A geochemical study of lacustrine sediments: towards palaeo-climatic reconstructions of high Andean biomes in Colombia

Boom, A.

Citation for published version (APA):
CHAPTER 6

Cutan, a common aliphatic biopolymer in cuticles of drought-adapted plants.


Organic geochemistry (accepted)

The aliphatic biopolymer cutan, previously only known from *Agave americana* and *Clivia miniata*, was isolated in high purity from the leaf cuticles of several drought-adapted plants. These plants have succulent leaves with thick cuticles, and all but one are CAM plants. Following a chemical workup procedure, the cutan biopolymer has been identified using pyrolysis-GC/MS. These findings shed new light on the function of this biopolymer, in particular the occurrence of this in drought-adapted plants. In addition, a possible explanation arises for an outstanding debate on the origin of aliphatic biopolymers in certain fossil leaves and fossil terrestrial plant deposits. We propose that the presence of cutan in the fossil record, may relate to a high contribution of drought-adapted plants, such as CAM plants.
6.1. Introduction

Higher plants biosynthesise plant-specific, resistant biopolymers. Lignin, suberin and cutin are well known examples. Lignin is especially prevalent in woody tissues, while suberin is a lignin-like cell wall constituent that serves to make the cell wall water and air tight. It is present in root tissue and it is also the main constituent of cork tissue (Kolattukudy, 1980). Cutin is a polyester present in the cuticles of plant leaves and fruits consisting mainly of \( \text{C}_{16} \text{-C}_{18} \) hydroxy-fatty acid moieties, and protects against microbial attack and serves to make plant leaves water and air tight (Kolattukudy, 1976).

Investigations of the chemical composition of plant cuticles resulted in the discovery of a new biopolymer, cutan, which is highly aliphatic and resistant to degradation (Nip et al., 1986; Nip et al., 1986; Tegelaar et al., 1989). The function and structure of this biopolymer is still poorly understood. Upon flash pyrolysis, this biopolymer yields a highly characteristic series of long-chain \( n \)-alkenes and \( n \)-alkanes. Cutan has so far only been positively identified in two plant species, *Agave americana* and *Clivia miniata*. Tegelaar et al. (1989) proposed a tentative structure of cutan based upon the results from flash pyrolysis, FT-IR and \(^{13}\)C NMR spectroscopic techniques: a polysaccharide-like structure, with ether-bound long aliphatic side chains. The importance of non-ester bonds in cuticles of *Clivia* sp. was already recognized by Schmidt and Schönherr (1982). Data published by de Leeuw et al. (1991) indicated that the polysaccharides cannot be a part of the macromolecular structure. Also, McKinney et al. (1996) found no evidence for polysaccharide units in cutan, neither by pyrolysis nor by \(^{13}\)C NMR studies. In addition, tetramethylammonium hydroxide thermochemolysis released exclusively fatty acid and aromatic fragments. Based on these results, the authors proposed a different structure, in which aromatics moieties are part of the biopolymer. However, these authors and recently Villena et al. (1999) also showed that the aromatic moieties can be removed completely upon purification.

Flash pyrolysates of fossil leaf cuticles often show similar distribution patterns of \( n \)-alkanes and \( n \)-alkenes as pyrolysates of cutan do (Nip et al., 1986), suggesting that cutan is an important chemical component of these fossil leaves. Ancient terrestrial organic deposits often contain a large contribution of aliphatic signal upon pyrolysis, e.g. coal (Kralert et al., 1995). However, questions arise on the origin of these cutan-like signals in sediments. The fossil record of cutan-containing plants like *Agave americana* and *Clivia miniata* does not exist and these two plants alone can, therefore, not explain the widespread occurrence of cutan in the fossil record (Möslle et al., 1998). In addition, the physiological function of cutan remains unknown.

In this paper, we present several new examples of plant cuticles that do contain cutan. These plants were selected based on their adaptation to drought. We, therefore, assume that cutan has a physiological function as an effective water resistant and airtight layer. This puts the list of cutan-containing plants in an entirely new perspective that may have an impact on our interpretation of the cutan fossil record.
6.2. Materials and Methods

6.2.1. Plant material

Leaves of *Podocarpus* sp. were sampled from a garden in Chia (Colombia) and dried. Leaves of *Clusia multiflora* were collected in the Paramo de la Rusia in Boyacá, (Colombia) and sun dried. Leaves of *Clusia rosea* were obtained from a greenhouse-cultivated specimen. Sun dried cuticles of exotic *Agave americana* were sampled in Greece. Leaves of *Cereus* sp. were sampled from NIOZ houseplants. Leaves of an unidentified epiphytic orchid, which had been cultivated in a greenhouse, were sampled and kept in the refrigerator for a few days prior to treatment. Control plants were sampled: a grass species, *Clethra* sp., an ericaceous species and *Myrsine guyanense*. The control plants were all sampled in the field in Colombia.

