

drugs were dissolved in water and absorbed onto 6.35-mm Schleicher & Schuell disks and placed on the seeded plates. For antagonism studies, various amino acids and di- and tripeptides were codisked with the peptide drugs.

Acknowledgment. We thank Drs. George Dunn and David Berges for helpful suggestions during the preparation of this paper and Dr. Charles Gilvarg for his excellent advice during the progress of this research.

Registry No. Boc-DL-*m*-FPhe, 87184-23-4; DL-*m*-FPhe, 2629-54-1; Boc-DL-*m*-FPhe-OSu, 87184-24-5; Boc-DL-*m*-FPhe-L-Ala-L-Ala, 87184-25-6; L-Ala-L-Ala, 1948-31-8; DL-*m*-FPhe-L-

Ala-L-Ala, 87184-26-7; L-*m*-FPhe-L-Ala-L-Ala, 87184-16-5; D-*m*-FPhe-L-Ala-L-Ala, 87184-17-6; Cbz-L-*m*-FPhe-L-Ala-L-Ala-OBzl, 87184-27-8; L-Ala-L-Ala-OBzl-HCl, 69871-83-6; Cbz-L-*m*-FPhe, 49759-64-0; DL-*m*-FPhe-L-Met-L-Met, 87184-29-0; Boc-DL-*m*-FPhe-L-Met-L-Met, 87184-28-9; L-*m*-FPhe-L-Met-L-Met, 87184-18-7; D-*m*-FPhe-L-Met-L-Met, 87184-19-8; Cbz-L-*m*-FPhe-L-Ala-OBzl, 87184-30-3; L-Ala-OBzl, 17831-01-5; L-*m*-FPhe-L-Ala, 87184-20-1; DL-*m*-FPhe-*N*-carboxyanhydride, 87184-31-4; DL-*m*-FPhe-L-Ala, 87184-32-5; L-Ala, 56-41-7; D-*m*-FPhe-L-Ala, 87184-21-2; L-Ala-L-Ala-DL-*m*-FPhe, 87184-34-7; Boc-L-Ala-L-Ala-OSu, 67818-94-4; Boc-L-Ala-L-Ala-DL-*m*-FPhe, 87184-33-6; L-Ala-L-Ala-L-*m*-FPhe, 87184-22-3; L-Ala-L-Ala-D-*m*-FPhe, 87184-35-8; L-*m*-FPhe, 19883-77-3.

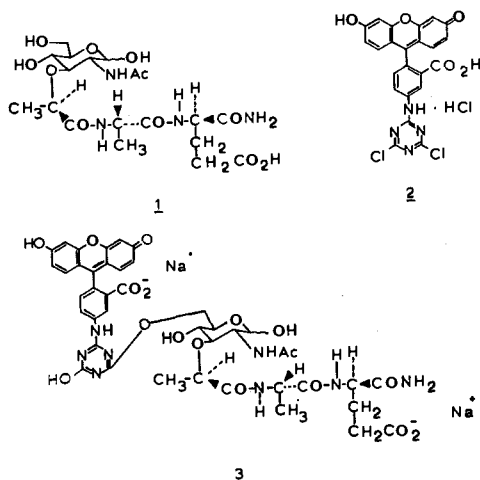
Synthesis of a Biologically Active Fluorescent Muramyl Dipeptide Congener

C. K. Hiebert,[†] W. C. Kopp,[‡] H. B. Richerson,[‡] and C. F. Barfknecht*[†]

Department of Internal Medicine, College of Medicine, and Division of Medicinal Chemistry-Natural Products, College of Pharmacy, The University of Iowa, Iowa City, Iowa 52242. Received April 15, 1983

A fluorescent-labeled muramyl dipeptide (MDP) has been prepared to probe immunoadjuvant cellular interactions. *N*-Acetylmuramyl-L-alanyl-D-isoglutamine (1) was synthesized in improved yield and reacted with 2-(fluoresceinylamino)-4,6-dichloro-*s*-triazine (DTAF, 2) to give the fluorescent adduct DTAF-MDP (3), attached through the 6-position of the sugar moiety. Adjuvant activity was assessed by using two different *in vitro* assays, macrophage spreading, and inhibition of macrophage migration. Both assays indicated that the apparent adjuvant activity of 3 is comparable to that of 1.

