dihydroxyacetone-P to 1-alkyl-*sn*-glycero-3-P which is subsequently deacylated.

Discussion

In order to provide corroborative evidence indicating an exchange of hydrogen on DHAP during glyceryl ether synthesis, it would be essential to demonstrate that hydrogen from the aqueous environment becomes bound to the glyceryl ether molecule at C-3 of the DHAP moiety. The data presented here indicate that each molecule of *O*-alkyl lipid formed in the presence of tritiated water acquires tritium activity consistent with the addition of 1 H/mol of *O*-alkyl lipid formed. The data also indicate that the tritium is gained at C-3 of DHAP of *O*-alkyl-DHAP.

In addition to being consistent with the findings of others that acyl-DHAP is a precursor of *O*-alkyl lipid, other facets of this study can be summarized as follows. In the *Tetrahymena* system, acyl-DHAP leads to the formation of acyl-DHA and to DHA; the formation of DHA proceeds *via* a hydrogen exchange and is CoA dependent; in the presence of hexadecanol, acyl-DHAP is preferentially used in *O*-alkyl lipid formation, a reaction, or a series of reactions, which involve a hydrogen exchange; the preferential utilization of acyl-DHAP in *O*-alkyl lipid formation is reflected in reduced DHA formation. We do not consider the data presented to be evidence that acyl-DHA is necessarily the only precursor of DHA.

Acknowledgments

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4-Sphingenine Derivatives in Wheat Flour Lipids†

Raili Laine and Ossi Renkonen*

ABSTRACT: *erythro*-4-Sphingenine and *erythro*-sphinganine were isolated from hydrolysates of wheat flour cerebrosides. Derivatives of both these bases, containing cis or trans double bonds at C_8, were also isolated. Argentation chromatography, gas–liquid chromatography, infrared spectroscopy, partial hydrazine reduction, ozonolysis and hydrogenolysis were used for identification of the new bases. The presence of a trans double bond at C_4 is typical of animal long-chain bases, but has not previously been described in plant systems.

In sphingoglycolipids of plants the known long-chain bases (LCB)[1] are derivatives of sphinganine and 4*D*-hydroxysphinganine (Carter *et al.*, 1961a). On the other hand, the LCB typical of animal systems, 4-sphingenine, has not been found in higher plants. The present report shows that wheat flour cerebrosides contain 4-sphingenine and its derivatives.

Materials and Methods

Isolation of Wheat Long-Chain Bases. Two samples of wheat flour were studied: 2.3 kg of Manitoba II (1969) and 1.5 kg of White Rose (1970), both of which were obtained from the State Granary (Helsinki). They had not been subjected to any previous chemical treatment.

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[1] Abbreviation used is: LCB, long-chain bases. The nomenclature used for the LCB's is that proposed by the IUB Commission (1967).

The lipids were extracted with chloroform–methanol (2:1, v/v). The extract was partitioned in the biphasic system chloroform–methanol–water (8:4:3) (Folch *et al.*, 1957) and the lipids were recovered from the lower layer. The glycerolipids were destroyed with mild alkaline methanolysis and unchanged ceramide hexosides together with sterol glucosides were then isolated from the methanolysates by column chromatography on silicic acid, as described previously (Renkonen and Hirvisalo, 1969). In addition to ceramide monohexosides small amounts of three slowly migrating glycolipids were revealed in this fraction with thin-layer chromatography (tlc). In three experiments (White Rose and Manitoba) this mixture was hydrolyzed with $Ba(OH)_2$ according to Morrison and Hay (1970). In two experiments (Manitoba) this mixture was subjected to Smith degradation as described elsewhere (Renkonen, 1969); the liberated ceramides were then cleaved by alkaline methanol as described by Carter *et al.* (1961b).

The LCB's were isolated from the hydrolysates with preparative tlc on silica gel G using a modified system of Sambasivarao and McCluer (1963) with chloroform–methanol–7 N NH_4OH (60:20:3) as solvent.

Reference Long-Chain Bases. A purified preparation of 4-sphingenine was obtained from bovine brain sphingomyelin as described previously (Renkonen and Hirvisalo, 1969). Catalytic hydrogenation gave sphinganine. *threo*-Sphinganine was a synthetic sample obtained from Dr. E. F. Jenny, Basel, Switzerland. 4*D*-Hydroxysphinganine was a gift from Dr. H. E. Carter, University of Illinois, Urbana, Ill.