6.2.2. Cuticle isolation

About 2 to 3 leaves were used from each plant species studied; only one large leaf was used from the Orchid and *Cereus*. The cuticles from *Agave* were collected from a large number of leaves. The leaves from the Orchid and the exceptionally succulent *Cereus* were not dried; instead, their cuticles were removed using a scalpel, and remaining leaf tissue was scraped off. The dried leaves of the other samples and the manually cleaned cuticles were added to a solution of oxalic acid and ammonium oxalate. The mixture was heated until boiling point and stirred for 24 h (Tegelaar et al., 1989). The soaked leaves were taken out of the solution and rinsed with water. By means of a sharp scalpel and tweezers, the cuticles were separated from the leaves. The cuticles were spread out and the remaining tissues were manually removed with the scalpel until a visually clean transparent cuticle remained. The dry weight of the cuticle samples was about 1 to 2 grams. The control plants yielded substantially less (<1 gram).

6.2.3. Cutan isolation

The dried cuticles were extracted with dichloromethane (DCM) and methanol. The extract was discarded. Hydrolysable material was removed by mild acid hydrolysis. The cuticles were transferred into a 5 ml trifluoroacetic acid solution (2 M) and refluxed for 2 h. They were subsequently washed with water until the aqueous phase turned neutral and with 5 ml methanol (3x), and then dried. A more aggressive acid hydrolysis was applied to remove all remaining hydrolysable material. To this end the dried residue was transferred into 5 ml sulphuric acid (12 M) and was stirred for 2 h at room temperature. The residue was subsequently washed with water, methanol and DCM and dried. This residue was refluxed in 5 ml sulphuric acid (2 M) for 1 h. The residue was rinsed with water until the aqueous phase turned neutral and subsequently washed with 5 ml of methanol (3x), yielding ca. 50 mg of material. Ultimately, the residue was saponified for 2 h in a refluxing methanolic solution of KOH (2M, 4% H$_2$O). Then the residue was extracted with 5 ml methanol (2x), 5 ml methanol/DCM (1:1, 2x), 5ml DCM (2x), yielding ca. 30 mg of whitish powder,
which was used for cutan analyses. The control plants: grass, *Clethra* sp., the ericaceous plant and *Myrsine guyanense* leaves did not yield a white residue, but only a trace of blackish material.

6.2.4. Curie-temperature pyrolysis-gas chromatography mass-spectrometry (Py-GC/MS).

Py-GC/MS was performed using a Hewlett Packard 5890 Series II gas chromatograph with a FOM-3LX pyrolysis unit, interfaced to a VG Autospec Ultima mass spectrometer operating at 70eV, resolution 1000 with a mass range of m/z 800-50 and a cycle time of 1.8 s. A small amount of the sample was applied to a ferromagnetic wire with a Curie-temperature of 610°C. The GC was equipped with a fused silica capillary column (25 m x 0.32 mm) coated with CP-Sil 5 (film thickness 0.40? im), with helium as a carrier gas.

6.3. Results

We have performed a sequential hydrolysis method, adapted from Blokker *et al.* (1999) and Tegelaar (1989), to facilitate separation of the non-hydrolysable part of the cuticle from the hydrolysable part (e.g. cutin). If instead a harsh treatment such as refluxing sulphuric acid would have been applied, the procedure might have produced artefacts, because of the large amount of compounds being released at once. By stepwise increasing the hydrolysis potential we aimed to minimise the formation of artefacts and isolate a pure non-hydrolysable residue. All plant specimens except the control set yielded a whitish powder residue at the final stage of the procedure in good yield (between 1 and 3% of the isolated cuticle dry weight).

Additional electron microscopy on the *Clusia multiflora* and *Agave americana* residues revealed that some particles with cell wall structures remained intact (not shown). The majority of the material was, however, entirely amorphous. These remaining fragments showing cell structure indicate that the residue is not completely purified, and that other cell wall constituents might still be present albeit in relatively small amounts. Thus, we may expect traces of other components (e.g. cellulose) in our isolated cutan fractions, but it only will be a very small proportion of the total.

Py-GC/MS enables the identification of fragments formed upon pyrolysis, thus revealing structural elements of the biopolymer. The flash pyrolysates of the residues from the investigated plants show a very strong aliphatic nature (Figures 1 and 2) and are lacking any traces of cutin-derived monomers (typically C\(_{16}\) and C\(_{18}\) fatty acids, Kolattukudy, 1976) indicating that our work-up procedure completely removed the ester-bound cutin monomers. The flash pyrolysates are dominated by C\(_7\) to C\(_{33}\) n-alkenes and n-alkanes.

