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[C0039]

Synthesis of New Antibody Hypervariable Loop Mimetics Using a D-Pro-L-Apro Template

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Abstract

The aim of this work is to develop new approaches for the synthesis of protein epitope mimetics. These should be conformationally well defined molecules, that accurately mimick the stuctures and properties of exposed surface regions of peptides and proteins. Here we describe the synthesis and application of a dipeptide template, comprising L-4-aminoproline (Apro) and D-proline (D-Pro), to induce a stable b-hairpin conformation in a loop mimetic based upon the L3 complementarity determining region (CDR) of the anti-haemagglutinin antibody HC19. The loop mimetic comprises the cyclic peptide cyclo-(Leu-Trp-Tyr-Ser-Asn-His-Trp-Val-D-Pro-Apro-), which by NMR is shown to adopt a stable and well defined ß-hairpin conformation in DMSO solution. The observed conformation is essentially identical to that found for the L3 CDR in the crystal structure of the antibody Fab fragment.

1. Introduction

Antibodies are well known for their ability to recognize and bind virtually any organic molecule. The immune system exploits the immunoglobulin fold for the generation of a large combinatorial library of proteins, each having a binding site composed of surface loops, whose chemical and physical properties together confer a unique ability to bind antigens. In attempting to develop synthetic molecules able to bind specifically to the surfaces of proteins, we are interested in the design of small molecule mimetics of antibody recognition loops, or so-called hypervariable or complementarity determining regions (CDRs). In particular, we have focused on mimetics of the second and third

CDRs of the heavy (H) and light (L) chains of IgG antibodies, which adopt well defined b-hairpin structures [1]. In previous work [2], we showed that an accurate mimetic (**1**) of the L3 ß-hairpin loop from the antibody HC19, specific for an influenza virus hemagglutinin, could be prepared by transplanting the loop from the immunoglobulin framework onto a D-Pro-L-Pro template (see **Figure-1**). The resulting molecule (**1**) was shown by NMR and MD to populate a stable ß-hairpin conformation in DMSO solution, which closely mimics that found in the high resolution crystal structure of the antibody Fab fragment.

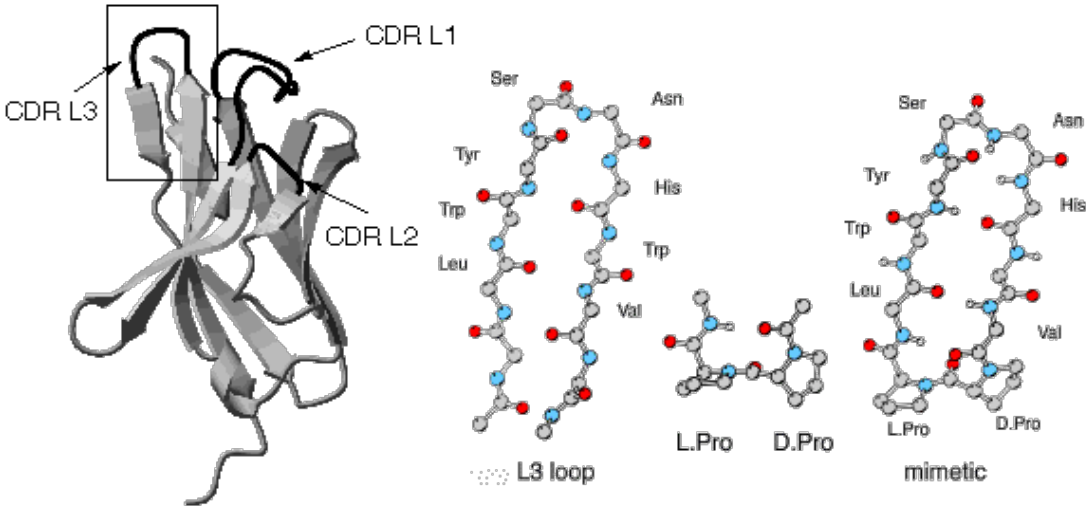
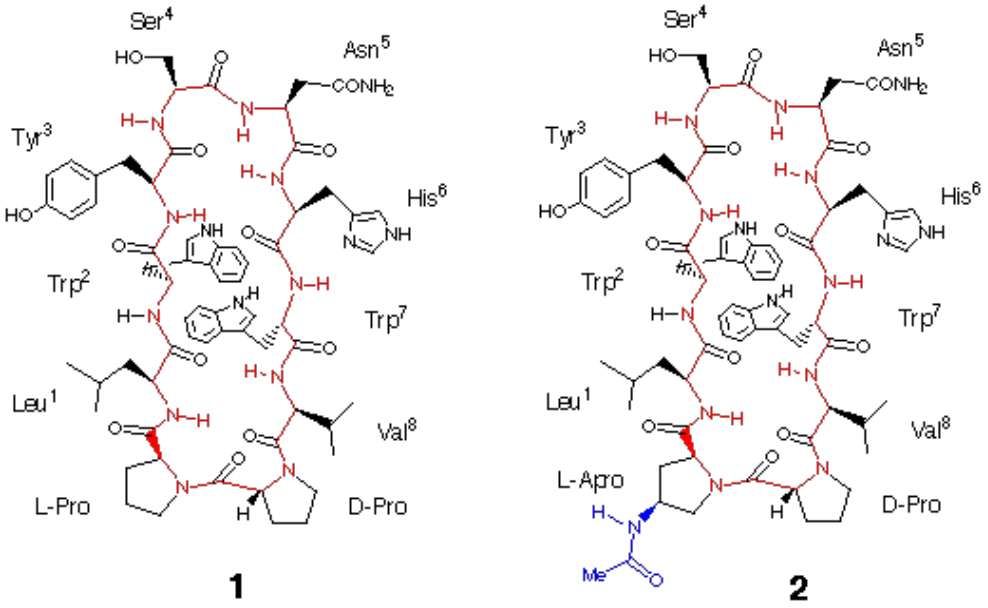


