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Report

Formulation of Vaccine Adjuvant Muramyldipeptides (MDP). 2. The Thermal Reactivity and pH of Maximum Stability of MDP Compounds in Aqueous Solution

Michael F. Powell,[1,2] Linda C. Foster,[1,3] Allyn R. Becker,[1] and William Lee[1,3]

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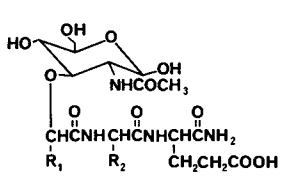
The degradation of muramyldipeptides (MDPs) in aqueous solution obeys the rate law $k_{obs} = k_{H^+}a_{H^+} + k_o + k_{HO^-}a_{HO^-}$ and the Arrhenius equation. For example, the rate constants for degradation of *N*-acetylmuramyl-L-threonyl-D-isoglutamine, 3, at 25°C are $k_{H^+} = 2.3 \times 10^{-6}\ M^{-1}\ sec^{-1}$, $k_o = 8.2 \times 10^{-10}\ sec^{-1}$, and $k_{HO^-} = 0.19\ M^{-1}\ sec^{-1}$. The degradation rates are dependent on the side-chain substituents; it is predicted that sterically hindered MDP compounds will show an extended shelf life in aqueous solution. Product studies in the weakly acid pH region (where the pH of maximum stability occurs) show that MDP compounds degrade largely by hydrolysis of the dipeptide side chain. These data show that MDP 3 exhibits a shelf life (t_{90}) of greater than 2 years in aqueous solutions of pH 4–4.5, the pH of maximum stability.

KEY WORDS: muramyldipeptide; hydrolysis kinetics; parenteral formulation; high-pressure liquid chromatography (HPLC).

INTRODUCTION

Muramyldipeptide (MDP; 1) makes up a portion of the repeating subunit of peptidoglycans (1,2) and is the smallest fragment that can substitute for mycobacteria in Freund's complete adjuvant (3,4). Because this small molecule elicits both cell-mediated and humoral immune responses and stimulates carbon clearance by the reticuloendothelial system and nonspecific immunity against infections (5), it has enjoyed a decade of popularity as a trial vaccine adjuvant. Unfortunately, 1 is pyrogenic (6,7) and shows some local toxicity in the form of inflammation and granulomas at the injection site (8,9). The design of MDP vaccine adjuvants involves the modification of the peptide side chain for separation of the desirable and undesirable properties. A considerable degree of separation has been found, in that some muramyl dipeptides show reduced toxicity without lessened immunoactivity (10–12). This was the rationale for the design of MDPs 2–4 (Scheme I).



	R_1	R_2
1	CH_3	CH_3
2	H	CH_3
3	CH_3	$CH(OH)CH_3$
4	CH_3	CH_2CH_3

Scheme I

The design of a superior vaccine adjuvant formulation involves optimizing both the MDP component and, surprisingly, the delivery vehicle. Not only must the vehicle be optimized for biological activity, but also it must provide a stable environment for the MDP component. Many of the vehicles used are water-in-oil (w/o) emulsions comprised of nonionic block polymer surfactants (13–15). In our hands, MDP in water-in-oil emulsions resides largely in the aqueous phase, thus making MDP susceptible to hydrolytic degradation. To date, the thermal reactivity of MDPs in aqueous solutions has gone unreported, and a dearth of information exists on the optimal formulation pH. To fill this void, we studied the pH and temperature dependence for the reaction of MDPs 2–4. Herein, we also show that the substituents on the dipeptide side chain govern the rate of MDP degradation.

