Communications to the editor

BIOCONVERSION OF RIBOSTAMYCIN (SF-733)

I. ISOLATION AND STRUCTURE OF 3 (OR 1)-N-CARBOXYMETHYLRIBOSTAMYCIN

Sir:

Microbial transformation of aminoglycosidic antibiotics has been the recent subject of extensive investigation, in particular, in connection with the mechanism of bacterial resistance against antibiotics. As the result, N-acetylation, O-phosphorylation and O-adenylation were recogized as inactivation processes of antibiotics.[1] It was found later that N-acetylation and O-phosphorylation of aminoglycosidic antibiotics were carried out by Streptomycetes[2] as well.

We wish to report in this communication a new type of bioconversion, that is, N-carboxymethylation of ribostamycin[3] (**1**) by *Streptomyces ribosidificus* which is the producer of the antibiotic.[4]

Cultivation: *S. ribosidificus* 25-83 was cultivated with shaking at 28°C in a medium containing 2.0 g of $NaNO_3$, 1.0 g of K_2HPO_4, 0.5 g of KCl, 0.018 g of $FeSO_4 \cdot 7H_2O$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 20.0 g of D-xylose and 0.5 g of yeast extract per liter. When other sugars, except D-xylose, were used as a carbon source, no bioconversion product was detected in the cultured broth.

Production was carred out using a 50-liter jar fermenter, and ribostamycin was fed three times at levels of 100 μg/ml at 42, 52 and 72 hours, to obtain the bioconversion product in high yield. The fermentation was stopped 92 hours after inoculation, and the mycelium was removed by filtration. Biosynthesis of ribostamycin in the medium containing D-xylose yielded insufficient product.

Purification: Broth filtrate (28.5 liters) was passed through a column of Amberlite IRC 50 (Na^+ type) and eltuted with 1N NH_4OH. Evaporation of the eluate gave a residue (18 g), which was chromatographed over Amberlite CG 50 (NH_4^+ type) developing with 0.03 N NH_4OH. Fractions showing brown color by the ninhydrin reagent were collected and concentrated to dryness to give a crude product (1.25 g). The crude product (1.0 g) was dissolved in water and passed through a column of Dowex 1×2 (OH^- type). The eluate with 1 N HCl was neutralized, diluted with water and passed through a column of CM-Sephadex C-25 which was previously equilibrated with 0.1 M NaCl. The bioconversion product was eluted by 0.4 M NaCl. The elutate was desalted by treating with Amberlite CG 50 (NH_4^+ type), and 370 mg of pure N-carboxymethylribostamycin (**2**) was obtained as white powder.

Properties and Structure: N-Carboxymethylribostamycin (**2**) showed m. p. 172~178°C, $[\alpha]_D^{24}$ +63° (*c* 1.32, H_2O), and an elementary analysis: *C* 43.8, H 6.5, N 10.1 %; Calcd. for $C_{19}H_{36}N_4O_{12}$: C 45.5, H 7.0, N 10.9 %. It migrated toward an anode more slowly than compound **1** upon paper electrophoresis (PEP) at pH 9.0, and moved again more slowly than **1** on paper chromatography (PPC) developed with *n*-butanol-pyridine-acetic acid-water (6:4:1:3). Compound **2** lacked antibiotic activity. It showed no UV maximum in water, and its IR spectrum was similar to that of 5″-carboxyribostamycin.[5]

The ^1H NMR spectrum in D_2O was similar to that of **1**, showing two anomeric proton signals at δ 5.58 and 5.35, and methylene protons at δ 2.60 and 3.05.

Treatment of **2** with acetic anhydride in methanol gave crystalline tetra-N-acetyl derivative (**3**), m. p. 219~220°C, and $[\alpha]_D^{24}$ +15° (*c* 1.04, H_2O). The ^1H NMR spectrum of **3** in D_2O exhibited four acetyl signals at δ 2.06 (2), 2.04 (1) and 1.98 (1).

Diazomethane treatment of **3** in methanol

Fig. 1.

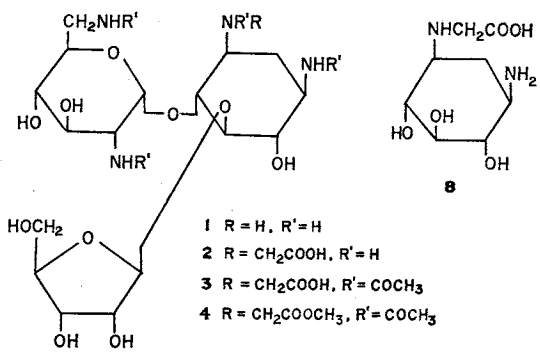
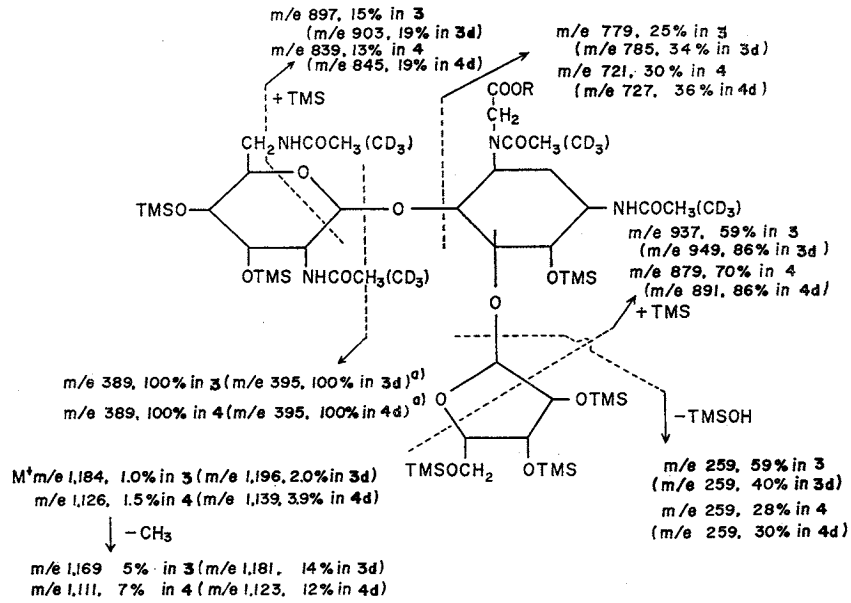


