Identification and mode of formation of hopanoid nitriles
in archaeological soils

Pierre Adam,^{a*} Philippe Schaeffer,^a Gaby Schmitt,^a Lucile Bailly,^a Blandine
Courel,^a Margaux Fresnais,^a Carole Fossurier,^{b,c} Michel Rohmer^d

^a *Laboratoire de Biogéochimie Moléculaire, Institut de Chimie de Strasbourg UMR 7177, Université
de Strasbourg-CNRS, E.C.P.M., 25 rue Becquerel, 67200 Strasbourg, France*

^b *Centre archéologique INRAP, 5 rue Holweck, 21000 Dijon, France*

^c *UMR 7268 Anthropologie bio-culturelle, Droit, Ethique & Santé ADES, Aix
Marseille Université, CNRS, EFS*

^d *Institut de Chimie de Strasbourg UMR 7177, CNRS-Université de Strasbourg, 4 rue Blaise Pascal,
F-67000 Strasbourg, France*

* Corresponding author: Tel: +33 (0)3 68 85 28 04; fax: +33 (0)3 68 85 27 99.

E-mail address: padam@unistra.fr (Pierre Adam).

ABSTRACT

In the course of the investigation of the lipid extracts from archaeological soils from two Merovingian tombs (7th Century, Ichtratzheim, France) in an agricultural area, a series of N-containing hopanoids was detected. They were postulated to correspond to C₃₁-C₃₃ hopanoid nitriles on the basis of mass spectrometry. The C₃₂ homologue was unambiguously identified by comparison with a standard obtained by synthesis. The mode of formation of the compounds was investigated using laboratory simulation experiments involving either N-acetyl-aminobacteriohopanetriol as a model compound representative of bacterial hopanoids with polyfunctionalized side chains or C₃₂ hopan-32-al. The experiments led us to propose that the formation of the nitriles from C₃₅ biohopanoid polyols could result from a two step process involving initially an oxidation step leading to the shortening of the functionalized side chain and a second step during which N is incorporated. In the context of the soils containing archaeological wood remains from tombs and found in an agricultural area, oxidants would certainly be available, notably in the form of O₂ diffusing from the surface or as H₂O₂ produced during wood degradation by wood-rotting fungi. Possible N sources could be residual N from the decomposing body within the tomb or manure and fertilizers used for field amendment.

The performed laboratory experiments gave also better insight in the diagenetic processes leading to the side-chain shortening of the hopanoids. In particular, it could be shown that C₃₁ hopanoids also derive from tetrafunctionalized hopanoids upon diagenesis and not solely from pentafunctionalized hopanoids as generally suggested in the literature.

Key words : Hopanoids, Nitriles, Soils, Ammonium, Hopanoid side-chain shortening, Nitrogen incorporation

1. Introduction

Given their ubiquity in the sub-surface, their specific bacterial origin, and their hydrocarbon skeleton prone to isomerization upon dia- and catagenesis, hopanoids have been proven to be useful biomarkers for biogeochemical studies dealing with microbial diversity in sediments (e.g. Talbot and Farrimond, 2007; Sáenz et al., 2011), climatic change (e.g. Cooke et al., 2008a, 2009), geochemical processes (e.g. Birgel and Peckmann, 2008), (palaeo)environmental assessment (e.g. Ourisson and Albrecht, 1992; Peters et al., 2005), early life evolution (e.g. Summons et al., 1999), oil-source or oil-oil correlation studies (e.g. Seifert and Moldowan, 1978; Peters et al., 2005), or maturation studies (e.g. Seifert and Moldowan, 1980; Peters et al., 2005). Regarding hopanoids in recent environments, the number of studies devoted to their occurrence and significance in soils is rather limited (e.g. Ries-Kautt and Albrecht, 1989; Bull et al., 1998; Winckler et al., 2001; Shunthirasingham and Simpson., 2006; Cooke et al., 2008b; Xu et al., 2009; Rethemeyer et al., 2010; Zhu et al., 2011) compared with those dealing with sedimentary settings. Whereas investigation of hopanoids in soils can give clues, notably, to bacterial diversity (e.g. Cooke et al., 2008b; Xu et al., 2009; Rethemeyer et al., 2010; Zhu et al., 2011) or to the relative contribution of bacterial biomass to soil organic matter (e.g. Shunthirasingham and Simpson, 2006), their use as molecular markers in soils is limited by a lack of knowledge concerning their specific diagenetic transformations in soils and their dependence on environmental conditions.

In this context, we came across a series of C₃₁-C₃₃ unknown hopanoids while investigating the lipid extracts from archaeological soils containing wood remains from Merovingian tombs (7th Century, Ichtratzheim, SE France). The compounds (**1-3**; numbers refer to structures in the Appendix; Fig. 1) were shown to bear a nitrile on the side chain, as demonstrated by synthesis of a reference C₃₂ compound (**2**). Furthermore, laboratory

experiments using model hopanoids (4-5) were carried out in order to investigate their possible mode of formation, which led us to propose different mechanistic pathways explaining N incorporation and side chain shortening in biohopanoids that might occur during early diagenesis in oxidative environments.

2. Experimental

2.1. Samples

The samples comprised three archaeological soils containing wood remains and which were collected in 2011 from two Merovingian tombs dating back to the 7th Century in an archaeological site at Ichtratzheim (NE France; Fossurier, 2013).

2.2. Analytical methods

2.2.1. Gas chromatography (GC)

GC was carried out with a Hewlett Packard 6890 gas chromatograph equipped with an on-column injector, flame ionization detector and a HP-5 fused silica column (30 m x 0.32 mm; 0.25 µm film thickness). H₂ was the carrier gas (constant 2.5 ml/ min) and the oven was programmed as follows: 70-200 °C (10 °C/min), 200 °C-300 °C (4 °C min⁻¹), isothermal at 300 °C.

