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Nuclear magnetic resonance of lipid A—the influence of solvents on spin relaxation and spectral quality

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Abstract

Nuclear magnetic resonance (NMR) spectroscopy of lipid A is limited by rapid transversal relaxation and subsequent line broadening caused by the tendency of these glycolipids to form aggregates in all solvents. To examine the influence of solvents on NMR spectra, hexa-acyl lipid A from *Escherichia coli* F515 was investigated. Line widths at half height, longitudinal relaxation times, and transversal relaxation times were measured in different solvents, lipid A concentrations, and temperatures. Chloroform-d, dioxane-d_8, and pyridine-d_5 each mixed with 25% methanol-d_4 as well as sole DMSO-d_6 and 0.1 M triethylamine-d_{15} (TEA-d_{15}) in D_2O caused good spectral resolutions and allowed structure analysis. ROESY and HMBC spectra gave an insight into the influence of transversal relaxation times on spectral quality in two-dimensional spectra. Solvent depending differences of interglycosidic NOEs indicated dissimilarities of the conformations in the interglycosidic linkage and allowed conclusions about the lipid A solution state.

Keywords: NMR; Lipid A; Deuterated solvents; Spin relaxation; Spectral resolution

1. Introduction

Lipopolysaccharides (LPS, endotoxins), located in the outer membrane of Gram-negative bacteria, are potent stimulators of the mammalian immune system. Lipid A, which constitutes its lipid component, is the endotoxically active moiety of LPS. It has recently been shown that lipid A activation of the Toll-like receptor 4 (TLR4) induces mammalian endotoxin signaling (Alexander and Rietschel, 2001; Raetz and Whitfield, 2002). Slight variations in the lipid A acylation pattern, however, cause extensive differences of the effect. Some isolated lipid A show endotoxic activities when tested in human mononuclear cells, whereas others have LPS antagonistic activities or rather no effects (Zähringer et al., 1994; Alexander and Rietschel, 2001). These different stimulations are supposed to be caused by varying lipid A intrinsic conformations (Seydel et al., 2000). The actual focus of interest is the mechanism of lipid A binding to TLR4, which likely involves other proteins like CD14 and MD2 (Raetz and Whitfield, 2002; Henneke and Golenbock, 2002). To elucidate this molecular binding mechanism, a systematic knowledge about configuration, conformation, and aggregation state of different

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lipid A from several bacterial strains is necessary. To investigate such structural features nuclear magnetic resonance (NMR) spectroscopy is a powerful analytical method. The common NMR-methods for such lipid A structure analysis were described (Agrawal et al., 1994) and most recent advances in this field have been reviewed (Zähringer et al., 1999). However, strong tendency of the amphiphilic lipid A to form aggregates restricts the NMR spectroscopic resolutions.

Three approaches were used to circumvent this problem. Aqueous solutions containing sodium dodecyl sulfate were utilized to minimize the micellar size, which, however, only caused minor improvements of the spectral resolution (Strain et al., 1983a,b; Takayama et al., 1983a,b; Oikawa et al., 2000). Therefore in a second approach lipid A was derivatized to yield more soluble products, which allowed an indirect determination of the lipid A configuration (Takayama et al., 1983a,b; Oikawa et al., 2000). In the third approach intact lipid A was dissolved in mixtures of organic solvents like chloroform-d, methanol-d_4, DMSO-d_6, or pyridine-d_5 leading to a reasonable line resolution, which made structure determination possible (Baltzer and Mattsby-Baltzer, 1986; Wang and Hollingsworth, 1995; Zähringer et al., 1999, 2001; Ribeiro et al., 1999). However, no systematic correlation between the used solvent mixtures and the spectral resolution was determined.

Therefore in this paper several solvents are systematically investigated with respect to numerous NMR parameters and conditions for an optimal line resolution of lipid A spectra were determined. Furthermore, variation of lipid A configuration in some solvents are investigated and an explanation for unreliable signal intensity in HMBC as reported by Ribeiro et al. (1999) is elaborated.

2. Materials and methods

2.1. Growth of bacteria, LPS extraction, lipid A preparation, and purification

The *E. coli* F515 strain was grown aerobically in a casein peptone medium using standard conditions and the bacteria were killed, washed, and dried as described earlier (Schmidt et al., 1970; Schlecht, 1975). LPS was extracted by the phenol–chloroform–petroleum ether method and the last precipitate was isolated (Galanos et al., 1969). Crude lipid A was obtained from 250 mg LPS batches by hydrolysis (0.1 M acetate, pH 4.4, 100 °C, 1 h). The hydrolysate was cooled on ice, adjusted to pH 8.5 with aqueous triethylamine (TEA), dialyzed, and freeze-dried. Hexa-acyl lipid A was obtained from fractions of 20.0 mg crude lipid A by preparative layer chromatography on silica gel plates (2.0 mm, 20 cm × 20 cm, silica gel $60F_{254}$, Merck) with chloroform/methanol/water 20:15:3 (v/v/v). The separated hexa-acyl lipid A fraction was resuspended in 2.0 ml distilled water, adjusted to pH 8.5 by addition of 0.36 M TEA, and dialyzed six times for 12 h against distilled water. The retentate was lyophilized to give purified hexa-acyl lipid A.

2.2. Sample preparation for NMR spectroscopy

Purified hexa-acyl lipid A (60.0 mg) was suspended in 30 ml distilled water at 0 °C, dissolved by addition of 0.36 M aqueous TEA, and precipitated adding 0.1 M HCl until pH dropped to ∼2. After centrifugation (2500 × *g*, 15 min, 4 °C) the residue was dissolved in 15 ml chloroform/methanol 4:1 (v/v) and washed five times with distilled water. The solvent was removed in vacuo and the residue was dried over P_4O_{10}. The fully protonated lipid A (2.0 mg each) was dissolved in 0.5 ml of the deuterated solvent and transferred into 5 mm high precision NMR sample tubes (Promochem). Triethylamine-d_{15} (TEA-d_{15}) or α,α,α-tris-(hydoxymethyl)-methylamine-d_8 (Tris-d_8) was added to the D_2O solution and the pH was adjusted adding a few microliter 1.0 M NaOD or 1.0 M DCl in D_2O, respectively. During addition the pH value of the solution was controlled and did not change to inadequate high or low pH values. The total volume of the sample was only slightly increased. All samples were not degassed, but treated in an ultra sonic bath (Bandolin Sanorex RX100) for 15–30 min directly before measurement.

