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CMP–KDO Synthetase: Overproduction and Application to the Synthesis of CMP–KDO and Analogs

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Abstract—CTP:CMP-3-deoxy-*manno*-octulosonate cytidylyltransferase (CMP–KDO synthetase, EC 2.7.7.38) has been cloned and overexpressed in *Escherichia coli*. The structure gene was amplified from the total DNA of *E. coli* K-235 through the primer-directed polymerase chain reaction. The gene was then cloned into lambda ZAP vector at the EcoRI and XbaI restriction sites and overexpressed in *E. coli* Sure strain at a level approximately 400 times as much as that produced in the host strain. Application of the enzyme to the synthesis of cytidine 5'-monophospho-3-deoxy-D-*manno*-2-octulosonic acid (CMP–KDO) and analogs was studied. Of several KDO analogs tested, 5-fluoro-2-keto-3,5-dideoxyoctulosonic acid (5-FKDO) was found to be a good substrate of the enzyme, and the product (CMP-5-FKDO) was prepared and characterized, representing the first stable CMP–KDO analog prepared enzymatically to date. The natural enzyme product, CMP–KDO, was however quite unstable ($t_{1/2} \approx$ 19 min, in 50 mM $MgCl_2$, 0.2 M Tris buffer, pH 9.0). A mechanism for the decomposition of CMP–KDO involving the hydrogen bonding interactions between the OH groups of C-5 and C-7 (and/or C-8) and the phosphate oxygens was proposed.

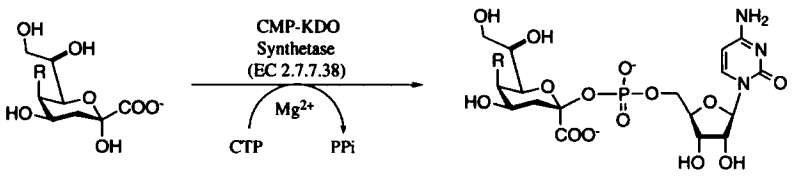
Introduction

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria and composed of lipid A, core oligosaccharide and O-antigen.[1] The eight-carbon sugar KDO (3-deoxy-D-*manno*-octulosonic acid) serves as a linker between the core sugar and lipid A.[2] KDO is incorporated into LPS via a membrane bound KDO transferase[3] which requires CMP-KDO as the sugar donor. The enzyme responsible for the synthesis of CMP–KDO is CMP–KDO synthetase which was first found in *E. coli* and was further purified and investigated by Ray *et al.*[4] The enzymatic reaction requires Mg^{2+} for activity (Scheme 1). The gene encoding the *E. coli* enzyme was cloned and sequenced[5,6] and was composed of 0.7 kb nucleotides[5b] with a molecular weight about 28,000 Da. Since CMP–KDO synthetase is a unique enzyme found in Gram-negative bacteria, and the formation of CMP–KDO is the rate limiting step of the LPS biosynthetic pathway,[2,4] tremendous efforts have been devoted to the development of inhibitors of this enzyme.[7,8] In order to study the specificity and to develop inhibitors of the enzyme, we report here the overproduction of this enzyme from *E. coli* and the study of its application to the synthesis of CMP–KDO and analogs. Until now, no one had isolated CMP–KDO or related analogs from the enzymatic reactions due to the instability of CMP–KDO in aqueous solution. The 5-deoxy-5-fluoro derivative of CMP-KDO prepared by this enzymatic reaction is, however, very stable and can be easily isolated and characterized. A mechanistic rationale for the difference in the stability between CMP–KDO and CMP–FKDO is proposed.

Results and Discussion

Primer design and PCR amplification

As shown in Table 1, the primer KDO5 contained an EcoRI restriction site, a Shine Dalgarno sequence, a start codon and the N-terminal hexapeptide sequences of the gene. The primer KDO3 contained an XbaI restriction site, stop codons, and the C-terminal hexapeptide sequence of the gene. As shown in Figure 1, the PCR products contained only one major band corresponding to a molecular weight of 0.7 kb which was similar to the one previously reported.[5a]



KDO (R = OH)
5-FKDO (R = F)

CMP-KDO (R = OH)
CMP-5-FKDO (R= F)

Scheme 1. Reaction catalyzed by CMP–KDO synthetase.

