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End group modification of pullulan

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Pullulan was selectively functionalized at its reducing terminus via reductive amination of the terminal aldehyde with 2-(4-nitrophenyl)ethylamine. The nitro group serves as a precursor for an amino group which can be converted into a reactive isothiocyanate group. The latter can be coupled with amines.

(Keywords: pullulan; reductive amination; end group)

INTRODUCTION

Hydrophilic polymers having a reactive end group are of interest for the modification of proteins, biomaterial surfaces and for the preparation of prodrug derivatives. In that respect extensive work has been devoted to the use of end group functionalized polyoxyethylenes[1–4]. Recently, the end group modification of dextrans has been reported as a tool for coupling with bioactive molecules[5]. Dextran is known as a biocompatible polysaccharide and finds clinical application as a blood substituent. It is also frequently used for the preparation of macromolecular prodrugs[6–8]. For a number of biomedical applications there is an interest in pullulan which is composed of $\alpha(1\rightarrow6)$ linked maltotriose units. Pullulan derivatives are used in the synthesis of coatings for liposomes[9–17]. This polysaccharide contains one reducing end group which can be used to introduce end group functionalities. Up to now, only limited data have been available about the conversion of pullulan into reactive derivatives. In recent papers, we have reported on the periodate oxidation[18], the chloroformate activation[19] and the succinoylation[20] of pullulan. The present study aims to develop methods for preparing end group functionalized pullulan derivatives.

EXPERIMENTAL

Materials and instruments

Pullulan was obtained from Sigma Chemical Company (St. Louis, MO, USA) and 1-(4-aminophenyl)ethylamine was obtained from Janssen Chemical Products (Beerse, Belgium).

^{1}H n.m.r. spectra were recorded on a Bruker 360 MHz apparatus. U.v. spectra were recorded on a UV-Uvikon 810 apparatus (Kontron Instruments).

Methods

Hydrolysis of pullulan. Pullulan (1 g, 6.2 mmol) was dissolved in 0.1 M HCl (50 ml). The solution was stirred for 3 h at 80°C and was then precipitated in methanol and filtered. After drying, the molecular weight was determined by analytical g.p.c. on TESEK G-4000, G-3000, G-2000 and G-1000 columns (8 × 250 mm, 10 μm), using pullulan standards for the calibration.

Synthesis of 1-[(4-nitrophenyl)ethylamino]pullulan. Pullulan (1 g, 6.2 mmol) was dissolved in dimethylsulfoxide (DMSO; 5 ml). 2-(4-Nitrophenyl)ethylamine (680 mg, 4.1 mmol) and 130 mg molecular sieves (4 Å) were added. After flushing with argon for 3 min, the reaction mixture was stirred for 24 h at 37°C. Then $NaBH_4$ (16.6 mg, 0.44 mmol) was added. The reaction mixture was stirred for 24 h at 37°C. The viscous brown mixture was cooled in an ice bath and H_2O (10 ml) was added. The pH of the mixture was adjusted to 5.5 by adding glacial acid (0.4 ml). After centrifugation, the supernatant was dialysed against water for 18 h and freeze dried.

^{1}H n.m.r. (D_2O, 360 MHz) δ: 5.4–5.5 [$\alpha(1\rightarrow4)$ acetal proton of pullulan (2H)]; 4.98 [$\alpha(1\rightarrow6)$ acetal proton (1H)]; 7.5 [aromatic protons *ortho* of the nitro group (2H)]; 8.2 (aromatic protons *meta* of the nitro group).

U.v. ($\lambda = 278$ nm, $\varepsilon = 8.6 \times 10^3$ l mol^{-1} cm^{-1}): degree of conversion 99%.

Synthesis of 1-[(4-isothiocyanatophenyl)amino]pullulan. 1-[(4-Aminophenyl)ethylamino]pullulan (0.3 g, 1.9 mmol) was dissolved in a mixture of water (30 ml) and 0.1 N was added. The suspension was stirred under H_2 atmosphere (276 kPa) for 6 h, filtered and freeze dried. The resulting powder was dried at 1 mmHg over P_2O_5 at 80°C for 18 h.

^{1}H n.m.r. (D_2O, 360 MHz) δ: 5.4–5.5 [$\alpha(1\rightarrow4)$ acetal proton of pullulan (2H)]; 4.98 [$\alpha(1\rightarrow6)$ proton of pullulan (1H)]; 6.9 [aromatic protons *ortho* of the amino group (2H)]; 7.2 [aromatic protons *meta* of the amino group (2H)].