2.2.2. GC-mass spectrometry (GC-MS)

GC-MS was carried out with a Thermo Trace gas chromatograph coupled to a Thermo TSQ Quantum mass spectrometer. The source was at 180 °C. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV. GC separation was performed with a HP5-MS column (30 m x 0.25 mm; 0.1 µm film thickness) using He as carrier gas. The temperature

program was: 70 °C (1 min), 70 °C-200 °C (10 °C min⁻¹), 200 °C-300 °C (4 °C min⁻¹), isothermal at 300 °C.

2.2.3. Nuclear magnetic resonance (NMR)

The NMR spectra of compounds **2** and **5** were recorded on a Bruker Avance I 500 MHz spectrometer (500 MHz for ¹H; 125 MHz for ¹³C). The chemical shifts are reported in ppm relative to tetramethylsilane with the solvents used as internal standard [CHDCl₂, δ¹H 5.32 ppm; CD₂Cl₂, δ¹³C 53.8 ppm (**2**) or pyridine-D₄: δ¹H 7.22, 7.58, 8.74 ppm; pyridine-D₅: δ¹³C 123.9, 135.9, 150.2 ppm (**5**)] and the coupling constants are expressed in Hz.

2.3. Extraction and fractionation of lipid extract from archaeological wood remains

The archaeological samples were extracted using sonication (25 min) with CH₂Cl₂/CH₃OH (1:1, v/v; 2 x 400 ml). The crude extracts obtained from filtration on celite were successively acetylated (Ac₂O/pyridine 1:1 v/v, 60 °C, 2 h) and, after removal of the solvent and excess reagent under reduced pressure, esterified using diazomethane in Et₂O. The derivatized extract was fractionated using liquid chromatography on silica gel, eluting successively with CH₂Cl₂/EtOAc (8:2 v/v) and CH₂Cl₂/CH₃OH (1:1 v/v), leading respectively to a moderately polar fraction analysed using GC-MS, and a polar fraction not further investigated.

2.4. C₃₂ hopanoid nitrile **2** (Fig. 2)

C₃₂ hopan-32-al **4** was first prepared by way of oxidation of 35-aminobacteriohopane-32,33,34-triol **6a** (isolated from a culture of a *Streptomyces* sp.) with H₅IO₆ according to the procedure described by Rohmer et al. (1984). A 30% solution of NH₃ in water (250 µl) and a solution of I₂ (12 mg, 47.4 µmol, 1.43 eq.) in tetrahydrofuran (THF; 250 µl) were successively added under stirring to C₃₂ hopan-32-al **4** (15 mg, 33.0 µmol) in THF (500 µl).

The mixture was stirred at room temperature (2 h), poured into a saturated solution of sodium thiosulfate (10 ml) and the organic mixture extracted with CH₂Cl₂ (2 x 10 ml). The organic extract was evaporated to dryness under vacuum and chromatographed over a silica gel column (cyclohexane/CH₂Cl₂, 1/1, v/v), yielding the C₃₂ hopanoid nitrile **2** (11 mg, 24.4 μmol, 74%).

Compound 2. GC-MS (EI, 70 eV, see also Fig. 4b) *m/z* (relative intensity) 451 (M⁺, 4%), 436(7), 369(14), 230(100), 202(10), 191(73), 149(15), 95(24). ¹H-NMR (500 MHz; CD₂Cl₂) : 0.73 (s, 3H), 0.80 (s, 3H), 0.83 (s, 3H), 0.88 (s, 3H), 0.960 (d, *J*=6.0 Hz, CH₃-29), 0.965 (s, 6H), 2.24 (ddd, *J*=5.0, 8.5, 17.5 Hz, 1H, -CH₂CN), 2.36 (ddd, *J*=8.5, 8.5, 17.5 Hz, 1H, -CH₂CN). ¹³C-NMR (125 MHz; CD₂Cl₂): 14.9, 16.2, 16.3, 16.8, 17.0, 19.3, 19.3, 19.6, 21.5, 21.9, 23.4, 24.6, 28.1, 30.3, 32.1, 33.7, 33.9, 34.3, 37.0, 38.0, 40.9, 42.0, 42.3, 42.4, 42.7, 44.9, 46.3, 49.9, 51.0, 55.0, 56.7, 120.9.

2.5. *N*-acetyl-35-aminobacteriohopane-32,33,34-triol **5**

35-Aminobacteriohopane-32,33,34-triol tetraacetate **6b** (36 mg; 50.4 μmol) was treated with Amberlyst A26 resin (242 mg) in CH₃OH/Et₂O (14 mL, 1/1; v/v) for 2.5 h (Reed et al., 1981; Neunlist et al., 1985). *N*-acetyl-aminobacteriohopanetriol **5** (28 mg; 47.7 μmol; 95%) was obtained after filtration (cotton + sand) and evaporation of the solvent.

Compound 5. APCI-MS *m/z* (relative intensity) 588 ([M+H]⁺, 100%), 570 ([M+H-H₂O]⁺, 5%), ¹H-NMR (500 MHz; pyridine-D₅) : 0.71 (s, 3H, CH₃), 0.83 (s, 6H, 2 CH₃), 0.90 (s, 3H, CH₃), 0.97 (s, 3H, CH₃), 0.98 (s, 3H, CH₃), 1.07 (d, *J*=6.0 Hz, 3H, CH₃-29), 2.10 (s, 3H, -COCH₃), 4.11 (m, 3H, -CH-OH), 4.37 (s, 1H, -CH₂-NHAc), 4.46 (s, 1H, -CH₂-NHAc), 6.37 (s, *J*=3.0 Hz, 1H, -OH), 6.49 (d, *J*=3.0 Hz, 1H, -OH), 6.57 (d, *J*=3.0 Hz, 1H, -OH), 8.99 (s, 1H, -NH-). ¹³C-NMR (125 MHz; Pyridine-D₅): 16.4, 16.4, 17.0, 17.1, 19.3, 19.3, 20.8, 21.6, 22.1, 23.3, 23.4, 24.5, 28.3, 31.1, 32.7, 33.7, 33.8, 33.9, 34.3, 37.6, 37.9, 40.8, 42.2, 42.2,

145 42.3, 42.6, 44.2, 44.9, 47.1, 49.9, 51.0, 55.0, 56.7, 74.9, 75.1, 75.8, 172.1.

