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SYNTHESIS AND FAST-ATOM-BOMBARDMENT–MASS SPECTROMETRY OF *N*-ACETYLMURAMOYL-L-ALANYL-D-ISOGLUTAMINE (MDP)

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ABSTRACT

N-Acetylmuramoyl-L-alanyl-D-isoglutamine (MDP) was synthesized by a series of condensations of appropriate reagents, followed by hydrogenolysis. Each intermediate step resulted in a stable, crystalline product. D-Isoglutamine 4-benzyl ester was condensed with *N*-(*tert*-butoxycarbonyl)-L-alanine *N*-hydroxysuccinimide ester, to give *N*-(*tert*-butoxycarbonyl)-L-alanyl-D-isoglutamine benzyl ester. Condensation of L-alanyl-D-isoglutamine benzyl ester with *N*-acetyl-1-*O*-benzyl-4,6-*O*-benzylidenemuramic acid, followed by hydrogenolysis, gave MDP. The synthetic scheme was shown to be capable of producing gram quantities of highly pure MDP, as well as a few of its analogs. The synthetic MDP was characterized by analytical and biological methods, and it was found that the use of fast-atom-bombardment–mass spectrometry may greatly simplify the characterization process.

INTRODUCTION

The immunoadjuvant activity of bacterial cell-walls was first observed[1] in 1956. Since that time, more than modest commitment to research on the immunopotentiating properties of whole cells and cell extacts of mycobacteria has ensued[2]. Concomitantly, pursuit began toward isolation and identification of an adjuvant-active component. This was successfully concluded with the identification of *N*-acetylmuramoyl-L-alanyl-D-isoglutamine (MDP)* as the minimum structure required for the observed activity[3,4]. This discovery has led to the synthesis of MDP and a large number of MDP analogs for the investigation of their immunological properties[5–7].

Through numerous immunological evaluations of the synthetic analogs of MDP, there has been a delineation of specific structural constraints that allow expression of adjuvant activity[2]. Once the intimacies of the total structure necessary

*N-[2-*O*-(2-Acetamido-2,3-dideoxy-D-glucopyranose-3-yl)-D-lactoyl]-L-alanyl-D-isoglutamine.

for immunoadjuvant activity were known, the mode of action of MDP could then be addressed. To monitor conveniently the biological fate of MDP, the radiolabelling of MDP is to be regarded as essential at the onset. With the continuing development of synthetic pathways leading to MDP, procedural modifications to existing synthetic schemes can accomplish a specific radiolabel[8–10]. To have general utility, such a synthetic scheme should have (*1*) the convenience of intermediates possessing long-term stability, (*2*) flexibility in the substitution of reagents, and (*3*) the ability to produce a highly pure product, in quantities ranging from milligrams to grams. Although numerous syntheses for MDP and its analogs have been reported, insufficient convenience or flexibility has rendered most of them highly specific and non-general in scope. Herein, we report a synthetic scheme which, with minimum attention, will produce up to gram quantities of highly pure MDP or its analogs. Final products were characterized by traditional analytical and biological methods, and it is shown that characterization of the final product derived from this synthetic scheme may be greatly simplified through the use of fast-atom-bombardment (f.a.b.)–mass spectrometry.

EXPERIMENTAL

General. — Melting points were determined with a Fisher–Johns melting-point apparatus (Fisher Scientific, Pittsburgh, PA) and are uncorrected. Elemental analyses were performed by the Microanalytical Laboratory of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, U.S.A. Optical rotations were determined with a DIP-181 Digital Polarimeter (Japan Spectroscopic Company, Ltd., Tokyo, Japan). Whatman GF/F glass-microfiber filters were used for filtrations. Sephadex LH-20 column-packing was obtained from Pharmacia Fine Chemicals (Piscataway, NJ), and components eluted from Sephadex columns were monitored by using an LKB Model 2138 Uvicord S detector (Bromma, Sweden) operated at a wavelength of 206 nm.

