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Structural elucidation of fungal polysaccharides isolated from the cell wall of *Plectosphaerella cucumerina* and *Verticillium* spp.

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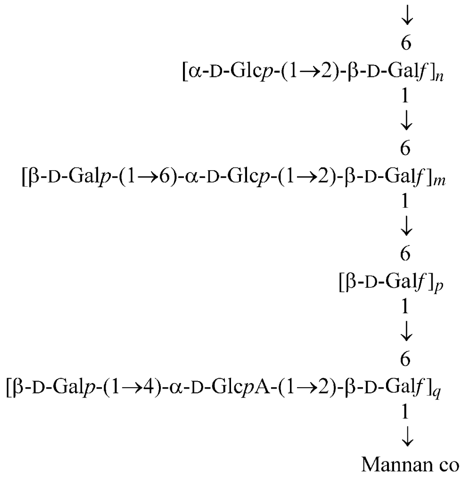
Abstract—The structure of acidic fungal polysaccharides isolated from the cell wall of *Plectosphaerella cucumerina*, *Verticillium dahliae*, and *V. albo-atrum* has been investigated by chemical analysis, methylation analysis, and 1D and 2D ^{1}H and ^{13}C NMR spectroscopy. The polysaccharides have an idealized repeating block of the type:



$$\begin{array}{r}
\downarrow \\
6 \\
[\alpha\text{-D-Glc}p\text{-}(1\rightarrow2)\text{-}\beta\text{-D-Gal}f]_n \\
1 \\
\downarrow \\
6 \\
[\beta\text{-D-Gal}p\text{-}(1\rightarrow6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow2)\text{-}\beta\text{-D-Gal}f]_m \\
1 \\
\downarrow \\
6 \\
[\beta\text{-D-Gal}f]_p \\
1 \\
\downarrow \\
6 \\
[\beta\text{-D-Gal}p\text{-}(1\rightarrow4)\text{-}\alpha\text{-D-Glc}p\text{A-}(1\rightarrow2)\text{-}\beta\text{-D-Gal}f]_q \\
1 \\
\downarrow \\
\text{Mannan core}
\end{array}$$

linked to a small mannan core (<15%), where $n = 13$, $m = 13$, $p = 5$, and $q = 8$ for *P. cucumerina*, and $n = 16$, $m = 16$, $p = 6$, and $q < 1$ for both *Verticillium* species.

Keywords: Fungi; *Plectosphaerella*; *Verticillium*; Polysaccharides; NMR Spectroscopy

1. Introduction

The alkali-extractable and water-soluble fungal polysaccharides (F1SS), which are minor components of the cell wall (2–8%), differ in composition and structure among genera and, in certain cases, among groups of species of a genus.[1] Polysaccharide moieties similar to that of the F1SS polysaccharides have been shown to occur in

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glycoproteins.[2,3] The complex carbohydrates of these molecules are antigenically relevant[4–8] and serve different biological functions, one of the most important of which is its participation in cell–cell and/or cell–host recognition phenomena.[9]

The cosmopolitan fungus *Plectosphaerella cucumerina* can become a severe plant pathogen under appropriate conditions. Nevertheless, its systematics, as well as that of *Plectosporium tabacinum*, its anamorph, are problematic.[10,11] Historically, *P. cucumerina* has been included in the Hypocreales[12,13] but Uecker,[11] based on ascoma development, concluded that it should be placed in the Sordariales.

The polyphyletic genus *Verticillium* is the anamorph of several genera belonging to the Hypocreales.[14,15]

Here we report on the novel structure of the polysaccharides F1SS of *P. cucumerina*, *V. dahliae*, and *V. albo-atrum* and discuss the taxonomic placement of these species on the basis of their structures.

2. Results

The alkali-extracted water-soluble cell-wall polysaccharides (F1SS) amounted to 5–7% of the dry cell-wall material in the three species. They are composed of mannose (6%), galactose (60%), and glucose (26%) in *P. cucumerina*, as shown by gas–liquid chromatography (GLC), and mannose (14%), galactose (54%), and glucose (32%) in both species of *Verticillium*. In addition, 6–10% of uronic acids were detected by the carbazole test. Absolute configuration analysis showed the D configuration for all of the sugars.

