# Structure elucidation of $C_{80}$, $C_{81}$ and $C_{82}$ isoprenoid tetraacids responsible for naphthenate deposition in crude oil production†

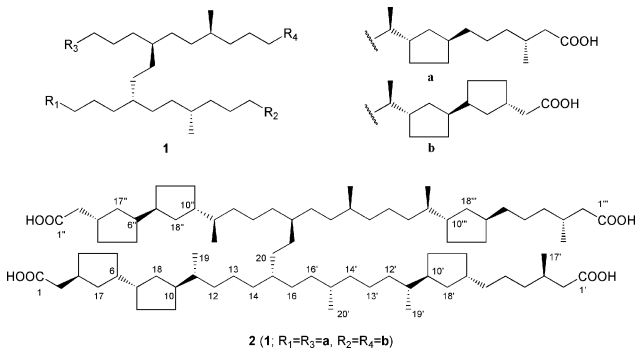
**Bjart F. Lutnaes,*[a] Jostein Krane,[b] Ben E. Smith‡[c] and Steven J. Rowland[c]**

***Received 30th January 2007, Accepted 16th April 2007***
***First published as an Advance Article on the web 10th May 2007***
**DOI: 10.1039/b701462g**

A series of $C_{80}$ isoprenoid 20-bis-16,16′-biphytanyl tetraacids has previously been found to be responsible for calcium naphthenate scaling in crude oil processing. This paper describes the structure elucidation by high-field NMR spectroscopy of the structures of the series of homologous $C_{80}$ tetraacids containing 4–8 five-membered rings. In addition, the structures of methyl-substituted $C_{81}$ and $C_{82}$ analogues containing 7 and 8 five-membered rings have been determined for the first time. The biosynthetic implications are discussed.

## Introduction

In a previous communication, we described the first structure elucidation of the $C_{80}$ isoprenoid 20-bis-16,16′-biphytanyl tetraacids, which are responsible for the formation of naphthenate deposits in crude oil production.[1] The tetraacids were assigned the general structure **1**, Scheme 1, with the side groups **a** and **b** present in various amounts to give a mixture of tetraacids with 4, 5 and 6 rings. The main tetraacid, that contained 6 rings was assigned the structure **2** (**1**; $R_1 = R_3 =$ **a**, $R_2 = R_4 =$ **b**), but other combinations of the side groups, giving other regioisomers could not be ruled out.




**Scheme 1**

Calcium naphthenate formation in production equipment is among the most challenging obstacles to high production regularity for oilfields where acidic crude oils are produced and water breakthrough has occurred.[2] Therefore, offshore oilfields are particularly affected. The solution today is to add chemical inhibitors to the well stream to prevent calcium naphthenate deposition. Still, production shut-downs are required for manual cleaning of the oil–water separators and other affected production equipment.

The presence of similar $C_{40}$ isoprenoid hydrocarbons, in petroleum and crude oils is well documented,[3] as is shorter-chain degradation products of these.[4]

However, until the structure determination of the tetraacids **1** from naphthenate deposits,[1] similar $C_{80}$ isoprenoid structures had only been reported as cell-wall lipids in the thermophilic archaea *Methanothermus fervidus*,[5] *Pyrococcus horikoshii* OT3,[6,7] *Thermococcus celer*,[7] *T. guaymasensis*[7] and *T. waitopuensis*,[7] although even then without a complete structure assignment. The $C_{80}$ isoprenoid lipids may constitute up to 50% of the total lipid content in the organism.[7] Therefore, and because of the abundance of similar $C_{40}$ isoprenoids in the membrane lipids of Archaea, it was concluded that the $C_{80}$ tetraacids **1** responsible for naphthenate deposition were of archaeal origin.[1]

Recently, the isomeric composition of the tetraacids **1** has been studied in more detail.[8] Separation of isomers of permethylated tetraacids was achieved by both high-temperature GC and preparative HPLC. The HPLC fractions were analysed by ESI–MS, and it was found that in addition to the tetraacids with 4, 5 and 6 five-membered rings, analogues containing 7 and 8 rings were also present. In addition, $C_{81}$ and $C_{82}$ analogues of the tetraacids with 7 and 8 rings were detected for the first time.[8]

The relative stereochemistry was assigned based on coupling constants and NOEs for the rings. The absolute stereochemistry was assigned from the structures of 16,16′-biphytanes found in archaeal lipid membranes, for which the stereochemistry of all chiral carbons have been proven by total synthesis for the acyclic[9] and the tetracyclic[10] compounds (GDGT-0 and GDGT-4).

