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A simple assay for 3-deoxy- α -manno-octulosonate cytidyltransferase (KdsB) and its use as a pathway screen

Li Yi^{1,2}, Melvin S. Velasquez¹, Tod P. Holler¹, and Ronald W. Woodard^{1,*}

¹Department of Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109

Abstract

This paper describes the adaptation of a simple colorimetric assay for inorganic pyrophosphate to the enzyme 3-deoxy- α -manno-octulosonate cytidyltransferase (CMP-KDO synthetase, KdsB, E.C. 2.7.7.38), a key enzyme in the biosynthesis of lipopolysaccharide (LPS) in Gram-negative organisms. This assay is particularly useful because it can be combined with the malachite green assay for inorganic phosphate to form an assay system capable of determining inorganic phosphate and inorganic pyrophosphate in the same solution (the MG/EK assay). This assay system has the potential for simultaneous screening of the 3-deoxy- α -manno-octulosonate (KDO) biosynthesis pathway. We test this potential using two enzymes, KdsB and KdsC, involved in the biosynthesis and utilization of the key bacterial 8-carbon sugar, KDO.

Keywords

Inorganic phosphate determination; pathway screen; 3-deoxy- α -manno-octulosonate cytidyltransferase (KdsB); 3-deoxy- α -manno-octulosonic acid (KDO)

Screening multiple enzymes in a single well, sometimes referred to as combinatorial enzymology, has some distinct advantages in the search for novel therapeutics, particularly for antibacterials. Though screening libraries of small molecules for individual enzyme inhibitors has proven to be an effective way to search for drug leads for many diseases, it has been a disappointing approach for the discovery of antibacterials [1]. One reason for this disappointing record may be that key enzymes in a bacterial biosynthetic pathway form catalytic complexes that behave differently than their isolated counterparts. Targeting an

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* To whom correspondence should be addressed **Corresponding Author Details** Ronald W. Woodard Department of Medicinal Chemistry University of Michigan 428 Church Street Ann Arbor, MI 48109-1065 Tel. +1 734-764-7366 FAX: +1 734-647-8430 rww@umich.edu.

²Current Address: Genentech, 1 DNA Way, South San Francisco, CA 94080

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Li Yi li.yi.um@gmail.com Phone +1 650 225 1108 FAX N/A Current affiliation Genentech Street Address 1 DNA Way, South San Francisco CA 94080

Melvin S. Velasquez velamel@umich.edu Phone +1 734 763 0119 FAX +1 734 647 8430 Current affiliation Department of Medicinal Chemistry, University of Michigan, Street Address 428 Church Street, Ann Arbor, MI 48109-1065

Tod P. Holler hollert@umich.edu Phone +1 734 763 0119 FAX +1 734 647 8430 Current affiliation Department of Medicinal Chemistry, University of Michigan, Street Address 428 Church Street, Ann Arbor, MI 48109-1065

Ronald W. Woodard rww@umich.edu Phone +1 734 763 7366 FAX +1 734 647 8430 Current affiliation Department of Medicinal Chemistry, University of Michigan, Street Address 428 Church Street, Ann Arbor, MI 48109-1065

individual enzyme in a screen might therefore select sub-optimal compounds and miss compounds that inhibit key bacterial protein-protein interactions. Another distinct advantage to combinatorial enzymology is its ability to lower up-front costs. Obtaining sufficient quantities of substrate for high-throughput screening of individual enzymes in complex bacterial pathways is both difficult and expensive. By assaying several enzymes simultaneously, this problem is reduced to the acquisition of the first substrate. Examples of this combinatorial approach include a high-throughput screen of the murein biosynthetic pathway using a single assay containing six enzymes [2], and a heptose biosynthetic pathway screen employing a single assay to screen five enzymes [3].

