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SYNTHESIS, BIOLOGICAL ACTIVITY AND MOLECULAR MODELLING OF THE 53-54 KETOMETHYLENE ANALOGUE OF HEL(52-61)

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Abstract

The synthesis of the 53-54 ketomethylene isostere of HEL(52-61), an antigenic decapeptide, was realised by convergent Fmoc solid-phase peptide synthesis. In addition to the target peptide, an unexpected hydroxysuccinyl by-product was recovered. The two ketomethylene epimers did not exhibit any stimulating or competitive activity. Molecular modelling studies of the analogues-MHC-II I-A^k complex suggested a departure from the canonical polyproline II conformation which could account for the absence of binding to I-A^k molecule.

Exploration of peptide-MHC (Molecular Histocompatibilty Complex)-TcR (T cell Receptor) molecular interactions has been developed over the last few years due to accumulative data on the sequences of natural peptides bound to MHC molecules, combinatorial peptide chemistry and mainly X-ray crystallographic studies [1]. If peptide-MHC class I (MHC-I) interactions are well understood with the elaboration of predictive binding motifs [2], less information have been collected for peptide-MHC class II (MHC-II) interactions. The length of peptides eluted from MHC-II molecules is 11 to 23-24 residues which is related to the structure of the MHC-II binding cleft. Contrary to MHC-I molecule, the MHC-II binding groove is opened at both extremities allowing protrusion of both peptide termini. Hydrogen bonding between the peptide main chain and side chains of MHC-II residues is conserved through murine MHC-II and HLA molecules which constrains the peptide to adopt a polyproline II type conformation [3]. Allele-specific binding is determined by polymorphism in the

peptide binding groove with 4-5 pockets accommodating specific peptide side chains.

The use of pseudopeptides bearing modified amide bond is an attractive strategy to gain insight into the rules governing the interactions between peptide and MHC-II molecules. We have used this strategy on an hen egg white lysozyme (HEL)-derived murine model, HEL(52-61) [4], an immunogenic peptide for murine MHC-II I-A^k restricted T-cell clones, by replacing the 53-54 amide bond of the model by *trans* alkene and reduced amide bonds [5]. Biological tests have shown that the *trans* ethylenic isostere is presented by MHC-II I-A^k molecule and recognised by HEL(52-61)-specific T-cell clones contrary to the reduced analogue which presents neither stimulating nor competing activity. We report herein the synthesis of the corresponding 53-54 ketomethylene isostere of HEL(52-61) by convergent solid-phase peptide synthesis, its biological activity and molecular modelling studies of isosteres-MHC-II I-A^k complex.

Material and Method

Fluorenyl-oxy-carbonyl(Fmoc)-protected amino acid derivatives were purchased from France Biochem (Meudon, France) and Neosystem (Strasbourg, France). Solvents and reagents were reagent grade and used without further purification. Melting points were performed on a Köfler hot-stage apparatus and were not corrected. IR spectra were carried out on a Perkin-Elmer FT-IR 1600 spectrophotometer. Optical rotations were determined with a Roussel-Jouan type 71 digital polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a Brucker AC 200 spectrometer. High resolution electrospray mass spectrometry (HRESMS) was performed by Quad Service (Poissy, France). Low resolution ESMS, MS-MS and LC-MS were carried out at the Vrije Universiteit Brussel (VUB) (Brussels, Belgium). Ascending TLC was performed on Merck precoated plates of silica gel 60 F_{256}. All products but peptides **4** and **5** were located by UV (254 nm), with iodine vapour or bromophenol blue/0.1 M NaOH for acids and purified by silica gel chromatography (Matrex silica 60 Å 35-70 μm).

