Structural analysis of rebaudioside A derivatives obtained by \textit{Lactobacillus reuteri} 180 glucansucrase-catalyzed trans-\(\alpha\)-glucosylation

Gerrit J. Gerwig \textsuperscript{a,b,1}, Evelien M. te Poele \textsuperscript{a,1}, Lubbert Dijkhuizen \textsuperscript{a,*}, Johannis P. Kamerling \textsuperscript{a,b}

\textsuperscript{a} Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747 AG, Groningen, The Netherlands
\textsuperscript{b} NMR Spectroscopy, Bijvoet Center for Biomolecular Research, Utrecht University, Padualaan 8, 3584 CH, Utrecht, The Netherlands

**Abstract**

The wild-type Gtf180-\(\Delta\)N glucansucrase enzyme from \textit{Lactobacillus reuteri} 180 was found to catalyze the \(\alpha\)-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 \(\alpha\)-\(\alpha\)-glucosylated at the steviol C-19 \(\beta\)-\(\beta\)-glucosyl moiety (55% conversion). The main product is a mono-(x1 \(\rightarrow\) 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 (x1 \(\rightarrow\) 3)- and (x1 \(\rightarrow\) 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.

© 2017 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

The leaves of the \textit{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 (\(\sim\)5–20% w/w of dried leaves) and rebaudioside A (\(\sim\)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 stevios, 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 \textit{Stevia} sweeteners is their slight bitterness and unpleasant (metallic) aftertaste, experienced by more than half of the human population.

For natural steviol glycosides with \(\beta\)-\(\beta\)-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-ΔN 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-ΔN 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-ΔN 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-ΔN glucansucrase enzyme and sucrose

Inspection of the molecular structure of rebaudioside A (RebA, 13-[β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl]-16-ene-19-oic acid) (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-ΔN 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-ΔN 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

Fig. 1. Structures of stevioside (Ste) and rebaudioside A (RebA), occurring as main components in the leaves of the Stevia rebaudiana plant.

Fig. 2. Composite structure of the extracellular polysaccharide EPS180 from the lactic acid bacterium Lactobacillus reuteri 180 [29].
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 structural information about the generated RebA derivatives, fractions F1–F9 were subjected to methylation analysis and NMR spectroscopy.

For an explanation of the code system used, see Fig. 4.

2.2. Structural analysis of HPLC fraction F1

NMR analysis of fraction F1 confirmed the presence of acceptor substrate RebA (Fig. 5, *H NMR spectrum of F1, identical to Supplementary Information Fig. S3; Tables 1 and 2). Methylation analysis (Table 3) revealed the presence of terminal Glc2 and 2,3-di-O-substituted Glc2 in the molar ratio of 3:1, as expected for RebA.

2.3. Structural analysis of HPLC fraction F2

Methylation analysis of F2 (RebA_+1Glc) (Table 3) showed a 3:1 molar ratio for terminal Glc2 and 2,3-di-O-substituted Glc2, indicating that the additional Glc2 residue is attached to a terminal Glc2 residue of RebA. The detection of a 6-mono-O-substituted Glc2 residue suggested a product with a Glc2(1→6) extension.

The *H NMR spectrum of F2 (Fig. 5; enlarged anomic region in Supplementary Information Fig. S6) exhibited the typical steviol core signal pattern as seen for RebA (F1). Besides the four β-anomeric *H signals related to RebA (Glc1, δH 4.910; Glc2, δH 4.700; Glc3, δH 4.801; Glc4, δH 4.699), one α-anomeric *H resonance (δH 4.865; J1,2 3.7 Hz), partly overlapping with one steviol C17 proton, was observed, stemming from a new α-linked Glc residue (Glc5).

The latter signal correlated with a 13C resonance at δC 99.5 in the HSQC spectrum (Fig. 6).

