# MECHANISM AND STEREOCHEMISTRY OF THE BIOSYNTHESIS OF 2-DEOXYSTREPTAMINE AND NEOSAMINE C†

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(Received for publication December 1, 1988)

Feeding experiments with D-[6,6-$^2H_2$]-, D-(6*R*)-[6-$^2H_1$]- and D-(6*S*)-[6-$^2H_1$]glucose in the fermentation of *Streptomyces ribosidificus*, followed by field desorption MS and $^2H$ NMR analyses of the resulting labeled ribostamycin samples, clearly demonstrated that 1) both hydrogens of the C-6 hydroxymethyl group of D-glucose are stereospecifically incorporated into the C-2 position of 2-deoxystreptamine and 2) the *pro S* hydrogen of the C-6 position of D-glucose is stereospecifically removed during the elaboration of neosamine C in the biosynthesis of ribostamycin. A plausible mechanism of formation of the deoxy-*scyllo*-inosose, an early precursor to 2-deoxystreptamine, is suggested to be analogous to the dehydroquinate synthesis in the shikimate pathway and the conversion of the C-6 hydroxymethyl group of D-glucose into the aminomethyl group of neosamine C is likely to involve a dehydrogenation step to a formyl group.

Biosynthesis of antibiotics has been extensively studied for their medical importance and structural uniqueness. However, there are many questions yet to be addressed to complete our knowledge of the detailed mechanisms of the biochemical processes involved in these antibiotics' production.[1,2] This field will continue to offer intriguing problems, especially in conjunction with the rapid progress of genetic manipulation techniques in the actinomycetes.[3,4]

We have studied from these standpoints the biosynthesis of the typical and structurally unique microbial secondary metabolites 2-deoxystreptamine and neosamine C, found in a class of aminocyclitol antibiotics. The genetics and biosynthetic mechanisms of these antibiotics have been attracting wide attention.[5~10] This paper provides a full account of our studies on the biochemical mechanisms and stereochemistry, necessary preliminaries to interdisciplinary research on genetics and enzymology, during the bio-conversion of D-glucose into 2-deoxystreptamine and neosamine C in ribostamycin produced by *Streptomyces ribosidificus*.[11,12]

The studies carried out earlier in various laboratories with isotope-tracer techniques and/or idi-

† This paper is dedicated to Professor KENNETH L. RINEHART on the occasion of his 60th birthday in appreciation of his outstanding leadership in the field of aminocyclitol antibiotics.

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otrophs, blocked mutants, clearly established that 2-deoxystreptamine is biosynthesized from D-glucose through 2-deoxy-*scyllo*-inosose, but not from *myo*-inositol.[13~17] The latter is utilized to form streptidine and actinamine, which are fully substituted aminocyclitols.[7] Therefore, a question being tackled in the present study was the precise mechanism and stereochemistry by which the 2-deoxy-*scyllo*-inosose precursor is formed from D-glucose at the very beginning of 2-deoxystreptamine biosynthesis. Although the later stages, starting from 2-deoxy-*scyllo*-inosose, in the biosynthesis of 2-deoxystreptamine have been studied somewhat by cell free systems,[18,19] even now, no enzyme involved in this particular biochemical process has been isolated nor has a cell free reaction system been established. We, therefore, took advantage of deuterium labeling and $^2$H NMR analyses in the present study. Similarly, although conversion of D-glucose into neosamine C *via* D-glucosamine was firmly established,[20] no intermediate in this process has been isolated nor has a detailed mechanism of the functional group modification from a hydroxymethyl group into an aminomethyl group been clarified. For this problem also, deuterium tracer and $^2$H NMR methodology must be quite effective and it seemed worthwhile to demonstrate the usefulness in this study.

