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OCCURRENCE AND BIOSYNTHESIS OF CYCLOPENTENYL FATTY ACIDS IN LEAVES AND CHLOROPLASTS OF FLACOURTIACEAE

ANNETT G. REHFELDT,* ERHARD SCHULTE† and FRIEDRICH SPENER*

* Institut für Biochemie, Universität Münster, Orléansring 23a, D-4400 Münster, W. Germany. † Institut für Lebensmittelchemie, Universität Münster, Piusallee 7, D-4400 Münster, W. Germany

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Key Word Index *Caloncoba echinata*: Flacourtiaceae, leaves; chloroplasts, cyclopentenyl fatty acids; occurrence; biosynthesis.

Abstract -Cyclopentenyl fatty acids, the unusual fatty acids occurring naturally in certain Flacourtiaceae, have been detected for the first time in leaves of various plants belonging to the tribes Pangieae, Oncobeae and Flacourtieae. In leaves and chloroplasts of *Caloncoba echinata* (Oncobeae) cyclopentenyl fatty acids are synthesized from aspartate plus pyruvate or glutamate plus acetate. The biogenesis of the cyclopentene ring occurs from a C_7 compound, that may be formed by either pair of substrates.

INTRODUCTION

In recent years cyclopentenyl fatty acids have gained attention for several reasons. (1) Their restricted occurrence in the tribes Pangieae and Oncobeae may be related to chemotaxonomy [1, 2]; (2) from a biochemical point of view, the unusual structure of these fatty acids prompted studies on their biosynthesis [3–5]; (3) derivatives of cyclopentenyl fatty acids served as models to study the molecular action of chiral aliphatic branched-chain pheromones [6].

Earlier, evidence was presented for the following biosynthetic path in seed and leaf tissues of *Hydnocarpus anthelminthica* and *Caloncoba echinata*: cyclopentenylglycine → cyclopentenyl carboxylic acid (aleprolic acid) → cyclopentenyl fatty acids [3–5]. The natural occurrence of non-proteinogenic cyclopentenylglycine was demonstrated recently [7]. In the present communication we report the occurrence of cyclopentenyl fatty acids in leaves of various Flacourtiaceae in the search for tissues suited for biochemical investigations. The role of primary metabolites as precursors of the 'secondary' cyclopentenyl fatty acids was then studied using leaves and chloroplasts of *C. echinata*.

RESULTS AND DISCUSSION

Occurrence

A method involving capillary-GC was worked out for the rapid determination of straight-chain and cyclic fatty acids occurring in leaves of Flacourtiaceae. Using the polar stationary phase FFAP at 175°, Me esters of cyclopentenyl fatty acids having n carbon atoms were eluted from the capillary column between $n + 1$ and $n + 2$ Me esters of straight-chain fatty acids. With the aid of a newly developed interface, the glass capillary column was connected to a MS, allowing unequivocal identification of cyclopentenyl fatty acid Me esters by their characteristic fragments at m/e 67 and 82 [8, 9]. The application of capillary-GC to the analysis of samples containing Me esters of straight-chain and cyclic fatty acids replaced the tedious two-step chromatographic procedures described earlier [4, 5, 10].

The results of GC–MS analyses of total fatty acids from leaves of Flacourtiaceae are summarized in Table 1, showing the proportions of cyclopentenyl fatty acids as percentages of total fatty acids. As expected, proportions of cyclic fatty acids were generally low, however, exceptions were noted for *F. inermis* (8.7%) and *F. cataphracta* (22.6%). Depending on the time of harvest the amounts of endogenous cyclic fatty acids may vary to some extent. Thus, proportions of cyclic fatty acids in *H. anthelminthica* leaves were found earlier to vary between 0.5 and 1.5%, whereas those in *C. echinata* leaves were 4.4% [10]. The choice of genera examined was an arbitrary one, it was limited by the availability of specimens. The results show that cyclopentenyl fatty acids are natural products occurring not only in Pangieae and Oncobeae, but also in Flacourtieae, which indicates a far broader distribution of cyclopentenyl fatty acids than presently known. Further work is needed in order to discern a pattern of chemotaxonomical relevance. Moreover, the detection of cyclopentenoid cyanogenic glycosides in Passifloraceae [11] may perhaps extend the distributional pattern. Cyclopentenylglycine, which is a known precursor of cyclopentenyl fatty acids [5], has been suggested to be a precursor for the cyclopentenoid cyanoglycosides as well [12, 13].

