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Treatment of Experimental Allergic Encephalomyelitis (EAE) by a Rationally Designed Cyclic Analogue of Myelin Basic Protein (MBP) Epitope 72–85

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Abstract—In this report the rational design, synthesis and pharmacological properties of an amide-linked cyclic antagonist analogue of the guinea pig myelin basic protein epitope $MBP_{72–85}$ are described. Design of the potent cyclic analogue was based on 2D NOESY nuclear magnetic resonance and molecular dynamics studies carried out in the linear antagonist $Ala^{81}MBP_{72–85}$. The cyclic antagonist completely prevented the induction of experimental allergic/autoimmune encephalomyelitis when coinjected with linear and cyclic agonist analogues $MBP_{72–85}$ and cyclo(2–9)$MBP_{72–85}$.

Experimental autoimmune encephalomyelitis (EAE) is one of the best studied experimental animal models of multiple sclerosis (MS).[1–3] It is a useful in vivo system for the therapeutic management of disease characterized by tissue damage and mediated by autoimmune T-cells. In Lewis rats immunized with guinea pig MBP protein, encephalitogenic T cells recognize the $MBP_{72–85}$ epitope dominating the immune response.[4]

The linear analogue Gln^1-Lys^2-Ser^3-Gln^4-Arg^5-Ser^6-Gln^7-**Asp^8**-Glu^9-Asn^{10}-Pro^{11}-Val^{12} ($MBP_{72–85}$) has been found to induce EAE, while substitution of the Asp residue at position 8 with Ala resulted in an analogue ([Ala^{81}]$MBP_{72–85}$) that prevented the induction of EAE by its parent peptide.[5,6] Such peptides may interfere with the formation of the trimolecular complex MHC–peptide–TCR and therefore can actively inhibit disease.[7,8]

One approach in the development of more stable molecules is the synthesis of cyclic analogues.[9,10] Cyclization is known to restrict the number of possible conformations, allowing the possibility to diminish the unfavored conformations from approaching the receptor site. Furthermore, cyclization of peptide sequences results in increased metabolic stability, potency, receptor selectivity and bioavailability, all of them reflecting a better pharmacological profile.[11,12] In particular cyclic peptides have been used in several cases as synthetic immunogens,[13] potent vaccines for diabetes,[14] antigens for herpes simplex virus,[15] transmembrane ion channels,[16] inhibitors of HIV-1 Tat-TAR interactions in human cells,[17] of α-amylase,[18] pancreatic trypsin[19] and as protein stabilizers.[20] The appropriate design of cyclic analogue by connecting the two least important residues for activity without causing drastic changes in the conformation of active peptide results in a rigid geometry of the cyclic peptide enhancing the binding affinity compared to the linear counterpart. The engineering of stable peptides is of great technological and economic importance, since the limited stability of peptides often severely restricts their medical and industrial application.