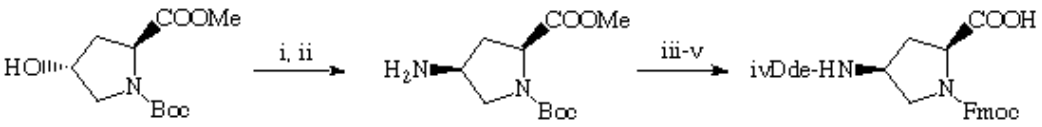
Figure-1. The variable region of the light chain of the antibody is shown as a ribbon diagram (PDB structure file 1GIG). The L3 loop to be mimicked is shown in the centre. The residues Leu-Trp-Tyr-Ser-Asn-His-Trp-Val are transplanted from the protein to the template to give the mimetic **1** [2].

In the present work, we sought to incorporate a new functional group into the D-Pro-L-Pro template, that would allow mimetics to be coupled together, or conjugated to other molecules. One convenient solution to this problem, is to replace the L-Pro residue in the template by (2*S*, 4*S*)-4-aminoproline (Apro), itself readily available from (4*S*)-4-hydroxyproline, and to use the new 4-amino group as the site for conjugation. We describe here the synthesis of the new mimetic **2**, and NMR studies of its solution conformation. These studies have shown that the modified template can be incorporated into a b-hairpin mimetic, which mimics accurately the conformation of the CDR loop in the intact HC19 antibody.



2. Results and Discussion

Synthesis. An orthogonally protected Apro ((2S, 4S)-ivDde-4-amino-1-Fmoc-pyrrolidine-2-carboxylic acid) was synthesized as shown in **Figure-2**. This building block is suitably protected for solid-phase peptide synthesis using Fmoc-chemistry [3], and the new amino function can be deprotected when required by treatment with hydrazine [4].