EXPERIMENTAL

Materials. Compounds 2–4 were prepared at the Institute of Organic Chemistry at Syntex (Palo Alto, Calif.); the synthetic details of these and other MDP compounds are described elsewhere (16,17). The compounds 2-mercaptoethanol, orthophthalaldehyde (OPA), *N*-acetylmuramic acid, *N*-acetyl-desmethylmuramic acid, alanine, isoglutamine, glutamic acid, and threonine were of reagent grade (or better) and were used without further purification. Buffer solutions of carbonate, acetate or phosphate, HCl, KCl, and NaCl were reagent grade (Aldrich or Mallinckrodt) and were also used without further purification. The mobile phase was prepared using high-pressure liquid chromatographic (HPLC)-grade methanol and distilled deionized water.

HPLC Method. Amino acid analysis was carried out

[1] Institute of Pharmaceutical Sciences, Syntex Research, Palo Alto, California 94304.

[2] To whom correspondence should be addressed. Present address: Cytel Corporation, 12520 High Bluff Drive, San Diego, California 92130.

[3] Present address: California Biotechnology, Mountain View, California 94303.

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using an HP 1090 HPLC system equipped with a programmable autoinjector (see Amino Acid Analysis). The separation and kinetic analyses of MDP compounds from their degradation products were carried out using an HPLC system consisting of a Micromeritics Model 725 autoinjector, a Model 110A Altex pump, a Model 770 Spectra Physics spectrophotometric detector, and an SP 4000 computing integrator. The reverse-phase (RP) HPLC method provided a linear response throughout the range of 0.10–10 μg MDP analogue injected. The method was as follows: column, Altex Ultrasphere ODS (25 × 0.46 cm, 5 μm); mobile phase, 2% methanol/98% 0.10 *M* phosphate buffer adjusted to pH 3 with H_3PO_4; flow rate, 1–1.5 ml/min; and detection, 210 nm. Stability specificity was shown by (i) first-order disappearance of the reactant HPLC peaks to baseline, (ii) spectral similarity of the leading and trailing edges of the HPLC reactant peak (which shows that the HPLC peak is not two or more peaks compressed together), and (iii) HPLC mobile-phase adjustment.

Each MDP compound eluted in two peaks by this method because of the resolution of the α and β anomers on the HPLC column. Typical retention volumes for the α and β anomers of 2 and 4 were approximately 26 and 16 ml, respectively; similarly the α and β anomers of 3 showed retention volumes of 14 and 6 ml. Kinetic samples were equilibrated in mobile phase for 2 hr before HPLC analysis; this was sufficient time for mutarotation to reach equilibrium. Following this, drug concentrations were quantitated using either the α- or the β-anomer peak. A small amount of peak tailing between the α- and the β-anomer peaks was observed and is probably due to slow mutarotation of the anomers on the column. To detect *N*-acetylmuramic acid and its desmethyl derivative, the HPLC method used for the product studies was slightly different from that used for the previous kinetic studies. In this case, a totally aqueous mobile phase of 0.1 *M* phosphate buffer at pH 3 was used. The elution volumes were 4.5 and 8.5 ml for the *N*-acetylmuramic acid anomers and 3.5 and 5.0 ml for the *N*-acetyl-desmethylmuramic acid anomers.

Amino Acid Analysis. Amino acid analysis was carried out using orthophthalaldehyde (OPA) derivatization with detection at 338 nm. Reaction solutions were neutralized using an equivalent volume of 0.1 *N* KOH and then derivatized with OPA and 2-mercaptoethanol (18). The reagents were mixed automatically and reproducibly by the HPLC autoinjector. Control solutions (containing no amino acids or MDP) and amino acid reference solutions were analyzed similarly. The HPLC conditions were similar to those above, except that a solvent gradient was used. The solvent gradient was ramped linearly from 0.1 *M* NaOAc, 0.01 *M* NaH_2PO_4, 0.01 *M* Na_2HPO_4, 2% CH_3CN at zero time to ~40% CH_3CN in 25 min. By this method, the retention volumes for the OPA derivatives of glutamic acid, isoglutamine, threonine, and alanine were 11.0, 17.5, 18.5, and 19.4 ml, respectively.