In this paper, we describe the structure elucidation of the individual $C_{80}$ 20-bis-16,16′-biphytanyl tetraacids containing 4–8 five-membered rings and of the novel $C_{81}$ and $C_{82}$ analogues, using high-field NMR spectroscopy.

[a]*Department of Chemistry, Norwegian University of Science and Technology (NTNU), Trondheim, NO-7491, Norway. E-mail: bjartl@chem.ntnu.no; Fax: +47 7359 4256; Tel: +47 7359 4097*
[b]*Department of Chemical Engineering, NTNU, Trondheim, NO-7491, Norway*
[c]*Petroleum and Environmental Geochemistry Group, University of Plymouth, Drake Circus, Plymouth, UK PL4 8AA, E-mail: srowland@plymouth.ac.uk*
† Electronic supplementary information (ESI) available: ESI MS and NMR spectra. See DOI: 10.1039/b701462g
‡ Present address: Oil Plus Ltd, Hambridge Road, Newbury, Berkshire, UK RG14 5SS.

## Results and discussion

### Structures of $C_{80}$ 20-bis-16,16′-biphytanes

In the preliminary communication, the $C_{80}$ tetraacids were assigned the general structure **1** based on 2D NMR techniques (COSY, ROESY, HSQC, HMSC, HSQC-TOCSY and 1,1-ADEQUATE).[1] The relative stereochemistry of the cyclopentane rings was assigned from the ROESY spectrum and coupling constants.

The major 20-bis-16,16′-biphytane, containing 6 five-membered rings, was illustrated with the structure **2**, which is one of four possible regioisomers, with the rings differently located in the molecule.

The general structure assigned to the hexacyclic compound was confirmed by the NMR data ($^1$H, $^{13}$C, DEPT135, NOESY, multiplicity-edited HSQC, HMBC and H2BC[11]) obtained for the corresponding fraction obtained by preparative HPLC of the tetramethyl esters.[8] The NMR data for the hexacyclic $C_{80}$ isoprenoid tetramethyl ester are shown in Table 1 in the ESI†. Since the amount of material available in the purified fraction was much lower than in the original study of the tetraacid mixture, the 1,1-ADEQUATE technique, which had proved very useful, was replaced by the H2BC technique; H2BC offers the advantage that only coupling to vicinal carbons are observed. Another advantage of this technique is higher sensitivity, as the magnetization is transferred between protons (COSY-HSQC), rather than between carbon atoms as in 1,1-ADEQUATE. The disadvantage is that quaternary carbon atoms cannot be detected, and that the resolution is much lower.

However, as was observed in the spectra of the mixture, there are two sets of signals for most of the carbon atoms, with an intensity ratio of *ca.* 2 : 3, see Fig. 1b. The difference in chemical shifts between the two sets were largest for the carbon atoms around the C10–C11/C10′–C11′ bonds. Three possible reasons were envisaged, i) there are two rotamers with different conformation around the C-10–C-11 bond, ii) there are stereoisomers with inverted stereochemistry probably at C-10 or C-11, and iii) there are regioisomers with the bicyclic end-unit located close to, or away from, the bridge cross-linking the two biphytane moieties.

