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Regioselective enzymatic acylation of *N*-acetylhexosamines

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Abstract

A careful choice of the reaction conditions (solvent, enzyme, acylating agent) allowed an efficient regioselective acylation of *N*-acetylhexosamines. 6-*O*-Acyl derivatives of 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc), 2-acetamido-2-deoxy-D-galactopyranose (GalNAc) and 2-acetamido-2-deoxy-D-mannopyranose (ManNAc) have been isolated from regioselective esterifications catalysed by the protease subtilisin in CH_3CN–DMSO 8:2 in good yields.

Keywords: *N*-Acetylhexosamines; Regioselectivity; Enzymatic acylation; Subtilisin; DMSO

1. Introduction

Enzymatic regioselective acylation of sugars is a well-established methodology nowadays. From the pioneering works of Klibanov and co-workers, published in the late 1980s [1], several papers described the selective esterification of mono-, di- and trisaccharides, and of their derivatives [2], the modification of natural glycosides [3] and of insoluble polysaccharides [4]. More recently, these biotransformations have been exploited to support the chemo-enzymatic synthesis of small oligosaccharides. In the first approach, lactose derivatives orthogonally protected with esters removable under different conditions were used for the efficient synthesis of milk oligosaccharides [5]. Bi-enzymatic approach to specific oligosaccharides based on the sequential use of lipases/proteases and glycosidases was proposed by us. The selective protection of reactive primary OH's by lipase/protease-catalysed acylation enabled to direct the action of glycosidases towards a single secondary OH of an acceptor [6]. In this way, trisaccharide *iso*-globotriose was synthesized in a simple three-step sequence [6a].

Our major present interest aims at the synthesis and characterisation of chitooligomer derivatives for application in immunology [7]. We have demonstrated recently the β-*N*-acetylhexosaminidase catalysed synthesis of 6-*O*,*N*,*N*′-triacetylchitobiose and 6′-*O*,*N*,*N*′-triacetylchitobiose using *p*-nitrophenyl 6-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside as a selectively protected sugar donor and 6-*O*-acetyl-2-acetamido-2-deoxy-D-glucopyranose as a selectively protected sugar acceptor [6b]. To extend this methodology to the preparation of other chitooligomers we have started a systematic investigation on the enzymatic acylation of different aminosugars and this report summarises the results achieved.

2. Experimental

2.1. Materials and methods

Pyridine, acetone, vinyl acetate, *N*,*N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO) and acetonitrile were

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from Aldrich. Trifluoroethyl acetate and trichloroethyl butyrate were prepared according to the standard procedure [8]. All other chemicals were of analytical grade.

The following enzymes were used for biocatalysed reactions: lipase PS from *Burkholderia cepacia* (Amano) adsorbed on celite (Hyflo Super Cell celite, Fluka) [9], lipase B from *Candida antarctica* immobilized on macroporous acrylic resin (Novozym 435, Novo-Nordisk), lipase from porcine pancreas (Sigma), lipase A-6 from *Aspergillus niger* and lipase CE-5 from *Humicola lanuginosa* (Amano). Protease N and proleather (both from *Bacillus subtilis*, Amano), alcalase (Novo-Nordisk) and subtilisin (protease type VIII from *Bacillus licheniformis*, Subtilisin Carlsberg, Sigma). Prior to use the latter enzymes were pH-adjusted (7.8) and lyophilised from a water solution containing K_2HPO_4 (0.5 g/g enzyme in 50 ml H_2O) or KCl (KCl, 4.9 g; subtilisin, 50 mg; K_2HPO_4, 50 mg; H_2O, 200 ml) or the substrate (GlcNAc, 200 mg; subtilisin, 100 mg; K_2HPO_4, 20 mg; H_2O, 10 ml). Subtilisin-CLECs were from Amano. All enzymatic reactions were paralleled with the controls void of enzymes to exclude spontaneous non-enzymatic acylations.

GC analyses were performed with a capillary cross-linked methyl silicone gum column (HP-1, 25 m × 0.32 mm × 0.52 μm film tickness, Hewlett-Packard). Thin-layer chromatography (TLC) was carried out on Merck precoated 60 F_{254} plates, detection was performed with Hanessian reagent ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 42 g; $Ce(SO_4)_2$, 2 g; H_2SO_4 conc., 62 ml made up to 1 l of H_2O). Flash column chromatography was performed on silica gel 60 (40–63 μm, Merck). NMR spectra were measured on a Varian INOVA-400 spectrometer (399.89 MHz for ^{1}H, 100.55 MHz for ^{13}C) and Bruker (type of NMR spectrometer used in Milan) in d_bDMSO, CD_3OD or D_2O (see text) at 30 °C. The residual solvent signal was used as an internal reference (DMSO: δ_H 2.50, δ_C 39.6; CD_3OD: δ_H 3.33, δ_C 49.3); internal acetone was used in D_2O solutions (δ_H 2.03, δ_C 30.5). Digital resolution used justified quoting the proton chemical shifts to three and carbon chemical shifts to two decimal places. The ^{13}C chemical shifts given to one decimal place are HMQC and HMBC readouts. Reported assignments are based on COSY, TOCSY, HMQC, and HMBC experiments. 1D-TOCSY was used to obtain ^{1}H NMR paramerers in the cases of overlap. The anomeric configuration throughout the *manno*- series is based on diagnostic [10] direct couplings ^1J(C-1,H-1) obtained from coupled-HMQC. The acylation sites were determined by HMQC (protons at the acylated carbon and the α-protons of the acyl are coupled to the same carbonyl). Supporting arguments were provided by downfield acylation shifts both in ^{1}H and ^{13}C NMR spectra. With the exception of non-reducing sugars (**3e**, **3f**) and the *p*NP glycoside **4a**, all compounds were mixtures of anomers. However, as the ^{1}H NMR spectra were measured from fresh solutions, the relative proportions of individual components might not reflect the equilibrium composition [11,12]. Moreover, these values are solvent dependent [13].

