Biosynthesis of the Pyrethrins: Unsaturated Fatty Acids and the Origins of the Rethrolone Segment

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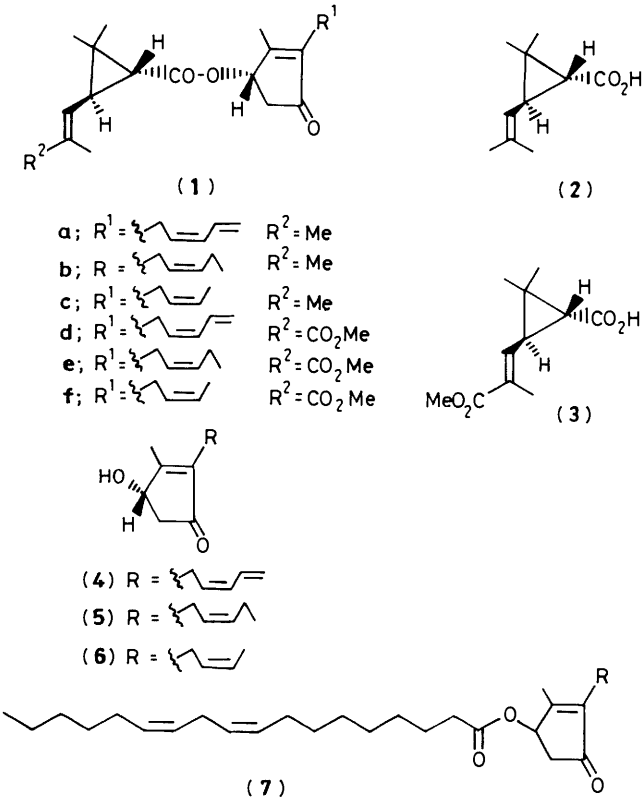
The biosynthetic origins of the rethrolone segment of the natural pyrethrins may be associated with linoleic or linolenic acid, and three hypotheses are outlined. By administration of suitable [2-^{14}C]- and [10-^{14}C]-labelled fatty acids, coupled with studies of their subsequent degradation, these hypotheses should be distinguishable. Administration of [10-^{14}C]oleic acid to an homogenate of *Chrysanthemum cinerariaefolium* achenes gave an incorporation ratio into the rethrolones relative to chrysanthemic acid of 1 to 0.5—0.25. This, and the results of ozonolysis, seem best interpreted as indicating that oleic acid is degraded to acetate before incorporation. [2-^{14}C]Linoleic acid was synthesized and administered, producing a label distribution between the rethrolones and chrysanthemic acid of 1 : 0.14—0.08. Kuhn-Roth analysis indicates again a strong background of degradation to acetate, though there is more labelling in the cyclopentenone methyl and its accompanying C-3 than would be expected from a pure acetate pathway.

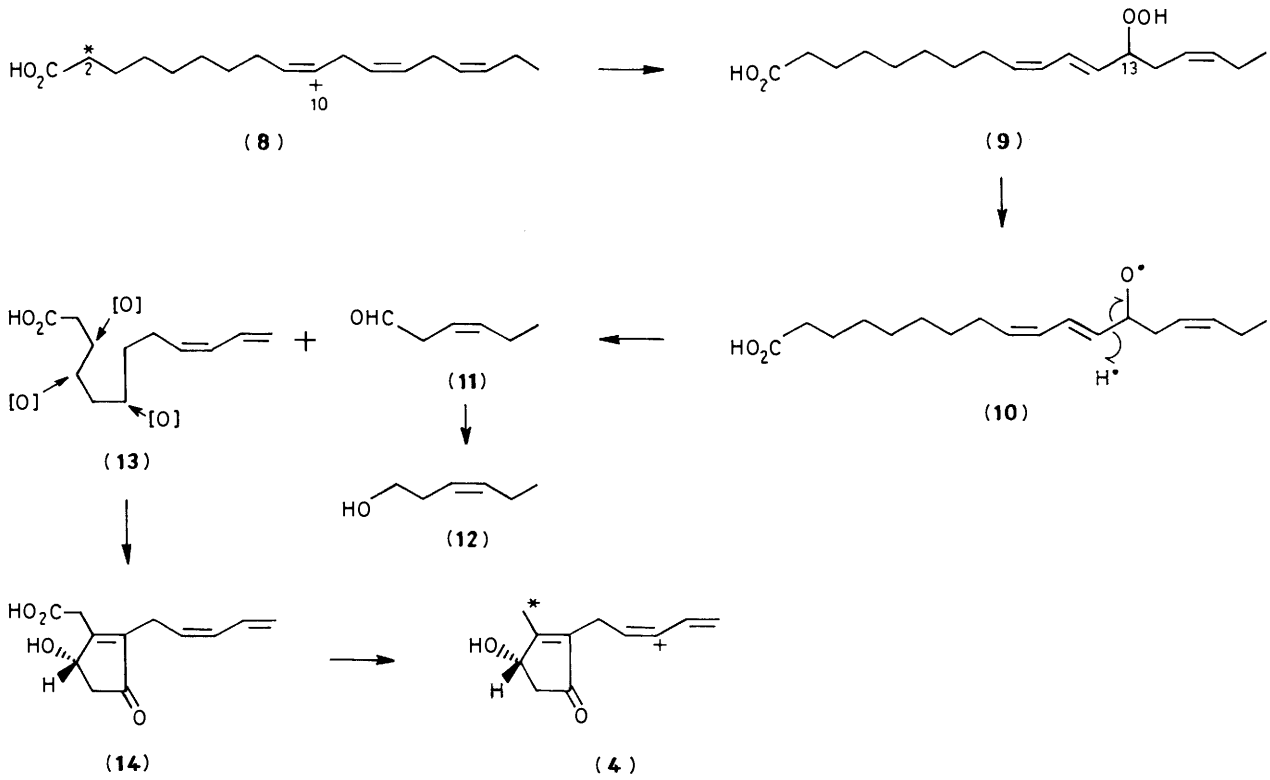
The pyrethrins (**1a**—**f**)[1] are well known as a group of insecticidal esters occurring in the flowers of pyrethrum, *Chrysanthemum cinerariaefolium* (Compositae). Little is known of the biosynthesis of the cyclopropane-containing acid portions of the molecules, chrysanthemic acid (**2**) and pyrethric acid (**3**), and still less about the cyclopentenolone, or rethrolone, segments. The latter have three side-chain types, pyrethrolone (**4**), jasmolone (**5**), and cinerolone (**6**).[1] Beyond an early report that labelled acetate is incorporated into the rethrolones,[2] there has been little information or speculation about their origins.

