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Pullulans produced by strains of *Cryphonectria parasitica*—II. Nuclear magnetic resonance evidence

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Abstract

The structure of pullullan-like polysaccharides produced as exocellular material by different strains of *Cryphonectria parasitica*, the fungus responsible for chestnut tree cankers, was investigated with nuclear magnetic resonance (NMR) techniques. ^{13}C, mono- and bidimensional ^{1}H, and ^{1}H–^{13}C heteronuclear correlated NMR spectra (HSQC and HMBC) were recorded. Advanced analysis of the NMR spectra allowed the main resonance of the atoms in the maltotriose and in the maltotetraose repeat units of pullulan-like polysaccharides from *C. parasitica* to be recognised with confidence. In all cases investigated, the presence of large amounts of α-(1→6) maltotetraose subunits was evidenced, in addition to the α-(1→6) maltotriose subunits, corresponding to the repeating unit of pullulan produced by *Aureobasidium pullulans* and other fungi. The results were in agreement with other data from this laboratory, obtained with independent techniques. The belief that in 'pullulans' the maximum amount of α-(1→6) maltotetraose subunits is about 7% can thus be considered as definitely outdated.

Keywords: Pullulan(s); Structure; *Cryphonectria parasitica*; Exopolysaccharide(s); NMR spectroscopy

1. Introduction

In a previous paper on this Journal (Forabosco et al., in press), the characterization of pullulans produced by three strains of *Cryphonectria parasitica* (Murrill) M.E. Barr was reported. The study was carried out with gel permeation chromatography, gas–liquid chromatography, capillary viscosimetry and static laser light scattering. The strains of *C. parasitica* were CP159 (virulent), isolated from a bark sample taken from canker margins of chestnut trees (*Castanea sativa* Mill.), CP263 (hypovirulent), isolated from cankers of recovering stands in Southern Italy (Rionero, Basilicata), and CP102, with intermediate virulence, obtained by segregation of monoconidial culture of hypovirulent strains in vitro. This cultural variant was never isolated from naturally infected chestnut plants.

It is well established that the maltotriose trimer α-(1→4)Glu*p*-α-(1→4)Glu*p*-α-(1→6)Glu*p*- represents the main repeat unity of pullulan produced by *Aureobasidium pullulans* (de Bary) G. Arnaud, a mitosporic fungus formerly called *Pullularia pullulans* (de Bary) Berkhout (syn.: *Dematium pullulans* de Bary) (Gibbs & Seviour, 1996; Leathers, 2003). However, other structures, and in particular the tetramer or maltotetraose α-(1→4)Glu*p*-α-(1→4)Glu*p*-α-(1→4)Glu*p*-α-(1→6)Glu*p*-, may be present in the polymeric chain of pullulan (Wallenfels, Keilich, Bechtler & Freudenberger, 1965). According to Carolan, Catley and McDougal (1983), the different repeating units should be randomly distributed throughout the molecule. In addition, according to other authors (Catley, Ramsay & Servis, 1986), 7% represents the maximum extent to which maltotetraose subunits have been detected so far. This is the reason why in the literature the term 'pullulan' is currently used for both the 'polymaltotriose' produced by *A. pullulans* and the polysaccharide varieties, similar to the pullulan, produced by other microbial species.

The present study aimed at (i) confirming with an independent technique the previous evidence from this laboratory, according to which the cited strains of *C. parasitica* produced 'pullulan-like polysaccharides' with much higher contents in the maltotetraose units than what is reported in the literature, (ii) verifying with modern techniques the structure proposed for these pullulan-like polysaccharides and (iii) elucidating the location of the maltotetraose units along the polysaccharide chain.

For this study, ^{13}C, mono- and bidimensional ^{1}H, and ^{1}H–^{13}C heteronuclear correlated NMR spectra (Heteronuclear

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Single-Quantum Coherence spectra, HSQC, and high resolution Heteronuclear Multiple Bond Correlation spectra, HMBC) of polysaccharides dissolved in D_2O were recorded. Throughout this paper, the discussion refers to the general structure and labels shown in Fig. 1, where the (trimeric) structure of the repeat unity of the common pullulan is indicated by the sequence *A*-*B*-*C* and the maltotetraose unity is built up by the four glucose rings A'-B'-B''-C'. Accordingly, the glycosidic linkages to the subsequent glucose rings are α-(1→4), α-(1→4), α-(1→6) for *A*, *B*, and *C* and α-(1→4), α-(1→4), α-(1→4), α-(1→6) for A', B', B'' and C', respectively.

In other words, the general structure of the linear glucans here called pullulan-like polysaccharides is supposed to be built up by trimers *A*-*B*-*C*, with the possible insertion of another glucose between *B* and *C*. This insertion was found to modify the chemical environment and hence the chemical shift of some atoms of the glucose rings *A*, *B* and *C*, that therefore in the tetrameric unity were named A', B' and C', respectively (Fig. 1(b)). The glucose ring present in the maltotetraose unity and absent from the maltotriose unity was named B' because its topological situation should not differ significantly from that of the ring named *B* in the trimer.

No other structures, as detected in some 'pullulans' (Leathers, 2003), were found in the polysaccharides from *C. parasitica*.

2. Experimental section

2.1. Materials

The pullulan-like polysaccharides were obtained by Prof. L. Sparapano and coworkers at the University of Bari, Italy, as exocellular material from three different strains of *C. parasitica*. A preceding paper (Forabosco, Bruno, Sparapano, Liut, Marino & Delben, in press) reported the description of the strains, the conditions for the production of the exopolysaccharides, the procedures for their extraction from the culture medium, and finally the methods used for their purification. The polysaccharides were labelled with the name of the producing fungal strain, plus 'up' or 'lo' to indicate the 'upper' or 'lower' fractions obtained in the purification of the polysaccharide, respectively. In addition, the presence of glucose or sucrose as the carbon source in the mineral culture medium was indicated with the letters 'G' or 'S', respectively.

The following samples were studied with NMR: CP159upG, CP159loG, CP159loS, CP263upG, CP263loG, CP263loS and CP102loS. For comparison purposes, the commercial pullulans purchased from Hayashibara Co. (Okajama, Japan), Pullulan PI-20, lot 01207, and from Sigma (USA), Pullulan P4516, lot 078H1060, were also studied.

