Design and Synthesis of a Potent Cyclic Analogue of the Myelin Basic Protein Epitope MBP_{72-85}: Importance of the Ala^{81} Carboxyl Group and of a Cyclic Conformation for Induction of Experimental Allergic Encephalomyelitis

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Experimental allergic encephalomyelitis (EAE) is induced in susceptible animals by immunodominant determinants of myelin basic protein (MBP), such as guinea pig sequence MBP_{72-85}. Two linear and one cyclic analogues based on MBP_{72-85} have been synthesized and evaluated for EAE induction in Lewis rats. The linear peptide Gln^1-Lys^2-Ser^3-Gln^4-Arg^5-Ser^6-Gln^7-Asp^8-Glu^9-Asn^{10}-Pro^{11}-Val^{12} (**1**) was found to induce EAE, while substitution of the Asp residue at position 8 with Ala resulted in an analogue (**2**) which suppressed the induction of EAE by its parent peptide. Nuclear magnetic resonance studies of analogue **1** in dimethyl sulfoxide (DMSO) using TOCSY/ROESY techniques revealed a head-to-tail intramolecular interaction (ROE connectivity between βVal^{12}-γGln^1), indicating a pseudocyclic conformation for the immunogenic peptide **1**. A conformational model was developed using NMR constraints and molecular dynamics. Based on this model, a novel amide-linked cyclic analogue has been designed and synthesized by connecting the side-chain amino and carboxyl groups of Lys and Glu at positions 2 and 9, respectively, of linear analogue **1**. The cyclic analogue Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Pro-Val-NH_2 (**3**) had similar activity to the linear peptide **1**, and the EAE effects induced by cyclic analogue **3** were completely suppressed by co-injection with the Ala^{81}-substituted analogue **2** in Lewis rats. The similar potencies of analogues **1** and **3** support the proposed cyclic comformation suggested for analogue **1** from NMR studies and computer modeling and provides the basis for designing more potent molecules with improved properties such as increased resistance to degradation.[15] The present findings suggest that a cyclic conformation for the MBP_{72-85} epitope positions the carboxyl group of Asp^{81} correctly and presumably other side groups of the peptide such as Arg^{78} in a manner which enables functional binding of the trimolecular complex MHC−peptide−T cell receptor resulting in EAE.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterized by local T cell and macrophage infiltrates, demyelination, and loss of neurologic function.[1,2] MS is generally considered to be an autoimmune disease caused by neuroantigen-specific CD4+ T cells.[1,2] Candidate autoantigens include constituents of the myelin sheath such as myelin basic protein (MBP) and proteolipid protein (PLP).[3−6] Modern approaches toward the therapeutical management of MS involve the design and use of peptide analogues of disease-associated myelin epitopes to induce peripheral T cell tolerance.[7−9] Experimental autoimmune encephalomyelitis (EAE), one of the best-studied experimental animal models of MS,[1] represents an invaluable in vivo system for the evaluation of such therapeutic approaches. EAE is a CD4+ T cell-mediated disease that can be induced by immunization with MBP or PLP proteins or peptide epitopes.[10] In Lewis rats immunized with guinea pig MBP protein, encephalitogenic T cells which recognize the 72−85 amino acid sequence MBP_{72-85} dominate the immune response.[10,11] The assumption has been that disease can be modulated with peptides that interfere with the formation of the trimolecular complex MHC−peptide−T cell receptor,[12] although there is gathering evidence that analogues of disease-associated epitopes can actively inhibit disease through the activation of antigen-specific regulatory T cells.[7,13,14] Peptide therapy, however, is hindered due to the sensitivity of peptides to proteolytic enzymes. Continuous infusions and therefore prohibitive amounts of peptides are necessary to elicit the necessary biological response.

To address the need for more stable molecules,[15,16] a cyclic analogue which could maintain the biological function of the original peptide, yet could also be able to elicit a response in pharmacological quantities, was designed, synthesized, and evaluated for activity in the EAE system. Design of this cyclic analogue was based

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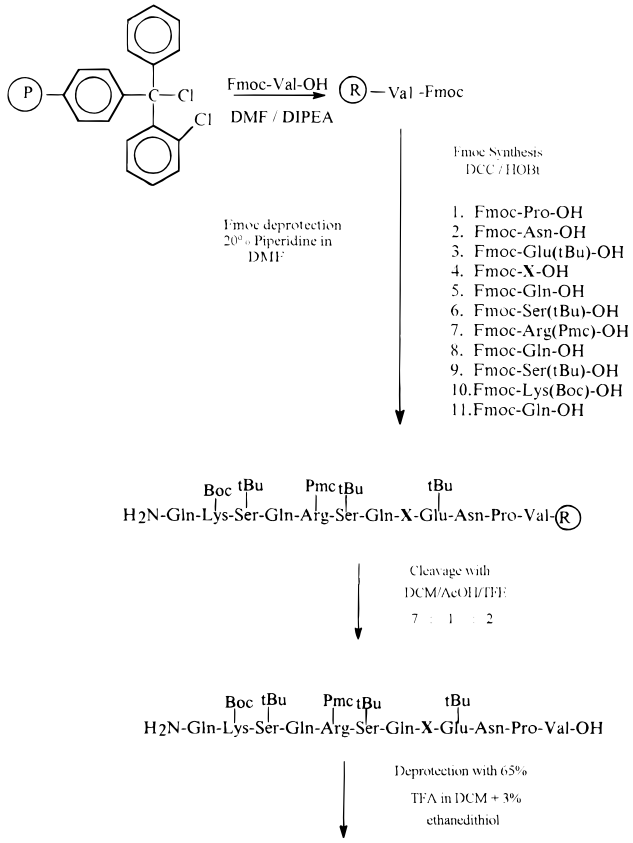
Scheme 1. Synthetic Procedure for Linear MBP Analogues **1** and **2**



H_2N-Gln-Lys-Ser-Gln-Arg-Ser-Gln-X-Glu-Asn-Pro-Val-OH

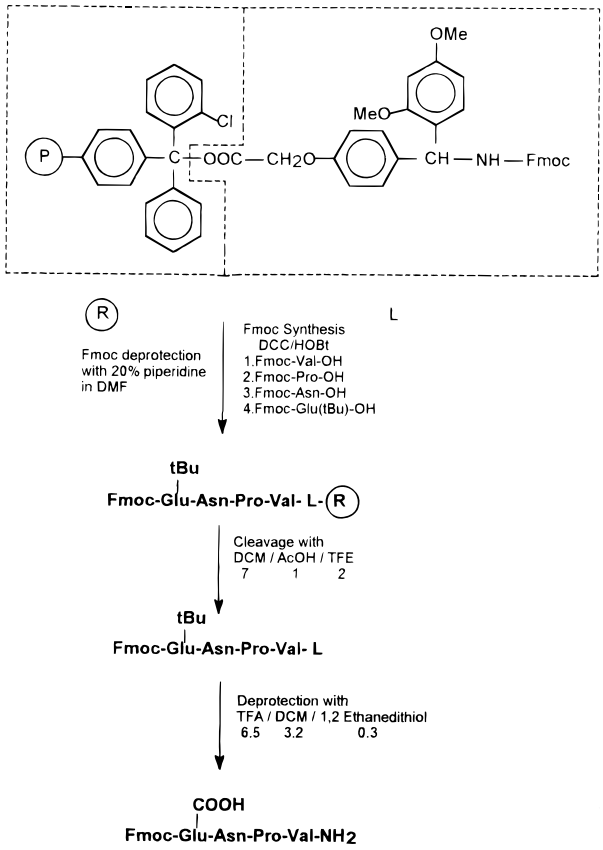
X: Asp (Analogue 1)
Ala (Analogue 2)

