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BIOSYNTHESIS OF (2*E*,4*E*,6*E*)-5-ACETOXYMETHYLTETRADECA-2,4,6-TRIENOIC ACID IN *EREMOPHILA OPPOSITIFOLIA*

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Key Word Index—*Eremophila oppositifolia*; Myoporaceae; 5-acetoxymethyltetradecatrienoic acid; fatty acid biosynthesis.

Abstract—Evidence is presented indicating that the triple conjugated branched chain fatty acid from *Eremophila oppositifolia* R.Br. arises by the acetate–malonate pathway with the side chain carbon atom originating from the *S*-methyl group of methionine.

INTRODUCTION

The occurrence of (2*E*,4*E*,6*E*)-5-acetoxymethyltetradeca-2,4,6-trienoic acid (**1**) in *E. oppositifolia* R.Br. [1] is yet another example of a compound isolated from a member of the Myoporaceae that is of interest from both the chemical and biogenetic points of view. Other notable examples include the tricyclic diterpene eremolactone [2] from *E. freelingii*, the eremophilane group of sesquiterpenes from *E. mitchelli* [3] and more recently the tricyclic sesquiterpenes from *E. georgii* [4] which appear to be close relatives of the zizaene based compounds [5]. In each of these cases there is no simple fit to the isoprene rule and they must therefore arise by rearrangement of some suitable intermediate.

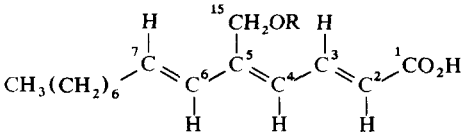
With respect to the *E. oppositifolia* acetoxy acid, the occurrence of this compound as a major component of the leaf extract is in itself interesting since other conjugated ethylenic hydroxy acids such as α-kaurolenic (**2**) and dimorphecolic acid (**3**) [6] occur as components of seed oils. Moreover, the all *trans* nature and terminal positioning of the conjugated unsaturation units is without precedent†. Finally, the presence and positioning of the carbon atom of the side chain, which gives a basic skeleton containing an odd number of carbon atoms is unusual, but also serves to suggest two possible biogenetic approaches to the molecules.

The first of these involves the *de-novo* biosynthesis of long chain, even numbered fatty acids by the acetate–malonate pathway. Introduction of the C-1 side chain could occur either by incorporation of a propionate unit (as methyl malonyl CoA) during chain extension or by methylation involving *S*-adenosylmethionine [7, 8]. In the former case, however, the methyl group would be expected to be at C-4 or C-6.

The second approach, involves the formation of an aromatic intermediate (e.g. **4**) followed by oxidative cleavage and further transformations to yield the desired acid.

The two pathways can be distinguished by determining the origin of the methylene attached to C-5. For an aromatic precursor, incorporation of NaOAc [2-^{14}C] would label this methylene carbon with ^{14}C, while for the alternative process incorporation of L-methionine [*methyl*-^{3}H] would result in tritium labelling of the methylene group.

The objectives of the present study were firstly to determine the best method for feeding labelled precursors for incorporation by the plant and then to define by which of these basic pathways the acetoxy acid arises.



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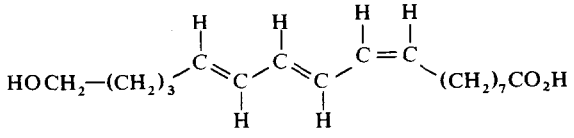
10 R = H



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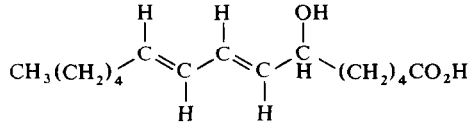

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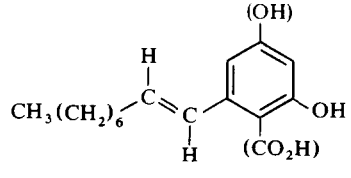

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†The isolation and identification of (2*E*,4*E*,6*Z*)-deca-2,4,6-trienoic acid from *Euphorbia pulcherrima* Willd. has been reported recently [12].

