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Biosynthesis and Elongation of Short- and Medium-Chain-Length Fatty Acids

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Short- and medium-chain-length fatty acids (FAs) are important constituents of a wide array of natural products. Branched and straight short-chain-length FAs originate from branched chain amino acid metabolism, and serve as primers for elongation in FA synthase-like reactions. However, a recent model proposes that the one-carbon extension reactions that utilize 2-oxo-3-methylbutyric acid in leucine biosynthesis also catalyze a repetitive one-carbon elongation of short-chain primers to medium-chain-length FAs. The existence of such a mechanism would require a novel form of regulation to control carbon flux between amino acid and FA biosynthesis. A critical re-analysis of the data used to support this pathway fails to support the hypothesis for FA elongation by one-carbon extension cycles of α-ketoacids. Therefore, we tested the hypothesis experimentally using criteria that distinguish between one- and two-carbon elongation mechanisms: (a) isotopomer patterns in terminal carbon atom pairs of branched and straight FAs resulting from differential labeling with [^{13}C]acetate; (b) [^{13}C]threonine labeling patterns in odd- and even chain length FAs; and (c) differential sensitivity of elongation reactions to inhibition by cerulenin. All three criteria indicated that biosynthesis of medium-chain length FAs is mediated primarily by FA synthase-like reactions.

The broad structural diversity of short- and medium-chain length fatty acids (scFAs and mcFAs, respectively) and their derivatives is incorporated into a wide array of biomolecules as components of antibiotics, insect pheromones, and plant storage lipids (Denoya et al., 1995; Laakel et al., 1994; Tang et al., 1994; Giblin-Davis et al., 1996; Schal et al., 1994; Pennanec' et al., 1991; Charlton and Roeloffs 1991; Knapp et al., 1991; Thompson et al., 1990; Hartman and Reimann, 1989). Understanding the biosynthesis of these compounds is critical both to understanding their regulation and designing strategies for their manipulation.

scFAs and mcFAs are also found in sugar polyesters secreted by Solanaceous plants as defensive agents against a wide array of insect herbivores and pathogens (Gentile and Stoner, 1968; Gentile et al., 1968, 1969; Juvik et al., 1982, 1994; França et al., 1989). These polyesters are composed of either Glc or Suc to which as many as five or six FAs, respectively, may be esterified (Schumacher, 1970; Severson et al., 1985; King et al., 1986, 1988, 1990; King and Calhoun, 1988; Shinozaki et al., 1991; Shapiro et al., 1994). The acyl substituents exhibit a remarkable degree of species-specific structural diversity: They range in length from 3:0 to 12:0, and include straight-chain, *iso*-branched, and *anteiso*-branched FAs with both odd and even numbers of carbon atoms.

The biosynthesis of branched-chain FAs has been extensively investigated in bacteria (Oku and Kaneda, 1988; Kang et al., 1997a, 1997b; Zelles, 1997). Iso- and anteiso-branched FAs 14 to 17 carbon atoms long are derived from α-keto derivatives of Leu, Val, and Ile, which serve as short-chain primers for elongation. In this model, NAD^+- and CoA-dependent branched-chain ketoacid dehydrogenase provides acyl-CoA primers through oxidative decarboxylation of these ketoacid precursors. A FA synthase (FAS) system then elongates these three- to five-carbon primers utilizing malonyl-CoA as a substrate. As an alternative to this model, Oku and Kaneda (1988) proposed that decarboxylation by branched-chain ketoacid decarboxylase, rather than oxidative decarboxylation, provides an aldehyde-based primer for elongation; however, evidence for such aldehyde derivative products of decarboxylation has not been obtained.

In plants, *iso*-branched scFAs of sugar polyesters are similarly derived from branched-chain amino acid metabolism: Val and Leu are incorporated into *i*4:0 and *i*5:0 acids (2-methylpropionic and 3-methylbutyric acid, respectively) through a process of transamination and oxidative decarboxylation of the resulting 2-oxoacid (Fig. 1; Kandra and Wagner, 1990; Walters and Steffens, 1990; Luethy et al., 1997).

Similar to the *iso*-branched scFAs, *ai*5:0 (2-methylbutyric acid) is derived from Thr through conversion into 2-oxobutyric acid by Thr dehydratase, followed by a multienzyme conversion into 2-oxo-3-methylpentanoic acid (also derived by transamination of Ile) and subsequent decarboxylation (Fig. 1; Walters and Steffens, 1990). In addition, the incorporation of *iso*- and *anteiso*-branched scFAs and mcFAs into sugar polyesters is sensitive to chlorsulfuron, an inhibitor of acetolactate synthase, a key enzyme in branched-chain amino acid metabolism (Kandra et al., 1990; Walters and Steffens, 1990). Biogenesis of straight and *iso*-branched mcFAs was proposed to occur either through de novo initiation and extension (for *n*-fatty acids) or through utilization of *i*4:0 or *i*5:0 primers to generate even or odd chain-length *iso*-branched mcFAs, respectively (Walters and Steffens, 1990).

