High-resolution proton nuclear magnetic resonance characterization of seminolipid from bovine spermatozoa

Juan G. Alvarez,[1,†] Bayard T. Storey,[*,†] Mark L. Hemling,[**] and Robert L. Grob[††]

Departments of Physiology* and Obstetrics and Gynecology,† University of Pennsylvania, School of Medicine, Philadelphia, PA 19104; Smith Kline and French Laboratories, King of Prussia, PA; and Department of Chemistry,†† Villanova University, Villanova, PA

Abstract The high-resolution one- and two-dimensional proton nuclear magnetic resonance (^{1}H-NMR) characterization of seminolipid from bovine spermatozoa is presented. The ^{1}H-NMR data was confirmed by gas–liquid chromatography–mass spectrometric analysis of the partially methylated alditol acetates of the sugar unit, mild alkaline methanolysis of the glyceryl ester, mobility on normal phase and diphasic thin-layer chromatography (HPTLC), and fast atom bombardment mass spectrometry (FAB-MS). The structure of the molecule corresponds to 1-O-hexadecyl-2-O-hexadecanoyl-3-O-β-D-(3'-sulfo)-galactopyranosyl-*sn*-glycerol. —**Alvarez, J. G., B. T. Storey, M. L. Hemling, and R. L. Grob.** High-resolution proton nuclear magnetic resonance characterization of seminolipid from bovine spermatozoa. *J. Lipid Res.* 1990. **31:** 1073–1081.

Supplementary key words ^{1}H-NMR analysis • FAB–mass spectrometry

Although the different phospholipid components of mammalian spermatozoa have been well characterized, the chemical characterization of the different glycolipid classes present in mammalian spermatozoa has not yet been established.

A sulfated glycoglycerolipid that seems to be unique to spermatozoa is found in substantial quantities in mammalian spermatozoa examined to date (2). It has been designated seminolipid and has the general composition: 1-O-alkyl-2-O-acyl-3-O-β-D-(3'-sulfo)-galactopyranosyl-*sn*-glycerol. The isolation from boar sperm was reported by Ishizuka, Suzuki, and Yamakawa (3) and independently from rat sperm by Kornblatt, Schachter, and Murray (4). In a more recent report, a partial characterization of seminolipid from bovine spermatozoa was presented (5). The basic stereochemistry of this class of glycolipid, as obtained from human sperm, was established by Ueno, Ishizuka, and Yamakawa (6). The di-C16 seminolipid containing the 1-O-hexadecyl, 2-O-hexadecanoyl groups was synthesized by Gigg (7), which confirmed the particulars of the seminolipid structure.

The recent introduction of high-resolution one-dimensional (1-D) proton nuclear magnetic resonance (^{1}H-NMR) spectroscopic techniques, the development of 2-dimensional (2-D) NMR methods, including 2-D J-correlated spectroscopy (COSY); and fast atom bombardment mass spectroscopy, have provided a new and convenient approach to glycolipid characterization.

Despite the extensive characterization of the seminolipid from various mammalian species, a comprehensive ^{1}H-NMR characterization of seminolipid from spermatozoa has never been reported. In this report we present the characterization of seminolipid from bovine spermatozoa by high-resolution 1-D and 2-D ^{1}H-NMR spectroscopy along with gas–liquid chromatography–mass spectrometric analysis of the fatty acid methyl esters and partially methylated alditol acetates, mobility on HPTLC, and FAB–mass spectrometry.

MATERIALS AND METHODS

Reagents

The glycolipid standards from bovine brain including sulfatide (SF), galactosyl ceramide (GalCer), GM3, GD1a, and the orcinol ferric chloride spray reagent were purchased from Sigma Chemical Co. (St. Louis, MO). Precoated silica gel HP-K high performance plates

Abbreviations: GLC, gas–liquid chromatography; sulfatide, GalCer I^3-sulfate; GalCer, Gal (β1-1) ceramide; GM3, II^{3}NeuAc-LacCer; GD1a, IV^{3}NeuAc,II^{3}NeuAc-GgOse$_4$Cer (1); FAB–MS, fast atom bombardment–mass spectrometry; HPTLC, high performance thin-layer chromatography; SF, sulfatide.

[1]To whom reprint requests should be addressed at: Department of Obstetrics and Gynecology, 574 Dulles Building, 3400 Spruce Street, Hospital of the University of Pennsylvania, Philadelphia, PA 19104-6080.

(10 × 10 cm, 250 μm thickness) were obtained from Whatman Inc. (Clifton, NJ). Precoated diphasic plates (10 × 10 cm, 250 μm thickness), consisting of NH_2-modified and silica gel hemiplates (see below for more detailed information), and silica gel (60A pore, 35–75 μm particle size) for column chromatography were purchased from Analtech Inc. (Newark, DE). DEAE-Sephadex A-25 was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Solvents were chromatographic grade from EM Science. Inorganic salts were from J. T. Baker (Phillisburg, NJ) and of the highest purity available.

Sample preparation

Thirteen ejaculates from seven different bulls were collected at the Dairy Breeding Center, University Park, PA. The seminal plasma was separated by centrifugation at 800 *g* for 20 min and the resulting pellet was resuspended in 50 ml of isotonic saline. The sperm suspension was centrifuged at 800 *g* for 20 min and the supernatant was aspirated. The final pellet (40 ml of packed sperm with a total concentration of 7×10^{10} cells) was then stored at −20°C until extraction was performed.

