Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 357–360

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Sequestration of Bacterial Lipopolysaccharide by Bis(Args) Gemini Compounds

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Received 7 May 2001; accepted 7 November 2001

Abstract—Gemini compounds of the type N^{α},N^{ω}-bis(N^{α}-lauroyl arginine)α,ω-alkylenediamides or bis(Args) bind bacterial lipopolysaccharide and neutralize endotoxic activity in in vitro tumor necrosis factor-α and nitric oxide release assays. Sequestration of lipopolysaccharide results in protection in a murine model of endotoxemia. However, the bis(Args) compounds are cytotoxic by virtue of being highly membrane-active. The development of less surface-active analogues may yield potentially therapeutically useful compounds for the treatment of Gram-negative sepsis.

Introduction

Gram-negative sepsis is a serious and common clinical problem, and is the primary cause of mortality in the intensive care unit worldwide[1] accounting for some 200,000 fatalities in the US annually.[2] Despite tremendous strides in antibacterial chemotherapy, mortality due to septic shock has essentially remained unchanged at about 45%,[3] reflecting the absence of specific therapy aimed at the underlying pathogenetic mechanisms. The primary trigger in the Gram-negative septic shock syndrome is endotoxin, a constituent of the outer membrane of all Gram-negative bacteria. Endotoxins [or lipopolysaccharides (LPS)] consist of a polysaccharide portion and a glycolipid called lipid A. The structurally highly conserved lipid A[4] is the toxic moiety of LPS,[5,6] and sequestration of LPS by molecules designed to bind lipid A is a logical and attractive target for drug development.

The anionic amphiphilic nature of lipid A enables it to interact with a variety of cationic hydrophobic ligands.[7–9] In our ongoing efforts to develop small molecules that would specifically bind lipid A, we have converged on linear cationic amphipathic molecules possessing terminal, protonatable cationic groups positioned so as to be able to simultaneously interact with the glycosidic phosphates on lipid A,[10,11] as well as appropriately positioned apolar moieties to enable hydrophobic interactions with the polyacyl domain of lipid A. Noteworthy examples are homologated spermine **1**[12] and DOSPER **2**, a dialkyl-polyamine conjugate.[13] We now report the LPS-binding and -neutralizing activities of a new class of cationic amphipathic compounds of the *gemini* type derived from the arginine, the N^{α},N^{ω}-bis(N^{α}-lauroyl arginine)α,ω-alkylenediamides or bis(Args), $C_6(LA)_2$, **3** and $C_9(LA)_2$, **4**.[14] The compound N^{α}-lauroyl arginine methyl ester (LAM), **5** is the single chain counterpart of **3** and **4**.[15] These compounds provide useful leads for the rational design of nontoxic, and yet effective endotoxin sequestrants.

Results and Discussion

Efficient synthesis of **3** and **4** using BOP (benzotriazole-1-yloxytris(dimethylamino)phosphonium hexafluophosphate reagent in the presence of Dabco (1,4-diazabicyclo[2.2.2]octane) as activating base allowed the simultaneous condensation of N^{α}-lauroyl-L-nitroarginine[16] to both primary amino groups of α,ω-alkylenediamine in dry CH_2Cl_2 permitting average yields of 90%.[14] Dabco and BOP impurities were eliminated by washing with diethyl ether, and protected N^{α},N^{ω}-bis (N^{α}-lauroyl-L-nitroarginine) α ω-alkylenediamide compounds were obtained with a purity of >99% by several recrystallizations from hot methanol or from a mixture of formic acid and water. Catalytic hydrogenation was

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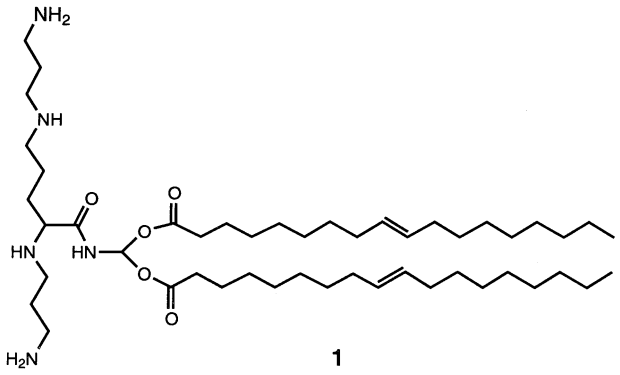
0960-894X/02/$ - see front matter
PII: S0960-894X(01)00749-1



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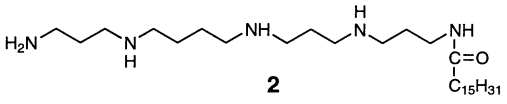

