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# The structure of the O-polysaccharide from *Pseudomonas stutzeri* OX1 containing two different 4-acylamido-4,6-dideoxy-residues, tomosamine and perosamine

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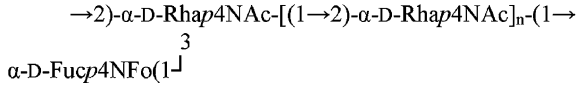
**Abstract**—The structure of the O-polysaccharide from the lipopolysaccharide of *Pseudomonas stutzeri* OX1 was determined by chemical procedures and by 1D and 2D NMR spectroscopy. The analysis revealed the presence of a heterogeneous polymer made by 4-acetamido-4,6-dideoxy-D-mannopyranose (D-Rha*p*4NAc) and 4-formamido-4,6-dideoxy-D-galactopyranose (D-Fuc*p*4NFo). The combination of chemical and NMR analyses indicates that the heterogeneity of the polymer depends on its non-stoichiometric glycosylation by Fuc4NFo, as shown below:




The structure of the heterogeneous polymer was confirmed by Smith degradation that significantly simplified the structure of the O-polysaccharide, allowing for the isolation and identification of a linear homopolymer of Rha*p*4NAc.


*Keywords:* *Pseudomonas stutzeri* OX1; Lipopolysaccharide; NMR spectroscopy; 4-Acetamido-4,6-dideoxy-mannose; 4,6-Dideoxy-4-formamido-galactose

## 1. Introduction

*Pseudomonas stutzeri* OX1 is a Gram-negative microorganism, originally isolated from activated sludges of a wastewater treatment plant.[1] Its survival in such contaminated habitat has been attributed to its ability to use aromatic compounds like phenol, dimethylphenol, cresol and *o*-xilene,[1] as a source of carbon and energy. These molecules are converted by a complex series of enzymatic systems[2–4] into dihydroxylated derivatives, which are subsequently transformed into citric acid cycle[5] intermediates. To date, some of these enzymatic systems have been purified and characterised, namely, toluene-*o*-xylene monooxygenase,[2,3] phenol hydroxylase[4] and catechol 2,3 dioxygenase.[6] It has been hypothesised[7] that, besides these specific metabolic pathways, microorganisms like *P. stutzeri* must have developed other biochemical features for the survival under harsh conditions such as the presence of organic solvents at high concentrations.

Previous studies have already shown that the outer membrane of Gram-negative bacteria is involved in adaptation mechanisms to hostile habitats,[7,8] and that changes occur in inner and outer membranes,[9] for example, fatty acids and phospholipids composition, under different growth conditions. However, few studies have

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been carried out on the role of lipopolysaccharides (LPSs) in these accommodation mechanisms.

LPSs are the main constituents of the outer leaflet of the outer cell membrane of Gram-negative bacteria, hence directly interacting with external environment.[9] Typical smooth form lipopolysaccharide (S-LPS) structure is constituted by three chemically and biogenetically distinct regions:[10,11] a glycolipid moiety, the Lipid A; an oligosaccharide region, the core region; a polysaccharide, the O-specific chain (O-polysaccharide, O-chain). LPSs in their rough form (R-LPS) lack the O-specific chain and are known as lipooligosaccharides (LOS).

*P. stutzeri* OX1 was grown under different conditions and we found that LPS was synthesised only in cells grown in rich medium whereas, interestingly, cells grown on phenol as the only carbon source did not produce any LPS but instead a lipooligosaccharide that was isolated and characterised.[12,13] This paper describes the structural elucidation of the polysaccharide fraction from the LPS of *P. stutzeri* OX1.

## 2. Results and discussion

Cells of *P. stutzeri* OX1 grown in rich medium were extracted using the hot phenol–water procedure.[14] Two phases were obtained, dialysed, lyophilised and analysed by SDS-PAGE.[15] The LPS was detected in the phenol phase and further purified by digestion with nucleases, protease and by gel-permeation chromatography. The lipopolysaccharide was hydrolysed using mild acidic conditions, and the O-polysaccharide fraction (**PS1**) was collected in the supernatant, after centrifugation. Compositional analysis, carried out by GC–MS analysis of the acetylated *O*-methyl glycoside and of the alditol acetate derivatives, yielded two different 4-amino-4,6-dideoxy-hexoses. Methylation analysis showed the presence of the derivatives of 2-substituted 4-amino-4,6-dideoxy-hexopyranose, 2-3-di-substituted 4-amino-4,6-dideoxy-hexopyranose, terminal 4-amino-4,6-dideoxy-hexopyranose (ratio 3.7:1:0.9, detector response). Monosaccharides were finally identified as D-perosamine, 4-amino-4,6-dideoxy-D-mannose (D-Rha4N) and D-tomosamine, 4-amino-4,6-dideoxy-D-galactose (D-Fuc4N) by their comparison with authentic standards.

