A Linoleic Acid (8R)-Dioxygenase and Hydroperoxide Isomerase of the Fungus Gaeumannomyces graminis

Biosynthesis of (8R)-Hydroxylinoleic Acid and (7S,8S)-DiHydroxylinoleic Acid from (8R)-Hydroperoxylinoleic Acid*

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The fungus Gaeumannomyces graminis metabolized linoleic acid extensively to (8R)-hydroperoxylinoleic acid, (8R)-hydroxylinoleic acid, and three- (7S,8S)-dihydroxylinoleic acid. When G. graminis was incubated with linoleic acid under an atmosphere of oxygen-18, the isotope was incorporated into (8R)-hydroxylinoleic acid and 7,8-dihydroxylinoleic acid. The two hydroxyls of the latter contained either two oxygen-18 or two oxygen-16 atoms, whereas a molecular species that contained both oxygen isotopes was formed in negligible amounts. Glutathione peroxidase inhibited the biosynthesis of 7,8-dihydroxylinoleic acid. These findings demonstrated that the diol was formed in negligible amounts. Glutathione peroxidase inhibited the biosynthesis of 7,8-dihydroxylinoleic acid. These findings demonstrated that the diol was formed from (8R)-hydroperoxylinoleic acid by intramolecular hydroxylation at carbon 7, catalyzed by a hydroperoxide isomerase. The (8R)-dioxygenase appeared to metabolize substrates with a saturated carboxylic side chain and a 9Z-double bond. G. graminis also formed ω-2- and ω-3-hydroxy metabolites of the fatty acids. In addition, linoleic acid was converted to small amounts of nearly (65% R) racemic 10-hydroxy-8,12-octadecadienoic acid by incorporation of atmospheric oxygen. An unstable metabolite, 11-hydroxylinoleic acid, could also be isolated as well as (13R,13S)-hydroxy-(9E,9Z), (11E)-octadecadienoic acids and (9R,9S)-hydroxy-(10E), (12E,12Z)-octadecadienoic acids. In summary, G. graminis contains a prominent linoleic acid (8R)-dioxygenase, which differs from the lipoygenase family of dioxygenases by catalyzing the formation of a hydroperoxide without affecting the double bonds of the substrate.

The fungus Gaeumannomyces graminis is a fungal parasite of wheat. It causes the "take all disease" (1), which is responsible for damage to crops throughout the world, G. graminis and take all disease have been subject to numerous studies (1), but the metabolism of linoleic acid and other common fatty acids of plants by G. graminis has attracted little attention. Sih et al. (2) reported that G. graminis metabolizes arachidonic acid by ω-2- and ω-3-hydroxylation, but linoleic acid was not investigated. In retrospect, this may have been an omission.

* The abbreviations used are: (8R)-HODE, (8R)-hydroxyoctadeca-9Z,12Z-dienoic acid; 9-HODE, 9-hydroxyoctadeca-10E,12Z-dienoic acid; 10-HODE, 10-hydroxyoctadeca-9Z,12Z-dienoic acid; 11-HODE, 11-hydroxyoctadeca-9Z,12Z-dienoic acid; 12-HODE, 12-hydroxyoctadeca-9Z,12Z-dienoic acid; 13-HODE, 13-hydroxyoctadeca-9Z,12Z-dienoic acid; 14-HODE, 14-hydroxyoctadeca-9Z,12Z-dienoic acid; DiHODE, dihydroxyoctadecadienoic acid; DiHOME, dihydroxyoctadecadienoic acid; DihOTrE, dihydroxyoctadecatrienoic acid; GC-MS, gas chromatography-mass spectrometry; HPODE, hydroperoxyoctadecadienoic acid; HPLC, high-performance liquid chromatography; MeS, trimethylsilyl; RP, reverse-phase; RP-HPTLC, reverse-phase high performance thin-layer chromatography; SP, straight-phase; CP, chiral-phase; EGTA, [ethylenebis(oxyethylenenitril)]tetraacetic acid.

