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Stereochemistry of oxidized fatty acids generated during catalytic oxygenation of lauric acid and unsaturated analogs by plant microsomes

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The capacity of microsomes from aminopyrine-induced Jerusalem artichoke (*Helianthus tuberosus* L.) to oxidize saturated and unsaturated fatty acids has been investigated using lauric acid and a series of unsaturated lauric acid analogs (7-, 8-, 9- and 10-dodecenoic acids) as radiolabeled substrates. In the presence of NADH, lauric acid was mono-hydroxylated principally at carbon 9. Steric analysis of this product showed a low enantiomeric excess of 28%. Mono-hydroxylated and mono-epoxidated reaction products were formed from the unsaturated analogs. The epoxidation/hydroxylation ratio was related to the position of the double bond in the aliphatic chain. The oxidation of 7-dodecenoic acid (7-DDNA) and 10-DDNA produced mainly 9-hydroxy-7-DDNA and 9-hydroxy-10-DDNA plus minor amounts of 7,8-epoxy- or 10,11-epoxylauric acid, respectively. In contrast, 8- and 9-DDNAs yielded essentially 8,9-epoxy- and 9,10-epoxylauric acids and smaller amounts of 10-hydroxy-9-DDNA and 8-hydroxy-9-DDNA, respectively. The optical purity and the absolute configuration of the major metabolites were investigated. Epoxidation of *Z* 8-DDNA and *Z* 9-DDNA occurs with high enantiomeric excesses. When the double bond was in the *Z* configuration, (8*S*,9*R*)/(8*R*,9*S*) 8,9-epoxylauric acid (93/7) or (9*R*,10*S*)/(9*S*,10*R*) 9,10-epoxylauric acid (89/11) were produced. In contrast, when the double bond was in the *E* configuration, steric analysis showed an enantiomeric ratio of 52/48 for *E* 8,9-epoxide and of 59/41 for *E* 9,10-epoxide. *Z* 7-DDNA led to the formation of 98% of the 9(*S*)-hydroxy-*Z* 7-DDNA enantiomer, while 9-hydroxy-*Z* 10-DDNA derived from *Z* 10-DDNA was 35% (*R*) and 65% (*S*).

Olefin; Fatty acid; Epoxidation; Hydroxylation; Stereochemistry; Cytochrome *P*-450; Induction; Microsome; Higher plant

1. INTRODUCTION

Plants contain numerous hydroxy- and epoxy fatty acids [1,2], some of which may play important biological roles [3–6]. To date, few studies on the stereoselectivity of oxygen transfer occurring in cytochrome *P*-450 reactions during fatty acid oxidation have been performed in plants [7]. Interest in the stereochemistry of *P*-450-catalyzed fatty acid oxidation had been stimulated by the fact that oxidized arachidonic acid derivatives exhibit several biological activities in mammals. The chirality of metabolites generated both in vitro and in vivo seems to be under *P*-450 enzyme control [8,9]. In plants, we have previously reported that microsomes from Jerusalem artichoke (*Helianthus tuberosus* L.) contain a cytochrome *P*-450 system that catalyses both in-chain hydroxylation of lauric acid [10] and epoxidation of *Z* and *E* 9-dodecenoic acids (DDNA) [11]. Similarly, we have found that the metabolism of unsaturated lauric acid analogs described in this paper involved microsomal *P*-450-dependent systems (to be published). Herein, we examine the stereochemical characteristics of the major hydroxy- and epoxy reaction products generated upon incubation of microsomes from aminopyrine-induced Jerusalem artichoke tubers with NADPH and radiolabeled 7-, 8-, 9- and 10-DDNA and lauric acids.

Abbreviations: RP-HPLC, reverse phase high pressure liquid chromatography; GC-MS, gas chromatography coupled to mass spectrometry; *n*-DDNA, dodecenoic acid with a number locating the position of double bond.

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2. MATERIALS AND METHODS

2.1. *Plant materials*

Jerusalem artichoke tubers (*Helianthus tuberosus* L., cv. Blanc commun) were sliced and aged in a 20 mM aqueous solution of aminopyrine for 48 h. Microsomal fractions were prepared as already described [11].