Studies into the adjuvant active fractions of lysozyme digested mycobacteria cell walls led Ellouz et al.^{1,2} to propose that *N*-acetylmuramyl-L-alanyl-D-isoglutamine (1)



(muramyl dipeptide, MDP) is the minimum structure required which expresses the full spectrum of adjuvant activity when substituted for mycobacteria in Freund's complete adjuvant (FCA). MDP is known to have many effects on both the humoral- and cell-mediated immune systems, and MDP has been implicated in the stimulation of macrophages.³⁻⁷

The total synthesis^{2,8-11} and structure-activity relationship (SAR) studies¹¹⁻¹⁵ have been performed on MDP. SAR studies revealed several interesting aspects that are relevant to the studies reported here. The C-6 hydroxyl moiety on the muramyl portion of the molecule can be acylated without changing the adjuvant activity.¹³ The C-6 hydroxyl group can be converted to an amino function without affecting the activity.¹⁴ The amino acid L-alanine

can be replaced by various L amino acids without a significant change in activity.¹⁵ The γ -glutamyl carboxyl group can be converted to an ester or amide or coupled to other amino acids without a change in the biological activity.¹¹

Radiolabeled derivatives of MDP have been prepared^{16,17} to study the distribution of MDP in the body and to help determine the mechanisms of action for the different adjuvant and immunogenic properties. MDP was found to be distributed throughout the body without substantial

- (1) Ellouz, F.; Adam, A.; Clorbaru, R.; Lederer, E. *Biochem. Biophys. Res. Commun.* 1974, 59, 1317.
- (2) Merser, C.; Sinaý, P.; Adam, A. *Biochem. Biophys. Res. Commun.* 1975, 66, 1316.
- (3) Parant, M. *Springer Semin. Immunopathol.* 1979, 2, 101.
- (4) Damals, C.; Parant, M.; Chedid, L. *Cell. Immunol.* 1977, 34, 49.
- (5) Cummings, N. P.; Pabst, M. J.; Johnson, R. B., Jr. *J. Exp. Med.* 1980, 152, 1659.
- (6) Pabst, M. J.; Johnston, M. J., Jr. *J. Exp. Med.* 1980, 151, 101.
- (7) Wahl, S. M.; Wahl, L. M.; McCarthy, J. B.; Chedid, L.; Mergenhagen, S. E. *J. Immunol.* 1979, 122, 2226.
- (8) Kusumoto, S.; Ikenaka, K.; Shiba, T. *Bull. Chem. Soc. Jpn.* 1979, 52, 1177.
- (9) LeFrancier, P.; Choay, J.; Derrien, M.; Lederman, I. *Int. J. Pept. Protein Res.*, 1977, 9, 249.
- (10) Schwartzman, S. M.; Ribl, E. *Prep. Biochem.* 1980, 10, 255.
- (11) Kotani, S.; Watanabe, Y.; Kinoshita, F.; Shimono, T.; Morisaki, I.; Shiba, T.; Kusumoto, S.; Tarumi, Y.; Ikenaka, K. *Biken J.*, 1975, 18, 105.
- (12) Chedid, L.; Lederer, E. *Biochem. Pharmacol.* 1978, 27, 2183.
- (13) Kotani, S.; Kinoshita, F.; Morisaki, I.; Shimono, T.; Okunaga, T.; Takada, H.; Tsujimoto, M.; Watanabe, Y.; Kato, K.; Shiba, S.; Kusumoto, S.; Okada, S. *Biken J.* 1977, 20, 95.
- (14) Hasegawa, A.; Okumura, H.; Kiso, M.; Azuma, I.; Yamamura, Y. *Agric. Biol. Chem.* 1980, 44, 1309.
- (15) Adam, A.; Devys, M.; Souvannavong, V.; LeFrancier, P.; Choay, J.; Lederer, E. *Biochem. Biophys. Res. Commun.* 1976, 72, 339.
- (16) Kusumoto, S.; Ikenaka, K.; Shiba, T. *Tetrahedron Lett.* 1977, 4055.
- (17) Pichat, L.; Tostan, J.; LeFrancier, P.; Sinaý, P.; Lederer, E. *J. Labelled Compd. Radiopharm.* 1980, 17, 153.

[†] College of Pharmacy.

