Enzymatic Carbocycle Formation in Microbial Secondary Metabolism. The Mechanism of the 2-Deoxy-*scyllo*-inosose Synthase Reaction as a Crucial Step in the 2-Deoxystreptamine Biosynthesis in *Streptomyces fradiae*

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The mechanism of 2-deoxy-*scyllo*-inosose synthase reaction, a carbocycle formation step from D-glucose-6-phosphate in the biosynthesis of a major 2-deoxystreptamine aglycon of clinically important aminoglycoside antibiotics, was investigated with a partially purified enzyme fraction from neomycin-producing *Streptomyces fradiae* IFO 13147. Singly and doubly labeled D-[4-^{2}H]- and D-[4-^{2}H,3-^{18}O]glucose-6-phosphate were used for chase experiments, and the 2-deoxy-*scyllo*-inosose product was analyzed by ^{2}H-NMR and GC-MS. The deuterium label at C-4 of the substrate appeared to be retained at C-6 of the product without scrambling the doubly-labeled isotopes. Since the oxidative process with the aid of NAD^+ is essential, which was reported previously, the hydride abstraction and returning appear to take place within the same glucose molecule. These results strongly suggest that this carbocycle formation is catalyzed by a single 2-deoxy-*scyllo*-inosose synthase enzyme with a catalytic requirement of NAD cofactor, the mechanism of which is closely resembled to the dehydroquinate synthase in the shikimate pathway.

Aminoglycoside antibiotics are among those clinically important and a major structural feature is the existence of characteristic aminocyclitol aglycons.[1] In view of the structure and biosynthesis of aminocyclitols, aminoglycoside antibiotics are classified into two classes. One is those that contain a fully-substituted aminocyclitol such as streptomycin, hygromycin, fortimicin, *etc.* The aminocyclitols in these antibiotics were shown or proposed to be biosynthesized from *myo*-inositol,[2,3] which is ubiquitous in animals, plants, and microorganisms. On the other hands, the other major class of aminoglycoside antibiotics such as kanamycin, neomycin, ribostamycin, butirosin, *etc.* contain a unique aminocyclitol, 2-deoxystreptamine (**1**), which is found only in these antibiotics and is thus a typical product of microbial secondary metabolism.

The biosynthesis of 2-deoxystreptamine (**1**) has been attracting attention. It has been well-established that **1** is biosynthesized *via* non-aminogenous cyclitol 2-deoxy-*scyllo*-inosose (**2**), which is a product of intramolecular C–C bond formation between C-1 and C-6 of D-glucose (**3**).[4] Previously, we demonstrated that this crucial cyclization of **3** proceeds stereospecifically by the use of whole cell tracer technology with chiral deuterium labeling of D-glucose and ^{2}H-NMR spectroscopy for the biosynthesis of ribostamycin by *Streptomyces ribosidificus*,[5] and we proposed a mechanism of the cyclization reaction and a responsible enzyme as "2-deoxy-*scyllo*-inosose synthase". The proposed mechanism of 2-deoxy-*scyllo*-inosose synthase reaction is shown in Scheme 1. The first step is an oxidation at the C-4 position of the substrate D-glucose-6-phosphate (**4**), and an elimination of a phosphate group from the "activated" ulose is followed to form an enol or enolate intermediate. Subsequent reduction at C-4, followed by an aldol-type intramolecular condensation between C-1 and C-6 or *vice versa* gives rise to the product **2**. The reaction mechanism was further examined recently with a cell-free system of *Streptomyces fradiae* IFO 13147, which produces neomycins.[6] In this study **4** was verified to be a true substrate, and requirement of NAD^+ was elucidated. The mechanism of this reaction appears to closely resemble to that of dehydroquinate synthase in the shikimate pathway for the biosynthesis of aromatic amino acids in microorganisms and plants. These findings may raise an intriguing question as to the comparative biochemistry and evolution of microbial secondary and essential metabolisms.

To get more insight into the mechanism and enzymology of 2-deoxy-*scyllo*-inosose synthase, we undertook the chase experiments of the fate of the C-4 hydrogen of D-glucose-6-phosphate using isotopically single-labeled and doubly-labeled materials. Previously, Akhtar *et al.* reported that the C-4 hydrogen of D-glucose was not incorporated during the biosynthesis of **1** in the whole cells of *S. fradiae* (producing neomycins) and suggested involvement of an oxidoreduction at C-4 of the substrate.[7] This was seconded by our recent observation of the requirement of NAD^+ for the formation of **2** at the cell-free level, as mentioned above.[6] On the other hands, in the dehydroquinate synthase reaction, the corresponding C-5 hydrogen (on the β-position to the phosphate ester) is retained throughout the reaction despite the observa-

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(5) (a) Kakinuma, K.; Ogawa, Y.; Sasaki, T.; Seto, H.; Ōtake, N. *J. Am. Chem. Soc.* **1981**, *103*, 5614–5616. (b) Kakinuma, K.; Ogawa, Y.; Sasaki, T.; Seto, H.; Ōtake, N. *J. Antibiot.* **1989**, *42*, 926–933.

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(7) (a) Goda, S. K.; Akhtar. M. *J. Chem. Soc. Chem. Commun.* **1987**, 12–14. (b) Goda, S. K.; Akhtar, M. *J. Antibiot.* **1992**, *45*, 984–994.