146 2.6. Laboratory simulation experiments

147 To *N*-acetyl-35-aminobacteriohopane-32,33,34-triol **5** (resp. C₃₂ hopan-32-al **4**) (13 mg; resp.
148 6 mg) in acetonitrile (28 ml) was added iron acetylacetonate (29 mg) and a 10% aqueous
149 solution of ammonia (15 mL). H₂O₂ (30%, 3 ml) was slowly added over 15 min. under
150 stirring under an air atmosphere. After 2 h, H₂O₂ (2 ml) was slowly added over 10 min, and
151 the reaction mixture stirred for 1 h. Water (20 ml) was added and the reaction mixture
152 extracted successively with CH₂Cl₂ (2 x 30 ml) and EtOAc (30 ml) after acidification (pH <
153 2) with a 10% HCl solution and each extract was washed with water. The organic extracts
154 were then combined and the solvent removed under vacuum. The crude reaction mixture was
155 acetylated at 60 °C (2 h, Ac₂O/pyridine 1:1 v/v) and, after removal of the solvent and excess
156 reagent under reduced pressure, the mixture was esterified using diazomethane in Et₂O. In the
157 case of the experiment involving **4**, the crude mixture obtained was analysed using GC-MS
158 without further purification. In the case of the experiment with **5**, the derivatized extract was
159 fractionated by way of liquid chromatography on silica gel, eluting successively with CH₂Cl₂
160 and EtOAc, leading respectively to a moderately polar fraction analysed using GC-MS, and a
161 polar fraction comprising mainly 35-aminobacteriohopane-32,33,34-triol tetraacetate **6b** (12
162 mg) as evidenced from probe MS.

163 3. Results and discussion

164 3.1. Identification of hopanoid nitrile **2** and related compounds

165 GC-MS investigation of the apolar derivatized fraction separated after derivatization
166 from the solvent extract from the three soil samples revealed a predominance of linear lipid
167 series (*n*-alkanes, *n*-alcohols, *n*-acids) along with steroids (sterols, stanols), higher plant

triterpenes and hopanoids (Fig. 3a). Detailed investigation of the distribution of the hopanoids (m/z 191 fragmentogram, Fig. 3b) showed the presence of various oxygenated hopanoids comprising hopanoid ketones, alcohols and acids, as well as uncommon difunctionalized hopanoids tentatively assigned as, respectively, C₃₃ diol **7** based on comparison with published mass spectra (Peiseler and Rohmer, 1991; Watson and Farrimond, 2000; Saito and Suzuki, 2007) and C₃₃ hydroxyacid **8** based on interpretation of the fragmentation (methyl ester and acetate; Fig S1). Such compounds likely represent diagenetic products of bacteriohopanepolyols (e.g. Saito and Suzuki, 2007; Sessions et al., 2013), although compound **8** might also correspond to a biological lipid since unsaturated analogues have been found in *Acetobacter aceti* ssp. *xylinum* (Peiseler and Rohmer., 1991). In addition to these oxygenated hopanoids, novel hopanoids, characterized by the occurrence in their mass spectra of fragments typical of hopanoids (m/z 191, m/z 369), together with intense even fragments at m/z 216 + $n \times 14$ and of odd molecular ions ($[M^+]$ 437 + $n \times 14$, $n = 0-2$; Fig. 4) could also be detected and were postulated to correspond to C₃₁-C₃₃ hopanoid nitriles (**1-3**).

In order to unambiguously identify this novel series of hopanoids, a standard of the C₃₂ homologue **2** was synthesized using a procedure adapted from that described by Talukdar et al. (2001) for the synthesis of nitriles from aldehydes. Synthesis was based on treatment of the C₃₂ hopanoid aldehyde **4** with molecular I₂ and aqueous NH₃ (Fig. 2), leading to nitrile **2**. The synthetic standard coeluted on GC (HP5-MS column) with the geochemical compound. In addition, the two compounds had superimposable mass spectra, confirming the identification of **2**.

3.2. Origin of hopanoid nitriles – general procedure of laboratory simulation experiments

The formation of hopanoid nitriles from biohopanoid polyols could result from a two step process comprising (i) oxidation leading to shortening of the functionalized side chain and formation of intermediate aldehydes such as **4**, able to further incorporate N, and (ii) a step in which N is incorporated. The latter step implies that a relatively abundant source of N must be available. In the context of archaeological soils comprising wood remains from tombs, found in an agricultural area, oxidants required for the first step would certainly be available, notably in the form of O₂ diffusing from the surface or as H₂O₂ produced during wood degradation by wood rotting fungi (Koenigs, 1974). Possible sources of N for the second step could be residual N from the decomposing body in the tomb (Filley et al., 2001), or manure and fertilizers used for field amendment (e.g. Paul and Beauchamp, 1993; Deng et al., 2006). In this respect, Erman et al. (2000) showed that nitriles can be formed from aldehydes using NH₃ and H₂O₂ as reagent. Alternatively, one might also envisage the possible role of other inorganic N species such as NH₂OH which also occur in soil (Bremmer et al., 1980, McLain and Martens, 2005) since a nitrile might also be formed by dehydration of aldoximes originating from the reaction between aldehydes and hydroxylamine (Dewan et al., 2006; Chill and Mebane, 2009).

