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SYNTHETIC INHIBITORS OF GALACTOCEREBROSIDE BIOSYNTHESIS

RAMESH C. ARORA AND NORMAN S. RADIN

Mental Health Research Institute, University of Michigan, Ann Arbor, Mich. 48104 (U.S.A.)

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SUMMARY

1. The brain enzyme which forms galactosyl ceramide ("cerebroside") from UDPGal and hydroxy ceramide was assayed *in vitro* with a rat brain preparation. Various synthetic substances resembling hydroxy ceramide were tested as possible inhibitors. Strong inhibition was obtained with *N*-bromoacetyl DL-erythro-3-phenyl-2-amino-1,3-propanediol. The degree of inhibition was found to be independent of length of incubation, and kinetic analysis indicated that the amide acted as a non-competitive inhibitor with respect to either substrate.

2. Deletion of either one of the two hydroxyl groups in the amide weakened the inhibitory effect, as did replacement of the bromine atom by hydrogen, chlorine, or iodine.

INTRODUCTION

As part of a program to develop methods for influencing the deposition of myelin, we have been synthesizing compounds which might act as inhibitors of the enzymes involved in sphingolipid metabolism. We have recently described compounds which inhibit or stimulate galactocerebroside β -galactosidase^{1,2}. These are fatty acyl amides of amino alcohols and thus resemble ceramide, which can be described as *N*-acyl 3-alkyl-2-amino-1,3-propanediol. Replacement of the alkyl group by an aromatic group led to inhibitory activity and replacement of the alkyl group by a 2-methyl group led to stimulatory activity.

Another enzyme carries out the reverse reaction, the transfer of galactose from UDPGal to ceramide^{3,4}. Unlike the galactosidase, which appears to act about equally well with cerebroside containing hydroxy and nonhydroxy fatty acids⁵, the galactosyltransferase shows a marked preference for ceramides containing 2-hydroxy fatty acids. It thus seemed appropriate to synthesize compounds which resemble the lipoidal substrate in possessing a substituent in the 2-position of the acyl group. This paper describes the inhibitory action of such compounds on the UDPGal: ceramide galactosyltransferase of rat brain.

MATERIALS AND METHODS

Materials

Acyl halides and norephedrine (DL-*erythro*-3-phenyl-2-amino-3-propanol) were bought from Eastman Organic Chemicals. L-Phenylalaninol (L-3-phenyl-2-amino-1-propanol) was obtained from Gallard-Schlesinger Chemical Mfg. Corp. DL-*erythro*-3-phenyl-2-amino-1,3-propanediol and its *p*-nitro derivative were gifts from Parke, Davis and Co.

The reagents used in the enzyme assay have been described before³.

Synthesis of inhibitors

1 mmole of norephedrine (188 mg) was dissolved in a mixture of 3 ml of tetrahydrofuran (freshly distilled from KOH) and 5 ml of 50% sodium acetate·3H₂O solution (w/v). While stirring this mixture we added 1.4 mmoles of chloroacetyl chloride (0.1 ml) in 2 ml tetrahydrofuran; half of this was added 5 min after the first portion. After stirring for 2 h we left the mixture in the cold room overnight and then isolated the amide by partitioning with 18 ml chloroform and 2 ml methanol. The lower layer was washed with water until neutral, then evaporated in vacuo to an oil. The amide showed a single spot after thin-layer chromatography on silica gel PF-254 (Brinkmann-Merck) with chloroform-methanol-acetic acid (88:4:8, by vol.), using a chromate-sulfuric spray.

Similar amides were prepared by the same procedure from bromoacetyl bromide, 2-bromopropionyl chloride, and 3-bromopropionyl chloride. All the norephedrine and phenylalaninol derivatives showed a single spot on thin-layer chromatography, but the derivatives of phenylaminopropanediol contained a nonpolar impurity which required a purification by silica gel column chromatography. The impurity was eluted with benzene-chloroform (1:1), and the amides were eluted with chloroform-methanol (98:2).