2.2. *Measurement of enzyme activities*

The lauric acid in-chain hydroxylase from artichoke was assayed as previously described [10,11]. The standard assay contained, in a final volume of 0.2 ml, 0.1–1 mg microsomal protein, 0.1 M sodium phosphate buffer, pH 7.4, 1 mM NADPH, 6.7 mM glucose-6-phosphate, 0.4 U glucose-6-phosphate dehydrogenase, 2.12 μM [1-^{14}C]lauric acid (59 Ci/mol) and aqueous sodium laurate to make the final concentration 100 μM. The same procedure was used when [1-^{14}C]DDNAs (17.7

Ci/mol) were incubated with microsomes except that a 100 μM final concentration was obtained using only radiolabeled substrates. The reactions were stopped after 20 min by adding 200 μl of acetonitrile containing 0.2% (v/v) acetic acid. Aliquots of the supernatant were injected into a RP-HPLC column after spooning proteins, or spotted on silica gel plates.

2.3. *Synthesis and purification of* n-[1-^{14}C]*DDNAs*

Radiolabeled compounds were synthesized according to established procedures [12,13]. The chemical synthesis led to a mixture of *Z* and *E* isomers showing the following *Z*/*E* ratios: 70/30 for 7-DDNA, 80/20 for 9-DDNA and 80/20 for 10-DDNA. 8-DDNA was over 93% *Z*. Separation of *Z* and *E* isomers was performed by RP-HPLC as already described [11,12]. Racemic samples of epoxides were obtained from their parent radiolabeled DDNAs using *m*-chloroperoxybenzoic acid. The corresponding diols were prepared by acidic hydrolysis of the epoxides. All compounds were purified by TLC and RP-HPLC according to published procedures. All reaction products gave satisfactory ^{1}H NMR (200 MHz).

The total synthesis of enantiomerically pure (8*R*,9*S*)-epoxydodecanoic acid methyl ester proceeded in eight steps, with the optically active epoxide introduced via an asymmetric Sharpless reaction as summarized below. All intermediates were characterized by ^{1}H and ^{13}C NMR, their infra-red spectra were recorded, and optical rotation was measured for optically active products. 1,7-Heptanediol was reacted with aqueous HBr and was continuously extracted with hexane over 2 days to yield crude 7-bromo-1-heptanol in 82% yield [13]. The bromo-alcohol was then oxidized to 7-bromo-heptanoic acid with potassium permanganate in aqueous sulfuric acid. The resulting acid was purified by silica gel chromatography yielding 88% of pure 7-bromo-heptanoic acid. The bromo-acid was added dropwise to the di-anion of propargylic alcohol at −30°C to give crude 10-hydroxy-8-decynoic acid [14], which was purified after conversion to its methyl ester with diazomethane, yielding 58% of 10-hydroxy-8-decynoic acid methyl ester. The hydroxy-alcyne was then hydrogenated to 10-hydroxy-*Z* 8-decanoic acid (90% yield) by means of P-2 Nickel [15,16]. In the next step, a Sharpless reaction [17] using (+)diethyltartrate gave 71% of 10-hydroxy-(8*R*,9*S*)-epoxydecanoic acid. The epoxy-alcohol was oxidized to the corresponding aldehyde using the Collins oxidation conditions [18] in the presence of cellite (62% yield). Wittig reaction with one equivalent of the ylide of ethyltriphenylphosphonium bromide in the presence of 20% HMPA (hexamethylphosphoramide) in THF yielded 55% of (8*R*,9*S*)-epoxy-10-dodecenoic acid methyl ester. Hydrogenation of the double bond was performed by means of 2,4,6-triisopropyl-benzenesulfonyl-hydrazide [19] yielding 92% of optically pure (8*R*,9*S*)-epoxydodecanoic acid methyl ester. The (9*R*,10*S*)-epoxydodecanoic acid methyl ester was synthesized following a similar procedure but starting with 1,8-octanediol and using methyltriphenylphosphonium bromide in the Wittig reaction.

