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Synthesis of α and β anomers of UDP-*N*-acetylmuramic acid †

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ABSTRACT

A convenient synthesis of UDP-*N*-acetylmuramic acid, a key compound for the study of the cytoplasmic synthetases of bacterial peptidoglycan, is described. Separation of its two anomers was carried out by HPLC. The α anomer is identical with natural UDP-*N*-acetylmuramic acid. Both anomers are substrates for the L-alanine-adding enzyme from *Escherichia coli* with K_m values of 0.13 mM (α) and 2 mM (β).

INTRODUCTION

Peptidoglycan is an essential constituent of the cell wall of eubacteria. Its biosynthesis is a complex process involving many steps, both in the cytoplasm and in the cell membrane[2]. UDP-*N*-acetylmuramyl derivatives are substrates for several cytoplasmic synthetases of this pathway[3–5], and are therefore required for detailed study of these enzymes. UDP-*N*-acetylmuramic acid (UDP-MurNAc) and its derivatives are not commercially available and have to be prepared from bacterial cells in which they accumulate under specific conditions[6]. However, such procedures are long and tedious, and yields are often low[7]. An alternative approach is to synthesize UDP-MurNAc and to use it as starting material for the in vitro enzymatic preparation of the other UDP-MurNAc-containing precursors. To date, two syntheses of UDP-MurNAc have been published: (i) Twenty-five years ago, Heymann et al. reported the chemical synthesis of UDP-MurNAc by coupling uridine 5′-monophosphomorpholidate to MurNAc 1-phosphate[8]. However, the description of the steps leading to MurNAc 1-phosphate was incomplete; furthermore, the authors could not separate the two anomers of UDP-MurNAc.

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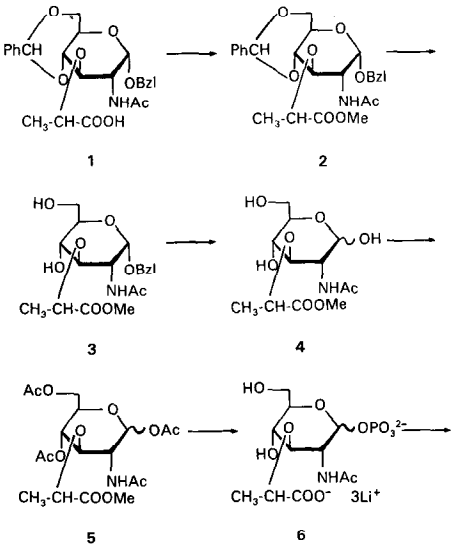

α-UDP-MurNAc + β-UDP-MurNAc

Scheme 1.

(ii) Very recently, Benson et al. described the enzymatic synthesis of UDP-MurNAc from UDP-*N*-acetylglucosamine[9]. Their procedure is simple, and readily available substrates are used (UDP-*N*-acetylglucosamine, enolpyruvate phosphate, NADPH). However, purified UDP-*N*-acetylglucosamine enolpyruvyl transferase and UDP-*N*-acetylenolpyruvylglucosamine reductase are required.

Herein, we describe a total chemical synthesis of UDP-MurNAc using commercially available starting material and reagents.

RESULTS AND DISCUSSION

The starting material for our synthesis (Scheme 1) is commercially available benzyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-α-D-glucopyranoside (**1**). Esterification by diazomethane gave the corresponding methyl ester **2** (refs. 10 and 11). The benzylidene group was removed by boiling in 60% acetic acid[10], affording diol **3** after silica gel column chromatography. 2-Acetamido-2-deoxy-3-*O*-[(*R*)-1-(methoxycarbonyl)ethyl]-D-glucopyranose **4** was prepared by removal of the benzyl group by catalytic hydrogenolysis and obtained as an oil, as already described[12,13]. Peracetylation of **4** with acetic anhydride in pyridine afforded the amorphous 2-acetamido-1,4,6-tri-*O*-acetyl-2-deoxy-3-*O*-[(*R*)-1-(methoxycarbonyl)ethyl]-α,β-D-glucopyranose **5**. TLC in several solvent systems showed two spots, the lower being much more intense than the upper; they probably correspond to the 9:1 mixture of α and β anomers described by