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was bought from Supelco, Inc. N,O-Bis(Si(CH3)2)acetamide was from Stohler Isotope Chemicals Inc. (Rutherford, NY). 18O2 (96%, 100 ml) was from Ion (Mt. Marion, NY). Cartridges with octadecasilane silica or with silica were from Waters (Milford, MA). Precoated TLC plates with silica gel (DC-Fertigplatten Kieselgel 60 F, thickness of 0.25 or 0.5 mm) or with octadecasilane silica (HPLTC-Fertigplatten RP-18; Merck). Racemization (19) and of 9-HODE and their E, E-isomers were obtained by autoxidation and purified by SP-HPLC (13). Al2O3 was from BDH Chemicals Ltd. (Poole, United Kingdom). (135)-HODE was obtained by biosynthesis using soybean lipoxigenase (type IV, Sigma) (6). Glutathione peroxidase (from bovine erythrocytes) was from Sigma.

cultivation—G. graminis was cultivated at room temperature (22 °C) in culture broth, which contained, per liter, 20 g of d-glucose and 5 g each of NaCl, K2HPO4, Bacto-Soytone, and yeast extract. Casamino acid medium was prepared similarly, but 5 g of casamino acids replaced the yeast extract. The medium was stirred gently, and the fungus was typically grown for at least 5 days under aerobic conditions. The fungus was harvested by filtration through a nylon net (100 μm). The mycelia were washed with saline, weighed wet, and either used directly or stored at −80 °C.

Preparation of Supernatants—The mycelia of G. graminis were sonicated, and the 100,000 × g supernatant was obtained with a modified polytron (17). Alternatively, the frozen mycelia were minced in a mortar (at +4 °C) with Al2O3 (half the weight of the fungus) (14) and −1.5 volumes of buffer (0.05 M Tris-HCl, pH 7.4, with 1 mM EDTA and 1 mM EGTA). The material was centrifuged (10,000 × g, 10 min at 4 °C), and the supernatant (0.05 mg of protein/mL) was used.

Incubation with Mycelia—The mycelia were incubated in 0.1 M sodium borate buffer, pH 8.2, with the fatty acids (added in a small volume of ethanol) for 2 h at room temperature (7). Mycelia (0.5–10 g, wet weight) were incubated with 7–50 mg of the fatty acid in 7–150 ml of borate buffer. In small-size experiments, only 30 mg of mycelia were incubated with 1–1°C(18:2-6)-0.4 μCi or 2 μg of exogenous 18:2-6 (60 μl) in 0.8 ml of borate buffer (30 min was usually sufficient for extensive metabolism of the substrate). Incubations under an atmosphere of O2 were performed after repeated evacuation and purging with nitrogen. The incubations were terminated by filtration, and the medium was acidified to pH 3 or, in some experiments, to pH 5 and extracted either with ethyl acetate or on a cartridge of octadecasilane silica as described (7). Over 20 incubations were performed with 18:2-6 and mycelia.

Incubation with Supernatants—The 100,000 × g supernatant was incubated with [18O]18:2-6 as described (7). NADPH (1 mM) was added in some experiments to assess formation of cytochrome P-450 metabolites. The 10,000 × g supernatant was incubated with [13C]18:2-6 (1.2 μCi/ml), glutathione (1 mM), and 0–10 units/ml glutathione peroxidase for 5 min on ice and for 20 min at 22 °C (water bath).

Separation of Metabolites—After extractive isolation, metabolites formed from 18:2-6 and [13C]18:2-6 were analyzed by RP-HPLC and SP-HPLC as described (7) or after methylation by preparative TLC or HPLC as described (18). The enantiomers of 9-HODE and 13-HODE methyl esters were separated by CP-HPLC as described (20).

Other Analyses—Radioactivity was determined by liquid scintillation (Beckman LS2800) using ACS (Amersham International) as a scintillation mixture. Protein concentrations were determined as described by Bradford (21) with bovine albumin as a standard.

RESULTS

Metabolism of 18:2-6 by G. graminis: Separation of Metabolites by RP-HPLC and TLC

18:2-6 was extensively metabolized both by the cell-free supernatant and by mycelia to mono- and dihydroxy compounds. In both cases, two of the main metabolites were (8R)-HODE and 7,8-DiHODE, which are identified below. Mycelia also converted 18:2-6 to ω2- and ω3-hydroxy metabolites as well as to ω2- and ω3-hydroxy metabolites of (8R)-HODE. These metabolites were also cell-free supernatants and NADPH.

