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How to broaden the applicability of the high potency sweeteners steviol glycosides: from enzymatic glycosylation to recombinant production

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Abstract

Because of the increasing prevalence of obesity, cardiovascular diseases and type-2-diabetes and the rising consumer demand for natural food ingredients, the natural high potency sweeteners steviol glycosides have a great potential in the production of a wide range of zero and reduced calorie food products and beverages. Steviol glycosides have a sweetness between 50 and 450 times greater than that of sucrose, are non-caloric and do not induce a glycaemic response. However, besides sweetness, almost half of the human population experiences an unpleasant bitter aftertaste of steviol glycosides. This perception of bitterness is caused by genetic variation in the two involved human bitter taste receptors. Therefore, there is a need to improve the organoleptic properties of steviol glycosides. Recent studies show a general interest in enzymatic glycosylation of stevioside, the most abundant steviol glycoside, in order to improve its organoleptic properties. Enzymatic glycosylation of stevioside is already reported successful in protocols using several different enzyme-glycosyl-donor combinations, including cyclodextrin glucanotransferase (CGTase) with either soluble starch or cyclodextrin, Biozyme L and maltose, α -amylase and starch or pullulanase and pullan. Literature searches showed that six different glycosylated steviosides have been reported to comprise improved organoleptic properties. However, only a minor fraction of studies on enzymatic glycosylation of stevioside determined the chemical structures of the obtained products and human sensory evaluations were seldom included. Future research has to explore whether the extent of reduced bitterness of glycosylated steviosides compared to stevioside is enough to make stevioside applicable for a larger part of the human population. In addition, future research should give insight in the extent of calorie increase as well. However, there could also be a need to adopt a new approach. Since 2011, eight patents describing recombinant production of steviol glycosides have been published. In the most recent patent application, it is stated that recombinant production of the minor steviol glycoside rebaudioside M, which comprises better organoleptic properties than stevioside, could be highly cost effective. However, it remains to be seen if this promise will be fulfilled and whether consumers will accept recombinantly produced sweeteners.

Keywords: *high potency sweeteners, steviol glycosides, enzymatic glycosylation, recombinant production*

Introduction

In Paraguay, the leaves of the plant *Stevia rebaudiana* Bertoni have already a centuries-old history of use as a sweetener and medicine (Risso et al., 2014). Around the 80s, as a response to increasing health awareness, the utility of this plant came also apparent in other parts of the world (Brandle et al., 1998; SCF Scientific Committee on Food, 1984). The sweetness of the leaves arises from the presence of natural high potency sweeteners, the steviol glycosides. These glycosides are non-caloric and do not induce a glycaemic response. In addition, they seem also to comprise valuable potential health benefits, such as decreasing the blood sugar level, inhibiting atherosclerosis, anti-hyperglycemic, anti-hypertensive, anti-inflammatory, anti-cancer and

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immune stimulating (Li et al., 2013). For these reasons, steviol glycosides have a great potential in the production of a wide range of zero and reduced calorie food products and beverages. In this way, steviol glycosides could be useful in the fight against the current high prevalence of obesity, cardiovascular diseases and type-2-diabetes. Furthermore, it will also answer the rising consumer demand for natural food ingredients (Hellfritsch et al., 2012).

Steviol glycosides have a sweetness between 50 and 450 times greater than that of sucrose, depending on the steviol glycoside and the sensory protocol applied (Li et al., 2012; O'Donnel & Kearsley, 2012; Risso et al., 2014; Gasmalla et al., 2014). Since the recent approval of purified steviol glycosides for consumption in the United States and the European Union (Risso et al., 2014), it is permitted to use steviol glycosides in various food products such as biscuits, jams, chocolates, ice-creams, baked foods, soft drinks and fruit drinks, sauces, sweet corn, yoghurt, soya products and as dietary supplements and table-top sweeteners (Gasmalla et al., 2014). The major suppliers in the stevia sectors are the companies Cargill (www.cargill.com) and PureCircle (www.purecircle.com) and their greatest success to date has been achieved within the soft drinks market (Thomas., 2012). However, the application of steviol glycosides is limited. Besides sweetness, almost half of the human population experiences an unpleasant bitter aftertaste of steviol glycosides. The ability and the intensity of this bitter aftertaste arises from genetic variation in the two bitter taste receptors that mediate this taste, the human taste receptors type 2 member 4 and member 14, hTAS2R4 and hTAS2R14 (Hellfritsch et al., 2012; Risso et al., 2014).

For this reason, the demand to improve the organoleptic properties of steviol glycosides is rising. Currently, in many steviol glycosides containing products, the sugar maltodextrin is added in order to compensate the bitter aftertaste. In 1984 Dubois et al. found that the bitter taste of steviol glycosides may be eliminated by increasing their molecular hydrophilic character through chemical substitution of polar groups (Dubois et al., 1984). Nowadays, several studies have shown that enzymatic glycosylation eliminates the bitter aftertaste as well (Lobov et al., 1991, Yamamoto et al., 1994, Abelian et al., 2004, Kochikian et al., 2006, Jung et al., 2007, Jaitak et al., 2009, Li et al., 2012, Ye et al., 2013; Musa et al., 2014; Lu et al., 2014 and Ye et al., 2014). Besides, Cargill, in collaboration with the Swiss biotechnology company Evolva (www.evolva.com), has recently published a patent application on the production of steviol glycosides via microbial fermentation. This approach potentially allows highly cost-effective production of desired steviol glycosides.

In this report, recent innovations and their challenges concerning enzymatic glycosylation of steviol glycosides are covered. Furthermore, by means of their comparison, suggestions for future research were made. In addition, the strategy to improve the utility of steviol glycosides by recombinant production is also covered. Hence, the aim of this report was to consider the potential approaches for improving the applicability of steviol glycosides.

Chemical structures of steviol glycosides and their relation with bitter aftertastes

Many different steviol glycosides have been detected from different cultivars. As of October 2014, at least 38 steviol glycosides of *Stevia rebaudiana* Bertoni have been identified (Ceunen et al., 2013; Montoro et al., 2014; Ibrahim et al., 2014). These compounds are all glycosides of the diterpenoid skeleton *ent*-13-hydroxyl kaur-16-en-19-oic acid, known as steviol (figure 1) (Brandle et al., 1998). The steviol glycoside stevioside is the most abundant compound of the steviol glycosides. The most common composition of steviol glycosides of the wild variety is stevioside (5–10%), rebaudiosides A (2–5%) and C (1%), dulcoside A (0.5%), rebaudiosides D, E, and F (0.2%), and steviolbioside (0.1%), which are listed in table 1 (Ceunen et al., 2013).

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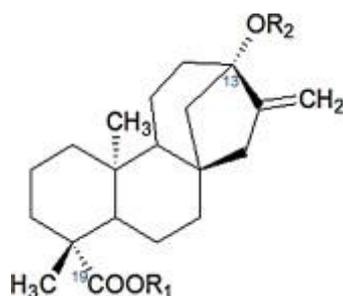


Figure 1. The diterpenoid skeleton of steviol glycosides, named steviol. The positions of C13 and C19 are indicated in the figure. R₁ and R₂ indicate separate glycone chains of the different steviol glycosides.

The glycosylation of steviol at both C13 and C19 is essential for the intensity of the sweetness of a steviol glycoside. Both the separate R₁ and R₂ glycone lengths and the number of total β-glycosyl residues correlate with sweetness. This is demonstrated by human sensory studies performed with fifteen trained tasters biased by selecting subjects who were sensitive to the bitter taste of the minor steviol glycoside rubusoside (Hellfritsch et al., 2012). They found an increased sweetness of rebaudioside D in comparison with the other steviol glycosides and an increased sweetness of rebaudioside A compared to stevioside (table 1). Replacing glucose by rhamnose reduces the sweet taste of steviol glycosides, which was demonstrated by the reduced sweetness of dulcoside A compared with stevioside and the reduced sweetness of rebaudioside C compared with rebaudioside A (table 1) (Hellfritsch et al., 2012). In table 1, the sweetness of the steviol glycosides is ranked from sweetest to less sweet (1-6). Rebaudioside E and rebaudioside F are not included in the ranking due to difficulties in their purification (Hellfritsch et al., 2012).

