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Articles

Importance of Specific Hydrogen-Bond Donor−Acceptor Interactions for the Key Carbocycle-Forming Reaction Catalyzed by 2-Deoxy-*scyllo*-inosose Synthase in the Biosynthesis of 2-Deoxystreptamine-Containing Aminocyclitol Antibiotics

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A crucial enzyme in the biosynthesis of the 2-deoxystreptamine aglycon of clinically important aminocyclitol antibiotics is 2-deoxy-*scyllo*-inosose synthase (DOIS), which converts ubiquitous D-glucose 6-phosphate (G-6-P) into the specific carbocycle 2-deoxy-*scyllo*-inosose. Among all the oxygenated carbons of the substrate, C-1, -4, -5, and -6 are directly involved in the chemical transformation. To get insight into the roles of C-2 and C-3 hydroxy groups, 2-deoxy-2-fluoro-, 3-deoxy-3-fluoro-, 2-amino-2-deoxy-, and 3-amino-3-deoxy-D-glucose 6-phosphates (2-F-G-6-P, 3-F-G-6-P, 2-NH_2-G-6-P, and 3-NH_2-G-6-P, respectively) were subjected to the DOIS reaction as probe, since a fluorine substituent generally acts as a hydrogen-bond acceptor, and an ammonium functionality derived physiologically from an amino group as a hydrogen-bond donor. Among those tested, 2-F-G-6-P and 3-NH_2-G-6-P were used as substrates by DOIS and were converted into the corresponding deoxyfluoro- and aminodeoxy-*scyllo*-inososes, respectively. In contrast, 3-F-G-6-P and 2-NH_2-G-6-P were inactive in the cyclization reaction. Clearly, DOIS recognizes the G-6-P substrate through specific hydrogen-bonding interactions, i.e., through a hydrogen-donating group for C-2 and an accepting group for C-3 of the substrate. Modeling of DOIS based on the structure of evolutionary-related dehydroquinate synthase is also described.

Introduction

2-Deoxy-*scyllo*-inosose synthase (DOIS) catalyzes the conversion of ubiquitous D-glucose 6-phosphate (G-6-P) into specific 2-deoxy-*scyllo*-inosose (DOI) as the initial key step in the biosynthesis of clinically important 2-deoxystreptamine-containing aminocyclitol antibiotics including neomycins, kanamycins, paromomycins, ribostamycin, xylostasin, butirocins, gentamicin, sagamicin, sisomicins, etc.[1,2] Subsequent biosynthetic conversion of 2-deoxy-*scyllo*-inosose to the matured medicinally active antibiotics involves the formation of 2-deoxystreptamine and a series of glycosidations to the diaminocyclitol.[3−8] We have

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(1) Rinehart, K. L., Jr.; Stroshane, R. M. *J. Antibiot.* **1976**, *29*, 319−353.
(2) *Aminocyclitol antibiotics*; Rinehart, K. L., Jr., Suami, T., Eds.; ACS Symposium Series No. 125; American Chemical Society: Washington, DC, 1980.

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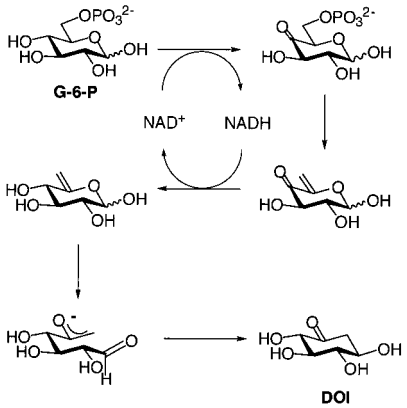


Figure 1. Reaction mechanism of the 2-deoxy-*scyllo*-inosose synthase.

been involved for quite some time in the enzymological studies of the first committed biochemistry in the 2-deoxystreptamine biosynthesis, and several key issues have already been addressed. The plausible mechanism of the conversion of D-glucose into 2-deoxy-*scyllo*-inosose was first suggested by isotope-tracer technology with [6,6-$^2H_2$]-, (6*R*)-[6-^{2}H]-, and (6*S*)-[6-^{2}H]-D-glucose using whole cell fermentation of ribostamycin-producing *Streptomyces ribosidificus*.[9] The mechanism was later confirmed by the kinetic isotope effect experiments using partially purified preparations of the responsible DOIS derived from neomycin-producing *Streptomyces fradiae* and butirocin-producing *Bacillus circulans*.[10,11] The mechanism appeared to be mechanistically similar to that of the dehydroquinate synthase reaction in the shikimate pathway. DOIS catalyzes the multistep direct cyclization of G-6-P into the six-membered carbocycle DOI as illustrated in Figure 1. Subsequently, we were successful in purifying the key enzyme, DOIS from *B. circulans* SANK72073.[12] Recently, the structural gene (*btrC*) for DOIS were identified and heterologously overexpressed in *Escherichia coli*.[13]

With the overproduced DOIS enzyme (BtrC) in hand, we envisaged that DOIS has significant applicability for the preparation of various deoxycyclitols, which appear to be useful in several ways. First of all, the product DOI was envisioned to be conveniently converted into catechol or other benzenoids by rather simple chemical reactions, because of its unique unsymmetrical structure that differs from *myo*-inositol. Catechol is an important industrial chemical used as an antioxidant and a starting material for flavors and fragrances, for medical and agricultural agents, and for other things. In fact, we have recently shown a potential two-step process to catechol from D-glucose comprising one-pot incubation of D-glucose with recombinant BtrC (DOIS) and hexokinase, followed by reductive dehydration of the intermediary DOI with hydroiodic acid.[14] Second, DOIS may facilitate a novel approach to deoxygenated or other functionalized cyclitols as a useful chiral pool for the enantioselective synthesis of various physiologically active chiral molecules.[15] To explore these potentials in directed ways, more precise and detailed features of the chemistry and substrate recognition of DOIS must be clarified.