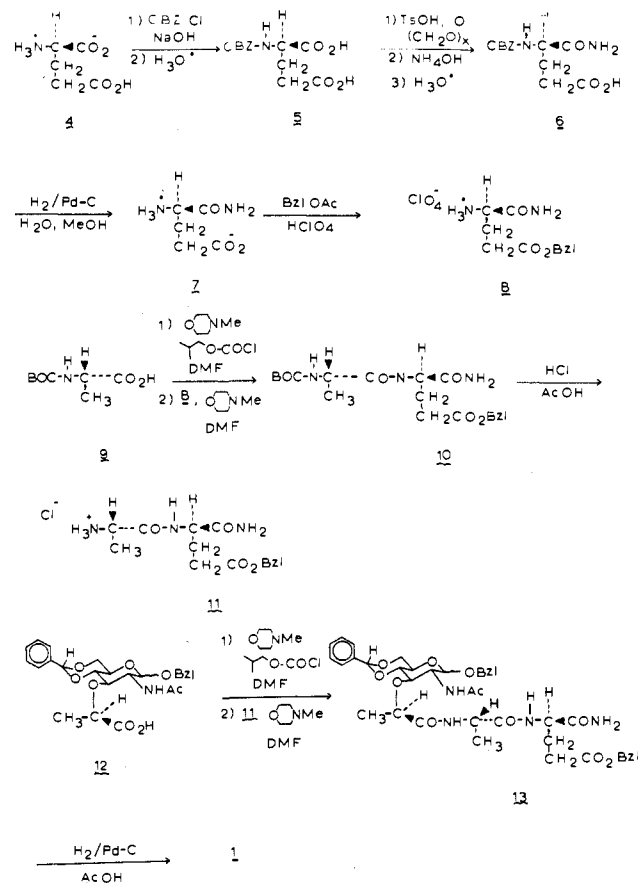
[‡] College of Medicine.

localized concentrations¹⁸ and rapidly eliminated unchanged in the urine. These studies were useful in examining the residence time of MDP in the body and in understanding some of the metabolic fate of MDP, but the studies were not helpful in elucidating the mechanism by which MDP exerts its biological properties.

A fluorescent-labeled MDP to visualize the binding of MDP to various cells would be useful to study immunoadjuvant cellular interactions in experimental model systems.¹⁹ It is essential that such a fluorescent derivative possess adjuvant potency similar to the parent compound for the binding studies to be meaningful. The effect of the dye molecule on the observed pharmacological activity (due to changes in binding, distribution, efficacy, etc.) is important. The preparation of fluorescent-labeled derivatives of small molecules that retained high biological activity was accomplished by Hazum and co-workers,²⁰ who prepared fluorescent-labeled enkephalins for studying opiate receptors.²¹ Since the C-6 hydroxyl function on the muramyl portion of MDP can be modified with complete retention of adjuvant activity,^{13,14} a fluorescent label attached through the C-6 hydroxyl group of muramyl dipeptide theoretically could retain adjuvant activity. Attempts failed in this laboratory to react the C-6 hydroxyl of MDP with the dye, fluorescein isothiocyanate (FITC), using a variety of methods. Although aryl isothiocyanates will react with alcohols, the reaction is slow. In the reaction of MDP with FITC, solvents that would solvate MDP react with the FITC faster than MDP. We report here that 2-(fluoresceinylamino)-4,6-dichloro-*s*-triazine (DTAF, 2) was successfully coupled to MDP via the dichlorotriazinyl moiety, which is known to react with alcohols in the presence of water^{22,23} to yield the fluorescent adduct DTAF-MDP (3), and that the resultant congener retained *in vitro* biological activity.