ivDde = 1-(4,4-dimethyl-2,6-dioxocyclo-hex-1-ylidene)-3-methylbutyl

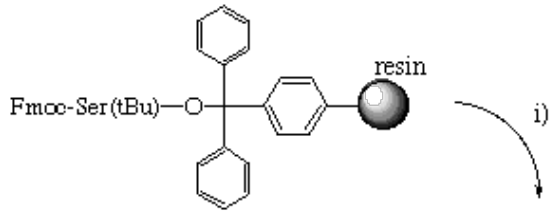
Reagents: i) PPh_3, EtOOC-N=N-COOEt, PhthNH, THF (88%); ii) NH_2NH_2, EtOH; iii) LiOH, H_2O/MeOH; iv) ivDde-OH (51% 3 steps); iv) TFA/CH_2Cl_2 2:1; v) Fmoc-Succ, iPr_2NEt, CH_2Cl_2 (93% 2 steps).

Figure-2

The linear precursor of the hairpin mimetic was assembled by solid-phase Fmoc-methodology, such that the hairpin-constraining template (the dipeptide unit, L-Apro-D-Pro) is introduced near the middle of the peptide chain. This is important to enforce a backbone conformation conducive to efficient macrocyclization, which is subsequently performed in solution [2,5,6]. Hence, in planning the synthesis of **2**, the cyclic mimetic was cleaved retrosynthetically between Ser^4 and Asn^5, and the solid-phase synthesis was initiated with Ser^4.

The synthesis of the linear precursor of **2**, outlined in **Figure-3**, was carried out on *Novasyn TGT* resin. All amino acids, including the orthogonally protected Apro were coupled sequentially following the standard Fmoc methodology. After cleavage from the resin, the fully side-chain protected peptide was subjected to cyclization in solution with HATU/HOAt for activation [7]. The cyclization proceeds in close to quantitative yield, as monitored by reverse-phase HPLC. Removal of the ivDde-protecting

group by hydrazine could also be followed by reverse phase HPLC and was complete in 1h at room temperature. The free amine was finally capped with acetic anhydride, although clearly a wide range of other acylating agents could also be used. Finally, all side chain protecting groups were removed under standard conditions with TFA.



H-Asn(Mtt)-His(Trt)-Trp(Boc)-Val-pro-Apro(ivDde)-Leu-Trp(Boc)-Tyr(tBu)-Ser(tBu)—O—RESIN

ii-vi)

2

Reagents: i) solid-phase peptide synthesis using amino acid and HBTU/HOBt (4 equiv.), iPr_2NEt, in DMF; and 20% piperidine in DMF for Fmoc removal; ii) $AcOH/CH_2Cl_2/MeOH$ 5:4:1; iii) HATU, HOAt, iPr_2NEt, DMF; iv) NH_2-NH_2 in DMF; v) Ac_2O, DMF; vi) TFA, iPr_3SiH, H_2O 95:2.5:2.5.

Figure-3

Conformational analysis. The solution conformation of the mimetic was studied by NMR spectroscopy in d_6-DMSO. The 1D ^1H NMR spectrum recorded at 600 MHz reveals a large spectral dispersion of the H-C(a) protons, and also of the NH resonances (**Figure-4**), indicative of a defined conformation. The ^3J (a,NH) coupling constants for most residues are > 8.5 Hz, consistent with the presence of b-sheet structure [8]. The three backbone peptide NH groups for Leu^1, Tyr^3 and Val^8, which according to structure calculations and MD simulations (see **Table**) are involved in H-bonding between peptide amide groups across a b-hairpin, are also those that have significantly slower H/D exchange rates.

