^{13}C-NMR STUDIES ON RIBOSTAMYCIN AND ITS RELATED COMPOUNDS

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The ^{13}C-NMR spectra of ribostamycin and its related compounds were measured, and the correlation of chemical shifts and structures was discussed. With the aid of the ^{13}C-NMR technique, the structures of two bioinactivated compounds from ribostamycin have been determined.

The ^{13}C-NMR technique has now emerged as a potent tool for the structural investigation of carbohydrates. Although ^{13}C-NMR spectra of neutral sugars[1], steroids[2], amino acids[3] and many other compounds were hitherto examined, the field of amino sugars and amino cyclitols has remained little investigated, and no systematic study has been reported except for fragmentary reports on some aminoglycoside antibiotics[4a,4b].

In the present paper, the ^{13}C-NMR spectra, with full assignments of all the ^{13}C signals, are described for an aminoglycosidic antibiotic ribostamycin (SF-733) (**4a**) and related compounds, including 2-deoxystreptamine (**1a**), neamine (**2a**), paromamine (**3**), di-N-acetyl-2-deoxystreptamine (**1b**), mono-N-acetyl-2-deoxystreptamine (**1c**) and tetra-N-acetylneamine (**2b**).

All the compounds examined in this report gave well resolved ^{13}C-spectra, in contrast to ^{1}H-spectra which showed serious overlapping of signals, and a slight structural change brought about distinct ^{13}C signal shifts. For the spectral assignment, the ^{13}C resonance shifts caused by the change of pD in D_2O and by N-acetylation of the amino groups were utilized.

Finally, the ^{13}C-NMR technique has been applied successfully to the determination of the structures, especially the site of modification, of two kinds of new derivatives (SF-733D and SF-733X) of ribostamycin inactivated by *Streptomyces ribosidificus*.

Results and Discussion

The ^{13}C-chemical shifts of ribostamycin (**4a**) and its related compounds (**1a**, **1b**, **1c**, **2a**, **2b** and **3**) are summarized in Table 1. As shown in Fig. 1, the ^{13}C-NMR spectrum of **4a** gave a well-resolved spectrum in which peaks of two anomeric carbons (C-1′ and 1″), a methylene carbon (C-2), an aminomethyl carbon (C-6′), a hydroxymethyl carbon (C-5″) and two other methine carbons bearing amino groups (C-1 and 3) could easily be assigned based on the general rules for ^{13}C-NMR chemical shifts[5]. On the other hand, it was rather difficult to assign other carbons, in particular those densely crowded in the middle region. This difficulty was overcome by utilizing the assignments of the constituent units (**1a** and **2a**) and an analogue (**3**), and the regular shift of ^{13}C signals accompanied by changing the pD and by N-acetylation.

The spectral assignment of 2-deoxystreptamine (**1a**) was unequivocal from the consideration of

Fig. 1. ^{13}C-NMR spectrum of ribostamycin (**4a**)