2.3. NMR spectroscopy

All spectra were recorded on an AVANCE DRX 600 spectrometer (Bruker) using the XWINNMR 2.6 software. Irradiation and measurement frequencies were 600.1 MHz for ^1H and 125.1 MHz for ^{13}C and the temperature was adjusted between 280 ± 0.05 K

and 330 ± 0.05 K. Proton spectra were recorded with a ^{1}H-pulse angle of 90° and an acquisition of 16k data points. After zero filling to 32k data points the free induction decay was Fourier transformed to spectra with a spectral range of 6000 Hz. For the determination of spectral resolution the line width at half height was determined for each single spin isochromat in the investigated multiplets. The average spin isochromat line width of half height was calculated for each multiplet. Longitudinal relaxation time constants (T_1) were determined by the inversion recovery method (Vold et al., 1968) and the transversal relaxation times (T_2) were measured with the spin-echo sequence (CPMG; Meiboom and Gill, 1958). All two-dimensional homonuclear spectra were recorded with 2k data points in the F_2-dimension and 256–512 experiments in F_1-dimension (Bax and Davis, 1985; Neuhaus and Williamson, 1989; Hurd, 1990). For TOCSY and ROESY mixing times of 100 and 225 ms were used, respectively (Bax and Davis, 1985; Neuhaus and Williamson, 1989). After zero filling to 512 data-points in F_2-dimension and appropriate sinusoidal multiplication in both dimensions a Fourier transformation led to spectra with a range of 4800 Hz in both dimensions. The heteronuclear two-dimensional ^{1}H/^{13}C-HMQC and ^{1}H/^{13}C-HMBC spectra were measured with 2k data points in the F_2-dimension and 128–384 experiments in F_1-dimension (Willker et al., 1993). The heteronuclear spectra were processed and transformed like the homonuclear spectra and had a final spectral range of 30,000 Hz in the F_1-dimension (^{13}C) and 4800 Hz in the F_2-dimension (^{1}H).

The following internal standards for ^{1}H and ^{13}C were used: chloroform-d (chloroform, ^{1}H: 7.24 ppm, ^{13}C: 77.0 ppm); DMSO-d_6 (DMSO, ^{1}H: 2.49 ppm, ^{13}C: 39.5 ppm); DMF-d_7 (DMF, ^{1}H: 2.74 ppm, ^{13}C: 30.1 ppm); dioxane-d_8 (dioxane, ^{1}H: 3.53 ppm, ^{13}C: 66.6 ppm); pyridine-d_5 (pyridine, ^{1}H: 7.19 ppm [H-2], ^{13}C: 123.5 ppm [C-2]); D_2O (3-(trimethylsilyl)-propanoic acid-d_5, ^{1}H: 0.0 ppm, ^{13}C: 0.0 ppm).

