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Report

Formulation of Vaccine Adjuvant Muramyldipeptides (MDP). 1. Characterization of Amorphous and Crystalline Forms of a Muramyldipeptide Analogue

Tai Wah Chan[1,2] and Allyn Becker[1]

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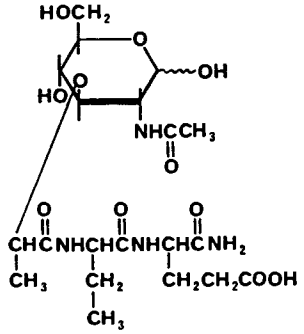
A relatively nonhygroscopic crystalline form of the glycopeptide, *N*-acetylmuramyl-L-α-aminobutyryl-D-isoglutamine (I), containing approximately one molecule of water was prepared from amorphous material. The crystalline material, consisting of a mixture of the α and β anomers, exhibited better physical and chemical stability than the lyophilized amorphous material. The α/β-anomer ratios of I in both the crystalline and the amorphous state were approximately equal but different from that in solution.

KEY WORDS: muramyldipeptide; hydrate; crystalline modification; mutarotation.

INTRODUCTION

N-[2-Acetamido-3-*O*-(D-ethyl-1-carbonyl)-2-deoxy-D-glycose]-L-alanyl-D-isoglutamine (muramyldipeptide; MDP) was proposed in 1974–1975 to be the minimum structure required to express the full spectrum of adjuvant activity when substituted for mycobacteria in Freund's complete adjuvant (1,2). Since that time considerable synthetic efforts, coupled with bioassays (3–9), have resulted in chemical modifications of MDP with improved immunoadjuvant properties and reduced toxicity. Although from a pharmaceutical standpoint crystalline material is probably most desirable, with only a few exceptions (6–8,10,11), synthetic MDP analogues have been reported to be amorphous powders.

N-Acetylmuramyl-L-α-aminobutyryl-D-isoglutamine (I) has been under development at Syntex as an agent for stimulation of nonspecific resistance to infection. During preformulation studies crystalline I was prepared from the initially synthesized amorphous material. Herein we describe our studies comparing the properties of amorphous and crystalline I.



Scheme I

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

[2] To whom correspondence should be addressed.

EXPERIMENTAL

Materials

N-Acetylmuramyl-L-α-aminobutyryl-D-isoglutamine (I) was obtained from the Institute of Organic Chemistry at Syntex (12) as a lyophilized powder. The methanol used for high-performance liquid chromatography (HPLC) was distilled-in-glass grade. All other chemicals were reagent grade.

Apparatus

X-Ray powder diffraction patterns were obtained on a General Electric X-ray diffractometer with Ni-filtered CuK_α radiation. The scanning angle was from 5 to 30° 2θ, at 2°/min. Differential scanning calorimetry (DSC) thermograms were recorded on a Perkin–Elmer DSC-2. The heating rate was 10°C/min. A nitrogen purge was maintained throughout each run. Thermogravimetric analysis (TGA) was conducted with a Perkin–Elmer TGS-2 thermogravimetric balance. The heating rate was 2.5°C/min, and a dry nitrogen purge was maintained throughout each run. Microscopic observations were made with a Leitz Ortholux II POL-BK polarized light microscope. Hot-stage microscopy was conducted with a Leitz polarized light microscope in conjunction with a Mettler FP52 hot stage equipped with a Mettler FP5 control unit. The anomer ratio of the MDP was determined using an HPLC system consisting of a Spectra Physics Model 3500 pump operated at 1.0 ml/min, a Schoeffel variable-wavelength detector monitoring at 210 nm, an SP 4000 integration system, and an Altex Ultrasphere 5μ ODS column (250 × 4.6 mm).

Preparation of Crystalline Modification of I

Two methods were employed to prepare crystalline material of I. (i) The compound (~500 mg) was dissolved in

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0.5 ml of water. After equilibration at 25°C for 2 days the solution turned into a white paste of fine needles which was then filtered and air-dried. (ii) Approximately 200 mg of I in an open vial was placed in a desiccator containing a saturated solution of sodium acetate (relative humidity, 76%). The powder first turned into a viscous solution and then solidified to a white solid mass. After 5 days the solid was removed and ground to a powder.