Preparation of Long-Chain Base Derivatives. Triacetyl LCB's were prepared as described elsewhere (Renkonen and Hirvisalo, 1969). *N*-Acetyl LCB's were obtained by subjecting the triacetyl compounds to mild alkaline methanolysis, whereas free bases were obtained from triacetyl and *N*-acetyl derivatives by strong alkaline methanolysis under conditions described by Carter *et al.* (1961b). Trimethylsilyl ethers of free or *N*-acetyl bases were prepared by silylation procedure of Carter and Gaver (1967).

Oxidation Procedures. For ozonolysis of the LCB's a procedure developed by Privett and Nickell (1966) for fatty acids was used. The triacetyl LCB's (300 μg) were dissolved in 0.3 ml of highly purified pentane and cooled to $-70°$; 1.0 ml of ozone saturated pentane at $-70°$ was added. After 30 min at $-70°$, excess ozone was removed with a stream of dry nitrogen. The reaction mixtures were reduced with triphenylphosphine (Stein and Nicolaides, 1962) and injected directly into the gas–liquid chromatograph (glc). To obtain quantitative results an internal standard (dodecanal) was added to the LCB prior to ozonolysis and glc.

Reduction Procedures. Catalytic hydrogenation and partial hydrazine reduction were carried out and the reaction products were analyzed as described previously (Renkonen and Hirvisalo, 1969).

Hydrogenolysis of Long-Chain Bases Containing Trans Double Bond at C_4. Hydrogenolysis of 3-*O*-acetyl groups of 4-sphingenine and its derivatives (Carter *et al.*, 1947a) was carried out as described previously (Renkonen and Hirvisalo, 1969).

Chromatographic Methods. Various forms of tlc and glc were carried out essentially as described previously (Renkonen and Hirvisalo, 1969). The reduction products of ozonolysis were analyzed on a 4-m glc column of 15% EGSS-X on Gas Chrom P; isothermal runs were carried out at 100–190°.

Physical Measurements. Infrared (ir) spectra were recorded with a Perkin-Elmer Infracord type 457; the samples were in the form of a film on potassium bromide.

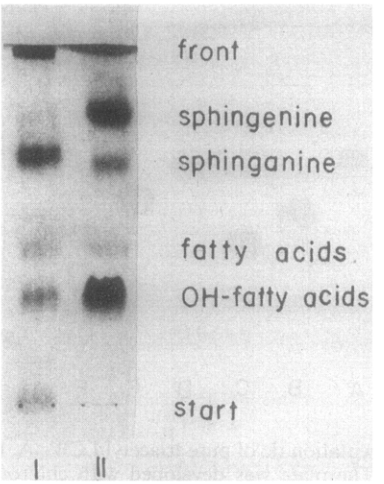

FIGURE 1: Thin-layer chromatography of $Ba(OH)_2$ hydrolysate of "alkali-stable" glycolipids of wheat flour: (I) $Ba(OH)_2$ hydrolysate of wheat flour glycolipids; (II) mixture of reference compounds 4-sphingenine, sphinganine, 4*D*-hydroxysphinganine, palmitic acid, and 2-hydroxyhexadecanoic acid (4*D*-hydroxysphinganine and palmitic acid overlap in this picture). The silica gel G plate was developed with chloroform–methanol–7 N NH_4OH (60:20:3) and stained with 20% ammonium hydrogen sulfate (Ziminski and Borowski, 1966).

Specific rotations were determined at 27° with a Perkin-Elmer polarimeter Type 141, using a thermostated tube 10 cm long and 0.9 ml in volume.

Results

Isolation of the Long-Chain Bases. The alkali-stable, lipophilic glycolipid fraction amounted to 0.036% (Manitoba) and 0.038% (White Rose) of the flour weight of our samples. After treating the glycolipids with $Ba(OH)_2$ the hydrolysate revealed on tlc a small sphingenine fraction and a strong sphinganine fraction in addition to a fast-migrating band of sterols (Figure 1). 4*D*-Hydroxysphinganine and related LCB's were not found in the hydrolysates although they are known to be constituents of wheat cerebrosides (Carter *et al.*, 1961a; MacMurray and Morrison, 1970). Morrison has recently found that alkaline treatment of ceramides containing unsubstituted fatty acids and 4*D*-hydroxysphinganine gives low and variable recoveries of the LCB's (private communication).