In order to test this possibility, we performed two laboratory simulation experiments involving either C₃₂ hopan-32-al **4** or *N*-acetyl-35-aminobacteriohopane-32,33,34-triol **5**, a hopanoid with a polyfunctionalized side chain representative of bacterial hopanoids, in the presence of an oxidant (H₂O₂) and NH₃ as a source of N under an air atmosphere. Iron acetylacetonate was added as catalyst to induce formation of hydroperoxyl radicals (HOO•) from H₂O₂, the latter being able to initiate autoxidation (e.g. Pignatello, 1992; Sychev and Isak, 1995; Kwan and Voelker, 2002). CH₃CN was chosen as a solvent since it is miscible with water and compatible with oxidative conditions (Martin and Garrone, 2003; Pavan et al., 2005).

3.2.1 Laboratory simulation experiments involving C₃₂ hopan-32-al **4**

Hopanoid aldehydes such as C₃₂ hopan-32-al **4** are considered geochemical transformation products formed during oxidative degradation of the side chain of bacteriohopanepolyols and have been detected in sediments (e.g. Dastillung et al., 1980) and can be formed during geomimetic autoxidation experiments involving biological hopanoid polyols (Bisseret and Rohmer, 1995; Bisseret et al., 1997). Aldehydes are labile species able to react with a variety of organic and inorganic compounds. The absence of C₃₂ hopan-32-al **4** in the investigated soils does therefore not exclude this compound as a potential reaction intermediate but rather reflects its high reactivity.

In our laboratory experiment, several oxygenated hopanoids were formed from **4** (Fig. 5). They comprised, beside unreacted C₃₂ hopan-32-al **4**, hopanoid acids (C₃₁ - C₃₂), hopanoid ketones (C₂₇, C₂₉), as well as the C₃₁ aldehyde **9** and alcohol **10** resulting from shortening of the side chain. In this respect, it is interesting to note that the C₃₁ hopanol **10** and related C₃₁ acids found in sediments are sometimes interpreted as diagenetic transformation products of pentafunctionalized bacteriohopanepolyols (e.g. Saito and Suzuki, 2007). Our experiment suggests that, depending on environmental conditions, they might also derive from tetrafunctionalized hopanoids via C₃₂ intermediates such as C₃₂ hopan-32-al **4**. The C₃₃ hopanoid methylketone **11** is most likely an artefact resulting from the partial alkylation of C₃₂ hopan-32-al **4** by reaction with CH₂N₂ (Gutsche, 1954). Besides the predominant oxygenated compounds, C₃₁ and C₃₂ nitriles (**1**, **2**) could indeed be observed (Fig. 5), validating our hypothesis of a potential role of C₃₂ hopan-32-al **4** in the formation of C₃₁ and C₃₂ hopanoid nitriles from bio/geohopanoids. A mechanistic scheme explaining the origin of the various hopanoids formed in the experiment involving C₃₂ hopan-32-al **4** is presented in Fig. 6. The different mechanisms proposed for the formation of oxygenated degradation

products are based on those reported by Marteau et al. (2013) for the oxidative degradation of fragrant aldehydes. Concerning the processes leading to the C₃₂ nitrile **2** from C₃₂ hopan-32-al **4**, the mechanisms might involve formation of an intermediate aldimine **12**, which is further oxidized to nitrile **2**. Alternatively, one might also envisage that the formation of the nitriles involves addition of NH₂OH, possibly formed to some extent by oxidation of NH₃ by H₂O₂ (Mantegazza et al., 1999), followed by elimination of water of the formed intermediate aldoxime **13** (Dewan et al., 2006; Chill and Mebane, 2009).

The oxidative degradation, leading to hopanoids with shortened side chains, is most likely induced under the conditions used by way of formation of a radical HOO• formed by reaction of H₂O₂ with Fe³⁺ ions (e.g. Pignatello, 1992; Sychev and Isak, 1995; Kwan and Voelker, 2002). This radical is able to abstract a hydrogen atom from organic compounds, leading to organic radicals and thus initiating autoxidation. In the case of C₃₂ hopan-32-al **4** (Fig. 6), reaction with HOO• might first lead to an acyl radical (Chatgililoglu et al., 1999) by abstraction of the hydrogen atom from the aldehyde functionality. This intermediate radical might either undergo decarbonylation (Chatgililoglu et al., 1999; Marteau et al., 2013) leading to a C₃₁ hopanyl radical which might, notably, react with oxygen and lead, after a reactional sequence (Marteau et al., 2013), to formation of the C₃₁ hopanol **10**. The latter can be further oxidized to the related C₃₁ aldehyde **9**, thereby opening a pathway to the C₃₁ hopanoid nitrile **1** via the C₃₁ aldimine **14** or aldoxime **15** as described for the C₃₂ hopan-32-al **4** (see above) or can lead to formation of the C₃₁ acid.

3.2.2 Laboratory simulation experiments involving *N*-acetyl-35-aminobacteriohopane-32,33,34-triol **5**.

In order to test whether hopanoid nitriles can also be formed from a hopanoid polyol with an extended side chain, we performed a simulation experiment using *N*-acetyl-35-

aminobacteriohopane-32,33,34-triol **5** as a model for biological hopanoids. Due to its very low solubility, being amphiphilic, a homogeneous phase could, however, not be obtained. The experiment was thus performed with a suspension of the starting material. After 3 h, the crude mixture was recovered as described in Section 3.2.1, derivatized and fractionated on a silica gel column, yielding a very low amount of a fraction having a polarity lower than that of the starting material and which was analyzed using GC-MS. The gas chromatogram (Fig. 7) shows that several short chain hopanoids (C₂₇-C₃₂) were formed, comprising hopanoid acids, alcohols (**10**), aldehydes (**4**, **9**) and nitriles (**1**, **2**) which were the same as those formed from C₃₂ hopan-32-al **4**. Their formation might therefore be explained by processes involving C₃₂ hopan-32-al **4** as intermediate (cf. Fig. 6). The mode of formation of C₃₂ hopan-32-al **4** from *N*-acetyl-35-aminobacteriohopane-32,33,34-triol **5** is illustrated in Fig. 8. The process might comprise oxidation of the alcohol at C-34 (Pathway A, Fig. 8), induced by H₂O₂ in the presence of Fe³⁺ ions (Martin and Garrone, 2003), leading to ketone **16**, followed by the cleavage of the C-32/C-33 bond by way of a retro-aldol type mechanism (Peiseler and Rohmer, 1991). Alternatively, cleavage of the C-32/C-33 bond in *N*-acetyl-35-aminobacteriohopane-32,33,34-triol **5** might have proceeded by oxidation of the alcohol at C-33 (not shown) followed by autoxidation of the C-32/C33 hydroxyketo group, as mentioned by Bisserset and Rohmer (1995) to account for the cleavage of the hopanoid side chain by autoxidation.

