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NAPHTHALIC ANHYDRIDE PREVENTS INHIBITION OF FATTY ACID ELONGATION BY THIOCARBAMATES

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; fatty acid elongation; thiocarbamate herbicides; pebulate; safener; naphthalic anhydride.

Abstract—The thiocarbamate herbicide, pebulate, and its more potent herbicidal form, pebulate sulphoxide, selectively inhibit the formation of very long-chain fatty acids (VLCFAs) in young barley leaves without significantly affecting *de novo* synthesis of palmitate and stearate. The aim of this study was to investigate the action of two safening chemicals on the inhibition of VLCFA synthesis by these thiocarbamate compounds. We examined the action of the monooxygenase inhibitor, 1-aminobenzotriazole, a substance which could potentially reduce the oxidation of pebulate (*S*-propylbutylethyl thiocarbamate) to its sulphoxide form. Treatment of young barley leaves with this solution (up to 50 mg l^{-1}), however, was unable to block the specific inhibitory effects of pebulate or its sulphoxide derivative on VLCFA synthesis. In contrast, the safener naphthalic anhydride, when applied as a seed dressing (0.5% w/w), was able to counteract the inhibitory effects of pebulate and pebulate sulphoxide on VLCFA biosynthesis. © 1998 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

The protective effects of safeners against herbicidal compounds were first discovered by Otto Hoffman in 1947 [1]. These chemical protectants have been found to improve the efficiency of existing herbicides by selectively eliminating crop injury without reducing the herbicidal potential, thereby permitting greater use of effective but less-selective herbicides [2]. Safeners may act in a number of different ways. They may, for example, act by competing with the herbicide for the same site of action [3, 4]. On the other hand, there is much accumulated evidence that safeners enhance herbicide metabolism through increased activities of cytochrome P_{450}, glutathione-*S*-transferases (GSTs) or glucose transferases and by raising glutathione levels [5]. For example, induction of cytochrome P_{450} mixed-function oxidases [6] and GSTs [7–9] have been often reported. In addition, safeners may enhance the activities of vacuolar transporters [10] indicating that vacuolar transport systems and, therefore, elimination of potentially toxic conjugates are an integral part of the detoxification mechanism.

Previous work from our group has demonstrated the susceptibility of very long-chain fatty acid (VLCFA) synthesis (fatty acid elongation) to the thiocarbamate, pebulate, and its more potent herbicidal form, pebulate sulphoxide [10, 11]. Sulphoxidation of thiocarbamates is thought to be important for their herbicidal activity and can be mediated by microsomal NADPH-dependent cytochrome P_{450} monoxygenases [12]. Data has also been published implicating the actions of peroxidases [13, 14] or, more recently, an unusual enzyme, the peroxygenase [15] in (sulph) oxidation. Conjugation of the sulphoxide derivative to glutathione and the subsequent formation of secondary conjugates [16–18] ultimately results in the formation of metabolites which are relatively non-toxic to the plant. Safening compounds, which could reduce the oxidation of pebulate to its sulphoxide by impairing activity of the oxidation enzymes or, alternatively, chemicals capable of enhancing the rate of removal of the more toxic sulphoxide compound, would be likely to protect the crop from thiocarbamate injury [19].

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Table 1. 1-Aminobenzotriazole (ABT) does not affect the labelling of barley leaf fatty acids from [1-^{14}C] acetate

	Fatty acid labelling (%)				
ABT concentration (mg l^{-1})	≤18	20:0	22:0	24:0	26:0
Experiment 1					
0	47.4 ± 0.7	4.5 ± 2.1	12.1 ± 0.3	9.1 ± 3.2	21.5 ± 1.7
50	43.1 ± 6.0	4.4 ± 1.6	14.6 ± 1.8	9.0 ± tr.	26.7 ± 7.8
Experiment 2					
0	82.3 ± 5.8	3.5 ± 0.5	8.3 ± 1.5	3.1 ± 0.4	2.9 ± 0.3
50	83.9 ± 3.9	2.5 ± 1.0	8.0 ± 0.9	2.8 ± 0.6	2.8 ± 1.1

Two experiments are shown with leaves from 10 or 8 day-old barley plants, respectively. Seeds were pre-treated with ABT solution for 24 h before germination in vermiculite. Results show means ± S.D. Fatty acids are abbreviated with the number before the colon showing the carbon number and the figure after giving the number of double bonds.