2.4. *Derivatization of oxidized metabolites*

An etheral solution of diazomethane was used for methylation. Silylation was performed with a silylating reagent (*N*,*O*-bis-(trimethlysilyl)trifluoroacetamide + 1% trimethylchlorosilane) in pyridine (1:1) for 10 min at 70°C. Hydrogenation of unsaturated analogs was achieved using Pd/charcoal as catalyst. Methylester derivatives of the synthetic 9(*R*)-hydroxylaurate and of enzymatically formed hydroxylaurates were derivatized with the chiral reagent (*S*)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) as described by Dale and Mosher [20] leading to a diastereoisomeric mixture (*R*,*S* and *S*,*S*).

2.5. *Chromatographic conditions*

Metabolites produced were resolved by silica TLC plates (G60 F254, Merck) which were developed in a system of diethyl ether/light petroleum (b.p. 40–60°C)/formic acid (70:30:1, v/v/v). Reaction products were located using a radiometer thin-layer scanner (Berthold LB 2723). The areas corresponding to epoxylauric acids (R_f 0.6–0.7) and mono-hydroxydodecenoic acids (R_f 0.42–0.50) from incubations with *n*-DDNAs and 8-, 9- and 10-hydroxylauric acids (as a bulk, R_f 0.54) from incubation with lauric acid, were scraped, eluted with a mixture of diethyl ether/hexane (1:1, v/v) and subjected to RP-HPLC analysis.

RP-HPLC analysis was performed as described [11,12] using an initial mobile phase of acetonitrile/water/acetic acid (25:75:0.2; v/v/v) at a flow rate of 2 ml/min. A linear gradient (0–100%) of 80% acetonitrile in aqueous acetic acid (0.2%) was applied 35 min after injection with the same flow rate to elute residual substrates. Radioactivity was monitored by a computerized on-line solid scintillation counter (Ramona-D RAYTEST, Germany). Each oxidized metabolite was collected and re-subjected to RP-HPLC analysis to obtain pure compounds.

Chiral phase-HPLC analysis was performed using a Chiracel OB column (4.6 × 250 mm, J.T. Baker Chemical Co.). Racemic *n*-epoxylauric acid methyl esters were resolved using a mixture of hexane/2-propanol/acetic acid 98:2:0.1 (v/v/v) with a flow rate of 1 ml/min. Stereoisomers from *Z* 8,9-epoxylaurate were eluted from the column at 20 min (8*S*,9*R*) and 25 min (8*R*,9*S*) and from *Z* 9,10-epoxylaurate at 20.5 min (9*R*,10*S*) and 27.5 min (9*S*,10*R*). Stereoisomers (not characterized) from corresponding *E* epoxides were eluted at 18 and 22 min for *E* 8,9-epoxide and at 18.5 and 24 min for *E* 9,10-epoxide. Retention times were 15 and 17.5 min for *Z* 7,8-epoxides and 19 and 23 min for *Z* 10,11-epoxides. Radioactivity was monitored as described above or eluted fractions (0.5 ml/fraction) were collected and radioactivity measured with a liquid scintillation counter.

Normal-phase HPLC analysis was performed using a silica 5 μm ultrasphere column (4.6 × 250 mm, Beckman Co.). The synthetic 9(*R*)-hydroxylauric acid was mixed with the enzymatically formed [1-^{14}C]9-hydroxylauric acid and the mixture was derivatized before injection onto the column. The resulting diastereoisomers (*R*,*S* and *S*,*S*) of 9-hydroxylaurate were eluted from the column at 22 min (*R*,*S*) and 26.5 min (*S*,*S*) with a mixture of hexane/ethyl acetate (99:1, v/v) at a flow rate of 2 ml/min. The synthetic diastereoisomer (*R*,*S*) was detected by UV light (260 nm) and the radioactivity of enzymatically formed enantiomers was monitored as described above. Similar retention times were obtained when diastereoisomers (not determined) from 8- and 10-hydroxylaurate derivatives were eluted from the column.