RESULTS AND DISCUSSION

In terms of the known chemistry of the acetoxy acid (**1**) [1], a combination of several reactions could be utilized to differentiate between the two biogenetic schemes. In principle, it was required to convert a sample of **1**, of known sp. act., to a crystalline derivative where the C-5 side chain carbon atom was retained, but the hydrogens were lost. The most readily accessible derivative proved to be 2-nonyladipic acid, and the reaction sequence used is outlined in Fig. 1. This figure also shows the transformations carried out to ascertain the distribution of the carbon label.

It is interesting to note that whereas (**1**) is stable in its normal environment within the intact leaf, attempts to purify it by multiple recrystallization only serve to make it susceptible to rapid air oxidation [1]. Accordingly, it was converted to a sufficiently stable derivative, the maleic anhydride adduct (**5**) and purified to constant sp. act. in that form. The adduct in turn, could be transesterified and the percentage of label in the labile acetoxy group determined. Hydrogenation of **1** followed by hydrolysis gave the crystalline, stable hydroxy acid (**6**) which in sequence was oxidized to 2-nonyladipic acid (**7**) with Jones' reagent. The acetoxy acid (**1**) could also be oxidized with ozone and the resulting *n*-octanoic acid (**8**) fragment isolated, purified as the *p*-bromophenylacyl ester (**9**) and the percentage of label in that portion of the original molecule ascertained.

