Unexpected Biosynthetic Precursors of Amarogentin − A Retrobiosynthetic ^{13}C NMR Study

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Dedicated to Professor Hiroyuki Inouye on the occasion of his 80th birthday (Sanju)

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A root culture of *Swertia chirata* (Gentianaceae) produced the natural bitter compound amarogentin in 0.1% (dry weight) yield. The biosynthesis of amarogentin was studied by in vivo experiments, using [1-^{13}C]glucose, [U-$^{13}C_6$]glucose, [1-^{13}C]acetate, [2-^{13}C]acetate, [7-^{13}C]benzoic acid, [ring-$^{13}C_6$]cinnamic acid, or [7-^{13}C]shikimic acid as precursors. Comparison between labeling patterns of amarogentin and amino acids showed that dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), serving as building blocks of the sweroside moiety, are biosynthesized predominantly (> 95%) by the recently discovered deoxyxylulose phosphate pathway. The biphenylcarboxylic acid moiety is biosynthesized by a polyketide-type pathway, with three units of acetyl-CoA and one unit of 3-hydroxybenzoyl-CoA, this being formed from an early shikimate pathway intermediate and not via cinnamic or benzoic acid.

Introduction

Amarogentin (**1**, Figure 1), a natural product from *Swertia* and *Gentiana* roots, was first isolated by Inouye and Nakamura.[1,2] The compound is used in alcoholic beverages (e.g. "Enzian Schnaps" in German) and, because of its extremely bitter taste, it has been proposed as an additive for soft drinks in place of quinine.[3,4] However, toxicological analysis should precede broader nutritional use, since high doses of amarogentin have been shown to induce mutagenic activity in *Salmonella typhimurium*[5] and to inhibit topoisomerase 1 in *Leishmania donovani*.[6]

The biosynthesis of amarogentin is incompletely understood. Incorporation experiments with ^{14}C-labeled acetate, phenylalanine, and benzoic acid had suggested a polyketide origin for the biphenylcarboxylic moiety, and 3-hydroxybenzoyl-CoA (presumed to originate from phenylalanine via cinnamic acid and benzoic acid) was proposed as a starter entity for polyketide formation.[7]

The sweroside moiety is structurally similar to secoiridoid alkaloids. On the basis of the well-known iridoid biosynthetic pathway,[8,9] the carbon skeleton of the sweroside moiety can be dissected into the C_5 precursors dimethylallyl pyrophosphate (**2**, DMAPP) and isopentenyl pyrophosphate (**3**, IPP) (Figure 2). Incorporation experiments with [^{14}C]acetate were interpreted in terms of DMAPP/IPP formation via mevalonate.[7] In the light of more recent investigations into the nonmevalonate terpene biosynthesis pathway in plants,[10−14] a reinvestigation of this claim appeared in order.

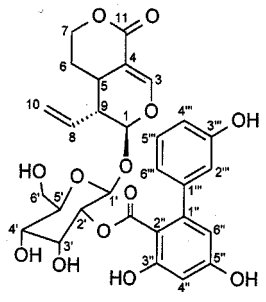

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Figure 1. Structure of amarogentin (**1**)

We have shown that complex biosynthetic problems can be addressed efficiently by quantitative comparison between isotope labeling patterns in secondary metabolites and primary metabolites (amino acids and nucleosides).[10,15−17] Application of this retrobiosynthetic approach (for reviews see refs.[18,19]) showed that the secoiridoid moiety of amarogentin is formed by the nonmevalonate isoprenoid biosynthesis pathway. The 3-hydroxybenzoic acid moiety was shown to be biosynthesized from an early shikimate intermediate, rather than from phenylalanine.

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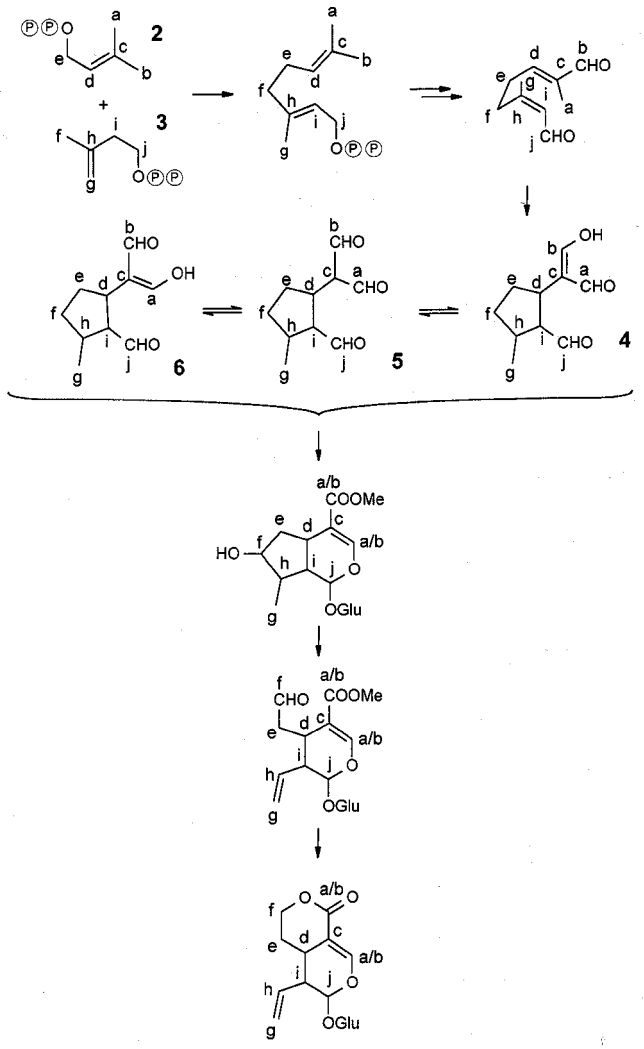

