Letters to the Editor

Synthesis of covalent conjugates of hexaarabinofuranoside with proteins and their testing as antigens for serodiagnosis of tuberculosis

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Specificity and sensitivity are crucial in diagnosis of tuberculosis. An insufficiently sensitive method will "miss" too many persons really sick with active tuberculosis. If the method is insufficiently specific, its use will give many false positive results among healthy people or among non-tubercular patients (the latter fact is not too critical for mass screening of population, because the primary diagnosis is always checked by alternative methods). Enzyme-linked immunosorbent analysis (ELISA), immunochromatography (IC), and dot-blotting technique using one or several recombinant protein antigens of *Mycobacterium tuberculosis* were developed in the recent time for serodiagnosis of tuberculosis. In particular, an array of recombinant antigens (38-, 63-, 64-, 14-, and 59-kDa) is successfully used in commercial serological assays TB-DOT (Upper Bio-tech, China, http://www.poct.cn) and TB-Check-1 (VEDA.LAB, France, http://www.vedalab.com); the declared assay specificity reaches 99.42%, and the sensitivity is 98.52% (see Ref. 1). However, it was found when using these tests that this level of sensitivity is reached only for certain human populations, which can be associated with national and ethnic peculiarities of the humoral immune response to tuberculosis.[2] For example, 11 recombinant proteins of *M. tuberculosis* have earlier[3] been obtained and used as antigens for the analysis of 320 sera of sick and healthy peoples from the central region of Russia. None of them provided the sensitivity higher than 55%, and various combinations of antigens increased this parameter by at most 7%.

It was assumed that an insufficient sensitivity is related to the absence of a carbohydrate component in the antigens used, since it was shown that antibodies to the carbohydrate part of *M. tuberculosis* lipoarabinomannan (LAM) were observed for the majority of tubercular patients.[4] Therefore, we attempted to synthesize (Schemes 1 and 2) covalent conjugates of the terminal hexaarabinofuranoside fragment of LAM, *viz.*, the main epitope of LAM,[5] with recombinant proteins of *M. tuberculosis* in order to

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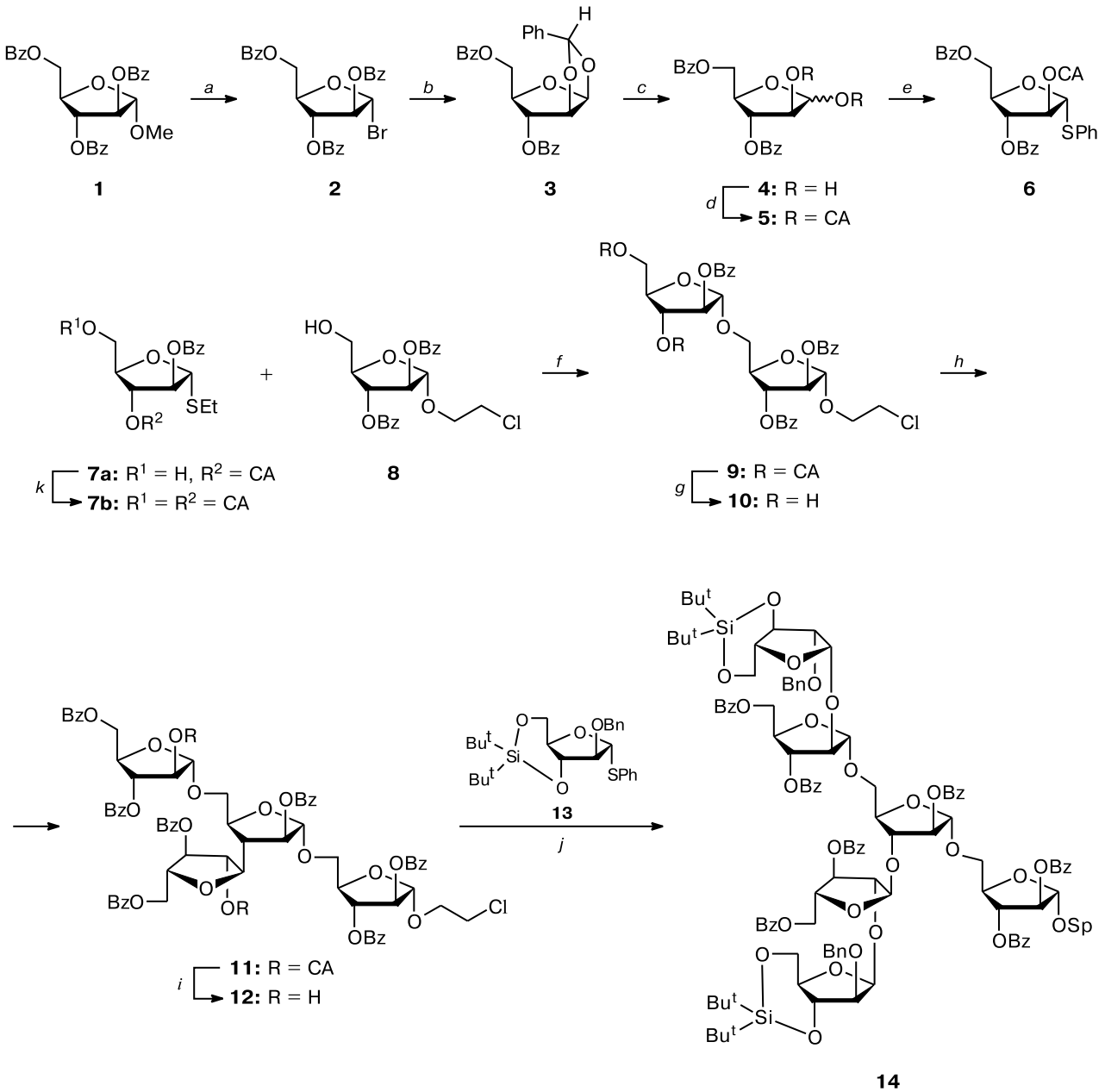
obtain *in vitro* glycosylated antigens for serodiagnosis of tuberculosis.

