Carbohydrate Polymers 75 (2009) 670–676

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

# Synthesis of 6-deoxy-6-chloro and 6-deoxy-6-bromo derivatives of scleroglucan as intermediates for conjugation with methotrexate and other carboxylate containing compounds

Francesco Marchetti, Massimo Bergamin, Susanna Bosi, Riaz Khan, Erminio Murano, Stefano Norbedo *

*Polymer Conjugation Department, EURAND S.p.A., Area Science Park, Padriciano 99, 34012 Trieste, Italy*

ARTICLE INFO

*Article history:*
Received 24 July 2008
Received in revised form 8 September 2008
Accepted 8 September 2008
Available online 18 September 2008

*Keywords:*
Polysaccharide conjugate
Methotrexate
Scleroglucan
Selective chlorination

ABSTRACT

Polysaccharides are widely used as carriers in the field of drug delivery. We present a methodology to obtain water soluble drug-conjugates based on scleroglucan. Selective C-6 halogenation gives access to C-6 esters; conjugates between methotrexate and scleroglucan are described, potentially useful for anti-tumour therapy or in rheumatoid arthritis treatment.



## 1. Introduction

Scleroglucan is a natural neutral polysaccharide produced by fungi of the *Sclerotium* genre and consisting of a (1,3)-β-D-glucan backbone with lateral (1 → 6) glucose residues on each third monomer (Sutherland, 1998) (Fig. 1). The regularity of the resulting tetramer depends on its natural source: for instance, commercially available scleroglucan from *Sclerotium rolfsii* is a highly regular polymer. In water, scleroglucan assumes a triple helix structure, which is retained over a wide range of temperature, pH and electrolyte concentration (Norisuye, Yanaki, & Fujita, 1980; Stokke, Falch, & Dentini, 2001). This structure imparts rod-like rigidity and is disrupted only at pH above 12 or using a solvent such as dimethylsulfoxide.

Chemical modification of scleroglucan is a valuable mean to obtain new compounds which may be relevant for different applications. Polysaccharides are, in general, ideal candidates for drug delivery systems since they are in general water soluble, biocompatible, non-toxic and multifunctional.

A number of chemical transformations of scleroglucan have been described. Controlled and selective oxidation gives *scleraldehyde* (Maeda et al., 2001), while further oxidation affords *sclerox* (Stokke, Elgsaeter, Smidsrød, & Christensen, 1995), which is a polycarboxylated form of scleroglucan with two carboxyls per tetramer, located on the lateral chain. Other charged derivatives were obtained by oxidation of the primary hydroxyl group (de Nooy, Rori, Masci, Dentini, & Crescenzi, 2000), by esterification with phthalic acid or by etherification with chloroacetic acid or chloroalkylamines (de Nooy et al., 2000). A neutral cyano-ethylated scleroglucan was also described (Gianni et al., 2002). Iodine labelling of scleroglucan at the primary hydroxyl group was achieved by way of the 6-*O*-tosylscleroglucan, formed under basic aqueous conditions followed by nucleophilic substitution by $^{127}I^-$ (Boeykens, Vázquez, Temprano, & Rosen, 2004). Reported reactions on scleroglucan are usually performed under strongly aqueous basic conditions, where it exists as single chains, fully exposed to reactants.

We are interested in using polysaccharides as carrier molecules for drugs in order to enhance their water solubility, their stability and efficacy, to reduce toxicity and to target the drug to the active site.

In this paper we present a strategy for selective modification of scleroglucan, based on a direct C-6 selective Vilsmeier halogenation followed by substitution with alkyl or aryl carboxylates. This reaction sequence affords scleroglucan esters, formed regioselectively on the primary alcoholic functions, with degrees of substitution varying up to one sugar per tetramer.

To demonstrate the versatility of the method, we present the synthesis of a scleroglucan–methotrexate conjugate, potentially useful for antitumour therapy or in rheumatoid arthritis treatment. We also report the synthesis of 6-*O*-benzoylscleroglucan and 6-*O*-butyroylscleroglucan; butyrate derivatives are known to exhibit antitumour activity against colon cancer (Coradini, Pellizzaro, Miglierini, Daidone, & Perbellini, 1999).

* Corresponding author. Tel.: +39 0403756611; fax: +39 0409220016.
*E-mail address:* stefano.norbedo@eurand.com (S. Norbedo).

0144-8617/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2008.09.007

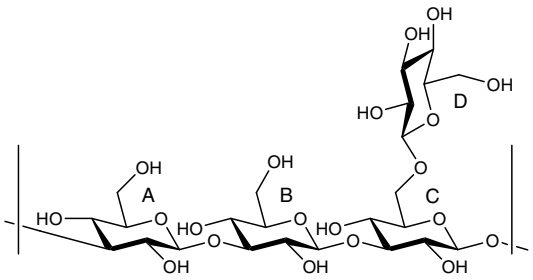



**Fig. 1.** The repeating unit of scleroglucan.

## 2. Materials and methods

Actigum CS11 from Degussa Texturant Systems was purified and depolymerized as described below. Methotrexate (MTX) was purchased from Fermion, Oulu (Finland), and used without further purification. All the reagents were purchased from Aldrich and Fluka and used without further purification. *N*,*N*-dimethylformamide (DMF), dimethylsulfoxide ($Me_2SO$), acetone, ethanol and isopropanol were purchased from Merck. *N*,*N*-dimethylformamide was distilled from BaO and stored over activated molecular sieves prior to use. Dimethylsulfoxide was stored over activated molecular sieves prior to use. Milli-Q water, produced by a Millipore ultra-pure water system, was used in final stages of dialysis or ultrafiltration. 10 g/mol cutoff membranes were used for dialysis. A Pall Centramate Ultrafiltration system, equipped with 10 g/mol cutoff membrane cassettes, was used for tangential flow filtration (TFF). Reaction vessels were oven-dried and cooled under nitrogen prior to use.

