ELSEVIER

Carbohydrate Polymers 24 (1994) 193–197

0144-8617/94/$07.00

# Acetylation of a β-1,6-branched β-1,3-glucan, yielding Schizophyllan-acetate

**André Albrecht & Udo Rau***

*Institute of Biochemistry and Biotechnology, Technical University of Braunschweig, Spielmannstr. 7, 38106 Braunschweig, Germany*

(Received 17 November 1993; revised version received 6 February 1994; accepted 7 February 1994)

Several enzymatic and chemical methods were employed to attempt to acylate the homoglucan Schizophyllan. The enzymatic attempts with an immobilized lipase and a protease failed because of the lack of a suitable organic solvent and for steric reasons. Of the chemical methods attempted, only strong acid catalysis with a large excess of a carboxyl compound led to acetylation. The degree of acetylation is discussed.

## INTRODUCTION

Schizophyllan, an extracellular, uncharged polysaccharide secreted by the wood-rotting basidiomycete *Schizophyllum commune*, consists of a (1 → 3)-linked β-D-glucopyranoside main chain with a (1 → 6)-linked β-D-glucopyranosyl group attached on average to every third residue of the main chain. In aqueous solutions, Schizophyllan possesses a rod-like, triple-helical structure with the side-chains pointing to the outside. In dimethylsulfoxide (DMSO) and in solutions with a pH 12·5, the glucan disintegrates to single, random-coiled chains (Norisuye *et al.*, 1980; Kashiwagi *et al.*, 1981; Van *et al.*, 1984; Lecacheux *et al.*, 1986). The conditions required for maximum glucan production are well known and described in detail by Rau *et al.* (1992*a*). Depending on the conditions of cultivation and downstream processing, the glucan can be obtained with molecular weights in the range from 6 to $20 \times 10^6$ g/mol$^{-1}$, equivalent to 9000–30000 repeating units (Fig. 1).

Due to its chemical structure, Schizophyllan shows many interesting properties. It has a high conformational stability at high temperatures (≤135°C), extremes of pH (1–12) and high salinity (15%). This stability, in combination with its high intrinsic viscosity, may enable its use in enhanced oil recovery (Rau *et al.*, 1992*b*).

Here we report the results of our attempts to acylate Schizophyllan. The esters of Schizophyllan could have interesting properties, if the properties of Schizophyllan itself on the one hand and those of sugar esters on the other, could be combined. Examples of acylated polysaccharides with a wide range of technical applications are cellulose esters such as cellulose acetate.

## EXPERIMENTAL

### Materials and methods

The molar concentrations of Schizophyllan are expressed in terms of repeating units (Fig. 1, molar mass: 648 g mol$^{-1}$). In calculating concentrations, the water content (about 10% (w/w) even after lyophilization) was considered.

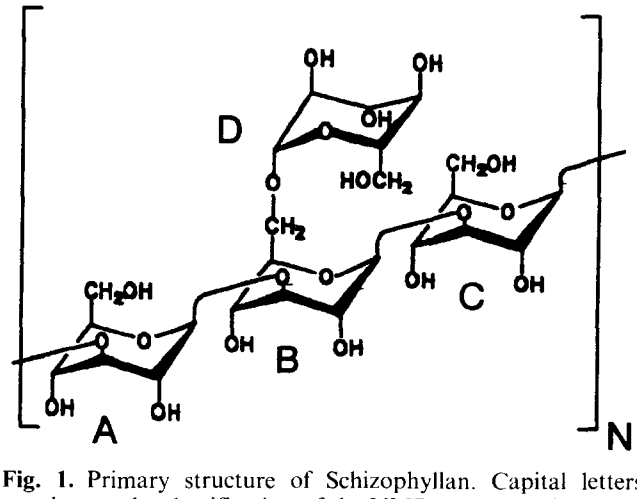


**Fig. 1.** Primary structure of Schizophyllan. Capital letters relate to the classification of the NMR signals (Table 1).

