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Cork Suberin Molecular Structure: Stereochemistry of the C_{18} Epoxy and *vic*-Diol ω-Hydroxyacids and α,ω-Diacids Analyzed by NMR

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Supporting Information

ABSTRACT: Suberin is the biopolyester that protects the secondary tissues of plants against environmental variability and aggressions. Cork suberin is composed mostly of C_{18} ω-hydroxyacids and α,ω-diacids, 9,10-substituted with an unsaturation, an epoxide ring, or a *vic*-diol group. Although determinant for suberin macromolecular structure, the stereochemistry of these monomers is poorly studied, sometimes with contradictory results. An NMR technique was used here to assign the configuration of the 9,10-epoxy and 9,10-diol groups in C_{18} suberin acids, comparing the chemical shifts of diagnostic ^{1}H and ^{13}C signals with the ones of model compounds, before and after conversion of the *vic*-diol group into benzylidene acetal derivatives. The relative configuration was proved to be *cis* in the C_{18} 9,10-epoxy and *threo* in the C_{18} 9,10-diol suberin acids. These monomers were present in suberin probably as racemic mixtures, as shown by polarimetry. The revealed stereochemistry allows the suberin macromolecule to be built as an ordered array of midchain kinked C_{18} acids, reinforced by intramolecular hydrogen bonding.

KEYWORDS: *suberin, cork, C_{18} 9,10-epoxy suberin acids, C_{18} 9,10-diol suberin acids, vic-diols stereochemistry determined by NMR, benzylidene acetal derivatives*

INTRODUCTION

Suberin is a lipid macromolecule found in suberized plant cell walls, which act as a frontier barrier toward environmental variability and aggressions.[1] Suberized cells comprise most of the periderm, the tissue that envelops plant organs of secondary growth, like tree trunks or potato tubers. Suberized cells also develop as wound tissue in plants, after injury or leaf fall, and in internal organs where water flow control is needed, like in the Casparian bands of root endodermis.[1] The epitome of suberized plant tissues is cork, and the outer bark of the cork-oak (*Quercus suber* L.), cork, is harvested in a renewable and sustainable way with growth cycles of 9 years. As a material, cork has a unique set of properties, which makes it used worldwide in an enormous number of products, for some of them without an alternative substitute.

The barrier and insulation properties of suberin, which are vital for plant survival, derive ultimately from its molecular and supramolecular structure in suberized cell walls. In spite of its importance and the significant quantity of knowledge already gathered [see comprehensive reviews in refs 2 and 3], many doubts remain about how suberin is built up as a macromolecule.

Suberin is known to be a glycerolipid polyester: when the ester bonds are broken, suberin depolymerization occurs, releasing its constituent "monomers". Besides glycerol, the major suberin monomers are long-chain aliphatic acids, collectively named as "suberin acids", which typically account for 80% or more of its depolymerization products.[4] Within the latter, two families of α,ω-bifunctional fatty acids are largely dominant: ω-hydroxyacids and α,ω-diacids. The fact that ω-hydroxyacids and α,ω-diacids have either hydroxyl or carboxylic acid linking groups at both chain ends, allows the macromolecular polyester to grow.[3]

Although all suberins studied so far from different plant species and tissues show the same basic composition pattern of glycerol, ω-hydroxyacids, and α,ω-diacids, they can be quite variable in the specific suberin acids of which they are made. Suberin ω-hydroxyacids and α,ω-diacids typically have even-numbered carbon chain lengths between C_{16} and C_{24}.[2,4] There are suberins with significant amounts of C_{16} acids, while others include C_{22} or longer acids as relevant monomers.[2] However, all analyzed suberins have C_{18} suberin acids in significant amounts, meaning that they play a major role in its macromolecular structure. These C_{18} suberin acids are specific because they all have a midchain "functional" group, at carbons C-9 and C-10: this can be a double-bond or an oxygen-containing substituent group, either an epoxide ring or two hydroxyl groups in vicinal positions (*vic*-diol). Because these secondary substituents impart chirality to the carbons they are attached to, these suberin acids can have different stereochemical configurations.

Understanding the stereochemistry of the C_{18} suberin acids is important for two main reasons: first, the spatial arrangement of the C_{18} monomers within the polyester macromolecule is dependent on their spatial configuration and conformation;[3] second, because these suberin acids are potentially interesting for a number of valuable industrial uses, their stereochemistry will also determine their properties, reactivity, and possible applications.[1,5]

The focus of the present work was the stereochemistry of the C_{18} suberin acids with oxygen-containing substituents, namely, 9,10-epoxide and 9,10-diol groups, found in cork suberin [the

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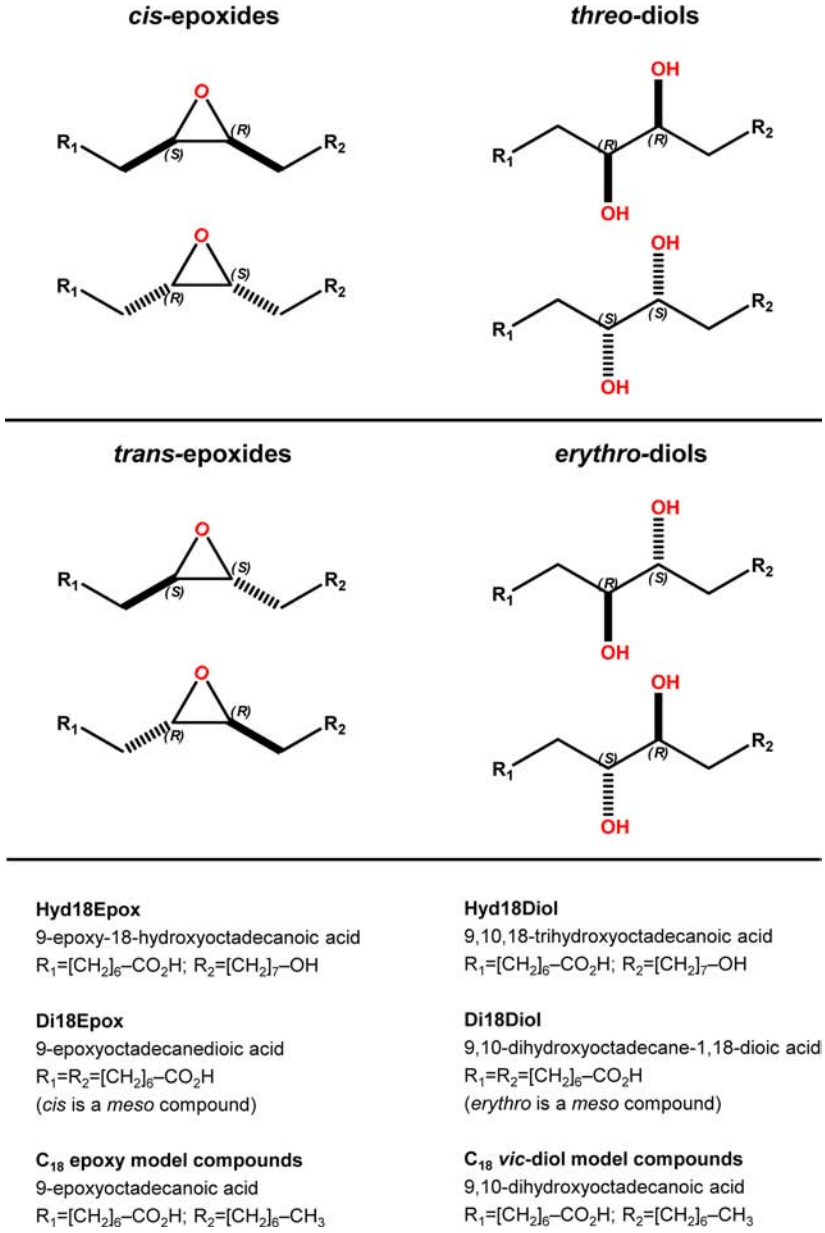