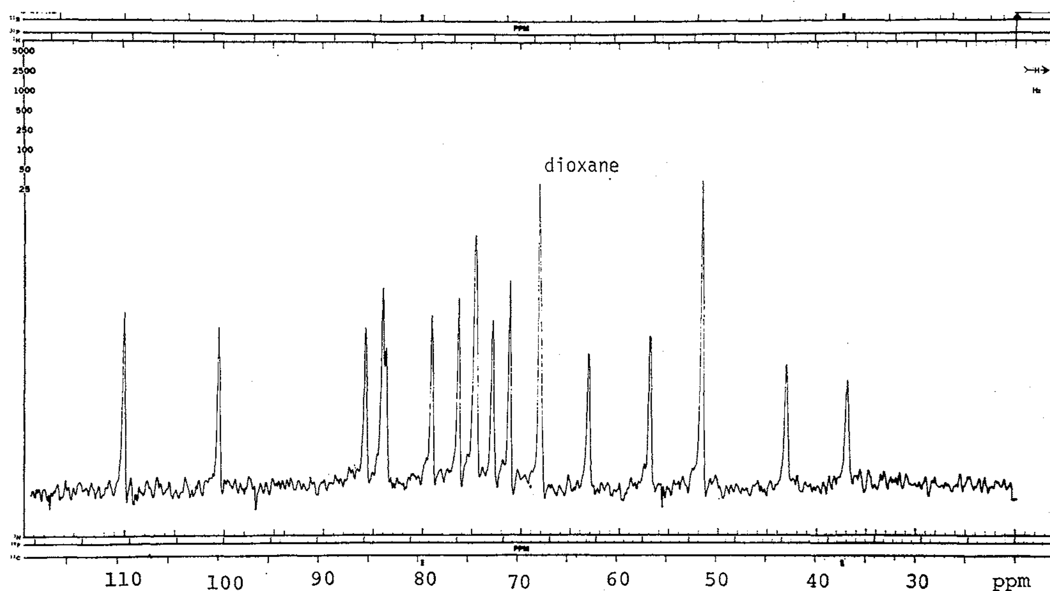


Table 1. ^{13}C-Chemical shifts of compounds **1a**~**4a**

Carbon	1a	1b	1c	2a	2b	3	4a
1	51.4	50.7	51.1	51.4	50.5	51.2	51.2
2	36.9	33.6	34.6	36.5	33.8	36.7	36.7
3	51.4	50.7	50.6	50.3	49.7	50.4	51.2
4	78.5	75.2	77.3	87.7	80.4	88.7	83.0
5	76.6	76.8	76.5	76.9	78.0	76.7	85.0
6	78.5	75.2	75.2	78.1	75.6	78.2	78.4
1′				101.5	98.4	101.9	99.8
2′				56.2	54.7	52.2	56.4
3′				74.4	71.5	74.6	74.1
4′				72.4	71.5	70.8	72.3
5′				73.4	71.5	73.8	73.9
6′				42.6	40.8	61.5	42.7
1″							109.1
2″							75.7
3″							70.5
4″							83.4
5″							62.6
$\underline{CH_3}CO$		23.2	25.0		23.1×3 23.6		
$CH_3\underline{CO}$		174.4	174.6		175.6 175.1 174.7 174.1		

the peak heights and the simple structure. In neamine (**2a**) and paromamine (**3**), an anomeric carbon appeared in the lowest field region. A signal appearing in the second lowest region, at 87.7 ppm in **2a** or 88.7 ppm in **3**, was assigned to C-4, because the C-4 is the site of glycosidation[6]. Other

Fig. 2. Correlation of the spectra of 2-deoxystreptamine (**1a**), paromamine (**3**), neamine (**2a**) and ribostamycin (**4a**)

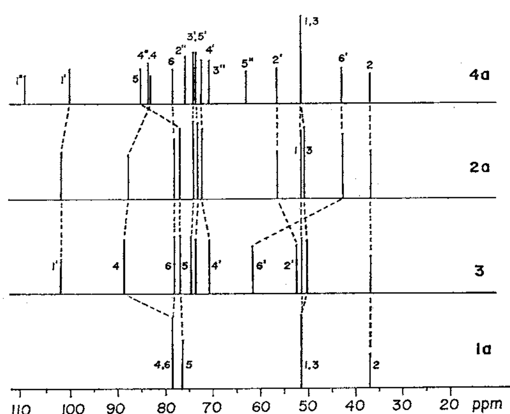
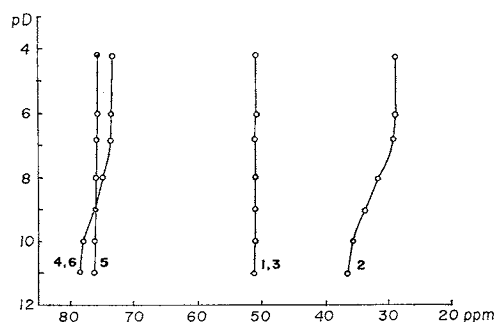


Table 2. ^{13}C-Chemical shifts of 2-deoxystreptamine (**1a**)

Carbon	pD						
	11.0	10.0	9.0	8.0	6.8	6.0	4.2
1, 3	51.4	51.4	51.3	51.2	51.2	51.2	51.1
2	36.9	36.0	34.2	32.0	29.5	29.2	29.2
4, 6	78.5	78.0	76.6	75.1	73.9	73.7	73.2
5	76.6	76.4	76.2	76.0	75.7	75.7	75.6

Fig. 3. Dependence on pD of chemical shifts for individual carbons of 2-deoxystreptamine (**1a**)



signals were reasonably assigned as shown.

