Synthesis and Immunostimulating Properties of Lipophilic Ester and Ether Muramyl Peptide Derivatives

Ghada Merhi,[†] Anthony W. Coleman,[‡] Jean-Philippe Devissaguet,[†] and Gillian M. Barratt*,[†]

Laboratoire de Pharmacotechnie-Biopharmacie, URA CNRS 1218, Université de Paris-Sud, Faculté de Pharmacie, 5 rue Jean Baptiste Clément, 92296 Châtenay-Malabry, France, and Institut de Biologie et Chimie des Protéines, CNRS UPR 412, 7 passage du Vercors, 69367 Lyon Cedex 07, France

Macrophages can become cytotoxic toward tumor cells when activated by immunomodulators. Three different muramyl peptides were synthesized: one hydrolyzable lipophilic ester derivative (MTP-Chol) and two nonhydrolyzable lipophilic ether derivatives (MTP-octadecane and MTP-heptadecafluorooctadecane). Activation of the RAW 264.7 cell line was studied by measuring nitrite production as an indication of NO-synthase activity. The lipophilic ester derivative, incorporated within nanocapsules, was as active as free muramyl dipeptide, whereas the lipophilic ether derivatives were unable to activate macrophages. MTP-octadecane in micellar form was not capable of inducing macrophage cytotoxicity either. These results indicate that lipophilic muramyl peptides need to be hydrolyzed to yield a hydrosoluble metabolite in order to activate macrophages.

Introduction

Cancer immunotherapy with macrophage activators has been suggested as a possible treatment for drug-resistant metastases.[9] Muramyl peptides, which are low molecular weight derivatives of bacterial peptidoglycan,[1] would be useful immunomodulators if the drawbacks of poor penetration into macrophages[25] and rapid elimination[20] could be overcome. Therefore, over the past 15 years a great deal of research has been devoted to producing lipophilic analogs, such as muramyltripeptide−phosphatidylethanolamine (MTP-PE)[14] and muramyltripeptide-cholesterol (MTP-Chol)[21] and incorporating them into colloidal drug delivery systems, which can be readily captured by macrophages. Liposomes were the first system to be studied,[14,21] followed later by polymeric nanocapsules.[17,18,29]

Many studies have shown that encapsulation greatly increases the macrophage-activating potential of muramylpeptides *in vitro*[2,5,14,24] and renders them effective against metastases *in vivo*.[4,10,29] Lipophilic derivatives are more readily incorporated into and retained within drug delivery systems, leading to increased efficacy.[21,22] The site of action of muramyl peptides is believed to be intracellular, since a photoactivable derivative was able to specifically label a cytoplasmic protein of 40−44 kDa.[26] The activity of colloidal delivery systems could therefore be explained as follows: particulate carriers are taken up by macrophages by phagocytosis and concentrated in the lysosomal compartment. Here, the muramyl peptide derivatives are released from the carriers and metabolized to a water-soluble derivative which can diffuse into the cytoplasm. In order to test the second part of this hypothesis, we have synthesized two new muramyl peptides in which the sugar−tripeptide is linked to a lipophilic moiety by a stable, ether linkage and have determined their biological activity.

Various mechanisms for the interaction of encapsulated muramyl peptides may be considered and these
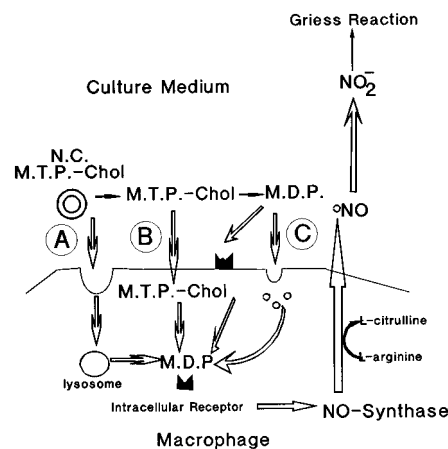

Figure 1. Possibles mechanisms of interaction between macrophages and MTP-Chol within nanocapsules.

are schematized in Figure 1. In order to test and define the actual mechanism of macrophage activation, the synthesis and biological testing of muramyl derivatives for which a specific pathway is blocked seems useful.

The active derivative used in our laboratory, *N*-(acetylmuramyl)-L-alanyl-D-isoglutamine-L-alanylcholesterol (MTP-Chol, Figure 2), has two possible cleavage sites: the amide bond between the L-alanyl spacer and the D-isoglutamine of muramyldipeptide and the ester linkage between the sugar−tripeptide and cholesterol. This latter bond would be a substrate for cholesterol esterases in the lysosomes, where colloidal carriers would be accumulated after phagocytosis. In order to determine the importance of this hydrolysis, we have synthesized two new muramyl peptides in which the sugar−tripeptide is linked to a lipophilic moiety by a stable ether linkage (Figures 3 and 4). Although ether linkages within phospholipids occur naturally, this sort of bond is not normally found in other lipids and would be expected to be more stable than an ester bond in the lysosomal environment. The macrophage-activating capacities of these compounds, formulated either as a micellar solution or as nanocapsules prepared from poly-(D,L-lactide), have been compared with those of MTP-