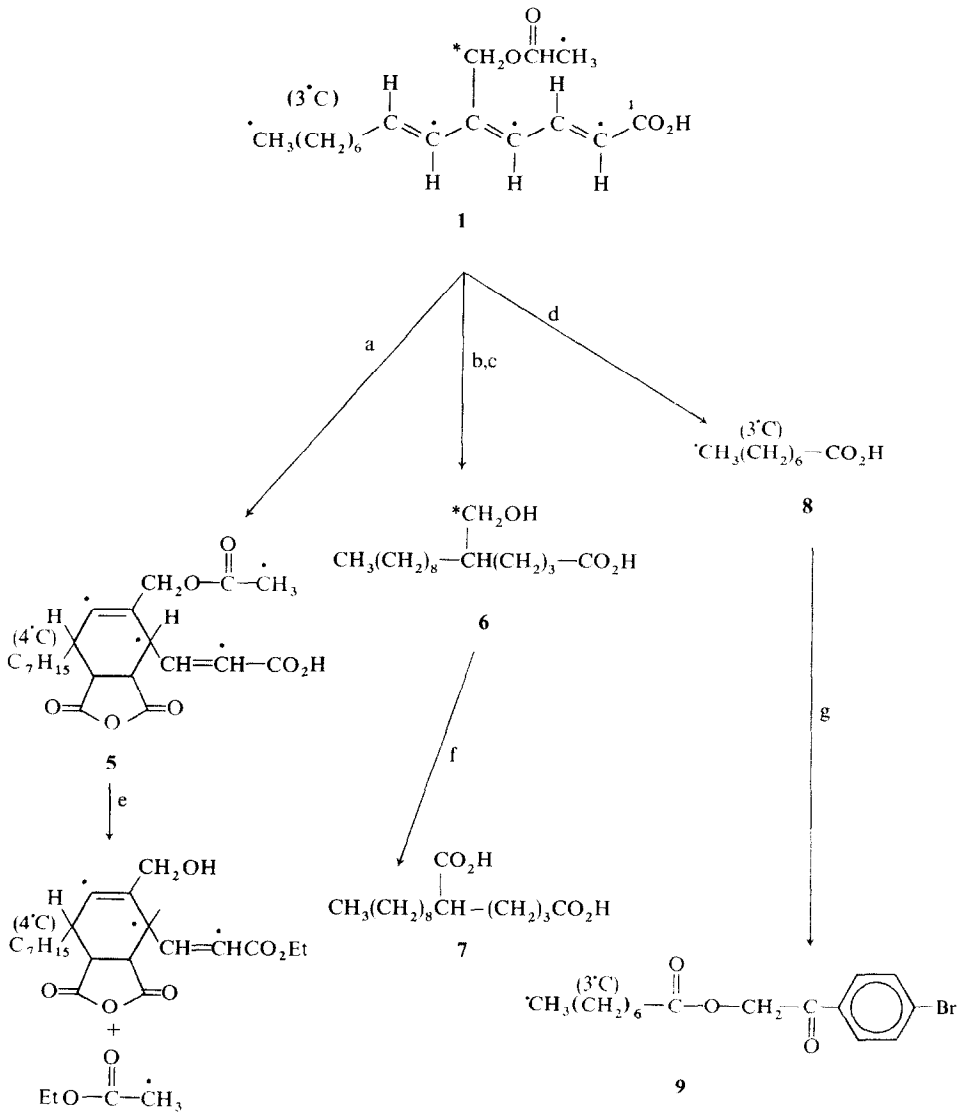

Fig. 1. Transformations carried out to determine the distribution of label in the acetoxy acid (**1**): a, maleic anhydride/Δ; b, H_2Pd/C; c, $^{\ominus}OH$; d, O_3; e, $EtOH/H^+/\Delta$; f, Jones' reagent; g, *p*-bromophenacyl bromide.

'C Indicates positions labelled by NaOAc[2-^{14}C].
*C Indicates positions labelled by L-methionine[methyl-^{3}H].

Table 1. Incorporation of NaOAc[2-^{14}C] into the acetoxy acid (**1**)

Feeding technique	Incubation time	Total radio activity fed	Sp. act. of 1† dpm/mol	dpm/mg	% Incorporation	Distribution of label in **1** C_8 unit	C-15 Acetate
Twigs (severed from plant)	48 hr	0.008 mCi/twig	1.4×10^8	4.7×10^2	2.7×10^{-2}		
	84 hr		1.9×10^8	6.4×10^2	3.6×10^{-2}		
Leaf tips (Intact plant)	0 days; following final feeding	0.012 mCi/branch	1.8×10^8	6.2×10^2	4.6×10^{-2}		
	1		2.0×10^8	6.8×10^2	5.2×10^{-2}		
	2		2.2×10^8	7.5×10^2	5.4×10^{-2}		
	4		3.3×10^8	1.1×10^3	8.3×10^{-2}		
Wick feeding	1 week (young plant)	0.10 mCi	3.3×10^9	1.1×10^4	2.7×10^{-1}	29%	25%
	*3 week (young plant)	0.10 mCi	1.1×10^{10}	3.9×10^4	6.2×10^{-1}	19%	12%
	5 week (estab. plant)	0.180mCi	5.9×10^8	2.0×10^3	7.1×10^{-2}	77%	4%

*Representative experimental parameters: total counts fed = 2.2×10^8 dpm; total recovered via ether extraction 1.4×10^7 dpm; 53% neutrals, 43% carbonate soluble; fr. wt of plant harvested = 2.4 g; wt of crude acid fraction isolated = 38 mg; wt of cold **1** added prior to reaction sequence (s) = 52 mg.

†Although the acetoxy acid (**1**) was recrystallized to constant sp. act. as the adduct (**4**) these results are all expressed in terms of the acetoxy acid and are corrected for any dilution factors.

Before attempting a definitive double labelling experiment however, different techniques for achieving labelled acetate uptake by the leaves were examined to determine the best method for obtaining the highest percentage incorporation.

Single labelling experiments

Incubation experiments with leaf discs or small twigs excised from the plant were used initially to show that labelled acetate was incorporated (Table 1). Although both methods were experimentally convenient, the incubation periods were severely restricted and major physical damage was caused to the leaves and stem respectively. Attention was then directed to methods utilizing the intact plant. When the tips were clipped from leaves of selected branches and the aqueous acetate solution applied to these points, slightly better incorporations were achieved (Table 1) than the previous method. However, the limiting factor appeared to be the slow rate of absorption of the solution by the leaf so that much of the applied radioactivity was deposited on the outside surface as the solvent evaporated. A fourth technique, employing the 'wick method'. [9] proved to be the best method since further increases in percentage incorporation were obtained, no damage was caused to the leaves and it provided total flexibility with respect to incubation times (Table 1).

Correlation of the three incorporation results with respect to incubation times for the wick feedings (Table 1) is difficult since the two young plants were undergoing rapid growth when compared with the established one, but they do suggest an initial rapid production and/or turnover of **1** during that part of the plant's life cycle. This thesis finds support from the results obtained when these same plants were re-used at a later date. The rapid growth had ceased and the incorporations found (Table 2) were similar to those for the established plant described above.

