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(54) Title: PEPTIDE ANALOGUES OF MYELIN BASIC PROTEIN EPITOPES IN THE TREATMENT OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE) AND MULTIPLE SCLEROSIS (MS)

(57) Abstract: This invention relates to novel linear and cyclic peptide analogues of Myelin Basic Protein epitopes (from guinea pig MBP_{72-85} and human MBP_{87-99}) and their conjugates with mannan and/or KLH useful in the treatment of Experimental Autoimmune Encephalomyelitis (EAE) and Multiple Sclerosis (MS). For the first time cyclic analogues of MBP epitopes have been synthesized and shown to prevent the development of EAE. There is gathering evidence that analogues of disease-associated epitopes can be conjugated to mannan and/or KLH and actively generate antigen specific regulatory CD4/CD8 T cells and Th1/Th2 cytokines.

TITLE

Peptide analogues of Myelin Basic Protein epitopes in the treatment of Experimental Autoimmune Encephalomyelitis (EAE) and Multiple Sclerosis (MS)

TECHNICAL FIELD OF INVENTION

This invention relates to novel linear and cyclic peptide analogues of Myelin Basic Protein epitopes and their conjugates with mannan and/or KLH useful in the treatment of Experimental Autoimmune Encephalomyelitis (EAE) and Multiple Sclerosis (MS).

INTRODUCTION BACKGROUND OF INVENTION

This invention discloses analogues of Myelin antigens and mannan/KLH conjugates for treatment of Multiple Sclerosis.

Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis /Animal model

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS) characterized by local T cell and macrophage infiltrates, demyelination and loss of neurologic function [Steinman, 1996, Martin, et al 1992]. MS is believed to be an autoimmune disease triggered by CNS-specific CD4 T lymphocytes. Candidate autoantigens include constituents of the myelin sheath, such as myelin basic protein (MBP) and proteolipid protein (PLP). Modern approaches towards the therapeutic management of MS involve the design and use of peptide analogues of disease-associated myelin epitopes to induce peripheral T cell tolerance [Hafler, et al 1995, Hohfeld, 1997, Ota, et al 1990]. Experimental Autoimmune Encephalomyelitis (EAE), one of the best studied experimental animal models of MS, is a useful *in vivo* system for the evaluation of such therapeutic approaches.

Inhibition of disease using guinea pig MBP epitope 72-85 analogues

In Lewis rats immunized with guinea pig MBP protein, encephalitogenic T cells recognizing the MBP_{72-85} epitope, dominated the immune response [Wauben, et al

1992]. In particular, the linear analogue Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Pro-Val (MBP_{72-85}) has been found to induce EAE, while substitution of the Asp residue at position 81 with Ala resulted in an analogue Gln-Lys-Ser-Gln-Arg-Ser-Gln-Ala^{81}-Glu-Asn-Pro-Val ($Ala^{81}MBP_{72-85}$) that prevented the induction of EAE by its parent peptide. Peptides participating in the trimolecular complex, MHC-peptide-TCR and cause an antagonist effect (ie. loss of T-cell activation) has a loss of H-bond contacts of the peptide side chains to the CDR3 loops of the TCR. In such an event this loss of H-bond contact causes an agonist peptide to become an antagonist peptide [Degano, et al 2000]. Peptide therapy, however, is hindered due to the sensitivity of peptides to proteolytic enzymes. Continuous infusions of prohibitive amounts of peptides are necessary to elicit the necessary biological response [Matsoukas, et al 1994, Matsoukas, et al 1996]. To address the need for more stable molecules, cyclic analogues that could maintain or suppress the biological function of the original peptide, yet could also elicit a response in pharmacological quantities, were designed, synthesized and evaluated for their activity in the EAE system as well as in human peripheral blood T cells [Tselios, et al 1998, Tselios, et al 1999]. Design of potent cyclic analogues was based on Nuclear Magnetic Resonance and Molecular Dynamics studies carried out in the agonist and antagonist linear analogues, MBP_{72-85} and $Ala^{81}MBP_{72-85}$ respectively. These studies revealed a head-to-tail intramolecular proximity (ROE connectivity between βVal^{12}-γGln^{1} in MBP_{72-85} and βPro^{11}H-γGln^{1} in $Ala^{81}MBP_{72-85}$), suggesting cyclic conformations for the two linear analogues in solution. These results led to the synthesis of the cyclic analogues: 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 (c-MBP_{72-85}) Gln^{1}-Lys^{2}-Ser^{3}-Gln^{4}-Arg^{5}-Ser^{6}-Gln^{7}-Ala^{8}-Glu^{9}-Asn^{10}-Pro^{11}-Val^{12}-NH_2 (c-$Ala^{81}MBP_{72-85}$) by connecting the ε amino group of Lys and γ carboxyl group of Glu at positions 2 and 9 [Tselios, et al 1999]. Cyclization is known to restrict the number of possible conformations, allowing the possibility to diminish the unfavored conformations for approaching the receptor site in a direct peptide-receptor interaction. The c-MBP_{72-85} analogue has comparable potency to MBP_{72-85} in inducing EAE in Lewis rats while the clinical and histopathological manifestations of disease induced by c-MBP_{72-85} are prevented by a linear antagonist ($Ala^{81}MBP_{72-85}$) [Tselios, et al 1999]. In our studies the encephalitogenic activity of MBP_{72-85} was completely prevented by the co-injection with c-$Ala^{81}MBP_{72-85}$. Furthermore, the cyclic analogues (c-MBP_{72-85} and c-$Ala^{81}MBP_{72-85}$) were assessed for their biological activities in human peripheral

blood T cells and their activity was comparable with that of linear agonist and antagonist analogues MBP_{72-85} and $Ala^{81}MBP_{72-85}$. The linear and cyclic forms of the agonist peptide MBP_{72-85} had the comparable effect on human T cell activation and proliferation and their effect was completely reversed by co-culturing of the cells with the linear or cyclic analogues of the antagonist peptide $Ala^{81}MBP_{72-85}$. The comparable potencies of linear and cyclic analogues MBP_{72-85} and $c\text{-}MBP_{72-85}$ as well as of analogues $Ala^{81}MBP_{72-85}$ and $c\text{-}Ala^{81}MBP_{72-85}$, indicate that a cyclic conformation of the MBP_{72-85} epitope together with a carboxyl group at position 81 is important for the function of the trimolecular complex, MHC-peptide-T cell receptor and for the activation of EAE specified T-cells.

Demyelination and inflammation in the spinal cord at day 15 after immunization with MBP_{72-85}, is completely prevented by the co-immunization of the $c\text{-}Ala^{81}$ MBP_{72-85}. 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 $c\text{-}Ala^{81}MBP_{72-85}$ shows a complete absence of inflammatory infiltrates and normal myelin structure. Cyclic analogues offer multiple advantages compared to linear counterparts, in terms of stability, duration of action and receptor selectivity.

Inhibition of disease using human MBP epitope 87-99 analogues

Structure-Activity studies based on the human MBP_{87-99} [Vergeli, et al 1996, Brocke, et al 1996] epitope (Val-His-Phe-Phe-Lys-Asn-Ile-Val-Thr-Pro-Arg-Thr-Pro) resulted in linear and cyclic analogues namely $[Arg^{91},Ala^{96}]MBP_{87-99}$ (Val-His-Phe-Phe-Arg^{91}-Asn-Ile-Val-Thr-Ala^{96}-Arg-Thr-Pro) and cyclo-(87, 99)$[Arg^{91},Ala^{96}]MBP_{87-99}$ (Val^{87}-His-Phe-Phe-Arg^{91}-Asn-Ile-Val-Thr-Ala^{96}-Arg-Thr-Pro^{99}) which completely prevented the induction of EAE when co-injected to Lewis rats together with encephalitogenic agonist MBP_{72-85}. Blockade of MBP_{72-85} induced EAE by the previous unrelated peptides could indicate that the mechanism of inhibition is not due to binding competition but rather due to the delivery of a negative signal by the antagonist which overcomes the agonist response possibly through the activation of antigen specific regulatory T cells.