Bacterial lipopolysaccharide (LPS) biosynthesis has long been considered an attractive target for antibiotic discovery because it is unique to Gram-negative bacteria and essential to their viability[4]. Two different chemical series that target LPS biosynthesis have been cited as precedent for further work. The first is a series of KdsB inhibitors [5], which prevent the attachment of 3-deoxy-D-manno-octulosonic acid (KDO) to the LPS precursor lipid IV_A. This series had problems with membrane permeability and never made it to the market. More recently, a series of LpxC inhibitors, which prevent the de-acetylation of an early intermediate in the biosynthesis of lipid IV_A, have shown activity against multiple Gram-negative organisms [6, 7]. However, there are concerns about the antibacterial spectrum of agents targeting LPS biosynthesis because viable strains of *Moraxella catarrhalis*[8], *Neisseria meningitidis*[9] and *Acinetobacter baumannii*[10] that completely lack LPS have either been prepared in the laboratory or, in the case of *A. baumannii*, discovered in clinical isolates resistant to the antibiotic colistin [10]. All of these isolates share a property—they display dramatically increased susceptibility to standard antibacterial agents. This hypersusceptibility to standard agents is shared by an *Escherichia coli* strain expressing lipid A, the minimal LPS structure required for viability in this organism[11]. Exploitation of this weakness in combination therapies could extend the lifetimes of a significant number of antibiotics. What is needed is an LPS biosynthesis inhibitor with a toxicity profile superior to colistin.

Targeting the 3-deoxy-D-manno-octulosonate (KDO) biosynthetic pathway is a promising source of this type of agent. KDO, which is found in Gram-negative bacteria and plants, but not in humans, serves as the bridge between lipid IV_A, which acts as the membrane anchor of LPS, and the inner oligosaccharide core of LPS. Biosynthesis of KDO requires four steps starting from the pentose pathway intermediate D-ribulose 5-phosphate (Ru5P). In *Escherichia coli*, these steps are catalyzed by enzymes arabinose 5-phosphate isomerase (KdsD, E.C. 5.3.1.13); 3-deoxy-D-manno-octulosonic acid 8-phosphate synthase (KdsA, E.C. 2.5.1.55); 3-deoxy-D-manno-octulosonic acid 8-phosphate phosphatase (KdsC, E.C. 3.1.3.45); and 3-deoxy-D-manno-octulosonate cytidylyltransferase (KdsB, E.C. 2.7.7.38), as depicted in Figure 1. As mentioned above, some success has been reported for KdsB [5]. A recent report describes x-ray crystal structures that may accelerate additional work in this area[12].

There are a limited number of options available to monitor the flux through this pathway. An existing assay quantifies the product of the pathway, CMP-KDO [13]. In order to distinguish CMP-KDO from the other sugars in the pathway, this assay requires an initial sodium borohydride reduction (to reduce KDO), an acid treatment (to hydrolyze CMP-KDO), and then a thiobarbituric acid colorimetric assay for the KDO that was originally coupled to CMP. This complex regime is clearly unsuitable for screening. Another possibility would be to consider looking at the consumption of CTP. While the consumption of ATP can be readily visualized in high-throughput mode by the use of luciferase technology, methods that would allow robust direct determination of CTP are in their infancy [14]. These facts led us to the conclusion that we require an assay that quantifies the inorganic pyrophosphate (PP_i)

produced in the final step that is robust even in the presence of inorganic phosphate (P_i) generated earlier in the pathway. So we have adopted an existing colorimetric PP_i assay [15-17] to measure KdsB activity and combined it with the traditional malachite green assay for P_i to obtain a system that can simultaneously measure P_i and PP_i . To test the assay system's potential to monitor the KDO biosynthesis pathway, we used the last two enzymes in the pathway, KdsC (KDO 8-phosphate phosphatase, EC 3.1.3.45) and KdsB (CMP-KDO synthetase, EC 2.7.7.38).

