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A New Sepharose Derivative Containing Covalently Bound *myo*-Inositol: Its Structure and Application

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myo-Inositol was covalently bound on an epoxy-activated sepharose. In order to elucidate the structure of the derivative of sepharose formed, in a model reaction *myo*-inositol was coupled to propylene oxide, whereby two compounds were obtained. By NMR-spectroscopy, ^{13}C-resonancespectroscopy and gas chromatography substance **I** could be characterized as a mixture of two isomers. Both are the product of a binding to the hydroxyl group on C-atom 2 of *myo*-inositol. The only difference seems to be the point of attachment to the side chain. Substance **II**, however, originates by substitution on an equitorial hydroxyl group. The application of this new affinity gel is reported.

(Keywords: Affinity chromatography; Epoxy coupling; Myo-inositol; Sepharose)

Ein neues Sepharose-Derivat mit kovalent gebundenem myo-Inosit: Struktur und Anwendung

myo-Inosit wurde an eine epoxy-aktivierte Sepharose kovalent gebunden. Zur Aufklärung der Struktur dieses Sepharosederivats wurde in einer Modellreaktion *myo*-Inosit an Propylenoxid gekoppelt, wobei zwei Substanzen erhalten wurden. Durch NMR-Spektren, ^{13}C-Resonanzspektren und Gaschromatographie konnte Substanz **I** als Gemisch zweier Isomerer charakterisiert werden. Beide sind durch Derivatisierung am C-Atom 2 des Inosits entstanden, sie unterscheiden sich nur im Ort der Bindung an der Seitenkette. Bei Substanz **II** ist hingegen die Substitution an einem äquatorialen O-Atom eingetreten.

Es wird über die Anwendung des neuen Affinitätsgels berichtet.

Introduction

In two previous publications the synthesis of affinity gels has been described, which were designed for the biospecific affinity chromatography of enzymes of *myo*-inositol metabolism. The first of

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the gels contains a residue of *epi*-inositol, an inositol stereochemically equivalent to *myo*-inositol at 5 of its 6 C-atoms[1]. The second gel contains a *myo*-inositol-2-phosphate residue bound as it's phosphoamide[2]. As will be shown later, one of the enzymes of phytic biosynthesis in *Lemna gibba*[3] did not bind to either of the above mentioned gels. For the isolation of this enzyme, which catalyzes the *ATP* dependent synthesis of *myo*-inositol monophosphate from *myo*-inositol, we tried to develop a new affinity gel. This gel should contain the complete structure of *myo*-inositol but should not carry a negative charge as the last mentioned gel does. The method ideally suited for this purpose was epoxy-activation and dircet coupling to *myo*-inositol.

Materials and Methods

Synthesis of the Sepharose Derivatives

A) 50 ml of preswollen and thoroughly washed Separose 4 B (Pharmacia) were treated with butanediol diglycidylether (EGA-Chemie, Steinheim/Albuch, Germany) according to *Porath*[4]. By this treatment we achieve crosslinking and "epoxy-activation", i.e. introduction of reactive epoxy groups, being covalently bound to the carbohydrate backbone. 5 ml of the product, or alternatively 5 ml of preswollen and washed epoxy-activated Sepharose 6 B (Pharmacia) were mixed with a solution of *myo*-inositol (0.4*M*) containing 2×10^6 dpm [U-^{14}C]*myo*-inositol (Radiochemical Centre, Amersham) in 0.01*M*-NaOH (10 ml) and shaken for 24 h at 25 °C.

The gel was then washed with 1 l of H_2O, excess H_2O removed by suction on a *Büchner* funnel and the bound radioactive material determined in an aliquot of the product as described below.

B) A second method for the coupling of *myo*-inositol to epoxy-activated Sepharose is the acid-catalyzed reaction in waterfree media.

2 g of lyophilyzed epoxy-activated Sepharose 6 B (Pharmacia) were treated with dry dimethylsulfoxide (*DMSO*) for 2 h. This led to normal swelling and did not destroy the bead structure of the Sepharose, visible in the microscope. The gel was filtered with suction and resuspended in *DMSO* 3 times and then sucked until any excess *DMSO* was removed. 2.75 g *myo*-inositol containing 2×10^6 dpm [U-^{14}C]*myo*-inositol (Radiochemical Centre, Amersham) were dissolved in 25 ml of *DMSO* and 1 ml of BF_3-etherate (45% solution, Merck, Darmstadt, Germany) was added with shaking. After 5 min the gel (3.4 g) was added. The mixture was shaken for 1 h at 25 °C. The gel was filtered with suction, resuspended in saturated $NaHCO_3$ and shaken at room temperature overnight. Finally, the gel was washed with 1 l of H_2O and the bound radioactivity determined by liquid scintillation counting.