Inhibition of disease through control of cytokine secretion using mannan conjugates of MBP epitopes

There is gathering evidence that analogues of disease-associated epitopes can be conjugated to mannan and/or KLH and actively generate antigen specific regulatory CD4/CD8 T cells and Th1/Th2 cytokines. The ability to alter the cytokine secretion of autoreactive T cell lines through peptide or mimetic treatment even in longstanding autoimmune disease indicates that cytokine therapy might have therapeutic benefits by switching the function of myelin reactive T cells such that they are non-pathogenic. Mannan conjugates of Myelin antigens in the oxidized or reduced form (with the participation or not of KLH) are suitable tools to control cytokine secretion.

Current peptide therapies for MS

Current peptide therapies of multiple sclerosis include treatment with Interferon's (Interferon beta-1α and Interferon beta-1β) and glatiramer acetate (copolymer-1) which is a synthetic protein comprised of the major aminoacids Glu, Gln, Lys, Arg of MBP. These immunomodulators have been approved by the FDA for patients with relapsing-remitting MS. Interferon's given by S.C. injections, reduce the frequency, severity and duration of exacerbation but their impact on preventing disability over the long-term is not yet established. Side effects also are common and consist of reactions at the injection site, fever, myalgia and blu-like syndrome. So far the reported benefits from the use of interferon's and copolymer are marginal and therefore the need for improved therapeutics are imperative. Another approach under clinical investigation for autoimmune suppression is the oral administration of autoantigens. In this study, orally administered antigens suppress autoimmunity in animal models, including EAE, collagen and adjuvant-induced arthritis, uveitis and diabetes in the non-obese diabetic mouse. Low doses of oral antigen induce antigen-specific regulatory T-cells which act by releasing inhibitory cytokines such as transforming growth factor-beta, interleukin-4, and interleukin-10 at the target organ. Thus, one can suppress inflammation at a target organ by orally administering an antigen derived from the side of inflammation, even if it is not the target of the autoimmune response. Initial human trials of orally administered antigen have shown positive findings in patients with MS and Rheumatoid Arthritis. A double-blind, placebo-controlled, phase III multi-center trial of oral myelin in relapsing-remitting multiple sclerosis patients is in progress, as are

phase II clinical trials investigating the oral administration of type II collagen in rheumatoid arthritis, S-antigen in uveitis and insulin in type I diabetes. This promising method has the oral administration advantage over the previous methods using interferons and copolymer-1. However, issues related to the peptide nature and cost of administered substance renders the non-peptide mimetic approach, even in its infancy, an attractive goal to pursue.

Strategies in the immunotherapy of Multiple Sclerosis and clinical perspectives

In the immunotherapeutic approach towards the development of therapeutic vaccines for MS the assumption is that MBP epitopes or their analogues can actively inhibit or prevent disease through the activation of antigen-specific regulatory T cells, or antibodies related to myelin sheath destruction. The myelin sheath is constituted from the proteins: MBP, Proteolipid Protein (PLP), Myelin Oligodentrocyte Glycoprotein (MOG) and heat-shock protein which are implicated in MS. Thus, epitopes of these Myelin sheath proteins are targets for immunotherapeutic techniques. In areas of inflammation in MS, antibodies against the minor protein MOG have been demonstrated. MOG antibodies were related to significant myelin disruption, probably by coating the myelin so that macrophages could engulf and destroy coated sections of myelin, blocking nerve impulses temporarily or permanently. Thus, we now know that antibodies do play a role in MS, and cooperate with antigen presenting cells in myelin destruction. Blocking the effects of these MOG antibodies with secondary antibodies or non-peptide mimetics might be an important avenue for future therapy.

Another direction in the immunotherapy of autoimmune diseases is the use of Multiple-Antigen Peptide (MAP) systems introduced by Tam [Tam 1990]. This system represents a novel approach to anti-peptide antibody production. It is build on a resin which bears a core of radial branching lysine dendrites on which a number of copies of a given peptide antigen can be synthesized. Lysine derivatives have been used for the solid phase synthesis of lysine cores suitable for the assembly of antigenic peptides. These peptides have found application in raising antibodies and in the preparation of synthetic vaccines. On a lysine core several different epitopes of a protein or of different proteins can be assembled to create the required antigenic synthetic protein. Following assembly of the peptide on the MAP core, the peptide is deprotected and cleaved from the support using standard techniques, yielding a highly immunogenic

macromolecular structure without the need for conjugation to a carrier protein. The MAP approach has been shown to yield higher antibody titres than using monomeric peptide-conjugates. Alternatively, the two epitopes can be synthesized on alternate branches of the lysine core, using Boc and Fmoc chemistry. T-cell and B-cell epitopes can also be combined sequentially within a single linear sequence.

Another challenging strategy in the immunotherapy of MS or other autoimmune diseases is the use of peptide poly-lysine analogues conjugated with mannan via KLH in its oxidized or reduced form to develop Th1/Th2 responses followed by release of appropriate cytokines [Apostolopoulos, et al 1995, Apostolopoulos, et al 1996, Apostolopoulos, et al 1998, Lofthouse, et al 1997]. The aim of this approach is the development of a therapeutic vaccine for prevention or control of disease. Presently, antigen peptide of MBP, PLP and MOG proteins conjugated to mannan (with or without KLH) in its oxidized and reduced form are under investigation. Assays to study their effect on cellular proliferation, antibody production and cytokines secretion being determined in Lewis rats and in human peripheral blood T-cells. These studies include the development of recently rationally designed constrained cyclic antagonist peptide analogues based on MBP epitopes 72-85 and 87-99 which suppresses the development of clinical E.A.E., CNS inflammation and demyelination. The use of oxidized or reduced mannan to develop a Th1 or Th2 response (and the appropriate cytokines) to Myelin peptides expressed in MS constitutes a novel strategy for the treatment of disease. Mannan has been investigated extensively for its ability to generate responses in several model systems. Its adjuvant function has been shown to stem, from its ability to target the mannose receptor an antigen presenting cells. Of particular interest, mannan conjugated to peptide under oxidizing conditions induces Th1-type immune responses, while peptides conjugated to mannan under reducing conditions generate Th2-type immune responses. Antibodies, CTL, tumor protection or the secretion of IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, TNF-alpha, IFN-gamma, GM-CSF, TGF-beta, cytokines have been measured by mRNA or in vitro culture of ATP/T-cells, after immunization with oxidized or reduced mannan-peptide conjugates.

REFERENCES

Apostolopoulos, V.; Lofthouse, S.; Popovski, V.; Chelvanayagam G.; Sandrin, M.; McKenzie I. *Nature Biot.* **1998**, 16, 278.

Apostolopoulos, V.; Pietersz, A.; McKenzie, I. *Vaccine*, **1996**, 14, 930.

Apostolopoulos, V.; Pietersz, A.; Loveland, B.; Sandrin, M.; McKenzie, I. *Proc. Natl. Acad. Science,* **1995**, 92, 10128.

Berezhkovskiy, L.; Pham, S.; Reich, P.; Deshpande, S. *J. Pept. Res.* **1999**, 54, 112.

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. *Nature* **1996**, 379, 343.

Bruggle, F.; Timmermans, M.; Van Unen, A.; Ten Hove, J.; Van De Werken, G.; Poolman, T.; Hoogerhout, P. *J. Pept. Sci.* **1999**, 5,381.

