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# Structure of a polysaccharide from a *Rhizobium* species containing 2-deoxy-β-D-*arabino*-hexuronic acid

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**Abstract**

The structure of the extracellular polysaccharide (EPS) produced by the *Rhizobium* sp. B strain isolated from atypical nodules on alfalfa has been determined using a combination of chemical and physical techniques (methylation analysis, high pH-anion exchange chromatography (HPAEC), mass spectrometry and 1-D and 2-D NMR spectroscopy). As opposed to the EPS from other strains of *Rhizobium*, the EPS from the sp. B strain contains D-Glc together with L-Rha and 2-deoxy-D-*arabino*-hexuronic acid. It is a polymer of a repeating unit having the following structure:

→4)-β-D-Glc*p*-(1→4)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→4)-2-deoxy-β-D-Glc*p*A-(1→

The polysaccharide also contains 0.6 *O*-acetyl groups per sugar which have not been located. 

*Keywords:* Extracellular polysaccharide; *Rhizobium* sp.; Structural analysis; 2-Deoxy-arabino-D-hexuronic acid

## 1. Introduction

A number of extracellular microbial polysaccharides find wide applications due to their rheological properties.[1] For instance, thickening and gelling properties are of great importance in food industry. Xanthan and gellan are among the most widely used polysaccharides in this field. They are produced by *Xanthomonas campestris*[2] and *Pseudomonas elodeae*[3] and their physical properties can be directly related to their structure.[4,5] Despite the availability of these polysaccharides, there is a need to identify and characterize new polysaccharides having specific rheological properties and potential applications. Interesting models for searching such polysaccharides are the bacteria from the *Rhizobiaceae* family. Indeed, these organisms produce a large variety of extracellular polysaccharides such as homopolymers of glucuronic acid[6] and heteropolymers containing either neutral sugars[7] or both neutral monosaccharides and uronic acids.[8–10] One of these polymers is secreted by the *Rhizobium* sp. B strain and forms highly viscous aqueous

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solutions. Despite the potential applications of this polysaccharide, its structure is still unknown. In the present study, we report on the use of a series of chemical analyses to characterize at the structural level this EPS from *Rhizobium* sp. B. The sugar composition of the polymer was shown to be significantly different by comparison to the EPS so far characterized from other *Rhizobium* strains. In particular, we have shown that the EPS from the B strain is made of a repeating unit containing 2-deoxy-D-*arabino*-hexuronic acid.

## 2. Results and discussion

The EPS was isolated from a liquid culture of *Rhizobium* sp. B strain by ethanol precipitation. It was successively purified by dialysis against distilled water and lyophilized. Protein and nucleic acids were not detected in the purified EPS by UV spectrometry. In addition, the elution profile on a DEAE column (HPLC fitted with a TSK DEAE gel) showed only one symmetrical peak indicating that no other polysaccharide was present in the sample.

HPAEC-PAD analysis of the trifluoroacetic acid hydrolysate revealed the presence of two sugars, i.e., Rhamnose and Glucose, in a ratio of 1:2. This was confirmed by GLC–MS (EI) analysis of the trimethylsilylated derivatives obtained from the TFA hydrolysate. Methylation analysis showed the presence of (1→4)-linked Glc, (1→3)-linked Glc and (1→4)-linked Rha, detected, respectively, as 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,4,5-tri-*O*-acetyl-6-deoxy-2,3-di-*O*-methyl-L-mannitol in a ratio of about 1:1:1. No per-*O*-methylated alditol acetates arising from a non-reducing terminal residue or a branch point was detected, indicating that the molecule is linear and has a $\overline{DP}_n$ higher than 100.

The $^1$H NMR spectrum of the native polysaccharide was complex due to partial acylation. A total deacylation was performed by heating the sample for 1 h in NaOD (pD 10) in order to make it more suitable for NMR studies. A content of 0.6 *O*-acetyl groups per sugar was determined by comparison of the H-1 integral in the anomeric region (4.6–5.2 ppm) with the free acetates integral (1.7–1.9 ppm).

