simplifying assumptions have been used, as previously pointed out and, moreover, continuing process studies may allow further cost reduction. However, the reduction in cost accomplished by using water as the solvent for the CFA reaction is substantial.

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The Oxygenated Fatty Acid of Calendula Seed Oil

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Abstract

The seed oil of *Calendula officinalis* L. contains a monohydroxy acid amounting to some 5% of its component acids. This acid has been isolated and shown to be D-(+)-9-hydroxy-10,12-octadecadienoic acid, probably 10-*trans*,12-*cis*.

Introduction

THE SEED OIL OF *Calendula officinalis* L. (family *Compositae*) has been examined in recent years in three laboratories. McLean and Clark (1) demonstrated that a high proportion of the fatty acids consisted of a conjugated trienoic acid, which was isolated in its isomerised all-*trans* form and shown to be 8,10,12-octadecatrienoic acid. Chisholm and Hopkins (2) isolated the acid in its natural configuration, proved conclusively that it was *trans*-8, *trans*-10,*cis*-12-octadecatrienoic acid and estimated that it comprised 47% of the oil. Earle et al. (3,4) in their examination of a large number of seed oils from *Compositae* and other families also noted the presence of conjugated trienoic acid in *Calendula* oil, estimated at 51–53% of the fatty acids, other components being palmitic (5%), stearic (2%), oleic (5.5%) and linoleic (34%) acids. In addition, they obtained a carbonyl value corresponding to 4% of the oil, if calculated as a C_{18} keto-acid, and also noted an apparent "dimorphecolic acid" (i.e., 9-OH-*trans*-10,*trans*-12-octadecadienoic acid) content of 4%, as determined by their hydrogen bromide titration method (4).

Long-chain keto-acids are very rare in nature and for this reason it was considered worthwhile to examine more closely any oxygenated acid present in *Calendula* oil. Only one oxygenated acid was detected. This was isolated and shown to be D-(+)-9-hydroxy-10,12-octadecadienoic acid, probably 10-*trans*,12-*cis*.

Experimental and Results

Seeds of *Calendula officinalis* L. were harvested from the garden of one of us, the original seed having been purchased from a reputable seedsman. The fresh seed (165 g) was finely ground and extracted three times with light petroleum at room temperature over a period of 24 hr. The solvent was removed from the clear filtered extract, at less than 30° in a rotary evaporator, to yield 16.7 g of oil. The oil was hydrolysed by standing overnight at room temperature with 5% ethanolic potassium hydroxide and nonsaponifiable material was removed by ether extraction. After careful acidification to pH 5 with 1 N sulfuric acid, the mixed acids were immediately extracted into diethyl ether. The ether solution was washed with water till neutral, dried and the solvent removed to yield 14.7 g of mixed fatty acids. Thin-layer chromatography (TLC), with ether-hexane-formic acid (50:50:1) as solvent, of the mixed acids alongside suitable standards revealed no trace of a keto acid component but a small amount of a hydroxy acid which had similar migration characteristics to a dimorphecolic acid standard.

The hydroxy acid fraction (914 mg, 6.2% of the total mixed acids) was concentrated in 70% aqueous ethanol by a three-funnel six-withdrawal distribution against hexane as stationary solvent. This concentrate was esterified with diazomethane and separated by preparative TLC with ether-hexane (1:1) as developing solvent, to yield 690 mg (4.7% of total mixed acids) of monohydroxy methyl ester which migrated as a single component on analytical TLC, with the same solvent system.

Spectrophotometry

The isolated hydroxy ester had an ultraviolet absorption maximum at 233mμ, $\Sigma = 23{,}150$, corresponding to a conjugated diene grouping. There was no measurable absorption in the conjugated triene region. The spectrum of a sample treated with anhydrous hydrogen bromide in ether showed some residual absorption at 233 mμ and a strong band with maxima at 260, 269 and 280 mμ indicating an all-*trans* conjugated triene group. This effect of hydrogen bromide treatment is characteristic of a vicinal hydroxydiene grouping and arises by partial dehydration to conjugated triene accompanied by partial substitution of bromine for hydroxyl (5).

The infrared spectrum of a dilute (ca. 0.3% w/v) solution of the hydroxy ester in carbon tetrachloride showed a single sharp band at 2.76 μ in the hydroxyl stretching region. This corresponded exactly to the intramolecularly associated hydroxyl band of a conjugated dienol grouping such as methyl dimorphecolate and differed from the dilute solution spectra of other unsaturated hydroxyl groupings (6). The infrared spectrum of a thin film of the ester showed bands of near equal intensity at 10.13 μ and 10.51

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μ which are characteristic of a *cis,trans-* or *trans, cis-*conjugated diene system (7).

The spectral evidence already pointed to a conjugated dienol grouping such as we had previously encountered in hydroxy acids from *Artemisia absinthium* and other seed oils (8). Chromatographic studies provided further evidence for this structure before it was unequivocally verified by degradation.