Figure 2. Biosynthesis of the sweroside moiety of amarogentin on the basis of established mechanisms of secoiridoid formation:[8,9] carbon atoms originating from DMAPP (**2**) and IPP (**3**) are indicated by a−e and f−j, respectively

Results and Discussion

NMR Signal Assignment

The ^{13}C NMR spectrum of amarogentin had previously been assigned by Ikeshiro and Tomito.[20] Since retrobiosynthetic NMR studies are entirely dependent on unequivocal signal assignments, we decided to obtain additional data using advanced two-dimensional NMR methods. On the basis of homocorrelation (COSY) and heterocorrelation (HMQC, HMBC) spectra, the assignments of 11 ^{13}C signals (specifically carbon atoms 1, 4, 1′, 2′, 3′, 2″, 4″, 3″, 5″, 2‴ and 4‴) required revision. ^{1}H and ^{13}C chemical shift values, together with $^{1}H^{13}C$ and $^{13}C^{13}C$ coupling constants, are summarized in Table 1. Supporting evidence for the novel assignments were obtained by INADEQUATE spectroscopy of a multiply ^{13}C-labeled amarogentin sample (from the incorporation experiment with fully ^{13}C-labeled glucose), affording carbon connectivities for 15 pairs of directly adjacent carbon atoms (Figure 3, Table 1).

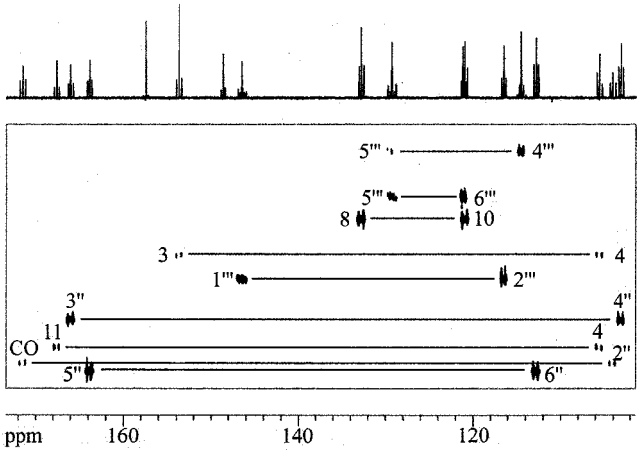

Figure 3. Part of a two-dimensional INADEQUATE spectrum of amarogentin obtained from the experiment with [U-$^{13}C_6$]glucose: the one-dimensional ^{13}C NMR spectrum of the same sample is superimposed on top of the figure

Incorporation Experiments

Root cultures of *Swertia chirata* described in this study produced amarogentin at a relatively high level (about 1 mg per g of dry cell mass). The roots could be grown in culture medium containing glucose instead of sucrose without loss of viability or amarogentin productivity.

The ^{13}C NMR signals of amarogentin from a tracer experiment with a mixture of [U-$^{13}C_6$]glucose and natural abundance glucose proffered at a ratio of 1:20 (w/w) are shown in Figure 4. Specifically, the top part of the Figure shows signals from the biphenylcarboxylic moiety, and the bottom part shows signals from the sweroside moiety. It is immediately obvious that the signals displayed in the Figure show satellites indicative of $^{13}C^{13}C$ coupling through one or multiple bonds. Several signals indicate the presence of different multiply ^{13}C-labeled molecular species in the amarogentin sample. As an example, the NMR signal of C-9 shows ^{13}C-coupled satellites indicative of simultaneous ^{13}C coupling to C-1 (coupling constant 40.7 Hz) and to C-7 (coupling constant 4.2 Hz) (Figure 4). It can be concluded that a $^{13}C_3$ fragment must have been diverted to the sweroside moiety from the proffered [U-$^{13}C_6$]glucose (Figure 5A). This finding is not consistent with a mevalonate origin via acetyl-CoA, since the mevalonate pathway cannot contribute fragments comprising more than two carbon atoms. It is also obvious that the contributed three-carbon moiety must have been fragmented by an intramolecular rearrangement reaction. On the other hand, a 3-carbon fragment could have been contributed by the nonmevalonate IPP biosynthesis pathway from [U-$^{13}C_3$]glyceraldehyde 3-phosphate via [3,4,5-$^{13}C_3$]1-deoxy-D-xylulose 5-phosphate, [1,3,4-$^{13}C_3$]2-C-methyl-D-erythritol 4-phosphate, and [1,2,4-$^{13}C_3$]IPP. More detailed evidence supporting this hypothesis is presented below.

