Steviol Glycosides: Chemical Diversity, Metabolism, and Function

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Steviol Glycosides: Chemical Diversity, Metabolism, and Function

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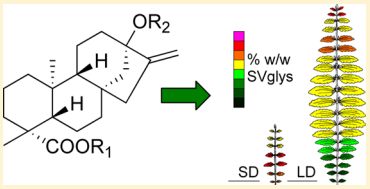
ABSTRACT: Steviol glycosides are a group of highly sweet diterpene glycosides discovered in only a few plant species, most notably the Paraguayan shrub *Stevia rebaudiana*. During the past few decades, the nutritional and pharmacological benefits of these secondary metabolites have become increasingly apparent. While these properties are now widely recognized, many aspects related to their in vivo biochemistry and metabolism and their relationship to the overall plant physiology of *S. rebaudiana* are not yet understood. Furthermore, the large size of the steviol glycoside pool commonly found within *S. rebaudiana* leaves implies a significant metabolic investment and poses questions regarding the benefits *S. rebaudiana* might gain from their accumulation. The current review intends to thoroughly discuss the available knowledge on these issues.



INTRODUCTION

In recent years, the development of natural sweeteners has been moving up a gear in order to provide alternatives for sucrose (saccharose) that are preferably noncalorific, noncariogenic, and generally healthy. The need for alternative sweeteners is expected to increase further, as metabolic disorders such as type-II diabetes and obesity are becoming ever more prevalent. One of the most promising alternatives constitutes a large family of highly sweet diterpenoid glycosides named steviol glycosides. These are found typically in large amounts within the leaves of *Stevia rebaudiana* (Bertoni) Bertoni, a rhizomatous perennial shrub of the family Asteraceae (tribe Eupatorieae), which is native to the Amambay Cordillera in Northeast Paraguay. It is by far the best known member of the genus *Stevia*, which includes a total of 220−230 species.[1] Besides *S. rebaudiana*, steviol glycosides have been reported in three other species, namely, the Mexican *Stevia phlebophylla* A. Gray,[2] the Chinese blackberry *Rubus suavissimus* S. Lee (Rosaceae),[3] and the Japanese perennial *Angelica keiskei* (Miq.) Koidz. (Apiaceae).[4]

Recent reviews have mainly focused on the chemical, agronomic, pharmacological, toxicological, and nutritional aspects of *S. rebaudiana* and steviol glycosides.[5−13] The current review discusses thoroughly some of the lesser known aspects regarding the in vivo and in vitro metabolism of steviol glycosides, and their relationship with overall plant physiology. After a brief overview of the main secondary metabolites isolated from *S. rebaudiana*, the chemical diversity of all known steviol glycosides extracted from plant sources is discussed, including the methodologies used for their detection, isolation, and characterization. Next, a comprehensive overview is given of the biochemistry and physiology related to their biosynthesis and accumulation in *S. rebaudiana*. This includes the question of how the accumulation of steviol glycosides in *S. rebaudiana* is influenced by various abiotic factors. Since they can accumulate up to 25% of the leaf dry matter, the presence of such large amounts of secondary metabolites poses questions concerning the benefits *S. rebaudiana* might gain from their accumulation. So far, no clear physiological function has been associated unambiguously with the large pool of steviol glycosides, but several hypotheses have been proposed. These will be reviewed in the last section, followed by a short conclusion and prospects for future research on this remarkable species.

CHEMICAL CONSTITUENTS OF *STEVIA REBAUDIANA*

For the past few decades, *S. rebaudiana* has been the subject of extensive phytochemical analysis, during which time a large number of molecular structures have been identified. Most studies were done on the isoprenoids and phenolic compounds, but many other constituents such as macro- and micronutrients, fatty acids, and vitamins have also been found in *S. rebaudiana*.[12,14,15]

Members of most major isoprenoid structural types have been detected in *S. rebaudiana*. Diterpenes have so far been found in at least 9% (or 20 species) of the *Stevia* species, occurring mainly as *ent*-kauranes and *ent*-labdanes.[16] Besides the steviol glycosides, which are *ent*-kaurane-type diterpene glycosides, several *ent*-labdanes have been detected within *S. rebaudiana* leaves[17,18] and flowers,[19] and include the non-glycosylated sterebins A−N.[18,20−22] The methanolic leaf extract of *S. rebaudiana* has yielded several triterpenes and sterols, such as phytosterols,[23,24] nonsweet steroid glycosides,[25] and lupeol esters.[17] Callus cultures obtained from the leaves were shown to produce stigmasterol.[26] The profile of other lipids such as pigments, fatty acids, phospholipids, and glycolipids has also been described.[27,28]

The number of compounds detected within the essential and volatile oils of *S. rebaudiana* ranges from 30 to over 300, which, in total, make up about 0.4−3.5% w/w of dried leaves.[29−40] One study reported three times more essential oils in the inflorescence compared to vegetative leaves.[31] Although their individual composition was markedly dependent on the ontogenetic stage and genotype,[36] the sesquiterpenes caryophyllene α-oxide and spathulenol were usually among the most

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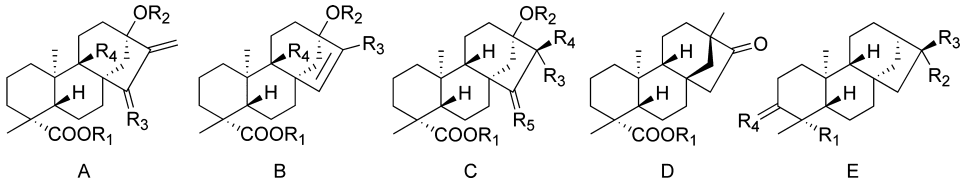

Figure 1. Aglycones of the major *ent*-kaurane glycosides extracted from *Stevia rebaudiana* and *Rubus suavissimus*.

Table 1. List of Known Steviol Glycosides Extracted from *Stevia rebaudiana*[a]

trivial name	AG[b]	R_1	R_2	R_3	R_4	formula	mass[c]	ref[d]
steviol	A	H	H	H_2	H	$C_{20}H_{30}O_3$	318.22	84
steviolmonoside	A	H	Glcβ1−	H_2	H	$C_{26}H_{40}O_8$	480.27	71
steviol-19-*O*-β-D-glucoside	A	Glcβ1−	H	H_2	H	$C_{26}H_{40}O_8$	480.27	85
rubusoside	A	Glcβ1−	Glcβ1−	H_2	H	$C_{32}H_{50}O_{13}$	642.33	71
rebaudioside A family [Glcβ(1−2)[±Glcβ(1−3)]Glcβ1−]								
steviolbioside	A	H	Glcβ(1−2)Glcβ1−	H_2	H	$C_{32}H_{50}O_{13}$	642.33	70
stevioside	A	Glcβ1−	Glcβ(1−2)Glcβ1−	H_2	H	$C_{38}H_{60}O_{18}$	804.38	58
−	A	Glcβ(1−2)Glcβ1−	Glcβ1−	H_2	H	$C_{38}H_{60}O_{18}$	804.38	82
rebaudioside E	A	Glcβ(1−2)Glcβ1−	Glcβ(1−2)Glcβ1−	H_2	H	$C_{44}H_{70}O_{23}$	966.43	86
rebaudioside B	A	H	Glcβ(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{38}H_{60}O_{18}$	804.38	70
rebaudioside A	A	Glcβ1−	Glcβ(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{44}H_{70}O_{23}$	966.43	70
rebaudioside D	A	Glcβ(1−2)Glcβ1−	Glcβ(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{50}H_{80}O_{28}$	1128.48	86
rebaudioside I	A	Glcβ(1−3)Glcβ1−	Glcβ(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{50}H_{80}O_{28}$	1128.48	71
rebaudioside M	A	Glcβ(1−2)[Glcβ(1−3)]Glcβ1−	Glcβ(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{56}H_{90}O_{33}$	1290.54	71
−	A	Glcβ1−	Glcβ(1−6)Glcβ(1−2)Glcβ1−	H_2	H	$C_{44}H_{70}O_{23}$	966.43	72
rebaudioside L	A	Glcβ1−	Glcβ(1−6)Glcβ(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{50}H_{80}O_{28}$	1128.48	71
rebaudioside C family [Rhaα(1−2)[±Glcβ(1−3)]Glcβ1−]								
dulcoside A	A	Glcβ1−	Rhaα(1−2)Glcβ1−	H_2	H	$C_{38}H_{60}O_{17}$	788.38	87
dulcoside B	A	H	Rhaα(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{38}H_{60}O_{17}$	788.38	71
rebaudioside C	A	Glcβ1−	Rhaα(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{44}H_{70}O_{22}$	950.44	88
[dulcoside B]								87
rebaudioside H	A	Glcβ1−	Glcβ(1−3)Rhaα(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{50}H_{80}O_{27}$	1112.49	71
rebaudioside K	A	Glcβ(1−2)Glcβ1−	Rhaα(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{50}H_{80}O_{27}$	1112.49	71
rebaudioside J	A	Rhaα(1−2)Glcβ1−	Glcβ(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{50}H_{80}O_{27}$	1112.49	71
rebaudioside N	A	Rhaα(1−2)[Glcβ(1−3)]Glcβ1−	Glcβ(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{56}H_{90}O_{32}$	1274.54	71
rebaudioside O	A	Glcβ(1−3)Rhaα(1−2)[Glcβ(1−3)]Glcβ1−	Glcβ(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{62}H_{100}O_{37}$	1436.59	71
[Glcβ(1−3)Glcβ1−] family								
rebaudioside G	A	Glcβ1−	Glcβ(1−3)Glcβ1−	H_2	H	$C_{38}H_{60}O_{18}$	804.38	71
rebaudioside F family [Xylβ−]								
−	A	Glcβ1−	Xylβ(1−2)Glcβ1−	H_2	H	$C_{37}H_{58}O_{17}$	774.37	73
rebaudioside F	A	Glcβ1−	Xylβ(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{43}H_{68}O_{22}$	936.42	89
−	A	Glcβ1−	Glcβ(1−2)[Xylβ(1−3)]Glcβ1−	H_2	H	$C_{43}H_{68}O_{22}$	936.42	73
−	A	Xylβ(1−6)Glcβ1−	Glcβ(1−2)Glcβ1−	H_2	H	$C_{43}H_{68}O_{22}$	936.42	74
[Fruβ−] family								
−	A	Glcβ1−	Glcβ(1−2)[Fruβ(1−3)]Glcβ1−	H_2	H	$C_{44}H_{70}O_{23}$	966.43	75
[Glcα−] family								
−	A	Glcα(1−2)Glcα(1−4)Glcβ1−	Glcβ(1−2)Glcβ1−	H_2	H	$C_{50}H_{80}O_{28}$	1128.48	76
−	A	Glcβ1−	Glcα(1−3)Glcβ(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{50}H_{80}O_{28}$	1128.48	77
−	A	Glcβ1−	Glcα(1−4)Glcβ(1−3)[Glcβ(1−2)]Glcβ1−	H_2	H	$C_{50}H_{80}O_{28}$	1128.48	77
[6-deoxyGlcβ−] family								
−	A	Glcβ1−	6-deoxyGlcβ(1−2)Glcβ1−	H_2	H	$C_{38}H_{60}O_{17}$	788.38	78
−	A	Glcβ1−	6-deoxyGlcβ(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{44}H_{70}O_{22}$	950.44	78
−	A	6-deoxyGlcβ1−	Glcβ(1−2)[Glcβ(1−3)]Glcβ1−	H_2	H	$C_{44}H_{70}O_{22}$	950.44	79

[a]The steviol glycosides have been grouped in families (as per Ohta et al.[71]) and further ordered according to their glycosylation pattern. Abbreviations: AG, aglycone; Fru, fructose; Glc, glucose; Rha, rhamnose; Xyl, xylose. [b]The aglycone refers to that given in Figure 1. [c]Exact mass given in amu. [d]The first report on their in vivo detection and characterization.

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Table 2. List of Known Isomers and Glycosylated Forms of Oxidized Steviol Extracted from *Stevia rebaudiana*[a]

trivial name	AG[b]	R_1	R_2	R_3	R_4	R_5	formula	mass[c]	ref[d]
–	B	Glcβ1–	Glcβ(1–2)Glcβ1–	CH_3	H		$C_{38}H_{60}O_{18}$	804.38	74
–	B	H	Glcβ(1–2)[Glcβ(1–3)]Glcβ1–	CH_3	H		$C_{38}H_{60}O_{18}$	804.38	80
–	B	Glcβ1–	Glcβ(1–2)[Glcβ(1–3)]Glcβ1–	CH_3	H		$C_{44}H_{70}O_{23}$	966.43	81
–	B	Glcβ1–	Glcβ(1–2)Glcβ1–	CH_2OH	H		$C_{38}H_{60}O_{19}$	820.37	74
–	B	Glcβ1–	Glcβ(1–2)[Glcβ(1–3)]Glcβ1–	CH_2OH	H		$C_{44}H_{70}O_{24}$	982.43	81
–	B	Glcβ1–	Glcβ(1–2)Glcβ1–	CHO	H		$C_{38}H_{58}O_{19}$	818.36	74
–	C	H	Glcβ(1–2)[Glcβ(1–3)]Glcβ1–	OH	CH_3	H_2	$C_{38}H_{62}O_{19}$	822.39	80
–	C	Glcβ1–	Glcβ(1–2)[Glcβ(1–3)]Glcβ1–	OH	CH_3	H_2	$C_{44}H_{72}O_{24}$	984.44	72
isosteviol-19-*O*-β-D-glucoside	D	Glcβ1–					$C_{26}H_{40}O_8$	480.27	80

[a]Abbreviations: AG, aglycone; Glc, glucose. [b]The aglycone refers to that given in Figure 1. [c]Exact mass given in amu. [d]The first report on their in vivo detection and characterization.

abundant essential oil components detected in the leaves. Essential oils extracted from the inflorescence additionally tend to contain large amounts of *trans*-β-farnesene and nerolidol. These two compounds are thought to be largely responsible for the specific flavor of the dried herb and inflorescence, together with other minor nonoxygenated sesquiterpenes, and monoterpenes such as β-pinene and limonene.[41] In the roots, about 60 essential oil components have been detected, of which α-longipinene, α-isocomene, and modheph-2-ene are the major representatives.[42]

Besides isoprenoids, many phenolic compounds have been identified in the leaf extracts of *S. rebaudiana*, including flavonol glycosides,[43−49] and a large variety of coumarins and cinnamic acid derivatives.[50−52] It has been suggested that these phenolic compounds could affect the organoleptic properties of *Stevia*-based products. Another compound that might influence their taste is steviamine, a bitter, water-soluble, indolizidine alkaloid, which was not only the first alkaloid to be extracted from *S. rebaudiana*, but also the first of its kind ever to be isolated from species in the family Asteraceae.[53]

STEVIOL GLYCOSIDES. OCCURRENCE AND DIVERSITY

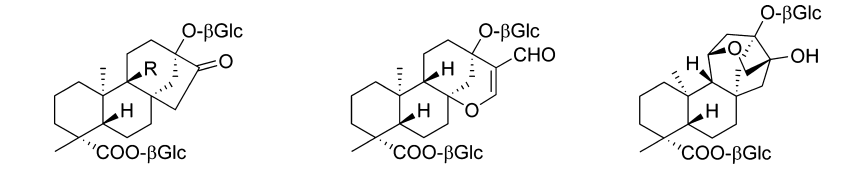
***Stevia rebaudiana* (Bertoni) Bertoni.** After the discovery of the species by Western science in 1887,[1,54] its sweet principles were initially related to glycyrrhizin,[55] and, shortly afterward, isolated in a crude fashion for the first time.[56,57] In 1931, the chemical formula of stevioside (**37**), the most abundant steviol glycoside, was derived by two French chemists. From a series of (non)enzymatic hydrolysis experiments they described the glycosidic nature of **37**, and the acidic properties of its degradation products, steviol (**19**) and isosteviol (**43**, an *ent*-beyerane-type diterpene formed by acid catalysis of steviol glycosides).[58−61] However, their structural elucidation proved to be a challenge.[62−67] When the structure of **37** was shown to be a sophoroside, it was only the second such structure to have been found in Nature, with the first one being kaempferol sophoroside from *Sophora japonica*.[64] The absolute stereochemistry of **19**, **37**, and **43** was ultimately fully determined by Mosettig et al.[68]

For 45 years, stevioside (**37**) would be regarded as the only steviol glycoside present in the leaves of *S. rebaudiana*, until researchers from the University of Hiroshima obtained rebaudiosides A (**38**) and B (**34**) from a methanolic leaf extract.[69,70] By the early 21st century, nine steviol glycosides had been identified within *S. rebaudiana* leaves, namely, stevioside (**37**), rebaudiosides A (**38**), B (**34**), C (**42**), D (**40**), E (**39**), and F (**31**), dulcoside A (**41**), and steviolbioside (**36**). Extensive selection procedures have yielded cultivars with large differences in total steviol glycoside content as well as percentage steviol glycoside compositions, but the most common composition of the wild variety, calculated on a dry weight basis, is often reported as follows: stevioside (**37**) (5–10% w/w), rebaudiosides A (**38**) (2–5%) and C (**42**) (1%), dulcoside A (**41**) (0.5%), rebaudiosides D (**40**), E (**39**), and F (**31**) (0.2%), and steviolbioside (**36**) (0.1%).