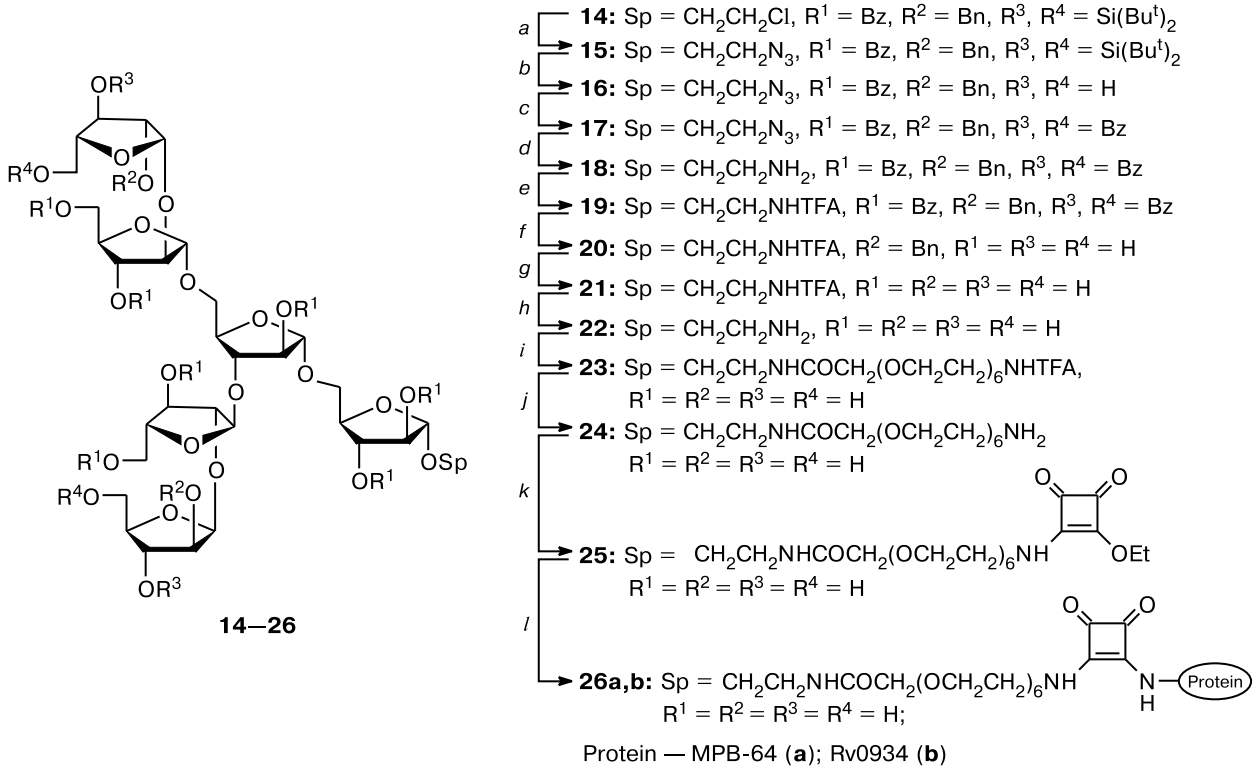
We synthesized hexasaccharides **22** and **24** as glycosides with the functionalized spacer-aglycones (see Schemes 1 and 2). The role of the spacer-aglycone is not limited by the spatial separation of the carbohydrate ligand from the polymer support only, which is required for efficient binding of the carbohydrate ligand to the corresponding receptor. The nature of the spacer-aglycone that links the oligosaccharide haptene to the polymer support can