In a similar way as described for RebA in the Supplementary Information, using 2D NMR spectroscopy (TOCSY with different mixing times, ROESY and HSQC, Fig. 6), the *H/13C chemical shifts of the steviol core (Table 1) and the five Glc residues (Table 2) were assigned. The *H and 13C chemical shift sets of Glc2, Glc3 and Glc4 are nearly identical to those of F1 (RebA), suggesting that no modifications had occurred in the carbohydrate moiety at the

---

Table 1

| Carbon| F1 | F2 | F3\[1] | F3\[2] | F4 | F4\[4S] | Ste | RebA (C5D5N) |
|-------|----|----|------|------|----|-------|-----|-------------|
| Number| *H | *H | *H | *H | *H | *H | *H | *H |
| 1     | 0.81 41.6 | 0.81 41.6 | 0.82 41.5 | 0.81 41.6 | 0.82 41.5 | 0.78 41.8 | 0.82 41.3 | 0.78 41.2 |
| 2     | 1.81 | 1.82 | 1.80 | 1.81 | 1.82 | 1.78 | 1.82 | 1.77 |
| 3     | 1.40 20.3 | 1.30 20.3 | 1.30 20.3 | 1.30 20.3 | 1.30 20.4 | 1.25 20.4 | 1.31 20.5 | 1.45 19.9 |
| 4     | 1.75 | 1.60 | 1.51 | 1.52 | 1.51 | 1.37 | 1.60 | 2.22 |
| 5     | 1.07 38.8 | 1.04 38.9 | 1.06 38.9 | 1.06 38.9 | 1.06 38.9 | 0.88 40.0 | 1.06 38.6 | 1.03 37.3 |
| 6     | 2.07 | 2.06 | 2.04 | 2.04 | 2.04 | 1.96 | 2.05 | 2.35 |
| 7     | 1.15 58.1 | 1.11 58.1 | 1.14 58.1 | 1.14 58.1 | 1.15 58.0 | 0.94 57.9 | 1.14 57.9 | 1.05 57.8 |
| 8     | 1.60 22.8 | 1.68 22.8 | 1.82 22.9 | 1.82 22.9 | 1.84 22.9 | 1.85 22.9 | 1.65 22.8 | 1.92 22.6 |
| 9     | 1.80 | 1.80 | 1.80 | 1.80 | 1.80 | 1.80 | 1.82 | 2.46 |
| 10    | 1.41 42.5 | 1.39 42.5 | 1.40 42.4 | 1.41 42.5 | 1.41 42.5 | 1.37 42.3 | 1.41 42.2 | 1.41 42.2 |
| 11    | 1.50 | 1.51 | 1.49 | 1.49 | 1.49 | 1.47 | 1.47 | 1.30 |
| 12    | 0.98 54.7 | 0.97 54.8 | 0.97 54.8 | 0.97 54.8 | 0.97 54.7 | 0.95 55.0 | 0.96 54.8 | 0.88 54.5 |
| 13    | 1.70 23.1 | 1.70 22.9 | 1.66 22.9 | 1.66 22.9 | 1.68 23.0 | 1.67 22.5 | 1.78 22.9 | 1.68 22.1 |
| 14    | 1.82 | 1.83 | 1.80 | 1.81 | 1.80 | 1.80 | 1.83 | 1.68 |
| 15    | 1.49 38.2 | 1.47 38.1 | 1.48 38.2 | 1.48 38.2 | 1.48 38.1 | 1.48 39.1 | 1.47 37.7 | 2.00 37.3 |
| 16    | 1.90 | 1.89 | 1.88 | 1.88 | 1.88 | 1.88 | 1.89 | 2.25 |
| 17    | 1.42 45.7 | 1.41 45.6 | 1.41 45.8 | 1.41 45.8 | 1.40 45.8 | 1.42 46.1 | 1.40 45.8 | 1.41 45.0 |
| 18    | 2.12 | 2.10 | 2.10 | 2.10 | 2.10 | 2.10 | 2.15 | 2.66 |
| 19    | 2.07 48.5 | 2.03 48.5 | 2.05 48.6 | 2.05 48.5 | 2.05 48.5 | 2.05 48.8 | 2.05 48.4 | 2.05 48.2 |
| 20    | 4.875 105.7 | 4.870 105.8 | 4.875 105.6 | 4.875 105.7 | 4.875 105.7 | 4.875 105.7 | 4.872 105.4 | 5.01 105.1 |
| 21    | 5.065 | 5.060 | 5.040 | 5.040 | 5.038 | 5.038 | 5.044 | 5.035 |
| 22    | 1.175 | 29.6 | 1.174 | 29.5 | 1.175 | 29.6 | 1.174 | 29.4 |
| 23    | 20.827 | 16.8 | 0.830 | 16.8 | 0.827 | 16.9 | 0.822 | 16.9 |

