Exploring the glucosylation potential of glucansucrases

Devlamynck, Tim Nick

Chapter 7

Enzymatic transglucosylation of neohesperidin dihydrochalcone: glucansucrase Gtf180-ΔN-Q1140E as biocatalyst for the glycodiversification of sweet glycosides

Tim Devlamynck[1,2], Gerrit Gerwig[3], Wim Soetaert[1], Lubbert Dijkhuizen[2]
University of Ghent[1], University of Groningen[2], University of Utrecht[3]

Abstract

Increasing rates of obesity and diabetes type 2 lead to a rapidly growing number of health-conscious consumers, fueling the demand for low-calorie food products. Natural high-intensity sweeteners (HIS), of which steviol glycosides [e.g. rebaudioside A (RebA)] extracted from the leaves of *Stevia rebaudiana* are best known, have a competitive edge over synthetic HIS such as aspartame and acesulfame K, suffering from a bad reputation among consumers. Over the years, several other natural compounds have been proposed as possible products for the HIS market of the future. An interesting target is the flavor enhancer neohesperidin dihydrochalcone (NHDC). The main disadvantages of NHDC are its low water solubility at room temperature, resulting in a relatively low bioavailability, and an unpleasant lingering aftertaste. This chapter reports the α-glucosylation of NHDC with glucansucrase Gtf180-ΔN-Q1140E from *Lactobacillus reuteri* 180, using sucrose as donor substrate, in an attempt to improve its sensory and physicochemical properties. Structure elucidation of the novel glucosylated products revealed that NHDC was glucosylated at the β-D-glucosyl unit and the α-L-rhamnosyl unit, through an (α1→6) and (α1→4) linkage, respectively. The obtained conversion degree of 65% offers a good starting point for future improvements by reaction engineering. The α-glucosylated NHDC products displayed much higher water solubilities than NHDC and retained strong anti-oxidant capacities, enabling their use as nutraceuticals. Sensory analysis by a trained panel revealed limited bitterness suppressing effects of NHDC and its α-glucosylated products on RebA, in contradiction to previous reports.

1. Introduction

Previously we demonstrated the potential of the glucansucrase enzyme Gtf180-ΔN and in particular its Q1140E mutant[97] to trans-α-glucosylate rebaudioside A (RebA) and stevioside as a means to improve their sensory properties[68-70]. In addition to these steviol glycosides from *Stevia rebaudiana*, also neohesperidin dihydrochalcone (NHDC, Figure 1) has attracted considerable attention from the food industry, due to its intensive sweetness. Later on, NHDC's remarkable properties as flavor enhancer, as such contributing to the mouth feel of several food products and/or suppressing the bitterness displayed by other compounds, expanded the interest in this versatile molecule[246-248].

First discovered in 1963 by Horowitz and Gentili[246], NHDC is industrially produced by hydrogenation of neohesperidin (hesperetin 7-*O*-neohesperidoside), a flavanone glycoside isolated from citrus fruits such as mandarin, orange and grapefruit[249]. Recently, NHDC was found to naturally occur as a minor constituent in the plant *Oxytropis myriophylla*[250] and in the bark of the tree *Eysenhardtia polystachya*[251]. Classified as a semisynthetic sweetener, NHDC is roughly 340 times sweeter than sucrose and was approved as sweetener by the European Union in 1994. Similarly to other highly sweet glycosides such as glycyrrhizin (*Glycyrrhiza glabra*, liquorice) and most steviol glycosides, NHDC displays a lingering liquorice aftertaste limiting its application as the sole sweetener in foods and beverages[252]. NHDC is best applied in sweetener blends: it displays strong synergistic effects when combined with other sweeteners such as saccharin, aspartame, and RebA[253]. The total sweetness intensity of such mixtures is greater than the theoretical sum of the intensities of the individual components[254]. The use of NHDC thus provides a substantial economic benefit, as much less of the other sweetener needs to be supplied.

In addition, NHDC finds many applications as a flavor enhancer in a wide range of food –and pharmaceutical products, as such identified by the E number E959[255-257]. A typical example concerns the enhancement of the creaminess of

dairy products such as yogurt and ice cream. Moreover, NHDC is particularly effective in masking the bitterness displayed by several other compounds, such as limonin and naringin from citrus fruits. A patent application from 2012 even claims that the bitterness and astringency exhibited by RebA and monk fruit extract are very effectively suppressed by the addition of 10 ppm NHDC[258]. Also the pharmaceutical industry makes good use of this remarkable NHDC characteristic to reduce the bitterness of several drugs in tablet form, such as the antipyretic paracetamol[259]. More recently, several studies have attributed strong antioxidant[260-262], hepatoprotective (preventing damage to the liver)[263] and prebiotic[264] properties to NHDC. In the context of all these possible applications, the main disadvantage of NHDC is its low water solubility at room temperature (0.4 g/L), resulting in a relatively low bioavailability and limiting its application as sweetener in water based dispersions, such as syrups and jams[265].

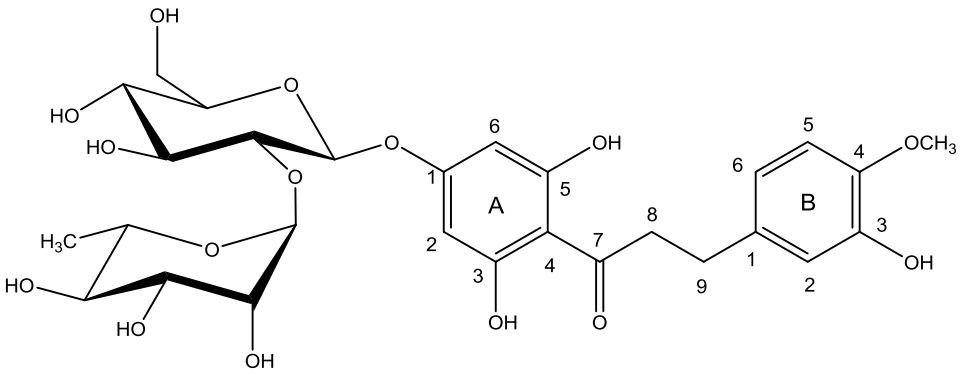


Figure 1. Chemical structure of neohesperidin dihydrochalcone (NHDC) with carbon atom numbering as applied in the structure elucidation. The numbering used is arbitrary (similar to Caccia et al. 1998[266]) as there is no consensus in literature.

NHDC contains 9 hydroxy groups (Figure 1): 3 of rhamnose, 3 of glucose and 3 of the aglycon (2 on the A-ring and 1 on the B-ring). In theory, all these hydroxy groups can be enzymatically glycosylated, depending on the biocatalyst and its substrate specificity and regioselectivity. To our knowledge, NHDC glycosylation has been reported only once: by applying maltogenic amylase from *Bacillus stearothermophilus*, NHDC was maltosylated at the β-D-glucosyl unit through an

(α1→6) linkage, resulting in a 700-fold increase of solubility and a 7-fold decrease of sweetness[267]. Previously, we reported the trans-α-glucosylation of a wide variety of compounds, ranging from catechol to more complex molecules such as steviol glycosides, with Gtf180-ΔN from *Lactobacillus reuteri* 180 and derived mutants, highlighting the broad acceptor substrate specificity of this enzyme[68-70,110]. This chapter reports the transglucosylation of NHDC with Gtf180-ΔN-Q1140E, yielding three new-to-nature α-glucosylated NHDC derivatives. A detailed structural analysis is provided, a necessity for the evaluation of the quality and safety of novel food products. In addition, the sensory properties, anti-oxidant capacities and solubilities of the α-glucosylated NHDC products were compared to NHDC.

