Bioorganic & Medicinal Chemistry 21 (2013) 6718–6725

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Divergent and convergent synthesis of polymannosylated dibranched antigenic peptide of the immunodominant epitope MBP(83–99)

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ARTICLE INFO

Article history:
Received 8 May 2013
Revised 17 July 2013
Accepted 5 August 2013
Available online 13 August 2013

Keywords:
Di-branched antigenic peptide
Experimental autoimmune encephalomyelitis
Divergent synthesis
Convergent synthesis
Microwave-assisted synthesis
Mannosylation

ABSTRACT

Multiple antigenic peptide (MAP) systems are dendrimeric structures bearing multiple copies of identical or different peptide epitopes, and they have been demonstrated to show enhanced immunogenicity. Herein, we report the direct (divergent) and indirect (convergent) synthesis, using contemporary synthetic approaches, of a di-branched antigenic peptide (di-BAP) containing the immunodominant epitope MBP(83–99), which is implicated in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS). The direct synthesis (di-BAP 1) was performed using microwave irradiation. The indirect synthesis (di-BAP 2) was carried out performing an efficient chemoselective coupling reaction through the formation of a thioether bond. Both di-BAPs were conjugated to polysaccharide mannan since mannosylation is a promising technique to achieve modulation in immune response. The conjugation was achieved through free amino groups of both di-BAPs via the formation of Schiff bases. The mannan-conjugated di-BAPs were further evaluated in vivo in a prophylactic vaccination protocol, prior to EAE induction in Lewis rats.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease of the central nervous system (CNS), believed to be mediated by an autoimmune T cell response directed to proteins of the myelin sheath, such as myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG). Experimental autoimmune encephalomyelitis (EAE) is the best studied animal model for MS. EAE is mediated by CD4+ T lymphocytes and shares many of the features seen in MS.[1,2] EAE is induced by immunization with myelin antigens emulsified in complete Freund's adjuvant (CFA). Depending upon the immunization protocol and background of mice used, EAE can take an acute, chronic progressive or relapsing–remitting course. EAE can be induced in Lewis rats by immunization with encephalitogenic guinea pig or rat MBP epitopes.[3] More specifically, previous studies have shown that the MBP(83–99) epitope is bound to the DR2 molecule expressed on inflammatory cells.[4] EAE induction with MBP peptides tends to induce EAE with considerable inflammation in the CNS, but with little or no demyelination which is a hallmark of human MS.[5,6]

A variety of therapeutic approaches have been developed in order to modulate immune responses, such as the use of altered peptide ligands (APLs) of immunodominant myelin epitopes in which the primary TCR contact residues are substituted. APLs may suppress the EAE symptoms triggering different immunological responses.[7,8] Another approach in the induction of tolerance is the administration of myelin or immunodominant myelin epitopes,[9] such as Dirucotide. Dirucotide is a synthetic peptide with a sequence corresponding to hMBP(82–98), which is the immunodominant epitope in MS patients with HLA haplotype DR2 and/or DR4. However, its further clinical use was terminated because it did not meet the primary endpoint of delaying disease progression

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http://dx.doi.org/10.1016/j.bmc.2013.08.008

during the two-year MAESTRO-01 Phase III trial in patients with secondary progressive multiple sclerosis (SPMS).[10]

It is evident that there is a compelling need for the development of improved protocols for the treatment of MS. One current experimental approach is to control this autoimmune disease through immunization promoting the induction of T regulatory cells (Tregs) or changing the immune response of patients from Th1 (IFN-γ and TNF-α) to Th2 (IL-4, IL-5, IL-10 and TGF-β) via the secretion of anti-inflammatory cytokines. Mannan derived from *Saccharomyces cerevisae* is a polysaccharide (polymannose) which possesses a backbone of α (1→6)-linked D-mannose units substituted by side chains containing α (1→2)- and α (1→3)-linked D-mannose units (Fig. 1).[11] Mannan has successfully been used as a carrier to target T cell antigens to the mannose receptor (MR) of macrophages and dendritic cells (DCs) leading to the induction of Th1 or Th2 immune responses in cancer immunotherapy protocols.[12–15]

The low immune responses of peptides could be overcome using a carrier protein such as bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), tetanus toxoid (TT) and others,[16] an approach which can present some disadvantages, such as the production of irrelevant anti-protein carrier antibodies due to the immunodominance of the carrier, and the insufficient stability and sometimes altered antigenic properties of the peptide moiety because of the conjugation process.[17] A successful approach in the increase of the immunogenicity of single peptides has been the use of dendrimeric systems,[18–20] such as multiple antigenic peptide (MAP) systems. MAPs were developed by Tam[21] and contain an inner core matrix consisting of sequential lysine layers (known as generations) or other bifunctional units, to which multiple copies of identical or different[22] peptide epitopes are attached. Based on literature data, MAPs exhibit several advantages in comparison to the corresponding peptides or peptide polymers, due to their high molar ratio of antigen to core molecule and their three-dimensional structure: (i) they do not require further conjugation to a carrier protein,[21] (ii) they are more stable to proteolytic degradation,[23] (iii) they present enhanced immunogenicity,[21,24–26] and (iv) they may produce site-specific antibodies.[27]

MAPs can be synthesized using a direct (divergent) or an indirect (convergent) approach. In the direct approach, multiple copies of peptide epitopes can be synthesized by standard stepwise solid-phase peptide synthesis (SPPS) directly on the resin-bound core using Boc chemistry,[21] Fmoc chemistry,[28] or a combination thereof.[24] The introduction of microwave irradiation in peptide synthesis can be useful even in the case of hindered MAPs synthesis, since it has been successfully applied as a fast and efficient way to synthesize difficult and large peptide sequences.[29–31]

The indirect approach includes the separate synthesis of the core and the peptide epitopes, which are coupled using a chemoselective reaction.[23,28,32–34] The advantages of this approach are: (i) the possibility of using partially protected or fully unprotected fragments which present better solubility, even in aqueous solution, and (ii) the fragments can be purified in a step before the coupling reaction, thus decreasing the possibilities of any by-products formation.

