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STRUCTURAL CHARACTERIZATION OF ALIPHATIC, NON-HYDROLYZABLE BIOPOLYMERS IN FRESHWATER ALGAE AND A LEAF CUTICLE USING RUTHENIUM TETROXIDE DEGRADATION

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Key Word Index—RuO_4 oxidation; resistant non-hydrolyzable biopolymers; *Botryococcus braunii*; hydroxy fatty acids; cutan; *Agave americana*; algaenan; *Tetraedron minimum*

Abstract—Aliphatic, non-hydrolyzable biopolymers were subjected to RuO_4-oxidation in order to examine the potential of this method in revealing details on their structures. The method was tested on model compounds first and found to cleave alkyl chains of aromatic moieties, double bonds and ether bonds. Oxidation of the biopolymer cutan derived from cuticles of *Agave americana* and the algaenans isolated from the cell walls of the freshwater algae, *Botryococcus braunii* (race A and L) and *Tetraedron minimum*, resulted in the formation of specific oxidation products. In the case of *B. braunii*, the results confirmed previously suggested structures. Results obtained for cutan and *T. minimum* algaenan enabled, for the first time, the reconstruction of the building blocks of these biopolymers. These blocks consist of unsaturated long-chain hydroxy fatty acids, which are cross-linked *via* ether-bonds or ester-bonds.

INTRODUCTION

The major part of sedimentary organic material consists of insoluble, highly complex macromolecules, operationally defined as kerogen. It has become progressively clear that kerogens may, for a substantial part, be derived from the selective preservation of resistant, non-hydrolyzable biopolymers, which occur in the cell walls of marine and freshwater microalgae, and the cuticles of leaves (for a review see [1]). These biopolymers are usually present in low amounts (1–2% of dry biomass) in extant organisms but, since they are very resistant against microbial and chemical degradation, they are enriched upon sedimentation and burial. Analysis on a molecular level is only possible if the resistant biopolymer undergoes some treatment which depolymerizes the macromolecular network. The small molecules obtained in this way may be analyzed by techniques like gas chromatography–mass spectrometry (GC–MS). Analytical pyrolysis (Py) GC–MS techniques have revealed a great deal of information and showed that most of these polymers are aliphatic in nature, as was also clear from ^{13}C NMR analyses (e.g. [2, 3]).

Studies of the aliphatic, non-hydrolyzable biopolymers have, until now, mostly been performed by using techniques like flash pyrolysis and only rarely using chemical degradation. Ruthenium tetroxide (RuO_4) is a very common oxidation reagent, and Stock and co-workers used this reagent on coals and macerals [4, 5], whilst Trifilieff [6] used it to characterize asphaltene and resin fractions of mineral oils. More recently, Boucher *et al.* [7, 8] and Standen *et al.* [9, 10] used RuO_4 degradation to study kerogens. In the present work, this reagent is used to characterize insoluble, non-hydrolyzable biopolymers isolated from the cell walls of the freshwater algae, *Botryococcus braunii* and *Tetraedon minimum* (so-called algaenans; [1]) and the terrestrial plant, *Agave americana* (cutan; [11]).

RESULTS AND DISCUSSION

To test the effect of the RuO_4 reagent on the different types of linkages potentially present in biopolymers, several model compounds (Figure 1) were subjected to oxidation. Alkyl aromatic and unsaturated