The $^1$H-1D NMR spectrum of the O-deacylated EPS (Fig. 1) showed that the repeating unit contained one or several residues in addition to the three sugars identified by methylation analysis. In the anomeric region, four proton signals were observed. Two of these at $\delta$ 4.51 and 4.69, and having a $J_{H\text{-}1,H\text{-}2}$ value of 7.9 Hz, were attributed to H-1 of the β-D-glucopyranose residues (respectively, C and A residues). One signal at $\delta$ 5.11 with a $J_{H\text{-}1,H\text{-}2}$ value of 1.8 Hz was assigned to H-1 of the α-L-rhamnopyranose residue (B residue). The fourth signal at $\delta$ 4.77 corresponded to a broad doublet with an apparent $J_{H\text{-}1,H\text{-}2}$ value of 9.5 Hz (D residue).

The high field region contained a signal at $\delta$ 1.29 assigned to the methyl group of the L-rhamnopyranose, and two signals characteristic of the protons of a methylene group were present at $\delta$ 1.59 and 2.32.

The 1-D-$^{13}$C NMR spectrum (data not shown) revealed four signals in the anomeric region at $\delta$ 101.05, 101.7, 103.4 and 104.1. These data are in agreement with the presence of a repeating unit composed of four sugars. The signals at $\delta$ 103.4 and 104.1 with a $^1J_{C\text{-}1,H\text{-}1}$ value of 162.2 Hz correspond to C-1 of the β-glucopyranose sugars. The signal at $\delta$ 101.7 with a $^1J_{C\text{-}1,H\text{-}1}$ value of 166.6 Hz was assigned to C-1 of the α-rhamnopyranose residue. The fourth signal at $\delta$ 101.5 with a $^1J_{C\text{-}1,H\text{-}1}$ value of 162.3 Hz is characteristic of the β-anomeric configuration. The signals at $\delta$ 17.8, 38.2 and 175.8 showed, respectively, the presence of a methyl group from rhamnose, a methylene and a carboxyl group. These data suggest that the D residue is probably an acidic deoxy-sugar.

An attempt was made to determine the nature of the deoxy-uronic acid after hydrolysis by TFA of the native polymer. The uncommon sugar could not be released by this method, most presumably because of its sensitivity to acid hydrolysis. The degradation of 2-deoxy-sugar after acid hydrolysis suggests that the D residue is a 2-deoxy-uronic acid. Indeed, sensitivity to acid hydrolysis is shown

for 2-deoxy-sugars.[11] The carboxyl-reduced polymer (1 mg) was methanolyzed at 80 °C with 0.5 M HCl in methanol (2.5 mL). Aliquots were removed at different times (15, 30 min, 2 and 20 h) and then dried under an argon flow. Analysis by GLC–MS (EI) of the trimethylsilylated derivatives of the released monosaccharides revealed the presence of signals similar to those obtained with a methyl pertrimethylsilyl-2-deoxy-glucoside standard. The intensity of the signals varied with methanolysis time. Significant signals of a silylated methyl 2-deoxy-1-glucoside were obtained in the sample methanolyzed during 15 min. Altogether these results suggest that the repeating unit of the polymer was composed of one α-L-rhamnopyranose residue, two β-D-glucopyranose residues with (1→3) and (1→4) linkages and one 2-deoxy-β-glucuronic acid.

GLC–MS (EI) analysis of the *R*(−)butan-2-ol glycosides was performed as described by Gerwig et al.[12,13] It showed that rhamnose and glucose were respectively in the L and D configuration. Analysis of the pertrimethylsityl-2-butyl-glycoside to determine the configuration of the 2-deoxy-*arabino*-hexose from the reduced EPS was unsuccessful due to the sensitivity of the sugar to the hydrolytic conditions used.

The 1D NMR spectrum of the polysaccharide from *Rhizobium* sp. B showed a great similarity with that from *S. paucimobilis* exopolysaccharide I-886[14] which contains a 2-deoxy - β - D - *arabino* - hexopyranuronic acid residue. The chemical shifts of the acidic residue were close to the corresponding value reported for S. *paucimobilis* exopolysaccharide I-886.[14] This indicates that the *arabino* and D-absolute configuration of the acidic residue is the same as in S. *paucimobilis* exopolysaccharide I-886.