In the present study, we report both the microwave-assisted direct synthesis and, through a chemoselective reaction, the indirect synthesis of a di-BAP, containing MBP(83–99), one of the main immunodominant epitopes implicated in EAE and MS. The synthesized di-BAPs were further conjugated to polysaccharide mannan since mannosylation has been considered a promising technique to achieve modulation of the immune response.[35,36]

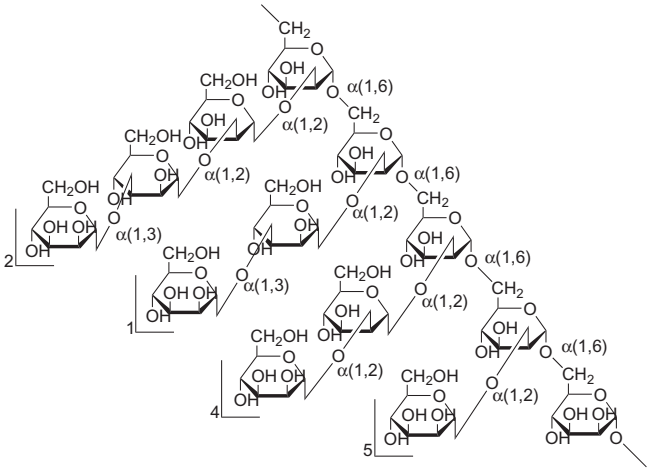


Figure 1. Structure of mannan from *Saccharomyces cerevisae*.[11]

2. Materials and methods

2.1. Materials

2-Chlorotrityl chloride polystyrene resin (1% DVB, 200–400 mesh) was purchased from CBL-Patras (Patras, Greece). Wang resin was purchased from Novabiochem (Bad Soden, Germany) Fmoc-protected-amino acids were purchased from CBL-Patras (Patras, Greece) either from Novabiochem (Bad Soden, Germany). Solvents and other reagents were purchased from Merck (Darmstadt, Germany), Sigma–Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland) and Panreac (Barcelona, Spain).

Mini-cleavages were performed on a Discover™ S Class single-mode microwave reactor equipped with Explorer-48 autosampler (CEM Corporation, Matthews, NC) using a frequency of 2450 MHz, while peptide synthesis was performed on a Liberty™ Microwave Peptide Synthesizer (CEM Corporation, Matthews, NC).

The determination of resin loading capacity was achieved spectrophotometrically using a UV/Visible Spectrophotometer Cary 4000.

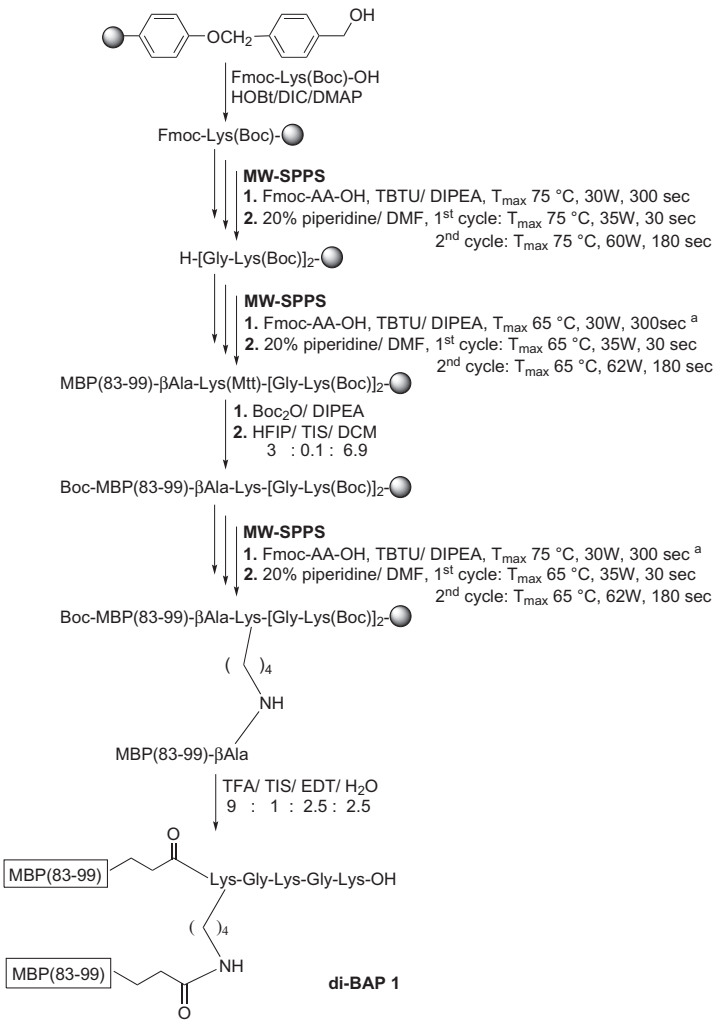
Mannan (polymannose from *Saccharomyces cerevisiae*) was purchased from Sigma–Aldrich Ltd (Steinheim, Germany) and the PD-10 columns (Sephadex G-25 M) used for the purification of mannan were purchased from Amersham Biosciences (Uppsala, Sweden).

The Complete Freund's Adjuvant (CFA) was purchased from Sigma–Aldrich Ltd (Steinheim, Germany) and it was supplemented with H37Ra *Mycobacterium tuberculosis* by Difco (Detroit, MI).