Pyrethrins-I (**1a**—**c**) and pyrethrins-II (**1d**—**f**) are localized in the achenes of the flower-head where they occur together with substantial amounts of fatty acids. Combined fatty acids represent up to 40% (w/w) of pyrethrum oleoresin, and the presence of palmitic, stearic, linoleic, and linolenic acids have been reported.[3] Furthermore, the pyrethrins are found with rethrolone fatty acid esters such as the palmitate or linoleate (**7**) esters.[4] It would not be surprising therefore if there were a biosynthetic relationship between rethrolones and natural unsaturated fatty acids, and we have set out to find whether experimental evidence supporting such a view could be obtained.



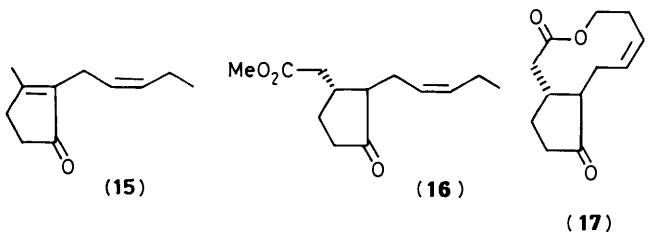
The biosynthesis of rethrolones (**4**)—(**6**) might proceed by one of three types of route. The first, outlined in Scheme 1, may be described as a 'methyl-end shortening' and is designated MES-A. Linoleic or linolenic acid might be the substrate and the process is illustrated using the latter (**8**). Lipoxygenase action leads to the 13-hydroperoxide (**9**), which then decomposes to give (**10**); this leads to (*Z*)-hex-3-enal (**11**) with formation of a pyrethrolone-like diene system (**13**). Oxidation steps (in unspecified order), aldol condensation, and decarboxylation of a vinylogous β-keto-acid *e.g.* (**14**) then lead to pyrethrolone (**4**). Reduction would lead to jasmolone (**5**) though the origins of the further chain shortening which gives the minor component cinerolone (**6**) remain obscure. The shortened methyl-end fragment (*Z*)-hex-3-enal (**11**) and its reduction product (*Z*)-hex-3-enol (**12**) are known to derive from linolenic acid *via* hydroperoxide decomposition.[5] Similarly hexanal and hexanol are produced from linoleic acid, and all four six-carbon compounds are widely distributed in green plants. Leaf alcohol does occur with jasmone (**15**) in the flowers of *Jasminium grandiflorum* (Oleaceae),[6] and also found with jasmone are other compounds, such as (**16**) and (**17**)[7] which are reminiscent of (**14**). Jasmone has been converted into jasmolone by microbiological oxidation at C-4.[8]

A different biosynthetic route from linolenic acid to the rethrolones is summarised in Scheme 2. Fundamentally this requires chain shortening from the carboxyl end (CES), which might occur early to give (**18**:n = 1), or later, from (**22** or **24**; n = 7); three β-oxidations are involved. Lipoxygenase-initiated reactions paralleling those in prostaglandin biosynthesis[9] lead *via* (**19**) and (**20**) to (**21**). Hydrogen transfer to the latter radical is then envisaged, instead of trapping by oxygen at the allylic position as occurs in prostaglandin biosynthesis. Isomerisation of the *endo*-peroxide to the ketol (**22**), dehydration to (**24**) and isomerisation of the double bond—all reactions well recognized in prostaglandin biosynthesis—lead to the ketone (**23**).



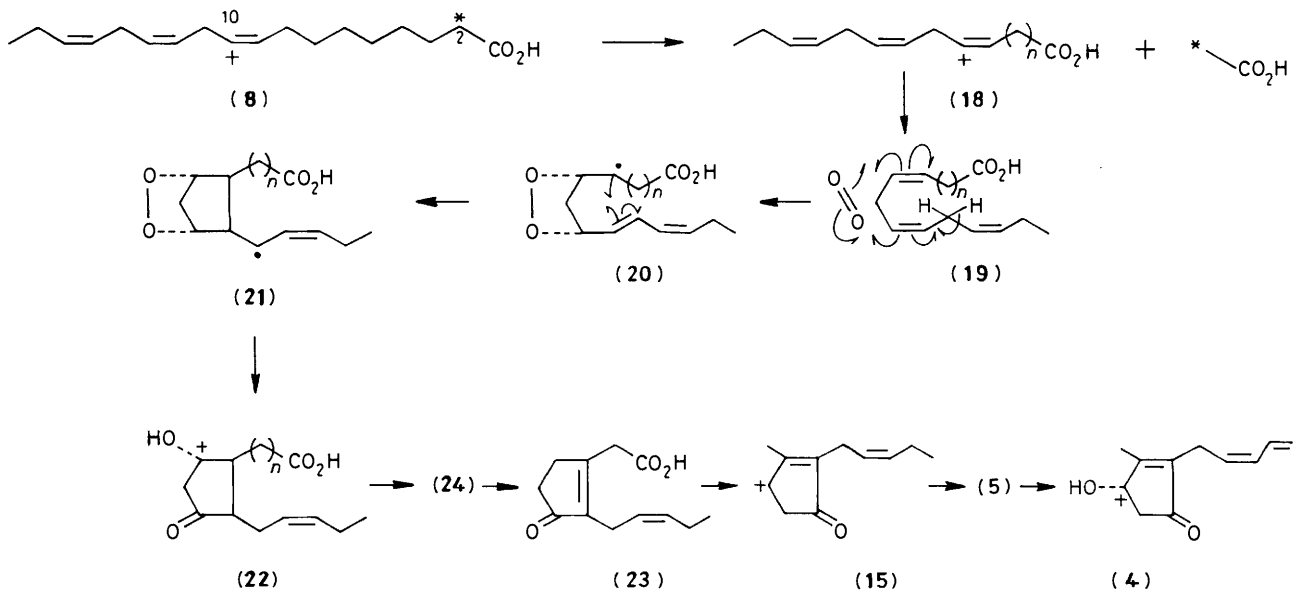
Scheme 1. Hypothesis for the formation of the rethrolones by 'methyl-end shortening' (MES-A)