Apparently, DOIS catalyzes the most sophisticated conversion of biologically ubiquitous G-6-P. As depicted in Figure 1, the initial NAD^+-dependent dehydrogenation takes place at C-4 of G-6-P. The next elimination of a phosphate from C-5 and C-6 gives an enone function on the glucose backbone, and the hydride at C-4 of the strongly associated NADH then comes back to the original C-4 position of the intermediate to afford a cyclic enol ether. The final hemiacetal opening at C-1, followed by aldol-type intramolecular condensation between C-6 and C-1, gives DOI. Among all, the oxygen functionalities at C-1, -4, -5, and -6 are involved directly in the reaction. Intriguing, then, are the roles of the hydroxy groups at C-2 and C-3. As already described, the C-3 oxygen is retained throughout the DOIS reaction.[10,11] Also reported previously was that the DOIS from *B. circulans* accepted 2-deoxy- and 3-deoxy-D-glucose 6-phosphates as substrate to convert into the corresponding dideoxy-*scyllo*-inosose products. Although the conversion was less effective (3−4% of the G-6-P substrate), the hydroxy groups at C-2 and C-3 of G-6-P seemed to be relevant for the substrate recognition.[16]

In this paper, we describe precise analysis of the function of C-2 and C-3 hydroxy groups of the substrate and conversely the substrate recognition of DOIS by means of substrate analogues having either a fluorine or an amino substituent.

Results and Discussion

The importance of hydrogen bonding in the enzyme−substrate interaction is well-recognized. The precise role of C-2 and C-3 hydroxy groups of the G-6-P substrate in the DOIS reaction has been tackled in this context. Deoxyfluoro sugars have been widely used in the studies of binding interaction between carbohydrate substrates and enzymes[17] because the substitution of a hydroxy group by a fluorine has little effect on steric bulkiness. But, the key feature is the high electron negativity of fluorine, which may restrict the hydrogen bonding capability. Thus, a fluorine substituent cannot donate a hydrogen bond and can only act as a hydrogen-bond acceptor.[17−19] In contrast to a fluorine substituent, an ammonium group in place of a hydroxy group plays the

(3) Rinehart., K. L., Jr.; Malic, J. M.; Nystrom, R. S.; Stroshane, R. M.; Truitt, S. T.; Taniguchi, M.; Rolls, J. P.; Haak, W. J.; Ruff, B. A. *J. Am. Chem. Soc.* **1974**, *96*, 2263.
(4) Daum, S. J.; Rosi, D.; Goss, W. A. *J. Antibiot.* **1977**, *30*, 98.
(5) Furumai, T.; Takeda, K.; Kinumaki, A.; Ito, Y.; Okuda, T. *J. Antibiot.* **1979**, *32*, 891.
(6) Fujiwara, T.; Takahashi, Y.; Matsumoto, K.; Kondo, E. *J. Antibiot.* **1980**, *33*, 824.
(7) Kase, H.; Iida, T.; Odakura, Y.; Shirahata, K.; Nakayama, K. *J. Antibiot.* **1980**, *33*, 1210.
(8) Yamauchi, N.; Kakinuma, K. *J. Antibiot.* **1992**, *45*, 774.
(9) (a) Kakinuma, K.; Ogawa, Y.; Sasaki, T.; Seto, H.; Otake, N. *J. Am. Chem. Soc.* **1981**, *103*, 5614. (b) Kakinuma, K.; Ogawa, Y.; Sasaki, T.; Seto, H.; Otake, N. *J. Antibiot.* **1989**, *42*, 926.
(10) Yamauchi, N.; Kakinuma, K. *J. Org. Chem.* **1995**, *60*, 5614.
(11) Kudo, F.; Yamauchi, N.; Suzuki, R.; Kakinuma, K. *J. Antibiot.* **1997**, *50*, 424.
(12) Kudo, F.; Hosomi, Y.; Tamegai, H.; Kakinuma, K. *J. Antibiot.* **1999**, *52*, 81.
(13) Kudo, F.; Tamegai, H.; Fujiwara, T.; Tagami, U.; Hirayama, K.; Kakinuma, K. *J. Antibiot.* **1999**, *52*, 559.
(14) Kakinuma, K.; Nango, E.; Kudo, F.; Matsushima, Y.; Eguchi, T. *Tetrahedron Lett.* **2000**, *41*, 1935.
(15) Kiddle, J. J. *Chem. Rev.* **1995**, *95*, 2189.
(16) Iwase, N.; Kudo, F.; Yamauchi, N.; Kakinuma, K. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 2396.
(17) (a) *Fluorinated carbohydrates: chemical and biochemical aspects*; Taylor, N. F., Ed.; ACS Symposium Series No. 374; American Chemical Society: Washington, DC, 1988. (b) Tsuchiya, T. *Adv. Carbohydr. Chem. Biochem.* **1990**, *48*, 91. (c) Dax, K.; Albert, M.; Ortner, J.; Paul, B. J. *Curr. Org. Chem.* **1999**, *3*, 287.

