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STRUCTURE AND BIOSYNTHESIS OF β-DIKETONES IN BARLEY SPIKE EPICUTICULAR WAX

by

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The structure and composition of the β-diketones in barley spike and carnation leaf epicuticular waxes have been determined by gas liquid chromatography-mass spectrometry. Barley wax contained, in addition to the well known hentriacontan-14,16-dione, small amounts of tritriacontan-16,18-dione, nonacosan-12,14-dione and nonacosan-14,16-dione. Five β-diketones were identified in carnation wax: nonacosan-10,12-dione, nonacosan-12,14-dione, hentriacontan-12,14-dione, tritriacontan-12,14-dione and tritriacontan-14,16-dione. The biosynthesis of the β-diketones was studied by incorporating various precursors into tissue slices of barley spikes with awns removed. Incorporation of [1-^{14}C]-pentadecanoic acid labelled a novel C_{30} β-diketone which upon cleavage contained the ^{14}C in the C_{16-30} end. This confirms that the carbon chain of the C_{31} β-diketone is formed in vivo by an elongation mechanism which proceeds from the C_{31} toward the C_1 end of the molecule. A comparison of the position of label within the β-diketone chains synthesized from C_{12}, C_{14}, C_{16} and C_{18} labelled precursors revealed that the β-keto acyl elongase which is responsible for β-diketone carbon chain synthesis has the following specificities: C_{14} or C_{16} chains serve equally well as primers; C_{12} chains are first elongated to C_{14} or C_{16} before they can serve as primers; C_{18} chains can not serve as primers. After protection of the β-diketo groups by the β-keto acyl elongase either 7 or 8 additional C_2 units are added before the presumed decarboxylation to yield the complete β-diketone molecule.

Abbreviations: GLC = gas liquid chromatography, HEA = hexane:diethyl ether:acetic acid, MS = mass spectrometry, TLC = thin layer chromatography, TMSi = trimethylsilyl

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1. INTRODUCTION

Biosynthesis of the long chain lipids present in plant epicuticular waxes occurs in the following way. With the aid of an elongation complex, C_{16} or longer fatty acyl precursors are elongated to specific chain lengths by the successive addition of C_2 units from malonyl-CoA. The latter then: (a) enter the decarboxylation pathways leading to hydrocarbons, secondary alcohols and ketones; (b) enter the reduction pathways giving rise to aldehydes, primary alcohols and esters; or (c) are dissociated from the elongation complex to yield free acids (4, 5, 13, 26, 27). While hydrocarbons, esters, aldehydes, primary alcohols, and free fatty acids are important components of barley spike waxes they occur in minor amounts compared to the β-diketone and hydroxy-β-diketone lipids (23, 25, 26).

Initial experiments with tracers suggested that the carbon chain of the C_{31} β-diketone was also synthesized by an elongation mechanism (16). This has been supported by the recent results from experiments in which the effects of inhibitors on the distribution of label within the carbon chain has been ascertained (15). That the elongation pathway leading to the β-diketones differed from that giving rise to the hydrocarbons was originally suggested from a comparison of the chemical composition of the waxes on barley mutants (eceriferum) (23, 26, 27). This has also been supported by the results from the recent inhibitor plus tracer studies (15). Furthermore the biosynthetic pathways leading to hydrocarbons and β-diketones seem to diverge when the chain has 16 carbons, since labelled palmitate but not stearate could be incorporated into the β-diketones, while both substrates were good precursors for the hydrocarbons (15).

The present paper explores the ability of various precursors to serve as substrate for the β-diketone synthesis. In order to interpret the results of these experiments it was necessary to identify the minor β-diketones which earlier experiments indicated must be present in barley spike wax (23). This identification was carried out by gas liquid chromatography-mass spectrometry. The precursor studies reveal certain specificities of the β-diketone elongation system and confirm that they are formed by an elongation mechanism.

2. MATERIALS AND METHOD

2.1. General

Techniques of plant culture and location of the radioactive lipids on the thin layer plates (radio-TLC) are described elsewhere (14, 15). The amounts of radioactivity in the lipid classes separated by thin layer chromatography (TLC) are expressed as the average of three independent experiments. The distributions of label among the various chain lengths have been calculated from at least three injections into the radio-gas liquid chromatograph.

2.2. Chemicals

The isotopes used included sodium [1-^{14}C]-acetate (58 mCi·mmole^{-1}), sodium [2-^{14}C]-acetate (58.8 mCi·mmole^{-1}), sodium[1-^{14}C]-hexanoate (58 mCi·mmole^{-1}), [1-^{14}C]-lauric acid (32 mCi·mmole^{-1}), [1-^{14}C]-myristic acid 45 mCi·mmole^{-1}), [1-^{14}C]-palmitic acid (56 mCi·mmole^{-1}), [9,10-^{3}H]-palmitic acid (500 mCi·mmole^{-1}), [1-^{14}C]-stearic acid (58 mCi·mmole^{-1}) and [9,10-^{3}H]-stearic acid (500 mCi·mmole^{-1}), which were purchased from the Radiochemical Centre (Amersham, England); [1-^{14}C]-palmitoyl-CoA (60 mCi·mmole^{-1}) and [1-^{14}C]-stearoyl-CoA (50.4 mCi·mmole^{-1}) which were supplied by New England Nuclear (Boston, Massachusetts); [1-^{14}C]-pentadecanoic acid (60 mCi·mmole^{-1}) which was obtained from DHOM Products (North Hollywood, California) and [2-^{14}C]-palmitic acid (19.2 mCi·mmole^{-1}) which came from Applied Science (Pennsylvania). Chloroform stabilized with amylene (Merck, Darmstadt, West Germany) was used throughout.

2.3. Incorporation of substrates into tissue slices

The barley mutant *cer-u*^{69} was used because it lacks hydroxy-β-diketones and has a compensatory increase in the amount of β-diketone per spike (23, 26). The procedure employed was essentially as described previously (15) but with the following changes: tissue slices prepared from two spikes with awns removed were incubated with the appropriate substrate in a total volume of 10 ml for 3 hours. The epicuticular waxes only were recovered by

extraction with 40 ml of chloroform for 30 sec (11). Ten replicate experiments were made with each substrate.

