Synthesis and Evaluation of Esters of N,N-Bis(2-chloroethyl)-*p*-aminophenol and N,N-Bis(2-bromoethyl)-*p*-aminophenol as Potential Antitumor Agents

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A series of benzoate esters of N,N-bis(2-chloro- (or bromo-) ethyl)-*p*-aminophenol have been synthesized and evaluated as antitumor agents against the Walker 256 carcinosarcoma to further test the hypothesis that hydrolysis of the ester linkage may be a prerequisite for antitumor activity. The 2,6-dimethylbenzoate of 4-(N,N-diethylamino)phenol was not hydrolyzed by crude rat liver esterases, and the toxicities and antitumor activities of the corresponding mustards were much reduced relative to the parent unsubstituted benzoate. These results lend some support to the previously expressed hypothesis that hydrolysis to the free phenolic mustards is a necessary step for antitumor activity. In a series of *meta*- and *para*-substituted benzoates of 4-(N,N-diethylamino)phenol, hydrolysis by the rat liver esterase preparation was dependent upon the electronic effects of the substituents but this dependence did not extend to the toxicities or antitumor activities of the corresponding nitrogen mustards.

As part of an investigation into the design of nitrogen mustard with latent activities it was decided to synthesize substituted benzoate esters of 4-[N,N-bis(2-chloro- (or bromo-) ethyl)amino]phenol. Ross[2a] has shown that the benzoate and acetate of 4-[N,N-bis(2-chloroethyl)amino]phenol, although chemically less reactive, were more effective against the Walker 256 tumor system than the parent 4-[N,N-bis(2-chloroethyl)amino]phenol. Hebborn and Danielli[2b] showed that the Walker tumor contains enzymes capable of hydrolyzing these esters and concluded that the esters were converted *in vivo* to the free phenol and that the latter exerted the antitumor action. The greater degree of selectivity of these esters, as indicated by their superior chemotherapeutic index relative to the parent phenol, was in part attributed to activation by esterases in the tumor cells. However, this conclusion was not substantiated completely and the possibility remained that the more favorable antitumor action of esters of 4-[N,N-bis(2-chloroethyl)amino]phenol was perhaps due to a more favored transport into the tumor cells where they could exert an alkylating effect *in toto*.

In an attempt to distinguish between these possibilities, the 2-methyl- and 2,6-dimethylbenzoates of 4-[N,N-bis(2-chloroethyl)amino]phenol and 4-[N,N-bis-(2-bromoethyl)amino]phenol were prepared and their activities against the Walker 256 system were determined and compared with the unsubstituted benzoate. If hydrolysis of these compounds to the parent phenol is a prerequisite for effective biological activity then the *ortho*-substituted compounds should be less toxic than the parent benzoate because of their less rapid hydrolysis.

On the assumption that the antitumor activities of esters of 4-[N,N-bis(2-chloro- (or bromo-) ethyl)amino]-phenol do depend upon enzymatic hydrolysis it was decided to determine whether any general relationship existed between the ease of enzymatic hydrolysis, the toxicities, antitumor activities, and the electronic effects of substituents in a series of *meta*- and *para*-substituted benzoate esters of 4-[N,N-bis(2-chloro-(or bromo-) ethyl)amino]phenol.

Experimental Section[3]

Synthetic Procedures.—Where one method is used to prepare more than one compound in a series, a procedure is described and a summary of the details for other members of the series is presented in Tables I–V.

4-[N,N-Bis(2-chloroethyl)amino]phenol Hydrobromide (I).—The following procedure proved to be considerably more convenient than that described in the literature.[4] Benzyl 4-[N,N-bis(2-chloroethyl)amino]phenyl ether (13.0 g, 0.04 mole), prepared from benzyl 4-[N,N-bis(2-hydroxyethyl)amino]phenyl ether[4] by the method of Cohen and Tipson,[5] was stirred at room temperature in 32% HBr–AcOH (100 ml) for 4 hr. The solution was poured into Et_2O (1000 ml), and the precipitate was filtered and crystallized from AcOH to give I, mp 202–210°, in 75% yield. The analysis was not completely satisfactory, probably due to halogen exchange in the above procedure, but this material proved to be completely satisfactory for subsequent reactions. *Anal.* ($C_{10}H_{14}BrCl_2NO$) Br: calcd, 25.37; found, 26.91, 27.01.

4-[N,N-Bis(2-bromoethyl)amino]phenol hydrobromide (II) was prepared similarly to I and had mp 214–215° (AcOH), in 77% yield. *Anal.* ($C_{10}H_{14}Br_3NO$) C, H, Br.

4-[N,N-Bis(ethyl- (or 2-haloethyl-) amino]phenyl benzoates (1–49, Tables I–III) were prepared by esterifying the appropriate phenol with the substituted acid chloride in the presence of either pyridine or Et_3N.

