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# SOLUTION CONFORMATIONS OF THE IMMUNOMODULATOR MURAMYL PEPTIDES

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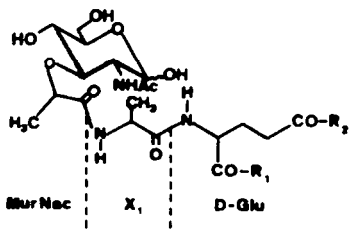
ABSTRACT - MDP, Murabutide and the MDP[D-Ala] analogue have been studied dissolved in dimethylsulfoxyde by means of $^1$H-n.m.r., including the 2D.2Q $^1$H-$^1$H inadequate and NOESY (Nuclear Overhauser Effect Spectroscopy) experiments. The results confirm the "S" shaped conformation constituted by two adjacent ß turns in MDP. The first turn is a tight "type II ß turn" and takes place around the MurNac (N-acetylmuramic acid) moiety of MDP and Murabutide and is stabilized by a $C_{10}$ hydrogen bonding between the Ala NH (i + 3) and the acetamido C = O (i). The second turn is centered around L-Ala-D-iGln and requires the α-carboxamide group of D-iGln for its stabilization and thus does not occur in Murabutide. The L-Ala —>D-Ala inversion in MDP[D-Ala] produces substantial conformational modifications leading to a cyclic structure with the iGln α-carboxamide protons and the acetamido methyl group in close spatial proximity. The dissociation of the adjuvant, antiinfection and pyrogenic activities in the 3 compounds is well depicted by these variations of conformations.

## INTRODUCTION

Our recent $^1$H-n.m.r. studies of MDP (MurNac-L-Ala-D-iGln) 1, the smallest immunostimulating entity of mycobacterial cell wall, and of its therapeutically promising analogue, Murabutide (Mur Nac-L-Ala-D-Gln-OnBu) 2, have revealed a common conformational structure, namely a type II ß turn involving the MurNac-L-Ala moiety in the two glycopeptides (Figure 1) (1). Both glycopeptides and numerous derivatives were found to stimulate non-specific resistance against bacterial infection, while others, as for instance MDP[D-Ala] (MurNac-D-Ala-D-iGln) 3, appeared inactive in this respect (2).




| | $X_1$ | $R_1$ | $R_2$ |
|---|---|---|---|
| 1 | L-Ala | $NH_2$ | OH |
| 2 | L-Ala | $O(CH_2)_4H$ | $NH_2$ |
| 3 | D-Ala | $NH_2$ | OH |

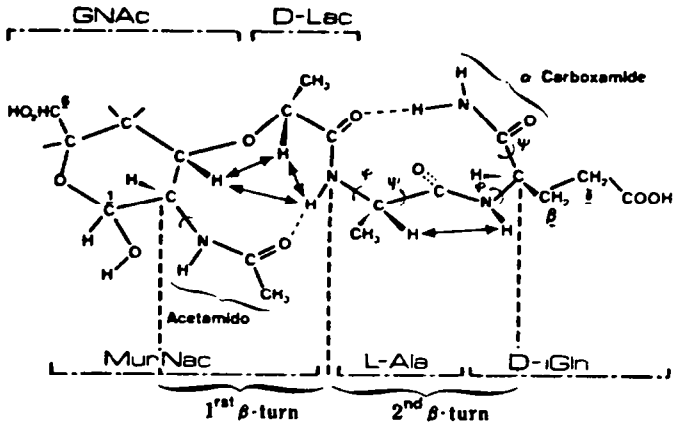



Figure 1 : Conformation of MurNac-L-Ala-D-iGln

Further $^1$H-n.m.r. studies were undertaken, using some analogues chemically modified on the L-alanyle site ($X_1$) and inactive against *k. pneumoniae* challenge, which suggest a relationship between the expression of such a biological activity and the existence of the first turn (3). Here we report direct evidence from 2-D Noesy experiments on the β turn common to MDP and Murabutide, and the origin of its destabilization in MDP[D-Ala] (MurNac-D-Ala-D-iGln) 3.