2. Materials and methods

2.1. Production and purification of glucosylated NHDC

The production of glucosylated NHDC (Ingrizo) was performed at 50 mL scale in a shake flask, in duplicate. Samples were analyzed by HPLC. The temperature and agitation rate were set at 37 °C and 150 rpm, respectively. Gtf180-ΔN-Q1140E[97] (5 U/mL), produced as described previously[70], was incubated in a 20% ethanol solution containing 20 g/L NHDC and 125 mM sucrose supplemented with 25 mM sodium acetate (pH 4.7) and 1 mM $CaCl_2$. One unit (U) of enzyme activity corresponds to the conversion of 1 µmole sucrose (used for hydrolysis and transglucosylation) in a solution of 100 mM sucrose, 25 mM sodium acetate (pH 4.7) and 1 mM $CaCl_2$ at 37 °C.

After completion of the reaction (i.e. when a steady state was reached), glucosylated NHDC was isolated from the reaction mixture by adsorption onto Lewatit VP OC 1064 MD PH macroporous resin. A washing step with water removed the residual sugars and α-glucan oligosaccharides. Desorption of glucosylated NHDC was achieved with 70% isopropanol. The resulting mixture was evaporated *in vacuo* and subsequently applied onto a Reveleris X2 flash

chromatography system, applying a Reveleris amino cartridge (40 µm) with water (solvent A) and acetonitrile (solvent B) as the mobile phase. Following gradient elution was used: 99% solvent B (0-2 min), 99-35% solvent B (2-20 min), 35% solvent B (20-25 min). The collected fractions were evaporated *in vacuo* and subsequently freeze dried to remove the residual water.

2.2. MALDI-TOF-mass spectrometry

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on an AximaTM mass spectrometer (Shimadzu Kratos Inc., Manchester, UK), equipped with a Nitrogen laser (337 nm, 3 ns pulse width). Positive-ion mode spectra were recorded using the reflector mode at a resolution of 5000 FWHM and delayed extraction (450 ns). Accelerating voltage was 19 kV with a grid voltage of 75.2%. The mirror voltage ratio was 1.12 and the acquisition mass range was 200-2000 Da. Samples were prepared by mixing on the target 1 µL sample solutions with 1 µL aqueous 10% 2,5-dihydroxybenzoic acid in 40% acetonitrile as matrix solution, containing 0.1% TFA.

2.3. Methylation analysis

Samples were permethylated using CH_3I and solid NaOH in DMSO, as described by Ciucanu and Kerek (1984)[227], then hydrolyzed with 2 M TFA (2 h, 120 °C) to give the mixture of partially methylated monosaccharides. After evaporation to dryness and redissolving in H_2O, reduction was performed with $NaBD_4$ (2 h, room temperature). After neutralization with 4 M acetic acid and removal of boric acid by co-evaporation with methanol, the samples were acetylated with acetic anhydride/pyridine (1:1, 30 min, 120 °C). After evaporation to dryness and re-dissolving in dichloromethane, the mixtures of partially methylated alditol acetates (PMAAs) were analyzed by gas-liquid chromatography – electron ionization – mass spectrometry (GLC-EI-MS) on an EC-1 column (30 m x 0.25 mm, Alltech), using a gas chromatograph mass spectrometer (GCMS-QP2010 Plus from Shimadzu Kratos Inc.) and a temperature gradient (140-250 °C at 8 °C/min)[228].

2.4. NMR spectroscopy

Resolution-enhanced 1D/2D 500-MHz $^{1}H/^{13}C$ NMR spectra were recorded in D_2O on a Bruker DRX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) at a probe temperature of 310 K. Data acquisition was done with Bruker Topspin 2.1. Before analysis, samples were exchanged twice in D_2O (99.9 atom% D, Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization, and then dissolved in 0.6 mL D_2O. Suppression of the deuterated water signal (HOD) was achieved by applying a WEFT (water eliminated Fourier transform) pulse sequence for 1D NMR experiments and by a selective pre-saturation pulse of 1 s during the relaxation delay in 2D experiments. The 2D TOCSY spectra were recorded using an MLEV-17 (composite pulse devised by Levitt et al., 1982) mixing sequence with spin-lock times of 20, 50, 100 and 200 ms. The 2D ^{1}H-^{1}H ROESY spectra were recorded using standard Bruker XWINNMR software with a mixing time of 200 ms. The carrier frequency was set at the downfield edge of the spectrum in order to minimize TOCSY transfer during spin-locking. Natural abundance 2D ^{13}C-^{1}H HSQC experiments (^{1}H frequency 500.0821 MHz, ^{13}C frequency 125.7552 MHz) were recorded without decoupling during acquisition of the ^{1}H FID. The NMR data were processed using the MestReNova 9 program (Mestrelab Research SL, Santiago de Compostella, Spain). Chemical shifts (δ) are expressed in ppm by reference to internal acetone (δH 2.225 for ^{1}H and δC 31.07 for ^{13}C).

2.5. Solubility measurements

The solubilities of NHDC and α-glucosylated NHDC products were determined in 250 µL of ultrapure water, incubated in a thermoblock at 25 °C. Products were added until clear precipitation was noticeable, after which the samples were vortexed multiple times and allowed to equilibrate for 24 h. The supernatants were diluted in ethanol and subsequently subjected to HPLC analysis. Calibration was accomplished using the appropriate standard curves, obtained after purification of the glycosylated products as described in 2.1. All analyses were performed in duplicate.

2.6. Determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is widely used to determine the anti-oxidant potential of various compounds. DPPH possesses an unpaired valence electron susceptible to scavenging by molecules displaying anti-oxidant activity, a chemical reaction which can be followed spectrophotometrically[268].

The DPPH radical-scavenging activity was evaluated by adding 100 µL of methanol, containing various concentrations of NHDC and α-glucosylated NHDC products (10-1000 µM), to 200 µL of methanol supplemented with 200 µM DPPH. The samples were incubated at room temperature during 15 min in complete darkness, after which their absorbance was measured at 517 nm. The (glucosylated) NHDC concentration required to reduce the absorbance by 50% (EC_{50}) was calculated by linear regression (of the linear part) of the absorption curves. Analyses were performed in duplicate. Butylated hydroxytoluene (BHT) was included in the experiments to serve as comparison.