## Materials and Methods

Site-specifically and/or stereospecifically deuterated substrates were prepared chemically. D-[6,6-$^2H_2$]Glucose was prepared from D-glucurono-6,3-lactone according to the literature procedure.[21] Both D-(6*R*)- and D-(6*S*)-[6-$^2H_1$]glucose were synthesized by the method already described.[22] The ribostamycin-producing strain was *S. ribosidificus*. Fermentation was carried out aerobically at 28°C in a 500-ml Erlenmeyer flask containing 80 ml of the production medium on a rotary shaker. The production medium was composed of glucose 2.5%, Bacto-soyton 3.0%, distillers solubles 0.5%, $MgSO_4 \cdot 7H_2O$ 0.25%, and $FeSO_4 \cdot 7H_2O$ 0.0005% in distilled water. The pH was adjusted before autoclaving to 7.5. The inoculum size was 2%, which was obtained from a 48-hour seed culture. The deuterated substrate, D-[6,6-$^2H_2$]glucose (2.0 g), was added aseptically to the culture 48 hours after inoculation and fermentation was continued for 7 days. Chirally deuterated substrates, D-(6*R*)- and D-(6*S*)-[6-$^2H_1$]glucose (1.0 g each), were added to the culture separately 24 hours after inoculation and fermentation was also conducted for 7 days.

Isolation and purification of the deuterium enriched specimens of ribostamycin were carried out according to literature procedures guided by antibacterial assay against *Bacillus subtilis* PCI 219 and ninhydrin reaction.[22] The filtered broth was passed through a column of Amberlite IRC-50 ($NH_4^+$ form), which was then washed well with deionized water. The adsorbed antibiotic was eluted with 1 N $NH_4OH$. The crude sample was further purified by repeated chromatography on Amberlite CG-50 ($NH_4^+$ type) with gradient elution (0.1 N $NH_4OH$ to 0.8 N $NH_4OH$) to yield chromatographically homogeneous labeled samples of ribostamycin. Identification of the specimens obtained by these feeding experiments was carried out by comparison with an authentic specimen using TLC. Visualization of the chromatogram was performed by ninhydrin reaction as well as bioautography against the same test organism.[23,24] An enriched sample (180 mg) was obtained by supplementation of D-[6,6-$^2H_2$]glucose. Similarly, 129 mg of enriched ribostamycin was obtained by supplementation of D-(6*S*)-[6-$^2H_1$]glucose and 99 mg of enriched antibiotic from D-(6*R*)-[6-$^2H_1$]glucose.

Field desorption (FD)-MS were recorded on a Hitachi M-80 spectrometer. $^1$H and $^2$H NMR spectra were obtained with a Jeol GX-400 spectrometer. The chemical shifts were calculated from the solvent signal. For $^1$H NMR, the spectrometer was operated at 400 MHz using a sample (free base) dissolved in deuterated water and the solvent signal was used as an internal standard located at 4.8 ppm. The $^2$H NMR spectra were obtained at 61.46 MHz as a solution in distilled water and the natural abundance deuterium signal of the solvent was used as an internal standard at 4.8 ppm.

## Results

The FD-MS of a ribostamycin sample (free base) obtained by supplementation of D-[6,6-$^2H_2$]-glucose showed a significant peak at *m/z* 457 (M+H+2 amu) as depicted in Fig. 1. The non-labeled

Fig. 1. FD-MS of a deuterium enriched ribostamycin sample prepared by feeding of D-[6,6-$^2H_2$]glucose.