2.4. Preparative thin layer chromatography

The initial separation of the total epicuticular wax classes was carried out as described previously (14, 15) except that chloroform was the developing solvent instead of benzene. The β-diketones, aldehydes and esters were extracted as a single group (15) and were separated using »hybrid« copper acetate plates (15) with chloroform as the developing solvent. The β-diketones isolated from each set of replicate experiments were combined. An aliquot of each was retained for quantitating the distribution and amounts of radioactivity in the wax classes as described in section 2.5. The isolated β-diketones were checked for impurities by radio-TLC in the following two TLC systems: a, silica gel H with HEA (hexane:diethyl ether:acetic acid; 70:30:2; v/v/v) as the developing solvent and b, »hybrid« copper acetate silica gel H plates with chloroform as the developing solvent. The purity was always better than 99%.

2.5. Determination of radioactivity among the lipid classes

Label in the hydrocarbons was assayed as described previously (15). The aliquot containing the β-diketones, aldehydes and esters was chromatographed both on a 5 × 20 cm silica gel H plate and a 5 × 20 cm »hybrid« copper acetate plate (15). Chloroform served as the developing solvent in both cases. The amount of label in the three wax classes was quantitated by liquid scintillation counting as described previously (15). A small amount of radioactivity (< 1% of the total) was found at the origin on the silica gel H plate. An equivalent amount was subtracted from the amount of radioactivity found at the origin on the copper acetate plate where the β-diketones were retained to obtain the reported, actual amount of label in the β-diketones.

The counting efficiency was 93% and 42% for ^{14}C and ^{3}H, respectively, as determined from ^{14}C and ^{3}H-labelled toluene. The presence of copper acetate did not influence the ^{14}C counting, but the counting efficiency of the ^{3}H samples was reduced to 16%.

2.6. Gas liquid chromatography (GLC)

GLC was carried out using a Hewlett-Packard 5840 A series gas chromatograph with a flame ionization detector. A stainless steel column, 152 cm × 3.2 mm, containing 5% SE30 on Anakrom ABS 110/120-mesh (Analabs) was used. The column was operated isothermally at 240°C for the separation of the β-diol bis-trimethylsilyl (TMSi) ethers.

2.7. Gas liquid chromatography-mass spectrometry (GLC-MS)

An VG Micromass type 7070 F instrument was used with an ionizing potential of 70 eV and an ionizing current of 100 μA. The β-diketones were analysed as their β-diol bis-TMSi ethers, which were separated on a 150 cm × 2 mm column containing 5% OV-1 on Chromosorb W 80/100-mesh. The column temperature was increased from 250° to 270°C when the shortest chain β-diol bis-TMSi ether had been eluted.

2.8. Radio-gas liquid chromatography (radio-GLC)

The analyses were performed with a Packard 427 series gas chromatograph fitted with a 10:1 splitter, the minor arm of which led to the flame ionization detector while the major arm led to a Packard 894 series gas proportional counter. The signal from the flame ionization detector was fed to a Spectra-Physics System I computing integrator, while the signal from the gas proportional counter was fed to a Packard 7240 series digital integrator. The same type column was employed as described in section 2.6. The following temperature programs were used: 110–200°C, 2°C·min^{-1}, for the methyl esters of the β-diketone fatty acid moieties, isothermal at 240°C for the β-diol bis-TMSi ethers, and 40–240°C, 2°C·min^{-1}, for the butyl esters of the fatty acids resulting from α-oxidation of the fatty acid moieties obtained by the iodoform cleavage of the β-diketones. Counting efficiency for the gas proportional counter was calculated from the following relationship:

$$\text{percentage efficiency} = \frac{(C-B) \times 100}{dpm \times V/FR}$$

(C-B) represents the counts (C) minus background (B) recorded by the integrator (Packard 7240) when a known number of disintegrations per minute (dpm) are injected into the radio-GLC. V = volume of the gas proportional detector tubing, and FR = total flow rate through the detector tubing. Counting efficiency was 71% and 28% for ^{14}C and ^{3}H, respectively. At the sensitivity setting employed for the gas proportional counter as little as 150 dpm could be accurately assayed in a single peak.

2.9. Preparative gas liquid chromatography

Preparative GLC was used to separate the C_{29}, C_{31} and C_{33} β-diketones labelled with [1-^{14}C]-myristic acid. The Packard 427 series gas chromatograph described in section 2.8. was employed. The major effluent from the column splitter was led through teflon tubing (1.55 mm, internal diameter) cooled by liquid N_2. The sample was trapped in a short column (1.5 cm) of 1% Dexsil 300 on 100/120-mesh Supelcoport (Supelco, Pennsylvania) in the centre of the length of the teflon tubing. The trapped lipid was eluted with chloroform. Injection of known amounts of methyl [1-^{14}C]-palmitate followed by liquid scintillation counting gave an average trapping efficiency of 96% for the three separate runs. Re-injections of 10,000 dpm aliquots of the isolated C_{29} and C_{31} β-diol bis-TMSi ethers into the radio-GLC revealed that they were pure, that is, only a single radioactive peak was detectable in each case.

2.10. Chemical reactions

2.10.1. Iodoform cleavage of the β-diketones

The β-diketones were cleaved by the iodoform method previously described by NETTING and VON WETTSTEIN-KNOWLES (16). The resulting fatty acid moieties were analysed by radio-GLC as their methyl ester derivatives.

2.10.2. Preparation of the β-diol bis-TMSi ether derivatives

The β-diketones were incubated with 10 mg $NaBH_4$ in 99% ethanol at 35°C for 18 hours (7, 14). The resulting β-diols were purified by TLC with chloroform containing 2% ethanol (v/v) as the developing solvent (see Figure 1). After extraction from the TLC plates, the β-diols were