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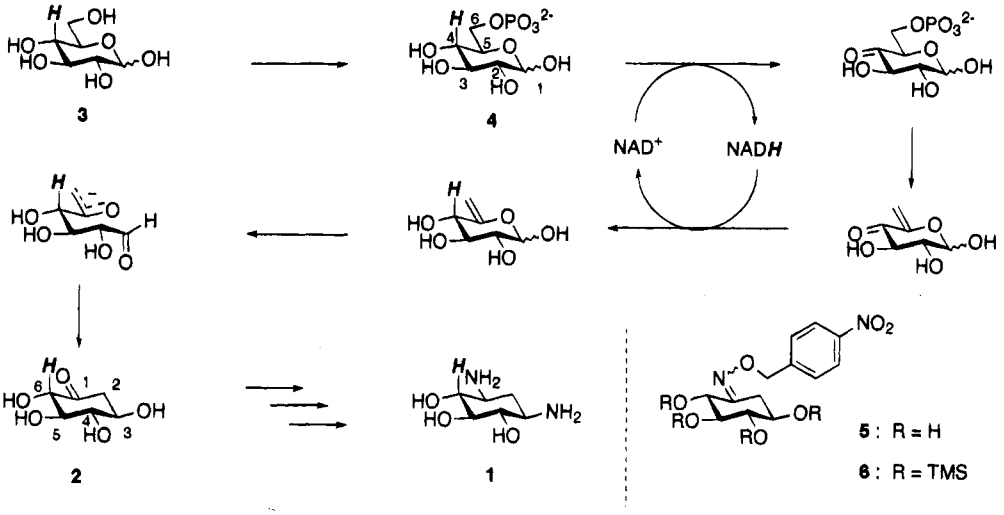
Scheme 1. Proposed Mechanism of 2-Deoxy-*scyllo*-inosose Synthase Reaction in the Biosynthesis of 2-Deoxystreptamine



tion of involvement of oxidoreduction there.[8] The fate of the C-4 hydrogen of the substrate **4** is therefore crucial as to unequivocally address the precise mechanism of the 2-deoxy-*scyllo*-inosose synthase reaction and its similarity or dissimilarity to the dehydroquinate synthase reaction. Herein, we report the first synthesis of D-[4-^{2}H,3-^{18}O]-glucose and its phosphate as well as the chase experiments with a partially purified enzyme from *S. fradiae,* implying that a single enzyme is involved in the multistep transformation catalyzed by 2-deoxy-*scyllo*-inosose synthase.[9]

Result and Discussion

Deuterium Labeling Experiment. The fate of the hydrogen at C-4 of **4** in the "2-deoxy-*scyllo*-inosose synthase" reaction was first studied using D-[4-^{2}H]glucose-6-phosphate with a partially purified enzyme system. [4-^{2}H]-**4** was prepared according to the literature procedures,[10,11] and [6,6-^{2}H$_2$]-**4** was used as a positive control. The enzyme reaction product was converted to *O*-(4-nitrobenzyl) oxime **5**.[6] After purification of the labeled product **5** by preparative HPLC, each ^{2}H-NMR spectrum was recorded. The pertinent region of the spectrum are shown in Figure 1, along with the ^{1}H-NMR of the nonlabeled oxime **5**. The ^{1}H-NMR signals of nonlabeled **5** were unequivocally assigned. The chemical shifts of two protons of the C-2 methylene of **5** were significantly different due to the anisotropy of the benzene ring of the (4-nitrobenzyl) oxime (δ 2.58, dd, J = 9.8 and 14.1 Hz and 3.89, dd, J = 4.9 and 14.1 Hz), which was useful for differentiation of the corresponding signal in the ^{2}H-NMR spectra. The spectrum of the enzyme reaction product from the [6,6-^{2}H$_2$]-**4** substrate showed characteristic two ^{2}H-NMR signals of equal intensity at the methylene portion (δ 2.54 and 3.81). This result was quite consistent with the previous observation in the biosynthesis of ribostamycin with the whole cell system of *S. ribosidificus*, that two C-6 protons of **3** were stereospecifically incorporated into the methylene portion of **1**.[5] From the proposed reaction mechanism, the C-4 hydrogen in **4** was anticipated to be retained on C-6 in **2**, and this was really the case. The ^{2}H-NMR spectrum of the product derived from [4-^{2}H]-**4** cleanly demonstrated a single ^{2}H signal at δ 4.65, which was attributable to the C-6 methine of **5**, based on comparison with the ^{1}H-NMR spectrum of nonlabeled **5** (δ 4.71, d, J = 7.6 Hz). Thus, it appears that the C-4 hydrogen in **4** was retained on the same position, to which it originally attached, of the six-membered carbocyclic ring product **2**.

