Glucosylsterol and acylglucosylsterol of snake epidermis: structure determination

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Abstract The structures of two classes of glycolipids, acylglucosylsterol and glucosylsterol, from snake epidermis have been determined by chemical, spectroscopic, and gas-liquid chromatographic methods. The acylglucosylsterol consists of a glucose molecule attached to cholesterol and an ester-linked fatty acid on carbon 6 of the sugar. The major ester-linked fatty acids are palmitic, stearic, and oleic. The glucosylsterol consists of glucose attached to cholesterol. These unusual glycolipids may play a role in epidermal barrier function in snakes and it is concluded that these sterol glycosides may remain in the reptiles as a vestige of a more primitive barrier-forming mechanism.—**Abraham, W., P. W. Wertz, R. R. Burken, and D. T. Downing.** Glucosylsterol and acylglucosylsterol of snake epidermis: structure determination. *J. Lipid Res.* 1987. **28:** 446–449.

Supplementary key words epidermis • sterol glycoside • NMR

It is now well established that lipids comprise the cutaneous permeability barrier in terrestrial vertebrates (1–3). In mammalian, avian, and reptilian skin these lipids are packaged into lamellar granules within the viable epidermal cells and are subsequently extruded into the extracellular compartment on completion of differentiation (4, 5). This extruded lipid forms multiple bilayer sheets between the completely keratinized cells in the outer layers of the epidermis (6) and provides the barrier to water loss (7, 8) which is essential for life on dry land (9).

Extensive studies on the lipids of mammalian epidermis have inplicated an unusual glycolipid, an acylglucosylceramide, in the assembly of the epidermal lamellar granules (10). This same lipid has recently been isolated from avian epidermis (11) and has been tentatively identified in phase IV snake epidermis but is not found in the skins of fish or frogs (12), neither of which form lamellar granules (4). Occurrence of the acylglucosylceramide, therefore, appears to be closely associated with the ability to form lamellar granules.

In addition to the acylglucosylceramide, bird epidermis contains unusual classes of glycolipids not found in mammals—glucosylsterols and acylglucosylsterols (11). It has been noted that some of the molecular attributes which are thought to be of functional importance for the acylglucosylceramide are also found in the acylglucosylsterol, and it was suggested that the latter may represent a vestige of a more primitive barrier-forming mechanism. If these suggestions are correct, then it might be expected that reptiles, which are precursors of birds in an evolutionary sense, would also contain these unusual glycolipids. The principal goal of the present investigation was to determine whether glucosylsterols and acylglucosylsterols are present in reptile epidermis.

MATERIALS AND METHODS

Animals

Naturally shed epidermis was obtained from four bull snakes (Colubridae *Pituophis melanoleucus sayi*). Cast skins were frozen within 48 hr of shedding.

Isolation of glycolipids

The shed skins were extracted in 3 successive mixtures of chloroform–methanol (C–M) 2:1, 1:1, 1:2 (v/v), each for 2 hr with the last mixture heated to 45°C. The extracts were combined and evaporated to dryness under reduced pressure. The lipids were redissolved in C–M 2:1 and applied to a 0.5-mm-thick layer of silica gel 60H (E. M. Reagents, Darmstadt, West Germany) on a 20 × 20 cm glass plate. The chromatograms were developed in C–M–acetic acid 190:9:1 (v/v/v). The plate was sprayed with an ethanolic solution of 8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt (10 mg/100 ml of 95% ethanol) and viewed under ultraviolet light. Two glycolipid bands (R_f values 0.2 and 0.05) were scraped from the plate and the lipids were

Abbreviations: GLC, gas-liquid chromatography; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; FAME, fatty acid methyl esters.

eluted with C-M-water 50:50:1 (v/v/v). These glycolipid fractions were acetylated and rechromatographed using hexane-ether-acetic acid 30:70:1 (v/v/v). The acetates were found to be chromatographically pure in several solvent systems. Visual comparison with analytical chromatograms of lipid mixtures of known composition indicated that each of the two glycolipid fractions amounted to 1-2% of the original lipid mixture, which contained large amounts of nonpolar lipids that were probably not components of the intercellular lipid lamellae.

Analysis of lipids

Each of the glycolipids was hydrolyzed by treatment with 1 N HCl in methanol containing 10 M water for 18 hr at 60°C (13). The reaction mixture was dried under a stream of nitrogen, and the residue was partitioned between chloroform and water. Sterols, free fatty acids, and fatty acid methyl esters (FAME) in the chloroform layer from the above partition were isolated by preparative TLC. Sterols were acetylated and analyzed by GLC on a 12-meter BP-1 silica capillary column (Scientific Glass Engineering, Inc., Austin, TX) using cholesteryl acetate and cholestanyl acetate as standards. The free fatty acids were methylated by treatment with excess 10% BCl_3 in methanol (Sigma Chemical Co., St. Louis, MO) at 60°C for 1 hr. FAME were analyzed by GLC on a 50-meter CP SIL 88 capillary column (Chrompack Inc., Bridgewater, NJ) under programmed temperature conditions (160-220°C, + 2°/min) and compared with FAME standards.