Reagents. — Reagents were obtained from the following sources, and used without purification: *N*-acetyl-1-*O*-benzyl-4,6-*O*-benzylidenemuramic acid (**6**), *N*-(*tert*-butoxycarbonyl)-L-alanine *N*-hydroxysuccinimide ester, and *N*-acetylmuramoyl-L-alanyl-D-isoglutamine (MDP) (Sigma Chemical Company, St. Louis, MO); *N*-(*tert*-butoxycarbonyl)-D-isoglutamic acid 4-benzyl ester (**2**; Vega Biochemicals, Tucson, AZ, U.S.A.); benzotriazol-1-ol hydrate (Chemical Dynamics Corporation, South Plainfield, NJ); L-alanine, 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (Boc-On), *N*-hydroxysuccinimide, 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide. (DAEC), dicyclohexylcarbodiimide, trifluoroacetic acid, and *p*-toluenesulfonic acid monohydrate (Aldrich Chemical Company, Milwaukee, WI); hydrogen (Matheson Gas Products, Dorsey, MD); and palladium black (Engelhard Industries, Newark, NJ). Benzotriazol-1-ol ammonium salt was prepared according to Bajusz *et al.*[11]. Other laboratory

chemicals used in this work were A.C.S.-certified, reagent-grade materials, or the equivalent, and were used without purification.

Thin-layer chromatography (t.l.c.). — This was performed on glass plates coated with Silica Gel G (Fisher Scientific Company, Pittsburgh, PA) and developed with either 18:2:1 (v/v/v) chloroform–methanol–acetic acid or 1:1:1:1 (v/v/v/v) 1-butanol–ethyl acetate–acetic acid–water, followed by detection with ninhydrin spray, iodine vapor, or sulfuric acid charring.

9.6-MPa Liquid chromatography (l.c.). — For glycopeptide samples, this was performed, upon injection of the sample, by using a Waters Associates (Milford, MA) U6K injector, onto a Varian (Palo Alto, CA) AX-10 Micropack column (4 mm × 30 cm) at 50°. The column was eluted with 2:3 acetonitrile–triethylammonium trifluoroacetate, pH 3.1, at 1 mL/min by using twoWaters Model M-45 pumps controlled by a Waters Model 660 Solvent Programmer, and a pressure of 9.6 MPa. Components eluted from the column were detected at 206 nm by employing an LKB Model 2138 Uvicord S detector (Bromma, Sweden).

High-field, mass spectrometry. — Mass spectra were recorded with a Kratos MS-50 mass spectrometer fitted with a high-field magnet and equipped with a Kratos DS-55 data system (Data General Nova 4X minicomputer, employing the RDOS operating system). Electron-impact, mass spectra were scanned from samples introduced directly into the ion source on a ceramic-tipped, sample-probe shaft (Vacumetrics, Inc., Ventura, CA). The ion-source temperature was initially set at 100°, and then raised to 200°; mass spectra were scanned every 25 s. The accelerating potential was 8 kV, the ionizing potential was 70 eV, and the ionizing current was 400 μA.

F.a.b.–mass spectra[12–14]. — These were scanned from underivatized samples introduced directly into the ion source as colloidal suspensions in a glycerol sample-matrix (Alfa Products, Danvers, MA), placed on a gold-plated copper, sample-probe tip; bombardment was achieved with a 40-μA beam of 9-keV xenon (Matheson, Dorsey, MD) fast atoms generated in a Saddle–Field neutral-beam gun (Ion Tech, Ltd., Teddington, England).

N-(tert-*Butoxycarbonyl*)-L-*alanine*[15]. — To a stirred, aqueous solution of L-alanine (0.89 g, 10.0 mmol) were added triethylamine (2.1 mL, 15 mmol), 1,4-dioxane (6 mL), and Boc-On (2.71 g, 11 mmol). The resulting mixture became a clear, yellow solution within 15 min, and was stirred for an additional 3 h at room temperature. Water and ethyl acetate were then added, and the aqueous layer was separated, washed with ethyl acetate, and acidified with cold, M hydrochloric acid. The product was then extracted from the aqueous phase with ethyl acetate, and the extract dried (magnesium sulfate), and evaporated under diminished pressure. The solid residue was recrystallized from ethyl acetate–hexane, to yield *N*-(*tert*-butoxycarbonyl)-L-alanine as fine, white crystals (1.31 g, 69%); m.p. 81–82° (lit.[16] m.p. 83–84°).

