Kinetic Isotope Effect and Reaction Mechanism of 2-Deoxy-*scyllo*-inosose Synthase Derived from Butirosin-producing *Bacillus circulans*[†,††]

FUMITAKA KUDO, NORIAKI YAMAUCHI, RIEKO SUZUKI
and KATSUMI KAKINUMA*

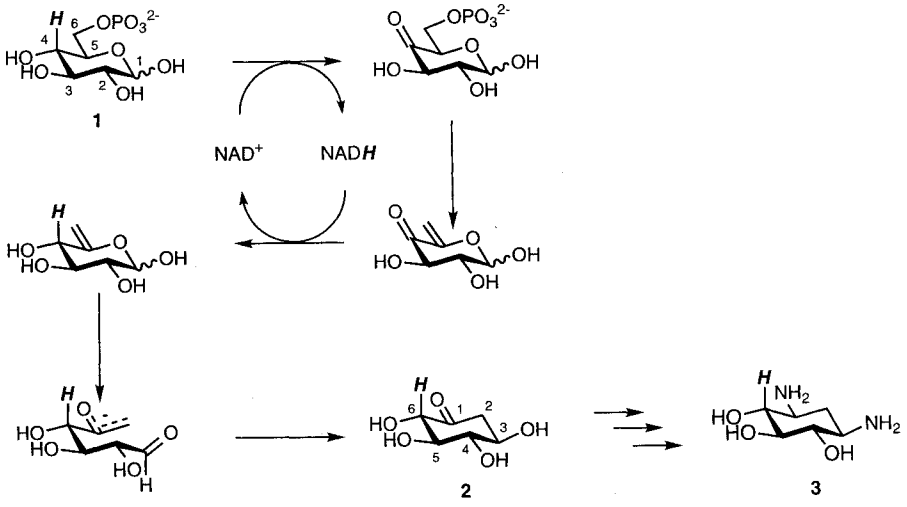
Department of Chemistry, Tokyo Institute of Technology,
O-okayama, Meguro-ku, Tokyo 152, Japan

(Received for publication February 17, 1997)

The mechanism of 2-deoxy-*scyllo*-inosose synthase reaction, a carbocycle formation step from D-glucose-6-phosphate in the biosynthesis of the 2-deoxystreptamine aglycon of clinically important aminocyclitol antibiotics, was investigated with a partially purified enzyme from butirosin-producing *Bacillus circulans* SANK 72073. Nonlabeled and double-labeled D-[4-^{2}H, 3-^{18}O]glucose-6-phosphate were used for cross-over experiment, and the oxime-TMS ether derivative of the 2-deoxy-*scyllo*-inosose product was analyzed by GC-MS. The deuterium label at C-4 of the substrate appeared to be retained at C-6 of the inosose product without scrambling of the double-labeled isotopes. Since the transient reduction of NAD^+ cofactor was proved to be essential in the 2-deoxy-*scyllo*-inosose reaction, the hydride abstraction and returning appeared to take place within the same glucose molecule. The observed kinetic isotope effect was estimated to be $k_H/k_D=2.4$. These results strongly suggest that this carbocycle formation is catalyzed by a single 2-deoxy-*scyllo*-inosose synthase enzyme with catalytic requirement of NAD^+, the mechanism of which appears to be resembled closely to the 2-deoxy-*scyllo*-inosose synthase in the *Streptomyces fradiae*.

2-Deoxy-*scyllo*-inosose synthase is a crucial enzyme in the biosynthesis of a major group of clinically important aminoglycoside antibiotics, in that the enzyme catalyses the intramolecular carbocyclization of D-glucose-6-phosphate (**1**) into the first non-aminogenous cyclitol 2-deoxy-*scyllo*-inosose (**2**).[1~3] The latter is ultimately transformed into 2-deoxystreptamine (**3**) as the central and characteristic aminocyclitol aglycon in these antibiotics.[4] The reaction mechanism of this carbocycle-forming enzyme has attracted wide attention for quite some time, because **3** is only found in these antibiotics as a product of microbial secondary metabolism.[5] We have recently proposed the mechanism of this key enzyme reaction, as shown in Scheme 1, by the use of a partially purified enzyme derived from a neomycin-producing *Streptomyces fradiae* IFO 13147.[6]

Scheme 1. Proposed mechanism of 2-deoxy-*scyllo*-inosose synthase reaction in the biosynthesis of 2-deoxystreptamine.



