A Highly Reactive Sulfur Mustard Gas Derivative for Localized Infusion Studies

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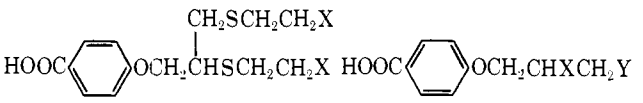
The preparation of *p*-[2,3-di(2-bromoethylthio)-*n*-propyloxy]benzoic acid is described. Measurements of the rate of hydrolysis of this compound in aqueous solution at 2, 14, and 20° indicate that the half-life of the agent under physiological conditions will be 4.8 sec. The results of some preliminary biological tests are reported.

In connection with studies of tumor growth inhibition by localized infusion techniques, a water-soluble highly reactive difunctional alkylating agent was required. It was desirable that the agent should have a half-life of a few seconds under physiological conditions. 2-Halogenoethylamino compounds were considered but such derivatives of aliphatic amines suffer from the disadvantage that, although cyclization to an ethylenimmonium ion is rapid, the subsequent reaction of this ion with nucleophilic centers is relatively slow. Similar derivatives of aromatic amines, the so-called "aromatic nitrogen mustards," do not form such stable ethylenimmonium ions but the initial rate of halogen release is not adequate. It was therefore decided to examine 2-halogenoethylthio derivatives which do not suffer from these disadvantages.

If halogenoethyl groups are attached to the same sulfur atom, very high reactivity cannot be achieved because of the mutual deactivating effect of the halogen atoms; for example, the half-life of di(2-chloroethyl) sulfide (sulfur mustard gas) is about 3 min. in water at 37°.[1] For this reason a molecule containing two isolated halogenoethylthio groups appeared to be desirable. The incorporation of an acidic group that would be dissociated at physiological pH would confer water solubility. It was also expected that such a group would promote localized protein adsorption as observed by Linford in the case of *p*-di(2-chloroethyl)aminophenylbutyric acid (chlorambucil).[2] These considerations led to the synthesis of *p*-[2,3-di(2-bromoethylthio)-*n*-propyloxy]benzoic acid (I, X = Br).[3,4]

Methyl *p*-hydroxybenzoate was condensed with 3-chloro-1,2-propanediol in the presence of alkali to give a product which on hydrolysis yielded *p*-(2,3-dihydroxy-*n*-propyloxy)benzoic acid (II, X = Y = OH). An attempt to convert II (X = Y = OH) into the 2,3-dibromo derivative using concentrated aqueous hydrobromic acid led to the formation of a bromohydrin, probably II (X = OH; Y = Br).

The required intermediate, *p*-(2,3-dibromo-*n*-propyloxy)benzoic acid (II, X = Y = Br) was readily obtained by the action of bromine on *p*-allyloxybenzoic acid in chloroform solution. This dibromide was condensed with 2-mercaptoethanol in the presence of alkali to yield the diol I (X = OH). Treatment of the dihydroxy acid (I, X = OH) with cold concentrated aqueous hydrobromic acid afforded the dibromide (I, X = Br). An improved yield of the required compound was obtained by the action of dry HBr on the diol in chloroform solution.



In order to assess the rate of reaction of the dibromoethylthio acid (I, X = Br) under physiological conditions, the rate of hydrolysis of the carbon–bromine linkage was determined at 2, 14, and 20° in aqueous solution at pH 8.3. The rate of reaction at 37° was derived by extrapolation. Because of the rapid hydrolysis of the dibromoethylthio acid in aqueous media it was not possible to determine the dissociation constant by direct potentiometric titration. The related dihydroxy acid (I, X = OH) has pK_a = 4.4, and replacement of hydroxyl by bromine at a point distant from the carboxyl group should not significantly modify this value. The derived half-life of 4.8 sec. at 37° and pH 8.3 therefore refers to the anionic form of the acid that will exist under physiological conditions.