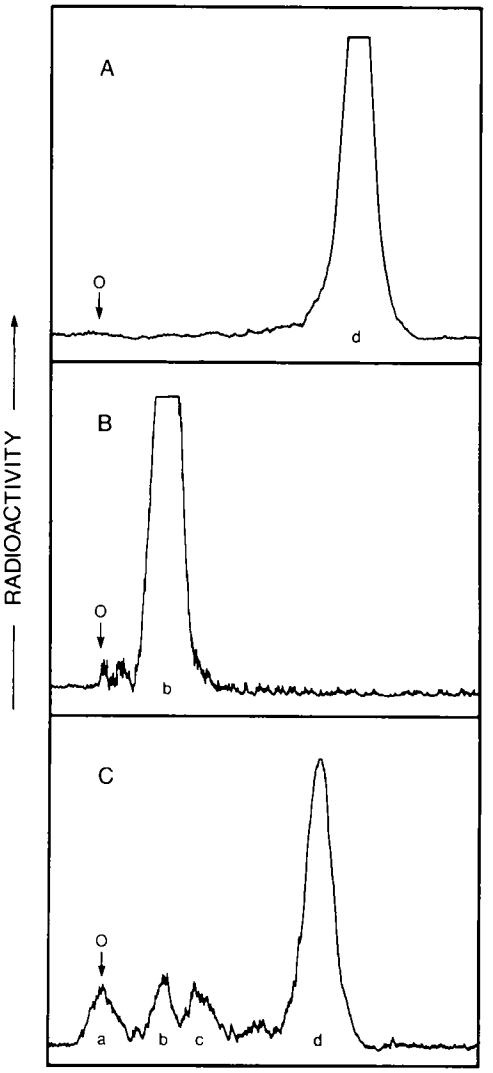

Figure 1. Efficiencies of the reduction of β-diketones to β-diols by $NaBH_4$, and the oxidation of β-diols to β-diketones by $K_2Cr_2O_7$, as assayed by radio-TLC on silica gel H plates and liquid scintillation counting. A, β-diketones before treatment with $NaBH_4$. B, after treatment with $NaBH_4$. C, the C_{31} β-diol after treatment with $K_2Cr_2O_7$ (see section 2.9 and *2.10.3*). HEA was the developing solvent in A and C, while chloroform:ethanol (98:2; v/v was the developing solvent in B. O, origin; a, unknown; b, β-diols; c fatty acids; and d, β-diketones.

carefully dried. The bis-TMSi ether derivatives were prepared by treating the diols with 100–200 µl BSA (bis-trimethylsilylacetamide, Pierce, Illinois) in pyridine (1:1, v/v) for ½ an hour at 40°C. The conversion was 100% as determined from radio-TLC and radio-GLC of aliquots of the β-diol substrate and the bis-TMSi ether product.

2.10.3. Oxidation of β-diols to β-diketones

To determine the structure of the C_{29} and C_{31} β-diketones labelled with [1-^{14}C]-myristic acid, the C_{29} and C_{31} β-diol bis-TMSi ether derivatives were isolated by preparative GLC and converted to β-diketones, and the distribution of radioactivity between the two ends of the molecule was determined as described in section 2.8. and *2.10.1.*

The C_{29} (160,000 cpm) and the C_{31} (114,000 cpm) β-diol bis-TMSi ethers were dissolved in 1 ml of petroleum ether (bp, 40–60°C) and hydrolysed to β-diols by incubating with 1 ml of 5 N H_2SO_4 at 40°C for 5 min. The β-diols were extracted with 3 × 3 ml of petroleum ether, and the combined extracts were washed three times with 2 ml of water. The β-diols were converted to β-diketones by oxidation with $K_2Cr_2O_7$ as described by VON WETTSTEIN-KNOWLES and NETTING (24), for the oxidation of secondary alcohols to ketones. The reaction was allowed to proceed for 2 hours at 25°C with gentle shaking. The products of the reaction were separated by TLC on silica gel H plates using HEA as developing solvent (see Figure 1). The total yield of β-diketone was approx. 60%.

2.10.4. Chemical α-oxidation

The fatty acids obtained from the cleavage of the β-diketones were subjected to chemical α-oxidation as described by NETTING (17) except for the following modifications: After the oxidation by $KMnO_4$ approximately 100 µg arachidic acid (C_{20}) was added to give a better precipitation of the fatty acid oxidation products by ammonia. The fatty acids were butylated by reaction with 100 µl BF_3-butanol reagent (Supelco, Pennsylvania) at 90°C for 20 min. After cooling to 0°C, excess BF_3 was destroyed by addition of 1 ml 25% NaCl and the butyl esters were recovered by extraction with 3 × 2 ml of n-pentane. The combined extracts were concentrated to 30 µl and the radioactive distribution of the butyl esters was determined by radio-GLC.

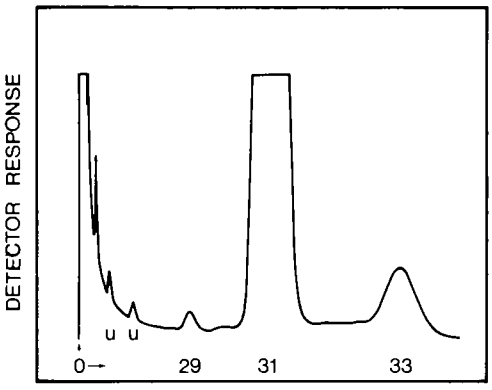

Figure 2. GLC pattern of β-diketones from barley spike epicuticular wax analysed as their β-diol bis-TMSi ether derivatives on a 5% SE 30 column. The number on each peak represents the carbon chain lengths. O, origin. U, unknowns.

3. RESULTS

3.1. Composition and structural analyses of β-diketones in epicuticular waxes of barley and carnation

The β-diketones isolated from spike epicuticular wax of the barley mutant *cer-u*[69] were converted to their β-diol bis-TMSi ethers and subjected to GLC analysis. The three peaks shown in Figure 2 were found to co-chromatograph with the known C_{29}, C_{31} and C_{33} β-diketones of carnation, Dianthus caryophyllus (7, 8). This indicated that, in addition to the earlier identified hentriacontan-14,16-dione (9, 23), at least two other β-diketones with different chain lengths were present in barley spike epicuticular wax.

To confirm the chain lengths and determine the positions of the carbonyl groups within the chains, the β-diol bis-TMSi ether derivatives of the β-diketones were analysed by GLC-MS. A recent analysis (7) of such derivatives of β-diketones has shown the following mass ions to be significant for a structural analysis: M^+-15 (CH_3) and M^+-90 (Me_3SiOH) reveal the mole-

Table I.
Structural characterization of the β-diketones from the waxes of barley *cer-u*[69] spikes and carnation leaves.

