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SYNTHESIS OF NEW ACYLPSEUDOPEPTIDES ANALOGOUS TO *N*-ACETYLMURAMYL DIPEPTIDE (MDP).

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Abstract

The synthesis of new non-glycosylated derivatives of MDP is described. Acylpseudopenta- or hexapeptides, of general formula R_1-XxxΨ[CH_2O]-D(L)-Ala-Ala-D-Glu[Lys(R_2)-NHEt]-NH_2, were obtained in two configurational forms on the α-carbon of the pseudo-alanyl (or lactyl) residue. The *N*-terminal part of these compounds (R_1-XxxΨ[CH_2O]-D(L)-Ala) mimics some of the essential chemical functions of *N*-acetylmuramic acid (Xxx = Gly or Ser) and is substituted either by a lauroyl, a *t*-butyloxycarbonyl or an acetyl moiety (R_1). R_2 is either an acetate counter-ion or a glycyl residue. The diastereoisomers were resolved by high performance liquid chromatography (HPLC) and their absolute configuration was determined by proton nuclear magnetic resonance (NMR) in dimethylsulfoxide (DMSO). This technnique evidenced furthermore two hydrogen bonds present in the structure of MDP.

Introduction

Since the disovery of the immunostimulating properties of *N*-acetylmuramyl dipeptide (MDP)[1], numerous synthetic analogs were synthesized with the aim of increasing its immunological capacities and of establishing structure - activity relationships[2]. Some non-glycosylated analogs of MDP bearing the dipeptides H-Ala-D-Glu-OH or H-Ala-D-Glu-NH_2 also demonstrate good immunomodulating capacities when their N-terminal ends are substituted by a hydrophobic lauroyl or heptanoyl residue replacing the *N*-acetylmuramic acid moiety as in the lauroyl tetrapeptide (LTP)[3] or in the heptanoyl peptide FK-565[4], two semi-synthetic active compounds. These lipophilic MDP derivatives have anti-infectious and/or anti-tumoral activities[5]; the alkyl chains being the structural keys resulting in active derivatives.

In the cell wall of many gram+ bacteria the γ-carboxyl function of glutamic acid is subtituted by *meso*-diaminopimelic acid (*meso*-DAP). However, Ellouz *et al* (1974)[1] and Kotani *et al* (1975)[6] showed that some bacteria, such as *Micrococcus roseus* or *Staphylococcus aureus*, have a cell-wall peptidoglycan bearing in this position a L-lysyl residue yielding Mur*N*Ac-Ala-D-Glu(Lys)-NH_2 after partial hydrolysis, which showed good immunostimulating activity.

According to these results and to the recent demonstration of the presence of cell-surface and intracellular MDP receptors[7] in B-lymphocytes and macrophages respectively, we prepared non-glycosylated analogs of MDP substituted on their γ-carboxyl end by a L-lysyl residue and on their *N*-terminal extremity by an acylpseudodipeptidic moiety mimicking different essential chemical functions of *N*-acetylmuramic acid.

Recently, Fermandjian *et al* (1987)[8] and Sizun *et al* (1988)[9] studied the conformation of MDP in DMSO by NMR. These investigations led them to propose a model in which an "S" tridimensional structure results from two successive β-turns stabilized by two hydrogen bonds, and a structure - activity relationship. In a similar way, it was interesting to examine by NMR if non-glycosylated acylpseudopeptides would be able to adopt the same conformational characteristics in DMSO as MDP.

In this paper, the synthesis and the HPLC and NMR characterizations of twelve new pseudopeptides bearing a methylene-oxy surrogate $\Psi[CH_2O]$ linkage, are described. To study the configurational influence of the lactyl (or pseudo-alanyl) residue, we developed a synthesis strategy whereby each pseudopeptide was obtained as a racemate.

Results and Discussion

The structure of the newly synthesized molecules is presented in Fig. 1.

