Structural analysis of rebaudioside A derivatives obtained by Lactobacillus reuteri 180 glucansucrase-catalyzed trans-α-glucosylation

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Abstract
The wild-type Gtf180-ΔN glucansucrase enzyme from Lactobacillus reuteri 180 was found to catalyze the α-glucosylation of the steviol glycoside rebaudioside A, using sucrose as glucosyl donor in a trans-glucosylation process. Structural analysis of the formed products by MALDI-TOF mass spectrometry, methylation analysis and NMR spectroscopy showed that rebaudioside A is specifically α-α-glucosylated at the steviol C-19 β-D-glucosyl moiety (55% conversion). The main product is a mono-(α1 → 6)-glucosylated derivative (RebA-G1). A series of minor products, up to the incorporation of eight glucose residues, comprise elongations of RebA-G1 with mainly alternating (α1 → 3)- and (α1 → 6)-linked glucopyranose residues. These studies were carried out in the context of a program directed to the improvement of the taste of steviol glycosides via enzymatic modification of their naturally occurring carbohydrate moieties.

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1. Introduction

The leaves of the Stevia rebaudiana BERTONI plant contain a high variety of sweet substances, called steviol glycosides [1,2], and so far more than 40 different structures have been elucidated (see review Ref. [3]). Stevioside (5–20% w/w of dried leaves) and rebaudioside A (2–5% w/w of dried leaves) are the most abundant components (Fig. 1), tasting about 200–300 times sweeter than sucrose (0.4% aqueous solution). Structurally, steviol glycosides have ent-13-hydroxykaur-16-en-19-oic acid as aglycone, called steviol, but are differing in carbohydrate composition at the C-13-tert-hydroxyl and C-19-carboxyl functions.

Due to the growing awareness and concerns for human health related to excessive consumption of sugar (sucrose), the application of steviol glycosides as non-caloric bio-alternatives for sucrose and as substitutes for artificial (synthetic) sweeteners is strongly promoted nowadays [4–8]. Since a couple of years, steviol glycosides have been permitted for use as food additive and sweetener in the USA [9] and in Europe (E960) [10,11]. However, despite their intense sweetness and diverse beneficial pharmacological properties [12–15], the main drawback for successful commercialization of Stevia sweeteners is their slight bitterness and unpleasant (metallic) aftertaste, experienced by more than half of the human population.

For natural steviol glycosides with β-D-glucopyranosyl units as constituents, it has been reported that the ratio of the number of glucose units at the C-13 site to that at the C-19 site of the steviol core has a relationship with the sweetness as well as with the quality of taste of the steviol glycosides [16,17]. To improve the
taste, especially for food applications, chemical and enzymatic modifications of the carbohydrate moieties of specific steviol glycosides have been investigated, and showed promising results [3,16,18–25].

Glucansucrase enzymes from probiotic lactic acid bacteria, when incubated with sucrose as donor/acceptor substrate, produce (sucrose-linked) α-D-glucans with different linkage types depending on the specific strain/specific glucansucrase used [26]. In the presence of sucrose plus non-sucrose acceptor substrates, these enzymes additionally catalyze the formation of oligosaccharide/glycoconjugate products [27]. Previously, it has been shown that the glucansucrase enzyme Gtf180 from Lactobacillus reuteri 180 and its recombinant truncated Gtf180-DN derivative were able to synthesize from sucrose an α-D-glucan (EPS180) with ~69% (α1/6) and ~31% (α1/3) linkages, the latter being present both in the main chain, although not in a successive way, and as branching points (Fig. 2) [28]. More recently, it has been shown that EPS180 also contains low amounts (<1%) of (α1/4) linkages [29], whereas incubations with mutant Gtf180-DN enzymes led to bioengineered (1/3,1/4,1/6)-α-D-glucans with up to 12% (α1/4) linkages [29–31]. For the latter polysaccharides, Glc(α1 → 4) and Glc(α1 → 4)Glc(α1 → 4) units were found to occur in terminal positions.

With a focus on enzymatic modifications of steviol glycosides to produce derivatives with improved organoleptic properties, we have incubated rebaudioside A with sucrose and the wild-type Gtf180-DN glucansucrase enzyme from Lb. reuteri 180. Here, we present detailed structural analyses by MALDI-TOF mass spectrometry, methylation analysis and 1D/2D 1H/13C NMR spectroscopy of obtained α-D-glucopyranosylated products.