Table 1. The most common composition of the wild variety of the *Stevia rebaudiana* Bertoni leaf (Ceunen et al., 2013).

	Composition ^a	R ₁ (C19)	R ₂ (C13)	Formula	Mass ^b	Rank of sweetness ^c
Steviol	-	H	H	C ₂₀ H ₃₀ O ₃	318.22	ND
Stevioside	5-10%	Glcβ1-	Glcβ(1-2) Glcβ1-	C ₃₈ H ₆₀ O ₁₈	804.38	3
Rebaudioside A	2-5%	Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	C ₄₄ H ₇₀ O ₂₃	966.43	2
Rebaudioside C	1%	Glcβ1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	C ₄₄ H ₇₀ O ₁₇	950.44	4
Dulcoside A	0.5%	Glcβ1-	Rhaα(1-2) Glcβ1-	C ₃₈ H ₆₀ O ₁₇	788.38	5
Rebaudioside D	0.2%	Glcβ(1-2) Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	C ₅₀ H ₈₀ O ₂₈	1128.48	1
Rebaudioside E	0.2%	Glcβ(1-2) Glcβ1-	Glcβ(1-2) Glcβ1-	C ₄₄ H ₇₀ O ₂₃	966.43	ND
Rebaudioside F	0.2%	Glcβ1-	Xylβ(1-2)[Glcβ(1-3)]Glcβ1-	C ₄₃ H ₆₈ O ₂₂	936.42	ND
Steviolbioside	0.1%	H	Glcβ(1-2) Glcβ1-	C ₃₂ H ₅₀ O ₁₃	642.33	6

R₁ and R₂ refer to Figure 1. Abbreviations: Glc, D-glucopyranosyl (glucose); Rha, L-rhamnopyranosyl (rhamnose); Xyl, D-xylopyranosyl (xylose); ND, not determined. ^a Calculated on a dry weight basis (w/w). ^b Exact mass in Dalton (Ceunen et al., 2013). ^c The relative rank of sweetness from sweetest to less sweet (1-6) according to the study of Hellfritsch et al. (Hellfritsch et al., 2012).

The bitter taste of steviol glycosides is also correlated with the total number of β-glycosyl residues. In the study of Hellfritsch et al. steviol glycosides bearing few β-glycosides, such as steviolbioside, showed lower bitter threshold values in vivo and higher bitter intensities compared to the glycosides with many β-glycosyl residues, such as rebaudioside D. In agreement, the weakest bitter taste was elicited by rebaudioside D. However, the correlation between structure and bitterness is less evident in comparison with the correlation between structure and sweetness intensity (Hellfritsch et al., 2012).

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respectively, according to the 1000 genomes project phase 1 (dbSNP, 2014). Risso et al. showed that this hTAS2R4 SNP is specifically associated with the ability to perceive the bitter aftertaste, while this hTAS2R14 SNP is associated with the perceived intensity of bitterness (Risso et al., 2014).

The G allele of this hTAS2R4 SNP causes an amino acid substitution at residue 96 (figure 2), resulting in a valine to leucine change, while the G allele of the hTAS2R14 SNP is a synonymous substitution, it does not cause an amino acid substitution. Both variations do not alter the secondary structure of the receptors. However, while variation of the hTAS2R14 SNP was not linked with any protein change, the hTAS2R4 SNP is found to be often co-inherited with two other mutations. These two mutations are closely located to the hTAS2R4 SNP on the same chromosome, which reduces the change of recombination between these mutations. The two mutations are found in the haplotype map of the human genome corresponding to northern and western Europeans (CEU) and are located in the hTAS2R4 gene and in the gene of hTAS2R5, known as rs2233998 and rs2227264 respectively (Risso et al., 2014; dbSNP, 2014). Both mutations are found to be associated with eating behaviour (Dotson et al., 2010) and besides, this hTAS2R5 SNP has been proved to be related to the perception of bitterness of espresso coffee (Hayes et al., 2011; Risso et al., 2014). Importantly, in contrast to the hTAS2R4 SNP rs2234001, the hTAS2R4 SNP rs2233998 is likely to alter the hTAS2R4 receptor. It causes an amino acid change of residue 7 (figure 2), in which a phenylalanine residue is substituted into a serine residue (dbSNP, 2014). This substitution of residue 7 results in the replacement of a phenyl-group by a hydroxyl-group. Therefore, together with the fact that residue 7 is located in one of the transmembrane domains, a possible scenario to correlate this polymorphism to the capability of perceiving the bitter aftertaste of steviol glycosides, is that this mutation changes the characteristics of the hTAS2R4 binding pocket.

Enzymatic glycosylation of steviol glycosides

In order to eliminate the bitter aftertaste of steviol glycosides, increasing the lengths of the glycone chains of the steviol glycosides by enzymatic transglycosylation is a promising approach. Enzymatic transglycosylation has been a commonly used safe and green methodology in both food and medicine chemistry in order to make glycosyl acceptors more hydrophilic or give them a different taste (Lu et al., 2014). For enzymatic glycosylation, different enzyme classes can be used including Leloir glycosyl transferases, trans-glycosidases, glycoside phosphorylases and glycoside hydrolases (Desmet et al., 2012). Furthermore, a broad-range of glycosyl donors can be used. Enzyme and glycosyl donor combinations that were already effective for glycosylation of stevioside, the most abundant steviol glycoside, include alternansucrase with sucrose (Musa et al., 2014), pullulanase with pullan, Biozyme L with maltose (Lobov et al., 1991), α -amylase with starch (Ye et al., 2013; Ye et al., 2014), dextrin dextranase (DDase) with maltodextrin (Yamamoto et al., 1994) and cyclodextrin glucanotransferase (CGTase) with either maltodextrin, cyclodextrin or starch (Abelian et al., 2004; Kochikian et al., 2006; Jung et al., 2007; Jaitak et al., 2009; Li et al., 2012; Lu et al., 2014)

The highest stevioside conversion reported reached 90%. This was obtained using microwave accelerated transglycosylation (MAR) with a CGTase produced from an alkalophilic strain of *Bacillus firmus* in combination with the glycosyl donor cyclodextrin. MAR includes enzymatic transglycosylation under microwave-assisted irradiation, which means that the reaction mixture was held in a microwave oven at 80 W and 50 °C for 1 min (Jaitak et al., 2009). The highest stevioside conversion reported carried out by traditional techniques reached 77.11% using a commercial CGTase and corn starch hydrolysate (Li et al., 2013). Another high conversion of 76% was obtained using a CGTase from alkalophilic *Bacillus sp. BL-12* with the glycosyl donor maltodextrin (Jung et al., 2007). In agreement, Lu et al found that CGTase with either gelatinized or hydrolysed starches or cyclodextrin as glycosyl donors presented all similar high transglycosylation activities to stevioside. In

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contrast, reported yields of the other enzymatic glycosylation couples are relatively low: they vary between 38.3% and 48.2%. Therefore, CGTase with either maltodextrin, cyclodextrin or starches are considered as the most effective glycosyl donor and enzyme combinations for the transglycosylation of stevioside (Lu et al., 2014; Li et al., 2013).