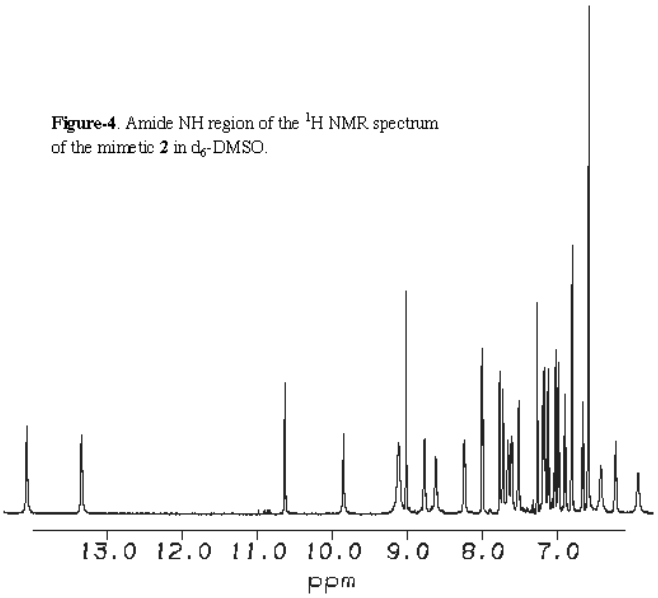


Figure-4. Amide NH region of the ^1H NMR spectrum of the mimetic **2** in d_6-DMSO.

The most striking evidence for a stable b-hairpin conformation, however, comes from NOESY spectra. These reveal an extensive network of long-range NOE connectivities involving residues far apart in the sequence, but spatially close together on opposite sides of the hairpin. This includes, for example, a strong NOE between the H-C(a) protons in Trp^2 and Trp^7 (distance restraint 2.4 Å).

Since the NMR analysis indicates that the mimetic adopts a well defined conformation, average solution structures were calculated by dynamic simulated annealing [9], with distance restraints derived from relative NOE build-up rates. A family of low energy structures were generated (**Figure-5**), that fullfil almost all of the distance restraints (see below), and possess a well defined b-hairpin

backbone geometry (**Figure-6**).

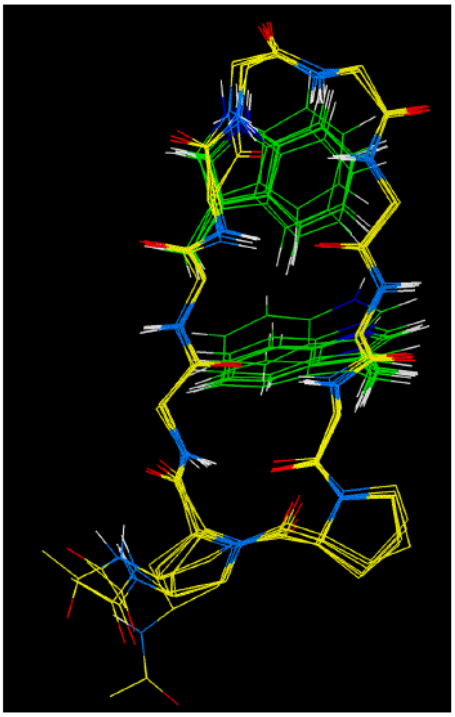


Figure-5. Superimposition of a group of low energy NMR structures of mimetic **2** determined by simulated annealing. N atoms in blue, O atoms in red. The D-Pro-Apro template is at the bottom.