## RESULTS

The 2D double quantum $^1$H-$^1$H inadequate spectrum of Murabutide dissolved in dimethylsulfoxide is given in Figure 2, together with the 1D $^1$H-spectrum. The experiment provides a useful procedure which has allowed the univocal assignment of all the protons of the molecule, including those belonging to the minor β-anomer population, even for the sugar moiety. The 2 quantum skew diagonal ($w_1 = 2w_2$) is drawn on the 2D map. Each of the direct connectivities NH —> α-H, α-H —> βH...in

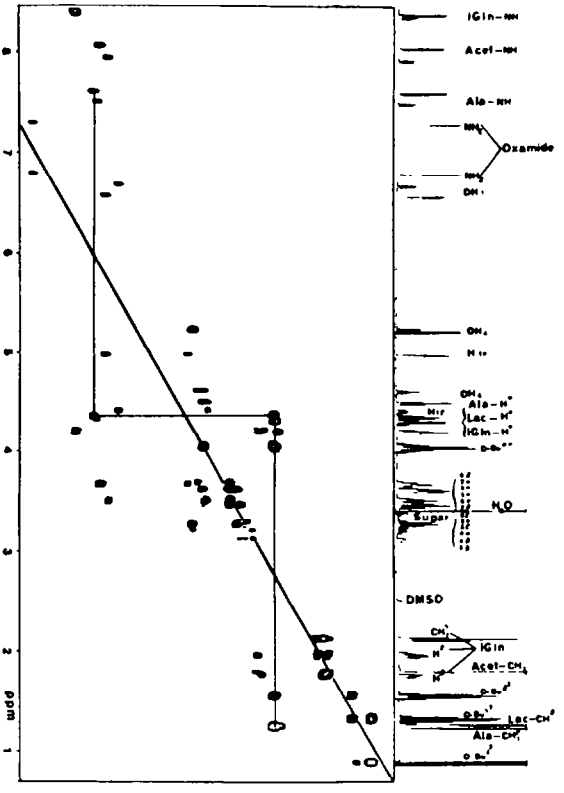



Figure 2 : $^1$H-Inadequate spectrum of Murabutide in dimethyl-sulfoxide, 20°C, chemical shifts from internal TMS.

the aminoacids and H-1 —> H-2 —> H-3 —> H-4 —> H-5 —> H-6,6' in the sugar is manifested by a pair of signals symmetrically disposed relative to the skew diagonal at the $W_2$ positions of the two interacting spins. The assignments of the Ala protons is given as an example.

The 2D $^1$H-NOESY contourplot maps, cross sections corresponding to αNH protons of Murabutide and MDP[D-Ala] dissolved in dimethylsulfoxide are given in Figures 3 (a) and 3 (b) respectively.

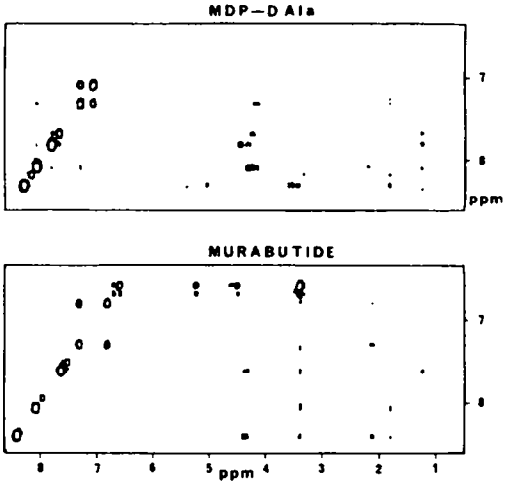



Figure 3 : NOESY contour plots spectra for the NH cross sections of
a) MDP[D-Ala]
b) Murabutide
Dimethylsulfoxide, 20°C, chemical shifts from internal TMS.