2.7. Sensory analysis

The sensory analysis was performed in individual tasting booths at the UGent Sensolab (Belgium) by a trained panel (9 persons). In short, taste (sweetness, liquorice, astringency and bitterness) was evaluated by swirling the sample in the mouth for 5 sec after which the sample was expectorated. Aftertaste was evaluated 10 sec after swallowing the solution. Lingering based on the maximum taste intensity was rated 1 min later. Sucrose reference solutions (5%, 7.5% and 10% sucrose, scoring 5, 7.5 and 10, respectively) were provided. Water (Spa Reine**)** and plain crackers were used as palate cleansers between sampling. Following sweetened solutions were tasted: 588 mg/L rebaudioside A (RebA, Tereos PureCircle Solutions), 588 mg/L RebA + 10 ppm NHDC, 588 mg/L RebA + 10 ppm monoglucosylated NHDC (NHDC-G1), and 588 mg/L RebA + 10 ppm diglucosylated NHDC (NHDC-G2). All samples were evaluated in duplicate.

Statistical analyses were performed with SPSS 23 (SPSS Inc., Chicago, USA). All tests were done at a significance level of 0.05. One-Way ANOVA was used to investigate any significant difference between the solutions. Testing for equal variances was executed with the Modified Levene Test. When conditions for equal variance were fulfilled, the Tukey test was used to determine differences between samples[210]. In case variances were not equal, a Games-Howell post-hoc test was performed[211].

3. Results and discussion

3.1. Production of glucosylated NHDC

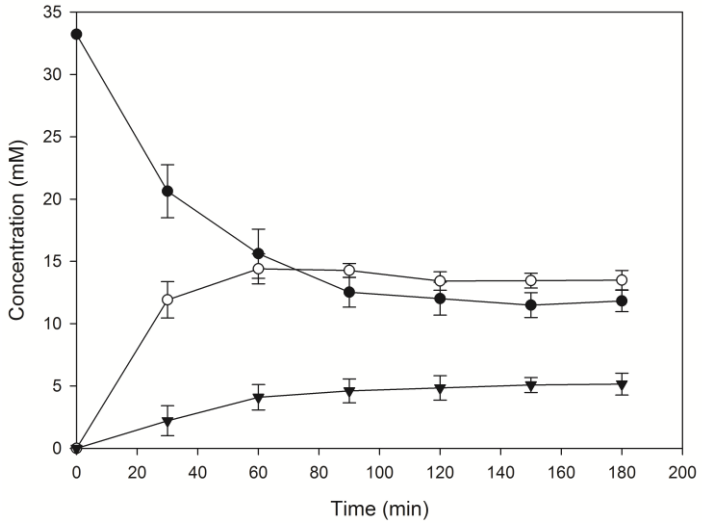


Figure 2. HPLC analysis of the synthesis of α-D-glucosides of NHDC by Gtf180-ΔN-Q1140E in time (33 mM NHDC; 125 mM sucrose; 20% v/v EtOH; 5 U/mL enzyme). ● NHDC, ○ NHDC-G1, ▼ NHDC-G2. Sucrose was depleted after 180 min.

As NHDC is only sparingly soluble in water (0.4 g/L), a cosolvent had to be supplied to the glycosylation reaction mixture in order to avoid suboptimal yields and conversion degrees. Indeed, as demonstrated in chapter 3, higher concentrations of acceptor substrate generally result in higher conversion degrees. Gtf180-ΔN-Q1140E retained its activity in solutions with up to 20% v/v

EtOH (Figure S1). The reaction mixture was consequently composed of 20% v/v EtOH, containing 33 mM NHDC (maximal amount that could be dissolved), and 125 mM sucrose, enough donor substrate to drive the reaction to completion. The incubation of 5 U/mL Gtf180-ΔN-Q1140E in this reaction mixture resulted in a NHDC conversion degree of 64.4% (Figure 2). HPLC analysis also revealed that at least two major products were synthesized, 62% NHDC-G1 and 38% NHDC-G2.

3.2. Structural characterization of glucosylated NHDC products

NHDC was enzymatically transglycosylated by Gtf180-ΔN-Q1140E, at first sight resulting in the formation of 2 major products (Figure 2). However, purifying the reaction mixture with flash chromatography resulted in the isolation of three major glucosylated products (P1, P2, P3), of which P1 and P2 were formed in equal amounts. MALDI-TOF mass spectrometric analysis revealed the same quasi-molecular mass peaks *m/z* 796.5 $[M+Na]^+$ and *m/z* 812.5 $[M+K]^+$ for P1 and P2, indicating NHDC+1Glc (MW = 774 Da), as compared to NHDC (MW = 612 Da) showing *m/z* 634.5 $[M+Na]^+$ and *m/z* 650.6 $[M+K]^+$. P3 showed *m/z* 958.6 $[M+Na]^+$ and *m/z* 974.6 $[M+K]^+$, indicating NHDC+2Glc (MW = 936 Da). In other words, NHDC-G1 consists of products P1 and P2, whereas NHDC-G2 was equal to P3.

In order to obtain information about the linkage pattern of the carbohydrate moieties of the products, methylation analysis was performed (Table SI). For NHDC, the expected terminal Rhamnose and 2-substituted Glucose in molar ratio 1:1 was found. Additionally, in P1 and P2, the presence of 4-substituted Rhamnose and 2,6-disubstituted Glucose was observed, respectively. P3 showed both of the latter residues. The minor amounts of →6)Glc*p*(1→ and →2)Glc*p*(1→ found by methylation analysis indicate that the products were not 100% pure.

Different NMR spectroscopic techniques (^{1}H- and ^{13}C-NMR, TOCSY, ROESY and HSQC) were used to investigate the structures of the transglucosylated

NHDC derivatives. Firstly, NMR spectroscopy was performed with the acceptor substrate NHDC in D_2O to obtain reference data (Figure 3). In spite of the reported low solubility of NHDC in water, D_2O samples were easily obtained. It has to be noted that the ^1H chemical shifts are strongly affected by temperature and sample concentrations (probably due to self-association of NHDC in D_2O).

