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Pathway Analysis of Branched-Chain Ester Biosynthesis in Apple Using Deuterium Labeling and Enantioselective Gas Chromatography−Mass Spectrometry

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The biosynthesis of volatile esters by Red Delicious apples was investigated by incubating fruit tissue with deuterated flavor precursors at various times after controlled atmosphere (CA) storage and measuring deuterium incorporation into branched-chain ester volatiles. 2-Methylbutyl acetate was the only volatile not significantly reduced by CA storage. Conversion of 2-methylbutanol to 2-methylbutyl acetate and of 2-methylbutanoic acid to ethyl 2-methylbutanoate and to hexyl 2-methylbutanoate was limited by the availability of 2-methylbutyl substrates but not by acetyl-CoA, ethanol, or hexanol, respectively. The enzymatic activity required for these reactions declined during CA storage. The conversion of 2-methylbutanoic acid to 2-methylbutanol was also substrate limited, but enzymic activity appeared stable in storage. Biosynthesis of both 2-methylbutanoic acid and 2-methylbutanol, from isoleucine, was severely depressed under CA storage. The reduced metabolism of isoleucine to 2-methylbutanoyl-CoA may be the primary reason for reduced branched-chain ester synthesis in CA-stored Red Delicious apples. Enantioselective gas chromatography−mass spectrometry confirmed that the chirality of (*S*)-2-methylbutyl acetate derives from L-isoleucine with the other enzymes in this pathway not being enantiospecific. Treatment of tissue samples with 2-methylbut-2*E*-enal gave only (*S*)-2-methylbutyl acetate, indicating that biosynthesis was not via tiglyl-CoA.

KEYWORDS: ***Malus domestica*** **Borkh.; Red Delicious; apple; CA storage; biosynthetic pathways; flavor; aroma; deuterium labeling; enantioselective; GC-MS**

INTRODUCTION

The most common volatile flavor and aroma compounds of fruit are straight and branched-chain alkyl esters produced from fatty (*1*−*4*) and amino acids (*5*). Branched-chain esters derived from 3-methylbutyl, 2-methylbutyl, and 2-methylpropyl alcohols and acids are important contributors to the flavors of many fruits including melons (*6*), strawberries (*7*), and bananas (*8*). Ethyl 2-methylbutanoate (**16**) [aroma threshold of 0.1 ppm in water (*9*)], which has "intense apple-like, green and fruity" flavor attributes (*9*), and 2-methylbutyl acetate (**15**) [flavor threshold of 5 ppb in water (*9*)], with banana and apple aroma attributes, are major contributors to the flavors of many apple cultivars (*1*, *10*−*12*).

The biosynthesis of the 3-methylbutyl, 2-methylbutyl, and 2-methylpropyl esters proceeds from the amino acids, leucine, isoleucine, and valine, respectively, in apples (*5*, *13*) and strawberries (*7*), melons (*14*), and bananas (*15*). The operation of these biosynthetic pathways is supported by numerous feeding experiments where the addition of intermediates enhanced the concentrations of specific aroma compounds (*3*−*5*, *7*). Thus, the addition of d_{10}-L-isoleucine (**1a**) to the peel of Red Delicious apples resulted in deuterium-labeled 2-methylbutanol (**13a**), 2-methylbutyl, 2-methylbutanoate, and 2-methylbut-2-enyl esters (*5*) (**Figure 1**). Recently, a number of alcohol acyl-CoA transferases (AAT), which catalyze the last step in the biosynthesis of ester volatiles, have been characterized in strawberries (*16*), apples (*17*−*20*), and melons (*21*). These enzymes can catalyze reactions involving a variety of acyl-CoA and alcohol substrates.

Volatile production by fruit is also dependent on the variety, maturity, storage, and environmental conditions and is linked to ripening, the climacteric rise in respiration, and the production of ethylene by the fruit (*22*, *23*). In apple, the activity and expression of an AAT, but not of an alcohol dehydrogenase (ADH), is dependent on ethylene, suggesting an involvement of ethylene in ester biosynthesis (*17*). Suppression of ethylene action by the use of 1-methylcyclopropene (*23*, *24*) or of ethylene biosynthesis using transgenic apple trees (*25*) or ethylene synthesis inhibitors (*26*) reduces the production of branched and straight-chain esters and alcohols. Storage of apples under controlled atmosphere (CA) can result in both the enhancement (*27*−*29*) and the suppression of particular flavor volatiles (*2*, *27*, *29*, *30*). The largest effects are produced with extended storage times under low oxygen (0.5%) and high CO_2 concentrations (6%) (*30*). Thus, ultralow O_2 (ULO) CA storage

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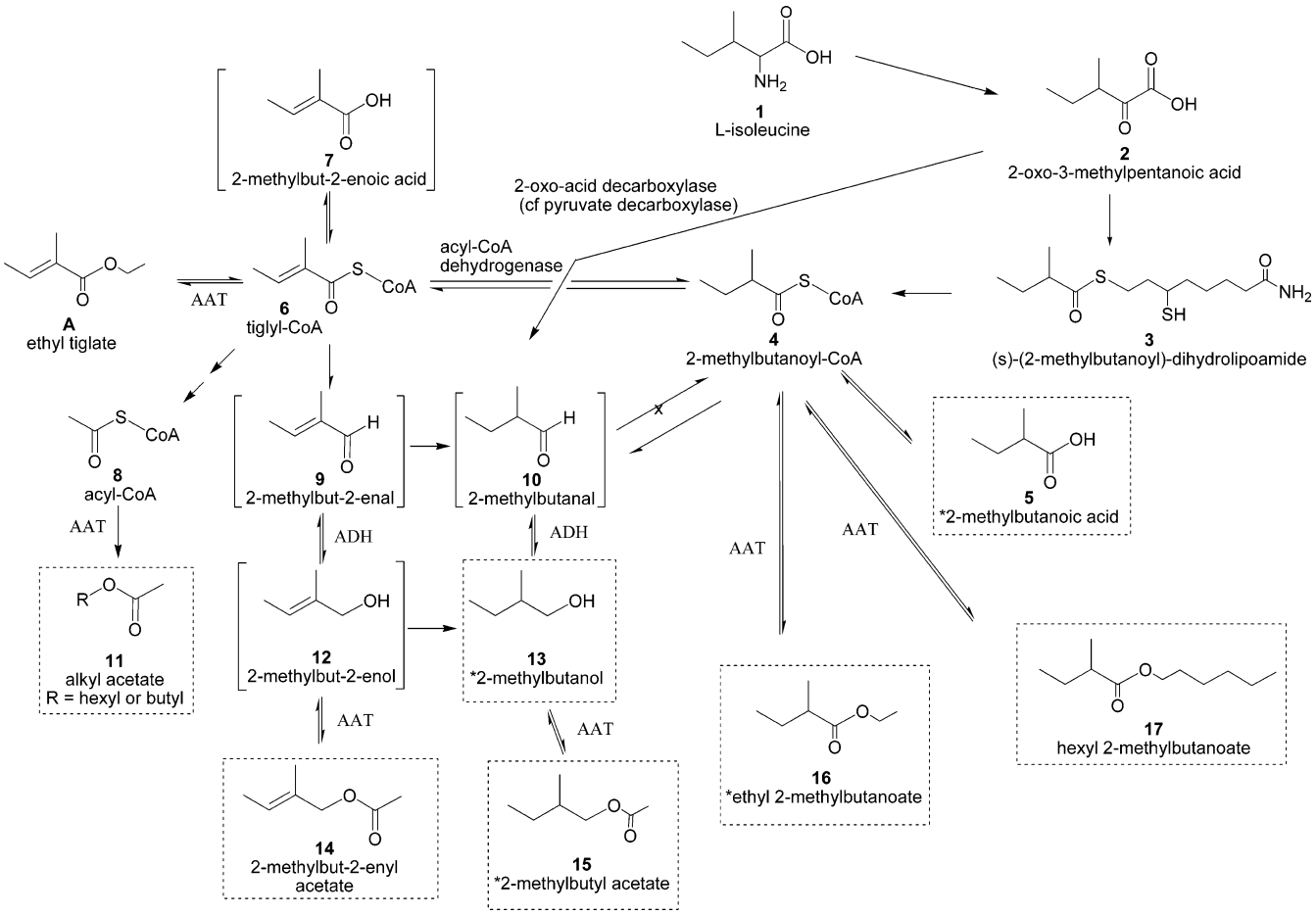