Biosynthesis

Our studies on the biosynethesis of cyclopentenyl fatty acids indicated cyclopentenylglycine and its transamination product cyclopentenylglyoxylate to be precursors of cyclopentenyl fatty acids and excluded cyclization mechanisms via straight-chain unsaturated fatty acids or

Table 1. Cyclopentenyl fatty acids in leaves of Flacourtiaceae

Tribe Subtribe		Cyclopentenyl fatty acids*
Pangieae	*Hydnocarpus anthelminthica*	1.5
	H. kurzii	0.9
	Kiggelaria africana	1.8
Oncobeae	*Caloncoba echinata*	1.9
	Oncoba spinosa	0.5
	Xylotheca kraussiana	0.5
Flacourtieae		
Flacourtiinae	*Flacourtia inermis*	8.7
	F. indica	0.6
	F. cataphracta	22.6
	F. sepiaria	0.4
	F. jangomas	0.7
	Azara celastrina	0.3
	A. lanceolata	0.9
	A. petiolaris	0.6
	A. microphylla	0.5
	Arechavaletaia uruguayensis	0.5
	Dovyalis caffra	0.8
	D. hebecarpa	0.9
Idesiinae	*Idesia polycarpa*	0.7
	Poliothyrsis sinensis	0.4

* Expressed in wt % of total fatty acids.

polyketides [5, 9]. Consequently, the intermediate formation of straight-chain C_7 compounds suitable for C_5 ring cyclization was postulated. Three hypotheses, outlined below, were tested by incubating radioactively labelled substrates with leaves and chloroplasts of *C. echinata* and by subsequent quantitation of the label incorporated into cyclopentenyl fatty acids.

$C_4 + C_3$ *hypothesis.* In accordance with the first steps of lysine biosynthesis in higher plants, the condensation of aspartate and pyruvate leads to α,ε-diaminopimelate, which may be transaminated to α,ε-diketopimelate. Reduction of one carboxyl group to the aldehyde may furnish a compound capable of cyclizing via intramolecular aldol condensation to a C_5 ring structure. Removal of the oxygen functions attached to the ring affords cyclopentenylglyoxylate, the precursor of aleprolate and cyclopentenyl fatty acids.

$C_5 + 2 \times C_1$ *hypothesis.* α-Ketopimelate may be obtained by a two-fold C_1 chain elongation of α-ketoglutarate with acetyl CoA. After reduction of α-ketopimelate to the semialdehyde, intramolecular aldol condensation may form 2′-hydroxycyclopentylglyoxylate, which is dehydrated to cyclopentenylglyoxylate. In our experiments we applied α-amino acids rather than α-keto acids, however, the former were rapidly converted to the latter by the action of ubiquitous transaminases [14].

A biosynthetic scheme via C_7 metabolites of the shikimate pathway was no longer considered when it became clear that radioactivity from labelled shikimate was not incorporated into cyclopentenyl fatty acids. Radioactivity from labelled glucose was found only in the aliphatic chain of the cyclic fatty acids.

