Chemical modification of pullulan: 3. Succinoylation

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The present paper describes the succinoylation of pullulan by reaction with succinic anhydride in dimethylsulfoxide as solvent and *N,N'*-dimethylaminopyridine as catalyst. Nuclear magnetic resonance analysis indicated that the carboxylic group is preferably introduced at the C-6 hydroxyl groups. A series of succinoylated pullulan derivatives are prepared with degrees of substitution ranging from 0 to 100%. Activation of the carboxylic groups with *N,N'*-carbonyldiimidazol and subsequent coupling with amines is discussed.

(Keywords: succinic anhydride; pullulan; structural analysis)

INTRODUCTION

The macromolecular carrier–drug approach has attracted considerable interest in recent years as a means of increasing the duration of activity through slow release[1]. Several polysaccharides are being used as carrier molecules in the preparation of polymeric drugs[2]. In this connection, pullulan possesses several properties which would make it a feasible carrier candidate and has been selected for the preparation of polymer–bioactive agent adducts[3–5]. For the preparation of polymer–drug conjugates, conversion of the polysaccharide into a reactive derivative is usually required. Various methods have been elaborated to transform the original polymer into a reactive derivative in order to make possible the attachment of bioactive compounds that lack the appropriate functionality[6–15]. The activation of polysaccharides with succinic anhydride has been described in the literature[16,17].

The aim of this work is to report the incorporation of carboxylic groups into pullulan by reaction with succinic anhydride. A series of succinoylated pullulan derivatives were prepared with a degree of substitution ranging from 0 to 100%. Another objective of this work is to assign the site of substitution after reaction of pullulan with succinic anhydride. Nuclear magnetic resonance (n.m.r.) was the preferred technique. The present paper also describes the activation of the carboxylic groups introduced on pullulan by reaction with *N,N'*-carbonyldiimidazol. The resulting derivative can be coupled with amines. 1-Aminopropan-2-ol is used as a model amine because the methyl group of the amine is easily detected in ^{1}H n.m.r.

MATERIALS AND METHODS

Materials and instruments

Pullulan was obtained from Sigma Chemical Company (St Louis, MO, USA) and was dried over phosphorus pentoxide before use. Succinic anhydride was purified by dissolving in chloroform and refluxing for 6 h. 4-Dimethylaminopyridine (Janssen Chemical, Beekse, Belgium) was used without further purification. Dimethylsulfoxide (DMSO) was dried and distilled before use. ^{1}H n.m.r. spectra were run on a Brücker WH 360 spectrometer.

Methods

Synthesis of pullulan monosuccinate ester derivatives (100% acylation). To a solution of 1 g pullulan (6.2 mmol) in 15 ml DMSO is added 2.5 g succinic anhydride (25 mmol) in 10 ml DMSO. The solution is thermostatted at 40°C and 0.28 g (2.6 mmol) 4-dimethylaminopyridine is added while stirring. The reaction mixture is kept at 40°C for 24 h. The reaction product was isolated by precipitation in a fivefold volume of a mixture of ethanol and ether (1:1, v/v). The dried precipitate was dissolved in 10 ml water and the solution was injected onto a preparative gel filtration column (300 ml, Sephadex G-25, eluent water, flow rate 2 ml min^{-1}, detection 400 refractive index). The polymer fraction was collected and freeze dried. The degree of esterification was determined by titrimetric analysis, ^{1}H n.m.r. spectroscopy and infra-red (i.r.) analysis. The same reaction conditions were used for the partial succinoylated pullulan derivatives.

Coupling of 1-aminopropan-2-ol with pullulan monosuccinate. To a solution of 0.15 g pullulan monosuccinate (95% succinoylation) in 5 ml DMSO at 25°C was added, while stirring, 0.21 g *N,N'*-carbonyldiimidazol (1.28 mmol). After 1 h. 0.15 g 1-aminopropan-2-ol (1.28 mmol) was added and stirring was continued for 48 h. The reaction mixture was diluted with an equal volume of water and injected on a preparative gel filtration column (Sephadex G-25). The polymer fraction was collected and freeze dried.

