COMBIMICINS, NEW KANAMYCIN DERIVATIVES BIOCONVERTED BY SOME *MICROMONOSPORA*S

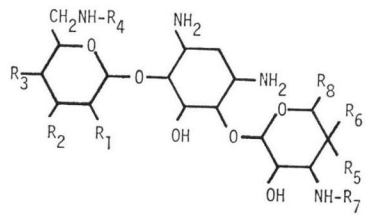
Sir:

Recently various new aminoglycoside derivatives were obtained by a technique called "mutational biosynthesis" or "mutasynthesis".[1~3] However, most of them were derivatives which could be produced by the action of deoxystreptamine-negative mutants of aminoglycoside producing strains (idiotrophs) upon addition of some synthetic analogs of deoxystreptamine to the medium. That is, they were aminoglycosides in which the deoxystreptamine moiety was replaced with those synthetic analogs.[4~7,1,8~13] By noting the fact that most of gentamicins are 3′,4′-dideox compounds, we attempted to utilize gentamicin producing organisms or their mutants for the conversion of kanamycins to 3′,4′-dideoxykanamycins. To our surprise, we obtained a new series of compounds which are characterized by 4″-C-methylation as well as 3′,4′-dideoxygenation in the kanamycin molecule.

The new series of compounds, combimicins (named after their hybridized structure of kanamycins and gentamicins), were obtained by growing a new gentamicin producer, *Micromonospora* sp. K-6993 which was isolated by us from a soil sample, or its gentamicin non-producing mutant Y-41 (registered as ATCC 31348 and 31349 respectively) in the presence of kanamycins. From kanamycin B were obtained combimicin B_1 (I) and combimicin B_2 (II), and from kanamycin A was derived combimicin A_2 (III).

In a representative run, the strain Y-41 was shake-cultured in a medium containing 3.5% soybean meal, 5.0% dextrin, 0.7% calcium carbonate and 0.000025% cobaltous chloride (pH 7.0) at 29°C for 120 hours, to which kanamycin B was added at a concentration of 300 μg/ml 24 hours after the start of fermentation. Antibiotics in the broth filtrate were isolated by adsorption at pH 7.0 on Amberlite IRC 50 resin (NH_4^+ form) followed by elution with 1 N ammonium hydroxide solution. Active fractions thus obtained were chromatographed over Amberlite CG 50 (NH_4^+ form) using a gradient elution with 0~0.7 N ammonium hydroxide. Monitored by a silica gel thin-layer chromatography developed with chloroform - methanol - 28% ammonium hydroxide (20: 15: 8) and bioautographed against *Escherichia coli* K-12, fractions giving an Rf of 0.36 were collected and further purified by a silica gel column chromatography eluted with chloroform - methanol - 28% ammonium hydroxide (20: 15: 8), passed through Dowex 1×2 column (OH^- form) to remove impurity, and again subjected to the above Amberlite CG 50 gradient column chromatography in succession. Active fractions thus obtained were lyophilized to give a pure powder of combimicin B_2 (II). When frac-

Chart 1. Structures of combimicins.



	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
Kanamycin A	OH	OH	OH	H	OH	H	H	CH_2OH
Kanamycin B	NH_2	OH	OH	H	OH	H	H	CH_2OH
Gentamicin C_{1a}	NH_2	H	H	H	CH_3	OH	CH_3	H
Combimicin B_2 (II)	NH_2	H	H	H	CH_3	OH	CH_3	CH_2OH
Combimicin B_1 (I)	NH_2	H	H	CH_3	CH_3	OH	CH_3	CH_2OH
Combimicin A_2 (III)	OH	H	H	H	CH_3	OH	CH_3	CH_2OH

Table 1. Physicochemical properties of combimicins.

Combimicin	B_2	B_1	A_2
Appearance	white powder	white powder	white powder
Melting point (°C)	143~146	108~111	129~131
$[\alpha]_D^{25}$ (*c* 1, H_2O)	+142°	+128°	+147.5°
Elemental analysis (%)	Found Calcd.	Found Calcd.	Found Calcd.
C	48.06 48.28	49.51 49.30	48.59 48.18
H	8.96 8.71	9.31 8.87	8.80 8.49
N	14.11 14.07	13.52 13.69	11.15 11.24
Molecular formula	$C_{20}H_{41}N_5O_8 \cdot H_2O$	$C_{21}H_{43}N_5O_8 \cdot H_2O$	$C_{20}H_{40}N_4O_9 \cdot H_2O$
Molecular weight (M^+)	479	493	480
^1H NMR (D_2O, δ)	(with 26% ND_4OD)	(with 26% ND_4OD)	
4″-C-methyl	1.28 (3H, s)	1.30 (3H, s)	1.29 (3H, s)
6′-N-methyl	—	2.60 (3H, s)	—
3″-N-methyl	2.55 (3H, s)	2.70 (3H, s)	2.56 (3H, s)
1″-anomeric proton	5.06 (1H, d, J=4.2)	5.06 (1H, d, J=4.2)	5.12 (1H, d, J=4.2)
1′-anomeric proton	5.10 (1H, d, J=3.4)	5.10 (1H, d, J=3.2)	5.27 (1H, d, J=3.3)
Rf on silica gel TLC			
Solvent I*	0.36	0.43	0.39
Solvent II*	0.05	0.09	0.06