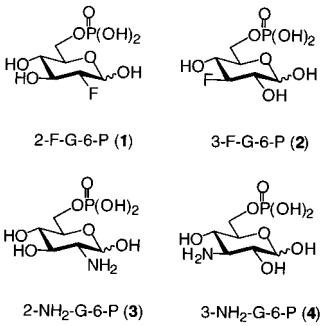
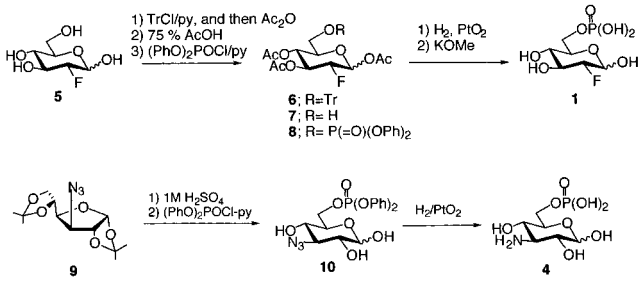


Figure 2. Structures of 2-F-G-6-P (**1**), 3-F-G-6-P (**2**), 2-NH_2-G-6-P (**3**), and 3-NH_2-G-6-P (**4**).

Scheme 1



role of hydrogen-bond donor but cannot accept a hydrogen bond. Aminodeoxy sugar phosphate generally exists in an aqueous solution as a zwitterionic form having a protonated amino group under physiological conditions.[20] Thus, when a hydroxy group of carbohydrate substrate acts as a hydrogen-bond donor in the enzyme−substrate interaction, an aminodeoxy sugar analogue can be accepted by the enzyme, but a deoxyfluoro sugar cannot be recognized. Conversely, when a hydroxy group of sugar substrate acts as a hydrogen bond acceptor, the reverse should be the case. In the case that a hydroxy group is of minor importance in substrate recognition, replacement of the hydroxy group by a fluorine or an amino substituent would not affect significantly in the enzyme reaction. To elucidate the precise role of C-2 and C-3 hydroxy groups, the DOIS reaction was analyzed with a series of deoxyfluoro- and aminodeoxy sugars, e.g., 2-deoxy-2-fluoro-, 3-deoxy-3-fluoro-, 2-amino-2-deoxy-, and 3-amino-3-deoxy-D-glucose 6-phosphates **1**−**4** (2-F-G-6-P, 3-F-G-6-P, 2-NH_2-G-6-P, and 3-NH_2-G-6-P, respectively) (Figure 2).

All the necessary substrates were acquired as follows. Although 2-F-G-6-P (**1**) had been enzymatically prepared previously by phosphorylation of 2-deoxy-2-fluoroglucose with ATP/hexokinase,[19] we chemically synthesized in this study mainly by adopting the literature procedures as depicted in Scheme 1.[21] Thus, easily available 2-deoxy-2-fluoro-D-glucose **5**[22] was converted conveniently to a 6-*O*-trityl ether in a standard manner, and the remaining hydroxy groups were then protected as acetate ester in one pot. Selective removal of the trityl group by acid gave **7**, and subsequent treatment with diphenyl phosphorochloridate in the presence of base afforded **8**. The protecting groups were successively removed by catalytic hydrogenolysis and base hydrolysis to yield 2-F-G-6-P (**1**). 3-F-G-6-P (**2**) was synthesized basically according to the previous method.[23] 2-NH_2-G-6-P (**3**) (D-glucosamine 6-phosphate) was commercially available and was used without further purification.[24] The synthesis of 3-NH_2-G-6-P (**4**) was performed in this study starting from the well-known 3-azido-3-deoxy-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose **9**[25] and is also shown in Scheme 1. After acid hydrolysis, the resulting 3-azido-D-glucose **10** was directly phosphorylated with diphenyl phosphorochloridate, and subsequent catalytic hydrogenolysis gave 3-NH_2-G-6-P (**4**).

Incubation of these substrate analogues was carried out as already described with a recombinant DOIS (BtrC).[13] The reaction products were converted into the corresponding *p*-nitrobenzyl oxime derivative by treatment with *O*-(*p*-nitrobenzyl)hydroxylamine for fluoro sugars and into the N-acetylated *p*-nitrobenzyl oxime derivatives for amino sugars by treatment with acetic anhydride, followed by *O*-(*p*-nitrobenzyl)hydroxylamine.[10,11] The resulting derivatization mixtures were subjected to HPLC analysis. The HPLC profiles of each incubation are shown in Figure 3. The formation of the enzymatic cyclization products from the 2-F- and 3-NH_2-G-6-P substrates was firmly detected, and each reaction product was clearly characterized. Thus, a large-scale incubation of 2-F- and 3-NH_2-G-6-P with DOIS was separately carried out, and the reaction products were converted into the *p*-nitrobenzyl oxime and the N-acetylated *p*-nitrobenzyl oxime derivatives, respectively, as described above. The derivatized reaction products were purified by preparative TLC and HPLC. The structures of these products were fully characterized by spectroscopic means including DQF−COSY and HMQC spectra, which are summarized in Table 1. These data well confirmed the structures of the enzymatic reaction products being 2-deoxy-4-fluoro- and 2-deoxy-5-amino-*scyllo*-inososes from the 2-F- and 3-NH_2-G-6-P, respectively. These results clearly indicated that DOIS was able to recognize 2-F- and 3-NH_2-G-6-P as substrate and the reaction proceeded in a usual manner. Further, the kinetic parameters were estimated for these substrate analogues in the DOIS reaction, and the results are shown in Table 2. While both analogues were less active as substrate compared with G-6-P, this could be due to the difference of overall electrostatic interactions between the enzyme and the analogues.