Chaloin, L.; Mery, J.; Van Mau, N.; Divita, G.; Heitz, F. *J. Pept. Sci.* **1999**, 5, 381.

Degano, M.; Garcia, K.; Apostolopoulos, V.; Rudolph, M.; Teyton, L.; Wilson, I. *Immunity*, **2000**, 12, 251.

Hafler, D.; Weiner, H. *Immunol. Rev.* **1995**, 144, 75.

Hohfeld, R. *Brain.* **1997**, 120, 865.

Iwai, H.; Pluckthun, A. *FEBS Lett.* **1999**, 459, 166.

Kromboritis, E., Hatridakis, G., Barlos, K. *J. Biol. Chem.,* **1998,** 273, 10874

Kurtzke, F. *Neurology,* **1983**, 33, 1442.

Lofthouse, S.; Apostolopoulos, V.; Pietersz, G.; Wenjun, L.; McKenzie, I. *Vaccine,* **1997,** 15, 14, 1586.

Martin, R.; McFarland, H.; McFarlin, D. *Ann. Rev. Immunol.* **1992**, 10,153

Matsoukas, J.; Hondrelis, J.; Agelis, G.; Barlos, K.; Gatos, D.; Ganter, R.; Moore, D.; Moore, G. *J. Med. Chem.* **1994**, 37, 2958.

Matsoukas, J.; Panagiotopoulos, D.; Keramida, M.; Mavromoustakos T.; Yamdagni, R.; Wu, Q.; Moore, G.; Saifeddine, M.; Hollenberg M. *J. Med. Chem.* **1996**, 39, 3585.

Mezo, G.; Majer, Z.; valero, L.; Andreu, D.; Hudecz, F. *J. Pept. Sci.* **1999**, 5, 272.

Oligino, L.; Lung, T.; Sastry, L.; Bigelow, J.; Cao, T.; Curran, M.; Burke, R.; Wang, S.; Krag, D.; Roller, P.; King, R. *J. Biol. Chem.* **1997**, 272, 29046.

Ota, K.; Matsui, M.; Milford, E.; Mackin, G.; Weiner, H.; Hafler, D. *Nature (Lond)* **1990,** 346, 183.

Scott, P.; Abel-Santos, E.; Wall, M.; Wahnon, C.; Benkovic, J. *Proc. Natl. Acad. Sci. USA* **1999**, 16,16.

Steinman, L. *Cell* **1996**, 85, 299.

Tam, P.; Clavijo, P.; Lu, Y.; Nussenzweig, S.; Nussenzweig, S.; Zavala, F. *J. Exp. Med* **1990**, 171, 299.

Tamilarasu, N.; Huq, I.; Rana, T. *Bioor. Med. Chem. Let.* **2000**, 10, 971.

Tselios, T.; Probert, L.; Kollias, G.; Matsoukas, E.; Roumelioti, P.; Alexopoulos, K.; Moore, G.; Matsoukas J. *Amino Acids* **1998**, 14, 333.

Tselios, T.; Probert, L.; Daliani, I.; Matsoukas, E.; Troganis, A.; Gerothanassis, P.; Mavromoustakos, T.; Moore, G.; Matsoukas J. *J. Med. Chem.* **1999**, 42, 1170.

Vergeli, M.; Hemmer, B.; Utz, U.; Vogt, A.; Kalbus, M.; Tranquill, L.; Conlon, P.; Ling, N.; Steinman, L.; McFarland, H.; Martin R. *Eur. J. Immunol.* **1996**, 26, 2624.

Wauben, M.; Boog, C.; Zee, R.; Joosten, I.; Scihlief, A.; Eden, W. *J. Exp. Med.* **1992**, 176, 667.

STATE OF ART OF INVENTION

A. CONFORMATIONAL MODELS

A1. Conformational model of guinea pig MBP_{72-85}

A Cyclic conformation and the role of Asp/Arg interaction in triggering activity, is disclosed in this application.

To address the need for more stable molecules, cyclic analogues which could maintain the biological function of the original peptide, yet could be able to elicit a response in pharmacological quantities, were designed, synthesised and evaluated for activity in the EAE. system. Design of the cyclic analogues of guinea pig MBP epitope 72-85 was based on Nuclear Magnetic Resonance and Molecular Dynamics studies carried out in the Guinea Pig agonist linear MBP_{72-85} epitope. These studies revealed a head to tail intramolecular proximity (ROE connectivity between βVal12-γGln1) suggesting a cyclic conformation for MBP_{72-85} (Fig. 1). We therefore, synthesised the cyclic analogues 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 (1) and Gln^1-Lys^2-Ser^3-Gln^4-Arg^5-Ser^6-Gln^7-Ala^8-Glu^9-Asn^{10}-Pro^{11}-Val^{12}-NH_2 (2) by connecting the ε amino group of Lys and γ carboxyl group of Glu at positions 2 and 9. The cyclic analogue 1 was assessed for its biological activity in the EAE. system and its activity was comparable with that of linear agonist. EAE induced by cyclic analogue 2 was completely suppressed by the co-injection of the $Ala^{81}MBP_{72-85}$ antagonist analogue. The comparable potencies of linear and cyclic analogues, indicate that the encephalitogenic linear peptide participates in the trimolecular complex with a cyclic conformation in which the carboxyl group of Asp at position 81 together with the guanidino group of Arg residue may play an important role for activation of this complex. Replacement of Asp by Ala results in disruption of interaction and antagonist activity.

A2. Conformational model of human MBP_{87-99}

Design of the cyclic analogues of Human MBP epitope 87-99 was based on Nuclear Magnetic Resonance and Molecular Dynamics. These studies revealed a head to tail intramolecular proximity (ROE connectivity between NHArg97-αVal87) indicating a pseudocyclic conformation for the immunogenic peptide MBP_{87-99}. Similarly NMR studies of [Arg^{91}, Ala^{96}] MBP_{87-99} (antagonist) revealed a head to tail intramolecular

interaction (ROE connectivity between αPhe89-δ_2Pro99).

B. ADVANTAGES OF CYCLIC ANALOGUES OVER LINEAR

Cyclization of amino acid sequences results in increased metabolic stability, potency, receptor selectivity and bioavailability all of them reflecting a better pharmacological profile [Scott, et al 1999, Oligino, et al 1997]. In particular cyclic peptides have been used in several cases as synthetic immunogens [Bruggle, et al 1999], potent vaccine for diabetes [Berezhkovskiy, et al 1999], antigens for Herpes Simplex Virus [Mezo, et al 1999], transmembrane ion channels [Chaloin et al 1999], inhibitors of HIV-1 Tat-TAR interactions in human cells [Tamilarasu, 2000], of α-amylase, pancreatic tripsin and as protein stabilizer [Iwai, et al 1999].

Furthermore, advantages of cyclic analogues over their linear counterpart include (i) The cyclic analogues are more stable molecules and thus more resistant to enzymatic degradation, a quality that makes them attractive candidates as drug leads. (ii) It is an intermediate step towards the rational design and development of a non-peptide drug for oral administration, which is the ultimate goal of this work and technology. (iii) The conformation of the cyclic analogues is fixed compared to the conformational flexibility characterizing the linear counterparts. The active conformation of the potent linear peptides, which are very important for further drug development, has been identified through combined SAR, NMR and Dynamics studies.

To our knowledge, this is the first time that cyclic analogues of MBP epitopes have been synthesized and shown to prevent the development of EAE.

