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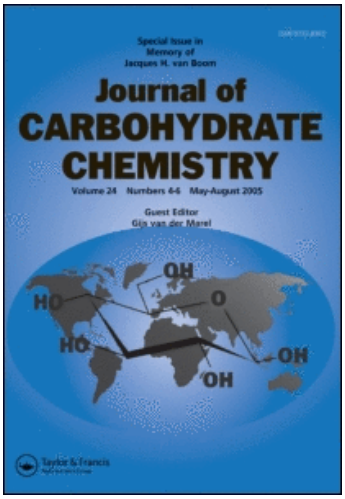

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STRUCTURAL ANALYSIS OF SUCCINOYLATED AND CHLOROFORMATE ACTIVATED PULLULAN:NMR STUDY IN DMSO SOLUTION

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ABSTRACT

A new technique is proposed in order to assign the substitution sites of pullulan after a reaction. The OH proton resonances of pullulan are identified in DMSO solution, after complete analysis of its proton spectrum using Correlated Spectroscopy (COSY) and Homonuclear Hartmann-Hahn (HOHAHA) experiments.

INTRODUCTION

A number polysaccharides have been used as carrier molecules in pharmaceutical applications, including the preparation of polymer prodrugs.[1] There has been an increasing interest in the use of pullulan for the design in drug delivery systems, e.g., for the preparation of macromolecular prodrug derivatives.[2] Direct coupling of polysaccharides with drugs is only possible if the latter have the appropriate functionality. In most cases, the carrier molecule or the drug must be transformed into a suitable reactive derivative enabling covalent coupling.

As part of an ongoing project on macromolecular prodrugs, various methods for the activation of pullulan have been studied, including succinoylation and chloroformate activation. The objective of this study was to assign the site of substitution after a reaction of the glucan with either succinic anhydride or 4-nitrophenyl chloroformate. NMR was the technique of choice.

The region of the OH proton resonances in the ^{1}H NMR spectra of D-glucose, methyl glucosides, glucobioses and glucotrioses has been exhaustively studied by Casu and coworkers.[3,4] Pullulan is a linear glucan consisting of a repeating trisaccharide unit, containing two $\alpha(1\rightarrow4)$ linkages and one $\alpha(1\rightarrow6)$ linkage. The glucoside units in the repeating trisaccharide are labelled Glc I, Glc II and Glc III respec-

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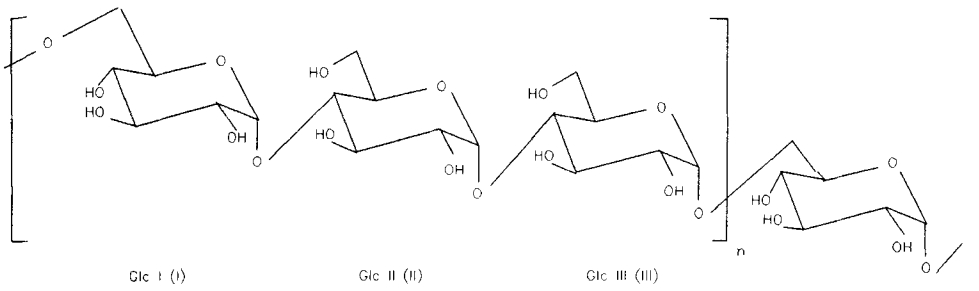
tively. The ^{1}H and ^{13}C NMR parameters of pullulan in D_2O have been published.[5]



The presumed complete set of proton chemical shifts of pullulan in D_2O was obtained from Correlated Spectroscopy (COSY), Relayed Coherence Transfer (RCT) and double RCT experiments.[5] Although with these techniques the connectivities between the protons on C-1, C-2, C-3 and C-4 were unequivocally assigned in the three moieties, the assignments of H-5, H-6A and H-6B in the appropriate units was not straightforward from these experiments. The ^{13}C NMR chemical shifts[6] were recently verified by HMQC (Heteronuclear Multiple Quantum Coherence, or Reverse Heteronuclear Correlated Experiment).[5] So far no such data in DMSO-d_6 have been reported. DMSO-d_6 is the medium of choice for an NMR study of pullulan, since the OH resonances can be studied in this medium.

