The Biogenesis of Fruit Flavors: A Continuing Story

Ralf G. Berger, Institut für Lebensmitteltechnologie und Analytische Chemie, der Technischen Universität München

The very first comprehensive studies on the chemical identity of volatile flavors from fruit already considered their biochemical source and function. Investigating *Bartlett* pear volatiles, Heinz and Jennings[1] speculated as early as in 1966 that esters of unsaturated C_8 to C_{12} fatty acids arise during fatty acid synthesis. In the senescing fruit a repeating cycle "ceases at some certain chain length," thus "releasing the intermediate medium-chain acids" for re-esterification with methanol or ethanol.

Similarly, "a plausible case" was constructed by oxidation of the C_{18}-polyunsaturated unconjugated fatty acids by the action of a lipoxygenase (LOG). Some years later, Creveling and Jennings[2] found that most of the double bond positions in pear volatiles were "consistent with those that would be derived from β-oxidation of the unsaturated fatty acids found in pear mitochondrial particles."

While the subcellular origin of *Bartlett* pear volatiles still remains obscure, some progress has been achieved in the field: principal pathways of acyl metabolites, of amino acids, and of phenylpropanoids have been established in feeding studies using preparations from fruits and labeled and nonlabeled substrates.[3-5]

Besides the basic scientific interest inherent in attaching the biogenesis of volatiles to general biochemical pathways, some research efforts were guided by the idea of producing better-flavored food. With a better biochemical understanding, it would seem easier to improve existing agricultural treatments, and storage and processing technologies, ultimately resulting in products with improved quality.

The present paper will likewise emphasize the applied aspects of some selected recent results highlighting the morphological and subcellular site of flavor biogenesis, and flavor-producing cell cultures.

Enrichment of fruit flavors by PA-storage

The experimental basis of studies on the biogenesis of fruit flavors is the common observation that preparations from fruit when incubated under "aged tissue" conditions are able to take up exogenous substrates and include them into pathways of flavor formation. This approach was recently transferred to intact fruits which were stored in the presence of volatile precursors of flavor compounds.

Apples of the cv. *Jonathan,* which are rich in butyl esters, were stored in a controlled atmosphere containing n-butanol (5mM/10L x kg, 2 d). Then, a cylindrical tissue was radially excised using a cork borer, the tissue divided into 6 zones, and the concentration of volatiles measured for different depths along the cross-section (Figure 1).

The concentration gradient from peel to core was factor 2 for precursor butanol, factor 35 for the medium polar products, butyl esters, and factor 1100 for α-farnesene. These results clearly demonstrated the epidermal localization of the genesis of some apple volatiles.[6]

0272-2666/90/0003-3301$04.00/00

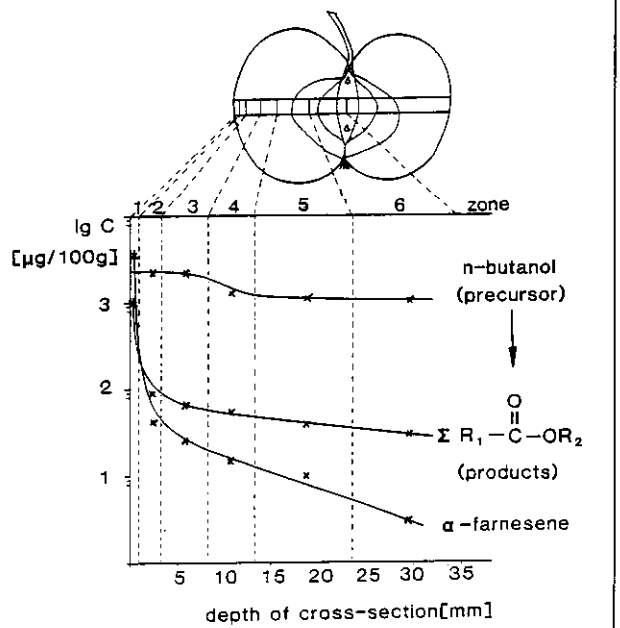

Figure 1. Concentration profiles of precursor and products along the cross-section of precursor atmosphere (PA) stored apples: The subepidermal tissue is the site of ester formation in the fruit.

