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Synthesis of *N*-Acetylmuramic Acid Derivatives as Potential Inhibitors of the D-Glutamic Acid-Adding Enzyme

Geneviève Auger, Jean van Heijenoort and Didier Blanot

Orsay (France), Enveloppes Bactériennes et Peptides, CNRS URA 1131, Université de Paris-Sud

Claude Deprun

Orsay (France), Equipe
"Interaction Ions-Surfaces et Matériaux", IPN, CNRS-IN_2P_3, Université de Paris-Sud

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Abstract Compounds containing *N*-acetyl-D-muramic acid and (L-1-aminoethyl)phosphonic acid were designed as potential inhibitors of the D-glutamic acid-adding enzyme of the biosynthesis of bacterial peptidoglycan. 2-Acetamido-2-deoxy-3-*O*-[(*R*)-2-propionyl-(L-1-aminoethyl)phosphonic acid]-D-glucopyranose (**3**) was synthesized. 2-Acetamido-1,4,6-tri-*O*-acetyl-2-deoxy-3-*O*-[(*R*)-2-propionyl-(L-1-aminoethyl)phosphonic acid dimethyl ester]-α,β-D-glucopyranose (**9**) was also prepared and was submitted to the MacDonald reaction in order to introduce a phosphate group on the anomeric position. A complex mixture of phosphorylated or/and methylated derivatives of **3** was obtained. They were purified by h.p.l.c. and characterized by analyses of hexosamine, amino acid and labile phosphate, and by plasma desorption mass spectrometry. Neither **3** nor its derivatives inhibited the D-glutamic acid-adding enzyme from *Escherichia coli*.

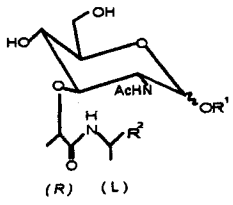
The D-glutamic acid-adding enzyme [UDP-*N*-acetylmuramoyl-L-alanine: D-glutamate ligase (ADP-forming), EC 6.3.2.9.] is a highly specific enzyme belonging to the biosynthetic pathway of bacterial peptidoglycan [1]. It catalyzes the following reaction:

UDP-MurNAc-L-Ala + D-Glu + ATP ⇌ UDP-MurNAc-L-Ala-D-Glu + ADP + P_i

(MurNAc = *N*-acetyl-D-muramic acid, 2-acetamido-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-D-glucopyranose). As the other enzymes of peptidoglycan biosynthesis, it is a potential target for antibacterial agents [2].

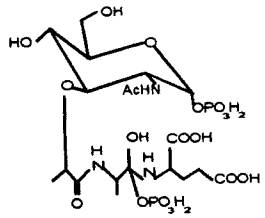
Although unknown, the reaction mechanism of the D-Glu-adding enzyme can be postulated by analogy with enzymes catalyzing the same type of reaction, *viz.* glutamine synthetase [3] and γ-glutamylcysteine synthetase [4]: the carboxyl group of alanine would be activated by ATP to give an acyl phosphate intermediate, which would undergo the nucleophilic attack of D-Glu to yield a tetrahedral transition state. Such a rationale has been successfully used for the design of inhibitors of two other enzymes of peptidoglycan biosynthesis, the *meso*-diaminopimelic acid-adding enzyme [5, 6] and the D-alanine: D-alanine ligase [7, 8].

Since 2-acetamido-2-deoxy-3-*O*-[(*R*)-2-propionyl-L-alanine]-α-D-glucopyranosyl phosphate **1** (Scheme 1) is a substrate for the D-Glu-adding enzyme [9], it seemed reasonable to assume that simple derivatives of MurNAc, substituted by the phosphonic analogue of L-alanine [(L-1-aminoethyl)phosphonic acid, L-Ala*P*], might act as analogues of the tetrahedral transition state **2** (Scheme 1) and thereby inhibit the enzyme. In this paper, we wish to describe the synthesis of phosphonic derivative **3** (Scheme 1). The subsequent introduction of a phosphate group on the anomeric position of the MurNAc moiety led to a complex mixture of derivatives of **3**, which were purified and characterized.