An alternative method to obtain the LCB's, involving the Smith degradation of the wheat glycolipids and subsequent alkaline cleavage of the resulting ceramides, also gave a sphingenine as well as a sphinganine fraction. This treatment destroys the trihydroxy LCB's completely.

The two LCB fractions were isolated from the hydrolysate by preparative tlc. The 4-sphingenine fraction amounted to 2.5 ± 0.9% (mean ± SD) of the alkali-stable glycolipids, whereas the larger sphinganine fraction amounted to 8.5 ± 2.8% (mean ± SD).

The two LCB fractions were acetylated and subjected to argentation tlc. Both fractions revealed three components which were separated by preparative tlc and weighed. The six compounds (A–F) were obtained in almost pure form, as shown in Figure 2. Table I shows their relative amounts. Infrared spectra (Figure 3) confirmed that B–F were sphingosine-like molecules; the sample of A was too small and too

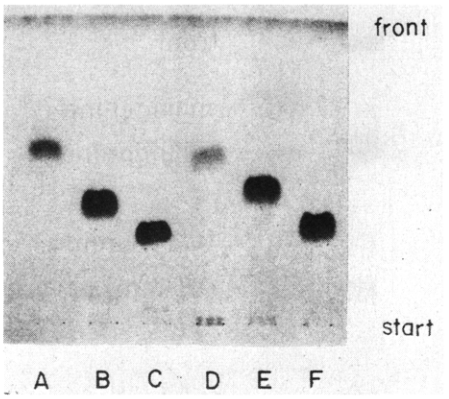

FIGURE 2: Argentation tlc of pure triacetyl LCB's A–F isolated from wheat flour. The plate was developed with chloroform–methanol (98:2) and stained with 20% ammonium hydrogen sulfate.

impure for a satisfactory spectrum, however. It is noteworthy that compounds B, C, and E had absorption peaks at about 970 cm^{-1} corresponding to trans double bonds. The spectrum of F was almost identical with that of *cis*-14-sphingenine (Renkonen and Hirvisalo, 1969).

Structural Analysis of the Long-Chain Bases. The mobility of the acetylated wheat LCB's on argentation tlc revealed some correlations helpful in the subsequent structural analysis. All three components of the sphingenine fraction had a pair in the sphinganine fraction which had the same mobility (Figure 2). The double bond at C_4 does not affect the mobility of the LCB's in this system, whereas the chain double bonds do (Renkonen and Hirvisalo, 1969). Therefore, it appeared likely already at this stage of the work that identical chain structures beyond C_4 were present in A and D on one hand, in B and E on the other hand, and also in the third pair, C and F. The only difference within each pair would thus be the presence or absence of the C_4 double bond.

Comparison with reference compounds on argentation tlc revealed that triacetyl A and D had a similar mobility than triacetyl-4-sphingenine and sphinganine. Triacetyl B and E had a mobility similar to that of triacetyl-4,*cis*-14-sphingadienine, but triacetyl C and F were clearly slower. This led us first to think, incorrectly, that C and F have two chain double bonds beyond C_4.