NMR spectra were recorded for $D_2O$ or dimethylsulfoxide–$d_6$ solutions on a Varian UNITY-Inova 500 equipped with an inverse $^1$H/broadband probehead fitted with a Z-gradient coil ($^1$H frequency 499.80 MHz, $^{13}$C frequency 125.69 MHz) or on a Varian Mercury-VX200 equipped with a direct $^1$H/broadband probehead ($^1$H frequency 199.98 MHz, $^{13}$C frequency 50.29 MHz). $^1$H NMR spectra were recorded with both a standard pulse sequence and filtering macromolecular species by diffusion weighing, using a Doneshot pulse sequence. Molecular weight distributions were determined with a HPSEC/MALS/RI/UV system composed by a Jasco PU-2080 HPLC pump equipped with an Alfatech autosampler, TSK PWxl (Tosoh Bioscience) G6000 + G5000 + G3000 columns and a TSK PWxl guard column (Tosoh Bioscience), a MALS DAWN EOS detector (Wyatt Technology Co., USA), a UV spectrophotometric detector 875-UV (Jasco) and an Interferometric Refractometer Optilab rEX detector (Wyatt Technology Co., USA), using 0.15 M NaCl solution as eluant. HPLC analyses were performed on an Agilent 1100 chromatographic system, equipped with a Phenomenex Synergi 4μ Max-RP80 A column and a diode array detector, using phosphate/citric acid buffer (pH 6) and acetonitrile mixtures as eluant. Water content was determined by TGA on a Perkin Elmer Thermogravimetric Analyzer TGA7.

## 3. Results and discussion

### 3.1. Depolymerization of scleroglucan

Commercial Actigum CS11 was purified by stirring in water at 40 °C for 24 h, followed by precipitation in ethanol, redissolution in water, extensive dialysis against Milli-Q water and freeze-drying. Purified scleroglucan was depolymerized using hydrogen peroxide under basic aqueous conditions, in order to combine the breaking of triple helix structures with radical statistic chain cleavage (Hjerde, Stokke, Smidsrød, & Christensen, 1998). We found a good dependence of the degree of depolymerization on the reaction time. Most importantly, the obtained samples presented good polidispersity values (1.9–2.7), and the degree of branching was shown to be fully conserved by NMR analysis (Misaki, Kakuta, Sasaki, Tanaka, & Miyaji, 1981). Table 1 shows how the molecular weight of scleroglucan can be tuned by controlled hydrolysis.

### 3.2. Halogenation

The reaction of scleroglucan (1), with a combination of methanesulfonyl chloride and DMF (Vilsmeier reaction) resulted in the selective replacement of primary hydroxyl groups by chloride. This reagent has been successfully used for the chlorination of primary hydroxyl groups of polysaccharides such as amylose (Cimecioglu, Ball, Kaplan, & Huang, 1994), pullulan (Mocanu, Constantin, & Carpov, 1996), laminaran (Khan, Bosco, Konowicz, Stucchi, & Rizzo, 1996) and cellulose (Ishii, Ishizu, & Nakano, 1977; Matsui, Ishikawa, Kamitakahara, Takano, & Nakatsubo, 2005). An attempt to rationalize the reaction of this combination of reagents with alcohols was made (Cimecioglu et al., 1994; McCormick, Dawsey, & Newman, 1990). A possible side reaction is mesylation of the polysaccharidic hydroxyl groups, and a proper choice of experimental conditions can promote either reaction pathway, affording mesylates or halides (McCormick et al., 1990; Mocanu et al., 1996).

The excellent reported C-6 selectivity is due to the more pronounced reactivity of the primary hydroxyl groups with respect to secondary ones, and to the particular mechanism, involving an $S_N2$ substitution by halide, which is disfavoured on ring carbons.

Applying these conditions, this behaviour was confirmed also for scleroglucan (Scheme 1).

In a first set of chlorination reactions we assessed the dependency of the outcome of the reaction on temperature and reaction time. Low molecular weight scleroglucan ($10^5$–$2 \times 10^5$) and a fixed excess of mesylchloride (30 eq per repeating tetramer) were used in a series of reactions carried out for 20 h at temperatures ranging from 40 to 80 °C (Table 2, entries 2a to 2f).

The degree of substitution (DS) is here defined as the fraction of chlorinated primary hydroxyl groups, or in general the fraction of substituted primary positions (three primary positions per tetramer).

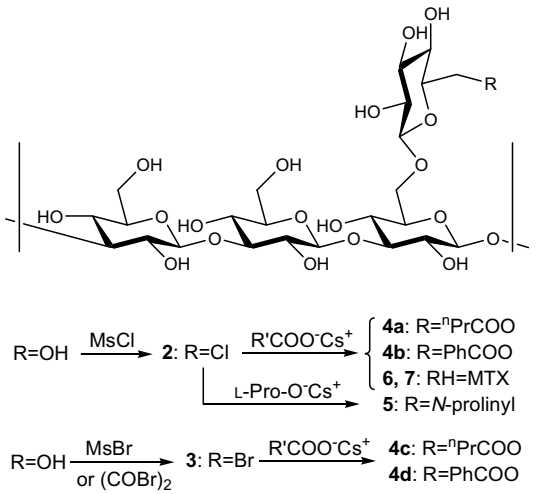
In all these reactions the only observed product was 6-chloro-6-deoxy-scleroglucan and mesylates were not detected. The structure was confirmed by NMR and quantification of primary chlorinated hydroxyl groups was obtained from quantitative decoupled $^{13}$C NMR spectra (Fig. 2). The peak of $CH_2Cl$ is present at 44 ppm in the $^{13}$C NMR spectrum, accompanied by a decrease of the intensity of the peak of $CH_2OH$ at 61 ppm.