In recent years, researchers have detected many more steviol glycosides, present in trace quantities in dried leaf extracts originating from different cultivars.[71−82] As of April 2013, 34 steviol glycosides have been identified in *S. rebaudiana*, together with eight isomers or glycosylated forms of oxidized steviol (**19**) (Figure 1, Tables 1 and 2). In addition, the monoglucosyl ester of isosteviol (**43**) has also been isolated.[80] Several more steviol glycosides were detected by LC-MS but not yet fully characterized.[83] No report has been published yet on the organoleptic properties of these newly described steviol glycosides.

Isolation and Analysis of Steviol Glycosides. Many protocols have been developed for the extraction of steviol glycosides from plant material. Most of them start with a water- or alcohol-based extraction, followed by several purification steps using, for example, pigment-removing agents, partitioning procedures (often involving the extraction of steviol glycosides from the aqueous phase with *n*-butanol),[90] solid-phase extraction, and column chromatography. The application of supercritical fluid extraction[91,92] and nanofiltration[93] for steviol glycoside extraction still requires optimization to obtain higher purification levels or yields. Steviol glycosides have been purified further by extensive recrystallization, often coupled with preparative RP-HPLC to obtain highly pure compounds. Their subsequent characterization involved not only extensive MS and NMR studies but also the analysis of the cleaved-off sugars by a combination of alkaline (C-19 sugar moieties), and acid hydrolysis (C-13 and C-19 sugar moieties).[72−81] Specific methods have also been developed for the extraction of steviol glycosides or their metabolites from blood, urine, and feces,[94−96] as well as from food.[97]

Since the 1930s, a large number of methodologies have been developed to separate and detect the various steviol glycosides and their derivatives, both qualitatively and quantitatively. Initially, analytical methodology was primarily based on the detection of decomposition products and on chemical derivatization. Degradation of steviol glycosides was usually done by acid hydrolysis to obtain isosteviol (**43**)[60,61] or alkaline hydrolysis to obtain steviolbioside (**36**).[63] Various enzymatic procedures with, for example, hesperidinase, pectinase, and



suavioside O (R=H; $C_{31}H_{48}O_{14}$, M_r 644.30)[149]
9-hydroxysuavioside O (R=OH; $C_{31}H_{48}O_{15}$, M_r 660.30)[149]

suavioside P ($C_{32}H_{48}O_{15}$, M_r 672.30)[149]

suavioside M ($C_{32}H_{50}O_{15}$, M_r 674.31)[149]

Figure 2. Unusual suaviosides extracted from *Rubus suavissimus*.

Table 3. List of Known Steviol Glycosides and Other *ent*-Kaurane Glycosides Extracted from *Rubus suavissimus*[a]

trivial name	AG[b]	R_1	R_2	R_3	R_4	R_5	formula	mass[c]	ref[d]
steviol	A	H	H	H_2	H		$C_{20}H_{30}O_3$	318.22	151, 152
steviolmonoside	A	H	Glcβ1−	H_2	H		$C_{26}H_{40}O_8$	480.27	143
rubusoside	A	Glcβ1−	Glcβ1−	H_2	H		$C_{32}H_{50}O_{13}$	642.33	3
15β-hydroxyrubusoside	A	Glcβ1−	Glcβ1−	β-OH	H		$C_{32}H_{50}O_{14}$	658.32	149
15-oxorubusoside	A	Glcβ1−	Glcβ1−	O	H		$C_{32}H_{48}O_{14}$	656.30	149
suavioside B	A	Glcβ1−	Glcβ1−	H_2	OH		$C_{32}H_{50}O_{14}$	658.32	143
suavioside Q_1	A	Glcβ1−	Glcα(1−6)Glcβ1−	H_2	H		$C_{38}H_{60}O_{18}$	804.38	149
suavioside R_1	A	Glcβ1−	Glcβ(1−6)Glcβ1−	H_2	H		$C_{38}H_{60}O_{18}$	804.38	149
suavioside S_1	A	Glcβ1−	Galα(1−6)Glcβ1−	H_2	H		$C_{38}H_{60}O_{18}$	804.38	150
suavioside C_1	A	Glcβ1−	*trans-p*-coumaroyl^{6}Glcβ1−	H_2	H		$C_{41}H_{56}O_{15}$	788.36	143
suavioside D_1	A	Glcβ1−	caffeoyl^{6}Glcβ1−	H_2	H		$C_{38}H_{60}O_{18}$	804.38	143
suavioside Q_2	A	Glcα(1−6)Glcβ1−	Glcβ1−	H_2	H		$C_{38}H_{60}O_{18}$	804.38	149
suavioside R_2	A	Glcβ(1−6)Glcβ1−	Glcβ1−	H_2	H		$C_{38}H_{60}O_{18}$	804.38	149
suavioside S_2	A	Galα(1−6)Glcβ1−	Glcβ1−	H_2	H		$C_{38}H_{60}O_{18}$	804.38	150
suavioside C_2	A	*trans-p*-coumaroyl^{6}Glcβ1−	Glcβ1−	H_2	H		$C_{41}H_{56}O_{15}$	788.36	149
suavioside D_2	A	caffeoyl^{6}Glcβ1−	Glcβ1−	H_2	H		$C_{38}H_{60}O_{18}$	804.38	143
suavioside J	B	Glcβ1−	Glcβ1−	CH_2OH	H		$C_{32}H_{50}O_{14}$	658.32	143
9-hydroxysuavioside J	B	Glcβ1−	Glcβ1−	CH_2OH	OH		$C_{32}H_{50}O_{15}$	674.31	149
suavioside H	B	Glcβ1−	Glcβ1−	CHO	H		$C_{32}H_{48}O_{14}$	656.30	143
9-hydroxysuavioside H	B	Glcβ1−	Glcβ1−	CHO	OH		$C_{32}H_{48}O_{15}$	672.30	149
suavioside F	C	H	Glcβ1−	CH_3	OH	H_2	$C_{26}H_{42}O_9$	498.28	143
suavioside G	C	Glcβ1−	Glcβ1−	CH_3	OH	H_2	$C_{32}H_{52}O_{14}$	660.34	143
suavioside I	C	Glcβ1−	H	CH_2OH	OH	H_2	$C_{26}H_{42}O_{10}$	514.28	143
suavioside L	C	Glcβ1−	Glcβ1−	CH_2OH	H	H_2	$C_{32}H_{52}O_{14}$	660.34	149
15-oxosuavioside L	C	Glcβ1−	Glcβ1−	CH_2OH	H	O	$C_{32}H_{50}O_{15}$	674.31	149
15-oxo-16-*epi*-suavioside L	C	Glcβ1−	Glcβ1−	H	CH_2OH	O	$C_{32}H_{50}O_{15}$	674.31	149
16β-hydroxysuavioside L	C	Glcβ1−	Glcβ1−	CH_2OH	OH	H_2	$C_{32}H_{52}O_{15}$	676.33	149
16α-hydroxysuavioside L	C	Glcβ1−	Glcβ1−	OH	CH_2OH	H_2	$C_{32}H_{52}O_{15}$	676.33	149
paniculoside IV	E	COO-β1Glc	OH	OH	H_2		$C_{26}H_{42}O_9$	498.28	143
suavioside A	E	CH_3	CH_2O-β1Glc	OH	α-OH		$C_{26}H_{44}O_8$	484.30	147
suavioside E	E	COO-β1Glc	OH	CH_2OH	H_2		$C_{26}H_{42}O_9$	498.28	143
suavioside K	E	COO-β1Glc	OH	CH_2O-β1Glc	H_2		$C_{32}H_{52}O_{14}$	660.34	149
sugeroside	E	CH_3	CH_2O-β1Glc	OH	O		$C_{26}H_{42}O_8$	482.29	147

[a]Abbreviations: AG, aglycone; Gal, galactose; Glc, glucose; Rha, rhamnose; Xyl, xylose. [b]The aglycone refers to that given in Figure 1. [c]Exact mass given in amu. [d]The first report on their in vivo detection and characterization.

takadiastase, were also used to obtain steviol (**19**), steviolbioside (**36**), and other catabolites.[63,98−100] Early analysis of these compounds involved TLC coupled with densitometry,[69,101] while other protocols involved the specific detection of isosteviol (**43**), either colorimetrically[102,103] or via gas-chromatographic detection of its methyl ester.[104]

Separation of underivatized steviol glycosides became more important after the discovery of rebaudioside A in 1975. From the late 1970s on, steviol glycosides have increasingly been analyzed by HPLC due to the easiness of sample preparation and the more satisfactory separation of stevioside (**37**), rebaudioside A (**38**), and other steviol glycosides, compared to methodologies based on TLC.[105−109] Eventually, highly specific HPLC-based analytical methods have evolved for the separation and quantitation of the different steviol glycosides with ever higher resolution and sensitivity, using a variety of different instrumentation (e.g., RP-HPLC,[110] 2D-HPLC,[111] ultra-HPLC,[112] and 2D-ultra-HPLC[48]), columns (e.g., C_{18},[94,113] NH_2,[24,114] and HILIC[115−117]), mobile phases (e.g., AcCN and MeOH) with[113] or without[110] gradient elution, and detection

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systems (e.g., UV,[113] MS,[112] and amperometry[118]). Besides HPLC-based methods, other analytical protocols include, for example, high-performance TLC,[119] overpressured layer chromatography,[120] capillary electrophoresis,[121,122] high-speed counter-current chromatography,[123] 2D-GC,[124] quantitative ^{1}H NMR,[125,126] near-infrared reflectance spectroscopy,[127−129] and square-wave polarography.[130] Recently, ambient ionization MS techniques such as desorption electrospray ionization (DESI) have been applied successfully for the direct analysis of steviol glycosides in *S. rebaudiana* leaves with minimal sample preparation.[131,132]

Most HPLC-based methodologies use an external standard as reference for quantitation, usually rebaudioside A (**38**) or stevioside (**37**) of >99% purity. Recently, an internal standard has been developed for steviol glycoside analysis, namely, the 19-*O*-*β*-D-galactopyranosyl ester of steviolmonoside (**35**).[133] Use of an internal standard allows for the correction of losses due to sample cleanup and is independent of errors in injection volume or detector sensitivity. Likewise, dihydroisosteviol is used as an internal standard for the analysis of free steviol (**19**) in both plant material and food, involving a fluorometric detection of the methoxycoumarinyl ester derivative of **19**.[84,134] When applied to the measurement of isosteviol (**43**), formed after acid hydrolysis of any steviol glycosides present, this method was shown to be an effective way to measure directly the amount of steviol equivalents in food.[135]

***Rubus suavissimus* S. Lee (Rosaceae).** *Rubus suavissimus* S. Lee, Chinese blackberry, is native to the Jinxiu Dayaoshan mountain range in Guangxi, People's Republic of China. The plant was first described in detail by Lee,[136] after it had been erroneously considered as taxonomically equal to the nonsweet *R. chingii* Hu.[3] The leaves of both species are palmately five- or seven-lobed, which, for a total of 378 Chinese *Rubus* species, only occurs otherwise in *R. reflexus* var. *lanceolobus*.[137] Lu[138] corrected the botanical name to *R. chingii* var. *suavissimus* (S. Lee). However, the plant is still referred to as *R. suavissimus*.

The leaves of *R. suavissimus* accumulate rubusoside (**33**) to about 5% w/w of their dry weight.[3] It was also isolated from the fruits (0.018% w/w),[139] but appeared absent in the roots.[140] Rubusoside (**33**) was the first diterpene glycoside ever extracted from a species in the Rosaceae family. In a survey of 39 *Rubus* species, the presence of diterpene glycosides appeared limited to the leaves of *R. suavissimus* (*ent*-kaurane skeleton) and *R. chingii* (*ent*-labdane skeleton).[141] Instead, the leaves of most *Rubus* species characteristically synthesize ursane-type triterpene glycosides,[142] whereas in *R. suavissimus* and *R. chingii* those compounds are mainly found in the roots.[140,143] In *S. rebaudiana*, the presence of **33** has been described only very recently.[71,85] Previously, it had been suggested that in vivo synthesis of **33** in *S. rebaudiana* is unlikely due to the apparent inability of purified UGT protein extracts to glucosylate **33**.[144] On the other hand, **33** was effectively synthesized by cell suspension cultures of *Eucalyptus perriniana* and *Coffea arabica* after incubation with steviol (**19**) (10.6 and 7.5% w/w, respectively).[145]

Besides rubusoside, steviolmonoside (**35**) was also first isolated from *R. suavissimus* before it was identified in *S. rebaudiana*, occurring at concentration levels of 0.004 to 0.08% w/w.[143,146] Among 14 leaf samples, the content of **35** varied the most, when compared to four other marker compounds for *R. suavissimus*.[146]

In the 1990s, researchers from the University of Hiroshima purified a series of *ent*-kaurane-type diterpene glycosides, named suaviosides, from methanolic leaf extracts of *R. suavissimus* (Figures 1 and 2, Table 3).[143,147−150] These include 10 true steviol glycosides, which have so far not been identified in *S. rebaudiana* (series 1 and 2 of suaviosides C, D, and Q−S). The molecules attached to the steviol (**19**) skeleton vary from glucose and galactose to caffeoyl and coumaroyl moieties. Although most of them have a neutral or bitter taste, some are very sweet (suaviosides Q−S). Because their content within the leaves is rather low (maximum about 0.05% w/w for individual suaviosides), they are economically less interesting economically than the steviol glycosides from *S. rebaudiana*.

Steviol (**19**) itself was also extracted from the leaves of *R. suavissimus*.[151,152] Starting from 10 kg of dry material, Wang and Lu[151] obtained 40 mg of **19**, or 4 μg/g dry wt, which falls in the same range as the amount of **19** detected in dry leaves of *S. rebaudiana* (0.3−30 μg/g dry wt).[84,85,134]

***Stevia phlebophylla* A. Gray (Asteraceae).** The Mexican species *Stevia phlebophylla* A. Gray was first collected by American botanist Edward Palmer on the shaded hillsides near the Rio Blanco, Jalisco, Mexico, in 1886.[153] Three collections were made before 1902, when the species was presumed extinct.[154] Recently, the species resurfaced during a study on the cytogeographical distribution of Mexican *Stevia* species.[155] In the literature, *S. phlebophylla* has been mentioned only sporadically[153,154,156,157] before it was included in an organoleptic evaluation of 110 different *Stevia* species.[158,159] An 1889 herbarium specimen of *S. phlebophylla* held at the Field Museum of Natural History, Chicago, IL (voucher specimen Pringle 2291),[158] was among 18 species tasting slightly sweet. After screening all 110 *Stevia* species for the presence of steviol glycosides, only *S. phlebophylla* was found to contain traces of stevioside (**37**).[2] In the same study, the long stability of steviol glycosides was confirmed by the detection of stevioside (**37**), and rebaudiosides A (**38**) and C (**42**) in a 1919 specimen of *S. rebaudiana*. The presence of **37** in *S. phlebophylla* was shown by analytical TLC and HPLC, and by GC/MS detection of isosteviol methyl ester, which is typically formed after acid hydrolysis of steviol glycosides and subsequent methylation.[2] However, a recent re-evaluation of two herbarium specimens of *S. phlebophylla* A. Gray, held at the New York Botanical Garden (1889 specimen Pringle 2291 and 1902 specimen Pringle 9981), failed to detect any steviol glycosides, based upon extensive HPLC and MS analyses.[160] Eluting at an almost similar retention time as **37**, a xylosylated derivative of acetylated suavioside E, 16*β*-hydroxy-17-acetoxy-*ent*-kauran-19-oic acid-(6-*O*-*β*-D-xylopyranosyl-*β*-D-glucopyranosyl) ester (**44**), was isolated and identified by ^{1}H and ^{13}C NMR spectroscopy. After acid hydrolysis, a compound was obtained having a molecular mass similar to isosteviol (**43**), but with a slightly different fragmentation pattern in MS. It was suggested that the carbocation formed under acid catalysis is not stabilized by a Wagner−Meerwein rearrangement as with steviol glycosides, but possibly by a hydride shift leading to a C-17 aldehyde.[160] To confirm unambiguously that *S. phlebophylla* does not produce steviol glycosides, this C-17 aldehyde should be coanalyzed with the acid hydrolysate of the original herbarium specimen of *S. phlebophylla* in which stevioside (**37**) was apparently detected. Nevertheless, it is plausible that *S. rebaudiana* remains the only known steviol glycoside-producing *Stevia* species, with only 50% of them so far having been investigated.[2,158,159] Of the approximately 14 *Stevia* species occurring in Paraguay, five have not yet been tested