The following connectivities are observed :

a) In Murabutide :

i) Ala-NH (7.60 ppm) :
Lac α-H (4.29 ppm)
sugar H-3 (3.44 ppm)

and ii) Glx-NH (8.40 ppm) :
Ala α-H (4.35 ppm)
Glx ϑ-H,H' (2.11 ppm)

b) In MDP[D-Ala] :

i) Ala-NH (7.81 ppm) :
Lac α-H (4.46 ppm)
iGln NH (8.03 ppm)
Acetamido NH (8.26 ppm)

ii) iGln-NH (8.03 ppm) :
Ala α-H (4.31 ppm)
iGln ϑ-H,H' (2.23 ppm)
Ala β-$CH_3$ (1.24 ppm)
Oxamide $NH_\alpha$ (7.30 ppm)

iii) Acetamido NH (8.26 ppm) :
sugar H-1 (5.05 ppm)
sugar H-2 (3.55 ppm)
sugar H-3 (3.45 ppm)

and iv) Oxamide $NH_\alpha$ (7.30 ppm) :
iGln α-H (4.18 ppm)
Acetamido $CH_3$ (1.85 ppm)

The NOESY spectrum of MDP which is not shown here displays the following connectivities :

i) Ala-NH (7.63 ppm) :
Lac α-H (4.26 ppm)

ii) iGln-NH (8.17 ppm) :
Ala α-H (4.26 ppm)
iGln ϑ-H,H' (4.31 ppm)
Oxamide $NH_\alpha$ (7.33 ppm)

iii) Acetamido NH (8.05 ppm) :
sugar H-1 (4.95 ppm)
sugar H-2 (3.66 ppm)
sugar H-3 (3.44 ppm)

In addition, in MDP, the Lac αH (4.26 ppm) and the sugar H-3 (3.44 ppm) protons manifest a very strong connectivity.

Chemical shifts of the backbone protons of the 3 glycopeptides in the α-and β-anomeric forms, are given in Table 1.

| Moiety | Proton | MDP | | Murabutide | | MDP [D-Ala] | |
|---|---|---|---|---|---|---|---|
| | | α | β | α | β | α | β |
| GlcNac | H-1 | 4.95 | 4.41 | 4.92 | 4.40 | 5.05 | 4.81 |
| | H-2 | 3.66 | 3.5 | 3.64 | 3.47 | 3.55 | - |
| | Ac-NH | 8.05 | 7.93 | 8.06 | 7.93 | 8.26 | 8.12 |
| | Ac-$CH_3$ | 1.78 | 1.77 | 1.78 | 1.77 | 1.824 | 1.813 |
| D-Lactoyl | Hα | 4.26 | 4.13 | 4.29 | 4.18 | 4.46 | 4.55 |
| $X_1$ | NH | 7.63 | 7.51 | 7.60 | 7.49 | 7.81 | 7.68 |
| | Hα | 4.26 | 4.22 | 4.35 | 4.31 | 4.31 | 4.25 |
| $X_2$ | NH | 8.17 | 8.10 | 8.40 | 8.37 | 8.03 | 7.95 |
| | Hα | 4.136 | 4.144 | 4.18 | | 4.18 | |

Table 1 : Proton chemical shifts for the glycopeptides dissolved in $d_6$-DMSO at 20°C, in ppm/TMS.

The coupling constants $^3J$ HCαNH and the NH T° coefficients in the backbone are given in Table 2.