In a previous study of fatty acid elongation, we found that 1-aminobenzotriazole (ABT) was able to produce a protective response against the inhibitory effect of the thiocarbamate, diallate [20]. Here we report the results from experiments in which we used ABT and another compound reported to give protection against some thiocarbamates [7], naphthalic anhydride (NA). We tested antagonistic effects against pebulate and its sulphoxide metabolite (cf. [10, 11]) and used young barley leaves as the experimental system, since this tissue is capable of good rates of fatty acid elongation which is also susceptible to inhibition by thiocarbamates.

RESULTS AND DISCUSSION

ABT has been demonstrated previously to impair cytochrome P_{450} mixed-function oxidase activity by irreversibly alkylating the protohaem group [21]. The effects of this monooxygenase inhibitor on total incorporation of radioactivity from [1-^{14}C]acetate into fatty acids of young barley leaves are shown in Table 1. Increasing concentrations of ABT in the range up to 50 mg l^{-1} neither reduced total labelling nor had any significant effect on the pattern of radioactive fatty acids. This result agreed with previous data using a similar system [20] and showed that the monooxygenase inhibitor did not interfere with uptake of the precursor or the subsequent fatty acid biosynthetic reactions. Since no adverse effects were observed, we used ABT at 50 mg l^{-1} in all subsequent experiments, this concentration being thought to be sufficiently high to be antagonistic towards herbicides metabolised by the cytochrome P_{450} monooxygenase system [20].

Incubation of barley leaves with 25 μM pebulate or pebulate sulphoxide caused a selective decrease in VLCFA labelling. As noted before [11], pebulate sulphoxide was the more potent inhibitor of fatty acid elongation. In contrast, the labelling of fatty acids, such as palmitate, stearate and oleate (which were made *de novo*), was not inhibited by the thiocarbamates (Table 2). This result again emphasised the selective effect of thiocarbamates against fatty acid elongation [22, 23]. Separate elongase systems have been demonstrated in plants, which are selective for different chain length substrates (see Refs. [24] and [25]). The elongation system involved in the elongation of stearate to arachidate may be less sensitive to inhibitors by thiocarbamate compounds that the further elongation of arachidate (Table 2).

Pre-incubation of the barley leaves with a solution of ABT for 4 h failed to have any effect on

Table 2. 1-Aminobenzotriazole (ABT) cannot block the inhibitory effects of pebulate or pebulate sulphoxide on fatty acid elongation by barley leaves

		Fatty acid labelling (dpm × 10^{-3})				
ABT Treatment (mg l^{-1})	Herbicide (concentration)	≤18	20:0	22:0	24:0	26:0
0	none	68.6 ± 13.9	2.9 ± 1.0	6.9 ± 1.5	2.6 ± 0.6	2.4 ± 0.2
0	pebulate (25 μM)	88.8 ± 14.0	1.7 ± 1.0	2.2 ± 1.2†	1.2 ± 0.4†	1.4 ± 0.4†
0	pebulate sulphoxide (25 μM)	120.4 ± 5.0	1.6 ± 0.9	1.8 ± 0.7†	0.2 ± 0.2†	tr ± tr†
50	none	92.8 ± 12.7	2.8 ± 1.0	8.8 ± 3.9	3.1 ± 1.8	3.1 ± 2.1
50	pebulate (25 μM)	81.7 ± 16.9	0.8 ± 0.2	3.2 ± 1.4	0.5 ± 0.3†	0.5 ± 0.4†
50	pebulate sulphoxide (25 μM)	116.3 ± 12.2	tr ± tr†*	tr ± tr†*	tr ± tr†*	tr ± tr†*

Results show means ± S.D. (n = 3). tr = < 50 dpm. Seeds were allowed to imbibe water or a 50 mg l^{-1} solution of ABT for 24 h at 20°C before sowing in vermiculite. Barley plants were then grown for a further 9 days, leaves detached and incubated for 4 h in the presence of [1-^{14}C] acetate (with herbicides) (see Experimental).

*Significantly different from no ABT treatment by Student's t-test ($p < 0.05$).

†Significantly different from no herbicide controls ($p < 0.05$).