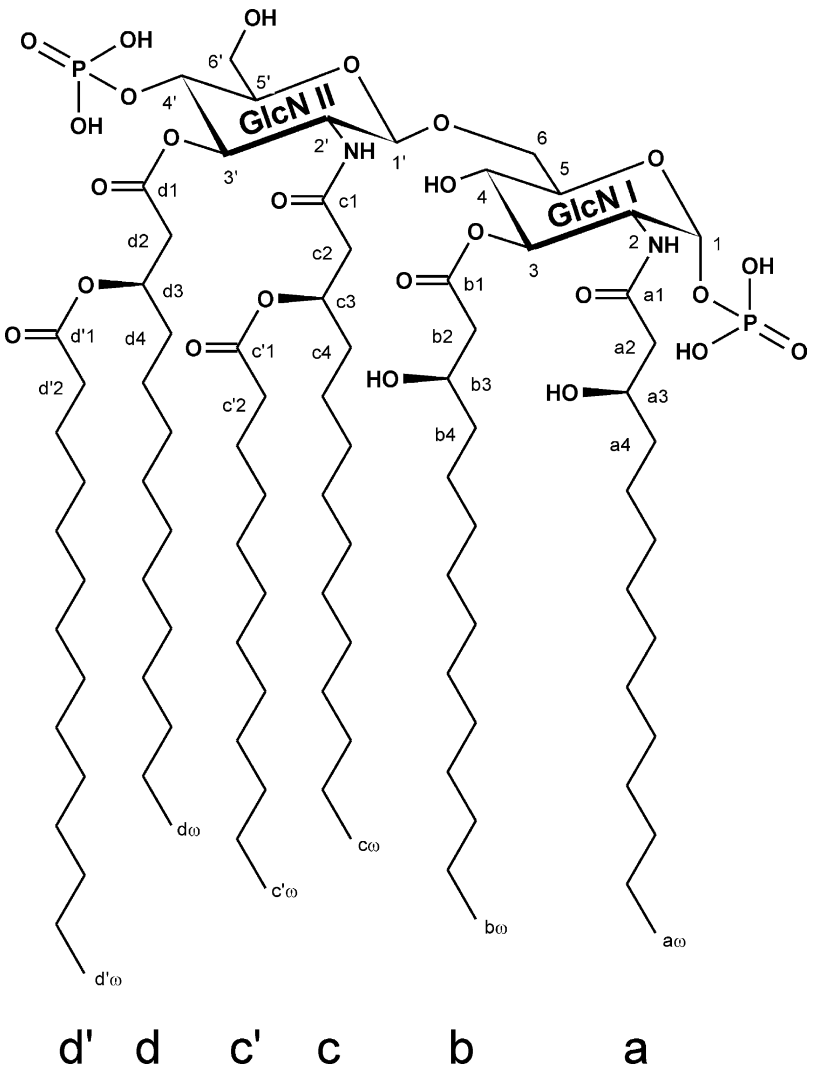
3. Results

Hexa-acyl lipid A from *E. coli* F515 was easy to obtain in 50 mg scale using standard isolation protocols and its primary structure had been unequivocally determined by analysis and synthesis (Imoto et al., 1985; Wang and Hollingsworth, 1995). Therefore it was used as model compound in this study. Its structure is shown in Scheme 1 where several atoms are indicated to assign their signals in the NMR spectra. For line form and relaxation analysis the protons H-1, H-1′ and H-d2, and all methyl group protons in the fatty acids were investigated, as they represent the hydrophilic and hydrophobic regions in the molecule, respectively.

3.1. Solitary organic solvents

From the variety of deuterated organic solvents only chloroform-d and DMSO-d_6 had been used as single solvents for NMR measurements of lipid A (Wang and Hollingsworth, 1995; Zähringer et al., 2001). Whereas chloroform-d caused very poor line resolution, the spectra in DMSO-d_6 showed reasonable line width at half height and good signal separation.

In this study it was found that DMSO-d_6 at 330 K was the best single solvent for lipid A NMR spectra, causing line widths at half height of ∼3.5 Hz of single isochromats and transversal relaxation (T_2) times of ∼0.2 s (Fig. 1, Table 1a). Traces of water in the solution, however, caused a broad signal at ∼3.0 ppm and the DMSO signal at 2.49 ppm overlapped the H-d2 signal (Fig. 1). Spectra of lipid A in DMF-d_7 at 330 K showed slightly poorer resolution and shorter transversal relaxation times. The water now resonated at 3.5 ppm and two solvent signals overlapped some signals of the lipid A spectrum. Lipid A spectra recorded in dioxane-d_8 and pyridine-d_5 at 300 K showed similar line resolution as in DMF-d_7 at 330 K. In dioxane-d_8 the spectra were disturbed by water and dioxane signals in the region of carbohydrate proton signals, whereas in pyridine-d_5 the pyridine signals did not overlap any lipid A signal. In pyridine-d_5 traces of OD^{-}-ions caused slight saponification of esters and amides, which was detected by TLC after 48 h. The detected line widths at half height as well as the transversal and longitudinal relaxation times of the investigated lipid A protons in all these single solvents are summarized in Table 1a. In agreement with earlier reported results (Wang and Hollingsworth, 1995) spectra taken in chloroform-d led to much poorer



Scheme 1.

line resolution. Hexa-acyl lipid A is furthermore only slightly soluble in acetone-d_6, acetonitrile-d_3, 2,2,2-trifluoroethanol-d_3, and methanol-d_4 which were therefore not suitable for NMR measurements of lipid A.

3.2. Mixtures of chloroform-d with protic solvents

Although hexa-acyl lipid A spectra taken in chloroform-d did not show sufficient line resolution, mixtures of chloroform-d with protic solvents were used several times and caused reasonable line widths at half height (Baltzer and Mattsby-Baltzer, 1986; Zähringer et al., 1999; Ribeiro et al., 1999).

Hence chloroform-d/methanol-d_4 was tested with lipid A concentration of 4.0 mg ml^{-1} and caused reasonable line resolution in solvent ratios from 9:1 to 1:1 (v/v) at 300 K. Within these solvent ratios lipid A signals showed ~3.5 Hz line width at half height and the transversal relaxation times were ~0.2 s (Fig. 1, Table 1b). In comparison the longitudinal relaxation times (T_1) were about one magnitude

