxide. Compound II-1 (antibiotic G-418) was isolated from the first enriched fractions 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, succes-

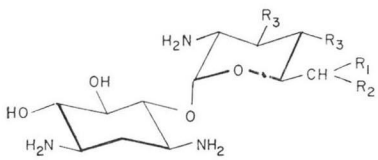
Fig. 2. Structures of compounds



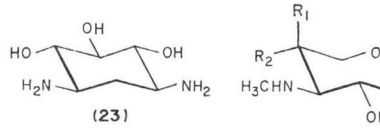
	R_1	R_2	R_3	R_4	R_5	R_6	R_7
2	H	OH	NH_2	OH	H	OH	OH
3	CH_3	OH	NH_2	OH	OH	CH_3	$NHCH_3$
4	CH_3	OH	NH_2	OH	H	OH	$NHCH_3$
5	H	OH	NH_2	OH	OH	CH_3	$NHCH_3$
6	H	OH	NH_2	OH	H	OH	$NHCH_3$
7	CH_3	OH	NH_2	OH	OH	H	$NHCH_3$
9	H	OH	NH_2	OH	OH	H	$NHCH_3$
10	H	NH_2	OH	OH	OH	CH_3	$NHCH_3$
11	CH_3	NH_2	OH	OH	OH	CH_3	$NHCH_3$
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	$NHCH_3$







	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



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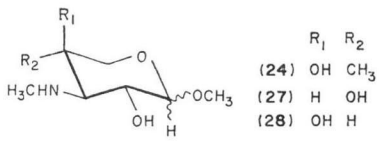

	R_1	R_2
(24)	OH	CH_3
(27)	H	OH
(28)	OH	H

Table 2. Physical and chemical properties of gentamicin minor components

Structure	Compound number	Identical	Formula	MW	$[\alpha]_D^{20}$ (1%, water)	PC	TLC	
						Rf rel. B_1 System A	Rf System B	Rf System C
1	I-1	New compound	$C_{20}H_{36}N_4O_7$	444	+144°	1.60	0.93	0.16
2	I-2	Gentamicin A_2	$C_{17}H_{33}N_3O_{11}$	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	$C_{13}H_{27}N_3O_6$	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	JI-20A	$C_{19}H_{39}N_5O_9$	481	—	0.39	0.14	0.76
13	IV-4	JI-20B	$C_{20}H_{41}N_5O_9$	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	$C_{18}H_{36}N_4O_{10}$	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	$C_{13}H_{28}N_4O_4$	304	+92°	1.12	0.59	0.27
21	VII-5	New compound	$C_{18}H_{37}N_5O_7$	435	+144°	0.71	0.48	0.20
22	VII-6	Gentamine C_{1a}	$C_{12}H_{26}N_4O_4$	290	+97°	0.82	0.42	0.28

sively. 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 chromatographed 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 was 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-Sephadex 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 $C_{1a} \sim C_2$ 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 with gentamicin C_{2a}[22].

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 1×2 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.

Properties and Identification of the Components

All compounds isolated are water-soluble, basic substances. The free bases are white hygroscopic powders. Some of them were crystallized as hydrate or ethanol solvate. The removal of water and solvent is very difficult and in certain cases the free bases easily absorbe the atmospheric CO_2, forming carbonates. For that very reasons it is difficult to obtain satisfactory and reproducible analytical data. Some properties of the components isolated (except gentamicin C components) are shown on Table 2. The structures of all isolated and well characterized minor components, including the new compounds, are illustrated on Fig. 2. (**1** ~ **22**).

By the usual procedure free bases yield per-N-acetyl derivatives giving more characteristic analytical values. Furthermore, the compounds were characterized by PMR and mass spectra of free bases, per-N-acetyl derivatives, and per-N-acetyl-per-N,O-methyl derivatives, respectively.* On strong acidic hydrolysis (6 N hydrochloric acid, 100°C, 4 hours) all compounds gave 2-deoxystreptamine (**23**) identified by paper chromatography and IR spectroscopy. On the acidic hydrolysis of the per-N-acetyl-per-N,O-methyl derivatives of **1** ~ **7**, **9** ~ **17**, **19**, and **21** gave N,N′-diacetyl-2-deoxy-

* Only selected data are given.