Synthesis of N$^\alpha$*-Boc-Asp(*t*Bu)-(*R,S*)Tyr(*t*Bu)Ψ[$COCH_2$]Gly-OH* ***2***

N$^\alpha$-Boc-Asp(*t*Bu)-(*R,S*)Tyr(*t*Bu)Ψ[$COCH_2$]Gly-OMe **1**

To a solution of dipeptide *N*$^\alpha$-Boc-Asp(*t*Bu)-Tyr(*t*Bu)-OH (1.29 g, 2.54 mmol) and the anhydride of monomethyl succinate (1.8 g, 7.32 mmol) in dichloromethane (30 ml) was added *N,N'*-dimethylaminopyridine (0.029 g, 0.229 mmol) and 450 μl (2.23 mmol) of triethylamine. The mixture was stirred at reflux for 90 min at which time the mixture was dried *in vacuo*. The solution was then stirred for 45 min at r.t. with 5% $NaHCO_3$ (25 ml), extracted with ethyl acetate (3 × 75 ml) and washed with 0.1 M acetic acid (2 × 50 ml), brine (50 ml) and water (50 ml). The organic layer was dried over Na_2SO_4, filtered and evaporated. Finally, the residue was purified by flash chromatography (silica gel, petrol ether/ ethyl acetate 7:3) yielding 1.25 g of **1** as a yellow paste (2.16 mmol, 90% yield).

Compound **1**: $[\alpha]_D^{28°C}$ +13.5 (c 1.48; $CHCl_3$). IR-FT (film, ν cm^{-1}): 3339 (ν NH); 2978 (ν_{as} CH_3); 2932 (ν_{as} CH_2); 1784, 1727 and 1674 (ν CO); 1609, 1509 et 1456 (ν C=C arom); 1390; 1365; 1238; 1165; 899; 847. ^{1}H

NMR (200 MHz, $CDCl_3$, δ ppm): 1.25 (s, 9H, *t*Bu); 1.36 (s, 18H, 2*t*Bu); 2.41-3.00 (m, 8H, 4 CH_2); 3.56 and 3.60 (2 s, 3H, OCH_3); 4.37 (m, 1H, CHα); 4.67 (m, 1H, CHα); 5.70 (m, 1H, NH); 6.82 (2d, ^3J = 8.36 Hz and ^3J = 8.28 Hz, 2H, 2H arom Tyr); 7.01 (2 d, ^3J = 8.36 Hz and ^3J = 8.28 Hz, 2H, 2H arom Tyr); 7.17 (m, 1H, NH). ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 27.74 ($3CH_3$ *t*Bu); 28.02 ($3CH_3$ *t*Bu); 28.55 ($3CH_3$ *t*Bu); 34.84 (CH_2); 35.04 (CH_2); 36.13 and 36.20 (CH_2); 37.10 (CH_2); 51.48 (OCH_3); 58.98 (Cα); 59.98 (Cα); 78.09 (Cq *t*Bu); 80.06 (Cq *t*Bu); 81.31 (Cq *t*Bu); 124.02 (2CH arom Tyr); 129.41 and 129.47 (2CH arom Tyr); 130.78 (Cq arom Tyr); 154.13 (CO arom Tyr); 155.22 (CO *t*Boc); 170.55 (CO); 170.72 and 170.76 (CO); 172.57 and 172.64 ($\underline{C}OOCH_3$) ; 206.40 and 206.64 (CO Tyr).

N^{α}-Boc-Asp(*t*Bu)-(*R,S*)Tyr(*t*Bu)Ψ[$COCH_2$]Gly-OH **2**

A solution of ester **1** (1.3 g, 2.249 mmol) in 20 ml MeOH was cooled to 0°C and 10 ml of 1 M NaOH was slowly added under stirring. The mixture was then stirred for 5 h at 0°C. At this time, the solution was neutralised at 0°C with 1 M HCl and MeOH removed under vacuum. The aqueous layer was then extracted with ethyl acetate (3 × 100 ml). The organic layers were dried over Na_2SO_4, filtered and evaporated. The residue was finally purified by flash chromatography (silica gel, petrol ether/ethyl acetate 8:2) to afford 1.14 g of acid **2** as a yellow solid (2.02 mmol, 90% yield).