+ and * show the ^{14}C labelling positions

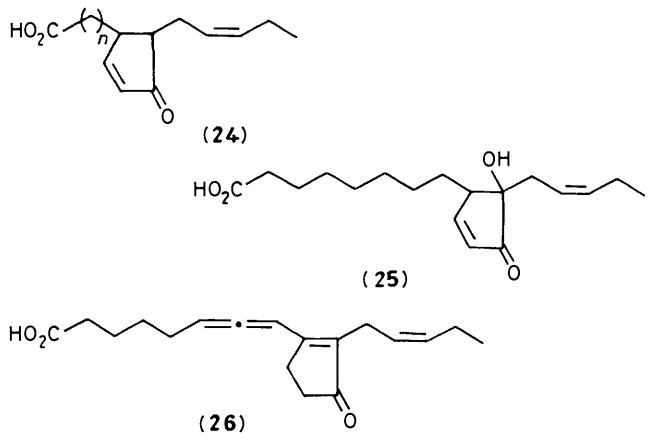


Decarboxylation to jasmone (**15**) and 4-hydroxylation leads to jasmolone (**5**). Formation of pyrethrolone (**4**) and of cinerolone (**6**), would require dehydrogenation of the terminal side-chain and loss of a carbon atom, respectively.

There is good precedent for a plant pathway of this general type, since linoleic 13-hydroperoxide is converted by flax-seed enzymes into 12-oxophytodienoic acid (**24**; $n = 7$),[10] and the latter has been shown to undergo hydrogenation, β-oxidation, and epimerisation to give jasmonic acid (**16**) in several plant



Scheme 2. Hypothesis for the formation of the rethrolones by 'carboxyl-end shortening' (CES)

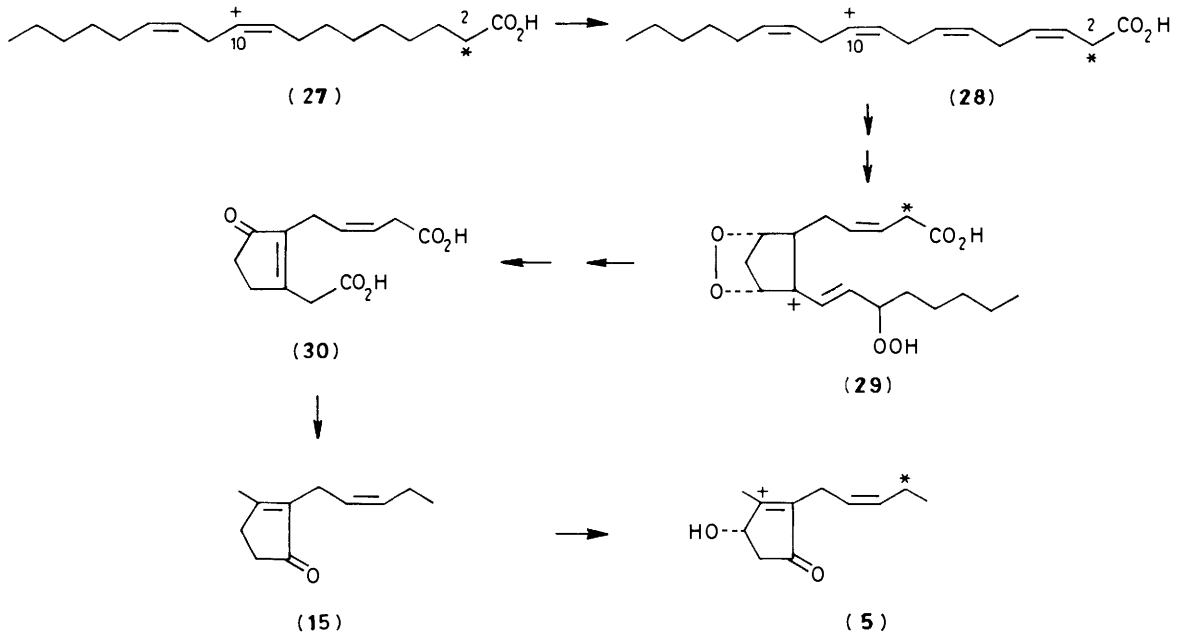


species.[11] In addition, cyclopentenones, *e.g.* (**24**) and (**25**), possibly formed by this pathway, have recently been reported in *Chromolaena spp.* (Compositae),[12] and the related (**26**) has been found in a moss, *Dicranum scoporium*,[13] so compounds of this type are quite widely distributed in plants.

In connection with jasmone biosynthesis, Demole[6] has recently suggested a mode of formation which involves methyl-end shortening but is basically different from the MES-A mechanism in that the carboxyl-containing end ultimately becomes the alkenyl side chain (Scheme 3). We designate this MES-B. It should be noted however that the mechanism requires further dehydrogenation of linoleic acid (**27**) towards the carboxy end to give (**28**), and that such dehydrogenation is more characteristic of animal than plant processes.[14] The tetraene acid is converted into (**29**) by routes analogous to those in prostaglandin biosynthesis: isomerisation to the ketol, dehydration and double bond migration, and oxidation of the hydroperoxide side chain follow, leading to (**30**). A decarboxylation and then reduction of the carboxy group terminating the unsaturated side chain is proposed, leading to jasmone (**15**).

Inspection of Schemes 1—3, or consultation of Tables 3 and 6, shows that administration of suitable [^{14}C]-fatty acids labelled in the 2-position and in the 10-position should give experimental results which allow discrimination between the three schemes, at least in theory. It is along these lines that our present efforts have been directed.

Pyrethrum presents a difficult subject for biosynthetic study. In the English climate it flowers for only about a fortnight in the year towards the end of June, and tracer administration has to be compressed within this period. We have used direct feeding to cut flower heads with four or five disc-florets open (the time of maximum pyrethrin production), vacuum infiltration to dissected achenes, and administration to an homogenized dissected achene preparation in phosphate buffer, pH 6.96. All three methods give only low incorporations of precursors and therefore in the present work we have used fresh achene homogenate in all the experiments. A further difficulty arises from the fact that six pyrethrins are being produced in the biosynthesis. The major group, the pyrethrins-I (**1a**—**1c**), can be satisfactorily separated from the pyrethrins-II (**1d**—**1f**) by column chromatography, but the separation of pyrethrin-I (**1a**), jasmolin-I (**1b**), and cinerin-I (**1c**) is difficult, quite apart from the fact that small amounts of product with low radioactivities are involved. We have therefore treated this mixture of pyrethrins-I as an entity. G.l.c. analysis (OV-17 on Chromosorb W) showed that the proportions of pyrethrins in our flowers were fairly constant: pyrethrin-I ($C_{21}H_{28}O_3$: *M*, 328.46), 78%; jasmolin-I ($C_{21}H_{30}O_3$: *M*, 330.47) 13%; and cinerin-I ($C_{20}H_{28}O_3$: *M*, 316.44), 9%.