In *P. cucumerina*, methylation analysis of the reduced polysaccharide gave the derivatives corresponding to terminal glucopyranose and galactopyranose, 6-O- and 2,6-di-O-substituted galactofuranose, 6-O-substituted glucopyranose, and 6-O-substituted mannopyranose, and 4-O-substituted glucopyranuronic acid (Table 1).

Methylation analyses were also performed on both species of *Verticillium*, giving similar components to those found in *P. cucumerina*, although traces of the derivative corresponding to terminal glucopyranuronic acid were identified (<1%), instead of that of 4-O-substituted Glc*p*A (Table 1).

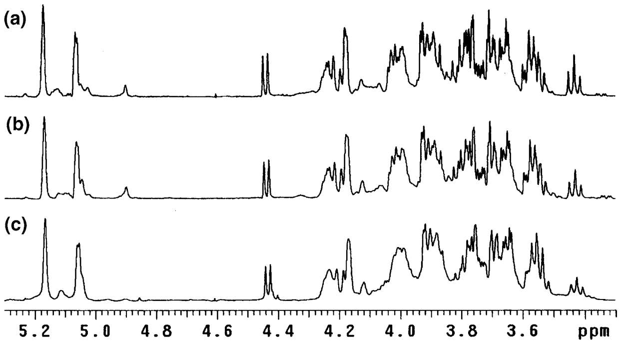


Figure 1. ^{1}H NMR spectra (D_2O, 40 °C, 300 MHz) of alkali-extracted water-soluble cell-wall polysaccharides F1SS isolated from: (a) *V. dahliae*, (b) *V. albo-atrum*, and (c) *P. cucumerina*.

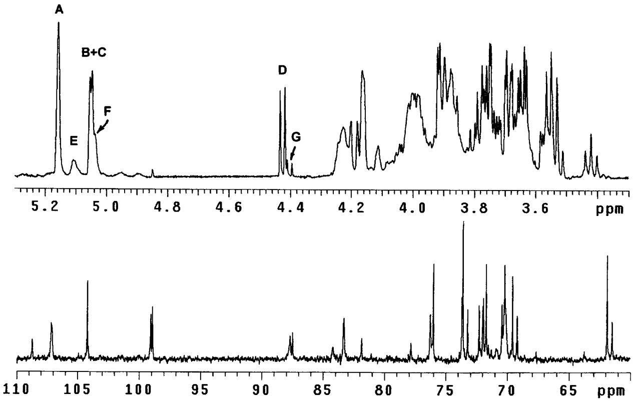


Figure 2. (a) ^{1}H NMR spectrum (D_2O, 40 °C, 500 MHz) and (b) ^{13}C NMR spectrum (D_2O, 40 °C, 125 MHz) of F1SS polysaccharide isolated from *P. cucumerina*. The anomeric peaks in the first spectrum have been labeled **A**–**G**.

The ^{1}H NMR spectra of the polysaccharides in the acid form were very similar in the three species (Fig. 1). Accordingly, the polysaccharide from *P. cucumerina* was then selected for further studies.

The high-resolution ^{1}H NMR spectrum (Fig. 2a) contained at least three major (5.16, 5.06, and 4.43 ppm) and three minor anomeric signals (5.12, 5.04, and 4.41 ppm).

The ^{13}C NMR spectrum (Fig. 2b) exhibited four major (107.1, 104.2, 99.0, and 98.9 ppm) and two minor

Table 1. Linkage types deduced from methylation analysis of F1SS polysaccharides

Linkage type	*V. albo-atrum* CECT 2693	*V. dahliae* CECT 2694	*P. cucumerina* CBS 137.33
Glc*p*-(1→	16.2	16.0	17.6
Glc*p*A-(1→	0.8	0.9	—
Gal*p*-(1→	22.7	20.6	22.3
→4)-Glc*p*A-(1→	—	—	8.3
→6)-Glc*p*-(1→	17.8	23.6	13.6
→6)-Gal*f*-(1→	4.5	2.3	2.0
→2,6)-Gal*f*-(1→	38.0	36.6	36.2

(108.7 and 104.1 ppm) anomeric singlets. Therefore one of the major anomeric signals in the ^1H NMR spectrum should contain two overlapping protons.