Figure 1. Biosynthetic pathways to the formation of branched-chain ester volatiles in Red Delicious apples. Boxed compounds were identified as products of isoleucine catabolism by deuterium labeling experiments in the current and previous studies (*5*). Bracketed compounds are nondeuterated precursors, which increased the concentrations of 2-methylbutanoic acid, or 2-methylbutanol, or 2-methylbutyl acetate. Compound names designated with asterisks, **5**, **13**, **15**, and **16**, were produced in deuterium-labeled form on injection of apple fruit with ethyl d_4-tiglate (**A**) (*38*).

reduces concentrations of branched and straight-chain esters with a concomitant increase of ethyl esters (*28*, *29*), and ULO storage at high CO_2 concentrations (3%) further suppresses the production of branched-chain esters (*2*). Reduced volatile production is believed to result from a limiting supply of immediate precursors rather than from degradation or inactivation of the biosynthetic enzymes AAT and ADH (*31*, *32*), as treatment of fruit or tissue sections with the vapors of aldehydes, alcohols, and carboxylic acids significantly enhances concentrations of the corresponding esters (*3*, *22*, *33*). While production of ethyl 2-methylbutanoate by McIntosh and Cortland apples was severely suppressed during low ethylene CA storage, after ripening, CA-stored fruit produced substantially greater amounts of ethyl 2-methylbutanoate than fruit ripened at 20 °C immediately after harvest (*27*). Similarly, Rome apples produced more 2-methylbutyl acetate after low oxygen CA storage than after cool storage (*31*).

The biochemical basis for these effects remains obscure. Inhibition by high CO_2 concentrations of the tricarboxylic acid cycle, from which most amino acid precursors are derived, has been hypothesized (*2*). Jonagold apples stored under a ULO and high CO_2 (6%) regime (*30*) contained lower concentrations of ATP. AAT activity was reduced and absent in Rome apples stored at 1.0 and 0.5% oxygen for 9 months but recovered during storage in air (*31*). The recovery of volatile biosynthesis capability after CA storage was more pronounced for the amino acid than for the fatty acid metabolism-derived volatiles (*2*).

The aim of this research was to obtain a better understanding of the behavior, during CA storage, of the biochemical pathways leading to volatile ester production and to establish the biochemical basis for the reduced production of branched-chain esters by apples during CA storage. Precursor treatment of Red Delicious apples, over 5 months of CA storage, was used to identify the specific biochemical steps limiting volatile production. Enantioselective gas chromatography−mass spectrometry (GC-MS) was used to establish additional interconnections in the pathways for volatile ester biosynthesis.

MATERIALS AND METHODS

Plant Material. Early commercial harvest Red Delicious (April 7, 1999) apples (*Malus domestica*) of count size 125 (~75 mm diameter) were obtained from orchards in Hawke's Bay, New Zealand. Soluble solids at harvest were 13.3% Brix [standard error of the mean (SEM) = 0.1] using a hand-held refractometer (Atago P1, Tokyo), and firmness was 7.03×10^4 N (SEM = 0.1×10^4) using an 11.1 mm probe on a hand-held penetrometer. Fruits were judged preclimacteric based on the relative starch index of 3.3 (SEM = 0.2) measured using the starch iodine pattern method (*34*). The fruit was stored under CA (1 °C, 3 kPa CO_2, 3 kPa O_2, and 95 kPa N_2) for 5 months during which time fruits were periodically removed and immediately analyzed for their ability to produce flavor volatiles. Fruits for experiments with nondeuterated flavor precursors were obtained at eating ripeness from a local supermarket.

Chemicals. All chemicals were obtained from the Aldrich Chemical Co. unless otherwise stated. Pentane and Et_2O (Analar, BDH) were

Table 1. Deuterium-Labeled Flavor Precursors Added to Apple Skin Tissue Sections and Labeled Flavor Compounds Measured in Solvent Extracts from These Tissue Samples

precursor solution no.	deuterated flavor precursors in aqueous stock solutions	precursor concn in 4.2 mL assay system (μM)	deuterated flavor compounds measured
1 (alcohols)	d_3-hexanol (**18b**)	150	d_3-hexyl 2-methylbutanoate (**17c**)
	2-d_3-methylbutanol (**13b**)	150	2-d_3-methylbutyl acetate (**15b**)
2 (acids)	2-d_3-methylbutanoic acid (**5b**)	435	ethyl 2-d_3-methylbutanoate(**16b**)
			hexyl 2-d_3-methylbutanoate (**17b**)
			2-d_3-methylbutanol (**13b**)
			2-d_3-methylbutyl acetate (**15b**)
	d_4-acetic acid (**19b**)	2430	2-methylbutanoate d_3-acetate (**15c**)
3 (isoleucine)	d_{10}-L-isoleucine (**1a**)	1636	d_9-2-methylbutanoic acid (**5a**)
			ethyl-d_9-2-methylbutanoate (**16a**)
			hexyl-d_9-2-methylbutanoate(**17a**)
			d_9-2-methylbutanol (**13a**)
			d_9-2-methylbutyl acetate (**15a**)

purified by distillation over CaH_2 and passage through neutral alumina (Ajax Chemicals) that had been activated by heating for 10 h at 350 °C. Racemic and (*S*)-2-methylbutyl acetate were synthesized from racemic and (*S*)-2-methylbutanol (*5*). d_{10}-Isoleucine was obtained from Cambridge Isotope Laboratories (Woburn, MA).