We found that the entire range of DS from 0 to 1 can be obtained working between 40 and 80 °C. When working at temperatures above 60 °C we observed a dramatic fall in the quantity of recovered water soluble product. Moreover, products obtained under these conditions present a relevant percentage of formate groups, deriving from hydrolysis of the immonium complex (inter-

**Table 1**
Scleroglucan depolymerization dependence on hydrolysis time

| | *t* (min) | Yield % | $M_W$ (g/mol) | PI |
|---|---|---|---|---|
| **1a** | n.a. | n.a. | 1200 | 2.7 |
| **1b** | 20 | 90 | 500 | 2.5 |
| **1c** | 40 | 90 | 246 | 2.3 |
| **1d** | 60 | 90 | 116 | 2.1 |
| **1e** | 80 | 60 | 82 | 1.9 |

One percent solutions of scleroglucan in 1.5 N NaOH were treated with 6.3 mL of 30% $H_2O_2$/g of scleroglucan.
**1a** is untreated purified scleroglucan.




**Scheme 1.** Synthesis of halogenated scleroglucan and ester or amino derivatives thereof. Only one primary hydroxyl group is shown to take part in the reactions for convenience: all primary hydroxyl groups are statistically involved in reactions, independently on their position in the repeating tetramer.

**Table 2**
Dependence of scleroglucan chlorination upon temperature and time

| | *t* (h) | *T* °C | DS | Yield % |
|---|---|---|---|---|
| **2a** | 20 | 40 | 0 | 95 |
| **2b** | 20 | 50 | 0.20 | 95 |
| **2c** | 20 | 55 | 0.28 | 90 |
| **2d** | 20 | 60 | 0.35 | 60 |
| **2e** | 20 | 65 | 0.57 | 40 |
| **2f** | 20 | 80 | 1.00 | 20 |
| **2g** | 6 | 50 | 0.04 | 97 |
| **2h** | 25 | 50 | 0.23 | 96 |
| **2i** | 30 | 50 | 0.25 | 94 |
| **2j** | 72 | 50 | 0.30 | 93 |

DS are averaged values from repeated reactions and were found to lie within a ±0.02 range.
Yields are averaged values from repeated reactions.

mediate of halogenation; Cimecioglu et al., 1994), which can be hydrolyzed only by prolonged basic aqueous treatment. Most of the product was recovered as a water insoluble solid; NMR analysis of these solids in dimethylsulfoxide–$d_6$ generally showed very high chloride contents.

At this stage of the work we considered water solubility of halogenated intermediates to be critical for the solubility of the resulting esters, since the latter would not stand prolonged aqueous basic treatment in order to regain solubility. Thus, in another set of reactions, temperature was fixed at 50 °C to maximize yield, and reaction time was varied. Table 2 (entries 2g–2j and 2b) summarizes the results for these reactions, highlighting a smooth dependence of DS upon reaction time.

A plateau of 0.30 for DS was reached with prolonged reaction times. Albeit limited in DS range, we found these conditions an optimal compromise to produce chlorinated scleroglucan in high yield and with a good chloride content. A DS of 0.30 means roughly one chlorinated glucose per tetramer, optimal for carrier–drug conjugation applications.

The chlorination reactions do not seem to depend on the molecular weight of the starting material: when a sample of scleroglucan with $M_w = 1.2 \times 10^6$ was treated at 50 °C for 20 h, it gave a DS of 0.17, in good agreement with the previous results.

For the synthesis of 6-bromo-6-deoxyscleroglucan we considered again the Vilsmeier reagent combination, i.e. methanesulphonyl bromide/DMF.

The use of mesyl bromide gave homogeneous suspensions, but nevertheless required rather high temperatures to get appreciable results (Table 3). A basic treatment was required at the end of the reaction to eliminate formates, at pH 9. In this case, contrary to mesyl chloride, a strong excess leads to water insoluble products. For instance, when making **3a**, we recovered an insoluble solid in 70% yield whose DS, measured in dimethylsulfoxide, is comparable with that of the soluble fraction. In subsequent trials we gradually reduced the amount of reagent, observing an increase in yield at the expense of DS, attaining a good compromise for products **3c** and **3d**. The structure of the products was confirmed by NMR (see Fig. 2 for a $^{13}C$ NMR spectrum).

### 3.3. Esterification

The obtained halogenated scleroglucans could be converted into esters by reacting them with the cesium salts of organic acids in dimethylsulfoxide. Carboxylates were formed *in situ* by adding solid cesium carbonate to a dimethylsulfoxide solution containing modified scleroglucan and an organic acid in a threefold molar excess with respect to the tetramer. The results for coupling to butyric and benzoic acid are listed in Table 4.

We found that cesium butyrate is an optimal nucleophile for this reaction, able of displacing all halogen groups, even starting from less reactive **2j**, while cesium benzoate, under the same conditions, substitutes only one third of total chlorides from **2j** and around half bromides from **3c**.

Interestingly, reactions with unprotected amino acids seem to prefer amine formation with respect to ester formation. For instance, 6-deoxy-6-chlorosclerogluca**n 2b** (DS 0.20), when treated with excess proline and cesium carbonate in dimethylsulfoxide, afforded exclusively the tertiary amine **5** (DS 0.20 in amine, Scheme 1) with the final effect of introducing charged carboxylates on the scleroglucan chain.

In order to demonstrate the applicability of our strategy to the production of pharmaceutically relevant bioconjugates, we studied the esterification with methotrexate, a folate analogue antimetabolite used for the treatment of many neoplastic disorders, psoriasis, psoriatic arthritis, rheumatoid arthritis and Crohn's disease. Methotrexate (Fig. 3) presents a dicarboxylic glutamic portion, and in its dianionic form the γ-carboxylate is the more reactive nucleophile, being less acidic and less hindered than the α-carboxylate.

We thought that use of excess diacid, combined with higher reactivity on the γ-carboxylate, should minimize any possible cross-linking, so, on a first instance, we did not consider selective carboxylate protection before conjugation.