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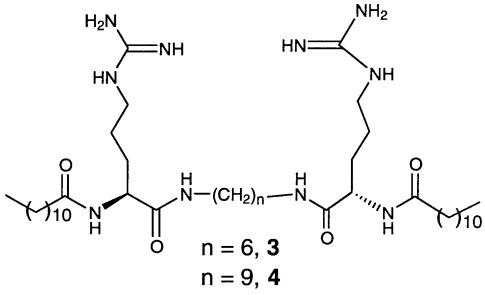

n = 6, 3
n = 9, 4

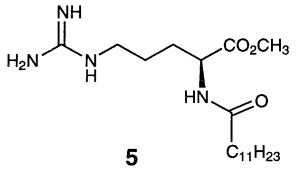

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Table 1. Protective effect of 4 in D-galactosamine-sensitized mice challenged with LPS

LPS CHALLENGE (ng/mouse)	Bis(Args) **4** dose (μg/mouse)		
	0	10	40
0	—	0/5	0/5
20	5/5	7/10	0/10
40	5/5	9/10	6/10

Female, outbred, CF-1 mice (22–28 g) were injected intraperitoneally with 800 mg/kg D-galactosamine and 20 ng LPS, followed by a separate injection of **4** in sterile saline. Lethality was scored at 24 h. Numbers denote dead/total.

performed in MeOH/formic acid mixture, and deprotected **3** and **4** compounds were re-purified by preparative column chromatography. The purity of compounds was verified by capillary electrophoresis, FAB-EM, and NMR.[14] The single chain compound **5**[15] was used as a non-gemini control for **3** and **4**.

We were interested in evaluating **3** and **4** for endotoxin-sequestering activity since the highly basic, protonatable bis-guanidinium functionalities separated by a polymethylene spacer provide for excellent recognition of the bis-phosphates on the lipid A glycosidic backbone[11] while the terminally placed alkyl chains permit apposition to the polyacyl hydrophobic domain of lipid A without the steric hindrance anticipated in molecules such as DOSPER.[12,13] The availability of the monomeric compound **5** afforded confirmation of our observations that two cationic functions separated by an optimal distance of about 1.4 nm was required for high binding affinity and efficacy of neutralization of endotoxicity via simultaneous recognition of the lipid A phosphates.[13] Furthermore, the amide linkages of the acyl substituents are susceptible to hydrolytic cleavage,[14] and are thus favorable from a toxicological point of view.

Cells such as macrophages respond to the presence of LPS by producing proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and nitric oxide (NO), which act in concert to mediate tissue damage, resulting in the shock state.[17] Sequestration of LPS results in attenuated cellular activation, and can therefore be indirectly measured by inhibition of TNF-α and NO secretion.[10,11,13] The inhibition of proinflammatory mediator production by **3** and **4** was measured murine macrophage-like cell line J774.A1. Monomeric **5** served as a control for the gemini compounds while polymyxin B (PMB), a cyclic decapeptide antibiotic[18] know to inhibit endotoxicity[19–21] and **1** were reference compounds. TNF-α was assayed by specific ELISA (Fig. 1), and NO was measured as nitrite using the Griess assay (Fig. 2).[12,13] It is clear that potency of LPS neutralization in both assays is in the order PMB > **1** > **4** > **3** > > **5**.

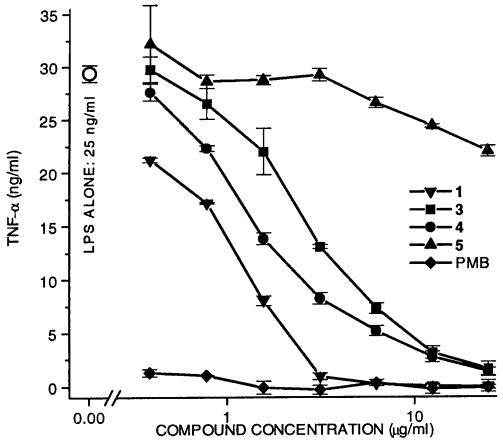

Figure 1. Inhibition of TNF-α production by J774.A1 cells stimulated for 18 h with *E. coli* O111:B4 LPS (25 ng/mL) in the presence of graded doses of compounds. TNF-α was measured by ELISA. Data points represent mean ± SD determined on quadruplicate samples of a single, representative experiment.

