61. S. Harkar, T. K. Razdan, and E. S. Waight, Phytochemistry, 23, 2893 (1984).
62. C. B. Rogers, Phytochemistry, 27, 3217 (1988).

OXIDIZED LIPIDS OF COTTON-PLANT LEAVES

S. D. Gusakova, S. G. Yunusova, and A. I. Glushenkova

UDC 582.796:581.45:547.915

Free fatty acids and diacylglycerols containing them have been isolated from the leaves of a cotton plant of a wilt-resistant variety. The composition and structures of the hydroxylipids have been determined by a combination of the methods of IRS, UVS, GLC, and the mass spectrometry of TMS derivatives, and changes in the composition of the hydroxy acids taking place on artificial infection of the plant with the fungus Verticillium dahliae Kleb. have been revealed.

On the penetration of a parasitic fungus into plant tissues, lipid peroxide oxidation processes (LOPs) are activated in them [1]. The LOP metabolites include a broad set of oxygenated fatty acids (OFAs), which have been isolated in recent years from the vegetative organs of resistant and susceptible varieties of rice [2], tomatoes [3], and the plant Phleum pratense [4]. In the resistance varieties, OFAs are present in healthy tissues, and in the susceptible varieties they are biosynthesized in response to infection stress. The OFAs that have been described possess a pronounced fungitoxicity and are capable in low concentrations of inducing systemic resistance in varieties susceptible to infection.

We have carried out research studies of the OFAs in young healthy leaves of a cotton plant of the resistant variety 175F (sample I) and in leaves of the same plant artificially infected with cotton wilt (sample II).

The surface lipids were first removed from the freshly-gathered leaves. After nonlipid impurities had been eliminated from the cell lipids, these lipids were separated with the aid of CC on silica gel into neutral and polar fractions. The neutral lipids were separated into classes by rechromatography using CC and TLC. According to TLC results in system 1 and qualitative reactions with picric acid and 2,4-dinitrophenylhydrazine, the neutral lipids I and II contained no epoxy or oxo derivatives. The fractions corresponding in their chromatographic mobility to a mixture of 1,2(1,3)-diacylglycerols (DAGs) and hydroxylipids (R_f 0.3) amounted for sample I to 2.1 and for sample II to 2.9 mg/g of dry tissue substance.

To determine the classes of the hydroxylipids, parts of the fractions of samples I and II were converted into trimethylsilyloxy (TMS) derivatives, and these were analyzed by mass spectrometry.

The mass spectra of the TMS derivatives of hydroxylipids I and II had no fundamental qualitative differences. In them the strongest peaks were those of the ion $(M - 31)^+$ with m/z 371-411 formed as the result of the breakdown of the di-TMS ethers of hydroxy acids with the general formula $CH_3(CH_2)_nCHOTMS(CH_2)_mCOOTMS$. The peaks of the M^+ ions with m/z 358-470 and the $(M - 15)^+$ ions with m/z 343-455 had low intensities and the mass numbers of these peaks showed that some of the main components of these fractions were free monohydroxy acids with chain lengths of from 12 to 20 carbon atoms and from 0 to 2 double bonds.

Another series of strong peaks included those of $(M - 15)^+$ ions with m/z 625, 651, and 677 and of $(M - 90)^+$ ions with m/z 550, 576, and 602, which were assigned to the main high-mass fragments from the breakdown of the TMS ethers of DAGs [5] of the 16:0–16:0-TMS, 18:1–16:0-TMS, and 18:1–18:1-TMS types. The peaks of M^+ (m/z 640, 666, and 692), $(M - 103)^+$ with

Institute of Chemistry of Plant Substances, Uzbekistan Republic Academy of Sciences, Tashkent. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 31-35, January-February, 1992. Original article submitted April 29, 1991.

0009-3130/92/2801-0023$12.50

m/z 537, 563, 589, and $(M - CH_2OCOR)^+$ with m/z 371 and 397 related to derivatives of the same lipids. Furthermore, the spectra of I and II contained low-intensity ions of the above-mentioned types of fragments for the TMS-DAGs but with mass numbers corresponding to the di-TMS ethers of DAGs with residues of the unsubstituted 16:0, 16:1, and 18:1 acids and of hydroxy acids (M^+ 724-780). In the di-TMS-DAGs I the main hydroxyacyl residues were those of the OH-14:0, OH-15:1, OH-16:1, and OH-18:1 acids, and the di-TMS-DAGs II those of the OH-18:1 acid.

