Chemical modification of pullulan: 2. Chloroformate activation

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The present paper describes the chloroformate activation of pullulan by reaction with 4-nitrophenyl chloroformate. N.m.r. analysis indicated that the ester formation takes place preferentially at the C6 hydroxy groups of pullulan. It was further demonstrated that activation gives linear carbonates, aliphatic carbonates including cyclic five-membered carbonates, and non-strained aliphatic carbonates. All of these carbonates react with amines to yield the corresponding urethane derivatives. The 4-nitrophenyl chloroformate activation of pullulan is an easy method for obtaining amine-containing pullulan derivatives.

(Keywords: 4-nitrophenyl chloroformate; pullulan; aminated pullulan; structural analysis; n.m.r. study)

INTRODUCTION

Several polysaccharides are being used as carrier molecules in the preparation of polymeric drugs[1]. For the preparation of polymer–drug conjugates, conversion of the polysaccharide into a suitable reactive derivative is usually required. A large number of methods are available to activate polysaccharides[2]. The activation of polysaccharides with chloroformates has been described before in the literature[3–15]. The present paper describes the chloroformate activation of pullulan and the preparation of an amine-containing pullulan derivative.

MATERIALS AND METHODS

Materials and instruments

Pullulan was obtained from the Sigma Chemical Company (St Louis, MO, USA) and was dried over phosphorus pentoxide before use. 4-Nitrophenyl chloroformate (Aldrich, Bornem, Belgium) and 4,4-dimethylaminopyridine (Janssen Chimica, Beerse, Belgium) were used without further purification. Dimethylsulfoxide and pyridine (Janssen Chimica) were dried and distilled before use. ^{1}H n.m.r. spectra were run on a Bruker WH 360 spectrometer.

Methods

4-Nitrophenyl chloroformate activation of pullulan. 4-Nitrophenyl chloroformate activated pullulan derivatives with different degrees of activation were prepared by reacting the parent polymer with varying amounts of the chloroformate. The reaction time in each case was 4 h. As an example, the preparation of a derivative with a degree of substitution of 30% is described in the following.

4-Nitrophenyl chloroformate (0.92 g, 4.6 mmol) was added to a solution of pullulan (1 g, 6.17 meq anhydroglucoside units) in 60 ml of a dry DMSO/Py mixture (1/1 by volume) thermostatted at 0°C. To this solution was added 4,4-dimethylaminopyridine (DMAP) (3.4 mg, 0.276 mmol). The reaction mixture was stirred for 4 h at 0°C and subsequently added to an excess of dry ethanol/ether (1/1 by volume). The white precipitate was collected and washed with an excess of ethanol and finally dried under vacuum. A degree of activation of 25 mol% was obtained.

4-Nitrophenyl chloroformate activation of pullulan as a function of time. To a solution of dry pullulan (2 g, 12.35 mmol) in 120 ml dry DMSO/Py (1/1 by volume) thermostatted at 0°C were added 4-nitrophenyl chloroformate (1.8 g, 9.2 mmol) and DMAP (0.12 g, 0.92 mmol). The mixture was kept at 0°C and stirred at regular time intervals. Then, 10 ml of the solution were withdrawn and added to a 50 ml ethanol/50 ml ether mixture. The precipitate was collected and washed with an excess of ethanol and dried under vacuum.

Determination of the aromatic carbonate content. Activated pullulan (5–10 mg) was dissolved in 10 ml of 0.1 N NaOH. The concentration of phenolate was determined spectrophotometrically. The 4-nitrophenolate moiety has a $\lambda_{max} = 402$ nm and a molar extinction coefficient $\varepsilon_M = 18\,400\ \mathrm{l\,mol^{-1}\,cm^{-1}}$.

Determination of the total carbonate content. Activated pullulan (5–10 mg) was dissolved in 10 ml of 0.1 N $Ba(OH)_2$ solution. The mixture was boiled for 0.5 h under a nitrogen atmosphere. After cooling, the reflux condenser was rinsed with 10 ml of CO_2-free water and the solution was titrated with 0.1 N HCl using phenolphthalein as indicator.

Coupling of the activated pullulan derivative with 2-hydroxypropylamine. Activated pullulan (0.5 g, 3.2 mmol; degree of activation 25 mol%) was dissolved in DMSO/Py

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and a two-fold excess of 2-hydroxypropylamine was added. After 48 h at room temperature the reaction product was precipitated in ethanol/ether (1/1 by volume), filtered and washed with ethanol and ether. For further purification, the product was dissolved in water and the solution was injected onto a preparative g.p.c. column (G-25 Sephadex). The product was then freeze dried. The degree of substitution was determined by ^{1}H n.m.r. spectroscopy.