2.6. *Gas Chromatography-mass spectrometry*

GC-MS analysis was carried out after methylation and silylation of products as described elsewhere [11]. Spectra were obtained in the EI mode at 70 or 20 eV for epoxide derivatives. Characterization of metabolites was performed by comparing retention times and mass spectra of metabolites with authentic references.

3. RESULTS AND DISCUSSION

At present, only limited studies are available concerning microsomal plant enzymes involved in fatty acid oxidation, and information on the chirality of metabolites generated are scarce. Herein we report the structure and the stereochemistry of novel metabolites produced from a series of unsaturated lauric acid analogs. Our results are summarized in Fig. 1. Microsomes from animopyrine-induced Jerusalem artichoke tubers [11] were used throughout this work. Lauric acid was hydroxylated at C8 (11%), C9 (62%) and C10 (27%) positions. These proportions are identical within error limits to those determined in incubations with microsomes from manganese- or phenobarbital-induced artichoke tubers [10].

For the first time absolute configurations of metabolites generated from a plant system catalyzing in-chain hydroxylations of medium-chain fatty acids have

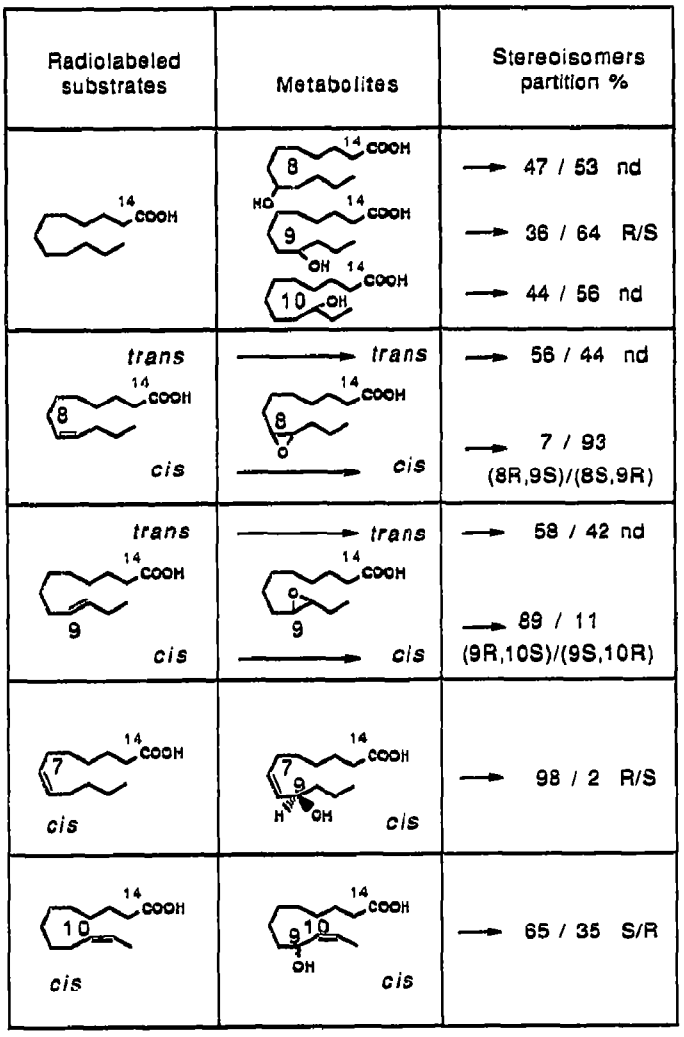

Fig. 1. Stereochemistry of major metabolites generated from lauric acid and unsaturated analogs incubated with *H. tuberosus* microsomes and NADPH. Values are the percent enantiomer distributions calculated from at least two different experiments with S.D. < 10%. ND, not determined.

been established. The absolute configuration of 9-hydroxylauric acid was found to be 64% (*S*) and 36% (*R*). Similar stereoselectivity had been observed in mammals for the (w-1)-hydroxylation of capric acid [21] and of lauric acid [22]. In addition, we demonstrate that the enantiomeric composition of 8-hydroxylaurate and 10-hydroxylaurate was almost racemic (enantiomeric excesses of 6 and 12%, respectively).