In most studies of enzymatic glycosylation of stevioside, Kochikian et al., 2006; Jung et al., 2007; Li et al., 2013; Lu et al., 2014 and Ye et al., 2014, determination of the composition of the obtained products was lacking. In those studies molecular weights were determined in order to determine the extent of glycosylation of the products. However, the extent of glycosylation is not sufficient to establish whether an improved product is formed. The organoleptic properties are determined by both regioselectivity and linkage specificity. Through identification of CGTase glycosylated steviosides by proton and carbon nuclear magnetic resonance spectroscopy (^1H and ^{13}C NMR), Fukunaga et al. found that both mono- and di-trans- α -1-4-glycosylation of stevioside at the C13-position (R^1 in figure 3), gave highly improved products with remarkable improvement in both the intensity and quality of the sweetness (compounds 1 and 12) (table 2). However, in contrast, both mono- and di-trans- α -1-4-glycosylation at the C19 position (R^2 in figure 3), compounds 2 and 11, deteriorate the sweetness: both gave an increased bitter aftertaste and a lower sweetness intensity (Fukunaga et al., 1989). The fact that determination of the extent of glycosylation is not sufficient is further emphasized by the fact that, in contrast to mono- α -1-4-glycosylation at the C19 position, both mono- α -glycosylation of the C6 hydroxyl group and the C2 hydroxyl group at the C19-position, obtained by using the commercial enzyme Biozyme L and maltose and α -amylase and starch respectively, led to a remarkable improvement in the quality of taste (compounds 4 and 6) (Lobov et al., 1991; Ye et al., 2013). In turn, mono- α -1-3-glycosylation at the C19 position, obtained by using Biozyme L and maltose, did increase the bitter aftertaste (compound 5). Biozyme L is classified as a β -amylase which produces maltose from amylose. The unexpected α -1-6 and α -1-3-glycosylation might have been caused by α -glucosidase contaminating this commercial enzyme (Lobov et al., 1991). Furthermore, in contrast to di- α -1-4-glycosylation at the C19 position, di- α -1-2- α -1-4-glycosylation at the C19 position, obtained by using the commercial α -amylase BAN 480L and starch, led to both improvement of taste and an increase in sweetness (Ye et al., 2013). Other determined structures that were tested on taste include mostly tri-glycosylated steviosides which were not remarkably changed according to their sweetness or quality of taste (Fukunaga et al., 1989) (table 2). This suggested that highly substituted steviosides do not improve the quality of taste. However, neither structure determination studies nor taste studies were performed on tetra- and penta- glycosylated steviosides. However, both tetra- and penta- glycosylation of stevioside have been observed as well (Li et al., 2013; Lu et al., 2014).

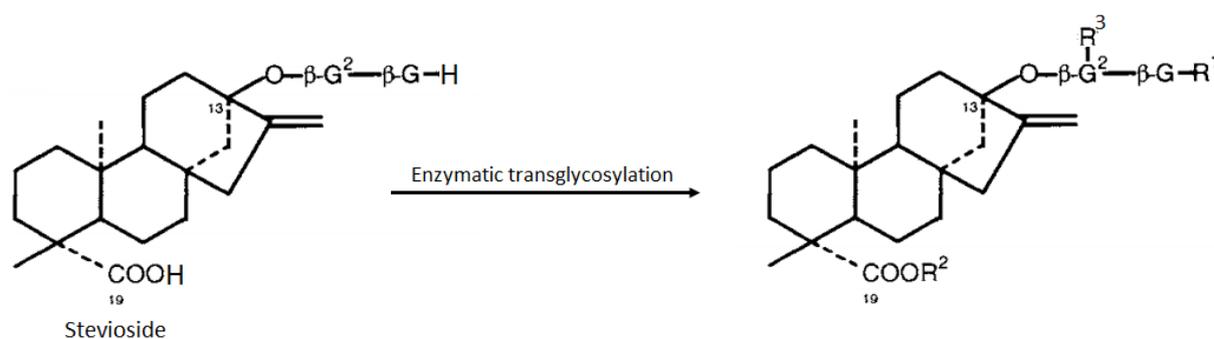


Figure 3. Structure of stevioside before and after enzymatic transglycosylation. See table 2 for possible substitutions of R^1 , R^2 and R^3 . Figure adapted from Lobov et al., 1991.

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Table 2. Reported structures of glycosylated stevioside by using different methods of enzymatic glycosylation

Identified compounds	R ¹ (C13-position)	R ² (C19-position)	R ³ (C13 position)	Enzym/donor combination ^a	Bitter aftertaste
1	α -1-4-glc	H	H	1, 2, and 3a	Reduced ^c
2	H	α -1-4-glc	H	1	Bitter aftertaste ^c
3	α -1-6-glc	H	H	3b, 4 and 6	ND
4	H	α -1-6-glc	H	3b	Reduced ^d
5	H	α -1-3-glc	H	3b	Bitter ^d
6	H	α -1-2-glc	H	5	Reduced ^e
7	H	α -1-2-glc- α -1-4-glc	H	5	Reduced ^e
8	α -1-6- α -1-3-glc	H	H	6	ND
9 ^b	H	H	α -1-6-glc	4	ND
10 ^b	α -1-6-glc	H	α -1-6-glc	4	ND
11	H	α -1-4-glc- α -1-4-glc	H	1, 2 and 3a	Bitter ^c
12	α -1-4-glc- α -1-4-glc	H	H	1 and 3a	Reduced ^c
13 ^b	α -1-6-glc	H	α -1-6-glc	4	ND
14	H	α -1-4-glc- α -1-4-glc- α -1-4-glc	H	1	Slightly reduced ^c
15	α -1-4 glc α -1-4-glc- α -1-4-glc	H	H	1	Slightly reduced ^c
16	α -1-4-glc	α -1-4-glc	H	1	Bad aftertaste ^c
17	α -1-4-glc	α -1-4-glc- α -1-4-glc	H	1	Bad aftertaste ^c
18	α -1-4-glc- α -1-4-glc	α -1-4-glc	H	1	Bad aftertaste ^c

R¹, R² and R³ refer to Figure 3. Abbreviations: Glc, D-glucopyranosyl (glucose); ND. Not determined. **a** These structures were obtained using **(1)** CGTase and soluble starch (Fukunaga et al., 1989); **(2)** CGTase and cyclodextrin (Jaitak et al., 2009); **(3a)** pullulanase and pullan (Lobov et al., 1991) **(3b)** Biozyme L and maltose (Lobov et al.,1991); **(4)** DDase and maltodextrin (Yamatomo et al., 1994) **(5)** α -amylase and starch (Ye et al., 2013) **(6)** Alternansucrase and sucrose (Musa et al., 2014) **b** Improvement of taste was not examined. Quality of taste according to human sensory studies performed by **c** Fukunaga et al., 1989 **d** Lobov et al., 1991 and **e** Ye et al., 2013.

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It should be emphasized that human sensory experiments of glycosylated steviosides were performed in different manners, which makes the results not readily comparable. Furthermore, in the studies of Fukunaga et al. and Yamamoto et al. taste panels contained only five and six tasters respectively (Fukunaga et al., 1989; Yamamoto et al., 1994). The human sensory experiment by Ye et al. was performed with a larger taste panel of seventeen tasters (Ye et al., 2013). Besides, in none of these human sensory experiments, taste panels were beforehand tested on the ability to perceive the bitter aftertaste of stevioside and, as a matter of course, not tested on the hTAS2R4 and hTAS2R14 polymorphisms associated with perception of this bitter aftertaste. However, according to the results, mono- and di-trans- α -1-4-glycosylation of stevioside specific at the C13-position and mono- α -1-6-glycosylation, mono- α -1-2-glycosylation and di- α -1-2- α -1-4-glycosylation at the C19 position provide the most promising products (figure 4). These products comprise compounds 1, 4, 6, 7 and 12 of table 2 and resulted from either CGTase and soluble starch or cyclodextrin, Biozyme L and maltose, α -amylase and starch or pullulanase and pullan (table 2). Unfortunately, quality of taste of both mono- α -1-6-glycosylation at the C13 position at R¹ and R³ was not examined, while, in view of the similarities with the improved products, these compounds are promising as well.

