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SYNTHESIS OF GLYCINATED GLYCOCONJUGATES BASED ON 1-THIOGLYCOSIDES AND THEIR PRELIMINARY STUDIES AS POTENTIAL IMMUNOMODULATORY FACTOR

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Abstract: The biological importance of lipopolysaccharides (LPS), components of bacterial cell wall has not been explained sufficiently. The glycine present in these structures could play an important role in the immunological response after bacterial infections and during sepsis. In our studies we obtained synthetic and stable substituted glycinated 1-thioglycosides derivatives of monosaccharides, e.g., D-glucose or D-galactose as well as disaccharides, e.g., melibiose and lactose. The conditions of acylation reactions were validated and specific products were separated by using chromatography methods. Their structures were confirmed by NMR. These compounds were conjugated with carrier proteins e.g., bovine serum albumin and horse myoglobin. Prior to conjugation proteins were modified with glycidol to create the protein-diol intermediates and subsequent periodate oxidation of the glycol moieties to generate the reactive aldehyde functionalities. Modified and formylated carrier proteins were conjugated with acylated thioglycosides in the presence of sodium cyanoborohydride. Subsequently, the products obtained were analyzed in SDS-PAGE and separated by using HW-55S gel-filtration chromatography. The immunoreactivity of selected glycinated glycoconjugates were studied in ELISA assays with specific anti-aminoacylated glyconjugate antibodies obtained after rabbit immunization with *Escherichia coli* K12 C600 core oligosaccharide glycine-containing glycoconjugate. The differences in the immunoreactivity of different glycinated 1-thioglycosides were observed. The received glycine-acylated glycoconjugates could mimic the non-sugar substituents localized in various bacterial LPS. These synthetic compounds could be candidates for their use as glycoconjugate vaccines in protection against serious bacterial infections, e.g., sepsis.

Keywords: glycine epitope, lipopolysaccharide, glycinated 1-thioglycosides, glycoconjugates, bacterial infections, sepsis

Vaccinations may provide an efficient and effective way to prevent persistent and severe infectious diseases in humans (1, 2). The increasing resistance of bacterial pathogens to antibiotics is the inspiration to the searching of new and immunologically active epitopes in composed antigens. These epitopes should be able to modulate immunological response through antibodies production, what is a promising way to obtain long-lasting immunological protection.

Lipopolysaccharide (LPS) moiety is of great importance in the pathogenesis of sepsis and septic shock and the glycine localized in their structure can play an important role in the immunological response after bacterial infections (3–5). The glycine residue is one of the non-sugar substituents localized in core part of bacterial LPS from various pathogenic strains e.g., *Neisseria meningitidis, Escherichia coli*, *Hafnia alvei*, *Shigella flexneri*, *Salmonella typhimurium*, or *Haemophilus influenzae* (reviewed in 4). Structural and functional studies under glycine presence in the bacterial lipopolysaccharide (LPS) are complicated because of its lability. Biological importance of labile sugar-aminoacylated structures based on glycine has not been explained sufficiently so far. When creating

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epitope, this structure probably plays an essential role in the immunomodulatory reactions during bacterial infections (4, 6). We suppose that synthetic thioglycosides are proper candidates for using them in the new glycoconjugates synthesis in the combination with modified protein carriers (7–9). Stable substituted glycinated 1-thioglycosides were synthesized and structurally characterized by NMR method (9). The different synthetic glycinated 1-thioglycosides were conjugated with proteins by the implementation of reductive amination method (10). It is a very useful strategy which requires that one of the two species to be conjugated should carry aldehyde functions, while the other species should be an amine-containing compound (10). Synthetic glycinated 1-thioglycosides based on monosaccharides and disaccharides used in these studies contain free functional amine group in the short linker structure (Scheme 1). Obtained different glycine-acylated glycoconjugates as the model structures may mimic the common epitopes in different bacterial endotoxins. Such synthetic antigens could be applied for broadly reactive antibodies production which would be able to neutralize endotoxin biological activity (4).

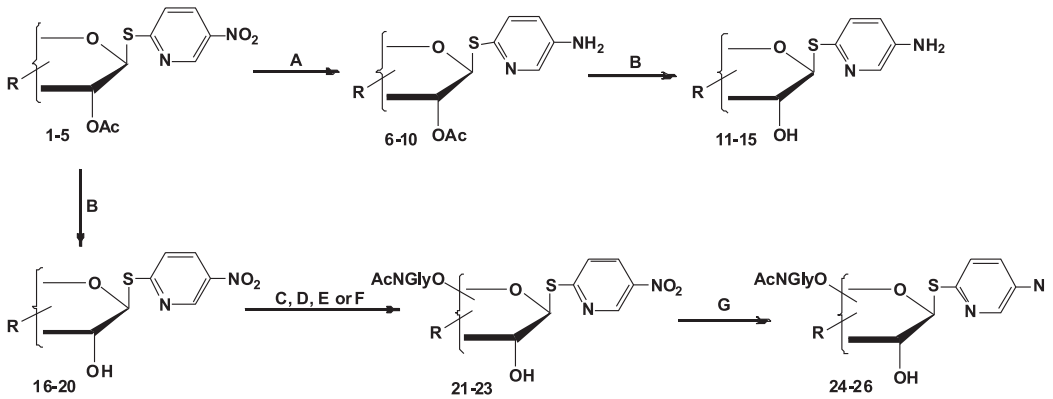
Bovine serum albumin (BSA) and horse muscle myoglobin (MYO) as carrier proteins were used in our experiments. There are many reports on BSA as an effective carrier protein used for glycoconjugate vaccine synthesis, considering the fact that BSA is one of the cheapest commercially available protein used as a common model protein for various basic studies and has broad practical applications (11–14). For example, unique *Vibrio cholerae* LPS-protein conjugates based on BSA were able to induce significant levels of antigen-specific Ig isotypes, especially of IgG and IgM (15–17). On the other hand, *Shigella sonnei* O-specific oligosaccharide-core-BSA conjugates (O-SPC) revealed some immunogenicity and the coupling reaction with BSA was carried out at a neutral pH and room temperature (18).

Obtained in our laboratory synthetic sugar-protein compounds could be used as glycoconjugate vaccines in protection against serious bacterial infections.

EXPERIMENTAL

Chemicals and reagents

All chemicals (e.g., albumin from bovine serum, myoglobin from equine skeletal muscle, glycidol, 2,4,6-trinitrobenzene sulfonic acid (TNBS), ammonium acetate, sodium cyanoborohydride) used in experiments were of analytical grade and purchased from Sigma-Aldrich (Poland) or ACROS Organics (Geel, Belgium). The aluminum sheets coated with silica gel 60 F_{254} for TLC experiments



Scheme 1. Synthesis of non-glycinated or glycinated derivatives of 5-amino-2-pyridyl-1-thioglycosides

were from Merck. The Bio-Gel P-4 and resin HW-55S for column chromatography were purchased from Bio-Rad (France) and Toyopearl (Tosoh Bioscience, Japan), respectively.

Reagents for electrophoresis were from Bio-Rad (France). ELISA experiments were performed by using serum obtained after rabbit immunization with *E. coli* K12 C600 core oligosaccharide glycine-containing glyconjugate kindly obtained from Institute of Immunology and Experimental Therapy in Wrocław (Poland). Secondary antibodies peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) were purchased from Jackson ImmunoResearch, Biokom (Poland).

UV/VIS measurements were performed by using spectrophotometer Jasco (Model V-530, Jasco Inc., Japan). The optical densities in ELISA assay were read at $\lambda = 492$ nm in a Labsystems Multiskan MS Microplate Reader.

The ^{1}H-NMR and ^{13}C NMR spectra were recorded for solutions in $CDCl_3$ or CD_3OD with Varian spectrometer at 300 MHz or 600 MHz, using TMS as the internal standard. NMR solvents were purchased from ACROS Organics (Geel, Belgium). Chemical shifts (δ) are expressed in ppm and coupling constants (J) in Hz. The following abbreviations were used to explain the observed multiplicities: s, singlet; d, doublet; dd, doublet of doublets; ddd, doublet of doublet of doublets; t, triplet; dd~t, doublet of doublets looking as triplet; m, multiplet; b, broad. Optical rotations were measured on JASCO P-2000 Series polarimeter using a sodium lamp (589.3 nm) at room temperature. Reactions were monitored by TLC. TLC plates were inspected under UV light ($\lambda = 254$ nm) and developed by charring after spraying with 10% H_2SO_4 in EtOH. Column chromatography was performed on silica gel 60 (70–230 mesh, Fluka) developed with either toluene:AcOEt or $CHCl_3$:MeOH solvent systems. Organic solvents were evaporated on a rotary evaporator under diminished pressure at 50°C.

General procedures

Synthesis of (5-nitro-2-pyridyl) per-*O*-acetyl-1-thio-β-D-glycosides derivatives of D-glucose (**1**), D-galactose (**2**), maltose (D-Glc-α(1>4)-D-Glc) (**3**), lactose (D-Gal-β(1>4)-D-Glc), (**4**) and melibiose (D-Gal-α(1>6)-D-Glc) (**5**) was performed according to previously published procedures (8). 4-(4,6-Dimethoxy-(1,3,5)-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) was obtained by the procedure implementation described by Kunishima (19).

Procedure A: reduction of nitro group in per-*O*-acetylated 1-thioglycosides aglycone

Compounds **1**–**5** (1 eqv.) were dissolved in CH_2Cl_2. To the resulting solution acetic acid and zinc powder (8 eqv.) were added. The whole mixture was stirred at room temperature. The reaction was monitored by TLC on silica gel plates using toluene:AcOEt (1:1, v/v) solvent system. After completion of reaction, zinc was filtered off, the reaction mixture was diluted with CH_2Cl_2 and washed with brine (3×10 mL). The organic layer was dried over anhydrous $MgSO_4$, the adsorbent was filtered off and the filtrate was concentrated to give crude products (**6**–**10**) which were purified by column chromatography.