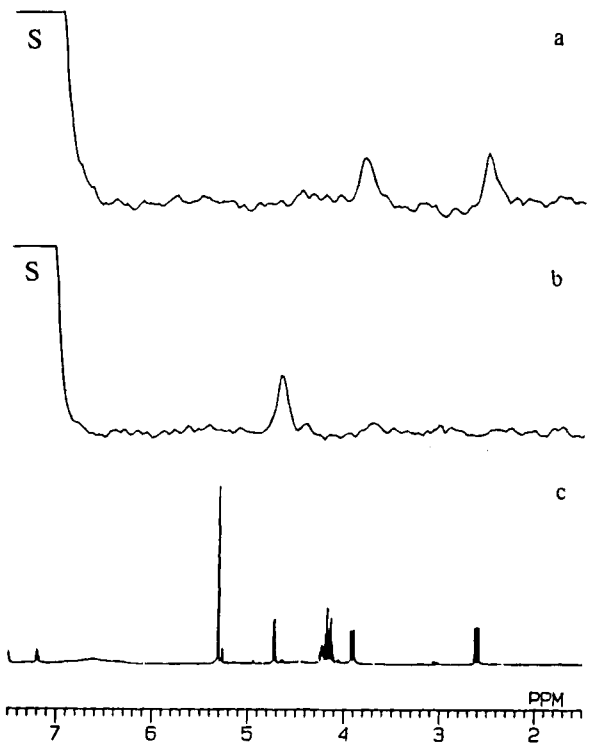


Figure 1. ^{2}H NMR spectra (76.77 MHz, C_5H_5N) of **5** derived from the enzyme reaction products and ^{1}H NMR spectrum (500 MHz, C_5D_5N) of the nonlabeled standard: (a) (^{2}H) the product from [6,6-^{2}H$_2$]-**4**; (b) (^{2}H) the product from [4-^{2}H]-**4**; (c) (^{1}H) nonlabeled standard **5**. "S" is the natural abundance deuterium signal of the solvent.

The retention of deuterium under these conditions was further confirmed by GC-MS analysis of tetra-*O*-trimethylsilyl (TMS) derivative **6** of the oxime **5**. The

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(9) A portion of this work has appeared in a preliminary form. See, Yamauchi, N.; Kakinuma, K. *J. Antibiot.* **1993**, *46*, 1916–1918.
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Table 1. Relative Intensities of the Mass Spectra of 6 Obtained from the Enzyme Reaction of Deuterium Single-Labeled Substrate and Synthetic Standard[a,b]

substrate, *m/z*	D-glucose-6-phosphate	D-[4-^{2}H]glucose-6-phosphate	chemically synthesized [6-^{2}H]-2-deoxy-*scyllo*-inosose
599	0	0	0
600	100	56.6	0
601	50.3	100	100
602	26.3	50.6	48.3
603	10.5	26.3	28.9

[a] Mass spectral scanning was performed in triplicate for each case. [b] Similar results were obtained in duplicate enzyme preparation and incubation.

conversion yield of each substrate was first estimated by quantitative HPLC analysis[6] to be around 10% in the cell-free incubation of 1 mM of the substrate in the presence of 2 mM of NAD^+ cofactor. No endogenous or residual 2-deoxy-*scyllo*-inosose was observed under these conditions. Interestingly, the yield of 2-deoxy-*scyllo*-inosose formation was significantly (~40%) varied (10.1 % from nonlabeled substrate; 6.0% from [4-$^2H_2$]-**4**, 2 h incubation with 1 mL of enzyme solution). The mass spectral intensities (molecular ion region) of each enzyme reaction product are shown in Table 1. A control spectrum of the enzyme reaction product from nonlabeled substrate showed molecular ion peak at *m/z* 600 and characteristic fragment ion peaks at *m/z* 585 ($(M - CH_3)^+$), 464 ($(M - CH_2Ar)^+$), 448 ($(M - OCH_2Ar)^+$) (Ar = *p*-NO_2Ph) (spectrum not shown). Essentially no ion at *m/z* 599 ($(M - 1)^+$) was observed. The mass spectrum of the enzyme reaction product derived from [4-^{2}H]-**4** showed the ions at *m/z* 600 and 601, which indicated the enzyme reaction product was a mixture of monodeuterated and nonlabeled **2**. As a synthetic standard of putative product, [6-^{2}H]-**2** was prepared chemically by the Ferrier reaction from the same intermediate in the synthesis of [4-^{2}H]-**4** substrate[12,13] and was converted to the TMS-ether **6** ([6-^{2}H]-**6**). The mass spectrum of [6-^{2}H]-**6** showed a molecular ion peak at *m/z* 601 but no ion at *m/z* 600. This result indicated that $(M - 1)^+$ fragmentation ion was not formed under these mass spectral conditions. Therefore, these results obtained from the incubation with [4-^{2}H]-**4** suggested that about 56% of the product was in fact deuterated. Thus, the C-4 hydrogen of the substrate appears to be retained throughout the multistep enzyme reaction. Further, since the starting [4-^{2}H]-**4** was almost 96% deuterium enriched, a kinetic isotope effect seemed to operate in the enzyme reaction resulting in reduction of the deuterium enrichment in the product.[14] The observation made by Akhtar *et al.* can be rationalized similarly.[7] As mentioned above, the 2-deoxy-*scyllo*-inosose synthase requires NAD^+ (not $NADP^+$) for the responsible reaction.[6] Since the oxidation states of the substrate **4** and the product **2** are the same, both oxidation and reduction must take place during the biosynthesis of 2-deoxy-*scyllo*-inosose. The site of the oxidation–reduction was strongly suggested to be at C-4 of the substrate based upon the results of Akhtar *et al.* as well as our own described above. The next question to be addressed was whether the hydrogen transfer is intra- or intermolecular. In other words, the abstracted C-4 hydrogen could come back to the original substrate molecule or it transfers into a different molecule with scrambling. The precise mechanism of this hydrogen transfer further provides a clue as to whether a single enzyme is responsible for the overall reaction or certain dissociable enzymes are involved. We tackled this question by using intramolecularly ^{2}H- and ^{18}O-double labeled substrate, based on the assumption that 3-mass unit difference between the labeled and nonlabeled substrate is significant enough to differentiate the hydrogen transfer mechanisms, even under the influence of kinetic isotope effect.