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* Author to whom correspondence should be addressed. Tel. + 33 1 46 83 56 27. Fax + 33 1 46 61 93 34.
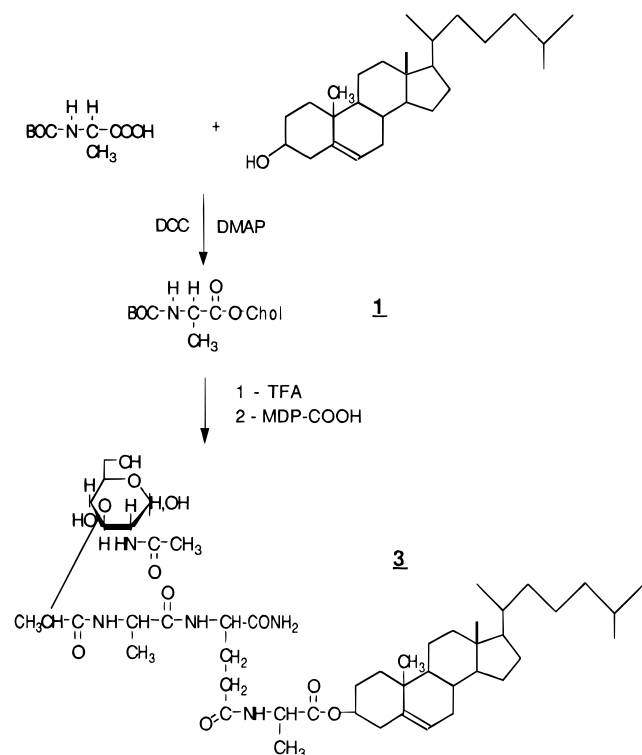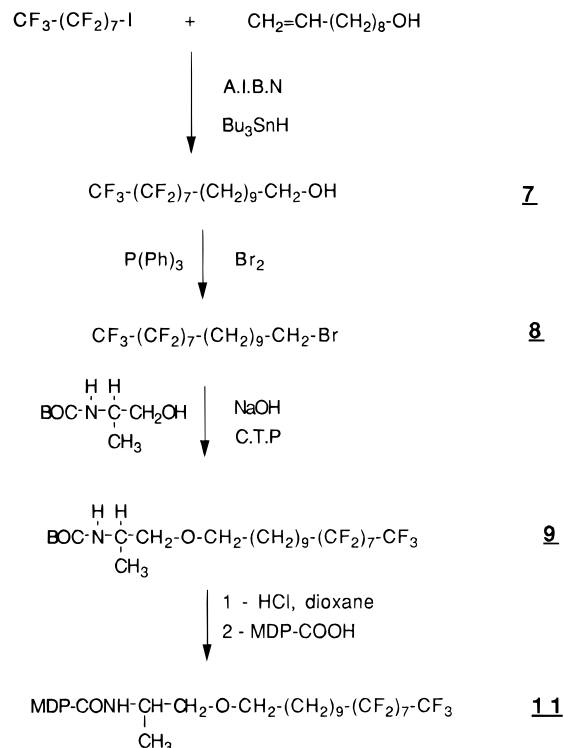
Figure 2. Synthesis of MTP-Chol.
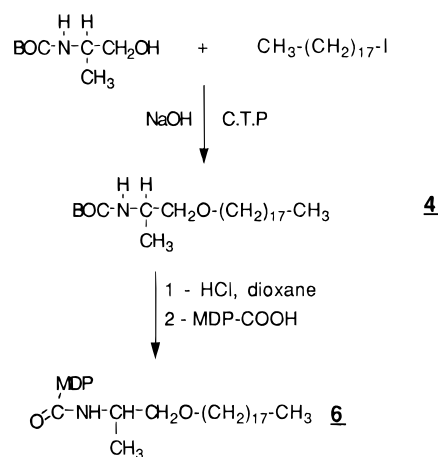
Figure 3. Synthesis of MTP-octadecane.

Chol. As a criterion of macrophage activation, we chose the induction of NO-synthase (NOS, EC 1.14.23) activity, since this is a major effector of macrophage-mediated cytostatic activity in rodent systems, responsible for antimicrobial, antiparasitic, and antitumoral effects.[8,18,23]

Results and Discussion

1. Synthesis of Compounds. MDP-L-alanylcholesterol (MTP-Chol, **3**, Figure 2) was synthesized from MDP as described by Phillips et al.[21] The products **6** and **11** were synthesized via the procedures given in Figures 3 and 4. In the case of MTP-octadecane, Boc-alaninol was coupled to 1-iodooctodecane via a Freedman coupling[12] and purified by chromatography. The Boc protecting group was removed by hydrochloric acid/dioxane, and then use of a mixed anhydrides coupling followed by column chromatography gave the desired **6** in 48% yield. For MTP-[heptadecafluorooctadecane], the mixed fluorohydrocarbon chain was prepared by


Figure 4. Synthesis of MTP-heptadecafluorooctadecane.

radical coupling of iodoperfluorooctane with 9-decen-1-ol, followed by tributyltin hydride reaction.[3,19] The bromo derivative was prepared by a modified Wiley reaction.[27] The subsequent coupling of Boc-alaninol and the MDP as for MTP-octadecane led to the preparation of **11** with a low yield (16%) in the final synthetic step. The yield in the peptide coupling could not be improved under a variety of conditions (data not shown). All products were characterized by ^{1}H (400 MHz) NMR and were pure by TLC.