Incubation with Deuterium-Labeled Precursors. Volatile production by fresh fruit was determined immediately before and after 22, 51, 94, and 149 days of CA storage. Twenty fruits were used for each set of volatile experiments. After they were warmed to room temperature for 6 h, apples were peeled with a potato peeler, 60 g of peel was cut into 2−3 mm squares, and the peel was rinsed twice in 200 mL of pH 6.45 MES (11 mM) buffer containing 0.33 M mannitol (Sigma), 0.68 mg/L chloramphenicol (Sigma), and 0.11 mg/L cycloheximide (Sigma) (*5*). Washed peel (2.0 g) was suspended in 2.5 mL of this buffer in a 20 mL glass scintillation vial (Wheaton). Deuterated precursors were prepared as stock solutions in water at concentrations of 6.3 mM 2-d_3-methylbutanol (**13b**) and d_3-hexanol (**18b**), 1.02 M d_4-acetic acid (**19b**), 183 mM 2-d_3-methylbutanoic acid (**5b**), and 142 mM d_{10}-L-isoleucine (**1a**). Aliquots (100, 10, 10, and 50 μL, respectively) of the stock solutions were added to the vials, which were sealed and incubated at 25 °C for 16 h. Final concentrations of precursors in the assay system are shown in **Table 1**. Five replicates of each treatment and control (buffer only) sample were used. SEMs were calculated for the five replicates, and Student's *t* tests were used (Microsoft Excel) to determine whether incubation with the deuterated precursors significantly increased the levels of the "target" compounds. Structures of deuterated precursors and their biosynthetic products are shown in **Figure 2**.

Aroma volatiles were extracted from the buffer by adding 5 mL of pentane/Et_2O (50:50) to each scintillation vial, which was resealed, and the contents were mixed on an orbital shaker for 30 min. The slurry was frozen at −18 °C overnight, and the pentane/Et_2O phase was decanted, filtered through glass wool, and then filtered through a 0.5 μm stainless steel high-performance liquid chromatography sample filter (Upchurch Scientific, WA) using a gastight syringe. Volatiles were measured by comparison with authentic compounds as external standards using selected ion monitoring (**Table 2**) GC-MS with a QP5050A instrument (Shimadzu Scientific Instruments, Kyoto). The authentic standards were ethyl 2-d_3-methylbutanoate (**16b**, 93%), 2-d_3-methylbutanoic acid (**5b**, 95%), 2-d_3-methylbutanol (**13b**, 97%), 2-d_3-methylbutyl acetate (**15b**), 2-methylbutyl acetate (**15**) (*7*), ethyl 2-methylbutanoate (**16**, Acros, 99%), 2-methylbutanoic acid (**5**, >98%), 2-methylbutanol (**13**, >98%), and hexyl 2-methylbutanoate (**17**, 99.8%) synthesized from 2-methylbutanoic acid and hexanol. The remaining compounds listed in **Table 2** were identified based upon their elution slightly before their nondeuterated counterparts and diagnostic ions identified previously (**13a**, **15a**, **15c**, **16a**, **17a**, and **17b**) (*7*). Separations were achieved on a 60 m × 0.25 mm i.d., 0.25 μm, ZB-Wax column (Phenomenex, Torrance, CA). The injector temperature was 220 °C, and the He pressure ramp was 70 kPa for 10 min, 0.2 kPa/min to 77 kPa, 1.2 kPa/min to 110 kPa, and held for 15 min. The oven ramp was 25 °C for 10 min, 1 °C/min to 55 °C, 5 °C/min to 200 °C, 15 °C/min to 220 °C, and held for 20 min.

Incubation with Nonlabeled Branched-Chain Precursors. 2-Methylbut-2*E*-enal (**9**), 2-methylbutanal (**10**), and 2-methylbut-2*E*-enol (**12**) were added to each of two separate samples of Red Delicious apple skin in buffer, as above, to produce concentrations of ca. 250 μM. 2-Methylbut-2*E*-enoic (tiglic) acid (**7**) was used at 40 μM.

Chirality of 2-Methylbutyl Acetate (15) Produced from 2-Methylbut-2E-enal (9). Solutions of volatiles in pentane/Et_2O were analyzed on a 30 m × 0.25 mm i.d., 0.25 μm, β-Dex 325 enantioselective GC column (Supelco) in a Agilent Technologies 6890N GC coupled to a Waters GCT time-of-flight mass spectrometer. Injections were 1 min splitless at 200 °C, and the He flow was 1 mL/min. The oven ramp was 30 °C for 1 min, 1 °C/min to 80 °C, and 10 °C/min to 230 °C. Under these conditions, (*S*)-2-methylbutyl acetate eluted 17 s before (*R*)-2-methylbutyl acetate.

Chirality of 2-Methylbutyl Acetate (15) Produced from Racemic Ethyl 2-Methylbutanoate (16). Whole fruits were exposed for 2 h to the vapor from a 50 μL sample of ethyl 2-methylbutanoate in sealed 1.5 L headspace jars, which were then left open (aired) for 1 h before resealing and headspace sampling onto Tenax-TA traps (*5*). Separations were conducted on a 30 m × 0.25 mm i.d., 0.25 μm, Cyclodex-β enantioselective GC column (J&W) in a HP5890 GC with flame ionization detection detection. Injections were 1 min splitless at 220 °C, and the detector was at 240 °C. The oven ramp was 30 °C for 12 min, 3 °C/min to 100 °C, 5 °C/min to 150 °C, 15 °C/min to 220 °C, and held for 10 min. Peak identities were confirmed by coinjection with racemic and (*S*)-2-methylbutyl acetate.

RESULTS AND DISCUSSION

The relative importance of precursor availability and enzyme activities on volatile ester biosynthesis in CA-stored Red Delicious apples was assessed by periodically removing batches of apples from CA storage and feeding them both branched and straight-chain flavor precursors. Concentrations of flavor precursors for feeding experiments were chosen so as to exceed endogenous concentrations but not to produce toxic symptoms (reduced volatile production). As the skin is the primary source of volatile production in apples (*17*, *19*, *35*), with decreasing volatile production toward the center of the fruit (*36*), the skin was used as a model for the whole fruit. CA storage of fruit for 21 days resulted in a reduction in the concentrations of all of the volatiles measured except 2-methylbutyl acetate (**15**) (**Figures 3**−**5**). This effect was particularly marked for hexyl 2-methybutanoate (**17**) (**Figure 3B**) and 2-methylbutanoic acid (**Figure 5C**), which were reduced ca. 10- and 300-fold, respectively.