The $^{1}$H NMR spectrum of **PS1** (Fig. 1) appeared rather simple. At low fields, two sharp singlet signals were visible, at 8.16 and 7.86 ppm, deriving from the two possible isomers (*Z* and *E*, respectively) of a *N*-formyl group. The different amount of the two species (relative ratio 9:1) can be explained on the basis of the higher stability of the *Z* isomer. In addition, five anomeric signals were present in the spectrum, all appearing as broad singlets, relative to five different spin systems, **A**–**E**, at 5.16, 5.13, 5.07, 5.03 and 4.98 ppm, respectively,

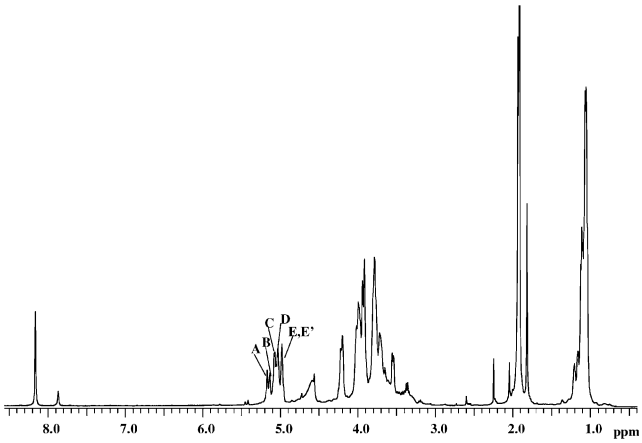



**Figure 1.** $^{1}$H NMR of the O-polysaccharide from *P. stutzeri* OX1 (**PS1**). Anomeric signals are designated by capital letters.

in a relative ratio 0.5:0.5:1:1:1. Beside the ring proton region at 4.3 and 3.3 ppm, other significant signals were present, at 1.92–1.98 ppm belonging to *N*-acetyl groups, and between 1.13 and 1.03 ppm, belonging to methyl groups of the 6-deoxy-hexoses. Despite the quite simple appearance of the spectrum, 2D NMR spectra analysis pointed to the presence of a heterogeneous product. On the basis of DQF-COSY, TOCSY and NOESY spectra, the full proton resonances of the spin systems **A**–**E** could be assigned (Table 1), whereas the interpretation of the $^{1}$H, $^{13}$C HSQC spectrum allowed for the assignment of all $^{13}$C resonances. Spin systems **A** and **B** appeared to have the same chemical nature. For both residues, the small $^{3}J_{1,2}$ coupling constant value (about 1.8 Hz) deduced from the DQF-COSY spectrum, together with the diagnostic H-5/C-5 chemical shift values,[16] suggested α-*manno* configuration. In the TOCSY spectrum, starting from H-2 proton signals, all correlations with the ring proton and with methyl proton signals were visible. In the HSQC spectrum, both H-4 **A** and H-4 **B** (3.93 and 3.92 ppm) correlated to a nitrogen-bearing carbon signal (52.1 ppm) leading to the identification of **A**–**B** residues as perosamine, 4-amino-4,6-dideoxy-mannose (Rha4N). A comparison of carbon resonances of **A** and **B** with unsubstituted residues,[17,18] gave evidence of a downfield shift of C-2 and C-3 signals for both residues indicating substitution at O-2 and O-3. Thus, **A** and **B** spin systems were both identified as 2,3-di-substituted α-perosamine. Similar considerations could be made for spin systems **C** and **D**, which were both finally identified as 2-substituted α-perosamine owing to the downfield glycosylation of their C-2 signal. Residue **E** was identified as unsubstituted 4-amino-4,6-dideoxy-α-galactose (α-Fuc4N). Its *galacto*-configuration was inferred on the basis of coupling constant values of ring protons, in particular, $^{3}J_{3,4}$ (3 Hz). In fact, H-5 of **E** spin system was only detectable by NOESY experiment since in the TOCSY