However, for petunia (*Petunia hybrida*) and species in the genus *Nicotiana*, which synthesize FAs extended from an *ai*5:0 primer to form, for example, *ai*6:0 and *ai*7:0 (3-

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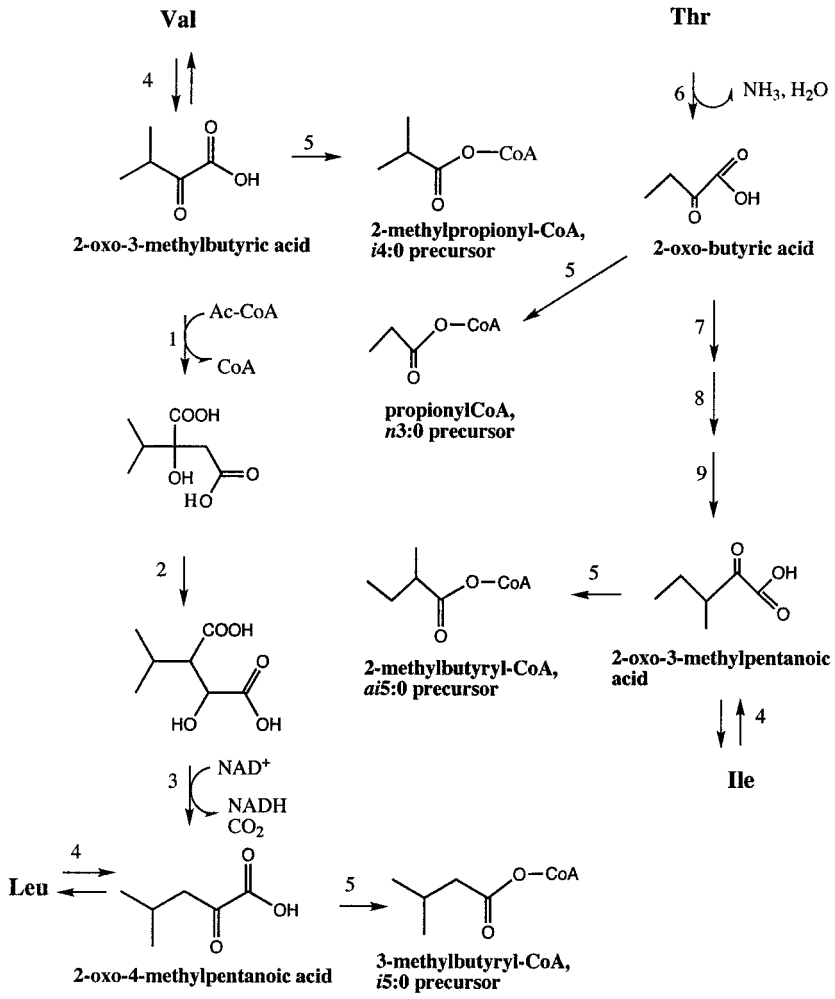

Figure 1. Role of branched-chain amino acid metabolism in the biosynthesis of scFA primers. The one-carbon elongation reaction represented by reactions 1, 2, and 3 (IPMS, IPMDH, and IPMDCase, respectively) were proposed by Kroumova et al. (1994) to also carry out further elongation reactions yielding FAs up to C12. Other enzymes or enzyme complexes include: 4, aminotransferase; 5, branched-chain oxoacid dehydrogenase complex; 6, Thr dehydratase; 7, aminohydroxy acid synthase; 8, acetolactate synthase; and 9, dihydroxyacid dehydratase.

methylpentanoic and 4-methylhexanoic acid, respectively; Shapiro et al., 1994; Son et al., 1994), it is less clear how a two-carbon elongation mechanism such as FAS could give rise to the even chain-length *ai*6:0 product. A novel route for FA biosynthesis was suggested in which the one-carbon extension reactions of branched amino acid biosynthesis, i.e. the Leu pathway, were hypothesized to carry out a much broader range of reactions, including the elongation reactions leading to biosynthesis of all mcFAs (Fig. 1; Kroumova et al., 1994). The authors propose that one- to eight-carbon-atom elongation of 2-oxoacids (α-ketoacids) derived from the cognate amino acids is catalyzed by 2-isopropylmalate synthase (IPMS), 3-isopropylmalate dehydratase (IPMDH) and 3-isopropylmalate dehydrogenase (IPMDCase), without the involvement of ketoacyl synthases or associated reactions of a FAS complex. The proposed mechanism results in the addition of acetate at each condensation event, followed by oxidative decarboxylation of the terminal carboxylate, and leads to formation of a series of elongated FAs varying by one-carbon increments. A scheme in which extension occurs in one-carbon increments provides a plausible explanation for *ai*6:0 and *ai*7:0 elongation from *ai*5:0; however, Kroumova et al. (1994) suggest that αKAE also controls biosynthesis of iso-branched and normal-chain FAs of both odd and even carbon length.

The initial substrate for the Leu pathway is 2-oxo-3-methylbutyric acid (Fig. 1). The α-ketoacid (2-oxoacid) elongation (αKAE) model requires that IPMS, IPMDH, and IPMDCase accept, in addition to the terminal isopropyl group of 2-oxo-3-methylbutyric acid, both *n*- and branched alkyl substituents ranging up to 11 carbons in length. Therefore, if IPMS, IPMDH, and IPMDCase were multifunctional enzymes capable of accepting an extremely wide range of alkyl substituents, this would require a far greater degree of integration of amino and FA metabolism than has been previously understood. Control of both amino acid and FA biosynthesis by this complex would raise novel questions with respect to substrate level regulation and cell-type-specific regulation of IPMS to effect amino acid rather than FA biosynthesis or vice versa. In addition, this would impose a very complex regulation of carbon flux between Leu biosynthesis and flux through *iso*-, *anteiso*-branched, and straight-chain FAs ranging from 3:0 to 12:0.