1,3

	R^1	R^2
1	PO_3H_2 (α)	COOH
3	H (α β)	$PO(OH)_2$

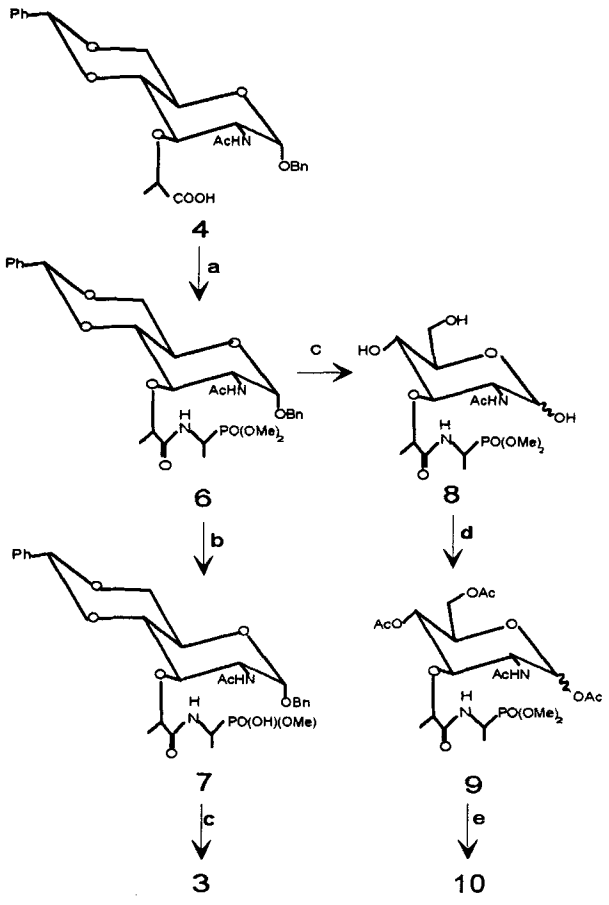


2

Scheme 1

Results

Our synthesis scheme (Scheme 2) was originally designed for the direct preparation of 2-acetamido-2-deoxy-3-*O*-[(*R*)-2-propionyl-(L-1-aminoethyl)phosphonic acid]-D-glucopyranose **3**. For this purpose, commercial benzyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-[(*R*)-1-

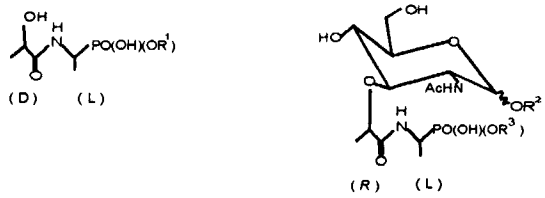


a) 1.HOSu, DCCI; 2.Et_3 N, 5; b) 1.LiOH, H_2O, THF; 2. HCl; c) H_2/Pd, AcOH; d) Ac_2O, pyridine; e) 1. H_3PO_4, 55°C; 2. LiOH, H_2O, THF.

Scheme 2

carboxyethyl]-2-deoxy-α-D-glucopyranoside **4** was coupled with dimethyl (L-1-aminoethyl)phosphonate **5**, yielding protected derivative **6**. However, treatment of **6** with Me_3SiBr (a reagent known to remove both alkyl groups of dialkyl phosphonates [10]) resulted in considerable degradation [1]. Therefore, another route was followed. Removal of one methyl group with alkali [11] afforded **7**. Catalytic hydrogenolysis in acetic acid and h.p.l.c. purification yielded a compound consisting in two interconvertible anomers. Its NMR spectrum revealed the disappearance of the benzyl and benzylidene groups, but also of the methyl ester; therefore, this compound was tentatively identified as **3**. This was later confirmed by its co-elution with **10E** (*vide infra*). It was obvious that the prolonged exposure to the acidic conditions used during the hydrogenolysis was responsible for the cleavage of the methyl ester.

In an attempt to synthesize derivatives containing a phosphate group on the anomeric position (*i.e.* analogues of **1**), compound **6** was hydrogenated in acetic acid, yielding **8**. The NMR spectrum showed that the two methyl groups of the dimethyl phosphonate were intact: therefore, the cleavage of a methyl phosphonate by acetic acid occurs only for monomethyl phosphonates. Peracetylation with acetic anhydride in pyridine yielded **9**, which was submitted to the action of molten phosphoric acid at 55 °C, followed by treatment with lithium hydroxide. Such a procedure, originally proposed by MacDonald [12], had already been successfully used for the synthesis of MurNAc 1-phosphate [13, 14]. Reverse-phase h.p.l.c. analysis of the product **10** revealed the presence of at least 9 compounds, which were purified by semi-preparative h.p.l.c. in triethylammonium formate buffer (Fig. 1). The identity of each compound was determined : i) by analyses of muramic acid (Mur), Ala*P* and labile phosphate; ii) by plasma desorption mass spectrometry (PDMS); iii) in some cases, by ^{1}H-NMR; iv) by h.p.l.c. analysis of the products formed after specific reactions (hydrolysis with 0.1 M HCl, removing α and β phosphate groups [15]; mild hydrolysis with 10 mM sodium acetate pH 4.5, removing only β phosphate groups [16]; β-elimination reaction with ammonium hydroxide, yielding the lactoyl moiety from MurNAc derivatives unsubstituted on the anomeric position [17]). The results of the analyses are shown in Table 1 and 2 and the proposed structures for **10A–H** are given on Scheme 3.