Procedure B: deprotection of (5-amino-2-pyridyl) or (5-nitro-2-pyridyl) per-*O*-acetylated 1-thioglycosides

Compounds **1**–**10** (1 eqv.) were suspended in MeOH. To the resulting mixture 1 M methanolic solution of MeONa (1 eqv.) was added. The whole mixture was stirred at room temperature. The reaction was monitored by TLC on silica gel plates using $CHCl_3$: MeOH (5:1, v/v) solvent system. After completion of reaction, reaction mixtures were neutralized by adding ion exchange resin Amberlyst 15. The resin was filtered off and the organic layers were concentrated with small amount of silica gel in order to prepare samples of crude products (**11**–**20**) for purification by column chromatography.

Procedure C: acylation of (5-nitro-2-pyridyl) 1-thioglycosides with *N*-acetyl-L-glycine using DCC as coupling agent

To a solution of compound **16** (0.32 mmol) and DCC (0.35 mmol) in dry pyridine (5 mL) an equimolar amount of *N*-acetyl-L-glycine (0.32 mmol) was added. The mixture was stirred at room temperature. The reaction was monitored by TLC on silica gel plates using $CHCl_3$: MeOH (5:1, v/v) solvent system. After 24 h, the reaction mixture was concentrated and crude products were purified by column chromatography with $CHCl_3$/MeOH 100:1 → 10:1 solvent system.

Procedure D: acylation of (5-nitro-2-pyridyl) 1-thioglycosides with *N*-acetyl-L-glycine using DCC/DMAP system as coupling agent

To a solution of compound **16** (0.32 mmol), DCC (0.35 mmol) and DMAP (0.03 mmol) in dry pyridine (5 mL), an equimolar amount of *N*-acetyl-L-glycine (0.32 mmol) was added. The mixture was stirred at room temperature. The reaction was mon-

itored by TLC on silica gel plates using $CHCl_3$: MeOH (5:1, v/v) solvent system. After 24 h the reaction mixture was concentrated and crude products were purified by column chromatography with $CHCl_3$/MeOH 100:1 → 10:1 solvent system.

Procedure E: acylation of (5-nitro-2-pyridyl) 1-thioglycosides with *N*-acetyl-L-glycine using DCC/HOBt system as coupling agent

To a solution of compound **16** (0.32 mmol), DCC (0.35 mmol) and HOBt (0.03 mmol) in dry pyridine (5 mL), an equimolar amount of *N*-acetyl-L-glycine (0.32 mmol) was added. The mixture was stirred at room temperature. The reaction was monitored by TLC on silica gel plates using $CHCl_3$: MeOH (5:1, v/v) solvent system. After 24 h, the reaction mixture was concentrated and crude products were purified by column chromatography with $CHCl_3$/MeOH 100:1 → 10:1 solvent system.

Procedure F: acylation of (5-nitro-2-pyridyl) 1-thioglycosides with *N*-acetyl-L-glycine using DMT-MM as coupling agent

To a solution of compound **16** (0.32 mmol in dry THF (3 mL), an equimolar amount of *N*-acetyl-L-glycine (0.32 mmol) was added. To this mixture an equimolar amount of DMT-MM (0.32 mmol) and NMM (0.32 mmol) were added. The mixture was stirred at room temperature. The reaction was monitored by TLC on silica gel plates using $CHCl_3$: MeOH (5:1, v/v) solvent system. After 24 h, the reaction mixture was concentrated and crude products were purified by column chromatography with $CHCl_3$/MeOH 100:1 → 10:1 solvent system.

Procedure G: reduction of glycinated (5-nitro-2-pyridyl) 1-thioglycosides

Compounds **21**–**23** (1 eqv.) were dissolved in MeOH. To the resulting solution acetic acid and zinc powder (8 eqv.) were added. The whole mixture was stirred at room temperature. The reaction was monitored by TLC on silica gel plates using $CHCl_3$: MeOH (2:1, v/v) solvent system. After completion of reaction, zinc was filtered off, the reaction mixture was neutralized with Et_3N and concentrated to give crude products (**24**–**26**) which were purified by column chromatography.

(5-Amino-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (**6**)

(5-Nitro-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside **1** (1.95 g, 4 mmol) in CH_2Cl_2 (60 mL) with addition of acetic acid (34 mL) and zinc powder (1.31 g, 20 mmol) were submitted to the general procedure **A** described above. Reaction time: 20 min. Product **6** (1.51 g, 83%) was obtained as a light yellow solid after purification by column chromatography with toluene:AcOEt (8:1 to 1:2, v/v) solvents system. $[\alpha]_D^{20} = -9.9^o$ (c = 1.1, $CHCl_3$), m.p. 65–66°C; ^{1}H NMR ($CDCl_3$, δ_H, ppm): 2.01, 2.03, 2,04, 2.05 (4×s, 12H, CH_3CO), 3.40–3.95 (bs, 2H, NH_2), 3.77 (ddd, 1H, *J* = 2.3 Hz, *J* = 4.6 Hz, *J* = 10.0 Hz, H-5), 4.09 (dd, 1H, *J* = 2.3 Hz, *J* = 12.5 Hz, H-6a), 4.25 (s, 1H, *J* = 4.6 Hz, *J* = 12.5 Hz, H-6b), 5.12 (d, 1H, *J* = 9.8 Hz, H-4), 5.13 (dd~t, 1H, *J* = 10.5 Hz, H-2), 5.30 (dd~t, 1H, *J* = 9.3 Hz, H-3), 5.40 (d, 1H, *J* = 10.5 Hz, H-1), 6.91 (dd, 1H, *J* = 2.7 Hz, *J* = 8.4 Hz, H-4_{pyr}), 7.14 (d, 1H, *J* = 8.4 Hz, H-3_{pyr}), 7.99 (d, 1H, *J* = 2.7 Hz, H-6_{pyr}). ^{13}C NMR ($CDCl_3$, δ_C, ppm): 20.60, 20.62, 20.73 (CH_3CO), 62.04 (C-6), 68.34 (C-4), 69.65 (C-2), 74.12 (C-3), 75.78 (C-5), 83.60 (C-1), 122.77, 126.04, 137.34, 141.50, 141.98 (C_{pyr}), 169.47, 169.53, 170.17, 170.66 (C=O).

(5-Amino-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside (**7**)

(5-Nitro-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside **2** (1.95 g, 4 mmol) in CH_2Cl_2 (60 mL) with addition of acetic acid (34 mL) and zinc powder (1.31 g, 20 mmol) were submitted to the general procedure **A** described above. Reaction time: 30 min. Product **7** (1.64 g, 90%) was obtained as a light yellow solid after purification by a column chromatography with toluene:AcOEt (8:1 to 1:2, v/v) solvents system. $[\alpha]_D^{20} = 20.2^o$ (c = 2.4, $CHCl_3$), m.p. 92–93°C; ^{1}H NMR ($CDCl_3$, δ_H, ppm): 1.99, 2.00, 2.05, 2.16 (4×s, 12 H, CH_3CO), 3.40–3.60 (bs, 2H, NH_2), 3.99 (ddd~dt, 1H, *J* = 6.4 Hz, *J* = 0.8 Hz, H-5), 4.11 (dd, 1H, *J* = 6.4, *J* = 11.2 Hz, H-6a), 4.13 (dd, 1H, *J* = 7.0 Hz, *J* = 11.2 Hz, H-6b), 5.13 (dd, 1H, *J* = 3.4 Hz, *J* = 9.3 Hz, H-3), 5.35 (dd~t, 1H, *J* = 10.2 Hz, H-2), 5.42 (d, 1H, *J* = 10.2 Hz, H-1), 5.46 (dd, 1H, *J* = 0.8 Hz, *J* = 3.4 Hz, H-4), 6.91 (dd, 1H, *J* = 3.0 Hz, *J* = 8.4 Hz, H-4_{pyr}), 7.23 (d, 1H, *J* = 8.4 Hz, H-3_{pyr}), 8.01 (d, 1H, *J* = 3.0 Hz, H-6_{pyr}).^{13}C NMR ($CDCl_3$, δ_C, ppm): 20.61, 20.65, 20.70, 20.84 (CH_3CO), 61.29 (C-6); 67.16 (C-4), 67.31 (C-3), 72.10 (C-2), 74.37 (C-5), 84.20 (C-1), 122.78, 125.89, 137.37, 141.43, 142.31 (C_{pyr}), 169.74, 170.07, 170.31, 170.35 (C=O).

(5-Amino-2-pyridyl) per-*O*-acetyl-1-thio-β-maltoside (**8**)

(5-Nitro-2-pyridyl) per-*O*-acetyl-1-thio-β-maltoside **3** (3.5 g, 4 mmol) in CH_2Cl_2 (70 mL) with addition of acetic acid (35 mL) and zinc powder (1.31 g, 20 mmol) were submitted to the general

procedure **A** described above. Reaction time: 10 min. Product **8** (1.60 g, 47%) was obtained as a light yellow solid after purification by a column chromatography with $CHCl_3$:MeOH (80:1 to 40:1, v/v) solvents system. $[\alpha]_D^{20} = 55.6^o$ (c = 0.5, $CHCl_3$), m.p. 71–74°C; ^{1}H NMR ($CDCl_3$, δ_H, ppm): 2.01, 2.02, 2.05, 2.09, 2.10 (5×s, 21H, CH_3CO), 3.40–4.50 (bs, 2H, NH_2), 3.78 (ddd, 1H, *J* = 2.4 Hz, *J* = 4.1 Hz, *J* = 9.5 Hz, H-5_{glu}), 3.95 (m, 1H, H-5'$_{glu}$), 4.03 (dd~t, 1H, *J* = 9.3 Hz, H-4'$_{glu}$), 4.04 (dd, 1H, *J* = 1.9 Hz, *J* = 12.5 Hz, H-6'b$_{glu}$), 4.18–4.30 (m, 2H, H-6a$_{glu}$, H-6'a$_{glu}$), 4.42 (dd, 1H, *J* = 2.4 Hz, *J* = 12.0 Hz, H-6b$_{glu}$), 4.85 (dd, 1H, *J* = 3.9 Hz, *J* = 10.5 Hz, H-2'$_{glu}$), 4.98 (dd~t, 1H, *J* = 9.7 Hz, H-4$_{glu}$), 5.05 (dd~t, 1H, *J* = 9.6 Hz, H-3'$_{glu}$), 5.36 (dd~t, 1H, *J* = 9.9 Hz, H-2$_{glu}$), 5.38 (d, 1H, *J* = 10.5 Hz, H-1$_{glu}$), 5.39 (dd~t, 1H, *J* = 10.3 Hz, H-3$_{glu}$), 5.41 (d, 1H, *J* = 3.9 Hz, H-1'$_{glu}$), 6.91 (dd, 1H, *J* = 2.9 Hz, *J* = 8.3 Hz, H-4$_{pyr}$), 7.13 (d, 1H, *J* = 8.5 Hz, H-3$_{pyr}$), 8.01 (d, 1H, *J* = 2.9 Hz, H-6$_{pyr}$). ^{13}C NMR ($CDCl_3$, δ_C, ppm): 20.55, 20.65, 20.76, 20.87 (CH_3CO), 61.46, 63.01 (C-6$_{glu}$, C-6'$_{glu}$), 67.99, 68.46, 69.30, 69.96, 70.59, 72.77, 76.00 (C-2$_{glu}$, C-3$_{glu}$, C-4$_{glu}$, C-5$_{glu}$, C-2'$_{glu}$, C-3'$_{glu}$, C-4'$_{glu}$, C-5'$_{glu}$), 83.20 (C-1$_{glu}$), 95.53 (C-1'$_{glu}$), 122.71, 126.00, 141.33, 142.09, 160.22, (C_{pyr}), 169.39, 169.73, 169.87, 170.06, 170.41, 170.49 (C=O).