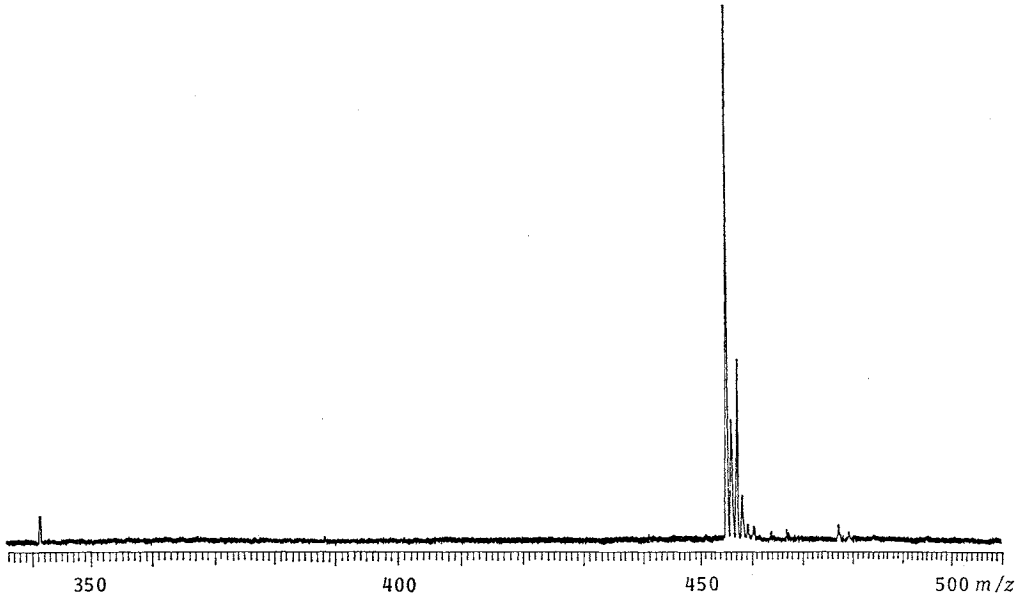
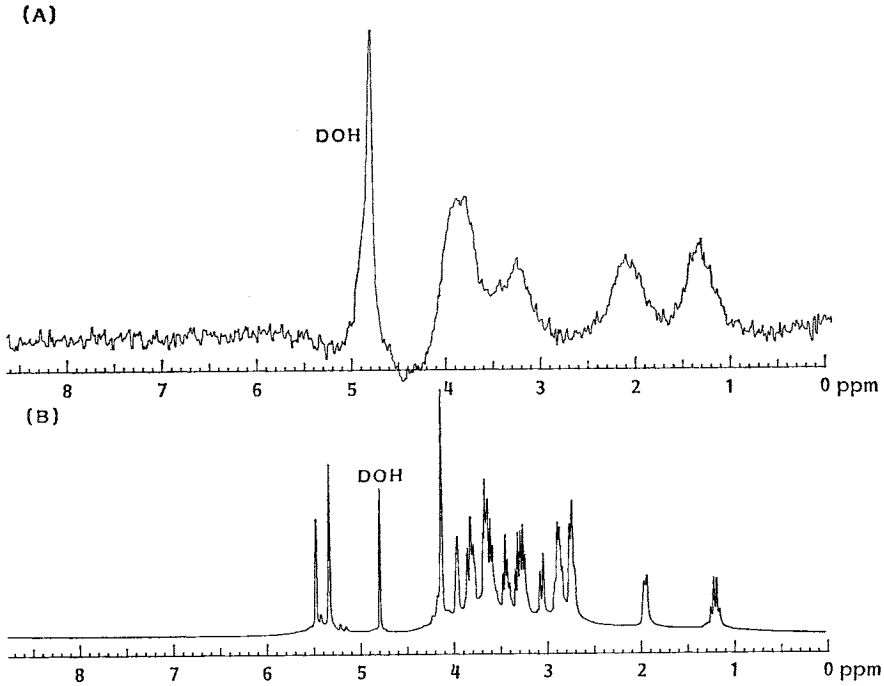



Fig. 2. $^1H$ and $^2H$ NMR spectra of the deuterium enriched sample prepared by feeding of D-[6,6-$^2H_2$]-glucose.

(A): $^2H$ NMR spectrum in $H_2O$ at 61.46 MHz, (B): $^1H$ NMR spectrum in $^2H_2O$ at 400 MHz.