The bromine content of bromoacetyl 3-phenyl-2-amino-1,3-propanediol (m.p. 160–161 °C) was found by the Spang Microanalytical Laboratory to be 27.8% (theor. for C₁₁H₁₄O₃NBr is 27.8% Br). The infrared spectrum in a KBr pellet contained a complex system of peaks (Fig. 1). The two characteristic amide peaks are visible at 6.03 and 6.45 μm. While the small peak at 5.7 μm is suggestive of contamination with an ester, this peak is often seen with other lipoidal amides. The expected strong hydroxyl peak at 3.05 μm is visible, as well as many bands from the benzene ring.

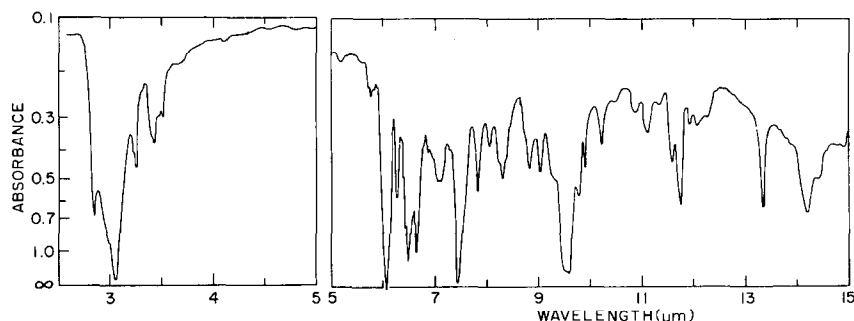


Fig. 1. Infrared spectrum of *N*-bromoacetyl DL-*erythro*-3-phenyl-2-amino-1,3-propanediol.

Bromoacetyl norephedrine ran at R_F 0.51 in the thin-layer chromatographic system described above while the reference compounds, hydroxy ceramide and non-hydroxy ceramide ran at R_F 0.46 and 0.78, respectively. Bromoacetyl phenylamino-propanediol ran at 0.26.

The iodo derivatives were prepared from the bromo derivatives by exchange. 1 mmole of bromo compound was refluxed 2 h with 1.5 mmoles of NaI in 5 ml of acetone. The precipitate of NaBr was removed by filtration, the acetone was removed by evaporation, and the iodo compound was washed by partitioning between chloroform and water. It exhibited a single spot on thin-layer chromatography.

Enzyme assay

UDPGal and dithiothreitol were purchased from Calbiochem. [^{14}C]UDPGal came from New England Nuclear Corp.

Rats of the Sprague-Dawley strain, 16 days old, were killed by decapitation and the brains were homogenized with 6 vol. of 0.25 M sucrose containing 10 mM nicotinamide. After addition of 3 vol. more of homogenizing solution, we centrifuged the suspension for 15 min at $11000 \times g$. Microsomes were prepared from the supernatant by centrifugation at $105000 \times g$ for 45 min. The particles were washed by suspension in water and highspeed centrifugation. The preparation was then lyophilized and stored under vacuum over P_2O_5 overnight at -20°C . We then extracted the dried microsomal material four times with cold acetone, each time for 15 min, removing the extracts by centrifugation. Most of the solvent was removed from the pellet by airdrying in the cold room 1 h, and the powder was stored *in vacuo* as above.

To assay the inhibitory effect of the synthetic compounds, we evaporated a solution of amide to dryness (usually 60 μmoles) in a 13 mm \times 100 mm screw cap test tube. To the tube was then added a solution of hydroxy ceramide³ in benzene (0.4 mg) and a suspension of microsomal acetone powder (2 mg) in 0.1 ml benzene. The mixture was evaporated to dryness with a stream of nitrogen. To this was added 0.2 ml of a solution containing 0.5 μmole of ATP, 10 μmoles of Tris-HCl (pH 7.4), 0.2 μmole dithiothreitol, 0.4 μmole EDTA, 1 μmole nicotinamide, 1 μmole MgCl_2 and 16 nmoles of UDPGal (40000 cpm). The insoluble material was suspended in the liquid by immersing the capped test tubes in a cold bath in an ultrasonic cleaner, then shaken 2 h at 37°C .

