VOLUME 69, NUMBER 3

JOC Articles

FEBRUARY 6, 2004

Active Site Mapping of 2-Deoxy-*scyllo*-inosose Synthase, the Key Starter Enzyme for the Biosynthesis of 2-Deoxystreptamine. Mechanism-Based Inhibition and Identification of Lysine-141 as the Entrapped Nucleophile[§]

Eriko Nango,[†] Tadashi Eguchi,[‡] and Katsumi Kakinuma*[,†]

Department of Chemistry and Department of Chemistry and Materials Science, Tokyo Institute of Technology, O-okayama, Meguro-ku, Tokyo 152-8551, Japan

kakinuma@chem.titech.ac.jp

Received May 24, 2003

A key enzyme in the biosynthesis of clinically important aminoglycoside antibiotics including neomycin, kanamycin, gentamicin, etc. is 2-deoxy-*scyllo*-inosose synthase (DOIS), which catalyzes the carbocycle formation from D-glucose-6-phosphate to 2-deoxy-*scyllo*-inosose (DOI). To clarify its precise reaction mechanism and crucial amino acid residues in the active site, we took advantage of a mechanism-based inhibitor carbaglucose-6-phosphate (pseudo-DL-glucose, C-6-P) with anticipation of its conversion to a reactive α,β-unsaturated carbonyl intermediate. It turned out that C-6-P clearly showed time- and concentration-dependent inhibition against DOIS, and the molecular mass of the resulting modified-DOIS with C-6-P was 160 mass units larger than that of native DOIS. Thus, the expected α,β-unsaturated intermediate appeared to trap a specific nucleophilic group in the active site through the Michael-type 1,4-addition. The covalently modified amino acid residue was determined to be Lys-141 by means of enzymatic digestion and subsequent LC/MS and LC/MS/MS of the digest. Also discussed are the role of Lys-141 in the substrate recognition and the reaction pathway and comparison with evolutionary related dehydroquinate synthase.

Introduction

2-Deoxystreptamine (DOS)-containing aminoglycoside antibiotics including neomycin, kanamycin, gentamicin, and ribostamycin are clinically important antibacterial agents.[1,2] Improvement of these antibiotics has long been a subject of intensive studies. An important biochemical approach to this end included mutasynthesis or mutational biosynthesis, which utilized idiotrophic mutants of producing strains and supplementation of appropriate analogues of biosynthetic intermediates.[3,4] Particularly focused at one time were those mutants which were unable to biosynthesize DOS.[5−10] Most of the chemical pathways from D-glucose to DOS and ultimate antibiotics have been elucidated so far, mostly through the isotope-tracer studies,[1,2,11,12] however, any biosynthetic enzymes and relevant genes have not been characterized until recently.

2-Deoxy-*scyll*o-inosose (DOI) synthase (DOIS) is the key enzyme in the biosynthesis of DOS, which catalyzes the cyclization of D-glucose-6-phosphate (G-6-P) into the six-membered carbocycle DOI[11,12] as the first intermediate in the DOS biosynthesis.[1,6,7,13,14] The reaction mech-

[§] This paper is dedicated to Dr. Kenneth L. Rinehart, professor emeritus of the University of Illinois, on the occasion of his 75th birthday.

* To whom correspondence should be addressed.

[†] Department of Chemistry.

[‡] Department of Chemistry and Materials Science.

(1) Rinehart, K. L., Jr.; Stroshane, R. M. *J. Antibiot.* **1976**, *29*, 319−353.

(2) Rinehart, K. L., Jr.; Suami, T., Eds. *Aminocyclitol antibiotics*; ACS Symp. Ser., No. 125; American Chemical Society: Washington, DC, 1980.

(3) Shier, W. T.; Rinehart, K. L., Jr.; Gottlieb, D. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *63*, 198−204.

(4) Nagaoka, K.; Demain, A. L. *J. Antibiot.* **1975**, *28*, 627−635.

(5) Daum, S. J.; Rosi, D.; Goss, W. A. *J. Am. Chem. Soc.* **1977**, *99*, 283−284.

(6) Daum, S. J.; Rosi, D.; Goss, W. A. *J. Antibiot.* **1977**, *30*, 98−105.

(7) Furumai, T.; Takeda, K.; Kinumaki, A.; Ito, Y.; Okuda, T. *J. Antibiot.* **1979**, *32*, 891−899.

(8) Kase, H.; Iida, T.; Odakura, Y.; Shirahata, K.; Nakayama, K. *J. Antibiot.* **1980**, *33*, 1210−1212.

(9) Fujiwara, T.; Takahashi, Y.; Matsumoto, K.; Kondo, E. *J. Antibiot.* **1980**, *33*, 824−829.

(10) Fujiwara, T.; Takahashi, Y.; Matsumoto, K.; Kondo, E. *J. Antibiot.* **1980**, *33*, 836−841.

(11) Kakinuma, K.; Ogawa, Y.; Sasaki, T.; Seto, H.; Otake, N. *J. Am. Chem. Soc.* **1981**, *103*, 5614−5616.

(12) Kakinuma, K.; Ogawa, Y.; Sasaki, T.; Seto, H.; Otake, N. *J. Antibiot.* **1989**, *42*, 926−933.

10.1021/jo034706y CCC: $27.50
Published on Web 09/23/2003

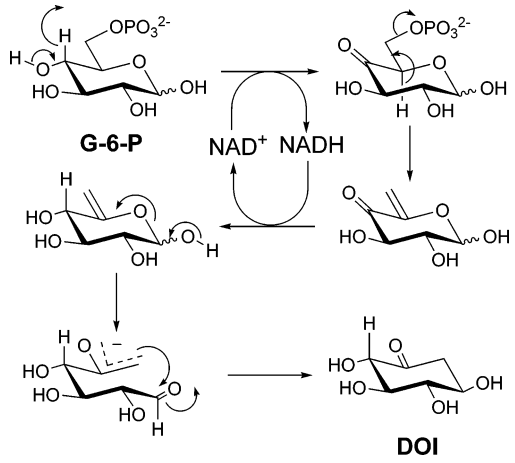


FIGURE 1. Reaction mechanism of DOIS.

anism of DOIS was already elucidated as shown in Figure 1 by crossover experiments using an isotopically double-labeled substrate with partially purified enzyme preparations derived from butirosin-producing *Bacillus circulans* (*B. circulans*) SANK 72073 and neomycin-producing *Streptomyces fradiae* IFO 13147.[15,16]

