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IDENTIFICATION OF 16-HYDROXYOXOHEXADECANOIC ACID MONOMERS IN PLANT CUTINS*

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Key Word Index—*Citrus limon*; Rutaceae; *Physalis peruviana*; Solanaceae; *Ribes nigrum*; Grossulariaceae; cutin; ketohydroxy fatty acids; 16-hydroxy-10-oxohexadecanoic acid; structural analysis; positional isomerism; MS.

Abstract—The structure of a new hydroxyketo fatty acid which occurs as a major monomer of *Citrus limon* fruit cutin has been determined by IR, NMR and MS. The monomer was shown to be a mixture of positional isomers of 16-hydroxyoxohexadecanoic acid with the 10-oxo isomer predominating. Substantial amounts of the 9-oxo isomer were present together with smaller quantities of the 8- and 7-isomers. The same compounds were also found to be important constituents of the fruit cutins of *Physalis peruviana* and *Ribes nigrum*.

INTRODUCTION

THE MONOMERIC units of plant cutins, the lipid polymers of cuticular membranes, are mainly C_{16} and C_{18} hydroxy fatty acids. However, recent work has shown that additional oxygen containing groups may also be present, the most common being epoxides which occur in the C_{18} monomers.[1-4] Kolattukudy has also demonstrated the existence of aldehydic compounds in two cutins using a combination of reduction, reductive depolymerisation and deuterium labelling; 9-hydroxy-16-oxohexadecanoic acid was the major component of embryonic shoots of *Vicia faba*[5] and 9,10-epoxy-18-oxooctadecanoic acid was identified in immature apple fruits.[6]

The present paper describes the isolation and characterization of a new C_{16} hydroxyoxo monomer from the cutin of mature fruits of *Citrus limon* and its identification also in the fruit cutins of *Physalis peruviana* and *Ribes nigrum*.

RESULTS AND DISCUSSION

In the course of our investigations on the depolymerisation products of plant cutins a compound was found in certain species which did not correspond with any of the previously recorded monomers. In lemon fruit cutin this compound comprised *ca* 50% of the total monomers, the remainder being mainly dihydroxyhexadecanoic acid.

* Part VI in the series "The Composition of Plant Cutins". For Part V see HOLLOWAY, P. J. (1973) *Phytochemistry* **12**, 2913.

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[2] WALTON, T. J. and KOLATTUKUDY, P. E. (1972) *Biochemistry* **11**, 1885.
[3] HOLLOWAY, P. J. and DEAS, A. H. B. (1973) *Phytochemistry* **12**, 1721.
[4] HOLLOWAY, P. J. (1973) *Phytochemistry* **12**, 2913.
[5] KOLATTUKUDY, P. E. (1972) *Biochem. Biophys. Res. Commun.* **49**, 1040.
[6] KOLATTUKUDY, P. E. (1973) *Lipids* **8**, 90.

The unknown compound was well resolved from other monomers by TLC and chromatographed (R_f 0·3) below the ω-hydroxymonobasic methyl ester fraction (R_f 0·45) suggesting that it was probably monohydroxy in nature. The GLC R_t of the TMS ether derivative on SE30 was the same as that of the TMS ethers of methyl 18-hydroxyoctadec-9-enoate and dimethyl 8-hydroxyhexadecane-1,16-dioate but on OV210 was longer than either of these two compounds. The GLC data indicated that the compound could be either C_{16} or C_{18} in chain length and contained a functional group more polar than either $-OSi(Me)_3$ or $-COOMe$. Preparative TLC of the depolymerization products from lemon fruit cutin afforded a chromatographically pure sample of the unknown compound which was then analysed in detail.

The IR spectrum of the isolated compound was indicative of a long-chain saturated aliphatic compound and also showed the presence of free hydroxyl and two strong carbonyl absorptions, one corresponding with an ester (1740 cm^{-1}) and the other with a ketone (1720 cm^{-1}). The presence of a ketone and not an aldehyde was supported by the absence of any absorption near 2720 cm^{-1} (aldehydic C–H stretching). The oxo function in the compound was further confirmed by the formation of a 2,4-dinitrophenylhydrazone using a TLC test.[7]

Corroborative information was obtained from the NMR spectrum which showed a triplet τ 6·34 (2H) indicative of a primary alcohol group and a triplet τ 7·68 (2H) together with a singlet τ 6·32 (3H) characteristic of a methyl ester of a monobasic acid. The spectrum showed no signals for an aldehyde group (τ 0–1·0) but a triplet τ 7·60 (4H) attributable to protons on the two methylene groups α to a keto group; a similar splitting pattern was observed in the spectrum of tricosan-12-one. The broad multiplets occurring at τ 8·43 and τ 8·70 (together 20H) were characteristic of a straight chain aliphatic structure, the difference in chemical shift of the two signals probably arising from the combined deshielding effects of the keto and ester functions on the protons of methylene groups β, γ ... to the keto group.

