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GMDP: unusual physico-chemical and biological properties of the anomeric forms

Elena A. Meshcheryakova,* Konstantin S. Mineev, Pavel E. Volynski, Tatiana M. Andronova and Vadim T. Ivanov

Disaccharide containing unit of peptidoglycan from bacterial cell wall, *N*-acetyl-D-glucosaminyl-*N*-acetylmuramyl-L-alanyl-D-glutaminamide (glucosaminyl-muramyl-dipeptide) registered in Russia as an immunomodulatory drug, is shown to participate in slow equilibrium of α and β anomeric forms. Data of NMR spectra and molecular dynamics indicate that the α-anomer predominantly acquires a folded conformation stabilized by intramolecular hydrogen bond between the alanyl carbonyl and muramyl NH proton. The β-form displays a considerable fraction of extended, non-hydrogen bonded structures. In the standard immunoadjuvant test system, the α-form is practically inactive, and the activity of the equilibrium mixture with $\alpha:\beta = 68:32$ ratio is due to the presence of β-anomer. Such unique α–β selectivity of biological action must be considered at the design of related immunoactive glycopeptides. Copyright © 2015 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: glycopeptide; anomers; equilibrium; NMR; computer simulation; adjuvant; activity

Introduction

The standard building block of bacterial cell wall, *N*-acetyl-D-glucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamin (glucosaminyl-muramyl-dipeptide, GMDP), displays along with other muramyl peptides a broad spectrum of biological effects [1–6]. Since 1995, it was used in Russia as an oral therapeutic for treatment of immunodeficient states of various etiologies under the trade mark Licopid (number 95/211/4 in the Russian Federation Government Register of medicines). By analogy with other related glycopeptides, in particular *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) [7], it could be assumed that GMDP should form in solution a dynamic equilibrium of anomeric α-isomer and β-isomer with axial orientation of the C_1 muramic hydroxyl in the α-isomer and equatorial orientation in the β-isomer (Figure 1). In the present work, the assumption was experimentally confirmed, the anomeric forms isolated, and equilibrium kinetics studied. The conformational states of α-anomer and β-anomer were studied by spectroscopic techniques (circular dichroism and NMR-proton magnetic resonance) and molecular dynamics. Immunomodulatory action of α-enriched and β-enriched GMDP samples was measured in the standard adjuvant test system. The α-isomer proved practically inactive, the β-form being fully responsible for the adjuvant activity of GMDP. The unique α–β selectivity of GMDP action was correlated with conformational preferences of the two anomers.

Materials and Methods

Chromatography

Preparative GMDP fractionation was carried out on the preparative Knauer HPLC system supplied with reverse phase 100 × 600 mm column, filled with Polygoprep 100-30, C18 (Macherey-Nagel, PA, USA). La Chrom Hitachi instrument supplied with autosampler and reverse phase Macherey-Nagel Nucleosil C18, 123 × 3 mm column was used for analytical purposes.

Circular dichroism spectra were recorded on a Jasco J-500A, (Japan) instrument in water (pH 4.0) at 23°, 0.3 mg/ml concentration, cell path 0.5 mm.

NMR Spectroscopy

Nuclear magnetic resonance spectra were recorded on 800-MHz Avance III Bruker spectrometer, (Germany) equipped with (^{1}H, ^{13}C, and ^{15}N) triple resonance TCI cryoprobe. ^{1}H spectra were measured with 10-s relaxation delay using WATERGATE [8] technique to suppress the strong signal of the solvent protons. ^{1}H chemical shifts were measured relative to H_2O protons, the chemical shift of the signal being arbitrarily chosen as 4.75 ppm at 30°. Content of α-anomer and β-anomer was determined from intensities of corresponding signals from N–Ac and C^{α}–CH_3 groups of muramic acid in 1D NMR spectra by line shape fitting performed in TOPSPIN software (Bruker Biospin GMBH, Germany). Chemical shift assignment and interproton interaction measurements were performed by 2D NMR spectroscopy. 2D DQF COSY [9] and 2D NOESY [10] (mixing time 250 ms) spectra were recorded with the use of WATERGATE