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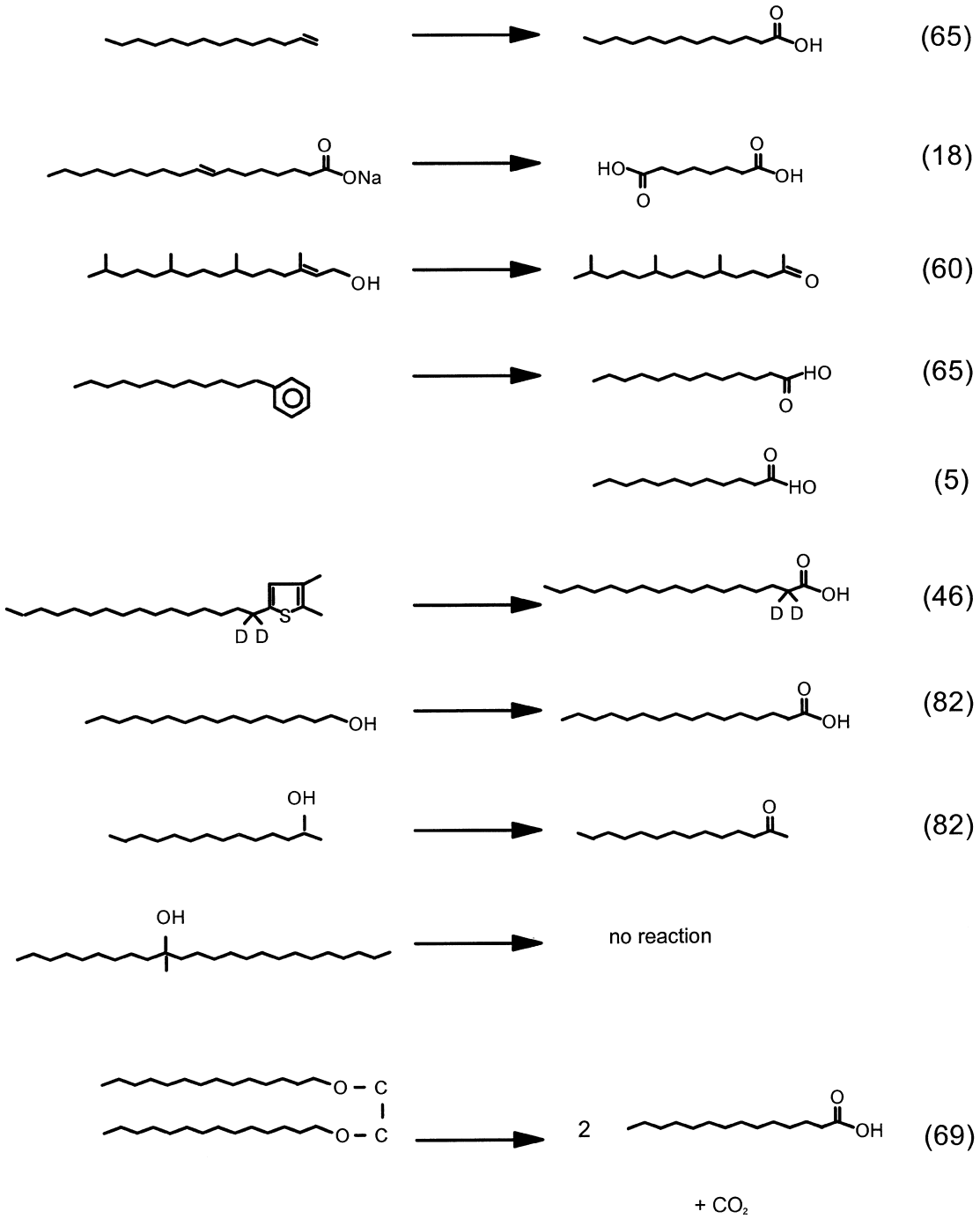

Fig. 1. RuO_4-oxidation products of model compounds. Numbers in brackets indicate % yields.

compounds were oxidized, whereby aromatic rings are completely oxidized (presumably to CO_2) resulting in an acid possessing one carbon atom more than the aliphatic side-chain of the starting aromatic substrate, consistent with previous observations (e.g., [6, 10]). In the case of a double bond, cleavage takes place between the two carbon atoms, where the double bond is positioned and two aliphatic acids are formed or, in the case of a terminal double bond, an aliphatic acid and CO_2. Alcohols yield different products upon RuO_4-oxidation depending on the carbon atom to which the hydroxyl group is attached. Primary alcohols yield acids, secondary alcohols yield ketones and tertiary alcohols do not react at all. Importantly, ether lipids are also cleaved to yield acids. Blanks were performed to detect possible contaminations which were found to consist mostly of the C_{16} and C_{18} fatty acids in a typical, *ca* 2:1 ratio.