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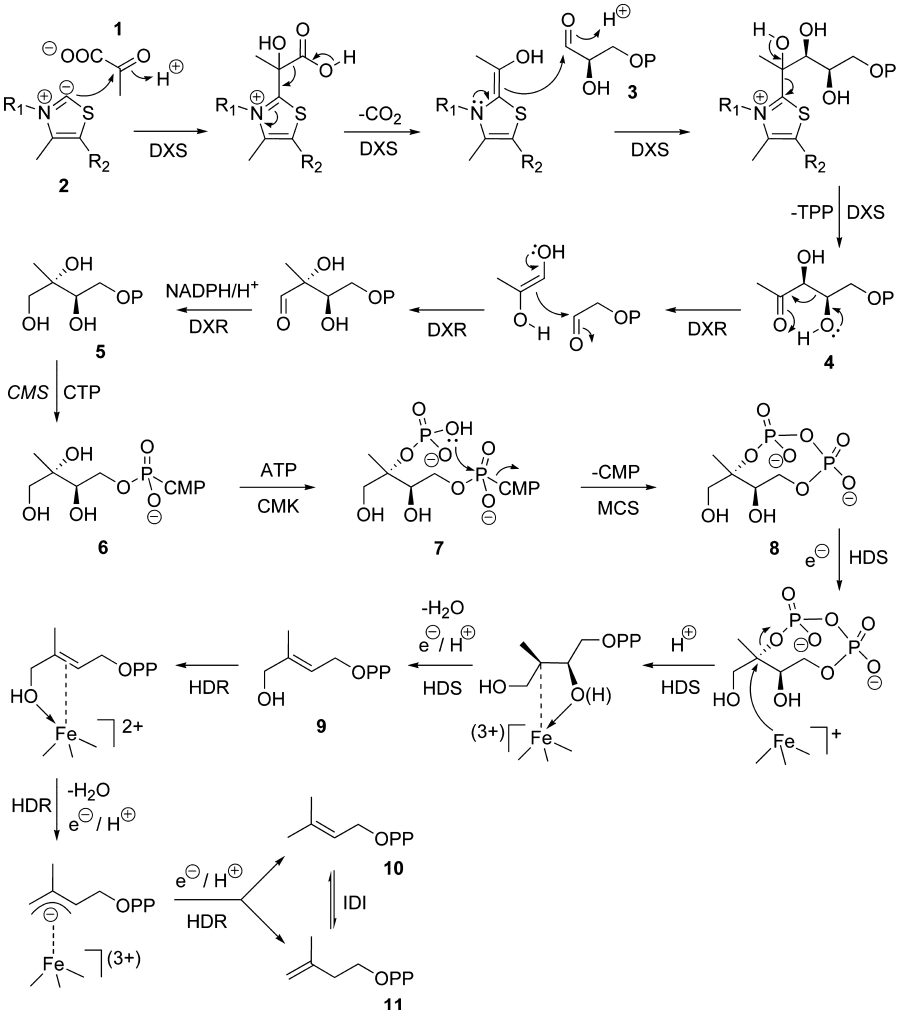
Scheme 1. Methylerythritol 4-Phosphate Pathway Leading to Isopentenyl Pyrophosphate (11)[a]



[a]Abbreviations: CMS: 4-diphosphocytidyl-2-*C*-methyl-D-erythritol synthase; DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS: 1-deoxy-D-xylulose 5-phosphate synthase; HDR: (*E*)-4-hydroxy-3-methylbut-2-enyl pyrophosphate reductase; HDS: (*E*)-4-hydroxy-3-methylbut-2-enyl pyrophosphate synthase; IDI: isopentenyl pyrophosphate isomerase; MCS: 2-*C*-methyl-D-erythritol 2,4-cyclopyrophosphate synthase; TPP: thiamine pyrophosphate.

for the presence of steviol glycosides, namely, *S. amambayensis*, *S. apensis*, *S. estrellensis*, *S. parvifolia*, and *S. rojassii*.[2,161]

***Angelica keiskei* (Miq.) Koidz. (Apiaceae).** Using IR and both ^{1}H and ^{13}C NMR spectroscopic analyses, Zhou et al.[4] identified octa-acetylated rubusoside in a purified ethanolic leaf extract of the Japanese perennial *Angelica keiskei* (Miq.) Koidz. Starting from 20 kg of dried leaves, about 2.2 g of the steviol glycoside was obtained, or 0.011% w/w. Previously, this molecule had been chemically analyzed in a biotransformation study with steviol (**19**).[145]

Occurrence of Steviol (19). Apart from *S. rebaudiana* and *R. suavissimus*, **19** has been identified in few other plant species. Extensive GC/MS analyses of the seeds of *Cucurbita maxima* revealed its presence in the cellulase-treated endosperm of seeds with maturity index 61%.[162] Steviol (**19**) was additionally isolated from purified hexane extracts of the roots of several Indian mangroves, including *Bruguiera gymnorhiza* (L.) Savigny (Rhizophoraceae) (7 μg/g dry wt),[163] *Bruguiera cylindrica* (L.) Blume (Rhizophoraceae) (43 μg/g dry wt),[164] and *Ceriops decandra* (Griff.) W. Theob. (Rhizophoraceae) (13 μg/g dry wt).[165] In the latter species, isosteviol (**43**) was also found, at 8 μg/g dry wt. Besides the recent discovery of its monoglucosylester in *S. rebaudiana* leaves,[80] **43** has been reported in only one other species, namely, *Mirabilis jalapa* L. (Nyctaginaceae) (104 μg/g dry wt in the tubers).[166]

BIOSYNTHESIS OF STEVIOL GLYCOSIDES

After the diterpenoid nature of steviol (**19**) was discovered,[62] the involvement of the classic acetate−mevalonic acid pathway in its biosynthesis was investigated, using labeled precursors. While labeled acetate could be successfully incorporated in the steviol (**19**) skeleton,[98] incorporation yields of labeled mevalonic acid were none[98] to very limited.[167] Further research claimed effective incorporations of *ent*-kaurene (**15**) and *ent*-kaurenoic acid (**18**) into **19**.[167,168] The discovery of the alternative methylerythritol 4-phosphate pathway in the beginning of the 1990s[169] made it clear that the lack of incorporated label in certain types of isoprenoids is due to the evolutionary divergence of the two biosynthetic pathways.[170] While the cytosolic acetate−mevalonic acid pathway is mainly involved in the biosynthesis of sesqui-, tri-, and polyterpenes, the plastidial methylerythritol 4-phosphate pathway contributes

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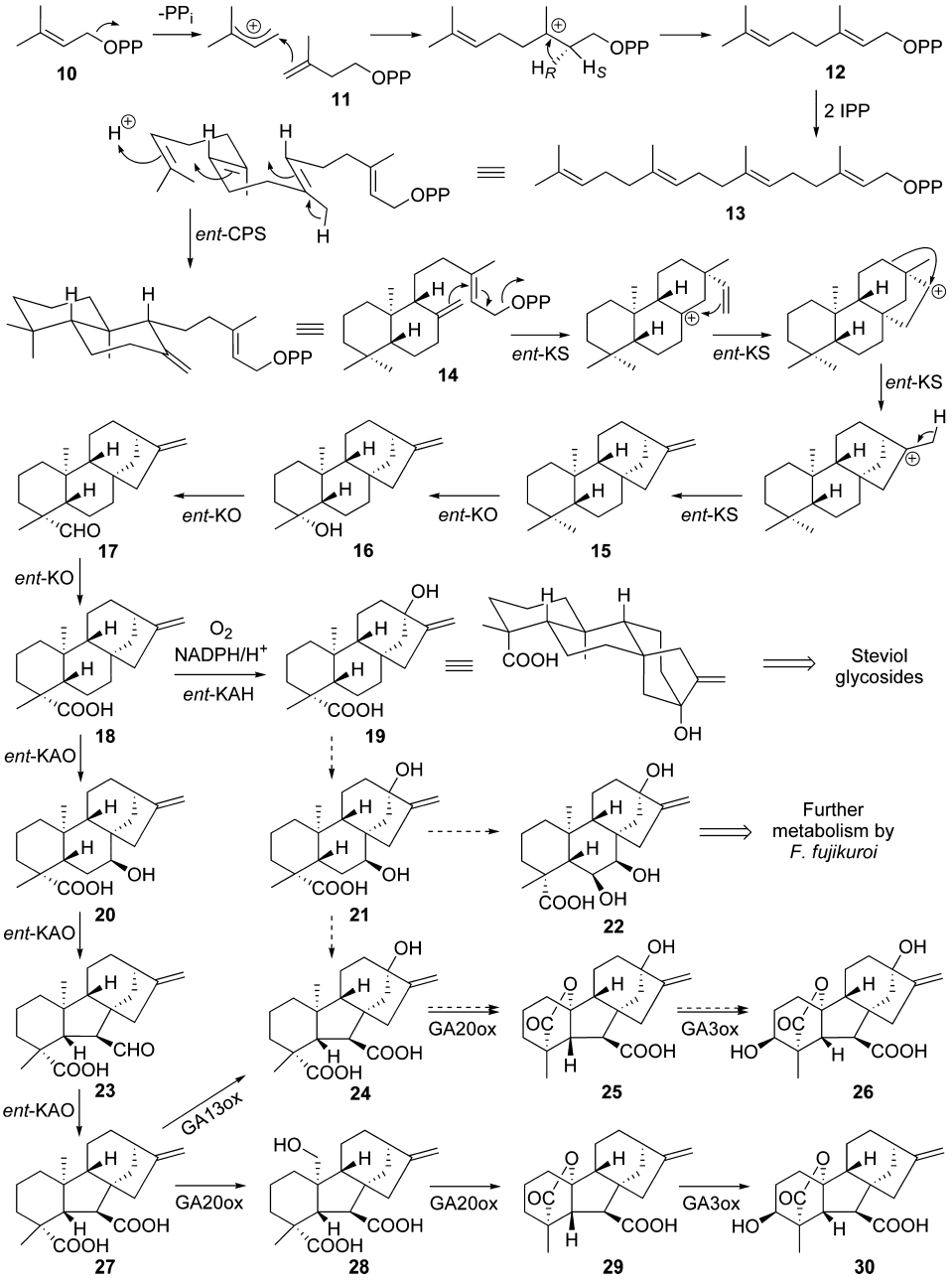
Scheme 2. Biosynthetic Pathway to Steviol (19) and the Gibberellins[a]



[a]Marked by dashed lines is a possible alternative route to C-13 hydroxylated gibberellins in plants (from steviol (**19**) to GA_{20} (**25**) and GA_1 (**26**)), which, at the same time, includes some of the major metabolites obtained after incubation of **19** by *Fusarium fujikuroi*, mutant B1-41a. Abbreviations: *ent*-CPS: *ent*-copalyl pyrophosphate synthase; *ent*-KAH: *ent*-kaurenoic acid 13-hydroxylase; *ent*-KAO: *ent*-kaurenoic acid oxidase; *ent*-KO: *ent*-kaurene oxidase; *ent*-KS: *ent*-kaurene synthase; GA2ox: GA 2-oxidase; GA3ox: GA 3-oxidase; GA13ox: GA 13-oxidase; GA20ox: GA 20-oxidase.

to the synthesis of mono-, di-, and tetraterpenes.[170−172] Extensive analysis of the intramolecular labeling patterns of incorporated [1-^{13}C]-glucose finally revealed the involvement of the methylerythritol 4-phosphate pathway in the early steps of the biosynthesis of **19** (Scheme 1).[173] The discovery of a minimal level of crosstalk between both pathways[174] might explain the contradictory results obtained earlier with labeled mevalonic acid[98,167] as the result of label contamination. By analyzing the intramolecular isotopic distribution instead of measuring the presence or absence of label, a clear distinction between both pathways can be made.

In the first step of the methylerythritol 4-phosphate pathway, pyruvate (**1**) and glyceraldehyde 3-phosphate (**3**) are converted to 1-deoxy-D-xylulose 5-phosphate (**4**), in a reaction catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase via its coenzyme thiamine pyrophosphate (**2**). This deoxy sugar is then reduced by 1-deoxy-D-xylulose 5-phosphate reductoisomerase to 2-*C*-methyl-D-erythritol 4-phosphate (**5**) involving a reverse aldol−aldol reaction coupled with a reduction by NADPH. After a cytidylation (**6**), followed by a phosphorylation and cyclization (**7**), 2-*C*-methyl-D-erythritol 2,4-cyclopyrophosphate (**8**) is formed. In the next step, this molecule is reduced to (*E*)-4-hydroxy-3-methylbut-2-enyl pyrophosphate (**9**) by a complex

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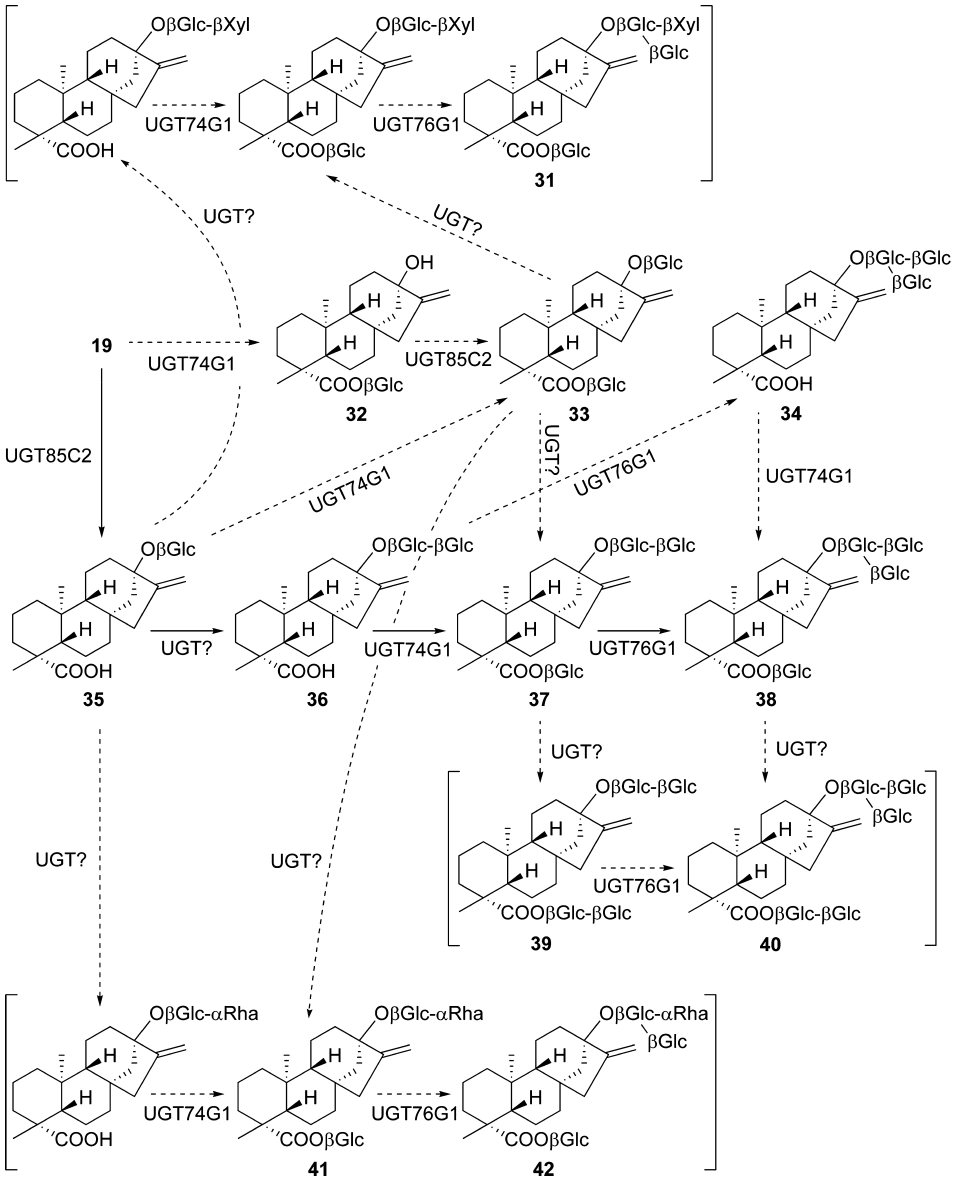
Scheme 3. Biosynthetic Pathways of the Major Steviol Glycosides[a]



[a]Solid lines represent the main biosynthetic steps known to occur in vivo; dashed lines are either biosynthetic steps that are catalyzed in vitro but may not be favored in vivo (the pathways to stevioside (**37**) and rebaudioside A (**38**) via rubusoside (**33**) and rebaudioside B (**34**), respectively) or hypothetical steps that have not yet been characterized in vitro nor in vivo (the pathways to dulcoside A (**41**) and rebaudiosides C (**42**), D (**40**), E (**39**), and F (**31**)). The latter have been placed between brackets.

bioorganometallic reaction mechanism involving direct iron−carbon interactions of **8** with the [4Fe-4S] cluster-containing domain of (*E*)-4-hydroxy-3-methylbut-2-enyl pyrophosphate synthase.[175] A second reduction, catalyzed by (*E*)-4-hydroxy-3-methylbut-2-enyl pyrophosphate reductase via a similar mechanism,[176,177] leads to both isopentenyl pyrophosphate (**11**) and dimethylallyl pyrophosphate (**10**), usually in a ratio of 85:15.[178]

In the next phase of isoprenoid biosynthesis, monomers of **11** are used to construct the building blocks of the different isoprenoid families. Steviol (**19**) is a diterpene, of which the building block, *trans*-geranylgeranyl pyrophosphate (**13**), consists of four units of **11** (Scheme 2). Each unit is attached to another by a series of electrophilic additions, involving the stereospecific loss of a proton. The final such addition leading to **13** is catalyzed by *trans*-geranylgeranyl pyrophosphate synthase. Due to the prochirality of **13**, the subsequent protonation-initiated intramolecular cyclization leads to two main enantiomeric series, the normal and *ent* series. Both are diastereoisomers with opposite configurations at C-5, C-9, and C-10, which results in the *ent* series having negative $[\alpha]_D$ values, contrary to the normal series.[179,180]

In the case of steviol glycosides, this initial cyclization of **13** leads to *ent*-copalyl pyrophosphate (**14**), a reaction that is catalyzed by a terpene cyclase, *ent*-copalyl pyrophosphate synthase. An ionization-initiated cyclization of **14** by *ent*-kaurene synthase eventually leads to an *ent*-kauranyl cation that is stabilized by a Wagner−Meerwein rearrangement (1,2-alkyl shift) to form the tetracyclic *ent*-kaurene (**15**). In *S. rebaudiana*, this molecule was found to be relatively volatile as it is emitted from the headspace.[181] In the leaves, **15** was detected at 0.7 μg/g dry wt.[38]