On RP-HPLC, dihydroxy metabolites of [13C]18:2-6 eluted after 4–5 min (8,16-DIHOODE and 8,17-DIHOODE) and 8–9 min (mainly 7,8-DIHOODE), whereas the monohydroxy metabolites eluted after 14–17 min (mainly 8-HODE, 16-HODE, and 17-HODE). TLC analysis of methylated products formed by a large-scale incubation of 18:2-6 with mycelia showed six major bands in addition to the unmetabolized substrate. Rp values are given in Table I. The most polar band contained 8,17-DIHOODE methyl ester, the next 7,8-DIHOODE and 8,16-DIHOODE methyl esters, the third 17-HODE methyl ester, the fourth 8-HODE methyl ester and small amounts of slightly less polar products (16-HODE, 10-HODE, and 11-HODE methyl esters), the fifth 9-HODE and 13-HODE methyl esters, and the sixth, least polar band 8-HPHODE methyl ester. Bands 4–6 were only partly resolved. The monohydroxy metabolites of one representative incubation were roughly quantified by integration of a gas chromatogram, 8-HODE consti-
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TABLE I

| TLC band        | Rp value | C-value |
|-----------------|----------|---------|
| Band 1, 8,17-DiHODE | 0.07     | 21.7    |
| Band 2          |          |         |
| 7,8-DiHODE      | 0.12     | 20.8    |
| 8,16-DiHODE     | 0.31     | 20.4    |
| Band 3, (17R)-HODE | 0.37 (0.35-0.39) | 19.1 |
| Band 4          |          |         |
| (8R)-HODE       | 0.37 (0.35-0.39) | 19.1 |
| 10-HODE         | 0.41 (0.39-0.43) | 19.8 |
| 11-HODE         | 0.44 (0.43-0.46) | 19.6 |
| 16-HODE         |          |         |
| Band 5          |          |         |
| 9-HODE          | 0.41 (0.39-0.43) | 19.8 |
| 13-HODE         |          |         |
| Band 6, (8R)-HPODE | 0.44 (0.43-0.46) | 19.6 |

*These compounds were separated by RP-HPTLC (methanol/water, 9:1) with Rp = 0.56 for (7S,8S)-DiHODE methyl ester and Rp = 0.69 for 8,16-DiHODE methyl ester; Rp = 0.42 for 8-HODE methyl ester.

In contrast to this complex mixture of metabolites formed by mycelia, incubation of the cell-free supernatant with [14C] 18:2n-6 (and without NADPH) resulted in only three bands of radiolabeled metabolites on TLC as shown in Fig. 1A. These were identified as 7,8-DiHODE, 8-HODE, and 8-HPODE methyl esters by cochromatography with standards. The [13C]-labeled material marked 8-HPODE methyl ester in Fig. 1A (8-HPODE-Me) co-migrated with (8R)-HODE methyl ester on TLC when reduced with SnCl₂. The formation of 7,8-DiHODE could be completely inhibited by glutathione peroxidase (see Fig. 1B and text below).

Identification of (8R)-Dioxygenase Metabolites of 18:2n-6

(8R)-HPODE Methyl Ester — This compound was identified in band 6 on TLC. Treatment with SnCl₂ changed its polarity on TLC to that of (8R)-HODE methyl ester. GC-MS analysis of the same amount of material of band 6 before and after reduction with SnCl₂ and derivatization is shown in Fig. 2. Chemical reduction with SnCl₂ increased the amount of (8R)-HODE methyl ester dramatically.