Monitoring of Moisture Uptake with Time by Thermogravimetric Analysis

The sample chamber of the TGA apparatus was maintained at a constant relative humidity by passing in nitrogen which had been bubbled through a saturated sodium acetate solution (relative humidity, 76%). The relative humidity of the atmosphere over the sample was approximately equal to that of a saturated salt solution. The weight of a sample of amorphous I was monitored for 25 hr at 76% relative humidity and ambient temperature.

HPLC Analytical Methods

Two reverse-phase HPLC analytical methods were developed for the quantitation of I. Method I was used for studying the degradation of the compound. Method II was used for determining the anomer ratio of I in solution under conditions where degradation of I was not observed. The mobile phase for Method I was 9% methanol–91% 0.1 *M* potassium dihydrogen phosphate buffer adjusted to a pH of 3.0 with concentrated phosphoric acid. Method II used a mobile phase consisting of 15% methanol–85% 0.1 *M* potassium dihydrogen phosphate adjusted to a pH of 3.0 with concentrated phosphoric acid. For both methods a linear relationship was obtained between the detector response and the amount of compound injected over the range of 0.1 to 10 μg. Typical chromatograms of undegraded I using Methods I and II are shown in Figs. 1A and B, respectively.

RESULTS AND DISCUSSION

The lyophilized powder of I consisted of irregular isotropic glass-like plates (Fig. 2A). An X-ray powder diffraction pattern of this solid showed a broad band that is characteristic of amorphous materials (Figure 3A). Materials obtained by crystallization from a supersaturated solution at room temperature (Method I) and by exposure of the amorphous material to high humidity (Method II) for a few days were crystalline as indicated by powder X-ray diffraction (Figs. 3B and C) and polarizing microscopy. The crystals obtained by Method I were fine needles, whereas the crystals obtained by Method II were agglomerates of birefringent rods (Fig. 2B).

Thermal Analysis

Amorphous I softened and melted at 70–75°C. The differential scanning calorimetry (DSC) thermogram showed a broad endotherm at 45–129°C (Fig. 4A). By hot-stage microscopy, using a drop of silicone oil under the glass coverslip to facilitate observation of gaseous evolution, crystalline I was found to release bubbles at about 120°C and then decompose at 158.6°C. The DSC thermogram of the crystalline material prepared by both methods showed two endothermic transitions: one at 80–130°C and the other at 175°C (Fig. 4B). The first endotherm was due to the release of water of hydration, and the second endotherm was due to melting. The first DSC transition was found to be reversible

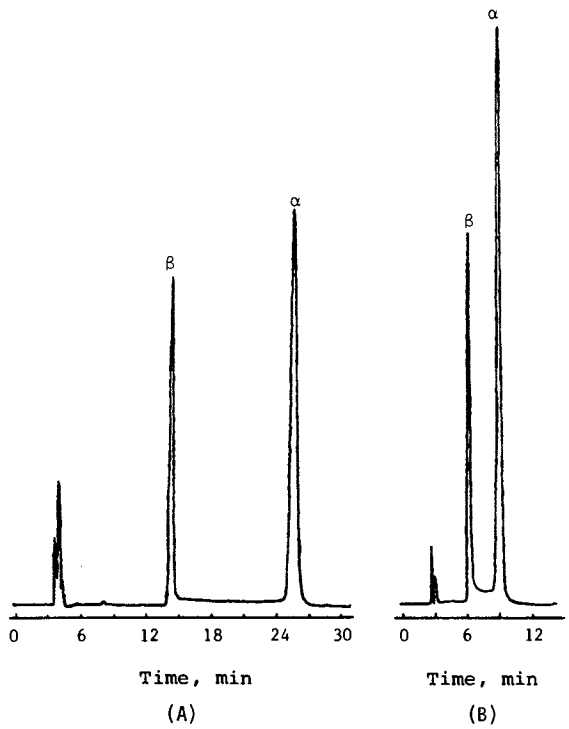


Fig. 1. Typical HPLC chromatogram of an undegraded sample of I using (A) Method I and (B) Method II.

