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

Rutger S. van der Hoeven and John C. Steffens*

Department of Plant Breeding and Biometry, 252 Emerson Hall, Cornell University, Ithaca, New York 14853

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 [13C]acetate; (b) [13C]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 dehydrase 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 i4:0 and i5:0 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, a i5:0 (2-methylbutyric acid) is derived from Thr through conversion into 2-oxo-butyric acid by Thr dehydratase, followed by a multi-enzyme 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 i4:0 or i5:0 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 i5:0 primer to form, for example, a i6:0 and a i7:0 (3-
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 ai6:0 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 ai6:0 and ai7:0 elongation from ai5:0; 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 substrates, 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 aKAE model posed a number of problems. Therefore, we chose to critically examine the existence of aKAE 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 peduncles were gently blotted with paper towels and a diagonal cut was made on the stem. The stem was inserted through a 3-cm Petri dish filled with a solution of labeled substrate. Substrates were gently blotted with paper towels and a diagonal cut with elongation by a FAS-based mechanism: enrichment of the m/z 89 isotopomer when either [1-$^{13}$C]- or [2-$^{13}$C]acetate is incorporated during FA biosynthesis can be unambiguously assessed. The biogenesis of normal, iso- 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 aKAE 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 predic-
tions (Table I). For the iso-branched FAs of *L. pennellii* and *N. umbratica*, i5:0 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. i8:0 and i10:0 (6-methylheptanoic and 8-methylnonanoic acid) are extension products from an i4:0 primer (Walters and Steffens, 1990).

Both i8:0 and i10:0 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

**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 [13C]acetate is incorporated during FA biosynthesis can be unambiguously assessed. The biogenesis of normal, iso- 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.

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Both i8:0 and i10:0 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 incorporated.
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 \( d_{8}-\text{Val} \) incorporation into \( d_{7}-i_{10:0} \) (Walters and Steffens, 1990). In accordance with this, the FAS-like extension of \( d_{9}-i_{5:0} \) (\( d_{9}-3\)-methylbutyric acid), which is derived from \( d_{10}-\text{Leu} \) 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 \( \alpha \)-KAE event: enrichment of \( m/z \) 89 when \([2-13C]\)- and \([U-13C]\)acetate are incorporated, and no enrichment when \([1-13C]\)acetate 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.

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 \([1-13C]\) or \([2-13C]\)acetate is incorporated, and \( m/z \) 90 enrichment when \([U-13C]\)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 \([1-13C]\), \([2-13C]\), or \([U-13C]\)acetate 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.

Analysis of \([U-13C]\)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
The fatty acids n4:0, n5:0, and n6:0 ethylacetic acid is obscured by the solvent peak (hexane), and is therefore not accounted for in calculating the mol % fatty acid composition.

For *N. umbratica*: n3:0; 3.13, i4:0; 5.67, ai 5:0; 17.92, i5:0, 5.52, ai 6:0; 51.37, i6:0; 5.03, ai 7:0; 8.10, n7:0; 1.45, i8:0; 1.44, n8:0; 0.37. The fatty acids n4:0, n5:0, and n6:0 <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.), n5:0; 3.61, i5:0; 34.54, n6:0; 8.84, i6:0; 11.96, n7:0; 19.35, nCB: 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 i4:0 and ai 5:0 cannot undergo McLafferty rearrangement to m/z 88 and related isotopomers (Fig. 1). ND, Not detectable (<0.01% enrichment).

### Table 1. Percent enrichment of FA-derived m/z 89 and 90 isotopomers after incorporation of differentially [13C]-labeled acetates

<table>
<thead>
<tr>
<th>FA Source</th>
<th>[1-13C]</th>
<th>[2-13C]</th>
<th>[U-13-C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z 89</td>
<td>m/z 90</td>
<td>m/z 89</td>
<td>m/z 90</td>
</tr>
<tr>
<td>n5:0</td>
<td>P.h.</td>
<td>0.21</td>
<td>0.01</td>
</tr>
<tr>
<td>n6:0</td>
<td>P.h.</td>
<td>0.20</td>
<td>0.04</td>
</tr>
<tr>
<td>n8:0</td>
<td>P.h.</td>
<td>0.39</td>
<td>0.04</td>
</tr>
<tr>
<td>n10:0</td>
<td>P.h.</td>
<td>0.28</td>
<td>0.04</td>
</tr>
<tr>
<td>n11:0</td>
<td>N.u.</td>
<td>0.40</td>
<td>0.14</td>
</tr>
<tr>
<td>n12:0</td>
<td>L.p.</td>
<td>0.29</td>
<td>0.05</td>
</tr>
<tr>
<td>ai6:0</td>
<td>N.u.</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ai7:0</td>
<td>N.u.</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