2. Macrophage Activation Experiments. Compound **6** was prepared as a micellar solution. No activation of RAW 264.7 cells was observed (Figure 5). In contrast, a micellar solution of MTP-Chol gave a significant increase in nitrite production at a concentration of 10^{-6} M.[30]

We then wanted to compare the ability of compound **6** to induce NOS activity in RAW 264.7 macrophages after incorporation into nanocapsules. A centrifugation−ultrafiltration technique followed by HPLC showed that the percentage of encapsulation of **6** within nanocapsules was about 95%.

Compound **6** was unable to induce a significant increase in nitrite production (Figure 6). As a positive control, MDP was used as equivalent concentrations. In this case, nitrite production was induced in a dose-dependent fashion, confirming that muramyl peptides alone can induce NOS in this cell line.[23] This is at variance with results obtained using rat alveolar macrophages,[2,18] where a strong synergy between muramyl peptides and bacterial endotoxin (LPS) was observed. A probable explanation is that the RAW 264.7 cell line is extremely sensitive to LPS[23] and since the fetal calf serum used was not guaranteed low in endotoxin, there was probably sufficient present to constitute a second signal. In contrast to compound **6**, compound **3** (MTP-Chol) within nanocapsules was as active as MDP (Figure 7).
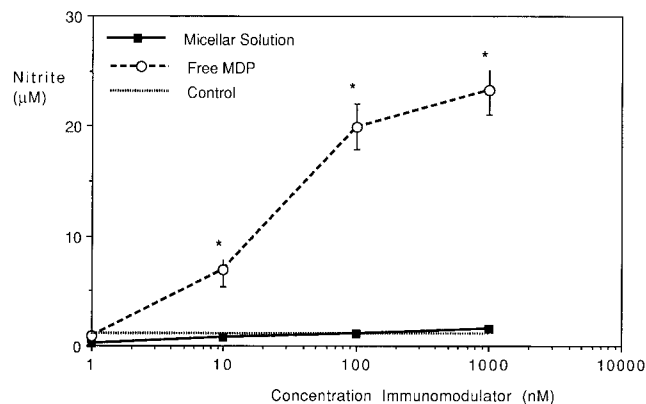
Figure 5. Nitrite production by RAW 264.7 cells induced by MTP-octadecane micelles. Values are the mean ± standard deviations of at least six wells. *Significant difference from control, $p < 0.001$, Student's two-tailed t-test.
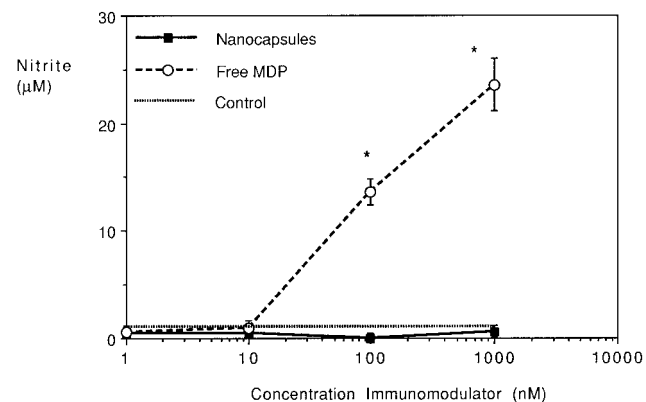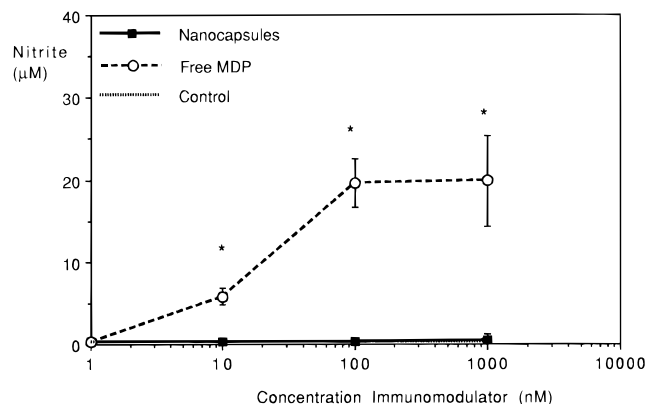

