BIOCONVERSION OF RIBOSTAMYCIN (SF-733). II

ISOLATION AND STRUCTURE OF 3-N-ACETYLRIBOSTAMYCIN, A MICROBIOLOGICALLY INACTIVE PRODUCT OF RIBOSTAMYCIN PRODUCED BY *STREPTOMYCES RIBOSIDIFICUS*

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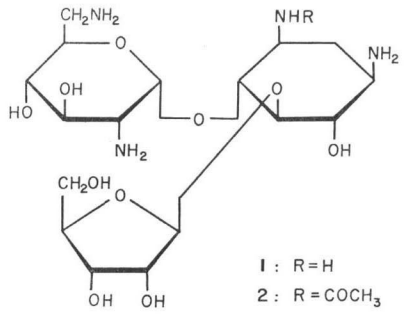
The isolation and structure determination of 3-N-acetylribostamycin, a microbiologically inactive derivative, produced enzymatically from ribostamycin by *Streptomyces ribosidificus* is described. The location of the acetyl group was established by mass and NMR spectrometry of the new compound and its derivatives, and by optical rotation studies conducted on N-ethoxycarbonyl-2-deoxystreptamine. The latter compound was obtained by partial acid hydrolysis of tri-N-ethoxycarbonyl-N-acetylribostamycin.

The isolation and structure of 3-(or 1-) N-carboxymethyl ribostamycin, a microbiologically inactive derivative of ribostamycin (**1**) produced by *Streptomyces ribosidificus* was reported in the previous paper[1]. This paper describes the isolation and structure of a second inactive derivative of **1**, 3-N-acetylribostamycin (**2**), produced by the same organism. Ribostamycin is a metabolite of *S. ribosidificus* when the latter is grown under normal fermentation conditions.

Isolation of 3-N-Acetylribostamycin (2)

A crude preparation containing **2** was obtained from the fermentation broth of *Streptomyces ribosidificus* cultured on a D-xylose containing medium with ribostamycin as substrate[1], by adsorption on Amberlite IRC-50 (Na^+ type) resin followed by elution with 1 N NH_4OH. Further purification was obtained by readsorption on Amberlite CG-50 (NH_4^+ type) resin, and by developing successively with 0.03 N, 0.1 N and 0.2 N NH_4OH. From the 0.03 N NH_4OH eluate were obtained 3-(or 1-) N-carboxymethyl ribostamycin[1] and arginine. 3-N-Acetylribostamycin (**2**) was eluted just before ribostamycin (**1**) with 0.2 N NH_4OH.



Complete separation of **2** from **1** was accomplished by preparative PPC developed with BuOH-pyridine-AcOH-H_2O (6 : 4 : 1 : 3). One more chromatographic step on Amberlite CG-50 (NH_4^+ type) resin gave a pure sample of **2**, which was distinguishable from **1** by TLC and GLC. Compound **2** reacted to form a tri-N-salicylidene SCHIFF base (**2a**). The CD curve of **2a** in MeOH was differed from that of tetra-N-salicylidene ribostamycin (**1a**), showing a negative COTTON effect at 316 nm, in contrast to a positive one for **1a**. Compound

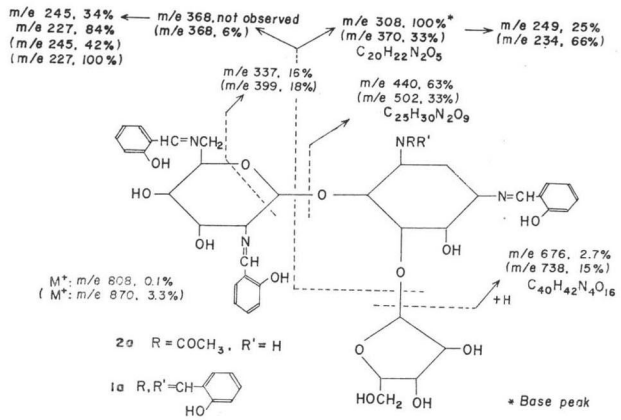
2 was practically devoid of biological activity; a 20 mg/ml solution showed weak activity against *Bacillus subtilis* (26 mm) and *Pseudomonas tabaci* (12 mm) by paper disc assay.