Thus, the secondary (minor) class of hydroxylipids of cotton-plant leaves were acylhydroxyacylglycerols. No fragments relating to the breakdown of hydroxyacyldiacylglycerol derivatives were detected in the spectrum.

The ions present in the spectra in the regions of moderate mass numbers enable a preliminary conclusion to be made concerning the structure of the main hydroxy acids present in the free form. Thus, no ions with the composition $[CHOTMS(CH_2)_nOCOTMS]^+$ (fragments B), where n = 7-3, were observed in the spectrum of I and II, which showed the absence of α-cleavage (in relation to CHOTMS) on the side of the CH_3-ends of the molecules of the di-TMS derivatives of hydroxy acids and, consequently, the absence of CH_2-separated CH=CH and CHOH groups. Low-intensity ions of the saturated fragment B with m/z 331 (n = 8) and of unsaturated fragments with m/z 343 (n = 9) and 357 (n = 10) corresponded to locations of the CHOTMS group at carbon atoms 10, 11, and 12. Fragments A $[CH_3(CH_2)_nCHOTMS]^+$ were, in the main, monounsaturated and had m/z values of from 143 to 241. According to these results, the hydroxy acids were monoenic with the olefinic bond predominantly in the allyl position with respect to the CHOH group.

For the further investigation of the hydroxy acids, parts of the fractions I and II were hydrolyzed with alcoholic alkali. Analysis of the hydrolysis products by the TLC method in system 1 (with ricinoleic acid as standard) showed the presence of free unsubstituted acids (R_f 0.5) and monohydroxy acids (R_f 0.3). After methylation of the hydrolysis products, under the same chromatographic conditions spots of the methyl esters (MEs) of unsubstituted acids and of monohydroxy acids with R_f 0.87 and 0.42 respectively, were obtained, and these were isolated by preparative TLC. The yields of the MEs of the hydroxy acids were 0.7 mg/g from sample I and 1.3 mg/g from II.

The composition of the MEs of the unsubstituted acids isolated as the result of the saponification of the DAGs and of the hydroxy-DAGs of fractions I and II, according to GLC analysis, are given below. It can be seen that in the infected leaves (II) the fatty acids of this fraction were more saturated and contained a smaller amount of the 18:1 species than those from the healthy leaves (I):

Acid	Diacyl- and hydroxyacylglycerols, % of the weight of the acids	
	I	II
12:0	Tr.	Tr.
14:0	0.5	Tr.
16:0	29.5	34.2
16:1	1.2	2.3
17:0	0.9	1.8
18:0	1.6	3.0
18:1	52.4	42.8
18:2	10.1	12.5
18:3	3.8	3.4
Σ_{sat}	32.5	39.0
Σ_{unsat}	67.5	61.0

The MEs of the hydroxy acids were analyzed with the aid of UVS and IR spectrometry and, in the form of TMS ethers, by GLC and mass spectrometry. The UV spectrum of the MEs of the hydroxy acids revealed weak absorption of cis,trans-conjugated dienols in the $\lambda_{max}^{ethanol}$ 234 nm region. In the IR spectrum, there were weak bands of vibrations of cis,trans-conjugated dienes at 948 and 985 cm^{-1}, and stronger bands (particularly in the MEs of the hydroxy acids II) of an isolated trans-olefinic bond at 965 cm^{-1} and of hydroxy groups at 3200-3600 cm^{-1}.