In addition to the regulatory questions posed by a dual functionality of IPMS, IPMDH, and IPMDCase in Leu biosynthesis and FA biosynthesis by elongation of 2-oxoacids, we found that the evidence presented for the existence of

the αKAE model posed a number of problems. Therefore, we chose to critically examine the existence of αKAE in FA biosynthesis using stable isotope-labeling techniques in conjunction with differentially ^{13}C- or ^{2}H-labeled substrates and gas chromatography-mass spectrometry (GC-MS).

MATERIALS AND METHODS

Materials

L-Val-d_8 and [U-^{13}C]Thr were purchased from Cambridge Isotope Laboratories (Andover, MA). L-Leu-d_{10} was purchased from MSD Isotopes (Claire-Pointe, Quebec). Cerulenin (2,3 epoxy-4-oxo-7, 10 dodecadienamide), [1-^{13}C]acetate, [2-^{13}C]acetate, [U-^{13}C]acetate, and TBA-HSO_4 (tetrabutylammonium-hydrogensulfate) were purchased from Sigma (St. Louis). PFBBr (pentafluorobenzylbromide) was purchased from Pierce Chemical (Rockford, IL).

Precursor Administration

Terminal branch tips of *Lycopersicon pennellii* (LA 716), *Nicotiana umbratica*, and petunia (*Petunia hybrida* cv Falcon Red) were removed from the plants and placed in water until further use. Plant material was immersed for 4 to 5 s in anhydrous ethanol with careful agitation to remove trichome exudate, after which time the shoots were immediately immersed in water to remove residual ethanol. Samples of the ethanol wash were saved as exudate reference samples. After the water wash, shoots and peduncels were gently blotted with paper towels and a diagonal cut was made on the stem. The stem was inserted through a layer of Parafilm stretched across a 5-cm Petri dish filled with a solution of labeled substrate. Substrates were composed of 5 mM [^{2}H]amino acid or [^{13}C]NaOAc solutions in deionized water. Optionally the substrate solutions were provided with 100 μM cerulenin. Incubations were performed for 24 h under a 75 W incandescent lamp placed approximately 15 cm from the shoots. Every 6 to 8 h the substrate reservoir was replenished with deionized water. Cerulenin incubations were provided with a fresh 100 μM solution after the first 6 h of incubation. Samples were prepared by rinsing the shoots in 20 mL of anhydrous ethanol for 4 to 5 s as described above.

Product Analysis

The ethanol extracts were evaporated at 30°C under vacuum, and dissolved in 600 μL of CH_2Cl_2. The FA constituents of the sugar polyesters were trans-esterified to ethylesters with sodium ethoxide. Sample preparation and GC conditions were as described by Walters and Steffens (1990). A 1-μL aliquot was used for GC injection.

[U-^{13}C]Thr-labeled samples were derivatized using PFBBr. A 100-μL sample in CH_2Cl_2 was evaporated to dryness under a continuous flow of N_2. Sugar polyesters were treated with 50 μL of 1 M NaOH for 1 h at RT. One-hundred microliters of TBA-HSO_4 and 200 μL of 0.06 M PFBBr in CH_2Cl_2 were then added and the derivatization was allowed to proceed for 25 min with frequent vortexing. GC analysis of [^{13}C]acetate- or [^{2}H]amino acid-labeled FA ethyl esters employed a Hewlett-Packard 5890 gas chromatograph (model 5890, Hewlett-Packard, Palo Alto, CA), equipped with either a flame-ionization or mass-selective detector (model 5970, Hewlett-Packard) in the selected ion monitoring (SIM) mode, run as described in Walters and Steffens (1990). A DB-FFAP capillary column (30 m, 0.24 mm in diameter, J&W Scientific, Folsom, CA) was used. The oven was programmed to hold at 60°C for 5 min and then increase to 250°C at 10°C/min.

RESULTS AND DISCUSSION

Incorporation of Differentially Labeled [1-, 2-, U-^{13}C]Acetates

We used stable isotopes and SIM-GC-MS to assess how acetate is incorporated into *n*-, *iso*-, and *anteiso*-branched FAs. FAs were derivatized as ethyl esters to facilitate observation of acetate incorporation into the carboxy-terminal two carbon atoms of each FA. In electron ionization (EI)-MS these two atoms are retained in the major McLafferty rearrangement product, *m/z* 88 (Fig. 2A; McLafferty, 1959; Ryhage and Stenhagen, 1963). Therefore, by monitoring *m/z* 88 and its +1-atomic mass unit and +2-atomic mass unit isotopomers (*m/z* 89 and *m/z* 90, respectively), the means by which differentially labeled [^{13}C]acetate is incorporated during FA biosynthesis can be unambiguously assessed. The biogenesis of normal, *iso*-branched, and *anteiso*-branched scFAs and mcFAs can be visualized as taking place by three possible routes; two distinct patterns of labeling with [1-^{13}C]-, [2-^{13}C]-, or [U-^{13}C]acetate can be predicted:

1. De novo biosynthesis through FAS-mediated reactions and extension of *n*-, branched-, even-, or odd-chain-length primers via FAS-mediated reactions predicts enrichment of the *m/z* 89 isotopomer when either [1-^{13}C]- or [2-^{13}C]acetate is incorporated, and enrichment of the *m/z* 90 isotopomer when [U-^{13}C]acetate is incorporated.