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Following its formation, **15** then crosses the plastidial membrane and is transferred to the endoplasmatic reticulum, where it is oxidized to *ent*-kaurenoic acid (**18**) by a P450 monooxygenase, *ent*-kaurene oxidase. This reaction occurs in three steps, via the respective alcohol (**16**) and aldehyde (**17**). Situated at an important branching point, **18** can be hydroxylated at C-7 to *ent*-7α-hydroxykaurenoic acid (**20**) and further to the gibberellins, whereas hydroxylation at C-13 leads to steviol (**19**) and the steviol glycosides. In some plants (e.g., *Cucurbita maxima*), part of the pool of **18** is metabolized to kaurenolides, which possess a [C-4/C-6]-γ-lactone bridge, or to fujenoic acids (*seco*-ring B kaurenoids derived from **20**).[182−184] In gibberellin biosynthesis, the formation of **20** is immediately followed by a ring B contraction to GA_{12}-aldehyde (**23**) and further oxidation to GA_{12} (**27**). These reactions are catalyzed by a membrane-associated CYP450 enzyme, *ent*-kaurenoic acid oxidase. As the common precursor to all gibberellins, **27** then undergoes hydroxylation on either C-13 or C-20. Activity of GA13ox leads to GA_{53} (**24**) and the C-13 hydroxylated gibberellins, whereas GA20ox activity yields GA_{15} (**28**) and the non-C-13 hydroxylated gibberellins. While GA20ox is a soluble 2-oxoglutarate-dependent dioxygenase, the nature of GA13ox remains unclear.[185] In rice, two P450 enzymes have been identified that convert GA_{12} (**27**) into GA_{53} (**24**), one of which also possesses C-13 hydroxylating activity toward *ent*-kaurenoic acid (**18**), thus forming steviol (**19**).[186] In the next steps, gibberellins from both series are oxidized by GA20ox to GA_{20} (**25**) and GA_9 (**29**), which are both C_{19} gibberellins. The final step in the formation of bioactive gibberellins is the 3β-hydroxylation of GA_{20} (**25**) and GA_9 (**29**) to GA_1 (**26**) and GA_4 (**30**), respectively, by the dioxygenase GA3ox. In order to fine-tune the regulation of the pool of bioactive gibberellins, several deactivation mechanisms exist, the most prevalent being 2β-hydroxylation, which is catalyzed by the dioxygenase GA2ox. Other mechanisms include the formation of methyl or glucosyl ester derivatives,[187] or the conversion of gibberellins to their 16α,17-dihydrodiols via the 16α,17-epoxide, the latter reaction which, in rice, is catalyzed by CYP714D1.[188] Interestingly, *Arabidopsis thaliana* contains at least two CYP714 enzymes showing this activity, one of which, CYP714A2, is an ortholog of the steviol synthase discovered in rice, and similarly C-13 hydroxylates *ent*-kaurenoic acid (**18**) to steviol (**19**).[186,189−192] Overexpression of both leads to dwarfism, with CYP714A1 producing the most severe effect.[193]

Recently, the major genes involved in the methylerythritol 4-phosphate pathway in *S. rebaudiana* were isolated and functionally characterized.[194−196] Other characterized genes in the biosynthesis of steviol (**19**) include *trans-geranylgeranyl pyrophosphate synthase*,[195] *ent-copalyl pyrophosphate synthase* and *ent-kaurene synthase*,[197] *ent-kaurene oxidase*,[144] and *ent-kaurenoic acid 13-hydroxylase*.[198−201] Both *ent-kaurene synthase* and *ent-kaurene oxidase* are duplicated in *S. rebaudiana* and, together with *ent-copalyl pyrophosphate synthase*, highly expressed in mature leaves. This is contrary to the pattern observed in other plants, where they are mainly involved in gibberellin biosynthesis and are only highly expressed in the very young, actively growing leaves.[144,197,202] Whether one copy is used for gibberellin synthesis and the other for steviol glycoside production is not known. Comparatively, two copies of *ent-copalyl pyrophosphate synthase* have been found in rice, one of which is involved in gibberellin biosynthesis and the other in phytoalexin biosynthesis.[203,204] Nevertheless, it appears that the biosyntheses of steviol glycosides and gibberellins are spatially and temporally separated in order to prevent possible interference with normal gibberellin metabolism.[197]

Initial research erroneously located *ent*-kaurenoic acid 13-hydroxylase in the stroma of the chloroplast.[205−207] Efforts to clone the gene based upon the published N-terminal sequence of the enzyme led to fructose 1,6-biphosphate aldolase.[6,8] Later, several sequences were published on the GenBank database, all belonging to a protein of 476 amino acids denoted as *ent*-kaurenoic acid 13-hydroxylase and classified as CYP716D4 (GenBank accessions ABD60225, ACD93722, and ACL10147). Recent studies on this protein involved structural analysis, 3D computational modeling, and expression studies in *E. coli*.[208,209] Semiquantitative RT-PCR analysis showed its expression in roots, stems, leaves, and flowers, with high transcript abundance in the latter two organs.[208] A molecular docking study found good affinity of this enzyme with steviol (**19**).[209] The gene was again ascribed as *ent-kaurenoic acid 13-hydroxylase* in an in vitro study on the effect of sucrose treatments on the transcription of steviol glycoside biosynthetic genes.[210] However, no functional characterization of the enzyme has been reported so far, and its inactivity toward *ent*-kaurenoic acid (**18**) in the WAT11−WAT21 yeast expression system has been suggested.[211] This expression system was specifically designed to test the functionality of CYP450 enzymes.[212]

After the formation of steviol (**19**), a series of glycosylations takes place in the cytosol, leading to the large family of the steviol glycosides (Scheme 3). Glycosylations usually stabilize, detoxify, and solubilize metabolites and are often considered as the end point of their biosynthetic pathways. In the case of steviol glycosides, these reactions are catalyzed by cytosolic UDP-dependent glycosyltransferases (UGTs). They transfer a sugar residue from an activated donor (mostly UDP-glucose) to an acceptor molecule. In leaf mesophyll of *S. rebaudiana*, the synthesis of UDP-glucose by UDP-glucose pyrophosphorylase was mainly located in the cytoplasm, with some activity retained in the Golgi body.[213] Plant UGTs are known to exert a broad substrate specificity, which is generally limited by a regiospecificity.[214] This was seen, for example, in cell suspensions of *Digitalis purpurea*, which were able to produce steviolbioside (**36**) and stevioside (**37**) after incubation with steviol (**19**).[215] In *S. rebaudiana*, more than a dozen UGTs have been detected,[216−220] yet only three have so far been clearly shown to contribute to steviol glycoside biosynthesis.[198,219,221]

Glycosylation of steviol (**19**) preferentially begins at the C-13 hydroxy group,[216] producing steviolmonoside (**35**) in a reaction catalyzed by UGT85C2 (Scheme 3). Next, **35** is glucosylated at the C-2′ of its C-13 glucose moiety to form steviolbioside (**36**). The UGT involved has so far eluded functional characterization, although a candidate UGT has been reported (UGT91D2).[222] A UGT74G1-catalyzed glucosylation of the C-4 carboxylic acid moiety of **36** then yields stevioside (**37**), followed by a UGT76G1-catalyzed glucosylation of the C-3′ of the C-13 glucose to form rebaudioside A (**38**). Recently, two UGTs having 98% homology with UGT76G1 were reported,[220,223] at least one of which showed a similar glucosylating activity toward **37**.[223]

Although stevioside (**37**) and rebaudioside A (**38**) are the most common steviol glycosides, the latter may not be the final product of the pathway. Further glycosylations likely take place, as steviol glycosides have been described bearing up to seven glycosyl moieties (rebaudioside O), thus considerably complicating the biosynthetic pathway. Additionally, no rhamnosyl- or

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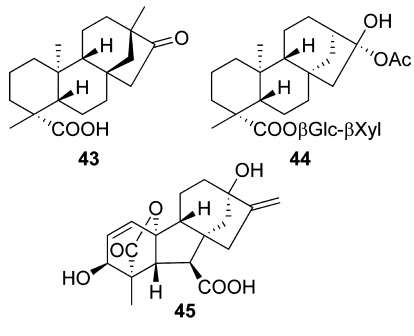
xylosyltransferases have been described yet, leaving the biosynthesis of, for example, dulcoside A (**41**) and rebaudioside C (**42**), or rebaudioside F (**31**), respectively, incomplete. Although the unknown UGTs involved perform similar actions (glycosylations with UDP-glucose, UDP-rhamnose, and UDP-xylose on the C-2′ of the C-13-glucose moiety), they presumably constitute different enzymes. UGTs usually have very strict sugar donor specificities even though their substrate specificity is markedly lacking selectivity.[224] Since the formation of rebaudioside C (**42**) from dulcoside A (**41**) involves the glucosylation of C-3′ of the C-13 glucose, this reaction is possibly catalyzed by UGT76G1.[225] In a similar way, this enzyme, together with UGT74G1, might be involved in the biosynthesis of rebaudiosides F (**31**) and D (**40**) (Scheme 3). Their involvement was further implied recently in patents describing the recombinant production of steviol glycosides, some of which reportedly used *A. thaliana* sequences in combination with UGT91D2 to enable the biosynthesis of **41**, **42** (*RHM2*), and **31** (*UXS3* or *UGD1*).[222,226−229]

The pathway described above is thought to occur mainly in planta, yet incubations with purified UGTs revealed a wider range of potential biosynthetic routes that also include rubusoside (**33**) and rebaudioside B (**34**) (Scheme 3). These assays yielded higher amounts of intermediates than are present within *S. rebaudiana* leaves, especially **33**.[144] Yet as previously noted, the inability of crude[216,218] or purified protein extracts[144] to use **33** as a substrate suggested that the glucosylation pathway via steviol-19-*O*-*β*-D-glucopyranosyl ester (**32**) and **33** does not occur in vivo or at best merely occurs as a relatively unproductive shunt. The latter hypothesis may be favored because of the recent detection of **33** in *S. rebaudiana* leaf extracts.[71,85,230] Possibly, the pathway via **33** is prevented by a rapid metabolic channeling from steviol (**19**) to steviolbioside (**36**) via steviolmonoside (**35**). In fact, at least some degree of regulation of the multiple glycosylations can be assumed because the UGTs are not likely to occur just freely in the cytoplasm together with all their potential substrates, especially given their lack of strict substrate specificities.[8] It was suggested that glycosylation by UGT85C2 is a key regulatory step of steviol glycoside biosynthesis due to a linear correlation between *UGT85C2* transcription and total steviol glycoside content observed in the upper leaves of plants growing under short-day conditions.[231] A regulation of some kind might also explain the significantly linear correlations observed between the transcript levels of *ent-kaurenoic acid 13-hydroxylase* and the three *UGTs* during vegetative growth.[232] One possible mode of regulation involves metabolon formation. However, extensive coexpression studies and FRET analyses of the three UGTs and *ent*-kaurene oxidase did not find indications of metabolon formation or substantial molecular interactions, indicating that any such mechanism is at best a weak and highly transient phenomenon.[144] Moreover, a prevention of rubusoside (**33**) synthesis by metabolon formation implies the involvement of the as yet undiscovered UGT catalyzing the formation of steviolbioside (**36**) and thus cannot be ruled out. Possibly, *ent*-kaurenoic acid 13-hydroxylase might also be involved. While a shunt via **33** may not be favored in the production of stevioside (**37**) and rebaudioside A (**38**), its role as an intermediate in the biosynthesis of rebaudioside C (**42**) and rebaudioside F (**31**) can also not be ruled out and awaits further elucidation (Scheme 3).[71]

While the exact role of rubusoside (**33**) in vivo is not clear yet, the presence of rebaudioside B (**34**) and, to a minor degree, steviolbioside (**36**) in leaf extracts has often been regarded as being mostly an artifact from the extraction procedure due to partial hydrolysis of rebaudioside A (**38**) or stevioside (**37**).[99] The C-19 glycosyl ester linkage is known to be the most heat-sensitive bond.[233] This was verified by the recent observation that **34** and **36** concentrations are very low in water extracts, but may increase during subsequent purification steps.[125]

After the formation of rebaudioside A (**38**) and stevioside (**37**), it is generally thought that they are stored in the vacuole.[8,234,235] In a subcellular fractionation experiment, 90% of the cellular **37** was located in the 12 000 g of supernatant, which contained vacuolar inclusions and soluble cytoplasm. The remaining 10% was equally divided in the other fractions, inclusive of nuclei, chloroplasts, mitochondria, Golgi, endoplasmatic reticulum, and microbodies.[236] Nothing is known yet about the mechanism facilitating vacuolar sequestration. Although a trans-Golgi network-independent membrane vesicle-mediated transport to the vacuole has been described for certain flavonoids (mainly anthocyanins)[237,238] and alkaloids,[239] such a mechanism seems an unlikely explanation for steviol glycoside transport, as they are being produced in the cytoplasm. Their translocation across the tonoplast likely involves steviol glycoside-sensitive transporter proteins, which are energized either by the transmembrane electrochemical gradient [secondary carriers such as multidrug and toxin efflux (MATE) transporters] or directly by the hydrolysis of ATP [ATP-binding cassette (ABC) transporters].[8] Secondary activated carriers have been implicated in the vacuolar transport of endogenously glycosylated compounds, whereas ABC transporters are often involved in the detoxification of xenobiotics. This way, the same molecule can be transported by different mechanisms in various plant species, depending on its species specificity. Such a phenomenon was observed with, for example, the flavone glucoside saponarine apigenin 6-*C*-glucosyl-7-*O*-glucoside, which is specific to barley, but not to *A. thaliana*.[240] MATE transporters are currently thought to play a larger role in the vacuolar sequestration of secondary metabolites than do ABC transporters and have been implicated in the transport of proanthocyanidin precursors[241,242] and alkaloids.[243]

In *S. rebaudiana*, only one study has so far reported transporter activities. Using cytochemical lead precipitation, ATPase activities were found to increase in the tonoplast as leaves matured, with simultaneous decreases occurring in the plasmalemma. Both activities decreased rapidly in senescent leaves.[244] Several candidate genes coding for MATE and ABC transporters have been found in an annotated EST collection, some of which may be involved in steviol glycoside transport.[8]



ACCUMULATION OF STEVIOL GLYCOSIDES IN *STEVIA REBAUDIANA*

The accumulation of steviol glycosides within *S. rebaudiana* has emerged as a dynamic process, complicating the extrapolation of results obtained with one cultivar, grown under specific conditions, to the species level. This is at least partially caused by a significant genetic variability. Considerable levels of variance are seen not only between plants of the same cultivar, but even between similar plants in the same developmental stage. For example, in a field study with 300 *S. rebaudiana* plants, total steviol glycoside content varied between 0.5 and 3.7% w/w at the seedling stage and between 6.7 and 18.6% w/w at harvest time.[245] Even within a population of in vitro propagated plants, a significant variation in steviol glycoside levels remained, necessitating the pooling of large amounts of leaves to obtain reliable results.[230,232] Nevertheless, despite these issues, a few general observations can be made regarding steviol glycoside accumulation in *S. rebaudiana*.

In Vivo Accumulation Dynamics. Scientific reports on the accumulation of steviol glycosides and their seasonal variation in *S. rebaudiana* were first published in the early 1970s.[102,104,246] These and later investigations have shown that steviol glycosides accumulate mostly within the leaves, followed by flowers, stems, seeds, and roots.[230,247] In leaves, steviol glycosides are synthesized during vegetative growth until their maximal level is reached between the end of flower bud formation and initial flowering.[104,230,248] In the northern hemisphere this coincides with the end of the summer period or early autumn. During flowering, a 20 to 50% decrease in steviol glycoside levels has been reported,[104,247,249−255] while others have rather observed a stagnation.[246,256] In cultivars exhibiting a strong outgrowth of lateral shoots, absolute and % w/w steviol glycoside yield per plant might even significantly increase during flowering.[230] These variations not only are related to the use of different genotypes or methodologies but are also strongly influenced by photoperiod. For example, using the same cultivar and methodology, steviol glycoside levels decreased about 25% in plants flowering under short-day regimes of 8 h, while a stagnation was seen in those under 16 h long days.[230] Successive harvesting plays a role in steviol glycoside production as well, with one report noting 40% more stevioside (**37**) in leaves after a second harvest.[257]

The level of steviol glycosides not only changes throughout ontogeny but also differs spatially with the plant canopy. The % w/w steviol glycoside content is usually highest in young, actively growing leaves near the apex and steadily decreases toward the lower, senescent leaves.[217,230,247,248] However, the reverse pattern was seen in plants flowering under 8 h short days.[230] When expressed as absolute amounts (e.g., mg per leaf), the spatial pattern of steviol glycoside levels is somewhat similar to the distribution of leaf dry wt, with the highest amount usually measured in midstem leaves and the lowest levels near the apex and base of the plant.[230]

Besides variations in total steviol glycoside levels, their individual composition can also vary significantly, depending on genotype, ontogeny, and photoperiod. Due to the commercialization of rebaudioside A (**38**) as the preferred steviol glycoside for sweetening purposes, intensive selection and cultivation practices have yielded cultivars with markedly altered steviol glycoside compositions in their leaves, both in vitro and in vivo.[24,71,258,259] One parameter to evaluate cultivars for the commercial extraction of **38** is the ratio of its concentration against that of stevioside (**37**) within the leaves. Within the same cultivar, this ratio appeared to be relatively stable during vegetative growth, but did increase about 35% in the lower leaves during reproductive development. Similarly to the individual steviol glycoside pools, this pattern was also influenced by photoperiod and genotype.[230]

In the stems, the highest steviol glycoside content is usually found near the apex (up to 1.5% w/w) and gradually decreases to about 0.5% w/w near the base.[230,247] Compared with leaves, the individual composition of the steviol glycoside pool is different in the stems and is likewise influenced by genotype and photoperiod.[230] Bondarev et al.[247] noted equal amounts of stevioside (**37**) and rebaudioside A (**38**) in the stems, a composition that, in other investigations, was only noted at the end of vegetative growth.[230] Both proportions decreased rapidly during reproductive development, while those of rebaudioside C (**42**) and dulcoside A (**41**) increased significantly. On the other hand, Higashi et al.[217] measured 2-fold more stevioside (**37**) than rebaudioside A (**38**) in stems.

