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Useful applications of DOSY experiments for the study of mushroom polysaccharides

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Dedicated to Professor R. Suau on the occasion of his 60th birthday

Abstract—DOSY analysis has been performed on different mushroom extracts and fractions with the aim to describe a general method for the study of high molecular weight polysaccharides. These NMR experiments can be exploited to monitor the fractionation pathways performed on crude extracts in order to isolate polysaccharides. DOSY can also rapidly verify the purity of the isolated compounds, as well as evaluate their molecular size. In spite of the complexity of DOSY spectra of mixtures, this NMR technique seems to be a valid analytical tool that could be adopted as a routine method for the study of polysaccharides from different sources.

Keywords: Diffusion ordered spectroscopy (DOSY); NMR chromatogram; Natural mixtures; Mushroom polysaccharides

1. Introduction

Protocols for the structural elucidation of mushroom polysaccharides are well established.[1,2] NMR spectroscopy is nowadays a primary tool for structural analysis. Recently, several papers addressed the use of diffusion ordered spectroscopy (DOSY)[3–5] as a key NMR experiment that provides relevant information for the study of natural mixtures.[6,7] The possibility to use DOSY as 'NMR chromatography'[3,8,9] seems to be still undervalued in the field of natural products and polysaccharides structural analysis. Some authors have discussed the similarities of DOSY and size exclusion chromatography. The shape and size of a molecule are important variables for both techniques.[10] DOSY can be also safely used for molecular weight evaluation of unknown, purified constituents by preparing a calibration curve with known compounds.[11] DOSY has recently been applied to the study of complex systems, like liquid foods.[12,13] In this context, we herein present the results of the application of a DOSY-based protocol performed on different mushroom extracts, paying particular attention to their polysaccharide derivatives. It is noteworthy that diffusion-type experiments can resolve carbohydrate mixtures[14] and provide polysaccharide analyses.[15]

Mushrooms comprise a vast and yet largely untapped source of potential pharmaceutical products.[2] The medicinal use of hot water extracts from basidiomycetes is known in folk medicine, especially in East Asian countries.[16] Important pharmacological activities like anticancer and immunomodulatory are due to the high molecular weight polysaccharides from the cell walls, either free or complexed with proteins[17] that can be extracted with hot water. With the aim to develop and improve a methodology for the rapid screening of the species and further purification of the components, we have studied different crude extracts and fractions from three edible and medicinal mushroom species (*Agaricus bisporus* (J.Lge) Imbach, *Pleurotus ostreatus* (Jacq.: Fr.) Kumm, and *Leatiporus sulphureus* (Bull.: Fr.)

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Murr.) as test cases. The obtained results highlighted the efficacy of DOSY analysis, especially in monitoring the fractionation and purification pathways.

2. Results and discussion

The powdered fruiting bodies of *A. bisporus* were extracted as described by Mizuno.[1] The protocol represents a general method to increase the yield of high molecular weight polysaccharide derivatives from mushroom material, repeatedly heating in 80% ethanol, to remove small secondary metabolites, then extracting the residue with hot water (100 °C for 3 h) to obtain uncharged polysaccharides. After removal of the solvents, the obtained crude extracts were labelled ABe (ethanol) and ABw (water), respectively. The ABw fraction was further processed by ultrafiltration with a 5 kDa protein M_r cut-off membranes, thus producing a filtrate and retained material (dubbed ABw_f and ABw_r, respectively). ^{1}H NMR and 2D-DOSY analyses were performed on all these crude extracts and fractions with the aim to verify the effectiveness and drawbacks of DOSY. The ^{1}H NMR analyses (Fig. 1, left) supplied relevant data concerning the general chemical composition of the contents of the analysed samples. Further information was deduced from 2D-DOSY experiments, and in particular from the projections along the diffusion dimension (Fig. 1, right). In Figure 1, higher (less negative) values of $\log D$ correspond to smaller molecular weight compounds, with the corresponding peaks appearing to the left of the traces. The smallest detected component in each mixture is due to the residual HDO from the solvent at $\log D = -8.70$. The position of HDO is important since it reports the viscosity of the sample. High concentrations of polysaccharides are viscous and will have modify the values of $\log D$. Peaks due to the higher molecules appear to the right (smaller values of $\log D$). Thus, it is possible to directly assess the fact that the more abundant compounds in the ABe extract are small secondary metabolites detected around $\log D = -9.25$. In contrast, ABw extract is effectively enriched in polysaccharide derivatives with ^{1}H NMR signals mostly in the carbohydrate region and showing smaller values of $\log D$ (ranging from -9.20 to -10.20) for the major compounds in the mixture. Nevertheless, some traces of small molecules are still detected in this extract around $\log D = -9.20$. After the ultra-filtration step, the amount of these small molecules was strongly reduced in the ABw_r fraction. Further insights were gained with experiments on ABw extracts shown in Figure 2, where the data were acquired using different DOSY conditions. By increasing the diffusion time and reducing the gradient range by only using high values of gradient strength (see Section 3), a DOSY spectrum was obtained (Fig. 2A) in which the signals of the small molecular weight compounds were effectively filtered out, including those of the solvent (Fig. 2). In this manner, an estimate of the diffusion coefficient and size (related to the calibration curve—see Supplementary data) of the larger molecules was obtained, since overlap with the proton signals of the smaller constituents was minimised. Nevertheless, the diffusion coefficients, and therefore the corresponding

