Preparation and analysis of benzoylated cerebrosides

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Abstract The benzoylation of cerebrosides with benzoyl chloride and with benzoic anhydride is described, and the derivatives that contained hydroxy and nonhydroxy fatty acids were isolated by high pressure liquid chromatography (HPLC). Studies on the structures of these derivatives and the products formed by mild alkaline hydrolysis are reported. Reaction of cerebrosides containing nonhydroxy fatty acids with benzoyl chloride results in amide-acylation in addition to normal *O*-acylation. Mild alkali treatment of the *N*-diacyl derivative results in the formation of *N*-benzoyl psychosine. Derivatization with benzoic anhydride avoids amide-acylation. These derivatives are useful for the HPLC analysis of cerebrosides.

Supplementary key words high pressure liquid chromatography · benzoyl chloride · benzoic anhydride · nuclear magnetic resonance

The analysis of cerebrosides and other glycolipids by high pressure liquid chromatography (HPLC) becomes a practical and convenient procedure if derivatives are prepared that allow the utilization of a sensitive ultraviolet detector. A system for the separation of neutral glycolipids by HPLC of their benzoylated derivatives has been reported (1). These derivatives were prepared by reaction with benzoyl chloride in a manner similar to that described by Acher and Kanfer (2). They demonstrated that benzoylated cerebrosides could be readily separated into two fractions by silicic acid column chromatography. It was originally thought that these derivatives were only *O*-benzoyl derivatives and that the parent cerebrosides could be regenerated by catalytic deacylation with sodium methoxide in methanol. We present evidence now which indicates that in addition to normal *O*-acylation, amide-acylation also occurs with cerebrosides that contain nonhydroxy fatty acids. The resulting diacylamine leads to the formation of *N*-benzoyl psychosine after mild alkaline treatment as shown in Fig. 1. Derivatization with benzoic anhydride avoids significant amide-acylation, and this procedure is suitable in the HPLC analysis of cerebrosides with nonhydroxy fatty acids (NFA-CR) and of cerebrosides with hydroxy fatty acids (HFA-CR).

MATERIALS AND METHODS

"Beef brain cerebroside" was obtained from Koch-Light Laboratories, Colnbrook, Buckinghamshire, England. Cerebroside II was prepared by Acher and Kanfer (2). *N*-Stearoyldihydroglucocerebroside was obtained from Miles Laboratories, Inc., Kankakee, Ill. *N*-α-Hydroxy lignoceryl psychosine and nonhydroxy fatty acid cerebroside were kindly supplied by Dr. Y. Kishimoto (Eunice Kennedy Shriver Center, Waltham, Mass.). Benzoyl chloride was obtained from Eastman Kodak Co. and benzoic anhydride from Aldrich Chemical Co. Thin-layer plates precoated with silica gel G (250 μm) were obtained from Analtech, Inc. Free and benzoylated cerebrosides on TLC plates were detected by charring the plates with 40% (v/v) H_2SO_4 and by visualization under UV light. HPLC was conducted with the apparatus previously described (1) or with a Perkin-Elmer model 1220 liquid chromatograph in conjunction with a Laboratory Data Control ultraviolet monitor (model 1285). A 50 cm × 2.1 mm ID stainless steel column "dry packed" with Zipax (controlled surface porosity beads, Instrument Products Division, E. I. du Pont de Nemours and Co., Wilmington, Del.) was utilized for HPLC. IR spectra were obtained with a Beckman model IR-33 instrument. NMR spectra were obtained with a Varian HA-100 spectrometer equipped with Fourier transform accessories.

Samples of cerebrosides (0.1–1.0 mg) were benzoylated with 0.6 ml of benzoyl chloride–pyridine 1:5 (v/v) for 1 hr at 60°C; similar quantities were benzoylated with 0.6 ml of 5% benzoic anhydride in pyridine for 18 hr at 110°C. Two procedures were utilized to purify the benzoylated products. Procedure A: the reaction mixture was dried under a stream of nitrogen and the residue was dissolved in 5 ml of hexane and successively washed with 3

Abbreviations: HPLC, high pressure liquid chromatography; NFA-CR, cerebrosides with nonhydroxy fatty acids; HFA-CR, cerebrosides with hydroxy fatty acids; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; GLC, gas–liquid chromatography.