† Biochemical studies on 2-deoxy-*scyllo*-inosose, an early intermediate in the biosynthesis of 2-deoxystreptamine Part VI. For Part V. See ref. 6.

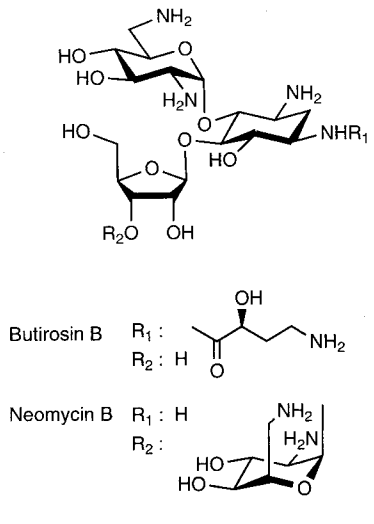
†† This paper is dedicated to Professor NOBORU OTAKE on the occasion of his 70th birthday.

Described in this paper are detailed insights into the reaction mechanism of 2-deoxy-*scyllo*-inosose synthase derived from the butirosin-producing *Bacillus circulans*,[7] which is taxonomically distinguishable from the neomycin-producing *Streptomyces fradiae*. The over-all pathway of the butirosin biosynthesis was previously reported by others.[3,8]

The biosynthesis of aminocyclitol antibiotics has been extensively studied, mostly by the use of whole cells of the producing microorganisms as well as non-producing blocked (idiotrophic) mutants.[2,3,8,9] In addition, significant progress on the genetic level has appeared for the biosynthetic studies of inositol-derived aminocyclitol antibiotics such as streptomycin[10] and fortimicin.[11] However, in contrast, the enzymes involved in the biosynthesis of these antibiotics have mostly been remained unclear except for some particular enzymes, *e.g.* transamination enzymes of relevant inososes.[12] Apparently, precise features of the responsible biosynthetic enzymes, in addition to the genetic background, are particularly important for further development and application of new technology based upon the microbial secondary metabolism. Further, of significance is comparative studies as well, which may give rise to some clue to the understanding of molecular evolution of the microbial secondary metabolism.

We have been involved in the biochemical studies on the chemically simple but important enzyme reaction in the biosynthesis of **3** by focusing particularly on 2-deoxy-*scyllo*-inosose synthase derived from *Bacillus circulans* in addition to that of *Streptomyces fradiae*. Described here is our recent studies of the kinetic isotope effect observed in the reaction of 2-deoxy-*scyllo*-inosose synthase, which has been partially purified from the cells of butirosin-producing *Bacillus circulans*.

Fig. 1. Structures of butirosin and neomycin.



Materials and Methods

Bacterial Strain and Detection of the 2-Deoxy-*scyllo*-inosose Synthase Activity

A butirosin-producing organism *Bacillus circulans* SANK 72073 was maintained on agar slant of the IFO 802 medium consisting of polypeptone (Wako) 1%, yeast extract (Oxoid) 0.2%, $MgSO_4 \cdot 7H_2O$ 0.1%, agar 1.5%, pH 7.0. The 2-deoxy-*scyllo*-inosose synthase activity was detected by the HPLC method as described previously.[13] The enzyme incubation was carried out with D-glucose-6-phosphate 5 mM, NAD^+ 5 mM and $CoCl_2$ 5 mM in 50 mM Tris-HCl buffer (pH 7.5) for 12 hours at 37°C.

Bacterial Cultivation and Purification of the 2-Deoxy-*scyllo*-inosose Synthase

A saline suspension (0.9% NaCl, 1 ml) of *Bacillus circulans* SANK 72073 from a stock slant was inoculated into a 100 ml of soy bean meal (SBM) medium consisting of 2% SBM extract solution[3] (20 g of soy bean meal was extracted with 1 liter of distilled water by autoclaving for 20 minutes at 120°C, and then centrifuged at 7,000 rpm for 30 minutes) with supplemented 1% glycerol (pH 7.5 to 7.7) in a 500-ml Erlenmeyer flask. Cultures were grown on a rotary shaker at 28 to 29°C. After 3 days' cultivation, the cells were harvested by centrifugation (7,000 rpm × 1 hour, 4°C) and were successively washed with 0.5 M NaCl solution and twice with 50 mM Tris-HCl buffer (pH 7.5). The cells were then suspended in Tris-HCl buffer (0.5 g of wet cells per 1 ml of the buffer), and then sonicated for 10 minutes at 0°C in an iced water bath by a Branson sonifier Type 250. The sonicate was centrifuged at 7,000 rpm for 1 hour and the resulting supernatant was then fractionated by $(NH_4)_2SO_4$ precipitation in a standard manner.

