Chemical Synthesis of α-L-Fucopyranosylceramide and Its Analogues and Preparation of Antibodies Directed to This Glycolipid†

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ABSTRACT: A novel glycolipid, α-L-fucopyranosylceramide, was previously isolated and characterized from metastatic human adenocarcinoma [Watanabe, K., Matsubara, T., & Hakomori, S. (1976) *J. Biol. Chem. 251*, 2385-2387]. For further investigation of the pathobiological significance of this glycolipid with its specific antibodies, the antigen glycolipid α-L-fucopyranosylceramide was chemically synthesized by Königs-Knorr-type condensation of 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl bromide and a ceramide, followed by removal of benzyl groups by catalytic hydrogenation. α-L-Fucopyranosyl-*N*-palmitoylethanolamine and 2,3,4-tri-*O*-benzyl-α-L-fucopyranosylsphingosine were also synthesized. The antibodies prepared against a synthetic α-L-fucopyranosylceramide included in liposome reacted very well with the synthetic as well as the natural α-L-fucopyranosylceramide isolated from human adenocarcinoma, but the kinetics of the complement-dependent liposome lysis and the complement fixation pattern with the synthetic fucosylceramide were significantly different from those with the natural fucosylceramide. The natural fucosylceramide showed a much weaker immunogenicity than the synthetic fucosylceramide. The remarkable difference in liposome lysis, complement fixation, and immunogenicity between the synthetic and the natural fucosylceramide must be due to the difference in ceramide structures. The results indicate that the strength of antigenicity and immunogenicity of glycolipids may greatly depend on ceramide structure, whereas the specificity is solely determined by the sugar moiety. The anti-α-L-fucosylceramide antibodies display cross-reactivity with galactosylceramide, ceramide, and liposome alone that was eliminated by absorption with galactosylceramide liposomes.

A novel glycolipid, α-L-fucopyranosylceramide, was previously isolated from the metastatic lesion of the colonic human adenocarcinoma, which was absent in normal tissue (Watanabe et al., 1976). For a further study on distribution of fucosylceramide by an immunohistological technique and for a possible antibody-mediated targeting of chemotherapeutic drugs to human cancer as was demonstrated in mice tumor models (Urdal & Hakomori, 1980), efforts have been made to produce the specific antibodies to α-L-fucosylceramide. However, since the source of the material is very limited and the natural product isolated from tumor tissue was always contaminated with a small quantity of galactosylceramide, we have synthesized α-L-fucopyranosylceramide and its analogues. The synthetic α-L-fucopyranosylceramide was used as the immunogen to obtain the antibodies directed to this synthetic compound. The reactivities of the glycolipids with liposome lysis and complement fixation have been compared between the synthetic and the naturally occurring fucosylceramides and their analogues.

Materials and Methods

Preparation of Materials and Synthesis of α-L-Fucopyranosylceramide and Its Analogues. Ceramide. Bovine brain cerebroside (1 g) was oxidized with periodic acid, followed by Smith degradation according to the procedure of Carter et al. (1961), giving 380 mg of ceramide, which was identified with an authentic sample on TLC,[1] R_f 0.71 (in chloroform-methanol, 3:2 v/v).

Sphingosine was prepared from cerebroside by acid hydrolysis in aqueous methanol following a procedure of Sweeley & Moscatelli (1959): R_f value on TLC 0.14 (in chloroform-methanol-water, 65:25:4 v/v).

N-(Dichloroacetyl)sphingosine. Sphingosine (200 mg) was reacted with methyl dichloroacetate according to the procedure described by Shapiro & Sheradsky (1963), giving 192 mg of *N*-(dichloroacetyl)sphingosine: mp 89-91 °C; R_f on TLC 0.79 (in chloroform-methanol-water, 65:25:4 v/v); MS *m/e* 239 [$CH_3(CH_2)_{12}CH{=}CHCH(OH)$], 170 ($Cl_2CHCONHCH{-}CH_2OH$).

2,3,4-Tri-O-benzyl-1-O-(p-nitrobenzoyl)-β-L-fucopyranose. This was prepared through four steps from L-fucose according to the procedure described by Dejter-Juszynski & Flowers (1971) except benzyl bromide instead of benzyl chloride was used for blocking hydroxyl groups of L-fucose: overall yield from L-fucose 15.3% of the theoretical; mp 123-124 °C (lit. 120-122 °C) after crystallization from methanol; IR spectra λ_{max} 1736 (C=O), 1087 (C-O).

2,3,4-Tri-O-benzyl-α-L-fucopyranosyl Bromide. This compound was prepared from 2,3,4-tri-*O*-benzyl-1-*O*-(*p*-nitrobenzoyl)-β-L-fucopyranose and hydrogen bromide immediately prior to use according to the procedure of Dejter-Juszynski & Flowers (1971) and used immediately without isolation.

2,3,4-Tri-O-benzyl-α-L-fucopyranosylceramide (I, Figure 1). A solution of ceramide (67 mg, 0.1 mmol), tetraethylammonium bromide (21 mg, 0.1 mmol), and diisopropylethylamine (20 μL, 0.1 mmol) in 5 mL of dichloromethane and 1.2 mL of dimethylformamide was stirred for 30 min at room temperature in the presence of 1 g of molecular sieve (4 Å). Then a solution of freshly prepared 2,4,6-tri-*O*-benzylfucopyranosyl bromide (0.12 mmol) in 7 mL of dichloromethane was added to the above solution. The reaction

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[1] Abbreviations: TLC, thin-layer chromatography; MS, mass spectrometry or spectra; NMR, nuclear magnetic resonance spectroscopy or spectra; Me_2SO, dimethyl sulfoxide.