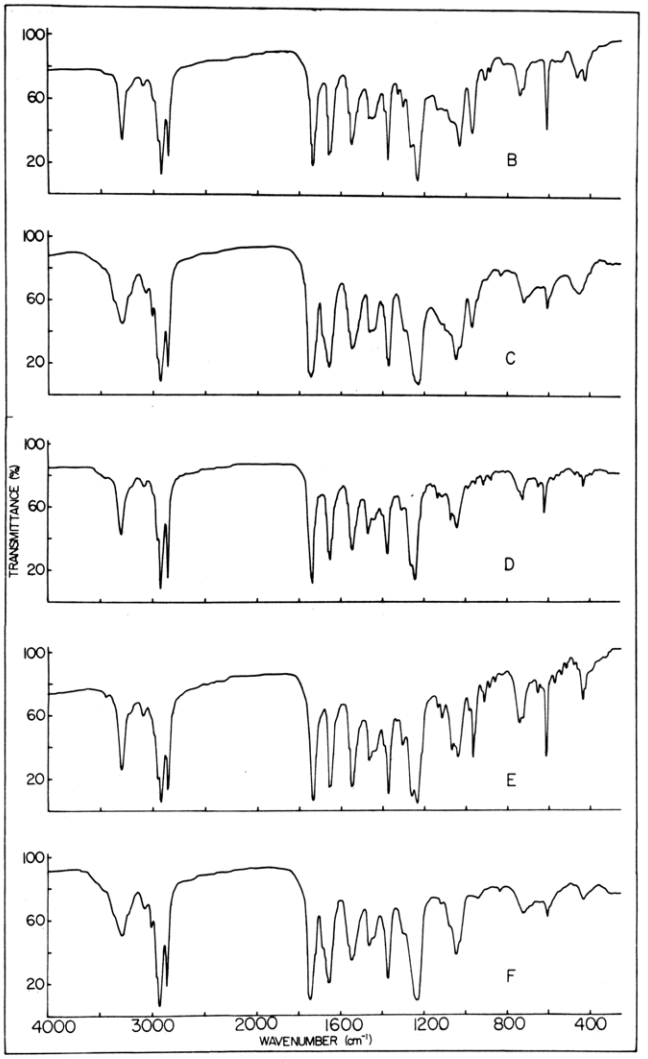

FIGURE 3: Infrared spectra of the acetylated LCB's A–F of wheat.

TABLE I: Dihydroxy Long-Chain Bases Isolated from Wheat Flour Cerebrosides.[a]

	%
4-Sphingenine derivatives	
A	3.0 ± 1.3
B	6.0 ± 0.6
C	14.0 ± 1.0
Sphinganine derivatives	
D	9.6 ± 1.9
E	14.9 ± 5.6
F	52.5 ± 5.2

[a] Three separate fractionations were carried out on argentation tlc with both the 4-sphingenine and the sphinganine fractions. The overall yields varied between 71 and 98%. The results give the percentage (± SD) of each component as compared to the sum of the six isolated LCB's.

The idea of identical chain structures in the sphingenine and sphinganine series was also supported, indirectly, by similar weight ratios between the individual members of the two series; A:B:C was approximately the same as D:E:F (Table I).

Gas–Liquid Chromatography of the Bases. Gas–liquid chromatography of hydrogenated *N*-acetyl-*O*-trimethylsilyl derivatives of the six LCB's on SE-30 revealed that the sphinganine derivative was the main component in all cases. This indicated that the LCB's were all straight-chain C_{18} compounds. The minor compounds amounted probably to less than 10% in all cases, and the most prominent among the minor peaks seemed to be the straight-chain C_{16} compound.

Gas–liquid chromatography separated the derivatives of *threo*-sphinganine and *erythro*-sphinganine (Carter and Gaver, 1967). Table II shows that all of the hydrogenated wheat LCB's had retention times identical with *erythro*-sphinganine, and therefore could be assigned the erythro configuration at C_2–C_3.

Gas–liquid chromatography of the derivatives of the native LCB's on SE-30 (Table II) revealed that compound A was

TABLE II: Relative Gas–Liquid Chromatographic Retention Times for *N*-Acetyl-*O*-trimethylsilyl derivatives of Long-Chain Bases on SE-30.[a]

LCB	Derivatives of Native LCB's	Derivatives of Hydrogenated LCB's
A	0.91	1.00
B	0.86	1.00
C	0.82	1.00
D	1.00	1.00
E	0.93	1.00
F	0.90	1.00
Sphinganine	1.00	1.00
4-Sphingenine	0.91	1.00
rac-threo-Sphinganine	0.88	0.88
4,*cis*-14-Sphingadienine	0.91	1.00

[a] Relative to *N*-acetyl-*O*-trimethylsilylsphinganine. Conditions for glc: 4-m 3% SE-30 column, temperature 220°.

identical with 4-sphingenine, and compound D with sphinganine. The retention times of B and C as related to A were similar to those of E and F in relation to D. This supported further the assumption that the pairs B and E and C and F had identical chain structures beyond C_4.

