Lipid A Precursor from *Pseudomonas aeruginosa* Is Completely Acylated Prior to Addition of 3-Deoxy-α-manno-octulosonate*

(Received for publication, October 30, 1987)

Robert C. Goldman,† Colette C. Doran, Sunil K. Kadam, and John O. Capobianco

*From the Anti-infective Research Division, Pharmaceutical Discovery, Abbott Laboratories, Abbott Park, Illinois 60064*

The early steps in the assembly of lipopolysaccharide (LPS) start with UDP-N-acetyl-d-glucosamine (1), a molecule which is also used in the assembly of peptidoglycan. A molecule of 3-OH-C14:0 is transferred from acyl carrier protein to the 3-position of the N-acetyl-d-glucosamine residue, followed by removal of the N-linked acetyl group and further acylation at this site with another molecule of 3-OH-C14:0. This yields UDP-2,3-diacyl-GlcN (2). A portion of UDP-2,3-diacyl-GlcN is hydrolyzed to UDP + 2, 3-diacyl-GlcN then 2,3-diacyl-GlcN condenses with a molecule of UDP-2,3-diacyl-GlcN, and the product is phosphorylated (3, 4) to yield the basic disaccharide structure of lipid A, O-(2-amino-2-deoxy-β-D-glucopyranosyl)-(1→6)-2-amino-2-deoxy-α-D-glucose, acylated at positions 2, 3, 2', 3' with 3-OH-C14:0, and bearing phosphate at positions 1 and 4' (IV₅). Compound IV₅ is one of the lipid A precursor species which accumulated when a temperature-sensitive *kdsA* mutant of *Salmonella typhimurium* was incubated at the restrictive temperature (5). Thermal inhibition of KDO-phosphate synthetase, the product of the *kdsA* gene, effectively blocks further synthesis of KDO and causes accumulation of four major species of putative lipid A precursors (5). Since precursor species IV₅ accumulates first and to the greatest extent in both *S. typhimurium* and *Escherichia coli*, it likely represents the normal *in vivo* acceptor for KDO. The other precursor species which appear only after IV₅ has accumulated several hundred-fold in the inner membrane probably represent aberrant reactions occurring in response to inhibition of metabolite flow through the pathway.

Lipid A precursor from *S. typhimurium* is an acceptor of two KDO molecules *in vitro* (6), and recently a species with a single KDO was detected both *in vitro* and *in vivo* (7). Although the species containing a single KDO was not a precursor to the species containing two KDO molecules, it was a transient metabolite. Although purely speculative at present, the species containing a single KDO could be a direct precursor to LPS and thus represent a subfraction of LPS containing a single KDO. A single KDO apparently links lipid A to other core sugars in some Gram-negative bacteria (8, 9).

Our knowledge of the early stages of LPS assembly is limited to *S. typhimurium* and *E. coli* for two major reasons: (i) the genetic approach is limited due to lack of methods for directly selecting for mutations in the pathway, and (ii) the direct biochemical analysis of the pathway is difficult because intermediates are present in low levels and are rapidly turning over. However, we recently reported a new class of synthetic antibacterial agents (10) which specifically inhibit 3-keto-D-manno-octulosonate incorporation in Gram-negative bacteria. The new agents consist of competitive inhibitors of CMP-KDO synthetase, α-C-[1,5-anhydro-7-amino-2,7-dideoxy-D-manno-heptopyranosyl]carboxylate (II) and α-C-[1,5-anhydro-7-amino-2,7-dideoxy-D-manno-heptopyranosyl]carboxylate (I) which are delivered to bacteria as peptide prodrugs. These prodrugs are transported by the oligopeptide permease system and hydrolyzed by intracellular peptides which release the free inhibitor.

In this report, we have used these new antibacterial agents.

---

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed.

‡ The abbreviations used are: LPS—lipopolysaccharide; CMP-KDO synthetase, CTP-CMP-3-deoxy-α-manno-octulosonate cytidylyltransferase; KDO, 3-deoxy-D-manno-octulosonate; α-C-[1,5-anhydro-8-amino-2,7,8-trideoxy-D-manno-octopyranosyl]carboxylate, III, I amide linked to the carboxyl terminus of anilalanine; II, α-C-[1,5-anhydro-7-amino-2,7-dideoxy-D-manno-heptopyranosyl]carboxylate; IV, II linked to the carboxyl terminus of anilalanine; HPLC, high performance liquid chromatography.
to examine the early stages in assembly of LPS in diverse species of Gram-negative bacteria and to show that, although similarities exist, there are species-specific differences. In particular, the major lipid A precursor species from *Pseudomonas aeruginosa* is completely acylated with all of the fatty acids present on mature LPS prior to addition of KDO.