Plant Material	β-diketones		Relative intensities of $CH_3(CH_2)_xCHO^+SiMe_3$ fragment ions[c]						Position of carbonyl groups
	chain length[a]	%[b]	m/e 229	257	285	313	341	369	
cer-u[69]	29[d]	0.4	0	32	100	43	0	0	12, 14; 14, 16
	31	95.8	0	0	100	88	0	0	14, 16
	33	3.8	0	0	0	100	0	0	16, 18
Carnation	29[d]	11.2	59	100	0	78	33	0	10, 12; 12, 14
	31	83.1	tr	100	tr	tr	100	tr	12, 14
	33[d]	5.7	0	49	74	0	72	19	12, 14; 14, 16
Brassica spp.[e]	29		0	0	100	0	0	0	14, 16

a) Determined from GLC-MS analyses of the β-diol bis-TMSi ether derivatives (see text).
b) Quantitated via GLC analyses (weight %) of the β-diol bis-TMSi ether derivatives.
c) The MS were taken at the center of each GLC peak. Intensities are relative to base peak in each MS.
d) The relative intensities of the fragments differed from the start, the center and the end of the GLC peaks, indicating that the column was partly separating the isomers present. In all cases the most symmetrical isomer was the first to elute.
e) Data from (7).

cular formular, $C_nH_{2n}(OSiMe_3)_2$. The fragments $CH_3(CH_2)_xCHO^+SiMe_3$ arise from cleavage on both sides of the methylene group positioned between the two TMSi groups (7). The number of the latter type fragments, which are the largest peaks in the MS, depends on whether or not the β-diketone was a symmetrical or asymmetrical molecule as well as on the number of isomers present.

The M^+-15 and M^+-90 mass ions for the three β-diketone peaks were at 569 and 494 m/e, 597 and 522 m/e, and 625 and 550 m/e confirming the chain lengths as C_{29}, C_{31} and C_{33}, respectively. In all cases the relative intensities of these ions was less than 1 % of the base peak in the respective chromatogram.

In Table I the relative intensities of the $CH_3(CH_2)_xCHO^+SiMe_3$ fragments are given. These data allow the following deductions to be drawn. The two longer β-diketones consist of single isomers, namely hentriacontan-14,16-dione (Figure 3, I) and tritriacontan-16,18-dione (II). The shortest β-diketone, however, appears to be a mixture of two isomers, namely nonacosan-14, 16-dione (III) and nonacosan-12,14-dione (IV). An estimate of the relative amounts of III and IV could not be made because of the small amount of material present.

Nonacosan-14,16-dione (III) has also been analysed by HOLLOWAY et al. (7). Their results are included in Table I for comparison. With respect to the fragmentation pattern at the lower end of the MS, they are typical for long chain aliphatic compounds and the present analyses were very similar to those reported by the latter and other authors (7, 19–21).

Nonacosan-12,14-dione (IV) has been reported in carnation (7). To strengthen the identification of the same compound (IV) in barley wax, the β-diketones from carnation were isolated and analysed in the same way as the barley material. The chain length distribution is very similar to that given by HORN and LAMBERTON (8). The complex mixture of fatty acid moieties obtained by cleavage of the total β-diketones from carnation prohibited HORN and LAMBERTON from assigning unambiguous structures to the minor components (8). This is now possible from the $CH_3(CH_2)_xCHO^+SiMe_3$ fragments shown in Table I. In addition to the major component (7, 8), hentriacontan-12,14-dione (Figure 3, VI) four minor β-diketones can be identified in the

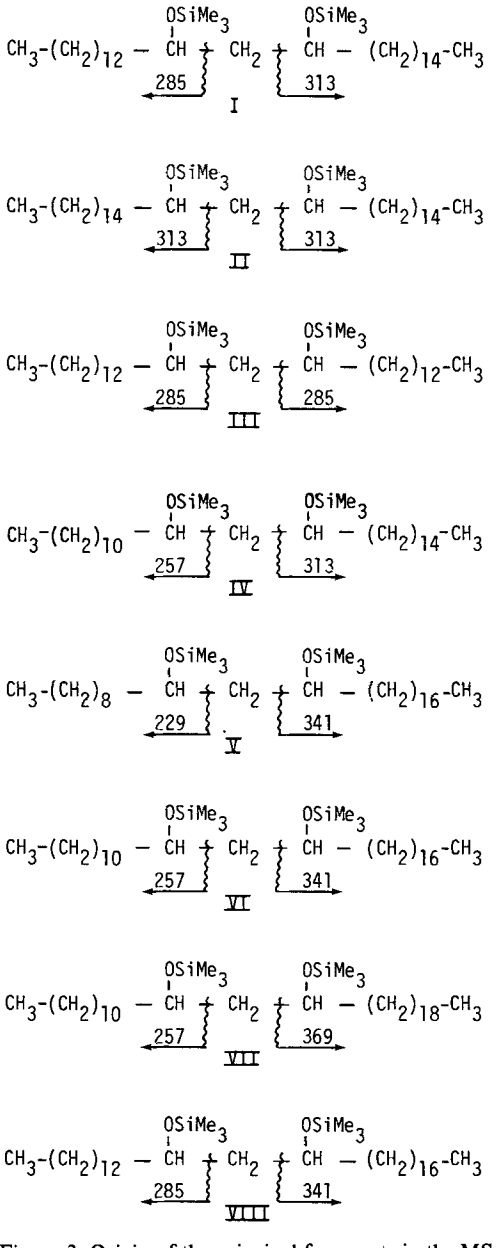

Figure 3. Origin of the principal fragments in the MS of the β-diol bis-TMSi ether derivatives of eight β-diketones from H. vulgare and D. caryophyllus. I = hentriacontan-14,16-dione; II = tritriacontan-16,18-dione; III = nonacosan-14,16-dione; IV = nonacosan-12,14-dione; V = nonacosan-10,12-dione; VI = hentriacontan-12,14-dione; VII = tritriacontan-12,14-dione; and VIII = tritriacontan-14,16-dione.

carnation wax namely, nonacosan-12,14-dione (IV), nonacosan-10,12-dione (V), tritriacontan-12,14-dione (VII) and tritriacontan-14,16-dione (VIII). Only the largest (VI) and one of the minor (IV) components were identified by a recent GLC-MS analysis (7). That compounds V, VII and VIII in the carnation wax were not previously detected may have two explanations: Firstly, the genotype of the present plants may differ from those investigated before (7). Secondly, they are minor components which could have been lost in preparation of the bis-TMSi ether derivatives.