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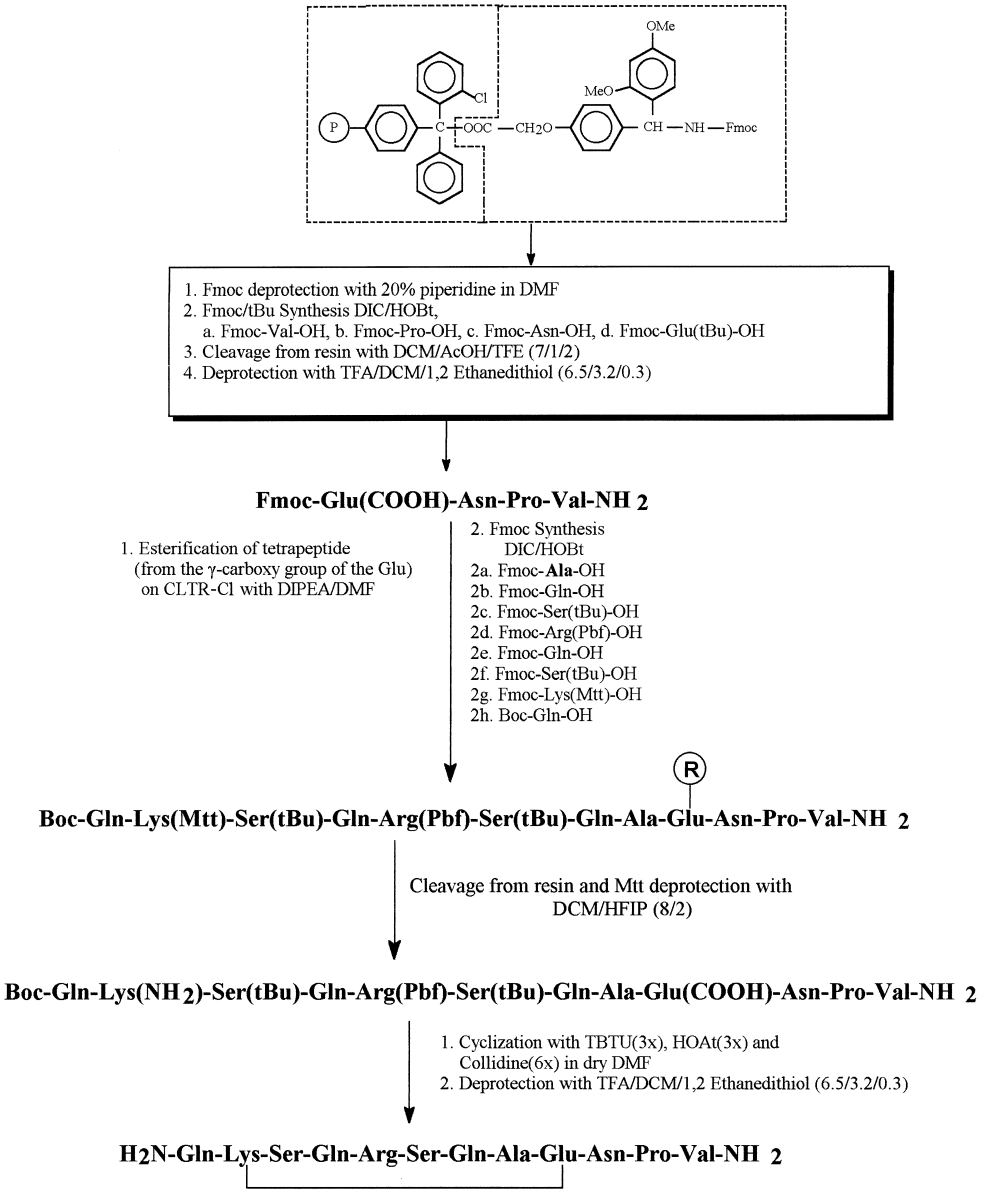
Our group has been involved in recent years in the design and synthesis of cyclic analogues for important peptides such of angiotensin II,[21] thrombin receptor peptides[22] and myelin basic protein[10] as important intermediate steps towards the design of nonpeptide mimetics for treating hypertension,[21,23] thrombosis and/or angiogenesis[22] and multiple sclerosis. In a recent work, the synthesis of a cyclic analogue of $MBP_{72–85}$ was reported[10] in an attempt to better understand the stereoelectronic requirements for agonist activity. This analogue was found to be almost equipotent with the linear one in inducing EAE. While the synthesis of agonists aids in the exploring of pharmacophores in the drug design, the synthesis of antagonists may help in the treatment of MS. For this reason, in this work we designed, synthesized, and evaluated for its activity in the EAE system, a cyclic analogue that could suppress the biological function of the original peptide, yet could also elicit a response in pharmacological quantities. Design of potent cyclic antagonist, cyclo(2–9)[Ala^{81}]$MBP_{72–85}$ was based on nuclear magnetic resonance and molecular dynamics studies carried out in the antagonist linear analogue [Ala^{81}]$MBP_{72–85}$ respectively.