N-(tert-*Butoxycarbonyl*)-L-*alanine* N-*hydroxysuccinimide ester*[17]. — *N*-(*tert*-Butoxycarbonyl)-L-alanine (1.89 g, 10 mmol) was added to a solution of *N*-hydro-

xysuccinimide (1.27 g, 11 mmol) and 3-[3-(dimethylamino)propyl]-1-ethylcarbodiimide (DAEC; 2.11 g, 11 mmol) in 2-propanol (5 mL), and the mixture was stirred overnight at room temperature. The solution was evaporated under diminished pressure, water was added to the residue, and the aqueous phase was extracted with ethyl acetate. The extract was successively washed with water (twice), 0.1M sodium hydrogencarbonate, and water (twice), dried (magnesium sulfate), and evaporated under diminished pressure. The solid residue was recrystallized from 2-propanol, to yield *N*-(*tert*-butoxycarbonyl)-L-alanine *N*-hydroxysuccinimide ester as fine, white crystals (1.16 g, 59%); m.p. 286–288°.

N-(tert-*Butoxycarbonyl*)-D-*isoglutamine benzyl ester* (**2**). — To a cooled solution of *N*-(*tert*-butoxycarbonyl)-D-isoglutamic acid 4-benzyl ester (**1**; 2.36 g, 7.3 mmol) and benzotriazol-1-ol ammonium salt (1.06 g, 8.0 mmol) in *N,N*-dimethylformamide (20 mL) was added dicyclohexylcarbodiimide (1.44 g, 7.0 mmol), with stirring. The mixture was allowed to warm to room temperature, and was stirred for an additional 24 h. The resulting 1,3-dicyclohexylurea was removed by filtration, and the filtrate was diluted with water (100 mL). The product was extracted from the aqueous phase with ethyl acetate, and the extract was washed successively with aqueous, 5% sodium hydrogencarbonate, aqueous 0.1M hydrochloric acid, and water, dried (sodium sulfate), and evaporated under diminished pressure. The solid residue was triturated with hexane, and then recrystallized from ethyl acetate–hexane, to yield compound **2** as fine, white crystals (2.0 g, 85%); m.p. 122–124° (lit.[18] m.p. 124°), $[\alpha]_D^{25}$ +1.5° (*c* 1.1, acetic acid) {lit.[18] $[\alpha]_D^{25}$ +2.0 ±0.5° (*c* 1.0, methanol)}; t.l.c. in 18:2:1 (v/v/v) chloroform–methanol–acetic acid, R_F 0.78.

N-(tert-*Butoxycarbonyl*)-L-*alanyl*-D-*isoglutamine benzyl ester* (**4**). — A solution of compound **2** (1.75 g, 5.4 mmol) and *p*-toluenesulfonic acid (0.99 g, 5.2 mmol) in cold trifluoroacetic acid (20 mL) was stirred for 20 min. The excess of trifluoroacetic acid was evaporated under diminished pressure and diethyl ether was added. The resulting precipitate (**3**) was filtered off, dried in a vacuum desiccator, dissolved in oxolane (100 mL), and the solution cooled. Triethylamine (0.73 mL) and *N*-(*tert*-butoxycarbonyl)-L-alanyl *N*-hydroxysuccinimide ester (1.57 g, 5.5 mmol) were added, and the mixture was stirred for 24 h at 0°, allowed to warm to room temperature, stirred for an additional 24 h, and evaporated under diminished pressure. A solution of the residue in ethyl acetate was washed successively with aqueous 5% sodium hydrogencarbonate, aqueous 0.1M hydrochloric acid, and water, dried (sodium sulfate), and evaporated under diminished pressure. The solid residue was triturated with hexane, and then recrystallized from ethyl acetate–hexane, to yield compound **4** as fine, white crystals (1.45 g, 68%); m.p. 139–140° (lit.[18] m.p. 140°), $[\alpha]_D^{25}$ −8.9° (*c* 1.0, methanol) {lit.[18] $[\alpha]_D^{25}$ −9.0 ±0.5° (*c* 1.0, methanol)}; t.l.c. in 18:2:1 (v/v/v) chloroform–methanol–acetic acid, R_F 0.68.