Table 1 also contains the results for the distribution of the acetate label found in the acetoxy acid from the wick feeding experiments. Although physiological variations between the plants used cannot be estimated, two points deserve further comment. Firstly, the decrease in percentage counts in the acetate group of **1** as incorporation time increases suggest the group to be labile within the plant, probably by way of transesterification processes. This is in accord with the hydroxytrienoic acid (**10**) being the other major crystalline

Table 2. Double labelling experiments with NaOAc[2-^{14}C] and L-methionine[*methyl*-^{3}H] into the acetoxy acid (**1**)

Experiment	Radioactivity fed (mCi)*	Sp. Act. of **1** dpm/mol	dpm/mg	Fr. wt of plant Harvested (wt of **1** isolated)	% Incorporation	Hydroxyacid (**6**) dpm/mol	Diacid (**7**)† dpm/mol
1	^{14}C 0.10	3.2×10^8	1.1×10^3	4.0 g (29 mg)	1.5×10^{-2}		
	^{3}H 0.0125	6.5×10^6	2.2×10^1		2.3×10^{-3}		
2	^{14}C 0.05	4.1×10^8	1.4×10^3	3.5 g (28 mg)	3.6×10^{-2}		
	^{3}H 0.05	5.6×10^7	1.9×10^2		4.8×10^{-3}		
3	^{14}C 0.0125	3.2×10^7	1.1×10^2	3.5 g (29 mg)	1.2×10^{-2}		
	^{3}H 0.10	4.1×10^8	1.4×10^3		1.7×10^{-2}		
4	^{14}C 0.10	6.7×10^8	2.3×10^3‡†	6.9 g (93 mg)§	9.7×10^{-2}	5.0×10^8	5.1×10^8
	^{3}H 0.40	9.7×10^8	3.3×10^3		3.4×10^{-2}	10.0×10^8	9.4×10^7

* Incubation period 3 weeks.

†25% of ^{14}C counts present in C-15 acetate (from transesterification procedure).

‡90.6% of ^{3}H counts lost upon oxidation.

§87 mg of cold **1** added prior to reaction sequences; 17.3 mg and 16.6 mg of **6** and **7** were recovered after final recrystallisation.

component isolatable from large scale plant extractions [10]. Secondly, the enhanced importance of the C_8 unit in the 5 week feeding trial (Table 1) with respect to the other results suggests the presence of a C_8 metabolic pool. Although attempts to isolate labelled *n*-octanoic acid directly from the plant by ether extraction after diluting with cold material, were unsuccessful it was later shown to be present by GC–MS analysis as a steam volatile component of a fraction obtained by saponification of the neutral extract.

Double labelling experiments

Initially, three double labelling experiments were carried out using different NaOAc[2-^{14}C]:L-methionine-[*methyl*-^{3}H] ratios. Both labels were incorporated in each case (Table 2) and these experiments served to suggest an acetate:methionine feeding ratio of 1:4 if similar sp. act. were to be obtained for ^{3}H and ^{14}C. The experiment was therefore repeated using this ratio (Expt 4), and the resulting labelled acetoxy acid purified and hydrogenated and oxidized as shown in Fig. 1. The results (Table 2) demonstrated clearly that the C-5 side chain carbon atom did originate from methionine. The 9.4% of tritium counts remaining in the diacid (**7**) are readily accounted for by isotopic scrambling during the hydrogenation reaction [11].

Thus, while this fatty acid is biosynthesized via the acetate–malonate pathway, with the side chain being introduced as a C-1 unit from methionine, there remains the question as to what stage it is incorporated and oxidized. Moreover, the sequence and means by which the unsaturation units are introduced are still biosynthetically interesting problems awaiting solution.

EXPERIMENTAL

Radioactivity measurements were made using a liquid scintillation counter with 2,5-diphenyloxazole (0.7%) 2,2-*p*-phenylene-*bis*-(5-phenyloxazole) (0.05%) and naphthalene (5%) in freshly distilled dioxan as the scintillation fluid. Quench corrections were made by the channel ratios method. A radiochromatogram scanner was used for scanning of TLC plates (Si gel 0.25 mm thick). NaOAc[2-^{14}C] (55.1 mCi/mM) and L-methionine [*methyl*-^{3}H] (11 Ci/mM) were obtained from the Radiochemical Centre, Amersham, England. MS were recorded at 70 eV and abundances are quoted as a percentage of the base peak. PMR spectra were measured at 60 or 90 MHz and chemical shifts are quoted on the δ scale with reference to TMS as internal standard.