on nuclear magnetic resonance and molecular dynamics studies carried out in the agonist linear analogue **1**. These studies revealed a head-to-tail intramolecular proximity (ROE connectivity between βVal^{12}-γGln^{1}) suggesting a cyclic conformation for analogue **1**. We, therefore, synthesized the cyclic 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}-NH_2 by connecting the ϵ amino group of Lys and the γ carboxyl group of Glu at positions 2 and 9. Cyclization of a linear molecule is known to restrict its number of possible conformations making it possible to determine which of the conformations observed in solution best approximates the receptor-bound conformation. The cyclic analogue was assessed for its biological activity in the EAE system, and its activity was comparable with that of linear agonist analogue **1**. EAE induced by cyclic analogue **3** was completely suppressed by the co-injection of the Ala^{81}MBP antagonist analogue **2**. The comparable potencies of linear and cyclic analogues **1** and **3** indicate that the encephalitogenic linear peptide **1** participates in the trimolecular complex with a cyclic conformation in which the carboxyl group of Asp at position 81 plays an important role for activation of this complex.

Results

Scheme 1 shows the synthesis of linear peptide analogues **1** and **2** by Fmoc methodology using the trityl

Scheme 2. Synthetic Procedure for Fmoc-Glu(COOH)-Asn-Pro-Val-NH_2



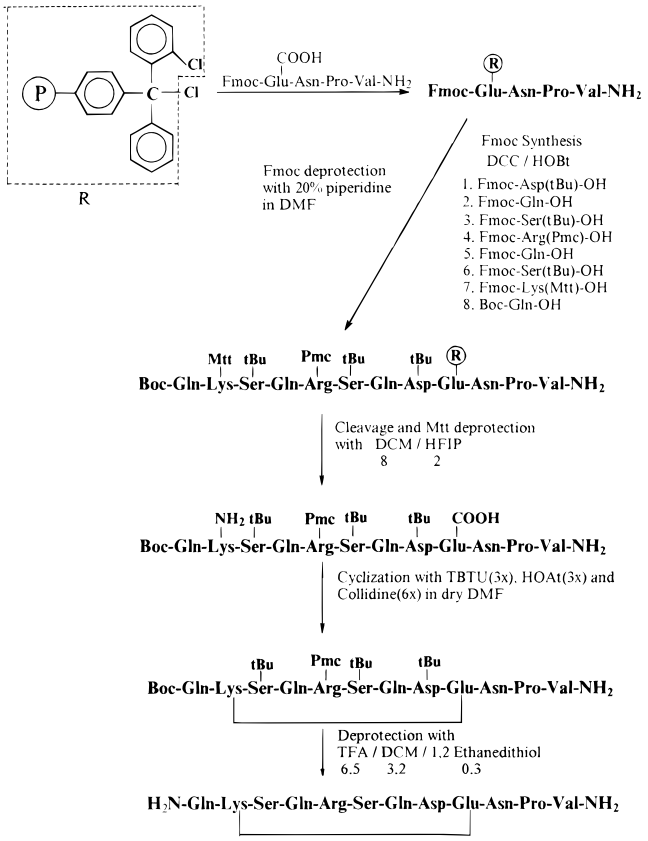
chloride resin. Schemes 2 and 3 show the synthesis of cyclic analogue **3** in which residues at positions 2 (Lys) and 9 (Glu) are involved in cyclization. Figures 1 and 2 show clinical scores for peptide analogues **1**−**3** in the EAE assay. Figures 3 and 4 show low-energy conformers of linear and cyclic peptides **1** and **3** depicted from dynamics experiments. Table 1 shows chemical data of linear and cyclic MBPs.

Chemistry. The linear receptor-activating MBP peptides (Glu-Lys-Ser-Gln-Arg-Ser-Glu-X-Glu-Asn-Pro-Val, X = Asp, Ala) (analogues **1** and **2**) were synthesized using solid-phase methods and conventional 9-fluorenylmethyloxycarboxyl (Fmoc) procedures followed by purification to homogeneity using HPLC chromatography (Scheme 1). The cyclic analogue **3**, based on the guinea pig MBP$_{72-85}$ motif, was synthesized using the 2-chlorotrityl chloride resin (CLTR)[11] that was used previously for the synthesis of novel cyclic amide-linked analogues of angiotensins II and III[17,18] as well as for the synthesis of the thrombin receptor SFLLR motif.[19−21] A novel strategy for synthesis and cyclization was followed. Thus, first Fmoc-Glu(COOH)-Asn-Pro-Val-NH_2 was synthesized by Fmoc synthesis using a linker-resin shown in Scheme 1. This was possible through cleavage from resin with the splitting reagent dichloromethane/acetic acid/trifluoroethanol (DCM/AcOH/TFE) (7/1/2) and deprotection with trifluoroacetic acid/dichloromethane/ethanedithiol (TFA/DCM/EDT) (6.5/3.2/0.3). The peptide obtained was then attached to chlorotrityl resin through the Glu γ carboxyl group. Subsequent stepwise synthesis led to the linear protected tetrapeptide attached to resin. Cleavage of protected peptide from the resin and Mtt deprotection of

Table 1. Chemical Data on Linear and Cyclic MBP_{72-85} Analogues

compd structure	MW (FABMS)	TLC R_f BPAW	TLC R_f CMA	amino acid analysis Gln	Lys	Ser	Glu	Arg	Asp	Asn	Pro	Val	Ala
1, Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Pro-Val	1415	0.08	0.29	2.90	0.92	1.96	0.97	1.00	0.98	0.96	0.98	0.95	
2, Gln-Lys-Ser-Gln-Arg-Ser-Gln-Ala-Glu-Asn-Pro-Val	1371	0.09	0.34	2.94	0.97	1.97	0.98	1.00		0.92	0.97	0.96	1.02
3, Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Pro-Val-NH_2	1395	0.15	0.58	2.93	0.94	1.96	0.94	1.00	0.97	0.92	0.93	1.04	

Scheme 3. Synthetic Procedure for Cyclic MBP_{72-85} (Analogue **3**)



Lys as well as tBu deprotection of Glu afforded a free Lys side-chain amino group and a free Glu side-chain carboxyl group available for the coupling−cyclization step. Successful cyclization of the precyclic linear peptide was possible by the coupling of the Lys and Glu side chains of the linear peptide motif, at positions 2 and 9 as outlined in Schemes 2 and 3.