(8R)-HODE Methyl Ester — This compound was identified in band 4. The mass spectrum (Me₃Si ether methyl ester derivative) was as previously reported (7). In addition, some GC-MS analysis showed the presence of an isomer with a slightly larger C-value (Fig. 2A). The mass spectrum of this compound and (8R)-HODE methyl ester was almost identical, and it was tentatively identified as a Z,E-isomer of 8-HODE methyl ester. Incubation under an atmosphere of oxygen-18 led to the incorporation of this isotope into 8-HODE as shown by GC-MS analysis (Fig. 2A). The incorporation of oxygen-18 into (8R)-HODE was ~48% (average value calculated from the intensity of several structural ions). It is noteworthy that (8R)-HODE also is an endogenous constituent of the fungus (see below).

7,8-DiHODE Methyl Ester — The material in band 2 was derivatized to its Me₃Si ether methyl ester and analyzed by mass spectrometry. The major metabolite of band 2 was identified as 7,8-DiHODE methyl ester, but this band also contained smaller amounts of 8,16-DiHODE methyl ester (see below); the two compounds were separated by capillary gas chromatography. The mass spectrum (Fig. 2A) of the former compound was obtained and showed signals at m/z 470 (M⁺), 455 (M⁺ - 15), 439 (M⁺ - 31), 381 (MH⁺ - 90), and 231 (CH₃OOCH₂CH=O⁺-Si(CH₃)₃, the base peak) as well as many other ions (e.g. m/z 73, 127, 199, 269, and 291). The molecular ion and the fragmentation indicated a dihydroxy metabolite of 18:2n-6. The metabolite also formed an n-butylboronic acid derivative. The mass spectrum (C-
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FIG. 3. Mass spectrometric analysis of products formed from 18:2n-6 by mycelia of G. graminis under oxygen-18 gas. A, mass spectrum of (8R)-HODE; B, mass spectrum of (7S,8S)-DiHODE (left) and magnified signals in the upper mass range (right). Me$_3$Si (TMS) ether methyl ester derivatives, electron impact ionization. The value of 22.0 showed important signals at m/z 392 (5%, M$^+$), 361 (2%, M$^+$ - 31), 335 (5%, M$^+$ - 57, loss of C$_{3}$H$_{6}$O), 308 (base peak, M$^+$ - 84, loss of C$_{3}$H$_{4}$BO), 294 (90%), 290 (20%, C$_{4}$H$_{9}$B(OH)$_{2}$), and 277 (30%, M$^+$ - (84 + 31)) and, in the lower mass range, at m/z 136 (80%), 157 (60%), and 179 (85%). This indicated a 1,2- or 1,3-dihydroxy metabolite. The Me$_3$Si ether methyl ester derivative of the hydrogenated compound (Fig. 4B) showed ions at m/z 474 (M$^+$), 459 (M$^+$ - 15), 443 (M$^+$ - 31), 385 (M$^+$ - 90), 301 (CH$_{3}$OCC(CH$_{3}$)$_{2}$CH=O-Si(CH$_{3}$)$_{3}$), and 243 (M$^+$ - 231) as well as many other ions (e.g. m/z 113, 171, 199, 289, and 304). The base peak was m/z 73. These data are in accordance with the compound being 7,8-DiHODE methyl ester.

Identification of Monooxygenase Metabolites of 18:2n-6—Monooxygenase metabolites were formed when all the fatty acids were incubated with mycelia or with the 100,000 × g supernatant and NADPH (7). 17-HODE and 16-HODE Methyl Esters—17-HODE methyl ester was identified in band 3, whereas 16-HODE methyl ester was identified as a minor compound in band 4 (together with the main product, (8R)-HODE methyl ester). The mass spectra of their Me$_3$Si ether methyl ester derivatives and their C-values were as published (7). The C-values of the cyclic carbonate derivative of 2,3-dihydroxyxundecane-1,11-dioates were 16.68 and 16.66, respectively. From this experiment, it was obvious that 7,8-DiHODE had the threeo-configuration at C(7)-C(8) and also that the compound possessed a double bond in the A' position. Since this compound was formed from (8R)-HPODE, its absolute configuration was deduced to be (7S,8S)-dihydroxylinoleic acid. The presence of a hydroxyl at carbon 7 changes the priority of the carbon atoms according to the Cahn-Ingold Prelog nomenclature. The position of the 8-hydroxyl is thus the same in (8R)-HODE and (7S,8S)-DiHODE.
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HODE was confirmed to be $R$ (in agreement with the absolute configuration of (19R)- and (18R)-hydroxyeicosatetraenoic acids formed from arachidonic acid by $\omega_2$- and $\omega_3$-hydroxyl-ation of this fungus) (22).

