NOTE

PREPARATION AND SOME MICROBIOLOGICAL PROPERTIES OF NOVEL KANAMYCIN-GLUCOSIDE DERIVATIVES

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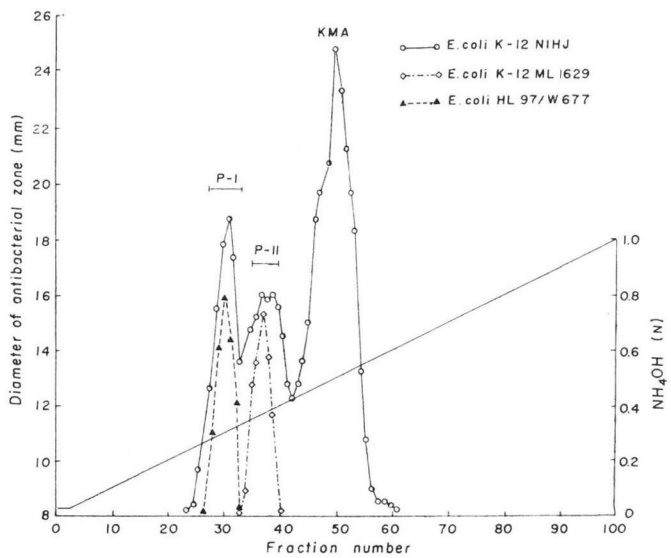
Some years ago, glycosyl derivatives of kanamycin A (KMA) were obtained enzymatically by ENDO *et al.*[9] and chemically by PERLMAN *et al.*[10]. Although their chemical and biological properties were partially described, their precise chemical properties and antibacterial activities against drug-resistant bacteria producing specific aminoglucosides-inactivating enzymes were not mentioned.

In this study, we have prepared new reduced types of KMA-glucosides and their chemical and microbiological properties have been investigated. At first, we tried to prepare the enzymatically-derived compounds by the method of ENDO *et al.*[9]. Utilizing the identical method, we prepared the glucosyl derivative of KMA by transglucosylase activity of Clarase®. We detected a glucosyl derivative possessing the same properties as described by them. However, this compound could not be regarded as the O-glucoside, reported in the case of neamine[7], because analysis in high-voltage paper electrophoresis (pH 1.8, 220 volts/cm, about 40 mA/cm, for 30 minutes, with Model HV 5000-3 of Savant Instruments, Inc.) and in silica gel thin-layer chromatography (using $CHCl_3$-MeOH-NH_4OH, 1:3:2), showed unchanged mobility and failed to detect reducing sugar after hydrolysis of this derivative with purified α-glucosidase from *Aspergillus niger* or *Rhizopus delemar*. An additional reason against the existence of an O-glucoside is that boiling the derivative in phenylhydrazine hydrochloride solution has liberated free KMA with the precipitation of phenylglucosazone. All our attempts to synthesize the O-glucosides of KMA using maltose as a glucosyl donor have been unsuccessful by enzymes such as purified α-glucosidase from *Aspergillus niger* or *Aspergillus oryzae* or *Rhizopus delemar* which are found to associate with partial transglucosylase activity and by crude cyclodextrin glycosyltransferase produced by *Bacillus macerans*.

We have tried to prepare different type of glucosides of KMA by the following method: 1.0 g KMA (free base) and 4.0 g glucose were dissolved in 10 ml of 0.02 N NH_4OH and after gradual addition of 1.0 g $NaBH_4$ with stirring, the reaction mixture was permitted to stand for 5 hours at 45°C. In order to degrade excess

Fig. 1. Chromatography of $NaBH_4$-treated glucosyl kanamycin A on an Amberlite CG-50 column (2.0×18 cm).

The material prepared by the method described in the text was applied to CG-50 column equilibrated with 0.02 N NH_4OH. Elution was carried out with 1,000 ml of linear gradient of 0.02~1.0 N NH_4OH. Antibacterial activity (Diameter of inhibitory zone, mm) was determined bioautographically by paper-disk method.



$NaBH_4$, the pH of the solution was adjusted to pH 2.0 with HCl and after standing for 15 minutes at room temperature, 300 ml of MeOH was added slowly to the solution. The resulting precipitate was separated from the solution by filtration and dissolved again in 50 ml of distilled water. After adjustment of the pH to 8 with NaOH, the solution was then passed through Amberlite CG-50 resin (NH_4^+ cycle, 2.0 × 18 cm) and eluted with 1,000 ml of gradient of 0.02~1.0 N NH_4OH. By bioautography using paper-discs, we observed mainly three fractions possessing antibacterial activity against *Escherichia coli* K-12 NIHJ as shown in Fig. 1. Using kanamycin-resistant strain, *E. coli* HL97/W677 as test organism inactivating KMA by 6′-N-acetylation, only fraction P-I possessed antibacterial components. In a similar manner, only fraction P-II was active against *E. coli* K-12 ML1629 that inactivates KMA by 3′-O-phosphorylation[5,6]. The peak possessing the highest activity against *E. coli* K-12 NIHJ was identified as unreacted KMA by high-voltage paper electrophoresis and thin-layer chromatography. The respective fractions of P-I and P-II were pooled and after the lyophilization, the crude materials were dissolved in distilled water and purified by rechromatography on Amberlite CG-50 (NH_4^+ cycle, 1.2 × 20 cm) using the gradient of 0~0.5 N NH_4OH. After lyophilization of active fractions, purified P-I (~128 mg) and P-II (~109 mg) were obtained.

