IDENTIFICATION OF 16-OXO-9-HYDROXY HEXADECANOIC ACID A NOVEL MONOMER, AS A MAJOR COMPONENT OF THE BIOPOLYMER CUTIN IN EMBRYONIC VICIA FABA

P. E. Kolattukudy
Department of Agricultural Chemistry
Washington State University
Pullman, Washington 99163

Received September 28,1972

Summary: 16-oxo-9-hydroxy hexadecanoic acid and 16-oxo-10-hydroxy hexadecanoic acid were identified as major components of the cutin of the embryonic shoot of germinating Vicia faba by hydrogenolysis with $LiAlH_4$, deuterolysis with $LiAlD_4$ and treatment with $NaBD_4$ followed by hydrogenolysis and analysis of the products by a combination of gas-liquid chromatography and mass spectrometry.

The structural component of the plant cuticle is a polymer of hydroxy fatty acids called cutin. Hydrolytic or reductive cleavage of the polymer followed by gas-liquid chromatography in combination with mass spectrometry showed that the monomers consist of a C_{16} family and C_{18} family of hydroxy acids (1,2,3). The former contains mainly ω-hydroxy palmitic acid and 9,16 or 10,16-dihydroxy palmitic acid. In V. faba leaves 10,16-dihydroxy palmitic acid was identified to be the major component (4). In this communication identification of 16-oxo-9-hydroxy hexadecanoic acid as a major component of the cutin of the embryonic shoots of V. faba is reported. This aldehyde is a novel component of cutin and it does not appear to have been reported in any other natural source.

EXPERIMENTAL

Vicia faba seeds were germinated at 75° for 6 days. The embryos were separated from the cotyledons and the shoot portion was excised. The tissue was washed 6 times with distilled water and homogenized in

This work was supported in part by National Science Foundation Grant GB 23081. Scientific paper 3923 , Project 2001, Research Center, College of Agriculture, Washington State University, Pullman, Washington.

Copyright © 1972 by Academic Press, Inc.
All rights of reproduction in any form reserved.

water with an Omni-Mixer for 1 minute and centrifuged at 3000 x g for 5 minutes. The pellet was homogenized with water in a Ten-Broek homogenizer and centrifuged. The soluble lipids were thoroughly extracted from the pellet as described before (4). The final residue was washed with tetrahydrofuran and then refluxed with $LiAlH_4$ or $LiAlD_4$ (> 99 atom%D) in tetrahydrofuran, the lipid products were recovered, and the C_{16} triol was isolated from the hydrogenolysate by thin-layer chromatography as described elsewhere (4). The C_{16} triol fraction was treated with N,O-bis(trimethylsilyl)acetamide at 90° for 15 minutes, excess reagent was removed with a stream of N_2 and the product was analyzed by a combination of gas-liquid chromatography and mass spectrometry (5). In order to reduce aldehyde functions suspected to be present in the polymer the final pellet obtained from the above procedure was treated with $NaBD_4$ (> 98 atom%D) in methanol for 2 hours at room temperature with vigorous stirring. The suspension was centrifuged, the soluble lipids were removed from the residue as before, the final residue was hydrogenolyzed with $LiAlH_4$, the C_{16} triol was isolated and analyzed (5). Gas-liquid chromatography was done with a Varian Aerograph Mode 328 gas chromatograph attached to a Perkin-Elmer Hitachi RMU6D mass spectrometer with a Bieman separator interphase. The details of the experimental conditions are under Figure 1.

RESULTS AND DISCUSSION

Pea seeds appeared to develop at least some of the enzymes involved in the cuticular wax biosynthesis early in the germination process (6). It is possible that during the early stages of germination the embryo also develops the enzymes involved in the synthesis of the hydroxy fatty acid polymer, cutin. In my attempts to use germinating V. faba seeds to obtain a cell-free synthesis of cutin, a novel monomer was encountered in this tissue.

