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DESIGN, SYNTHESIS AND BIOLOGICAL PROPERTIES OF STRUCTURALLY NON-RELATED ENDOTOXIN INHIBITORS

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Abstract: In order to test the role of the supramolecular structure of lipopolysaccharides (LPS) in determining endotoxicity, two synthetic inhibitors, EI1 and EI2, which are structurally unrelated to lipid A, were designed, synthesized and examined for their ability to inhibit the biological activities of LPS. Both molecules were capable of inhibiting the activity of lipopolysaccharides in standard assays. The syntheses and aspects of the biological activities are described here.

Lipopolysaccharides (LPS) or endotoxins are complex lipid-linked carbohydrate molecules which reside in the outer membranes of gram negative bacteria. LPS contains three major structural domains. These are the lipid A (lipid-linked anchor region), R-core (short oligosaccharide region) and O-antigen chain (repeated polysaccharide region). One of these three components, the lipid A (Figure-1) which in the *Enterobacteriaceae* is usually a glucosamine disaccharide bearing two phosphate groups and six fatty acid chains, is now known to be responsible for most biological activities (fever and lethal shock in higher animals) of LPS.[1] Although the biomechanism of lipid A at the molecular level has sparked a lot of interest, what structural aspects of lipid A are responsible for its biological activities is still not well understood.

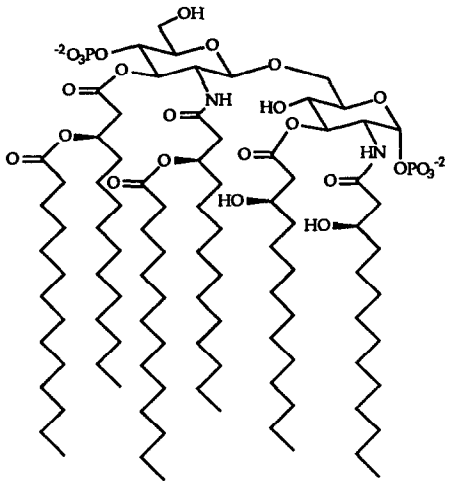


Figure-1

Lipid A of *E. coli*.

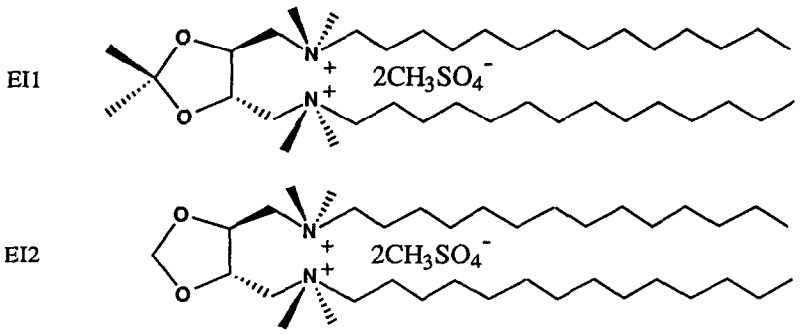
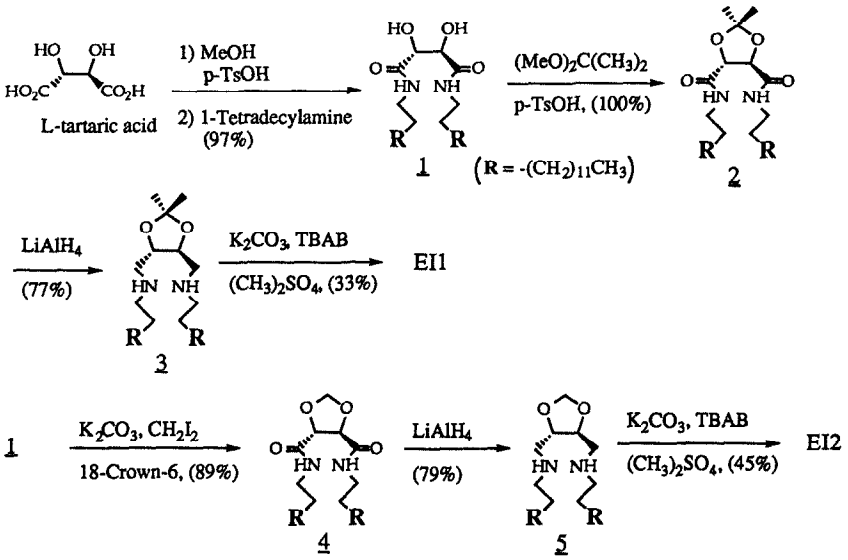


