Novel inhibitors of the condensing enzymes of the Type II fatty acid synthase of pea (*Pisum sativum*)

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The type II fatty acid synthases (FASs) of higher plants (and *Escherichia coli*) contain three condensing enzymes called β-ketoacyl-ACP synthases (KAS), where ACP is acyl-carrier-protein. We have used novel derivatives of the antibiotic thiolactomycin to inhibit these enzymes. Overall *de novo* fatty acid biosynthesis was measured using [1-^{14}C]acetate substrate and chloroplast preparations from pea leaves, and [1-^{14}C]laurate was used to distinguish between the effects of the inhibitors on KAS I from those on KAS II. In addition, the activities of these enzymes, together with the short-chain condensing enzyme, KAS III, were measured directly. Six analogues were tested and two, both with extended hydrocarbon side chains, were found to be more effective inhibitors than thiolactomycin. Incubations with chloroplasts and direct assay of the individual condensing enzymes showed that all three compounds inhibited the pea FAS condensing enzymes in the order KAS II > KAS I > KAS III. These results demonstrate the general activity of thiolactomycin and its derivatives against these FAS condensation reactions, and suggest that such compounds will be useful for further detailed studies of inhibition and for use as pharmaceuticals against Type II FASs of pathogens.

Key words: thiolactomycin analogues, plant fatty acid synthesis.

INTRODUCTION

Fatty acid synthesis is catalysed by the concerted action of acetyl-CoA carboxylase and fatty acid synthase (FAS). In plants, FAS is located in the plastids where it exists as a type II multienzyme complex [1]. Several of the component proteins of this FAS exist as isoforms. Amongst these are the β-ketoacyl-ACP synthases (KASs or condensing enzymes), where ACP is acyl-carrier-protein. Three forms of KAS, designated KAS I, KAS II and KAS III, are found. The first purification of KAS I and/or KAS II from plants was achieved in the 1980s using spinach [2,3], barley [4] or parsley [5]. KAS I is known to be capable of condensing malonyl-ACP with acyl-ACP primers of between 2 and 14 carbons. Thus, it yields palmitate as its longest chain product. KAS II is responsible for producing stearate. These two condensing enzymes can also be differentiated on the basis of their sensitivity to inhibitors such as cerulenin and arsenite [1,6].

There has been some attention paid to which partial reaction of the FAS complex might be rate-limiting. For many years it was thought that acetyl-CoA: ACP acyltransferase was a good candidate [7]. However, the discovery in *Escherichia coli* that the initial condensation reaction was catalysed by a third condensing enzyme, which used acetyl-CoA directly [8], meant that the situation had to be reassessed. This short-chain condensing enzyme has been found in plants [9], is called KAS III and seems to control the speed of the initial reactions of FAS [10,11]. Its presence renders the acetyl-CoA:ACP acyltransferase protein redundant and, indeed, the latter acyltransferase was shown also to be a partial reaction of the *E. coli* short-chain condensing enzyme [8].

Early experiments with the antibiotic thiolactomycin showed that it selectively inhibited Type II, dissociable, FASs. In the *E. coli* system, acetyl-CoA:ACP acyltransferase and condensing enzymes were thiolactomycin-sensitive [12,13]. Thiolactomycin has been used to inhibit plant enzymes and the KAS IIIs from avocado [14] and spinach [9] were shown to be sensitive, although an oxidative breakdown product may have been responsible for some of the effects [10]. We showed that, in peas, all three condensing enzymes were inhibited by thiolactomycin [15]. Since KAS III is not inhibited by cerulenin [9,11,14], the classic inhibitor of *de novo* synthesis, it is of considerable interest to find alternative chemicals which are effective.

Moreover, thiolactomycin may have utility in the treatment of diseases caused by protozoan parasites of the phylum Apicomplexa, such as *Plasmodium* spp. (the causative agents of malaria) [16]. Alternative and more potent compounds could be of particular use in this regard. Therefore, we have studied the effect of thiolactomycin analogues against fatty acid synthesis in detail and, in this paper, report their effect on the individual condensation (KAS) reactions of pea FAS.