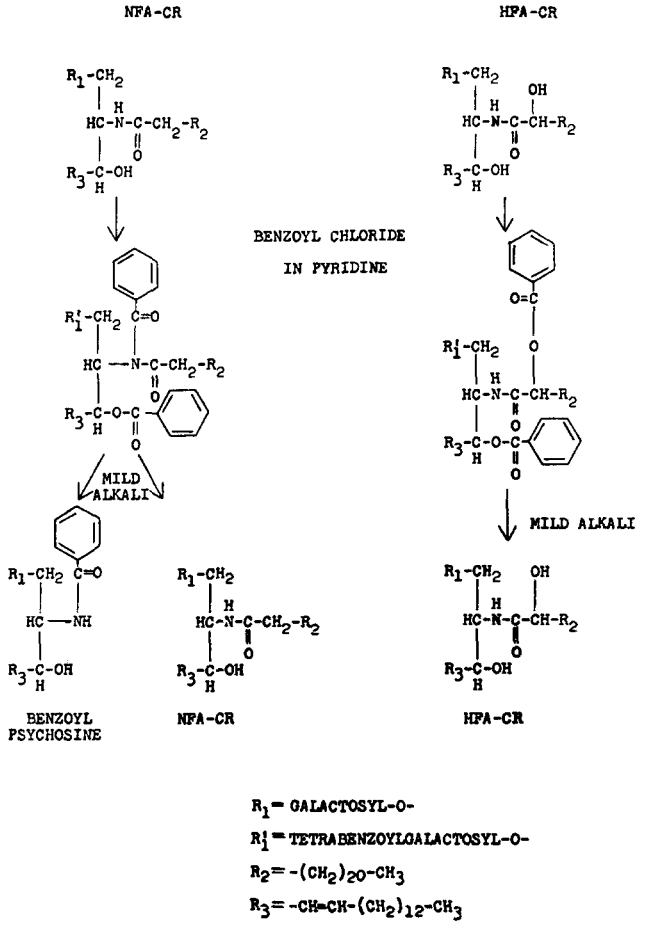


Fig. 1. The formation of benzoylated cerebroside derivatives by reaction with benzoyl chloride and their degradation with mild alkali.

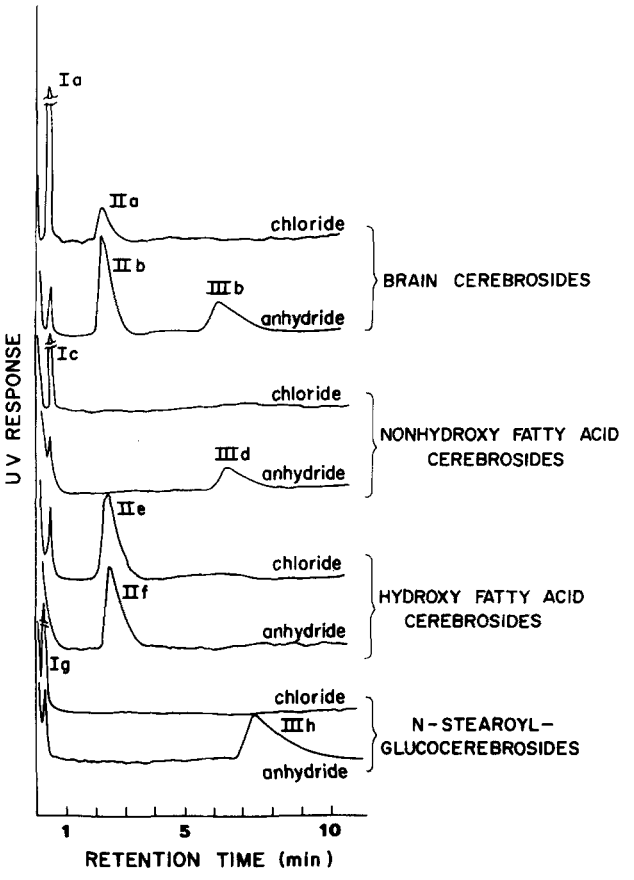


Fig. 2. HPLC analysis of benzoylated cerebrosides. Samples benzoylated with benzoyl chloride (chloride) or benzoic anhydride (anhydride) were injected into the HPLC apparatus and eluted with 0.13% MeOH in pentane at a flow rate of 1.5 ml/min.