2.2. Methods

2.2.1. Microwave-assisted solid-phase peptide synthesis (MW-SPPS) of di-BAP 1

Di-BAP **1** was prepared in solid phase on a Wang resin (1 mmol/g) under microwave irradiation following the Fmoc/*t*Bu strategy (Scheme 1). The resin was manually functionalized with the first N^{α}-Fmoc (9-fluorenylmethyloxycarbonyl)-protected amino acid, Fmoc-Lys(Boc)-OH. Wang resin (830 mg) was pre-swollen in 8 mL of DCM for 30 min. A mixture of Fmoc-Lys(Boc)-OH (319 mg, 0.68 mmol), HOBt (126 mg, 0.82 mmol), and DIC (105 μL, 0.68 mmol) was dissolved in minimum volume of DMF (approximately 1.5 mL) and then added to Wang resin. Subsequently, DMAP (8.3 mg, 0.068 mmol) was dissolved in minimum volume of DMF, it was added to the resin, and the resin was stirred for 3 h at rt.[37] The resin was successively washed with DMF, DCM, and MeOH (5 times each), and dried overnight under vacuum. The loading of the resin (0.12 mmol/g resin) was determined spectro-



Scheme 1. Microwave-Assisted Solid Phase Peptide Synthesis (MW-SPPS) of di-BAP **1**. Arg and His residues were coupled using different conditions as reported in the paper.

photometrically (301 nm, ε: 7800 M^{-1} cm^{-1}) from the amount of the adduct dibenzofulvene-piperidine formed after treatment of a small amount of Fmoc-Lys(Boc)-resin with piperidine/DCM/MeOH. The remaining active sites of the resin were acetylated by stirring with a mixture of Ac_2O/pyridine/DCM (2:3:5 v/v/v) at rt for 30 min, and subsequently it was thoroughly washed with DCM, DMF, and MeOH (5 times each).

The resin-bound polylysine core was synthesized using microwave irradiation on Fmoc-Lys(Boc)-Wang resin at 0.1 mmol scale. Branching was achieved by the incorporation of the Fmoc-Lys(Mtt)-OH derivative. After Fmoc deprotection, followed the addition of a β-Ala spacer and then the first epitope MBP(83–99) was synthesized.

The Fmoc protecting group was removed by treatment with a solution of 20% piperidine in DMF. All coupling reactions of the appropriate amino acid residue (0.2 M in DMF, 5 equiv) were performed with TBTU (0.5 M in DMF, 5 equiv) and DIPEA (2 M in NMP, 10 equiv). Since Mtt group can be partially removed at high temperatures, after the introduction of Fmoc-Lys(Mtt)-OH in the sequence, the coupling and deprotection steps were carried out at 65 °C instead of 75 °C. Each Fmoc-deprotection microwave cycle was characterized by two steps; the first one was for 30 sec at 65 °C and 35 W, and the second one for 180 sec at 65 °C and 62 W. All coupling reactions were for 300 s at 65 °C and 30 W. In order to avoid racemization of His and formation of δ-lactam of Arg, during coupling, particular coupling cycles were used. The coupling cycle of His consisted of two steps; the first one was for 120 s at 25 °C and 0 W, and the second one for 240 s at 50 °C and 30 W.[38] Arg residues were coupled twice. The first coupling was a two-step cycle. The first step of the first cycle was for 1500 s at 25 °C and 0 W, while the second step was for 300 s at 65 °C and 25 W. The second coupling cycle was for 300 s at 65 °C and 30 W.[39]

When the synthesis of the first MBP(83–99) epitope was accomplished, the N^{α}-terminus was protected with Boc_2O (11 equiv) and DIPEA (11 equiv) at rt for 2 h. Subsequently, the Mtt orthogonally-protected lysine residue was deprotected using HFIP/TIS/DCM solution (3:0.1:6.9 v/v/v) at rt for 8 h and, after the addition of a β-Ala spacer, the second epitope MBP(83–99) was synthesized stepwise on the core using microwave irradiation at 75 °C as mentioned above, apart from His (50 °C). In order to monitor the synthesis, microwave-assisted mini-cleavages were performed after coupling of the Fmoc-β-Ala and Fmoc-Pro residues on the N^{ε}-group of Lys and the intermediate products were characterized by UPLC-MS. For the microwave-assisted mini-cleavage, the Discover™ S Class single-mode microwave reactor (CEM) was used, and the microwave cycle was performed for 15 min at 45 °C, 15 W, with cooling, using a TFA/TIS/EDT/H_2O solution (94:1:2.5:2.5 v/v/v/v).

Di-BAP cleavage from the resin and removal of the amino acids side chain protecting groups were carried out with the solution used in mini-cleavages for 5 h at rt. The solvent was partially

evaporated, and the crude di-BAP **1** was precipitated with cold diethyl ether and collected after filtration (0.24 g).

The crude di-BAP **1** was subjected to a UPLC analysis (Waters ACQUITY Ultra Performance LC system equipped with UV detector) using an Acquity C18 BEH column (1.7 μm, 2.1 × 100 mm). Separation was achieved by gradient elution of 20% to 90% solvent B (solvent A = 0.1% TFA in H_2O; solvent B = 0.1% TFA in ACN) over 3 min at a flow rate of 0.45 mL/min and a column temperature at 40 °C (conditions I) (see Supplementary, Fig. S1 A).

The purification of di-BAP **1** was carried out on a semi-preparative RP-HPLC (Waters 600 solvent delivery system, combined with a UV 2487 Dual Wavelength Absorbance Detector) using a Jupiter Phenomenex C18 column (10 μm, 250 × 10 mm) at 230 and 254 nm. Separation was achieved by gradient elution of 20% to 70% solvent B (solvent A = 0.1% TFA in H_2O; solvent B = 0.1% TFA in ACN) over 40 min at a flow rate of 4 mL/min (conditions II).

After lyophilization, the pure di-BAP **1** was obtained in 20% yield (480 mg) and analyzed by analytical RP-HPLC (Waters system equipped with a 600E system controller, an operating system Millennium 2.1 and a Waters 996 photodiode array detector) using a C-18 Nucleosil column (5 μm, 250 × 5 mm) at 214 and 254 nm. Separation was achieved by gradient elution of 5% to 100% solvent B (solvent A = 0.1% TFA in H_2O; solvent B = 0.1% TFA in ACN) over 30 min at a flow rate of 1 mL/min (conditions III) (see Supplementary Information, Fig. S1 B).

