Studies on the structure of the plant wax nonacosan-10-ol, the main component of epicuticular wax conifers

Antonio J. Matas, Maria José Sanz, Antonio Heredia

a Departamento de Biología Molecular y Bioquímica, Grupo de Caracterización y Síntesis de Biopolímeros Vegetales, Facultad de Ciencias, Universidad de Málaga, E-29071 Málaga, Spain  
b Centro de Estudios Ambientales del Mediterráneo, Fundación CEAM, Parque Tecnológico, Charles Darwin 14, E-46980 Paterna, Valencia, Spain

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Abstract

The main component presents in the epicuticular waxes of needles of Pinus halepensis and the most of conifers, the secondary alcohol nonacosan-10-ol, has been investigated by X-ray diffraction and differential scanning calorimetry. The results obtained from these physical techniques permitted to establish a definitive structural model of the molecular arrangement of this wax, basically in good agreement with the model formulated by other authors from theoretical formulations. Biological implications of the proposed structure have been also formulated.

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1. Introduction

The surface of plant aerial parts are covered by the cuticle, a complex biopolymer which serves as a barrier between the plant and its environment. The cuticle acts as the first protective barrier against UV radiation and bacterial and fungal attacks [1]. A particular mechanism that plants have adopted to survive desiccation in a terrestrial environment is the production of waxes deposited into (intracuticular) and on (epicuticular) the cuticular layer. Cuticular waxes are a solid mixture of very long chain fatty acids, alcohols, aldehydes, esters and \( n \)-alkanes [2]. In some plants, other compounds such as cyclic terpenoids and phenolics can also be present. These mixtures of nonpolar compounds are deposited at continuous or very frequent intervals of time in the outer surface of leaves and fruits forming crystalline and amorphous structural regions following a mechanism that still constitutes an unresolved paradigm of plant lipid research. Moreover, cuticular waxes constitute the main barrier limiting the transport across the plant–atmosphere interface. This property allows waxes to control cuticular transpiration, foliar uptake of xenobiotics and resistance against fungi [3].

In spite of the biological importance of these lipidic components, scarce attention has been made on cuticular waxes. During the last years only a few studies were focused to stress biophysical aspects of plant waxes. In this sense, it has been established that epicuticular crystalline–amorphous structures are mainly the final result of the physical–chemical properties of the wax rather than a consequence of a specific transport process [3]. X-ray diffraction and calorimetric techniques have been used to explore the structure of some type of plant waxes [4] and, more recently, thermodynamics interpretation of the self-assembly process of wax formation, based on the measurement of specific heat of distinct plant waxes mixtures, has been formulated by our research group [5].

In the present work, we report how the combined use of the physical techniques above mentioned may be used to complete our knowledge on the molecular arrangement and structure of one singular type of plant wax: the secondary alcohol nonacosan-10-ol (Fig. 1, see attached structure). This alcohol is the main component of Pinaceae plant waxes and its biological importance is mainly ascribed to the role of the shape and morphology of the aggregates and crystals, in form of characteristic tubules that form the nonacosan-10-ol on the conifer needles. Changes in the morphology of this wax have been taken as an indication of ageing of leaf
2. Material and methods

2.1. Isolation, identification and quantification of nonacosan-10-ol

One-year-old twigs of Pinus halepensis were samples in spring 2000 from trees in the Els Ports area, north of Castellón province from different plots. Samples were collected from the upper third of the tree; five branches per tree were collected including all geographic orientations. Cuticular waxes were extracted from almost four hundreds of twigs. They were carefully washed once in CHCl₃ for 30 s at room temperature. The resulting solution was dried, filtered and the organic solvent removed under reduced pressure. Cuticular waxes were separated into their different compound classes by preparative thin layer chromatography (silica gel) using benzene as the mobile phase. A band containing the secondary alcohol (Rᵣ 0.55) was removed from the plates, eluted and analysed by combined gas chromatography and mass spectrometry (GC–MS) in a Hewlett-Packard 5890 (USA), equipped with a flame ionisation detector, with a methyl silicon capillary column. For derivatization, the samples were dissolved in 100 μl of N,O-bis(trimethylsilyl)acetamide (Sigma, France) and 100 μl of dry pyridine for 30 min at 60 °C. Temperature programme: 2 min at 60 °C, 50 °C·min⁻¹ to 200 °C, 3 °C·min⁻¹ to 300 °C, carrier gas He. Peak areas were recorded by electronic integration. In the case of wax samples, all dominating compounds were identified and they comprised the 85% of the chromatogram area. Single components were identified by comparing their mass spectra with those from authentic samples, the NBS library of mass spectra or from previously published data.