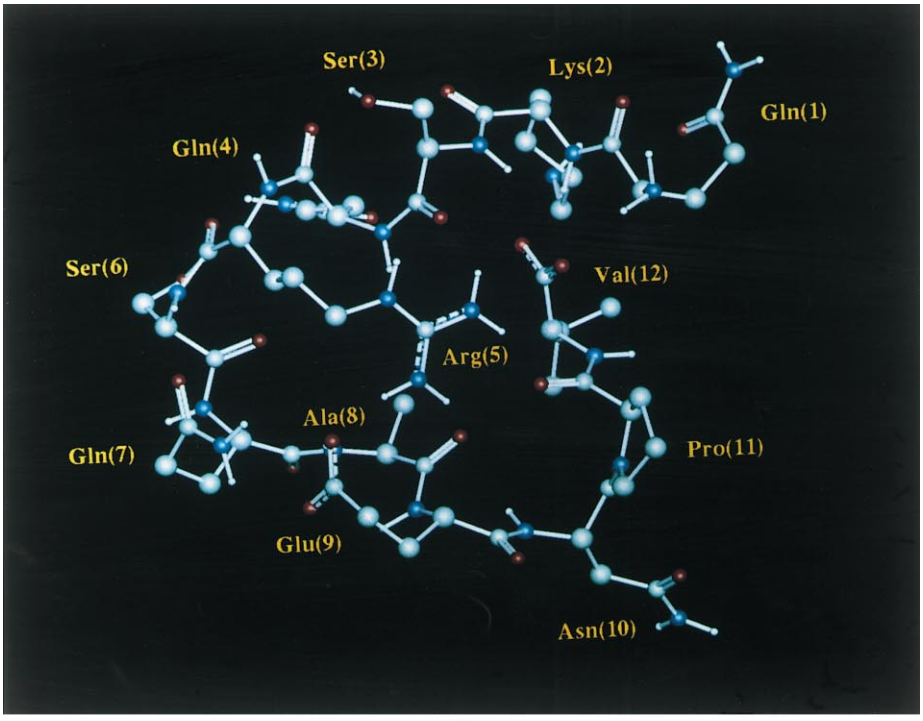
Table 1. Observed NOEs that impose cyclic conformation for the analogue [Ala^{81}]$MBP_{72–85}$. These NOEs were used as constraints for the building of a molecular model. Consequently, the molecular model was used to the design of cyclo(2–9)[Ala^{81}]$MBP_{72–85}$ (M: medium, S: strong)

NOE connectivities	Intensity of connectivity	Distance range used as a constraint (Å)
βAsn^{10}–βSer^{6}	M	3.0–3.5
βAsn^{10}–αSer^{6}	S	2.5–3.0
βGlu^{9}–γVal^{12}	M	3.0–3.5
γPro^{11}–γGln^{1}	M	3.0–3.5
δLys^{2}–*n*NHArg^{5}	S	2.5–3.0

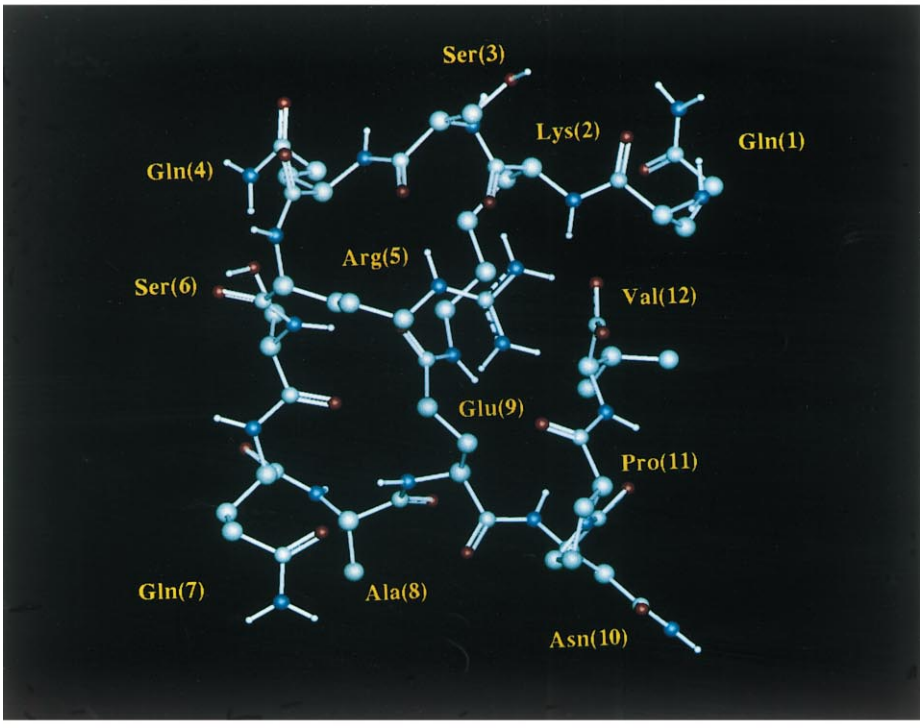


Scheme 1. Synthetic procedure for cyclic analogue cyclo(2–9)[Ala^{81}]$MBP_{72–85}$.