B1. Cyclic analogues of human MBP_{87-99} epitope inducing and suppressing the developmeat of EAE

The MBP_{72-85} peptide (25μg) induced an acute monophasic disease with a peak clinical score at day 13 after the initial injection, followed by complete recovery in all animals by day 18. Coinjection of [Arg^{91}, Ala^{96}]MBP_{87-99} (500μg) with the potent agonist peptide MBP_{72-85} (25μg) completely prevented the development of EAE. (Fig. 2) demonstrating that this linear antagonist is a potent inhibitor of disease induced by linear analogue MBP_{72-85}. Further modification and cyclization resulted in two cyclic

antagonist peptides. The Lys side chain and C-terminus amide-linked cyclic analogue, cyclo-(91, 99) [Ala^{96}] MBP$_{87-99}$ (Val-His-Phe-Phe-Lys^{91}-Asn-Ile-Val-Thr-Ala^{96}-Arg-Thr-Pro^{99}) had low inhibitory activity in the EAE system while the amide-linked cyclic analogue, cyclo-(87, 99) [Arg^{91}, Ala^{96}] MBP$_{87-99}$ was a strong inhibitor of EAE when co-administered with MBP$_{72-85}$ (Fig. 2). Co-injection of any cyclic analogue (500μg) with MBP$_{72-85}$ (25μg) did not inhibit EAE activity. However all cyclic analogues shown in Fig. 3 except cyclo-(1, 5) Phe^{1}-Ala-Arg-Gln-Acp^{5} resulted in delay of onset of EAE (from day 13 to day 15) but not its severity (Fig. 3).

B2. Cyclic analogue c-Ala^{81}MBP$_{72-85}$ of guinea pig MBP 72-85 epitope potently inhibits MBP$_{72-85}$-induced EAE in Lewis rats

Analogues MBP$_{72-85}$ and c-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 and to evaluate the activity of the cyclic antagonist. When c-Ala^{81}MBP$_{72-85}$ was co-injected with MBP$_{72-85}$ the clinical signs of EAE were completely prevented (Fig. 4), demonstrating that c-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. In contrast, spinal cord section taken from rats immunized with MBP$_{72-85}$ and Ala^{81}MBP$_{72-85}$ showed the complete absence of inflammation and demyelination.

C. MANNAN/KLH CONJUGATES OF MBP EPITOPES (LINEAR OR CYCLIC/AGONISTS OR ANTAGONISTS) IN THE IMMUNOTHERAPY OF EAE IN RATS: IMPLICATIONS FOR ITS USE IN MS

Mannan has been used as a successful carrier to target peptides to the macrophage/dentritic cell mannose receptor. Upon binding, MHC class I or MHC class II presentation of peptides is generated; stimulating either CTL/A6 or Th1/Th2 immune responses. Preliminary results suggest that conjugations of reduced mannan to cyclic

antagonist/agonist peptides are more potent than cyclic analogues alone. Further investigations are being done to measure cytokines and T cells after immunization of oxidized/reduced mannan conjugates to cyclic MBP analogues.

DETAILED DESCRIPTION OF WORK

Synthesis of guinea pig MBP cyclic peptides

Cyclo-(2, 9)MBP_{72-85}: Gln-Lys^2-Ser-Gln-Arg-Ser-Gln-Asp^{81}-Glu^9-Asn-Pro-Val-NH_2

Cyclo-(2, 9) $Ala^{81}MBP_{72-85}$: Gln-Lys^2-Ser-Gln-Arg-Ser-Gln-Ala^{81}-Glu^9-Asn-Pro-Val-NH_2

The synthesis of Fmoc-Glu(COOH)-Asn-Pro-Val-NH_2 was performed starting with H_2N-Linker(Rink)-2-chlorotrityl chloride resin. The Fmoc/tBu strategy and a single coupling protocol with N,N Diisopropylcarbodiimid/1-Hydroxybenzotriazole (DIC/HOBt) in dimethylformamide (DMF) was used through all syntheses. The amino acids were: Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-Asn-OH and Fmoc-Glu(tBu)-OH. The protected peptide on the resin was treated with the splitting mixture dichloromethane/acetic acid/2,2,2 trifluoroethanol (DCM/AcOH/TFE) (50 ml, 7:1:2) for 1 h at room temperature to remove the peptide from the resin. The mixture was filtered and the resin washed with the splitting mixture (X2) and DCM (X3). The solvent was removed on a rotary evaporator and the obtained oily product precipitated from dry diethyl ether as a white solid. The protected peptide-linker material was treated with 65% trifluroacetic acid (TFA)+3% ethanedithiol (EDT) in DCM for 4h to deprotect Glu from tBu and to liberate the amidated tetrapeptide fragment from linker. 2-Chlorotrityl chloride resin in dry DMF was stirred in a round bottom flask. Diisopropylethylamine (DIPEA) and Fmoc-Glu(COOH)-Asn-Pro-Val-NH_2 added and the solution was stirred for 45 min at room temperature. A mixture of methanol (MeOH) and DIPEA (8/2) was then added for endcaping and the resulting mixture was stirred for another 10 min at room temperature. The Fmoc-Glu(Resin)-Asn-Pro-Val-NH_2 was used for the synthesis of the linear precursor peptide following the protocol previously described [Tselios, et al 1999]. The amino acids used in Fmoc synthesis were: Fmoc-Ala-OH, FmocAsp(tBu)-OH, Fmoc-Gln-OH, Fmoc-Ser(tBu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Mtt)-OH and the last amino acid was Boc-Gln-OH. The completed peptide on resin was dried in vacuo and then treated with the splitting mixture DCM/1,1,1,3,3,3 hexafluoro-2-

propanol (8/2) for 6h at room temperature to remove the peptide from the resin and for the deprotection of Lys from Mtt. The mixture was filtered and the resin was washed with the splitting mixture (X2) and DCM (X3). The solvent was removed on a rotary evaporator and the obtained oily product precipitated from cold dry diethyl ether as a white solid. Cyclization of linear protected peptide was achieved using O-benzotriazol-1-yl-N,N,N′,N′-tetramethyluronium tetrafluoroborate (TBTU), 2,4,6 collidine, 1 hydroxy 7-azabenzotriazol and dry DMF as solvent. The solution of peptide, 2,4,6 collidine and 1 hydroxy 7-azabenzotriazol in dry DMF was added dropwise to a solution of TBTU in DMF for 2h and the solution was stirred for 3 h. The protected cyclic peptide was treated with 65% TFA in DCM + 3% EDT for 5 h at room temperature (Fig. 5). The crude peptide product was purified further by preparative HPLC.

Synthesis of human MBP cyclic peptides

Cyclo-(87, 99) [Arg^{91}, Ala^{96}] MBP$_{87-99}$ (Val^{87}-His-Phe-Phe-Arg^{91}-Asn-Ile-Val-Thr-Ala^{96}-Arg-Thr-Pro^{99})

Cyclo-(91, 99) [Ala^{96}] MBP$_{87-99}$ (Val^{87}-His-Phe-Phe-Lys^{91}-Asn-Ile-Val-Thr-Ala^{96}-Arg-Thr-Pro^{99})