Scheme 1



Reagents, conditions, and yields: *a*. 32% HBr/AcOH; *b*. $NaBH_4$, MeCN (90% over two steps); *c*. 90% TFA, CH_2Cl_2; *d*. $(ClCH_2CO)_2O$, *sym*-collidine, CH_2Cl_2, 96%; *e*. PhSH, $BF_3 \cdot Et_2O$, CH_2Cl_2, 0 °C, 3 h, 83%; *f*. NIS, TESOTf, MS 4 Å, CH_2Cl_2, −20 °C, chromatography on SiO_2, 76%; *g*. Py; H_2O, 70 °C, 2 h, chromatography on SiO_2, 89%; *h*. **6**, NIS, AgOTf, MS 4 Å, CH_2Cl_2, −40 °C → +4 °C, 4 h, chromatography on SiO_2, 84%; *i*. Py, H_2O, 70 °C, 2 h, chromatography on SiO_2, 89%; *j*. NIS, AgOTf, MS 4 Å, CH_2Cl_2, −40 °C, 1 h, chromatography on SiO_2 (HPLC), 55%; *k*. $(ClCH_2CO)_2O$, *sym*-collidine, CH_2Cl_2, chromatography on SiO_2, 87%.

Scheme 2



Reagents, conditions, and yields: *a*. NaN_3, DMF, 70 °C, 30 h, 97 %; *b*. *n*-Bu_4NF, THF, 12 h, chromatography on SiO_2 (HPLC), 77%; *c*. BzCl, Py, chromatography on SiO_2, 80%; *d*. PPh_3, THF—H_2O (10 : 1), 0 °C → 20 °C; *e*. $(CF_3CO)_2O$, NEt_3, THF, gel chromatography on Bio-Beads S×3 (toluene), chromatography on SiO_2, 73% over two steps; *f*. NaOMe, MeOH, ~20 °C, 30 h, gel chromatography on Sephadex LH-20 (MeOH); *g*. H_2, Pd/C, MeOH—H_2O, 28 °C, 12 h, gel chromatography on Sephadex LH-20 (MeOH), 41% over two steps; *h*. NaOH, MeOH; *i*. $HOOCCH_2(OCH_2CH_2)_6NHTFA$, DMT-MM, *N*-methylmorpholine, MeOH, gel chromatography on Sephadex LH-20 (MeOH), 86%; *j*. NaOH, MeOH; *k*. diethyl squarate, H_2O, pH 7.5, chromatography on Sep-Pak C18 (gradient H_2O→MeOH); *l*. protein MPB-64 (**27a**) or Rv0934 (**27b**), pH 9, dialysis (water).

also determine both the specificity of the elicited antibodies and the possibility of efficient immunization.[6] In this work, for conjugation with proteins we used hexaarabinofuranoside **24** with a long, flexible, and hydrophilic spacer which contained the oligo(ethylene glycol) fragment.