**Table 1.** $^{1}H$ and $^{13}C$ NMR chemical shifts (ppm) of the intact O-polysaccharide from *P. stutzeri* OX1 (**PS1**) and of **PS1** after Smith degradation (**PS2**) [Chemical shifts are relative to acetone ($^{1}H$, 2.225 ppm, $^{13}C$, 31.45 ppm at 30 °C)]

| Sugar residue | 1 | 2 | 3 | 4 | 5 | 6 | C=O | $CH_3$(H) |
|---|---|---|---|---|---|---|---|---|
| **PS1** | | | | | | | | |
| **A** | 5.16 | 4.22 | 3.91 | 3.93 | 3.91 | 1.11 | — | 1.92 |
| α-2,3-Rha4NAc | 100.9 | 73.9 | 73.4 | 52.1 | 68.6 | 17.6 | 175.2 | 23.1 |
| **B** | 5.13 | 4.23 | 3.90 | 3.92 | 3.91 | 1.12 | — | 1.98 |
| α-2,3-Rha4NAc | 101.0 | 74.8 | 73.4 | 52.1 | 68.8 | 17.4 | 174.9 | 21.0 |
| **C** | 5.07 | 3.98 | 3.94 | 3.76 | 3.69 | 1.06 | — | 1.92 |
| α-2-Rha4NAc | 100.7 | 77.7 | 69.2 | 53.7 | 69.1 | 17.5 | 175.7 | 22.8 |
| **D** | 5.03 | 4.02 | 3.91 | 3.77 | 3.70 | 1.06 | — | 1.92 |
| α-2-Rha4NAc | 101.2 | 77.6 | 69.3 | 53.7 | 69.1 | 17.5 | 175.7 | 22.8 |
| **E** | 4.97 | 3.54 | 3.80 | 4.20 | 3.98 | 1.03 | — | 8.16 |
| α-Fuc4NFo | 96.1 | 68.9 | 69.2 | 53.0 | 66.3 | 16.3 | 166.2 | — |
| **E′** | 4.97 | 3.48 | 3.74 | 4.18 | 3.94 | 1.04 | — | 7.86 |
| α-Fuc4NFo | 95.7 | 68.6 | 69.6 | 53.2 | 66.2 | 16.4 | 169.6 | — |
| **PS2** | | | | | | | | |
| α-2-Rha4NAc | 5.05 | 4.03 | 3.95 | 3.80 | 3.73 | 1.07 | — | 1.94 |
| | 100.9 | 77.4 | 68.3 | 53.5 | 68.9 | 17.2 | 175.6 | 22.5 |

spectrum the low $^{3}J_{4,5}$ impaired any magnetisation transfer over H-4. The anomeric coupling constant value (3.2 Hz) of residue **E** clearly indicated α-orientation. In analogy with **A**–**D** spin systems, H-4 **E** correlated to a nitrogen bearing carbon that resonated at 53.0 ppm.

In the $^{1}H$, $^{13}C$ HMBC spectrum scalar correlations were found between H-4 of **A**–**D** and carboxyl group signals around 175 ppm and between these latter and methyl signals at about 2 ppm, thus indicating that all the Rha4N residues are *N*-acetylated. On the other hand, Fuc4N **E** showed a cross peak between H-4 and a carboxyl group signal shielded at 166.2 ppm which, in turn, is correlated to the formyl proton at 8.16 ppm, thus confirming the presence of the *Z* isomer of formyl group at *N*-4 of **E** residue. A minor series of signals in the COSY and TOCSY spectra, relative to the same anomeric proton at 4.97 ppm, described another spin system (**E′**), which was identified as Fuc4NFo, that is, a modified residue **E**, in which the *N*-formyl group was attached as *E* isomer. In fact, in the HMBC spectrum, H-4 **E′** and the formyl proton at 7.86 ppm both correlated to the carboxyl signal at 169.6 ppm.

