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DISTRIBUTION AND BIOSYNTHESIS OF STEARIDONIC ACID IN LEAVES OF *BORAGO OFFICINALIS*

GARETH GRIFFITHS,* ELIZABETH Y. BRECHANY,† FRANCES M. JACKSON,‡ WILLIAM W. CHRISTIE,§ STEN STYMNE‖ and A. KEITH STOBART‡

Department of Molecular and Environmental Physiology, Horticulture Research International, Wellesbourne, Warwickshire, CV35 9EF, U.K.; †Hannah Research Institute, Ayr, Scotland, U.K.; ‡School of Biological Sciences, University of Bristol, Bristol BS8 1UG, U.K.; ‡Scottish Crop Research Institute, Invergowrie, Scotland, U.K.; ‖Swedish University of Agricultural Sciences, Department of Plant Breeding Research, Svalof S268 31, Sweden

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Abstract—An octadecatetraenoic acid was present as a major fatty acid component of the leaf lipids of borage. Gas chromatography–mass spectrometry of its picolinyl esters gave unsaturation centres at carbons Δ^6, Δ^9, Δ^{12}, and Δ^{15} and confirmed its identity as stearidonic acid (SDA; octadecatetraenoic acid, C18:4, $\Delta^{6,9,12,15}$. The chloroplast galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) were particularly rich in SDA. SDA was absent, however, from the plastid phospholipid, phosphatidylglycerol (PG). Stereochemical analysis of the fatty acids in leaf MGDG and phosphatidylcholine (PC) showed that both SDA and γ-linolenic acid (GLA) were almost exclusively located at carbon *sn*-2 of these complex lipids. In time-course studies with excised seed cotyledons induced to green by light treatment, SDA appeared in the galactolipids before its detection in PC suggesting that its major site of synthesis in the leaf was prokaryotic and largely located in the chloroplasts. Borage seed microsomes, which have high Δ^6 desaturase (Δ^6des) activity, catalysed the synthesis of SDA from exogenously supplied α-linolenic acid (ALA). Linseed cotyledons which have an active Δ^{15}des, on the other hand, could not convert exogenously supplied GLA to SDA. These observations suggest that SDA is formed from ALA via Δ^6des activity at carbon sn-2 of MGDG and not from GLA and subsequent Δ^{15}-desaturation.

INTRODUCTION

An octadecatetraenoic acid (18:4; stearidonic acid, SDA) has been reported in a number of species in the Boraginaceae [1], Ranunculaceae [2], Onagraceae [3] and Grossulariaceae [4]. Seed membrane and storage lipids of these species are also characterized by the presence of γ-linolenic acid (18:3 $\Delta^{6,9,12}$; GLA) [5]. Δ^6 fatty acids are of particular value to the pharmaceutical and health food industry [6, 7] and, hence, there is interest in their biosynthesis. We have previously shown that in borage seeds, GLA is synthesized microsomally and that the Δ^6 desaturase (Δ^6des) is particularly active with linoleoyl (18:2 $\Delta^{9,12}$) substrate esterified to position *sn*-2 of *sn*-phosphatidylcholine (PC) [5]. Work with leaf tissue from other species has shown that Δ^{15}-desaturase (Δ^{15}des) activity, responsible for the synthesis of α-linolenate (18:3 $\Delta^{9,12,15}$; ALA) resides with highest activity in the chloroplast and utilizes linoleate (LA) esterified to complex lipids, particularly monogalactosyldiacylglycerol (MGDG) [8]. In addition to *de novo* polyenoic fatty acid biosynthesis in the chloroplasts, numerous studies have shown that this organelle also imports endoplasmic reticulum (ER) synthesized lipids, giving rise to prokaryotic and eukaryotic lipid configurations within the plastid [9, 10]. In order to synthesize SDA, it is necessary to have Δ^6- and Δ^{15}-desaturation of LA. Here we extend our earlier observations [11] and report on the distribution of SDA in complex lipids of borage and studies on its biosynthesis.