2. Results and discussion

2.1. Incubation of rebaudioside A with the Gtf180-DN glucansucrase enzyme and sucrose

Inspection of the molecular structure of rebaudioside A (RebA, 13-[2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl]oxy]ent-kaur-16-en-19-oic acid β-D-glucopyranosyl ester) (Fig. 1) shows four Glc residues (Glc1, Glc2, Glc3 and Glc4) with a total of fourteen free hydroxyl groups, which can act as acceptors for transglucosylation reactions. In view of the reported enzymatic activity of the wild-type Gtf180-DN glucansucrase enzyme from Lb. reuteri 180 (Section 1) [28–31], at first instance, elongations at HO-3, HO-4 and HO-6 are expected.

RebA (50 mM) was incubated at 37°C with 10 U/mL wild-type Gtf180-DN enzyme in sodium acetate buffer, pH 4.7, containing 1.0 M sucrose. After 3 h, a second batch of 1.0 M sucrose was added to the reaction mixture, and the incubation was prolonged for 21 h. After removal of glucose, fructose, gluco-oligo/polysaccharide (products of the “natural activity” of the enzyme), protein material, and residual sucrose from the reaction mixture via solid-phase extraction, HPLC analysis of the (α-glucosylated) RebA mixture showed a complex pattern of peaks, as visualized in Fig. 3. Fraction F1 had the same retention time as the acceptor substrate RebA. For further analysis, fractions F1–F9 were isolated.

MALDI-TOF-MS analysis of the fractions F1–F9 showed a series of quasi-molecular ions [M+Na]⁺, in accordance with an extension of RebA (F1) with one (F2) to eight (F9) glucose residues, respectively (Supplementary Information Fig. S1). However, in view of the HPLC peak clusters within some fractions, groups of isomeric components with the same molecular mass can be expected. Integration of the HPLC peaks in Fig. 3 revealed that 55% of RebA...
was converted into glucosylated products [mainly F2 (25%) and minor amounts of F3 to F9 (in total 30%)]. To obtain detailed structural information about the generated RebA derivatives, fractions F1–F9 were subjected to methylation analysis and NMR spectroscopy.

As literature NMR data of RebA were mostly available for solvent systems differing from D2O [e.g. C5D5N, C5D5N spectroscopy. F1 functions structural information about the generated RebA derivatives, fractions F1–F9 were subjected to methylation analysis and NMR spectroscopy.

Fig. 3. HPLC profile after a 24-h incubation of RebA (50 mM) with wild-type Gtf180-2N enzyme (10 U/mL) and sucrose (1.0 M was added at t = 0 and 3 h) at pH 4.7 and 37°C.

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Table 2

<table>
<thead>
<tr>
<th>Residue</th>
<th>F1</th>
<th>F2</th>
<th>F3′1</th>
<th>F3′2</th>
<th>F4</th>
<th>F4-4S</th>
<th>Ste</th>
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<td>3.35</td>
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<tr>
<td>H-8</td>
<td>3.35</td>
<td>3.35</td>
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<td>3.35</td>
<td>3.35</td>
<td>3.35</td>
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</table>

* In ppm relative to the signal of internal acetone (δ 2.225 for 1H and 31.07 for 13C).

* For F3′2 the δ values of the major component RebA-G2b are given. The δ values of the anomic protons of the carbohydrate moieties at the steviol C-19 site of the minor component RebA-G2c are: Glc1 H1, δ1H 5.410; Glc6 H1, δ1H 4.857; Glc6 H1, δ1H 5.265.

* Substituted carbon positions are indicated in italics.
2.4. Structural analysis of HPLC fraction with maltose as donor substrate in the extension of Glc1 copyranosyl-3-bioconversion reaction of RebA into rebaudioside D [Glc(1 → 2,3-di-O-substituted Glc) is RebA with a Glc-O-kaur-16-en-19-oic acid 6-β-D-glucopyranosyl-(1 → 2)-anomeric signals of this moiety are found at \( \delta_{H-1} 5.426/ \delta_{C} 67.5; \) RebA-G1 (peak area ratio 3:7), which were isolated for further analysis. MALDI-TOF-MS revealed identical spectra for F3P1 and F3P2 (peak area ratio 3:7), which were isolated for further analysis. MALDI-TOF-MS revealed identical spectra for F3P1 and F3P2 ([M + Na]+, \( m/z \) 1312.7; [M + K]+, \( m/z \) 1329.5), reflecting in both cases the attachment of two Glc residues at RebA (in view of the foregoing at the steviol C-19 site). Methylation analysis of both fractions showed a significant difference between F3P1 and F3P2 for the amounts of 3-, 4- and 6-mono-O-substituted Glc derivatives (Table 3). Furthermore, the \( ^1H \) NMR spectra of F3P1 and F3P2 are clearly different (Fig. 7; enlarged anomeric regions in Supplementary Information Fig. S8).