The biopolymers isolated from the resistant outer cell-walls of the green microalgae *B. braunii*, so-called PRB-algaenans, have been well studied by NMR and pyrolysis (e.g., [3, 13]). In our study, we treated the algaenans isolated from *Botryococcus braunii* race A (PRB A) and race L (PRB L). This classification in races is based on the presence of specific lipids; race A biosynthesizes high amounts of botryals, whilst race L biosynthesizes high amounts of lycopadienes [3, 13]). RuO_4-oxidation of PRB A resulted in the formation of C_8–C_{14} diacids with the C_{14} α,ω-dicarboxylic acid dominating (Table 1) and the C_8 and C_9 α,ω-dicarboxylic acids also present in relatively high amounts. These products can be correlated to the structure of PRB A, as proposed by Gelin *et al.* [3], with the botryal structure as the monomeric unit (Figure 2). Through biochemical oxidation of the double bonds, the linear polymer may form epoxides, yielding ether bonds which can, thus, cross-link the macromolecule. RuO_4-oxidation of the carbon–carbon bonds at the proposed sites of the functionalities (double bonds and vicinal ether bonds) of the polymer will exactly yield the most abundant products encountered in the chemical degradation mixture. The yields of the respective products are, however, low Tab. 1, despite the fact that they seem to be representative of the biopolymer. In total, the compounds represent *ca* 11%. Reasons for this are unclear although it may be suggested that either the reagent is not effective on the whole macromolecule or that high amounts of non-detectable lower-$M^{+\cdot}$ acids or CO_2 are formed. Despite this low recovery, the experiment still provides both additional evidence for the proposed structure

Table 1. Amounts of compounds released ($mg\,g^{-1}$) by RuO_4-oxidation of biopolymers.

Compounds	*Agave americana*	*B. braunii*
Fatty acids		
C_{18}	1.3	—
C_{26}	1.0	—
C_{27}	1.8	—
C_{28}	2.7	—
C_{30}	3.8	—
C_{32}	3.2	—
α,ω-Dicarboxylic acids		
C_8	—	5
C_9	2.7	13
C_{11}	—	1
C_{12}	—	3
C_{13}	—	11
C_{14}	—	73
C_{27}	1.0	—
C_{28}	1.3	—
C_{29}	2.0	—
C_{30}	2.0	—
C_{31}	2.9	—
C_{32}	2.4	—