Besides C₂₇-C₃₂ hopanoids, compounds with C₃₃ and C₃₄ skeletons were also observed (Fig. 7). They comprised the C₃₃ hopanoid hydroxyacid **8** (cf. Section 3.1 and see Fig. S1a, Supplementary data for mass spectrum) and a C₃₃ compound tentatively assigned as hopanoid hydroxynitrile **17** (analyzed as the acetate), based on interpretation of its mass spectrum (Fig. S1b). Its formation (Fig. 8) is postulated to proceed via the related C₃₃ hydroxyaldehyde **18** and hydroxyimine **19** by way of processes similar to those described to account for the

formation of the C₃₂ nitrile **2** (Fig. 6). The intermediate **18** could, however, not be detected in the products formed in the experiment with compound **5**. In this respect, we have not excluded the possibility that compound **20** (Fig. 7, 8) results from addition of CH₂N₂ to C₃₃ hydroxyaldehyde **18**. Based on the mass spectrum of the related acetate (Fig. S2a), it might correspond to either a hydroxyketone or a hydroxyepoxide (Fig. 7, 8).

The formation of intermediate **18** might be explained by side chain cleavage (Fig. 8, Pathway B), affecting an intermediate C₃₅ aldehyde **21** formed by oxidation of the NH₂ group at C-35. Amines are indeed likely to be oxidized by H₂O₂ to their related imines or oximes (e.g. Sudhakar Reddy and Jacobs, 1993; Sudhakar Reddy and Sayari, 1994). The latter can then be hydrolyzed under aqueous conditions to form aldehyde **21**. The C₃₃ aldehyde **18** would then result from cleavage of the intermediate C₃₅ hopanoid aldehyde by way of retro-aldol type reactions (Fig. 8, Pathway C, Peiseler and Rohmer, 1991) or from successive oxidative processes (Fig. 8, Pathway D, cf. Fig. 6 and Marteau et al., 2013), resulting in progressive shortening of the side chain via all the intermediate C₃₄-C₃₂ aldehydes (**22**, **18**, **4**). Finally, oxidation of **22** leads to the hydroxyacid **23** (Fig. 8) which was tentatively assigned on the basis of MS (Fig. S2b, Supplementary data).

If we consider the mechanisms involved in the formation of the hopanoid nitriles based on our laboratory experiments performed on both C₃₂ hopan-32-al **4** (Fig. 4), *N*-acetyl-35-aminobacteriohopane-32,33,34-triol **5** (Fig. 6), and the identification of the reaction intermediates, it appears that the nitriles such as **1-3** might be formed starting not only from *N*-acetyl-35-aminobacteriohopane-32,33,34-triol **5** but also from any other bacterial C₃₅ hopanepolyol. The formation of the C₃₀ nitrile homologue is also expected to occur starting from pentafunctionalized biohopanoids.

3.3. *N* incorporation into biolipids

The N containing geohopanoids identified represent the second example of N-containing geohopanoids. Indeed, there is only a single report of the tentative assignment of N-containing geohopanoids in oils from the Liaohe Basin, NE China (Oldenburg et al., 2004). The reported structures were postulated to correspond to pyridinohopanes **24** and quinolinohopanes **25** on the basis of MS. The laboratory simulation experiments with C₃₂ hopan-32-al **4** and N-acetyl-aminobacteriohopanetriol **5** as model compounds give clues to the processes involved in the geochemical incorporation of N into organic matter, which might possibly also have played a role in the formation of the N containing geohopanoids reported by Oldenburg et al. (2004). In particular, N incorporation might have occurred by reaction of inorganic nucleophilic N species such as NH₃ with hopanoid diagenetic intermediates bearing carbonyl group(s) in the side chain, leading to formation of imines. The easy reaction of NH₃ or amines with carbonyls to form imines under aqueous conditions has been observed by Amrani et al. (2007) in experiments involving various aldehydes and ketones as model compounds, aimed at investigating the possible role of NH₃ or amines in the formation of S cross-linked geomacromolecules. The imines formed as intermediates might then further react, depending on the prevailing diagenetic conditions, to form, notably, (i) nitriles under oxidative conditions (in the case of aldimines), (ii) pyridines or pyrroles by way of cyclization/aromatization (e.g. involving amination of 1,4 or 1-5-dicarbonyls), or (iii) amines by way of reduction of the C=N double bond in reductive settings. The latter have, however, not been reported.

In any case, availability of N is certainly a key factor for the diagenetic formation of N compounds. High amounts of N were most likely available for the formation of hopanoid nitriles in the soil samples and could originate either from the decomposing body within the tomb (Filley et al., 2001), or from manure or ammonium fertilizers used for field amendment (e.g. Paul and Beauchamp, 1993; Deng et al., 2006). In this context, one would expect the

formation of N compounds to be favored in sediments from eutrophic coastal areas and lakes (e.g. van Luijn et al., 1999).