Specific Rotation of the Long-Chain Bases. Specific rotations could be determined only for the acetylated compounds E and F and for the completely saturated triacetyl F. All three had specific rotations close to that reported for acetylated sphinganine (Table III). This proved that compound F must have the *D* configuration at C_2. The same applies also for compound E as it differs structurally from sphinganine (see below) in the same way as dehydrophytosphingosine differs from phytosphingosine, and this difference does not affect the specific rotation (Carter and Hendrickson, 1963; Carter *et al.*, 1954).

Hydrogenolysis of Triacetyl Long-Chain Bases. The LCB's which have an olefinic double bond in allylic position to an *O*-acetoxy group lose this group upon catalytic hydrogenation (Carter *et al.*, 1947a). The resulting molecules, acetylated 3-deoxysphinganines, can be detected conveniently by tlc (Renkonen and Hirvisalo, 1969). Acetylated 3-deoxysphinganine was indeed formed, together with acetylated sphinganine, when acetylated compounds A, B, and C were hydrogenated with platinum as catalyst. In contrast, acetylated compounds

TABLE III: Specific Rotation of Triacetyl Long-Chain Bases in Chloroform.

LCB	$[\alpha]_D^{27}$ (deg)
E (*c*, 0.61)	+17.7
F (*c*, 0.61)	+17.3
F hydrogenated (*c*, 0.45)	+16.4
Sphinganine[a] (*c*, 1.0)	+18.0
4-Sphingenine[a] (*c*, 1.0)	−11.7

[a] Carter *et al.* (1947b); $[\alpha]_D^{28}$.

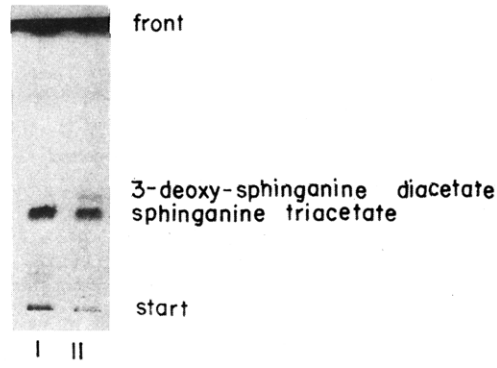

FIGURE 4: Thin-layer chromatography of reaction products obtained by treating acetylated LCB's with platinum and hydrogen: (I) products of LCB-F, a sphinganine derivative; (II) products of LCB-C, a sphingenine derivative. The silica gel G plate was developed twice with chloroform–methanol (99:1) and stained with 20% ammonium hydrogen sulfate.

D, E, and F did not form acetylated 3-deoxysphinganine under these conditions, but yielded only acetylated sphinganine. An example of the reaction mixtures on tlc plates is shown in Figure 4. These experiments agree with the tlc mobilities of the native LCB's on untreated silica gel, and suggest that compounds A, B, and C contain a double bond at C_4, whereas compounds D, E, and F do not.

Hydrazine Reduction of Compound F. The number of double bonds was determined in compound F since it was available in larger amounts than the others. The different double bonds in LCB are reduced at approximately similar rates with hydrazine (Renkonen and Hirvisalo, 1969). Therefore, the mixture resulting after partial reduction of a dienoic LCB contains a fully saturated LCB, two monoenoic LCB's, and unreacted dienoic LCB. In contrast, the reaction mixture after the partial reduction of a monoenoic LCB contains only two

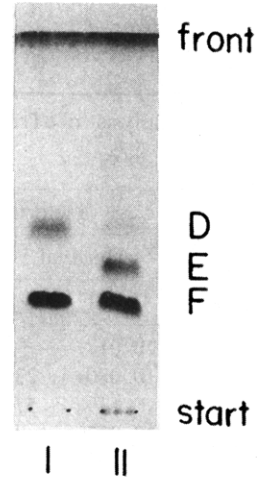

FIGURE 5: Argentation tlc of reaction products obtained in partial reduction of triacetyl F with hydrazine hydrate: (I) the reaction mixture obtained from LCB-F (after reacetylation); (II) a model mixture consisting of: triacetyl D (= triacetylsphinganine), triacetyl E, and triacetyl F. The plate was developed with chloroform–methanol (98:2) and stained with 20% ammonium hydrogen sulfate.