N,N′-5-O-trimethylstreptamine identified by microanalytical data, mass spectrum (*m/e*: 288), optical inactivity, and by direct comparison of authentic material prepared from kanamycin.

Hydrolysis of compound I-2 with 6 N hydrochloric acid for 1 hour, followed by analysis of the hydrolysate by paper chromatography, confirmed the presence of 2-glucosamine and xylose. Methanolysis (4 hours) of this compound afforded paromamine (**25**) and methyl D-xyloside identified by direct comparisons with the authentic materials. The PMR data of compound I-2 (δ 2.8, $H_{2'}$, $J_{2'\sim3'}=10$ Hz; 5.0, $H_{1''}$, $J_{1''\sim2''}=2.5$ Hz; 5.2, $H_{1'}$, $J_{1'\sim2'}=4$ Hz) were similar to the data published for gentamicin A_2.[8] The PMR spectra (δ 1.98, 3H, s; 2.02, 3H, s; 2.05, 3H, s) and analytical data of tri-N-acetate as well as the mass spectra (M=721; *m/e* 530, *445*, 260, 228) of the per-N-acetyl-per-methyl derivative also confirm the identity of compound I-2 with gentamicin A_2 (**2**).

Methanolysis (2 hours) of compounds II-1 and II-3 produced methyl garosaminide (anomeric mixture) (**24**) which was identified by chromatographic and mass spectroscopic methods (M=191; *m/e* 160, 159, 118, 115, 100, 74, *73*) with the authentic material isolated from methanolysate of gentamicin C[23]. The investigation of its crystalline N-acetyl derivative (β-anomer: mp 185~190°C, $[\alpha]_D^{20}$ +210°) confirmed this assignment[24]. The other products of methanolysis of compounds II-1 and II-3 were 6′-methylparomamine (**26**) and paromamine (**25**), respectively; **25** was identified by direct comparison with authentic material. The structure of **26** was established by its elemental composition ($C_{13}H_{27}N_3O_7$), hydrolytic cleavage (2-deoxystreptamine, 6-methyl-2-glucosamine), PMR (δ 1.20, 3H, d; 5.28, 1H, d), and mass spectrum (*m/e*: 233, 232, 217, 203, *191*, 176, 173, 163, 145, 72, 71, 59). The PMR spectra of compounds II-1 (δ 1.20, 3H, s; 1.21, 3H, d; 2.52, 3H, s; 5.14, 1H, d; 5.28, 1H, d) and II-3 (δ 1.22, 3H, s; 2.54, 3H, s; 3.80, 2H, brs; 5.15, 1H, d; 5.30, 1H, d) as well as the mass spectra of the per-N-acetyl-per-methyl derivative of compounds II-1 (M=790; *m/e*: 699, 602, 544, *500*, 274, 230, 142, 129) and II-3 (M=776; *m/e*: 699, 602, 530, *500*, 260, 230, 129), confirmed the identity of the above mentioned materials with antibiotic G-418 (**3**) and gentamicin X_2 (**5**), respectively.