ml each of 95% methanol saturated with Na_2CO_3, 0.6 N HCl in 95% methanol, and 95% methanol. The hexane layer was dried under a stream of nitrogen. Procedure B: 5 ml of methanol was added to the reaction mixture, which was then refluxed for 30 min; the solution was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 3 ml of 50% methanol saturated with K_2CO_3. The methanol–water was extracted with 5 ml of chloroform. The lower phase was then successively extracted with 3 ml each of 50% methanol, 0.5 N HCl in 50% methanol, and 50% methanol. The final lower phase was evaporated to dryness and the residue was dissolved in a small volume of hexane. The benzoylated derivatives of HFA-CR and NFA-CR were isolated by preparative HPLC. The benzoylation procedure was scaled up 10-fold, approximately 1 mg of the benzoylated derivatives was injected on the Zipax column, and the column was eluted with 0.2% methanol in hexane. The peaks were collected manually, and the process was repeated several times to accumulate sufficient quantities of the separated compounds. Purity of the separated derivatives was determined by rechromatography of an aliquot of the accumulated fractions. The mild alkaline hydrolysis products of the benzoylated cerebrosides were prepared by a modification of the procedure described by Vance and Sweeley (3). The benzoylated lipid was dissolved in 1 ml of 0.6 N methanolic NaOH, and an equal volume of chloroform was added. The solution was allowed to stand for 1 hr at room temperature, after which 1.7 ml of water and 3.4 ml of chloroform were added. The phases were mixed, and the lower phase was washed with methanol–water 1:1 and evaporated to dryness under a stream of nitrogen.

RESULTS

Chromatographic behavior of benzoylated cerebrosides

Benzoylated "beef brain cerebrosides" were prepared by the benzoyl chloride and the benzoic anhydride methods, and the products were compared by HPLC. The results are shown in Fig. 2. Peaks Ia and IIa were obtained from the benzoyl chloride preparation, whereas peaks IIb

TABLE 1. TLC of lipid products obtained after mild alkaline treatment of individual benzoylated cerebrosides

Spots Observed	HPLC Peaks						
	Ia	IIa	IIIb	Ic	IIId	IIe	IIf
A	+	−	+	+	+	−	−
B	−	+	−	−	−	+	+
C	+	−	−	+	−	−	−

HPLC peaks, shown in Fig. 2, were collected and hydrolyzed as described in the text. The lipid products were analyzed by TLC as described in the legend to Fig. 3, and the presence (+) or absence (−) of spots A, B, and C (as shown in Fig. 3) was noted.

and IIIb were obtained from the benzoic anhydride preparation. Peaks IIa and IIb were shown to cochromatograph in the HPLC system and by TLC in benzene–ethyl acetate 9:1.

Similar amounts of the following samples were benzoylated with benzoyl chloride and benzoic anhydride: *N*-stearoyldihydroglucocerebroside, hydroxy fatty acid cerebroside (*N*-α-hydroxy lignoceryl psychosine), and nonhydroxy fatty acid cerebroside. The HPLC patterns are shown in Fig. 2, and analogous results were obtained by TLC in benzene–ethyl acetate 9:1. It can be seen that the type of product formed from the NFA-CR depends upon the benzoylation reagent used, whereas the products formed from HFA-CR are apparently the same for both reagents. These results demonstrate that peaks I and III (Fig. 2) are benzoylation products of NFA-CR, whereas peak II is the derivative formed from HFA-CR. The derivatives formed from the glucocerebroside with either the chloride or anhydride are separable from the galactocerebroside (NFA-CR) derivatives. The minor unlabeled peaks with retention times less than 1 min are presumably small amounts of the *N*-benzoylated derivatives (see below).

Mild alkaline hydrolysis products

"Beef brain cerebrosides" were benzoylated separately with benzoyl chloride and with benzoic anhydride. The benzoylated products were subjected to mild alkali hydrolysis in methanolic NaOH, and the lipid products were compared with the original beef brain cerebrosides by TLC. These results are shown in Fig. 3. It is apparent that a new product (spot C) was formed as a result of benzoylation with benzoyl chloride and subsequent treatment with mild alkali. The new product (spot C) was shown to have chromatographic properties identical with cerebroside II prepared by Acher and Kanfer (2). Examination of the individual benzoylated derivatives isolated by preparative HPLC (see Table 1) revealed that spots A and C (Fig. 3) originated from peaks labeled I (Fig. 2), whereas only spot B originated from peaks labeled II. The benzoylated products formed from the reaction with benzoic anhydride, peaks II and III, gave rise to spots B (HFA-CR) and A (NFA-CR), respectively. These results indicate that