Kinetics. To obtain pseudo-first-order kinetics, the buffer concentration (~0.005–0.15 *M*) was always maintained in large excess over the drug concentration ($1–25 \times 10^{-4}$ *M*). In all experiments, buffer solutions were prepared shortly before use, then serially diluted, and the pH of each serial dilution was determined at the reaction temperature. The pH meter and electrode were calibrated at the same temperatures. Except for acid solutions greater than 0.1 *M*, the ionic strength was maintained constant at 0.15 *M* by the addition of sodium chloride or potassium chloride. Solutions of pH 2 or less were made up using hydrochloric acid of a known concentration; the pH's of these solutions were calculated from known activity coefficients or H_0 values (19,20). Stock solutions of 2, 3, or 4 were prepared in water and stored in the dark at 4°C when not in use. For the slower kinetic runs or those carried out at elevated temperatures, 100 ml of reaction solution and a small amount of the drug stock solution (~0.1–0.5 ml) were mixed well before 5-ml aliquots of the mixture were transferred to amber ampoules, flame sealed, and temperature equilibrated. At known time intervals, ampoules either were removed from the temperature bath and refrigerated or were assayed immediately by HPLC against a freshly prepared reference solution of drug. Upon removal of the last sample, all of the stored samples were analyzed on the same day. Faster reaction rates were obtained by removing aliquots from a single reaction vessel at given time intervals, immediately quenching with acetate buffer to a final pH of ~4–5, and then assaying as before. In a typical experiment, 8–12 samples were analyzed, and the peak area integrations were converted to concentrations or percentage remaining values by the use of linear response calibration curves determined earlier for 2–4. pH measurements were also carried out to ensure that the solutions maintained their buffer capacity during the course of the reaction.

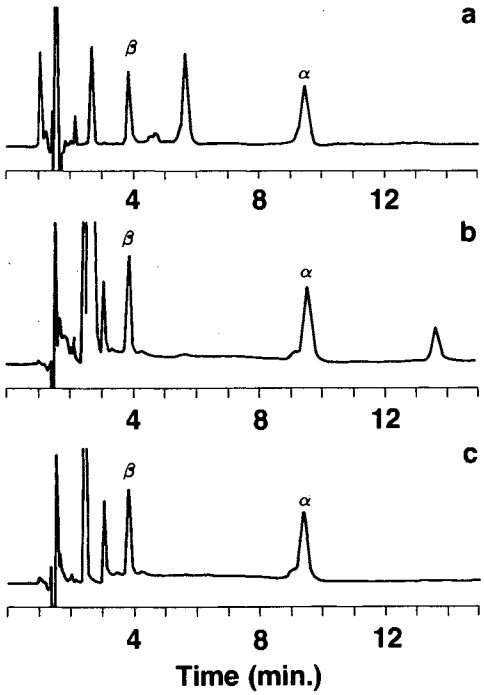


Fig. 1. HPLC chromatograms for the degradation of 3 at (a) pH 1 (60°C, 50% drug remaining), (b) pH 6 (80°C, 41% drug remaining), and (c) pH 8.6 (60°C, 50% drug remaining). The flow rate was 1.5 ml/min; the α and β isomers of 3 are indicated above.

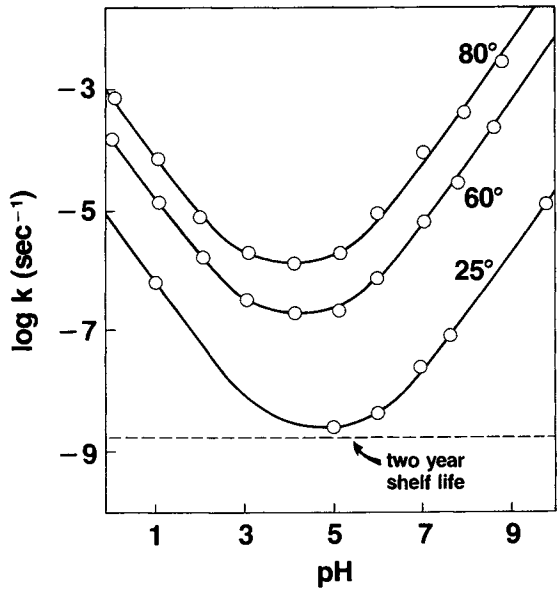


Fig. 2. pH–rate profile for the degradation of muramyldipeptide 2 in aqueous solution.