For the synthesis of precyclic human MBP cyclic analogues, we resorted to the Fmoc/tBu methodology utilizing the 2 chlorotrityl chloride resin. The peptide synthesis was achieved using DIC / HOBt in DMF and the N^{α}-NH_2 of amino acids were protected with the Fmoc group. The side chain of amino acids were protected as following: Trt for His, Pbf for Arg, tBu for Ser, Thr, Asp, Glu, Boc for Lys, as regarding the cyclic analogue cyclo-(91, 99)[Ala^{96}] MBP$_{87-99}$ (by Nε-NH_2 of Lys and C-terminous) we used Mtt protected group because it easily removed with the mixture HFIP(1,1,1,3,3,3 hexafluoro-2-propanol)/DCM (8/2) in which the peptide cleavages from the resin. Otherwise, in the cyclo-(87, 99)[Arg^{91}, Ala^{96}]MBP$_{87-99}$ the side chain of Lys was protected with Boc group. The completed protected linear peptides on resin were dried in vacuo and then treated with the splitting mixture DCM/HFIP (8/2) for 7h at room temperature to remove the peptide from the resin and for the deprotection of Lys from Mtt in the cyclo-(91, 99)[Ala^{96}] MBP$_{87-99}$. Each one of the linear protected peptides was dissolved in DMF and was added collidine, HOAt. This mixture was added dropwise in a solution of TBTU in DMF for 8 hours. The cyclization was determined by TLC and analytical reversed phase HPLC. The solvent was removed

under reduced pressure affording a light yellow oily residue. The cyclic protected peptide (purity ≥90%) precipitated from H_2O and dried in vacuo for 16h. The cyclic protected peptide was treated with 65% TFA in DCM and 3% ethanodithiol as scavanger for 4 hours at room temperature. The resulting solution was concentrated to a small volume and the final free peptide was precipitated as a light yellow amorphous solid added diethylether (purity ≥80%). Peptide purity was assessed by analytical HPLC reruns, thin layer chromatography (TLC) and mass spectrometry (ESIMS) (Fig. 6).

Conjugation of peptide to mannan and/or KLH

1mg/ml mannan (0.1M phosphate buffer pH 6.0) is oxidized to a poly-aldehyde by treating with 100μl 0.1M sodium periodate, for 1 hour at 4°C. 10μl ethanedithiol is added for 30 minutes at 4°C to stop oxidation. The mixture is passed through a PD-10 column and the mannan fraction is collected. The PD-10 column pre-calibrated with 0.1M carbonate buffer pH 9.0. The void volume of the PD-10 column is 2.5ml, the oxidized mannan (1ml) is added, then is added 1.5ml 0.1M carbonate buffer pH 9.0. The following 2ml are collected. For the conjugation to mannan, 1mg of each peptide [Linked to KLH or Lys residues added (Lys-Gly-Lys-Gly-Lys-Gly-Lys-Gly-Lys-Gly)] is allowed to react with oxidized mannan overnight at room temperature. The antigen will be conjugated to mannan in the oxidized form (contains aldehydes and Schiff bases) (Scheme 2). For conjugation of antigen to mannan in the reduced form (reducing the aldehydes to alcohol's and Schiff baces to amines): Oxidized mannan antigen complex is reacted with 1 mg sodium borohydride for 3 hours at room temperature. The conjugation is used with no further purification (Fig. 7).

Induction-inhibition and assessment of EAE

Inbred Lewis rats bred and maintained in the animal facility of the Hellenic Pasteur Institute were used in all experiments. Female rats (220g) were immunised with linear MBP_{72-85} (30μg) (n=10, as positive control), or MBP_{72-85} (30μg) plus the cyclic analogue c-$Ala^{81}MBP_{72-85}$ (500μg) or [Arg^{91},Ala^{96}]MBP_{87-99} (n=5) in 200μl of an emulsion containing equal volumes of peptide diluted in sterile saline and Freund's complete adjuvant (Difco, USA) containing 4 mg/ml heat-killed *M. tuberculosis* (H37Ra) (Difco). Immunisation 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 following: 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. For histological analyses, mice were anaesthetised with ether, bled and perfused with PBS (pH 7.4) (PBS) followed by 4% paraformaldehyde in PBS. Spinal cord was dissected out and fixed overnight in 4 % paraformaldehyde in PBS at 4°C before been embedded in paraffin. Paraffin sections were stained with Luxol fast blue and Nuclear Fast Red for visualisation of demyelination and inflammation respectively.

WHAT IS CLAIMED

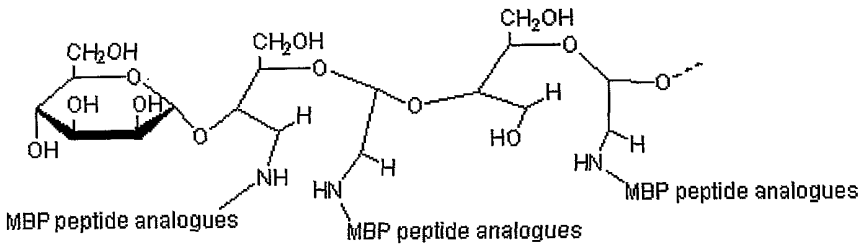
1. A conformational model of linear epitope sequence from guinea pig MBP_{72-85} and human MBP_{87-99}.

2. The cyclic agonist(cyclo-MBP_{72-85}) and cyclic antagonist(cyclo-$Ala^{81}MBP_{72-85}$) peptide derived from guinea pig MBP epitope 72-85 for the treatment of Multiple Sclerosis (MS).

3. The antagonist linear peptides $[X^{91}, Y^{96}]MBP_{87-99}$ derived from human MBP epitope 87-99 where X= Arg, Lys, Asn, Ala, Orn and Y= Ala, Gly, Val, Pro for the treatment of Multiple Sclerosis (MS).

4. The antagonist cyclic peptides cyclo(87-99) $[X^{91}, Y^{96}]MBP_{87-99}$, cyclo(91-96) $[X^{91}, Y^{96}]MBP_{87-99}$ derived from human MBP epitope 87-99 where X, Y as in claim 3 for the treatment of Multiple Sclerosis (MS).

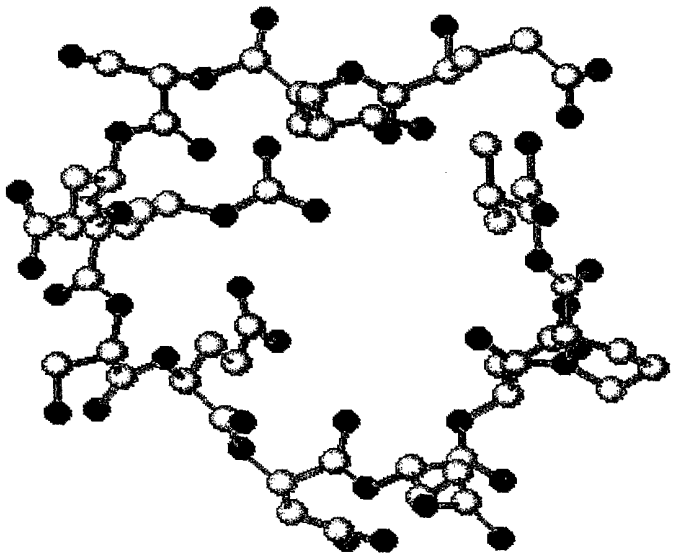
5. The Mannan/KLH conjugates with linear MBP_{72-85} agonist, linear/cyclic $Ala^{81}MBP_{72-85}$ antagonists and linear/cyclic $[X^{91}, Y^{96}]MBP_{87-99}$ antagonists in the oxidised and reduced form of mannan where X, Y as in claim 3, 4 for the treatment of Multiple Sclerosis (MS).