(5-Amino-2-pyridyl) per-*O*-acetyl-1-thio-β-lactoside (**9**)

(5-Nitro-2-pyridyl) per-*O*-acetyl-1-thio-β-lactoside **4** (3.5 g, 4 mmol) in CH_2Cl_2 (70 mL) with addition of acetic acid (35 mL) and zinc powder (1.31 g, 20 mmol) were submitted to the general procedure **A** described above. Reaction time: 10 min. Product **9** (1.43 g, 42%) was obtained as a light yellow solid after purification by a column chromatography with $CHCl_3$: MeOH (80:1 to 40:1, v/v) solvents system. $[\alpha]_D^{20} = -107.0^o$ (c = 1.7, $CHCl_3$), m.p. 95–98°C; ^{1}H NMR ($CDCl_3$, δ_H, ppm): 1.96, 2.03, 2.04, 2.05, 2.06, 2.07, 2.15 (7×s, 21H, CH_3CO), 3.69 (ddd, 1H, *J* = 1.8 Hz, *J* = 5.3 Hz, *J* = 10.0 Hz, H-5$_{glu}$), 3.83 (dd~t, 1H, *J* = 9.4 Hz, H-4$_{glu}$), 3.88 (m, 1H, H-5'$_{gal}$), 4.06–4.18 (m, 3H, H-6a$_{glu}$, H-6'a$_{gal}$, H-6'b$_{gal}$), 4.43 (dd, 1H, *J* = 1.8 Hz, *J* = 12.3 Hz, H-6b$_{glu}$), 4.47 (d, 1H, *J* = 8.2 Hz, H-1'gal), 4.95 (dd, 1H, *J* = 3.5 Hz, *J* = 10.0 Hz, H-3'$_{gal}$), 5.05 (dd, 1H, *J* = 8.8 Hz, *J* = 10.0 Hz, H-2$_{glu}$), 5.11 (dd, 1H, *J* = 7.8 Hz, *J* = 10.5 Hz, H-2'$_{gal}$), 5.28 (dd~t, 1H, *J* = 9.4 Hz, H-3$_{glu}$), 5.34 (d, 1H, *J* = 10.5 Hz, H-1$_{glu}$), 5.35 (m, 1H, H-4'$_{gal}$), 6.90 (dd, 1H, *J* = 2.9 Hz, *J* = 8.4 Hz, H-4$_{pyr}$), 7.12 (d, 1H, *J* = 8.4 Hz, H-3$_{pyr}$), 7.99 (d, 1H, *J* = 2.9 Hz, H-6$_{pyr}$). ^{13}C NMR ($CDCl_3$, δ_C, ppm): 20.45, 20.59, 20.71, 20.76 (CH_3CO), 60.83, 62.24, 66.61, 69.04, 70.67, 70.99, 73.90, 76.19 (C-2$_{glu}$, C-3$_{glu}$, C-4$_{glu}$, C-5$_{glu}$, C-6$_{glu}$, C-2'$_{gal}$, C-3'$_{gal}$, C-4'$_{gal}$, C-5'$_{gal}$, C-6'$_{gal}$), 83.42 (C-1$_{glu}$), 100.97 (C-1'$_{gal}$), 122.71, 125.86, 137.31, 141.29, 160.84 (C_{pyr}), 169.03, 169.66, 169.73, 170.02, 170.11, 170.32 (C=O).

(5-Amino-2-pyridyl) per-*O*-acetyl-1-thio-β-melibioside (**10**)

(5-Nitro-2-pyridyl) per-*O*-acetyl-1-thio-β-melibioside **5** (3.5 g, 4 mmol) in CH_2Cl_2 (70 mL) with addition of acetic acid (35 mL) and zinc powder (1.31 g, 20 mmol) were submitted to the general procedure **A** described above. Reaction time: 10 min. Product **10** (0.61 g, 18%) was obtained as a light yellow solid after purification by a column chromatography with $CHCl_3$:MeOH (80:1 to 40:1, v/v) solvents system. $[\alpha]_D^{20} = 78.8^o$ (c = 1.5, $CHCl_3$), m.p. 130–132°C; ^{1}H NMR ($CDCl_3$, δ_H, ppm): 1.99, 2.01, 2.05, 2.06, 2.08, 2.11 (6×s, 21H, CH_3CO), 3.52 (dd, 1H, *J* = 1.0 Hz, *J* = 10.5 Hz, H-6a$_{glu}$), 3.72 (dd, 1H, *J* = 7.8 Hz, *J* = 10.5 Hz, H-6b$_{glu}$), 3.77–3.98 (m, 4H, H-5$_{glu}$, H-5'$_{gal}$, H-6'a$_{gal}$, H-6'b$_{gal}$), 4.93 (dd~t, 1H, *J* = 9.8 Hz, H-4$_{glu}$), 4.97 (m, 1H, H-4'$_{gal}$), 5.01 (d, 1H, *J* = 3.4 Hz, H-1'$_{gal}$), 5.07 (dd, 1H, *J* = 3.2 Hz, *J* = 10.7 Hz, H-2'$_{gal}$), 5.09 (dd, 1H, *J* = 9.3 Hz, *J* = 10.5 Hz, H-2$_{glu}$), 5.16 (dd, 1H, *J* = 3.4 Hz, *J* = 10.7 Hz, H-3'$_{gal}$), 5.32 (dd~t, 1H, *J* = 9.3 Hz, H-3$_{glu}$), 5.62 (d, 1H, *J* = 10,5 Hz, H-1$_{glu}$), 6.93 (dd, 1H, *J* = 2.7 Hz, *J* = 8.3 Hz, H-4$_{pyr}$), 6.98 (d, 1H, *J* = 8.3 Hz, H-3$_{pyr}$), 8.19 (d, 1H, *J* = 2.7 Hz, H-6$_{pyr}$). ^{13}C NMR ($CDCl_3$, δ_C, ppm): 20.58, 20.65, 20.74, 20.84, 20.90 (CH_3CO), 62.10, 66.07, 67.01, 67.52, 67.92, 68.28, 69.05, 69.62, 74.12 (C-2$_{glu}$, C-3$_{glu}$, C-4$_{glu}$, C-5$_{glu}$, C-6$_{glu}$, C-2'$_{gal}$, C-3'$_{gal}$, C-4'$_{gal}$, C-5'$_{gal}$, C-6'$_{gal}$), 81.90 (C-1$_{glu}$), 95.43 (C-1'$_{gal}$), 123.06, 124.27, 137.94, 141.481, 141.96, (C_{pyr}), 169.59, 170.06, 170.18, 170.30, 170, 36, 170.55 (C=O).

(5-Amino-2-pyridyl) 1-thio-β-D-glucopyranoside (**11**)

(5-Amino-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside **6** (0.556 g, 1.2 mmol) in MeOH (40 mL) with addition of 1 M methanolic solution of MeONa (1.2 mL, 1.2 mmol) were submitted to the general procedure **B** described above. Reaction time: 30 min. Product **11** (0.12 g, 35%) was obtained as a light yellow solidifying oil after purification by a column chromatography with $CHCl_3$:MeOH (8:1 to 4:1, v/v) solvents system. $[\alpha]_D^{20} = -6.8^o$ (c = 0.6, MeOH); ^{1}H NMR (CD_3OD, δ_H, ppm): 3.10–3. 80 (m, 11H, H-2, H-3, H-4, H-5, H-6a, NH_2, 4×OH), 3.83 (dd, 1H, *J* = 1.9 Hz, *J* = 12.1 Hz, H-6b), 4.67 (d, 1H, *J* = 9.7 Hz, H-1), 7.02 (dd, 1H, *J* = 2.9 Hz, *J* = 8.5 Hz, H-4$_{pyr}$), 7.38 (dd, *J* = 0.6 Hz, 1H, *J* = 8.5 Hz, H-3$_{pyr}$), 7.89 (dd, 1H, *J* =

0.6 Hz, *J* = 2.9 Hz, H-6_{pyr}). ^{13}C NMR (CD_3OD, δ_C, ppm): 58.30 (C-6), 2.85, 71.31, 73.83, 79.45 (C-2, C-3, C-4, C-5), 82.04 (C-1), 123.80, 129.84, 137.35, 141.68, 145.56 (C_{pyr}).