In some cases it is relatively easy to assign the substitution site in glucan derivatives. It is well recognized that in the ^{1}H NMR spectrum of a carbohydrate, acetylation or benzoylation cause a downfield shift of 1 - 1.5 ppm on the ring proton at the carbon of substitution.

As a case study, acetylation and benzoylation of dextran has been exhaustively studied by Gagnaire and Vignon.[7] Unfortunately, in the crowded region δ 3.30-3.80 ppm, the resonances can not always be differentiated, because of incomplete esterification. Incomplete esterification gives ^{13}C NMR spectra too complex to interpret with great certainty.

For these reasons we have developed an alternative technique to interpret ^{1}H NMR spectra from such acylated polysaccharides. It was anticipated that, provided exchange phenomena were eliminated and the solutions were kept very dry, the identification of OH proton resonances in DMSO and the differences in the integration of the OH resonance before and after the reaction may provide statistical information on the acylation site. The spectral region for the OH proton resonances and the resonances for the anomeric protons is less crowded and is

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completely isolated from the resonance of the other skeleton protons. In this study we have explored the possibilities of employing this technique with pullulan.

RESULTS AND DISCUSSION

The ^{1}H NMR data of pullulan in DMSO-d_6 are given in **Table 1**. The COSY 45 experiment plot from pullulan in DMSO-d_6 is given in **Figure 1**. Here the connectivities with the OH proton resonances are indicated. In **Figure 2** the HOHAHA spectrum of pullulan is given. Throughout the text the chemical shift values as extracted from the HOHAHA spectrum are used, which may be slightly different from those seen in the COSY spectrum (different concentrations were used and the spectra were recorded on different spectrometers).

1. EXTRACTION OF THE ^{1}H NMR PARAMETERS

The extraction of ^{1}H NMR chemical shifts is in principle straightforward by tracing the connectivities between the ring protons of each moiety starting from the anomeric protons in a COSY 45 experiment. Unfortunately, because of the width of the pullulan signals due to high molecular weight (M_n = 50032 and M_w = 99550) the cross peaks are very broad and overlap. Verification of the assignments is therefore necessary. Because of the poor resolution of the proton resonances, assignments based on connectivity with carbon resonances by a ^{13}C, ^{1}H heteronuclear correlated experiment, was not possible for the samples under study (although there are reports in the literature on useful HMQC experiments that might be employed).[5] For the assignments of the ^{1}H NMR resonances, COSY and HOHAHA experiments were used. The resonances of the anomeric protons were identified first.

Comparison of the ^{1}H NMR spectrum of pullulan in DMSO-d_6 and in DMSO-d_6 with traces of TFA (in order to shift the OH proton resonances towards higher frequencies) showed that the anomeric protons resonate at δ 4.98, 5.03 and 4.67 ppm, respectively. Usui et al.[8] and De Bruyn et al.[9] have studied the chemical shifts of the glucosidic protons in maltose and isomaltose, and it was reported that in D_2O the H-1 signal for a $\alpha(1\rightarrow4)$ linkage is found at δ 5.40 and for an $\alpha(1\rightarrow6)$ linkage at δ 4.99 ppm. In DMSO-d_6 these values shift about 0.3/0.4 ppm upfield. It can be concluded that the resonances for the anomeric protons at δ 5 belong to Glc I and II; the resonance at δ 4.67 to Glc III.

Knowing the resonances of the anomeric protons, identification of the resonances of H-2 in each ring is possible. The COSY 45 spectral plot of pullulan in DMSO-d_6 shows that the resonances at δ 4.98 and δ 4.67 have a connectivity with resonances close to δ 3.27, the resonance at δ 5.03 has a connectivity with the resonance at δ 3.34. These resonances show a connectivity with the OH resonances at δ 5.55 and a com-

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TABLE 1. 1H NMR data of α-D-glucopyranose, Me α-D-glucopyranoside and pullulan in DMSO-d_6.

