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SYNTHESIS OF 2-N-(HEXADECANOYL)-AMINO-4-NITROPHENYL PHOSPHORYLCHOLINE-HYDROXIDE, A CHROMOGENIC SUBSTRATE FOR ASSAYING SPHINGOMYELINASE ACTIVITY

Andrew E. GAL and Frank J. FASH

Developmental and Metabolic Neurology Branch, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20014, USA

Received June 5, 1975, accepted September 24, 1975

2-N-(Hexadecanoyl)-amino-4-nitrophenyl phosphorylcholine-hydroxide a compound resembling sphingomyelin is synthesized. It is cleaved by sphingomyelinase to the chromogenic N-acylaminonitrophenyl moiety. Phospholipase C preparations do not hydrolyze this compound. The starting material is 2-amino-4-nitrophenol which when acylated with palmitoyl chloride yields the hexadecananilide. Reaction with β-bromoethylphosphoryldichloride gives the phosphate which is quaternized with trimethylamine to give the title compound.

1. Introduction

Accumulation of sphingomyelin in the Niemann-Pick disease is caused by the deficiency of sphingomyelinase [1]. The determination of the activity of this enzyme in small tissue samples has previously necessitated the use of radioactive sphingomyelin [2]. The degree of the enzymatic hydrolysis was determined by counting the radioactivity in the polar part of the hydrolysate which contains [N-methyl-^{14}C]-phosphorylcholine. More recently ditritiumsphingomyelin was used by Harzer and Benz [3] who measured the liberated radioactive dihydroceramide by scanning thin-layer chromatograms.

It was of interest to prepare a compound that has a chemical and structural resemblence to sphingomyelin and would yield a chromogenic moiety after reaction with sphingomyelinase. Such a simplified procedure is particularly useful for routine determinations and it is beneficial from the clinical chemist's viewpoint. The artificial substrate proposed by Brady et al. [4], which is the same as the title compound, corresponds to these requirements. It is dissimilar to sphingomyelin only by having an aromatic ring instead of a long aliphatic chain and a nitro group replacing the primary hydroxyl one carbon removed. This compound was synthesized and its use for the assay of sphingomyelinase was extensively studied. As the result of this research we could demonstrate that it would advantageously replace the radioactive substrate being equally sensitive and specific as radioactive sphingomyelin [5].

The synthesis of the title compound (5) was accomplished in three steps (fig. 1). By reacting 2-amino-4-nitrophenol (*1*) with palmitoyl chloride 2′-hydroxy-5′-nitrohexadecananilide (*2*) was obtained in good yield. The sodium or potassium salts of compound (*2*) were then prepared and were subsequently reacted in benzene with β-bromoethylphosphoryldichloride which was prepared according to Jean [6]. Hirt and Berchtold [7] used β-bromoethylphosphoryldichloride for the synthesis of lecithin and Rabinsohn et al. [8] for the preparation of sphingomyelin. The products of the phosphorylation reaction are, depending whether one or both molecules of chlorine of the β-bromoethyldichloride reacts with the phenolates, 2′-(β-bromoethylphosphoryl)-5′-nitrohexadecananilide (*3*) or 2′-(β-bromoethylphosphoryl)-bis-5′-nitrohexadecananide. These two compounds can be easily separated due to the solubility of compound (*3*) in water as an alkali salt. It has been found that the "bis" compound can be transformed into the acidic (*3*) through mild hydrolytic conditions. Modifications of the synthesis by replacing the sodium by the potassium salt or by reverse addition of the reagents does not alter the proportion of these two reaction products. Phosphorylation with β-chloroethylphosphoryldichloride used by Shapiro et al. [9] for the synthesis of dihydrosphingomyelin and prepared according to Renshaw and Hopkins [10] yielded the analogous chlorine containing compound (*4*). A corresponding "bis" compound was not isolated. The quaternization of compound (*3*) with trimethylamine was carried out by the procedure of Eibl et al. [11] in methylethyl ketone at 60°C for one day. Higher temperatures and longer reaction time diminishes the yield. The reaction product, a quaternary bromide as a trimethylammonium salt, could be transformed into the hydroxide (*5*) by treatment with a mixture of weak acidic and basic ion exchange resins following a similar procedure developed by Baer et al. [12] for the synthesis of lecithin. The identity of the product was established by elemental analysis and by its hydrolysis to phosphorylcholine. Also NMR spectroscopy in dimethylsulfoxide-d_6 confirmed the formula in de-