Figure 1. Structural representation of the stereochemistry of the C_{18} 9,10-epoxy and C_{18} 9,10-diol acids: possible relative (*cis*/*trans* and *erythro*/*threo*) and absolute configurations.

stereochemical analysis of the C_{18} 9-unsaturated has been presented and discussed elsewhere[6]]. Cork suberin has significant amounts of C_{18} 9,10-epoxy and C_{18} 9,10-diol suberin acids, together representing ca. 44% of the suberin mass.[4,7] They include the C_{18} epoxy-*ω*-hydroxyacid, 9,10-epoxy-18-hydroxyoctadecanoic (from now on referred to as Hyd18Epox), the C_{18} epoxy-*α*,*ω*-diacid, 9,10-epoxy-octadecane-1,18-dioic (Di18Epox), the C_{18} diol-*ω*-hydroxyacid, 9,10,18-trihydroxy-octadecanoic (Hyd18Diol), and the C_{18} diol-*α*,*ω*-diacid, 9,10-dihydroxyoctadecane-1,18-dioic (Di18Diol). Each of these epoxide and *vic*-diol suberin acids has two asymmetric carbons and therefore can exist in the form of up to four different stereoisomers (Figure 1). The suberin epoxyacids can have a *cis* or *trans* relative configuration, each one with two possible absolute configurations in the form of enantiomeric pairs: *S*,*R* or *R*,*S* for the *cis* isomer and *S*,*S* or *R*,*R* for the *trans* isomer (Figure 1). Because of the molecule symmetry, *cis*-Di18Epox is a *meso* compound since the two enantiomers are identical (Figure 1). The relative configuration of the C_{18} 9,10-diol suberin acids can be described using the Fischer projection, as *threo* (with possible absolute configurations of *R*,*R* or *S*,*S*) or *erythro* (*R*,*S* or *S*,*R*) (Figure 1). The *erythro*-Di18Diol is also *meso* because the *R*,*S* and *S*,*R* forms are indistinguishable (Figure 1).

Except for a few pioneering works dating back to the middle of the last century, the stereochemistry of these C_{18} 9,10-epoxy and C_{18} 9,10-diol acids in suberin have never been thoroughly studied. The relative configuration of the Hyd18Diol (by then also known as "phloionolic acid") and Di18Diol ("phloionic acid") was first studied by Seoane and co-workers.[8−11] By comparing the melting points of *erythro* and *threo* *vic*-diols synthesized through stereospecific reactions from unsaturated acids of known stereochemistry, with the ones isolated from cork, and following the empiric rule that *erythro* isomers have

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higher melting points than the corresponding *threo* isomers, the *threo* configuration was proposed for both the Hyd18Diol and Di18Diol suberin acids.[9,11,12] On the contrary, in a more recent publication, both *threo* and *erythro* Hyd18Diol and Di18Diol suberin acids were identified in cork, although it was not mentioned how the configuration was assigned.[13] In this latter work, cork suberin was depolymerized by methanolysis and the solubilized suberin monomers analyzed by GC-MS, with the assigned *threo* and *erythro* forms chromatographically resolved; on average, for both *vic*-diols, the *threo* and *erythro* forms were found in almost identical amounts.[13]

The only known study on the relative stereochemistry of the C_{18} 9,10-epoxy suberin acids was also from Seoane and co-workers.[14] These authors isolated the Hyd18Epox and Di18Epox acids from cork suberin and hydrolyzed the epoxide rings into *vic*-diols; the latter showed melting points similar to the Hyd18Diol and Di18Diol suberin acids, to which these same authors had previously assigned a *threo* configuration, as discussed above; due to the stereospecific *anti*-hydroxylation reaction used in the hydrolysis, a *cis* configuration was assigned to the epoxide suberin acids.[14]

In order to get unambiguous and definite results on the stereochemistry of the C_{18} 9,10-epoxy and C_{18} 9,10-diol suberin acids from cork, in the present work we assessed this question using NMR-based techniques. NMR has been successfully used for the determination of both the relative and absolute stereochemistry of organic compounds.[15−17] The general strategies include the use of chiral solvents or additives, without covalent linking to the stereoisomer(s) under analysis; the use of chiral derivatizing agents, with covalent linkage to the substrates, sometimes using the two enantiomers of the CDA; or the use of the nuclear Overhauser effect (NOE) through space interactions between nuclei, which can show the atoms' relative positions, particularly in rigid systems. In the case of the two C_{18} 9,10-epoxy and the two C_{18} 9,10-diol suberin acids under consideration here, their stereochemistry analysis by NMR faced a number of situations: each compound has two stereogenic carbons that can, in the case of the 9,10-diols, rotate around the σ bond linking them therefore with many possible spatial conformations; the chemical neighborhood on both sides of the chiral centers are identical methylene chains up to seven carbons away, giving symmetrical effects in many approaches; there was no previous safe knowledge if one or two of the possible relative configurations were present, and if both, in what relative proportions, and neither if each of the former includes one or two of its possible enantiomers (Figure 1).

The analysis of the stereochemical configuration of vicinal secondary hydroxyls with symmetric alkyl substituents, like the *vic*-diols we are dealing with here, was successively achieved by NMR, after the esterification of both hydroxyls with the *R* and *S* enantiomers of arylmethoxyacetic acids; the chemical shifts of the protons linked to the chiral centers in the (*R*,*R*)- and (*S*,*S*)-derivatives, namely, the sign of the δR-δS differences for the same proton, allowed the unequivocal assignment of the absolute configuration of those diols.[16,18] This same approach was essayed with the suberin acids *vic*-diols, but the methyne protons linked to the hydroxyls, after esterification with *R*- and *S*-methoxyphenylacetic acid (MPA), showed as unresolved broad multiplets whose chemical shifts were identical in the (*S*,*S*)- and (*R*,*R*)-MPA derivatives (data not shown).

To allow an unambiguous assignment of the suberin acids' relative configuration, the approach followed here was to compare the most diagnostic ^{1}H and ^{13}C chemical shifts of the C_{18} 9,10-epoxy and C_{18} 9,10-diol suberin acids with the ones of model compounds with known relative stereochemistry. The model compounds used included the C_{18} 9,10-epoxy and C_{18} 9,10-diol substituted octadecanoic (stearic) acids, structurally identical to the suberin acids in what concerns the stereogenic carbons and their vicinity (Figure 1). Identical C_{18} *vic*-diol model compounds were used, but starting from C_{18} 9-unsaturated and C_{18} 9,10-epoxy compounds with known configuration and transforming them into *vic*-diols with determined stereochemistry through stereospecific reactions, to check the validity of the latter.

The NMR analysis was applied to the suberin acids and model compounds as such but also carried out after converting the *vic*-diols into benzylidene acetals (BzAc), a group which is normally used to protect secondary *vic*-diols.[19] The analysis of the suberin epoxyacids stereochemistry was also done using this same derivative, after conversion of the epoxides into *vic*-diols through a stereospecific reaction. The advantage of the use of BzAc derivatives is that the five-sided ring they form with the *vic*-diol has restricted conformational mobility, thus keeping the carbons and protons of interest for the NMR stereochemistry analysis in relatively fixed positions.

The implications of the stereochemistry of the C_{18} midchain oxygenated suberin acids for the macromolecular arrangement of suberin are discussed, and a model is proposed based on such results.

MATERIALS AND METHODS

Cork C_{18} 9,10-Epoxy and C_{18} 9,10-Diol Suberin Acids. The two C_{18} 9,10-epoxy and the two C_{18} 9,10-diol suberin acids, ω-hydroxyacids (identified by the prefix Hyd) and α,ω-diacids (Di), in the form of methyl esters, were obtained from cork after a methanolysis depolymerization reaction. These compounds were isolated and purified through a process of successive extraction and purification steps (method under patent submission) and obtained with the following purities as determined by GC-MS analysis: 9-epoxyoctadecanedioic acid, dimethyl ester (Di18Epox_Me), 95%; 9-epoxy-18-hydroxyoctadecanoic acid, methyl ester (Hyd18Epox_Me), 97%; 9,10-dihydroxyoctadecanedioic acid, dimethyl ester (Di18-Diol_Me), 99%; and 9,10,18-trihydroxyoctadecanoic acid, methyl ester (Hyd18Diol_Me), 99%. The four C_{18} suberin acids were characterized by mass spectrometry (EIMS), FTIR, and 1D NMR (^{1}H and ^{13}C), and 2D NMR (COSY, HSQC, and HMBC) spectroscopy.