In order to establish the monosaccharide sequence within the repeating unit, interresidual dipolar correlations in the NOESY (Fig. 2) and scalar long-range correlations in the $^{1}H$, $^{13}C$ HMBC spectra were used. The anomeric proton of **A** residue (H-1 **A**) gave NOE effect with H-1 **C** and H-2 **C**, whereas H-1 **C** gave NOE effect with H-1 and H-2 **A**, thus indicating that **A** and **C** residues are both 2-substituted and arranged in a disaccharide structure. Likewise, **B** and **D** spin systems could be attributed to the same disaccharide structure, although in a different magnetic environment. Furthermore, no connections between **A**–**C** and **B**–**D** fragments were visible in the NOESY spectrum. The anomeric proton of **E** residue (H-1 **E**) gave a dipolar correlation with both H-3

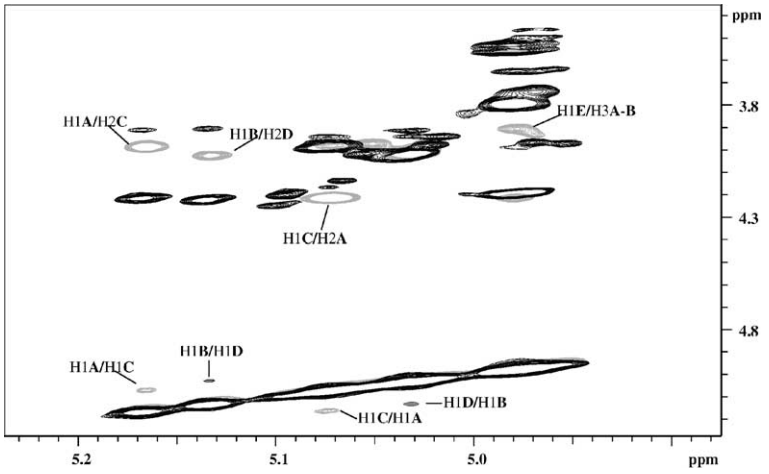



**Figure 2.** Zoom of the TOCSY (black) and NOESY (grey) spectra of **PS1**. Annotations refer to interresidual cross-peaks of spin systems reported in Table 1.

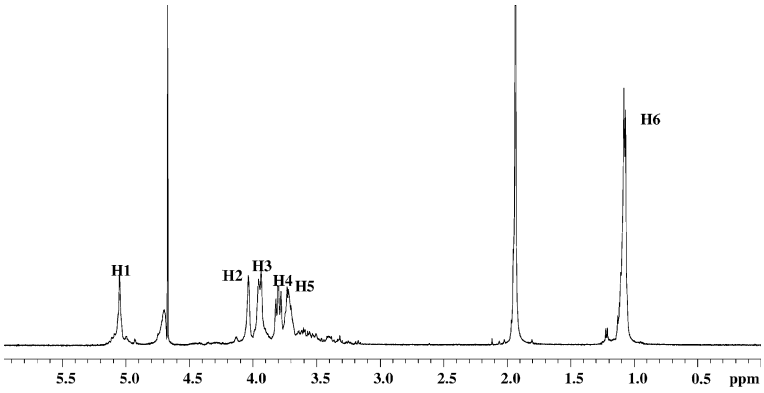
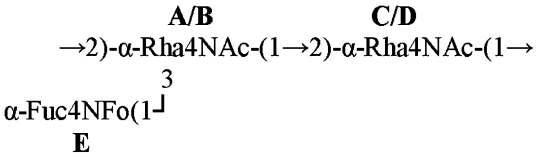



**Figure 3.** $^{1}$H NMR of **PS2**, obtained from Smith degradation of **PS1**. Capital letters indicate ring protons.

of **A** and **B**, thus confirming our previous conclusions (see above), that is, that **A** and **B** are 2,3-disubstituted residues and **E** residue is attached to their O-3 position.

$^{1}$H, $^{13}$C HMBC spectrum contained scalar long-range correlations between H-1 **A**/C-2 **C**, H-1 **C**/C-2 **A**, H-1 **B**/C-2 **D**, H-1 **D**/C-2 **B**, H-1 **E**/C-3 **A**-**B**. Thus, it can be concluded that in product **PS1** two identical moieties are present, whose difference cannot be derived from the data above. Their structure can be drawn as

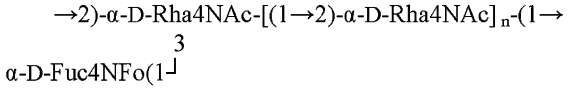



The formyl group, present as pair of isomers, was an expected source of magnetic heterogeneity, but the 1:9 ratio between *E* and *Z* isomers in the $^{1}$H NMR spectrum could not be the only origin for **A**/**B** and **C**/**D** duets because most of the heterogeneity was expected and found on Fuc4N residue (**E**/**E**′), to which the formyl group is directly attached.