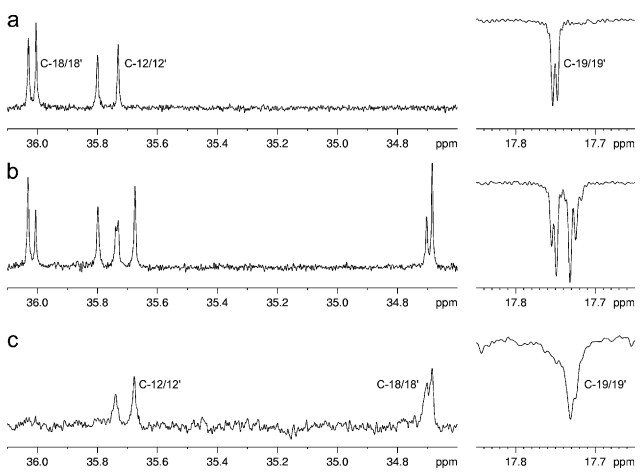
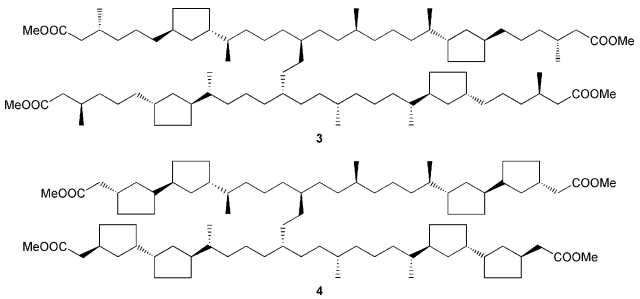



**Fig. 1** 150 MHz DEPT135 spectra ($CDCl_3$, 298 K) of selected regions for a) tetracyclic, b) hexacyclic, and c) octacyclic $C_{80}$ isoprenoid tetramethyl esters. Signals from $CH_2$ are phased positive and $CH_3$ negative.

If the two sets of signals were caused by the existence of two stable conformers, it was expected that the population of these two conformers would be dependent on the temperature.

Therefore, experiments at higher temperature (323 K) were carried out. No changes in the populations of the two signals were observed. The experiments revealed, however, that the temperature change resulted in changes in the $^{13}$C chemical shifts, particularly of the central carbon atoms C-15, C-16 and C-20 of *ca.* 0.3 ppm, indicating changes in the spatial arrangement of the molecules.

The second explanation cannot be probed for the acyclic parts of the molecule without total synthesis of the possible stereoisomers. However, a prerequisite for the third possible explanation to hold true is that the two sets should have equal intensities for the pure tetracyclic compound **3** and the octacyclic compound **4**, Scheme 2, as regioisomers is not possible for these compounds, since all the side chains have 1 or 2 five-membered rings, respectively. If, on the other hand, these compounds showed the same intensity ratio between the two sets of signals as the tetraacid mixture, it would leave only the second explanation.



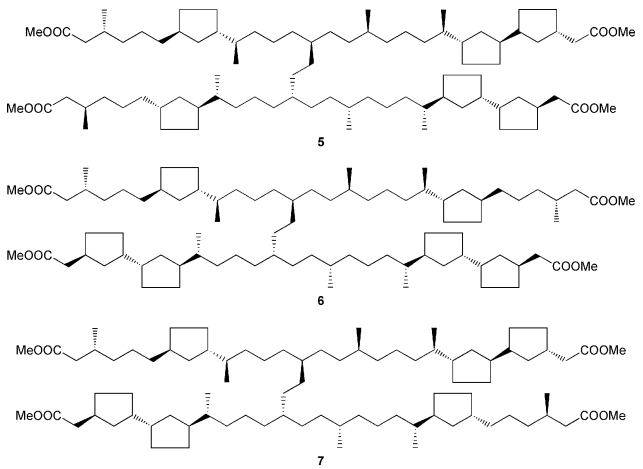

**Scheme 2**

The $^1$H and $^{13}$C chemical shifts of **3** and **4** were assigned from the HSQC spectra combined with the assignment for the hexacyclic compound, Table 1 in the ESI†. This also made it possible to unambiguously assign some signals within the spectrum of the hexacyclic compound, for which overlapping in the HSQC spectrum made differentiation impossible, *e.g.* at C-12, Fig. 1. Fig. 1 also shows the signals due to the methyl groups at *ca.* 17.74 ppm (C-18/18′). While the intensity of the two sets of signals is 2 : 3 for the hexacyclic compound, it is 1 : 1 for **3**, in agreement with the third explanation, that the double set of signals is caused by regioisomerism.