On the other hand, microsomes incubated with unsaturated analogs and NADPH catalyzed epoxidation as well as allylic hydroxylation (Table I) without rearrangement and with retention of the stereochemistry of the double bond (Fig. 1). The absolute configurations of *Z* 9,10-epoxylaurates formed from *Z* 9-dodecenoate was a mixture of 89% (9*R*,10*S*) and 11% (9*S*,10*R*) enantiomers. In contrast, the parent *E* 9,10-epoxylaurate

Table I

Distribution of *n*-hydroxydodecenoic acids and *n*-epoxylauric acids generated by *H. tuberosus* microsomes incubated with 7- to 10-dodecenoic acids

Substrates	Metabolites (%)	
	Epoxides	Hydroxy
7-DDNA	12 ± 3	88 ± 2
8-DDNA	81 ± 3	19 ± 4
9-DDNA	88 ± 7	12 ± 3
10-DDNA	24 ± 2	76 ± 2

Values are the means ± S.D., calculated from at least three different experiments. Results are expressed as percent of products formed. Hydroxylations occur: at C9 carbon for 7- and 10-DDNA; at C8 for 9-DDNA and C10 for 8-DDNA.

was a nearly racemic mixture exhibiting a low enantiomeric excess of 18%. These results are in agreement with those recently reported by Fahlstadius [7] who found a mixture of 78% (9*R*,10*S*) and 22% (9*S*,10*R*) enantiomers for the *Z* 9,10-epoxylaurate and a mixture of 57% (9*S*,10*S*) and 43% (9*R*,10*R*) enantiomers for the *E* 9,10-epoxylaurate. In addition, our results show that the *Z* 8,9-epoxylaurate produced from microsomal incubation of *Z* 8-DDNA was a mixture of 93% (8*S*,9*R*) and 7% (8*R*,9*S*) enantiomers. In contrast, the *E* 8,9-epoxylauric acid was generated with only 12% enantiomeric excess. These results demonstrate that shifting the double bond by one carbon position (i.e. 8-DDNA to 9-DDNA) results in the epoxidation of the opposite enantiotopic face of the double bond. In contrast, the *E* forms are oxidized on both *Re* and *Si* faces with a similar efficiency. These results are in sharp contrast with those observed with model porphyrins catalyzing epoxidation of *Z* and *E* olefins. Further interpretation of our results is difficult in the light of the complexity of the different possible reaction mechanisms of epoxydation as proposed in [23]. Interestingly, the 9-hydroxy-*Z* 7-DDNA generated from *Z* 7-DDNA was in a large enantiomeric excess of 98% (*R*) and 2% (*S*). In contrast, the 9-hydroxy-*Z* 10-dodecenoate from *Z* 10-DDNA was 65% (*S*) and 35% (*R*).

Although the absolute configuration of minor metabolites generated from *Z* 7-DDNA and *Z* or *E* 10-DDNA was not assigned, the enantiomeric excesses of epoxides have been calculated. Chiral analysis of *Z* 7,8-epoxy, *Z* and *E* 10,11-epoxides generated from their parent unsaturated fatty acids showed that each epoxide constituted enantiomeric mixtures exhibiting excesses of 28, 38 and 36%, respectively.

In conclusion, oxidation of lauric acid and a series of unsaturated (delta 7–10) analogs by microsomes from aminopyrine-induced *H. tuberosus* occurred with a high regioselectivity and concerned three carbon positions (C8–C10) of substrates. Microsomes catalyzed (through cytochromes *P*-450-dependent reactions) hydroxylation