TOCSY, HSQC and ROEY measurements carried out on HPAEC fraction F3P1 afforded the assignment of the \( ^1H \) and \( ^13C \) resonances stemming from the steviol aglycone (Table 1) and the carbohydrate moieties (Table 2). The \( ^1H \) and \( ^13C \) data of the steviol core correspond with those found for RebA (F1) and RebA-G1 (F2). The same holds for the \( ^1H \) and \( ^13C \) signals of the trisaccharide moiety at the steviol C-13 site, indicating that no modifications had occurred at this site, in agreement with the alkaline-treatment results of F3. The \( \beta \)-anomeric signals of this moiety are found at \( \delta_{H-1} 4.720/\delta_{C} 103.8 \) (Glc2), \( \delta_{H-1} 4.810/\delta_{C} 103.5 \) (Glc3) and \( \delta_{H-1} 4.700/\delta_{C} 103.8 \) (Glc4). The remaining carbohydrate signals in the \( ^1H \) and \( ^13C \) anomeric regions represent the \( \beta \)-anomeric signals of Glc1 (steviol C-19 site) at \( \delta_{H-1} 5.426/\delta_{C} 95.7 \) and the overlapping \( \alpha \)-anomeric signals of Glc5 and Glc6 at \( \delta_{H-1} 4.88/\delta_{C} 99.5 \) (H-1’s also overlap with one of the steviol C-17 protons, \( \delta_{H} 4.875 \). The downfield chemical shift value of Glc1 C-6 (\( \delta_{C} 67.5; \) RebA Glc1 C-6: \( \delta_{C} 62.2 \) is typical for a terminal Glc(\( \alpha \)1 → 3) or Glc(\( \alpha \)1 → 4) residue (see fraction F3P2, Section 2.4), supports the absence of such extensions at Glc1.

To confirm the exclusive introduction of the Glc residue (Glc5) at the steviol C-19 glucosyl moiety (Glc1) of RebA, and not at the steviol C-13 trisaccharide part, fraction F2 was subjected to alkaline conditions (1.0 M NaOH, 2.5 h, 80 °C), which should specifically cleave the C-19 carboxyl-glucosyl ester linkage, leaving the glycosylation of the steviol C-13 part intact. To check the reaction conditions, the alkaline-induced transition of RebA (F1) into rebaudioside B (RebB; Fig. 4; Tables 1 and 2) was carried out as a positive control. The formed product from F2 (denoted as F1-4S), isolated via reversed phase column chromatography, was investigated by MALDI-TOF-MS ([M + Na]+, \( m/z \) 827.3), methylation analysis (terminal Glcp: 2,3-di-O-substituted Glcp = 2:1) and NMR spectroscopy (1D, TOCSY, HSQC, ROESY; presence of Glc2, Glc3 and Glc4, absence for the amounts of 3-, 4- and 6-mono-O-substituted Glc residues, indicating that no further branching had occurred (Table 3). The presence of 3-, 4- and 6-mono-O-substituted Glcp (ratio 11:3:19) indicated elongation of terminal Glc residues, and suggested the occurrence of a mixture of compounds, which was supported by the 1D \( ^1H \) NMR spectrum of F3 (Fig. 5). Alkaline treatment of F3 yielded only RebB (F1-4S; Supplementary Information Fig. S7), demonstrating that extensions had only occurred at the steviol C-19 site.

