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Structural investigation of a fucoidan containing a fucose-free core from the brown seaweed, *Hizikia fusiforme*

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Abstract—A fucoidan, obtained from the hot-water extract of the brown seaweed, *Hizikia fusiforme*, was separated into five fractions by DEAE Sepharose CL-6B and Sepharose CL-6B column chromatography. All five fractions contained predominantly fucose, mannose and galactose and also contained sulfate groups and uronic acid. The fucoidans had MWs from 25 to 950 kDa. The structure of fraction F32 was investigated by desulfation, carboxyl-group reduction, partial hydrolysis, methylation analysis and NMR spectroscopy. The results showed that the sugar composition of F32 was mainly fucose, galactose, mannose, xylose and glucuronic acid; sulfate was 21.8%, and the MW was 92.7 kDa. The core of F32 was mainly composed of alternating units of →2)-α-D-Man(1→ and →4)-β-D-GlcA(1→, with a minor portion of →4)-β-D-Gal(1→ units. The branch points were at C-3 of →2)-Man-(1→, C-2 of →4)-Gal-(1→ and C-2 of →6)-Gal-(1→. About two-thirds of the fucose units were at the nonreducing ends, and the remainder were (1→4)-, (1→3)- and (1→2)-linked. About two-thirds of xylose units were at the nonreducing ends, and the remainder were (1→4)-linked. Most of the mannose units were (1→2)-linked, and two-thirds of them had a branch at C-3. Galactose was mainly (1→6)-linked. The absolute configurations of the sugar residues were α-D-Man*p*, α-L-Fuc*p*, α-D-Xyl*p*, β-D-Gal*p* and β-D-Glc*p*A. Sulfate groups in F32 were at C-6 of →2,3)-Man-(1→, C-4 and C-6 of →2)-Man-(1→, C-3 of →6)-Gal-(1→, C-2, C-3 or C-4 of fucose, while some fucose had two sulfate groups. There were no sulfate groups in either the GlcA or xylose residues.

Keywords: Fucoidan; Structure; Brown seaweed; *Hizikia fusiforme*

1. Introduction

Fucoidans, polysaccharides that contain a number of fucose and sulfate ester groups, are one of the main constituents of brown seaweeds. For the past decade fucoidans have been extensively studied due to their numerous biological activities, among them anticoagulant, antiviral and antihypertensive activities.[1–6]

Hizikia fusiforme is a kind of brown seaweed that mainly grows in the temperate seaside areas of the northwest Pacific, including China, Japan and Korea. It has been widely used as Chinese herbal medicine and health food for hundreds of years.[7–9] Fucoidan of *H. fusiforme* is a very important material valued for its various biological activities. However, there are only few studies on its chemical composition, and its structure is not precisely known.[10–13] The aim of this work was to study in detail the structure of the fucoidan isolated from *H. fusiforme*.

2. Results and discussion

2.1. Extraction and purification of fucoidan

The chemical composition of powdered *H. fusiforme* is as follows: moisture, 12.2%; ash, 15.4%; protein, 12.3%; fat, 1.6%; algin, 28.4% and fucoidan, ~2.7%. A fucoidan was extracted from the powder of *H. fusiforme*

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with water at 70 °C and purified through ethanol precipitation. Further elimination of algin was achieved by using $CaCl_2$. The yield of fucoidan from alga powder was 0.78%, and its fucose content was 18.6%. The chemical composition of fucoidan isolated from *H. fusiforme* is reported in Table 1. The results showed that the main sugars of fucoidan were fucose, mannose and galactose, along with other major components, uronic acids and sulfate.

2.2. Fractionation of fucoidan

Ion-exchange chromatography on a DEAE Sepharose CL-6B column separated the fucoidan into three peaks (Fig. 1). The major fraction, F3, was graded into three fractions (F31, F32 and F33) by gel-filtration chromatography on a Sepharose CL-6B column (Fig. 2). The chemical compositions of the five fractions are presented in Table 1. The results showed that all the five fractions predominantly contained fucose, mannose and galactose, and also contained sulfate and uronic acid, plus a small amount of protein. These had MWs from 25 to 950 kDa.

2.3. Structure of F32

Fractions F31, F32 and F33 produced a symmetrical peak on gel-filtration chromatography and a single band on agarose gel electrophoresis (Fig. 3). Because the content of F32 is higher and its chemical composition is representative in the five fractions, F32 was the first fraction selected to study its structure.

2.3.1. Periodate oxidation and Smith degradation. The molar ratios of the products of F32 after Smith degradation are shown in Table 2. 1,2-Propanediol is the product of 1,2-disubstituted fucose and rhamnose, which also produce glycerol at the same time. So about one-third of fucose (11.8/(11.8 + 23.4)) is 1,2-disubstituted. The 1,4-disubstituted hexose produces threitol, and the 1,4-disubstituted hexose and the deoxyhexose produce ethylene glycol after Smith degradation. The larger amounts of ethylene glycol (6.5%) and the smaller amounts of threitol (1.1%) mean that most of the 1,4-disubstituted sugars are derived from fucose and xylose. Mannose and galactose are stable from the effects of periodate oxidation, indicating that most of them are 1,3-disubstituted or have more than two hydroxyl groups substituted.

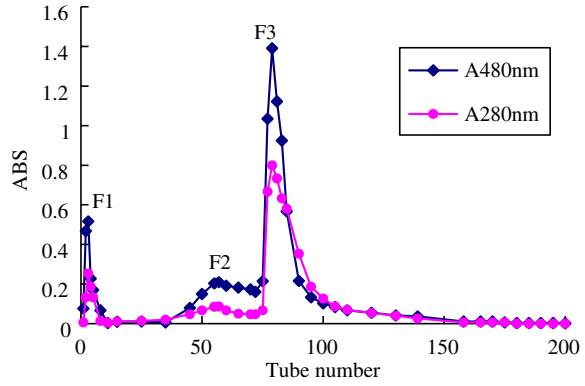

Figure 1. Chromatogram of fucoidan on a DEAE Sepharose CL-6B column.