MATERIALS AND METHODS

Materials

Malachite green carbinol hydrochloride, ammonium molybdate tetrahydrate, sodium sulfite, sodium metabisulfite, and sodium pyrophosphate tetrabasic decahydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anhydrous sodium phosphate monobasic, 1-amino-2-naphthol-4-sulfonic acid, and β -mercaptoethanol (β -ME) were obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA). NuncTM polystyrene 96-well flat bottom assay microplates were from Fisher Scientific (Pittsburgh, PA, USA). Absorbances were determined using a Thermomax UV/Vis plate reader from Molecular Devices (Sunnyvale, CA, USA). Assays at 37 °C were performed using a PTC-200 thermal cycler from MJ research (Waltham, MA, USA). KDO8P was synthesized enzymatically as previously described [18]. Hexa-histidine-tagged enzyme KdsC was over-expressed in *E. coli* and purified as previously described [18]. The KdsC was judged > 95% pure by SDS-PAGE on a 12% gel.

Preparation and assay of KdsB

The *kdsB* gene was cloned into a vector carrying an N-terminal hexa-histidine tag, pT7-LOH [19], which was used to transform *E. coli* BL21(DE3) cells. Cells were grown in 2xYT medium supplemented with 100 μ g/ml ampicillin to an optical density at 600nm of 0.6, then induced to express KdsB by the addition of IPTG to a final concentration of 0.2mM. After induction, cells were grown overnight at 17 °C, then harvested by centrifugation. Crude extract of the cells was obtained by sonication in buffer containing 20 mM Tris-HCl, pH 7.8, 500 mM NaCl, and 5 mM imidazole. The clarified supernatant was loaded on a Ni-NTA column (HisBind[®], Novagen) and eluted at 250 mM imidazole. The purified protein was dialyzed in 10 mM Tris-HCl, pH 7.5 and stored at -80 °C. Protein concentration was determined by Bradford protein assay (Bio-Rad). The protein was judged > 95% pure by SDS-PAGE on a 12% gel.

KdsB activity was assayed in a 96-well plate format using the eikonogen (EK) assay as follows. Each reaction mixture (40 μ L) contained 100 mM glycine-NaOH buffer, pH 10, 5 mM $MgCl_2$, 1 mM CTP, 1 mM KDO, and KdsB. The reaction was initiated by the addition of the enzyme and incubated at 37°C for 1 min. An equal volume (40 μ L) of ice-cold 100% ethanol was added to quench the reaction. Portions of each quenched mixture (40 μ L) were transferred to a flat-bottom microplate for reading. PP_i released in the reaction mixture was quantified by the EK method as described below.

Steady-state kinetic parameters of KdsB were measured by varying KDO concentration (0 – 2500 μ M) at a fixed concentration of 3mM CTP, and by varying CTP concentration (0 – 2500 μ M) at a fixed concentration of 3mM KDO. Saturations curves were fit to the Michaelis-Menten equation by non-linear regression using Origin 8.5 software (OriginLab, Northampton, MA USA). KdsB saturation curves were performed as above (pH 10, 5mM $MgCl_2$) and also at pH 9.3 (glycine-NaOH buffer) in the presence of 10 mM $MgCl_2$, which are the standard assay conditions used in previous work [13, 20].

KdsC assays

Individual KdsC assays were performed in a solution (40 μ L) containing 100 mM glycine-NaOH, pH 10, 5 mM $MgCl_2$, 0.2 mM KDO8P, and KdsC enzyme. The reaction was initiated by the addition of the enzyme, incubated at 37 $^{\circ}C$ for 1 min, and quenched by addition of 40 μ L of ice-cold 100% ethanol. A 40 μ L portion of each quenched mixture was transferred to a flat-bottom microplate, and its P_i concentration was determined using the MG assay described below. Steady-state kinetic parameters of KdsC at pH 10 were measured by varying KDO8P concentration (0–2000 μ M) in the reactions. Saturations curves were fit to the Michaelis-Menten equation by non-linear regression using Origin 8.5 software (OriginLab, Northampton, MA USA).