---

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

* As 13C data have been deduced from HSQC measurements, 13C chemical shifts in D2O are missing for C-6, C-8, C-10, C-13, C-16 and C-19.

* For comparison, NMR data of RebA recorded in C5D5N at 271 K (99.96 atom% D; solvent calibration: δH 3.410; δC 70.0) are included [35].
steinol C-13 site. The TOCSY Glc5 H-1 track (δH 4.865) showed the complete scalar coupling network H-1,2,3,4,5,6a,6b, and combined with the Glc5 C-1—C-6 set of chemical shifts, a terminal Glc (δH 6) unit is indicated. Based on the inter-residue ROESY cross-peaks between Glc5 H-1 (δH 4.865) and Glc1 H-6a/b (δH 3.87/3.69) (Fig. 6), combined with the 13C downfield shift of ~5 ppm for Glc1 C-6 (δC 67.0; RebA Glc1 C-6: δC 62.2) (HSQC spectrum, Fig. 6), a Glc5 (δH 6) Glc1 disaccharide element could be established. Note that Glc5 H-1 of the steviol core ester-bound β-isomalトate in F2 (δH 4.865, T = 334 K) resonates clearly upfield from the anomeric proton of the terminal Glc (δH 6) residue in free β-isomalトate (δH 4.46; T = 300 K) [40]. The absence of ~3 Glcp (1→ or 4 Glcp (1→ elements in the methylation analysis of F2 (Table 3) together with the absence of a δH-anomeric 1H signal at δH ~5.27,
2.4. Structural analysis of HPLC fraction

presence of Biozyme L20.

glycosylation reaction with maltose as donor substrate in the
extension of Glc1 copyranosyl-3-anosyl ester, and will be called RebA-G1 (Fig. 4).

F2 (RebA+1Glc), F3P1 (RebA+2Glc), F3P2a (RebA+2Glc), F3P2b (RebA+2Glc) and F4 (RebA+3Glc)] and alkaline-treated fractions F1–F4 = RebB (fraction F1-4S). [Glc1], etc. are denotations for the glucose residues attached at the steviol C-19 site.

Fig. 4. Structures of RebA (F1), α-glucosylated RebA products [fractions F2 (RebA+1Glc), F3P1 (RebA+2Glc), F3P2a (RebA+2Glc), F3P2b (RebA+2Glc) and F4 (RebA+3Glc)] and alkaline-treated fractions F1–F4 = RebB (fraction F1-4S). [Glc1], etc. are denotations for the glucose residues attached at the steviol C-19 site.

typical for a terminal Glc (α1 → 3) or Glc (α1 → 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) at 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 revealing identical spectra for F1 (Fig. 7; enlarged anomeric regions in Supplementary Information Fig. S8) 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 Glc (ratio 11:3:19) indicated elongation of terminal Glc residues, and suggested the occurrence of a mixture of compounds, which was supported by the 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 Glcp 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).