Table 3. Effect of naphthalic anhydride (NA) on the incorporation of radioactivity from [1-^{14}C] acetate into fatty acids of young barley leaves

	Fatty acid labelling (% total)			
NA concentration (μM)	≤18	20:0	23:0	24:0
0	36.5 ± 6.3	11.0 ± 3.2	7.5 ± 2.1	35.0 ± 1.4
0.05	37.8 ± 6.1	14.0 ± 2.3	9.3 ± 0.4	32.1 ± 3.1
0.10	35.7*	11.2*	11.7*	36.3*

Results are means ± S.D. (n = 3) except *(single analysis only). Leaves from 10-day-old plants were pre-incubated with different solutions of NA for 4 h before incubating with [1-^{14}C] acetate, as described in Experimental section.

the subsequent inhibition of fatty acid elongation by pebulate or its sulphoxide (data not shown). Therefore, we investigated whether longer-term changes in fatty-acid synthesis could be caused by ABT. Seeds were allowed to imbibe the reagent for increasing periods (up to 24 h) before subsequent growth in vermiculite (see Experimental). This had no effect on fatty-acid labelling nor did it prevent the inhibition of fatty acid elongation by pebulate and pebulate sulphoxide (Table 2). In fact, pre-treatment with ABT significantly exacerbated the inhibition by pebulate sulphoxide and may have increased that by pebulate, though the latter effect was not statistically significant. As before, there were no changes in the labelling of fatty acids ($< C_{18}$) made *de novo* (Table 2).

The failure of ABT to protect VLCFA synthesis from inhibition by pebulate sulphoxide contrasts with our previous results (under similar conditions) for barley and other monocotyledons for another thiocarbamate, diallate [20]. With the latter, we found that the monooxygenase inhibitor was capable of reversing completely the selective inhibition caused by the thiocarbamate herbicide diallate and suggested that ABT could be acting by blocking formation of the respective sulphoxide. Since the mechanism for oxidation of thiocarbamates to sulphoxides has been reported to be due to a number of different enzymes, depending on the compound tested [2, 12–15], then it is quite possible that ABT-sensitive systems are not involved in the metabolism of pebulate. ABT-sensitive systems include peroxygenase activities [26] in addition to P_{450}-mediated enzymes. Clearly, ABT would not affect the efficiency of pebulate if other enzymes were involved in its sulphoxidation. In any case, ABT would not be expected to alter the potency of pebulate sulphoxide and, indeed, it did not (Table 2).

Early studies with the safener naphthalic anhydride (NA) (see Ref. [7]), revealed that this chemical enhanced the rate of EPTC metabolism in corn seedlings and it was predicted that the safener conferred protection by activating those enzymes involved in EPTC inactivation. Lay and Casida [8] were the first to demonstrate that safener actions against the thiocarbamate EPTC in corn involved increases in the level of glutathione and raised glutathione *S*-transferase activity although NA may also increase the activity of cytochrome P_{450} monooxygenases. Increases in glutathione *S*-transferase activity, in particular, have been used to explain the action of many safeners against thiocarbamates [2, 5, 19].

Different concentrations of NA were incubated with young barley leaves to enable us to investigate whether the compound itself affected fatty acid labelling from [1-^{14}C]acetate. Concentrations of NA up to 0.1 mM were used and were found to have no effect on the amount or pattern of fatty acid labelling (Table 3). Therefore, we felt we could use similar solutions of the compound for protective activity against pebulate and, more particularly, its sulphoxide. [Moreover, in further experiments, involving longer exposures and/or treatment of seeds with dry powder rather than solutions (see later), NA *per se* never showed any significant effect on fatty acid labelling].

When young barley leaves were pre-incubated for a 4 h period in the presence of 0.1 mM NA prior to incubations with pebulate or pebulate sulphoxide, no protective effect was found on fatty acid elongation (data not shown). This was similar to the experiments with ABT outlined above. Therefore, we used a longer exposure of seeds to 0.1 mM NA solution (24 h) to see whether this treatment had any protective effect. However, under these experimental conditions, also, NA failed to prevent the specific inhibition of fatty acid elongation by pebulate (data not shown) and, especially, by pebulate sulphoxide (Table 4). Thus, if NA acts as a safener by enhancing detoxification of the sulphoxide metabolite in barley, then the experimental conditions used for the experiment shown in Table 4 were insufficient to demonstrate such an action.