The final di-BAP **1** was identified by Micromass® Q-Tof MICRO™ mass spectrometer (Waters) equipped with an ESI source (see Supplementary Information, Fig. S1 C).

2.2.2. Synthesis of di-BAP 2 via thioether chemoselective ligation

2.2.2.1. Synthesis of bromoacetylated polylysine core 3. The protected polylysine core H-Lys-[Gly-Lys(Boc)]2-CLTR **3a** (Scheme 2) was prepared on 862 mg of a manually functionalized 0.58 mmol/g Fmoc-Lys(Boc)-CLTR[40,41] under microwave irradiation at 0.5 mmol scale, following the MW-SPPS protocol for 2-chlorotrityl resin (CLTR) we previously reported.[29] Branching was achieved by the incorporation of the Fmoc-Lys(Fmoc)-OH derivative.

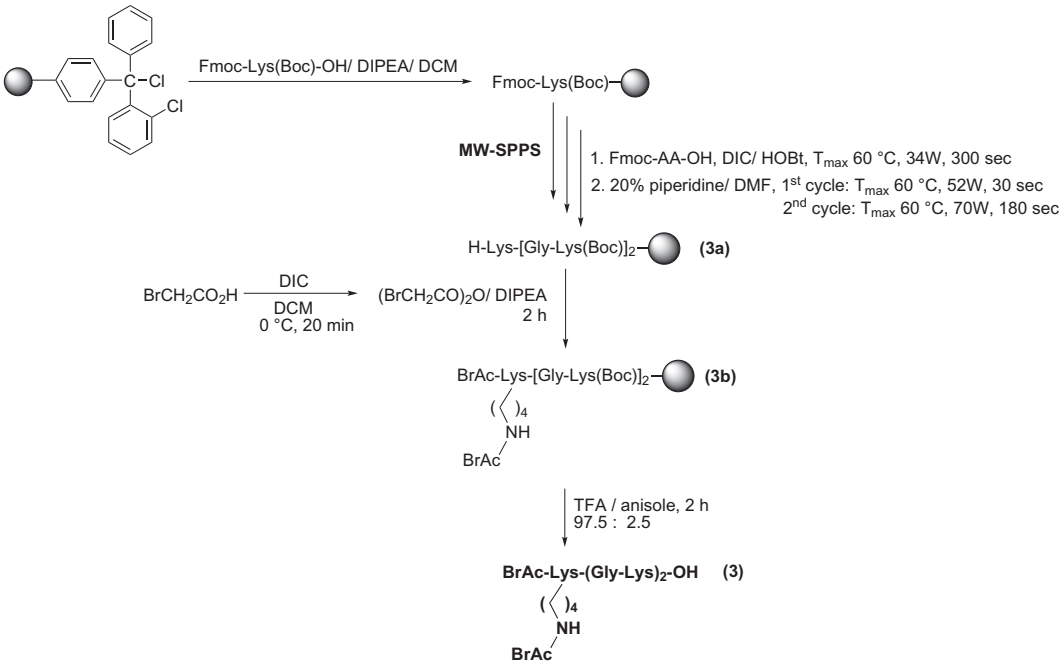
After N^{α},N^{ε}-di-Fmoc deprotection of the N-terminal Lys residue, bromoacetic acid was coupled to the protected polylysine core on the solid support using symmetric anhydride chemistry. Initially the core-resin was swollen in DMF for 30 min. Preparation of the bromoacetic anhydride was carried out by dissolving bromoacetic acid (278 mg, 2 mmol) in 5 mL dry DCM. The solution was cooled to 0 °C, and subsequently DIC (156 μL, 1 mmol) was added. The mixture was stirred for 20 min. The precipitated diisopropylurea (DIU) was removed by filtration.[42] The bromoacetic anhydride was added to the resin and after 15 min DIPEA (340 μL, 2 mmol) was also added to the reaction mixture and it was stirred for 2 h at rt. The $(BrCH_2CO)_2$-Lys-[Gly-Lys(Boc)]2-CLTR **3b** was subsequently filtered and washed with DCM (1 × 5 mL), DMF (3 × 5 mL), *i*-PrOH (2 × 5 mL) and *n*-hexane (1 × 5 mL), and dried under vacuum. The completion of the reaction was verified by Kaiser test.

The bromoacetylated polylysine core **3** was then cleaved by treating **3b** with a TFA/anisole solution (97.5:2.5 v/v) for 2 h at rt. The resin was filtered, the solvent was partially evaporated, and the crude peptide **3** was precipitated from cold diethyl ether and collected by filtration (0.27 g).

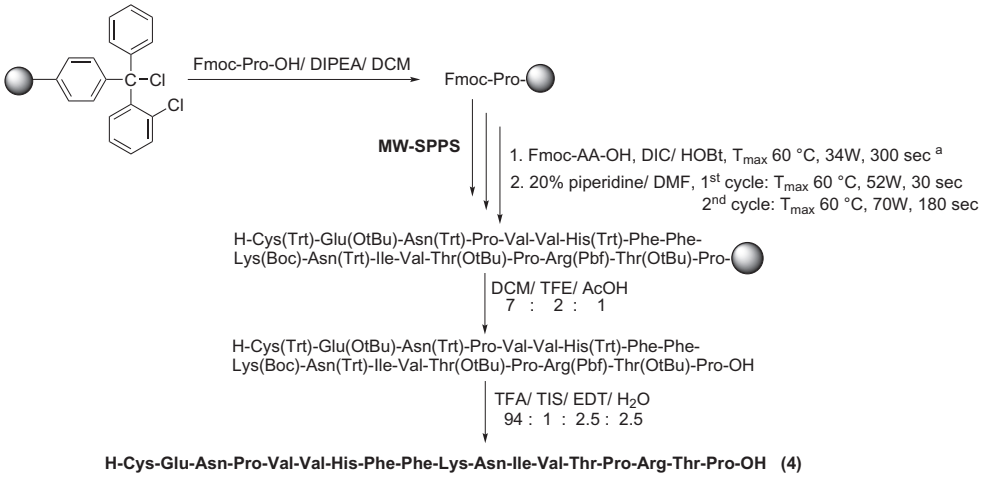
The purification of the crude peptide **3** was carried out on a semi-preparative RP-HPLC (Waters 600 solvent delivery system, combined with a Waters 996 photodiode array detector) using a Nucleosil C-18 column (7 μm, 250 × 10 mm) at 230 nm and 254 nm. Separation was achieved by gradient elution of 5% to 50% solvent B (solvent A = 0.1% TFA in H_2O; solvent B = 0.1% TFA in ACN) over 45 min at a flow rate of 3 mL/min (conditions IV).