Figure 6. Nitrite production by RAW 264.7 cells induced by MTP-octadecane within nanocapsules. Values are the mean ± standard deviations of at least six wells. *Significant difference from control, $p < 0.001$, Student's two-tailed t-test.
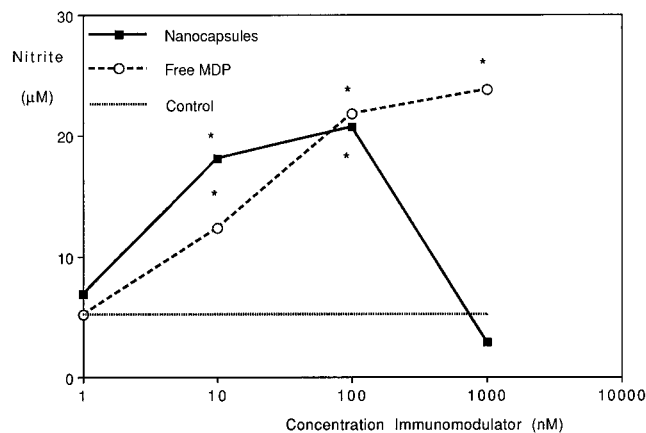
Figure 7. Nitrite production by RAW 264.7 cells induced by MTP-Chol within nanocapsules. Values are the mean ± standard deviations of at least six wells. *Significant difference from control, $p < 0.001$, Student's two-tailed t-test.

Compound **11**, which is more hydrophobic and more stable than **6**, was also unable to induce a significant increase in nitrite production (Figure 8).

These results indicate that compounds **6** and **11** cannot activate macrophages in their intact form and that they are not metabolized by macrophages to an active compound. The presence of an ether bond between the hydrophilic and hydrophobic parts of the compounds synthesized prevents the liberation of a muramyl tripeptide. In contrast, hydrolysis of the amide bond be-

Figure 8. Nitrite production by RAW 264.7 cells induced by MTP-heptadecafluorooctadecane within nanocapsules. Values are the mean ± standard deviations of at least six wells. *Significant difference from control, $p < 0.001$, Student's two-tailed t-test.

tween L-alanine and D-isoglutamine could occur, generating MDP. In our experimental system, however, this hydrolysis does not seem to occur to any great extent, because if free MDP were produced at a concentration of 10^{-7} M or more, it would have stimulated NOS synthase activity as in the positive controls.

A systematic study of the ability of different alkyl esters of MDP-L-alanine to protect mice against *Klebsiella pneumoniae* has been made.[15] The eicosyl (C20) derivative was particularly effective, showing that the presence of a long alkyl chain, as in compound **6**, did not in itself reduce biological activity. The tripeptide MDP-L-alanine was also active in this *in vivo* system. In the work of Level, only ester-linked compounds were synthesized. The importance of a biodegradable linkage between the muramyl tripeptide and the lipophilic portion was shown by the observation that a sterically hindered N-methyl-L-alanyl residue, in this case coupled to a diglyceride moiety, led to a reduction in the biological activity compared to that of the reference compound with L-alanine as the spacer.[15]

Yapo et al.[28] studied the *in vivo* fate of the natural tripeptide N-(acetylmuramyl)-L-alanyl-D-isoglutamyl-*meso*-2,2'-diaminopimelic acid in the mouse. They recovered ^{14}C-labeled *meso*-2,2'-diaminopimelic acid in the urine, showing that the final amide linkage could be hydrolyzed in mammalian systems despite the presence of unusual amino acids. In contrast, intravenously administered MDP was excreted without metabolism,[20] and has also been shown to be stable in liver lysosomal fraction[6] and in human monocytes.[16] Fogler et al.[11] also found [3H]nor-MDP intact in the plasma and urine, but detected metabolism in the liver. In the work reported by Yapo et al.[28] the tripeptide was, however, unable to protect mice against *Klebsiella* infection, whereas MDP was effective, suggesting that metabolism might occur in the kidneys but not at the site of action of MDP (in this case, probably alveolar macrophages).

The susceptibility of compounds **6** and **11** to enzymes would be different from that of a soluble tripeptide because of the presence of the ether-linked hydrophobic chain. Apart from solubility considerations (these compounds form micelles in aqueous media), the liberation of MDP would require an endopeptidase rather than an exopeptidase activity. Under these conditions, it is possible that hydrolysis of the N-terminal amide bond

to yield a desmuramyl peptide might occur more rapidly than release of intact MDP.

The importance of lysosomal metabolism in the activity of MTP-Chol has been shown in experiments performed in the presence of monensin.[18] This ionophore dissipates the pH gradient which exists between the lysosomes and the cytoplasm and thus reduces acid hydrolase activity and pH-dependent receptor−ligand dissociation. Under these conditions, the ability of nanocapsules containing MTP-Chol to induce NOS activity in rat alveolar macrophages was significantly reduced. Interestingly, monensin also reduced the activity of MDP. It seems that protonation of the C-terminal carboxyl group is necessary to allow the uncharged form to diffuse into the cytoplasm.[18]

In conclusion, the synthesis of two new derivatives of MDP has allowed us to confirm the hypothesis that the lipophilic derivatives described in the literature are in fact prodrugs; the results underline the importance of the presence of a labile linkage that is readily cleaved by the macrophage enzyme system. The prodrugs allow the immunomodulator to be prepared in colloidal or micellar forms, which gain access to macrophages more readily (by phagocytosis) than soluble derivatives which are taken up by fluid-phase pinocytosis, but once inside the cell, they must be degraded to small, water-soluble molecules in order to evoke a biological response.