3.2. Incorporation of labelled substrate into β-diketones and hydrocarbons

Previous studies showed that [1−^{14}C]-labelled acetate, lauric, myristic, palmitic and stearic acids were incorporated into β-diketones and hydrocarbons in a barley tissue slice system (15). This study has been repeated and extended to include eight additional precursors. The amounts of label incorporated into the β-diketones from the 13 different substrates are shown in Table II. The amounts of label in the hydrocarbons are also included to allow comparison with the earlier results (15). Palmitic acid, its CoA derivative and shorter chain fatty acids were all incorporated into β-diketones, whereas only trace amounts of label from stearic acid or stearoyl-CoA were incorporated into the β-diketones. By contrast the hydrocarbons were as well as or better labelled by the C_{18} than the shorter substrates. In the present study the efficiency of incorporation of label from acetate and stearic acid into the β-diketones is reduced compared to the earlier results (15). This reflects the improved method of quantitating label in the β-diketones as described in section 2.5. The efficiency of incorporation of label from lauric, myristic and palmitic acids is very much superior to that from acetate or stearic acid in agreement with the earlier results. The present results thus extend and corroborate the previous observations and the conclusion drawn therefrom; namely, that the last common intermediate in β-diketone and hydrocarbon biosynthesis is a carbon chain of 16 carbon atoms.

Very interestingly the incorporation of label from [1−^{14}C]-pentadecanoic acid (C_{15}) into the

Table II.

Incorporation of labelled precursors into β-diketones and hydrocarbons by tissue slices prepared from two *cer-u*[69] spikes with awns removed[a].

Substrate	μCi	dpm × 10^{-3}[b]	
		β-diketones	hydrocarbons
[2-^{14}C]-acetate	15	16	30
[1-^{14}C]-acetate	15	30	50
[1-^{14}C]-hexanoate	15	19	61
[1-^{14}C]-lauric acid	15	208	31
[1-^{14}C]-myristic acid	15	284	47
[1-^{14}C]-pentadecanoic acid	15	94	8
[1-^{14}C]-palmitic acid	15	236	40
[2-^{14}C]-palmitic acid	10	146	36
[9, 10-^{3}H]-palmitic acid	50	300	247
[1-^{14}C]-palmitoyl-CoA	3.3	147	18
[1-^{14}C]-stearic acid	15	tr[c]	41
[9, 10-^{3}H]-stearic acid	50	tr	375
[1-^{14}C]-stearoyl-CoA	3.3	tr	61

a) Procedures for isolation and quantitation of the labelled epicuticular wax classes are given in Materials and Methods and (14, 15).

b) The counts per min obtained by liquid scintillation counting were converted to disintegration per min (dpm) using the counting efficiencies given in section 2.5.

c) Less than 500 dpm.

Table III.

Distribution of radioactivity in β-diketones isolated from tissue slices prepared from the spikes minus the awns of the mutant *cer-u*[69] fed various labelled precursors[a].

Substrate	β-diketones						Total dpm injected[b]
	C_{27}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}	
	(Radioactivity %)						
[2-^{14}C]-acetate	0	1.3	0	95.2	0	3.5	34220
[1-^{14}C]-hexanoate	0	6.9	0	90.4	0	2.6	40890
[1-^{14}C]-lauric acid	2.7	12.7	0	84.6	0	tr[c]	31630
[1-^{14}C]-myristic acid	0	52.5	0	47.5	0	tr	29370
[1-^{14}C]-pentadecanoic acid	0	0	97.6	0	2.4	0	15320
[1-^{14}C]-palmitic acid	0	0	0	98.7	0	1.3	36530
[2-^{14}C]-palmitic acid	0	0	tr	97.0	0	3.0	26240
[9, 10-^{3}H]-palmitic acid	0	2.2	3.3	94.5	0	tr	88570

a) The β-diketones were isolated from 10 incorporation experiments (see Materials and Methods) and aliquots analysed as their β-diol bis-TMSi ethers.

b) The total dpm in a single injection was calculated using the calibration procedure of NETTING and BARR (18).

c) tr ≤ 200 dpm.

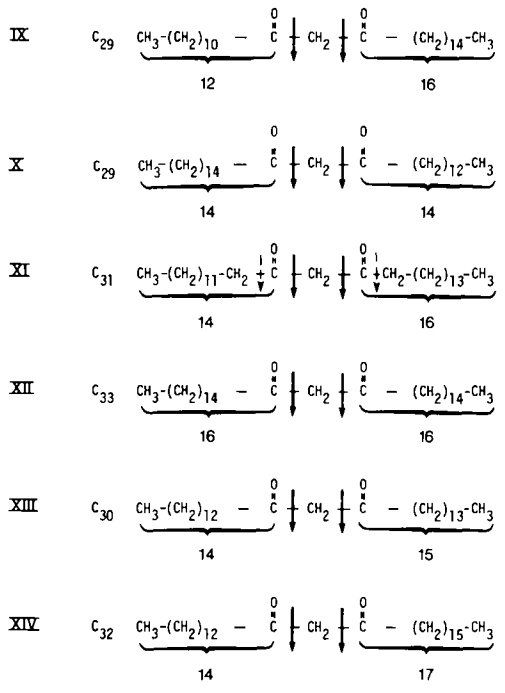

Figure 4. Sites of cleavage of six (IX–XIV) β-diketones during the iodoform reaction. The major cleavage sites are indicated with bold faced arrows. The resulting fatty acid moieties from the six individual β-diketones are shown in braces. Probable minor cleavage sites are indicated with dashed arrows only in structure XI (see text).

β-diketones was several fold greater than that from either [1–^{14}C]- or [2–^{14}C]-acetate. However, [1–^{14}C]-pentadecanoic acid was by far the least efficient hydrocarbon precursor.

3.3 Distribution of radioactivity in β-diketones labelled with various precursors

To examine the mode of β-diketone synthesis more closely, the β-diketones labelled with selected precursors were isolated by preparative TLC and the distribution of radioactivity in the different chain lengths was determined by radio-GLC of their β-diol bis-TMSi ether derivatives. The results are shown in Table III. The incorporation of acetate and the three palmitic acid substrates into the β-diketones resulted in a distribution of radioactivity very similar to the mass distribution (cf. Table I). As the chain length of the substrate is increased up to 14 carbon atoms, the proportion of radioactivity in the C_{29} β-diketone is also progressively increased. The maximum amount was 53% of the label when myristic acid was supplied as the precursor. When lauric acid served as the substrate, an additional radioactive peak was observed in the radio-GLC trace of the β-diketone derivatives. A semilog plot of apparent carbon number vs retention time from an isothermal radio-GLC analysis gave a straight line, indicating that the four radioactive peaks belonged to a homologous series. The newly appearing compound was thus tentatively identified as a C_{27} β-diketone.

In contrast to the results with the seven precursors described above, pentadecanoic acid labelled none of the naturally occurring β-diketones, but instead 98 percent of the radioactivity was present in a peak located between a C_{29} and a C_{31} β-diketone. A preliminary identification based on its retention time relative to internal standards indicated that this was a C_{30} β-diketone. A minor peak that is assumed to be a C_{32} β-diketone was also present.