Compound **2**: $[\alpha]_D^{28°C}$ +3.7 (c 1.08 ;$CHCl_3$). FT IR (film, ν cm^{-1}): 3500-2500 (ν OH); 3338 (ν NH); 3055 (ν NH et ν CH arom); 2981 (ν_{as} CH_3); 2932 (ν_{as} CH_2); 1717 (ν CO); 1611 and 1507 (ν C=C); 1420; 1394 ; 1368; 1266; 1162; 890; 738 and 704 (γ CH arom). ESPMS (positive mode, %): 588 (33, $[M+H+Na]^+$); 587 (100, $[M+Na]^+$); 566 (30, $[M+2H]^+$); 565 (87, $[M+H]^+$); 532 (25, $[M+2H+Na\text{-}tBu]^+$); 531 (83, $[M+Na+H\text{-}tBu]^+$); 510 (26, $[M+3H\text{-}2tBu]^+$); 509 (91, $[M+2H\text{-}2tBu]^+$); 453 (31); 409 (24); 312 (31); 225 (48); 178 (25); 130 (26). ^1H NMR (200 MHz, DMSO-d_6, δ ppm): 1.24 (s, 9H, *t*Bu); 1.36 (s, 18H, 2 *t*Bu); 2.35-2.50 (m, 4H, $COC\underline{H_2}C\underline{H_2}COOH$); 2.67 (m, 2H, $CH_2\beta$); 2.98 (m, 2H, $CH_2\beta$); 4.24 (M, 1H, Hα); 4.40 (M, 1H, Hα); 6.79-6.86 (2 d, ^3J = 7.43 Hz and ^3J = 8.23 Hz, 2H, 2H arom Tyr); 6.99 (d, 1H, ^3J = 7.57 Hz, NH); 7.09 (d, ^3J = 8.23 Hz, 2H, 2H arom Tyr); 8.20 (m, 1H, NH). ^{13}C NMR (50 MHz, DMSO-d_6, δ ppm): 27.70 ($3CH_3$ *t*Bu); 28.18 ($3CH_3$ *t*Bu); 28.61 ($3CH_3$ tBu); 34.04 (CH_2); 34.73 (CH_2); 36.8 (CH_2); 37.51 (CH_2); 59.30 and 59.55 (Cα); 59.79 (Cα); 77.65 (Cq *t*Bu); 78.23 (Cq *t*Bu); 80.05 and 80.13 (Cq *t*Bu); 123.52 (2CH arom Tyr); 129.62 and 129.68 (2CH arom Tyr); 132.32 and 132.38 (Cq arom Tyr); 153.54 (CO arom Tyr); 155.21 (CO *t*Boc); 169.94 and 169.53 (CO); 171.78 (CO); 173.63 (COOH); 207.83 (CO Tyr).

Synthesis of the ketomethylene analogue $Tyr^{53}\Psi[COCH_2]Gly^{54}$HEL(52-61)

Solid-phase synthesis of the ketomethylene peptide was carried out with a Novasyn GEM semi-automatic continuous flow synthesiser on a NovaSyn TGA resin (0.15 mmol, subst. 0.25 mmol/g). Loading of first residue (Arg) on resin was performed with the symmetrical anhydride method. Couplings were mediated by benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP)/1-hydroxybenzotriazole (HOBt)/diisopro-pylethylamine (DIEA) mixture in dimethylformamide (DMF) for all amino acids but Asn