P_i determination using malachite green (MG assay)

The standard malachite green (MG) assay [21] was modified for 96-well plate format. The MG assay reagent was prepared by mixing malachite green solution (0.09% w/v malachite green carbinol hydrochloride dissolved in deionized (18M Ω) water) and ammonium molybdate solution (8.4% w/v ammonium molybdate in 11M HCl) in a 3:1 ratio. A 200 μ L aliquot of the MG assay reagent was added to each well containing 40 μ L of test sample, and incubated at room temperature for 5 min to develop color. Absorbance was recorded at 650 nm. Each microplate contained 84 sample wells and 12 control wells. The control wells consisted of three sets of four wells, each set containing a serial dilution of a standard aqueous solution of NaH_2PO_4 .

PP_i determination using the eikonogen reagent (EK assay)

The eikonogen reagent (EK) assay was developed based on literature precedent [15–17]. Three assay reagents were prepared: (1) ammonium molybdate solution (2.5% w/v ammonium molybdate in 2.5 N H_2SO_4); (2) eikonogen reagent (0.25 g sodium sulfite, 14.65 g sodium metabisulfite, and 0.25 g 1-amino-2-naphthol-4-sulfonic acid (eikonogen) in 100 mL hot ddH₂O); (3) aqueous β -ME (0.5M). The eikonogen reagent was prepared in hot deionized (18M Ω) water and always warmed prior to use in order to ensure homogeneity. During the assay, test samples (40 μ L) were transferred from the assay mixture into a 96-well microplate. A 50 μ L portion of ammonium molybdate reagent was added to each well, followed by 70 μ L of a 2:5 mixture of eikonogen reagent and β -ME that was freshly prepared before use. After thorough mixing, the plate was incubated either at 37 $^{\circ}C$ for 10 min, or at ambient temperature for 20 min, to allow the color to develop. This color was stable for 24 h. Absorbance was measured at 595 nm. Each microplate contained 84 sample wells and 12 control wells. The control wells consisted of three sets of four wells each. The sets of four wells contained serial dilutions of an aqueous solution of $Na_4P_2O_7$.

The MG/EK assay for simultaneous determination of P_i and PP_i

The P_i /PP_i co-assay was performed by dividing the test sample into two identical 40 l aliquots, testing one in the MG assay (above) and the other in the EK assay (above). The concentration of P_i was determined from the MG assay, using absorbance at 650 nm (A_{650}) and the molar absorption coefficient ($\epsilon_{P_i, MG}$) of $3.12 \times 10^4 M^{-1}cm$ (equation 1). The concentration of PP_i was calculated using equation 2 below. The absorbance at 595 nm (A_{595}) comes directly from the EK assay. The molar absorption coefficient for phosphate in the EK assay ($\epsilon_{P_i, EK}$) was $4.83 \times 10^3 M^{-1}cm^{-1}$ and the molar absorption coefficient for PP_i in the EK assay ($\epsilon_{PP_i, EK}$) was $2.27 \times 10^4 M^{-1}cm^{-1}$.

$$[P_i] = A_{650} / \epsilon_{P_i, MG} \quad (1)$$

$$[PP_i] = \{A_{595} - ([P_i] \cdot \epsilon_{P_i-EK})\} / \epsilon_{PP_i-EK} \quad (2)$$

The KdsC/KdsB combination assay

The KdsC/KdsB assay was performed in 96-well, polypropylene, skirted PCR microplates (Fisher). In a typical assay, the test compound (1 μ L in DMSO or water) was mixed with the combined enzymes (20 μ L in 100mM glycine-NaOH, pH 10, containing 0.1mg/mL bovine serum albumin and 5mM $MgCl_2$), then allowed to incubate at 37 °C for 10 minutes. At this point, the substrates KDO8P and CTP (1 mM each, 20 μ L in 100 mM glycine-NaOH, pH 10, containing 0.1mg/mL bovine serum albumin and 5mM $MgCl_2$) were added to initiate the reaction. The final reaction conditions were therefore 100 mM glycine-NaOH, pH 10, 0.1 mg/mL bovine serum albumin, 5mM $MgCl_2$, 0.5 mM KDO8P, 0.5 mM CTP, and enzyme concentrations as described in the individual figure legends. At the specified quench time, 40 μ L of ice-cold 10% trichloroacetic acid in water was added to stop the reaction. A 40 μ L aliquot of the stopped reaction was transferred to a clear, flat-bottom polystyrene 96-well microplate for the EK assay, and a 25 μ L aliquot was transferred to a second clear, polystyrene flat-bottom 96-well microplate (Costar) for the MG assay. When performing time-course measurements, aliquots awaiting assay were kept in a single microplate on ice until the last time point was taken and then analyzed together.