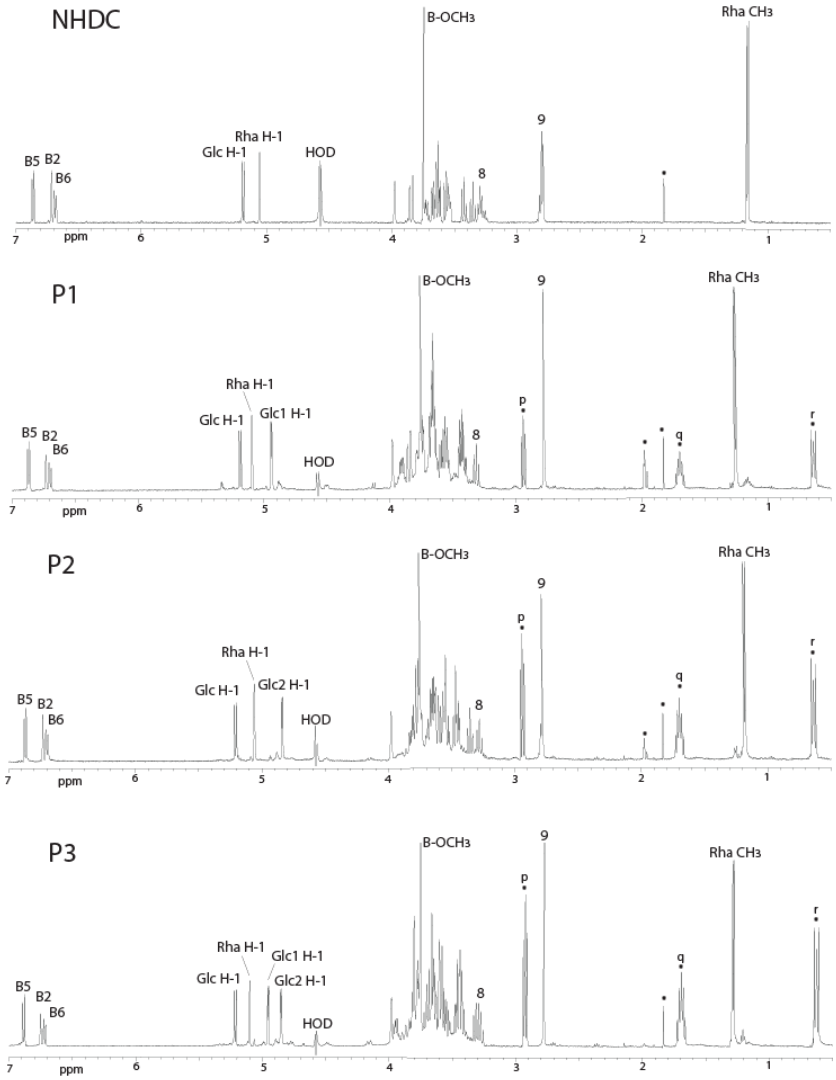


Figure 3. 1D 500-MHz ^1H NMR spectra of NHDC, P1, P2 and P3, recorded in D_2O at 310 K. * resonance signals stemming from contamination(s).

The 500-MHz ^{1}H NMR spectrum (Figure 3) of NHDC showed the signals of the aromatic protons at δ 6.87 [d, *J*=8.2 Hz, B5], at δ 6.72 [s, *J*=2.0 Hz, B2], and at δ 6.70 [d, *J*=8.2 Hz, B6], together with two methylene groups [δ 3.29 (C8) and δ 2.81 (C9)] and a methoxy group [δ 3.75 (B-OCH_3)], typical for the dihydrochalcone skeleton[266]. The aromatic proton signals of A2 and A6 (see NHDC structure, Figure 1), initially found at δ 5.99, had disappeared due to exchange with deuterium from the solvent (D_2O) after prolonged time at 310 K[269]. Also, the C8 proton, in α position to the carbonyl group (C7), is susceptible to deuterium exchange via the keto-enol route, consequently, ^{13}C correlations in HSQC experiments will not be observed. Concerning the carbohydrate moiety, the ^{1}H NMR spectrum exhibited signals due to one β-D-glucopyranosyl unit [δ 5.185 (d, *J*=7.6 Hz, Glc H-1)] and one α-L-rhamnopyranosyl unit [δ 5.059 (br s, *J* <2 Hz, Rha H-1) and δ 1.168 (d, *J*=6.1 Hz, Rha CH_3)]. The assignment of the signals (Table SII) was made through 2D NMR TOCSY experiments, using different mixing times, and in combination with ROESY and HSQC (Figures S2 and S3). Indeed, the obtained NMR data of NHDC (Table SII) were in agreement with She et al. (2011)[250] and Caccia et al. (1998)[266].

The 500-MHz ^{1}H NMR spectrum (Figure 3) of fractions P1 and P2 showed an extra α-anomeric proton at δ 4.936 (*J*=3.7 Hz) and at δ 4.845 (*J*=3.7 Hz), respectively, stemming from two differently attached glucose units, denoted as Glc1 and Glc2. Compared to NHDC, the NMR spectrum of fraction P3 showed signals stemming from both Glc1 and Glc2 (δ 4.955: *J*=3.7 Hz and δ 4.855: *J*=3.7 Hz). The resonance signals indicated with * in Figure 3 (e.g. p, q and r, having mutual TOCSY connections) are stemming from contamination(s). The total assignment of NMR signals (Table SII) was made through 2D NMR TOCSY, HSQC and ROESY experiments in a similar way as for NHDC (Figures S4-9), revealing that Glc1 and Glc2 were attached to NHDC at the α-L-rhamnopyranosyl unit through an (α1→4) linkage and at the β-D-glucopyranosyl unit through an (α1→6) linkage, respectively (Figure 4). In other words, P3 contained both the newly formed (α1→4) linkage present in P1 and the (α1→6) linkage introduced in P2. The results are in agreement with the methylation analyses (Table …). To

our knowledge, these α-glucosylated NHDC derivatives have not been described before and can thus be considered new-to-nature.

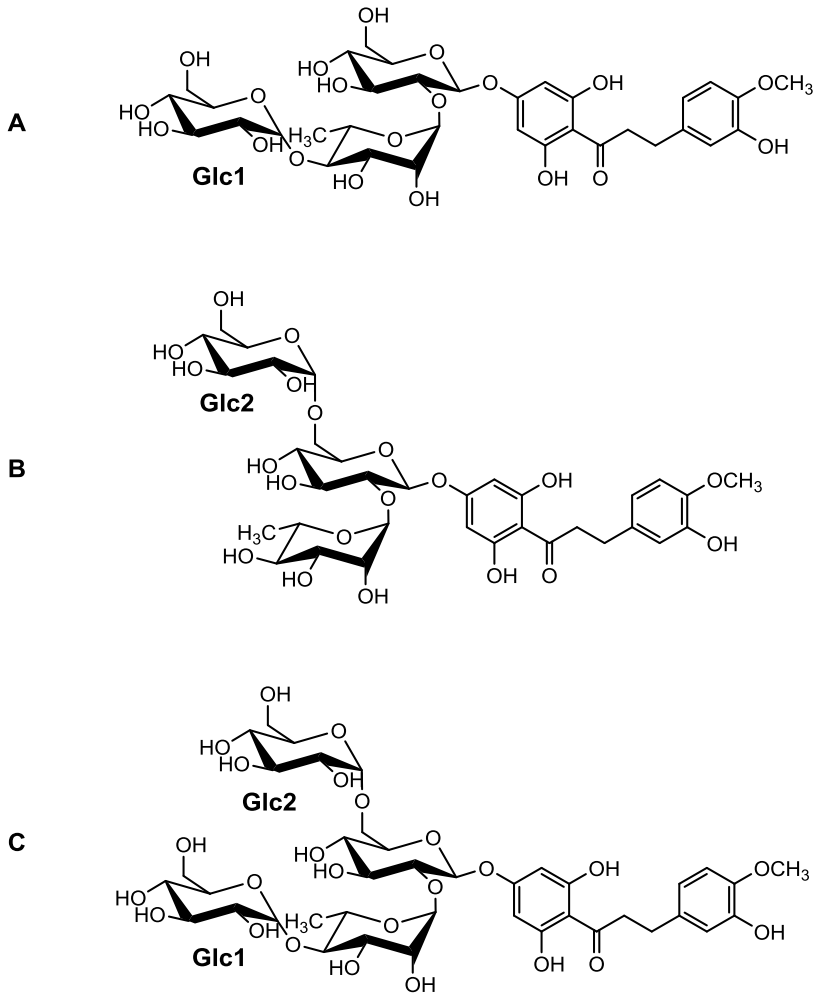
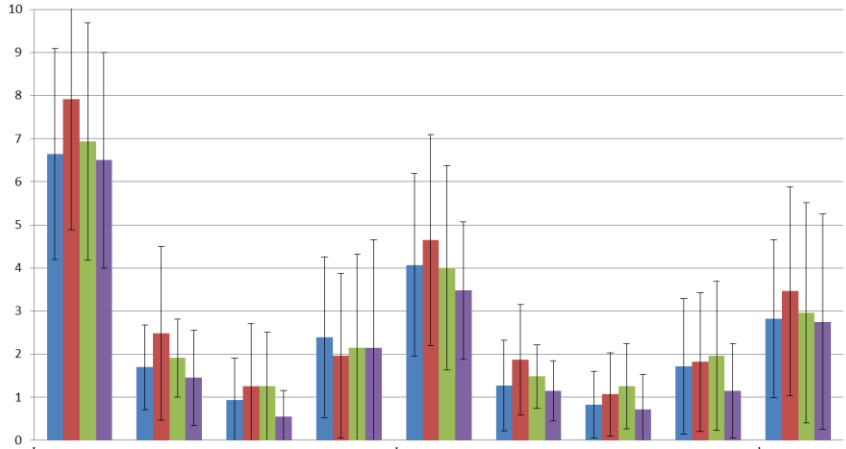