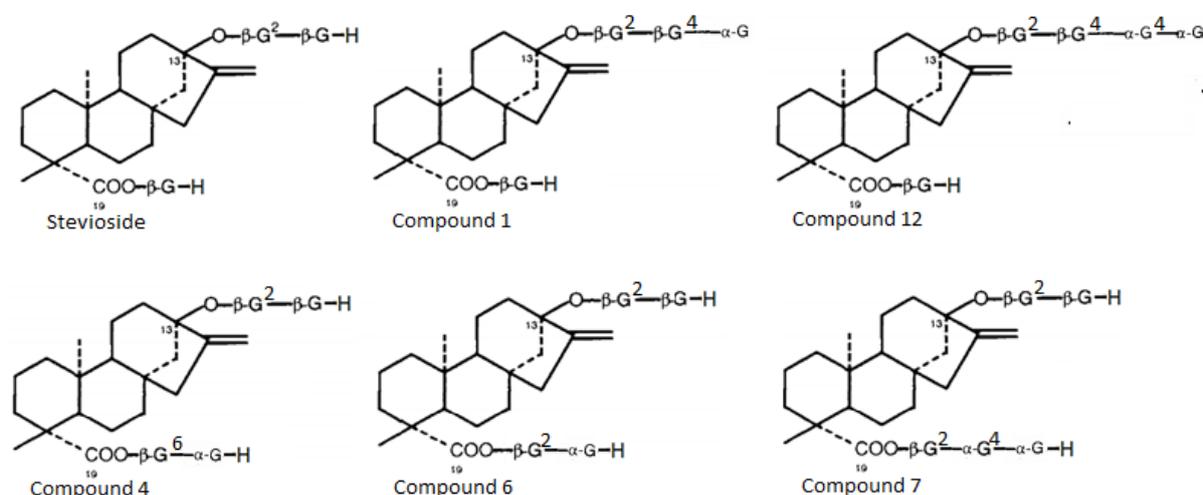


Figure 4. Stevioside and the promising glycosylated steviosides that are shown to have a significant reduced bitter aftertaste. The compound numbers refer to table 2.

Advantageously, in all enzymatic glycosylation studies of stevioside a very poor yield of highly substituted steviosides was found. The two improved products obtained by using α -amylase and starch, compounds 6 and 7, accounted even for 96 g of the 100 g of the total trans-glycosylated steviosides (Ye et al., 2014). This means, even though the transglycosylation yield was only 38.3%, the yield of actual improved steviosides was 36.8%. In the glycosylation studies of stevioside with CGTase, mono- and di-glycosylated steviosides were the main products as well. In the study of Jaitak et al, using MAR, the yield of the promising compound 1, mono-trans- α -1-4-glycosylated stevioside, was as high as 66%. However, unfortunately, the yield of possibly improved steviosides of other CGTase studies comprising compounds 1, 2 and 11, are not reported. Glycosylation with DDase and maltodextrin and pullulanase and pullan gave also mono- and di-glycosylated steviosides as main products (Yamamoto et al., 1994). In contrast, Biozyme L and maltose provided a relatively high amount of tri-glycosylated steviosides. The poor yield of highly substituted steviosides might be caused by hydrolysis of glycosylated steviol by the enzymes themselves (Li et al., 2013).

The obtained glycosylated steviosides are significantly different from the naturally occurring steviol glycosides. While the naturally occurring steviol glycosides contain only β -glycosyl residues, the

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enzymes used for glycosylation of stevioside introduce α -glycosyl residues. Moreover, taken stevioside as starting point, naturally occurring steviol glycosides contain specifically β -1-2-glycosidic linkages at the C19 position and β -1-3-glycosidic linkages at the R³ C13 position (table 1 and figure 3), while the obtained glycosylated steviosides contain mainly α -1-4- or α -1-6-glycosidic linkages at the C19 or the R² C13 position (table 2). If we restrict ourselves to the improved products of figure 4 and compare them with the naturally occurring steviol glycosides, compound 6, mono- α -1-2-glycosylated stevioside, comes closest to the natural occurring steviol glycosides. It is similar to rebaudioside E, which is a mono- β -1-2-glycosylated version of stevioside (figure 5). Both rebaudioside E and compound 6 have a reduced bitter aftertaste compared to stevioside (Ye et al., 2013). Moreover, both compounds have a similar sweetness as stevioside. Apparently, the difference in stereochemistry of the glycosidic bond does not influence sweet and bitter taste reception to a great extent. However, the extent of reduced bitterness may not be comparable due to the use of different human sensory methods.

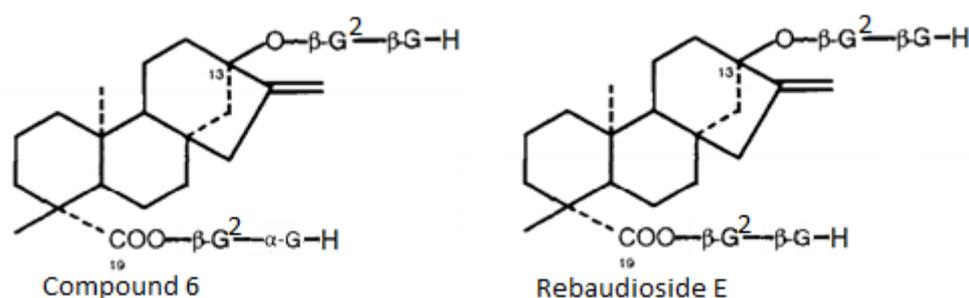


Figure 5. The structures of the promising compound 6 and the steviol glycoside rebaudioside E. Both compounds comprise better organoleptic properties than stevioside.

Importantly, the characteristic of steviol glycosides to be non-caloric is due to the fact that β -glycosidic linkages are resistant to hydrolysis by human digestive enzymes. In contrast, α -1-4-glycosidic linkages are hydrolysed by human digestive enzymes. Trans-glycosylation of steviol glycosides by for instance CGTase will therefore increase the caloric content of the steviol glycosides, which will not answer the consumers demand for low and zero calorie food products. In order to answer this demand, introduction of α -amylase resistant glycosidic linkages, such as α -1,2 and α -1,6, observed in compounds 4 and 6 (figure 4), are therefore more desirable (Leemhuis et al., 2014). Besides, introduction of β -glycosyl residues by using glycosylation enzymes involved in the biosynthesis of steviol glycosides could also be a possibility to avoid calorie increase. In vivo glycosylation reactions of steviol glycosides are catalysed by cytosolic UDP-dependent glycosyl transferases (UGTs), which belong to the class of Leloir glycosyl transferases. Prakash et al. showed already the conversion of rebaudioside A to rebaudioside D (table 1) by the glycosyl transferase UGTSL2. Besides rebaudioside D, two other steviol glycosides were obtained as minor products, named rebaudioside D2 and M2 (figure 6A). Furthermore, using the glycosyl transferase UGT76G1, rebaudioside I, a minor natural steviol glycoside, was produced from rebaudioside A as well (figure 6B) (Prakash et al., 2014).

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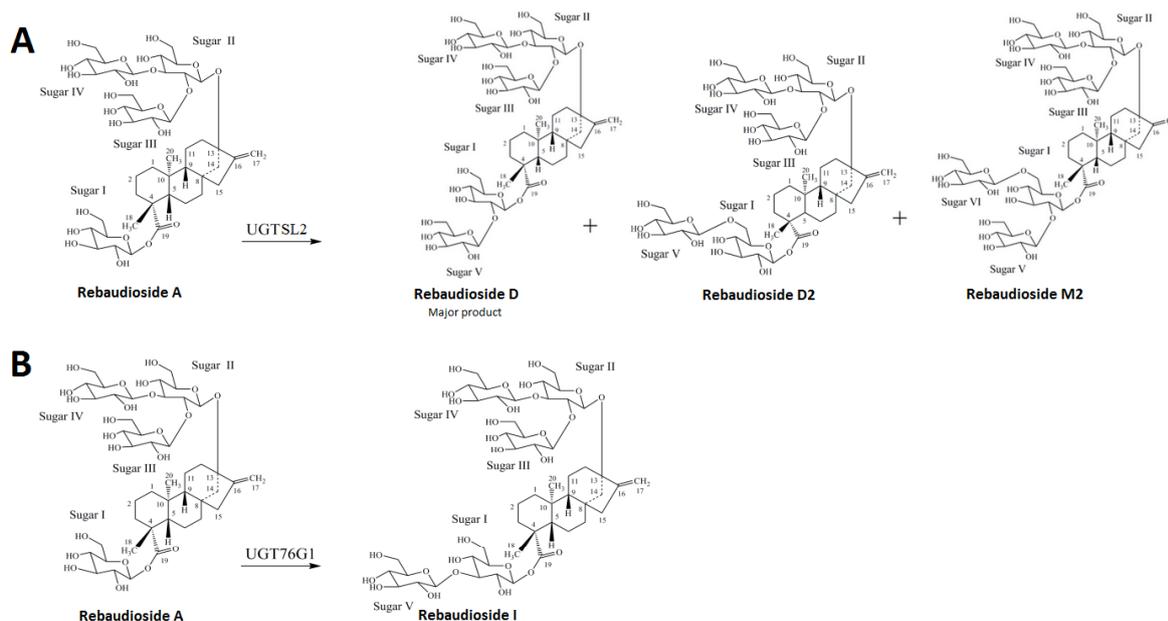