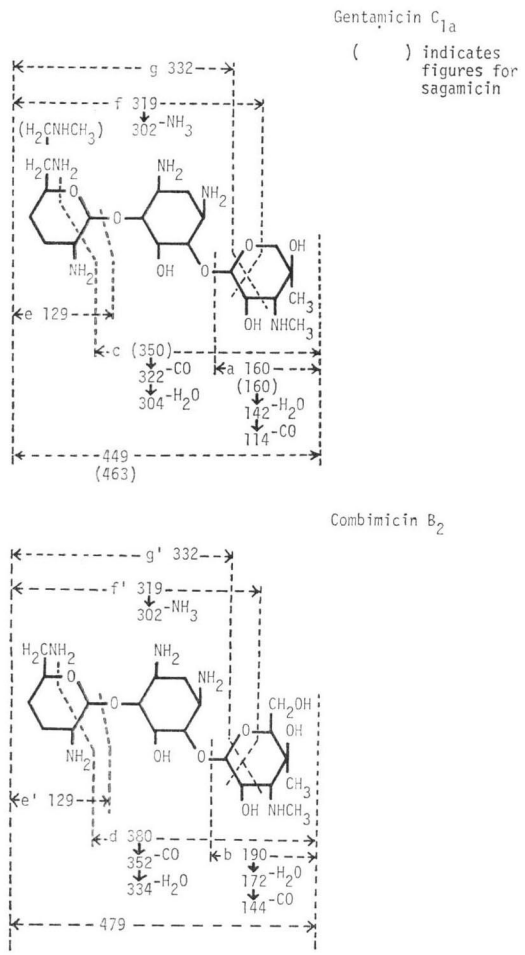
* Solvent I: chloroform - methanol - 28% ammonium hydroxide (20 : 15 : 8);
Solvent II: chloroform - methanol - 28% ammonium hydroxide (2 : 1 : 1, lower phase).
Rf's for kanamycin A, kanamycin B and gentamicin C_{1a} were 0.04, 0.05 and 0.42 with solvent I; 0.00, 0.00 and 0.08 with solvent II respectively.

tions giving an Rf of 0.43 were processed, combimicin B_1 (**I**) was obtained as a pure powder. Upon adding kanamycin A into the broth instead of kanamycin B and by processing the fractions giving an Rf of 0.39 on the same system, combimicin A_2 (**III**) was purified as a pure substance.

Combimicin B_2 (**II**) was obtained as a colorless amorphous powder melting at 143~146°C. It showed an $[\alpha]_D^{25}$ of +142° (*c* 1, H_2O). Its molecular weight was determined to be 479 by mass spectrometry. Elemental analysis was: Found, C 48.06%, H 8.96%, N 14.11%, Calculated for $C_{20}H_{41}N_5O_8 \cdot H_2O$, C 48.28%, H 8.71%, N 14.07%. Physicochemical properties of combimicin B_1 (**I**) and combimicin A_2 (**III**) are given in Table 1 along with the above-described data of combimicin B_2. Their ^1H NMR chemical shifts and Rf's on silica gel TLC with two solvent systems are also presented in the same table.

The structure of combimicin B_2 was established by comparison of the mass and nuclear magnetic resonance spectra of combimicin B_2 (**II**), gentamicin C_{1a} and sagamicin which had been shown to be the 6′-N-methyl derivative of gentamicin C_{1a}.[15] The mass spectrum of combimicin B_2 closely resembled those reported on gentamicin C_{1a} and sagamicin[14,15]. Combimicin B_2 gave a molecular ion at *m/z* 479, which suggested a molecular formula of $C_{20}H_{41}N_5O_8$ in satisfactory agreement with the elemental analysis. Gentamicin C_{1a} was known to give a molecular ion at *m/z* 449 ($C_{19}H_{39}N_5O_7$). Thus combimicin B_2 was shown to give a 30 mass units larger molecular ion which corresponded to the presence of an additional CH_2O group. Accordingly, gentamicin C_{1a} was reported to have a fragment at *m/z* 160 and sagamicin to give fragments at *m/z* 160, 142 and 114 resulting from cleavage of the garosamine moiety (Chart 2. a), while combimicin B_2 gave fragments at *m/z* 190, 172 and 144 (Chart 2. b) which were 30 mass-units larger than those corresponding peaks of gentamicin C_{1a} and sagamicin. Further, sagamicin gave fragments at *m/z* 350, 322 and 304 (Chart 2. c) corresponding to the pseudodisaccharide consisting of 2-deoxystreptamine and garosamine, while combimicin B_2 gave fragments