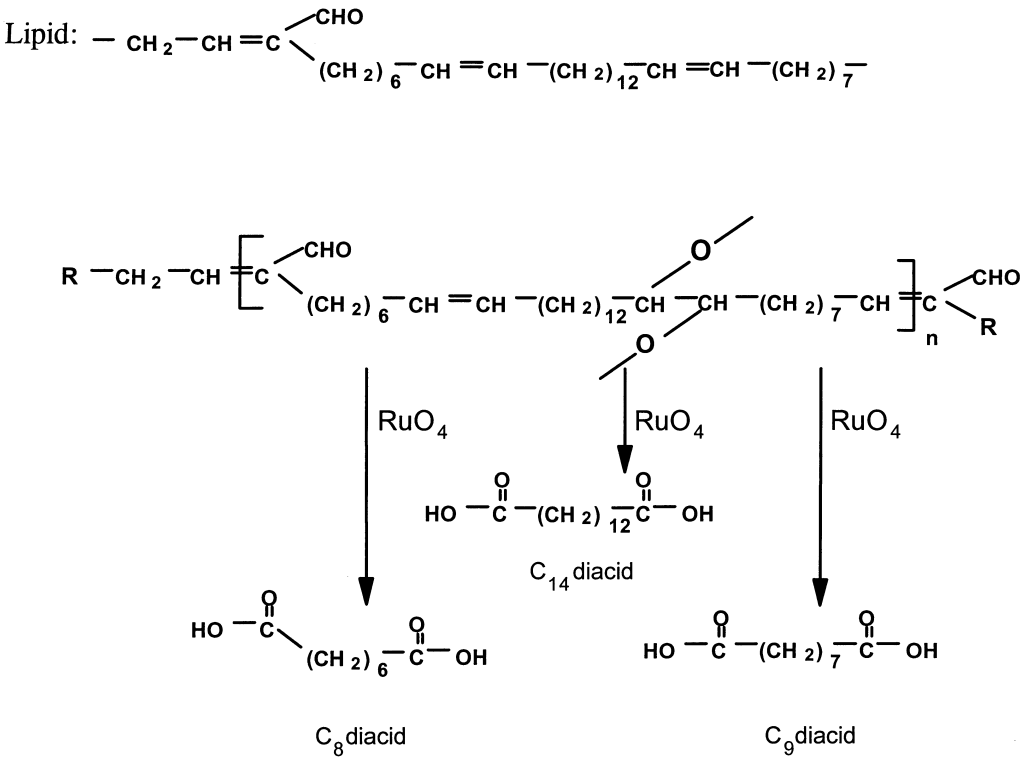

Fig. 2. Rationale for formation of RuO_4-oxidation products formed from PRB A. The presumed building block of the biopolymer is indicated at the top.

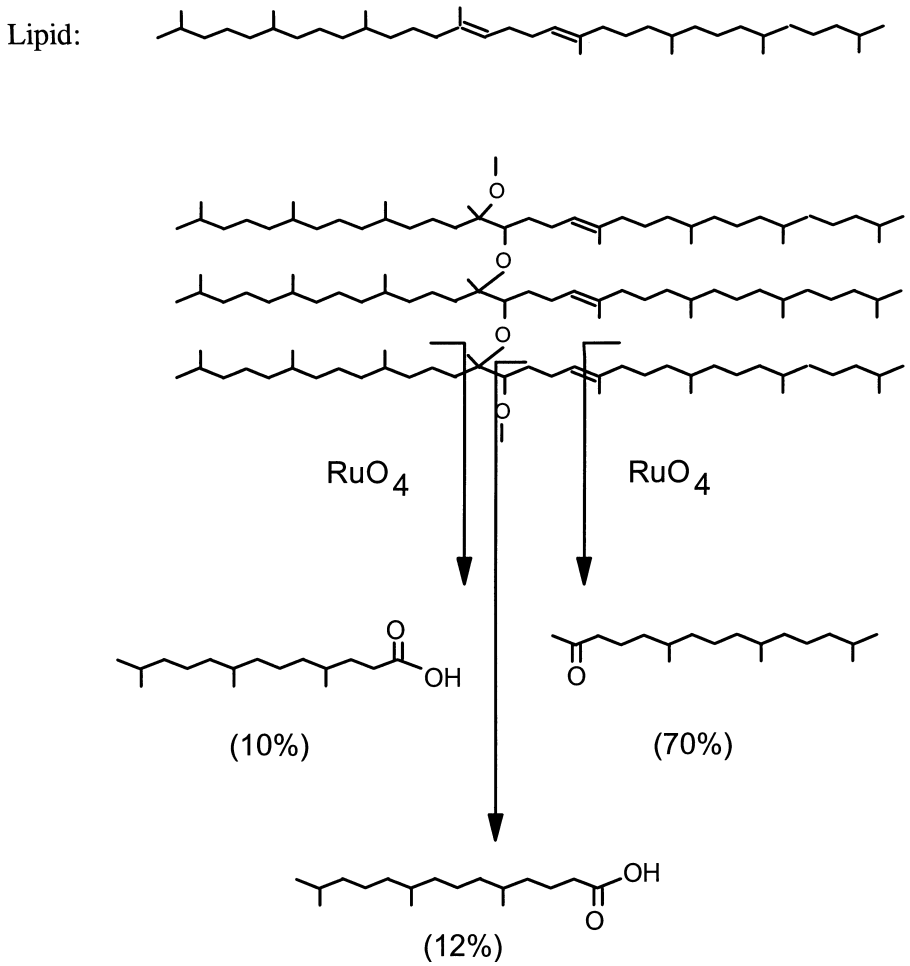

Fig. 3. Rationale for formation of RuO_4-oxidation products formed from PRB L. The presumed building block of the biopolymer is indicated at the top. Numbers in brackets indicate relative amounts of formed products.

of the PRB A and demonstrates the potential of the RuO_4-oxidation method in revealing the building blocks of the biopolymer.