In contrast, the major species from other enteric Gram-negative species contains only 3-OH-C14:0, and lacks the non-hydroxy fatty acids characteristic of mature LPS.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**—Bacterial strains used in this study are listed in Table I. Bacteria were grown at 37 °C in defined medium (11) containing 0.2% glucose, 0.5 mM N-acetylglucosamine and 1 mM leucine, except where indicated.

**Induction of Lipid A Precursor and Analysis by Radiolabeling**—Bacteria from an overnight culture were inoculated into fresh medium containing 0.2% glucose, 0.5 mM N-acetylglucosamine, and 1 mM leucine, except where indicated.

**Analysis of Radiolabeled Fatty Acids**—Fatty acids attached to lipid A precursor were radiolabeled by protocol (see ii), except 100 µCi of labeled acetate was added to 10 ml cultures. Precursors were extracted from bacteria as described above and purified by chromatography on Silica Gel H. Specific precursor peaks were extracted from silica gel using chloroform/methanol/H₂O (63:33:4), containing 0.1 N HCl, which was evaporated to dryness under a stream of nitrogen. Fatty acids were released by acid hydrolysis (0.5 ml of 4 N HCl at 100 °C in a sealed, nitrogen-flushed tube). Free fatty acids were resuspended in 2 volumes of methanol containing 0.1% sodium dodecyl sulfate and 5% mercaptoethanol (v/v) at 100 °C. The residue was resuspended in chloroform for application to Silica Gel H plates. Plates were developed in a solvent of chloroform, pyridine, and 88% formic acid, and tritium counts determined with a Packard Tri-Carb scintillation counter. Peak tubes were pooled and extracted into chloroform as outlined above.

**Pulse-Chase Experiments**—Cells were grown in defined medium at 37 °C to an A₅ₐ₀ value of 0.3–0.5, drug (III, 50 µg/ml) was added for 5 min, followed by pulse labeling with [³H]N-acetylglucosamine (21 µCi/ml) for 10–30 min. Cells were rapidly chilled and resuspended in medium containing 5 µCi/ml of unlabeled N-acetylglucosamine, and resuspended in medium lacking drug and radiolabel, but containing 5 mM unlabeled N-acetylglucosamine. Samples were taken at the end of the pulse period and at designated times following chase.

**Physical Procedures**—Glucosamine content was determined after hydrolysis with 4 N HCl at 100 °C for 14 h by a modification of the Elson-Morgan reaction (18, 19). Phosphodiesters were determined by the Ames procedure (20) and protein estimated by the method of Lowry et al. (21). Fatty acids were determined by gas chromatography.
Lipid A Precursor from P. aeruginosa

5219

(7) after conversion to their methyl esters with methanolic HCl (100 °C/17 h). A standard fatty acid mix was prepared from the lipid A of P. aeruginosa K799. Confirmation of peaks was accomplished by mass spectrometry on a Hewlett-Packard model 5985A, gas chromatography/quadrupole mass spectrometer (Packard Instrument Co.) operated at 70 eV ionization energy, 300 micro A filament, and a source temperature of 200 °C. Both electron and chemical (NH₃) impact were utilized to generate mass molecular ions [M + H]⁺ and [M + NH₄]⁺.

The mass molecular ion (M − H)⁺ for DEAE-cellulose peak I and for the lipid A standard were determined on a Kratos MS-50 mass spectrometer (AEI/Kratos) in the negative mode. Samples (10−20 µg) in chloroform/methanol (4:1) were mixed with tetrathylene glycol on the instrument probe. A neutral beam of xenon was used and the translational energy varied between 6−8 kV. Data were collected at 30 s/decade scan rate over a mass range of 500−1800 daltons.