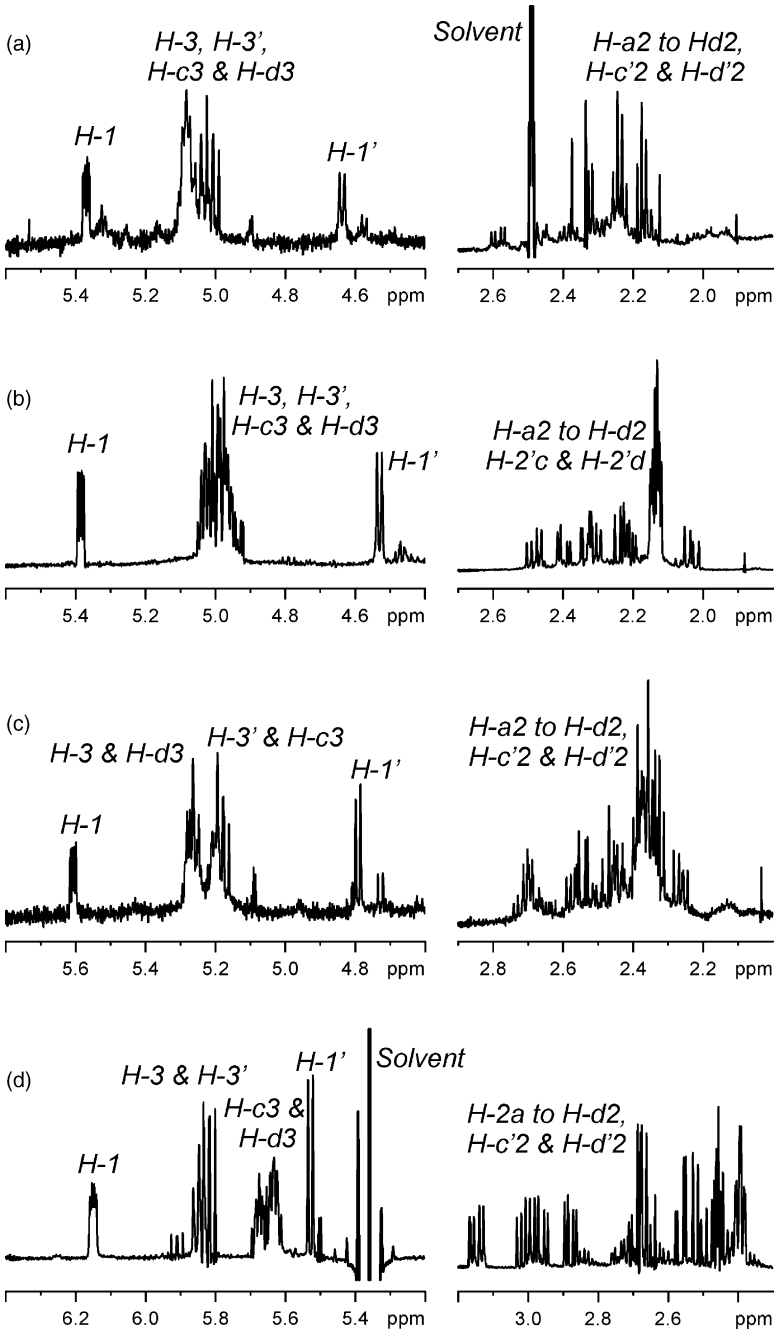


Fig. 1. Parts of the hexa-acyl lipid A ^{1}H-NMR spectra in five selected solvents: (a) DMSO-d_6, 330 K; (b) chloroform-d/methanol-d_4 4:1 (v/v), 280 K; (c) dioxane-d_8/methanol-d_4 4:1 (v/v), 300 K; (d) pyridine-d_5/methanol-d_4 4:1 (v/v), 300 K; (e) D_2O (0.1 M TEA-d_{15}), 310 K. Shown are the protons signals H-1, H-1′, H-3, and H-3′ from the backbone and H-a2 to H-d2, H-c′2, H-d′2; H-c3, and H-d3 from the fatty acids, which represent lipophilic and hydrophilic parts in lipid A, respectively.

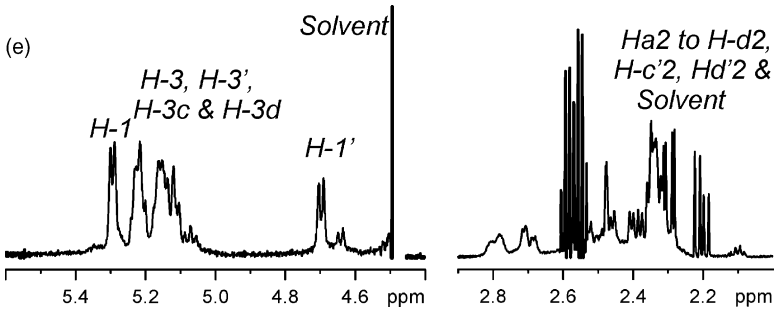


Fig. 1. (*Continued*).

longer. The T_1 times of the investigated protons were in the range of about 2.1–5.0 s (Table 1b). At a chloroform-d/methanol-d_4 ratio of 4:1 (v/v) increasing lipid A concentrations from 0.5 to 16.0 mg ml^{-1} were tested. The concentration rise caused a decrease of the T_2 time and consequently an increase of the line width at half height, whereas the longitudinal relaxation times remained constant. A temperature decrease from 300 to 280 K did not influence the spectral resolution and all lipid A and solvent signals showed nearly constant chemical shifts. The signal of residual HDO in the spectrum, however, shifted like HDO in a methanol/water NMR thermometer and could therefore be "adjusted" to a spectral region where it did not overlap other signals. Traces of DCl in the solution, however, led to slight saponification of esters and amides, which was detected by TLC after 48 h. The measured T_1 times, T_2 times, and line width at half height of lipid A protons in chloroform-d/methanol-d_4 mixtures under varying conditions are summarized in Table 1b.

A mixture of chloroform-d, methanol-d_4, and D_2O was optimized earlier to a ratio of 2:3:1 (v/v/v) for NMR investigations of lipid A (Ribeiro et al., 1999). Therefore the influence of a varying D_2O amount in this solvent system was tested in this study. The variation was kept in the ratio from 2:3:0 (v/v/v) to 2:3:1 (v/v/v) to remain a monophasic mixture (Bligh and Dyer, 1959). While the HDO signal shifted from 3.90 ppm in 2:3:0 (v/v/v) to 4.26 ppm in 2:3:1 (v/v/v), the transversal relaxation times of all investigated nuclei decreased and in accordance the line widths at half height consequently increased (Table 1c).

3.3. Mixtures of aprotic solvents with methanol-d_4

As the addition of 25% (vol.) methanol-d_4 to a lipid A solution in chloroform-d considerably improved the spectral quality, a similar addition to lipid A solutions in other aprotic deuterated solvents was inevitable. In dioxane-d_8 and pyridine-d_5 this addition caused a distinct improvement of the spectral resolution, comparable to the one in chloroform-d/methanol-d_4 (Fig. 1, Table 1d). Remarkable, the signal-to-noise ratio in all of these mixtures was worse than in DMSO-d_6. The determined line width at half heights as well as the longitudinal and transversal relaxation times of the investigated lipid A protons are summarized in Table 1d. An addition of methanol-d_4 to lipid A solutions in DMSO-d_6 or DMF-d_7 did not affect the spectral quality and the solubility of lipid A in acetonitrile-d_3 and acetone-d_6 was not enhanced by addition of methanol-d_4.