Chart 2. Mass spectral fragmentation of combimicin B_2 and gentamicin C_{1a}.



at *m/z* 380, 352 and 334 (Chart 2. d), which were again 30 mass-units larger. By using kanamycin B as material, these results suggested that combimicin B_2 was an analog of gentamicin C_{1a} differing only in the presence of an additional CH_2O group in the garosamine moiety as shown in Chart 1 (**II**). This structure was consistent with the fact that gentamicin C_{1a} and combimicin B_2 gave identical fragments at *m/z* 129 (Chart 2. e, e') corresponding to the purpurosamine moiety and at *m/z* 319, 302 (Chart 2. f, f') and at 332 (Chart 2. g, g') corresponding to the pseudodisaccharide comprising purpurosamine and 2-deoxystreptamine.

Additional evidence for the location of C-methylation was obtained by comparison of the ^{13}C-NMR spectra of combimicin B_2 (**II**) and gentamicin C_{1a}. As seen from Table 2, carbon resonances of both compounds are virtually unchanged from each other except for the chemical shifts at C-5'' and C-4'' and the appearance of a new signal at 61.2 ppm in combimicin B_2 which gave rise to a triplet in an off-resonance study, and suggested the presence of the expected C-6'' O-substituted methylene. Accordingly, the C-5'' signal of combimicin B_2 changed to a doublet and showed a downfield shift of 7.4 ppm, and suggested the occurrence of a substitution at this atom. The C-4'' signal also showed a 1.5 ppm downfield shift. Thus the structure given in Chart 1 (**II**) was established for combimicin B_2, although configuration at C-4'' remains to be elucidated and is now under study. ^{1}H-NMR results were also consistent with this structure. Further, by similar mass spectral and ^{1}H-NMR studies, structures given in Chart 1 (**I**) and (**III**) were assigned to combimicins B_1 and A_2 respectively.

Combimicins have strong antibacterial activities against Gram-positive and negative organisms. Results are presented in Table 3. Combimicin B_2 showed less ototoxicity than the

Table 2. ^{13}C Chemical shifts of combimicin B_2 and gentamicin C_{1a} (ppm, 26% ND_4OD in D_2O).

	GM-C_{1a}	CBM-B_2		GM-C_{1a}	CBM-B_2		GM-C_{1a}	CBM-B_2
C-1	52.1	51.4	C-1′	102.8	102.9	C-1″	101.9	101.3
C-2	37.2	37.2	C-2′	51.3	51.4	C-2″	70.5	70.6
C-3	51.0	50.9	C-3′	27.6	27.4	C-3″	64.8	65.4
C-4	89.1	90.0	C-4′	28.9	28.9	C-4″	73.4	74.9
C-5	75.6	76.0	C-5′	71.8	71.8	C-5″	69.1	76.5
C-6	88.1	88.7	C-6′	46.6	46.6	C-6″		61.2
						3″-N-CH_3	38.4	38.6
						4″-C-CH_3	23.4	22.5

GM: gentamicin, CBM: combimicin.

Table 3. Antibacterial activities of combimicins.

	Combimicin			Kanamycin	
	B_2	B_1	A_2	A	B
Staphylococcus aureus ATCC 6538P	0.19	0.19	0.78	0.78	0.39
Staphylococcus aureus Smith	0.19	0.39	3.13	3.13	0.78
Staphylococcus aureus Ohnuma	0.19	0.19	3.13	1.56	0.78
Staphylococcus aureus (PC, SM, TC, KM, Mac-resistant)	0.19	0.19	6.25	>100	50
Salmonella enteritidis 1891	0.39	0.78	1.56	1.56	1.56
Proteus vulgaris OXK US	0.78	0.78	3.13	6.25	3.13
Escherichia coli NIHJ	0.78	0.78	1.56	3.13	1.56
Escherichia coli K-12 ML 1629	1.56	1.56	3.13	>100	>100
Escherichia coli K-12 R-5	25	1.56	>100	>100	100
Pseudomonas aeruginosa ATCC 8689	0.78	3.13	6.25	>100	100
Pseudomonas aeruginosa 99	3.13	3.13	12.5	100	50
Pseudomonas aeruginosa GN-315	100	1.56	>100	>100	100

Medium: Heart infusion agar. PC: Penicillin. SM: Streptmycin. TC: Tetracycline. KM: Kanamycin. Mac: Macrolide antibiotics.

gentamicin complex. After 4 weeks' intramuscular administration of 100 mg/kg per day, the gentamicin complex gave rise to the disappearance of the pinna reflex for the whole range of frequencies tested in 4 of 7 guinea pigs, while combimicin B_2 showed disappearance of the reflex only in a range higher than 10 kHz in one of 4 animals. Details will be reported elsewhere.

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