Table 1. The designed primer sequences for the amplification of CMP–KDO synthetase gene

Primer KDO5
5'- ATATTGAATTCTAAACTAGTCGCCAAGGAGACAGTC
EcoR I — Shine Dalgarno sequence
ATAATG AGTTTTGTGGTCATT
Start — Gene N-terminal

Primer KDO3
5'- GCGCTCTAGA CTA GCGCATTTCAGCGCGAAC
Xba I — Stop — Gene C-terminal

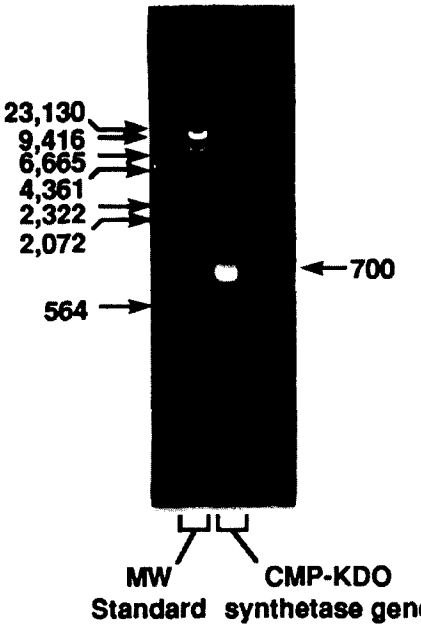


Figure 1. Amplification of CMP–KDO synthetase gene by PCR (analyzed by Agarose 0.8% gel).

Construction of the expression vector and screening for the positive clones

After ligation of the digested vector and inserts, the DNA was packaged to form the phage particles, which were used to infect *E. coli* XL1-Blue on LB agar plates containing X-gal and IPTG (isopropylthio-β-D-galactoside). The white plaques containing the inserts were cored and excised. The colonies were further grown on LB medium, induced with IPTG and then used for enzyme activity assay. Out of 30 plaques selected, 26 showed positive enzyme activity. The clone which gave the highest enzyme activity was chosen for the enzyme production and assigned as wKDO-9. The plasmid CMPKDO-9 (Fig. 2) contains the CMP–KDO synthetase gene controlled by the lac promoter which can be induced by the addition of IPTG.

Expression and purification of cloned CMP–KDO synthetase

The cloned wKDO-9 was grown in LB rich medium and induced with IPTG overnight at room temperature as described previously.[9] A very low activity was observed

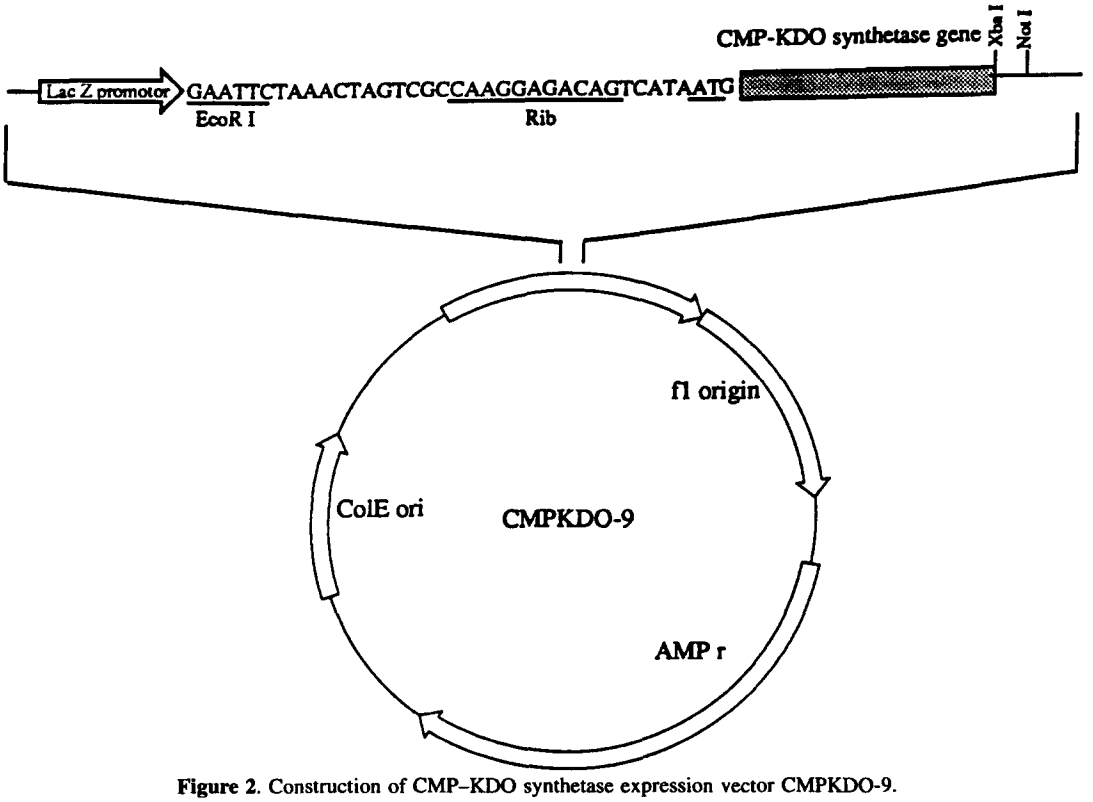


Figure 2. Construction of CMP–KDO synthetase expression vector CMPKDO-9.