The use of the 2-chlorotrityl resin, as well as of mild conditions (DCM/AcOH/TFE, 7/1/2) for cleaving the peptide−resin bond, allowed peptide release from the resin and the subsequent cyclization of the desired protected peptide. Cyclization of the protected peptide was achieved using *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU), 1-hydroxyl-7-azabenzotriazole, and 2,4,6-collidine in dimethylformamide (DMF)[19,20] (Scheme 3). The cyclization reaction was monitored using the ninhydrin test, and the reaction mixture was resolved by thin-layer chromatography with a *n*-butanol/acetic acid/water (4/1/1) solvent system. The protected cyclic analogue was then deprotected with 65% trifluoroacetic acid (TFA) + 3% EDT in DCM (Schemes 2 and 3). The free cyclic analogue was purified by high-performance liquid chromatography (HPLC), and its structure was confirmed by amino acid analysis and fast atom bombardment mass spectroscopy (M^+ =

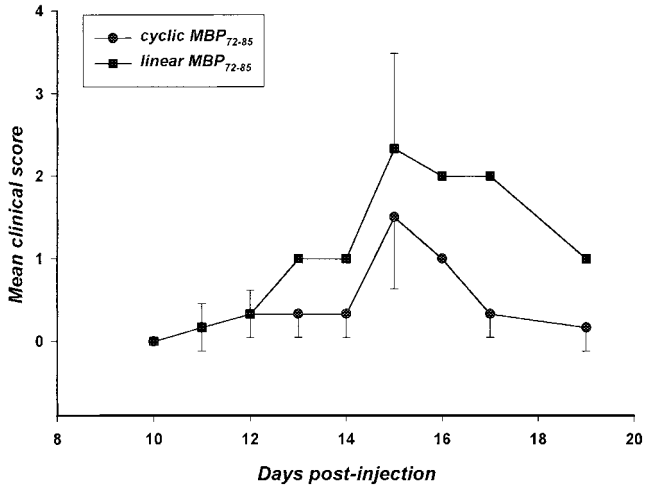


Figure 1. Induction of EAE by linear and cyclic MBP_{72-85} analogues. EAE was induced by footpad injection of 30 μg of linear MBP_{72-85} (analogue **1**) ($n = 6$) as positive control or 30 μg of cyclic MBP_{72-85} (analogue **3**) ($n = 7$). Results are expressed as mean clinical score values ± SEM. Statistical analysis of EAE induced by each analogue showed there was no statistical difference by Student's *t*-test ($p > 0.07$ at day 15).

1395). The biological profile of the cyclic peptide was then evaluated by its ability to induce EAE in Lewis rats and by co-injection with antagonist $Ala^{81}MBP_{72-85}$ (Figures 1 and 2).

Activity of the Cyclic MBP_{72-85} Peptide Analogue 3 in the EAE Model and Coinjection with Antagonist $Ala^{81}MBP_{72-85}$ Linear Analogue 2. Cyclic MBP_{72-85} was found to induce EAE in Lewis rats with a similar time course and potency ($p > 0.07$ at peak of EAE) as linear MBP_{72-85} when immunized at the same concentration (Figure 1). Both the linear and cyclic peptides induced an acute monophasic disease with a peak clinical score at day 15 following the initial injection and eventual complete recovery in all animals. The ability of the linear analogue **2** ($Ala^{81}MBP_{72-85}$) to inhibit the disease induced by cyclic peptide was also studied. It has been previously shown that this analogue completely prevents disease induction by linear MBP_{72-85}.[1] Here it is shown that the co-injection of $Ala^{81}MBP_{72-85}$ analogue with the cyclic analogue **3** completely prevented the development of EAE (Figure 2) ($p < 0.01$ at peak of disease) demonstrating that this linear antagonist **2** is a potent inhibitor of disease induced by linear analogue **1** or cyclic analogue **3** in Lewis rats.

Conformational Studies of Linear MBP_{72-85} Analogue 1. Amino acid spin system was identified by locating networks of characteristic connectivities in the 2D COSY and 2D TOCSY spectra.[22−24] Long-range assignments were made through ROESY experiment using a 350-ms mixing time.[23,24] The NOE connectivities observed for analogue **1** define a cyclic conformation for

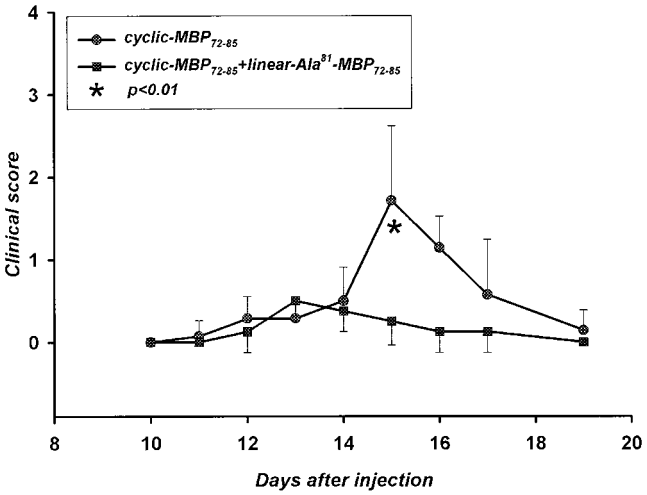


Figure 2. Prevention of EAE induced by cyclic MBP$_{72-85}$ with analogue Ala^{81}MBP$_{72-85}$. EAE was induced by footpad injection of 30 μg of cyclic MBP$_{72-85}$ (analogue **3**) ($n = 7$) and was prevented when the same quantity of analogue **3** was co-injected with 500 μg of the linear antagonist peptide Ala^{81}MBP$_{72-85}$ (analogue **2**) ($n = 4$). Results are expressed as mean clinical score values $\pm$ SD, and statistical comparison between the two groups of mice using student's *t*-test showed a significant inhibition of disease induction by analogue **2** ($p < 0.039$ at day following disease induction). Statistical analysis by Student's *t*-test revealed that disease inhibition by Ala^{81}MBP$_{72-85}$ was statistically significant ($p < 0.01$ at day 15).