It has been noted in ^{1}H-NMR spectroscopy that protonation and N-acetylation of amino groups in amino sugars cause considerable downfield shift of the signal for the methine bearing the amino group[7]. It was surprising, however, that a similar study of the ^{13}C spectra revealed the most pronounced effect on the β-carbons rather than the α-carbons relative to an amino group. For example, the chemical shifts of C-2, 4 and 6, which are adjacent to the aminomethylene carbons in **1a** (Fig. 3 and Table 2) were shifted upfield (5.3~7.7 ppm) by protonation, while the signals of C-1 and 3, α to the amino groups, and that of C-5, γ to the amino groups, underwent little shift. The magnitude of the upfield shift was dependent upon the degree of protonation. This is clearly seen in Fig. 3, where the chemical shifts of C-4 and C-6 in **1a** increase continuously as the pD of the solution is raised. Marked upfield shifts (2.5~7.3 ppm) of β carbons to amino groups occurred also on N-acetylation as shown in Figs. 4 and 5 and Table 1.

Fig. 4. Correlation of the spectra of 2-deoxystreptamine (**1a**), di-N-acetyl-2-deoxystreptamine (**1b**) and mono-N-acetyl-2-deoxystreptamine (**1c**)

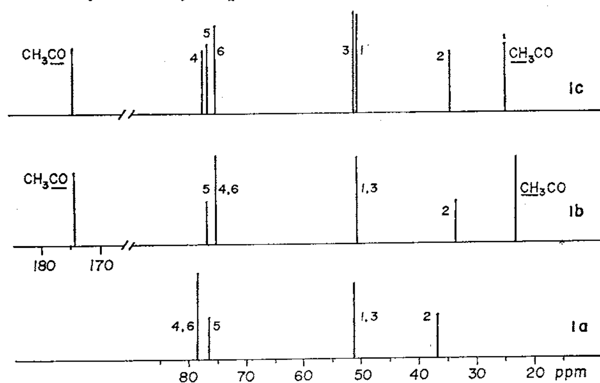


Fig. 5. Correlation of the spectra of neamine (**2a**) and tetra-N-acetylneamine (**2b**)

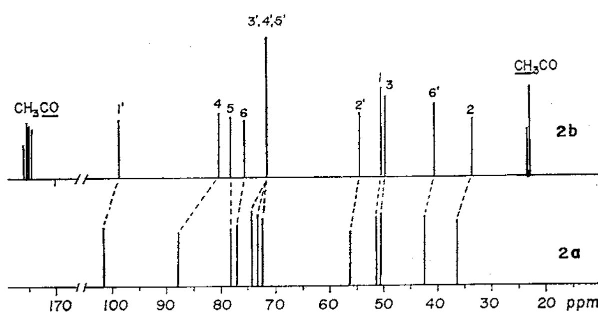


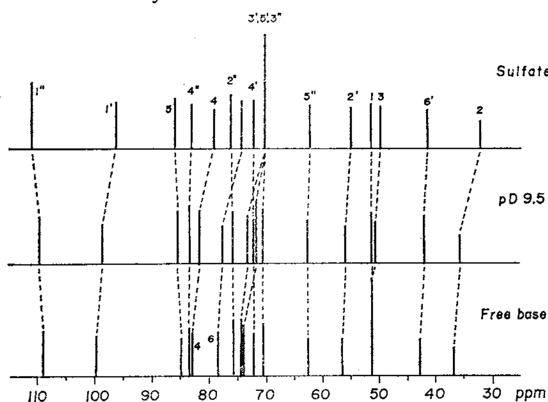
Table 3. ^{13}C-Chemical shifts of compounds **4a**~**4d**