Compounds II-4, III-3, and V-2 are isomeric substances (Table 2). Based upon the analytical data and mass spectra the empirical formula of $C_{18}H_{36}N_4O_{10}$ was calculated. The methanolysis (36 hours) of both compounds II-4 and III-3 afforded paromamine (**25**) and an anomeric mixture of methyl pentosides. These methyl pentosides were isomeric (empirical formula: $C_7H_{15}NO_4$). The methyl glycoside from compound II-4 was identified by its physical (β-anomer: mp 138°C, $[\alpha]_D^{20}$ −57°), chemical (oxidation with periodate), PMR (δ 2.46, NCH_3, s; 2.75, H_3, dd, $J_{2\sim3}=10$ Hz, $J_{3\sim4}=8.5$ Hz; 3.4, H_4, dt, $J_{4\sim5_e}=5$ Hz, $J_{4\sim5_a}=10$ Hz, $J_{3\sim4}=8$ Hz; 3.45, OCH_3, s; 4.72, H_1, d, $J_{1\sim2}=3.5$ Hz), and mass spectral properties (M=177; *m/e*: 146, 145, 115, *104*, 100, 86, 74, 73, 72) with methyl gentosaminide (**27**)[25]. The investigation of its crystalline N-acetyl derivative (β-anomer: mp 180°C, $[\alpha]_D^{20}$ −60°) confirmed the identity also. The methyl glycoside from compound III-3 proved to be identical on the basis of its optical rotation value ($[\alpha]_D^{20}$ +152°), PMR (δ 2.34, NCH_3, s; 2.80, H_3, dd, $J_{2\sim3}=10$ Hz, $J_{3\sim4}=3$ Hz; 3.42, OCH_3, s; 4.1, H_4, brs; 4.80, H_1, d, $J_{1\sim2}=4$ Hz), and mass spectral properties (M=177; *m/e*: 146, 145, 115, *104*, 86, 74, 73) with 4-*epi*-methyl gentosaminide (**28**)[17]. **28** was also isolated from the methanolysate (3 hours) of compound V-3, besides of 2-deoxystreptamine (**23**) and methyl 6-amino-6-deoxy-α-D-glucopyranoside[26]. Hydrolysis of compound V-3 with 6 N hydrochloric acid (2 hours) also indicated the presence of 6-amino-6-deoxy-D-glucose in the hydrolysate in contrast with compounds II-4 and III-3, which gave 2-amino-2-deoxy-D-glucose (2-glucosamine) on the acidic hydrolysis. On the basis of the above findings it seems apparent that compounds II-4, III-3, and V-3 are

identical with gentamicin A (**6**), gentamicin A_1 (**9**), and gentamicin A_3 (**16**), respectively. Detailed PMR and mass spectroscopic investigations led to the same conclusion. The PMR spectra of compounds II-4 (δ 2.51, 3H, s; 5.06, 1H, d; 5.30, 1H, d), III-3 (δ 2.40, 3H, s; 4.12, brs; 5.10, 1H, d; 5.26, 1H, d), and V-2 (δ 2.42, 3H, s; 5.15, 1H, d; 5.32, 1H, d), moreover the mass spectra of per-N-acetyl-per-methyl derivatives of compounds II-4 (M=762; *m/e*: 685, 588, 530, 486, 260, 232, 228, *216*, 142, 129), III-3 (M=762; *m/e*: 685, 588, *530*, 486, 260, 232, 228, 216, 187, 142, 129), and V-2 (M=762; *m/e*: 620, 530, 486, 260, 232, 228, *216*, 187, 142) correlated well with the formerly determined structures of these compounds[7].

The methanolysis (4 hours) of compounds IV-1, IV-2, IV-3, and IV-4 alike yielded methyl garosaminide (**24**). From the methanolysate of compound IV-1, methyl 6-amino-6-deoxy-α-D-glucopyranoside hydrochloride ($[\alpha]_D^{20}$ +130°) was isolated. Compound IV-2 gave a similar methyl glycoside which was identified by analytical ($C_8H_{17}NO_5$), and physical ($[\alpha]_D^{20}$ +82°) data, PMR (δ 1.24, 3H, d; 1.75, 1H, m; 4.85, 1H, d), and mass spectra as methyl 6-amino-6-deoxy-6-methyl-α-D-glucopyranoside. Acidic hydrolysis (1 hour) of compound IV-1 afforded 6-amino-6-deoxy-D-glucose, 2-deoxystreptamine (**23**), and garosamine (identified by chromatography) as main products, and O-α-D-6-amino-6-deoxy-glucopyranosyl-2-deoxystreptamine (6**AD**), garamine (**8**), and 5-aminomethylfurfural (degradation product of 6-aminoglucose) as minor products. 6**AD** was identified by PMR and mass spectrum (*m/e*: 306, 287, 232, 204, 203, 191, 162, 145, 130, *71*), and by direct comparison with authentic material isolated from the partial hydrolysate of kanamycin A. The hydrolytic products from compound IV-2 were the same except that instead of 6-amino-6-deoxy-D-glucose (and its derivatives) their 6-methyl homologues were present. The final proof of identity of compounds IV-1 and IV-2 with gentamicin B (**10**), and gentamicin B_1 (**11**), respectively was provided by spectroscopic studies. The PMR spectra of compounds IV-1 (δ 1.26, 3H, s; 2.55, 3H, s; 5.12, 1H, d; 5.36, 1H, d), and IV-2 (δ 1.14, 3H, d; 1.24, 3H, s; 2.52, 3H, s; 5.10, 1H, d; 5.36, 1H, d) together with the mass spectra of per-N-acetyl-per-methyl derivatives of compounds IV-1 (M=776; *m/e*: 689, 634, 530, 260, *230*, 228, 198, 142), and IV-2 (M=790; *m/e*: 703, 634, 544, *274*, 242, 230, 198) gave more evidence of these identities.

