BIOCONVERSION OF RIBOSTAMYCIN (SF-733). III.

FORMATION, STRUCTURE AND SYNTHESIS OF 3-N-CARBOXYMETHYL RIBOSTAMYCIN*

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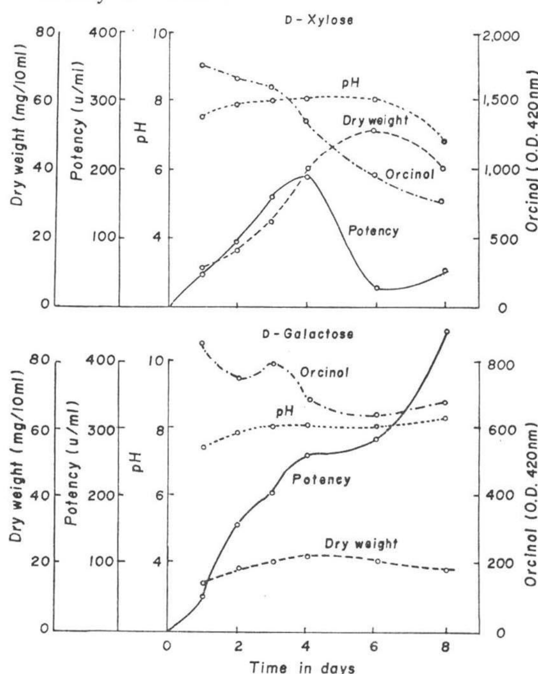
A new inactivated product of ribostamycin (SF-733), 3-N-carboxymethyl ribostamycin, was obtained from the broth of *Streptomyces ribosidificus* which was grown on a medium containing D-xylose. Detection and some biochemical mechanism of N-carboxymethylation were discussed, and structure of 3-N-carboxymethyl ribostamycin was proposed based on the chemical degradation and synthesis.

The inactivation processes of antibiotics by the producing strains have often observed in the fermentation of aminoglycosidic antibiotics. For instance, N-acetylation is observed in case of kanamycin[1], neomycin[2] and ribostamycin[3] fermentation, and N-phosphorylation is observed in the neomycin fermentation[4], and O-phosphorylation is observed in the streptomycin fermentation[5,6]. In the previous communication[7], we have added a new type of inactivating process of antibiotic, N-carboxymethylation of ribostamycin[8] in the cultured broth of *Streptomyces ribosidificus*[9] which was grown on a medium containing D-xylose.

The present paper describes formation of 3-N-carboxymethyl ribostamycin in the various conditions and the structure determination and synthesis of 3-N-carboxymethyl ribostamycin.

Fig. 1. Time course of ribostamycin formation by the medium containing D-xylose or D-galactose.

Growth was expressed as dry weight (110°C, 2 hours) of cells. Sugar was determined by orcinol method and expressed as the optical density at 420 nm.



Ribostamycin formation in the medium containing D-xylose

As already reported[9], *S. ribosidificus* is able to utilize common sugars including D-xylose. Fig. 1 shows time course of ribostamycin formation in the presence of D-xylose compared with that of D-galactose. The maximum formation of ribostamycin in the D-

* Isolation and structure of 3- (or 1-) N-carboxymethyl ribostamycin have been briefly communicated[7].

xylose culture broth was observed at 4 days and after that sudden decrease of potency occurred. These phenomena were not observed in case of another sugars including D-galactose.

Detection of an inactivated product of ribostamycin, 3-N-carboxymethyl ribostamycin

The broth filtrate growing on D-xylose for 6 days was subjected to Amberlite IRC-50 (Na^+ type) and 1 N NH_4OH eluate was subjected to paperchromatography. The result is shown in Fig. 2.

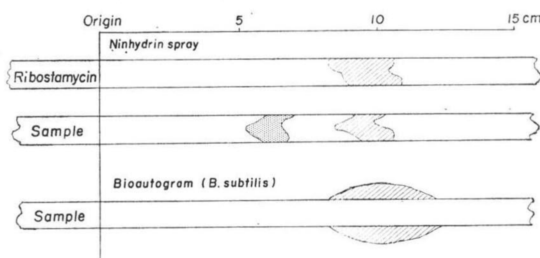


Fig. 2. Detection of 3-N-carboxymethyl ribostamycin on paperchromatogram.