Figure 6. Bioconversions of rebaudioside A to rebaudioside D, D2, M2 (**A**) and I (**B**) using the UDP-dependent glycosyl transferases UGTSL2 and UGT76G1 respectively. Figure adapted from Prakash et al., 2014.

Biosynthesis of steviol β -glycosides

Steviol glycosides are derived from the non-mevalonate pathway or methylerythritol 4-phosphate pathway (MEP pathway). In *Stevia rebaudiana* Bertoni, this metabolic route is catalysed by a subset of seven enzymes and leads to the C5 building block molecule, isopentenyl pyrophosphate (IPP), which is required for the synthesis of the diterpenoid skeleton steviol (Ceunen et al., 2013). A simplified model of this metabolic route leading to IPP is represented in figure 7, including the required metabolites, pyruvate and glyceraldehyde-3-phosphate, several enzymes, co-enzyme thiamine pyrophosphate (TPP) and other co-factors (figure 7).

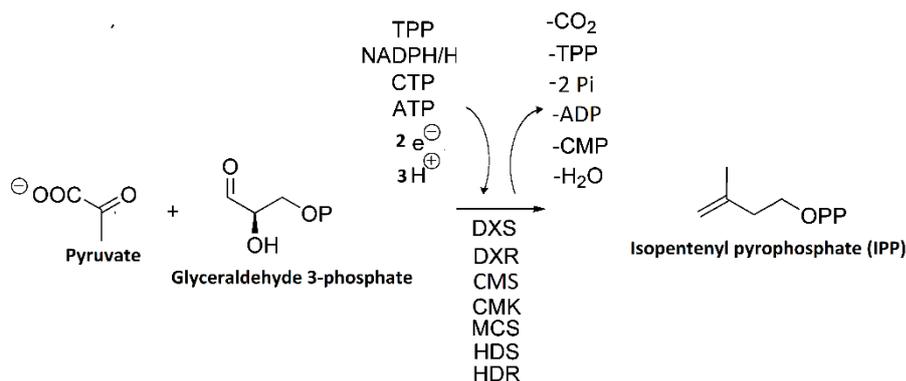


Figure 7. Methyl-Erythritol-4-Phosphate Pathway leading to Isopentenyl Pyrophosphate (IPP). Abbreviations: **DXS**: 1-deoxy-d-xylulose 5-phosphate synthase; **DXR**: 1-deoxy-d-xylulose 5-phosphate reductoisomerase; **CMS**: 4-diphosphocytidyl-2-C-methyl-d-erythritol synthase; **CMK**: 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase; **MCS**: 2-C-methyl-d-erythritol 2,4-cyclopyrophosphate synthase; **HDS**: (E)-4-hydroxy-3-methylbut-2-enyl pyrophosphate synthase; **HDR**: (E)-4-hydroxy-3-methylbut-2-enyl pyrophosphate reductase; **TPP**: co-enzyme thiamine pyrophosphate; **NADH**: Reduced nicotinamide adenine dinucleotide (NAD). **CTP**: cytidine triphosphate; **ATP**: adenine triphosphate; **ADP**: adenine diphosphate. **CMP**: cytidine monophosphate; **Pi**: inorganic phosphate.

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The building block of steviol, trans-geranylgeranyl pyrophosphate, consists of four units of IPP (figure 8). Each unit is attached to another by a series of electrophilic additions, involving a stereospecific loss of a proton. The final of such additions is catalysed by the enzyme trans-geranylgeranyl pyrophosphate synthase (GPPS) (Ceunen et al., 2013). The enzymes that are subsequently involved in the biosynthesis of steviol include *ent*-copalyl pyrophosphate synthase (*ent*-CPS), *ent*-kaurene synthase (*ent*-KS), *ent*-kaurene oxidase (*ent*-KO) and *ent*-kaurenoic acid 13-hydroxylase (*ent*-KAH) (figure 8).

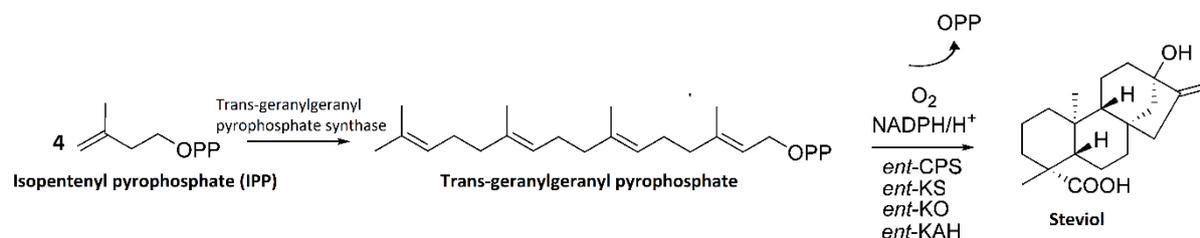


Figure 8. Biosynthetic pathway from IPP to steviol. Abbreviations: **OPP**: pyrophosphate; ***ent*-CPS**: *ent*-copalyl pyrophosphate synthase; ***ent*-KS**: *ent*-kaurene synthase; ***ent*-KO**: *ent*-kaurene oxidase; ***ent*-KAH**: *ent*-kaurenoic acid 13-hydroxylase.

After the formation of steviol, a series of glycosylation reactions take place, leading to the large family of steviol glycosides (Ceunen et al., 2013). As mentioned above, these reactions are catalysed by cytosolic UDP-dependent glycosyl transferases (UGTs), which belong to the class of Leloir glycosyl transferases. As sugar donor, UGTs use UDP-glucose, which is a glucose molecule coupled to a uridine diphosphate and is synthesised by the enzyme UDP-glucose pyrophosphorylase. Plant UGTs are known to exert a broad substrate specificity. In *Stevia rebaudiana* Bertoni, more than a dozen UGTs have been detected, yet only three have so far been clearly shown to contribute to steviol glycoside biosynthesis, including the glycosyl transferases UGT85C2, UGT74G1, UGT76G1 (Ceunen et al., 2013). However, three other UGTs also might play a role. These include two UGTs having 98% sequence similarity with UGT76G1 of which one, named UGTSr, showed a similar glycosylating activity towards stevioside as UGT76G1 (Madhav et al., 2013) and the glycosyl transferase UGT91D2, which is a candidate UGT for the glycosylation of steviolmonoside leading to steviolbioside (figure 9), because this glycosylation reaction has been shown in vitro (WO 2014122227; Ceunen et al., 2013).