The biosynthesized hydroxy cerebroside was isolated by the addition of 4 ml of chloroform-methanol (2:1, by vol.) containing 0.1 mg of a mixture of brain sphingolipids⁶, followed by 1 ml of 0.88% KCl in methanol-water (1:1, by vol.). The lower layer, after centrifugation of the mixture, was washed with 2×2 ml of methanol-water (1:1, by vol.), evaporated to dryness, and counted by liquid scintillation⁷. Percentage inhibition was calculated from the decrease in observed cpm compared with the control, inhibitor-free tubes. Triplicate assays were run and the control activities were about 7850 cpm, corresponding to 2.45 nmoles of cerebroside.

RESULTS

Inhibition of cerebroside synthesis

The ability of various haloacyl amides of DL-erythro-3-phenyl-2-amino-3-propanol to inhibit galactocerebroside synthesis is quite sensitive to the nature of the acyl

TABLE I

INHIBITION OF CERAMIDE GALACTOSYLTRANSFERASE BY AMIDES OF 3-PHENYL-2-AMINO-3-PROPANOL
Each value shown is the mean of a set of triplicate determinations. Assay procedure as in text.

Acyl group	Inhibition (%)	
	Run 1	Run 2
-COCH ₃	3	
-COCH ₂ Cl	10	8
-COCH ₂ Br	50	48
-COCH ₂ I	40	36
-COCH ₂ CH ₂ Br	24	20
-COCH ₂ CH ₂ I	12	10
-COCHBrCH ₃	18	14

group (Table I). Omission of the halogen group yields an inert compound and a chlorine atom in the acetyl group results in negligible activity. The best inhibitor is the bromoacetyl derivative. The effectiveness of the bromine atom is reduced by adding another C atom to the acyl group. The iodoacetyl amide is almost as active as the bromoacetyl, and here too an additional C atom weakens the effect.

The addition of a second hydroxy group (bromoacetyl DL-erythro-3-phenyl-2-amino-1,3-propanediol) raises the inhibition to 71% at 0.3 mM amide, but moving the single hydroxyl group to the 1-position brings the inhibition down to 33%. Adding a *p*-nitro group to the diol also weakens the effect (31% inhibition).

Nature of the inhibition

A comparison of the effects of the two best inhibitors at different concentrations of ceramide showed (Figs 2 and 3) that they act as noncompetitive inhibitors. The K_m of the enzyme with respect to ceramide was $2.1 \cdot 10^{-3}$ M (the molecular weight of the ceramide was estimated to be 650). A similar plot (Fig. 4) with different concentrations of UDPGal and the bromoacetyl diol showed that the inhibition was also noncompetitive with respect to the substrate sugar nucleotide. From the intercept on the left abscissa the K_m for the enzymatic binding of UDPGal was found to be $2.1 \cdot 10^{-4}$ M.

Although the iodo compounds were found to be less inhibitory than the bromo compounds (which should be less reactive chemically), it seemed possible that the