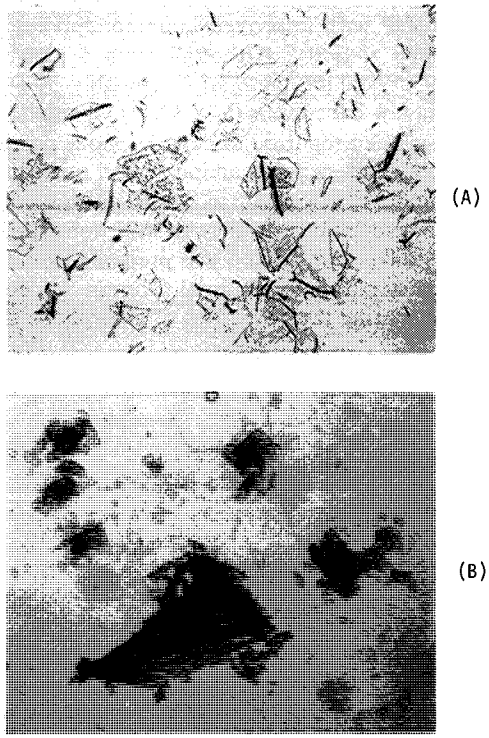


Fig. 2. Photomicrograph of (A) amorphous I and (B) crystalline I. 133×; reduced 35% for reproduction.

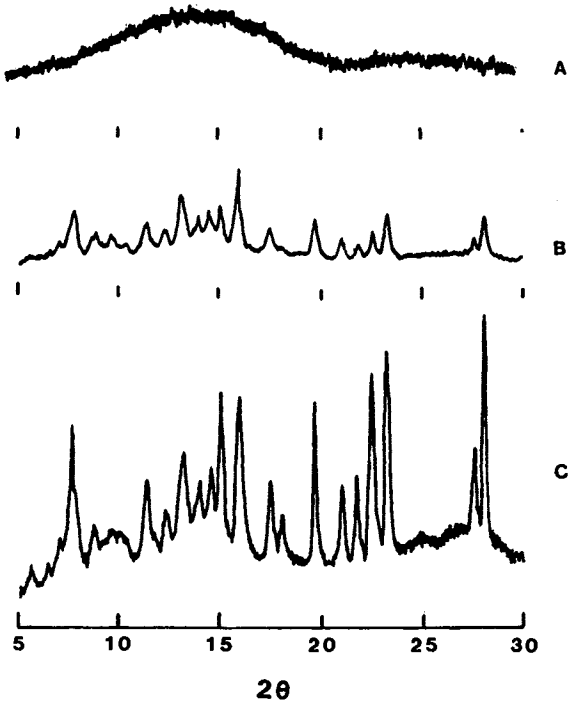


Fig. 3. X-Ray powder diffraction patterns of (A) amorphous I, (B) crystalline I obtained by crystallization from a supersaturated solution of I in water, and (C) crystalline I obtained by exposing amorphous I to high-humidity conditions (relative humidity, 76%).

by the following experiment. A small amount of crystalline material was heated in the DSC to 140°C (Fig. 5A) and then cooled to 40°C under nitrogen. The thermogram obtained on the resulting material did not show the initial transition, as the water of crystallization had already been removed (Fig. 5B). However, on exposure to air (relative humidity, ~40%) for 2 to 3 hr, the first endothermic transition was re-

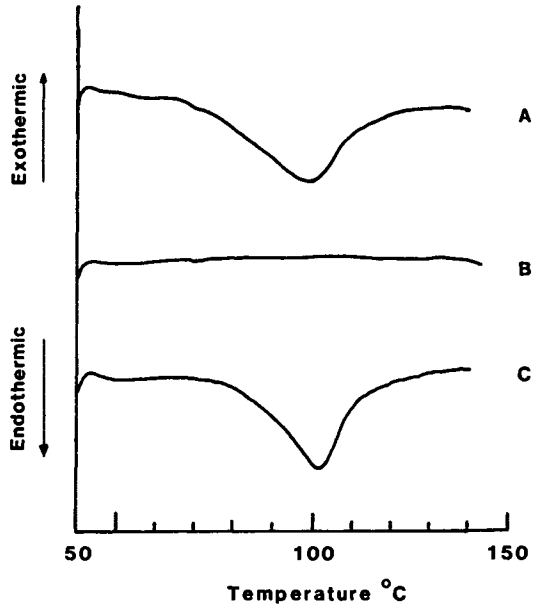


Fig. 5. DSC thermogram of (A) crystalline I, (B) the residue after heating crystalline I to 140°C and cooling under nitrogen, and (C) the dehydrated material by exposure to ambient atmospheric conditions (relative humidity, 40%).