RESULTS AND DISCUSSION

Analysis of the fatty acid content of the total lipids of young borage leaves (Table 1) showed substantial quantities of a constituent with properties (GC retention time, argentation TLC, products of hydrogenation) indicative of a C18:4 acid. Although early previous work reported a similar fatty acid [1], it was considered necessary, before proceeding with biochemical studies, to fully establish its identity by GC-mass spectrometry.

*Author to whom correspondence should be addressed.

Table 1. Fatty acids of borage leaf lipids (mol%)

Lipid	16:0	t16:0	18:0	18:1	18:2	γ-18:3	α-18:3	18:4
Total	12	2	2	3	9	6	43	23
MGDG	5	0	2	1	2	6	49	35
DGDG	15	0	4	2	2	5	59	13
PG	31	35	4	5	10	<1	15	0
PC	23	0	5	5	24	7	26	10
PE	32	0	6	4	29	7	16	6

Lipids extracted from leaf tissues and purified by TLC. After transmethylation, FAMEs of complex lipids analysed by GC. Similar results obtained in separate analyses on other occasions (typically >3% error of mean).

The picolinyl ester derivative of the putative SDA had a retention time on a methyl silicon phase (see Experimental) expected for such an acid. The mass spectrum (Fig. 1) had a prominent ion at *m/z* 367 and confirmed the anticipated empirical formula. The base peak at *m/z* 92, together with abundant ions at *m/z* 108, 151 and 164, are characteristic of most picolinyl ester derivatives [12]. If fragmentation proceeds from the terminal methyl group, the first significant ion is at *m/z* 352 with a gap of 14 mu to 338 for the next methylene group. A gap of 40 mu to *m/z* 299 is indicative of the first double bond with a gap of 26 mu between *m/z* 338 and 312 showing that it was at C-15 of the acyl chain. Further gaps of 14 and 26 mu in sequence, confirmed that the remaining double bonds were at C-12, C-9, and C-6, respectively, and that the compound was, therefore, the picolinyl ester of $\Delta^{6,9,12,15}$-octadecatetraenoic acid (SDA). This structure was further confirmed by a comparison with that of the same fatty acid from cod-liver oil [13], where the structure had been determined by other methods. Other positional isomers would have had very different spectra.

Stearidonic acid was also found as a component of the total leaf lipid fatty acids in other species of the Boraginaceae, viz., *Cynoglossum officinale* (houndstongue; 8%), *Anchusa officinali* (green alkanet; 13%), *Anchusa azuerea* (5%), *Myosotis palustris* (11%), *Echium vulgare* (vipers bugloss; 11%), *Pulmonaria officinalis* (common lungwort; 10%) and *Symphytum officinale* (comfrey; 16%).

The fatty acid composition of borage leaf lipids was determined (Table 1). Whilst the chloroplast lipids, MGDG and DGDG, contained substantial SDA it was, however, absent in phosphatidylglycerol (PG). The MGDG and DGDG also contained GLA, whereas only trace amounts of this fatty acid were present in PG. All three chloroplast lipids, on the other hand, contained ALA. Phosphatidylcholine (PC), found largely in the ER with traces in the chloroplast envelope [14], and phosphatidylethanolamine (PE), which is considered to be entirely extraplastidic [15], had both linolenic acid isomers and SDA. Stereospecific analysis of borage leaf PC and MGDG showed that the SDA and GLA were almost completely confined to position *sn*-2 (Table 2). The ALA, on the other hand, was particularly enriched at the *sn*-1 of MGDG and with lesser amounts at this position in PC. These observations are consistent with previous reports that the Δ^6des in borage seed microsomes catalyse desaturation with substrate at position *sn*-2 of complex lipids [5]. The virtual absence of GLA