2.2. ^1H NMR spectra

^1H NMR experiments were performed with 500 MHz Varian mod. VRX-UNITY INOVA and Bruker Avance 500 spectrometers, both operating at 500.13 MHz for ^1H and equipped with a multinuclear reverse 5 mm probe (TXI). The solutions were prepared dissolving 5 up to 9 mg of lyophylized pullulan per mL of D_2O (99.99% D_2O from Isotec Inc., Matheson, USA). Proton spectra were obtained with presaturation of the residual HOD signal during relaxation. The spectra were recorded at constant

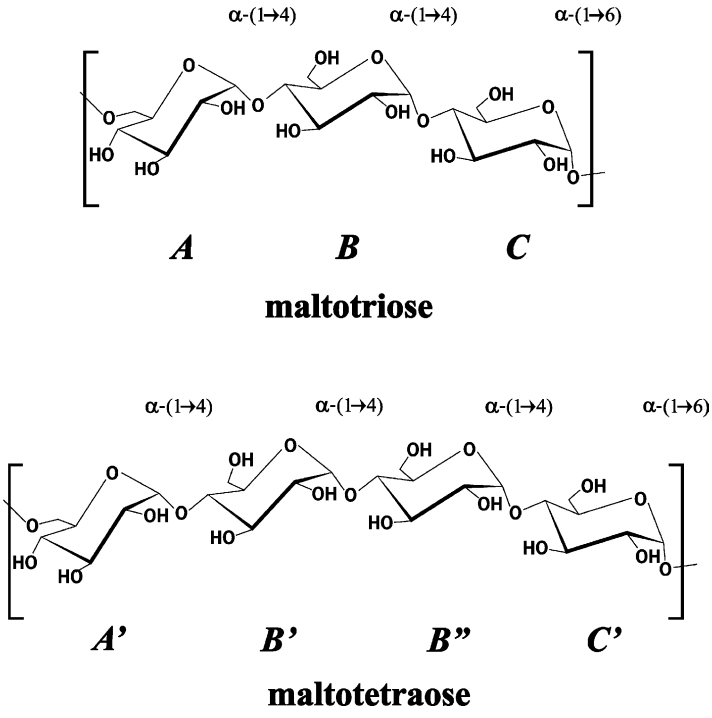

Fig. 1. Repeat units of pullulan and pullulan-like polysaccharides from *Cryphonectria parasitica*. The D-glucose *A*-*B*-*C* rings represent the (maltotriose) structure of pullulan from *Aureobasidium pullulans*. The insertion of the further ring B'' transforms the trimeric repeat unit in the (maltotetraose) tetrameric one, given by the glucose A'-B'-B''-C' rings.

temperature in the 25–70 °C range. The sweep width was about 12 ppm (6000 Hz), with a time domain size of about 16000. For each measurement, 128 scans were performed, with recycling time of about 3 s, i.e. 1 s for fixed delay and 2 s as the presaturation time. Chemical shift values were measured downfield from sodium trimethylsilyl propionate (TSP) used as the external standard.

In addition to monodimensional ^{1}H NMR spectra, conventional double-quantum-filtered COrrelated SpectroscopY (COSY), TOtal Correlation SpectroscopY (TOCSY) and Nuclear Overhauser Enhancement SpectroscopY (NOESY) spectra were recorded using matrices between 1 K×256 w and 2 K×512 w.

The TOtal Correlation SpectroscopY (TOCSY) spectra were acquired with 32 scans and 768 free induction decays in order to increase the resolution of the second dimension, $\omega 1$. The spectra were Fourier transformed onto a data matrix of 4 K×2 K with a phase shifted ($\pi/2$) square sine-bell function.

2.3. ^{13}C NMR spectra

^{13}C NMR experiments were performed with AC200 and AMX400 Bruker spectrometers, operating for ^{13}C at 50.3 and 100.61 MHz respectively, both equipped with a 10 mm multinuclear probe. Spectra were run at a probe temperature of 30 or 50 °C. The samples were dissolved in D_2O at a final concentration of 10 up to 30 mg mL^{-1}. The ^{13}C spectra were measured with proton decoupling during acquisition time. Recycle delay was 2 s and 3.000–10.000 scans were recorded. The spectra were obtained by applying the Fourier transform to the Free Induction Decay (FID), after exponential multiplication of 3.0 Hz.

The calibration was made using MeOH signal as the external standard at 51.75 ppm with respect to the TSP signal.

2.4. Heteronuclear correlated bidimensional NMR spectra

Two-dimensional gradient enhanced Heteronuclear Single-Quantum Coherence (HSQC) and high resolution Heteronuclear Multiple Bond Correlation (HMBC) spectra were recorded at 50 °C with carbon decoupling during acquisition time. The polarisation transfer delay ($\Delta = 1/[2\times {}^{1}J_{C-H}]$) was set with a $^{1}J_{C-H}$ coupling value of 150 Hz. 512 experiments were performed, and the data points were acquired on memory of 1 K. To enhance memory, the obtained matrix, with a size of 1 K×512 w, was zero filled to 4 K×2 K and treated by application of a squared cosine function prior to Fourier double transformation.

3. Results and discussion

3.1. Chemical shift assignments and identification of the polysaccharide repeat unity

The ^{13}C NMR spectra of the pullulan-like exopolysaccharide CP159upG and the Sigma commercial pullulan are in Fig. 2(a) and (b), respectively.

For the commercial pullulan, the assignments of the peaks were performed according to Gorin (1981) and to Benesi and Brant (1985), who in turn verified their interpretation using evidence from previous papers (Colson, Jennings & Smith, 1974; Heyraud, Rinaudo, Vignon & Vincendon, 1979; Morris & Hall, 1982). The presence in the spectrum of well defined and sharp peaks testified the high purity of the samples, as well as the absence of appreciable extent of oligomers. The peaks were 18 and showed similar intensities. The spectrum recorded was compatible with the expected structure for the pullulan, i.e. the polymaltotriose structure. The signals at about 102.8, 102.4 and 100.8 ppm, corresponding to α-(1→4), α-(1→4) and α-(1→6) glycosidic bonds of the glucose rings *A, B* and *C,* respectively (see Fig. 1), verified that the maltotriose unit