Fraction F3 was subfractionated by high-pH anion-exchange chromatography (HPAEC) on CarboPac PA-1, yielding two fractions F3P1 and F3P2 (peak area ratio 3:7), which were isolated for further analysis. MALDI-TOF-MS revealed identical spectra for F3P1 and F3P2 ([M + Na]+, \( m/z \) 1312.7; [M + K]+, \( m/z \) 1329.5), reflecting in both cases the attachment of two Glc residues at RebA (in view of the foregoing at the steviol C-19 site). Methylation analysis of both fractions showed a significant difference between F3P1 and F3P2 for the amounts of 3-, 4- and 6-mono-O-substituted Glc derivatives (Table 3). Furthermore, the \( ^1H \) NMR spectra of F3P1 and F3P2 are clearly different (Fig. 7; enlarged anomeric regions in Supplementary Information Fig. S8).
in accordance with the presence of a \( \alpha \rightarrow 6 \) Glc1\( \beta \) \( \rightarrow \) C-19 residue. As Glc5 C-6 showed the same downfield chemical shift as Glc1 C-6 (\( \delta_C \) 67.5) and Glc6' had similar \( ^{13} \)C values as terminal Glc5(\( \alpha \) \( \rightarrow \) 6) in F2 (Table 2), the monosaccharide sequence of the carbohydrate moiety at C-19 of F3P1 should be Glc6'(\( \alpha \) \( \rightarrow \) 6)Glc5(\( \alpha \) \( \rightarrow \) 6)Glc1(\( \beta \) \( \rightarrow \) (RebA-G2a) (Fig. 4). The latter sequence is further supported by the inter-residual ROESY cross-peaks between Glc6' H-1 (\( \delta_H \) 4.875) and Glc5 H-6a/b (\( \delta_H \) 3.88/3.65) and between Glc5 H-1 (\( \delta_H \) 4.875) and Glc1 H-6a/b (\( \delta_H \) 3.89/3.70). Also the methylation analysis data of F3P1 (Table 2) are in accordance with this structure. Note that Glc5 and Glc6' H-1 of the steviol core ester-bound \( \beta \) isomaltotriose in F3P1 (\( \delta_H \) 4.88, \( T \) = 334 K) resonates clearly.

Fig. 5. 500-MHz \(^1\)H NMR spectra of HPLC fractions F1, F2, F3 and F4, recorded in D\(_2\)O at 334 K. Enlarged anomeric regions are presented in Supplementary Information Fig. S6. Steviol core C-17, C-18 and C-20 protons are indicated in the spectrum of F1. For structures, see Fig. 4.
Table 3  
Methylation analysis of the carbohydrate moieties in RebA and α-glucosylated RebA products (fractions F1-F5 and F9).

<table>
<thead>
<tr>
<th>Alditol acetate</th>
<th>( R_t )</th>
<th>Structural feature</th>
<th>Peak area (%)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>F1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RebA</td>
</tr>
<tr>
<td>2,3,4,6-Hex(^a)</td>
<td>1.00</td>
<td>Glcp(1→)</td>
<td>74</td>
</tr>
<tr>
<td>2,4,6-Hex</td>
<td>1.16</td>
<td>→2Glc(1→)</td>
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<tr>
<td>3,4,6-Hex</td>
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<td>2,3,4,6-Hex</td>
<td>1.22</td>
<td>→6Glc(1→)</td>
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<tr>
<td>4,6-Hex</td>
<td>1.32</td>
<td>→2,3Glc(1→)</td>
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<tr>
<td>2,4-Hex</td>
<td>1.39</td>
<td>→3,6Glc(1→)</td>
<td>–</td>
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</table>

\(^a\) \( R_t \), retention time relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol (1.00) on GLC (see Section 4.5).
\(^b\) Averaged rounded-off values from methylation analyses carried out on fractions, isolated from three different incubations with Gtf180-D\(\text{N}\).
\(^c\) 2,3,4,6-Hex = 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylhexitol-1-d, etc.
\(^d\) tr = trace (<3%).

Fig. 6. HSQC, TOCSY (mixing time 200 ms) and ROESY spectra of the carbohydrate part of HPLC fraction F2 (RebA-G1), recorded in D\(\text{2}O\) at 334 K. In the HSQC spectrum, a\(^3\) means cross-peak H-3/C-3 of residue Glc2, etc.; assignments in red reflect the substituted positions of the residues. In the ROESY spectrum, the inter-residual cross-peaks confirming the Glc4(\(\beta\)1→3)Glc2, Glc3(\(\beta\)1→2)Glc2 and Glc5(\(\alpha\)1→6)Glc1 linkages are indicated with red boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
upfield from the anomeric protons of the terminal and internal Glc(x1 → 6) residues in free β-isomaltotriose (δH-1 ~4.96; T = 300 K) [40]. Furthermore, their H-5 chemical shifts can be used to discriminate between a terminal Glc(x1 → 6) and an internal → 6)Glc(x1 → 6) position: **Glc6** H-5, δH 3.64; **Glc5** H-5, δH 3.80.