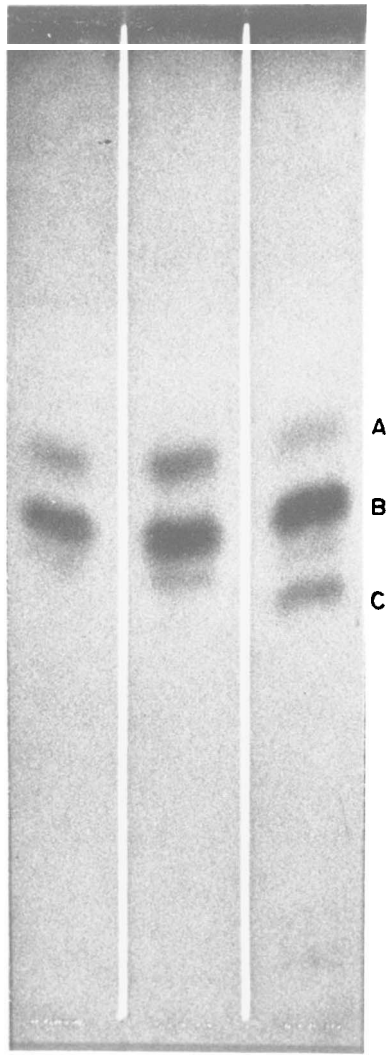


Fig. 3. TLC of lipid products obtained after mild alkaline treatment of benzoylated "beef brain cerebrosides": lane 1, original "beef brain cerebrosides"; lane 2, products from cerebrosides benzoylated with benzoic anhydride; lane 3, products from cerebrosides benzoylated with benzoyl chloride. Spot identification: *A*, NFA-CR; *B*, HFA-CR; *C*, benzoyl psychosine. The plate was developed in $CHCl_3$–CH_3OH–pyridine–H_2O, 40:10:1:1, and the spots were visualized after spraying the plates with 40% H_2SO_4.

the benzoyl derivatives of cerebrosides prepared with benzoic anhydride give rise to the original cerebrosides when subjected to mild alkaline hydrolysis but that the derivatives prepared with benzoyl chloride give rise to a new product (spot C, Fig. 3) when treated under these conditions. The new product originates from the benzoylated NFA-CR and is identical to cerebroside fraction II (2).

The fatty acid composition of the alkaline hydrolysis products was examined after methanolysis in 0.5 N anhydrous methanolic HCl as described previously (4). The products recovered from the benzoyl chloride derivatives contained roughly one-half the amount of nonhydroxy

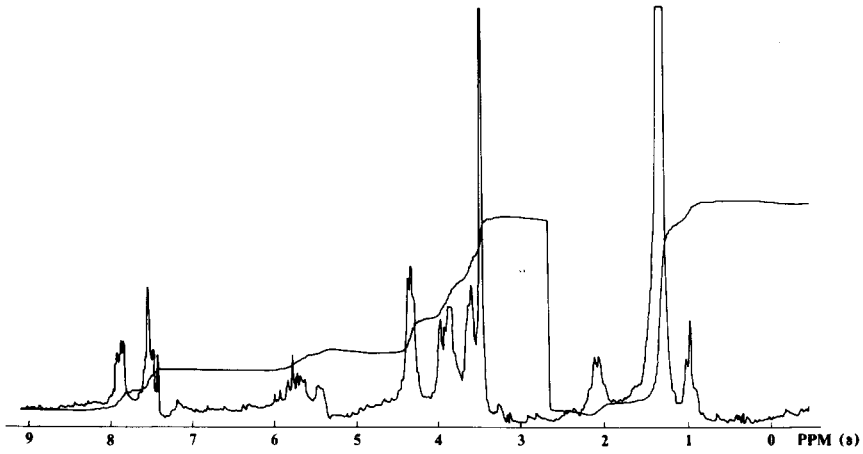


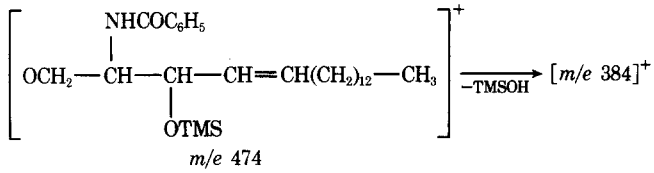
Fig. 4. The 100-MHz NMR spectrum of cerebroside II. The sample (10 mg), after deuterium exchange, was dissolved in 0.3 ml of $CDCl_3$–CD_3OD 9:1, and the spectrum was run at room temperature with a Varian HA-100 spectrometer equipped with Fourier transform accessories.

fatty acids as the original cerebrosides. The cerebrosides recovered from the benzoic anhydride derivatives had a fatty acid composition the same as the original cerebrosides except the relative amount of unsaturated fatty acids was decreased by about 50%. Rebenzoylation of these cerebrosides (with benzoic anhydride) resulted in benzoylated derivatives that had identical TLC and HPLC behavior as the original benzoylated cerebrosides.