The precipitates obtained by 45~70% $(NH_4)_2SO_4$ saturation was dialyzed overnight against the 50 mM Tris-HCl buffer (pH 7.5), and the dialysate was loaded onto a column (2.5 i.d. × 15 cm) of DEAE-cellulofine A-800 (Chisso) equilibrated with 50 mM Tris-HCl (pH 7.5). The adsorbed proteins were eluted by the same buffer with linear gradient of NaCl concentration from 0 M NaCl (300 ml) to 0.4 M NaCl (300 ml). The 2-deoxy-*scyllo*-inosose synthase activity was observed around the fraction of approximately 0.2 M NaCl. The active fractions were concentrated into *ca.* 3 ml by centrifugation (5,000 rpm × 30 minutes × several times)

with Centriprep-10 (Amicon). The concentrate was then loaded onto a column (2.5 i.d. × 8.5 cm) of Q Sepharose FF (Pharmacia) equilibrated with 50 mM Tris-HCl (pH 7.5). Elution was carried out again with the same buffer in a linear gradient manner from 0 M NaCl (200 ml) to 0.4 M NaCl (200 ml). The enzyme activity was observed in the fractions of *ca.* 0.2 M NaCl, which was in turn used for the tracer experiments of the 2-deoxy-*scyllo*-inosose synthase reaction.

Preparation of D-[6,6-$^2H_2$]-**1** and D-[3-^{18}O,4-^{2}H]-**1**

Isotopically labeled substrates, D-[6,6-$^2H_2$]-**1**[14,15] and D-[3-^{18}O,4-^{2}H]-**1**,[6,15] were prepared as already described.

Results and Discussion

Confirmation of Enzyme Activity

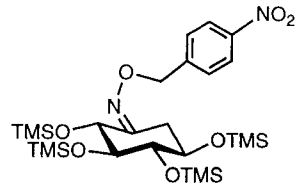
The 2-deoxy-*scyllo*-inosose synthase activity was clearly detected by the HPLC assay method in the enzyme solutions prepared by precipitation with $(NH_4)_2SO_4$ saturation (45~70%) from the supernatant of the disrupted *Bacillus circulans* cells. The activity was further confirmed by analyzing the reaction products from both nonlabeled **1** and [6,6-$^2H_2$]-**1** by means of GC-MS of the oxime-TMS ether derivative of **2** as already reported.[16] The spectrum of 2-deoxy-*scyllo*-inosose *p*-nitrobenzyloxime tetra-*O*-TMS ether (**4**) derived from the incubation of nonlabeled **1** showed an intense molecular ion peak at *m/z* 600 (Fig. 2-a), whereas the spectrum of the corresponding oxime-TMS ether derived from [6,6-$^2H_2$]-**1** demonstrated an intense molecular ion peak at *m/z* 602 (Fig. 2-b). This observation clearly indicated that the cell-free reaction system of *Bacillus circulans* in fact contained 2-deoxy-*scyllo*-inosose synthase.

Purification of the Enzyme

In order to elucidate the enzyme reaction more precisely and quantitatively, it was essential to remove any residue of **2** formed in the microbial cultivation. Previously, we encountered in some difficulty in evaluating the possible kinetic isotope effect in the earlier experiments using a partially purified enzyme of *Streptomyces fradiae*, and some ambiguity has been remained unresolved.[6] Therefore, the purification of the 2-deoxy-*scyllo*-inosose synthase enzyme from *Bacillus circulans* was pursued in this study as described in the Materials and Methods section.

