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Synthesis and biochemical evaluation of some novel *N*-acyl phosphono- and phosphinoalanine derivatives as potential inhibitors of the D-glutamic acid-adding enzyme

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A series of *N*-(5-phthalimidopentanoyl)-, *N*-[2-(2-ethoxy)acetyl]-, and *N*-(7-oxooctanoyl)-phosphono and phosphinoalanine derivatives has been synthesized and evaluated for inhibition of the D-glutamic acid-adding enzyme (MurD) of peptidoglycan biosynthesis.

1. Introduction

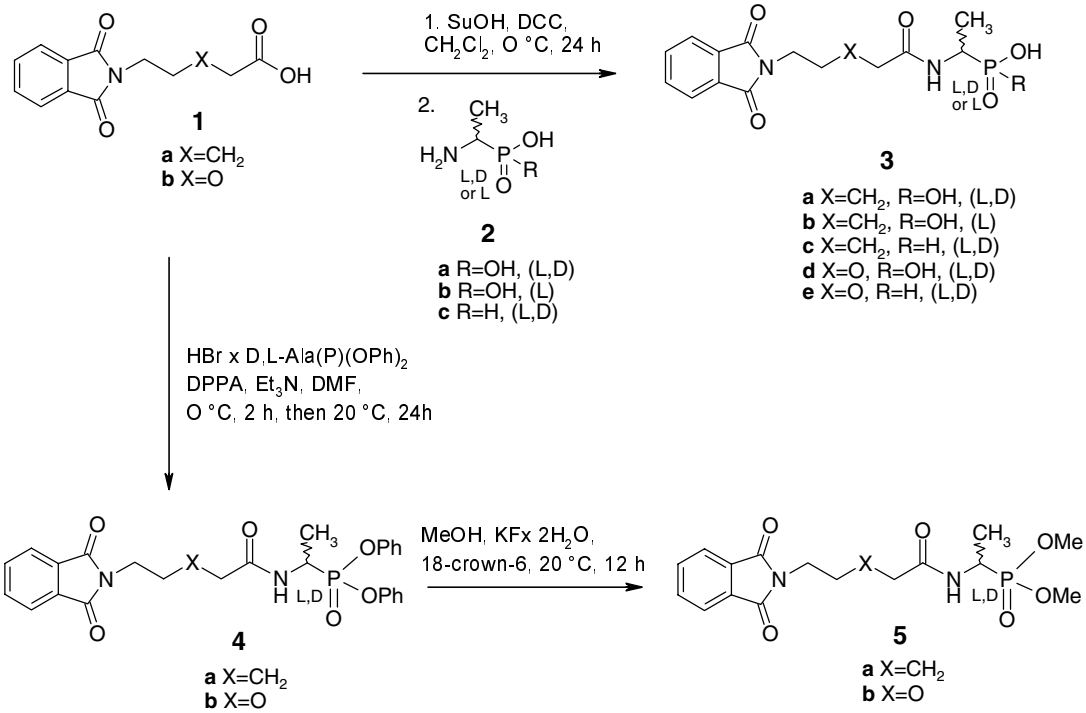
Peptidoglycan is a network of alternating *N*-acetyl muramic acid (MurNAc) and *N*-acetyl glucosamine units that are cross-linked by D-amino acid-containing tetrapeptides. It provides rigidity for the cell wall and protects bacteria from osmotic lysis [1]. Peptidoglycan biosynthesis is therefore an important target for antibiotic research [2]. Among the numerous steps of this pathway, the D-glutamic acid-adding enzyme (MurD), which catalyzes the formation of the peptide bond between uridine diphosphate-*N*-acetylmuramoyl-L-alanine and D-glutamic acid, has recently become important in the search for new antibiotics. Phosphonic and phosphinic analogs of its tetrahedral intermediate have been synthesized and evaluated [3–5]. During our research towards MurD inhibitors we wanted to synthesize new MurNAc-L-Ala analogs as potential inhibitors of the enzyme.