Chemistry. The preparation of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (1) follows a combination of the procedure of Kusumota et al.⁸ and the procedure of LeFrancier et al.⁹ The main differences between the two groups' approach to the synthesis of MDP are the route for preparing γ -benzyl-D-isoglutamine and the conditions used for peptide coupling. The preparation of the γ -benzyl ester of D-isoglutamine can be accomplished in high yields by using a modification of the method Kusumota and co-workers⁸ utilized in the preparation of the analogous γ -*tert*-butyl derivative. Higher yields were accomplished for the peptide-coupling reactions using the mixed anhydride coupling method than were accomplished using the carbodiimide/*N*-hydroxysuccinimide method. The former method is reported to give no racemization,²⁴ while some racemization has been observed with *N*-hydroxysuccinimide.²⁵ The overall yield of the seven-step reaction sequence starting from D-glutamic acid was 64%. This

Scheme I



compares favorably with the reported yields of 41⁸ and 28%.⁹

The general procedure used for the preparation of MDP is outlined in Scheme I. The amino function on D-glutamic acid is protected with benzyl chloroformate to give 5 (26). The cyclic anhydride of the protected D-glutamic acid was produced according to Straka and Zaoral²⁷ and reacted with aqueous ammonia to yield the protected isoglutamine 6. Removal of the benzyloxycarbonyl protecting group was accomplished by hydrogenolysis with 5% Pd/C in a methanol/water solvent system. The catalyst was filtered, and the solvent was evaporated *in vacuo* to give D-isoglutamine (7). By stirring D-isoglutamine at room temperature for 4 days in benzyl acetate in the presence of 70% perchloric acid, the corresponding perchlorate salt of γ -benzyl-D-isoglutamine (8) could be obtained. Boc-Ala (9) was prepared according to the procedure of Itoh and co-workers.²⁸ By using an isobutyl chloroformate and *N*-methylmorpholine coupling procedure,⁹ Boc-Ala was coupled with 8 to yield the protected dipeptide 10 in good yield. The optical activity of the protected dipeptide was comparable to literature values,²⁹ confirming that the two centers of asymmetry were not racemized in the course of the reactions performed on the amino acids. Deprotection of the terminal amino function, followed by coupling with benzyl-*N*-acetyl-4,6-*O*-benzylidene-muramic acid (Sigma Chemical Co.) (12) yielded the protected MDP (13). Hydrogenolysis of 13 in acetic acid over 5% Pd/C produced free *N*-acetylmuramyl-L-alanyl-D-isoglutamine (1). Puri-

- (18) Parant, M.; Parant, F.; Chedid, L.; Yapo, A.; Petit, J. F.; Lederer, E. *Int. J. Immunopharmacol.* **1979**, *1*, 35.
 (19) Richerson, H. B.; Suelzer, M. T.; Swanson, P. A.; Butler, J. E.; Kopp, W. C.; Rose, E. F. *Am. J. Pathol.* **1982**, *106*, 409.
 (20) Hazum, E.; Chang, K.-J.; Shechter, Y.; Wilkinson, S.; Cuatrecasas, P. *Biochem. Biophys. Res. Commun.* **1979**, *88*, 841.
 (21) Hazum, E.; Chang, K.-J.; Cuatrecasas, P. *Science* **1979**, *206*, 1077.
 (22) Chayen, R.; Gould, S.; Harell, A.; Stead, C. V. *Anal. Biochem.* **1971**, *39*, 533.
 (23) DeBelder, A. N.; Granath, K. *Carbohydr. Res.* **1973**, *30*, 375.
 (24) Beyerman, H. C.; DeLeer, E. W. B.; Floor, J. *Recl. Trav. Chim. Pays-Bas* **1973**, *92*, 481.
 (25) Fujino, M.; Kobayashi, S.; Obayashi, M.; Fukuda, T.; Shingawa, S.; Nishimura, O. *Chem. Pharm. Bull.* **1974**, *22*, 1857.
 (26) Carter, H. E.; Frank, R. L.; Johnston, H. W. "Organic Syntheses"; Wiley: New York, 1955; Collect. Vol III, p 167.

- (27) Straka, R.; Zaoral, M. *Collect. Czech. Chem. Commun.* **1977**, *42*, 560.
 (28) Itoh, M.; Haglwar, D.; Kamiya, T. *Tetrahedron Lett.* **1975**, 4393.
 (29) LeFrancier, P.; Bricas, E. *Bull. Soc. Chim. Biol.* **1967**, *49*, 1257.