The lyophilized pure peptide **3** was obtained in 60% yield (0.162 g) and analyzed by analytical RP-HPLC (conditions III). The final peptide **3** was identified by ESI-MS (Waters Micromass ZQ 4000 mass detector) (see Supplementary Information, Fig. S2 A).

2.2.2.2. Synthesis of the peptide epitope Cys-MBP(83–99) 4. Cys-MBP(83–99) **4** (Scheme 3) was prepared on 794 mg



Scheme 2. Synthesis of the bromoacetylated polylysine core **3**.



Scheme 3. Microwave-assisted solid phase peptide synthesis (MW-SPPS) of peptide **4**. Arg and His residues were coupled using different conditions as reported in the paper.

of a manually functionalized Fmoc-Pro-CLTR (0.63 mmol/g)[40,41] under microwave irradiation[29] on a 0.5 mmol scale.

The synthesized protected peptide on the resin was cleaved with the cleavage solution DCM/TFE/AcOH (7:2:1) for 1 h at rt, followed by deprotection using TFA/TIS/EDT/H_2O (94:1:2.5:2.5 v/v/v/v) for 5 h at rt. The solvent was partially evaporated and the crude product **4** was precipitated with cold diethyl ether and collected after filtration (0.81 g).

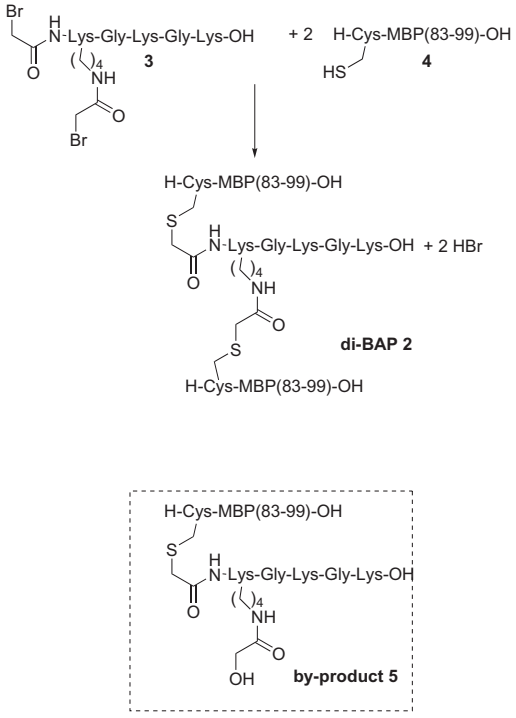
The purification was carried out on a semi-preparative RP-HPLC (Waters 600 solvent delivery system, combined with a Waters 996 photodiode array detector) using a Nucleosil C-18 column (7 μm, 250 × 10 mm) at 230 nm and 254 nm. Separation was achieved by gradient elution of 10 to 60% solvent B (solvent A = 0.1% TFA in H_2O; solvent B = 0.1% TFA in ACN) over 45 min at a flow rate of 3 mL/min (conditions V).

After lyophilization of the pure peptide **4**, a yield of 67.3% (0.545 g) was obtained. The final peptide **4** was further analyzed by analytical RP-HPLC (conditions III) and identified by ESI-MS (Waters Micromass ZQ 4000 mass detector) (see Supplementary Information, Fig. S2 B).

2.2.2.3. Ligation of peptides 3 and 4. For the thioether ligation reaction (Scheme 4), $(BrCH_2CO)_2$-Lys-(Gly-Lys)$_2$-OH **3** (0.8 mg, 1 μmol) and H-Cys-MBP(83–99)-OH **4** (4.2 mg, 2 μmol) were dissolved in 2 mL of a degassed solution of ACN/0.1 M borate buffer (pH 8.72) (1:3 v/v) and the solution was held under N_2 for 2 h at rt.[22] The completeness of the reaction was verified by analytical RP-HPLC (conditions III) (see Supplementary Information, Fig. S2 C). The solvent was partially evaporated and subjected to semi-preparative RP-HPLC purification (conditions V) to yield, after lyophilization, the final di-BAP **2** in 50% yield (2.4 mg, 0.5 μmol). The final di-BAP **2** was identified by a Micromass® Q-Tof MICRO™ mass spectrometer (Waters) equipped with an ESI source (see Supplementary Information, Fig. S3 A–B).

2.2.3. Conjugation of di-BAPs to mannan

Briefly, mannan (14 mg) was dissolved in 0.1 M sodium phosphate buffer (1 mL, pH 6.0), followed by the addition of 0.1 M sodium metaperiodate (100 μL) in phosphate buffer (pH 6.0) and incubated for 1 h at 4 °C. Ethylene glycol (10 μL) was added to the mixture and incubated for 30 min at 4 °C. The mixture (oxidized mannan) was passed through a PD-10 column (Sephadex G-25 M column, Amersham Biosciences, Sweden) pre-equilibrated with a 0.1 M bicarbonate buffer (pH 9.0) followed by addition of bicarbonate buffer (1.5 mL, pH 9.0). Oxidized mannan was isolated by collecting the following 2 mL of eluate, to which 1 mg of di-BAP



Scheme 4. Chemoselective ligation of **3** and **4** to give di-BAP **2**.