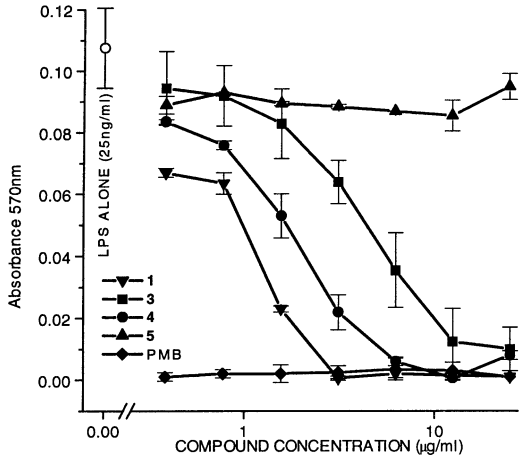

Figure 2. Inhibition of NO production by J774.A1 cells stimulated for 18 h with *E. coli* O111:B4 LPS (25 ng/mL) in the presence of graded doses of compounds. NO was measured as nitrite using Griess reagent. Data points represent mean ± SD determined on quadruplicate samples of a single, representative experiment.

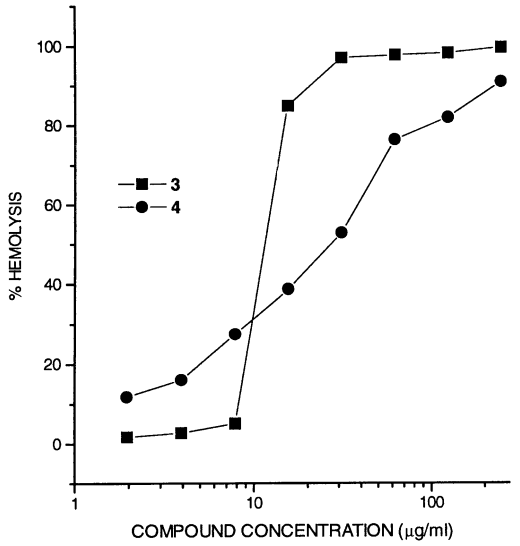

Figure 3. Hemolytic activity of **3** and **4**. O-positive, washed erythrocytes were suspended in 0.9% saline, final hematocrit and exposed to graded concentrations of compounds. Hemolysis was measured by absorbance at 578 nm and normalized to 100% hemolysis obtained by hypotonic lysis.[13]

The ED_{50} values for TNF-α inhibition for **1**, **3** and **4** are similar, being 0.9, 1.4, and 2.5 μg/mL, respectively, while that of 5 is indeterminately large (Fig. 1), values which are paralleled also in the NO inhibition assay (Fig. 2). We expected **4** to be less potent than **1**, and that of **3**, even less so, given that the nine (or six) carbon spacing is less than optimal for strong simultaneous coulombic interactions with lipid A phosphates.[12,13] Particularly noteworthy is that **5** is almost bereft of significant sequestration activity, emphasizing the obligatory requirement of two, optimally spaced cationic functions.[13] It is to be noted that the cytokine- and NO-inhibitory activities of **3** and **4** are clearly evident (Figs. 1 and 2, respectively) at concentrations well below that required to manifest in cytotoxicity; furthermore, that the inhibition of TNF-α and NO was not due to cytotoxicity was verified by cell viability assays following a 4 h exposure to **3** and **4** at concentrations ranging from 1 to 10 μg/mL (data not shown).

We evaluated **4** in a well-established murine model of endotoxic shock using outbred CF-1 mice sensitized to the lethal effects of LPS with D-galactosamine.[12] In this model, a challenge dose of 10 ng/mouse results in 100% lethality. Graded doses of 4 were administered by intraperitoneal injection to groups of animals concurrent with LPS challenge, the results of which are summarized in Table 1. At 40 μg/mouse, **4** is clearly protective even at four times the LD_{100} dose of LPS.

Although these results represent a significant step in our attempts to arrive at molecules that would be clinically useful, compounds such as **3** and **4**, are themselves unlikely to be of therapeutic value. The bis(Arg) compounds are gemini molecules with pronounced surface activity,[14–16] and are, indeed, cytotoxic in hemolysis assays (Fig. 3) and in other cytotoxicity assays (data not shown) by virtue of being membrane-active. However, this class of compounds offer an excellent point of departure with which to refine the design and development of specific, yet nontoxic LPS sequestrants. As mentioned before, the lability of the amide-linked hydrocarbon substituents is clearly preferable to *N*-alkylated compounds. As has been observed with gemini surfactants,[22,23] is likely that the replacement of the polymethylene spacer with a hydrophilic, H-bond donor rich spacer would considerably decrease surface activity and thereby reduce cytotoxicity.

Acknowledgement

This was supported by the MEC, project PPQ2000-1687-CO2-01.

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