Structure of 3-N-Acetylribostamycin (2)

The NMR spectrum of **2** in D_2O exhibited two anomeric proton signals at δ 5.60 ($J_{1,2}$ 4.0 Hz) and 5.39 ($J_{1,2}<1$ Hz), two methylene signals at δ 1.94 and 1.34, and a noticeable acetyl signal at δ 2.01. Except for the last-mentioned signal, the spectrum of **2** was very similar to that of **1**. The acetyl group must be associated with an acetamido group and not an ester, since the IR spectrum showed strong bands at 1630 cm^{-1} and 1560 cm^{-1} without any ester band.

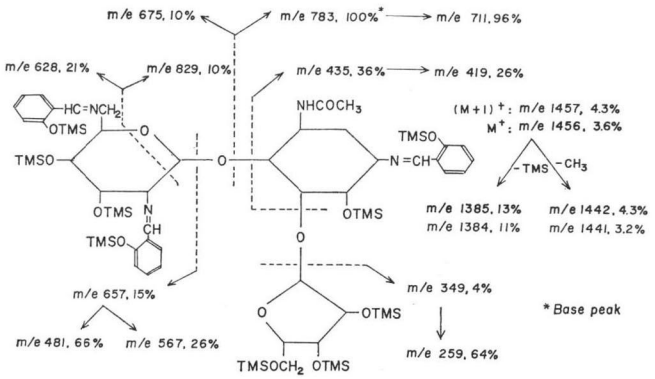
Location of the acetyl group was examined by mass spectrometry of N-salicylidene (**2a**) and N-salicylidene-O-TMS derivative (**2b**) summarized in Charts 1 and 2. The spectra were interpreted on the basis of the comparable fragmentation of the corresponding derivatives of the parent antibiotic (**1a** and **1b**) of known structure (Chart 1). Beside the M^+ at *m/e* 808, the spectrum of **2a** was characterized by the appearance of well-recognized peaks at *m/e* 676, 440, 337, 308, 249, 245 and 227. The peaks at *m/e* 245 and 227 could be ascribable to the N-salicylidene 2, 6-diaminoglucose (2, 6-AG) moiety as in **1a**. However, the fragment ions con-

Chart 1. Main fragmentation of N-salicylidene-N-acetylribostamycin (2a) and N-salicylidene ribostamycin (1a)* A base peak was arbitrarily selected from the peaks above *m/e* 200.



* Figures in parenthesis indicate fragment ions of **1a**.

Chart 2. Main fragmentation of N-salicylidene-N-acetyl-O-TMS-ribostamycin (2b). A base peak was arbitrarily selected from the peaks above *m/e* 250.



taining the 2-deoxystreptamine (2-DSA) portion at *m/e* 676, 440 and 308 were consistently shifted by *m/e* 62 to lower mass region than the corresponding ions from **1a**. This shift could be rationalized only if one of the two amino groups in DSA is acetylated in **2a**, thereby preventing formation of a SCHIFF base. The *m/e* 249 peak could be formed from N-acetyl-N-salicylidene 2-DSA by the loss of an acetamide group.

The same conclusion was derived from the mass spectrum of the N-salicylidene-O-TMS derivative (**2b**). When the fragmentation of **2b** was compared with that of N-salicylidene-O-TMS ribostamycin (**1b**)[13] (Chart 2), it was apparent that the 2-DSA containing fragment ions from **2b** including the M^+ and $(M+1)^+$ were shifted *m/e* 134 to lower mass region than the corresponding ions from **1b**. The 134 mass units corresponded to the difference between the O-TMS-salicylideneimino (192 mass unit) and acetamido group (58 mass unit). On the other hand, fragment ions from 2, 6-AG (*m/e* 657, 567, 481) and ribose moieties (*m/e* 345, 259) appeared without any mass shift.

Heating **2** with hydrazine hydrate at 125°C for 35 hours, followed by chromatography on Dowex 1×2 (OH^- type) resin gave **1** in 80% yield. Identity was confirmed by comparing Rf values on TLC and PPC, retention time (RT) on GLC, and bioactivity recovered with those of the authentic **1**. This indicated that no alteration of the framework of ribostamycin was involved in the formation of **2**.

The position of substitution of the acetyl group was suggested on the basis of ^1H NMR study. Table 1 shows the magnitude of the down-field shift of the H-1′ and H-2_a protons

Table 1. Deshielding effect of protonation on the chemical shift of the H-1′ and H-2_a protons in D_2O.