Natural glycosylation of steviol preferentially begins at the C13 hydroxyl group, producing steviolmonoside in a reaction catalysed by the glycosyl transferase UGT85C2 (figure 9). Next, steviolmonoside is glycosylated at the C2 hydroxyl of its C13 glucose moiety to form steviolbioside. The UGT involved in this step is not yet characterized, but as previously mentioned, UGT91D2 is reported as a candidate for this reaction. Glycosylation of the C4 carboxylic acid moiety of steviolbioside yields stevioside, which is catalysed by UGT74G1. A UGT76G1-catalyzed glycosylation of the C3 hydroxyl group of the C13 glucose takes place to form rebaudioside A (figure 9) (Ceunen et al., 2013).

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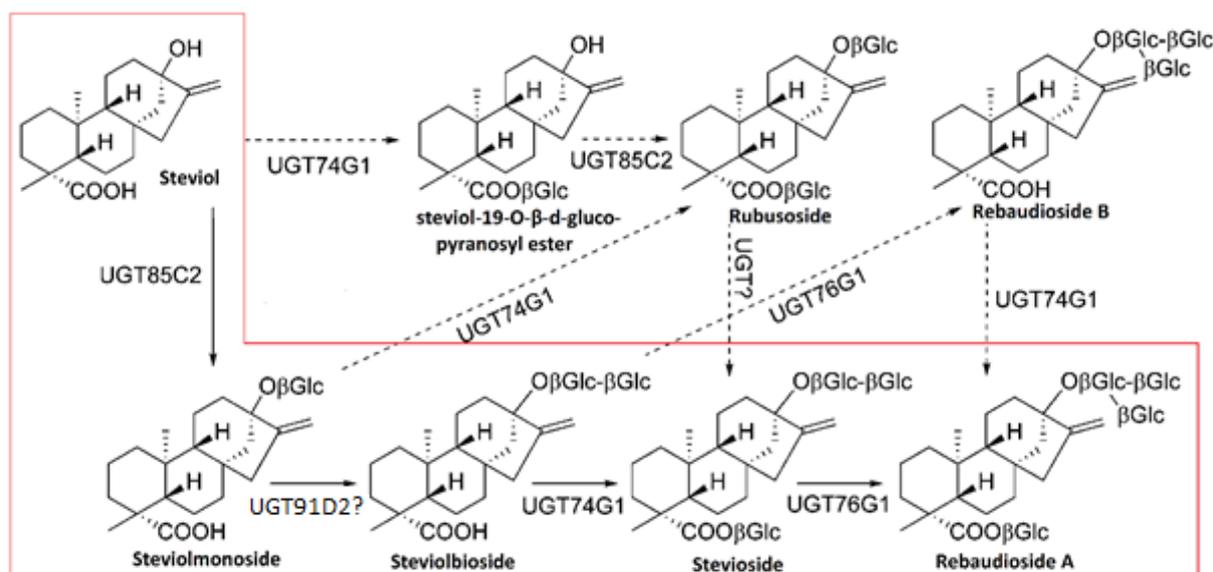


Figure 9. Biosynthetic pathways of major steviol glycosides, stevioside, rebaudioside A and steviolbioside via the minor steviol glycosides rubusoside, rebaudioside B, steviolmonoside and steviol-19-O-β-D-glucopyranosyl ester. Solid lines represent the main biosynthetic steps known to occur in vivo. The steviol glycosides involved are indicated with a red box. Dashed lines are biosynthetic steps that are catalysed in vitro but may not be favoured in vivo, comprising the pathways via rubusoside to stevioside and rebaudioside A and via rebaudioside B to rebaudioside A. (Ceunen et al, 2013) Figure adapted from Ceunen et al. 2013.

Although stevioside and rebaudioside A are the most common steviol glycosides, rebaudioside A is not the final product of this pathway. Further glycosylation reactions likely take place, leading to steviol glycosides such as rebaudioside D. Moreover, recently, a minor steviol glycoside has been described bearing up to seven β-glycosyl moieties, called rebaudioside O (Chaturvedula et al., 2013). However, further biosynthetic steps have not yet been found in vivo. Additionally, no rhamnosyl- or xylosyl-transferases have been described yet. Knowledge about the biosynthesis of dulcoside A, rebaudioside C and rebaudioside F remains therefore incomplete as well. The biosynthesis of the rhamnosylated and xylosylated steviol glycosides requires probably different enzymes. Even though plant UGTs have a broad substrate specificity, they usually have a very strict sugar donor specificity. In vitro, incubations of steviol glycosides with purified UGTs revealed a wider range of potential biosynthetic routes. Humphrey et al. reported biosynthetic routes leading to the minor steviol glycosides rubusoside and rebaudioside B (Humphrey et al., 2009) (figure 9). However, the glycosylation pathway via steviol-19-O-β-D-glucopyranosyl ester and rubusoside probably merely occurs as a relatively unproductive shunt, because of the reported inability of crude or purified protein extracts to use rubusoside as a substrate (Humphrey et al., 2009; Ceunen et al., 2013).

Since the formation of rebaudioside C from dulcoside A involves the glycosylation of the C3 hydroxyl group of the C13 glucose group, similar to the glycosylation of stevioside to rebaudioside A, this reaction is possibly catalysed by the glycosyl transferase UGT76G1 as well (figure 10A). In a similar way, this enzyme, together with UGT74G1, might be involved in the biosynthesis of rebaudiosides D and F (figure 10B and 10C). Moreover, the involvement of UGT74G1 and UGT76G1 in the biosynthesis of dulcoside A, rebaudioside C and rebaudioside F was further implied recently in patents describing the recombinant production of these steviol glycosides using sequences of UGT76G1 and UGT74G1 (US 20100297722; WO 2011060044; WO 2011153378; WO 2012075030; WO 2013110673; WO 2013022989; WO 2014086890; WO 2014122227; Ceunten et al., 2013).

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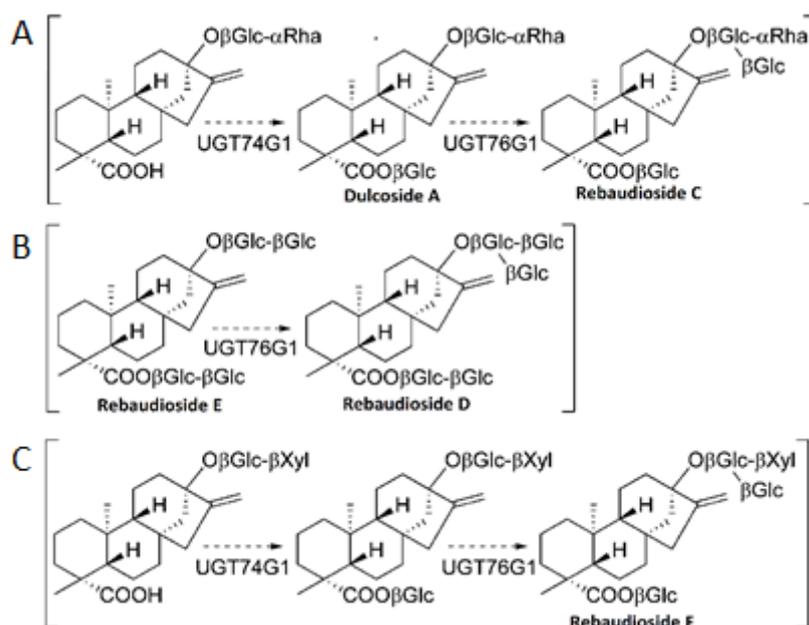


Figure 10. Hypothetical steps from dulcoside A to rebaudioside C (A), from rebaudioside E to rebaudioside D (B) and steps leading to rebaudioside F (C). The steps have not yet been characterized in vivo nor in vitro (Ceunen et al., 2013). Figure adapted from Ceunen et al. 2013.