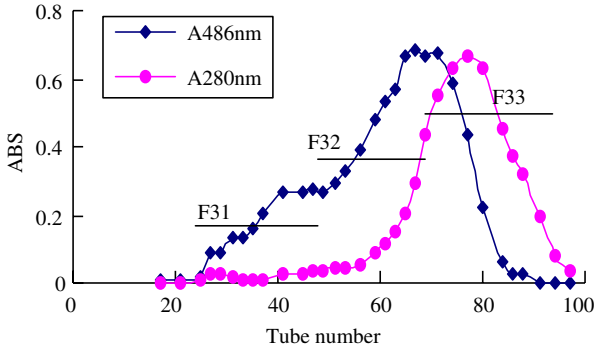

Figure 2. Chromatogram of F3 on a Sepharose CL-6B column.

2.3.2. Partial acid hydrolysis of DSF32. Because sulfate groups would disturb the accurate analysis of the mode of linkage and sequence analysis of the sugar residues, the sulfate groups of F32 were removed, and the product of desulfation was named DSF32. The S=O band at 1264 cm^{-1} of DSF32 almost disappeared, and its sulfate content dropped to ~0.5%, which indicated that sulfate

Table 1. Chemical composition of fucoidan and its fractions isolated from *H. fusiforme*

Sample	Fucose[a]	Uronic acid	Sulfate	Protein	MW (kDa)	Neutral sugars[b]						
						Fuc	Man	Gal	Xyl	Glc	Rha	Ara
Fucoidan	18.6	19.4	11.8	1.7	—	32.0	23.1	23.0	6.4	7.9	2.1	5.4
F31	31.2	13.4	23.9	1.7	776	44.5	17.3	33.0	3.1	1.0	1.1	—
F32	32.8	21.8	21.8	1.2	92.7	41.7	24.4	20.6	6.1	2.0	3.6	1.5
F33	18.8	32.4	3.3	1.4	25.9	37.6	27.0	22.4	5.3	4.5	2.0	1.2
F2	20.1	29.3	11.6	1.8	507	38.2	30.3	18.2	7.6	3.5	1.4	0.8
F1	22.1	17.4	19.3	2.4	959	29.6	29.3	22.7	7.9	8.8	0.9	0.8

[a] Determined by the method of Gibbons.[20]
[b] Determined by GLC.

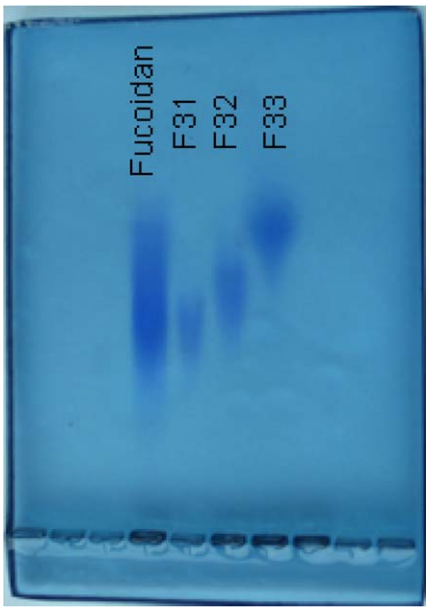

Figure 3. Agarose gel electrophoresis of fucoidans.

was almost completely removed. However, it was still very difficult to analyze the structure of DSF32 on account of its complicated chemical composition and larger MW. So DSF32 was partially hydrolyzed into simpler products whose structures were easier to analyze. Then according to the structure of a series of hydrolyzed products, ranging from simple to complex, the structure of DSF32 can be deduced. DSF32 was hydrolyzed sequentially by 0.03, 0.1 and 0.5 M trifluoroacetic acid (TFA), and the products were dialyzed using a membrane with a MW cutoff of 3500 Da. The retentates of dialysis were collected and lyophilized and named JL0.03, JL0.1 and JL0.5, respectively.

2.3.3. Structural analysis of JL0.5. The yield of JL0.5 from DSF32 was 26.9%, and its MW is 1.1 kDa. The sugar composition of JL0.5 before and after carboxyl-group reduction is given in Table 3. The uronic acids in the polysaccharide were first reduced to their neutral sugars through carboxyl-group reduction, and then the neutral sugars were determined by GLC analysis. Comparing the change of the neutral sugars before and after carboxyl-group reduction, the composition of the uronic acids can be calculated.[14] The results showed that the primary sugars of JL0.5 were mannose (46.4%) and glucuronic acid (50.5%), the proportion being about 1:1. There was also a minor proportion of galactose.

Methylation results of JL0.5 and its carboxyl-group reduced product (R-JL0.5) are reported in Table 4. There are also small fragments of →2,6)-Man-(1→, →2,3)-Man-(1→ and →2,4)-Gal-(1→ in R-JL0.5, which are not listed in Table 4; their total relative molar ratio is 0.4. By comparing the methylation results of JL0.5 and R-JL0.5, it can be confirmed that the Glc of Glc-(1→ and →4)-Glc-(1→ in R-JL0.5 is the carboxyl-group reduced product of GlcA. The methylation results showed that JL0.5 is mainly composed of →2)-Man-(1→ and →4)-GlcA-(1→, as well as a little →4)-Gal-(1→ and GlcA-(1→, and there were small branches at C-3 or C-6 of →2)-Man-(1→ and at C-2 of →4)-Gal-(1→.

The ^{13}C NMR spectrum of JL0.5 is shown in Figure 4. There are five peaks in the range δ 90–110, which means JL0.5 has five anomeric carbons. They are named A, B, C, D and E, and their chemical shifts are 104.9, 104.3, 104.1, 101.2 and 94.6, respectively. A strong signal at δ 176.4 is easily assigned to the carboxyl group of a uronic acid, which indicates that there are a significant number of uronic acids in JL0.5.

The ^{1}H NMR and HSQC spectra of JL0.5 are presented in Figures 5 and 6. From the HSQC spectrum five anomeric protons are found, in which proton A is covered by a water peak. Their anomeric configurations can be deduced by their chemical shifts and $^{3}J_{1,2}$ values (Table 5).