MATERIALS AND METHODS

Materials

Fatty acid standards were obtained from Nu-Chek (Elysian, MN, U.S.A.) and specialized reagents from Sigma–Aldrich. *E. coli* strain B paste was purchased from Porton Products (Salisbury, Wiltshire, U.K.). Radiolabelled fatty acids were obtained from Amersham International. Pea seeds (*P. sativum* cv. Onward), purchased from Nutting (Leicester, U.K.), were germinated and grown at 20 °C with 650 μE/s per m^2 illumination and a 12 h light/dark cycle. Leaves were harvested from plants around 14 days-old for chloroplast isolation. Thiolactomycin and its derivatives were synthesized by a procedure based on that

Abbreviations used: ACP, acyl-carrier-protein; FAS, fatty acid synthase; KAS, β-ketoacyl-ACP synthase.

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of Wang and Salvino [17]. They were a kind gift from K. Lindner of AgrEvo U.K. Ltd. (Saffron Walden, Essex, U.K.).

Isolation of ACP

ACP was isolated from *E. coli* strain B paste by the method of Majerus et al. [18]. The purity was checked by SDS/PAGE.

Preparation of [^{14}C]acyl-ACPs

Acyl-ACP synthase was purified from *E. coli* [19] and used to prepare [1-^{14}C]lauroyl-ACP and [1-^{14}C]palmitoyl-ACP [20] to be used as substrates for β-ketoacyl-ACP I and II respectively.

Chloroplast incubations

Leaves were harvested from peas 14 days after sowing. Intact chloroplasts were isolated using a Percoll density gradient [21], as modified in [22]. Once isolated, chloroplasts were kept in the dark on ice and used as soon as possible. Chloroplasts were quantified by chlorophyll measurement [23]. After a pre-incubation in the light for 10 min at 25 °C, the chloroplast preparations were incubated with [1-^{14}C]acetate, as described in [22]. When inhibitors were used, these were added to the incubation tubes in methanol and the solvent evaporated under nitrogen before the pre-incubation [15]. Reactions were stopped by the addition of KOH (6 %, final concn.) and heating at 70 °C for 30 min in Teflon-capped tubes. Fatty acids were extracted and analysed by radio-GLC [22], with quantification using RAMONA Rachel software (Lab Logic, Sheffield, U.K.).

Leaf disc incubations

Leaf discs were cut from 14-day-old pea leaves with a no. 6 cork borer and floated adaxial side uppermost in water until used. Up to 5 discs were added to 5 ml of incubation medium [24] in a 25 ml conical flask. Inhibitor solutions were dried down in the flasks (see above) before the incubation medium was added. Pea discs were added, adaxial side-uppermost and then incubated with vacuum infiltrated [1-^{14}C]laurate (3.7 kBq) in the light for 6 h at 25 °C. At the end of incubation, discs were washed with several changes of water before being heated in propan-2-ol in a Teflon-capped tube at 70 °C for 30 min. Lipids were extracted [25] by a modification of the method of Garbus et al. [26]. Fatty acid methyl esters were produced and analysed by radio-GLC [22] (see above).

Assay of KAS enzymes

KAS I and II were assayed under the conditions described for the spinach enzymes [2], except that NADH and NADPH were both added at 5 mM. A 40–80 % saturated $(NH_4)_2SO_4$ fraction of the leaf soluble proteins was used as the enzyme source. [1-^{14}C]-Lauroyl-ACP and [1-^{14}C]palmitoyl-ACP were used as substrates for KAS I and KAS II respectively. After extraction, the fatty acids were analysed by radio-GLC. Enzyme activities were calculated from the specific radioactivities of the substrates. No allowance was made for endogenous acyl-ACPs since these were shown to be negligible by the linearity of the substrate–velocity curve at low [1-^{14}C]acyl-ACP concentrations. Final concentrations of acyl-ACPs used were approx. 0.2 mg/ml, levels at which the reactions were directly dependent on enzyme protein.

KAS III was assayed using the following reagents (final concentrations shown): 10 μM [1-^{14}C]acetyl-CoA (1.85–2.22 GBq·mol^{-1}), 5 μg ACP (*E. coli*), 1 mM NADH, 2 mM NADPH, 10 mM malonyl-CoA, and 10 μg purified *E. coli* malonyl-CoA:ACP transacylase [27] in 0.1 M Tris/HCl, pH 7.5. 10–20 μg of protein was added to a final incubation volume of 50 μl. The reaction was stopped with 10 % TCA (5 % final concn.) and the pellets washed and counted [14]. KAS III activity was taken as the amount of radiolabel incorporated into the acid-precipitated protein following stimulation by the addition of malonyl-CoA and malonyl-CoA:ACP transacylase [14].