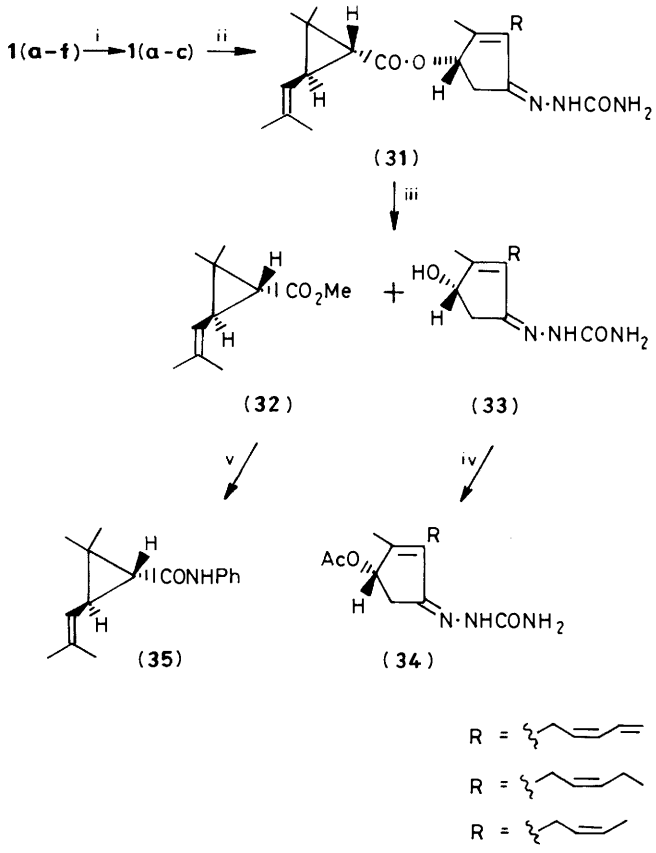
Scheme 3. Hypothesis for the formation of the rethrolones by 'methyl-end shortening' second version (MES-B)

Unless intensive purification is undertaken, meaningless high precursor incorporations are recorded. In our work the pyrethrins-I were isolated as crystalline semicarbazones (**31**) and then purified by reversed-phase h.p.l.c. (C_{18} column, MeOH–H_2O). Methanolysis then cleaved the ester giving methyl chrysanthemate (**32**) and the mixture of rethrolone semicarbazones (**33**).[15] These were purified further by conversion into their crystalline acetyl derivatives (**34**), and then counted and used for degradation in this form (Scheme 4). Methyl chrysanthemate was hydrolysed, converted into its acid chloride and then into the crystalline anilide (**35**), and counted.

Table 1. Administration of sodium [10-^{14}C]oleate to pyrethrum achene homogenate

	Expt. 1 Sodium [2-^{14}C]Acetate	Expt. 2 Sodium [10-^{14}C]Oleate	Expt. 3 Sodium [10-^{14}C]Oleate[e]
Wt. (g) of achenes in homogenate	50	50	50
Radioactivity administered (μCi)	25.0	25.0	25.0
Wt. of crude pyrethrins-I (mg)[a]	88	114	54
Wt. of pyrethrins-I semicarbazones (mg)[b]	32.1	37.9	37.0
Wt. of rethrolone semicarbazone acetates (mg)[c]	6.8	5.7	5.2
D.p.m./mg of above	902	1 866	1 246
Specific activity of above (μCi/mmol)[d]	0.11	0.23	0.16
% Incorporation of above	11×10^{-3}	19×10^{-3}	12×10^{-3}
Wt of (+)-*trans*-chrysanthemanilide (mg)	2.4	2.8	2.7
D.p.m./mg of above	704	918	1 096
Specific activity of above (μCi/mmol)	0.08	0.10	0.12
% Incorporation of above	3.1×10^{-3}	4.6×10^{-3}	5.3×10^{-3}

[a] After column chromatography. [b] After h.p.l.c. [c] After t.l.c. [d] Uses 'mol wt' of 276.5 representing the rethrolone semicarbazone acetate mixture. [e] With cofactors (see Experimental).



Scheme 4. Isolation of labelled pyrethrins-I and derivatives of the rethrolones and chrysanthemic acid.

Procedures: i Silica gel chromatography. ii Semicarbazide hydrochloride–NaOAc–pyridine, then C_{18}-reversed phase h.p.l.c. iii NaOMe–MeOH. iv Pyridine–Ac_2O, then t.l.c. v *a* NaOH, *b* $SOCl_2$, *c* $PhNH_2$, Me_3N.

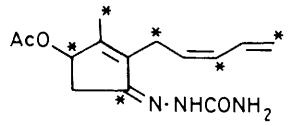
The first administration experiments were carried out using [10-^{14}C]oleic acid (sodium salt). Oleic acid is dehydrogenated to linoleic and linolenic acid in higher plants[14] and [10-^{14}C]oleic acid is readily available *via* the Wittig reaction. Results are given in Table 1. Expt. 1 is a control using sodium [2-^{14}C]acetate. Expt. 2 uses unsophisticated achene homogenate, while in Expt. 3, cofactors (CoA,ATP,NADPH) were added. The results of the two experiments are consistent with an incorporation of the acid of *ca.* 2×10^{-2}% into the rethrolone derivatives, close to that of the acetate control. Table 2 shows that in the MES-A mechanism all the radioactivity should reside in the side-chain carbon atoms, which can be ozonised[16] to give short-chain volatile aldehyde products. The CES and MES-B mechanisms should bring about no side-chain incorporation. Table 3 shows that no clear-cut results of this kind are obtained. In both Experiments, although the radioactive balance is poor, probably through polymerisation and formation of volatile materials which do not react with 2,4-dinitrophenylhydrazine, a 35:65 distribution between the volatile aldehyde derivatives and the non steam-volatile materials was found.

Table 2. Comparison of degradative results from the administration of [10-^{14}C]oleic acid with biosynthetic hypotheses.

