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Structural elucidation of the capsular polysaccharide of *Bacteroides fragilis* strain 23745M1

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Abstract

The capsule of *Bacteroides fragilis* (ATCC23745) consists of two distinct polysaccharides, the separation of which could not be accomplished. The mouse-passaged strain (23745M1), however, yielded a preponderant polysaccharide which was isolated and purified. Using mainly high resolution NMR spectroscopy, the structure of the polysaccharide was elucidated and it is composed of the following repeating unit:

→ 3-β-D-Glc*p*NAc-(1 → 3)-α-L-Rha*p*-(1 → 4)-α-L-Fuc*p*-(1 →
2
↑
1
α-L-D-Yer-(1 → 6)-α-D-Glc*p*

where α-L-D-Yer is 3,6 dideoxy-4-*C*-(L-*glycero*-4′-hydroxyethyl)-α-D-*xylo*-hexopyranoside.

Keywords: Polysaccharide; *Bacteroides fragilis*; NMR

1. Introduction

Bacteroides fragilis is the most common obligately anaerobic bacterial species isolated from serious human infections [1], and studies conducted in animals have

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demonstrated that its capsule, identified as a high molecular weight polysaccharide [2], is an important virulence factor [3]. The capsule has been found to promote the formation of intra-abdominal abscesses even in the absence of viable bacteria [3], and, when injected in animals, the polysaccharide is also able to induce in them a T-cell dependent response that provides protection against subsequent challenge with live bacteria [4]. Therefore, a precise definition of the *B. fragilis* polysaccharide is essential to understand the structural basis of these important biological properties.

The capsule of *Bacteroides fragilis* (NCTC9343) is unusual in that it consists of two distinct polysaccharides [5] of which the structures of both have been elucidated [6]. Another strain of *Bacteroides fragilis* (ATCC23745), which also promotes the formation of intra-abdominal abscesses, has been described [3]. This strain also produces two polysaccharides, although in this case the separation of the two polysaccharides proved to be intractable. Following mouse passage, however, it was possible to isolate and purify one polysaccharide from the mouse-passaged strain (23745M1), the structural elucidation of which is reported herein. Although neither the mouse passaged-strain nor the polysaccharide were now able to induce intra-abdominal abscesses, the isolated polysaccharide was still able to bind to a monoclonal antibody made to the pre-mouse passaged organism (unpublished results).

2. Results and discussion

Four sugars, D-glucose, 2-acetamido-2-deoxy-D-glucose, L-fucose and L-rhamnose were identified by sugar analysis. Yersiniose was then identified by ^{1}H and ^{13}C NMR spectral analysis which thus revealed that *B. fragilis* polysaccharide consisted of five

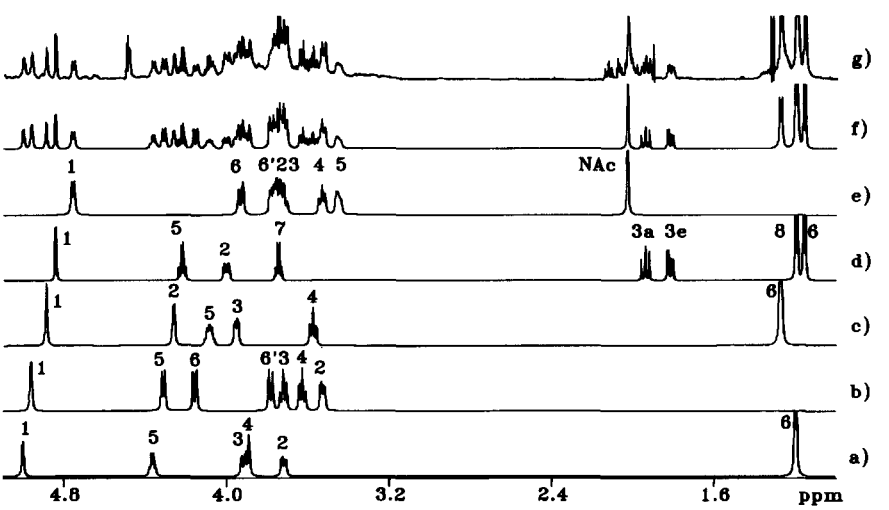

Fig. 1. Proton spectra of the polysaccharide in D_2O at 325 K showing the spin simulation for each residue (**a**–**e**), the simulated spectrum (f) and the experimental spectrum (g). The HOD resonance is at 4.488 ppm.