A 2D ^1H–^{13}C HMQC spectrum showed seven cross-peaks in the anomeric region, revealing that the doublet at 5.06 ppm contained two protons. A series of 2D homo- (DQCOSY, TOCSY) and ^1H–^{13}C hetero- (HMQC, HSQC-TOCSY) NMR experiments (see Fig. 3a and b) led to complete assignment of all the proton and carbon signals of the four main residues, labeled **A**–**D** from low to high field, and most signals of the three minor residues, labeled **E**–**G** (see Fig. 2a and Table 2).

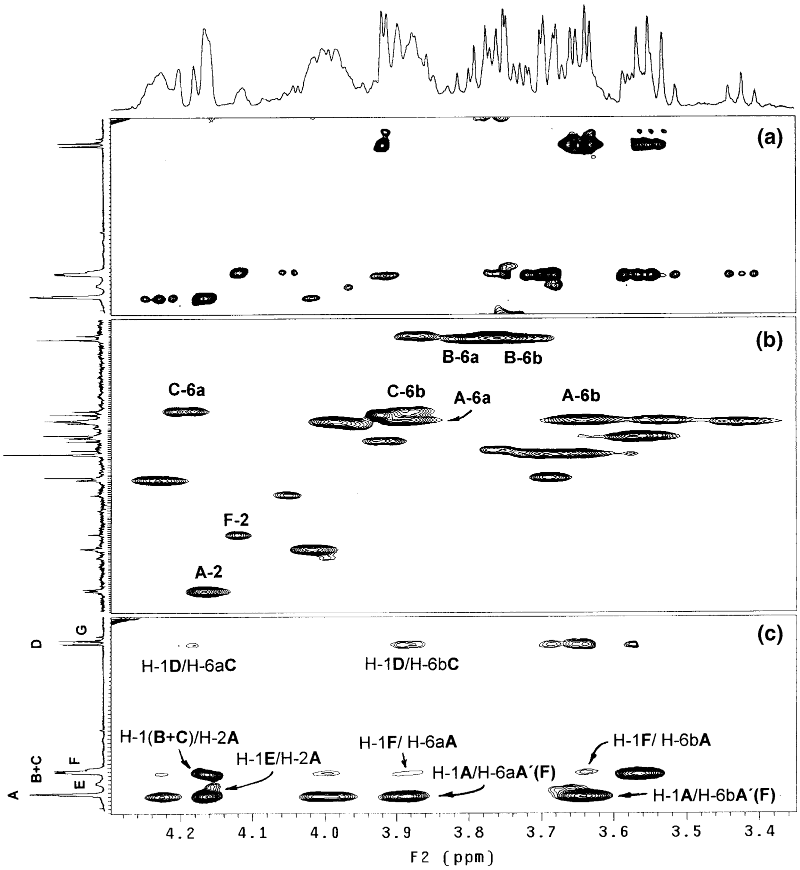


Figure 3. Key region of the 2D- (a) TOCSY, (b) HMQC, and (c) NOESY NMR spectra of the polysaccharide F1SS isolated from *P. cucumerina*. Relevant crosspeaks have been labeled.

Table 2. ^1H and ^{13}C NMR chemical shifts (δ) at pD = 3.0 for the alkali-extractable water-soluble cell-wall polysaccharide isolated from *Plectosphaerella cucumerina*

Residue		Proton or carbon[a]						
		1	2	3	4	5	6a	6b
A	H	5.16	4.16	4.23	4.01	3.98	3.89	3.64
	C	107.1	**87.6**	76.2	83.2	70.4	**70.1**	
B	H	5.06	3.57	3.70	3.42	3.75	3.86	3.75
	C	99.0	71.9	73.6	70.4	73.2	61.4	
C	H	5.06	3.56	3.71	3.54	3.92	4.20	3.89
	C	98.9	71.9	73.6	70.1	72.2	**69.1**	
D	H	4.43	3.56	3.65	3.92	3.69	3.78	3.72
	C	104.2	71.9	73.5	69.5	76.0	61.9	
E	H	5.12	3.66	3.69	3.97			
	C	99.2	71.3					
F	H	5.04	4.12	4.05	4.00		~3.89	~3.64
	C	108.7	81.8	77.8	84.2		~**70.2**	
G	H	4.40	3.56	3.65	3.92			
	C	104.1	71.8	73.5				