* *Correspondence to: Elena A. Meshcheryakova, M. M. Shemyakin & Yu. A. Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia. E-mail: meshcheryakova.elena@mail.ru*

M.M.Shemyakin & Yu. A. Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia

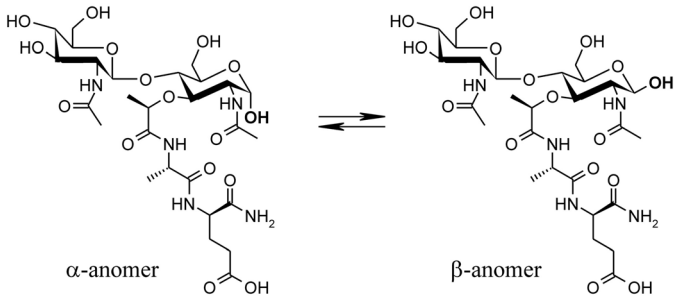

Figure 1. Equilibrium of GMDP anomers.

[8] for solvent suppression. $^{3}J_{NH-CH}$ scalar couplings were measured by fitting 2D cross peaks in NOESY [10] spectra with two Lorentsian peaks in Wolfram Research Mathematica software. Kinetics of amide proton exchange to solvent protons was assessed by 1D CLEANEX [11] spectrum (20-ms mixing delay). Magnitudes of peaks in the spectra were normalized by the integral of the most intense signal, and the resultant values were considered as relative exchange rates for the corresponding amide protons.

Computer Simulations

Molecular dynamics analysis was carried out for GMDP analog, methyl *N*-acetylmuramyl dipeptide (MeMDP) with the *N*-acetylglucosaminyl carbohydrate residue replaced with methyl radical. The starting state of the system was constructed as follows. MeMDP in a random conformation was placed into a cubic box and solvated by TIP3P ($\sim 7 \times 10^3$) molecules of water [12]. The system was equilibrated by energy relaxation via 5×10^4 steps of the steepest descent minimization followed by heating from 5 K to the temperature of simulation (310 K) during 500 ps MD run. After that, 100 ns collection MD runs were carried out. All simulations were performed with the use of GROMACS 4.5 package [13] and the ff99SB-ILDN force field [14], with a time step of 2 fs and imposed 3D periodic boundary conditions, in the NVT ensemble at 310 K. A twin-range (12/20 Å) spherical cutoff function was used to truncate non-bonded interactions. The system was coupled through the v-rescale thermostat [15] to a temperature bath with 0.1-ps coupling constant. Coordinates were collected at 10-ps interval.

Immunoadjuvant Activity Test

BALB/c mice, weighing 17–20 g, female, six animals per group, were immunized i.p. in 0.2 ml of saline with 25 μg/animal ovalbumin immediately followed by injection of 100 μg/animal of GMDP in 0.2 ml of saline (the sample prepared directly before the injection). The control group received only ovalbumin. Two boostings with 12.5 μg of ovalbumin were carried out over two week intervals. One week after the final boosting, antisera were collected, and the antiserum from each animal was analyzed by standard ELISA [16]. The local ethics committee approved the study. The results of triplicate independent experiments were presented as mean values ±SE, using Student's *t*-test.

Results and Discussion

Preparation of α-enriched and β-enriched GMDP Samples

A total of 26.0 g of crude GMDP obtained as described in [17] by hydrogenation of the respective benzyl ester was applied in 1000 ml of phase A (water) to the preparative HPLC column. Elution was carried out with 200 ml/min flow rate of phase A for 10 min followed by linear gradient of 0% to 20% of phase B (60% v/v EtOH–H_2O). GMDP fractions 1 and 2 containing, respectively, 6.0 and 3.0 g were collected at time intervals shown in Figure 2 and freeze dried. About 1 mg of each sample in 1 ml of water was applied to the analytical HPLC column and eluted at 0.7 ml/min flow rate with the 0.05% trifluoroacetic acid/80% acetonitrile gradient. In both cases, two identical components were found with 82 : 18 ratio in fraction 1 and 5 : 95 ratio in fraction 2. As follows from the NMR spectra (see succeeding text), sample 1 is the enriched β-anomer and sample 2 is predominantly the α-anomer of GMDP.