Chemicals—All radiolabels were purchased from Amersham Corp. DEAE-cellulose (DE52) was obtained from Whatman and methanolic HCl kits were purchased from Alltech Assoc. Inc. KDO analogs were synthesized at Abbott Laboratories.³ Compound I (α-C-[1,5-anhydro-8-amino-2,7,8-trideoxy-D-manno-octopyranosyl](carboxylate) and Compound II (α-C-[1,5-anhydro-7-amino-2,7-dideoxy-D-manno-heptopyranosyl]carboxylate) were linked to the carboxyl terminus of alanylalanine yielding compounds III and IV, respectively (U.S. Patents 4,613,589 and 4,613,590). All other chemicals were from Sigma.

RESULTS AND DISCUSSION

Induction of Lipid A Precursor in P. aeruginosa—Growth ceased and new glucosamine containing metabolites accumulated (Fig. 1, A and B) following treatment of P. aeruginosa K799 with III (I amide linked to the carboxyl terminus of alanylalanine). Intraspecific synthesis of III releases the aCMP-KDO synthetase inhibitor I which inhibits aCMP-KDO synthetase from P. aeruginosa with a kᵣ of 0.5 µM. The major component migrated on Silica Gel H between phospholipid and lipid A precursor IV, (relative position shown by A) purified from P. aeruginosa strain A5007 (data not shown).

Fatty acid content was determined by HPLC. The major component contained 3-OH-C10:0, C12:0, or 3-OH-C10:0, respectively, from the major phospholipid. Two fragments (M + H)⁺ were predicted to arise from cleavage of the glucosamine residue. Two fragments (M + H)⁺ were consistent with loss of either 2-OH-C12:0 in one case, C12:0 in the other, two anionic fragments would be predicted to arise from cleavage of the glycosidic bond. Two fragments (M + NH₄⁺ − 2H)⁺ were 1617 daltons, based on previous studies of lipid A from this and other strains of P. aeruginosa (12, 22, 23). A major ion [M − H]⁻ of mass 1616, was observed by fast atom bombardment mass spectroscopy which is consistent with our proposed structure (Fig. 4). Less abundant ions at 1600 [M − H]⁻ and 1632 [M − H]⁻ are consistent with subspecies where (i) both the nonreducing and reducing glucosamine residues contain C12:0 as the acyloxyacyl fatty acid (1600 [M − H]⁻), (ii) both the nonreducing and reducing glucosamine residues contain 2-OH-C12:0 as the acyloxyacyl fatty acid (1632 [M − H]⁻). The component at 1616 [M − H]⁻ could thus represent two components, where (i) the nonreducing and reducing glucosamines contain C12:0 and 2-OH-C12:0 as the acyloxyacyl fatty acids, respectively, or (ii) the nonreducing and reducing glucosamine residues contain 2-OH-C12:0 as the acyloxyacyl fatty acid. Fragments at 1418, 1434, and 1446 [M − H]⁻ are consistent with loss of either 2-OH-C12:0, C12:0, or 3-OH-C12:0, respectively, from the major species of 1617 daltons. If the reducing glucosamine actually consists of two components differing in the acyloxyacyl fatty acid (2-OH-C12:0 in one case, C12:0 in the other), two anionic fragments would be predicted to arise from cleavage of the glycosidic bond. Two fragments (M + NH₄⁺ − 2H)⁺ were

³ P. Lartey, D. Riley, R. Hallas, W. Rosenbrook, Jr., D. Norbeck, D. Gramporinik, W. Kohlenrenner, N. Wideburg, and A. Perret, manuscript in preparation.
Fig. 2. Analysis of fatty acids contained in the drug-induced metabolite. Cells were grown and treated with compound III as described in Fig. 1. At the time of drug addition, [3H]acetate was added to 20 μCi/ml. After 3 h, cells were harvested and extracted as described in Fig. 1. The major new metabolite (fraction 13 of Fig. 1) was eluted from the silica gel plate using chloroform/methanol/H2O (63:33:4). Following evaporation to dryness, fatty acids were released by acid hydrolysis (A), or base hydrolysis (B), extracted into hexane, and analyzed by HPLC on a C18 column. Radioactivity in the column effluent was monitored using a Radiomatics flow detector.