*quasi*-molecular ion peak (base peak) was observed at *m/z* 455 (M+H). The intensity of the former was about 33.7% of the latter, while the ion at *m/z* 456 was about 21.7% of the *quasi*-molecular ion. The ions ($M^+$+Na) and ($M^+$+Na+2 amu) were also observed at *m/z* 477 and 479, respectively. These data clearly indicated incorporation of deuterium from the supplemented deuterated glucose. The $^1H$ and $^2H$ NMR spectra of the sample are shown in Fig. 2. The $^1H$ NMR spectrum confirmed the product to be ribostamycin. Relevant protons were assigned as follows. The signals due to protons on C-2 of 2-deoxystreptamine were assigned to those observed at $\delta$ 1.19 (br q, $J$=12.5 Hz, axial) and at $\delta$ 1.94 (td, $J$=4 and 13 Hz, equatorial). The protons on C-6′ of neosamine C appeared as a pair of dd at $\delta$ 2.82 ($J$=3 and 14 Hz) and at $\delta$ 3.02 ($J$=7 and 14 Hz). In the D-ribose moiety, protons of the C-5″ hydroxymethyl group were observed as another pair of dd at $\delta$ 3.65 ($J$=6.4 and 12.5 Hz) and at $\delta$ 3.83 ($J$=3 and 12.5 Hz). However, the enrichment of deuterium was not high enough to estimate the decrease of signal intensities which might have conceptually been expected. The $^2H$ NMR spectrum showed four broad signals and assignments of the signals were made by comparison with the corresponding $^1H$ NMR chemical shifts, though there are small differences of the corresponding chemical shifts. A signal at $\delta$ 1.3 was assigned to the axial deuterium at the C-2 position of 2-deoxystreptamine, and a signal at $\delta$ 2.1 to the equatorial deuterium at the same C-2 position. A signal at $\delta$ 3.3 was assigned to a deuterium at the C-6′ aminomethyl group of neosamine C and a strong signal at $\delta$ 3.8 was assigned to the C-5″ hydroxymethyl group of the D-ribose moiety. Intensities of these signals were approximately 1 : 1 : 1 : 2, respectively.

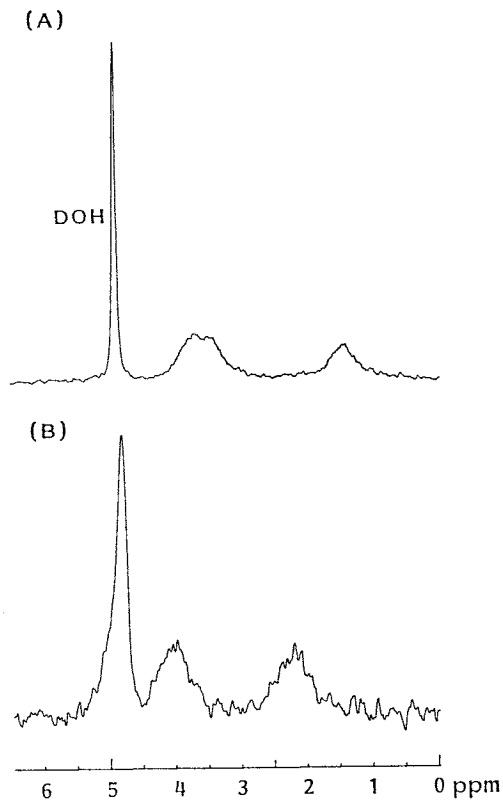



Fig. 3. $^2H$ NMR spectra of the deuterium enriched ribostamycin samples.

(A): A sample obtained by feeding of D-(6*R*)-[6-$^2H_1$]glucose, (B): a sample obtained by feeding of D-(6*S*)-[6-$^2H_1$]glucose.