Coupling of the activated pullulan carbonate with ethylenediamine. Pullulan (1 g, 6.2 mmol) was dissolved in 60 ml DMSO/Py and 4-nitrophenyl chloroformate (0.92 g, 4.6 mmol) was added. To this solution DMAP (3.4 mg, 0.276 mmol) was added. The reaction mixture was stirred for 4 h at 0°C. After 4 h, the activated polymer was added to a 50-fold excess of ethylenediamine and stirred for 48 h at room temperature. After 48 h, the polymer was precipitated in ethanol/ether (1/1 by volume), filtered and washed with ethanol and ether. For further purification, the product was dissolved in H_2O and dialysed against double-distilled water. After two days, the product was freeze dried. The degree of substitution was determined by ^{1}H n.m.r. spectroscopy and the *o*-phthaldialdehyde (OPA) method.

Determination of the amine content in pullulan N-(*2-aminoethyl*)*carbamate.* First, 0.25 ml of sample solution, 0.75 ml of double-distilled water, 1.5 ml of reagent solution A (borate buffer at pH 10+0.05 vol% 2-mercaptoethanol) and 0.5 ml of reagent solution B (10 ml ethanol + 40 ml water + 20 mg *o*-phthaldialdehyde) were mixed thoroughly. After 10 min at room temperature, the absorption at $\lambda = 334.5$ nm was measured against a reagent blank. The amine content was calculated from a calibration curve obtained from glycine standards.

RESULTS AND DISCUSSION

Introduction

Pullulan is a linear polysaccharide which is produced by the yeast-like fungus *Aureobasidium pullulans* (*Pullularia pullulans*)[16–18]. It consists of α-1,6-linked maltotriose units (*Figure 1*).

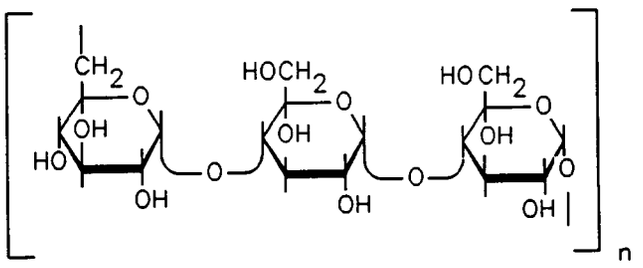

Figure 1 Structure of pullulan

Chloroformates are interesting reagents for the activation of polymers with hydroxy functions. The carbonate groups introduced react with amines to form the corresponding urethane derivatives (*Figure 2*).

This method is also of interest for the preparation of derivatives of pullulan.

4-Nitrophenyl chloroformate activation of pullulan

The activation of dextran with 4-nitrophenyl chloroformate was introduced by Vasil'ev[8] and was studied in our laboratory by Vandoorne[19]. It was shown by the latter that during activation different types of carbonate esters are formed. The aromatic 4-nitrophenyl carbonate esters can react with neighbouring hydroxy groups to give five-membered cyclic carbonates. Similar reactions are anticipated subsequent to the chloroformate activation of pullulan (*Figure 3*).

Characterization of the carbonate esters in 4-nitrophenyl chloroformate activated pullulan

The 4-nitrophenyl chloroformate activated pullulan was characterized by u.v. spectrophotometry[20]. The amount of 4-nitrophenolate after hydrolysis with sodium hydroxide was determined. The u.v. spectrum of 4-nitrophenolate shows an absorption maximum at $\lambda_{max} = 402$ nm with a molar extinction coefficient $\varepsilon_M = 18\,400$ l mol^{-1} cm^{-1}.

The degrees of substitution of pullulan for different ratios of chloroformate to pullulan (reaction time 4 h) are summarized in *Table 1*.

The total carbonate content can be determined quantitatively by hydrolysis with $Ba(OH)_2$ and back-titration of the excess $Ba(OH)_2$ with HCl[6,7]. The results are in *Table 1*.

In a preliminary study, the chloroformate activation was followed as a function of time. The maximum degree of substitution was reached after 4 h.

Coupling of the 4-nitrophenyl chloroformate activated pullulan with amines

Reaction with a model amine, e.g. 2-hydroxypropylamine. Reaction of pullulan with 4-nitrophenyl chloroformate leads to the formation of the pullulan (4-nitrophenyl)carbonate derivative which readily reacts with 2-hydroxypropylamine to form the corresponding carbamate derivative (*Figure 4*).