In contrast, no trace of the enzymatic reaction product was detected whatsoever from 3-F- and 2-NH_2-G-6-P. Furthermore, these analogues showed no inhibitory activity against the DOIS reaction (data not shown). Apparently, the enzyme does not accept these analogues in the active site at all. It appears, therefore, that the presence of hydroxy groups at C-2 and C-3 is important for the substrate interaction.

The results obtained in the present work are quite remarkable by the fact that the complementary results

(18) (a) Kovacs, T.; Pabuccuoglu, A.; Lesiak, K.; Torrence, P. F. *Bioorg. Chem.* **1993**, *21*, 192. (b) Chapeau, M. C.; Frey, P. A. *J. Org. Chem.* **1994**, *59*, 6994. (c) Percival, M. D.; Withers, S. G. *Biochemistry* **1992**, *31*, 498−505. (d) Withers, S. G.; Street, I. P.; Percival, M. D. *J. Am. Chem. Soc.*, **1988**, 59. (e) Street, I. P.; Armstrong, R. C.; Withers, S. G. *Biochemstry* **1986**, *25*, 6021.

(19) Bessell, E. M.; Foster, A. B.; Westwood, J. H. *Biochem. J.* **1972**, *128*, 199.

(20) Clarke, H. B.; Datta, S. P.; Rabin, B. R. *Biochem. J.* **1955**, *59*, 209.

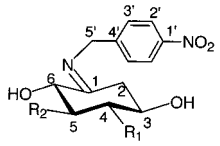
(21) Lardy, H. A.; Fischer, H. O. *J. Biol. Chem.* **1946**, *164*, 513.

(22) Kovác, P. *Carbohydr. Res.* **1986**, *153*, 168.

(23) Wright, J. A.; Taylor, N. F. *Carbohydr. Res.* **1974**, *32*, 366.

(24) Purchased from Sigma.

Table 1. Spectroscopic Data for the Derivatives of Enzymatic Reaction Products



	from 2-F-G-6-P (**1**): R_1 = F, R_2 = OH		from 3-NH_2-G-6-P (**4**): R_1 = OH, R_2 = NHAc	
	EI-MS *m/z* 530 (M^+, as trisTMS ether) δ_F (*J* in Hz), −193.8 (ddt, 52, 6, 13)		FAB-MS *m/z* 354 ($M^+ + 1$)	
	δ_H (*J* in Hz)	δ_C (*J* in Hz)	δ_H (*J* in Hz)	δ_C
1		156.0 (d, 2)		158.0
2	3.51 (dt, 14.3, 5.5)	29.3 (d, 9)	3.45 (dd, 13.9, 4.9)	30.6
	1.93 (dd, 14.3, 11.5)		2.02 (dd, 13.9, 10.4)	
3	3.70 (tdd, 11.5, 8.5, 5.5)	68.6 (d, 19)	3.51 (tdd, 13.9, 8.2, 4.9)	72.5
4	4.22 (dt, 51.5, 8.5)	98.4 (d, 180)	3.44 (dd, 9.0, 8.2)	76.7
5	3.47 (dt, 14.0, 8.5)	76.0 (d, 18)	3.68 (t, 9.2)	60.0
6	4.08 (d, 8.5)	73.7 (d, 9)	4.10 (d, 9.2)	73.3
1′		147.1		148.1
2′	8.21 (d, 8.8)	124.5	8.21 (d, 8.8)	125.2
3′	7.58 (d, 8.8)	129.6	7.60 (d, 8.8)	130.3
4′		148.9		149.6
5′	5.28 (s)	75.6	5.25 (s)	76.2
			1.97 (s, CH_3)	23.7 (CH_3)
				174.8 (C═O)

Table 2. Kinetic Parameters of the Substrate Analogues with DOIS

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
2-F-G-6-P (**1**)	2.1	0.050	24
3-NH_2-G-6-P (**4**)	1.4	0.014	10
G-6-P[a]	0.21	1.0	4800

[a] From ref 13.

were obtained by replacement of hydroxy groups at the C-2 and C-3 positions with either a fluorine or an amino group. As to the C-2 hydroxy group, substitution by an amino group (2-NH_2-G-6-P) resulted in loss of affinity to DOIS, whereas 2-F-G-6-P showed a moderate substrate activity, thereby strongly suggesting that the fluorine, thus the original C-2 hydroxy group, acts as hydrogen-bond acceptor. In contrast, the results concerning to the 3-position showed that replacement of the hydroxy group by fluorine diminished the binding affinity to DOIS and substitution by an amino group maintain the substrate activity, which well demonstrated that the C-3 hydroxy group of the substrate G-6-P acts as hydrogen-bond donor.