1 or **2** was added and allowed to react overnight at rt in the dark. Reduced mannan-di-BAP **1** or reduced mannan-di-BAP **2** complexes were prepared by treating the oxidized ones with sodium borohydride (1 mg/mL oxidized conjugate) for 3 h at rt.[12,43–45]

In the current work, the conjugation of the di-BAPs to mannan was verified by analytical RP-HPLC (conditions III). In particular, firstly a sample of di-BAP **1** or **2** which contained the same concentration of the di-BAP in the final conjugate (0.5 mg/mL) was subjected to RP-HPLC analysis, and then, at the same conditions, was injected the related conjugate. The absence of the peak corresponding to the di-BAP alone, without mannan, revealed the complete conjugation of the di-BAPs to mannan (data not shown).

2.3. EAE induction and evaluation

Experiments were performed in female Lewis rats of three months of age that were bred and maintained under specific

pathogen-free conditions in the Experimental Animal Facility of the Hellenic Pasteur Institute. di-BAP **1** conjugated with oxidized mannan (equivalent to 700 μg mannan and 30 μg di-BAP **1** per injection) or di-BAP **2** conjugated with reduced mannan (equivalent to 700 μg mannan and 30 μg di-BAP **2** per injection) or oxidized mannan (equivalent to 700 μg mannan and 30 μg di-BAP **2** per injection) or as controls, unconjugated reduced mannan (700 μg per injection), unconjugated oxidized mannan (700 μg per injection),or PBS vehicle were administered to groups of rats while they were under *isoflurane sedation*, in a prophylactic protocol by s.c. tail base injection two days prior to induction of EAE. EAE was induced by s.c. injection of an emulsion containing 40 μg of MBP(74–85) dissolved in 50 μl of saline and 50 μl of CFA (Sigma–Aldrich) supplemented with H37Ra *Mycobacterium tuberculosis* (Difco) at a final concentration of 4 mg/ml. Rats were evaluated daily for weight loss and clinical score. Scoring was based on clinical signs according to the following parameters; (1) floppy tail; (2) mild paraparesis; (3) severe paraparesis; (4) tetraparesis or moribund condition.[46] Rats were allowed free access to food and water throughout the experiment and all animal procedures were approved by institutional review boards and national authorities and conformed to European Union guidelines.

3. Results and discussion

In this study, the divergent and convergent synthetic procedures of di-BAPs using the immunodominant epitope MBP (83–99) and their effect upon EAE development were studied. Thus, we synthesized a MAP with two branches, containing the immunodominant epitope MBP(83–99), which is implicated in EAE, following both a direct (MW-SPPS) and an indirect (chemoselective ligation) synthetic approach. The C-terminus of both di-BAP **1** and **2** consists of an alternate Gly-Lys motif which acts as a linker between the MAP and mannan, through the formation of Schiff bases on the available free amino functions.

The MW-assisted solid-phase synthetic strategy described could be used for the synthesis of large peptides or MAPs with different peptides in a poly-lysine core. We combined the benefits of microwave irradiation leading to the rapid and efficient synthesis of difficult peptide sequences and MAPs. Mannosylation is a promising technique to achieve modulation of immune response. The lack of immunogenicity of the tested analogues could be overcome using two or three immunodominant epitopes of myelin proteins on a poly-lysine core. It is known that many different epitopes of myelin proteins have been recognized by the encephalitogenic T cells obtained in MS patients. There is not only one antigen responsible for the appearance of the disease. The protection of mice using mannan-peptide conjugation is antigen specific and it induces durable peptide-specific T cell tolerance in mice. Moreover, the mannan conjugated with mucin 1, a tumour cell surface protein, has been previously successfully used (CVacTM product) as a therapeutic vaccine for the treatment of ovarian cancer.

3.1. MW-assisted SPPS of di-BAP 1

The step by step synthesis of di-BAP **1** using conventional RT-SPPS protocols presented difficulties in terms of slow and incomplete couplings and Fmoc-removal reactions, probably due to chain aggregation. Thus, we turned to MW-SPPS, which may allow the fast stepwise synthesis of large and difficult peptide sequences[29–31] Di-BAP **1** was synthesized on a Wang resin, following the standard Fmoc/tBu strategy, using a Liberty™ microwave peptide synthesizer (CEM). A low resin substitution (0.12 mmol/g resin) was chosen in order to prevent steric hindrance and chain aggregation, which increase proportionally to the peptide chain. TBTU and DIPEA were used during the coupling as activating and base reagents respectively, while Fmoc removal was performed by treatment with 20% piperidine in DMF.

In order to reach the desired branching level, Fmoc-Lys(Mtt)-OH was used in the appropriate position, as Mtt group can be selectively removed under mild acidic conditions. Since Mtt group can be partially removed at high temperatures, after the introduction of Fmoc-Lys(Mtt)-OH in the sequence, the rest coupling and deprotection steps were carried out at a lower temperature (65 °C instead of 75 °C).

After the completeness of the MW-SPPS of the first MBP(83–99) epitope on the N^{α} amino group of the resin-bound lysyl core matrix, the N^{α}-terminus of MBP(83–99)-core was capped with Boc_2O. The Mtt orthogonal protecting group was then removed and the second copy of the MBP(83–99) epitope was synthesized stepwise on the N^{ε} amine group of the lysine, under microwave irradiation.

In order to prevent steric hindrance, β-Ala was used as a spacer between the core and the two epitope copies. The process of the di-BAP **1** synthesis was monitored by microwave-assisted mini cleavages and subsequent UPLC-MS analysis.