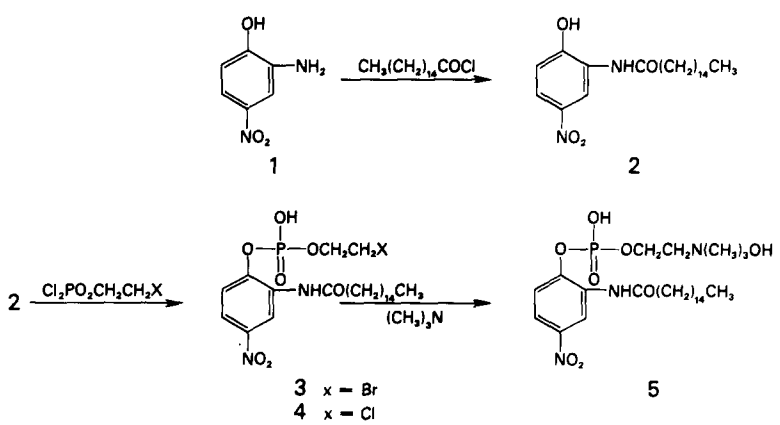

Fig. 1. Formulae and flow diagram for the synthesis of 2-N-(hexadecanoyl)-amino-4-nitrophenyl phosphorylcholine-hydroxide.

tail. Due to micelle formation a NMR spectrum could not be obtained in D_2O. When the chloro isomer (*4*) was reacted with trimethylamine only traces of the quaternary compound (*5*) could be obtained.

II. Experimental

A. Materials and methods

Trimethylamine and argon were used from lecture bottles (Matheson Gas Products). Methyl ethyl ketone was dried over Drierite and then distilled over molecular sieve 3A. The ion exchange resins Rexyn 102 (H) 100–200 mesh, and Rexyn 203 (OH), 16–50 mesh, were purchased from Fisher Sci. Co., AG-3-X4A chloride form, 100–200 mesh, from Bio Rad was transformed into the OH form with 0.5 N sodium hydroxide (weight/vol = 1 : 10). The resins were made in the laboratory. The various compounds on the layer were washed with a large volume of methanol and then dried under diminished pressure.

Melting points were taken on a Thomas-Hoover melting point apparatus and are corrected. Silica gel G and MN 300 cellulose plates were supplied by Analtech Inc. Silica gel H (EM. Labs. Inc.) plates were visualized by charring with ammonium bisulfate according to Gal [13] or by using the phospholipid spray solution developed by Vaskovsky and Kostetsky [14]. Visible absorption was measured on a Gilford 240 spectrophotometer. Infrared spactra were obtained with a Perkin-Elmer 621 infrared grating spectrophotometer on KBr disks (1.5 mg sample/300 mg KBr). The NMR spectra were taken on a Varian XL-100-15 spectrometer operating at 100 MHz.

B. 2'-Hydroxy-5'-nitrohexadecananilide (*2*)

Palmitoylchloride 22.6 g (82 mmol) was added with stirring at 25°C to a solution of 12.3 g (80 mmol) of 2-amino-4-nitrophenol in 190 ml pyridine. After 72 hr at room temperature the pyridine was removed under reduced pressure. The last traces of pyridine were distilled with toluene (twice with 50 ml) and the residue dried at 40°C (0.1 mmHg, 4 hr). The product was scraped off the flask and was stirred for 2 hr with 600 ml of water and then filtered. The residue was dried and recrystallized from 2 liters of chloroform. Yield 25 g (80%); mp 146–148°C. The compound is soluble in acetone, methyl ethyl ketone, tetrahydrofuran, chloroform, and pyridine and slightly soluble in methyl and ethyl alcohol, ethyl acetate and ethyl ether. On thin-layer chromatography in chloroform–methanol (50 : 1) it had R_f 0.3 (2-amino-4-nitrophenol R_f 0.1). The infrared spectrum displayed bands at 2920, 2850 (alkane), 1650 (anilide), 1527, 1345 ($-NO_2$), 1430 (alkane) and 1070 cm^{-1} (trisubstituted benzene?).