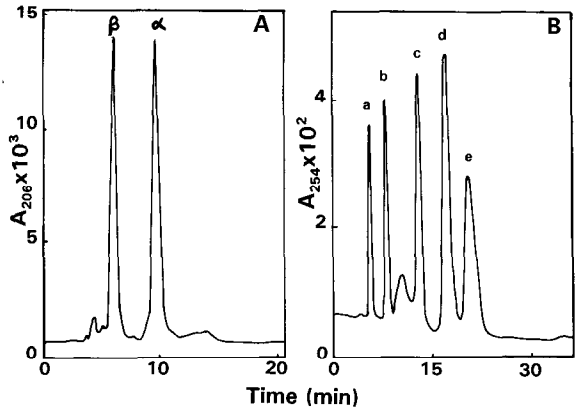

Fig. 1. Analytical HPLC of synthetic MurNAc 1-phosphate (A) and crude UDP-MurNAc (B). Column: Nucleosil $5C_{18}$ 150×4.6 mm. Mobile phase: 50 mM ammonium phosphate, pH 3.5. Flow rate: 0.5 mL/min. See Experimental for peak identification.

Carroll[12]. A phosphate group was then introduced at the anomeric position by treatment with 8 equivalents of molten phosphoric acid at 55°C (refs 8 and 14); removal of the protecting groups with lithium hydroxide yielded the lithium salt of MurNAc 1-phosphate **6**. Analysis by reverse-phase HPLC (Fig. 1A) revealed the presence of two compounds present in nearly equal amounts. The less polar component co-eluted with MurNAc 1-phosphate prepared from natural UDP-MurNAc treated with snake-venom pyrophosphatase[15]: it was thus identified as the α anomer, the more polar peak being the β anomer. The material contained 71% of MurNAc 1-phosphate. It was used without further purification for the next stage.

MurNAc 1-phosphate was transformed into its tri-*n*-octylammonium salt and coupled, under anhydrous conditions, with commercial uridine 5′-monophosphomorpholidate[8,16]. The reaction was allowed to proceed for 6 days; on the 3rd day, one more equivalent of uridine 5′-monophosphomorpholidate was added. Analytical HPLC of the crude product (Fig. 1B) revealed the presence of five main components, which were separated first by gel filtration on Sephadex G-25 (ref 17), then by reverse-phase HPLC in ammonium formate buffer[6], permitting the isolation of the α and β anomers of UDP-MurNAc (15.8 and 7.7 μmol, respectively). Their identities were established by comparison with authentic samples, by analyses (Table I) for uridine, phosphate, and muramic acid (Mur), and by ^{1}H NMR spectroscopy (δ H-1, 5.64 and 5.06 for the α and β anomers, respectively). UDP-α-MurNAc was homogeneous by high-performance thin-layer chromatography (HPTLC) in two solvent systems and by analytical HPLC at 262 or at 214 nm; its R_f and retention time values were identical with those of natural UDP-MurNAc. Owing to the lability of the β-glycosidic bond[8], UDP-β-MurNAc contained traces of UDP; this relative instability could explain its lower yield.

TABLE I

Analytical data for the anomers of UDP-MurNAc

Compound	Yield (μmol)	Ratios of uridine to Mur to phosphate		HPLC		Analytical HPLC [a] (min)
		Calcd	Found	$R_f(D)$	$R_f(E)$	
UDP-α-MurNAc	15.8	1.0:1.0:2.0	1.0:1.18:1.95	0.67 [b]	0.20 [b]	15.4 [b]
UDP-β-MurNAc	7.7	1.0:1.0:2.0	1.0:1.16:2.13	0.61	0.18	12.2
				(0.09) [c]	(0.06) [c]	(5.5) [d]

[a] Column: Nucleosil $5C_{18}$ 150×4.6 mm; eluent: 50 mM ammonium phosphate, pH 3.5; flow rate: 0.5 mL/min. [b] Identical with natural UDP-MurNAc. [c] Faint spot of UDP. [d] Small peak of UDP.