Incubation with Alcohol Precursors: 2-d_3-Methylbutanol (13b) and d_3-Hexanol (18b). Incubation of tissue samples with 2-d_3-methylbutanol at 149 μM (concentrations 4−12-fold greater than the endogenous concentrations in the control tissue samples) significantly ($P < 0.05$) increased 2-methylbutyl

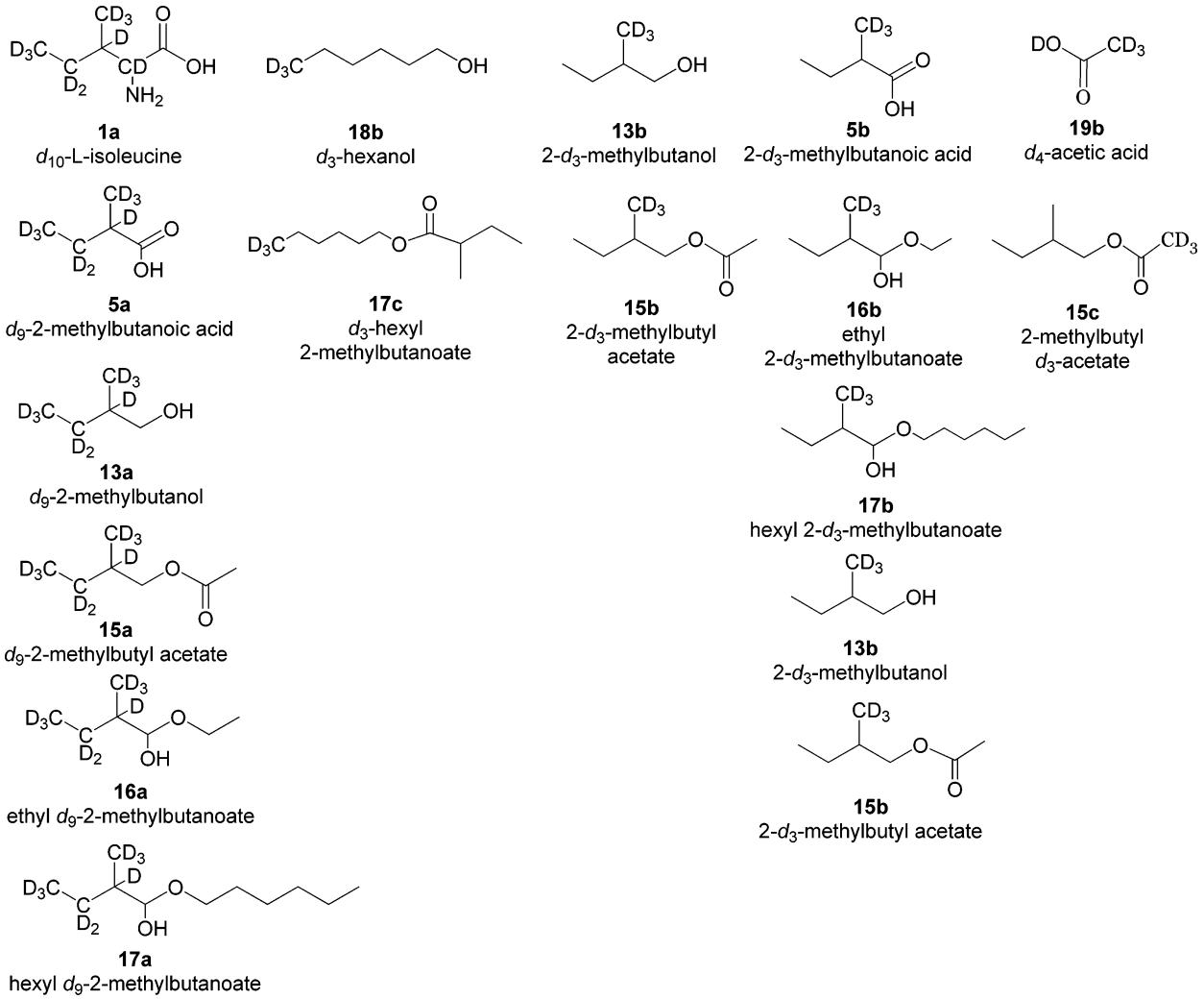

Figure 2. Structures of deuterated precursors and biosynthetic products.

acetate (**15**) concentrations from two- to six-fold above those in the control samples (**Figure 3A**), with the additional ester produced being d_3-labeled (*5*). The addition of d_4-acetic acid (**19b**) with 2-d_3-methylbutanol (**13b**) did not further increase production of 2-methylbutyl acetate (data not shown), implying that the alcohol precursor was limiting. The AAT activity in the fruit was sufficient to acetylate additional alcohol at all times during the storage trial, but the capacity to produce additional 2-methylbutyl acetate declined as the storage period continued, indicating a progressive loss of AAT activity. In their study of an AAT enzyme (MdAAT2) from Golden Delicious apple skin, Li et al. (*19*) similarly found that decreased ester production was correlated with reduced AAT activity and with decreased concentrations of precursors, although the concentration of the MdAAT2 protein was maintained at a relatively high level.

In contrast, overall production of hexyl 2-methylbutanoate (**17**) in CA-treated fruit was, at best, nine-fold below that of the fresh fruit and did not increase on addition of 2-d_3-methylbutanol (**13b**) (**Figure 3B**). The production of hexyl 2-d_3-methylbutanoate (**17b**) requires oxidation of 2-d_3-methylbutanol (**13b**) to 2-d_3-methylbutanoyl-CoA before esterification (**Figure 1**) and while this process occurs to a minor extent in Granny Smith apples, it was not observed previously in Red Delicious apples (*5*). In the present study, while Red Delicious apple skin produced 2−6-fold more 2-d_3-methylbutyl acetate (**15b**) than 2-methylbutyl acetate when incubated with 2-d_3-methylbutanol (**13b**), no 2-d_3-methylbutanoic acid (**5b**), ethyl 2-d_3-methylbu-

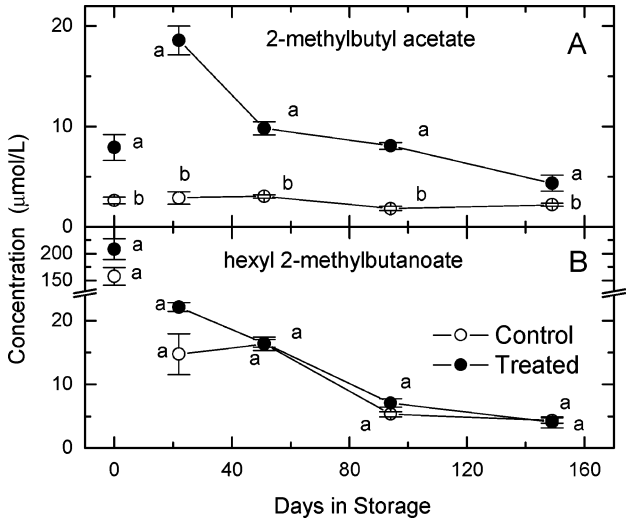

Figure 3. Concentration (mean ± SEM) of volatiles produced by Red Delicious apple peel incubated for 16 h in buffer with 2-d_3-methylbutanol and d_3-hexanol. Hollow symbols represent product concentrations in control (untreated) samples. Solid symbols represent product concentrations (deuterated + nondeuterated) in samples treated with the deuterated alcohol precursor. Points at zero days are values for fresh fruit at the beginning of the storage trial. Means at each time point with the same lower case letter are not significantly different ($P > 0.05$). The deuterated hexyl 2-methylbutanoate in **B** was produced from d_3-hexanol.