RESULTS AND DISCUSSION

The basic structure of the thiolactomycin ring was retained but the side chain modified in the analogues (Figure 1). In thiolactomycin the side chain is 4 carbons long with a methyl branch. Two double bonds prevent flexibility in the chain. For the analogues, most of the structural changes in the chain were either alterations in unsaturation or the length of the chain. Compounds 3 and 5 contained an epoxy or a phenyl group, respectively (Figure 1).

In order to evaluate their activity, thiolactomycin or its analogues were tested for general effects on the *de novo* synthesis of fatty acids by using isolated pea chloroplasts and [1-^{14}C]acetate substrate (Table 1). Thiolactomycin produced significant inhibition of total fatty acid synthesis at 100 μM or higher concentrations. This agreed with previous data for pea [15]. In

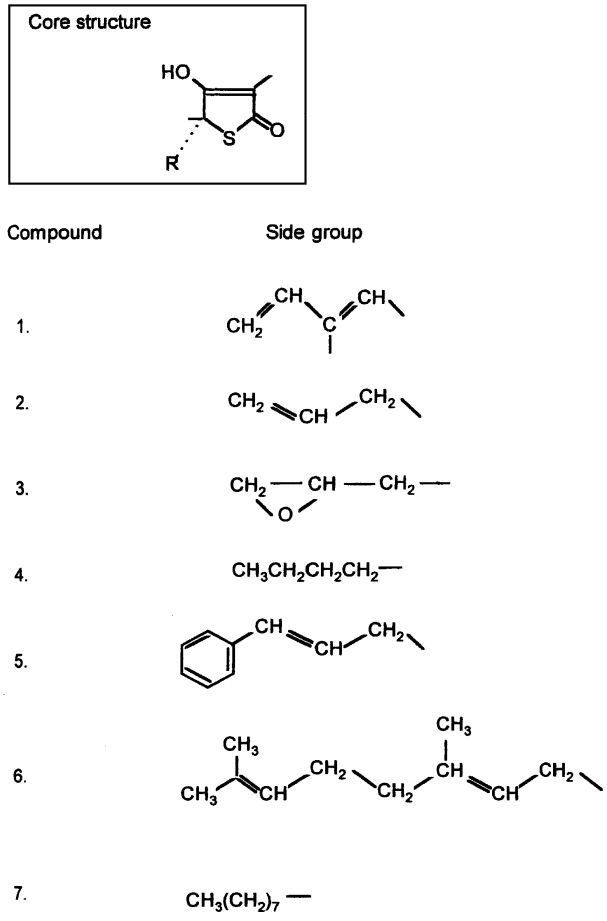

Figure 1 Structures of thiolactomycin (compound 1) and its analogues used in the studies

Table 1 Effect of thiolactomycin or its analogues on fatty acid synthesis from [1-^{14}C]acetate by isolated pea leaf chloroplasts

Results are expressed as % of controls. Means ± S.D. ($n = 3$) are shown. n.d., non detected; n.m., not measured. * Donates significant difference from control (Student's t test) $P < 0.05$. A representative experiment is shown but similar results were obtained for compounds 2–4 in two separate experiments and in at least three separate experiments for compounds 1 and 5–7. See Figure 1 for the structures of thiolactomycin (compound 1) or its analogues (compounds 2–7).

	Inhibitor concentration (μM)			
Compound used	1000	100	10	1
1	14 ± 2*	19 ± 4*	90 ± 5	97 ± 4
2	106 ± 3	101 ± 3	n.m.	n.m.
3	105 ± 6	99 ± 3	n.m.	n.m.
4	88 ± 4*	106 ± 6	97 ± 5	92 ± 5
5	39 ± 6*	95 ± 2	102 ± 3	103 ± 3
6	n.d.*	25 ± 4*	80 ± 2*	91 ± 4
7	n.d.*	6 ± 1*	50 ± 3*	95 ± 2

further experiments (results not shown) an IC_{50} for thiolactomycin against chloroplast total fatty acid synthesis was obtained of approx. 170 μM. As discussed before [15], the sensitivities of bacterial or plant FASs to thiolactomycin vary considerably, and the pea system seems to be rather insensitive compared to avocado, for example [14].

Analogues 2 and 3 had no significant effect on fatty acid labelling at any concentration, while compounds 4 and 5 produced significant inhibition only at 1 mM concentrations (with very little inhibition by analogue 4). Two analogues, compounds 6 and 7, gave better inhibition of total chloroplast fatty acid synthesis than thiolactomycin. This was especially true for analogue 7 which gave around 50 % inhibition at 10 μM (Table 1). Both analogues contained extended hydrocarbon side chains (Figure 1) and were able to completely inhibit fatty acid synthesis at 1 mM.