Both UDP-MurNAc anomers were assayed as substrates for the reaction catalyzed by the L-alanine-adding enzyme:

UDP-MurNAc + L-Ala + ATP ⇄ UDP-MurNAc-L-Ala + ADP + P_i

The enzymatic activity was measured by following the appearance of UDP-MurNAc-L-[^{14}C]Ala under the conditions described by Liger et al.[18]. A partially purified preparation of the enzyme, originating from an over-producing strain of *Escherichia coli*, was used. Both anomers were substrates for the reaction (Fig. 2). K_m Values for the α and β anomers were 0.13 and 2 mM, respectively. The former value was comparable with that determined for natural UDP-MurNAc (0.17 mM).

Data concerning the effect of structural analogues of UDP-MurNAc-containing precursors on peptidoglycan synthetases are scarce. To the best of our knowledge, only dihydrouridine[7,15] and 5-fluorouridine[19] derivatives have been studied and shown to be good substrates. The present results constitute the first evidence that

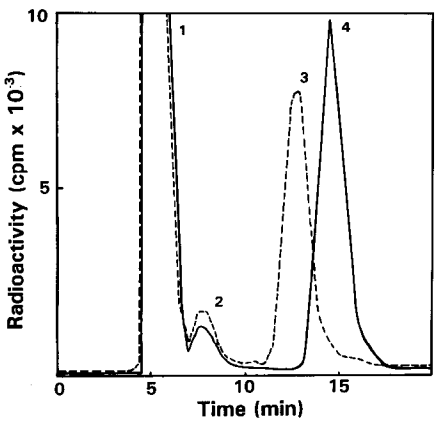

Fig. 2. Elution pattern of the radioactivity for assays of addition of L-[^{14}C]Ala to UDP-α-MurNAc (———) and UDP-β-MurNAc (– – – – –). Column: Nucleosil $5C_{18}$ 150×4.6 mm. Mobile phase: 50 mM ammonium formate, pH 4.3. Flow rate: 0.5 mL/min. Peak 1, alanine; peak 2, impurity present in commercial L-[^{14}C]Ala; peak 3, UDP-β-MurNAc-L-Ala; peak 4, UDP-α-MurNAc-L-Ala.

the β anomer of a UDP-MurNAc-containing precursor may be a substrate for a peptidoglycan synthetase.

In summary, we have described a convenient synthesis of UDP-MurNAc; purification by HPLC offers an easy way of separating the α and β anomers. Synthetic UDP-MurNAc, which can be prepared on a multimilligram scale by our procedure, can serve as starting material for the in vitro enzymatic preparation of the other UDP-MurNAc-containing precursors. These reactions can be carried out with a crude bacterial extract[18,20] or, better, with the corresponding synthetases totally or partially purified from over-producing strains[21–23]. By this approach, the facile preparation of highly radioactive precursors is also possible.