One of the first studies on the reproductive organs reported up to 18% w/w stevioside (**37**) within the flowers.[249] This amount was refuted by others, who found between 1 and 4.7% w/w.[19,102,247,256,260] Within these organs, most steviol glycosides accumulate within the green involucres (5−7% w/w), with minor amounts present in petals (0.7% w/w), pistils (0.6% w/w), and achenes (0.2−1.5% w/w).[230] Some researchers mainly found stevioside (**37**) in the flowers and the seeds,[19,247] while others noted a very high ratio of rebaudioside A (**38**) to stevioside (**37**) (around 4−5), especially in juvenile white petals and achenes. These values decreased to 1.3 and 3.5 in withering petals and ripened achenes, respectively.[230] Analysis of the achenes for **37** showed its presence in the embryo and achene wall of both light-colored, fertile and dark-colored, sterile achenes.[261]

In the roots, steviol glycoside content usually comprises 0.1% w/w or even less.[217,247] One report mentions a 7-fold increase in % w/w steviol glycoside content after the transition to reproductive development (from 0.05 to 0.35% w/w).[230] At the same time, the ratio of rebaudioside A (**38**) to stevioside (**37**) in the roots increased from 0.4 to 2.5, while the proportion of rebaudioside C (**42**) increased to about 50%. On the other hand, others have detected only **37** in the roots,[217] or equal amounts of **37**, **38**, and **42**,[247] while some could not detect steviol glycosides at all.[256,262] In comparison, hairy root cultures of *S. rebaudiana* were unable to produce steviol glycosides, indicating an impaired biosynthetic capacity in roots,[263] even though these organs are able to express a capacity for carbon autotrophy.[264] The overall lack of significant steviol glycoside production in *S. rebaudiana* roots is consistent with the very low transcription of *ent-copalyl pyrophosphate synthase*, *ent-kaurene synthase*, *ent-kaurene oxidase*, and the three known *UGTs* in these organs.[144,197] Possibly, the expression of the former three genes is mainly limited to that involved in gibberellin biosynthesis occurring in the root tip.[197,265]

On the basis of a positive correlation between gland distribution and steviol glycoside content in the leaves, both in vivo and in vitro, it was suggested that steviol glycosides may be secreted in glandular trichomes throughout ontogeny.[266,267] *S. rebaudiana* bears two kinds of nonglandular trichomes ("long hair" and "short hair"), besides a peltate glandular type.[33,267−269] Both kinds are found on leaves and inflorescences, but they are generally larger on the latter.[267] Any steviol glycosides present in these structures may have been

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synthesized in situ, as the glandular head bears at least three pairs of chloroplast-containing cells. Colorimetric assays revealed the presence of phenolic compounds, sesquiterpenes, and alkaloids within the trichomes of *S. rebaudiana*.[33] Significant amounts of terpenoids were found only in the subcuticular space of the peltate glands[33] and might possibly be related to the previously mentioned *ent*-kaurene (**15**) emissions occurring in this species.[181] Monteiro et al.[268] suggested that the trichomes of *S. rebaudiana* contain sesquiterpene lactones to protect the plant against herbivory. These compounds have been found in several *Stevia* species,[270,271] but none have so far been reported in *S. rebaudiana*. Regardless, while some diterpenes are known to accumulate in glandular trichomes,[272] there is no direct proof yet for the accumulation of steviol glycosides in these structures.

Besides the analysis of steviol glycosides in *S. rebaudiana*, the content of free steviol (**19**) has also been reported recently.[84,85,134] Measured in μg/g dry wt or ppm, **19** was detected in reproductive apexes (up to 37 ppm), leaves (up to 30 ppm), stems (<5 ppm), and roots (<2 ppm), although the latter could not be unambiguously verified by MS.[134] The large difference between the amount of **19** and that of its glycosidic conjugates indicates that **19**, although synthesized in large amounts, is continuously and rapidly glycosylated to stevioside (**37**) and rebaudioside A (**38**). In the leaves, the main pattern of its spatiotemporal distribution that was largely independent of the spatial position of the leaf, photoperiod, or genotype, was a decline seen after flower opening.[134] Possibly, this phenomenon is related to a significant decrease of the transcript levels of *ent-kaurene synthase*, *ent-kaurenoic acid 13-hydroxylase* and the three *UGTs* during the transition to flowering, after they had remained constant throughout vegetative growth in both young and mature leaves.[232] Whereas this would at least result in a stagnation of steviol glycoside levels, it does not explain the mechanism of their subsequent photoperiod-dependent decline as previously noted.

Research has focused not only on the patterns of steviol glycoside accumulation but also on its relationship with other parameters. Early work reported linear relationships between leaf biomass on one hand and branch number, plant height, leaf area, and leaf thickness on the other hand.[273−276] Positive correlations were also found between stevioside (**37**) levels and leaf area or root number,[277] while others did not find a relationship between the content of **37** and leaf yield or the leaf to stem ratio.[278]

When the steviol glycoside pool is considered, there appears to be little agreement on the correlation between the amounts of individual steviol glycosides measured within the leaves, despite their known biosynthetic relationship. Some researchers claimed the amounts of rebaudioside A (**38**) and stevioside (**37**) are not related.[248,273,276,279] Others found negative correlations between **37** and **38** and between rebaudioside C (**42**) and dulcoside A (**41**) (r = −0.70),[245] while positive correlations between **37** and **41** and between **38** and **42** have also been reported (r = 0.82−0.90).[245,276] Under 16 h long days, an inverse correlation was noted between the ratio of **38** to **37** on one hand and leaf dry matter and steviol glycoside content per leaf on the other.[230] Previously, this ratio was found to be correlated only to leaf thickness (r = 0.81).[276] These contradictory results again make it difficult to state general principles on steviol glycoside metabolism at the species level. At the same time, they denote the remarkable versatility of growth morphology and steviol glycoside accumulation across different genotypes. In one study, correlations between **37**, **38**, **41**, and **42** showed a high variability, depending on the prevalence of glucosylated (**37**, **38**) over rhamnosylated steviol glycosides (**41**, **42**) in the total steviol glycoside pool of individual plants.[245] Part of the contradictions can also be explained by the amount of data points included in the correlation analysis and by the way of data representation. Regarding the latter, less significant correlations were found in case steviol glycoside amounts were expressed as amount per unit biomass (% w/w) than when they were calculated as amounts per leaf.[160] As such, strong significant correlations were found between the amounts of **37**, **38**, and **42** (r > 0.90), albeit in a photoperiod-dependent way (see the Photoperiodism section).[160,230]

The above-mentioned observations make it clear that the observed accumulation of steviol glycosides is a complex and dynamic process, reflecting an underlying physiology and biochemistry that is currently not yet fully understood. As discussed below, the picture is complicated even further by the influence of various abiotic parameters on the accumulation of steviol glycosides.

In Vitro Accumulation Dynamics. The effect of propagation techniques on the steviol glycoside content of *S. rebaudiana* has been investigated on several occasions. While some reports claim that steviol glycoside levels are not affected by the method of propagation,[280] others have indicated less heterogeneity of growth patterns and steviol glycoside profile in in vitro propagated plants, compared to plants grown from seeds, cuttings, and stem tip cultures.[281,282] Moreover, total steviol glycoside levels were 5−10-fold less in leaves and stems of in vitro plants, although their individual composition was similar to those cultivated in vivo.[266,282,283]

In vitro culturing of explants is usually done on a Murashige and Skoog medium supplemented with auxins and cytokinins. Under these conditions, one study reported a dependency of de novo synthesis of steviol (**19**) on root development, whereas *both* shoots and roots were capable of steviol glycoside production.[284] Two explanations were suggested: a stevioside (**37**) precursor or derivative was produced in the roots and transported to the leaves for further metabolism, or the roots produced a factor required to elicit biosynthesis of **37** in the leaves. This dependency seen in vitro seems remarkable given the very low steviol glycoside levels commonly reported for in vivo roots. Later research refuted these findings, as a clear relationship was found between steviol glycoside accumulation and shoot rather than root development,[266] the latter which itself depends on photoperiod and leafiness of the shoot.[285]

Several studies have been done on the steviol glycoside biosynthesis in callus and cell suspension cultures. These cultures were able to synthesize only minor amounts of steviol glycosides, with large variations noted during culture growth.[282,286,287] In calli, only equal stevioside (**37**) and rebaudioside B (**34**) and minor steviolbioside (**36**) amounts were measured, whereas cell suspensions mainly contained **37**, with no steviol glycosides present in the medium.[282] Hsing et al.[260] observed a 50% decrease in the content of **37** during a period of dedifferentiation, even though the calli still contained concentration levels of **37** up to 16% w/w, which is remarkably high. When incubated with steviol (**19**), callus cultures were shown to produce **37**.[288] Also in *R. suavissimus*, calli were found to produce rubusoside (**33**),[289] which was further promoted by blue light illumination after 28 days in the dark.[290] On the other

hand, blue light had no effect on the production of **37** in *S. rebaudiana* calli, nor did white or red light.[282]

Steviol Glycoside Biosynthetic Capacity and Differentiation. The limited capability of calli and cell suspensions to synthesize steviol glycosides seems closely related to their level of differentiation. The involvement of the methylerythritol 4-phosphate pathway and the vacuolar sequestration of steviol glycosides further indicate that on the subcellular level steviol glycoside biosynthetic capacity is linked with plastid and vacuole development. While plastids occur in all major plant organs, their differentiation to mature chloroplasts appears crucial for steviol glycoside biosynthesis to have a maximal output. Indeed, in situ hybridization studies have shown a much higher transcription of *ent-copalyl pyrophosphate synthase* and *ent-kaurene synthase* in leaves compared to roots that was further limited to mesophyll and palisade parenchyma.[197] No transcript signals were seen in the epidermal, trichomal, and vascular tissues. Furthermore, in the young, upper leaves, or during the early vegetative stages of some species, ground tissue has not yet differentiated in palisade and spongy parenchyma.[291,292] This differentiation process has not been described in *S. rebaudiana*, but it is likely to play a role as well in the consolidation of the steviol glycoside biosynthetic pathway during early leaf development.

The morphological differentiation of both mesophyll and callus tissue has been investigated in a series of ultrastructural studies.[293−297] A correlation between organelle differentiation and the capacity for steviol glycoside biosynthesis was indirectly observed in cell cultures whose steviol glycoside content appeared not to be related with that in the donor plants.[282] In addition, this correlation was observed both in calli[298] and in vivo (in both space and time; Table 4).[234,299,300]

The importance of cellular and tissue differentiation in relation to the steviol glycoside biosynthetic capacity was seen further in callus manipulations. Increase of fresh and dry cell biomass of callus cultures by temperature, light intensity, or nutrient composition did not induce steviol glycoside production,[301] contrary to callus differentiation.[282,298] The dependency of steviol glycoside biosynthesis on differentiation might also explain the lesser steviol glycoside amounts measured in in vitro plants. Chloroplasts of epidermal and mesophyll leaf cells of in vitro plants have small thylakoids and exhibit markedly decreased functional activity compared to in vivo plants. These cells usually have a weakly developed endoplasmatic reticulum and Golgi apparatus with little contact between the organelles, indicating little metabolic activity.[283,302] It was also shown that etiolated in vitro cultures, which initially contained only proplastids, were devoid of any steviol glycoside biosynthetic activity, whereas transferring them to the light stimulated both chloroplast formation and steviol glycoside biosynthesis.[283]

Influence of Abiotic Factors on Steviol Glycoside Accumulation. Plant growth not only is determined by the genotype but is heavily influenced by a wide range of biotic and abiotic factors, such as light, day length, temperature, moisture, nutrient availability, soil types, and rhizosphere chemistry. How these factors influence the growth and development of *S. rebaudiana* has been the topic of several extensive reviews.[7,275,303,304] This section concerns the effect of some of those parameters on steviol glycoside accumulation.

Photoperiodism. *S. rebaudiana* is a short-day (SD) plant with a critical day length of about 12 to 13 h.[248,305−307] Therefore, *S. rebaudiana* will flower precociously when

Table 4. Relationship between Stevioside (37) Content and the Differentiation of Plastids and Vacuoles in *Stevia rebaudiana*

differentiation level		organ or tissue			content of stevioside (**37**)
		in vitro	in vivo		
plastids	vacuoles	callus	spatially	ontogenetically	
large, fully developed with complex thylakoid membranes, many grana and dense stromae	highly vacuolized; morphologically specialized vacuoles with inclusions (granules and vesicles) in vivo	slow-growing,[a] compact, green	mature, middle leaves	mesophyll of late vegetative plants (large membranous system; maximal level of vacuolar inclusions during floral initiation)	high
small with simple thylakoid membranes, few grana and lamellae; sometimes with starch grains	relatively small, high number, translucent with a round or elliptical shape	slow-growing, compact, yellow	young, upper leaves	mesophyll of early vegetative plants (small vacuoles connect to the central vacuole)	medium
simple structures with few lamellae and a reduction and/or disintegration of thylakoid membranes	decomposition and disappearance of vacuolar inclusions	fast-growing, loose, yellow	lower, senescent leaves	mesophyll of flowering plants (thin layer of electron-dense material at the tonoplast)	low

[a]With or without shoot formation.

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cultivated in SD conditions of <12 h. Yet considerable differences have been noted between cultivars concerning the time of flowering, the number of flowers, and the optimal photoperiod to achieve the highest percentage of flowering plants.[160,256,306,308] Long-day conditions on the other hand prolong vegetative growth by prohibiting early flowering, thus increasing internode length, leaf area, leaf biomass, total carbohydrate content, protein levels, and absolute steviol glycoside levels.[309] The optimal photoperiod for a high steviol glycoside yield was reportedly 16 h for diploid *S. rebaudiana* plants and 20 h for tetraploids.[310] Besides its effect on overall steviol glycoside levels, photoperiod also has a significant influence on the characteristics of their accumulation. Although the total steviol glycoside yield was much higher under 16 h long days, 30% higher levels of steviol glycoside production per unit biomass were noted under 8 h short days throughout the vegetative growth stage. At the same time, the rate of leaf production was 27% higher.[134,230] These results seem contradictory to earlier research reporting 42% lower steviol glycoside production per unit biomass and 16% lower rate of leaf production under short days.[309] The discrepancies are possibly due to differences in plant cultivation (seed-grown[309] or in vitro propagated[230]), while the rate of leaf production can further be influenced by genotype,[311] temperature,[312] and rate of plant height increase,[313] of which the latter, in *S. rebaudiana*, is influenced by ploidy.[314]

The recent notion of an increased rate of leaf production under short days was supported by a 2-fold higher level of leaf glucose when compared to similar plants under long days.[315] Short-day conditions usually result in higher photosynthetic rates and increased starch synthesis during the day, while starch mobilization during the night occurs at a lesser, more controlled rate.[316] The carbon flux directed toward the biosynthesis of secondary metabolites is likely to be more strictly regulated under these conditions. In *S. rebaudiana*, this flux is mainly divided between the shikimic acid and steviol glycoside biosynthetic pathways.[317] Besides an increased photosynthetic rate, mitotic activity in the apical region is known to increase after five short-day cycles, indicating an early formation of the transitional apex in this species.[318,319] Floral induction is likely to cause further starch mobilization due to the increased sink requirements of the apical region, resulting in higher carbohydrate influx from the source leaves.[320,321] Interestingly, under long days, a sharp increase in glucose levels was observed in the middle to upper leaves at the end of vegetative growth, possibly acting as a metabolic trigger for the phase transition to reproductive development.[315] These leaves were situated near the sink-to-source boundary, as derived from the diurnal carbohydrate variation in *S. rebaudiana*[315] and from the hypothesis that in situ photosynthesis becomes significantly contributory at 80% of full leaf expansion.[322]

A more controlled carbon flux toward the biosynthesis of steviol glycosides during short days of 8 h may explain part of the dependence of steviol glycoside accumulation patterns on photoperiodism. Under short days, total steviol glycoside levels were significantly more correlated with the amount of glucose per leaf (r = 0.80 and 0.46 for short and long days, respectively)[315] and, when expressed as steviol equivalents,[10,135] with the level of free steviol (**19**) (r = 0.52 and 0.16 for short and long days, respectively).[134] Earlier, linear correlations have been reported between steviol glycoside levels and total soluble carbohydrates within leaves of *S. rebaudiana* grown in field conditions under photoperiods of 12 to 13 h.[296] When plants were grown from seeds under a 16 h photoperiod, they showed an inverse correlation between total soluble carbohydrate content and leaf dry matter.[323] This again indicates the versatility of these correlations and their dependence on the allocation of available carbon.