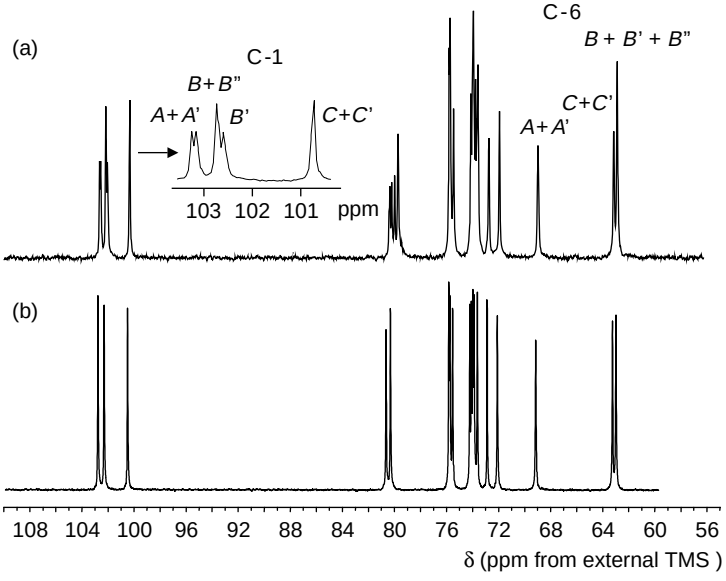

Fig. 2. ^{13}C NMR spectra of (a) CP159upG and (b) Sigma pullulan from *A. pullulans*.

corresponded to the building block of the polysaccharide chain (Gibbs & Seviour, 1996; Leathers, 2003). The absence of oligomers was confirmed by the absence of the signals, in the region between 93 and 97 ppm, due to the carbon atoms of the chain reducing ends.

In the case of the pullulan-like polysaccharides produced by *C. parasitica*, the anomeric signals of the rings *A* and *B* were split in two components (Fig. 2(a)), exhibiting different intensities, while the corresponding signal of *C* remained unchanged. Taking as unitary the anomeric signal of the residue $C+C'$, that appeared at 100.83 ppm, the intensities of the two components of the signals of *A* and *B* were 0.5 : 0.5 and 1 : 0.5, respectively. They were interpreted as due to the anomeric carbon atoms of *A* and *B* in the maltotriose and maltotetraose repeat units, respectively, namely *A* and A', and *B*, B' and B'', respectively.

The peaks around 80 ppm were generated by the resonance of the C-4 atoms of the rings *B* and *C*, involved in the glycosidic bonds. In the case of the commercial pullulans, that have the polymaltotriose structure, for the C-4 atoms only two peaks were present, at 80.87 and 80.52 ppm, and the areas under the peaks were similar (Fig. 2(b)). The peaks were interpreted as due to the resonance of the C-4 atoms in the rings *B* and *C*, respectively.

On the contrary, for the pullulan-like polysaccharides from *C. parasitica* four resonance peaks were detected in this region (Fig. 2(a)). Attributing to the highest of them an intensity equal to unity, the areas under the other peaks corresponded to about half unity. The four C-4 signals were interpreted as due to the resonance of atoms belonging to the glucoses *B*, B', B'' and *C*, respectively. So far, it was impossible to assign the peaks due to the C-4 atoms of these glucose rings with higher precision. Conversely, the signals at 80.20 and 80.46 ppm were interpreted as due to the C-4 atoms belonging to the *C* and C' rings, respectively. It is worth pointing out that, on passing from the commercial pullulans to the polysaccharides produced by *C. parasitica*, the signal of the C-4 atom of the ring *C* was shifted toward higher fields by as much as 0.4 ppm.

For all the polysaccharides investigated, the signal due to resonance of the C-4 atoms of the rings *A* and A', that are not engaged in any glycosidic bond, was detected at about 72 ppm.

All these signals were also detected in the high resolution HSQC spectrum (Fig. 3(a)). Their relative intensities, calculated by integration of HSQC cross-peaks (Guerrini, Naggi, Guglieri, Santarsiero & Torri, 2005), agreed with those measured by the monodimensional carbon spectrum. As shown by the anomeric region of high resolution HSQC spectrum (Fig. 3(b)), the two components of the residue *B* had similar proton chemical shift, whereas the proton chemical shifts of *A* and A' differed of about 5 Hz. As expected, the *A*, *B* and *C* glucose rings showed different signals pattern in the TOCSY spectrum (Fig. 4) due to the different glycosidic linkages in which they are involved.

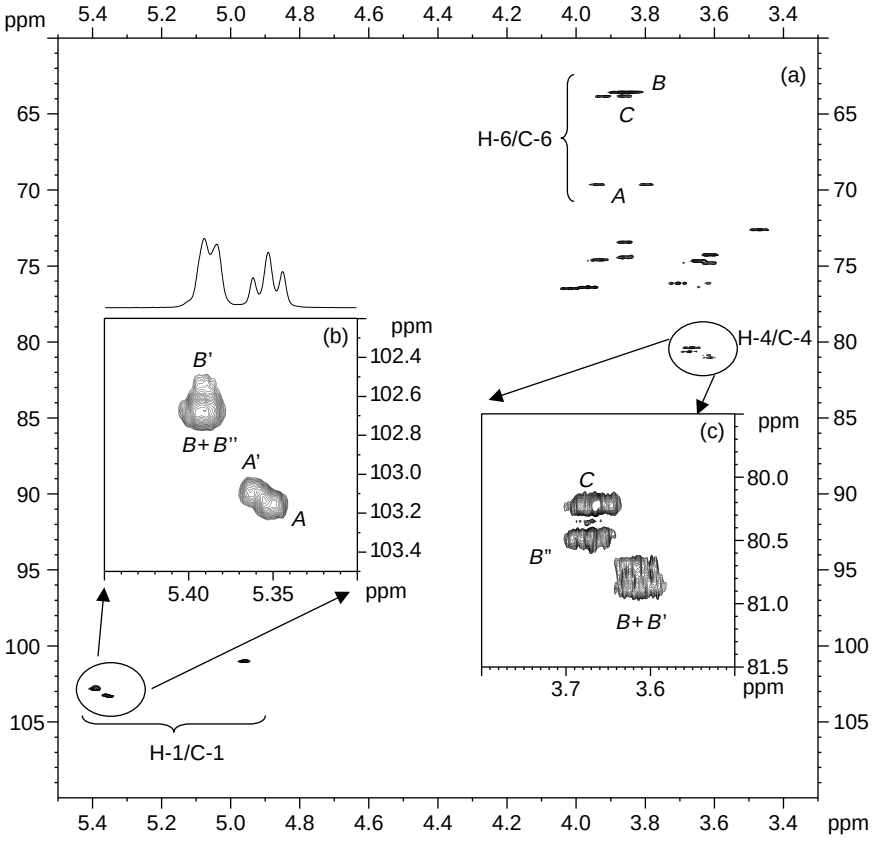

Fig. 3. (a) Heteronuclear Single-Quantum Coherence (HSQC) spectrum of CP263upG and magnification of (b) the anomeric nuclei region and (c) the C-4 atoms region.