In the aqueous matrix of the fruit, the diffusion-driven equilibration was slow for apolar and rapid for polar constituents, such as butanol.

The rapid turnover of exogenous alkyl substrates to carboxylic esters with fruity odours stimulated the development of a novel storage process to accumulate volatile flavors in fruit. This process was termed PA- (= precursor atmosphere) storage corresponding to the well known CA- (= controlled atmosphere) storage.

A two-day incubation of *Red Delicious* apples in the presence of ethanol as a precursor resulted in tremendous concentration increases of up to factor 200 for certain ethyl esters. The extremely strong flavor of the precursor treated apples renders them almost inedible, but enables processing to flavor enriched juices, concentrates or dried products.

As a result, the alkylation reactions of PA-storage compensate for losses of endogenous impact components which may have been caused by one-sided breeding efforts, by improper storage and transport, or by the often inevitable thermal processing steps. To obtain a sufficient carry-over of the accumulated volatile flavors into the products, the ester hydrolyzing activities must be completely inactivated by an initial HTST step; otherwise the first-order reaction kinetics of ester hydrolysis will take care that the original gain is quickly brought to nothing.

Successful PA-storage was performed with a number of ester coined fruits, such as apple, pear, strawberry, and banana.[7]

Metabolism of C_8-diols in apple fruit

In the course of studies on the PA-storage of various apple cvs., two main compounds (concentrations of 10 to 100 mg/kg) were detected in the extracts. They could not be immediately identified by routine GC/MS analysis. This was surprising, as the volatile composition of apple, the economically most important fruit of Western Europe, was thoroughly investigated by many researchers.

Control experiments confirmed the biological origin of the unknowns. Microderivatization, IR and NMR spectroscopy, and chemosynthesis lead to the identification of 1,3-octanediol and (5Z)-octenediol.

Both diols are less interesting from a flavor point of view, but possess distinct antifungal properties.[8] They accumulate during fruit ripening, partly occurring as glycosides,[8,9] and might be precursors of other, more flavor-active C_8-compounds found in apple. Hydroxy esters, monoenols and dienols may be formally derived by simple hydration, alkylation, and redox reactions.

In agreement with an anticipated enzymatic formation, chiral gas chromatography showed the diols to possess high optical purities.[8] Based on literature data and comparative measurements of the optical rotation of the diacetoxy derivative, Schreier assigned the R-configuration to the chiral carbon in three position. A formation of the octanediol in the course of fatty acid synthesis was, therefore, concluded.[9]

Alternatively, a LOG-initiated degradation of linoleic and linolenic acid, respectively, might be suggested, easily explaining the concurrent formation of the (5Z)-analogue compound. A LOG-dependent biosynthesis of the closely related 1-octen-3-ol, a constituent of mushroom and fish flavor, was established by Grosch by contacting fungal enzymes with a 10-hydroperoxy-(8E, 12Z)-octadecadienoate.[10]

Subcellular sites of flavor generation

Remarkable results obtained by Tressl using passion fruit showed that the hydroxybutanoates in the yellow cv. were mainly of the (S)-(+)-configuration, whereas the (R)-(−)-form predominated in the purple cv.[11] The (S)- configuration corresponds to a β-oxidative formation, and the (R)- configuration to fatty acid synthesis or to a non-epimerized hydroxyacyl intermediate of β-oxidation. This, as well as the findings with octanediol, shows that there are several possible reactions leading to chiral acyl metabolites. Thus, the involvement of fatty acid synthesis vs β-oxidation should be assessed with great care.