Anal. $C_{22}H_{36}N_2O_4$ (392.55). Calcd.: C, 67.32; H, 9.25; N, 7.14.

Found: C, 67.48; H, 9.08; N, 7.43 .

1. Sodium salt of (2)

To a suspension of 5.9 g (15 mmol) of 2′-hydroxy-5′-nitrohexadecananilide (*2*) in 150 ml benzene was added 30 ml of a 0.5 M methanolic sodium methoxide. Compound (*2*) went into solution in this mixture. After stirring for 2 hr at room temperature and 24 hr at 4°C the precipitated red sodium salt was filtered and washed with benzene-methanol (6 : 1) and then with benzene. The product was dried 6 hr at 60°C in vacuum. Yield 5.5 g (89%); mp 193°C (decomp.). The visible spectrum of a solution of thsi salt in ethanol displayed a single λ max at 415 nm (ε 15, 000).

Anal. $C_{22}H_{35}N_2NaO_4$ (414.53). Calcd.: C, 63.74; H, 8.51; N, 6.75 .

Found: C, 62.91; H, 8.48; N, 6.71 .

2. Potassium salt of (2)

To a solution of 1.12 g (20 mmol) of potassium hydroxide in 50 ml of ethyl alcohol was added 7.85 g (20 mmol) of (*2*). After evaporating the solvent the residue was recrystallized from benzene-ethyl alcohol (20 : 1). The orange colored salt was filtered and dried (0.1 mmHg, 8 hr over potassium hydroxide). Yield 7.5 g (90%); mp 168°C (decomp.).

Anal. $C_{22}H_{35}KN_2O_4$ (430.64). Calcd.: C, 61.35: H, 8.19; N, 6.50.

Found: C, 60.66; H, 8.16; N, 6.31 .

C. 2′-(β-Bromoethylphosphoryl)-5′-nitrohexadecananilide (3)

(a) The sodium salt of 2′-hydroxy-5′-nitrohexadecananilide 4.15 g (10 mmol) was suspended in 400 ml of benzene. The suspension was stirred and heated to boiling and benzene 100 ml, was collected through a concentrator condenser. The temperature of the mixture was lowered to 25°C and 4.84 g (20 mmol; 2.64 ml) of β-bromoethylphosphoryldichloride was added. The slightly yellow solution was stirred for 1 hr and then refluxed for 24 hr. After cooling to 10°C, 10 ml (0.56 mol) of water was added and stirring was continued for 72 hr at room temperature. The precipitate 4.2 g, was filtered and washed with 100 ml of benzene. The dried and pulverized product was then suspended in 90 ml of 0.2 M sodium acetate buffer (pH 5.0) and stirred for 3 hr, filtered through a coarse filter paper and the filtrate was acidified with 90 ml of 1 N hydrochloric acid and extracted twice with 180 ml of ethyl acetate. After evaporation of the solvent, the residue, 2.7 g was recrystallized from 70 ml of acetone to yield 2.2 g (38%) of (*3*); mp 117–118°C. The compound is water soluble as a sodium salt. The solubility in 0.2 M sodium acetate buffer (pH 5.) is 100 mg per ml, it is soluble in methyl and ethyl alcohol, ethyl acetate, acetone, methyl ethyl ketone, tetrahydrofuran, benzene and cloroform and slightly soluble in ethyl ether.On thin layer chromatography in chloroform–methanol–wa-

ter (75 : 25 : 3) it had R_f 0.5 (Compound (*2*) had R_f 0.95). The infrared spectrum displayed the following additional bands when compared with the spectrum of compound (*2*), 1210 (P = O stretch), 1005, 950, 920 (P = O(OH)), 730, 560 cm^{-1} (C–Br?).