Double-Labeling (^{18}O and ^{2}H) Experiment. The labeled compound chosen for the mechanistic probe of the enzyme reaction was [4-^{2}H,3-^{18}O]-**4**, because the hydrogen of C-4 was crucial and the oxygen at C-3 seemed to be inert to the enzyme reaction. Despite the significant potential of carbohydrates specifically enriched with oxygen isotopes for the biochemical studies, their preparative methods have not been well developed. Most of the reported methods are based on the exchange reaction of labeled H_2O and sugar substrate, involving oxidation–isotope exchange–reduction, enzymatic exchange, or molybdate-catalyzed exchange and rearrangement.[15-17] For example, a preparation of [3-^{17}O]-**3** was reported by Gorin *et al.*, which involved an acetal–water exchange reaction of 1,2:5,6-di-*O*-isopropylidene-α-D-*ribo*-hexofuranuro-3-ulose (**7**).[15]

This procedure, however, seemed to be inappropriate to prepare D-[4-^{2}H,3-^{18}O]-**3** regardless to the order of the isotope introduction. If deuterium was introduced first, the deuterium was replaced afterward during the oxygen exchange. If the oxygen was incorporated first, introduction of deuterium must require multistep transformation and be tedious unless $^2H_2{}^{18}O$ is available. One of the most straightforward deuteration methods at C-4 of D-glucose is based on the base-catalyzed exchange reaction of the corresponding hydrogen of **7** with pyridine–D_2O.[18] In addition, chemistry of **7** has been well established, *i.e.* the C-3 carbonyl group can easily be reduced, and subsequent S_N2 displacement at C-3 with an oxygen-nucleophile yields the desired *gluco*-isomer.

The synthesis of [4-^{2}H,3-^{18}O]-**3** is outlined in Scheme 2. Most crucial was the preparation of a suitable ^{18}O-labeled nucleophile, and the material of choice here was [$^{18}O_2$]sodium benzoate, which was prepared, according to the method reported by Kobayashi *et al.*,[19] from benzotrichloride (α,α′,α′-trichlorotoluene) and [^{18}O]H_2O (96.4% ^{18}O, a product of ISOTEC Inc., Miamisburg, OH), the least expensive and commercially available [^{18}O]enriched

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(13) Yamauchi, N.; Kakinuma, K. *J. Antibiot.* **1992**, *45*, 756–766.
(14) The observed quantitative "isotope effects", estimated from the mass spectral analyses, was somewhat too high than those generally expected. The reason for these results are not clear at the moment. Although quantitative analyses have not been carried out, we would suspect that nonlabeled **4** was remained in the enzyme during the preparation of the cell-free system. Further, a certain number of enzymes relating to **4** must exist in the enzyme preparation, which might be the source of nonlabeled **4**. Since the conversion yield from the supplemented **4** was quite low (~10%), the residual nonlabeled **4** as an enzyme–substrate complex, even in a small amount, could undergo preferential conversion into the nonlabeled product **2** in good yield. This would significantly affect the isotope ratio of the product.
(15) Gorin, P. A. J.; Mazurek, M. *Carbohydr. Res.* **1978**, *67*, 479–483.
(16) Caprioli, R. M.; Seifert, W. E., Jr. *Biochem. Biophys. Acta* **1973**, *297*, 213–219.
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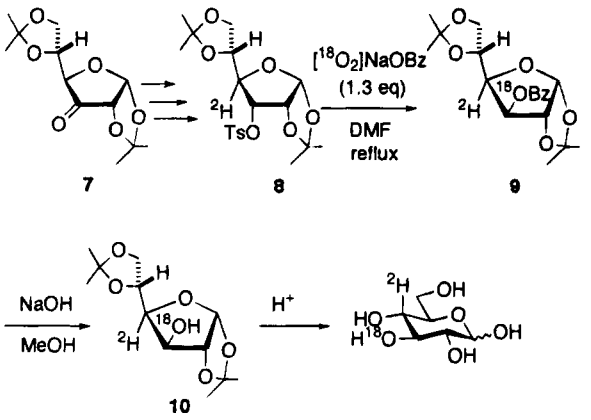
Scheme 2