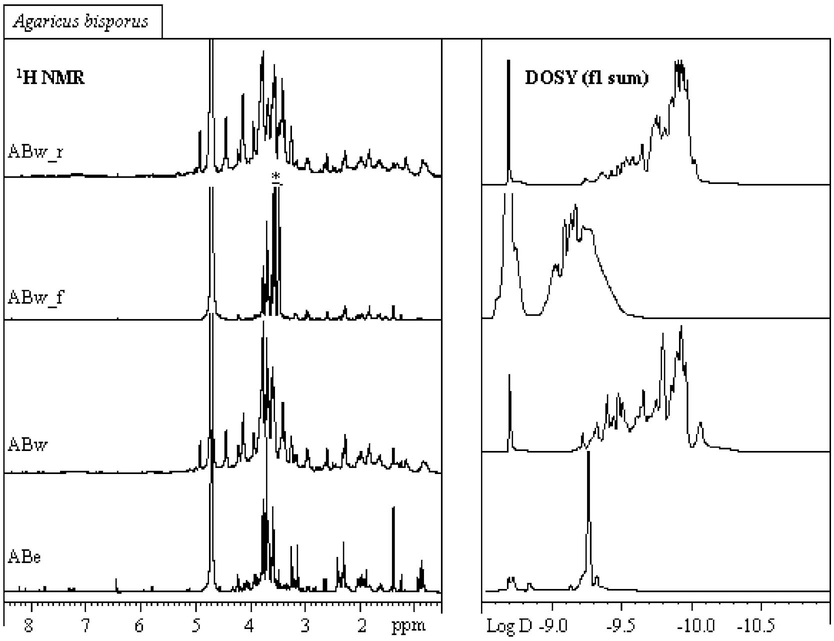

Figure 1. ^{1}H NMR spectra (left) and DOSY (right) experiments on different extracts and fractions (see Section 3) of *A. bisporus*. Although full 2D-DOSY analyses were performed, only the sum of the projections in the diffusion dimension is shown. (*) Glycerol.

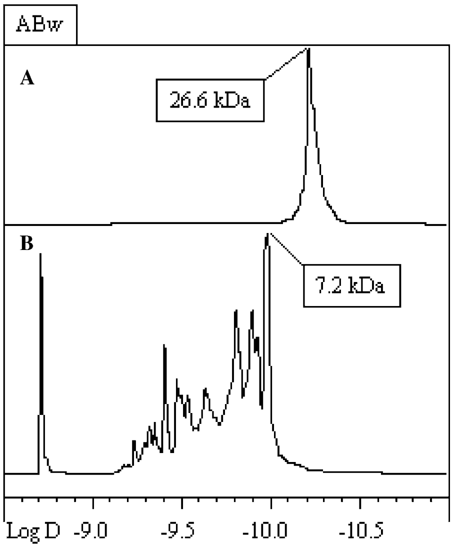

Figure 2. Projection of the diffusion dimension of the crude ABw extract performed with different DOSY parameters. (A) Higher values of diffusion time and reduced gradient range by only using high values of gradient strength (see Section 3). (B) Projection of the diffusion dimension of the same sample collected under 'standard' conditions. Molecular weight values were derived from a calibration curve prepared with different dextrans from 10 to 500 kDa (Fig. S1).

molecular weight values shown in Figure 2, are only approximate. Molecular weight values were derived from the calibration curve obtained with different dextrans (Figure S1 in Supplementary data; calibrations curves were derived using different DOSY acquisition parameters—see Section 3).