8,1\textsuperscript{7}DiHODE Methyl Ester—The main product in band 1 was identified as 8,17-DiHODE methyl ester by the mass spectrum of its Me\textsubscript{3}Si ether derivative. The mass spectrum showed signals at $m/z$ 470 (M\textsuperscript{+}), 455 (M\textsuperscript{+} - 15), 439 (M\textsuperscript{+} - 31), 380 (M\textsuperscript{+} - 90), 284 (a characteristic ion, possibly formed through rearrangement), 327 (cleavage between C-7 and C-8, a weak signal), and 117 (cleavage between C-16 and C-17) as well as many other ions (e.g. $m/z$ 147, 197, 211, 237, 271, and 368). The base peak was $m/z$ 73. The $d_9$-Me\textsubscript{3}Si ether methyl ester derivative and the mass spectrum of the compound after hydrogenation (Me\textsubscript{3}Si ether methyl ester derivative) gave consistent results with the compound in question being 8,17-DiHODE methyl ester.

8,1\textsuperscript{6}DiHODE Methyl Ester—8,16-DiHODE methyl ester was identified in TLC band 2 by the mass spectrum of its Me\textsubscript{3}Si ether derivative. It could be separated from 7,8-DiHODE methyl ester by RP-HPTLC (Table I) or as free acids by RP-HPLC (see above). The mass spectrum showed signals at $m/z$ 470 (M\textsuperscript{+}), 455 (M\textsuperscript{+} - 15), 439 (M\textsuperscript{+} - 31), 380 (M\textsuperscript{+} - 90), 284 (a characteristic ion, possibly formed through rearrangement), 327 (loss of CH\textsubscript{3}OOC-(CH\textsubscript{2})\textsubscript{6}-CHOSi(CH\textsubscript{3})\textsubscript{3}), and 131 (CH\textsubscript{3}CH\textsubscript{2}CH=O-Si(CH\textsubscript{3})\textsubscript{3}) and other ions (e.g. $m/z$ 147, 171, 217, 237, and 351). The base peak was $m/z$ 73. The $d_9$-Me\textsubscript{3}Si ether methyl ester derivative and the mass spectrum of the compound after hydrogenation (Me\textsubscript{3}Si ether methyl ester derivative) supported the structure.

Identification of Minor Metabolites of 18:2n-6

10-HODE Methyl Ester—This compound was one of the many compounds in the upper half of band 4, and it could be purified from (8R)-HODE methyl ester (but not from 16-HODE methyl ester) by repeated TLC. The mass spectrum is shown in Fig. 5A (left). The hydroxyl oxygen was derived from the atmosphere as shown by the incorporation of 43% oxygen-18 (Fig. 5A, right). Hydrogenation yielded 10-hydroxyoctadecanoic acid (Fig. 5B). Ozonolysis of the (-)-menthoxycarbonyl derivative of 10-HODE methyl ester yielded the (-)-menthoxycarbonyl derivative of malate. The absolute configuration was 65% (R)-malate and 35% (S)-malate. Ozonolysis also determined the position of the double bonds, and the metabolite was thus identified as 10-hydroxyoctadec-8,12-dienoic acid.

11-HODE Methyl Ester—This compound could be isolated from incubations, which were extracted at pH 5-6. At pH 3, 11-HODE is rearranged to racemic 13-HODE and 9-HODE.\textsuperscript{2} The structure of 11-HODE was determined from GC-MS analysis (Me\textsubscript{3}Si ether methyl ester derivative) before and after hydrogenation (Fig. 5, C and D) and by its C-value. The mass spectrum showed characteristic ions at $m/z$ 382 (M\textsuperscript{+}), 367 (M\textsuperscript{+} - 15), 351 (M\textsuperscript{+} - 31), 292 (M\textsuperscript{+} - 90), 225 (loss of C(1)-

\textsuperscript{2}Hamberg, M., Gerwick, W. H., and Åsen, P. A. (1992) Lipids, in press.
and the mass spectrum showed two strong and informative signals at m/z 201 (CH$_3$OCC(CH$_3$)$_4$CH=O$^+$-Si(CH$_3$)$_3$) and 287 (M$^+$ - 99, loss of C(11)-C(18)). These data were consistent with those from the original description of 11-HODE.