Prior to the discussion of individual results, some general conclusions can be drawn from the numerous experiments conducted. The percentage of endogenous cyclopentenyl fatty acids in leaves and chloroplasts was *ca* 2% of total fatty acids, which corresponded quite well with the percentage of *de novo* synthesized cyclopentenyl fatty acids from acetate-[1-^{14}C] in either case. In contrast, different incorporation rates were obtained after incubations with aleprolate-[1-^{14}C]. The low radioactivity (4.8%) found in cyclic fatty acids of leaves indicated a rapid degradation of the substrate to labelled acetate and its reuse for *de novo* synthesis of mostly straight-chain fatty acids [4]. In chloroplasts, however, aleprolate-[1-^{14}C] was almost exclusively elongated to long-chain cyclic fatty acids (86%). Obviously, the multidirectional metabolism of the substrate in detached leaves can be kept to a minimum when working with chloroplasts. The results of these experiments reveal that leaves and chloroplasts synthesize straight-chain and cyclic fatty acids, provided appropriate starter molecules are furnished for the fatty acid synthetase(s).

After incubation of leaves and chloroplasts with a single substrate according to the proposed $C_4 + C_3$ and $C_5 + 2 \times C_1$ pathways, a *de novo* synthesis of cyclic fatty acids was hardly recognized, whereas with combinations of substrates, as shown in Tables 2 and 3, a significant amount of cyclic fatty acid synthesis was observed. Control experiments were carried out with leaves and chloroplasts

Table 2. Precursors of cyclopentenyl fatty acids in *Caloncoba echinata* leaves*

Substrates	Amounts (μCi)	Amounts (nmol)	Incorporation into total fatty acids/g fr. tissue (nmol)	Incorporation into total fatty acids/g fr. tissue (nmol%)	Radioactivity in cyclopentenyl fatty acids (%)
Acetate-[1-^{14}C]	1	1000	210.8	21.08	2.2
D,L-Aleprolate-[1-^{14}C]	1	100	9.6	9.6	4.8
Pyruvate-[3-^{14}C]	1	48.8	20.5	42.0	17.9
+ L-Aspartate		48.8			
L-Aspartate-[U-^{14}C]	1	4.9	0.10	1.96	18.1
+ Pyruvate		4.9			
α,ε-Diaminopimelate-[1,7-^{14}C]	1	34	0.01	0.04	6.3
L-Glutamate-[U-^{14}C]	1	4.22	0.01	0.26	11.6
+ Acetate		4.22			
D,L-α-Aminoadipate-[6-^{14}C]	1	21.3	0.01	0.04	13.9
+ Acetate		21.3			

*Mean of three determinations within ±5%.

Table 3. Precursors of cyclopentenyl fatty acids in *Caloncoba echinata* chloroplasts*

Substrates	Amounts (μCi)	Amounts (nmol)	Incorporation into total fatty acids/mg chlorophyll (nmol)	Incorporation into total fatty acids/mg chlorophyll (nmol%)	Radioactivity in cyclopentenyl fatty acids (%)
Acetate-[1-^{14}C]	1	1000	30	3.0	2.1
D,L-Aleprolate-[1-^{14}C]	1	100	0.5	0.5	86.6
Pyruvate-[3-^{14}C]	1	48.8	0.12	0.25	5.2
+ L-Aspartate		48.8			
L-Aspartate-[U-^{14}C]	1	4.9	0.01	0.11	27.1
+ Pyruvate		4.9			
α,ε-Diaminopimelate-[1,7-^{14}C]	1	34	0.03	0.08	17.6
L-Aspartate-[U-^{14}C]	1	5.43	0.01	0.13	5.8
+ Acetate		5.43			
L-Glutamate-[U-^{14}C]	1	4.22	0.01	0.18	37.8
+ Acetate		4.22			
D,L-α-Aminoadipate-[6-^{14}C]	1	21.3	0.02	0.09	21.7
+ Acetate		21.3			

* Mean of three determinations within ±5%.

of hazelnut (*Corylus avellana*), a plant which does not contain endogenous cyclopentenyl fatty acids [4]. Regardless of the substrate combinations tested, cyclopentenyl fatty acids were not synthesized by preparations from this plant.