Fig. 3. Purification of the major new metabolite. Cells were grown as described in Fig. 1, and treated with compound III to induce accumulation of the new metabolite. Following delipidation, the new metabolite was extracted into chloroform and then transferred into 90% phenol/methanol (1:2) which was applied to a column of DEAE-cellulose equilibrated with methanol containing 1% (v/v) acetic acid. Material was eluted with a linear gradient of 0–1 M ammonium acetate in equilibration solvent. Fractions were collected and monitored for radioactivity (C) and ammonium acetate concentration (Δ). Material eluting between 0.2–0.3 M ammonium acetate was collected for further analysis.

observed at m/z 826 and 842, which is consistent with the above hypothesis. These data are similar to those reported for lipid A of Chromobacterium violaeceum (22). The identity of the fatty acids (2-OH-C12:0, 3-OH-C12:0, C12:0, and 3-OH-C10:0) were confirmed by tandem gas chromatography/fast atom bombardment mass spectroscopy (Table III). Mass molecular ions [M + NH₄⁺]⁺ at m/z 220, 232, and 248 corresponded to fatty acid methyl esters of 3-OH-C10:0, C12:0, and 2-OH-C12:0 or 3-OH-C12:0. Electron impact fragments [M + H⁺]⁺ at m/z 103 indicative of 3-OH groups were observed with gas chromatography peaks corresponding to 3-OH-C10:0 and 3-OH-C12:0 methyl esters. The peak corresponding to C12:0 methyl ester produced m/z 74 and m/z 87 indicative of aliphatic methyl esters. Finally, the peak representing 2-OH-C12:0 methyl ester produced m/z 171 [CH₃(CH₂)₉-COH + OH] and m/z 90 [HO-CH = COH-OCH₃]⁺.

Control experiments demonstrated that the new phosphorylated and acylated glucosamine metabolites were only detectable following treatment with III. No fatty acids derived from lipid A were detectable by direct gas chromatographic analysis of chloroform extracts from control cells, whereas 12:0, 3-OH-10:0, 3-OH-12:0, and 2-OH-12:0 were all present from analogous extracts of drug-treated cells (data not shown). A second series of control experiments used radiola-
beling of control cells to high specific activity with [3H]acetate (10 μCi/ml) followed by extraction chromatography on Silica Gel H, and analysis of the chromatographic regions between the position of IV, and phospholipid. No 12:0, 3-OH-10:0, 3-OH-12:0, or 2-OH-12:0 fatty acids were detected by HPLC analysis (data not shown). These control experiments show that the new lipid A derivative only appears following treatment with III, and that it is not a cleavage product of mature LPS generated during extraction.

The New Lipid A Derivative Accumulates in the Inner Membrane and Is a Precursor to LPS—The new lipid A derivative accumulated predominantly in the inner membrane following pulse labeling with [3H]N-acetylglucosamine (Fig. 5, A and B), with only 5-10% being found in the outer membrane fractions. The membrane fractions observed were nearly identical to those previously reported (17), consisting of inner membrane, M band, and outer membrane. Although we obtained slight fractionation of the outer membrane into subfractions (Fig. 5A), there was no significant difference in the small amounts of lipid A derivative present in the two outer membrane fractions analyzed. In addition, less than 5% of the pulsed radiolabel was present in the M band, and it was thus not investigated further. These data are consistent with the hypothesis that the new lipid A derivative is assembled in the inner membrane, but is not capable of rapid translocation to the outer membrane. This observation is similar to the results reported for S. typhimurium (24) and suggests that, in both Salmonella and Pseudomonas, preassembled lipid A is not capable of rapid translocation to the outer membrane. If true, removal of drug should allow addition of lipid A to complete LPS. Following removal of drug, 50-80% of the pulsed radioactivity present in the new lipid A derivative chased into LPS (Table IV), with a concurrent decrease of the lipid A derivative from the inner membrane (Fig. 5B). These data show that the new lipid A derivative is a precursor to LPS.