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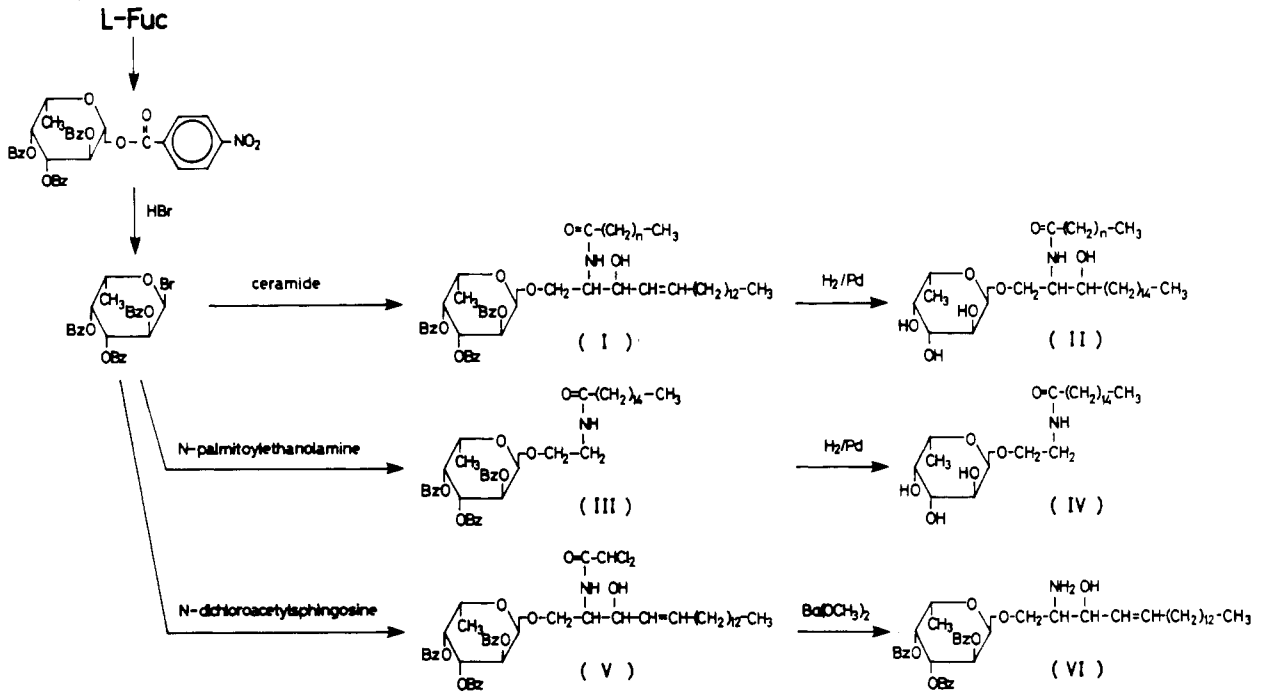


FIGURE 1: Various steps for synthesis of α-L-fucopyranosylceramide and its analogues.

was monitored by TLC (benzene–ethyl acetate, 4:1 v/v). After 9 days, the reaction mixture was diluted with dichloromethane (30 mL) and filtered through filter-aid (Celite) to remove insoluble materials. The filtrate was washed successively with water, with 0.5 N HCl, and twice with saturated sodium chloride. The filtrate was dried with $MgSO_4$ and then concentrated in vacuo. The resulting light brown syrup (116 mg) was chromatographed on a column of Bio-Sil A (1 × 100 cm) with a benzene–ethyl acetate system (4:1 to 1:4 v/v) for gradient elution.

The third fraction was characterized by MS and NMR spectrometry as 2,4,6-tri-*O*-benzyl-α-L-fucopyranosylceramide (compound I): 40 mg, 32% yield based on the given quantity of 2,3,4-tri-*O*-benzyl-1-*O*-(*p*-nitrobenzoyl)-α-L-fucopyranose. The TLC R_f value in benzene–ethyl acetate–acetone (5:5:2) was 0.68. The TLC R_f values in the same solvent of various fractions obtained from the reaction mixture and their yields were 2,3,4-tri-*O*-benzyl-1-*O*-(*p*-nitrophenyl)-α-L-fucopyranose (the starting sugar derivatives) 0.76 (14 mg, 20% recovery), 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl bromide 0.63 (20 μg, 42% recovery), and ceramide 0.34 (6 mg, 9% recovery). These products were characterized by MS and NMR; see Results.

α-L-Fucopyranosylceramide (II, Figure 1). A solution of compound I (34 mg) in 15 mL of ethanol was hydrogenated with 5% Pd/C (40 mg) for 20 h. The reaction mixture was filtered and evaporated to dryness, giving white crystals of α-L-fucopyranosylceramide in a quantitative yield (23 mg): TLC R_f 0.53 (in chloroform–methanol–water, 65:25:4 v/v). R_f values of galactosylceramide from bovine brain are 0.50 and 0.45, and the R_f value of α-L-fucopyranosylceramide from human adenocarcinoma is 0.52, under the same conditions. Normal and α-hydroxylated fatty acids of synthetic fucosylceramide were analyzed by gas chromatography–mass spectrometry (Laine et al., 1974). The product was characterized by mass spectrometry as the acetyl derivative (see Results).

Reactivity of α-L-Fucopyranosylceramide and β-Galactopyranosylceramide with Trityl Chloride. In order to test whether the L-fucosyl residue was exclusively linked to the primary hydroxyl group of ceramide by the Königs–Knorr-type reaction, we subjected 100 μg of the synthetic α-L-fucosylceramide extensively dried on phosphorus pentoxide to tritylation with 2 mg of trityl chloride in 200 μL of dried pyridine in a sealed tube for 48 h at room temperature, followed by heating at 100 °C for 3 h (Barker, 1963). β-Galactosylcerebroside was tritylated under the same conditions. The reaction mixtures were evaporated with toluene to dryness, dissolved in chloroform and repeatedly partitioned with water, and examined by thin-layer chromatography on zinc silicate plates in benzene–acetone (2:1 v/v). Trityl glycolipids were detected under ultraviolet light as well as by the orcinol reaction. Trityl cerebroside (trityl ether of the C_6 primary OH of the galactose residue) gave a band with an R_f of 0.1, while unreacted trityl chloride and tritylcarbinol gave fast-migrating bands (R_f 0.9–1). Fucosylceramide did not give any slow-migrating bands similar to trityl cerebroside.

N-Palmitoylethanolamine. The condensation reaction of palmitic acid (5.36 g, 20 mmol) with ethanolamine (1.22 g, 20 mmol) for 1 h at 120 °C under reduced pressure with an aspirator gave white crystals: 3.84 g (65% yield), crystallized from ethanol; mp 93–95.5 °C (lit. mp 94.4 °C; Reid, 1937); IR absorption peaks at 3210 (OH), 3150 (NH), 1639 (C=O, amide), and 1052 (δ-OH).