Synthesis of 1-[(4-isothiocyanatophenyl)amino]pullulan. 1-[(4-Aminophenyl)ethylamino]pullulan (0.3 g, 1.9 mmol) was dissolved in a mixture of water (30 ml) and 0.1 N $NaHCO_3$ (30 ml). The solution was cooled in an ice bath and excess thiophosgene was added (1.09 g, 9.5 mmol). The mixture was stirred for 30 min at room temperature. The reaction mixture was extracted with diethylether (3 × 75 ml) and the aqueous layer was

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dialysed against water for 16 h at 0°C and finally freeze dried.

Coupling of 1-[(4-isothiocyanatophenyl)amino]pullulan with 2-hydroxypropylamine. 1-[(4-Isothiocyanatophenyl)-amino]pullulan (100 mg, 0.6 mmol) was dissolved in 10 ml DMSO/pyridine solution. A five-fold excess of 2-hydroxypropylamine (3 mmol, 0.15 ml) was added. The reaction mixture was stirred for 24 h. The reaction product was precipitated in methanol/ether (1/1 v/v) and applied on a preparative G-25 column. The polymer fraction was collected and freeze dried. The same procedure was used for preparing the reaction product by coupling with ethylenediamine or ε-aminocapronic acid.

^{1}H n.m.r. (D_2O, 360 MHz) δ: 5.4–5.5 [α(1→4) acetal proton of pullulan (2H)]; 4.98 [α(1→6) acetal proton of pullulan (1H)]; 1.2 (methyl protons of 2-hydroxypropyl-amine).

Coupling of the reducing end group of pullulan (without activation) with amines. (1) Coupling with ethylenediamine. To a solution of pullulan (0.25 g, 1.5 mmol) in buffer solution (7.5 ml, pH 5), excess ethylenediamine (15 meq, 0.9 g) was added. The reaction mixture was stirred for 1 h at room temperature. $NaBH_4$ (0.15 mmol, 5.7 mg) and then $NaCNBH_3$ (0.15 mmol, 9.4 mg) were added. After 24 h the reaction product was precipitated in methanol/ether and injected on a G-15 column.

^{1}H n.m.r. (D_2O, 360 MHz) δ: 2.9 [methylene protons of ethylenediamine (2H)]; 3.3 [methylene protons of ethylene diamine (2H)]; 5.4–5.5 [α(1→4) acetal proton of pullulan (2H)]; 4.98 [α(1→6) acetal proton of pullulan (1H)].

(2) Coupling with ε-aminocapronic acid. The same procedure was followed as described for the coupling with ethylenediamine.

^{1}H n.m.r. (D_2O, 360 MHz) δ: 2.2 [methylene protons α of the carboxylic group (2H)]; 1.7 [methylene protons β of the carboxylic group (2H)]; 1.6 [methylene protons γ of the carboxylic group (2H)]; 1.4 (methylene group β of the amino group); 3.1 (methylene group α of the amino group).

Table 1 Hydrolysis of pullulan in 0.1 M HCl at 80°C

Pullulan	M_w	M_n	M_w/M_n
Commercial	85 400	53 400	1.59
1 h hydrolysis	8050	5400	1.48
3 h hydrolysis	4450	3400	1.32
5 h hydrolysis	3290	2800	1.16

RESULTS AND DISCUSSION

Hydrolysis of pullulan

Commercial pullulan has a high molecular weight ($M_w = 83\,400$ and $M_n = 53\,500$). In order to study the end group modification of pullulan, low molecular weight pullulan is preferred. Therefore, pullulan was hydrolysed in 0.1 M HCl at 80°C. The results are given in *Table 1.*

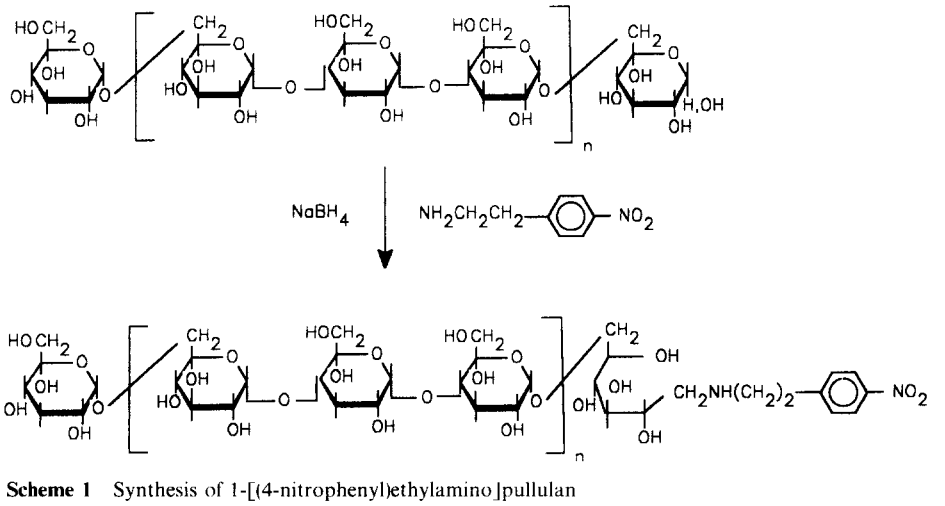
More structural information about the chemical shifts of the reducing end group was obtained by ^{1}H n.m.r. analysis. Two signals were observed from the anomeric proton of the reducing end group: an α-linked proton at $\delta = 5.22$ and a β-linked proton at $\delta = 4.63$ ppm. Similar values have been reported for dextran[21].

