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Mild acid hydrolysis of sulfated fucans: a selective 2-desulfation reaction and an alternative approach for preparing tailored sulfated oligosaccharides

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Sulfated fucans from marine invertebrates have simple, linear structures, composed of repeating units of oligosaccharides. Most of these polysaccharides contain 3-linked fucosyl units, but each species of invertebrate has a specific pattern of sulfation. No specific enzyme able to cleave or to desulfate these polysaccharides has been described yet. Therefore, we employed an alternative approach, based on mild acid hydrolysis, in an attempt to obtain low molecular-weight derivatives from sulfated fucans. Surprisingly, we observed that sulfated fucans from *Lytechinus variegatus* and *Strongylocentrotus pallidus* (but not the sulfated fucans from other species) yield by mild acid hydrolysis oligosaccharides with well-defined molecular size as shown by narrow bands in polyacrylamide gel electrophoresis (PAGE). The sulfated oligosaccharides obtained by mild acid hydrolysis were purified by gel-filtration chromatography, and their structures were identified by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy, revealing an identical chemical composition for all oligosaccharides. When we followed the acid hydrolysis by ^{1}H-NMR spectroscopy, we found that a selective 2-desulfation occurs in the fucans from *S. pallidus* and from *L. variegatus*. The reaction has two stages. Initially, 2-sulfate esters at specific sites are removed. Then the desulfated units are cleaved, yielding oligosaccharides with well-defined molecular size. The apparent requirement for the selective 2-desulfation is the occurrence of an exclusively 2-sulfated fucosyl unit linked to or preceded by a 4-sulfated residue. Thus, a homofucan from *Strongylocentrotus franciscanus* resists desulfation by mild acid hydrolysis, because it lacks the neighboring 4-sulfated unit. Overall, our results show a new approach for desulfating sulfated fucans at specific sites and obtaining tailored sulfated oligosaccharides.

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Introduction

Sulfated fucans are among the most widespread nonmammalian-sulfated polysaccharides distributed in nature. They occur in algae (Percival and McDowell, 1967; Nishino *et al*., 1991) and marine invertebrates, particularly in echinoderms, such as sea cucumbers (Mourão and Bastos, 1987; Vieira and Mourão, 1988) and sea urchins (SeGall and Lennarz, 1979, 1981; Glabe *et al*., 1982). In the case of the sea urchins, such compounds are extracted from the egg jelly layer of female gametes. These sulfated polysaccharides are composed of simple, linear structures, and repeated units that differ principally in the pattern of sulfation. Several of these sulfated fucans have the α(1→3)-glycosidic linkage between 2-, 4-, and 2,4-sulfated fucopyranose residues (Mulloy *et al*., 1994; Alves *et al*., 1998; Pereira *et al*., 1999; Vilela-Silva *et al*., 1999, 2002). These singular structural characteristics confer an opportunity on the sulfated α-L-fucans of marine invertebrates to reveal the mechanism of their biological actions (Mourão and Pereira, 1999; Pereira *et al*., 1999; Hirohashi *et al*., 2002). On the other hand, algal-sulfated fucans are difficult tools for studying the biological activities because of their complex and heterogeneous structures (Pereira *et al*., 1999; Berteau and Mulloy, 2003; Mourão, 2004).

In addition to the simplicity of structures, the possibility of preparing low molecular-weight derivatives from the sulfated fucans of echinoderms makes it simple to study the relation between molecular size and biological activity (Pomin *et al*., 2005; Matsubara *et al*., 2005). However, a limited number of techniques for molecular reduction have been described. The enzymes that cleave the sulfated polysaccharide core lead to a rapid decrease in the molecular masses of these polymers (Kitamura *et al*., 1992), and the enzymatic kinetics or the biochemical properties of these fucanases are still unclear. Besides the influence of the molecular size, another important study of sulfated fucans is to reveal the significant role of the sulfated positions in the biological actions in mammalian systems (Pereira *et al*., 2002). One way to analyze the influence of these sulfated groups is to remove them with specific sulfatases. However, only one report has described a sulfatase able to cleave specifically certain sulfate groups of marine-sulfated fucans, and this enzyme acts only on disaccharide units (Daniel *et al*., 2001).

To overcome these limitations, in a previous work, we employed an alternative method based on mild acid hydrolysis

to specifically desulfate and depolymerize the sulfated fucans at determinated sites. We observed that 0.01 M HCl is able to produce specific-sulfated oligosaccharides from the sulfated fucan of *Lytechinus variegates* after a selective 2-sulfate removal. Using the oligosaccharides of well-defined molecular size obtained by this procedure, we were able to identify the minimum size that allowed this sulfated fucan to interact with heparin cofactor II, a plasmatic inhibitor of thrombin, the most important protease of hemostasis (Pomin *et al.*, 2005).

However, in the above study, we were unable to clarify the structural requirements for the selective desulfation and the specific cleavage. Some questions still remain as What specific structural characteristics are responsible for the 2-desulfation? Which glycosidic linkage is preferentially cleaved by mild acid hydrolysis to prepare oligosaccharides of well-defined molecular size? Here, we extend our studies of hydrolysis with acid to sulfated fucans from other species with regular and repetitive structures, differing only in the 2- and 4-sulfation pattern (Figure 1). Surprisingly, the sulfated fucan from the *Strongylocentrotus pallidus* was able to produce tailored oligosaccharides, in the same way described for *Lytechinus variegatus*. Two other species, *Strongylocentrotus purpuratus* and *Strongylocentrotus franciscanus*, were cleaved unspecifically, yielding heterogeneous-sulfated oligosaccharides. Correlating the chemical structures of these sulfated fucans with their susceptibilities to desulfation and the capacity for producing well-defined oligosaccharides, we were able to elucidate better the reaction mechanism of the mild acid hydrolysis of the sulfated fucans.

