THE CHEMICAL SYNTHESIS OF DINITROPHENYL DERIVATIVES OF SERINE AND ETHANOLAMINE

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Summary: The chemical synthesis of fluorodinitrophenylethanolamine, fluorodinitrophenylserine, seryldinitrophenylethanolamine, dinitrophenylbis-ethanolamine and dinitrophenylbis-serine has been achieved. These dinitrophenyl derivatives were synthesized to serve as reference compounds for the study of nearest neighbor analysis of amino-phospholipids in the erythrocyte membrane.

The erythrocyte membrane contains appreciable quantities of phosphatidylethanolamine (PE) and phosphatidylserine(PS) (1,2). These amino-phospholipids appear to be localized primarily on the inner surface of the erythrocyte membrane (3,4,5) and up to 20 percent of these phospholipids become cross-linked to membrane proteins by difluorodinitrobenzene (DFDNB). The cross-linking of amino-phospholipids to each other can give some idea of the nearest neighbor frequency of these lipids in the membrane. We examined this problem with the cross-linking agent DFDNB. (6) In order to identify the various cross-linked phospholipids it was necessary to chemically synthesize the five dinitrophenyl derivatives of serine and ethanolamine, these being as follows: fluorodinitrophenylethanolamine, fluorodinitrophenylserine, seryldinitrophenylethanolamine, dinitrophenylbis-serine and dinitrophenylbis-ethanolamine. To our knowledge only dinitrophenylbis-ethanolamine has been synthesized previously (7). These aforementioned dinitrophenyl derivatives are possible products which result from the HCl hydrolysis of dinitrophenylated lipids obtained by treatment of the erythrocyte membrane with DFDNB.

DFDNB-1,5-difluoro-2,4-dinitrobenzene; FDNB-1-fluoro-2,4-dinitrobenzene; PE-phosphatidylethanolamine; PS-phosphatidylserine.

Methods and Reagents

1-Fluoro-2,4-dinitrophenyl-5-(N)-ethanolamine. (FDNP-Ethanolamine) M.W. 245

122 mg (2 mmoles) of ethanolamine (Eastman Kodak) were dissolved in 45 ml of distilled water, to which were added 300 mg of $NaHCO_3$ and 408 mg (2 mmoles) of DFDNB (Sigma) dissolved in 5 ml of methanol. The DFDNB in methanol was added slowly over a 1 hr. period at 4°C. This low temperature was used to minimize formation of the bis compound (8). After two hours the solution was brought to 23°C, cooled, acidified to pH 4.0 with 6N HCl and cooled on ice. The FDNB-ethanolamine crystallized as yellow needles which were isolated by centrifugation and recrystallized from acetone-water 1:1 v/v. The yield was 279 mg (57%); m.p. 145-146°C. (uncorrected). Mass spectral analysis gave a parent peak having a molecular weight of 245. Thin layer chromatography gave a single component having Rf value of 0.87. Thin layer chromatography was carried out on silica gel-coated glass plates (Merck & Co., Darmstadt, SG5763) using a solvent of chloroform-methanol-water 65:25:4 v/v. This system was used for all the compounds given below.

1-Fluoro-2,4 dinitrophenyl-5(N)-serine (FDNP-serine) M.W. 289

204 mg (1 mmole) of DFDNB were dissolved in 15 ml of acetone-water 2:1 v/v. To this solution were added 300 mg of $NaHCO_3$ in 5 ml of water. 75 mg (0.71 mmole) of D,L-serine (Nutritional Biochem.) in 5 ml of water were added slowly over a period of 1 hr. at 23°C. The reaction did not proceed very far at 4°C as was the case with ethanolamine, therefore a temperature of 23°C was used. After 30 minutes the acetone was removed in vacuo in a rotary evaporator at 40°C. The remaining solution was acidified with concentrated HCl to pH 3.0 and extracted with ether. The ether extract was washed once with water and evaporated to dryness in vacuo. The oily residue was recrystallized from ether:benzene 1:1 v/v, yielding 79 mg (38%) of yellow needles, m.p. 164-165 (uncorrected). Mass spectral analysis gave a parent peak having molecular weight of 289. Thin layer chromatography gave a single component having Rf value of 0.33. Treatment with methanolic-HCl for 2 hrs. at 50°C gave the

TABLE I

Properties of Dinitrophenyl Derivatives of Serine and Ethanolamine

Compound	Rf[a]	M.P.	λ max nm	Molar Ext. Coeff.[b]
FDNP-ethanolamine	0.87	145-146	333	1.4×10^4
FDNP-serine	0.33	164-165	333	1.4×10^4
DNP-bis ethanolamine	0.71	206-207	331	2.4×10^4
DNP-bis-serine	0.07	decomposes	339	2.4×10^4
serine-DNP-ethanolamine	0.28	decomposes	335	2.4×10^4

[a]The Rf values obtained by TLC in chloroform-methanol-water 65:25:4 as explained in the text.

[b]The DNP-bis-serine was not isolated in sufficient purity for measurement of its molar extinction coefficient. The dimethyl ester of DNP-bis-serine was prepared and found to have a molar extinction coefficient of 2.4×10^4 at 325 nm. The methyl ester of serine-DNP-ethanolamine had a molar extinction coefficient of 2.4×10^4 at 330 nm.

methyl ester derivative having Rf value of 0.90.