A pre-spacer approach was used in the synthesis of hexasaccharide **24**. This is based on the preparation of protected hexasaccharide **14** as 2-chloroethyl glycoside, removal of the protecting groups, and modification of the aglycone to 2-aminoethyl glycoside **22** followed by its coupling with $HOOCCH_2(OCH_2CH_2)_6NHTFA$ (see Ref. 7) and liberation of the amino group. This approach is extremely divergent and, unlike the synthetic schemes used earlier[8] for the synthesis of oligosaccharide fragments of LAM, makes it possible to obtain a set of hexaarabinofuranosides with various spacer-aglycones from the single hexasaccharide precursor without repeating the optimization of the non-trivial steps of the glycosidic bond formation. The rational scheme of assembling protected hexasaccharide **14** includes two steps of bis-glycosylation: with the formation of 1,2-*trans*-glycosidic bonds [2+1+1] and 1,2-*cis*-glycosidic bonds [4+1+1]. The hydroxyl groups to be further glycosylated were protected with chloroacetyl groups, which can selectively be removed in the presence of the *O*-benzoyl protecting groups.

The known methyl 2,3,5-tri-*O*-benzoyl-α-D-arabinofuranoside (**1**)[9] was transformed into 3,5-di-*O*-benzoyl-1,2-*O*-benzylidene-β-D-arabinofuranose (**3**), which served as a key monosaccharide building block for the synthesis of phenyl 3,5-di-*O*-benzoyl-2-*O*-chloroacetyl-1-thio-α-D-arabinofuranoside (**6**) (see Scheme 1) containing the selectively removable chloroacetyl protecting group for subsequent chain elongation at O(2). (1-5)-Linked disaccharide **10** was obtained in 76% yield by glycosylation of 2-chloroethyl 2,3-di-*O*-benzoyl-α-D-arabinofuranoside (**8**)[10] with thioglycoside **7b** (synthesized by chloroacetylation of alcohol **7a**)[10] followed by removal of the chloroacetyl groups. The glycosylation of the synthesized disaccharide **10** with thioglycoside **6** afforded tetrasaccharide **11** in 84% yield. The ^{13}C NMR spectrum of tetra-

saccharide **11** contains signals for the anomeric carbon atoms of the α-linked arabinofuranose residues (δ_C 105.30, 105.47, 105.51, and 105.57), and the ^{1}H NMR spectrum exhibits signals for four anomeric protons (δ_H 5.27 (2 H), 5.37, and 5.44). The monosaccharide residues linked by 1,2-*cis*-glycosidic bonds were introduced into tetrasaccharide **12** with two hydroxyl groups by glycosylation with thioglycoside **13** (see Ref. 8a) to form an anomeric mixture of hexasaccharides in 95% yield. The required β,β-linked isomer of hexasaccharide **14** was isolated by HPLC in a yield of 55%. Subsequent steps included modification of the aglycone in the hexasaccharide (**14**→**15**), desilylation (**15**→**16**), benzoylation of the free hydroxyl groups (**16**→**17**), reduction of the azido group in the aglycone to the amino group (**17**→**18**), trifluoroacetylation of the amino group (**18**→**19**), *O*-deacylation (**19**→**20**), hydrogenolysis (**20**→**21**), and liberation of the amino group to form unprotected hexasaccharide **22** with the aminoethyl aglycone, which was coupled with $HOOCCH_2(OCH_2CH_2)_6NHTFA$[7] to form amide **23** in 86% yield (15% based on protected hexasaccharide **14**) ($[\alpha]_D^{28}$ +36.0 (*c* 0.44, MeOH); MS (ESI): *m/z* 1288.5024 [M + NH_4]. Calculated for $C_{48}H_{81}F_3N_2O_{33}$ [M + NH_4]: 1288.5012). The ^{13}C NMR spectrum of hexasaccharide **23** contains signals for two anomeric carbon atoms of the β-linked arabinofuranose residues (δ_C 102.62 and 102.72) and four anomeric carbon atoms of the α-linked arabinofuranose residues (δ_C 107.27, 107.57, and 109.73 (2 C)); the ^{1}H NMR spectrum exhibits signals for two anomeric protons of the β-linked arabinofuranose residues (δ_H 5.03 (2 H, br.s) and four anomeric protons of the α-linked arabinofuranose residues (δ_H 4.90 (s), 4.96 (s), 5.09 (s), and 5.17 (s)), whose presence confirms the structure of the synthesized compound. Removal of the *N*-trifluoroacetyl protecting group in amide **23** by alkaline hydrolysis gave spacered hexasaccharide **24**, which was used without isolation for the conjugation with proteins.