Direct experimental evidence for the subcellular

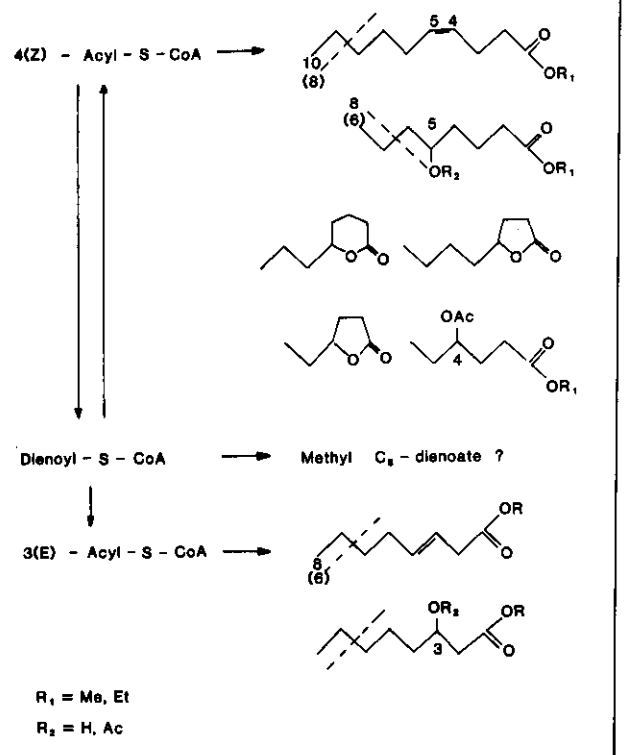

Figure 2. According to the classical β-oxidation scheme the degradation of (2E,4Z)-decadienoate yields (2Z)-octenoate and acetyl-CoA. The alternative dienoyl-CoA reductase pathway found in pineapple fruit yielded (3E)- and (4)-unsaturated, and C_{n-2}-saturated products.

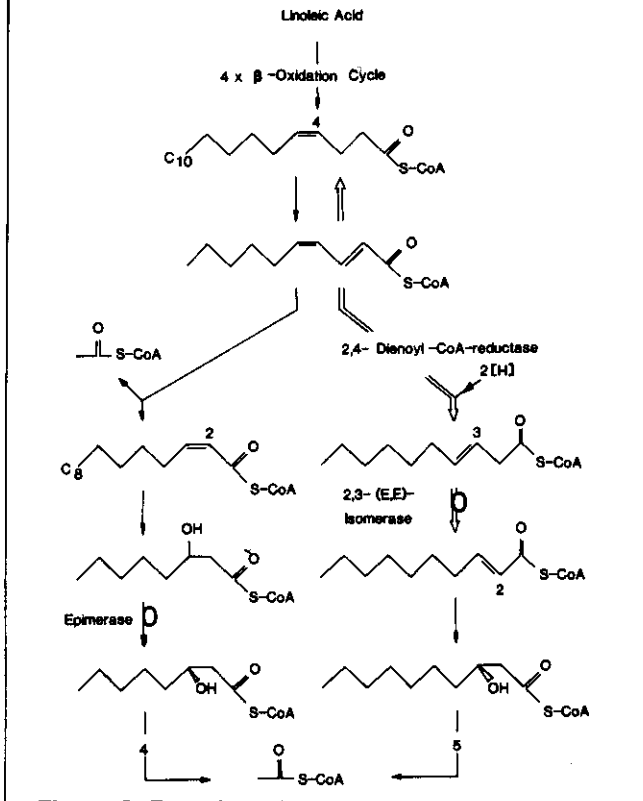

Figure 3. Based on the peroxisomal dienoyl-CoA pathway (see Figure 2) a wide diversity of products may be formally derived. These compounds are actually major constitutents of the polar fractions of pineapple flavor.

site of flavor generation, such as for the operative pathway, was generally lacking. Only recently, experiments with pineapple tissue revealed for the first time a subcellular site of flavor formation.[12,13]

Segments of pineapple fruit were incubated in separate experiments with various unsaturated C_6-acyl moieties. The main products observed, butanoates and (3E)-hexenoates, do not fit into the classical β-oxidation scheme, but are consistent with the dienoyl-CoA reductase pathway (Figure 2).

The classical pathway via 3-hydroxyacyl-CoA epimerase would degrade a (2E, 4)-dienoyl intermediate to a (2)-unsaturated compound and acetyl-CoA. The alternative pathway via 2,4-dienoyl-CoA reductase, by contrast, yielding a 3E-intermediate followed by a C_{n-2}-saturated compound, would explain the mentioned metabolites found in pineapple.