source. The isotope enrichment of the product was determined to be 95% by negative FAB-MS. The other component, [4-^{2}H]-3-*O*-(*p*-toluenesulfonyl)-1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose (**8**), was prepared from nonlabeled **7** according to the Liu's procedure.[20] The crucial displacement reaction was performed with 1.3 equiv of [$^{18}O_2$]sodium benzoate in *N,N*-dimethylformamide. The reaction underwent cleanly in 74% yield to give a product **9**, and 12% of the starting material **8** was recovered. Subsequent hydrolysis of **9** afforded **10**, the ^{18}O- and ^{2}H-enrichment of which was determined with EI-MS spectrum by monitoring the characteristic $(M - 15)^+$ ion of the product **10**. Thus, the ratio of [4-^{2}H,3-^{18}O]-, [3-^{18}O]-, [4-^{2}H]-**10** and nonlabeled material was estimated to be 92:4:3:1. The product was a mixture of ~95% enriched molecules as to each ^{18}O- and ^{2}H-isotope. Essentially, no exchange of deuterium took place during the displacement and debenzoylation. The product was then hydrolyzed by acid treatment to give [4-^{2}H,3-^{18}O]-**3**, quantitatively. It should be pointed out at this stage that the present method involving nucleophilic displacement with labeled sodium benzoate is an easy, versatile, and economical way for the preparation of the labeled compounds with oxygen isotopes. The final chemical derivatization gave [4-^{2}H,3-^{18}O]-**4**,[11] which was used for the incubation with 2-deoxy-*scyllo*-inosose synthase.

The incubation of [4-^{2}H,3-^{18}O]-**4** was carried out similarly to the case of singly labeled substrate. Two kinds of substrate preparation was subjected to the incubation with a partially purified enzyme, one of the substrate was solely [4-^{2}H,3-^{18}O]-**4**, and the other was a mixture of the labeled **4** and nonlabeled substrate in 7:3 ratio, in the presence of NAD cofactor. A pertinent molecular ion region of the mass spectra of the oxime-TMS ether products derived from each substrate mixture is shown in Figure 2. The intensities of relevant ions are summarized in Table 2.

Spectrum a (from the solely labeled substrate) (Figure 2) clearly demonstrates an intense molecular ion at *m/z* 603, which is 3-mass units higher than the nonlabeled component (*m/z* 600). Also observed are characteristic fragment ions such as *m/z* 585/588 ($(M - CH_3)^+$), 464/467 ($(M - CH_2Ar)^+$), 448/451 ($(M - OCH_2Ar)^+$). These ions indicate that the enzyme reaction product be comprised from two compounds, *i.e.* the double-labeled (^{2}H and ^{18}O) product was major and the nonlabeled was

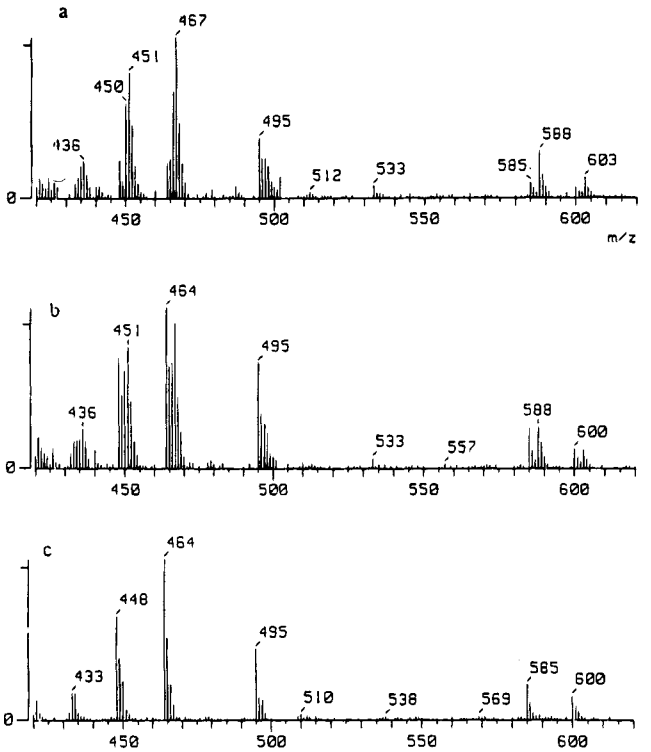


Figure 2. Mass spectra (molecular ion region) of **6** obtained from the enzyme reaction of double-labeled substrate: (a) the product from [4-^{2}H,3-^{18}O]-**4**; (b) the product from a 7:3 mixture of [4-^{2}H,3-^{18}O]- and nonlabeled **4**; (c) the product from nonlabeled **4**.

Table 2. Relative Intensities of the Mass Spectra of 6 from the Enzyme Reaction of Double-Labeled Substrate[a]

substrate, *m/z*	[4-^{2}H,3-^{18}O]-**4** only	[4-^{2}H,3-^{18}O]-**4** and nonlabeled **4** (7:3)	nonlabeled **4**
599	1.1	3.2	0
600	50.5	100	100
601	43.0	52.2	53.5
602	38.0	42.0	32.8
603	100	95.7	17.2
604	49.9	51.8	4.5
605	21.5	24.1	0
606	8.2	10.3	0

[a] Mass spectral scanning was performed in triplicate for each case.

minor. The formation of significant amount of nonlabeled product is probably due to the kinetic isotope effect as described above.[14]

Spectrum b (from the combined substrate of the labeled- and nonlabeled) again shows the ion peaks at *m/z* 600 and *m/z* 603 with almost equal intensity. Importantly, significantly intensified ions above the natural abundance isotope levels were not observed at *m/z* 601 and 602. This observation also suggests the enzyme reaction product from the combined substrate was essentially a mixture of double-labeled product and nonlabeled product. If the enzyme reaction were to proceed through intermolecular hydrogen transfer, scrambling of the isotope-label must have been taken place and significant ions should have been observed at *m/z* 601 and 602. This was actually not the case. Thus, the hydrogen transfer from a double-labeled substrate to a nonlabeled intermediate was at least negligible. These results strongly indicate that the hydrogen transfer during the 2-deoxy-*scyllo*-inosose formation involves an essentially intramolecular process within the enzyme