with the host strain *E. coli* Sure, and a weak activity was detected without the addition of IPTG to the clone wKDO-9. A significant increase of CMP–KDO activity was, however, obtained after the induction of IPTG. Approximately 0.04 U mL^{-1} of the enzyme activity was observed, corresponding to a 400-fold increase of activity compared to the original host strain (Table 2). CMP–KDO synthetase was purified by column chromatography as shown in Table 3. The low yield of enzyme recovery after matrix Blue B may be due to the low binding capacity of the gel. After gel filtration, the enzyme was almost pure as judged by the SDS-polyacrylamide gel electrophoresis (Fig. 3), and the molecular weight (28,000) was in agreement with the 700 kb nucleotide sequence.[5] The specific activity was determined to be 10.1 U mg^{-1} which was comparable with the natural enzyme (9.3 U mg^{-1})[4] but lower than the recombinant enzyme (20.1 U mg^{-1}) reported by Goldman *et al.*[5] This difference may be due to the differences in enzyme preparation and assay conditions. In our overexpression system, a ribosome binding site at the optimal distance for expression of the cloned sequence[10] was used to replace the original ribosome binding site in the original sequence.[6] This strategy was successfully used in the overproduction of CMP–*N*-acetyl-neuraminic acid synthetase in *E. coli*.[11] Several affinity ligands were tested for the purification of CMP–KDO synthetase. Orange A dye which was shown to bind CMP–*N*-acetylneuraminic acid synthetase specifically[12] was not able to bind this enzyme well. Only Blue B dye gave better specific binding; however, the concentration of ligand in the gel is too low to be useful for large-scale processes. Work is in progress to develop a more practical procedure for the purification of this enzyme.

Synthesis of CMP–KDO and analogs

The following compounds had been tested as substrates for CMP–KDO synthetase: 5-fluoro-2-keto-3,5-dideoxy-octulosonic acid (5-FKDO),[13] 5-*epi*-KDO,[13] 5-deoxy-KDO,[13] 3*R*-FKDO[14] and L-KDN[15] (Fig. 4). It was found that, with the exception of L-KDN, the others are substrates for CMP–KDO synthetase.[16] The activity of CMP–KDO synthetase towards 5-FKDO was measured[9] (45 U mL^{-1}) and was found to be comparable to KDO. Interestingly, the product CMP-5-FKDO was very stable and no significant decomposition was observed even after 3 days, while CMP–KDO is quickly decomposed ($t_{1/2} \approx 19$ min) under the same condition (0.2 M Tris buffer, pH 9.0, 50 mM $MgCl_2$). The unexpected stablity prompted us to isolate and characterize this product.

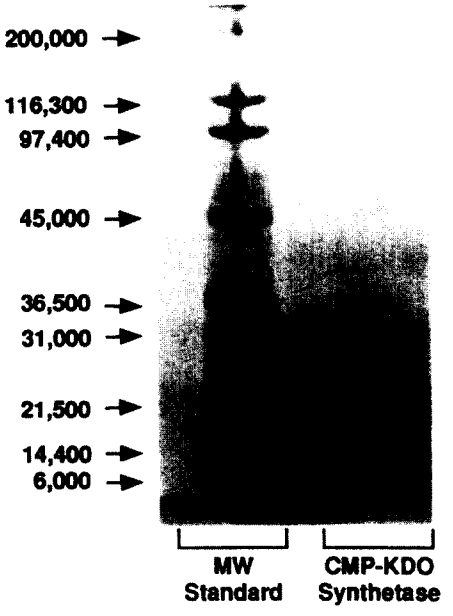


Figure 3. SDS–polyacrylamide gel electrophoresis of the purified CMP–KDO synthetase.

Table 2. Expression of CMP–KDO synthetase in *E. coli* strains

Strains	CMP-KDO synthetase activity (Unit/ml culture)
Sure (host)	<0.0001
wKDO-9 (Without IPTG induction)	0.003
wKDO-9 (With IPTG induction)	0.04

The ^1H and ^{13}C NMR data of this product fully supported the structure as depicted in Scheme 1. The coupling constant $J_{C\text{-}1'',H\text{-}3''ax}$ (*ca* 6 Hz) observed at C-1" (δ 173.47) in the gated proton decoupled ^{13}C NMR spectrum indicatesthe *trans* relationship of C-1" and H-

Table 3. Purification of CMP–KDO synthetase from cell free extract of *E. coli* strain wKDO-9 (from a 2 L fermentation medium)

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)
Cell free extract	5680	165.0	0.03	100
DEAE sepharose CL-6B	650	99.7	0.15	60
Blue B affinity	24	36.4	1.50	22
Superose 12 gel	2	20.2	10.1	12