2.2. Sugars derivatisation for GC analysis

A sample of the enzymatic reaction solution (30 μl) was diluted with pyridine (30 μl) and reacted with 1,1,1,3,3,3-hexamethyldisilazane (30 μl) and CF_3COOH (3 μl). After 5 min, 5 μl of this final solution was injected into the GC. Conditions: for **1** and its derivatives: oven temperature at 200 °C for 3 min, then from 200 to 300 °C with a heating rate of 5 °C/min.

2.3. Acetylation of 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc, *1*) in DMF

GlcNAc (**1**, 500 mg, 2.26 mmol) was dissolved in DMF (22.5 ml) and trifluoroethyl acetate (2.5 ml). Protease N (750 mg) was suspended and the reaction was shaken at 40 °C (250 rpm) and monitored by TLC (AcOEt:MeOH:H_2O = 10:2:0.4). After 6 days the enzyme was filtered off. Flash chromatography (eluent: AcOEt:MeOH:H_2O = 10:1:0.3) gave **1a** (370 mg, 1.41 mmol, 62% yield).

*2-Acetamido-6-*O*-acetyl-2-deoxy-*D*-glucopyranose* (**1a**): mp 179–181 °C. ^{1}H NMR (DMSO + D_2O): 1.85 (3H, s, NAc), 2.03 (3H, s, Ac), 3.12 (1H, t, J = 8 Hz), 3.55 (2H, m), 3.80 (1H, dd, J_1 = 7 Hz, J_2 = 2 Hz, H-5), 4.05 (1H, dd, J_1 = 11 Hz, J_2 = 7 Hz, H-6b), 4.25 (1H, dd, J_1 = 11 Hz, J_2 = 2 Hz, H-6a), 4.90 (1H, d, J = 3 Hz, H-1), 7.68 (1H, d, J = 10 Hz, NH). NMR (DMSO): 20.7 (Ac), 22.5 (NAc), 54.1 (C-2), 63.9 (C-6), 69.2 (C-5), 70.1 (C-3), 70.9 (C-4), 90.5 (C-1), 169.9 (NCO), 170.6 (CO).

2.4. Optimisation of the reaction conditions

(a) GlcNAc (20 mg) was dissolved in 200 μl DMSO. Different organic cosolvents (700 μl) as well as trifluoroethyl acetate (100 μl) were added, followed by the addition of protease N (30 mg). The suspensions were shaken at 45 °C and at definite times, samples were taken and analysed by GC as described in 2.2. Data reported in Fig. 1.
(b) Reagents and analytical procedures were as described previously. The following amounts of enzymes were used: protease N, 30 mg; subtilisin, 5 mg; alcalase,

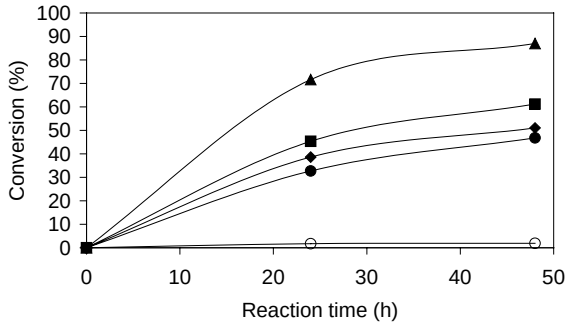


Fig. 1. Acetylation of GlcNAc catalysed by protease N in organic solvents (*tert*-amyl alcohol (◆), dioxane (■), acetonitrile (▲), acetone (●), DMF (○)) containing 20% (v/v) DMSO.

Table 1
Conversion (%)[a] of GlcNAc (**2**) during the regioselective acetylation catalysed by proteases and lipases suspended in various organic solvents mixtures

Enzyme	Solvent–DMSO (8:2, v/v)		
	t-Amyl alcohol	Acetonitrile	Dioxane
Proteases			
Protease N	51	87	61
Subtilisin	95	67	48
Proleather	72	73	7
Alcalase	12	17	3
Lipases			
Novozym 435	51	72	11
Porcine pancreatic lipase	6	8	1
Lipase PS	48	7	3
Chromobacterium viscosum lipase	13	15	1
Humicola lanuginosa lipase	1	1	1

[a] Degrees of conversion were evaluated after 48 h by GC (see Section 2).

20 mg; proleather, 30 mg; Novozym 435, 10 mg; porcine pancreatic lipase, 50 mg; lipase PS on celite, 50 mg; lipase CE-5, 30 mg; *Chromobacterium viscosum* lipase, 50 mg. Data reported in Table 1.

2.5. Acetylation of 2-acetamido-2-deoxy-D-galactopyranose (GalNAc, 2) in acetonitrile–DMSO

GalNAc (**2**, 500 mg, 2.3 mmol) was dissolved in DMSO (4 ml) followed by the addition of acetonitrile (13 ml) and trifluoroethyl acetate (2 ml). Subtilisin lyophilised with K_2HPO_4 (200 mg) was suspended and the mixture was shaken at 45 °C for 3 days monitoring the reaction by TLC (AcOEt:MeOH:H_2O = 10:2:0.4). The enzyme was filtered off, the solvent evaporated and the residue purified by flash chromatography (eluent: first AcOEt to remove residual DMSO, then AcOEt:MeOH:H_2O = 10:2:0.4) to afford products **2a** (315 mg, 1.2 mmol, 51%) and **2b** (31 mg, 0.11 mmol, 5%).