Subsequent NMR spectroscopy analyses (1-D TOCSY, 2-D COSY and 2-D HMQC),

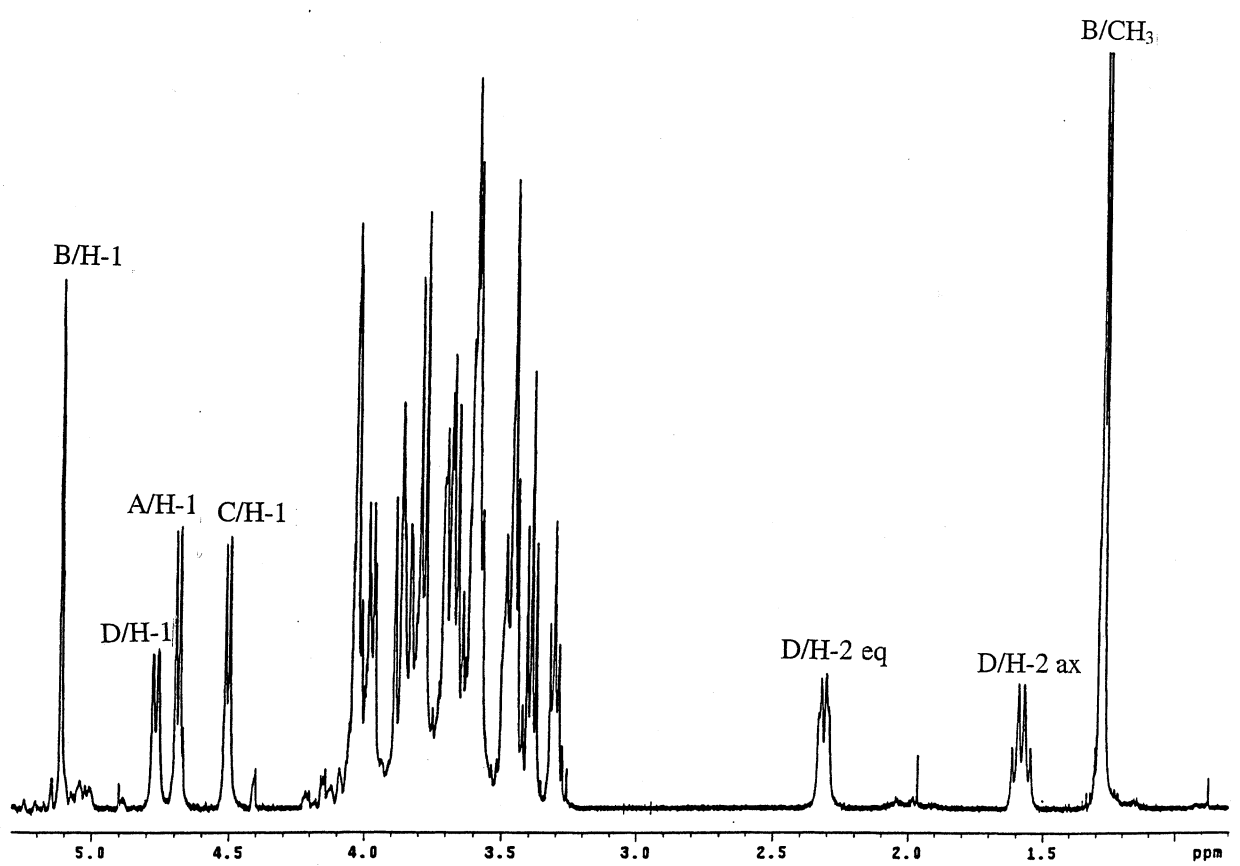


Fig. 1. 1-D $^1$H NMR spectrum (500 MHz, 60 °C) of the deacylated polysaccharide (10 g/L in $D_2O$). [A/H-1], [B/H-1], [C/H-1], [D/H-1]: H-1 in A, B, C and D residues; [D/H-$2_{eq,ax}$]: H-2 equatorial and axial in D residue; [B/$CH_3$]: protons from methyl group in B residue.

Table 1
$^1$H NMR assignments in the *Rhizobium* sp. B deacylated polysaccharides in $D_2O$

| Residue | Chemical shifts (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | H-1 | H-2 | H-3 | H-4 | H-5 | H-6a | H-6b |
| A | 4.69 (7.9) | 3.31 | 3.61 | 3.59 | 3.49 | 3.85 | 3.70 |
| B | 5.11 (1.8) | 4.03 | 3.98 | 3.67 | 4.03 | 1.29: $CH_3$ | |
| C | 4.51 (7.9) | 3.40 | 3.59 | 3.46 | 3.48 | 3.88 | 3.69 |
| D | 4.77 (9.5) | 1.59 (ax) 2.32 (eq) | 3.81 | 3.60 | 3.79 | | |

Coupling constants ($^3J_{H\text{-}1,H\text{-}2}$ in Hz) are given in brackets. A: →4)-β-D-Glc*p*-(1→; B: →4)-α-L-Rha*p*-(1→; C: →3)-β-D-Glc*p*-(1→; D: →4)-2-deoxy-β-D-Glc*p*A-(1→.