2,3,4-Tri-O-benzyl-L-fucopyranosyl-N-palmitoylethanolamine (III, Figure 1). The condensation of *N*-palmitoylethanolamine (90 mg, 0.30 mmol) with freshly prepared 2,3,4-tri-*O*-benzylfucopyranosyl bromide (0.35 mmol) was carried out under the same conditions as the preparation of compound I, as described above. After 10 days, the reaction mixture was purified by chromatography on silica gel (Bio-Sil A, 1 × 30 cm) with benzene–ethyl acetate (1:1 v/v) as the elution solvent. The third fraction was characterized by mass spectrometry as 2,3,4-tri-*O*-benzylfucopyranosyl-*N*-palmitoylethanolamine (52 mg, 48% yield) in addition to the recovered compounds *N*-palmitoylethanolamine (31 mg, 34% yield) and 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl bromide (180 mg, 45% yield): TLC R_f for compound III 0.74 and for 2,3,4-tri-*O*-benzyl-α-fucopyranosyl bromide 0.55 (in benzene–ethyl acetate, 1:1 v/v); for the MS of this product, see Results.

α-L-Fucopyranosyl-N-palmitoylethanolamine (IV, Figure 1). Hydrogenation of compound III (54 mg) with Pd/C (40

mg) gave α-L-fucopyranosyl-*N*-palmitoylethanolamine as white crystals, 28 mg (83% yield). For the MS and NMR of this product, see Results.

2,3,4-Tri-O-benzyl-L-fucopyranosyl-N-(dichloroacetyl)-sphingosine (V, Figure 1). Compound V was prepared by the same procedure as the preparation of compound I. The crude product was purified by silica gel chromatography (1.5 × 60 cm) with benzene–ethyl acetate (3:2 v/v) as the elution solvent. From 0.12 mmol (50 mg) of *N*-(dichloroacetyl)sphingosine (a) and an excess amount of 2,3,4-tri-*O*-benzylfucopyranosyl bromide, which was converted from 0.15 mmol of 2,3,4-tri-*O*-benzyl-1-*O*-(*p*-nitrobenzoyl)-α-L-fucopyranose (b), 40 mg of 2,3,4-tri-*O*-benzyl-L-fucopyranosyl-*N*-(dichloroacetyl)-sphingosine was obtained; 25 mg of a and 13 mg of b were recovered as unreacted: TLC R_f 0.71 in benzene–ethyl acetate–acetone (5:5:2 v/v); for MS data, see Results.

2,3,4-Tri-O-benzyl-L-fucopyranosylsphingosine (VI, Figure 1). A solution of compound V (20 mg) in 6.5 mL of 90% aqueous ethanol containing 1 mL of 0.25 M barium methoxide was warmed at 70 °C for 3 h. The reaction mixture, after evaporating the ethanol under a nitrogen stream, was extracted with 40 mL of chloroform and then successively washed with 5 mL of 0.1 M acetic acid and twice with 10 mL of saturated sodium chloride solution, dried with sodium sulfate, and concentrated in vacuo. The residue was coevaporated with toluene, giving a pale yellow syrup (21 mg, almost quantitative yield), which is ninhydrin positive: TLC R_f 0.16 for VI and 0.68 for V (in chloroform–methanol, 9:1 v/v).

Mass spectra (MS) were determined with a Finnigan Model 3000 mass spectrometer with a data system.

Nuclear magnetic resonance spectra (NMR) were determined with a JEOL FX-100 instrument in deuterated chloroform ($CDCl_3$). Since the quantity of the sample was limited, the scan was repeated 50–1000 times to accumulate the intensities of peaks.

Antibodies directed to synthetic α-L-fucopyranosylceramide, natural α-L-fucopyranosylceramide isolated from human adenocarcinoma, and β-D-galactopyranosylceramide were prepared according to the modified procedure of Nagai & Ohsawa (1974). Liposomes were prepared from ethanol rather than tetrahydrafuran. Synthetic L-fucosylceramide (4 mg) or natural L-fucosylceramide (2 mg), 4 times the weight of egg yolk lecithin, and 30 times the weight of cholesterol were dissolved in 0.75 mL of ethanol and warmed if necessary. The solution was added drop by drop into 8.25 mL of physiological saline under vigorous stirring. The whole mixture was placed in an ultrasonic bath for 30 s to 1 min, followed by centrifugation at 10000*g* for 30 min. The supernatant fluid was discarded and the precipitate was suspended in 1 mL of a 1% solution of methylated bovine serum albumin (BSA) in physiological saline. The suspension was allowed to stand at 4 °C for 18 h and then mixed with 1 mL of the complete Freund adjuvant with an enforced amount of *Tuberculus bacilli* (10 mg/mL). A heavy emulsion (2–2.5 mL) was prepared, and aliquots were injected into New Zealand or San Juan rabbits at several locations. After 12 days, the second booster injection was made with the same quality and quantity as that of the first antigen emulsions. Antisera were taken at seven different times (on day 30, 50, 65, 83, 100, 150, and 170 after the initial injection) and treated with the Sepharose column coupled with methylated BSA to eliminate anti-methylated BSA antibodies.

Determination of Antigen–Antibody Reaction. The titer of antibodies in native or purified sera was determined by the liposome lysis assay according to the method described by Six et al. (1974). Complement fixation was carried out with microtiter plates as previously described (Lennette, 1969).