Model Compounds. C_{18} 9,10-Epoxy and C_{18} 9,10-diol monoacids of known relative stereochemistry were used as model compounds and purchased as pure standards: *cis*-9,10-epoxyoctadecanoic acid, 99%, from Sigma-Aldrich (Lisboa, Portugal); rac-*trans*-9,10-epoxyoctadecanoic acid, 98%, and rac-*threo*-9,10-dihydroxyoctadecanoic acid, 98%, from Santa Cruz Biotechnology (Heidelberg, Germany); and *erythro*-9,10-dihydroxyoctadecanoic, 97%, from Larodan Fine Chemicals (Malmoe, Sweden). The C_{18} 9-unsaturated monoacids, *cis*-oleic acid methyl ester and *trans*-elaidic acid methyl ester, 99%, were purchased from Sigma-Aldrich. Two other compounds with a *vic*-diol group substituted in an alkyl chain of known relative stereochemistry, were used, namely, *erythro*-5,6-dodecanediol, 98%, and *threo*-5,6-dodecanediol, 96%, purchased from TCI Europe (Zwijndrecht, Belgium). All solvents were of HPLC grade and obtained from Merck (Lisboa, Portugal).

GC-MS Analysis. *Separation of cis/trans Isomeric Pairs.* *cis*- and *trans*-9,10-epoxyoctadecanoic acid mixtures were prepared for GC-MS analysis from equimolar solutions of each of the two acids as follows: 1.0 mg (0.3 mmol) of *cis*-9,10-epoxyoctadecanoic acid was diluted in 130 μL of pyridine and 130 μL of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA); an identical solution was prepared for

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trans-9,10-epoxyoctadecanoic acid; aliquots of 100 μL of each solution were mixed in a vial to which 800 μL of pyridine was added.

Separation of erythro/threo Isomeric Pairs. Solutions for GC-MS analysis of equimolar mixtures of *threo*- and *erythro*-9,10-dihydroxyoctadecanoic acid, and *threo*- and *erythro*-5,6-dodecanediol were identically prepared as described above.

Analysis of Suberin Acids. Solutions of each of the suberin acids, 9-epoxyoctadecanedioic acid, dimethyl ester (Di18Epox_Me), 9-epoxy-18-hydroxyoctadecanoic acid, methyl ester (Hyd18Epox_Me), 9,10-dihydroxyoctadecanedioic acid, dimethyl ester (Di18Diol_Me), and 9,10,18-trihydroxyoctadecanoic acid, methyl ester (Hyd18Diol_Me) were prepared for GC-MS analysis by dissolving 1.0 mg of each suberin acid in the TMS-derivatizing solution of 130 μL of pyridine and 130 μL of BSTFA.

GC-MS Conditions. All of the above solutions were injected in a 7890A gas chromatographer coupled to a 5975C mass spectrometer detector (Agilent Technologies, USA) in the following chromatographic conditions: the column was DB5-MS (60 m, internal diameter 0.25 mm, film thickness 0.25 μm); the oven program was with an initial temperature of 200 °C, followed by a temperature increase to 250 °C at 8 °C/min, and then to 300 °C at 3 °C/min; the final temperature was kept for 15 min; injections were made in splitless mode, with an injector temperature of 300 °C. Mass spectrometer conditions were electron ionization, 70 eV; source temperature, 230 °C; and quadrupole temperature, 150 °C; the transfer line temperature was kept at 310 °C.

Hydroxylation of the C_{18} 9-Unsaturated Monoacids into C_{18} 9,10-Diols. *vic*-Diols of determined stereochemistry were obtained through a stereospecific *syn*-hydroxylation of the C_{18} 9-unsaturated monoacid model compounds, namely, *cis*-oleic acid methyl ester (converted to the *erythro*-9,10-diol) and *trans*-elaidic acid methyl ester (converted to the *threo*-9,10-diol) by permanganate oxidation following a procedure adapted from Bhushan et al.[20]

Cetyltrimethylammonium Permanganate (CTAP) Preparation. $KMnO_4$ (5.0 g (31.7 mmol)) was dissolved in 160 mL of water. In another flask, 12.6 g (34.7 mmol) of cetyltrimethylammonium bromide (CTAB) was dissolved in 155 mL of water during 1 h with stirring. This CTAB solution was added slowly and dropwise to the $KMnO_4$ solution for 1 h, with strong stirring. The mixture was allowed to further react for 30 min and the resulting violet precipitate recovered by filtration on a G3-porosity glass filter and washed with water. This residue (CTAP) was dried on a vacuum oven over P_4O_{10}. Dry CTAP (10.2 g) was ground to a fine powder and kept in a refrigerator.

Hydroxylation Reaction. *cis*-Oleic acid methyl ester (1.0 g (3.4 mmol)) was dissolved in 10 mL of dichloromethane, to which a solution of 6.8 g (16.9 mmol) of CTAP dissolved in 102 mL of dichloromethane was added dropwise, and the mixture allowed to react for 100 h at room temperature. The reaction mixture was filtered on a G3-porosity glass filter, 50 mL of dichloromethane added to the filtered solution, and the organic solution washed with 3 × 150 mL of water. After dichloromethane removal in a rotary evaporator, *erythro*-9,10-dihydroxyoctadecanoic acid methyl ester was recovered in a reaction yield of 37%. The same procedure was followed to hydrolyze *trans*-elaidic acid methyl ester: 0.9 g (3.0 mmol) was dissolved in 9 mL of dichloromethane, and 6.1 g (15.2 mmol) of CTAP dissolved in 92 mL of dichloromethane was added. The recovered organic phase included *threo*-9,10-dihydroxyoctadecanoic acid methyl ester in a reaction yield of 90%.

Purification of the C_{18} 9,10-Diols Obtained from the Hydroxylation of the C_{18} 9-Unsaturated Monoacids. Each of the C_{18} 9,10-diols obtained was purified by medium pressure liquid chromatography (MPLC) as follows: the solid material recovered from the organic phases was dissolved in chloroform/ethyl acetate 7:3 and applied to a VersaFlash silica column, 40 × 75 mm, and eluted with the same solvent at a flow rate of 50 mL/min. From the mixture enriched in *erythro*-9,10-dihydroxyoctadecanoic acid methyl ester, a fraction with 82.0 mg of the latter was recovered in a purity of 99%. From the mixture enriched in *threo*-9,10-dihydroxyoctadecanoic acid methyl ester, 131.5 mg of the latter was recovered in a purity of 99%.