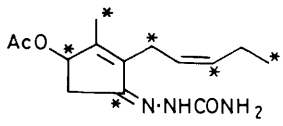
	% Radiolabelling in ozonolysis products	
	In 2,4-dinitrophenyl-hydrazones of volatile aldehydes	In non-volatile products
Found:		
Expt. 2	34.1	65.9
Expt. 3	36.4	63.6
Requires:		
Scheme 1 (MES-A)	100	0
Scheme 2 (CES)	0	100
Scheme 3 (MES-B)	0	100
Acetate incorporation:		
via MES-A	32	68
via CES	32	68
via MES-B	20	80

Clues as to how this degradative result might have been attained are to be found in Table 1. In both experiments, 2 and 3, the incorporation of radioactivity into the chrysanthemic anilide is a half to a quarter of that into the rethrolones-I. Chrysanthemic acid is undoubtedly terpenic in origin and the terpene precursor must have been formed *via* the degradation of oleic acid to labelled acetate, and synthesis of mevalonic lactone from this. There is thus a strong suspicion that the incorporation into the rethrolones is achieved by degradation of the [10-^{14}C]oleate to acetate. Comparison with Expt. 1 lends

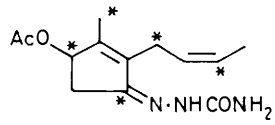
MES-A (or CES) labelling from * $MeCO_2H$ (see schemes 1 and 2)



Pyrethrolone Deriv.



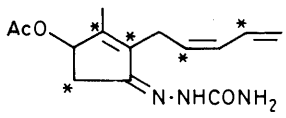
Jasmolone Deriv.



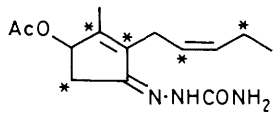
Cinerolone Deriv.

Side-chain volatiles: 2/6 x 78 % | 2/6 x 13 % | 1/5 x 9 % = 32·1 %

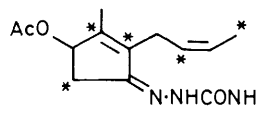
MES-B



Pyretholone



Jasmolone Deriv.



Cinerolone Deriv.

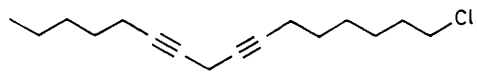
Side-chain volatiles: 1/5 x 78 % | 1/5 x 13 % | 1/5 x 9 % = 20 %

Scheme 5. Calculation of labelling carried by steam distillable aldehydes formed by ozonolysis of the side-chain of the rethrolones mixture, as biosynthesized from [2-^{14}C]acetate by three different mechanisms.

Table 3. Radioactivity of rethrolone semicarbazone acetates from administration of [10-^{14}C]oleic acid, and the ozonolysis products.

	Expt. 2	Expt. 3
Wt. (mg)[a] of rethrolone semicarbazone acetates ozonised	5.7	5.2
Total radioactivity (d.p.m.) of sample	10 636	6 479
Wt (mg) of volatile aldehydes from ozonolysis (as 2,4-dinitrophenylhydrazones)	7.2	6.4
Total radioactivity (d.p.m.) of sample	1 922	1 267
Radioactivity of starting sample (%)	18.1	19.6
Total recovered radioactivity (%)	34.1	36.4
Wt (mg) of involatile products from ozonolysis	3.1	2.9
Total radioactivity (d.p.m.) of sample	3 726	2 221
Radioactivity of starting sample (%)	35.0	34.3
Total recovered radioactivity (%)	65.9	63.6

[a] Rescaled after removal of sample for counting.



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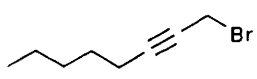

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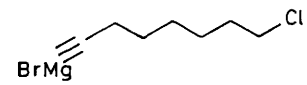

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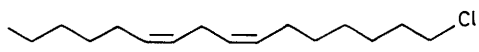

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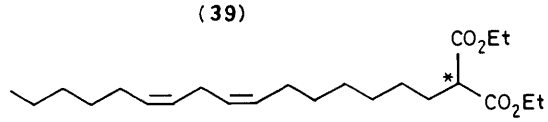

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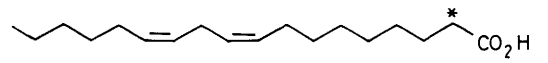

(41)

weight to this view: incorporations of [2-^{14}C]acetate are very similar to those of Expts. 2 and 3.

[10-^{14}C]Oleate would be degraded to [2-^{14}C]acetate. We can calculate the distribution of radioactivity between the volatile aldehydes and the non-volatile material for reincorporation of [2-^{14}C]acetate *via* the above pathways as shown in Scheme 5. Though the experimental results can only represent a rough approximation because of assumptions made, it is clear that degradation to acetate before incorporation is very probable (Table 2).

In the second group of experiments, recorded in Table 4, it was decided not to rely on *in situ* 12,13-dehydrogenation of oleic acid but to use instead [2-^{14}C]linoleic acid, capable of acting more directly as the substrate. For this, the halogeno-7,10-diyne (**36**) was required and this was made by the copper-catalysed coupling of the chloroacetylene magnesium bromide (**37**) and the prop-2-ynyl bromide (**38**).[17] The resulting diyne (**36**) was semi-hydrogenated using Lindlar catalyst and the methylene interrupted diene (**39**) was converted into the iodide and allowed to react with diethyl [2-^{14}C]malonate. The product (**40**) was hydrolysed and decarboxylated to give [2-^{14}C]linoleic acid (**41**).

The acid (**41**) was administered to pyrethrum achene homogenate in two experiments (Expts. 5 and 6) along with an acetate control experiment (Expt. 4). Expt. 5 involves unsophisticated achene homogenate whereas in Expt. 6 poly(vinylpyrrolidone) and dithiothreitol were added to complex phenols and reduce quinones respectively. In addition ATP, NAD^+, and $NADP^+$ along with divalent metal ions were added. As in the oleic acid experiments there is incorporation into the chrysanthemum segment of the molecule, though smaller than that into the rethrolone segment.

From Table 6, if the [2-^{14}C]linoleic acid were incorporated intact by the MES-A mechanism, all the label should reside in the cyclopentenolone methyl group of all three rethrolone derivatives (**4**)—(**6**). It should be distilled quantitatively as acetic acid in a Kuhn-Roth terminal methyl determination.[18] In a pure CES scheme there should be no labelling anywhere in the rethrolones except inasmuch as the labelled [2-^{14}C]acetic acid formed by β-oxidation (Scheme 2) is distributed on alternate

Table 4. Administration of sodium [2-^{14}C]linoleate to pyrethrum achene homogenate.