The crude product was analyzed by UPLC (conditions I) (see Supplementary Information, Fig. S1 A), and purified by semi-preparative RP-HPLC (conditions II) to give, after lyophilization, the final di-BAP **1** in 10.5% total yield (Table 1). The pure di-BAP **1** was analyzed by analytical RP-HPLC (conditions III) and identified by a Micromass® Q-Tof MICRO™ mass spectrometer (Waters) equipped with an ESI source (theoretical average mass: 4613.3272; observed mass: 4613.1524) (see Supplementary Information, Fig. S1 B–C).

3.2. Synthesis of bromoacetylated polylysine core 3

The protected peptide **3a** was synthesized on CLTR-Cl[40,41] using the standard Fmoc/tBu methodology under microwave irradiation. In order to avoid premature cleavage of the peptide from the CLTR due to its high sensitivity at elevated temperatures and after performing repetitive cycles of microwave, we adopted the modified MW-SPPS conditions previously reported by us.[29] DIC/HOBt was used as coupling reagent, while the Fmoc removal was performed by treatment with 20% piperidine in DMF. Branching was achieved by the incorporation of Fmoc-Lys(Fmoc)-OH derivative on the resin-bound lysyl core matrix. After deprotection of Fmoc-Lys(Fmoc)-OH followed bromoacetylation of the polylysine core by using bromoacetic acid, DIC and DIPEA. The completeness of the reaction was verified by Kaiser test. The cleavage of the bromoacetylated polylysine core **3** from the resin and the removal of the amino acids side chain protecting groups were carried out by treatment with a 97.5% TFA solution in the presence of anisole as a scavenger. Thiol or silyl type scavengers were not used in order to avoid their reaction with the bromine moieties. Also, in order to avoid the hydrolysis of the bromine, the cleavage reaction was reduced to 2-2.5 h.

The crude product was subsequently purified by semi-preparative RP-HPLC (conditions IV) to give after lyophilization the final bromoacetylated polylysine core **3** in 43% total yield (Table 1). The pure core **3** was analyzed by analytical RP-HPLC (conditions III) and identified by ESI-MS (see Supplementary Information,

Table 1
List of peptides and di-BAPs synthesized by direct and indirect approach

Peptide	Purification yield (%)	Total yield (%)	t_R[a] (min)	M_{calc} (Da)
di-BAP **1**	20.0	10.5	16.74	4613.35
Peptide **3**	60.0	42.7	10.60	758.50
Peptide **4**	67.3	52.0	16.92	2098.43
di-BAP **2**	50.0	50.0	16.53	4793.53

[a] Conditions III.

Fig. S2 A). The existence of both bromine moieties in the final product **3** was verified by the presence of three peaks; one corresponding to the M of the peptide, a second one of a double height of the previous peak corresponding to the M+2, and a third one of the same height of the first peak corresponding to the M+4.

3.3. MW-SPPS of the peptide epitope *Cys*-MBP(83–99) 4

MBP(83–99) was synthesized bearing a cysteine in its N^{α}-terminus in order to react further with the bromine moieties of the core **3**. *Cys*-MBP(83–99)**4** was prepared on CLTR-Cl[40,41] using the standard Fmoc/tBu methodology under microwave irradiation and according to the modified MW-SPPS protocol for CLTR-Cl[29] DIC/HOBt was used as coupling reagent, while the Fmoc removal was performed by treatment with 20% piperidine in DMF.

As with histidine and arginine, also cysteine requires special attention during MW-SPPS, since it is prone to racemization. Cysteine racemization has been attributed to base-catalyzed α-carbon proton abstraction during coupling. The level of racemization can be decreased by using weaker bases and avoiding preactivation in phosphonium or aminium salt mediated coupling protocols.[47] Additionally, in order to overcome increases in racemization from excess microwave energy, coupling temperature was reduced to 50 °C.[38]

The crude product was subsequently purified by semi-preparative RP-HPLC (conditions V) to give, after lyophilization, the final peptide **4** in 52% total yield (Table 1). The pure peptide **4** was analyzed by analytical RP-HPLC (conditions III) and identified by ESI-MS (see Supplementary Information, Fig. S2 B).

3.4. Synthesis of di-BAP 2 via Thioether Chemoselective ligation

Since the MW-SPPS resulted in a 10.5% total yield of di-BAP **1**, we turned our attention to chemoselective ligation techniques which are considered to be an efficient route for obtaining dendrimeric constructs in high yields and purity.

A number of chemoselective ligation methods have been used to synthesize large and complex peptide vaccines. In a previous comparative study, Zeng et al.[22] evaluated the yields and ease to carry out three different chemoselective techniques, such as oxime bond, thioether bond and disulfide bond formation and they found that thioether bond formation was the simplest method, giving the highest yield of immunogen. Taking this in consideration as well as the fact that thioether bonds are stable in the physiological pH range, we decided to follow this method for the synthesis of di-BAP **2**.

Thioether ligation relies on the highly chemoselective reaction between thiols and haloacetyl groups at neutral to mildly alkaline pH. Thus, it is critical to perform the reaction in a pH range of 8.5, in order to ensure that no other functional groups of the peptide sequence, such as lysine, react with the haloacetyl groups. Another critical step is to keep the reaction under anaerobic conditions in order to prevent the thiol peptide from forming disulfide dimers.

Ligation of peptides **3** and **4** proceeded almost quantitively to give di-BAP **2** (see Supplementary Information, Fig. S2 C). After 2 h, the starting peptide **3** could not be detected by analytical RP-HPLC (conditions III), while in the mixture were present small quantities of the peptide **4** and of the by-product **5**, i.e. the peptide **3** ligated with one of the two bromine moieties of the core **4**, while the other bromine was hydrolyzed (see Supplementary Information, Fig. S2 C, Scheme 4).

The purification of the di-BAP **2** was performed by semi-preparative RP-HPLC purification (conditions V) after partial evaporation. The pure di-BAP **2** was obtained in 50% total yield after lyophilization (Table 1), and it was analyzed by analytical RP-HPLC (conditions III) and identified by a Micromass® Q-Tof MICRO™ mass spectrometer (Waters) equipped with an ESI source (theoretical average mass: 4793.5294; observed mass: 4793.5560) (see Supplementary Information, Fig. S3 A–B).