Figure-2
Structures of inhibitors



Scheme-1
Syntheses of EI1 and EI2

Most work to date[2] has focused on finding out what the structural requirements for endotoxicity are by removing structural components from lipid A and then comparing its biological activities with that of the parent

lipid A molecule. Such studies give only limited information about the molecular basis for endotoxicity since the supramolecular structure or packing arrangement of LPS might also be a determinant. It thus became necessary to design molecules which would interact with the LPS aggregates at the level of the head group and the fatty acid chains. We therefore designed two molecules, EI1 and EI2 (Figure-2) which have dense positive charges to interact with the negatively charged head group of lipid A, and tested their biological activities as inhibitors of LPS activity. We envisioned that the charged head groups function to maintain the supramolecular structure of lipid A and to initiate binding of lipid A to host cell membranes. Molecules, such as EI1 and EI2, with bulky charged centers and long alkyl chains would alter the lipid A or LPS counter ion arrangement and rearrange its packing structure thus affecting biological activity. They have no carbohydrate groups and, therefore, can not possibly function by blocking LPS binding sites.

EI1 was prepared from L-tartaric acid according to Scheme-1. Tartaric acid diamide 1[3] was prepared in two steps from dimethyltartrate. Although the one step coupling of L-tartaric acid with 1-decylamine was attempted with DCC, ammonolysis of the diester turned out to be the best way because of the easier work up. No further purification was needed. Isopropylidene acetal 2 was prepared in excellent yield[4] and used in the next step without purification. Reduction of 2 was performed by lithium aluminium hydride to give diamine 3.[5] Methylation of 3 was performed in heterogeneous solution to give EI1 in poor yield (33%),[6] but, because of the isolation procedure, the purity of the product was so high that no further isolation or purification step was needed after washing the reaction mixture once with water. Methylene acetal 4[7] could not be prepared by any acid catalyzed acetalization method (e.g. formalin-HCl, paraformaldehyde-HCl, $CH_2(OMe)_2$-(p-TsOH), $CH_2(OMe)_2$-BF_3, $CH_2(OMe)_2$-P_2O_5). Base catalyzed acetalization of 3 with CH_2I_2-K_2CO_3 gave 4. Diamine 5[8] was prepared by the same procedure as that of 3. EI2[9] was then prepared by the same procedure as that of EI1 from 5.

The biological tests of EI1 and EI2 were very informative. Both completely inhibited the endotoxin activity of LPS in the LAL (*Limulus* amoebocyte lysate) assay.[10,11] The LAL assay is an endotoxin-induced coagulation reaction of the blood of the horseshoe crab, *Limulus polyphemus*. It is a rapid method widely used for endotoxin detection. We utilized a chromogenic assay that relies upon the ability of endotoxin to activate a serine protease, found in the lysate, which will cleave p-nitroaniline conjugated to a peptide substrate. This results in chromophore release, with the amount of chromophore proportional to the amount of endotoxin present. In this assay, at a concentration of 75 μg/ml, both molecules completely inhibited the LAL response normally produced by two endotoxin units of *E. coli* LPS. Even though the inhibitors, EI1 and EI2 inhibited the LAL response, they did not block binding of LPS to the host cell in fluorescence microscopy analyses. We wanted to determine the ability of both EI1 and EI2 to prevent binding of fluorescently labeled LPS from *Salmonella typhimurium* to the cell line RAW264.7, a murine, Abelson leukemia virus transformed macrophage.[12] The binding(observed by fluorescence microscopy) to the RAW264.7 cells actually increased in the presence of both EI1 and EI2. This clearly indicated that these substances facilitated the incorporation of the lipopolysaccharide into the cell membranes ruling out the possibility that simple electrostatic blocking was taking place. A change of supramolecular structure is therefore suggested.