2. Investigations, results and discussion

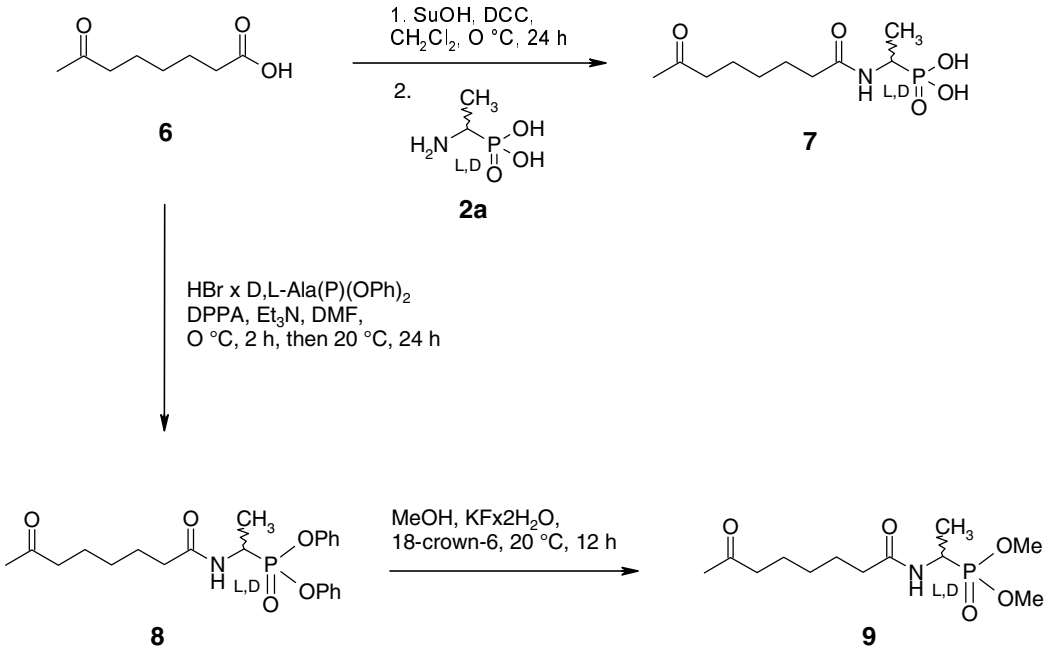
We replaced L-Ala with its phosphonic and phosphinic analogs and, based on the previous research of muramyl dipeptide (MDP) analogs as potential immunomodulating compounds, three carboxylic acids were selected as simple replacements for the MurNAc moiety. The aromatic parts of 5-phthalimidopentanoic and 2-(2-phthalimidoethoxy)acetic acid can be considered as a π electronic substitute for the MurNAc moiety, as recently demonstrated by design and synthesis of several immunologically active phthalimido MDP analogs [6, 7]. 7-Oxooctanoic acid has also been successfully employed as a replacement for the MurNAc fragment in acyclic apyrogenic MDP analog LK-409 [8]. Taking advantage of our experience in the synthesis of phosphonamidate MDP analogs [9], we prepared several new *N*-acyl derivatives of phosphonic and phosphinic alanines and assayed them for MurD inhibition.

Carboxylic acids **1a**–**b** and **6** were coupled with racemic (1-aminoethyl)phosphonic acid (L,D-Ala(P)) **2a** using the *N*-hydroxysuccinimide ester method [10] to give racemic phosphonic acids **3a**, **3d**, and **7**, respectively. Using the same method, L-phosphonic acid **3b** was prepared from L-Ala(P) **2b**, and racemic phosphinic acids **3c** and **3e** were

Scheme 1



Scheme 2



prepared from the corresponding phosphinic acid **2c**. The desired products **3a-e** (Scheme 1) and **7** (Scheme 2) were obtained in moderate yields after purification by semi-preparative RP-HPLC.

Carboxylic acids **1a**–**b** and **6** were also coupled with racemic diphenyl (1-aminoethyl)phosphonate hydrobromide using diphenylphosphorylazide as a coupling reagent. The resulting diphenylphosphonates **4a**–**b** and **8** were finaly transesterified with KFx2 H_2O/18-crown-6/methanol system [11] to give dimethylphosphonates **5a**–**b** (Scheme 1) and **9** (Scheme 2).