which was incorporated as tetrafluorophenyl ester derivative. Removal of the Fmoc group before each coupling was performed by a 20% solution (v/v) of piperidine in DMF for 10 min. Monitoring was done using the Kaiser test. Coupling of protected peptide **2** (3 equiv./resin) was performed manually with BOP 3 equiv./HOBt 3 equiv./DIPEA 6 equiv. for 18 h at room temperature using an in-house apparatus. Release of peptides from resin was carried out by treatment of the resin at room temperature with 20 ml/g of a freshly prepared solution of trifluoroacetic acid (TFA)/phenol/H_2O/ethanedithiol (EDT) /thioanisole/triisopropylsilane (TIS) 81.5:5:5:2.5:5:1 mixture [6] for 15 h so as to achieve complete cleavage of the Mtr protecting group. The peptides were precipitated from the cleavage solution after filtration of the resin, by addition of cold diethyl ether (100 ml/g), dissolved in 0.1% TFA/30% acetonitrile (CH_3CN)/H_2O and finally lyophilised. LC-MS analysis of the crude peptide was performed on a Kontron HPLC system coupled with a Fisons VG Quattro II mass spectrometer in positive mode on a Vydac 218TP54 C18 column (250 × 4.6 mm, 5 μm particle size, 300 Å pore size). Elution gradient was 0%-60% B in 30 min at a flow rate of 1 ml/min where A was H_2O and solvent B 60% CH_3CN/H_2O with detection at 215 nm. Purification of the crude peptide was performed on a Kontron HPLC 400 system equipped with a Vydac 218TP1022 C18 reverse-phase preparative column (250 × 22 mm, 10 μm particle size, 300 Å pore size).The elution gradient was 10%-45% B in 45 min at a flow rate of 16 ml/min where A was triethylammonium phosphate (TEAP) pH 2.25 and solvent B was 60% CH_3CN/TEAP pH 2.25. Pure fractions were pooled and desalinated with an elution gradient of 15% B-42% B in 45 min at a flow rate of 16 ml/min where A was TFA 0.1%/H_2O and solvent B 0.08% TFA/60% CH_3CN/H_2O. Detection was at 210 nm.

Asp-(*R,S*)TyrΨ[$COCH_2$]Gly-Ile-Leu-Gln-Ile-Asn-Ser-Arg **4**

More polar isomer **4a** (10.4 mg, 0.008 mmol, 5.9% yield):

LC-MS: t_R = 17.85 min. ESMS (positive mode, %): 1177.6 ($[M+H]^+$, 1); 589.7 ($[M+2H]^{2+}$, 100).

Less polar isomer **4b** (12.9 mg, 0.01 mmol, 7.3% yield):

LC-MS: t_R = 17.95 min. ESMS (positive mode, %): 1177.5 ($[M+H]^+$, 1); 589.7 ($[M+2H]^{2+}$, 100).

HO-$N^{\alpha 55}$-Suc-Ile-Leu-Gln-Ile-Asn-Ser-Arg **5** (23.9 mg, 0.054 mmol, 16.9% yield):

LC-MS: t_R = 17.64 min. ESMS (positive mode, %): 943 ($[M+H]^+$, 100); 491 (26); 472 ($[M+2H]^{2+}$, 26); 342 (20); 328 (79). HRESMS (positive mode, %): 943.520652 ($[M+H]^+$, 100); $C_{40}H_{71}N_{12}O_{14}$ requires 943.521271.

Biological test

Anti-HEL I-A^k-restricted 2A11 and 3A9 mouse cell hybridomas recognising the HEL(52-61) peptide were co-cultured with $H2^k$ mouse CH27 B cells in the absence or presence of serial dilution of peptides as reported [4]. The level of T cell stimulation was determined by measuring the IL-2 release using the CTL-L2 bioassay. The results were expressed as stimulated peptide concentration (Spc) where Spc was the graphically determined peptide concentration giving 50% of the maximal T cell stimulation. For competition experiments, serial dilutions of the competing peptide were mixed with a concentration of the HEL(52-61) peptide giving a