The over-all reaction of DOIS is comprised of five steps. Initial NAD^+-dependent dehydrogenation at C-4 of the G-6-P substrate, followed by elimination of a phosphate from C-6, gives an enone functionality on the glucose backbone. Then, the hydride, which is derived from the initial dehydrogenation and resides at the associated NADH, comes back to the original C-4 position of the intermediate. Subsequent hemiacetal ring opening and the final aldol-type intramolecular condensation between C-6 and C-1 give rise to DOI. This DOIS reaction was pointed out to be mechanistically similar to that of dehydroquinate synthase (DHQS) catalyzing the cyclization of 3-deoxy-D-*arabino*-heputulosonate-7-phosphate (DAHP) to dehydroquinate (DHQ) in the shikimate pathway,[17] but dissimilarity between DOIS and DHQS was also found, particularly in the stereochemistry of over-all reactions.[11,12,18]

Recently, we isolated DOIS from the above-mentioned *B. circulans* SANK72073,[19] and the structural gene (*btrC*) was subsequently cloned and overexpressed in *Escherichia coli* (*E. coli*).[20] Certain homology (25−29%) was observed between *B. circulans* DOIS and several DHQSs on the amino acid level. With the recombinant DOIS (BtrC) in hand, we envisaged high potential of DOIS and DOI product for the preparation of various useful resources, particularly of benzenoid compounds. We successfully demonstrated a short-step conversion of D-glucose into catechol by the chemoenzymatic approach with DOI.[21] Preparation of hydroquinone from DOI has been additionally described recently by others.[22]

To explore the potential of DOIS and DOI for antibiotic improvement and chemical manufacturing, more precise and detailed features of the chemistry and the substrate recognition of the DOIS active site are indispensable. Along this line, we have described very recently the substrate recognition of DOIS using substrate analogues. Specific hydrogen bond accepting and donating interactions between the hydroxy groups at C-2 and C-3 of G-6-P and the enzyme were shown to be responsible for the recognition.[23]

To identify crucial amino acids in the active site, and to get closer insight into the enzyme catalysis, we took advantage of a mechanism-based inhibitor.[24,25] In this study, carbaglucose-6-phosphate (pseudo-DL-glucose, C-6-P) was designed as an inhibitor to be involved in ready formation of a specific covalent bond with the surrounding enzyme framework. Through the first-half reaction by DOIS, C-6-P is envisioned to be transformed into an α,β-unsaturated carbonyl intermediate, which can be trapped in a specific nucleophilic group of the active site through 1,4-addition. The covalently modified amino acid residue should subsequently be identified by LC/MS and LC/MS/MS. In this paper, we describe detailed analysis of the DOIS reaction and the DOIS active site with a mechanism-based inhibitor C-6-P. Similarity and dissimilarity between DOIS and DHQS are also discussed accordingly.

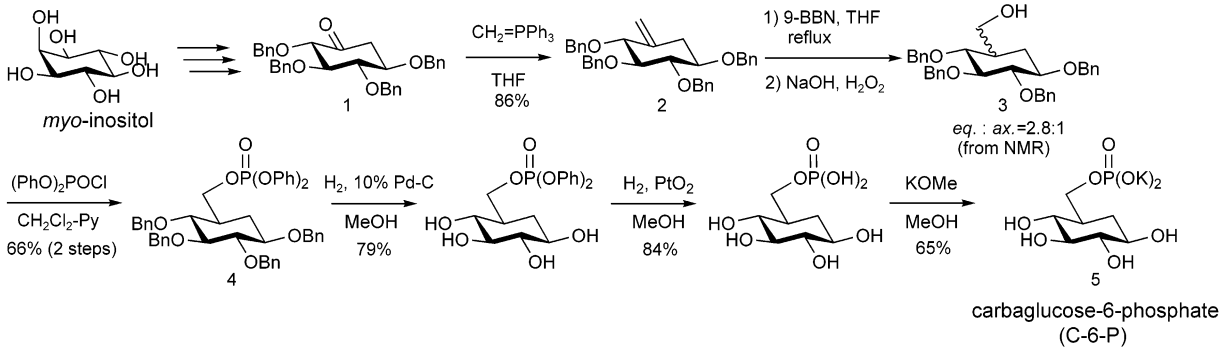
Results and Discussion

To get insight into the active site of DOIS, we designed a carbaglucose analogue as a mechanism-based inhibitor to trap a relevant amino acid residue. Carbaglucose was prepared previously by several groups, and their multistep syntheses started either with *exo*-2,*endo*-3-diacetoxy-*endo*-5-acetoxymethyl-7-oxabicyclo[2.2.1]heptane, D-glucuronolactone, or with dehydroshikimic acid.[26−28] We developed a new facile synthetic procedure for C-6-P from protected racemic DOI (**1**) as shown in Scheme 1, since this starting material had previously been synthesized in our group.[29]

Transformation of **1** into C-6-P was rather straightforward. Thus, **1** was subjected to Wittig reaction with methylenetriphenylphosphorane in THF. In a reaction under 90 mM concentration of the substrate **1**, the product yield was rather low, because elimination and subsequent aromatization took place. Under a diluted concentration of 40 mM, the product (**2**) was obtained in 86% yield without aromatization. Subsequent hydrobo-

(13) Yamauchi, N.; Kakinuma, K. *J. Antibiot.* **1992**, *45*, 767−773.
(14) Yamauchi, N.; Kakinuma, K. *J. Antibiot.* **1992**, *45*, 774−780.
(15) Yamauchi, N.; Kakinuma, K. *J. Org. Chem.* **1995**, *60*, 5614−5619.
(16) Kudo, F.; Yamauchi, N.; Suzuki, R.; Kakinuma, K. *J. Antibiot.* **1997**, *50*, 424−428.
(17) Yamauchi, N.; Kakinuma, K. *J. Antibiot.* **1993**, *46*, 1916−1918.
(18) Nango, E.; Kudo, F.; Eguchi, T.; Kakinuma, K. *Chem. Lett.* **2003**, *32*, 438−439.
(19) Kudo, F.; Hosomi, Y.; Tamegai, H.; Kakinuma, K. *J. Antibiot.* **1999**, *52*, 81−88.
(20) Kudo, F.; Tamegai, H.; Fujiwara, T.; Tagami, U.; Hirayama, K.; Kakinuma, K. *J. Antibiot.* **1999**, *52*, 559−571.
(21) Kakinuma, K.; Nango, E.; Kudo, F.; Matsushima, Y.; Eguchi, T. *Tetrahedron Lett.* **2000**, *41*, 1935−1938.
(22) Hansen, C. A.; Frost, J. W. *J. Am. Chem. Soc.* **2002**, *124*, 5926−5927.
(23) Eguchi, T.; Sasaki, S.; Huang, Z.; Kakinuma, K. *J. Org. Chem.* **2002**, *67*, 3979−3984.
(24) Silverman, R. B. *Mechanism Based Enzyme Inactivation: Chemistry and Enzymology*; CRC Press Inc.: Boca Raton, FL, 1988.
(25) Walsh, C. T. *Annu. Rev. Biochem.* **1984**, *53*, 493−535.
(26) Ogawa, S.; Tsukiboshi, Y.; Iwasawa, Y.; Suami, T. *Carbohydr. Res.* **1985**, *136*, 77−89.
(27) Yoshikawa, M.; Murakami, N.; Yokokawa, Y.; Inoue, Y.; Kuroda, Y.; Kitagawa, I. *Tetrahedron* **1994**, *50*, 9619−9628.
(28) Entwistle, D. A.; Hudlicky, T. *Tetrahedron Lett.* **1995**, *36*, 2591−2594.
(29) Yamauchi, N.; Kakinuma, K. *J. Antibiot.* **1992**, *45*, 756−766.