2. Biosynthesis through the αKAE pathway or through the FAS-mediated extension reactions coupled to one-carbon chain-shortening events predicts enrichment of the *m/z* 89 isotopomer when either [2-^{13}C]- or [U-^{13}C]acetate is incorporated, but no enrichment when [1-^{13}C]acetate is incorporated (Fig. 2B).

The data can be interpreted directly from these predictions (Table I). For the *iso*-branched FAs of *L. pennellii* and *N. umbratica*, *i*5:0 shows *m/z* 89 enrichment with [2-^{13}C]- and [U-^{13}C]acetate and no enrichment with [1-^{13}C]acetate, in accordance with its IPMS-based biosynthesis from 2-oxo-3-methylbutyric acid; oxidative decarboxylation of 2-oxo-4-methylpentanoic acid results in loss of the C1-carboxyl derived from acetyl-CoA. *i*8:0 and *i*10:0 (6-methylheptanoic and 8-methylnonanoic acid) are extension products from an *i*4:0 primer (Walters and Steffens, 1990). Both *i*8:0 and *i*10:0 show a pattern of incorporation consistent with elongation by a FAS-based mechanism: enrichment of *m/z* 89 when [1-^{13}C] or [2-^{13}C]acetate is incorporated, and *m/z* 90 enrichment when [U-^{13}C]acetate is

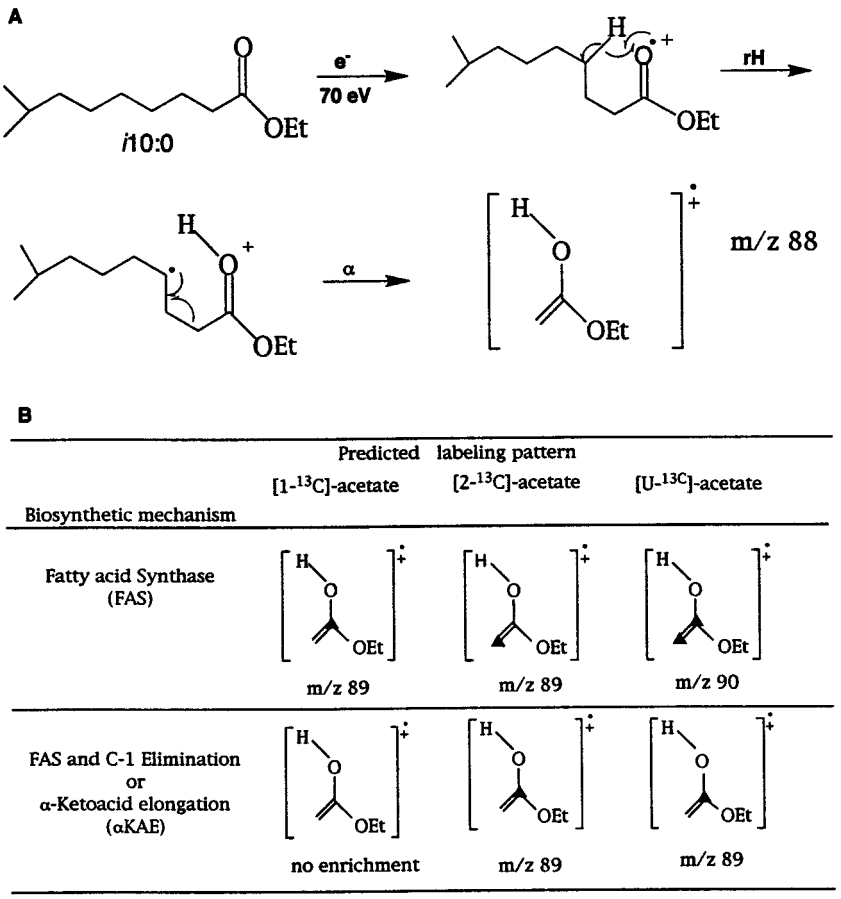

Figure 2. A, McLafferty rearrangement of FA ethyl esters in EI-MS permits sampling of isotopic enrichment in the carboxy-terminal two-carbon fragment. B, Biosynthetic mechanism of FA elongation predicts distinct patterns of [^{13}C]-enrichment (▲) of FA carboxy-terminal two-carbon fragments (*m/z* 88 isotopomer) after incorporation of differentially labeled [^{13}C] acetates into either *m/z* 89 or *m/z* 90. Consecutive incorporation of two labeled acetates was not detectable by SIM-MS, because at incorporation rates of approximately 1%, this results in an insignificantly low enrichment of 0.01%. Also note that in the αKAE model, both [2-^{13}C]- and [U-^{13}C]acetate incorporation predicts enrichment of the *m/z* 89 isotopomer in either C1 or C2. Randomization of label would result in *m/z* 89 enrichment regardless of whether [1-^{13}C]-, [2-^{13}C]-, or [U-^{13}C]actetate is administered. For example, when petunia was used in these studies, *m/z* 89 was enriched in all FAs analyzed, regardless of initial position of the heavy atom in the precursor.