Results and discussion

Production of well-defined oligosaccharides from sulfated fucans by mild acid hydrolysis is influenced by the particular structure of the polysaccharides

Mild acid hydrolysis is an apparently nonspecific method for depolymerizing the sulfated polysaccharides. Even so, all of the sulfated fucans (Figure 1) maintained with 0.01 M HCl at 60°C for 1 h showed a reduction in molecular size (Figure 2), as previously reported for another sulfated fucan (Pomin *et al.*, 2005). Only the fucans of the species *S. pallidus* and *L. variegatus* produced specifically tailored oligosaccharides, as shown by their multiple metachromatic bands in polyacrylamide gel electrophoresis (PAGE). For the other species, although the molecular masses of the polysaccharides were reduced, no narrow bands appeared in the PAGE, indicating a heterogeneous mixture of oligosaccharides. Thus, the formation of well-defined molecular size oligosaccharides is notably dependent on the particular structure of the sulfated polysaccharides. As shown in Figure 1, all of the sulfated α-L-fucans used have the α(1→3)-glycosidic linkage, differing only in the pattern of sulfation (2-sulfate, 4-sulfate/substitutions). Thus, the specific cleavage of the polysaccharides by mild acid hydrolysis was influenced specifically by their pattern of sulfation.

Yielding of specific-sulfated oligosaccharides by the mild acid hydrolysis of the sulfated fucans, and increase of their proportion with the course of time

The PAGE shown in Figure 3A and B reveals the same higher electrophoretic mobility for the narrow bands after

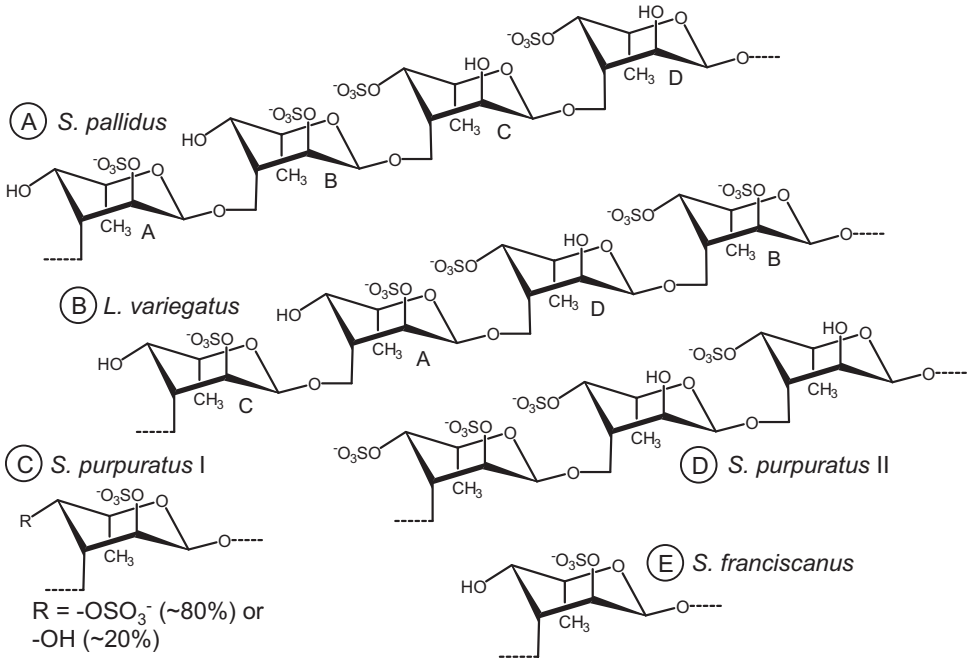

Fig. 1. Structures of sulfated α-L-fucans from sea urchin egg jelly coat. These polysaccharides consist of 3-linked, α-L-fucose units with 2-sulfate/4-sulfate substitutions. All these fucans are composed of repetitive oligosaccharides. Four types of fucose units are designated by letters A–D in the fucans from *Strongylocentrotus pallidus* and from *Lytechinus variegatus*. The species *Strongylocentrotus purpuratus* has two types of repetitive sulfated fucan (indicated as I and II).

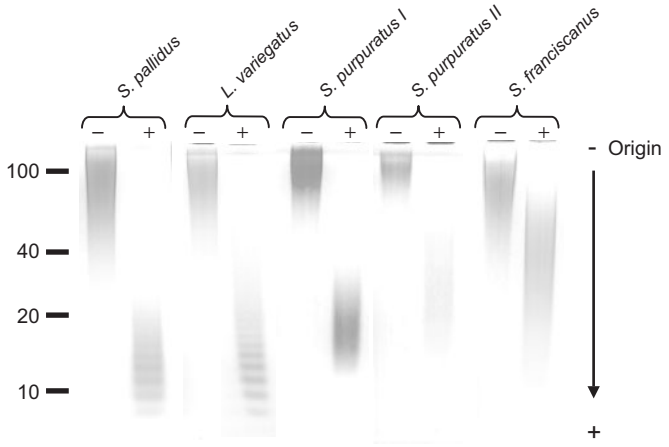

Fig. 2. Mild acid hydrolysis of different sulfated fucans. The sulfated fucans (100 μg of each) were dissolved in 100 μL of distilled water (–) or aqueous 0.01 M HCl solution (+) and heated at 60°C for 1 h. The pH of the solution was then adjusted to 7.0 with the addition of 100 μL of ice-cold 0.01 M NaOH. The samples (10 μg of each) were analyzed by PAGE, as described under *Materials and methods*. The molecular weights (kDa) of standard compounds are indicated at the left. These standards are high molecular-weight dextran sulfate (≥100 kDa), chondroitin 4-sulfate from bovine trachea (~40 kDa), dermatan sulfate from pig skin (~20 kDa), and low molecular-weight dextran sulfate (~10 kDa).