The $^2H$ NMR spectrum of an enriched specimen produced by feeding of D-(6*S*)-[6-$^2H_1$]glucose is displayed in Fig. 3. Two signals are observed in equal intensities at $\delta$ 2.1 and at $\delta$ 3.9. The former signal indicates stereospecific incorporation of deuterium into the equatorial position of the C-2 methylene group of the 2-deoxystreptamine moiety and the latter suggested deuterium incorporation into the C-5″ position of the D-ribose moiety. No signal was observed either at $\delta$ 1.3 or at $\delta$ 3.3.

By contrast, deuterium incorporation from D-(6*R*)-[6-$^2H_1$]glucose was observed at $\delta$ 1.3, $\delta$ 3.3 and at $\delta$ 3.7. The latter two signals were not well resolved though. The signal at $\delta$ 1.3 indicated specific incorporation of deuterium into the axial position of the C-2 methylene group of 2-deoxystreptamine. The C-6′ aminomethyl group of neosamine C was also labeled with deuterium, as in-

dicated by the signal at $\delta$ 3.3.

## Discussion

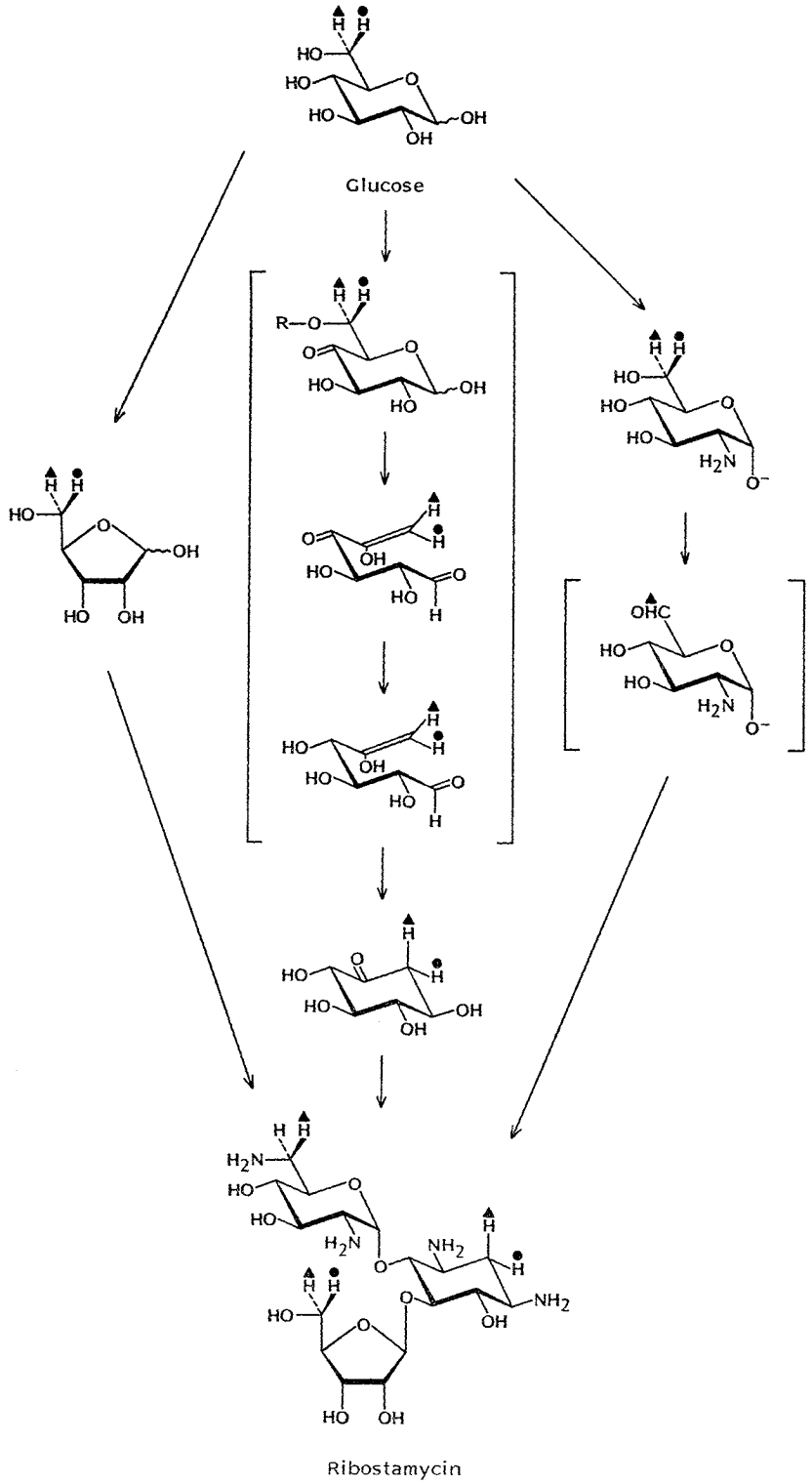
The precursor-product relationship for the biosynthesis of neomycins, closely related antibiotics to ribostamycin, was worked out by RINEHART and co-workers using $^{13}C$ tracer methodology.[13] Feeding experiments with D-[6-$^{13}C$]glucose and D-[1-$^{13}C$]glucosamine to the fermentation of *Streptomyces fradiae*, followed by $^{13}C$ NMR analyses of the resulting enriched neomycin demonstrated that: 1) The C-2 carbon and the C-1 carbon of 2-deoxystreptamine are derived from C-6 and C-1 of D-glucose, respectively, 2) the hydroxymethyl group of the D-ribose moiety is derived partially from C-6 of D-glucose and 3) the aminomethyl groups of neosamines B and C are also derived from C-6 of D-glucose.