Seminolipid extraction and purification

The frozen pellet was thawed in a water bath at 37°C, resuspended in 50 ml of distilled water (W), and subjected to mechanical disruption with an Ultra-Turrax polytron homogenizer for 2 min. Then 134 ml of methanol (M) was added and the homogenate was stirred for 1 h at 4°C, followed by addition of 67 ml of chloroform (C) and stirring for 30 min at 4°C (final C-M-W ratio was 4:8:3, v/v/v). The organic liquid phase was separated by centrifugation at 800 *g* for 5 min, and the resulting pellet was extracted twice as indicated above. The combined supernatants were evaporated to dryness and then partitioned by addition of 50 ml of a mixture of C-M-W 4:2:1 (v/v/v), followed by centrifugation at 800 *g* for 5 min. The aqueous upper phase (AUP) was aspirated and a volume of Folch's theoretical upper phase (8) (C-M-0.74% KCl 1:10:10, v/v/v) equal to the AUP obtained was added to the organic lower phase, vortex-mixed, and centrifuged at 800 *g* for 5 min. This procedure was repeated five times. The final organic lower phase was evaporated to dryness under rotatory evaporation, redissolved in 2 ml of C-M-W (30:60:8, v/v/v) (solvent 1), and then loaded onto a DEAE-Sephadex A-25 column (acetate form, 1 × 10 cm), prepared according to Ledeen, Yu, and Eng (9). The neutral fraction was eluted with 100 ml of solvent 1. The acidic fraction containing the seminolipid was then eluted with 100 ml of C-M-0.8 M sodium acetate 30:60:8, evaporated to dryness, partitioned with 35 ml of a mixture of C-M-W 4:2:1 (v/v/v), and centrifuged at 800 *g* for 5 min. The resulting lower phase containing the seminolipid was aspirated, evaporated to dryness, redissolved in 1 ml of a mixture of dichloromethane-acetate (D-A) 1:1 (v/v), and loaded onto a silica gel column (1 × 10 cm) packed in the same solvent. Then 50 ml fractions of D-A (1:1, v/v) were collected and the eluted lipids were monitored by high performance thin-layer chromatography (HPTLC) (see below). The fractions containing the pure seminolipid were combined (total yield of 11 mg), evaporated to dryness, and stored at −20°C until further analysis.

Mild alkaline methanolysis

One hundred μg of the purified seminolipid was hydrolyzed at 40°C for 1 h in 1 ml of 0.2 N anhydrous methanolic-NaOH. The methanolysate was then neutralized, partitioned with 7 ml of C-M-W 4:2:1 (v/v/v), centrifuged at 800 *g* for 5 min, and the lower phase was aspirated and evaporated to dryness. The hydrolysate (lyso-seminolipid + fatty acid methyl ester) was then redissolved in 1 ml of methanol and the fatty acid methyl esters were extracted with 3 ml of n-hexane. This procedure was repeated three times. The hexane extracts were combined, evaporated to dryness, and redissolved in 20 μl of n-hexane. The fatty acid methyl esters were analyzed by GLC on a WCOT capillary column (Supelco-Wax-10) with initial and final temperatures in the column oven of 150°C and 250°C, respectively, programmed to increase at a rate of 10°C/min. The analysis was performed on a Varian 3700 instrument equipped with a flame ionization detector operated at a temperature of 260°C. Fatty acid methyl ester peaks were identified by comparison of retention times of standard mixtures and quantitated using a Hewlett-Packard 3392A integrator, using methyl heptadecanoate as the internal standard.

Solvolysis of sulfate ester

One hundred μg of the purified seminolipid was dried in vacuo in a desiccator over blue silica gel, dissolved in 1 ml of reagent grade dioxane, and heated at 100°C for 30 min. This treatment effectively removed the sulfate ester moiety of sulfatide as monitored by HPTLC. Dioxane was removed in vacuo and the resulting residue was partitioned (8). After centrifugation the lower phase containing the desulfated seminolipid was aspirated, evaporated to dryness, and stored at −20°C.

High performance thin-layer chromatography

Aliquots (4 μl each) of the purified seminolipid, lyso seminolipid, sulfatide standard, and desulfated seminolipid, dissolved in C-M 1:1 (v/v) at a concentration of 1 mg/ml, were streaked on Whatman silica gel plates as a thin band 5 mm from the lower edge of the plate. Aliquots (4 μl each) of GD1a, GM3, seminolipid, desulfated seminolipid, and GalCer, dissolved in the same solvent (1 mg/ml), were streaked on Analtech diphasic plates.

The Analtech diphasic plates consist of an NH_2-modified silica gel lower hemiplate (5 × 10 cm) with anion exchange properties, an interphase, and a normal phase silica gel neutral upper hemiplate (5 × 10 cm). The denomination of lower and upper derives from the fact that the samples are applied to the NH_2-modified hemiplate and thus, in an ascending development mode, the NH_2-modified half becomes the lower hemiplate (10). The plates were then dried, predeveloped in C–M 1:1 (v/v) to 10 mm from the lower edge of the plate, dried, and placed in 4 × 13 × 14 cm size tanks using C–M–W 65:30:8 (v/v/v) (solvent 2) for the Whatman plates and C–M–0.6 M $CaCl_2$–0.12 M sodium acetate 55:45:4:6 (v/v/v/v) (solvent 3) for the Analtech plates as the mobile phases. After development the plates were thoroughly dried, sprayed with the orcinol ferric chloride reagent, and placed in a Frigidaire microwave oven for 5 min at setting 9 (11). The stained chromatograms were then scanned with a Shimadzu CS-9000 spectrodensitometer at 500 nm in the transmission mode.