This strategy of destroying the supramolecular structure of LPS will become more prominent in the future since it has recently been demonstrated that LPS activity is correlated with or determined by the symmetry of the array structures which it forms.[13]

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3. N,N'-bis-tetradecyl-(2R,3R)-tartaric acid diamide (1): To a solution of L-tartaric acid (7.0g, 46.6 mmole) in methanol (50 ml) were added p-TsOH (0.2g) and anhydrous magnesium sulfate (5g). After stirring at room temperature for 18 hours, sodium bicarbonate (1.0g) was added to the reaction mixture. All precipitate was filtered away using celite and the filtrate was concentrated under reduced pressure. The residue was redissolved and passed through a short silica gel column (CH_2Cl_2-MeOH : 20-1), the suspension was concentrated and the concentrate was treated with 1-tetradecylamine (24g, 0.11 mole) and MeOH (150 ml) at 70°C for 20 hours. After cooling the reaction mixture to room temperature, the precipitated product was collected by filtration. The filter cake was washed twice with MeOH (50 ml) and dried *in vacuo*. Yield ; 25.3g (97%). TLC ; R_F = 0.32 (CH_2Cl_2-MeOH : 20-1). (For NMR analysis, the product was acetylated in acetic anhydride and pyridine because of its poor solubility in the usual NMR solvents. The diacetate of the product was then analyzed by NMR spectroscopy.) ^{1}H-NMR (300 MHz, $CDCl_3$); δ 0.85(6H, t, J = 7 Hz, CH_3), 1.20-1.48(48H, m, CH_2), 2.13(6H, s, CH_3), 3.10-3.40(4H, m, NCH_2), 5.56(2H, s, CH). ^{13}C-NMR (75.5 MHz, $CDCl_3$); δ 39.8($NHCH_2$), 72.2(CH), 166.1(C-carbonyl), 169.5(C-carbonyl).
4. N,N'-bis-tetradecyl-2,3-O-isopropylidene-(2R,3R)-tartaric acid diamide (2): To a suspension of 1 (16g, 28.6 mmole) in 2,2-dimethoxypropane (150 ml) was added p-TsOH (0.2g). After stirring at room temperature for 4 hours, 50 ml of the solvent was removed from the reaction mixture by fractional distillation, and the residual solution was cooled to room temperature and treated with anhydrous potassium carbonate (1.0g). After evaporating all 2,2-dimethoxypropane from the reaction mixture, the residue was treated with distilled water (100 ml) and the precipitate formed was collected by filtration. The filter cake was dissolved in ether (200 ml) and the resultant ethereal solution was filtered through celite to remove a small amount of precipitate in the

solution. The filtrate was dried over anhydrous magnesium sulfate and concentrated. The residue was used for the next reaction without further purification. Yield ; 17.6g (100%). TLC ; R_F = 0.50 (CH_2Cl_2-MeOH : 40-1). ^{1}H-NMR (300 MHz, $CDCl_3$); δ 0.85(6H, t, J = 6 Hz, CH_3), 1.21-1.55(48H, m, CH_2), 1.47(6H, s, $C(CH_3)_2$), 3.27(4H, td, J = 6 and 6 Hz, NCH_2), 4.48(2H, s, CH).