RuO_4-oxidation of the algaenan of *Botryococcus braunii* race L (PRB L) resulted in the formation of only a few products. The major product was identified as a C_{18} isoprenoid ketone. Furthermore, three C_{15}–C_{17} isoprenoid monoacids are present and a series of C_8 to C_{14} α,ω-dicarboxylic acids with a straight-chain carbon skeleton. Gelin *et al.* [3] proposed the structure shown in Fig. 3 for the algaenan PRB L, based on pyrolysis data and with lycopadiene as the building block. Our results are in complete accordance with this proposed structure. Oxidation resulting in carbon–carbon bond cleavage at the functionalities proposed will result in the products found in the reaction mixture Fig. 3. It is interesting to note that small amounts of the same straight-chain C_8 to C_{14} fatty acids are found, as with the RuO_4-oxidation of PRB A. This may indicate the presence of small amounts of PRB A in our PRB L sample. Again, the results provide additional evidence for the proposed structure of PRB L and demonstrates the selectivity of the RuO_4-method.

Oxidation of the algaenan isolated from *T. minimum* resulted in the formation of several specific α,ω-dicarboxylic acids [Fig. 4(a)]. The low M_+ dicarboxylic acids are dominated by C_9–C_{11} α,ω-dicarboxylic acids, whilst the higher $M^{+\cdot}$ α,ω-dicarboxylic acids are mainly the C_{21}, C_{23} and C_{25} homologues. The structure of the algaenan is until now unknown but studies of the ester-bound lipid fraction have revealed relatively high amounts of C_{30}, C_{32} and C_{34} Δ^9 ω-hydroxyacids [14]. Similarly to the algaenans of *B. braunii*, the algaenan of *T. minimum* may have these particular α,ω-dicarboxylic acids as building blocks, whereby either ester or ether bonds link the aforementioned hydroxyacids to each other. Furthermore, the double bonds may be biochemically oxidized to form ether bonds which, in turn, will cross-link the polymer. The proposed structure of the algaenan of *T. minimum* will yield, upon RuO_4 oxidation, those products which are predominantly encountered in the reaction mixture (Figure 5). It thus seems likely that