Extraction. A sample of (2*E*,4*E*,6*E*)-5-acetoxymethyltetradeca-2,4,6-trienoic acid (**1**) for the preparation of compounds required for radioactivity dilution procedures was isolated from *E. oppositifolia* as described in ref. [1]. Since facilities for obtaining both PMR and MS data were not generally available at the time the structural elucidation work was reported [1], for the sake of completeness and reference, these are now recorded for the present samples.

(i) (2E,4E,6E)-5-*Acetoxymethyltetradeca-2,4,6-trienoic acid* (**1**). PMR: (CCl_4, 90 MHz) δ 11.93 (—CO_2H, *s*); 7.75 (3-H, *dd*, J_{3-4} = 12 Hz; J_{3-2} = 15 Hz); 6.22 (4-H, *d*, J_{4-3} = 12 Hz); 6.08 and 5.92 (6 and 7-H, *br m*); 5.87 (2-H, *d*, J_{2-3} = 15 Hz); 4.9 (15-CH_2, *br s*, *W*/2 = 5 Hz); 2.13 (8-CH_2, *br m*, *W*/2 = 20 Hz); 2.02 (acetate); 1.30 (9 → 13 CH_2, *br s*, *W*/2 = 6.5 Hz); 0.88 (14-CH_3; *br t*, J_{14-13} ~ 5.5 Hz). MS (50°) *m/e* (%), 294 (M^+, 5), 234 (M^+-HOAc, 40), 105 (100).

(ii) *Maleic anhydride adduct* (**5**). PMR: ($CDCl_3$, 60 MHz) δ 7.68 (3-H, *dd*, J_{3-2} = 16 Hz, J_{3-4} = 9.5 Hz); 6.05 (2-H, *d*, J_{2-3} = 16 Hz); 5.92 (6-H, *m*, *W*/2 = 5 Hz); 4.52 (15-CH_2, *m* *W*/2 = 7 Hz), 2.06 (acetate); 1.33 (9 → 13-CH_2, *br s*, *W*/2 = 8 Hz), 0.89 (14-CH_3, *br t* J_{14-13} = 5 Hz). MS: (100°) *m/e* (%), 392 (M^+, 2), 374 (15), 350 (10), 332 (M^+-HOAc, 100).

(iii) 5-*Hydroxymethyltetradecanoic acid* (**6**). PMR: ($CDCl_3$, 60 MHz) δ 7.12 (—OH, *br s*, *W*/2 = 4 Hz); 3.56 (CH_2OH, *m*, *W*/2 = 7 Hz); 2.35 (2-CH_2, *br t*, J_{2-3} = 7 Hz); 1.25 ([—CH_2—]$_n$, *br s*, *W*/2 = 6 Hz); 0.89 (14-CH_3, *br t*, J_{14-13} = 5 Hz). MS: (50°) *m/e* (%) 240 (M^+-H_2O, 5), 228 (100).

(iv) 2-*Nonyladipic acid* (**7**). PMR: ($CDCl_3$, 60 MHz) δ 10.48 (2 × CO_2H, *br s*, *W*/2 = 12 Hz); 2.38 (2-H, *m*, *W*/2 = 10 Hz), 1.25 ([—CH_2]$_n$, *br s*, *W*/2 = 5 Hz); 0.88 (—CH_3, *br t*, *J* = 5 Hz). MS: (70°) *m/e* (%), 273 (M^+ + 1, 1); 128 (100).

Plant sources. Young plants of *E. oppositifolia* for tracer studies, 4 mm high were collected on two separate occasions 19 km east of Southern Cross, Western Australia. These were subsequently potted and maintained under greenhouse conditions at the University of W.A., Nedlands. At the times of the radiolabelling feeding expts one set of plants had been kept for 18 months and were well established while the other was used *ca* 2.5 months following collection, during a period of rapid growth. Samples of plant material for extraction purposes were obtained from the same location.