End group modification of pullulan

Synthesis of 1-[(4-isothiocyanatophenyl)ethylamino]-pullulan. In a first step the reducing end group of pullulan was modified by reductive amination with 1-(4-nitrophenyl)ethylamine in DMSO solution (*Scheme 1*). The macromolecular derivative was characterized by u.v. spectroscopy ($\lambda = 278$ nm, $\varepsilon_M = 8.6 \times 10^3$ l mol^{-1} cm^{-1}) and ^{1}H n.m.r. spectroscopy. The coupling yield was 100%. The aromatic nitro group was subsequently reduced with H_2 on 10% Pd/C as catalyst giving the corresponding amine (conversion 100%). Characterization of the derivative was performed by ^{1}H n.m.r. spectroscopy (*Scheme 2*).

Finally, the aniline function of the reducing end group was converted to a reactive thioisocyanate group by reaction of 1-[(4-aminophenyl)ethylamino]pullulan with thiophosgene (*Scheme 3*).

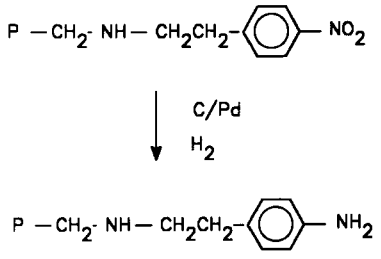
Coupling of 1-[(4-isothiocyanatophenyl)ethylamino]-pullulan with amines. (1) Coupling with a model amine (e.g. 2-hydroxypropylamine). 1-[(4-Isothiocyanatophenyl)-



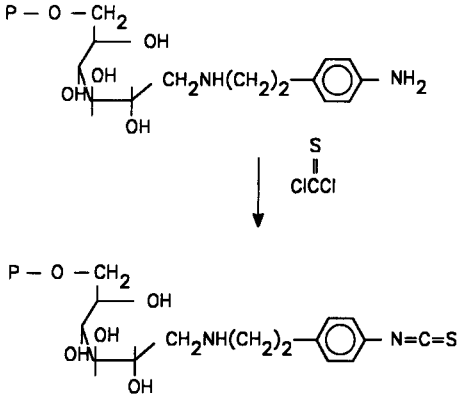
Scheme 1 Synthesis of 1-[(4-nitrophenyl)ethylamino]pullulan

ethylamino]pullulan can be coupled with amines via the thioisocyanate reactive end group. As model amine 2-hydroxypropylamine was selected (*Scheme 4*). The structure of the reaction product was analysed by ^1H n.m.r. It was determined from the data that the degree of substitution was complete.

(*2*) *Coupling with ethylenediamine and ε-aminocapronic acid.* One objective of this study was the synthesis of amino or carboxylic acid terminated pullulan. Polysaccharide derivatives with free amino functions or free carboxyl groups are interesting materials for further coupling, e.g. with bioactive agents. For the introduction of amino end groups ethylenediamine was used whereas ε-aminocapronic acid was used for the introduction of a carboxylic end group. The coupling method was identical to that described for the model amine. The reaction products were characterized by ^1H n.m.r. The degree of substitution for both derivatives was complete (*Scheme 5*).