Table 2. The products of F32 after Smith degradation and native F32

F32	Ethylene glycol	1,2-Propanediol	Glycerol	Threitol	Rha	Ara	Xyl	Fuc	Man	Glc	Gal
After	6.5	11.8	12.2	1.1	1.9	0.8	1.0	23.4	21.5	1.7	18.2
Native					3.6	1.5	6.1	41.7	24.4	2.0	20.6

Table 3. Sugar composition of partially hydrolyzed products of DSF32 before and after carboxyl-group reduction

Sugar	JL0.5		JL0.1		JL0.03		DSF32	
	Native	Reduced	Native	Reduced	Native	Reduced	Native	Reduced
Man	92.8	46.4	63.9	46.9	38.6	39.9	25.9	24.5
Gal	6.5	2.2	21.1	10.5	28.3	18.3	22.5	16.0
Glc	0.6	51.1	2.5	35.8	1.7	28.3	1.1	22.2
Xyl			6.7	4.1	11.0	5.7	9.2	3.5
Fuc			3.5	1.6	16.2	6.9	38.5	32.8
Rha			2.3	1.0	2.7	1.0	2.0	0.7
Ara					1.5	0.3	0.8	0.4

Table 4. Partially methylated alditol acetates of F32, DSF32 and partially hydrolyzed products of DSF32

Alditol	Relative mole ratio					
	JL0.5	R-JL0.5	R-JL0.1	R-JL0.03	DSF32	F32
2,3,4-Me_3-Xyl			2	6	4	4
2,3,4-Me_3-Fuc				4	20	5
2,3-Me_2-Fuc				2	5	6
2,4-Me_2-Fuc				2	4	2
2,3-Me_2-Xyl				1	2	1
3,4-Me_2-Fuc					1	5
2,3,4,6-Me_4-Glc		2	2	3		
2,3,4,6-Me_4-Gal			2	4	3	2
2-Me-Fuc						5
3-Me-Fuc						8
3,4,6-Me_3-Man	16	15	30	23	10	2
Pentacetate Fuc						11
2,3,6-Me_3-Gal	1	1	4	5	2	2
2,3,6-Me_3-Glc		10	27	23		
2,3,4-Me_3-Gal			4	10	7	3
4,6-Me_2-Man			2	4	20	5
2,6-Me_2-Gal						2
3,6-Me_2-Gal			1	2	1	
3,4-Me_2-Gal				3	3	
6-Me-Man						1
3-Me-Gal						2
2,4-Me_2-Gal						8
2-Me-Gal						1
4-Me-Man						10
3-Me-Man						4

Combined with the spectra of the COSY-45 (Fig. 7) and HSQC of JL0.5, the chemical shifts of protons and carbons can be determined as shown in Table 6. According to the chemical shifts of the carbons, the sugar composition and the methylation analysis of JL0.5, its sugar residues can be assigned as A, →4)-β-D-Gal-(1→; B, β-D-GlcA-(1→; C, →4)-β-D-GlcA-(1→; D, →2)-α-D-Man-(1→ and E, →2)-α-D-Man.[15] By comparing these results with the methylation analysis, there appears to be an excessive amount of the sugar residue E (→2)-α-D-Man) in the NMR spectrum. This can be explained that the methyl glycoside formed during the methylation of the mannose residue at the reducing end will be hydrolyzed in further hydrolysis reactions, the hydroxy of C-1 then becomes free, and then it is acetylated in a later reaction. So there is only →2)-Man-(1→ but no →2)-Man in the methylated products.

From the C/H coupled peaks found in HMBC spectrum of JL0.5 (Fig. 8), namely D1/C4, C1/D2, B1/D2 and C1/A4, it can be deduced that H-1 of residue D is correlated with C-4 of residue C, H-1 of residue C is correlated with C-2 of residue D, H-1 of residue B is correlated with C-2 of residue D and H-1 of residue C is correlated with C-4 of residue A. From these results, together with the results of sugar composition and methylation analyses of JL0.5, its structure can be proposed as the following:

2.3.4. Structural analysis of JL0.1. JL0.1 is the product of F32 hydrolyzed by 0.1 M TFA. The sugar composition of JL0.1 is mainly mannose, GlcA and galactose, with a small amount of xylose, fucose and rhamnose present (Table 3). R-JL0.1 is the carboxyl-group reduced product of JL0.1, and its methylation results are reported in Table 4. In comparison with the methylation results of R-JL0.5, R-JL0.1 shows an increase in the amount of Xyl-(1→, Gal-(1→ and →6)-Gal-(1→; moreover, a little of the →2)-Man-(1→ has a branch at C-3, and a little of the →4)-Gal-(1→ has a branch at C-2. So it can be deduced that Xyl-(1→, Gal-(1→ and →6)-Gal-(1→ are linked with →2,3)-Man-(1→ and →2,4)-Gal-(1→, and they were missed when hydrolyzing with 0.5 M TFA.

2.3.5. Structural analysis of JL0.03. JL0.03 is the product of F32 hydrolyzed by 0.03 M TFA. The sugar composition of JL0.03 is mainly mannose, GlcA and galactose, with a small amount of xylose, fucose, rhamnose and arabinose present (Table 3). R-JL0.03 is the carboxyl-group reduced product of JL0.03, and its methylation results are given in Table 4. By comparing the methylation results with those of R-JL0.1, R-JL0.03 adds the methylated products of Fuc, and the proportion of Xyl-(1→ and →6)-Gal-(1→ also increases. The branch points are at C-3 of →2)-