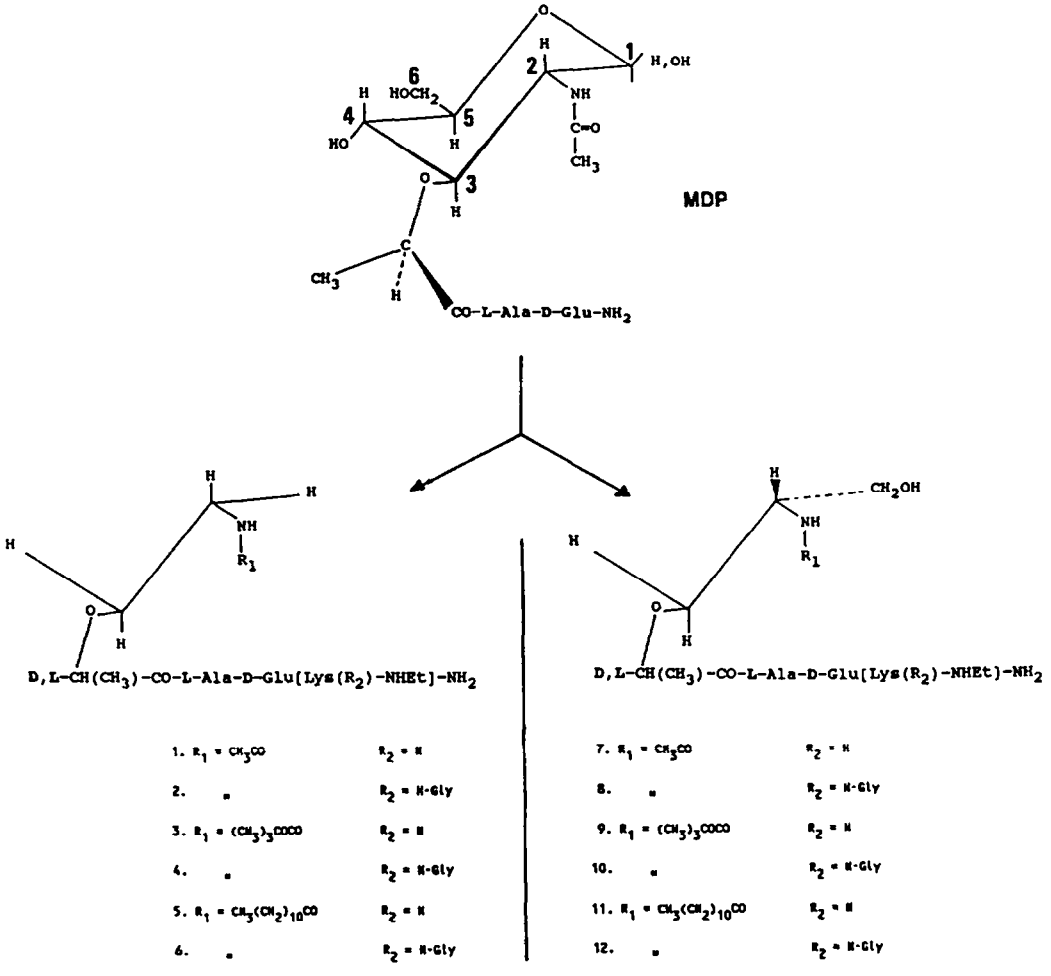

FIGURE 1: Structure of the pseudopeptides synthesized.

Formally, starting with the dipeptide H-Ala-D-Glu-NH_2, present in MDP , we realized different original substitutions involving *N*- and *C*-terminal parts. The γ-carboxylic function of glutamic acid was amidated either by H-Lys(Z)-ethylamide or by the dipeptide H-Lys(Z-Gly)-NHEt. The ethylamide group provides an irreversible blockage of the lysine α-carboxy-lic acid and the glycyl residue introduces an α-amine function less basic than the ϵ-amine of the lysyl residue.

The *N*-terminal extremity was acylated by the pseudodipeptides R_1-GlyΨ[CH_2O]-D,L-Ala-OH or R_1-SerΨ[CH_2O]-D,L-Ala-OH which mimic the carbons 2 and 3, and 1, 2 and 3 of *N*-acetyl-muramic acid respectively, as shown on Fig. 1. R_1 groups corresponding to acetyl (Ac), *t*-butyloxycarbonyl (Boc) or lauroyl (Laur) residues were chosen by taking into account the results obtained from non-glycosylated acylpeptides or lipopeptides, analogs of MDP.

The preparation of pseudopenta- and pseudohexapeptides was carried out in the following procedure. First, the peptide H-Ala-D-Glu[Lys(X)-NHEt]-NH_2, where X represents Z or Z-Gly, was synthesized by classical peptide chemistry. The peptides H-Ala-D-Glu[Lys(Z)-NHEt]-NH_2 and H-Ala-D-Glu[Lys(Z-Gly)-NHEt]-NH_2 were both synthesized following a common pathway up to the dipeptide Boc-D-Glu[Lys(Z)-NHEt]-NH_2. Afterwards, this compound was selectively deprotected by Boc removing, to obtain the peptide where X = Z or, by elimination of the Z group to further yield the other peptide where X = Z-Gly (Fig. 2).

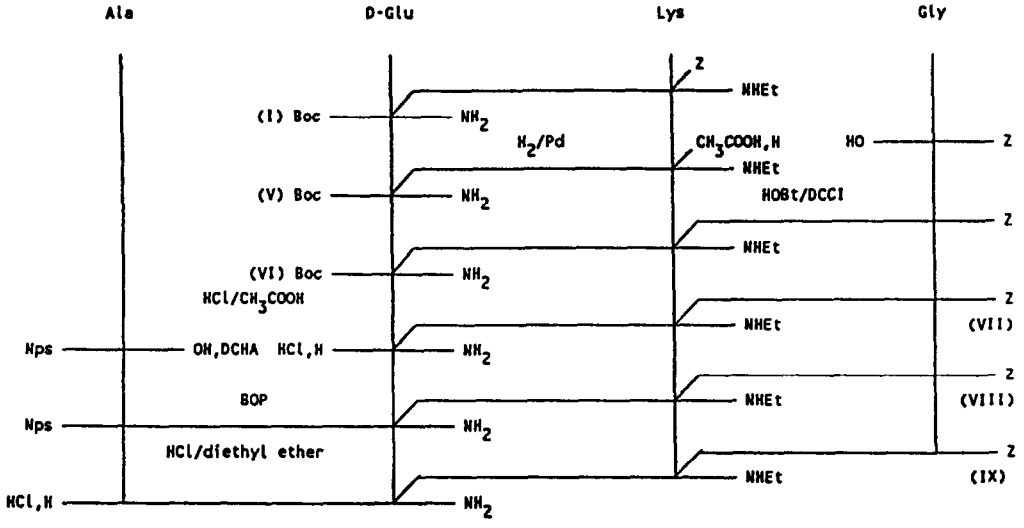

FIGURE 2: Strategy of synthesis of the tetrapeptide HCl,H-Ala-D-Glu[Lys(Z-Gly)-NHEt]-NH_2.

Secondly, the pseudodipeptides Boc-XxxΨ[CH_2O]-D,L-Ala-OH, where Xxx is Gly or L-Ser, were prepared according to two distinct pathways previously described by Rubini *et al* (1986)[10] and Breton *et al* (1990)[11] respectively. The acid labile Boc group could be further replaced by an acetyl or a lauroyl group easily introduced by using their corresponding anhydrides.

Finally, the *N*-terminal end of the tripeptide H-Ala-D-Glu[Lys(Z)-NHEt]-NH_2 or of the tetrapeptide H-Ala-D-Glu[Lys(Z-Gly)-NHEt]-NH_2 was acylated by the pseudodipeptides yielding the protected pseudopenta- (**1, 3, 5, 7, 9** and **11**) and pseudohexapeptides (**2, 4, 6, 8, 10** and **12**) which were finally deprotected through a single step (Fig. 3).

R_1-YyyΨ[CH_2O]-D,L-Ala-OH + HCl,H-Ala-D-Glu[Lys(R_2')-NHEt]-NH_2

DMF | BOP

R_1-YyyΨ[CH_2O]-D,L-Ala-Ala-D-Glu[Lys(R_2')-NHEt]-NH_2

CH_3OH/CH_3COOH | H_2/Pd

R_1-XxxΨ[CH_2O]-D,L-Ala-Ala-D-Glu[Lys(R_2)-NHEt]-NH_2

FIGURE 3: Synthesis of acylpseudopenta- and acylpseudohexapeptides carried out by a fragment coupling methodology followed by a deprotection reaction. R_1 = Ac, Boc or Laur ; R_2 = H or H-Gly- ; R_2' = Z or Z-Gly ; Xxx = Gly or Ser ; Yyy = Gly or Ser(Bzl).