In detached leaves (Table 2), the overall incorporation of radioactivity from substrates of the proposed $C_4 + C_3$ and $C_5 + 2 \times C_1$ pathways into total fatty acids was generally much lower than that from acetate-[1-^{14}C] and aleprolate-[1-^{14}C], yet the percentage of radioactivity found in cyclopentenyl fatty acids was much higher. Substrates of the $C_4 + C_3$ pathway, i.e. aspartate + pyruvate and their condensation product α,ε-diaminopimelate, served equally well as precursors for cyclic fatty acids as those of the $C_5 + 2 \times C_1$ pathway, i.e. glutamate + acetate and α-aminoadipate + acetate, respectively.

In experiments with chloroplasts (Table 3), a higher percentage of radioactivity was found in cyclopentenyl fatty acids, compared to experiments with whole leaves. For example, the percentage of radioactivity in cyclopentenyl fatty acids originating from aspartate-[U-^{14}C] went up to 27.1% of total fatty acids, from glutamate-[U-^{14}C] to 37.8%. The chloroplast experiments demonstrate that this organelle does not only possess a fatty acid synthetase capable of producing cyclopentenyl fatty acids from aleprolate, but is also equipped with all enzymes responsible for the biogenesis of the cyclopentene ring.

At present, a decision between the two pathways proposed for the biosynthesis of cyclopentenyl fatty acids

cannot be made, since several metabolic interrelationships may exist. Thus, pyruvate dehydrogenase has been recognized as a plastidal enzyme complex [15, 16] and may convert pyruvate to acetyl CoA. On the other hand, aspartate may be transaminated to oxaloacetate and thus the two substrates of the $C_4 + C_3$ pathway become substrates for a C_1 chain elongation. It can be seen from Table 3 that aspartate + acetate do not serve as good as precursors for cyclopentenyl fatty acids as glutamate + acetate or α-aminoadipate + acetate. Apparently, extramitochondrial C_1 chain elongations require α-ketodicarboxylic acids having five carbon atoms or more. It is interesting to note that in yeast the stereochemistry of oxaloacetate elongation—catalyzed by enzymes of the citric acid cycle—is different from that of corresponding reactions with higher homologues outside the mitochondria [17]. Lysine metabolism represents an additional link between the two pathways, as aspartate + pyruvate give rise to lysine, which in the course of its degradation furnishes α-ketoadipate, again a building block of the $C_5 + 2 \times C_1$ pathway.

The results of the present study reveal for the first time the metabolic link between primary metabolites and the secondary cyclopentenoid products. This biosynthetic pathway is localized in chloroplasts of green *C. echinata* leaves. The scope of mechanisms envisaged for cyclopentene ring formation could be confined to two alternatives. Regardless of the pathway involved, the first cyclopentenoid intermediate must be cyclopentenylglyoxylate. This intermediate is either transformed to aleprolate, thus giving rise to cyclopentenyl fatty acids as reported here, or transaminated to non-proteinogenic cyclopentenylglycine. The latter pathway was demonstrated in a concurrent study [7]. Recent experiments showed that α-ketopimelate, a mandatory intermediate of the $C_5 + 2 \times C_1$ pathway, is a precursor of cyclopentenyl fatty acids (Tober and Spener, unpublished results).