Table 2. Target and Reference Ions Used for Selected Ion GC-MS Measurement of Deuterium-Labeled Branched-Chain Compounds from Apple Skin Tissue Sections Incubated with Deuterium-Labeled Flavor Precursors

compound	*m/z* target ion	*m/z* reference ions
ethyl 2-methylbutanoate (**16**)	102.1	85.1
ethyl 2-d_3-methylbutanoate (d_3-methyl) (**16b**)	105.1	88.1, 60.1
d_5-ethyl 2-methylbutanoate (d_5-ethyl) (**16**)	108.1	85.1, 50.1
d_8-ethyl 2-methylbutanoate (d_5-ethyl + d_3-methyl)	110.1	88.1, 60.1, 50.1
ethyl d_9-2-methylbutanoate (d_9-2-methylbutanoate) (**16a**)	107.1	94.2, 66.1
2-methylbutyl acetate (**15**)	43.1	73.1, 78.2
2-d_3-methylbutyl acetate (d_3-methyl) (**15b**)	43.1	73.1
2-methylbutyl d_3-acetate (d_3-acetate) (**15c**)	46.1	70.1
d_6-2-methylbutyl acetate (d_3-methyl + d_3-acetate)	46.1	73.1
d_9-2-methylbutyl acetate (d_9-2-methylbutyl) (**15a**)	43.1	70.1
2-methylbutanol (**13**)	57.1	70.1
2-d_3-methylbutanol (d_3-methyl) (**13b**)	60.1	59.1, 73.1
d_9-2-methylbutanol (**13a**)	66.1	64.2, 78.2
2-methylbutanoic acid (**5**)	74.1	87.1
2-d_3-methylbutanoic acid (d_3-methyl) (**5b**)	77.1	90.1, 60.1
d_9-2-methylbutanoic acid (**5a**)	79.1	66.1
hexyl 2-methylbutanoate (**17**)	103.1	94.2
hexyl 2-d_3-methylbutanoate (d_3-methyl) (**17b**)	106.1	88.1
d_3-hexyl 2-methylbutanoate (d_3-hexyl) (**17c**)	103.1	87.1, 59.1
d_6-hexyl 2-methylbutanoate (d_3-methyl + d_3-hexyl)	106.1	87.1, 88.1
hexyl d_9-2-methylbutanoate (d_9-2-methylbutanoate) (**17a**)	112.2	94.2

tanoate (**16b**), or hexyl 2-d_3-methylbutanoate (**17b**) was detected, suggesting again that this oxidation reaction does not occur in Red Delicious apples. Hexyl 2-methylbutanoate production decreased with continued storage and was not significantly increased by incubation with d_3-hexanol although low concentrations of d_3-hexyl 2-methylbutanoate (**17b**) were produced (**Figure 3B**). In contrast, hexyl acetate production showed a 2−3-fold increase ($P < 0.05$, data not shown) in response to incubation with d_3-hexanol (**18b**) (*4*), suggesting that hexyl acetate production is limited by the availability of hexanol and probably not by acetyl-CoA, as addition of d_4-acetic acid (**19b**) had little effect.

Incubation with 2-d_3-Methylbutanoic Acid (5b). 2-Methylbutanoic acid (**5**) is biosynthetically related to ethyl 2-methylbutanoate (**16**) and hexyl 2-methylbutanoate (**17**) via 2-methylbutanoyl-CoA (**4**), but conversion to 2-methylbutyl acetate requires reduction to 2-methylbutanal and 2-methylbutanol (**Figure 1**). Incubation of apple skin with 2-d_3-methylbutanoic acid (**5b**) increased ethyl 2-methylbutanoate 5−10-fold above its endogenous concentrations (**Figure 4A**). Including d_6-ethanol (16.5 mM) with the 2-d_3-methylbutanoic acid gave only a minimal further increase (1−1.5-fold) in ethyl 2-methylbutanoate concentrations (data not shown), although a substantial amount of the deuterated ethanol was incorporated into the ester (as d_5-ethyl 2-d_3-methylbutanoate). This suggests that 2-methylbutanoic acid, rather than ethanol, is limiting for the production of ethyl 2-methylbutanoate, akin to the production of 2-methylbutyl acetate being limited by the availability of isoleucine-derived 2-methylbutanol rather than acetic acid. The increase in ethyl 2-methylbutanoate production in response to added substrate appeared relatively constant over the storage period, suggesting stability of the corresponding AAT enzyme.

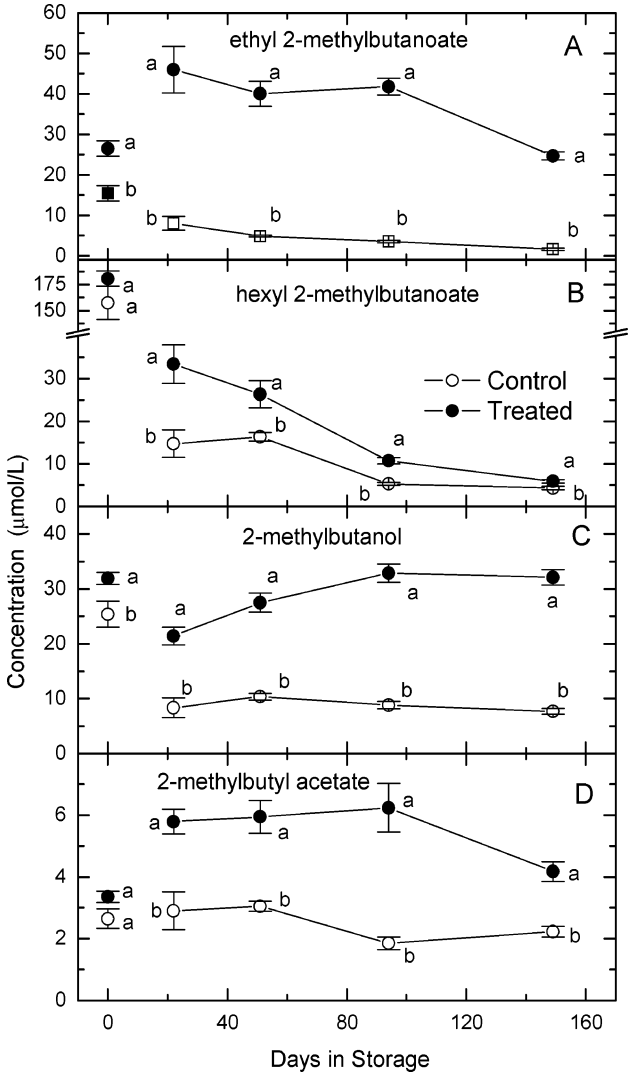

Figure 4. Concentration (mean ± SEM) of volatiles produced by Red Delicious apple peel incubated for 16 h in buffer with 2-d_3-methylbutanoic acid. Hollow symbols represent product concentrations in control (untreated) samples. Solid symbols represent product concentrations (deuterated + nondeuterated) in samples that were treated with 2-d_3-methylbutanoic acid. Points at zero days are values for fresh fruit at the beginning of the storage trial. Means at each time point with the same lower case letter are not significantly different ($P > 0.05$).