Hydrolysis of the C_{18} 9,10-Epoxyacids into C_{18} 9,10-Diols. A stereospecific *anti*-hydroxylation converting the epoxides into *vic*-diols, namely, the *cis*-9,10-epoxides into *threo*-9,10-diols and the *trans*-9,10-epoxides into *erythro*-9,10-diols, was applied to the C_{18} 9,10-epoxide model compounds (*cis*- and *trans*-9,10-epoxyoctadecanoic acids) and to the C_{18} 9,10-epoxy suberin acids (Hyd18Epox_Me and Di18-Epox_Me). The *anti*-hydroxylation reaction was carried out by an acid-catalyzed hydrolysis, described as follows: 50.1 mg (0.2 mmol) of *cis*-9,10-epoxyoctadecanoic acid was dissolved in 5 mL of tetrahydrofuran and 5 mL of water, and 500 μL of sulphuric acid was added dropwise. The mixture reacted at room temperature for 72 h with stirring, and after the reaction completion, 20 mL of water and 20 mL of dichloromethane were added and the organic phase recovered. After solvent removal, 42 mg of *threo*-9,10-dihydroxyoctadecanoic acid in a purity of 72% was recovered from the organic phase and further purified in 0.5 mm TLC silica plates with hexane/isopropyl alcohol/formic acid 70:30:1. The band with R_f = 0.8 was collected, extracted with methanol/dichloromethane 1:1, giving 12.0 mg of *threo*-9,10-dihydroxyoctadecanoic acid methyl ester with a purity of 90%. The same procedure was applied to 10.0 mg (0.03 mmol) of *trans*-9,10-epoxyoctadecanoic acid with a final recovery of 10.0 mg of *erythro*-9,10-dihydroxyoctadecanoic acid (88% pure); 17.8 mg (0.05 mmol) of Hyd18Epox_Me, resulting in 16.0 mg of recovered organic phase with Hyd18Diol in 98% purity, which was methylated afterward as described below; and 62.8 mg (0.2 mmol) of Di18Epox_Me resulting in 44.7 mg of Di18Diol in 97% purity, also further methylated.

Methylation of the Free Acids. Some of the purchased model compounds, as well as their synthesized derivatives, when in the form of free acids, were methylated (to avoid signal overlapping with diagnostic signals of interest in the NMR spectra) in methanol with 1% sulphuric acid, for 3 h, in reflux at 80 °C.

Derivatization of *vic*-Diol Groups into Benzylidene Acetals (BzAc). All *vic*-diol compounds were converted to the corresponding BzAc derivatives, including the C_{18} 9,10-diol suberin acids (Di18Diol_Me and Hyd18Diol_Me); the equivalent C_{18} 9,10-diols obtained from the *anti*-hydroxylation of the C_{18} 9,10-epoxy suberin acids (Di18Epox_Me and Hyd18Epox_Me); the purchased *erythro*- and *threo*-C_{18} 9,10-dihydroxyoctadecanoic acids; the latter two obtained from (i) the *syn*-hydroxylation of the model compounds *cis*-oleic acid methyl ester and *trans*-elaidic acid methyl ester and (ii) from the *anti*-hydroxylation of the model compounds *trans*- and *cis*-C_{18} 9,10-epoxyoctadecanoic acids; and the model compounds C_{12} *erythro*-5,6-dodecanediol and C_{12} *threo*-5,6-dodecanediol.

BzAc Derivatization Reaction. To prepare the BzAc derivatives, a procedure adapted from McElhanon et al.[21] was used. To a known quantity of each *vic*-diol compound, benzaldehyde dimethyl acetal (α,α-dimethoxytoluene) and *p*-toluenesulfonic acid monohydrate were added in molar proportions of 100× and 0.1×, respectively. Dimethylformamide was added up to a total volume of 3 mL/0.1 mmol of the *vic*-diol compound. A few beads of molecular sieve were added, and the solution mixture was allowed to react in an oil bath at 100 °C for 24 h, in reflux with stirring. The reaction mixture was then partitioned in 20 mL of water/20 mL of dichloromethane, the organic phase recovered, and analyzed by GC-MS to control the reaction yield.

Purification Steps. The *vic*-diol BzAc derivatives were isolated and purified from the corresponding organic phases by MPLC (in the same system described above) and, when necessary, also by TLC to a minimum of 98% purity. Di18Diol_Me BzAcs (one from the suberin acid Di18Diol_Me and the other from the hydrolysis of the suberin acid Di18Epox_Me) were purified in hexane/ethyl acetate 9:1; Hyd18Diol_Me BzAcs (one from the suberin acid Hyd18Diol_Me and the other from the hydrolysis of the suberin acid Hyd18-Epox_Me) were purified in chloroform/ethyl acetate 9:1; *threo*-9,10-dihydroxyoctadecanoic acid_Me BzAcs (one from the model compound *threo*-9,10-dihydroxyoctadecanoic acid_Me, a second from the hydroxylation of *cis*-oleic acid methyl ester, and a third from the hydrolysis of the *cis*-9,10-epoxyoctadecanoic acid) were purified in chloroform/ethyl acetate 7:3; *erythro*-9,10-dihydroxyoctadecanoic acid_Me BzAcs (one from the model compound *erythro*-9,10-dihydroxyoctadecanoic acid_Me, a second from the hydroxyla-

tion of *trans*-elaidic acid methyl ester, and a third from the hydrolysis of the model compound *trans*-9,10-epoxyoctadecanoic acid) were purified in chloroform/ethyl acetate 7:3 (the latter two BzAc derivatives were lost in the purification steps and were not used for the NMR analysis); *threo*-5,6-dodecanediol BzAc and *erythro*-5,6-dodecanediol BzAc were purified in hexane/ethyl acetate 9:1.

DSC Analysis. For melting point determination, the suberin acids Hyd18Epox_Me, Di18Epox_Me, and Di18Diol_Me were analyzed by differential scanning calorimetry (DSC). The thermograms were acquired on a Maia DSC 200 F3 (Netzsch, Germany) in a temperature range from −50 °C to +90 °C, with a heating rate of 20 °C/min. The suberin acid samples, previously dried in a vacuum oven, were weighed in aluminum pans (5.0 mg of Hyd18Epox_Me, 5.8 mg of Di18Epox_Me, and 9.6 mg of Di18Diol_Me), and cooled to −50 °C with liquid nitrogen. DSC was calibrated with an expanded uncertainty of measurement error <0.5 °C.

FTIR Analysis. FTIR spectra were acquired from the four suberin acids and from the 9,10-epoxy and the *vic*-diol model compounds, either in ATR or transmission mode, depending on its physical state at room temperature, in an Alpha-P spectrometer (Bruker Optik, Germany). ATR spectra were obtained from liquid and semisolid samples in a diamond cell with a pressure clamp; transmission spectra were obtained from solid samples in KBr pellets after grinding to a fine powder (1.5 mg of sample with 200 mg of KBr). Thirty-two scans were acquired per spectrum with a resolution of 4 cm^{-1} in a wavenumber range from 4000 cm^{-1} to 400 cm^{-1}.

Raman Analysis. Raman spectra were obtained from the cork suberin 9,10-epoxyacids, Di18Epox_Me and Hyd18Epox_Me, and from the 9,10-diol cork suberin acid, Di18Diol_Me, in a system comprising a double monochromator Spex 1403 (Horiba, Japan), with an argon ion laser line at 514.5 nm, model 2016 (Spectra-Physics, USA), and a R928 photomultiplier detector (Hamamatsu Photonics, Japan). The spectra were acquired at room temperature in 90° geometry, with a spectral resolution of 4 cm^{-1}.

NMR Analysis. NMR spectra, including the 1D ^{1}H and ^{13}C, together with 2D correlation experiences (COSY, HSQC, and HMBC), were obtained from the suberin acids, model compounds, synthesized *vic*-diols, and all BzAc derivatives. The NMR spectra were acquired on an Avance II+ 600 spectrometer (Bruker Biospin, Germany), with frequency resonances of 600.13 MHz for protons and 150.96 MHz for carbons, equipped with a cryoprobe and pulse gradient units, able to produce magnetic field pulsed gradients in the z-direction of 56.0 G/cm. All samples were solubilized in deuterated chloroform with 0.03% of TMS, with an approximate concentration of 5 mg/500 μL, and the spectra acquired at a temperature of 300 K. The TMS signal (0.00 ppm) was used to calibrate the chemical shifts in ^{1}H spectra, and chloroform (77.00 ppm) was used as reference in ^{13}C spectra. The NMR spectra were processed in MestReNova, version 8.0.1 (Mestrelab Research, Spain).

Polarimetry Analysis. The specific rotation of the suberin acids, Hyd18Epox_Me, Di18Epox_Me, Hyd18Diol_Me, and Di18Diol_Me was measured in a Perkin-Elmer 241 MC Polarimeter, equipped with a sodium lamp (589 nm) and a 1 mL quartz cell with 1 dm of length. Sample concentration was between 5 and 10 mg/mL in dichloromethane and the measurements acquired at room temperature (ca. 25 °C).