Table 1
NMR data [a] for the *B. fragilis* polysaccharide in D_2O at 325 K

	→ 4)-α-L-Fuc*p*	→ 6)-α-D-Glc*p*	→ 2,3)-α-L-Rha*p*	α-L-D-Yer	→ 3)-β-D-Glc*p*NAc
	a	**b**	**c**	**d**	**e**
H-1	5.00 (5.20)	4.959 (5.23)	4.888 (5.12)	4.842 (4.78)	4.756 (4.72)
H-2	3.717 (3.77)	3.528 (3.54)	4.260 (3.92)	4.002 (4.09)	3.754 (3.65)
$H\text{-}3^{ax}$	3.915 (3.86)	3.719 (3.72)	3.952 (3.81)	1.941 (1.92)	3.721 (3.56)
$H\text{-}3^{eq}$				1.821 (1.88)	
H-4	3.877 (3.81)	3.628 (3.42)	3.579 (3.45)		3.533 (3.46)
H-5	4.361 (4.20)	4.308 (3.84)	4.085 (3.86)	4.219 (4.21)	3.453 (3.46)
H-6	1.196 (1.21)	4.153 (3.84)	1.274 (1.28)	1.157 (1.23)	3.931 (3.91)
H-6′		3.782 (3.76)			3.776 (3.75)
H-7				3.747 (3.82)	
H-8				1.196 (1.28)	2.030 (2.06)
$J_{1,2}$	3.8	4.1	1.8	3.5	8.5
$J_{2,3a}$ $J_{2,3e}$	10.3	9.8	3.6	11.5, 5.0	10.3
$J_{3a,4}$ $J_{3a,3e}$	3.4	9.3	9.1	–, −13.0	8.9
$J_{4,5}$ $J_{7,8}$	0.6	9.3	9.8	−, 6.5	10.3
$J_{5,6}$ $J_{5,6'}$ $J_{6,6'}$	6.6	1, 1, −11	6.5	6.5	2, 5, −12.3
C-1	100.66 (93.1)	98.31 (93.0)	100.20 (94.8)	97.96 (99.9)	103.70 (95.9)
C-2	69.21 (69.1)	72.27 (72.5)	76.63 (71.8)	65.93 (68.0)	56.54 (57.9)
C-3	69.75 (70.3)	74.19 (73.8)	79.42 (71.0)	30.97 (31.4)	81.63 (74.8)
C-4	82.28 (72.8)	70.25 (70.7)	72.27 (73.2)	75.86 (76.6)	69.93 (71.1)
C-5	67.93 (67.1)	71.48 (72.4)	70.58 (69.1)	67.67 (68.1)	76.50 (76.82)
C-6	16.15 [b] (16.3)	65.38 (61.8)	17.23 (17.7)	13.28 (13.7)	61.90 (61.9)
C-7				70.38 (71.0)	175.35 (175.5)
C-8				16.44 [b] (16.7)	23.23 (23.1)

[a] Chemical shift in ppm and $J_{H,H}$ in Hz. Chemical shifts of appropriate monosaccharides, yersiniose [9] and others [14] are in parentheses.
[b] Similarly labelled assignments are interchangeable.

different pyranosides. The residues were labelled **a–e** according to the decreasing order of the chemical shift for the anomeric protons (Fig. 1). The ^1H signals were assigned in a series of 1D TOCSY experiments [7]. ^{13}C NMR chemical shifts were assigned using a ^1H-^{13}C shift-correlated experiment [8]. Relevant NMR data for the polysaccharide and their corresponding monosaccharides are given in Table 1. The nuclear Overhauser effects (Fig. 2), obtained from 1D NOESY experiments [7], were used for the determination of the anomeric configuration of sugars, the sequence of sugars and the absolute configuration of yersiniose.

Residue **a** was assigned to α-L-fucopyranose. A $^3J_{H1,H2}$ of 3.8 Hz indicated the α-configuration for this sugar. The $^3J_{H,H}$ coupling pattern for the ring protons included small couplings to H-4, thus indicating the galactopyranosyl configuration. A doublet at 1.196 ppm assigned to H-6 and integrating for three protons demonstrated that this sugar had a 6-deoxy function. Examination of chemical shift differences for the carbon signals of this residue as compared to values in the corresponding monomer indicated that C-4 was shifted 9.5 ppm downfield, thus suggesting that residue **a** was substituted at O-4.