All synthesized compounds were assayed as inhibitors of purified MurD from *Escherichia coli* [12]. The phthalimido derivatives **3a**, **3b**, **3d** and **5b** were found to slightly inhibit the MurD activity at 1 mM (8, 9, 14 and 7% inhibition, respectively). This work confirms our preceding results [3, 4] which showed that the D-glutamic acid part of the tetrahedral intermediate is necessary for a good inhibition. Nevertheless, if we compare compounds **3a**–**d** and **5b** with our previous series of compounds (L-Ala(P) acylated with muramoyl-type moieties) devoid of inhibitory activity [3], we can conclude that the 2-(2-phthalimidoethoxy)acetyl or 5-phthalimidopentanoyl group impart to phosphonic alanine a certain affinity for MurD. This should lead to the synthesis of more potent inhibitors of MurD with the incorporated "D-Glu" fragment.

3. Experimental

3.1. Equipment and chemicals [9]

3.2. General procedure for the synthesis of 3a–e and 7 using active ester method

A mixture of the appropriate carboxylic acid **1a**–**b**, or **6** (12 mmol) and *N*-hydroxysuccinimide (12 mmol) in 50 ml of dioxane was treated with dicyclohexylcarbodiimide (12 mmol) at 0 °C and stirred at RT overnight. The precipitated dicyclohexylurea was filtered off, the filtrate was evaporated and the residue crystallized from ethanol. The resulting ester (10 mmol) was dissolved in a mixture of 10 ml water and 10 ml DMF and cooled to 0 °C. The corresponding acid **2** [13, 14] (11 mmol) and Et_3N (24 mmol) were added and the resulting mixture was stirred at 0 °C for 24 h. The solvent was evaporated under reduced pressure and the residue was purified by semipreparative RP-HPLC on a Vydac 218TP1022 column (25 × 2.2 cm); elution was performed at 7 ml/min with a gradient of methanol in 0.1% trifluoroacetic acid. All the results of elemental analyses were in an acceptable range.

3.2.1. (1-L,D) {1-[N-(5-Phthalimidopentanoyl)amino]ethyl}phosphonic acid (**3a**)

Yield 28% (two steps). m.p. 158–161 °C. ^{1}H NMR (DMSO-d_6) δ (ppm): 1.11 (dd, J = 7.3 Hz, J = 15.8 Hz, 3 H, CH_3), 1.40–1.65 (m, 4 H, 2 CH_2), 2.13 (t, J = 6.8 Hz, 2 H, CH_2CO), 3.60 (t, J = 6.8 Hz, 2 H, NCH_2), 3.90–4.20 (m, 1 H, CH), 7.70–7.90 (m, 5 H, NH and aromatic). ^{31}P NMR (DMSO-d_6) δ (ppm): 22.9. FAB-MS m/z 355 $(M + H)^+$.
$C_{15}H_{19}N_2O_6P$ (354.3) × H_2O

3.2.2. (1-L) {1-[N-(5-Phthalimidopentanoyl)amino]ethyl}phosphonic acid (**3b**)

Yield 27% (two steps). Hygroscopic, softens at 60 °C. ^{1}H NMR (DMSO-d_6) δ (ppm): 1.14 (dd, J = 7.1 Hz, J = 15.8 Hz, 3 H, CH_3), 1.40–1.65 (m, 4 H, 2 CH_2), 2.13 (t, J = 6.8 Hz, 2 H, CH_2CO), 3.55 (t, J = 6.8 Hz, 2 H, NCH_2), 3.90–4.20 (m, 1 H, CH), 7.70–7.90 (m, 5 H, NH and aromatic). ^{31}P NMR (DMSO-d_6) δ (ppm): 22.9. FAB-MS m/z 355 $(M + H)^+$. $[\alpha]_D^{20} = -19.95$ (c = 0.47, MeOH).
$C_{15}H_{19}N_2O_6P$ (354.3) × 0.7 H_2O