N-*Acetyl-1*-O-*benzyl-4,6*-O-*benzylidenemuramoyl*-L-*alanyl*-D-*isoglutamine benzyl ester* (**7**). — A solution of compound **4** (1.34 g, 3.3 mmol) and *p*-toluenesulfonic acid (0.63 g, 3.3 mmol) in cold trifluoroacetic acid (15 mL) was stirred for 20 min. The excess of trifluoroacetic acid was evaporated under diminished pressure,

and diethyl ether was added to the residue. The resulting precipitate (**5**) was filtered off, dried in a vacuum desiccator, dissolved in a mixture of *N,N*-dimethylformamide (3 mL) and oxolane (27 mL), and the solution cooled. To it were added, with stirring, triethylamine (0.46 mL), compound **6** (1.56 g, 3.8 mmol), benzotriazol-1-ol (0.51 g, 3.8 mmol), and dicyclohexylcarbodiimide (0.68 g, 3.3 mmol). The mixture was allowed to warm to room temperature, stirred for 24 h, and evaporated under diminished pressure. The residue was triturated with water, and the solid filtered off, and recrystallized from ethanol, to yield compound **7** as fine, white crystals (2.0 g, 80%); m.p. 218–220° (lit.[19] m.p. 226.5–227.5°), $[\alpha]_D^{25}$ +84.4° (*c* 0.5, *N,N*-dimethylformamide) {lit.[19] $[\alpha]_D^{25}$ +88.5° (*c* 2.1, dimethylformamide)}; t.l.c. in 18:2:1 (v/v/v) chloroform–methanol–acetic acid, R_F 0.75.

N-*Acetylmuramoyl*-L-*alanyl*-D-*isoglutamine* (**8**, *MDP*). — To a solution of compound **7** (0.250 g, 0.33 mmol) in glacial acetic acid (50 mL) was added palladium black and the compound was hydrogenolyzed for 3 to 5 days in the usual way, the progress of the hydrogenolysis being monitored by t.l.c. using any of the aforementioned solvent systems. The catalyst was filtered off, and, after addition of water (30 mL), the filtrate was evaporated under diminished pressure. The residue was dissolved in a small volume of 0.1M acetic acid and then applied to a column (2.5 × 85 cm) of Sephadex LH-20 which was developed with the same solvent. The fractions corresponding to the main peak were pooled and lyophilized. The lyophilized material was rechromatographed under the same conditions, to yield compound **8** (MDP) as fine, white crystals (0.078 g, 50%), $[\alpha]_D^{25}$ +30.4° (*c* 0.3, water; after 24 h) {lit.[19] $[\alpha]_D^{25}$ +33.1° (*c* 0.51, water; after 25 h)}; t.l.c. in 1:1:1:1 (v/v/v/v) 1-butanol–ethyl acetate–acetic acid–water, R_F 0.51.

Anal. Calc. for $C_{19}H_{32}N_4O_{11} \cdot H_2O$: C, 44.70; H, 6.71; N, 10.98. Found: C, 44.93; H, 6.62; N, 11.05.

Using the foregoing synthetic scheme as a guide, *N*-acetylmuramoyl-L-alanine (MMP) and *N*-acetylmuramoyl-L-alanyl-D-isoglutaminyl-L-lysin amide (MTP–NH_2) were similarly synthesized.

N-*Acetylmuramoyl*-L-*alanine* (*MMP*). — L-Alanine benzyl ester (1.18 g, 6.6 mmol) (to be used as the peptide moiety) was prepared, and condensed as before with compound **6**. The resulting product was subjected to hydrogenolysis as previously described, and, after processing, yielded MMP as a colorless oil (0.342 g, 14% overall); $[\alpha]_D^{25}$ +28.8° (*c* 0.2, water; after 24 h) {lit.[19] $[\alpha]_D^{17}$ +30.9° (*c* 0.51, water; after 25 h)}; t.l.c. in 1:1:1:1 (v/v/v/v) 1-butanol–ethyl acetate–acetic acid–water, R_F 0.53.