TABLE 1. Composition of the Hydroxy Acids of the Leaves of a Healthy (I) and a Wilt-Infected (II) Cotton Plant of Variety 175-F

Hydroxy acid	GLC				Mass spectra, m/z			
	% of the mass of the TMS ethers of the MEs				Di-TMS	TMS ethers of the MEs		
	peak	RRT*	I	II	M^+	M^+	fragment A A (%, I;II)	fragment B
9-OH-10-12:1	1	0.24	2,6	2,1	358	300	143	285
9-OH-10-13:1	2	0.33	5,8	4.9	372	314	157	"
OH-14:0	3	0.44	11,3	5,8	388	Abs.	—	—
9-OH-10-14:1					386	328	171	285
9-OH-15:0	4	0,57	14,6	12.2	402	Abs.	187	259
9-OH-10-15:1					400	342	185	285
OH-16:0	5	0,75	15,2	9,1	416	Abs.	—	—
9-OH-10-16:1					414	356	199	285
9-OH-10, 12-16:2	6	0.98	14,3	4,7	412	351	197 (13; 8)	311 (1; 1)
13-OH-9, 11-16:2							213	285
9-OH-10-17:1	7	1,10		6,8	428	370	173	299
12-OH-9-17:1	8	1,15†	2,3	2,7			215	273
10-OH-18:0					444	Abs.	187	301
12-OH-18:0								
9-OH-10-18:1								
11-OH-9-18:1	9	1,3	22,2	36,2	442	381	227 (17; 100)	285 (9; 29)
10-OH-8-18:1							241 (13; 37)	271 (15; 89)
8-OH-9-18:1								
9-OH-12-18:1	10	1,47	2,5	6,3	442	384	227	259
12-OH-9-18:1							187	299
9-OH-10, 12-18:2	11	1,80	3,5	2,2	440	382	225	311
12-OH-9, 11-18:2								
OH-20:0	12	2,22	5,7	7,0	472	Abs.	—	—
1-OH-12-20:1					470	412	227	313

*Relative to the 18:0 ME.
†Partially resolved peak.

When the TMS ethers of the MEs of the hydroxy acids I and II were chromatographed with the aid of Ag^+-TLC in system 2, in both cases two spots were obtained, with R_f 0.77 and 0.7. Under the same conditions, the model TMS of the ME of the 12-OH-9Z-18:1 acid had R_f 0.68, and that of the 12-OH-18:0 acid had R_f 0.72. In view of the higher mobility of trans isomers on an Ag^+-impregnated sorbent [6], the spot with R_f 0.77 was assigned to ethers of trans isomers of hydroxy acids.

The mass spectra of the TMS ethers of the MEs of hydroxy acids I and II were the usual ones for these derivatives [7]. According to the characteristic high-mass fragments, the qualitative compositions of the hydroxy acids correlated with that revealed on the basis of the mass spectra of the di-TMS derivative.

The quantitative compositions of hydroxy acids I and II were established by chromatographing the TMS ethers of the MEs by the GLC method on a polar phase. The components were identified by means of the combined results of chromatographic and mass-spectrometric analyses. These results are given in Table 1.

The positions of the CHOTMS groups in the saturated hydroxy acids present in the mixture in minor amounts could not be determined reliably because of the complexity of the mass spectra.

The OH-14:0, OH-16:0, and OH-20:0 acids were detected in the mass spectra of the TMS ethers from the $(M - 15)^+$ and $(M - 31)^+$ fragments.

It can be seen from Table 1 that the hydroxy acids of the healthy cotton plant leaves consisted of 24 mainly monoenic components, among which the isomeric OH-18:1 acids predominated. The homologous OH-14:1, OH-15:1, and OH-16:1 acids were present in appreciable amounts, while the proportion of dienic hydroxy acids was insignificant. Of the four isomeric OH-18:1 acids, according to the intensities of the peaks of fragments A and B (in the mass spectrum of the TMS derivatives of the MEs) [7], in sample I the amounts of the 9-OH- and 10-OH-18:1 acids (m/z 227 and 271) were higher.

In the infected leaves, the amount of hydroxy acids was 1.8 times higher than in the healthy tissues (see above), and in the mixture of hydroxy acids of II the proportion of

hydroxyoctadecenoic acids, mainly the 9-OH and 10-OH isomers, was higher by approximately the same factor (1.6). Infection of the cotton plant with the fungal pathogen did not affect the qualitative compositions of the hydroxylipids and of the hydroxy acids of the leaves. It must be mentioned that the OH-17:1, OH-18:2, 9(12)OH-12(9)-18:1, and 10-OH-18:0 acids are components of the structure of the hydroxyacyldiacylglycerols present in seeds of the cotton plant Gossypium hirsutum (Tashkent-1 variety) [8] and its wild form G. mexicanum var. nervosum [5].