3.4. Distribution of label between the two ends of the β-diketone molecule

The mode by which selected precursors were incorporated into the two ends of the β-diketone molecule was then examined. This necessitated the cleavage by the iodoform reaction of the mixture of β-diketones synthesized from each given substrate. The origin of the C_{12}, C_{14} and C_{16} fatty acid moieties which can be formed from the four β-diketones normally synthesized by *cer-u*[69] spikes are shown as formular IX to XII, in Figure 4. The resulting mixtures of fatty acid moieties from each experiment were analysed by radio-GLC as their methyl esters and the results are shown in Table IV. Incorporation of acetate gave a distribution of radioactivity quite similar to the mass distribution. As the chain length of the substrate increased from 2 (acetate) to 14 (myristic acid) carbon atoms, the relative amount of label in the C_{14} fatty acid moieties increased from 48 to 77% and that in the C_{16} fatty acid moieties decreased from 48 to 21%. Palmitic acid labelled almost exclusively the C_{16} fatty acid moieties of the β-diketones, although

Table IV.

Distribution of label among fatty acid moieties obtained by the cleavage of β-diketones synthesized from various fatty acids[a].

Substrate	Fatty acid moieties						Total dpm injected[b]
	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{17}	
	(Mol %)[c]						
	1.0	2.3	43.7	3.1	49.9		
	(Radioactivity %)[d]						
[1–^{14}C]-acetate	0	1.8	48.2	2.2	47.7	0	25610
[1–^{14}C]-hexanoate	0	1.2	58.8	1.3	38.7	0	26950
[1–^{14}C]-lauric acid	3.5	2.5	59.4	2.0	32.7	0	58700
[1–^{14}C]-myristic acid	0	0	77.3	1.4	21.3	0	46760
[1–^{14}C]-pentadecanoic acid	0	0	0	97.2	0	2.8	12030
[1–^{14}C]-palmitic acid	0	0	0	0	100	0	35370
[2–^{14}C]-palmitic acid	0	0	0	6.1	93.9	0	47370
[9, 10–^{3}H]-palmitic acid	0	0	1.1	5.2	93.8	0	114800

[a]) Data are for aliquots from the same incorporation experiments analysed in Table III.
[b]) See legend to Table III.
[c]) The amount of a fatty acid in n moles was determined by the method of NETTING and BARR (18).
[d]) The labelling patterns of the fatty acids were determined by radio-GLC of their methyl esters.

some radioactivity was observed in the C_{15} fatty acid moieties when [2–^{14}C]- and [9, 10–^{3}H]-palmitic acids were fed to the tissue slices.

In contrast to the seven substrates described above, pentadecanoic acid labelled primarily a C_{15} β-diketone fatty acid moiety. Co-GLC with standards was used to identify this fatty acid. A small amount of label was also present in a C_{17} β-diketone fatty acid moiety. The proposed origin of these fatty acid moieties from the C_{30} and C_{32} β-diketones are shown in formulas XIII and XIV of Figure 4.

The small amount of mass in the C_{13} and C_{15} fatty acid moieties is observed both when the β-diketones are cleaved by base hydrolysis (15) and by the iodoform reaction. It has previously been suggested (16) that these C_{13} and C_{15} fatty acid moieties could result from the cleavage of a C_{29} β-diketone (nonacosan-13, 15-dione). Such a C_{29} isomer was not detected by GLC-MS analysis in the present study. The C_{13} and C_{15} fatty acid moieties, however, might conceivably arise from cleavage on the outer sides of the β-diketo group, as illustrated by the dashed arrows in XI (Figure 4).

3.5. Distribution of radioactivity within individual β-diketones labelled with [1–^{14}C]-myristic acid

As was shown in Table III feeding myristic acid to the tissue slices resulted in two major radioactive β-diketones. The determination of the distribution of radioactivity between the two ends of these two different β-diketone molecules necessitated the separation of the C_{29} and C_{31} β-diketones by preparative GLC. The results of the radio-GLC analysis of the fatty acid moieties obtained by cleavage of the isolated C_{29} and C_{31} β-diketones are shown in Table V. The C_{29} β-diketone was only labelled in the C_{14} fatty acid moiety. Thus the structure of the C_{29} β-diketone was deduced to be nonacosan-14,16-dione. Since this molecule is symmetrical it is not possible to distinguish between the C_{1-14} and C_{16-29} ends, nor to verify whether myristic acid was incorpotated into one or both ends of the molecule. The radioactivity in the C_{31} β-diketone was unexpectedly evenly distributed between the C_{14} and C_{16} fatty acid moieties.

Table V.

Distribution of radioactivity between the ends of two β-diketones (X and XI, Figure 4) labelled with [1-^{14}C]-myristic acid[a].

β-diketone[b]	Fatty acid moiety[c]	
	C_{14}	C_{16}
	(Radioactivity %)	
C_{29}	100	0
C_{31}	50	50

a) Data are from an aliquot of the same [1-^{14}C]-myristic acid experiment analysed in Tables III and IV.

b) The β-diketones were isolated by trapping their β-diol bis-TMSi ether derivatives by preparative GLC, see section 2.9.

c) The β-diol bis-TMSi ethers were converted to β-diketones as described in section *2.10.3*. The β-diketones were cleaved by the iodoform reaction and the resulting fatty acid moieties were analysed by radio-GLC as their methyl esters.

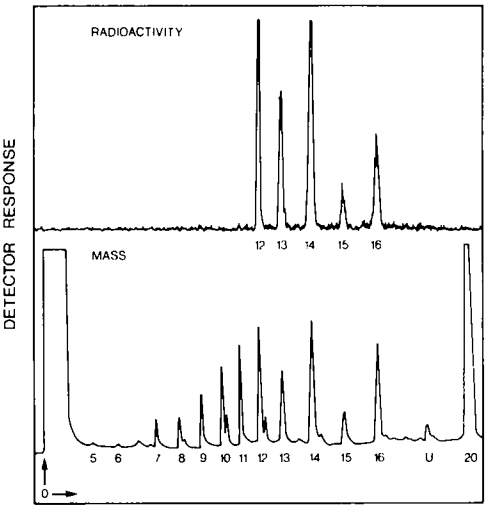

Figure 5. Radio-GLC pattern of the butyl esters obtained from chemical α-oxidation of the β-diketone fatty acid moieties (section *2.10.4*). The β-diketones had been labelled with [1-^{14}C]-lauric acid. The composition of the fatty acid moieties before the α-oxidation can be found in Table IV. The number on each peak represents the chain length. The C_{20} (arachidic acid) was added as a carrier during the α-oxidation (see section *2.10.4*). O, origin; U, unknown.