Conclusions

A novel series of C₃₁-C₃₃ nitrogen-containing hopanoids was detected in lipid extracts from archaeological soils from two Merovingian tombs (7th Century, Ichtratzheim, France) located in an agricultural area. They were shown to correspond to hopanoid bearing a terminal nitrile group on the side chain, as demonstrated by synthesis of a C₃₂ reference compound. Furthermore, their possible mode of formation was investigated using laboratory experiments. The latter revealed that hopanoid nitriles can result from N (NH₃) incorporation into either C₃₅ bacteriohopanepolyols (e.g. aminobacteriohopanetriol **6a**) or into hopanoid alteration products (e.g. C₃₂ hopanoid aldehyde **4**). The experiments led us to propose that the formation of the hopanoid nitriles from C₃₅ biohopanoid polyols could result from a two step process. The first step corresponds to the oxidative alteration of the functionalized side chain of biohopanoids, which is certainly favored in soils where various oxidants such as O₂ or H₂O₂ peroxide are available. The N incorporation step which follows depends on the availability of N which, in soils from tombs, might have originated from the decomposing body within the tomb or from manure and fertilizers used for field amendment. The performed laboratory experiments gave also better insight in the processes leading to the diagenetic shortening of the hopanoid side-chain. In particular, it could be shown that C₃₁ hopanoids might also derive from tetrafunctionalized hopanoids whereas these compounds are often interpreted as resulting from the diagenetic transformation of pentafunctionalized bacteriohopanepolyols.

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References

- Amrani, A., Turner, J.W., Ma, Q., Tang, Y., Hatcher, P.G., 2007. Formation of sulfur and nitrogen cross-linked macromolecules under aqueous conditions. *Geochimica et Cosmochimica Acta* 71, 4141-4160.
- Birgel, D., Peckmann, J., 2008. Aerobic methanotrophy at ancient marine methane seeps: A synthesis. *Organic Geochemistry* 39, 1659-1667.
- Bisseret, P., Rohmer, M., 1995. From bio- to geohopanooids: an efficient abiotic passage promoted by oxygen in the presence of cuprous chloride. *Tetrahedron Letters* 36, 7077-7080.
- Bisseret, P., Baron, A., Rodier, C., Neunlist, S., Rohmer, M., 1997. Geomimetic autoxidation of biohopanooids: a route to bis-hopanooids, potential new sedimentary molecular fossils. *Tetrahedron Letters* 38, 3905-3908.
- Bremner, J.M., Blackmer, A.M., Waring, S.A., 1980. Formation of nitrous oxide and dinitrogen by chemical decomposition of hydroxylamine in soils. *Soil Biology & Biochemistry* 12, 263-269.

382 Bull, I.D., van Bergen, P.F., Poulton, P.R., Evershed, R.P., 1998. Organic geochemical
 383 studies of soils from the Rothamsted Classical Experiments-II, soils from the hoosfield spring
 384 barley experiment treated with different quantities of manure. *Organic Geochemistry* 28, 11-
 385 26.

386 Chatgililoglu, C., Crich, D., Komatsu, M., Ryu, I., 1999. Chemistry of acyl radicals.
 387 *Chemical Reviews* 99, 1991-2069.

388 Chill, S.T., Mebane, R.C., 2009. A facile one-pot conversion of aldehydes into nitriles.
 389 *Synthetic Communications: An International Journal for Rapid Communication of Synthetic*
 390 *Organic Chemistry* 39, 3601-3606.

391 Cooke, M.P., Talbot, H.M., Wagner, T., 2008a. Tracking soil organic carbon transport to
 392 continental margin sediments using soil-specific hopanoid biomarkers: a case study from the
 393 Congo fan (ODP site 1075). *Organic Geochemistry* 39, 965-971.

394 Cooke, M.P., Talbot, H.M., Farrimond, P., 2008b. Bacterial populations recorded in
 395 bacteriohopanepolyol distributions in soils from Northern England. *Organic Geochemistry* 39,
 396 1347-1358.

397 Cooke, M.P., van Dongen, B.E, Talbot, H.M., Semiletov, I., Shakhova, N., Guo, L.,
 398 Gustafsson, Ö., 2009. Bacteriohopanepolyol biomarker composition of organic matter
 399 exported to the Arctic Ocean by seven of the major Arctic rivers. *Organic Geochemistry* 40,
 400 1151-1159.

401 Dastillung, M., Albrecht, P., Ourisson, G., 1980. Aliphatic and polycyclic ketones in
 402 sediments. C₂₇-C₃₅ ketones and aldehydes of the hopanes series. *Journal of Chemical*
 403 *Research (S)*, 166-167.

404 Deng, S.P., Parham, J.A., Hattey, J.A., Babu, D., 2006. Animal manure and anhydrous
 405 ammonia amendment alter microbial carbon use efficiency, microbial biomass, and activities
 406 of dehydrogenase and amidohydrolases in semiarid agroecosystems. *Applied Soil Ecology* 33,
 407 258-268.

408 Dewan, S.K., Singh, R., Kumar, A., 2006. One pot synthesis of nitriles from aldehydes and
 409 hydroxylamine hydrochloride using sodium sulphate (anhyd) and sodium bicarbonate in dry
 410 media under microwave irradiation. *Arkivoc*, 41-44.

411 Erman, M.B., Snow, J.W., Williams, M.J., 2000. A new efficient method for the conversion
 412 of aldehydes into nitriles using ammonia and hydrogen peroxide. *Tetrahedron Letters* 41,
 413 6749-6752.

414 Filley, T.R., Blanchette, R.A., Simpson, E., Fogel, M.L., 2001. Nitrogen cycling by wood
 415 decomposing soft-rot fungi in the « King Midas tomb », Gordion, Turkey. *Proceedings of the*
 416 *National Academy of Sciences of the United States of America* 98, 13346-13350.

417 Fossurier, C., 2013. Habitat âge du Bronze, parcellaire antique et nécropole altomédiévale -
 418 occupation humaine à Ichtratzheim de la fin du Bronze final au début du Xe siècle de notre
 419 ère - Ichtratzheim, Bas-Rhin, ZAC Niederfeld. Excavation Report, INRAP GES, Dijon.