		R^2	R^3
10A R^1 = H	**10C**	PO_3H_2 (β)	H
10B R^1 = Me	**10D**	Me(β)	H
	10E	H(αβ)	H
	10F	PO_3H_2(α)	H
	10G	Me(β)	Me
	10H	PO_3H_2(α)	Me

Scheme 3

The most abundant compounds, **10A** and **10B**, did not contain Mur; their NMR and mass spectra showed that they corresponded to [L-1-(D-lactoylamino)ethyl]-phosphonic acid [$(M-H)^-$ = 196] and its monomethyl ester [$(M-H)^-$ = 210], respectively. They were certainly produced during the lithium hydroxide step, by β-elimination [18] or C-O cleavage [19] of MurNAc derivatives whose anomeric position was unsubstituted.

All the other compounds contained Mur. Compound **10E** did not contain labile phosphate. In analytical h.p.l.c., it appeared as a mixture of interconvertible anomers [2], whose retention times were identical with

[1] Degradation also occurred to a lesser extent when **9** was treated with Me_3SiBr.

[2] Peak E on Fig. 1 corresponded to the α anomer. The β anomer was eluted during the sensitivity change (arrow on Fig. 1).

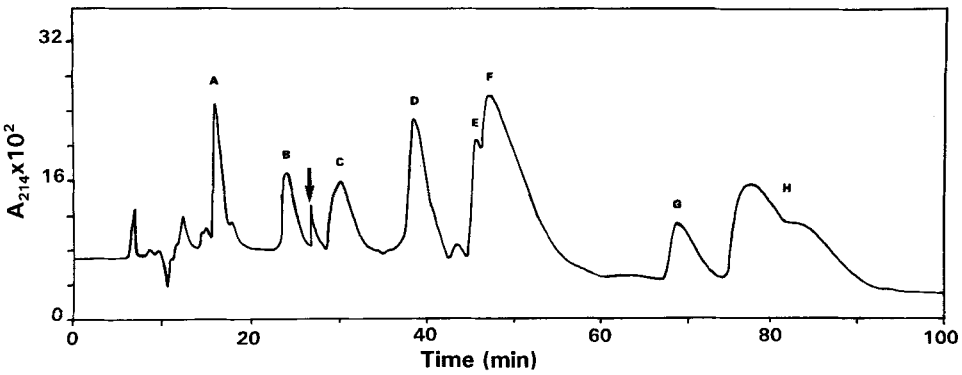


Fig. 1. Purification of compounds **10A-H** by semi-preparative h.p.l.c. Column: Nucleosil 10 C_{18} (300 × 7.8 mm). Mobile phase: 20 mM triethylammonium formate, pH 4.0. Flow rate: 1.5 ml/min. Detection at 214 nm (from the arrow, the sensitivity was multiplied by 10). After each run, the column was washed with acetonitrile for 30 min.

Table 1. Analytical data for compounds **10A-H.**

Compound	Amount (μmol)	Mur	Analysis AlaP	Analysis labile phosphate	Analytical h.p.l.c. (min) $R_t(E)$	Analytical h.p.l.c. (min) $R_t(F)$	m/z (relative abundance)
10A[a]	40.1	0	[b]	0	17.1	11.2	196(35,$[M-H]^-$), 79(91,PO_3^-), 63(100,PO_2^-)
10B[c]	31.0	0	[b]	0	28.6	14.2	210(26,$[M-H]^-$), 196(3,$[M-Me]^-$), 79(77,PO_3^-), 63(100,PO_2^-)
10C	2.1	1	0.92	0.93	34.8	15.7	479(3,$[M-H]^-$), 381(7,$[M-H-H_3PO_4]^-$), 311(2), 265(4), 97(34, $H_2PO_4^-$), 79(100,PO_3^-), 63(58,PO_2^-)
10D	5.3	1	0.95	0.05	40.3	20.3	413(24,$[M-H]^-$), 399(5$[M-Me]^-$), 215(2), 208(3), 199(8), 192(3), 183(7), 171(5), 169(4), 157(4), 146(6), 79(86,PO_3^-), 63(100,PO_2^-)
10E	3.0	1	1.06	0	27.0 & 52.9 (1:2)	13.8 & 19.8 (1:2)	421(2,$[M + Na-2H]^-$), 339(11,$[M-H]^-$), 217(7), 183(7), 97(100)[d], 81(23), 80(57), 79(68,PO_3^-), 63(56,PO_2^-)[e]
10F$_1$[f]	4.0	1	0.87	1.01	69.0	20.3	501(4,$[M + Na-2H]^-$), 479(13,$[M-H]^-$), 462(3), 381(9,$[M-H-H_3PO_4]^-$), 265(3), 199(3), 181(2), 177(8,$[H_3PO_4]PO_3^-$), 159(10,$[HPO_3]PO_3^-$), 143(2), 97(26,$H_2PO_4^-$), 81(4), 80(6), 79(100,PO_3^-), 64(1), 63(53,PO_2^-)
10F$_2$	2.9	1	0.88	1.05	69.0	21.9	479(2), 472(2),413(1), 411(1), 397(1), 383(3), 201(14), 199(7), 177(6), 159(5), 156(7), 143(3), 97(37), 80(16), 79(100), 63(56)[g]
10G	3.1	1	0.94	0.07	69.5	29.8	427(5,$[M-H]^-$), 422(5), 413(1,$[M-Me]^-$), 399(1,$[M-2Me + H]^-$), 397(1), 383(1), 379(1), 311(5), 291(8), 277(14), 265(6), 233(5), 219(5), 217(7), 201(11), 97(100)[d], 81(2), 80(62), 79(62,PO_3^-), 64(13), 63(56,PO_2^-)
10H[h]	16.1	1	1.03	0.91	111.5	27.7	515(5,$[M + Na-2H]^-$), 493(11,$[M-H]^-$), 395(2,$[M-H-H_3PO_4]^-$), 381(1,$[M-Me-H_3PO_4]^-$), 199(2), 177(4,$[H_3PO_4]PO_3^-$), 159(4,$[HPO_3]PO_3^-$), 97(28,$H_2PO_4^-$), 79(100,PO_3^-), 64(2), 63(53,PO_2^-)