*2-Acetamido-6-*O*-acetyl-2-deoxy-*D*-galactopyranose* (**2a**): mp 101–102 °C. ^{1}H NMR (D_2O)-α-pyranose:β-pyranose:α-furanose:β-furanose = 50:41:2:7. α-Pyranose: 1.833 (3H, s, NAc), 1.904 (3H, s, OAc), 3.713 (1H, dd, $J_{1,2}$ = 3.2 Hz, $J_{2,3}$ = 11.1 Hz, H-3), 3.817 (1H, dd, $J_{2,3}$ = 3.2 Hz, $J_{3,4}$ = 1.0 Hz, H-4), 3.925 (1H, dd, $J_{1,2}$ = 3.7 Hz, $J_{2,3}$ = 11.1 Hz, H-2), 4.017 (1H, dd, $J_{5,6a}$ = 9.1 Hz, $J_{6a,6b}$ = 12.9 Hz, H-6a), 4.068 (1H, m, H-5), 4.096 (1H, dd, $J_{5,6b}$ = 4.8 Hz, $J_{6a,6b}$ = 12.2 Hz, H-6b), 5.017 (1H, d, $J_{1,2}$ = 3.7 Hz, H-1); β-pyranose: 1.831 (3H, s, NAc), 1.908 (3H, s, OAc), 3.514 (1H, dd, $J_{2,3}$ = 10.8 Hz, $J_{2,3}$ = 3.3 Hz, H-3), 3.666 (1H, dd, $J_{1,2}$ = 8.4 Hz, $J_{2,3}$ = 10.8 Hz, H-2), 3.757 (1H, dd, $J_{3,4}$ = 3.3 Hz, $J_{4,5}$ = 1.3 Hz, H-4), 3.684 (1H, m, H-5), 4.435 (1H, d, $J_{1,2}$ = 8.4 Hz, H-1); α-furanose: 5.946 (1H, d, $J_{1,2}$ = 5.6 Hz, H-1); β-furanose: 5.875 d (1H, $J_{1,2}$ = 4.2 Hz, H-1). ^{13}C NMR (D_2O): α-pyranose: 20.4 (OAc), 22.1 (NAc), 50.3 (C-2), 64.2 (C-6), 67.4 (C-3), 68.2 (C-5), 68.7 (C-4), 91.3 (C-1), 174.1 (6-C=O), 174.9 (2-C=O); β-pyranose: 20.4 (OAc), 22.1 (NAc), 53.8 (C-2), 64.2 (C-6), 67.9 (C-4), 71.0 (C-3), 72.4 (C-5), 96.7 (C-1), 174.1 (6-CO), 174.9 (2-CO).

*2-Acetamido-6-*O*-acetyl-2-deoxy-*D*-galactofuranose* (**2b**): mp 95–96 °C. ^{1}H NMR (D_2O): α-furanose:β-furanose:α-pyranose:β-*pyranose* = 71:6:13:10; α-furanose: 1.912 (3H, s, Ac), 3.768 (1H, m, H-5), 3.786 (1H, dd, $J_{3,4}$ = 7.4 Hz, $J_{4,5}$ = 4.7 Hz, H-4), 3.946 (1H, dd, $J_{5,6a}$ = 6.3 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6a), 4.036 (1H, dd, $J_{5,6b}$ = 3.8 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6b), 4.108 (1H, dd, $J_{2,3}$ = 9.6 Hz, $J_{3,4}$ = 7.4 Hz, H-3), 4.327 (1 H, dd, $J_{1,2}$ = 4.7 Hz, $J_{2,3}$ = 9.6 Hz, H-2), 5.953 (1H, d, $J_{1,2}$ = 4.7 Hz, H-1); β-furanose: 5.886 (1H, d, $J_{1,2}$ = 4.6 Hz, H-1);. α-pyranose: 5.017 (1H, d, $J_{1,2}$ = 3.7 Hz, H-1); β-pyranose: 4.434 (1H, d, $J_{1,2}$ = 8.4 Hz, H-1). ^{13}C NMR (D_2O): 20.3 (OAc), 21.7 (NAc), 57.4 (C-2), 65.3 (C-6), 69.0 (C-5), 71.6 (C-3), 82.5 (C-4), 94.3 (C-1).

2.6. Acetylation of 2-acetamido-2-deoxy-D-mannopyranose (ManNAc, 3) in acetonitrile–DMSO

ManNAc (3, 500 mg, 2.3 mmol) was dissolved in DMSO (4 ml), followed by the addition of acetonitrile (13 ml) and trifluoroethyl acetate (2 ml). Subtilisin lyophilised with K_2HPO_4 (200 mg) was suspended and the mixture was shaken at 45 °C for 2 days monitoring the reaction by TLC (AcOEt:MeOH:H_2O = 10:2:0.4). The enzyme was filtered off, the solvent evaporated and the residue purified by flash chromatography (eluent: first AcOEt to remove residual DMSO, then AcOEt:MeOH:H_2O = 10:1:0.5) to give products **3a** (345 mg, 1.3 mmol, 59%) and **3b** (95 mg, 0.30 mmol, 13%).