compound	chemical shift (ppm)										
	H-1	H-2	H-3	H-4	H-5	H-6A	H-6B	OH-2	OH-3	OH-4	OH-6
α-**D**-glucopyranose	4.91	3.11	3.42	3.04	3.55	3.59	3.45	4.43	4.61	4.75	4.34
Me α-**D**-glucopyranoside	4.52	3.19	3.39	3.05	3.31	3.62	3.45	4.67	4.72	4.83	4.43
pullulan											
→6)-Glc*p*-α(1→4) (I)	4.98	3.28	3.40	3.06	3.75	3.65	3.50	5.0*	5.0	5.0	-
→4)-Glc*p*-α(1→4) (II)	5.03	3.34	3.64	3.27	3.64	3.90	3.50	5.6	5.5	-	4.7
→4)-Glc*p*-α(1→6) (III)	4.68	3.27	3.71	3.37	3.50	3.60	3.60	5.6*	5.5	-	4.5

* May be reversed

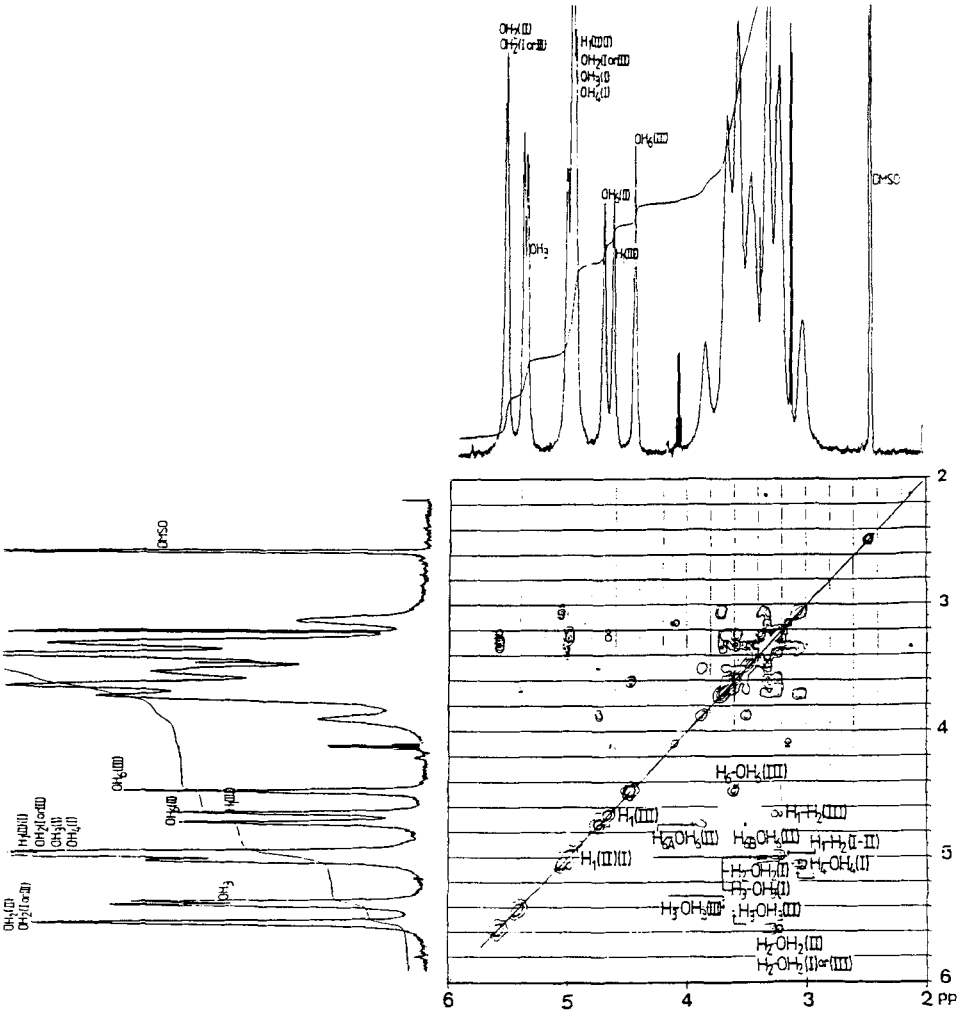

FIG. 1. Plot from COSY 45 experiment of pullulan in DMSO-d6

plex pattern at δ 5.0. The other resonances in the region δ 4.4-5.5 must be attributed to the three OH-3 proton resonances, the two OH-6 proton resonances of II and III and the OH-4 resonance of I.