The amino acid sequence of the recombinant DOIS (BtrC) is similar to a certain extent to the sequences of dehydroquinate synthase (DHQ synthase) in the shikimate pathway, e.g., DHQ synthase of *E. coli* (26.5% in 324 aa overlap), of *Emericella nidulans* (26.4% in 276 aa overlap), and of *Bacillus subtilis* (34.1% in 264 aa overlap).[13] The three-dimensional structure of DHQ synthase derived from *E. nidulans* was recently elucidated by X-ray crystallography.[26] Actually, the *E. nidulans* DHQ synthase is a portion of a large multimodular polypeptide, which is different from DOIS;[26] however the catalytically relevant amino acids in the metal binding and substrate recognition sites seem to be conserved in BtrC, the 3-D structural feature of the substrate recognition of DOIS is proposed by modeling studies as depicted in Figure 4.[27] The results suggest a model complex where the hydrogen-bond-accepting carboxylate groups of Glu-183 and Asp-135 interact with the 3-hydroxy group of

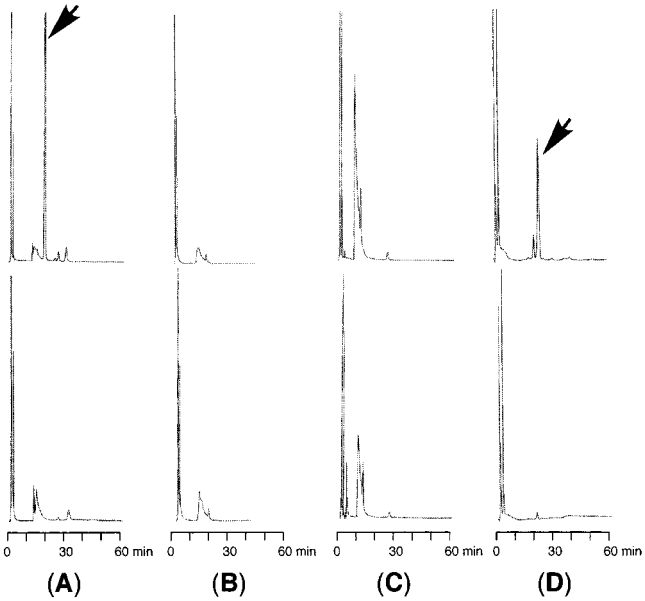


Figure 3. HPLC profiles of DOIS reaction products from substrate analogues. Top: reaction product. Bottom: control (incubations were carried out without the enzyme). (A) Reaction product from 2-F-G-6-P (**1**). (B) Reaction product from 3-F-G-6-P (**2**). (C) Reaction product from 2-NH_2-G-6-P (**3**). (D) Reaction product from 3-NH_2-G-6-P (**4**). An arrow indicates the peak of enzyme reaction product.

(25) Brimacombe, J. S.; Bryan, J. B.; Husain, A.; Stacey, M.; Tolley, M. S. *Carbohydr. Res.* **1967**, *3*, 318.

(26) Carpenter, E. P.; Hawkins, A. R.; Frost, J. W.; Brown, K. A. *Nature* **1998**, *394*, 299.

(27) Molecular modeling study was carried out by using the Homology program,[28] and structural refinement was done by CHARMm,[29] implemented in Insight II (ver. 98.0, Accelry Inc., San Diego).

(28) Greer, J. *Proteins*, **1990**, *7*, 317.

(29) (a) Brooks, B. R.; Bruccoleri, R. E.; Olafson, B. D.; States, D. J.; Swaminathan, S.; Karplus M. *J. Comput. Chem.* **1983**, *4*, 187. (b) MacKerell, A. D.; Brooks, Jr., B.; Brooks, C. L., III; Nilsson, L.; Roux, B.; Won, Y.; Karplus M. The Energy Function and Its Parametrization with an Overview of the Program. In *The Encyclopedia of Computational Chemistry*; Schleyer, P. v. R., Ed.; John Wiley & Sons: Chichester, 1998; Vol. 1, p 271.

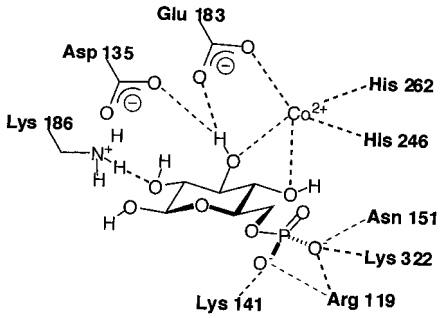


Figure 4. Plausible model of substrate recognition of 2-deoxy-*scyllo*-inosose synthase.

G-6-P. In turn, the hydrogen-bond-donating ammonium group of Lys-186 may recognize the 2-hydroxy group of the substrate. More detailed analysis of the interaction between substrate and DOIS is under investigation.

In conclusion, the present study clearly demonstrates that specific and directional hydrogen-bonding interactions between the hydroxy groups at C-2 and -3 of the G-6-P substrate and the active center of the DOIS enzyme play a important roles in DOIS reaction. It should thus be emphasized that the most sophisticated chemistry upon ubiquitous G-6-P catalyzed by DOIS really requires all the oxygen.