Figure 4. Chemical structures of α-glucosylated NHDC products: **A** NHDC-P1, **B** NHDC-P2 and **C** NHDC-P3.

3.3. Sensory analysis of glucosylated NHDC products

NHDC is not only a high-intensity sweetener (340 times sweeter than sucrose), it is reported to suppress the bitterness displayed by other sweeteners such as RebA. A sensory analysis of aqueous solutions sweetened with RebA and 10

ppm NHDC, NHDC-G1, and NHDC-G2 was performed by a trained panel, evaluating 9 different taste attributes (Figure 5).



Sample	Taste				Aftertaste				Lingering
	Sweet	Liquorice	Astringent	Bitter	Sweet	Liquorice	Astringent	Bitter	
RebA	6.6^{A}	1.7^{A}	0.9^{A}	2.4^{A}	4.1^{A}	1.3^{A}	0.8^{A}	1.7^{A}	2.8^{A}
RebA + NHDC	7.9^{A}	2.5^{A}	1.3^{A}	2.0^{A}	4.6^{A}	1.9^{A}	1.1^{A}	1.8^{A}	3.5^{A}
RebA + NHDC-G1	6.9^{A}	1.9^{A}	1.3^{A}	2.1^{A}	4.0^{A}	1.5^{A}	1.3^{A}	2.0^{A}	3.0^{A}
RebA + NHDC-G2	6.5^{A}	1.5^{A}	0.6^{A}	2.2^{A}	3.5^{A}	1.2^{A}	0.7^{A}	1.2^{B}	2.8^{A}

Figure 5. Sensory analysis of RebA, RebA + 10 ppm NHDC, RebA + 10 ppm NHDC-G1 and RebA + 10 ppm NHDC-G2. Sweetness, bitterness and off-flavors were rated on a scale of 15. Mean values are given in the table. ^{A,B}: different letters indicate significant differences ($p<0.05$) between solutions following one-way ANOVA and post-hoc test.

In general, the trained panel perceived only slight differences between the various solutions. The claimed bitterness suppressing effect of NHDC was detected by the trained panel, however, the difference with the NHDC-less RebA solution was not significant. Only the addition of 10 ppm NHDC-G2 resulted in a significant reduction of RebA's bitter aftertaste. A possible explanation is the addition of too little NHDC, although Sun et al.[258] recommended a similar dose of 10 ppm. Nevertheless, the solution containing RebA + 10 ppm NHDC was perceived as sweeter than the NHDC-less RebA solution, confirming NHDC's synergistic effect. Slight increases in astringency, liquorice taste, and lingering

were also observed, typical consequences of adding NHDC. Interestingly, the addition of NHDC-G1 and NHDC-G2 instead of NHDC reduced these undesired side-effects. However, as the trained panel was not able to perceive significant differences between the various solutions, investigation of the appropriate dose is required to obtain conclusive results. The limited availability of the trained panel prevented further experimenting. In any case, it appears that α-glucosylation of RebA by Gtf180-ΔN-Q1140E[68-70] is a much more effective strategy to decrease its bitterness than the addition of (glucosylated) NHDC. In this context, analysis of the sensory properties of a solution containing (glucosylated) NHDC and glucosylated RebA (RebA-G) is also an interesting option.

3.4. Solubility of glucosylated NHDC products

The low solubility of NHDC prevents possible applications as nutraceutical, to exploit its strong anti-oxidant properties. Determination of the solubilities in water of NHDC and its α-glucosylated products revealed an impressive 800-fold increase of solubility for NHDC-G1, whereas NHDC-G2 was even more soluble (Table I). Previously, maltosylation of NHDC at the β-D-glucosyl unit, introduced through an (α1→6) linkage, resulted in a 700-fold increase of solubility[267]. An alternative approach consisted of the cosolubilization of NHDC with sodium saccharin, resulting in a 200-fold increase of solubility, significantly lower than the improvement obtained after glycosylation[265].

3.5. Anti-oxidant properties of glucosylated NHDC products

In general, glycosylation of anti-oxidants increases their solubility in water, however, the antioxidant capacity typically decreases[139]. The ability of NHDC and α-glucosylated NHDC products to scavenge DPPH was consequently evaluated (Table I). Although glucosylating NHDC clearly reduced the scavenging activity (EC_{50}), NHDC-G1 and NHDC-G2 still showed excellent anti-oxidant capacities, displaying EC_{50}'s similar to the established anti-oxidant butylated hydroxytoluene (BHT) which possessed an EC_{50} of 35.2 μM (determined experimentally).

Considering the substantially improved water solubility of α-glucosylated NHDC (NHDC-G, composed of NHDC-G1 and NHDC-G2) compared to NHDC, NHDC-G can be considered a more promising and useful anti-oxidant.

Table I. Solubility in water and DPPH radical-scavenging activity (EC_{50}) of NHDC and α-glucosylated NHDC products. EC_{50} is the (glucosylated) NHDC concentration required to reduce the absorbance of a solution with 200 µM DPPH at 517 nm by 50%.