	Expt. 4 Sodium [2-^{14}C]acetate	Expt. 5 Sodium [2-^{14}C]linoleate	Expt. 6 Sodium [2-^{14}C]linoleate[c]
Wt. (g) of achenes in homogenate	50	75	75
Radioactivity administered (μCi)	25	39.3	39.3
Wt. of crude pyrethrins-I (mg)[a]	104	98	61
Wt. of pyrethrin-I semicarbazones (mg)[b]	29.6	39.1	22.4
Wt. of rethrolone semicarbazone acetates (mg)[c]	4.7	6.3	3.9
D.p.m./mg of above	708	1 448	2 413
Specific activity of above (μCi/mmol)[d]	0.088	0.18	0.30
% Incorporation of above	6×10^{-3}	11×10^{-3}	10×10^{-3}
Wt. of (+)(*trans*-chrysanthemanilide (mg)	1.8	1.9	0.8
D.p.m./mg of above	420	749	822
Specific activity of above (μCi/mmol)	0.046	0.082	0.090
% Incorporation of above	1.3×10^{-3}	1.6×10^{-3}	0.75×10^{-3}

[a-e] See Table 1.

Table 5. Radioactivity of rethrolone semicarbazone acetates from administration of [2-^{14}C]linoleic acid and the Kuhn-Roth products

	Expt. 5	Expt. 6
Wt. (mg)[a] of rethrolone semicarbazone acetates for Kuhn-Roth	6.3	3.9
Total radioactivity (d.p.m.) of sample	9 122	9 411
Total radioactivity (d.p.m.) of sodium acetate from Kuhn-Roth	2 331	3 441
Radioactivity (d.p.m.) of starting sample (%)	25.6	36.6
Total recovered radioactivity (d.p.m.) (%)	30.6	47.7
Total radioactivity (d.p.m.) of Kuhn-Roth residue after distillation	5 283	3 774
Radioactivity (d.p.m.) of starting sample (%)	57.9	40.1
Total recovered radioactivity (d.p.m.) (%)	69.3	52.3

[a] Rescaled after removal of sample for counting.

Table 6. Comparison of degradative results from the administration of [2-^{14}C]linoleic acid with biosynthetic hypotheses

	% Radioactivity in Kuhn-Roth experiments	
	In Kuhn-Roth volatile acetic acid	In non-volatile products
Found:		
Expt. 5	25.5 [30.6]	[69.4]
Expt. 6	36.6 [47.7]	[52.3]
Requires:		
Scheme 1 (MES-A)	100	0
Scheme 2 (CES)	0	0
Scheme 3 (MES-B)	9	13
Acetate incorporation:		
via MES-A	18.8	
via CES	18.8	
via MES-B	21.8	

carbons throughout the rethrolones. In the third scheme MES-B, labelling will occur on the side chain. In the pyrethrolone molecule the labelling will disappear (chromic acid oxidation to CO_2), and in jasmolone it will appear as propionic acid which should not distil under the strict analytical conditions of the Kuhn-Roth labelled distillable acetic acid resulting only from the cinerolone side chain.

For Expt. 5 titration of the acetic acid produced gave a C(Me) value of 13.5% (calc. for the rethrolone acetate semicarbazone mixture: 11.5%). The whole sample of titrated acetic acid was then counted and found to contain 25.5% of the activity of the starting rethrolone acetate semicarbazones. (The undistilled residues from the Kuhn-Roth experiment were also counted and the distribution of label between the acetic acid and these was 31:69. Because of loss of radioactivity by oxidation to escaped gases however, this is probably not a useful ratio to consider). The radioactivity of the distilled acetic acid is inconsistent with clean operation of any of the three hypothetical schemes considered and is again best explained by degradation of the initial [2-^{14}C]linoleic acid followed by reincorporation as acetate (Table 6). Nonetheless, the radioactivity of the acetic acid was slightly higher than required by any of the acetate reincorporation pathways.

In Expt. 6 the experimental C(Me) figure was 12.6% (calc. 11.5%) and the radioactivity contained in the distilled Kuhn-Roth acetic acid was 36.6% of the original rethrolone acetate semicarbazone mixture. This suggests some additional operation of the MES-A pathway. However, in view of the many experimental difficulties with the pyrethrins system such a view can be very tentative, at most. Any such incorporation is taking place against a strong obscuring background of degradation to acetate and re-synthesis, and interpretations require caution. It is hoped however, that the present work has brought to light some of the difficulties involved and will provide an entry for further experimentation with rethrolone biosynthesis.

Experimental

Preparation of Pyrethrum Homogenates and Administration of Radiolabelled Precursors.—The achenes from those flowers of *C. cinerariaefolium* having three or four rows of disc florets open were removed by dissection (50 g or 75 g for each experiment) and homogenised (Waring blender) in phosphate buffer solution (300 ml, pH 6.96). The dark suspension was transferred to a flask (500 ml) and the radiolabelled substrate administered as its sodium salt. The flask was lightly stoppered (air admitted) and shaken for 40 h, when the homogenate was extracted with light petroleum (×6), dried ($MgSO_4$), and evaporated to yield a yellow oil consisting of impure pyrethrins. Column chromatography on silica gel allowed isolation of the impure pyrethrins-I (R_F 0.82) on t.l.c. using ether–hexane (1:1) as eluant.

Preparation and Purification of Pyrethrins-I Semicarbazones.—In a typical experiment impure labelled pyrethrins-I

(0.088 g, 0.23 mmol) were dissolved in ethanol (acetone free), and semicarbazide hydrochloride (0.15 g, 1.3 mmol) was added, followed by sodium acetate (0.1 g, 1.2 mmol). Water (0.2 ml) and pyridine (0.1 ml) were added and the suspension was stirred until all the solid had dissolved. The solution was placed under nitrogen, kept at 0 °C for 24 h and the crystals which had deposited were filtered off (Willstatter nail) and washed with a little cold water before being dried *in vacuo* (first-crop material). The filtrate was diluted with distilled water (to 10 ml) and extracted with ethyl acetate; the extract gave, after drying, a pale yellow gum.

Both the first-crop material and the recovered gum were purified by semi-preparative h.p.l.c. [C_{18}-reverse phase, eluting with methanol–water (9:1)]. After removal of the solvent at low temperature under reduced pressure, off-white crystals of purified pyrethrins-I semicarbazones (32 mg) were obtained, m.p. 116—119 °C, $\nu_{max.}$($CHCl_3$), 1 720 cm^{-1}, $\lambda_{max.}$(EtOH) 257.5sh (ε 8.230), 263 (29 600), and 271sh nm (8 230). The n.m.r. spectrum (250 MHz, $CDCl_3$) showed characteristic resonances for a mixture of pyrethrin-I, jasmolin-I, and cinerin-I semicarbazones. (Spectroscopic data are quoted from an unlabelled run.)