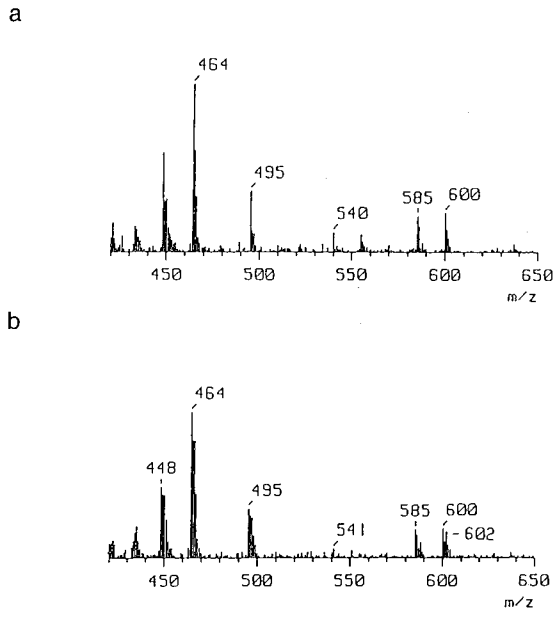
Fig. 2. Mass spectra (molecular ion region) of **4** obtained from the enzyme reaction of **1**.

(a) The product from nonlabeled **1**, (b) the product from [6,6-$^2H_2$]-**1**.



4

TMS-oxime derivative of 2-deoxy-*scyllo*-inosose



Cross-over Experiment with Double-labeled Substrate

The enzymatically active fraction obtained from the Q Sepharose chromatography was subjected to the cross-over experiment using nonlabeled and double-labeled substrates.

A major issue here was whether C-4 hydrogen retains in the original substrate molecule or it transfers into a different molecule with scrambling. As already reported, the 2-deoxy-*scyllo*-inosose synthase reaction requires the presence of NAD^+, which clearly means the involvement of oxidation (dehydrogenation).[13] Further, since the oxidation states of the substrate and the reaction product are just the same, a reduction step must also been involved in the over-all enzyme reaction. It was already pointed out that the most plausible site of oxidation is C-4, since the oxidation at C-4 is advantageous in activating the C-5 position for the removal of proton resulting in enhancement of the phosphate elimination. This crucial

chemistry was tackled by using double-labeled substrate, D-[3-^{18}O,4-^{2}H]-**1**, with a partially purified 2-deoxy-*scyllo*-inosose synthase enzyme from *Bacillus circulans*.

If the C-4 hydrogen transfer takes place in a round-trip fashion from the substrate to the cofactor and the subsequent returning from the cofactor to the product within the same enzyme molecule without dissociation of any intermediates and cofactors, one should only observe, in the incubation of a mixture of D-[3-^{18}O,4-^{2}H]-**1** and nonlabeled **1**, the products corresponding to the substrate components of molecular weight *m/z* 600 (from nonlabeled) and *m/z* 603 (from double-labeled) on the GC-MS analysis. On the other hand, if any intermediates and/or a cofactor would dissociate from the enzyme active site, one would simultaneously observe, in addition to the nonlabeled and double-labeled products, the products of *m/z* 601 (containing only ^{2}H-label) and *m/z* 602 (containing only ^{18}O-label) formed by molecular scrambling through cross-over.

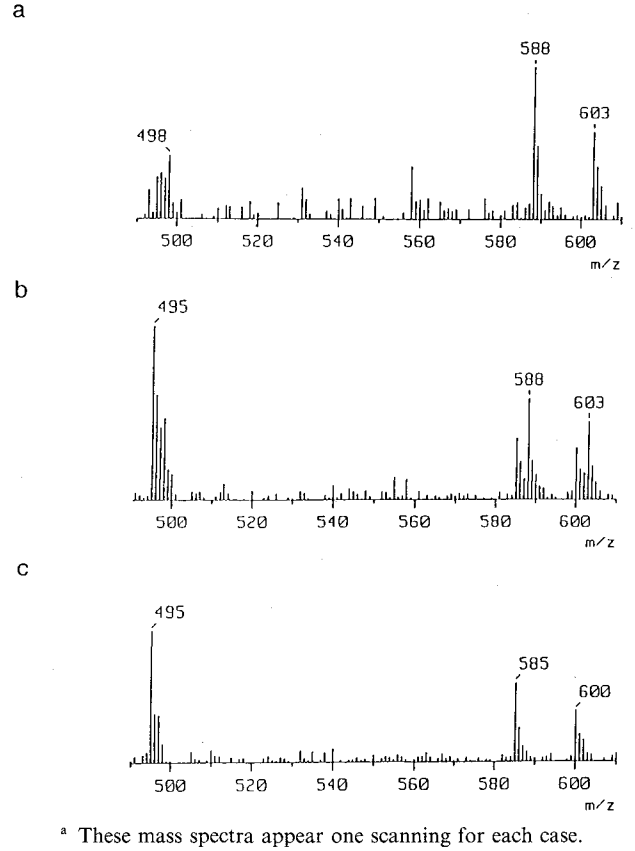
Two kinds of substrate preparation was actually subjected to the incubation in the presence of NAD^+ cofactor with a partially purified enzyme, one of the substrate was solely double-labeled substrate, and the other was a mixture of the double-labeled and nonlabeled substrate in a 7 : 3 ratio. Pertinent molecular ion regions of the mass spectra of the oxime-TMS ether derivatives of the incubation products from each substrate mixture are shown in Fig. 3. The intensities of the resulting relevant ions are summarized in Table 1.