RESULTS AND DISCUSSION

Pullulan is a linear polysaccharide produced by the yeast-like fungus *Aureobasidium pullulans* (*Pullularia*

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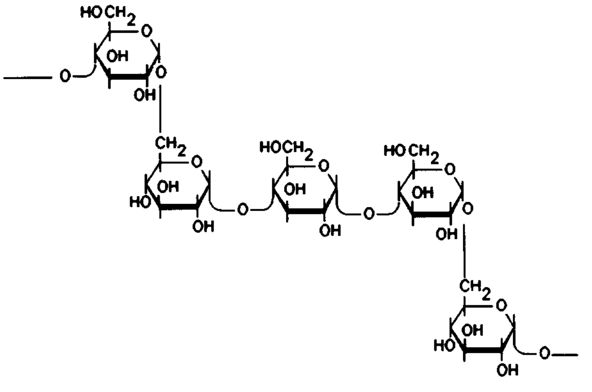

Figure 1 Structure of pullulan

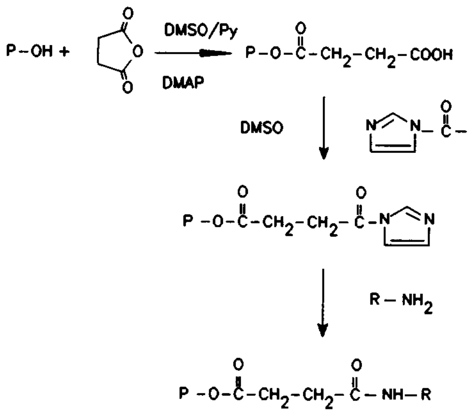

Figure 2 Reaction of a polysaccharide with succinic anhydride, activation with *N,N'*-carbonyldiimidazol and subsequent reaction with amines

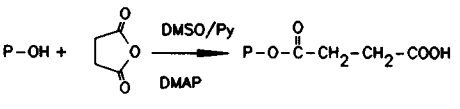

Figure 3 Reaction of pullulan with succinic anhydride

pullulans)[3-5]. It consists of (1-6)-α-maltotriose units (*Figure 1*).

A carboxylic group can easily be introduced on the polysaccharide by reaction of the polysaccharide with succinic anhydride. The carboxylic groups can then be activated, for example by reaction with *N,N'*-carbonyldiimidazol, and subsequently coupled with amine-containing substances, e.g. drug molecules (*Figure 2*).

Synthesis of partially and totally succinoylated pullulan derivatives

Monosuccinate esters of pullulan, with different degrees of acylation, can be prepared by controlled reaction of pullulan with succinic anhydride (*Figure 3*).

The succinoylation of dextran and inulin was described before in the literature[18,19]. It was demonstrated that 4-dimethylaminopyridine is a useful catalyst. In this work the succinoylation was performed in DMSO at 40°C for 24 h. 4-Dimethylaminopyridine was added as a catalyst.

The degree of esterification can be controlled by the ratio of succinic anhydride to pullulan and the reaction conditions. The degree of succinoylation was determined by titrimetric analysis of the carboxylic groups as well as by ^1H n.m.r. spectroscopy. The results are shown in *Table 1*. The amount of carboxylic group in succinoylated pullulan was determined titrimetrically. These results are also given in *Table 1*.

The monosuccinate esters of pullulan were further characterized by ^1H n.m.r. spectroscopy in D_2O. The signals at $\delta = 2.6$–2.7 ppm are due to the methylene protons of succinic anhydride (4H). The anomeric protons of pullulan resonate at $\delta = 4.9$ ($\alpha(1\rightarrow6)$) and at $\delta\,5.3$–5.5 ($\alpha(1\rightarrow4)$). The degree of substitution was calculated from the integrations of these signals and is shown in *Table 1*.

The formation of monosuccinate esters of pullulan was confirmed by i.r. analysis (*Table 2*).

Coupling of succinate ester of pullulan with 1-aminopropan-2-ol

The succinate derivatives of pullulan were transformed into the corresponding reactive imidazolide by treatment with *N,N'*-carbonyldiimidazol (CDI), a coupling reagent that has been used for the preparation of esters and amides[20-22]. Reaction of CDI with a carboxylic group results in a reactive ester that can easily react with alcohols or amines giving rise to the corresponding esters or amides (*Figure 4*).