In previous experiments [15] we showed, indirectly, that thiolactomycin inhibited all three pea KAS enzymes. In fact, thiolactomycin appeared to be most effective against KAS II, which is responsible for chain-lengthening of palmitate to stearate and, therefore, controls the ratio of C16:C18 fatty acids synthesized by plants [1]. In the present study, examination of the fatty acid products labelled in the chloroplast incubations (Table 2) showed that, for thiolactomycin and the two most effective analogues, compounds 6 and 7, labelling of 18-carbon acids was especially affected, indicating again that KAS II was more sensitive than the other condensing enzymes. However, because chloroplast labelling is most effective with [1-^{14}C]acetate, it is not possible to assess the inhibition of KAS I separately from KAS III using this system because the FAS product of the latter (butyroyl-ACP) is utilized by KAS I for chain-lengthening to palmitate [1].

Table 2 Pattern of fatty acids synthesized by pea leaf chloroplasts in the presence of 100 μM thiolactomycin or its analogues

Incubations were carried out with [1-^{14}C]acetate as described in the Materials and methods section. In this particular experiment, thiolactomycin and compounds 6 and 7 produced 77 %, 75 % and 91 % inhibition of total labelling respectively (see Table 1 also). Means ± S.D ($n = 3$) shown. Statistical difference, in comparison to the control, was estimated by Student's t test. Fatty acid abbreviations: 14:0, myristate; 16:0, palmitate; 18:0, stearate; 18:1, oleate.

	Radioactive fatty acids (% total products)				
	14:0	16:0	18:0	18:1	Others
Control	3 ± 2	44 ± 4	6 ± tr.	43 ± 3	4 ± 2
+ Thiolactomycin	11 ± 3 ($P < 0.1$)	64 ± 3 ($P < 0.02$)	4 ± 2	15 ± 1 ($P < 0.005$)	6 ± 2
+ Compound 6	10 ± 1 ($P < 0.05$)	74 ± 5 ($P < 0.02$)	2 ± 1 ($P < 0.025$)	11 ± 2 ($P < 0.005$)	3 ± 1
+ Compound 7	10 ± 3 ($P < 0.1$)	76 ± 6 ($P < 0.02$)	2 ± 2 ($P < 0.1$)	7 ± 3 ($P < 0.005$)	5 ± 2

Table 3 Synthesis of fatty acids from [1-^{14}C]laurate by pea leaf discs in the presence of 100 μM thiolactomycin or its analogues

For identity of compounds used see Figure 1. Means ± S.D. ($n = 3$) shown. Statistical analysis was by Student's t test with P values (compared to control) shown in the Table.

			Fatty acid labelling (d.p.m. × 10^3)	
	Compound added	Total synthesis (% control)	14C and 16C products	18-carbon products
	None (control)	100 ± 3	39.6 ± 5.5	32.4 ± 8.4
	1	44 ± 3 ($P < 0.01$)	29.5 ± 1.0	n.d.† ($P < 0.01$)
	2	151 ± 10 ($P < 0.05$)	66.3 ± 1.9 ($P < 0.05$)	42.4 ± 2.4
	3	134 ± 3 ($P < 0.02$)	33.8 ± 2.8	62.7 ± 9.6
	4	80 ± 3 ($P < 0.05$)	29.9 ± 7.4	27.6 ± 2.1
	5	110 ± 3	49.1 ± 12.3	30.1 ± 2.5
	6	24 ± 2 ($P < 0.01$)	17.3 ± tr. ($P < 0.01$)	n.d.† ($P < 0.01$)
	7	5 ± 3 ($P < 0.01$)	–*	–*

*Insufficient radioactivity for analysis of products by radio-GLC.
† n.d., none detected.