Chemical properties were compared with KMA (free base) by thin-layer chromatography using Yamato-Replate 50[R] with $CHCl_3$ - MeOH - NH_4OH (1 : 3 : 2) as developing solvent. By spraying with ninhydrin reagent, Rf values of P-I, P-II and KMA were determined as 0.67, 0.61 and 0.75, respectively. By boiling treatment with phenylhydrazine hydrochloride, neither P-I nor P-II formed a glucosazone precipitate which is associated with the liberation of KMA. This result was in contrast to the phenomenon observed in the glucosyl derivatives obtained by the method described by previous authors[9,10].

Depending on the method reported by OGAWA *et al.*[1~4], P-I and P-II were hydrolyzed by refluxing their MeOH solution saturated with HCl gas for 10 hours and after the 40-fold concentration, the products were analyzed by paper-chromatography (developing solvent; *n*-BtOH - AcOH - H_2O, 4 : 1 : 2.5). After spraying with ninhydrin reagent, Rf values of the resulting

Table 1. *In vitro* antibacterial activity of P-I and P-II compared with KMA, DKB and GMCx.

Test organism	Resistance mechanism	Minimal inhibitory concentration (MIC)* (μg/ml)				
		P-I	P-II	KMA	DKB	GMCx
Escherichia coli HL97/W677	6′-N-acetyl	25	>200	200	100	3.12
〃 R5/W677	〃	25	>200	200	12.5	3.12
〃 JR88	3-N-acetyl	12.5	12.5	12.5	12.5	>200
〃 LA290/R55	2″-O-adenylyl	50	6.25	50	100	50
〃 K-12 ML 1629	3′-O-phosphoryl	>200	25	>200	3.15	3.15
〃 K-12 NIHJ	sensitive	6.25	6.25	<0.8	0.8	0.8
〃 10536	〃	6.25	6.25	<0.8	<0.8	0.8
Pseudomonas aeruginosa GN315	6′-N-acetyl	>200	>200	>200	>200	12.5
〃 130	3-N-acetyl	>200	50	>200	12.5	>200
〃 3796	〃	>200	>200	>200	>200	>200
〃 39	〃	>200	50	200	12.5	3.12
Proteus inconstans 164	2′-N-acetyl	25	50	12.5	>200	50
Staphylococcus aureus FDA 209P	sensitive	6.25	6.25	<0.8	1.56	<0.8
Bacillus subtilis ATCC 6633	〃	6.25	3.12	<0.8	<0.8	<0.8
Bacillus mycoides	〃	12.5	25	3.12	0.8	0.8

* One loopful of 10^8 cells/ml was inoculated. MICs were determined by a standard serial dilution agar method. See the text in detail.

spots were compared with those derived from KMA. In the case of P-I, a new ninhydrin positive and periodate-sensitive spot having Rf 0.18 was detected with simultaneous loss of the spot (Rf 0.39) originating from 6-amino-6-deoxyglucose in KMA. On the other hand, from P-II, a new spot having Rf 0.15 was demonstrated along with the disappearance of the spot (Rf 0.26) derived from 2-deoxystreptamine. This new spot was also sensitized with ninhydrin and periodate treatments.

Analysis of P-I and P-II gave C 43.76%, N 8.54% and C 45.63%, N 8.37% respectively. The C/N ratio of 5.12 and 5.42 were close to the C/N ratio of 5.14 expected for the case of glucose to KMA, 1: 1.

These results suggested that P-I were modified by one mole of glucose at the moiety of 6-amino-6-deoxyglucose and P-II was modified at the 1- or 3-NH_2 group of 2-deoxystreptamine in KMA respectively. Postulation might be permissible that P-I is polyhydroxy-alkylated derivative of KMA modified at 6-NH_2 group of 6-amino-6-deoxyglucose and P-II was modified one at either the 1- or 3-NH_2 group of 2-deoxystreptamine.

Antibacterial activity of P-I and P-II was compared with KMA, dibekacin (DKB) and gentamicin C complex (GMCx) against some aminoglycosides-resistant strains known to produce specific inactivating enzyme. Minimal inhibitory concentration (MIC) was determined after incubation for 18 hours by standard serial two-fold dilution method using Heart infusion agar (Eiken Chemical Co., Ltd.) as follows. One loopful of 10^8 cells/ml grown in Tryptosoya broth (Nissui Pharmaceutical Co., Ltd.) was inoculated onto agar-plates. As presented in Table 1, compared with KMA, P-I had an improved potency against *E. coli* HL97/W677 and *E. coli* R5/W677 both possessing 6′-N-acetyltransferase but had lower activity against almost all the sensitive strains. P-II had considerably low level of MIC (6.25 μg/ml) against *E. coli* LA290/R55 producing 2″-O-adenylyltransferase responsible for KMA-, DKB- and GMCx-resistance. P-II was more effective than KMA and P-I against *E. coli* K-12 ML1629 encoding 3′-O-phosphoryl transferase and *Ps. aeruginosa* 130 and *Ps. aeruginosa* 39 both producing 3-N-acetyltransferase but the activity of P-II was lower than DKB. Against another sensitive strains, P-II had also lesser inhibitory effects than KMA as well as DKB or GMCx.

Antibacterial spectra of P-I and P-II also indicate that the former was modified at 6′-NH_2 group of 6-deoxy-6-amino-glucose moiety and the latter was probably at 1-NH_2 group of 2-deoxystreptamine moiety like amikacin (BB-K8)[8]. The identification of the absolute structure of these has been now undertaken.

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