Paperchromatography was carried out by a solvent system, BuOH-pyridine-AcOH-H_2O (6:4:1:3) for 5 days.

Effect of various carbon sources on the formation of 3-N-carboxymethyl ribostamycin

Though 3-N-carboxymethyl ribostamycin was detected in the medium containing D-xylose, an experiment was made to determine the effect of other sugars on the formation of 3-N-carboxymethyl ribostamycin. Ribostamycin in the concentration of 400 μg/ml was added to 6-day culture broths which had grown on various sugars and the results were shown in Table 1. The 3-N-carboxymethyl ribostamycin was clearly observed in the medium containing pentoses, but dropped significantly in case of hexoses and oligosaccharides. These findings may suggest that a pentose metabolism is responsible for the formation of 3-N-carboxymethyl ribostamycin.

Table 1. Effect of various carbon sources on the formation of 3-N-carboxymethyl ribostamycin.

Carbon source	4 days	6 days			After incubation			CM-Rm* Formation
	Growth	Growth	pH	Potency (u/ml)	pH	Potency (u/ml)	Difference (u/ml)	
D-Glucose	+++	+++	7.2	1	7.2	140	261	±
D-Galactose	+++	+++	7.8	160	8.2	348	212	−
D-Mannose	+++	++	8.2	175	8.2	375	200	−
D-Fructose	+++	+++	6.0	3	6.2	224	182	−
D-Ribose	+++	+++	5.8	0	5.9	140	260	+
D-Xylose	+++	++	8.0	19	8.0	332	87	++
L-Xylose	+	+	7.0	0	7.1	300	100	+
L-Arabinose	+++	+++	8.4	0	8.3	36	364	−
Sucrose	+++	+++	6.0	1	6.0	140	261	−
Maltose	+++	+++	7.0	22	7.2	290	132	−
N-Acetyl-D-glucosamine	+++	+++	8.4	11	8.6	300	111	−
Starch	+++	+++	8.2	82	8.4	350	132	−
Glycerol	+++	+++	5.6	9	6.0	208	201	−

* CM-Rm: 3-N-carboxymethyl ribostamycin

Effect of C-2 compounds on the formation of 3-N-carboxymethyl ribostamycin

The results of this experiment is shown in Table 2. All the C-2 compounds except glyoxylic acid stimulated slightly the formation of 3-N-carboxymethyl ribostamycin. However,

Table 2. Effect of C-2 compounds on the formation of 3-N-carboxymethyl ribostamycin.

C-2 compound	Cultured broth				IRC-50 treatment			CG-50	PPC
	Mycelium		Filtrate		Effluent	Eluate		Eluate count (dpm) $\times 10^4$	CM-Rm* count (dpm) $\times 10^3$
	Volume	Count (dpm) $\times 10^5$	Count (dpm) $\times 10^6$	Potency (unit)	Count (dpm) $\times 10^6$	Count (dpm) $\times 10^6$	Potency (unit)		
Acetic acid	+	1.8	2.9	8,800	0.4	1.7	5,900	4.7	6.6(5.0)
Oxalic acid	+++	0.6	2.7	4,300	1.0	1.4	2,900	3.7	6.0(4.4)
Glyoxalic acid	++	0.2	2.8	12,000	2.0	0.6	4,200	1.7	0.8(0)
Glycine	+	3.1	2.9	8,000	0.3	1.6	8,100	4.8	8.1(6.5)
Glycolic acid	++	1.8	2.6	7,200	0.4	1.4	6,300	4.8	8.7(7.2)
None	+++	2.7	2.9	7,600	0.7	1.5	6,500	2.4	1.6(0)

* CM-Rm: 3-N-carboxymethyl ribostamycin

considering the utilization as a carbon source for growth by the strain, these results did not indicate the direct insertion of the C-2 unit into the carboxymethyl group of 3-N-carboxymethyl ribostamycin.