[a] NMR: δ 1.27 (dd, 3H, CH_3 Ala*P*), 1.37 (d, 3H, CH_3 Lac, J = 7 Hz), 3.96 (m, 1H, CH Ala*P*), 4.24 (q, 1H, CH Lac, J = 7 Hz).

[b] Only compound detected by amino acid and hexosamine analysis.

[c] NMR: δ 1.31 (dd, 3H, CH_3 Ala*P*), 1.36 (d, 3H, CH_3 Lac, J = 7 Hz), 3.55 (d, 3H, PO(OH)(OCH_3), J = 11 Hz), 4.15 (m, 1H, CH Ala*P*, 4.24 (q, 1H, CH Lac, J = 7 Hz).

[d] Since the compound does not contain labile phosphate, this peak does not correspond to $H_2PO_4^-$.

[e] The fragment resulting from the loss of one molecule of water was obscured by a broad peak covering m/z 377–387. In the positive-ion spectrum, it appeared as a sharp peak at m/z 383.

[f] NMR: δ 1.29 (dd, 3H, CH_3 Ala*P*), 1.40 (d, 3H, CH_3 Lac, J = 7 Hz), 2.01 (s, 3H, NAc), 3.51–4.14 (m, 7H, H-2,3,4,5,6,6'Mur + CH Ala*P*), 4.26 (q, 4H, CH Lac, J = 7 Hz), 5.39 (m, 1H, H-1 Mur).

[g] Positive-ion spectrum: m/z (relative abundance) 662(38), 647(8), 522(6), 495(2), 452(14), 437(36), 415(33), 397(100), 383(21).

[h] NMR: δ 1.29 (dd, 3H, CH_3 Ala*P*), 1.37 (d, 3H, CH_3 Lac, J = 7 Hz), 1.99 (s, 3H, NAc), 3.45–4.08 (m, 9H, H-2,3,4,5,6,6'Mur + PO(OH)(OCH_3)), 3.56 (d, 3H, PO(OH)(OCH_3), J = 11 Hz), 4.12 (m, 1H, CH Ala*P*), 4.21 (q, 4H, CH Lac, J = 7 Hz), 5.32 (m, 1H, H-1 Mur).

those of the product of hydrogenolysis of **7**. Its identification to 2-acetamido-2-deoxy-3-*O*-[(*R*)-2-propionyl-(L-1-aminoethyl)phosphonic acid]-D-glucopyranose **3** was substantiated by the β-elimination reaction, which transformed it totally into **10A**, and by its mass spectrum [$(M-H)^-$ = 399].

Table 2. Results of specific reactions for characterization of compounds **10A-H**.

Compound	0.1M HCl, 100 °C, 10 min	10mM AcONa, pH 4.5, 100 °C, 1h	4M NH_4OH, 37 °C, 5h
10B	→ **10A** (100 %)	→ **10B** (75 %) + **10A** (25 %)	unchanged
10C	→ **10E** (100 %)	→ **10E** (100 %)	n.d.[a]
10D	→ **10D** (65 %) + **10E** (35 %)	n.d.	unchanged
10E	n.d.	n.d.	**10A** (100 %)
10F$_1$	→ **10E** (100 %)	unchanged	unchanged
10F$_2$	→ **10E** (100 %)	→ **10E** (20 %) + 2 peaks[b]	unchanged
10G	→ **10D** (60 %) + **10E** (40 %)	n.d.	unchanged
10H	→ **10E** (100 %)	→ **10H** (70 %) + **10F$_1$** (30 %)	n.d.

[a] n.d., not done.

[b] $R_t(E)$ 45.7 & 81.0 min, $R_t(F)$ 18.1 & 26.8 min, ratio 1:2.7.