5. N,N'-bis-tetradecyl-1,4-diamino-(2S,3S)-dihydroxybutane isopropylidene acetal (3): To a solution of 2 (9.4g, 15.6 mmole) in dry ether (50 ml) was added lithium aluminium hydride (2.0g, 52.6 mmole). After refluxing for 16 hours, ethylacetate (3 ml) was added to the reaction mixture to quench the reaction. The reaction mixture was treated with additional ether (400 ml) and 20% aqueous NaOH solution (200 ml) at room temperature for 20 hours. The organic layer was isolated and dried over anhydrous magnesium sulfate, filtered and the filtrate concentrated. The concentrate was treated with pet. ether (200 ml). The precipitate formed was removed by filtration and the filtrate was concentrated. The concentrate was subjected to flash column chromatography (CH_2Cl_2-MeOH-$(Et)_2NH$: 40-2-1). Yield ; 6.9g (77%). TLC ; R_F = 0.20 (CH_2Cl_2-MeOH-$(Et)_2NH$: 40-2-1). ^{1}H-NMR (300 MHz, $CDCl_3$); δ 0.85(6H, t, J = 7 Hz, CH_3), 1.20-1.50(48H, m, CH_2), 1.36(6H, s, CH_3), 2.61(4H, t, J = 6 Hz, NCH_2), 2.73(2H, dd, J = 10 and 4 Hz, CH_2N), 2.82(2H, dd, J = 10 and 6 Hz, CH_2N), 3.82-3.90(2H, m, CH). ^{13}C-NMR (75.5 MHz, $CDCl_3$); δ 50.0(CH_2NH), 51.9(CH_2NH), 79.0(CH), 109.0(OCO).
6. N,N'-bis-tetradecyl-1,4-dimethylamino-(2S,3S)-dihydroxybutane isopropylidene acetal di-methylsulfate salt (EI1 $2CH_3SO_4^-$): To a solution of 3 (8.7g, 15.0 mmole) in dichloromethane (40 ml) were added dimethyl sulfate (7.2g, 57.1 mmole), anhydrous potassium carbonate (9.46g, 68.6 mmole) and tetrabutylammonium bromide (0.1g). After stirring at room temperature for 18 hours, the reaction mixture was treated with distilled water (100 ml) for 18 hours. The organic layer was isolated and dried over anhydrous magnesium sulfate. After filtration, the filtrate was concentrated and the residue was dried *in vacuo*. Yield ; 4.2g (33%). ^{1}H-NMR (300 MHz, $CDCl_3$); δ 0.80(6H, t, J = 7 Hz, CH_3), 1.15-1.30(44H, m, CH_2), 1.40(6H, s, CH_3), 1.60-1.80(4H, m, CH_2), 3.16(6H, s, NCH_3), 3.18(6H, s, NCH_3), 3.30-3.40(4H, m, NCH_2), 3.60(6H, s, $CH_3SO_4^-$), 3.77(2H, dd, J = 13 and 7 Hz, CH_2N), 3.98(2H, dd, J = 13 and 1 Hz, CH_2N), 4.42(2H, m, CH). ^{13}C-NMR (75.5 MHz, $CDCl_3$); δ 51.5, 54.2, 58.5, 64.2, 66.0, 72.6(CH), 112.8(OCO).
7. N,N'-bis-tetradecyl-2,3-O-methylene-(2R,3R)-tartaric acid diamide (4): To a suspension of 1 (1.32g, 2.35 mmole) in dichloromethane (50 ml) were added anhydrous potassium carbonate (2.2g, 16 mmole) and 18-crown-6 (0.27g, 1.0 mmole). To this solution was added diiodomethane (1.5g, 5.6 mmole) at room temperature. The reaction mixture was refluxed for 18 hours. When the reaction was complete, the reaction mixture was cooled to room temperature and treated with distilled water (50 ml). The organic layer was isolated and dried over anhydrous magnesium sulfate. After filtration, the filtrate was concentrated and the residue was subjected to flash column chromatography. Yield ; 1.2g (89%). TLC ; R_F = 0.50 (CH_2Cl_2-MeOH : 30-1). ^{1}H-NMR (300 MHz, $CDCl_3$); δ 0.85(6H, t, J = 6 Hz, CH_3), 1.20-1.55(48H, m, CH_2), 3.26(4H,m, NCH_2), 4.44(2H, s, CH), 5.11(2H, s, OCH_2O). ^{13}C-NMR (75.5 MHz, $CDCl_3$); δ 77.5(CH), 96.1(OCH_2O), 169.0(C-carbonyl).
8. N,N'-bis-tetradecyl-1,4-diamino-(2S,3S)-dihydroxybutane methylene acetal (5): The procedure for the preparation of 5 was the same as that employed for the preparation of 3. Yield ; 79%. TLC ; R_F = 0.20 (CH_2Cl_2-MeOH-$(Et)_2NH$: 40-2-1). ^{1}H-NMR (300 MHz, $CDCl_3$); δ 0.85(6H, t, J = 6 Hz, CH_3), 1.20-1.55(48H, m, CH_2), 2.62(4H, t, J = 6 Hz, NCH_2), 2.75(2H, dd, J = 12 and 3 Hz, CH_2N), 2.83(2H,