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

n3:0, n9:0, and n11:0 FAs occurring in sugar polyesters. Together, these data suggest that Thr is converted to the n3:0 primer via Thr dehydratase to yield 2-oxo-butyric acid and oxidative decarboxylation to yield propionyl-CoA. The n3:0 primer is then elongated by FAS-like mechanisms to yield propionyl-CoA. The n3:0 primer is then elongated by FAS-like mechanisms to yield propionyl-CoA. The n3:0 primer is then elongated by FAS-like mechanisms to yield propionyl-CoA. The n3:0 primer is then elongated by FAS-like mechanisms to yield propionyl-CoA. The n3:0 primer is then elongated by FAS-like mechanisms to yield propionyl-CoA. The n3:0 primer is then elongated by FAS-like mechanisms to yield propionyl-CoA. The n3:0 primer is then elongated by FAS-like mechanisms to yield propionyl-CoA. The n3:0 primer is then elongated by FAS-like mechanisms to yield propionyl-CoA. The n3:0 primer is then elongated by FAS-like mechanisms to yield propionyl-CoA. The n3:0 primer is then elongated by FAS-like mechanisms to yield propionyl-CoA. The n3:0 primer is then elongated by FAS-like mechanisms to yield propionyl-CoA. The n3:0 primer is then elongated by FAS-like mechanisms to yield propionyl-CoA. The n3:0 primer is then elongated by FAS-like mechanisms to yield propionyl-CoA. The n3:0 primer is then elongated by FAS-like mechanisms to yield propionyl-CoA.
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 n3:0, n4:0, n5:0, and n7:0 (up to 50% of incorporation) and much less efficiently in n8:0 (less than 5%). As demonstrated earlier, n7:0 and n8:0 are extended in two-carbon increments (Table I) in which n5:0 and n4:0, respectively, are implicated as intermediates, thereby accounting for the incorporation of the isotopically enriched n3:0 primer. The biogenesis of n4:0 remains uncertain. Clearly, n3:0 is extended to n5:0 and n7:0. We propose that n5:0, derived from one cycle of FAS elongation of n3:0, undergoes C1 elimination to form n4:0, which then serves as a primer for FAS-like elongation to n8:0 (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 d9-Val into d7-i10:0 is inhibited 48% by cerulenin, and its incorporation into d7-i11:0 is inhibited approximately 70% (after incorporation of d8-Val into d7-i5:0). As shown previously, d10-Leu is incorporated into d9-i5:0 and elongated in two-carbon increments exclusively into d9-i9:0 and d9-i11:0 (Walters and Steffens, 1990). Incorporation of d10-Leu into d9-i11:0 is also inhibited approximately 60% by cerulenin (Table III). Cerulenin also inhibits the incorporation of [U-13C]acetate into n10:0, i10:0, and n12:0 by 81%, 67%, and 51%, respectively, while having no effect on incorporation into shorter FAs (data not shown).

Incorporation of d9-Val and d10-Leu into i4:0 and i5:0 is not significantly affected by cerulenin treatment. However, incorporation into i9:0 is increased substantially as a result of cerulenin treatment when either d9-Val or d10-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 i9:0 may be related to the involvement of a KAS III- or KAS IV-like enzyme in the initial elongation of primers; increased incorporation into i9:0 (in length equivalent to n8:0) 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

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### Table II. Percent M+3 enrichment of fatty acids resulting from [U-13C]Thr incorporation

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>L. pennellii</th>
<th>N. umbratica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anteisobranched</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ai5:0*</td>
<td>11.6</td>
<td>20.1</td>
</tr>
<tr>
<td>ai6:0*</td>
<td>NDb</td>
<td>20.1</td>
</tr>
<tr>
<td>ai7:0</td>
<td>ND</td>
<td>16.3</td>
</tr>
<tr>
<td>Straight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n3:0</td>
<td>27.8</td>
<td>56.0</td>
</tr>
<tr>
<td>n4:0</td>
<td>ND</td>
<td>53.9</td>
</tr>
<tr>
<td>n5:0</td>
<td>ND</td>
<td>39.3</td>
</tr>
<tr>
<td>n6:0</td>
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<td>ND</td>
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<tr>
<td>n7:0</td>
<td>ND</td>
<td>30.6</td>
</tr>
<tr>
<td>n8:0</td>
<td>ND</td>
<td>4.9</td>
</tr>
<tr>
<td>n9:0</td>
<td>42.0</td>
<td>ND</td>
</tr>
<tr>
<td>n10:0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>n11:0</td>
<td>35.8</td>
<td>ND</td>
</tr>
<tr>
<td>n12:0</td>
<td>0</td>
<td>ND</td>
</tr>
</tbody>
</table>

* The isomeric ai5:0 and i5:0, and similarly ai6:0 and i6:0 are not separable as PFB esters in this GC system. Accordingly, enrichment of ai5:0 and ai6:0 is underestimated, as Thr is not incorporated in isobranched FAs (33).

b ND, Fatty acid not detectable.

### Table III. Effect of cerulenin on percent incorporation of d9-Val and d10-Leu into fatty acids

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>d9-Val</th>
<th>d10-Leu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerulenin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* 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 [1-14C]- and [2-14C]acetate. According to their proposed scheme, [2-14C]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 [1-14C]acetate is extensively randomized prior to incorporation, then randomization of [2-14C]acetate must also be assumed. Randomization would preclude the conclusion that [2-14C]acetate is differentially incorporated into FAs.

In fact, their data also strongly indicate randomization of [2-14C]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 acetaldheyde and pyruvate to form acetalactic 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 carbonyl 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 [2-14C]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 minute 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 [1-14C]acetate labels pyruvate invalidates the basis for concluding that αKAE-based elongation reactions contribute to the synthesis of any FA (Kroumova et al., 1994).

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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