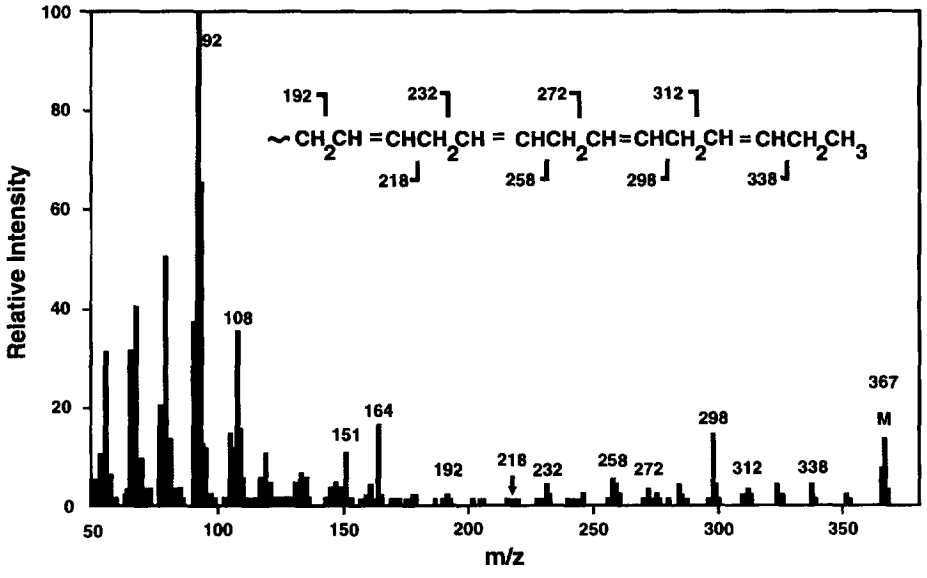

Fig. 1. GC-mass spectrum of picolinyl ester derivative of stearidonic acid.

Table 2. Positional distribution of fatty acids in MGDG and PC in borage leaves (mol%)

Lipid	16:0	t16:0	18:0	18:1	18:2	γ-18:3	α-18:3	18:4
MGDG								
sn-1	10	0	3	1	3	1	82	0
sn-2	<1	0	<1	<1	1	10	10	78
PC								
sn-1	52	0	9	10	13	2	13	1
sn-2	5	0	2	16	19	11	36	11

MGDG and PC purified by TLC and hydrolysed with phospholipase A_2. Lyso-derivatives (representing fatty acids at position *sn*-1) and unesterified fatty acids (liberated from position *sn*-2) were purified by TLC, transmethylated and the FAMEs analysed by GC.

and SDA in PG is particularly noteworthy, this phospholipid being exclusively synthesized within the chloroplast [16] through the 16:3 prokaryotic pathway [9] with predominantly saturated fatty acids at the *sn*-2 position and, hence, little substrate for Δ^6 desaturation.

Seeds of many oil-seed species lack chloroplasts as well as the ability to synthesise ALA, i.e. Δ^{15}des activity. The developing cotyledons of borage are a case in point. They are cream in colour and contain proplastids with little or no chloroplast complex lipid. If the developing cotyledons are removed from the seed and exposed to light they green and produce chloroplasts (see ref. [17]). Such tissue provides, therefore, an experimental system in which the synthesis of ALA and chloroplast lipid can be induced in the light (Table 3). The membrane lipids of pregreened cotyledons are rich in LA, GLA with no ALA or SDA. With greening, however, MGDG and DGDG became the predominant complex lipids and contained GLA, ALA and SDA. Whilst some ALA also appeared in the phospholipids, these have no detectable SDA. These results suggest that SDA is synthesized within the developing chloroplasts by the Δ^{15}- and Δ^6-desaturases acting on LA esterified to the galactolipids. The relatively low levels of ALA in PC and PE compared with those of the galactolipids further implies that the major site of the Δ^{15}des resides in the chloroplast and can utilize LA at both positions *sn*-1 and -2 of MGDG.

The isolation of active microsomes from leaf tissue, uncontaminated by plastidic membranes is extremely difficult. In order, therefore, to elucidate further SDA biosynthesis, microsomal membranes prepared from the cotyledons of developing borage seed were incubated with radiolabelled linoleoyl-CoA or ALA-CoA under desaturating conditions (Table 4). Borage microsomes have Δ^6des activity [5, 18] and catalysed the conversion of [^{14}C]LA to GLA. The microsomes also converted the [^{14}C]ALA to SDA. These results indicate that a membrane preparation with Δ^6 desaturase activity, and with no Δ^{15}-desaturase, will introduce a double bond into exogenously supplied ALA at the Δ^6-position to yield the tetraenoic acid.