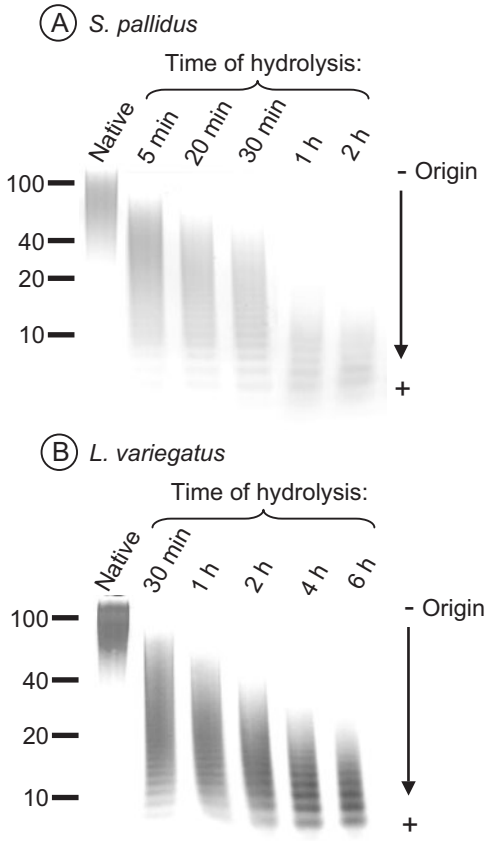

Fig. 3. Mild acid hydrolysis of sulfated fucans from *Strongylocentrotus pallidus* (**A**) and from *Lytechinus variegatus* (**B**) after different periods of time. Each sample (500 μg dry weight) was dissolved in 500 μL of aqueous 0.01 M HCl and heated at 60°C. After different periods of time (5 min, 20 min, 30 min, 1 h, and 2 h for the fucan from *S. pallidus* [**A**] and 30 min, 1 h, 2 h, 4 h, and 6 h for the fucan from *L. variegatus* [**B**]), aliquots of 100 μL of each sample were removed, and the pH was adjusted to 7.0 with the addition of 100 μL of ice-cold 0.01 M NaOH. The samples (10 μg of each) were analyzed by PAGE, as described under *Materials and methods*. The molecular weights (kDa) of standard compounds are indicated at the left, as described in legend of Figure 2.

different periods of time of hydrolysis. In fact, these defined metachromatic bands indicate that, as the time of hydrolysis proceeds, the same sulfated oligosaccharides are produced for each species. Although the cleavage of sulfated fucan from *S. pallidus* was faster than that from *L. variegates* (Figure 3A and B), both species showed the capacity to increase the proportion of low molecular-weight oligosaccharides in longer periods of hydrolysis, in detriment of the proportion of the high molecular-weight polysaccharides. The critical time to yield oligosaccharides from *S. pallidus* was after 30 min of hydrolysis (Figure 3A), while for *L. variegatus* it was 4 h (Figure 3B). For both species, these sulfated oligosaccharides ranged from <10 kDa to ~20 kDa.

The well-defined molecular size oligosaccharides obtained by mild acid hydrolysis have different molecular weights but the same chemical structure

The first step to characterize the structures of the sulfated oligosaccharides from the fucan of *S. pallidus* was to purify them. Thus, the oligosaccharides produced after 1 h of mild acid hydrolysis were fractionated by gel-filtration chromatography on Bio-Gel P10 (Bio-Rad Laboratories, Hercules, CA) (closed circles in Figure 4A). The oligosaccharides were separated, identified by their metachromatic property, and pooled as I→V. As we increased the time course of hydrolysis to 2 h (open circles in Figure 4A), the same elution profile was noticed, and the proportion of low molecular-weight masses rose as the high molecular-weight masses decreased. No oligosaccharides were identified by the phenol–sulfuric acid reaction after the elution of the smaller oligosaccharide (designated 'I' in Figure 4A), excluding the existence of possible desulfated oligosaccharides not assayed because of the absence of metachromatic property. PAGE of the purified fractions from the gel-filtration chromatography (Figure 4B) showed bands of a well-defined size, as already noted in Figure 3A. The absence of appropriate standards to compare with the electrophoretic mobility of the oligosaccharides I→V did not allow us to estimate precisely their respective molecular masses. However, the ^{1}H-nuclear magnetic resonance (NMR) spectrum of the sulfated oligosaccharides I→V (data not shown) revealed the same chemical structure for all of them, as exemplified for oligosaccharide IV (Figure 4C). Their structure will be described in full detail next.

Promotion of an exclusive desulfation of the 2-sulfated fucosyl unit necessarily linked to or preceded by a 4-sulfated residue in the sulfated fucans by the mild acid hydrolysis

In an attempt to identify the chemical modifications that occur in the polysaccharides during the mild acid hydrolysis, we followed the reaction by one dimensional ^{1}H-NMR