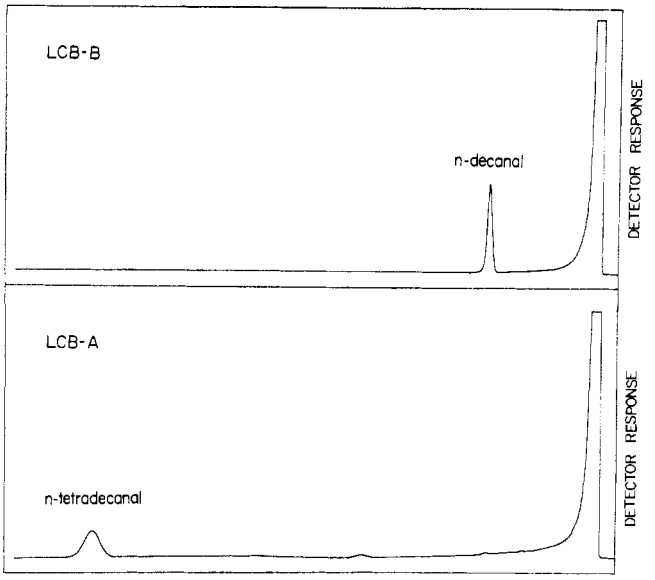

FIGURE 6: Gas–liquid chromatography of aldehydes derived from reductive ozonolysis of triacetyl LCB's in pentane. Conditions for glc: 4-m 15% EGSS-X on Gas Chrom P, temperature 140°.

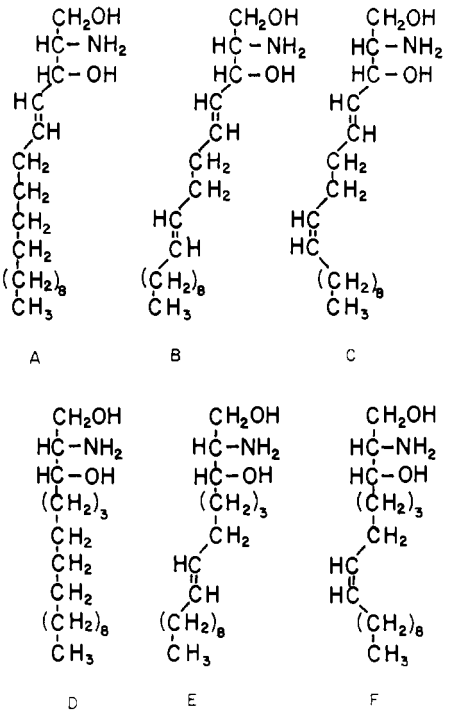

FIGURE 7: Suggested structures for dihydroxy long-chain bases from wheat.

compounds, the saturated LCB and the monoenoic LCB. When acetylated compound F was treated with hydrazine and the product analyzed by argentation tlc, only two spots were found. The first corresponded to unreacted compound F and the second to sphinganine (Figure 5). Thus compound F contains only one olefinic double bond. This result was a surprise; we had expected to find two double bonds in F since it migrated more slowly than 4,*cis*-14-sphingadienine on argentation tlc. Obviously the location of the double bond along the chain is of major importance for the LCB's as well as for other lipid components (Morris and Nichols, 1970) in determining their mobility on argentation tlc. The fact that F is a monoenoic LCB suggests that C, and also B and E, may contain only one double bond beyond C_4.

Ozonolysis of Triacetyl Long-Chain Bases. Reductive ozonolysis of the LCB's and analysis of the monoaldehydes formed allowed the location of the double bonds. Examples of the glc tracings obtained are shown in Figure 6, and the quantitative results are collected in Table IV. Compound D yielded only a small amount of decanal. The result is consistent with the presumption that D is sphinganine contaminated with about 15% of compound E. Compound E itself gave decanal in a high yield. Thus, its double bond must be located at C_8. An ir spectrum (Figure 3) suggests that this double bond has the trans geometry. Compound F also gave decanal in a very good yield. Thus it has the olefinic linkage at C_8 just as compound E. However, the ir spectrum and argentation tlc showed that in F, the linkage has the cis geometry.

Authentic 4-sphingenine yielded only tetradecanal under the experimental conditions used. However, the yield was only one-fourth of that obtained in the cleavage of the C_8-double bond of compounds E and F. Clearly, therefore, the rates of ozonolysis of different olefinic bonds are different in LCB's.