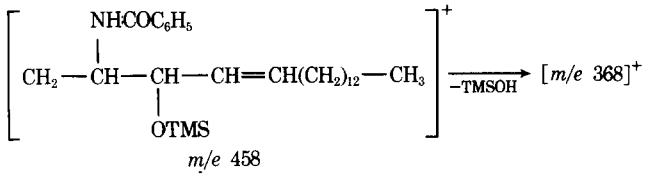
Identification of cerebroside fraction II as *N*-benzoyl psychosine

Acher and Kanfer (2) demonstrated that benzoylated cerebrosides, prepared with benzoyl chloride in pyridine, could be readily separated into two fractions (eluates 1 and 2) by silicic acid column chromatography. When eluate 1 was treated with 1 N sodium methoxide in methanol, a precipitate (cerebroside I) formed, and the material obtained from the mother liquor (cerebroside II) was reported to be a cerebroside that contained mainly short-chain and unsaturated nonhydroxy fatty acids.

A sample of cerebroside fraction II, obtained from Dr. Julian Kanfer, was dissolved in methanol, filtered, and precipitated with 10 vol of acetone. A portion of this material was sent to Dr. Charles C. Sweeley (Michigan State University, East Lansing, Mich.) for mass spectrometric analysis. Direct probe analysis of the trimethylsilyl ether derivative revealed a strong ion at *m/e* 311 for $[CH_3(CH_2)_{12}CH{=}CHCHOTMS]^+$, indicating that the sphingosine portion was normal, and the usual ions at *m/e* 451 and 361 indicated the presence of a normal hexose residue. There were a number of peaks that suggested the presence of a benzoyl group, the clearest being a strong ion at *m/e* 105 for the benzoyl group itself. The presence of the benzoyl group as an amide derivative of psychosine was evidenced by the presence of the following ions:



m/e 474



m/e 458

The mass spectra data suggest that the cerebroside fraction II is the *N*-benzoyl derivative of psychosine.

The GLC behavior of cerebroside II was examined by Dr. Roger Laine in Dr. Sweeley's laboratory. The trimethylsilyl ether derivative was chromatographed on an OV-101 (0.05% on AC 110, 120–140) column programmed from 150 to 300°C at 10°C/min. The material eluted just after psychosine and long before cerebrosides. It also gave only one peak, indicating the absence of various fatty acids.

A 100-MHz NMR spectrum of cerebroside II (Fig. 4) was obtained by Dr. Brian Sykes (Dept. of Chemistry, Harvard University, Cambridge, Mass.). Aromatic resonances seen at 7.5–8.0 ppm provided further evidence for the presence of a benzoyl residue. Examination of the integration curve revealed that the ratio of aromatic protons to methyl protons (1.00 ppm) was 1.8; the value expected for *N*-benzoyl psychosine was 5/3, or 1.7. Thus, the NMR data provide evidence for the presence of one benzoyl group per sphingosine residue in this compound. The NMR spectra of the other debenzoylated product (cerebroside I) indicated the absence of any benzoyl residues. The UV spectra of cerebroside fraction II showed a λ_{max}^{EtOH} of 225 nm and ϵ_{max} of 10,355.