The obtained diastereoisomer pairs were easily identified by NMR, particularly in the area of the amido protons where the same protons of each isomer have slightly different chemical shifts. This is illustrated by the example in Fig.4. As shown in Fig.5, analytical scale HPLC can also be used for this purpose. Moreover, these two methods permit a relative dosage of each diastereoisomer in the same pair.

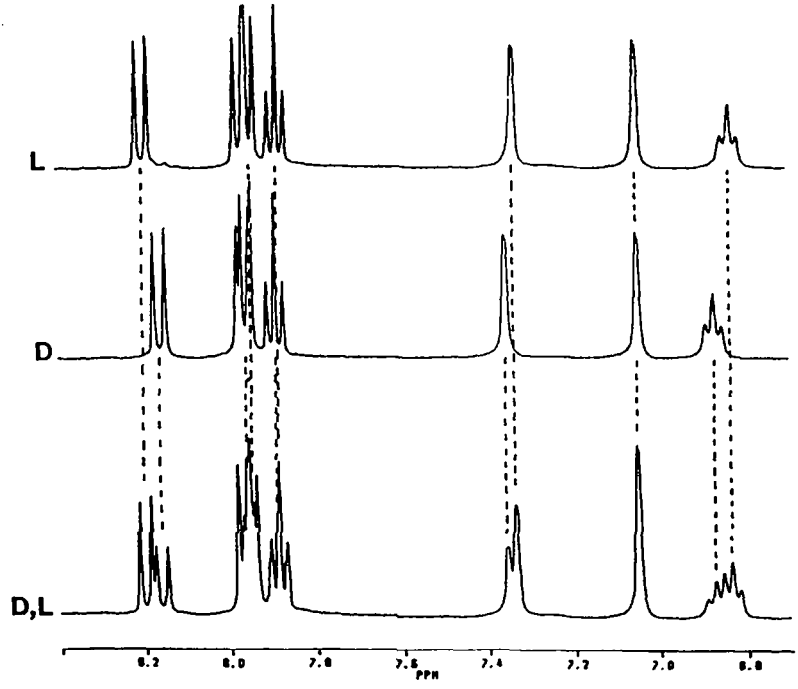

FIGURE 4: NMR spectra (300 MHz) in DMSO d_6, at 300 K of:
- Boc-GlyΨ[CH_2O] Ala-Ala-D-Glu(Lys-NHEt)-NH_2, CH_3COOH
- Boc-GlyΨ[CH_2O] D-Ala-Ala-D-Glu(Lys-NHEt)-NH_2, CH_3COOH
- Boc-GlyΨ[CH_2O] D,L-Ala-Ala-D-Glu(Lys-NHEt)-NH_2, CH_3COOH

in the area of the amido protons.

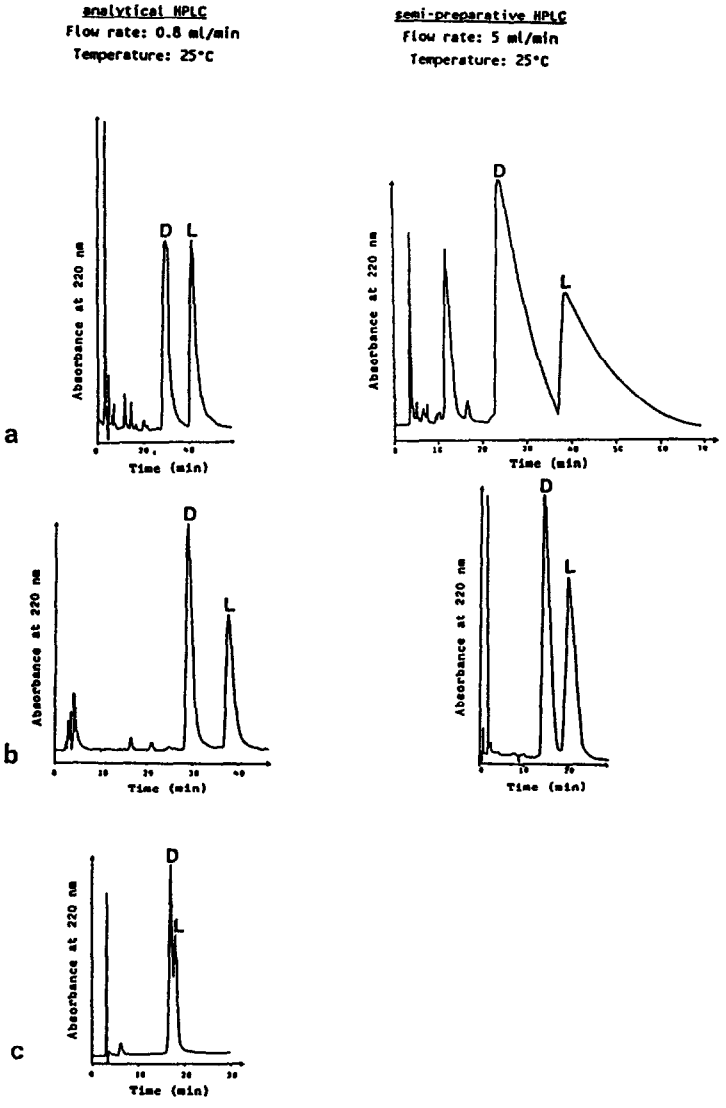

FIGURE 5: Analytical and semi-preparative HPLC chromatograms of:
a- Ac-GlyΨ[CH_2O]-D,L-Ala-Ala-D-Glu(Lys-NHEt)-NH_2, CH_3COOH
b- Boc-SerΨ[CH_2O]-D,L-Ala-Ala-D-Glu(Lys-NHEt)-NH_2, CH_3COOH
c- Laur-GlyΨ[CH_2O]-D,L-Ala-Ala-D-Glu[Lys(H-Gly)-NHEt]-NH_2, CH_3COOH.