SCHEME 1. Synthesis of C-6-P



ration of **2** with 9-BBN under reflux yielded a mixture of two diastereoisomeric alcohols (eq:ax = 2.8:1), which were chromatographically unseparable. The mixture was immediately converted into diphenyl phosphates and the resulting phosphates were able to be separated by silica gel column chromatography to give diphenyl phosphate (**4**). Deprotection by two-step catalytic hydrogenation, first using Pd−C and then PtO_2, afforded the desired DL-C-6-P (**5**).

With this postulated inhibitor in hand, the inhibition assay was first carried out with purified DOIS. As expected, when the DOIS-inhibitor reaction mixture was subjected to gel filtration to separate DOIS from the inhibitor, the DOIS activity could not be recovered at all. These results strongly suggested covalent-bond formation between the enzyme and the inhibitor. Accordingly, an inactivation assay comprised of preincubation of DOIS with C-6-P, subsequent dilution, and assay of residual enzyme activity was next pursued to see time- and concentration-dependent inactivation.

As shown in Figure 2, C-6-P was clearly found to be a specific mechanism-based inhibitor. The half-time ($t_{1/2}$) for inactivation at each inhibitor concentration was plotted against 1/[C-6-P], referred to as a Kitz−Wilson plot (Figure 3),[30] to provide K_I and k_{inact} values for C-6-P.

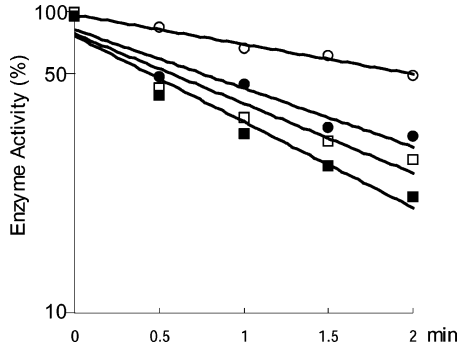


FIGURE 2. Time-dependent inactivation of DOIS by C-6-P. A solution (25 μL) of DOIS (54 μM), C-6-P (250−6000 μM), 5 mM NAD^+, and 50 mM Tris-HCl−0.2 mM $CoCl_2$ (buffer A, pH 7.7) was preincubated at 46 °C for 1−2 min. The reaction mixture was diluted 40-fold with the same buffer A for subsequent assay of residual activity. An assay mixture (total 50 μL) containing the diluted preincubation solution, 5 mM NAD^+, 5 mM D-glucose-6-phosphate, and buffer A was incubated at 46 °C for 15 min. C-6-P concentrations were 100 (open circle), 800 (closed circle), 1600 (open square), and 2400 μM (closed square).

In fact, C-6-P was found to be remarkably inhibitory against DOIS (K_I = 224 μM, $k_{inact} = 3.22 \times 10^{-3}$ s^{-1}). As compared with K_m (210 μM) for the substrate G-6-P, C-6-P was recognized similarly as G-6-P. On the other hand, the k_{inact} value for C-6-P suggested rather slow inactivation in comparison with normal catalysis, k_{cat} (1.0 s^{-1}), for the substrate G-6-P. This may probably be due to the slow 1,4-addition reaction of a nucleophilic residue to the inhibitor. To confirm such covalent bond formation between C-6-P and DOIS, LC/MS analysis was carried out with the reacted enzyme. The molecular mass of the enzyme−inhibitor adduct was calculated by deconvolution with software provided by the instrument manufacturer. Significant change of the molecular mass of DOIS before and after incubation with C-6-P is shown in Figure 4. The resulting mass of DOIS bound with C-6-P was estimated to be 40768 Da, which was ca. 160 mass units larger than that of the native enzyme (40608 Da), although the unreacted enzyme remained because of brief reaction time. This mass difference clearly indicated that the covalent bond formation took place between DOIS and C-6-P through direct addition without any dehydration. The possibility of nonselective formation of an imine adduct with a ketone intermediate could be ruled out because dehydration should have been involved in this case.

C-6-P was in fact a mechanism-based irreversible inhibitor and its reaction with DOIS was proposed accordingly as shown in Scheme 2. Thus, after the initial oxidation at C-4 and subsequent elimination of a phosphate, C-6-P was converted within the enzyme into an α,β-unsaturated methylene cyclohexanone, which was

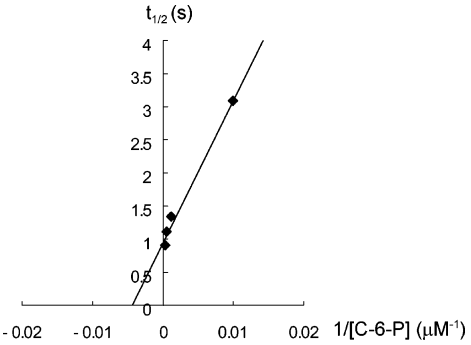


FIGURE 3. Kitz and Wilson plot for the inactivation of DOIS by C-6-P. Half-times ($t_{1/2}$) for inactivation at each inhibitor concentration were estimated from Figure 2 and plotted against 1/[C-6-P].

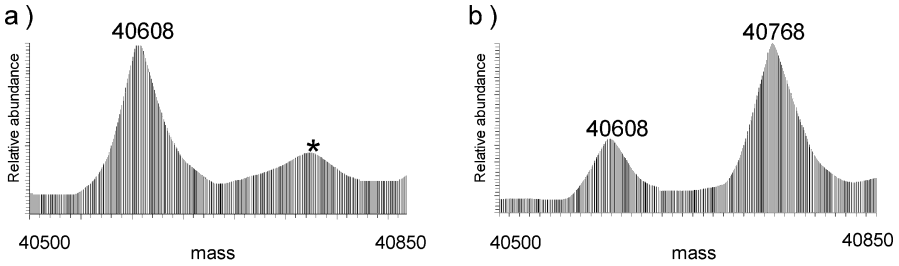
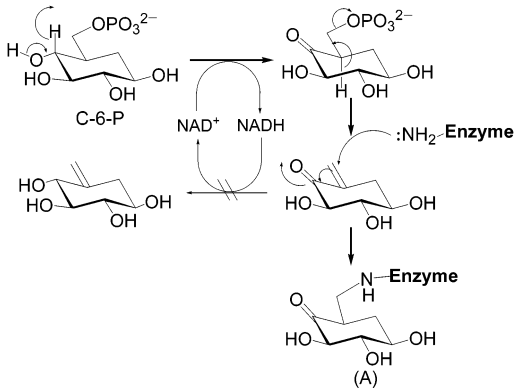


FIGURE 4. The molecular masses of (a) DOIS and (b) DOIS after reaction with C-6-P. An aliquot of a DOIS/C-6-P reaction product was loaded onto a Senshu Pak PEGASIL-300 C4P column, which was connected on-line to a mass spectrometer (Finnigan LCQ). Elution was carried out with a mixture consisting of acetonitrile/water (9:1) containing 0.1% HCOOH and 0.01% TFA. The molecular masses were estimated by deconvolution with BioWorks 1.0 software. The asterisk (∗) indicates unknown noise derived from deconvolution.