Feeding techniques. (i) *Leaf discs.* Leaves (≃ 0.1 g) from the terminal branches of the growing plant were sliced into thin discs and incubated under sunlight for 4 hr at room temp. in aq. NaOAc[2-^{14}C] (0.125 ml, 0.0025 mCi) soln. The discs were then washed with H_2O, crushed and extracted with Et_2O. The Et_2O extract was washed with 4% aq. Na_2CO_3 soln and the carbonate solubles recovered by acidification to pH 4 (HCl) and extraction with Et_2O. The crude carbonate fraction was chromatographed on a Si gel TLC plate in di-*iso*propyl ether–Me_2CO–HOAc (93:6:1) and the plate scanned for radioactivity. The results indicated incorporation of the labelled acetate into the acetoxy acid (**1**) (ii) *Twigs.* Small twigs (~ 1 g) were cut *ca* 8 cm back from the growing tips and stood in aq. NaOAc[2-^{14}C] soln (0.4 ml, 0.008 mCi). After uptake of this soln an equivalent amount of H_2O was added. When this had been absorbed the twigs were extracted and the acetoxy acid (**1**) was separated by prep.-TLC and purified by recrystallization to constant sp. act. through its maleic anhydride adduct (**5**). Incorporation times of 48 and 84 hr gave adducts with sp. act. of 1.37×10^8 and 1.90×10^8 dpm/mol, respectively. (iii) *Leaf tips.* Leaf tips of selected branches of a plant were clipped and aq. NaOAc-[2-^{14}C] applied dropwise at these points over a period of 5 days (0.012 mCi/branch). The branches were harvested at periods of 1,2 and 4 days after the final feedings and the acetoxy acid adducts (**5**) found to have sp. act. of 2.0×10^8, 2.23×10^8 and 3.36×10^8 dpm/mol, respectively. (v) *Wick feeding.* A small diameter hole (0.6 mm) was bored in a branch and threaded with cotton sewing thread. One end of the thread was cut level with the branch and the other placed in a vessel containing an aq. soln of the compound(s) to be fed. The average infusion time for 1 ml of soln was 12 hr. After uptake of the radiolabelled compound(s) 3 × 1 ml portions of H_2O were fed to wash the vessel and wick. After the desired incubation period, the branch was harvested by cutting 5 cm below the wick. Branches harvested from other parts of the plant, and checked for radioactivity, indicated that back flow of the fed compounds did not occur to any significant extent. The acetoxy acid was isolated from the 'fed' branch as before and divided into portions for the subsequent transformation reactions. Data from these expts are presented in Tables 1 and 2 for the single and double labelling situations respectively.

Chemical transformations (i) *Maleic anhydride adduct* (**5**). The acetoxy acid (**1**) and maleic anhydride (1:1 molar ratio) in C_6H_6 (1 ml/100 mg of **1**, average reaction scale of 30 mg) were heated at 120° for 7 hr under N_2 in a sealed ampoule. The adduct **5** was isolated by prep.-TLC and recrystallized from C_6H_6–petrol (ii) *Transesterification of* (**5**). An EtOH soln, maintained at 50 ml, of the adduct (known wt and sp. act.) and *p*-TsOH (trace) was distilled in 5 ml aliquots until the residual act. of the soln was constant. The percentage act. of the adduct arising from the side chain acetoxy group was then calculated from both the distillate and residue. (iii) *Ozonolysis of* (**1**). The acid (**1**, *ca* 30 mg)

was dissolved in $CHCl_3$ (3 ml), cooled to $-15°$ and treated with excess O_3. The $CHCl_3$ was evapd and the residue dissolved in Me_2CO (5 ml) and treated with Jones' reagent for 30 min. Dilution with H_2O and Et_2O extraction yielded *n*-octanoic acid (**8**) which was purified by prep.-TLC. The *n*-octanoic acid was derivatized as the *p*-bromophenylacyl ester (**9**) and recrystallized to constant sp. act. (iv) *Hydrogenation and hydrolysis of* (**1**). 5-Hydroxymethyltetradecanoic acid was prepared as described in ref. [1] and recrystallized to constant sp. act. from petrol. (v) *Oxidation of 5-hydroxymethyltetradecanoic acid* (**6**). 2-Nonyladipic acid was prepared as previously described [1] and recrystallized to constant sp. act. from petrol.

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