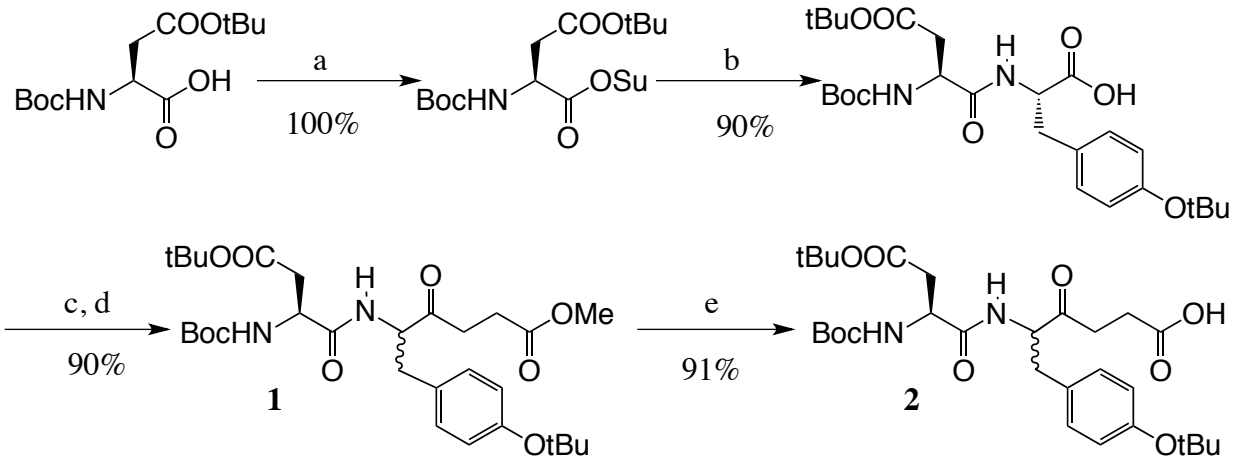
suboptimal T cell stimulation.

Molecular modelling

Molecular modelling was performed with the SYBYL package version 6.2 (Tripos Inc., St Louis, MO, USA). Starting from the HLA-DR1/HA crystal structure [7] deposited in the Protein Data Bank, code 1DLH, we constructed a model of the I-A^k molecule. All hydrogen atoms are explicitly taken into account throughout the calculations. The force field used is Koll_all, a clone of Amber [8] by Tripos. A dielectric function of 4d was used in combination with a non-bonded interactions cut-off of 8 Å. All calculations were performed on Origin 2000 (R10000) and O2 (R5000) Silicon Graphics workstations. Energy minimisation was performed in two steps beginning with 1000 iterations using the steepest descent algorithm followed by complete minimisation using the conjugate gradient algorithm until the RMS gradient of the potential energy was less than 0.1 kcal/mol.$Å^2$. I-A^k backbone was set as an aggregate. Initial velocities were also taken from a Boltzmann distribution and readjusted every 25 fs. The system was progressively heated to 300 K during the first ps. Then, the temperature was being held at 300 K for the rest of the simulation by coupling the system to a heat bath [9] using a temperature coupling constant of 0.1 ps. The SHAKE algorithm [10] was used to constrain all bond lengths to their equilibrium values with a bond length tolerance of 2.5×10^{-4}. Coordinates, energies and velocities were collected and saved every 1000 fs for the longest dynamics. The energy minimisation was performed the same way as above. In order to save computational time, only the $\alpha 1$ and $\beta 1$ domains of the MHC-II I-A^k molecule were taken into account. This approximation was shown not to alter the accuracy of molecular dynamic simulations [11].

Results

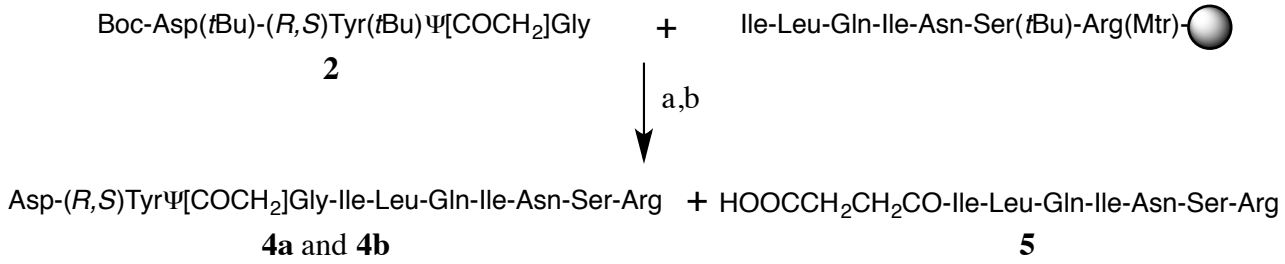
Our strategy for the synthesis of $Tyr^{53}\Psi[COCH_2]Gly^{54}$HEL(52-61) required the coupling of a suitable protected ketomethylene moiety onto HEL(55-61). We initially planned to use the N^{α}-Fmoc-Asp(*t*Bu)-(*R,S*)Tyr(*t*Bu)$\Psi[COCH_2]$Gly-OH molecule synthesised by a modified Dakin-West reaction [12]. Unfortunately, we were unable to achieve the coupling of the Fmoc-protected molecule by solid phase peptide synthesis. This failure could be ascribed to the basic condition used for deprotection of the N-terminal Fmoc protecting group. Potential formation of imine between the N-terminal amino group and the ketomethylene carbonyle during the final deprotection stage might lead to degradation or instable products. As an alternative, we took advantage of the fact that the Asp residue was the last one in the HEL(52-61) sequence to envisage direct coupling of the N^{α}-Boc-protected compound **2**. This ketomethylene acid **2** was synthesised by the same Dakin-West strategy [12] with good overall yield as shown in figure 1. We were unable to separate the two isomers of **1** or **2** by flash chromatography.



