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Designed Glucopeptides Mimetics of Myelin Protein Epitopes As Synthetic Probes for the Detection of Autoantibodies, Biomarkers of Multiple Sclerosis

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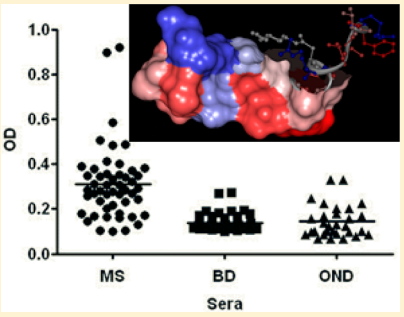
Supporting Information

ABSTRACT: We previously reported that CSF114(Glc) detects diagnostic autoantibodies in multiple sclerosis sera. We report herein a bioinformatic analysis of myelin proteins and CSF114(Glc), which led to the identification of five sequences. These glucopeptides were synthesized and tested in enzymatic assays, showing a common minimal epitope. Starting from that, we designed an optimized sequence, SP077, showing a higher homology with both CSF114(Glc) and the five sequences selected using the bioinformatic approach. SP077 was synthesized and tested on 50 multiple sclerosis patients' sera, and was able to detect higher antibody titers as compared to CSF114(Glc). Finally, the conformational properties of SP077 were studied by NMR spectroscopy and structure calculations. Thus, the immunological activity of SP077 in the recognition of specific autoantibodies in multiple sclerosis patients' sera may be ascribed to both the optimized design of its epitopic region and the superior surface interacting properties of its C-terminal region.



INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) whose pathogenesis has not been yet elucidated, even if an autoimmune mechanism against myelin antigens is thought to contribute to its etiopathogenesis.[1] However, in spite of extensive research efforts, a clear-cut identification of the native autoantigen(s) has not been accomplished. The most extensively studied putative autoantigens are components of normal CNS myelin,[2] and special attention has been devoted to the role of post-translational modifications.[3] In spite of this uncertainty, immunological characterization of autoantibodies circulating in patients' sera as disease biomarkers has been strongly pursued, aiming at the development of simple and reliable diagnostic/prognostic tools, a still largely unmet medical need.

To overcome the limits inherent to the uncertainty about the relevant native autoantigens, we have successfully applied a "reversed approach" toward the selection of synthetic antigenic probes able to recognize specific autoantibodies in patients' sera in a simple immunoenzymatic assay.[4] Accordingly, a designed synthetic glucosylated peptide, termed CSF114(Glc), was shown to detect specific autoantibodies in a subpopulation of MS patients.[5] Subsequently, the sequence of the original peptide was further optimized, with special attention to the conformational propensity of the designed active glucopeptides, in the context of the solid-phase immunoenzymatic assay.[6,7] However, although the practical value of these glucopeptides has been clinically demonstrated,[8] the problem of the identification of the native autoantigen(s) recognized by anti-CSF114(Glc) autoantibodies is still open. Immunochemical analyses performed on sections of CNS samples from normal individuals demonstrated that anti-CSF114(Glc) antibodies (affinity purified IgG or sera containing IgM) showed a diffuse immunostaining on myelin sheaths and oligodendrocytes but not on non-CNS human tissues, thus confirming the CNS specificity of anti-CSF114(Glc) antibodies.[4] On the basis of the observations that the endogenous antigen(s) target of the anti-CSF114(Glc) antibodies is(are) located in the CNS and specifically on the myelin sheath, we performed a sequence alignment analysis of myelin proteins and CSF114-(Glc). Because the recognition of the synthetic antigen CSF114(Glc)

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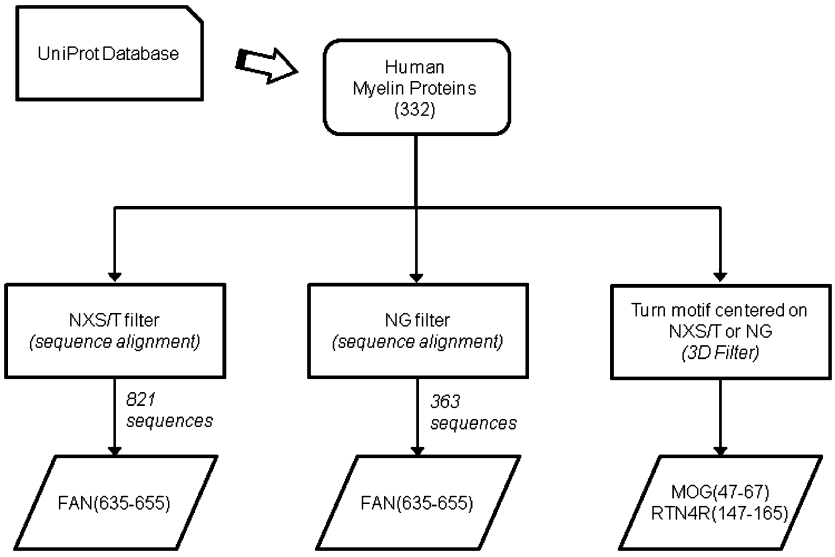

Figure 1. Workflow used for the bioinformatic approach: three different and independent filters were applied in a parallel strategy to compare domain sequences contained in human myelin proteins with CSF114(Glc).

by MS specific autoantibodies is hypothesized to be dependent on the peptide sequence and on its β-turn conformation,[5,7] we performed both a sequence and a 3D alignment study of CSF114(Glc). The homology results were instrumental for the design of a second-generation antigenic probe. We describe here the design of the new glucopeptide, termed SP077, as well as its immunological and conformational properties.

RESULTS AND DISCUSSION

Bioinformatic Approach. To identify the CNS proteins mimicked by the synthetic antigenic probe CSF114(Glc), we first selected the human myelin proteins from the UniProt database.[9] The collection of the 332 human myelin proteins was analyzed by applying three different and independent filters, two based on sequence alignments and one on the comparison of 3D structures (Figure 1).

One sequence alignment was performed by considering the antigenic elements present in the CSF114(Glc) primary structure and essential for the antibody recognition. The *N*-glucosylated Asn residue is the fundamental requirement necessary to detect the specific antigenic activity. It is known that the consensus sequence for *N*-glycosylation of proteins is characterized by the presence of the tripeptide Asn−Xaa−Ser/Thr (where Xaa is any amino acid except Pro). This consensus sequence is a minimum requirement, and it is not always sufficient to predict the *N*-glycosylation of proteins in physiological conditions. Yet, CSF114(Glc) is mimicking a neo-autoantigen generated in a pathological condition and thus the first criterion used for the sequence alignment was based on the selection of the myelin proteins containing the Asn−Xaa−Ser/Thr tripeptide (where Xaa is any amino acid). The Asn−Xaa−Ser/Thr filter led to the selection of 154 proteins, which were then subjected to the sequence alignment with CSF114(Glc) centering Asn(Glc) on the Asn residue of the consensus tripeptide. A total of 821 sequences, selected with this criterion, were compared with CSF114(Glc). The number of amino acids identical to the ones of CSF114(Glc) for each sequence is reported in Figure 2. The highest homology corresponded to an 8/21 amino acid identity (38%) and was found only for the 635−655 domain of the factor associated with neutral sphingomyelinase activation (FAN).