The coupled pullulan derivatives were characterized by ^{1}H n.m.r. spectroscopy. In the ^{1}H n.m.r. spectrum of the reaction product, the methyl protons of the 2-hydroxypropyl moiety show up as a doublet at $\delta = 1.1$ ppm. From the integration of the n.m.r. signals

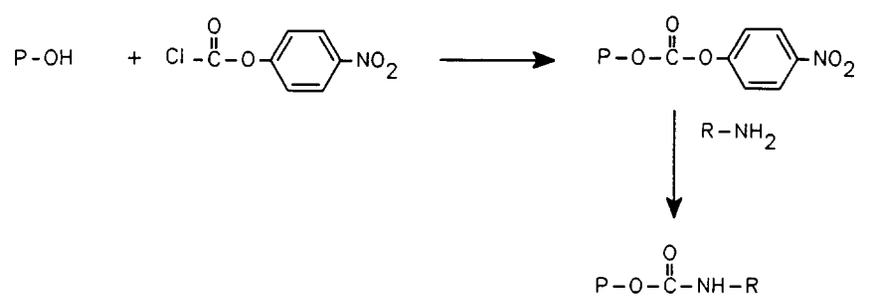

Figure 2 Reaction of a polysaccharide with 4-nitrophenyl chloroformate and an amine

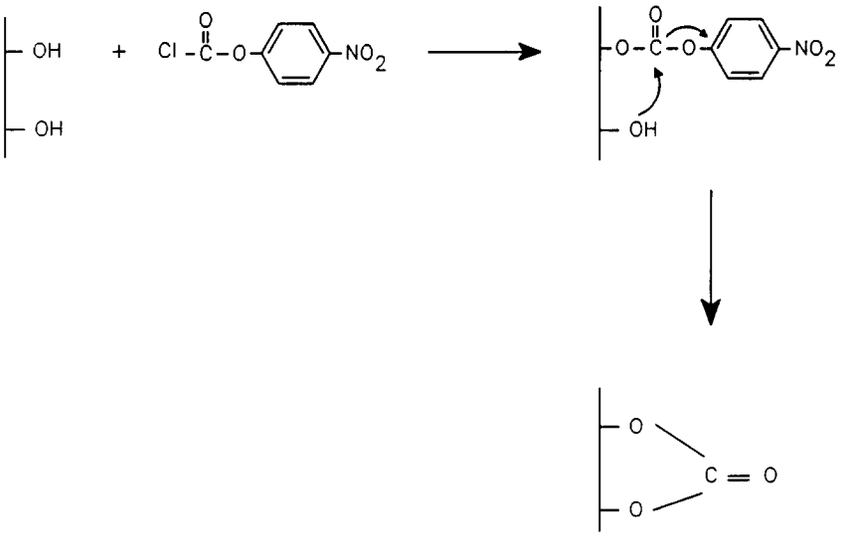

Figure 3 Formation of five-membered cyclic carbonates

Table 1 Degree of substitution of activated pullulan, percentage of linear carbonates and percentage of carbonates in total

Chloroformate (g)/pullulan (g)	Degree of substitution (%)[a]	Linear carbonates (%)	Total carbonates (%)
0.13	5	0.25	3.3
0.28	10	1	10.6
0.43	15	2	14
0.58	20	2.5	19.1
0.74	25	3.4	23
0.92	30	4.7	27

[a] Degree of substitution (%) is defined as the amount of substitution per 100 anhydroglucoside units

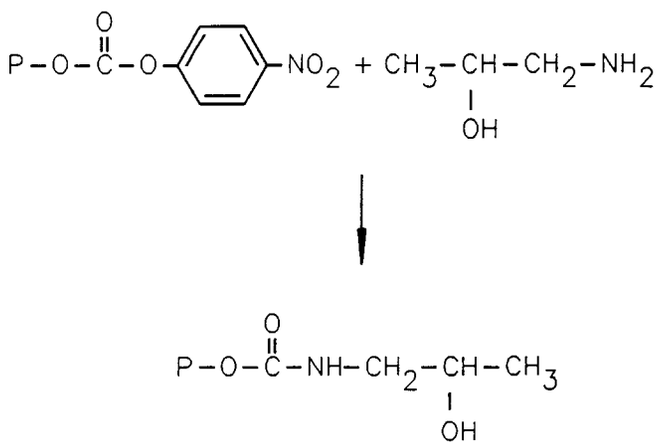

Figure 4 Reaction of pullulan (4-nitrophenyl)carbonate with 2-hydroxypropylamine

the degree of modification can be easily calculated. The results are shown in *Figure 5*.