Table 2
$^{13}$C NMR assignments in the *Rhizobium* sp. B deacylated polysaccharide in $D_2O$

| Residue | Chemical shifts (ppm) | | | | | |
|---|---|---|---|---|---|---|
| | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
| A | 104.1 (162.2) | 74.8 | 75.4 | 79.8 | 76.04 | 61.1 |
| B | 101.7 (166.6) | 71.4 | 71.3 | 82.2 | 68.5 | 17.77: $CH_3$ |
| C | 103.4 (162.2) | 74.9 | 83 | 69.12 | 76.7 | 61.9 |
| D | 101.05 (162.3) | 38.2 | 70.3 | 83.15 | 76.7 | |

$^1J_{C\text{-}1,H\text{-}1}$ values (Hz) are given in brackets. A: →4)-β-D-Glc*p*-(1→; B: →4)-α-L-Rha*p*-(1→; C: →3)-β-D-Glc*p*-(1→; D: →4)-2-deoxy-β-D-Glc*p*A-(1→.

Table 3
HMBC experiments on the *Rhizobium* sp. B deacylated polysaccharide in $D_2O$

| Residue | Anomeric atom | | $J_{H-C}$ connectivities to | | Residue atom |
|---|---|---|---|---|---|
| | $\delta$ ($^1$H) | $\delta$ ($^{13}$C) | $\delta$ ($^1$H) | $\delta$ ($^{13}$C) | |
| A | 4.69 | 104.1 | 3.67 | 82.2 | B: C-4, B: H-4 |
| B | 5.11 | 101.7 | 3.59 | 83, 83 | C: C-3, C: H-3/C: C-3 |
| C | 4.51 | 103.4 | 3.60 | 83.15 | D: C-4, D: H-4 |
| D | 4.77 | 101.05 | 3.59 | 79.8, 79.8 | A: C-4, A: H-4/A: C-4 |

$^2J_{H-C}$ and $^3J_{H-C}$ connectivities for the anomeric atoms (H-1 and C-1) of the deacylated polymer. A: →4)-β-D-Glc*p*-(1→; B: →4)-α-L-Rha*p*-(1→; C: →3)-β-D-Glc*p*-(1→; D: →4)-2-deoxy-Glc*p*A-(1→.

confirmed the identity of the D residue as 2-deoxy-*arabino*-Hex*p*A by comparison with the previous chemical shifts obtained for the exopolysaccharide from S. *paucimobilis* I-886. $^1$H and $^{13}$C chemical shifts deduced from these analyses are summarized in Tables 1 and 2.

2-D NOESY and HMBC experiments were carried out to determine the sequence of the sugars in the repeating unit and to verify the type of the different linkages.

HMBC experiments (Table 3) revealed correlations between C-1 in the rhamnose residue ($\delta$ 101.7) and H-3 in the glucose residue noted C ($\delta$ 3.59), as well as between C-1 of the 2-deoxy-hexuronic acid ($\delta$ 101.05) and H-4 in the glucose residue noted A ($\delta$ 3.59). Correlations were detected between the anomeric carbon from the glucose residue noted A ($\delta$ 104.1) and H-4 in the rhamnose residue ($\delta$ 3.67), and between C-1 of the glucose residue noted C ($\delta$ 103.4) and H-4 in the 2-deoxy-hexuronic acid ($\delta$ 3.60).

2-D NOESY experiments showed a correlation between resonances at $\delta$ 1.29 (protons from the rhamnose methyl group) and $\delta$ 4.69 (H-1 in the (1→4)-linked glucose), between $\delta$

5.11 (H-1 of the rhamnose) and $\delta$ 3.59 (H-3 of the (1→3)-linked glucose) and between $\delta$ 4.51 (H-1 of the (1→3)-linked glucose) and $\delta$ 3.60 (H-4 of the 2-deoxy-hexuronic acid residue). The anomeric proton of the 2-deoxy-hexuronic acid residue ($\delta$ 4.77) showed a correlation with a resonance signal at $\delta$ 3.59 (H-4 of the (1→4)-linked glucose).