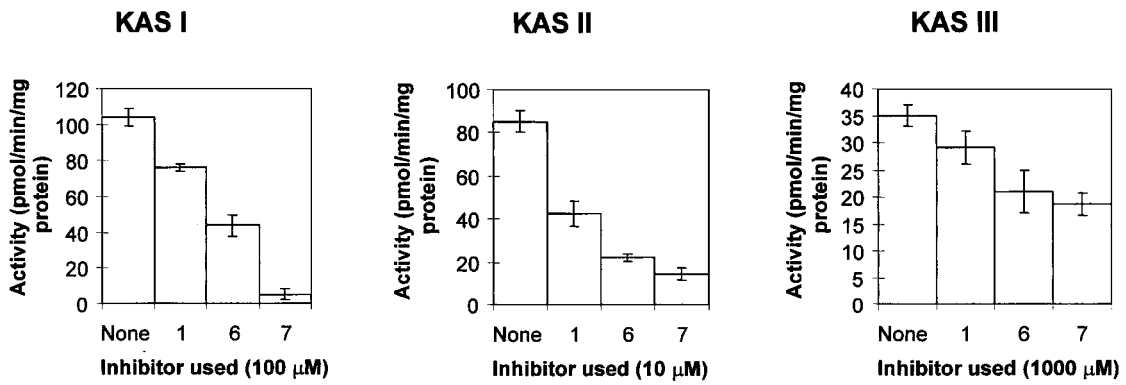

Figure 2 Effect of thiolactomycin or compounds 6 and 7 (see Figure 1) on the activity of pea KAS I, II and III

The assays were made using a 40–80% $(NH_4)_2SO_4$ precipitate of the soluble fraction from a total leaf homogenate, in the presence of 10–1000 μM inhibitor, as described in the Materials and methods section. Means $\pm$ S.D. ($n = 3$) are shown.

Accordingly, we carried out experiments using leaf discs and [1-^{14}C]laurate as substrate. Laurate is effectively taken up by such tissue preparations [15] and, because it is efficiently activated to its ACP-derivative, is incorporated into long-chain fatty acids [28] through the action of KAS I and KAS II and the other partial reactions of FAS. The major radiolabelled products detected were palmitate, oleate and linoleate, with smaller amounts of palmitoleate and stearate. In order to simplify interpretation of the data, the labelled fatty acid products have been grouped into 16-carbon and 18-carbon compounds (Table 3). Since the latter are only made via the activity of KAS II (together with desaturases), their labelling is a good measure of the activity of this condensing enzyme.

Two compounds, analogues 2 and 3, produced significant increases in fatty acid labelling (Table 3). This was due to increased uptake of [1-^{14}C]laurate into the discs. The uptake was not affected by any of the other analogues (results not shown). Thiolactomycin and compounds 6 and 7 again produced highly significant decreases in total fatty acid synthesis. In fact, the inhibition of incorporation from [1-^{14}C]laurate was broadly comparable to that from [1-^{14}C]acetate in the chloroplast incubations (Table 1), except that compound 5 (with a phenyl group) produced some effects against *de novo* synthesis but none against laurate metabolism. For thiolactomycin and analogue 6, no 18-carbon products could be detected (Table 3), demonstrating that KAS II was very sensitive to inhibition by the latter compound. In the experiment shown in Table 3 there was insufficient radioactivity for radio-GLC analysis of the products formed in the presence of compound 7. However, in further experiments using 25 μM concentrations of this analogue, labelling of 18-carbon products was completely inhibited, indicating a strong effect on KAS II for compound 7 also (results not shown).

In order to test specifically the most effective inhibitors against the three condensing enzymes, we assayed them directly. All compounds were tested against KAS III; compounds 2–4 were without effect, compound 5 produced 38% inhibition at 1 mM. The activities of thiolactomycin and compounds 6 and 7 are shown in Figure 2. From the results obtained with chloroplasts and leaf discs, it appeared that KAS I was more sensitive to inhibition than KAS III, while KAS II was the most sensitive. Therefore, having obtained moderate inhibition of KAS III at 1 mM concentrations of thiolactomycin and compounds 6 and 7, we tested 100 μM concentrations against KAS I and 10 μM concentrations against KAS II (Figure 2). In agreement with the previous results (Tables 1–3), the three condensing enzymes showed an order of sensitivity of KAS II > KAS I > KAS III towards thiolactomycin and its derivatives. In addition, compound 7, with an octanyl side chain, was the most effective inhibitor in all cases (Figure 2).

In summary, we have shown that all three condensing enzymes of pea FAS are sensitive to thiolactomycin and various analogues. KAS II is particularly sensitive. Substitution of the isopentadienyl side chain of thiolactomycin with longer alkyl chains (in particular an octanyl group) formed more effective inhibitors. The latter compounds should prove of future use in detailed studies of plant KASs perhaps in experiments similar to those recently reported for cerulenin with the *E. coli* KAS II [29]. They will also be of use for the inhibition of other condensation reactions (such as those of the malaria parasite, *Plasmodium* spp. [16]) which are inhibited by thiolactomycin.