and epoxidation of all substrates tested with total retention of the configuration. *Z* and *E* unsaturated analogs are metabolized with similar efficiency. Steric analysis showed that some metabolites were generated with a high degree of enantioselectivity while others were produced as nearly racemic mixtures. Unsaturation in *Z* configuration implicating the C9 carbon leads to the corresponding *Z* epoxides with a large enantiomeric excess. In contrast, the *E* epoxides produced were almost racemic. Interestingly and within error limits, *Z* 7-DDNA gave rise to a quasi optically pure 9*R*-hydroxy-*Z* 7-dodecenoate (98%) but the stereochemistry of 9-hydroxy-*Z* 10-dodecenoate, generated from the regioisomer *Z* 10-DDNA, was 35% (*R*) and 65% (*S*). Our results demonstrate that the oxidation of the allylic carbon C9 of these two substrates leads to the same chiral induction but with very contrasted enantiomeric excess. Thus, it appears that the position and the configuration of the double bond determines not only the structure of metabolites generated but also the face of attack. Although further investigations are necessary for the understanding of the chirality of reactions which may be due to the contribution of multiple forms of *P*-450, the system studied here provides a highly interesting example of the chemo-, regio- and stereospecificities of fatty acid oxidation by microsomal enzymes from plant.

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REFERENCES

[1] Badami, R.C. and Patil, K.B. (1981) Prog. Lipid Res. 19, 119–153.
[2] Sessa, D.J., Gardner, H.W., Kleiman, R. and Weisleder, D. (1977) Lipids 12, 613–619.
[3] Koiwai, A. and Matsuzaki, T. (1988) Phytochemistry 27, 2827–2830.
[4] Kato, T., Yamaguchi, Y., Hirano, T., Yokoyama, T., Uyehara, T., Namai, T., Yamanaka, S. and Harada, N. (1984) Chem. Lett. 409–412.
[5] Kolattukudy, P.E., Walton, T.J. and Kushwaha, R.P.S. (1971) Biochem. Biophys. Res. Commun. 42, 739–744.
[6] Kolattukudy, P.E. (1980) In: The Biochemistry of Plants. Lipids: Structure and Function (P.K. Stumpf and E.E. Conn, eds.) pp. 571–663, Academic Press, New York.
[7] Fahlstadius, P. (1991) Phytochemistry 30, 1905–1907.
[8] Capdevilla, J.H., Karara, A., Waxman, D.J., Martin, M.V., Falck, J.R. and Guengerich, F.P. (1990) J. Biol. Chem. 265, 10865–10871.
[9] Falck, J.R., Lumin, S., Blair, I., Dishman, E., Martin, M.V., Waxman, D.J., Guengerich, F.P. and Capdevila, J.H. (1990) J. Biol. Chem. 265, 10244–10249.
[10] Salaün, J.-P., Benveniste, I., Reichhart, D. and Durst, F. (1981) Eur. J. Biochem. 119, 651–655.
[11] Salaün, J.P., Weissbart, D., Durst, F., Pflieger, P. and Mioskowski, C. (1989) FEBS Lett. 246, 120–126.
[12] Weissbart, D., Salaün, J.-P., Durst, F., Pflieger, P. and Mioskowski, C. (1992) Biochim. Biophys. Acta (in press).
[13] Degering, E.F. and Boatright, G.J. (1950) J. Am. Chem. Soc. 72, 5137–5139.
[14] Cossy, J. and Pete, J.P. (1986) Tetrahedron Lett. 27, 573–574.
[15] Brown, C.A. and Ahuja, W.K. (1973) J. Chem. Soc. Chem. Commun. 553–554.
[16] Brown, C.A. and Ahuja, W.K. (1973) J. Org. Chem. 38, 2226–2230.
[17] Katsuki, T. and Sharpless, K.B. (1980) J. Am. Chem. Soc. 102, 5976–5978.
[18] Ratcliffe, R.W. (1975) Org. Synth. 55, 84–86.
[19] Cusack, N.J., Reese, C.B. and Roozpeikar, B. (1972) J. Chem. Soc. Chem. Commun. 1132–1133.
[20] Dale, J.A. and Mosher, H.S. (1973) J. Am. Chem. Soc. 95, 512–519.
[21] Hamberg, M. and Björkhem, I. (1971) J. Biol. Chem. 246, 7411–7416.
[22] Azerad, R., Boucher, J.L., Dansette, P. and Delaforge, M. (1990) J. Chromatog. 498, 293–302.
[23] Ostovic, D. and Bruice, T.C. (1988) J. Am. Chem. Soc. 111, 6511–6517.