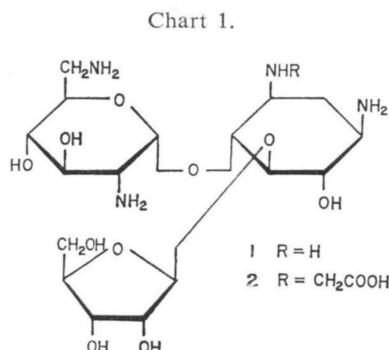
Close to N-carboxymethylation, LANCINI *et al.*[9] reported O-carboxymethylation in the biosynthesis of rifamycin B from rifamycin SV. A carboxymethyl group in case of rifamycin B was derived from pentose cycle and not from C-2 compound. Therefore, pentose metabolism may play a role on the biosynthesis of carboxymethyl group.

Addition of acetate unit through a carboxyl group is the well-known biochemical reaction, but the insertion of an acetate unit through a methylene to an amino group has not been mentioned in the literatures. In this connection, it was interesting to see that besides 3-N-carboxymethyl ribostamycin, 3-N-acetylribostamycin[3] was formed simultaneously in the inactivation processes by *S. ribosidificus*.

Structure of 3-N-carboxymethyl ribostamycin (2)

As already reported[7], the structure of carboxymethyl ribostamycin was proposed as 3- (or 1-) N-carboxymethyl ribostamycin in which the substitution position of the carboxymethyl group has not been determined unambiguously. The gross structure of **2** deduced at first on the basis of mass spectrometric evidence* was confirmed chemically by the identification of methylriboside liberated by methanolysis of **2** and isolation of optically active N-carboxymethyl-2-DSA by acid hydrolysis. In order to prove the 3-N-carboxymethyl substitution on 2,3-N-acetyl ribostamycin[3] of known structure was subjected to N-carboxymethylation by treating with glyoxylic acid and sodium borohydride. Acid hydrolysis of resulting N-carboxymethyl derivative gave

Chart 1.



* Details of mass spectrometry of ribostamycin and the related compounds will be published elsewhere.

Table 3. Separation of a mixture of N-carboxymethyl ribostamycin.

Fraction	82~85	90~95	101~113	114~124	223~245
NH_4OH eluent	0.05	0.05	0.05	0.05	0.1N
Dry weight	810 mg	1.22 g	700 mg	270 mg	370 mg
Rf on TLC[1]	0.81 p[3]	0.77 p[4] 0.68 b[5]	0.66 p 0.47 p	0.47 b	0.16 p
Rf on PPC[2]	0.49 b	0.79 p[4] 0.64 pb[5]	0.82 p 0.77 b	0.77 b	1.00 p
Ratio[6]	tri-di (2.5:1)	di-mono (1.5:1)	mono	mono	mono

1) Silicagel TLC developed with $CHCl_3$-MeOH-4%NH_4OH (2:1:1)
2) Developed descendingly with *n*-PrOH-Pyr.-AcOH-H_2O (15:10:3:12) on Toyo No. 50 filter paper. Rf values indicated were relative to that of Rm (1.00).
3) ninhydrin color: p, purple b, brown pb, purplish brown
4) a main spot
5) a minor spot
6) Ratio of tri-, di, and mono-N-carboxymethyl derivatives as determined from the relative peak intensities in the MS spectra of N-acetyl-O-TMS derivatives.

the 1-N-carboxymethyl-2-DSA* whose IR spectrum was almost identical with that of N-carboxymethyl derivative from **2**. These two compounds showed almost same magnitude of optical rotation, but opposite sign ($[\alpha]_D^{25}-29.2°$ for 1-N-carboxymethyl-2-DSA and $[\alpha]_D^{25}+27.8°$ for acid hydrolyzate of **2**). Therefore, 3-N-carboxymethylation in **2** was established.

As an additional support for 3-N-carboxymethylation, N-carboxymethyl-N-*p*-methoxybenzyl-2-DSA, which was prepared from **2** showed CD maximum at 272 nm with $[\theta]-475$. This could be favorably comparable to that of 1-N-*p*-methoxybenzyl-2-DSA ($[\theta]_{278}-508$)[11], but definitely different from that of 3-N-*p*-methoxybenzyl-2-DSA ($[\theta]_{237}+402$)[12].