*2-Acetamido-6-*O*-acetyl-2-deoxy-*D*-mannopyranose* (**3a**): colourless oil: ^{1}H NMR (CD_3OD): α-furanose:β-furanose:α-pyranose:β-pyranose = 5.4.76.15; α-pyranose: 2.029 (3H, s, 2-Ac), 2.078 (3H, s, 6-Ac), 3.542 (1H, dd, $J_{3,4}$ = 9.6 Hz, $J_{4,5}$ = 9.9 Hz, H-4), 3.998 (1H, ddd, $J_{4,5}$ = 9.9 Hz, $J_{5,6a}$ = 7.0 Hz, $J_{5,6b}$ = 2.2 Hz, H-5), 4.022 (1H, dd, $J_{2,3}$ = 4.8 Hz, $J_{3,4}$ = 9.6 Hz, H-3), 4.263 (1H, dd, $J_{1,2}$ = 1.7 Hz, $J_{2,3}$ = 4.8 Hz, H-2), 4.275 (1H, dd, $J_{5,6a}$ = 7.0 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6a), 4.388 (1H, dd, $J_{5,6b}$ = 2.2 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6b), 5.026 (1H, d, $J_{1,2}$ = 1.7 Hz, H-1); β-pyranose: 2.072 (3H, s, 2-Ac), 2.078 (3H, s, 6-Ac), 3.411 (1H, dd, $J_{3,4}$ = 9.2 Hz, $J_{4,5}$ = 9.6 Hz, H-4), 3.480 (1H, ddd, $J_{4,5}$ = 9.6 Hz, $J_{5,6a}$ = 2.2 Hz, $J_{5,6b}$ = 7.0 Hz, H-5), 3.674 (1H, dd, $J_{2,3}$ = 4.4 Hz, $J_{3,4}$ = 9.2 Hz, H-3), 4.417 (1H, dd, $J_{5,6a}$ = 2.2 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6a), 4.279 (1H, dd, $J_{5,6b}$ = 7.0 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6b), 4.429 (1H, dd, $J_{1,2}$ = 1.7 Hz, $J_{2,3}$ = 4.4 Hz, H-2), 4.873 (1H, d, $J_{1,2}$ = 1.7 Hz, H-1); α-furanose: 5.264 (1H, d, J = 5.7 Hz, H-1); β-furanose: 5.243 (1H, d, J = 5.0 Hz, H-1). ^{13}C NMR (CD_3OD)-α-pyranose: 21.04 (6-Ac), 22.87 (2-Ac), 55.65 (C-2), 65.79 (C-6), 69.58 (C-4), 70.56 (C-3),

71.70 (C-5), 94.99 (C-1), 173.26 (6-CO), 174.44 (2-CO); β-pyranose: 21.01 (6-Ac), 23.07 (2-Ac), 56.05 (C-2), 65.70 (C-6), 69.38 (C-4), 74.55 (C-3), 76.40 (C-5), 95.37 (C-1), 173.26 (6-CO), 175.98 (2-CO).

2-Acetamido-3,6-di-O-*acetyl-2-deoxy*-D-*mannopyranose* (**3b**): colourless oil: ^{1}H-NMR (d_bDMSO): 1.90, 1.95 and 2.05 (3H each, s, 3 × Ac), 3.62 (1H, t, J = 9.8 Hz, H-4), 3.92 (1H, br t, J = 8.5 Hz, H-5), 4.15 (1H, dd, J_1 = 12.6 Hz, J_2 = 7.8 Hz, H-6a), 4.23 (1H, br d, J = 4.64 Hz, H-2), 4.35 (1H, br d, J = 12.6 Hz, H-6b), 4.84 (1H, br s, H-1α), 5.02 (1H, dd, J_1 = 4.6 Hz, J_2 = 9.8 Hz, H-3). ^{13}C-NMR (DMSO) δ: 20.6, 20.7 and 22.3 (3 × Ac), 50.4 (C-2), 63.9 (C-6), 64.2 (C-4), 69.9 (C-5), 71.6 (C-3), 92.4 (C-1), 170.6 and 171.2 (2 × CO).

2.7. Butanoylation of 2-acetamido-2-deoxy-D-*mannopyranose (ManNAc, **3**) in acetonitrile–DMSO*

ManNAc (**3**, 250 mg, 1.1 mmol) was dissolved in DMSO (2 ml) followed by the addition of acetonitrile (6.5 ml) and trichloroethyl butyrate (1 ml). Subtilisin lyophilised with K_2HPO_4 (100 mg) was suspended and the mixture was shaken at 45 °C. At various intervals, the course of the reaction was monitored by TLC (AcOEt:MeOH:H_2O = 10:2:0.4). When the desired point of conversion was reached (after 1 day), the enzyme was filtered off. Evaporation of the solution and flash column chromatography (eluent: first AcOEt for removing residual DMSO, followed by AcOEt:MeOH:H_2O = 10:0.5:0.2) gave pure products **3c** (257 mg, 77.9 %) and **3d** (46 mg, 11.5 %).

2-Acetamido-6-O-*butyryl-2-deoxy*-D-*mannopyranose* (**3c**): colourless oil. ^{1}H NMR (CD_3OD): α-furanose:β-furanose:α-pyranose:β-pyranose = 5:4:77:14; α-pyranose: 0.977 (3H, t, J = 7.4 Hz, 6-$COCH_2CH_2C\mathbf{H}_3$), 1.672 (2H, m, 6-$COCH_2C\mathbf{H}_2CH_3$), 2.028 (3H, s, 2-Ac), 2.351 (2H, t, J = 7.4 Hz, 6-$COC\mathbf{H}_2CH_2CH_3$), 3.540 (1H, dd, $J_{3,4}$ = 9.6 Hz, $J_{4,5}$ = 9.9 Hz, H-4), 3.996 (1H, ddd, $J_{5,6b}$ = 2.2 Hz, $J_{5,6a}$ = 7.0 Hz, $J_{4,5}$ = 9.9 Hz, H-5), 4.025 (1H, dd, $J_{2,3}$ = 4.7 Hz, $J_{3,4}$ = 9.6 Hz, H-3), 4.271 (1H, dd, $J_{5,6a}$ = 7.0 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6a), 4.413 (1H, dd, $J_{5,6b}$ = 2.2 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6b), 4.255 (1H, dd, $J_{1,2}$ = 1.7 Hz, $J_{2,3}$ = 4.7 Hz, H-2), 5.031 (1H, d, $J_{1,2}$ = 1.7 Hz, H-1); β-pyranose: 0.981 (3H, t, J = 7.4 Hz, 6-$COCH_2CH_2C\mathbf{H}_3$), 1.677 (2H, m, 6-$COCH_2C\mathbf{H}_2CH_3$), 2.072 (3H, s, 2-Ac), 2.351 (2H, t, J = 7.4 Hz, 6-$COC\mathbf{H}_2CH_2CH_3$), 3.483 (1H, ddd, $J_{4,5}$ = 9.9 Hz, $J_{5,6a}$ = 7.2 Hz, $J_{5,6b}$ = 2.2 Hz, H-5), 3.409 (1H, dd, $J_{3,4}$ = 9.2 Hz, $J_{4,5}$ = 9.9 Hz, H-4), 3.677 (1H, dd, $J_{2,3}$ = 4.4 Hz, $J_{3,4}$ = 9.2 Hz, H-3), 4.281 (1H, dd, $J_{5,6a}$ = 7.2 Hz, $J_{6a,6b}$ = 11.8 Hz, H-6a), 4.425 (1H, dd, $J_{1,2}$ = 1.7 Hz, $J_{2,3}$ = 4.4 Hz, H-2), 4.433 (1H, dd, $J_{5,6b}$ = 2.2 Hz, $J_{6a,6b}$ = 11.8 Hz, H-6b), 4.874 (1H, d, $J_{1,2}$ = 1.7 Hz, H-1); α-furanose: 5.266 (1H, d, $J_{1,2}$ = 5.7 Hz, H-1); β-furanose: 5.244 (1H, d, $J_{1,2}$ = 5.0 Hz, H-1). ^{13}C NMR (CD_3OD)-α-pyranose:14.22 (6-$COCH_2CH_2\mathbf{C}H_3$), 19.70 (6-$COCH_2\mathbf{C}H_2CH_3$), 22.90 (2-Ac), 37.18 (6-$CO\mathbf{C}H_2CH_2CH_3$), 55.67 (C-2), 65.58 (C-6), 69.60 (C-4), 70.54 (C-3), 71.75 (C-5), 94.90 (C-1), 174.40 (2-CO), 175.74 (6-CO); β-pyranose: 14.22 (6-$COCH_2CH_2\mathbf{C}H_3$), 19.70 (6-$COCH_2\mathbf{C}H_2CH_3$), 23.10 (2-Ac), 37.15 (6-$CO\mathbf{C}H_2CH_2CH_3$), 56.37 (C-2), 65.53 (C-6), 69.41 (C-4), 74.55 (C-3), 76.44 (C-5), 95.31 (C-1), 175.58 (2-CO), 175.64 (6-CO); α-furanose: 102.10 (C-1); β-furanose: 97.29 (C-1).