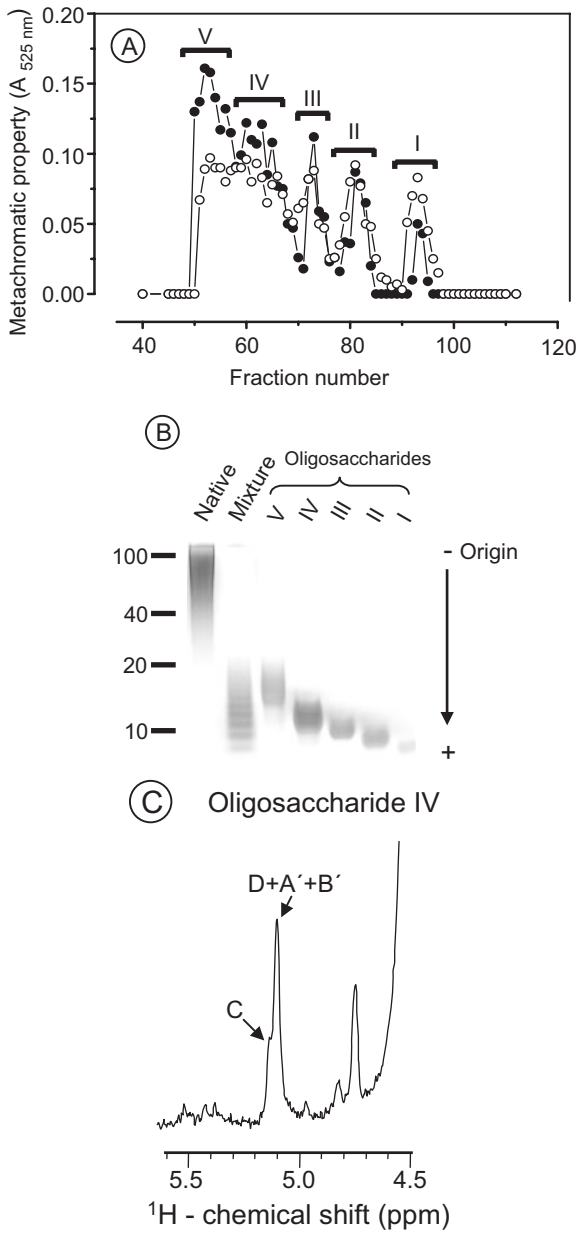

Fig. 4. Size fractionation and ^{1}H-NMR spectroscopy of the sulfated oligosaccharides from *Strongylocentrotus pallidus*. (**A**) Sulfated fucan (5 mg) partially hydrolyzed with 1 mL of 0.01 M HCl at 60°C during 1 h (closed circles) or 2 h (open circles) was applied to a Bio-Gel P10 column (200 × 0.9 cm), equilibrated with aqueous 10% ethanol, containing 1.0 M NaCl. The fractions (1 mL) were eluted as described under *Materials and methods*, and their metachromatic properties were assayed. The fractions containing the oligosaccharides (as indicated by the horizontal bars) were pooled, freeze-dried, desalted, and dissolved in distilled water. (**B**) Intact sulfated fucan, a mixture of unfractionated oligosaccharides, and five major fractions obtained by Bio-Gel P10 (10 µg of each) were applied to a 12% polyacrylamide gel and analyzed as described in the legend of Figure 2. The molecular weights (kDa) of standard compounds are indicated at the left. (**C**) Expansions of the 5.6–4.5 ppm regions of the ^{1}H-NMR spectrum at 600 MHz of the purified oligosaccharide IV obtained by Bio-Gel P10 column. The spectrum was recorded at 60°C for samples in 99.9% D_2O. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm. The residual water signal has been suppressed by presaturation. The respective anomeric signals are indicated by arrows (Figure 1). All the oligosaccharides (I→V) showed the same ^{1}H-NMR spectra (data not shown) as exemplified for oligosaccharide IV.

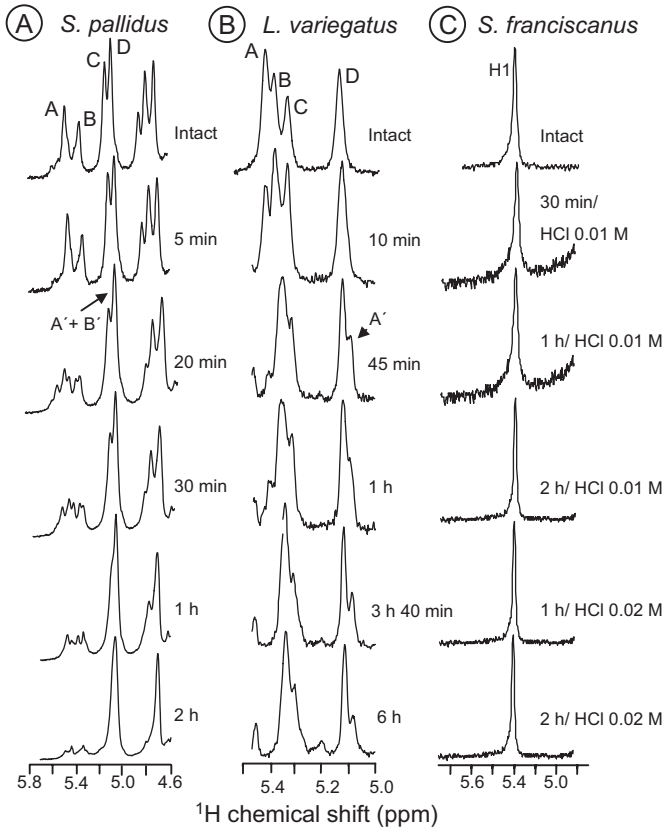

Fig. 5. Expansions of the anomeric regions of the ^{1}H-NMR spectra at 600 MHz of the sulfated fucans from *Strongylocentrotus pallidus* (**A**), *Lytechinus variegatus* (**B**), and *Strongylocentrotus franciscanus* (**C**) at different periods of mild acid hydrolysis. The sulfated fucans (3 mg of each) were dissolved in 1.0 mL of 99.9% D_2O containing 0.01 M HCl (for all sulfated fucans) or 0.02 M HCl (exclusively for *S. franciscanus*) in a 5-mm inner diameter NMR tube. These solutions were heated at 60°C, and ^{1}H-NMR spectra were recorded at different periods of time, as indicated. The A′ and B′ indicate desulfated residues.

spectroscopy (Figure 5A–C). The fucans from *S. pallidus*, *L. variegatus*, and *S. franciscanus* were incubated with 0.01 M HCl inside the NMR tube, and several ^{1}H spectra were recorded at different periods of time.

In the case of the sulfated fucan from *S. pallidus* (Figure 5A), the first 20 min of hydrolysis showed a regression of the ^{1}H-anomeric signals of the residues A and B (Figure 1A) with a parallel increase in the signal A′ + B′ + C + D, as indicated by their respective integrals (Figure 6A). The mild acid hydrolysis tends to form only one peak resonating at ~5.18 ppm, as observed in the ^{1}H-NMR spectrum of 2 h of hydrolysis. This wide peak represents all the ^{1}H-anomeric signals of the molecule (A′ + B′ + C + D), identified in total correlation spectroscopy (TOCSY) spectrum (Figure 7B) and in ^{1}H/^{13}C heteronuclear multiple quantum coherence (HMQC) spectrum (Figure 7D) of the unfractionated oligosaccharides obtained by 1 h of hydrolysis. The ^{1}H-anomeric signals of A′ and B′ both indicate desulfated residues, confirmed by the displacement of their H1 signals to high field shifts (~0.34 ppm to A′ and ~0.25 ppm to B′) (Figure 7A versus B and C versus D), and the disappearance of their