It has been reported that the allylic monoenic hydroxy acids 9-OH-10E-18:1 and 10-OH-8E-18:1 detected in the vegetative organs of the plant Phleum pratense infected by the pathogenic fungus Epichloe typhina possess toxic properties against another fungal parasite Cladosporium phlei [4].

The authors concerned put forward a hypothesis of the existence of an enzyme system participating in the formation of these hydroxy acids from the 18:1 acid, although the lipoxygenases described hitherto are specific mainly for the 18:2 acid and, in some cases, for more unsaturated acids [9].

Another possible pathway for the formation of allylic hydroxy monoenes is nonenzymatic photosensitive oxidation of the 18:1 acid taking place in the presence of photosensitizers by a free-radical mechanism with the participation of molecular O_2 and/or by an "ene" mechanism on interaction with $^1O_2$ [10].

In addition to chlorophyll, it is possible to assign to the photosensitizers the polyphenolic pigment gossypol that is present in cotton-plant leaves [11] and possesses photodynamic properties [12].

It has been established that on the photosensitive oxidation of the 18:1 acid by molecular O_2 in a model system four isomeric hydroxyperoxides – 8-OOH, 9-OOH, 10-OOH, and 11-OOH – are formed, while oxidation by singlet oxygen $^1O_2$ gives only the 9-OOH and 10-OOH isomers [10]. The presence in cotton-plant leaves of all four isomeric allylic OH-18:1 acids with a predominance of the 19-OH- and 10-OH-18:1 species may be the result of a combined occurrence of these two types of nonenzymatic reactions.

EXPERIMENTAL

UV spectra were taken on a Hitachi instrument in ethanol, IR spectra on a UR-10 instrument in a film, mass spectra on a MKh-1310 instrument with direct introduction of the sample at an ionizing voltage of 50 V, a collector current of 40 μA, and temperatures of the ionization chamber and of the evaporator bulb of 150 and 80°C, respectively.

GLC was performed as described in [13], using for identification the TMS ethers obtained in this investigation and literature information.

For TLC we used Silufol UV 254 preprepared plates (Kavalier, Czechoslovakia) and silica gel L 5/40 (Czechoslovakia) with the addition of 10% of $CaSO_4$; the impregnated sorbent contained 20% of $AgNO_3$; preliminary activation of the layer at 105°C for 1 h; systems 1 – hexane–diethyl ether (7:3, v/v); and 2 – benzene.

The plants were grown in a hothouse against an artificial infection background to the phase of 3-4 true leaves and were infected in the root neck with a mixture of biotypes A and B of the fungus Verticillium dahliae Kleb. at a concentration of conidia and endospores of $2.5 \cdot 10^4$ in 1 ml of inoculum. The infected plants were kept in an artificial-climate chamber against a temperature-wilt background [14]. After the appearance of chlorotic spots on the leaves, the plants were removed from the soil, and the leaves were separated under laboratory conditions and were treated as described in [15]. The isolated cell lipids were separated by CC on silica gel, the fraction of hydroxylipids being eluted with the hexane–ether (92:8, v/v) system.

Alkaline hydrolysis was carried out with a 10% aqueous methanolic solution of KOH [H_2O–MeOH (1:9, v/v)] with the addition of ten parts of the alkaline solution to one part of sample.

The fatty acids were methylated with diazomethane. The silylation of the fraction of hydroxylipids and of the hydroxy acid methyl esters was performed by dissolving a weighed sample (10 mg) in pyridine (1.0 ml) and then added hexamethyldisilazane (0.8 ml) and trimethylchlorosilane (0.4 ml) and keeping the mixture for 1-2 h [16]. The residues of the

reagent were evaporated off at 40°C in a rotary evaporator. The TMS ethers were extracted with diethyl ether, and the completeness of the reaction was evaluated by TLC in system 1.

