Naturally occurring long-chain β-hydroxyketones

HARALD H. O. SCHMID and PATRICIA C. BANDI

The Hormel Institute, University of Minnesota, Austin, Minnesota 55912

ABSTRACT A fraction comprising about 0.7% of the extractable surface lipids of cabbage (*Brassica oleracea*) leaves was isolated and identified as a mixture of isomeric β-hydroxyketones consisting mainly of 14-keto-16-hydroxynonacosane and 15-keto-13-hydroxynonacosane.

SUPPLEMENTARY KEY WORDS ketones · ketols · secondary alcohols · cyclic acetals · acetone ketals

THE SURFACE WAXES of plants contain aliphatic constituents characterized by very long odd-numbered carbon chains. They are usually hydrocarbons, ketones, and secondary alcohols (1), but β-diketones (2) and hydroxy-β-diketones (3) have also been identified. The biosynthesis of some of these lipids and their possible biogenetic relationships have been the subject of speculation (4, 5) and, more recently, of experimentation (6, 7).

Leaves of *Brassica oleracea* (cabbage, broccoli) have been used for metabolic experiments. Major constituents of their surface wax are hydrocarbons, ketones, and secondary alcohols consisting mainly of C_{29} compounds and homologous series of even-numbered fatty acids, primary alcohols, and wax esters (5, 6). We have recently found appreciable amounts of *n*-triacontanal and other long-chain aldehydes in the surface wax of *Brassica oleracea* leaves (8).

Most of the current evidence (6, 7, 9) supports the view of an "elongation–decarboxylation" pathway in the biosynthesis of the C_{29} compounds. So far all attempts to demonstrate interconversions between hydrocarbons, ketones, and secondary alcohols have failed (9).

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

Obviously, the exact location of the functional groups and the possible occurrence of positional isomers is of major importance if interconversions between these lipid classes are considered. However, structural analyses have produced conflicting evidence. In the early work of Channon and Chibnall (4) the major ketone in cabbage leaves was identified as the symmetrical 15-ketononacosane. Recent studies (6, 10) confirmed this structure. However, Purdy and Truter (5) had found mixtures of two isomeric ketones having the functional groups in the 10- and 15-positions. They also reported the occurrence of the corresponding secondary alcohols as well as two isomeric ketols, namely 10-hydroxy-15-ketononacosane and 15-hydroxy-10-ketononacosane.

A minor lipid class of *Brassica oleracea*, presumably the 10,15-ketols reported by Purdy and Truter, was found to incorporate radioactivity from acetate-^{14}C as well as from ^{14}C-labeled long-chain fatty acids (6).[1] Although this lipid class amounted to only 0.7% of the total surface lipids, it contained up to 5% of the radioactivity incorporated within relatively short time periods.[1]

In view of the relatively rapid biosynthesis of this lipid and its possible role as a precursor of other lipid classes, we isolated the material in pure form and identified it as a mixture of isomeric β-hydroxyketones. In this communication we describe the structure and physical properties of this new lipid and compare its isomer composition to that of the ketones and secondary alcohols isolated from the same wax.

EXPERIMENTAL METHODS

Materials

Fatty acids were purchased from The Hormel Institute Lipids Preparation Laboratory, Austin, Minn. 15-

[1] Bandi, P. C., and H. H. O. Schmid. In preparation.

Ketononacosane was prepared from cabbage surface wax by preparative TLC (hexane–diethyl ether 95:5). The preparation was pure by TLC and greater than 95% pure by GLC. The structure of the ketone was ascertained by degradation via a Beckmann rearrangement as well as by infrared and mass spectroscopy.