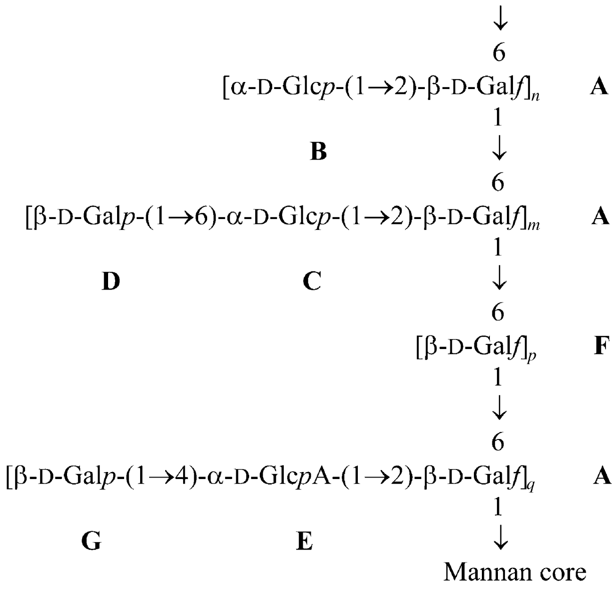
[a] Underlined bold numbers represent glycosylation sites.

Observation of the anomeric proton coupling constants and comparison of the values obtained with those of model compounds[16–18] permitted the assignment of **A** to 2,6-di-O-substituted β-Gal*f*; **B**, to terminal α-Glc*p*; **C**, to 6-O-substituted α-Glc*p*; **D** and **G**, to terminal β-Gal*p*; **E**, to 4-O-substituted α-Glc*p*A, and **F**, to 6-O-substituted β-Gal*f*. The anomeric configuration of the residues was inferred from the proton coupling constants and also from the chemical shifts of the anomeric carbons. Thus, galactofuranoses **A** and **F** showed $^3J_{1,2} \leqslant 2$ Hz, which are indicative of the β configuration (compare with α-Gal*f* values $J_{1,2} > 4$ Hz[19]), **B** and **C** showed $^3J_{1,2} = 3.7$ Hz, demonstrative of the α configuration, and **D** and **G** exhibited $^3J_{1,2} = 7.8$ Hz, which reveal the β configuration for both units. The coupling constant of **E** was deduced to be around 3.8 Hz, compatible with the α configuration.

Concerning the connection of the different units, a 2D-NOESY experiment (mixing time = 300 ms, Fig. 3c) contained, in addition to the expected intraring signals, the relevant crosspeaks gathered in Table 3, from which the presence of fragments **B**→2**A**→6**A**′(**F**), and **D**→6**C**→2**A**→6**A**′ were deduced, where **A**′ represents a second unit of **A**. Analogously, an HMBC experiment showed the relevant crosspeaks indicated in Table 4, thus unambiguously confirming the linkages deduced from the NOESY spectrum.

Connection of the small terminal β-Gal*p* unit (**G**), most probably linked to the 4-*O*-Glc*p*A residue (**E**), could not be clearly stated on the basis of NMR data, due to the paucity of the amounts of **G** and **E**. However, the fact that both residues were in analogous proportion, and the absence of signals **E** and **G** in the spectra of both *Verticillium* species, where traces of terminal glucuronic acid were detected in the methylation analyses instead of 4-*O*-Glc*p*A, seem to provide consistent support for the presence of the fragment **G**→4**E** in the structure of *P. cucumerina*.