The FD-MS spectrum of the enriched specimen obtained in this study by feeding of D-[6,6-$^2H_2$]-glucose showed a significant ion at *m/z* 457 (Fig. 1), which is two mass units higher than the *quasi*-molecular ion (*m/z* 455) of nondeuterated molecules. The three structural units of ribostamycin are supposed to be formed independently and the antibiotic molecule is then constructed by a series of reactions assembling these units. Although all of these structural units are derived from D-glucose as described above, dilution of the labeled substrate during the culture must be taken into account. The probability of simultaneous enrichment of two structural units in a single molecule may not be quite high, hence, the two-mass units difference of the *quasi*-molecular ion mentioned above can suggest intact incorporation of the C-6 methylene group of D-glucose into one of the structural units. However, location of such enrichment could not be determined only with these mass spectral data. The $^2H$ NMR spectrum of this enriched specimen showed four signals (Fig. 2). Incorporation of deuterium into both the axial and equatorial positions on the C-2 methylene group of 2-deoxystreptamine demonstrated that the bond breaking at the C-6 position of D-glucose during the formation of a six-membered carbocyclic intermediate was not one of the two C-H bonds or the C-C bond between C-6 and C-5, but rather the C-O bond instead. Furthermore, stereospecific incorporation of the chirally deuterated substrates, D-(6*R*)- and D-(6*S*)-[6-$^2H_1$]glucose, as evidenced by the $^2H$ NMR spectra in Fig. 3, indicated that no randomization of deuterium incorporation took place, which reinforced the above discussions. Consequently, these data firmly ruled out two hypothetical pathways; 1) cyclization of D-glucose into a *myo*-inositol- or *myo*-inosose-like intermediate, as in the biosynthesis of streptidine, followed by deoxygenation and 2) deoxygenation of D-glucose into a 6-deoxyhexose type intermediate and cyclization. First of all, any *myo*-inositol like intermediate cannot account for the incorporation of both of the hydrogens on C-6 of D-glucose, since one hydrogen should have been eliminated during the formation of a fully substituted six-membered carbocyclic ring. Secondly, if deoxygenation at C-6 of D-glucose would take place first, incorporation of the deuterium cannot be stereospecific, because a hydrogen should have been introduced from the medium and, thereby, labeling should have been randomized.