*To whom correspondence should be addressed.
Dedicated to Prof. Fritz Wagner on his 65th birthday.

*Preparation of Schizophyllan*

Schizophyllan was produced and isolated as described by Rau *et al.* (1992*a*). The dia-filtrated aqueous solution was preserved by adding 0·5% formic acid and stored at 4°C. Aliquots were lyophilized, pulverized in a mortar and then dried over phosphorous pentoxide. This powder was used in all experiments unless otherwise stated.

In order to use Schizophyllan as a substrate for enzymatic transformations, it was immobilized on silica-gel (Riedel-de Haën, Seelze, Germany) to increase the surface area. Native Schizophyllan (50 mg) and 1000 mg of silica-gel were dissolved in about 10 ml of water, stirred for at least 24 h, distilled on a rotary evaporator as far as possible and finally lyophilized. The solid was pulverized in a mortar and dried over phosphorous pentoxide.

After the reaction, the powder was dissolved in an aqueous 7·5% KBr solution and stirred until an increase in viscosity indicated the removal of the glucan. The supernatant was lyophilized and analyzed by IR-spectroscopy.

*Lipozyme™*

Lipozyme™ is the immobilized lipase from *Mucor miehei* (E.C. 3.1.1.3.) and described in detail by Svanholm (1989) and Eigtved and Hansen (1986). It was purchased from Novo, Denmark.

*Protease N*

Protease N is a crude preparation of subtilisin, a protease of *Bacillus subtilis* (E.C. 3.4.21.14.). It was purchased from Amano Pharmaceutical Co., Japan.

In order to obtain maximum catalytic activity, 20 g of the enzyme were dissolved in 200 ml 0·1 M phosphate buffer solution, pH 7·8, and the decreasing pH readjusted. Finally, the solution was lyophilized and the enzyme pulverized in a mortar. This procedure was described by Carrea *et al.* (1989).

*Other chemicals*

All other chemicals used were of analytical grade (Fluka or Merck). Solvents were used without further purification with the exception of drying over molecular sieves.

Butanoic acid trifluorethylester was prepared by a general procedure described by Höfle *et al.* (1978) and Steglich (1969).

**Reactions**

*Enzymatic experiments*

(i) Lipozyme™: 500 mg Schizophyllan or 2·5 g of the immobilized Schizophyllan were incubated with an excess of dodecanoic acid or ethylmyristate, respectively. Lipozyme™ (200 mg) was added to each of the four mixtures. The reactions were carried out in open 50-ml Erlenmeyer flasks by shaking at 250 rpm and 60°C. After 24 h the enzyme was inactivated by adding a few ml of dry methanol (60°C for 1 h). The native and immobilized Schizophyllan were separated from the enzyme by sedimentation in a narrow plastic tube, using ethanol as the separation phase. After removal of the immobilized glucan from the silica-gel and drying, the samples were analyzed by IR-spectroscopy.

(ii) Protease N: to 10 ml of the Schizophyllan suspended in dry dimethylformamide (DMF) at a concentration of 0·5 g litre$^{-1}$ ($7·7 \times 10^{-6}$ mol repeating units), $1·54 \times 10^{-5}$ mol butanoic acid methylester or -trifluoroethylester were added. Protease N (0·5 g) was added at the start and after three days. The reactions were carried out in sealed 50-ml reaction flasks, samples were withdrawn at the beginning and after five days and analyzed by gas chromatography. Controls were performed to exclude the loss of fatty acid ester, through evaporation, adsorbance on the enzyme or reaction without the catalyst. The reaction proceeded at 45°C in an orbital shaker at 100 rpm.