a. DME, *N*-hydroxysuccinimide, DCC, 0°C, 24 h b. DMF/DMSO, DIPEA, Tyr(*t*Bu), r.t., 5 h c. Anhydride of monomethyl succinate, DMAP cat., Et_3N, CH_2Cl_2, reflux, 90 min d. Evaporation then $NaHCO_3$ 5%, r.t., 45 min e. MeOH, NaOH 1 M, 0°C, 5 h

Figure 1.

Coupling of acid **2** by solid phase peptide synthesis on HEL(55-61) was successfully realised with BOP/HOBt/DIPEA for 18 h at room temperature using 3 equiv. of ketomethylene moiety **2** - Figure 2.



a. BOP/HOBt/DIPEA, DMF, 18 h b. TFA/phenol/H_2O/EDT/thioanisole/TIS 81.5:5:5:2.5:5:1, 15 h

Figure 2.

After cleavage from the resin and precipitation, the LC-MS analysis of the crude peptide showing three closed peaks eluted at t_R 17.64, 17.85 and 17.95 min with $[MH]^+$ pseudomolecular ions at m/z 943, 589.7 and 589.7 respectively. The two less polar peptides fitted with the two ketomethylene epimers **4a** and **4b**. The major more polar product was unexpected and a high resolution mass analysis showed that it could be a succinyl derivative of HEL(55-61). As a matter of fact, this by-product revealed to be identical by RP-HPLC analysis and MS-MS experiments with a sample of HO-$N^{\alpha 55}$-Suc-HEL(55-61) **5** synthesised by amidation of HEL(55-61) on solid phase with monotertiobutyl succinate and subsequent cleavage. Formation of this derivative is difficult to rationalise because it would imply an oxidation of (*R,S*)Tyr^{53}Ψ[$COCH_2$]Gly^{54}HEL(52-61) during the synthesis. Hypothetical N-terminal amidation of HEL(55-61) by remaining monomethyl succinate from the N^{α}-Boc-Asp(*t*Bu)-(*R,S*)Tyr(*t*Bu)-Ψ[$COCH_2$]Gly-OMe synthesis could be ruled out as ^{1}H NMR spectra of isolated compounds **1** and **2** did not show departure of integration in the methylenes area.

Ketomethylene peptides **4a** and **4b** were tested for their ability to stimulate 2A11 and 3A9 MHC-II I-A^k restricted T cell clones [4] and/or to compete for presentation of natural HEL(52-61) to these T cells .- Table 1.

Entry		Peptide sequence										2A11 Spc[a]	3A9 Spc	2A11 Cpc[b]	3A9 Cpc
		52	53	54	55	56	57	58	59	60	61				
3		Asp	Tyr	Gly	Ile	Leu	Gln	Ile	Asn	Ser	Arg	8 nM	4 μM	-	-
4a	-	-[$COCH_2$]-		-	-	-	-	-	-	-		n.s.[c]	n.s.	syn.[d]	syn.
4b	-	-[$COCH_2$]-		-	-	-	-	-	-	-		n.s.	n.s.	n.c.[e]	n.c.
6[f]		-	-[CH_2NH]-		-	-	-	-	-	-	-	n.s.	n.s.	n.c.	syn.
7[g]		-	-[E,CH=CH]-		-	-	-	-	-	-	-	25 μM	30 μM	-	-

[a] Spc: 50% Stimulating Peptide Concentration. [b] Cpc: 50% Competing Peptide Concentration. [c] n.s.: no stimulation at a concentration of at least 100 μM. [d] syn.: synergy with HEL(52-61) above 10 μM. [e] n.c.: no competition against HEL(52-61) at a concentration of at least 100 μM. [f] see [5] and [16]. [g] see [5]. Data represent the average of two independent experiments.