Determination of the Gel Bound Radioactive Material

For this purpose the gel was freed of excess H_2O by suction. (The swelling factor thus observed was 22 ± 2.) 0.3 g of the resulting product were mixed with 0.5 ml concentrated HCl in a scintillation vial and gently warmed until a clear solution was obtained. Now 0.5 ml H_2O and 10 ml scintillation mixture were added. The

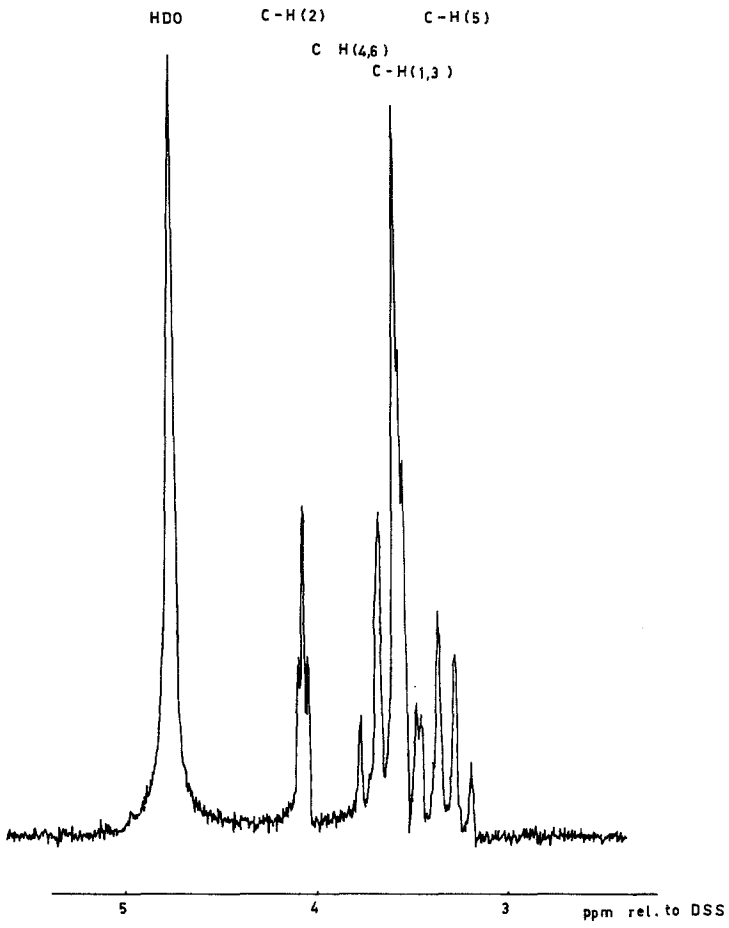


Fig. 1. Proton magnetic resonance spectrum of *myo*-inositol taken at 100 MHz. For the numbering system see formulas **1**—**3**. Only C—H protons can be seen

precipitate formed could be redissolved by shaking overnight at room temperature. The resulting clear mixture was measured in a Beckman LS-230 scintillation counter. The composition of the scintillation mixture was as follows: 667 ml Toluene (p. A. Merck); 333 ml Triton X-100 (Serva, Heidelberg); 5.5 g *PPO* (Sigma, St. Louis); 0.1 g *POPOP* (Sigma, St. Louis). To determine the quench in the liquid scintillation counting 0.3 g pure Sepharose 4 B, 0.5 ml HCl, 0.5 ml H_2O, 3×10^5 dpm of ^{14}C-ethanol (Radiochemical Centre, Amersham) and 10 ml scintillation mixture were treated and measured as above. The counting efficiency was 80%. Repeated measurements of the same sample showed that chemiluminescence did not disturb our method.