*Chemical experiments*

(i) Acetylation: 200 mg glucan (lyophilized, not pulverized) were soaked in sufficient volume of conc. acetic acid for about 10–15 h. Excess acid was removed by filtration. Addition of 9·5 ml acetic anhydride and 0·5 ml conc. sulfuric acid started the reaction, which was performed under slow stirring at room temperature. After 5 h the glucan was precipitated with three volumes of isopropanol and subsequently separated by centrifugation. The precipitate was resuspended in and then washed with isopropanol ten times to remove the acetic acid; finally the glucan was dried and analyzed by IR and NMR spectroscopy.

(ii) Four experiments were carried out in pyridine: 550 mg ($7·7 \times 10^{-4}$ mol repeating units) Schizophyllan were added to 20 ml of ice-cold pyridine. Decanoic acid chloride and -imidazolide, the latter prepared by a procedure described by Staab (1957) served as the carboxyl compounds. The first was used in equimolar ratio and in ten-fold excess, the second in equimolar ratio and three-fold excess. The carboxyl compound was dissolved in 10 ml chloroform and added dropwise. The reaction mixture was stirred and kept under a nitrogen atmosphere. After the whole carboxyl compound was added, the mixture was equilibrated to room temperature. After 2 days, the glucan was isolated by filtration, washed

with ethanol, dried and analyzed by IR-spectroscopy.

(iii) Two experiments were carried out in DMSO: to 20 ml of a glucan/DMSO solution (*c*: 10 mmol repeating units, $2 \times 10^{-4}$ mol absolute), 278 ml ($2 \times 10^{-3}$ mol) or 2·78 mol ($2 \times 10^{-2}$ mol) triethylamine benzoylchloride was added dropwise in 10- and 100-fold molar excess. Precipitation and purification was as described in (i) above and the resultant material was analyzed by IR and UV.

**Instrumentation**

For the determination of the reaction turnover with protease N in DMF, quantitative gas-chromatographic measurements were performed using a 438 A chromatograph from Chrompack, Frankfurt am Main, Germany, a CB WAX 52 capillary column (Chrompack) and a temperature program as follows: 60–170 C; +10 C min$^{-1}$ A 1 ml sample was taken from the reaction flask. The fatty acid ester was extracted with 1 ml *n*-hexane After phase separation, 1 $\mu$l of the hexane phase was injected into the GC.

IR-spectra were recorded on a Perkin-Elmer (Überlingen, Germany) 1600 series FT-IR-spectrometer in a range from 2000 to 500 cm$^{-1}$.

UV/VIS spectra were recorded using the 'UV-2100' from Shimadzu, Japan.

Proton-decoupled $^{13}$C-NMR spectra were recorded in deuterated DMSO at 80 C and 150 MHz using a Bruker WM-600 spectrometer.

## RESULTS AND DISCUSSION

In general, there are two possible approaches to the preparation of sugar esters. A lot of work has been spent on the *chemical synthesis* of sugar esters (i.e. Osipow *et al*., 1956; Osipow & Rosenblatt, 1967; Feuge *et al*., 1970). As there are many disadvantages, such as coloring of the products or the impossibility of reaching both high regioselectivity and high yields when no blocking groups are used, recent studies focus on *enzymatic synthesis* of esters. The standard reaction is carried out in a solvent such as *n*-hexane, the alcohol and the carboxyl compound are dissolved in equimolar ratios and the enzyme is added. For thermodynamic purposes the solvent has to be nearly free of water. The enzyme needs only a small amount of water to maintain its active structure. Higher yields of product can be obtained by varying the molar ratios. The enzymes used for these transformations are lipases (E.C. 3.1.1.3.) which can catalyze both esterification and transesterification reactions (Svanholm, 1989). The advantages of enzymatic synthesis are mild reaction conditions and high regioselectivity. Although there is no reported work on the enzymatic acylation of polysaccharides, we decided to treat Schizophyllan in this way. A regioselective acylation of the protruding side-chain group seemed possible.