The low S/N ratio in the DEPT135 spectrum of **4** makes integration of the signals inaccurate. However, combined with integrals from the HSQC spectrum, it became clear that the presence of the $C_{81}$ and $C_{82}$ analogues affects the intensities of this signal. The lower intensity of the signal at 35.738 ppm compared to that of the signal at 35.676 ppm identified this signal as belonging to the methylene group that is close to the bridge between the two biphytane moieties, *vide infra.*

In the spectrum of the hexacyclic compound, the intensity of these two signals is approximately 3 : 2, with highest intensity for the signal at 35.739 ppm. This implies that two out of five end groups with the bicyclopentyl moiety are located close to the cross-linking bridge, and thereby that three out of five end groups with only one cyclopentyl moiety are located close to the bridge. The hexacyclic compound is thus better represented with structure **5**, Scheme 3, than with the originally assigned structure **2**. Assuming

a statistical distribution of the four side groups, structure **5** accounts for 34% and structure **2** only 6% of the hexacyclic compound. Compounds **6** and **7** contribute 30% each. The presence of pentacyclic and heptacyclic tetraacids demonstrate that different lengths of the two biphytane moieties are possible, but whether the different length of the biphytane moieties in **6** affects the probability for formation of this compound is not known. Thus, it is also possible that only **2** and **5** are present in the mixture, in a 2 : 3 ratio.




**Scheme 3**

The chemical shifts for the pentacyclic and the heptacyclic $C_{80}$ bisbiphytanes were also assigned from their HSQC spectra, and the integrals of the signals were a linear combination of the signals from **3** and **5**, and **4** and **5**, respectively, as expected.

### Structures of $C_{81}$ and $C_{82}$ 20-bis-16,16′-biphytanes

The presence of minor amounts of $C_{81}$ and $C_{82}$ compounds in the mixture of 20-bis-16,16′-biphytanyl tetraacids was first discovered in the fractions of the $C_{80}$ tetraacid with 7 and 8 rings.[8] Minor amounts were also present in the fractions containing 6 and 5 rings. To the best of our knowledge, such analogues to the archaeal membrane tetraether lipids have not been reported in the literature.

However, a $C_{40}$ 16,16′-biphytane compound containing an extra carbon atom has been reported previously in immature crude oil from the Be′eri seep.[12] The extra carbon atom, found to belong to a methyl group, was located between C-11 and C-11′, but the exact position could not be determined. Unpublished results regarding the presence of an extra methyl group at C-13 in acyclic biphytane from a methanogenic bacterium were also mentioned.[12]

The HPLC fractions of heptacyclic and octacyclic (**4**) tetraacid methyl esters contained $C_{80}$, $C_{81}$ and $C_{82}$ compounds in 1 : 0.75 : 0.15 and 0.75 : 1 : 0.15 ratios, respectively, as judged from ESI MS data. The ESI MS spectrum of the mixture of octacyclic 20-bis-16,16′-biphytanyl tetraacid methyl esters is shown in Fig. 2. Assuming that the extra carbon atom is located at a specific position, the intensity of the signals from the side-chain containing the extra carbon atom should be only approximately 15% of that of side chains without the extra carbon atom.

Indeed, the 800 MHz HSQC spectra of the two fractions containing the $C_{81}$ and $C_{82}$ compounds did only show one set of extra signals, in addition to those expected for the $C_{80}$ compound.

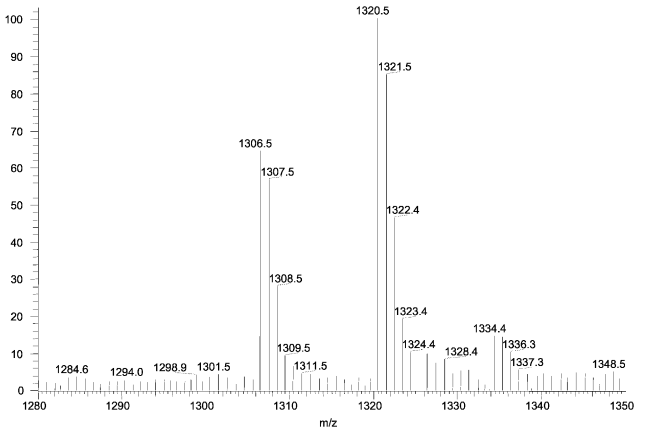



**Fig. 2** ESI MS spectrum of the mixture of $C_{80}$ ($m/z$ 1306 [M + Na]), $C_{81}$ ($m/z$ 1320) and $C_{82}$ ($m/z$ 1334) octacyclic 20-bis-16,16′-biphytanyl tetraacid methyl esters.

