Ceramide-like synthetic amides that inhibit cerebroside galactosidase

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Abstract Amides resembling ceramide (fatty acyl sphingosine) were synthesized and tested for their effects on rat brain cerebrosidase (galactosyl ceramide β-galactosidase). The best inhibitor was *N*-decanoyl DL-*erythro*-3-phenyl-2-aminopropanediol, which exhibited a K_i of 0.4 mM. A Lineweaver-Burk plot indicated that the amide acted as a noncompetitive inhibitor, presumably by attachment to a site other than the substrate-active site. Preincubation did not affect the degree of inhibition, and inhibition was independent of incubation duration; these observations suggest that the inhibitor does not combine with the enzyme irreversibly. Structural variations produced decreased inhibitory activity: loss of one of the hydroxyl groups, replacement of the aromatic side chain with an aliphatic or substituted phenyl group, or isomeric inversion of the 3-hydroxyl group. It appears that the best activity is obtained with a substance most closely resembling natural ceramide. The cerebrosidases of rat spleen, kidney, and liver are also inhibited by the same amide.

Supplementary key words *N*-decanoyl 3-phenyl-2-amino-propane-1,3-diol and homologs · *N*-decanoyl 3-phenyl-2-amino-propan-3-ol and homologs · *N*-decanoyl 3-phenyl-2-amino-propan-1-ol and homologs · *N*-decanoyl 3-(*p*-nitro-phenyl)-2-amino-propane-1,3-diol · *N*-decanoyl 3-(*m*-nitro-phenyl)-2-amino-propane-1,3-diol · *N*-decanoyl 3-(*p*-bi-phenyl)-2-amino-propane-1,3-diol · *N*-decanoyl 2-aminopro-panol and homologs · *N*-decanoyl 2-amino-butan-1-ol and homologs · *N*-decanoyl 1-amino-propan-2-ol and homologs · galactocerebrosidase of rat brain, liver, kidney, and spleen

CEREBROSIDASE is a lysosomal hydrolase which acts on brain cerebrosides to produce galactose and ceramide (1–3). Compounds similar to ceramide might conceivably act as inhibitors of the enzyme by combining with the lipid-binding region of the substrate-active site. As part of a continuing study of the physiological role of cerebrosides in brain, we have synthesized a number of amides as potential inhibitors and tested them for activity in vitro, preparatory to testing their effects in vivo. Particularly studied were compounds related to 3-phenyl-2-amino-1,3-propanediol, which bears considerable resemblance to sphingosine. Sphingosine could be named 3-pentadecenyl-2-amino-1,3-propanediol, and the double bond in the natural lipid occurs in the same position as one of the double bonds of the benzene ring (disregarding resonance). The presence of two asymmetric carbon atoms in these substances results in the existence of D- and L-*erythro* and D- and L-*threo* forms; natural sphingosine is the D-*erythro* form (4).

The influence of isomerism, side chains, fatty acid structure, and hydroxyl groups has been studied. Some of the compounds tested proved to be stimulatory, and they will be described separately.

MATERIALS AND METHODS

Preparation of inhibitors

3-Phenyl-2-amino-1,3-propanediol was a gift from Parke, Davis & Co., Ann Arbor, Mich., as were the nitro and phenyl derivatives of this compound. The aliphatic amines and 3-phenyl-2-amino-3-propanol (norephedrine) were bought from Eastman Organic Chemicals, Rochester, N.Y.; norephedrine is the DL-*erythro* compound. 3-Phenyl-2-amino-1-propanol (L-phenylalaninol) was obtained from Fluka AG through Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N.Y. Fatty acids and acyl halides of high quality were obtained from commercial sources. Tetrahydrofuran was freshly distilled from KOH.

Abbreviations: I, 3-phenyl-2-amino-1-propanol; II, 3-phenyl-2-amino-3-propanol; III, 3-phenyl-2-amino-1,3-propanediol; IV, the *p*-nitro derivative of III; V, the *m*-nitro derivative of III; VI, the *p*-phenyl derivative of III; VII, 2-aminopropanol; VIII, 2-amino-2-methylpropanol; IX, 2-amino-2-methyl-propanediol; X, 2-amino-1-butanol; XI, 1-amino-2-propanol.