Carbohydrate composition and anomeric linkages

Two hundred μg of the purified seminolipid and sulfatide standard were permethylated according to the procedure of Hakomori (12). Then the permethylated glycolipids were hydrolyzed separately in 80% acetic acid containing 0.7 N HCl at 70°C for 18 h, reduced with $NaBH_4$, and acetylated with acetic anhydride (13). The resulting partially O-methylated alditol acetates were resolved by GLC on a 3% ECNSS-M column maintained at 140°C, and identified by mass spectrometry on a GLC–MS Shimadzu LKB-9000 instrument, operated under the following conditions: electron energy 70 eV; accelerating voltage, 3.5 kV; ionizing current, 60 μA; and temperature of molecular separator and ion source, 230° and 270°C, respectively.

Proton NMR spectroscopy

The purified seminolipid was dried in vacuo in a desiccator over blue silica gel for at least 24 h prior to analysis, and the resulting residue was dissolved in 0.5 ml of freshly prepared Me_2-SO-d_6 to yield 4 mM sample solutions. The samples were then transferred to 200 × 5 mm NMR tubes (Wilmad Glass Co., Buena, NJ). The spectra obtained in this solvent are referred to as nondeuterated; the spectra obtained in Me_2-SO-d_6-D_2O (98:2 v/v) as deuterated. Proton spectra were obtained on a Bruker AM-500 (500 MHz) spectrometer equipped with an Aspect 3000 computer, operating in the Fourier-transform mode with quadrature detection. Probe temperature was 298° × 2°K. Integrated, one-dimensional spectra were obtained over a spectral range of 5 KHz (0–10 ppm) and 16,000 sampling points. Chemical shifts are expressed in ppm and referenced to internal dimethylsulfoxide (δ 2.486). Proton NMR interpretations are based on those reported by Dabrowski, Egge, and Hanfland (14), Iida et al. (15), Gasa et al. (16), and Harris and Turvey (17).

The two-dimensional (2-D) correlated spectroscopy (COSY) spectra (16, 18) were obtained using 2K × 2K matrix of data. The sine bell function was applied during data processing and the matrix was symmetrized following Fourier transformation to remove most of the artefacts.

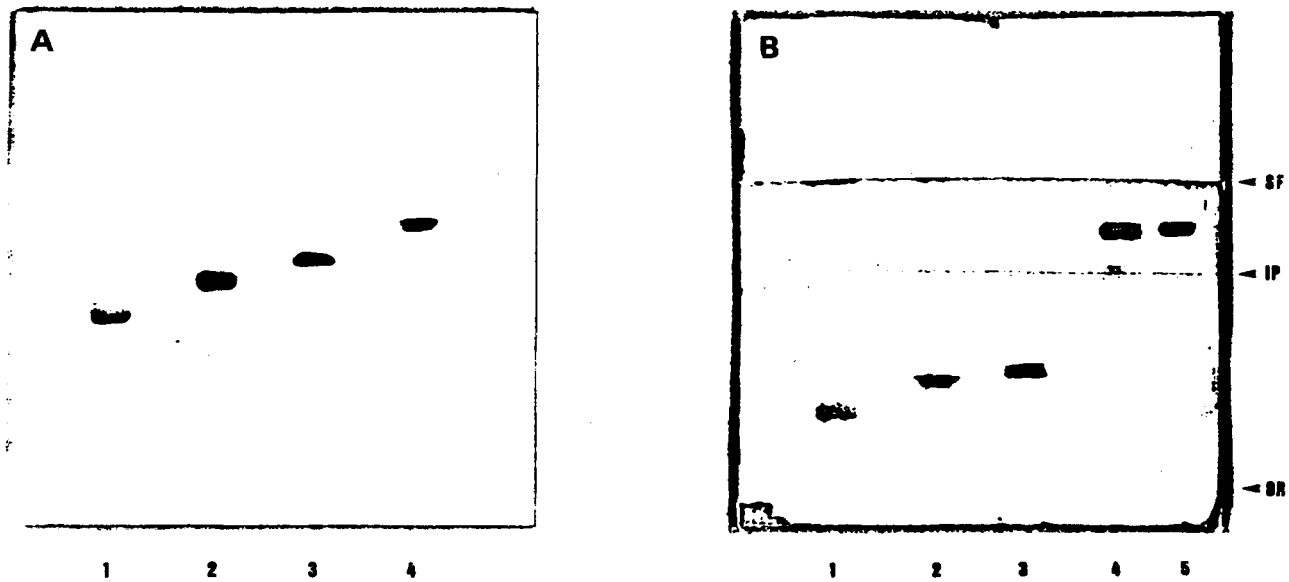


Fig. 1. Orcinol-sprayed HPTLC chromatogram of seminolipid from bovine spermatozoa. A) Lyso-seminolipid (1), sulfatide (2), seminolipid (3), and desulfo-seminolipid (4) (4 μg of each) were separated on a Whatman HP-K plate using solvent 2 as the mobile phase (see Materials and Methods). B) GDIa (1), GM3 (2), seminolipid (3), desulfo-seminolipid (4), and GalCer (5) (4 μg of each), were separated on an Analtech diphasic plate using solvent 3 as the mobile phase. SF, solvent front; OR, origin; IP, interphase.