The spectrum obtained from the incubation of solely double-labeled substrate demonstrates an intense molecular ion at *m/z* 603 (Fig. 3-a). This observation indicates that only double-labeled product was enzymatically synthesized from the double-labeled substrate. The spectrum obtained from the incubation of a mixture of the double-labeled and nonlabeled substrates shows the ion peaks of *m/z* 600 and *m/z* 603 in almost equal intensity (Fig. 3-b). Importantly, no particular ions significantly intensified beyond the natural abundance level were observed at *m/z* 601 and 602. In other words, the intensities of these ions were within the range of natural isotopomers of the ions of *m/z* 600. These results thus verified that the enzyme reaction product from the combined substrate was essentially a mixture of double labeled and nonlabeled products without a presence of either singly ^{2}H- or ^{18}O-labeled product.

The observation that the product from the incubation of a 7 : 3 mixture of double-labeled and nonlabeled substrate was almost a 1 : 1 mixture of the double-labeled and non-labeled product, clearly indicates the involvement of kinetic isotope effect. This may, in turn, support that the reaction (oxidoreduction) at C-4 of substrate is crucial in the 2-deoxy-*scyllo*-inosose synthase reaction in *Bacillus circulans*. Further, by assuming the extent of conversion is basically due to the kinetic isotope effect, the isotope effect was estimated to be approximately 2.4

Fig. 3. Mass spectra (molecular ion region) of **4** obtained from the enzyme reaction of double-labeled substrate.[a]

(a) The product from [4-^{2}H,3-^{18}O]-**1**, (b) the product from a 7 : 3 mixture of [4-^{2}H,3-^{18}O]- and nonlabeled **1**, (c) the product from nonlabeled **1**.



[a] These mass spectra appear one scanning for each case.

Table 1. Relative intensities of the mass spectra of **4** obtained from the enzyme reaction of double-labeled substrate.[a,b]

Substrate *m/z*	[4-^{2}H, 3-^{18}O]-**1** only	[4-^{2}H, 3-^{18}O]-**1** and nonlabeled **1** (7 : 3)	Nonlabeled **1**
600	7	91	100
601	14	50	54
602	14	47	26
603	100	100	17
604	59	54	8
605	37	28	1
606	13	20	0

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

[b] Similar results were obtained in duplicate enzyme preparation and incubation.

for the net reaction based on 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 kinetic isotope effect of 2-deoxy-*scyllo*-inosose synthase reaction in *Bacillus circulans* may fall into the same range of the isotope effect estimated for the same enzyme derived from *Streptomyces fradiae* ($k_H/k_D=2.7$).[6]

Thus, the results described above strongly suggest that "2-deoxy-*scyllo*-inosose synthase" in *Bacillus circulans* is a single enzyme and catalyzes the multistep reaction which involves the transient reduction of NAD^+ cofactor and aldol-type carbocycle formation as observed in the *Streptomyces fradiae* enzyme.

Acknowledgments

This work was financially supported by a Grant-in Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, and by "Research for the Future" Program from the Japan Society for the Promotion of Science. Gratitude are also due to Sankyo Co., Ltd. for the donation of *Bacillus circulans* and to Fuji Seiyu Ltd. for the gift of soy bean meal used in this study.