In the region between 5% and 30% activation there is a linear relationship between the amount of 4-nitrophenyl chloroformate used, the degree of activation and the degree of substitution after reaction with the amine. The results are shown in *Figure 5*.

Preparation of aminated pullulan. As shown in *Figure 5*, 4-nitrophenyl carbonate activated pullulan can be used as an intermediate in the preparation of aminopullulan. Slow addition of the activated pullulan carbonate to a large excess of multifunctional amine

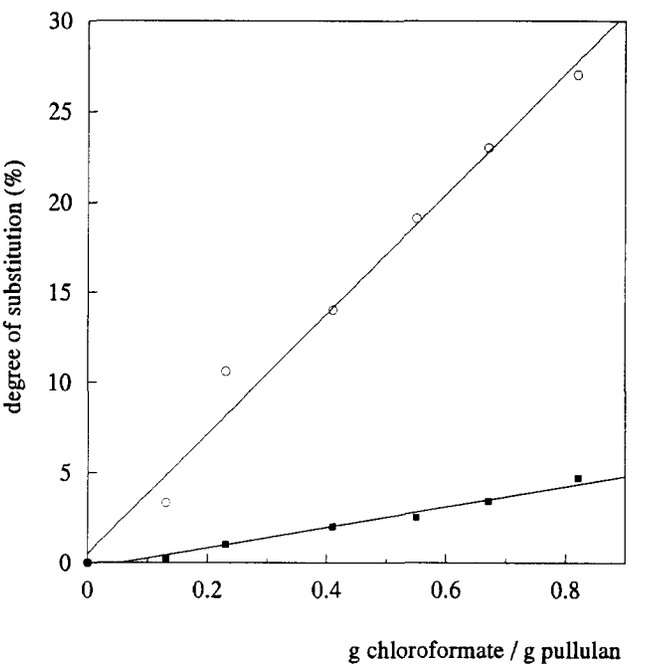

Figure 5 4-Nitrophenyl carbonate substitution (■) and 2-hydroxypropylamine content (○) as functions of the amount of 4-nitrophenyl chloroformate used for the activation

resulted in a pullulan derivative containing primary amines (*Figure 6*).

The degree of substitution was determined by ^{1}H n.m.r. spectroscopy and by means of *o*-phthaldialdehyde (OPA). The degree of substitution was found to be 16.6% by n.m.r. and 17.2% by OPA.

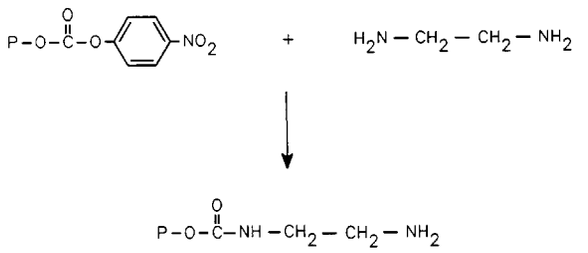

Figure 6 Reaction of pullulan (4-nitrophenyl)carbonate with ethylenediamine

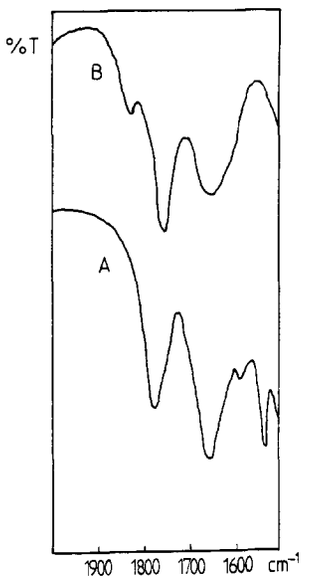

Figure 7 I.r. spectra of 4-nitrophenyl chloroformate activated pullulan prepared in DMSO/Py with DMAP (curve A) and triethylamine (curve B) as catalyst. In curve A there is an absorption maximum at 1765 cm^{-1} from the aromatic carbonates. In curve B there is an absorption maximum at 1805 cm^{-1} from the five-membered ring carbonates and another at 1740 cm^{-1} from the non-strained aliphatic carbonates

Side-reaction during the 4-nitrophenyl chloroformate activation: formation of aliphatic carbonates

The data gained from total carbonate analysis as well as the analysis of the product of the reaction with 2-hydroxypropylamine indicate that the total degree of modification is larger than could be ascertained from u.v. analysis of the activated pullulan. An explanation for these reactive groups is that they are aliphatic carbonates. The i.r. spectrum of activated pullulan is shown in *Figure 7*.