Table 1

The introduction of a ketomethylene moiety at the 53-54 junction showed contrary to the *trans* ethylenic counterpart [5] a deleterious effect on T cells stimulation. Even at concentration of 100 μM, the two ketomethylene peptides were unable to stimulate 3A9 and 2A11 T cells. This lack of stimulation activity was probably ascribed to the poor ability of the ketomethylene peptides **4** to bind to MHC-II I-A^k molecules since they could not compete with the presentation of the natural peptide. Interestingly, peptides **4a** and **5** showed a synergy behaviour on competition activity with HEL(52-61). These peptides, unable by their own to stimulate the T cell hybridoma, increased the stimulation level of these T cells in the presence of a suboptimal concentration of the cognate HEL[52-61] peptide. Such a synergy has been observed previously in other combination of cognate and non-cognate peptides including peptide derived from the 81-90 invariant chain segment adjacent to the 89-101 segment able to bind to an MHC-II binding cleft [13]. Indeed, beside their peptide binding groove, MHC-II molecules are likely to display an accessory peptide binding site as shown by the ability of invariant chain to bind to MHC-II molecules solely through the interaction of the 81-90 invariant chain segment with MHC-II molecules outside the peptide binding cleft [14][15]. It is hypothesised that peptide binding to this accessory peptide binding site has allosteric effect on the binding of antigen peptide to the cleft [13]. Accumulative evidence showed that this secondary peptide binding site is selective for a subset of peptides for which some key residues have been mapped (reviewed in [13]). From our data, this accessory binding site seems to accommodate HEL(52-61) analogues with a ketomethylene or reduced bond at the junction 53-54 but the configuration at the Tyr53 position must be set for the ketomethylene peptide.

We used molecular modelling techniques [16] to fit these pseudopeptides in the MHC-II I-A^k binding groove. Firstly, a bound conformation of the antigenic peptide HEL(52-61) associated to the MHC-II I-A^k was modelled by homology with the 3D structure of hemagglutinin HA(306-318)/HLA-DR1 complex [7]. Secondly, the 53-54 peptide bond was replaced manually by the corresponding moiety. Molecular dynamics experiments without any hydrogen bonding constraints over 100 ps showed that there was a marked folding of

the first three N-terminal residues for the reduced peptide and the ketomethylene peptide **4** ((*S*)Tyr configuration): Root Mean Square Distance of the whole alpha backbone between the starting and the ending structure of the molecular dynamics were respectively 1.572 Å and 0.851 Å. The most important difference between these pseudopeptides was the overall conservation of the polyproline II type conformation for the *trans* ethylenic peptide and the deformation of the peptide backbone from the starting structure for the reduced peptide and the ketomethylene peptide **4** - see figure 3.