From all the combined data, an idealized structure of the polysaccharide is proposed to be



$$\begin{array}{rl}
\downarrow & \\
6 & \\
[\alpha\text{-D-Glc}p\text{-}(1\rightarrow 2)\text{-}\beta\text{-D-Gal}f]_n & \mathbf{A}\\
1 & \\
\mathbf{B}\qquad\qquad \downarrow & \\
6 & \\
[\beta\text{-D-Gal}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 2)\text{-}\beta\text{-D-Gal}f]_m & \mathbf{A}\\
1 & \\
\mathbf{D}\qquad\qquad\qquad \mathbf{C}\qquad\qquad \downarrow & \\
6 & \\
[\beta\text{-D-Gal}f]_p & \mathbf{F}\\
1 & \\
\downarrow & \\
6 & \\
[\beta\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{A-}(1\rightarrow 2)\text{-}\beta\text{-D-Gal}f]_q & \mathbf{A}\\
1 & \\
\mathbf{G}\qquad\qquad\qquad \mathbf{E}\qquad\qquad \downarrow & \\
\text{Mannan core} &
\end{array}$$

Table 3. Connectivity between residues in *P. cucumerina*, as revealed by NOESY experiments

	Residue						
	A	B	C	D	G	E	F
H-1 of **A** (2,6 Gal*f*)	H-6a+b	H-1	H-1				H-6a+b
H-1 of **B** (*t*-Glc*p*)	H-2						
H-1 of **C** (6-Glc*p*)	H-2						
H-1 of **D** (*t*-Gal*p*)			H-6a+b				
H-1 of **E** (4-Glc*p*)	H-2						
H-1 of **F** (6-Gal*f*)	H-6a+b						H-6

Table 4. Connectivity between residues in *P. cucumerina*, as revealed by an HMBC experiment

	Residue						
	A	B	C	D	G	E	F
H-1 of **A** (2,6 Gal*f*)	C-6						C-6
C-1 of **A** (2,6 Gal*f*)	H-6a+b						
H-1 of **B** (*t*-Glc*p*)	C-2						
C-1 of **B** (*t*-Glc*p*)	H-2						
H-1 of **C** (6-Glc*p*)	C-2						
C-1 of **C** (6-Glc*p*)	H-2						
H-1 of **D** (*t*-Gal*p*)			C-6				
H-1 of **F** (6-Gal*f*)	C-6						
C-1 of **G** (*t*-Gal*p*)			H-6a				

where the subindices are around $n = 13$, $m = 13$, $p = 5$, and $q = 8$ for *P. cucumerina*, and $n = 16$, $m = 16$, $p = 6$, and $q < 1$ for both *Verticillium*. In addition, in both ^{1}H NMR spectra of *Verticillium* spp., a signal at 4.91 ppm and other small signals between 5.0 and 5.3 ppm could be observed (Fig. 1), the first corresponding to 6-O-substituted α-Man*p* and those at lower field to the 2-O-substituted α-Man*p* units forming part of the mannan core, as have been previously observed in several fungal F1SS polysaccharides.[1] The subindices *n*–*q*, deduced by using integration of the anomeric protons of the NMR spectrum, represent the proportions of the corresponding side chains in the polysaccharide and not the real sequence, since the possibility of finding the positions that each side chain hold along the galactofuranan backbone is still beyond reach.

3. Discussion

Polysaccharides F1SS from several hypocrealean fungi have a small α-(1→6)-mannan core and long chains of β-(1→6) galactofuranose, almost fully branched at O-2 by different side chains. Usually, one type of branch is neutral, containing a single glucopyranose residue, and the other is acidic, with single residues of glucuronic acid,[16] di- or tri-saccharides of glucuronic acid and β-mannopyranose[3,17,20] or disaccharides of glucuronic acid and β-glucopyranose.[1,21] The repeating unit of the polysaccharide from *P. cucumerina* has similar features, but terminal β-galactopyranose residues are incorporated into half of the neutral chains and to all the acidic chains (see structure above). The polysaccharides of *V. dahliae* and *V. albo-atrum* lack the terminal galactopyranose residue in the acidic chain. These findings indicate that the three species analyzed here are closely related, which is in agreement with the results of DNA analysis.[22]

These structures relate the three species studied to one of the lineages of hypocrealean fungi, characterized by their F1SS polysaccharides with a β-(1→6)-galactofuranose main chain. This structure differs from other lineages of this order whose F1SS polysaccharide has a backbone of β-(1→6)-β-(1→5)-galactofuranose.[23,24]

Uecker[11] has suggested the relationship of *P. cucumerina* with members of the Sordariales. The polysaccharides F1SS from genera belonging to this order consist of a chain of α-(1→6)-mannopyranose partially substituted at the O-2 positions by single residues of β-galactofuranose.[25–27] Therefore, since they are unambiguously different from the polysaccharides analyzed herein, it can be safely concluded that *P. cucumerina* and the two species of *Verticillium* belong to the Hypocreales and that they are phylogenetically unrelated to the Sordariales.