The experimental conditions are:

a- Solvent: H_2O/CH_3CN (95/5) + 0.1% of ammonium acetate, analytical HPLC: Lichrospher 100 RP-18 column (250 x 4 mm), pressure: 110 bars, injected quantity: 0.02 mg/0.02 ml, semi-preparative HPLC: Lichrosorb RP-18 column (250 x 10 mm), pressure: 115 bars, injected quantity: 25 mg/0.2 ml.

b- Solvent: $H_2O/CH_3OH/CH_3CN$ (85/2/13) + 0.1% of ammonium acetate, analytical HPLC: Lichrospher 100 RP-18 column (250 x 4 mm), pressure: 107 bars, injected quantity: 0.02 mg/0.02 ml, semi-preparative HPLC: Lichrospher CH 18/2 column (250 x 10 mm), pressure: 113 bars, injected quantity: 4 mg/0.05 ml.

c- Solvent: $H_2O/CH_3OH/CH_3CN$ (35/30/35) + 0.1% of ammonium acetate, Kromasil C-18 column (250 x 4 mm), pressure: 89 bars, injected quantity: 0.02 mg/0.02 ml.

TABLE I : Physico-chemical characteristics of the acylpseudopeptides R_1-XxxΨ[CH_2O]-D(L)-Ala-Ala-D-Glu[Lys(R_2)-NHEt]-NH_2, CH_3COOH.

Pseudopeptides (a)	Configuration (b)	$[\alpha]^{25}_{546}$ (c=1, H_2O)	melting point (°C)	k' (c)	Rf (d)
R_1 = CH_3CO R_2 = H Xxx = Gly 1	L	- 27.4°	55 - 56	15.23 (7, III)	0.15 (E)
	D	- 6.7°	119 - 120	10.71 (7, III)	
R_1 = CH_3CO R_2 = H-Gly Xxx = Gly 2	L	- 33.8°	41 - 43	4.68 (6, III)	0.44 (F)
	D	- 7.1°	149 - 151	3.31 (6, III)	
R_1 = Boc R_2 = H Xxx = Gly 3	L	- 33.6°	190 d	15.52 (1, III)	0.25 (C)
	D	- 10.6°	40 - 41	12.28 (1, III)	
R_1 = Boc R_2 = H-Gly Xxx = Gly 4	L	- 32.6°	170 - 172	6.20 (2, I)	0.45 (D)
	D	- 8.7°	186 - 188	4.77 (2, I)	
R_1 = $CH_3(CH_2)_{10}CO$ R_2 = H Xxx = Gly 5	L	n.d.	218 - 219	4.31 (3, I)	0.18 (D)
	D	n.d.		4.04 (3, I)	
R_1 = $CH_3(CH_2)_{10}CO$ R_2 = H-Gly Xxx = Gly 6	L	n.d.	193 - 195	11.17 (4, II)	0.44 (D)
	D	n.d.		10.08 (4, II)	
R_1 = CH_3CO R_2 = H Xxx = Ser 7	L	n.d.	n.d.	5.37 (1, IV)	0.15 (A)
	D	- 3.6°	200 d	4.82 (1, IV)	
R_1 = CH_3CO R_2 = H-Gly Xxx = Ser 8	L	- 31.7°	32 - 34	10.18 (6, III)	0.41 (B)
	D	- 14.6°	44 - 46	6.56 (6, III)	
R_1 = Boc R_2 = H Xxx = Ser 9	L	- 36.7°	74 - 75	4.33 (1, I)	0.35 (C)
	D	- 13.2°	95 - 96	3.18 (1, I)	
R_1 = Boc R_2 = H-Gly Xxx = Ser 10	L	- 34.1°	51 - 53	5.97 (1, I)	0.27 (D)
	D	- 61.9°	70 - 72	4.37 (1, I)	
R_1 = $CH_3(CH_2)_{10}CO$ R_2 = H Xxx = Ser 11	L	n.d.	189 - 190	3.55 (5, I)	0.66 (E)
	D	n.d.			
R_1 = $CH_3(CH_2)_{10}CO$ R_2 = H-Gly Xxx = Ser 12	L	n.d.	172 - 174	n.d.	0.77 (D)
	D	n.d.		n.d.	

(a) numeric designation used in Fig.1.

(b) configuration of lactyl residue.

(c) analytical column (250 x 4 mm), flow rate: 0.8 ml/min, pressure between 90 and 130 bars, applied quantity: 0.02 mg/0.02 ml, temperature: 25°C.

HPLC solvents:

1: Solvent H_2O/CH_3CN (85/15) + 0.1% of ammonium acetate.
2: Solvent $H_2O/CH_3OH/CH_3CN$ (81/4/15) + 0.1% of ammonium acetate.
3: Solvent H_2O/CH_3CN (60/40) + 0.1% of ammonium acetate.
4: Solvent H_2O/CH_3CN (70/30) + 0.1% of ammonium acetate.
5: Solvent $H_2O/CH_3OH/CH_3CN$ (35/30/35) + 0.1% of ammonium acetate.
6: Solvent H_2O/CH_3CN (92/8) + 0.1% of ammonium acetate.
7: Solvent H_2O/CH_3CN (95/5) + 0.1% of ammonium acetate.

HPLC columns:

I: Kromasil C-18 column.
II: Hi-Pore reversed phase RP-304 column.
III: Lichrospher 100 RP-18 column.
IV: Lichrosorb RP-Select B column.

(d) CCM solvents:

A: Solvent $CHCl_3/CH_3OH/NH_3$ (28% in water) (4/4/0.3).
B: Solvent $CH_3OH/CHCl_3/H_2O/CH_3COOH$ (4/2/1/1).
C: Solvent $CHCl_3/CH_3OH/NH_3$ (28% in water) (8/4/0.5).
D: Solvent $CHCl_3/CH_3OH/NH_3$ (28% in water) (8/4/0.2).
E: Solvent ethyl acetate/pyridin/water (20/10/11).
F: Solvent $CH_3OH/CH_3COOH/H_2O$ (4/1/1).

n.d. not determined.

With the objective of studying the role of the pseudo-alanine α-carbon configuration on immunomodulating activity, the acylpseudopeptides had to be optically pure. The semi-preparative scale HPLC allowed this difficult purification step. Optimal conditions of eluant mixture and flow rate were determined at the analytical scale. The acylating groups (R_1) have a strong effect on the retention time and the resolution: acetylated and *t*-butyloxycarbonylated isomers were well-separated on a C-18 column (Fig. 5a and b). However, under approximatively the same conditions, lauroylated diastereisomers which are hardly resoluted at the analytical scale (Fig. 5c) cannot be purified at a preparative scale.