3.4. Lipid A in aqueous solutions of amines

Recording lipid A spectra in D_2O seemed to be obvious, as water is the natural solvent, but the amphiphilic glycolipids formed micelles in water, which made the quality of the spectra rather poor. Nevertheless, the spectral resolution could be improved by addition of deuterated Tris-d_8 or TEA-d_{15}. In both cases a 0.1 M concentration of the amine at 310 K and 30 min ultra sonification led to the best spectral resolution in D_2O (Fig. 1, Table 1e). The water signal did not overlap any lipid A signal, but small signals of the amines appeared. The 50 times molar surplus of the amine caused pD values of 10.9 in Tris-d_8 and 11.3

Table 1
Measured line width at half height (median of all single isochromates in a multiplet), longitudinal relaxation time (T_1), and transversal relaxation time (T_2) of lipid A in several solvents

Solvent(s)	Ratio (v/v)	Concentration (mg)	Temperature (K)	Line width (Hz)				T_1 (s)				T_2 (s)			
				H-1	H-1′	H-d2	Me	H-1	H-1′	H-d2	Me	H-1	H-1′	H-d2	Me
Part a															
DMSO-d_6		4.0	330	3.0	3.6	3.2	4.8	2.1	2.8	4.3	2.3	0.10	0.20	0.20	0.60
DMF-d_7		4.0	330	n.d.[a]	n.d.[a]	4.2	5.1	7.2	6.4	3.6	1.0	>0.05	>0.05	0.25	0.60
$CDCl_3$		4.0	300	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	2.8	>0.05	>0.05	>0.05	0.50
Dioxane-d_8		4.0	300	n.d.[a]	3.2	n.d.[b]	2.3	3.0	3.4	4.0	2.1	0.07	0.15	0.15	0.50
Pyridine-d_5		4.0	300	n.d.[a]	6.0	6.0	n.d.[b]	3.6	3.6	5.0	2.1	0.07	0.20	0.20	0.60
Part b															
$CDCl_3/CD_3OD$	4:1	4.0	300	n.d.[a]	3.3	2.9	2.4	3.8	4.2	5.0	2.1	0.07	0.20	0.22	0.75
	3:2	4.0	300	n.d.[a]	3.8	2.9	3.0	4.2	4.5	5.0	2.1	0.07	0.20	0.22	0.65
	2:3	4.0	300	n.d.[a]	n.d.[b]	3.3	3.6	4.7	n.d.[b]	5.0	2.1	0.07	n.d.[b]	0.20	0.65
	4:1	0.5	300	2.7	3.6	2.8	2.1	3.7	3.8	5.0	2.1	n.d.[c]	n.d.[c]	n.d.[c]	0.75
	4:1	2.0	300	n.d.[a]	3.5	2.6	2.0	3.7	3.3	5.0	2.1	0.10	0.20	0.20	0.75
	4:1	8.0	300	n.d.[a]	4.1	3.7	2.2	3.4	3.3	5.0	2.2	0.07	0.17	0.18	0.75
	4:1	16.0	300	n.d.[a]	6.4	5.4	2.3	3.4	3.3	5.0	2.3	0.06	0.16	0.17	0.75
	4:1	4.0	280	2.7	3.4	3.0	2.1	2.6	3.1	4.4	2.9	0.10	0.20	0.17	0.50
	4:1	4.0	290	n.d.[a]	3.2	3.0	2.1	2.9	3.7	4.8	2.3	0.07	0.20	0.22	0.70
Part c															
$CDCl_3/CD_3OD/D_2O$	2:3:1	4.0	300	n.d.[a]	7.0	3.8	4.5	10.0	n.d.[b]	5.3	2.1	>0.05	>0.05	0.08	0.40
Part d															
Dioxane-d_8/CD_3OD	4:1	4.0	300	2.5	2.5	3.4	2.5	2.5	2.9	4.3	2.1	0.15	0.23	0.20	0.70
Pyridine-d_5/CD_3OD	4:1	4.0	300	n.d.[a]	3.1	2.4	n.d.[b]	2.9	3.6	5.2	2.5	0.10	0.23	0.30	0.60
Part e															
D_2O (0.1 M Tris)		4.0	310	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	4.3	4.0	n.d.[a]	3.3	>0.05	>0.05	0.10	0.30
D_2O (0.1 M TEA)		4.0	310	n.d.[a]	7.0	n.d.[a]	n.d.[a]	2.6	1.9	4.3	2.6	0.07	0.15	0.15	0.50

The spectral parameters are listed for the protons H-1, H-1′, H-d2 and the methyl groups.
[a] Not detectable because of small coupling constants.
[b] Not detectable because of signal overlap with other signals.
[c] Not detectable because of low signal-to-noise ratio.

in TEA-d_{15}. Addition of DCl to adjust the pD to ~7 caused a deterioration of the spectral resolution. Despite the high pD value no NMR detectable decomposition of lipid A was observed during 24 h. However, a thin layer chromatogram, which was taken after 48 h, indicated that the hexa-acyl lipid A was slightly decomposed.

3.5. Influence of transversal relaxation time on signal intensity

The short transversal relaxation times caused problems recording two-dimensional lipid A spectra that require long transversal magnetization transfer. This was demonstrated on the signal intensity in ROESY spectra where the initial linear rate approximation of the growing ROE at short mixing times was blotted out by cross relaxation at relative short mixing times of ~150 ms. Fig. 2 shows the ROEs between the proton H-1′ and protons H-5′, H-6a, and H-6b at different mixing times with an optimal signal intensity at ~200 ms.

