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Tunable Enzymatic Synthesis of the Immunomodulator Lipid IV_A To Enable Structure–Activity Analysis

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Abstract

The Lipid A family of glycolipids, found in the outer membranes of all Gram-negative bacteria, exhibits considerable structural diversity in both lipid and glycan moieties. The lack of facile methods to prepare analogues of these natural products represents a major roadblock in understanding the relationship between their structure and immunomodulatory activities. Here we present a modular, cell-free multienzymatic platform to access these structure–activity relationships. By individually purifying 19 *Escherichia coli* proteins and reconstituting them in vitro in the presence of acetyl-CoA, UDP-*N*-acetylglucosamine, NADPH, and ATP, we have developed a system capable of synthesizing Lipid IV_A, the first bioactive intermediate in the Lipid A pathway. Our reconstituted multienzyme system revealed considerable promiscuity for orthologs with distinct substrate specificity, as illustrated by swapping enzymes from distantly related cyanobacterial and *Pseudomonas* species. Analysis of the agonistic and antagonistic activities of the resulting products against the THP-1 human monocytic cell line revealed hitherto unrecognized trends, while opening the door to harnessing the potent biological activities of these complex glycolipid natural products.

Lipid A, the hydrophobic anchor of bacterial lipopolysaccharide (LPS, also known as endotoxin), is not only an essential component of the outer membrane of all Gram-negative bacteria, but also one of the most potent small-molecule elicitors of the eukaryotic innate immune response. Humans have evolved multiple proteins to recognize this glycolipid with high affinity, including lipopolysaccharide binding protein (LBP), CD14, the TLR4-MD2

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Author Contributions

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ASSOCIATED CONTENT

Supporting Information

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Materials, methods, and supporting figures (PDF)

The authors declare no competing financial interest.

complex, caspase 4, and caspase 5.¹ Notwithstanding impressive advances in the chemical- and biosynthesis of these natural products,^{2–4} the availability of facile methods to prepare structurally defined analogues remains a major roadblock in our understanding of their remarkable biological properties. Here we present a novel approach to access these structure–activity relationships.

Our target for this study was Lipid IV_A, the first bioactive intermediate in the Lipid A biosynthetic pathway (Figure 1). In humans, Lipid IV_A is an antagonist of Lipid A by virtue of its ability to bind to the transmembrane receptor TLR4-MD2 without eliciting a response. Synthetic analogues of Lipid IV_A have been investigated as potential treatments of severe sepsis,⁵ viral infections,⁶ myocardial and renal ischemia-reperfusion injury,⁷ chronic LPS-mediated airway disease,⁸ contact lens-associated corneal infection,⁹ and liver diseases.¹⁰ Further, the TRIF-biased TLR4 agonist monophosphoryl Lipid A is clinically approved for use as a vaccine adjuvant (Cervarix, Fendrix).¹¹

The building blocks for Lipid IV_A biosynthesis are acetyl-CoA and UDP-*N*-acetylglucosamine (UDP-GlcNAc) with NADPH and ATP as essential cofactors. By individually purifying 19 *E. coli* proteins and reconstituting them in vitro along with these substrates and cofactors (see Supporting Information), we have developed a chemically defined system capable of synthesizing Lipid IV_A at yields and purity required for a cell culture-based investigation of structure–activity relationships.

The biosynthetic pathway of Lipid IV_A (Figure 1) can be operationally divided into four modules. Module A, comprised of AccC, AccA, AccD, and *holo*-AccB (with a tethered biotin cofactor), converts acetyl-CoA into malonyl-CoA. In the presence of NADPH, acetyl-CoA and malonyl-CoA initiate synthesis of a fatty acyl chain via the collaborative activity of FabH, FabD, FabG, FabA or FabZ, FabI, and *holo*-ACP (which harbors a tethered phosphopantetheine arm) (Module B). Additional malonyl-CoA and NADPH equivalents are consumed by FabB or FabF, FabD, FabG, FabA or FabZ, FabI, and *holo*-ACP (Module C) to elongate the butyryl-ACP thioester product of module B. Whereas this pathway generates fatty acyl chains of varying lengths and degrees of reduction, a fourth module (Module D) composed of six enzymes (LpxA, LpxC, LpxD, LpxH, LpxB, and LpxK) intercepts the 3-hydroxymyristoyl-ACP intermediate to yield Lipid IV_A.

To ascertain the activity of purified enzymatic components of the Lipid A biosynthetic pathway, 5 μ M each of LpxA, LpxC, LpxD, LpxH, LpxB, and LpxK were mixed with 10 μ M *holo*-AccB, 10 μ M *holo*-ACP, and 1 μ M each of all of the other enzymes shown in Figure 1. Control reactions individually lacking LpxK, LpxK/B, LpxK/B/H, LpxK/B/H/D, and LpxK/B/H/D/C were also performed. As shown in Figure S1, high-resolution LC-ESI-MS analysis verified formation of the expected products, including Lipid IV_A, in each reaction mixture. Importantly, the desired peak was the major product of each assay mixture; prior intermediates from the Lipid IV_A biosynthetic pathway were undetectable.