Physico-chemical characteristics of newly synthesized optically pure acylpseudopenta- and hexapeptides are summarized in Table I.

The absolute configuration of the lactyl moiety in pseudopeptides bearing serine residues (Xxx = Ser) was determined as previously described[11]. For the acylpseudopeptides containing a glycyl residue (Xxx = Gly), their absolute configuration was assigned as follows: knowing that the formation of mixed ethers occured generally through an SN2 mechanism with inversion of configuration, we synthesized the enantiomer Ac-GlyΨ[CH_2O]-D-Ala-OH starting from (S) 2-chloro propionic acid, the optical purity of which had been controlled. Having a pseudodipeptide with a defined configuration allowed the synthesis of the acylpseudopentapeptide Ac-GlyΨ[CH_2O]-D-Ala-Ala-D-Glu(Lys-NHEt)-NH_2, CH_3COOH (1, **D**) following the procedure already described. This peptide was purified by semi-preparative HPLC (Fig.6) under experimental conditions presented in Fig. 5a. The comparison of chromatograms from Fig. 5a and Fig. 6 enabled us to assign the absolute configurations of the two isomers. In Fig. 6, the second minor peak, corresponding to the acylpseudopentapeptide Ac-GlyΨ[CH_2O]Ala-Ala-D-Glu(Lys-NHEt)-NH_2, CH_3COOH (1, **L**), may be explained by an incomplete inversion of configuration on the lactyl residue (82% instead of 100%) which takes place during the mixed ether formation involving the participation of the vicinal carboxylate group.

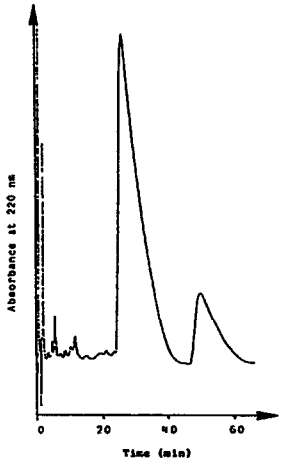

FIGURE 6: Semi-preparative HPLC chromatogram of Ac-GlyΨ[CH_2O]-D-Ala-Ala-D-Glu(Lys-NHEt)-NH_2, CH_3COOH (1, D); the pseudodipeptidic linkage Ψ[CH_2O] of which was synthesized from (S) 2-chloro propionic acid. The experimental conditions used are described in Fig. 5a legend.

NMR investigations were further carried out to obtain conformational informations about these optically pure pseudopeptides in DMSO solution. A classical approach to study the existence of hydrogen bonds involving amide protons consists in recording amide proton chemical shifts at various temperatures. Protons exposed to the solvent and not involved in intramolecular interactions show a $\Delta\delta/\Delta T$ ratio of approximately -6.10^{-3} p.p.m./K. On the other hand, pro-

tons which are involved in intramolecular interactions, such as hydrogen bonds, give appreciably lower absolute values. Six different acylpseudopeptides were selected for this study. Results were reported in Table II.

TABLE II: Influence of the temperature variation upon chemical shifts of the different amide protons from some synthesized pseudo-peptides.

Acylpseudopeptides		Chemical shift variations related to the temperature $\Delta\delta/\Delta T$ ($\times 10^3$) ppm/K					
Number (a)	Configuration	NH Glu	αNH Lys	NH Ala	NH ethylamide	NHtrans NH_2	NHcis NH_2
1	L	- 5.4	- 6.0	- 5.4	- 5.6	- 4.1	- 5.7
1	D	- 5.3	- 5.4	- 4.0	- 5.3	- 3.9	- 5.1
3	L	- 6.5	- 7.2	- 5.3	- 6.6	- 4.3	- 5.2
3	D	- 6.7	- 7.3	- 5.4	- 6.7	- 4.4	- 5.3
9	L	- 6.8	- 5.5	- 7.2	- 6.5	- 4.5	- 5.7
9	D	- 6.3	- 7.1	- 4.1	- 6.4	- 4.4	- 5.8

The α NH proton of glycine or serine gives in all cases values between -5.5 and $-8.0 \cdot 10^{-3}$ ppm / K
(a) see Fig. 1 for numeric designation.

For each analyzed pseudopeptide, it appears that the NH trans proton from the NH_2 residue could be systematically involved in a hydrogen bond as shown for MDP[9]. On the contrary, the NH cis proton from the same amide group would be in contact with the solvent.

As shown on Fig. 7 the NH alanine proton from the pseudopentapeptide Boc-SerΨ[CH_2O]-D-Ala-Ala-D-Glu(Lys-NHEt)-NH_2,CH_3COOH (9, D) could be implicated in an intramolecular interaction, whereas this proton in Boc-SerΨ[CH_2O]Ala-Ala-D-Glu(Lys-NHEt)-NH_2, CH_3COOH (9, L) would not have the same surrounding. The lactyl (or pseudoalanyl) α-carbon configuration would act here as an essential element influencing the conformation of this type of molecule. Chapman *et al*[12] in 1982 and Fermandjian *et al*[8] in 1987 also observed a hydrogen bond involving the NH alanine proton of MDP solubilized in DMSO.

Taking these preliminary results into account, it is possible that, as in the MDP conformation in DMSO, we simultaneously find, for a few acylpseudopeptides, two hydrogen bonds which lock the conformation according to an "S" tridimensional structure corresponding to two successive β-turns (Fig. 8). The alanine amide proton and the carbonyl of acetyl or *t*-butyloxycarbonyl moieties or the serine hydroxyl group could form a first hydrogen bond (1st β-turn). In regard to the NH trans proton from NH_2 group, it could participate in a hydrogen bond involving the carbonyl of the pseudoalanine (or lactyl) residue (2nd β-turn). As for MDP, where lactyl is in the D configuration, only the newly synthesized molecules which bear a D-pseudoalanine (or D-lactyl) moiety seem capable of simultaneously presenting these two interactions. These two hydrogen bonds were both found to be weaker than in MDP.

The immunostimulating properties of these non-glycosylated derivatives of MDP are now under investigation *in vitro*.