The methanolysis of mixture of compounds IV-3 and IV-4 (2 hours) afforded methyl garosaminide (**24**), neamine (**29**), and 6′-methylneamine (**30**), which were separated on ion-exchange chromatography. These products were identified by direct comparison with authentic compounds and by PMR (**30**: δ 1.24, 3H, d; 5.35, 1H, d), and mass spectral studies (**30**: M=336; *m/e*: 319, 301, 231, 217, 203, 193, 191, 175, 163, *145*, 71, 59; **29**: M=322; *m/e*: 305, 287, 217, 203, 191, 161, *145*, 72, 71, 59). On the basis of the above findings and of the PMR spectra of free bases (IV-4: δ 1.24, 3H, s; 1.24, 3H, d; 2.50, 3H, s; 5.08, 1H, d; 5.34, 1H, d), and their N-pentaacetyl derivatives, as well as the mass spectra of their per-N-acetyl-per-methyl derivatives (IV-4: M=831; *m/e*: 731, 675, 585, *500*, 315, 283, 230, 129) compounds IV-3 and IV-4 were found to be identical with antibiotics JI-20A (**12**) and JI-20B (**13**), respectively.

Compounds III-2 (garamine) (**8**), V-1 (sisomicin) (**14**), VI-1 (gentamicin C_1), VI-2 (gentamicin C_2), VI-3 (gentamicin C_{1a}), VII-2 (gentamine C_1) (**18**), VII-4 (gentamine C_2) (**20**), and VII-6 (gentamine C_{1a}) (**22**) were identified by physical, chemical and spectroscopic investigations and by direct chromatographic comparison with authentic materials.

The structural determination of new compounds was carried out by similar methods. Compound V-2 (3″-N-demethylgentamicin C_2) was prepared by chemical demethylation from gentamicin

C_2. The unique structure of compound I-1 was determined by detailed mass spectrometric and ^{13}C-NMR studies. The methanolysis of compounds III-1 (6′-methylgentamicin A_1), VII-1 (4″-demethylgentamicin C_1), VII-3 (4″-demethylgentamicin C_2), and VII-5 (4″-demethylgentamicin C_{1a}) gave methyl 4-*epi*-gentosaminide, while compound II-2 (6′-methylgentamicin A) yielded methyl gentosaminide. The pseudodisaccharide type products of methanolysis were 6′-methylparomamine (II-2, III-1), gentamine C_2 (V-2, VII-3), gentamine C_1 (VII-1), and gentamine C_{1a} (VII-5). The details of these studies will be reported in the near future in this Journal.

All compounds isolated show antimicrobial activity against Gram-positive, Gram-negative, and *Mycobacteria*. Gentamicin B showed striking activity against *Mycobacteria* in both *in vitro* and *in vivo* experiments. The details of biological investigations will be published in separate communications.

The biosynthesis of gentamicin and sisomicin has been investigated by several authors[27,28]. It is of interest to consider how the above reported numerous compounds fit into the possible biosynthetic schemes and, in particular, the significance of sisomicin and some new compounds (I-1, V-2) in the biosynthesis of gentamicins. These problems require further investigations.

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