Table I. Effect of MDP on Macrophage Spreading

MDP source	concn, $\mu\text{g/mL}$	% spreading (no. of plates examined)
saline control		46.6 (5)
DTAF-MDP	0.01	45.0 (3)
	0.10	68.5 (4)
	1.0	74.3 (3)
	10.0	73.3 (3)
I-MDP ^a	0.01	50.0 (2)
	0.10	72.5 (2)
	1.0	73.0 (2)
	10.0	80.0 (1)
P-MDP ^b	0.01	62.5 (2)
	0.10	80.0 (2)
	1.0	75.5 (2)
	10.0	79.0 (1)
L,L-MDP ^c	0.01	48.0 (1)
	0.10	56.0 (1)
	1.0	51.5 (2)
	10.0	76.0 (1)

^a Muramyl dipeptide prepared in our laboratory.

^b Commercially available MDP from the Institut Pasteur, Paris. ^c *N*-Acetylmuramyl-L-alanyl-L-isoglutamine, commercially available from Calbiochem-Behring, La Jolla, CA.

fication with Amberlite CG400 anion-exchange resin (converted to the acetate form) was accomplished by eluting the MDP with 0.01 M acetic acid. Optical rotation measurements of our synthetic muramyl dipeptide compared favorably with the literature value.⁹ Examination of the portions of the ¹³C NMR spectra of MDP that relate to the asymmetric carbons of the dipeptide disclosed the presence of only one of the possible four diastereomers.

The fluorescent-labeled MDP was prepared by reacting MDP with DTAF in an aqueous 0.4 M Na₂CO₃/acetone solution at 45 °C for 6 h. The adduct was lyophilized and eluted twice on a Sephadex G-15 gel filtration column (*M_r* cutoff 1200). This procedure afforded a homogeneous fluorescent adduct. Proton NMR (D₂O) indicated an aromatic proton to MDP methyl proton ratio of 3:2, which is consistent with the nine nonexchangeable aromatic protons in DTAF and the two methyl groups in MDP, consistent with 1:1 ratio of fluorescent label to parent compound.

Biological Results and Discussion

In addition to the capacity of MDP to serve as the minimal adjuvant active moiety in Freund's complete adjuvant, MDP has been reported to have a number of in vitro biological activities. We chose to focus on the ability of MDP to induce spreading of cultured guinea pig peritoneal exudate macrophages³⁰ and to inhibit their migration from capillary tubes.³¹ Previous studies have demonstrated a direct correlation between the ability of MDP isomers to inhibit macrophage migration and their ability to act as adjuvants.^{31,32}

Macrophage spreading is defined as pronounced cell elongation or distinct membrane ruffling of rounded cells. The fluorescently labeled DTAF-MDP demonstrated significant spreading (Table I) at a concentration as low as 0.1 $\mu\text{g/mL}$. The maximum percent of the macrophages that exhibited spreading was 80%. DTAF-MDP was

Table II. Effect of MDP on Migration of Guinea Pig Peritoneal Exudate Cells

MDP source	concn, $\mu\text{g/mL}$	migration index				
		expt 1	expt 2	expt 3	expt 4	expt 5
DTAF-MDP	0.01	73	84	62		
	0.10	102	52	27	51	74
	1.0	63	60	18	49	70
	10.0				53	74
I-MDP ^a	0.01	119	64	52		
	0.10	68	61	17	62	
	1.0	70	68	12	52	
	10.0				63	
P-MDP ^b	0.01		64	34		
	0.10		64	34	54	
	1.0		63	26	58	
	10.0				52	65
L,L-MDP ^c	0.01	92				
	0.10	88				
	1.0	114				
	10.0	108				

^a Muramyl dipeptide prepared in our laboratory.

^b Commercially available MDP, from the Institut Pasteur, Paris. ^c *N*-Acetylmuramyl-L-alanyl-L-isoglutamine, commercially available from Calbiochem-Behring, La Jolla, CA.

comparable to our unlabeled MDP and the commercially available MDP (Institut Pasteur) at concentrations above 0.1 $\mu\text{g/mL}$. The commercially available *N*-acetylmuramyl-L-alanyl-L-isoglutamine (L,L-MDP, Calbiochem) is not active as an adjuvant. As seen in the table, very little increase in the percent of macrophages spreading was observed until a concentration of 10 $\mu\text{g/mL}$ is used. The results of this in vitro assay indicate that DTAF-MDP has the potential for being an active adjuvant.