Experimental Section

General Information. Deuteriochloroform (99.8% atom enriched, Merck) or deuterium oxide (99.8% atom enriched, Merck) was used for NMR solvent. NMR chemical shifts were reported in δ value based on internal TMS (0 ppm) or solvent signal ($CDCl_3$ $\delta_C = 77.0$; D_2O $\delta_H = 4.65$) as references. When D_2O was used, dioxane ($\delta_C = 67.4$) was used as an internal standard. Kieselgel 60 (70−230 mesh, Merck) was used for standard column chromatography or Kieselgel 60 (230−400 mesh, Merck) for a flash chromatography. Preparative thin-layer chromatography was carried out with Merck Kieselgel 60 F_{254} (0.5 mm). All reactions, except for catalytic hydrogenation reaction, were carried out in an inert (Ar or N_2) atmosphere.

Protein concentration was quantitated by the Lowry−Folin method with bovine serum albumin as standard. Enzyme preparation was carried out at 4 °C.

1,3,4-Tri-*O*-acetyl-2-deoxy-2-fluoro-6-*O*-triphenylmethyl-D-glucopyranose (6). To a solution of **5**[22] (237 mg, 1.30 mmol) in pyridine (1.5 mL) was added triphenylmethyl chloride (543 mg, 1.95 mmol). The reaction mixture was stirred at 40 °C for 5 h. Acetic anhydride (2 mL) was then added, and the reaction mixture was further stirred for an additional 30 min. The mixture was diluted with ether, and water was added. The organic layer was separated, and the aqueous layer was extracted with ether. The combined organic layer was washed with 1 M HCl, saturated aqueous $NaHCO_3$, and brine and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by column chromatography with hexanes−EtOAc (5:1) to give **6** (634 mg, 89%, α/β = ca.1:0.9): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.45−7.10 (m, aromatic), 6.57 (t, J = 3.9 Hz, H-1, α-anomer), 5.77 (dd, J = 8.1, 3.2 Hz, H-1, β-anomer), 5.45 (dt, J = 12.1, 9.5 Hz, H-3, α-anomer), 5.31 (dt, J = 14.2, 9.2 Hz, H-3, β-anomer), 5.26 (t, J = 10.2 Hz, H-4, α-anomer), 5.20 (t, J = 9.6 Hz, H-4, β-anomer), 4.75 (ddd, J = 49, 9.6, 3.9 Hz, H-2, β-anomer), 4.50 (dt, J = 51, 8.5 Hz, β-anomer), 3.97 (ddd, J = 10.3, 3.7, 2.5 Hz, H-5, α-anomer), 3.71 (ddd, J = 9.8, 3.9, 2.4 Hz, H-5, β-anomer), 3.34 (dd, J = 10.5, 2.5 Hz, H-6), 3.33 (dd, J = 10.5, 2.5 Hz, H-6), 3.06 (dd, J = 10.9, 3.9 Hz, H-6), 3.02 (dd, J = 10.9, 3.9 Hz, H-6), 2.21 (s), 2.17 (s), 2.08 (s), 2.04 (s), 1.75 (s), 1.74 (s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.1, 170.0, 168.9, 168.8, 168.6, 168.5, 143.2, 128.5, 127.6, 126.9, 91.2 (d, J = 24 Hz), 88.4 (d, J = 22 Hz), 86.3 (d, J = 190 Hz), 86.4, 86.3, 86.1 (d, J = 193 Hz), 73.8, 72.9 (d, J = 19 Hz), 70.9 (d, J = 19 Hz), 70.7, 67.9 (d, J = 7 Hz), 67.5 (d, J = 7 Hz), 61.2, 60.8, 20.6, 20.5, 20.4, 20.3, 20.2. Anal. Calcd for $C_{31}H_{31}O_8F$: C,67.63; H, 5.68. Found: C, 67.82; H, 5.94.

1,3,4-Tri-*O*-acetyl-2-deoxy-2-fluoro-D-glucopyranose (7). A solution of **6** (195 mg, 0.356 mmol) in AcOH−H_2O (4 mL, 3:1) was stirred at 80 °C for 1 h. The reaction mixture was cooled to room temperature, and the solvent was removed under vacuum to give a residue, which was purified by column chromatography with hexanes−EtOAc (1:1) to yield **7** (105 mg, 95%, α/β = ca.1:1): ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.42 (d, J = 3.9 Hz, H-1, α-anomer), 5.80 (dd, J = 8.1, 3.2 Hz, H-1, β-anomer), 5.60 (dt, J = 12, 9.6 Hz, H-3, α-anomer), 5.45 (dt, J = 14, 9.3 Hz, H-3, β-anomer), 5.07 (t, J = 10 Hz, H-4, α-anomer), 5.04 (t, J = 9.5 Hz, H-4, β-anomer), 4.65 (ddd, J = 49, 9.8, 3.9 Hz, H-2, α-anomer), 4.45 (ddd, J = 51, 9.1, 8.1 Hz, H-2, β-anomer), 3.96−3.85 (m, H-5, α-anomer), 3.83−3.65 (m), 3.63−3.52 (m), 2.65 (br, 6-OH), 2.20 (s), 2.18 (s), 2.12 (s), 2.11 (s), 2.08 (s), 2.07 (s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 170.1, 170.0, 169.8, 168.9, 168.8, 91.0 (d, J = 24 Hz), 88.2 (d, J = 22 Hz), 88.1 (d, J = 191 Hz), 86.0 (d, J = 193 Hz), 74.7, 72.3 (d, J = 19 Hz), 71.7, 70.1 (d, J = 20 Hz), 67.8 (d, J = 7 Hz), 67.5 (d, J = 7 Hz), 60.4, 60.3, 20.7, 20.7, 20.6, 20.5, 20.5. Anal. Calcd for $C_{12}H_{17}O_8F$: C,46.76; H, 5.56. Found: C, 46.69; H, 5.80.