In a first set of reactions, reaction time was kept constant at 48 h while temperature was varied between 25 °C and 80 °C. The starting activated scleroglucan **2c** had a chloride DS of 0.28. Quantification of linked methotrexate was best performed by means of HPLC, first analyzing a water solution of the scleroglucan ester to measure any free methotrexate left and then analyzing a solution of hydrolyzed conjugate (2 h in 0.1 N NaOH solution at room temperature) to measure the total quantity of methotrexate. Bound methotrexate could then be determined by difference. HPLC results were supported by $^1H$ NMR analysis (Fig. 4 shows a 2D HSQC spectrum of a conjugate) and by DOSY (Diffusion Ordered SpectroscopY) NMR measurements, which clearly showed the drug bound to the polymer.

As can be seen from Table 5 (**6a**–**e**), the reaction takes place at an optimum rate at 80 °C, giving a linked methotrexate DS of 0.20, corresponding to the displacement of 70% of initial chloride groups. Higher temperatures/longer reaction times were avoided in order to minimize degradation of the polymer.

In an effort to increase the loading in methotrexate, we reconsidered the chlorination of scleroglucan at higher temperatures,

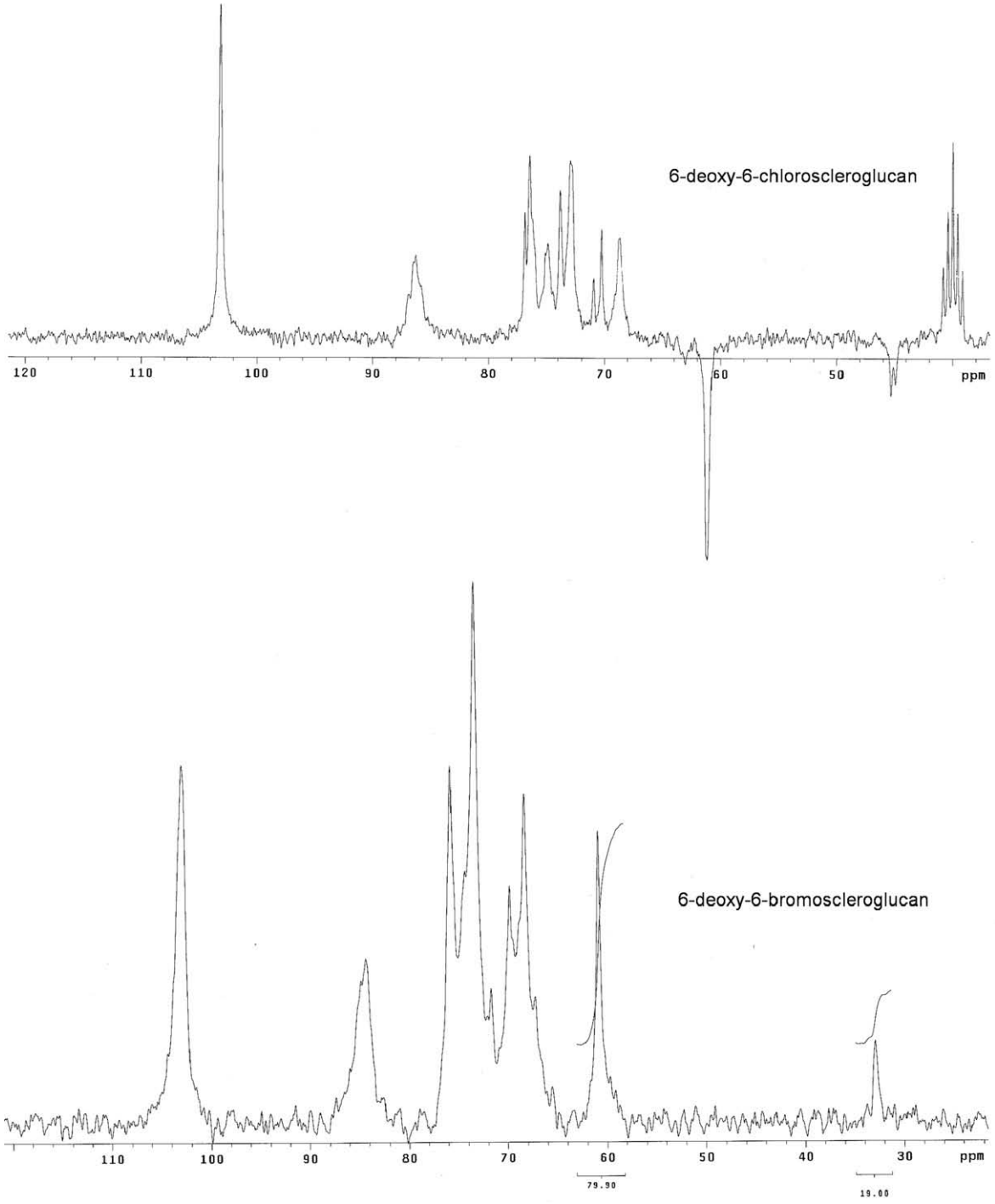



**Fig. 2.** DEPT-135 NMR spectrum of 6-deoxy-6-chlorosclerogluca in dimethylsulfoxide–$d_6$ and $^{13}C$ NMR spectrum of 6-deoxy-6-bromosclerogluca in $D_2O$.

skipping the basic work-up used to deformilate. Thus, scleroglucan with a $M_w$ of 500 kg/mol was treated with excess mesylchloride, at 60 °C for 20 h and quenched with aqueous NaOH to pH 7. The resulting product, as expected, was soluble only in $Me_2SO$ and

**Table 3**
Bromination of scleroglucan by mesyl bromide

| | MsBr eq | DS | Yield % |
|---|---|---|---|
| **3a** | 40 | 0.60 | 10 |
| **3b** | 20 | 0.47 | 28 |
| **3c** | 10 | 0.25 | 75 |
| **3d** | 8 | 0.23 | 82 |
| **3e** | 4 | 0.00 | 64 |

Reaction temperature was 70 °C and reaction time was 16 h for all entries.