In a similar way as described for **F3P1**, TOCSY, HSQC and ROESY measurements carried out on HPAEC fraction **F3P2** afforded the assignment of the 1H/13C resonances stemming from the steviol aglycone (Table 1) and the carbohydrate moieties (Table 2). The steviol core NMR data of **F3P2** are similar to those of **F3P1**. The same holds for the 1H and 13C signals of the carbohydrate moiety at the steviol C-13 site (for the anomeric regions: **Glc2**, δH-1 4.715, δC-1 97.6; **Glc3**, δH-1 4.810, δC-1 103.6; **Glc4**, δH-1 4.702; δC-1 103.9). These results, together with the alkaline-treatment data of **F3**, indicated that also in **F3P2** the carbohydrate moiety at the steviol C-13 site was not modified. The remaining carbohydrate signals in the anomeric region of the 1H NMR spectrum of **F3P2** (Fig. 7; enlarged anomeric region in Supplementary Information Fig. S8) represent a heterogeneous β-anomeric signal of **Glc1** (steviol C-19 site) at δH ~5.42, a heterogeneous α-anomeric signal at δH ~4.88 (**Glc5**), overlapping with one of the C-17 steviol protons, and a heterogeneous α-anomeric signal at δH ~5.28 (**Glc6**). The **Glc1** H-1 signal is clearly built up from two doublets at δH 5.425 and 5.410 (J1,2 8.3 Hz; peak ratio 2.7:1.0), the **Glc5** H-1 signal from two doublets at δH 4.873 and 4.857 (J1,2 4.1 Hz; peak ratio 2.8:1.0), and the **Glc6** H-1 signal from two doublets at δH 5.275 and 5.265 (J1,2 4.3 Hz; peak ratio 2.8:1.0), suggesting the presence of two compounds in

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**Fig. 7.** 500-MHz 1H NMR spectra of HPAEC fractions **F3P1** and **F3P2**, recorded in D2O at 334 K. ø means contamination. Enlarged anomeric regions are presented in Supplementary Information Fig. S8. For structures, see Fig. 4.
fraction F3P2. As H-1 signals of terminal Glc(α1 → 3) and terminal Glc(α1 → 4) residues, when present in a major/minor mixture, are difficult to distinguish by their chemical shifts [compare nigerotriose and maltotriose (300 K) [40]], the δ values of Glc H-1 at 5.275 and 5.265 ppm could reflect the presence of both possibilities.

Inspection of the NMR data of F3P2, obtained from combined TOCSY and HSQC experiments (Fig. 8, HSQC spectrum plotted at a high level), showed a downfield Glc C-1 signal at δC 67.0, in agreement with a 6-substituted Glc residue and a downfield Glc C-3 signal at δC 81.5, in agreement with a 3-substituted Glc residue [compare with β-isomaltose (α-2-6-α-Glc(→1-6)-β-α-Glc(→ C-6, δC 61.4; C-6, δC 66.6)] and α-nigerose (α-2-6-α-Glc(→1-3)-α-2-6-α-Glc(→ C-3, δC 73.7; C-3, δC 80.6)] [42]. ROESY experiments showed inter-residual cross-peaks between Glc6 H-1 (δH 5.27) and Glc5 H-3 (δH 3.79) and between Glc5 H-1 (δH 4.87) and Glc1 H-6a/b (δH 3.91/3.69). Intra-residual H-1-H-2 cross-peaks for Glc5 and Glc6 confirmed their α-configurations. Based on these experiments, it was concluded that the major component (~74%) in fraction F3P2 contained the Glc6(α1 → 3)Glc5(α1 → 6)Glc1(β1 → glycan at the steviol C-19 site (RebA-G2b) (Fig. 4). Taking into account the methylation analysis of F3P2 (Table 3), showing ~75% 3-O-mono-substituted Glcp and ~25% 4-O-mono-substituted Glcp, it is suggested that the minor glycan at the steviol C-19 site is Glc6(α1 → 4)Glc5(α1 → 6)Glc1(β1 → (RebA-G2c) (Fig. 4). In summary, the three components present in fraction F3 occur in a molar ratio RebA-G2a:RebA-G2b:RebA-G2c of about 40:47:13. Note that anomeric chemical shift differences exist between terminal Glc(α1 → 3)/Glc(α1 → 4) residues in free oligosaccharides [terminal Glc(α1 → 3): δH1.1 -5.36; terminal Glc(α1 → 4): δH1.1 -5.40; T = 300 K] [40] and steviol core ester-bound trisaccharides [Glc6(α1 → 3), δH1.1 -5.275; Glc6(α1 → 4), δH1.1 -5.265; T = 334 K].