(5-Amino-2-pyridyl) 1-thio-β-D-galactopyranoside (12)

(5-Amino-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside **7** (0.463 g, 1 mmol) in MeOH (40 mL) with addition of 1 M methanolic solution of MeONa (1 mL, 1 mmol) were submitted to the general procedure **B** described above. Reaction time: 25 min. Product **12** (0.11 g, 37%) was obtained as a light yellow solidifying oil after purification by a column chromatography with $CHCl_3$:MeOH (8:1 to 4:1, v/v) solvents system. $[\alpha]_D^{20} = 11.3^o$ (c = 0.5, MeOH); ^{1}H NMR (CD_3OD, δ_H, ppm): 3.10–3.92 (m, 12H, H-2, H-3, H-4, H-5, H-6a, H-6b, NH_2, 4×OH), 4.62 (d, 1H, *J* = 9.9 Hz, H-1), 7.05 (dd, 1H, *J* = 2.8 Hz, *J* = 8.5 Hz, H-4_{pyr}), 7.38 (d, 1H, *J* = 8.5 Hz, H-3_{pyr}), 7.89 (d, 1H, *J* = 2.8 Hz, H-6_{pyr}). ^{13}C NMR (CD_3OD, δ_C, ppm): 58.64 (C-6), 62.89, 72.03, 73.68, 79.36 (C-2, C-3, C-4, C-5), 83.11 (C-1), 122.89, 129.12, 137.45, 142.12, 148.47 (C_{pyr}).

(5-Amino-2-pyridyl) 1-thio-β-maltoside (13)

(5-Amino-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-maltoside **8** (0.6 g, 0.8 mmol) in MeOH (30 mL) with addition of 1 M methanolic solution of MeONa (0.8 mL, 0.8 mmol) were submitted to the general procedure **B** described above. Reaction time: 25 min. Product **13** (0.12 g, 52%) was obtained as a light yellow solidifying oil after purification by a column chromatography with $CHCl_3$:MeOH (10:1 to 1:1, v/v) solvents system. $[\alpha]_D^{20} = 30.7^o$ (c = 0.3, MeOH); ^{1}H NMR (CD_3OD, δ_H, ppm): 3.19–3.91 (m, 21H, H-2_{glu}, H-3_{glu}, H-4_{glu}, H-5_{glu}, H-$6a_{glu}$, H6b_{glu}, H-2'$_{glu}$, H-3'$_{glu}$, H-4'$_{glu}$, H-5'$_{glu}$, H-6'a_{glu}, H6'b_{glu}, NH_2, 7×OH), 4.67 (d, 1H, *J* = 9.8 Hz, H-1_{glu}), 5.15 (d, 1H, *J* = 3.7 Hz, H-1'$_{glu}$), 7.03 (dd, 1H, *J* = 2.9 Hz, *J* = 8.5 Hz, H-4_{pyr}), 7.38 (dd, 1H, *J* = 0.6 Hz, *J* = 8.5 Hz, H-3_{pyr}), 7.89 (dd, 1H, *J* = 0.6 Hz, *J* = 2.9 Hz, H-6_{pyr}). ^{13}C NMR (CD_3OD, δ_C, ppm): 62.33, 62.72 (C-6_{glu}, C-6'$_{glu}$), 71.48, 73.52, 74.20, 74.78, 75.08, 79.27, 80.67, 80.79 (C-2_{glu}, C-3_{glu}, C-4_{glu}, C-5_{glu}, C-2'$_{glu}$, C-3'$_{glu}$, C-4'$_{glu}$, C-5'$_{glu}$), 88.40 (C-1_{glu}), 102.81 (C-1'$_{glu}$), 123.73, 129.99, 137.39, 142.10, 158.44 (C_{pyr}).

(5-Amino-2-pyridyl) 1-thio-β-lactoside (14)

(5-Amino-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-lactoside **9** (0.4 g, 0.54 mmol) in MeOH (20 mL) with addition of 1 M methanolic solution of MeONa (0.54 mL, 0.54 mmol) were submitted to the general procedure **B** described above. Reaction time: 25 min. Product **14** (0.096 g, 39%) was obtained as a light yellow solidifying oil after purification by a column chromatography with $CHCl_3$:MeOH (10:1 to 1:1, v/v) solvents system. $[\alpha]_D^{20} = -18.0^o$ (c = 0.5, MeOH); ^{1}H NMR (CD_3OD, δ_H, ppm): 3.18–3.92 (m, 21H, H-2_{glu}, H-3_{glu}, H-4_{glu}, H-5_{glu}, H-$6a_{glu}$, H6b_{glu}, H-2'$_{gal}$, H-3'$_{gal}$, H-4'$_{gal}$, H-5'$_{gal}$, H-6'a_{gal}, H-6'b_{gal}, NH_2, 7×OH), 4.33 (d, 1H, *J* = 7.2 Hz, H-1'$_{gal}$), 4.67 (d, 1H, *J* = 9.9 Hz, H-1_{glu}), 7.02 (dd, 1H, *J* = 2.7 Hz, *J* = 8.7 Hz, H-4_{pyr}), 7.37 (d, 1H, *J* = 8.7 Hz, H-3_{pyr}), 7.89 (d, 1H, *J* = 2.7 Hz, H-6_{pyr}).

(5-Amino-2-pyridyl) 1-thio-β-melibioside (15)

(5-Amino-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-melibioside **10** (0.4 g, 0.54 mmol) in MeOH (20 mL) with addition of 1 M methanolic solution of MeONa (0.54 mL, 0.54 mmol) were submitted to the general procedure **B** described above. Reaction time: 25 min. Product **15** (0.088 g, 36%) was obtained as a light yellow solidifying oil after purification by a column chromatography with $CHCl_3$:MeOH (10:1 to 1:1, v/v) solvents system. $[\alpha]_D^{20} = -175.0^o$ (c = 0.4, MeOH); ^{1}H NMR (CD_3OD, δ_H, ppm): 3.20–3.90 (m, 21H, H-2_{glu}, H-3_{glu}, H-4_{glu}, H-5_{glu}, H-$6a_{glu}$, H6b_{glu}, H-2'$_{gal}$, H-3'$_{gal}$, H-4'$_{gal}$, H-5'$_{gal}$, H-6'a_{gal}, H-6'b_{gal}, NH_2, 7×OH), 4.78 (d, 1H, *J* = 9.8 Hz, H-1_{glu}), 4.80 (d, 1H, *J* = 3.7 Hz, H-1'$_{gal}$), 7.07 (dd, 1H, *J* = 2.9 Hz, *J* = 8.5 Hz, H-4_{pyr}), 7.37 (dd, 1H, *J* = 0.5 Hz, *J* = 8.5 Hz, H-3_{pyr}), 7.90 (dd, 1H, *J* = 0.5 Hz, *J* = 2.9 Hz, H-6_{pyr}).

(5-Nitro-2-pyridyl) 1-thio-β-D-glucopyranoside (16)

(5-Nitro-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside **1** (2.4 g, 5 mmol) in MeOH (40 mL) with addition of 1 M methanolic solution of MeONa (5 mL, 5 mmol) were submitted to the general procedure **B** described above. Reaction time: 30 min. Product **16** (2.30 g, 95%) was obtained as a white solid after purification by a crystallization from ethyl alcohol. $[\alpha]_D^{20} = 110.2^o$ (c = 0.5, MeOH); m.p. 59–62^{o}C; ^{1}H NMR (CD_3OD, δ_H, ppm): 3.33–3.52 (m, 3H, H-2, H-3, H-4), 3.47 (ddd, 1H, *J* = 2.2 Hz, *J* = 5.4 Hz, *J* = 9.3 Hz, H-5), 3.66 (dd, 1H, *J* = 5.6 Hz, *J* = 12.2 Hz, H-6a), 3.86 (dd, 1H, *J* = 1.9 Hz, *J* = 12.0 Hz, H-6b), 5.45 (d, 1H, *J* = 9.5 Hz, H-1), 7.57 (dd, 1H, *J* = 0.5 Hz, *J* = 9.5 Hz, H-3_{pyr}), 8.40 (dd, 1H, *J* = 2.7 Hz, *J* = 9.0 Hz, H-4_{pyr}), 9.21 (dd, 1H, *J* = 0.5 Hz, *J* = 2.7 Hz, H-6_{pyr}).^{13}C NMR (CD_3OD, δ_C, ppm): 62.68. (C-6), 71.24, 73.58, 79.80, 82.32 (C-2, C-3, C-4, C-5), 85.23 (C-1), 123.12, 132.55, 143.19, 145.71, 167,75 (C_{pyr}).

(5-Nitro-2-pyridyl) 1-thio-β-D-galactopyranoside (**17**)

(5-Nitro-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside **2** (5 g, 11 mmol) in MeOH (50 mL) with addition of 1 M methanolic solution of MeONa (11 mL, 11 mmol) were submitted to the general procedure **B** described above. Reaction time: 30 min. Product **17** (4.95 g, 98%) was obtained as a white solid after purification by a crystallization from ethyl alcohol. $[\alpha]_D^{20} = -86.6^o$ (c = 0.6, MeOH); m.p. 144–147°C; ^{1}H NMR (CD_3OD, δ_H, ppm: 3.59 (dd, 1H, *J* = 3.2 Hz, *J* = 9.3 Hz, H-3), 3.67–3.73 (m, 3H, H-5, H-6a, H-6b), 3.77 (dd~t, 1H, *J* = 9.3 Hz, H-2), 3.95 (d, 1H, *J* = 3.2 Hz, H-4), 5.39 (d, 1H, *J* = 9.8 Hz, H-1), 7.61 (dd, 1H, *J* = 0.9 Hz, *J* = 9.0 Hz, H-3_{pyr}), 8.39 (dd, 1H, *J* = 2.7 Hz, *J* = 9.0 Hz, H-4_{pyr}), 9.20 (dd, 1H, *J* = 0.9 Hz, *J* = 2.7 Hz, H-6_{pyr}).