Further experiments were designed to determine whether SDA could be formed from GLA in a system with Δ^{15} desaturase activity and capable of ALA production. Unfortunately, *in vitro* Δ^{15}des activity is extremely labile and difficult to obtain. Experiments, therefore, were conducted with the developing cotyledons of linseed, a tissue accumulating triacylglycerols (TAG) particularly rich in ALA. Cotyledon slices were incubated with either [^{14}C]oleate (OL), -LA or -GLA. The results (Table 5) show that the OL and LA were readily utilized in ALA synthesis. No radioactive SDA, however, was formed from the labelled GLA.

In our initial studies, we speculated that the GLA synthesized at position *sn*-2 of PC in the ER was desaturated, after transfer of the diacylglycerol (DAG)

Table 3. Fatty acid composition of lipids in borage seeds exposed to light (mol%)

Time (hr)	Lipid	16:0	18:0	18:1	18:2	γ-18:3	α-18:3	18:4
0	PC	30.1	3.3	11.9	39.0	15.6	n.d.	n.d.
	PE	31.6	5.9	6.5	46.8	9.2	n.d.	n.d.
MGDG, DGDG, PG		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
115	MGDG	14.6	2.4	7.8	28.6	24.3	18.4	4.0
	DGDG	22.7	5.2	3.4	20.0	11.7	34.3	2.7
	PC	30.0	3.9	10.8	31.8	20.4	3.0	n.d.
	PE	46.7	6.4	5.3	28.2	9.8	3.0	n.d.
	PG	65.5*	1.5	5.7	15.1	4.5	7.6	n.d.

Developing borage cotyledons were excised from seed coats and exposed to light for the time indicated. Lipids were extracted, purified by TLC and analysed as FAME by GC. Similar results obtained in separate experiments on other occasions (typically >5% error of mean).

*16:0 also includes *t*16:1 in PG only.

n.d.: not detected.

Table 4. Utilization of [^{14}C]linoleoyl-CoA and [^{14}C]linolenoyl-CoA in microsomal membranes prepared from developing seeds of borage

Substrate	Radioactivity incorporated (nmol)			
	18:2	γ-18:3	α-18:3	18:4
[^{14}C]18:2-CoA	77.8 (65)	42.2 (35)	n.d.	n.d.
α-[^{14}C]18:3-CoA	—	—	153 (77)	46.6 (23)

Microsomal membranes (equivalent to 110 nmol PC) were incubated for 3 hr with NADH (2 μmol), 10 mg BSA, 1000 units catalase, 66 mM sucrose, in the presence of either [^{14}C]18:2-CoA (specific activity 2500 dpm nmol^{-1}) or α-[^{14}C]18:3-CoA (2868 dpm nmol^{-1}), total vol. 1 ml. Lipids extracted, transmethylated and FAMEs purified by $AgNO_3$-TLC. Radioactivity was determined by liquid scintillation counting. Figures in parentheses expressed as %.

n.d.: not detected.