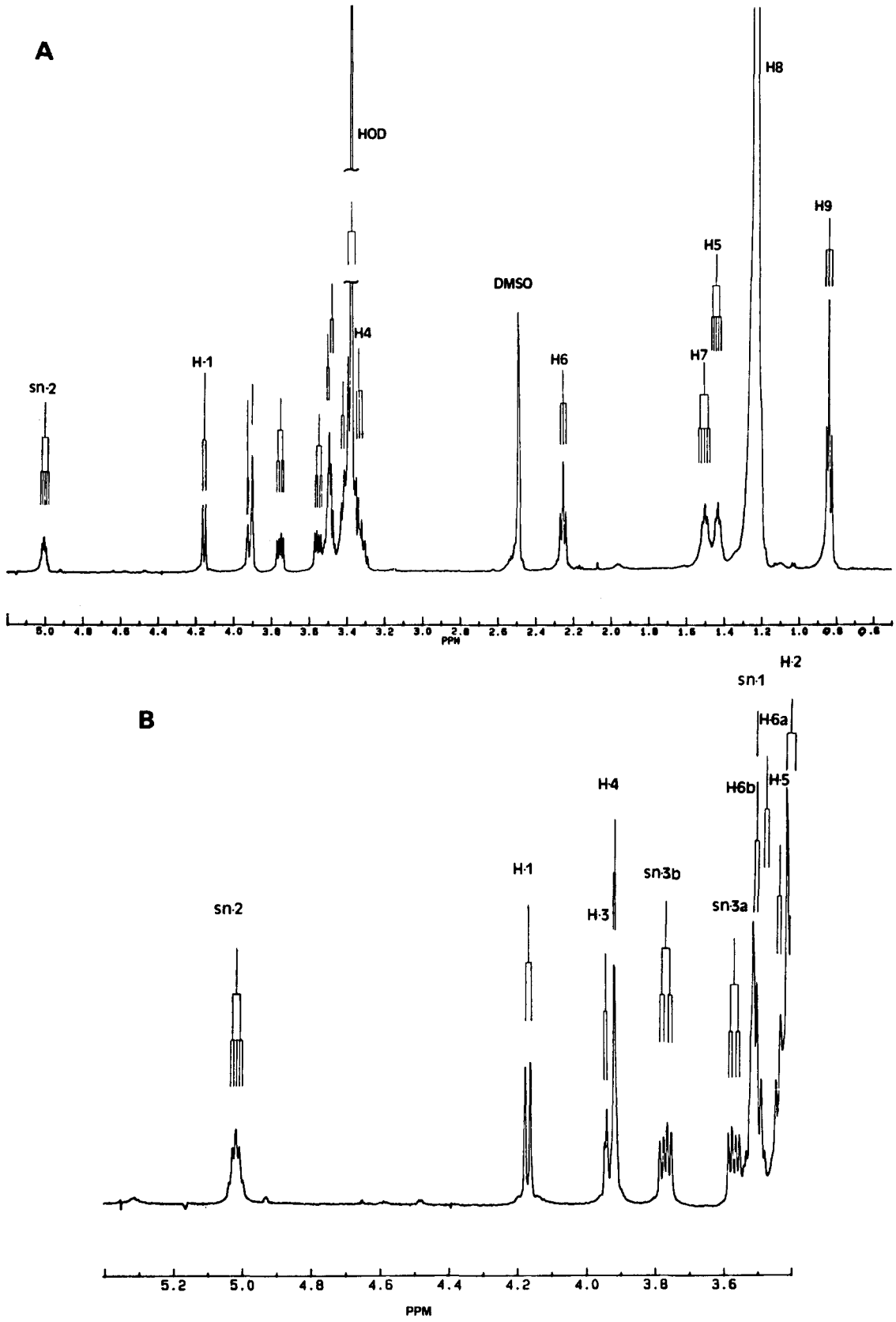


Fig. 2. Integrated one-dimensional 500 MHz proton NMR spectrum of deuterated seminolipid from bovine spermatozoa. A) Total spectrum between 0.5 and 5.2 ppm. B) Expanded subspectrum corresponding to the down-field region between 3.4 and 5.2 ppm. The sugar ring protons are labeled with dashed numbers.

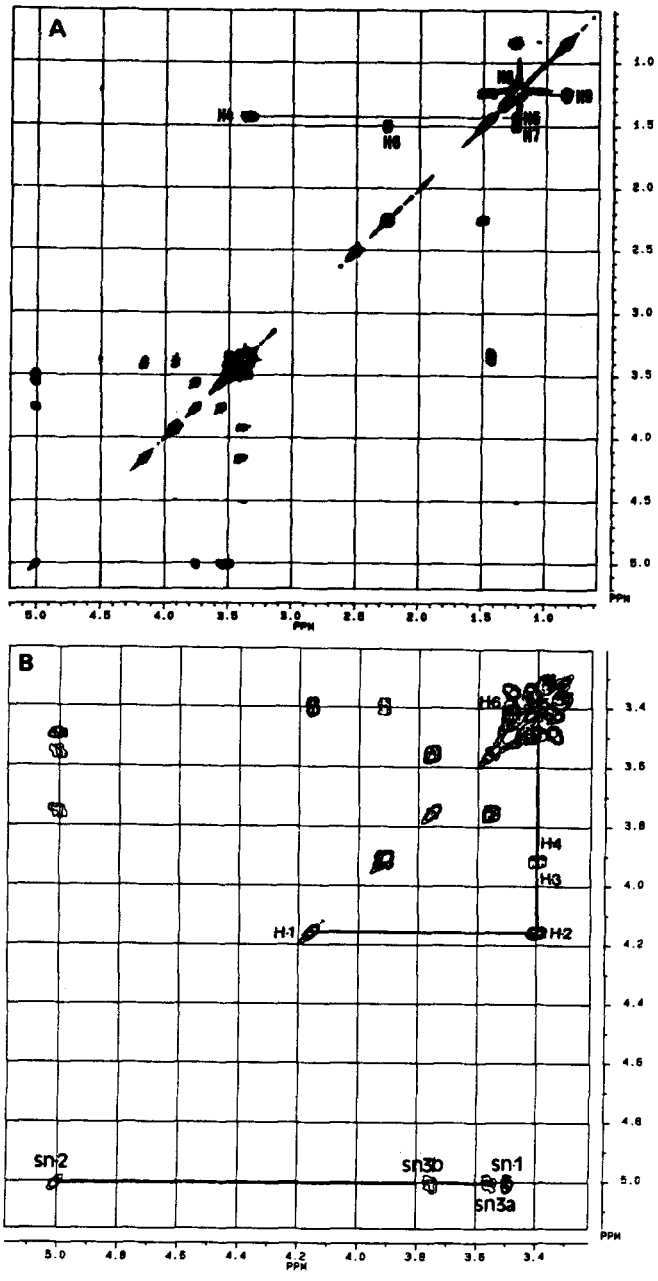


Fig. 3. Contour plot of a two-dimensional COSY spectrum of seminolipid from bovine spermatozoa. A) Contour plot of the same region shown in Fig. 1a. B) Contour plot of the subspectrum between 3.3 and 5.2 ppm.