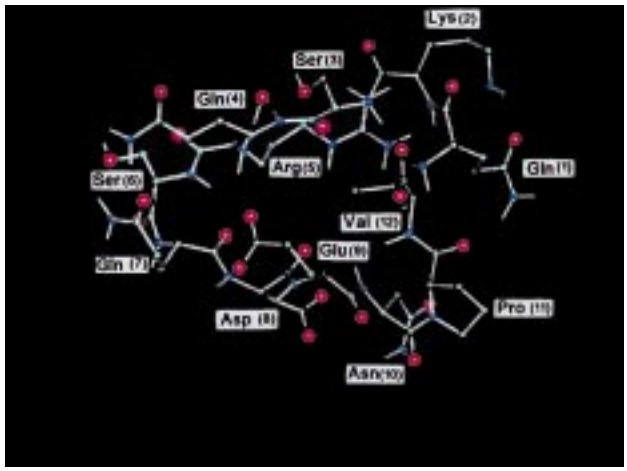


Figure 3. Low-energy conformer of compound **1** derived from dynamics experiment. The most important features of this model are its cyclic form and the interaction between Arg^5-Lys^2 amino acids.

this molecule. More specifically, the NOE connectivities which impose such a cyclic conformation are ωNHArg^5-δLys^2, NHGln^1-αPro^{11}, and βVal^{12}-γGln^1. A constructed model based on the distance constraints due to NOE connectivities for compound **1** is shown in Figure 3. The proposed molecular modeling (Figure 3) anticipates that the synthesis of a cyclic analogue by connecting side chains of Lys at position 2 and Glu at position 9 would not greatly affect its activity. Furthermore, such a synthesis would restrict the conformation of these two amino acids and may ultimately lead to a peptide with better biological profile. The synthesis of **3** indeed gave an active compound almost equipotent as **1**. This is a good example of where molecular modeling leads to the rational design of a cyclic molecule (Figure 4) which supports the proposed model.

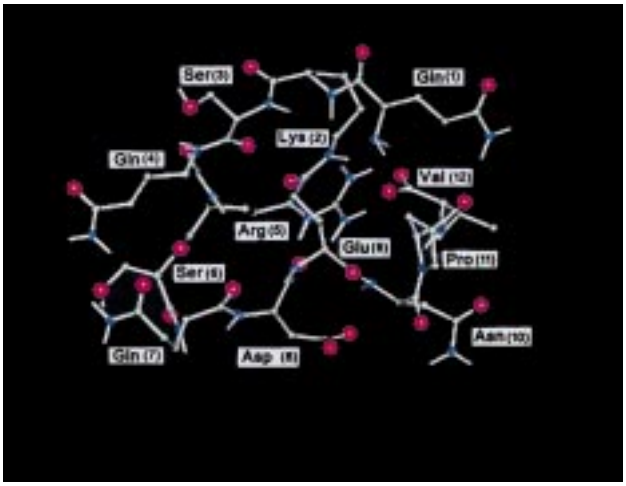


Figure 4. Low-energy conformer of cyclic compound **3** derived from dynamics experiment. An important electrostatic interaction between the guanidino group of Arg^5 and the carboxylate group of Asp^8 is observed.

Discussion

Development of alternative molecules that mimic the immunomodulatory activity of MBP epitope peptides and maintain an advantage over regular peptides in terms of stability is a necessary step before these molecules can be used for therapeutic purposes. There are two approaches in the development of such molecules. One is the design and synthesis of nonpeptides or small semimimetic peptides with immunomodulatory activity.[15,16] This design is based on the knowledge of the immunodominant amino acid residues of the MBP$_{72-85}$ epitope and involves the construction of a chemical moiety in which the important pharmacophoric groups are incorporated.[25-27] This approach has been pursued and may lead to important pharmaceuticals for treating encephalomyelitis and possibly MS.[15,16] The other approach is the design and synthesis of potent cyclic MBP$_{72-85}$ analogues which offer several advantages such as increased resistance to metabolic degradation and restriction of conformational flexibility. Constrained peptide analogues of high potency offer important information regarding the bioactive conformation assumed by the peptide and could be very useful in drug design for the development of nonpeptide mimetics. In this report, the Fmoc synthesis and biological activity of two linear and a cyclic peptide analogue derived from the guinea pig MBP$_{72-85}$ peptide that induces transient EAE in Lewis rats are described. Linear compound **1** induces EAE, while replacement of Asp at position 81 with Ala results in a peptide analogue which suppresses EAE induced by analogue **1** when co-injected, indicating the importance of a carboxyl group at position 81 for activity.[7] Cyclic analogue **3** has been demonstrated to induce EAE with potency comparable to that exerted by linear analogue **1**. The cyclic analogue **3** was designed based on the conformational properties of the agonist linear analogue **1**, which assumes a cyclic conformation as revealed from NMR studies. Indeed, one of the major findings of this study was that the ROESY data in compound **1** indicated a proximity of the N- and C-terminal domains. The molecular dynamics approach, in which the structure was munipulated to fulfill the observed ROE between βVal^{12} and γGln^1,

yielded a cyclic conformational model (Figure 3). In this model the major observed ROEs have been taken into account.

Another important ROE is the one that brings into spatial proximity the amino acids Arg^5 and Lys^2 (Figure 3). In fact, dynamics experiments show that Arg^5 is a very flexible amino acid which can adopt a spatial proximity with Lys^2, Gln^1, Asp^8, and Glu^9 amino acids. These results are congruent with the observed NOEs. The importance of the Arg residue for immunogenic activity has been previously demonstrated for small semimimetic molecules,[15] wherein the Arg guanidino group forms a salt bridge with a carboxylate group.[16] Accordingly, in these potential interactions of the Arg side chain in MBP_{72-85}, the most interesting is the one with Asp^8. In other low-energy conformers derived from dynamics experiments (not shown) Arg^5 clusters with Asp^8 amino acid. This interaction is also observed in the cyclic compound **3** (Figure 4). These observations may in part give a molecular explanation for the antagonist properties of compound **2** which possesses an Ala^8 instead of an Asp^8 amino acid. Such substitution interrupts the significant electrostatic interaction between the guanidino group of Arg^5 and the carboxylate group of Asp^8 amino acids. The cyclic model of linear analogue **1** allowed us to design and synthesize the cyclic analogue **3** which was found to induce EAE activity comparable to that exerted by linear counterpart **1**. EAE induced by the cyclic analogue was completely suppressed when it was co-injected with the $Ala^{81}MBP_{72-85}$ linear analogue **2** which is known to suppress EAE induced by linear MBP_{72-85} in Lewis rats.[7]