moiety to the chloroplast, by Δ^{15}des to form SDA at position *sn*-2 of MGDG [19]. This was recently supported by Williams *et al.* [10] studying the effects of temperature acclimation in 18:4 plants and in which all of the DAG moieties were considered to be cytosolic (or eukaryotic) in origin. The results obtained here, however, from the borage and linseed experiments indicate that the sequence of desaturation is from LA → ALA → SDA. It would appear that GLA is a metabolic end product and cannot be converted further to SDA. Both the Δ^6 and Δ^{15}des compete for LA and in those cellular compartments with a high Δ^{15}des activity, i.e. the chloroplast, the generation of ALA predominates. This subsequently could be converted to SDA by Δ^6des which may also reside in the chloroplast. Conversely, where Δ^6des predominates, i.e. the ER, GLA will accumulate with little SDA formation. The detection of SDA in MGDG and its absence in PC in the greening cotyledons (Table 3) would be consistent with both the Δ^{15} and Δ^6des being present in the chloroplast of borage leaves, although no attempt was made to establish the presence of isoforms in other cellular compartments. There are also a number of other alternative possibilities to explain the distribution of SDA in the leaf lipids. For example, SDA could be rapidly removed from PC and transferred to the galactolipid or that GLA is transferred from PC to galactolipid and then desaturated by a plastidic desaturase which is different from the ER one in its substrate specificity. Since the borage seed Δ^6des gene has recently been cloned [20] and is most probably the ER enyme [18], expressing it in *Arabidopsis* mutants deficient in either ER or plastidic Δ^{15}-desaturation (FAD3 [21], FAD7 [22] and FAD 8 [23] mutants) should yield definitive answers on the pathway(s) operating to produce SDA. Alternatively, expressing the Δ^6des in tobacco and targeting it to the ER and/or chloroplast should also yield valuable information on SDA formation and the inter-relationship between prokaryotic and eukaryotic lipid biosynthesis.

The observation that SDA in PC is formed from ALA and not GLA also raises questions regarding substrate recognition. The Δ^{12}-desaturase may align with the double bond at position Δ^9 and the methyl end of the fatty acid [24]. Here, however, the Δ^{15}des may be locating the first double bond from the carboxyl end in GLA resulting in no formation of SDA. The ER Δ^6des, on the other hand, may recognize the acyl chain

Table 5. Utilization of [^{14}C] fatty acids in developing cotyledons of linseed

^{14}C fatty acid substrate and lipid analysed	Radioactivity incorporated (nmol)				
	18:1	18:2	γ-18:3	α-18:3	18:4
Total lipid:					
[^{14}C]18:1	4.1 (64)	1.7 (27)	n.d.	0.6 (9)	n.d.
[^{14}C]18:2	—	2.4 (66)	n.d.	1.2 (34)	n.d.
[^{14}C]18:3	—	—	3.6 (100)	n.d.	n.d.
PC:					
[^{14}C]18:1	0.3 (37)	0.4 (50)	n.d.	0.1 (13)	n.d.
[^{14}C]18:2	—	0.2 (53)	n.d.	0.2 (47)	n.d.
[^{14}C]18:3	—	—	0.4 (100)	n.d.	n.d.

Developing cotyledons (0.2 g fr. wt) were incubated for 4 hr in KPi buffer with the NH_4 salts of either [^{14}C]18:1, [^{14}C]18:2 or [^{14}C]18:3. Extracted lipids purified by TLC, transmethylated and FAMEs separated by $AgNO_3$-TLC and radioactivity determined by liquid scintillation counting. Figures in parentheses expressed as %.

n.d.: not detected.

from the double bond at postion 9 and the carboxyl end, and this would result in the formation of SDA from ALA.

EXPERIMENTAL

Chemicals. [1-^{14}C]Oleic acid (octadeca-9-enoic acid; 2.07 MBq mol^{-1}), [1-^{14}C]linoleic acid (octadeca-9,12-dienoic acid; 2.18 MBq mol^{-1}), α-[1-^{14}C]linolenic acid (octadeca-9,12,15-trienoic acid, 2.08 MBq mol^{-1}) were obtained from Amersham. γ-[1-^{14}C]Linolenic acid (octadeca-6,9,12-trienoic acid, 1.9 MBq mol^{-1}) was prepared enzymatically as described below. Catalase (thymol-free, 11 000 units mg^{-1}), BSA (fraction V, fatty acid-free), CoASH, NADH, phospholipase A_2 from Indian cobra (*Naja naja*) venom, *Rhizopus arrhizus* lipase and various fatty acids were purchased from Sigma. [1-^{14}C]18:2-CoA (specific radioactivity 66 Bq nmol^{-1}) and [1-^{14}C]18:3-CoA (specific radioactivity (48 Bq nmol^{-1}) were synthesized from their mixed anhydrides as described in ref. [25].