Anal. $C_{24}H_{40}BrN_2O_7P$ (579.46) . Calcd.: C, 49.75; H, 6.96; N, 4.83; P, 13.79 .

Found: C, 49.80; H, 7.26; N, 4.66; P, 13.70.

The buffer-insoluble compound was mainly the starting material (*2*).

(b) The synthesis described above was repeated by using the potassium salt of (*2*). The yield of (*3*) was 2.1 g (36%); mp 117–118°C.

(c) β-Bromoethylphosphoryldichloride 4.84 g (20 mmol; 2.64 ml) was added to 300 ml anhydrous benzene. To this solution was added with mechanical stirring at 25°C 4.15 g (10 mmol) of 2′-hydroxy-5′-nitrohexadecananilide sodium salt during 3 hr (12 portions). After refluxing the mixture for 2 hr it was cooled to 10°C and 10 ml (0.56 mol) of water was added. The mixture was stirred for 72 hr at room temperature. The precipitate was worked up as described in the method (a). The yield of (*3*) was 2.1 g (36%); mp 117–118°C.

D. 2′-(β-Bromoethylphosphoryl)-bis-5′-nitrohexadecananilide

The mother liquors of the reaction mixtures of the reactions described above (a, b, and c) were cooled to 4°C and the precipitate formed was recrystalized from benzene and from acetone successively. Yield, 3.6 g (12.6%), mp 94–95°C. The compound was insoluble in 0.2 M sodium acetate buffer (pH 5.0). Thin-layer chromatography was carried out on silica gel H plates in the solvent system chloroform–methanol (3 : 1) it had R_f 0.4 (compound (*3*) had R_f 0.5).

Anal. $C_{46}H_{74}BrN_4O_{10}P$ (953.99) . Calcd.: C, 57.92; H, 7.82; N, 5.87; Br, 8.37 .

Found: C, 57.63; 58.12; H, 8.00, 8.03; N, 6.01, 5.85; Br, 8.32 .

E. Hydrolysis of 2′-(β-bromoethylphosphoryl)-bis-5′-nitrohexadecananilide

A solution 239 mg (0.25 mmol) of 2′-(β-bromoethylphosphoryl)-bis-5′-nitrohexadecananilide in 4 ml of ethanol and 4 ml of 0.5 N ammonium hydroxide solution was heated in a sealed ampoule for 4 hr at 80°C. The solvent and the excess of ammonium hydroxide were evaporated and the residue was stirred with 5 ml of 0.2 M sodium acetate buffer and filtered. The filtrate was acidified with 5 ml of 1 N hydrochloric acid and extracted twice with 5 ml of ethyl acetate. The residue which was obtained after evaporation was recrystallized from 2 ml of acetone. Yield 70 mg (48%); mp 113–117°C. The product of the hydrolysis was 2′-(β-bromoethylphosphoryl)-5′-nitrohexadecananilide (*3*). This was proven by mp, mixed mp and by thin-layer chromatography

F. 2'-(β-Chloroethylphosphoryl)-5'-nitrohexadecananilide (4)

This compound was prepared following the synthesis of compound (*3*) by method (a), using 3.94 g (20 mmol; 2.46 ml) of β-chloroethylphosphoryldichloride. The precipitate from benzene at room temperature was subsequently recrystallized from benzene and isopropyl ether; yield 1.6 g (30%); mp 122–124°C. The compound is soluble in 0.2 M sodium acetate buffer (pH 5.0) to the extent of 100 mg per ml. Thin-layer chromatography in chloroform–methanol–water (75 : 25 : 3) it had R_f 0.5.

Anal. $C_{24}H_{40}ClN_2O_7P$ (535.01). Calcd.: C, 53.88; H, 7.54; N, 5.24; Cl, 6.63 .

Found: C, 53.44; H, 7.77; N, 5.17; Cl, 6.22 .