TOCYS, HSQC and ROESY measurements carried out on HPAEC fraction F3P1 afforded the assignment of the 1H/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 β-anomeric signals of this moiety are found at δH 4.720/δC 103.5 (Glc2), δH 4.810/δC 103.5 (Glc3) and δH 4.700/δC 103.8 (Glc4). The remaining carbohydrate signals in the 1H and 13C anomeric regions represent the β-anomeric signals of Glc1 (steviol C-19 site) at δH 5.426/δC 95.7 and the overlapping α-anomeric signals of Glc5 and Glc6 at δH 4.88/δC 99.5 (H-1’s also overlap with one of the steviol C-17 protons, δH 4.875). The downfield chemical shift value of Glc1 C-6 (δC 67.5; RebA Glc1 C-6: δC 62.2) is
in accordance with the presence of a \(\alpha\rightarrow 6\)Glc1(\(\beta\)1 \(\rightarrow\) 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(\(\alpha\)1 \(\rightarrow\) 6) in F2 (Table 2), the monosaccharide sequence of the carbohydrate moiety at C-19 of F3P1 should be Glc6'(\(\alpha\)1 \(\rightarrow\) 6)Glc5(\(\alpha\)1 \(\rightarrow\) 6) Glc1(\(\beta\)1 \(\rightarrow\) (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.

Fig. 5. 500-MHz \(^1\)H NMR spectra of HPLC fractions F1, F2, F3 and F4, recorded in D₂O 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).

| Alditol acetate | $R_t$ | Structural feature | Peak area (%) |
|----------------|------|-------------------|---------------|
|                |      |                   | F1  | F2  | F3  | F3'1| F3'2| F4  | F5  | F9  |
|                |      | RebA              |     |     |     |     |     |     |     |     |
|                |      | +1Glc             |     |     |     |     |     |     |     |     |
| 2,3,4,6-Hex    | 1.00 | Glc(1 → 2)       | 74  | 61  | 50  | 50  | 49  | 45  | 45  | 38  | 26  |
| 2,4,6-Hex      | 1.16 | Glc(1 → 3)       |     |     |     |     | 11  | 15  | 11  | 23  | 32  |
| 3,4,6-Hex      | 1.18 | Glc(1 → 4)       |     |     |     |     | 3   | tr  | 5   | tr  | tr  |
| 2,3,4,6-Hex    | 1.22 | Glc(1 → 6)       |     |     |     |     | 19  | 19  | 33  | 16  | 29  |
| 4,6-Hex        | 1.32 | Glc(1 → 2,3)     | 26  | 20  | 17  | 17  | 15  | 15  | 12  | 8   |
| 2,4-Hex        | 1.39 | Glc(1 → 3,6)     |     |     |     |     |     |     | tr  | 3   | 4   |

$R_t$, retention time relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol (1.00) on GLC (see Section 4.5).

Averaged rounded-off values from methylation analyses carried out on fractions, isolated from three different incubations with Gtf180-DN.

2,3,4,6-Hex = 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylhexitol-1-d, etc.

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 D2O at 334 K. In the HSQC spectrum, $\gamma_3$ 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(β1 → 3)Glc2, Glc3(β1 → 2)Glc2 and Glc5(α1 → 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(α1 → 6) residues in free β-isomaltotriose ($\delta_{\text{H-1}}$ ~4.96; $T = 300$ K) [40]. Furthermore, their H-5 chemical shifts can be used to discriminate between a terminal Glc(α1 → 6) and an internal → 6)Glc(α1 → 6) position: Glc6 H-5, $\delta_{\text{H}}$ 3.64; Glc5 H-5, $\delta_{\text{H}}$ 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 $^1$H/$^{13}$C 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 $^1$H and $^{13}$C signals of the carbohydrate moiety at the steviol C-13 site (for the anomeric regions: Glc2, $\delta_{\text{H-1}}$ 4.715, $\delta_{\text{C-1}}$ 97.6; Glc3, $\delta_{\text{H-1}}$ 4.810, $\delta_{\text{C-1}}$ 103.6; Glc4, $\delta_{\text{H-1}}$ 4.702; $\delta_{\text{C-1}}$ 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 $^1$H 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 $\delta_{\text{H}}$ ~5.42, a heterogeneous α-anomeric signal at $\delta_{\text{H}}$ ~4.88 (Glc5), overlapping with one of the C-17 steviol protons, and a heterogeneous α-anomeric signal at $\delta_{\text{H}}$ ~5.28 (Glc6). The Glc1 H-1 signal is clearly built up from two doublets at $\delta_{\text{H}}$ 5.425 and 5.410 ($J_{1,2}$ 8.3 Hz; peak ratio 2.7:1.0), the Glc5 H-1 signal from two doublets at $\delta_{\text{H}}$ 4.873 and 4.857 ($J_{1,2}$ 4.1 Hz; peak ratio 2.8:1.0), and the Glc6 H-1 signal from two doublets at $\delta_{\text{H}}$ 5.275 and 5.265 ($J_{1,2}$ 4.3 Hz; peak ratio 2.8:1.0), suggesting the presence of two compounds in