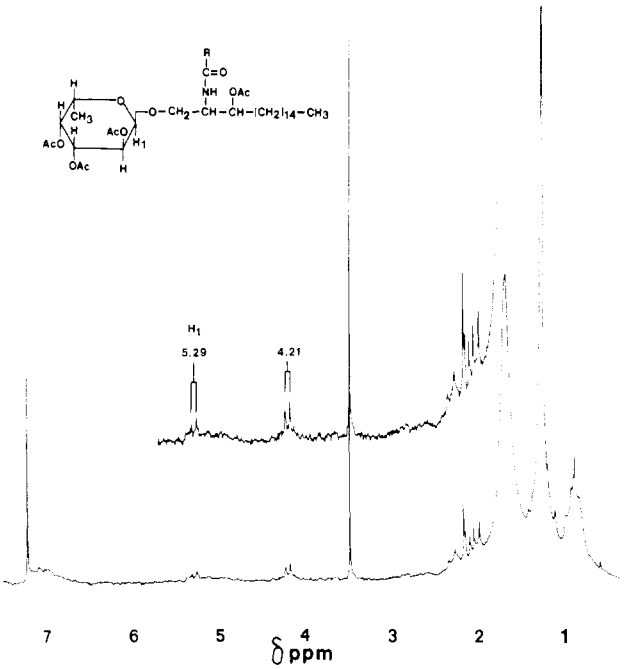


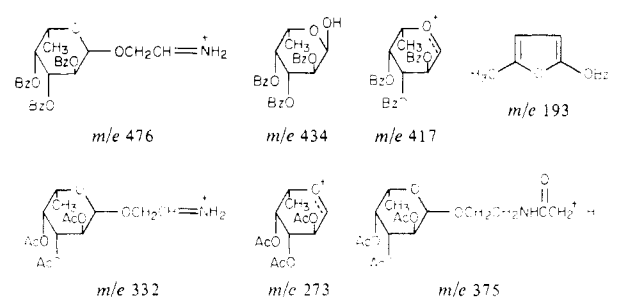
FIGURE 2: NMR spectra of α-L-fucopyranosylceramide *O*-acetate.

Results

Characterization of Synthetic Compounds. Six compounds have been synthesized according to the scheme shown in Figure 1 and the procedure described under Materials and Methods. These compounds have been characterized by MS and NMR as described below. Significantly, MS of compounds I–V indicated that the fucosyl residue is linked at the C_1 primary hydroxyl of sphingosine, exclusively. This was further assessed by failure in detecting the trityl ether derivative of α-L-fucopyranosylceramide (compound II); i.e., the compound did not contain a primary hydroxyl group. NMR of compounds I–V inicated that the fucosyl residue of all these compounds is linked through an α-glycosidic linkage, by the presence of a doublet at δ 5.29 (J = 5 Hz) for the acetylated compound II (Figure 2), and similar doublets with a similar chemical shift value and coupling constant have been found for other compounds, as described below.

MS Data of α-L-Fucopyranosyl Derivatives. (a) The MS of compound I (Figure 1) was characterized by *m/e* 476,* 434,* 310 (417 − BzlOH), 239 [$CH_3(CH_2)_{14}C{=}O^+$ or $CH_3(CH_2)_{12}CH{=}CHCHO^+H$], 253 [$CH_2{=}C(OBz)CH{=}O^+Bz$], 240 (BzOCH=CHOBz), and 193* (for the assignment of the mass numbers with an asterisk, see footnote 2). (b) The MS of α-L-fucopyranosylceramide (II, Figure 1) as

[2] Mass number assignments for α-L-fucopyranosyl derivatives are as follows:



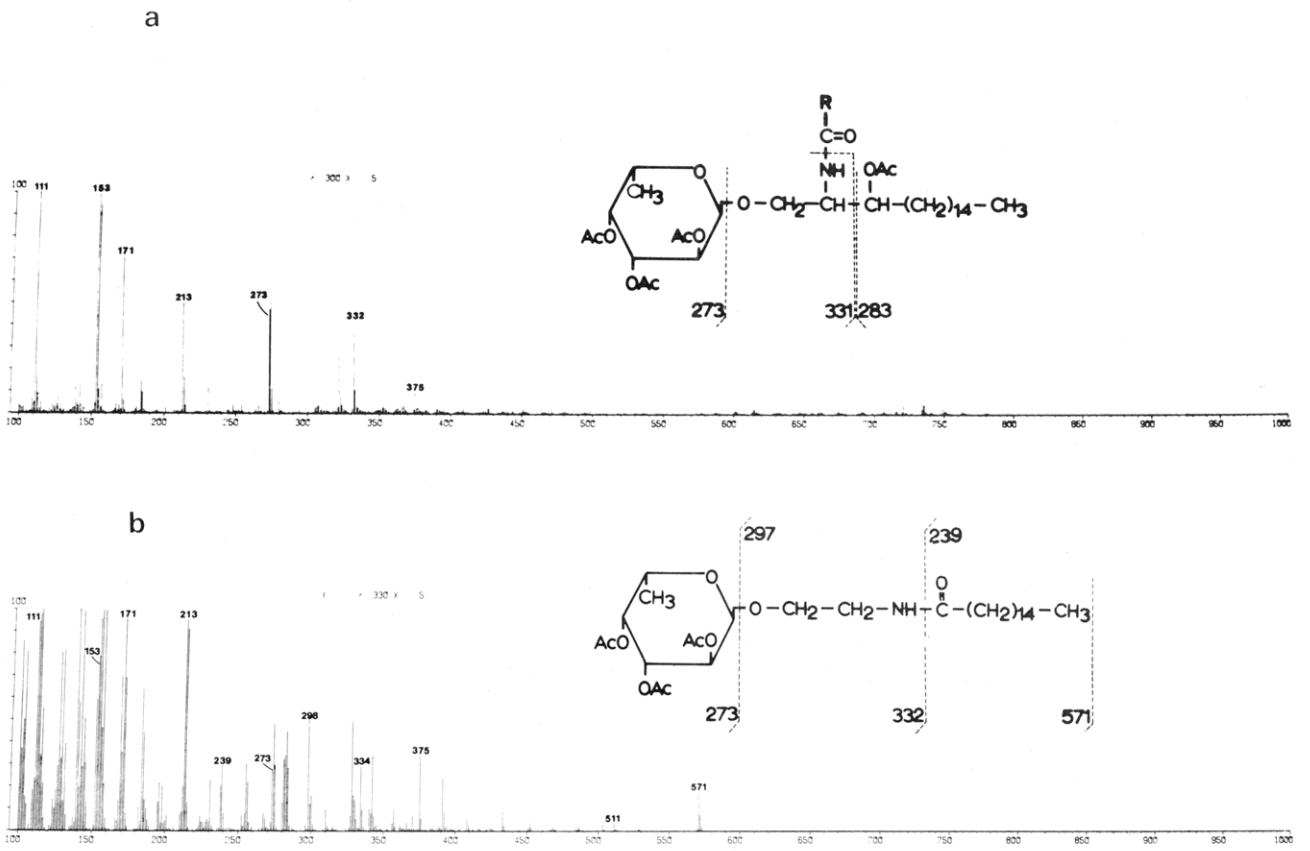


FIGURE 3: (a) Mass spectra of compound II and (b) that of compound IV.