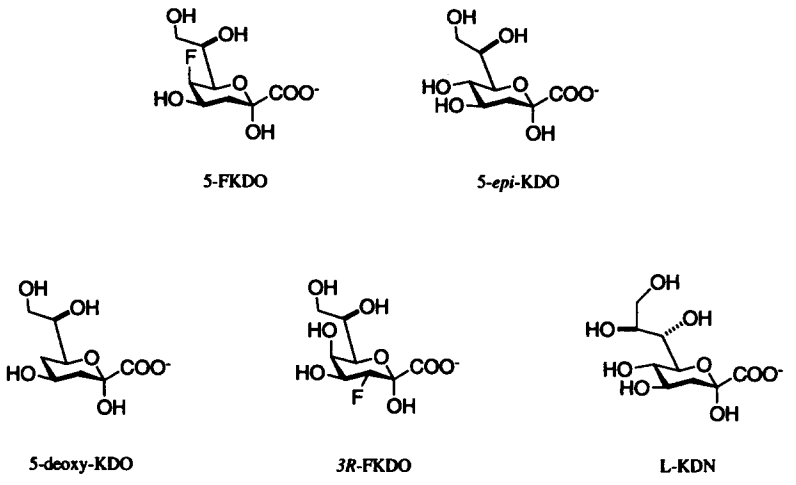


Figure 4. Compounds tested as substrates for CMP–KDO synthetase.

3"ax (Fig. 5),[17,18] suggesting the β-linkage (equatorial orientation in $^5C_2$ conformer) of the glycosidic bond. This result is consistent with the proposed β-linkage in CMP–KDO.[18,19]

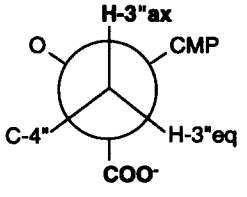


Figure 5. *trans* Relationship of C-1" and H-3"ax in CMP–KDO.

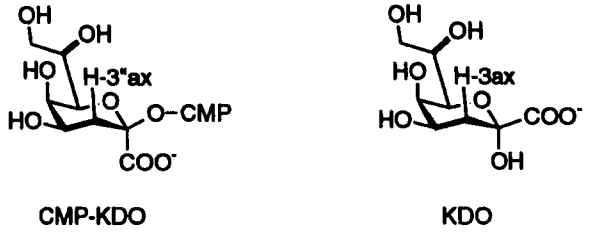
Stability study of CMP–KDO and analogs

^{1}H NMR was used to monitor the stability of CMP–KDO and analogs based on the diagnostic signal of H-3"ax of CMP–KDO (or analogs): a triplet with large coupling constants due to the presence of *trans* and geminal couplings, $J_{3"ax,3"eq} = J_{3"ax,4"} = 12–13$ Hz. Although the H-3"ax of CMP–KDO is very similar to the H-3ax of KDO in the ^{1}H NMR spectrum, they can be easily distinguished by the chemical shift: δ 2.10–2.20 for CMP–KDO and δ 1.85–1.95 for KDO (Fig. 6).

In agreement with the literature,[18,19] CMP–KDO gradually decomposed to CMP and KDO, and the decomposition was a first-order reaction (Equations 1 and 2).

$$d\,[\text{CMP–KDO}] / dt = -kt \quad (1)$$

$$\ln ([\text{CMP–KDO}]_t) = \ln ([\text{CMP–KDO}]_0) - kt \quad (2)$$



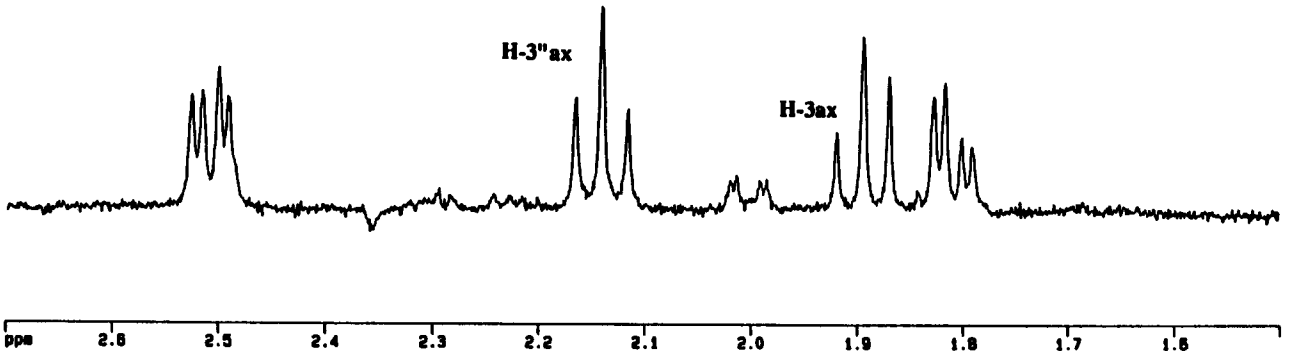


Figure 6. ^{1}H NMR Spectrum of CMP–KDO and KDO, indicating different signals between H-3"ax of CMP–KDO and H-3ax of KDO.