Compounds **10C**, **10F$_1$**, **10F$_2$** and **10H** contained labile phosphate. **10F$_1$** and **10H** remained mainly unchanged upon AcONa hydrolysis, indicating that they were α anomers. NMR and mass spectrometry data led to their identification to 2-acetamido-2-deoxy-3-*O*-[(*R*)-2-propionyl-(L-1-aminoethyl)phosphonic acid]-α-D-glucopyranosyl phosphate [$(M\text{-}H)^-$ = 479] and its monomethyl ester [$(M\text{-}H)^-$ = 493], respectively. Small amounts of **10F$_1$** observed in the AcONa hydrolyzate of **10H** certainly originated from the partial cleavage of the methyl phosphonate in these conditions (*cf.* **10B** partly transformed into **10A**). **10C** was totally transformed into **10E** by AcONa hydrolysis: therefore, its structure was 2-acetamido-2-deoxy-3-*O*-[(*R*)-2-propionyl-(L-1-aminoethyl)phosphonic acid]-β-D-glucopyranosyl phosphate, which was confirmed by its mass spectrum [$(M\text{-}H)^-$ = 479]. **10F$_2$** was totally cleaved by AcONa hydrolysis, yielding a small amount of **10E** plus two new peaks. The presence of **10E** suggested the partial cleavage of a methyl phosphonate, as seen above. It was tempting to conclude that the two accompanying peaks corresponded to the anomers of 2-acetamido-2-deoxy-3-*O*-[(*R*)-2-propionyl-(L-1-aminoethyl)phosphonic acid monomethyl ester]-D-glucopyranose and that **10F$_2$** was therefore 2-acetamido-2-deoxy-3-*O*-[(*R*)-2-propionyl-(L-1-aminoethyl)-phosphonic acid monomethyl ester]-β-D-glucopyranosyl phosphate. However, the corresponding molecular ion [$(M\text{-}H)^-$ = 493] could not be seen on its negative-ion mass spectrum; it appeared on the positive-ion one (MH^+ = 495), but the presence of uninterpretable high-mass ions suggested that the product was impure.

Compounds **10D** and **10G** contained no labile phosphate. However, since they displayed a single peak in analytical HPLC and remained unchanged in β-elimination conditions, their anomeric position was substituted. The fact that **10G** was transformed into **10D** upon HCl hydrolysis (*cf.* **10B** → **10A**) and the examination of the mass spectra led us to propose methyl 2-acetamido-2-deoxy-3-*O*-[(*R*)-2-propionyl-(L-1-aminoethyl)phosphonic acid]-D-glucopyranoside [$(M\text{-}H)^-$ = 413] and its monomethyl ester [$(M\text{-}H)^-$ = 427] for **10D** and **10G**, respectively. The relatively high proportion (35–40 %) of **10E** formed upon HCl hydrolysis suggested a β anomeric configuration (in the same conditions, model compound α-methyl MurNAc remained intact, whereas the β anomer yielded 60 % of MurNAc). Methyl glycosides **10D** and **10G** might arise from the action of methyl phosphate, formed during the MacDonald reaction by acidolysis of the methyl phosphonate function, but further experiments would be necessary to verify this hypothetical mechanism.

Compounds **3, 8** and **10A–H** were assayed at 1 mM as inhibitors of the D-Glu-adding enzyme form *Escherichia coli*. None of them significantly inhibited the reaction whatever the conditions used (direct assay of inhibition or assay with preincubation).

Discussion

Our results illustrate the difficulty of synthesizing MurNAc derivatives containing dialkyl phosphonates. It is obvious that the heterogeneity of the product obtained by the MacDonald reaction arises from the presence of the dimethyl phosphonate group, which is partially cleaved by phosphoric acid, yielding both free phosphonic acid (**10A, C, D, E** and **F$_1$**) and monomethyl phosphonate (**10B, G, H** and perhaps **F$_2$**); furthermore, possible formation of methyl glycosides (**10D** and **G**) complicates the mixture. It seems evident that the MacDonald reaction, if efficient for simple sugars [12–14], cannot be used for the phosphorylation of more complex derivatives.

The fact that no compound inhibits the enzyme is surprizing. Three explanations can be given: i) the reaction pathway does not include a tetrahedral transition state; ii) the tetrahedral transition state does exist, but it is not stabilized or tightly bound by the enzyme, as it is the case for pea seed glutamine synthetase [20]; iii) our compounds mimic too imperfectly the transition state owing to the absence of key structural elements

(*e.g.* D-Glu.). Possible solutions for obtaining powerful inhibitors might be the synthesis of acyl phosphate analogues [8] or of phosphinic acid derivatives [7].