Dynamics of $\alpha \leftrightarrow \beta$ GMDP Equilibrium

After dissolving samples 1 and 2 in water (1 mg/ml, 25°, pH 3.7), the content of anomeric forms measured by analytical HPLC gradually changes after reaching ~150 min the equilibrium $\alpha : \beta$ ratio of 68 : 32 or $\alpha/\beta = 2.12$ (Figure 3). The respective mutarotation constant, 1.37×10^{-2}, followed from the slope of semilogarithmic plot shown in Figure S1 (Supporting Information). Both the time required for reaching the equilibrium and the $\alpha : \beta$ equilibrium ratio found for GMDP are close to respective MDP values – 3 h for reaching the equilibrium and 53.6 : 26.8 or $\alpha/\beta = 2.00$ [18]; in [19], $\alpha/\beta = 2.04$ was found. The anomerization rates of sample 2 in physiological solution and in 10% mice blood serum were indistinguishable from the rate shown in Figure 3.

We also found that in 96% ethanol at 25° C, the α/β ratio is 85.3 : 14.7, i.e. less polar medium shifts the equilibrium in favor of the α-isomer. Relative to the solution state, the freeze-dried samples 1 and 2 are conformational quite stable, although at long storage, the anomeric ratio gradually shifts to the aqueous equilibrium state: after 2 years at −20° C, the content of α-form in sample 2 dropped from 95% to 85% while the content of β-form in sample 1 dropped from 82% to 63%.

CD Spectra

The spectra of samples 1 and 2 as well as the computed spectra for the pure α-isomer and β-isomer have similar shape in the 210–240-nm range with weak $n - \pi$ effects at ~235 nm (Figure S2). At shorter wavelengths ($\pi - \pi$ transitions), the effects are more intense and of opposite sign. Although quite distinct, these spectra do not provide any concrete information on the conformational preferences of the anomers.

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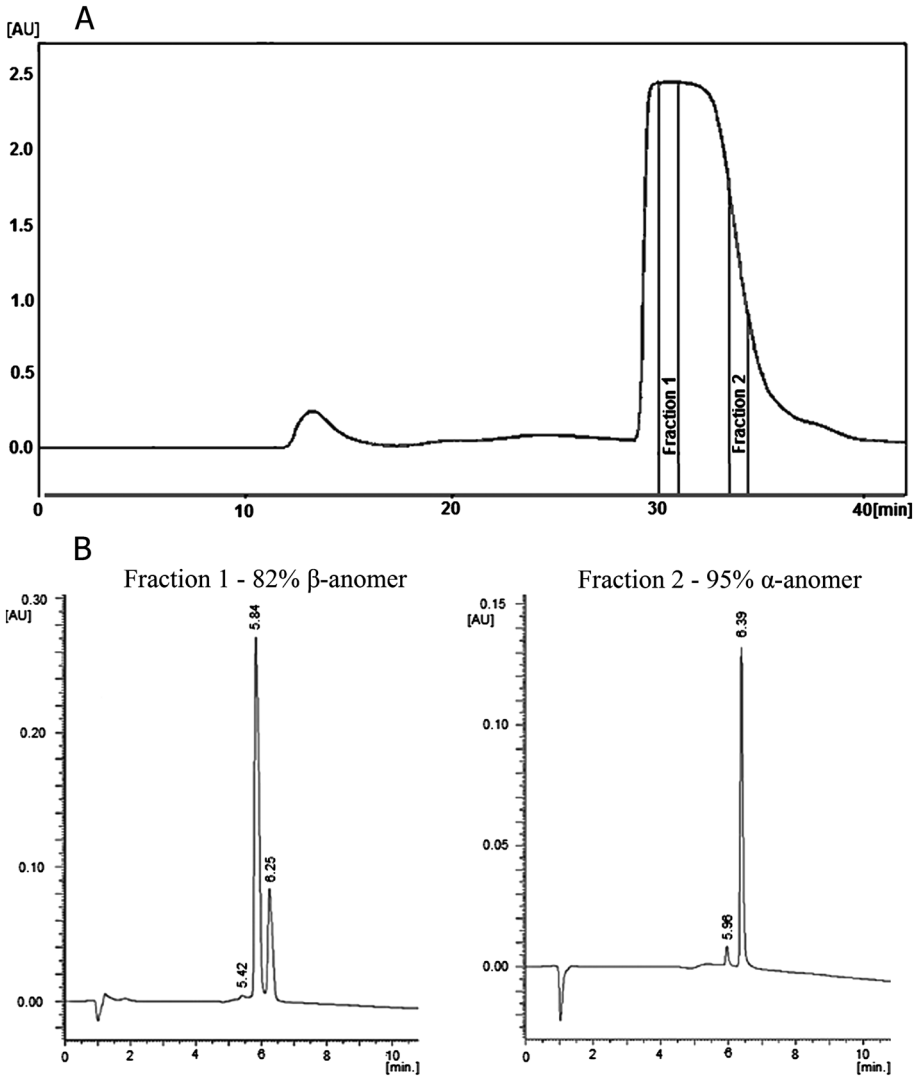