Negative ion fast atom bombardment mass spectrometry

The FAB mass spectrum of seminolipid was obtained on a VG ZAB-HF spectrometer with a high field magnet and VG 11/250 data system. An Ion Tech saddle field atom source was operated at 9 kev and a discharge current of 1 mA. Samples were dissolved (10 μg/μl) in methylene chloride–methanol (ca. 1:1) and 2 μl was added to ca. 2 μl of triethylenetetramine as matrix on a stainless steel FAB target ribbon. The samples were introduced into the spectrometer through the FAB/FD probe inlet, in line with the flight axis (19).

RESULTS AND DISCUSSION

High performance thin-layer chromatography

The orcinol-sprayed chromatogram of purified seminolipid from bovine spermatozoa, mild alkali-treated seminolipid, desulfated seminolipid, and sulfatide standard is shown in **Fig. 1A.** The migration of all four components on normal phase silica gel is consistent with seminolipid isolated from sperm of other mammalian species. Glycolipid separation on diphasic silica gel has recently become available (10) (see Materials and Methods). This HPTLC modality provides additional information concerning not only the acidic character of the glycolipid but also its degree of purity. As shown in Fig. 1B, the seminolipid migrated as a single component slightly ahead of GM3 on the NH_2-hemiplate. This indicates that it is also an acidic but less polar glycolipid. Solvolysis of the sulfate ester resulted in loss of its acidic character migrating now with GalCer in the neutral silica upper hemiplate (Fig. 1B).

Sugar analysis

GLC-MS analysis of the partially methylated alditol acetates of the purified seminolipid and sulfatide standard indicated the presence of a 1,3-di-O-acetyl-2,4,6-O-tri-O-methylgalactitol, consistent with the ^{1}H-NMR characterization of seminolipid and the structure of seminolipid (data not shown).

GLC analysis of fatty acid methyl esters

The GLC chromatogram of the n-hexane extract of mild alkali-treated seminolipid (see Materials and Methods) revealed the presence of 96% palmitic acid methyl ester and 3.2% stearic acid methyl ester (data not shown).

Proton 1-D and 2-D NMR spectroscopy

Fig. 2A shows the 1-D spectrum of the purified seminolipid from bovine spermatozoa. Outside of the region between 3 and 4 ppm in which most of the sugar ring resonances reside, several classes of resonances can be identified. In the up-field alkyl region, a 6-proton alkyl methyl triplet can be seen at 0.83 ppm, which corresponds to the terminal methyl groups of the alkyl ether and fatty acid moieties (H9) (Fig. 2A). A 50-proton multiplet is found at 1.24 ppm corresponding to the alkyl methylene chain (H8). Two 2-proton quintets are found at 1.4 ppm (H5) and 1.5 ppm (H7) which are characteristic of the β protons to the ether oxygen and ester carbonyl, respectively. A 2-proton triplet at 2.25 ppm (H6) corresponds to

TABLE 1. Chemical shifts for methylene and methine protons of seminolipid from bovine spermatozoa

	Proton Chemical Shifts (ppm)								
Residue	H-1	H-2	H-3	H-4	H-5	H-6	H-7	H-8	H-9
I	4.164	3.402[a]	3.931	3.904	3.424	3.485(A) 3.502(B)			
II	3.552(a) 3.754(b)	5.012	3.500	3.351	1.400	2.253	1.502	1.241	0.833

[a]Exact chemical shift obscured by HOD resonance.

the α protons to the carbonyl ester and a 2-proton triplet (H4) at 3.35 ppm corresponds to the α protons to the ether oxygen (Fig. 2A), consistent with the assignments reported by Ishizuka et al. (3). The absence of vinyl proton resonances between 5.2 and 5.6 ppm is consistent with the GLC analysis of the fatty acid moieties (15).

In the down-field region, proton resonances corresponding to the glycerol moiety protons are found (Fig. 2B). Two 1-proton doublet of doublets resonances at 3.75 ppm (sn-3b) and 3.55 ppm (sn-3a) could be ascribed the *sn*-3 methylene protons. A characteristic 1-proton quintet at 5.01 ppm (sn-2) is seen corresponding to the glycerol methine proton (Fig. 2B), in good agreement with the assignment reported by Ishizuka et al. (3). A 2-proton resonance at 3.50 ppm corresponds to the glycerol *sn*-1 methylene protons (Fig. 2B).

In the down-field anomeric region a well-resolved 1-proton doublet is detected at 4.16 ppm (H-1) (Fig. 2B). Consideration of the chemical shift and coupling constant (Tables 1 and 2) of this anomeric proton indicates that it is consistent with a deshielded β-D-galactopyranosyl residue (3, 14). In order to assign the sugar ring proton resonances in the multiproton envelope (3–4 ppm), and the connectivities of the methylene and methine protons corresponding to the lipid moiety, 2-D COSY spectra acquisition was required. Analysis of the spectra shown in **Fig. 3A and 3B** using integration data and process of elimination as guides allowed the identification of both sugar and lipid proton resonances (**Table 1**). The ^4C conformation of the sugar unit could be established from the coupling constants (**Table 2**), as explained previously by Dabrowski et al. (14) for the unsubstituted galactosyl unit: small values for $J_{3,4}$ and $J_{4,5}$ (3.1 and 1.2 Hz) correspond to gauche interactions of the pairs H-3/H-4 and H-4/H-5, respectively. From the near identical $J_{5,6A}$ and $J_{5,6B}$ coupling constants of 5.4 and 5.2 Hz, respectively (Table 2) it can be inferred that the hydroxymethylene group is freely rotating around the C^5–C^6 bond (14).