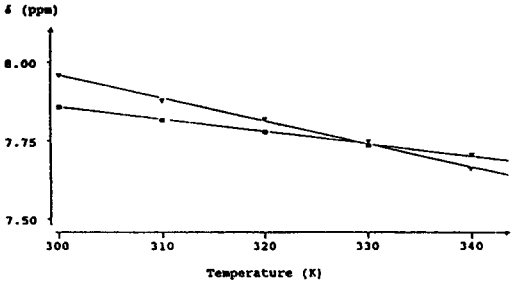

FIGURE 7: Variations of chemical shifts of NH alanine protons from L ( ) and D ( ) isomers of the pseudopentapeptide Boc-SerΨ[CH_2O]-D,L-Ala-Ala-D-Glu(Lys-NHEt)-NH_2,CH_3COOH related to the temperature.

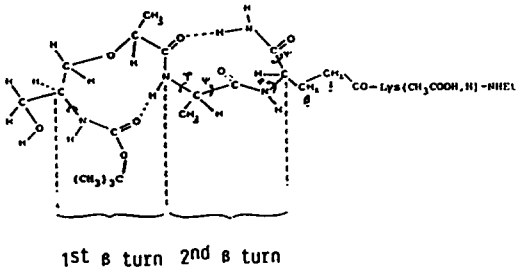

FIGURE 8: Conformational model of pseudopentapeptide Boc-SerΨ[CH_2O]-D-Ala-Ala-D-Glu(Lys-NHEt)-NH_2, CH_3COOH (9, D).

Experimental section

Chemical methods

All the amino acids used were of the L configuration unless otherwise stated. Amino acids derivatives were obtained from Novabiochem (Laufelfingen, Switzerland). Isobutylchloroformate, ethylchloroformate, acetic anhydride, dicyclohexylamine, *N*-methylmorpholine, *N*-hydroxysuccinimide (HOSu), *N,N'*-dicyclohexylcarbodiimide (DCCI) and palladium-barium sulfate (10% Pd) hydrogenation catalyst were obtained from Merck (Darmstadt, FRG); 2-nitrobenzenesulfenyl chloride, diisopropylethylamine (DIEA), triethylamine (TEA) and 1-hydroxybenzotriazol (HOBt) were purchased from Aldrich (Strasbourg, France); 2-*t*-butyloxycarbonyloxylmino-2-phenylacetonitrile (Boc-ON), lauric anhydride, benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium-hexafluorophosphate (BOP), anhydrous ammonia, and ethylamine were purchased from Ega-Chemie (Steinheim/Albuch, FRG), Sigma (Saint-Louis, MO, USA), Richelieu Biotechnologies (Saint-Hyacinthe, Canada), Setic-Labo (Paris, France) and Prolabo (Paris, France) respectively. All solvents used for synthesis were freshly distilled. Compound purity was monitored by thin layer chromatography on silica gel 60 F-254 precoated plastic-backed plates provided by Merck. The diastereoisomers were separated by HPLC using a Waters (Saint-Quentin-en-Yvelines, France) equipment at the analytical scale and a Gilson (Villiers-le-Bel, France) equipment at the semi-preparative scale. 250 x 4 mm analytical columns used were packed, with Lichrospher 100 RP-18 (C-18) (5 μm), Lichrosorb RP-Select B (C-8) (endcapped) (5 μm) from Merck, with Hi-pore reversed phase (C-4) (endcapped) from Bio-Rad (Paris, France) or with Kromasil C-18 (endcapped) from Eka-Nobel (Surte, Sweden). 250 x 10 mm columns packed with, Lichrospher 100 CH-18/2 (endcapped) (5 μm) or Lichrospher 100 RP-18 (7 μm) from Merck, were used at the semi-preparative scale. HPLC grade acetonitrile and methanol were purchased from SDS (Peypin, France) and ultra pure water was obtained from a Millipore station. 300 MHz NMR spectra were recorded with a Brüker AM 300 spectrometer coupled to an Aspect 3000 computer. Dry samples were dissolved in DMSO d_6 (99,8%) from CEA (Saclay, France). Chemical shifts were expressed as p.p.m. from tetramethylsilane (Me_4Si = 0.00).

Synthesis of Boc-D-Glu[Lys(Z)-NHEt]-NH_2 (I)

Boc-D-Glu[Lys(Z)-NHEt]-NH_2 (I)

3.44 g (10 mmol) of HCl,H-Lys(Z)-NHEt, prepared as previously described[13], were dissolved in 44 ml of a solvent dimethoxyethane/water (10:1). 1.54 ml (11 mmol) of TEA and 3.43 g (10 mmol) of Boc-D-Glu(OSu)-NH_2, obtained according the procedure described by Lefrancier and Bricas[14], were successively added. After overnight stirring at room temperature, 50 ml of water were added to precipitate the product. Purification was carried out by recrystallisation in hot isopropanol. The protected dipeptide was filtered off and dried in vacuo on P_2O_5. We obtained 3.74 g (yield: 70%). m.p. = 219 - 220°C ; $[\alpha]^{25}_{546}$ = - 13.5° (c=1, DMF) ; Rf = 0.52 in the system $CHCl_3/CH_3OH$ (5/1).

Synthesis of the tripeptide HCl,H-Ala-D-Glu[Lys(Z)-NHEt]-NH_2 (IV)

HCl,H-D-Glu[Lys(Z)-NHEt]-NH_2 (II)

(II) was obtained from (I) following acid hydrolysis of the Boc group by HCl/acetic acid. To 3.28 g (6.1 mmol) of (I) in 18 ml of acetic acid, 22 ml (39.6 mmol) of 1.8N HCl solution in acetic acid were added. After 20 min stirring, the product was precipitated in cold diethyl ether, filtered off and dried in vacuo on KOH. 2.75 g were obtained (yield: 96%).

m.p. = 184 - 185°C ; $[\alpha]_{546}^{25}$ = - 14.5° (c=1, CH_3OH) ; Rf = 0.69 in the system ethyl acetate/pyridine/H_2O (20/10/11) ; silver titration: 98%.