Another sequence alignment was performed on the collection of 332 human myelin proteins because the antigen/antibody affinity is altered by the amino acids flanking the Asn(Glc) residue. In fact, it was previously reported that the antigenic activity of CSF114(Glc) is due to the presence of a type I′ β-turn and can be modulated slightly changing the residues flanking Asn(Glc).[6] It is known that a type I′ β-turn is favored in proteins by the presence of the following preferred residues: Tyr, Ile, and Val at position *i*; Asp, Asn, and Gly at *i*+1; Gly at *i*+2; charged and polar residues at *i*+3.[10] Therefore, considering that position *i*+1 is fundamental for the activity of CSF114(Glc) and corresponds to Asn(Glc), the second filter applied to the database of the human myelin proteins was based on the analysis of the presence of the Asn(*i*+1)−Gly(*i*+2) dipeptide. This second parallel filter has been selected also considering the results previously reported by Petrescu et al., which showed that Gly and Val are the only two amino acids found in position *i*+1 of *N*-glycosylated Asn by a statistical analysis performed on 506 glycoproteins.[11] The filter based on the Asn−Gly dipeptide was thus selected by combining the prevalence of amino acids flanking Asn in a type I′ β-turn and the prevalence of amino acids flanking Asn in *N*-glycosylated proteins.

The selection of the myelin proteins containing the Asn−Gly dipeptide led to the identification of 129 proteins. The comparison with CSF114(Glc) was performed on 363 sequences and also with this criterion the best alignment was obtained with 8/21 amino acid identity (38%). The highest homology score corresponded to the sequence FAN(635−655), already identified also by the Asn−Xaa−Ser/Thr filter (Figure 2).

FAN (UniProtKB/Swiss-Prot accession number: Q92636) is an ubiquitous protein that activates and regulates the production of ceramide by neutral sphingomyelinase (N-SMase) in response to tumor necrosis factors (TNF), CD40L, and Δ9-tetrahydrocannabinol.[12] FAN is an adaptor protein that couples the p55 TNF-receptor (TNF-R55/TNFR1) to N-SMase.[13] The FAN sequence contains a beige and Chédiak−Higashi (BEACH) domain, a glucosyltransferases, Rab-like GTPase activators and myotubularins (GRAM) domain, and a C-terminal WD repeat domain.[14] The WD-repeat (Trp-Asp repeat), consisting of a conserved core of 23−41 amino acids and encoding a structural motif,

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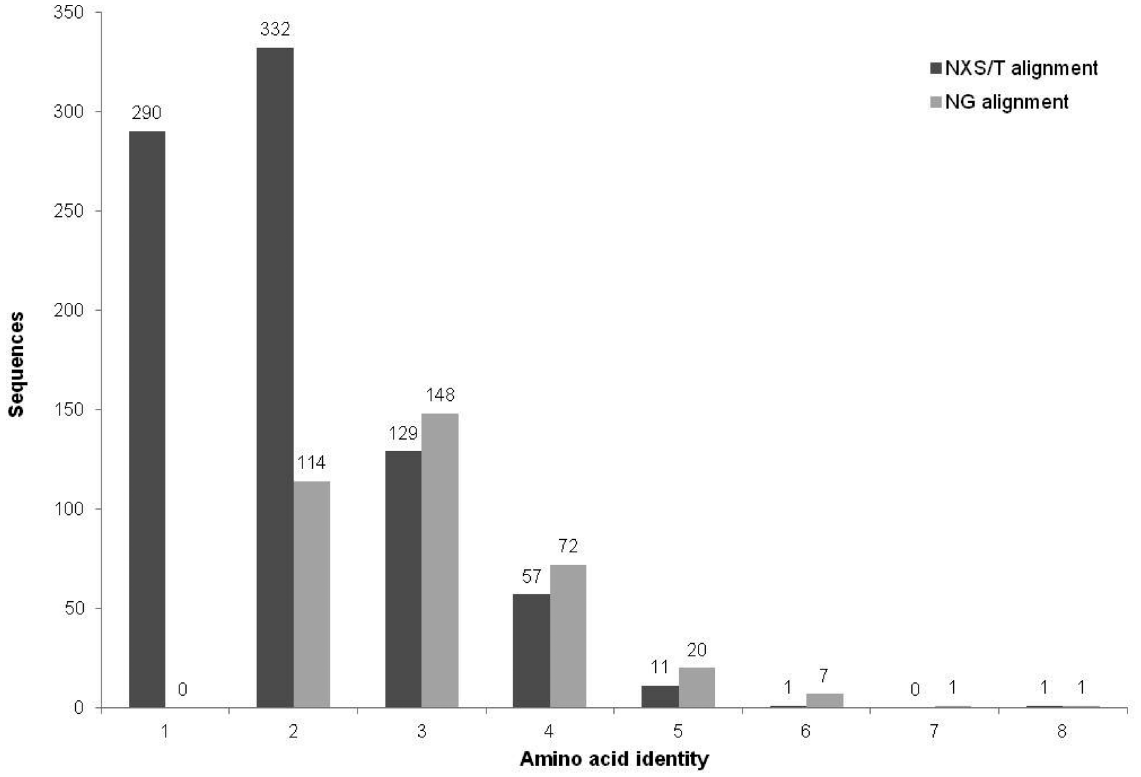

Figure 2. Distribution of the amino acid identity for the sequence alignment of CSF114(Glc) with the human myelin proteins containing the *N*-glycosylation consensus sequence (dark gray) and the human myelin proteins containing the Asn-Gly dipeptide (light gray).

is responsible for N-SMase activation in FAN. The sequence FAN(635−655) identified by the alignment with CSF114(Glc) is located inside the first of six WD-repeat domains. Up to now, to the best of our knowledge, the *N*-glycosylation of Asn-641 has not been reported even if the residue is contained in a consensus sequence.

For the 3D alignments, the database containing the human myelin proteins was limited to those possessing a secondary structure already characterized and reported in the RCSB PDB archive.[15] The secondary structures were then analyzed to select turn motifs comprising or in the proximity of a *N*-glycosylation consensus sequence or of an Asn-Gly motif. The homology of the selected structural domains was then evaluated in comparison with CSF114(Glc) conformation. Using this criterion, two proteins have been selected: myelin oligodendrocyte glycoprotein (MOG, UniProtKB/Swiss-Prot accession number: Q16653) and reticulon-4 receptor (RTN4R, nogo receptor, UniProtKB/Swiss-Prot accession number: Q9BZR6).