Figure 2. Chromatographic separation of GMDP anomers: (A) preparative scale; (B) analytical control.

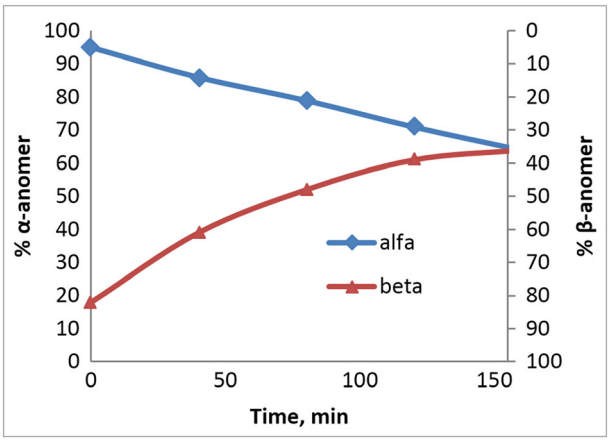

Figure 3. Time dependence of GMDP anomeric equilibrium.

NMR Spectra

H^1 NMR spectra of GMDP were measured in D_2O/H_2O (9 : 1), at approximately 5 mg/ml, 30° C, at pH 4.0 and pH 7.5. All spectra were taken 4–5 h after dissolving the sample, i.e. at the established anomeric equilibrium. The observed spectra display superposition of the spectra of individual monomers with their considerably differing chemical shifts, especially in the area of MurNAc signals (Figure 4). The respective chemical sifts and $^3J_{NH-CH}$ coupling constants are shown in Tables S1 and 1. Besides, the intensity of interproton contacts was measured at pH 7.5 (Table S2) as well as the kinetics of NH-solvent exchange (Table 2). Assignment of spectral signals to α and β forms followed from the value of MurNAc H1–H2 vicinal coupling constants, which must be lower in the α-form (approximately 2–4 Hz as cited in [20]; 3.1 Hz found in GMDP) and higher in the β-form (7–9 Hz theoretically; 8.6 Hz found in GMDP). Strong or medium interproton interactions $C_1H–C_2H$ in one of the forms and $C_1H–C_3H$, $C_1H–C_5H$ in the others as seen in Table S3 and shown in Figure 5 also confirm the anomeric assignments given in Tables 1, 2, S1, and S2 as well as in Figures 2–4. In accordance with the aforementioned HPLC data, the intensity ratio of the muramic NAc and $C^{\alpha}–CH_3$ α and β signals was 70 : 30. High values of the $^3J_{NH-CH}$ constants in the β-MurNAc residue and in the GlcNAc residue (not participating in the $\alpha \leftrightarrow \beta$ equilibrium) point to *trans* orientation of NH–CH protons, while in the α-MurNAc residue, the respective values are approximately 2 Hz lower, indicating deviation from *trans*-conformation [21].