Addition of 2-d_3-methylbutanoic acid (**5b**) (**Figure 4B**) increased amounts of hexyl 2-methylbutanoate approximately two-fold ($P > 0.05$) above those in the control sample, consistent with the results from feeding d_3-hexanol (**18b**) (**Figure 3B**) in that the availability of 2-methylbutanoyl-CoA, rather than hexanol, limits hexyl 2-methylbutanoate production. As with the d_3-hexanol incubation experiments (**Figure 3B**), the quantities of hexyl 2-methylbutanoate produced on incubation with 2-d_3-methylbutanoic acid (**5b**) were always below those in the fresh fruit and the concentrations, and increases seen on precursor feeding decreased further as the storage trial pro-

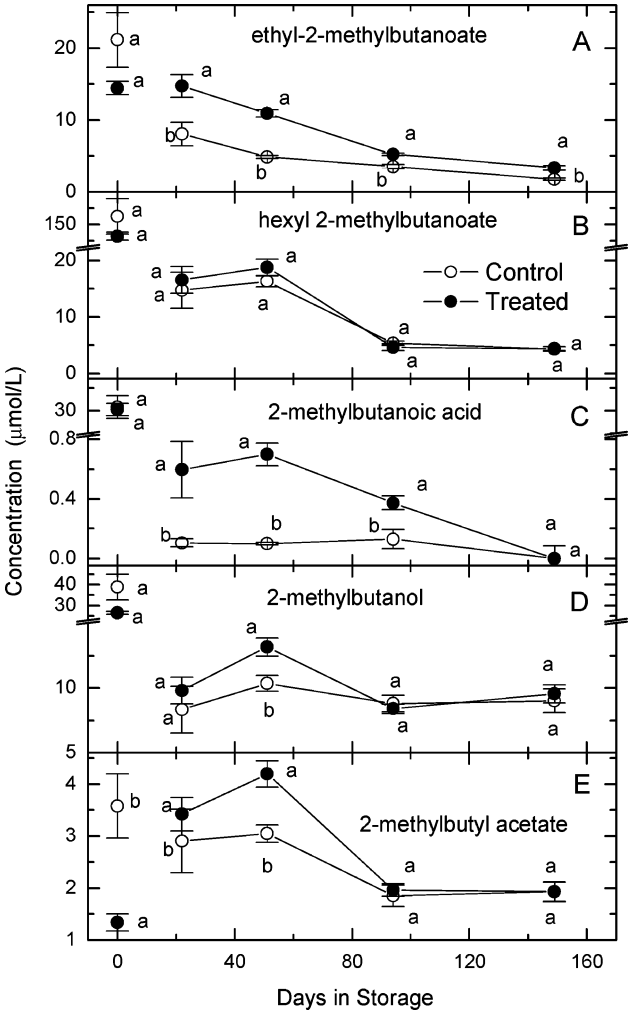

Figure 5. Concentration (mean ± SEM) of volatiles produced by Red Delicious apple peel incubated for 16 h in buffer with d_{10}-L-isoleucine. Hollow symbols represent product concentrations in control (untreated) samples. Solid symbols represent product concentrations (deuterated + nondeuterated) in samples that were treated with d_{10}-L-isoleucine. Points at zero days are values for fresh fruit at the beginning of the storage trial. Means at each time point with the same lower case letter are not significantly different ($P > 0.05$).

ceeded. The very low production of hexyl 2-methylbutanoate by CA-stored apples and the failure of the apples to increase their rate of its production markedly upon treatment with its biosynthetic precursors suggest that hexyl 2-methylbutanoate is produced by different AAT enzymes with differing specificities to those responsible for production of the other two esters (*18*, *20*).

Production of 2-d_3-methylbutanol (**13b**) from 2-d_3-methylbutanoic acid increased total 2-methylbutanol concentrations to 3−4-fold above those in the control samples. The ability to produce 2-d_3-methylbutanol remained effectively constant throughout the storage trial (**Figure 4C**), indicating a stable level of enzymic activity. As expected, 2-d_3-methylbutyl acetate behaved similarly to 2-d_3-methylbutanol but with more modest two-fold concentration increases over control samples (**Figure 4D**). From these results, it is clear that production of 2-methylbutyl acetate will increase with 2-methylbutanoic acid concentrations but this response is not as great as it is for ethyl 2-methylbutanoate production (**Figure 4A**).

Incubation with the Amino Acid Precursor: d_{10}-L-Isoleucine (1a). Incorporation of d_{10}-L-isoleucine into the branched-chain volatiles produced by Red Delicious skin resulted in the production of d_9-esters (**Figure 2**), at amounts similar to those of the endogenous flavor volatiles (**Figure 5**), but with lower concentrations of deuterium incorporation as compared with experiments using deuterated 2-methylbutanol and 2-methylbutanoic acid (**Figures 3** and **4**). The concentration of isoleucine added to the buffer was 10-fold (1600 μM) greater than that of the alcohols (150 μM) and four-fold greater than that of the added 2-methylbutanoic acid (435 μM). Levels of the endogenous flavor volatiles were not changed by feeding isoleucine at this concentration.

Significantly more ethyl 2-methylbutanoate was produced (two-fold) in the d_{10}-isoleucine-incubated CA samples, but incubation with d_{10}-isoleucine gave no significant increase in the concentrations of hexyl 2-methylbutanoate (**Figure 4A**,**B**). The lower incorporation of labeled precursor into hexyl 2-methylbutanoate relative to ethyl 2-methylbutanoate parallels the results obtained on feeding 2-d_3-methylbutanoic acid (**Figure 4B**) and may result from differing AAT activities or specificities (*18*).

The total concentration of 2-methylbutanoic acid in the d_{10}-isoleucine-treated tissue was up to seven-fold greater than that in the control tissue samples during the first 50 days of CA storage (**Figure 5C**). Even so, 2-methylbutanoic acid concentrations were always very much lower (0.03−2%) in CA-stored fruit skin than in the skin of fresh fruit and these reduced concentrations correlated with lower concentrations of 2-methylbutanol and hexyl 2-methylbutanoate (**17**). The addition of 2-methylbutanoic acid, however, did not greatly increase either alcohol or ester concentrations (**Figure 4C**,**D**), and the very low concentrations of 2-methylbutanoic acid in the isoleucine-incubated fruit were not reflected in very low concentrations of ethyl 2-methylbutanoate (**16**) or 2-methylbutyl acetate (**Figure 5**), suggesting efficient but substrate-limited conversion of 2-methylbutanoic acid precursor to these esters.