G. 2-N-(Hexadecanoyl)-amino-4-nitrophenyl phosphorylcholine-hydroxide (5)

Trimethylamine 13.4 g (0.23 mol) was dissolved in 100 ml methyl ethyl ketone at 0°C under anhydrous condition giving a 1.85 M solution.

In a 50 ml ampoule was placed 1.6 g (2.76 mmol) of compound (*3*). The ampoule was cooled in ice, flushed with argon and 40 ml of the trimethylamine solution was added. The sealed ampoule was heated for 24 hr at 60°C cooled to room temperature, opened and the solvent and the trimethylamine were evaporated with a stream of nitrogen at 40°C. Acetone, 20 ml, was added and evaporation was continued. The residue was transferred into a round bottom flask with 40 ml of ethyl alcohol and, after evaporating the alcohol it was stirred for 48 hr with 120 ml of ethyl acetate filtered, washed with 20 ml of this solvent and dried (0.1 mmHg. 8 hr over potassium hydroxide). This material weighing 1.3 g was recrystallized from 33 ml of acetone. Yield 1.1 g (57%); mp 130–131°C. It gave a positive Beilstein test for bromine.

Anal. 2-N-(Hexadecanoyl)-amino-4-nitrophenyl phosphorylcholine-bromide trimethylamine salt; $C_{30}H_{58}BrN_4O_7P$ (697.69):

Calcd.: C, 51.64; H, 8.38 ; N, 8.03. Found: C, 51.18; H, 8.49; N, 7.60.

The trimethylamine salt 1.3 g was dissolved in 130 ml of 95% methanol. After the addition of 5 g of AG-3X-4A in OH form and 5 g of Rexyn 102 H ion exchange resins the mixture was magnetically stirred for 2 hr, filtered and washed thoroughly with 240 ml of 95% methanol. The filtrate was evaporated and the residue was recrystallized from 50 ml of acetone; yield 880 mg (55%); mp 182–184°C. This compound is soluble in water (33 mg per ml at room temperature); a 0.05 M aqueous solution has a pH 5.6. It has about the same solubility in chloroform and methyl ethyl ketone as in water while it is less soluble in acetone, ethyl acetate, ethyl ether or tetrahydrofuran. Thin-layer chromatography: chloroform–methanol–water (6 : 4 : 1) it had R_f 0.8. The infrared spectrum (fig. 2) displayed the following ad-

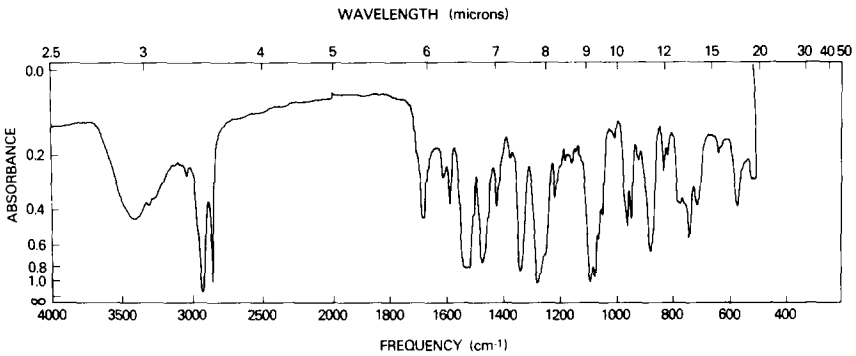

Fig. 2. Infrared spectrum of 2-N-(hexadecanoyl)-amino-4-nitrophenyl phosphorylcholine hydroxide.

ditional bands when compared with the spectrum of the compound (*3*): 1470 ($-CH_3$ deformation, linked to nitrogen), 1080, 1095 cm^{-1} (aliph. $-CH_2-$). The infrared spectra of choline iodine showed three intense bands: 1470, 1080 and 950 cm^{-1}. Absorption at these wavelengths were also reported in the literature [15]. The NMR spectra (fig. 3) in DMSO-d_6 with TMS as reference showed the following aromatic resonances: A doublet at δ 9.04 with only meta coupling ($J_{1,2}$ = 2.8 HZ); a doublet of doublets at δ 7.96 ($J_{2,3}$ = 9 HZ, $J_{2,1}$ = 2.8 HZ); a doublet at δ 7.54 ($J_{3,2}$ = 9 HZ). The aliphatic region contained a multiplet at δ 4.25 ($-CH_2-$), a multiplet at δ 3.6 ($-CH_2-$), a singlet at 3.18 [$N^+(CH_3)_3$], a clearly defined triplet at δ 2.4 ($-CH_2-$), the methylene envelope at δ 1.24 of 22 protons and the terminal methyl, a board