Carbon	**4a**	**4a***	**4b**	**4c**	**4d**
1	51.2	51.3	51.4	50.4	50.6
2	36.7	35.6	32.0	33.3	33.4
3	51.2	50.7	49.8	49.0	49.0
4	83.0	81.6	79.2	76.9	76.5
5	85.0	85.5	86.0	85.8	86.3
6	78.4	77.7	74.4	74.7	74.3
1′	99.8	98.7	96.4	96.9	96.9(d)
2′	56.4	56.0	55.0	54.4	54.0
3′	74.1	71.6	70.0	71.3	71.4
4′	72.3	72.3	72.0	71.3	71.4
5′	73.9	73.1	70.0	71.3	71.4
6′	42.7	42.0	41.4	41.2	40.7
1″	109.1	109.6	111.0	109.6	110.8(d)
2″	75.7	75.9	76.1	75.8	87.7
3″	70.5	70.5	70.0	70.6	81.7
4″	83.4	83.3	83.3	83.4	86.3
5″	62.6	62.5	62.1	62.6	63.3
$\underline{CH_3}CO$				23.0×4	23.2×4
$CH_3\underline{CO}$				175.2	175.2
				174.9	174.7×2
				174.5	174.0
				174.1	
$-O{>}\underline{C}{<}CH_3$ / $-O{>}\ \ {<}CH_3$					113.8(s)
$-O{>}C{<}\underline{CH_3}$ / $-O{>}\ \ {<}\underline{CH_3}$					25.0 26.7

* pD 9.5

Letters in parentheses gave off resonance data.

Fig. 6. Correlation of the spectra of a free base, a sulfate and a partially protonated form of ribostamycin



Based on these observation on model compounds, it was now possible to fully assign the ^{13}C spectrum of **4a**.

Comparison (Fig. 2) of the spectrum **4a** with those of **1a**, **2a** and **3** confirmed the signal assignment of the carbons C-1, 2, 3, 6, 1′, 2′, 1″, 3″ and 5″ in **4a**. Except for anomeric carbons, C-4 and C-5 were expected in the lowest region and two of three signals in the region of 83.0~85.0 ppm might be assignable to them.

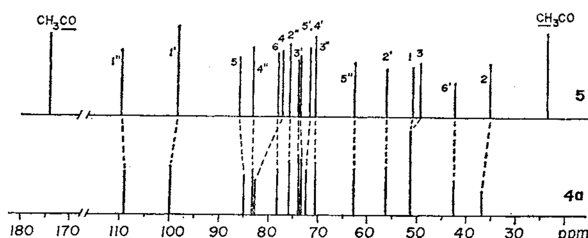
Utilizing the regular upfield shifting described above, the carbon signals from the ribose moiety, which is devoid of an amino group, could be distinguished from those from the other two moieties. The ^{13}C signals of C-2″, 3″, 4″ and 5″ remained essentially unshifted upon protonation and N-acetylation, but other carbons, except for the carbons γ to amino groups (C-5 and 4′) showed marked upfield shifts. A distinction between C-4 and C-5 was also possible by the application of the same technique. A ^{13}C-signal at 83.0 ppm in the free base showed a noticeable upfield shift (3.8 ppm) in the sulfate, and hence was assigned to C-4 which is a β carbon to the C-3 amino group. On the other hand, a signal at 85.0 ppm which showed negligible shifting either by protonation or N-acetylation could be assignable to C-5. Since the C-6 signal of **1a**, **2a** and **3** appeared in a narrow region at 78.1~78.5 ppm, the signal at 78.4 ppm in **4a** was assigned to C-6. Furthermore, the assignment of the ribose carbons was consistent with the reported data for uridine[8], and the reported data[9] for methyl α-D-glucopyranoside were employed for the 2,6-diaminoglucose moiety. Comparison of the two spectra of tetra-N-acetylribostamycin (**4c**) and its 2″,3″-O-isopropylidene derivative (**4d**) confirmed the assignment of C-4″ of ribose. Owing to O-alkylation in **4d**, C-2″ and 3″ were shifted downfield, but the signal assigned to C-4″ was not shifted.

The structure of SF-733D (5)

SF-733D was one of the metabolites of ribostamycin inactivated by *Streptomyces ribosidificus*[10].