Compound A gave tetradecanal, in about the same yield as 4-sphingenine. In addition, some decanal was found, which suggests that A was contaminated with compound B or C. Compounds B and C, in turn, gave only decanal, indicating that they both have double bonds at C_8, just like E and F. B and C have additional double bonds at C_4, and we expected to find some tetradecenal in their ozonolysis products, but it was not found. This may be explained by the slow attack of the LCB double bond at C_4.

The C_8 double bond in compound B is probably of trans geometry, and that of compound C of cis geometry. This is suggested by the ir spectrum of B, which shows in Figure 3 a higher absorption at 970 cm^{-1} than does the spectrum of 5-sphingenine (Renkonen and Hirvisalo, 1969). The spectrum of C, in turn, shows trans olefinic absorption similar to that

TABLE IV: Reductive Ozonolysis of Triacetyl Long-Chain Bases.

LCB	Sample Size (μmol)	Yield of Aldehydes: Decanal μmol	Decanal %	Tetradecanal μmol	Tetradecanal %
Triacetyl A	0.194	0.017	9	0.032	16
Triacetyl B	0.350[a]	0.080	23		0
Triacetyl C	0.315	0.179	57		0
Triacetyl D	0.217	0.017	8		0
Triacetyl E	0.374	0.194	52		0
Triacetyl F	0.370	0.260	70		0
Triacetyl-4-sphingenine	0.370	0.000	0	0.047	13

[a] The sample was obviously contaminated with silicic acid, and an erroneously large weight was recorded.

of 4-sphingenine. These findings are also consistent with the relative mobility of B and C on argentation tlc.

Discussion

The six LCB structures suggested by the present findings are shown in Figure 7. The presence of the double bond at C_4 in plant LCB's (compounds A, B, and C), the presence of dienoic LCB's in plants (compounds B and C), and the presence of a cis double bond at C_8 of a LCB (compounds C and F) are new contributions of this study. Sphinganine (compound D) has been described previously as a component of wheat flour lipids (Carter *et al.*, 1961a; MacMurray and Morrison, 1970), and a dihydroxy base with an isolated double bond was described by Carter *et al.* (1961a). MacMurray and Morrison (1970) then observed that this LCB has an isolated double bond at C_8. No previous data are available on the geometry of this double bond, but the present findings reveal both trans (compound E) and cis double bonds (compound F) at this position.

Long-chain bases are generally saturated or monoenoic molecules, and only few dienoic LCB's have been detected from natural sources (Karlsson, 1970a,b). The 4,*cis*-14-sphingadienine of human plasma (Karlsson, 1967; Polito *et al.*, 1968; Renkonen and Hirvisalo, 1969), the 4,8-sphingadienine of sea anemone (Karlsson, 1970c) and oyster (Hayashi and Matsubara, 1970, 1971), and the eicosasphinga-4,11-dienine of scorpion (O'Connor *et al.*, 1970) are among the better known examples. Singh and Privett (1970) obtained two unsaturated C_{16}-aldehydes by periodate cleavage of soybean LCB's; these had longer glc retention times than 2-hexadecenal on EGSS-X. They may have derived from LCB's identical with our compounds B and C.

Long-chain base biosynthesis in higher plants is not at present understood (Stoffel, 1971; Morell and Braun, 1972). However, the presence of the same LCB's in wheat as in yeast (sphinganine and hydroxysphinganine) and oyster (4-sphingenine and 4,8-sphingadienine) suggests similar mechanisms of formation. Using oyster microsomes Hammond and Sweeley (1973) have obtained evidence for condensation of palmitoyl coenzyme A with serine and subsequent desaturation of the 3-ketosphinganine followed by reduction of the keto group. This sequence of reactions could explain the formation of the trans double bonds found in wheat LCB's, but it does not explain the synthesis of the cis olefinic bonds. An additional desaturation or isomerization mechanism may thus be operative in wheat. The isolated double bonds at C_{11} (O'Connor *et al.*, 1970) and at C_{14} (Renkonen and Hirvisalo, 1969) in natural LCB's may also be synthesized by mechanisms slightly different from those in oysters. Another possibility is that the cis double bond at C_8 in wheat LCB's are artifacts resulting from the milling process. To our knowledge the flour samples had not been subjected to any previous chemical treatment which might have caused isomerization of the olefinic bonds.

Acknowledgments

The ir spectra were recorded by Mr. L. Kalervo.

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