Preparation and Crude Fractionation of Lipids

Fresh mature cabbage was obtained from a local farm. The lipids were extracted by immersing the leaves in chloroform for about 20 min. The solvent was removed under reduced pressure in a rotary evaporator. Water was decanted and the residual water was removed by azeotropic distillation with small amounts of chloroform. The lipids were fractionated (11) on layers of Silica Gel H, 30 g per 20 × 20 cm plate, using hexane–diethyl ether 70:30 as the developing solvent in unlined tanks. Bands containing the ketones, secondary alcohols, and a band above and including the leading edge of the primary alcohols were scraped off and eluted. The lipids were further purified by TLC.

Thin-Layer Chromatography

All lipids and most of the reaction products were purified by TLC on layers of Silica Gel H, 0.5 mm thick, using mixtures of hexane–diethyl ether as the developing solvents in tanks lined with filter paper. The hydroxyketone fraction was separated completely from the primary alcohols by chromatography with hexane–diethyl ether 80:20. Fractions were made visible by spraying the plates with a 0.1% ethanolic solution of 2′,7′-dichlorofluorescein. The bands were scraped off and eluted with chloroform. Chromatography of the hydroxyketone fraction was repeated until a pure product was obtained (Fig. 1).

Gas–Liquid Chromatography

A Victoreen 4000 instrument equipped with a flame ionization detector was used. Columns 180 cm long, 0.4 cm I.D., packed with Gas-Chrom P, 80–100 mesh containing 18% ethylene glycol succinate (Hi-EFF-2BP, Applied Science Laboratories Inc., State College, Pa.) were used at 180°C and 200°C. Columns 190 cm long, 0.2 cm I.D., packed with Anakrom ABS 90–100 mesh containing 6% silicone gum rubber SE-30 (Analabs, Inc., North Haven, Conn.), were operated at 285°C. Peak areas were measured by triangulation.

Spectroscopy

Infrared spectra were recorded with a Perkin–Elmer Model 21 spectrophotometer. Carbon disulfide was used as a solvent except in the ranges 2300–2000 cm^{-1} and 1650–1400 cm^{-1}, where tetrachloroethylene was used. Nuclear magnetic resonance spectra were taken in $CDCl_3$ with a Varian A-60A spectrometer using tetramethylsilane as the internal standard. Mass spectra were recorded on a Hitachi Perkin–Elmer RMU-6-D instrument equipped with the direct insertion system M6-150; the spectra were taken at an ionization potential of 70 ev.

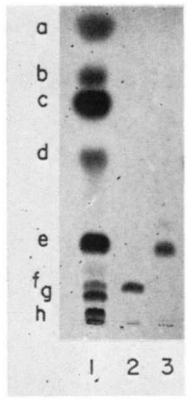


FIG. 1. Thin-layer chromatogram of cabbage wax (*1*), hydroxyketones (*2*), and secondary alcohols (*3*). Individual fractions are: hydrocarbons (*a*), wax esters (*b*), ketones (*c*), aldehydes (*d*), secondary alcohols (*e*), hydroxyketones (*f*), primary alcohols (*g*), and fatty acids (*h*). Silica Gel H; hexane–diethyl ether 90:10.

Polarimetry

The optical rotation was measured with a Bendix automatic polarimeter using chloroform as the solvent.

Reactions

Hydroxyketones were reduced to diols and ketomesylates to secondary alcohols with lithium aluminum hydride in anhydrous diethyl ether at reflux temperature. Long-chain diols were acetylated with acetic anhydride in pyridine at 80°C for 2 hr. Hexadecanal (12) was prepared from the corresponding alcohol via the mesylate (13). Free fatty acids were converted to their methyl esters by reaction with methanol–HCl (5%) at 80°C for 2 hr.

Acetone Ketals. Long-chain diols were converted to their isopropylidene derivatives by reacting them for 30 min with anhydrous acetone containing 1% perchloric acid at room temperature. Yields were 50–70%. The isopropylidene derivatives were purified by TLC (hexane–diethyl ether 90:10).