LITERATURE CITED

1. L. D. Keppler and A. Novacky, Phytopathology, No. 1, 104 (1986).
2. T. Kato, Y. Yamaguchi, T. Hirano, T. Yokohama, T. Uyehare, T. Namai, S. Yamanaka, and N. Harada, Chem. Lett., No. 3, 409 (1984).
3. A. Vernenghi, J. Einhorn, G. Kunesch, C. Malosse, T. Ramiandrasoa, and A. Ravise, Chem. Abstr., 104, No. 15, 126723 (1986).
4. H. Koshino, S. Togiya, T. Yoshihara, S. Sacamura, T. Shimanuki, T. Sato, and A. Tajimi, Tetrahedron Lett., 28, No. 1, 73 (1987).
5. S. G. Yunusova, S. D. Gusakova, N. N. Stepanichenko, Sh. Kholmuradova, and A. I. Glushenkova, Khim. Prir. Soedin., 57 (1987).
6. W. W. Christie, Lipid Analysis, Pergamon Press, Oxford (1973), p. 120.
7. R. Kleiman and G. F. Spencer, J. Am. Oil Chem. Soc., 50, 31 (1973).
8. S. G. Yunusova, S. D. Gusakova, and Ya. V. Rashkes, Khim. Prir. Soedin., 436 (1984).
9. H. W. Gardner and D. Weisleder, Lipids, 7, No. 3, 191 (1972).
10. A. W.-S. Chan, J. Am. Oil Chem. Soc., 54, No. 3, 100 (1977).
11. A. S. Sadykov and A. I. Ismailov, Dokl. Akad. Nauk UzSSR, No. 3, 40 (1959).
12. A. A. Tyshchenko, S. D. Gusakova, I. N. Nazarova, and A. I. Glushenkova, Khim. Prir. Soedin., 354 (1987).
13. S. D. Gusakova and D. T. Asilbekova, Khim. Prir. Soedin., No. 6 (1981).
14. Yu. I. Ikramov, D. A. Musaev, S. M. Berdiev, and S. A. Usmanov, Methodological Instructions for a Rapid Temperature Method for the Early Evaluation and Selection of a Cotton Plant for Resistance to Cotton Wilt [in Russian], Minselkhoz, UzSSR, Tashkent (1981).
15. S. G. Yunusova, S. D. Gusakova, Kh. T. Mirzaazimova, A. I. Glushchenkova, S. A. Usmanov, and Yu. Ikramov, Khim. Prir. Soedin., 285 (1989).
16. F. D. Gunstone and H. R. Schuler, Chem. Phys. Lipids, 15, 174 (1975).

COUMARINS OF *Smyrniopsis aucheri*

Z. R. Dzhafarov, Z. A. Kuliev, A. D. Vdovin, A. A. Kuliev, V. M. Malikov, and N. M. Ismailov

UDC 947.918

Chemical transformations and spectral characteristics have enabled us to establish the structure and configurations of three coumarins: (+)-2',2'-dimethyl-3'β-hydroxy-3',4'-dihydropyrano(5',6':6,7)coumarin (I); (+)-4'β-hydroxy-5'β-(1-hydroxy-1-methylethyl)-4',5'-dihydrofuro(2',3':6,7)coumarin (II); and (+)-4'β-hydroxy-5'-(1-glucopyranosyloxy-1-methylethyl)-4',5'-dihydrofuro(2',3':6,7)-coumarin (III) isolated from the roots of *Smyrnopsis aucheri* Karjag. This is the first time that these compounds, which have been called smyrinol, smyrindiol, and smyrindioloside, have been detected in nature.

Continuing investigations of smyrniopsis roots collected in the flowering and fruit-bearing phase of the plant we have studied the physicochemical and spectral parameters of the substances isolated previously [1] and have established structures of another three coumarin compounds: (I-III).

The presence in the UV spectrum of smyrinol (I) of absorption maxima at 210, 225, 250, 260, 300, and 325 nm showed that the substance was a 6,7-substituted coumarin [2]. Its IR

Institute of Chemistry of Plant Substances, Uzbekistan Republic Academy of Sciences, Tashkent. Azerbaidzhan State University, Baku.

Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 36-40, January-February, 1992. Original article submitted December 24, 1990; revision submitted August 16, 1991.

0009-3130/92/2801-0027$12.50