MOG is a potential autoantigen for MS and has been identified as target antigen of demyelinating autoantibodies in MS animal models.[16−19] It is a transmembrane glycoprotein characterized by an extracellular domain of 125 amino acids exposed on the surface of myelin sheath. The extracellular domain of MOG comprises a single Ig-like motif and is natively *N*-glycosylated at position 31.[20] The 3D alignment of the structure of the extracellular domain of rat MOG (PDB ID: 1PKO)[21] with CSF114(Glc) led to the identification of the C′−C″ loop, which comprises the residues 47−67 characterized by the presence of an Asn−Gly dipeptide. Moreover, MOG(47−67) and CSF114(Glc) share the same type I′ β-hairpin structure with the NG dipeptide at the tip of the hairpin.[5,20]

Some interest was also aroused by the sequence 97−117 of MOG. Residues 97−117 are comprised in the FG loop of MOG. The FG loop was identified as a dominant component of the epitope recognized by the antigen-binding fragment (Fab) of the MOG-specific demyelinating monoclonal antibody 8−18C5.[20] Furthermore, it has been recently reported to contain one immunodominant region target of the pathogenic anti-MOG antibodies in MS animal models.[22] In the crystal structure of the extracellular domain of MOG (PDB ID: 1PKO), the FG loop forms a hairpin loop classified as a II′-β turn. This structure was previously shown by us to be suitable for interaction with anti-CSF114(Glc) antibodies.[6] In the FG loop, the second position of the type II′ β-turn is occupied by His-103, which results in a strained loop conformation with dihedral angles of His-103 in forbidden regions of the Ramachandran plot. Thus, a linear peptide with the FG loop sequence would be unable to reproduce this strained loop structure. To overcome this problem and to introduce the *N*-glycosylation site, we replaced the His−Ser sequence of the FG loop with a Gly−Asn sequence which is known to stabilize a type II′ β-turn.[10,23]

The conformational comparison of CSF114(Glc) with the crystal structure of RTN4R (PDB ID: 1P8T)[24] focused on the sequence 147−165, which is natively *N*-glycosylated at position 153. RTN4R is a leucine-rich repeat protein characterized by a globular structure, which is mainly expressed in brain and in gray matter. RTN4R plays a key role as inhibitor of axonal growth and of myelin regeneration after an axonal lesion.[25] RTN4R is a receptor for reticulon-4 (nogo protein), oligodendrocyte myelin glycoprotein, and myelin associated glycoprotein.[26] Among these myelin proteins associated to RTN4R receptor, the oligodendrocyte myelin glycoprotein (OMG, UniProtKB/Swiss-Prot accession number: P23515) is characterized by a secondary structure similar to RTN4R itself. Therefore, we selected also the sequence OMG(162−180), which is highly homologous to RTN4R(147−165) and thus to CSF114(Glc) and contains an *N*-glycosylation

motif in proximity to Asn in position 168. OMG is a protein expressed on the surface of oligodendrocytes, and it is involved in myelin sheath formation.[27] It was recently reported that OMG can induce a severe form of the experimental autoimmune encephalitis (EAE), the most commonly used MS animal model.[28]

Peptide Synthesis. According to the sequence and the 3D alignments of human myelin proteins with CSF114(Glc), the following sequences were selected (Table 1): FAN(635−655), MOG(47−67), MOG(97−117), RTN4R(147−165), and OMG(162−180). To evaluate the immunological activity of the sequences selected from the alignment study, the following glucopeptides were synthesized (Table 2): [Asn^{641}(Glc)]FAN(635−655), [Asn^{53}(Glc)]MOG(47−67), [Gly^{103}, Asn^{104}(Glc)]-MOG(97−117), [Asn^{153}(Glc)]RTN4R(147−165), and [Asn^{168}(Glc)]OMgp(162−180).

Table 1. Sequences Selected from the Sequence and 3D Alignments of CSF114(Glc) vs Homologous Human Myelin Proteins

peptide	sequence
CSF114(Glc) (**1**)	T P R V E R **N G** H S V F L A P Y G W M V K
FAN(635−655)	G I T V S R **N G S** S V F T T S Q D S T L K
MOG(47−67)	V V H L Y R **N G** K D Q D G D Q A P E Y R G
MOG(97−117)	T C F F R D H S Y Q E E A A M E L K V E D
RTN4R(147−165)	T F R D L G **N L T** H L F L H G N R I S
OMG(162−180)	T L I N L T **N L T** H L Y L H N N K F T

The glucopeptides **2**−**6** were synthesized by a 9-fluorenylmethoxycarbonyl (Fmoc)/tBu solid-phase peptide strategy introducing the Fmoc-Asn(GlcOAc4)-OH building block during the stepwise synthesis as previously reported.[6] All glucopeptides were purified by high-performance liquid chromatography (HPLC, purity ≥95%) and characterized by electrospray ionization mass spectrometry (ESI-MS, Table 2).

Immunological Activity. Competitive enzyme-linked immunosorbent assay (ELISA)[29] was used to analyze the anti-CSF114(Glc) autoantibody binding affinities of the newly synthesized peptides. The data shown in Figure 3 indicate that all the new glucopeptides show full inhibition of anti-CSF114(Glc) IgGs present in a significant reference MS patient serum, thus indicating that they share a common minimal epitope with the parent glucopeptide. However, very minor differences in affinity are observed by evaluating the IC_{50} of glucopeptides **1**−**6** (Table 3).

Table 2. HPLC and ESI-MS Data of the Synthetic Glucopeptides 2−7

no.	glucopeptide	sequence	HPLC R_t (min)	HPLC gradient (%)[a]	yield (%)	ESI-MS (m/z) $[M + 2H]^{2+}$ found (calcd)
2	[Asn^{641}(Glc)]FAN(635−655)	GITVSR**N**(**Glc**)GSSVFTTSQDSTLK	7.3	20−50	3	1174.63 (1174.74)
3	[Asn^{53}(Glc)]MOG(47−67)	VVHLYR**N**(**Glc**)GKDQDGDQAPEYRG	11.0	10−40	2	1290.72 (1290.84)
4	[Gly^{103},Asn^{104}(Glc)]MOG(97−117)	TCFFRDG**N**(**Glc**)YQEEAAMELKVED	9.3	20−60	1	1329.70 (1329.91)
5	[Asn^{153}(Glc)]RTN4R(147−165)	TFRDLG**N**(**Glc**)LTHLFLHGNRIS	8.0	30−60	10	1187.86 (1187.82)
6	[Asn^{168}(Glc)]OMG(162−180)	TLINLT**N**(**Glc**)LTHLYLHNNKFT	8.3	30−70	2	1217.47 (1217.35)
7	SP077	TFRVLR**N**(**Glc**)GTSVFLHPNKWTVK	12.0	20−50	5	1332.37 (1332.18)

[a]HPLC solvents: A, 0.1% trifluoroacetic acid (TFA) in H_2O; B, 0.1 TFA in CH_3CN. Gradients of B solvents were performed in 20 min.