β-D-GlcA-(1→[2)-α-D-Man-(1→4)-β-D-GlcA-(1→]n_1→4)-β-D-Gal-(1→

[4)-β-D-GlcA-(1→2)-α-D-Man-(1→]n_2→4)-β-D-GlcA-(1→2)-α-D-Man

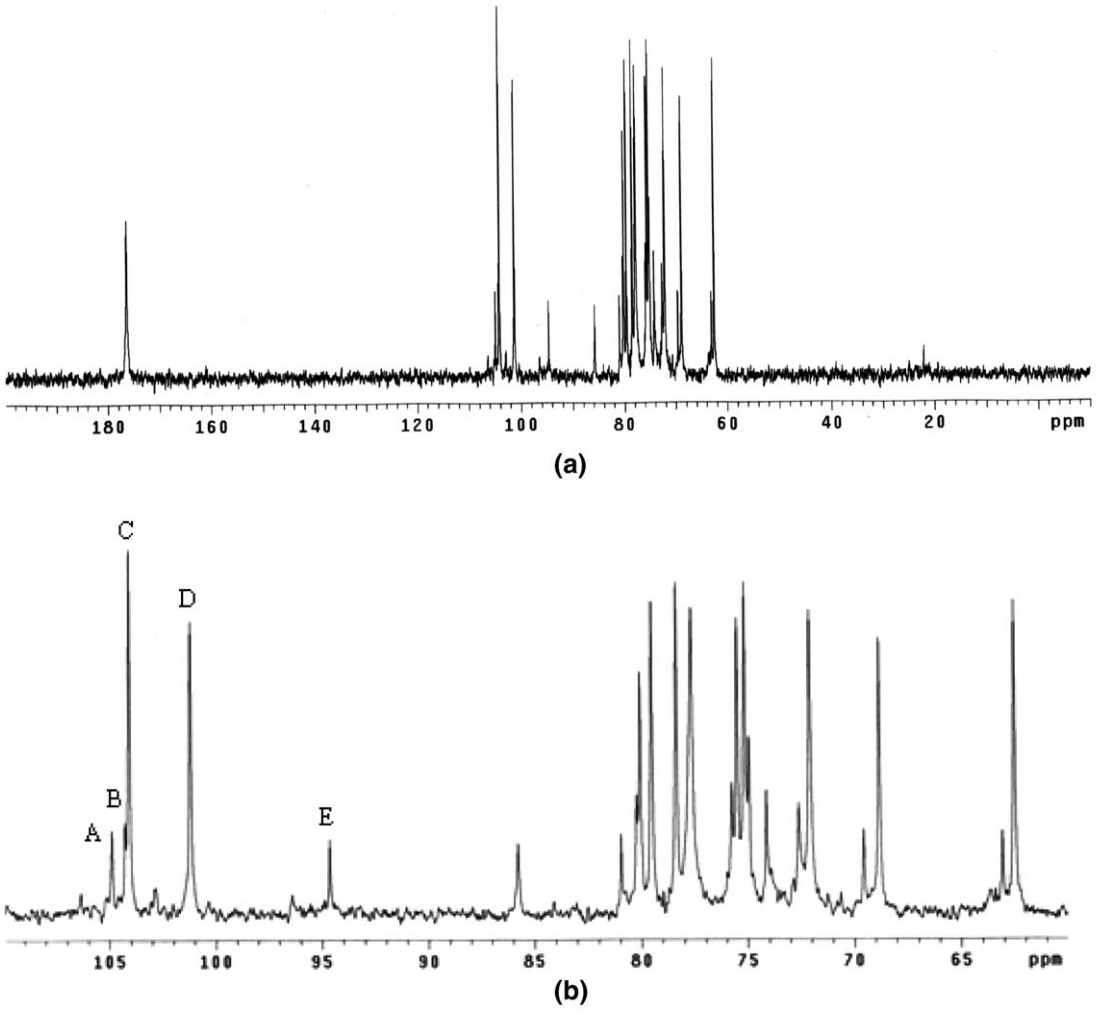

Figure 4. ^{13}C NMR spectrum of JL0.5: (a) 0–160 ppm; (b) expanded region 60–110 ppm.

Man-(1→, at C-2 of →4)-Gal-(1→ and at C-2 of →6)-Gal-(1→.

2.3.6. Structural analysis of DSF32. The sugar composition of DSF32 is very similar to F32, and its fucose content increases noticeably in comparison with that of JL0.03 (Table 3). The methylation results for DSF32 are presented in Table 4. The results show that about two-thirds of the fucose units are at the nonreducing ends, so they are easily cleaved when hydrolyzing, and the others are (1→4)-, (1→3)- and (1→2)-linked. About two-thirds of the xylose units are at the nonreducing ends, and the others are (1→4)-linked. Mannose is mainly (1→2)-linked; furthermore, two-thirds of the mannose residues have branches at the C-3 position. The majority of the galactose residues are (1→6)-linked. As for the size of the sugar rings, they are all in the pyranoid form except 2,3-Me_2-Xyl, which may be →4)-Xyl*p*-(1→ or 5)-Xyl*f*-(1→ and cannot be confirmed by methylation.

2.3.7. Structural analysis of F32. After obtaining the structure of DSF32, the next step was to confirm the position of the sulfate group in F32. The position of the sulfate group can be judged by IR, methylation and NMR studies.[16,17] But our studies showed that, besides the C–O–S vibration in 820–850 cm^{-1} region, there was also a C–H bending vibration of the sugar reducing end, which caused complications in interpreting the IR data that would lead to an erroneous result for sulfate position assignment if based on IR data alone.[18] Therefore, we compared the methylation results of F32 and DSF32. The methylation results for F32 are shown in Table 4. By comparing the methylation results of F32 and DSF32, it can be deduced that sulfate groups are mainly at C-6 of →2,3)-Man-(1→, at C-4 and C-6 of →2)-Man-(1→ and at C-3 of →6)-Gal-(1→. For fucose, the sulfate groups are at C-2, C-3 or C-4, while some fucoses have two sulfate groups. In addition, there is no sulfate group in xylose.