2.5. Structural analysis of HPLC fraction F4

Methylation analysis of HPLC fraction F4 (RebA+3Glc) showed the presence of 6- and 3-mono-O-substituted Glcp in the molar ratio 2.6:1.0; furthermore, the molar ratio of terminal and 2,3-di-O-substituted Glcp is 3:1 (Table 3). Although the integration of the anomeric protons in the 1H NMR spectrum of F4 (Fig. 5) suggest the presence of one major component, in view of the mentioned structures present in fraction F3, it would be expected that also fraction F4 contains a mixture of different compounds with the same molecular mass. As already discussed for F2 and F3, also in F4 the extra α-linked Glc residues are only located at the steviol C-19 site [alkaline treatment of F4 yielded only RebB (F1-4S; Supplementary Information Fig. S7)]. The 1H/13C NMR assignments of the F4 steviol aglycone and carbohydrate moieties, derived from TOCSY, HSQC and ROESY measurements, are presented in Tables 1 and 2, respectively (spectra not shown).

Inspection of the 1H NMR spectrum of F4 (Fig. 5) revealed the characteristic peak pattern of the steviol core, comprising the two steviol H-17 signals in the carbohydrate anemic region and further signals in the upfield region 0.80 -2.20 ppm (Table 1). In the anomeric region, the three β-anomeric proton resonances stemming from the carbohydrate moiety at the steviol C-13 site [Glc2: δH1.1 4.700 (with δC 197.6); Glc3: δH1.1 4.802 (with δC 103.6); Glc4: δH1.1 4.700 (with δC 103.7)] are detected (Table 2). The Glc1 H-1 - H6b (H-1, δH 5.425) and C-1-C-6 (C-6, δC 67.0) chemical shift sets correspond with the → 6)Glc (β1 → C-19 residue, just like in F2 and F3. The three α-anomeric signals reflect the glycan extension at Glc1. The Glc7 1H and 13C sets are in accordance with a terminal Glc(α1 → 6) residue (e.g. H-1, δH 4.875, J1,2 < 4 Hz; C-1, δC 99.3; C-3, δC 74.5; C-6, δC 62.5; compare with the sets of Glc5 in F2 and Glc6 in F3P1). The Glc5 1H and 13C sets indicate an internal → 3) residue (e.g. H-1, δH 4.875, J1,2 < 4 Hz; C-1, δC 99.2; C-3, δC 82.3; C-6, δC 62.5; compare with the sets of Glc5 in F3P2). Finally, the Glc6 1H and 13C sets show the presence of an internal → 6) residue (e.g. H-1, δH 5.259, J1,2 < 4 Hz with C-1, δC 101.2; H-5, δH 4.09 with C-5, δC 72.2; C-3, δC 75.0; C-6, δC 67.0). Furthermore, ROESY experiments revealed inter-residual cross-peaks between Glc7 H-1 (δH 4.875) and Glc6 H-6a/b (δH 3.94/3.64), between Glc6 H-1 (δH 5.259) and Glc5 H-3 (δH 3.77) and between Glc5 H-1 (δH 4.875) and Glc1 H-6a/b (δH 3.89/3.70). Taken together, the various results of fraction F4 indicated the presence of a major product (~73%), having a carbohydrate moiety consisting of Glc7(α1 → 6)Glc6(α1 → 3)Glc5(α1 → 6)Glc1(β1 → (RebA-G3a) at the steviol C-19 site, i.e. an elongation of RebA-G2b with a Glc(α1 → 6) residue (Fig. 4). Note that Glc6 H-1 (δH 5.259, 334 K) of the steviol core ester-bound glycan in F4, oriented in an internal → 6)Glc(α1 → 3) position, resonates upfield from the anomeric proton of internal → 6)Glc(α1 → 3) residues (δH1.1 -5.33, T = 300 K) in similar free oligosaccharides, e.g. Glc(α1 → 6)Glc(α1 → 3)Glc(α1 → 6)Glc [28]. Taking into account composite models of α-α-glucons generated from sucrose using wild-type and mutant Gtf180-ΔAn1 glucansucrases as biocatalyst [28,30,31], the
structure of one of the expected minor products is hypothesized to be an elongation of RebA-G2a with a Glc(\(1 \rightarrow 6\)) residue [Glc7(\(1 \rightarrow 6\))Glc6(\(1 \rightarrow 6\))Glc5(\(1 \rightarrow 6\))Glc1(\(1 \rightarrow \)).