Table 1. NMR spectroscopic data of amarogentin isolated from root cultures of *S. chirata*

Position	Chemical shifts, ppm		Coupling constants, Hz		^{13}C Abundance (%) of amarogentin labeled from			
	δ^{13}C	δ^{1}H	J_{HH}	J_{CC}	[1-^{13}C]acetate	[2-^{13}C]acetate	[1-^{13}C]glucose	[7-^{13}C]shikimate
CO	171.5			77.4 (2′′)	21.05	3.04	1.16	1.31
11	167.6			73.0 (4)	0.90	0.75	2.33	1.28
3′′	166.0			70.8 (4′′)	4.84	0.87	1.16	1.21
5′′	163.9			62.4 (6′′)	4.33	0.71	1.13	1.09
3′′′	157.5			–	1.69	1.37	1.39	1.09
3	153.7	7.43 (d)	2.6 (5)	78.1 (4)	1.19	1.05	2.22	1.07
1′′	148.7			54.7 (1′′′), 2.6 (2′′′)	1.44	2.69	1.25	8.12
1′′′	146.6			61.3 (2′′′), 54.7 (1′′)	1.13	0.80	1.02	1.09
8	132.9	5.44 (dt)	17.1 (10′), 9.9 (10, 9)	69.7 (10)	0.89	1.14	1.11	1.06
5′′′	129.4	7.17 (t)	7.8 (4′′′, 6′′′)	56.9 (4′′′, 6′′′)	1.25	1.14	1.01	1.16
6′′′	121.2	6.72 (d)	7.8 (5′′′)	57.3 (5′′′), 2.6 (4′′′)	0.97	0.98	2.82	1.03
10	121.0	5.23 (m)		69.7 (8)	0.97	0.98	3.33	1.11
2′′′	116.5	6.70 (m)		61.7 (1′′′), 2.6 (1′′)	1.21	1.03	3.62	1.04
4′′′	114.6	6.77 (dd)	8.0 (5′′′), 2.6	57.6 (5′′′), 2.6 (6′′′)	1.22	0.94	1.21	0.98
6′′	112.9	6.16 (d)	2.6 (4′′)	62.6 (5′′)	0.89	6.22	3.47	1.01
4	105.6			73.0 (11), 78.2 (3)	0.94	1.53	1.09	1.29
2′′	104.1			77.4 (CO)	1.86	12.18	3.62	1.34
4′′	103.2	6.29 (d)	2.6 (6′′)	70.8 (3′′)	1.21	8.34	3.34	1.08
1	97.2	5.39 (d)	1.6 (9)	40.9 (9)	1.02	1.09	2.93	1.10
1′	96.8	4.28 (d)	7.9 (2′)	48.2 (2′)	1.00	0.94	4.96	1.07
5′	78.4	3.07 (m)		43.6 (6′, 4′)	1.08	0.98	1.00	1.10
3′	74.9	2.81 (t)	9.2 (2′, 4′)	nd	1.02	1.10	1.42	1.05
2′	74.7	4.72 (dd)	9.5 (3′), 7.9 (2′)	nd	1.02	1.10	1.06	1.06
4′	71.7	3.22 (t)	9.3 (3′, 5′)	41.1 (5′)	1.13	1.23	1.20	1.06
7	69.6	4.36 (m)		4.4 (9)	1.19	1.14	1.25	1.03
		4.24 (m)						
6′	62.5	3.83 (dd)	12.0 (6′), 2.1 (5′)	43.6 (5′)	1.21	1.07	2.58	0.97
		3.60 (dd)	12.0 (6′), 6.0 (5′)					
9	43.5	2.58 (ddd)	9.4 (8), 5.4 (5), 1.4 (1)	40.7 (1), 4.2 (7)	1.06	1.14	1.02	1.08
5	28.7	2.74 (m)		34.7 (6)	1.03	1.03	1.02	1.05
6	25.9	1.69 (m)		34.7 (5)	1.28	1.00	2.77	0.95
		1.58 (qd)	12.8 (6, 5, 7), 4.3 (7)					