EXPERIMENTAL

General methods.—Melting points were taken on a Tottoli apparatus (Büchi, Switzerland) in open capillary tubes and are uncorrected. Optical rotations were measured with a Perkin–Elmer 141 polarimeter. Uridine was determined by ultraviolet absorption at 262 nm using ϵ_M 9890 M^{-1} cm^{-1} (ref 24). Phosphate was assayed according to Chen et al.[25] as modified by Ames and Dubin[26]. Muramic acid was determined after acid hydrolysis (6 M HCl, 95°C, 16 h) with a Biotronik LC-2000 analyzer (Biotronik, Frankfurt/Main, Germany); muramic acid standards were hydrolyzed under the same conditions[21]. ^1H NMR spectra at 400 MHz were recorded in D_2O at the Service de RMN of the Institut de Chimie des Substance Naturelles (Gif-sur-Yvette, France) on a Bruker WM400 apparatus; chemical shifts are relative to 4,4-dimethyl-4-silapentanoate-2,2,3,3-d_4. TLC and HPTLC were performed on precoated plates of Silica Gel $60F_{254}$ and on HPTLC plates of Silica Gel $60F_{254}$ (10×10 cm), respectively (Merck, Darmstadt, Germany), with the following solvent systems: *A*, 120:40:12:48 1-butanol–pyridine–AcOH–water; *B*, 15:1 $CHCl_3$–MeOH; *C*, 10:5:2 *n*-hexane–$CHCl_3$–MeOH; *D*, 7:3 EtOH–1 M ammonium acetate; *E*, 6:1:3 1-propanol–water–28% ammonium hydroxide. Compounds were located with a UV lamp, by charring with H_2SO_4, or by reaction with chlorine–*N*,*N*,*N'*,*N'*-tetramethyl-4,4'-diaminodiphenylmethane[27]; phosphate-containing compounds were revealed with the reagent of Hanes and Isherwood[28].

Compounds.—Benzyl *N*-acetyl-4,6-*O*-benzylidene muramic acid (benzyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-D-glucopyranoside) **1** and uridine 5'-monophosphomorpholidate 4-morpholine-*N*,*N'*-dicyclohexylcarboxamidine salt were from Sigma (St. Louis, MO; compounds B 5757 and U 6000, respectively). The optical rotation of the muramic acid derivative ($[\alpha]_D^{20}$ + 110° (*c* 0.5, MeOH)) corresponded to that of the α anomer[10,11]. Crystalline phosphoric acid was from Fluka (Buchs, Switzerland). Pyridine was dried by refluxing with lime followed by fractional distillation; it was stored over molecular sieves. Tri-*n*-octylamine (Sigma) was redistilled before use. Natural UDP-MurNAc was prepared according to Flouret et al.[6]. L-[^{14}C]Alanine (5.55 TBq/mol) was purchased from CEA (Saclay, France). Nucleotide pyrophosphatase (from *Crotalus adaman-*

teus venom) was from Sigma. L-Alanine-adding enzyme (sp. activity 181 nmol/min/mg protein) was partially purified from an over-producing strain of *E. coli*[29].

Benzyl 2-acetamido-4,6-O-benzylidene-2-deoxy-3-O-[(R)-1-(methoxycarbonyl)-ethyl]-α-D-glucopyranoside (**2**).—To a solution of benzyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-α-D-glucopyranoside **1** (991 mg, 2.1 mmol) in MeOH (20 mL), a solution of CH_2N_2 (3.5 mmol) in ether (20 mL) was added dropwise at 0°C. The mixture was left for 15 min at 0°C, and the solvents were evaporated. The residue was crystallized from MeOH to yield **2** (834 mg, 82%); TLC: R_f (*A*) 0.93; R_f (*C*) 0.66; mp 214–216°C; $[\alpha]_D^{20}$ + 119° (*c* 0.95, $CHCl_3$); lit. mp 212–213°C; $[\alpha]_D^{21}$ + 94° (*c* 0.70, $CHCl_3$)[10]; mp 213–214°C; $[\alpha]_D^{25}$ + 100° (*c* 1.09, $CHCl_3$)[11].

Benzyl 2-acetamido-2-deoxy-3-O-[(R)-1-(methoxycarbonyl)ethyl]-α-D-glucopyranoside (**3**).—A solution of **2** (745 mg, 1.53 mmol) in 60% AcOH (20 mL) was heated for 30 min in a boiling water bath. After cooling to room temperature, the solution was evaporated to give a glassy solid. Traces of AcOH and PhCHO were removed by codistillation with water. The product was then chromatographed on a column of Silica Gel 60 (35–70 mesh, Merck; 70 g) with 15:1 $CHCl_3$–MeOH. The fractions containing **3** were evaporated, and the residue was triturated in hexane to yield **3** (338 mg, 60%); TLC: R_f (*A*) 0.85; R_f (*B*) 0.28; mp 121–123°C; $[\alpha]_D^{20}$ + 153° (*c* 0.91, $CHCl_3$); lit. mp 120–122°C; $[\alpha]_D^{28}$ + 137° (*c* 0.94, $CHCl_3$)[10].