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Experimental

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. Elemental analyses were performed at the Service Central d'Analyses du CNRS (Vernaison, France). Labile phosphate was assayed by the colorimetric method of Chen *et al.* [21], the preliminary ashing step being replaced by hydrolysis with 1 M HCl at 100 °C for 15 min. Mur and Ala*P* were determined after acid hydrolysis (6 M HCl, 95 °C, 16 h) with a Biotronik LC-2000 amino acid analyzer (Biotronik, Frankfurt/Main, Germany); Mur standards were hydrolyzed in the same conditions. ^{1}H-NMR spectra at 400 MHz were recorded in D_2O at the Service de RMN of the Institut de Chimie des Substances 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. Mass spectra were recorded on a DEPIL time-of-flight mass spectrometer [22]. Flight distance was 40 cm, acceleration voltage was ±15 kV, and time bins were 1 or 2 ns per channel. Counting time varied between 15 min and 2 h. Samples (30 μg) were dissolved in 30 μl of water/ethanol 1:1 (v/v), desalted with Dowex-50 ($Me_2NH_2^+$) resin, and sprayed onto aluminized mylar disks. Masses were determined from peak centroid calculation. Mass calibration was based on H^- (1.00837) and CN^- (26.00362) for negative-ion and on H^+ (1.00728) and Na^+ (22.98922) for positive-ion spectra. Intensities were calculated relative to a base peak equivalent to 100. Except noted otherwise, only the negative-ion spectra are presented, since they display less fragmentation. T.l.c. was performed on precoated plates of silica gel 60 (E. Merck, Darmstadt, Germany) with the following solvent systems (v/v): *A*, 1-butanol/pyridine/acetic acid/water 120:40:12:48; *B*, 1-butanol/acetic acid/water 3:1:1; *C*, *n*-hexane/chloroform/methanol 10/5/2; *D*, *n*-hexane-chloroform/methanol 2/1/1. Compounds were located by charring with H_2SO_4, or by reaction with ninhydrin or with chlorine-*N,N,N',N'*-tetramethyl-4,4'-diaminodiphenylmethane [23]. Analytical h.p.l.c. was carried out on Nucleosil $5C_{18}$ (250 × 4.6 mm) at 0.5 ml/min with the following mobile phases: *E*, 50 mM triethylammonium phosphate, pH 4.0; *F*, same buffer/acetonitrile 98:2 (v/v). Detection was performed at 206 nm.

Protected MurNAc derivative **4** was from Sigma (St. Louis, MO). Crystalline phosphoric acid and L-Ala*P* were from Fluka (Buchs, Switzerland). UDP-MurNAc-L-Ala and D-Glu-adding enzyme were prepared according to Michaud *et al.* [8] and Pratviel-Sosa *et al.* [24], respectively. DL-[^{14}C]Glu (2 GBq/mmol) was purchased from NEN (Du Pont de Nemours, Les Ulis, France).

Dimethyl (L-1-amino)ethylphosphonate, Oxalate (**5**)

L-Ala*P* (2 g, 16 mmol) was transformed into its benzyloxycarbonyl derivative as described for the racemate [25]; yield 100 %; mp. 108–111 °C; t.l.c.: $R_f(B)$ 0.63. Esterification with trimethylorthoformate at 105 °C [26, 27] afforded oily dimethyl (L-1-benzyloxycarbonylamino)ethylphosphonate; yield 99%, t.l.c.: $R_f(A)$ 0.88. The benzyloxycarbonyl group was removed by catalytic hydrogenolysis, and the product was crystallized from methanol/ether as the oxalate. Yield: 2.69 g (70 %); mp. 128–130 °C; $[\alpha]_D$ -0.4° (*c* 1, MeOH); t.l.c.: $R_f(A)$ 0.52, $R_f(D)$ 0.44.

$C_4H_{12}NO_3P.C_2H_2O_4$ (243.2)	Calcd:	C 29.64	H 5.80	N 5.76	P 12.74
	Found:	C 29.79	H 5.72	N 5.90	P 12.58

*Benzyl 2-acetamido-4,6,*O*-benzylidene-2-deoxy-3-*O*-[(R)-2-propionyl-(L-1-aminoethyl)phosphonic Acid Dimethyl Ester]-α-D-glucopyranoside* (**6**)

Protected MurNAc derivative **4** (1 g, 2.12 mmol) and *N*-hydroxysuccinimide (305 mg, 2.65 mmol) were dissolved in THF (20 ml). The solution was cooled in ice, and *N,N'*-dicyclohexylcarbodiimide (438 mg, 2.12 mmol) was added. The mixture was stirred for 3h at 0 °C and for 1 h at room temperature. The precipitate of dicyclohexylurea was filtered off and the resulting solution was cooled again. Triethylamine (0.32 ml, 2.33 mmol) and **5** (462 mg, 1.90 mmol) were added, and the temperature of the mixture was allowed to increase gradually to room temperature. After overnight stirring, the solvent was evaporated. The residue was taken up in ethyl acetate (120 ml), washed with 5 % $KHSO_4$ (4 × 30 ml), 1 M $NaHCO_3$ (4 × 30 ml) and brine (3 × 30 ml), and dried over $MgSO_4$. The solvent was evaporated, and the product was purified by silica gel column chromatography in hexane/chloroform/methanol 10:5:2 (v/v). Crystallization: ethyl acetate/hexane. Yield 741 mg (64 %); mp. 176–180°; $[\alpha]_D$ + 82.5 °C (*c* 0.5, methanol); t.l.c.: $R_f(A)$ 0.89, $R_f(C)$ 0.40.