A more serious problem was the "lack" of signals in HMBC spectra of lipid A that was reported earlier (Ribeiro et al., 1999). In the present study the HMBC spectra of lipid A (16 mg ml^{-1}, chloroform-d/methanol-d_4 4:1 (v/v)) showed detectable ^2J- and ^3J-couplings between carbon atoms and the according protons that possessed transversal relaxation times above ~0.1 s. These protons were mainly located in the fatty acids of the hexa-acyl lipid A. In opposite protons with transversal relaxation times below ~0.1 s showed low or undetectable cross correlation to the according carbon atoms. All protons in the backbone possessed such low T_2 times and therefore did not show any reasonable signals in the

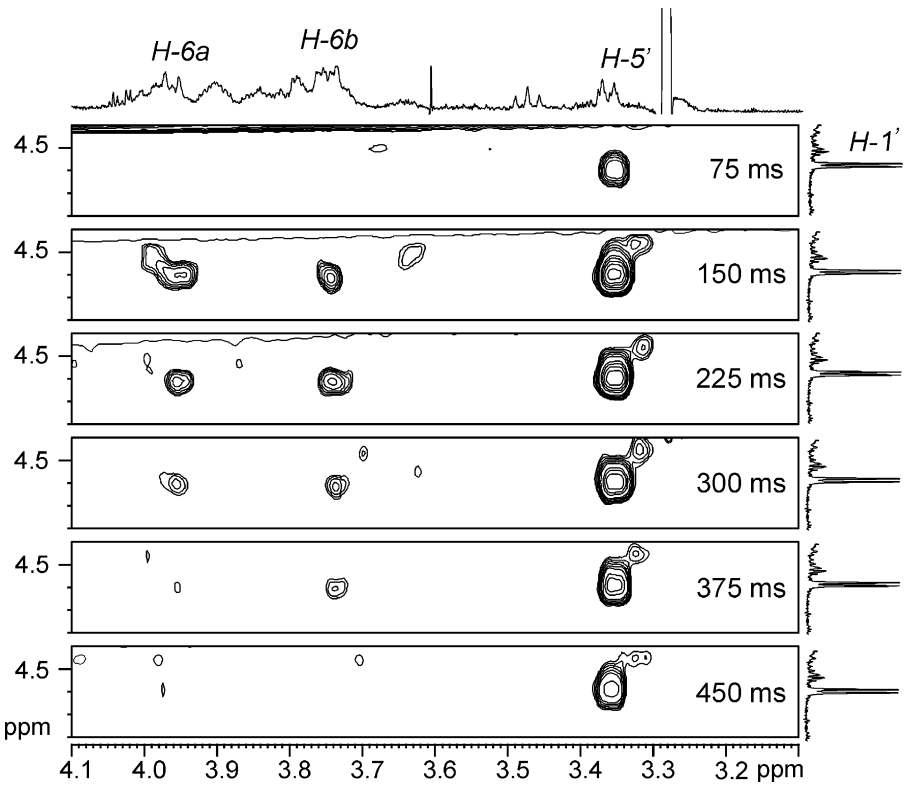


Fig. 2. Parts from ROESY spectra of lipid A in chloroform-d/methanol-d_4 4:1 (v/v) at different mixing times that indicate the correlation between mixing time and signal intensity. Shown are the ROEs between H-1′ and the protons H-5′, H-6a, and H-6b.

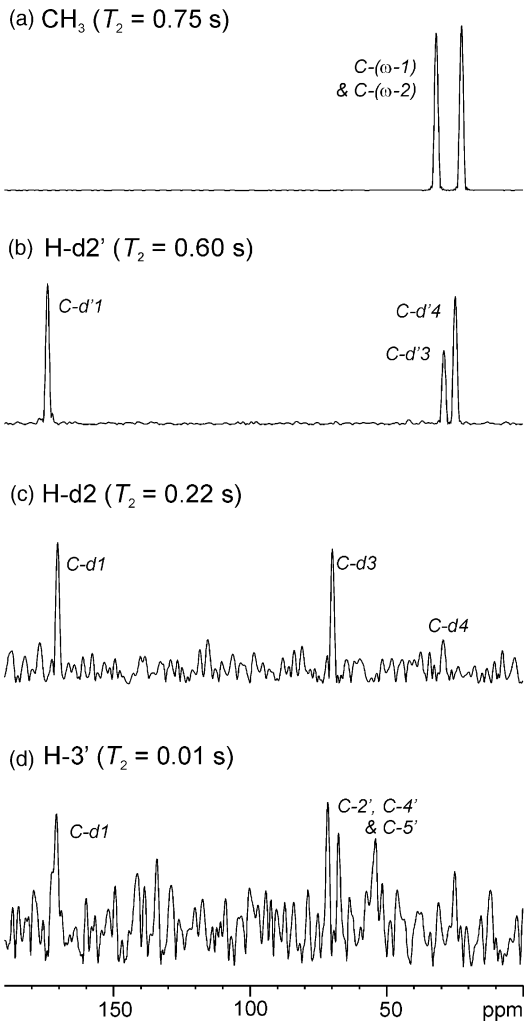


Fig. 3. ^{13}C-Traces of the HMBC spectrum in chloroform-d/methanol-d_4 4:1 (v/v) indicating the ^2J- and ^3J-couplings of selected protons to according carbon atoms. (a) Protons of the terminal methyl groups ($T_2 = 0.75$ s) to carbon atoms $\omega - 1$ and $\omega - 2$ with an excellent *S*/*N* ratio. (b) H-d2′ ($T_2 = 0.60$ s) to carbon atoms C-d1′, C-d3′ and C-d4′ with a good *S*/*N* ratio. (c) H-d2 ($T_2 = 0.22$ s) to carbon atoms C-d1, C-d3 and C-d4 with poor *S*/*N* ratio. (d) H-3′ ($T_2 = 0.01$ s) to carbon atoms C-d1, C-2′, C-4′ and C-5′ with a very poor *S*/*N* ratio.

HMBC spectra. Fig. 3 presents HMBC traces of ^{13}C atoms, which show correlations to some investigated protons. A coherence between the signal intensities and the transverse relaxation time of the affiliated protons is obvious.

3.6. Influence of solvents on secondary structure

The conformation and aggregation state of hexa-acyl lipid A varied distinctly in solvents that caused good spectral resolution. These structural alterations are exemplary indicated by the interglycosidic NOEs of H-1′ to the protons in glucosamine I (Fig. 4). While NOEs from H-1′ to H-6a and H-6b possessed different intensities in all tested solvents, in DMSO-d_6 one of these two NOEs was undetectable. Furthermore, in pyridine-d_5/methanol-d_4 4:1 (v/v) an additional NOE from H-1′ to H-5 was detected, indicating a higher flexibility of lipid A in this solvent.