6. A method for the synthesis of guinea pig cyclic analogues as in claim 2.

7. A method for the synthesis of human cyclic analogues as in claim 4.

8. A method for the synthesis of peptides-KLH/Mannan conjugates as in claim 5.



MBP $_{72\text{-}85}$

Figure 1

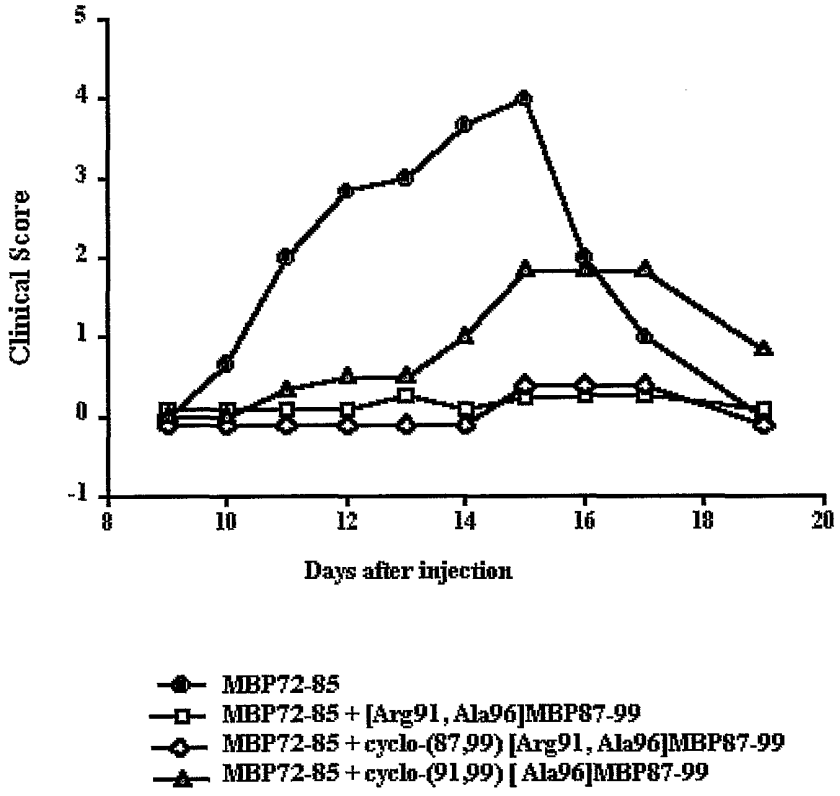
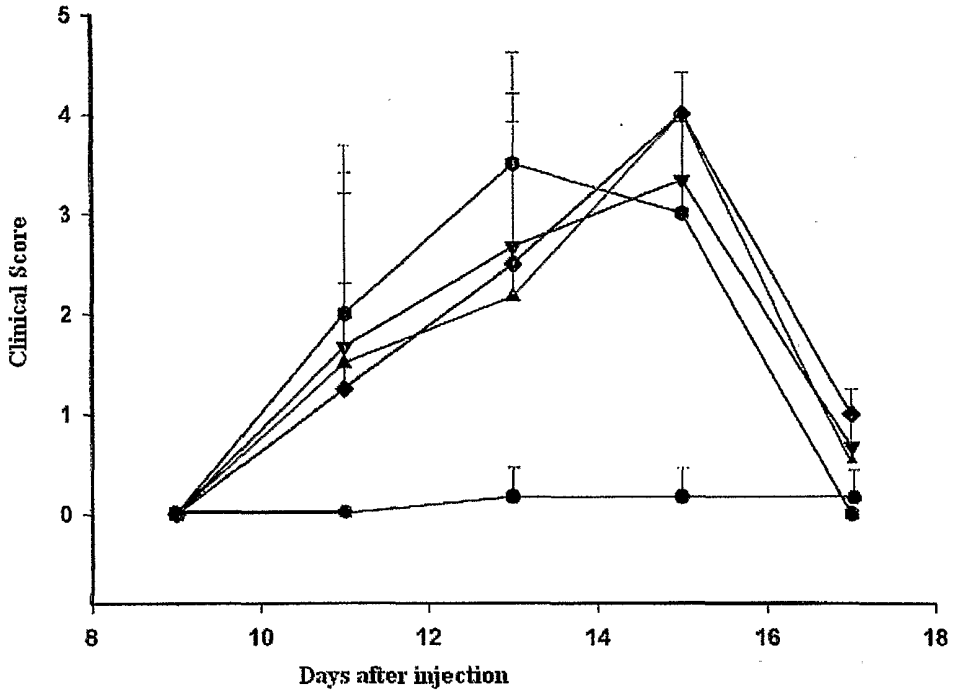

Clinical Score
5
4
3
2
1
0
-1
8
10
12
14
16
18
20
Days after injection
MBP72-85
MBP72-85 + [Arg91, Ala96]MBP87-99
MBP72-85 + cyclo-(87,99) [Arg91, Ala96]MBP87-99
MBP72-85 + cyclo-(91,99) [Ala96]MBP87-99

Figure 2


Clinical Score
5
4
3
2
1
0
8
10
12
14
16
18
Days after injection

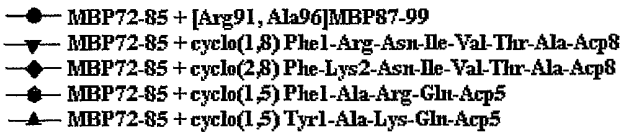

MBP72-85 + [Arg91, Ala96]MBP87-99
MBP72-85 + cyclo(1,8) Phe1-Arg-Asn-Ile-Val-Thr-Ala-Acp8
MBP72-85 + cyclo(2,8) Phe-Lys2-Asn-Ile-Val-Thr-Ala-Acp8
MBP72-85 + cyclo(1,5) Phe1-Ala-Arg-Glu-Acp5
MBP72-85 + cyclo(1,5) Tyr1-Ala-Lys-Gln-Acp5

Figure 3

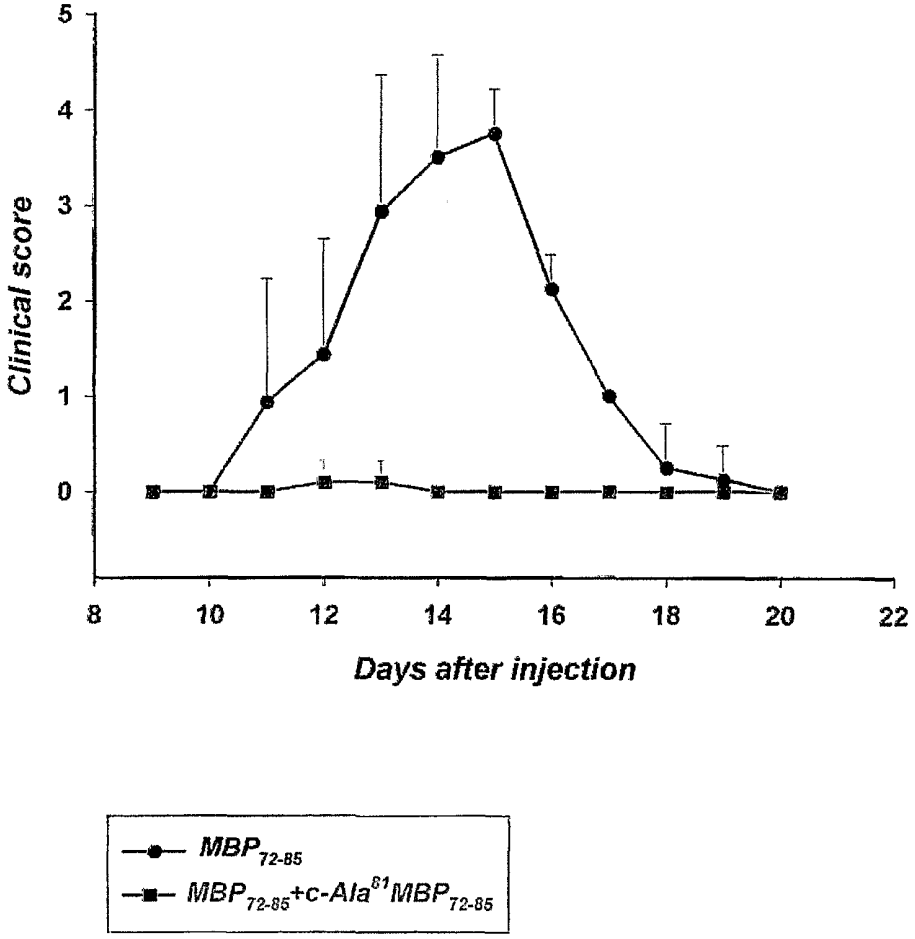
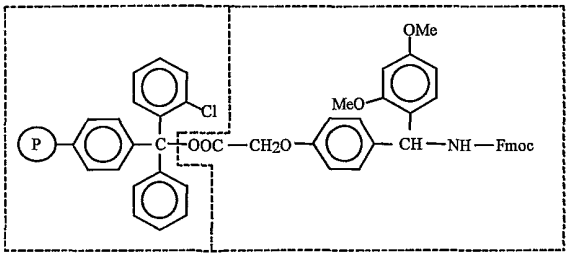