NMR spectra

Proton NMR spectra were recorded on a Bruker WM-360 spectrometer operating in the Fourier transform mode. Three mg of the sample was dissolved in 0.5 ml of solvent ($CDCl_3$ or a 2:1 (v/v) mixture of $CDCl_3$-CD_3OD). Acetylation of the free hydroxyl groups improved the quality of the spectra of these glycolipids. The residual proton signal from $CDCl_3$ (7.2 ppm) was used as internal reference to determine the chemical shifts. The coupling constants were determined by a series of decoupling experiments. The absence of significant unassigned signals indicated that both of the lipid fractions were virtually free from contamination by other classes of lipids.

Chemical modification

Each of the glycolipids was saponified by treatment with excess chloroform-methanol-10 N NaOH 2:7:1 (by volume) for 1 hr at 60°C. The reaction mixtures were then neutralized with 2 N HCl and the products were extracted into chloroform. Acetylation of the free hydroxyl groups was carried out in pyridine-acetic anhydride 1:1 (v/v) warmed to 40°C for 2 hr and then evaporated to dryness under a stream of nitrogen.

RESULTS

The less polar of the two glycolipids was saponifiable, indicating the presence of an ester-linked fatty acid. **Fig. 1** shows the proton NMR spectra of the two glycolipids, viz., acylglucosylsterol (Fig. 1A) and glycosylsterol (Fig. 1B) in the acetylated form. Identification of the sugars, the sterol, and the position of attachment of the ester-linked fatty acid were based on proton NMR spectra. Fig. 1A indicates the presence of a sugar, predominantly or entirely glucose. The anomeric proton is at 4.53 ppm as a doublet with a coupling constant (J_{12}) of 8 Hz indicating a β-glucosidic linkage. The protons on carbons 2, 3, and 4 give rise to triplets between 4.85 ppm and 5.2 ppm with coupling constants ranging from 8 to 9 Hz (J_{12}, J_{23}, J_{34}, and J_{45}). The methylene protons attached to the sugar ring are non-equivalent and appear as two doublets of doublets at 4.06 and 4.2 ppm with coupling constants of 12.5 Hz (J_{6A6B}) and 3.2 Hz (J_{56}). These confirm the presence of a β-D-glucoside. The methylene protons attached to the sugar ring of the native acylglucosylsterol are shifted upfield to around 3.5 ppm after saponification, indicating the location of the ester linkage on the 6-hydroxyl group of the sugar. No other sugar ring proton signals of the acylglucosylsterol were significantly altered by saponification. The proton NMR spectrum of the acetylated glucosylsterol (Fig. 1B) closely resembles Fig. 1A, indicating the presence of glucose as the major or sole sugar component in the glucosylsterol. The methyl groups from the sterol moiety in the acetylated glycolipid, in the region 0.62 to 0.95 ppm (Figs. 1A and 1B), and the olefinic proton at 5.3 ppm, indicate that the sterol is cholesterol. GLC of sterol acetates from both the glycolipids gave a single peak corresponding to cholesterol. **Table 1** shows the composition of the esterified fatty acids in the acylglucosylsterol.

DISCUSSION

The results of the present study demonstrate the presence of glucosylsterol and acylglucosylsterol in cast skins from the bull snake. Similar glycolipids have previously been identified in plant sources (14, 15) and in chicken epidermis (11). The molecular details of the sterol glycosides reported in the present work differ only slightly from those found in the chicken. The acylglucosylsterols from both sources contained glucose as the major sugar component, with fatty acid ester-linked to the 6-hydroxyl group, and, in both cases, the principal fatty acids were palmitic, stearic, and oleic. The main difference between the sterol glycosides from the two classes of vertebrates is that those from the snake contain cholesterol as the only component while those from the bird contain substantial proportions of cholestanol in addition to cholesterol (11).