From hydrolysis with NaOH and u.v. analysis of the amount of 4-nitrophenolate and a separate hydrolysis with $Ba(OH)_2$ and back-titration, the amounts of aromatic carbonates and aliphatic carbonates can be determined. The total amount of carbonate can thus be calculated.

We can conclude that the 4-nitrophenyl chloroformate activation of pullulan results in different reactive derivatives. First of all, we have the formation of aromatic carbonates as shown in *Figure 8*.

As is observed for dextran, reaction of the 4-nitrophenyl carbonate with a neighbouring OH group leads to a five-membered ring carbonate (*Figure 9*).

The other type of carbonate observed in the i.r. spectrum is most likely formed by reaction of 4-nitrophenyl chloroformate with other OH groups to give non-strained aliphatic carbonates. Since the molecular weight of the activated pullulan is not markedly increased (data not shown), and since no insolubilization is observed, these carbonates are most likely the result of intramolecular reactions, presumably between neighbouring units.

Conclusion. The 4-nitrophenyl chloroformate activation of pullulan is an easy method for obtaining reactive derivatives.

N.m.r. study of 4-nitrophenyl chloroformate activated pullulan

The objective of this study was to ascertain the sites of the pullulan molecule at which 4-nitrophenyl chloroformate reacts. N.m.r. was the preferred technique for this study.

It was anticipated that, as long as exchange phenomena were excluded and the solutions were kept very dry, the identification of the OH proton resonances in DMSO solution and the differences in the integrations of the OH proton resonances before and after the reaction might provide statistical information on the substitution site. The region of the OH proton resonances is less crowded and is completely isolated from the resonances for the other ring protons. The n.m.r. techniques used were

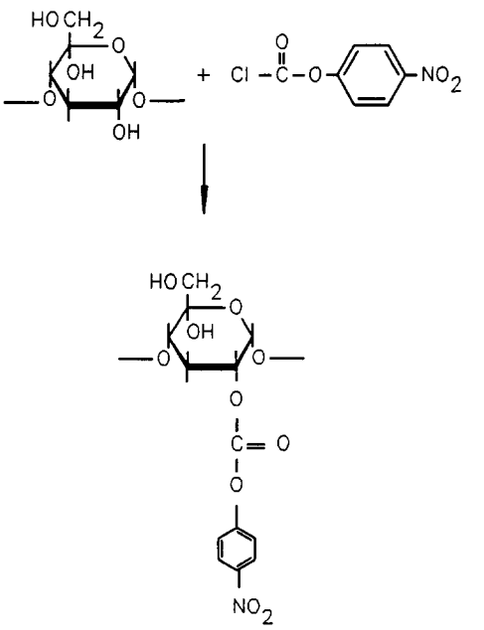

Figure 8 Formation of aromatic carbonates

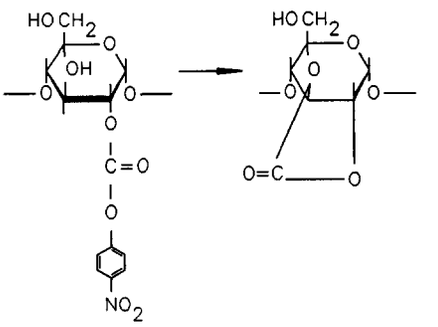

Figure 9 Formation of five-membered ring carbonates

COSY 45 (correlated spectroscopy) and HOHAHA (Homonuclear Hartmann–Hahn)[21].

Results. In the ^{1}H n.m.r. spectrum of pullulan in DMSO, the signals for the OH6 protons are found at $\delta = 4.7$ and $\delta = 4.5$ ppm. In the ^{1}H n.m.r. spectrum of a partially activated pullulan, we observe a decrease in integration of the two OH6 signals only.

It must be pointed out that the integration of the H1 protons is used as a reference. In comparison with the integration of the H1 resonance we see no changes in the integrations of the other protons except those for the two OH6 resonances.

For carbohydrates, it is known that the secondary alcohol hydroxy groups at C2 are more reactive than the hydroxy groups at C3 and C4. Pullulan has two primary alcohol hydroxy groups and seven secondary alcohol hydroxy groups per maltotriose unit. It is also known that primary alcohol hydroxy groups are more reactive than secondary alcohol hydroxy groups. The n.m.r. study shows that during activation, the hydroxy groups at C6 react first.

The conclusion is that it is possible to determine the site of substitution in pullulan by reaction with 4-nitrophenyl chloroformate and subsequent n.m.r. analysis of the product.

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