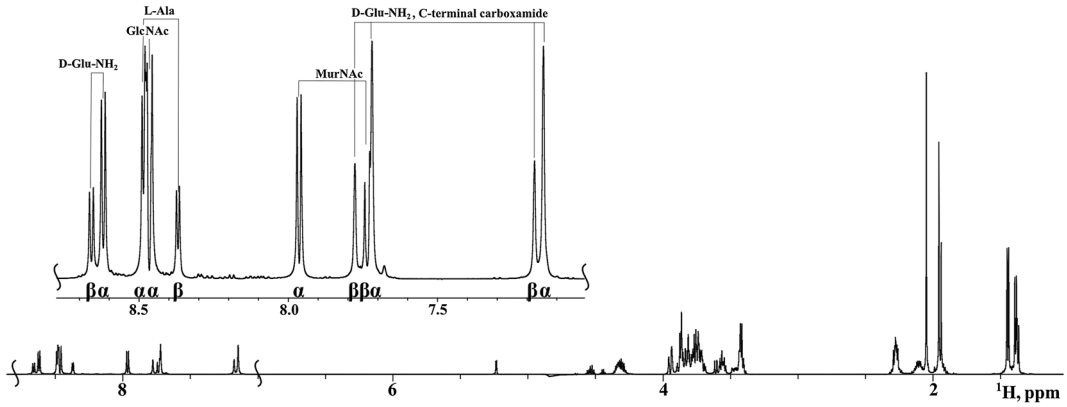

Figure 4. NMR spectra of GMDP.

Table 1. $^{3}J_{NH-CH}$ constants (Hz) of GMDP

Residue	pH 4.0		pH 7.5	
	α	β	α	β
GlcNAc	10.3	10.3	11.2	11.2
MurNAc	7.8	9.8	8.8	9.8
Ala	5.3	5.3	5.3	5.1
D-Glu(NH_2)	8.0	7.8	8.1	8.3

GMDP, glucosaminyl-muramyl-dipeptide.

Table 2. Relative exchange rates of GMDP amide protons (H_2O, pH 7.5)

Anomer	Residue					
	GlcNAc	MurNAc	Ala	D-Glu (NH_2)	D-Glu (NH_2) (NH2-1)	D-Glu (NH_2) (NH2-2)
α	13	1	65	100	49	49
β	13	13	46	100	59	49

GMDP, glucosaminyl-muramyl-dipeptide.

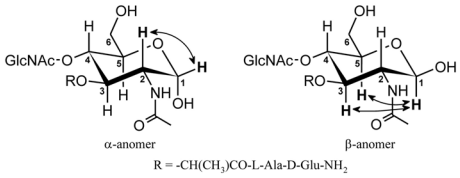

Figure 5. Anomer specific interatomic contact in GMDP as obtained from NMR spectra.

Comparison of NH-solvent proton exchange rates (Table 2) unexpectedly revealed a strong, more than tenfold suppression of exchange rates of the muramyl NH proton in the α-form comparing with the β-form, indicating lower availability of the NH proton to the solvent. As a rule, such effect is ascribed to the formation of intramolecular hydrogen bonds involving that proton. In summary, the NMR data provide clear evidence of conformational transition within the MurNAc residue accompanying the $\alpha \leftrightarrow \beta$ equilibrium of GMDP.