Compound screening and titration

Individual compounds were screened using the KdsB/KdsC combination assay described above, at 10 mM final concentration. Results were expressed as percent enzyme activity remaining in the well. Titration data were fit either to a 4-parameter logistic model (equation 3, below) or a two-parameter logistic (equation 4, below) using GraFit 5.0 (Erithacus Software) and the equation:

$$\begin{aligned} y &= (A - B) / (1 + (x/IC_{50})^h) \\ y &= 100 / (1 + (x/IC_{50})^h) \end{aligned} \quad (3)$$

Where y is the % inhibition, x is the compound concentration, A is the range maximum, B is the range minimum, and h is the Hill slope.

RESULTS

The Malachite Green (MG) and Eikonogen (EK) assays

The malachite green assay was chosen for this work because it does not require the presence of another enzyme. The drawback is that it destroys the sample, so simultaneous measurement of P_i and PP_i will require us to either divide the sample into two aliquots or run identical reactions in parallel. Figure 2A shows the response of the malachite green (MG) assay to increasing concentrations of added NaH_2PO_4 (P_i) and of added $Na_4P_2O_7$ (PP_i). These data show that the MG assay is not responsive to PP_i . It only gives a color in response to the concentration of P_i . The absorbance readings are linear in the range of 0-22 nmoles of P_i , and from the slope of the regression line, a molar absorption coefficient for the detection of P_i in the MG assay (ϵ_{P_i-MG}) of $3.12 \times 10^4 M^{-1} cm^{-1}$ can be calculated.

To measure PP_i , we chose to explore the eikonogen (EK) assay [15-17], a colorimetric method that measures PP_i without generating P_i and has never before been used as a KdsB assay. Figure 2B shows the response of the EK assay to increasing concentrations of added NaH_2PO_4 (P_i) and of added $Na_4P_2O_7$ (PP_i). As had been the case with the MG assay, the absorbance readings are linear in the range of 0-22 nmoles of P_i or of PP_i . A low detection

limit (nmole levels of P_i and/or PP_i) was achieved in both assays. The response to each analyte is linear, and from these slopes we calculate molar absorption coefficients of $4.83 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ for P_i ($\epsilon_{P_i\text{-EK}}$) and $2.27 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for PP_i ($\epsilon_{PP_i\text{-EK}}$).

The EK assay is suitable for analysis of KdsB

Hexa-histidine-tagged KdsB from *E. coli* was cloned by PCR, over-expressed in *E. coli* BL21(DE3) cells and purified by metal-chelate chromatography as described in the Materials and Methods. As the reported optimal assay conditions of KdsB [13, 20] and KdsC [18] differ, we proposed to perform the co-assay at pH 10 and Mg^{2+} concentration of 5 mM, which should allow the two enzymes to catalyze at similar rates. Kinetic constants for KdsB were determined using substrates KDO and CTP at both the reported optimal pH (9.3) and Mg^{2+} concentration (10mM), and at the proposed co-assay pH (10) and Mg^{2+} concentration (5mM). The EK assay was used to follow the progress of these reactions. As seen in Figure 3, the EK assay provides good quality data that can be easily fit. The kinetic constants derived from these data are in Table 1. KdsB shows a modest 2-fold increase in apparent K_M for each substrate, and a modest 2-fold increase in apparent k_{cat} conditions shift from its reported optimum to the proposed co-assay conditions.