Residue **b** was assigned to α-D-glucopyranose based on a $^3J_{H1,H2} = 4.1$ Hz and only large $^3J_{H,H}$ values of 9–10 Hz for the ring protons. Its C-6 signal was shifted downfield

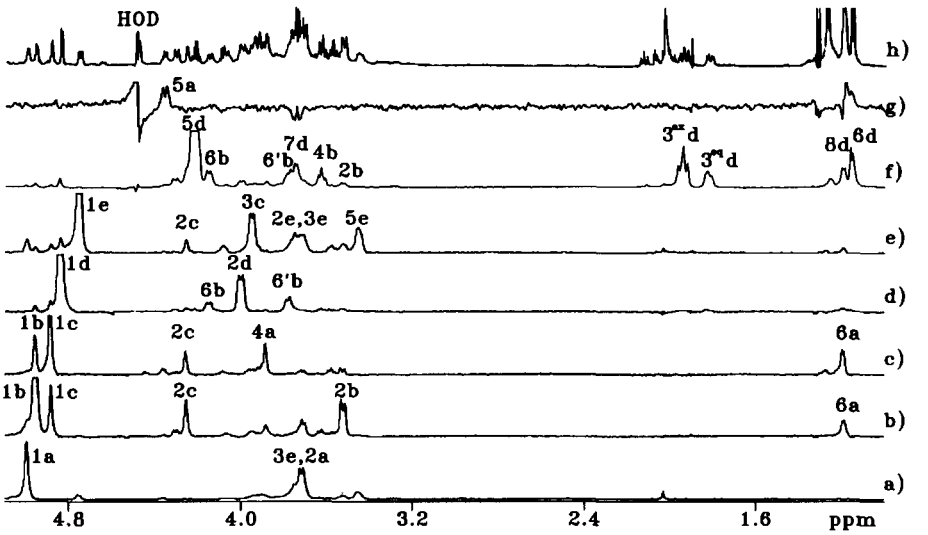

Fig. 2. 1D NOESY spectra for the anomeric protons of each residue (**a**–**e**) and the H-5**d** resonance (f). The 1D NOESY-TOCSY spectrum (g) was obtained using the NOE transfer from H-1**b** signal followed by a TOCSY transfer from 1.196 resonance (H-6**a**).

by 3.6 ppm in comparison to C-6 of its corresponding monomer, indicating linkage to O-6 of residue **b**.

Residue **c** was assigned to α-L-rhamnopyranose. The small magnitudes of $^{3}J_{H1,H2}$ and $^{3}J_{H2,H3}$ indicated a pyranose ring system having a *manno* configuration. A doublet at 1.274 ppm, integrating for three protons, demonstrated that this residue had a 6-deoxy function. The intraresidue NOE between H-1 and H-2 and the absence of the H-1–H-3 and H-1–H-5 NOEs demonstrated that it had the α-configuration. Comparison of the chemical shifts of this unit in the polysaccharide with those of its corresponding monomer showed significant deshielding of C-2 and C-3 signals (4.8 and 8.4 ppm, respectively) indicative of two linkages at O-2 and O-3.

Residue **d** was assigned to 3,6-dideoxy-4-*C*-(L-*glycero*-4′-hydroxyethyl)-α-L-*xylo*-hexose (α-L-D-Yer). The α-configuration of **d** was determined from the value of $^{3}J_{H1,H2}$ (3.5 Hz) and from the chemical shift of C-3 of the methylene group in the ring at 30.97 ppm characteristic of the α form of yersiniose [9]. Comparison of the ^{13}C NMR spectrum with the ^{1}H-^{13}C shift correlated spectrum showed a quaternary carbon C-4 at 75.86 ppm representing the branch point of yersiniose. The similarity of the chemical shifts of this residue in the polysaccharide with those of the corresponding monomer indicated that it was a terminal residue. Due to the lack of readily available reference compounds, as explained below, an attempt was also made to establish the absolute configuration of residue **d** by simulation of interresidue NOEs.