incorporated. The labeling patterns for *i*8:0 (in *N. umbratica*) and *i*10:0 (in *L. pennellii*) are consistent with two and three cycles, respectively, of FAS-like extension from an *i*4:0 primer, as suggested by the previous observation of d_8-Val incorporation into d_7-*i*10:0 (Walters and Steffens, 1990). In accordance with this, the FAS-like extension of d_9-i5:0 (d_9-3-methylbutyric acid), which is derived from d_{10}-Leu by transamination and oxidative decarboxylation, results exclusively in the formation of d_9-*i*9:0 and d_9-*i*11:0 (7-methyloctanoic and 9-methyldecanoic acid [Walters and Steffens, 1990; this paper]). In contrast, the labeling of *i*6:0 (4-methylpentanoic acid) in *N. umbratica* resembles that predicted for a chain-shortening or αKAE event: enrichment of *m/z* 89 when [2-^{13}C]- and [U-^{13}C]acetate are incorporated, and no enrichment when [1-^{13}C]acetate is provided.

Similarly, the branched acids *ai*6:0 and *ai*7:0 (3-methylpentanoic and 4-methylhexanoic acid) of *N. umbratica* exhibit a labeling pattern consistent with that predicted by either terminal C1 elimination of FAS-extended products or αKAE. Both *ai*6:0 and *ai*7:0 show enrichment of *m/z* 89 when [2-^{13}C]- and [U-^{13}C]acetate are incorporated, and none when [1-^{13}C]acetate is incorporated. It is possible, as an alternative mechanism for the αKAE-model, that in *N. umbratica* *i*6:0 originates from one cycle of FAS-like elongation of *i*5:0 to *i*7:0, followed by C-1 elimination to yield *i*6:0. By analogy, in this species *ai*5:0 may be elongated to *ai*7:0 to yield *ai*6:0 after C-1 elimination. Subsequently, *ai*6:0 would serve as a primer to yield *ai*7:0 through a similar process.

Even- and odd-numbered *n*-FAs ranging in length from 7:0 to 12:0 in *L. pennellii* and *N. umbratica* precisely follow the pattern predicted by FAS, with enrichment of *m/z* 89 when [1-^{13}C] or [2-^{13}C]acetate is incorporated, and *m/z* 90 enrichment when [U-^{13}C]acetate is incorporated. This is consistent with a de novo origin of the even-chain-length FAs, and for the odd-chain-length FAs is consistent with a two-carbon elongation of an odd-carbon-chain-length primer supplied by Thr, through its conversion to 2-oxobutyric acid followed by oxidative decarboxylation (Walters and Steffens, 1990; Kroumova et al., 1994).

In contrast to *L. pennellii* and *N. umbratica*, enrichment of the *m/z* 89 isotopomer occurs regardless of whether [1-^{13}C], [2-^{13}C]-, or [U-^{13}C]acetate is administered to petunia. This pattern of labeling is consistent only with a high degree of label randomization.

Analysis of [U-^{13}C]Thr Incorporation

An interesting question concerns the identity of the primers used to elongate FAs of odd and even carbon atom chain length. For example, Table I shows that odd-carbon-chain-length *n*-FAs are extended from an odd-chain-length primer by a FAS-like mechanism. We have previously shown that feeding Thr to *L. pennellii* elevated the levels of

Table I. *Percent enrichment of FA-derived m/z 89 and 90 isotopomers after incorporation of differentially [^{13}C]-labeled acetates*

The abundance of *m/z* 89 and 90 isotopomers relative to the *m/z* 88 fragment was calculated, and enrichment was obtained by subtracting the ratios for *m/z* 89, and 90 fragments of the control sample. The *m/z* 88 McLafferty rearrangement fragment allowed analysis of the abundant (>1 mol %) branched and *n*-FAs of *L. pennellii (L.p.)* and *N. umbratica* (*Nu.*). Mol % fatty acid composition (FID detection) in *L. pennellii* (LA 716) sugar polyesters: *n*3:0; 0.18, *i*4:0; 50.62, *ai*5:0; 19.38, *i*5:0; 3.64, *i*9:0; 0.16, *n*9:0; 0.03, *i*10:0; 13.73, *n*10:0; 8.36, *i*11:0; 0.40, *n*11:0; 0.07, *i*12:0; 0.38, *n*12:0; 3.05. For *N. umbratica*: *n*3:0; 3.13, *i*4:0; 5.67, *ai*5:0; 17.92, *i*5:0, 5.52, *ai*6:0; 51.37, *i*6:0; 5.03, *ai*7:0; 8.10, *n*7:0; 1.45, *i*8:0; 1.44, *n*8:0; 0.37. The fatty acids *n*4:0, *n*5:0, and *n*6:0 <0.5% in *N. umbratica* were only detectable by SIM GC-MS. The acetyl group constitutes 40.3 mol % of total acyl groups in *N. umbratica* sugar polyesters (Shinozaki et al. 1991); however, in these GC-experiments ethylacetic acid is obscured by the solvent peak (hexane), and is therefore not accounted for in calculating the mol % fatty acid composition. The mol % distribution for the straight acyl groups in Petunia cv, Falcon Red (*P.h.*). *n*5:0; 3.61, *i*5:0; 34.54, *n*6:0, 8.84, *i*6:0; 11.96, *n*7:0; 19.35, *n*C8: 21.70. However, Petunia cv Falcon Red also contains a number of branched fatty acids and minor amounts of other straight fatty acids, which are not accounted for here. Note that for structural reasons *i*4:0 and *ai*5:0 cannot undergo McLafferty rearrangement to *m/z* 88 and related isotopomers (Fig. 1). ND, Not detectable (<0.01% enrichment).