3.2.3. (1-L,D) {1-[N-(5-Phthalimidopentanoyl)amino]ethyl]phosphinic acid (**3c**)

Yield 30% (two steps). m.p. 121–124 °C. ^{1}H NMR (DMSO-d_6) δ (ppm): 1.15 (dd, J = 7.3 Hz, J = 15.1 Hz, 3 H, CH_3), 1.43–1.63 (m, 4 H, 2 CH_2), 2.15 (t, J = 6.8 Hz, 2 H, CH_2CO), 3.56 (t, J = 6.6 Hz, 2 H, CH_2N), 3.80–4.10 (m, 1 H, CH), 6.70 (d, J = 533 Hz, P–H), 7.80–7.90 (m, 4 H, aromatic), 8.10 (d, J = 7.9 Hz, NH). ^{31}P NMR (DMSO-d_6) δ (ppm): 28.8. FAB-MS m/z 339 $(M + H)^+$.
$C_{15}H_{19}N_2O_5P$ (338.3) × 0.7 H_2O

3.2.4. (1-L,D) (1-{N-[2-(Phthalimidoethoxy)acetyl]amino}ethyl)phosphonic acid (**3d**)

Yield 31% (two steps). m.p. 178–180 °C. ^{1}H NMR (DMSO-d_6) δ (ppm): 1.17 (dd, J = 7.3 Hz, J = 5.8 Hz, 3 H, CH_3), 3.68 (t, J = 5.6 Hz, 2 H, CH_2O), 3.80 (t, J = 5.6 Hz, 2 H, NCH_2), 3.90 (s, 2 H, CH_2CO), 3.94–4.10 (m, 1 H, CH), 7.27 (d, J = 9.8 Hz, 1 H, NH), 7.80–7.90 (m, 4 H, aromatic). ^{31}P NMR (DMSO-d_6) δ (ppm): 22.0. FAB-MS m/z 357 $(M + H)^+$.
$C_{14}H_{17}N_2O_7P$ (356.3) × 0.5 H_2O

3.2.5. (1-L,D) (1-{N-[2-(Phthalimidoethoxy)acetyl]amino}ethyl)phosphinic acid (**3e**)

Yield 28% (two steps). m.p. 130–133 °C. ^{1}H NMR (DMSO-d_6) δ (ppm): 1.19 (dd, J = 7.5 Hz, J = 16.2 Hz, 3 H, CH_3), 3.68 (t, J = 5.4 Hz, 2 H,

CH_2O), 3.80 (t, J = 5.4 Hz, 2 H, NCH_2), 3.85–4.00 (m, 3 H, CH and CH_2CO), 6.75 (d, J = 537 Hz, 1 H, PH), 7.75 (d, J = 8.7 Hz, 1 H, NH), 7.80–7.90 (m, 4 H, aromatic). ^{31}P NMR (DMSO-d_6) δ (ppm): 28.8. FAB-MS m/z 341 $(M+H)^+$.
$C_{15}H_{19}N_2O_5P$ (340.3) × 0.7 H_2O

3.2.6. (1-L,D) 1-[(7-Oxooctanoyl)amino]ethylphosphonic acid (**7**)

Yield 29% (two steps). As an oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.25–1.45 (m, 5 H, CH_3 and CH_2), 1.50–1.70 (m, 4 H, 2 CH_2), 2.12 (s, 3 H, CH_3CO), 2.30 (t, 2 H, J = 7.1 Hz, $COCH_2$), 2.45 (t, 2 H, J = 7.1 Hz, $COCH_2$), 4.20–4.40 (m, 1 H, CH), 8.45 (br s, 1 H, NH). ^{31}P NMR (DMSO-d_6) δ (ppm): 20.4. FAB-MS m/z 266 $(M + H)^+$.
$C_{10}H_{20}NO_5P$ (265.2) × 1.5 H_2O

3.3. General procedure for the synthesis of diphenylphosphonates 4a–b and 8

To a stirred solution of appropriate carboxylic acid **1a–b** or **6** (3.71 mmol), and diphenyl 1-aminoethylphosphonate hydrobromide [15] (3.71 mmol) in dry DMF (10 ml), diphenylphosphorylazide (4.45 mmol) and triethylamine (8.16 mmol) were added at 0 °C. After stirring for 2 h at this temperature and overnight at RT, EtOAc (200 ml) was added and the solution was washed subsequently with 10% citric acid, H_2O, saturated $NaHCO_3$, H_2O and saturated NaCl solution (70 ml each). The organic phase was dried ($MgSO_4$) and the solvent removed under reduced pressure.