The most obvious differences were the presence of two methylene groups at 41.4 ppm and 44.5 ppm and a methyl group at 21.4 ppm.

The parts of the HSQC spectra of tetracyclic compound **3**, hexacyclic compound **5**, the 7-ring fraction and the 8-ring fraction containing the signals due to the methylene groups in the C-13 and C-13′ positions are shown in Fig. 3.

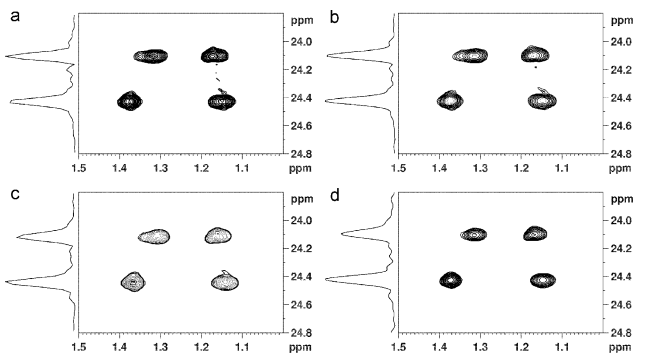



**Fig. 3** Parts of HSQC spectra (800 MHz, $CDCl_3$, 298 K) showing the C-13/C-13′ methylene groups at *ca.* 24.1 and 24.4 ppm, respectively, a) the tetracyclic $C_{80}$ compound **3**; b) the hexacyclic $C_{80}$ compound **5**; c) the heptacyclic $C_{80}$, $C_{81}$, $C_{82}$ mixture, and d) the octacyclic $C_{80}$, $C_{81}$, $C_{82}$ mixture. The sum projections of the signals are shown to the left.

The two last of these contain the $C_{81}$ and $C_{82}$ analogues in addition to the $C_{80}$ compounds. For these compounds, the intensity of the signals at 24.1 ppm, originating from the C-13 methylene group close to the bridge between the two biphytanes, is clearly lower than that of the C-13 methylene group further away from the bridge. The same was observed for the methylene groups in the C-14 position.

These observations support a structural change in the C-12–C-14 region rather than in the C-12′–C-14′ region of the octacyclic compound.

The situation is more complex for the methylene groups in a C-12/C-12′ position. Due to the additional proximity to the cyclopentyl or bicyclopentyl moiety, the carbon atoms in this position give rise to four different $^{13}C$ chemical shifts, as shown in Fig. 1. Because of the small difference in chemical shifts of these four signals, they could not be differentiated by 2D NMR

methods at 600 MHz. At 800 MHz, however, it was possible to distinguish between the various signals from the C-12/C-12′ methylene moieties, as shown in Fig. 4.

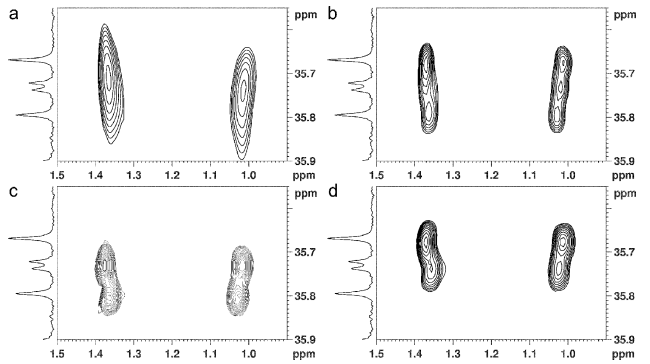



**Fig. 4** Part of HSQC (298 K, $CDCl_3$) spectrum showing the resolution obtained at 800 MHz of the signals from the C-12/C-12′ methylene moieties, a) hexacyclic $C_{80}$ compound at 600 MHz, b) hexacyclic $C_{80}$ compound, c) tetracyclic $C_{80}$ compound, and d) octacyclic $C_{80}$, $C_{81}$, $C_{82}$ mixture, all at 800 MHz. The projections show the DEPT135 spectrum of the hexacyclic $C_{80}$ compound, which was recorded at 150 MHz for $^{13}C$. The signals are assigned in Fig. 1.