## DISCUSSION

Conformational aspects :

a) The first β turn in MDP and Murabutide

We recently reported that MDP in dimethylsulfoxide adopted a "S" shaped conformation consisting of two adjacent turns (Figure 2). In this study we characterized a turn in both MDP and Murabutide which involves the MurNac-L-Ala segment, and mimics the type II β turn found in L-D depsipeptides. This turn is stabilized by a $C_{10}$ hydrogen bonding between the Ala-NH proton (i + 3 of β turns) and the acetamido carbonyl group (playing the role of i residue of the β turn). The relevant backbone $^3J$ HCαNH and NH T° coefficients are given in Table 2. The possible replacement of an L-amino acid by a GlcNac (N-acetylglucosamine) moiety in a type II β turn constitutes an interesting novelty which could be exploited in the design and the synthesis of new peptide analogues. Further insight into the properties of this turn are given by the NOESY experiments performed in the present work (Figure 3a). The cross-peaks between the Ala-NH proton (residue i+3) and the sugar H-3 proton (replacing the C=O oxygen of residue i+1) and Ala-NH (residue i+3) and the Lac α-proton (i+2) mentioned in the Results section, indicates that these three protons are in spatial proximity in the molecule of Murabutide (Figure 2) : this arrangement is consistent with the formation of a tight turn around the hinge MurNAC, similar to a β turn of type II. The same features are encountered in MDP consistently with the existence in the first part of this molecule of a β turn similar to that found in Murabutide.

The situation, however, is completely modified in the MDP[D-Ala] analogue. The NOESY spectrum still displays the Ala-NH —> Lac α-H connectivity, but the one Ala-NH —> sugar H-3 interaction apparently does not exist anymore in this analogue. It is replaced by a new

connectivity, Ala-NH —> acetamido NH, which probably results by the rotation of the acetamido group around the sugar C-2 ⟩— NH acetamido bond. This in turn leads to the disruption of the $C_{10}$ hydrogen bonding observed in MDP and Murabutide, which is consistent with the high NH temperature coefficient of the Ala residue in MDP[D-Ala] (Table 2).

| Compound | Acetamido group | | | | $X_1$ | | | | $X_2$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $\Delta\delta$NH/ $\Delta$T JNH-$C_2$H | | | | $\Delta\delta$NH/ $\Delta$T JNH-$C_\alpha$H | | | | $\Delta\delta$NH/ $\Delta$T JNH-$C_\alpha$H | | | |
| | α | ß | α | ß | α | ß | α | ß | α | ß | α | ß |
| MDP | -5.30 | -2.25 | 7.95 | 8.70 | -2.60 | -0.72 | 7.03 | 6.93 | -5.30 | -5.30 | 8.20 | 8.30 |
| Murabutide | -5.30 | -2.00 | 7.96 | 8.55 | -2.60 | -0.70 | 7.90 | 7.74 | -5.00 | -4.90 | 7.70 | 7.53 |
| MDP [D-Ala] | -4.00 | -4.30 | n.o. | n.o. | -3.30 | -1.60 | 7.20 | 7.10 | -5.60 | -3.10 | 7.00 | 7.10 |

Table 2 : $^1H$-$^1H$ coupling constants (Hz) and temperature dependence coefficients of the amide protons (X $10^{-3}$ ppm/$K^{-1}$) for the glycopeptides dissolved in $d_6$-DMSO.

The chemical shifts of the backbone protons in the MurNac-Ala segment shown in Table 1 also are very sensitive to the ß turn destabilization produced by the chirality inversion of the Ala residue in MDP[D-Ala].

b) The second ß turn in MDP

The $^3J$ HCαNH values found for L-Ala (7.03 Hz) and D-iGln (8.20 Hz) are consistent with the occurrence in the second part of the MDP molecule of a type II-ß turn identical to the ß turns observed in L-D peptides. Although the stabilizing $C_{10}$ hydrogen bonding (Figure 2) is not detected by temperature experiments, the conformational changes shown by the L-Ala residue when the α-carboxamide group at the C-terminus is replaced by another group (case of Murabutide in this work and also of MDP[D-Gln] and MDP[D-Glu] (3)) suggest the implication of the α-carboxamide protons in the stabilization of the second ß turn in MDP. For instance, the rotation around the Ala Cα ⟩— N bond in Murabutide, which is consistent with the increase of $^3J$ HCαNH in the Ala residue of this analogue (Table 2) may be related to the loss of the 4 ——> 1 hydrogen bonding Lac C=O...H-NH-CO-D-iGln. However, this can occur without a change in the Ala Ψ and Glx φ angles at the hinge of the ß turn (Figure 2), a result consistent with the appearance of a strong cross-peak between the Glx NH proton and the Ala α-proton (≈ cis arrangement) in the Murabutide NOESY spectrum (Figure 3a) and only a weak cross-peak between the Glx NH proton and the Glx α-proton (≈ trans arrangement). These connectivities are also found in the MDP spectrum which, in addition, indicates that in the iGln residue of MDP the α-carboxamide E proton is located near