Clinical score
5
4
3
2
1
0
8
10
12
14
16
18
20
22
Days after injection
MBP72-85
MBP72-85+c-Ala81MBP72-85

Figure 4



1. Fmoc deprotection with 20% piperidine in DMF
2. Fmoc/tBu Synthesis DIC/HOBt,
   a. Fmoc-Val-OH, b. Fmoc-Pro-OH, c. Fmoc-Asn-OH, d. Fmoc-Glu(tBu)-OH
3. Cleavage from resin with DCM/AcOH/TFE (7/1/2)
4. Deprotection with TFA/DCM/1,2 Ethanedithiol (6.5/3.2/0.3)

Fmoc-Glu(COOH)-Asn-Pro-Val-NH_2

1. Esterification of tetrapeptide (from the γ-carboxy group of the Glu) on CLTR with DIPEA/DMF

2. Fmoc Synthesis DIC/HOBt
2a. Fmoc-**Ala**-OH
2b. Fmoc-Gln-OH
2c. Fmoc-Ser(tBu)-OH
2d. Fmoc-Arg(Pbf)-OH
2e. Fmoc-Gln-OH
2f. Fmoc-Ser(tBu)-OH
2g. Fmoc-Lys(Mtt)-OH
2h. Boc-Gln-OH

Boc-Gln-Lys(Mtt)-Ser(tBu)-Gln-Arg(Pbf)-Ser(tBu)-Gln-Ala-Glu(R)-Asn-Pro-Val-NH_2

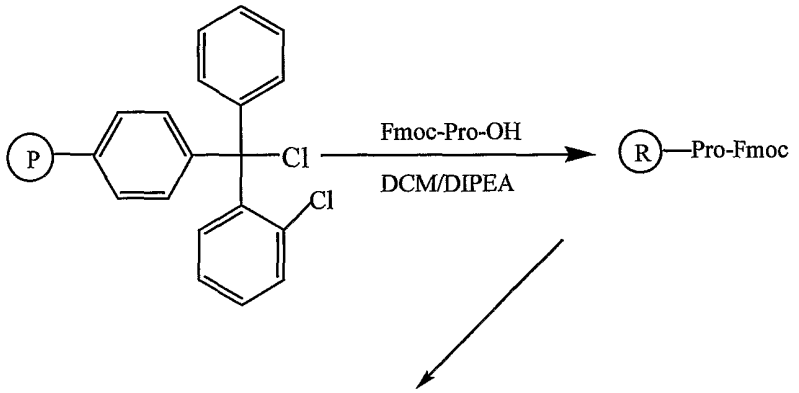
Cleavage from resin and Mtt deprotection with DCM/HFIP (8/2)

Boc-Gln-Lys(NH_2)-Ser(tBu)-Gln-Arg(Pbf)-Ser(tBu)-Gln-Ala-Glu(COOH)-Asn-Pro-Val-NH_2

1. Cyclization with TBTU(3x), HOAt(3x) and Collidine(6x) in dry DMF
2. Deprotection with TFA/DCM/1,2 Ethanedithiol (6.5/3.2/0.3)

H_2N-Gln-Lys-Ser-Gln-Arg-Ser-Gln-Ala-Glu-Asn-Pro-Val-NH_2

Figure 5



A) Fmoc Deprotection with 20% piperidine in DMF
B) Fmoc Synthesis using DIC/HOBt
1. Fmoc-Thr(tBu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH, Fmoc-Thr(tBu)-OH, Fmoc-Val-OH, Fmoc-Ile-OH, Fmoc-Asn-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Phe-OH, Fmoc-Phe-OH, Fmoc-His(Trt)-OH, Fmoc-Val-OH
2. Fmoc-Thr(tBu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH, Fmoc-Thr(tBu)-OH, Fmoc-Val-OH, Fmoc-Ile-OH, Fmoc-Asn-OH, Fmoc-Lys(Mtt)-OH, Fmoc-Phe-OH, Fmoc-Phe-OH, Fmoc-His(Trt)-OH, Boc-Val-OH
C) Cleavage from resin for analogue 1, cleavage from resin and Mtt deprotection for analogue 2 with HFIP/DCM (2/8)

1. H_2N-Val-His(Trt)-Phe-Phe-Arg(Pbf)-Asn-Ile-Val-Thr(tBu)-Ala-Arg(Pbf)-Thr(tBu)-Pro-OH
2. Boc-Val-His(Trt)-Phe-Phe-Lys(NH_2)-Asn-Ile-Val-Thr(tBu)-Ala-Arg(Pbf)-Thr(tBu)-Pro-OH

D) Cyclization with TBTU(3x), HOAt(3x) and Collidine(6x) in dry DMF
E) Deprotection with TFA/DCM/1,2 Ethanedithiol (6.5/3.2/0.3)

1. Val-His-Phe-Phe-Arg-Asn-Ile-Val-Thr-Ala-Arg-Thr-Pro

2. H_2N-Val-His-Phe-Phe-Lys-Asn-Ile-Val-Thr-Ala-Arg-Thr-Pro

1. cyclo-(87,99)[Arg^{91}, Ala^{96}] MBP_{87-99}
2. cyclo-(91,99)[Ala^{96}] MBP_{87-99}