(5-Amino-2-pyridyl) 1-thio-β-maltoside (**18**)

(5-Nitro-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-maltoside **3** (5 g, 6.45 mmol) in MeOH (80 mL) with addition of 1 M methanolic solution of MeONa (6.45 mL, 6.45 mmol) were submitted to the general procedure **B** described above. Reaction time: 20 min. Product **18** (1.3 g, 43%) was obtained as a light yellow solid after purification by a column chromatography with $CHCl_3$:MeOH (10:1 to 2:1, v/v) solvents system. $[\alpha]_D^{20} = 4.0^o$ (c = 1.6, MeOH); m.p. 201–202°C; ^{1}H NMR (CD_3OD, δ_H, ppm): 3.23–3.93 (m, 19H, H-2_{glu}, H-3_{glu}, H-4_{glu}, H-5_{glu}, H-$6a_{glu}$, H$6b_{glu}$, H-$2'_{glu}$, H-$3'_{glu}$, H-$4'_{glu}$, H-$5'_{glu}$, H-$6'a_{glu}$, H-$6'b_{glu}$, 7×OH), 5.26 (d, 1H, *J* = 3.8 Hz, H-$1'_{glu}$), 5.49 (d, 1H, *J* = 10.0 Hz, H-1_{glu}), 7.57 (dd, 1H, *J* = 0.6 Hz, *J* = 8.9 Hz, H-3_{pyr}), 8.41 (dd, 1H, *J* = 2.7 Hz, *J* = 8.9 Hz, H-4_{pyr}), 9.22 (dd, 1H, *J* = 0.6 Hz, *J* = 2.7 Hz, H-6_{pyr}).^{13}C NMR (CD_3OD, δ_C, ppm): 62.11, 62.73 (C-6_{glu}, C-$6'_{glu}$), 71.49, 73.20, 74.17, 74.80, 75.08, 79.51, 80.58, 80.88 (C-1_{glu}, C-2_{glu}, C-3_{glu}, C-4_{glu}, C-5_{glu}, C-$2'_{glu}$, C-$3'_{glu}$, C-$4'_{glu}$, C-$5'_{glu}$), 102.82 (C-$1'_{glu}$), 123.20, 132.58, 143.19, 145.75, 167.55 (C_{pyr}).

(5-Amino-2-pyridyl) 1-thio-β-lactoside (**19**)

(5-Nitro-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β- lactoside **4** (3.3 g, 4.25 mmol) in MeOH (60 mL) with addition of 1 M methanolic solution of MeONa (4.25 mL, 4.25 mmol) were submitted to the general procedure **B** described above. Reaction time: 120 min. Product **19** (1.0 g, 50%) was obtained as a light yellow solid after purification by a column chromatography with $CHCl_3$:MeOH (10:1 to 2:1, v/v) solvents system. $[\alpha]_D^{20} = 2.0^o$ (c = 0.5, MeOH); m.p. 180–182°C; ^{1}H NMR (CD_3OD, δ_H, ppm: 3.42–3.99 (m, 12H, H-2_{glu}, H-2_{glu}, H-3_{glu}, H-4_{glu}, H-5_{glu}, H-$6a_{glu}$, H-$6b_{glu}$, H-$2'_{gal}$, H-$3'_{gal}$, H-$4'_{gal}$, H-$5'_{gal}$, H6'a_{gal}, H-6'b_{gal}), 4.39 (d, 1H, *J* = 7.3 Hz, H-$1'_{gal}$), 5.51 (d, 1H, *J* = 10.0 Hz, H-1_{glu}), 7.56 (d, 1H, *J* = 8.8 Hz, H-3_{pyr}), 8.41 (dd, 1H, *J* = 2.7 Hz, *J* = 8.8 Hz, H-4_{pyr}), 9.22 (d, 1H, *J* = 2.7 Hz, H-6_{pyr}).^{13}C NMR (CD_3OD, δ_C, ppm): 61.77, 62.56 (C-6_{glu}, C-$6'_{gal}$), 70.30, 72.55, 74.75, 76.13, 77.13, 79.17, 79.63, 80.02, 81.04 (C-1_{glu}, C-2_{glu}, C-3_{glu}, C-4_{glu}, C-5_{glu}, C-$2'_{gal}$, C-$3'_{gal}$, C-$4'_{gal}$, C-$5'_{gal}$), 104.97 (C-1_{gal}), 123.61, 135.69, 141.25, 145.01, 168.40 (C_{pyr}).

(5-Amino-2-pyridyl) 1-thio-β-melibioside (**20**)

(5-Nitro-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-melibioside **5** (3.2 g, 4.3 mmol) in MeOH (80 mL) with addition of 1 M methanolic solution of MeONa (4.3 mL, 4.3 mmol) were submitted to the general procedure **B** described above. Reaction time: 25 min. Product **20** (0.64 g, 52%) was obtained as a light yellow solid after purification by a column chromatography with $CHCl_3$:MeOH (10:1 to 1:1, v/v) solvents system. $[\alpha]_D^{20} = 30.6^o$ (c = 0.3, MeOH); m.p. 168–171°C; ^{1}H NMR (CD_3OD, δ_H, ppm): 3.20–3.90 (m, 21H, H-2_{glu}, H-3_{glu}, H-4_{glu}, H-5_{glu}, H-$6a_{glu}$, H$6b_{glu}$, H-$2'_{gal}$, H-$3'_{gal}$, H-$4'_{gal}$, H-$5'_{gal}$, H-6'a_{gal}, H-6'b_{gal}, NH_2, 7×OH); 4.78 (d, 1H, *J* = 9.8 Hz, H-1_{glu}); 4.80 (d, 1H, *J* = 3.7 Hz, H-$1'_{gal}$); 7.07 (dd, 1H, *J* = 2.9 Hz, *J* = 8.5 Hz, H-4_{pyr}); 7.37 (dd, 1H, *J* = 0.5 Hz, *J* = 8.5 Hz, H-3_{pyr}); 7.90 (dd, 1H, *J* = 0.5 Hz, *J* = 2.9 Hz, H-6_{pyr}).

***N*-Acetylglycinated (5-nitro-2-pyridyl) 1-thio-β-D-glucopyranoside** (**21**)

Procedure C: mixture of monoglycinated products was isolated in 31% yield.

Procedure D: mixture of monoglycinated products was isolated in 40% yield.

Procedure E: mixture of monoglycinated products was isolated in 12% yield.

Procedure F: mixture of monoglycinated products was isolated in 38% yield. ^{1}H NMR (CD_3OD, δ_H, ppm): 1.99, 2.02, 2.03, 2,04 (4×s, 3H, CH_3CO), 3.30–4.54 (m, 8H, H-2, H-3, H-4, H-5, H-6a, H-6b, CH_2), 5.48 (d, 0.69H, *J* = 9.5 Hz, H-1), 5.53 (d, 0.15H, *J* = 10.01 Hz, H-1), 5.61 (d, 0.07H, *J* = 10.3 Hz, H-1), 5.74 (d, 0.09H, *J* = 10.5 Hz, H-1), 7.59 (m, 1H, H-3_{pyr}), 8.45 (m, 1H, H-4_{pyr}), 9.24 (m, 1H, H-6_{pyr}).^{13}C NMR (CD_3OD, δ_C, ppm): 22.23 (CH_3CO), 41.98 (CH_2NH), 62.68. (C-6), 71.23, 73.58, 79.79, 82.32 (C-2, C-3, C-4, C-5), 85.22 (C-1), 123.12, 132.56, 143.19, 145.72, 167.74 (C_{pyr}), 173.82 (C=O).

***N*-Acetylglycinated (5-nitro-2-pyridyl) 1-thio-β-D-galactopyranoside** (**22**)

Procedure D: mixture of monoglycinated products was isolated in 48% yield. ^{1}H NMR

(CD_3OD, δ_H, ppm): 1.97, 1.98, 2.01, 2,02 (4×s, 3H, CH_3CO), 3.51–4.35 (m, 8H, H-2, H-3, H-4, H-5, H-6a, H-6b, CH_2), 5.39 (d, 0.84H, J = 10.0 Hz, H-1), 5.49 (d, 0.05H, J = 9.8 Hz, H-1), 5.54 (d, 0.07H, J = 9.7 Hz, H-1), 5.63 (d, 0.04H, J = 9.7 Hz, H-1), 7.60 (m, 1H, H-3_{pyr}), 8.42 (m, 1H, H-4_{pyr}), 9.21 (m, 1H, H-6_{pyr}).[13]C NMR (CD_3OD, δ_C, ppm): 22.23 (CH_3CO), 42.05 (CH_2NH), 65.46 (C-6), 70.37, 76.36, 77.91, 81.00, 85.75 (C-2, C-3, C-4, C-5), 101.38 (C-1), 123.16, 132.51, 143.15, 145.66, 167.86 (C_{pyr}), 167.85, 171.17 (C=O).

_N_-Acetylglycinated (5-nitro-2-pyridyl) 1-thio-β-melobioside (23)

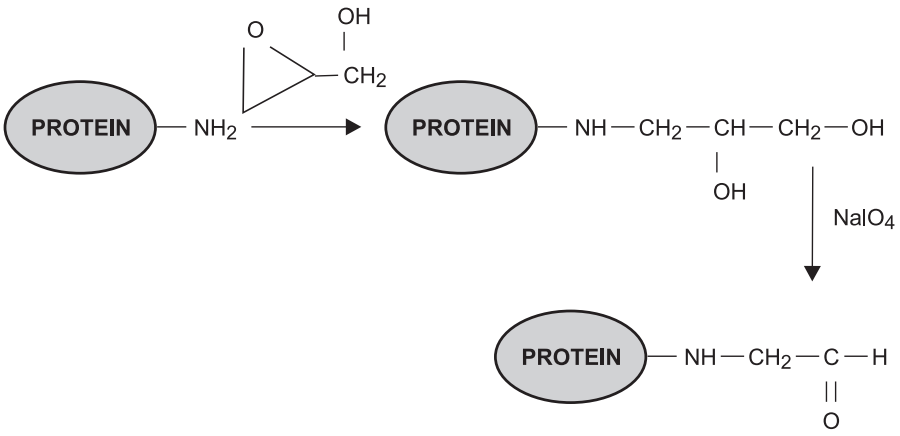
Procedure D: mixture of monoglycinated products was isolated in 48% yield. [1]H NMR (CD_3OD, δ_H, ppm): 1.98 (s, 3H, CH_3CO), 3.23–3.94 (m, 14H, H-2_{glu}, H-3_{glu}, H-4_{glu}, H-5_{glu}, H-$6a_{glu}$, H6b_{glu}, H-2'$_{glu}$, H-3'$_{glu}$, H-4'$_{glu}$, H-5'$_{glu}$, H-6'a_{glu}, H6'b_{glu}, CH_2), 4.85 (d, 1H, J = 3.7 Hz, H-1_{gal}), 5.41 (d, 1H, J = 9.7 Hz, H-1_{glu}), 7.62 (d, 1H, J = 8.9 Hz, H-3_{pyr}), 8.44 (dd, 1H, J = 2.7 Hz, J = 8.9 Hz, H-4_{pyr}), 9.21 (dd, 1H, J = 2.7 Hz, H-6_{pyr}).[13]C NMR (CD_3OD, δ_C, ppm): 22.09 (CH_3CO), 41.95 (CH_2NH), 62.76, 67.80 (C-6_{glu}, C-6'$_{glu}$), 70.37, 71.14, 71.50, 71.61, 72.13, 73.57, 79.85, 80.62 (C-2_{glu}, C-3_{glu}, C-4_{glu}, C-5_{glu}, C-2'$_{gal}$, C-3'$_{gal}$, C-4'$_{gal}$, C-5'$_{gal}$), 85.29 (C-1_{glu}), 100.08 (C-1'$_{gal}$), 123.73, 129.99, 137.39, 142.10, 158.44 (C_{pyr}). 167.85, 171.17 (C=O).