Two methods were used for the acylations. The phenyl derivatives were prepared by a modified version of the Shapiro-Flowers procedure (5). In this procedure, 1 mmole of amino alcohol was dissolved in a mixture of 3 ml of tetrahydrofuran and 5 ml of 50% (w/v) sodium acetate·$3H_2O$. A solution of acyl chloride (1.2 mmoles in 2 ml of tetrahydrofuran) was added while stirring, and stirring was continued for 2 hr; the mixture was then kept in the cold overnight. The amide was isolated by partitioning with added solvents (24 ml of chloroform, 12 ml of methanol, and 9 ml of water) and the material in the lower layer was washed with water and purified by column chromatography on silica gel (Unisil, Clarkson Chemical Co., Williamsport, Pa.). Nonpolar impurities were removed with benzene–chloroform 1:1 and the amides were eluted with chloroform containing 1 or 2% methanol. Fractions were examined by thin-layer chromatography on silica gel PF-254 (Brinkmann-Merck), using chloroform–methanol–acetic acid 88:4:8. The purified compounds showed a single spot or a trace of a second spot.

Elemental analysis of *N*-octanoyl 3-phenyl-2-amino-1-propanol showed satisfactory agreement with theoretical values:

Analysis: $C_{17}H_{27}O_2N$ (mp 71–72°C);
calculated: C, 73.87; H, 9.85
found: C, 73.64; H, 9.74

For the decanoyl derivative of *erythro*-phenylamino-propanediol:

Analysis: $C_{19}H_{32}O_3N$ (mp 100–101°C);
calculated: C, 70.77; H, 10.00
found: C, 71.02; H, 9.6

Infrared spectra of some of the compounds showed the expected peaks for amide and hydroxyl groups; no ester absorption peak was visible. All the compounds appear to be new. Melting points (except for low-melting compounds) are listed in Table 3.

Acylation of the aliphatic amino alcohols was carried out in dimethylformamide, as recommended for similar acylations (6, 7). 2 mmoles of amino alcohol was dissolved in 5 ml of dimethylformamide (dried over CaH_2) and, with stirring, 1 mmole of acyl halide was added dropwise, without solvent. The mixture was stirred 2 hr, then purified as above.

Elemental analysis of *N*-myristoyl 2-methyl-2-amino-1-propanol:

Analysis: $C_{18}H_{37}O_2N$ (mp 48–49°C);
calculated: C, 72.19; H, 12.45
found: C, 72.24; H, 12.3

The elemental analyses were carried out by Spang Microanalytical Laboratory, Ann Arbor.

The yields in the two procedures were over 50%; most were above 65%. The inhibitors were stored over desiccant at room temperature, but solutions in chloroform–methanol were stored at −20°C.

Enzyme assay

The enzyme was prepared as the pH 3 precipitate from brains of rats weighing 80–100 g as described before (8) and was assayed by a simplified procedure (9). The synthetic compound to be tested was evaporated from solution on the walls of the incubation tube (generally 0.3 μmole) and the radioactive cerebroside emulsion was added, followed by diluent, citrate buffer, and enzyme suspension.

The substrate was prepared according to the procedure used in preparing [^{3}H]lactoside (10), except that the initial oxidation step was carried out in a more dilute solution. Brain cerebrosides (33 mg) were dissolved in 33 ml of tetrahydrofuran and 33 ml of phosphate buffer, then shaken gently for 4 hr with 0.5 ml of galactose oxidase solution. This portion of enzyme contained 500,000 KABI units, which assayed to be 250 units in the Worthington modification of the assay method of Avigad et al. (11). An additional portion of oxidase was added (0.2 ml), incubation was continued overnight, and the aldehyde was processed as described before.

The inhibitions are reported, in percentages, as the decrease in activity caused by the added amide compared with a control incubated without amide.

RESULTS

Inhibition by aromatic amides

The effect of fatty acyl group chain length is shown for the amides of amine IV in Table 1. All compounds tested were found to be slightly inhibitory, with the largest effect coming from amides of intermediate length fatty acids, such as decanoate. No sharp differences between optical isomers were seen. The naturally occurring antibiotic chloromycetin (*N*-dichloroacetyl amide of the D-*threo* isomer of IV) yielded only 4% inhibition.

Since decanoate was one of the most effective acids, a variety of decanoates were tested (Table 2). The best inhibitor was DL-*erythro*-III, which resembles ceramide the most closely of all the compounds in possessing the *erythro* configuration and the 1,3-propanediol grouping. Omission of the hydroxyl at position 1 caused complete loss of inhibitory power (compound II) and omission of the 3-OH caused a lesser loss (compound I).

Comparison of the nitro derivatives (IV) also shows the *erythro* form to be more effective than the *threo* forms.