The $^{1}$H NMR spectrum indicated that 2-substituted Rha4N (**C**/**D** units) is present in higher amount with respect to nodal Rha4N **A**/**B** (anomeric signal ratio, **A**:**B**:**C**:**D**, 1:1:2:2) and this would suggest that **PS1** heterogeneity does not only depend on *E* and *Z* isomers ratio but also on the presence of a longer and not distinguishable chain of 2-substituted Rha4N residues and that this latter, in particular, causes the splitting of each Rha4N in two signals.

In order to simplify the intricate structure of **PS1** and to further support the structural hypothesis we have advanced above, a Smith degradation was carried out on the polymer. After purification of the products by gel permeation chromatography on a TSK HW-40 column, a product, was collected, which eluted in the void volume. The compositional and 2D NMR analyses (Fig. 3, Table 1) of this fraction indicated the presence of a highly regular polysaccharide (**PS2**) built up of 2-substituted α-Rha4N, in which the heterogeneity was completely removed. As expected on the basis of the data collected on **PS1**, only terminal Fuc4N residues possessed a *vic*-diol functional group, prone to oxidation by periodate reagent, and consequently it has eliminated from the **PS1** by the Smith degradation.

On the basis of the **PS2** structure, the conclusion can be drawn that most of the heterogeneity of **PS1** is due to non stoichiometric glycosylation by the Fuc4N residue in a linear chain of 2-substituted α-Rha4N. Thus, we can confidently affirm that the O-polysaccharide obtained from the LPS of *P. stutzeri* OX1 has the following structure:




However, since both compositional analysis and anomeric signal integration in the $^{1}$H NMR spectrum of **PS1** were suggestive of the presence of a regular polymer, it is evident that our data do not allow to discriminate between the following structural possibilities: (i) two different repeating units are present in two regular polysaccharides, or (ii) only one polysaccharide is present, consisting of a monosaccharide backbone to which a side chain monosaccharide is attached in a non-full stoichiometric fashion. Any attempt to separate by gel permeation chromatography the two putative coexisting polysaccharides failed.

The isolation and structural characterisation of this novel O-polysaccharide from *P. stutzeri* OX1 LPS confirms previous findings of the presence of non-regular polymers in the outer membrane of Gram-negative bacteria, as in the case of several O-polysaccharide chains isolated from LPS of other *Pseudomonas* and *Xanthomonas* strains.[19–22] Moreover, we have shown that the non-regularity of the structure of the polysaccharide fraction from the LPS of *P. stutzeri* OX1 depends on Fuc4N

residues linked in a non-stoichiometric fashion. This confirms recent data on the occurrence of carbohydrate residues unsystematically attached to a carbohydrate chain,[21,22] which are used to mask the repetitiveness of the polymer besides usual non-carbohydrate groups (acetyl, phosphate, methyl groups).

## 3. Experimental

### 3.1. Bacterial growth, isolation of LPS and O-polysaccharide

Cells were routinely grown in liquid rich Luria-Bertani medium, at 27 °C for 10 h up to about 1 $OD_{600}$/mL. Cells were recovered by centrifugation (6000 rpm, 15 min, 4 °C), washed with water and lyophilised (5.230 g of dried cells). Dried cells were suspended in water and extracted three times with the hot phenol–water procedure.[14] After extraction the two phases were collected, dried and analysed by SDS-PAGE. Gels were stained with silver nitrate as described.[15] LPS was identified in the phenol extract on the basis of the typical ladder-like migration pattern and purified after nuclease and protease digestions and by GPC chromatography on a Sephacryl HR-400. This procedure allowed for the purification of a pure LPS fraction (yield: 42 mg, 8.0% of bacterial dry mass). In order to obtain the O-polysaccharide chain, the LPS (30 mg) was hydrolysed with aq 1% AcOH for 2 h at 100 °C and centrifuged (11,000 rpm, 4 °C, 1 h). The supernatant (27 mg, 90% of LPS) was purified by the gel-permeation chromatography on a Sephacryl S300-HR column (90 cm × 1.5 cm) using 0.05 M ammonium bicarbonate as eluent. Elution was monitored with a Waters differential refractometer.