_N_-Acetylglycinated (5-amino-2-pyridyl) 1-thio-β-D-glucopyranoside (24)

N-acetylglycinated (5-nitro-2-pyridyl) 1-thio-β-D-glucopyranoside **21** (0.28 g, 0.68 mmol) in MeOH (20 mL) with addition of acetic acid (6 mL) and zinc powder (0.23 g, 3.4 mmol) were submitted to the general procedure **A** described above. Reaction time: 15 min. Product **24** (0.087 g, 33%) was obtained as a light yellow solidifying oil after purification by a column chromatography with $CHCl_3$:MeOH (8:1 to 1:2, v/v) solvents system. [1]H NMR (CD_3OD, δ_H, ppm): 2.01, 2.04 (2×s, 3H, CH_3CO), 3.30–4.02 (m, 8H, H-2, H-3, H-4, H-5, H-6a, H-6b, CH_2), 5.29 (m, 1H, H-1), 7.23 (dd, 1H, J = 2.7 Hz, J = 8.5 Hz, H-4_{pyr}), 7.44 (d, 1H, J = 8.5 Hz, H-3_{pyr}), 7.98 (d, 1H, J = 2.7 Hz, H-6_{pyr}), 8.46 (bs, 1H, NH). [13]C NMR (CD_3OD, δ_C, ppm): 22.11 (CH_3CO), 46.93 (CH_2), 61.02 (C-6), 69.59, 72.11, 77.41, 80.20 (C-2, C-3, C-4, C-5), 86.73 (C-1), 125.65, 128.61, 136.87, 141.03, 143.46 (C_{pyr}), 179.21 (C=O).

_N_-Acetylglycinated (5-amino-2-pyridyl) 1-thio-β-D-galactopyranoside (25)

N-acetylglycinated (5-nitro-2-pyridyl) 1-thio-β-D-galactopyranoside **22** (0.14 g, 0.34 mmol) in MeOH (10 mL) with addition of acetic acid (3 mL) and zinc powder (0.12 g, 1.7 mmol) were submitted to the general procedure **A** described above. Reaction time: 20 min. Product **25** (0.032 g, 49%) was obtained as a light yellow solidifying oil after purification by a column chromatography with $CHCl_3$:MeOH (10:1 to 1:2, v/v) solvents system. [1]H NMR (CD_3OD, δ_H, ppm): 1.99 (s, 3H, CH_3CO), 3.46–4.00 (m, 8H, H-2, H-3, H-4, H-5, H-6a, H-6b, CH_2), 4.66 (d, 1H, J = 9.5 Hz, H-1), 7.03 (dd, 1H, J = 2.7 Hz, J = 8.5 Hz, H-4_{pyr}), 7.38 (d, 1H, J = 8.5 Hz, H-3_{pyr}), 7.88 (d, 1H, J = 2.7 Hz, H-6_{pyr}), 8.53 (bs, 1H, NH).



Scheme 2. Preparation method of formylated protein derivatives

_N_-Acetylglycinated (5-amino-2-pyridyl) 1-thio-β-melibioside (26)

N-acetylglycinated (5-nitro-2-pyridyl) 1-thio-β-melibioside **23** (0.3 g, 0.52 mmol) in MeOH (20 mL) with addition of acetic acid (5 mL) and zinc powder (0.18 g, 2.6 mmol) were submitted to the general procedure **A** described above. Reaction time: 15 min. Product **26** (0.055 g, 19%) was obtained as a light yellow solidifying oil after purification by a column chromatography with $CHCl_3$:MeOH (10:1 to 1:2, v/v) solvents system. ^{1}H NMR (CD_3OD, δ_H, ppm): 2.06 (s, 3H, CH_3CO), 3.34–4.21 (m, 14H, H-2$_{glu}$, H-3$_{glu}$, H-4$_{glu}$, H-5$_{glu}$, H-6a$_{glu}$, H6b$_{glu}$, H-2'$_{gal}$, H-3'$_{gal}$, H-4'$_{gal}$, H-5'$_{gal}$, H-6'a$_{gal}$, H-6'b$_{gal}$, CH_2), 4.91 (d, 1H, J = 3.9 Hz, H-1'$_{gal}$), 5.07 (d, 1H, J = 10.0 Hz, H-1$_{glu}$), 7.23 (dd, 1H, J = 2.7 Hz, J = 8.5 Hz, H-4$_{pyr}$), 7.38 (d, 1H, J = 8.5 Hz, H-3$_{pyr}$), 8.00 (d, 1H, J = 2.7 Hz, H-6$_{pyr}$). ^{13}C NMR (CD_3OD, δ_C, ppm): 21.93 (CH_3CO), 41.53 (CH_2), 61.34, 66.35 (C-6$_{glu}$, C-6'$_{gal}$), 68.55, 69.43, 69.76, 69.84, 70.91, 71.97, 72.32, 77.60, 85.99 (C-1$_{glu}$, C-2$_{glu}$, C-3$_{glu}$, C-4$_{glu}$, C-5$_{glu}$, C-2'$_{gal}$, C-3'$_{gal}$, C-4'$_{gal}$, C-5'$_{gal}$), 98.08 (C-1'$_{gal}$), 125.26, 127.19, 137.76, 142.22, 142.92 (C_{pyr}), 171.48, 181.21 (C=O).

Modification of albumin and myoglobin to formylated derivatives

The preparation of formylated protein derivatives was performed as shown on Scheme 2. Carrier proteins, e.g., bovine serum albumin or horse myoglobin (0.01 g) were dissolved in 0.1 M sodium carbonate-sodium bicarbonate buffer, pH 9.2 and then glycidol was added (at final concentration 1.1 M). Obtained mixtures were stirred at room temperature for 72 h and the modified proteins were purified by extensive dialysis (membrane 8–10 kDa) in 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2. Dialysis was continued against water MiliQ by using the Amicon Ultra Centrifugal Filter (50 kDa for BSA and 10 kDa for MYO, Millipore). The concentration of the purified protein-diol derivatives were determined spectrophotometrically at λ = 280 nm, according to (10). Obtained products were lyophilized and frozen. The efficiency of generation reactive diols on amino groups in proteins was determined by the colorimetric method with 2,4,6-trinitrobenzenesulfonic acid (TNBS), which allowed to estimate the nonmodified free amino groups in tested proteins (20). Samples (0.4 g) of the modified or unmodified protein (as a control) were dissolved in 0.1 M borate buffer, pH 9.2 and incubated with 1% (w/v) TNBS at room temperature for 1 h and the absorbance at 420 nm was measured in UV/VIS spectrophotometer.

Further, protein-diol intermediates were oxidized by a 0.01 M sodium periodate in 0.1 M citrate-phosphate buffer pH 6.0, in order to prepare the formylated proteins. The mixtures of protein-diols (in the same buffer as above) and sodium periodate solution were stirred at room temperature in the dark for 60 min. Excess of periodate was quenched by adding ethylene glycol (4:1, v/v) for 10 min of incubation, then the formylated proteins (10 mg) were purified on Bio-Gel P-4 gel filtration column using 0.01 M citrate buffer, pH 6.0 as eluent. The eluted fractions were monitored at 280 nm and pure formylated proteins underwent lyophilization.

Conjugation of different glycinated 1-thioglycosides with carrier proteins

Solutions of different glycinated 1-thioglycosides as well as non-glycinated derivatives based on monosaccharide or disaccharides were preincubated in 0.1 M phosphate buffer, pH 6.3 with formylated BSA and MYO proteins. The optimal molar ratios of formylated proteins to glycinated 1-thioglycosides as well as non-glycinated derivatives were established as 1:200 and 1:35 for preparing BSA and MYO glyconjugates, respectively. All mixtures were carefully stirred for 5 h at room temperature in the dark. The volume of 0.01 mL of 5 M sodium cyanoborohydride stock solution in the conjugation buffer was added to 1.0 mL of incubation mixtures. The reactions were performed for 18 h in the dark at room temperature. At the end, 0.02 mL of 3 M ethanolamine stock solution was added to 1.0 mL of incubation mixtures for blocking of unreacted aldehydes. After 10 min of incubation, the solutions were filtered to 0.05 M ammonium acetate pH 6.8 and frozen. Products were purified by gel-filtration on HW-55S column (1.5 × 55 cm) (21) using the equilibration buffer of 0.05 M ammonium acetate pH 6.8 and fractions monitoring for protein content at 280 nm and for the carbohydrates with colorimetric (at λ = 490 nm) phenol-sulfuric acid method (22). Then, selected fractions were pooled and lyophilized.

SDS-PAGE analysis of glycoconjugates

The SDS-PAGE analysis of obtained products was performed according to Laemmli (23). Samples of 20 μg of proteins were incubated for 5 min at 95°C in 0.068 M Tris-HCl buffer pH 6.8 containing 2% (w/v) SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.025% bromophenol blue. Electrophoresis was performed in 0.025 M Tris-HCl, 0.192 M glycine and 0.1% SDS electrode buffer, pH 8.3, at 20 mA in 4% stacking gels and at 35–60 mA in 10%

Table 1. The sugar content in glycoconjugates purified by gel-filtration on HW-55S column.