Synthesis of the Model Compound

4.2 g *myo*-inositol and an equimolar amount of propylene oxide (Merck, Darmstadt) were dissolved in 90 ml of 0.63*M*-NaOH and shaken overnight at 25 °C. The mixture was deionized with a column of Dowex 50 (H^+ form), evaporated to dryness in vacuo and extracted with hot chloroform-methanol (1:1 *v/v*). The extract was evaporated to dryness, dissolved in acetone/H_2O (85:15 *v/v*) and fractionated on a column (2 × 50 cm) of cellulose powder MN 2100 (Macherey & Nagel, Düren) in acetone/H_2O (85:15). Besides *myo*-inositol two substances, which together comprised the bulk of the inositol derivatives, could be isolated in chromatographically pure form. Substance **I**: 120 mg, $Rf = 0.1$ (ascending thin layer chromatography on cellulose plates, acetone/H_2O; 85:15). Substance **II**: 10 mg, $Rf = 0.13$. Both substances were colourless oils which did not crystalize.

Structure Determination of the Model Compounds, NMR Experiments

All NMR spectra were taken on a Varian XL 100-15 (100 MHz) NMR spectrometer by FT-mode for low amounts of substances. The samples were dissolved in D_2O and evaporated to dryness repeatedly to remove traces of H_2O and then taken up in D_2O. In the proton magnetic resonance spectrum of *myo*-inositol (Fig. 1), showing only the resonances of the C—H protons, the triplett at 4.08 ppm is conspicuous. It is shifted downfield from the bulk of the resonances pointing to an equatorial position[5] and its small coupling constant (2.5 Hz) indicates axial-equatorial coupling. These facts suggest that we are dealing with C—H (2) (using the numbering system of Formula **1**, the whole molecule existing in a chair conformation with only one axial OH-group (**1**). Definite proof for this line assignment was gained by selectively decoupling the ^{13}C-^1H spin coupling of the corresponding downfield line in the ^{13}C-spectrum of *myo*-inositol (spectrum not shown here). By similar methods the other three signals in the proton magnetic resonance spectrum of *myo*-inositol could be assigned and the influence of substituents on the oxygen atoms on the chemical shifts of the C—H-protons could be studied[6]. But these arguments are not necessary for the structure determination of substance **I**. As the molecule contains a plane of symmetry, only four resonances can be observed: C—H(2) (1 proton), C—H (1, 3) (2 protons), C—H (4, 6) (2 protons), and C—H (5) (1 proton). The assignments are indicated in Fig. 1. The proton magnetic resonance spectrum of substance **I** (Fig. 2) shows that only one of the four resonances corresponding to the resonances of *myo*-inositol is shifted significantly with respect to these, namely the triplett assigned to C—H (2). The integration of the spectrum shows that only 1 molecule of propylene oxide has reacted (the methyl resonance at 1.19 ppm corresponds to only one CH_3-group). There are two other new resonances: one corresponding to one proton, with a chemical shift of 4.03 ppm [C—H (8) in Fig. 2] and one corresponding to two protons, somewhat obscured in the region of C—H (5) [CH_2 (7) in Fig. 2]. On the basis of these assignments substance **I** most probably has the structure **2a**, **b**. The spin-spin coupling constants for C—H (5), and C—H (4, 6) are still characteristic for axial-axial coupling (about 10 Hz)[5]. This means that the substituent in the 2-position does not cause the molecule to flip to the alternative chair conformation. It is still in the same conformation as *myo*-inositol. According to the literature[7] only the isomer **2a** should occur when the coupling is performed in aqueous alcali. The spectrum (Fig. 2) also does not indicate a mixture of two isomers, the ^{13}C-resonance spectrum, however, clearly shows 12 resonances. We would expect 7 resonances

for one pure isomer and 14 for a mixture of the two, but apparently the two structural isomers **2a** and **b** which can be formed by our reaction are so similar that not even ^{13}C-resonance spectroscopy can resolve all of the resonances.

We silylated substance **I** and tried to resolve the two supposed isomers with the help of gas chromatography (for details see below). The main peak