A methylation of RF32 (carboxyl-group reduced product of F32) was also done, and there was a large number of →4)-Glc-(1→ units, with very few other fragments of methylated glucose. By comparing the methylation results for F32, it can be deduced that GlcA

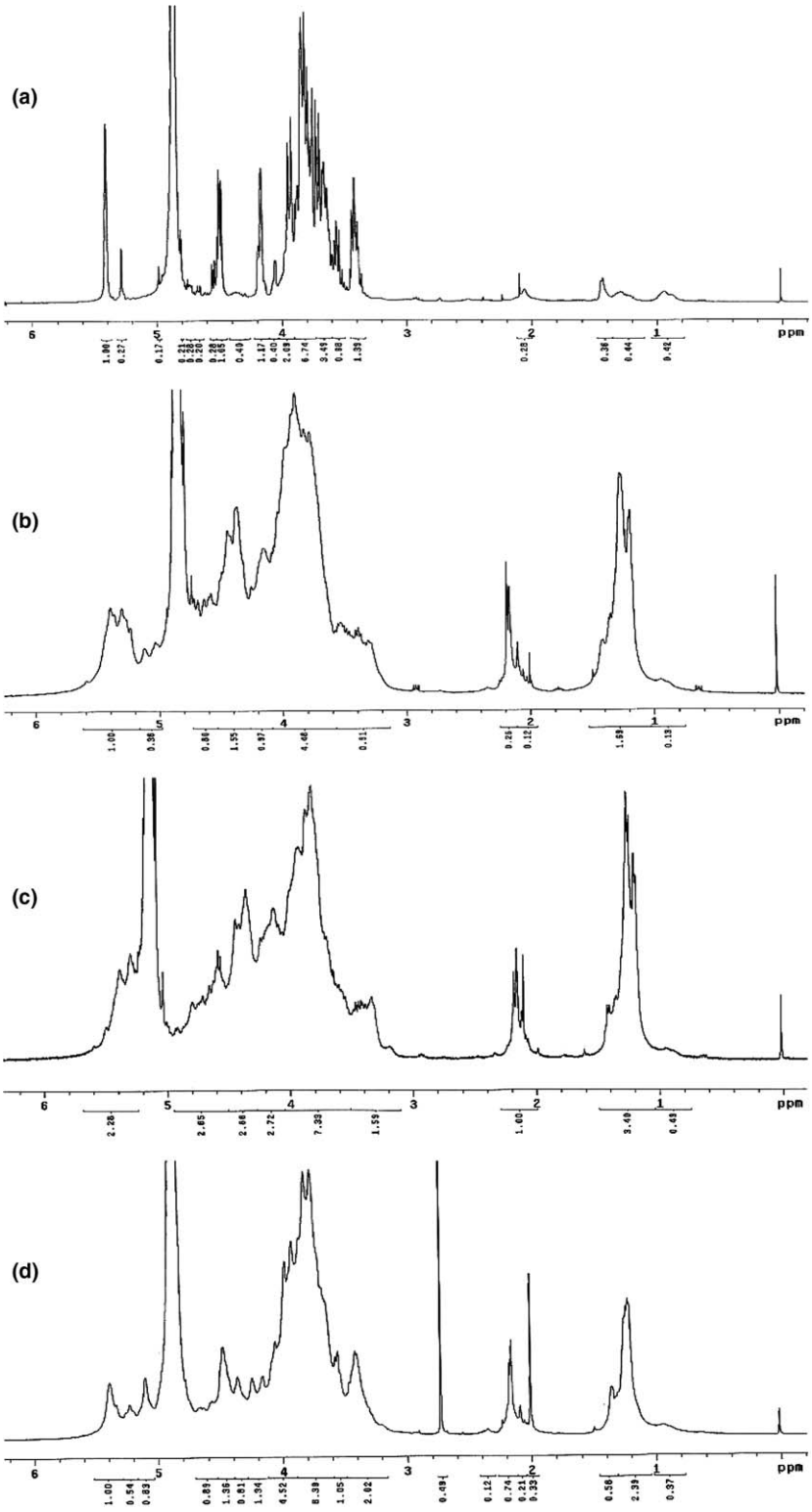

Figure 5. ^{1}H NMR Spectra of (a) JL0.5, (b) F32, (c) F32 after adding DCl, and (d) DSF32.

is mainly (1→4)-linked, and there is no sulfate group in GlcA.

The ^{1}H NMR spectrum of F32 is shown in Figure 5b. The ^{1}H NMR spectrum of F32 after adding DCl (Fig. 5c) gives a signal at δ 4.8–5.0, which is covered by the HDO peak in Figure 5b. By combining the analysis results with those of JL0.5, it can be inferred that δ 4.49–4.44 is the signal of β-D-Glc*p*A, δ 4.44–4.36 is the

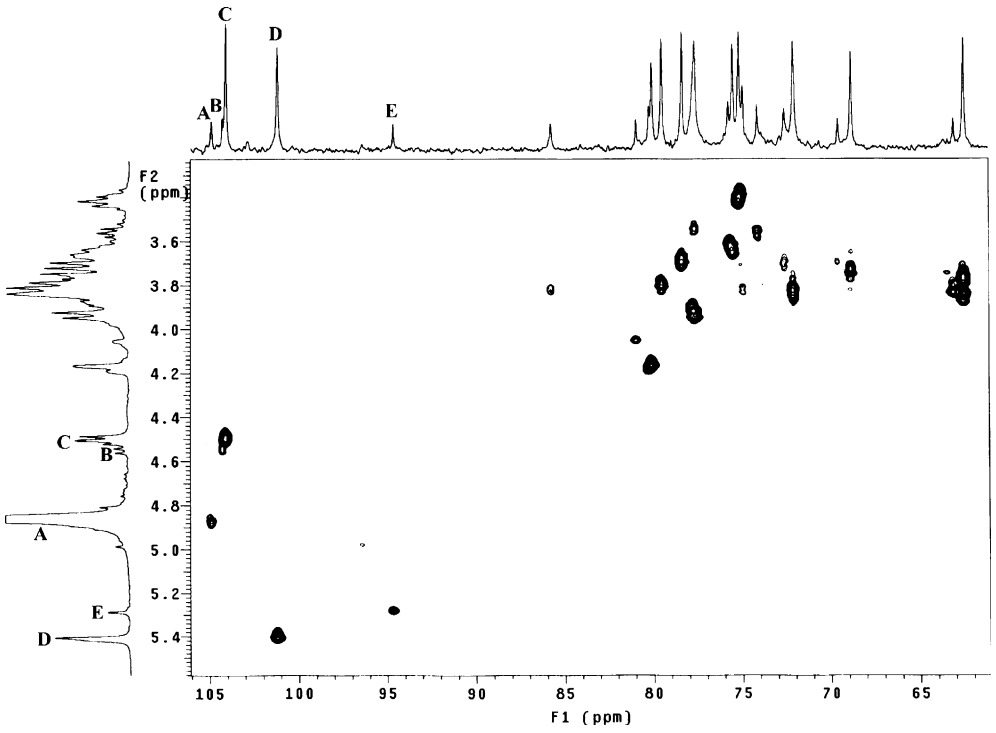

Figure 6. HSQC spectrum of JL0.5.