Cyclization of peptides has proved to be a very valuable tool in the design of analogues with resistance to metabolic degradation in other systems. However, this approach requires at least two structural changes to be made within a single analogue, and it is important that the two residues to be connected should be the least important for biological activity. Structure−activity studies have shown that the MBP_{72-84} peptide analogue induces EAE in Lewis rats and that single alanine-substituted peptide analogues at positions K^{74}, S^{75}, R^{78}, Q^{80}, D^{81}, E^{82}, and P^{84} resulted in significant reduction of the proliferative responses of a T cell line that is specific for the MBP_{72-85} peptide.[7] Since the peptides that bind to MHC class II molecules have been determined to involve a minimum of nine amino acid residues which satisfy a particular motif, the design of a cyclic mimetic that would maintain their functional role in vivo is quite challenging. Cyclization at positions 2 and 9 results in an analogue (**3**) which is highly potent (estimated to possess 80% encephalitogenic potency when it is compared to parent linear analogue **1**) indicating that the Lys and Glu residues at positions 2 and 9 are not so important to elicit the onset of EAE in Lewis rats. We have chosen Lys and Glu at positions 2 and 9 for cyclization since in the linear agonist peptide,[7] single alanine substitution abolishes agonist activity. The potency of the cyclic analogue confirms the validity of our choise. NMR studies on linear and cyclic peptides generally demonstrate that cyclization of peptide results in restriction of backbone conformation but usually has a minimal effect in the freedom of motion of the side chains (other than those included in the cyclic bridge formation). Accordingly, the availability of amino acid side chains for interaction with another molecule (such as MHC) should not be impeded although the relative positioning of the side chains in space would be fixed to some degree in the cyclic peptide. Apparently our cyclic analogue of MBP_{72-85} is recognized and is bound by MHC molecules and also by T cell receptors mediating the EAE response, perhaps implying that a near-cyclic conformation for MBP_{72-85}, rather than a linear one, is responsible for the pathogenic effects of the epitope peptide. The delayed response and slightly weaker clinical score of **3** compared to analogue **1** may be due to conformational deviations. This is in line with the cyclic conformation for the potent linear analogue **1** found by TOCSY and ROESY NMR techniques, in which an interresidue ROE interaction between βVal^{12}-γGln^1 was observed. To test the validity of this ROE interaction, NMR experiments were carried out at lower concentration (1 mg/0.33 mL of solvent). The low-dilution experiment proved that intermolecular head-to-tail association is primarily the cause of this ROE phenomenon.

Conclusion

In conclusion this research is aimed at the elucidation of the conformational characteristics of the guinea pig encephalitogenic MBP_{72-85} sequence, which would allow the design of analogues with improved properties. Structure−activity studies have shown the importance of the Asp COOH group at position 81 for potency,[7] while NMR and theoretical calculations indicate a head-to-tail spatial proximity suggesting a cyclic conformation for the sequence. To confirm this conformation, a cyclic analogue has been designed and synthesized. Cyclization of linear MBP analogue **1** between residues 2 (Lys) and 9 (Glu) has resulted in a potent cyclic analogue revealing structural requirements necessary for activity. Apparently, the side chains of residues 2 and 9 are not critical for activity. The comparable potencies of linear and cyclic analogues **1** and **3** support a cyclic conformation assumed by the MBP_{72-85} sequence while approaching the T cell receptor. Thus, a cyclic conformation of MBP_{72-85} together with a carboxyl group at position 81 appears to be important for the function of the trimolecular complex MHC−peptide−T cell.

Experimental Section

HPLC/TLC/FABMS/ESIMS/Amino Acid Analysis. Preparative HPLC for linear and cyclic MBP analogues was performed with a Waters system equipped with a 600E system controller using a Lichrosorb RP-18 reversed-phase preparative column (250 × 10 mm) with 7-μm packing material.[19,20] Separations were achieved with a stepped linear gradient of acetonitrile (AcN) (0.08% TFA) in water (0.08% TFA) over 60 min at a flow rate of 3 mL/min. The crude peptide material (20 mg) was dissolved in methanol/water (450 μL), and this solution was injected using a Waters U6K injector with a 2.0-mL sample loop. Fractions were manually collected at 0.5-min intervals; the elution time of the major product was 25−30 min. Elution of the peptide was determined simultaneously from the absorbances at 230 and 214 nm (Waters 996 photodiode array detector). Fractions containing the major peptide peak were pooled, and acetonitrile was removed using a rotary evaporator. After lyophilization, the product was stored at −20 °C. Peptide purity was assessed by analytical HPLC reruns (Nucleosil-120 C18, 250 × 4.0 mm), thin-layer chromatography (TLC), and mass spectrometry (FABMS, ESIMS). Appropriate

molar ratios of amino acids were observed for compounds **1**−**3** (Table 1). Compositional analysis data were collected from 6 M HCl hydrosylates (150 °C, 1 h) using a Beckmann 6300 high-performance analyzer. The TLC solvent systems used were as follows: *n*-butanol/acetic acid/water (4/1/1) (BAW), toluene/acetic acid/methanol (7/1.5/1.5) (TAM), *n*-butanol/pyridine/acetic acid/water (15/10/3/6) (BPAW), and chloroform/methanol/ammonia (CMA). FABMS spectra were run on an AEI M29 mass spectrometer. The FAB gun was run at 1-mA discharge current at 8 kV. The FAB matrix used was a mixture of dithiothreitol−dithioerythritol (6:1) (Cleland Matrix).[28] The ESIMS spectra were run on a TSQ 7000 spectrometer (electrospray mode) by direct infusion. A solution of the sample (1 μg/1 μL) in 50:50 methanol:water containing 0.5% CH_3COOH was introduced in the ESI probe at a flow rate of 3 μL/min with a Harvard syringe. The capillary temperature was 200 °C and the sheath gas 45 units, while the spray needle voltage was +4.5 kV.

Synthesis of Peptide Analogues of MBP$_{72-85}$: Gln-Lys-Ser-Gln-Arg-Ser-Gln-X-Glu-Asn-Pro-Val, X = Asp (analogue 1), Ala (analogue 2). The linear peptides were prepared on 2-chlorotrityl chloride resin (0.7 mmol Cl^-/g) using novel solid-phase peptide synthetic method.[17,19,29,30] The first N^{α}-Fmoc (9-fluorenylmethyloxycarboxyl)-protected amino acid [Fmoc-Val-OH (1 equiv)] was coupled to the resin in 1 h in the presence of diisopropylethylamine (DIPEA) (3.2 equiv) in *N*,*N*-dimethylformamide (DMF). The remaining peptide chain was assembled by sequential couplings of the following Fmoc-protected amino acids (2.5 equiv): Fmoc-Pro-OH, Fmoc-Asn-OH, Fmoc-Glu(tBu)-OH, Fmoc-Ala-OH, Fmoc-Asp(tBu)-OH, Fmoc-Gln-OH, Fmoc-Ser(tBu)-OH, Fmoc-Arg(Pmc)-OH, and Fmoc-Lys(Boc)-OH in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) (3.75 equiv) and 1-hydroxybenzotriazol(HOBt) (3.5 equiv) in DMF for 4 h. The completeness of each coupling was verified by the Kaiser test and TLC in system (BAW and TAM). The Fmoc protecting groups were removed by treatment with piperidine (20% in DMF, 2 × 10 min). The protected peptide resin was then cleaved with the splitting solution dichloromethane/acetic acid/2,2,2-trifluoroethanol (DCM/AcOH/TFE, 7/1/2) for 1 h at room temperature. The solvent was removed on a rotary evaporator, and the obtained oily product precipitated from cold dry diethyl ether as a white solid (>90% pure by reversed-phase HPLC at 214 nm). Deprotection, using 65% TFA + 3% EDT in DCM for 4 h (Scheme 1), afforded the final free peptides which were purified by preparative HPLC.