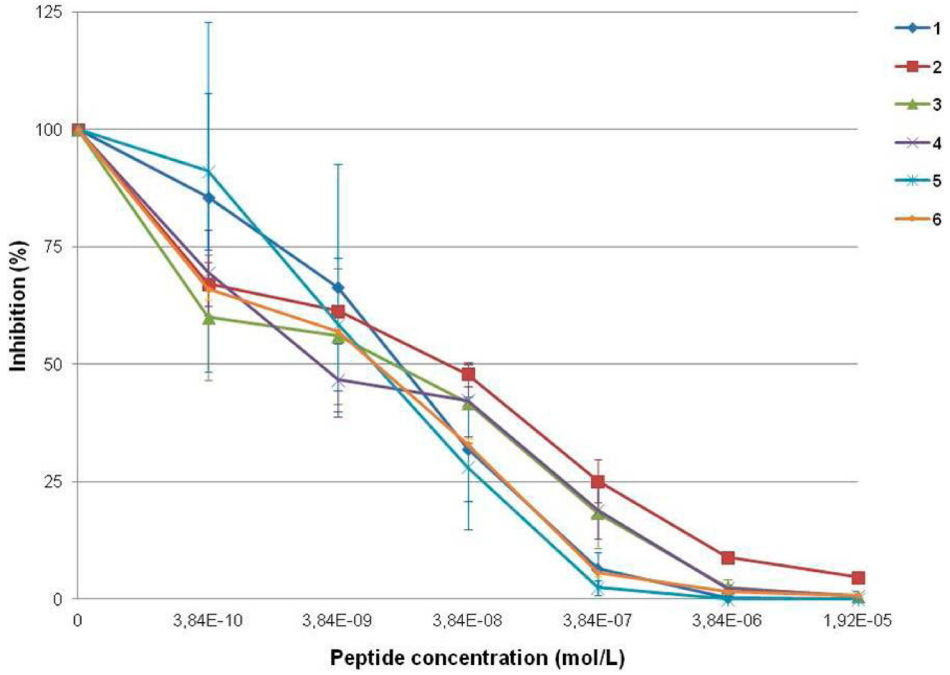

Figure 3. Inhibition curves of anti-CSF114(Glc) antibodies with glucopeptides **2**−**6** compared with CSF114(Glc) (**1**) in a competitive ELISA. The results are expressed as the percentage of inhibition of a representative MS serum (ordinate axis). The concentrations of the peptides used as inhibitors are on the abscissa.

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Table 3. IC_{50} Data of the Synthetic Glucopeptides 1−6 Determined by Inhibition Curves

glucopeptide	IC_{50} (IC_{50} range)[a] (nM)	$logIC_{50}$±SD[b]
CSF114(Glc) (**1**)	20 (9.5−43)	−7.69 ± 0.10
[Asn^{641}(Glc)]FAN(635−655) (**2**)	139 (49−392)	−6.86 ± 0.14
[Asn^{53}(Glc)]MOG(47−67) (**3**)	120 (56−254)	−6.92 ± 0.10
[Gly^{103},Asn^{104}(Glc)]MOG(97−117) (**4**)	106 (7.5−481)	−6.97 ± 0.36
[Asn^{153}(Glc)]RTN4R(147−165) (**5**)	9.5 (2.3−39)	−8.02 ± 0.19
[Asn^{168}(Glc)]OMG(162−180) (**6**)	36 (24−53)	−7.44 ± 0.05

[a]Range determined as 95% confidence interval of the IC_{50} to express the asymmetrical uncertainty. [b]Standard deviation (SD) determined on the log scale.

Design, Synthesis, and Immunological Activity of Glucopeptide SP077. Starting from the observation that the new glucopeptides **2**−**6**, selected by sequence or structural homology with CSF114(Glc), recognize specific autoantibodies in MS sera with similar affinities, we decided to design an optimized sequence showing a higher degree of homology with both the parent glucopeptide **1** and the five fragments of CNS protein deduced using the bioinformatic approach. The goal of this research was to obtain a new designed peptide, characterized by a higher homology to potential MS autoantigens (human myelin proteins) and by the chemical and structural features of the synthetic probe CSF114(Glc) necessary for the detection of MS-specific autoantibodies. The design of a new sequence was thus aimed at improving the biological activity of CSF114(Glc) by increasing its homology to potential MS autoantigens.

Therefore, glucopeptide SP077 was designed according to the following criteria: (a) inclusion of the *N*-glycosylation consensus site (Asn−Xaa−Ser/Thr); (b) Asn(Glc) residue is maintained in position 7; (c) residues of CSF114(Glc) which are present in the same position in at least one of the CNS sequences are conserved (i.e., Thr-1, Arg-3, Val-4, Arg-6, Gly-8, Ser-10, Val-11, Phe-12, Leu-13, and Lys-21); (d) residues which are recurrent in the same position in at least two CNS sequences are replaced, as compared to CSF114(Glc) (i.e., Phe-2, Leu-5, Thr-9, His-14, Asn-16, Lys-17, and Thr-19); (e) whereas a specific repetition is not evident, residues originally present in CSF114(Glc) are conserved (i.e., Pro-15, Trp-18, and Val-20). Therefore, the final sequence of SP077 is: Thr-Phe-Arg-Val-Leu-Arg-Asn(Glc)-Gly-Thr-Ser-Val-Phe-Leu-His-Pro-Asn-Lys-Trp-Thr-Val-Lys.

SP077 was synthesized, purified, and tested in solid-phase ELISA (SP-ELISA) for its ability to detect diagnostically relevant IgM autoantibodies in MS patients sera.[5] Figure 4 reports the IgM titers measured in SP-ELISA on 50 representative MS patients' sera, using glucopeptides **1** and **7** as antigens. The newly designed antigen SP077 clearly detects higher antibody titers, as compared to CSF114(Glc). Specificity was verified using 50 normal blood donors and 30 other neurological diseases patients; SP077 showed negligible immunoreactivity toward IgM antibodies (Abs) of normal blood donor (BD) and other neurological disease (OND) patients, thus confirming to specifically recognize IgM in sera of a subpopulation of MS patients.