A. Using Pyridine.—4-[N,N-Bis(2-bromoethyl)amino]phenol hydrobromide (II, 1.1 g, 0.003 mole) was suspended in dry pyridine (10 ml) and warmed in a steam bath. To the warm solution was added 3-chlorobenzoyl chloride (500 mg, 0.003 mole). After 45 min at room temperature, H_2O (10 ml) was added followed by sufficient HCl (10%) to neutralize the pyridine. The precipitated ester was filtered and recrystallized.

B. Using Et_3N.—4-[N,N-Bis(2-bromoethyl)amino]phenol was liberated from its HBr salt by the addition of $NaHCO_3$ solution and extraction (Et_2O). The free phenol (650 mg, 0.002 mole) was dissolved in Et_3N (10 ml) and 4-methoxybenzoyl chloride (450 mg, 0.0026 mole) was added. After 20 min at room temperature, the ester was isolated according to the procedure described under A.

Enzyme Assay.—The source of the esterase employed was a crude rat liver homogenate. Male Holtzman rats were decapitated and the liver was rapidly removed, placed on ice, weighed, minced,

(1) (a) Department of Biochemical Pharmacology; (b) Department of Biochemistry; (c) Center for Theoretical Biology.

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(3) Melting points were determined with a Thomas–Kofler hot stage and are corrected. Analyses are by Dr. A. Bernhardt, Mulheim, West Germany, and, where indicated only by symbols of the elements, the results are within ±0.4% of the calculated values.

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TABLE I

PHYSICAL DATA FOR SUBSTITUTED BENZOATE ESTERS OF 4-[N,N-BIS(2-BROMOETHYL)AMINO]PHENOL

$XC_6H_4COOC_6H_4N(CH_2CH_2Br)_2$-*p*

No.	X	Method[a]	Mp, °C	Yield, %	Solvent[b]	Formula[c]
1	H	A	101.5–102	59	a	$C_{17}H_{17}Br_2NO_2$
2	2-Me	A	102–106.5	62	b	$C_{18}H_{19}Br_2NO_2$
3	2,6-Me_2	B	88.5–90	45	b	$C_{19}H_{21}Br_2NO_2$
4	2-C_2H_5	A	80–82	38	a	$C_{19}H_{21}Br_2NO_2$
5	2-C_3H_7-*i*	A	77–79	30	a	$C_{20}H_{23}Br_2NO_2$
6	3-CH_3	A	70–72	65	b	$C_{18}H_{19}Br_2NO_2$
7	4-CH_3	A	111–112.5	58	c	$C_{18}H_{19}Br_2NO_2$
8	3-Cl	A	117.5–119.5	63	a	$C_{17}H_{16}Br_2ClNO_2$
9	4-Cl	A	132–136.5	57	a	$C_{17}H_{16}Br_2ClNO_2$
10	3-MeO	A	85.5	79	a	$C_{18}H_{19}Br_2NO_3$
11	4-MeO	B	113–117.5	54	a	$C_{18}H_{19}Br_2NO_3$
12	3-CF_3	A	81.5–84	65	b	$C_{18}H_{16}Br_2F_3NO_2$
13	4-CF_3	A	92.5–93.5	66	b	$C_{18}H_{16}Br_2F_3NO_2$
14	3-NO_2	A	82–87.5	45	a	$C_{17}H_{16}Br_2N_2O_4$
15	4-NO_2	A	117–118	64	a	$C_{17}H_{16}Br_2N_2O_4$
16	4-C_6H_5	A	138	50	a	$C_{23}H_{21}Br_2NO_2$
17	4-C_7H_{15}-*n*	B	71–75	40	b	$C_{24}H_{31}Br_2NO_2$
18	4-C_9H_{19}-*n*	B	64–65	65	b	$C_{26}H_{35}Br_2NO_2$

[a] A, esterification in pyridine; B, esterification in Et_3N. [b] Solvent: a, C_6H_6–ligroin (bp 50–80°); b, ligroin (bp 100–120°); c, ligroin (bp 50–80°). [c] All compounds were analyzed for C, H, Br, N.

TABLE II

PHYSICAL DATA FOR SUBSTITUTED BENZOATE ESTERS OF 4-[N,N-BIS(2-CHLOROETHYL)AMINO]PHENOL

$XC_6H_4COOC_6H_4N(CH_2CH_2Cl)_2$-*p*

No.	X	Method	Mp, °C	Yield, %	Solvent[a]	Formula[b]
19	H	A	Known[a]		a	
20	2-Me	A	69–71	65	b	$C_{18}H_{19}Cl_2NO_2$
21	2,6-Me_2	A	57.5–59	41	b	$C_{19}H_{21}Cl_2NO_2$
22	4-Me	A	85–87	59	b	$C_{18}H_{19}Cl_2NO_2$
23	3-Br	A	68–69	38	a	$C_{17}H_{16}BrCl_2NO_2$
24	4-Br	A	71–73	42	a	$C_{17}H_{16}BrCl_2NO_2$
25	3-Cl	A	103	65	a	$C_{17}H_{16}Cl_3NO_2$
26	3-F	A	94–96	67	b	$C_{17}H_{16}Cl_2FNO_2$
27	4-F	A	88–90	74	b	$C_{17}H_{16}Cl_2FNO_2$
28	4-MeO	A	76–78	41	a	$C_{18}H_{19}Cl_2NO_3$
29	3-NO_2	A	66–68	40	a	$C_{17}H_{16}Cl_2N_2O_4$

[a] Solvent: a, C_6H_6–ligroin (bp 50–80°); b, ligroin (bp 100–120°). [b] All compounds were analyzed for C, H, Cl, N.