Experimental Procedures

Materials. Muramyl dipeptide (MDP) was obtained from the Institut Choay (Montrouge, France). Poly(D,L-lactide), molecular weight 32 000, was obtained from Boehringer Ingelheim. Synperonic PE/F68 was from ICI, ethyl oleate from Aldrich, and soya-bean lecithin (Epikuron 170) from Lucas Meyer (Hamburg, Germany). Solvents and other reagents were obtained from Prolabo (Courbevoie, France).

Tissue culture products were obtained from Gibco (Eragny, France), tissue culture flasks and 24- or 96-well plates from Nunc (Polylabo, Strasbourg, France).

Solvents were distilled under nitrogen over the appropriate drying agents. All chromatography was carried out on silica gel S 0.032−0.063 mm (Riedel de Haën), and thin-layer chromatography was carried out on silica gel 60F$_{254}$ plates (Merck). All ^{1}H and COESY (200 and 400 MHz) and ^{13}C (50 MHz) NMR spectra were recorded with a Brucker Ac200 spectrometer.

3-*O*-[(*tert*-Butyloxycarbonyl)-L-alanyl]cholesterol (1). Boc-L-alanine (0.946 g, 5 mmol), 1.5 g (4 mmol) of cholesterol, and 0.488 g of 4-(dimethylamino)pyridine were dissolved in a mixture of solvent DMF and THF (20/5) (v/v). Dicyclohexylcarbodiimide (1.11 g, 5.4 mmol) was added. The reaction mixture was stirred at room temperature for 18 h. The solvent was evaporated under vacuum. The residue was dissolved in CH$_2$Cl$_2$, and the organic layer was extracted from NaHCO$_3$ (1 M), 5% w/v KHSO$_4$, and water, evaporated under vacuum, and purified by column chromatography. Yield: 66%. R_f: 0.25 (toluene/ether, 10/1). ^{1}H NMR (CDCl$_3$): 0.65 ((CH_3)$_3$COCONHCH(CH_3)CO$_2$, H of cholesterol, broad multiplet, 56H), 4.25 (CH(CH$_3$)CO$_2$, t, 1H, J = 6 Hz), 4.5 (CO$_2$CH(Chol), broad multiplet, 1H), 5.05 (OCONH, d, 1H, J = 7.3 Hz), 5.35 (C=C(H)C, d, 1H, J = 5 Hz). Anal. (C$_{35}$H$_{59}$NO$_4$) C, H, N.

3-*O*-(L-Alanyl)cholesterol (2). Trifluoroacetic acid (2 mL) was added to 0.95 g (1.67 mmol) of compound **1**. The solution was stirred for 3 h in an ice bath. The acid was evaporated under vacuum, and the product was dissolved in water. A solution of NaOH (0.1 N) was added until a basic pH (∼9) was reached. The aqueous phase was extracted from CH$_2$Cl$_2$, and the organic layer was evaporated and dried under vacuum. Yield: 90%. ^{1}H NMR (CDCl$_3$): 0.65 (H$_2$NCH(CH_3)CO$_2$, H of cholesterol, broad multiplet, 47H), 3.55 (CH(CH$_3$)CO$_2$, q, 1H,

J = 6.5 Hz), 4.65 (CO$_2$CH(Chol), broad multiplet, 1H), 5.35 (C=C(H)C, d, 1H, J = 5.3 Hz). Anal. (C$_{30}$H$_{51}$NO$_2$) C, H, N.

3-*O*-(*N*-(Acetylmuramyl)-L-alanyl-D-isoglutamyl-L-alanyl)cholesterol (3). MDP (0.2 g, 4.06 × 10^{-4} mol) was dissolved in 10 mL of anhydrous DMF. The reaction mixture was maintained at −15 °C. *N*-Methylmorpholine (45 μL, 4.06 × 10^{-4} mol) was added. After 1 h at −15 °C, 53 μL (4.06 × 10^{-4} mol) of isobutyl chloroformate was added. After 2 h at −15 °C, a solution of 0.183 g (4.06 × 10^{-4} mol) of **2** dissolved in 10 mL of anhydrous chloroform was added dropwise. The reaction mixture was stirred for 1−2 h at −15 °C and left overnight at room temperature. The solvent was evaporated under vacuum, and the residue was purified by column chromatography. Yield: 49%. R_f: 0.21 and 0.34 (MeOH/CHCl$_3$, 1/4). Anal. (C$_{49}$H$_{81}$N$_5$O$_{12}$·3H$_2$O) H, N; C: calcd, 63.11; found, 59.03. COESY and ^{1}H NMR (400 MHz, pyridine-d_5): 0.65 (H of cholesterol, CH_3 (lactyl), 2 CH_3 (alanine), broad multiplet, 62H), 2.1 (CH_3CONH, s, 3H), 2.3 ((CH_2)α (isoglutamine), broad multiplet, 2H), 2.7 ((CH_2)β (isoglutamine), broad multiplet, 2H), 4.0 (2 CH (alanine), CH (lactyl), CH (isoglutamine), H$_2$, H$_3$, H$_4$, H$_5$, H$_6$, H′$_6$, OH_4, OH_6, CO$_2$CH(Chol), C=C(H)C, broad multiplet, 14H), 6 (H$_1$, d, 1H, J = 3.1 Hz), 6.2 (OH_1, broad singulet, 1H), 8.2 (CONH_2, s, 1H), 8.4 (CONH_2, s, 1H), 9.05 (CH$_3$CONH, CONH (alanyl), t, 2H, J = 6.1 Hz), 9.25 (CONH (alanyl), d, 1H, J = 6.8 Hz), 9.55 (CONH(isoglutamine), d, 1H, J = 6.8 Hz).