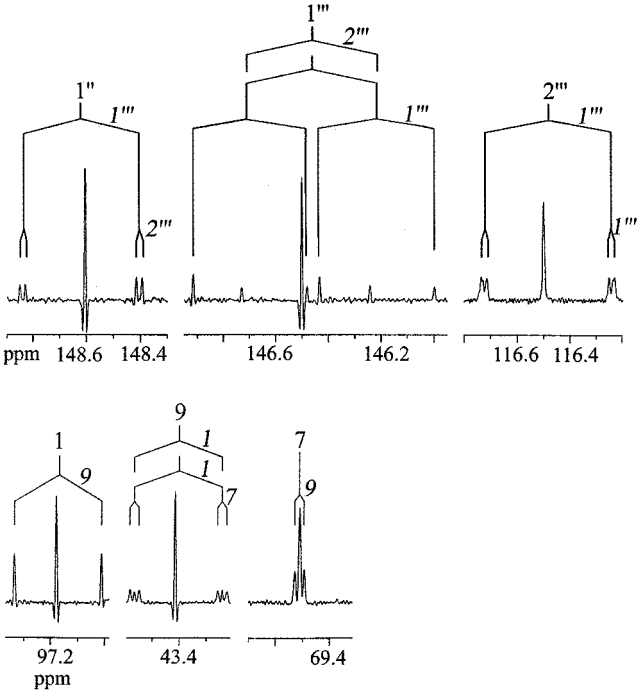

Figure 4. ^{13}C NMR signals of amarogentin obtained from the experiment with [U-$^{13}C_6$]glucose: $^{13}C^{13}C$ couplings are indicated

Three pairs of jointly transferred ^{13}C atoms (CO/C-2′′, C-3′′/C-4′′, C-5′′/C-6′′) observed in the dihydroxybenzoate moiety suggest a polyketide origin. The labeling signature of the hydroxyphenyl moiety is more complex and comprises one 4-carbon block and two 3-carbon blocks, one of which extends into the adjacent dihydroxybenzoate ring system (Figure 5A). As shown in detail below, this is indicative of the hydroxyphenyl moiety having its origin in an early shikimate pathway intermediate.

All amarogentin isotopomers derived from the analysis of carbon-carbon coupling constants (Table 1), as well as from the two-dimensional INADEQUATE experiment (Figure 3, Table 1), are shown schematically in Figure 5A. It should be noted that the Figure represents a superposition of twelve $^{13}C_2$ isotopomers, four $^{13}C_3$ isotopomers, and one $^{13}C_4$ isotopomer. The numbers in Figure 5A indicate the relative fractions of each isotopomer.

It is interesting to note that the glucose moiety present in amarogentin shows evidence of intense remodeling through glycolysis/glycogenesis as well as the pentose phosphate pathway. There is no evidence whatsoever for the intact incorporation of the proffered [U-$^{13}C_6$]glucose.

The experimentally determined isotope distributions in amarogentin produced from the experiments with [1-^{13}C]glucose, [1-^{13}C]-, [2-^{13}C]acetate, and [7-^{13}C]shikimate as precursors are summarized in Table 1 and Figure 5B, C, and D, respectively. No ^{13}C enrichment was observed in amarogentin produced from experiments with [7-^{13}C]benzoate and [ring-$^{13}C_6$]cinnamic acid as precursors.

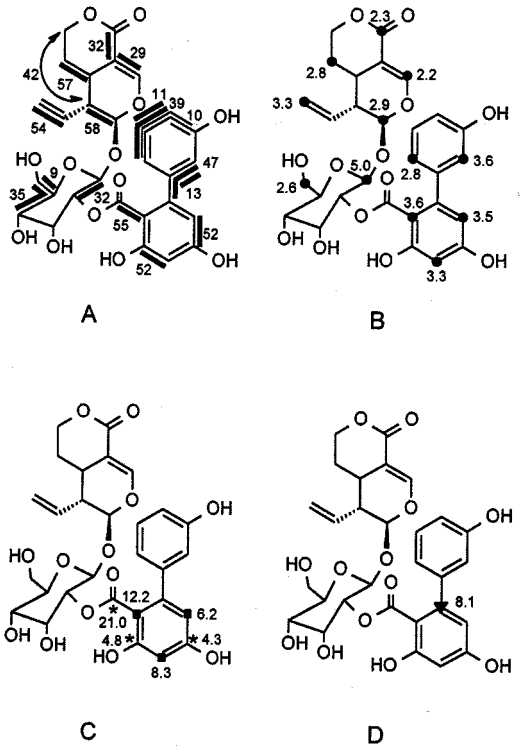

Figure 5. ^{13}C labeling patterns of amarogentin: (A) obtained from the experiment with [u-$^{13}C_6$]glucose; bold lines indicate ^{13}C-labeled isotopomers with directly adjacent ^{13}C atoms, the arrow indicates [1,7,9-$^{13}C_3$]amarogentin, and numbers represent fractions of ^{13}C-coupled satellites in the global NMR signal of a given carbon atom (as percentage); (B) obtained from the experiment with [1-^{13}C]glucose; filled circles indicate significantly ^{13}C-enriched atoms (> 2% ^{13}C) and numbers represent absolute ^{13}C abundances; (C) obtained from the experiments with ^{13}C-labeled acetates; asterisks indicate significantly ^{13}C-enriched atoms from [1-^{13}C]acetate, squares indicate significantly ^{13}C-enriched atoms from [2-^{13}C]acetate, and numbers represent absolute ^{13}C abundances; (D) obtained from the experiment with [7-^{13}C]shikimate; the filled triangle indicates [1′′-^{13}C]amarogentin and the number indicates absolute ^{13}C-abundance

Analysis of Labeling Patterns by Means of a Retrobiosynthetic Approach

Biosynthetic hypotheses can be subjected to a rigorous test by prediction of the amarogentin labeling pattern from the labeling patterns of central metabolic intermediates. These can be derived in turn from experimentally determined amino acid labeling patterns, using procedures that have been described elsewhere (for reviews see refs.[18,19]).