To facilitate kinetic analysis of the reconstituted Lipid IV_A system, we sought to develop a continuous assay in which NADPH consumption by FabG and FabI could be measured by UV spectrometry (Figures S2–S3). In a control experiment utilizing acyl-ACP thioesterase

TesA from *E. coli* as a release mechanism, Modules A–C exhibited a collective turnover rate of 40 min⁻¹ (Figure 2). When TesA was replaced with equimolar ratios of LpxA, LpxC, LpxD, LpxH, LpxB, and LpxK, we anticipated that 3-hydroxymyristoyl-ACP would be redirected by LpxA, LpxC, and LpxD at some frequency. However, two questions arose. First, to what extent would NADPH consumption capture the activity of LpxA/C/D, given that *holo*-ACP was present in substantial stoichiometric excess? Second, was the LpxA-catalyzed acyl transfer reaction thermodynamically unfavorable, as had been previously proposed?¹²

In experiments containing LpxA/C/D, modules A–C exhibited a turnover rate of 99 min⁻¹ for at least 20 turnovers (Figures 2 and S3). LpxC was confirmed as the first committed step toward Lipid IV_A synthesis (Figure 2 and S3). In the absence of a release mechanism, a turnover rate of 26 min⁻¹ was observed, presumably due to the presence of excess ACP intended to mimic protein concentrations observed in *E. coli*.¹³ The excess ACP supported NADPH consumption for approximately two ACP elongations, where one elongation is defined as conversion of *holo*-ACP to C₁₈-ACP (Figure S3).

It is known that the reconstituted fatty acid synthase from *E. coli* produces saturated as well as *cis*-(ω -7) monounsaturated fatty acyl-ACP species.¹¹ Since biosynthetically derived Lipid A in *E. coli* is exclusively derived from saturated fatty acids, at the outset of this study we expected that unsaturated fatty acyl-ACP species would be dead-end products in our reconstituted system for enzymatic Lipid IV_A synthesis. Remarkably, however, Lipid IV_A analogues with one or more monounsaturated 3-OH-C₁₄ fatty acyl chains were observed in substantial amounts in addition to the fully saturated natural product (Figure S1). This observation suggested that both LpxA and LpxD have tolerance for unsaturated as well as saturated fatty acyl chains, prompting us to revisit the structure of Lipid A synthesized by *E. coli*. *E. coli* DH5 α was grown under standard conditions (37 °C), and Lipid A was extracted using an established protocol.¹⁴ Consistent with earlier reports, only the fully saturated natural product was observed (Figure S4), suggesting that *E. coli* harbors a regulatory mechanism to suppress incorporation of monounsaturated acyl chain substrates into the natural product. The precise nature of this regulatory mechanism remains to be elucidated, but we hypothesize it relates to the cocistronic expression of the genes encoding LpxA, LpxD and FabZ in *E. coli* (Figure S5). Other observations also suggest the activities of FabZ and the enzymes responsible for Lipid A synthesis may be coregulated.¹⁵ Therefore, we sought a means of enriching for fully saturated Lipid IV_A analogues through rational modifications to the platform's enzymatic components, as outlined below.

In previous studies with reconstituted fatty acid synthases, we had also observed that, when *E. coli* FabZ and FabA were replaced with FabZ from the cyanobacterium *Synechococcus* sp. PCC7002, saturated fatty acids were the dominant products.¹⁶ We therefore attempted to engineer the composition of enzymatically synthesized Lipid IV_A by an analogous manipulation. As shown in Figure 3A, fully saturated Lipid IV_A was by far the most abundant product when *E. coli* FabZ and FabA were replaced by *Synechococcus* FabZ. Unexpectedly, however, the product of this reconstituted system predominantly harbored C₁₂ hydroxyacyl chains in lieu of C₁₄ acyl chains. With increasing concentrations of the FabB ketosynthase, it was expected that unsaturated C₁₄ analogues of Lipid IV_A would be the

dominant products in the presence of *E. coli* FabA and FabZ;¹⁷ indeed, this was found to be the case (Figure 3B).

To further test structure modification strategies, *E. coli* LpxA/C/D was replaced with *P. aeruginosa* LpxA/C/D. LpxA and LpxD orthologs from *P. aeruginosa* are known to be selective for C₁₀ and C₁₂ hydroxyacyl chains, respectively.¹⁸ While a major reaction product harbored the expected acyl chains, several unanticipated products were also observed (Figure 3C). These side reactions can be explained by the ability of *P. aeruginosa* LpxD to incorporate C₁₄ hydroxyacyl chain.¹⁸ Together, these findings suggest that, while the role of LpxA and LpxD as “hydrocarbon rulers” in selecting acyl chains with appropriate lengths has been explored in isolation,^{19–23} the fully reconstituted system may shine new light on the control of chain length *in vivo*.