When safeners are used in agriculture, they are often used as seed dressings. We had not used such treatments initially because of difficulties in quantifying the safener exposure. However, with the failure of NA solutions to safen the thiocarbamate effects, we tried seed-dusting. Using amounts of NA (see Table 5) which had been used for maize (e.g. [27]), we dusted the seeds prior to planting in moist vermiculite. The vermiculite was kept moist, taking care not to disturb the covering of NA on the surface of the seed coat because the pre-emergent phase of growth (where the coleoptile emerges through the soil layer containing the herbicide) has been reported to be the period when safening action is most critical (see Ref. [7]). When the seedings were 10 days old, leaf blades were detached and incubated with pebulate or pebulate sulphoxide. Analysis of the radiolabelled fatty acids revealed

Table 4. Naphthalic anhydride solutions are ineffective at preventing the inhibition of fatty acid elongation by pebulate sulphoxide (PS)

NA concentration (mM)	Herbicide (concentration)	Fatty acid labelling (dpm $\times 10^{-3}$)				
		≤18	20:0	22:0	24:0	26:0
0	none	105.1 ± 8.2	1.8 ± 2.1	6.3 ± 2.0	2.6 ± 1.1	2.6 ± 1.1
0	PS (25 μM)	93.4 ± 28.1	1.1 ± 0.3	1.2 ± 0.8	0.2 ± 0.3	tr $\pm$ tr
0.1	PS (25 μM)	70.6 ± 25.2	0.2 ± 0.2	0.3 ± 0.2	tr $\pm$ tr	tr $\pm$ tr

Results show means $\pm$ S.D. ($n = 3$). tr. = trace < 50 dpm. Seeds were pre-treated with naphthalic anhydride solution for 24 h and then grown for a further 9 days in vermiculite. Control seeds were pre-treated with water only. Leaves were removed from the 10-day-old plants and incubated with [1-^{14}C] acetate (and with pebulate sulphoxide) for 4 h as described in the Experimental section.

that pre-treatment of seeds with NA alone in this manner had no significant effect on the total radiolabelling of fatty acids from [1-^{14}C]acetate. It did, however, reverse the inhibition of fatty acid labelling caused by both pebulate and pebulate sulphoxide (data not shown).

The protective action of NA was then examined in more detail. NA alone had no significant effect on the labelling of either fatty acids made *de novo* or those formed by elongation. Pebulate and pebulate sulphoxide caused severe inhibition of fatty acid elongation, as expected (Table 5). No significant inhibition of the labelling of fatty acids of C_{18} or less (made *de novo*) was seen but, because of the high rate of elongation, total incorporation of radioactivity into fatty acids was inhibited significantly (Table 5). Treatment with NA was able to successfully reverse the inhibition of fatty acid elongation by both pebulate and pebulate sulphoxide, so that labelling of VLCFAs was not significantly different from controls (Table 5).

If NA safens by up-regulating glutathione *S*-transferases involved in the detoxification of thiocarbamate sulphoxide [2, 5, 19], then its protective effect against both pebulate and pebulate sulphoxide (Table 5) can be easily explained. Its activity would contrast with a compound that prevented activation of the thiocarbamate to its sulphoxide. In this latter case, one would expect the inhibitory action of pebulate sulphoxide itself to be unimpared.

Table 5. Dusting seeds with naphthalic anhydride (NA) prevents the inhibitory effects of thiocarbamates on the incorporation of radioactivity from [1-^{14}C] acetate into the very long chain fatty acids of barley leaves

NA concentration (% dry wt.)	Herbicide	Fatty acid labelling (dpm $\times 10^{-3}$)		
		total	≤C18	VLCFAs
0	none	11.4 ± 1.5	4.8 ± 0.7	6.6 ± 2.1
0	P (25 μM)	6.1 ± 1.1*	3.8 ± 0.9	2.3 ± 0.3*
0	PS (25 μM)	5.1 ± 0.5*	4.0 ± 0.5	1.1 ± 0.2*
0.5	none	12.9 ± 0.9	4.7 ± 0.6	8.2 ± 2.6
0.5	P (25 μM)	$10.2 \pm$ tr	4.6 ± 0.3	5.5 ± 0.4
0.5	PS (25 μM)	10.8 ± 1.2	4.7 ± 0.3	6.0 ± 2.0

Pebulate (P) or pebulate sulphoxide (PS) were used at 25 μM in the incubation solutions. Leaves were from 10 day-old plants. Safener was added as a dusting before germination (see Experimental). Results show means $\pm$ S.D. ($n = 3$).

*Significantly different from untreated barley by Student's *t*-test, $p < 0.05$.