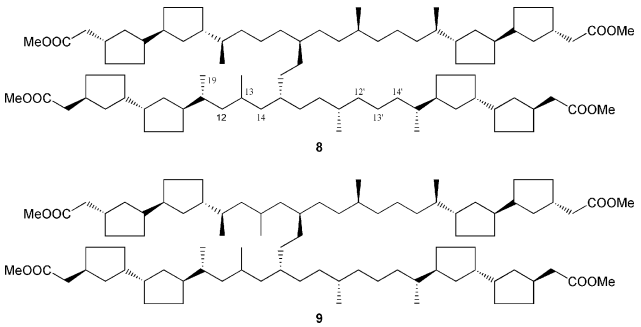
Since there is only one extra set of signals in the HSQC spectrum of the mixture containing both the $C_{81}$ and $C_{82}$ compounds, the extra carbon atom must be located in a chemically equivalent position in the $C_{81}$ and the $C_{82}$ analogues.

Finally, the presence of the extra methyl group was assigned by a 2D HSQC-TOCSY spectrum of the fraction containing the $C_{81}$ and $C_{82}$ analogues of the octacyclic compound (**8** and **9**, Scheme 4). Correlations were observed between the new methyl group, the methine group (C-13) it was attached to, and the downfield-shifted methylene groups C-12 and C-14. The C-12 methylene group also showed further correlations to the C-19 or C-19′ methyl group. Combined with the intensity data above, the additional methyl group was assigned to the C-13 position close to the bridge between the two 16,16′-biphytanes.

### Biosynthetic implications

The current picture for the biosynthesis of $C_{40}$ biphytane moieties in archaeal tetraether lipids is not clear, but the data suggest a radical mechanism, where two digeranylgeranylglyceryl phosphate (DGGGP, **10**, Scheme 5) moieties are coupled in a radical mechanism.[13]

Later, it was shown that inhibition of the central coupling by addition of the allylamine terbinafine leads to accumulation of




**Scheme 4**

glycerophophatidylcaldarchaetidylglycerol (**11**), and not **10**.[14] This may suggest that **10**, or its unsaturated analogue, is the reactive species in the coupling reaction.

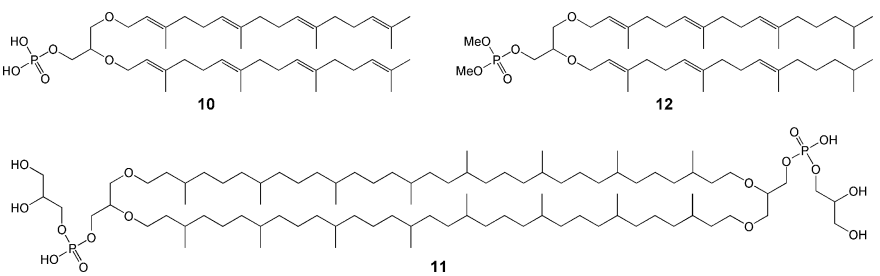
This reduced form of **10**, which contains no double bonds, was also claimed to undergo central coupling to yield the $C_{40}$ biphytane moiety. This result appears to be in contradiction with the finding of Eguchi *et al.*,[13] that 14,15-dihydroDGGGP dimethyl ester (**12**), with a saturated terminal double bond, cannot react to give the unsaturated 72-membered ring compound corresponding to **11**.

Regardless of whether the radical is in a stabilised allylic position, both these reaction paths may explain the cross-linking of two biphytanes to form the $C_{80}$ bisbiphytanes, subsequent to the cyclisation to give the 72-membered ring, a consecutive formation of radicals on the central methyl groups provides an opportunity for a central cross-linking to the $C_{80}$ bisbiphytane determined for the tetraacids.