Residue **e** was assigned to 2-acetamido-2-deoxy-β-D-glucopyranose. The large value of $^{3}J_{H1,H2}$ (8.5 Hz) demonstrated that residue **e** had the β configuration. Large $^{3}J_{H,H}$ (~ 10 Hz) for the remaining ring protons enabled the assignment of the *gluco* configuration to **e**. The chemical shift of 56.54 ppm for the C-2 signal demonstrated the

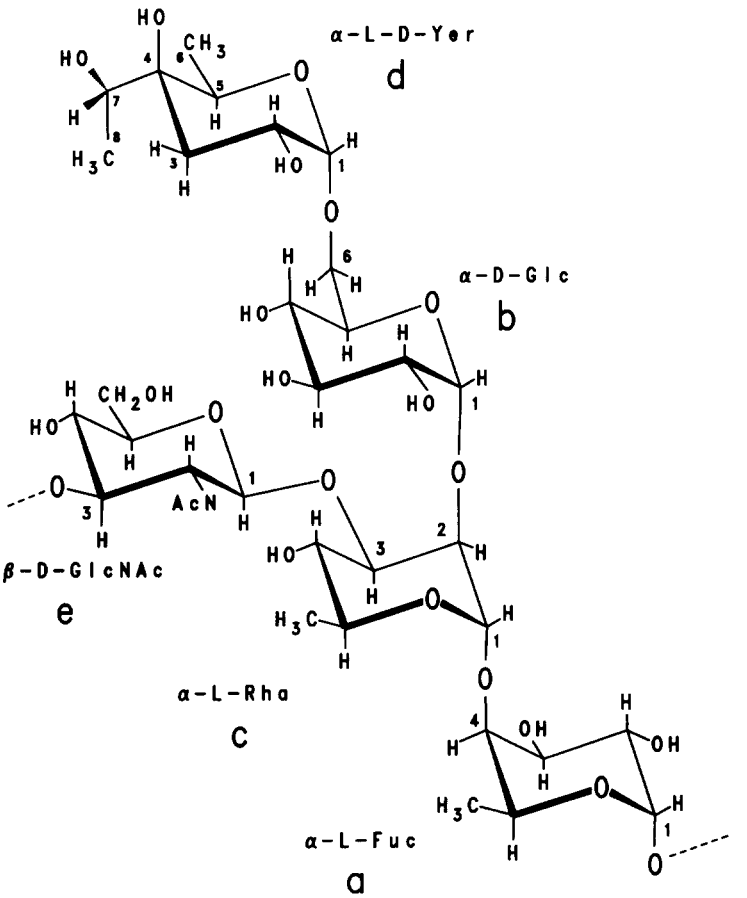

Fig. 3. Structure of the repeating unit of the *B. fragilis* polysaccharide.

presence of an amino function, and signals at 23.23 ppm (NAc-CH_3) and 175.35 ppm (NAc-CO) suggested that it was *N*-acetylated. The C-3 signal was shifted downfield by 6.8 ppm, indicating that residue **e** was substituted at O–3.

Structure of the polysaccharide.—The structure of the polysaccharide is shown in Fig. 3. The nuclear Overhauser effect (Fig. 2) was used to obtain sequence information because of the close proximity between the anomeric proton and proton attached to the carbon across the glycosidic linkage. The NOE between H-1**a** and H-3**e** indicated that residue **a** (α-Fuc) was linked to O-3 of residue **e** (β-GlcNAc). Residue **b** was linked to O-2 of residue **c** (α-Rha) as deduced from the NOE between H-1**b** and H-2**c**. H-1**c** had NOE's with H-2**c** and H-4**a** establishing the α-(1 → 4) linkage for the **c-a** unit (α-Rha-(1-4)-α-Fuc). The NOEs between H-1**d**, H-6**b** and H-6**b** established that residue **d** (α-Yer) was linked to O-6 of residue **b** (α-Glc). Residue **e** was linked to O-3 of residue **c** (α-Rha) as indicated by the enhancement of the H-3**c** resonance upon irradiation of H-1**e**. The structure of the polysaccharide (Fig. 3) deduced from NOE data is also in accord with glycosylation shifts observed in the ^{13}C NMR spectrum (Table 1).

Table 2
NOE data for α-Yer-(1 → 6)-α-D-Glc (**d**–**b**) used to determine the absolute configuration of yersiniose and the calculated NOE for α-L-D-Yer-(1 → 6)-α-D-Glc (LDD) and α-D-L-Yer-(1 → 6)-α-D-Glc (DLD)

NOE	Experimental [a]	Calculated	
		LDD	DLD
H-5**d**/H-3^{ax}**d**	28	18	19
H-5**d**/H-3^{eq}**d**	14	10	10
H-5**d**/H-4**b**	14	29	10
H-5**d**/H-2**b**	1	2	6
H-1**b**/H-6**d**	0	0	6

[a] ±50% experimental error.