Sucrose levels have shown larger variations in their relationship with dry matter and total steviol glycoside levels.[315] This may be due to not only the altered carbohydrate metabolism related to floral induction but also the function of sucrose as a vehicle for transport and storage of glucose. Intriguingly, a recent report suggested a role of sucrose as an enhancer of the transcription of steviol glycoside biosynthetic genes.[210] Whether the large sucrose fluctuations during vegetative growth under short days are related to the concomitant higher variations in steviol glycoside levels is currently not known. These variations were mainly due to stevioside (**37**), overall resulting in higher, but widely varying rebaudioside A (**38**) to stevioside (**37**) ratios during this stage and significantly less correlations between the levels of rebaudiosides A (**38**) or C (**42**) and stevioside (**37**) (r = 0.53 and 0.36, respectively). At the same time, **38** and **42** remained strongly correlated (r = 0.93).[160,230]

Taken together, it is possible that the altered carbohydrate metabolism is reflected by a less homeostatically balanced steviol glycoside pool during vegetative growth under short-day conditions, when compared to long days. Whether these variations are also reflecting alterations in UGT and vacuolar transporter activities is not known. Alternatively, the differences in fluctuations of rebaudioside A (**38**) and stevioside (**37**) may be linked to the differences observed in their respective reactive oxygen species (ROS) scavenging activities.

Steviol glycosides are known to be potent ROS scavengers.[324−326] While both have similar scavenging activities toward hydroxyl radicals, stevioside (**37**) is a stronger scavenger (IC_{50} = 1.5 mM) than rebaudioside A (**38**) for superoxide radicals (IC_{50} = 2.5 mM).[326] An enhanced degradation of **37** was also indicated in in vitro plants treated with polyethylene glycol-induced drought stress together with paclobutrazol. In these plants, total steviol glycoside levels decreased dramatically, while the ratio of **38** to **37** significantly increased.[327]

Regardless, given these observations, the higher biosynthetic rate of total steviol glycosides measured under short-day conditions seems remarkable. At the same time, floral opening resulted in much sharper declines in total steviol glycoside levels in the upper leaves, resulting in a reversed spatial distribution of % w/w steviol glycoside levels compared to those flowering under long days of 16 h.[230] Whether these observations indicate that steviol glycosides are being used as a carbon source for reproductive development under conditions where photosynthesis becomes more limiting awaits further elucidation.

These investigations show clearly that long-day conditions are highly beneficial for optimal plant growth and steviol glycoside production. Under natural conditions, long-day conditions of 16 h or more are found only during the summer period above 50° latitude. Yet in these regions, other abiotic factors such as temperature and irradiance are generally not ideal for the field production of *S. rebaudiana*.[328,329] One way to solve the issue of providing long-day regimes during short-day conditions is by directing photoreceptor responses. As light plays a tremendous role in plant growth, influencing photomorphogenesis by photoreceptor stimulation has become a major topic in plant physiology. Plants in which flowering is

initiated on the basis of photoperiod (or more specifically, the duration of the night), such as *S. rebaudiana*, are particularly sensitive to light-induced changes. Early experiments in this field used interruptions of the night with 1 h of weak incandescent light.[256,307] These conditions successfully sustained vegetative growth, while *S. rebaudiana* plants under normal 8 to 12 h photoperiods flowered early.[307,308] The shortest vegetative growth stage was seen during 8 h photoperiods, whereas plants under 12 h showed the highest flowering rate.

In order to optimize photoreceptor activation, red light was used recently instead of white light.[160,330] Red light activates phytochrome, a light-sensitive sensor pigment, which, in its active form, can dramatically alter the normal plant response to day length conditions. Phytochrome usually exists in two conformational states, which can rapidly interconvert. The inactive form, Pr, absorbs red light at A_{max} 650−670 nm, forcing it to undergo a rapid *cis*−*trans* conformational change to Pfr. This active form can again be inactivated by far-red illumination (A_{max} 705−740 nm). During long nights, Pfr is slowly and independently from light converted back to Pr (dark conversion). When the Pfr to Pr ratio is below a critical threshold, floral induction will be triggered (in short-day plants such as *S. rebaudiana*) or inhibited (in long-day plants such as *A. thaliana*). The complex chain of biochemical signals from the activity of Pfr, present in the leaves, to the induction or inhibition of homeotic genes in the apical meristem has not yet been fully elucidated, but has been extensively researched in, for example, *A. thaliana*[331] and rice.[332] When applied to *S. rebaudiana*, plants subjected to a midnight interruption of 5 min red light while growing under a photoperiod of 8 h was sufficient to mediate a phytochrome-regulated inhibition of early flowering normally seen under those conditions.[160,330] In fact, after nine weeks, a more than 3-fold higher steviol glycoside yield was obtained when compared to control plants (152 and 47 mg per plant, respectively).[160] Although these experiments were done under controlled greenhouse conditions, the method requires only a minimal amount of equipment and energy input, therefore allowing a scaling up to field level. Moreover, a cheap, commercially available LED strip with a wavelength around 631 nm proved equally effective as specialized modules of 660 nm.[160,330]

No significant differences were seen in the composition of the steviol glycoside pool and the rebaudioside A (**38**) to stevioside (**37**) ratio between red light-treated and control plants, suggesting that red light influences total rather than individual steviol glycoside amounts. This may possibly infer an influence of red light on early genes in the biosynthetic pathway rather than specific UGTs. However, in other plants, this influence is usually exerted on the last steps of the gibberellin biosynthetic pathway (e.g., on 3β-hydroxylation in *A. thaliana* and lettuce).[265,333] It is equally possible that the increased steviol glycoside levels in red light-treated plants are the result of the temporary inhibition of flowering rather than a direct consequence of Pfr action on the steviol glycoside biosynthetic pathway.

Light Intensity. The natural habitat of *S. rebaudiana* lies in a mountainous, subtropical region of South America, where the plant thrives in a warm, humid, and sunny climate. When plants were cultivated under 60% shading, dry leaf biomass production per plant declined about 60%, with each plant growing only half the amount of paired leaves compared to control plants.[334] Flowering was also affected, as shown by a delayed anthesis coupled with a lower percentage of flowering plants and lower flowering rate.[334,335] Increased shading regimes did not significantly alter the total % w/w steviol glycoside level or the rebaudioside A (**38**) to stevioside (**37**) ratio.[335] However, due to the diminished biomass production under shade, absolute steviol glycoside yield per plant was likely to be lower.

Besides shading, *S. rebaudiana* growth is also affected by the changes in solar radiation, not only due to variations in latitude but also because of the annual shift in solar altitude. Already by 1976, the inverse correlation between percentage stevioside (**37**) content and latitude was reported (r = −0.61 for n = 12).[336] Additionally, when photoperiodism changes from short to long days while at the same latitude, the intensity of irradiation becomes an increasingly important determinant affecting leaf biomass accumulation.[328,337] In one experiment, an irradiation of 100 W/m^2 during a 16 h photoperiod doubled the dry leaf biomass per plant when compared to an irradiation of 50 W/m^2 under the same photoperiod.[328] For in vitro production of steviol glycosides, optimal irradiance was shown to be 35 to 45 W/m^2, which was almost 3-fold lower than that for in vivo plants.[310]

Temperature. The effect of temperature on the growth of *S. rebaudiana* has been investigated on several occasions. Early work suggested that the optimal temperature range is 15−30 °C, although plants have survived temperatures as low as −3 °C.[102,273] Optimal growth and stevioside (**37**) production were obtained under a 25/20 °C (day/night) regime.[329] Although the % w/w content of **37** was lower in this regime compared to the control greenhouse condition (9.2 and 10.1% w/w, respectively), total yield per plant of **37** was 50% higher due to a concomitant increase in leaf biomass. Under a 15/10 °C regime, 40 or 60% lower values were measured for % w/w content of **37** and total leaf biomass, respectively, compared to plants grown under the 25/20 °C regime.[304,329] Nepovím et al.[280] nevertheless concluded that temperature is not an important parameter for the in vivo production of **37**.

Nutrients. Due to the economic importance of *S. rebaudiana*, many studies have reported on its nutrient requirements,[7] but relatively few have focused on the association between nutrient supply and steviol glycoside accumulation. Overall nutrient requirements are low to moderate due to the adaptation of the plant to relatively poor soils in its natural habitat.[7] Changes in the soil pH (4−8) affected plant growth without altering % w/w steviol glycoside levels.[308] Similarly, application of N-, P-, or K-based fertilizers did not change steviol glycoside levels, but did cause precocious flower budding.[279,338−340] Visual symptoms of nutrient deficiency in *S. rebaudiana* include changes in leaf color to dark green (P, B), yellow (N, K), or pale green (chlorosis; S, Mg), sometimes mottled (K) or with dark necrotic spots (Ca, K, S), and an impairment of overall growth (N, P). These deficiencies reduced steviol glycoside levels within the leaves, with the most severe decreases generally observed in Ca, K, and S deficiencies.[308,341−343] On the other hand, addition of 5 ppm B to hydroponically grown plants resulted in 50% higher yields of stevioside (**37**) and rebaudioside A (**38**).[344,345] Addition of Zn may also increase steviol glycoside levels significantly.[308] Studies with in vitro cultures further showed that the addition of 3−5% sucrose could effectively increase steviol glycoside production.[210,346]

Catabolism of Steviol Glycosides. As previously mentioned, maximal steviol glycoside levels are generally reached around the phase transition to flowering, followed by a decrease or stagnation, depending on photoperiodic

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Table 5. Overview of Bioassays Performed on Steviol (19)

bioassay	dose per plant	method[a]	bioactive response[b]	reference
dwarf *Zea mays* L. mutant *an-1* leaf sheath elongation	50−100 μg	microdrop	+	369
dwarf *Zea mays* L. mutant *d*-1 leaf sheath elongation	0.1−300 μg	microdrop	−	368, 369, 400
	100 μM	root dip	−[c]	366
dwarf *Zea mays* L. mutant *d*-2 leaf sheath elongation	50−100 μg	microdrop	−	369
dwarf *Zea mays* L. mutant *d*-3 leaf sheath elongation	0.1−300 μg	microdrop	−	368, 400
dwarf *Zea mays* L. mutant *d*-5 leaf sheath elongation	0.1−300 μg	microdrop	+[d]	368, 369, 400, 408
	100 μM	root dip	+[c]	366
dwarf *Oryza sativa* L. mutant Tan-ginbozu leaf sheath elongation	0.2−2 μg	microdrop	+[e]	371
	10 μg	microdrop	+	401
	50−100 μg	microdrop	−	369
	620 μM	shoot dip	+[f]	409
	100 μM	root dip	+	401
dwarf *Pisum sativum* L. stem elongation	50−100 μg	microdrop	−	369
dwarf *Pisum sativum* L. *var.* Meteor stem elongation	0.1−10 μg	microdrop	−	368
dwarf *Pisum sativum* L. *var.* Progress n° 9 stem elongation	0.1−100 μg	microdrop	−[g]	366
dwarf *Pisum sativum* L. *var.* Progress n° 9 (*lele* genotype) stem elongation	10 μg	microdrop	+[h]	373
dwarf *Pisum sativum* L. *var.* Alaska (*Le* genotype) stem elongation	10 μg	microdrop	+[i]	373
Lactuca sativa L. *var.* Grand Rapids hypocotyl elongation	1−1000 μM	seed imbibition	−	400
Lactuca sativa L. *var.* Arctic King hypocotyl elongation	0.001−10 μg	seed imbibition	−	368
Lactuca sativa L. *var.* Gigante I-1797 hypocotyl elongation	3−300 μM	seed imbibition	+[j]	374
	10 pM to 1 μM	seed imbibition	+[k]	367
Cucumis sativus L. hypocotyl elongation	50−100 μg	microdrop	−	369
Cucumis sativus L. *var.* Perfection Ridge hypocotyl elongation	0.1−10 μg	microdrop	−	368
Cucumis sativus L. *var.* Meio Longo hypocotyl elongation	30−300 μM	seed imbibition	(+)	374
Cucumis sativus L. *var.* Grand Rapids seed germination	10−300 μM	seed imbibition	−[l]	400
Avena sativa L. *var.* Brighton mesocotyl elongation	0.01−10 μM	mesocotyl dip	−	400
Pharbitis nil (L.) Choisy seedling growth	50−100 μg	microdrop	−	369
Phaseolus vulgaris L. *var.* Pintado seedling growth	50 μg	microdrop	+	374
Hordeum vulgare L. *var.* Kikaihadaka aleurone bioassay	0.1 ng to 10 μg[m]	paper disk	+[n]	372
	10 pM to 1 μM	seed imbibition	+[k]	367
Kalanchoe gastonis-bonnieri Raym.-Hamet & H. Perrier bolting	ND[o]	ND	−	374
Kalanchoe gastonis-bonnieri Raym.-Hamet & H. Perrier floral induction	ND	ND	−	374
Anemia phyllitidis (L.) Sw. antheridium formation	≤160 μM	ND	−	410
dwarf *Thlaspi arvense* L. mutant EMS-141 stem growth	1 μg	microdrop	−[p]	411
Vitis vinifera L. *var.* Venus grape development	100−200 μM	shoot dip	(+)	367

[a]Four main methods have been used: (1) microdrop application of a few μL of EtOH solution containing a specific amount of the compound on the first grown leaves, sometimes repeatedly during a few days; (2) seed imbibition during incubation in aqueous solutions of the compound; (3) dipping the shoots of young seedlings in aqueous solutions of the compound for a short period of time; (4) dipping the roots in aqueous solutions of the compound for a period of 1−2 days. [b]+ active, (+) slightly active, − inactive. [c]Using the barley endosperm, dwarf pea, and dwarf corn bioassays on the acidic ethyl acetate fraction of methanol extracts, or seedling diffusates, from treated and untreated *d-1* and *d-5* mutants, a small but significant increase in endogenous gibberellin content was found in both mutants after steviol (**19**) application. [d]≤1% gibberellic acid (**45**). [e]≤0.1% gibberellic acid (**45**). [f]Reversed by 0.1 μM prohexadione, not by 100 μM ancymidol. [g]60% growth restoration in etiolated plants treated with growth inhibitor AMO-1618 or CCC. [h,i]Bioactivity only seen in etiolated plants that is either [h]reduced or [i]not after red light treatment. [j]At 300 μM, under 24 h light or dark. [k]Bioactive potency of **19** exceeds that of gibberellic acid (**45**) at the lowest concentrations tested. [l]Slight inhibition. [m]Per endosperm. [n]Maximal bioactivity observed with a 50 ng dose after two days. [o]No data given. [p]Except on petiole growth in cold-treated plants.

conditions.[230,248] The mechanism behind this decrease is not well understood. Recent research[230] has refuted earlier hypotheses attributing the steviol glycoside decline to the presence of dry senescent leaves with much lower steviol glycoside content,[262] or to the decrease in leaf biomass due to reproductive development.[347]

It is not clear whether a direct relationship exists between the steviol glycoside decline and the increasing carbohydrate demands related to shortening photoperiods and earlier floral induction. The complex transition from vegetative growth to reproductive development results in a series of morphological, physiological, and biochemical alterations, often including a partial reallocation of available carbon resources. For example, the decline of the phenolic acid rosmarinic acid in *Mentha* spp. upon flowering appears to be due to a reallocation of its carbon to the production of lignin and suberin needed for stem strengthening and to flavonoid production for flowering.[348] In *S. rebaudiana*, any reallocation of the carbon fixed within the steviol glycoside skeleton would require either an in situ degradation,[349] or their transport to the reproductive organs[247,249] or the roots[160] for further metabolism. Concerning the first possibility, no indications were found for a steviol glycoside-specific glycosidase activity within the leaves, despite extensive research.[160] In plants, most β-glycosidases are found within the vacuole, the same compartment where steviol glycosides are thought to be stored. Besides the possibility that steviol glycoside-specific glycosidases are simply not present in *S. rebaudiana*, the enzymes might also be spatially separated from the steviol glycosides. In some plants, the spatial separation of the glycosidase from its glycoside constitutes an

important defense mechanism against various pests. After cell disruption by, for example, insects, enzymatic degradation releases the active defense compounds from their glycosidic forms, thus enabling their antiherbivory function.[350,351]

For long-distance transport to occur from the leaves to the reproductive organs or the roots, a reversible transport of steviol glycosides over the tonoplast is required. Such a mechanism has been described for a number of complex molecules, including glycosylated triterpenes,[352,353] but has not yet been reported for steviol glycosides. Ultrastructural studies have shown that, compared to other species in the Asteraceae family, *S. rebaudiana* mesophyll cells have rather large vacuoles[354] containing specialized vesicles and granules during late vegetative and early reproductive development that disappear again during flowering and in senescent leaves. These vesicles and granules possibly contain crystalline steviol glycosides.[234,299] In addition, electron-dense material was observed near the tonoplast in mesophyll cells from flowering plants (Table 4).[300] Whether all these observations are related to transport or even to steviol glycosides at all is not known. In addition, it is likely that flowers are fully capable of synthesizing their own steviol glycosides, as they possess the ability to undergo the MEP pathway.[355] Nevertheless, until proven otherwise categorically, the transport of certain amounts of steviol glycosides to the reproductive organs cannot be ruled out. For instance, ecdysteroids are synthesized in young leaves of *Ajuga reptans*, after which they accumulate in reproductive organs.[356]

The observation of a 7-fold increase in % w/w steviol glycoside levels in *S. rebaudiana* roots during reproductive development suggests transport to the roots for further metabolism.[230] An ontogeny-dependent long-distance transport of terpenoids to these organs has been described in a few cases. At the onset of flowering, up to 75% of the monoterpene menthone present in mature leaves of *Mentha piperita* is rapidly reduced and glucosylated to neomenthyl glucoside, which is then transported to the roots and further catabolized to acetyl-CoA necessary for the synthesis of sterols and acyl lipids.[357] An analogous catabolic pathway was discovered for the monoterpene camphor in mature leaves of *Salvia officinalis*.[358,359] Possibly, the labdane diterpene marrubiin undergoes similar catabolism in *Marrubium vulgare*.[272] Whether this also occurs in *S. rebaudiana* is currently not known. The presence of steviol glycoside-specific glycosidase activity in the roots has not yet been reported. Intriguingly, an exploratory analysis of EDTA-facilitated phloem exudates of *S. rebaudiana* plants in different ontogenetic stages revealed a 6-fold higher amount of steviol glycosides after 24 h in plants showing 10% flowering, when compared to vegetative control plants.[160]

Regardless of the mechanism, the catabolism of steviol glycosides is likely to yield steviol (**19**) as an intermediate step. However, instead of a (transient) increase of the levels of **19**, an overall decrease was measured upon flower opening,[134] which was related to declining transcript levels of its biosynthetic genes.[232] The lack of any evidence for the enzymatic degradation of steviol glycosides might possibly indicate a nonenzymatic degradation by ROS. As already noted, steviol glycosides are known to be potent ROS scavengers.[324−326] Endogenous ROS levels are transiently increased during the stressful transition to reproductive development,[360] often resulting in increased levels of antioxidants and antioxidant enzymes.[361] Hydrogen peroxide is even postulated to be involved in floral induction.[361,362] In *S. rebaudiana*, a transient increase in peroxidase activity was measured in leaves of flowering plants.[363] Considering that short-day conditions can result in higher ROS production, as observed in, for example, tobacco,[364] the ROS-scavenging activity of steviol glycosides offers a possible explanation for the decline in their levels after floral induction under short days.