The ^{1}H-NMR and MS analyses indicated that SF-733D was a 3- or 1-N-acetylribostamycin. In the present investigation, the ^{13}C-NMR spectrum of SF-733D (**5**) was examined in order to determine the position of the N-acetyl group. As shown in Fig. 7, the resonances due to an N-acetyl group were observed at 174.0 and 23.5 ppm, together with an upfield shift of the C-3 (or C-1) signal. This indicated that one of the two amino groups in the 2-deoxystreptamine moiety was N-acetylated. For reference, ^{13}C-NMR of mono-N-acetyl-2-deoxystreptamine (**1c**) showed N-acetyl signals at 25.0 and 174.6 ppm, together with a similar upfield shift of C-3 (or C-1). The position of N-acetylation could be determined to be the C-3 amino group since the resonance of C-4 in **5**, which is β to the C-3 amino group, underwent a distinct upfield shift (5.9 ppm), apparently caused by N-acetylation of the C-3 amino group. On the other hand, the resonance of C-6, which is β to the C-1 amino group was almost unchanged. Consequently, the structure of SF-733D (**5**) was determined to be 3-N-acetylribostamycin.*

Fig. 7. Correlation of the spectra of ribostamycin (**4a**) and SF-733D (**5**)



The structure of SF-733X (**6**)

SF-733X was another metabolite of ribostamycin bioinactivated by *S. ribosidificus*. ^{1}H-NMR

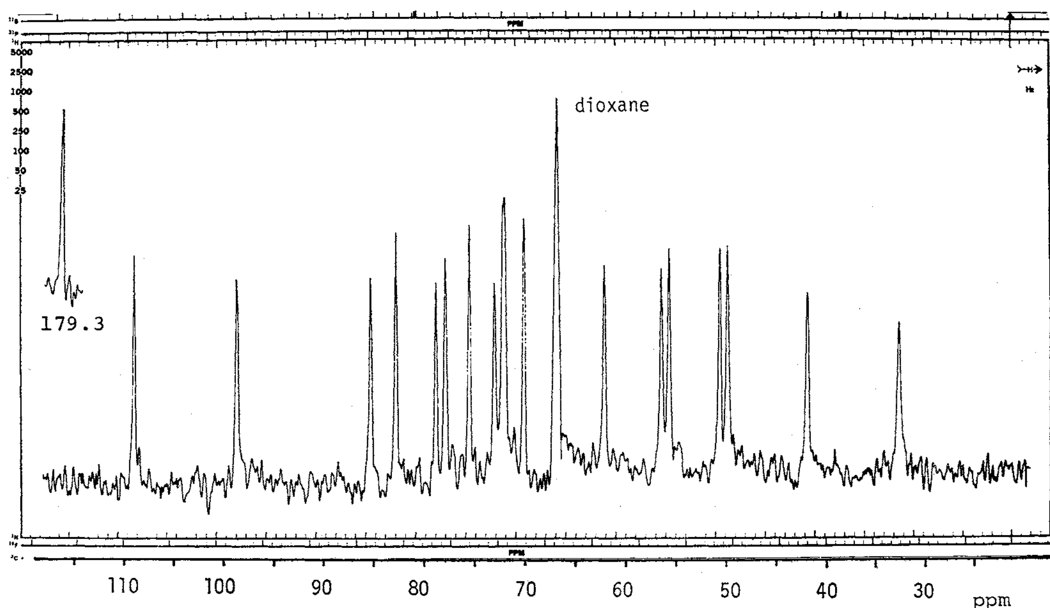
Table 4. ^{13}C-Chemical shifts of compounds **1d**~**6**

Carbon	**1d***	**1d****	**4a**	**5**	**6**	**6*****
1	51.5	51.2(d)	51.2(d)	51.0	51.1	51.1(d)
2	34.2	27.4(t)	36.7(t)	35.3	33.2	26.9(t)
3	57.7	57.5(d)	51.2(d)	49.4	57.0	54.4(d)
4	76.6	72.4	83.0	77.1	79.3	77.6
5	76.6	75.6	85.0	85.9	85.8	84.7
6	78.5	72.6	78.4	78.1	78.4	73.4
1′			99.8(d)	98.5	99.1	97.4(d)
2′			56.4(d)	56.3	56.2	54.4(d)
3′			74.1	74.0	73.5	71.6
4′			72.3	71.7	72.5	69.3
5′			73.9	73.5	72.7	69.8
6′			42.7(t)	42.3	42.5	41.5(t)
1′′			109.1	109.6	109.4	111.8(d)
2′′			75.7	75.9	76.0	76.4
3′′			70.5	70.7	70.6	70.9
4′′			83.4	83.3	83.3	83.1
5′′			62.6(t)	62.7	62.7	61.3(t)
-NHC̲H₂COOH	50.6	47.0(t)			50.4	47.6(t)
-NHCH₂C̲OOH	180.0	170.6			179.3	171.9
-NHCOC̲H₃				23.5		
-NHC̲OCH₃				174.0		

* pD 11.0, ** pD 2.0, *** pD 4.5 Letters in parenthesis gave "off resonance" data.