420 Gutsche, C.D., 1954. The reaction of diazomethane and its derivatives with aldehydes and
 421 ketones. *Organic Reactions* 8, 364-429.

422 Koenigs, J.W., 1974. Production of hydrogen peroxide by wood-rotting fungi in wood and its
 423 correlation with weight loss, depolymerization, and pH changes. *Archives of Microbiology*
 424 99, 129-145.

425 Kwan, W.P., Voelker, B.M., 2002. Decomposition of hydrogen peroxide and organic
 426 compounds in the presence of dissolved iron and ferrihydrite. *Environmental Science &*
 427 *Technology* 36, 1467-1476.

428 Mantegazza, M.A., Petrini, G., Spanò, G., Rivetti, F., 1999. Selective oxidations with
 429 hydrogen peroxide and titanium silicalite catalyst. *Journal of Molecular Catalysis A:*
 430 *Chemical* 146, 223-228.

431 Marteau, C., Ruyffelaere, F., Aubry, J.-M., Penverne, C., Favier, D., Nardello-Rataj, V., 2013.
 432 Oxidative degradation of fragrant aldehydes. Autoxidation by molecular oxygen. *Tetrahedron*
 433 69, 2268-2275.

434 Martin, S.E., Garrone, A., 2003. Efficient solvent-free iron(III) catalyzed oxidation of
 435 alcohols by hydrogen peroxide. *Tetrahedron Letters* 44, 549-552.

436 McLain, J.E.T., Martens, D.A., 2005. Nitrous oxide flux from soil amino acid mineralization.
 437 *Soil Biology & Biochemistry* 37, 289-299.

438 Neunlist, S., Holst, O., Rohmer, M., 1985. Prokaryotic triterpenoids. The hopanoids of the
 439 purple non-sulphur bacterium *Rhodomicrobium vannielii*: an aminotriol and its aminoacyl
 440 derivative, *N*-tryptophanyl and *N*-ornithinyl aminotriol. *European Journal of Biochemistry*
 441 147, 561-568.

442 Oldenburg, T.B.P., Huang, H., Donohoe, P., Willsch, H., Larter, S.R., 2004. High molecular
 443 weight aromatic nitrogen and other novel hopanoid-related compounds in crude oils. *Organic*
 444 *Geochemistry* 35, 665-678.

445 Ourisson, G., Albrecht, P., 1992. Hopanoids 1. Geohopanoids: the most abundant natural
 446 products on earth? *Accounts of Chemical Research* 25, 398-402.

447 Paul, J.W., Beauchamp, E.G., 1993. Nitrogen availability for corn in soils amended with urea,
 448 cattle slurry, and solid and composted manures. *Canadian Journal of Soil Science* 73, 253-
 449 266.

450 Pavan, C., Legros, J., Bolm, C., 2005. Iron-catalyzed oxidation of cycloalkanes and
 451 alkylarenes with hydrogen peroxide. *Advanced Synthetic Catalysis* 347, 703-705.

452 Peiseler, B., Rohmer, M., 1991. Prokaryotic triterpenoids. (22*R*,32*R*)-34,35-
 453 dinorbacteriohopane-32,33-diols from *Acetobacter aceti* ssp. *xylinum*: New bacteriohopane
 454 derivatives with shortened side-chain. *Journal of the Chemical Society, Perkin Transaction I*,
 455 2449-2453.

456 Peters, K.E., Walters, C.C., Moldowan, J.M., 2005. *The Biomarker Guide, Second Edition.*
 457 Vol. 2. Biomarkers and Isotopes in Petroleum Exploration and Earth History, Cambridge
 458 University Press, Cambridge, United Kingdom.

459 Pignatello, J.J., 1992. Dark and photoassisted Fe³⁺-catalyzed degradation of chlorophenoxy
 460 herbicides by hydrogen peroxide. *Environmental Science & Technology* 28, 944-951.

461 Reed, L.A. III, Risbood, P.A., Goodman, L., 1981. Catalytic deacetylation of sugars with
 462 strong base ion-exchange resins; an alternative to the Zemplén procedure. *Journal of the*
 463 *Chemical Society, Chemical Communications*, 760-761.

464 Rethemeyer, J., Schubotz, F., Talbot, H.M., Cooke, M.P., Hinrichs, K-U, Mollenhauer, G.,
 465 2010. Distribution of polar membrane lipids in permafrost soils and sediments of a small
 466 Arctic catchment. *Organic Geochemistry* 41, 1130-1145.

467 Ries-Kautt, M., Albrecht, P., 1989. Hopane-derived triterpenoids in soils. *Chemical Geology*
 468 76, 143-151.

469 Rohmer, M., Bouvier-Navé, P., Ourisson, G., 1984. Distribution of hopanoid triterpenes in
470 prokaryotes. *Journal of General Microbiology* 130, 1137-1150.

471 Sáenz, J.P., Eglinton, T.I., Summons, R.E., 2011. Abundance and structural diversity of
472 bacteriohopanepolyols in suspended particulate matter along a river to ocean transect. *Organic*
473 *Geochemistry* 42, 774-780.

474 Saito, H., Suzuki, N., 2007. Distributions and sources of hopanes, hopanoic acids and
475 hopanols in Miocene to recent sediments from ODP Leg 190, Nankai Trough. *Organic*
476 *Geochemistry* 38, 1715-1728.

477 Seifert, W.K., Moldowan, J.M., 1978. Applications of steranes, terpanes and monoaromatics
478 to the maturation, migration and source of crude oils. *Geochimica et Cosmochimica Acta* 42,
479 77-95.

480 Seifert, W.K., Moldowan, J.M., 1980. The effect of thermal stress on source-rocks. Quality as
481 measured by hopane stereochemistry. In: Douglas A.G., Maxwell J.R. (Eds.), *Advances in*
482 *Organic Geochemistry*. Pergamon Press. Oxford, pp. 710-724.