1,3,4-Tri-*O*-acetyl-2-deoxy-6-*O*-diphenyloxyphosphoryl-2-fluoro-D-glucopyranose (8). Diphenyl phosphorochloridate (3.51 mL, 16.8 mmol) was added to a solution of **7** (2.6 g, 8.43 mmol) in CH_2Cl_2 (15 mL) and pyridine (5 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1.5 h. The mixture was diluted with ether, and water was added. The organic layer was separated, and the aqueous layer was extracted with ether. The combined organic layer was washed with 1 M HCl, saturated aqueous $NaHCO_3$, and brine and then dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by column chromatography with hexanes−EtOAc (1:1) to yield **8** (3.6 g, 78%, α/β = ca.1:1.1): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38−7.12 (m, aromatic), 6.36 (d, J = 3.9 Hz, H-1, α-anomer), 5.85 (t, J = 8.1, 3.2 Hz, H-1, β-anomer), 5.54 (dt, J = 12, 9.5 Hz, H-3, α-anomer), 5.42 (dt, J = 14, 9.2 Hz, H-3, β-anomer), 5.08 (t, 10 Hz, H-4, α-anomer), 5.07 (t, 9.8 Hz, H-4, β-anomer), 4.55 (ddd, J = 53, 9.8, 3.9 Hz, H-3, α-anomer), 4.51−4.20 (m), 4.18−4.08 (m, H-5, α-anomer), 3.98−3.85 (m, H-5, β-anomer), 2.09 (s), 2.07 (s), 2.05 (s), 2.01 (s), 2.00 (s), 1.97 (s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.7, 169.5, 169.0, 168.9, 168.3, 168.1, 150.1, 150.0, 129.5, 125.1, 119.8, 119.7, 119.6, 90.6 (d, J = 25 Hz), 87.8 (d, J = 190 Hz), 87.7 (d, J = 22 Hz), 85.4 (d, J = 193 Hz), 72.5 (d, J = 7 Hz), 72.2 (d, J = 19 Hz), 70.1 (d, J = 20 Hz), 69.6 (d, J = 7 Hz), 67.2 (d, J = 7 Hz), 67.0 (d, J = 7 Hz), 65.9 (d, J = 6 Hz), 65.8 (d, J = 6 Hz), 20.2, 20.1, 20.0. Anal. Calcd. for $C_{24}H_{26}O_{11}FP$: C, 53.34; H, 4.85. Found: C, 53.26; H, 5.06.

2-Deoxy-2-fluoro-D-glucose 6-phosphate Potassium Salt (1). To a solution of **8** (703 mg, 1.30 mmol) in methanol (3 mL) was added PtO_2 (146 mg). The mixture was stirred vigorously for 6 h under hydrogen atmosphere. The catalyst was removed by filtration. The filtrate was evaporated to yield 1,3,4-tri-*O*-acetyl-2-deoxy-2-fluoro-D-glucopyranose 6-phosphate (505 mg, quant). To a solution of the obtained 1,3,4-tri-*O*-acetyl-2-deoxy-2-fluoro-D-glucopyranose 6-phosphate (505 mg, 1.30 mmol) in methanol (2 mL) was added a solution of potassium methoxide in methanol (5 mL, 0.653 mmol/ml) at 0 °C. The mixture was stored overnight at 0 °C. The precipitate was collected by centrifugation and was then washed successively with 2-propanol, EtOH−ether (4:1), EtOH−ether (1:1), EtOH−ether (1:4), and ether to give **1** (375 mg, 85%): ^{1}H NMR (300 MHz, D_2O) δ 5.28 (d, J = 3.2 Hz, H-1, α-anomer), 4.28 (ddd, J = 50, 9.1, 3.5 Hz, H-2, α-anomer), 4.03 (t, J = 8.3 Hz, H-2, β-anomer), 3.98−2.75 (m); ^{13}C NMR (100 MHz, D_2O) δ 95.3 (d, J = 23 Hz), 94.6 (d, J = 181 Hz), 91.6 (d, J = 184 Hz), 91.0 (d, J = 21 Hz), 76.2 (d, J = 7 Hz), 74.4 (d, J = 17 Hz), 71.6 (d, J = 17 Hz), 71.5 (d, J = 7 Hz), 69.7 (d, J = 8 Hz), 69.5 (d, J = 7 Hz), 63.4 (d, J = 4 Hz), 63.3 (d, J = 3 Hz).