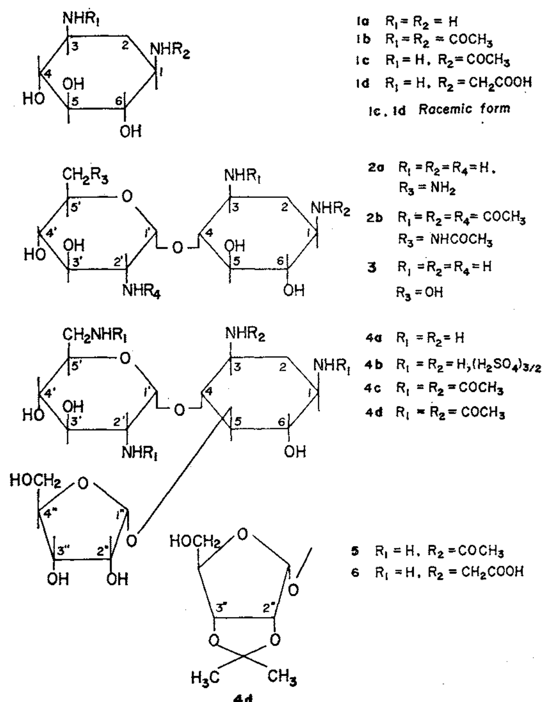
* K. L. RINEHART, Jr. *et al.* isolated mono-N-acetyl-neomycins[17], neomycins LP_1 and LP_2 in which C-3 amino groups of deoxystreptamine moiety of neomycins C and B were N-acetylated.

Fig. 8. ^{13}C-NMR spectrum of SF-733X (6)



and MS analyses and chemical degradation indicated that SF-733X was a 3-N- or 1-N-carboxymethyl-ribostamycin[11].

^{13}C-NMR was now applied in order to prove the position of the carboxymethyl group. The ^{13}C-NMR spectrum of SF-733X (**6**) (Fig. 8) showed an additional two carbons, at 179.3 and 50.4 ppm, compared with that of **4a**. Further ^{13}C-NMR investigation including an "off resonance" experiment (Table 4) and pD change revealed that the resonance at 179.3 ppm was the signal of a carboxyl carbon, and that the resonance at 50.4 ppm was due to the methylene carbon sandwiched between a carboxyl and an amino group. This assignment was supported by the spectrum of synthetic mono-N-carboxymethyl-2-deoxystreptamine (**1d**)[12] (Table 4), which showed the resonances of a carboxymethyl group at 180.0 and 50.6 ppm in alkaline solution. These signals were shifted upfield by 9.4 and 3.6 ppm upon acidification, just as were the corresponding signals of **6** (7.4 and 2.8 ppm). Thus, the N-carboxymethyl group was independently proven by ^{13}C-NMR.



Of the two signals at 51.1 and 57.0 ppm assignable to C-1 and 3, the resonance at 51.1 ppm could be assigned to C-1, because the resonance position of C-1 remained constant in all the compounds examined in this work and little

affected by protonation and N-acetylation. In contrast, the resonance of C-3 moved upward in **4a** by protonation. Thus the ^{13}C-NMR decided in favor of C-3 substitution by a carboxymethyl group. Independent evidence for C-3 substitution came from the behaviours of the C-4 and 6 signals. The signals at 85.8, 83.3 and 78.4 ppm in **6** were assignable to C-5, 4″ and 6, because they were very close to the C-5, 4″ and 6 signals at 85.0, 83.4 and 78.4 ppm in **4a**. Upon protonation of the amino groups, the C-6 signal in **6** showed an upfield shift of 5.0 ppm which was comparable to the shift (4.0 ppm) of C-6 in **4a**. Therefore, the signal at 79.3 ppm was assignable to C-4, 3.7 ppm higher than that in **4a**. Since N-alkylation of an amino group has been reported to cause an upfield shift of a β carbon to the amino group[4a,5], the higher chemical shift of the C-4 signal could easily be explained if the C-3 amino group was carboxymethylated.