![Fig. 7. 500-MHz $^1$H NMR spectra of HPAEC fractions F3P1 and F3P2, recorded in D$_2$O at 334 K. ø means contamination. Enlarged anomeric regions are presented in Supplementary Information Fig. S8. For structures, see Fig. 4.](image-url)
fraction F3P2. As H-1 signals of terminal Glc(a1 → 3) and terminal Glc(a1 → 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 δH 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 at δC 67.0, in agreement with a 6-substituted Glc1 residue, and a downfield Glc C-3 signal at δC 81.5, in agreement with a 3-substituted Glc3 residue [compare with β-isomaltose (α-D-GlcP1(→1-6)β-D-GlcP: C-6, δC 61.4; C-6, δC 66.6) and α-nigerose (α-D-GlcP1(→1-3)α-D-GlcP: 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(a1 → 3)Glc5(a1 → 6)Glc1[b1 → 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(a1 → 4)Glc5(a1 → 3)Glc1[b1 → (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(a1 → 3)/Glc(a1 → 4) residues in free oligosaccharides [terminal Glc(a1 → 3): δH1-1 -5.36; terminal Glc(a1 → 4): δH1-1 -5.40; T = 300 K] [40] and steviol core ester-bound trisaccharides [Glc6(a1 → 3), δH1-1 -5.275; Glc6(a1 → 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 anomic region and further signals in the upfield region 0.80–2.20 ppm (Table 1). In the anomic region, the three β-anomeric proton resonances stemming from the carbohydrate moiety at the steviol C-13 site [Glc2: δH1-1 4.700 (with δC1 97.6); Glc3: δH1-1 4.802 (with δC1 103.6); Glc4: δH1-1 4.700 (with δC1 103.7)] are detected (Table 2). The Glc1 H-1–H-6b (H-1, δH 5.425) and C-1–C-6 (C-6, δC 67.0) chemical shift sets correspond with the → 6)Glc(b1 → C-19 residue, just like in F2 and F3. The three α-anomeric signals reflect the glycan extension at Glc1. The Glc1 1H and 13C sets are in accordance with a terminal Glc(a1 → 6) residue (e.g. H-1, δH 4.875, J1,2 < 4 Hz; C-1, δC 59.3; 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 59.2; 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 Glc6 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(a1 → 6)Glc6(a1 → 3)Glc5(a1 → 6)Glc1[b1 → (RebA-G3a) at the steviol C-19 site, i.e. an elongation of RebA-G2b with a Glc(a1 → 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)Glcn(a1 → 3) position, resonates upfield from the anomeric proton of internal → 6)Glc(a1 → 3) residues (δH -5.33, T = 300 K) in similar free oligosaccharides, e.g. Glc(a1 → 6) Glc(a1 → 3)Glc(a1 → 6)Glc [28]. Taking into account composite models of α-α-glucans generated from sucrose using wild-type and mutant Gtf180-ΔM glucanucrases 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(\(\alpha_1 \rightarrow 6\)) residue [Glc7(\(\alpha_1 \rightarrow 6\))Glc6(\(\alpha_1 \rightarrow 6\))Glc5(\(\alpha_1 \rightarrow 6\))Glc1(\(\beta_1 \rightarrow \)].