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REFERENCES

1 Harwood, J. L. (1996) Biochim. Biophys. Acta **1301**, 7–56
2 Shimakata, T. and Stumpf, P. K. (1982) Proc. Natl. Acad. Sci., U.S.A. **79**, 5808–5812
3 Shimakata, T. and Stumpf, P. K. (1983) Arch. Biochem. Biophys. **220**, 39–45
4 Hoj, P. B. and Mikkelsen, J. D. (1982) Carlsberg Res. Commun. **47**, 119–141
5 Schuz, R., Ebel, J. and Hahlbrock, K. (1982) FEBS Lett. **140**, 207–209
6 Harwood, J. L. (1988) Annu. Rev. Plant Physiol. Plant Mol. Biol. **39**, 101–138
7 Stumpf, P. L. and Shimaka, T. (1983) in Biosynthesis and Function of Plant Lipids (Thomson, W. W., Mudd, J. B. and Gibbs, M., eds.), pp. 1–16, Waverly Press, Baltimore
8 Jackowski, S. and Rock, C. O. (1987) J. Biol. Chem. **262**, 7927–7931
9 Jaworski, J. G., Clough, R. C. and Barnum, S. R. (1989) Plant Physiol. **90**, 41–44
10 Jaworski, J. G., Clough, R. C., Barnum, S. R., Post-Beittenmiller, D. and Ohlrogge, J. B. (1990) in Plant Lipid Biochemistry, Structure and Utilization (Quinn, P. J. and Harwood, J. L., eds.), pp. 97–104, Portland Press Ltd., London
11 Walsh, M. C., Klopfenstein, W. E. and Harwood, J. L. (1990) Phytochemistry **29**, 3797–3799
12 Nishida, I., Kawaguchi, A. and Yamada, M. (1984) Plant Cell Physiology **25**, 265–268
13 Nishida, I., Kawaguchi, A. and Yamada, M. (1986) J. Biochem. (Tokyo) **99**, 1447–1454
14 Gulliver, B. S. and Slabas, A. R. (1994) Plant Mol. Biol. **25**, 179–191
15 Jones, A. L., Dancer, J. E. and Harwood, J. L. (1995) Phytochemistry **39**, 511–514

16 Waller, R. F., Keeling, P. J., Donald, R. G. K., Striepen, B., Handman, E., Lang-Unnasch, N., Cowman, A. F., Besra, G. S., Roos, D. S. and McFadden, G. I. (1998) Proc. Natl. Acad. Sci., U.S.A. **95**, 12352–12357
17 Wang, C-L. J. and Salvino, J. M. (1984) Tetrahedron Lett. **25**, 5243–5246
18 Majerus, P. W., Alberts, A. W. and Vagelos, P. R. (1969) Methods Enzymol. **14**, 43–50
19 Rock, C. O. and Cronan, J. E. (1981) Methods Enzymol. **71**, 163–168
20 Rock, C. O., Garwin, J. L. and Cronan, J. E. (1981) Methods Enzymol. **71**, 397–403
21 Mills, W. R. and Joy, K. W. (1980) Planta **148**, 75–83
22 Walker, K. W. and Harwood, J. L. (1985) Biochem. J. **226**, 551–556
23 Arnon, D. I. (1949) Plant Physiol. **24**, 1–15
24 Jones, A. L., Dancer, J. E. and Harwood, J. L. (1994) Biochem. Soc. Trans. **22**, 258S
25 Smith, K. L., Douce, R. and Harwood, J. L. (1982) Phytochemistry **21**, 569–573
26 Garbus, J., De Luca, H. F., Loomans, M. E. and Strong, F. M. (1963) J. Biol. Chem. **238**, 59–63
27 Verwoert, I. I. G. S., Verbree, E. C., van der Linden, K. H., Nijkamp, H. J. J. and Stuije, A. R. (1992) J. Bacteriol. **174**, 2851–2857
28 Cho, S. H. and Thompson, G. A. (1987) in The Metabolism, Structure and Function of Plant Lipids (Stumpf, P. K., Mudd, J. B. and Nes, W. D., eds.), pp. 623–629, Plenum Press, New York
29 Moche, M., Schneider, G., Edwards, P., Dekesh, K. and Lindqvist, Y. (1999) J. Biol. Chem. **274**, 6031–6034

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