RESULTS AND DISCUSSION

Spectroscopic Analysis and Stereochemistry of the C_{18} 9,10-Epoxy Suberin Acids. *GC-MS and DSC Analyses.* The two C_{18} 9,10-epoxy suberin acids were obtained from cork as methyl esters, after suberin depolymerization by methanolysis, with purities, as determined by GC-MS, of 95% for Di18Epox_Me and 96% for Hyd18Epox_Me. The EIMS of methyl esters and TMS derivatives of suberin acids have been previously presented and discussed.[22] The calculated Kovats indexes were for Di18Epox_Me 2355 and for Hyd18Epox_Me 2369. Their melting points and fusion enthalpies were determined by DSC, respectively, as 40.7 °C and 57.4 kJ/mol for Hyd18Epox_Me and 21.7 °C and 46.2 kJ/mol for Di18Epox_Me. Literature values for the melting point of Hyd18Epox_Me is 38 °C, and Di18Epox_Me is reported as an oil at room temperature.[14]

A first approach to check if these epoxyacids were present in cork suberin both as *cis*- and *trans*-isomers or only in one of the two possible relative configurations was made by GC-MS analysis, comparing with model compounds. The latter, C_{18} *cis*-9,10-epoxyoctadecanoic acid and C_{18} *trans*-9,10-epoxyoctadecanoic acid, both as methyl esters, were mixed and baseline separated in the chromatographic conditions described in Materials and Methods. Each of the two suberin C_{18} 9,10-epoxyacids was then analyzed in the same chromatographic conditions, including solution concentration and injection volume, but only one peak was recognizable in the chromatograms. This gave evidence that the two suberin epoxyacids, Di18Epox and Hyd18Epox, were present in cork suberin probably in only one of the forms, either *cis* or *trans*.

FTIR and Raman Analyses. The *cis* or *trans* configuration of epoxide rings can be tentatively assessed by vibrational spectroscopy, namely, FTIR and Raman. The diagnostic bands used for this purpose derive from the epoxide ring vibrations: in epoxides substituted in alkyl chains, typically, for *cis*-isomers this band is observed in the range of 785−865 cm^{-1} and for *trans*-isomers at 860−950 cm^{-1}.[23] In the *cis* and *trans* model compounds analyzed here, these bands were conspicuous: in the C_{18} *cis*-epoxyacid, it was found at 847 cm^{-1} and in the C_{18} *trans*-epoxyacid at 877 cm^{-1} (see Supporting Information, Figure SM1). With respect to the two suberin epoxyacids, a relatively strong band at 844 cm^{-1} was observed in Hyd18Epox_Me, and a medium to weak band at 837 cm^{-1} was present in Di18Epox_Me (Supporting Information, Figure SM2). The former can probably be assignable to a *cis*-epoxide ring vibration, but the latter, due to its weak intensity, was ambiguous. However, in both suberin acids (and also in the two model compounds) there were a number of weak intensity bands in the window assignable to the ring vibrations of *cis*- and *trans*-epoxides, and therefore, no definite conclusion can be drawn about their configuration based only on the FTIR analysis.

The Raman spectra analysis was also inconclusive: both Hyd18Epox_Me and Di18Epox_Me showed absorption bands in the window of the *cis*- and *trans*-epoxide ring vibrations; however, some of these bands could also be assignable to skeletal vibrations of the alkyl chains, which are stronger in Raman than in FTIR spectra.

NMR Analysis. The two suberin acids, Di18Epox_Me and Hyd18Epox_Me, and the two model compounds of known relative configuration, C_{18} *cis*-epoxyacid and C_{18} *trans*-epoxyacid, were analyzed by NMR including ^{1}H, ^{13}C, and 2D correlation techniques, namely, COSY, HSQC, and HMBC. The chemical shifts of the protons and carbons relevant for the discussion of the *cis* or *trans* configuration, namely, of the epoxide methines (C-9 and C-10) and of the adjacent methylenes (C-8 and C-11), are presented in Table 1. A first approach for the assignment of the *cis* or *trans* configuration would be the coupling constants between the two epoxide methine protons. However, in the proton spectra of all analyzed epoxides these protons showed up as a broad unresolved multiplet, as would be expected due to the similarity of the alkyl substituents on the epoxide ring. A second approach would be the values of the chemical shifts of protons and carbons directly

Table 1. Chemical Shifts (ppm) of ^{1}H and ^{13}C Methines and Adjacent Methylenes in C_{18} 9,10-Epoxyacids from Cork Suberin and Model Compounds

	epoxide methines (C-9 and C-10)		adjacent methylenes (C-8 and C-11)	
	^{1}H	^{13}C	^{1}H	^{13}C
C_{18} 9,10-epoxy suberin acids				
cis-Di18Epox_Me	2.89	57.07	1.49	27.74
cis-Hyd18Epox_Me	2.89	57.16 and 57.19	1.50	27.78 and 27.79
C_{18} 9,10-epoxy model compounds				
cis-9,10-epoxyoctadecanoic acid	2.91	57.23 and 57.28	1.50	27.77 and 27.81
trans-9,10-epoxyoctadecanoic acid	2.66	58.90 and 58.95	1.51	32.07 and 32.11
$\delta^{cis\text{-epox}}-\delta^{trans\text{-epox}}$	+0.25	−1.67 and −1.67	−0.01	−4.30 and −4.30

linked to the epoxide group, due to different shielding in the *cis* and *trans* environments. In fact, a significant difference was found between the chemical shifts of the methine epoxide protons, in the *cis* and *trans* model compounds analyzed, with a $\Delta H^{\delta cis\text{-}\delta trans}$ of +0.25 ppm (Table 1). This comparative shielding of the *trans*-epoxide methine protons was also observed in a number of other *cis*/*trans* isomers of C_{18} epoxy fatty acids, with the epoxide group in different chain positions.[24,25] This systematic upfield effect might be imputable to the fact that the methine protons in *trans*-isomers are spatially closer to the methylene protons located on the other side of the epoxide ring, in comparison to the *cis*-isomers, where such proximity does not exist (Figure 2). In this way, having in mind the

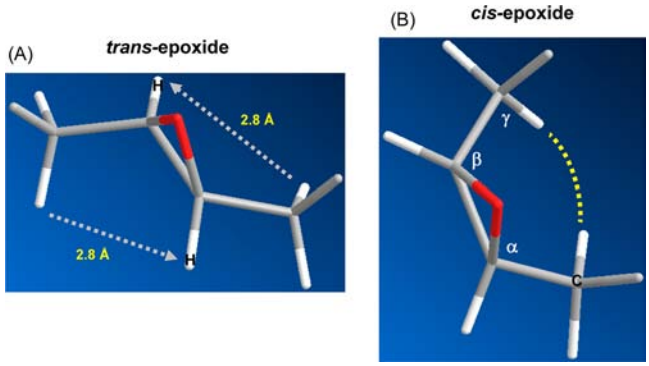

Figure 2. NMR chemical shift effects in *cis*- and *trans*-epoxides. (A) In *trans*-epoxyacids, shielding of the epoxide methine protons (marked) by the opposite methylenes. (B) In *cis*-epoxyacids, shielding of the adjacent methylene carbons (one marked) due to the γ-effect of the "substituent" group three carbons away (the opposite adjacent methylene).

structural similarity with the model compounds analyzed, the chemical shifts of the methine epoxide protons of suberin epoxyacids could be seen as indicative of a *cis* configuration: 2.89 ppm both for Di18Epox_Me and Hyd18Epox_Me, a value very close to 2.91 ppm observed in the *cis* model compound and farther apart from the 2.66 ppm of the corresponding *trans* isomer (Table 1).