The key experiment, the conversion of a 2,4-unsaturated acyl compound, was repeated using deuterated sorbate, and the expected products were found labeled. The labeling pattern of the substrate was retained in the products indicating a concerted protonation/deprotonation reaction by the reductase. By repeating this pathway on the C_8 and C_{10}-level a wide diversity of products may be formally derived, most of which are actually major constituents of the polar fractions of pineapple flavor: 3- and 4-enoates, 3-, 4-, and 5-hydroxy and acetoxy esters, and 4- and 5-alkanolides (Figure 3).

The activity and peroxisomal localization of the dienoyl-CoA reductase pathway was demonstrated in *E. coli*,[14] *Candida tropicalis*,[15] and mammalian liver.[14,16]. The experimental proof in pineapple tissue posed some difficulties, e.g., all attempts using sucrose density gradient centrifugation failed. Finally, a protocol including five centrifugation steps and a Percoll density gradient for separating the cell organelles yielded active mitochondria and peroxisomes.

With the conditions applied, a significant amount of mitochondria seemed to be disrupted, as indicated by high activities of fumarase in the denser fractions. This debris, however, may rather be attributed to the initial homogenization step when the tough fruit tissue has to be mechanically disintegrated.

Upon feeding [D_5]-(2E, 4E)-hexadienoyl-CoA to organelle fractions, the expected products of the dienoyl-CoA reductase pathway were exclusively

detected in the peroxisomal fraction. The mitochondrial fractions with and without precursor and the peroxisomal fraction without precursor were devoid of mass fragments diagnostic of deuterated (3E)- and (4E)-hexenoates and of deuterated butanoates.

The synthetic events of the dienoyl-CoA reductase pathway could well reflect the homeostatic requirements of a senescing tissue: in combination with the auxillary activities of β-oxidation they could help the plant cell to avoid critical levels of medium-chain fatty acids which may otherwise accumulate in the course of membrane degradation up to detrimental levels. As the biogenesis of peroxisomes is effectively induced in procaryotic and eukaryotic cells by chemical treatments, a better characterization of the plant peroxisomal system could open up new avenues to flavor improved fruits.

Bioflavors from microbial and plant cell cultures

Studies on the biogenesis of fruit flavors are not only a continuing story, they are just going through a renaissance because of the recent surge of interest in flavor-producing cell cultures. Microbial and plant cell cultures are being discussed as future independent sources of volatile flavors.[17]

Among the microbial cultures, some highly developed filamentous fungi proved to be active producers of volatile flavors.[18] Optimizing the yields is not only a matter of precursor feeding, appropriate bioreactor design and downstreaming, but is also affected by regulation phenomena.

When, e.g., submerged cultured *Polyporus durus*, a basidiomycete producing 4-octanolide, was supplemented with various saturated or unsaturated fatty acids thought to be alkanolide precursors,[19] no promoting effects were found. Only the addition of a synthetic coconut oil fraction, called Miglyol, markedly enhanced the biosynthesis of alkanolides.[20]

This beneficial effect may be traced back to a controlled liberation of the fatty acid substrate by a fungal exolipase. The mono and diacyl-glycerols formed may mediate the uptake of substrate. The lactonization of the primary products, 4-hydroxy acids, is favored by the dramatic drop of the pH (from pH 6 to pH < 3) during the lag phase. As a result the volatile product is removed from its equilibrium of formation. Some alkanolide producing microbial cultures reach the 1 g/l threshold now,[21] and first industrial applications have been published.

All attempts to accumulate significant amounts of volatile flavors in plant cell cultures have failed so far. This inability is usually discussed in terms of a lacking morphological differentiation, as intact plants store their volatile secondary metabolites in oil glands, resin ducts, or in specialized tissues. On the other hand, our experiments to simulate accumulation sites by adding synthetic organic or inorganic polymers showed limited success.

However, when growing heterotrophic cultures of *Citrus* sp., e.g., were supplied with the desired volatile products, terpenols, a rapid catabolism was observed. This means that, if the plant cells in vitro synthesized terpenes at all, accumulation would have been prevented by the much faster degradation.