The labeling patterns of tyrosine (**7**) and leucine (**11**) obtained on hydrolysis of cell mass from the experiment with [U-$^{13}C_6$]glucose (Figure 6) are qualitatively similar to those found with numerous other plants supplied with a mixture of [U-$^{13}C_6$]glucose and natural abundance glucose.[10,16,21,22] The side chain of tyrosine (**7**) reflects the labeling pattern of phosphoenolpyruvate (**8**), from which it is obtained biosynthetically by the shikimate pathway of aromatic amino acid biosynthesis. Moreover, phosphoenolpyruvate (**8**) and glyceraldehyde 3-phosphate (**10**) should have very similar labeling patterns in view of their metabolic proximity in the carbohydrate catabolic pathways. Similarly, the labeling pattern of acetyl-CoA (**12**) and that of hydroxyethyl-TPP (“activated acetaldehyde”, **13**) can be inferred from the labeling pattern of leucine (**11**) on the basis of the unique leucine biosynthetic pathway.

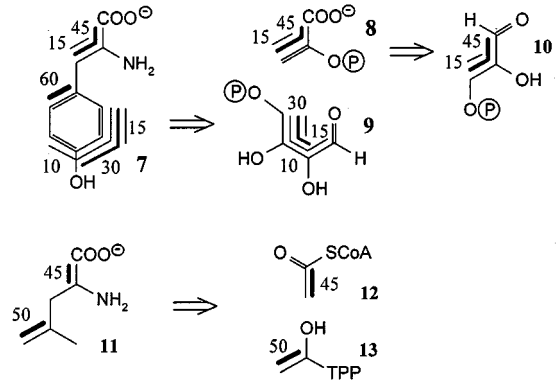

Figure 6. Retrobiosynthetic analysis of tyrosine (**7**) and leucine (**11**) from the experiment with [U-$^{13}C_6$]glucose: bold lines indicate ^{13}C-labeled isotopomers with directly adjacent ^{13}C atoms; numbers represent fractions of ^{13}C-coupled satellites for a given multiply ^{13}C-labeled isotopomer in the global NMR signal of a given carbon atom (as a percentage); on the basis of well-known mechanisms of tyrosine and leucine biosynthetic pathways, the labeling patterns of central intermediary metabolites were reconstructed as indicated by the retro-arrows

Figure 7 illustrates the predicted labeling patterns for the sweroside moiety of amarogentin produced from IPP (**3**) biosynthesized from 1-deoxyxylulose 5-phosphate (**14**) (prediction A) and from mevalonate (**15**) (prediction B). The predictions from the central intermediates derived above are based on known IPP biosynthesis pathways in plants (for reviews, see refs.[12−14]), as well as on the known pathway

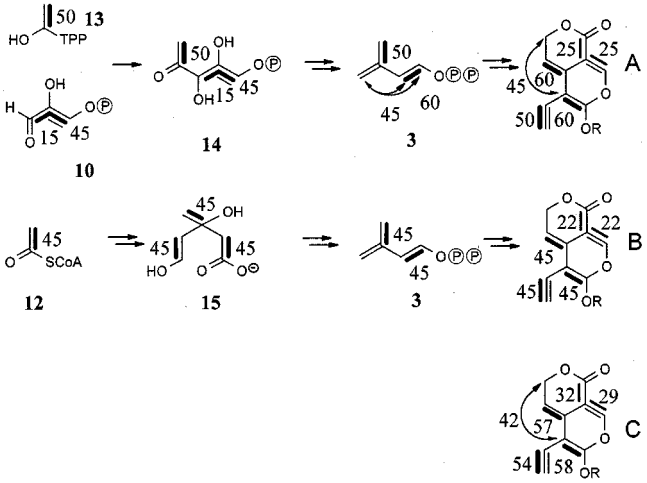

Figure 7. Observed and predicted labeling patterns for the sweroside moiety of amarogentin from the experiment with [U-$^{13}C_6$]glucose: bold lines indicate ^{13}C-labeled isotopomers with directly adjacent ^{13}C atoms; numbers represent fractions of ^{13}C-coupled satellites for a given multiply ^{13}C-labeled isotopomer in the global NMR signal of a given carbon atom (as a percentage); arrows indicate $^{13}C_3$ isotopomers with two directly adjacent ^{13}C atoms and one ^{13}C atom connected by long-range coupling; the labeling patterns of hydroxyethyl-TPP (**13**), glyceraldehyde 3-phosphate (**10**), and acetyl-CoA (**12**) were reconstructed from the labeling patterns of amino acids (see Figure 6); (A) prediction on the basis of the deoxyxylulose phosphate pathway of IPP/DMAPP biosynthesis; (B) prediction on the basis of the mevalonate pathway of IPP/DMAPP biosynthesis; (C) observed