$C_{29}H_{39}N_2O_{10}P$ (606.6)	Calcd:	C 57.42	H 6.48	N 4.62	P 5.11
	Found:	C 57.62	H 6.35	N 4.86	P 4.87

*2-Acetamido-2-deoxy-3-*O*-[(R)-2-propionyl-(L-1-aminoethyl)-phosphonic Acid]-D-glucopyranose* (**3**)

To a suspension of **6** (40 mg, 0.066 mmol) in THF (1 ml), 0.5 M lithium hydroxide (0.356 ml, 0.178 mmol) was added in small portions while the mixture became clearer. After overnight stirring at room temperature, 1 M HCl (0.178 ml, 0.178 mmol) was added. The solvents were evaporated and the residue was taken up in water. Upon acidification to pH 2 with 10 mM HCl at 0 °C, **7** precipitated; yield 20 mg (51 %), t.l.c.: $R_f(A)$ 0.60, with minor impurities. 18 mg (0.030 mmol) were hydrogenated in glacial acetic acid (4 ml) for 52 h in the presence of 5 % Pd/C. After filtration of the catalyst and evaporation of the solvent, the product precipitated from methanol/ether. It was purified by semi-preparative h.p.l.c. on μBondapak C_{18} (300 × 7.8 mm) in 50 mM ammonium formate, pH 4.6, at 1.5 ml/min. Two peaks (retention times: 9.5 and 12 min) were collected. Both displayed the same Mur to Ala*P* ratio and each gave the original chromatographic profile after re-injection. They were pooled and lyophilized. Yield 3 mg, 6.7 μmol determined by Mur and Ala*P* analysis (22 %); t.l.c.: $R_f(A)$ 0.15; h.p.l.c.: $R_t(E)$ 27.0 min and 52.9 min (1:2 ratio), $R_t(F)$ 13.8 min and 19.8 min (1:2 ratio).

NMR: δ 1.28 (m, 3H, CH_3 Ala*P*), 1.42 (2d, 3H, CH_3 Lac α + β), 2.05 (s, 3H, NAc), 3.45–4.05 (m, 7H, H-2,3,4,5,6,6' Mur+CH Ala*P*), 4.25 (q, 0.4 H, CH Lac β), 4.31 (q, 0.6H, CH Lac α), 4.7 (H-1 Mur β, partly covered by HDO), 5.20 (d, 0.6H, *J* = 3.5Hz, H-1 Mur α):

2-Acetamido-2-deoxy-3-O-[(R)-2-propionyl-(L-1-aminoethyl)-phosphonic Acid Dimethyl Ester]-D-glucopyranose (**8**)

Compound **6** (370 mg, 0.61 mmol) was hydrogenated in glacial acetic acid (35 ml) for 60 h in the presence of 5 % Pd/C. After filtration of the catalyst and evaporation of the solvent, the product precipitated from methanol/ether. Yield 279 mg (87 %); mp. 110 °C; $[\alpha]_D$ + 37.0° (*c* 0.5, MeOH); t.l.c.: $R_f(A)$ 0.72, $R_f(B)$ 0.39; h.p.l.c.: $R_t(F)$ 69 and 121 min [3]. $C_{15}H_{29}N_2O_{10}P.3CH_3OH$ Calcd: C 41.22 H 7.88 N 5.34 P 5.90 (524.5) Found: C 40.90 H 7.65 N 5.57 P 5.65

NMR: δ 1.43 (d, 3H, CH_3 Lac), 1.47 (m, 3H, CH_3 Ala*P*), 2.04 (s, 0.4H, NAc β), 2.05 (s, 0.6H, NAc α), 3.45–4.03 (m, 12H, H-2,3,4,5,6,6' Mur + $PO(OCH_3)_2$), 3.86 (2d, 6H, *J* = 11 Hz, $PO(OCH_3)_2$ α + β), 4.26 (q, 0.4H, CH Lac β), 4.35 (q, 0.6H, CH Lac α), 4.53 (2q, 1H, CH Ala*P* α + β), 4.71 (d, 0.4H, *J* = 9 Hz, H-1 Mur β), 5.22 (d, 0.6H, *J* = 3.5 Hz, H-1 Mur α).

2-Acetamido-1,4,6-tri-O-acetyl-2-deoxy-3-O-[(R)-2-propionyl-(L-1-aminoethyl)phosphonic Acid Dimethyl Ester]-α,β-D-glucopyranose (**9**)

To a solution of **8** (230 mg, 0.44 mmol) in pyridine (9 ml), Ac_2O (2.7 ml) was added, and the mixture was kept overnight at room temperature. After evaporation, the oily residue was taken up in ethyl acetate; upon addition of hexane, amorphous **9** precipitated. Yield 243 mg (100 %), t.l.c.: $R_f(A)$ 0.71; in solvent system *D*, the two anomers could be distinguished, with R_f 0.65 (strong) and 0.68 (weak).