^{13}C-NMR spectroscopy of **2** was described in the separate paper[13].

Synthesis of 3-N-carboxymethyl ribostamycin (2)

Condensation of ribostamycin (**1**) with glyoxylic acid in aqueous solution followed by reduction with sodium borohydride gave a mixture of N-carboxymethyl derivatives. Separation of the mixture was effected by chromatography on Dowex 50W×2 (NH_4^+ type) developing with dilute ammonia. Tri-, di- and mono-N-carboxymethyl derivatives and the starting material (**1**) were eluted in that order. Rechromatography on Dowex 50W×2 (NH_4^+ type) gave two mono-N-carboxymethyl derivatives (*A* and *B*), each as a single compound on TLC and PPC. The mono-N-carboxymethyl derivative (*B*) eluted last showed identical Rf values on TLC, PPC, the same retention time (RT) on GLC, and superimposable ^{1}H and ^{13}C-NMR and mass spectra with those of **2** obtained from the natural source. In contrast to *B* which was devoid of bioactivity, compound *A* eluted before *B* showed biological activity, about 20% of that of **1**. Mass analysis of *A* in the form of its N-acetyl-O-TMS derivative indicated that a main component was a positional antipode of **2**, *i.e.* 1-N-carboxymethyl ribostamycin.

* The authors wish to thank to Dr. S. KONDO, Institute of Microbial Chemistry, for his suggestion on the preparation of 1-N-carboxymethyl-2-DSA from 3-N-acetylribostamycin.

Experimental

Instrumental analysis: NMR spectra were measured at 100 MHz with a Varian XL-100 spectrometer with DDS as internal standard in D_2O. Chemical shifts were given on the δ scale. IR spectra were recorded with a Hitachi Model 215 IR spectrometer in KBr tablet. GLC was carried out using a Hewlett-Packard Gas Chromatography Model 402 equipped with a glass column of 0.75% OV-1, and developed with helium. Samples for GLC were trimethylsilylated with TMS-Pz (Tokyo Kasei Kōgyō) at room temperature for 30 minutes.

Cultivation: *Streptomyces ribosidificus* 25-83 was cultured as described in the previous paper[7].

Assay of 3-N-carboxymethyl ribostamycin: 3-N-Carboxymethyl ribostamycin was detected as follows: the broth filtrate was subjected to Amberlite IRC-50 (Na^+ type), and the NH_4OH eluate was applied on PPC. As shown in Fig. 2, a new ninhydrin-positive but bioinactive spot was observed on a paperchromatogram at R_{Rm} 0.65. This spot showed brown color by the ninhydrin reagent immediately after heating, but it turns purple gradually. 3-N-Carboxymethyl ribostamycin was assayed comparing visually the intensity of color developed with the ninhydrin reagent on a paperchromatogram.

An experiment to determine the precursor of a carboxymethyl group was carried out by the radioisotope technique using ^3H-ribostamycin which was kindly supplied from Dr. K. UMEMURA of our laboratories as follows: ^3H-ribostamycin was incubated with the cells which had previously grown on the D-xylose-containing medium for 4 days, containing 0.1% of D-xylose and 1.0% of neutralized C-2 compound for 40 hours, and the broth filtrate was applied on a column of Amberlite IRC-50 (Na^+ type). Radioactive fractions eluted with 0.03N NH_4OH were collected, mixed with cold carboxymethyl ribostamycin and subjected to paperchromatography developed with BuOH-pyridine-AcOH-water (6:4:1:3). A sport of 3-N-carboxymethyl ribostamycin developed by the ninhydrin reagent was cut off from a paperchromatogram and counted for the radioactivity using the toluene scintillator. The C-2 compounds tested were those derived from the glyoxylic acid cycle.