Recombinant production of steviol glycosides

Another approach in order to obtain steviol glycosides comprising good organoleptic properties includes recombinant production of desired steviol glycosides via microbial fermentation. In this way, steviol glycosides comprising good organoleptic properties, such as rebaudioside D, can be obtained up to 1 g/L from one microbial culture after approximately five days (WO 2014122227). The most preferable host cell is stated as the eukaryotic cell *Saccharomyces cerevisiae*, but other microbial host cells are suitable as well (WO 2013110673). Currently, eight patent applications of four different applicants describing recombinant production of steviol glycosides have been published (Washington University: US20100297722; University Of Massachusetts: WO 2011060044 and WO 2012075030; DSM (www.dsm.com): WO 2013110673; Evolva: WO 2011153378, WO 2013022989, WO 2014086890 and WO 2014122227). The necessity of recombinant production of steviol glycosides is explained by one main reason. The extracts of the Stevia plant contain contaminants such as plant-derived compounds that contribute to off-flavours and the mixture of steviol glycosides in the Stevia plant is highly variable. This requires purification of desired steviol glycosides, such as rebaudioside D. However, purification of steviol glycosides from the Stevia plant has proven to be labour intensive and inefficient (Hellfritsch et al., 2012; Gasmalla et al., 2014; WO 2014122227). The fact that the Stevia plant contains variable mixtures of steviol glycosides in combination with their limited purification contribute to a restricted yield of desired steviol glycosides. To overcome this problem by cultivation has been proven to be severely limited. The applicants of the published patents suggest that a recombinant production system could be a potential solution. They promise a production of high yields of desired steviol glycosides, with less plant-based contaminants and lower costs due to avoidance of plant breeding and the labour intensive and inefficient purification of steviol glycosides.

Four of the eight patents are held by the Swiss biotechnology company Evolva, including the most recent patent that was published this year (WO 2014122227). Evolva is focusses on sustainable, fermentation-based approaches to ingredients for health, wellness and nutrition and in 2013 Cargill and Evolva entered into an agreement to jointly develop and commercialize fermentation-based steviol glycosides. The two companies are especially focused on the steviol glycoside rebaudioside M.

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Rebaudioside M is a minor steviol glycoside, making up less than 0.1 percent of the wild variety of the *Stevia rebaudiana* Bertoni leaf (Ceunen et al., 2013). This makes commercial use through extraction not feasible concerning both production-scale and costs. However, rebaudioside M comprises good organoleptic properties. It bears six β -glycosyl moieties of which three at the C13 position of steviol and three at the C19 position, which is one glycosyl moiety more at the C19 position compared with rebaudioside D. It possess a 2-O- β -glucopyranosyl-3-O- β -glucopyranosyl- β -glucopyranosyl (Glc β (1-2)[Glc β (1-3)]Glc β 1-) unit on both the C13 and the C19 position (Chaturvedula et al., 2013) (figure 11). Prakash et al. determined rebaudioside M to be 200–350 times sweeter than sucrose. They also experimentally showed that this steviol glycoside possesses a reduced perception of bitterness compared to rebaudioside A. Its aftertaste was evaluated as only slightly bitter by human sensory assays using a taste panel of 60 tasters (Prakash et al., 2013).

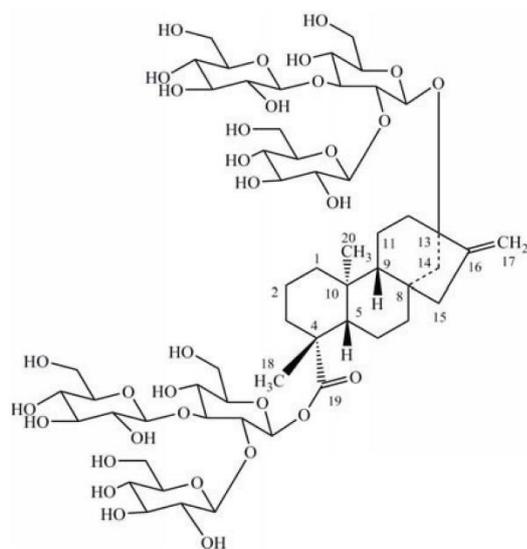


Figure 11. Molecular structure of rebaudioside M.

In order to obtain fermentation-based desired steviol glycosides, such as rebaudioside M, a host cell should at least express the previously described enzymes involved in the biosynthesis of steviol glycosides. Isoprenoids, such as the diterpenoid steviol, are found in every type of living organism and all larger isoprenoids are derived from the building block molecule IPP. Therefore, IPP is made in every living cell. It can be made through two chemical pathways, the previously described non-mevalonate pathway and the mevalonate pathway, which is an alternative route present in higher eukaryotes and some bacteria. This means that for the first part of steviol biosynthesis almost every organism can be used as a host. However, as mentioned above, the host should express another subset of genes including the genes required for synthesizing steviol, comprising the genes encoding a trans-geranylgeranyl pyrophosphate synthase (GGPPS); an *ent*-copalyl pyrophosphate synthase (*ent*-CPS); an *ent*-kaurene oxidase (*ent*-KO); an *ent*-kaurene synthase (*ent*-KS) and the steviol synthase *ent*-KAH polypeptide (figure 8). Furthermore, the host should also express the UDP-dependent glycosyl transferases UGT85C2, UGT74G1, UGT76G1 and UGT91D2 and an UDP-glucose pyrophosphorylase (figure 9).

As previously mentioned, no enzymes have been described yet that perform further glycosylation of rebaudioside A *in vivo* in order to obtain steviol glycosides like rebaudioside D and M. However, in the most recent patent describing recombinant production of steviol glycosides (WO 2014122227), production of hexa-glycosylated steviol is mentioned. *In vitro*, purified UGT76G1 in the presence of rebaudioside D and UDP-glucose revealed production of the hexa-glycoside rebaudioside M (WO

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2014122227). Moreover, production of rebaudioside M in recombinant steviol-glycoside producing yeast strains, containing the previously reported UGTs, was observed as well. However, the inventors mentioned that this was only observed when the UGT EUGT11 was expressed at high levels. High levels of EUGT11 expression resulted in production of more C19 β 1-2-di-glucosides, such as rebaudioside E and rebaudioside D, which can serve as substrate for UGT76G1 to form rebaudioside M (WO 2014122227) (figure 12). EUGT11 is a UDP-glycosyl transferase of the organism *Oryza sativa Japonica*, commonly known as Asian rice (Gene ID: 4333835). The above results suggest that UGT76G1 can further glycosylate Rebaudioside D, resulting in rebaudioside M (WO 2014122227).

As described above, UGT76G1 may be involved in the biosynthesis of rebaudioside D from rebaudioside E in vivo (figure 10B). In the recent patent (WO 2014122227), in vitro production of rebaudioside D from rebaudioside E by UGT76G1 is mentioned. The inventors mentioned furthermore the production of rebaudioside E from stevioside with UGT74G1 glycosylation. In addition, besides rebaudioside M production via rebaudioside E, an UGT74G1 catalysed step of rebaudioside M production via rebaudioside A is also announced (WO 2014122227) (figure 12).

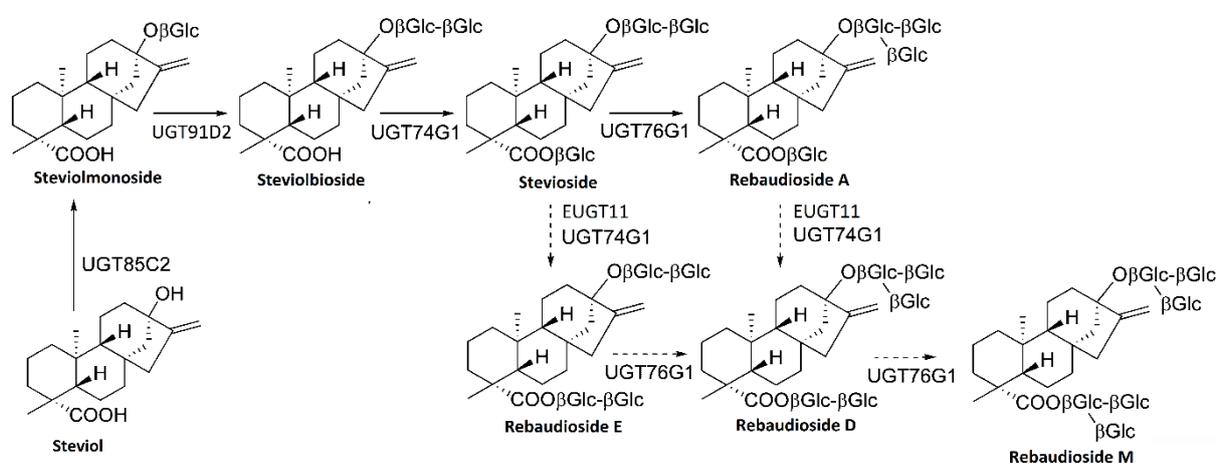


Figure 12. Recombinant biosynthetic route from steviol to rebaudioside M claimed by the biotechnology company Evolva.