In the COSY experiment a horizontal line through the collapsing resonances for H-2 of the moieties I and III encounters a crosspeak at δ 3.40 and δ 3.71, respectively, which are ascribed to H-3 resonances.

In DMSO solution the H-3 resonance at δ 3.40 has a crosspeak with the complex pattern of OH proton resonances at δ 5.0 and with the resonance at the highest field, namely δ 3.06, which can be assigned to H-4

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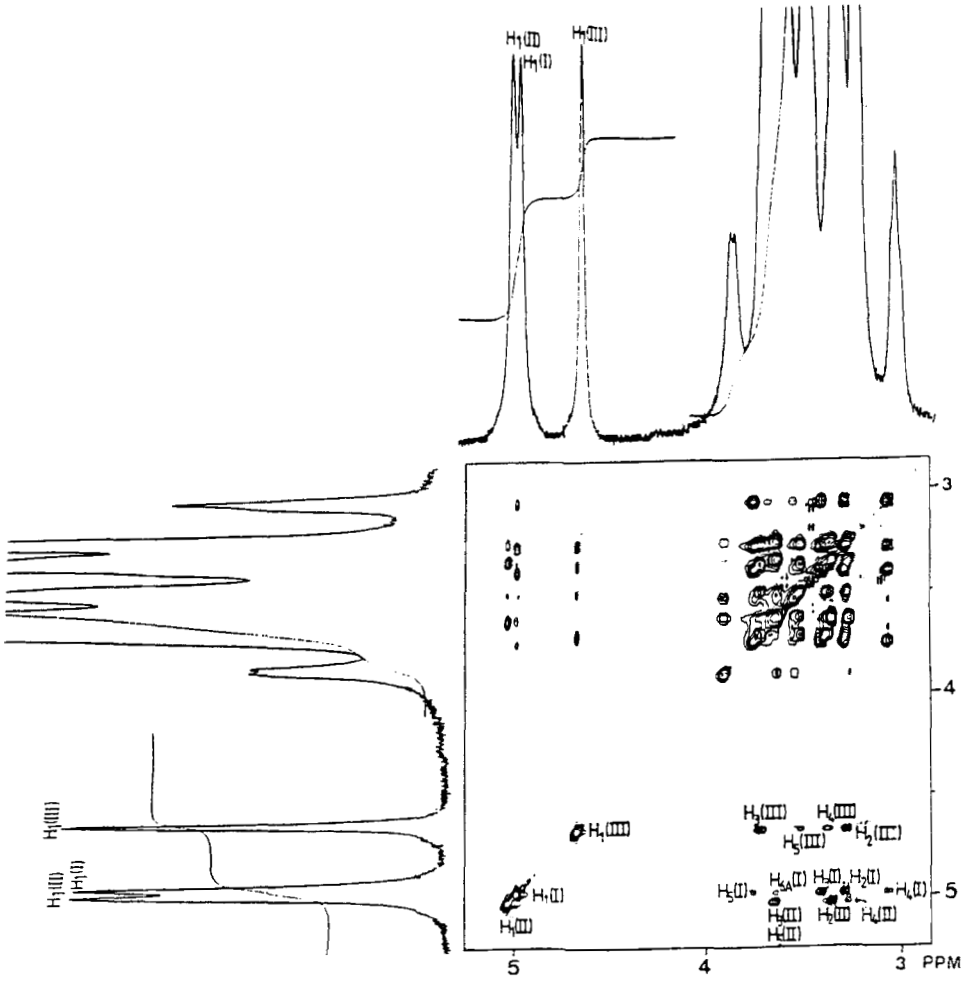

FIG. 2. HOHAHA experimental plot of pullulan in DMSO-d6

of the same moiety. The latter proton gives a crosspeak with a resonance at δ 3.75, which must consequently be assigned to H-5. Finally the resonance at δ 3.75 gives a cross peak with the H-6 resonances at δ 3.65 and 3.50.