TABLE 1. Inhibition of cerebrosidase by fatty acid amides of *p*-nitrophenylaminopropanediol (amine IV)

Fatty Acid	L-*threo* Isomer	D-*threo* Isomer	DL-*erythro* Isomer
18:0	8 (92–93°C)[a]	11 (94–95°C)	11 (119–120°C)
16:0	15 (86–88°C)	17 (87–88°C)	
14:0	21 (78–79°C)	19 (80–82°C)	
12:0	22 (62–63°C)	19 (58–60°C)	
10:0	18	15	26 (110–112°C)
8:0	(112–113°C)	16 (113–114°C)	11 (135–136°C)
6:0			6 (107–108°C)

Data are percentages of inhibition compared with control incubations. Inhibitor concentration was 0.3 mM.

[a] Numbers in parentheses are melting points of the amides.

Movement of the nitro group to the *meta* position (V) eliminated the inhibitory effect but elongation of the lipoid segment of the amino alcohol had little effect (VI vs. III and IV).

Conceivably, the inhibitory amides might act after first being hydrolyzed by an amidase present in our crude enzyme preparation. A test with 0.3 μmole of amine *erythro*-III resulted in 12% inhibition, and decanoic acid gave 3% inhibition. It is unlikely that these small effects could account for the much larger inhibition by the same concentration of the amide.

Preincubation of the decanoates of III and IV *erythro* compounds at 37°C for 30 min with the enzyme, diluent, and citrate buffer did not affect the degree of inhibition.

Several amides of DL-*erythro*-II were prepared using halogenated acyl groups: chloroacetyl, bromoacetyl, iodoacetyl, 3-bromopropionyl, and 3-iodopropionyl. These were tested by preincubating as above prior to adding the substrate and incubating for 3 hr. All five compounds were found to be inactive, evidently because their short chain length kept them from binding to the enzyme. They were, however, active inhibitors toward a related enzyme, UDPGal:ceramide galactosyltransferase.[1]

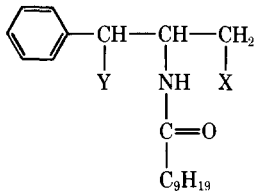
Inhibition by aliphatic amides

The fatty acid amides of 2-amino-1-butanol exhibited only weak inhibitory activity. The decanoyl to palmitoyl derivatives yielded 10–11% inhibition, and the stearate derivative, only 5%. Comparison of the decanoates of amines VII to XI showed these amides to be weakly inhibitory or, in the case of VIII and IX, to be stimulatory. The poorest inhibitor was derived from XI, in which the amino group is on a primary carbon instead of a secondary carbon. It seems likely that the sequence: primary alcohol, secondary amide, secondary alcohol, lipid group is essential for good binding to the enzyme. Table 3 lists the melting points of the inert compounds.

The ceramides formed from natural substrate were found to be slightly inhibitory. A mixture of non-hydroxy ceramides (prepared chemically from brain cerebrosides) yielded 9% inhibition and a mixture of hydroxy ceramides yielded 3% inhibition. It is evident that a benzene ring is much more effective than a straight aliphatic chain.

Tris base, which markedly inhibits a spleen preparation active in hydrolyzing glucocerebroside (12), was rather inert in our system. Our cerebrosidase preparation contained 8 mM Tris in each incubation tube; additional 20 mM Tris yielded 9% inhibition.

TABLE 2. Inhibition of cerebrosidase by decanoyl amides of various aromatic amino alcohols

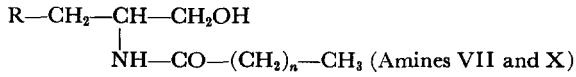


Amine	Aromatic Substitution	−X (1)	−Y (3)	Configuration	Inhibition	Melting Point
					%	°C
I		OH	H		18	81–82
II		H	OH	DL-*erythro*	3	65–66
III		OH	OH	D-*threo*	15	94–95
III		OH	OH	L-*threo*	17	92–93
III		OH	OH	DL-*erythro*	48	100–101
IV	*p*-nitro	OH	OH	D-*threo*	12	
IV	*p*-nitro	OH	OH	L-*threo*	18	
IV	*p*-nitro	OH	OH	DL-*erythro*	26	110–112
V	*m*-nitro	OH	OH	D-*threo*	2	135
VI	*p*-phenyl	OH	OH	L-*threo*	12	121–122

Each value is the mean of two separate experiments, each run in duplicate.

[1] Arora, R. C., and N. S. Radin. Unpublished observations.