The chain length of the compound and the position of the keto group were both determined by MS. The MS showed weak ions corresponding with M^+-18 and M^+-31 and a stronger ion at M^+-31-18 (M^+ 300) indicating a methyl ester of a C_{16} monohydroxyoxo-monobasic acid, and exhibited the characteristic fragmentation pattern of long-chain keto-esters[8,9] with ions formed by α and β-cleavage with respect to the keto group (Table 1). These fragment ions were more intense in the 20 eV spectrum than that at 70 eV (see experimental). The α-cleavage ions *m/e* 199 and 129 and the corresponding β-ions *m/e* 214 and 144 showed that the keto group was in the 10-position but the presence of ions *m/e* 185, 143, 200 and 158 indicated that a significant amount of the 9-keto isomer was also present. The spectrum also showed intense ions derived from the primary α- or β-cleavage fragments, those containing the hydroxyl group readily losing water and those containing the methyl ester group methanol (Table 1). Fragment ions probably derived from γ-cleavage were also present in the MS. Confirmation of the MW of the compounds were obtained from the MS of the TMS ether derivatives which showed an intense M^+-15 peak (M^+ 372) with smaller peaks at M^+-31 and M^+-47. The major fragment ions of these derivatives (Table 1) showed that both the 9- and 10-keto isomers were present and confirmed the terminal position of the hydroxyl group.

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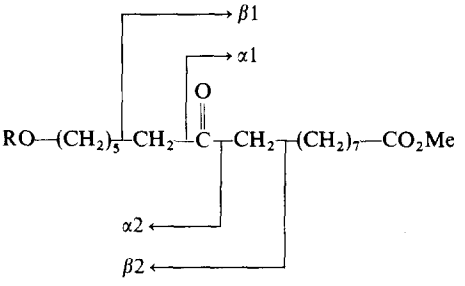
[8] RYHAGE, R. and STENHAGEN, E. (1960) *Ark. Kemi* **15**, 545.
[9] KLEIMAN, R. and SPENCER, G. F. (1973) *J. Am. Oil Chemists' Soc.* **50**, 31.

Reduction of the compounds with $NaBH_4$ gave a complete conversion to methyl dihydroxyhexadecanoate which on GC–MS analysis as the TMS ether derivative was found to contain 73% of the 10,16-isomer, 16% of the 9,16-, 9% of the 8,16- and 2% of the 7,16-isomers, thus providing confirmation of structure and an assessment of the positional isomer content of the original keto-acids. The positional isomer content of the reduced compounds was also very similar to that of the dihydroxyhexadecanoic acid fraction present in the cutin which was determined at the same time. Treatment of the isolated lemon fruit cuticular membrane with $NaBH_4$ prior to depolymerization resulted in the disappearance of the keto-compounds and an increase in the methyl dihydroxyhexadecanoate content of the depolymerization products. This result established that the keto-compounds were present in the original cutin polymer and were not formed as a result of the depolymerization procedures.

TABLE 1. MS FRAGMENTATION OF METHYL 16-HYDROXY-10-OXOHEXADECANOATE AND ITS TMS ETHER DERIVATIVE



Ion	R = H *m/e*	R = Si(Me)$_3$ *m/e*
α1 ^{+}CO–$(CH_2)_8$–CO_2Me	199	199
	199–MeOH → 167	
β1 [$^{\cdot}CH_2$–CO–$(CH_2)_8$–CO_2Me + H]$^+$	214	214
	214–MeOH → 182	
α2 RO–$(CH_2)_6$–CO^{+}	129	201
	129–H_2O → 111	201–Si(Me)$_3$OH → 111
β2 [RO–$(CH_2)_6$–CO–$\dot{C}H_2$ + H]$^+$	144	216
	144–H_2O → 126	216–Si(Me)$_3$OH → 126

Methyl 16-hydroxy-10-oxohexadecanoate and related positional isomers were identified in the depolymerization products of cape gooseberry and blackcurrant fruit cutins by TLC and GLC and by GC–MS of the TMS ether derivatives. In the former, the compounds comprised up to 23% of the total monomers and MS showed that about equal amounts of the 9- and 10-isomers were present together with appreciable quantities of the 8-isomer.

The monomers from blackcurrant fruit cutin contained about 14% of methyl 16-hydroxy-oxohexadecanoate and its isomer content was very similar to that of the same compound in lemon fruit cutin.

The natural occurrence of 16-hydroxyoxohexadecanoic acids has not been previously recorded and the compounds are another example of the constituents peculiar to cutin. Keto-fatty acids in general are rarely found as plant lipid constituents although they do occur in the seed oil of *Cuspidaria pterocarpa*[10] as C_{24}, C_{26} and C_{28} unsaturated homologues and can be formed from linoleic acid by the combined action of lipoxygenase and hydroperoxide isomerase.[11] Although the keto compounds of cutin have been analysed in only three species there is enough evidence to suggest that they are derived from the oxidation of dihydroxyhexadecanoic acid. Support for this hypothesis is found in the existence of positional isomers *per se* and in the very similar isomer contents of the dihydroxy and ketohydroxy compounds present in the same cutin. Another oxidation product of dihydroxyhexadecanoic acid, monohydroxyhexadecane-1,16-dioic, is also a common minor constituent of several cutins[12,13] and a similar positional isomer relationship exists between the two classes of hydroxy fatty acid. Biological oxidation may occur at the monomer stage prior to polymerization or in the cutin polymer itself by reaction of unesterified hydroxyl groups.