Other hypotheses that the formation occurs in a reductive coupling, *i.e.* requiring an unsaturated substrate and resulting in a saturated product, seem less likely to explain the formation of the $C_{80}$ compounds, as the unsaturated reaction sites required for the cross-coupling are consumed in the first coupling.

The enzymatic reduction of the double bonds in DPPPG (**10**) to form the saturated isoprenoid chain found in archeal lipid membranes has recently been investigated.[15] It was found that a single enzyme is responsible for the enantioselective reduction, and that both FAD and NADH were required for the activity.

The NMR data obtained here suggest that there is only one epimer of each individual tetraacid present, as there is only one set of signals in each of the $^{13}C$ spectra. This is also supported by the HPLC data, since a single chromatographic peak is obtained for each, whereas epimers might be expected to be separable by chromatography. However, it cannot be entirely ruled out that epimers might have overlapping $^{13}C$ spectral signals and identical chromatographic behaviour.




**Scheme 5**

Indeed, further knowledge about the presence of epimers might provide important information about the origin of the tetraacids. Partial or even complete epimerisation might be expected if the tetraacids were present during the petroleum-formation process. It is possible that epimerisation at some or all of the chiral centres in the acids might occur with increasing geological maturity – as is well known for other chiral isoprenoids. If, on the other hand, the tetraacids are of a more recent biological origin, epimerisation may not yet have occurred and the presence of a single or restricted number of epimers would then be expected.

Thermophilic archaea are known to biosynthesise tetraether lipids with a number of different cyclopentane rings, and the number seems to increase with increasing growth temperature.[16,17] If the present tetraacids are assumed to derive from such ethers, the identification and measurement of the different proportions of 4–8 ring acids, such as that established herein, may allow estimates of past geological temperatures to be made, which is important for geological reconstructions in petroleum prospecting and perhaps for helping to establish the temperature maximum of the 'deep biosphere'.[18,19]

## Experimental

### Samples

Samples of tetraacids from oil fields in West Africa and the Heidrun field on the Norwegian continental shelf used for the elucidation of the general structure for the $C_{80}$ tetraacids[1] were methylated by heating the samples in dioxane after addition of methanol and $BF_3$–diethyl etherate. The yield of tetraacids in the extraction of the naphthenate deposit was approximately 15%. However, optimisation of the yield was not attempted.

The permethylated tetraacids were fractionated according to the number of rings (5 samples with 4–8 rings), in addition to two fractions of the 7-ring and 8-ring compounds, which were enriched in the novel $C_{81}$ and $C_{82}$ analogues by semi-preparative HPLC as described previously.[8] The HPLC-ELSD chromatograms shown in Fig. 13 in the ESI† demonstrates the purity of the fractions analysed by NMR and MS.

### NMR spectroscopy

600 MHz 1D $^1$H and DEPT135 spectra of all samples, as well as NOESY, 1D $^{13}$C, multiplicity-edited HSQC, HMBC and H2BC[11] spectra of the hexacyclic compounds (**2**, **5–7**), and a 2D HSQC-TOCSY (75 ms mixing time) of the fraction containing the $C_{81}$ and $C_{82}$ analogues, were recorded on a Bruker Avance 600 NMR instrument equipped with a TCI CryoProbe. 800 MHz $^1$H and $^1$H–$^{13}$C HSQC spectra of all samples were recorded on a Varian Inova 800 NMR instrument using a TXI probe.

All spectra were recorded at 298 K in $CDCl_3$. Signals were calibrated against residual $CHCl_3$ at 7.27 ppm for $^1$H and 77.2 ppm for $^{13}$C.

Processing and analysis of all spectra were performed using the Bruker TopSpin 1.3 software. All 2D spectra were zero-filled in both dimensions. In addition, forward linear prediction was employed in the indirect dimension.

## Conclusions

Establishing the structures of the tetraacids is important not only from a geological and biological perspective, for helping to establish the palaeotemperatures of petroleum systems and for studying the temperature of the deep biosphere (*vide infra*), but also in an engineering perspective for predicting the phase behaviour of the troublesome naphthenate (tetraacid) salt deposits. Clearly the structural features of the acids may influence their conformations (*cf.* ref. 1) and this in turn may influence the cross-linking behaviour of the acids with metal cations and even the accessibility of the protic head groups during titrimetric estimations, particularly if the conformations are influenced by temperature, which seems likely.