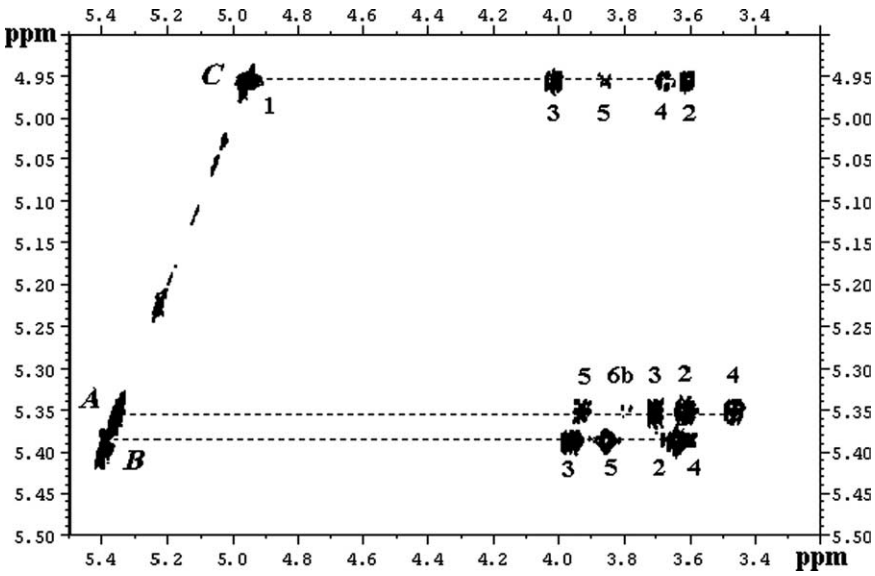

Fig. 4. High resolution TOtal Correlation SpectroscopY (TOCSY) of CP263upG.

It should be noted that the signals recorded at 5.39, 5.35 and 4.95 ppm in the monodimensional ^{1}H NMR spectrum (Fig. 5), markedly differing in chemical shift, were interpreted as due to the anomeric H atoms belonging to a 4-substituted and α-(1→4) linked glucose residue, to a 6-substituted and α-(1→4) linked residue and to a 4-substituted α-(1→6) linked residue, respectively, i.e. to *B*, *A* and *C* rings, respectively (Fig. 1).

As shown in Fig. 1, the glucose residues *B*, *B′* and *B″* are different because they are characterised by three different situations in the type of linkage of the adjacent residues. In fact, while the residues *B* and *B″* show the same profile in the direction of the reducing end, being α-(1→4) and α-(1→6) the linkages between the subsequent glucose rings, two α-(1→4) glycosidic linkages follow the residue *B′* in the same direction. Therefore, the most intense signals observed in the carbon spectrum in the region of the anomeric atoms should be given by the C atoms of the rings having the former topological situation, i.e. *B* and *B″*, whereas the weaker signal agreed with an α-(1→4) glucose residue placed in the latter sequence, i.e. the ring *B′*, that is present in the tetramer.

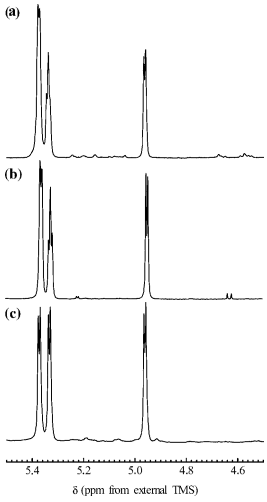

Fig. 5. Region of the anomeric protons of ^{1}H NMR spectra of (a) CP159loS, (b) CP263upG, and (c) Hayashibara pullulan.

To evaluate the influence of the structural environment on the resonance of the anomeric carbon atoms of the glucose residues *A* and *A′*, and *C* and *C′*, respectively, a longer chain sequence, bearing three glycosidic linkages, had to be taken into account. Toward the reducing end, the residue *A*, belonging to the maltotriose structure, is followed by two α-(1→4) and one α-(1→6) glycosidic linkages, while *A′*, belonging to the maltotetraose repeat unity, is followed by three α-(1→4) glycosidic linkages. The two different sequences were believed to generate the two signals in the ^{13}C NMR spectrum at 103.07 and 103.13 ppm, respectively (Fig. 2(a)). The sequences in which the glucose residues *C* and *C′* are involved do not differ up to three glycosidic linkages, that are in both cases α-(1→6), α-(1→4), α-(1→4). For this reason a single anomeric signal was generated, as it is shown in the carbon spectrum (Fig. 2(a)).

The proposed structures, i.e. the presence of maltotriose and maltotetraose repeat units in the polysaccharide chain, would be compatible with five C-4 atom signals. As previously mentioned, in the carbon spectrum of pullulan-like polysaccharides produced by *C. parasitica* four signals were observed, one of which having double intensity with respect to the others (Fig. 2(a)). The poor resolution of 2D (HMBC and HSQC–NOESY) spectra did not allow the complete assignments in this spectral region to be performed. It should only be recalled that for the assignments of the peaks generated by C-4 carbon atoms the glycosidic linkages must be taken into account toward both the reducing and non-reducing end directions.

The peaks in the higher field region were interpreted as due to the resonance of the C-6 atoms. In particular, the peak at 69.46 ppm was assigned to the resonance of the C-6 atoms of *A*, that are involved in the α-(1→6) glycosidic bond, while the corresponding signals of the C-6 atoms of the rings *C* and *B* were detected at 63.65 and 63.38 ppm, respectively. It is easily

Table 1
Chemical shift values for pullulan-like exopolysaccharides from *Cryphonectria parasitica* as indicated by ^{1}H (upper rows) and ^{13}C (lower rows) monodimensional NMR spectra