So far, only DMSO is known as an organic solvent for Schizophyllan (Kashiwagi *et al*., 1981), but DMSO inactivated most enzymes and all lipases by stripping of the essential water mono-layer. This is characteristic of most organic solvents with $\log P < 2$. $\log P$ is defined as the common logarithm of the partition coefficient of a given substance in an octanol/water system. It is used as a measure of the hydrophobicity of solvents: the higher the $\log P$, the more hydrophobic the solvent (Laane *et al*., 1987). Hence a suitable organic solvent for Schizophyllan with $\log P > 2$ had to be found. Unfortunately all attempts to dissolve the glucan in a suitable solvent failed; even the detour via 'inclusion' of other organic solvents as suggested for cellulose by Batzer and Lohse (1976) or sonification of the suspension led to no positive results. Only when using fresh lyophilized Schizophyllan and stirring it for several weeks in DMF could a colloid or microgel of Schizophyllan be obtained. Esterification of Schizophyllan with lipases in this solvent is impossible because the $\log P$ of DMF is −1·0.

Adelhorst *et al*. (1990) and Björkling *et al*. (1989) report the preparation of sugar esters in a solvent-free process by mixing the sugar compound, the fatty acid, and an immobilized lipase at 60–80 C. Due to immobilization, the enzyme tolerates these temperatures. Hence the reaction can occur in the molten state. However, this procedure is described only for mono- and disaccharides. We used Schizophyllan in the pulverized and the immobilized state. The immobilization procedure was that used by Berger *et al*. (1992), who immobilized hydrophilic diols in order to transform them by lipases in hydrophobic solvents. Unfortunately, in our work no modification could be detected by IR-spectroscopy. In summary, it is impossible to acylate Schizophyllan by lipases under the above conditions. The reasons are the lack of a suitable solvent and the inaccessibility of the substrate to the enzyme. Schizophyllan seems to be too large and even the protruding side-chain residues are unapproachable. This might be different if the side-chain residue was longer or if the polymer could be acetylated when it was in the disordered stage, but temperatures higher than 80 C would cause thermal inactivation of the enzyme.

As sugars have a poor solubility in organic solvents where lipases have activity, some work was carried out to find acylating enzymes which work in solvents appropriate for sugars. Carrea *et al*. (1989), and Riva *et al*. (1988), report the acylation of mono- and disaccharides in DMF by subtilisin, a protease. As subtilisin is expensive, a crude preparation of subtilisin called protease N was tried and found to have the same specifity. Protease N accepts other nucleophiles in

addition to water, e.g. alcohols, and can therefore be used for transesterification reactions (Zaks & Klibanov, 1988). As Schizophyllan forms a colloid in DMF, we tried to esterify the glucan by protease N. Butanoic acid and esters were used because of the enzyme's substrate specificity for short fatty acid esters. The activity with octanoic acid methylester is only 8% compared to butanoic acid methylester. In addition to the methylester, the trifluoroethylester was used because of its excellent leaving-group but no decrease in the concentration of the butanoic acid esters was detectable, indicating that no reaction has taken place. Again, the size of the Schizophyllan molecule seems to be the problem.

Several attempts were made to acylate Schizophyllan chemically. The acylation of mono- and disaccharides in organic solvents like pyridine is well known (Einhorn *et al.*, 1898). The sugar compound and the acylating reagent, such as a fatty acid chloride, are dissolved in the solvent and the reaction mixture is heated. Addition of a base such as triethylamine shifts the equilibrium to the side of the products by utilizing $H^+$ and forming triethylamine hydrochloride, for example. It has to be emphasized that this reaction condition enables homogenous catalysis. In contrast, Schizophyllan could not be acetylated in pyridine and, surprisingly, not even in DMSO where the condition for homogenous catalysis was given.

An acetylation of Schizophyllan was possible under the strong conditions of acid-catalysis and a distinct excess of acetic anhydride. The IR-spectrum clearly shows a signal at 1750 $cm^{-1}$, indicating the formation of an ester bond (data not shown); this result was confirmed by the NMR spectrum (Fig. 2).