Scheme 1 shows the synthetic pathway of cyclo(2–9)[Ala^{81}]MBP$_{72–85}$. Thus, first Fmoc-Glu-Asn-Pro-Val-NH_2 was synthesized by Fmoc/*t*Bu synthesis using 'linker' (Rink Bernatowitz)-resin(2-chlorotrityl chloride/CLTR). The obtained peptide was then attached to chlorotrityl resin through the Glu γ carboxyl group. Subsequent step-wise synthesis led to the linear Fmoc-protected tetrapeptide attached to resin. Fmoc/*t*Bu methodology was used to synthesize the linear protected peptide on the chlorotrityl resin. Cleavage of protected peptide from resin and Mtt deprotection of Lys afforded a free Lys side chain amino group and a free Glu side



(A)



(B)

Figure 1. Low-energy conformers of antagonist analogues, Ala^{81}MBP$_{72–85}$ (A) and cyclo(2–9)[Ala^{81}]MBP$_{72–85}$ (B) derived by dynamics experiment.

chain carboxyl group available for the coupling–cyclization step. Successful cyclization of the linear peptide, was possible by the coupling of the Lys and Glu side chains of the linear peptide motif, at positions 2 and 9. The cyclization reagents used were *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU), 1-hydroxyl-7-azabenzotriazole and 2,4,6-collidine in dry dimethylformamide (DMF) as solvent.[10,22] These conditions were proved to be optimal for highest yield. The unprotected cyclic analogue was purified by high performance liquid chromatography (HPLC) and its structure was confirmed by fast atom bombardment mass spectroscopy.

Structure elucidation and the conformational properties of the Ala^{81}MBP$_{72–85}$ under study were obtained through ^{1}H–^{1}H NOESY experiment.[21,23] The critical NOE connectivities observed for analogue Ala^{81}MBP$_{72–85}$ define