Atom (H or C)	A[a]	$A^{(a)}$	B	B'	B''	C	C'
1	5.357	5.346	5.386	5.386	5.386	4.955	4.955
	103.07	103.13	102.66	102.52	102.66	100.83	100.83
2	3.608	3.608	3.637	3.637	3.637	3.606	3.606
	74.64	74.64	74.50	74.50	74.50	74.10	74.10
3	3.699	3.699	3.961	3.961	3.961	4.014	4.014
	75.96	75.96	76.23	76.23	76.23	76.31	76.31
4	3.465	3.461	3.664[b]	3.605[c]	3.615[c]	3.664[b]	3.673[c]
	72.44	72.44	80.20[b]	80.84[c]	80.68[c]	80.20[b]	80.46[c]
5	3.927	3.927	3.851	3.851	3.851	3.855	3.855
	74.42	74.42	74.26	74.26	74.26	73.26	73.26
6	3.938/3.796[d]	3.938/3.796[d]	3.878/3.833[d]	3.878/3.833[d]	3.878/3.833[d]	3.923/3.859[d]	3.923/3.859[d]
	69.46	69.46	63.38	63.38	63.38	63.65	63.65

The D-glucose rings (first row) are labelled as indicated in Fig. 1
[a] The chemical shift assignments of A and A' can be reversed.
[b] Assignments done by comparison with the pullulan ^{13}C spectrum (Fig. 2(b)).
[c] The chemical shift assignments for the atoms H-4 and C-4 of B', B'' and C' can be reversed.
[d] Chemical shift values of the diasterotopic protons *a* and *b*.

observed that for the pullulan-like polysaccharides produced by *C. parasitica* the area under the peak at 63.38 ppm was one third higher than those under the peaks at 63.65 and 69.46 ppm. This difference should be significant in determining the sequence of maltotriose and maltotetraose repeat units along the polymer chain and will be taken again in the following Section 3.3.

The peaks at 63.38, 63.65 and 69.46 ppm should be generated by the resonance of the C-6 atoms of the $B+B'+B''$ rings, of the $C+C'$ rings, and finally of the $A+A'$ rings, respectively. No split was observed in the signals of the C-6 atoms, very likely because the hydroxy-methyl groups have a higher degree of freedom than the CH groups of the pyranosidic rings.

The HSQC spectrum of CP263upG (Fig. 3(a)) supports the assignment of C-6 atoms done by the analysis of the ^{13}C NMR spectrum. The diasterotopic effect, observed for the *a* and *b* H-6 atoms (Table 1), is stronger when the corresponding C-6 atom is involved in the glycosidic linkage (residue *A*), being the phenomenon due to a restriction of the conformational freedom. This effect, also observed for H-6 atoms of both residues *B* and *C*, gets stronger, as the distance of the ring from the 1–6 glycosidic linkage decreases.

The information given by the ^{13}C NMR spectra was used to identify the resonance of the anomeric protons in the ^{1}H NMR spectra of our polysaccharides using the bidimensional HSQC eteronuclear technique. The study of the HSQC–TOCSY spectrum (Fig. 6) and the mononuclear ^{1}H TOCSY spectrum (unreported) allowed the following assignments in the monodimensional ^{1}H NMR spectra.

In the region between 4.9 and 5.4 ppm, the peaks were due to the resonance of the anomeric H atoms only (Fig. 5). More precisely, for the pullulan-like polysaccharides from *C. parasitica* the peaks at 5.39, 5.35 and 4.95 ppm were due to the resonance of the anomeric protons of the $B+B'+B''$, $A+A'$, and $C+C'$ rings, respectively (Fig. 5(a) and (b)). The peak at about 5.35 ppm, although it resembled a triplet, was formed by two different resonance signals generated by the protons of the rings A and A', involved in the trimeric and tetrameric sequences, respectively. The chemical shift patterns of the anomeric protons in the A and A' residues, such as in B and B' residues, did not show significant differences, so indicating the presence of the same type of linkages and substitution in the pairs B, B' and A, A'. In particular, the chemical shifts of the two H-1 peaks of A and A' differed from each other as much as 5 Hz, as measured on a high resolution TOCSY spectrum obtained through a matrix of 4 K and a linear prediction procedure of 1.5 Hz (Fig. 4). This interpretation was supported by the observation that splitting of the peaks due to the presence of the ring B'' in the polymer structure was detected only for the anomeric atoms (both C-1 and H-1) and for the H-4 nuclei, whose chemical shifts differed as much as about 1.5 Hz, while the peaks due to the resonance of all the other atoms remained unchanged on passing from the commercial pullulans to the polysaccharides produced by *C. parasitica*.

In the same experiment, a further complexity was observed, that confirmed the relationship between signal multiplicities

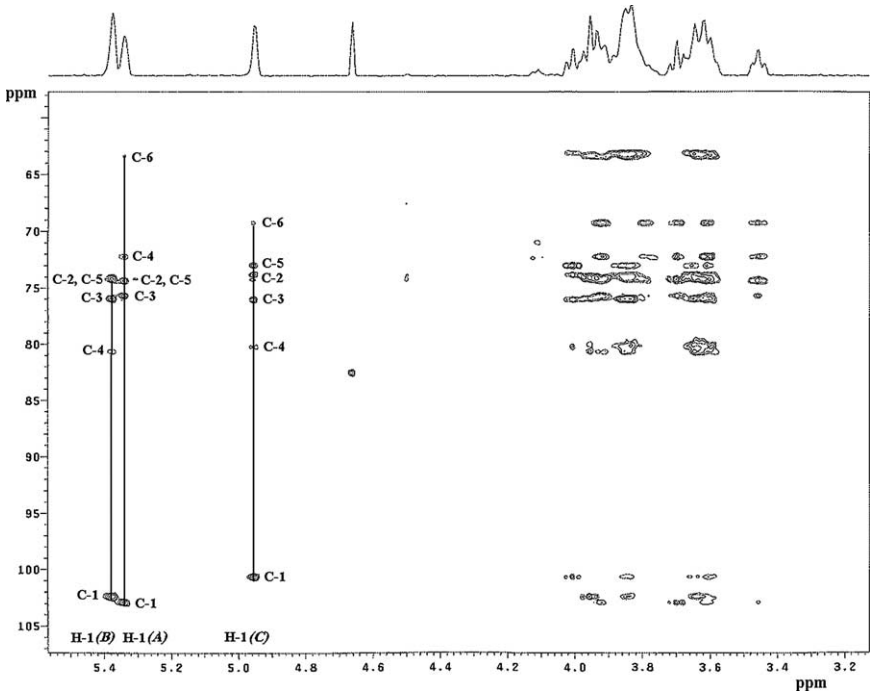

Fig. 6. Heteronuclear Single-Quantum Coherence–TOtal Correlation SpectroscopY (HSQC–TOCSY) spectrum of CP159upG. The correlation between the C atoms of the glucose residues *A*, *B* and *C* is shown.

