FOUR FUNGITOXIC C-18 HYDROXY UNSATURATED FATTY ACIDS
FROM STROMATA OF <u>EPICHLOE TYPHINA</u>

Hiroyuki Koshino, Satoshi Togiya, Teruhiko Yoshihara, Sadao Sakamura
Department of Agricultural Chemistry, Faculty of Agriculture
Hokkaido University, Sapporo 060, Japan
Tadayuki Shimanuki
National Grassland Research Institute, Nishinasuno 329-27, Japan
Tohru Sato, Akitoshi Tajimi
Hokkaido National Agricultural Experiment Station, Sapporo 061-01, Japan

Summary: Four fungitoxic C-18 hydroxy unsaturated fatty acids (1)~(4) have been isolated
from stromata of <u>Epichloe typhina</u>. Their structures including the absolute configuration
have been elucidated from spectral data.

The timothy plant (<u>Phleum pratense</u>) infected by a phytopathogenic fungus, <u>Epichloe
typhina</u>, has been found to be resistant against another timothy leaf spot disease pathogen
<u>Cladosporium phlei</u> [1]. We have initiated a study of the fungitoxic compounds of the stroma
of the fungus which has been called choke, because it is likely that such compounds act as
fungitoxins against <u>C</u>. <u>phlei</u>, and isolated three fungitoxic sesquiterpenes, chokol A, B and
C, from timothy chokes[2]. This paper describes the isolation and structure determinations
of four fungitoxic C-18 hydroxy unsaturated fatty acids (1)~(4).

The <u>n</u>-hexane soluble fraction from 70 % EtOH extract of chokes (20 Kg) was fractionated
by repeats of SiO$_2$ column and further by Lobar RP-8 column (80 % MeOH - H$_2$O) chromato-
graphies. Purification by HPLC on μBONDAPAK C-18 column using 60 % CH$_3$CN - H$_2$O gave
compound 1. The fraction containing compounds 2~4 was methylated with CH$_2$N$_2$ and purified
by HPLC[3], resulting in the isolation of methyl esters 2a~4a. TLC bioautography[4] with
<u>Cladosporium herbarum</u> was employed to monitor the activity.

Compound 1, $[\alpha]_D^{23}$ -2.4° (c=0.25, EtOH), had a molecular formula of C$_{18}$H$_{32}$O$_3$, from
FD-MS m/z 297 (MH$^+$) and the high resolution EI-MS m/z 278 (M$^+$ - H$_2$O, 278.2250, Calcd.
278.2245). The IR spectrum exhibited the presence of carboxyl (3300~2500, 1720 cm^{-1}) and
hydroxyl groups (3370 cm^{-1}). The ^{1}H-NMR (500 MHz, CDCl$_3$) spectrum[5] showed the presence of
an allyl hydroxyl group, 4.09 ppm (1H, ddd), and two olefinic double bonds, 5.36 and 5.55
ppm (J=10.8 Hz) for <u>cis</u>, and 5.48 and 5.65 ppm (J=15.1 Hz) for <u>trans</u> double bonds.
The 3H triplet signal at 0.89 ppm was assigned to the terminal methyl group and the 2H
triplet at 2.34 ppm indicated that the carboxyl group was present at the other terminal.
These data suggested that 1 was a hydroxy unsaturated fatty acid having a straight skeleton,
and the results of detailed spin decoupling experiments revealed the presence of the
partial structures shown in Fig. 1.

The positions of the hydroxyl group and double bonds were determined by mass fragmen-
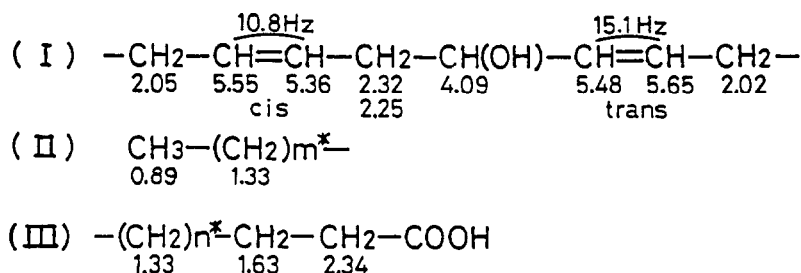tations of methyl ester (1a) obtained by CH$_2$N$_2$ treatment. EI-MS spectrum[6] of 1a exhibited
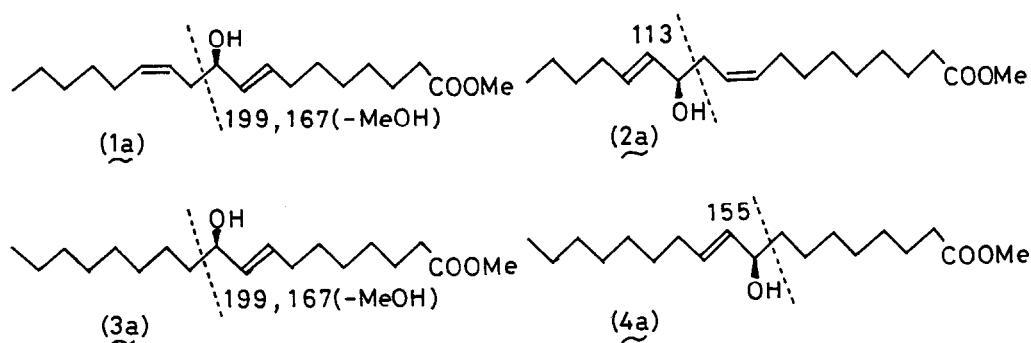
$$(I) \quad -CH_2-\overset{10.8Hz}{\overbrace{CH=CH}}-CH_2-CH(OH)-\overset{15.1Hz}{\overbrace{CH=CH}}-CH_2-$$