Cyclic Acetals. The diols derived from the hydroxyketones of *Brassica* as well as commercial 2,4-pentanediol and 1,4-butanediol were reacted with hexadecanal according to established procedures (14). Of the long-chain diols, 7 mg was reacted with 15 mg of hexadecanal in the presence of 3 mg of *p*-toluene–sulfonic acid. The reaction was almost complete within 4 hr as checked by TLC. The cyclic acetals were purified by TLC (hexane–diethyl ether 98:2).

Degradation of Hydroxyketones. All intermediate products were checked and purified by TLC. (*a*) *Preparation of ketomesylates:* Hydroxyketones, 15 mg, were dissolved in 3 ml of pyridine. The solution was chilled in an ice bath and 0.1 ml of methanesulfonyl chloride was added slowly with stirring. The solution was stirred at room temperature for 3.5 hr. Water was added and the lipids were extracted with diethyl ether. The ether phase was washed with 2 N H_2SO_4, water, 5% $NaHCO_3$, and water, then dried over Na_2SO_4. (*b*) *Reduction of the ketomesylates* (10–12 mg) with lithium aluminum hydride gave secondary alcohols in almost 95% yield. (*c*) *Oxidation of secondary alcohols:* The sample, 10–15 mg, was dissolved in 3 ml of diethyl ether, and 5 ml of 5 N H_2SO_4 containing 8% potassium dichromate was added; the mixture was then shaken at room temperature for 3 hr. The dichromate solution was then replaced with a fresh solution and the mixture was shaken overnight. The ether phase was separated off and washed twice with water. (*d*) *Preparation of oximes* (6): The ketones, 8–10 mg, were dissolved in 3 ml of pyridine–absolute ethanol, 1:1; 40 mg of hydroxylamine–HCl was added and the solution was held at 100°C for 1 hr. (*e*) *Beckmann rearrangement:* The oximes were dissolved in a mixture of 2.5 ml of glacial acetic acid and 0.5 ml of concentrated H_2SO_4. The solution was stirred at reflux temperature for 1.5 hr. After cooling, water was added, and the products were extracted with chloroform. The chloroform phase was washed several times with water. (*f*) *Hydrolysis of amides:* Amides were dissolved in 2 ml of an ethanolic solution of KOH (2%) and reacted at 180°C for 10 hr in a sealed ampule enclosed in a steel vessel. After cooling, 2 ml of water was added, the mixture was acidified with 2 N HCl, and the products were extracted with diethyl ether–hexane 3:1. (*g*) *Fatty acids* were purified by TLC using hexane–diethyl ether–acetic acid 90:10:0.5, and were then converted to their methyl esters.

Oxidative Cleavage of Hydroxyketones. The sample was treated as described above for the oxidation of secondary alcohols with dichromate–sulfuric acid. Free fatty acids were isolated by preparative TLC (hexane–diethyl ether–acetic acid 90:10:1) and converted to their methyl esters with methanol–HCl without prior elution from the adsorbent.

RESULTS AND DISCUSSION

A minor lipid class slightly less polar than primary alcohols and tentatively identified as ketols (hydroxyketones) was isolated from the surface wax of *Brassica oleracea* leaves. A total of 50 mg of purified material was obtained from about 45 kg of cabbage leaves. A thin-layer chromatogram of the total lipids and the isolated fraction is shown in Fig. 1.

The white crystalline material was optically active and exhibited a specific rotation of $[\alpha]_D^{26} = +1.5$. A gas–liquid chromatogram (SE-30, 285°C) of the hydroxyketones showed only one major peak. The infrared spectrum showed the absorption bands expected for a long-chain hydroxyketone: The C=O stretching absorption was found at 1710 cm^{-1} (s). Absorption bands characteristic of an alcohol function were those at 3600 (m), 1260 (m), and 1100 cm^{-1} (m). Additional bands due to C—H stretching vibrations of the methylene groups were at 2925 (s) and 2850 cm^{-1} (m), and at 1470 cm^{-1} (s) for the C—H deformation. The C—H deformation of the methyl groups was found at 1375 cm^{-1} (m), and the rocking mode of the carbon chain was at 718 cm^{-1} (m).