TABLE 3. Melting points of aliphatic amides found to be noninhibitory



R—CH_2—CH—CH_2OH
|
NH—CO—$(CH_2)_n$—CH_3 (Amines VII and X)

R	n	Melting Point
		°C
H	16	90–91
H	14	83–84
H	12	73–74
H	10	64–65
H	8	51
CH_3	16	89–90
CH_3	14	87–88
CH_3	12	78–79
CH_3	10	70–71
CH_3	8	62–63
CH_3—CH—CH_3		
OH NH—CO—$(CH_2)_n$—CH_3		(Amine XI)
	16	82–83
	14	79–80
	12	70–71
	10	64–65
	8	51–52

Kinetic analysis of inhibition

The nature of the inhibition by decanoyl DL-*erythro*-III was evaluated by means of the conventional graphic method (Fig. 1). The intersection of the two curves on the baseline indicates that the inhibitor acts noncompetitively. The K_m for the enzyme–cerebroside complex was found to be about 22 μM, a little better than the value of 27 μM previously observed (2). The difference may be accounted for by the omission of oleate in the previously used assay system.

The K_i for the inhibitor was determined by a plot according to Dixon (13) (Fig. 2). The K_i was about

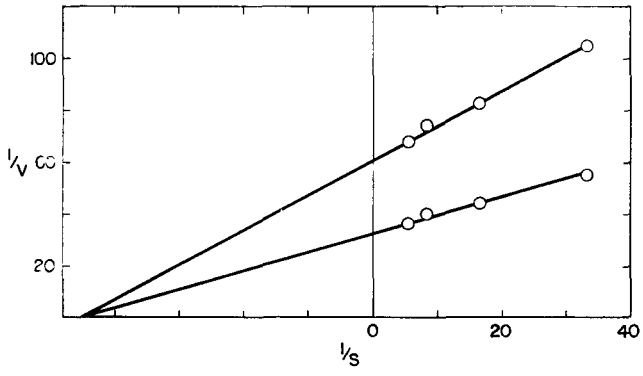


FIG. 1. Lineweaver-Burk plot relating substrate concentration to reaction rate. The incubation tubes differed not only in the amount of cerebroside but also (in the same proportion) in the amount of detergents, Tween 20 and Myrj 59. The reaction rates are in μmoles of galactose released in a 3-hr incubation. Upper curve, incubations with 0.3 μmole of *N*-decanoyl DL-*erythro*-phenylaminopropanediol; lower curve, controls.

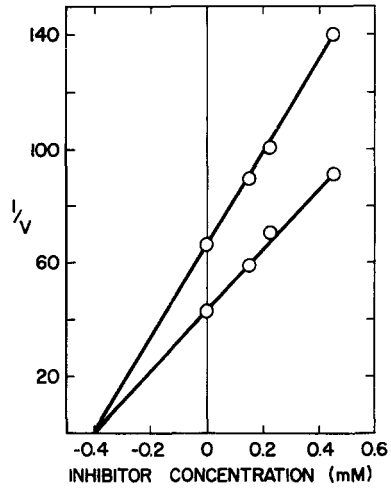


FIG. 2. Inhibition of cerebrosidase as a function of the concentration of decanoyl DL-*erythro*-phenylaminopropanediol. Reaction rate is defined in Fig. 1. Upper curve, incubations with 25 μg of cerebroside; lower curve, incubations with 150 μg of cerebroside. Tween 20 and Myrj 59 concentrations were proportional to the cerebroside concentrations.

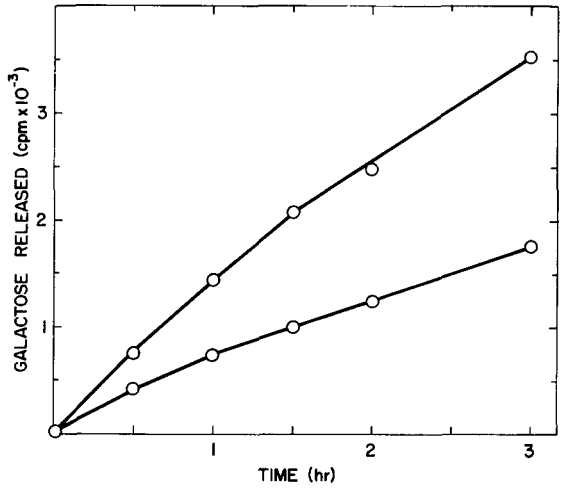


FIG 3. Amount of cerebroside hydrolyzed under standard conditions as a function of time. Upper curve, controls; lower curve, with 0.3 μmole of decanoyl phenylaminopropanediol. 1000 cpm = about 2.86 nmoles.

4×10^{-4} M and the intersection of the two lines again showed that the inhibitor acts noncompetitively.

A time study with 0.3 μmole of the same inhibitor (Fig. 3) showed that the degree of inhibition is similar at all points, as early as 30 min. There was a slight nonlinearity in the control and inhibited systems.