4. Discussion

All protons in hexa-acyl lipid A possessed transversal relaxation (T_2) times that were about one magnitude shorter than their longitudinal relaxation (T_1) times. As the T_2 times of all lipid A protons were belittled in a similar extend, the triggering effect equably influenced the whole lipid A molecule. Scalar relaxation of the first kind cannot effect the T_2 times in such an intensive and consistent extend, as only a few protons in lipid A showed a chemical exchange in protic solvents. Also scalar relaxation of the second kind cannot be the cardinal effect, because the investigated protons did not show significant couplings to the ^{14}N nuclei in the glucoseamines, which are the only nuclei in the molecule that possess very rapid T_1 relaxation. Furthermore, the portion of proton scalar relaxation caused by scalar coupled nuclei with spin $I = 1/2$ is negligible in case of the longitudinal relaxation. However, the transversal relaxation generated by these coupled nuclei constitutes an enumerable part of the total transversal relaxation. Based on the approximate relaxation times of the coupled nuclei an average of about 5–15% scalar relaxation can be estimated for the observed protons. Therefore the major part of the transversal relaxation in lipid A was caused by dipole–dipole interactions.

The transversal relaxation time of large, slowly tumbling molecules and aggregates is shorter than their longitudinal relaxation time, whereas nuclei of small, rapidly tumbling molecules possess nearly identical transversal and longitudinal relaxation times in low viscosity solvents. Therefore the reciprocal correlation

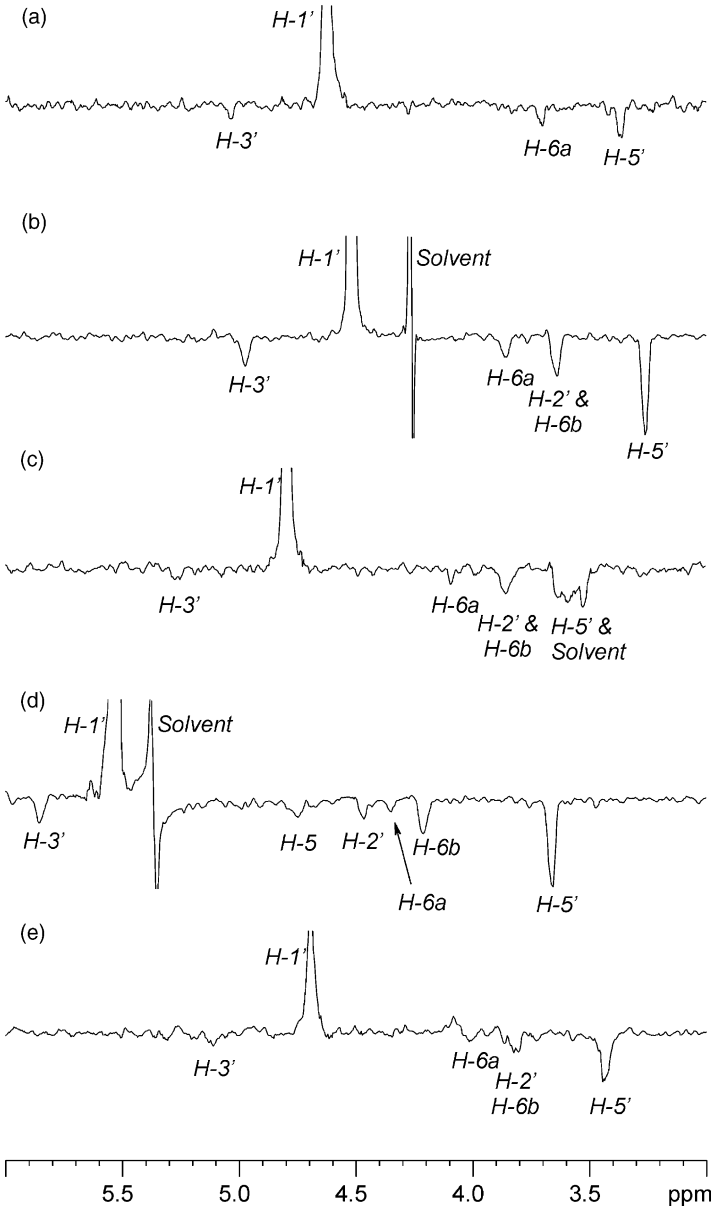


Fig. 4. ^{1}H-Traces of H-1′ from the ROESY spectra in five selected solvents: (a) DMSO-d_6; (b) chloroform-d/methanol-d_4 4:1 (v/v); (c) dioxane-d_8/methanol-d_4 4:1 (v/v); (d) pyridine-d_5/methanol-d_4 4:1 (v/v); (e) D_2O (0.1 M TEA-d_{15}). Mixing time was 225 ms in all spectra. Indicated are the intra- and interglycosidic NOEs of H-1′ to other protons in the lipid A backbone.

between the transversal relaxation and the line width at half height cause inevitably poor line resolution in spectra of large molecules and aggregates. Hexa-acyl lipid A in its fully protonated form, however, has a molecular mass of 1798.39 Da, not leading to any distinct differences between the two relaxation times when the molecule is completely solvated. Therefore the 10-fold difference between them indicated an aggregation of the lipid A in all tested solvents.

The total transversal relaxation times (T_2^*) calculated from the line width at half height and the measured transversal relaxation times (T_2), which were caused by genuine relaxation processes, allow a calculation of the transversal relaxation time portions caused by field inhomogeneities ($T_{2(\Delta B)}$). For that calculation the equation $(T_2^*)^{-1} = (T_2)^{-1} + (T_{2(\Delta B)})^{-1}$ was used (Claridge, 1999). The resulting $T_{2(\Delta B)}$ portions of the lipid A protons varied from 0.1 to 0.4 s depending on the different solvent systems and investigated lipid A protons. These $T_{2(\Delta B)}$ portions were significantly smaller than those of solvent protons, which had all been longer than 2 s. This finding indicates local field inhomogeneities around the lipid A molecules, explainable when lipid A forms aggregates.