2.2. X-ray diffraction

X-ray diffraction patterns were obtained with a Siemens D-501 automated diffractometer (Siemens, Germany) using graphite-monochromated CuKα radiation. The diffractograms were generally recorded between 10 and 40° (2θ) in 0.05° steps, counting 4 s per point at 40 kV and at 20 mA. The diffractometer permitted temperature control of the sample.

2.3. Calorimetry

All experiments were performed in a Shimadzu DSC-50 differential scanning calorimeter (DSC, Shimadzu Corp., Japan) with computer-aided data analysis. Aluminium DSC pans containing around 1.5 mg per sample were loaded into the calorimeter at room temperature. For each run, the thermograms were recorded during heating at 2 °C·min⁻¹, from 15 to 100 °C, and post-cooling to room temperature at the same rate using liquid nitrogen as cooler. To obtain the thermogram of a sample the baseline was subtracted form the data obtained from each sample.

2.4. Molecular modelling

Molecular modelling was made using the HyperChem software package (Hypercube, Waterloo, Canada). Molecular mechanics was used to model to investigate the conformation and electronics effects of the molecular arrangement of the secondary alcohol. The start point was the optimised structure of a single molecule of S-nonacosan-10-ol and further overall molecular geometry after the combination of...
some molecules was taken from one previously reference (see Section 4 for more detail). Initial bad contacts between different hydrocarbon chains and different parts of the same chain were relieved by energy minimisation computation using the Fletcher–Reeves method until a gradient of 0.08 kJ nm$^{-1}$. Molecular structures of final optimised structures were visualised using WebLab Viewer Pro (Molecular Simulations Inc., San Diego, USA).

3. Results

3.1. Chemical analysis

Nonacosan-10-ol was the most important (up 58%, w/w of the identified wax components) single component of epicuticular waxes extracted from $P.$ halepensis needles. Purification by thin layer chromatography and further analysis by gas chromatography–mass spectrometry increased the purity to 99%. Mass spectra of purified sample confirmed the identity of the wax compound. The corresponding trimethylsilyl derivative showed typical molecular mass fragments of 481, 369 and 229 (main molecular fragment) corresponding to nonacosan-10-ol [2].

3.2. Calorimetric behaviour of nonacosan-10-ol

It is known that important structural information on amorphous, crystalline behaviour and first and second order phase transition of molecules and polymer can be reached by DSC [4]. The typical heating and cooling thermograms of a sample of purified nonacosan-10-ol is shown in Fig. 1. In spite of the assumption that we are working with a pure substance, two endothermic peaks were recorded in the heating thermogram. These two peaks, located at 54 and 75 °C, correspond to two clear first-order transitions attributed to the melting of wax sample. Cooling thermograms showed considerable hysteresis, with two exothermic peaks at 68 and 50.5 °C, demonstrating the crystallinity of the sample investigated. The presence of two different thermal events for a pure substance, with a net difference of 21 °C, is notable indicating a polymorphic behaviour of this lipid crystalline compound. It is obvious that the enthalpy of recrystallization, calculated from the peak area located at 75 °C, was lower than the corresponding melting transition observed for the initial heating scan. It is a clear indication that the initial solid phase formed by cooling from the melted state is a metastable phase. With connections by hydrogen bonds between hydroxyl groups belonging to different molecules. In this model (Fig. 2, see attached structure), obtained after theoretical discussion on the molecular geometry of the wax without any experimental evidence, adjacent layers will interact by van der Waals interactions between the methylene chains. Assuming this model, the β peak could be assigned to the average distance between chains of molecules linked by hydrogen bonds. A recent structural study, using scanning tunneling microscopy, on the self assembly of monolayer of nonacosan-10-ol physisorbed on a graphite surface showed that the monolayer consists of parallel pairs of molecules of a width of 0.45 nm that are coupled by hydrogen bond [9]. These results are in good agreement with experimental data reported in this work.