A significant difference between the *cis* and *trans* model compounds was also observed in the ^{13}C NMR spectra, namely, in the carbons of the methylenes adjacent to the epoxide ring (C-8 and C-11). In fact, the *cis*-methylene carbons were comparatively more shielded with a $\Delta C^{\delta cis\text{-}\delta trans}$ of −4.30 ppm (Table 1). Differences of this order of magnitude were also found in the equivalent methylene carbons in several other C_{18} epoxyacids between *cis*- and *trans*-isomers.[26] This higher shielding observed in the C-8 and C-11 carbons in *cis*-isomers might be attributed to the so-called "gamma-effect", an empirical observation in which the chemical shift of a carbon is affected by a spatially close substituent located three bonds away.[27] In *cis*-epoxides "the substituent group" is the methylene group on the opposite side of the epoxide ring, gamma-positioned to the carbon under consideration; this spatial proximity and the van der Waals interactions believed to be behind the "gamma-effect" only exist in *cis*-isomers (Figure 2). Theoretical calculations taking into account bond length compression or stretching, or angular distortions, imparted by the steric effects of the substituent groups in the chemical shift, were able to predict to some extent the comparative shielding of gamma-carbons in similar alkyl-substituted *cis*-epoxides, when compared to *trans*-epoxides.[28]

The chemical shifts of the epoxide methine carbons (C-9 and C-10) and of the epoxide vicinal carbons (C-8 and C-11) both in Hyd18Epox_Me and Di18Epox_Me were very similar to those of the *cis* model compound (Table 1), thus reinforcing the hypothesis that the suberin epoxyacids had a *cis* configuration.

Spectroscopic Analysis and Stereochemistry of the C_{18} 9,10-Diol Suberin Acids. *GC-MS and DSC Analyses.* The C_{18} 9,10-diol cork suberin acids were extracted from cork suberin and purified up to 95%, in the case of Di18Diol_Me, and 97% in the case of Hyd18Diol_Me, as controlled by GC-MS. The calculated Kovats indexes were, respectively, 2522 and 2528. The melting point of Di18Diol_Me, measured by DSC, was 75.0 °C, with a fusion enthalpy of 42.5 kJ/mol. To check if the *vic*-diol suberin acids were present in cork both in the *erythro* and *threo* forms or only on one of them, the separation of the model compounds C_{18} *threo*-9,10-dihydroxyoctadecanoic acid and C_{18} *erythro*-9,10-dihydroxyoctadecanoic acid, which are structurally very close to the C_{18} 9,10-diol suberin acids, was essayed by GC. A 1:1 mixture of the two latter model compounds was successfully separated by GC with baseline resolution, with a difference in the retention times of 12 s (data not shown). Each of the C_{18} 9,10-diol suberin acids, analyzed exactly in the same GC conditions, afforded a single peak, suggesting that only one of the two possible relative configurations, either *threo* or *erythro*, was found in cork suberin.

NMR Analysis. The C_{18} 9,10-diol suberin acids were analyzed by NMR, together with two pairs of *threo* and *erythro* model compounds, namely, C_{18} *threo*- and *erythro*-9,10-dihydroxyoctadecanoic acids and C_{12} *threo*- and *erythro*-5,6-dodecanediols. The chemical shifts of protons and carbons of the C-9 and C-10 (C-5 and C-6 in the case of C_{12} diols) methines, where the hydroxyl groups are linked, which are relevant for the configuration discussion, are presented in Table 2. The differences in these chemical shifts between the *threo* and *erythro* model compounds are presented in Table 3.

A systematic difference was observed between the chemical shifts of the *erythro*- and *threo*-methine protons in the *vic*-diol model compounds, with a $\Delta H^{\delta erythro\text{-}\delta threo}$ of +0.19 ppm (Table 3). To understand this difference, we have to consider the possible conformations of the *vic*-diol group due to the rotation around the σ bond that links the two hydroxyl-substituted carbons and therefore the different magnetic environments in each case (Figure 3). A study carried out in 2,3-butanediol, a

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Table 2. Chemical Shifts (ppm) of ^{1}H and ^{13}C Methines in *vic*-Diols of Cork Suberin Acids (C-9 and C-10) and Model Compounds (C-9 and C-10 or C-5 and C-6), with the *vic*-Diol as a Free Group and as a Benzylidene Acetal (BzAc) Derivative

	free *vic*-diol group		benzylidene acetal (BzAc) derivative[f]			
	^{1}H	^{13}C	^{1}H	^{13}C	H_{BzAc}	C_{BzAc}
C_{18} 9,10-diol suberin acids						
threo-Di18Diol_Me	3.40	74.46	3.75	81.52 and 82.80	5.86	102.59
threo-Hyd18Diol_Me	3.41	74.46 and 74.48	3.75	81.52 and 81.54 82.80 and 82.83	5.86	102.60
threo-Di18Diol_Me[a]	3.40	74.48	3.75	81.52 and 82.80	5.86	102.59
threo-Hyd18Diol_Me[b]	3.40	74.47 and 74.49	3.75	81.52 and 81.53 82.80 and 82.82	5.86	102.59
C_{18} 9,10-diol model compounds						
threo-9,10-dihydroxyoctadecanoic acid	3.38	74.35 and 74.40	3.74	81.55 and 81.57 82.82 and 82.85	5.86	102.56
erythro-9,10-dihydroxyoctadecanoic acid methyl ester	3.60	74.71 and 74.76	4.11	78.74 and 78.77 79.20 and 79.28	5.77	103.05
threo-9,10-dihydroxyoctadecanoic acid methyl ester[c]	3.41	74.48 and 74.54	3.75	81.53 and 81.56 82.80 and 82.86	5.86	102.58
erythro-9,10-dihydroxyoctadecanoic acid methyl ester[d]	3.60	74.63 and 74.70				
threo-9,10-dihydroxyoctadecanoic acid[e]			3.75	81.53 and 81.56 82.80 and 82.85	5.86	102.58
C_{12} 5,6-diol model compounds						
threo-5,6-dodecanediol	3.41	74.51 and 74.53	3.76	81.55 and 81.57 82.84 and 82.85	5.87	102.57
eryhtro-5,6-dodecanediol	3.60	74.68 and 74.70	4.12	79.28	5.77	103.08

[a]Obtained from the hydrolysis of the cork suberin acid C_{18} *cis*-Di18Epox_Me. [b]Obtained from the hydrolysis of the cork suberin acid C_{18} *cis*-Hyd18Epox_Me. [c]Obtained from the *syn*-hydroxylation of the model compound C_{18} *trans*-elaidic acid methyl ester. [d]Obtained from the *syn*-hydroxylation of the model compound C_{18} *cis*-oleic acid methyl ester. [e]Obtained from the hydrolysis of the model compound C_{18} *cis*-9,10-epoxyoctadecanoic acid. [f]H_{BzAc} and C_{BzAc} are the ^{1}H and ^{13}C chemical shifts of the acetal methine in the benzylidene group.

Table 3. Chemical Shift Differences (Δppm) in ^{1}H and ^{13}C of *vic*-Diol Methines (from Suberin Acids and Model Compounds) with the *vic*-Diol Group in the Free Form and as the Benzylidene Acetal (BzAc) Derivative[a]

	$\Delta\delta^{Ef\text{-}Tf}$	$\Delta\delta^{EBzAc\text{-}TBzAc}$	$\Delta\delta^{TBzAc\text{-}Tf}$	$\Delta\delta^{EBzAc\text{-}Ef}$
^{1}H				
Hyd18Diol_Me (cork suberin)			+0.34	
Di18Diol_Me (cork suberin)			+0.35	
5,6-dodecanediol	+0.19	+0.36	+0.35	+0.52
9,10-dihydroxy-octadecanoic acid_Me	+0.19	+0.36	+0.34	+0.51
^{13}C				
Hyd18Diol_Me (cork suberin)			+7.07 and +8.34	
Di18Diol_Me (cork suberin)			+7.06 and +8.34	
5,6-dodecanediol	+0.17 and +0.17	−3.57 and −2.28	+7.05 and +8.32	+4.58 and +4.60
9,10-dihydroxy-octadecanoic acid_Me	+0.22 and +0.23	−3.59 and −2.79	+7.07 and +8.29	+4.48 and +4.05

[a]Ef, *Erythro* with free *vic*-diol group; Tf, *Threo* with free *vic*-diol group; EBzAc, *Erythro* with *vic*-diol group in the form of benzylidene acetal derivative; TBzAc, *Threo* with *vic*-diol group in the form of benzylidene acetal derivative.