The H-4 and H-3 signals of α-D-glucopyranose in DMSO-d_6 are found at δ 3.04 and δ 3.42, respectively. De Bruyn et al.[9] have shown that in the case of a 1(α→n) linkage (n= 2,3,4), the proton on the linked non-anomeric carbon and the axial protons on the neighbouring carbons, show a downfield shift of 0.10 to 0.35 ppm compared to α-D-glucopyranose.[9] The chemical shifts values for H-2, H-3 and H-4 for this

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ring indicate that they must belong to a unit that is not substituted at C-4. However, H-5 is found at δ 3.75. This is at a much higher frequency than the value found for H-5 in the parent α-**D**-glucopyranose. Furthermore no connectivity is seen with an OH proton resonance in the region of δ 3.65. This moiety must be 6-linked and α(1→4) linked. From the connectivities in the spectrum in DMSO it seems that H-4(I) connects with an OH-proton resonance at δ 5.0. These assignments for ring moiety I are easily verified in the HOHAHA spectrum after consideration of the cross section through δ 3.06 (H-4 I), where HOHAHA peaks are encountered at δ 3.28 (H-2 I), 3.40 (H-3 I), 3.50 (H-6B I), 3.65 (H-6A I), 3.75 (H-5 I) and 4.98 (H-1 I).

The site of the two collapsing H-2 resonances shows a further connectivity with a resonance at δ 3.71, assigned to H-3 of a second ring. Taking into consideration the proposal of De Bruyn et al.[9], the chemical shift value for this H-3 proton shows that it belongs to a moiety that is 4-linked (the δ value for H-3 in α-**D**-glucopyranose being 3.42). In the COSY 45 spectral plot of pullulan, the resonance at δ 3.71 gives a crosspeak at δ 3.37, assigned to H-4 of the second ring. This resonance is found at a higher frequency than found for the same H-4 proton in the parent sugar (**Table 1**). The resonance at δ 3.37 encounters a crosspeak at δ 3.50, ascribed to H-5. After having the signals from the third moiety, an OH proton resonance at δ 4.45 ppm remains unassigned but shows a connectivity with two (probably) collapsing resonances at δ 3.60. The H-6 resonances for this moiety (specified to be III) are found close to δ 3.60. These assignments are verified by the HOHAHA spectrum. A cross section through δ 4.68 (H-1 III) provides HOHAHA peaks at δ 3.27 (H-2 III), δ 3.71 (H-3 III), δ 3.37 (H-4 III) and δ 3.50 (H-5 III). In this slice the HOHAHA peaks for the H-6 protons are missing but a slice through δ 3.50 clearly shows the collapsing resonances at δ 3.60 for these protons.

The assignments of moiety II is straightforward from the COSY experiment. The resonance for H-2 (δ 3.34) gives a connectivity with a resonance at δ 3.64 (H-3), the latter having a connectivity with a resonance at δ 3.27 (H-4) which in turn is connected with a resonance at δ 3.64 (H-5). This resonance is connected with the resonance at δ 3.90 (crosspeak is missing in the COSY 45 spectrum, probably because the vicinal coupling constant $J_{5,6A}$ is expected to be 2Hz and is smaller than the resolution) and one at δ 3.50. In the COSY 45 experiment the crosspeak between the resonances for H-6A and H-6B is clearly present. These two H-6 resonances show a further connectivity with the OH proton resonance at δ 4.75. The assignments in the COSY spectrum are verified by the HOHAHA experiment. A slice through δ 3.90 (attributed to H-6A of II) indicates additionnal HOHAHA peaks at δ 3.64 (H-5 II), 3.5 (H-6B II) and 3.27 (H-4 II). A slice at δ 3.27 iden-

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tifies HOHAHA peaks at δ 3.64 (H-3 II), 3.34 (H-2 II) and 5.03 (H-1 II). A slice through δ 5.03 verifies the mentioned resonances, except for the resonance for H-6A II, that is missing.