2-Acetamido-3,6-di-O-*butyryl-2-deoxy*-D-*mannopyranose* (**3d**): colourless oil: ^{1}H NMR (CD_3OD): α-pyranose:β-pyranose = 83:17; α-pyranose 0.964 (3H, t, J = 7.4 Hz, 3-$COCH_2CH_2C\mathbf{H}_3$), 0.983 (3H, t, J = 7.4 Hz, 6-$COCH_2CH_2C\mathbf{H}_3$), 1.602–1.725 (4H, m, 3-$COCH_2C\mathbf{H}_2CH_3$, 6-$COCH_2C\mathbf{H}_2CH_3$), 2.003 (3H, s, 2-Ac), 2.305 (2H, t, J = 7.3 Hz, 3-$COC\mathbf{H}_2CH_2CH_3$), 2.362 (2H, t, J = 7.3 Hz, 6-$COC\mathbf{H}_2CH_2CH_3$), 3.718 (1H, dd, $J_{3,4}$ = 10.0 Hz, $J_{4,5}$ = 10.0 Hz, H-4), 4.121 (1H, ddd, $J_{4,5}$ = 10.0 Hz, $J_{5,6a}$ = 7.0 Hz, $J_{5,6b}$ = 2.3 Hz, H-5), 4.319 (1H, dd, $J_{5,6a}$ = 7.0 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6a), 4.417 (1H, dd, $J_{5,6b}$ = 2.3 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6b), 4.480 (1H, dd, $J_{1,2}$ = 1.7 Hz, $J_{2,3}$ = 4.6 Hz, H-2), 4.994 (1H, d, $J_{1,2}$ = 1.7 Hz, H-1), 5.224 (1H, dd, $J_{2,3}$ = 4.6 Hz, $J_{3,4}$=10.0 Hz, H-3); β-pyranose: 0.969 (3H, t, J = 7.4 Hz, 3-$COCH_2CH_2C\mathbf{H}_3$), 0.983 (3H, t, J = 7.4 Hz, 6-$COCH_2CH_2C\mathbf{H}_3$), 1.602–1.725 (4H, m, 3-$COCH_2C\mathbf{H}_2CH_3$, 6-$COCH_2C\mathbf{H}_2CH_3$), 2.038 (3H, s, 2-Ac), 2.298 (2H, t, J = 7.3 Hz, 3-$COC\mathbf{H}_2CH_2CH_3$), 2.362 (2H, t, J = 7.3 Hz, 6-$COC\mathbf{H}_2CH_2CH_3$), 3.589 (1H, ddd, $J_{4,5}$ = 9.4 Hz, $J_{5,6a}$ = 7.0 Hz, $J_{5,6b}$ = 2.1 Hz, H-5), 3.638 (1H, dd, $J_{3,4}$ = 9.5 Hz, $J_{4,5}$ = 9.4 Hz, H-4), 4.319 (1H, dd, $J_{5,6a}$ = 7.0 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6a), 4.438 (1H, dd, $J_{5,6b}$ = 2.1 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6b), 4.581 (1H, dd, $J_{1,2}$ = 1.7 Hz, $J_{2,3}$ = 4.3 Hz, H-2), 4.857 (1H, dd, $J_{2,3}$ = 4.3 Hz, $J_{3,4}$ = 9.5 Hz, H-3), 4.973 (1H, d, $J_{1,2}$ = 1.7 Hz, H-1). ^{13}C NMR (CD_3OD): α-pyranose: 14.21 (3-$COCH_2CH_2\ \mathbf{C}H_3$, 6-$COCH_2CH_2\mathbf{C}H_3$), 19.52 (3-$COCH_2\mathbf{C}H_2CH_3$), 19.70 (6-$COCH_2CH_2CH_3$), 22.73 (2-Ac), 37.17 (6-$CO\mathbf{C}H_2CH_3CH_3$), 37.34 (3-$CO\mathbf{C}H_2CH_3CH_3$), 52.92 (C-2), 65.45 (C-6), 67.03 (C-4), 71.82 (C-5), 73.47 (C-3), 95.01 (C-1), 173.83 (2-CO), 175.01 (3-CO), 175.75 (6-CO); β-pyranose: 14.24 (3-$COCH_2CH_2\mathbf{C}H_3$, 6-$COCH_2CH_2\mathbf{C}H_3$), 19.40 (3-$COCH_2\mathbf{C}H_2CH_3$), 19.70 (6-$COCH_2\mathbf{C}H_2CH_3$), 22.91 (2-Ac), 37.14 (6-$CO\mathbf{C}H_2CH_2CH_3$), 37.26 (3-$CO\mathbf{C}H_2CH_2$-CH_3), 53.31 (C-2), 65.33 (C-6), 66.73 (C-4), 76.14 (C-3), 76.38 (C-5), 94.71 (C-1), 174.6 (2-CO), 175.33 (3-CO), 175.64 (6-CO).