The correlation between the autoantibodies detected using the two glucopeptides **1** and **7** was analyzed to evaluate the fine specificity of CSF114(Glc) and SP077 in this subgroup of MS patients (Figure 5). The results show a significant and strong correlation between anti-CSF114(Glc) IgM and anti-SP077 IgM ($r = 0.73$, $p > 0.0001$). In fact, it was found that a 76% of MS sera are positive to CSF114(Glc) and SP077. A 14% of MS sera are positive only to CSF114(Glc) and not to SP077.

Taken together, these results indicate that the designed antigen SP077 is as effective as CSF114(Glc) in recognizing highly specific autoantibodies in MS patients' sera in the context of a solid-phase immunoenzymatic assay. Accordingly, we decided to study the conformational behavior of this glucopeptide and of the parent compound in a surface-mimicking environment.

Conformational Studies. *Choice of the Solvent System.* NMR structure of CSF114(Glc) was previously studied in HFA/water mixture.[7] This solvent mixture is a stabilizing agent which

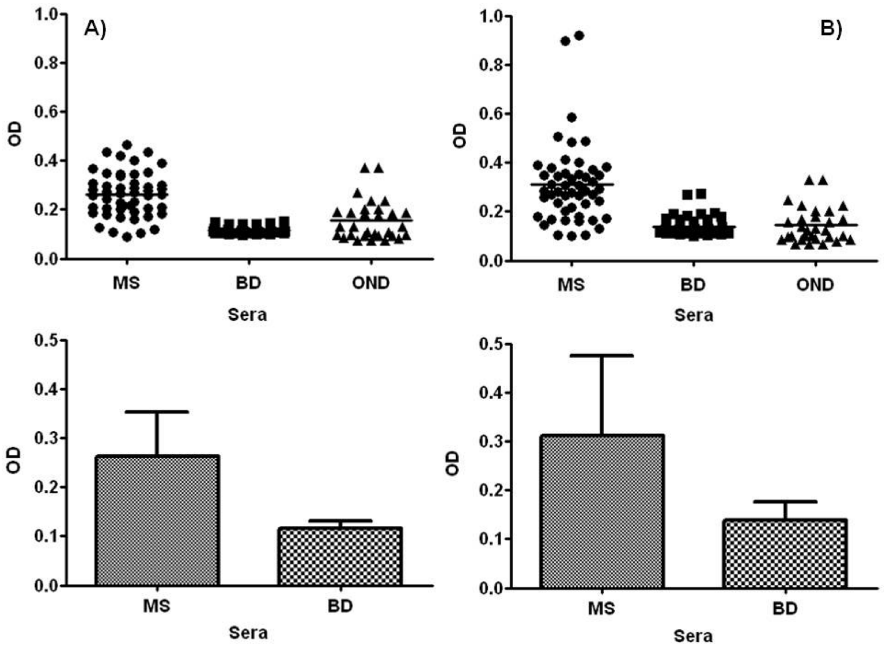

Figure 4. Autoantibodies recognition in MS patients' sera in SP-ELISA. The results are reported as IgM antibody titers to glucopeptides CSF114(Glc) **1** (A) and SP077 **7** (B) in 50 MS, 50 BD, and 30 OND sera.

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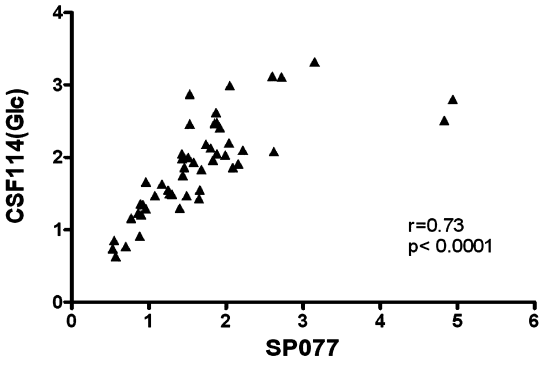

Figure 5. Graph reporting the correlation between IgM index in serum to CSF114(Glc) and to SP077 in 50 MS patients. The IgM antibody titer was expressed as IgM index, calculated by dividing the absorbance of each MS serum sample by the 95th percentile of the normal BD sera group. A result that was greater than 1.0 (i.e., greater than the 95th percentile of the normal BD sera group) is defined as positive.

increases the intrinsic tendency of the amino acid sequence to fold in defined secondary structures.[30] A plethora of data[30−32] show that water−fluoroalcohol constitutes an isotropic medium enveloping the full peptide in a hydrophobic environment where structural preorganization may occur according to the amino acid composition. In this respect, HFA/water mixture may be considered a suitable system to study the conformation of antibody−antigen interaction site. In view of the optimization of antigenic peptide for diagnostic application on ELISA support, interface solution made up of micelle aggregates may be more adaptive, providing a nonisotropic medium where the surface of the micelle forces the peptide to assume specific orientations.[33,34] This medium is important to study not only antibody−antigen interaction site, but also the region interacting with support.

Micelle solutions indeed have been extensively used as a biomimetic interface to study the structural basis of antibody−antigen reaction and constitute an accepted mimic of the interface where antibody−antigen reaction occurs.[35] Finally, from a technical viewpoint, micellar solutions are well suited for solution NMR spectroscopy as they tumble sufficiently quickly to result in high-resolution spectral lines.

NMR Spectroscopy. One-dimensional proton spectra were recorded in water and in the presence of increasing dodecylphosphatidylcholine (DPC)/sodium dodecylsulphate (SDS) (90/10 M:M) amounts, ranging from premicellar to concentrated DPC/SDS micelle solutions. One-dimensional proton spectra were recorded in ^{1}H water solution and in ^{2}H water solution to monitor the amide NH hydrogen−deuterium exchange. Two-dimensional proton spectra of SP077 and CSF114(Glc) were recorded in water and in DPC/SDS (90/10 M:M) mixed micelles. Double quantum filtered-correlation spectroscopy (DQF-COSY), total correlated spectroscopy (TOCSY),[36] and nuclear Overhauser effect spectroscopy (NOESY)[37] 2D spectra were recorded, and complete assignments of the proton spectra of SP077 and CSF114(Glc) (see Supporting Information) were achieved by the standard Wüthrich procedure[38] using the SPARKY software package.[39]

In the Supporting Information, the H/D exchange rates of backbone NHs are also reported, indicating that NH of residues exhibiting slow H/D exchange correspond to those involved in regular turn−helical structure.

CHα chemical shifts are known to be diagnostic of peptide and protein secondary structures. According to the chemical shift index (CSI),[40] groups of four (not necessarily consecutive) CHα showing N 0.1 ppm upfield shifted values, as compared to random coil values, are diagnostic of helix conformations. Regions characterized by nonconsecutive CHα values close or low-field shifted, as compared to the CHα random coil values, are diagnostic of coil conformations.[40] In agreement with this analysis, the trends of CHα chemical shift difference for CSF114(Glc) and SP077 in DPC/SDS micelle solutions evidence the presence of numerous CHα upfield shifts consistent with the preponderance of peptides in turn−helical conformations.