3-Azide-3-deoxy-6-*O*-diphenyloxyphosphoryl-D-glucose (10). A solution of **9**[25] (1.05 g, 3.68 mmol) in 1 M H_2SO_4 (6 mL) was stirred at 40 °C for 6 h. The mixture was neutralized by addition of Amberlite IRA-410 anion-exchange resin (OH^- form). After the resin was filtered, the solution was concentrated to dryness. The resulting residue (750 mg) was dissolved in pyridine (12.1 mL), and diphenyl phosphorochloridate (0.77 mL, 4.01 mmol) was added at 0 °C. The mixture was stirred at room temperature for 30 min. Water and $CHCl_3$ were added, the organic layer was separated, and the aqueous layer was extracted with $CHCl_3$. The combined organic layer was dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by column chromatography with CH_2Cl_2−methanol (10:1) to give **9** (440 mg, 47%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.20 (dd, J = 7.6, 8.0 Hz), 3.26−3.37 (m), 3.39 (t, J = 9.2 Hz), 3.47 (m), 3.61, (t, J = 9.6 Hz), 3.96 (m), 4.35−4.54 (m), 5.14 (d, J = 4.0 Hz, H-1, β-anomer), 7.18−7.22 (12H, m), 7.31−7.36 (8H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 129.8 (d, J = 5 Hz), 125.7, 125.6, 120.2 (d, J = 5 Hz), 120.02, 119.98, 119.97, 119.94, 96.9, 91.8, 73.8, 70.3, 70.28, 70.24, 68.2, 68.0, 67.6 (d, J = 5.8), 67.4, 65.9. Anal. Calcd for $C_{18}H_{20}N_3O_8P$: C, 49.43; H, 4.6; N, 9.61. Found: C, 49.52; H, 4.91; N, 9.54.

3-Amino-3-deoxy-D-glucose 6-Phosphate (4). To a solution of **10** (420 mg, 0.961 mmol) in water−acetic acid (2:1, 18 mL) was added PtO_2 (250 mg). The mixture was vigorously stirred for 12 h under hydrogen atmosphere. The catalyst was removed by filtration. The filtrate was concentrated to dryness, and the residue was crystallized from water−acetone to give **4** (220 mg, 84%): ^{1}H NMR (300 MHz, D_2O) δ 3.04 (dd, J = 10.2, 10.5 Hz, H-3, β-anomer), 3.22 (dd, J = 10.2, 10.7 Hz, H-3, α-anomer), 3.28 (dd, J = 7.8, 10.5 Hz, H-2, β-anomer), 3.59 (dd, J = 10.2, 10.7 Hz, H-4, α-anomer), 3.60 (dd, J = 3.5, 10.7 Hz, H-2, α-anomer), 3.61 (dd, J = 9.7, 10.2 Hz, H-4, β-anomer), 3.76−3.85 (m), 3.88−3.99 (m); ^{13}C NMR (75.5 MHz, D_2O) δ 31.07, 55.59, 58.34, 63.9, 66.1 (d, J = 6.1), 67.4, 68.8, 71.2, 71.1 (d, J = 7.4), 76.4 (d, J = 7.4), 92.1, 97.0. Anal. Calcd for $C_6H_{14}NO_8P$: C, 27.80; H, 5.44; N, 5.40. Found: C, 27.51; H, 5.74; N, 5.11.

Preparation of Recombinant DOIS from *E. coli*. Purified enzyme was prepared according to the method of Kudo et al.[13,14]

HPLC Analysis of DOIS Reaction with Substrate Analogues. An aliquot of a purified enzyme solution (65 μL, 2 μM) was added to a mixture of a substrate analogue (1.2−20 mM), NAD^+ (5 mM), and $CoCl_2$ (2 μM) in Tris−HCl buffer (pH 7.0, 100 μL). Incubation was carried out at 46 °C for 15 min. The reaction was terminated by adding 100 μL of methanol for fluorodeoxy analogues (**1** and **2**) and 100 μL of acetic anhydride for aminodeoxy analogues (**3** and **4**). For the latter reaction of **3** and **4**, each mixture was stirred at 46 °C for 30 min and then lyophilized, and methanol (100 μL) was added. To each methanolic solution was added 20−50 μL of *O*-(4-nitrobenzyl)hydroxylamine hydrochloride (NBHA) in pyridine (5 mg/mL). The resulting mixture was then heated to 60 °C for 1 h, and then the solvent was removed by flushing the air. The residue was dissolved in 100 μL of methanol, and the mixture was centrifuged (10 000 rpm × 10 min). The supernatant was filtered. An aliquot of each supernatant (5 μL) was analyzed on an HPLC apparatus equipped with a TSKgel ODS-80TM CTR column (4.6 mm i.d. × 10 cm, TOSOH). The elution was monitored by UV absorbance at 262 nm. The mobile phase consisted of 20% of methanol in deionized water. The reaction kinetics data were graphically analyzed by Lineweaver−Burk double-reciprocal plots.

Isolation of the Enzyme Reaction Products from Large-Scale Incubation. A purified enzyme solution (3 mL, 2 μM) was added to a mixture of a substrate analogue (5 mM), NAD^+ (5 mM), and $CoCl_2$ (2 μM) in a Tris−HCl buffer (pH 7.0, 4.7 mL). Incubation was carried out 46 °C for 30 min. The reaction was terminated by adding 5 mL of methanol for fluorodeoxy analogue **2** and 7.7 mL of acetic anhydride for aminodeoxy analogue **3**. For the reaction of **3**, the mixture was stirred at 46 °C for 1 h and then lyophilized, and methanol (7.7 mL) was added. To each methanolic solution was added 1.5 mL of a NBHA solution in pyridine (5 mg/mL). The mixture was heated to 60 °C for 2 h. The precipitate was removed by filtration, and the filtrate was concentrated. The residue was purified by preparative thin-layer chromatography with $CHCl_3$−MeOH (5:1), and then HPLC (Senshu Pak ODS column, 20 mm i.d., 25 cm, 20−50% CH_3CN in H_2O, 4 mL/min).

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