3.3.1. Diphenyl (1-L,D) {1-[N-(5-phthalimidopentanoyl)amino]ethyl} phosphonate (**4a**)

Yield 58%. m.p. 143–145 °C (crystallized from ethanol). ^{1}H NMR (DMSO-d_6) δ (ppm): 1.42 (dd, 3 H, J = 7.5 Hz, J = 17.5 Hz, CH_3), 1.45–1.60 (m, 4 H, CH_2CH_2), 2.10–2.20 (m, 2 H, CH_2CO), 3.45 (t, 2 H, J = 6.6 Hz, NCH_2), 4.61–4.78 (m, 1 H, CH), 7.05–7.20 (m, 6 H, aromatic), 7.30–7.40 (m, 4 H, aromatic), 7.80–7.90 (m, 4 H, phthalimido), 8.51 (d, 1 H, J = 9 Hz, NH). ^{31}P NMR (DMSO-d_6) δ (ppm): 20.9. FAB-MS m/z 507 $(M + H)^+$.
$C_{27}H_{27}N_2O_6P$ (506.5)

3.3.2. Diphenyl (1-L,D) (1-{N-[2-(phthalimidoethoxy)acetyl]amino}ethyl) phosphonate (**4b**)

Yield 50%. m.p. 105–108 °C (crystallized from ethanol). ^{1}H NMR (DMSO-d_6) δ (ppm) 1.45 (dd, 3 H, J = 7.5 Hz, J = 18 Hz, CH_3), 3.65 (t, 2 H, J = 5.3 Hz, CH_2O), 3.80 (t, 2 H, J = 5.3 Hz, NCH_2), 3.94 (s, 2 H, CH_2CO), 4.62–4.80 (m, 1 H, CH), 7.25–7.80 (m, 6 H, aromatic), 7.32–7.42 (m, 4 H, phthalimido), 8.15 (d, 1 H, J = 9.4 Hz, NH). ^{31}P NMR (DMSO-d_6) δ (ppm): 20.2. FAB-MS m/z 509 $(M + H)^+$.
$C_{26}H_{25}N_2O_7P$ (508.5)

3.3.3. Diphenyl (1-L,D) 1-[(7-oxooctanoyl)amino]ethylphosphonate (**8**)

Yield 51%. As an oil (CC on silica gel, eluent: EtOAc/hexane 3 : 1). ^{1}H NMR (DMSO-d_6) δ (ppm) 1.12–1.24 (m, 2 H, CH_2), 1.35–1.55 (m, 7 H, CH_3 and 2 CH_2), 2.30 (s, 3 H, CH_3CO), 2.12 (t, 2 H, J = 7.2 Hz, $COCH_2$), 2.33 (t, 2 H, J = 7.4 Hz, $COCH_2$), 4.63–4.80 (m, 1 H, CH), 7.10–7.25 (m, 6 H, aromatic), 7.35–7.45 (m, 4 H, aromatic), 8.45 (d, 1 H, J = 9.4 Hz, NH). ^{31}P NMR (DMSO-d_6) δ (ppm): 20.0. FAB-MS m/z 418 $(M + H)^+$.
$C_{22}H_{28}NO_5P$ (417.4)

3.4. General procedure for the synthesis of dimethylphosphonates 5a–b and 9

Diphenyl phosphonate **4a–b** or **8** (1.35 mmol) and KF × 2 H_2O (13.50 mmol) were dissolved in MeOH (20 ml) and 18-crown-6 (20 mg) was added. The mixture was heated until boiling for 10 min and left at RT overnight. The solvent was removed under reduced pressure and the oily residue mixed with water (20 ml). The transesterification product was then extracted into EtOAc (3 × 10 ml). The collected organic extracts were dried ($MgSO_4$), evaporated in vacuo and the residue was purified on a silica gel column (eluent: CH_2Cl_2/acetone 1 : 1).