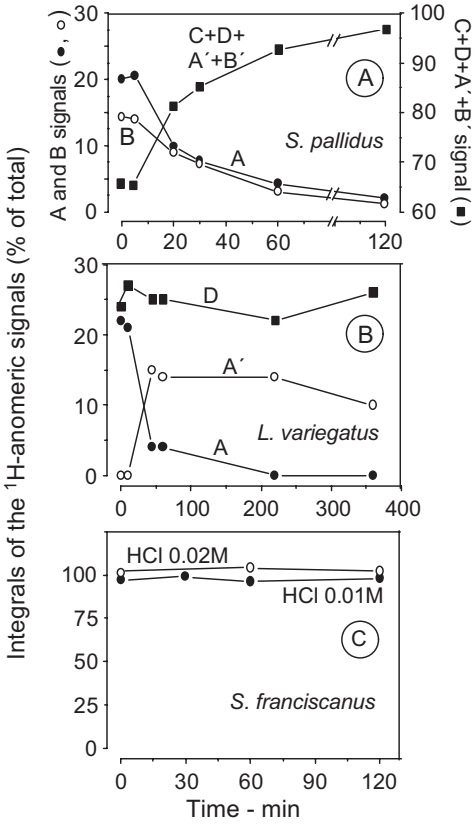

Fig. 6. ^{1}H-anomeric signals modifications in sulfated fucans during different periods of the mild acid hydrolysis determined by their respective integrals (% of total area of the anomeric peaks). The anomeric protons of residues (A, B, and C + D + A′ + B′) of the fucan from *Strongylocentrotus pallidus* (**A**) and of the residues (A, A′, and D) of the fucan from *Lytechinus variegatus* (**B**), indicated in Figure 5, were quantified by their integrals when incubated with 0.01 M HCl at 60°C. The A′ and B′ correspond to desulfated residues. The only ^{1}H-anomeric signal of the fucan from *Strongylocentrotus franciscanus* (**C**) was also integrated in incubations with 0.01 M and 0.02 M HCl at 60°C.

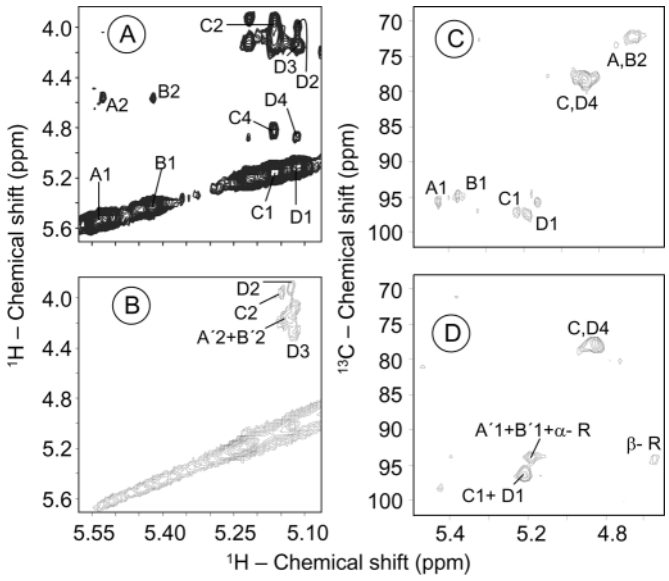

Fig. 7. Part of the TOCSY spectra (**A** and **B**) and the ^{1}H/^{13}C HMQC spectra (**C** and **D**) of native sulfated fucan from *Strongylocentrotus pallidus* (**A** and **C**) and of the mixture of oligosaccharides obtained by mild acid hydrolysis during 1 h (**B** and **D**). The reducing α- and β-anomeric signals of the nonsulfated and sulfated fucose units (Table II) range from 5.50 to 5.20 and from 4.73 to 4.55 ppm, respectively. α-R and β-R are anomeric signals of the reducing ends from nonsulfated fucose units. Oligosaccharide IV shows the same spectra as in **B** and **D**.

H2 signals from the ^{1}H/^{13}C HMQC spectra of the native fucan (Figure 7C versus D). These data agree with those previously reported for a desulfated fucan: high field shifts of ~0.3 and ~0.2 ppm to H1 of desulfated A and B residues, respectively (Table I). In contrast, no alterations were observed in HMQC spectrum of the anomeric signals of C and D residues (Figure 7D versus C; Table I). Both these signals represent 4-sulfated residues (Figure 1A), indicating clearly that 4-O-sulfated fucose units resist desulfation when incubated with acid.

In conclusion, the mild acid hydrolysis promotes in the sulfated fucan from *S. pallidus* a selective 2-desulfation. We can deduce that the predominant-repeating structure found in the products after 20 min of acidification is composed of two consecutive 2-desulfated fucosyl units linked to two following 4-sulfated fucose residues. Thus, the spectra obtained from the oligosaccharides purified by Bio-Gel P10 column (Figure 4C) are similar to those observed in the unfractionated sample after 1 h of hydrolysis (Figure 5A). These data indicate that all oligosaccharides produced by mild acid hydrolysis have the same structure.

Oligosaccharide IV showed the same HMQC spectrum as the mixture of oligosaccharides (not shown but similar to Figure 7D). In this case, the signal of β-anomeric proton at 4.55 ppm is ~3% of the total anomeric protons. Assuming an equilibrium of 4:6 between the α- and β-forms of reducing fucoses, we can estimate that signals of non-reducing α-protons that resonate between 5.18 and 5.22 ppm are ~97% of the total anomeric protons. This indicates that oligosaccharide IV contains ~20 fucose units per reducing terminal, that is, approximately five repeating tetrasaccharide units. Of course this is a rough calculation, but it emphasizes that the oligosaccharides obtained by mild acid hydrolysis are still high molecular-size compounds. These values are not too far from the molecular sizes estimated by PAGE (Figures 3A and 4B).

The same procedure was used to analyze the hydrolysis of the sulfated fucan from *L. variegatus* (Figure 5B). The ^{1}H-anomeric signal of the first 2-sulfated residue (designated as A in Figure 1B) decreases after 45 min with a concomitant appearance of the new anomeric signal of a desulfated residue (indicated A′). The integrals of anomeric signals of A and A′ residues interchange proportionally (Figure 6B). These data indicate not only the 2-desulfation, but, in this case, that a specific 2-sulfate ester is removed. The second residue, indicated as C signal (Figure 5B), resisted hydrolysis, probably because of the influence of its adjacent 2,4-disulfated fucose residue (Figure 1B).