The explanations for the shifts (Table 1) are as follows:

1: downfield-shift through $\beta$-effect (compared to native Schizophyllan);
2: C-3 (D) of the native glucan not identifiable;
3: upfield-shift through two $\beta$-effects;
4: upfield-shift through two $\beta$-effects;
5: the new signals indicate the creation of a carbonyl-C and a methyl group, respectively.

In Table 1 the signals of the native and acetylated Schizophyllan are compared to those of scleroglucan, a glucan excreted by the fungus *Sclerotium rolfsii* having

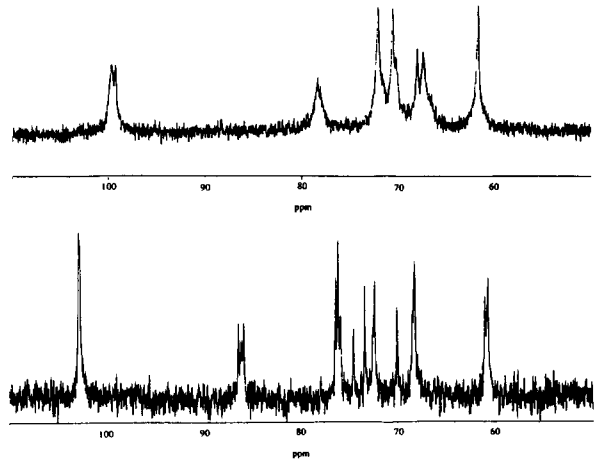


**Fig. 2.** Proton-decoupled $^{13}$C-NMR spectra of the native (below and acetylated (above) Schizophyllan.

identical molecular structure. There are slight differences in the signals of scleroglucan and the native Schizophyllan. Nevertheless, the signals of acetylated Schizophyllan show distinct shifts which are comparable with scleroglucan, the only exception being the C-6 (A/C/D). In this case, a downfield-shift of approximately 1 ppm is to be seen when compared with native Schizophyllan, but the signal is nearly identical with the corresponding signals of the scleroglucan. The findings of our laboratory would suggest complete acetylation, but the facts that the signal of C-6 (A/C/D) cannot be interpreted unequivocally as well as the large peaks of the acetylated Schizophyllan, are in contradiction to this suggestion. Therefore a clear statement on the degree of acetylation is impossible. A study of an acetylated scleroglucan could resolve this.

The mechanism of acylation might be the same as suggested for the formation of cellulose acetate (Eicher & Fischer, 1975): the concentrated acetic acid penetrates the native Schizophyllan, so the reagents have better access. It is assumed that the hydrogen bonds are cleaved or at least loosened. The sulfuric acid serves as a catalyst and forms intermediate esters in a fast and quantitative way and as the reaction proceeds these are replaced successively by acetyl groups.

Further investigations to determine essential characteristics, such as the molecular weight or tertiary structure, failed because until now no solvent for Schi-

**Table 1. Comparison of the $^{13}$C-NMR signals of scleroglucan (Rinaudo, 1982), native (Münzer, 1989) and acetylated Schizophyllan (compare with Fig. 1)**

| Classification | Scleroglucan [ppm] | Native [ppm] | Acetylated [ppm] |
|---|---|---|---|
| C-6 (A/C/D/) | 61·85/61·90/62·05 | 60·64/60·77/60·94 | 61·7[1] |
| C-3 (D) | 77·55 | 74·59 | n.i.[2] |
| C-5 (A/B/C/D) | 75·7/77·3/77·05/77·3 | 75·91/76·17/76·44/76·17 | 72·0[3] |
| C-3 (A/B/C) | 87·65/87·15/86·9 | 85·82/86·11/86·43 | 78·5[4] |
| C-carbonyl | | | 168*[5] |
| C-$CH_3$ | | | 21·0*[5] |

zophyllan acetate has been found. These data would be interesting, because under the strong conditions of acid catalysis a cleavage of the main chain or a split-off of the side chain might have occurred. There was slight solubility in DMSO, as shown by the NMR spectrum. Even in water, no solubility could be obtained.