In contrast to ethyl 2-methylbutanoate, 2-methylbutanol and 2-methylbutyl acetate concentrations in the tissue samples showed little response to feeding d_{10}-isoleucine (**Figure 5D**,**E**). Although comparable amounts of labeled precursor moved through each branch of the pathway from 2-methylbutanoic acid, the high endogenous concentrations of 2-methylbutanol and 2-methylbutyl acetate in the fruit skin meant that the total concentrations of these volatiles were not significantly increased.

Investigation of Alternate Biosynthetic Pathways. In addition to pathway restriction, the operation of alternate pathways and switching between them may contribute to the complex behavior of flavor volatiles during CA storage. The "standard" pathway (**Figure 1**) from isoleucine (**1**) is via 2-methylbutanoyl-CoA (**4**), which is reduced to 2-methylbutanal (**10**) (*37*) and then to 2-methylbutanol (**13**). In a second possible route (the Ehrlich pathway), 2-methylbutanal (**10**) is produced directly from **2** by a 2-oxo-acid decarboxylase (cf. pyruvate decarboxylase) (*37*). The production of 2-methylbutanoic acid (**5**) and ethyl 2-methylbutanoate (**16**) by this pathway would then require oxidation of 2-methylbutanal (**10**) to 2-methylbutanoyl-CoA (**4**). In *Saccharomyces cerevisiae*, 2-methylbutanol (**13**) may be produced from isoleucine by both a pyruvate decarboxylase and a nonpyruvate decarboxylase pathway (*37*). In the present study and previously (*5*), the pathway from 2-methylbutanal (**10**) to 2-methylbutanoyl-CoA (**4**) was found to be inactive in Red Delicious apples. Therefore, if the Ehrlich pathway occurs in these fruit, the operation of the "standard" pathway is also required to produce the 2-methylbutanoate compounds (**4**, **5**, **16**, and **17**). A third pathway, where tiglyl-CoA (**6**) is formed

Table 3. Increases (Times Endogenous Concentrations) of 2-Methylbutanoic Acid (**5**), 2-Methylbutanol (**13**), and 2-Methylbutyl Acetate (**15**) in Red Delicious Apple Tissue Slices Resulting from Incubation with Unsaturated Biosynthetic Precursors[a]

	ratio of compounds in incubated samples/controls		
precursor added	compound **5**	compound **13**	compound **15**
2-methylbut-2*E*-enoic acid (**7**)	16.7	2.8	3.5
2-methylbut-2*E*-enal (**9**)		16	8.6
2-methylbutanal (**10**)		6	6.5
2-methylbut-2*E*-enol (**12**)		3.5	5

[a] Blank entries, not detected.

by oxidation of **4** or indirectly from **10** via **9** (**Figure 1**), is implied in apples by the conversion of ethyl d_4-tiglate to ethyl d_4-2- methylbutanoate and to d_4-2-methylbutyl acetate (*38*) and by the formation of small amounts of a number of deuterated 2-methylbut-2-enyl products from d_{10}-isoleucine (**1a**), ethyl 2-d_3-methylbutanoate (**16b**), and 2-d_3-methylbutanoic acid (**5b**) (*5*). The intermediacy of **9** and **12** is implied by the identification of **14** in apple fruit (*5*). Additional biosynthetic products of isoleucine found in Red Delicious apples were hexyl and butyl d_3-acetate (d_3-**11**), presumably via d_3-acetyl-CoA (**8**) from d_7-tiglyl-CoA (**6**) (*38*).

To elucidate the involvement of 2-methylbut-2-enyl (tiglyl) compounds in alternate pathways for the biosynthesis of 2-methylbutyl acetate, unlabeled forms of compounds **7**, **9**, **10**, and **12** were incubated with apple skin. All four compounds significantly increased the concentrations of both 2-methylbutanol and 2-methylbutyl acetate above those found in the control samples (**Table 3**), thus establishing their potential intermediacy in the biosynthesis of 2-methylbutyl acetate. Incubation with 2-methylbut-*E*2-enoic acid (**7**) also markedly increased concentrations of 2-methylbutanoic acid (**5**), supporting the interconversion between **4** and **6** (*38*). The reduction of 2-methylbutanal (**10**) to 2-methylbutanol (**13**) also supports involvement of aldehyde (**10**) in the "standard" pathway, consistent with the general thesis that aldehydes are intermediates between carboxylic acids and alcohols (*4*, *39*).

The chirality of the 2-methylbutyl acetate produced in these reactions provides an additional probe of the biosynthetic pathways leading to its synthesis. Incubation of Granny Smith, Golden Delicious, and Red Delicious apples with ethyl d_4-2-methylbut-2-enoate (ethyl d_4-tiglate, **A**) (*38*) gave d_4-2-methylbutanol, d_4-2-methylbutyl acetate, d_4-2-methylbutanoic acid, and ethyl 2-methyl-d_4-butanoate consistent with the conversion of tiglyl-CoA to 2-methylbutanoyl-CoA. However, significant amounts of both the *R* and the *S* enantiomers of 2-methylbutyl acetate and of ethyl 2-methylbutanoate were produced, implying that the reduction of the tiglyl-CoA double bond to form 2-methylbutanoyl-CoA was not particularly enantiospecific. Natural 2-methylbutyl acetate and ethyl 2-methylbutanoate from apples have the *S* configuration (*40*) with the operation of the tiglate pathway being proposed as the source of small amounts (ca. 2%) of (*R*)-ethyl 2-methylbutanoate also found in apples (*38*).

Incubation of Red Delicious skin with racemic ethyl 2-methylbutanoate resulted in the production of racemic 2-methylbutyl acetate (**Figure 6A**,**B**), demonstrating that the chirality of the 2-methylbutyl acetate in apple derives from that of isoleucine and that all of the later enzymes in the pathway for its biosynthesis are not enantiospecific. Incubation of Red Delicious apple skin with 2-methylbut-2*E*-enal, however, produced a nine-fold increase of 2-methylbutyl acetate (**Table 3**) with only the *S* enantiomer (>90%) being detected by enantioselective GC-MS (**Figure 6C**). This confirms that the major biosynthetic pathway from 2-methylbut-2*E*-enal to 2-methylbutyl acetate does not occur via tiglyl-CoA (**6**) but probably through reduction to 2-methylbut-2*E*-enol (**12**) and to 2-methylbutanol or via 2-methylbutanal. This is also consistent with the formation of 2-methylbut-2-enyl compounds in Red Delicious (*5*) via the tiglyl-CoA ↔ 2-methylbutanoyl-CoA interconversion (**Figure 1**).