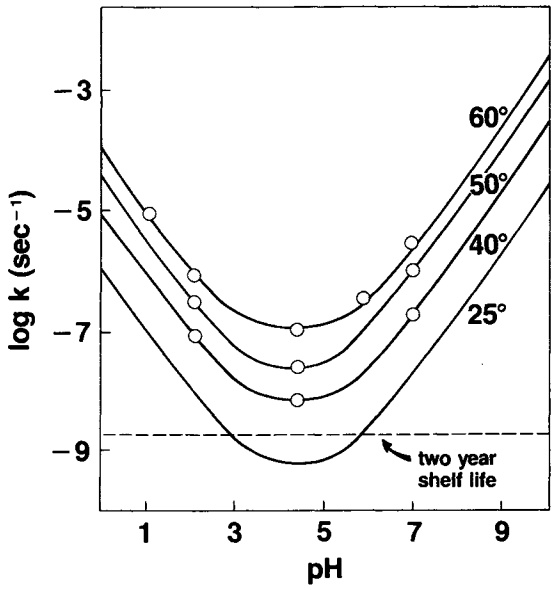


Fig. 3. pH–rate profile for the degradation of muramyldipeptide 3 in aqueous solution. The lower line for the degradation of 3 at 25°C is calculated from the Arrhenius parameters obtained from the 40–60°C data. The 40 and 50°C data were fitted according to Eq. (1) such that the slopes of +1 and −1 for the acid and base regions were assumed by analogy with the other curves having more data points.

RESULTS AND DISCUSSION

In this paper, the second in a series of four on the formulation of muramyldipeptide vaccine adjuvants (21–23), we report the effect of the solution pH and reaction temperature on the degradation of MDP compounds 2–4. We are interested in the aqueous chemical stability of MDPs because it allows us to delineate the factors governing the shelf life of our MDP emulsion formulations under development. Further, these studies show how the degradation rate is affected by the dipeptide substituents, giving insight into the degradation mechanisms.

The degradation of MDP compounds 2–4 was followed by stability-specific (or stability-indicating) reversed-phase HPLC. The degradation product profile was dependent on the pH, as shown for compound 3 in Fig. 1. For most experiments, reactions were followed for more than two half-lives with observed first-order drug loss. Mutarotation did not complicate the degradation kinetics inasmuch as mutarotation was at least 10^3-fold faster than the rate of chemical degradation (23). This rate difference precludes a buildup of one of the anomers that could result in biphasic kinetics if only one of the anomers were followed. The effect of buffer catalysis on the degradation rate was minimized by carrying out reactions at a low buffer concentration, usually 0.025 *M* or less. Control experiments of variable buffer concentrations (0.01–0.1 *M*) at different pH's demonstrated that general buffer catalysis in this reaction was negligible. The pseudo-first-order rate constants were used to construct the pH–rate profiles in Figs. 2 and 3. The secondary rate constants for 4 were determined at 80°C only; for this MDP compound, $k_{H^+} = 7.5 \times 10^{-6}\ M^{-1}\ sec^{-1}$, $k_o = 1.5 \times 10^{-6}\ sec^{-1}$, and $k_{HO^-} = 0.42\ M^{-1}\ sec^{-1}$. Inspection of these data show that MDP degradation follows the rate law of Eq. (1), where the rate constants k_{H^+}, k_o, and k_{HO^-} are the