Analysis of Lipid A Precursor from Enteric Gram-negative Bacteria—New glucosamine metabolites accumulated (Fig. 6, A-D) when Serratia, Providencia, Citrobacter, and Enterobac-

**FIG. 5.** Cellular location of the major new lipid A derivative. Cells (P. aeruginosa K799) were grown in defined medium at 37 °C and treated with compound III (50 μg/ml) at time 0. After 5 min, cells were pulse-labeled with 21 μCi/ml of [3H]N-acetylglucosamine for 15 min. The culture was rapidly chilled in an ice-water bath, washed twice by centrifugation, and resuspended in fresh medium lacking drug and radiolabel. Samples were taken at the end of the pulse and at 30 and 60 min following initiation of the chase. outer and inner membranes were isolated by sucrose density gradient centrifugation following lysis using a French press; IM, inner membrane; M, middle band; OM1 and OMII, subfractions of the outer membrane. A, outer and inner membranes were isolated by sucrose density gradient centrifugation following lysis using a French press; IM, inner membrane; M, middle band; OM1 and OMII, subfractions of the outer membrane. B, inner membranes from the pulse and 60-min chase sample were dialyzed against H2O, lyophilized, and glycolipids were extracted into chloroform for analysis by thin layer chromatography on Silica Gel H. pulse sample; —, 60-min chase sample.

**TABLE IV**

| Condition  | Precursor | LPS |
|------------|-----------|-----|
| Pulse      | 4302      | 28342 |
| Chase, 30 min | 873 (-3429)* | 31566 (+3224)* |
| Chase, 60 min | 847 (-3455)* | 31758 (+3416)* |

* Net loss of counts/min in lipid A precursor.
* Net increase of counts per min in LPS.

**FIG. 6.** Analysis of new metabolites in various Gram-negative bacteria. Cells were grown in defined medium at 37 °C to an A660 value of 0.2-0.3. Compound IV (50 μg/ml) and [3H]N-acetylglucosamine (4 μCi/ml) were added simultaneously, followed by incubation at 37 °C for 3 h when growth stasis occurred. Glycolipids were extracted into chloroform and analyzed by chromatography on Silica Gel H. A, A. P. stuartii; B, Citrobacter sp.; C, E. aerogenes or E. cloacae; D, S. marcescens. The arabic numerals 2, 3, and 4 over peaks of radioactivity show the relative position of lipid A derivatives III, IIa, and IVa, respectively, isolated from S. typhimurium strains RY 106 or 109 incubated at the restrictive temperature (see text).
ster species were treated with IV (II amide-linked to the carboxyl terminus of alanylalanine). In each case, the major component co-migrated with lipid A precursor species IVs prepared from S. typhimurium or E. coli. The structure of IVs is O-(2-amino-2-deoxy-β-D-glucopyranosyl)-(1→6)-(2-deoxy-α-D-glucose, acylated at positions 2, 3, 2', and 3' with 3-OH-C14:0 groups, and bearing phosphate at positions 1 and 4'. (5): Species IIIa of S. typhimurium is a derivative of IVs which contains phosphoethanolamine attached to the 4'-phosphate. Species IA is identical to IVs, except for attachment of phosphoethanolamine to the 4'-phosphate and attachment of aminopentose to the 1-phosphate. Species IIa is identical to IVs except for attachment of aminopentose to the 1-phosphate. The minor components from Serratia and Providencia comigrated with species IIa of S. typhimurium, while those from E. coli, Enterobacter, and Citrobacter comigrated with species IIIa from S. typhimurium. Both the major and minor components were radiolabeled with [32P]phosphate and [3H]acetate. The major components contained 3-OH-C14:0 as the only fatty acid (Fig. 7, A and B). The major components in each case are likely to have the same structure as species IVs from S. typhimurium, while the minor components probably reflect metabolites of the major component which only appear when the pathway is inhibited.2