Anal. Calc. for $C_{14}H_{24}N_2O_9 \cdot H_2O$: C, 43.98; H, 6.85; N, 7.33. Found: C, 44.14; H, 6.97; N, 7.51.

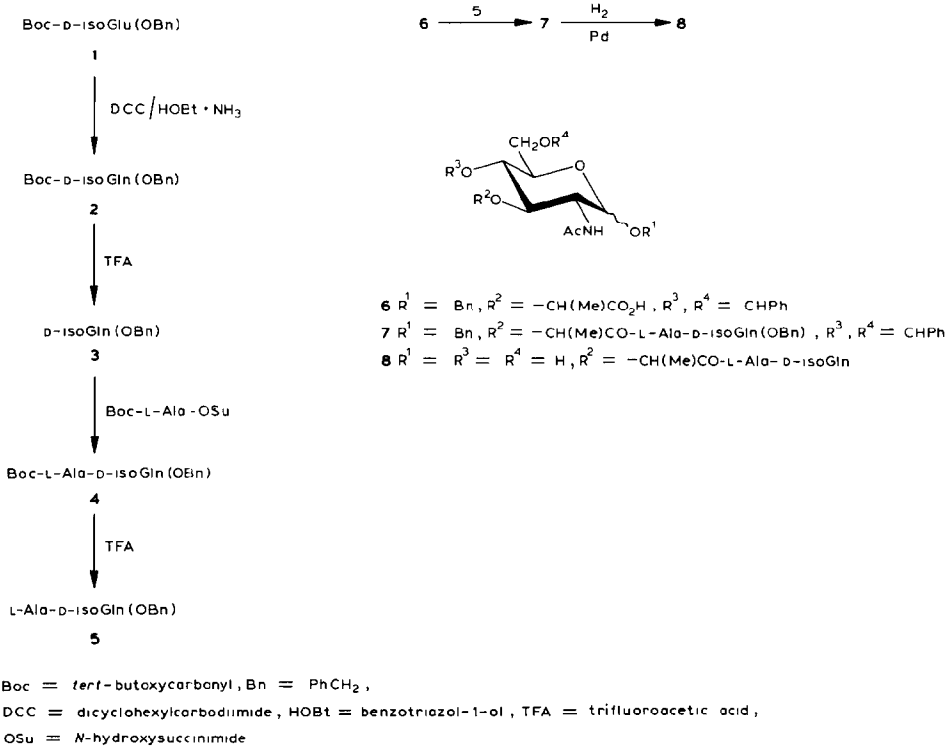
N-*Acetylmuramoyl*-L-*alanyl*-D-*isoglutamine*-L-*lysinamide* (*MTP–NH_2*). — L-Alanyl-D-isoglutaminyl-6-*N*-(benzyloxycarbonyl)-L-lysinamide (0.948 g, 2.1 mmol), to be used as the peptide moiety, was prepared, and condensed as before with compound **6**. The product was subjected to hydogenolysis as previously described, and, after processing, yielded MTP–NH_2 as a colorless oil (0.385 g, 29%

overall); $[\alpha]_D^{25}$ +14.9° (*c* 0.2, water; after 24 h); t.l.c. in 1:1:1:1 (v/v/v/v) 1-butanol–ethyl acetate–acetic acid–water, R_F 0.20.

Anal. Calc. for $C_{25}H_{45}N_7O_{11} \cdot AcOH \cdot H_2O$: C, 46.48; H, 7.37; N, 14.05. Found: C, 46.26: H, 7.44; N, 13.81.

RESULTS AND DISCUSSION

Synthesis. — A typical, synthetic approach to muramoyl peptides[7] is generally comprised of four distinct operations, including (*1*) the preparation of a protected *N*-acetylmuramide, (*2*) the preparation of a protected peptide, (*3*) coupling of the protected carbohydrate and protected peptide moieties, and (*4*) deprotection, and purification. The choice of reagent(s) in any of these operations frequently dictates the usage of reagents in the other steps, and thus each step merits brief discussion (see Scheme 1).



Scheme 1. Synthesis of *N*-acetylmuramoyl-L-alanyl-D-isoglutamine (**8**).