The above analysis applies to the samples in which all exchangeable protons were substituted by deuterium. Although the spectra of nondeuterated seminolipid is more complex it provides, however, additional structural information. A particularly useful application, in conjunction with the positional analysis of the sugar ring protons, is the determination of the position of substitution at the sugar unit. As shown in **Fig. 4** the presence of three sugar hydroxyl signals can be identified in the down-field region between 4.4 and 5.0 ppm. The 1-proton triplet found at 4.54 ppm is characteristic of the hydroxymethylene hydroxy group (OH^6) (14). The 1-proton doublet seen at 4.46 ppm could be assigned to an axial hydroxyl at C-4 (OH^4) (14) considering that H-4 is the only equatorial proton present in the sugar unit. The 1-proton doublet hydroxyl resonance at 4.93 ppm cannot be unequivocally assigned to either OH^2 or OH^3 on the sole basis of its chemical shift. The difference in the chemical shift between the protons of the sulfated galactosyl unit of seminolipid and the unsubstituted galactosyl unit (14) was largest with H-3 ring protons (0.654 ppm) (Table 1) mainly due to the deshielding effect by the electronegative sulfate group to the geminal proton, supporting that the location of the sulfate group is at the C-3 position (15–17).

TABLE 2. Apparent coupling constants for seminolipid proton resonances

	Proton-Proton Coupling Constants (± 0.05 Hz)								
Residue	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{5,8}$	$J_{6,7}$	$J_{7,8}$	$J_{8,9}$
I	7.72	8.8	3.10	1.20	5.4(A) 5.2(B)				
II	6.50	6.10				6.67	6.90	6.70	7.18

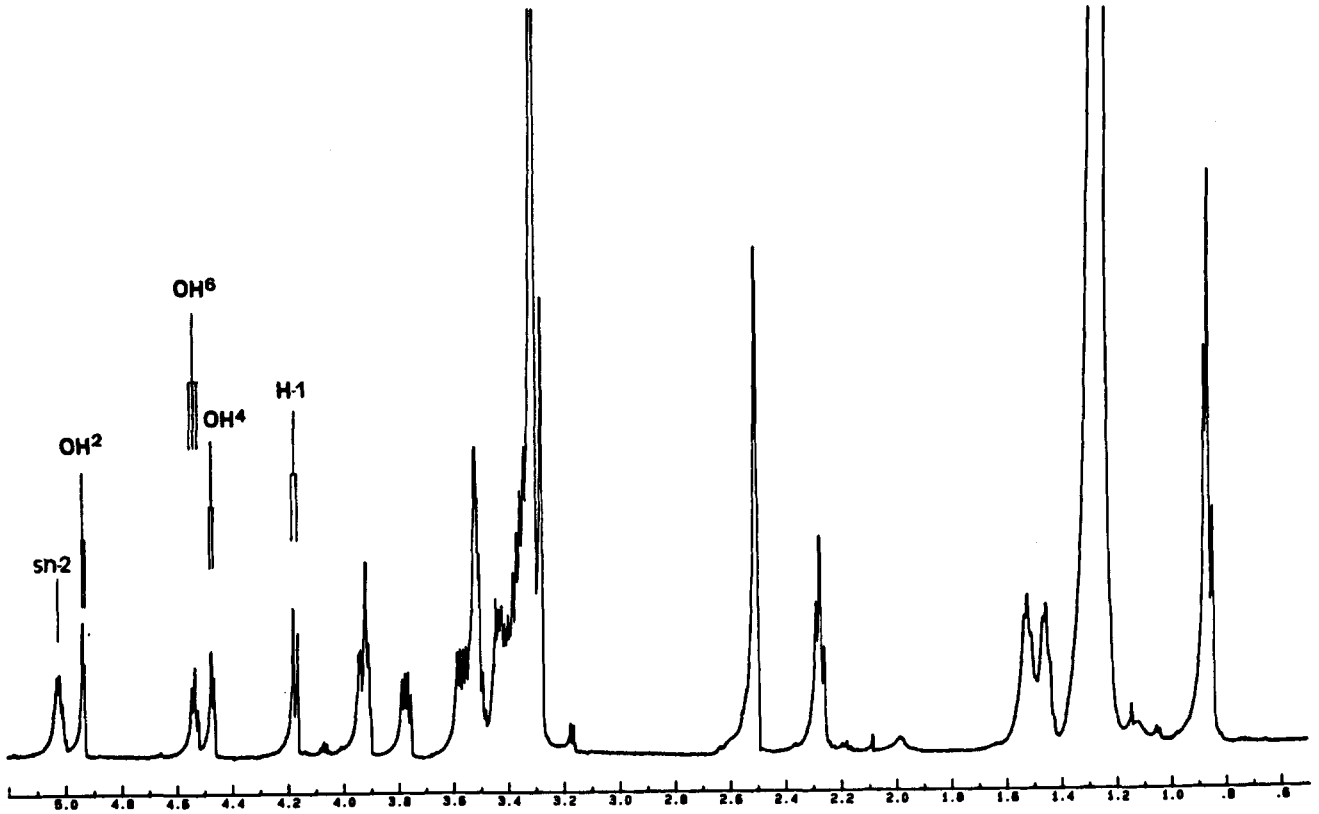


Fig. 4. One-dimensional 500 MHz proton NMR spectrum of non-deuterated seminolipid from bovine spermatozoa. Only the resonances corresponding to the glycerol methine proton (*sn*-2) and the sugar ring hydroxyls and anomeric proton (H-1) are indicated.