an acetylated derivative was characterized by m/e 332 (331 + 1), 273,* 213 (273 − AcOH), 171 (213 − CH_2=C=O), 153 (213 − AcOH), and 111 (153 − CH_2=C=O). The MS of this compound is shown in Figure 3a. (c) The MS of compound III (Figure 1) was characterized by m/e 434,* 418 (417* + 1), 310, 202, 239, 253, 240, and 183. Six masses are the same as was given by compound I. (d) The MS of compound IV (Figure 1) as an acetylated derivative gave m/e 571 (M^+), 511 (M^+ − 60), 273,* 213 (273 − AcOH), 171 (213 − CH_2=C=O), 153 (213 − AcOH), 111 (153 − CH_2=C=O), 239 [$CH_3(CH_2)_{14}C{=}O^+$], 281 [$CH_3(CH_2)_{14}CONHCH_2CH_2$], and 375.* The MS of this compound is shown in Figure 3b. (e) The MS of compound V gave m/e 417,* 310 (417 − BzlOH), 202 (310 − BzlOH), 193,* 239 [$CH_3(CH_2)_{12}CH{=}CHCHO^+OH$], 253 [$CH_2{=}C(OBz)CHO^+Bz$], and 204 (BzOCH=CHOBz).

NMR Data of α-L-Fucopyranosyl Derivatives. (a) NMR of α-L-fucopyranosylceramide tetraacetate was determined in $CDCl_3$. Scanning 1024 times gave the following signals (δ values): 0.89 (triplet, J = 4 Hz, from the terminal CH_3), 1.23 (doublet, J = 7 Hz, from the fucose CH_3), 1.25 [broad singlet, from $-(CH_2)_n-$], 1.68 (singlet, from acetyl group), 2.05 (triplet, J = 7 Hz, from CH_2CO), 3.48 (singlet, from NH), 4.21 (doublet, J = 6 Hz, from $CH_2CON{=}$), and 5.29 (doublet, J = 5 Hz, from the anomeric H_1 of fucose). The spectra of this compound are shown in Figure 2.

(b) NMR of α-L-fucopyranosylceramide tribenzyl ether was determined in $CDCl_3$. Scanning 64 times gave the following signals (δ values): 0.88 (triplet, J = 5 Hz, from the terminal CH_3), 1.20 (doublet, J = 7 Hz, from the fucose CH_3), 1.25 (broad singlet, from $-(CH_2)_n-$), 1.84–2.18 (from $-CH_2CO$ and $-CH_2C{=}$), 3.46 (−NH), 4.76 (singlet, from $-CH_2Ph$), and 5.34 (doublet, J = 5 Hz, from the anomeric H_1 of fucose).

(c) NMR of α-L-fucopyranosyl-*N*-palmitoylethanolamine triacetate was determined in $CDCl_3$. Scanning 256 times gave the following signals (δ values): 0.89 (triplet, J = 5 Hz, from the terminal CH_3), 1.16 (doublet, J = 7 Hz, from the fucose CH_3), 1.28 (singlet, from the CH_2 chain), 1.60 (singlet, from acetyl), 2.00–2.08 (from $-CH_2CO$), 2.18 (singlet, from −NH), 3.49 (singlet, from $-OCH_2CH_2N{=}$), and 5.29 (doublet, the anomeric H_1 of fucose; the coupling constant was not measurable).

(d) NMR of α-L-fucopyranosyl-*N*-palmitoylethanolamine was determined in Me_2SO-d_6. Scanning 1024 times gave the following signals (δ values): 0.86 (triplet, J = 5 Hz, from the terminal CH_3), 1.07 (doublet, J = 6 Hz, from the fucose CH_3), 1.25 (singlet, from $-CH_2-$), 2.04 (triplet, J = 2 Hz, from $-CH_2CO$), 3.32 (singlet, from $-OCH_2CH_2N{=}$), and 5.38 (from the anomeric H_1 of fucose; the coupling constant was not measurable).

Fatty Acid Composition of Synthetic α-L-Fucosylceramide. This was mainly found to be C20, C22, and C24 normal fatty acids: C18:0 5%, C20:0 8%, C22:0 28%, C24:0 20%, C20:1 5%, C22:1 2%, C24:1 10%, C20:0 α-OH 2%, C22:0 α-OH 4%, and C24:0 α-OH 16%.

Characterization of Antibodies.[3] The antibody titers directed to synthetic α-L-fucopyranosylceramide rapidly increased after the first injection of the glycolipid liposome; however, further increase of the titer was not obvious by booster injection. Antisera, thus obtained, contained antibodies directed not only to fucosylceramide but also to liposome components and ceramide; therefore, absorption with liposomes

[3] Two New Zealand rabbits and one San Juan rabbit were immunized with synthetic fucosylceramide, and one New Zealand rabbit was immunized with galactosylceramide. Two New Zealand rabbits were immunized with tumor fucosylceramide. Antibody responses in the three rabbits to synthetic fucosylceramide were equally greater than those of the two rabbits to tumor fucosylceramide. However, only one serum from each group was used for further characterization as described in this section.