Figure 6 summarizes the nuclear Overhauser effect (NOE) connectivities observed in the NOESY spectra of SP077 and

Figure 6. Short and medium range NOE effects of CSF114(Glc) (top), SP077 (bottom) in DPC/SDS 90:10 M/M. Colored secondary structure labeling are reported according to the legends.

CSF114(Glc) in DPC/SDS mixed micelles. The diagrams show a regular pattern of NOEs for both the peptides, indicating the prevalence of canonical secondary structures. Specifically, regular sequential CHα-NH(i,i+1) and NH-NH(i,i+1) NOE effects and several i,i+2 and i,i+3 medium range NOE correlations are present along different triads of the sequence, with systematic helical diagnostic α,β (i,i+3) effects observable in the central portion of both SP077 and CSF114(Glc) sequence.

Structure Calculation. The structure calculations of SP077 and CSF114(Glc) in DPC/SDS mixed micelles were performed using CYANA software[41] based on NOE data. These data were transformed into interprotonic distances and imposed as restraints in the calculation. Among 50 calculated structures, the resulting best 20 were selected according to the lowest values of their target functions. These structures were subjected to further minimization procedures with the SANDER module of AMBER 5.0 software,[42,43] using CYANA-derived restraints. Figure 7 shows the NMR structure bundle of SP077 and CSF114(Glc).

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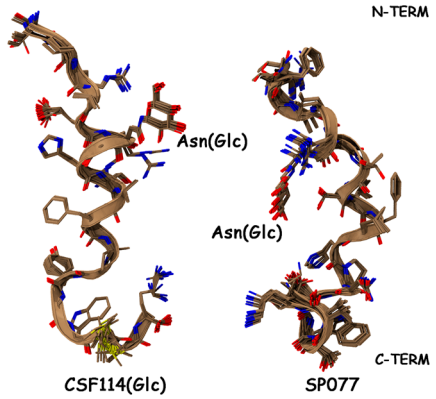

Figure 7. NMR structure bundles of CSF114(Glc) and SP077. NMR structures were calculated on the basis of NOE data collected at 600 MHz, 300 K.

The structures show a high degree of structural overlap (RMSD < 0.2) in the superimposed region, evidencing that the structural features have been defined with precision.

PROMOTIF analysis of structure bundles[44] confirmed the presence of canonical secondary structures with backbone dihedral angles located in allowed regions of Ramachandran plots (see Supporting Information). A summary of the secondary structure characterizing the peptides under scrutiny is reported in Figure 6. According to a quantitative evaluation of backbone dihedral angles (PROMOTIF software), α-helical structures are present in the central portions of both SP077 and CSF114(Glc), with γ-turns located in a short segment of C-terminal sequences.

The comparison of CSF114(Glc) structures in HFA/water[6,45] and in micelle solution shows a common tendency to assume turn conformations in correspondence of the glucosylated Asn. The central and C-terminal portions are different because in these regions CSF114(Glc) presents turn−helix structure in micelle solution but extended conformation in HFA/water mixture to form a general β-hairpin structure.

Study of Peptide−Micelle Interaction: Fluorescence Measurements. Once observed that the micelle surface is able to induce and stabilize helical conformations, to study the positioning of CSF114(Glc) and SP077 on the micelle surface, tryptophan fluorescence of both peptides in water and in DPC/SDS micellar solutions was registered (Supporting Information). The fluorescence intensities of some fine vibronic structures in the tryptophan fluorescence spectrum show strong environmental dependence.[45−48] In particular, the emission maximum shifts from 354 to 329 nm when going from water to an apolar medium.

Trp emission fluorescence spectra of CSF114(Glc) and SP077 in water are typical of the aqueous environment (λ_{max} = 354 nm), indicating that Trps are exposed to the aqueous medium. The addition of DPC/SDS surfactants causes a change of the Trps quantum yield which could be ascribed to peptide−micelle interaction.[49] The limited extent of the shift indicates that the Trps are greatly exposed to the solvent, suggesting a peptide positioning at the micelle surface rather than inside the inner hydrophobic core.

The monitoring of Trps fluorescence of both peptides at surfactant concentrations ranging from premicellar to concentrated micellar solution shows fluorescence intensity similar for water and dilute surfactant solutions, indicating almost negligible interaction between each peptide and DPC/SDS monomers. At the point of micelle formation, a sharp fluorescence variation is observed, indicating interaction of the peptides with the micelle aggregates.

The ability of CSF114(Glc) and SP077 to interact with the micelle aggregates rather than the single detergents is confirmed in NMR experiments, where chemical shift analysis indicates that ^{1}H chemical shifts of backbone NHs are significantly modified in response to the addition of micellar amounts of SDS/DPC surfactants (data not shown).

Surface Analysis. Analysis of side chain orientations indicates that although the segments carrying Asn(Glc) in both peptides are comprised in a turn−helical structure, they are characterized by different exposure of the Glc moiety. Whereas in CSF114(Glc) the sugar is clearly exposed to the solvent medium, in SP077 it appears involved in a network of H-bond interactions with the side chains of hydrophilic residues Thr-9, Ser-10, and His-14 (Figures 7 and 8). These findings point to the importance

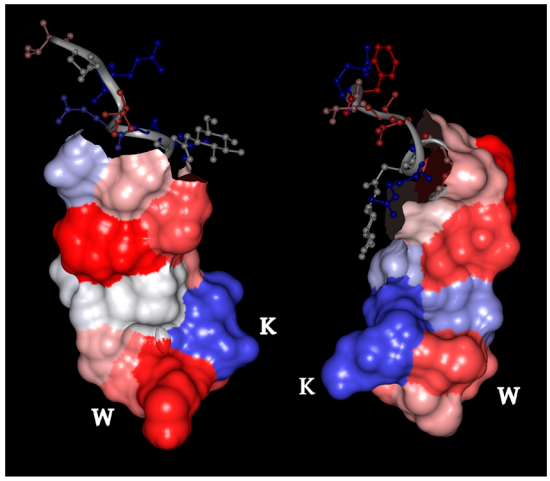

Figure 8. Molecular surfaces of CSF114(Glc) (A) and SP077 (B). Atomic surfaces are colored according to the hydrophobicity of the amino acid side chains.

of the residues in proximity of the glycosylated Asn in orienting the exposure of the sugar and justify the observed high incidence of hydrophobic residues in the i−3 to i+3 range of Asn(Glc) in glycosylated proteins. Petrescu et al. actually showed that in glycosylated antigenic proteins including Asn(Glc), the presence of hydrophobic residues around Asn(Glc) generates a microenvironment favoring the interactions of the sugar with the medium.[11]

Figure 8 shows the molecular surfaces of CSF114(Glc) (A) and SP077 (B) characterized by an hydrophobic/hydrophilic distribution consistent with the specific amino acid sequences. The above-mentioned different exposure of the sugar moieties in the two molecules is evident in this figure. Noticeable, there are significant differences in the C-terminal regions of both peptides, which include the positioning of the side chains of Trp and Lys residues. These two amino acids, typically present in membrane active peptides,[50] are able to promote the contact with membrane surfaces via hydrophobic and electrostatic interactions, respectively. The presence of these residues at the C-terminus of both peptides suggests the possibility that this portion mediates the interaction of both peptide antigens with the ELISA plate surface, which is fundamental for the correct exposure of the glucosylated epitope toward the antibody.