13-HODE and 9-HODE Methyl Esters—13-HODE methyl ester and its E,E-isomer were the main constituents of band 5, which also contained smaller amounts of 9-HODE methyl ester (and in some experiments, its E,E-isomer). 9-HODE and 13-HODE methyl esters were not separated by capillary gas chromatography, but were resolved by SP-HPLC. CP-HPLC showed that these compounds were nearly racemic. Furthermore, relatively little oxygen-18 (<20%) was incorporated into 13-HODE, its E,E-isomer, and 9-HODE. This was in contrast to 43–47% oxygen-18, which was incorporated into (8R)-HODE, 10-HODE, or 7,8-DiHODE in the same experiment. This indicated that 9-HODE and 13-HODE could have been formed by autoxidation of 18:2-6 during the isolation procedure or by another mechanism. TLC analysis of 18:2-6 showed no evidence of autoxidation products, but small amounts cannot be excluded (13) and could also be formed after termination of the experiment. Nevertheless, this finding led to the attempts described above to isolate 11-HODE, which can be rearranged nonenzymatically to 9-HODE and 13-HODE as discussed above.

Substrate Requirements of 18:2-6 (8R)-Dioxygenase and Hydroperoxide Isomerase

The substrate requirements were assessed by incubation of mycelia with a series of C$_8$ and C$_9$ fatty acids. The products were first analyzed by TLC and then by GC-MS. 18:2-6 appeared to be more efficiently metabolized by the (8R)-dioxygenases than by the other substrates as judged from TLC. Practically all fatty acids were metabolized by w$_2$- and/or w$_3$-hydroxylation, and these metabolites were identified by GC-MS.

9Z-18:1n-9—Metabolites of oleic acid, which could be separated by TLC into three bands, were identified by their mass spectra as 7,8-DiHODE (R$_f$ value of 0.12, C-value of 20.8), 17-HOME (R$_f$ value of 0.31), and 8-HOME (R$_f$ value of 0.37, C-value of 19.0). 7,8-DiHODE (Me$_3$Si ether methyl ester derivative) had a mass spectrum similar to that of 7,8-DiHOME, but some ions were increased by 2 mass units, e.g. at m/z 457 (M$^+$ - 15), 441 (M$^+$ - 31), and 383 (M$^+$ - 90), whereas other ions were unchanged, e.g. m/z 231 (CH$_3$OOC-CH$_3$-CH=O$^+$-Si(CH$_3$)$_3$), the base peak), 73, 113, 147, and 199.

18:3n-3—Incubations of 18:3n-6 resulted in formation of at least five products. They were separated by TLC, and the three major metabolites were identified by their mass spectra as 7,8-DiHOME (R$_f$ value of 0.12, C-value of 20.7), 8-HOME (R$_f$ value of 0.37, C-value of 19.2), and 17-HOME (R$_f$ value of 0.31; Ref. 7). The mass spectrum of 8-HOME was reported previously (7).

Mass Spectrum of 7,8-DiHOTrE—The Me$_3$Si ether methyl ester derivative had a mass spectrum similar to that of 7,8-DiHODE, but some ions were decreased by 2 mass units. The spectrum showed signals at m/z 468 (M$^+$), 453 (M$^+$ - 15), 437 (M$^+$ - 31), 379 (M$^+$ - 90), and 231 (CH$_3$OOC-(CH$_3$)$_5$-CH=O$^+$-Si(CH$_3$)$_3$), the base peak) as well as many other ions (e.g. m/z 73, 113, 147, 171, 217, and 269).

11Z-18:1n-7—cis-Vaccenonic acid was metabolized to 10-hydroxy-cis-vaccenonic acid with an R$_f$ value of 0.42, but the 9,10-diol of cis-vaccenonic acid could not be detected.