The oxidative degradation of geraniol was established by detecting geranic and citronellic acids, and, after some ten minutes more, detecting branched-chain cleavage products. This catabolic route was further substantiated by a concurrent increase in respiration,[7] and the synthesis of labeled sterols and carotenoids.[22] Slowing down this catabolism will be the primary objective in establishing better yielding cultures. In vitro plant cells will continue to be the most challenging and the most promising subject of future research into bioflavors.

Finally, it should be emphasized that progress in flavor research during the past two decades was greatly stimulated by methodological and instrumental advances. More recent examples are the separation of volatile enantiomers, such as the chiral octadiols, or of deuterated and nondeuterated analogues using high resolution capillary columns.

Similar mutual dependencies of basic and applied science can be predicted for the future flavor research.

Acknowledgment: *This paper was based on a lecture presented as part of the ACS "Symposium on Analytical Methods in Agriculture and Food Chemistry—A tribute to Walter G. Jennings"; Miami, Fl, 9/12-13, 1989. Funding provided by the ACS, the Federal Minister of Research and Technology (BMFT), Bonn, and the Federal Minister of Agriculture (BML), Bonn via AIF and Forschungskreis der Ernêhrungs-industrie is gratefully acknowledged. Essential support came from F. Drawert, Z. Akkan, G.R. Dettweiler, H. Kollmannsberger, and K. Neuhêuser are thanked for collaboration.*

References

1. D E Heinz and W G Jennings, *J Food Sci* **31**, 69-80 (1966)
2. R K Creveling and W G Jennings, *J Agric Food Chem* **18,** 19-24 (1970)
3. P Schreier, *Chromatographic Studies of the Biogenesis of Plant Volatiles,* W Bertsch, W G Jennings and R E Kaiser, eds, Heidelberg: H thig (1984)
4. *Biogeneration of Aromas,* T H Parliment and R Croteau, eds, Wash DC: ACS Symp Ser 317 (1986)
5. *Bioflavour '87,* P Schreier, ed, Berlin: W de Gruyter (1988)
6. M Knee and S G S Hatfield, *J Sci Food Agric* **32,** 593-600 (1981)
7. F Drawert in *Bioflavour '87,* P Schreier, ed, Berlin: W de Gruyter (1988) pp 3-32
8. R G Berger, G R Dettweiler and F Drawert, *Dtsch Lebensm Rdsch* **84,** 344-347 (1988)
9. W Schwab, G Scheller, D Gerlach and P Schreier, *Phytochemistry* **28,** 157-160 (1989)
10. W Grosch, *Lebensm Gerichtl Chemie* **41,** 40-46 (1987)
11. R Tressl and W Albrecht in *Biogeneration of Aromas,* T H Parliment and R Croteau, eds, Wash DC: ACS Symp Ser 317, (1986) pp 114-133
12. R G Berger and H Kollmannsberger in *Topics in Flavor Research,* R G Berger, P Schreier and S Nitz, eds, Marzling Eichhorn (1985) p 305-320
13. R G Berger, G R Dettweiler and F Drawert, *Phytochemistry,* submitted
14. V Dommes, W Luster, M Cvetanovic and W-H Kunau, *Eur J Biochem* **125,** 335-339 (1982)
15. P Dommes, V Dommes and W-H Kunau, *J Biol Chem* **256,** 10846-10848 (1983)
16. H Osmundsen, J Cervenka and J Bremer, *Biochem J* **208,** 749-755 (1982)
17. R G Berger and F Drawert in *Flavour Science and Technology,* M Martens, G A Dalen and H Russwurm Jr, eds, Chichester: Wiley (1987) pp 199-215
18. R G Berger, F Drawert and S H! drich in *Bioflavour ' 87,* P Schreier, ed, Berlin: W de Gruyter (1988) pp 415-434
19. C S Tang and W G Jennings, *J Agric Food Chem* **16,** 252-254 (1968)
20. R G Berger, K Neuh! user and F Drawert, *Z Naturforsch* **41c,** 963-970 (1986)
21. G -F-Kapfer, R G Berger and F Drawert, *Biotechnol Lett* in press (1989)
22. S Paisarnrat and C Ambid in *Topics in Flavour Research,* R G Berger, P Schreier and S Nitz, eds, Marzling: Eichhorn (1985) pp 321-333