Preparation of a protected N-*acetylmuramide.* — Although different derivatives have been used for protecting *N*-acetylmuramide, the 1-*O*-benzyl-4,6-*O*-benzylidene derivative has been the most frequently utilized[7]. Different

methodologies for deblocking procedures have been reported, although that most commonly used has been a one-step removal of all of the protecting groups by catalytic hydrogenolysis[7,20]. However, the catalyst can be, and frequently is, stereoselective in the hydrogenolysis, thus requiring elaborate synthetic schemes in order to produce stereospecific protection of the muramoyl moiety. However, it will be shown that a slight modification in the hydrogenolysis procedure allows the use of a commercial mixture of stereoisomers, thus avoiding the need for the longer synthetic schemes.

Preparation of a protected peptide. — Preparation of *tert*-butoxycarbonyl (Boc) derivatives of amino acids was accomplished by the usual methods[15,17]. The amidation of *tert*-butoxycarbonyl-L-glutamic acid 4-benzyl ester was performed by reaction with the amine salt of benzotriazol-1-ol[21,22], a transformation previously accomplished with gaseous ammonia in the presence of isobutyl chloroformate[23,24]. Both procedures preserve the stereochemistry of the product; however, the former, employing a homogeneous reaction-mixture, tends to be more convenient. This allows for easier handling of the reagent when working with small quantities of material.

A wide variety of (*tert*-butoxycarbonyl)-amino acid esters of *N*-hydroxysuccinimide is commercially available, and they can also be conveniently synthesized from readily available materials, thus allowing a high degree of freedom in the synthesis of a desired, protected peptide. If an amino acid residue that is uncommon, modified, or labelled in some way, should be desired in the final product, homogeneous-solution chemistry minimizing the handling of reagents could be used to advantage.

Dicyclohexylcarbodiimide was used in coupling appropriately deprotected amino acid derivatives. The reaction was performed in the presence of benzotriazol-1-ol for aiding in preservation of the stereochemistry of the reaction[21,22]. Alternatively, isobutyl chloroformate has been used for the same purpose[23,24].

Thus, at each intermediate step in the preparation of the protected peptide, stable, crystallizable materials could be isolated, thus circumventing the need to use materials as soon as they had been prepared[7].

Coupling of the protected carbohydrate and protected peptide moieties. — The coupling of the protected carbohydrate and protected peptide was mediated by dicyclohexylcarbodiimide, using, as before, benzotriazol-1-ol to aid in preservation of the stereochemistry of the reaction. It should be noted that suitably protected *N*-acetylmuramide is commercially available, thus easing some of the burdens of synthesis. However, it should also be noted that the least expensive and least-used commercial *N*-acetylmuramide derivative is a mixture of the anomers. Usually, a synthetic scheme is employed in which one anomer is preferentially synthesized, for reasons involved when considering deprotection. The commercially available mixture of anomers could, perhaps, be separated chromatographically before coupling with a protected peptide. However, for reasons to be discussed, it is unnecessary to engage in lengthier synthetic-schemes or tedious separation-procedures in

order to obtain the single anomers. The mixture can be used to advantage, as it is easier to synthesize the mixture than either anomer singly, and little difficulty is encountered in deprotection.

Deprotection. — Deprotection has usually been accomplished with a supported, palladium catalyst[25], a material that necessitates use of either the α or the β anomer of the protected carbohydrate. This has been necessary, as deprotection of anomeric mixtures has resulted in mixtures of under- and over-hydrogenated products that can, in part, be attributed to different rates of hydrogenolysis of the anomers. Because the anomeric mixture is commercially available, its use in hydrogenolysis could dramatically improve the overall procedure of the MDP synthesis. Therefore, the hydrogenolysis procedure warranted investigation.

Successful hydrogenolysis is often dependent upon the composition of the support for the palladium catalyst. The support is usually derived from charcoal, coal, organic polymers, silica, or alumina[26,27], which have different reactivities towards the substrate. Thus, the yields from a given hydrogenolytic procedure may differ greatly for only slightly modified substrates. Previous schemes for the synthesis of MDP chose the α anomer, as it is easier to prepare than the β anomer, although the time needed for complete hydrogenolysis is much longer[7]. If a mixture of the anomers were to be used, the selectivity of the supported palladium catalyst toward the β anomer would allow formation of over-hydrogenation products from the product of hydrogenolysis of the β anomer during the time required for hydrogenolysis of the α anomer.