2.05 5.55 5.36 2.32 4.09 5.48 5.65 2.02

cis 2.25 trans

$$(II) \quad CH_3-(CH_2)m^*-$$

0.89 1.33

$$(III) \quad -(CH_2)n^*CH_2-CH_2-COOH$$

1.33 1.63 2.34

Fig. 1. *m+n = 6

(1a)

(2a)

(3a)

(4a)

the prominent peaks, m/z 199 ($C_{11}H_{19}O_3$) and m/z 167 ($C_{10}H_{15}O_2$), caused by cleavage adjacent to the hydroxyl group[7]. Thus a structure of compound 1 was elucidated to be 10-hydroxy-8E, 12Z-octadecadienoid acid.

Methyl ester (2a) of compound 2 showed $[\alpha]_D^{23}$ -6.25° (c=0.32, EtOH) and the same molecular formula of $C_{19}H_{34}O_3$ as 1a. In the ^{1}H-NMR spectrum[8] it was indicated to possess the same partial structures (I~III) as compound 1. These results suggested that compound 2 was different from 1 by the combination of partial structures. EI-MS spectrum[8] of 2a showed an intensive ion peak at m/z 113 ($C_7H_{13}O$). Thus a structure of compound 2 was led to be 12-hydroxy-9Z,13E-octadecadienoic acid.

Methyl ester (3a) of compound 3 showed $[\alpha]_D^{23}$ -1.48° (c=0.27, EtOH) and a molecular formula of $C_{19}H_{36}O_3$ (M^+ 312). The ^{1}H-NMR spectrum[9] exhibited the presence of an allyl hydroxyl group, 4.03 ppm (1H, m), and a trans double bond, 5.44 and 5.62 ppm (J=15.4 Hz). EI-MS spectrum[9] of 3a showed the same fragmentations as 1a, (m/z 199 and 167). Based on the above spectral data the structure of compound 3 was determined as 10-hydroxy-8E-octadecenoic acid.

Methyl ester (4a) of compound 4, $[\alpha]_D^{23}$ -2.14° (c=0.28, EtOH), had the same molecular formula $C_{19}H_{36}O_3$ as 3a. The ^{1}H-NMR spectrum[10] of 4a was similar to that of 3a. But the EI-MS fragmentations[10] were different from 3a and had a strong peak at m/z 155 ($C_{10}H_{19}O$). On the basis of these evidences compound 4 was identified as 9-hydroxy-10E-octadecenoic acid, an isomer of 3.

The absolute configurations of four hydroxy fatty acids were determined by CD spectra of the corresponding benzoates[11] which were prepared with ($C_6H_5CO)_2O$, Et_3N and catalytic amounts of dimethylaminopyridine. All the CD spectra exhibited negative Cotton effect at

229 nm, thus indicating the R-configuration of the chiral carbon in each compound[12].

To our knowledge there have been investigations on spectral data of derivatives of 1~4 which were formed by photosensitized oxidation[7,13]. But the absolute stereochemistry and the isolation of 1~4 from natural source have never been reported.

Compound 1 and methyl esters 1a~4a inhibited the growth of C. herbarum at the amount of more than 50~100 μg / one spot on TLC plate[4,14].

Recently several C-18 oxygenated unsaturated fatty acids have been reported as the self-defensive substances in rice plants against rice blast disease[15]. The fatty acids (1~4) also can be regarded as the substances related to resistant mechanism of infected timothy plants by Epichloe typhina against Cladosporium phlei.

It is generally known that lipoxygenase enzymes from various plant sources catalyze the formation of 9S- and 13S-hydroperoxides from linoleic acid and do not oxidize oleic acid. In this study presumably the isolated fatty acids 1 and 2 are respectively formed by oxidation at C_{10} and C_{12} position of linoleic acid. And further, formation of fatty acids 3 and 4 indicates the oxidation of oleic acid. So occurrence of those acids (1~4) in the timothy chokes suggests the participation of an enzyme system differed from that in rice plants for instance.

REFERENCES AND FOOTNOTES

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2. T. Yoshihara, S. Togiya, H. Koshino, S. Sakamura, T. Shimanuki, T. Sato and A. Tajimi, Tetrahedron Lett., 26, 5551 (1985).