SCHEME 2. Proposed Inhibition Mechanism of C-6-P



then attacked by a nearby nucleophilic residue in the active site to afford a 1,4-addition product (**A**).

We turned our attention then to identification of the specific residue bound to C-6-P. The key approach to this involved proteolytic digestion of the DOIS−C-6-P adduct, followed by LC/MS and LC/MS/MS analyses of the resulting digests. The initial attempt at digestion of native DOIS without any prior treatment suggested significant resistance of DOIS to various peptidases. To improve the susceptibility of DOIS to enzyme digestion, reductive carboxymethylation (RCM) was undertaken to cleave disulfide bonds and to protect the resulting thiol groups of four cysteines. The RCM-treated DOIS−C-6-P adduct was easily digested by proteases. Another encountered problem was that undesired β-elimination of the bound amino acid residue took place during RCM, probably because of rather strong alkaline conditions. To circumvent such β-elimination, the DOIS−inhibitor adduct was first treated, prior to RCM, with $NaBH_4$ to reduce its intermediary saturated ketone to an alcohol functionality.

Tryptic digests of DOIS−C-6-P adduct were first analyzed closely by LC-MS. Predicted tryptic fragments are tabulated in Table 1. Individual molecular masses were comprehensively calculated for each LC fraction, except for those peptide fragments having smaller molecular mass below 300 amu. While almost all of the anticipated fragments were observed, the fragments corresponding to residues 120−150 expected for the cleavage at Arg-120, Lys-141, and Lys-150 were not detected. These results strongly suggested the binding of C-6-P to a residue in this region. LC/MS/MS analysis of the tryptic digest was attempted to determine the binding site without success, since the crucial fragments were larger than 2000 Da. A hydrolytic enzyme was then switched to chymotrypsin, which afforded rather smaller peptide fragments, well suited for MS/MS analysis; however, an alternative difficulty in identifying relevant peptides was encountered because of the complexity due to the formation of too many fragments. To surmount this problem, we took advantage of differential detection on mass spectra between isotope-labeled and nonlabeled specimen. A labeled inhibitor [6,6-$^2H_2$]-C-6-P was synthesized by using deuteriomethylenetriphenylphosphorane in a similar way to that of unlabeled inhibitor, and the reaction with DOIS was carried out similarly. After RCM modification and digestion of the adduct with chymotrypsin, peptide fragments showing 2-amu difference were surveyed in the HPLC elutes having the same retention time.

A chymotrypsin fragment being eluted at ca. 40 min showed a molecular ion of *m*/*z* 1032.6 (derived from DOIS bound with nonlabeled-C-6-P) or an ion of *m*/*z* 1034.6 (labeled-C-6-P bound DOIS) as shown in Figure 5. From these results, the inhibitor-binding site was suggested to be within the 139−146 fragment SIKQAVNL. Ultimate determination of the C-6-P binding site was furnished

TABLE 1. Molecular Masses of Tryptic Digests[a]

fragment	predicted (monoisotopic mass)	obsd[b]	fragment	predicted	obsd[b]
1−4	479.6	480.2	226−236	1313.9	1313.6
5−11	910.01	910.4	237−237	146.19	ND
12−31	2402.66	1201.9[c]	238−238	146.19	ND
32−59	3197.47	1599.0[c]	239−275	3839.8	1920.0[c]
60−67	918.15	918.6	276−279	472.54	ND
68−75	957.01	957.3	280−297	2198.5	1099.8
76−86	1261.4	1261.6	298−307	1067.9	1067.6
87−94	784.91	785.4	308−322	1800.4	901.2
95−95	174.2	ND	323−323	174.2	ND
96−119	2220.64	1110.7	324−327	479.58	479.4
120−141	**2253.66**	**ND**	328−357	3173.7	3173.7
142−150	**917.03**	**ND**	358−359	473.61	474.3
151−160	1225.41	1225.6	360−363	515.61	515.2
161−167	854.96	855.4	364−368	317.34	ND
168 − 175	898.03	898.4			
176−178	373.45	ND			
179−186	908.1	908.3			
187−196	1203.38	120.3.6			
197−212	1828.9	1828.7			
213−225	1571.81	1571.6			

[a] After the inhibitor-treated DOIS was digested with trypsin, the resulting tryptic peptide fragments were exhaustively analyzed by MS as described in Figure 5. [b] ND; not detected. [c] Multivalent ions.

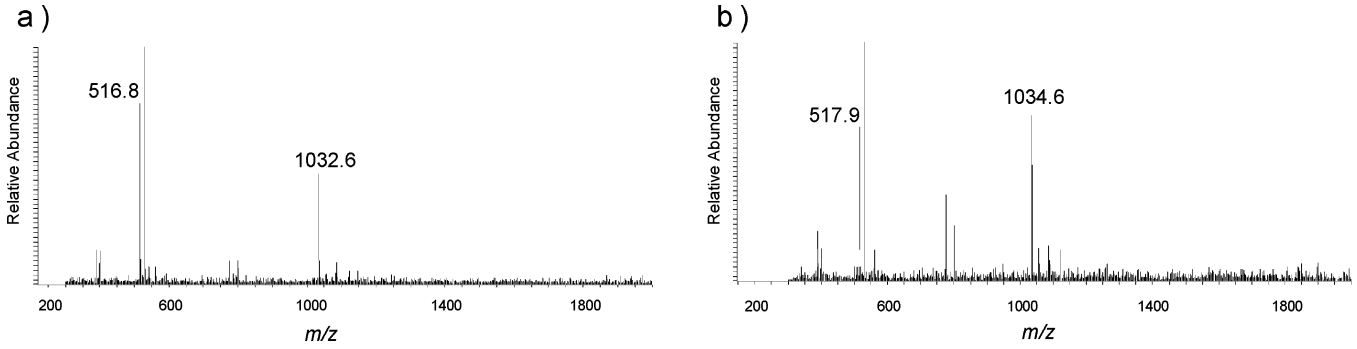


FIGURE 5. LC/MS differential analysis to identify a peptide entrapped with either C-6-P or deuterated C-6-P. The covalently modified DOIS was reduced with $NaBH_4$, reductively carboxymethylated, and then digested with chymotrypsin. Each digest was loaded onto a Senshu Pak PEGASIL-300 ODS-2 column, which was connected on-line to a mass spectrometer. The column was first washed with acetonitrile/water (1:9) containing 0.1% TFA for 10 min at a flow rate of 50 μL/min. Elution was then carried out with a linear gradient of 10−15% acetonitrile in water for 10 min, followed by a linear gradient of 15−20% acetonitrile in water for 40 min and then 20−100% acetonitrile for 20 min; mass spectra of a targeted peptide fragment binding with (a) C-6-P and (b) [6,6-$^2H_2$]-C-6-P.