We have found that palladium black effects deprotection of a variety of anomeric mixtures of MDP analogs. This procedure does not require prior separation, or directed synthesis, of a given anomer of the muramic acid. Furthermore, the hydrogenolysis is reproducible for several MDP analogs.

Although the use of palladium black resulted in reproducible deprotection of anomeric mixtures, with minimal formation of side products, prehydrogenation of the catalyst was necessary for formation of a consistent product. The duration of hydrogenolysis was found to be relatively noncritical: the optimal time appeared to be between 3 and 5 days. Longer times gave over-hydrogenated side-products, and shorter times resulted in an only partially deprotected product. The ability to use anomeric mixtures greatly simplifies the choice of a scheme for synthesizing the protected carbohydrate moiety, allowing greater flexibility, and lessening the effort expended in the synthesis.

General characterization. — Physical constants, including melting point, elemental analysis, optical rotation, and thin-layer chromatographic (t.l.c.) behavior, compared favorably with previous reports[15–19] for the synthetic *N*-acetylmuramoyl-L-alanyl-D-isoglutamine (MDP) and its reaction intermediates. The synthesized material was shown, by 9.6-MPa l.c. (see Fig. 1), to possess a retention index and purity comparable to those of commercially available MDP. Further verification of the correctness of the chemical structure assigned was demonstrated by electron impact (e.i.)–mass spectrometry (m.s.) of the *N,O*-permethylated deriva-

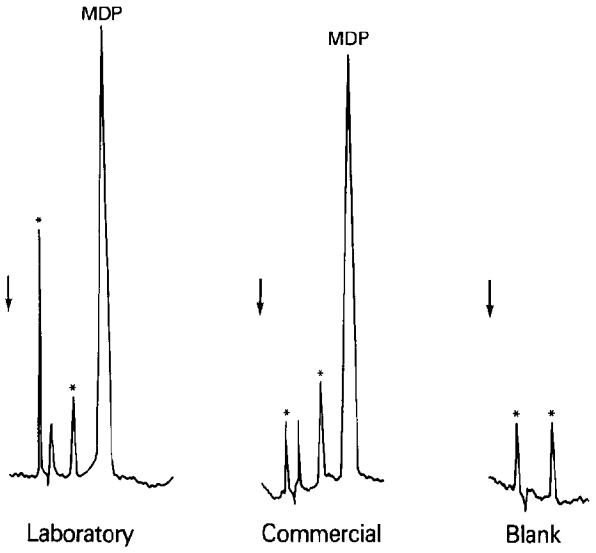
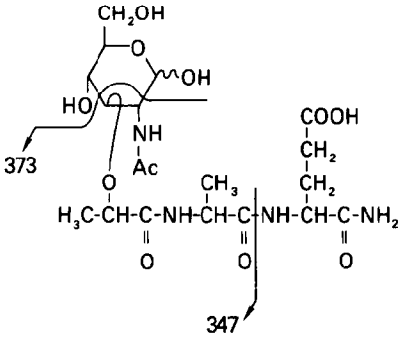


Fig. 1. Liquid chromatography, at 9.6 MPa, of synthetic and commercial *N*-acetylmuramoyl-L-alanyl-D-isoglutamine (**8**). [Conditions: Varian AX-10 Micropack (30 cm × 4 mm); temp., 50°; eluant, 3:2 acetonitrile–0.01M triethylammonium trifluoroacetate, pH 3.1; flow rate, 1 mL/min; detector, 206 nm; range 0.1.]