1-*O*-[(*tert*-Butyloxycarbonyl)-L-alanyl]octadecane (4). Boc-L-alaninol (0.5 g, 2.85 mmol) was dissolved in 25 mL of anhydrous THF. NaOH (0.57 g, 14.25 mmol) and bromide tetrabutylammonium (0.285 mmol) were added. After the addition of 1.9 g (5.7 mmol) of octadecanoyl bromide, the reaction mixture was stirred at room temperature for 18 h. The solvent was evaporated under vacuum. The residue was dissolved in CH$_2$Cl$_2$, and the organic layer was extracted from water (2 × 25 mL), evaporated under vacuum, and purified by column chromatography. Yield: 75%. R_f: 0.53 (acetone/hexane, 1/12). ^{1}H NMR (CDCl$_3$): 0.75 (CH_3(CH$_2$)$_{16}$, t, 3H, J = 6 Hz), 1.1 ((CH$_3$)$_3$COCONHCH(CH_3)CH$_2$O, CH$_3$(CH_2)$_{16}$, (CH_3)$_3$COCONH, broad multiplet, 44H), 3.2 (CH(CH$_3$), CH_2-OCH_2, broad multiplet, 5H). Anal. (C$_{26}$H$_{53}$NO$_3$) C, H, N.

1-*O*-(L-Alanyl)octadecane, Chlorhydrate (5). To 0.335 g of **4** (7.845 × 10^{-4} mol) was added 4 mL of a solution of hydrogen chloride 4 M in 1,4-dioxane. The mixture was stirred for 4 h at room temperature. After the removal of the solvent, the resulting white solid was dried under high vacuum. Yield: 63%. ^{1}H NMR (CDCl$_3$): 0.75 (CH_3(CH$_2$)$_{16}$, t, 3H, J = 6 Hz), 1.1 (HCl, H$_2$NCH(CH_3)CH$_2$O, CH$_3$(CH_2)$_{16}$, broad multiplet, 35H), 3.4 (HCl, H$_2$NCH(CH$_3$), CH_2OCH_2, broad multiplet, 5H), 8.2 (NH$_3$$^+Cl^-$, broad peak, 3H). Anal. (C$_{21}$H$_{46}$NO·HCl) C, H, N.

1-*O*-(*N*-(Acetylmuramyl)-L-alanyl-D-isoglutamyl-L-alanyl)octadecane (6). MDP (0.2 g, 4.06 × 10^{-4} mol) was dissolved in 10 mL of anhydrous DMF. The reaction mixture was maintained at −15 °C. *N*-Methylmorpholine (45 μL, 4.06 × 10^{-4} mol) was added. After 1 h at −15 °C, 53 μL (4.06 × 10^{-4} mol) of isobutyl chloroformate was added. After 2 h at −15 °C, a solution of 0.147 g (4.06 × 10^{-4} mol) of **5** and 57 μL (4.06 × 10^{-4} mol) of triethylamine dissolved in 10 mL of anhydrous chloroform was added dropwise. The reaction mixture was stirred for 1−2 h at −15 °C and left overnight at room temperature. The solvent was evaporated under vacuum, and the residue was purified by column chromatography. Yield: 48% R_f: 0.25 and 0.33 (MeOH/CHCl$_3$, 1/4). Anal. (C$_{40}$H$_{76}$N$_5$O$_{11}$·3H$_2$O) H, N; C: calcd, 59.92; found, 56.05. COESY and ^{1}H NMR (400 MHz, pyridine-d_5): 0.6 (CH_3-(CH$_2$)$_{16}$CH$_2$O, t, 3H, J = 6.5 Hz), 0.9 (CH$_3$(CH_2)$_{16}$CH$_2$O, CH_3 (lactyl), 2 CH_3 (alanine), broad multiplet, 41H), 1.9 (CH_3-CONH, s, 3H), 2.3 ((CH_2)α (isoglutamine), broad multiplet, 2H), 2.6 ((CH_2)β (isoglutamine), broad multiplet, 2H), 3.25 (CH$_2$OCH_2(CH$_2$)$_{16}$CH$_3$, broad multiplet, 2H), 3.7 (CH_2OCH$_2$-(CH$_2$)$_{16}$CH$_3$, broad multiplet, 2H), 4.0 (CH(CH$_3$)CH$_2$OCH$_2$, CONHCH(CH$_3$)CONH-, CH (lactyl), CH (isoglutamine), H$_2$, H$_3$, H$_4$, H$_5$, H$_6$, H′$_6$, OH_4, OH_6, broad multiplet, 12H), 5.9 (H$_1$, d, 1H, J = 3.2 Hz), 8.5 (CONHCH(CH$_3$)CH$_2$OCH$_2$, d, 1H, J = 6.5 Hz), 8.6 (CONHCH(CH$_3$)CONH, d, 1H, J = 6.5 Hz), 8.85

(CH$_3$CONH, d, 1H, J = 6.5), 9.9 (CONHCH(CONH$_2$)CH$_2$, d, 1H, J = 6.5 Hz).