Biological Data.—In our standard screening test against the subcutaneously implanted Walker rat carcinosarcoma,[5] a single intraperitoneal injection of 4 mg./kg. of *p*-[2,3-di(2-bromoethylthio)-*n*-propyloxy]benzoic acid in Arachis oil on the day following implantation produced 50% inhibition of tumor growth. Dr. L. Cobb reports that, in preliminary experiments in which 10^6 cells of the Walker carcinosarcoma 256 (Chester Beatty strain) was injected into the muscle of the hind limb of Wistar rats and the resulting tumor was treated 5 days later with a single injection of 1.6 mg./kg. of the dibromo acid dissolved in dimethyl sulfoxide into the artery supplying the tumor, a significant cytotoxic effect on the tumor was observed. A similar dose administered by the intraperitoneal route was without effect. The LD_{50} of the dibromo acid in 250-g. male Wistar rats was between 10 and 15 mg./kg.

(1) W. C. J. Ross, "Biological Alkylating Agents," Butterworth and Co. Ltd., London, 1962, p. 11.

(2) J. H. Linford, *Can. J. Biochem. Physiol.*, **40**, 137 (1962).

(3) Seligman and his colleagues have examined a large number of bis(2-halogenoethylthio) derivatives as agents for intraarterial regional chemotherapy [see, for example, B. Witten, C. E. Williamson, J. I. Miller, S. Sass, S. P. Kramer, L. E. Goodman, A. Alfohn, and A. M. Seligman, *Cancer Chemotherapy Rept.*, **No. 16**, 515 (Feb. 1962)] but our approach differs in that we are aiming at a stable linkage between the alkylating centers, since a resistant cross linkage is probably necessary for carcinostatic activity, and we are also seeking to introduce water-solubilizing and protein-binding groups.

(4) Bromine was chosen as the halogen atom in view of the higher reactivity of bromides as compared with chlorides and iodides [W. C. J. Ross, ref. 1, p. 105; S. Sass, C. E. Williamson, S. P. Kramer, L. E. Goodman, A. Ulfohn, A. M. Seligman, and B. Witten, *J. Med. Chem.*, **8**, 14 (1965)].

(5) The protocol for this carcinostatic assay is given by T. A. Connors, B. C. V. Mitchley, V. M. Rosenauer, and W. C. J. Ross, *Biochem. Pharmacol.*, **13**, 395 (1964).

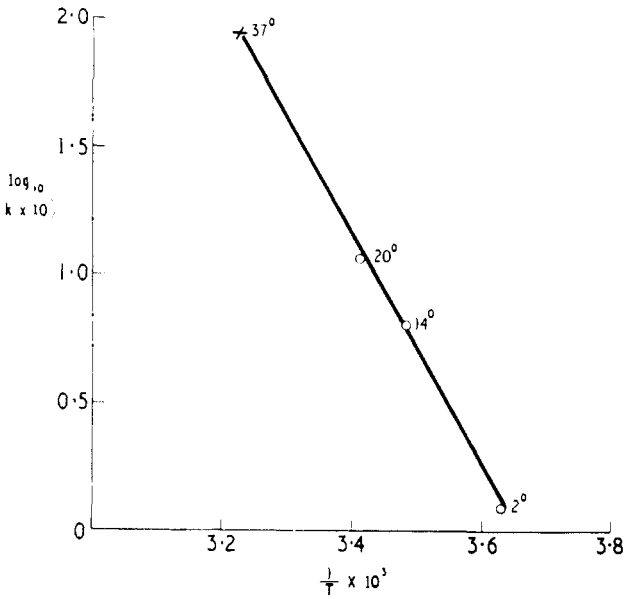


Figure 1.—Plot of log ($k \times 10$) against ($1/T$) $\times 10^3$ for the hydrolysis of *p*-[2,3-di(2-bromoethylthio)-*n*-propyloxy]benzoic acid.

when administered in dimethyl sulfoxide into the peritoneal cavity, the intrasaphenous vein, or the intrailiac artery.

Experimental Section[6]

***p*-(2,3-Dihydroxy-*n*-propyloxy)benzoic Acid (II, X = Y = OH).**—Methyl *p*-hydroxybenzoate (15.2 g.) was dissolved in a solution of sodium (2.3 g.) in ethanol (100 ml.). After the addition of 3-chloro-1,2-propanediol (11.05 g., 8.35 ml.), the mixture was stirred at 35° for 16 hr. and then at 60° for 20 hr. The precipitated NaCl was removed by filtration, and the filtrate was evaporated to dryness. The residue was heated under reflux with aqueous NaOH (200 ml., 1 *N*). An excess of concentrated HCl was then added, and the solution was concentrated. The dihydroxy acid which separated was recrystallized from methyl isobutyl ketone as prisms, m.p. 150°, yield 14 g. This material is apparently a hydrate for only after repeated crystallizations from the ketone could the melting point be raised to 166°; lit.[7] m.p. 168°.