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Taken together, these data indicate that the differences in antigenic activity displayed by SP077 and CSF114(Glc) may be explained on the basis of the observed structural features. The two peptides, although characterized by similar secondary structure, differ for the exposure of the sugar moiety and for the surface properties of the C-terminal portion.

CONCLUSIONS

Our results showed that the synthetic glucopeptide CSF114-(Glc) is characterized by a sequence and a structure similarity to the five domains **2**–**6** of the human myelin proteins selected by three different parallel bioinformatic strategies. Starting from the observation that the glucopeptides **2**–**6** inhibit anti-CSF114-(Glc) IgG autoantibodies, we have designed SP077 an optimized sequence characterized by a higher degree of homology with both CSF114(Glc) and the five domains **2**–**6** of the selected human myelin proteins. The optimized sequence SP077 detects high antibody titers in MS patients, which have been shown to significantly correlate to anti-CSF114(Glc) MS-specific autoantibodies. The slightly superior immunological activity of SP077, as compared to CSF114(Glc) in the recognition of specific autoantibodies in MS patients sera, may be ascribed to both the optimized design of its epitopic region, represented by the glucosylated Asn and the neighboring residues and the superior surface interacting properties of its C-terminal region.

Interestingly, these results confirm that when considering the interaction of an antibody with a complex peptide antigen, it should be taken into account that residues not directly involved in the interaction may modulate the epitope exposure.[51]

EXPERIMENTAL SECTION

Bioinformatic Approach. The amino acid sequence of human myelin proteins were retrieved from UniProt database (www.uniprot.org)[9] using as keyword "*myelin*" and selecting "human" as organism. The collection of the 332 human myelin proteins (Supporting Information), containing SwissProt reviewed and Trembl not reviewed proteins, was then used for the sequence and the 3D alignments.

Sequence Alignments. The amino acid sequences of the 332 human myelin proteins containing the Asn–Xaa–Ser/Thr tripeptide (where Xaa is any amino acid) or the Asn–Gly dipeptide were identified and then submitted to the alignment with glucopeptide **1** using the BioEdit Sequence Alignment software version 7.0.9.0 (6/27/07).[52] The alignments were performed centering the Asn residue of CSF114(Glc) with the selected Asn sequence to be aligned, considering only the amino acid identity and not introducing any gap.

Three-Dimensional Alignments. The database containing the 332 human myelin proteins was limited to those 3D characterized in the RCSB PDB archive (www.pdb.org).[15] The selected PDB files were compared with glucopeptide **1** NMR structure[5] by the use of the software DaliLight for the pairwise alignment of protein structures from the European Molecular Biology Laboratory—European Bioinformatics Institute (EMBL-EBI) Web site (www.ebi.ac.uk/Tools/structure/dalilite/).[53,54]

Chemistry: Materials and Methods. Protected L-amino acids and Wang resins preloaded with the first Fmoc-amino acid were purchased from Iris Biotech Gmbh (Marktredwitz, Germany). Peptide synthesis grade dimethylformamide (DMF) was purchased from Scharlau (Barcelona, Spain), hydroxybenzotriazole (HOBt) and *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU) were purchased from Iris Biotech, and TFA, dichloromethane (DCM), piperidine, *N*-methylmorpholine (NMM), and *N*,*N*-diisopropylethylamine (DIPEA) were purchased from Sigma Aldrich (Milan, Italy). The characterization of the products was performed by ultraperformance liquid chromatography on an ACQUITY ultra performance liquid chromatography (UPLC, Waters Corporation, Milford, Massachusetts) coupled to a single-quadrupole ESI-MS (Micromass ZQ) using a ACQUITY BEH C18 column (2.1 mm × 50 mm; 30 °C; flow rate, 0.45 mL/min) with an acetonitrile–water mobile phase containing TFA (0.1% v/v). All peptides were purified by preparative HPLC on a Waters model 2545 equipped with a single-quadrupole ESI-MS (model 3100, Waters) and an ultraviolet–visible (UV/vis) detector (model 2487, Waters) using a xBridge C18 column (19 mm × 50 mm) at 20 mL/min according to the methods described in Table 2. The purity of the peptides was analyzed on the previously described Waters model 2545 by using a xBridge C18 column (4.6 mm × 50 mm) at 1.2 mL/min with a mixture of eluents: (a) 0.1% TFA in H_2O (Milli-Q) and (b) 0.1% TFA in CH_3CN.

Peptide Synthesis. Solid-phase peptide synthesis was performed on the Apex 396 automatic peptide synthesizer (Aapptec, Matthews, NC), following the Fmoc/tBu strategy. All the reactions were performed in a sealed reaction block under N_2, where mixing is provided by a vortex and filtration is performed by a N_2 flow. Fmoc deprotections were performed with a 20% piperidine solution in DMF. Each coupling cycle included the following four steps: Fmoc-deprotection (25 min), washings with DMF (5 × 2 min), acyl coupling (45 min), and washings with DMF (3 × 2 min). For the deprotection of the second residue, a fast protocol is used to avoid diketopiperazine formation (3 × 5 min). For the coupling reaction were used: a 0.5 M solution of the Fmoc-protected amino acid and HOBt in DMF (4 equiv), a 0.5 M solution of TBTU in DMF (4 equiv), and a 4 M solution of NMM in DMF (8 equiv). Fmoc-L-Asn(GlcAc4)OH (2.5 equiv) that was synthesized as previously reported[5] was coupled using HOBt (1.5 equiv), TBTU (1.5 equiv), and NMM (3 equiv) in DMF for 1.5 h. After the removal of the last Fmoc-residue, the resin was washed with DCM and dried under a N_2 flow for 30 min. The cleavage from the resin and side chain deprotection was performed by the use of a TFA/thioanisole/ethanedithiol/phenol/H_2O mixture (94/2/2/2/2 v/v/v/v/v) (reagent K). The resin was treated for 2.5 h with reagent K (1 mL/100 mg of resin) at room temperature. The resin was filtered off, and the solution was concentrated by flushing with N_2. The peptides were precipitated from cold Et_2O and then lyophilized. The deprotection of the hydroxyl functions of sugar moieties was performed by the addition of 0.1 M methanolic NaOMe solution to a solution of the lyophilized peptides in dry MeOH (1 mL/100 mg of resin) until pH 12 was reached. After 3 h, the reaction was quenched by the addition of concentrated HCl until pH 7 was reached; the solvent was evaporated under vacuum, and the residue was lyophilized. Peptides were purified at a purity level ≥95% and characterized as described above (Table 2).