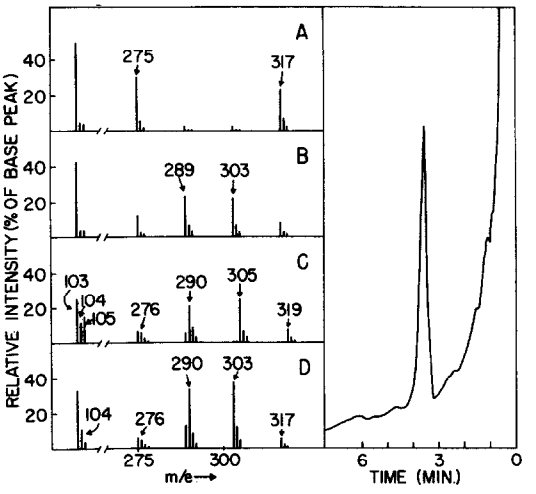

Figure 1. Gas-liquid chromatogram of the trimethylsilyl ether of the C_{16}-triol fraction (right) and partial mass spectra of the C_{16} triol from (A) hydrogenolysate of mature V. faba leaf cutin, (B) hydrogenolysate of cutin from embryonic tissue of V. faba, (C) deuterolysate ($LiAlD_4$) of cutin from embryonic V. faba, (D) hydrogenolysate of $NaBD_4$-treated cutin from embryonic V. faba. The flame ionization detector response obtained with all four C_{16}-triol samples were identical and the one shown here represents a typical pattern. A coiled glass column (147.0 x 0.31 cm o.d.) packed with 3% OV-1 on Gas Chrom Q at 225° with 45 ml/min He was used. Mass spectra were recorded at the top of each chromatographic peak with 70 eV ionizing voltage and the base peak in each spectrum was at m/e 73.

The C_{16} triol fraction isolated from the hydrogenolysate of the insoluble residue obtained from embryonic shoots of 6 day-old V. faba showed one sharp symmetrical peak on gas-liquid chromatography (Fig. 1). Mass spectrum of this component showed a weak molecular ion at m/e 490 and weak ions at m/e 475 (M^+-CH_3), 400 ($M^+-(CH_3)_3SiOH$) and 385 ($M^+-CH_3-(CH_3)_3SiOH$) as observed before in the spectrum of 1,7,16-trihydroxy hexadecane obtained from V. faba leaf cutin (5). Similarly the other characteristic ions at m/e 73, 75, 147, 149 were also observed. However, the major α-cleavage ions were at m/e 289, and 303 with a less intense

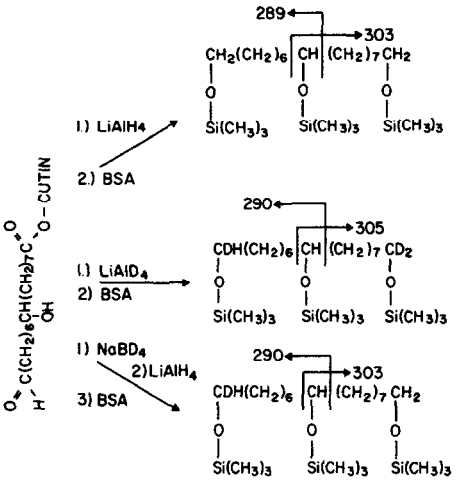

Figure 2. Chemical treatments of the cutin and the major α-cleavage ions expected from the C_{16} triol derived from 16-oxo-9-hydroxypalmitic acid by each treatment. Similar cleavages of the triol derived from the 10-hydroxy isomer were also observed as discussed in the text. BSA - N,O-bis(trimethylsilyl)acetamide.

---

pair at m/e 275 and 317 while the corresponding spectrum from leaf cutin showed major ions at m/e 275 and 317 (Fig. 1). From these results it is clear that the position of the in-chain hydroxyl group in the embryonic tissue is different from that in the leaf cutin. From the relative intensities of the ion pairs at 275, 317 and 303, 317, it is estimated that 70% of the C_{16} triol from the embryonic tissue was 1,8,16-trihydroxy hexadecane while only about 10% of that from the leaf was this isomer (5).

The reductive cleavage simultaneously converts the carboxyl groups in the polyester to primary alcohols and therefore the carboxyl end of the monomers cannot be distinguished from the ω-hydroxyl end of the parent acid. Furthermore any other function susceptable to $LiAlH_4$ would also have gone undetected. In order to overcome this difficulty $LiAlD_4$-deuterolysis product was examined. The mass spectrum of the C_{16} triol derived by deuterolysis showed a molecular ion,