***p*-(3-Bromo-2-hydroxy-*n*-propyloxy)benzoic Acid (II, X = OH; Y = Br).**—The above dihydroxy acid (2 g.) in aqueous HBr (10 ml., *d* 1.7) was heated on a steam bath for 1.5 hr. The solid obtained on cooling and diluting with water was recrystallized from water as plates, m.p. 153–155°. Repeated crystallization from aqueous methanol raised the melting point to 159–160°.

Anal. Calcd. for $C_{10}H_{11}BrO_4$: C, 43.7; H, 4.0; Br, 29.1. Found, C, 44.1; H, 4.0; Br, 29.9.

***p*-(2,3-Dibromo-*n*-propyloxy)benzoic Acid (II, X = Y = Br).**—Bromine (2.86 ml.) in chloroform (20 ml.) was slowly added to a stirred suspension of *p*-allyloxybenzoic acid[8] (9.9 g.) in $CHCl_3$ (100 ml.). The solution was warmed slightly to initiate the reaction which then proceeded with rapid decolorization of the added bromine. The suspended solid soon passed into solution but as more bromine was added reprecipitation occurred. When the addition was complete, the solution was stirred without heating for a further 0.5 hr. and then after dilution with petroleum ether (b.p. 40–60°) the solid was collected by filtration. The dibromo acid was recrystallized by dissolving in hot ethanol (200 ml.) and adding water until a turbidity formed; slow cooling then gave thick plates, m.p. 162–164°, yield 14 g. For analysis a specimen was dried at 100° (0.1 mm.).

Anal. Calcd. for $C_{10}H_{10}Br_2O_3$: C, 35.5; H, 3.0; Br, 47.3; neut. equiv., 338. Found: C, 36.1; H, 3.2; Br, 47.0; neut. equiv. by titration with 0.1 *N* NaOH, 336.

***p*-[2,3-Di(2-hydroxyethylthio)-*n*-propyloxy]benzoic Acid (I, X = OH).**—A mixture of dibromo acid (3.38 g.), mercaptoethanol (4.2 ml.), anhydrous Na_2CO_3 (3.71 g.), and water (25 ml.) was heated under reflux for 3 hr. The cooled solution was acidified with HCl (70 ml., 1 *N*), and the sticky solid which separated was collected. After trituration with ether the product was crystallized from methyl isobutyl ketone; it formed plates, m.p. 86–88°, yield 2 g. The acid appears to form a hydrate which tenaciously retains water but an anhydrous specimen was obtained by drying at 55° (0.1 mm.). When this material was extracted in a Soxhlet apparatus with dry ether, clusters of plates separated from the ether solution.

Anal. Calcd. for $C_{14}H_{20}O_5S_2$: C, 50.6; H, 6.1; S, 19.3; neut. equiv., 332.4. Found: C, 50.7; H, 6.1; S, 19.2; neut. equiv. by titration with 0.1 *N* NaOH, 329.

***p*-[2,3-Di(2-bromoethylthio)-*n*-propyloxy]benzoic Acid (I, X = Br). A.**—The above dihydroxy acid (5 g.) was stirred in concentrated aqueous HBr (15 ml., *d* 1.7) for 20 hr. at 25°. The insoluble material was collected by filtration, mixed with anhydrous $CaCl_2$, and extracted with dry ether. The extract was evaporated in a stream of dry air, and the residue repeatedly was extracted with hot petroleum ether (b.p. 60–80°). The cooled extracts gave the dibromo acid, m.p. 98–100°, yield 2 g.

B.—Dry HBr was passed into a suspension of the dihydroxy acid (20 g.) in boiling chloroform (400 ml.) until a clear solution was obtained. After the addition of charcoal, the solution was filtered and concentrated. Petroleum ether (b.p. 60–80°) was added to the hot solution until a turbidity formed. Slow cooling then gave clusters of flattened needles, m.p. 105–109°, not depressed by admixture with the product obtained by method A; yield 12.4 g.