## Acknowledgements

Prof. G. Karlsson and Prof. V. Orekhov are thanked for access to, and assistance with, the 800 MHz instrument at The Swedish NMR Centre at Göteborg University.

## References

1 B. F. Lutnaes, Ø. Brandal, J. Sjöblom and J. Krane, *Org. Biomol. Chem.*, 2006, **4**, 616.
2 T. D. Baugh, K. V. Grande, H. Mediaas, J. E. Vindstad and N. O. Wolf, *Proceedings–SPE 7th Int. Symp. on Oilfield Scale*, Aberdeen, SPE93011, Society of Petroleum Engineers (www.spe.org/elibrary), 2005.
3 J. M. Moldowan and W. K. Seifert, *Science*, 1979, **204**, 169; S. Schouten, M. J. L. Hoefs, M. P. Koopermans, H.-J. Bosch and J. S. Sinninghe Damste, *Org. Geochem.*, 1998, **29**, 1305.
4 J. Albaigés, J. Borbón and W. Walker, II, *Org. Geochem.*, 1985, **8**, 293; B. Chappe, W. Michaelis and P. Albrecht, *Phys. Chem. Earth*, 1980, **12**, 265.
5 H. Morii, T. Eguchi, M. Nishihara, K. Kakinuma, H. König and Y. Koga, *Biochim. Biophys. Acta*, 1998, **1390**, 339.
6 A. Sugai, Y. Masuchi, I. Uda, T. Itoh and Y. H. Itoh, *J. Jpn. Oil Chem. Soc.*, 2000, **49**, 695.
7 A. Sugai, I. Uda, Y. H. Itoh and T. Itoh, *J. Oleo Sci.*, 2004, **53**, 41.
8 B. E. Smith, P. A. Sutton, C. A. Lewis, B. Dunsmore, G. Fowler, J. Krane, B. F. Lutnaes, Ø. Brandal, J. Sjöblom and S. J. Rowland, *J. Sep. Sci.*, 2007, **30**, 375.
9 C. H. Heathcock, B. L. Finkelstein, T. Aoki and C. D. Porter, *Science*, 1985, **229**, 862; C. H. Heathcock, B. L. Finkelstein, E. T. Jarvi, P. A. Reddy and C. R. Hadley, *J. Org. Chem.*, 1988, **53**, 1922.
10 E. Montenegro, B. Gabler, G. Paradies, M. Seemann and G. Helmchem, *Angew. Chem., Int. Ed.*, 2003, **42**, 2419.
11 N. T. Nyberg, J. Ø. Duus and O. W. Sφrensen, *J. Am. Chem. Soc.*, 2005, **127**, 6154.
12 R. Y. P. Burhan, J. M. Trendel, P. Adam, P. Wehrung, P. Albrecht and A. Nissenbaum, *Geochim. Cosmochim. Acta*, 2002, **66**, 4085.
13 T. Eguchi, Y. Nishimura and K. Kakinuma, *Tetrahedron Lett.*, 2003, **44**, 3275.
14 N. Nemonto, Y. Shida, H. Shimada, T. Oshima and A. Yamagishi, *Extremophiles*, 2003, **7**, 235.
15 Y. Nishimura and T. Eguchi, *J. Biochem.*, 2006, **139**, 1073.
16 A. Gliozzi, G. Paoli, M. De Rosa and A. Gambakorta, *Biochim. Biophys. Acta*, 1983, **735**, 234.
17 I. Uda, A. Sugai, Y. H. Itoh and T. Itoh, *Lipids*, 2001, **36**, 103.
18 R. J. Parkes, B. A. Cragg, S. J. Bake, J. M. Getliff, K. Goodman, P. A. Rochelle, J. C. Fry and S. M. Harvey, *Nature*, 1994, **371**, 410.
19 R. J. Parkes, B. A. Cragg, A. J. Weightman, G. Webster, C. J. Newberry, T. G. Ferdelman, J. Kallmeyer, B. B. Jorgensen, I. W. Aiello and J. C. Fry, *Nature*, 2005, **436**, 390.