**Table 4**
Formation of scleroglucan esters by halogen displacement

| | Reactant | Halogen, DS | $T$ (°C) | $t$ (h) | Ester | Ester DS | Yield % |
|---|---|---|---|---|---|---|---|
| **4a** | **2j** | Cl, 0.30 | 80 | 40 | Butyric | 0.30 | 75 |
| **4b** | **2j** | Cl, 0.30 | 80 | 40 | Benzoic | 0.10 | 80 |
| **4c** | **3c** | Br, 0.47 | 60 | 19 | Butyric | 0.47 | 83 |
| **4d** | **3c** | Br, 0.25 | 60 | 19 | benzoic | 0.12 | 93 |

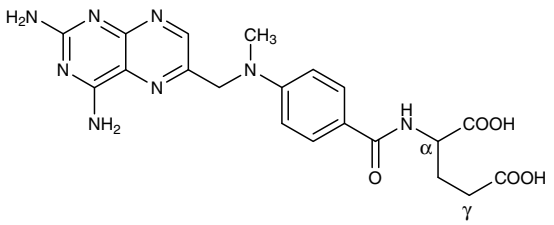



**Fig. 3.** Methotrexate.

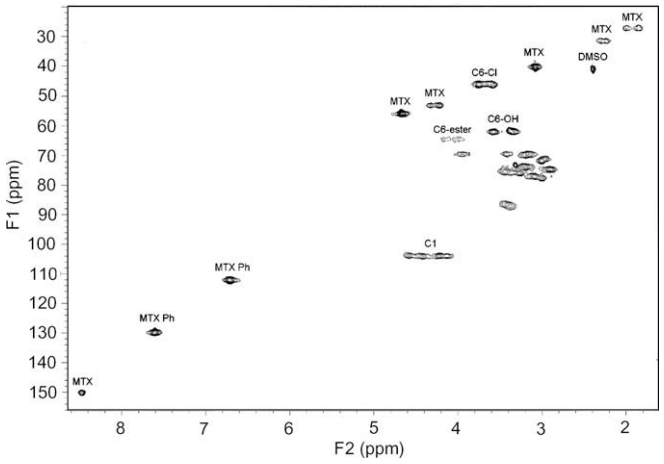



**Fig. 4.** $^{1}H$–$^{13}C$ heterocorrelated HSQC spectrum of a scleroglucan–MTX conjugate. F1 is the $^{13}C$ dimension, F2 is the $^{1}H$ dimension. The cross-peaks due to formation of an ester in C-6 are visible at 64 ppm ($^{13}C$).

**Table 5**
Dependence of MTX conjugation upon temperature

| | $T$ °C | $t$ (h) | MTX w/w% | DS | Yield |
|---|---|---|---|---|---|
| **6a**[a] | 25 | 48 | 0.0 | 0.00 | 100 |
| **6b**[a] | 50 | 48 | 2.3 | 0.011 | 75 |
| **6c**[a] | 60 | 48 | 5.6 | 0.028 | 70 |
| **6d**[a] | 70 | 48 | 8.5 | 0.044 | 70 |
| **6e**[a] | 80 | 48 | 30 | 0.20 | 75 |
| **7a**[b] | 60 | 24 | 20.0 | 0.12 | 90 |
| **7b**[b] | 60 | 48 | 21.8 | 0.13 | 92 |
| **7c**[b] | 80 | 24 | 35.5 | 0.26 | 95 |
| **7d**[b] | 80 | 48 | 48.0 | 0.44 | 94 |

[a] Conjugations performed on hydrolyzed and isolated intermediate.
[b] Conjugations performed directly on crude, non-hydrolyzed intermediate.

showed a DS of 0.73 for chloride and the presence of formate esters. This was used directly for coupling with methotrexate using cesium carbonate in $Me_2SO$. Four reactions (Table 5, **7a**–**d**) were carried out, changing temperature and reaction time.

At 60 °C, substitution reaches a plateau for DS at 0.13, while at 80 °C displacement is more extensive, giving a DS of up to 0.43 after 48 h, corresponding to the substitution of 59% of initial chloride groups. Water solubility of these samples is good for compounds **7a** and **7b**, while it drops down with the higher loadings. To impart good water solubility we succinylated conjugate **7c** (MTX DS of 0.26) using excess succinic anhydride in DMF, in the presence of triethylamine and DMAP. As expected, succinylation resulted in good water solubility.

## 4. Conclusions

We have developed a methodology for selective C-6 modification of scleroglucan. C-6 halogenated intermediates can then be converted to C-6 esters. Good reproducibility and control were achieved on halogen (and consequently ester) loading, maintaining chain integrity and good to high yields. A scleroglucan–methotrexate ester was synthesized to show how water soluble drug–polysaccharide conjugates can be produced by this method.

## 5. Experimental

### 5.1. Purification of actigum CS11

A mass of 10.0 g of commercial scleroglucan were suspended in 1.8 L of Milli-Q water and mechanically stirred for 24 h at 40 °C. The resulting suspension was concentrated in a rotary evaporator to half of the volume and precipitated in 3 L of ethanol. After filtration, the solid was suspended in water and dialyzed against Milli-Q water. After freeze-drying, 9.5 g of a white solid were recovered.

### 5.2. Depolymerized scleroglucan 1a–e

A mass of 8.9 g purified scleroglucan was suspended in 500 mL of 1.5 N NaOH solution under mechanical stirring. A volume of 56 mL of 30% hydrogen peroxide solution was then added and the mixture was stirred at room temperature for 40 min. A volume of 300 mL of 1.5 N NaOH solution was then added. After 20 min the mixture was heated to 60 °C and stirred for the required time. Then it was neutralized with 3 M HCl solution, dialyzed against Milli-Q water and freeze dried to obtain a white solid.