Molecular Dynamics Study: Correlation with NMR Data

Complementary information on the conformational equilibrium of GMDP anomers was obtained from the analysis carried out for the analog, MeMDP, in which the GlcNAc residue (whose NMR parameters are indifferent to the anomeric state of the parent molecule) is replaced for simplicity with methyl radical. The results of simulation study summarized in Table 3 show that independently of MurNAc C_1 hydroxyl orientation, the MurNAc NH proton can form hydrogen bonds with any of the three carbonyl oxygens in the peptide chain (Figure 6). Of the three possible H-bonded structures, the 14-membered cycle formed by alanyl carbonyl is the most stable. The analysis denied any essential formation of hydrogen bonds between the MurNAc C_1 hydroxyl and the MurNAc carbonyl (hypothetically shown in Figure 6), as well as of hydrogen bonds between the same anomeric hydroxyl and peptide chain carbonyls. According to the analysis, the MurNAc NH proton is relatively unavailable to hydration in the α-form comparing with the β-form (15.6% and 43.0% hydration, respectively). This result at least partially explains the aforementioned reduced exchange rate in the α-form. On the whole, the analysis revealed two major structural MeMDP clusters of which the first, occupying over 90% of the total time in the α-form, has a relatively compact, folded structure stabilized by the NH(MurNAc)-CO(Ala) hydrogen bond and the second, comprising over 35% of the β-form, has an extended, conformationally mobile structure not containing intramolecular

Table 3. Simulation data. H-bonding (%) in the GMDP model

H-bond	α	β
NH(MurNAc)-CO(Lac)	7.6	2.1
NH(MurNAc)-CO(Ala)	50.3	34.4
NH(MurNAc)-CO(Glu)	1.3	1.5
NH(MurNAc)-O(water)	15.6	43.0
OH1(MurNAc)-CO(Ala)	2.1	0
OH1(MurNAc)-CO(Glu)	2.9	0
OH1(MurNAc)-COO(Glu)	1.3	0
OH1(MurNAc)-CO(MurNAc)	0	2.0
OH1(MurNAc)-O(water)	79	79

GMDP, glucosaminyl-muramyl-dipeptide.

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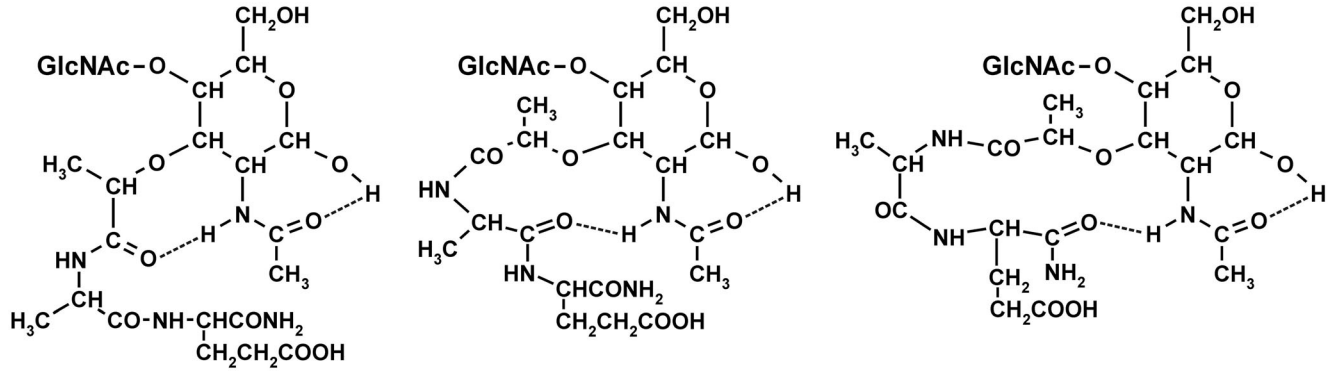

Figure 6. H-bonded versions of GMDP structure as obtained from MD simulation.