Developing a co-assay of KdsB and KdsC

Hexa-histidine-tagged KdsC from *E. coli* was cloned by PCR, over-expressed in *E. coli* BL21(DE3) cells and purified by metal-chelate chromatography as described in the Materials and Methods. Kinetic constants for KdsC were determined at pH 10 and 5 mM Mg^{2+} concentration using the MG assay (Table 1). KdsC, at pH 10, exhibits an apparent K_M for KDO and an apparent k_{cat} that are comparable to the kinetic constants determined for KdsB at pH 10. With both enzymes in hand, we next tested the ability of the combined MG and EK assays to determine the concentrations of P_i and PP_i simultaneously. A series of equimolar mixtures of P_i and PP_i were prepared, then tested first using the MG assay, and then using the EK assay. The concentration of P_i was determined from the MG assay using the molar absorption coefficient ($\epsilon_{P_i\text{-MG}}$) determined above. With this value in hand, the PP_i concentration was calculated from the response of the EK assay using the molar absorption coefficients $\epsilon_{P_i\text{-EK}}$ and $\epsilon_{PP_i\text{-EK}}$, as described in the Materials and Methods. A comparison of the theoretical and actual concentrations is shown in Figure 4. The combined MG/EK assay accurately determines the concentrations of P_i and PP_i in synthetic mixtures.

At this point we began to develop a version of the assay that is amenable to screening at higher throughput. For the sake of convenience and sensitivity, we sought to eliminate the use of ethanol as a reaction quench, to lower the enzyme concentrations, and to lengthen the duration of the assay. We found that 10% trichloroacetic acid in water, added at the same volume as we had been adding ethanol, gave good quenching without the problem of evaporation. Lowering the enzyme concentrations to nanomolar levels required the addition of 0.1 mg/mL bovine serum albumin to the assay buffer. Fortunately, the lowering of the enzyme concentration also extended the duration of the linear phase of the reaction. To optimize the concentration of each of the enzymes and determine the time-point most suitable for the quench, a 2-dimensional array of experiments was performed in which both enzyme concentrations were varied, and a time-course was performed at each of the enzyme concentration combinations. We tested four concentrations of KdsC (10, 20, 40 and 60 nM) and four concentrations of KdsB (25, 50, 75 and 100 nM), and sampled at 5, 10, 15 and 20 min. The rate of appearance of phosphate was linearly dependent upon the concentration of KdsC (Figure 5A), but was not greatly affected by the concentration of KdsB. The rate of appearance of pyrophosphate was dependent upon both the concentration of KdsC and the concentration of KdsB (Figure 5B). At fixed KdsC concentration, the rate of appearance of pyrophosphate is linearly related to the KdsB concentration, and increasing

the KdsC concentration accelerated the rate of pyrophosphate formation. Optimal screening conditions require the ability to measure phosphate and pyrophosphate simultaneously during the linear phase of their formation. We chose to pursue the conditions presented in Figure 5C. Using 60nM KdsC, 100nM KdsB, and a 20 minute reaction time, the concentration of phosphate and pyrophosphate are both reliably measurable and reasonably close to the linear phase of the time-course.

We next sought to test the assay with compounds. Unfortunately, samples of known KdsB inhibitors [5] were unavailable, so we tested the assay system in two separate ways. In the first test we titrated the trivial inhibitor EDTA, which inhibits both KdsC and KdsB by chelating the magnesium ions necessary for their activity. The results of the titration, shown in Figure 6, yielded an IC_{50} of 3.8 μ M with a Hill slope of 1.09 for inhibition of KdsC, and an IC_{50} of 1.5 μ M with a Hill slope of 5.43 for the inhibition of KdsB. Using the standard assay protocol, the EDTA encountered (and chelated) 5 mM Mg^{2+} in the 20 μ L aliquot of enzyme solution, and was then forced to compete for the 5 mM Mg^{2+} added with the 20 μ L aliquot containing substrates. Perhaps some KdsC turnover took place at concentrations of EDTA that were able to completely shut down subsequent turnover of KdsB. In the second test of the assay, we screened a small number of nucleotides, biologically relevant carbohydrates and phosphosugars at 10mM concentration, hoping to find a weak inhibitor. In addition to the inhibition of P_i and PP_i production, we measured the P_i contamination in each of the compounds. None of the compounds we assayed were selective inhibitors of either KdsC or KdsB (Figure 7). We discovered, however, that compounds containing a significant amount of contaminating P_i , like the UTP we used, do not give meaningful results in portion of the assay measuring inhibition of P_i production. Ignoring the contaminating P_i results in an apparent stimulation of P_i production (Figure 7, white bars), while subtracting out the contaminating P_i would yield a significant apparent inhibition (subtract grey bars from white bars in Figure 7). Since the production of PP_i was not inhibited, subtraction of the contaminating phosphate would lead to the absurd conclusion that P_i production (and therefore KDO production) was inhibited, but PP_i production (and therefore the activation of KDO) was not. Since we do not anticipate that a significant percentage of the compounds in screening libraries are contaminated with this level of P_i , we do not anticipate this being an issue in small molecule screening.