Compound	ΔH-1′ ($\delta_{\text{free base}} - \delta_{\text{TFA}}$*)	ΔH-2_a ($\delta_{\text{free base}} - \delta_{\text{TFA}}$)
N-Acetylribostamycin (**2**)	0.30	0.35
Ribostamycin (**1**)	0.66	0.83
Mono-N-acetyl-2-deoxy-streptamine[3]		0.35
2-Deoxystreptamine		0.74
4-O-(2, 6-Diamino-2, 6-dideoxy-α-D-glucopyranosyl)-1-amino-1-deoxy-*scyllo*-inositol	0.35	

* Trifluoroacetic acid salt

caused by the protonation of the neighboring amino group.[2] It was found that the paramagnetic shifts of H-1′ and H-2_a of **2** were about half of those of **1** and 2-DSA, but comparable to those of mono-N-acetyl-2-DSA[3] and 4-O-(2, 6-diamino-2, 6-dideoxy-α-D-glucopyranosyl)-1-amino-1-deoxy-*scyllo*-inositol* lacking the C-3 amino group of streptamine. This not only indicated the mono-N-acetylation of the 2-DSA moiety, but also favored 3-N-acetylation, because if the N-acetylation occurred at C-1, the paramagnetic shift of H-1′ should be much closer to

* EZAKI, N.; S. INOUYE & T. NIIDA: This compound, m.p. 278~280°C was isolated from fermentation broth of a *Streptomyces*. The structure was elucidated by the isolation of optically inactive 1-N-acetyl-2, 3, 5, 6,-tetra-O-methyl-*scyllo*-inosamine and 2, 6-N-acetamido-2, 6-dideoxy-3, 4-di-O-methyl-α-D-glucopyranose from hydrolysis of the tri-N-acetyl-hexa-O-methyl derivative. Full details will be published later.

that of ribostamycin (0.66 ppm) due to the positive charge on nitrogen at C-3, which is specially more near to H-1′ than that at C-1. 3-N-Acetylation was supported by the ^{13}C NMR spectroscopy.[8] The resonance of C-4, which is β to the C-3 acetamido group showed upfield shifting (5.9 ppm) as compared to that of **1**. Furthermore, chemical proof of 3-N-acetylation was obtained by the isolation of levorotatory 1-N-ethoxycarbonyl-2-DSA[4] after hydrolysis of tri-N-ethoxycarbonyl derivative of **2**, according to KONDO's procedure[5].

N-Acetylation of the aminoglycosidic antibiotics is a well-known phenomenon. RINEHART[6] has reported the isolation of 3-N-acetyl derivatives of neomycins B and C (LP_B and LP_C) from the fermentation broth of *Streptomyces fradiae* while MURASE *et al.*[7] reported the isolation of mono- and di-N-acetylated antibiotics (NK-1013-1 and NK-1013-2) from a mutant of *Streptomyces kanamyceticus*. Unfortunately, no details of the latter study were published. N-Acetylation reactions of aminoglycosidic antibiotics observed to date include 6′-N-acetylation of ribostamycin, kanamycins, neomycins and gentamicins by acetyltransferases from *Pseudomonas aeruginosa*[8] and from *Escherichia coli* carrying R-factors[9,10] and 3-N-acetylation of gentamicins by an enzyme from *Pseudomonas aeruginosa*[8] and by *Escherichia coli* carrying an R-factor[4]. The result presented in this paper suggests the possible presence of a 3-N-acetyltransferase in ribostamycin-resistant bacteria.

Experimental

General methods

NMR spectra were measured at 100 MHz with a JNM-4H-100 spectrometer with DDS as an internal standard in D_2O. Chemical shifts were given on the δ scale. CD curve were recorded with a JASCO model ORD/UV-5 instrument at 25°C, and IR spectra with Hitachi Model 215 IR spectrophotometer in KBr tablet. Trimethylsilylation of the compounds subjected to mass and GLC analysis was achieved by reacting with TMS-PZ (Tōkyō Kasei Kōgyō) at 50°C for 30 minutes. GLC was carried out at 295°C using a Hewlett-Packard Gas chromatograph Model 402 equipped with 0.75% OV-1 (0.4×120 cm). Mass spectra were recorded on a JMS-O1SG double focussing mass spectrometer at 75 eV using electric recording for the low-resolution data and photographic recording for the high-resolution data.