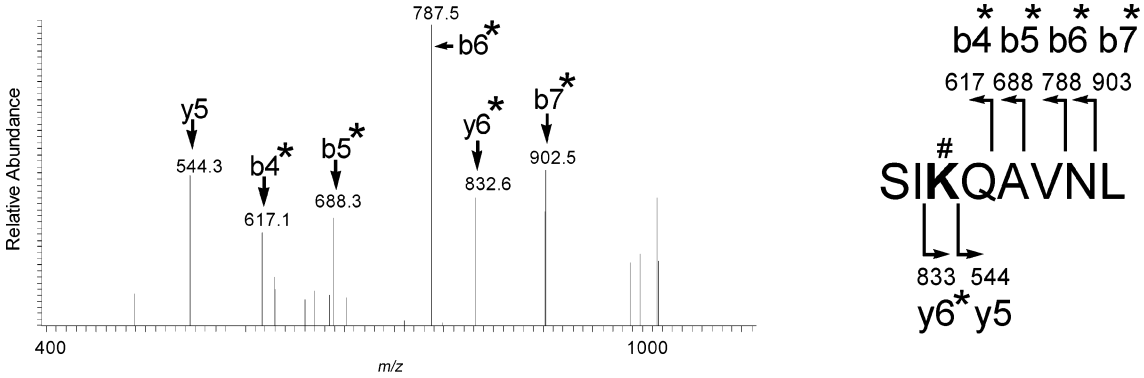


FIGURE 6. MS/MS analysis of the covalently modified fragment SIKQAVNL 139−146. A chymotrypsin digest of inhibitor-treated DOIS was subjected to LC/MS as described in Figure 5. The ion having *m*/*z* 1032.6 was selected and scanned for MS/MS analysis. Asterisks (∗) indicate the inhibitor-containing fragment; pound signs (#) indicate the inhibitor-labeled amino acid.

by LC/MS/MS analysis. The precursor ion of *m*/*z* 1032.6 (nonlabeled) was selected and subjected to collision-induced fragmentation. The product ions obtained are shown in Figure 6.

An ion of *m*/*z* 544.3 was assigned as a y-series fragment ion of Gln-Ala-Val-Asn-Leu (y5). Other ions, *m*/*z* 617.1, 688.3, 787.5, and 902.5, were completely consistent with the expected b-series ions derived from peptides having the inhibitor attached, because all these ions were 160 amu higher than those expected for nonsubstituted peptides, and these ions clearly corresponded to Ser-Ile-Lys-Gln (b4), Ser-Ile-Lys-Gln-Ala (b5), Ser-Ile-Lys-Gln-Ala-Val (b6), and Ser-Ile-Lys-Gln-Ala-Val-Asn (b7), respectively. Further, the ion of *m*/*z* 832.6 was attributable to another y-series ion, Lys-Gln-Ala-Val-Asn (y6), having the inhibitor attached. All these data, particularly the difference between y5 and y6, allowed us to conclude that Lys-141 was the site of binding with C-6-P. In addition, the mass numbers of all these fragment ions confirmed that the binding of DOIS with the C-6-P inhibitor certainly took place through a Michael-type 1,4-addition reaction, but not through an imine formation. Although the bonding site on C-6-P has not been rigorously determined, the most plausible site appears to be the emerged-exomethylene group after elimination of phosphate. Prior to the aforementioned 1,4-addition, Lys-141 has to be changed into a free amino group in the productive enzyme from an ammonium ion form in the resting enzyme, most probably by base catalysis vide post.

In our recent study, specific hydrogen bonding interactions were shown to be important in the DOIS−substrate complex formation, and possible interactions in the DOIS active site were postulated by modeling studies based on the crystallographic structure of a cousin enzyme dehydroquinate synthase (DHQS) of the shikimate pathway.[23] DOIS (BtrC) and microbial DHQSs have marginal homology (25−29%) on the amino acid level and the responsible reactions are chemically similar to each other.[20] The postulated DOIS−substrate interaction (Figure 7) suggested that Lys-141 locates in the vicinity of the C-6 phosphoryloxymethyl group of G-6-P. The results obtained in the present study appear to confirm the relevance of the postulated DOIS−substrate interaction and a certain similarity of the amino acid arrangement in the active site of DOIS to that of DHQS.

Despite the above-mentioned similarity of DOIS to DHQS, significant differences should also be pointed out. One difference is that a similar carbacyclic analogue of DHQS corresponding to C-6-P for DOIS was reported to be a reversible inhibitor rather than a mechanism-based

(30) Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245.

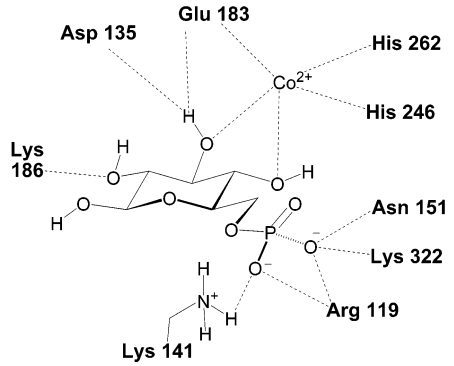


FIGURE 7. Plausible model of substrate recognition in the active site of DOIS.