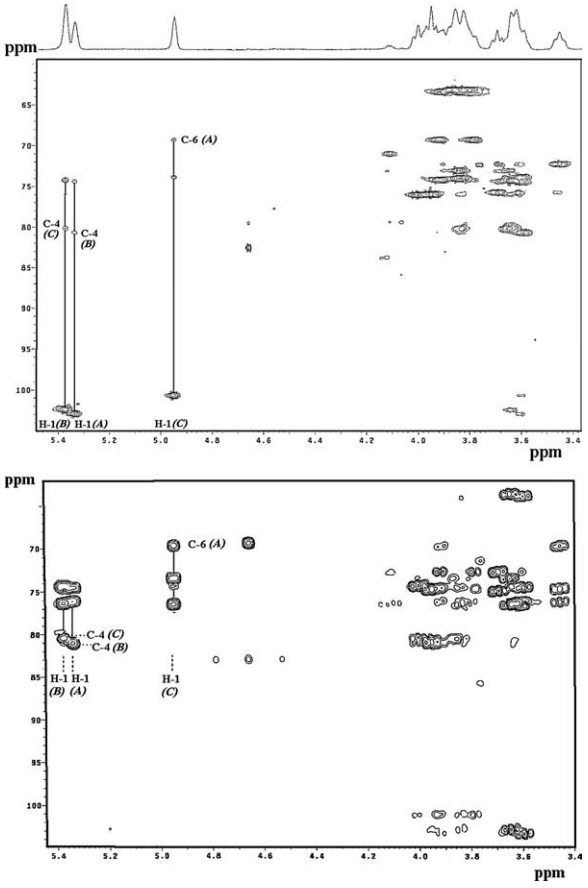

Fig. 7. (a) Heteronuclear Single-Quantum Coherence–Nuclear Overhauser Enhancement SpectroscopY (HSQC–NOESY) spectrum of CP159upG; (b) Heteronuclear Multiple Bond Correlation (HMBC) spectrum of the same sample. The long-range proton-carbon correlation across the glycosidic linkages is shown.

and structural inhomogeneity. In fact, the doublet at 5.39 ppm also showed a poor symmetry in the signal, so suggesting a signal complexity. Using the MaxEnt elaboration procedure, the peak analysis gave two signals as a result, one of which prevalent, having a difference in the chemical shift of about 1 Hz.

In the case of the commercial pullulans, the monodimensional ^{1}H NMR spectrum showed three identical doublets, due to the resonance of the H atoms belonging to the *B*, *A* and *C* glucose rings (Fig. 5(c)).

The assignments of the peaks due to resonance of the protons of the other rings were performed by bidimensional COSY and TOCSY spectra.

Finally, the H atoms bound to the C-4 atoms of the ring *A* were also identified. This and all other attributions were done using the spectrum HSQC–TOCSY reported in Fig. 6. The assignments were confirmed by the values found with the integration of the volumes under the peaks in the bidimensional ^{1}H HSQC spectra, performed as reported elsewhere (Guerrini et al., 2005).

The complete list of values of the identified chemical shifts is in Table 1.

The HSQC–NOESY and the HMBC spectra of CP159upG are reported in Fig. 7(a) and (b), respectively. These spectra are generated by the interactions between the atoms through space and along the glycosidic bonds, respectively, and allowed us to define the sequence of the rings. According to the previously reported assignments, these techniques agreed indicating the following correlation as the most relevant in the anomeric spectral region. The H-1 atoms of *A* correlate with the C-4 atoms of *B*, the H-1 atoms of *B* correlate with the C-4 atoms of *C*, and the H-1 atoms of *C* correlate with the C-6 atoms of *A* (Fig. 7). As a consequence, the structure suggested for the

pullulan-like polysaccharides produced by different strains of *C. parasitica* was a chain built up by *A*-*B*-*C* and A'-B'-B''-C' repeat units, characterised by the glycosidic bonds indicated in Fig. 1.

The correlation between the other components of *A* and *B* was not possible, because of the insufficient resolution of the two-dimension experiments.

The results here presented were considered as reliable, since the ^{13}C NMR spectra of all polysaccharides produced by *C. parasitica* were comparable from the qualitative point of view, with the important difference in the relative values of the areas under the peaks attributed to the resonance of the C-1 and C-6 atoms. Indeed, the bidimensional HSQC spectrum, reported in Fig. 3 for CP263upG, was also recorded for the pullulan-like exopolysaccharides obtained from other fungal strains, so confirming their structural peculiarity.

3.2. Maltotriose/maltotetraose ratio along the polysaccharide chain

The analysis of the NMR spectra was also used to get information on the extent of the maltotetraose sequences along the chain of the pullulans. The computations were performed mainly using the peaks given by the anomeric protons in the ^{1}H NMR spectra. The ^{13}C NMR resonance of C-1 and C-6 atoms were also used. The signals of corresponding atoms were considered as suitable for quantitative analyses, because chemically equivalent carbon atoms were only compared and the proton decoupling was only performed during the acquisition time and not during the relaxation time. In the case of the C-6 ^{13}C NMR spectra, the calculations were performed with both the intensities of the peaks, and the areas under them.

As is well known, the commercial pullulans, that have maltotriose as the repeat unity, do not indicate different relaxations in the chain. The areas under the three ^{1}H NMR peaks due to the anomeric atoms were identical (Fig. 5(c)). The presence of a couple of identical peaks due to resonance of the C-1 atoms of each glucose ring in the ^{13}C NMR spectra reinforced the indication that no structures different from the trimer were present along the chain.