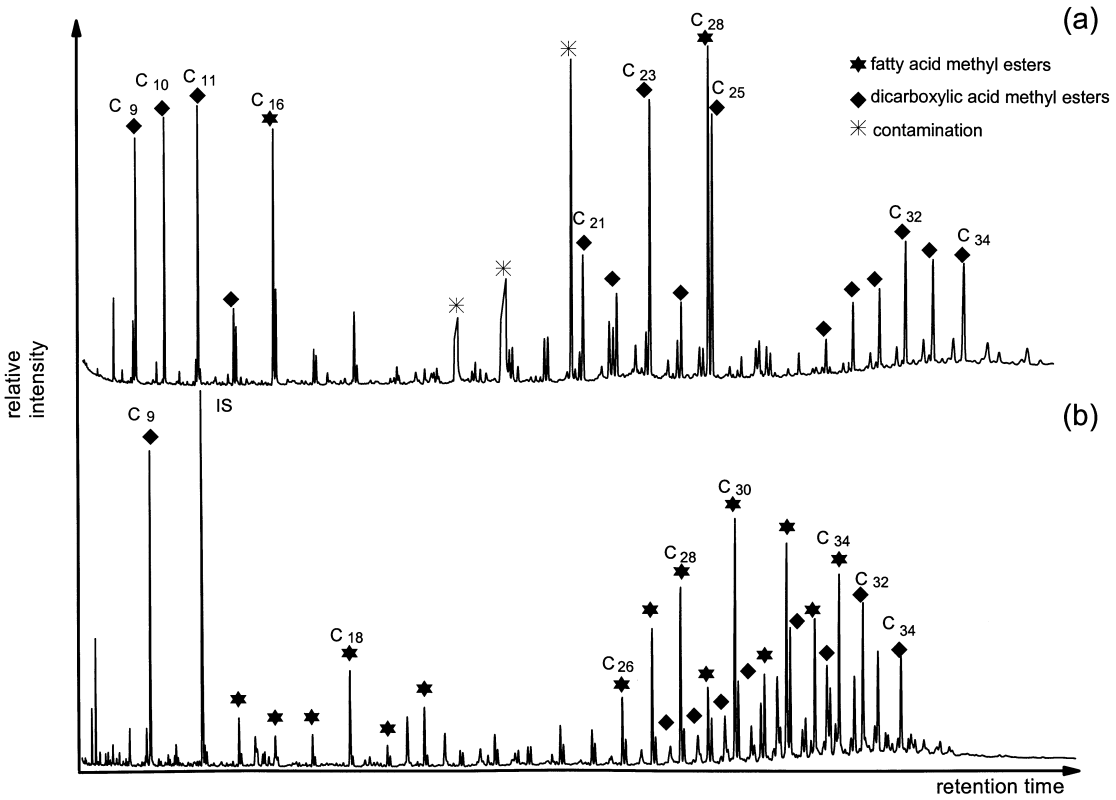

Fig. 4. Gas chromatogram of RuO_4-oxidation products (after derivatisation with diazomethane) of (a) algaenan isolated from *Tetraedron minimum* and (b) cutan isolated from cuticles of *Agave americana*.

C_{30}, C_{32} and C_{34} unsaturated ω-hydroxyacids are the building blocks for the algaenan of *T. minimum*. It should be noted that products like C_{16} and C_{28} monoacids, present in relatively high amounts in the reaction mixture, cannot be derived from the proposed monomeric unit of the polymer. Since these compounds are monoacids, they may have been linked by an ether-bond or by an ester-bond which somehow, possibly due to steric protection, has survived the alkaline hydrolysis. They probably represent terminal units of the polymer. The structure proposed is in agreement with the pyrolysis and ^{13}C NMR results of the algaenan [15]; the pyrolysis products were mainly *n*-alkenes and *n*-alkanes, and signals in the ^{13}C NMR spectra were dominated by those from CH_2 carbon atoms, thus confirming the aliphatic character of the biopolymer.

Cuticular membranes and outer barks of terrestrial plants can also contain insoluble, non-hydrolyzable biopolymers. RuO_4 oxidation of such a biopolymer present in cuticles of *A. americana*, so-called cutan [11], yielded a number of mono- and α,ω-dicarboxylic acids [Fig. 4(b)]. The main α,ω-dicarboxylic acid was C_9, with smaller amounts of C_{26}–C_{34} α,ω-dicarboxylic acids. Furthermore, relatively high amounts of C_{26}–C_{34} fatty acids with a high, even-over-odd carbon-number-predominance were present. Unfortunately, yields (Tab. 1) were, again, not very high and the major compounds detected account for only up to *ca* 3 wt%. This may be predominantly due to the release of short-chain acids and CO_2, which escape our analytical window. Standen [10] also performed RuO_4 oxidation on cutan and obtained a somewhat different product distribution. The α,ω-dicarboxylic acids were also dominated by C_9, but no high amounts of C_{26}–C_{34} di- or fatty acids were detected. Instead, a series of fatty acids with a keto-group at an undetermined position and with relatively long chain-lengths were detected. These differences may be due to different reaction conditions used for RuO_4-oxidation, which can have significant effects on the final product distribution [10].