FA	Source	Isotopomer					
		[1-^{13}C]		[2-^{13}C]		[U-^{13}-C]	
		m/z 89	*m/z* 90	*m/z* 89	*m/z* 90	*m/z* 89	*m/z* 90
Iso-branched							
*i*5:0	*L.p.*	ND	ND	1.93	0.11	2.27	0.02
*i*5:0	*N.u.*	0.05	ND	0.67	0.01	0.60	0.02
*i*5:0	*P.h.*	0.03	ND	0.71	ND	1.19	0.04
*i*6:0	*P.h.*	0.28	ND	1.37	0.20	1.49	0.20
*i*6:0	*N.u.*	0.01	ND	0.90	0.09	0.94	0.10
*i*8:0	*N.u.*	0.33	0.01	0.40	0.01	0.09	0.45
*i*10:0	*L.p.*	1.10	0.12	1.30	0.14	0.55	1.00
Anteiso-branched							
*ai*6:0	*N.u.*	ND	ND	0.08	0.01	0.93	0.08
*ai*7:0	*N.u.*	ND	ND	0.80	0.09	0.81	0.10
Straight-chain							
*n*5:0	*P.h.*	0.21	0.01	1.03	0.17	1.23	0.15
*n*6:0	*P.h.*	0.20	0.04	1.35	0.17	0.82	0.14
*n*8:0	*P.h.*	0.39	0.04	1.36	0.21	1.55	0.21
*n*10:0	*P.h.*	0.28	0.04	1.00	0.18	2.16	0.23
*n*7:0	*N.u.*	0.40	0.14	0.58	0.09	0.08	0.54
*n*8:0	*N.u.*	0.57	ND	0.69	0.07	0.17	0.70
*n*10:0	*L.p.*	0.89	0.09	1.20	0.11	0.73	0.91
*n*12:0	*L.p.*	0.29	0.05	1.19	0.09	0.02	0.70

*n*3:0, *n*9:0, and *n*11:0 FAs occurring in sugar polyesters. Together, these data suggest that Thr is converted to the *n*3:0 primer via Thr dehydratase to yield 2-oxo-butyric acid and oxidative decarboxylation to yield propionyl-CoA. The *n*3:0 primer is then elongated by FAS-like mechanisms to *n*9:0 and *n*11:0. Similarly, when acetolactate synthase is inhibited by chlorsulfuron, *n*3:0, *n*9:0, and *n*11:0 become significant constituents of sugar polyesters (Walters and Steffens, 1990). This is consistent with elongation of an 2-oxobutyric acid-derived *n*3:0 primer by three and four cycles of FAS-like extension to *n*9:0 and *n*11:0, respectively.

In contrast, the αKAE pathway predicts that both odd- and even-chain-length FAs are derived from single-carbon extension reactions acting upon a Thr-derived *n*3:0 primer (Walters and Steffens, 1990). Therefore, we examined the ability of [U-^{13}C]Thr to serve as a primer for normal and *anteiso*-branched scFAs and mcFAs (Table II). In this case we prepared pentafluorobenzyl esters to maximize the appearance of FA molecular ions by EI-MS. Because incorporation of amino acids into sugar polyester acyl substituents is far more efficient than incorporation of acetate, SIM analysis was sensitive enough to detect the less-abundant straight-chain FAs of length ≥ *n*3:0 in *N. umbratica* (*n*4:0–*n*8:0), and *n*9:0 and *n*11:0 in *L. pennellii*, in addition to abundant *anteiso*-branched and straight FAs in both species (legend of Table II). Incorporation of [U-^{13}C]Thr, containing four ^{13}C atoms, into FAs leaves three ^{13}C atoms to form the backbone of both *n*3:0 (^{13}C loss by oxidative decarboxylation of 2-ketobutyrate) and *ai*5:0 through the Ile pathway (^{13}C loss by oxidative decarboxylation of 2-oxo-3-methylpentanoic acid). Therefore, SIM analysis followed the molecular and M+3 isotopomer ions of PFB-derivatized FAs.