On the contrary, the ^{1}H NMR spectra of the pullulan-like polysaccharides produced by different strains of *C. parasitica* showed pronounced differences between each other in the areas under the peaks due to the anomeric hydrogen atoms of the D-glucose *A*, *B* and *C* rings. Fig. 5(a) and (b) reports the ^{1}H NMR spectra of CP159upG and CP263upG, respectively. For each spectrum, the areas under the peaks due to the resonance of the anomeric protons of $A+A'$, $\mathrm{Area}_{(A)}$, and $C+C'$, $\mathrm{Area}_{(C)}$, must be equal, therefore in the computation their average value, $\mathrm{Area}_{(A,C)}$, was mostly used, being:

$$\mathrm{Area}_{(A,C)} = \frac{1}{2}[\mathrm{Area}_{(A)} + \mathrm{Area}_{(C)}] \quad (1)$$

Differently, the area under the peak at about 5.39 ppm was somewhat higher in both cases, because of the presence of the ring B'' in addition to the rings $B+B'$. The percentage of the maltotetraose structure, *X*, was computed by the following expression:

$$X = 100\frac{[\mathrm{Area}_{(B)} - \mathrm{Area}_{(A,C)}]}{\mathrm{Area}_{(A,C)}} \quad (2)$$

where $\mathrm{Area}_{(B)}$ is the area under the peak due to the resonance of the anomeric hydrogen atoms of *B*, B' and B''. In a few cases, the deconvolution of the peaks due to the resonance of the anomeric protons of the $B+B'+B''$ and $A+A'$ rings was difficult because an extended overlapping. In these cases, the computation of *X* was done using the following expression:

$$X = 100\frac{[\mathrm{Area}_{(A,B)} - 2\mathrm{Area}_{(C)}]}{\mathrm{Area}_{(C)}} \quad (3)$$

being $\mathrm{Area}_{(A,B)}$ the total area under the peaks due to the resonance of the anomeric protons on *A*, A', *B*, B' and B''.

The volumetric integration of the signals in the bidimensional ^{1}H HSQC spectra (Guerrini et al., 2005), confirmed both the assignments of the peaks, as stated above, and the computed values of *X*.

The values of *X* for the pullulan-like polysaccharides from *C. parasitica* are in Table 2, where they are compared with the corresponding values found with methylation analysis and already published (Forabosco *et al*., in press).

Analogous computations were done using the ^{13}C NMR peaks due to resonance of the C-6 atoms. The peaks appeared in the 63–70 ppm range and were well separated. In this case, the percentage of the maltotetraose structure was computed using the expression (4):

$$X = 100[F_{(B)} - F_{(C)}]/F_{(C)} \quad (4)$$

where $F_{(B)}$ is a function related to the peak at higher field, due to the resonance of the C-6 atoms belonging to the glucose rings *B*, B' and B'' rings, and $F_{(C)}$ is the same function related to the peak at lower field, due to the resonance of the C-6 atoms belonging to the glucose *C* and C' rings. The functions *F* stem for the intensities of the related peaks or, alternatively, the values of the areas under them.

The values of the percentage of maltotetraose obtained by ^{13}C NMR spectra are not reported in Table 2 because they are not completely reliable for the reasons explained before.

Table 2
Percentage of the tetramer in the pullulan-like polysaccharides

Sample	NMR[a]	GC[b]
CP159upG	86	–
CP159loG	87	89
CP159loS	66	75
CP263upG	47	42
CP263loG	–	52
CP263loS	46	37
CP102loS	58	50

[a] Determination made using the areas under the peaks of the anomeric atoms in the ^{1}H NMR spectra.

[b] Determination made with gaschromatographic measurements (Forabosco et al., in press).

However, they were found to be in appreciable agreement with the corresponding values computed using the ^{1}H NMR spectra.

3.3. Determination of the polysaccharide structure

The homogeneity exhibited by our ^{13}C NMR spectra and the sharpness of the peaks (see the ^{13}C NMR spectrum of CP159upG in Fig. 2(a) as a sample case) indicated the presence of regular structures along the polymer chains of the pullulans under study. In fact, a random distribution of maltotriose and maltotetraose sequences along the polysaccharide chain should generate much broader peaks.

Two regular structures were considered as the most reliable ones, the former being a regular alternation of the trimeric and tetrameric sequences along the chain, the latter being the presence of blocks of maltotriose units, followed by blocks of maltotetraose units. The authors believe that the former structure is much more probable for three reasons. First, the already discussed differences in the intensities of the peaks due to the resonance of the C-6 atoms and in the areas under them was believed to suggest the presence of alternating maltotriose and maltotetraose sequences in the structure of these polysaccharides. Secondly, a block structure should be made up of a large amount of repeat units, that could only eliminate the effects due to the change in the sequence in the NMR spectra. In other words, random distribution along the polymer chain of short trimeric and tetrameric sequences would generate much broader peaks than those recorded because of the change in the sequence of the repeat units. The third evidence was given by the same intensities exhibited by the signals due to the resonance of C-1 and C-4 atoms of the ring *A* in the ^{13}C NMR spectrum of CP263upG, that were recorded at 103.1 and 72.4 ppm, respectively.

On this basis, the evaluation of the structure was tentatively proposed for CP263upG. In the ^{1}H NMR spectrum of this polysaccharide (Fig. 5(b)), two small doublets were detected, at 4.64 and 5.23 ppm. They are due to the resonance of the reducing β and α anomers, respectively.

Indeed, similar peaks were also found in the spectra of the other pullulans from *C. parasitica*, but they were mostly too small or irregular to be taken into account for sensible calculations. It should be underlined that the ^{1}H NMR technique appeared much more sensible and effective in detecting the oligomers present in the polysaccharide solutions than the ^{13}C NMR one, by which the reducing ends anomeric atoms were not detected at all (Fig. 2(a)).

If $\mathrm{Area}_{(\alpha,\beta)}$ is the total area under the peaks at 4.64 and 5.23 ppm and $\mathrm{Area}_{(C)}$ is the area under the peaks at 4.96 ppm, due to the H-1 atoms in the *C* rings, from the expression:

$$\frac{\mathrm{Area}_{(\alpha,\beta)}}{\mathrm{Area}_{(\alpha,\beta)} + \mathrm{Area}_{(C)}} \times 100 \qquad (5)$$

the percentage of reducing ends with respect to the total anomeric protons is obtained, that for CP263upG was 4.65. Should the polymer chain built up by 100 repeat units, both maltotriose and maltotetraose, the total number of anomeric protons would be $3\times100+44=344$, being 44 the average percentage of maltotetraose structures for this polysaccharide (Table 2). Dividing 344 by 4.65, one obtains 74, that is the average number of glucose rings per polymeric chain. This value gives an average molecular weight of the order of 10^{4} g mol^{-1}, that is compatible with the shape of the NMR spectra recorded with this sample. The disagreement with the molecular weight data already published (Forabosco et al., in press) can be attributed to depolymerisation of the polysaccharides due to the prolonged high temperature of NMR experiments (up to 70 °C) and/or the activity of specific enzymes, as happened for similar systems (unpublished evidence from our laboratory).