Synthesis of Fragment Fmoc-Glu(COOH)-Asn-Pro-Val-NH$_2$. Fmoc-linker-2-chlorotrityl resin (4 g, 0.55 mmol/g) was used for the synthesis of the fragment precursors, following protocols previously described.[29,30] The amino acids used in Fmoc synthesis of tetrapeptide were Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-Asn-OH, and Fmoc-Glu(tBu)-OH. The protected peptide on the resin (5.15 g) was treated with the splitting mixture dichloromethane/acetic acid/2,2,2-trifluoroethanol (50 mL, 7/1/2) for 1 h at room temperature to remove the peptide from the resin. The mixture was filtered off and the resin washed with the splitting mixture (×2) and DCM (×3). The solvent was removed on a rotary evaporator, and the obtained oily product was precipitated from dry diethyl ether as a white solid (1.55 g, 85%). The protected peptide-linker material was treated with 65% TFA + 3% EDT in DCM (4 mL) for 4 h to deprotect Glu from tBu and to liberate the amidated tetrapeptide fragment from linker. The resulting solution was concentrated under vacuum to a small volume (0.5 mL). The final free peptide (1.25gr) was precipitated as a light-yellow amorphous solid by the addition of diethyl ether (Scheme 2).

Preparation of Fmoc-Glu(resin)-Asn-Pro-Val-NH$_2$. 2-Chlorotrityl chloride resin (5 g, 1.5 mequiv of Cl^-/g of resin) in dry DMF (35 mL) was stirred in a round-bottom flask. Diisopropylethylamine (DIPEA) (3.3 mL, 8.28 mmol) and Fmoc-Glu(COOH)-Asn-Pro-Val-NH_2 (1.25 g, 1.84 mmol) were added, and the solution was stirred for 45 min at room temperature. A mixture of MeOH (0.8 mL) and DIPEA (0.2 mL) was then added for endcaping, and the mixture was stirred for another 10 min at room temperature. The Fmoc-Glu(resin)-Asn-Pro-Val-NH_2 was filtered, subsequently was washed with DMF (3 × 10 mL), 2-propanol (iPrOH) (2 × 10 mL), and *n*-hexane (2 × 10 mL), and dried in vacuo for 24 h at room temperature (Scheme 3). The loading of the amino acid per gram of substituted resin was 0.35 mmol of amino acid/g of resin, calculated by weight and amino acid analysis.

Synthesis of Protected Linear Peptide: Boc-Glu-Lys-(NH_2)-Ser(tBu)-Gln-Arg(Pmc)-Ser(tBu)-Gln-Asp(tBu)-Glu-(COOH)-Asn-Pro-Val-NH$_2$. Fmoc-Glu(resin)-Asn-Pro-Val-NH_2 was used for the synthesis of the linear precursor peptide following the protocol previously described.[29,30] The amino acids used in Fmoc synthesis were Fmoc-Asp(tBu)-OH, Fmoc-Gln-OH, Fmoc-Ser(tBu)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Lys(Mtt)-OH, and Boc-Gln-OH. The completed peptide on resin was dried in vacuo and then treated with the splitting mixture dichloromethane (DCM)/1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (8/2) for 6 h at room temperature to remove the peptide from the resin and for the deprotection of Lys from Mtt.[31] The mixture was filtered off, and the resin was washed with the splitting mixture (×2) and DCM (×3). The solvent was removed on a rotary evaporator, and the obtained oily product was precipitated from cold dry diethyl ether as a white solid (Scheme 3).

Cyclization of Protected Peptide. To a solution of linear protected peptide (200 mg, 0.1 mmol) in dry DMF (40 mL) were added 2,4,6-collidine (0.08 mL, 0.6 mmol) and 1-hydroxy-7-azabenzotriazole (40.8 mg, 0.3 mmol). The solution was added dropwise to a solution of *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) (96.4 mg, 0.3 mmol) in dry dimethylformamide (80 mL) for 2 h, and the solution was stirred for 3 h. The reaction was followed by the ninhydrin test on TLC using *n*-butanol/acetic acid/water (4/1/1) as an elutant. The solvent was removed from the reaction mixture under reduced pressure affording a light-yellow oily residue. The cyclic protected peptide was precipitated from H_2O and was dried in vacuo for 12 h.[19,20]

Preparation of Free Cyclic Peptide: H$_2$N-Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Pro-Val-NH$_2$. The dried residue from the cyclization reaction was treated with 65% trifluoroacetic acid in dichloromethane + 3% ethanedithiol for 5 h at room temperature. The resulting solution was concentrated under vacuum to a small volume (0.5 mL). Several drops of methanol were added, and the final free cyclic peptide was precipitated as a light-yellow amorphous solid by the addition of diethyl ether. The crude peptide product was purified further by preparative HPLC as outlined above.[17,19,20] Purification was verified by analytical HPLC.

Induction and Suppression of EAE Using MBP Analogues 1−3. Inbred Lewis rats, bred and maintained in the animal facility of the Hellenic Pasteur Institute under SPF conditions, were used in all experiments. Female rats (200 g) were immunized with linear MBP$_{72-85}$ analogue **1** (30 μg) (n = 6, as positive control), cyclic MBP$_{72-85}$ analogue **3** (30 μg) (n = 7), or cyclic MBP$_{72-85}$ analogue **3** (30 μg) plus Ala^{81}MBP$_{72-85}$ linear peptide analogue **2** (500 μg) (n = 4) in 200 μL of an emulsion composed of equal volumes of Freund's complete adjuvant (Difco) CFA containing 4 mg/mL heat-killed *M. tuberculosis* (H37Ra) (Difco) and PBS. Immunization was performed subcutaneously in the two hind foot pads and repeated 7 days later with the same dosage. Rats were examined daily for clinical signs of EAE and scored as follows: 0, no clinical disease; 0.5, weight loss; 1, tail weakness; 2, paraparesis of hindlimbs; 3, paraplegia of hindlimbs; 4, paraplegia with forelimb weakness, moribund; 5, death. PBS/CFA-injected animals served as negative controls.

NMR Spectroscopy. NMR experiments were performed using a Bruker AMX-400 MHz NMR spectrometer. Five milligrams of compound **1** was dissolved in 0.4 mL of DMSO-d_6. The chemical shifts are reported relative to the undeuterated fraction of the methyl group of DMSO-d_6 at 2.50 ppm with respect to TMS. One-dimensional spectra were recorded with a sweep width of 4.600 Hz and 32K (zero-filled to 64K) data points and by methods previously described.[23,24,32] The TOCSY

experiment performed for resonance assignment provided contour plots which were symmetrized with respect to diagonal. The parameters for the $^1H-^1H$ TOCSY spectrum were spectral width 3623 Hz, acquisition 0.28 s, relaxation delay 1 s, 48 transient and 4 dummy scans for each of 512 increments. The FIDS were zero-filled to 1K data points prior to FT. Sinebell window function was used for resolution enhancement. The phase sensivity (TPPI mode) $^1H-^1H$ TOCSY spectra[33,34] were acquired with a 100-ms spin−lock pulse. The 2D $^1H-^1H$ ROESY spectrum[35,36] was acquired with a 350-ms mixing time and was carried out with concentrations of 5 or 1 mg of compound **1** in 0.4 mL of DMSO in order to verify the head-to-tail ROEs.