### 5.3. 6-6-Chloro-deoxyscleroglucan 2a-j

A mass of 4.3 g (6.6 mmol) of depolymerized scleroglucan 1d-5−5 °C and 5.2 mL (66 mmol) of methanesulfonyl chloride were added dropwise. After 30 min the mixture was heated to the required temperature and stirred for the required time (see Table 2). Then it was cooled with an ice-bath and quenched with 200 mL of water. pH was brought to neutrality by addition of 1 M NaOH solution. The mixture was dialyzed against Milli-Q water, solid residues were filtered off and the solution was freeze-dried to afford white to off-white solids. The DS in chloride was determined from integration of the peak at 61 ppm ($CH_2OH$) and the peak at 45 ppm ($CH_2Cl$) in the $^{13}C$ NMR spectra. Letters A, B, C, D in the list of NMR signals specify the rings of the repeating tetramer (see Fig. 1). $^{13}C$ NMR ($\delta$) ($Me_2SO$-$d_6$): 44.8 ($CH_2Cl$), 45.2 ($CH_2Cl$), 61.0 (C-6; A, B, D), 68.7 (C-4, A, B, C; C-6, C), 70.1 (C-4, D), 73.2 (C-2, A, B, C), 74.0 (C-2, D), 75.0 (C-5, C), 76.3 (C-5, A, B, D), 77.0 (C-3, D), 86 (b, C-3, A, B, C), 103.4 (C-1, A, B, C, D).

### 5.4. 6-Bromo-6-deoxyscleroglucan 3a

A mass of 987 mg (1.52 mmol) of depolymerized scleroglucan 1e was dissolved in 45 mL of DMF by stirring for 1 h at room temperature under nitrogen. The resulting solution was cooled to −10 °C and 5.00 mL (61.4 mmol) of methanesulfonyl bromide were added dropwise. After stirring for 30 min, the mixture was gradually heated to 70 °C during 2 h. After stirring for 16 h the mixture was cooled to room temperature and poured in portions into 200 mL of a 1:1 mixture of ice and saturated $NaHCO_3$ solution. The pH was adjusted to 9 with 1.5 N NaOH and the suspension was stirred overnight. Solids were filtered, washed with water and dried under vacuum to give 783 mg (75%) of a white solid. The filtrate was neutralized with 1 N HCl, ultrafiltered against water, concentrated in a rotary evaporator and freeze-dried to give 109 mg (10.4%) of a pale brown solid. The DS in bromide was determined from integration of the peak at 61 ppm ($CH_2OH$) and the

peak at 33 ppm ($CH_2Br$) in the $^{13}C$ NMR spectra. Letters A, B, C, D in the list of NMR signals specify the rings of the repeating tetramer (see Fig. 1). $^{13}C$ NMR ($\delta$) ($D_2O$): 33.0 ($CH_2Br$), 61.0 (C-6; A, B, D), 68.5 (C-4, A, B, C; C-6, C), 70.0 (C-4, D), 73.8 (C-2, A, B, C), 74.3 (C-2, D), 76.0 (C-5, A, B, C, D; C-3, D), 84.5 (b, C-3, A, B, C), 103.0 (C-1, A, B, C, D).

### 5.5. 6-Bromo-6-deoxyscleroglucan 3b

Following the same procedure for the synthesis of **3d**, starting from 889 mg (1.37 mmol) of **1e** in 45 mL of DMF and 2.25 mL (27.7 mmol) of methanesulfonyl bromide, 296 mg (28%) of white solid were recovered freeze-drying the water-soluble filtrate. No appreciable water-insoluble solids were recovered during filtration. $^{13}C$ NMR spectrum was similar to that of **3a**.

### 5.6. 6-Bromo-6-deoxyscleroglucan 3c

Following the same procedure for the synthesis of **3d**, starting from 500 mg (0.772 mmol) of **1e** in 25 mL of DMF and 0.63 mL (7.7 mmol) of methanesulfonyl bromide, 394 mg (75%) of white solid were recovered freeze-drying the water-soluble filtrate. No appreciable water-insoluble solids were recovered during filtration. $^{13}C$ NMR spectrum was similar to that of **3a**.

### 5.7. 6-Bromo-6-deoxyscleroglucan 3d

Following the same procedure for the synthesis of **3d**, starting from 401 mg (0.619 mmol) of 1e in 20 mL of DMF and 405 μL (4.95 mmol) of methanesulfonyl bromide, 345 mg (82%) of white solid were recovered freeze-drying the water-soluble filtrate. No appreciable water-insoluble solids were recovered during filtration. $^{13}C$ NMR spectrum was similar to that of **3a**.

### 5.8. 6-Bromo-6-deoxyscleroglucan 3e

Following the same procedure for the synthesis of **3d**, starting from 392 mg (0.605 mmol) of **1e** in 20 mL of DMF and 197 μL (2.42 mmol) of methanesulfonyl bromide, 395 mg (100%) of white solid were recovered freeze-drying the water-soluble filtrate. No appreciable water-insoluble solids were recovered during filtration. $^{13}C$ NMR spectrum was similar to that of **3a**.

### 5.9. 6-O-butyroyl-scleroglucan 4a

A mass of 150 mg (0.227 mmol) of **2j** was dissolved in 15 mL of $Me_2SO$, stirring under nitrogen at room temperature. A mass of 41 μL (0.455 mmol) of butyric acid and 81 mg (0.249 mmol) of solid anhydrous cesium carbonate were then added and the mixture was heated to 80 °C and stirred for 40 h. Then it was cooled to room temperature, poured into 100 mL of ice water (pH was checked and found to be around 6.5), filtered to remove traces of solids and ultrafiltered against water. After concentration in a rotary evaporator, it was freeze-dried to afford 120 mg (75%) of a yellow solid. IR (solid): $\nu$ 1740 (ester); $^1H$ NMR ($D_2O$): $\delta$ 1.05 (m, 2.7H, Pr), 1.73 (m, 1.8H, Pr), 2.51 (m, 1.8H, Pr), 3.4–4.75 (m, 24H), 4.84 (m, 4H, anomeric).