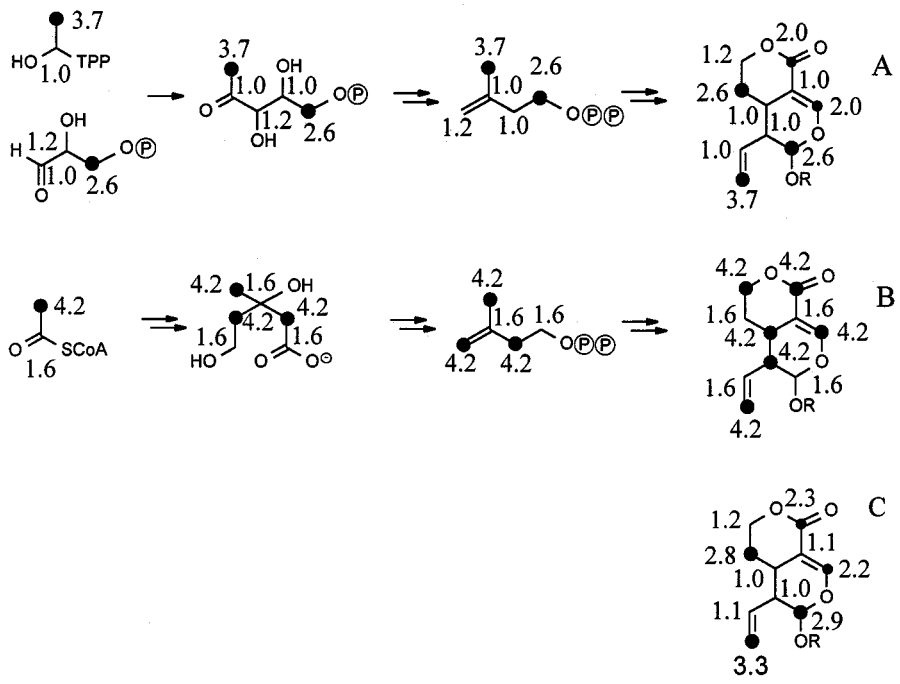

Figure 8. Observed and predicted labeling patterns for the sweroside moiety of amarogentin from the experiment with [1-^{13}C]glucose: filled circles indicate significantly ^{13}C-enriched atoms (> 2% ^{13}C) and numbers represent absolute ^{13}C abundances; for further details see Figure 7

of iridoid biosynthesis.[8,9] It should be noted that C-11 and C-3 of amarogentin are biosynthetically equivalent, due to the rapid equilibrium between the cyclopentane trialdehyde intermediate (**5**) and its tautomeric enol forms (**4**, **6**) (Figure 2). Consequently, the original methyl groups from the DMAPP building block are indistinguishable with respect to their label distribution. The match between the experimental data (Figure 7C) and the deoxyxylulose phosphate pathway prediction (Figure 7A) is excellent. Most notably, the predicted ^{13}C^{13}C coupling between C-9 and C-7 through three bonds was detected experimentally (Figure 4). On the other hand, the mevalonate prediction (Figure 7B) is clearly at odds with the experimental data.

The same retrodictive/predictive approach described in detail above for the experiment with [U-^{13}C$_6$]glucose can be applied for the experiment with [1-^{13}C]glucose (Figure 8). Again, the observed labeling pattern (Figure 8C) is perfectly in line with the prediction based on 1-deoxyxylulose 5-phosphate as an intermediate (Figure 8A) but not with the prediction based on mevalonate (Figure 8B). With allowance for the error limits of quantitative NMR analysis, the data show that more than 95% of the IPP and DMAPP precursor molecules for amarogentin are formed through 1-deoxyxylulose 5-phosphate.

In a similar manner, the amino acid labeling information can be used to predict the labeling of the biphenylcarboxylic moiety of amarogentin produced from an early shikimate intermediate (Figure 9A), as opposed to that from the biosynthetic reaction sequence involving phenylalanine, cinnamic acid, and benzoate as intermediates (Figure 9B). The carbon atoms 1, 2, and 7 of shikimate (**16**) reflect the labeling pattern of phosphoenolpyruvate. However, the carboxylic carbon atom of shikimate is lost in the later stages of aromatic amino acid biosynthesis, and consequently only two carbon atoms (C-4 and C-9 of phenylalanine, **18**) of the original 3-carbon block (contributed from phosphoenolpyruvate) can be transferred to the final product through this hypothetical pathway. As shown in Figure 9B, the phenolic ring of amarogentin should have a very complex labeling pattern if it were formed through intermediates possessing an inherently C_2-symmetric phenyl ring (such as