Compound	Solubility (mM)	EC_{50} (µM)
NHDC	0.6 ± 0.1	27.3 ± 4.0
NHDC-G1	490.9 ± 45.2	34.3 ± 1.7
NHDC-G2	> 600.0	48.7 ± 1.5

4. Conclusions

NHDC finds many applications as flavor enhancer (E959) in the food- and pharmaceutical industry due to its ability to suppress bitterness and astringency, for example in the antipyretic paracetamol[255-259]. The strong anti-oxidant capacity displayed by NHDC has further increased the interest in this versatile molecule[260-262]. NHDC's main drawback is its low solubility in water, which reduces its bioavailability but also its applicability as a high-intensity sweetener[265]. Gtf180-ΔN-Q1140E catalyzed glycosylation of NHDC, using sucrose as donor substrate, yielded three new-to-nature α-glucosylated NHDC products displaying improved solubilities and retained anti-oxidant capacities. Sensory analysis of the flavor enhancing effects of (α-glucosylated) NHDC on RebA by a trained panel revealed a slight decrease of bitterness and an increased sweetness. In future work different concentrations of (α-glucosylated) NHDC and RebA will be evaluated. This may provide valuable information about the appropriate doses that should be applied to obtain the optimal bitterness suppressing effect. Although the obtained conversion degree of roughly 65% was not of the same order as for RebA and stevioside glycosylation (95%)[68-70], it forms a good starting point for a profound optimization of the reaction conditions. Additionally, the screening of the available Gtf180-ΔN mutant library[99] may reveal variants with improved NHDC glycosylation potential.

5. Supplementary information

Table SI. Linkage analysis of the glycosyl moieties in NHDC and in α-glucosylated NHDC products.

PMAA	R_t[a]	Structural feature	Peak area (%)[b]			
			NHDC	P1	P2	P3
1,5-di-O-acetyl-2,3,4-tri-O-methyl-5-CH_3 pentitol	0.76	Rha*p*(1→	49	tr	28	tr
1,4,5-tri-O-acetyl-2,3-di-O-methyl-5-CH_3 pentitol	0.93	→4)Rha*p*(1→	-	25	tr	21
1,5-di-O-acetyl-2,3,4,6-tetra-O-methylhexitol	1.00	Glc*p*(1→	-	36	33	52
1,2,5-tri-O-acetyl-3,4,6-tri-O-methylhexitol	1.16	→2)Glc*p*(1→	51	32	4	3
1,5,6-tri-O-acetyl-2,3,4-tri-O-methylhexitol	1.22	→6)Glc*p*(1→	-	4	3	2
1,2,5,6-tetra-O-acetyl-3,4-di-O-methylhexitol	1.39	→2,6)Glc*p*(1→	-	3	32	22

[a] R_t, retention time relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol (1.00) on GLC.

[b] average values (no molar response/correction factors used).

Table SII. ^{1}H and ^{13}C chemical shift values (δ, ppm)[a] of NHDC and glycosylated derivatives P1, P2 and P3 in D_2O at 310 K. Substituted carbon positions are indicated in grey.

	NHDC		**P1**		**P2**		**P3**	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
B2	6.72	115.3	6.73	115.5	6.74	115.6	6.75	115.5
B5	6.87	112.7	6.87	112.7	6.88	112.9	6.88	112.8
B6	6.70	119.6	6.70	120.2	6.71	120.5	6.72	120.1
B-OCH_3	3.75	55.9	3.76	55.9	3.75	56.2	3.76	56.0
8	3.29	42.2	3.30	41.8	3.30	41.9	3.28	42.0
9	2.81	29.8	2.78	30.0	2.79	30.1	2.79	30.0
Glc βH-1	5.185	97.0	5.184	97.0	5.215	97.0	5.215	97.0
H-2	3.58	78.7	3.58	78.6	3.58	79.0	3.58	78.4
H-3	3.64	75.7	3.65	76.0	3.65	76.0	3.67	76.2
H-4	3.43	68.7	3.43	68.9	3.47	71.9	3.47	71.6
H-5	3.57	75.6	3.56	75.8	3.76	74.7	3.76	74.2
H-6a	3.85	60.3	3.85	60.4	3.80	66.3	3.81	66.3
H-6b	3.67		3.66		3.78		3.77	
Rha αH-1	5.059	101.0	5.085	100.4	5.065	101.3	5.103	100.5
H-2	3.98	69.9	3.97	70.3	3.98	70.4	3.98	70.2
H-3	3.64	69.7	3.74	68.5	3.65	70.6	3.77	68.2
H-4	3.36	71.6	3.42	81.0	3.36	71.8	3.43	81.0
H-5	3.74	68.7	3.91	67.5	3.76	68.5	3.95	67.6
CH_3	1.17	16.6	1.26	16.9	1.19	16.9	1.29	16.6
			Glc(α1→4)Rhaα				**Glc**(α1→4)Rhaα	
Glc1 αH-1	-	-	4.940	99.0	-	-	4.955	98.9
H-2	-	-	3.43	71.4	-	-	3.44	71.5
H-3	-	-	3.55	76.0	-	-	3.59	75.0
H-4	-	-	3.31	69.2	-	-	3.32	69.3
H-5	-	-	3.78	71.7	-	-	3.78	72.0
H-6a	-	-	3.77	60.1	-	-	3.78	60.2
H-6b	-		3.68		-		3.66	
					Glc(α1→6)Glcβ		**Glc**(α1→6)Glcβ	
Glc2 αH-1	-	-	-	-	4.845	98.1	4.855	97.8
H-2	-	-	-	-	3.46	70.5	3.46	71.0
H-3	-	-	-	-	3.68	72.9	3.71	73.0
H-4	-	-	-	-	3.29	69.5	3.28	69.3
H-5	-	-	-	-	3.56	71.9	3.57	72.0
H-6a	-	-	-	-	3.69	60.3	3.68	60.2
H-6b	-		-		3.62		3.62	

[a] In ppm relative to internal acetone (δ 2.225 for ^{1}H and δ 31.07 for ^{13}C).

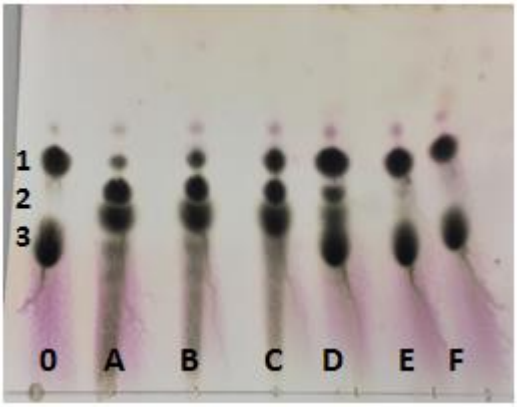


Figure S1. Transglycosylation activity of Gtf180-ΔN-Q1140E after a 1 h incubation in RebA glycosylation mixtures (84 mM RebA, 282 mM sucrose) containing **A** 0%, **B** 10%, **C** 20%, **D** 30%, **E** 40%, and **F** 50% EtOH. **1** RebA, **2** Monoglucosylated RebA (RebA-G1), **3** Sucrose.