### 5.10. 6-O-benzoyl-scleroglucan 4b

Following the same procedure for the synthesis of **4a**, starting from 300 mg (0.455 mmol) of **2j** in 30 mL of $Me_2SO$, 100 mg (0.909 mmol) of benzoic acid and 165 mg (0.506 mmol) of cesium carbonate, 270 mg (80%) of yellowish solid were obtained. IR (solid): $\nu$ 1720 (ester), 1603 (Ph); $^1H$ NMR ($D_2O$): $\delta$ *3.4–4.75* (m, 24H), 4.84 (m, 4H, anomeric), 7.67 (m, 0.60H, Bz), 7.80 (m, 0.30H, Bz), 8.18 (m, 0.60H, Bz).

### 5.11. 6-O-butyroyl-scleroglucan 4c

Following the same procedure for the synthesis of **4a**, starting from 83 mg (0.116 mmol) of **3f** in 10 mL of $Me_2SO$, 26 mg (0.29 mmol) of benzoic acid and 38 mg (0.116 mmol) of cesium carbonate, 69 mg (83%) of yellow solid were obtained. IR (solid): $\nu$ 1740 (ester); $^1H$ NMR ($D_2O$): $\delta$ 1.05 (m, 4.2H, Pr), 1.73 (m, 2.8H, Pr), 2.51 (m, 2.8H, Pr), 3.4–4.0 (m, 21.2H), 4.42 (m, 1H, $CH_2O$), 4.64 (m, 1.4H, $CH_2OCO$), 4.96 (m, 1.4H, $CH_2OCO$), 4.55 (m, 4H, anomeric).

### 5.12. 6-O-benzoyl-scleroglucan 4d

Following the same procedure for the synthesis of **4a**, starting from 150 mg (0.219 mmol) of **3f** in 15 mL of $Me_2SO$, 70 mg (0.79 mmol) of benzoic acid and 38 mg (0.72 mmol) of cesium carbonate, 139 mg (83%) of yellow solid were obtained. IR (solid): $\nu$ 1719 (ester), 1602 (Ph); $^1H$ NMR ($D_2O$): $\delta$ *3.4–4.75* (m, 24H), 4.84 (m, 4H, anomeric), 7.67 (m, 0.72H, Bz), 7.80 (m, 0.37H, Bz), 8.18 (m, 0.71H, Bz).

### 5.13. 6-(N-prolinyl)-6-deoxyscleroglucan 5

A mass of 300 mg (0.46 mmol) of **2b was** dissolved in 30 mL of $Me_2SO$, stirring under nitrogen at room temperature. A mass of 100 mg (0.93 mmol) of L-proline and 165 mg (0.51 mmol) of solid anhydrous cesium carbonate were then added and the mixture was heated to 80 °C and stirred for 48 h. Then it was cooled to room temperature, poured into 100 mL of ice water (pH was checked and found to be around 6), filtered to remove trace solids and ultrafiltered against water. After concentration in a rotary evaporator, it was freeze-dried to afford 247 mg (80%) of a white solid. DS (L-proline): 0.20. $^1H$ NMR ($D_2O$): $\delta$ 2.35 (m, 0.6H, $CH_2$ Pro), 2.1 (m, 1.2H, $CH_2$ Pro), 2.4 (m, 0.6H, $CH_2$ Pro), 3.1–4.25 (m, 24H), 4.7 (m, 4H, anomeric).

### 5.14. 6-O-methotrexylscleroglucan ***6a–e***

A mass of 150 mg (0.23 mmol) of 6-chloro-6-deoxyscleroglucan was added to 7.5 mL of $Me_2SO$, stirring under nitrogen at 80 °C for 1 h. After cooling to room temperature, a solution of 314 mg (0.69 mmol) of methotrexate in 3 mL of $Me_2SO$ and 225 mg (0.69 mmol) of solid cesium carbonate were added, and the resulting suspension was heated to the required temperature and vigorously stirred for the required time. Then it was cooled to room temperature and quenched with two volumes of water. After neutralization to pH 7 with 1 N HCl solution, the resulting solution was diluted with water, filtered through a class IV sintered glass filter and ultrafiltered by TFF. The purified solution was concentrated in a rotary evaporator and freeze-dried to afford a yellow solid. Letters A, B, C, D in the list of NMR signals specify the rings of the repeating tetramer (see Fig. 1). NMR integrations depend on the degree of substitution in MTX and vary for each compound. IR (solid): $\nu$ 1728 (ester). UV (nm): 246.3, 305.6. $^1H$ NMR ($D_2O$): $\delta$ 1.90 (m, $CH_2$ MTX), 2.04 (m, $CH_2$ MTX), 2.30 (m, $CH_2$ MTX), 2.83–3.54 (m), 3.10 (s, $CH_3$ MTX), 3.60 (m, $CH_2OH$), 3.63 (m, $CH_2Cl$), 3.68 (m, $CH_2Cl$), 3.97 (m, $CH_2O$), 4.01 (m, $CH_2OCO$), 4.1–4.6 (m, anomeric), 4.19 (m, $CH_2OCO$), 4.23 (m, $C_\alpha H$ MTX), 4.65 (s, $CH_2N$ MTX), 6.71 (m, Ar MTX), 7.60 (m, Ar MTX), 8.46 (s, Ar MTX). $^{13}C$ NMR ($\delta$) ($D_2O$): 27 ($CH_2$ MTX), 31.5 ($CH_2$ MTX), 41 ($CH_3$ MTX), 46 ($CH_2Cl$), 53 ($C_\alpha$ MTX), 56 ($CH_2N$ MTX), 61.6 (C-6, A, B, D), 65 (C-6-OCO), 70 (C-4, A, B, C; C-6, C), 72 (C-4, D), 75 (C-2, A, B, C), 76 (C-2, D), 76.5, 77, 78, 78.5, 87 (C-3, A, B, C), 104 (C-1, A, B, C, D), 112 (Ar, MTX), 130 (Ar, MTX), 150.5 (Ar, MTX).