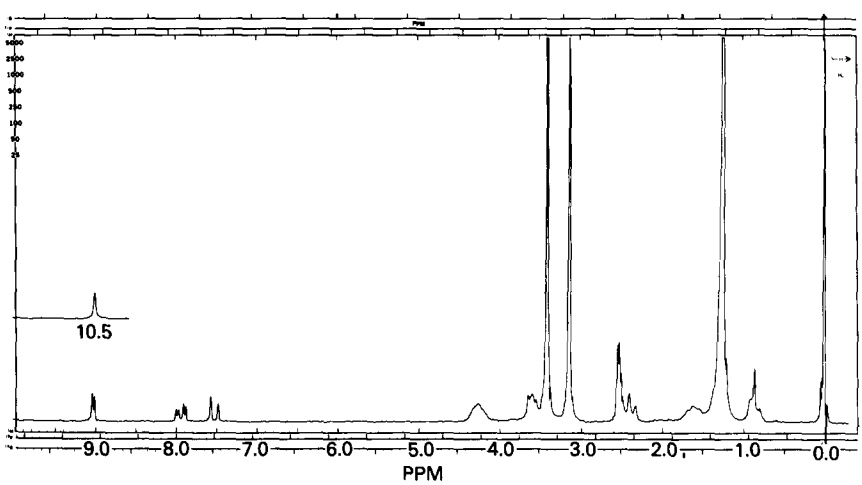

Fig. 3 NMR spectrum of 2-N-(hexadecanoyl)-amino-4-nitrophenylphosphorylcholine-hydroxide.

triplet at δ 0.86. The singlet at δ 10.5 presumably comes from a hydrogen bonded –NH–.

Anal. $C_{27}H_{50}N_3O_8P$ (575.69). Calcd.; C, 56.33; H, 8.75; N, 7.30: P, 5.38 .

Found: C, 56.36; H, 8.76; N, 7.33; P, 5.25 .

A 0.05 M solution of this compound in 1 N sodium hydroxide was kept 24 hr at 25°C, it showed only 6% decomposition. Heated with 2 N sodium hydroxide for 2 hr at 100°C it hydrolyzed quantitatively forming the insoluble sodium salt of compound (*2*).

H. Methanolysis of 2-N-(hexadecanoyl)-amino-4-nitrophenyl phosphorylcholine-hydroxide (5) with methanolic hydrochloric acid

A solution of 11.5 mg (0.02 mmol) of compound (*5*) in 1 ml of 1 N methanolic hydrochloric acid was heated in a sealed ampoule for 1 hr at 100°C. The solution was brought to room temperature and extracted twice with the same volume of hexane then quickly evaporated with nitrogen. The residue was dissolved in 1 ml of ethyl alcohol. An aliquot of this solution was compared with phosphorylcholine by thin-layer chromatography, on Mn 300 cellulose plates [16] in the solvent system acetic acid–n-propanol–water–phenol (1 : 2 : 1 : 1) it had R_f 0.8. Spots were visualized by the phospholipid spray. On silica-gel G plates R_f 0.2 was observed (detection by charring). The methanolysis resulted in near quantitative recovery of phosphorylcholine from product (*5*) as it was found by visual comparison of the spots after thin-layer chromatography with phosphorylcholine standard. Phosphorylcholine used as standard was prepared from phosphorylcholine–chloride calcium salt. An aqueous solution of this salt was treated with a mixture of Rexyn 203(OH) and of Rexyn 102(H), and the eluates were freeze-dried.

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