### 3.2. Compositional and methylation analysis

Monosaccharides were analysed as acetylated *O*-methyl glycoside and acetylated alditol derivatives. Absolute configuration determination was carried out on *O*-butyl glycoside derivatives as described.[23] Authentic samples of D-Fuc4N from *Agrobacterium* and D-Rha4N from *Citrobacter* were a gift of Dr. C. de Castro (Dept. of Organic Chemistry and Biochemistry, University of Naples Federico II) and Dr. Y. A. Knirel (Russian Academy of Science, Moscow), respectively. For the preparation of the *O*-methyl glycoside derivatives, **PS1** (1 mg) was dried over $P_2O_5$ for 16 h under vacuum in a desiccator, dissolved in 2 M methanol/HCl and kept at 80 °C for 16 h. The sample was then dried and acetylated with acetic anhydride (200 μL) and pyridine (200 μL) at 80 °C for 30 min. For the preparation of the acetylated alditol derivatives, the polysaccharide was hydrolysed with 10 M HCl at 80 °C for 30 min, dried under vacuum, reduced with $NaBD_4$ at room temperature for 16 h and acetylated as described above. Methylation was carried out as described.[24] After extraction with chloroform/water, the permethylated polysaccharide was collected in the organic phase, hydrolysed with 10 M HCl for 30 min at 80 °C, reduced and acetylated.[17,18] The partially methylated alditol acetates derivatives were analysed by GC–MS. The GC–MS analyses were performed using Hewlett–Packard 5970 instrument equipped with an SPB-5 capillary column (Supelco, 30 m × 0.25 i.d., flow rate of 0.8 mL/min; He as the carrier gas).

### 3.3. Smith degradation

An aliquot of the polysaccharide (20 mg) was degraded by Smith degradation.[25] The sample was dissolved into 2 mL of water to which 5 mL of 0.1 M $NaIO_4$ solution were added and kept at 4 °C for 72 h. The reaction was quenched by adding 26 μL of ethylene glycol, neutralised with 0.5 M NaOH and then reduced with $NaBH_4$, followed by dialysis. After freeze drying, the sample was subjected to mild acid hydrolysis with 6% acetic acid, at 100 °C for 2 h. Acid was removed under vacuum and the sample was purified by chromatography on a TSK HW-40 column.

### 3.4. NMR spectroscopy

1D and 2D $^1H$ NMR spectra were recorded on solutions containing 5 mg sample dissolved in 0.6 mL of $D_2O$, at 30 °C. $^1H$ and $^{13}C$ experiments were carried out using a Varian Inova 500 instrument. Spectra were calibrated with internal acetone [$\delta_H$ 2.225, $\delta_C$ 31.45].

Nuclear Overhauser enhancement spectroscopy (NOESY) was carried out using data sets ($t_1 \times t_2$) of 4096 × 1024 points, and 16 scans were acquired. A mixing time of 200 ms was used. Double quantum-filtered phase-sensitive COSY experiments were performed with 0.258 s acquisition time, using data sets of 4096 × 1024 points, and 64 scans were acquired. Total correlation spectroscopy experiments (TOCSY) were performed with a spinlock time of 80 ms, using data sets ($t_1 \times t_2$) of 4096 × 1024 points, and 16 scans were acquired. In all homonuclear experiments the data matrix was zero-filled in the F1 dimension to give a matrix of 4096 × 2048 points and was resolution enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. Coupling constants were determined on a first-order basis from 2D phase sensitive double quantum filtered correlation spectroscopy (DQF-COSY).[26] Heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments were measured using pulse field gradient programs in the $^1H$-detected mode via single quantum coherence with proton decoupling in the $^{13}C$ domain, using data sets of 2048 × 512 points, and 64 scans were

acquired for each $t_1$ value. Experiments were carried out in the phase-sensitive mode according to the method of States et al.[27] The $^{1}H$, $^{13}C$ HSQC experiment was carried out with carbon multiplicity editing. A 60 ms delay was used for the evolution of long-range connectivity in the HMBC experiment. In all heteronuclear experiments the data matrix was extended to $2048 \times 1024$ points using forward linear prediction extrapolation.[28,29]

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