The half life and decomposition rate of CMP–KDO (or analogs) were determined based on the remaining CMP–KDO (or analogs) in the mixture determined by ^{1}H NMR analysis (Fig. 7). Based on the results, it appears that the 5"-OH group of CMP–KDO plays a major role in the decomposition. A possible mechanism for the decomposition is that the 5"-OH and 7"-OH (and/or the 8"-OH) are hydrogen bonding to the phosphate oxygens to facilitate the glycosidic cleavage, which may proceed through a twist boat conformation in order to fulfill the stereoelectronic requirement[20] (Scheme 2). This mechanism is supported by the fact that KDO exists in aqueous solution as a mixture of chair and twist-boat conformers, and the twist-boat conformer **b** is believed to contribute to the formation of 1,5-lactone **c** in aqueous solution.[13]

This mechanism is also supported by the observation that CMP–5-FKDO is stable since the hydroxyl group at C5 is replaced with the F and the remote inductive effect of F would also strengthen the glycosidic linkage.[21–23] In addition, model studies suggest that in the twist-boat conformation of CMP–KDO, the 7" or 8"-OH is involved in H-bonding interaction with the phosphate group. The hydrogen-bonding interactions

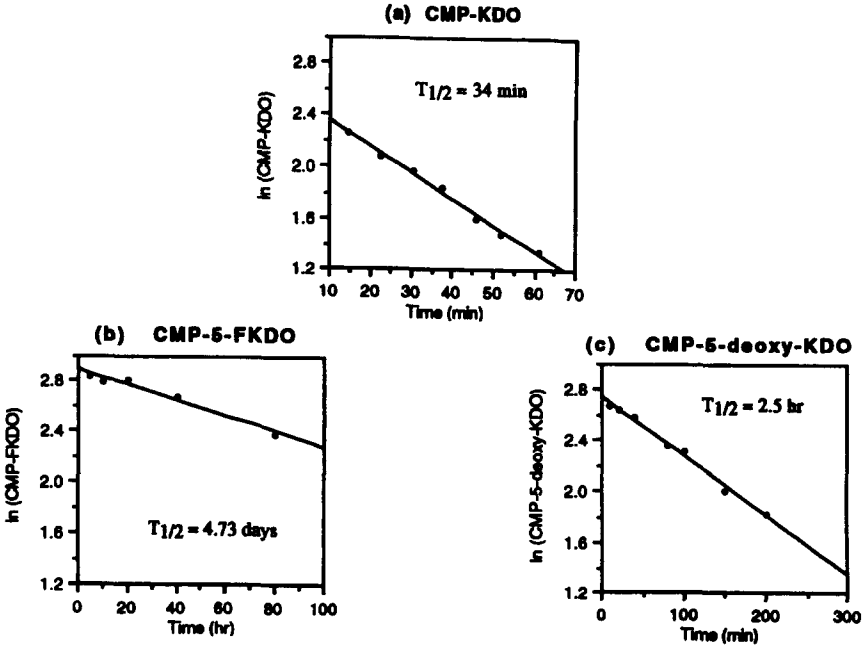
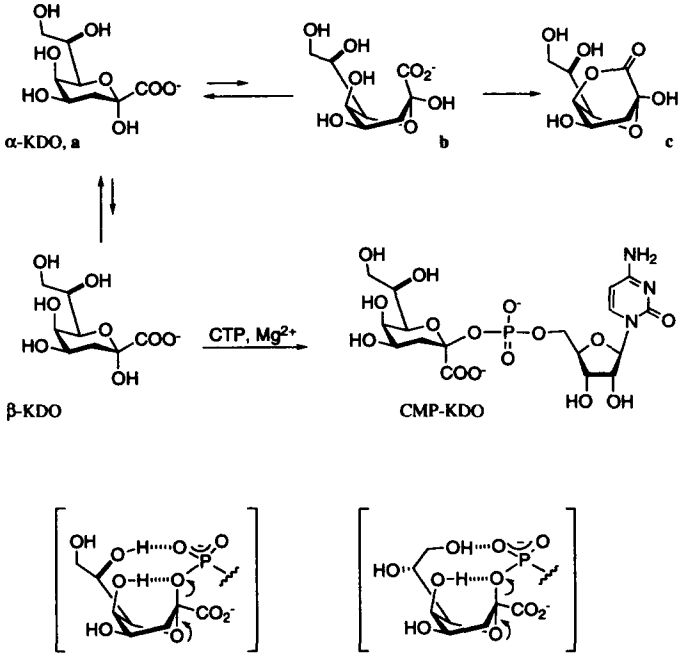


Figure 7. The decomposition rates of CMP–KDO and analogs in 20 mM $MgCl_2$ and 100 mM Tris–HCl (pH 7.5). These rates were determined based on the remaining CMP–KDO and analogs measured by ^{1}H NMR analysis (EtOH was used as the internal standard).



Scheme 2. Proposed mechanism of CMP–KDO decomposition.

contributed from the donors of 5"- and 7"-OH (or 8"-OH) are further supported by the observed solvent isotope effect.[24]

In conclusion, CMP–KDO synthetase has been prepared and used in the first synthesis of a CMP–KDO analog, CMP–5-FKDO. The high stability of CMP–5-FKDO, in comparison with CMP–KDO, led us to propose a mechanism for the decomposition of CMP–KDO. The unusual instability of CMP–KDO toward hydrolysis together with the information obtained with regard to the specificity of CMP–KDO synthetase should facilitate our development of inhibitors of the enzyme.