Furthermore, the inventors also mentioned the need for expression of one or more genes encoding a cytochrome P450 reductase. A cytochrome P450 reductase facilitates the cycling of NADP⁺ to regenerate NADPH. Both *ent*-KO and *ent*-KAH require NADPH for their activity (figure 7) and therefore activity of a cytochrome P450 reductase. Hence, by inclusion of a recombinant gene encoding a cytochrome p450 reductase, the activity of *ent*-KO and/or *ent*-KAH in a recombinant microorganism can be significantly increased (WO 2014122227).

All the required genes can be derived from different organisms. For example, suitable CPR enzymes and UDP-dependent glycosyl transferases include those made by both *Stevia rebaudiana* and *Arabidopsis thaliana*, a small flowering plant that is widely used as a model organism in plant biology. Furthermore, also non-plant genomes are suitable. For example, suitable GGPPS enzymes included those made by *Gibberella fujikuroi*, *Mus musculus*, *Thalassiosira pseudonana*, *Streptomyces clavuligerus*, *Sulfolobus acidocaldarius* and *Synechococcus sp* as well (WO 2014122227).

Discussion

Enzymatic glycosylation of the most abundant steviol glycoside, stevioside, in order to improve its organoleptic properties has been successful using several different enzyme-glycosyl-donor combinations, including CGTase with either soluble starch or cyclodextrin, Biozyme L and maltose, α -

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amylase and starch or pullulanase and pullan. The glycosylated steviosides that are reported to comprise improved organoleptic properties include mono- and di- α -1-4-glycosylated stevioside at the C13 position of steviol and mono- α -1-6-glycosylation, mono- α -1-2-glycosylation and di- α -1-2- α -1-4-glycosylation at the C19 position (figure 4). Using microwave accelerated transglycosylation (MAR) instead of traditional techniques resulted in the highest reported stevioside conversion.

Noteworthy, while α -tri-glycosylation of stevioside did not reduce the bitter aftertaste of stevioside, β -tri-glycosylation of stevioside, resulting in rebaudioside M, resulted in a reduced perception of bitterness compared to rebaudioside A and therefore compared to stevioside. This could be explained by differences in the glycosidic linkages. Proton and carbon nuclear magnetic resonance spectroscopy revealed that regioselectivity and linkage specificity of steviol glycosides are to a large extent related to their taste, while the difference in stereochemistry of the glycosidic bond does probably not influence sweet and bitter taste perception to a great extent, which can be seen by comparing rebaudioside E and the promising compound 9.

Unfortunately, in most enzymatic glycosylation studies the compositions of the obtained glycosylated steviosides have not been determined. Structure determination can especially give much information if human sensory evaluations are performed as well. However, the few studies that combined human sensory evaluations with structure determination used relatively small taste panels, comprising less than seventeen tasters. Moreover, these human sensory experiments of glycosylated steviosides were performed in different manners, which makes the results not readily comparable. Future human sensory studies should explore their exact improvement of taste. The studies should be performed with a large group of tasters and tasters should be biased by selecting subjects who are sensitive to the bitter aftertaste of steviol glycosides. The tasters should also be tested on the intensity of this perception, since both sensitivity and intensity of perception are correlated with human genetic variation.

In addition, an important issue concerning the applicability of enzymatic glycosylated stevioside comprises the caloric content of α -glycosylated steviosides. Until now, no study on calorie increase of the improved glycosylated steviosides has been done, even though the non-caloric characteristic of steviol glycosides plays an important role in their popularity. Therefore, future studies should give more insight in the caloric content of the improved glycosylated steviosides. Even though, CGTase with either maltodextrin, cyclodextrin or starches are considered as the most effective glycosyl donor and enzyme combinations for the transglycosylation of stevioside, the α -1-4-glycosidic linkages that they introduce will probably increase the caloric content of the steviol glycosides, which will not answer the consumers demand for low and zero calorie food products. In order to answer this demand, introduction of α -amylase resistant glycosidic linkages, such as α -1-2 and α -1-6 are therefore more desirable. Furthermore, if men want to avoid calorie increase, suitable enzymes that introduce β -glycosidic linkages could be searched for as well. B-glycosylation of rebaudioside A using UDP-glucose-dependent glycosyl transferases was already shown by Prakash et al. (Prakash et al., 2014). However, UDP-glucose is an expensive co-factor, which hampers their application in the laboratory and industry (Desmet et al., 2012).

In the future, it remains to be seen whether glycosylated steviosides will indeed provide a sufficient larger group of consumers. Moreover, enzymatic glycosylation should be more efficient than purification of minor glycosides such as rebaudioside M, which comprises better organoleptic properties than stevioside. By the applicants of the patents describing recombinant production of steviol glycosides it is stated that recombinant production of steviol glycosides will be highly cost-effective due to avoidance of plant breeding and the labour intensive and inefficient purification of steviol glycosides. However, steviol glycosides derived from recombinant production are far from natural. Therefore, because of the rising consumer demand for natural food ingredients,

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recombinant production could also result in a loss of consumers, instead of the only expected gain of consumers due to reduced bitter-aftertastes.

Currently, eight patent applications describing recombinant production of steviol glycosides have been published. However, complete studies showing recombinant production of steviol glycosides such as rebaudioside M have not yet been published. When publications become available that actually report recombinant production of steviol glycosides, it would be useful to compare recombinant production with enzymatic glycosylation of stevioside in terms of efficiency, cost, social acceptance and taste. Even though rebaudioside M was characterized as only slightly bitter, the question remains whether there is still enzymatic glycosylation necessary to further reduce this bitter aftertaste. Human sensory studies have to reveal this question by comparing rebaudioside M with the improved glycosylated steviosides of figure 4.

In conclusion, the increased demand of zero and reduced calorie food products and beverages together with the increased demand for natural food produces has raised the popularity of steviol glycosides in the use as high potency sweeteners. However, since almost half of the human population perceives a bitter aftertaste due to genetic variation of two human bitter taste receptors, improvement of the organoleptic properties of steviol glycoside is desirable. Enzymatic glycosylation of the steviol glycoside stevioside in order to improve its organoleptic properties has been successful using several different enzyme-glycosyl-donor combinations. Until now, six different glycosylated steviosides have been proven to reduce the bitter aftertaste of stevioside. However, future human sensory studies should explore their exact improvement of taste. The studies should be performed with a large group of tasters and the tasters should be tested on both the ability to perceive the bitter aftertaste of steviol glycosides and on the intensity of this perception, since both are correlated with human genetic variation. Comparison of the improved glycosylated steviosides with naturally occurring steviol glycosides can provide insight into the effect of glycosidic linkages on taste. Furthermore, studies on the binding pocket of the two bitter receptor involved, hTAS2R4 and hTAS2R14, will provide more insight about the relation between structure and taste. Because the biosynthetic pathway of steviol glycosides is already quite well elucidated, present patents show a general interest in the recombinant production of steviol glycosides. In the most recent patent application (WO 2014122227), it is stated that recombinant production of the minor steviol glycoside rebaudioside M could be highly cost effective. However, it remains to be seen whether this promise will be fulfilled and whether consumers will accept recombinantly produced sweeteners. By all means, studies on the above points would contribute to the development and improvement of steviol glycosides as high potency sweeteners.

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