3.4.1. Dimethyl (1-L,D) {1-[N-(5-phthalimidopentanoyl)amino]ethyl} phosphonate (**5a**)

Yield 66%. m.p. 121–123 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) 1.19 (dd, 3 H, J = 7.3 Hz, J = 16.8 Hz, CH_3), 1.42–1.64 (m, 4 H, CH_2CH_2), 2.15 (t, 2 H, J = 6.8 Hz, CH_2CO), 3.56 (t, 2 H, J = 6.6 Hz, NCH_2), 3.61 (d, 3 H, J = 10.5 Hz, $POCH_3$), 3.62 (d, 3 H, J = 10.5 Hz, $POCH_3$), 4.21–4.38 (m, 1 H, CH), 7.80–7.90 (m, 4 H, phthalimido), 8.15 (d, 1 H, J = 9.4 Hz, NH). ^{31}P NMR (DMSO-d_6) δ (ppm): 29.5. FAB-MS m/z 383 $(M + H)^+$.
$C_{17}H_{23}N_2O_6P$ (382.4)

3.4.2. Dimethyl (1-L,D) (1-{N-[2-(phthalimidoethoxy)acetyl]amino}ethyl) phosphonate (**5b**)

Yield 70%. m.p. 96–100 °C. ^{1}H NMR (DMSO-d_6) δ (ppm) 1.22 (dd, 3 H, J = 7.5 Hz, J = 18 Hz, CH_3), 3.62 (d, 3 H, J = 10.5 Hz, $POCH_3$), 3.63 (d, 3 H, J = 10.5 Hz, $POCH_3$), 3.68 (t, 2 H, J = 5.2 Hz, CH_2O), 3.80 (t, 2 H, J = 5.2 Hz, NCH_2), 3.92 (s, 2 H, CH_2CO), 4.22–4.38 (m, 1 H, CH), 7.75 (d, 1 H, J = 9.4 Hz, NH), 7.82–7.90 (m, 4 H, phthalimido). ^{31}P NMR (DMSO-d_6) δ (ppm): 29.5. FAB-MS m/z 385 $(M + H)^+$.
$C_{16}H_{21}N_2O_7P$ (384.3)

3.4.3. Dimethyl (1-L,D) 1-[(7-oxooctanoyl)amino]ethylphosphonate (**9**)

Yield 67%. As an oil. ^{1}H NMR (DMSO-d_6) δ (ppm) 1.18–1.25 (m, 5 H, CH_3 and CH_2), 1.38–1.55 (m, 4 H, 2 CH_2), 2.06 (s, 3 H, CH_3CO), 2.10 (t, 2 H, J = 7.1 Hz, $COCH_2$), 2.40 (t, 2 H, J = 7.3 Hz, $COCH_2$), 3.63 (d, 6 H, J = 10.5 Hz, $P(OCH_3)_2$), 4.25–4,40 (m, 1 H, CH), 8.15 (d, 1 H, J = 9 Hz, NH). ^{31}P NMR (DMSO-d_6) δ (ppm): 29.5. FAB-MS m/z 294 $(M + H)^+$. HR-MS m/z = 294.1418, calculated for $C_{12}H_{24}NO_5P$ = 294.1470.

3.5. Inhibition studies

Compounds **3a–e**, **5a**, **5b**, **7** and **9** were tested at 1 mM for their ability to inhibit the formation of UDP-MurNAc-L-Ala-D-[^{14}C]Glu as described previously [4]. The substrate concentrations were 5 mM ATP, 25 μM UDP-MurNAc-L-Ala and 25 μM D-[^{14}C]Glu. In order to improve the solubility of the compounds, the mixtures contained 5% (v/v) DMSO. Purified enzyme from *E. coli* JM83(pMLD58) [12] was used.

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