Isolation and purification of 3-N-carboxymethyl ribostamycin: Production of 3-N-carboxymethyl ribostamycin was carried out using a 50-liter jar fermenter with medium containing D-xylose supplemented with 0.05% yeast extract[7]. Under these conditions, ribostamycin production was not enough to obtain 3-N-carboxymethyl ribostamycin, so ribostamycin was fed three times at 42, 52 and 72 hours each 100 μg/ml. After 92 hours from inoculation, the cultivation was stopped and mycelium was removed by filtration.

The broth filtrate (450 μg/ml, 28.5 liters) was passed through a column of 4-liter Amberlite IRC-50 (Na^+ type) and eluted with 1 N NH_4OH. The fractions showing antibiotic activity were collected (*ca.* 4 liters) and evaporated to dryness to give a mixture of ribostamycin and 3-N-carboxymethyl ribostamycin (18 g). The separation of 3-N-carboxymethyl ribostamycin from ribostamycin was carried out with a column of Amberlite CG-50 (NH_4^+ type) (100 ml). Aqueous effluent and the 0.03 N NH_4OH eluate which showed brown color by the ninhydrin reagent were collected (500 ml) and concentrated to dryness to yield a crude 3-N-carboxymethyl ribostamycin. From 14.5 g of crude ribostamycin, 1.25 g of crude 3-N-carboxymethyl ribostamycin was obtained. Ribostamycin was recovered from the 0.1 N NH_4OH eluate. A crude 3-N-carboxymethyl ribostamycin (1.0 g) was dissolved in 10 ml of water, and passed through a column of Dowex 1×2 (OH^- type) (40 ml). The column was washed with water, and eluted with 1 N HCl. The ninhydrin-positive fractions were collected (50 ml), neutralized with 1 N NaOH, diluted to 300 ml with water and passed through a column of CM-Sephadex C-25 (100 ml) which was previously equilibrated with 0.1 M NaCl. The column was washed well with 0.1 M NaCl, and 3-N-carboxymethyl ribostamycin was eluted with 0.4 M NaCl. About 1 liter of the ninhydrin-positive fraction was diluted to 2 liters with water and passed through

a column of Amberlite CG-50 (NH_4^+ type) (20 ml). After washing with water, the column was eluted with 0.1 N NH_4OH. The ninhydrin-positive fractions were collected and concentrated to dryness to obtain 370 mg of 3-N-carboxymethyl ribostamycin as a white powder. m.p. 172~178°C, $[\alpha]_D^{24}+63°$ (*c* 1.32, H_2O)

Anal. Calcd. for $C_{19}H_{36}N_4O_{12}$: C 45.5, H 7.0, N 10.9
Found: C 43.8, H 6.5, N 10.1

It migrated toward an anode more slowly than ribostamycin upon paper electrophoresis using 0.1 M beronal buffer pH 9.0, and lacked in any antibiotic activity and showed no UV maximum in water. Its solubility and color reaction were similar to those of ribostamycin.

Preparation of tetra-N-acetyl carboxymethyl ribostamycin and its methyl ester: To a solution of **2** (130 mg) in MeOH (20 ml) was added five drops of acetic anhydride, and the mixture stood at room temperature overnight. Evaporation of solvent gave a residue, which was crystallized from H_2O-MeOH. Yield, 85 mg, m.p. 265~270°C (dec.) with sintering at 219~220°C. $[\alpha]_D^{24}+15°$ (*c* 1.04, H_2O) NMR(D_2O): 2.06 ($2C\underline{H}_3$), 2.04 ($C\underline{H}_3$), 1.98 ($C\underline{H}_3$)

Anal. Calcd. for $C_{27}H_{44}N_4O_{16}5H_2O$: C 42.1, H 7.05, N 7.3
Found: C 42.1, H 6.9, N 7.3

A portion of N-acetylcarboxymethyl ribostamycin was treated with excess of diazomethane in MeOH at room temperature for 30 minutes to yield, after evaporation of solvent and excess reagent, N-acetyl-3-N-carboxymethyl ribostamycin methyl ester. IR (KBr): 1735, 1640 cm^{-1}, NMR (D_2O): 3.45 ($OC\underline{H}_3$)