3.6. Chemical α-oxidation of the β-diketone fatty acid moieties

Since many plants contain active α- and β-fatty acid oxidizing enzymes, it was necessary to check that labelled precursors had not been degraded and subsequently incorporated into the β-diketones. The mixtures of C_{12-16} fatty acids obtained by the cleavage of the β-diketones arising from given incorporation experiments (see Figure 3 and Table IV) were subjected to chemical α-oxidation. The butylated products of

Table VI.

Distribution of radioactivity among the chemical α-oxidation products of the fatty acids moieties formed by cleavage of the β-diketones[a].

Substrate	Fatty acids[b]						Total dpm injected[c]
	C_{11}	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	
	(Radioactivity %)						
[1-^{14}C]-lauric acid	0	24.1	17.2	35.6	6.9	16.2	25380
[1-^{14}C]-myristic acid	0	0	0	69.8	7.8	22.5	51800
[1-^{14}C]-palmitic acid	0	0	0	0	0	100	10960
[2-^{14}C]-palmitic acid	0	0	0	0	28.0	72.0	15590

a) Cleavage of the β-diketones synthesized from given substrates resulted in the mixtures of fatty acid moieties shown in Table IV. Four of these mixtures were then subjected to chemical α-oxidation, and the distribution of label among the resulting fatty acids determined by radio-GLC.

b) Analysed as butyl esters.

c) See legend to Table III.

the α-oxidations were analysed by radio-GLC and the results are shown in Table VI. One example is illustrated in Figure 5. No radioactivity was found in oxidation products with chain lengths shorter than the particular [1-^{14}C]-labelled substrate used. That is, radioactivity from [1-^{14}C]-labelled C_{12}, C_{14} and C_{16} fatty acids was found exclusively in the C_{12-16}, C_{14-16} and C_{16} fatty acids, respectively. Label from [2-^{14}C]-palmitic acid was as expected, observed in both the C_{15} and C_{16} fatty acids, but no radioactivity was detected in shorter chain lengths.

On the other hand, feeding [9,10-^{3}H]-palmitic acid to the tissue slices gave rise to small amounts of labelled C_{29} and C_{30} β-diketones, while [2-^{14}C]- palmitic acid yielded a trace of a C_{30} β-diketone (Table III). This is presumably a result of α- and/or β-oxidation of the substrate and utilization of the resulting C_{15} and C_{14} fatty acids in the biosynthesis of the β-diketones. From these results the conclusion can be drawn that α- and/or β-oxidation of the radioactive substrates occurs, but that the released ^{14}C is not subsequently used in β-diketone synthesis at a level detectable by the used methodology.

4. DISCUSSION

Earlier investigations have led to the proposal that the carbon skeleton of hentriacontan-14,16-dione, the predominant β-diketone in barley waxes, is synthesized via elongation starting from the C_{31} end of the molecule and proceeding towards the C_1 end (16). More specifically, a C_2 unit is added to palmitic acid, perhaps the end product of fatty acid synthetase, to give a C_{18} β-keto acid intermediate. Protection of the β-diketo group and further elongation by 7 C_2 units leads to a C_{32} β-keto acid. The sequence of reactions starting from palmitic acid is postulated to be mediated by an elongation enzyme system hereafter termed a β-keto acyl elongase. A final decarboxylation of the C_{32} β-keto acid and release of the protection group gives the C_{31} β-diketone molecule (15). This is shown schematically in Figure 6, scheme A. The four schemes C to F illustrate the possible modes of synthesis of the three newly identified minor β-diketones, given that the plant uses the same basic mechanism for their synthesis. Since two of the β-diketones are symmetrical only one possible structure for each exists as shown in schemes C and D (Figure 6). For the asymmetrical β-diketone both schemes E and F are theoretically possible. Examination of schemes A, C, D, E and F reveals that only two variables are needed to account for synthesis of the four different barley β-diketones. Firstly, either a C_{12}, C_{14} or C_{16} chain can serve as a substrate for the β-keto acyl elongase giving rise to the β-keto acid intermediate. Secondly, either 6, 7 or 8 C_2 units may be added to the β-keto acid intermediate. The evidence from the present experimental results will be discussed below in relationship to the schemes of β-diketone synthesis illustrated in Figure 6.

Feeding [1-^{14}C]-myristic acid to the tissue slices resulted in the incorporation of large amounts of radioactivity into a C_{29} β-diketone. Cleavage of the molecule and α-oxidation of the resulting acids showed that all the label was present in the carboxyl carbon of a C_{14} fatty acid. The C_{29} β-diketone, therefore, is nonacosan-14,16-dione. These results lead to the conclusion that an exogenous precursor shorter than palmitic acid, the end product of fatty acid synthetase, can be directly used as substrate by the β-keto acyl elongase. That is, the latter enzyme system competes very effectively with the fatty acid synthetase for the exogenously supplied myristic acid. Subsequently, the addition of 7 C_2 units to the β-keto acid must occur, just as it does in the in vivo synthesis of the small amount of nonacosan-14,16-dione.

Relatively small amounts of label from [1-^{14}C]-lauric acid, however, were found in the C_{27} and C_{29} β-diketones and in the carboxyl carbon of a C_{12} fatty acid moiety produced by cleavage of these β-diketones. Lauric acid, thus, in contrast to myristic acid is poorly accepted by the β-keto acyl elongase as a primer for the synthesis of the β-diketones. Instead, lauric acid is elongated by the fatty acid synthetase and the resulting myristic and palmitic acids then serve as substrates for the β-keto acyl elongase. Since [1-^{14}C]-palmitic acid did not label the C_{29} β-diketone (nonacosan-12,14-dione) the synthesis of this compound most likely occurs via scheme E and not via F in Figure 6.