3. High performance liquid chromatography of methyl esters (1a~4a). Column: μPORASIL (7.8 mm x 30 cm); eluting solvent: 0.5 % ethanol-n-hexane; rate of solvent flow: 2.0 ml / min; wavelength of UV detector: 210 nm; retention time: 2a, 27 min 30 sec; 1a, 30 min 30 sec; 3a, 33 min; 4a, 34 min.

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5. ^{1}H-NMR (500 MHz, CDCl$_3$) of 1: δ 0.89 (3H, t, J=6.8 Hz), 1.33 (12H, m), 1.63 (2H, m), 2.02 (2H, m), 2.05 (2H, m), 2.25 (1H, dddd, J=14.2, 7.3, 6.4, 1.5 Hz), 2.32 (1H, dddd, J=14.2, 7.3, 6.4, 1.5 Hz), 2.34 (2H, t, J=7.6 Hz), 4.09 (1H, ddd, J=6.8, 6.4, 6.4 Hz), 5.36 (1H, ddddd, J=10.8, 7.3, 7.3, 1.5, 1.5 Hz), 5.48 (1H, dddd, J=15.1, 6.8, 1.5, 1.5 Hz), 5.55 (1H, ddddd, J=10.8, 7.3, 7.3, 1.5, 1.5 Hz), 5.65 (1H, dddd, J=15.1, 6.8, 6.8, 1.0 Hz).

6. EI-MS of 1a: m/z (rel. intensity); 292 [M^+ - H_2O] (5), 199 (41), 181 (12), 167 (87), 149 (18), 139 (37), 121 (51), 83 (47), 69 (47), 67 (50), 57 (100).

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8. Spectral data for 2a: ^{1}H-NMR (270 MHz, CDCl$_3$) δ 0.90 (3H, t, J=7.3 Hz), 1.30 (12H, m), 1.62 (2H, m), 2.03 (4H, m), 2.30 (2H, t, J=7.3 Hz), 2.18~2.38 (2H, m), 3.67 (3H, s), 4.08 (1H, br.ddd, J=6.6, 6.6, 6.6 Hz), 5.38 (1H, ddddd, J=11.0, 7.3, 7.3, 1.5, 1.5 Hz), 5.48 (1H, dddd, J=15.4, 6.6, 1.3, 1.3 Hz), 5.54 (1H, ddddd, J=11.0, 7.3, 7.3, 1.5, 1.5 Hz), 5.67 (1H, dddd, J=15.4, 6.6, 6.6, 0.7 Hz). EI-MS m/z; 292 [M^+ - H_2O] (6), 195 (5),

166 (12), 124 (12), 113 (72), 95 (53), 81 (27), 69 (29), 67 (33), 57 (100).

9. Spectral data for 3a: ^{1}H-NMR (270 MHz, CDCl$_3$) δ 0.88 (3H, t, J=7.0 Hz), 1.25~1.40 (18H, m), 1.40~1.60 (2H, m), 1.62 (2H, m), 2.02 (2H, br.ddd, J=6.6, 6.6, 6.6 Hz), 2.30 (2H, t, J=7.7 Hz), 3.67 (3H, s), 4.03 (1H, m), 5.44 (1H, dddd, J=15.4, 7.0, 1.1, 1.1 Hz), 5.62 (1H, dddd, J=15.4, 6.6, 6.6, 0.7 Hz). EI-MS m/z; 312 [M$^+$] (0.4), 294 (6), 199 (53), 167 (100), 139 (40), 121 (50), 57 (81).

10. Spectral data for 4a: ^{1}H-NMR (270 MHz, CDCl$_3$) δ 0.88 (3H, t, J=7.0 Hz), 1.25~1.40 (18H, m), 1.40~1.60 (2H, m), 1.62 (2H, m), 2.02 (2H, br.ddd, J=6.6, 6.6, 6.6 Hz), 2.30 (2H, t, J=7.7 Hz), 3.67 (3H, s), 4.03 (1H, m), 5.44 (1H, dddd, J=15.4, 7.0, 1.1, 1.1 Hz), 5.63 (1H, dddd, J=15.4, 6.6, 6.6, 0.7 Hz). EI-MS m/z; 312 [M$^+$] (0.2), 294 (5), 213 (12), 181 (32), 155 (72), 95 (69), 81 (80), 57 (100).

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12. CD spectra of each benzoate: 1b, λ ext (EtOH) 229 nm ($\Delta\epsilon$ - 0.03); 2b, λ ext (EtOH) 229 nm ($\Delta\epsilon$ - 0.03); 3b, λ ext (n-hexane) 229 nm ($\Delta\epsilon$ - 0.05); 4b, λ ext (EtOH) 229 nm ($\Delta\epsilon$ - 0.01). In the ^{1}H-NMR vicinal coupling constants between the olefinic and carbinyl protons were Jvic=7.0~7.3 Hz. Optical purity of these compounds was not ascertained, since the oxygenation of unsaturated fatty acids by lipoxygenase proceeds partly through nonenantioselectively[16].

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14. Each sample was spotted on TLC plates (Merck, Kieselgel 60, 0.5 mm thickness) and the diameter of each spot was 6~8 mm.

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