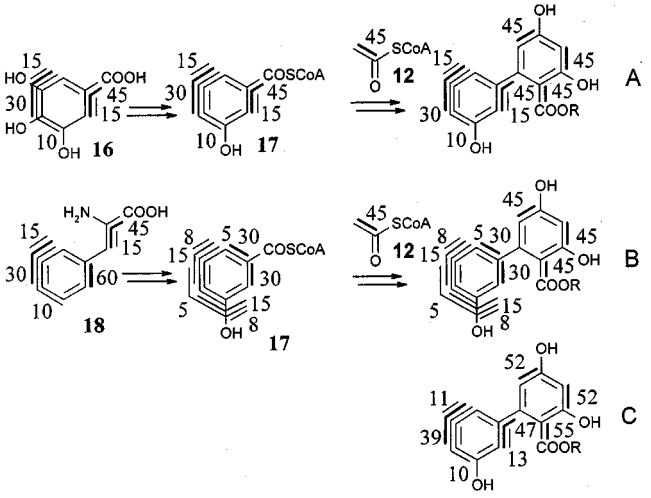

Figure 9. Observed and predicted labeling patterns for the biphenylcarboxylic moiety of amarogentin from the experiment with [U-^{13}C$_6$]glucose: (A) prediction based on a polyketide-type biosynthesis with three units of acetyl-CoA (**12**) and one unit of 3-hydroxybenzoyl-CoA (**17**) directly derived from an early shikimate intermediate, the labeling pattern of shikimate (**16**) was predicted from the labeling patterns of phosphoenolpyruvate (**8**) and erythrose 4-phosphate (**9**) (see Figure 6); (B) prediction based on a polyketide-type biosynthesis with three units of acetyl-CoA (**12**) and one unit of 3-hydroxybenzoyl-CoA (**17**) derived from phenylalanine (**18**); (C) observed; for more details, see Figure 7

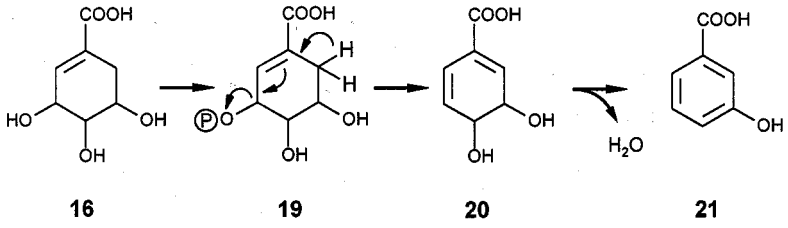

Figure 10. Hypothetical mechanism of 3-hydroxybenzoate formation in *S. chirata*: for details, see text

phenylalanine, cinnamate, and benzoate). The comparison of the experimental data (Figure 9C) with the prediction leaves no doubt whatsoever that amarogentin is derived from an early shikimate intermediate prior to the prephenate stage.

Independent verification for the shikimate origin of the 3-hydroxyphenyl moiety is provided by the efficient incorporation of [7-^{13}C]shikimate into position 1′′ of amarogentin (Figure 5D and Table 1.). In contrast, neither [7-^{13}C]benzoate, nor [U-$^{13}C_9$]cinnamic acid were incorporated into amarogentin at a significant rate.

A hypothetical mechanism for 3-hydroxybenzoate formation from an early shikimate intermediate is shown in Figure 10. Elimination of phosphate from shikimic acid 3-phosphate (**19**) might yield a diene intermediate (**20**), which could then be converted into 3-hydroxybenzoic acid (**21**) by dehydration. The CoA derivative of 3-hydroxybenzoic acid could then be used as a polyketide pathway starter moiety for the biphenyl moiety in amarogentin.

Conclusion

Information about the biosynthetic precursors of amarogentin could provide the basis for a biotechnological preparation of the bitter compound. The data presented in this paper quantitatively assess the biosynthetic precursors and give insights into the mechanisms of amarogentin biosynthesis. In contrast to earlier proposals, it is shown that the IPP and DMAPP required for the formation of the sweroside moiety are entirely or preferentially (> 95%) derived through the recently discovered deoxyxylulose phosphate pathway. Moreover, the putative starter unit, 3-hydroybenzoyl-CoA, involved in the polyketide biosynthesis of the biphenylcarboxylic moiety is biosynthesized (> 98%) via an early shikimate derivative, and not via phenylalanine as proposed earlier. It remains to be established whether minor fractions of mevalonate- or phenylalanine-derived metabolites might contribute to amarogentin biosynthesis.