It is important to observe that with the chemical shifts for H-2 (δ 3.34 for II and δ 3.27 for III), differentiation can be made between the two α(1-4) linked moieties, and the two OH-6 proton resonances of moiety II and III can be assigned with certainty.

The chemical shifts for the H-6 protons are not the values expected from glucobiose datas e.g., isomaltose.[9] The reason may be due to the conformation of the CH_2OR part.[10] Unfortunately the coupling constants ($J_{5,6A}$) can not be extracted, but the chemical shifts can be assigned. The H-6 resonances for the CH_2OH group compared to the CH_2OH group of III, are found at δ 3.60. The H-6 resonances compared to the CH_2O-α-D-glucopyranosyl part (II) are found at δ 3.90 and 3.50.

The OH proton resonances are assigned in a direct way and the assignments agree completely with the expectations from previous studies. The observations of St-Jacques et al.[11] are used for the amylose α(1→4) units. For amylose in DMSO the OH-2 resonances are at δ 5.50, OH-3 at δ 5.40 and OH-6 at δ 4.60. These values are confirmed by SECSY experiments of Taravel.[12] The values at the lower field region for the resonances of OH-2 of moiety II and OH-3 of moiety II with OH-2 of moiety III. The values for the resonances of OH-6 are expected at δ 4.5 and 5.7.

2. ASSIGNMENT OF THE SUBSTITUTION SITE IN TWO PULLULAN DERIVATIVES

The ^1H NMR spectrum of pullulan in DMSO is given in **Figure 1**. The signals from the OH-6 protons are found at δ 4.7 (moiety II) and δ 4.5 (moiety III) ppm. Pullulan has per 100 glucose units 233 secondary OH-groups and 67 primary OH-groups. For the reaction of pullulan with succinic anhydride, the equivalent of succinic anhydride added was less than the equivalent of primary alcohol groups. The substitution degree was about 20% (20 primary OH-groups of the 67 primary OH-groups have reacted). The same conditions are used for the reaction of pullulan with 4-nitrophenyl chloroformate. In the ^1H NMR spectrum of succinoylated pullulan, a decrease in integration of the two OH-6 signals for about 40-60% is observed. The integrations of the OH-2, OH-3 and OH-4 resonances remain unchanged. Also in the ^1H NMR spectrum of 4-nitrophenyl chloroformate activated pullulan, a decrease in integration of the two OH-6 signals for about 40-60% is observed. Because of the line broadening after reaction, only an apprximate quantification is possible.

The conclusion is that the OH-6 groups are the substitution site in pullulan during a reaction with succinic anhydride and 4-nitrophenyl chloroformate, while the reactivity of the other hydroxyl groups must be negligible.

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EXPERIMENTAL

Pullulan was obtained from Sigma Chemical Company (St. Louis, MO, USA) and was dried over phosphorous pentoxide at 90 °C during 48 h. The average molecular weight determined by analytical gel-permeation chromatography was: M_n= 50032, M_w = 99550. 4-Nitrophenyl chloroformate was obtained from Merck. It was used without further purification. Succinic anhydride was obtained from Fluka Chemika. It was purified by dissolving in dry chloroform and refluxing during 6 h. The warm solution was filtered and the filtrate was concentrated. 2-Hydroxypropylamine was obtained from Janssen Chemica (Beerse, Belgium) and distilled before use. (Methyl-sulfoxide)-d_6, 99.9% D was obtained from Aldrich Chemical Company, Inc.

The ^{1}H NMR spectra at 20 ° were obtained with a Bruker AM 500 spectrometer operating at 500.12 MHz, using a pulse angle of 19° and a resolution of 0.33 Hz/point.

The COSY 45 experiment involved the sequence 90°(^{1}H)-t_1-45°(^{1}H)-Acq.[13] The 90°(^{1}H) pulse was 7.90 μs and the relaxation delay was 1.5 ms. A π/2-shifted sine-bell function was used in each dimension. A 1 x 0.5 K matrix was obtained using 16 scans for a sweep width of 2994 Hz in the t_2 direction and 1497 Hz in the t_1 direction.