Nps-Ala-D-Glu[Lys(Z)-NHEt]-NH_2 (III)

2.65 g (5.6 mmol) of (II), 0.86 g (5.6 mmol) of monohydrated HOBt and 1.16 g (5.6 mmol) of DCCI were added in 60 ml of cold dimethylformamide (DMF) containing 2.37 g (5.6 mmol) of Nps-Ala-OH,DCHA synthesized according to Zervas *et al* (1963)[15]. After stirring for 1 hour at -10°C and overnight at room temperature, dicyclohexylurea was eliminated by filtration. The filtrate was concentrated under vacuum and precipitated in diethyl ether. The purification was achieved by recrystallisation in boiling isopropanol. 2.96 g of dry sample were harvested (yield: 80%).
m.p. = 235 - 236°C ; $[\alpha]_{546}^{25}$ = - 23.6° (c=1, DMF) ; Rf = 0.57 in the system $CHCl_3/CH_3OH$ (6/1).

HCl,H-Ala-D-Glu[Lys(Z)-NHEt]-NH_2 (IV)

To 2.80 g (4.2 mmol) of (III) in 80 ml of DMF, 2.90 ml (12.6 mmol) of 4.3N HCl in diethyl ether were added before stirring for 20 min. The hydrochloride salt was precipitated in diethyl ether and recrystallized in boiling isopropanol/diethyl ether (1:1). We obtained 2.16 g of dry compound (yield: 94%).
m.p. = 203 - 205°C ; $[\alpha]_{546}^{25}$ = - 8.0° (c=1, CH_3OH) ; Rf = 0.68 in the system ethyl acetate/pyridine/H_2O (20/10/11), silver titration: 99%.

Synthesis of the tetrapeptide HCl,H-Ala-D-Glu[Lys(Z-Gly)-NHEt]-NH_2 (IX)

Boc-D-Glu(Lys-NHEt)-NH_2, CH_3COOH (V)

To 5 g (9.3 mmol) of (I) in 54 ml of methanol containing water (10%), 534 µl (9.3 mmol) of acetic acid and 0.50 g of palladium - barium sulfate hydrogenation catalyst (10% Pd) were added. (I) was hydrogenated for 5 hours under stirring. The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in a minimal quantity of acetic acid to precipitate the product in cold hexane - ether mixture (1:1). We obtained 4.20 g of dry compound (yield: 97%).
m.p. = 162-164°C ; $[\alpha]_{546}^{25}$ = - 29.8° (c=1, CH_3OH) ; Rf = 0.10 in the system $CHCl_3/CH_3OH$/NaOH 1N in water (8/8/1) NMR spectra δ p.p.m. : 0.98 (3H, t, CH_3 ethyl), 1.25 (2H, m, γCH_2 Lys), 1.37 (9H, s, Boc), 1.43 (2H, m, δCH_2 Lys + 1H, m, $CH\beta CH_2$ Lys), 1.59 (1H, m, $CH\beta CH_2$ Lys), 1.68 (1H, m, $CH\beta CH_2$ Glu), 1.80 (3H, s, acetate), 1.88 (1H, m, $CH\beta CH_2$ Glu), 2.14 (1H, m, $CH\gamma CH_2$ Glu), 2.18 (1H, m, $CH\gamma CH_2$ Glu), 2.59 (2H, t, ϵCH_2 Lys), 3.06 (2H, m, CH_2 ethyl), 3.81 (1H, m, αCH Glu), 4.13 (1H, m, αCH Lys), 6.74 (1H, d, αNH Glu), 6.96 (1H, s, NHcis NH_2), 7.24 (1H, s, NHtrans NH_2), 7.92 (1H, t, NH ethylamide), 7.97 (1H, d, αNH Lys).

Boc-D-Glu[Lys(Z-Gly)-NHEt]-NH_2 (VI)

3.84 g (8.3 mmol) of (V) in 75 ml of DMF, neutralized by 1.16 ml (8.3 mmol) of TEA, were coupled with 1.73 g (8.3 mmol) of Z-Gly-OH, according to the procedure described for the preparation of (III), to yield (VI). 3.95 g were obtained (yield: 76%).
m.p. = 185-187°C ; $[\alpha]_{546}^{25}$ = - 15.0° (c=1, CH_3COOH) ; Rf = 0.56 in the system $CHCl_3/CH_3OH/CH_3COOH$ (85/20/5) ; NMR spectra δ p.p.m. : 0.98 (3H, t, CH_3 ethyl), 1.22 (2H, m, γCH_2 Lys), 1.38 (9H, s, Boc), 1.45 (2H, m, δCH_2 Lys + 1H, m, $CH\beta CH_2$ Lys), 1.58 (1H, m, $CH\beta CH_2$ Lys), 1.67 (1H, m, $CH\beta CH_2$ Glu), 1.84 (1H, m, $CH\beta CH_2$ Glu), 2.13 (1H, m, $CH\gamma CH_2$ Glu), 2.17 (1H, m, $CH\gamma CH_2$ Glu), 3.03 (2H, m, ϵCH_2 Lys + 2H, m, CH_2 ethyl), 3.56 (2H, d, αCH_2 Gly), 3.81 (1H, m, αCHGlu), 4.11 (1H, m, αCH Lys), 5.02 (2H, s, CH_2 benzyl), 6.73 (1H, d, αNH Glu), 6.95 (1H, s, NHcis NH_2), 7.23 (1H, s, NHtrans NH_2), 7.35 (5H, s, phenyl), 7.36 (1H, t, αNH Gly), 7.79 (1H, t, ϵNH Lys), 7.83 (1H, t, NH ethylamide), 7.88 (1H, d, αNH Lys).

HCl,H-D-Glu[Lys(Z-Gly)-NHEt]-NH_2 (VII)

(VII) was obtained following the same procedure employed for the preparation of (II). The Boc protecting group was removed from 2 g (3.4 mmol) of (VI) in 6 ml of acetic acid, by addition of 10.60 ml (16.9 mmol) of 1.59N HCl in acetic acid. The product was precipitated in diethyl ether, filtered and dried. 1.74 g of dry product were harvested (yield: 97%).
m.p. = 151 - 152°C ; $[\alpha]_{546}^{25}$ = - 12.5° (c=1, CH_3OH) ; Rf = 0.68 in the system ethyl acetate/pyridine/H_2O (20/10/11).