its α and NH protons in agreement with a turn formation at the C-terminal (Figure 2). Remarkably, the increase in the Ala φ angle in the Murabutide molecule has no disturbing effects on the stability of the first β turn as shown by the similarity of the Ala NH temperature coefficient in MDP and Murabutide (Table 2). In MDP[D-Ala] both the first β turn and the second β turn are modified in such a way that a completely new conformation is generated. The appearance of a new connectivity between the carboxamide $NH_\varepsilon$ proton and the acetamido methyl group in the NOESY spectrum indicates that the two ends of the MDP[D-Ala] molecule are in spatial proximity. The chirality change, L-Ala —> D-Ala, in the glycopeptide therefore has inverted the orientation of the C-terminal iGln residue such that the molecule adopts a cyclic conformation instead of the "S" shaped conformation found in MDP.

Conformation - Activity relationships :

The present $^1$H-n.m.r. studies indicate that the MurNac moiety can readily accomodate a "type II β turn" in MDP and Murabutide. Manifestation of the antiinfection activity in MDP and Murabutide requires the integrity of this turn, while the undesirable pyrogenic activity is abolished in Murabutide apparently with the defection of the second turn. The two compounds, however, display about the same adjuvant properties. The change of the conformation in the MDP[D-Ala] molecule with disappearance of the "S" shaped structure and its replacement by a cyclic structure results in a complete loss of activity in this analogue.

## CONCLUSION

We have shown the existence of important similarities between the turns occuring in glycopeptides and in peptides. The L,D,L,D alternation of asymmetric Cα-carbons in the glycopeptide backbone, with the sugar C2-carbon mimicking the L, Cα-carbon of amino acid residues, confers a "S" shaped conformation to the MDP molecule. The conformation-activity relationships indicate that any structural change performed in the glycopeptide, modifying the "S" conformation, produces measurable variation of the biological activities. These notions emphasize the importance of conformational analysis in the series of MDP for the design of more potent analogues.

## EXPERIMENTAL

Materials :

The synthesis of MDP, MDP[D-Ala] (4) and Murabutide (5) have been reported previously.

$^1$H-n.m.r. experiments :

The n.m.r. samples were recorded on Bruker AM500 and MSL300 spectrometers associated to Aspect 3000 computers. For the $^1$H 500 MHz spectra a spectral window of 5000 Hz was chosen. The 16K FID was resolution enhanced by Lorentzian-gaussian function, zero filled to 32 K and Fourier transformed. The data set for the 2D-experiments consisted of 256 time increment (128-256 scans each 2K FID) with 1 sec recycle delay. The $t_1$ dimension was zero filled to 1K. For Inadequate $^1$H-$^1$H experiments, 128 times increment and 0.03 sec for double quantum delay were chosen. The resulting data matrix ($t_1$, $t_2$) was double Fourier transformed to give 2D-spectrum in the frequency domain ($f_1$, $f_2$).

Noesy experiments were performed at 300 MHz. NOE was nul at 500 MHz. The mixing time was 400 msec (same order of magnitude as the average $t_1$). A 5% random variation was used to eliminate J cross-peaks.

Experiments were run at 295° K using 10 mM solutions of samples in $d_6$-DMSO (99.99% $^2H$). TMS was used as an internal reference. Temperature variation experiments were performed between 295° K and 330° K.

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