The macrophage migration inhibition assay (Table II) focuses on the ability of MDP to directly inhibit the migration of macrophages. The migration index was determined as follows with an index of 80 being considered significantly inhibited:

$$\text{migration index} = \frac{\text{mean of test migration}}{\text{mean of control migration}} \times 100$$

Each experiment number in the table represents the use of a different animal. Although there is variability from animal to animal, the general trend is apparent: DTAF-MDP exhibits significant inhibition at comparable concentration levels as either commercial or our unlabeled MDP. It is also important to note that the inactive unlabeled L,L-MDP shows no significant inhibition of migration at any of the concentrations used.

Experimental Section

Melting points were obtained on a Thomas-Hoover Unimelt capillary melting point apparatus and are uncorrected. Polarimetric measurements were obtained on a Perkin-Elmer 141 polarimeter. Proton NMR spectra were generated on a Varian EM 360 1H NMR. Microanalysis was performed by Galbraith Laboratories, Inc., Knoxville, TN. Sephadex G-15 (Sigma Chemical Co.) was used for column chromatography.

γ -Benzyl Isoglutamate Perchlorate (8). To 300 mL of benzyl acetate were added 3.36 g (22.6 mmol) of 7 and 3.0 mL of 70% perchloric acid. The reaction was stirred at room temperature for 4 days and precipitated with 600 mL of anhydrous Et₂O, and the product was collected by filtration. Recrystallization from MeOH-Et₂O afforded 7.65 g (99%) of 8: mp 171–172 °C; $[\alpha]_{\text{D}}^{25} -13.4^\circ$ (MeOH). Anal. (C₁₂H₁₆N₂O₃·HClO₄) C, H, N.

γ -Benzyl *N*-(*tert*-Butyloxycarbonyl)-L-alanyl-D-isoglutamate (10). Compound 10 was prepared according to the procedure of LeFrancier et al.⁹ yield 4.73 g (77.5%); mp 139–140 °C (lit.²⁹ mp 140 °C); $[\alpha]_{\text{D}}^{25} -9.1^\circ$ (MeOH) [lit.²⁹ $[\alpha]_{\text{D}}^{25} -9.0^\circ$ (MeOH)]. Anal. (C₂₀H₂₉N₃O₆) C, H, N.

(30) Tanaka, A.; Nagao, S.; Imai, K.; Mori, R. *Microbiol. Immunol.* 1980, 24, 547.

(31) Yamamoto, Y.; Nagao, S.; Tanaka, A.; Koga, T.; Onoue, K. *Biochem. Biophys. Res. Commun.* 1978, 80, 923.

(32) Nagao, S.; Tanaka, A.; Yamamoto, Y.; Koga, T.; Onoue, K.; Shiba, T.; Kusumoto, K.; Kotani, S. *Infect. Immun.* 1979, 24, 308.

N-Acetylmuramyl-L-alanyl-D-isoglutamine (1). By the procedure for LeFrancier et al.,⁹ compound 13 was hydrogenolized over 5% palladium on charcoal to give 1: yield 2.427 g (97.9%); mp 134 °C dec; a portion (200 mg) of 1 was eluted on an Amberlite CG400 column (converted to acetate form) with 0.01 M AcOH and then lyophilized: $[\alpha]^{22}_D +43.5^\circ$ (AcOH) [lit.⁹ $[\alpha]^{25}_D +44^\circ$ (AcOH)]; NMR ($\text{Me}_2\text{SO}-d_6$) δ 1.23 (m, 6 H, Ala- CH_3 , Mur- CH_3 superimposed) 1.83 (s, 3 H, NHCOCH_3), 1.90 (m, 2 H, $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 2.10 (m, 2 H, $\text{CH}_2\text{CO}_2\text{H}$), 8.46 (d, 1 H, CHNH), 8.86 (d, 1 H, CRHNH). Anal. ($\text{C}_{19}\text{H}_{32}\text{N}_4\text{O}_{11}\cdot\text{AcOH}\cdot 2\text{H}_2\text{O}$) C, H, N.