2.8. 2-Acetamido-1,6-di-O-acetyl-2-deoxy-α-D-mannopyranose (***3e***)

2-Acetamido-6-O-acetyl-2-deoxy-D-mannopyranose (**3a**) (200 mg, 0.8 mmol) was dissolved in pyridine (1 ml). Acetone (8 ml), trifluoroethyl acetate (2 ml) and Novozym 435 (200 mg) were added, the reaction mixture was shaken at 45 °C and monitored by TLC (AcOEt:MeOH:H_2O = 10:2:0.4 and $CHCl_3$:MeOH:H_2O = 9:1.5:0.1). After 7 days,

the enzyme was filtered off. Evaporation of the solvent and flash chromatography (eluent:$CHCl_3$:MeOH:H_2O = 9:1.5:0.1) gave product **3e** in 18.4% yield (42 mg).

2-Acetamido-1,6-di-O-*acetyl-2-deoxy-α*-D-*mannopyranose* (**3e**): colourless oil. ^{1}H NMR (d_bDMSO): 1.886 (3H, s, 2-NAc), 2.008 (3H, s, 6-OAc), 2.077 (3H, s, 1-OAc), 3.539 (1H, dd, $J_{3,4}$ = 9.2 Hz, $J_{4,5}$ = 9.7 Hz, H-4), 3.653 (1H, ddd, $J_{4,5}$ = 9.7 Hz, $J_{5,6a}$ = 7.0 Hz, $J_{5,6b}$ = 2.1 Hz, H-5), 3.763 (1H, dd, $J_{2,3}$ = 4.9 Hz, $J_{3,4}$ = 9.2 Hz, H-3), 4.041 (1H, ddd, $J_{1,2}$ = 2.1 Hz, $J_{2,3}$ = 4.9 Hz, $J_{2,NH}$ = 7.9 Hz, H-2), 4.097 (1H, dd, $J_{5,6a}$ = 7.0 Hz, $J_{6a,6b}$ = 11.9 Hz, H-6a), 4.256 (1H, dd, $J_{5,6b}$ = 2.1 Hz, $J_{6a,6b}$ = 11.9 Hz, H-6b), 5.746 (1H, d, $J_{1,2}$ = 2.1 Hz, H-1), 7.774 (1 H, d, $J_{2,NH}$ = 7.9 Hz, NH). ^{13}C NMR (d_bDMSO): 20.7 (1-Ac, 6-Ac), 22.4 (2-Ac), 51.4 (C-2), 63.6 (C-6), 66.5 (C-4), 67.9 (C-3), 72.7 (C-5), 91.9 (C-1), 168.6 (1-CO), 169.8 (2-CO), 170.3 (6-CO).

2.9. *2-Acetamido-1,6-di*-O-butyryl-2-deoxy-D-mannopyranose (*3f*)

2-Acetamido-6-*O*-butyryl-2-deoxy-D-mannopyranoside (**3c**) (150 mg, 0.5 mmol) was dissolved in acetone (3 ml). Trichloroethyl butyrate (1 ml) and Novozym 435 (100 mg) were added and the mixture was shaken at 45 °C. The course of the reaction was monitored by TLC (AcOEt:MeOH:H_2O = 10:1:0.3) and after 4 days the enzyme was filtered off. Evaporation of the solution and flash chromatography (eluent:AcOEt:MeOH:H_2O = 10:0.5:0.2) gave 37.4 mg of product **3f** (19.4 %).

2-Acetamido-1,6-di-O-*butyryl-2-deoxy*-D-*mannopyranose* (**3f**): brownish oil. ^{1}H NMR (CD_3OD): 0.962 (3H, t, J = 7.4 Hz, 6-$COCH_2CH_2C\mathbf{H}_3$), 1.005 (3H, t, J = 7.4 Hz, 1-$COCH_2CH_2C\mathbf{H}_3$), 1.649 (2H, m, 6-$COCH_2C\mathbf{H}_2CH_3$), 1.700 (2H, m, 1-$COCH_2C\mathbf{H}_2CH_3$), 2.046 (3H, s, 2-Ac), 2.326 (2H, t, J = 7.3 Hz, 6-$COC\mathbf{H}_2CH_2CH_3$), 2.400 (2H, t, J = 7.2 Hz, 1-$COC\mathbf{H}_2CH_2CH_3$), 3.622 (1H, dd, $J_{3,4}$ = 9.5 Hz, $J_{4,5}$ = 9.9 Hz, H-4), 3.816 (1H, ddd, $J_{4,5}$ = 9.9 Hz, $J_{5,6a}$ = 7.3 Hz, $J_{5,6b}$ = 2.2 Hz, H-5), 3.975 (1H, dd, $J_{2,3}$ = 4.9 Hz, $J_{3,4}$ = 9.5 Hz, H-3), 4.265 (1H, dd, $J_{5,6a}$ = 7.3 Hz, $J_{6a,6b}$ = 11.9 Hz, H-6a), 4.279 (1H, dd, $J_{1,2}$ = 1.8 Hz, $J_{2,3}$ = 4.9 Hz, H-2), 4.404 (1H, dd, $J_{5,6b}$ = 2.2 Hz, $J_{6a,6b}$ = 11.9 Hz, H-6b), 5.962 (1H, d, $J_{1,2}$ = 1.8 Hz, H-1). ^{13}C NMR (CD_3OD): 14.15 (1-$COCH_2CH_2\mathbf{C}H_3$), 14.20 (6-$COCH_2CH_2\mathbf{C}H_3$, 19.67 (1-$COCH_2\mathbf{C}H_2CH_3$), 19.72 (6-$COCH_2\mathbf{C}H_2CH_3$), 22.78 (2-Ac), 37.16 (1-$CO\mathbf{C}H_2CH_2CH_3$, 6-$CO\mathbf{C}H_2CH_2CH_3$), 53.82 (C-2), 65.15 (C-6), 68.95 (C-4), 70.56 (C-3), 74.39 (C-5), 93.69 (C-1), 173.15 (1-CO), 174.49 (2-CO), 175.56 (6-CO).