PDMS: m/z (relative abundance) 553 (8, $[M-H]^-$), 539 (18, $[M-Me]^-$), 525 (1, $[M-2Me + H]^-$), 511 (2, $[M-Ac]^-$), 497 (2, $[M-Me-Ac + H]^-$), 479 (2), 224 (3), 109 (33), 99 (31), 87 (24), 80 (28), 79 (100, PO_3^-), 73 (36), 71 (73), 64 (7), 63 (93, PO_2^-).

Phosphorylation of **9**: *Compounds* **10A-H**

The acetylated derivative **9** (200 mg, 0.36 mmol) and crystalline phosphoric acid (283 mg, 2.89 mmol) were dried *in vacuo* for 48 h over $Mg(ClO_4)_2$. Phosphoric acid was melted at 55 °C, **9** was added, and the temperature was maintained at 55 °C for 3 h with continuous evacuation. The dark brown mixture was taken up in dry THF (1.7 ml). The solution was added to cold 1M LiOH (10.3 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 product was precipitated three times from methanol/ether. The aqueous solution (1 ml) of the residue (80 mg) was passed through a column of AG50W-X2 (NH_4^+) and eluted with water. Lyophilization resulted in 68 mg of crude **10**, which displayed several peaks in analytical h.p.l.c.

[3] The two peaks were separated by a plateau (ratio of the areas of peak 1 to peak 2 to plateau, 1:5.7:10.3). This phenomenon has already been observed for late-eluting muramyl peptides [28].

Purification of **10A-H**

Compounds **10A-H** were separated by semi-preparative h.p.l.c. on Nucleosil $10C_{18}$ in triethylammonium formate buffer (Fig. 1)[4] Peaks **E** and **F**, which were poorly resolved, were further purified in the same system. Two compounds, $\mathbf{F_1}$ and $\mathbf{F_2}$, revealed in peak **F** by analytical h.p.l.c. in acetonitrile-containing mobile phase F_1, were separated by semi-preparative h.p.l.c. in this mobile phase. Yield and analytical data for **10A-H** are given in Table 1 [for NMR spectroscopy, the samples were previously treated with AG50W-X2 (NH_4^+) resin in order to remove triethylamine].

Specific Reactions for Characterization of **10A-H**

Samples of compounds **10A-H** (30 mmol) were submitted to the following treatments: i) 0.1 M HCl (200 µl) at 100 °C for 10 min, followed by neutralization with 1 equivalent of ammonium hydroxide; or ii) 10 mM sodium acetate, pH 4.5 (200 µl), at 100 °C for 1 h; or iii) 4 M ammonium hydroxide (200 µl) at 37 °C for 5 h, followed by neutralization with 1 equivalent of acetic acid. After lyophilization, the residues were taken up in water (100 µl), and aliquots (5–10 nmol) were analyzed by h.p.l.c. (Table 2).

Enzyme Assays

i) Direct assay of inhibition. Compounds were tested for their ability to inhibit the synthesis of UDP-MurNAc-L-Ala-D-[^{14}C]Glu in a mixture (final volume : 50 µl) containing 0.1 M Tris/HCl buffer (pH 8.6), 5 mM $MgCl_2$, 5 mM ATP, 7.5 µM UDP-MurNAc-L-Ala, 15 µM D-Glu, DL-[^{14}C]Glu (3 KBq), 1 mM compound, and purified enzyme [25 µl in 20 mM potassium phosphate, 1 mM 2-mercaptoethanol, 0.1 mM $MgCl_2$, 5 % (v/v) glycerol, pH 7.0]. The mixture was incubated for 30 min at 37 °C, and the reaction was stopped by addition of 10 µl glacial acetic acid. The mixture was lyophilized and taken up in the h.p.l.c. elution buffer. The radioactive substrate and product were separated by reverse-phase h.p.l.c. with a Nucleosil $5C_{18}$ column (150 × 4.6 mm) as stationary phase, and isocratic elution at a flow rate of 0.5 ml/min with 50 mM ammonium formate, pH 4.6 [29, 30]. Detection and quantification were performed with the h.p.l.c. radioactivity monitor LB 506 C-1 (Berthold, Bad Wildbad, Germany) using Quinckszint Flow 2 scintillator (Zinsser Analytic, Maidenhead, UK) at 1.5 ml/min.

ii) Assay with preincubation. The enzyme was preincubated for 15 min at 37 °C with the compound (1 mM) in a mixture containing Tris/HCl, ATP and $MgCl_2$ (total volume: 40 µl). The reaction was initiated by addition of a mixture (10 µl) of the substrates. The remaining part of the assay was run as described above.

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[4] In spite of the peculiar shape of its peak, compound **10H** was homogenous (see Table 1).

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Address for correspondence:
Dr. D. Blanot
Enveloppes Bactériennes et Peptides
CRNS URA 1131
Bâtiment 432
Université de Paris-Sud
F-91405 Orsay, France