483 Sessions, A.L., Zhang, L., Welander, P.V., Doughty, D., Summons, R.E., Newman, D.K.,
484 2013. Identification and quantification of polyfunctionalized hopanoids by high temperature
485 gas chromatography-mass spectrometry. *Organic Geochemistry* 56, 120-130.

486 Shunthirasingham, C., Simpson, M.J., 2006. Investigation of bacterial hopanoid inputs to soils
487 from Western Canada. *Applied Geochemistry* 21, 964-976.

488 Sudhakar Reddy, J., Jacobs, P.A., 1993. Selective synthesis of oximes with primary aliphatic
489 amines and hydrogen peroxide in the presence of catalytic quantities of titanium silicalite
490 molecular sieves. *Journal of the Chemical Society, Perkin Transactions I*, 2665-2666.

491 Sudhakar Reddy, J., Sayari, A., 1994. Oxidation of primary amines over vanadium silicalite
 492 molecular sieve, VS-1. *Catalysis Letters* 28, 263-267.

493 Summons, R.E., Jahnke, L.L., Hope, J.M., Logan, G.A., 1999. 2-Methylhopanoids as
 494 biomarkers for cyanobacterial oxygenic photosynthesis. *Nature* 400, 554-557.

495 Sychev, A.Y., Isak, V.G., 1995. Iron compounds and the mechanisms of the homogeneous
 496 catalysis of the activation of O₂ and H₂O₂ and of the oxidation of organic substrates. *Russian*
 497 *Chemical Reviews* 64, 1105-1129.

498 Talbot, H.M., Farrimond, P., 2007. Bacterial populations recorded in diverse sedimentary
 499 biohopanoid distributions. *Organic Geochemistry* 38, 1212-1225.

500 Talukdar, S., Hsu, J.L., Chou, T.C., Fang, J.M., 2001. Direct transformation of aldehydes to
 501 nitriles using iodine in ammonia water. *Tetrahedron letters* 42, 1103-1105.

502 Watson, D.F., Farrimond, P., 2000. Novel polyfunctionalised geohopanoids in a recent
 503 lacustrine sediment (Priest Pot, UK). *Organic Geochemistry* 31, 1247-1252.

504 Winkler, A., Haumaier L., Zech, W., 2001. Variation in hopanoid composition and abundance
 505 in forest soils during litter decomposition and humification. *Organic Geochemistry* 32, 1375-
 506 1385.

507 Van Luijn, F., Boers, P.C.M., Lijklema, L., Sweerts, J.-P.R.A., 1999. Nitrogen fluxes and
 508 processes in sandy and muddy sediments from a shallow eutrophic lake. *Water Research* 33,
 509 33-42.

510 Xu, Y., Cooke, M.P., Talbot, H.M., Simpson, M.J., 2009. Bacteriohopanepolyol signatures of
 511 bacterial populations in Western Canadian soils. *Organic Geochemistry* 40, 79-86.

512 Zhu, C., Talbot, H.M., Wagner, T., Pan, J.M., Pancost, R.D., 2011. Distribution of hopanoids
513 along a land to sea transect: Implications for microbial ecology and the use of hopanoids in
514 environmental studies. *Limnology and Oceanography* 56, 1850-1865.

515

516 **Figure captions**

517 **Fig. 1.** Structures of C₃₁-C₃₃ hopanoid nitriles (**1-3**) identified in archaeological soils
518 containing wood remains from Merovingian tombs (7th Century, Ichtratzheim, France).

519 **Fig. 2.** Synthesis of C₃₂ hopanoid nitrile **2** from 35-aminobacteriohopane-32,33,34-triol **5**.

520 **Fig. 3.** (a) Gas chromatograms (GC-MS) of an apolar fraction from the lipid extract from an
521 archaeological soil containing buried wood (SP79-SO) from a Merovingian tomb (7th
522 Century, Ichtratzheim, France), **X**: contaminant, (b) *m/z* 191 mass fragmentogram showing
523 distribution of hopanoids from the sample. Carboxylic acids were analysed as methyl esters
524 and alcohols as acetates.

525 **Fig. 4.** Mass spectra (EI, 70eV) of (a) C₃₁ (**1**) and (b) C₃₂ (**2**) hopanoid nitriles.

526 **Fig. 5.** Mass fragmentogram (*m/z* 191) showing distribution of hopanoids formed upon
527 treatment of C₃₂ hopan-32-al **4** with NH₃ and H₂O₂. Carboxylic acids were analysed as methyl
528 esters and alcohols as acetates.

529 **Fig. 6.** Mode of formation of hopanoids from treatment of C₃₂ hopan-32-al **4** with NH₃ and
530 H₂O₂. The structures in red were identified in the reaction mixture after degradation. The
531 structures in blue correspond to postulated intermediates.

532 **Fig. 7.** Mass fragmentogram (*m/z* 191) showing distribution of hopanoids from treatment of
533 N-acetyl-35-aminobacteriohopane-32,33,34-triol **5** with NH₃ and H₂O₂. Carboxylic acids
534 were analysed as methyl esters and alcohols as acetates. The hydroxyacid **23** in the fraction is
535 not shown in the Fig. (retention time 61 min).

536 **Fig. 8.** Mode of formation of hopanoids (C₃₂–C₃₅) from treatment of *N*-acetyl-35-
537 aminobacteriohopane-32,33,34-triol **5** with NH₃ and H₂O₂. The structures in red were

538 detected in the reaction mixture after degradation. The structures in blue correspond to
539 postulated intermediates.

Appendix

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig.7

Fig.8

Supplementary Data

Fig. S1. Mass spectra (electron impact, 70eV) of (a) C_{31} hydroxyacid (**8**) and (b) C_{33} hydroxynitrile (**17**) (as methyl esters and/or acetates)

Fig. S2. Mass spectra (electron impact, 70eV) of (a) C_{34} hopanoid (**20**) and (b) C_{34} dihydroxyacid (**27**) (as acetates and methyl esters).