Chart 1. Principal fragmentation of O-TMS derivatives of tetra-N-acetyl-carboxymethylribostamycin (**3**), its N-deutero-acetyl analog (**3d**), tetra-N-acetyl-carboxymethylribostamycin methyl ester (**4**), its N-deuteroacetyl analog (**4d**).

a) The base peak was arbitrarily selected from the peaks above *m/e* 250.



yielded a methyl ester (**4**), which showed an IR band at 1730 cm^{-1}, indicative of an ester carbonyl. Hence the presence of a carboxyl group in **2** was indicated.

The structure of **2** was first revealed by mass spectrometry. Though **2** itself was not amenable to this technique, mass spectra of trimethylsilyl (TMS) derivatives of **3** and **4** (**5** and **6** respectively) gave M^+ at *m/e* 1,184 and 1,126, respectively. The fragmentations were analyzed using a comparison with the spectrum of N-acetyl-O-TMS-ribostamycin (**7**) and also by the deuterium exchange technique. The results are summarized in Chart 1. Fragment ions arising from ribose and 2,6-diaminoglucose moieties of **7** were observed as such in the spectra of **5** and **6**, but the deoxystreptamine fragments of the latter were shifted to 130 or 72 mass units to higher mass region than those of the corresponding deoxystreptamine fragments of **7**. The increased mass units, 130 or 72, were shown to be due to $C_5H_{10}O_2Si$ or $C_3H_4O_2$ by high resolution mass data. Accordingly, **2** was shown to possess the complete structure of **1** with an extra substituent ($C_2H_2O_2$), at the deoxystreptamine portion. Considering the formation of tetra-N-acetyl-hexa (or hepta)-O-TMS derivative and the presence of a carboxyl group, the $C_2H_2O_2$ must be the carboxylmethyl group attached to one of two amino groups of the deoxystreptamine moiety. These results were fully supported by mass spectrum of the N-salicylidene derivative and by ^{13}C NMR spectroscopy. N-Salicylidene SCHIFF bases of **1** and **2** gave, after O-trimethylsilylation, strong M^+ at *m/e* 1,590 and 1,544, respectively. The difference of mass units could be rationalized only if one of four amino groups in **2** was a secondary or tertiary amino group that is incapable of forming a SCHIFF base. As described in detail in a separate paper,[6] ^{13}C NMR of **2** gave two extra peaks at δ_{TMS} 179.3 and 50.4 which were absent in the spectrum of **1**. The lower position of δ_{TMS} 179.3 signal clearly indicated the carbonyl carbon, while the signal at δ_{TMS} 50.4 was close to that of the methylene carbon of sarcosine (δ_{TMS} 52.5).

In order to confirm the proposed structure, **2** was methanolyzed with 1 N HCl-methanol at room temperature overnight. Liquid and gas chromatographic examination of the ethanol-soluble methanolysate showed the presence of methyl riboside, being identified through comparison with an authentic sample prepared from **1**. The mass spectrum of the ethanol-insoluble portion showed, after O-trimethylsilylation, M^+

at *m/e* 850 and fragmentation compatible with N-carboxymethyl-O-TMS-neamine. The structure was further supported by mass spectra of tetra-N-acetyl-N-methoxycarbonylmethylneamine-O-acetate (M^+ at *m/e* 730) and its O-deuteroacetate (M^+ at *m/e* 743).

Vigorus acid hydrolysis of **3** with 6 N HCl at 100°C for 3 hours yielded N-carboxymethyl-deoxystreptamine (**8**), m. p. 180~185°C., 2, 6-diaminoglucose and N-carboxymethylneamine, but deoxystreptamine was not detected. These compounds were obtained by preparative paper-chromatography using the solvent system described above.

Compound **8** was identified through comparison with the authentic sample which was prepared by reaction deoxystreptamine with one mole of Na-glyoxalate followed by reduction with $NaBH_4$. Both natural and synthetic compounds showed identical Rf values on PPC and TLC, and the same migration rate on PEP and IR spectrum.

The position of the carboxymenthyl group at either N-1 or N-3 was not determined definitely, but ^{13}C NMR favoured the N-3 substitution.[6]

To the best of our knowledge, this is the first report on N-carboxymethylation in the field of biochemical reaction. Only in the case of biosynthesis of the carboxyformidoyl group kasugamycin, a reaction of some resemblance has been suggested between amino group of kasugamine and metylene of glycine.[7] It is interesting to note that acetic acid is introduced *via* the methyl group, instead of carboxyl group which is commonly observed in biochemical reactions. Synthetic proof of compound **2** and the investigation of the biochemical bases of this reaction are now in progress.

MICHIO KOJIMA
SHIGEHARU INOUYE
TARŌ NIIDA

Research Laboratories of Meiji Seika Kaisha, Ltd., Morooka-cho, Kohoku-ku, Yokohama, Japan

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