Preparation of DTAF-Labeled MDP (3). MDP (1; 100 mg, 0.2 mmol) was dissolved in 0.4 M Na_2CO_3 (2.5 mL). The MDP solution was added to a solution of DTAF (118.0 mg, 0.2 mmol) in acetone (30 mL) and heated at 45 °C for 3 h. The acetone was removed under reduced pressure, leaving an orange oil. H_2O (2.5 mL) was added, and the crude product was eluted twice on a Sephadex G-15 column to give pure MDP-DTAF: yield 92.3 mg (46.9%); NMR (D_2O) δ 1.20 (m, 6 H, Ala- CH_3 , Mur- CH_3 superimposed), 1.85 (s, 3 H, NHCOCH_3), 1.90 (m, 2 H, CH_2), 2.10 (m, 2 H, CH_2), aromatic protons appear from δ 6.0 to 8.0 as overlapping multiplets integrating out to 9 protons. Anal. ($\text{C}_{42}\text{H}_{41}\text{N}_8\text{O}_{17}\text{Na}$) C, H, N.

Biological Methods. Macrophage Recovery. Outbred guinea pigs obtained from a local supplier were used as the source of peritoneal exudate cells. An inflammatory exudate was induced by intraperitoneal injection of 30 mL of mineral oil 3–5 days before sacrifice. The animals were euthanized by barbiturate overdose, and cells were recovered as follows. An abdominal midline incision was made, and sterile saline containing 5 units/mL of heparin was poured through the retracted incision site. The opening was clamped, and the abdominal cavity was kneaded externally. The fluid was removed by insertion of a sterile perforated tube, the contents of which were then transferred by pipet to a sterile collection bottle. This peritoneal lavage procedure was repeated three times. The oil layer was removed, and cells were collected by centrifugation. Recovered cells were washed 3 times in Hank's balanced salt solution and resuspended in RPMI-1640 tissue culture media supplemented with 5% heat-inactivated newborn calf serum, 1 mM L-glutamine, 20 mM HEPES buffer (pH to 7.3), 50 units/mL of penicillin, and 50 $\mu\text{g}/\text{mL}$ of streptomycin.

Macrophage Spreading Assay. Macrophage spreading was performed as described by Tanaka et al.³⁶ Peritoneal exudate

cells were diluted to $5 \times 10^5/\text{mL}$ in supplemented RPMI-1640 media, and 1.8 mL of the cell suspension was added to a series of 35-mm Falcon culture dishes. Each plate then received 0.2 mL of MDP diluted to give final concentrations ranging from 10.0 to 0.01 $\mu\text{g}/\text{mL}$. Control plates received 0.2 mL of diluent. Plates were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 4 h. The culture fluid was decanted, and the plate was washed 3 times with warm Hank's balanced salt solution to remove non-adherent cells. The plates were immediately fixed by addition of 10% neutral buffered formalin. The plates were blind coded, and spreading was assessed by a single observer using phase contrast microscopy. Spreading was defined as pronounced cell elongation or distinct membrane ruffling of rounded cells. Five representative fields were examined, and a minimum of 100 cells were counted, from which a percentage of spread cells was determined.

Macrophage Migration Inhibition Assay. Macrophage migration in the presence or absence of MDP was assessed by a standard capillary tube assay³¹ using cells cultured in the supplemented RPMI-1640 media described above. Sterile 50- μL hematocrit tubes were filled with cells at a concentration of $2 \times 10^7/\text{mL}$, and one end was sealed with Critoseal. The cells were pelleted, and the tube was broken at the cell-fluid interface. The packed cells were placed in the wells of a leukocyte migration plate (Sterilin, Oxford, England), which had been filled with media with or without MDP. For each MDP concentration tested, the assay was run in quadruplicate. The chambers were covered with glass cover slips and incubated for 18 h at 37 °C in a humidified 5% CO_2 atmosphere. The cell migration pattern was projected onto a screen, where it was traced. The area of the projection pattern was measured on an MOD digitizer (Carl Zeiss Inc.). A migration index was determined as follows, and an index of less than 80 was considered to be significant inhibition.

$$\text{migration index} = \frac{\text{mean migration area of test}}{\text{mean migration area of control}} \times 100$$

Acknowledgment. This research was supported by NIH Research Grants HL 19873 and HL 22676.

Registry No. 1, 53678-77-6; 2, 87137-43-7; 3-2Na, 87137-44-8; 4, 6893-26-1; 5, 63648-73-7; 6, 19522-39-5; 7, 19522-40-8; 8-HClO₄, 87137-45-9; 9, 15761-38-3; 10, 18814-49-8; 11-HCl, 59524-62-8; 12, 87173-13-5; 13, 87173-14-6.