The present results were quite consistent with the previous proposals. 2-Deoxy-*scyllo*-inosose was suggested to be a very early intermediate in the biosynthesis of 2-deoxystreptamine based on the extensive studies from various laboratories using idiotrophic mutants of the gentamicin-producing organism *Micromonospora purpurea* VIb-3P, studied by DAUM *et al.*,[14] the butirosin-producing *Bacillus circulans*, studied by the Tanabe group and the Takeda group,[15,16] and the sagamicin-producer *Micromonospora sagamiensis*, studied by KASE *et al.*[17] The results discussed above clearly showed that the C-O bond of the hydroxymethyl group of D-glucose was replaced by the C-C bond between C-1 and C-2 of 2-deoxystreptamine.

Taking into account all of the available evidences, we propose the following mechanism. D-Glucose or its 6-phosphate is converted into an enol or enolate-type intermediate by a lyase-like enzyme and then such an intermediate cyclizes by an aldol-reaction to 2-deoxy-*scyllo*-inosose. This is analogous to the dehydroquinate synthase reaction to form dehydroquinate from 3-deoxy-*arabino*-heptulosonic acid 7-phosphate in the shikimate pathway.[25] Involvement of an intermediate like a hexos-4-ulose to facilitate a $\beta$-elimination reaction by a hypothetical lyase-like enzyme may further be postulated, which is again similar to the shikimate pathway. This proposal has actually been sup-

Fig. 4. The mechanism and stereochemistry of the biosyntheses of individual components of ribostamycin. R represents H or a phosphate group.



ported recently by an independent study by AKHTAR *et al.*,[26] and GODA and AKHTAR.[27]

Incorporation of deuterium into the D-ribose unit from all of the substrates tested is not surprising because the D-ribose unit of neomycin was already demonstrated to be derived in part by way of the hexose monophosphate pathway from D-glucose.[13] Interesting to note was that the incorporation ratios of D-glucose into D-ribose and 2-deoxystreptamine seemed to be quite similar. This only means that the dilution ratios of these units by nondeuterated counterparts are similar and does not suggest by any means that the metabolic pools of these two structural units or their turnover rates are equal.

Concerning the biosynthesis of neosamine C, feeding experiments with chirally deuterated substrate, D-(6*S*)- and D-(6*R*)-[6-$^2H_1$]glucose indicated that the *pro S* hydrogen of the C-6 hydroxymethyl group of D-glucose was stereospecifically replaced with a hydrogen derived from the medium and the *pro R* hydrogen was retained in neosamine C as can be seen in the $^2H$ NMR spectra (Fig. 3). These results strongly suggest a 6-aldehydo-hexose intermediate, which may subsequently be transformed through an unknown transamination mechanism into a 6-amino-6-deoxy-hexose unit. An analogous 6-ulose formation in primary metabolism is the uridine diphosphoglucose (UDPG) dehydrogenase reaction, which is a 4-electron NAD-linked oxidation of UDPG to UDP-glucuronic acid.[28] During this reaction, UDP-$\alpha$-D-*gluco*-hexodialdose is formed. The proposed mechanism of the neosamine C formation has been recently supported and the stereochemistry of the transamination step has been elucidated.[29,30]

The results described above bear out for the first time the precise mechanism of the biosynthesis of 2-deoxystreptamine containing antibiotics including the cryptic stereochemistry of the first stage of cyclization of D-glucose during the formation of 2-deoxystreptamine as well as in the conversion of the hydroxymethyl group into the aminomethyl group of neosamine C, as illustrated in Fig. 4. Also demonstrated is the usefulness of deuterium chiral labeling and $^2H$ NMR methodology to study the microbial metabolism from precise mechanistic standpoints.

Acknowledgment

This work was financially supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture.

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