FUNCTION OF STEVIOL GLYCOSIDES

The presence of very large amounts of secondary metabolites poses questions regarding the benefits *S. rebaudiana* might gain from their accumulation, especially given the considerable metabolic cost involved.[365] Whether steviol glycosides have increasingly adapted a physiological function throughout evolution is still subject to much debate. However, several hypotheses have been postulated.

Steviol (19) as a Precursor of C-13 Hydroxylated Gibberellins. At the end of the 1950s, when the structural similarity between steviol (**19**) and gibberellins became clear, Prof. Erich Mosettig was the first to propose a gibberellin-like physiological activity of **19**.[366] Starting in the 1960s, researchers applied an extensive series of bioassays on **19** (Table 5). One of the most specific of these bioassays was the reversal of genetic dwarfing. Dwarf mutants produce little or no gibberellins during the seedling stage or do not use them efficiently for growth. Exogenous application of gibberellins or bioactive gibberellin-like compounds restores their growth to a certain level of the wild-type. Other bioassays focused more on their physiological effects on stem elongation, seed germination, or the induction of α-amylase in endosperm tissue.

Research on the bioactivity of **19** has made it clear that its bioactive response depends on the type of bioassay and cultivar used but also on light treatment and the concentration applied. This may explain contradictory results obtained with similar bioassays. For instance, in lettuce hypotocyls, **19** was significantly more active than gibberellic acid (**45**) at $\leq 10^{-10}$ M, whereas the opposite was seen at higher concentrations.[367] Yet despite the concentration-dependent fluctuations noted in some bioassays, overall bioactivity seemingly increased in the sequence of the biosynthetic pathway: *ent*-kaurene (**15**) (0−0.1×) < *ent*-kaurenol (**16**), *ent*-kaurenoic acid (**18**), *ent*-7α-hydroxykaurenoic acid (**20**) (0.1−1×) ≤ steviol (**19**) (1×) ≪ gibberellic acid (**45**) (100−1000×).[368−372] In dwarf peas, steviol (**19**) did not promote growth[368,369] except after etiolation and addition of the growth retardant AMO-1618, CCC,[366] or paclobutrazol.[373] The effect of these growth retardants could only be *partially* counteracted by application of **19**. This was also noted in beans, where application of **19** after growth retardation by CCC promoted stem elongation and the accumulation of dry matter.[374] In the etiolated peas, **19** was active only in relatively large concentrations and did not restore growth to the height of the untreated dark controls as did gibberellic acid (**45**).[366] Furthermore, Pfr formation (by red light treatment) reduced the bioactivity of **19** in etiolated dwarf peas, but not in etiolated wild-type plants.[373]

Many of the results from these bioassays are consistent with the hypothesis that steviol (**19**) needs to be converted to a gibberellin-like substance in order to become active and thus acts as a gibberellin precursor rather than a gibberellin analogue. A comprehensive list of indications for this hypothesis is given below:

(1) In *Zea mays*, steviol (**19**) is inactive in the dwarf mutant *d-1* but active in the *d-5* mutant. The *d-5* mutation blocks the conversion of *ent*-copalyl pyrophosphate (**14**) to *ent*-kaurene

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(**15**),[375] whereas the *d-1* mutation blocks the 3β-hydroxylation from GA_{20} (**25**) to GA_1 (**26**).[376] Intriguingly, a small but significant increase in endogenous gibberellin content was found in *both* mutants after steviol (**19**) application.[366] It appears that **19** is not active on itself, but can be metabolized to the highly active GA_1 (**26**), possibly through *ent*-7α,13-dihydroxykaurenoic acid (**21**), GA_{53} (**24**), and GA_{20} (**25**; Scheme 2).[373] Furthermore, GA_{20} (**25**) activity decreases in the following order: Tan-ginbozu dwarf rice (1 ng/seedling), *d-5* dwarf maize (100 ng/seedling), *d-1* dwarf maize (negligible). Taken together, this may indicate that steviol (**19**) is converted to gibberellin in many or all bioassays, only some of which are sensitive to the gibberellin(s) produced.[377] In case GA_{20} (**25**) would have been active in *d-1*, the lack of bioactivity of steviol (**19**) might have been due to an inability to convert **19** to **25**.

(2) Coincidently, GA_{20} (**25**) remains the only gibberellin ever identified in *S. rebaudiana*, occurring at a concentration of 1.2 μg per kg fresh matter of *S. rebaudiana* tissue (stems and leaves).[377,378] This is comparable to its concentrations reported in other species in the Asteraceae family.[379,380] In case the major gibberellins in *S. rebaudiana* were not hydroxylated at C-13, steviol (**19**) would be an unlikely precursor because dehydroxylation seems unlikely to occur.[377] Compared to the amount of steviol (**19**) (0.3−30 μg/g dry wt), GA_{20} (**25**) occurs in 25−2500 times lower concentrations. If the presence of **25** in *S. rebaudiana* suggests that steviol (**19**) acts as a putative precursor, only a very small fraction of the total pool of **19** is implicated. Still, direct conversion of labeled **19** to gibberellins in *S. rebaudiana* was not successful.[377] Furthermore, exogenously applied **19** to *S. rebaudiana* did not affect stem elongation or parthenocarpic fruit induction, contrary to gibberellic acid (**45**),[374] and also failed to stimulate fruit development.[305] Presumably the rapid glycosylation of **19** prevented the incorporation of label into gibberellins, thereby inactivating its bioactive properties. Bioassays in which **19** did show activity possibly lack this rapid and effective detoxification mechanism, resulting in **19** being treated as a precursor to C-13 hydroxylated gibberellins. Other possible explanations include the lack of enzymes in *S. rebaudiana* to convert **19** to bioactive gibberellins or the conversion of **19** to certain C-13 hydroxylated gibberellins that happen to be inactive in *S. rebaudiana*.[377]

(3) Fungal enzymes are capable of converting steviol (**19**) to a series of C-13 hydroxylated gibberellins common to higher plants.[381,382] Incubation of *Fusarium fujikuroi* mutant B1-41a with **19** initially yielded *ent*-7α,13-dihydroxykaurenoic acid (**21**), which was further metabolized to a series of compounds, including GA_1 (**26**), GA_{53} (**24**), and GA_{18} (Scheme 2).[381,383] This mutant is 97.5% effectively blocked at the step between *ent*-kaurenal (**17**) and *ent*-kaurenoic acid (**18**).[384] The metabolism of steviol (**19**) in *F. fujikuroi* B1-41a even proved to be a practical route to obtain rather inaccessible C-13 hydroxylated gibberellins.[383] In the wild-type strain LM-45-399, GA_{20} (**25**) rather than GA_1 (**26**) predominates after incubation with steviol (**19**),[385] which is probably due to the competition of **19** and its metabolites with endogenous intermediates for the same enzymes.[386] When these wild-type cultures were incubated with **19** in a medium containing inhibitors of gibberellin biosynthesis, a very similar metabolism was observed to that in mutant B1-41a.[387,388] Further research reported that the production of gibberellin analogues was prevented by C-19 methylation[381,386,389] or C-13 acetylation of **19**,[390] while being relatively insensitive to structural changes in rings C and D of the natural substrate, *ent*-kaurenoic acid (**18**).[386,390] The fungal enzymes involved in gibberellin biosynthesis are characterized by low substrate specificities and generally accept *ent*-kaurenoic acid (**18**) as well as steviol (**19**). In plants, the diversity of naturally occurring gibberellins argues for the utilization of the same enzyme systems to make identical transformations on different substrates. Thus, a lack of enzyme specificity in plants argues for the presence of a pathway to gibberellins via steviol (**19**). Nonetheless, there are also important differences in the pathways and enzymes involved, indicating that gibberellin biosynthesis has evolved independently in vascular plants and fungi, with little or no horizontal gene transfer.[391] For example, in *F. fujikuroi*, 3β-hydroxylation is catalyzed by a CYP450 that acts on GA_{12}-aldehyde (**23**), early in the biosynthetic pathway. In plants, this hydroxylation is the final step and is catalyzed by a 2-oxoglutarate-dependent dioxygenase.

(4) *ent*-7α,13-Dihydroxykaurenoic acid (**21**), an important intermediate between steviol (**19**) and C-13 hydroxylated gibberellins formed in *F. fujikuroi* mutant B1-41a, was also detected in the endosperm of *C. maxima*, together with **19**.[162] Additionally, incubation of the microsomal fraction of the endosperm with [^{14}C]GA_{12}-aldehyde (**23**) in the presence of ancymidol yielded GA_{53} (**24**) among other compounds, indicating the presence of a C-13 hydroxylating activity.[392] It is therefore possible that, at least in the investigated tissue, steviol (**19**) is an intermediate in the formation of C-13 hydroxylated gibberellins.

(5) Further clues that C-13 hydroxylation can take place prior to B-ring contraction comes from the roots of two *Bruguiera* species, from which *ent*-kauren-13-hydroxy-19-al and *ent*-kauren-13,19-diol were isolated, together with steviol (**19**).[163,164] Occurring at 4−40 μg/g dry wt, they represent the C-13 hydroxylated series of the common three-stage oxidation step from *ent*-kaurene (**15**) to *ent*-kaurenoic acid (**18**), of which the intermediates *ent*-kaurenol (**16**) and *ent*-kaurenal (**17**) were also isolated from the same roots. The coexistence of both series is significant because it implies the possibility of a divergence between the non-C-13 and C-13 hydroxylated gibberellins occurring as early as *ent*-kaurene (**15**), at least in the species investigated.

(6) As of April 2013, 136 gibberellins are known to occur naturally,[393] of which only a few have intrinsic biological activity in vascular plants. The main structural requirements for intrinsic growth stimulatory activity are that the molecule is a C_{19} gibberellin, with a [C-4/C-10]-γ-lactone bridge and a C-6 carboxylic acid, and that it possesses a functional group at C-3 (usually a β-hydroxy but a double bond such as in GA_5 is also possible) but no 2β-hydroxy group.[394] The structural requirements for florigenic activity are subtly different than those for stem elongation and include the presence of a C−C double bond at either C-1/C-2 or C-2/C-3, while 3β-hydroxylation is not required.[395] Less understood requirements include the *exo*-methylene moiety and the B-ring contraction. Since steviol (**19**) lacks most requirements, it can be presumed it has no intrinsic gibberellin activity.

Despite these indications that steviol (**19**) can act as a precursor to C-13 hydroxylated gibberellins, there are some indirect clues pointing otherwise:

(1) Hedden et al.[396] noted that steviol (**19**) is likely to be a terminal branch product from *ent*-kaurenoic acid (**18**) and thus unrelated to the gibberellic acid pathway. In comparison, there is no evidence either that *ent*-12α- and *ent*-15α-hydroxykaurenoic acid are in vivo precursors of *ent*-12α- and *ent*-15α-

hydroxylated gibberellins, respectively, even though some of these gibberellins can be generated in vitro by feeding cultures of *F. fujikuroi* mutant B1-41a with these hydroxylated *ent*-kaurenoic acid derivatives.[392,396,397]

(2) Even if the biosynthetic apparatus can convert steviol (**19**) to gibberellins, it may not occur in vivo due to the uncommon structure of **19**. Comparatively, feeding *Pisum sativum* seedlings with [^{3}H]GA_{14} revealed an alternative pathway to GA_1 ($GA_{14} \rightarrow GA_{18} \rightarrow GA_{38} \rightarrow GA_{23} \rightarrow GA_1$).[398] However, neither GA_{14} nor its metabolites (except for GA_1) have been identified as native in *P. sativum* seedlings, casting doubts on the relevance of this proposed pathway in vivo. Nonetheless, the postfeeding biosynthesis of these unnatural metabolites is significant because it confirms the limited substrate specificity of some of the enzymes involved.

(3) Due to the narrow occurrence of steviol (**19**) in natural sources, a shunt to gibberellins via **19**, if it occurs, would appear to be only a byway to gibberellin synthesis.[399] Furthermore, one might speculate that other plants in which GA_{20} (**25**) has been identified might also contain steviol (**19**), but so far it has eluded detection. This might be due to its highly transient nature, even though in *S. rebaudiana*, free steviol (**19**) is present at 25−2500-fold higher levels compared to GA_{20} (**25**).

It is clear that further elucidation is warranted concerning the physiological role of steviol (**19**). On several occasions, the bioactivity of steviol glycosides was also investigated. Stevioside (**37**) was biologically inactive within the maize *d-1*, *d-3*, and *d-5* mutants[400] and had no effect on the germination of *S. rebaudiana* itself, as well as *Lactuca sativa* L., *Rumex obtusifolius* L., *Cucumis anguria* L., and *Cucumis melo* L.[261] On the other hand, various steviol glycosides, such as stevioside (**37**), rebaudiosides A (**38**), B (**34**), and C (**42**), and dulcoside A (**41**), were bioactive in rice seedling, lettuce hypocotyl, and barley seed assays, although to a lesser extent compared to steviol (**19**).[401,402] The bioactive response varied only little between the different steviol glycosides, thus implicating their common aglycone, steviol (**19**), as its major cause. Possibly, glycosylation inhibited the bioactivity of **19**, while in some assays, at least part of the applied steviol glycosides was converted back to **19**.