Table 5. Anomeric protons of JL0.5

Anomeric proton	A	B	C	D	E
Chemical shift	4.870	4.545	4.495	5.406	5.287
$^3J_{1,2}$	>7[a]	7.6	7.6	0	1.2
Configuration	β	β	β	α	α

[a] $^3J_{1,2}$ of A was inferred by the intensity of A1/A2 coupled peak in COSY-45 spectrum (Fig. 7).

signal of β-D-Gal*p*, δ 5.39–5.23 are the signals of α-D-Man*p*, α-D-Xyl*p* and α-L-Fuc*p*, δ 5.12–5.02 is the signal of α-L-Fuc*p* whose O-2 is substituted, and the signals of the 6-CH_3 of fucose are around δ 1.26.[15] According to the chemical shifts of the anomeric protons, it can be confirmed that 2,3-Me_2-Xyl in Table 4 is pyranoid →4)-Xyl-(1→.

The ^{1}H NMR spectrum of DSF32 is given in Figure 5d. After desulfation the peak at δ 5.10 in DSF32 is higher than that for F32, suggesting that the O-2s of many fucoses are sulfated. After sulfation of the O-2 of fucose, the δ H-1 will shift to low field. The strong peak in δ 4.48 corresponds to β-D-Glc*p*A.

From the NMR data, it can be inferred that Man, Fuc and Xyl are of the α anomeric configuration, and GlcA and Gal are of the β anomeric configuration. According to the structures of JL0.5, JL0.1, JL0.03 and DSF32, adding the position of sulfate groups and anomeric configurations of sugar residues, we can propose the structure for F32 as shown in Figure 9; however, other variations are possible.

There is 1.2% protein in F32, whose linkage type with glycan is also a part of the structure of F32. After β elimination in NaOH, the absorption at 240 nm increased, indicating that the linkage type between the glycan and protein was an *O*-glycosidic bond.[19] After β-elimination in NaOH containing $NaBH_4$, the amounts of Thr and Ser in the proportion of the total amino acids of F32 decreased from 10.0% and 7.6% to 6.7% and 6.8%, respectively, suggesting that the reducing ends of the sugars are linked mainly through *O*-glycosidic bonds with Thr, along with a few Ser residues.

3. Experimental

3.1. Materials

The brown seaweed *H. fusiforme* was collected in Dongtou (Zhejiang Province, China, May 2001). It was washed with tap water, lyophilized and milled.

3.2. Analytical methods

Fucose was determined by the Gibbons method.[20] Uronic acid (as GlcA) was measured by the carbazole sulfuric acid method.[21] Sulfate was assayed by the Dodgson and Price method,[22] and protein by the Lowry method.[23] Algin was determined by the carbazole colorimetric method.[24] Neutral sugars were analyzed, after H_2SO_4

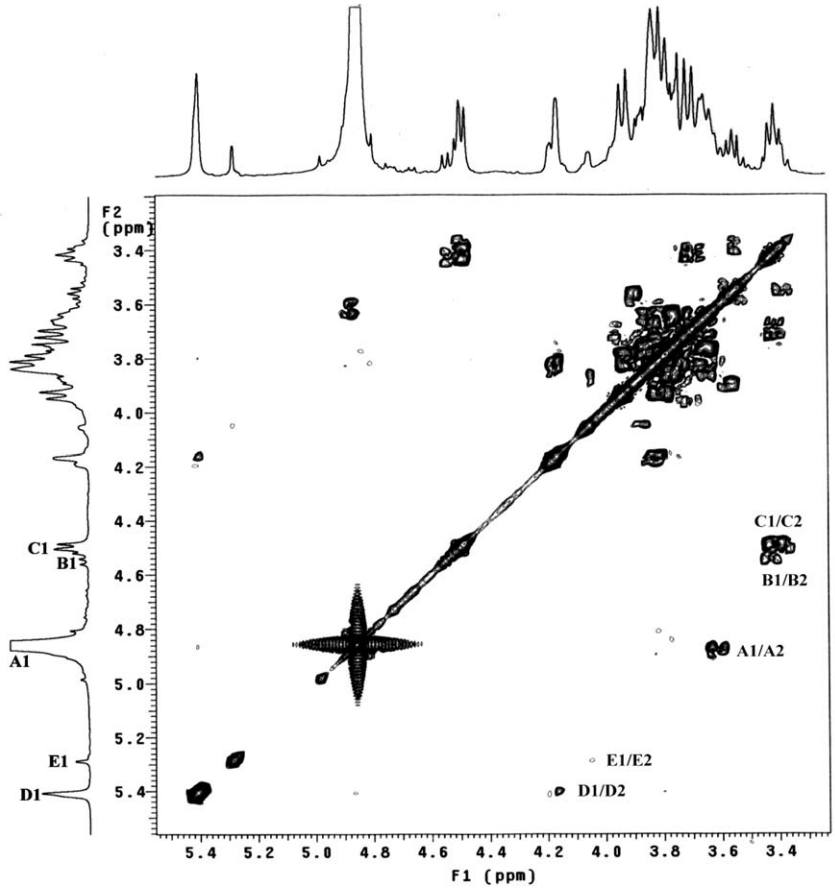

Figure 7. COSY-45 spectrum of JL0.5.