## ACKNOWLEDGEMENT

We thank Dr V. Wray of the Gesellschaft für Biotechnologische Forschung mbH, Braunschweig, for recording the NMR spectra.

## REFERENCES

Adelhorst, K., Björkling, F., Godtfredsen, S.E. & Kirk, O. (1990) *Synthesis*, 112–5.

Batzer, H. & Kohse, F. (1976). *Hüthig & Wepf Vlg.* Basel Heidelberg, p. 123.

Berger, M., Laumen, K. & Schneider, M.P. (1992). *Biotechn. Lett.*, **14**(7), 553–8.

Björkling, F., Godtfredsen, S.E. & Kirk, O. (1989). *J. Chem. Soc., Chem. Commun.*, 934–5.

Carrea, G., Riva, S. & Secundo, F. (1989). *J. Chem. Soc. Perkin. Trans. I*, 1057–61.

Eicher, T. & Fischer, W. (1975) In *Ullmanns Enzyklopädie, 4. Aufl.*, Vol 9, Vlg. Chemie, Weinheim Bergstr.

Eigtved, P. & Hansen, T.T. (1986). Novo Industri A.S, 2880 Bagsvaerd, Denmark, **A-05948**.

Einhorn, A. & Hollandt, F. (1898). *Liebigs Ann. Chem.*, **301**, 95.

Feuge, R.O., Zeringue, Jr, H.J. Weiss, T.J. & Brown, M. (1970) *J. Am. Oil Chem. Soc.*, **47**, 56–60.

Höfl, G., Steglich, W. & Vorbrüggen, H. (1978). *Angew. Chem.*, **90**, 602–15.

Kashiwagi, Y., Norisuye, T. & Fujita, H. (1981) *Macromolecules*, **14**, 1220–5.

Laane, C., Boeren, S., Vos, K. & Veeger, C. (1987) *Biotechnol. Bioengng.*, **30**, 81–7.

Lecacheux, D., Mustiere, Y. & Panaras, R. (1986). *Carbohydr. Polym.*, **6**, 477–92.

Münzer, S. (1989). PhD Thesis, Technical University of Braunschweig, Braunschweig, Germany.

Norisuye, T., Yanaki, T. & Fujita, H. (1980). *J. Polym. Sci., Polym. Phys.*, **18**, 547–58.

Osipow, L.I. & Rosenblatt, W. (1967). *J. Am. Oil Chem. Soc.*, **47**, 307–9.

Osipow, L.I., Snell, F.D., Marra, D. & York, W.C. (1956). *Ind. Eng. Chem.*, **48**, 1459.

Rau, U., Olszewski, E. & Wagner, F. (1992*a*). *GIT*, **4**, 331–7.

Rau, U., Haarstrick, A. & Wagner, F. (1992*b*). *Chem. Ing. Tech.*, **64**, 576–7.

Rinaudo, M. (1982). *Carbohydr. Polym.*, **2**, 135–44.

Riva, R., Chopineau, J., Kieboom, A.P.G. & Klibanov, A.M. (1988). J. Am. Chem. Soc., **110**, 584–9.

Staab, H.A. (1957). *Liebigs Ann. Chem.*, **609**, 83–8.

Steglich, W. (1969). *Angew. Chem.*, **23**, 1001.

Svanholm, H. (1989). Novo Industri A.S, 2880 Bagsvaerd, Denmark, **A-06052**.

Van, K., Asakawaw, T. & Teramoto, A. (1984). *Polym. J.*, **16**(1), 61–9.

Zaks, A. & Klibanov, A.M. (1988) *J. Biol. Chem.*, **263**(7), 3194–201.