### 2.6. Structural analysis of HPLC fractions F5-F9

As is evident from the HPLC profile in Fig. 3, fractions F5 (RebA+Glc) to F9 (RebA+Glc) represent complex mixtures of RebA derivatives. Inspection of the 1D 1H NMR spectra of F5 to F9 (spectra not shown) learned that the steviol core regions (0.80–2.20 ppm) are identical and the carbohydrate bulk regions (3.20–4.20 ppm) similar to those in the 1D 1H NMR spectra of F3 and F4. When treated with alkali, each fraction was converted into RebB (F1-4S, Supplementary Information Fig. S7), meaning that also in the case of F5 to F9 \(\alpha\)-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 (\(\alpha_1 \rightarrow 6\))- and (\(\alpha_1 \rightarrow 3\))-linked Glc residues. More specifically, the spectra showed relative increase in intensities of the H-1 resonance at \(\delta_{H} 5.25\) and the H-5 resonance at \(\delta_{H} 4.10\), derived from internal \(\rightarrow 6\) Glc(\(\alpha_1 \rightarrow 3\)) residues, and the H-1 resonance at \(\delta_{H} 4.57\), derived from terminal Glc(\(\alpha_1 \rightarrow 6\)) and internal \(\rightarrow 6\) Glc(\(\alpha_1 \rightarrow 6\)) residues. Methylation analysis of these high-molecular-mass fractions showed an increase of (\(\alpha_1 \rightarrow 6\)) and (\(\alpha_1 \rightarrow 3\)) linkages towards almost equal molar amounts (Table 3; only traces of 4-linked Glcp were observed), suggesting the preference of the wild-type Gtf180-\(\Delta\)N enzyme for the synthesis of alternating (\(\alpha_1 \rightarrow 6\))(\(\alpha_1 \rightarrow 3\)) linkages in the major formed RebA derivatives. Furthermore, the finding of low amounts of 3,6-di-O-substituted Glcp residues in the methylation 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-\(\alpha\)-glucosylations, cyclodextrin glycosyltransferase systems, introducing elongations with \(\alpha\)-Glc-(1 \rightarrow 4) units at both the steviol C-13 and C-19 sites of stevioside, rubusoside and rebaudioside A (RebA), gained great attention. In these bioconversions, glucose donors such as cyclo-dextrins, maltodextrins and starches are used. But also other \(\alpha\)-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 mono-, three di- and three tri-\(\alpha\)-glucosylated stevioside derivatives. Two products were characterized in detail, showing elongations of the terminal Glc(\(\beta_1 \rightarrow 2\)) residue of the \(\beta\)-sophorosyl disaccharide at the steviol C-13 site with a Glc(\(\alpha_1 \rightarrow 6\)) unit and with a Glc(\(\alpha_1 \rightarrow 3\))Glc(\(\alpha_1 \rightarrow 6\))Glc(\(\alpha_1 \rightarrow 3\)) trisaccharide [43-45]. When incubated with sucrose alone, alternansucrase produces a glucan with alternating (\(\alpha_1 \rightarrow 3\)) and (\(\alpha_1 \rightarrow 6\)) linkages, called alternan [46].