Table 6. Chemical shift data for JL0.5

Residue	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
A	4.87/104.90	3.64/75.76	3.84/74.96	4.19/80.23	3.81/74.96	3.84/63.07
B	4.55/104.28	3.42/75.16	3.56/74.13	3.94/77.68	3.72/72.60	–/176.36
C	4.50/104.09	3.42/75.16	3.69/78.39	3.80/79.54	3.92/77.68	–/176.36
D	5.41/101.21	4.16/80.08	3.82/72.10	3.75/68.83	3.64/75.52	3.78,3.86/62.51
E	5.29/94.65	4.05/80.95	3.78/72.10	3.69/69.55	3.64/75.52	3.78,3.86/62.51

hydrolysis, by GLC as their aldononitrile acetates, using an Agilent 6890 instrument with an HP-1 capillary column (30 m × 0.25 μm). The polysaccharide was hydrolyzed with 6 M HCl (110 °C, 24 h), and the amino acid was measured using an Agilent 1100 HPLC. GLC–MS analysis of partially methylated alditol acetates was performed on a Finnigan Trace GC–MS instrument equipped with an OV-1701 capillary column (30 m × 0.25 mm), using a temperature of 150–250 °C raised at 3 °C/min, and held at 250 °C for 10 min.

3.3. Extraction

The powdered seaweed was extracted with water three times (70 °C, 2 h, 1:10 seaweed:water) and centrifuged at 3000 rpm for 15 min. The supernatant was filtered through a nylon cloth and then ultrafiltered using a membrane with a MW cutoff of 3000 Da. The retentate was concentrated, and EtOH was added to produce a final concentration of 75%. The precipitate was washed with EtOH, acetone and Et_2O, then dried at 40 °C. The dry product (2 g) was dissolved in 80 mL of water and mixed with 3 mL of 3 M $CaCl_2$, then filtered through a nylon cloth. EtOH was added to the filtrate to produce a final concentration of 75%. The precipitate was washed with EtOH and acetone, then dried at 40 °C. A purified fucoidan was thus obtained after these procedures.

3.4. Fractionation

Fucoidan (800 mg) was dissolved in 80 mL of distilled water, and after centrifugation the supernatant was applied to a DEAE Sepharose CL-6B column

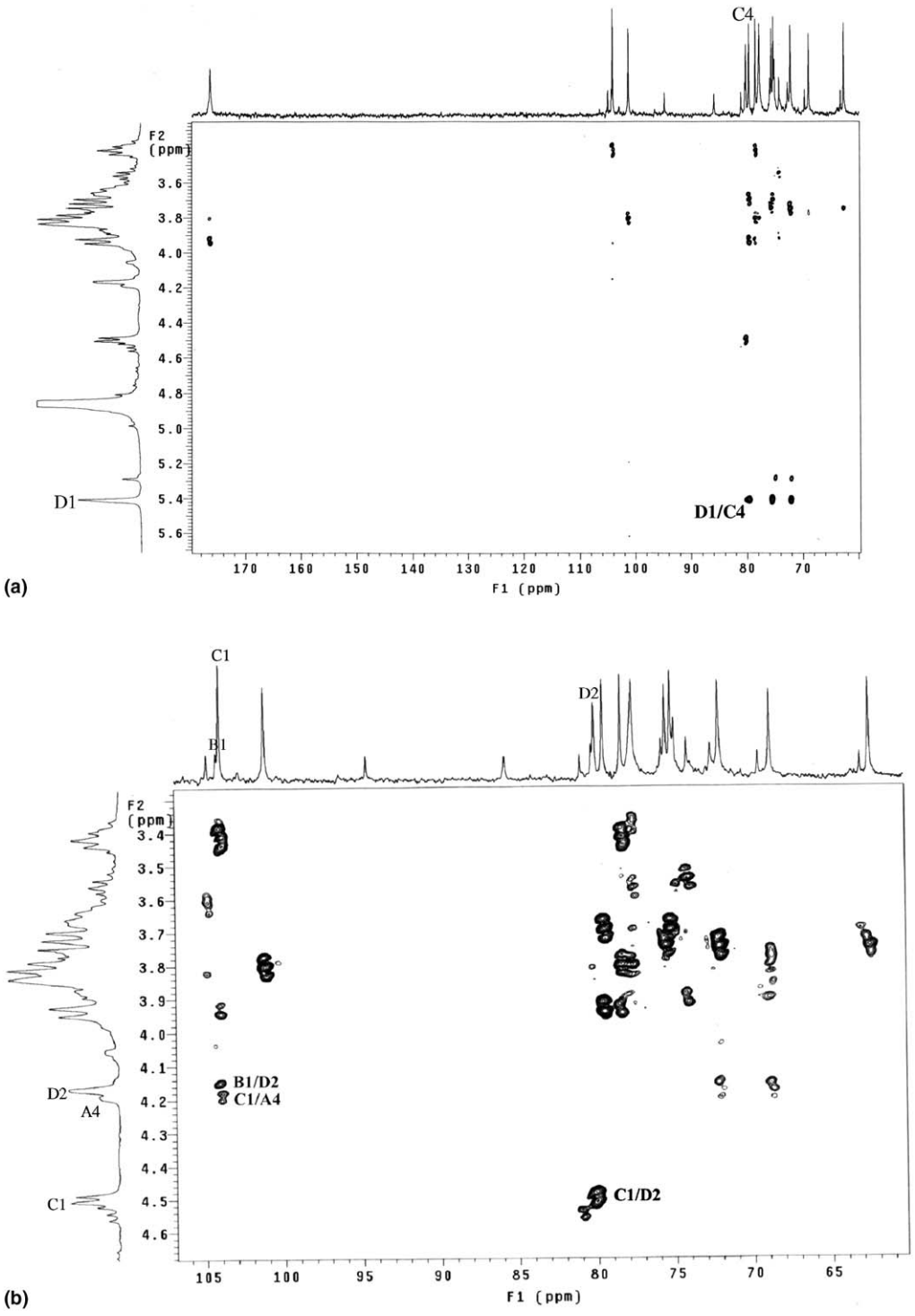

Figure 8. HMBC spectrum of JL0.5: (a) full spectrum; (b) expanded, enlarged region.