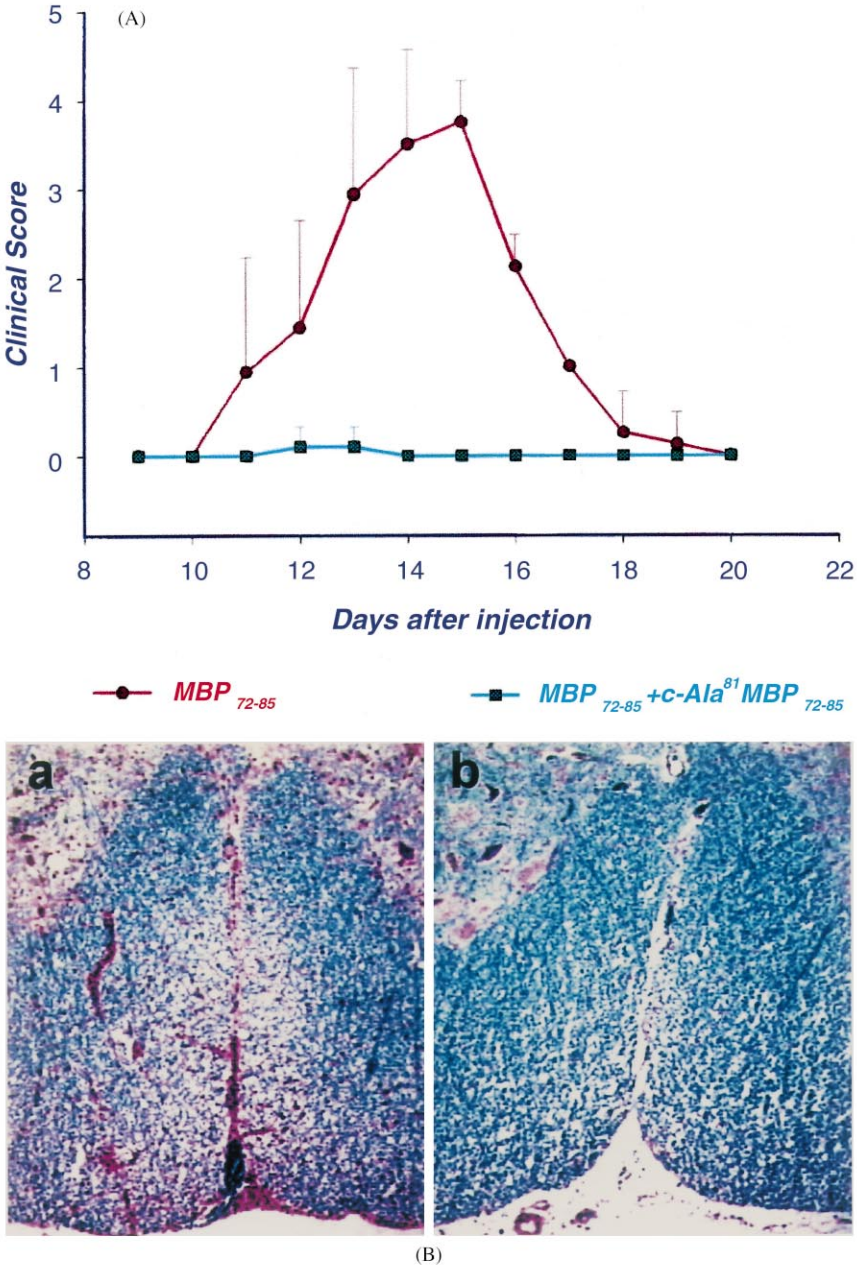


Figure 2. EAE can be suppressed by co-immunization with MBP$_{72–85}$ and cyclo(2–9)[Ala^{81}]MBP$_{72–85}$. (A) Animals were immunized with the linear encephalitogenic peptide MBP$_{72–85}$ alone (circles) or were co-immunized with MBP$_{72–85}$ and cyclo(2–9)[Ala^{81}]MBP$_{72–85}$ at a ratio of 1:17 (squares) and assessed daily for signs of clinical disease. (B) Demyelination and inflammation in the spinal cord at day 15 after immunization with MBP$_{72–85}$ (a), is completely prevented by the co-immunization of the cyclo(2–9)[Ala^{81}]MBP$_{72–85}$ (b). Spinal cord sections of an MBP$_{72–85}$-immunized rat showing multiple perivascular infiltrates (densely stained by Nuclear Fast Red), and patchy demyelination (loss of continuity of Luxol Fast Blue staining). In contrast, sections from a rat co-immunized with MBP$_{72–85}$ and cyclo(2–9)[Ala^{81}]MBP$_{72–85}$ shows a complete absence of inflammatory infiltrates and normal myelin structure (b).

a cyclic conformation for the molecule and are shown in Table 1. This conformation together with an Ala residue at position 8 are requirements for antagonist activity

The constructed model for compound [Ala^{81}]MBP$_{72–85}$ was based on the distance constraints revealed from NOE connectivities as well as on computational analysis and is shown in Figure 1A. Figure 1B shows a low-energy conformer of cyclic analogue cyclo(2–9)[Ala^{81}]MBP$_{72–85}$ derived from dynamics experiment.

The obtained model anticipates that the synthesis of a cyclic analogue through side chains connection of Lys at position 2 and Glu at position 9 would not greatly affect the activity of antagonist [Ala^{81}]MBP$_{72–85}$. Furthermore, such a synthesis would restrict the conformation and may ultimately lead to a peptide with better biological profile. The synthesis of cyclic analogue cyclo(2–9)[Ala^{81}]MBP$_{72–85}$ indeed gave active compound as [Ala^{81}]MBP$_{72–85}$. This is an example where molecular modelling leads to a rational design of a useful candidate.

In Figure 2 are presented the biological data of analogues in the EAE system. As it has been recently described,[10] analogues MBP$_{72–85}$ and cyclo(2–9)MBP$_{72–85}$ induce EAE when injected subcutaneously in Lewis rats. Both these analogues induce an acute monophasic disease with a peak clinical score at day 15 following the initial injection, and eventual complete recovery in all animals. In the present study, MBP$_{72–85}$ was used to induce EAE (Fig. 2A) and to evaluate the activity of the cyclic antagonist. When cyclo(2–9)[Ala^{81}]MBP$_{72–85}$ was co-injected with MBP$_{72–85}$ the clinical signs of EAE were completely prevented (Fig. 2A), demonstrating that cyclo(2–9)[Ala^{81}]MBP$_{72–85}$ is a powerful antagonist of MBP$_{72–85}$-induced EAE in Lewis rats. The result was confirmed at the histological level. Histopathological examination of spinal cord sections taken from MBP$_{72–85}$-injected animals, which were sacrificed at the peak of the disease, showed extensive perivascular and parenchymal inflammation throughout the length of the spinal cord as well as demyelination demonstrated by focal loss of luxol fast blue-stained myelin (Fig. 2B). In contrast, spinal cord section taken from rats immunized with MBP$_{72–85}$ and cyclo(2–9)[Ala^{81}]MBP$_{72–85}$ showed the complete absence of inflammation and demyelination.

The equal activity of these peptides with their linear congeners assigns the cyclic antagonist, cyclo(2–9)-[Ala^{81}]MBP$_{72–85}$ as putative drug lead for the development of potent molecules with improved pharmacological properties and increased degradation resistance in the research for the immunotherapy of multiple sclerosis (MS).

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