Anal. Calcd. for $C_{14}H_{18}Br_2O_3S_2$: C, 36.7; H, 4.0; Br, 34.9; S, 14.0. Found: C, 36.9; H, 4.0; Br, 35.0; S, 14.1.

The titration equivalent of the dibromo acid could not be determined by direct titration with alkali because of the rapid hydrolysis of the carbon–bromine bond. However, if thiosulfate is present in the solution, reaction with this powerful nucleophile takes preference over hydrolysis and the equivalent is readily determined. At the same time the alkylating potential of the molecule can be assessed by back-titrating unreacted thiosulfate.

The dibromo acid (211 mg.) (I, X = Br) in ethanol (20 ml.) was added to aqueous sodium thiosulfate (20 ml., 0.1 *N*); 4.65 ml. of 0.1 *N* NaOH was required to titrate the acid (phenolphthalein indicator) indicating an equivalent weight of 454 (calcd. 458). After warming the solution for a short time the excess thiosulfate was titrated with 0.1 *N* iodine solution (starch indicator), 10.9 ml. being required. Thus 9.1 ml. of 0.1 *N* thiosulfate was required for reaction with the bromine atoms; the theoretical value if both bromine atoms interact with the anion is 9.2 ml.

TABLE I

HYDROLYSIS OF *p*-[2,3-DI(2-BROMOETHYLTHIO)-*n*-PROPYLOXY]-BENZOIC ACID IN AQUEOUS SOLUTION AT 2°, pH 8.3

Time (t), min.	0.1 *N* NaOH added (x)	log [$a/(a - x)$]
1	0.042	0.069
2	0.074	0.130
3	0.099	0.186
4	0.121	0.240
5	0.139	0.290
6	0.154	0.337
7	0.169	0.387
∞	0.28 (= a)	

TABLE II

RATE CONSTANTS AND HALF-LIVES FOR THE HYDROLYSIS OF *p*-[2,3-DI(2-BROMOETHYLTHIO)-*n*-PROPYLOXY]BENZOIC ACID

Temp., °C.	k, min.$^{-1}$	$t_{1/2}$
2	0.124	5.6 min.
14	0.634	1.1 min.
20	1.16	36 sec.
37	8.7	4.8 sec.

(6) Melting points were determined with a Townson and Mercer heated metal block apparatus and are corrected.

(7) W. Bradley and O. Stephenson, British Patent 619,403 (March 9, 1949).

(8) (a) J. B. Cohen and H. W. Dudley, *J. Chem. Soc.*, **97**, 1732 (1910); (b) L. Claisen and O. Eisleb, *Ann.*, **401**, 21 (1913); (c) Höchster Farbw., German Patent 423,031 (1925); P. Friedlander, "Fortschritte der Teerfarben fabrikation und verwandter Industriezweige," Vol. 15, Springer-Verlag, Berlin, 1928, p. 1608.

Rates of Hydrolysis of the Dibromo Acid (I, X = Br) at 2, 14, and 20°.—The hydrolysis rates were determined by measuring the rate of H^+ formation from the reaction

$$RSCH_2CH_2Br + H_2O \longrightarrow RSCH_2CH_2OH + H^+ + Br^-$$

All the measurements were made with the automatically recording Radiometer Titrigraph fitted with a glass electrode. Reaction solutions were maintained at the appropriate temperatures in jacketed vessels connected to a thermostated water bath. For each series of runs approximately 70 mg. of the dibromo acid was dissolved in 10 ml. of dry acetone. A 1-ml. aliquot of this solution was added to 20 ml. of water at the required temperature and containing the exact equivalent of decinormal NaOH required to neutralize the carboxyl group (approximately 0.2 ml.). Decinormal NaOH was automatically added to maintain the pH of the solution at its initial value of 8.3. The rate of addition was recorded on a time-scale graph.

Table I shows details of results obtained in a typical run. Dibromo acid (6.55 mg.) dissolved in 1 ml. of acetone was added at zero time to 20 ml. of water at 2° containing 0.14 ml. of 0.1 *N* NaOH. A plot of log $[a/(a - x)]$ against t gave a straight line for all runs showing that the reaction is first order with respect to dibromo acid. In the case of the data in Table I, the slope of this line is 0.053 and this equals $k/2.303$. Hence the rate constant is 0.124 min.$^{-1}$.