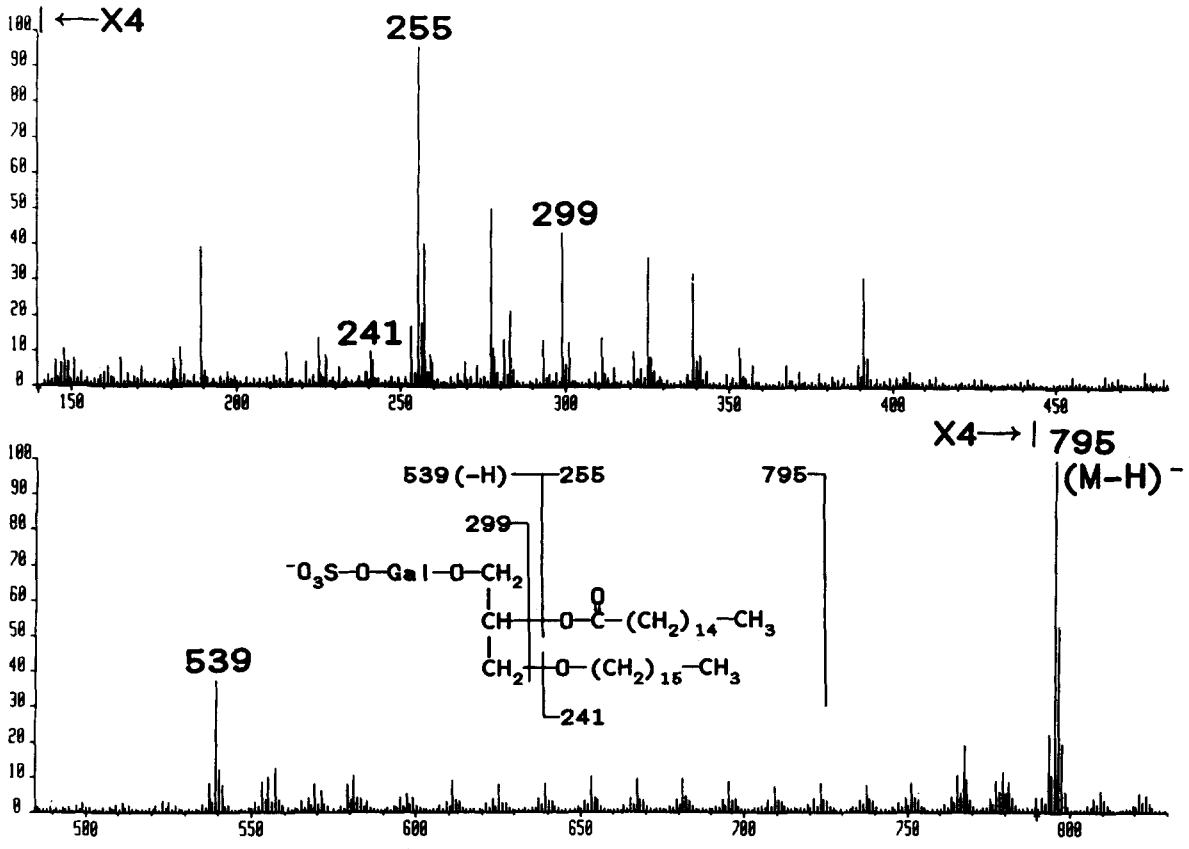
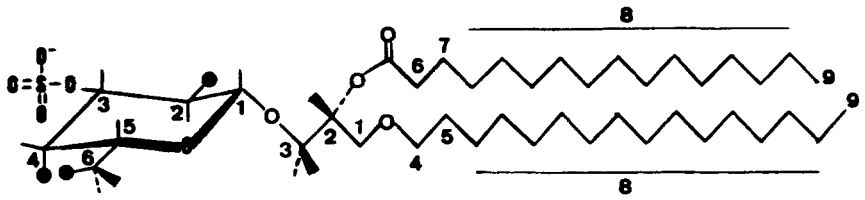


Fig. 5. Negative ion FAB mass spectrum of seminolipid from bovine spermatozoa.



I II

Fig. 6. Structure of seminolipid from bovine spermatozoa. The sugar and glycerolipid units are labeled as I and II, respectively. Filled circles (●) correspond to the hydroxy groups of the sugar unit.

Fast atom bombardment mass spectrometry

The FAB mass spectrum of the seminolipid is shown in **Fig. 5.** The ion at *m/z* 795 is consistent with the pseudomolecular ion of seminolipid containing palmitic acid as the fatty acid (*m/z* 255) and chimyl alcohol as the alkyl ether (*m/z* 241). The fragment ion at *m/z* 299 was assigned to the loss of cetyl alcohol and palmitic acid from the pseudomolecular ion. Although the assignments for the ions at *m/z* 795 and 255 are the same as those reported by Kushi, Handa, and Ishizuka (20) for the negative secondary ion mass spectrum of seminolipid from boar spermatozoa, the ion at *m/z* 241 could also be ascribed to $(HSO_3\text{-}Gal\text{-}2H)^-$ as indicated by the same authors. These results are also consistent with the structure reported by Ishizuka et al. (3) corresponding to the seminolipid from boar spermatozoa.