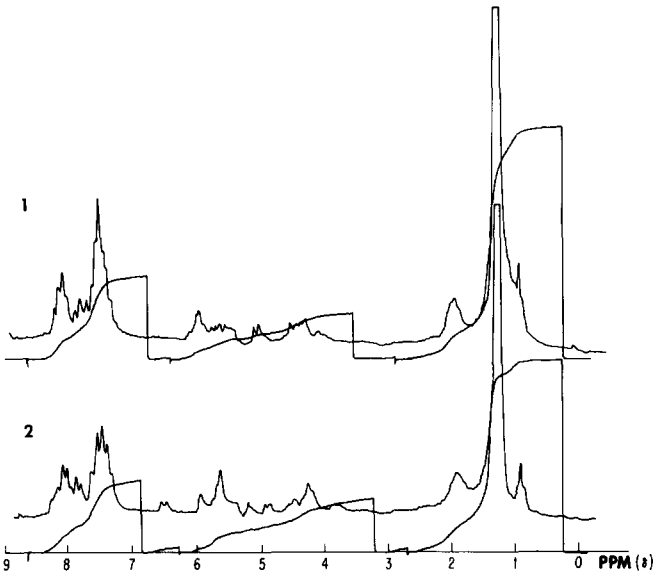


Fig. 5. Fourier transformation 100-MHz NMR spectra of the benzoylated derivatives of NFA-CR (trace 1) and HFA-CR (trace 2) prepared by reaction with benzoyl chloride. The spectra were obtained on 10 mg of each derivative in 0.4 ml of CCl_4. Each spectra represents 256 transients, with 0.4 sec acquisition time per transient and sweep width of 1000 Hz.

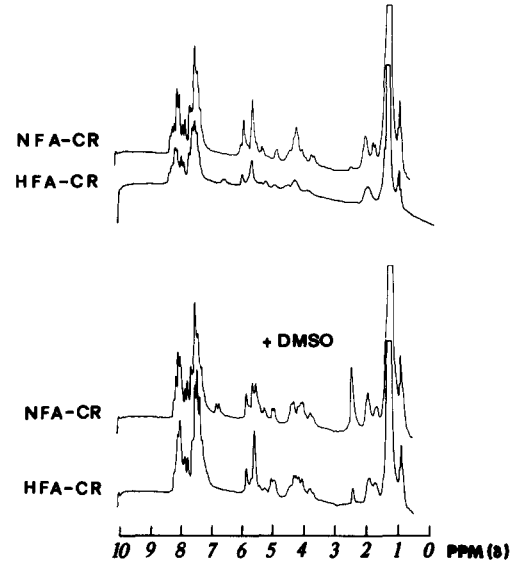


Fig. 6. Fourier transformation 100-MHz NMR spectra of the benzoylated derivatives of NFA-CR and HFA-CR prepared by reaction with benzoic anhydride. The spectra were obtained on 10 mg of each derivative in 0.4 ml of CCl_4 and after the addition of 3 drops of dimethyl sulfoxide. Each spectrum represents 100 transients, with 1.0 sec acquisition time per transient and sweep width of 1000 Hz.

A preparation of psychosine (prepared from beef brain cerebroside) was obtained from Dr. Yasuo Kishimoto. This material was *N*-benzoylated with 2% benzoyl chloride in tetrahydrofuran–50% aqueous sodium acetate 5:4 (v/v) for 90 min at room temp. 5 vol of chloroform–methanol 2:1 was added to the reaction mixture followed by 1 vol of H_2O. After mixing, the upper phase was discarded and the lower phase was washed twice with 50% methanol. The dried material from the lower phase, benzoyl psychosine, was approximately 95% pure as judged by TLC. The synthetic benzoyl psychosine cochromatographed with cerebroside fraction II in two solvent systems (chloroform–methanol–pyridine–water 40:10:1:1, and chloroform–methanol–water 70:20:3), and the two materials had identical IR and UV spectra.

We conclude from the data presented above that cerebroside II is *N*-benzoyl psychosine rather than a cerebroside containing mainly short-chain and unsaturated nonhydroxy fatty acids as reported by Acher and Kanfer (2). It seems likely that the fatty acids initially reported as components of this material originated from a minor cerebroside contaminant.

Spectrometric analysis of the benzoylated cerebrosides

10 mg of each of the NFA-CR and HFA-CR benzoyl derivatives formed in the reaction with benzoyl chloride (peaks Ia and IIa, Fig. 1) were dissolved in 0.4 ml of CCl_4 for NMR analysis. These spectra are shown in Fig. 5. The resonance signals have been assigned as follows: 7.2–8.2 ppm, aromatic protons; 6.5 ppm, secondary amide proton; 4.9 ppm, anomeric proton of the galactose ring; and 0.8–2.0 ppm, methyl and methylene protons. The secondary amide resonance (6.5 ppm) is present in the HFA-CR spectra but is not seen in the NFA-CR spectra. Both the secondary amide proton and the anomeric proton appear as well-resolved doublets with coupling constants of 8 and 7 Hz, respectively. Recently, Martín-Lomas and Chapman (5) reported 220-MHz NMR spectra of acetylated galactocerebrosides that were amenable to first-order analysis. The chemical shift of the amide proton in Cl_3CD was 5.7 and 6.3 ppm for the NFA-CR and HFA-CR derivatives, respectively, and varied with concentration and solvent used. The coupling constant of —NH—CH— protons was reported as 8–9.5 Hz. Our data and signal assignments are entirely consistent with these 220-MHz studies.