Conjugate composition (No. of fraction after HW55-S)	Total sugar content* [%]
BSA-Glc/NAcGly (85–86)	38
BSA-Gal/NAcGly (55–69)	53
BSA-Lac/NAcGly(-) (51–63)	74
BSA-Mel/NAcGly (70–94)	43
MYO-Glc/NAcGly (91–104)	79
MYO-Mel/NAcGly (63–78)	81

*The total sugar content [%] was calculated by determination of sugar amount in studied fractions in phenol-sulfuric acid method in relation to protein content in the selected glycoconjugates fractions *vs.* protein amount (in mg) initially applied in the conjugation experiments.

or 12% separating gels, depending on BSA or MYO glycoconjugates type (MiniPROTEAN System, Bio-Rad). Next, the products were visualized by staining with Coomassie Brillant Blue R-250 (Park) and analyzed by using GelDoc™ XR+ System (Bio-Rad, France).

ELISA studies of synthetic glycoconjugates

In these experiments, the 96-well plates (MultiSorp™, Nunc) were coated with different synthetic glycoconjugates (1 μg/well) in 0.2 M carbonate buffer, pH 9.6 by incubation at 37°C for 3 h. Blocking was performed with 10% skimmed milk dissolved in TBS-T buffer (0.15 M Tris-HCl, 0.05 M NaCl, pH 7.4, containing 0.05% Tween) at room temperature for 2 h. Plates were washed three times with TBS-T. The serial dilutions of rabbit serum (0.1 mL/well) in PBS, pH 7.4 were added and incubated at temperature 4°C for 16 h. After washing three times with TBS-T, the peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) conjugate diluted 1:2000 in PBS was added to each well and incubation at room temperature for 2 h was performed. Then, after washing with TBS-T, 0.1 mL/well of *o*-phenylenediamine dihydrochloride substrate solution (30 mg OPD; 0.5 mL methanol; 10 mL of citric acid-sodium citrate buffer, pH 4.5; 0.01 mL H_2O_2) was added. After 15 min, the reaction was stopped with 0.05 mL/well of 40% sulfuric acid and the optical densities were read at λ = 492 nm using a Labsystems Multiskan MS Microplate Reader.

RESULTS AND DISCUSSION

Synthetic 1-thioglycosides derivatives of monosaccharides and disaccharides

(5-Nitro-2-pyridyl) 1-thioglycosides derivatives of monosaccharides such as D-glucose **1** and D-galactose **2** as well as disaccharides consisted of these two monosugars: maltose **3**, lactose **4** and melibiose **5** were prepared according to earlier published procedure (7–9).

For the preparation of (5-amino-2-pyridyl) 1-thioglycosides **6–10** compounds **1–5** were used as substrates and nitro group reduction procedure with zinc powder/acetic acid system in CH_2Cl_2 described by Roy and co-workers for 4-nitrophenyl 1-thioglycosides was applied (24). (5-Amino-2-pyridyl) per-*O*-acetyl-1-thioglycosides **6–10** were obtained in a good yields and then were submitted to deacetylation reaction using 1 M methanolic solution of MeONa as a base necessary for acetyl groups removing. Unprotected products **11–15** were isolated in a satisfying yields (Scheme 1). These compounds were prepared in order to compare an action of non-glycinated glycoconjugates and their glycinated analogues.

(5-Nitro-2-pyridyl) 1-thioglycosides **1–5** were also subjected to deprotection and as result of conducted reaction 1-thoglycosides **16–20** were obtained. These 1-thioglycosides were subjected to acylation reaction with *N*-acetylglycine (Scheme 1). Then, in obtained glycinated 1-thioglycosides the

nitro group was reduced into amino group. Direct acylation of (5-amino-2-pyridyl) 1-thioglycosides **11–15** with *N*-acetylglycine cannot be performed because in such reaction the amino group would be acylated earlier than sugar hydroxyl groups.

The direct construction of amide bond using acid and alcohol becomes feasible at high temperature (above 100°C), which may be troublesome when other sensitive functionalities are present within coupled compounds. Therefore, activation of carboxylic acid seems to be necessary. There are numerous commercially available coupling reagents including dicyclohexylcarbodiimides (DCC) (25) or plus additives such as hydroxybenzotriazole (HOBt) or 4-dimethylaminopyridine (DMAP) (26, 27). The carbodiimide reacts with the carboxylic acid to form O-acylisourea mixed anhydride which can react directly with amine to yield the desired amide. Unfortunately, isomerisation of reactive *O*-acylisourea into unreactive N-acylurea may also be observed. Addition of nucleophiles such as DMAP or HOBt hampers the side reaction.

The efficiency of 2-chloro-4,6-disubstituted-1,3,5-triazines in formation of the peptide bond was demonstrated by Kaminski and co-workers (28). Activated ester resulting from reaction of carboxylic acid with triazine derivative ("superactive ester") contains an excellent leaving group which can be displaced by an alcohol. Such reaction requires the presence of a tertiary amine in the reaction medium. A range of tertiary amines were tested and the best results were obtained when *N*-methylmorpholine (NMM) was applied (29).

Kunishima and co-workers found that 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMT-MM) can be formed and isolated in THF and then used as an efficient condensing agent facilitating formation amides and esters (19). Taking advantage of these information, acylation reactions of 1-thioglycoside **16** with *N*-acetyl-L-glycine using different procedures with DCC, DCC/DMAP, DCC/HOBT or DMT-MM as coupling agents were performed. The best results were obtained for acylation using DCC/DMAP coupling system. Substitution of N-acetylglycine in obtained product was observed at a few different positions (products were analyzed by using ^{1}H and ^{13}C NMR techniques). Developed procedure with DCC/DMAP coupling system was applied for acylation 1-thioglycosides **17** and **20**.

In case of acylation of D-glucose **21**, glycine substitution occurred in a few positions (4). In contrast to D-glucose as a modification substrate, dur-

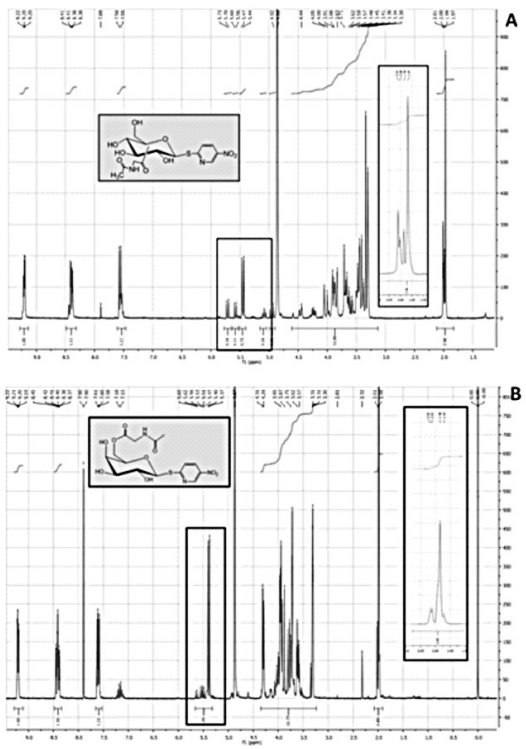


Figure 1. The ^{1}H NMR analysis of products formed after 1-thioglucoside (A) and 1-thiogalactoside (B) substitution by N-acetyl-L-glycine residue

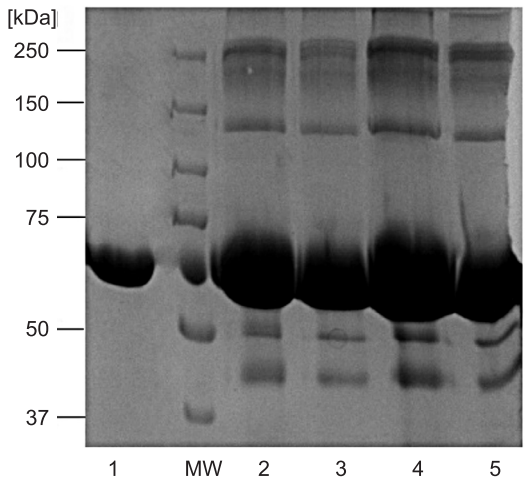


Figure 2. SDS-PAGE analysis of protein products obtained after conjugation of formylated BSA with various glycinated 1-thioglycosides based on glucose (Glc), galactose (Gal), melibiose (Mel): Mel/NAcGly (line 2); Glc/NAcGly (line 3); Gal/NAcGly (line 4); or not glycinated lactose derivatives: Lac/NAcGly(-) (line 5). Control sample: nonmodified BSA monomer – line 1; MW – molecular mass standard of proteins

ing D-galactose acylation reaction the main product **22** was substituted at C-6 position at sugar ring. Other isomeric products of 1-thiogalactoside acylation are present in insignificant amounts (Fig. 1A,B). Formation of main acylation product at C-6 position of galactose unit was observed.

In order to prepare obtained glycinated 1-thioglycosides **21–23** to conjugation, the nitro group present in aglycone of 1-thioglycosides was reduced into amino group. For these reaction earlier described procedure with zinc powder was applied. An only change was application of methanol in place of dichloromethane as a solvent. Unfortunately, formation of partially deglycinated by-products during the reaction was observed. Desired products were obtained in rather moderate yield.

Formylated proteins

Treatment of the stable protein-diol intermediates with sodium periodate resulted in the generation of reactive aldehyde functionalities through the oxidation of the glycol moieties (10). Both carrier proteins, e.g., BSA monomer and MYO, were subjected to synthesis of formylate derivatives. The efficiency of amino groups modification of BSA and MYO derivatives was estimated by comparison of the absorbance values for studied samples (modified proteins) with control samples (nonmodified proteins) at 92.1% and 86.3%, respectively.

In the next step, the oxidation of the glycol moieties with sodium periodate was performed for generation of reactive aldehyde functionalities. Desalted on Bio-Gel P-4 the formylated proteins were collected for conjugation experiments.

Glycoconjugates of different glycinated 1-thioglycosides with proteins

The carrier proteins modified by glycidol treatment next were coupled with amine group containing glycinated 1-thioglycosides based on monosaccharides and disaccharides. The mild oxidation of glycol intermediates method was applied in order to obtain the reactive aldehyde proteins derivatives.