A definitive probe on the assignment of the different basal spacings found in the diffraction pattern of the sample of nonacosan-10-ol could be achieved by monitoring the diffraction pattern of a sample of the secondary alcohol with temperature. Fig. 3 shows the different diffraction patterns during heating from room temperature to 100 °C followed by further cooling until room temperature. It can be observed that, while the peak located at 0.414 nm disappeared between 60 and 100 °C due to the melting of the hydrocarbon chains, the peak located at higher basal spacing, 0.453 nm, only clearly disappeared when the temperature of 80 °C was reached. At the same time, the above defined α

![Fig. 2. X-ray diffraction pattern of pure nonacosan-10-ol. Previously reported molecular arrangement of this molecule is also represented in the figure. For more details, see text.](image-url)
peak remained almost unchanged. The reestablishment of the original diffraction pattern after cooling of the sample again demonstrated the hysteresis of the cooling process.

4. Discussion

The above mentioned data may serve to built a more comprehensive molecular model of the nonacosan-10-ol. Simultaneous discussion of the calorimetric and diffraction data clarifies the model. Thus, the first endothermic peak recorded at 54 °C represents the melting of the hydrocarbon chains, whereas the second endothermic peak may be now clearly assigned to the melting of the molecule chains connected by hydrogen bonds. The enthalpy associated to these thermal events confirmed this assumption: the average energy of the first transition was 4.7 kJ mol⁻¹ and the energy of the second transition was 13.5 kJ mol⁻¹. These energies are in the range of typical van der Waals and hydrogen bonds interactions, respectively.

Taken into account the above discussed data, one putative molecular model of the special arrangement of the nonacosan-10-ol may be presented in Fig. 4. The model agrees the previously one proposed by Jetter and Riederer [8] being confirmed in the present work by data obtained using appropriate physical techniques. Moreover, the information obtained using these techniques may help to a better understanding on the biophysics of this plant wax and its physiological importance.

From a more biophysical point of view, we would like to stress the special arrangement that show the nonacosan-10-ol. This molecule, its special molecular arrangement, is able to show two typical thermal events that we can find in plant waxes: the thermal behaviours corresponding to the van der Waals and the hydrogen bond interactions. The conjunction of both interactions produces a relatively ordered molecular arrangement which is macroscopically manifested by the presence of tubular aggregates deposited on the stomata of most species of Pinaceae and other species [8]. Some causes of the alteration of epicuticular structures from tubules to flat platelets during the ageing of leaf surfaces and under the impact of air pollutants could be interpreted.
by taking into account the structural data presented here and the self-association formulation recently reported by our research group [5]. Independent of the contribution of other factors, the diffusion and/or sorption of other molecules can interfere in the molecular arrangement of this type of wax. Molecules as diterpene acids, reported as components in needles of conifer waxes [10] or other molecules as long chain fatty acids may cause this disruption. Moreover, potential hydrogen bond formation between wax molecules and these molecules of different structure and functional groups, could modify the molecular symmetry and arrangement of nonacosan-10-ol crystals resulting in the formation of less crystalline zones as a consequence of negative excess specific heat of these mixtures [5]. The structure of nonacosan-10-ol presented here is the final result of a delicate balance between weak interactions. Partial disruption of this equilibrium of interactions could irreversibly affect the overall structure and subsequent morphology. Thus, it is plausible that prolonged and elevated concentrations of such molecules could result in the rearrangement of the molecule of wax towards a less ordered structure. Current research is focused to check this hypothesis. In these experiments, mixtures of nonacosan-10-ol and long chain fatty acids will be analyse by DSC and X-ray diffraction in order to evaluate the changes in the two thermal transitions of the secondary alcohol. Similar experiments will be conducted on nanocosan-10-ol samples after controlled sorption of terpene molecules in closed chambers. Preliminary results seem to indicate the validity of this hypothesis.

5. Conclusions

The research presented here completes the knowledge on the special arrangement and structure of the secondary alcohol nonacosan-10-ol, the main component of the cuticular waxes of Pinaceae. It has been possible after the combination of the information submitted by powerful physical techniques as X-ray diffraction and calorimetry. The tubular arrangement that occurs in this fatty alcohol can be explained by the molecular structure showed in Fig. 4 which takes into account the occurrence of both, hydrogen bond and van der Waals interactions. The model proposed here suggests a possible explanation of the physical modification or alteration that takes place in complex epicuticular waxes during leaf ageing and after the interaction of air pollutants. Partial or total disruption of hydrogen bonds conduces to an irreversible modification of the structure and crystallinity of the nonacosan-10-ol.

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