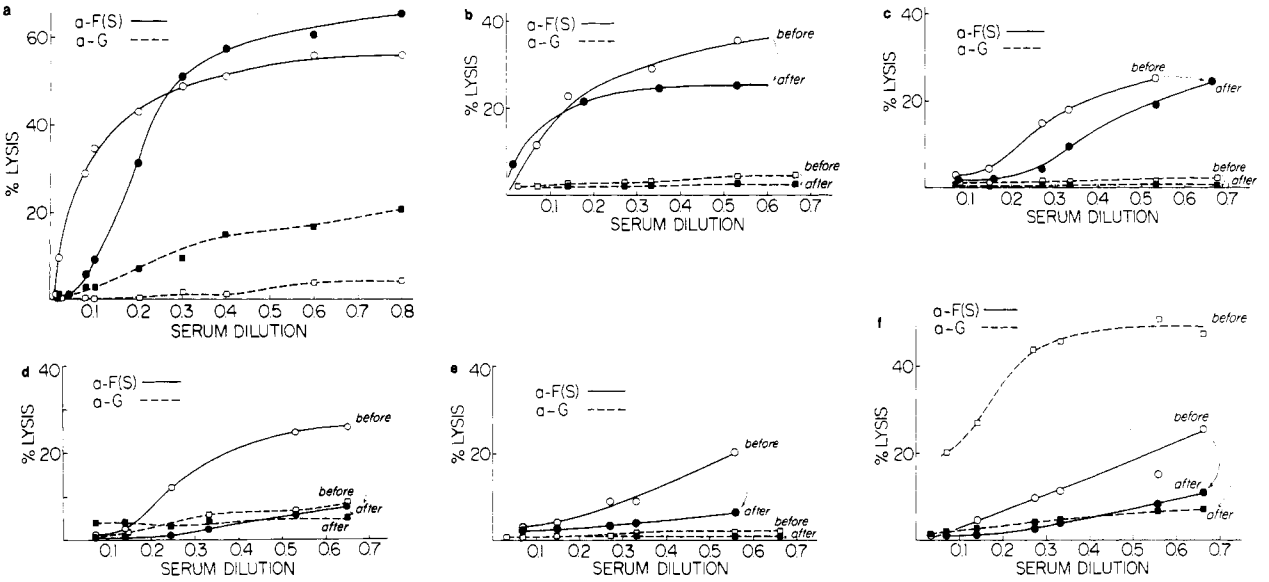


FIGURE 4: (a) The complement-dependent liposome lysis of ceramide liposomes by anti-synthetic α-L-fucoceramide, abbreviated as a-F(S), and by anti-galactosylceramide, abbreviated as a-G. The lysis curve of synthetic α-L-fucosylceramide liposome by a-F(S) is shown by open circles, whereas the lysis curve of tumor fucosylceramide liposome by a-F(S) is shown by solid circles. The lysis curve of synthetic fucosylceramide liposome by a-G is shown by open squares, and the lysis curve of tumor fucosylceramide liposome by a-G is shown by solid squares. Note that tumor fucosylceramide cross-reacts with anti-galactosylceramide (possibly due to contamination; see text). (b) The lysis curve of synthetic fucosylceramide liposome by a-F(S) is shown by open circles. The lysis curve of the same liposome by a-F(S) after absorption with galactosylceramide liposome is shown by solid circles. The lysis curve for synthetic fucosylceramide by a-G is shown by open squares, and that after absorption with galactosylceramide is shown by solid squares. (c) The lysis curve of tumor fucosylceramide liposome by a-F(S) is shown by open circles. The same lysis curve by the same antibody after absorption with galactosylceramide liposome is shown by solid circles. The lysis reactivity of synthetic fucosylceramide liposome by a-G is shown by open squares, and the same lysis reactivity by the same antibody after absorption by galactosylceramide is shown by solid squares. (d) The lysis curve of ceramide liposome by a-F(S) is shown by open circles, and the reactivity of the same ceramide liposome by the same antibody after absorption with galactosylceramide liposome is shown by solid circles. The reactivity of ceramide liposome by a-G is shown by open squares, and the reactivity of the same ceramide liposome by the same a-G antisera after absorption with galactosylceramide liposome is shown by solid squares. (e) The lysis curve of lecithin–cholesterol alone by a-F(S) is shown by open circles, and the same reactivity of the same liposome by a-F(S) after absorption with galactosylceramide liposome is shown by solid circles. The lysis reactivity of liposomes alone by a-G is shown with open squares, and that after absorption with liposome is shown by solid squares. (f) The lysis curve of galactosylceramide liposome by a-F(S), before and after absorption with galactosylceramide liposome, is shown by open circles and solid circles, respectively. The lysis reactivity of galactosylceramide liposome by a-G, before and after absorption with galactosylceramide liposome, is shown by open squares and solid squares, respectively.

containing galactosylceramide was necessary to eliminate nonspecific antibodies. Anti-synthetic fucosylceramide antibodies after absorption with galactosylceramide did not react with galactosylceramide, ceramide, or liposome alone and showed a specific reaction with α-L-fucopyranosylceramide.

The antiserum (7A3) to the synthetic fucosylceramide reacted well with both the synthetic α-L-fucosylceramide and the natural α-L-fucosylceramide isolated from human cancer (Figure 4a, open and solid circles), whereas the antiserum (7A2) to β-D-galactosylceramide did not react to the synthetic fucosylceramide but cross-reacted significantly with α-L-fucosylceramide isolated from tumor (Figure 4a, open and solid squares). The anti-synthetic fucosylceramide (7A3, A2), however, reacted nonspecifically with galactosylceramide (Figure 4f, open circles and solid line), with ceramide (Figure 4d, open circles and solid line), and with lecithin–cholesterol liposomes (Figure 4e, open circles and solid line). All these nonspecific reactions were absorbed by treating antiserum with liposomes containing galactosylceramide (solid circles with solid line in Figure 4f; dotted lines with open and closed squares in Figure 4d,e). The anti-galactosylceramide antiserum (7A2) was not significantly cross-reacting with ceramide or liposomes alone (Figure 4d,e, dotted lines), and the specific reaction with galactosylceramide (Figure 4f, open squares and dotted line) was absorbed out by galactosylceramide liposome (Figure 4f, solid squares and dotted line). Absorption of anti-fucosylceramide antiserum with galactosylceramide liposome slightly reduced the reactivity (Figure 4b,c). The reduction of anti-fucosylceramide reactivity to the tumor fucosylceramide was slightly greater than that with synthetic fucosylceramide (Figure 4c).