Figure 3 shows the result of applying the NMR-based analyses, as presented in Figure 1, to the crude hot water extract of *P. ostreatus* (POw) and the two major fractions obtained after ultra-filtration with a 5 kDa M_r cut-off membrane, dubbed POw_r (retained) and POw_f (filtrate), respectively. The evaluation of the three resulting projections along the diffusion dimension permits the visualisation in the changes of apparent diffusion coefficient due to overlapping of chemical shifts resonances. For the POw extract, the overlapping of the proton signals of the high and low molecular weight compounds results in a major peak at $\log D = -9.35$, corresponding to a weighted 'average peak'. Nevertheless, this fractionation step for the POw extract did not render a significant improvement compared with the published method based on the precipitation of high molecular weight constituents in methanolic solution.[18] Thus, DOSY can be rapidly exploited to evaluate the effectiveness of the different strategies in the purification of a natural mixture.

Precipitation in methanolic solution was performed to separate the high and low molecular weight compounds of the crude hot water extract of *L. sulphureus* (LSw). Two fractions, labelled LSw_s and LSw_p (soluble and precipitate, respectively), were obtained. The same NMR procedures, as applied to previous samples were performed on these mixtures (Fig. 4). A broad, averaged peak was detected for LSw crude extract. Fraction LSw_s presented a similar profile to the LSw crude extract, while LSw_p fraction (representing a 4% of the total mass of LSw extract) showed a major peak at lower $\log D$ values, corresponding to a partially purified, high molecular weight polysaccharide. DOSY analysis could thus supply certain information about the purity of the sample. The presence of an ^{1}H NMR signal resonance at δ 5.30 ppm in the 2D-DOSY spectrum (right,

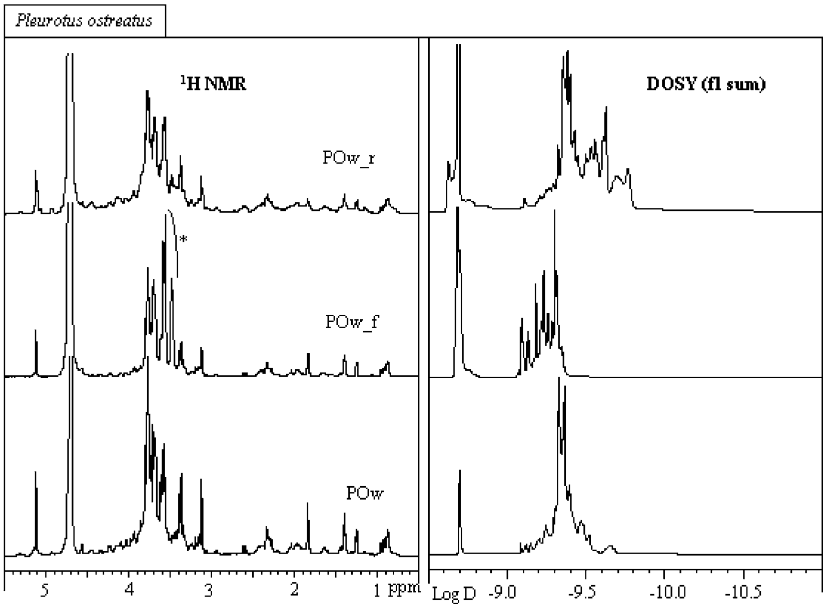

Figure 3. ^{1}H NMR spectra (left) and projection of the diffusion dimensions (right) on different extracts and fractions (see Section 3) of *P. ostreatus*. (*) Glycerol.