Plant material. Common borage (*Borago officinalis* L.) and linseed (*Linum usitatissimum*, var. Iduna) plants were grown from seed in a 16 hr photoperiod at 18° with an 8 hr dark period at 13°. Only young expanding leaves of borage were used in the lipid analyses. When seeds were used, flowers were hand pollinated and seeds were harvested 15–20 days after flowering. Leaves and seeds from other species of the Boraginaceae were obtained from The Botanical Garden, University of Uppsala, Sweden.

Analytical procedures. Lipids were extracted from leaf material, after removal of the major veins, by homogenization in an Ultra-Turax with MeOH–$CHCl_3$ according to a modification of the method of ref. [26]. The lower $CHCl_3$ phase, which contains the complex lipids and the non-esterified fatty acids, was removed and evapd to dryness under N_2. The residue was dissolved in a small vol. of $CHCl_3$ and polar lipids (PL) purified by TLC on silica gel 60 (Merck) in $CHCl_3$–MeOH–HOAc–H_2O (170:30:20:7) and quantified by FID-GC, with heptadecanoic acid as int. standard [5].

Positional analysis of fatty acids in PLs was performed by treatment of the lipid with phospholipase A_2 [5]. The intramolecular distribution of fatty acids in galactolipids was determined after hydrolysis with *Rhizopus arrhizus* lipase according to ref. [27].

Lipid samples (MGDG) for MS, were hydrolysed and the picolinyl esters derivatives prepd by reaction of the mixed anhydrides of the UFA and TFA with 3-(hydroxymethyl)-pyridine and subjected to GC-MS [13]. In brief, a fused capillary column (25 m × 0.2 mm), coated with a cross-linked methylsilicone (Hewlett Packard, U.K.) with He carrier gas was temp. programmed from 60 to 220 at 50 min^{-1} then to 260 at 1 min^{-1}. The column outlet was connected directly into the ion source of a mass-selective detector, operated at an ionization energy of 70 eV.

Enzyme assays. Microsomal prepns of developing borage seeds were obtained as described earlier [5]. Desaturase activities in microsomal frs were measured by incubating the microsomes (0.5 mg protein) at 25° for 3 hr with 200 nmol of either [1-^{14}C]18:2-CoA or [1-^{14}C]18:3-CoA, 2 μmol NADH, 1000 units of catalase, 66 mM sucrose and 10 mg of BSA in a final vol. of 1 ml with 0.1 M KPi buffer (pH 7.2). Incubations were termined and lipids extracted as described above. *In vivo* desaturation of [1-^{14}C]fatty acids in linseed cotyledons was followed by incubating young developing cotyledons (0.2 g fr. wt) with 10 nmol of the NH_4-salt of the [1-^{14}C]acids in 0.25 ml KPi buffer for 4 hr. After that time, cotyledons were rinsed in buffer to remove residual isotope and the lipids extracted in an Ultra-Turrax homogenizer in the medium described in ref. [26].

Synthesis of γ-[1-^{14}C]linolenic acid. [1-^{14}C]Linoleic acid (1 μmol), bound to 20 mg BSA, was incubated at 25° with microsomes (3 mg protein) prepd from developing borage cotyledons (see below), in the presence of NADH (8 μmol), ATP (4 μmol), $MgCl_2$ (2 μmol), CoASH (4 μmol), catalase (4000 units) and sucrose (250 μmol) in 0.1 M KPi (pH 7.2) in a total vol. of 4 ml. After 4 hr, the incubation was terminated by addition of 2 ml KOH (1 M) and the mixt. refluxed for 30 min. After acidification, unesterified fatty acids (UFA) were extracted in hexane and transmethylated [5]. The Me esters were separated by $AgNO_3$-TLC [27]. The gel containing the trienoic species was removed, eluted with $CHCl_3$ and the esters refluxed with KOH (1 ml, 1 M) for 30 min. After acidification, UFAs were extracted in hexane. The GLA was *ca* 97% pure as judged by TLC and radio-GC with a sp. act. of 1.9 MBq mol^{-1}.

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