Results of complement fixation are shown in Figure 5. Anti-synthetic fucosylceramide reacted specifically with the α-L-fucosyl residue on complement fixation, irrespective of its carrier molecule, although the degree and the pattern of the complement fixation showed a considerable difference. The extent of the complement fixation of anti-synthetic fucosylceramide with the synthetic fucosylceramide was much greater (Figure 5b,A) than with natural fucosylceramide (Figure 5b,B) and with α-L-fucopyranosyl-*N*-palmitoylethanolamine (Figure 5b,C). In contrast to the reactivity with the liposome lysis assay, there was no cross reaction demonstrated by complement fixation with galactosylceramide liposome and liposome alone (Figure 5a,B; Figure 5b,E,D). Similarly, anti-galactosylceramide did not cross-react with fucosylceramide on complement fixation. Antibody response to the natural α-L-fucosylceramide isolated from human tumor and included in liposomes was much weaker than to the synthetic α-L-fucosylceramide (data not shown).

Discussion

Previously we have isolated and characterized a novel glycosphingolipid, α-L-fucopyranosylceramide, from human adenocarcinoma. The glycolipid was isolated in one case from a large metastatic tissue in which a relatively large quantity of fucosylceramide was accumulated (Watanabe et al., 1976). Although fucosylceramide was detectable in the monohexosylceramide fraction of several cases of metastatic human

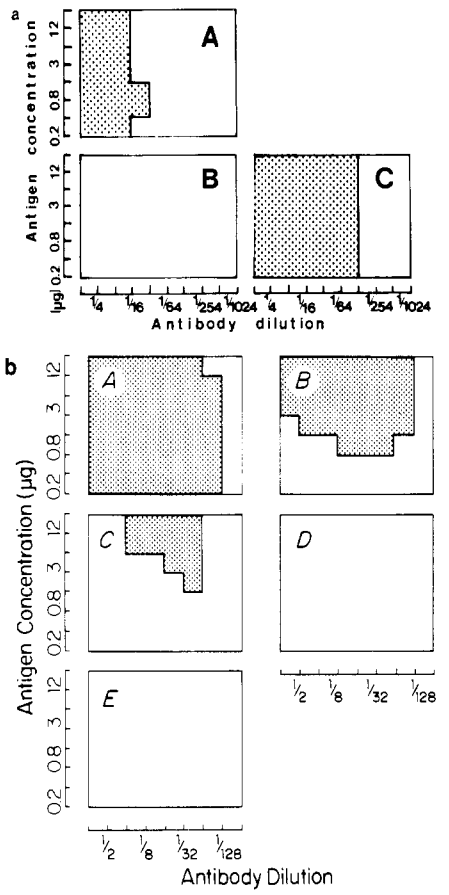


FIGURE 5: (a) The complement fixation patterns assayed on microtiter plates according to the procedure of Lennette (1969) with anti-fucosylceramide and anti-galactosylceramide. The ordinate is antigen concentration in micrograms per well; the abscissa, antibody dilution. The dotted shadow area indicates that which showed a complete inhibition of hemolysis. (A) The reactivity with synthetic fucosylceramide and with anti-synthetic fucosylceramide (7A3). (B) The reactivity of galactosylceramide and anti-synthetic fucosylceramide. (C) The reactivity of anti-galactosylceramide (7A2) with galactosylceramide. (b) The same complement fixation patterns as in (a). (A) The reactivity of anti-synthetic fucosylceramide and with synthetic fucosylceramide. (B) The reactivity of anti-synthetic fucosylceramide and with tumor fucosylceramide. (C) The reactivity of anti-synthetic fucosylceramide with α-L-fucopyranosyl-*N*-palmitoylethanolamine. (D) The reactivity of anti-synthetic fucosylceramide with liposome alone. (E) The reactivity of anti-synthetic fucosylceramide with galactosylceramide. The difference in the fixation pattern between Figure 5a,A, and Figure 5b,A, is due to the antigen dilution and to the quality of complement. These two experiments were carried out at different times.

adenocarcinoma, its chemical quantity was, in general, very low and was difficult to separate from the dominant quantity of glucosyl- and galactosylceramide. The best preparation still had a small amount of galactosylceramide contamination. Although the presence of fucosylceramide seems to be limited to the metastatic deposit of human adenocarcinoma, further extensive studies on the quantitative distribution in various tissue are necessary. A specific antibody directed to fucosylceramide should be obtained in order to perform an immunohistochemical study or radioimmunoassay. Since a pure fucosylceramide preparation completely free from galactosylceramide is difficult to obtain, this investigation was initiated.

For synthesis of fucosylceramide, a Königs–Knorr-type condensation reaction of 2,3,4-tri-*O*-benzyl-α-fucopyranosyl bromide with ceramide without a protective group at the C_3 hydroxyl of sphingosine was performed; a resulting fucosylation was found only at the C_1 primary hydroxyl group. The MS of tribenzylfucosylceramide (compound I) was characterized by the major mass *m/e* 476 (the ion for tribenzylfucosyl linked to a primary alcohol group of ethanolamine; see footnote 1) and the mass *m/e* 239 (the ion for the cleavage product of C_2 and C_3 of sphingosine; see footnote 1). The MS of compound II (fucosylceramide acetate) was characterized by the presence of *m/e* 375 and 332 (331 + 1) (possible structure; see footnote 1), but the chance of *m/e* 143 ($AcOCH_2CHNHCOCH_2$) and 129 ($AcOCH_2CHNHC{=}O$). These results indicate that fucosylation took place at the C_1-hydroxyl (primary hydroxyl) group of ceramide. Absence of a primary hydroxyl group in the synthetic fucosylceramide was further assessed by failure of tritylation with triphenylmethyl chloride (trityl chloride). NMR indicated the α-anomeric linkage of fucose.[4] However, the final product contained exclusively dihydrosphingosine (sphinganine), which was produced during hydrogenolysis of the benzyl group.

For synthesis of fucosylsphingosine, a similar condensation reaction with *N*-(dichloroacetyl)sphingosine and 2,3,4-tri-*O*-benzyl-α-fucopyranosyl bromide was performed. The fucosylation proceeded again through the C_1-hydroxyl rather than the C_3-hydroxyl group.

Synthesis of fucosylsphingosine was performed, aimed at further coupling of the amino group of sphingosine to a macromolecular carrier, which will enhance the specificity and reactivity of the α-L-fucosyl residue as an immunogen and antigen. The results of such application will be described elsewhere.