Molecular Modeling. To extend the observations made using NMR, a theoretical modeling approach was used. Theoretical calculations were performed as described previously[32,37,38] using a Silicon Graphics 4D/35 workstation and Quanta software of MSI. The structure of compound **1** was first minimized using a combination of steepest descent and conjugate gradient algorithms. The resulting minimized structure was imposed to adopt NOEs by applying distance constraints in combination with conjugate gradient minimization algorithm. Thus, in the created model the major NOE connectivities are within 2−5 Å apart. The minimization procedure was completed when no improvement in the model could be achieved (NOE distance constraints remained constant). Compound **3** was assumed to have identical conformation as **1**. Therefore, the structure of the cyclic peptide was created by cyclization through the amino acids 2 and 9 while keeping intact all the distance constraints of the linear peptide **1**. To further explore the low-energy conformers of the compounds **1** and **3**, molecular dynamics were applied at 300K using 1-, 2-, and 1-ps time frames for heating, equilibration, and simulation steps. One hundred structures from the simulated ones were minimized using 1000 iteration steps and a conjugate gradient algorithm. Details of the above techniques have been recently described.[32,37−39]

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Supporting Information Available: Figure of a selected region of the 2D $^1H-^1H$ 400-MHz ROESY NMR spectrum and a table with interresidue ROE connectivities for linear agonist MBP_{72-85} analogue **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Martin, R.; McFarland, H.; McFarlin, D. Immunological aspects of demyelinating diseases. *Annu. Rev. Immunol.* **1992**, *10*, 153−187.

(2) Steinman, L. Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. *Cell* **1996**, *85*, 299−302.

(3) Pette, M.; Fujita, K.; Wilkinson, D.; Altmann, D. M.; Trowsdale, J.; Giegerich, G.; Hinkkanen, A.; Epplen, J. T.; Kappos, L.; Wekerle, H. Myelin autoreactivity in multiple sclerosis: recognition of myelin basic protein in the context of HLA-DR2 products by T lymphocytes and multiple sclerosis patients and healthy donors. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 7968−7972.

(4) Ota, K.; Matsui, M.; Milford, E. L.; Mackin, G. A.; Weiner, H. L.; Hafler, D. A. T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. *Nature (London)* **1990**, *346*, 183−187.

(5) Martin, R.; Jaraquemada, D.; Flerlage, M.; Richert, J.; Whitaker, J.; Long, E. O.; McFarlin, D. E.; McFarland, H. F. Fine specificity and HLA restriction of myelin basic protein-spesific cytotoxic T cell lines from multiple sclerosis patients and healthy individuals. *J. Immunol.* **1990**, *145*, 540−548.

(6) Wucherpfennig, K. W.; Catz, I.; Hausmann, S.; Strominger, J. L.; Steinman, L.; Warren, K. G. Recognition of the immunodominant myelin basic protein peptide by autoantibodies and HLA-DR2-restricted T cell clones from multiple sclerosis patients. *J. Clin. Invest.* **1997**, *100*, 1114−1122.

(7) Wauben, M.; Boog, C.; Zee, R.; Joosten, I.; Scihlief, A.; Eden, W. Disease inhibition by major histocompatibility complex binding peptide analogues of disease-associated epitopes: More than blocking alone. *J. Exp. Med.* **1992**, *176*, 667−677.

(8) Hafler, D. A.; Weiner, H. L. Immunologic mechanisms and therapy in multiple sclerosis. *Immunol. Rev.* **1995**, *144*, 75−107.

(9) Hohfeld, R. Biotechnological agents for the immunotherapy of multiple sclerosis. Principles, problems and perspectives. *Brain* **1997**, *120*, 865−916.

(10) Zamvil, S. S.; Steinman, L. The T lymphocyte in experimental allergic encephalomyelitis. *Annu. Rev. Immunol.* **1990**, *8*, 579−621.

(11) Chou, Y.; Vandenbark, A.; Jones, R.; Hashim, G.; Offner, H. Selection of encephalitogenic rat T lymphocyte clones recognizing an immunodominant epitope on myelin basic protein. *J. Neurosci. Res.* **1989**, *22*, 181.

(12) Wucherpfennig, K.; Sette, A.; Southwood, S.; Oseroff, C.; Matsui, M.; Strominger, J.; Hafler, D. A. Structural requirements for binding of an immunodominant Myelin Basic Protein peptide to DR_2 isotypes and for its recognition by human T cell clones. *J. Exp. Med.* **1994**, *179*, 279−290.

(13) Vergelli, M.; Hemmer, B.; Utz, U.; Vogt, A.; Kalbus, M.; Tranquill, L.; Conlon, P.; Ling, N.; Steinman, L.; McFarland, H.; Martin, R. Differential activation of human autoreactive T cell clones by altered peptide ligands derived from myelin basic protein peptides (87−99). *Eur. J. Immunol.* **1996**, *26*, 2624−2634.

(14) Brocke, S.; Gijbels, K.; Allegretta, M.; Ferber, I.; Piercy, C.; Blankenstein, T.; Martin, R.; Utz, U.; Karin, N.; Mitchell, D.; Veroma, T.; Waisman, A.; Gaur, A.; Conlon, P.; Ling, N.; Fairchild, P.; Wraith, D.; O'Carra, A.; Farhman, G.; Steinman, L. Treatment of experimental encephalomyelitis with a peptide analogue of Myelin Basic Protein. *Nature* **1996**, *379*, 343−346.

(15) Tselios, T.; Probert, L.; Kollias, G.; Matsoukas, E.; Roumelioti, P.; Alexopoulos, K.; Moore, G.; Matsoukas, J. Design and synthesis of small semi-mimetic peptides with immunomodulatory activity based on Myelin Basic Protein (MBP). *Amino Acids* **1998**, *14*, 333−341.

(16) Pires, J.; Matsoukas, J.; Moore, G. Recognition of the encephalitogenic myelin basic protein (74−85) peptide by the immune-system: Importance of conformation. *Proc. West. Pharmacol. Soc.* **1998**, *41*, 193−195.

(17) Matsoukas, J.; Hondrelis, J.; Agelis, G.; Barlos, K.; Gatos, D.; Ganter, R.; Moore, D.; Moore, G. Novel synthesis of cyclic amide-linked analogues of angiotensins II and III. *J. Med. Chem.* **1994**, *37*, 2958−2669.

(18) Vlahakos, D.; Matsoukas, J.; Ancans, J.; Moore, G.; Iliodromitis, E.; Marathia, K.; Kremastinos, D. Biological activity of the novel cyclic angiotensin II analogue, [Sar^1,Lys^3,Glu^5]ANG II. *Lett. Pept. Sci. (LiPS)* **1996**, *3*, 191−194.