Chromatography

The hydroxy ester from *Calendula* oil was subjected to gas-liquid chromatography (GLC) on both polyethylene glycol adipate and Apiezon L stationary phases at temperatures of 182C and 204C, respectively, in Pye argon chromatographs. On both types of column a double peak was obtained, corresponding not to hydroxy esters but to *trans,trans,cis-* and *trans, trans,trans-*conjugated trienoic C_{18} esters. This is diagnostic for a conjugated dienol grouping which is dehydrated and partially isomerised during GLC (9). Samples of methyl dimorphecolate, run to verify that the column conditions would indeed lead to this result, gave chromatograms almost identical to those of the *Calendula* hydroxy ester on both stationary phases.

A portion of the hydroxy ester was hydrogenated and the product had identical relative retention times to 9-hydroxystearate on both columns. There is sufficient variation of the relative retention times of the isomeric hydroxystearates on these two stationary phases (10,11) to narrow the possibilities down to a C_{18}-hydroxy ester with the substitution between the 8- and 12-positions.

The natural hydroxy ester was also examined by TLC, using as standards methyl 9-OH-*trans,trans*-10,12-octadecadienoate from *Dimorphotheca* oil and 9-OH,*trans,cis*-10,12-octadecadienoate and 13-OH-*cis, trans*-9,11-octadecadienoate from *Artemisia* oil (8). These three hydroxy-diene isomers are separable on TLC (8,12) and the *Calendula* hydroxy ester migrated with the 9-OH-*trans,cis*-diene isomer on silica gel G with diethyl ether-hexane (1:1) as developing solvent. On a silver nitrate impregnated layer these two compounds again migrated together (cf. 12).

That the portion of the hydroxy ester which had been treated with anhydrous HBr for the ultraviolet studies had given conjugated trienoic and bromodienoic compounds was further indicated on TLC by disappearance of the hydroxy ester spot and its replacement by less polar compounds. This behaviour on TLC, like the change in ultraviolet spectrum, is indicative of a conjugated dienol grouping.

The hydrogenated hydroxy ester migrated exactly with standard 9-hydroxystearate on TLC. The isomeric 5- through 12-hydroxystearates have progressively increasing mobilities on TLC under the conditions used (12) and, since GLC had limited the possibilities to a hydroxystearate substituted between the 8- and 12-positions, it was thus almost certainly proved that the *Calendula* hydroxy ester was a 9-hydroxy-C_{18} derivative. This conclusion was reinforced by the fact that the melting point of the saturated ester (49–51C) was not depressed in admixture with authentic 9-hydroxystearate. There was no evidence of a 13-hydroxy isomer being present as in several other seed oils (2,8).

The microanalytical techniques so far described together pointed to the *Calendula* hydroxy acid being a C_{18}-acid having a *cis,trans*-conjugated diene system with a vicinal hydroxyl group in position 9. Final verification of this structure was obtained by classical degradative studies.

Oxidative Degradation

The unsaturated hydroxy ester was oxidised by permanganate-periodate (13), the acidic scission products were esterified with diazomethane and were identified by GLC on Apiezon L in comparison with standard monobasic and dibasic acid esters. Hexanoate, identified at 70C, was the sole monobasic fragment and azelate was the predominant dibasic product. Small proportions of shorter dibasic fragments which were present were considered to be due to over-oxidation. The hydroxydiene grouping was thereby shown to lie between the 9- and 13-positions in the C_{18}-chain, i.e., either 9-hydroxy-10,12-diene, as indicated by the chromatography studies, or 13-hydroxy-9,11-diene.

The position of the hydroxyl group was shown to be in the 9-position by oxidation of the hydrogenated ester with chromium trioxide in acetic acid (14). This gave predominantly C_9 and C_{10} monobasic acids and C_8 and C_9 dibasic acids, with very minor amounts of shorter chain homologues but no trace of C_{12} or C_{13} dibasic acids which would arise from 13-hydroxystearate.

Optical Activity

The sample of pure hydroxy ester subsequently used for all the analytical and degradative experiments herein described had a specific rotation of $[\alpha]_D^{20^\circ} = +3.6^\circ$ ($c = 19.4\%$ in $CHCl_3$) as measured with a normal polarimeter using a 2 dm narrow bore cell. This rotation is of the same sign and of comparable magnitude to values of $+4.5^\circ$ and $+5^\circ$ obtained for methyl dimorphecolate (11,15). A second sample of the hydroxy ester, isolated from a different batch of seeds, had the much lower rotation of $[\alpha]_{546.1\,m\mu}^{25^\circ} = +0.52^\circ$ ($c = 40\%$ in $CHCl_3$) when measured with an ETL/NPL Automatic Polarimeter, using a 2 cm cell. Pure methyl 9-hydroxystearate prepared from this second sample had $[\alpha]_{546.1\,m\mu}^{25^\circ} = -0.06^\circ$, which is of the same sign as, but considerably smaller than, the values of -0.32° and -0.29° which we obtained under the same conditions for the 9-hydroxystearates derived respectively from the 9-hydroxy-12-octadecenoate of *Strophanthus* oil and from methyl dimorphecolate. The discrepancies in these various specific rotation values indicate that the second hydroxy ester isolate had undergone considerable recemisation at some stage. We are unable to suggest a mechanism for this but can only record that the second isolate inadvertantly remained at room temperature for several weeks and required extensive repurification before these optical rotational measurements were made. As part of a study of the absolute optical configurations of a large number of natural monohydroxy acids (11), we are preparing a further sample of the *Calendula* hydroxy acid and we should have unequivocal rotation values in the near future.