11,11′,12,12′,13,13′,14,14′,15,15′,16,16′,17,17′,18,18′,18″-Heptadecafluorooctadecan-1-ol (7). Perfluorooctyl iodide (3.96 mL, 15 mmol) and 4 mL (22.5 mmol) of 9-buten-1-ol were placed in a three-necked flask equipped with a condenser, a nitrogen inlet, and a stopper. The mixture was homogenized by heating to 80 °C, then 37.5 mg of 2,2′-azoizobutyronitrile (AIBN) was added in small portions over the next 45 min, and the reaction mixture was stirred for another 5 h at 80 °C. The raw product and 0.246 g of AIBN were dissolved in 20 mL of absolute toluene under nitrogen. Tri-*n*-butyltin hydride (8 mL, 30 mmol) was added with a syringe via a rubber septum, a reflux condenser was placed on the flask, and the solution was poured into 200 mL of distilled methanol to decompose excess reducing agent. The solvent was evaporated to obtain a solid which was purified by recrystallization from toluene. Yield: 70%. ^{1}H NMR (CDCl$_3$): 1.1 (CF$_2$CH$_2$CH$_2$(CH_2)$_6$ and CH$_2$OH, broad singlet, 13H), 1.5 (CF$_2$CH$_2$CH_2 and CH_2CH$_2$OH, broad multiplet, 4H), 1.85 (CF$_2$CH_2, broad multiplet, 2H), 3.55 (CH_2-OH, t, 2H, J = 5.5 Hz). Anal. (C$_{18}$F$_{17}$H$_{21}$O) C, H.

1-Bromo-11,11′,12,12′,13,13′,14,14′,15,15′,16,16′,17,17′,-18,18′,18″-heptadecafluorooctadecane (8). Triphenylphosphane (1.5 g, 5.73 mmol) and bromine (0.295 ml, 5.73 mmol) were dissolved in 30 mL of anhydrous DMF. After 30 min at room temperature, 3 g (5.21 mmol) of **7** was added, and the solution was stirred at 60 °C for 15 h. The solution was poured into iced water (300 mL). The precipitate was collected by filtration and purified by crystallization from ethanol. Yield: 80%. ^{1}H NMR (CDCl$_3$): 1.1 (CF$_2$CH$_2$(CH_2)$_7$, broad multiplet, 14H), 1.75 (CF$_2$CH_2(CH$_2$)$_7$CH_2, broad multiplet, 4H), 3.35 (CH_2-Br, t, 2H, J = 6 Hz). Anal. (BrC$_{18}$F$_{17}$H$_{20}$) C, H.

1-*O*-[(*tert*-butyloxycarbonyl)-L-alanyl]-11,11′,12,12′,13,-13′,14,14′,15,15′,16,16′,17,17′,18,18′,18″-heptadecafluorooctadecane (9). This compound was prepared via a procedure analogous to that of compound **4**. Yield: 70%. R_f: 0.52 (acetone/hexane, 2/5). ^{1}H NMR (CDCl$_3$): 1.0 (Boc-NHCH-(CH_3), d, 3H, J = 6 Hz), 1.15 (CF$_2$(CH_2)$_9$, (CH_3)$_3$COCONH, broad multiplet, 27H), 3.2 (CH_2OCH_2, Boc-NHCH(CH$_3$), broad multiplet, 5H). Anal. (C$_{26}$F$_{17}$H$_{36}$NO$_3$) C, H, N.

1-*O*-(L-Alanyl)-11,11′,12,12′,13,13′,14,14′,15,15′,16,16′,17,-17′,18,18′,18″-heptadecafluorooctadecane, Chlorhydrate (10). This compound was prepared via a procedure analogous to that of compound **5**. Yield: 70%. ^{1}H NMR (CDCl$_3$): 1.15 (CF$_2$(CH_2)$_9$, HCl, H$_2$NCH(CH_3), broad multiplet, 21H), 3.3 (CH_2OCH_2, HCl, H$_2$NCH(CH$_3$), broad multiplet, 5H), 8 (NH$_3$$^+Cl^-$, broad peak, 3H). Anal. (C$_{21}$F$_{17}$H$_{29}$NO·HCl) C, H, N.