dd, J = 12 and 8 Hz, CH_2N), 3.82(2H, m, CH), 4.99(2H, s, OCH_2O). ^{13}C-NMR (75.5 MHz, $CDCl_3$); δ 49.8(CH_2NH), 51.0(CH_2NH), 78.5(CH), 94.0(OCO).

9. N,N'-bis-tetradecyl-1,4-dimethylamino-(2S,3S)-dihydroxybutane methylene acetal di-methylsulfate salt (EI2 $2CH_3SO_4^-$): The procedure for the preparation of EI2 was the same as that employed for the preparation of EI1. Yield ; 45%. ^1H-NMR (300 MHz, $CDCl_3$); δ 0.85(6H, t, J = 7 Hz, CH_3), 1.20-1.35(44H, m, CH_2), 1.65-1.83(4H, m, CH_2), 3.23(6H, s, NCH_3), 3.25(6H, s, NCH_3), 3.42(4H, t, J = 6 Hz, NCH_2), 3.68(6H, s, $CH_3SO_4^-$), 3.85(2H, dd, J = 12 and 7 Hz, CH_2N), 4.15(2H, dd, J = 12 and 1 Hz, CH_2N), 4.49(2H, m, CH), 5.15(2H, s, OCH_2O). ^{13}C-NMR (75.5 MHz, $CDCl_3$); δ 51.2, 51.3, 54.5, 64.0, 66.5, 73.0(CH), 112.8(OCO).
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11. LAL assay: All tests to screen for the presence of endotoxin were performed using the Whittaker Bioproducts QCL-1000 Quantitative Chromogenic LAL, Whittaker Bioproducts, Inc.(Walkersville, MD). All assays were run concurrently using *E. coli* 0111:B4 endotoxin as a reference standard. The total volume of reagents used per assay was 0.3 ml. Endotoxin inhibitors were incubated at a concentration of 25 μg/assay with *E. coli* 0111:B4 endotoxin at a concentration of 5.88×10^{-3} ng/assay, at room temperature for 30 min. prior to assaying. The assay was performed following the test kit instructions.
12. The effect of the interaction of EI1 or EI2 with LPS was studied by determining whether these substances blocked the binding of fluorescent-labeled LPS to cells from an immune cell line known to be activatable by LPS. The binding(observed by fluorescence microscopy) to the RAW264.7 cells(ATCC TIB71; W.C. Raschke, S. Baird, P. Ralph and I. Nakoinz,*Cell*, **1978**, *15*, 261.) actually increased in the presence of EI1 and EI2. The EIs were mixed with the labeled LPS prior to having contact with host cells. In the LAL assay system, the inhibitors were used at a concentration of 10^4 ng per assay, compared to *E. coli* LPS which was used at 10^{-3} ng per assay. Due to this large difference in concentration between endotoxin and EIs, and the concentration of LPS-dye conjugate that must be used in order to view LPS microscopically(5.5 mg/ml), we chose to use EIs at concentrations of 55 mg/ml, 5.5 mg/ml, and 0.55 mg/ml in the binding assays.
Cells labeled in the presence of EI1 and EI2 had increased dye uptake at 55 mg/ml EIs. Concentrations of EIs used at concentrations equivalent to LPS had little effect on prevention of binding. At one tenth the endotoxin concentration there was decreased binding.
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