2.10. *p-Nitrophenyl 2-acetamido-6*-O-acetyl-2-deoxy-*β*-D-galactopyranoside (*4a*)

p-Nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (**4**) (280 mg, 0.8 mmol) was dissolved in pyridine (21 ml), followed by addition of acetone (29 ml) and vinyl acetate (10 ml). Novozym 435 (200 mg) was added and the suspension was shaken (200 rpm) at 45 °C and monitored by TLC (AcOEt:MeOH:H_2O = 10:1:0.3). Enzyme addition was repeated after 2 days (100 mg) and after 4 days (50 mg). After 7 days, TLC analysis showed almost 100% conversion to a single product. The enzyme was filtered off and the product **4a** was obtained by crystallisation from pyridine in 53.3% yield (166 mg).

p-Nitrophenyl 2-acetamido-6-O-*acetyl-2-deoxy-β*-D-*galactopyranoside* (**4a**): amorphous white solid: ^{1}H-NMR (CD_3OD) 1.996 (3H, s, 2-NAc), 2.091 (3H, s, 6-OAc), 3.811 (1H, dd, $J_{2,3}$ = 10.7 Hz, $J_{3,4}$ = 3.3 Hz, H-3), 3.934 (1H, dd, $J_{3,4}$ = 3.3 Hz, $J_{4,5}$ = 1.2 Hz, H-4), 3.998 (1H, ddd, $J_{4,5}$ = 1.2 Hz, $J_{5,6a}$ = 4.6 Hz, $J_{5,6b}$ = 7.7 Hz, H-5), 4.237 (1H, dd, $J_{1,2}$ = 8.4 Hz, $J_{2,3}$ = 10.7 Hz, H-2), 4.290 (1H, dd, $J_{5,6a}$ = 4.6 Hz, $J_{6a,6b}$ = 11.5 Hz, H-6a), 4.368 (1H, dd, $J_{5,6b}$ = 7.7 Hz, $J_{6a,6b}$ = 11.5 Hz, H-6b), 5.234 (1H, d, $J_{1,2}$ = 8.4 Hz, H-1), 7.190 & 8.229 (4H, AA′BB′, ΣJ = 9.3 Hz, *p*NP). ^{13}C NMR (CD_3OD): 20.8 (OAc), 23.0 (NAc), 54.0 (C-2), 64.7 (C-6), 69.6 (C-4), 72.5 (C-3), 74.6 (C-5), 102.2 (C-1), 117.8 (2 C, 2 × C-*ortho*), 126.7 (2 C, 2 × C-*meta*), 144.2 (C-*para*), 163.8 (C-*ipso*), 172.7 (6-CO), 174.4 (2-CO).

3. Results and discussion

3.1. *Acylation of* N-acetylhexosamines

One of the main problems related to the enzymatic acylation of free mono- and disaccharides is the low solubility of these compounds in the organic solvents that are more suitable to preserve the catalytic activity of lipases and proteases. The use of polar solvents, like pyridine or DMF, substantially decreases the number of enzymes that can be employed. For instance, only the protease subtilisin, commercially available under different trade names, is significantly active in DMF [14]. The data on the effect of DMSO, another very good solvent for carbohydrates, are more contradictory. DMSO can dissolve proteins and thus it causes a significant enzyme denaturation and a consequent loss of activity [15]. On the other hand, there are recent reports that describe the acylation of sugars in a mixture of organic solvents containing a significant percentage of DMSO [16]. These latter solvent mixtures might also be suitable for the acylation of chitobiose, one of our future target compounds.

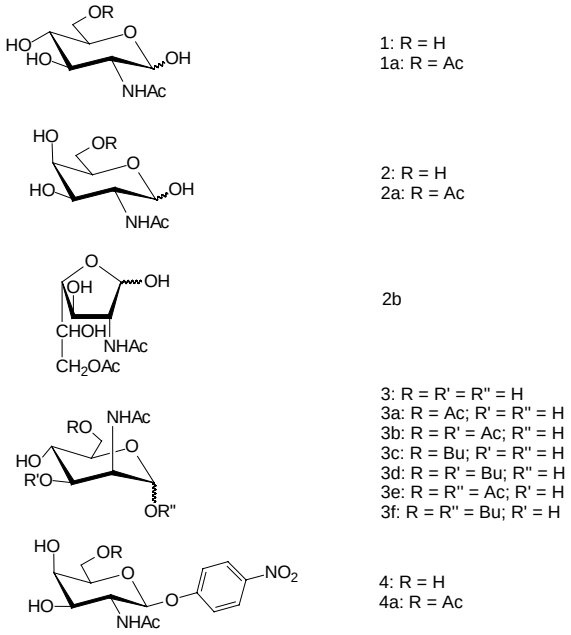
Our initial goal was the optimisation of the reaction conditions to achieve the selective acylation of the *N*-acetylhexosamines, such as 2-acetamido-2-deoxy-D-glucopyranose (**1**), 2-acetamido-2-deoxy-D-galactopyranose (**2**) and 2-acetamido-2-deoxy-D-mannopyranose (**3**). We chose the cheapest of these sugars as a reference compound and evaluated the performances of different enzymes under various reaction environments. As a “reference“ reaction we chose the acetylation of **1** in DMF catalysed by