Scheme 2 Synthesis of 1-[(4-aminophenyl)ethylamino]pullulan

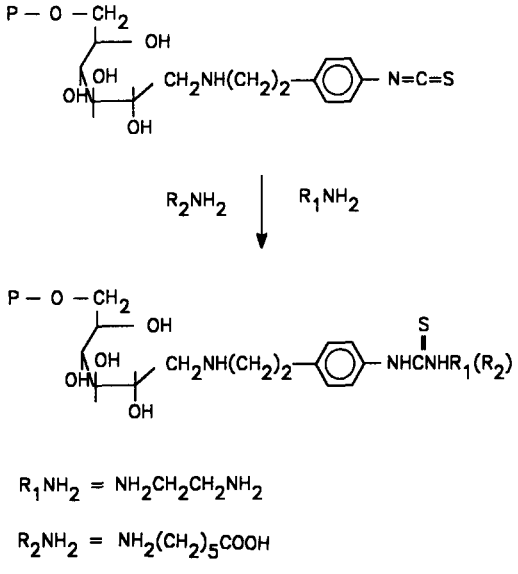


Scheme 3 Synthesis of 1-[(4-isothiocyanatophenyl)ethylamino]pullulan

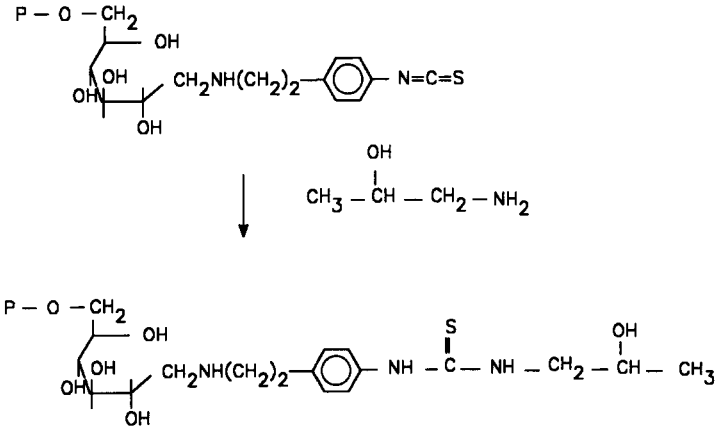
Reaction of the reducing end group of pullulan with amines

Instead of conversion of the reducing end group of pullulan into a reactive intermediate derivative, functionalization of the end group can be performed without previous activation. Reductive coupling with ethylenediamine and ε-aminocapronic acid can be applied. As reducing agents $NaBH_4$ and $NaCNBH_3$ were used. It has been reported that $NaCNBH_3$ is a selective reductant for Schiff bases whereas $NaBH_4$ can reduce aldehydes as well as Schiff bases[18,22] (*Scheme 6*).

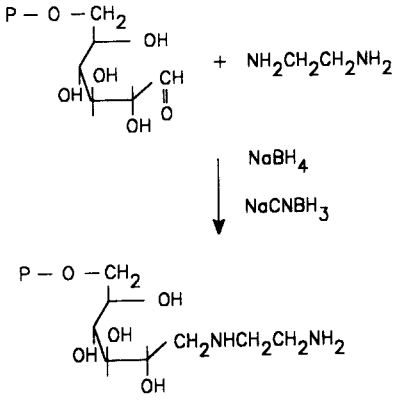
Pullulan was dissolved in a buffer (pH 5) and excess ethylenediamine was added. $NaBH_4$ and $NaCNBH_3$ were used as reducing agents. The degree of substitution in the final product was determined by ^1H n.m.r. spectroscopy. The results are given in *Table 2*. For the reaction with $NaBH_4$ the conversion was 90%. This is considered as a complete conversion. It was shown by the tetrazolium blue method that each polymer has one reducing end group[23]. From the data it was found that



Scheme 5 Coupling of 1-[(4-isothiocyanatophenyl)ethylamino]pullulan with ethylenediamine and ε-aminocapronic acid



Scheme 4 Coupling of 1-[(4-isothiocyanatophenyl)ethylamino]pullulan with 2-hydroxypropylamine



Scheme 6 Reaction of the reducing end group of pullulan with ethylenediamine

Table 2 Reductive amination of pullulan with ethylenediamine or ε-aminocapronic acid (conversion, %)

Reducing agent	Ethylenediamine	ε-Aminocapronic acid
$NaBH_4$	91	90
$NaCNBH_3$	88	85

the highest coupling yields were obtained using $NaBH_4$ as the reducing agent.

CONCLUSIONS

In this paper two methods have been reported for the modification of the reducing end group of pullulan. One approach was the reaction of the end group with 1-[(4-nitrophenyl)ethylamine] and subsequently coupling with thiophosgene. The reactive 1-[(4-isothiocyanatophenyl)ethylamino]pullulan can then be coupled with amines. A second method was the direct coupling of amines with the reducing end group of pullulan via reductive amination. The reducing agents used were $NaBH_4$ and $NaCNBH_3$. In both cases, high yield conversion of the reducing end group into an amino or carboxylic acid terminated end group was observed.

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