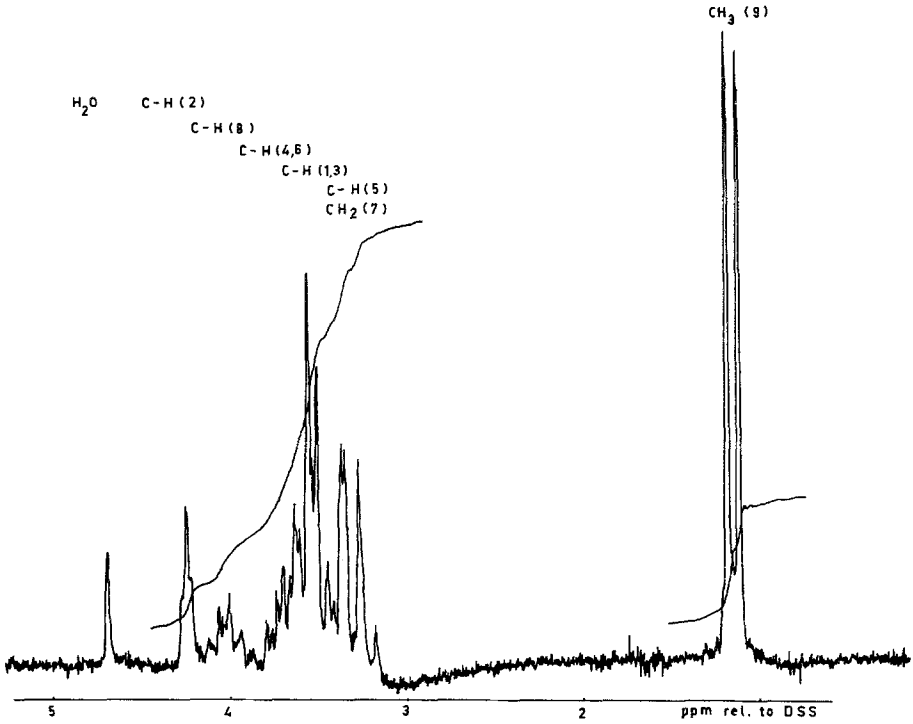


Fig. 2. Proton magnetic resonance spectrum of substance **I**

comprised 93% of the material present in **I** and was split into two parts with very similar retention times (difference = 0.3 min) and nearly equal intensity. From the proton resonance spectrum of substance **I** it can be concluded that both isomers are substituted at C-2 and the difference between them must be the point of attachment on the side chain. Therefore we propose for the two isomers the structures **2a** and **b**.

The proton magnetic resonance spectrum of substance **II** is shown in Fig. 3. The integration shows that **II**, which is minor product, is also monosubstituted. The position of the triplett ofC—H (2) (4.05 ppm), which isvery similar to C—H (2) in *myo*-inositol, indicates that substance **II** is substituted at one of the equatorial oxygens.