From the integration curves shown in Fig. 5, the ratio of combined methyl and methylene proton signals to the aromatic proton signals was found to be 2.7 for the HFA-CR derivative (trace 2) and 2.8 for the NFA-CR derivative (trace 1), indicating that the number of benzoyl residues in each is the same. The ratio of the aromatic proton peak area to the amide proton peak area (which has the same area as the β-anomeric proton) in the HFA-CR spectrum was determined to be 30.1 by the "cut-and-weigh" method. These data suggest that there are six benzoyl residues present in both compounds and that the NFA-CR derivative contains an amide-linked benzoyl group.

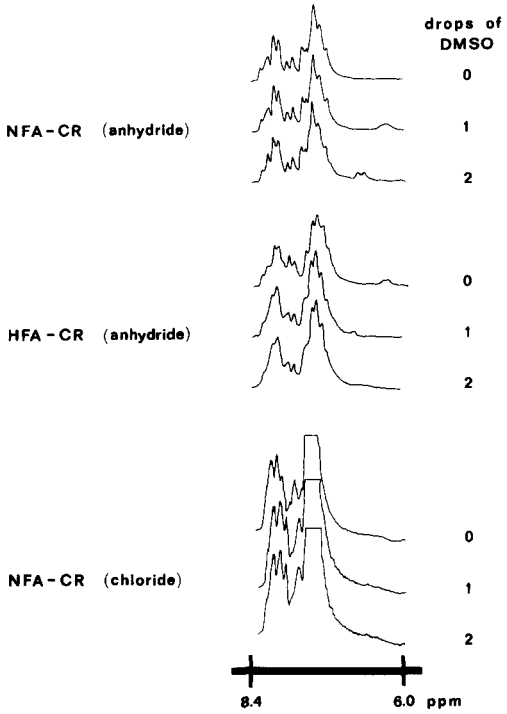


Fig. 7. Effect of dimethyl sulfoxide (DMSO) on the NMR spectra of benzoylated cerebrosides. The spectra were obtained as described in Fig. 6, except expanded partial spectra were recorded and the NFA-CR (chloride) spectra represent 250 transients. Note that DMSO caused the resonance signal attributable to the amide proton to shift downfield so that it is readily apparent at 6.8 ppm in the NFA-CR (anhydride) spectra. The amide proton signal originally exposed at about 6.3 ppm in the HFA-CR (anhydride) spectra is seen to move downfield and become obscured in the aromatic proton signals after addition of DMSO. The amplified spectra of the NFA-CR (chloride) derivative show the absence of a signal in this region either before or after the addition of DMSO.

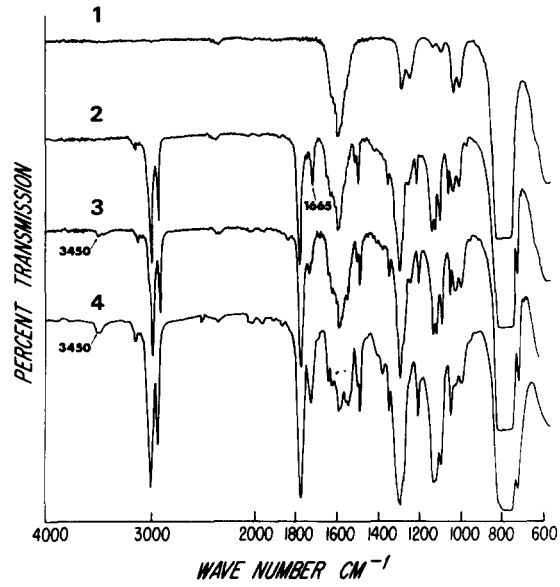


Fig. 8. IR spectra of the benzoylated derivatives of cerebrosides. The solvent (CCl_4) spectra is trace 1. The spectra of the NFA-CR and HFA-CR derivatives prepared by reaction with benzoyl chloride are traces 2 and 3, respectively; the NFA-CR derivative prepared with benzoic anhydride is trace 4. The spectra were recorded on 10 mg of each sample in approximately 0.5 ml of CCl_4 in a KCl liquid cell with a 0.18-mm light path. Identification of absorption bands: 3450 cm^{-1}, amide N—H stretching; 1665 cm^{-1}, diacylamine carbonyl stretching. The wave numbers of the absorption bands were corrected with reference to the CCl_4 absorption peaks.