Position of the Functional Groups Relative to Each Other

Reduction of the hydroxyketones with lithium aluminum hydride produced diols which showed the expected infrared spectrum and migration rate in TLC. The diols were acetylated and analyzed by GLC (SE-30, 285°C); the gas–liquid chromatogram of the diacetates showed only one major peak. As it was not possible to achieve cleavage of the diols with metaperiodate (15), vicinal hydroxy substitution was ruled out. However, the diols reacted with acetone to form compounds free of hydroxy groups as judged by their infrared spectrum and their migration rate in TLC. Both the diols and their acetone adducts were separable into two fractions by repeated development of the thin-layer chromatograms. A reduction of the hydroxyketones may have yielded isomeric diols with their hydroxy groups located close enough to each other to render the isomers separable by chromatography. Obviously, two types of sterically different diols would produce two conformationally different acetone ketals. Thus, the hydroxy groups were assumed to be in close proximity.

Proof that the hydroxy groups of the diols were located in β-position to each other was obtained by preparing their cyclic acetals of *n*-hexadecanal, and by comparing the NMR spectra of the resulting 2-pentadecyl-4,6-dialkyl-1,3-dioxane (Fig. 2) with those of appropriate standards.

The NMR spectrum in Fig. 2 shows a triplet at 4.51 ppm which can be attributed to an axial hydrogen at carbon-2 of a 1,3-dioxane system.[2] The same triplet at 4.51 ppm was found in the spectrum of 2-pentadecyl-4,6-dimethyl-1,3-dioxane. A corresponding triplet in the spectrum of 2-pentadecyl-1,3-dioxolane, the five-membered ring acetal derived from ethanediol, occurs at 4.88 ppm[2] and in the spectrum of the seven-mem-

[2] Baumann, W. J., and B. J. Weseman. Personal communication.

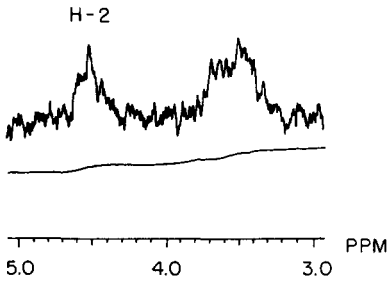
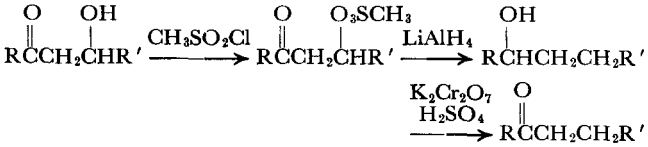


FIG. 2. Part of the NMR spectrum of the cyclic acetals of hexadecanal and the long-chain diols derived from the hydroxyketones of *Brassica*.

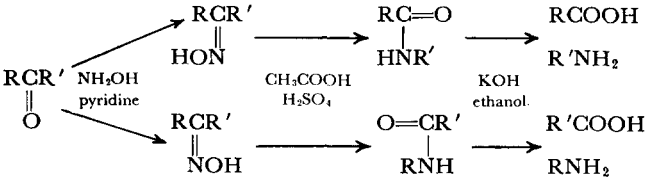
bered ring acetal derived from 1,4-butanediol at 4.62 ppm. Thus, structures other than those of 1,3-dioxanes for the cyclic acetals of the diols derived from the hydroxyketones of *Brassica* were ruled out.

Positions of the Keto Groups

The β-hydroxyketones were degraded in order to determine the position of the functional groups and to identify positional isomers. First, the hydroxy groups were removed via the mesylates (11) to obtain ketones:



When the ketones were analyzed by GLC, the chromatogram showed only one major peak with a retention time identical to that of 15-ketononacosane. The locations of the carbonyl groups were determined via the oximes (16) followed by a Beckmann rearrangement:



The fatty acids derived from the ketones are listed in Table 1; formation of C_{14}, C_{15}, and C_{16} acids shows that the keto groups of the β-hydroxyketones were located in positions 14 and 15 with relative amounts of about 65% and 35%, respectively.