(19) (a) Matsoukas, J.; Panagiotopoulos, D.; Keramida, M.; Mavromoustakos, T.; Yamdagni, R.; Wu, Q.; Moore, G.; Saifeddine, M.; Hollenberg, M. Synthesis and contractile activities of cyclic thrombin receptor-derived peptide analogues with a Phe-Leu-Leu-Arg motif: Importance of the Phe/Arg relative conformation and the primary amino group for activity. *J. Med. Chem.* **1996**, *39*, 3585−3591. (b) Panagiotopoulos, D.; Matsoukas, J.; Alexopoulos, K.; Zebeki, A.; Mavromoustakos, T.; Saifeddine, M.; Hollenberg, M. Synthesis and activities of cyclic thrombin-receptor-derived peptide analogues of the Ser_{42}-Phe-Leu-Leu-Arg_{46} motif sequence containing D-Phe and/or D-Arg. *Lett. Pept. Sci. (LiPS)* **1996**, *3*, 233−240.

(20) (a) Burgess, K.; Lim, D. Synthesis and solution conformation of cyclo[RGDRGD]: A cyclic peptide with selectivity for the $\alpha V\beta 3$ receptor. *J. Med. Chem.* **1996**, *39*, 4520−4526. (b) Heavner, G.; Audhya, T.; Doyle, D.; Tjoeng, F. S.; Goldstein, G. Biologically active conformations of thymopentin. *Int. J. Pept. Protein Res.* **1991**, *37*, 198−209.

(21) Matsoukas, J.; Hollenberg, M.; Mavromoustakos, T.; Panagiotopoulos, D.; Alexopoulos, K.; Yamdagni, R.; Wu, Q.; Moore, G. Conformational analysis of the thrombih receptor agonist peptides SFLLR and SFLLR-NH_2 by NMR: Evidence for a cyclic bioactive conformation. *J. Protein Chem.* **1997,** *16*, 113−131.

(22) Wurhrich, K. *NMR of Proteins and Nuclear Acids*; John Wiley and Sons: New York, 1986.

(23) Matsoukas, J.; Yamdagni, R.; Moore, G. J. ^1H NMR studies of sarmesin and [Des^1]sarmesin conformation in dimethyl sulfoxide by nuclear overhauser effect (NOE) enhancement spectroscopy: Folding of the N- and C-terminal domains. *Peptides* **1990**, *11*, 367−374.

(24) Matsoukas, J.; Bigam, G.; Zhou, N.; Moore, G. ^1H NMR studies of [Sar^1]angiotensin II conformation by nuclear overhauser effect spectroscopy in the rotating frame (ROESY): Clustering of the aromatic rings in dimethyl sulfoxide. *Peptides* **1990**, *11*, 359−366.

(25) Moore, G.; Smith, J.; Baylis, B.; Matsoukas, J. Design and pharmacology of peptide mimetics. *Adv. Pharmacol.* **1995**, *33*, 91−141.

(26) Giannis, A.; Rubsam, F. Peptidomimetics in Drug Design. *Adv. Drug Res*. **1997**, *29*, 1−78.
(27) Monos, D.; Soulika, A.; Argyris, E.; Gorga, J.; Stern, L.; Magafa, V.; Cordopatis, P.; Androulakis, I.; Floudas, C. HLA-peptide interactions: theoretical and experimental approaches. In *12th International histocompatibility conference, 'HLA diversity functional and medical implications'*; Charron, D., Ed.; EDK Sevres, 1997; Vol. II, Proceedings of 12th International Histocompatibility Conference.
(28) Hogg, A. M. Conversion of mass spectrometers for fast-atom bombardment using easily constructed components. *Int. J. Mass Spectrom. Ion Phys*. **1983**, *49*, 25−34.
(29) Barlos, K.; Gatos, D.; Hondrelis, J.; Matsoukas, J.; Moore, G.; Schafer, W.; Sotiriou, P. Preparation of new acid-labile resins of sec-alcohol type and their applications in peptide synthesis. *Liebigs Ann. Chem*. **1995**, 951−955.
(30) Barlos, K.; Gatos, D.; Schafer, W. Synthesis of prothymosin α-(ProT$_\alpha$)-α protein consisting of 109 amino acid residues. *Angew. Chem., Int. Ed. Engl*. **1991**, *30*, 590−593.
(31) Aletras, A.; Barlos, K.; Gatos, D.; Koutsogianni, S.; Mamos, P. Preparation of the very acid-sensitive Fmoc-Lys(Mtt)-OH. *Int. J. Pept. Protein Res*. **1995**, *45*, 488−496.
(32) Matsoukas, J.; Hondrelis, J.; Keramida, M.; Mavromoustakos, T.; Makriyiannis, A.; Yamdagni, R.; Wu, Q.; Moore, G. Role of NH_2-terminal domain of angiotensin II and [Sar^1]angiotensin II on conformation and activity: NMR evidence for aromatic ring clustering and peptide backbone folding compared to [Des^{1,2,3}]-angiotensin II. *J. Biol. Chem*. **1994**, *269*, 5303−5312.
(33) Eich, G.; Bodenhansen, G.; Ernst, R. Exploring nuclear spin systems by relayed magnetization transfer. *J. Am. Chem. Soc*. **1982**, *104*, 3731−3732.
(34) Davis, D. G.; Bax, A. Assignment of complex ^{1}H NMR spectra via two-dimensinal homonuclear Hartmann−Hahn spectroscopy. *J. Am. Chem. Soc*. **1985**, *107*, 2820−2821.
(35) Bothner, A.; Stephens, R.; Lee, J.; Warren, C.; Jeanloz, J. Structure determination of a tetrasaccharide: Transient nuclear overhauser effects in the rotating frame. *J. Am. Chem. Soc*. **1984,** *106*, 811−813.
(36) Bax, A.; Grzesiek. ROESY. In *Encyclopedia of NMR*; Grant, D. M., Harris, R. K., Eds.; Wiley: Chichester, 1996; pp 4157−4166.
(37) Mavromoustakos, T.; Yang, D. P.; Theodoropoulou, E.; Makriyannis, A. Studies of the conformational properties of the cannabimimetic aminoalkylindole pravadoline using NMR and molecular modeling. *Eur. J. Med. Chem*. **1995**, *30*, 227−234.
(38) Theodoropoulou, E.; Mavromoustakos, T.; Panagiotopoulos, D.; Matsoukas, J.; Smith, J. Superimposition of Potent Non-peptide AT$_1$ Receptor Antagonists with Angiotensin II. *Lett. Pept. Sci. (LiPS)* **1996**, *3*, 209−216.
(39) Moore, G.; Matsoukas, J. Charge-Transfer Interactions. In *Peptide Hormones in Hellenic Forum on Bioactive Peptides*; Cordopatis, P. A., Ed.; Crete University Press: Crete, Greece, **1997**; pp 161−170.

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