Should it be true that in the chain the trimeric and tetrameric sequences regularly alternate, as indicated by the sharpness of the NMR peaks and verified by the evidence recalled before, each chain would be constituted of 10 (trimer + tetramer) sequences, an eleventh trimer and finally one further ring. The constraints are verified as follows. In this case, the percentage of tetraose sequences is $100\times10/(10+11+0.33)=47$, that is very close to the value 46, that in turn is the average value found with the calculation based on NMR measurements (Table 2), and the number of total rings results $10\times4+11\times3+1=74$, as required.

4. Conclusions

^{1}H and ^{13}C NMR measurements, here presented and discussed as an extension of the study reported in a preceding work (Forabosco et al., in press), allowed the complete characterisation of the pullulan-like polysaccharides produced by three strains of *C. parasitica* exhibiting different virulence.

The spectra recorded indicated the presence of polymeric samples of high purity and ordered configuration. This permitted the small differences between different samples in the ^{1}H and ^{13}C NMR spectra to be detected. E.g. the resonance of the C-4 atoms in the 80 and 81 ppm region, respectively, showed at least four components, with relative intensities of 0.5 : 0.5 : 0.5 : 1, instead of the two signals present in the commercial pullulan spectrum (Fig. 2(b)).

Thanks to both monodimensional and bidimensional spectra, the complete assignment of the peaks was performed. In particular, the analysis of heterocorrelated spectra and the study of high resolution TOCSY spectra were found to be very useful.

All the spectral properties of the pullulan-like exopolysaccarides produced by *C. parasitica* described above agree with the presence of both maltotriose and maltotetraose sequences in the polysaccharide backbone (Fig. 1). The presence of the α-(1→4) linked glucose ring B'', that transforms the trimeric sequence into the tetrameric one, induces shift of the signals of the adjacent residues, that is more evident for the atoms involved in the glycosidic linkages.

On the basis of the NMR analysis, the structure of the exopolysaccharides produced by *C. parasitica* was confirmed to be that of modified pullulans. Although somewhat different for different producing strains of *C. parasitica*, the structure of such exopolysaccharides was verified to contain, in all cases, a

large amount of maltotetraose, that was much higher than so far indicated in the literature for these polysaccharides (Catley et al., 1986), in agreement with independent studies from this laboratory.

In addition, in the case of CP263upG the NMR analysis allowed to give indications on the regular sequences of the trimer (maltotriose) and the tetramer (maltotetraose) in the polymer chain.

The elucidation of the structure for the complete set of pullulans produced by the *C. parasitica* strains could be of great help to understand the possible role of the pullulans in the phytopatogenicity of the producing fungi against the infected plants. For this study, the preparation of pullulans with molecular weights not exceeding 10^4 g mol^{-1} seems to be mandatory. Only in that case, in fact, the ^{1}H NMR technique could show the peaks due to the reducing anomeric groups, that allows the computations here suggested and performed in the case of the sample CP263upG.

The correlation between the virulence of the strains of *C. parasitica* and the structure of pullulans produced by the fungus was out of the aims of the present study. However, the authors sincerely encourage researchers towards the suggested investigation, that could bear important evidence to the hypothesis presented by the authors in the preceding paper (Forabosco et al., in press) and to the different one suggested by Molinaro, Piscopo, Lanzetta and Parrilli (2002) on the possible role of polysaccharides in the phytopatogenesis.

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References

Benesi, A. J., & Brant, D. A. (1985). Trends in molecular motion for a series of glucose oligomers and the corresponding polymer pullulan as measured by ^{13}C NMR relaxation. *Macromolecules*, *18*, 1109–1116.

Carolan, G., Catley, B. J., & McDougal, F. J. (1983). The location of tetrasaccharide units in pullulan. *Carbohydrate Research*, *114*, 237–243.

Catley, B. J., Ramsay, A., & Servis, C. (1986). Observations on the structure of the fungal extracellular polysaccharide pullulan. *Carbohydrate Research*, *153*, 79–86.

Colson, P., Jennings, H. J., & Smith, I. C. P. (1974). Composition, sequence, and conformation of polymers and oligomers of glucose as revealed by carbon-13 nuclear magnetic resonance. *Journal of the American Chemical Society*, *96*, 8081–8086.

Forabosco, A., Sparapano, L., Bruno, G., Liut, G., Marino, D., & Delben, F. (in press). Pullulans produced by strains of *Cryphonectria parasitica*—I. Production and characterization of the exopolysaccharides. *Carbohydrate Polymers*.

Gibbs, P. A., & Seviour, R. J. (1996). Pullulan. In S. Dumitriu (Ed.), *Polysaccharides in medicinal applications* (pp. 59–86). New York: Marcel Dekker.

Gorin, P. A. J. (1981). ^{13}C-NMR spectroscopy of polysaccharides. *Advances in Carbohydrate Chemistry and Biochemistry*, *38*, 13–104.

Guerrini, M., Naggi, A., Guglieri, S., Santarsiero, R., & Torri, G. (2005). Complex glycosaminoglycans: Profiling substitution patterns by two dimensional NMR spectroscopy. *Analytical Biochemistry*, *337*, 35–47.

Heyraud, A., Rinaudo, M., Vignon, M., & Vincendon, M. (1979). Carbon-13 NMR spectroscopic investigation of α- and β-1,4-glucose homooligomers. *Biopolymers*, *18*, 167–185.

Leathers, T. D. (2003). Pullulan. In E. J. Vandamme, S. De Baets, & A. Steinbüchel (Vol. Eds.), *Biopolymers. Polysaccharides II: Polysaccharides from eukaryotes: Vol. 6* (pp. 1–25). Weinheim: Wiley–VCH.

Molinaro, A., Piscopo, V., Lanzetta, R., & Parrilli, M. (2002). Structural determination of the complex exopolysaccharide from the virulent strain of *Cryphonectria parasitica*. *Carbohydrate Research*, *337*, 1707–1713.

Morris, G. A., & Hall, L. D. (1982). Experimental chemical shift correlation maps for heteronuclear two-dimensional nuclear magnetic resonance spectroscopy. II. Carbon-13 and proton chemical shifts of α-D-glucopyranose oligomers. *Canadian Journal of Chemistry*, *60*, 2431–2441.

Wallenfels, K., Keilich, G., Bechtler, G., & Freudenberger, D. (1965). Untersuchungen an Pullulan. IV. Die Klärung des Strukturproblems mit physikalischen, chemischen und enzymatischen Methoden. *Biochemische Zeitschrift*, *341*, 433–450.