stored, indicating that the anhydrous solid had regained its water of hydration (Fig. 5C). Thermogravimetric analysis (TGA) of an air-dried crystalline sample showed that water was release on heating in two stages: an initial gradual loss of 1.5% of the total weight between 40 and 55°C, followed by a further weight loss of 4.09% between 60 and 130°C (the theoretical value for the monohydrate is 3.43%). The initial stage probably represented a slow release of loosely bound surface moisture, while the second stage could be attributed to the loss of one molecule of water of hydration with some adsorbed water. The TGA results thus suggest that the crystalline material was a monohydrate.

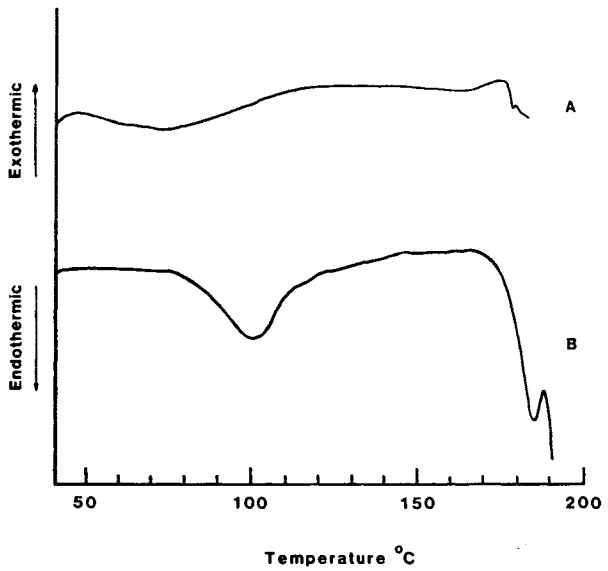


Fig. 4. DSC thermogram of (A) amorphous I and (B) crystalline I obtained by Method II.

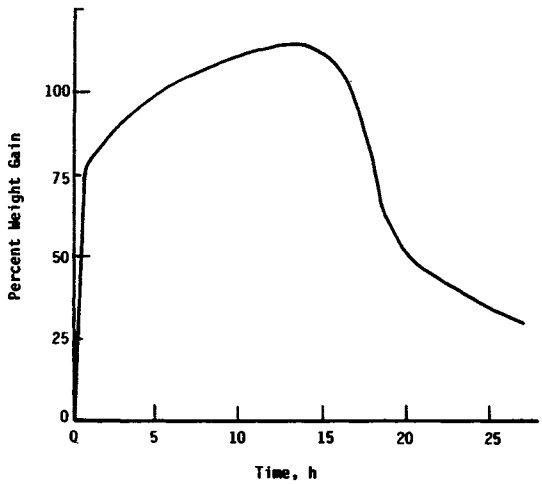


Fig. 6. Plot of weight gain versus time for a sample of amorphous I exposed to a relative humidity condition of 76% in the sample chamber of a thermogravimetric balance at room temperature.

Hygroscopicity of Amorphous I

The weight gain due to the absorption of moisture by amorphous material was followed over a period of 25 hr. As illustrated in Fig. 6, exposure of anhydrous amorphous material to a relative humidity of 76% resulted in an initial sharp gain in weight, followed by a slow decrease in weight after reaching a maximum. The final material was shown by powder X-ray diffraction to be identical to the crystalline material prepared by the methods described under Experimental. These observations demonstrated that the conversion of the amorphous to the crystalline form proceeded by the following pathway:

$$\text{Amorphous} \xrightarrow{+H_2O} \text{supersaturated solution} \xrightarrow{-H_2O} \text{crystalline}$$

The amorphous material turned into a syrupy solution when exposed to 76% relative humidity, whereas the crystalline material remained as a solid under similar conditions.