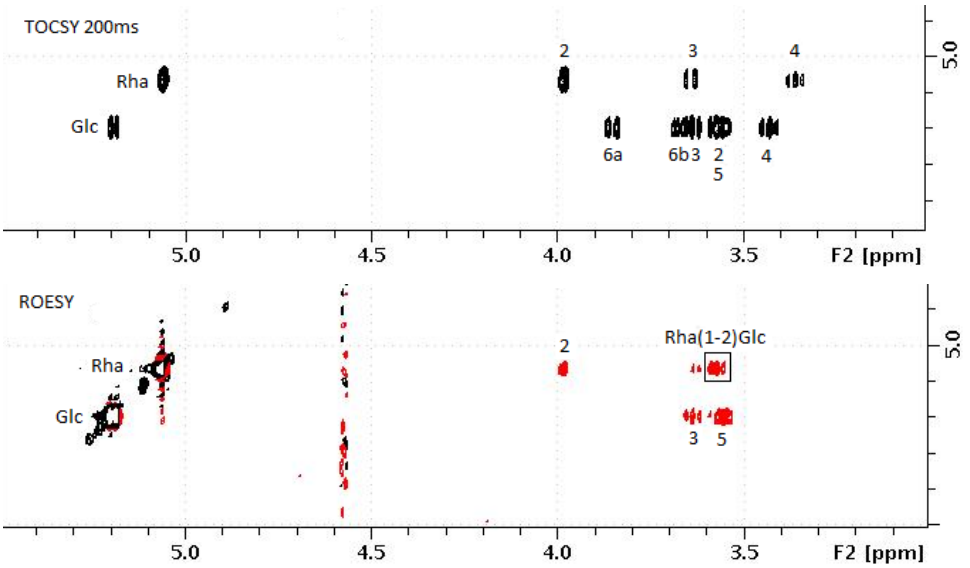


Figure S2. TOCSY (200 ms) and ROESY spectrum of the carbohydrate part of NHDC, recorded in D_2O at 310 K.

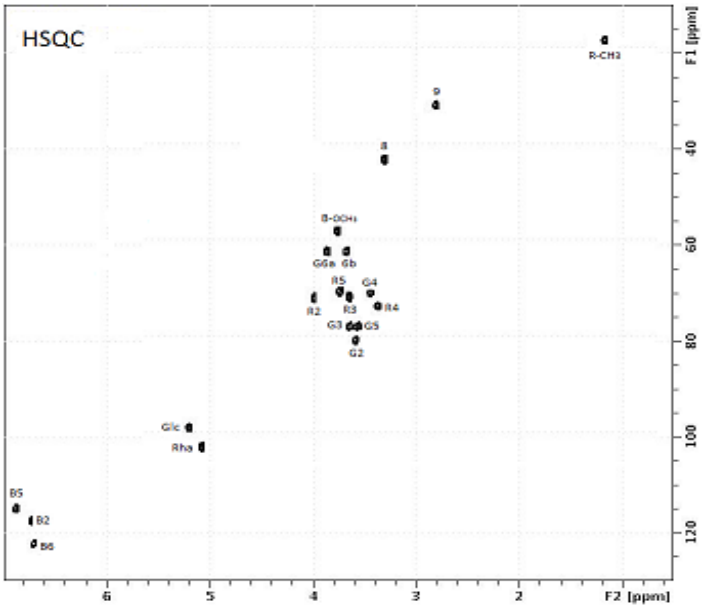


Figure S3. HSQC spectrum of NHDC, recorded in D_2O at 310 K.

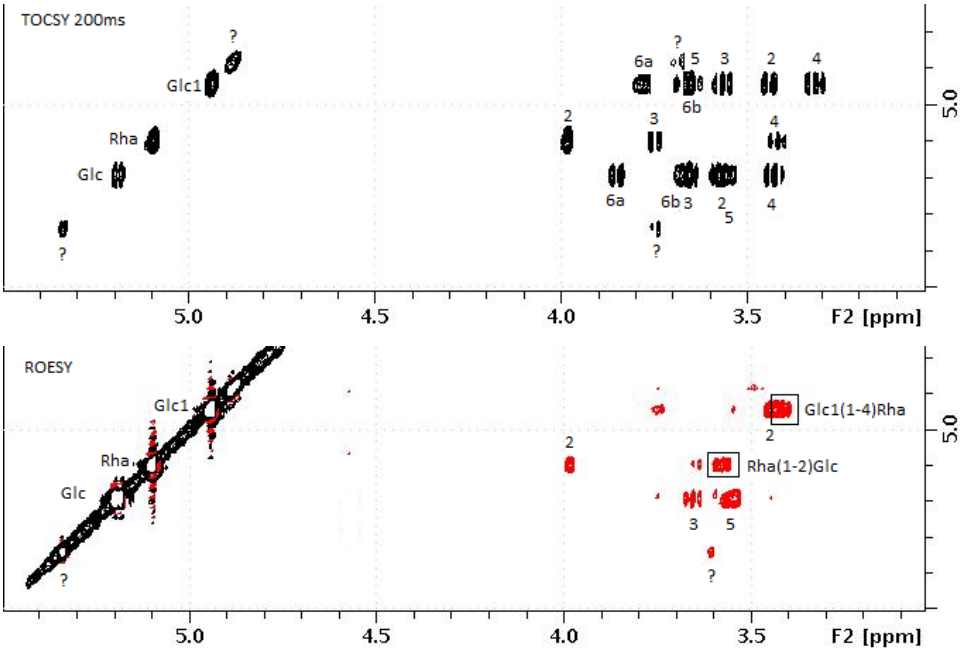


Figure S4. TOCSY and ROESY spectrum of the carbohydrate part of P1, recorded in D_2O at 310 K.

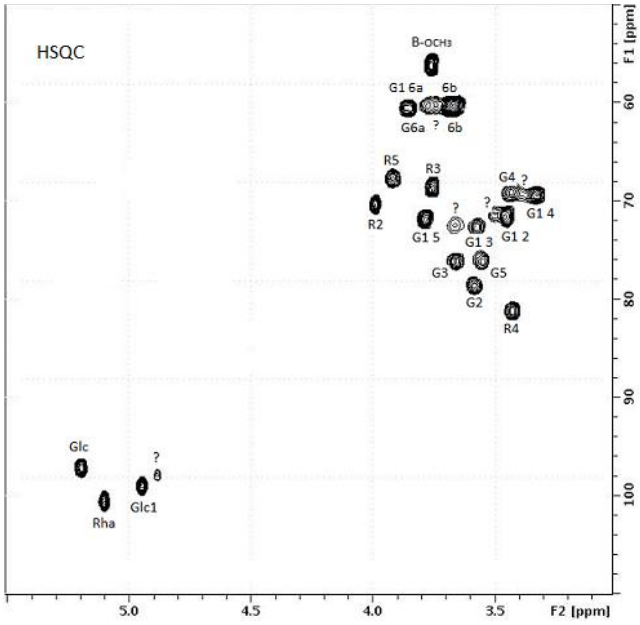


Figure S5. HSQC spectrum of the carbohydrate part of P1, recorded in D_2O at 310 K. ?, signals stemming from contamination(s).