Recent studies in our research group have shown that (mutant) Gtf180-\(\Delta\)N glucanase of L. 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-\(\Delta\)N glucanase 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 mut mutant Gtf180-\(\Delta\)N glucanase enzymes, molecular docking studies of the Gtf180-\(\Delta\)N–RebA complex, and sweetness/bitterness tests will be described elsewhere [te Poel et al., manuscript in preparation]. As a first step, the Glc(\(\beta_1 \rightarrow 2\))-C-19 residue of RebA was found to be elongated with a Glc(\(\alpha_1 \rightarrow 6\)) residue (RebA-G1). Further extensions, up to eight Glc units, comprised mainly Glc(\(\alpha_1 \rightarrow 6\)) and Glc(\(\alpha_1 \rightarrow 3\)) residues; in the trisaccharide, elongation evidence for a termination with a Glc(\(\alpha_1 \rightarrow 6\)) residue was found. The major higher \(\alpha\)-glucosylated RebA products are built up by elongation of the Glc(\(\alpha_1 \rightarrow 6\))Glc(\(\beta_1 \rightarrow 2\))C-19 moiety with alternating (\(\alpha_1 \rightarrow 3\))/(\(\alpha_1 \rightarrow 6\)) linked Glc units, accompanied by products with only (\(\alpha_1 \rightarrow 6\))-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-\(\Delta\)N [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 \(\alpha\)-\(\alpha\)-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-\(\Delta\)N 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 \(\mu\)mol 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-\(\Delta\)N. 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 33μi 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 \(\mu\)m NH2 semi-preparative chromatography column (250 mm × 10 mm, Phenomenex), using an UltiMate 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 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.
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 33 μ 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 solid NaOH in (CH3)2SO, as described previously [49], then hydrolyzed with 4 M acetic acid and boric acid was removed by repeated co-evaporation with methanol. The obtained partially methylated alditol samples were acetylated with 1:1 acetic anhydride-pyridine (30 min, 120 °C). After evaporation to dryness, the mixtures of partially methylated alditol acetates, dissolved in dichloromethane, were analyzed by GLC-EI-MS on an EC-1 column (30 m × 0.25 mm; Alltech), using a GCMS-QP2010 Plus instrument (Shimadzu Kratos Inc., Manchester, UK) and a temperature gradient (140–250 °C at 8 °C/min) [50].

4.6. Mass spectrometry

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on an Axima™ mass spectrometer (Shimadzu Kratos Inc.), 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 112 and the acquisition mass range was 200–6000 Da. Samples were prepared by mixing on the target 1-μL sample solutions with 1 μL aqueous 10% 2,5-dihydroxybenzoic acid in 70% acetonitrile as eluent, and lyophilized.

4.7. NMR spectroscopy

Resolution-enhanced 1D/2D 500–500 MHz 1H/13C NMR spectra were recorded in D2O on a Bruker DRX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University). To avoid overlap of anomic signals with the HOD signal, the spectra were run at 334 K. Data acquisition was done with Bruker Topspin 2.1. Before analysis, samples were exchanged twice in D2O (99.9 atom% D, Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization, and then dissolved in 0.6 mL D2O. It should be noted that during longer stay in aqueous solution, partial degradation of steviol glycosides can occur due to loss of the carbohydrate moiety from the steviol C-19 carboxyl group and possible formation of the Δ15-16 isomer and the Δ16-17 hydration products [51,52]. Therefore, fresh solutions of ~4 mg/mL (~4 mM) were used for all NMR measurements. Suppression of the HOD signal was achieved by applying a WEFT (water eliminated Fourier transform) pulse sequence for 1D NMR experiments and by a pre-saturation 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.) [53] mixing sequence with spin-lock times of 20, 50, 100 and 200 ms. The 2D 1H-1H 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 13C-1H HSQC experiments (1H frequency 500.0821 MHz, 13C frequency 125.7552 MHz) were recorded without decoupling during acquisition of the 1H 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).

Acknowledgements

This work was financially supported by EU project NOVOSIDES FP7-KBBE-2010-4–265854 (LD).