EXPERIMENTAL

Fruits of *Citrus limon* (cv. Adamopoulou) were supplied by Dr J. Procipiou, Agricultural Research Station, Rhodes, Greece and fruits of *Physalis peruviana* (cv. TN diploid) and *Ribes nigrum* (cv. Torr Cross) were obtained from plants grown at Long Ashton Research Station. Cuticular membranes were isolated using ammonium oxalate oxalic acid[14] and the extraction and depolymerization of isolated membranes carried out as previously described.[3,4] The TLC, PLC, GLC and GC–MS methods and equipment employed were also those used in earlier work.[3,4,15]

Isolation and characterization of methyl 16-hydroxyoxohexadecanoate. A sample of the compound (10 mg) was isolated from the depolymerization products of *C. limon* fruit cutin by PLC on silica gel ($CHCl_3$–EtOAc, 4:1). It gave a single spot on TLC and as the TMS ether one GLC peak RR_t (tetracosane) SE30 1·02 and RR_t (octacosane) OV210 1·18. IR ν_{max}^{film}: 3400 (OH), 2940, 2860, 1740 (–COOMe), 1720 (>C=O), 1470, 1440, 1370, 1250, 1200, 1175, 1060, 725 cm^{-1}. NMR 100 MHz ($CDCl_3$, internal standard TMS): τ 6·32 (*s*) $OC\underline{H}_3$, 6·34 (*t*, *J* 6Hz) $-C\underline{H}_2OH$, 7·60 (*t*, *J* 7Hz) $-C\underline{H}_2-\overset{\|}{C}-O$, 7·68 (*t*, *J* 7Hz) $-C\underline{H}_2-COOMe$, 8·43 (*m*) $-C\underline{H}_2-$, 8·70 (*m*) $-C\underline{H}_2-$. Assignment of NMR signals was based on comparisons with pure samples of methyl hexadecanoate, methyl octadecenoate, methyl 10,16-dihydroxyhexadecanoate, methyl 12-hydroxyoctadecanoate and tricosan-12-one. MS probe 20 eV *m/e* (rel. intensity): 282 (M^+-18) (3), 269 (M^+-31) (8), 251 (M^+-31-18) (22), 227 (12), 214 (70), 200 (19), 199 (43), 185 (20), 182 (20), 171 (22), 158 (23), 157 (65), 144 (41), 143 (24), 140 (28), 139 (45), 129 (46), 126 (100), 125 (66), 111 (71), 102 (37), 97 (55), 83 (67), 71 (53), 55 (37), 43 (23). MS probe 70 eV *m/e*: 282 (1), 269 (2), 251 (6), 227 (3), 214 (13), 200 (4), 199 (10), 185 (5), 182 (4), 171 (5), 157 (13), 144 (8), 139 (13), 129 (11), 126 (19), 125 (18), 111 (20), 102 (6), 97 (25), 85 (62), 83 (100), 71 (25), 69 (32), 55 (70), 43 (36). TMS ether GC–MS 70 eV *m/e*: 372 (M^+) (1), 357 (M^+-15) (53), 341 (M^+-31) (7), 325 (M^+-47) (17), 296 (m^* M^+-15 → M^+-47), 286 (7), 251 (9), 216 (9), 215 (9), 214 (26), 201 (16), 200 (7), 199 (15), 185 (13), 182 (5), 159 (30), 139 (20), 126 (45), 111 (26), 103 (31), 97 (34), 83 (52), 75 (81), 73 (82), 69 (66), 55 (100), 43 (35).

$NaBH_4$ reduction. To 2 mg methyl 16-hydroxyoxohexadecanoate was added 2·5 ml of a 0·2% soln of $NaBH_4$ in EtOH and the mixture stirred vigorously for 2 hr at 20°. After acidification with 5 ml of dil. HCl, the EtOH was removed under reduced pressure and the reduction products recovered by Et_2O extraction. The products were identified as a mixture of positional isomers of methyl dihydroxyhexadecanoate by chromatographic and MS comparison with an authentic sample of methyl 10,16-dihydroxyhexadecanoate. Positional isomer content

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was determined from the MS of the TMS ether derivatives using a published method.[15] Isolated cuticular membranes were treated with the $NaBH_4$ solution for 4 hr transferred to the HCl soln and stirred for a further 15 min. Before depolymerization the membranes were thoroughly washed with H_2O and MeOH.

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