Reaction of the succinate derivatives of pullulan (>97.8% acylation) with CDI results in the corresponding imidazolide derivative. The latter can react with amines. 1-Aminopropan-2-ol is chosen as the model amine because in ^1H n.m.r. the methyl protons are very well detected. The reaction was performed in DMSO at room temperature. The reaction product was characterized by ^1H n.m.r. spectroscopy. ^1H n.m.r. indicates that only an amide is formed and not the corresponding ester (reaction of the imidazolide derivative with the alcohol group of 1-aminopropan-2-ol).

In the ^1H n.m.r. spectrum of the reaction products, the methyl protons of the 2-hydroxypropyl groups show up as a doublet at $\delta = 1.1$ ppm. From the integration values

Table 1 Degree of acylation of pullulan

Succinic anhydride/ pullulan (wt/wt)	Degree of acylation (%)[a] Titrimetric analysis	^1H n.m.r.
0.09	4.55	6
0.18	8.90	10.6
0.37	18.00	17.50
0.93	54.70	–
1.86	97.80	–

[a] Degree of acylation is defined as the amount of substitution per 100 anhydroglucoside units

Table 2 I.r. data of succinoylated pullulan

Wavenumber (cm^{-1})		Functions
2500–3700	Carboxylic acid	O–H stretching
1745	Ester	C=O stretching
1715	Carboxylic acid	C=O stretching
1415	Carboxylic acid	Coupling of O–H deformation and C=O stretching
1160	Ester	C–C(=O)–O band of a saturated ester
950	Carboxylic acid	O–H deformation

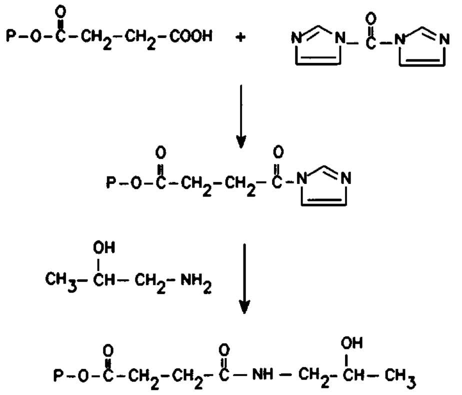

Figure 4 Reaction of CDI-activated pullulan with 1-hydroxypropylamine

of the n.m.r. signals, the degree of modification can easily be calculated.

The n.m.r. spectrum of the coupled product indicates that over 95% of the succinate carboxylic acids have reacted with 1-aminopropan-2-ol.

N.m.r. study of partially succinoylated pullulan

One of the objectives of this study was to assign the site of substitution after reaction of pullulan with succinic anhydride. N.m.r. was the technique of choice.

It was anticipated that, provided exchange phenomena are excluded and the solutions are kept very dry, the identification of the OH proton resonances in DMSO solution and the difference in integration of the OH resonances before and after the reaction may provide statistical information on the substitution site. The region of the OH proton resonance signals is less crowded and is completely isolated from the resonance signals for other ring protons. For this purpose COSY 45 (correlated spectroscopy) and HOHAHA (homonuclear Hartmann–Hahn)[23] n.m.r. techniques were used.

In the ^1H n.m.r. spectrum of pullulan in DMSO, the signals for the OH-6 protons appear at $\delta = 4.7$ and $\delta = 4.5$ ppm. The integration for the H-1 protons was used as reference. In partially succinoylated pullulan, one observes a decrease in integration for the two OH-6 signals.

It is known that for carbohydrates the secondary hydroxyl groups at C-2 are more reactive than the hydroxyls at C-3 and C-4. Pullulan has two primary hydroxyl groups and seven secondary hydroxyl groups per maltotriose unit. Moreover, it is known that, in general, primary hydroxyl groups are more reactive than secondary hydroxyl groups. This n.m.r. study shows that during succinoylation the hydroxyl groups at C-6 react preferably.

In conclusion, partial succinoylation of pullulan is an elegant method to introduce functionalities which can easily be coupled with amines. This activation method opens perspectives for the preparation of pullulan–drug conjugates.

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