[1-^{14}C]-myristic acid also proved to be a good precursor for a C_{31} β-diketone. Cleavage of the

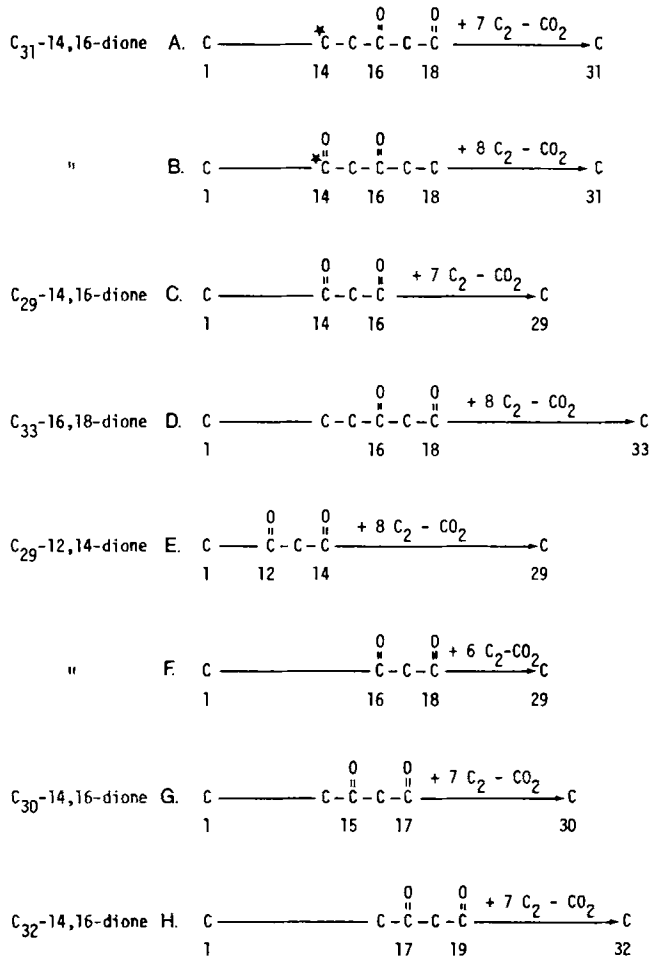

Figure 6. Possible modes of synthesis of eight β-diketones via elongation. Schemes A, C, D and E represent the normal in vivo biosynthesis in barley. Schemes B, G and H have been induced. Scheme F is included to account for all possibilities, but does not occur in vivo and has not been induced (see text). The numbering system is in the direction of induced elongation and is the opposite of the IUPAC nomenclature rules in A, F, G and H. C_{29} = nonacosan; C_{31} = hentriacontan; C_{33} = tritriacontan; C_{30} = triacontan; and C_{32} = dotriacontan. Position of ^{14}C within the C_{31} β-diketones after labelling with [1−^{14}C]-myristic acid is indicated by an asterisk.

latter followed by α-oxidation of the fatty acid moieties revealed that the label was in the carboxyl carbon of the C_{14} and in the third carbon of the C_{16} fatty acid. According to scheme A in Figure 6, myristic acid should first serve as a substrate for the fatty acid synthetase. After conversion to palmitate, the β-keto acyl elongase would yield a C_{31} β-diketone molecule with the label in the C_{16} fatty acid moiety. The unexpected labelling of the C_{14} fragment of the C_{31} β-diketone can be explained, however, by analogy with the synthesis of the C_{29}-diketone from [1−^{14}C]-myristic acid. That is, the β-keto acyl elongase accepts the myristic acid as a primer and synthesizes a C_{16} β-keto acid. Instead of the latter being elongated with 7 C_2 units to give the C_{29} β-diketone (Figure 6C), the addition of 8 C_2 units occurs as shown in scheme B of Figure 6. Addition of 8 C_2 units to a β-keto acid instead of 7 occurs in vivo in the synthesis of the C_{33} (tritriacontan-16,18-dione, Figure 6D). Thus, when [1−^{14}C]-myristic acid is fed to tissue

slices, two different C_{31} β-diketone molecules are formed as shown in Figure 6A and B. Cleavage of the β-diketone in scheme A yields label in the C_{16} acid whereas cleavage of the β-diketone in scheme B yields label in the C_{14} fatty acid moiety.

The present investigation shows that wax of barley as well as a number of other plant species (e.g. 6, 8, 20, 22) contains a homologous series of β-diketones whose members differ in chain length by two carbons. In this respect they are like many other wax lipid classes which have been shown to be synthesized via elongation (see 4, 5, 13). Perhaps the strongest experimental evidence obtained previously, to support the contention that the β-diketones are also synthesized via elongation (16), is the effect of preincubations with arsenite on incorporation of label from [2-^{14}C]-acetate (15). Label was found only in the C_{1-14} end, not in the C_{16-31} end of hentriacontan-14,16-dione (15). This is expected given that arsenite also here blocks elongation from 16 to 18 carbons as is well known for other fatty acid synthetic systems e.g. (2, 3, 10), and that the β-diketone is made via elongation starting from the C_{31} end and proceeding toward the C_1 end (16). Thus the label incorporated into the β-diketone chain is obtained by addition of labelled C_2 units to C_{18} or longer chains synthesized before addition of the arsenite (15). The results presented herein, in which [1-^{14}C]-pentadecanoic acid was used as a substrate for β-diketone synthesis prove the elongation hypothesis and leave no room for further doubt as to its correctness.

The basis for trying pentadecanoic acid as a substrate for β-diketone synthesis was the observation that [1-^{14}C]-myristic acid was such an effective precursor of nonacosan-14,16-dione. Given that the β-diketones are synthesized via elongation, the use of pentadecanoic acid as a substrate should result in formation of C_{30} and C_{32} β-diketones. This is the observed result. Almost all label from [1-^{14}C]-pentadecanoic acid was found in the novel C_{30} β-diketone. The label was located in a C_{15} fatty acid moiety formed upon cleavage. Thus the C_{15} fatty acid must serve as a substrate for the β-keto acyl elongase. The resulting C_{17} β-keto acid is then elongated by the addition of 7 C_2 units as shown in scheme G of Figure 6. The low amounts of radioactivity in the C_{32} β-diketone and in the C_{17} acid formed by cleavage implies that the pentadecanoic acid substrate may sometimes be first converted to a C_{17} fatty acid before serving as a substrate for the β-keto acyl elongase. Addition of 7 C_2 units as shown in scheme H of Figure 6 gives rise to the C_{32} β-diketone. No trace of label was detected in a C_{31} β-diketone. Thus a condensation (1, 12) between two moles of pentadecanoic acid plus one mole of malonyl-CoA did not take place. Therefore, one can now be confident that a similar condensation reaction does not lead to the label found in both the C_{14} and C_{16} fatty acid moieties arising from cleavage of the C_{31} β-diketone synthesized from [1-^{14}C]-myristic acid.

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