Experimental

^{13}C-NMR spectra

Proton noise decoupled ^{13}C-NMR spectra were recorded at a frequency of 25.2 MHz by means of a Varian XL-100-12 spectrometer using deuterium oxide in solution as the source of the lock signal. A Varian C-1024 time averaging computer was used to accumulate 150~2,000 scans of 2,500~5,000 Hz at a scan speed of 100 or 250 seconds. In general, 150~300 mg of the sample to be measured was dissolved in 0.8~1.2 ml of D_2O containing 2.0~5.0% of dioxane as an internal standard, and if necessary, the solution was adjusted to an appropriate pD before measurement. Chemical shifts were measured relative to the ^{13}C signal of dioxane and converted to parts per million from TMS using δ (dioxane) =67.4 ppm.

Materials

Preparation of SF-733D, SF-733X and mono-N-carboxymethyl-2-deoxystreptamine will be published in this Journal[10,12].

Preparation of mono-N-acetyl-2-deoxystreptamine (1c)

To a suspension of 2-deoxystreptamine free base (1.63 g) in MeOH (100 ml) was added dropwise acetic anhydride (1.8 ml) under ice-cooling. The resulting solution stood at room temperature for 2 hours, then was concentrated to dryness. The residue was dissolved in H_2O (150 ml) and passed through a column of Dowex 50W×2(NH_4^+) (4.5×21 cm). After washing with H_2O, the column was eluted with 0.04 N NH_4OH, 0.06 N NH_4OH and 0.08 N NH_4OH. Evaporation of the 0.06 N NH_4OH eluate gave mono-N-acetyl-2-deoxystreptamine (1.1 g), which was crystallized from MeOH, mp 229~230°C.

Anal. Calcd. for $C_8H_{16}N_2O_4$: C 47.1, H 7.9, N 13.7.
Found: C 46.7, H 8.4, N 13.5.

From the 0.08 N NH_4OH eluate 240 mg of the unreacted 2-deoxystreptamine was recovered.

Preparation of tetra-N-acetyl-2″,3″-O-isopropylideneribostamycin (4d)

To a solution of tetra-N-acetylribostamycin (4.36 g) in DMF (150 ml) were added 2,2-dimethoxypropane (5.0 ml) and *p*-toluenesulfonic acid (400 mg). The resulting solution stood overnight at room temperature and then was diluted with MeOH (200 ml). The reaction mixture was treated with Amberlite IRA 400 (OH^-) (100 ml) and concentrated to dryness to give crude tetra-N-acetyl-2″,3″-O-isopropylideneribostamycin, which was crystallized from ethanol-ethyl acetate (3.8 g) mp 178~180°C (dec.), $[\alpha]_D^{22}$+37.5°C (*c* 1, MeOH).

Anal. Calcd. for $C_{28}H_{46}N_4O_{14}$: C 50.8, H 7.0, N 8.5.
Found: C 50.6, H 6.8, N 8.3.

Other compounds employed in this work were prepared according to the procedures reported in the following literature references: 2-Deoxystreptamine (**1a**)[13], mp 220~221°C (dec.); di-N-acetyl-2-

deoxystreptamine (**1b**)[14] mp 290~291°C (dec.); neamine (**2a**)[14], mp 244~246°C (dec.); tetra-N-acetylneamine (**2b**)[14], mp 306~308°C (dec.); paromamine (**3**)[15], mp 237~239°C (dec.); ribostamycin (**4a**)[16], mp 193~195°C (dec.); tetra-N-acetylribostamycin (**4c**)[14], mp 203~205°C (dec.). Ribostamycin sulfate (**4b**) was supplied from Kawasaki Factory of this company.

All the compounds used in this study were homogeneous on paper chromatography (1-BuOH-pyridine-AcOH-H_2O, 6 : 4 : 1 : 3) or silica gel thin-layer chromotography ($CHCl_3$-MeOH-conc.-NH_4OH, 1 : 4 : 2 : 1).

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