A series of runs was made at 2, 14, and 20° and from the mean values of the rate constants at each temperature the rate constant at 37° was computed graphically using the Arrhenius equation: $2.303 \log k = -E/RT + \text{constant}$. The plot of $\log k$ against $1/T$ (°K.) gave a straight line (Figure 1). From the point on the graph whose abscissa corresponded to 37° the ordinate gave the required rate constant. Table II shows the measured rate constants and times for half-reaction ($t_{1/2}$ or "half-life") and the extrapolated values for 37°. The energy of activation of the reaction calculated from the slope of the line in Figure 1 is 20,700 cal.

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Reactivity of Some 2-*p*-Nitrophenoxy-1,3,2-dioxaphospholane 2-Oxides and -dioxaphosphorinane 2-Oxides[1]

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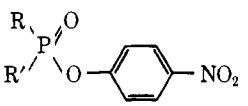
A number of 2-*p*-nitrophenoxy-1,3,2-dioxaphospholane 2-oxides and -dioxaphosphorinane 2-oxides have been prepared and examined for alkaline hydrolysis and anticholinesterase activity. Although the six-membered ring dioxaphosphorinane 2-oxides and the acyclic analog diethyl *p*-nitrophenyl phosphate (paraoxon) gave comparable rates of liberation of *p*-nitrophenol in aqueous NaOH, the cyclic esters were almost void of anticholinesterase activity. The five-membered ring dioxaphospholane 2-oxides gave instantaneous liberation of *p*-nitrophenol in water and were inactive as anticholinesterases.

The relationship between structure of phenyl esters of phosphoric, phosphonic, and phosphinic acids, their anticholinesterase properties, and toxicity to animals has been examined in great detail.[2-4] It has been shown that the inhibition of the cholinesterase enzymes is directly related to the reactivity of the phosphorus atom and correlations have been made between enzyme inhibition, alkaline hydrolysis rates, changes in P–O–aryl stretching frequencies, and Hammett σ-constants. The evidence shows that the organophosphorus poisons inhibit acetylcholinesterase and other esterases by phosphorylating the enzyme at one of the essential sites. Previous work from this and other laboratories have shown that in compounds of the general structure below, R and R′ may be varied to



considerable extent before extensive changes in the antiesterase properties become evident, provided the electrophilic character of the phosphorus atom is not altered to any large degree. For example, R and R′ may be alkyl or alkoxy groups of varying chain lengths and branching, and anticholinesterase activity is still maintained.

Steric factors must also be considered when assessing the reactivity of phosphorus esters, *e.g.*, when R or R′ is *t*-butyl, the compound is quite stable to alkaline hydrolysis, and anticholinesterase activity is low. In order to assess further the effect of steric factors in the inactivation of acetylcholinesterase by organophosphorus compounds, it was considered of interest to examine the activity of *p*-nitrophenyl esters in which the phosphorus atom was part of a ring system. Although five- and six-membered ring phosphorothionate esters of *p*-nitrophenol (2-sulfo-1,3,2-dioxaphospholanes and -phosphorinanes) have been proven to be poor anticholinesterases,[5] the corresponding oxygen esters have not been examined and their study seemed warranted since phosphorothionate esters are generally poor inhibitors because of the stabilizing effect of the sulfur atom. Further, five-membered ring cyclic phosphates are known to be quite susceptible to hydrolysis, but their activity toward cholinesterase has not been examined.

(1) Paper No. 1615, Citrus Research Center and Agricultural Experiment Station, University of California, Riverside, Calif. This investigation was supported in part by Public Health Service Research Grant CC-00038-07 from the Communicable Disease Center.

(2) W. N. Aldridge and A. N. Davison, *Biochem. J.*, **51**, 62 (1952).

(3) T. R. Fukuto and R. L. Metcalf, *J. Am. Chem. Soc.*, **81**, 372 (1959).

(4) G. Schrader, "Die Enwicklung neuer insektizider Phosphorsäure-Ester," Verlag Chemie, Weinheim, 1963.

(5) R. S. Edmundson and A. J. Lambie, *Chem. Ind.* (London), 1048 (1959).