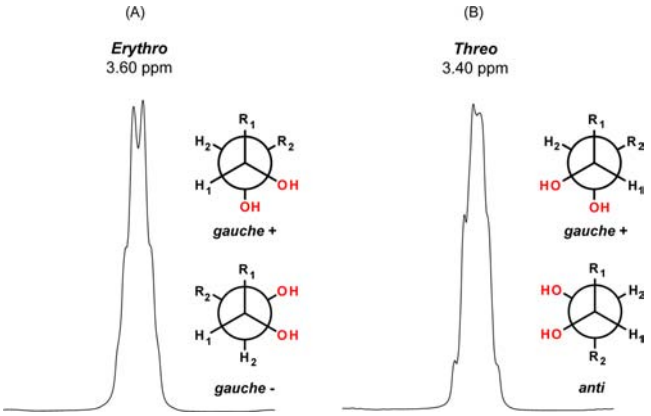

Figure 3. ^{1}H NMR multiplets of the *vic*-diol methine protons (H_1 and H_2) of *erythro* (A) and *threo* (B) model compounds (5,6-dodecanodiol) with the Newman projection conformations that allow hydrogen bonding between the hydroxyl groups (only *R,S-erythro* and *R,R-threo* shown).

much simpler *vic*-diol but with identical stereochemistry, analyzed the dominant conformers by ^{1}H NMR.[29] In low polarity solvents such as $CDCl_3$, both the *erythro* (*meso*-2,3-butanediol) and *threo* (±-2,3-butanediol) isomers preferred the conformations that allowed hydrogen bonding between the hydroxyl groups. These same conformational preferences can also be valid for the *vic*-diols in the present study, although they have much longer methylene chains attached to the hydroxyl-substituted chiral carbons. These conformations that approximate the vicinal hydroxyls (but avoid the eclipsed forms that would be sterically unfavorable) are shown in Figure 3, namely, *gauche*+ and *gauche*− in *erythro*, and *gauche*+ and *anti* in *threo* isomers. If these are the preferred conformations in the *vic*-diols we are dealing with here, they can explain the observed chemical shift differences between the *erythro*- and *threo*-isomers (Figure 3) because methine protons have different neighborhoods: in the *erythro* case, only one of the methine

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protons is spatially close to the opposite methylene group (H_1 to R_2 or H_2 to R_1, Figure 3A); but in the *threo* case, both methine protons are close to their opposite methylene groups (H_1 to R_2 and H_2 to R_1, Figure 3B). The spatially close opposite methylenes shield the methine protons, an effect stronger in *threo* isomers, thus explaining the difference of +0.19 ppm observed in *erythro* methine protons in model compounds (Table 3).

These assumed preferred conformations for *vic*-diols just discussed would also explain the comparative shielding observed in the carbons of the adjacent methylenes in *erythro*- versus *threo*-isomers, a difference of ca. −2 ppm, as measured in model compounds. A more significant "gamma-effect", similar to the one described above in *cis*-epoxides, exists in *erythro*-isomers due to the spatial proximity between R groups (R1 to R2), observed in both favored conformations; in *threo*-isomers, this proximity is only observed in one of the conformations (*gauche*+, Figure 3).

Comparing the chemical shifts of the *vic*-diol methine protons of Hyd18Diol_Me and Di18Diol_Me suberin acids with the ones of model compounds, we noticed that they are very similar to those of the *threo*-isomers (Table 2). However, because there is no direct proof of the preferred conformations around the *vic*-diol group in suberin acids and model compounds, some doubt can remain on the use of these chemical shift comparisons as proof of the relative configuration. To circumvent this situation, we reassessed the NMR analysis after converting the *vic*-diol groups into a cyclic structure, thus strongly diminishing the conformational variability as discussed below.

NMR Analysis of the *vic*-Diol Benzylidene Acetal (BzAc) Derivatives. All *vic*-diols, including the suberin acids, Hyd18Diol and Di18Diol, and the two pairs of *erythro* and *threo* model compounds, C_{18} 9,10-diol monoacids and C_{12} 5,6-dodecanediols, were derivatized as benzylidene acetals (BzAc), producing a "1,3-dioxolane" five-membered ring (Figure 4). Also, the suberin C_{18} 9,10-epoxyacids, Hyd18Epox and Di18Epox, and the C_{18} *cis*-9,10-epoxyacid model compound were hydrolyzed into C_{18} 9,10-diols and BzAc derivatized in the same way. The stereospecificity of the epoxide hydrolysis was used to confirm the *cis* or *trans* relative configuration of the suberin epoxyacids, as discussed below. Moreover, as a control,

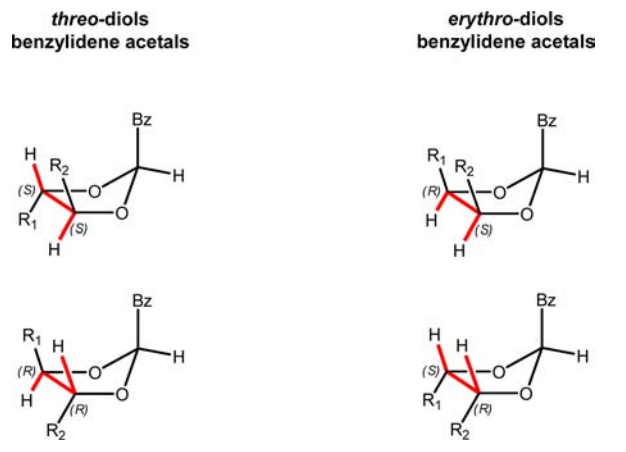

Figure 4. Benzylidene acetal derivatives of *vic*-diols shown in the envelope conformation. R_1 and R_2 are as described in Figure 1 (plus a CH_2 for each $(CH_2)_n$ group). [The quirality of the acetal carbon is not represented, depending on the R_1 and R_2 sustituents.]

erythro- and *threo*-C_{18} 9,10-diol monoacids, identical to the above model compounds, were obtained by the *syn*-hydroxylation of *cis*-oleic acid methyl ester and *trans*-elaidic acid methyl ester, respectively, and their BzAc derivatives also prepared. The NMR results of these BzAc derivatives with respect to the proton and carbon chemical shifts of the C-9 and C-10 (or C-5 and C-6) methines of the derivatized diols, as well as of the BzAc acetal methine (see Figure 4), are presented in Table 2.

Proton Spectra. Differences between the chemical shifts of the methine protons of *erythro*- and *threo*-*vic*-diol BzAc derivatives were conspicuous in model compounds, as shown in Table 3. As a rule, these methine protons in the *erythro*-BzAc derivatives had a chemical shift of ca. 4.12 ppm and in the *threo*-BzAc derivatives of ca. 3.75 ppm (Table 2), a difference of $\Delta H^{\delta erythroBzAc\text{-}\delta threoBzAc}$ of ca. +0.36 ppm (Table 3). Because this difference is significant and the chemical shift values are constant, this seems to be, by itself, a strong diagnostic feature to assign an *erythro* or *threo* configuration in these *vic*-diol structures.