2-Acetamido-2-deoxy-3-O-[(R)-1-(methoxycarbonyl)ethyl]-D-glucopyranose (**4**). —The benzyl glycoside **3** (224 mg, 0.59 mmol) in AcOH (30 mL) was hydrogenated for 7 h in the presence of 5% Pd–C (220 mg). The catalyst was filtered off, the solvent was evaporated, and the oily residue was dried in vacuo to yield **4** (248 mg, > 100%); TLC: R_f (*A*) 0.78; R_f (*E*) 0.73.

2-Acetamido-1,4,6-tri-O-acetyl-2-deoxy-3-O-[(R)-1-(methoxycarbonyl)ethyl]-α,β-D-glucopyranose (**5**).—To a solution of **4** in pyridine (6 mL), Ac_2O (1.8 mL) was added, and the mixture was kept at room temperature for 4.5 h. After evaporation, the oily residue was taken up in benzene; upon addition of hexane, amorphous **5** precipitated; yield 229 mg (89%); TLC: R_f (*B*) 0.84 (strong) and 0.86 (weak); R_f (*C*) 0.54 (strong) and 0.58 (weak).

N-*Acetylmuramic acid 1-phosphate* (**6**).—The acetylated derivative **5** (205 mg, 0.52 mmol) and crystalline phosphoric acid (407 mg, 4.15 mmol) were dried in vacuo for 48 h over $MgClO_4$. Phosphoric acid was melted at 55°C, **5** was added, and the temperature of the mixture was maintained at 55°C for 3 h with continuous evacuation. The dark brown mixture was taken up in dry THF (2.6 mL). The solution was added to cold 1 M LiOH (15.9 mL). The mixture was stirred overnight at 4°C, the precipitate of lithium phosphate was filtered off, and the pH of the filtrate was adjusted to 8 with 0.1 M HCl. A few drops of octyl alcohol were added, and the solvents were removed in vacuo. The residue was triturated in EtOH. After a few hours in the cold, **6** was collected by centrifugation and washed with a small volume of cold EtOH; yield 44 mg (22%); TLC: R_f (*D*) 0.20; R_f (*E*) 0.18

(positive reaction with Hanes and Isherwood's reagent). Analytical HPLC (Fig. 1A): two peaks (retention times: 6.0 and 9.6 min) in a 46 : 54 ratio; by integration of the two peaks and calculation relative to natural MurNAc 1-phosphate as standard, a 71% content in MurNAc 1-phosphate was determined. Anal. Calcd ratio of phosphate to Mur: 1.0 : 1.0; found: 1.0 : 1.31.