Positions of the Hydroxy Groups

Oxidation of the hydroxyketones with potassium dichromate–H_2SO_4 produced fatty acids, presumably via the β-diketones. Fatty acids were the only long-chain reaction products as judged by TLC, but their relative amounts were not well reproducible. However, the major fatty acids were always C_{13}, C_{14}, and C_{15} in the relative amounts of approximately 21, 58, and 20%, with small amounts (up to 3%) of C_{12} and C_{16} fatty acids.

TABLE 1 FATTY ACIDS OBTAINED FROM KETONES DERIVED FROM β-HYDROXYKETONES

Number of Carbon Atoms	Weight %
13	trace
14	34.0
15	32.4
16	33.6
17	trace

These results indicate that the two major hydroxyketones were 14-keto-16-hydroxynonacosane (I) yielding *only* C_{14} fatty acids, and 15-keto-13-hydroxynonacosane (II) yielding equal amounts of C_{13} and C_{15} fatty acids. The presence of a small amount of 14-keto-12-hydroxynonacosane would be consistent with the fatty acids obtained by oxidative cleavage.

$$\overbrace{CH_3(CH_2)_{12}CO}^{R^I}\ CH_2CHOH(CH_2)_{12}CH_3 \qquad \text{I}$$

$$\overbrace{CH_3(CH_2)_{13}CO}^{R^{II}}\ CH_2CHOH(CH_2)_{11}CH_3 \qquad \text{II}$$

Mass spectroscopy of the hydroxyketone fraction confirmed the chemical evidence. The spectrum showed a "molecular peak" at m/e 420 (1.43% relative intensity), corresponding to the molecular weight of a C_{29} hydroxyketone after the loss of one molecule of H_2O. Other prominent peaks were at m/e 211 (9.17%) and m/e 225 (4.73%), due to ions R^ICO and $R^{II}CO$. Their relative intensities support the approximate ratio of 2:1 for the major isomers. Other relatively intense peaks observed in the high mass unit region were at m/e 251 (3.74%), m/e 237 (6.70%), and m/e 223 (1.77%), which can be assigned to ions formed from the isomeric hydroxyketones through loss of water and an alkyl group R.

It is interesting to note that the positions of the hydroxy groups in the hydroxyketones did not fully correspond to those in the secondary alcohols from the same source. We isolated the secondary alcohols, oxidized them to the ketones, and determined the positions of their functional groups as described above. They consisted of two major isomers, 14-nonacosanol (42%) and 15-nonacosanol (58%), a finding which is in agreement with a very recent report by Macey and Barber (17). However, unlike the ketones reported by these authors (17), the ketone fraction isolated from the wax used in our experiments consisted almost exclusively of 15-ketononacosane, as previously reported by others (4, 6, 10).

Our results show that the keto and hydroxy groups in the bifunctional C_{29} compounds of cabbage surface wax as well as in those having only one functional group are all located near the center of the chain.

Metabolic studies using long-chain fatty acids as precursors[1] have confirmed the concept of chain "elongation–decarboxylation" (18) for the hydroxyketones as well as for the other C_{29} derivatives. A search for the corresponding keto, hydroxy, or hydroxyketo fatty acids which may occur in small amounts should help to provide additional information on the immediate precursors.

This investigation was supported in part by U.S. Public Health Service Research Grant No. CA 10155 from the National Institutes of Health; U.S. Public Health Service Research Grant No. HE 08214 from the Program Projects Branch, Extramural Programs, National Heart Institute; and by The Hormel Foundation. We thank Dr. R. T. Holman and Mr. H. Hayes for recording the mass spectrum. Miss Margaret Hasbargen provided valuable technical assistance.