Methanolysis of Pyrethrins-I Semicarbazones.—In a typical experiment, labelled pyrethrins-I semicarbazones (32 mg) were diluted with cold material (20 mg) and the product (52 mg, 0.14 mmol) was dissolved in dry methanol (1 ml) and stirred under nitrogen. Powdered sodium methoxide (10 mg, 0.18 mmol) was added to produce a light red solution which was stirred at 20 °C for 20 h. Potassium hydroxide solution (0.5 ml, 0.01M) was added and the mixture was kept for 30 min before being poured into brine (10 ml) and extracted with ethyl acetate. The basic aqueous portion was kept for recovery of chrysanthemic acid. The organic extracts were washed with water and brine, dried ($MgSO_4$), and evaporated to leave a light brown solid which was dissolved in dry pyridine (2 ml) and treated with acetic anhydride (0.6 ml, 4.2 mmol). The solution was stirred at 0 °C for 15 h under nitrogen, then poured into water (10 ml), and extracted with ethyl acetate. The combined organic extracts were washed with aqueous copper sulphate, water, and brine, before being dried and evaporated to give an oil. Purification by t.l.c. (R_F 0.27, eluant ethyl acetate) gave the purified rethrins-I semicarbazone acetates (6.8 mg) as an off-white solid, m.p. 103—107 °C, $\nu_{max.}$ 1 720 and 1 690 cm^{-1}; $\lambda_{max.}$ (EtOH) 263 nm (ε 27 400). The n.m.r. spectrum (250 MHz, $CDCl_3$) of the mixture of semicarbazone acetates showed resonances characteristic of pyrethrolone, jasmolone, and cinerolone semicarbazone acetates.

The aqueous basic fraction (above) was concentrated, acidified with 0.25M-hydrochloric acid, and extracted with ethyl acetate. Washing, drying, and evaporation of the extract gave (+)-*trans*-chrysanthemic acid (10 mg, 0.06 mmol) which was dissolved in dry tetrahydrofuran (THF) (0.5 ml) containing triethylamine (14 mg, 0.14 mmol). The solution was treated with thionyl chloride (8 mg, 0.07 mmol), cooled to 0 °C, and stirred for 30 min under nitrogen. Redistilled aniline (7 mg, 0.07 mmol) in dry THF was added and the mixture was stirred for 3 h. Brine (5 ml) and THF (2 ml) were then added and the organic layer was separated, washed, dried ($MgSO_4$), and evaporated to give an oil which solidified with time at room temperature. This material was purified by preparative t.l.c. [R_F 0.51, ethanol–light petroleum, (1:1) as eluant] to give (+)-*trans*-chrysanthem anilide (2.4 mg). The anilide had m.p. 111—112 °C, and was characterised by its 250 MHz n.m.r. spectrum; δ($CDCl_3$) 1.18 (3 H, s, 2-Me), 1.26 (1 H, d, 1-H), 1.31 (3 H, s, 2′-Me), 1.73 (6 H, s, 5- and 6-Me), 2.21 (1 H, dd, 3-H), 4.93 (1 H, d, 4-H), 7.09 (1 H, br, NH), and 7.27—7.56 (5 H, m, NPh).

Administration of Sodium [2-^{14}C]Acetate to Pyrethrum Achene Homogenate.—Sodium [2-^{14}C]acetate (25 μCi) was administered in phosphate buffer to the achene preparation according to the procedure above. After extraction and chromatography, impure pyrethrins-I (88 mg) were isolated. Formation of the semicarbazones and purification (h.p.l.c.) gave purified pyrethrins-I semicarbazone (32 mg). Methanolysis, derivatisation, and purification gave the rethrolone semicarbazone acetate (6.8 mg) (see Table 1).

Administration of [10-^{14}C]Oleic Acid to Pyrethrum Achene Homogenate.—[10-^{14}C]Oleic acid (25 μCi) was dissolved in 0.2M-sodium hydroxide and added to the buffered achene homogenate according to the general procedure. Work-up gave impure pyrethrins (114 mg). Formation of the semicarbazone and purification (h.p.l.c.) gave pyrethrins-I semicarbazone (38 mg). Methanolysis, derivatisation, and purification gave the rethrolone semicarbazide acetates (5.7 mg) (see Table 1).

In the second experiment [10-^{14}C]oleic acid (25 μCi) was similarly administered to the freshly prepared homogenate containing 10 mg each of coenzyme A, ATP, and NADPH. Following the general procedure gave impure pyrethrins-I (54 mg), converted into the pyrethrins-I semicarbazone (37 mg after h.p.l.c.). Methanolysis, derivatisation, and purification gave the rethrolone semicarbazide acetates (5.2 mg): see Table 1.

Ozonolysis of [^{14}C]-Labelled Rethrolone Semicarbazone Acetates Derived from Administration of [10-^{14}C]Oleic Acid.—Conditions for the ozonolysis were established using synthetic (±)-allethrolone semicarbazide acetate, m.p. 180—181 °C. Rethrolone semicarbazone acetates from the first labelled oleic acid experiment (5.7 mg, 0.23 μCi/mmol^{-1}) in dry methanol (1 ml) at −60 °C were subjected to ozonolysis under previously defined conditions (45 s). Dimethyl sulphide (0.1 mmol) was added (flask not purged with nitrogen to avoid loss of volatiles) and after stirring at −10 °C for 1 h, 0 °C for 1 h and 20 °C for 1 h, the volatile products were steam distilled into fresh 2,4-dinitrophenylhydrazine reagent (10%; 1 ml). After 24 h, the 2,4-dinitrophenylhydrazones of the volatile aldehydes were filtered off (7.2 mg) and counted (sodium borohydride was added to discharge the colour and reduce quenching) (Table 3). The residues from the steam distillation flask were also extracted (3.1 mg) and counted.

Following the same procedure, the rethrolone semicarbazone acetates from the second [10-^{14}C]oleic acid feeding (5.2 mg, 0.15 μCi/mmol) gave 2,4-dinitrophenylhydrazones of the volatile aldehydes (6.4 mg).

Administration of [2-^{14}C]Linoleic Acid to Pyrethrum Achene Homogenate.—Initially a control feeding experiment was done using sodium [2-^{14}C]acetate (25 μCi) as described above. Chromatography gave impure pyrethrins-I (104 mg). Formation of the semicarbazones and h.p.l.c. gave purified pyrethrin-I semicarbazones (29.6 mg), converted into the rethrolone acetate semicarbazones (4.7 mg). See Table 4 for details.