EXPERIMENTAL

Materials. Fresh leaves were obtained from: the Botanic Garden University of Hamburg, Germany, *Idesia polycarpa* Max., *Dovyalis caffra*, *Azara lanceolata* Hook. f., Botanic Garden University of Giessen, Germany, *Kiggelaria africana* L., *Azara celastrina* D. Don, *A. microphylla*, *Flacourtia cataphracta* Roxb., *F. sepiaria* Roxb., *Dovyalis hebecarpa* (Gardn.);; Botanic Garden Berlin–Dahlem, Germany, *Poliothyrsis sinensis* Oliv., *Oncoba spinosa* Forsk., *Flacourtia indica* (Burm. f.) Merr., *F. jangomas*, Botanic Garden University of Mayence, Germany, *Arechavaletaia uruguayensis* Speg., *Azara petiolaris* (D. Don) J. M. Johnst., *Xylotheca kraussiana* Hochst.; Botanic Garden University of Munich, *Flacourtia inermis* Roxb.: Foster Botanic Garden, Honolulu, Hawaii, *Hydnocarpus anthelminthica* Pierre, Lyon Arboretum University of Hawaii, Honolulu, Hawaii, *Hydnocarpus kurzii*; Belmonte Arboretum Wageningen, The Netherlands, *Caloncoba echinata* Oliv. Gilg.. *Corylus avellana* leaves were obtained locally.

Capillary-GC and MS. Leaf lipids were extracted according to ref. [18], converted to Me esters [19] and purified by TLC (Si gel G, hexane–Et_2O, 9:1). Fatty acid Me esters were analysed isothermally at 175° on a glass capillary column (40 m × 0.3 mm, i.d.), coated with FFAP; carrier gas H_2 (2.3 ml/min). Me esters were identified with the aid of reference compounds or by direct coupling of the capillary column to MS [20, 21]. The end of the coated glass capillary column was loosely connected, the end of the interface (heated glass capillary, 55 cm × 0.18 mm i.d., 1.59 mm o.d.) tightly connected with a 1.59 mm Swagelock union. The scavanger gas (He) was allowed to enter the union through extra metal tubing soldered to a small hole, which was drilled into the union. Solvent and fractions that must not reach the ion source were forced by the scavanger gas to leave the union at the side of the loosely connected glass capillary column. In addition, the scavenger gas prevented air from entering the ion source via the interface. The temp. of the ion source was 230°, spectra were scanned at 70 eV, and total ion current was set at 20 eV (double ion source).

Leaf expts. Detached leaves of *C. echinata* and of *C. avellana* were rinsed and wetted, and the substrates, dissolved in 0.5 ml H_2O, were taken up via the petiole under incandescent light. In addition, pre- and post-incubations (15 min each) with H_2O were carried out. Leaf lipids were then extracted and Me esters prepared as described above. Aliquots were taken to determine the radioactivity incorporated into total fatty acids by liquid scintillation counting. Carrier Me esters of cyclopentenyl fatty acids were added to the remaining soln and the mixture subjected to $AgNO_3$ chromatography (Si gel G/$AgNO_3$ 19:1 *w/w*, hexane–Et_2O 4:1, 4°) [22]. Individual Me ester fractions of straight-chain and cyclic fatty acids were isolated and the radioactivity of each fraction determined.

Chloroplast expts. Intact chloroplasts were isolated from 30–50 g portions of fr. leaves from *C. echinata* and *C. avellana* by the method of refs. [23] and [24], except that 845.1 mg $MnSO_4 \cdot H_2O$ were added to 10 ml suspension medium. The prepns were free of mitochondria, as shown by the lack of succinate: cytochrome-*c*-oxidoreductase (EC 1.3.99.1) [25]. Chlorophyll was determined according to ref. [26]. The chloroplast suspension (0.5 ml), stable for several hr at 4°, was combined with 0.5 ml cofactor mix (ATP, 4 mM; CoASH, 1 mM; NADPH, 0.2 mM; NADH, 0.2 mM) and aq. solns of substrates were added. Incubations were carried out at 27° for 1 hr under incandescent light with reciprocal shaking and stopped by the addition of 1 ml 10% KOH in 90% MeOH. After standing overnight at room temp., samples were acidified with 1.5–2 ml 2 N HCl. Fatty acids were extracted with 2 × 2 ml hexane per sample and converted to Me esters. Further work-up and determinations of radioactivity were carried out as described for leaf expts.

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