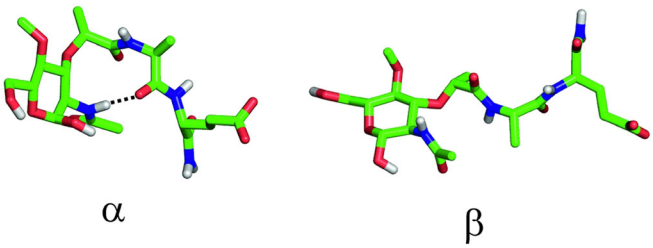

Figure 7. Dominating compact (α) and extended (β) structures of GMDP model as obtained from MD simulation.

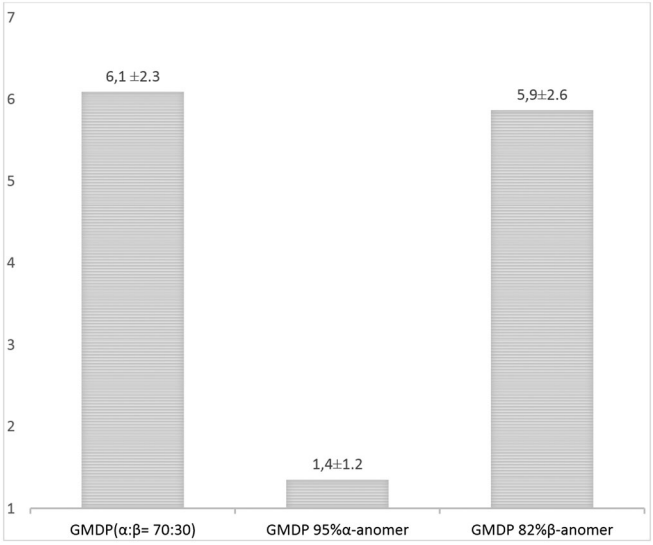

Figure 8. Stimulation of ovalbumin antibody production in BALB/c mice by GMDP samples with different $\alpha:\beta$ ratio (fold over control).

hydrogen bonds (Figure 7). Relative abundances and rotational angles (ϕ, ψ, and ω marked as shown in Figure S3) in the compact and extended α-form and β-form are presented in Table S3. More detailed dynamic presentation of hydrogen bonding and bond rotation in the GMDP model is given in Figures S4 and S5. Analogous behavior is assumed for GMDP itself.

Adjuvant Activity of GMDP Anomers

Biological activity of α-enriched and β-enriched GMDP samples 1 and 2 was evaluated in the test system implying stimulation of antibody formation in mice after immunization with ovalbumin. Immunization was carried out at 100 μg/animal dose of GMDP which in case of the equilibrium mixture of anomers (i.e. 68% of α-form and 32% of β) induces the maximal effect, approximately sixfold increase of antibody titers. Under these conditions, sample 2 with $\alpha:\beta$ ratio, 95 : 5, (corresponding to sixfold reduction of β-anomer concentration) was close to inactive, while sample 1 with 18 : 82 ratio (i.e. 2.6 times enriched with the β-form) retained full activity (Figure 8). In other words, the α-anomer showed negligible activity in the classic adjuvant test conditions, and the activity of the equilibrium mixture is entirely due to the presence of β-anomer. It is not clear if the inactivity of the α-anomer is related to transmembrane transport of GMDP and its intra-cell delivery problems or to the stage of receptor binding.

In any case, the functionally important steps involving GMDP apparently take place with rates significantly faster than the $\alpha\leftrightarrow\beta$ anomeric conversion.[‡]

Conclusion

Preparative separation of GMDP anomers allowed studying their individual conformational and biological properties. The α-anomer predominantly acquires a folded conformation stabilized by intramolecular CO···HN hydrogen bonds and is biologically inactive in the assay system. The biological activity of GMDP is linked to the β-anomer, which forms in aqueous solution a considerable fraction of extended structures. Such α–β anomer specificity of bioactive carbohydrate containing molecule to our knowledge has not been described so far in the available literature and should be considered in the design of novel preparations.

Conflicts of Interest

Authors declare no conflicts of interest.

Acknowledgements

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[‡]Earlier, we observed stimulation of peritoneal macrophages in mice 20 min after GMDP injection (unpublished).

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