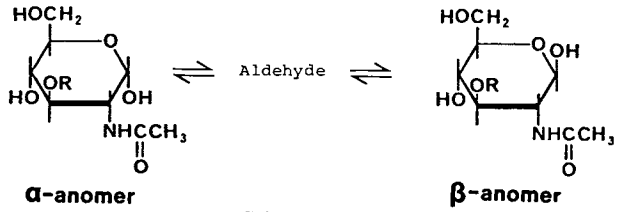
Chemical Stability of I

Under 76% relative humidity at 80°C crystalline I was found to exhibit significantly better chemical stability than the amorphous form (Table I).

Anomer Ratio of I in the Solid State

I can exist in both the α and the β anomeric forms. To determine the α/β-anomer ratio of solid I the rate of mutarotation of I in solution was studied.

The mutarotation between the two anomers can be described by Eq. (1).



Scheme II

^{13}C-Nuclear magnetic resonance (NMR) showed I to contain 67% of the α anomer and 33% of the β anomer[3] at equilibrium in dimethyl sulfoxide (DMSO). Since the anomer ratios of MDP and some of its analogues were shown to be same in both DMSO and water (13–16), we also expect that the anomer ratio for I would be similar in water.

Although HPLC showed that the two anomers eluted as separate peaks, the chromatogram did not return to baseline in the region between the two peaks by either HPLC method (Figs. 1A and B). However, a return to baseline was always obtained after elution of the second peak. This phenomenon is due to slow mutarotation of the two anomers during transit through the HPLC column.

Upon initially dissolving I in water and repeatedly analyzing the anomer ratio by HPLC, the first eluting peak was found to grow in area at the expense of the second eluting peak. The total area of integration was always constant, suggesting that the molar absorptivities of the two anomers were equal. After equilibrium was reached, subsequent injections into the HPLC showed no further changes in the anomer ratio. By area integration the equilibrium ratio of the first to the second eluting peak was approximately 1 to 2. This is in good agreement with the equilibrium ratio obtained by ^{13}C-NMR and suggests that the β anomer eluted

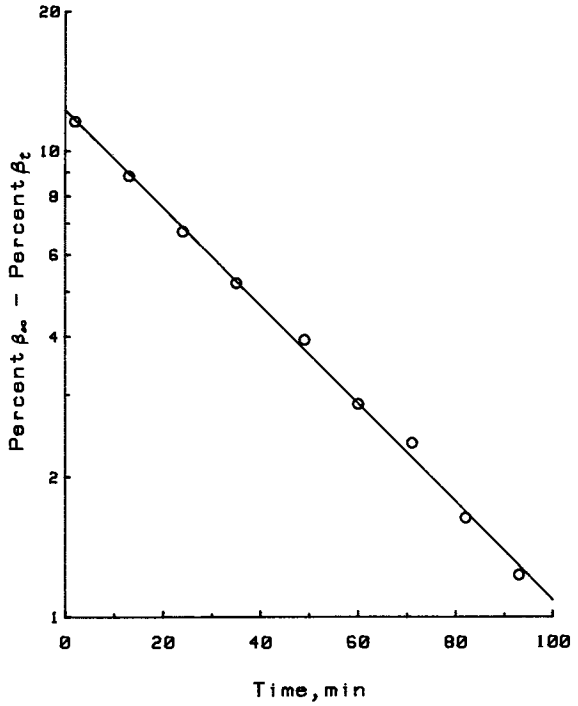


Fig. 7. A semilogarithmic plot for the mutarotation of amorphous I (0.10 mg/ml) at room temperature in 0.01 *M* acetate buffer (μ = 0.154 with added KCl) at pH 4.95.

[3] Syntex internal research report.

Table I. Stability of Amorphous and Crystalline I Stored for 4 Days at 80°C and 76% Relative Humidity[a]

Material	Percentage remaining
Amorphous	5.0
Crystalline[b]	83.0

[a] Assayed by HPLC Method I.
[b] Prepared by exposure to high humidity.