Isolation of 3-N-acetylribostamycin (2)

A broth filtrate (28.5 liters) obtained by the fermentation method described[1] was passed through a column of Amberlite IRC-50 (Na^+ type) resin (4 liters). Elution was effected with 1 N NH_4OH, and the eluate concentrated to dryness. The residue was dissolved in a small amount of H_2O, placed on a column of Amberlite CG-50 (NH_4^+ type) (100 ml), and developed with 0.03 N, 0.1 N, and 0.2 N NH_4OH, stepwisely. 3 (or 1)-N-Carboxymethyl ribostamycin, another metabolite, and arginine were eluted by 0.03 N NH_4OH. N-Acetylribostamycin (**2**) (362 mg) was eluted by 0.2 N NH_4OH just before ribostamycin (**1**). A part of this sample (300 mg) was further separated on preparative PPC (Tōyō Roshi No. 526 filter paper) developed with BuOH-pyridine-AcOH-H_2O (6:4:1:3). A band migrating faster than **1** was cut and eluted with H_2O. Evaporation of solvent from the eluate gave 250 mg of a residue. This was again purified by chromatography on Amberlite CG-50 (NH_4^+ type) resin using 0.08 N NH_4OH as a developing solvent. The final eluate was concentrated to dryness to give **2** as a white powder. m.p. 160°C $[\alpha]_D^{24}$+18° (*c* 1.0, H_2O)

Anal. Calcd. for $C_{19}H_{36}N_4O_{11}$: C 46.0, H 7.3, N 11.3
Found: C 45.4, H 7.9, N 11.0

On silica-gel TLC developed with lower phase of $CHCl_3$-MeOH-4% NH_4OH (2:1:1) and PPC with *n*-PrOH-pyridine-AcOH-H_2O (15:10:3:12), compound **2** moved faster than **1** (R_{Rm}

2.0 and 1.05). Relative retention time of **2** on GLC was RT_{Rm} 1.55.

Tri-N-salicylidene-3-N-acetylribostamycin (**2a**)

Treatment of **2** with an excess of salicylaldehyde in 50% methanol at room temperature for 3 hours, followed by precipitation from *iso*-PrOH - cyclohexane gave tri-N-salicylidene-N-acetylribostamycin (**2a**) in quantitative yield. m.p. 150°C with sintering at 120°~125°C. $[\alpha]_D^{24}$+49° (*c* 1.0, MeOH). CD_{max} in MeOH: 386 nm ([θ] 3,800), 316 (—6,700), 273 (5,100).

Anal. Calcd. for $C_{40}H_{45}N_4O_{14}$: C 59.4, H 6.0, N 6.9
Found: C 59.9, H 5.7, N 6.5

For reference, tetra-N-salicylidene ribostamycin (**1a**) was prepared by a similar procedure. m.p. *ca.* 135°C with sintering at 105°~115°C, $[\alpha]_D^{24}$+109° (*c* 1.0, MeOH). CD_{max} in MeOH: 380 nm ([θ] 4,500), 315 (5,800), 271 (23,000).

Anal. Calcd. for $C_{45}H_{50}N_4O_{14}$: C 62.1, H 5.8, N 6.4
Found: C 62.1, H 5.9, N 6.0

Isolation of 1-N-ethoxycarbonyl-2-DSA

A solution of **2** (620 mg) in H_2O (6.2 ml) was treated with ethoxycarbonyl chloride (470 mg) and $NaHCO_3$ (446 mg) for 4.5 hours. Excess of reagent was removed by extraction with ethyl ether, and the aqueous layer refluxed with an equal volume of conc. HCl for 40 minutes. After evaporation of solvent and HCl, the reaction mixture was applied on a Dowex 50W× 4 (H^+ type) resin (150 ml). Evaporation of the 0.3 N NH_4OH eluate gave a crude powder (190 mg), which was chromatographed on CM-Sephadex C-25 (50 ml), developing with 0.1 M ammonium formate. The effluent was cut into each 20-ml fractions, and fractions Nos. 14~21 gave after desalting with Dowex 50W×4 (H^+ type) resin, 1-N-ethoxycarbonyl-2-DSA (80 mg) as an amorphous powder. This powder (50 mg) was chromatographed on active carbon column (9.0 ml) and 10% EtOH eluate gave a pure colorless powder (23 mg). m.p. 193°~201°C (dec.) $[\alpha]_D^{25}$—8.9° (*c* 1.0, H_2O). By application of TACu method[12], $\Delta[M]_{436(TACu)}$ value was —720°. It showed $(M+1)^+$ at *m/e* 235. ^1H NMR in D_2O showed ethoxycarbonyl signals at δ 1.10 and 4.08, and C-2 methylene of 2-DSA at *ca.* δ 1.3 and 2.02, but no acetyl signal. It gave a single ninhydrin-positive spot at R_{DSA} 2.7 on silica-gel TLC developed with $CHCl_3$-EtOH-BuOH-17% NH_4OH (4:2:5:5).

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