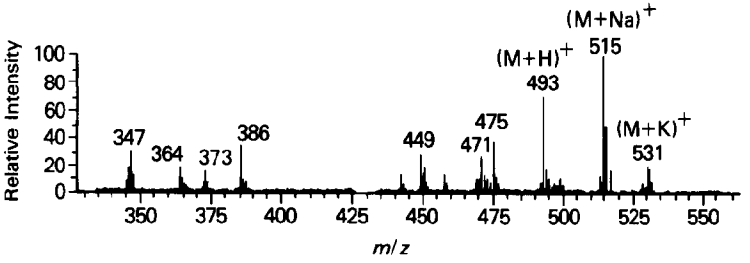


Fig. 2. Partial, postive f.a.b.–mass spectrum of *N*-acetylmuramoyl-L-alanyl-D-isoglutamine (**8**).

tive of MDP[28]. Ions having appropriate *m/z* values were observed for the molecular ion (*m/z* 618), and for fragments characteristic of the carbohydrate and peptide moieties[28]. Modifications in the reaction scheme (see the Experimental section) led to the successful synthesis of *N*-acetylmuramoyl-L-alanine (MMP) and *N*-acetylmuramoyl-L-alanyl-D-isoglutaminyl-L-lysinamide (MTP–NH_2), both of which were similarly characterized.

Synthetic *N*-acetylmuramoyl-L-alanyl-D-isoglutamine and *N*-acetylmuramoyl-L-alanyl-L-isoglutamine were tested for pyrogens, and, being found pyrogen-free, were then incubated with mouse-spleen cells. The appropriate stimulated response of *in vitro* incorporation of [^{3}H]thymidine was observed[29] for both the L-D and L-L forms of MDP.

Characterization by fast-atom-bombardment–mass spectrometry. — It is often desirable to obtain analytical data for a chemical compound without prior modification or derivatization. Fast-atom-bombardment (f.a.b.)–mass spectrometry[12] has been shown to be a suitable first tool in the analysis of a wide variety of unmodified chemical compounds[30], and was thus chosen for examination of MDP.

The positive f.a.b.–mass spectrum was recorded for underivatized MDP (2.5 μg, 5 nmol) (see Fig. 2). An aqueous solution of the compound was deposited in a small quantity of glycerol that had been placed on a gold-plated copper, sample-probe tip. The sample was then bombarded with a beam of 9-keV xenon neutral fast atoms. An equimolar mixture of sodium chloride and potassium chloride was added to induce attachment-ion formation, in order to aid in the interpretation of the resulting f.a.b.–mass spectrum[31]. As predicted, an ion at *m/z* 493, corresponding to the protonated molecular-ion of MDP, and the sodium- and potassium-attachment ions, at *m/z* 515 and 531, were present (see Fig. 2). Simple elimination of one molecule of water from the protonated molecular-ion gave rise to ions[32] observed at *m/z* 475. This peak probably has contributions from the "R" group of the D-isoglutamine residue, as well as O-1 of the muramoyl moiety. Protonation followed by loss of water at the former would result in an acylium ion, whereas the latter would lead to formation of an A-type of ion[33].

Ions that could be attributed to simple cleavages were also observed, at *m/z* 347 ("A_1" ion), *m/z* 364 ("NA_1" ion), and *m/z* 386 (the sodium-attachment of the "NA_1" ion)[34–36]. It is interesting that the ion at *m/z* 373 could be rationalized by presuming formation of an "H" fragmentation involving C-2 and C-3 of the carbohydrate moiety, as is frequently observed in electron-impact mass spectrometry of carbohydrates[33]. Because the sugar moiety of MDP is substituted at these positions, such an ion fragment is possible. However, in the present case, the fragmentation would be derived from the cationic, protonated molecular-ion, rather than the radical-cationic molecular-ion generated in e.i.-m.s. The mechanistic rationalization for f.a.b.–m.s. therefore predicts an *m/z* value (*m/z* 373 in the present case) that is one amu (the proton used in the ionization process) greater than the *m/z* value for the "H" fragment expected from e.i.-m.s.

CONCLUSIONS

It has thus been shown that the synthetic scheme presented here is capable of producing gram quantities of highly pure and biologically active *N*-acetylmuramoyl-L-alanyl-D-isoglutamine (MDP), and can be modified to produce analogs of MDP. A significant diminution in the length of the synthesis was obtained through the use of palladium black in the hydrogenolysis of a mixture of the anomers of protected MDP, thereby eliminating the need to synthesize single-anomer precursors. Fast-atom-bombardment–mass spectrometry of MDP revealed ions corresponding to the intact molecule, as well as structurally significant ion-fragments indicative of the peptide and carbohydrate moieties.

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