We have implicated the activity of thiocarbamate herbicides against fatty acid elongation in their well-known actions on surface wax formation [22, 23]. In our barley test system, there is no doubt that treatment with pebulate or pebulate sulphoxide (results not shown) has a dramatic effect on surface wax architecture (Fig. 1). Thus, the lobed cylindrical crystals apparent on the adaxial surface of untreated barley leaves become enlarged and more crenulate in appearance. For the abaxial surface, the lobed plates became less densely distributed and more lamellar and crenulate after treatment with pebulate [Fig. 1(c) and (d)]. Such a result is fully in keeping with the precursor role of VLCFAs in wax synthesis [28]. Indeed, thiocarbamates have recently been used to manipulate surface lipid composition [23].

To demonstrate further the safening activity of NA *in vivo*, seeds were dusted with NA and then germinated in the highest levels of pebulate or its sulphoxide that could be effectively dissolved in 2.5% (v/v) ethanolic solution. Thus, 5×10^{-4} M solutions of the thiocarbamates were used and applied to the barley seedlings throughout their growing period. Even at these very high concentrations, NA was still active in partially safening (Fig. 2) although only very limited growth was seen for pebulate-treated barley (not shown). However, even in this latter case, the NA-treated seeds contrasted markedly to the very effective herbicidal activity by both pebulate or its sulphoxide (Fig. 2) without the use of the safener. Moreover, for 2.5×10^{-5} M concentrations of herbicide (as used for the experiment shown in Table 5), NA dusting completely reversed the inhibitory action on plant growth (data not shown). It will also be seen clearly in Fig. 2, that NA treatment by itself had no detectable effect on either germination or on the growth rate of the barley variety used. This was also consistent with the lack of effect of NA on fatty acid biosynthesis (Tables 3 and 5).

The data described in our paper have shown that, under specific experimental conditions, a safener known to have activity towards thiocarbamate