For the HOHAHA experiment, recorded on a Bruker AM-500 spectrometer, a slightly modified MLEV 17 based experiment proposed by Bax and Davis[14] is used. The initial 90° pulse was replaced by a 10°x60°-x140°x composite pulse and the second trim puls was deleted. 5.81 kHz field strenght was used during the mixing period. The 90° puls was 43 μs. The trim pulse was 1.5 ms and the delay time was 1.5 ms. TPPI acquisition was used. A 2 x 2 K data matrix was obtained by 32 scans (using 4 dummy scans). In both directions the sweep width was 2590 Hz. No zero-filling was used but the resolution was enhanced by a π/4-sine-bell function in the F_1 direction. The carrier frequency was positioned at δ 4.22.

Reaction of pullulan with succinic anhydride. Pullulan (50 mg, 0.309 mmol), which has been dried for 48 h at 90 °C over phosphorous pentoxide, was dissolved in dry DMSO-d_6 (2 mL). Purified succinic anhydride (6.2 mg, 0.062 mmol) was added to the solution. The reaction mixture was stirred during 3 days at room temperature. The reaction was carried out under very dry conditions to avoid proton-deuterium exchange. The reaction was repeted four times and was found reproducible.

Reaction of pullulan with 4-nitrophenylchloroformate and coupling with 2-hydroxypropylamine. Pullulan (50 mg, 0.309 mmol), which has been dried over phosphorous pentoxide for 48 h at 90 °C, was dissolved in DMSO (2 mL). 4-Nitrophenyl chloroformate (30 mg, 150 mmol) was added to the solution. The reaction mixture was stirred during 4 h at

0 °C. After 4 h, an excess of 2-hydroxypropylamine was added to the solution and the reaction mixture was stirred during 48 h at room temperature. After 48 h, the reaction product was precipitated in dry 1/1 ethanol/diethylether (10 mL). The product was filtered and dried. After drying, the reaction product was dissolved in DMSO-d_6. The reaction was repeated four times and was found reproducible.

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REFERENCES

1. L. Molteni, *Drug Carriers in Biology and Medicine*, G. Gregoriadis, Eds.; Academic Press: London, *1979*, *p 107*.
2. *K. Kawahara, K. Ohta, H. Miyamoto, and S. Nakurama, Carbohydr. Pol.*, **4**, 335 (1984).
3. B. Casu and M. Reggiani, *Tetrahedron Lett.* , 2253 (1965).
4. B. Casu, M. Reggiani, G. Gallo, and A. Vigevani, *Tetrahedron*, **22**, 3061 (1966).
5. D. D. McInTyre and H. J. Vogel, *Starch/Stärke*, **43**, 69 (1991).
6. P. Colson, H. J. Jennings and I. C. P. Smith, *J. Am. Chem. Soc.* **96**, 8081 (1974).
7. D. Gagnaire and M. Vignon, *Makromol. Chem.* , **178**, 2321 (1977).
8. T. Usui, M. Yokoyama, N. Yamaoka, K. Matsuda, K. Tuzimura, H. Sugiyama, and S. Seto, *Carbohydr. Res.* , **33**, 105 (1974).
9. A. De Bruyn, M. Anteunis, and G. Verhegge, *Bull. Soc. Chim. Belges*, **84**, 721 (1975).
10. A. De Bruyn, *J. Carbohydr. Chem.* , **10**, 159 (1991).
11. M. St-Jacques, P. R. Sunderarajan, K. J . Taylor, and R. H. Marchessault, *J. Am. Chem. Soc.* , **98**, 4386 (1976).
12. F. R. Taravel and M. R. Vignon, *Polymer Bull.* , **7**, 153 (1982).
13. A. Bax, *Two-Dimensional Nuclear Magnetic Resonance in Liquids*, Delft University Press, 1982, p 78.
14. A. Bax and D. G. Davis, *J. Magn. Reson.* , **65**, 355 (1985).
15. R. Freeman, S. P. Kempsell, and M. H. Levitt, *J. Magn. Reson.* **38**, 453 (1980).

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