M^+-CH_3, $M^+-(CH_3)_3SiOH$ and $M^+-CH_3-(CH_3)_3SiOH$ 3 amus higher than that observed with hydrogenolysis, indicating incorporation of 3 deuterium atoms. The ion at m/e 105 ($CD_2OSi(CH_3)_3$) showed that 2 deuterium atoms were incorporated into one end of the molecule probably during the reductive cleavage of the ester. The major α-cleavage ion originating from this end of the molecule shifted from 303 to 305 and the minor one from 317 to 319 establishing the position of in-chain hydroxyl group at C-9 of the parent acid in the major component and C-10 in the minor component (Fig. 2). The occurrence of a relatively strong ion at m/e 104 ($CDHOSi(CH_3)_3$) indicated that the third deuterium was located on the carbon in the ω-position to the carboxyl group. This assignment was confirmed by the fact that the α-cleavage ions expected from this end of the molecule shifted from 275 to 276 and 289 to 290. These results indicate that the parent C_{16} molecule contained a carboxyl at one end and an aldehyde function on the other end. The absence of any α -cleavage ions carrying 3 deuterium atoms rules out the possibility that the molecule contained a keto group or an epoxide.

In order to confirm the presence of an aldehyde function in the native cutin the crude cutin preparation was treated with $NaBD_4$ at room temperature. If the structural assignment discussed above is correct, one deuterium should be incorporated into the aldehyde carbon and subsequent hydrogenolysis of the polymer with $LiAlH_4$ should give a C_{16} triol containing one deuterium on the carbon derived from the aldehyde. The mass spectrum of the C_{16} triol thus obtained showed that the ions at the high mass region were shifted by 1 amu and, as expected, an ion at m/e 104 was observed. Furthermore, the α-cleavage ions expected from the aldehyde end of the molecule were shifted from 275 to 276 and 289 to 290 confirming the presence of 16-oxo-9-hydroxy hexadecanoic acid and its 10-hydroxy isomer in the cutin of embryonic V. faba. However, the occurrence of significant α-cleavage ions at m/e 275 and 289 in the

spectrum of the C_{16} triol, derived from $LiAlD_4$ treatment and $NaBD_4$ treatment, indicated that the 9,16 and 10,16-dihydroxy palmitic acids were also present in this tissue. This communication represents the first report of an aldehyde containing hydroxy acid in the biopolymer cutin and the 16-oxo-9-hydroxy hexadecanoic acid identified here appears to be the first instance of its occurrence in nature.

The cutin from mature leaves contain 10,16-dihydroxy palmitic acid whereas in the embryonic tissue the major component is the 9 isomer. The biochemical implications of this change in the position of the in-chain substituent as well as the occurrence of an aldehyde function are not clear. The fact that aldehyde components have also been found in embryonic tissue, apical meristem, developing leaves and in fruits such as apple and tomato suggest that these components occur widely in plants. The occurrence of substantial amounts of aldehyde components appears to to the hallmark of young tissues suggesting a possible function. The aldehyde group may be used to anchor the cuticular polymer to the tissue by formation of cross linkages such as Schiff's base with an amino group of a protein or acetal with the hydroxyl group of a carbohydrate polymer of the cell wall. Only in the young tissue such linkages are necessary and as the tissue grows the bulk of the cutin synthesis would involve building of the polyester structure by esterification with hydroxy fatty acids. Consequently as the tissue develops the proportion of the aldehyde components should decrease. In fact 16-oxo-9-hydroxy hexadecanoic acid constituted nearly 50% of the monomers of cutin in the apical bud (less than 1 mm in length) of *V. faba* plants and this proportion gradually decreased as the leaves developed. In the fully mature leaves the oxo compound was only a minor component.

Oxidation of 9,16-dihydroxy palmitic acid appears to be the most likely mechanism for the biosynthesis of 16-oxo-9-hydroxy palmitic acid. Cell-free preparations of embryonic shoots of germinating *V. faba* has

been shown to catalyse w-hydroxy acid oxidation via an aldehyde intermediate (unpublished results). A detailed investigation into the occurrence, biosynthesis and function of the aldehyde components of cutin is in progress in this laboratory.

ACKNOWLEDGEMENT

I thank Linda Brown for technical assistance.

REFERENCES

1. Eglinton, G. and Hunneman, D. H., Phytochemistry, 7, 313 (1968).
2. Holloway, P. J. and Deas, A. H. B., Phytochemistry, 10, 2781 (1971).
3. Walton, T. J. and Kolattukudy, P. E., Biochemistry, 11, 1885 (1972).
4. Kolattukudy, P. E., Biochem. Biophys. Res. Commun., 41, 299 (1970).
5. Kolattukudy, P. E. and Walton, T. J., Biochemistry, 11, 1897 (1972).
6. Harwood, J. L. and Stumpf, P. K., Arch. Biochem. Biophys., 142, 281 (1971).