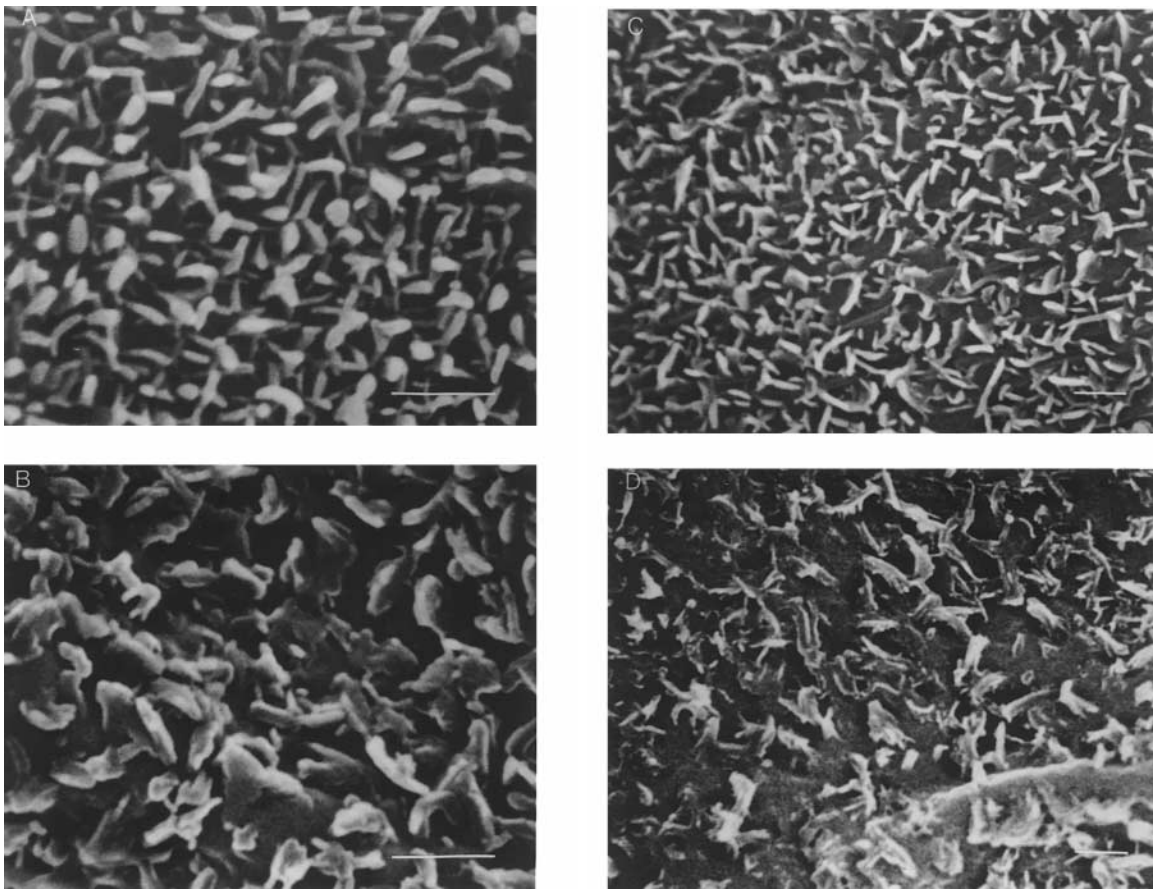

Fig. 1. Scanning electron micrographs of surface waxes on barley leaves following treatment with pebulate. Panel A shows the adaxial surface of untreated barley leaves and Panel B following treatment with 5×10^{-4} M pebulate. Panel C shows the abaxial surface of the untreated barley leaves and panel D following treatment with 5×10^{-4} M Pebulate. Barley seeds were soaked in water or herbicide solution for 24 h before growing in vermiculite for 7 days (see Experimental).

herbicides is able to protect against the actions of the same compounds towards fatty acid elongation. Such results add to previous evidence that fatty acid elongation is an important target for thiocarbamates [22, 23] and may help in the development of new agrochemicals.

EXPERIMENTAL

Plant material

Barley (*Hordeum vulgare* cv. Maris Otter) seeds were obtained from the Plant Breeding Institute, Cambridge. Seeds were grown for 8–10 days at 20°C in a 16 h day/8 h night cycle with 650 μmol s^{-1} m^{-2} illumination. When used as a dusting, the safener naphthalic anhydride (NA) (Aldrich) (0.5% w/w) was gently shaken with seeds (until they were evenly coated) prior to sowing in damp vermiculite.

Incubation and lipid extraction

Leaves were detached from seeds under water to prevent disruption of the transpiration stream. They were then incubated under conditions essentially as described previously [29]. Illumination was provided. For expts on the direct effects of safeners, a soln of the safening compound (ABT or NA) was used and the leaves pre-incubated for 4 h at 20°C before addition of [1-^{14}C]acetate (1 μCi per 5 leaves) and further incubated for 4 h at 20°C. Alternatively, in some experiments, safeners or safener solutions were used to treat seeds (see Results for details). To keep pebulate and its sulphoxide derivative in suspension, it was necessary to have EtOH present at a final conc. of 2.5% (v/v). Control experiments using this amount of EtOH showed that it did not affect fatty acid labelling (data not shown). At the end of the incubation period, leaves were rinsed briefly with H_2O to remove any unimbibed radiolabelled acetate.

Fig. 2. Effect of naphthalic anhydride (NA) against the inhibitory effects of pebulate sulphoxide on the growth of barley plants. The photograph shows (left to right): control, seeds dusted with 0.5% (w/w) NA prior to germination, seeds germinated in compost pre-treated with 5×10^{-4} M pebulate sulphoxide, seeds dusted with 0.5% (w/w) NA and then grown in compost pre-treated with 5×10^{-4} M pebulate sulphoxide.

Metabolism was terminated by the addition of 1.25 ml *iso*-PrOH and heating for 30 min at 70°C. Lipids were extracted using a modified Garbus extraction [30], where *iso*-PrOH replaced MeOH.

Lipid analysis

Fatty acid Me esters (FAMEs) were prepared from lipid extracts by transmethylation with 2.5% H_2SO_4 (v/v) in anhydrous MeOH for 2 h at 70°C [31]. Incorporation of a radiolabel into total lipid or FAME samples was determined by liquid scintillation counting. Aliquots of samples were evaporated to dryness under a stream of N_2 prior to the addition of Opti-flor scintillate (Canberra Packard). Counts were corrected for sample quenching by using the external standard channels ratio method. FAMEs were purified further using TLC on silica gel G with petrol (40–60°C)–Et_2O–HOAc (90:30:1) as solvent. These were analysed by GC connected via an efficient splitter to a gas-flow proportional counter. A glass column (4 mm i.d. × 1.5 m) packed with SP-2100 (5%, w/v) on Supelcoport (100–120 mesh) and a temperature programme (initial temperature 210°C for 10 min, then 4°C min^{-1} to 250°C) were used routinely for separations. Radiolabelled fatty acids were normally identified by comparison of their R_t values to standards but their structures were also confirmed by chemical degradation methods [32].

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