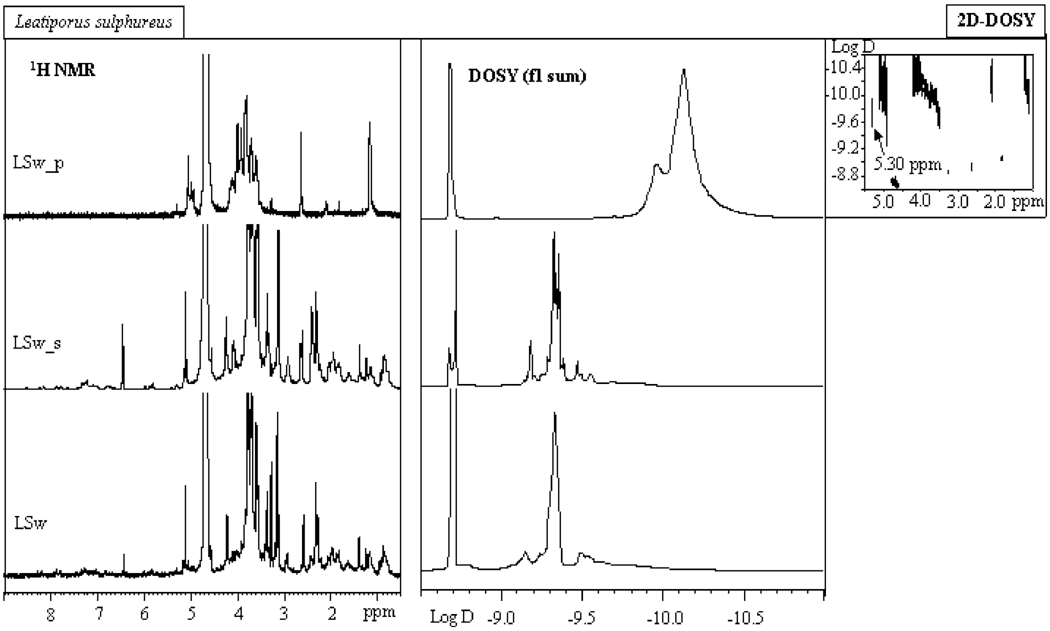

Figure 4. ^{1}H NMR spectra (left) and projection of the diffusion dimensions (right) on different extracts and fractions (see Section 3) of *L. sulphureus*. The 2D-DOSY spectrum of fraction LSw_p is also shown (right, top).

top) is due to the anomeric proton of an additional carbohydrate component, which has a higher diffusion coefficient with respect to the larger, major compound. Indeed, this hypothesis was confirmed by the 2D-DOSY experiment shown in Figure 5 (right), where increasing the diffusion time in the pulse sequence filtered out all the signals from the small molecular weight compounds. Here, the ^{1}H NMR signal resonance at δ 5.30 ppm was absent, the overlapping phenomena minimised, and a single peak at $\log D = -10.12$ was obtained (left), for which an approximate size of 15.4 kDa for this major derivative in LSw_p fraction was calculated by comparison to the molecular weight standards. Further, NMR-based analysis and comparison with the literature allowed the identification of this polysaccharide as a fucomannogalactan, previously isolated and identified by Alquini et al. from *L. sulphureus*.[19] The HSQC spectrum of fraction LSw_p is presented and described in Supplementary data (Fig. S2).

A further application of DOSY-based experiments to the study of mushroom polysaccharides is shown in Figure 6. Here, we show the comparison of the 1D-DOSY spectrum of crude LSw extract, using acquisition parameters to filter out low molecular weight components, with the regular 1D ^{1}H NMR spectra of LSw and LSw_p fractions. Due to its relative low concentration within the