Regardless of the bioactivity of steviol (**19**) or its relationship with the gibberellin biosynthetic pathway, it is clearly produced in massive amounts by the leaves of *S. rebaudiana*. A leaf with 10% w/w steviol glycosides and a rebaudioside A (**38**) to stevioside (**37**) ratio of 0.6 contains about 3.7% w/w steviol (**19**). Compared to the level of GA_{20} (**25**), the total pool of **19** present in an average *S. rebaudiana* leaf is at least 1 000 000-fold larger. Given the observation that the bioactivity of relatively large concentrations of **19** is about 100- to 1000-fold lower compared to that of gibberellic acid (**45**),[368,369,371,372] a gibberellin-overdose phenotype may result in the case where steviol (**19**) is not rendered inactive by rapid glycosylation (i.e., increased internode length, paler leaves, earlier flowering time). However, the severity of such overdose morphology largely depends on the regulatory mechanisms in place for maintaining gibberellin homeostasis. In fact, *ent*-kaurenoic acid oxidase appears to be an important rate-limiting step in the production of bioactive gibberellins. This was clearly seen in *A. thaliana* overexpressor lines of *ent-copalyl pyrophosphate synthase* and *ent-kaurene synthase*, where 1000-fold increases were noted in *ent*-kaurene (**15**) and *ent*-kaurenoic acid (**18**), whereas the levels of bioactive gibberellins remained virtually unchanged.[403]

Whether or not a lack of proper sequestration mechanisms would result in a distinct gibberellin overdose morphology, it would probably cause more fundamental disruptions on the subcellular level. Due to its lipophilicity (log P ≈ 1.7),[10] large amounts of free steviol (**19**) are likely to be toxic for the cellular environment, altering membrane protein bioactivities (IC_{50} = 40 μM in rat liver mitochondria),[404−407] and disrupting membrane permeability and rigidity. An excess of unconjugated **19** is reportedly even more toxic than similar levels of mono- and triterpenes.[145] The accumulation of steviol glycosides to such high concentration levels as seen in *S. rebaudiana* may thus represent an effective detoxification mechanism to cope with the huge influx of **19**. This way, its putative influence on the gibberellic acid pathway is minimized, while its anticipated toxic effects on the cellular environment are adequately quenched. As a side note, it should be stressed that, at the moment, any evidence is lacking for significant in vivo toxicity of **19** in animals as well as humans.[9,13]

Steviol Glycosides as Feeding Deterrents. Metivier and Viana[309] were the first to propose a hypothesis for the biological role of stevioside (**37**) as a defense mechanism against insect herbivory. At the time, other diterpenoids such as *ent*-kaurenoic acid (**18**) and *ent*-trachylobanoic acid were already known to inhibit growth rates in the moths *Homeosoma electellum* and *Heliothis virescens* by 50%.[412,413]

The unpalatability of *S. rebaudiana* was clearly observed in feeding experiments with the beetle *Epicauta adomaria*[309] and the grasshopper *Valanga irregularis*.[414] When offered *S. rebaudiana* along with leaves from other species, including *Capsicum*, *Amaranthus*, *Emilia*, and *Lycopersicum*, all except *S. rebaudiana* were consumed by *E. adomaria*. In a no-choice setup with only *S. rebaudiana* as food source, *E. adomaria* and *V. irregularis* even avoided the material and died from starvation within a few days.[309,414]

Nanayakkara et al.[415] further tested the feeding deterrent properties of steviol glycosides on the aphid *Schizaphis graminum*. Feeding deterrence was defined by the EC_{50} value, the concentration at which less than 50% of the aphids were feeding on the test compound compared to the control group. For steviol (**19**) and isosteviol (**43**), EC_{50} values were 140 and 115 ppm, respectively, whereas stevioside (**37**) and rebaudioside A (**38**) had minimal feeding deterrent activity, even at 650 ppm. However, this is still much less than the amounts found in fresh leaves (about 15 000 ppm, equivalent to 10% w/w steviol glycosides, calculated on a dry weight basis). An in vitro diet with up to 500 ppm steviol (**19**) did not deter larvae of *Heliothis virescens* Fabr. and *Aedes aegypti* L., indicating that the deterrent properties of **19** are insect-specific.[415]

Structurally, feeding deterrent activity of steviol (**19**) was diminished after acetylation or glycosylation of the C-13 hydroxy moiety or methylation of the C-4 carboxylic acid. Modification of the *exo*-methylene group or ring C/D rearrangement did not alter the antifeeding activity of **19**.[415] In comparison, the C-13 hydroxy and the C-17 *exo*-methylene moieties were both required for the mutagenicity of **19** toward *Salmonella typhimurium* TM677.[416−418]

Despite these observations in support of the feeding deterrent hypothesis, some reservations are needed. Not all investigated species avoided *S. rebaudiana* feeds, such as the mite *Tetranychus urticae*.[414] Soejarto[1] noted that the endemic population of *S. rebaudiana* is under pressure due to cattle grazing, indicating that mammalian herbivores graze on *Stevia*. In guinea pigs, feed intake was improved when 10% *S.*

rebaudiana leaves were added to stock feeds.[414] Likewise, several aquatic species were attracted to feeds enriched with stevioside (**37**), which was most likely due to the sweet taste of **37**, as sugar feeds had similar effects.[419] In a field inspection, several insects were found feeding on *S. rebaudiana* leaves, including *Pseudoplusia includens* and species of *Diabrotica*, *Spodoptera*, and *Schistocerca*.[420] Possibly, these insects were feeding on leaves with low steviol glycoside content.[414] However, the existence of cultivars with low steviol glycoside levels but with high resistance against various pests indicates that the response of *S. rebaudiana* to herbivory stress is more complicated. Besides low steviol glycoside levels, these cultivars reportedly contain high levels of phenols, which can also act as feeding deterrents.[421]

Another way in which steviol glycosides might defend against pests is by a phytoalexin-like mode of action. This would imply a rapid increase of steviol glycoside content after the onset of infection. However, elicitation of cell suspensions with the *S. rebaudiana*-specific pathogens *Alternaria steviae* and *Septoria steviae* did not result in an increase of steviol glycosides.[422] The question whether steviol glycosides act as a defense mechanism against pests thus remains largely unresolved. As suggested by Brandle and Telmer,[8] it may very well be that the only contribution of steviol glycosides to the fitness of *S. rebaudiana* is their sweetening properties, which perhaps do not deter many insects but clearly attract many humans, thus facilitating the spread and survival of this otherwise rare species.

Steviol Glycosides as Osmolytes. A variety of compounds, including secondary metabolites, act as osmoregulators and accumulate under conditions of water stress to allow the plant to adjust to water-stressed environments. For example, in transgenic tobacco plants, twice as much proline accumulated compared to the wild-type, resulting in improved plant growth under water and salt stress.[423]

Concerning the role of steviol glycosides in osmoregulation, information is scant. In its natural habitat, *S. rebaudiana* grows in regions with shallow water tables that are moist but not inundated.[7] The plant does not possess any xerophytic morphological features such as leathery leaves or a thick cuticle, indicating that any adaptation is limited to mild water stress.[414]

Theoretically, it is possible to calculate the contribution of steviol glycosides to the osmotic potential, ψ_s, of the cell. The following parameters for a random *S. rebaudiana* leaf were assumed: (1) moisture content of 85%; (2) calculated per dry wt, the leaf contains 10% w/w steviol glycosides, mostly stevioside (**37**) and rebaudioside A (**38**), of which the van't Hoff factor is 1; (3) the ratio of **38** to **37** is 0.6; (4) the steviol glycosides are uniformly distributed in the vacuolar sap of mesophyll cells. Using spinach leaves as a model,[424] it may be assumed that 90% of the leaf cellular space consists of mesophyll tissue, with vacuolar sap constituting 80% of the total mesophyll cell sap volume. Using these assumptions, the concentration of steviol glycosides in the vacuolar sap of mesophyll tissue of *S. rebaudiana* would be around 28.5 mM, or a contribution to ψ_s of −0.70 MPa at a temperature of 300 K (van't Hoff equation, $\psi_s = -ic_sRT$). Thus, steviol glycosides alone could not account for tolerance of water stresses higher than −0.70 MPa. Furthermore, considerable fluctuations in steviol glycoside levels within the leaf would be required for them to play a role in osmoregulation.[414] After eight days of imposing water stress, no significant changes in steviol glycoside content were observed, even though ψ_s of the leaf sap tripled to −3 MPa in the most stressed group.[414] However, only one stress cycle was imposed and only mature leaves were used in this experiment, which are known to exhibit decreased osmoregulation compared to young leaves.[425] When plants were subjected to eight weeks of continuous drought stress, steviol glycoside content declined about 30% in the most severe conditions, where soil moisture content was as low as 16%, just above the permanent wilting point of 14%.[308] Under extreme water stress, up to 5-fold less stevioside (**37**) was reported in dead leaves,[257,262] whereas others found no significant changes at all under similar conditions.[426] Taken together, these observations nevertheless cast doubts on a significant role of steviol glycosides as osmolytes.

Steviol Glycosides as an Energy Reserve. Vascular plants mainly use starch reserves for their short- and long-term energy demands, although oils and lipids in seeds may also be used. The possibility of a reallocation of the carbon fixed in steviol glycosides during reproductive development under short days was already discussed (see the Catabolism of Steviol Glycosides section). An important determinant for their availability for respiratory purposes is their turnover rate, which, for most terpenoids, is relatively low.[427] If steviol glycosides are used as an energy reserve, a hypothesis first proposed by Alvarez,[428] they should have a sufficiently large turnover. Pulse-chase labeling experiments with $^{14}CO_2$ revealed only little incorporations in stevioside (**37**) after 24 h,[429] and a limited turnover of the steviol glycoside pool was reported in both mature and immature *S. rebaudiana* leaves.[414] The relative stability of the steviol glycoside pool was further indicated by experiments where steviol glycoside levels did not change by dark stress of up to 10 days and only decreased after severe oxidative stress (2% H_2O_2 sprayed on the leaves).[160] In fact, it is likely that the latter result was caused by cell leakage due to the severity of the stress applied. In other experiments, a short-term exposure to elevated CO_2 resulted in a 50% increase in starch, while the steviol glycoside pool size remained constant.[414] Moreover, no diurnal changes were seen in steviol glycoside levels,[414,429] while starch decreased from 3.3 to 0.7% w/w during the night.[414] Taken together, the use of steviol glycosides as a short-term energy reserve seems unlikely. The hypothesis that they might have a long-term reserve function, e.g., to supply the energy demands during flowering and seed ripening, remains a possibility, as previously discussed.

Steviol Glycosides as Potent ROS Scavengers. While purified steviol glycosides may have potent ROS scavenging activities, the extent to which they play a role in the overall response of *S. rebaudiana* leaves to oxidative stress is not clear. In recent years, the antioxidant properties of *S. rebaudiana* leaves have been demonstrated in alcoholic[46,430,431] and water extracts.[326,432−434] High activities were also reported for the stems[326] and roots.[435] In calli, the antioxidant activities were found to be either more potent[433] or less potent[432] compared to the leaves. This contradiction might be related to differences in callus differentiation, resulting in altered metabolic responses to oxidative stress. Leaf extracts are capable of efficiently scavenging several kinds of ROS, including 2,2-diphenyl-1-picrylhydrazyl, hydroxyl, and superoxide radicals, in a comparable manner to *Gingko biloba* L. leaf extracts.[436] These properties have been related mainly to the presence of relatively large amounts of phenolic compounds,[430,437] or to inorganic salts.[438,439] A linear correlation was found between the total phenolic and flavonoid content on one hand and 2,2-diphenyl-1-picrylhydrazyl and hydroxyl radical scavenging activity on the

other.[432,434] In in vitro plants under polyethylene glycol-induced drought stress, phenolic compounds increased more than 10-fold, while at the same time, ROS scavenging capacities improved markedly.[327] However, the removal of most phenolic compounds from crude *S. rebaudiana* extracts with PVPP at the same time removed only 3, 25, or 45% of the hydroxyl, superoxide, and 2,2-diphenyl-1-picrylhydrazyl radical scavenging activities, respectively.[326] Active coal on the other hand removed not only most steviol glycosides from the extracts but also much more, though not all, ROS scavenging activities. These exploratory results indicate that ROS are scavenged mainly by compounds other than phenols.

In vitro assays with purified steviol glycosides revealed excellent scavenging activities against hydroxyl radicals, with IC_{50} values around 0.2 to 0.3 mM,[324−326] which was up to 20 times lower than for glucose and sucrose, but still more than quercetin (IC_{50} = 0.11 mM). Few differences were seen between the IC_{50} values of stevioside (**37**), rebaudioside A (**38**), and rubusoside (**33**), indicating that their antioxidant activity was mostly related to their common diterpene skeleton. While steviol glycosides clearly have the capacity to act as potent ROS scavengers, it is not known whether their in vivo metabolism is specifically adapted to constitute an integral part of the antioxidant response. It is possible that the role of steviol glycosides in the cellular antioxidant network is similar to the one recently proposed for fructans.[440] According to this model, phenolic compounds predominate in the vacuolar lumen, whereas fructans are more associated with the tonoplast. There, they capture hydroxyl radicals originating from the catalytic activity of type III vacuolar peroxidases on excess H_2O_2 levels due to increased stress conditions. The resulting sugar radical then moves to the vacuolar lumen, where it is either quenched by termination reactions or recycled at the cost of, for example, phenolic compounds. The ultrastructural observation of a thin layer of electron-dense material near the tonoplast in mesophyll cells of flowering *S. rebaudiana* plants lends credit to this hypothesis.[300] Yet, like many aspects discussed in this review, current information on this topic is too scant to deduce a clear interpretation for these observations.

CONCLUSIONS

Ever since the first scientific report on *S. rebaudiana* was published in 1899, the remarkable properties of its sweet principles have fascinated the scientific community. Nowadays, steviol glycosides, with rebaudioside A (**38**) in particular, are widely used commercially as a healthy, noncariogenic, zero-calorie alternative for sucrose. In addition, many studies with both animal models and human volunteers have shown clearly the beneficial pharmacological effects of stevioside (**37**) against type-II diabetes, hypertension, metabolic syndrome, and atherosclerosis.[10] Besides steviol glycosides, the leaves of *S. rebaudiana* are also known for their antioxidant properties, which are possibly related to a rich profile of various phenolic compounds.

In recent years, most genes involved in the biosynthetic pathway of the main steviol glycosides have been identified and characterized, but many questions still need answering. For example, the biosynthesis of the rhamnosylated and xylosylated steviol glycosides has not yet been elucidated. The apparent preference in vivo for the pathway to occur via steviolmonoside (**35**) and steviolbioside (**36**) instead of steviol-19-*O*-β-D-glucopyranosyl ester (**32**) and rubusoside (**33**) seems at odds with the coexistence of both routes in vitro. One plausible explanation, metabolon formation, has not been reported so far, but such a mechanism may still occur if it also requires *ent*-kaurenoic acid 13-hydroxylase or the unknown UGTs catalyzing the C-2′ glucosylation on the C-13 hydroxy moiety. Since steviol glycosides such as stevioside (**37**) act both as a cytosolic substrate and as an end-product stored in the vacuole, some level of coordination between the regulation of the steviol glycoside biosynthetic flux and vacuolar transporter activities can be presumed. An important role of alterations in transporter activities in the spatiotemporal variability of individual steviol glycoside ratios as well as their mutual correlations cannot be ruled out. Nevertheless, it is clear that the accumulation of steviol glycosides is strongly affected by ontogeny, which itself is influenced by photoperiod. Furthermore, it has become increasingly evident that the effects of day length, among other factors such as genotype, nutrients, temperature, and irradiance, should be taken into serious consideration during field production. Above all, the wide variability in observations made regarding steviol glycoside accumulation highlights the complexity of a large range of interacting biotic and abiotic factors underlying their cumulative effect on plant development. The ongoing development of new cultivars with diverse steviol glycoside profiles, meant for commercial applications, further impedes an easy deduction of scientific interpretations based on research data obtained with only one or two cultivars. By including a wider range of cultivars, preferably with wider variations in the ratio of rebaudioside A (**38**) to stevioside (**37**), the validity of any obtained results for the species *S. rebaudiana* could really be tested.

The decline in steviol glycoside levels during reproductive development has not been fully investigated so far. The manner in which this decline occurs depends on photoperiod and genotype. There is currently no unambiguous proof for an in vivo enzymatic or radical-initiated degradation of steviol glycosides nor for a transport to the roots or reproductive organs for further metabolism. One way to tackle this issue would be to track the ontogeny-dependent migration of labeled steviol glycosides for a considerable amount of time during a pulse-chase experiment after administering $^{14}CO_2$ to one or more leaves. The reversibility of steviol glycoside transport over the tonoplast might be tested by measuring the efflux of labeled steviol glycosides in isolated vacuoles. Similar work was done earlier to measure the reversible transport of oleanolic acid monoglucosides over the tonoplast.[352,353] The application of MS imaging to *S. rebaudiana* might also potentially increase our current knowledge on these issues dramatically. Using MALDI-TOF or MALDI-FTICR MS, changes in enzyme levels can be investigated on the tissue level, whereas nanoSIMS can be used to map the tissue-specific and intracellular fluctuations of steviol glycoside levels. The use of MS imaging in plants is a very recent development[441] and still requires much optimization of sample preparation. Yet, due to the large steviol glycoside pool in *S. rebaudiana* leaves, the spatial resolution of nanoSIMS should be sufficient to detect large variations in intracellular concentrations. An ultrahigh-resolution technique to map minimal changes in the metabolome of one cell has not yet been developed, although techniques such as LAESI and SMALDI are promising.[442]

So far, no clear function has been attributed to the steviol glycoside pool in *S. rebaudiana*. While steviol (**19**) shows clear bioactive responses related to gibberellins, the extent to which it acts as an in vivo precursor for C-13 hydroxylated gibberellins

remains uncertain. Likewise, there is no conclusive evidence either for a role of steviol glycosides in the protection against herbivory, for the reallocation of carbon sources during reproductive development, or for functioning as an important element of the cellular antioxidant network. No clear indications were found for a role of steviol glycosides as osmolytes. Perhaps the most concrete observation in this respect points to steviol glycosides as being a metabolic sink to quench the toxic effects related to the high levels of lipophilic steviol (**19**) otherwise piling up in the cytosol. The fact that **19** is produced in such large quantities raises questions regarding the genetic basis of this production capacity and how the steviol glycoside biosynthetic pathway has evolved from an evolutionary point of view. Two of the early genes in the pathway, *ent-kaurene synthase* and *ent-kaurene oxidase*, are known to be duplicated in *S. rebaudiana*, whereas *ent-copalyl pyrophosphate synthase* is single copy. Due to the upregulation of their expression in leaves, these genes were likely recruited to produce large amounts of steviol glycosides. Whether one copy is used for steviol glycoside synthesis and the other for gibberellin synthesis is not known, but the large difference in product accumulation in both pathways suggests a tight regulation. In order to trace the evolutionary origins of the steviol glycoside biosynthetic pathway, other *Stevia* species closely related to *S. rebaudiana* should be investigated. Based on an internal transcribed spacer sequence analysis, a phylogenetic tree was constructed with 70 *Stevia* species,[443] although most of them were of Mexican origin. From this tree, the species most closely related to *S. rebaudiana* are *S. velutinella* and members of a subclade of the perennials, including *S. connata*, *S. latifolia*, and *S. ovata*. Only *S. connata*, *S. incognita*, *S. ovata*, *S. latifolia*, *S. lehmannii*, and *S. seemannii* have so far been screened for the presence of steviol glycoside-related molecules, which gave negative results.[2] The incorporation and phytochemical investigation of more South American species may render a more complete picture of the evolutionary place of *S. rebaudiana* within the genus *Stevia* and the development of its remarkable sweeteners.

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Notes

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