Correlations in Table 3 confirmed the following structure for the repeating unit:

$$\rightarrow 4)\text{-}\beta\text{-}D\text{-}Glc_A p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-}L\text{-}Rha_B p\text{-}(1\rightarrow 3)\text{-}\beta\text{-}D\text{-}Glc p\text{-}(1\rightarrow 4)\text{-}2\text{-deoxy-}\beta\text{-}D\text{-}Glc_D p A\text{-}(1\rightarrow$$

The exopolysaccharide from the *Rhizobium* sp B strain is then a polymer of a repeating unit presenting an unusual structure for the *Rhizobiaceae* family and particularly for the species *Rhizobium*. The backbone contains an α-L-rhamnose residue which has been previously described in polymers from *Bradhyrhizobium elkanii*,[15] *japonicum*,[16] and some *Rhizobia* sp.[17,18] This monosaccharide is also found in a number of polysaccharides used in industry such as gellan and sphingans.[19,20] The 2-deoxy-D-*arabino*-hexuronic acid residue is the first 2-deoxy-acid sugar characterized in *Rhizobia* polysaccharides. So far, this monosaccharide has been found only in the exopolysaccharide from the *Sphingomonas paucimobilis* I-886 strain.[14] In fact, the repeating unit of the EPS from *Rhizobium* sp. B strain has a similar structure as the backbone of EPS I-886 from *S. paucimobilis*. EPS I-886 and EPS from *R.* sp. B strain contain *O*-acetyl groups in their native states. EPS I-886 also bears a disaccharide chain linked in the 6-position to the (1→3)-linked glucose and phosphate substituents.

The structure of the repeating unit of the EPS from *R.* sp. B strain is similar to the one of the backbone of polysaccharides belonging to the gellan gum family (Gellan,[19] Rhamsan,[20] Welan,[21] Sphingan S-657[4] or S-88[22]). Polysaccharides from this family are known as gelling or thickening agents. Since EPS B from *R.* sp. B strain gave viscous aqueous solutions and showed structural similarities with gellan gums, we propose to include this polysaccharide in the gellan gum family. However, further studies of its rheological properties must be investigated.

A modification or removal of one monosaccharide in the backbone structure modifies radically the rheological properties of polysaccharides.[5] It would therefore be interesting to investigate the rheological behavior of polysaccharides bearing a 2-deoxy-glucuronic acid (EPS from *Rhizobium* sp. B strain) instead of a glucuronic acid (gellan). Comparison studies on the solution properties of EPS from *Rhizobium* sp. B strain and EPS I-886 from *S. paucimobilis* I-886 should give some information about the effect of the side-chain structure on the rheological properties of these polysaccharides.

## 3. Experimental

*General methods.*—For GLC–MS (EI) analysis, a Hewlett–Packard 6980 MSD electronic impact (EI) instrument was used. Separation of alditol acetates or trimethylsilylated derivatives was performed using a HP1 methyl siloxane capillary column (25 m × 200 μm) with a temperature program increasing from 120 °C (10 min) to 210 °C at 2 °C/min, then to 240 °C (5 min) at 10 °C/min.

Sugars from dried methanolysate or hydrolysate (200 μg) were dissolved in Py (200 μL), and converted into $Me_3Si$ ether derivatives using the trimethylsilylated reagent (BSTFA and TMCS 1% from Pierce, 200 μL).

High performance anion-exchange chromatography (HPAEC) was performed on a Carbopac PA-1 column (4.5 × 25 mm) eluted with 16 mM NaOH at a flow rate of 1 mL/min for 41 min. Dried methanolysate or hydrolysate were dissolved in water and injected (20 μL). Detection was performed using a pulsed amperometric detector (Dionex).

*Production and purification of polysaccharide.*—The *Rhizobium* sp. B strain was isolated from atypical nodules on alfalfa in our laboratory (UPJV, Amiens, France).