These results indicate that lipid A is not completely solved but present as small micelles or aggregates in all tested solvents. Hence an improvement of spectral resolution requires an optimization of lipid A disaggregation.

4.1. Criteria for solvent selection

Five of the tested solvents disaggregated hexa-acyl lipid A quite well and caused reasonable NMR spectra. Single DMSO-d_6 had to be heated to 330 K to get well resolved spectra and the lipid A was therefore thermally affected. Furthermore, HDO and solvent signals partially overlapped the spectrum and the high boiling point of the solvent hindered recovery of the analyzed material. However, in DMSO-d_6 the best signal-to-noise ration of the lipid A proton signals was detected. Furthermore, a slow chemical exchange rate of the amide protons in DMSO-d_6 caused well resolved N–H proton signals suitable for detailed analysis of the lipid A acylation pattern (Janusch et al., 2002). In mixtures of dioxane-d_8/methanol-d_4 4:1 (v/v) the water signal and two solvent signals overlapped several lipid A signals and made structure determination difficult. Nevertheless the spectral resolution of the anomeric proton signals was the best in this solvent. Pyridine-d_5/methanol-d_4 4:1 (v/v) caused no overlapping signals and furthermore spread the lipid A spectrum as it is common for aromatic solvents. However, traces of water led to a high concentration of OD^--ions that catalyzed slow decomposition of lipid A. Chloroform-d/methanol-d_4 4:1 (v/v) was the most sensible unnatural solvent, because all signals were well resolved. No solvent signals overlapped the lipid A spectrum when using an appropriate temperature and recycling of the analyzed material was easy by removal of the solvents in a gentle vacuum.

As water is the natural solvent, the most interesting solvent for lipid A analysis was 0.1 M TEA-d_{15} in D_2O. The substitution of D^+-ions by $DN(C_2D_5)_3^+$ led to good line resolution and allowed structure determination in aqueous solvents, although a high pD caused slow decomposition of lipid A. These findings are in agreement with pH effects on solubility of LPS in H_2O reported by Mukerjee et al. (1999). Such investigations in the natural solvent are of actual interest, as the lipid A molecular binding mechanism to TLR4 has not yet been elucidated. After the importance of soluble receptor proteins CD14 and MD2 in the binding process has recently been shown (Henneke and Golenbock, 2002), the significance of the lipid A aggregation state and the involvement of other molecular species in this process have to be investigated. Therefore NMR of glycolipids in D_2O is a helpful task to discover the secondary lipid A structure and the dynamic process in binding it to TLR4 on mammalian macrophages.

4.2. Signal intensities in two-dimensional spectra

The short transversal relaxation time of hexa-acyl lipid A aggregates caused rapid transversal relaxation during evolution and mixing times in two-dimensional spectra and consequently diminished the signal intensities. COSY and HMQC spectra of lipid A were only slightly effected because of their short evolution periods. However, TOCSY and ROESY spectra were more intensively concerned, because they required mixing times of ~100 and ~250 ms, respectively. As shown in Fig. 2, an exact regulation of the mixing time is necessary to get optimal signal intensities.

Recording sensible HMBC spectra was nearly impossible. Like all $^1H/^{13}C$ heteronuclear correlation spectra they possessed a much lower signal intensity compared to the one in $^1H/^1H$ correlations, which was caused by the low absolute sensitivity of ^{13}C nuclei. The fast transversal relaxation of several lipid A protons during the HMBC evolution period of 60–100 ms furthermore diminished the magnetization to almost

undetectable amounts before the acquisition started. As all protons of the lipid A backbone showed such rapid transversal relaxation, they did not cause any reasonable signals in HMBC spectra and led to the lack of several cross peaks, which is shown in Fig. 3 and was reported earlier (Ribeiro et al., 1999). Therefore interglycosidic linkages in lipid A were superiorly determined by ROESY spectra (Ribeiro et al., 1999). Protons from the fatty acids in lipid A had a much slower transversal relaxation and showed signals of ^{2}J- and ^{3}J-couplings that can be helpful to determine fatty acid substitution pattern.

4.3. The secondary structure

For a comprehensive investigation of lipid A conformation molecular modeling is obligatory. However, NMR spectra provide several valuable and easy derivable information about the secondary structure and the aggregation state. For example, differences between the longitudinal and transversal relaxation times indicated that aggregates were formed in all solvents. Furthermore, the transversal relaxation time variation of some protons in different solvents (Table 1) indicated dissimilar tumbling of different molecular parts. For example, H-1 had a longer T_2 time in DMSO-d_6 than in pyridine-d_5/methanol-d_4 4:1 (v/v), while it is the other way round with the H-1′ and H-d2. These differences indicated dissimilar lipid A aggregation forms, as molecular parts on the aggregate surface possessed a higher flexibility and longer T_2 time than those in the center of the aggregate.

Particular NOEs gave insight into some details of lipid A conformation and aggregation state. In all solvents, for example, the intensity of the H-1′/H-6a-NOE differed from the intensity of the H-1′/H-6b-NOE, indicating a hindered flexibility of the interglycosidic bonds. However, in pyridine-d_5/methanol-d_4 4:1 (v/v) there was an additional interglycosidic NOE from H-1′ to H-5. Conformational prerequisite for the closeness between these nuclei was a higher flexibility of the interglycosidic linkage. This required a separate solvatization of the fatty acid chains at glucosamine I and at glucosamine II, indicating a lipid A solution state in pyridine-d_5/methanol-d_4 4:1 (v/v) that differed fundamentally from them in the other solvents.

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