To complete the structural elucidation of the polysaccharide, assignment of the absolute configuration of residue **d** was required. A comparison of the chemical shift data of **d** (Table 1) with that obtained from a series of known isomers of yersiniose [9] indicated that **d** was 3,6-dideoxy-4-*C*-(L-*glycero*-4′-hydroxyethyl)-α-D-*xylo*-hexopyranoside. These data, however, did not eliminate the possibility of an alternative assignment to the enantiomer 3,6-dideoxy-4-*C*-(D-*glycero*-4′-hydroxyethyl)-α-L-*xylo*-hexopyranoside. For the linkage α-Yer-(1 → 6)-α-D-Glc (**d**–**b**), the absolute configuration of glucose was unambiguously established by GC-MS. However, the absolute configuration of yersiniose could not be determined by this method because of the unavailability at that time of the previously synthesized [9] stereoisomers of yersiniose for use as standards. Therefore, an attempt was made to establish the absolute configuration of residue **d** using NOEs and potential energy calculations [6,10].

All the NOEs from Yer were investigated experimentally. Those which were useful for the determination of the absolute configuration of Yer are listed in Table 2. Due to the overlap of the H-6**a** and H-8**d** signals at 1.196 ppm, there was a possibility of an NOE between H-8**d** and H-1**b** or between H-6**a** and H-1**b** in the NOE spectra obtained with H-1**b** irradiation (Fig. 2b). This ambiguity was resolved by using a newly developed 1D analogue of the 3D-NOESY-TOCSY technique [11]. In this technique, the magnetization created at 1.196 ppm after the initial NOE step from H-1**b** was further transferred along the spin system during the consecutive selective TOCSY period (Fig. 2g). Since only the H-5**a** resonance was observed, the resonance at 1.196 ppm in Fig. 2b must be assigned to H-6**a**.

The experimental NOEs were then compared to the calculated ones for the enantiomers of Yer in the linkage α-Yer-(1 → 6)-α-D-Glc (**d**–**b**) (Table 2). Although the linkage between **d** and **b** involved an additional degree of freedom about the C-5–C-6 bond of **b**, the calculation was simplified to averaging only about two glycosidic torsion angles, since the O-6–C-6–C-5–H-5 torsion angle was restricted to the *trans* orientation, as indicated by the small values of $^3J_{H5,H6}$ and $^3J_{H5,H6'}$ (~ 1 Hz). The H-5**d** to H-4**b** NOE gave ambiguous results, since both were within the bounds of experimental error. However, the H-5**d** to H-2**b** NOE and H-1**b** to H-6**d** NOE indicate a distinct preference for the assignment of **d** to 3,6-dideoxy-4-*C*-(D-*glycero*-4′-hydroxyethyl)-α-L-*xylo*-hexopyranoside. However, because of the small magnitude of the NOEs involved, this assignment could not be made with absolute confidence. Therefore, the absolute

configuration of residue **d** was later confirmed by a comparison of the GC-MS analyses of the (±)-2-butyl glycosides of residue **d** with an authentic sample of yersiniose [9]. The lack of more definitive NOE data for this linkage (**d–b**) is probably due to the fact that **d** is terminal and thus has a higher degree of rotational freedom. In contrast, previous examples of the successful use of this type of analysis [6,10] have involved the collection of more definitive NOE data. This was probably achieved because the sugars investigated in these analyses were interchain residues and thus subject to more rotational restriction.

It is interesting to note that, by mouse passaging *B. fragilis* (ATCC 23745), it was possible to isolate one of its capsular polysaccharides which was identified by its reaction with an antiserum raised to the original strain. However both the mouse passaged organism (23745M1) and the isolated polysaccharide failed to induce intra-abdominal abscesses in rats. From the structure of the polysaccharide, it is possible to predict that it is not biologically active because it lacks carboxylate groups, which in conjunction with free amino groups are a structural requirement for biological activity [12]. In addition, it is probably unlikely that mouse passage could effect this type of structural change. Therefore, one must conclude that the activity, provided it is due to capsular polysaccharide, probably resides in the unisolated polysaccharide, which for the sake of consistency with this postulate, must have been structurally altered by mouse passage. Studies are in progress to isolate the other polysaccharide in order to confirm this postulate.