(3.5 × 30 cm). One fraction (F1) was eluted with distilled water, and then two fractions (F2 and F3) were eluted by a linear gradient 0.3→1.5 M NaCl. The elution was performed at a flow rate of 2 mL/min, 2 min/tube, monitored by UV absorption at 280 nm and a phenol–H_2SO_4 assay at 480 nm.[25] F1, F2 and F3 were collected and dialyzed against distilled water. After lyophilization, their yields were 23.1%, 7.3% and 48.0%, respectively. After removing free protein from F3 by the Sevag method,[26] F3 was further graded on a Sepharose CL-6B column (1.6 × 100 cm), eluted with 0.1 M NaCl at a flow rate of 25 mL/h, 10 min/tube, monitored by

Figure 9. Presumptive structure of F32. (1) Sulfate groups in F32 are at C-6 of →2,3)-Man-(1→, C-4 and C-6 of →2)-Man-(1→, C-3 of →6)-Gal-(1→, C-2, C-3 or C-4 of fucose, while some fucose have two sulfate groups. There is no sulfate group in GlcA and xylose. (2) There are also little Rha, Glc and Ara in F32, which are probably at side chains and ends. (3) The structure devised is just one of many possible.

UV absorption at 280 nm and a phenol–H_2SO_4 assay at 486 nm.[25] The elutant was separated into three parts: F31, F32 and F33. The three fractions were purified again on a Sepharose CL-6B column using the same conditions. After dialysis and lyophilization, yields of F31, F32 and F33 were 17.3%, 40.1% and 27.8%, respectively.

3.5. Determination of the molecular weight

Fractions F1, F2, F31, F32 and F33 were applied to a Sepharose CL-6B column (1.6 × 100 cm), eluted with 0.1 M NaCl and monitored by the phenol–H_2SO_4 method.[25] The column was calibrated using a pullulan series of different molecular weights.

3.6. Agarose gel electrophoresis

About 6 μg of each sample was applied to a 0.9% agarose gel in 0.06 M barbital buffer (pH 8.6) and subjected to electrophoresis at 60 V until the dye indicator (Cresol Red) migrated 4 cm from the origin. After electrophoresis, the gel was fixed with 0.1% cetyltrimethylammonium bromide for 1 h and stained with 0.1% Toluidine Blue in 0.1:5:5 HOAc–EtOH–water. The gel was de-stained with the same solution without toluidine.

3.7. Periodate oxidation and Smith degradation

Fraction F32 (20 mg) was dissolved in 50 mL of 15 mM $NaIO_4$ and kept in the dark at 4 °C for 7 days. Excess periodate was destroyed by the addition of 5 mL of ethylene glycol. The solution was then dialyzed against distilled water. $NaBH_4$ (100 mg) was added to the dialyzate, and the reaction mixture was maintained for 20 h in the dark at room temperature. After addition of HOAc to decompose the excess borohydride, the solution was dialyzed against distilled water and lyophilized. The polysaccharide thus obtained was hydrolyzed, derivatized and determined by GLC using the method for neutral sugars.

3.8. Desulfation

Fraction F32 (100 mg) was applied to a D001 (H^+) column and eluted with distilled water.[27] Acidic pH fractions were combined, and the pH of the solution was adjusted to pH 7.0 with pyridine, followed by lyophilization. The pyridinium salt of F32 was dissolved in 10 mL of 9:1 Me_2SO–MeOH. The reaction mixture was kept at 80 °C for 4 h, followed by cooling at room temperature. The reaction was terminated by the addition of 5 mL of water, and the mixture was then lyophilized. Loss of sulfate was monitored by the disappearance of the S=O

band at 1264 cm^{-1} and estimated by the method of Dodgson and Price.[22] The yield of desulfated product (named DSF32) was 65 mg.

3.9. Carboxyl-group reduction

A sample (30 mg) was dissolved in 3 mL of distilled water, followed by the addition of 60 mg of 1-ethyl-3-(3-dimethyaminopropyl)carbodiimide (EDC).[27] This solution was incubated at room temperature for 1 h under constant pH conditions (pH ~ 4.8) by the continuous addition of 0.4 M HCl. Then the reaction mixture was combined with 3 mL of freshly prepared 2 M $NaBH_4$ twice during the next 1.5 h, while maintaining the mixture at 50 °C. The reaction was terminated by the addition of HOAc, and the reaction mixture was dialyzed against distilled water. The dialyzate was then lyophilized, and 29 mg of carboxyl-group reduced product was obtained. The degree of reduction was estimated by the carbazole method.[21]

3.10. Methylation analysis

A sample was methylated according to the method of Needs and Selvendran.[28] The permethylated product was hydrolyzed with 2 M TFA (121 °C, 1 h), and the partially methylated sugars were reduced with $NaBH_4$ and converted to the alditol acetates.

3.11. Partial acid hydrolysis

DSF32 was treated with 0.03 M TFA at 100 °C for 1 h. The solution was dialyzed using a membrane with a MW cutoff of 3500 Da. The retentate was lyophilized and labelled JL0.03. JL0.03 was further hydrolyzed with 0.1 M TFA at 100 °C for 1 h, and dialyzed. The retentate was lyophilized and named JL0.1. Finally JL0.1 was treated with 0.5 M TFA at 100 °C for 1 h and dialyzed. The retentate was lyophilized and named JL0.5.

3.12. NMR spectroscopy

A 25–40 mg sample was dissolved in 0.5 mL of D_2O (99.9%), and then ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectra were obtained with a Mercury plus 400 spectrometer at 25 °C. All chemical shifts are reported relative to internal DSS. The parameters used for 2D experiments were as follows: COSY-45 [2048 × 2048 data matrix; relaxation delay 1.2 s; acquisition time 0.214 s; width 1199 Hz; 2D width 1199 Hz; sine bell 0.107 s]; gHSQC [1024 × 4096 data matrix; relaxation delay 1.2 s; acquisition time 0.3 s; width 1199 Hz; 2D width 6410 Hz; gauss apodization 0.099 s]; gHMBC [1024 × 8192 data matrix; relaxation delay 1.2 s; acquisition time 0.214 s; width 1199 Hz; 2D width 14,492 Hz; sine bell 0.107 s].

3.13. β-Elimination

(1) F32 was hydrolyzed in 0.2 M NaOH at 45 °C for 0.5 h, followed by scanning between 200 and 300 nm. (2) F32 was hydrolyzed in 0.2 M NaOH–1.0 M $NaBH_4$ at 45 °C for 24 h, and then the change of amino acids before and after elimination was determined.[19]

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2006.03.035.

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