Immunoenzymatic Assay. Sera were obtained for diagnostic purposes from patients who had given their informed consent, and samples were stored at −20 °C until use.

Inhibition ELISA. The antibody affinity was measured according to Rath et al.[29] in pure carbonate buffer (0.05 M, pH 9.6), and 96-well polystyrene ELISA plates (Nunc Maxisorp, Thermo) were coated with 1 μg/100 μL of glucopeptide **1** per well and were incubated at 4 °C overnight. The semisaturating sera dilution (1/200) was calculated from preliminary titration curves (absorbance, 0.7). At this dilution, sera were preincubated with increasing concentrations (3.84×10^{-11}, 3.84×10^{-10}, 3.84×10^{-9}, 3.84×10^{-8}, 3.84×10^{-7}, 3.84×10^{-6}, and 1.92×10^{-5} mol/L) of glucopeptides **1**–**7** for 1 h at room temperature. Unblocked antibodies were revealed by ELISA as follows: after the plates were washed five times with saline containing 0.05% Tween 20, 100 μL of alkaline-phosphatase-conjugated antihuman IgG (diluted 1/4000 in saline/0.05% Tween 20/10% fetal calf serum (FCS), Sigma Aldrich) was added to each well. After 3 h of incubation at room temperature, plates were washed five times and then 100 μL of a substrate solution consisting of 1 mg/mL *p*-nitrophenyl phosphate (Sigma Aldrich) in sodium carbonate buffer was added. After 30 min, the reaction was stopped with 1 M NaOH (50 μL), and the absorbance was read in a multichannel ELISA reader (Tecan Sunrise) at 405 nm. The antibody levels are expressed as absorbance in arbitrary units at 405 nm, and the antigenic probe concentration versus the percent absorbance is presented graphically.

Solid-Phase ELISA. In the direct binding assay, the experimental conditions were first optimized in terms of concentration of the glucopeptides **1** and **7** (10 or 20 μg/mL), coating buffer (0.05 M carbonate

buffer at pH 9.6 or 0.01 M phosphate buffer saline, PBS, at pH 7.4), and saturation reagent (bovine serum albumin, BSA, or FCS).

ELISA plates (Nunc Maxisorp, Thermo) were coated with glucopeptides **1** and **7** (10 μg/mL) in carbonate buffer and incubated overnight at 4 °C. Saturation was carried out with PBS containing 3% BSA (Sigma Aldrich) for 45 min at room temperature. Sera diluted 1:100 in PBS, 1% BSA, and 0.05% Tween-20 were incubated on the plates for 3 h at room temperature. After washings with saline containing 0.05% Tween 20, alkaline phosphatase conjugated antihuman IgM (Sigma Aldrich) diluted 1:500 was added to the wells, and the plates were incubated for 2 h at room temperature. Alkaline phosphatase activity was revealed with *p*-nitrophenyl phosphate in sodium carbonate buffer.

Sample Preparation for NMR Analysis. The samples for NMR experiments in mixed micelles of DPC/SDS (90/10 M:M) were prepared by dissolving an appropriate amount of peptide (1.5 mM) in a DPC/SDS water mixture. The DPC concentration used was 27 mM (27 times higher than DPC cmc),[51] and the molar DPC:SDS ratio was 90/10 (27 mM/3 mM) to mimic the ELISA solid surface. For NMR experiments, ^{d25}SDS and ^{d38}DPC were used.

NMR Analysis. NMR spectra were collected using a Bruker DRX-600 spectrometer at 300 K. One-dimensional NMR spectra were recorded in the Fourier mode with quadrature detection. The water signal was suppressed by low-power selective irradiation in the homogated mode. DQF-COSY, TOCSY, and NOESY[36,37,55] experiments were run in the phase-sensitive mode using quadrature detection in ω_1 via time-proportional phase increases of the initial pulse. Data block sizes were 2048 addresses in t_2 and 512 equidistant t_1 values. Prior to Fourier transformation, the time domain data matrices were multiplied by shifted $\sin^2$ functions in both dimensions. A mixing time of 70 ms was used for the TOCSY experiments. NOESY experiments were run with mixing times in the range of 100−300 ms. Qualitative and quantitative analyses of DQF-COSY, TOCSY, and NOESY spectra were achieved using SPARKY software.[39]

NMR Structure Calculations. Peak volumes were translated into upper distance bounds with the CALIBA routine from the CYANA software package.[41] The requisite pseudoatom corrections were applied for nonstereospecifically assigned protons at prochiral centers and for the methyl group. After discarding redundant and duplicated constraints, the final list of experimental constraints was used to generate an ensemble of 100 structures by the standard CYANA protocol of simulated annealing in torsion angle space implemented (using 6000 steps). No dihedral angle or hydrogen bond restraints were applied. The best 20 structures that had low target function values (0.83−1.19) and small residual violations (maximum violation = 0.38 Å) were refined by in vacuo minimization in the AMBER 1991 force field using the SANDER program of the AMBER 5.0 suite.[42,43] To mimic the effect of solvent screening, all net charges were reduced to 20% of their real values. Moreover, a distance-dependent dielectric constant ($\varepsilon = r$) was used. The cutoff for nonbonded interactions was 12 Å. NMR-derived upper bounds were imposed as semiparabolic penalty functions, with force constants of 16 kcal/mol Å. The function was shifted to be linear when the violation exceeded 0.5 Å. The best 10 structures after minimization had AMBER energies ranging from −441.4 to −391.1 kcal/mol. Final structures were analyzed using the Insight 98.0 program (Molecular Simulations, San Diego, CA).

■ ASSOCIATED CONTENT

Supporting Information

Database of the 332 human myelin proteins (XLS), NMR and fluorescence data of the analyzed peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

Abs, antibodies; BD, blood donor; CSI, chemical shift index; DIPEA, *N*,*N*-diisopropylethylamine; DPC, dodecylphosphatidylcholine; FAN, factor associated with neutral sphingomyelinase activation; FCS, fetal calf serum; HOBt, hydroxybenzotriazole; MS, multiple sclerosis; MOG, myelin oligodendrocyte glycoprotein; NMM, *N*-methylmorpholine; OMG, oligodendrocyte myelin glycoprotein; OND, other neurological disease; RTN4R, reticulon-4 receptor; SD, standard deviation; SDS, sodium dodecylsulphate; TBTU, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate

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