DISCUSSION

Responding to the need for antibacterials with novel mechanisms of action, we are in the process of developing a screen for inhibitors of the KDO biosynthesis pathway, which converts ribulose 5-phosphate into the bacteria-specific eight-carbon sugar KDO and activates it for transfer to lipid IV_A, a precursor of bacterial lipopolysaccharide. Lacking a convenient assay for the final carbohydrate product of the pathway, we developed a method to follow the phosphate and the pyrophosphate produced. Popular methods of measuring PP_i , like the inorganic pyrophosphatase-coupled assay [22] and the Pyrophosphate Reagent kit (Sigma) [23], are unsuitable because, in addition to adding another enzyme to the assay, they introduce P_i as a byproduct. Our method is unique in allowing the simultaneous measurement of these pathway products. The method, based upon the malachite green [21] and deikonogen [15-17] methods has proven to be amenable to 96-well microtiter plates, easy to perform, and accurate. The addition of BSA allowed us to perform the assay with 60nM KdsC and 100nM KdsB, potentially allowing us to accurately measure IC_{50} 's of 600 nM or more. We have shown that it is capable of titrating an inhibitor and have screened a small set of compounds. While we did not identify an inhibitor within this small set, we have discovered that contaminating phosphate can make the phosphate portion of the assay unreliable. This is somewhat ameliorated by the fact that the pyrophosphate portion of the assay is not significantly affected by phosphate contamination.

Expanding this assay to screen the entire KDO biosynthesis pathway is simple in principle, but is likely to be complex in execution. A key concern is the fact that KdsD and KdsA assays are normally run at pH 7.5, rather than the pH 10 used in this report, perhaps because of the potential instability of phosphoenolpyruvate and ribulose 5-phosphate at basic pH. To approach this issue, we will first construct an assay that couples KdsD with KdsA.

Finally, our method is applicable to systems beyond KDO biosynthesis. Generation of phosphate and pyrophosphate is found in other carbohydrate biosynthesis and activation pathways. A typical example is the pathway that allows incorporation of sedoheptulose 7-phosphate into bacterial lipopolysaccharide. Like many carbohydrate activation schemes, the activated molecule is a diphosphate, rather than a monophosphate like KDO-CMP. The diphosphate in the sedoheptulose case is formed through coupling of a sugar phosphate to ATP (Figure 8). Thus, two sequential steps in this pathway also generate a molecule of P_i and a molecule of PP_i .

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ABBREVIATIONS

α-ME	beta-mercaptoethanol
CMP-KDO	CMP-3-deoxy-D-manno-octulosonic acid
Pi	inorganic phosphate
PPi	inorganic pyrophosphate
KDO	3-deoxy-D-manno-octulosonic acid
KDO8P	3-deoxy-D-manno-octulosonic acid 8-phosphate
IPTG	isopropyl β -D-thiogalactopyranoside
LPS	lipopolysaccharide
PEP	phosphoenolpyruvate
Ru5P	ribulose 5-phosphate

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Figure 1.
Biosynthesis and activation of 3-deoxy-D-manno-octulosonate (KDO).