Recently, McKinney *et al.* [16] proposed a partial structure for cutan based upon ^{13}C NMR spectroscopy and pyrolysis in the presence of tetramethylammonium hydroxide (TMAH). It was suggested that an important structural moiety of cutan is comprised of long-chain fatty acids that are ester bound to tetrasubstituted benzene rings. These moieties were themselves attached, probably *via* C–C bonds, to an, as yet, undetermined cross-linked polymer. Using our RuO_4-degradation results, it is possible to propose a more detailed structure for a part of the cutan polymer (Fig. 6), though this model must be considered tentative, considering the low yields of oxidation products obtained (Tab. 1). The C_9 α,ω-

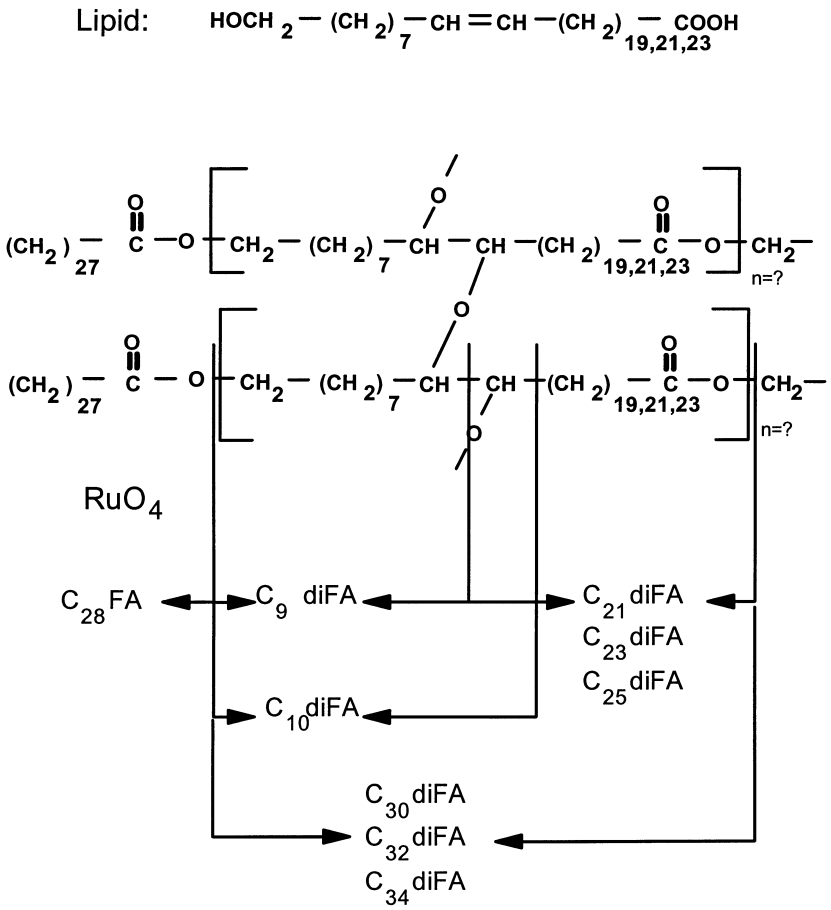

Fig. 5. Rationale for formation of RuO_4-oxidation products of proposed structure of the algaenan isolated from *Tetraedron minimum*. The presumed building block of the biopolymer is indicated at the top.

dicarboxylic acid may be generated from a C_7 alkyl moiety which is covalently bonded at both ends by benzyl moieties. The C_{26}–C_{34} α,ω-dicarboxylic acid may be generated from either C_{24}–C_{32} alkyl moieties covalently bonded to the benzene rings or *via* ester-bonds. Finally, the C_{26}–C_{34} fatty acids may be generated from terminal fatty acids ester-bound to benzene rings. It thus seems likely that cutan is partly built from C_9– and C_{26}–C_{32} alkyl moieties with C_{26}–C_{34} alkyl moieties at terminal positions. This may explain the highly aliphatic nature of the biopolymer as observed by ^{13}C NMR, since this polymer is built from units with relatively long alkyl chains. Furthermore, the pyrolysis products of cutan are dominated by a series of long-chain *n*-alkenes and *n*-alkanes [16, 17] which may be explained by the breakdown of the terminally attached C_{26}–C_{34} alkyl moieties, whilst the high abundance of α,ω-alkadienes in the pyrolysate may be explained by C_{24}–C_{32} alkyl chains which cross-link the aromatic moieties. It is interesting to note, in this respect, that pyrolysis only yields *n*-alkanes and *n*-alkenes up to C_{34}, consistent with the maximum chain-length of the terminal alkyl moieties. Pyrolysis in the presence of TMAH will probably result in the release of trihydroxybenzene moieties, C_{26}–C_{34} fatty acids and C_{26}–C_{34} dicarboxylic acids. The latter products were not observed by McKinney *et al.* [16], which is probably due to their highly polar nature and long retention times on the GC-columns used. Indeed, the authors noted that the amount of long-chain fatty acids released by their degradation method, was severely underestimated due to the poor chromatographic conditions.