4. Experimental

4.1. Microorganisms and culture media

The species used were: *P. cucumerina* (CBS 134.35), *Verticillium dahliae* (CECT 2694), and *V. albo-atrum* (CECT 2693). The microorganisms were maintained on slants of Bacto potato dextrose agar supplemented with 1 g L^{-1} of Bacto yeast extract (Difco). The basal medium and growth conditions have been previously described.[28]

4.2. Wall material preparation and fractionation

The preparation of wall material[29] and the fractionation procedure[30] were performed as previously described. The alkali-extracted and water-soluble fractions from the three species (100 mg) were subjected to gel-permeation chromatography under conditions previously described[30] in order to purify the F1SS polysaccharide.

4.3. Chemical analysis

For analysis of neutral sugars, the polysaccharides were hydrolyzed with 3 M trifluoroacetic acid (1 h at 121 °C). The resulting monosaccharides were converted into their corresponding alditol acetates[31] and identified and quantified by gas–liquid chromatography (GLC) using an SP-2380 fused silica column (30 m × 0.25 mm I.D. × 0.2 μm film thickness) with a temperature program (210–240 °C, initial time 3 min, ramp rate 15 °C min^{-1}, final time 7 min) and a flame-ionization detector.

The monosaccharides released after hydrolysis were also derivatized as devised by Gerwig et al.[32] and their absolute configuration was determined by GC–MS of the tetra-*O*-Me$_3$Si-(+)-2-butylglycosides using an SPB-1 fused silica column (30 m × 0.25 mm I.D. × 0.2 μm film thickness) with a temperature program (150–250 °C, initial time 3 min, ramp rate 2 °C min^{-1}, final time 30 min). The components of the sample were identified on the basis of their retention times and mass spectra.

4.4. Determination of uronic acid

The uronic acid was determined by using the carbazole reaction, with D-glucuronic acid as standard.[33]

4.5. Methylation analyses

Previously to methylation analysis, the carboxylic groups of uronic acids were reduced using NaBD$_4$ according to Taylor and Conrad.[34] The reduced polysaccharide was methylated following Ciucanu and Kerek's method[35] and hydrolyzed sequentially with 90% formic acid in water at 80 °C for 2 h and 5 M CF$_3$CO$_2$H at 100 °C for 5 h. The products were reduced with $NaBH_4$ and then acetylated as above. The corresponding partially methylated alditol acetates were examined by GC–MS using a SPB-1 column (30 m × 0.25 mm, 0.2 μm film thickness), a temperature program (160–210 °C, 1 min initial hold, 2 °C/min ramp rate) and a mass detector Q-mass from Perkin–Elmer. Quantifications were made according to peak area.

4.6. NMR analysis

A solution of each F1SS polysaccharide (pD $\approx$ 7.0) was stirred with Amberlite IR-120 to convert the sodium salt of uronic acids into the free carboxylic acids. The resulting acidic solutions (pD $\approx$ 3.0) were freeze-dried. Around 20 mg of this material was dissolved in D_2O (99.98%; 0.8 mL) followed by centrifugation (10,000*g*, 20 min). The supernatants ($\sim$0.7 mL) were used for recording H NMR spectra. The sample from *P. cucumerina* for 2D experiments was lyophilized, redissolved in D_2O (1 mL) and the process repeated twice for further deuterium exchange. The final sample was dissolved in 0.7 mL of D_2O (99.98% D).

^1H NMR spectra for F1SS polysaccharides were recorded at 40 °C on a Varian INOVA (300 MHz). 1D- and 2D- ^1H and ^{13}C NMR experiments for *P. cucumerina* were carried out at 40 °C on a Varian Unity 500 spectrometer with a reverse probe and a gradient unit. The 2D NMR experiments (DQF-COSY, TOCSY, NOESY, HMQC, HSQC-TOCSY, and HMBC) were performed by using the standard Varian software. Proton chemical shifts refer to residual HDO at δ 4.66 and carbon chemical shifts to internal acetone at δ 31.07.

Acknowledgments

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