In accordance with the data for acetate incorporation, we found incorporation of Thr into all *anteiso*-branched FAs, confirming that 2-oxo-3-methylpentanoic acid serves as a primer in the anteiso-branched pathway. Furthermore, we found that M+3 enrichment occurred exclusively in *n*3:0, *n*9:0, and *n*11:0 of *L. pennellii*. No Thr was incorporated into even chain-length FAs. Therefore, together with the evidence that acetate is incorporated intact into the carboxyl-terminal two carbon atoms of these FAs, it can be concluded that in *L. pennellii* odd-chain-length *n*-FAs are derived from the odd-chain-length *n*3:0 primer. Therefore, even-chain-length normal FAs are likely to be derived from

Table II. *Percent M+3 enrichment of fatty acids resulting from [U^{13}C]Thr incorporation*

Fatty Acid	*L. pennellii*	*N. umbratica*
Anteisobranched		
*ai*5:0[a]	11.6	20.1
*ai*6:0[a]	ND[b]	20.1
*ai*7:0	ND	16.3
Straight		
*n*3:0	27.8	56.0
*n*4:0	ND	53.9
*n*5:0	ND	39.3
*n*6:0	ND	ND
*n*7:0	ND	30.6
*n*8:0	ND	4.9
*n*9:0	42.0	ND
*n*10:0	0	ND
*n*11:0	35.8	ND
*n*12:0	0	ND

[a] The isomeric *ai*5:0 and *i*5:0, and similarly *ai*6:0 and *i*6:0 are not separable as PFB esters in this GC system. Accordingly, enrichment of *ai*5:0 and *ai*6:0 is underestimated, as Thr is not incorporated in isobranched Fas (33). [b] ND, Fatty acid not detectable.

de novo synthesis initiated from a two-carbon primer extended in two-carbon increments.

In contrast, *N. umbratica* showed Thr incorporation into a much wider array of FAs. This occurred at high efficiency in *n*3:0, *n*4:0, *n*5:0, and *n*7:0 (up to 50% of incorporation) and much less efficiently in *n*8:0 (less than 5%). As demonstrated earlier, *n*7:0 and *n*8:0 are extended in two-carbon increments (Table I) in which *n*5:0 and *n*4:0, respectively, are implicated to serve as intermediates, thereby accounting for the incorporation of the isotopically enriched *n*3:0 primer. The biogenesis of *n*4:0 remains uncertain. Clearly, *n*3:0 is extended to *n*5:0 and *n*7:0. We propose that *n*5:0, derived from one cycle of FAS elongation of *n*3:0, undergoes C1 elimination to form *n*4:0, which then serves as a primer for FAS-like elongation to *n*8:0 (Shine and Stumpf, 1974; Baardseth et al., 1987).

Inhibition of FA Elongation by Cerulenin

Because patterns of stable isotope labeling strongly indicated FAS-like mediation of mcFA elongation in sugar polyester mcFAs, we explored the sensitivity of this process to cerulenin, a specific inhibitor of β-ketoacyl synthase (KAS). A number of KAS-condensing enzymes have been identified in plants: KAS I, II, III, and IV (Shimakata and Stumpf, 1982; Jaworski et al., 1989; Dehesh et al., 1998). KAS I is capable of utilizing 2:0-ACP to 14:0-ACP as a substrate for elongation and is completely inhibited in vitro by 10 μM cerulenin. KAS II is primarily active with 14:0-ACP and 16:0-ACP as a substrate for elongation and is much less sensitive to cerulenin, i.e. 50% inhibition at 50 μM cerulenin (Shimakata and Stumpf, 1982). KAS III specifically synthesizes scFAs and is not sensitive to cerulenin (Jaworski et al., 1989). KAS III utilizes 6:0-ACP to form 8:0-ACP, and is inactive with 8:0- and longer acyl-ACPs as a substrate (Clough et al., 1992). Similarly, KAS IV has been shown to extend 6:0-ACP to 8:0-ACP in an extract of KAS IV overexpressing transgenic *Brassica* seeds in the presence of 100 μM cerulenin, but the further extension of 8:0-ACP to 10:0-ACP activity was strongly inhibited by cerulenin (Dehesh et al., 1998).

Incorporation of label into the elongated *iso*-branched and normal 10:0, 11:0, and 12:0 FAs was inhibited in vivo in the presence of cerulenin (Table III). Incorporation of d_8-Val into d_7-*i*10:0 is inhibited 48% by cerulenin, and its incorporation into d_7-*i*11:0 is inhibited approximately 70% (after incorporation of d_8-Val into d_7-*i*5:0). As shown previously, d_{10}-Leu is incorporated into d_9-*i*5:0 and elongated in two-carbon increments exclusively into d_9-*i*9:0 and d_9-*i*11:0 (Walters and Steffens, 1990). Incorporation of d_{10}-Leu into d_9-*i*11:0 is also inhibited approximately 60% by cerulenin (Table III). Cerulenin also inhibits the incorporation of [U-^{13}C]acetate into *n*10:0, *i*10:0, and *n*12:0 by 81%, 67%, and 51%, respectively, while having no effect on incorporation into shorter FAs (data not shown).