(20) (a) Rusell, R. N.; Liu, H.-w. *J. Am. Chem. Soc.* **1991**, *113*, 7777–7778. (b) Yu, Y.; Russel, R. N.; Thorson, J. S.; Liu, L.-d.; Liu, H.-w. *J. Biol. Chem.* **1992**, *267*, 5868–5875.

active site. In other words, the multistep reaction is apparently catalyzed by a single "2-deoxy-*scyllo*-inosose synthase" enzyme with a catalytic requirement of NAD cofactor.

It should be worth to take note about the kinetic isotope effect, although the present results may not allow a precise quantitative discussion. In the incubation either with the single deuterium labeled substrate or with the $^{2}H,^{18}O$ double-labeled substrate, formation of significant amount of nonlabeled product was observed. Further, the product from the incubation of a 7:3 mixture of the double-labeled and nonlabeled substrate was almost a 1:1 mixture of the double-labeled and nonlabeled product. These observations, together with the significant reduction of product formation in the incubation of [4-^{2}H]-**4**, apparently indicate involvement of kinetic isotope effect. This may, in turn, support that the reaction (oxidoreduction) at C-4 of the substrate is crucial in the 2-deoxy-*scyllo*-inosose synthase reaction. As pointed out above, Akhtar *et al.* previously reported that the C-4 hydrogen of **3** was not incorporated in **1** during the biosynthesis of neomycin with a whole cell system of *S. fradiae* by chasing [3-^{3}H], [3,4-^{3}H], and [U-^{14}C]glucose,[7] which can be rationalized similarly due to the isotope effect (tritium isotope effect is larger than deuterium isotope effect ($(k_H/k^3_H) = (k_H/k^2_H)^{1.44}$)).

Further, by assuming the extent of conversion is basically dependent to the kinetic isotope effect, the isotope effect was estimated to around 2.7 for the net reaction based on the intensities of each signal between m/z 600 to m/z 606 in the product from the incubation of the 7:3 mixture of labeled and nonlabeled substrate. The values of kinetic isotope effect of dehydroquinate synthase or other NAD-dependent oxidoreductases, which catalyze the reaction at C-4 of hexose derivatives or its equivalent such as hexose diphosphonucleotide oxidoreductases in the deoxysugar biosynthetic pathway, were reported to be in the range of $k_H/k^3_H = 1.8$ to 3.[8,21] For *myo*-inositol-1-phosphate synthase of several origins (plants or animals), which catalyzes a NAD-dependent oxidoreduction at C-5 of **4** (same substrate, but oxidoreduction occurs on the α-position to the phosphate), the isotope effect is about $k_H/k^3_H = 2.0-4.8$.[22] The kinetic isotope effect of the present 2-deoxy-*scyllo*-inosose synthase may fall into the same range of these isotope effects. In the dehydroquinate synthase reaction, catalytic requirement of NAD^+ was clearly shown by Knowles *et al.*[23] The same seems to be true in the present 2-deoxy-*scyllo*-inosose synthase reaction. Recently, polyketide synthase genes have been cloned and sequence homology to the fatty acid synthase genes have been substantiated, as originally anticipated from the similarity of the reaction mechanisms.[24] Biochemical as well as enzymatic comparative studies of 2-deoxy-*scyllo*-inosose synthase may thus be intriguing along this line.

Experimental Section

General. ^{1}H-NMR spectra were recorded at 270.16 or 500.12 MHz. ^{13}C-NMR spectra were recorded at 67.93 or 125.76 MHz. ^{2}H-NMR spectra were recorded at 76.77 MHz. For the ^{2}H-NMR spectra, natural abundance deuterium signal of C_5H_5N solvent (H-3 position, 7.20 ppm) was used as an internal standard. Mass spectra were obtained at ionization voltage of 70 eV. GC-MS spectra were recorded with a Shimadzu-LKB 9020DF spectrometer equipped with a OV-1 equivalent (12 m) column (single-labeling experiments), or with a JEOL-AX505HA spectrometer equipped with a SE-52 equivalent (30 m) column (double-labeling experiment) under these conditions; injector temperature 250 °C, ion source temperature 280 °C, He carrier (flow rate 50 mL/min). [4-^{2}H]-**4** was prepared chemically from [4-^{2}H]-**3** (96% deuterium enriched) according to the literature method.[10,11] The "workup as usual" refers to washing of the combined organic layer with 1 N HCl, saturated aqueous $NaHCO_3$, and brine, drying over anhydrous Na_2SO_4, and filtration and evaporation of the solvent. Other chemicals and biochemicals were purchased from commercial sources and used without further purification. Enzyme preparation was carried out at 4 °C. The protein content of an enzyme preparation was estimated by the Lowry's method.[25]