The higher shielding in the *threo* isomers can, as in the case of the epoxide methine protons, again be explained by the "substituent" effect of the opposite methylene group, which is spatially closer to the methine proton under consideration (Figure 4). Because of the restricted conformational mobility of the dioxolane ring, this effect would be stronger than the one observed in the underivatized *vic*-diols, being in fact almost double (+0.19 ppm in the underivatized *vic*-diols and +0.36 ppm in the BzAc derivatives, see Table 3). The effect of the phenyl group of the BzAc derivative in the methine chemical shifts of the derivatized *vic*-diols is harder to evaluate. Besides some conformational variability of the dioxolane ring, and the free rotation of the phenyl group around the acetal carbon, the BzAc derivates can have actually two different configurations. When the BzAc derivative is formed from the *vic*-diol and the precursor benzaldehyde dimethyl acetal, the acetal carbon of the latter becomes asymmetric, originating two new possible stereoisomers, the (*R*)-BzAc and the (*S*)-BzAc, thus locating the phenyl group in two possible opposite sides (Figure 4). Because in theory both reaction positions are identically probable, both enantiomers should exist in similar proportions, making the interpretation of the phenyl effect in the chemical shifts more complicated.

Another difference between the BzAc derivatives of *erythro*- and *threo*-*vic*-diols was observed in the BzAc structure itself, namely, in the acetal methine proton. In fact, in the model compounds analyzed, this proton had a chemical shift of 5.77 ppm in the *erythro*-isomers and 5.86 ppm in the corresponding *threo*-isomers (Table 2). This systematic difference of −0.09 ppm, showing that this proton in the *erythro*-BzAc derivatives is more shielded and the consistency of the chemical shift values in the model compounds analyzed, suggests that the chemical shift of the acetal proton in the BzAc derivatives of alkyl substituted *vic*-diols can also be a reliable way to assign the *erythro* or *threo* configuration.

^{13}C Carbon Spectra. The chemical shifts of the ^{13}C carbons in BzAc derivatives and their differences between the *erythro*- and *threo*-isomers, as observed in model compounds, also showed to be diagnostic of the relative configuration of *vic*-diols (Tables 2 and 3). To compare the ^{13}C chemical shifts, we have to consider that in the free *vic*-diol form there are different signals for the C-9 and C-10 carbons (or C-5 and C-6 in case of dodecanediols) because neither the suberin acids nor the model

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compounds are completely symmetric in relation to the *vic*-diol group. The exception is the Di18Diol_Me from suberin, which due to its complete symmetry only shows one signal for both C-9 and C-10 carbons. Comparing the ^{13}C chemical shifts of the *erythro* and *threo* forms of the underivatized model *vic*-diols, $\Delta C^{\delta erythro\text{-}\delta threo}$, a small average difference of ca. +0.2 ppm was observed (Table 3). This difference is probably too small for a safe assignment of the *vic*-diol configuration. However, much bigger differences were found when this comparison was made in the respective BzAc derivatives, either −2.8 or −3.6 ppm, depending on the carbon chemical shift considered (Table 3).

After the BzAc derivatization, the *vic*-diol methine carbons were significantly deshielded compared to its previous free hydroxyl underivatized forms, in an order of magnitude of ca. +8 ppm for the *threo* model compounds and ca. +4 ppm for the corresponding *erythro* (Table 3). This showed that the deshielding effect of the BzAc group in these carbons was stronger in the *threo* isomers. Nevertheless, the most diagnostic feature to assign the *erythro* or *threo* configuration for these *vic*-diols using ^{13}C NMR was simply the values of their chemical shifts: in the *vic*-diol BzAcs of *erythro* model compounds, the *vic*-diol carbons were in the window of 78.7 to 79.3 ppm, and in the *threo* equivalents, they were from 81.5 to 82.9 ppm (Table 2). Both ranges are short and unambiguously apart, making the configuration assignment safe. In both C_{18} 9,10-diol suberin acids, the chemical shifts of their *vic*-diol methine carbons as BzAc derivatives were in the *threo* range; identically, both C_{18} 9,10-epoxy suberin acids hydrolyzed into the corresponding C_{18} 9,10-diols had values corresponding to a *threo* relative configuration.

Stereochemistry of the C_{18} Suberin Acids and the Macromolecular Structure of Suberin. The results discussed above showed that the ^{1}H and ^{13}C chemical shifts of the methine protons and carbons of *vic*-diols substituted in alkyl chains derivatized as benzilidene acetals can unambiguously be used to assign their *erythro* or *threo* configuration. The comparison of these chemical shifts in the model compounds with the ones of cork suberin acids, Hyd18Diol_Me and Di18Diol_Me (Table 2), proved that both exist in only one relative configuration, which is *threo*. In the same way, comparing the ^{1}H and ^{13}C epoxide methine and methylene NMR chemical shifts of model compounds, with the ones of C_{18} 9,10-epoxy suberin acids, Hyd18Epox_Me and Di18-Epox_Me, the two latter compounds were shown to be concordant with a *cis* configuration. To confirm this assumption, both cork suberin and model epoxyacids were hydrolyzed by a stereospecific *anti*-hydroxylation, where *cis*-epoxides gave rise to *threo*-*vic*-diols, and *trans*-epoxides originated *erythro*-*vic*-diols. The chemical shifts of the hydrolyzed epoxyacids, either as free *vic*-diols or in the form of BzAc derivatives, showed them to be *threo*, definitely proving that the suberin epoxyacids had a *cis* configuration. The optical activity of these *cis*-epoxide and *threo*-*vic*-diol suberin acids measured by polarimetry was zero, showing that they probably exist as racemic mixtures of their corresponding pairs of enantiomers.

The stereochemistry of the C_{18} 9,10-epoxy and C_{18} 9,10-diol suberin acids in cork is *a priori* conditioned by the stereoselectivity of their biosynthetic pathways. Biosynthesis studies had shown that *cis*-oleic acid could be the precursor of C_{18} 9,10-epoxy and C_{18} 9,10-diol suberin acids,[2] following the stereochemically favored routes of oxidation of the *cis*-double bond to *cis*-epoxide and hydroxylation of the latter to *threo*-diol. This is in agreement with the configuration that was proved here for the C_{18} cork suberin acids as *cis*-9,10-epoxy and *threo*-9,10-diol.

Although practically nothing is known about how the suberin acids are three-dimensionally organized within the suberin macromolecule, at least a partial ordered arrangement is possible. As seen at the ultrastructural level, suberized cell walls show a composite lamellar structure, with alternate dark and translucent contrast, the latter with a regular thickness of about 3 nm.[30] An ordered packing of the C_{18} suberin acids was proposed to be the structural basis of these translucent lamellae.[3] Studies made by ^{13}C solid state NMR have shown that a part of the methylene chain in suberins was motionally restricted;[31,32] also a WISE NMR study in potato wound periderm found the most diagnostic suberin carbons in the vicinity of low mobility protons.[33] In both cases, the observed rigidity can be justified by an ordered "crystalline" phase.

Some fats can be regarded as a comparable situation to that of suberin, in the sense of being based on glycerol esterified to C_{18} midchain modified fatty acids. This is the case of triolein, the triacylglycerol of C_{18} *cis*-9-oleic acid, which in the solid phase packs the alkyl chains in an ordered manner, kinked at the double bond,[34] as favored by its *cis* configuration. In suberin, both the C_{18} 9,10-epoxide and the C_{18} 9-unsaturated acids[6] have a *cis* configuration, which will identically favor a similar bent molecular arrangement of their alkyl chains. Finally, if we bend in the same way the C_{18} *threo*-*vic*-diol suberin acids, they project their two hydroxyl groups to opposite directions, minimizing the steric hindrance between them. We should also bear in mind that these C_{18} midchain modified suberin acids must play a major role in cork suberin structure since they account for about 50% of all its long-chain monomers.[7] If all of these C_{18} suberin acids, with the midchain unsaturation, epoxide, and *vic*-diol group assume this kinked conformation, they could be conveniently packed in an ordered parallel way (see the Table of Contents Graphic in the Web edition). This packing would favor strong intramolecular bonding, namely, hydrogen bonding between epoxides and *vic*-diol groups. Ultimately, this molecular arrangement would allow an ordered macromolecular structure for suberin and justify the organized lamellar ultrastructure observed in cork cell walls.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

FTIR (ATR) spectra of model C_{18} 9,10-epoxyacids and C_{18} cork suberin 9,10-epoxyacids. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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