protease N (a crude preparation of subtilisin). As expected, the conversion was satisfactory and the product, isolated in 62% yield, was found to be 2-acetamido-6-*O*-acetyl-2-deoxy-D-glucopyranose (**1a**) according to ^{1}H and ^{13}C NMR (downfield shift of the C-6 signal; upfield shift of the C-5 signal). The same reaction was repeated using different polar solvents containing 20% (v/v) DMSO. As shown in Fig. 1, compared with pure DMF, reaction outcomes in these solvent mixtures were comparable or even better, the only exception being just the reaction DMF containing 20% DMSO, in which protease N was completely inactive.

The acetylation of **1** was repeated in three best solvent mixtures using different commercially available protease and lipase preparations, known to be able to catalyse sugar esterifications. The results, summarised in Table 1, clearly indicate that proteases — and particularly subtilisin — were more suitable for this biotransformation than lipases and that the use of a mixture acetonitrile–DMSO (80:20) gave better results with most of the enzymes tested. By increasing the amount of DMSO, the protease activities dramatically decreased, while lipases were inactivated and the acylation did not proceed at all.

Finally, the influence of different enzymatic preparations on the reaction performances were investigated. Lyophilisation of subtilisin pH-adjusted to 7.8 in the presence of an excess of KCl (to assure a higher hydration of the protein powder, [17]) or of the substrate GlcNAc (as a sort of substrate-imprinting of the active site, [16]) gave enzymatic preparation that proved to be by far less active than the standard enzyme lyophilised in the presence of K_2HPO_4. Apparently, the polarity of the reaction medium completely eliminated the activating effect of salts and substrates. Quite surprisingly, even CLEC preparations [18] were completely inactive in both DMF and acetonitrile–DMSO (80:20) and in dioxane–DMSO (80:20).

The best reaction conditions (subtilisin suspended in CH_3CN–DMSO, 8:2) were applied to the acylation of compounds **2** and **3**. In case of GalNAc, two monoesters, **2a** and **2b**, were isolated in 51% and 5% yields, respectively, and characterized by NMR. The main product **2a** was the expected 2-acetamido-6-*O*-acetyl-2-deoxy-D-galactopyranose. The set of vicinal coupling constants of the minor by-product **2b** $J_{1,2}$ to $J_{4,5}$ (4.7, 9.6, 7.4, and 4.7 Hz) did not match neither those of α-GalNAc nor β-GalNAc [19]. Downfield resonating C-4 (82.5 ppm) indicates its participation on an oxygen bridge formation (that implies a five-membered furanose ring). The $J_{1,2}$ coupling are distinctly different from those reported for β-Gal*f* [20] so that the structure of α-furanoside is proposed for this compound. The acetylation of ManNAc also gave two products, **3a** and **3b**, in 59% and 13% yields, respectively. The main product **3a** was the expected 2-acetamido-6-*O*-acetyl-2-deoxy-D-mannopyranose, as unambiguously determined by NMR. The by-product **3b** was 2-acetamido-3,6-di-*O*-acetyl-2-deoxy-D-mannopyranose.
The acetylation at C-3 OH was deduced from the downfield shift of the H-3 proton, which resonated at 5.02 ppm as a dd (J_1 = 9.8 Hz; J_2 = 4.6 Hz). The acylation of **3** was repeated using trifluoroethyl butanoate as an activated ester and the corresponding butanoyl derivatives **3c** and **3d** were recovered in good yields (see Section 2) (Scheme 1).



Scheme 1.

3.2. *Acylation of* N-acetylhexosamine derivatives

The solubility of the mono-acetylated aminosugars **2a** and **3a** was higher in organic solvents that are more suitable to preserve lipases activity. In order to evaluate the regioselectivity of these enzymes towards the secondary free OH's at C-3 and C-4 of **2a** and **3a**, these compounds were dissolved in the mixture of acetone-pyridine 8:1 containing trifluoroethyl acetate. The samples of lipases reported in Table 1 were then added to the reaction mixtures. The formation of a defined new product was observed only with 2-acetamido-6-*O*-acetyl-2-deoxy-D-mannopyranose using *C. antarctica* lipase B (Novozym 435). Compound containing three acetyls was isolated in 18% yield. It gave only one set of signals; H-1 resonance was shifted downfield and exhibited a heteronuclear coupling to a carbonyl (HMBC). Other acetylated carbons were C-2 and C-6. Therefore, the structure of 2-acetamido-1,6-di-*O*-acetyl-2-deoxy-α-D-mannopyranose was assigned to the compound **3e**. Similarly, Novozym 435-catalysed the butanoylation of **3c**, which gave 2-acetamido-1,6-di-*O*-butanoyl-2-deoxy-α-D-mannopyranose (**3f**) in 19% yield.

Finally, for further synthetic applications, we reacted *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (**4**) with Novozym 435 in a mixture of pyridine-acetone-vinyl acetate, in this case an almost quantitative conversion to the 6-*O*-acetate **4a** was observed.

4. Conclusions

We have shown that a careful choice of the reaction conditions (solvent, enzyme, acylating agent) can allow the efficient regioselective acylation of poorly soluble carbohydrates like *N*-acetylhexosamines. Our future work will focus on the enzymatic esterification of more complex aminosugars, like chitobiose and chitotriose, and on the exploitation of the monoacyl derivatives **2a**, **3a**, **3c**, **4a** as sugar acceptors in transglycosylation reactions prepared by enzymatic or by chemical synthesis.

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