Experimental Section

Materials: [1-^{13}C]- and [U-$^{13}C_6$]glucose were obtained from Omicron (South Bend, IN). [7-^{13}C]Benzoic acid, [1-^{13}C]bromoacetic acid, L-[U-$^{13}C_9$]phenylalanine, [1-^{13}C]-, and [2-^{13}C]acetate were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA). [7-^{13}C]Shikimic acid was synthesized from [1-^{13}C]bromoacetic acid and D-mannose according to refs.[23,24]

Preparation of [u-$^{13}C_9$]Cinnamic Acid: [U-$^{13}C_9$]Cinnamic acid was synthesized from 22 mg of L-[U-$^{13}C_9$]phenylalanine by the enzymatic action of 1.2 U of phenylalanine ammonia lyase in a total volume of 1.5 mL of 0.5 M sodium borate (pH = 8.5). The mixture was incubated at 30° C for 24 h, acidified with concentrated sulfuric acid to pH = 2.0, and extracted with diethyl ether (4 × 4 mL). The ether phase was concentrated and the [U-$^{13}C_9$]cinnamic acid was purified by preparative TLC (silica gel G 254) using toluene/acetic acid (8:1, v/v) as mobile phase. The R_f value was 0.7 (14.4 mg; yield, 77%).

Plant Cell Cultures: Root cultures of *Swertia chirata* were established[25] and grown in 1-L Erlenmeyer flasks containing Murashige and Skoog medium[26] with 3% glucose as sole carbon source. Root tissue (5 g, wet weight) was transferred to 250 mL of fresh medium. [1-^{13}C]- or [2-^{13}C]acetate were added to the culture medium at concentrations of 9.6 mM. [7-^{13}C]Benzoic acid or [U-$^{13}C_9$]cinnamic acid were added at concentrations of 0.1 mM, and [7-^{13}C]shikimic acid was added at a concentration of 0.5 mM. [U-$^{13}C_6$]Glucose was proffered together with a 20-fold excess of unlabeled glucose, and [1-^{13}C]glucose was proffered together with a 2.3-fold excess of unlabeled glucose. The cultures were grown under continuous incandescent light in a rotary shaker (100 rpm) at 28 °C for 21 d. The cells were harvested by filtration and subsequently washed with water.

Isolation of Amarogentin: The cultivated roots were frozen with liquid nitrogen and lyophilized (yield, 1.5 g dry weight per 250 mL of medium). The dry tissue was extracted three times with 200 mL of methanol under reflux. The extract was concentrated under reduced pressure, and the syrupy residue was loaded onto a column of silica gel (220−440 mesh, 1.8 × 20 cm). Stepwise elution with chloroform/methanol mixtures (30:1, 20:1, 15:1, 10:1, 6:1 and 4:1, v/v) afforded amarogentin in the 15:1 fraction. The solvent was evaporated under reduced pressure, the residue was dissolved in 4 mL of 55% aqueous methanol, and insoluble material was removed by filtration. The filtrate was subjected to preparative HPLC using a column of Nucleosil RP-18 (5 μm, 4.6 × 250 mm) which was eluted with 55% aqueous methanol at a flow rate of 3 mL min^{-1}. The eluent was monitored at 254 nm. Amarogentin had a retention volume of 60 mL. Fractions were combined and concentrated under reduced pressure. Typically, 4 mg of amarogentin was isolated from 4 g of root cells (dry weight).

Isolation of Amino Acids: The hydrolysis of biomass and the isolation of amino acids has been described earlier.[27] Typically, 1−10 mg of each amino acid was isolated from 4 g of root cells (dry weight).

NMR Spectroscopy: ^{1}H and ^{13}C NMR spectra were recorded at 500.13 MHz and 125.6 MHz, respectively, using a DRX 500 spectrometer from Bruker instruments, Karlsruhe, Germany. Two-dimensional INADEQUATE, HMQC, HMBC, and DQF-COSY experiments were performed with the aid of standard Bruker software (XWINNMR 1.3). Amarogentin was measured in [D$_4$]methanol.

Tyrosine was measured in D_2O (pH = 13), and leucine was measured in D_2O (pH = 1). Signals were referenced to solvent peaks or external trimethylsilylpropane sulfonate.

Estimation of ^{13}C Enrichment: The methods used for determining ^{13}C enrichment have been described in detail previously.[19] Briefly, ^{13}C NMR spectra of the isotopically labeled compound under study and of natural abundance material were recorded under the same experimental conditions. Integrals were determined for every ^{13}C NMR signal, and the signal integral for each respective carbon atom in the labeled compound was referenced to that of the natural abundance material, thus affording relative ^{13}C abundances for each position in the labeled molecular species. − In certain cases, these relative abundances can be converted to approximate absolute enrichment by assigning a value of 1.1% to the carbon atom with the lowest ^{13}C enrichment and referencing all other carbon atoms to that position. In other cases, absolute ^{13}C enrichment can be obtained for certain atoms from ^{13}C coupling satellites in the ^{1}H NMR spectra, provided that any hydrogen atom of the compound under study is a singlet or a doublet in which the coupling satellites can be determined to relatively high accuracy. − In NMR spectra of multiple-labeled samples displaying $^{13}C^{13}C$ coupling, each satellite in the ^{1}H-decoupled spectra is integrated separately. The integral of each respective satellite pair is then referenced to the total integrated signal of that respective carbon atom.

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