There is a slight odd-over-even carbon number predominance of the long-chain n-alkanes, indicating that there may be free alkanes present, which are being extracted thermally. The cutan fractions from *Clusia* and *Agave Americana* were tested for the
Figure 6.1. Gas chromatograms from the flash pyrolysates of the *Clusia rosea*, *Agave americana* and *Podocarpus* sp. residues (closed diamonds refer to n-alkene/alkane doublets and open circles refer to methyl ketones).
Figure 6.2. Gas chromatograms from the flash pyrolysates of the epiphytic orchid, Clusia multiflora and Cereus sp. residues. (closed diamonds refer to n-alkene/alkane doublets and open circles refer to methyl ketones).
presence of free n-alkanes by subjecting them to a subsequent extraction with DCM. The pyrolysates of the re-extracted cutan fractions showed the same n-alkane / n-alkene distributions. In addition, the extracts did not contain any n-alkanes. We, therefore, conclude that the observed odd-over-even predominance is indeed a feature of the flash pyrolysates. The Cereus cutan differs most from the others by the presence of several aromatic compounds and very high intensities of the C\textsubscript{31} and C\textsubscript{33} n-alkanes. In this specific case, this probably indicates impurities that are thermally extracted during pyrolysis. All other cutans show in principle the same characteristics, but the intensities of the individual peaks differ among the pyrolysates of the different plant species.

Mass chromatography of m/z 59 on the flash pyrolysates shows that methyl ketones are present from C\textsubscript{7} onwards. Long-chain (\textgtr C\textsubscript{30}) methyl-ketones are present with a stronger odd-over-even carbon number predominance and their chain lengths are in the same order of magnitude as those of the n-alkenes / n-alkanes. There is a slightly elevated abundance of the C\textsubscript{9} and C\textsubscript{10} methyl ketones.

6.4. Discussion

The data presented here show that the pyrolysis products from the aliphatic biopolymer consist predominantly of long-chain (up to C\textsubscript{33}) alkenes and alkanes, obviously making up the major part of the polymeric structure of cutan. This clearly distinguishes these samples from those with only cutin and other biopolymers. The isolation method that was applied here was exclusively focussed on removal of solvent extractable components, removal of cellulose and cleavage of ester bonds. A polymer like cutin, which is predominantly ester-bonded, is entirely removed using this method. This method was adapted from the original studies on the discovery of cutan (Nip et al., 1986; Nip M., et al., 1986; Tegelaar et al., 1989). For the isolation of cutan, Mösle et al. (1997) used strong oxidative conditions (H\textsubscript{2}O\textsubscript{2}/acetic acid at 65°C) to extract cutan, while the polymer itself never was described as to be resistant to strong oxidation. In fact, other oxidative methods such as ozonolysis (Villena et al., 1999) and ruthenium tetroxide treatment (Schouten et al., 1998) completely break down the polymeric structure, by cleaving ether-bonds which are present (Schmidt and Schönherr 1982). Thus, oxidative conditions must be avoided during cutan workup. This may also explain why Finch and Freeman (2001) did not find cutan in the CAM plant Kalanchoe, by using artificial diagenesis conditions. There is a strong possibility that cutan is not as resistant against harsh chemical conditions as is generally assumed and therefore will not always survive diagenesis.