Next, for the reductive amination sodium cyanoborohydride was used in order to complete reduction of the labile Schiff base intermediate to a chemically stable bond between aldehyde and amine functional groups. The optimal molar ratios of formylated proteins to glycinated 1-thioglycosides as well as non-glycinated derivatives were established as 1:200 and 1:35 for preparing BSA and MYO glycoconjugates, respectively. Calculations were based on the amount of free amine residue of basic amino acids in the native bovine albumin and horse myoglobin. The obtained product mixtures were analyzed by SDS-PAGE method. Representative BSA-derivatives contained glycoconjugates with molecular mass (M_W) 75, 95, 110, 165, 200, 230, 260 kDa (Fig. 2). In the mixture of MYO-derivatives, products with M_W 32, 130 and 150 kDa were detected (data not shown).

Mixtures of synthesized glycoconjugates were purified by HW-55S gel filtration in good yield. The more homogenous composition of products was observed for MYO-Glc/NAcGly derivatives than for BSA-Glc/NAcGly one (Fig. 3A,B, continuous lines). The selected glycoonjugate fractions after HW-55S purification were analyzed for total sugar content (Fig. 3A,B, dotted lines). The sugar component level was determined colorimetrically by phenol/sulfuric acid method and calculated from standard curve prepared for D-glucose. Obtained results were related to the amount of formylated protein taken for conjugation experiment. Table 1 contains data for the fractions collected after HW-55S chromatography and containing maximal level of total sugar. The samples of BSA-Glc/NAcGly and MYO-Glc/NAcGly were analyzed (Fig. 3) as well as BSA-Gal/NAcGly, BSA-Lac/NAcGly(-), BSA-Mel/NAcGly and MYO-Mel/NAcGly) fractions derived from other chromatographic separations (elution profiles from HW-55S not shown). The higher content of sugar component was observed for products based on BSA-galactose, than BSA-glucose, e.g., 53% and 38% for BSA-Gal/NAcGly (fractions 55–69) and BSA-Glc/NAcGly (fractions 64–73), respectively (Tab. 1 and Fig. 3A). The differences in the glycoconjugate sugar content were probably caused by the more homogenous substrate mixture formed during the substitution of 1-thiogalactoside with *N*-acetylglycine in comparison to heterogeneous 1-thioglucoside derivatives (see Fig. 1A,B).

The immunoreactivity of glycoconjugate fractions from Table 1 was tested in ELISA assay by using four dilutions (1:2500, 1:25000, 1:250000, 1:2500000) of rabbit serum (Fig. 4). The animal was immunized with *E. coli* K12 C600 core oligosaccharide glycine-containing glycoconjugate. The relative immunoreactivity of studied samples was referred to the control probe containing nonmodified albumin or myoglobin and corrected by the contribution of the reaction background.

Generally, the glycoconjugates with disaccharide-derived component demonstrated weaker immunoreactivity than monosaccharide one (BSA-Mel/NAcGly *vs.* BSA-Glc/NAcGly and BSA-

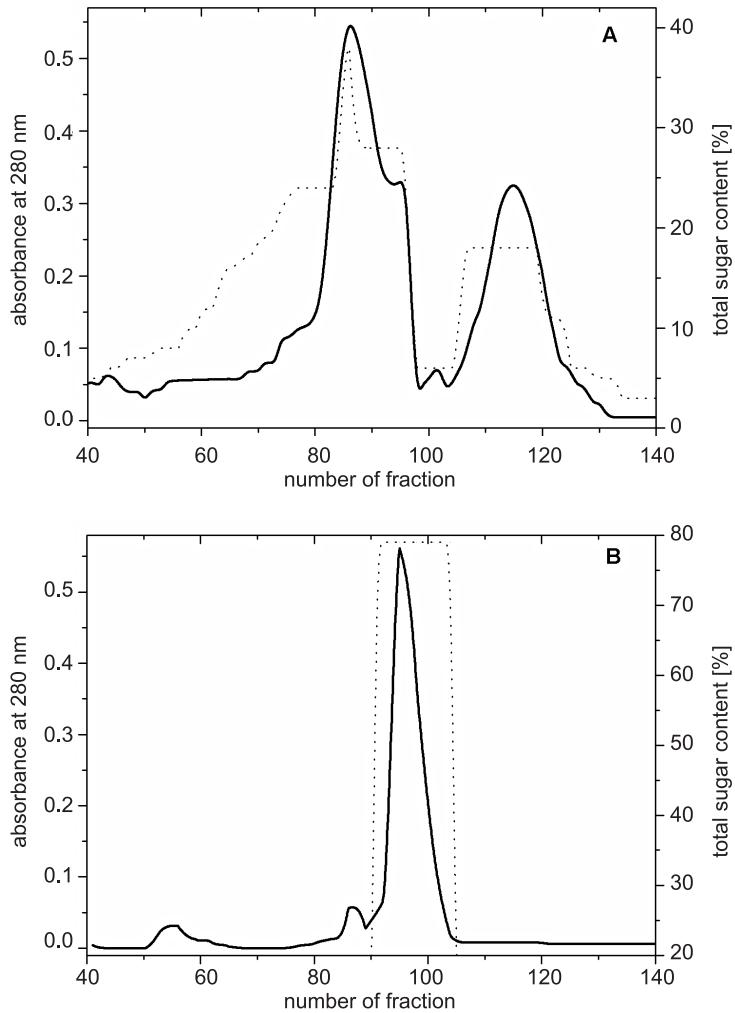


Figure 3. The HW-55S gel-filtration of product mixtures obtained after conjugation of formylated BSA or formylated MYO with glycinated 1-thioglycosides based on glucose: BSA-Glc/NAcGly (**A**) and MYO-Glc/NAcGly (**B**). Continuous line – profile of protein elution; dotted line – total sugar content [%]

Gal/NAcGly) (Fig. 4). For monosaccharide derivatives, the BSA-Glc/NAcGly was slightly more immunoreactive than BSA-Gal/NAcGly products. Moreover, the kind of carrier protein used in glycoconjugate synthesis probably could influence on the immunoreactivity differences for products containing the same sugar component (BSA-Glc/NAcGly *vs*. MYO-Glc/NAcGly and BSA-Mel/NAcGly *vs*. MYO-Mel/NAcGly). For this reason, the higher immunoreactivity of MYO-Glc/NAcGly and MYO-Mel/NAcGly glycoconjugates was observed for every four dilutions of used rabbit serum in comparison to analogous BSA glycoconjugates. For BSA-Lac/NAcGly(-) glycoconjugate, the immunoreactivity was practically imperceptible – in this glycoconjugate N-acetylglycine residue was not substituted in chemical synthesis (control glycoconjugate product). Furthermore, it is worth noting that the antigen used in the rabbit immunization for antibodies production contained BSA as a protein component and for this reason, the higher immunoreactivity was observed for BSA-based glycoconjugates.

In conclusions, the synthesis of stable glycinated 1-thioglycosides was performed and new glycoconjugates were obtained. Next, the presence of glycine epitope on synthesized glycoconjugates was verified by performing the immunoreactivity tests.

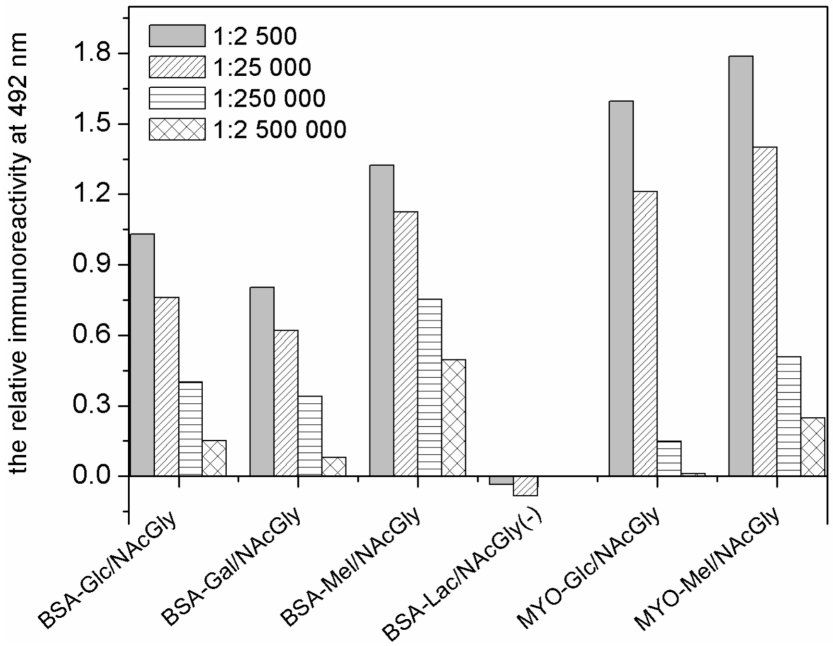


Figure 4. The relative immunoreactivity of obtained BSA- and MYO-glycoconjugates samples described in Table 1 after reaction with rabbit serum (in four dilutions 1 : 2500, 1 : 25000, 1 : 250000 and 1 : 2500000). Immunization of rabbit was performed with *E. coli* K12 C600 core oligosaccharide glycine-containing glycoconjugate

The differences in immunochemical properties of synthetic glycoconjugates were observed and they probably depend on the places of *N*-acetylglycine residue substitution in 1-thioglycosides and the kind of proteins carrier using in conjugation experiments.

Knowledge of the structure of the core domain of bacterial LPS is helpful in designing vaccines against many strains of Gram-negative bacteria.

Many research efforts are being directed towards searching for structural elements of LPS molecules common to many groups of pathogens. Finding a common epitope would be a basis for the development of a vaccine with a broad spectrum of specificity. The highly conserved structure of the endotoxin inner core justifies the search for a common epitope within this part of LPS. Different synthetic glycinated 1-thioglycosides based on monosaccharides and disaccharides obtained in our studies contain free functional amino group in the short linker structure. They should be applied in the glycine epitope glycoconjugate synthesis, which may be useful to vaccines construction against pathogenic bacterial strains. These preliminary results indicating rather low reactivity of studied anti-*E. coli* core LPS rabbit serum with synthesized compounds prompts for using these new conjugates as immunogens to obtain in animals specific antibodies which would allow the search for antigens with glycine-containing epitope.

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