Incorporation of d_8-Val and d_{10}-Leu into *i*4:0 and *i*5:0 is not significantly affected by cerulenin treatment. However, incorporation into *i*9:0 is increased substantially as a result of cerulenin treatment when either d_8-Val or d_{10}-Leu are administered. Together with the evidence for FAS-like activities driving elongation of FAs from straight and branched primers, the cerulenin insensitivity of elongation to *i*9:0 may be related to the involvement of a KAS III- or KAS IV-like enzyme in the initial elongation of primers; increased incorporation into *i*9:0 (in length equivalent to *n*8:0) would result from cerulenin inhibition of KAS I activities, which, from cerulenin sensitivity, appear to be responsible for further elongation to 10:0, 11:0, and 12:0 FAs.

There is no known mechanism by which cerulenin interferes with branched-chain amino acid biosynthesis; therefore, the proposed α-KAE pathway (Kroumova et al., 1994) is unlikely to contribute to the process of mcFA biosynthesis. Cerulenin sensitivity of FA elongation strongly indicates that FAS-like reactions are responsible for the synthesis of mcFAs of sugar polyesters. Together with the stable isotope labeling patterns employing differentially labeled acetate, branched amino acids, and Thr, there is little doubt that the biosynthesis of these mcFAs is a FAS-dependent process.

Our results are therefore at variance with those of Wagner and co-workers, who based the αKAE model on their results with *L. pennellii*, *N. glutinosa*, and *P. hybrida* (Kandra

Table III. *Effect of cerulenin on percent incorporation of d_8-Val and d_{10}-Leu into fatty acids*

Fatty Acid	d_8-Val		d_{10}-Leu	
	Cerulenin		Cerulenin	
	−	+	−	+
*i*4:0	38.4	34.4	ND[a]	ND
*i*5:0	25.8	34.2	78.2	80.0
*i*9:0	10.9	20.3	5.3	13.4
*i*10:0	17.5	9.1	ND	ND
*i*11:0	7.3	2.1	16.5	6.6

[a] ND, No detectable incorporation of Leu into these fatty acids in the presence or absence of cerulenin.

et al., 1990; Kroumova et al., 1994). However, in the present study, petunia completely randomized differentially labeled acetate, and no conclusion of any kind could be drawn. Furthermore the model for FA biosynthesis proposed by Wagner and co-workers depends on observation of differential incorporation of [1-^{14}C]- and [2-^{14}C]acetate. According to their proposed scheme, [1-^{14}C]acetate should not be incorporated into FAs due to the decarboxylation step catalyzed by IPMDCase (Kroumova et al., 1994). However, when they administered 1-labeled acetate, it was efficiently incorporated in FAs of petunia (Kroumova et al., 1994). This finding was reported to be consistent with "extensive randomization of this label" (Kroumova et al., 1994). The observation would seem to invalidate the study. The central assumption required of isotopic labeling is that biological systems do not discriminate between isotopically labeled molecules and unlabeled molecules, nor between different isotopically labeled versions of the same molecule. Therefore, if [1-^{14}C]acetate is extensively randomized prior to incorporation, then randomization of [2-^{14}C]acetate must also be assumed. Randomization would preclude the conclusion that [2-^{14}C]acetate is differentially incorporated into FAs.

In fact, their data also strongly indicate randomization of [2-^{14}C]acetate as well. Synthesis of isobutyric acid (2-methyl-propionic acid), analogous to biosynthesis of 2-oxo-3-methylbutyric acid in the formation of Val, is initiated via acetolactate synthase-catalyzed condensation of acetaldehyde and pyruvate to form acetolactic acid. Accordingly, the carbonyl carbon atom of 2-oxomethylbutyric acid, which becomes the carboxyl atom of isobutyrate, is derived from pyruvate. Therefore, the administration of labeled acetate should not result in significant carboxyl labeling of isobutyrate (in the absence of randomization). However, if one assumes randomization of labeled acetate into pyruvate, the prediction is that 25% of the label should reside in each carbon atom of isobutyrate. Indeed, the authors report that when [2-^{14}C]acetate is administered to *L. pennellii*, about one-quarter (27%) of the radioactivity recovered in isobutyrate resides in the carboxyl atom.

The experimental design of Kroumoun et al. (1994) requires that radioactivity does not partition into carbon atoms derived from the pyruvate primers utilized both by IPMS and by acetolactate synthase; analysis depends on the ratio of counts per mimute in the terminal carbon atom to total radioactivity of the molecule. Acceptance of the hypothesis that the authors' observed ratios match those predicted for αKAE depends on the presence of radioactivity exclusively in those atoms derived directly from acetate (Fig. 1 in Kroumova et al., 1994). Therefore, the observation that [2-^{14}C]acetate labels pyruvate invalidates the basis for concluding that αKAE-based elongation reactions contribute to the synthesis of any FA (Kroumova et al., 1994).

In the absence of evidence for the αKAE elongation model, we believe that a simpler explanation for the *ai*6:0, *ai*7:0 and *i*6:0, and *n*4:0 and *n*5:0 labeling patterns observed for *N. umbratica* may involve a C1 elimination mechanism occurring between some two-carbon elongations at the short-chain level (Shine and Stumpf, 1974; Baardseth et al., 1987).

Nevertheless, we have shown here that the structural diversity of sugar polyester acyl substituents is explicable on the basis of a combination of amino acid biosynthesis, FAS-like elongation of branched- and straight-chain primers provided by amino acid biosynthesis, and by de novo FA biosynthesis to yield the odd- and even-chain-length *n*- and branched FAs synthesized by these species.

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