Manuscript received 24 August 1970; accepted 13 November 1970.

References

1. Eglinton, G., and R. J. Hamilton. 1967. Leaf epicuticular waxes. *Science* (*Washington*). **156:** 1322–1335.
2. Horn, D. H. S., and J. A. Lamberton. 1962. Long-chain β-diketones from plant waxes. *Chem. Ind.* (*London*). 2036–2037.
3. Tulloch, A. P., and R. O. Weenink. 1969. Composition of the leaf wax of little club wheat. *Can. J. Chem.* **47:** 3119–3126.
4. Channon, H. J., and A. C. Chibnall. 1929. XXII. The ether-soluble substances of cabbage leaf cytoplasm. V. The isolation of *n*-nonacosane and di-*n*-tetradecyl ketone. *Biochem. J.* **23:** 168–175.
5. Purdy, S. J., and E. V. Truter. 1963. Constitution of the surface lipid from the leaves of *Brassica oleracea* (var. *capitata* [Winnigstadt]). III. Nonacosane and its derivatives. *Proc. Roy. Soc. Ser. B.* **158:** 553–565.
6. Kolattukudy, P. E. 1965. Biosynthesis of wax in *Brassica oleracea*. *Biochemistry*. **4:** 1844–1855.
7. Kolattukudy, P. E., R. H. Jaeger, and R. Robinson. 1968. Biogenesis of nonacosan-15-one in *Brassica oleracea:* dual mechanisms in the synthesis of long-chain compounds. *Nature* (*London*). **219:** 1038–1040.
8. Schmid, H. H. O., and P. C. Bandi. 1969. *n*-Triacontanal and other long-chain aldehydes in the surface lipids of plants. *Hoppe-Seyler's Z. Physiol. Chem.* **350:** 462–466.
9. Kolattukudy, P. E. 1970. Plant waxes. *Lipids*. **5:** 259–275.
10. Laseter, J. L., D. J. Weber, and J. Oró. 1968. Characterization of cabbage leaf lipids: *n*-alkanes, ketone, and fatty acids. *Phytochemistry*. **7:** 1005–1008.
11. Schmid, H. H. O., L. L. Jones, and H. K. Mangold. 1967. Detection and isolation of minor lipid constituents. *J. Lipid Res.* **8:** 692–693.
12. Mahadevan, V., F. Phillips, and W. O. Lundberg. 1966. Reactions of dimethyl sulfoxide with sulfonate esters of fatty alcohols. I. Synthesis of higher saturated and unsaturated fatty aldehydes. *Lipids*. **1:** 183–187.
13. Baumann, W. J., and H. K. Mangold. 1964. Reactions of aliphatic methanesulfonates. I. Syntheses of long-chain glycerol-(1) ethers. *J. Org. Chem.* **29:** 3055–3057.
14. Rao, P. V., S. Ramachandran, and D. G. Cornwell. 1967. Synthesis of fatty aldehydes and their cyclic acetals (new derivatives for the analysis of plasmalogens). *J. Lipid Res.* **8:** 380–390.
15. Baumann, W. J., H. H. O. Schmid, and H. K. Mangold. 1969. Oxidative cleavage of lipids with sodium metaperiodate in pyridine. *J. Lipid Res.* **10:** 132–133.
16. Furukawa, S. 1932. Studies on the constituents of "Ginkgo Biloba L." leaves. Part II. *Sci. Pap. Inst. Phys. Chem. Res. Tokyo*. **19:** 39–42.
17. Macey, M. J. K., and H. N. Barber. 1970. Chemical genetics of wax formation on leaves of *Brassica oleracea*. *Phytochemistry*. **9:** 13–23.
18. Kolattukudy, P. E. 1968. Biosynthesis of surface lipids. *Science* (*Washington*). **159:** 498–505.