Inhibition in other tissues

While galactosyl ceramide occurs predominantly in the brain, cerebrosidase occurs widely distributed throughout the body (1, 14, 15). The possibility that the brain

TABLE 4. Inhibition by decanoyl DL-*erythro*-phenylamino-propanediol of the cerebrosidase of various tissues

Tissue	Control Activity	Inhibition
	cpm	%
Rat brain	2020	20
Rat spleen	2677	36
Rat kidney	4099	23
Rat liver	831	37
Rabbit gray matter	377	10
Rabbit white matter	481	7

The rat tissue was obtained from 18-day-old rats, and the rabbit tissue from the brain of a 10-day-old rabbit.

hydrolase is different from extraneural hydrolase was tested by examining the effect of decanoyl phenylamino-propanediol (Table 4). In this experiment the tissue was simply lyophilized and homogenized in benzene, and 2-mg aliquots of the suspension were dried in the incubation tubes with the inhibitor. This procedure gives low activities with brain because of the dilution of the radioactive substrate with endogenous cerebroside.

As noted before with fresh tissue homogenates (1), liver contains relatively little cerebrosidase while in kidney there is relatively much more. All the rat tissues showed inhibition by the amide, although the amount was somewhat less than with the purified enzyme. This general response to the inhibitor indicates that cerebrosidase is the same in all organs. This conclusion comes also from the observation (16) that cerebrosidase is very weakly active in brain, liver, and spleen of humans suffering from Krabbe's disease. The rabbit tissues responded only slightly, and it is possible that our inhibitor is specific for the rat enzyme.

DISCUSSION

It appears that our active synthetic amides act as noncompetitive inhibitors, combining reversibly with an enzyme site that is different from the substrate-active site. This conclusion comes from the Lineweaver-Burk plots, the finding that preincubation with inhibitor did not affect the inhibition, and the observation that the degree of inhibition was independent of duration of incubation. The inhibitor does not affect the ability of the enzyme to bind the substrate, but does reduce the rate of its action. It is surprising to find that such an effector site requires a compound that resembles so closely, but not completely, the product of the enzyme's action on its natural substrate, ceramide.

One possible explanation is that the enzyme, after splitting the galactoside linkage, moves the lipoid product from the substrate-active site to the second site prior to release of the ceramide from the enzyme. The ceramide is rapidly released from the second site but not from the substrate-active site. If the inhibitor fits firmly in the second site, it may act as a noncompetitive inhibitor. Natural ceramide does not act as an inhibitor because it is so readily released from this site. Another possible explanation is that the inhibitor-sensitive site is normally occupied in the living animal by some natural ceramide derivative that acts as part of a physiological control system.

It seems likely that the D-*erythro* amides are more active than the L-*erythro* compounds, and attempts at resolving the isomers are planned.

The observation that "typical" kinetic properties were seen with a lipid substrate and lipid inhibitor may seem unexpected, especially since the substrate was added as an emulsion and the concentration of emulsifiers was changed together with the concentration of the substrate. However, a similar Lineweaver-Burk plot was obtained with this enzyme when the detergent concentrations were kept constant (2). It is likely that the detergents act simply to bring the substrate to the enzyme's active region rapidly; as the micelle approaches the active site the detergent molecules are squeezed away from the substrate molecule, which is pulled into close proximity to the enzyme. The detergent micelles may also act to emulsify the ceramide formed by the enzyme, thereby speeding the dissociation step. It would appear that the rate of substrate release from the micelle and of product emulsification is relatively fast compared with the hydrolytic step itself.

The role of the bile salt may be to combine with some effector site on the hydrolase, possibly polymerizing it to an active form. One should therefore expect that a minimal taurocholate concentration is required, independent of the emulsion concentration; we therefore kept this at the optimal level. The inhibitors, though rather insoluble in water, evidently dissolve fast enough (see Fig. 3) to combine with the enzyme like any nonlipoid substance.

On the basis of the above reasoning, one would expect a lipid substrate to be no different in its kinetic interaction with an enzyme than any water-soluble substrate, provided that the substrate is so well emulsified that the micelles diffuse rapidly to the enzyme surface and readily disgorge the substrate molecules.

Inhibitors previously reported for this enzyme include galactonolactone, which is apparently a general inhibitor of acidic galactosidases. At 0.8 mM concentration, it produced 89% inhibition (2). Our previous study also showed good inhibition with lactosyl ceramide, prepared from gangliosides by hydrolysis. Perhaps this is the natural controlling inhibitor postulated above. Glucosyl ceramide, which occurs in brain normally only in trace concentrations, produced only 7% inhibition at 0.3 mM concentration.

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