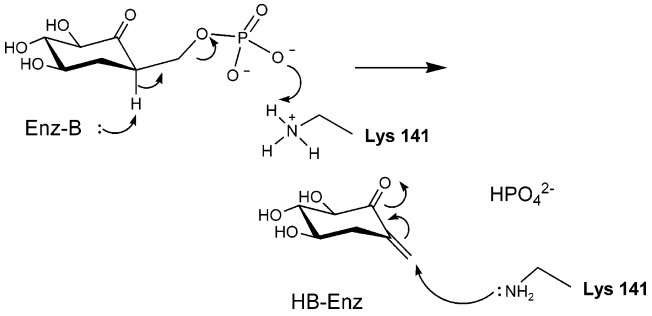


FIGURE 8. Proposed activation mechanism of Lys-141 in DOIS reaction with C-6-P.

covalent inhibitor.[31] Further, an important difference is found in the stereochemistry of the overall reaction. In the DHQS reaction, the phosphate elimination takes place in syn fashion by intramolecular self-catalysis and the ultimate aldol-reaction proceeds through a chair conformation.[31,32] On the other hand, anti elimination of a phosphate and aldol condensation through a chair conformation are postulated to the DOIS reaction. Although it is less likely, a mechanism comprising the syn elimination of a phosphate and aldol condensation through a boat conformation cannot be completely ruled out.[11,12,18]

Taking into account all the above-mentioned evidence, an activation mechanism of Lys-141 in the DOIS reaction is speculatively rationalized as follows (Figure 8). The phosphate elimination takes place in anti fashion by deprotonation with an as yet unidentified amino acid, which is different from that of DHQS, and then a proton of an ammonium ion of the peripheral Lys-141 is taken up by eliminating phosphate to form a free amino group on Lys-141. In the reaction of DOIS with C-6-P, nucleophilic 1,4-addition of the emerging free amino group to the more reactive α,β-unsaturated ketone takes place to a certain ratio (k_{inact} = 3.22 × 10^{-3} s^{-1}), probably accompanying slower reduction of the C-4 ketone due to some particular structural features of the ternary complex.

In the normal DOIS reaction with G-6-P, on the other hand, the intermediary α,β-unsaturated ketone may be less reactive toward the 1,4-addition and the reduction of the C-4 ketone may proceed smoothly, thus eliminating such a nucleophilic attack of the intermediary amino group of Lys-141. The amine form of Lys-141 in the normal reaction may have the important role of recovering the key basic residue functioning in the deprotonation at C-5 to facilitate a phosphate elimination in the second step of the DOIS reaction, although further studies appear to be necessary to prove these scenarios.

In conclusion, the present study precisely demonstrates that the carbacyclic analogue, C-6-P, is a potent mechanism-based and irreversible inhibitor, which is attacked by Lys-141 of DOIS in the enzyme reaction, resulting in the formation of a covalent bond. The results clearly support our recent postulation of the DOIS−substrate interaction, in which Lys-141 locates in the vicinity of a phosphate group of the G-6-P substrate and plays a key role in the reaction catalysis. Also discussed are significant similarities and dissimilarities between DOIS and DHQS, particularly in terms of the reaction mechanism and the structures of the active site.

Experimental Section

General. NMR chemical shifts were reported in δ value based on internal TMS (0 ppm) or solvent signal ($CDCl_3$ δ_C = 77.0; HDO δ_H = 4.65; CHD_2OD δ_H = 3.30, δ_C = 49.0) as reference. Dioxane was used as an internal standard (δ_C = 67.4) for D_2O solvent. In the case of ^{31}P NMR spectra, phosphoric acid was used as an external standard (δ_p = 0.00). For ^2H NMR spectra, the natural abundance deuterium signal of $CHCl_3$ solvent (δ_D = 7.26) was used as reference. All reactions, except for the catalytic hydrogenation reaction, were carried out in an inert (Ar or N_2) atmosphere.

Protein concentration was usually determined by the Lowry−Folin method with bovine serum albumin as standard. Sequencing Grade Modified Trypsin from porcine pancreas was purchased from Promega. α-Chymotrypsin TLCK treated from bovine pancreas was purchased from Sigma. All other chemicals were of the highest grade commercially available.

DL-2,4/3,5-2,3,4,5-Tetrabenzyloxy-1-methylenecyclohexane (2). To a solution of methyltriphenylphosphonium bromide (2.40 g, 6.54 mmol) in dry THF (46 mL) was added *n*-BuLi (3.68 mL, 1.54 M in hexane, 5.66 mmol) at −78 °C. The mixture was stirred for 20 min at −78 °C and then for 20 min at room temperature. To the mixture was then added dropwise a solution of 2,4/3,5-2,3,4,5-tetrabenzyloxycyclohexanone (2.28 g, 4.37 mmol) in dry THF (55 mL), which had been prepared as described previously.[29] The mixture was stirred for 20 min at −78 °C and then for 30 min at room temperature. The mixture was diluted with water and extracted with EtOAc (3 × 100 mL). The combined organic extract was successively washed with 2 M HCl, saturated aqueous $NaHCO_3$, and brine, and then dried over anhydr $MgSO_4$. After filtration and removal of the solvent, the residue was purified by silica gel column chromatography (hexanes−EtOAc, 40:1) to afford 1.93 g of **2** (85% yield): mp 82−85 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 2.05 (1H, t, *J* = 12.9 Hz), 2.72 (1H, dd, *J* = 5.1 and 12.9 Hz), 3.39 (1H, t, *J* = 9.0 Hz), 3.44 (1H, m), 3.62 (1H, t, *J* = 9.0 Hz), 3.92 (1H, d, *J* = 9.0 Hz), 4.80 (8H, m, PhC*H*$_2$O−), 4.97 (1H, d, *J* = 1.7 Hz), 5.24 (1H,d, *J* = 1.7 Hz), 7.30 (20H, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$) δ 36.3, 72.5, 73.5, 75.8, 80.4, 83.0, 85.0, 858, 127.5, 127.6,127.7, 127.9, 128.3, 128.3, 128.3, 138.3, 138.5, 128.8, 128.8, 140.1. Anal. Calcd for $C_{35}H_{36}O_4$: C 80.74, H 6.97. Found: C 80.97, H 7.18.

DL-1,3,5/2,4-2,3,4,5-Tetrabenzyloxy-1-diphenylphosphoryloxymethylcyclohexane (4). To a solution of **2** (1.87 g, 3.59 mmol) in dry THF (20 mL) was added 9-BBN (0.5 M THF solution, 23 mL, 10.8 mmol), and the mixture was stirred

(31) Widlanski, T.; Bender, S. L.; Knowles, J. R. *Biochemistry* **1989**, *28*, 7572−7582.
(32) Widlanski, T. S.; Bender, S. L.; Knowles, J. R. *J. Am. Chem. Soc.* **1987**, *109*, 1873−1875.