Figure 2. Calibration curves of (A) the malachite green assay and (B) the eikonogen assay in 96-well plateformat. Each sample contained 0-22 nmoles of P_i or PP_i in 40 μ L volume. All data points were measured in triplicate.

Figure 3. Steady-state kinetics of KdsB using the EK assay. (A) at pH 9.3, 10 mM Mg^{2+} ; (B) at pH 10, 5 mM Mg^{2+} ; The saturation curves are fit to the Michaelis-Menten equation. Lineweaver-Burk plots are shown in the inserts.

Figure 4.

Determination of P_i and PP_i in a 1:1 mixture. (A) The malachite green assay of the mixture; (B) the eikonogen assay of the mixture (the experimental measurements are shown in solid dots, the calculated P_i contribution is shown in open triangles, and calculated the PP_i contribution is shown in open squares); (C) comparison between the experimental P_i amounts (calculated from the MG assay) and the theoretical values; (D) comparison between the PP_i amounts (calculated by subtracting the P_i contribution from the total EK signal) and the theoretical values. All data points were measured in triplicate.

Figure 5.

(A) Production of P_i with low (20 nM, circles) and high (60 nM, squares) concentrations of KdsC in the presence of low (25 nM, open symbols) and high (100 nM, filled symbols) concentrations of KdsB (B) Production of PP_i with low (25 nM, circles) and high (100 nM, squares) concentrations of KdsB in the presence of low (20 nM, open symbols) and high (60 nM, filled symbols) concentrations of KdsC. (C) Timecourse of P_i production (open triangles) and PP_i production (filled triangles) using the enzyme concentrations selected for use in the standard assay protocol (60 nM KdsC, 100 nM KdsB).

Figure 6.

Inhibition of P_i formation (open triangles) and PP_i formation (filled squares) by the chelating agent EDTA. The P_i inhibition data were fit to a 2-parameter logistic (dashed line) and the PP_i data were fit to a 4-parameter logistic (solid line), as described in the Materials and Methods. The fit parameters for the P_i inhibition were: IC₅₀ = 3.8 M; Hill slope = 1.0886. The fit parameters for the PP_i inhibition were: Maximum = 94.9 % activity; Minimum = 0.25% activity; IC₅₀ = 1.5 μM; Hill slope = 5.43.

Figure 7.

Screening results for selected nucleotides and carbohydrates. The results for each compound are displayed as three bars. The grey bar represents the concentration of P_i contaminating the test compound, as a percent of the P_i concentration in an uninhibited reaction. The white bar represents the concentration of P_i produced in the presence of the test compound, as a percent of the P_i concentration in an uninhibited reaction, and the black bar represents the concentration of PP_i produced in the presence of the test compound, as a percent of the PP_i concentration in an uninhibited reaction. Abbreviations are: NeuAc, N-acetylneuraminic acid; MurAc, N-acetylmuramic acid; Mur, muramic acid; 2dGal, 2-deoxygalactose; GlucA6P, 6-phospho-D-glucuronic acid; G6P, glucose 6-phosphate; 2dG6P, 2-deoxyglucose 6-phosphate; Ri5P, ribose 5-phosphate; 2dRi5P, 2-deoxyribose 5-phosphate; F6P, fructose 6-phosphate; A5P, arabinose 5-phosphate.

Figure 8.
GmhB- and GmhC-catalyzed activation of D-glycero-D-manno-heptose in the biosynthesis of bacterial LPS

Table 1

Kinetic parameters of KdsC determined using the malachite green assay, and KdsB determined using the eikonogen assay.

	K_M (Kdo or Kdo8P) (mM)	K_M (CTP) (mM)	k_{cat} (s^{-1})
KdsB pH 9.3, 10 mM Mg^{2+}	0.28 ± 0.02	0.33 ± 0.04	12.5 ± 0.4
KdsB pH 10, 5 mM Mg^{2+}	0.52 ± 0.02	0.59 ± 0.03	21.9 ± 0.3
KdsC pH 10, 5 mM Mg^{2+}	0.37 ± 0.01		22.2 ± 2.1