3. Experimental

Bacterial strain and growth conditions.—*B. fragilis* reference strain 23745 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The strain used for this study was a spontaneous mutant isolated following serial passage of the prototype strain in mice. Identification of this strain as *B. fragilis* was confirmed by conventional methods. Aliquots of the prototype and mouse-passed strain were maintained frozen in peptone-yeast broth at −80°C. Frozen stocks were plated onto trypticase soy agar (Difco Laboratories, MI) plates supplemented with 5% sheep blood and incubated in Gas-pack jars or an anaerobic cabinet (BBL Microbiology Systems, MD) at 37°C for 48 h [5]. For extraction of capsular polysaccharide, organisms were grown in supplemented basal medium as previously described [5]. Anaerobiosis was maintained by flushing with N_2 and a pH of 7.2 was maintained by titration with 10 M NaOH.

Extraction of capsular polysaccharide.—Phenol–water extraction of *B. fragilis* capsular polysaccharide was carried out according to published methods [5]. Purification of the capsular polysaccharide was accomplished by gel filtration (Sephacryl S-400, Pharmacia, Uppsala, Sweden) using a deoxycholate–glycine–EDTA buffer [1]. Contaminating nucleic acids and proteins remaining after phenol–water extraction were removed by digestion with DNAase, RNAase, and pronase prior to chromatography.

Instrumental methods.—^{13}C and ^{1}H spectra were recorded at 325 K on a Bruker AMX 600 spectrometer using a 5 mm broad-band probe with the ^{1}H coil nearest to the

sample. The sample was 5 mg of polysaccharide in 0.5 mL of D_2O at neutral pH. Acetone was used as an internal chemical shift reference for ^1H NMR (2.225 ppm) and for ^{13}C NMR (31.07 ppm). Proton spin simulations were performed with the program LAOCN5 available in Dennis Hare's program FTNMR. 1D TOCSY and 1D NOESY [7] spectra were obtained using a half-Gaussian pulse [13] of 100 ms. Mixing times in the 1D TOCSY experiments varied between 20 and 160 ms and were set to 250 ms in the 1D NOESY experiments. For the NOESY spectra, 1024 scans were accumulated. The 1D analogue of the 3D NOESY–TOCSY experiment was performed using 95 ms half-Gaussian pulses for both selective NOESY and selective TOCSY transfer with mixing times of 200 ms and 47 ms, respectively, and 16384 scans. The ^{13}C spectrum was assigned using an ^1H-^{13}C shift correlated experiment [7].

Butanolysis and GC–MS.—The polysaccharides were first hydrolyzed with 4 M TFA for 1 h at 125°C, the solution was lyophilysed, and the released glycoses were butanolyzed with *R*-(−)-2-butanol (Aldrich) and TFA as the acid catalyst. Authentic standards of the individual sugars were butanolyzed with (±)-2-butanol. After trimethylsilylation with trimethylsilyltrifluoroacetamide in acetonitrile, the trimethylsilylated butyl glycoside derivatives were analyzed by GC–MS using a Hewlett–Packard 2985B system and a fused silica OV-17 capillary column (Quandree Corp.). The identity of each glycose derivative was established by comparison of its GLC retention time and MS with those of authentic reference samples. For determination of configuration of 3,6-dideoxy-4-*C*-(-*glycero*-4^1-hydroxyethyl)-*xylo*-hexose, NMR and potential energy calculations were also used.

Sugar analysis.—Polysaccharide was hydrolyzed for 5 h with 0.1 M H_2SO_4 at 80°C. After neutralization with barium carbonate and centrifugation, the supernatant was concentrated. The residue was dissolved in 2 mL H_2O, and the pH adjusted to 7–9 with 0.1 N NH_4OH. $NaBD_4$ (2 mg) was added and the mixture was kept at room temperature for 2 h. After neutralization with 50% acetic acid, the mixture was concentrated three times from CH_3OH and acetylated in pyridine with acetic anhydride. The sample was analyzed by GLC-mass spectrometry.

Calculations.—Ensemble-averaged NOE calculations and potential energy calculations were done as previously described [6,10], allowing for spin diffusion. The NOE was calculated for a 250 ms mixing time and a correlation time of 2.5 ns.

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