References

1) RINEHART, K. L., Jr.; J. M. MALIC, R. S. NYSTORM, R. M. STROSHANE, S. T. TRUITT, M. TANIGUCHI, J. P. ROLLS, W. J. HAAK & B. A. RUFF: Biosynthetic incorporation of [1-^{13}C]glucosamine and [6-^{13}C]glucose into neomycin. J. Am. Chem. Soc. 96: 2263～2265, 1974
2) DAUM, S. J.; D. ROSI & W. A. GOSS: Mutational biosynthesis by idiotrophs of *Micromonospora purpurea*. II. Conversion of non-amino-containing cyclitols to aminoglycoside antibiotics. J. Antibiotics 30: 98～105, 1977
3) FURUMAI, T.; K. TAKEDA, A. KINUMAKI, Y. ITO & T. OKUDA: Biosynthesis of butirosins. II. Biosynthetic pathway of butirosins elucidated from cosynthesis and feeding experiments. J. Antibiotics 32: 891～899, 1979
4) RINEHART, K. L., Jr. & R. M. STROSHANE: Biosynthesis of aminocyclitol antibiotics. J. Antibiotics 29: 319～353, 1976
5) RINEHART, K. L., Jr. & T. SUAMI (*Ed*): Aminocyclitol antibiotics. ACS Symposium Series No. 125. American Chemical Society, Washington D. C., 1980
6) YAMAUCHI, N. & K. KAKINUMA: Enzymatic carbocycle formation in microbial secondary metabolism. The mechanism of 2-deoxy-*scyllo*-inosose synthase reaction as a crucial step in the 2-deoxystreptamine biosynthesis in *Streptomyces fradiae*. J. Org. Chem. 60: 5614～5619, 1995
7) DION, H. W.; P. W. K. WOO, N. E. WILLMER, D. L. KERN, J. ONAGA & S. A. FUSARI: Butirosin, a new aminoglycoside antibiotic complex. Isolation and characterization. Antimicrob. Agents Chemother. 2: 84～88, 1972
8) FUJIWARA, T.; Y. TAKAHASHI, K. MATSUMOTO & E. KONDO: Isolation of an intermediate of 2-deoxystreptamine biosynthesis from a mutant of *Bacillus circulans*. J. Antibiotics 33: 824～829, 1980
9) KASE, H.; T. IIDA, Y. ODAKURA, K. SHIRAHATA & K. NAKAYAMA: Accumulation of 2-deoxy-*scyllo*-inosamine by a 2-deoxystreptamine-requiring idiotroph of *Micromonospora sagamiensis*. J. Antibiotics 33: 1210～1212, 1980
10) DISTLER, J.; K. MANSOURI, G. MAYER, M. STOCKMANN & W. PIEPERSBERG: Streptomycin biosynthesis and its regulation in Streptomycetes. Gene 115: 105～111, 1992
11) DAIRI, T.; T. OHTA, E. HASHIMOTO & M. HASEGAWA: Self cloning in *Micromonosporea olivasterospora* of *fms* genes for fortimicin A (astromicin) biosynthesis. Mol. Gen. Genet. 232: 262～270, 1992
12) LUCHER, L. A.; Y.-M. CHEN & J. B. WALKER: Reactions catalyzed by purified L-glutamine: keto-*scyllo*-inisitol aminotransferase, an enzyme required for biosynthesis of aminocyclitol antibiotics. Antimicrob. Agents Chemother. 33: 452～459, 1989
13) YAMAUCHI, N. & K. KAKINUMA: Confirmation of *in vitro* synthesis of 2-deoxy-*scyllo*-inosose, the earliest intermediate in the biosynthesis of 2-deoxystreptamine, using cell free preparations of *Streptomyces fradiae*. J. Antibiotics 45: 774～780, 1992
14) LEMIEUX, R. U. & J. D. JONES: The proton magnetic resonance spectra and tautomeric equilibria of aldoses in deuterium oxide. Can. J. Chem. 44: 249～262, 1966
15) LARDY, H. A. & H. O. L. FISCHER: Phosphoric esters of biological importance I. The synthesis of glucose-6-phosphate. J. Biol. Chem. 164: 513～519, 1946
16) YAMAUCHI, N. & K. KAKINUMA: Biochemical studies on 2-deoxy-*scyllo*-inosose, an early intermediate in the biosynthesis of 2-deoxystreptamine IV. A clue to the similarity of 2-deoxy-*scyllo*-inosose synthase to dehydroquinate synthase. J. Antibiotics 46: 1916～1918, 1993