Experimental

Reagents

The vector λLc1 which has been modified from Lambda ZAP vector (Stratagene Co.) was obtained from Dr R. A. Lerner in this institute.[10] All the chemicals were purchased from commercial sources as molecular biology grade reagents. CTP and KDO were purchased from Sigma Co. DEAE Sepharose CL-6B and Superose 12 Gel HPLC column were obtained from Pharmacia Co. (Piscataway, NJ) and Matrix Blue B was purchased from Amicon Co. (Danvers, MA).

Microorganisms

Escherichia coli strain K235 (ATCC 13207) was obtained from American Type Culture Collection. The *E. coli* XL1-Blue and Sure strains were purchased from Stratagene Co. (San Diego, CA). The microorganisms were maintained on LB (Luria–Bertani) medium. When host strains harbored with plasmids, the LB medium containing 100 μg mL^{-1} of ampicillin was used. Stock cultures were kept as cell suspension at –70 °C in 20% glycerol solution.

DNA manipulation

The DNA of *E. coli* strain K235 was extracted according to the method described by Maniatis *et al.*[25] Vector λLc1 was prepared as described by Huse *et al.*[10] Primers were custom synthesized and purified by acrylamide gel. The sequences of the primers KDO5 and KDO3 were shown in Table 1.

Amplification of the CMP–KDO synthetase gene

PCR amplification was performed in a 100 μL reaction mixture containing 1 μL (1 μg) of *E. coli* strain K235 DNA, 400 nmol each of primers KDO5 and KDO3, 200 μM of different dNTPs, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, and 2 units of *Thermus aquaticus* DNA polymerase. The reaction was overlayed with mineral oil and subjected to 30 cycles of amplifications. The cycle conditions were set as follows: denaturation, 94 °C for 1 min; annealing, 60 °C for 2 min; and elongation, 72 °C for 1.5 min. The primers were annealed with *E. coli* DNA at 94 °C for 2 min followed by slow cooling to room temperature prior to PCR amplification.

Construction of CMP–KDO synthetase expression vector

The DNA obtained from the PCR amplification was purified on 0.6% agarose gel. The DNA band corresponding to 0.7 kb was separated from the agarose gel and electro-eluted. The DNA was then extracted with phenol:chloroform and precipitated with ethanol overnight at –20 °C. The precipitated DNA was dissolved in an appropriate restriction enzyme buffer (H buffer) supplied by Boehringer Mannheim Biochemical Co. (Indianapolis, IN) and digested with EcoRI and XbaI each with 40 units μg^{-1} DNA at 37 °C for 2 h. The digested DNA was then recovered by phenol:chloroform extraction and ethanol precipitation (70% of final ethanol concentration containing 10% of 3 N sodium acetate, pH 5.2). After washing with 70% ethanol solution (–20 °C) and drying in speed vacuum, the DNA was resuspended in the TE buffer (10 mM Tris buffer and 1 mM EDTA, pH 7.5) to be used as insert. The arms were also prepared from the digestion of λLc1 with 20 units mg^{-1} DNA of EcoRI and XbaI and recovered with ethanol precipitation after extraction by phenol:chloroform. The insert was then ligated with the arms.[25] The resultant lambda DNA was then packaged with a packaging kit as suggested by the manufacturer (Stratagene Co., San Diego, CA) to form active phages.[25] The packaged phages were then used to infect *E. coli* XL1-Blue strain and plated on LB agar plates which contained X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG.

Screening for positive clones and excision to phagemid

The phage solution after packaging was used to infect the host strain *E. coli* XL1-Blue and plated on LB agar plates containing X-Gal (0.3 mg mL^{-1}) and IPTG (0.25 mg mL^{-1}). Since λLc1 contained LacZ gene in a multicloning site, the phages which contained inserts would not produce active galactosidase after the infection and therefore formed white plaques. The non-recombinanats will, however, produce the blue plaques.[25] The white plaques were then cored from agar plates and transferred to sterile microfuge tubes containing 500 μL of SM buffer (NaCl, 5.8 g; $MgSO_4 \cdot 7H_2O$, 2 g; 1 M Tris–Cl, pH 7.5, 50 mL; 2% gelatin solution, 5 mL in 1 L distilled water) and 20 μL of chloroform. For excision, 200 μL of the phage stock, 200 μL of XL1-Blue cells (OD_{660} = 1.0) and 2 μL of R408 helper phage (1×10^{11} pfu mL^{-1} from Stratagene Co.) were incubated at 37 °C for 10 min. The excised plasmids were plated on *E. coli* Sure cells and analyzed for expression of the enzyme after induction by IPTG. The colonies were inoculated in 10 mL of LB medium containing 100 μg mL^{-1} of ampicillin and incubated at 37 °C for 10 h. When the cell density reached OD_{660} ~ 0.8, IPTG was added (0.5 mM) and the culture was shaken at room temperature overnight. The cells were then harvested by centrifugation and lysed with

lysozyme (0.5 mg mL^{-1}). The supernatants were used for CMP–KDO synthetase activity assays. One clone which produced a high level of CMP–KDO synthetase activity was identified and designed as strain wKDO-9 and the plasmid was assigned as CMPKDO-9.