$$k_{obs} = k_{H^+}a_{H^+} + k_o + k_{HO^-}a_{HO^-} \quad (1)$$

coefficients for catalysis by hydronium ion, water (or a spontaneous reaction), and hydroxide ion, respectively. The activity of the hydronium ion, a_{H^+} (as measured by the glass pH electrode and approximately equal to $[H^+]$ under the experimental conditions used), and hydroxide ion, a_{HO^-} (as defined as $a_{HO^-} = K_w/a_{H^+}$), are used here instead of $[H^+]$ and $[HO^-]$. The secondary rate constants obtained by nonlinear least-squares analysis (24) and activation parameters derived from the data shown in Figs. 2 and 3 are given in Tables I and II. From the Arrhenius data for 2 and 3, the rate constants and shelf lives (t_{90}) at 25°C can be calculated. For example, the calculated shelf lives for 2 and 3 at 25°C and

Table I. Rate Constants and Activation Parameters for the Degradation of Muramyldipeptide 2 in Aqueous Solution

Kinetic pathway	Temp. (°C) 25	60	80	E_a (kcal mol^{-1})
k_{H^+} (M^{-1} sec^{-1})	6.1×10^{-6}	1.7×10^{-4}	8.9×10^{-4}	18.9 ± 0.1
k_0 (sec^{-1})	2.5×10^{-9}	1.6×10^{-7}	1.3×10^{-6}	23.7 ± 0.1
k_{HO^-} (M^{-1} sec^{-1})	0.19	5.1	19	17.8 ± 0.7

Table II. Rate Constants and Activation Parameters for the Degradation of Muramyldipeptide 3 in Aqueous Solution

Kinetic pathway	Temp. (°C) 25	40	50	60	E_a (kcal mol^{-1})
k_{H+} (M^{-1} sec^{-1})	2.3×10^{-6}[a]	8.7×10^{-6}	3.4×10^{-5}	1.0×10^{-4}	25.6 ± 1.2
k_0 (sec^{-1})	8.2×10^{-10}[a]	6.1×10^{-9}	2.0×10^{-8}	1.0×10^{-7}	29.3 ± 3.1
k_{HO^-} (M^{-1} sec^{-1})	0.19[a]	0.54	1.7	2.4	15.6 ± 4.4

[a] Calculated using the Arrhenius equation and the 40–60°C data. The relative error in the calculated 25°C rate constants is of the same magnitude as given for E_a in the last column.

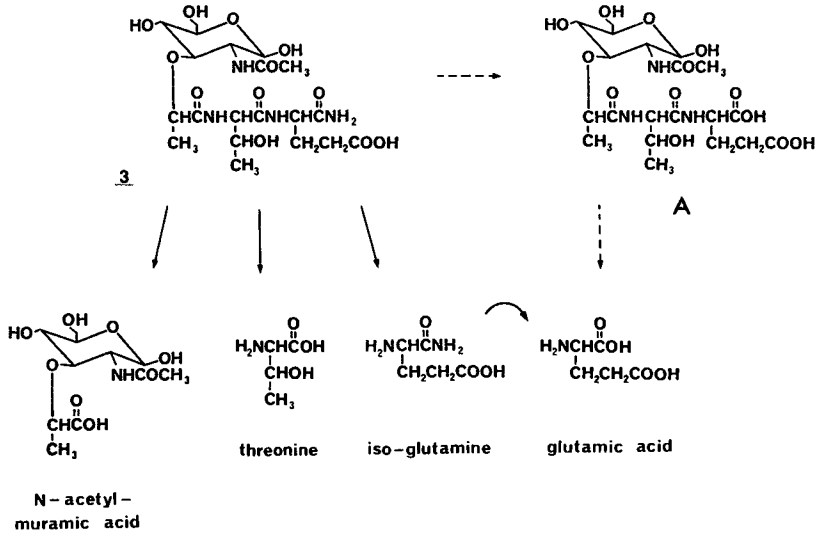
pH 4.5 are 1.2 and 5.4 years, respectively. The muramyldipeptides show maximum stability at pH 4–5; in this region these MDP compounds are approximately 10–100 times more stable than at pH 7. In comparison, the calculated RT shelf lives of 2 and 3 at pH 7.4 are 24 and 28 days, respectively.