The genes that encode MPB-64 and Rv0934 proteins of *M. tuberculosis* were cloned in *E. coli* by the polymerase chain reaction (PCR) using DNA of strain H37Rv and were expressed from plasmid pET under control of a strong promoter. Recombinant MPB-64 and Rv0934 proteins were purified by affinity chromatography. The conjugation of hexaarabinofuranoside **24** with the hydrophilic spacer-aglycone with MPB-64 (**27a**) and Rv 0934 (**27b**) proteins was carried out using the squaric acid fragment as a linker.[11] In the first step, the reaction of hexasaccharide haptene **24** with diethyl squarate in a 0.1 *M* phosphate buffer (pH 7.0) at ~20 °C (10 h) afforded monoethyl haptene squarate **25** (λ_{max} = 259 nm). After purification by reversed-phase chromatography on a Sep-Pak C18 cartridge (gradient water→MeOH), compound **25** was conjugated (separately) with proteins **27a** (λ_{max} = 274 nm) and **27b** (λ_{max} ≈ 265 nm, shoulder) in a 0.05 *M* borate buffer (pH 9.0) at +4 °C (60 h). Conjugates Ara_6-MPB-64 (**26a**) and Ara_6-Rv0934 (**26b**) were isolated by dialysis against water at +4 °C and stored in the frozen state. The inclusion of the squaric acid fragment and, hence, hexasaccharide into the obtained conjugates was indicated by a considerable increase in the absorption band intensity (λ_{max} = 277 nm) in the UV spectrum of conjugate **26a** compared to the starting protein **27a** (at the same concentration), as well as the appearance in the UV spectrum of conjugate **26b** of an intense absorption band (λ_{max} = 274 nm), which was absent from the spectrum of the starting protein **27b**.

The synthesized conjugates were used as antigens for ELISA of the sera of tubercular patients. We tested 200 sera collected in the central region of Russia: 100 sera from turbercular patients and 100 sera from healthy donors vaccinated with the anti-tuberculosis BCG vaccine. Artificial *M. tuberculosis* antigens **26a** and **26b** provided sensitivity of 68 and 55%, respectively, which exceeds the sensitivity for non-conjugated proteins **27a** and **27b** by more than 10%. Some decrease in the specificity of the obtained con-

Table 1. Results of determination of the sensitivity and specificity of recombinant proteins **27a,b** and their conjugates **26a,b** in ELISA with the sera from tubercular patients and from healthy donors[a]

Antigen	Sensitivity[b]	Specificity[c]
	(%)	
26a	68	80
26b	55	80
27a	55	84
27b	44	85

[a] We tested 200 sera collected in the central region of Russia: 100 sera from tuberculosis patients and 100 sera from healthy donors vaccinated with the anti-tuberculosis vaccine BCG. Testing was carried out by indirect ELISA. Antigens were immobilized in wells of 96-well plates (100 μL per well) from a concentration of 3 μg mL^{-1} in a carbonate buffer (pH 9.6) at 4 °C for 16 h. After washing off, samples of the sera diluted in a ratio of 1 : 200 were introduced into the wells and incubated for 1 h at 37 °C. The immune complexes formed were revealed by the peroxidase conjugate of antibodies against human IgG in the dilution 1 : 10 000. The immune complexes formed were detected with the substrate containing tetramethylbenzidine recording the absorbance at 450 nm when the reaction ceased. The cut-off point was determined by the results of ELISA of the studied proteins with the sera of the reference group as an average value of the detected absorbance of the sera of the reference group plus three standard deviations. The sensitivity and specificity of the diagnosis were determined from the value of cut-off.

[b] The diagnosis sensitivity of the antigens is the percent of positive samples in the serological assay of the sera from patients with the confirmed "tuberculosis" diagnosis.

[c] The diagnosis specificity of the antigens is the percent of negative (truly negative) responses in the serological assay of the sera of healthy donors or non-tubercular patients.

jugates (by ~5%) compared to that of non-conjugated proteins **27a** and **27b** should be mentioned: 80% in the case of conjugates **26a** and **26b** (see Table 1).

Thus, the introduction of LAM carbohydrate epitopes of *M. tuberculosis* (hexaarabinofuranoside) into tuberculosis protein antigens can increase the diagnostic sensitivity of the serological tuberculosis assays developed for the use in the central region of Russia, and the work in this direction seems promising.

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