1-*O*-(*N*-(Acetylmuramyl)-L-alanyl-D-isoglutamyl-L-alanyl)-11,11′,12,12′,13,13′,14,14′,15,15′,16,16′,17,17′,18,18′,18″-heptadecafluorooctadecane (11). This compound was prepared via a procedure analogous to that of compound **6**. Yield: 16%. R_f: 0.30 and 0.36 (MeOH/CHCl$_3$, 1/4). COESY and ^{1}H NMR (400 MHz, pyridine-*d*$_5$): 0.7 (CF$_3$(CF$_2$)$_7$(CH_2)$_9$-CH$_2$O, CH_3 (lactyl), 2 CH_3 (alanine), CH_3CONH, broad multiplet, 30H), 2.5 ((CH_2)α (isoglutamine), broad multiplet, 2H), 2.6 ((CH_2)β (isoglutamine), broad multiplet, 2H), 3.3 (CH$_2$-OCH_2(CH$_2$)$_9$(CF$_2$)$_7$CF$_3$, broad multiplet, 2H), 3.6 (CH_2-OCH$_2$(CH$_2$)$_9$(CF$_2$)$_7$CF$_3$, broad multiplet, 2H), 4.1 (CH(CH$_3$)-CH$_2$OCH_2, CONHCH(CH$_3$)CONH, CH (lactyl), CH (isoglutamine), H$_2$, H$_3$, H$_4$, H$_5$, H$_6$, H′$_6$, OH_4, OH_6, broad multiplet, 12H), 6.0 (H$_1$, d, 1H, J = 2.5 Hz), 8.0 (CONH_2, s, 1H), 8.3 (CONH_2, s, 1H), 8.5 (CONHCH(CH$_3$)CH$_2$OCH$_2$, d, 1H, J = 7.9 Hz), 9.0 (CONHCH(CH$_3$)CONH, CH$_3$CONH, broad multiplet, 2H), 9.6 (CONHCH(CONH$_2$)CH$_2$, d, 1H, J = 7.9 Hz). Anal. (C$_{40}$F$_{17}$H$_{59}$N$_5$O$_{11}$·3H$_2$O) C, H, N.

HPLC Analysis of MTP-Octadecane Nanocapsules. Compound **6** was assayed by HPLC using a Waters Microbondapak C18 column and UV detection at 215 nm. The mobile phase was methanol/water (92:8 v/v), and the flow rate 1 mL/min. The lower limit of sensitivity was 0.2 mg of compound **6** in an injection volume of 20 μL. The retention time was about 7.4 min.

These HPLC conditions allowed us to separate muramyl peptides from other nanocapsule components. Nonencapsulated MTP-octadecane was separated by ultrafiltration on Millipore "Ultrafree-MC units" (0.1 μm filter unit). The clear ultrafiltrates were then analyzed by HPLC. The MTP-octadecane content of the nanocapsules was calculated by the difference between the total drug concentration in the suspension and the free MTP-octadecane concentration measured in the ultrafiltrate.

Preparation of Micellar Solutions. A micellar solution of MTP-octadecane was prepared as follows: 2 mg of compound **6** was added to a solution of 100 mg of Poloxamer 188 in 10 mL of distilled water. The product dissolved to form a clear solution after ultrasound treatment of the preparation for 1 h at 50 °C.

Preparation of Nanocapsules. Nanocapsules containing muramyl peptides were prepared by interfacial deposition of PLA by the method of Devissaguet et al.[7] PLA (35 mg) was dissolved in 12.5 mL of acetone. Compound 3 (2 mg), compound **6**, or compound **11** and 50 mg of Epikuron 170 was dissolved in 0.25 mL of ethyl oleate and added to the acetone phase. This organic solution was then allowed to run slowly into 25 mL of distilled water containing 100 mg of a nonionic surfactant, Synperonic PE/F68, under moderate magnetic stirring. Nanocapsules were formed instantaneously. The acetone was removed and the preparation concentrated to 10 mL by evaporation under vacuum. Freshly prepared nanocapsules were used in all experiments. The mean particle diameter, measured by laser light scattering on a typical preparation (Nanosizer N4, Coultronics, Margency, France), was 200 ± 50 nm (mean ± standard deviation for 3 runs), with a polydispersity index of 0.07.

Cell Culture. The RAW 264.7 mouse monocyte−macrophage line (ECACC catalog number 91062702) was maintained as a adherent culture in RPMI-1640 medium supplemented with 10% decomplemented fetal calf serum, 2 mM L-glutamine, and antibiotics. Cells were passaged after detachment with trypsin−EDTA. For activation experiments, the cell suspension was adjusted to 4×10^5 viable cells/mL, estimated by Trypan blue exclusion, and plated into flat-bottomed 96-well plates at 200 μL/well. The cells were allowed to adhere for 2−3 h at 37 °C in a humidified 95% O$_2$−5% CO$_2$ atmosphere.

Macrophage Activation Experiments. The medium in 96-well plates was removed and replaced by medium containing the various activators at the desired concentration. The plates were incubated for a further 24 h; this was the induction phase. The medium was then removed and replaced with fresh medium without activators. After a further 24 h incubation period (effector phase) the medium was removed and a 100 μL aliquot was assayed for nitrite using the Griess reagent[13] in a microtiter plate assay, as an indication of NOS induction.

Statistical Analysis. The mean nitrite concentrations from at least three treated wells were compared with those in control wells by Student's two-tailed *t*-test.

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