EXPERIMENTAL

Samples

Cutan was isolated from the leaf cuticles *A. americana* as described previously [12]. Cutin was removed from the cuticles by saponification and polysaccharides by conc. H_2SO_4. The freshwater algae, *B. braunii* race A and L, were cultured as described previously [3]. *T. minimum* was cultured at room temp. in batches containing basal medium (13.6 g $NaHCO_3$, 4.0 g Na_2CO_3 and 0.5 g K_2HPO_4 in 0.5 l bidist. H_2O) with peptone. Algaenans were isolated as described elsewhere [3]. Briefly, freeze-dried biomass was extensively extracted ultrasonically and ester-bound material was removed by alkaline hydrolysis. Proteins

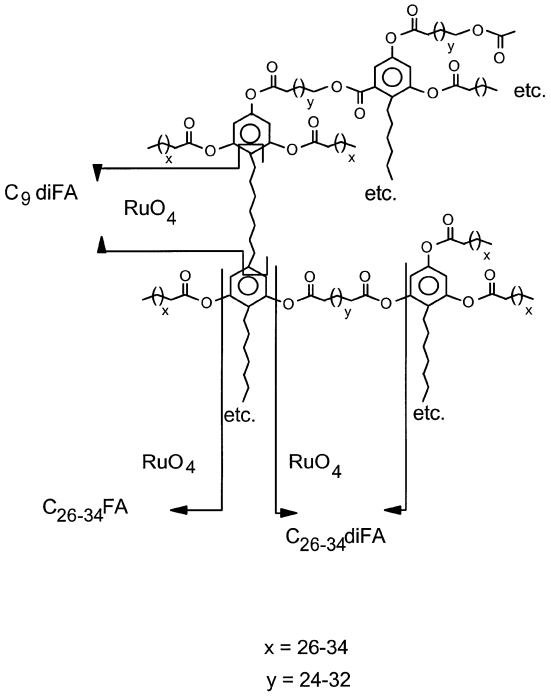

Fig. 6. Rationale for formation of RuO_4-oxidation products of proposed structure of the cutan isolated from *Agave americana*. The presumed bulding block of the biopolymer is indicated at the top.

and polysaccharides were removed using conc. H_2SO_4, yielding a final residue containing predominantly non-hydrolyzable polymeric material.

Ruthenium tetroxide oxidation

Typically, to *ca* 10 mg of biopolymer with a known amount of heptadecane (int. standard) was added 500 mg $NaIO_4$, 10 mg $RuCl_3.xH_2O$, 8 ml $CHCl_3$–MeCN (1:1) and 1 ml of H_2O. The reaction mixt. was stirred in an ultrasonic bath for 1 hr. After stirring for 8 hr. the reaction mixt. was extracted twice with CH_2Cl_2. The CH_2Cl_2-layer was dried ($MgSO_4$) and the solvent removed by rotary evap. Reaction products were methylated using CH_2CN_2 prior to analyses by GC.

GC and GCMS

FID GC was performed using an on-column injector and a fused silica capillary column (25 m × 0.32 mm) coated with CP Sil-5 (film thickness 0.12 μm), using He as carrier gas. Samples (dissolved in EtOAc) were injected at 70° and subsequently the oven was programmed to 320° at 10° min^{-1}, at which it was held for 10 min. GC–MS was carried out under similar conditions at 70 eV with a mass range m/z 40–800 and a cycle time of 1.8 s (resolution 1000).

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