In the first experiment [2-^{14}C]linoleic acid (34.3 μCi) was dissolved in 0.2M-sodium hydroxide solution and added to freshly prepared achene homogenate buffer solutions. Impure pyrethrins-I (0.098 g) were isolated, converted into the semicarbazones (39.1 mg after purification by h.p.l.c.), methanolysed, and converted into the rethrolone semicarbazide acetates (6.3 mg). For radiochemical results see Table 4.

For the second experiment the homogenate preparation was modified by adding Polyclar AT [8.0 g; commercial polyvinylpyrrolidone] and dithiothreitol (50 mg) to complex phenols and reduce quinones, respectively. Also added was ATP (10 mg), divalent metal ions ($MgCl_2$, $MnCl_2$, 10 mg of each) and NAD^+ and $NADP^+$ (5 mg of each). A solution of [2-^{14}C]linoleic

acid (39.3 μCi) in phosphate buffer was administered to the modified homogenate according to the general procedure. Impure pyrethrins-I (61 mg) were isolated, converted into their semicarbazones, and purified by h.p.l.c. (22.4 mg). Methanolysis, conversion into the acetate, and purification gave the rethrolone semicarbazide acetates (3.9 mg), see Table 4.

Kuhn-Roth Degradation of [^{14}C]-*Labelled Rethrolone Semicarbazone Acetates Derived from Administration of* [2-^{14}C]*Linoleic Acid.*—The standard microanalytical C-methyl determination was employed. Oxidative degradation of the rethrolone semicarbazone acetates from the first [2-^{14}C]linoleic acid feeding (above) (6.3 mg, 0.18 μCi/mmol^{-1}) gave a residue with total activity of 2.38×10^{-3} μCi (5 283 d.p.m.: counted by taking aliquots). The volatile acetic acid was titrated [13.53% C(Me); Calc. for the rethrolone semicarbazone acetate mixture, 11.5%] and the sodium salt was then evaporated to dryness and counted. The total activity was 1.05×10^{-3} μCi (2 331 d.p.m.).

The rethrolone semicarbazone acetate from the second administration experiment (3.9 mg, 0.30 μCi/mmol^{-1}) gave a residue with a total activity of 1.70×10^{-3} μCi (3 774 d.p.m.). The volatile acetic acid was titrated [12.63% C(Me); Calc. 11.52%] and the sodium salt was then evaporated to dryness and counted. The total activity was 1.55×10^{-3} μCi (3 441 d.p.m.).

(9Z,12Z)-[2-^{14}C]*Octadeca-9,12-dienoic Acid.*—16-Chlorohexadeca-6,9-diyne (1 g) in dry light petroleum, b.p. 40—60 °C (35 ml) was hydrogenated over Lindlar catalyst (0.5 g). After *ca.* 4 h, 171 ml of hydrogen had been absorbed (calc., 179 ml). The catalyst was filtered off and the solvent evaporated to leave a yellow oil (0.91 g) which was purified by bulb-to-bulb distillation. The chloride (1.8 g) in dry acetone (20 ml) was converted into the iodide by refluxing with sodium iodide (2.0 g) for 40 h. The iodide (1.45 g, 82%) darkened on exposure to light; δ (90 MHz, $CDCl_3$) 0.90 (3 H, t, Me), 1.09—1.92 (14 H, m, br, 7 × CH_2), 2.10 (4 H, m, CH_2CH=CH), 2.85 (2 H, m, CH=CHCH_2CH=CH), 3.21 (2 H, t, CH_2I), and 5.44 (4 H, m, CH=CH). A resonance at 3.54 (m, CH_2Cl) indicated the presence of *ca.* 5% of unchanged chloride. The (7Z,10Z)-1-iodohexadeca-7,10-diene was used for the next stage as quickly as possible.

Diethyl [2-^{14}C]malonate (250 μCi) was diluted to 46 mg with cold ester, dissolved in dry THF, cooled to 0 °C, and n-butyllithium in hexane (1.48M, 0.22 ml; 0.32 mmol) was added dropwise. The mixture was stirred at 20 °C for 30 min, and then (7Z,10Z)-1-iodohexadeca-7,10-diene (146 mg, 0.42 mmol) in dry THF (1 ml) was added and the mixture was refluxed under nitrogen (3 h). After cooling, potassium hydroxide (0.5 g) in ethanol–water (4:1; 1 ml) was added and the solution was stirred for 1 h. The THF was evaporated off and brine (5 ml) was added. Extraction with ether removed neutral material and after acidification to pH 2.0, further extraction with ether gave (8Z,11Z)-heptadeca-8,11-diene-1,1-dicarboxylic acid which solidified with time. This was decarboxylated by heating at 140 °/0.1 mmHg for 30 min. Distillation at 168—173 °C/0.1 mmHg gave a light yellow oil which was purified first by preparative t.l.c. [R_F 0.19, ether–hexane (2:4) as eluant] and then by C_{18}-reversed phase h.p.l.c. [methanol–water, (9.5:0.5) as eluant] to give (9Z,12Z)-[2-^{14}C]octadeca-9,12-dienoic acid (40.4 mg, 34%) as a colourless oil. The acid had a specific activity of 545 μCi/ mmol^{-1}, 4.32×10^6 d.p.m. mg^{-1} (total activity 78.6 μCi); δ_H (250 MHz, $CDCl_3$): 0.90 (3 H, t, Me), 1.20—1.48 (14 H, br m, 7 × CH_2), 1.67 (2 H, m, 3-CH_2), 1.94—2.19 (4 H, m, 8-CH_2 and 14 CH_2), 2.35 (2 H, t, 2-CH_2), 2.79 (2 H, dd, 11-CH_2), 5.40 (4 H, m, 9-H, 10-H, 12-H, 13-H), and 10.86—11.14 (1 H, br CO_2H); δ_C (250 MHz, $CDCl_3$): 14.04 (C_{18}), 22.58 (C_{17}), 24.66 (C_3), 25.65 (C_{11}), 27.21 (C_8, C_{14}), 29.08 (C_4, C_5, and C_6), 29.34 (C_{15}), 29.70 (C_7), 31.53 (C_{16}), 34.28 (C_2), 128.02 (C_{12}), 128.17 (C_{10}), 130.02 (C_9), 130.28 (C_{13}), and 179.79 (C_1).

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