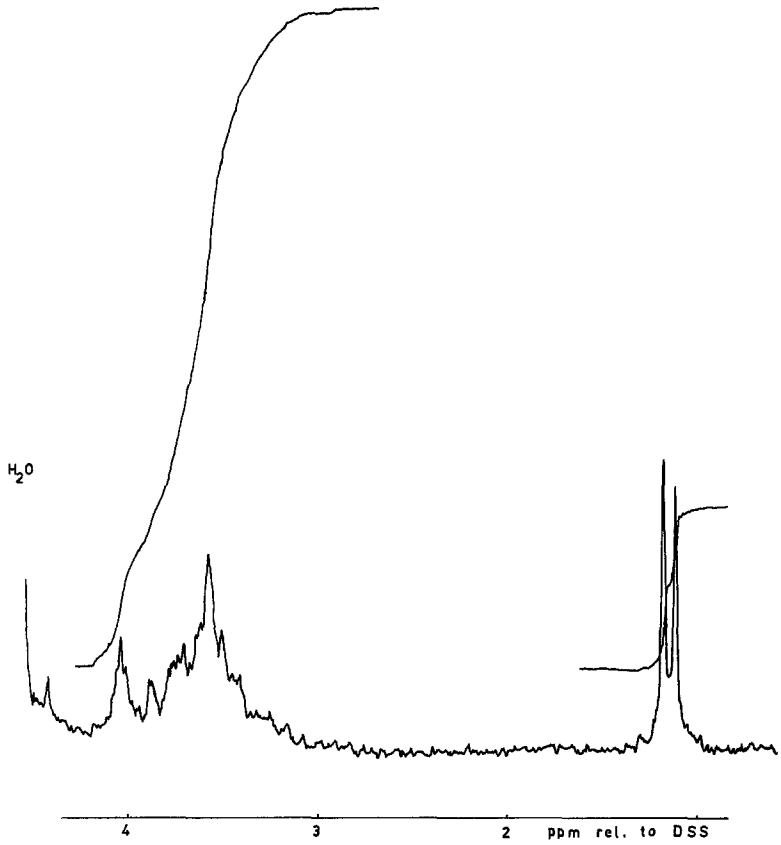


Fig. 3. Proton magnetic resonance spectrum of substance **II**

Gas Chromatography

Substance **I** was freed of traces of H_2O by evaporating with butanone-(2)/*n*-propanole (1:1 *v/v*) several times, silylated with *TSIM* for 10 min at 60 °C and injected into a Varian 2740 gas chromatograph. The column and liquid phase used were Chromosorb W-AW-DMCS (6 feet) and 3% OV-101, respectively. The temperature program was 100-240 °C. A Perkin-Elmer M 2 integrator was used. The whole system was originally developed for the separation of closely related sugars. To give an example, with this system the difference in the retention times of the two anomers of *D*-glucose is approximately 10 min, when treated in the same way as **I**.

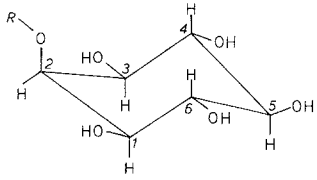
Results and Discussion

The concentration of *myo*-inositol in the Sepharose derivative synthesized in aqueous alcali was 5 mmol dm^{-3}. It was determined by measuring the incorporation of [U-^{14}C]-*myo*-inositol. The concen-

tration of *myo*-inositol in the gel synthesized in *DMSO*/BF_3 was 1.8 mmol dm^{-3}. These concentrations are well suited for enzyme purification but they are too low for the direct determination of the structure of the gels, i. e. of the point of attachment on the *myo*-inositol molecule.

For this reason we devised the model reaction described above, which applies, of course, only to the gel synthesized in aqueous alcali. There are two main differences between the gel reaction and the model reaction: 1) the concentration of NaOH is higher in the model reaction (it was impossible to obtain reasonable amounts of product in 0.01*M*-NaOH) and 2) the microenvironment in an aqueous Sepharose gel, with the epoxy component already immobilized, is different from the microenvironment of the reactants in solution. The structure of the model compound (substance **I**) should, however, follow our expectations, taking into account the preferred conformation of *myo*-inositol in aqueous solution. The attack of the epoxide occurs at the prominent single axial OH-group. This way of attack is probably not very much dependent on the exact concentration of NaOH and may not be too much influenced by the presence of the Sepharose macromolecule.

From these model experiments we conclude the structure **3** for our affinity gel. Of course we have to assume that the isomers found in the model experiments are also present. Other points of attachment may also occur (cf. substance **II**) but their amount should be less than 10% of all *myo*-inositol bound to the gel.



1 $R = -H$

2a $R = -CH_2-\overset{8}{C}HOH-\overset{9}{C}H_3$

2b $R = -\overset{8}{C}H\begin{matrix}\diagup \overset{7}{C}H_2OH \\ \diagdown \overset{9}{C}H_3\end{matrix}$

3a $R = -CH_2-CHOH-CH_2-O-(CH_2)_4-O-CH_2-CHOH-CH_2-O-\underset{|}{C}_{\text{Seph.}}-$

3b $R = -CH\begin{matrix}\diagup CH_2OH \\ \diagdown CH_2-O-(CH_2)_4-O-CH_2-CHOH-CH_2-O-\underset{|}{C}_{\text{Seph.}}-\end{matrix}$

When our gel is compared with the two inositol-containing gels previously synthesized in our laboratory, the great simplicity of the synthesis of the new gel should be stressed. Again the "solid phase modular approach", discussed by *Scheiner* and *Breitenbach*[2], is used.

To summarize the biological activity of our gel, we may say that it binds *myo*-inositol oxygenases (E.C. 1.13.99.1) from rat kidney and *Lemna gibba*. In this respect it shows the same activity as the gel described by *Koller* and *Hoffmann-Ostenhof*[1]. Recently it was possible to purify the *myo*-inositol oxygenase from rat kidney to homogeneity with the help of our gel[8]. *myo*-Inositol kinase (E.C. 2.7.1.64) from *Lemna gibba*, an enzyme which could not be previously purified in our laboratory and did not bind to the gels synthesized earlier, can be bound and purified with the help of our new affinity gel[3].

Up to now no data on the structure or biological activity of the affinity gel synthesized in *DMSO*/BF_3 are available. It will be interesting to see whether the point of attachment due to acid catalysis is different from that obtained under alcaline conditions.

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