for 20 min at room temperature. The mixture was heated under reflux for 5 h, and was then cooled to 0 °C. To the mixture were added water (5 mL), 3 M NaOH (10 mL), and aq 30% H_2O_2 (10 mL) at 0 °C, and the whole mixture was stirred for 50 min at room temperature. The mixture was then diluted with brine and extracted with EtOAc (3 × 100 mL). The combined organic extract was successively washed with 2 M HCl, saturated aqueous $NaHCO_3$, and brine, and then dried over anhydr $MgSO_4$. After filtration and removal of the solvent, the residue was purified by silica gel column chromatography (hexanes−EtOAc, 7:1 to 2:1) to afford 1.93 g of an unseparable mixture of **3** and its diastereoisomer (89% yield, 1β:1α = 2.8:1 from NMR): ^{1}H NMR of major isomer **3** (500 MHz, $CDCl_3$) δ 1.26 (1H, td, J = 11.1 and 13.3 Hz), 1.63 (1H, m), 1.78 (OH, br), 2.03 (1H, dt, J = 4.2 and 13.3 Hz), 3.42 (1H, dd, J = 9.0 and 11.1 Hz), 3.51 (1H, t, J = 9.0 Hz), 3.53 (1H, m), 3.56 (1H, t, J = 9.0 Hz), 3.63 (2H, m), 4.64 (1H, d, J = 11.6 Hz, PhCH*H*O−), 4.65 (1H, d, J = 10.8 Hz, PhCH*H*O−), 4.68 (1H, d, J = 11.6 Hz, PhCH*H*O−), 4.83 (1H, d, J = 10.8 Hz, PhCH*H*O−), 4.95 (1H, d, J = 10.9 Hz, PhCH*H*O−), 4.96 (1H, d, J = 10.9 Hz, PhCH*H*O−), 7.31 (20H, aromatic); ^{13}C NMR of **3** (125 MHz, $CDCl_3$) δ 29.9, 40.2, 64.4, 72.4 75.1, 75.7, 75.8, 80.1, 82.1, 86.0, 86.3, 127.5, 127.5, 127.6, 127.7, 127.8, 128.0, 128.2, 128.3, 128.4, 128.6, 138.2, 138.6, 128.7, 128.8. Anal. Calcd for $C_{35}H_{38}O_5$: C 78.04, H 7.11. Found: C 78.28, H 7.41.

To a solution of the diastereoisomeric mixture (1.39 g, 2.59 mmol) in CH_2Cl_2 (30 mL) and pyridine (10 mL) was added diphenylphosphoryl chloride (2.0 mL, 9.60 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 2 h. The mixture was then diluted with water and extracted with EtOAc (3 × 100 mL). The combined organic extract was washed with 2 M HCl, saturated aqueous $NaHCO_3$, and brine, and then dried over anhydr $MgSO_4$. After filtration and removal of the solvent, the residue was purified by silica gel column chromatography (benzene:ether, 50:1 to 25:1 to 10:1), which allowed separation of diastereoisomers, to afford 1.56 g of **4** (78% yield): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.35 (1H, q, J = 12.5 Hz), 1.74 (1H, m), 2.08 (1H, dt, J = 3.2 and 12.5 Hz), 3.36 (1H, t, J = 8.6 Hz), 3.49 (3H, m), 4.35 (2H, m), 4.72 (8H, m, PhC*H*$_2$O−), 7.30 (30H, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$) δ 29.2, 38.6 (d, J = 8.6 Hz), 68.7 (d, J = 6.2 Hz), 71.9, 74.9, 75.5, 76.6, 79.4, 79.4, 85.4, 85.8, 119.7, 119.8, 119.8, 119.8, 125.2, 125.2, 127.2, 127.3 127.4, 127.5, 127.6, 127.7, 128.0, 128.1, 128.2, 129.6, 128.0, 128.3, 138.4, 138.5, 150.2, 150.2, 150.3, 150.3; ^{31}P NMR (109 MHz, $CDCl_3$) δ −11.9. Anal. Calcd for $C_{47}H_{47}O_8P$: C 73.23, H 6.15. Found: C 73.13, H 6.32.

DL-Carbaglucose-6-phosphate Dipotassium Salt (C-6-P) (5). To a solution of **4** (1.10 g, 1.43 mmol) in methanol (7 mL) was added 10% Pd/C (113 mg). The mixture was vigorously stirred for 12 h under an H_2 atmosphere. Additional 10% Pd/C (30 mg) in methanol (3 mL) was supplemented to the mixture and stirring was continued for 23 h. After filtration and removal of the solvent, the residue (429 mg, 1.05 mmol) was further subjected to the second catalytic hydrogenation in a suspension of PtO_2 (72 mg) in methanol (15 mL). The whole mixture was vigorously stirred under an H_2 atmosphere for 2 days. The catalyst was removed by filtration and the solvent was removed under reduced pressure to give a residue (213 mg, 0.824 mmol), to which were added methanol (5 mL) and a solution of potassium methoxide (3.0 mL, 0.66 M, 2.0 mmol) at 0 °C. The mixture was stirred overnight at the same temperature. Precipitates were collected by centrifugation, and the product was then washed successively with 2-propanol, ethanol, ethanol−ether (1:1) and ether to give 179 mg of **5** (65% yield): ^{1}H NMR (300 MHz, D_2O) δ 1.30 (1H, q, J = 12.2 Hz), 1.56 (1H, m), 1.88 (1H, dt, J = 4.4, 12.2 Hz), 3.23 (3H, m), 3.44 (1H, m), 3.74 (2H, m); ^{13}C NMR (100 M Hz, D_2O) δ 32.6, 41.2 (d, J = 6.6 Hz), 66.4 (d, J = 4.9 Hz), 73.0, 74.1, 78.2, 78.9; ^{31}P NMR (109 M Hz, D_2O) δ 4.04. Anal. Calcd for $C_7H_{13}K_2O_8P$: C 25.15, H 3.92. Found: C 24.88, H 4.18.

DL-[6,6-$^2H_2$]-Carbaglucose-6-phosphate (6). DL-[6,6-$^2H_2$]-Carbaglucose-6-phosphate was synthesized by the same procedure as described above for the preparation of **5**, except that deuteriomethyltriphenylphosphonium bromide was used instead of methyltriphenylphosphonium bromide: ^{1}H NMR (400 MHz, D_2O) δ 1.23 (1H, q, J = 13 Hz), 1.51 (1H, m), 1.85 (1H, dt, J = 3.7, 13 Hz), 3.12 (2H, t, J = 9.3 Hz), 3.17 (1H, t, J = 9.3 Hz), 3.31 (1H, m); ^{13}C NMR (100 MHz, D_2O) δ 32.5, 40.1 (d, J = 6.6 Hz), 72.0, 73.1, 77.1, 78.0; ^{31}P NMR (109 MHz, D_2O) δ 4.65; ^{2}H NMR (61 MHz, H_2O) δ 3.62. HRMS (FAB) calcd for $C_7H_{11}{}^2H_2KO_8P$ 297.0111, found 297.0096

Preparation of Recombonant DOIS (BtrC) from Transformed *E. coli*. Purified BtrC was prepared according to a method adopted from that described by Kudo et al.[19] After chromatographic purification with a Hi Load 26/60 Superdex 200 pg (Pharmacia Biotech) on a FPLC (Pharmacia Biotech), the fractions containing BtrC were combined and concentrated into ca. 2 mL by centrifugation with a VIVASPIN 20-mL concentrater (Ieda Trading Corporation, Tokyo). The concentrate was then loaded onto a Hi Load 16/10 Phenylsepharose HP (Pharmacia Biotech), previously equilibrated with 5 mM Tris-HCl (pH 7.7) containing 1 M $(NH_4)_2SO_4$ and 0.2 mM $CoCl_2$, and the column was washed with the same buffer for 20 min. The adsorbed proteins were then eluted with 5 mM Tris-HCl (pH 7.7) containing 0.2 mM $CoCl_2$ with a linear gradient of $(NH_4)_2SO_4$ (concentration from 1 to 0 M) for 30 min. BtrC was eluted in the fractions of approximately 0 M $(NH_4)_2SO_4$.