To study the susceptibility of the 2-sulfate esters to desulfation caused by the mild acid hydrolysis, we followed possible structure modifications by several ^{1}H-NMR spectra in a homofucan constituted of 2-sulfated fucose units (Figure 5C). The only anomeric signal stayed unchanged, even at higher concentration of acid (0.02 M HCl). These results are supported by the unaffected integrals of the signals of the only constituent unit (Figure 6C). Thus, the sulfated fucan

Table I. Proton chemical shifts (ppm) for residues of α-fucose in native, chemically desulfated fucan, and mixture of oligosaccharides obtained by 1 h of mild acid hydrolysis of the fucan from *Strongylocentrotus pallidus*

	Native sulfated fucan[a]					Mixture of oligosaccharides obtained by 1 h of mild acid hydrolysis			
Próton	A	B	C	D	Desulfated[a]	A′	B′	C	D
H-1	5.52	5.43	5.20	5.12	5.23	5.18	5.18	5.22	5.14
H-2	**4.59**	**4.60**	4.01	4.06	4.09	4.10	4.10	4.06	4.06
H-3	4.18	3.18	4.19	4.19	4.09	4.09	3.15	4.11	4.11
H-4	4.12	4.12	**4.85**	**4.89**	4.13	4.13	4.13	**4.87**	**4.90**
H-5	4.69–4.56	4.47–4.56	4.46–4.56	4.46–4.56	4.43	4.40	4.41	ND	ND
H-6	1.32–1.43	1.32–1.43	1.32–1.43	1.32–1.43	1.38	1.30–1.40	1.30–1.40	1.30–1.40	1.3–1.4

ND, not determined.
The spectra were recorded at 600 MHz in 99.9% D_2O. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm for ^1H. Values in boldface type indicate positions bearing a sulfate ester. Similar chemical shifts were observed for the spectra of the purified oligosaccharide IV.
[a]Data from Vilela-Silva *et al*. (2002).

from *S. franciscanus* was observed totally resistant to desulfation, probably because of the absence of neighboring 4-sulfated fucose residue. It was not possible to follow chemical modifications by ^1H-NMR spectra of the fucans from *S. purpuratus* I and *S. purpuratus* II because of the limited amount of material.

Comparing the kinetics of acid hydrolysis of the sulfated fucans from *S. pallidus*, *L. variegatus*, and *S. franciscanus* (Figure 5A–C), we can conclude that the desulfation occurs exclusively on the 2-sulfated fucosyl unit obligatorily linked to or preceded by a 4-sulfated fucose unit. If the neighboring residue contains sulfation in the 2-position or in both 2- and 4-positions, as for *S. franciscanus* (only 2-sulfated neighboring units) and for *L. variegatus* (2,4-disulfated neighboring unit), respectively, the desulfation stops.

The glycosidic linkage of the desulfated fucose residue is more susceptible to acid cleavage than the sulfated units

It was already concluded that the oligosaccharides from *S. pallidus* produced in the course of the mild acid hydrolysis have a well-defined molecular size due to the narrow bands indicated by PAGE (Figures 2 and 3) and the elution profile in Bio-Gel P10 column (Figure 4A). Their preponderant chemical structure also remains unchanged as seen by ^1H-NMR spectra in Figure 4C. These observations suggest that this α-L-fucan is preferentially cleaved by HCl in a specific site. In an attempt to determine which glycosidic linkage is more susceptible to cleavage, we analyzed the unfractionated products of 1 h of mild acid hydrolysis (because all oligosaccharides have the same structure) using $^1H/^{13}C$ HMQC spectrum (Figure 7D). Therefore, the $^1H/^{13}C$ HMQC spectrum of the hydrolyzed products showed small, but detectable anomeric signals of reducing fucose units. These signals represent the equilibrium in aqueous solution between the α- and β-forms of the reducing ends (indicated as α-R and β-R in Figure 7D). Comparison between the ^1H and ^{13}C chemical shifts of the α- and β-reducing ends anomeric signals with standard compounds (Table II) clearly indicates that the glycosidic linkage was cleaved in a nonsulfated fucose unit. All these data are

Table II. Chemical shifts (ppm) for anomeric ^1H protons and ^{13}C carbons from reducing terminals of fucosyl units of the mixture of oligosaccharides obtained by 1 h of mild acid hydrolysis of the sulfated fucan from *Strongylocentrotus pallidus* and of standard compounds

	^1H chemical shifts[a]		^{13}C chemical shifts[a]	
	α-anomer	β-anomer	α-anomer	β-anomer
Mixture of oligosaccharides from 1 h of acid hydrolysis	**5.22**	**4.55**	**93.1**	**96.1**
Unsulfated fucose[b]	**5.22**	**4.55**		
Unsulfated fucose[c]	**5.20**	**4.55**	**93.1**	**97.1**
Fucose 2-sulfate[c]	5.49	4.69	93.2	97.6
Fucose 4-sulfate[b]	5.22	4.60		
Fucose 4-sulfate[c]	5.22	4.59	95.1	99.1
Fucose 2,4-sulfate[b]	5.49	4.73		

[a]The 600 MHz $^1H/^{13}C$ HMQC spectrum was recorded at 60°C. Chemical shifts are referenced to internal trimethylsilylpropionic acid at 0 ppm for ^1H and to methanol for ^{13}C. Values in boldface type indicate chemical shifts of nonsulfated fucosyl terminal units. Similar chemical shifts were observed for the spectrum of the purified oligosaccharide IV.
[b]Data from Mourão *et al*. (1996).
[c]Data from Daniel *et al*. (2001).