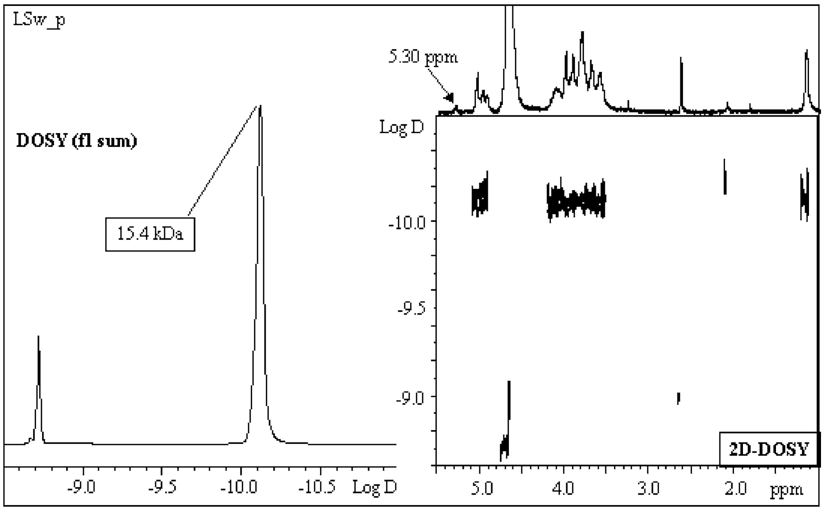

Figure 5. Projection of the diffusion dimension (left) and a 2D-DOSY experiment (right) of fraction LSw_p, performed by increasing big delta and little delta values (see Section 3). Molecular weight was derived from a calibration curve prepared with different dextrans from 10 to 500 kDa (Fig. S1).

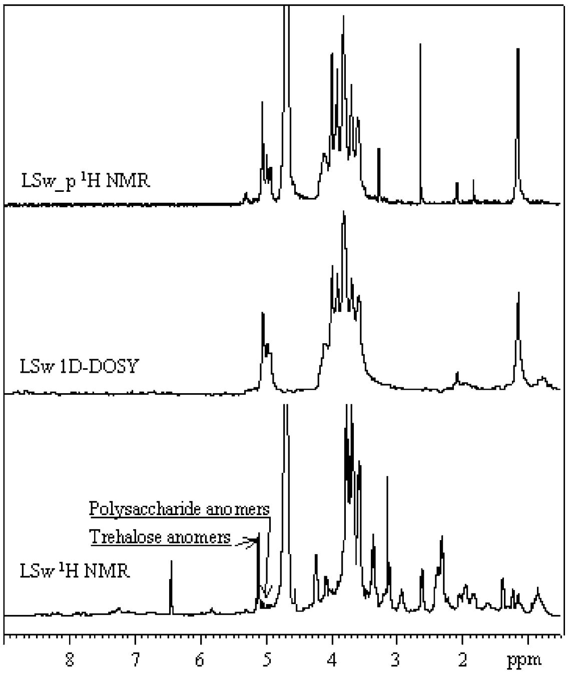

Figure 6. Comparison between the ^{1}H NMR spectra of LSw and LSw_p (bottom and top, respectively), with 1D-DOSY analysis of LSw (middle).

mixture, the proton signals of the fucomannogalactan were almost undetectable within the ^{1}H NMR of crude extract LSw. Indeed, the major anomeric signal in this spectrum is due to a trehalose derivative (identified by comparison with previous data[18]). However, the polysaccharide signals were readily identified in a diffusion edited 1D-DOSY spectrum, with the proper choice of the diffusion parameters to filter out the proton signals of the low molecular weight compounds. Comparison with the ^{1}H NMR spectrum of the LSw_p fraction confirmed the efficiency of this approach. 1D-DOSY experiments could be combined with other 1D or 2D NMR experiments[6] in order to acquire full spectral data of high molecular weight polysaccharides directly from the crude hot water extract of a mushroom species without the need of any purification step. DOSY experiments in general can supply other useful outputs for the research on natural mixtures, and further developments, probably in the form of 3D NMR experiments incorporating DOSY-filters,[20] can be optimised to produce a protocol to routinely use DOSY in natural product and polysaccharide chemistry.

3. Experimental

3.1. Mushroom material

The fruiting bodies of *A. bisporus* (J.Lge) Imbach (labelled AB) and *P. ostreatus* (Jacq.: Fr.) Kumm. (labelled PO) were bought directly from the market in Madrid, Spain, in January 2004. The fruiting bodies of *L. sulphureus* (Bull.: Fr.) Murr. (labelled LS) were collected in the Madrid area in October 2004 and identified by Dr. F. D. Calonge of the Royal Botanical Garden of the CSIC in Madrid, Spain.

3.2. Extraction

Mushroom materials were slowly dried (40 °C) and extracted as follows. The powdered fruiting bodies of AB (40 g) were repeatedly heated in 80% ethanol, obtaining an extract labelled ABe (20 g); then, the residue was extracted with hot water for 3 h to give an extract labelled ABw (3 g). The powdered fruiting bodies of PO and LS (10 g) were extracted with hot water for 3 h; the extracts were then lyophilised (yield ca 10%) and labelled POw and LSw, respectively.