2,4-Dinitrophenyl-1-(N)-ethanolamine-5-(N)-serine (Serine-DNP-ethanolamine)

M.W. 330

200 mg (0.82 mmole) of FDNP-ethanolamine were dissolved in 30 ml of methanol and mixed with 600 mg (5.7 mmoles) serine and 1 gm $NaHCO_3$ in 10 ml of water. The mixture was heated at 60°C for 3 hours, cooled and filtered to remove excess $NaHCO_3$ and excess serine. The filtrate was acidified with concentrated HCl to pH 3.0 and filtered. The filtrate was placed in a refrigerator at 5°C. A crystalline product (216 mg, 80% yield) was obtained after 24 hours. M.P. decomposes, beginning at 160°C. Thin layer chromatography showed one major component with an Rf value of 0.28. Treatment with methanolic-HCl

for 2 hrs at 50°C gave the methyl ester derivative, having an Rf value of 0.87.

2,4-Dinitrophenyl-1,5 (N,N) bis-ethanolamine (DNP-bis-ethanolamine), M.W. 286

1 gram (16.4 mmoles) of ethanolamine was added to 204 mg (1 mmole) of DFDNB in 1.5 ml of acetone. After standing at 23°C for 1 hr, 3 ml of water were added. The yellow precipitate which formed was isolated, washed three times with 3 ml of water and recrystallized from acetone-water 1:1 v/v. The yield was 162 mg (57%); m.p. 206-207 (uncorrected). Mass spectral analysis gave a parent peak having molecular weight 286. Thin layer chromatography gave a single component having Rf value 0.71. Waldkotter reported a m.p. of 211°C for his synthetic compound (7).

2,4-Dinitrophenyl-1,5-(N,N)-bis-serine (DNP-bis-serine) M.W. 374

A slurry of 204 mg (1 mmole) of DFDNB, 600 mg (5.7 mmoles) serine and 1 gm $NaHCO_3$ in 30 ml of methanol was heated with mixing for 7 hr at 60°C. The reaction mixture was cooled and filtered. The precipitate was washed with 10 ml of hot methanol. The combined filtrates were acidified with concentrated HCl to produce a flocculent precipitate. The precipitate was washed twice with chloroform and dried to yield 140 mg of yellow powder (yield - 37%). Attempts to crystallize this product were unsuccessful. The compound was thermally unstable such that melting point and mass spectral analysis were not definitive. Thin layer chromatographic analysis showed contamination by FDNP-serine. The Rf value of DNP-bis-serine was 0.07. Treatment of DNP-bis-serine with methanolic HCl for 2 hrs at 50°C gave the dimethyl ester derivative which had an Rf value of 0.80.

The dinitrophenylbis-serine derivative was prepared by the following alternate method: 204 mg (1 mmole) of DFDNB, 620 mg serine (6 mmoles) and 484 mg $NaHCO_3$ were dissolved in 25 ml of 70% methanol in water. Aliquots of the reaction mixture were analyzed by thin layer chromatography to follow the course of the reaction. The fluorodinitrophenylserine formed within 10 min. at 23°C. The dinitrophenylbis-serine required one hour to form at 50°C. The reaction mixture was acidified with concentrated HCl and extracted with ethyl

acetate. Preparative thin layer chromatography gave 108 mg of the dinitrophenylbis-serine (29% yield).

Attempts to crystallize the bis-serine-compound from water or organic solvents were unsuccessful. Crystallization was occasionally achieved from cold methanol-benzene but the crystals turned readily to a viscous oil. The bis-serine compound was photosensitive. It turned brown readily on exposure to sunlight. The monoserine compound FDNP-serine was also photosensitive but much less than the bis compound.

Partial separation of a mixture of dinitrophenyl derivatives of serine and ethanolamine by ethyl acetate extraction.

All the five derivatives mentioned above can be extracted from an acid aqueous medium (3N HCl) by ethyl acetate. However, if a solution of these derivatives is made alkaline with $NaHCO_3$, ethyl acetate will extract only FDNP-ethanolamine and DNP-bis-ethanolamine. Acidification of the remaining solution with concentrated HCl now permits the three serine derivatives (FDNP-serine, serine-DNP-ethanolamine and DNP-bis-serine) to be extracted into ethyl acetate. The hydroxyphenyl derivative of ethanolamine (HO-DNP-ethanolamine) is not extracted into ethyl acetate from an alkaline medium and behaves like the serine derivatives. This is due to the phenolic hydroxyl group which is ionized at pH 8.6.

Paper Electrophoresis of the DNP-derivatives

The DNP-derivatives of serine and ethanolamine were separated by paper electrophoresis on Whatman 3 MM filter paper at 300 v. for 1 hour using 2.5 mM Tris-19 mM glycine buffer pH 8.5 containing 1.3 mM EDTA. The mobilities (in cm) of the derivatives were as follows: toward the anode: FDNP-ethanolamine-1.4 cm; DNP-bis-ethanolamine-0.8 cm; toward the cathode: DNP-bis-serine-9.6 cm; FDNP-serine-6.1 cm; serine-DNP-ethanolamine - 4.8 cm.

The use of DFDNB for the neighbor analysis of PE and PS in the erythrocyte membrane is given in the companion paper (9).

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