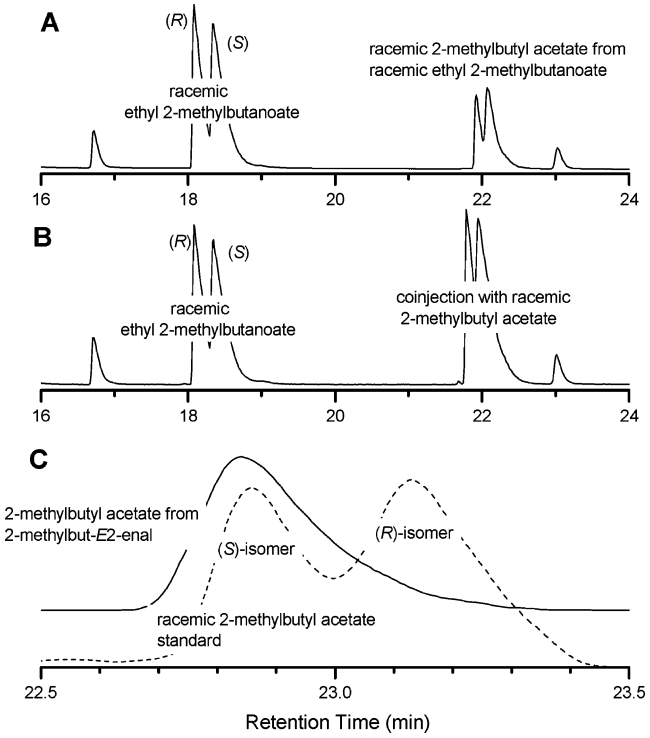

Figure 6. Enantioselective GC of 2-methylbutyl acetate produced by Red Delicious apple skin incubated (**A**) with racemic ethyl 2-methylbutanoate; (**B**) with racemic ethyl 2-methylbutanoate, as for **A**, with a coinjection of racemic 2-methylbutyl acetate; and (**C**) with 2-methylbut-2*E*-enal.

While the tiglate and the 2-methylbutanoate compounds in Red Delicious apples are interconvertible (*5*, *38*), reversibility in the biosynthesis of 2-methylbutyl acetate appears limited. The interconversion between 2-methylbutanol and 2-methylbutanoic acid favors 2-methylbutanol, and incubation with the 2-d_3-methylbutanol did not produce any 2-d_3-methylbutanoic acid or ethyl 2-d_3-methylbutanoate (herein and ref *5*). Oxidation of 2-methylbutanol through 2-methylbutanal to 2-methylbutanoyl-CoA does not seem to occur in Red Delicious fruit although these reactions occur minimally in Granny Smith (*5*). The ADH-mediated oxidation of 2-methylbutanol to 2-methylbutanal has been reported in *S. cerevisiae* (*41*). This nonreversibility in the biosynthesis of 2-methylbutyl acetate contrasts with the reported behavior for straight-chain ester volatiles where oxidation of alcohols and aldehydes through to the acid and subsequent CoA formation are known in Red Delicious fruit (*4*) and in other cultivars (*42*). Rowan et al. (*5*) also found that while Red Delicious apple tissues treated with d_{10}-isoleucine, 2-d_3-methylbutanoic acid, and ethyl 2-d_3-methylbutanoate all produced deuterated 2-methylbut-2-enyl compounds, treatment with 2-d_3-methylbutanol did not. However, in the present study, treatment with 2-methylbut-2*E*-enal (**9**) and 2-methylbut-2*E*-enol (**12**) increased the concentrations of both 2-methylbutanol and

2-methylbutyl acetate (**Table 3**). Therefore, it appears that these two unsaturated compounds (**9** and **12**) can be reduced to 2-methylbutanal (**13**) but that the reverse reaction does not occur, as is indicated in **Figure 1**.

In summary, suppression of ester volatile biosynthesis was observed for all compounds except 2-methylbutyl acetate from 21 days of CA storage and throughout the period of CA storage with the ability to synthesize branched-chain volatile compounds, particularly 2-methylbutanoic acid, from isoleucine, markedly suppressed with respect to fresh fruit (**Figure 5**). This suggests an overall reduction of pathway activity between L-isoleucine and 2-methylbutanoyl-CoA (**Figure 1**) occurring early in the storage period. The partitioning of labeled substrate from isoleucine between ethyl 2-methylbutanoate and 2-methylbutanol (and 2-methylbutyl acetate), and the increment that labeled compound makes to endogenous concentrations of these compounds, suggests that synthesis of ethyl 2-methylbutanoate, but not hexyl 2-methylbutanoate, is precursor limited in the CA fruit. The conversion of 2-methylbutanol to 2-methylbutyl acetate (**Figure 3A**) and of 2-methylbutanoic acid to ethyl 2-methylbutanoate and to hexyl 2-methylbutanoate (**Figure 4A**,**B**) was limited by the availability of the isoleucine-derived 2-methylbutyl substrate but not by acetyl-CoA, ethanol, or hexanol, respectively. The capacity of the fruit to effect these reactions declined during CA storage. A possible contributing factor is the decline in concentrations of isoleucine in fruit during storage (*43*). The concentration of 2-methybutanoic acid and the ability to synthesize hexyl 2-methylbutanoate were most severely affected by CA storage. The conversion of 2-methybutanoic acid to 2-methylbutanol (**Figure 4C**) was also substrate limited, but this enzymic activity was stable over the CA storage period. Reduction of aldehydes to alcohols is a rapid, high-capacity process in apple fruit (*4*, *42*), and the activity of the other steps in this part of the pathway would seem to be equally robust.

Analysis of the effects of CA storage on the conversion of isoleucine to 2-methylbutanoyl-CoA is complicated, as this latter compound that represents a branch point in volatile biosynthesis (**Figure 1**) was not measured directly. The biosynthetically closest observed intermediates are 2-methylbutanoic acid and 2-methylbutanol, both of which are severely reduced and substrate limited in CA-stored fruit (**Figure 5C**,**D**). The activity to metabolize isoleucine to 2-methylbutanoic acid also declined during storage. It therefore appears that reduced metabolism of isoleucine to 2-methylbutanoyl-CoA, and hence limitations on the availability of this compound for the synthesis of branched-chain esters, is the primary reason for the loss of ester synthesis in CA-stored Red Delicious fruit.

The chirality of the important flavor volatile, 2-methylbutyl acetate, is controlled by the first steps in its biosynthesis from L-isoleucine, with later steps being nonenantiospecific and allowing the production of racemic or potentially (*R*)-2-methylbutyl acetate by feeding suitable precursors to the fruit (**Figure 6**). The interconversion of tiglyl-CoA and 2-methybutanoyl-CoA is likewise nonenantiospecific (*38*). Treatment of tissue samples with 2-methylbut-2*E*-enal increased the concentration of (*S*)-2-methylbutyl acetate, indicating that this compound was not synthesized via tiglyl-CoA, which would have resulted in the production of significant amounts of both enantiomers (*38*). Analysis of this mixture of enantiospecific and nonspecific transformations provides an additional tool, along with deuterium labeling, for the investigation of this complex biosynthetic system.

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