Preparation of cell free extract from cloned strain

Bacteria were grown on LB rich medium (Bacto Tryptone, 25 g; yeast extract, 10 g; NaCl, 3 g; pH 7.0 in 4 L of deionized H_2O) containing 100 μg mL^{-1} ampicillin to mid-logarithmic phase (OD_{660} ~ 0.6–0.7) at 37 °C and then induced with 0.5 mM IPTG overnight at room temperature with shaking (250 rpm). The cultural broth was centrifuged (10,000 *g*, 20 min, 4 °C) and the cell pellet was washed with 500 mL of Tris buffer containing 20 mM Mg^{2+} and 0.2 mM DTT (pH 7.5) and resuspended in 1/50 volume of the same buffer. The cells were disrupted by a French pressure cell at 16,000 lb in^{-2} and centrifuged at 23,000 *g* for 60 min. The supernatant was used for enzyme assay and purification.

Enzyme purification

The enzyme was purified by ion exchange, affinity chromatography and gel filtration. The cell free extract (50 mL) obtained from a 2-L culture as described before was passed through a DEAE-Sepharose CL-6B column (3 cm × 35 cm) which was previously equilibrated with buffer (200 mM Tris–Cl, 20 mM Mg^{2+} with 0.2 mM DTT, pH 7.5). The enzyme was eluted with a linear gradient of NaCl (from 0 to 1 M) in the same buffer. The active fractions were collected and concentrated by ultrafiltration (M_r 10,000) and then dialyzed against 2 L of the same buffer as described before. The enzyme solution was then passed through a matrix gel Blue B (0.1 mg mL^{-1} dye) column (3 cm × 35 cm) and eluted with 0–1 M NaCl linear gradient in the same buffer. The active fractions were pooled and concentrated to 5 mL by ultrafiltration. The concentrated enzyme solution was then passed through an FPLC gel filtration column (Superose 12 HR 10/30) at a flow rate of 0.1 mL min^{-1}, and the active fractions were collected. The protein concentration was determined by the BCA assay kit (Pierce Co., Rockford, IL), and the purity of the protein was judged by SDS–PAGE (Phastsystem, Pharmacia Co.).

Enzyme assay

The activity of CMP–KDO synthetase was determined by the method described by Ray *et al.*[4] The enzyme was incubated in a 250 μL buffer containing 5.5 mM CTP, 2.8 mM KDO, 0.2 M Tris buffer, 20 mM $MgCl_2$ and 0.2 mM DTT, pH 9.0. After the mixture was incubated at 37 °C for 30 min, 50 μL of 1.6 M $NaBH_4$ was added to destroy excess KDO at room temperature for 15 min. The mixture was cooled on an ice bath and H_3PO_4 (50 μL) was added. After 5 min, the temperature was raised to 37 °C to cleave the phosphoester bond of CMP–KDO. The released KDO was oxidized with 50 μL of 0.2 M $NaIO_4$ at room temperature for 10 min, followed by addition of 400 μL of 4% $NaAsO_2$ in 0.5 N HCl. The solution was then transferred to a test tube containing 1 mL of 0.6% thiobarbituric acid in 0.5 M Na_2SO_4 and heated in boiling water for 15 min. After the solution was cooled, 1 mL of the solution was taken out and mixed with 1 mL of cyclohexanone. The mixture was shaken and centrifuged, and the upper layer was taken for the measurement at 549 nm (ε = 4.11 $mM^{-1}cm^{-1}$). One unit was defined as the formation of 1 μmol of CMP–KDO per minute under the assay conditions. The pure KDO purchased from Sigma Co. was used as standard.

Cytidine 5'-monophosphate-3", 5"-dideoxy-5"-fluoro-D-manno-octulosonate (CMP-5-FKDO)