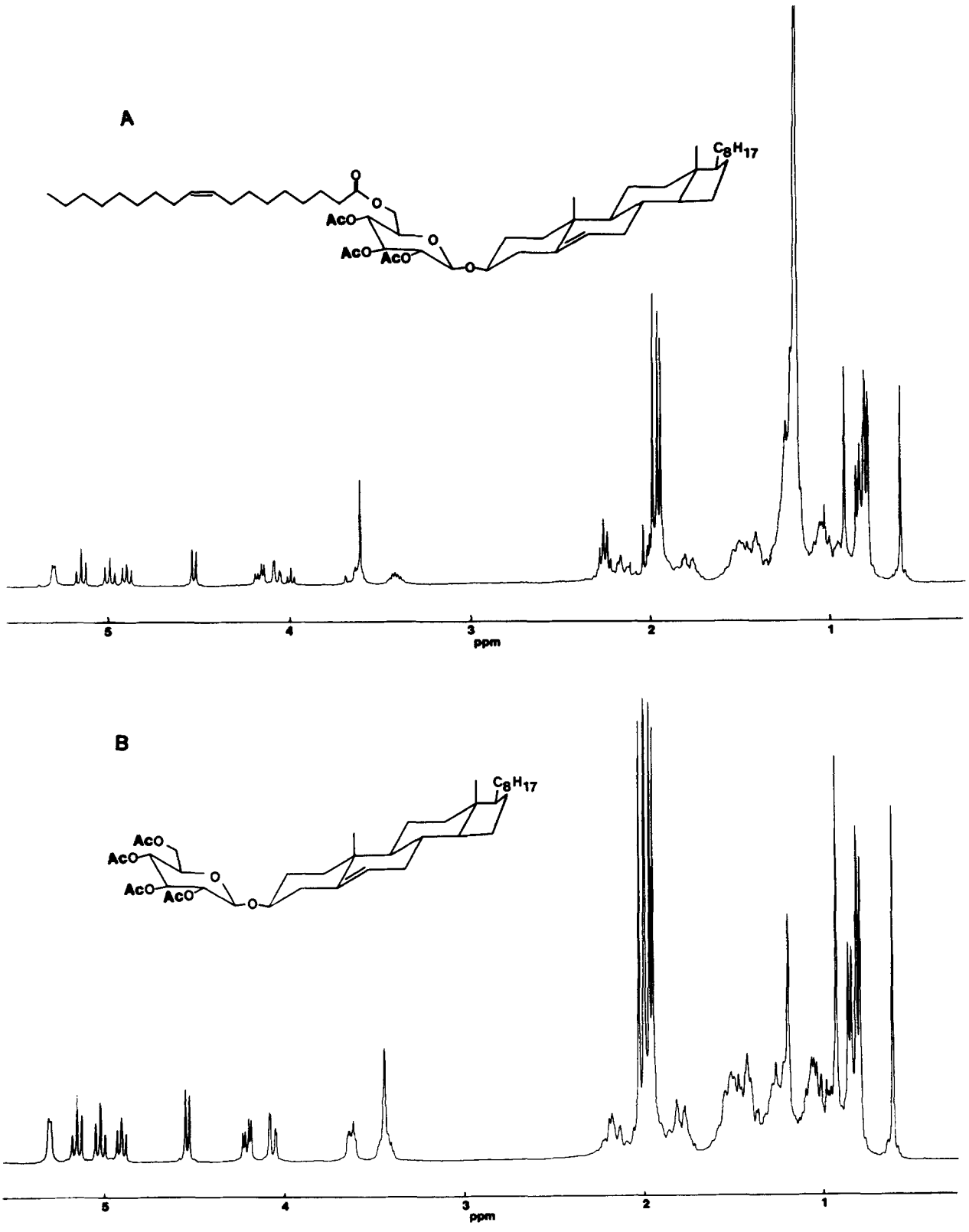

Fig. 1. Structures and proton NMR spectra of acetylated acylglucosylsterol (A) and glucosylsterol (B) from the snake epidermis. The free hydroxyl groups of the sugar in the native glycolipids were acetylated to improve the quality of the NMR spectra.

TABLE 1. Composition of fatty acids esterified to acylglucosylsterol

Carbon No.	Weight Percent
14:0	2.88
15:0	0.74
16:0	33.70
16:1	2.63
17:0	1.43
18:0	23.51
18:1	15.05
18:2	2.18
20:0	4.49
21:0	0.85
22:0	6.11
23:0	0.92
24:0	4.18
26:0	0.68
28:0	0.63

It should be pointed out that the sterol glycosides are not the only glycolipids found in snake epidermis. A recent survey of 24 species of snakes revealed that the cast skins of all of these species contained several components with thin-layer chromatographic mobilities similar to monohexosylceramides and glucosylsterols. Also, there were indications of one or more additional glycolipid components with a mobility similar to acylglycosylsterols in each of the species studied (16). In addition, acylglucosylceramide has been tentatively identified in the epidermis from snakes in phase IV of the skin growth and shedding cycle. Further work is necessary to identify these additional glycolipids.

It has been proposed that acylglucosylsterol, like acylglucosylceramide, may function as a molecular rivet which serves to hold together adjacent lamellar structures. This suggestion was based upon the observation that the acylglucosylsterol contains two hydrophobic moieties extending outward from a hydrophilic core, an arrangement that could permit the sterol and the fatty acyl chain to be inserted into two closely apposed bilayers while the central glucosyl group spans the intervening polar region. Such a molecular interaction could help to stabilize the intercellular lamellae that comprise the water barrier in birds and snakes.

A similar kind of interaction is thought to provide the driving force for formation of the stacks of membranous disks that are found within epidermal lamellar granules (17). However, this process is more strongly associated with the epidermal acylglucosylceramide than with the sterol glycosides, and it has been suggested that the sterol glycosides may remain in birds and reptiles as a vestige of a more primitive barrier-forming mechanism. The present results are in accord with this proposition.

This work was supported in part by a grant from the United States Public Health Service (AM 32374).

Manuscript received 26 August 1986 and in revised form 25 November 1986.

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