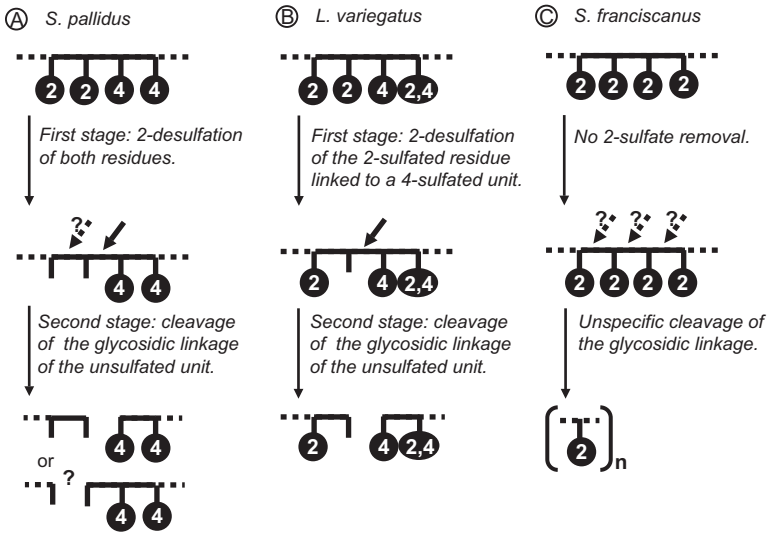

Fig. 8. Scheme summarizing the stages of mild acid hydrolysis of the sulfated fucans from *Strongylocentrotus pallidus* (**A**), *Lytechinus variegatus* (**B**), and *Strongylocentrotus franciscanus* (**C**). The numbers correspond to the sulfate positions in the fucose chain. Stippled arrows indicate the possible cleavage sites, and solid arrow indicates certainty cleavage site. In analogy with the ordered degradation already described for *L. variegatus*, the sulfated fucan from *S. pallidus* is probably cleaved after the second desulfated residue, yielding well-defined oligosaccharides. The oligosaccharides produced from *S. franciscanus* are totally homogeneous, constituted of only 2-sulfated fucose units and different exclusively by their molecular sizes.

coincident to the ordered degradation already described for the sulfated fucan of *L. variegatus* (Pomin *et al.*, 2005) (Figure 8B). In the case of the acid hydrolysis of *S. pallidus*, we were not able to determine which desulfated unit was cleaved (residues designated A′ or B′). But, certainly the cleavage did not occur after the 4-sulfated units (Table II).

Considering the particular characteristics of the acid hydrolysis method, like the selective 2-desulfation of a unit obligatorily linked to or preceded by a 4-sulfated fucose residue and the specific cleavage of the glycosidic linkage of the desulfated residue, we can trace a hypothetical analogy between the ordered degradation of the fucan from *S. pallidus* with that of the fucan from *L. variegatus*. Respecting these similarities, we deduce that probably the acid cleaves the glycosidic linkage of the second desulfated residue of the sulfated fucan from *S. pallidus* (Figure 8A and B). But, it was not possible to accomplish an appropriate experiment to ascertain which 2-sulfated residue has its glycosidic linkage cleaved, because of the absence of a sulfated fucan composed of a non-sulfated fucopyranose residue linked to a 4-sulfated fucose unit.

In the case of the fucan from *S. franciscanus*, we already observed an unspecific cleavage of the glycosidic linkage by the mild acid hydrolysis because of the widely dispersed electrophoretic bands (Figure 2). No 2-desulfation occurred in this fucan either (Figure 5C). These data suggest that probably the resistance to desulfation confers on this fucan a random cleavage of the linkage between the 2-sulfated fucose residues (Figure 8C). Overall, we can deduce that the glycosidic linkage of a desulfated unit is more labile to acid cleavage than one resistant to desulfation.

Conclusion

In synthesis, we presented evidence that mild acid hydrolysis, an apparently nonspecific approach, could be used as an alternative methodology to specifically desulfate and to produce sulfated oligosaccharides of a well-defined molecular size. In some specific cases, this approach showed the capacity to remove only the 2-sulfate esters, in an interesting way not described previously. After this first stage of the hydrolysis, a glycosidic linkage is preferentially cleaved (Figures 8A and B). This methodology depends on the particular sulfated pattern of the polysaccharides. Finally, the 2-sulfated fucose unit content in the sulfated polysaccharides influenced significantly the kinetics of the acid hydrolysis.

Some authors have reported that a desulfation method that differs from the mild acid hydrolysis, solvolysis in dimethyl sulfoxide (DMSO), promotes a first and complete 2-desulfation. Curiously, they show that 4-sulfate esters persist partially resistant to desulfation (Vilela-Silva *et al.*, 1999; Aquino *et al.*, 2005). These data corroborate ours, in that the 2-sulfate ester is more labile than a 4-sulfate group when they are exposed to harsh conditions.

Another relevant observation for the acid hydrolysis approach is that the desulfation in the fucan from *S. pallidus* was faster than in the fucan from *L. variegatus*, as seen by the earlier structural modifications analyzed by ^{1}H-NMR spectrum (Figure 5A versus B). Maybe this occurs because of the presence of two sites of desulfation in the first sulfated fucan (two 2-sulfated residues [A and B]). Like the desulfation, the formation of oligosaccharides with time shown in PAGE (Figure 3A versus B) was also faster. Another comparison is noticed between the yielding of sulfated oligosaccharides in PAGE of Figure 2. During 1 h of mild acid hydrolysis, all of the molecular sizes of the products from *S. pallidus* are smaller than 20 kDa. However, in the case of *L. variegatus*, there are some populations of not well-defined oligosaccharides with the weights above 20 kDa, and the products of the fucan from *S. franciscanus* ranged almost from ~60 to ~15 kDa. In addition, a clear difference can be noted in the molecular sizes of the products of the

acid hydrolysis from *S. purpuratus* I and *S. purpuratus* II. The different molecular weights of these oligosaccharides appear in the same time of hydrolysis (lanes 6 and 8 in Figure 2), indicates that the velocity of the hydrolytic cleavage is directly influenced by the particular structures of these sulfated fucans, mostly by the sulfated pattern.

An important point to be explained is how the presence of 4-sulfate groups increases the susceptibility of 2-sulfate esters of a preceding residue to hydrolysis. Such phenomenon can be related to intramolecular interactions. In fact, because of the acidic pH of the medium, many sulfate groups would be in a protonated form, so making possible the occurrence of intramolecular hydrogen bonds between two adjacent sulfate groups. These interactions would facilitate the removal of a sulfate group in a nucleophilic substitution reaction. Based on preliminary molecular modeling results on sulfated fucan disaccharides, and depending on the carbohydrate ring conformation, the molecular orientation necessary to supply such hydrogen bonds can be 100 kJ/mol less stable when only 2-sulfate groups are present than in molecules presenting a 4-sulfate group near a 2-sulfate group. In addition, this energy can be considerably bigger in great polysaccharide chains, because the entire molecule is less flexible than a simple oligosaccharide.[1] Regarding the increase in susceptibility to acid cleavage in a desulfated fucose unit compared with one of a nondesulfated unit, the sulfate groups can promote a steric and/or electrostatic protection of the glycosidic linkage of the polysaccharide. After its removal, the linkage becomes more susceptible to hydrolysis. These data are evidenced by the different degradation of the sulfated fucans from *S. pallidus*/*L. variegatus* and *S. franciscanus* (Figure 8).