2.6. Structural analysis of HPLC fractions F5-F9

As is evident from the HPLC profile in Fig. 3, fractions F5 (RebA+4Glc) to F9 (RebA+8Glc) represent complex mixtures of RebA derivatives. Inspection of the 1H NMR spectra of F5 to F9 (spectra not shown) learned that the stevior core regions (0.80–2.20 ppm) are identical and the carbohydrate bulk regions (3.20–4.20 ppm) similar to those in the 1H NMR spectra of F3 and F4. When treated with alkali, each fraction was converted into RebB (F1-45, Supplementary Information Fig. S7), meaning that also in the case of F5 to F9 α-glucosylation had only occurred at the steviol C-19 glucosyl moiety of RebA. The patterns of anomeric signals in the 1H NMR spectra of F5 to F9 are comparable with those of F3 and F4, indicating that the elongated carbohydrate chains at the steviol C-19 site of F5 to F9 are built up from (\(1 \rightarrow 6\)) and (\(1 \rightarrow 3\)) linked Glc residues. More specifically, the spectra showed relative increase in intensities of the H-1 resonance at δH 5.25 and the H-5 resonance at δH 4.10, derived from internal (\(1 \rightarrow 6\)) Glc([\(1 \rightarrow 3\)]) residues, and the H-1 resonance at δH 4.57, derived from terminal Glc([\(1 \rightarrow 6\)]) and internal (\(1 \rightarrow 6\)) Glc([\(1 \rightarrow 6\)]) residues. Methylolation analysis of these high-molecular-mass fractions showed an increase of (\(1 \rightarrow 6\)) and (\(1 \rightarrow 3\)) linkages towards almost equal molar amounts (Table 3; only traces of 4-linked Glcp were observed), suggesting the preference of the wild-type Gtf180-ΔN enzyme for the synthesis of alternating (\(1 \rightarrow 6\))([\(1 \rightarrow 3\]) linkages in the major formed RebA derivatives. Furthermore, the finding of low amounts of 3,6-di-O-substituted Glcp residues in the methylolation analysis of these fractions reflects also the possibilities of branching.

3. Conclusions

Over the years, several types of carbohydrate-active enzymes have been used in the glycosylation reactions of steviol glycosides (see review Ref. [3]). With respect to trans-α-glucosylations, cycloextrin glycosyltransferase systems, introducing elongations with α-α-Glcp(1→4) units at both the steviol C-13 and C-19 sites of steviolide, rubioside and rebaudioside A (RebA), gained great attention. In these bioconversions, glucose donors such as cycloextrins, maltodextrins and starches are used. But also other α-glucosidase transglycosylation systems were investigated, i.e. glucansucrase enzymes combined with sucrose as glucose donor.

Using alternansucrase from Leuconostoc citreum SK24.002 as biocatalyst, stevioside (Fig. 1) could be converted into a mixture of nine products, including three monos-, three dis- and three tri-α-glucosylated stevioside derivatives. Two products were characterized in detail, showing elongations of the terminal Glc([\(1 \rightarrow 2\)]) residue of the β-sophorosyl disaccharide at the steviol C-13 site with a Glc([\(1 \rightarrow 6\)]) unit and with a Glc([\(1 \rightarrow 3\)])Glc([\(1 \rightarrow 6\)]) Glc([\(1 \rightarrow 3\)]) trisaccharide [43–45]. When incubated with sucrose alone, alternansucrase produces a glucan with alternating (\(1 \rightarrow 3\)) and (\(1 \rightarrow 6\)) linkages, called alternan [46].

Recent studies in our research group have shown that (mutant) Gtf180-ΔN glucansucrase of Lb. reuteri strain 180 introduces glucose residues from sucrose into RebA, specifically at the C-19 site. In this report the structural analysis of the formed products with wild-type Gtf180-ΔN glucansucrase as biocatalyst has been described in detail. Structural biological aspects, including comparison of transglycosylation activities and differences in conversion percentages of wild-type and 82 mutant Gtf180-ΔN glucansucrase enzymes, molecular docking studies of the Gtf180-ΔN – RebA complex, and sweetness/bitterness tests will be described elsewhere [te Poel et al., manuscript in preparation]. As a first step, the Glc([\(1 \rightarrow 2\)])-linked Glc([\(1 \rightarrow 6\)]) residue of RebA was found to be elongated with a Glc([\(1 \rightarrow 6\)]) residue (RebA-G1). Further extensions, up to eight Glc units, comprised mainly Glc([\(1 \rightarrow 6\)]) and Glc([\(1 \rightarrow 3\]) residues; in the trisaccharide, elongation evidence for a termination with a Glc([\(1 \rightarrow 4\)]) residue was found. The major higher α-glucosylated RebA products are built up by elongation of the Glc([\(1 \rightarrow 6\)])Glc([\(1 \rightarrow 2\)])C-19 moiety with alternating (\(1 \rightarrow 3\))(\(1 \rightarrow 6\)) linked Glc units, accompanied by products with only (\(1 \rightarrow 6\)) linked sequences in lesser amounts. In the higher-molecular-mass components also 3,6-branching is indicated.