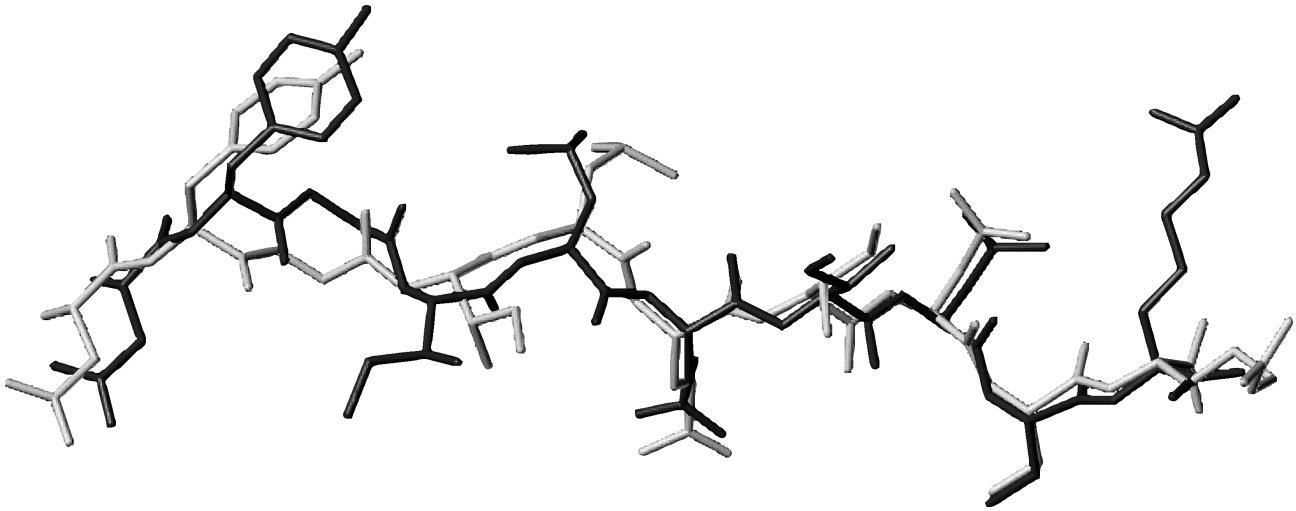


Figure 3. Superposition of HEL(52-61) (white) and the ketomethylene peptide **4** (black).

It is now established that Asp52 is a primary anchor residue for HEL(52-61) binding to MHC-II I-A^k molecule [17] and an adjacent conformational modification to this residue could preclude a tight binding to I-A^k at the P_1 pocket level. However, there is still an hydrogen bond occurring between the Asp residue and the Argα57 in the case of the ketomethylene peptide **4**. On the other hand, hydrogen bonds occurring between the Asp residue and the Argα56 side chain are observed in the cases of the reduced and *trans* ethylenic peptides. From comparison of the structural and electronic properties of the different analogues of HEL(52-61) and from these modelling results, it could be inferred that the ability of peptides with modified 53-54 amide bond to bind to MHC-II IA^k might not be related to the presence of hydrogen bonding between the peptide side chains or peptide backbone and MHC-II I-A^k residues but to the local conformational restriction brought about by the substitution mimicking the natural amide bond. Indeed, the hydrogen bond between the carbonyl of Tyr53 and Asnβ82 for HEL(52-61) is not longer present in the *trans* ethylenic analogue which however binds to MHC-II I-A^k. A greater local conformational mobility as in the reduced or the ketomethylene analogues could involve a global inability of the peptide to be shaped into the polyproline II type conformation during the dynamic process of peptide loading. Indeed, *in vitro*, long lived peptide-MHC-II I-A^k complexes occurred after a preliminary step of a rapidly formed, short lived complexes and these two complexes differ in their structure [18]. So, the hypothetical short lived complex of the ketomethylene or reduced peptides-MHC-II I-A^k dimers would not be allowed to evolve into the corresponding long lived peptides-MHC I-A^k complexes owing to the

conformational instability brought about by the ketomethylene or reduced bond.

In the context of MHC class II-TcR interactions, these biological and modelling results suggest that constrained pseudopeptides might be more efficient than analogues with an increased conformational mobility at the backbone level for blockade or activation of the immune recognition with the goal to obtain either agonist peptides for vaccination or antagonist scaffolds able to prevent auto-immune diseases.

Note added after submission of the manuscript. While our manuscript was under review, Fremont et coll. have published the first crystal structure of the HEL[50-62]-MHC class II I-A^k complex (Fremont D.H.; Monnaie D.; Nelson C.A.; Hendrickson W.A. and Unanue E.R. (1998) *Immunity*, *8*, 305-317). Our model of the HEL[52-61]- I-A^k complex is in good agreement with this crystallographic study predicting correctly the exposed and buried residues as well as six out of ten hydrogen bonds.

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