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# 2-Aminopyridine—a label for bridging of oligosaccharides HPLC profiling and glycoarray printing

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**Abstract** 2-Aminopyridine derivatives of oligosaccharides (OS-AP) were printed onto microchips by two different ways. The first method is based on direct covalent insertion of OS-AP in polyacrylamide gel 3D chip. The second method is based on conversion of OS-AP into more reactive OS-aminoalditol followed by covalent printing onto NHS-activated glass slides. This approach extends the range of saccharides suitable for covalent printing due to availability of commercial OS-AP and easy high-performance liquid chromatography separation of glycoprotein N-chains in form of AP derivatives.

**Keywords** 2-aminopyridine · Fluorescent label · Glycoconjugates · Glycochip · Oligosaccharides

**Abbreviations**

AP 2-aminopyridine
BSA bovine serum albumin
4-DMP 4-dimethylaminopyridine
DMSO dimethyl sulfoxide
Fmoc-Gly-SI N-(9-fluorenylmethoxycarbonyl)-glycine, succinimide ester
Ig immunoglobulin
YDS (Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1)$_2$-3,6Manβ1-4GlcNAcβ1-4GlcNAc
iM$_8$ iso-maltooctaose, (Glcβ1-6)$_8$
Mel melibiose, Galα1-6Glc
NP p-nitrophenol
OS oligosaccharide
NHS N-hydroxisuccinimide
PBS phosphate-buffered saline
pNPA poly(4-nitrophenyl acrylate)
Et$_3$N triethylamine
9OS (Galβ1-4GlcNAcβ1-2Manα1)$_2$-3,6Manβ1-4GlcNAcβ1-4GlcNAc

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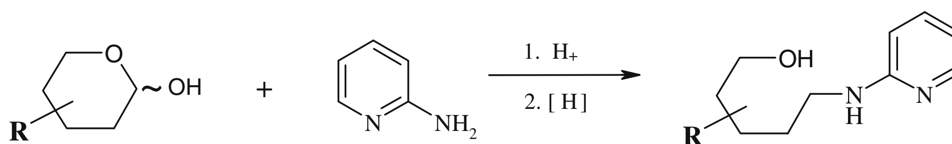
Modern printed glycoarrays are characterized by low oligosaccharide consumption, at the level of $10^{-12}$–$10^{-14}$ mole [1, 2]. Routine structure analysis of glycoprotein carbohydrate chains based on chromatographic separation of oligosaccharides is also typically performed within $10^{-10}$–$10^{-13}$ molar range. This coincidence opens an attractive prospect of using OS obtained after *analytical* HPLC for immobilization on a chip followed by assaying lectins and carbohydrate-binding antibodies. Fluorescent labeling of OS is especially attractive: on the one hand, it facilitates HPLC separation and makes OS detection more sensitive; on the other hand, it allows quantitative dosage of glycans during chip fabrication [3]. Therefore, general methodology could be the following: (1) carbohydrate chains are cleaved from protein core, (2) oligosaccharide pool is labeled with fluorescent reagent, (3) HPLC is performed, possibly in two-dimensional mode, *i.e.* using consequently two different columns, (4) after fluorometric

**Scheme 1** Reaction of OS labeling with 2-aminopyridine




detection and MS characterization, the collected peaks are diluted or concentrated to the optimal concentration, and (5) are placed into the microarrayer.

Obviously, the fluorescent label should be bifunctional [4], *i.e.* to have one functional group for OS labeling and another one as a tag for covalent binding to the chip surface. At a glance, it seems difficult to find a label both assisting good HPLC separation and satisfying the stringent requirements for the further carbohydrate-protein interaction; nevertheless, we succeeded in finding such a label—it is widely used 2-aminopyridine [5, 6].

Amino group in its composition possesses both functions mentioned above. Indeed, the product of reductive amination (Scheme 1) retains the amino group though it becomes a secondary one, *i.e.* much more chemically inert. Despite the decreased reactivity, we have found out two ways of AP-OS immobilization onto chip, direct and indirect ones.