4. Experimental

4.1. Steviol glycoside substrates and glucansucrase enzyme

Rebaudioside A (RebA) was purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands) and stevioside (Ste) from Wako Pure Chemical Industries (Osaka, Japan). Rebaudioside B (Rebb) was prepared by alkaline treatment of RebA (see Section 4.3). The Lactobacillus reuteri 180 glucansucrase enzyme Gtf180-ΔN [117-kDa N-terminally truncated (741 residues) fragment of the wild-type Gtf180 full-length protein] was produced and purified as described [47,48].

4.2. Preparation of α-α-glucosylated rebaudioside A products

Incubations of RebA (50 mM) were performed in 5 mL 25 mM sodium acetate (pH 4.7), containing 1 mM CaCl2, in the presence of 10 U/mL Gtf180-ΔN enzyme at 37 °C and 24 h. Two batches of 1.0 M sucrose donor substrate were added at t = 0 and t = 3 h, respectively. One unit (U) of enzyme is defined as the amount of enzyme required for producing 1 μmol fructose from sucrose per min in reaction buffer, containing 1.0 M sucrose at 37 °C. In this case, 1 U corresponded to 0.038 mg Gtf180-ΔN. The full rationale for these incubation conditions will be described in detail elsewhere [te Poel et al., manuscript in preparation]. The pool of glucosylated RebA products was isolated by solid-phase extraction (SPE) using a Strata-X 33μ Polymeric Reversed Phase column (Phenomenex, Utrecht, The Netherlands). Briefly, the SPE column was conditioned with 6 bed volumes methanol and subsequently equilibrated with 6 bed volumes de-ionized water. After loading of the sample, the column was washed with 6 bed volumes de-ionized water to remove enzyme, glucose, fructose, gluco-oligo/poly saccharides and residual sucrose. Then, the mixture of RebA products was eluted with 6 bed volumes 50% acetonitrile. Subsequently, the mixture was fractionated on a Luna 10 μm NH2 semi-preparative chromatography column (250 mm × 10 mm, Phenomenex), using an UltilMate 3000 HPLC system (ThermoFisher Scientific, Amsterdam, The Netherlands), equipped with a VWD-3000 UV-VIS detector (monitoring at 210 nm). Separations were obtained at a flow-rate of 4.6 mL/min under gradient elution conditions (solvent A = acetonitrile; solvent B = 0.025% aqueous acetic acid), starting with a 2-min isocratic step with 80% solvent A in B followed by a linear gradient of 80 to 50% solvent A in B over 38 min. The manually collected fractions were evaporated to dryness under a stream of nitrogen, and the residues were re-dissolved in de-ionized water and directly lyophilized. Fresh newly dissolved samples were used for analysis.

4.3. Alkaline hydrolysis

To release the carbohydrate moiety linked to the C-19 carboxyl group, 4 mg RebA and 4 mg of each transglycosylated product were...
individually dissolved in 1 mL 1.0 M NaOH and the solutions were heated at 80 °C for 2.5 h, then cooled down, and neutralized with 6 M HCl. The modified product fractions were isolated using Strata-X 33 μ Polymeric Reversed Phase columns (Phenomenex), as described in Section 4.2.

4.4. High-pH anion-exchange chromatography

High-pH anion-exchange chromatography (HPAEC) was performed on a Dionex DX500 workstation (Dionex, Amsterdam, The Netherlands), equipped with a CarboPac PA-1 column (250 × 9 mm; Dionex) and an ED40 pulsed amperometric detector, using a linear gradient from 30 mM to 265.2 mM sodium acetate in 100 mM NaOH (3 mL/min) over 26 min. Collected fractions were immediately neutralized with 4 M acetic acid, desalted on Strata-X 33μ Polymeric Reversed Phase columns (Phenomenex), using 50% aqueous acetonitrile as eluent, and lyophilized.

4.5. Methylation analysis

Steviol glycoside samples were permethylated using CH3I and solid NaOH in (CH3)2SO, as described previously [49], then hydrolyzed with 2 M trifluoroacetic acid (2 h, 120 °C) to give the mixture of partially methylated monosaccharides. After evaporation to dryness, the mixture, dissolved in H2O, was reduced with NaBD4 of partially methylated monosaccharides. After evaporation to solid NaOH in (CH3)2SO, as described previously[49], then hydrolyzed without decoupling during acquisition of the1H 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 1H and δC 31.07 for 13C).

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Appendix. Supplementary information

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.carres.2017.01.008.

References