Our study extends the list of plants that biosynthesise cutan considerably. All plants that we identified as cutan-containing plants have some degree of adaptation to drought. Although not all of them are adapted to arid conditions, each of them experiences periods of water shortage, hence they have developed CAM photosynthesis or thick water-proof cuticles to cope with the loss of water due to evaporation. The
aliphatic biopolymer cutan enhances the hydrophobic nature of these cuticles, preventing water from escaping from the leaves. Due to its very strong aliphatic nature it will be more effective than cutin. *Podocarpus* demonstrates that non-CAM plants are apparently able to biosynthesise cutan too. *Podocarpus* is a typical sclerophyll that, like CAM plants, has exceptionally thick cuticles. The cutan “fingerprint” is often found upon pyrolysis of fossil leaves (Larter 1984; Nip *et al.*, 1986; Mösle *et al.*, 1997). However in some cases this aliphatic signal is not predominant. This is possibly due to the lack of purification of the fossil samples. Cutan has never been extracted and isolated from these fossil leaves and generally it is assumed that selective preservation processes have purified the resistant aliphatic polymer. Moreover, studies towards cutan in living plants were always focused on plants that were strongly represented in the fossil record, and cutan has never been found in these plants. This leaves the question whether cutan in the fossil record really represents a biopolymer or a geo-polymer. The discovery of these new cutan-containing plants puts those of the fossil record into a new perspective. With these new examples and with the lack of cutan in the control plants it is clear that cutan is a true biopolymer and not an artefact resulting from workup. The occurrence of cutan in *Podocarpus* is in full agreement with the finding of cutan in Neogene fossil leaves of this plant (Wijninga, 1996), so far the only example of extant vs. fossil cutan. It demonstrates that also fossil cutan is in fact a preserved biopolymer and not a geo-polymer. CAM photosynthesis is probably very ancient, its occurrence is widespread throughout the plant kingdom. For example, several primitive *Isoëtes* species, a fern-like that has evolved during the Carboniferous and the epiphytic ferns from the family Polypodiaceae all have CAM photosynthesis. This widespread occurrence of these CAM plants in the present predicts that we might expect similar plants with likely similar cutan biopolymers within the geological record. It is, therefore, not unreasonable to assume that the presence of cutan in the fossil record points to plants with similar adaptations as described above to drought and or CAM photosynthesis, and our examples point to where we have to look for fossil examples of cutan.

Cutan may be used to study living relatives of plants that are known from the fossil record and thus reveal the evolutionary context of cutan and drought-adapted plants. *Welwitschia*, a living fossil, is a desert plant and also a CAM plant. *Ephedra* is also a drought-adapted relict of the past and has succulent-like leaves, its pollen record goes back into the Triassic (Benton, 1993). Plants related to *Cycas* also have thick leaves which are exceptionally tough and they also can cope with drought and can be traced back into the Carboniferous (Benton, 1993). We predict that these plants too will have the cutan biopolymer as a component of the cuticle. As mentioned earlier, the conifer *Podocarpus* represent the only living example of a plant with cutan that is well represented in the fossil record yet. Further studies may reveal more examples. It will be interesting to look at specific plants such as *Clusia*, which belongs to a very ancient lineage of angiosperm families, closely related to *Magnolia*, since Late Cretaceous fossils of *Clusia* leaves have been reported (Crepet and Nixon 1998). These may be an interesting target for an investigation towards fossil cutan. If such
fossils do indeed contain cutan as the living relatives of *Clusia* do suggest, they would offer a unique possibility to study for example the stable isotopic carbon isotopic signatures of ancient CAM plants, making cutan a highly potential palaeo-environmental indicator.

6.5. Conclusions

It is demonstrated that the biopolymer cutan is present in the cuticles of five plant species not studied before, which have in common that they are adapted to cope with severe drought. This indicates that cutan is not a rare plant biopolymer, but that it is probably a physiological adaptation to survive drought conditions. Therefore, it may only be present in those plants that are specialised in preventing water loss through evaporation. Since we also analysed grass, an ericaceous plant, *Myrsine guyanense* and *Clethra* sp. leaves, which do not have such adaptations and also do not yield a cutan residue using the same isolation procedure, we may conclude that cutan is not an artefact, but a real biopolymer. With the exception of *Podocarpus* sp. all the cutans found (and the cutan from *Clivia* from earlier studies) are from plant genera that contain CAM species, highly specialised to survive drought conditions. We assume that cutan is associated with the thick cuticle that protect these plants from water vapour escaping through the leaf, especially during the day, when environmental conditions are hot and dry. Plants operating in full CAM photosynthetic mode close their stomata during day time that and the presence of the aliphatic polymer cutan in these plants hints towards its possible function of water and air tight layer.

Furthermore we conclude that the cutan present in the geological record derives from the preserved biopolymer cutan. We, thus, propose that cutan present in the fossil record maybe derived from similar plants. When future research focused on the fossil counterparts of these plants confirms these results, then the occurrence of cutan could be interpreted as an environmental indicator.

6.6. Acknowledgements

We thank the Netherlands Organisation for Scientific Research (NWO) for financial support of this research (grant 750.196.16 to H. Hooghiemstra and J. J. Boon). Prof. T. van der Hammen is kindly thanked for providing access to his garden in Chia with indigenous *Podocarpus* trees and Prof. U. Lüttge (TU-Darmstadt) for providing leaves of *Clusia rosea*. Antoine Cleef (IBED, University of Amsterdam), Margaret Collinson (Royal Holloway) is thanked for performing electron microscopy on the *Clusia* and *Agave* samples and P. van Bergen (University of Utrecht) is thanked for useful discussions.
6.7. References


Wijninga V., 1996. Paleobotany and palynology of Neogene sediments from the high plain of Bogotá (Colombia). PhD University of Amsterdam.