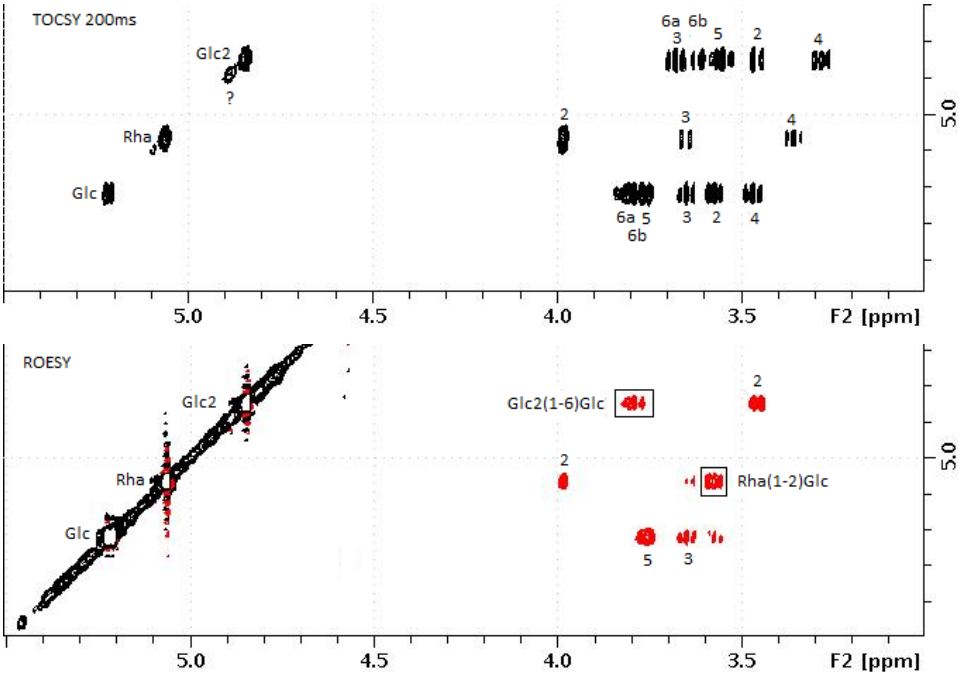


Figure S6. TOCSY and ROESY spectrum of the carbohydrate part of P2, recorded in D_2O at 310 K.

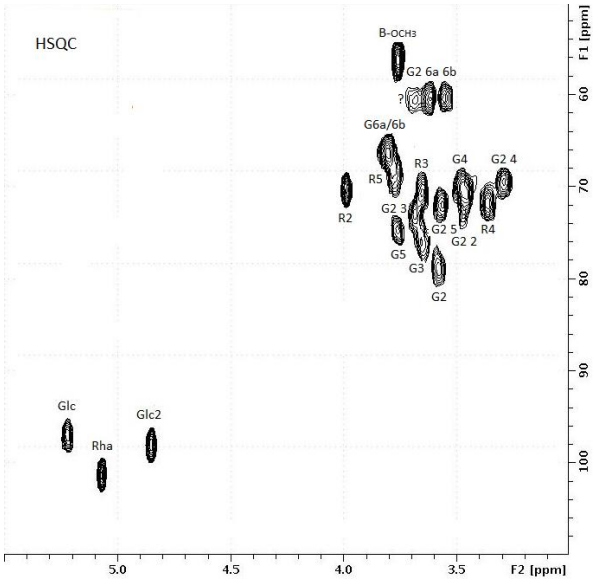


Figure S7. HSQC spectrum of the carbohydrate part of P2, recorded in D_2O at 310 K. ?, signals stemming from contamination(s).

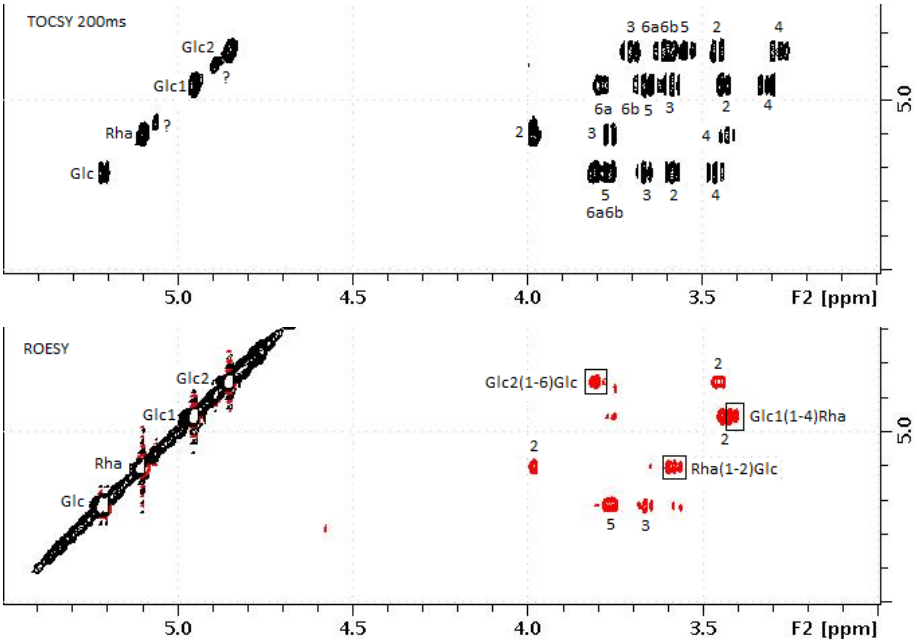


Figure S8. TOCSY and ROESY spectrum of the carbohydrate part of P3, recorded in D_2O at 310 K.

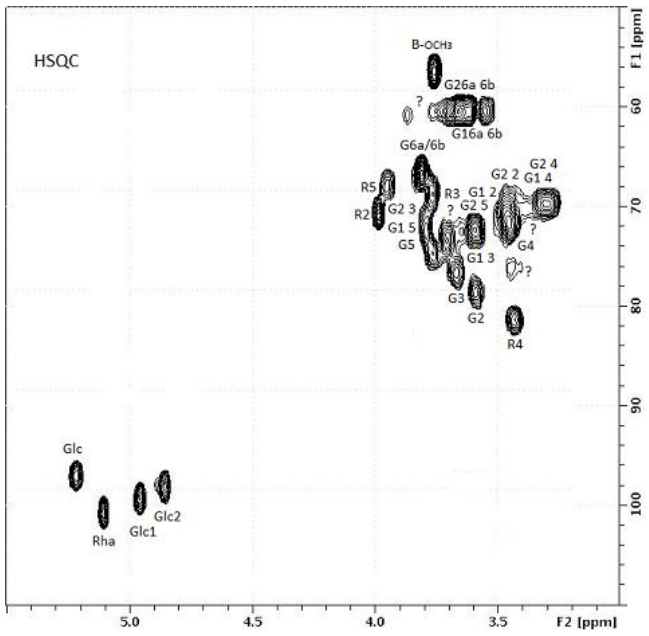


Figure S9. HSQC spectrum of the carbohydrate part of P3, recorded in D_2O at 310 K. ?, signals stemming from contamination(s).