Degradation of tetra-N-acetyl-3-N-carboxymethyl ribostamycin: A solution of N-acetyl carboxymethyl ribostamycin (50 mg) in 1 N HCl-MeOH (10 ml) was heated at 50°C for 30 minutes, and left at room temperature for 3 days. The reaction mixture was concentrated to dryness to afford a crude methanolyzate. The *iso*-PrOH extract of the residue showed identical mobility of TLC ($CHCl_3$-MeOH, 9:1) and identical retention time on GLC of its TMS derivative at 173°C, with those of an authentic sample of methyl riboside prepared from **1**. A part of *iso*-PrOH-insoluble portion that contained N-carboxymethyl neamine was precipitated from EtOH-ether. The carboxymethyl neamine fraction was refluxed with 3 N HCl for 4 hours. The hydrolyzate was separated on a Tōyō Roshi No. 50 filter paper, developed with BuOH-pyridine-AcOH-H_2O (6: 4: 1: 3) for 3 days. Three ninhydrin-positive spots were recognized at $R_{2,6AG}$ 0.16 (N-carboxymethyl neamine), 0.33 (N-carboxymethyl-2-DSA) and 1.00 (2, 6-diaminoglucose). Under the same condition, the hydrolyzate of N-acetylribostamycin showed three spots at $R_{2,6AG}$ 0.33 (neamine), 0.74 (2-DSA) and 1.00 (2, 6AG). A band showing $R_{2,6AG}$ 0.33 was eluted with H_2O, and the eluate passed through a short column of Amberlite CG-50 (NH_4^+ type). Elution with 0.05 N NH_4OH and evaporation of solvent gave a crystalline residue (5 mg). m.p. 245~250°C (dec.), $[\alpha]_D^{25}+27.8°$ (*c* 1.0, H_2O). It showed the same Rf values on PPC (BuOH-pyridine-AcOH-H_2O, 6:4:1:3) and silica-gel TLC (Rf 0.70, $CHCl_3$-MeOH-4% NH_4OH, 2:1:1), and almost identical IR spectrum as those of the synthesized sample of mono-N-carboxymethyl-2-DSA. A mixture of optically active N-carboxymethyl-2-DSA (10 mg) and excess anisaldehyde in 40% MeOH was heated at 80°C for 3 hours, reduced with $NaBH_4$ and concentrated to dryness. The residue was extracted with BuOH. Column chromatography of the BuOH-insoluble portion on Sephadex G-10 gave N-carboxymethyl-*p*-methoxybenzyl-2-DSA (5.5 mg). Silica-gel TLC (BuOH-AcOH-H_2O, 2:1:1) gave Rf 0.50 (Rf of carboxymethyl-2-DSA was 0.15), ORD (H_2O): $[\theta]_{278}^{\mathrm{through}}-350$, $[\theta]_{238}^{\mathrm{peak}}-300$, CD ($H_2O$): $[\theta]_{272}-475$.

Isolation of 1-N-carboxymethyl-2-DSA from N-carboxymethylated 3-N-acetylribostamycin: A solution of 3-N-acetylribostamycin[3] (2.5 g), glyoxylic acid monohydrate (5.0 g) and $NaHCO_3$ (4.5 g) in H_2O (100 ml) was stirring at room temperature for 4 hours, and then $NaBH_4$ (2.0 g) was added. The reaction mixture was stirring overnight (16 hours) at room temperature, its pH was adjusted to pH 7.0 with AcOH, and diluted with H_2O (100 ml). Elution with H_2O followed by evaporation gave 0.95 g of N-carboxymethylated 3-N-acetylribostamycin. An

N-carboxymethylated product (0.8 g) was dissolved in H_2O (4 ml) and conc. HCl (4 ml) was added. After heating at 100°C for 72 hours, the hydrolyzate was adsorbed on Dowex 50W×8 (H^+ type) column (30 ml), and the 0.5 N NH_4OH eluate gave a brown powder (450 mg). Further purification was carried out by chromatography on Dowex 50W×4 (H^+ type, 200~400 mesh) (70 ml), equilibrated with 0.35 M citrate-HCl buffer, pH 5.3 at 52°C. The resin column was developed with the same buffer at 52°C, the 1-N-carboxymethyl-2-DSA was eluted at the fractions No. 18~22 (15-ml cut). These fractions were combined and diluted to five-folds with H_2O and desalted on the Dowex 50W×8 (H^+ type) column (15 ml). The 0.5 N NH_4OH eluate was concentrated and addition of EtOH gave crystalline 1-N-carboxymethyl-2-DSA (40 mg). $[\alpha]_D^{25}-29.2°$ (*c* 1.0, H_2O).