Table II. Anomer Ratios of Amorphous and Crystalline I in Solution at pH 4.95

Material	% β_i[a]	β_i/α_i[b]	% β_∞[c]	$\beta_\infty/\alpha_\infty$
Amorphous	20.4 ± 1.1	0.26	32.6	0.49
Crystalline[d]	17.0 ± 1.0	0.20	32.8	0.49
Crystalline[e]	20.0 ± 1.1	0.25	32.9	0.49

[a] Percentage of the β anomer initially, with 95% confidence interval.
[b] The percentage of the α anomer is obtained by substraction from 100%.
[c] Percentage of the β anomer at equilibrium; average of two determinations.
[d] Prepared by precipitation from aqueous solution.
[e] Prepared by exposure to high-humidity conditions.

first in the HPLC system. This conclusion is also supported by the finding of Halls and co-workers (13) that the β anomer of MDP eluted prior to the α anomer by reverse-phase HPLC.

The mutarotation of I was found to obey first-order kinetics. A typical first-order plot of the difference between the percentage β anomer at equilibrium and the percentage at any particular time versus time is shown in Fig. 7. The anomer ratio of I in the solid state was calculated from the intercept of the plot at zero time. The initial and the equilibrium anomer ratios for amorphous and crystalline I in aqueous buffer at pH 4 are shown in Table II. While the percentage of the β anomer in solution at equilibrium (33% β anomer) was independent of the starting material, the percentage of the β anomer in the amorphous material was approximately equal to that in the crystalline material and lower than that in solution. Although the solid materials were enriched in the α anomer relative to that observed in solution, we were not able to obtain a crystalline form of I which contained only one of the two anomers. Thus the crystalline material obtained could be a mixture of two crystal forms, one containing the α anomer and the other the β anomer.

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REFERENCES

1. F. Ellouz, A. Adam, R. Ciorbaru, and E. Lederer. *Biochem. Biophys. Res. Comm.* 59:1317–1325 (1974).
2. C. Merser and P. Sinay. *Biochem. Biophys. Res. Comm.* 66:1316–1322 (1975).
3. S. Kotani, Y. Watanabe, F. Kinoshita, T. Shimono, I. Morisaki, T. Shiba, S. Kusumoto, Y. Tarumi, and K. Ikenaka. *Biken J.* 18:105–111 (1975).
4. S. Kotani, F. Kinoshita, I. Morisaki, T. Shimono, T. Okunaga, H. Takada, M. Tsujimoto, Y. Watanabe, K. Kato, S. Shiba, S. Kusumoto, and S. Okada. *Biken J.* 20:95–103 (1977).
5. L. Chedid and E. Lederer. *Biochem. Pharmacol.* 27:2183–2186 (1978).
6. A. Hasegawa, H. Okumura, H. Kiso, M. Kiso, I. Azuma, and Y. Yamamura. *Agr. Biol. Chem.* 44:1301–1308 (1980).
7. A. Hasegawa, H. Okumura, M. Kiso, I. Azuma, and Y. Yamamura. *Agr. Biol. Chem.* 44:1309–1313 (1980).
8. A. Hasegawa, Y. Hioki, M. Kiso, H. Okumura, and I. Azuma. *Carbohydr. Res.* 123:183–199 (1983).
9. A. Adam, M. Devys, V. Souvannavong, P. LeFrancier, J. Choay, and E. Lederer. *Biochem. Biophys. Res. Commun.* 72:339–346 (1976).
10. S. Kobayashi, T. Fukuda, I. Imada, M. Fujino, I. Azuma, and Y. Yamamura. *Chem. Pharm. Bull.* 27:3193–3196 (1979).
11. S. Kobayashi, T. Fukuda, H. Yukimasa, M. Fujino, I. Azuma, and Y. Yamamura. *Bull. Chem. Soc. Jpn.* 53:2570–2577 (1980).
12. G. Jones, J. Moffat, and J. Nestor. Ger. Pat. No. 2718010.
13. T. D. J. Halls, M. S. Raju, E. Wenkert, M. Zubu, P. Lefrancier, and E. Lederer. *Carbohydr. Res.* 81:173–176 (1980).
14. B. E. Chapman, M. Batley, and J. Redmond. *Aust. J. Chem.* 35:489–493 (1982).
15. H. Okumura, I. Azuma, M. Kiso, and A. Hasegawa. *Carbohydr. Res.* 117:298–303 (1983).
16. E. F. McFarland and C. Martinic. *Aust. J. Chem.* 36:1087–1096 (1983).