Figure 6

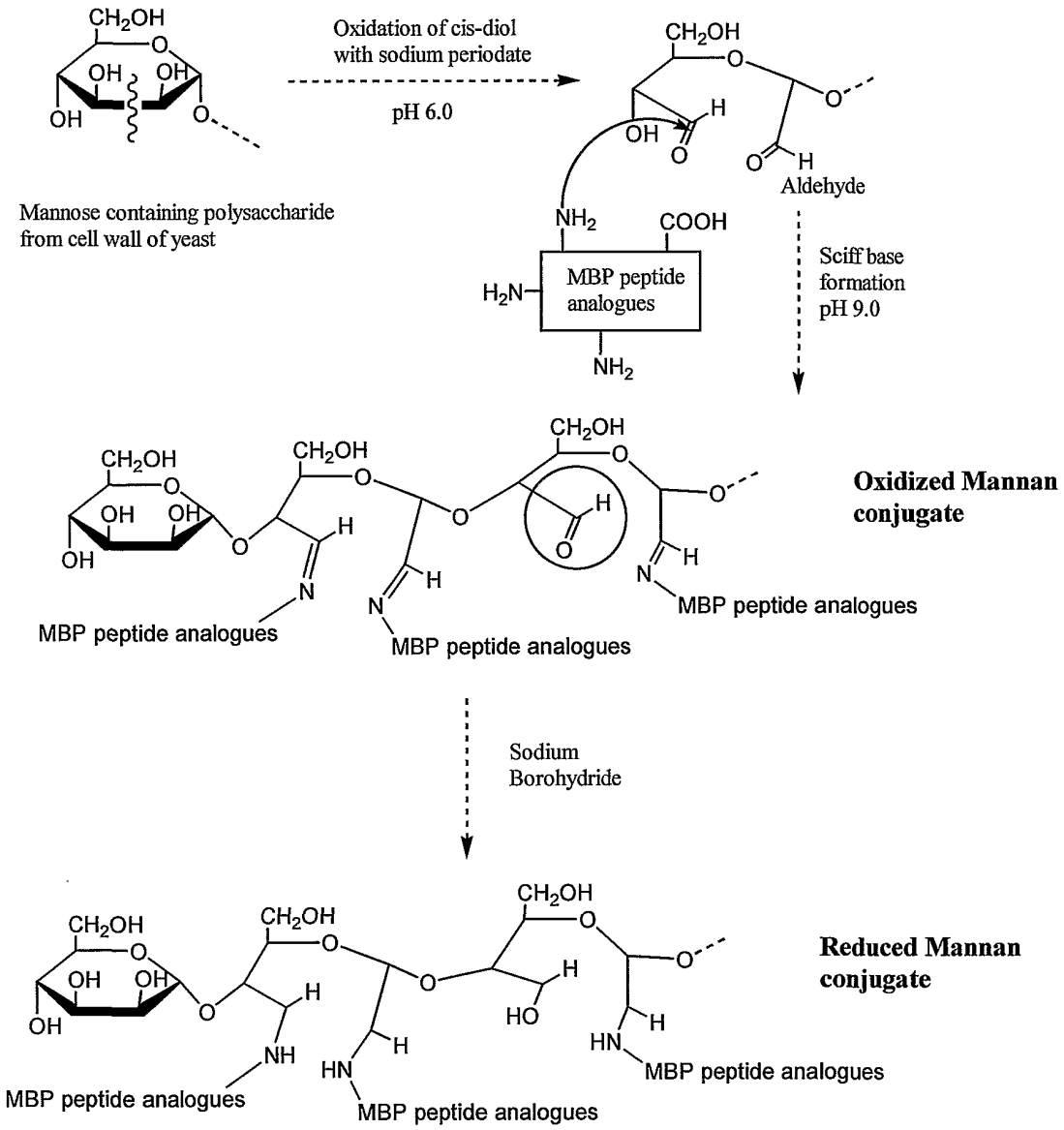
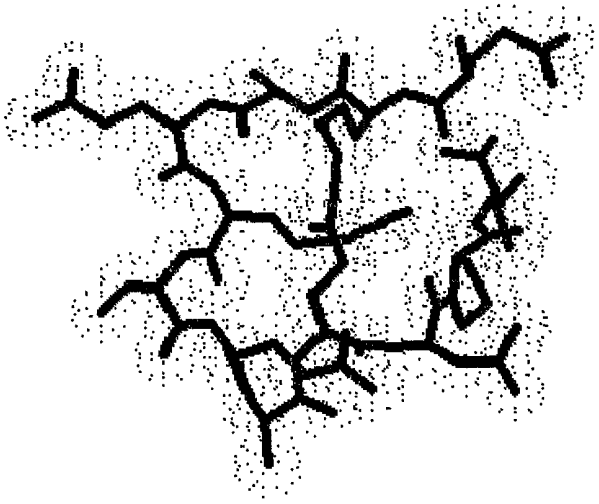

Oxidation of cis-diol
with sodium periodate
pH 6.0
Mannose containing polysaccharide
from cell wall of yeast
Aldehyde
MBP peptide
analogues
Sciff base
formation
pH 9.0
Oxidized Mannan
conjugate
MBP peptide analogues
MBP peptide analogues
MBP peptide analogues
Sodium
Borohydride
Reduced Mannan
conjugate
MBP peptide analogues
MBP peptide analogues
MBP peptide analogues

Figure 7



Cyclo(2, 9) $Ala^{81}MBP_{72-85}$: $Gln\text{-}Lys^{2}\text{-}Ser\text{-}Gln\text{-}Arg\text{-}Ser\text{-}Gln\text{-}Ala^{81}\text{-}Glu^{9}\text{-}Asn\text{-}Pro\text{-}Val\text{-}NH_2$

Figure 8

INTERNATIONAL SEARCH REPORT

In onal Application No
PCT/GR 01/00014

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, SEQUENCE SEARCH, EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TSELIOS T ET AL.: "Treatment of Experimental Allergic Encephalomyelitis (EAE) Induced by Guinea Pig Myelin Basic Protein Epitope 72-85 with a Human MBP87-99 Analogue and Effects of Cyclic Peptides" BIOORGANIC & MEDICAL CHEMISTRY, vol. 8, August 2000 (2000-08), pages 1903-1909, XP001041415	1,3
Y	the entire document, particularly page 1903; schemes 1 and 2; figures 1-3; page 1909, column 2, paragraph 2	4,5
	--- -/--	

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 December 2001	20/12/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Schmidt, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	TSELIOS T ET AL.: "Design and Synthesis of a Potent Cyclic Analogue of the Myelin Basic Protein Epitope MBP72-85: Importance of the Ala81 Carboxyl Group and of a cyclic conformation for Induction of Experimental Allergic Encephalomyelitis" JOURNAL OF MEDICAL CHEMISTRY, vol. 42, 16 March 1999 (1999-03-16), pages 1170-1177, XP002183552 cited in the application	1,2
Y	abstract; page 1170, right-hand column, last paragraph; page 1175, right-hand column; figures 1-3; table 1	4,5
Y	APOSTOLOPOULOS V ET AL.: "Cell-mediated immune responses to MUC1 fusion protein coupled to mannan" VACCINE, vol. 14, no. 9, 1996, pages 930-938, XP004069583 cited in the application page 931: "Conjugation of mannan to MUC1 fusion protein"; page 932: "Induction of DTH"; figure 6	5
X	WO 95 08572 A (THE BOARD OF TRUSTEES FOR THE LELAND STANFORD JUNIOR UNIVERSITY) 30 March 1995 (1995-03-30) claims 1,3,6	3
X	BROCKE S ET AL.: "Treatment of experimental encephalomyelitis with a peptide analogue of myelin basic protein" NATURE, vol. 379, 1996, pages 343-346, XP000601452 cited in the application page 343, column 2, paragraph 3; table 1	3

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 2,6 (completely) and 1,5,8 (partially)

   cyclo-MBP 72-85 and cyclo-'Ala-81!MBP 72-85, their conjugates and methods for their preparation

2. Claims: 3 (completely), 1,5,8 (partially)

   conjugated linear MBP 72-85 as well as linear peptides 'Ala-81!MBP 72-85 and 'X-91,Y-96!MBP 87-99, their conjugates and methods for their preparation

3. Claims: 4,7 (completely) and 5,8 (partially)

   cyclic peptides cyclo'X-91,Y-96!MBP 87-99, their conjugates and methods for their preparation

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 6-8

Present claims 6-8 relate to a method for the synthesis of MBP analogues as defined in claims 2,4 and 5.
Subject-matter of claims 6-8 lacks any technical feature within the meaning of Articles 5 and 6 PCT to such an extend as to render a meaningful search of said claims impossible.
Consequently, the search has been restricted to the products as disclosed in claims 1-5.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

formation on patent family members

Ir onal Application No
I ., GR 01/00014

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9508572 A	30-03-1995	AU 695801 B2	20-08-1998
		AU 7840694 A	10-04-1995
		CA 2172512 A1	30-03-1995
		EP 0720622 A1	10-07-1996
		JP 9502981 T	25-03-1997
		WO 9508572 A1	30-03-1995