Synthesis of racemic mono-N-carboxymethyl-2-DSA: To an aqueous solution (30 ml) of 2-DSA (2.35 g) were added glyoxylic acid monohydrate (1.88 g) and $NaHCO_3$ (1.76 g), and the mixture stirring at room temperature for 3 hours. $NaBH_4$ (500 mg) was added and stirring was continued for further 3 hours. The reaction solution was neutralized with AcOH, diluted with H_2O (200 ml), and passed through a column (5×22 cm) of Dowex 50W×2 (NH_4^+ type) resin. After washing with H_2O, the column was successively eluted with 0.06 N NH_4OH and 0.1 N NH_4OH. Evaporation of the 0.06 N NH_4OH eluate gave crystalline racemic mono-N-carboxymethyl-2-DSA (1.52 g), which was recrystallized from H_2O. m.p. 246~251°C (dec.) with sintering at 180~185°C. NMR in D_2O acidified with trifluoroacetic acid: 3.74 (-CH_2COOD).

Anal. Calcd. for $C_8H_{16}N_2O_5$: C 43.6, H 7.3, N 12.7
Found: C 43.6, H 7.4, N 12.6

From the 0.1 N NH_4OH eluate was recovered the unreacted 2-DSA (560 mg).

Synthesis of N-carboxymethyl ribostamycin: A solution of **1** (4.45 g), glyoxylic acid monohydrate (3.3 g) and $NaHCO_3$ (3.6 g) in H_2O (50 ml) was stirring at room temperature for 3 hours and then $NaBH_4$ (1.0 g) was added. After standing overnight, the reaction mixture was neutralized with AcOH, diluted with H_2O (380 ml), and applied on a column of Dowex 50W×2 (NH_4^+ type) (5×22 cm). After washing with H_2O, elution was effected with 0.05 N, 0.1 N and 0.2 N NH_4OH. Effluents were collected in 15 g fractions. Fractions No. 113~124 (compound *B*) showed m.p. *ca.* 200°C (dec.) and $[\alpha]_D^{25}+59°C$ (*c* 1.0, H_2O).

Anal. Calcd. for $C_{19}H_{36}N_4O_{12}$: C 44.5, H 7.1, N 10.9
Found: C 44.3, H 7.3, N 10.7

Its Rf values on PPC and TLC in Table 3, were indistinguishable from those of **2**. Furthermore, ^1H and ^{13}C-NMR spectra of compound ***B*** were superimposable to those of **2**.

A portion (690 mg) of fractions No. 90~95, which were shown to be a mixture, was once again chromatographed on Dowex 50W×2 (NH_4^+ type) (5×22 cm), developed with H_2O and 0.04 N NH_4OH. Effluents were collected in 15-g fractions. Evaporation of fractions No. 19~24 gave a biologically active residue (compound *A*) (230 mg). $[\alpha]_D^{21}+31°$ (*c* 0.97, H_2O).

Anal. Calcd. for $C_{19}H_{36}N_4O_{12}$: C 44.5, H 7.1, N 10.9
Found: C 44.0, H 7.3, N 10.5

A solution of 2,000 μg/ml of compound *A* showed the following bioactivity as assayed by the paper disc (8 mm) method: *Bacillus subtilis* 18 mm, *Bacillus stearothermophylus* 19 mm, *Staphylococcus aureus* FDA 209 P 14 mm, *Escherichia coli* 16 mm. It behaved as a single compound on silica-gel TLC (Rf 0.66, $CHCl_3$-MeOH-4%NH_4OH, 2:1:1) and PPC (R_{Rm} 0.82, *n*-PrOH-pyridine-AcOH-H_2O, 15:10:3:12).

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