10 mg of each of the NFA-CR and HFA-CR derivatives formed in the reaction with benzoic anhydride were dissolved in 0.4 ml of CCl_4 for NMR analysis. Secondary amide resonance was seen in HFA-CR spectra but, surprisingly, it was not visible in the NFA-CR spectra. Close inspection of the spectra suggested that the amide proton of the NFA-CR derivative might be observed in the 5–6 ppm region. In order to produce a downfield shift of the suspected amide proton signal, a series of spectra was obtained after the addition of several drops of dimethyl sulfoxide. The complete spectra of these derivatives before and after the addition of dimethyl sulfoxide are shown in Fig. 6. Partial spectra demonstrating the effect of increasing amounts of dimethyl sulfoxide are shown in Fig. 7. After the addition of dimethyl sulfoxide, the secondary amide proton peak of the NFA-CR derivative (anhydride) became distinct at 6.8 ppm, while that of the HFA-CR (anhydride) shifted downfield and became buried in the aromatic proton peaks. The effect of dimethyl sulfoxide on the spectra of the NFA-CR derivative formed by reaction with benzoyl chloride is also shown. No resonance attributable to a secondary amide proton was seen in the NFA-CR (chloride) spectra either before or after the addition of dimethyl sulfoxide. The ratio of the aromatic proton peak area to amide proton peak area for the HFA-CR (anhydride) derivative was approximately 1.2 times greater than that ratio for the NFA-CR (anhydride) derivative (Fig. 6). These NMR data suggest that the HFA-CR and NFA-CR derivatives have six and five benzoyl residues, respectively, and that there is no significant diacylamine formation in the reaction with benzoic anhydride.

The NFA-CR and HFA-CR derivatives formed from benzoyl chloride and the NFA-CR derivative formed from benzoic anhydride were dissolved in CCl_4 for IR spectrometry. These spectra are shown in Fig. 8. The absorption band at 3450 cm^{-1} seen in the spectra of benzoylated NFA-CR (anhydride) and HFA-CR (chloride) can be attributed to amide N—H stretching. The unique strong absorption band at 1665 cm^{-1} in the benzoylated NFA-CR (chloride) spectrum is characteristic of *N*-acyl-acylamino (diacylamine) compounds, as reported by Inch and Fletcher (6).

DISCUSSION

Inch and Fletcher (6) have reported that replacement of the nitrogen-attached proton in 2-acetylamine-2-deoxy-D-glucose derivatives by a benzoyl group to form the di-

acylamine readily occurs when benzoyl chloride in pyridine is employed as the acylating reagent. These workers were unable to detect diacylamine formation when benzoic anhydride was used. Upon alkaline treatment these diacylamine derivatives readily lose one of the nitrogen-attached acyl groups. Their work indicates that the *N*-acetyl and *N*-benzoyl groups compete for elimination with alkaline reagents, which results in the formation of a mixture of *N*-acetyl and *N*-benzoyl glucosamine derivatives.

The data presented above indicate that NFA-CR reacts in an analogous manner to form *N*-fatty acyl benzoyl psychosine derivatives when treated with benzoyl chloride in pyridine. The diacylamine degrades upon treatment with mild alkali to form roughly equal amounts of benzoyl psychosine and NFA-CR. The following evidence supports the diacylamine structure for the NFA-CR benzoyl chloride product: the material behaves as a single compound by TLC and HPLC; the IR spectrum shows a strong amide carbonyl stretching band at 1665 cm^{-1} as reported for diacylamines and no amide N—H stretching or bending and no OH stretching bands; the NMR spectrum indicates the absence of a secondary amide proton, and upon alkaline treatment the material gives rise to NFA-CR and benzoyl psychosine. No evidence was found for the presence of an *N*-benzoyl residue in the derivative formed from HFA-CR. The NMR studies of the derivatives formed from benzoic anhydride revealed that the amide proton of the benzoylated HFA-CR is downfield from the amide proton of the NFA-CR derivative. This shift is assumed to be a result of hydrogen bonding of the amide proton with the carbonyl oxygen of the benzoyl group on the fatty acid. Derivatization with benzoic anhydride does not lead to any significant amide-acylation, and the original cerebrosides can be regenerated by treatment of these benzoylated cerebrosides with mild alkali, although some loss of unsaturated fatty acids was noted.

The primary motivation for studying the structure of these derivatives has been their use for the HPLC analysis of glycolipids. Results on the quantitative aspects of the HPLC analysis of derivatized cerebrosides will be published elsewhere.

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