(12R)-Hydroxyoctadec-9Z-enoic Acid—Ricinoleic acid was oxygenated by mycelia to 8-hydroxyricinoleic acid. Small amounts of 7-hydroxyricinoleic acid were also found (R$_f$ values: 8.12-DHOME, 0.32; 7,12-DHOME, 0.38; 12,17-DHOME and 12,18-DHOME, 0.25). 7,8-Dihydroxyricinoleic acid could not be detected. The mass spectrum of the hydroxylated product was more informative with signals at m/z 459 (M$^+$ - 15), 389 (M$^+$ - 85), 384 (M$^+$ - 90), 331 (M$^+$ - 143, loss of CH$_3$OOC-(CH$_3$)$_3$), and 245 (CH$_3$OOC-(CH$_3$)$_5$-CH=O$^+$-Si(CH$_3$)$_3$) and, in the lower mass range, at m/z 187 and 73 (base peak).

7-Hydroxyricinoleic acid methyl ester was tentatively identified as a less polar metabolite than 8-hydroxyricinoleic acid methyl ester by TLC. It showed a mass spectrum (Me$_3$Si ether derivative) with a fragmentation in the upper mass range similar to that of 8-hydroxyricinoleic acid (M$^+$ - 15, M$^+$ - 31, MH$^+$ - 90). The 7-hydroxy metabolite showed a strong signal at m/z 231 (CH$_3$OOC-(CH$_3$)$_3$-CH=O$^+$-Si(CH$_3$)$_3$), and the base peak was m/z 187.

18:0, 9E-18:1n-9, 18:3n-6, 20:3n-6, 20:4n-6, 20:5n-3, and Methyl Ester of 18:2n-6—None of these fatty acids were substrates of the (8R)-dioxygenase.

Endogenous Hydroxy Fatty Acids of G. graminis

The endogenous hydroxy fatty acids were not studied systematically, but large amounts of (8R)-HODE and (7S,8S)-DiHODE were present in the fungus (extracted mycelia or the 100,000 × g supernatant) and were determined by GC-MS. Smaller amounts of 8-HOME, 7,8-DHOME, and 7,8-DIHOME were also found to be endogenous products of the fungus and could even be isolated from the growth medium after harvesting of the fungus. Our culture broth, based on yeast extract and hydrolyzed soy meal, presumably provided the fungus with 18:1n-9, 18:2n-6, and 18:3n-3.

Reproducibility

The enzymatic activity of the two strains of G. graminis (CBS 903 and 904) were compared for (8R)-dioxygenase activity with 18:2n-6 as a substrate. As judged from TLC, no qualitative difference between them could be noticed. (8R)-HODE and (7S,8S)-DiHODE were formed by both strains. The w$_2$- and w$_3$-hydroxylase activities of the same strain of G. graminis (CBS 903) varied considerably with time. Similar observations with cytochrome P-450 in fungi have been reported by many investigators (10, 14). This could be due to variations in oxygen tension, in the composition of the culture broth, and of the growth phase of the fungus (10). In one experiment with mycelia, 17-HODE and 16-HODE were very prominent relative to the formation of (8R)-HODE, possibly due to a technical mistake in the preparation of the culture broth (discolored due to a Maillard reaction). G. graminis was also grown on a more defined medium that was based on casamino acids instead of yeast extract. Under these conditions, G. graminis metabolized 18:2n-6 to (8R)-HODE and (7S,8S)-DiHODE in the same experiment or by another mechanism. TLC analysis of the endogenous hydroxy fatty acids was not studied systematically, but large amounts of (8R)-HODE and (7S,8S)-DiHODE were present in the fungus (extracted mycelia or the 100,000 × g supernatant) and were determined by GC-MS. Smaller amounts of 8-HOME, 7,8-DHOME, and 7,8-DIHOME were also found to be endogenous products of the fungus and could even be isolated from the growth medium after harvesting of the fungus. Our culture broth, based on yeast extract and hydrolyzed soy meal, presumably provided the fungus with 18:1n-9, 18:2n-6, and 18:3n-3.