The synthesis of fucosyl-*N*-palmitoylethanolamine was aimed at examining the extent of cross reaction with the ceramide moiety or the sphingosine moiety as this structure is identical up to the C_2 of sphingosine.

A successful antibody response was observed in San Juan rabbits to α-L-fucosylceramide-containing liposome. However, the antisera always contained antibodies also directed to ceramide and liposome itself. Absorption of such antisera with liposomes containing galactosylceramide resulted in a specific antibody directed to α-L-fucosylceramide. A significant difference in reactivities between synthetic and naturally occurring α-L-fucosylceramide must be ascribed to the difference of the ceramide moiety; i.e., synthetic ceramide has C_{18}-dihydrosphingosine (octadecasphinganine) and long-chain fatty acids (see above) in striking contrast to the natural α-L-fucosylceramide containing C_{18}- and C_{20}-sphingosine (octadecasphingenine and eicosasphingenine) and relatively short-chain fatty acids (mainly palmitic acid) (Watanabe et al., 1976). A much weaker reactivity of natural α-L-fucosylceramide liposome was observed in the liposome lysis assay at the lower antibody concentration as compared to the synthetic fucosylceramide liposome. A weaker reactivity of the natural fucosylceramide than that of the synthetic fucosylceramide was clearly demonstrated in the complement fixation reaction. The concept that the ceramide structure will affect the strength of the immunological reactivity of glycolipid, through the specificity as defined by the sugar structure, is the important basis of glycolipid antigenicity in general; this

[4] Hydrolyzability of fucosyl glycolipid by α-L-fucosidase of *Chalonia lampus* greatly varied due to the aglycon structure. The fucosyl residue of α-L-fucopyranosyl-*N*-palmitoylethanolamine was readily hydrolyzed in contrast to that of a synthetic α-L-fucopyranosylceramide which contained a long-chain fatty acid. Only a partial hydrolysis of fucosylceramide occurred even after a prolonged incubation as described before (Watanabe et al., 1976). However, all these synthetic fucosides gave a common spectrum for the α-anomeric proton (doublet, δ 5.29–5.35). Resistance to α-L-fucosidase may be introduced by the presence of a long-chain fatty acyl residue in the ceramide moiety.

idea was further strengthened by the observation that α-L-fucosylpalmitoylethanolamine showed a weaker reaction in complement fixation as compared to the synthetic fucosylceramide, although this analogue definitely reacts with anti-fucosylceramide antibodies.

The titer of antibody directed to synthetic α-L-fucosylceramide was higher than naturally occurring α-L-fucosylceramide. Therefore, the structure of the ceramide moiety also affects immunogenicity of the liposome. It is possible that ceramide without a double bond and with a longer fatty acid chain may form a more stable liposome than that with an unsaturated sphingosine (sphingenine) and with a shorter fatty acid chain. Antibodies directed to synthetic α-L-fucosylceramide cross-react with galactosylceramide, ceramide, and liposome alone; this may be because some antibodies may also be directed to the ceramide moiety and a part of the liposome structure as well, since the cross reactivities were removed readily by absorption with galactosylceramide liposomes. Anti-galactosylceramide did not react to the liposomes containing synthetic fucosylceramide but did react to those with natural fucosylceramide (Figure 4a, solid squares with dotted line). This indicates that galactosylceramide is present as a contaminant in the natural fucosylceramide isolated from adenocarcinoma.

Recently, monoclonal antibodies directed to glycolipids have been prepared (Young et al., 1979; Nowinski et al., 1980), and the technique for targeting of chemotherapeutic drugs to tumor cells through anti-glycolipid antibodies has been developed (Urdal & Hakomori, 1980). Logically, anti-α-L-fucosylceramide antibodies are useful reagents for targeting drugs in human cancer. Such efforts are under serious consideration.

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Analogues of 8-Hydroxy-5-deazaflavin Cofactor: Relative Activity as Substrates for 8-Hydroxy-5-deazaflavin-Dependent $NADP^+$ Reductase from *Methanococcus vannielii*†

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ABSTRACT: The 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase from *Methanococcus vannielii* was examined for its ability to catalyze the reduction of a number of 5-deazaflavin analogues of the natural cofactor. Comparison of the kinetic constants revealed certain substrate structure–reactivity relationships for the enzyme. The basic heterocyclic system of the natural cofactor 2,4-dioxopyrimido[4,5-*b*]quinoline was shown to be the minimum structural requirement since neither riboflavin nor 1,5-dideazariboflavin was reduced by the enzyme. The N-10 side chain of the natural cofactor was shown not to be essential since the enzyme could reduce 8-hydroxy-2,4-dioxopyrimido[4,5-*b*]quinoline. The study also indicated that there are some steric constraints at C-8 and C-7 with respect to interaction of the cofactor with the enzyme. Specifically, (a) the 8-methoxy derivative, in contrast to the 8-hydroxy compound, was not reduced and (b) the introduction of a substituent at C-7 resulted in a marked decrease in the rate of reduction. The importance of C-5 as the site for the electron entry was suggested by the finding that 5-methyldeazariboflavin was not reduced. The latter inhibited the reduction of 5-deazariboflavin.

A novel 8-hydroxy-5-deazaflavin cofactor (8-OH-5dFl)[1] that is abundant in methane-producing bacteria (Eirich et al., 1978; Ashton et al., 1979; Pol et al., 1980) serves as electron carrier in a formate–$NADP^+$ oxidoreductase system (Tzeng et al., 1975; Jones & Stadtman, 1980). In *Methanococcus vannielii* the 8-OH-5dFl, which is reduced by formate dehydrogenase and formate, serves as a cofactor for an enzyme (5-deazaflavin–$NADP^+$ reductase) that reduces $NADP^+$ to NADPH. The *M. vannielii* $NADP^+$ reductase specifically requires

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[1] Abbreviations: 8-OH-5dFl, 8-hydroxy-5-deazaflavin cofactor or coenzyme F_{420}; 5-deazaflavin–$NADP^+$ reductase, 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase; FMN, riboflavin 5′-phosphate; FAD, flavin adenine dinucleotide; NMR, nuclear magnetic resonance; ^1H NMR, proton nuclear magnetic resonance.

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