The *Rhizobia* cells were grown in 2 L Erlenmeyer flasks containing 1 L of *Rhizobium complete* (R.C.) medium[23] supplemented with sucrose (1% w/v). The incubation was performed during 72 h at 30 °C on a rotary shaker. The bacterial suspension was then centrifuged (53,900*g*, 40 min). The bacterial free

supernatant was supplemented with ammonium acetate (1 M) and the EPS was precipitated by addition of 2 vol isopropanol. The EPS precipitate was purified as described previously.[24]

*Carboxyl reduction.*—The native polysaccharide (2 mg) was dissolved in 0.02 M 2-(4-morpholino)-ethanesulfonic acid,[25,26] the pH was maintained at 4.75 with 4 M HCl and 1 - (3 - dimethylaminopropyl) - 3 - ethylcarbodiimide hydrochloride (30 mg) was added. The solution was stirred at rt for 90 min before addition of $NaBD_4$ (10 mg). The solution was kept at rt for 120 min and the pH was adjusted to 7. Reduction steps were performed under Ar. Then the solution was dialyzed overnight against water. The same carboxyl reduction cycle was performed three times on the polysaccharide samples.

*EPS hydrolysis.*—The native polysaccharide (1 mg) dissolved in 4 M TFA (1 mL) was heated at 100 °C for 4 h. The acid was removed by flushing the sample with air and the hydrolysate was dried under vacuum.

*EPS hydrolysis under reducing conditions.*—The carboxyl-reduced polysaccharide was hydrolysed at 100 °C with 0.5 M TFA for 2 h. During the hydrolysis 10 equiv of borane-4-methylmorpholine complex were added for instant reduction.[27]

*Methylation and peracetylation of the polymer.*—The native EPS (1 mg) was methylated according to the procedure of Hakomori.[28] The methylated polysaccharide was recovered by $CHCl_3$ extraction ($2 \times 0.5$ mL). The extract was washed with water ($15 \times 5$ mL) and dried under Ar. Hydrolysis of the methylated polysaccharide was performed as described above. The sugars from the hydrolysate were converted into partially methylated alditol acetates according to the procedure of Blakeney et al.[29]

*EPS methanolysis.*—The carboxyl-reduced polysaccharide (1 mg) was methanolysed at 80 °C with 0.5 M HCl in MeOH (2.5 mL) for 0.25, 0.5, 1, 2 and 20 h under Ar. The methanolysate was then dried under Ar.

*Absolute configurations of sugars.*—Absolute configuration of the monosaccharides from both of the native and the carboxyl-reduced polysaccharides was determined using the procedure described by Gerwig et al. [12,13]

*Preparation of the O-deacylated polymer.*—The polymer was dissolved in water (10% w/v) and deacylated according to Dantas et al.[30]

*NMR spectroscopy.*—1-D and 2-D NMR spectra of solutions in $D_2O$ (10 mg/mL) were recorded at 60 °C with a VARIAN UNITY Plus 500 spectrometer (500 MHz), except for proton-decoupled $^{13}C$ NMR spectra which were recorded with an AC 300 NB BRUKER instrument (300 MHz).

$^1H$ spectra were obtained with a spectral width of 1000 Hz, using a transmitter power of 56 dB, and a pulse length of 13.5 μs (90°). Ten scans with an acquisition time of 4 s were accumulated. Data were zerofilled to 32 K and a suppression of HOD signal was achieved by a presaturation for 1.5 s.

1-D TOCSY spectra were recorded using a selective (10 Hz) soft pulse sequence with an eburp1-256 shape, with selective power −5dB, pulse length of 186 ms and arrayed selective frequency. The mixing time was from 5 to 95 ms, The spectrum was collected with 4–76 transients and a relaxation delay of 0.7 s.

gCOSY experiments were carried out with the standard pulse sequence. The time-domain data set was of 2 K and four pulses were performed for each of the 512 experiments. The time-domain data sets were multiplied with a phase shifted sine bell. After zerofilling and Fourier transformation, data sets of $4 \times 4$ K were obtained.

2-D NOESY and TOCSY were performed using the method described above for gCOSY experiments, except for the number of transients which was 220. The mixing time for NOESY experiments was 300 ms.

2-D-$^{13}C$–$^1H$ HMQC and HMBC experiments with inverse detection were carried out at a $^1H$ frequency of 499.837 MHz (125.694 MHz for $^{13}C$) with a spectra width of 1000 Hz for $t_2$ and 14,000 for $t_1$, using the standard pulse sequences. The time-domain data sets were multiplied with a phase shift sine bell and after zerofilling and Fourier transformation. Data sets of $4 \times 4$ K were obtained.

Chemical shifts are given relative to external tetramethylsilane (TMS = 0 ppm) and the calibration was performed using the signals of the residual protons of the solvent as a secondary reference.

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