3.5. Conjugation of di-BAPs to mannan and monitoring of the reaction

Di-BAPs **1** and **2**, after lyophilization, were conjugated to polysaccharide mannan aiming in the diversion of the immune response from Th1 to Th2 in EAE. Conjugation of di-BAPs to mannan occurs via Schiff base formation between the free amino groups of the di-BAPs and the aldehyde of oxidized mannan. Reduction of oxidized mannan-di-BAPs complexes by treatment with $NABH_4$ results in the reduction of aldehydes to alcohols and of Schiff bases to alkylamines.

In the current work, the conjugation of the di-BAPs to mannan was verified by analytical RP-HPLC by the absence of the peak corresponding to the di-BAP alone (without mannan) in the final conjugates (data not shown).

3.6. Assessment of the prophylactic role of mannan-di-BAP 1 and mannan-di-BAP 2 conjugates on the development of MBP(74-85) induced EAE

To evaluate whether oxidized and reduced mannan- di-BAP **2** and oxidized mannan-di-BAP **1** conjugates can induce tolerance

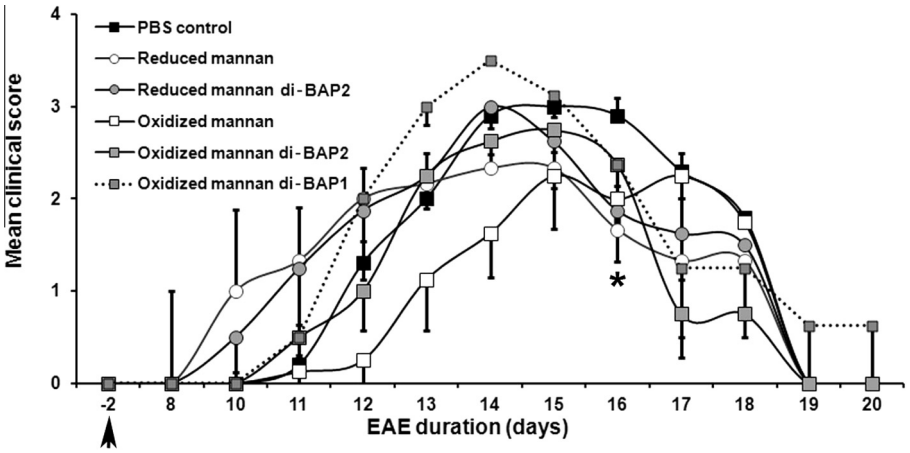


Figure 2. Immunization of Lewis rats pretreated with oxidized, reduced mannan-di-BAP **2** (n = 4 per group), oxidized mannan-di-BAP **1** (n = 4) or reduced (n = 3) mannan, and PBS vehicle control (n = 5) two days prior to the induction of EAE (arrow) (*, $p < 0.05$ for comparison between reduced mannan and PBS-treated groups).

in an MS-like disease, we used an MBP-EAE model inducible in Lewis rats by s.c. immunization with MBP(74–85) emulsified in CFA. Groups of rats were injected intradermally with dilute soluble oxidized or reduced mannan- di-BAP **2** or oxidized mannan di-BAP **1** conjugates (Fig. 2), unconjugated oxidized or reduced mannan or PBS 2 days prior to EAE induction. All rats developed an acute monophasic disease that was not significantly different to that developed in PBS-treated controls. Thus, mannan-conjugated di-BAP **1** and di-BAP **2** and unconjugated mannan-treated rats showed similar disease onset and progression of clinical symptoms as PBS-treated rats, except at one time point (day 16) where reduced mannan-vaccinated rats showed significantly reduced clinical signs. The finding that mannan MBP(83–99) conjugates did not protect against MBP(74–85)-induced EAE is consistent with previous data that mannosylated or mannan-conjugated myelin antigens (based on the MOG protein) induce T cell tolerance and protect against the development of EAE in a peptide-specific manner (*Tseveleki V.; Tselios T.; Friligou I.; Koutsoni O.; Emmanouil M.; Katsara M.; Vamvakas S.S.; Dotsika E.; Matsoukas J.; Apostolopoulos V.; Lassmann H.; Probert L. Immunotherapy of autoimmune encephalomyelitis using mannan-conjugated peptides to target dendritic cells: A pre-clinical study in mice. Unpublished results*). The tolerogenic potential of di-BAP **2** will need to be further tested upon the induction of MBP(83–99)-induced specific T cell responses.

4. Conclusions

In our study, di-branched antigenic peptide (di-BAP) syntheses were achieved following a direct (divergent) approach for di-BAP **1** using microwave irradiation and an indirect (convergent) approach for di-BAP **2** using a thioether chemical ligation method. The latter proved to be a more efficient synthetic strategy in terms of yield and purity, since it is a chemoselective reaction and the fragments herein used are firstly purified by HPLC. Moreover, the reaction proceeded smoothly without requiring any excess quantity of the peptide epitope. No solubility problems appeared but this is a parameter completely depending on the amino acid sequence of the peptide epitopes as well as on the branching level of the MAP, where steric hindrance is crucial. Also, the incorporation of the thioether linkage should not play any role in the biological effect, but this should be further evaluated.

Acknowledgments

We thank the Lifelong Learning Programme (LLP) Erasmus for the financial support of Dr. I. Friligou's mobility to PeptLab of the University of Florence. We also thank the Hellenic Republic, Ministry of Development, for the financial support of this study through the "Cooperation", 09SYN 21 609 program. Moreover we thank the Ente Cassa di Risparmio di Firenze for financial support to PeptLab of the University of Florence and the Agence Nationale de la Recherche for the Chaire d'Excellence Programme to A.M. Papini for the Project: PepKit at PeptLab@UCP.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.08.008.

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