Preparation of Partially Purified Enzyme from *Streptomyces fradiae* Cells. Harvested *S. fradiae* IFO 13147 cells (30 g, wet) were suspended in buffer A (50 mM Tris-HCl, pH 7.5, Co^{2+} and Mg^{2+} 0.2 mM, 20% v/v glycerol, 75 mL). The suspension was sonicated for 10 min and centrifuged at 10 000*g* for 30 min. The 30−45% ammonium sulfate precipitate was prepared as described previously.[6] The precipitate was dissolved in a minimum amount of buffer B (50 mM Tris-HCl, pH 7.5, Co^{2+} and Mg^{2+} 0.2 mM) and dialyzed against the same buffer for overnight. The crude enzyme fraction (15 mL) was chromatographed with DEAE-Cellulofine A-800 (1 cm i.d. × 20 cm, 15 mL) equilibrated with buffer B. The column was first washed with buffer B (45 mL), and the absorbed proteins were eluted with the same buffer by a linear gradient of NaCl concentration (0−0.4 M, 300 mL). Fractions (5 mL each) were collected, and the active fractions (around fraction no. 30) were used for the isotope tracer experiments.

Large Scale Preparation of a Partially Purified Enzyme and Incubation Method. Ammonium sulfate precipitate was prepared as mentioned above. A 60 mL volume of the dialyzed protein solution from 30−45% ammonium sulfate precipitate (prepared from 120 g of *S. fradiae* cells) was charged to DEAE-Cellulofine A-800 (1.6 cm i.d. × 25 cm) equilibrated with buffer B. The column was washed with 150 mL of buffer B, and the absorbed proteins were eluted with 300 mL of the same buffer by a linear gradient of NaCl concentration (0−0.4 M). Fractions (9 mL each) were collected, and the active fractions (around fraction no. 45) were used for the enzyme reaction. Deuterium-labeled **4** (5.3 mg, 15 μmol, 1 mM final) and NAD^+ (20.4 mg, 30 μmol, 2 mM final) were added to a portion of the combined active enzyme fraction (15 mL), and the whole was incubated for 2 h at 37 °C. After glucose oxidase-treatment and oxime derivatization,[6] products were purified by preparative HPLC (TSK-gel ODS 80T_MCTR, 4.6 mm i.d. × 10 cm, water−15% MeOH, flow rate 1 mL/min).

Standard Procedure for GC-MS Analysis. To a partially purified enzyme fraction (1 mL) were added labeled or nonlabeled **4** (1 μmol, 1 mM final) and NAD^+ (2 μmol, 2 mM final), and the whole was incubated for 2 h at 37 °C. After glucose oxidase treatment, the yield of 2-deoxy-*scyllo*-inosose from each labeled or nonlabeled **4** was estimated, after conversion to the *O*-(4-nitrobenzyl) oxime derivative, as reported.[6] The oxime product was dried and then trimethylsi-

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lylated by treatment with a mixture of 40 μL of pyridine, 10 μL of hexamethyldisilazane, and 10 μL of chlorotrimethylsilane.[26] The tetra-*O*-trimethylsilyl derivative of the oxime **6** appeared at a retention time of 9.4 min with a OV-1 equivalent (12 m) column (oven temp, started from 180 °C to 300 °C, +8 °C linear gradient), or at 15.6 min with a SE-52 equivalent (30 m) column (oven temp. from 120 °C to 320 °C, 20 °C linear gradient).

Preparation of [$^{18}O_2$]Sodium Benzoate. [$^{18}O_2$]sodium benzoate was prepared according to the procedure reported by Kobayashi *et al.*[18] A mixture of 1.25 g of benzotrichloride and 0.5 g of [^{18}O]H_2O (96.4% atom ^{18}O) was heated to 100 °C in a sealed tube, and the mixture was stirred at 100 °C for 24 h. The crystalline product was dried under reduced pressure, neutralized by the addition of aqueous 10% NaOH aqueous (ca. 2.5 mL), and the mixture was concentrated under reduced pressure. The residue was recrystallized by EtOH–water to give 939 mg of the product. FAB-MS (negative, intensity %) 121 (2.3), 123 (4.3), 125 (100), which was directly used for the next step.

[4-^{2}H,3-^{18}O]-3-*O*-Benzoyl-1,2;5,6-di-*O*-isopropylidene-α-D-glucofuranose (9). To a solution of [4-^{2}H]-3-*O*-(*p*-toluenesulfonyl)-1,2;5,6-di-*O*-isopropylidene-α-D-allofuranose (**8**) (1.65 g, 4.0 mmol)[19] in *N*,*N*-dimethylformamide was added [$^{18}O_2$]-sodium benzoate (782 mg, 5.2 mmol, 1.3 equiv), and the mixture was refluxed for 48 h. To the mixture was added a small amount of water, and the mixture was extracted with EtOAc three times (50 mL × 3). After workup as usual, the product was purified by silica gel column chromatography (50 g, hexane:EtOAc = 8:1 to 4:1) to give, along with 202 mg of recovered starting material (12.2% recover), 1.085 g of **9** (73.5%): $[\alpha]^{22}_D$ − 43.2 (*c* = 1.08, $CHCl_3$); IR ($CHCl_3$) 3020, 1705, 1385, 1215, 1090 cm^{-1}; FAB-MS (positive, intensity %) 371 (2.1), 370 ((M + H)$^+$, 8.6), 369 (1.2), 368 (2.8), 367 (0.8), 354 (25.2), 312 (65), 107 (100); ^{1}H-NMR (270 MHz, $CDCl_3$) δ 1.26 (3H, s), 1.32 (3H, s), 1.41 (3H, s), 1.56 (3H, s), 4.08 (1H, dd, *J* = 5.9 and 8.8 Hz), 4.11 (1H, dd, *J* = 5.9 and 8.8 Hz), 4.33 (1H, t, *J* = 5.9 Hz), 4.62 (1H, d, *J* = 3.9 Hz), 5.50 (1H, s), 5.94 (1H, d, *J* = 3.9 Hz), 7.47 (2H, aromatic), 7.60 (1H, aromatic), 8.03 (2H, aromatic); ^{13}C-NMR (67.9 MHz, $CDCl_3$) δ 25.19, 26.20, 26.74, 26.81, 67.22, 72.53, 76.53, 83.38, 105.14, 109.38, 112.34, 128.32, 128.53, 129.54, 129.70, 133.44, 165.14, 190.60. Anal. Calcd for $C_{19}H_{23}{}^2H_1O_5{}^{18}O_2$: C, 61.78; H + ^{2}H; 6.55. Found: C, 61.50; H + ^{2}H, 6.64.