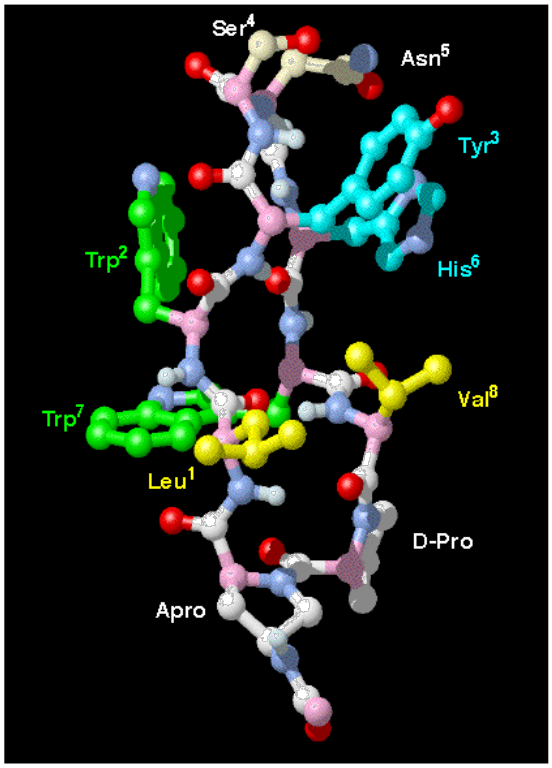


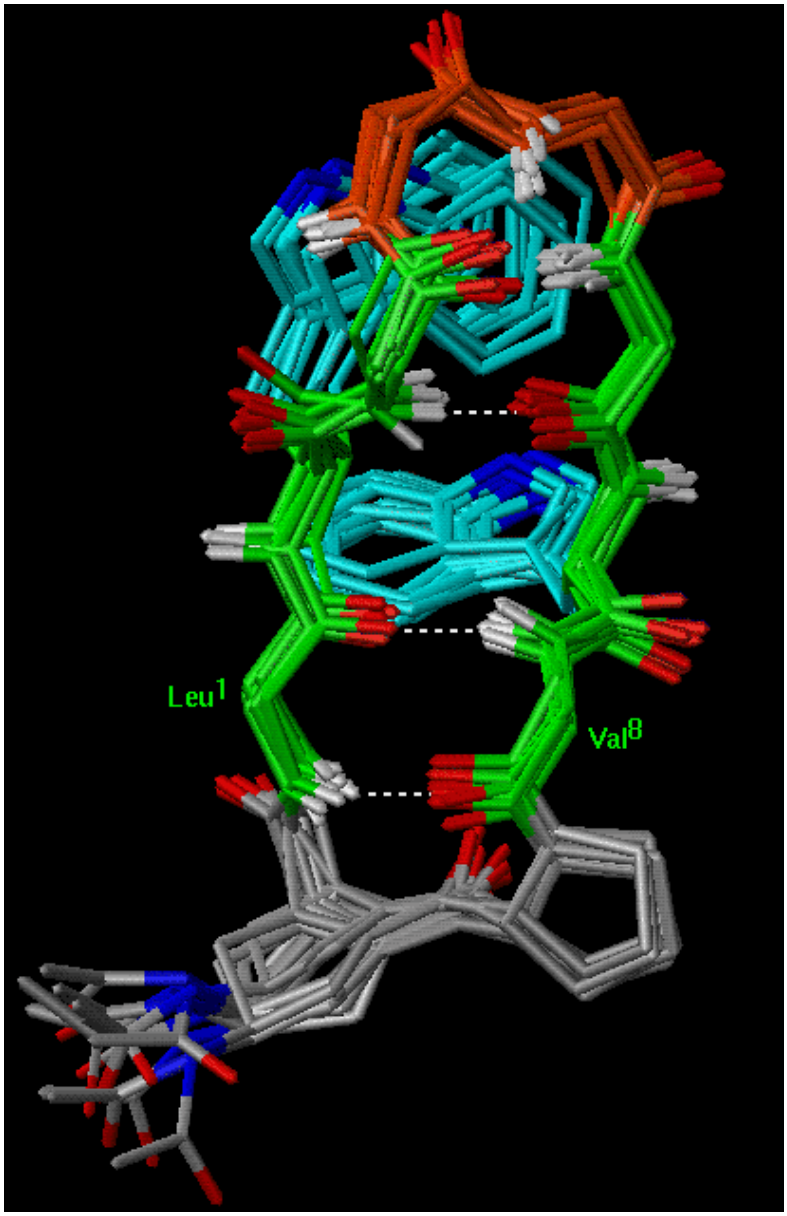
Figure-6. A ball-and-stick model of the solution structure of mimetic **2**. The D-Pro-Apro template is at the bottom.

An interesting feature of these structures is the close stacking of the indole groups of Trp^2 and Trp^7 on one side of the b-hairpin, as seen earlier for **1** [2]. The interaction of these indole rings is also supported by CD spectra recorded in MeOH and H_2O/MeOH (9:1), which include a characteristic strong exciton couplet at 225 nm.

The mimetic, however, is not rigid. Rather the NMR structures represent an average about which the molecule fluctuates. In particular, not all the NOEs involving the Trp^2 and Trp^7 side chains can be satisfied by one average NMR structure. For example, the Trp^7 HD1 proton shows NOEs to both Val^8 HN and to Trp^2 HA, which can best be explained if the Trp^7 indole is undergoing a 180^o flipping motion about the C(b)-C(g) bond, which is fast on the NMR time-scale.