A solution of 200 mM Tris–HCl buffer (pH 9.0, 5.3 mL) containing 50 mM of $MgCl_2$, 0.2 mM of dithiothreitol, CTP (Sigma C-9274, disodium salt dihydrate, 84.8 mg, 0.176 mmol, 30 mM of final concentration), and 5-FKDO (28.1 mg, 0.117 mmol, 20 mM of final concentration) was first prepared. To this solution was added inorganic pyrophosphatase (Sigma I-4503, 100 U mL^{-1} solution, 30 μL, 3 U) and CMP–KDO synthetase (300 μL, 15 U). The mixture was stirred overnight at 25 °C under Ar. The precipitated magnesium phosphate was removed by centrifugation. The supernatant was further centrifuged through Ultrafree-MC centrifuge tube (Nihon Millipore Kogyo, K. K., exclusion limit: 10,000 M_r) to remove proteins. The resulting solution was lyophilized and the residue was separated by preparative TLC (Merck Art 5744, 10 cm × 20 cm × 0.5 cm; × 6) using *i*-PrOH–H_2O–conc NH_3 solution (7:3:1). The band containing the desired product (R_f 0.33) was recovered by elution with *i*-PrOH–H_2O–conc NH_3 solution (30:20:1) and concentrated *in vacuo*. The residue was further purified by Biogel P-2 column (bed volume: 20 mL). The desired fraction eluted with H_2O–conc NH_3 solution (20:1) was lyophilized to give 28.7 mg (43% yield) of CMP–FKDO: ^1H NMR (500 MHz, D_2O) δ 2.30 (1H, *dd*, $J_{3"ax,3"eq}$ = 12.1, $J_{3"ax,4}$ = 12.3 Hz, H-3"ax), 2.62 (1H, *dd*, $J_{3"eq,4"}$ = 4.8, $J_{3"eq,3"ax}$ = 12.1 Hz, H-3"eq), 3.58 (1H, *dd*, $J_{6",7"}$ = 8.9, $J_{6",F}$ = 30.4 Hz, H-6"), 3.764 (1H, *dd*, $J_{8"b,7"}$ = 4.8, $J_{8"b,8"a}$ = 12.2 Hz, H-8"b), 3.80 (1H, *dd*, $J_{8"a,7"}$ = 3.0, $J_{8"a,8"b}$ = 12.2 Hz, H-8"a), 3.91 (1H, *ddd*, $J_{7",8"a}$ = 3.0, $J_{7",8"b}$ = 4.8, $J_{7",6"}$ = 8.9 Hz, H-7"), 3.95 (1H, *dddd*, $J_{4",5"}$ = 2.2, $J_{4",3"eq}$ = 4.8, $J_{4",3"ax}$ = 12.3, $J_{4",F}$ = 29.7 Hz, H-4"), 4.09 (1H, *ddd*, $J_{5'a,4'}$ = 2.6, $J_{5'a,P}$ = 5.3, $J_{5'a,5'b}$ = 11.8 Hz, H-5'a), 4.20 (1H, *ddd*, $J_{5'b,4'}$ = 2.3, $J_{5'b,P}$ = 4.4, $J_{5'b,5'a}$ = 11.8 Hz, H-5'b), 4.23 (1H, *dddd*, $J_{4',5'b}$ = 2.3, $J_{4',P}$ = 2.5, $J_{4',5'a}$ = 2.6, $J_{4',3'}$ = 5.0 Hz, H-4'), 4.27 (1H, *dd*, $J_{2',1'}$ = 4.4, $J_{2',3'}$ = 5.0 Hz, H-2'), 4.30 (1H, *dd*, $J_{3',2'}$ = 5.0, $J_{3',4'}$ = 5.0 Hz, H-3'), 4.82 (1H, *dd*, $J_{5",4"}$ = 2.2, $J_{5",F}$ = 51.4 Hz, H-5"), 5.97 (1H, *d*, $J_{1',2'}$ = 4.4 Hz, H-1'), 6.10 (1H, *br d*, $J_{5,6}$ = 7.5 Hz, H-5), 7.94 (1H, *d*, $J_{6,5}$ = 7.5 Hz, H-6); ^{13}C NMR (100 MHz, D_2O) δ ($C\underline{H}_3CN$ = 1.30) 34.84 (C-3"), 63.85 (C-8"), 64.84 (*d*, $J_{4',P}$ = 5.5 Hz, C-5'), 66.88 (*d*, $J_{6",F}$ = 18.5 Hz, C-6"),[a] 69.28 (*d*, $J_{3',P}$ = 3.3 Hz, C-3'), 69.78 (C-7"), 73.53 (*d*, $J_{4",F}$ = 17.5 Hz, C-4"),[a] 74.72 (C-2'), 83.25 (*d*, $J_{5',P}$ = 8.8 Hz, C-4'), 86.85 (*d*, $J_{5",F}$ = 179.5 Hz, C-5"), 89.58 (C-1'), 97.06 (C-5), 100.72 (*d*, $J_{2",P}$ =

8.0 Hz, C-2"), 141.89 (C-6), 158.24 (C-2), 166.64 (C-4), 173.47 (*d*, $J_{1'-P}$ = 7.0 Hz, C-1"); [a]assignment may be reversed. MS (FAB, M + H^+) 544.

Stability study of CMP–KDO and CMP–5-FKDO

CMP–KDO and CMP–5-FKDO were prepared by the CMP–KDO synthetase-catalyzed reaction as described above. After CTP was almost consumed, the precipitated magnesium phosphate was removed by centrifuge. The supernatant was further centrifuged through Ultrafree-MC centrifuge tube (Nihon Millipore Kogyo, K. K., exclusion limit: 10,000 M_r) to remove proteins and then transferred to an NMR tube with a coaxial insert (for external reference, D_2O). Each stability test was performed at 25 °C by ^1H NMR analysis.

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