UDP-N-Acetylmuramic acid (UDP-MurNAc).—Uridine 5′-monophosphomorpholidate 4-morpholine *N,N′*-dicyclohexylcarboxamidine salt (38 mg, 53 μmol) was dissolved in pyridine (5 mL) and evaporated to dryness in vacuo. Dissolution in pyridine and evaporation were repeated twice. Separately, an aqueous solution (0.5 mL) of **6** (29.1 mg, 53 μmol calculated from the HPLC analysis) was passed through a column of AG50W-X2 (pyridinium form) and eluted with water. The solvent was removed in vacuo, pyridine being added occasionally to maintain an alkaline pH. The residue was taken up in pyridine and tri-*n*-octylamine (75 μL, 173 μmol) was added. The solution was brought to dryness and the residue rendered anhydrous by dissolving it repeatedly (four times) in pyridine and evaporating the solvent in vacuo. Finally, the residue was redissolved in pyridine (~ 5 mL) and the solution added to uridine 5′-monophosphomorpholidate. The solvent was again evaporated in vacuo, pyridine (2.5 mL) was added, and the mixture was left at room temperature. After 50 h, more morpholidate (38 mg), previously dried by evaporation in pyridine, was added. At 145 h, the solvent was evaporated, and the residue was taken up in water (10 mL) containing sodium acetate (~ 20% more than the amount corresponding to the total tri-*n*-octylamine and 4-morpholine-*N,N′*-dicyclohexylcarboxamidine present). The solution was extracted three times with ether and lyophilized. The aqueous solution (4 mL) of the residue was passed through a column of AG50W-X2 (NH_4^+) and eluted with water. Lyophilization resulted in 68.4 mg of crude product, displaying 5 peaks upon analytical HPLC (Fig. 1B). Peaks a, b and d had the same retention times as UDP, UMP, and natural (α) UDP-MurNAc, respectively. The compounds were separated by gel filtration on Sephadex G-25 (120 × 1.8 cm), then by HPLC on μBondapak C_{18} (300 × 7.8 mm) in 50 mM ammonium formate, pH 3.5, at 1.5 mL/min. Analytical data (Table I) and ^1H NMR spectra showed that peaks c and d were the β and α anomers, respectively, of UDP-MurNAc. The total α + β (23.5 μmol) represented a 44% yield. Peak e did not contain Mur and was not further analyzed. ^1H NMR: α anomer: δ 1.33 (d, 1 H, *J* 7 Hz, CH_3 lactyl), 2.05 (s, 1 H, NAc), 3.40–3.97 (m, 6 H, H-2,3,4,5,6,6′ Mur), 4.08–4.50 (m, 6 H, H-2,3,4,5,5′ ribose + CH lactyl), 5.64 (m, 1 H, H-1 Mur), 5.99 (m, 2 H, H-5 uracil + H-1 ribose), 7.97 (d, 1 H, *J* 8 Hz, H-6 uracil); β anomer: δ 1.31 (d, 1 H, *J* 7 Hz, CH_3 lactyl), 2.04 (s, 1 H, NAc), 3.38–4.00 (m, 6 H, H-2,3,4,5,6,6′ Mur), 4.07–4.50 (m, 6 H, H-2,3,4,5,5′ ribose + CH lactyl), 5.06 (m, 1 H, H-1 Mur), 5.98 (m, 2 H, H-5 uracil + H-1 ribose), 7.95 (d, 1 H, *J* 8 Hz, H-6 uracil).

Preparation of N-acetylmuramic acid 1-phosphate from natural UDP-N-acetylmuramic acid.—Natural UDP-MurNAc (1.5 μmol) was dissolved in water (0.5 mL). 50 mM Tris · HCl buffer, pH 7.4 (1 mL), 1 M $MgCl_2$ (10 μL) and 1

mg/mL nucleotide pyrophosphatase (36 μL) were added. The mixture was incubated for 24 h at 37°C. The product was purified by HPLC on Nucleosil $10C_{18}$ (300 × 7.5 mm) in 50 mM ammonium formate, pH 3.5, at 2.7 mL/min. After lyophilization, 1.08 μmol was obtained (yield 72%). Analytical HPLC (conditions of Fig. 1A): one peak with a retention time of 9.6 min. Anal. Calcd ratio of phosphate to Mur: 1.0:1.0; found: 1.0:1.01.

L-*Alanine-adding enzyme assays.*—The enzymatic assay was performed according to Liger et al.[18], using 0.5 mM UDP-α- or -β-MurNAc. Radioactive substrate and product were separated by HPLC as previously described[18]. For the determination of the K_m values, 0.05–1 mM of the α-anomer and 0.2–3 mM of the β anomer were used, as well as 0.1 unit (α) and 1.8 unit (β) of enzyme.

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