CONCLUSIONS

The high-resolution 1-D and 2-D proton NMR analysis of seminolipid from bovine spermatozoa along with its chromatographic mobility on HPTLC, GLC-MS analysis of the partially methylated alditol acetates, GLC analysis of the fatty acid methyl esters obtained after mild alkaline methanolysis, and FAB-MS, resulted in its characterization as 1-O-hexadecyl-2-O-hexadecanoyl-3-O-β-D-(3'-sulfo)-galactopyranosyl-*sn*-glycerol (**Fig. 6**). This characterization is, however, ascribed under the assumption that the chirality of glycerol in seminolipid from bovine spermatozoa corresponds to that of the D-isomer, as reported by Ueno et al. (6) in seminolipid from human spermatozoa. Since chirality cannot be unequivocally established by proton NMR, the exact stereochemistry remains to be elucidated.

The authors wish to thank Dr. Robert K. Yu of the Department of Biochemistry, Medical College of Virginia, for critical review of this manuscript; and Dr. Gary J. Killian of the Diary Breeding Research Center, Penn State University for kindly providing the frozen specimens of bovine spermatozoa utilized in this study. We also thank Debbie Coffin and Barbara Brewer for excellent secretarial assistance in the preparation of this manuscript. This work was supported by NIH grants HD-15842 and HL-19737.

Manuscript received 31 October 1989 and in revised form 30 January 1990.

REFERENCES

1. IUPAC-IUB Commission of Biochemical Nomenclature. 1978. The Nomenclature of Lipids (Recommendations 1976). *J. Lipid Res.* **19:** 114-128.
2. Mann, T., and C. Lutwak-Mann. 1981. Male Reproductive Function and Semen. Springer Verlag, Berlin/Heidelberg/New York. 203-212.
3. Ishizuka, I., M. Suzuki, and T. Yamakawa. 1973. Isolation and characterization of a novel sulfoglycolipid, seminolipid, from boar testis and spermatozoa. *J. Biochem.* **73:** 77-87.
4. Kornblatt, M., H. Schachter, and R. K. Murray. 1972. Partial characterization of a novel glycerogalactolipid from rat testis. *Biochem. Biophys. Res. Commun.* **48:** 1489-1494.
5. Selivonchick, D. P., P. C. Schmid, V. Natarajan, and H. H. O. Schmid. 1980. Structure and metabolism of phospholipids in bovine spermatozoa. *Biochim. Biophys. Acta.* **618:** 242-254.
6. Ueno, K., I. Ishizuka, and T. Yamakawa. 1977. Glycolipid composition of human testis at different ages and the stereochemical configuration of seminolipid. *Biochim. Biophys. Acta.* **487:** 61-73.
7. Gigg, R. 1978. The alkyl ether as a protecting group in carbohydrate chemistry. Part 10. Synthesis of 3-O-(β-D-galactopyranosyl-3-sulphate-O-2-O-hexadecanoyl-1-O-hexadecyl-L-glycerol, 'seminolipid.' *J.C.S. Perkin I.* 712-718.
8. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497-509.
9. Ledeen, R. W., R. K. Yu, and L. F. Eng. 1973. Gangliosides of human myelin. Sialosylgalactosylceramide (G7) as a major component. *J. Neurochem.* **21:** 829-839.
10. Alvarez, J. G., and J. C. Touchstone. 1988. Separation of acidic and neutral glycolipids by diphasic thin-layer chromatography. *J. Chromatogr.* **436:** 515-516.

11. Alvarez, J. G., B. T. Storey, and J. C. Touchstone. 1989. In situ determination of malondialdehyde on thin-layer plates by fluorescence spectrodensitometry. *In* Planar Chromatography in the Life Sciences. J. C. Touchstone, editor. Wiley, New York. 45–58.
12. Hakomori, S. I. 1964. A rapid permethylation of glycolipid and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. *J. Biochem. (Tokyo).* **55:** 205–208.
13. Nakamura, K., Y. Hashimoto, M. Suzuki, J. Suzuki, and T. Yamakawa. 1984. Characterization of GM1b in mouse spleen. *J. Biochem.* **96:** 949–957.
14. Dabrowski, J., H. Egge, and P. Hanfland. 1980. High resolution nuclear magnetic resonance spectroscopy of glycosphingolipids. I. *Chem. Phys. Lipids.* **26:** 187–196.
15. Iida, N., T. Toida, Y. Kushi, S. Handa, P. Fredman, L. Svennerholm, and I. Ishizuka. 1989. A sulfated glucosylceramide from rat kidney. *J. Biol. Chem.* **264:** 5974–5980.
16. Gasa, S., M. Nakamura, A. Makita, M. Ikura, and K. Hikichi. 1985. Determination of the position of acetic and sulfuric esters in glycosphingolipids by two-dimensional proton-nuclear magnetic resonance spectroscopy. *Carbohydr. Res.* **137:** 244–252.
17. Harris, M. J., and J. R. Turvey. 1970. The conformations of glycoside sulphates in solution determined by NMR spectroscopy. *Carbohydr. Res.* **15:** 57–63.
18. Geyer, R., H. Geyer, S. Stirm, G. Hunsmann, J. Schneider, U. Dabrowski, and J. Dabrowski. 1984. Major oligosaccharides in the glycoprotein of Friend murine leukemia virus. Structure elucidation by one- and two-dimensional proton nuclear magnetic resonance and methylation analysis. *Biochemistry.* **23:** 5628–5637.
19. Hemling, M. E., R. K. Yu, D. Sedgwick, and K. L. Rinehart, Jr. 1984. Fast atom bombardment mass spectrometry of glycosphingolipids. Glycosphingolipids containing neutral sugars. *Biochemistry.* **23:** 5706–5713.
20. Kushi, Y., S. Handa, and I. Ishizuka. 1985. Secondary ion mass spectrometry for sulfoglycolipids: application of negative ion detection. *J. Biochem.* **97:** 419–428.