To demonstrate the promise of this multienzyme system for the synthesis of novel immunomodulatory glycolipids, product mixtures A–C (Figure 3) were tested against the multipotent human monocytic cell line THP-1, which is widely used to verify the ability of LPS to elicit the pro-inflammatory cytokine IL-6.²⁴ In each case the reaction products were further separated from residual quantities of contaminant LPS using mixed mode (anion exchange/normal phase) chromatography (Figure S105, Table S12).

Anti-inflammatory activities of product mixtures A–C were measured by coadministration with 1 ng/mL LPS (Figure S106) to cells that had been freshly differentiated into macrophage-like cultures by pretreatment with phorbol 12-myristate 13-acetate (PMA) (Figure 4A; see Supporting Information for experimental details). On the other hand, pro-inflammatory activities were measured by adding product mixtures alone to PMA-differentiated THP-1 cells (Figure 4B). All three products of enzymatic synthesis showed comparable LPS-antagonistic and TLR4-agonistic activity to a Lipid IV_A standard. In previous studies, Lipid IV_A showed modest agonistic activity at high concentrations.²⁵ Because glycolipids with dual antagonist–agonist properties are of considerable pharmacological interest (e.g., monophosphoryl Lipid A), we sought to study the immunomodulatory properties of product A in greater depth at a higher concentration (e.g., 100 ng/mL).

Using a multiplexed cytokine quantification assay (Figure S107, Table S13), we compared the agonistic activity of Product A to that of LPS derived from *E. coli* 0111:B4 strain and synthetic Lipid IV_A. As shown in Figure 4C and Figure S107, this product has a remarkable bias toward eliciting IL-22 and, to a lesser extent, TNF- α , compared to IL-6. Thus, Lipid IV_A can regulate immune response through cytokine modulation and merits careful analysis in future structure–activity studies. Forthcoming efforts to scale up these multienzyme synthetic systems and to improve the separation of close structural analogues synthesized within the same reaction mixture will aid in further analysis of their unique biological activities.

Not with standing these limitations, the stage is set for a structure–activity analysis of Lipid IV_A analogues. Beyond this application, the tool offers unprecedented access to probing the Lipid A biosynthetic pathway; studies are underway in the authors’ laboratory to

mechanistically understand the lack of unsaturation in the natural product. Further, as an essential, conserved pathway in many Gram-negative bacteria, these enzymes are an attractive antibiotic target. A long-term goal is to discover novel molecules that interfere with this biosynthetic pathway. Finally, Lipid A-like molecules have been discovered in plants²⁶ and are presumably involved in plant defense responses.²⁷ An enzymatic platform opens the door to investigating Lipid A-like molecules in organisms where genetic modifications are currently intractable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Enzymatic synthesis of *E. coli* Lipid IV_A. Modules A, B, and C synthesize 3-hydroxymyristoyl-ACP (3-OH-C₁₄-ACP), the acyl-ACP substrate for Lipid IV_A biosynthesis, in situ. Module D synthesizes Lipid IV_A. Protein components are in pink, while substrates and cofactors are in green.

Figure 2.

Effect of sequential addition of LpxA-LpxK on overall turnover rate. The assay mixtures (A–E) incrementally build the Lipid IV_A biosynthesis pathway by sequentially introducing 2 μM LpxA/C/D/H/B/K to Modules A–C, shown in Figure 1. (A) No Lpx enzymes, (B) 2 μM LpxA, (C) 2 μM LpxA/C, (D) 2 μM LpxA/C/D, and (E) 2 μM LpxA/C/D/H/B/K. As controls, (F) is the assay mixture, (E) lacking *holo*-ACP, and (G) corresponds to sample (A) plus 10 μM TesA. Turnover rate is normalized to the concentration of FabG. For representative progress curves, see Figure S3.

Figure 3.

Engineering Lipid IV_A structure by rational modification of the in vitro enzymatic system. The reaction mixture yielding Product A contained FabZ from *Synechococcus* sp. PCC 7002 in lieu of both *E. coli* FabA and FabZ. Product mixture B was synthesized in the presence of *E. coli* FabA and FabB at a ratio 1.5:10. Product mixture C was synthesized in the presence of LpxA/C/D from *P. aeruginosa* in lieu of *E. coli* LpxA/C/D. The reported values of relative abundance were calculated using area under extracted ion chromatograms. Dashed lines (red) indicate potential locations of double bonds. Abundance is normalized to the

compound denoted by an asterisk. All reaction products are mixtures, and only major products are shown. For supporting data, see Figures S6–104.

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Figure 4.

Immunomodulatory activity of enzymatically synthesized Lipid IV_A analogues. (A) Antagonistic and (B) agonistic activities of product mixtures A–C (Figure 3) were measured by quantifying IL-6 secreted by macrophage-like cells derived from the human THP-1 monocytic cell line. (C) Product A (100 ng/mL) appears to be a biased TLR4 agonist. For experimental details, see Supporting Information. Data shown is the mean of four or more biological replicates with error bars representing 95% confidence interval. Concentration values are order of magnitude estimates.