## Material and methods

2-aminopyridine (crystallized from hexane), anhydrous hydrazine, 4-dimethylaminopyridine, DMF, DMSO, Fmoc-Gly-SI, melibiose, $Et_3N$ and Tween-20 were from Fluka (Switzerland); iso-maltooctaose, Mel-$NHCOCH_2NH_2$, 9OS and 9OS-$NHCOCH_2NH_2$ were from Lectinity (Russia). Acetic acid, acetonitrile, ammonium acetate, methanol, and pyridine were from Reakhim (Russia). YDS-AP was from Glyence (Japan). Sodium cyanoborohydride was from Merck (Germany). $C_{18}$-silica and Sephadex LH-20 were from Pharmacia Biotech (Austria). Rh-nanoparticles were synthesized as described in [7]. pNPA [8] and *N*-biotinyl-caproic acid p-nitrophenyl ester [9] were synthesized as described earlier. Anti-Galα1-3Gal human antibodies were isolated using a disaccharide-exposing affinity adsorbent and their specificity was studied as described [10]. AP-labeled OSs were synthesized as described in [11, 12]. Conversion of Mel to the corresponding aminoalditol, Mel-ol-$NH_2$, was performed as described in [13]. Conversion of Mel-AP to the corresponding aminoalditol was performed according to the method described in [14]. Biotinylated *Erithrina cristagalli* lectin (ECL) was from Vector Labs (USA). Anti-human Ig(G+M) antibodies conjugated with peroxidase and mouse monoclonal anti-human IgG were from Southern Biotech (USA). Cy5-labeled anti-mouse antibodies were from Amersham Biosciences (USA). Alexa555 labeled streptavidin was from Pierce (USA).

*Conversion of OS-AP derivatives to the corresponding aminoalditols* was performed using Rh-nanoparticles. 250 pmol of 9OS-AP (obtained from YDS-AP using 0.05 M TFA[15]) in 200 μl water were hydrogenated with 0.3 μl (1 nmol) 3.8 M Rh-nanoparticles [7] solution at room temperature and atmospheric pressure, for 14 h. The mixture was centrifuged at 13400 g for 15 min and passed through 100 μl of $C_{18}$-silica. The product was eluted with 0.3 ml of water and evaporated using SpeedVac (Savant, USA). Then 0.2 ml of anhydrous hydrazine was added, incubated at 70°C for 3 min and evaporated as above. After co-evaporation with toluene (3×0.2 ml), the product was printed onto NHS-activated slides without further purification.

*3D hydrogel microchips* Hydrogel drop microchips with covalently immobilized saccharides were fabricated as described in [16]. Human antibodies against Galα1-3Gal in PBS (1:40) containing 0.3% BSA were added to a microchip (80 μl per chip) and the solution was incubated for 3 h at room temperature. After washing for 10 min with 0.01 M PBS, containing 0.1% Tween-20, mouse monoclonal anti-human IgG (1:1,000) were added and chips were kept for 3 h at room temperature, washed, and Cy5-labeled anti-mouse antibodies in PBS (1:1,000) were added (80 μl per chip) and kept for 2 h at room temperature. Results were read as described in [16].

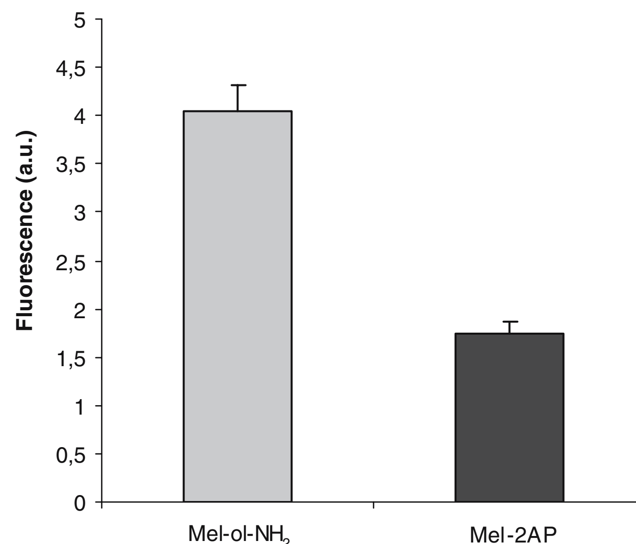



**Fig. 1** Comparison of antibody-binding potency of Mel-AP and Mel-ol-$NH_2$ immobilized onto 3D-gel microchip. Dilution of anti-αGal-antibodies is 1:40, saccharide content is about 10 pmol/per gel element

*Printed 2D array* Substances were printed onto NHS-activated glass slides as described in [1]. Slides were incubated with 10 μg/ml solution of biotinylated *Erithrina cristagalli* lectin in PBS, containing 0.5% Tween 20, at 80% humidity for 1 h at room temperature and successively washed by PBS with 0.1% Tween 20, PBS with 0.05% Tween, and finally with PBS. Then Alexa555 labeled streptavidin solution in PBS containing 0.5% Tween 20 (dilution 1:100) was added and incubated at 80% humidity for 1 h at room temperature. After washing with PBS containing 0.1% Tween 20 followed by water, fluorescence intensity was measured using ScanArray 5000 (PerkinElmer, USA).