[4-^{2}H,3-^{18}O]-1,2;5,6-Di-*O*-isopropylidene-α-D-glucofuranose (10). **9** (1.08 g, 2.9 mmol) was dissolved in a 5% NaOH–MeOH (10 mL) and was stirred for 15 min. The mixture was concentrated to *ca.* 5 mL and then $CHCl_3$ (50 mL) and water (20 mL) was added. The organic phase was separated, and the water phase was further extracted twice (50 mL × 2) with $CHCl_3$. After workup as usual, the products (842 mg) were purified by silica gel column chromatography (25 g, hexane:EtOAc = 2:1) to give 626 mg of **10** (81.4%): mp 105–107 °C; $[\alpha]^{22}_D$ −12.0 (*c* = 0.51, $CHCl_3$); IR ($CHCl_3$) 3400, 3000, 1385, 1075 cm^{-1}; EI-MS (intensity %) 250 (1.6), 249 (11.1), 248 ((M − 15)$^+$, 84.5), 247 (3.6), 246 (3.2), 245 (0.3), 190 (34), 101 (100); ^{1}H-NMR (270 MHz, $CDCl_3$) δ 1.32 (3H, s), 1.37 (3H, s), 1.44 (3H, s), 1.49 (3H, s), 3.14 (d, *J* = 4.0 Hz), 4.01 (1H, dd, *J* = 5.4 and 8.8 Hz), 4.15 (1H, dd, *J* = 5.4 and 8.8 Hz), 4.29 (1H, d, *J* = 3.9 Hz), 4.32 (1H, t, *J* = 5.4 Hz), 4.52 (1H, d, *J* = 3.4 Hz), 5.92 (1H, d, *J* = 3.4 Hz); ^{13}C-NMR (67.9 MHz, $CDCl_3$) δ 25.04, 26.05, 26.67, 26.71, 67.43, 72.90, 74.58, 80.71 (t, *J* = 22 Hz), 85.01, 105.09, 109.44, 111.67. Anal. Calcd for $C_{12}H_{19}{}^2H_1O_5{}^{18}O_1$: C, 54.75; H + ^{2}H, 7.66. Found: C, 54.45; H + ^{2}H, 7.64.

D-[4-^{2}H,3-^{18}O]Glucose. A mixture of **10** (614 mg, 2.3 mmol) and 10 mL of 0.1 M HCl was heated at 80 °C for 30 min. The mixture was neutralized with IRA-410 ion exchange resin (OH^- form) and the resin was removed by filtration. The filtrate was evaporated to dryness to give 427 mg of D-[4-^{2}H,3-^{18}O]-**3** (quant): mp 133–134 °C; $[\alpha]^{22}_D$ +73.5 (*c* = 0.24, water); ^{13}C-NMR (126 MHz, D_2O, 1,4-dioxane internal reference at 67.4 ppm) δ 61.38, 61.53, 72.17, 72.30, 73.49, 74.94, 76.49, 76.69, 92.89, 96.72. Anal. Calcd. for $C_6H_{11}{}^2H_1{}^{16}O_5{}^{18}O_1$: C, 39.34; H + ^{2}H, 6.60. Found: C, 39.19; H + ^{2}H, 6.68.

D-[4-^{2}H,3-^{18}O]Glucose-6-phosphate. D-[4-^{2}H,3-^{18}O]glucose was converted into its 6-phosphate (D-[4-^{2}H,3-^{18}O]-**4**) according to the literature procedure for the chemical synthesis of nonlabeled **4**.[11] $[\alpha]^{18}_D$ + 18.0 (c = 0.23, water); ^{13}C-NMR (126 MHz, D_2O, 1,4-dioxane internal reference at 67.4 ppm) δ 65.31 (two overlapped peaks), 71.06 (d, *J* = 7.3 Hz), 72.23, 73.33, 74.87, 75.36 (d, *J* = 7.3 Hz), 76.26, 93.01, 96.82. Anal. Calcd for $C_6H_{11}{}^2H_1{}^{16}O_5{}^{18}O_1{\cdot}H_2O$ (monohydrate): C, 20.16; H + ^{2}H, 3.67. Found: C, 19.85; H + ^{2}H, 4.07.

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