Conclusions—Our previous knowledge of the early stages of LPS assembly was restricted to a select set of E. coli and S. typhimurium laboratory strains. We have shown that although the early stages in LPS assembly are similar in diverse species of Gram-negative bacteria, there are significant differences which become apparent when the pathway is inhibited. The complexity of lipid A precursor species, which accumulate in S. typhimurium upon inhibition of the KDO pathway, is thus far unique. A major component apparently identical to IVs from S. typhimurium does not appear in other enteric Gram-negative species, however, only one other minor, more polar precursor species appeared. Species IVs accumulate first and to the greatest extent when S. typhimurium is treated with IVs, indicating that it is the normal in vivo acceptor of KDO. The much slower and less extensive accumulation of IA, IIa, and IIIa may thus reflect aberrant reactions which only occur when IVs has accumulated due to inhibition of KDO addition. Kinetic analysis of appearance of the two lipid A derivatives in E. coli was similar in that IVs appeared first and to the greatest extent. Since the component comigrating with IVs was the most abundant in all enteric species examined, the appearance of minor species likely reflects formation of metabolic side products due to inhibition of the normal pathway. The single species of nonenteric Gram-negative bacteria examined, two strains of P. aeruginosa, revealed a significantly difference compared to enteric organisms. The major precursor species accumulated was fully acylated, in contrast to the presence of only hydroxy fatty acids attached to the major precursor species of enteric species. These data are consistent with the following hypothesis. In P. aeruginosa, UDP-GlCNac is acylated at the 3 position with 3-OH-C14:0, at the 2-position (after deacylation) with 3-OH-C12:0, and finally the acyloxacyl fatty acid (2-OH-C12:0 or C12:0) is added to the 2-position 3-OH-C12:0 group. This would allow the observed heterogeneity in the structure of the nonreducing Gln. Hydrolysis of a portion of the derivatized Gln from UDP, yielding a source of the reducing Gln analogous to reactions in E. coli (1) would allow the observed heterogeneity in the reducing Gln. Alternatively, heterogeneity could arise following initial assembly of tetra-acyl-dissacharide 1-4'-bisphosphate, with subsequent addition of either C12:0 or 2-OH-C12:0 to both the reducing and nonreducing Gln residue. These differences likely reflect organism-specific pathways for the addition of fatty acids and KDO during the assembly of lipid A.

REFERENCES
1. Anderson, M. S., Bulawa, C. E., and Raetz, C. R. H. (1985) J. Biol. Chem. 260, 15536-15541
2. Bulawa, C. E., and Raetz, C. R. H. (1984) J. Biol. Chem. 259, 4846-4851
3. Ray, B. L., Painter, G., and Raetz, C. R. H. (1984) J. Biol. Chem. 259, 4852-4859
4. Ray, B. L., and Raetz, C. R. H. (1987) J. Biol. Chem. 262, 1122-1126
5. Raetz, C. R. H., Purcell, S., Meyer, M. V., Qureshi, N., and Takayama, K. (1985) J. Biol. Chem. 260, 16080-16088
6. Muson, R. S., Jr., Rasmussen, N. S., and Osborn, M. J. (1978) J. Biol. Chem. 253, 1503-1510
7. Cañohijano, J. O., Darveau, R. P., Goldman, R. C., Larley, P. A., and Pernet, A. G. (1987) J. Bacteriol. 169, 4030-4035
8. Shaw, D. H., Squires, M. J., Ishiguro, E. E., and Trust, T. J. (1986) Eur. J. Biochem. 161, 309-313
9. Strittmatter, W., Weckesser, J., Salimath, P. V., and Galanos, C. (1985) J. Bacteriol. 155, 153-158
10. Goldman, R. C., Kohlbrenner, W. F., Larley, P., and Pernet, A. G. (1987) Nature 328, 162-164
11. Neidhardt, F. C., Bloch, P. L., and Smith, D. F. (1974) J. Bacteriol. 119, 736-747
12. Kropinski, A. M., Kurio, J., Angus, B. L., and Hancock, R. E. W. (1982) Antimicrob. Agents Chemother. 21, 310-319
13. Rick, P. D., Fung, I., Wu, H., Osborn, M. J., and Pernet, A. G. (1987) J. Biol. Chem. 252, 4904-4912
14. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911-917
15. Nishijima, M., and Raetz, C. R. H. (1979) J. Biol. Chem. 254, 7837-7844
Lipid A Precursor from *P. aeruginosa*

16. Laemmli, U. K. (1970) Nature 227, 680–685
17. Hancock, R. E. W., and Nikaido, H. (1970) *J. Bacteriol.* 136, 381–390
18. Levvy, G. A., and McAllan, A. (1959) *Biochemistry* 73, 127–132
19. Reissig, J. L., Strominger, J. L., and LeLoir, L. F. (1955) *J. Biol. Chem.* 217, 959–966
20. Ames, B. N. (1966) *Methods Enzymol.* 8, 115–118
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
22. Wollenweber, H., Seydel, V., Lindner, B., Luderitz, O., and Rietschel, E. T. (1984) *Eur. J. Biochem.* 145, 265–272
23. Rietschel, E. T. (1976) *Eur. J. Biochem.* 64, 423–428
24. Osborn, M. J., Rick, P. D., and Rasmussen, N. S. (1980) *J. Biol. Chem.* 255, 4246–4251