Nps-Ala-D-Glu[Lys(Z-Gly)-NHEt]-NH_2 (VIII)

1.58 g (3.0 mmol) of (VII) in 38 ml of DMF, neutralized with 1.04 ml (6.0 mmol) of DIEA, were coupled with 1.26 g (3.0 mmol) of Nps-Ala-OH, DCHA using 1.72 g (3.9 mmol) of BOP reagent[16]. The product was precipitated in cold isopropanol and was harvested by filtration. 1.65 g were yielded (yield: 70%).
m.p. = 198 - 200°C ; $[\alpha]_{546}^{25}$ = - 22.1° (c=1, DMF) ; Rf = 0.52 in the system $CHCl_3/CH_3OH/CH_3COOH$ (85/20/5).

HCl,H-Ala-D-Glu[Lys(Z-Gly)-NHEt]-NH_2 (IX)

1.40 g (1.9 mmol) of (VIII) in 30 ml of DMF were treated according to the procedure used for the preparation of (IV).

m.p. = 138 - 140°C ; $[\alpha]_{546}^{25}$ = + 3.2° (c=1, CH_3OH) ; Rf = 0.60 in the system ethyl acetate/pyridine/H_2O (20/10/11) ; NMR spectra δ p.p.m. : 0.98 (3H, t, CH_3 ethyl), 1.22 (2H, m, γCH_2 Lys), 1.38 (3H, d, CH_3 Ala + 2H, m, δCH_2 Lys), 1.47 (1H, m, $CH\beta CH_2$ Lys), 1.57 (1H, m, $CH\beta CH_2$ Lys), 1.73 (1H, m, $CH\beta CH_2$ Glu), 1.96 (1H, m, $CH\beta CH_2$ Glu), 2.16 (2H, t, γCH_2 Glu), 3.03 (2H, m, ϵCH_2 Lys + 2H, m, CH_2 ethyl), 3.55 (2H, d, αCH_2 Gly), 3.91 (1H, m, αCH Glu), 4.12 (1H, m, αCH Lys), 4.20 (1H, m, αCH Ala), 5.02 (2H, s, CH_2 benzyl), 7.14 (1H, s, NHcis NH_2), 7.34 (5H, s, phenyl + 1H, t, αNH Gly), 7.48 (1H, s, NHtrans NH_2), 7.83 (1H, t, ϵNH Lys), 7.87 (1H, t, NH ethylamide), 7.96 (1H, d, αNH Lys), 8.19 (3H, s, NH_3^+ Ala), 8.62 (1H, d, αNH Glu).

Synthesis of the pseudopenta- and pseudohexapeptides

The acylpseudopenta- and acylpseudohexapeptides were prepared by coupling, with BOP reagent[16], between (IV) or (IX) respectively and the pseudodipeptides R_1-XxxΨ[CH_2O]-D,L-Ala-OH (R_1 = Ac, Boc or Laur ; Xxx = Gly or Ser(Bzl)) the synthesis of which has been already described[10], followed by a catalytic hydrogenolysis in presence of palladium-barium sulfate (10% Pd) (Fig. 3). Physico-chemical data concerning these new derivatives were reported in Table I.

NMR data of the pseudopentapeptides Ac-GlyΨ[CH_2O]Ala-Ala-D-Glu(Lys-NHEt)-NH_2,CH_3COOH (Ac-GlyΨLPP) and Ac-GlyΨ[CH_2O]-D-Ala-Ala-D-Glu(Lys-NHEt)-NH_2, CH_3COOH (Ac-GlyΨDHP) are:

Ac-GlyΨLPP (1, L): NMR spectra δ ppm: 1.00 (3H, t, CH_3 ethylamide), 1.22 (3H, d, CH_3 ΨAla), 1.25 (2H, m, γCH_2 Lys), 1.27 (3H, d, CH_3 Ala), 1.50 (2H, m, δCH_2 Lys + 1H, m, $CH\beta CH_2$ Lys), 1.64 (1H, m, $CH\beta CH_2$ Lys + 1H, m, $CH\beta CH_2$ Glu), 1.82 (3H, s, acetyl), 1.97 (1H, m, $CH\beta CH_2$ Glu), 2.14 (2H, t, γCH_2 Glu), 2.75 (2H, m, ϵCH_2 Lys), 3.07 (2H, m, CH_2 ethylamide), 3.22 (2H, m, αCH_2 Gly), 3.42 (2H, m, CH_2' Gly), 3.84 (1H, q, αCH ΨAla), 4.15 (1H, m, αCH Lys + 1H, m, αCH Glu), 4.32 (1H, m, αCH Ala), 7.10 (1H, s, NHcis NH_2), 7.31 (1H, s, NHtrans NH_2), 7.64 (3H, broad s, ϵNH_3^+ Lys), 7.84 (1H, t, NH ethylamide), 7.88 (1H, d, αNH Ala), 7.90 (1H, t, αNH Gly), 7.91 (1H, d, αNH Lys), 8.12 (1H, d, αNH Glu).

Ac-GlyΨDPP (1, D): NMR spectra δ ppm: 1.00 (3H, t, CH_3 ethylamide), 1.23 (3H, d, CH_3 ΨAla), 1.25 (2H, m, γCH_2 Lys), 1.27 (3H, d, CH_3 Ala), 1.50 (2H, m, δCH_2 Lys + 1H, m, $CH\beta CH_2$ Lys), 1.64 (1H, m, $CH\beta CH_2$ Lys + 1H, m, $CH\beta CH_2$ Glu), 1.82 (3H, s, acetyl), 1.97 (1H, m, $CH\beta CH_2$ Glu), 2.14 (2H, t, γCH_2 Glu), 2.75 (2H, m, ϵCH_2 Lys), 3.07 (2H, m, CH_2 ethylamide), 3.22 (2H, m, αCH_2 Gly), 3.42 (2H, m, CH_2' Gly), 3.84 (1H, q, αCH ΨAla), 4.14 (1H, m, αCH Lys + 1H, m, αCH Glu), 4.30 (1H, m, αCH Ala), 7.10 (1H, s, NHcis NH_2), 7.33 (1H, s, NHtrans NH_2), 7.64 (3H, broad s, ϵNH_3^+ Lys), 7.84 (1H, t, NH ethylamide), 7.88 (1H, d, αNH Ala), 7.91 (1H, d, αNH Lys), 7.94 (1H, t, αNH Gly), 8.06 (1H, d, αNH Glu).

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