### 5.15. Methotrexate 6-scleroglucanyl esters **7a–d**

A mass of 6 g (9.3 mmol) of **1b** were dissolved in 280 mL of DMF by stirring for 1 h at room temperature under nitrogen. The resulting solution was cooled to −5 °C and 7.2 mL (93 mmol) of methanesulfonyl chloride were added dropwise. After 30 min the mixture was heated to 60 °C and stirred for 20 h. Then it was cooled with an ice-bath, quenched with 200 mL of water and neutralized with 1 M NaOH solution. The mixture was dialyzed against Milli-Q water and freeze-dried to afford a solid which was directly used in the next step. A mass of 1.30 g (about 2 mmol) of this solid was added to 65 mL of $Me_2SO$, stirring under nitrogen at 80 °C for 1 h. After cooling to room temperature, a solution of 2.73 g (6.0 mmol) of methotrexate in 30 mL of $Me_2SO$ and 1.95 g (6.0 mmol) of solid cesium carbonate were added, and the resulting suspension was heated to the required temperature and vigorously stirred for the required time. Then it was cooled to room temperature and quenched with two volumes of water. After neutralization to pH 7 with 1 N HCl solution, the resulting solution was diluted with water, filtered through a class IV sintered glass filter and ultrafiltered by TFF. The purified solution was concentrated in a rotary evaporator and freeze-dried to afford a yellow solid. Spectra are comparable to those obtained for compounds **6a–e**.

## References


Boeykens, S. P., Vázquez, C., Temprano, N., & Rosen, M. (2004). Study of a novel labelled scleroglucan macromolecule. *Carbohydrate Polymers, 55*, 129–137.

Cimecioglu, A. L., Ball, D. H., Kaplan, D. L., & Huang, S. H. (1994). Preparation of amylose derivatives selectively modified at C-6. 6-Amino-6-deoxyamylose. *Macromolecules, 27*, 2917–2922.

Coradini, D., Pellizzaro, C., Miglierini, G., Daidone, M. G., & Perbellini, A. (1999). Hyaluronic acid as drug delivery for sodium butyrate: improvement of the antiproliferative activity on a breast-cancer cell line. *International Journal of Cancer, 81*, 411–416.

de Nooy, A. E. J., Rori, V., Masci, G., Dentini, M., & Crescenzi, V. (2000). Synthesis and preliminary characterisation of charged derivatives and hydrogels from scleroglucan. *Carbohydrate Research, 324*, 116–126.

Gianni, R., Delben, F., Liut, G., Bernstorff, S., Amenitsch, H., & Rizzo, R. (2002). Synthesis and conformational properties of cyanoethyl-scleroglucan. *Carbohydrate Polymers, 47*, 387–391.

Hjerde, T., Stokke, B. T., Smidsrød, O., & Christensen, B. E. (1998). Free-radical degradation of triple-stranded scleroglucan by hydrogen peroxide and ferrous ions. *Carbohydrate Polymers, 37*, 41–48.

Ishii, T., Ishizu, A., & Nakano, J. (1977). Chlorination of cellulose with methanesulphonyl chloride in *N*, *N* dimethylformamide and chloral. *Carbohydrate Research, 59*, 155–163.

Khan, R., Bosco, M., Konowicz, P. A., Stucchi, L., & Rizzo, R. (1996). Synthesis of 6-deoxy-6-halolaminarans and conversion of 6-chloro-6-deoxylaminaran into the 6-amino-6-deoxy derivative. *Carbohydrate Research, 292*, 39–46.

Maeda, H., Rambone, G., Coviello, T., Yuguchi, Y., Urakawa, H., Alhaique, F., et al. (2001). Low-degree oxidized scleroglucan and its hydrogel. *International Journal of Biological Macromolecules, 28*, 351–358.

Matsui, Y., Ishikawa, J., Kamitakahara, H., Takano, T., & Nakatsubo, F. (2005). Facile synthesis of 6-amino-6-deoxycellulose. *Carbohydrate Research, 340*, 1403–1406.

McCormick, C. L., Dawsey, T. R., & Newman, J. K. (1990). Competitive formation of cellulose *p*-toluenesulfonate and chlorodeoxycellulose during homogeneous reaction of *p*-toluenesulfonyl chloride with cellulose in *N*, *N*-dimethylacetamide-lithium chloride. *Carbohydrate Research, 208*, 183–191.

Misaki, A., Kakuta, M., Sasaki, T., Tanaka, M., & Miyaji, H. (1981). Studies on interrelation of structure and antitumor effects of polysaccharides: antitumor action of periodate-modified, branched (1 → 3)-β-D-glucan of auricularia auricula-judae, and other polysaccharides containing (1 → 3)-glycosidic linkages. *Carbohydrate Research, 92*, 115–129.

Mocanu, G., Constantin, M., & Carpov, A. (1996). Chemical reactions on polysaccharides, 5. Reaction of mesyl chloride with pullulan. *Die Angewandte Makromoleculare Chemie, 241*, 1–10.

Norisuye, T., Yanaki, T., & Fujita, H. (1980). Triple helix of a schizophyllum commune polysaccharide in aqueous solution. *Journal of Polymer Science. Polymer Physics Edition, 18*, 547–558.

Stokke, B. T., Elgsaeter, A., Smidsrød, O., & Christensen, B. E. (1995). Carboxylation of scleroglucan for controlled crosslinking by heavy metal ions. *Carbohydrate Polymers, 27*, 5–11.

Stokke, B. T., Falch, B. H., & Dentini, M. (2001). Macromolecular triplex zipping observed in derivatives of fungal (1 → 3)-β-D-glucan by electron and atomic force microscopy. *Biopolymers, 58*, 535–547.

Sutherland, I. W. (1998). Novel and established applications of microbial polysaccharides. *Trends in Biotechnology, 16*, 41–46.