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

[1] J.E. Brandle, A.N. Starratt, M. Gijzen, Can. J. Plant Sci. 76 (1998) 527–536.
[2] S. Ceunen, J.M.C. Geuns, J. Nat. Prod. 76 (2013) 1201–1228.
[3] G.J. Gerwig, E.M. te Poel, L. Dijkhuizen, J.P. Kamerling, Anal. Biochem. 273 (2000) 1–72.
[4] J.M.C. Geuns, Photochemistry 64 (2003) 913–921.
[5] J.M.C. Geuns, Phytochemistry 64 (2003) 913–921.
[6] S. Madan, S. Ahmad, G.N. Singh, K. Kohli, Y. Kumar, R. Singh, M. Garg, Indian J. Nat. Prod. Resour. 1 (2010) 267–286.
[7] S. Puru, D. Sharma, A.K. Tiwari, Biotechnol. Adv. 29 (2011) 781–791.
[8] E. Gupta, S. Purwar, S. Sundaram, G.K. Rai, J. Med. Plants Res. 7 (2013) 3343–3353.
[9] R.S. McQuate, Food Technol. 65 (2011) 6–13, 51.
[10] European food safety authority, EFSA J. 8 (1537) (2010) 1–84.
[11] EU Commission, Regulation (EU) No. 1131/2011, Off. J. Eur. Union L295 (2011) 205–211.
[12] G. Brahmacari, L.C. Mandal, R. Roy, S. Mondal, A.K. Brahmacari, Arch. Pharm. Chem. Life Sci. 1 (2011) 5–19.
[13] R. Lemus-Mondaca, A. Vega-Galvez, L. Zura-Bravo, K. Ah-Hen, Food Chem. 132 (2012) 1112–1132.
[14] S.K. Yadav, P. Gulera, Crit. Rev. Food Sci. Nutr. 52 (2012) 988–998.
[15] N. Shivanna, M. Naika, F. Khanum, V.K. Kaul, J. Diabetes Complicat. 27 (2013) 103–113.
[16] M. Dariset, K. Mizutani, R. Kasai, O. Tanaka, S. Kitahata, O. Sakada, S. Ogawa, F. Murakami, F.-H. Chen, Agric. Biol. Chem. 48 (1984) 2483–2488.
[17] U. Mani, G. Dubois, I. Prakash, Molecules 17 (2012) 4186–4196.
[18] G.E. Dubois, R.A. Stephenson, J. Med. Chem. 28 (1985) 93–98.
[19] Y. Fukunaga, T. Miyata, N. Nakayasu, K. Mizutani, R. Kasai, O. Tanaka, Agric. Biol. Chem. 53 (1989) 1603–1607.
[20] S.V. Lobov, R. Kasai, K. Ohtani, O. Tanaka, K. Yamashita, Agric. Biol. Chem. 55 (1991) 2959–2965.
[21] K. Ohtani, Y. Akawka, Y. Fujisawa, R. Kasai, O. Tanaka, K. Yamashita, Agric. Chem. Bull. 39 (1991) 3172–3174.
[22] K. Ohtani, Y. Akawka, H. Ishikawa, R. Kasai, S. Kitahata, K. Mizutani, S. Doi, M. Nakaura, O. Tanaka, Agric. Biol. Chem. 55 (1991) 449–453.
[23] O. Tanaka, Pure Appl. Chem. 69 (1997) 675–683.
[24] F. Ye, R. Yang, X. Hua, Q. Shen, W. Zhao, W. Zhang, IWT – Food Sci. Technol. 51 (2015) 524–530.
[25] I. Prakash, A. Markosyan, C. Bunders, Foods 3 (2014) 162–175.
[26] H. Leennhuis, T. Pijnjng, J.M. Dobruchowska, S.S. van Leeuwen, S. Král, B.W. Dijkstra, L. Dijkhuizen, J. Biotechnol. 163 (2013) 250–272.
[27] T. Desmet, W. Soetaert, P. Bojarov, V. Kren, L. Dijkhuizen, L. Dijkhuizen, L. Dijkhuizen, J. Agric. Chem. 49 (1989) 1607.
[28] S.S. van Leeuwen, S. Král, V. Kren, L. Dijkhuizen, J. Agric. Chem. 49 (1989) 1607.