3.3. Fractionation

Ultrafiltration (15,700*g*, 3 h, 25 °C) with 5 kDa M_r (protein) molecular weight cut-off membranes (Vivaspin 0.5 mL concentrator, Vivascience) was performed on 0.5 mL D_2O solutions at 5 mg/mL of ABw and POw extracts, thus obtaining the retained materials labelled ABw_r and POw_r, as well as the filtrates labelled ABw_f and POw_f, respectively. These four samples were diluted to 0.5 mL with D_2O for NMR analysis (impurities of glycerol from ultrafiltration membrane conditioners were observed in ABw_f and POw_f mixtures). LSw (50 mg) extract was dissolved in 5 mL of a methanol/water mixture (4/1), obtaining a soluble part and a precipitate that were separated by centrifugation. The precipitate was washed in 5 mL of methanol/water (4/1) and centrifuged, and this process was repeated three times to finally provide 48 mg of the soluble extract (LSw_s) and 2 mg of precipitate (LSw_p).

3.4. Preparation of the samples

Each NMR tube contained 0.5 mL of D_2O with the natural mixtures. For sample LSw_p, a concentration of 4 mg/mL was used. A saturated sample of LSw was prepared for the experiments shown in Figure 6, by dissolving 20 mg in 0.5 mL of D_2O and removing the precipitate to obtain a final concentration of ca. 30 mg/mL. The retained and filtered fractions from ABw and POw were obtained directly after ultrafiltration, as described above, without further evaluation of the relative amounts of samples. All other samples were prepared using ca. 2 mg/mL of the material.

3.5. NMR analyses

All the NMR analyses were performed on a Bruker Avance 500 MHz spectrometer, equipped with a triple resonance ^{1}H, ^{13}C, ^{15}N-probe. All experiments were performed at 298 K. Chemical shifts are given in ppm with respect to external DSS. The manufacturer's indirect referencing method was used for ^{13}C in the HSQC spectra (see Supplementary data). The 2D-DOSY experiments were usually carried out by recording 8 scans for each gradient step, with the ledbpg2s pulse sequence, a linear gradient of 32 steps between 2% and 95%, a diffusion time (big delta) of 0.1 s, and the length of the square diffusion encoding gradient pulses (little delta) of 1 ms, unless otherwise stated. A total acquisition time of ca. 8 min was thus employed. The standard Bruker protocol was used for processing in XWINNMR 3.5 or Topspin software. For the analysis of ABw sample, as shown in the top part of Figure 2, 16 scans, a linear gradient strength between 45% and 95%, big delta of 0.4 s, and little delta of 2 ms were used with an acquisition time of ca. 30 min. In contrast, for the analysis of sample LSw_p (Fig. 5), 8 scans, a linear gradient strength between 2% and 95%, big delta of 0.4 s and little delta of 2 ms were employed. The fitting of the diffusion dimension in the 2D-DOSY spectra was achieved using a two-parameter monoexponential fit (Bruker's DOSY 2D macro in XWINNMR 3.5). The sum of the projections in the diffusion dimension was displayed by using the standard f1sum command over the whole 2D-DOSY spectra. The analysis of these representations provides gross information on the molecular size of the constituents of the mixture. In these projections, the x-axis shows the values of $\log D$ ($D =$ diffusion coefficient) instead of retention time values, typical of classical chromatography. Diffusion edited ^{1}H NMR spectra (1D-DOSY) were acquired with the ledbpg2s1d pulse sequence, 256 scans, 80% gradient strength, big delta 0.4 s, and little delta 2 ms to give an acquisition time of ca. 13 min. HSQC (16 scans) was also performed using the standard gradient-enhanced sequence. A relaxation delay of 1.5 s was used, with 256 increments, and spectral widths of 12 ppm in f2 and 120 ppm (HSQC). 2K real data points in f2 were acquired. Squared cosine bells were used for processing.

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Supplementary data

The calibration curve obtained by DOSY analyses on different dextrans is presented in Figure S1. The HSQC spectra of fraction LSw_p is presented in Figure S2. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2005.11.008.

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