In order to study the degradation mechanism(s) that occurs at and below the pH region of maximum stability—specifically, the acid-catalyzed reaction—we examined the degradation products and reaction kinetics for 2 and 3 under acidic conditions. The degradation studies showed that the dipeptide side chain is susceptible to acid-catalyzed cleavage. For example, when the degradation of 2 and 3 was carried out at pH 1 and 60°C, amino acid analysis of the spent reaction solutions demonstrated the formation of alanine from 2 and threonine from 3 (Scheme II). Variable amounts of isoglutamine were also found, depending on the temperature and reaction time. This variability in isoglutamine production was probably caused by the further degradation of isoglutamine to give glutamic acid and other secondary products. Typically, the alanine and threonine product yields were approximately 50% of the theoretical amount.

Further, the products *N*-acetyl-desmethylmuramic acid (for 2) and *N*-acetylmuramic acid (for 3) were observed in the acid-catalyzed degradation reactions, in support of peptide cleavage. Reaction of 2 yielded a significant amount of *N*-acetyl-desmethylmuramic acid, indicating facile cleavage of the amide group closest to the sugar moiety. In contrast, reaction of 3 under the same conditions afforded only a trace of *N*-acetylmuramic acid (the corresponding degradation product for 3), presumably because of increased steric hindrance in 3 as compared with 2 for cleavage between the muramic acid moiety and the neighboring amino acid.

Another acid-catalyzed degradation pathway for MDP compounds may also occur. The terminal amide moiety should also be susceptible to hydrolysis, as based on rate constants given in the literature for similar compounds. For example, the k_{H+} for hydrolysis of acetamide (25) at 75°C is 10.3×10^{-4} M^{-1} sec^{-1}. Similarly, the degradation rate constant of Gly-NH_2 to Gly in the luteinizing hormone releasing hormone (LHRH) analogue, nafarelin (26), at 80°C is ~12.5×10^{-4} M^{-1} sec^{-1}. These are approximately the same as the k_{H+} for 2 at 80°C (see Table I); thus, it is likely that the acid hydrolysis of 2 or 3 should also produce the glutamic acid derivative of MDP (see A, Scheme II). Scheme II does not show the stepwise nature of acid-catalyzed MDP degradation; no synchronicity or preferred pathway should be inferred from the arrows in Scheme II.

That the k_{H+} rate constants for 2 and 3 are different also supports degradation involving the dipeptide side chain. (Reaction of only the muramyl group would not result in



Scheme II

such a noticeable substituent effect because the structural differences in 2 and 3 are in the side chain, several atoms away from the muramyl group.) Slower degradation rates for 3 (relative to 2) are observed because 3 has bulkier groups flanking the amide bonds (CH_3 vs H and CH_3CHOH vs CH_3) and thus amide hydrolysis is retarded. For acid-catalyzed reactions, it is unlikely that the polar nature of the substituent affects the rate because of opposing polar effects; increased electron density at the carbonyl carbon should aid protonation but hinder nucleophilic attack by water on the amidium ion. This balancing polar effect in amide hydrolysis has been reported previously (25).

In summary, we have demonstrated that muramyldipeptides are reasonably stable in aqueous solutions at room temperature and that sterically hindered MDPs (such as 3) may be expected to show a shelf life (or expiration dating period) of 2 years or more under the appropriate pH conditions. These stability data are essential for the development of vaccine adjuvant formulations containing MDP and show that adjuvant formulations containing 3 benefit from enhanced chemical stability (compared to other MDPs) due to a rate retardation in the neutral and acid-catalyzed reaction pathways.

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