## Results and discussion

First of all, we tested whether the secondary amino group in composition of AP-derivative could be acylated. To this end, octasaccharide $iM_8$-AP was used. Reaction of $iM_8$-AP with pNPA, *i.e.* acrylic acid fully activated with 4-nitrophenol did not proceed in standard conditions of primary aliphatic amines attachment [8, 17], *i.e.* at 37°C in the presence of triethylamine. Addition of *N*-acylation catalyst, 4-DMP, initiated the reaction that was completed in 4 h leading to neoglycoconjugate with OS content 20% mol. Interaction of $iM_8$-AP with 10-fold excess of biot-NH $(CH_2)_5$COONP, also led to quantitative acylation in presence of 4-DMP. However, both synthesized compounds were unstable in alkali conditions releasing initial AP-oligosaccharide, this evidencing that acylation in drastic conditions led to *O*- rather than *N*-acylation and that this way did not answer our purposes.

Nevertheless, we succeeded in AP-derivatives immobilization basing on other chemistries. The first one is nucleophilic addition to double bond followed by copolymerization reaction into polyacrylamide hydrogel [2, 16]. In the 3D hydrogel chip the drops of polyacrylamide gel (several nanoliters in volume) are coated onto glass surface. Saccharides containing primary amino groups or AP-derivatives were co-polymerized in a "one-pot" process. Typical amount of immobilized compound in such experiment was several picomol per gel element, that is, 2–3 orders of magnitude smaller than the amount of PAA-conjugates used for standard 96-well plate assays (nanomol per microplate well).

Immobilization efficiency of AP-derivatives was tested at the example of Mel-AP, which was compared to another melibiose derivative, Mel-ol-$NH_2$ (where reducing unit is also open, but amino group is primary). Interaction of anti-αGal-antibodies with Mel-AP was 2-fold lower than with Mel-ol-$NH_2$ (Fig. 1). The results were reproducible. These results demonstrate that the degree of Mel-AP insertion to 3D hydrogel is practically acceptable for reliable fluorescent reading.

2D printed glycoarray [1] is based on an acylation reaction. Typical amount of immobilized compound in such experiment was several femtomol per element (dot). As OS-AP derivatives are inert for *N*-acylation (see above), we converted them into compounds with elevated reaction ability. According to [14], pyridylamino-derivatives of N-glycan chains can be converted into corresponding aminoalditols. Efficiency of this reaction was tested by us at the examples of Mel-AP and 9OS-AP. In the first case, Mel-AP was hydrogenolyzed in presence of black palladium followed by hydrazinolysis as described in [14]. The obtained product was identified as 1-amino-1-deoxysugar by MS ($[M+H]^+$, *m/z* 344.1, cacld. 344.2). However, this catalyst proved to be inconvenient due to the difficulties with filtration, so 9OS-AP (250 pmol) has been hydrogenolyzed in the presence of nano-Rh particles, which can be removed by centrifugation more easily than palladium black. Only this method has been further used in this study. In both cases the yield of aminoalditols was about 30% according to HPLC data. The obtained 9OS-ol-$NH_2$ was printed on NHS-activated slides (2D array) [1], 9OS with glycyl spacer (9OS-Gly) was used as the control. The fact of immobilization on chip was confirmed by interaction with *Erithrina cristagalli* lectin. The oligosaccharides were bound by lectin in the similar degree (data not shown).

2D array is convenient as it allows working with picomol amounts of OS. Two orders of magnitude more ligand are necessary for the design of 3D-array, thus, 3D-approach becomes inapplicable in case of the work with picomol amounts of a ligand. At the same time, just due to the placement of 100-fold larger amount of oligosaccharide on the same surface as compared to 2D-array 3D-array produces more intense signal of better morphology. Thus, it is possible to immobilized AP-oligosaccharides on chip in two ways: (1) directly, by 3D-gel microchip technique, and (2) indirectly, by conversion into aminoalditol followed by printing onto activated 2D chip. Though the degree of immobilization in case of 3D chip, and conversion of AP-derivatives into aminoalditols in case of 2D chip have proven to be far from 100%, we consider the development of this direction to be prospective due to availability of AP-derivatives as commercial reagents, their easy separation with the help of HPLC, and well documented separation protocols for hundreds of AP-glycans [5, 6].

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