DISCUSSION

The main finding of this study is that (8R)-HODE is formed from an intermediate hydroperoxide, (8R)-HPHODE. This hydroperoxide may also be isomerized enzymatically to (7S,8S)-DiHODE. This metabolism of 18:2n-6 by G. graminis is summarized in Fig. 6. The sequence was deduced from...
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experiments under oxygen-18 gas and with glutathione peroxidase and from the isolation of 8-HPODE. Both (8R)-HODE and (7S,8S)-DiHODE occur endogenously in G. graminis when grown on soy meal and yeast extract.

Racemic 8-HODE was reported to possess antifungal activity, and the fungicidal effect of L. arvalis was attributed to its biosynthesis of 8-HODE (11). The mechanism of biosynthesis of 8-HODE by L. arvalis was not determined, but it seems possible that it is also derived from (8R)-HPODE. This study suggests that (8R)-HPODE, and possibly also (7S,8S)-DiHODE, should be analyzed for fungicidal and other biological activities. G. graminis may produce (8R)-HPODE for the same objective as L. arvalis, namely as a defense against certain fungi or other microorganisms.

The (8R)-dioxygenase differs from lipoxygenases in its mechanism of oxygenation. Lipoxygenases abstract a hydroxyl from one carbon atom and insert molecular oxygen after radical migration, whereas (8R)-dioxygenase catalyzes both substrate and position specificity as discussed above. However, 11-HODE was of particular interest for another reason. 11-HODE was unstable at an acidic pH, where it is rearranged to racemic 9-HODE and isomerase activity isomerase have slightly different substrate requirements, a point that will need further evaluation.

In agreement with previous reports, G. graminis also contains prominent 2α-(R)- and 3α-(R)-hydroxylase activities (2, 7, 22), and all fatty acids of this study were metabolized by these hydroxylases. These products are likely to be formed by cytochrome P-450 and have been described in a large number of fungi (10).

Certain oxygenated metabolites of polysaturated fatty acids can be formed by autoxidation, including (8R)-HODE. Autoxidation of 18:2n-6 will result in formation of small amounts of racemic 8-H(P)ODE (and 14-H(P)ODE), ~1% of the amount of Z,E- and E,E-isomers of 13-H(P)ODE and 9-H(P)ODE (6, 27). Other oxygenated products of 18:2n-6, which are not formed by lipid peroxidation in significant amounts, are 10-HODE and 11-HODE. These two compounds were isolated during incubations with G. graminis and were therefore likely to be formed enzymatically, although in relatively small amounts.

10-HODE was found to contain atmospheric oxygen in its hydroxyl. Bisosynthesis of 10-HPODE as well as enzymatic cleavage products of the hydroperoxide has been described in the mushroom Psalliota bispora (28, 29), but these metabolites could not be detected in G. graminis. The mechanism of biosynthesis of 11-HODE in G. graminis is unknown. (11R)-HODE was originally described as a metabolite of 18:2n-6 in a red alga, Lithothamnion corallioides, which does not contain (8R)-dioxygenase. Nevertheless, it is possible that this unstable metabolite in G. graminis might be a minor side product of the (8R)-dioxygenase that does not show an absolute substrate specificity as discussed above. However, 11-HODE was of particular interest for another reason. 11-HODE is unstable at an acidic pH, where it is rearranged to racemic 9-HODE and 13-HODE. The biosynthesis of 11-HODE could therefore to some extent explain why 13-HODE and 9-HODE, which were isolated from an incubation under oxygen-18, contained less oxygen-18 than the other oxygenated metabolites and were therefore racemic.

Lipoxygenase metabolites of polysaturated fatty acids are conveniently monitored during chromatography by the typical UV absorption of conjugated double bonds (23). 8-HPODE and its metabolites lack a conjugated double bond and are therefore more difficult to detect. Whether (8R)-dioxygenase or related dioxygenases are present in higher organisms cannot be determined at the present stage and is worthy of future investigations.

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Fig. 6. Summary of metabolism of 18:2n-6 by (8R)-dioxygenase and hydroperoxide isomerase of G. graminis.
Linoleic Acid (8R)-Dioxygenase and Hydroperoxide Isomerase

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