The significant points, however, are firstly that the natural ester is dextrorotatory like methyl dimorphecolate and secondly that the 9-hydroxystearates derived from the *Calendula* hydroxy ester, from dimorphecolate, and from the 9-hydroxy-12-octadecenoate from *Strophanthus* are all levorotatory. Baker and Gunstone (16) synthesised D-9-hydroxystearic acid by a stereochemically defined route and Bloch and Schroepfer (17) were able to show that this syn-

thetic acid was identical in rotation to the 9-hydroxystearic acid derived from the *Strophanthus* acid. The latter was thereby proved to have the D-configuration. We have similarly shown that dimorphecolic acid has the D-configuration (11) and, since the sign of rotation of the 9-hydroxystearate from the *Calendula* hydroxy acid is also negative it follows that it must have the D-configuration, i.e. (*S*) in the Cahn-Ingold-Prelog system (18).

Discussion

We have shown that the hydroxy acid which comprises some 5% of the total fatty acids of *Calendula officinalis* seed oil is D-(+)-9-hydroxy-10,12-octadecadienoic acid, one of the double bonds being *cis* and the other *trans*. This acid therefore is geometrically isomeric with dimorphecolic acid, the 9-hydroxy-*trans,trans*-10,12-octadecadienoic acid from *Dimorphotheca* oil. Like *Dimorphotheca* oil, *Calendula* oil does not contain any of the analogous 13-hydroxy-9,11-octadecadienoic acid isomer, as is the case with several other seed oils (2,8). Since this work was completed we have learned that the Peoria group have found what is probably the same acid amounting to some 65% of the acids of another species of the family *Compositae* (19).

We have not been able to determine if the conjugated diene system is *cis*-10,*trans*-12 or *trans*-10, *cis*-12 but we consider the latter configuration to be more likely from a consideration of possible biogenetic pathways. Thus, we (8,20) and others (3,4,15) have suggested in the past that these hydroxydienoic acids may be formed in nature from linoleic acid and that the structural correspondence of the hydroxydienoic isomers and the two series of conjugated trienoic acids (8,10,12- and 9,11,13-) suggests a common biosynthetic mechanism. These ideas have recently been elaborated by Gunstone (21) who proposes a generalised series of possible biological pathways to the various conjugated polyethenoid acids presently known to occur naturally. Although we consider that the 11-hydroxy-9,12-octadecadienoic acid intermediate proposed by Gunstone is neither necessary nor likely we concur with him in the belief that most of these conjugated unsaturated acids are derived from linoleic acid. If this is so, and we are currently engaged on biosynthetic experiments to try to clarify this (20), then one of the double bonds of linoleic acid must migrate into conjugation with the other. In so doing this bond will adopt the *trans*-configuration, as suggested by Gunstone (21) and as is the case in chemical autoxidation and in lipoxidase oxidation, and the bond which has not migrated will retain its *cis*-configuration. The 9-hydroxydiene acid would therefore have the *trans*-10,*cis*-12 configuration. That the 8,10,12-conjugated trienoic acid, which is the major component of *Calendula* oil and which may be considered to be derived from the hydroxydiene acid by dehydration, has the *trans*-10,*cis*-12 grouping in its conjugated system lends support to our conclusion that the *Calendula* hydroxy acid is D-(+)-9-hydroxy-*trans*-10,*cis*-12-octadecadienoic acid.

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Anatomical Variation in Fatty Acid Composition and Triglyceride Distribution in Animal Depot Fats[1]

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Abstract

The fatty acid composition and glyceride distribution of fatty acids in pork, beef and lamb depot fats from different localities within the same animal were determined by a combination of gas liquid chromatography and lipase hydrolysis techniques. The glyceride distribution was calculated according to the method of Vander Wal, based on the 1, 3-random and 2-random distribution pattern. Both fatty acid composition and glyceride structure were found to vary depending on the position within the animal from which the depot fat was obtained.

THE FATTY ACID COMPOSITION and glyceride type distribution of natural fats have been extensively studied by Hilditch and co-workers (1). These studies involved classical methods of fractional crystallization from suitable solvents followed by chemical analysis. The discovery that primary hydroxyl groups of glycerol esters of fatty acids are specifically cleaved by pancreatic lipase (2,3) permitted the detailed structural analysis of natural fats. Many theories of triglyceride distribution have been proposed and defended (4,5); and the 1, 3- random and 2- random distribution hypothesis of Vander Wal (5) has been shown to be applicable to a large number of depot fats. He proposed a method for calculating the distribution of saturated and unsaturated acyl groups in fats (5) from pancreatic lipase hydrolysis data. It is recognized from these studies, and those of others that the 2- position in the molecule of natural fats is generally occupied by a proportion of acyl groups

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