Certainly the methodology based on mild acid hydrolysis can be used as an efficient tool to study the relationship between molecular weight of the sulfated polysaccharides and their biological activities. However, the choice of this approach as a depolymerization procedure needs to be carefully analyzed. As described, the loss of the sulfated residues by solvolysis can promote a great decrease in the biological activity of the sulfated polysaccharide (Koyota *et al.*, 1997; Farias *et al.*, 2000). However, another report has used the mild acid hydrolysis to show that the reduction of the molecular mass can cause a decrease in the biological action of the sulfated fucan (Hirohashi and Vacquier, 2002). Probably, this loss of activity occurs not only because of the reduction of the molecular size, but may also be influenced by the desulfation at specific sites. Therefore, this report that used mild acid hydrolysis to establish the effect of the molecular weight on the biological activity needs to be reexamined.

Materials and methods

Extraction and purification of sulfated fucans

Adults of *S. pallidus*, *S. purpuratus*, *S. franciscanus* and *L. variegatus* were spawned into filtered sea water after intracelomic injection of 0.5 M KCl (~5 mL per specimen). The egg jellies were isolated from the female gametes by the pH 5.0 method, centrifuged, and lyophilized after dialysis against distilled water, as described previously (SeGall and Lennarz, 1979). The acidic polysaccharides were extracted from the jelly coats by papain digestion and partially purified by EtOH precipitation (Albano and Mourão, 1986). Sulfated fucans were purified by anion-exchange chromatography, and the purity was checked by agarose gel electrophoresis and NMR spectroscopy (Mulloy *et al.*, 1994; Alves *et al.*, 1998; Pereira *et al.*, 1999).

Mild acid hydrolysis of sulfated fucans

Sulfated fucans (5 mg) were dissolved in 1 mL of 0.01 M HCl and maintained at 60°C for different periods of time. After this depolymerization procedure, the pH was neutralized by the addition of 1 mL of ice-cold 0.01 M NaOH, as described previously (Pomin *et al.*, 2005). The amount of oligosaccharides formed was analyzed by PAGE, as described below. The oligosaccharides of *S. pallidus* were separated by gel-filtration chromatography and analyzed by PAGE.

PAGE

The native and low molecular-weight derivatives of the sulfated fucans (10 μg of each) were applied to a 12% 1-mm thick polyacrylamide gel slab in 0.02 M sodium barbital (pH 8.6) and run for ~45 min at 100 V. After the electrophoresis, the sulfated polysaccharides were stained with 0.1% toluidine blue in 1% acetic acid and washed for about 4 h in 1% acetic acid. The molecular masses of the low molecular-weight fragments of sulfated fucans were estimated by comparison with the electrophoretic mobility of standard compounds (Santos *et al.*, 1992; Pavão *et al.*, 1998; Pereira *et al.*, 1999; Pomin *et al.*, 2005). The standards used were high molecular-weight dextran sulfate (≥~100 kDa), chondroitin 4-sulfate from bovine trachea (~40 kDa), dermatan sulfate from pig skin (~20 kDa), and low molecular-weight dextran sulfate (~10 kDa).

Gel-filtration chromatography

The oligosaccharides formed after 1 and 2 h of mild acid hydrolysis of sulfated fucan from *S. pallidus* (5 mg) were fractionated on a Bio-Gel P10 column (200 × 0.9 cm), equilibrated with aqueous 10% ethanol, containing 1.0 M NaCl, as described previously (Pomin *et al.*, 2005). The fractions (1 mL) were eluted with the same solution at a flow rate of ~3 mL/h and assayed by their metachromatic property using 1,9-dimethylmethylene blue (Fardale *et al.*, 1986) and by phenol–sulfuric acid reaction (Dubois *et al.*, 1956). The fractions containing the various sulfated oligosaccharides were pooled, freeze-dried, and dissolved in 2.0 mL of distilled water. These solutions were desalted on a Superdex Peptide column (Amersham Biosciences, Piscataway, NJ), coupled to a fast protein liquid chromatography (FPLC) system. Fractions of 0.5 mL of these solutions were collected, and the oligosaccharides were detected by their metachromatic property. The fractions corresponding to the desalted oligosaccharides were pooled and freeze-dried.

[1]A more extensive study of the molecular modeling of sulfated fucan is now being undertaken by Dr. Hugo Verli. This study may help to understand in further detail the molecular mechanism of mild acid hydrolysis of these polysaccharides.

NMR experiments

^{1}H and ^{13}C spectra of the native sulfated fucan and its low molecular-weight derivatives were recorded using a Bruker DRX 600 apparatus (Bruker BioSpin GmbH, Rheinstetten, Germany) with a triple resonance probe. About 3 mg of each sample was dissolved in 0.5 mL of 99.9% D_2O (Cambridge Isotope Laboratory, Andover, MA). All spectra were recorded at 60°C with water suppression by presaturation. In some experiments, sulfated fucans (3 mg) were dissolved in 0.5 mL of 0.01 or 0.02 M HCl, prepared in 99.9% D_2O. The solution was put into an NMR tube, maintained at 60°C, and one dimensional ^{1}H-NMR spectra were recorded after different periods of time. TOCSY and $^{1}H/^{13}C$ HMQC spectra were recorded using states–time proportion phase incrementation for quadrature detection in the indirect dimension. TOCSY spectra were run with 4046×400 points with a spin-lock field of ~10 kHz and a mixed time of 80 ms. HMQC spectra were run with 1024×256 points and globally optimized alternating phase rectangular pulses for decoupling. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm for ^{1}H and to methanol for ^{13}C.

Supplementary Data

Supplementary data are available at *Glycobiology* online (http://glycob.oxfordjournals.org).

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Abbreviations

FPLC, fast protein liquid chromatography; HMQC, heteronuclear multiple quantum coherence; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; TOCSY, total correlation spectroscopy.

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