In order to explore how the structural properties inferred from NOE data might be influenced by motional averaging, molecular dynamics calculations were performed, using the GROMOS96 programs [10], both with and without distance restraints. These simulations suggest, that at least over several nanoseconds, the backbone of the mimetic remains in a well defined b-hairpin conformation, with only small fluctuations of backbone f and y angles, as illustrated in **Figure-7** and in the **Table**. A 180^o flipping of the indole ring in Trp^7 (*vide supra*) was, however, observed in the restrained MD simulations.

Structural results from restrained MD (6 ns at 300 K)						
Figure-7. Superimposition of 10 frames taken from the trajectory every 600ps (prepared with the program MOLMOL[11])	**Average f and y angles**			**H-bonds occurences**		
	residue	f	y	Donor	Acceptor	%



Leu[1]	-114.3 ± 12.9	143.2 ± 11.2	Leu[1] HN	Val[8] O	80
Trp[2]	-79.7 ± 11.8	132.7 ± 12.2	Tyr[3] HN	His[6] O	98
Tyr[3]	-128.2 ± 13.8	90.0 ± 15.3	Asn[5] HN	Tyr[3] O	87
Ser[4]	61.3 ± 11.0	-50.5 ± 15.3	His[6] HN	Tyr[3] O	51
Asn[5]	-135.6 ± 14.8	-7.2 ± 19.6	Val[8] HN	Leu[1] O	94
His[6]	-154.1 ± 17.9	161.6 ± 13.8	Rotating molecule		
Trp[7]	-92.1 ± 14.7	131.4 ± 15.2			
Val[8]	-114.5 ± 13.7	94.1 ± 9.9			
D-Pro[9]	58.9 ± 8.6	-120.6 ± 11.8	Animation of MD frames		
Apro[10]	-74.8 ± 12.1	-19.8 ± 19.5			

The new amino function in the L-Apro residue of the template, therefore, does not interfer with hairpin formation, and appears ideally placed to conjugate the loop mimetic to another carrier molecule.

Structural comparisons. - The goal of this work was to synthesize an accurate conformational mimetic of the L3 loop in the anti-hemagglutinin antibody HC19. A superimposition of a typical NMR solution structure of the mimetic, and the loop taken from the crystal structure of the Fab fragment [12,13] is shown in **Figure-8**. This demonstrates that the mimetic adopts essentially the same

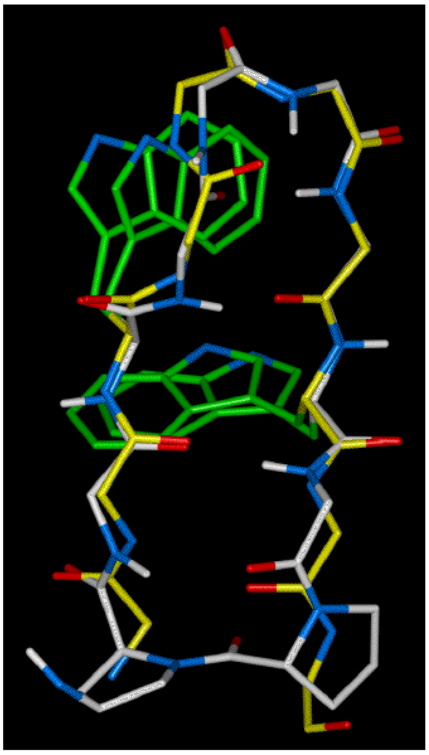


Figure-8: Superimposition of the solution structure of the mimetic (white) and the crystal structure of the antibody CDR L3 loop (yellow). N atoms are in blue, O atoms in red, the side chains of Trp^2 and Trp^7 are in green.

hairpin loop conformation as seen in the intact antibody. This excellent structural mimicry augers well for future applications of this technology, in the design of new biologically interesting protein epitope mimetics.

Acknowledgments

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