Inhibition Assay. For an inactivation assay, a solution (25 μL) of BtrC (55 μg), varying amounts of C-6-P (250−6000 μM), 5 mM NAD^+, and 50 mM Tris-HCl−0.2 mM $CoCl_2$ (pH 7.7, buffer A) was preincubated at 46 °C for 1−2 min. The reaction mixture was diluted 40-fold with the same buffer A for subsequent assay of residual activity. An assay mixture (total 50 μL) containing a diluted preincubation solution (10 μL) of 5 mM NAD^+, 5 mM D-glucose-6-phosphate, and buffer A was incubated at 46 °C for 15 min. The enzyme reaction was terminated by adding 50 μL of methanol. To the resulting mixture was added 10 μL of *O*-(4-nitrobenzyl)hydroxylamine hydrochloride in pyridine (2.5 mg/mL), and the whole mixture was then heated at 60 °C for 1 h. The amount of the resulting DOI oxime derivative was quantified by a HPLC method described previously.[20] The half-time ($t_{1/2}$) for inactivation at each inhibitor concentration was plotted against 1/[C-6-P], referred to as a Kitz and Wilson plot, and K_I and k_{inact} values were obtained by the following equation: $t_{1/2} = 0.69/k_{inact} + 0.69/k_{inact} \times K_I/I$ (ref 30) (I: the inactivator concentration, k_{inact}; the rate of inactivation, K_I; the concentration of the inactivator that produces half the maximal rate of inactivation).

LC/MS Analysis of C-6-P-Bound DOIS. A solution (total volume 700 μL) containing BtrC (1.4 mg), 5 mM NAD^+, 5 mM C-6-P, and buffer A was incubated at 46 °C for 1000 s. A portion (3 μL) of the supernatant was loaded onto a Senshu Pak PEGASIL-300 C4P column (1.0 mm i.d. × 15 cm, Senshu Scientific, Tokyo), which was connected on-line to a electrospray ionization (ESI) mass spectrometer (Finnigan LCQ). The column was first washed with acetonitrile/water (1:9) containing 0.1% HCOOH and 0.01% TFA for 10 min at a flow rate of 100 μL/min. Elution was carried out with a mixture consisting of acetonitrile/water (9:1) containing 0.1% HCOOH and 0.01% TFA. The elution was monitored with a UV detector at 280 nm, and mass spectra for each fraction were simultaneously scanned for a range from 300 to 2000 atomic mass units. The molecular mass was estimated by deconvolution with BioWorks 1.0 software (ThermoQuest).

Digestion of C-6-P-Bound DOIS. A mixture (total volume 1 mL) of BtrC (6 mg), 2 mM C-6-P, 5 mM NAD^+, and buffer A was incubated at 28 °C for 2 h. The reaction mixture was subjected to a Hitrap Desalting (Pharmacia Biotech), preequilibrated with 20 mM Tris-HCl (pH 8.1), at a flow rate of 0.5 mL/min. The elution was combined and lyophilized. The residue was dissolved in 50 mM Tris-HCl (pH 8.0) containing

10% sodium dodecyl sulfate and dithiothreitol (DTT, 4 mg). The mixture (total volume 1 mL) was treated at 37 °C for 1 h, and then a solution of $NaBH_4$−methanol (17 μL, 10 mg/mL) was added to the mixture at 0 °C. After 1 h, the whole was desalted and lyophilized. A portion of the residue (ca. 1 mg) was added to a solution (1.0 mL) of buffer B (0.5 M Tris-HCl, 7.0 M guanidine hydrochloride, 10 mM EDTA, pH 8.5) and DTT (760 μg), and the mixture was incubated at 37 °C for 3 h under an Ar atmosphere. To the reaction mixture was added iodoacetic acid (2.5 mg) in buffer B (25 μL) and the mixture was further incubated at 28 °C for 40 min. After being ultrafiltrated by Ultrafree, the reaction mixture was desalted with 20 mM NH_4HCO_3 (pH 8.1) by using a Hitrap Desalting. An aliquot of the eluted protein (total volume 103 μL, 70 μg) was digested with Sequencing Grade Modified Trypsin (1 mg/mL, 3 μL) in 50 mM acetic acid, 1 mM $CaCl_2$, and 100 mM Tris-HCl (pH 8.0) at 37 °C for 8 h. After lyophilization, the digest was dissolved in 20 mL of 0.1% TFA. In the case of chymotrypsin digestion, an aliquot of the eluted protein (total volume103 μL, 90 μg) was digested with α-chymotrypsin (5.2 μg) in 100 mM Tris-HCl (pH 8.0) at 37 °C for 8 h.

LC/MS Analysis of the Proteolytic Digests. A proteolytic digest of the inhibitor-treated BtrC was loaded onto a Senshu Pak PEGASIL-300 ODS-2 column (1.0 mm i.d. × 15 cm, Senshu Scientific, Tokyo), which was connected on-line to the ESI mass spectrometer. The column was first washed with acetonitrile/water (1:9) containing 0.1% TFA for 10 min at a flow rate of 50 μL/min. Elution was then carried out with a linear gradient of 10−15% acetonitrile in water for 10 min, followed by a linear gradient of 15−20% acetonitrile in water for 40 min and then 20−100% acetonitrile for 20 min. The elution was monitored with a UV detector at 210 nm and mass spectral data for each fraction were acquired for the range from 300 to 2000 atomic mass units. Ion-spray voltage was set at 4.5 kV and tube lens offset was 50 V. Scanning for full MS analysis and MS/MS analysis was carried out alternately. In the collision-induced dissociation mode, the trap was filled for up to 50 ms, depending on the number of ions entering the trap per unit of time. In this mode, the threshold to trigger ion selection was 5.0×10^4 and collision energy was 35−45%. Molecular weights and amino acid sequences of the peptides were attributed to the amino acid sequence estimated from the *btrC* gene, using the BioWorks software supplied with the LCQ and Protein Prospector (http://prospector.ucsf.edu).

Acknowledgment. This work was supported in part by a Grant-in-Aid for Scientific Research and the COE21 program from the Ministry of Education, Science, Sports, and Culture, Japan. Research Fellowships for Young Scientists support to E.N. from the Japan Society for the Promotion of Science is gratefully acknowledged.

JO034706Y