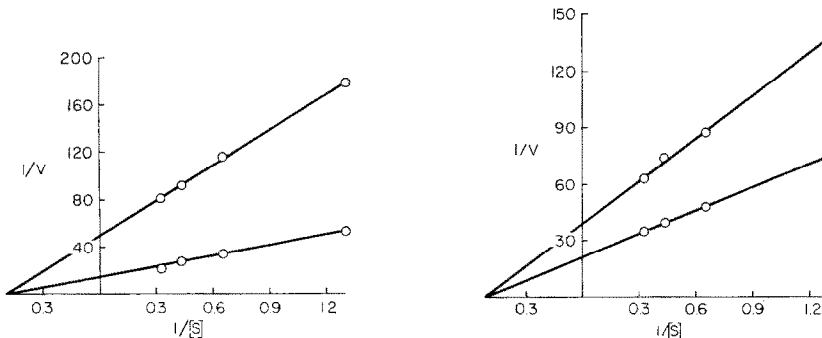


Fig. 2. A Lineweaver-Burk plot relating substrate concentration to reaction rate. $[S]$ is the concentration of hydroxy ceramide in $\mu\text{moles/ml}$. v is the radioactivity in the cerebroside formed in 2 h. Upper line: incubations in the presence of 0.3 mM bromoacetyl phenylaminopropanediol. Lower line: control incubations.

Fig. 3. A plot like that in Fig. 2 but with bromoacetyl norephedrine (upper line).

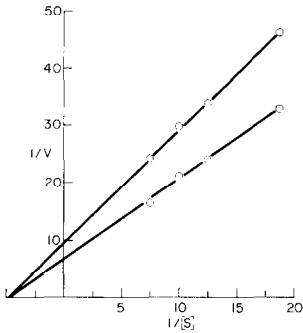


Fig. 4. A plot like that in Fig. 2 but with different UDPGal concentrations ($[S]$ is μ moles/ml). The inhibitor is 0.3 mM (upper line).

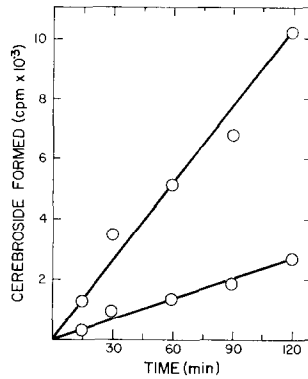


Fig. 5. The rate of cerebroside synthesis as a function of duration of the incubation. Lower line: incubation with added 0.3 mM bromoacetyl phenylamino propanediol. Upper line: control tubes.

inhibitors act by reaction of the halogen group with some group, such as a mercaptan, in the enzyme molecule. However, the effectiveness of the best inhibitor seems to be independent of time of incubation (Fig. 5).

DISCUSSION

Comparison of the inhibitory effectiveness of the various compounds tested indicates that the strongest effect is produced by the compound which most strongly resembles the lipoidal substrate, hydroxy ceramide. Both compounds possess the sequence, 2-amino-1,3-propanediol; both have a lipoidal group in the 3-position; the OH in the 3-position has the *erythro* configuration; and both have a bulky group in the 2-position of the acyl group. Omission of the bromide in the acyl group of the inhibitor renders it ineffective and omission of the hydroxyl in the acyl group of the substrate greatly reduces its effectiveness. A flaw in this comparison is the reduced inhibition seen with the 2-bromopropionyl derivative (Table I); one would expect the longer acyl group to be more effective since the acyl groups in the substrate are quite long.

From the early appearance of the inhibition by bromoacetyl phenylamino propanediol (Fig. 5), it would appear that no chemical reaction between the enzyme and bromoacetyl group is involved. From this observation and the appearance of the Lineweaver-Burk plots, it would seem that there is an effector-sensitive site on the galactosyltransferase which is different from the sites which bind the two substrates. It is interesting that a similar type of site is present in galactocerebroside galactosidase (ref. 1).

In a separate experiment we showed that the bromoacetyl compound inhibits the galactosyltransferase equally well when nonhydroxy ceramide is used as the galactose acceptor. This is additional evidence for the idea that a single transferase is involved in the formation of both types of galactocerebroside, hydroxy and nonhydroxy. Nonhydroxy ceramide is a much poorer galactose acceptor than the hydroxy analogue, a difference which seems consistent with the ineffectiveness of the synthetic inhibitors lacking the bulky bromine atom in the acetyl group.

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