TABLE III

PHYSICAL DATA FOR SUBSTITUTED BENZOATE ESTERS OF 4-(N,N-DIETHYLAMINO)PHENOL

$XC_6H_4COOC_6H_4NEt_2$-*p*

No.	X	Method	Mp, °C	Yield, %	Solvent[a]	Formula[b]
30	H	A	138	57	a	$C_{17}H_{19}NO_2$
31	2-Me	A	102–103.5	60	b	$C_{18}H_{21}NO_2$
32	2,6-(Me)	A	82–84	37	b	$C_{19}H_{23}NO_2$
33	2-Et	A	109	55	b	$C_{19}H_{23}NO_2$
34	2-Pr-*i*	A	85–86	40	b	$C_{20}H_{25}NO_2$
35	3-Me	A	110–111	65	a	$C_{18}H_{21}NO_2$
36	4-Me	A	115–116.5	60	a	$C_{18}H_{21}NO_2$
37	3-Cl	A	120–121.5	65	b	$C_{17}H_{18}ClNO_2$
38	4-Cl	A	99.5–102.5	35	b	$C_{17}H_{18}ClNO_2$
39	3-MeO	A	129–131	60	a	$C_{18}H_{21}NO_3$
40	4-MeO	B	116–117	58	a	$C_{18}H_{21}NO_3$
41	3-CF_3	A	83–85	25	a	$C_{18}H_{18}F_3NO_2$
42	4-CF_3	A	82–84	36	b	$C_{18}H_{18}F_3NO_2$
43	3-NO_2	A	100.5–103	30	b	$C_{17}H_{18}N_2O_4$
44	4-NO_2	A	119–121	30	b	$C_{17}H_{18}N_2O_4$
45	4-C_6H_5	A	125–129	70	b	$C_{23}H_{23}NO_2$
46	4-C_7H_{15}-*n*	B	85–87	74	b	$C_{24}H_{33}NO_2$
47	4-C_9H_{19}-*n*	B	67–69	70	b	$C_{26}H_{37}NO_2$

[a] Solvent: a, C_6H_6–ligroin (bp 50–80°); b, ligroin (bp 100–120°). [b] All compounds were analyzed for C, H, N.

TABLE IV

AMOUNT OF 4-(N,N-DIETHYLAMINO)PHENOL RELEASED BY ENZYMIC HYDROLYSIS OF SUBSTITUTED 4-(N,N-DIETHYLAMINO)PHENYL BENZOATES

$RC_6H_4COOC_6H_4N(C_2H_5)_2$-*p*

No.	R	Hammett σ value	Phenol released, μmoles	Incubation period, min
44	4-NO_2	+0.78	4.67	5
43	3-NO_2	+0.71	3.57	5
42	4-CF_3	+0.54	2.79	5
41	3-CF_3	+0.43	2.17	5
37	3-Cl	+0.37	2.07	5
38	4-Cl	+0.22	1.60	5
36	4-Me	−0.17	0.39	5
35	3-Me	−0.07	2.07	5
40	4-MeO	−0.27	0.32	5
39	3-MeO	+0.11	1.42	5
30	H	0	0.55	5
30	H	0	2.34	25
31	2-Me	--	1.15	25
32	2,6-Me_2	—	0.01	25

(0.5 ml, 30%) and centrifuged. The supernatant was removed, saturated with NaCl, and extracted with *n*-BuOH (2 ml). An 0.5-ml aliquot of the BuOH extract was made up to 5.75 ml with EtOH (4 ml, 20% aqueous solution), Folin reagent (0.25 ml), and Na_2CO_3 (1.0 ml, 10%). Color was developed by warming the tube to 95° for 5 min. The solution was clarified by centrifugation and the optical density was determined at 750 mμ using a Perkin-Elmer 202 spectrophotometer. The amount of 4-(N,N-diethylamino)phenol released was determined from a previously obtained standard curve. Control experiments were run in parallel to determine the amount of spontaneous hydrolysis of the compounds and the amounts of color contributed by the homogenate. In these experiments, conditions were identical with those described save that substrate or homogenate was absent. Additional control experiments demonstrated that the BuOH extraction technique gave recoveries of 90–100%.

Biological Test Methods.—Toxicity determinations were performed using male Swiss mice (20–25 g). The compound dissolved in cottonseed oil was administered by intraperitoneal injections to groups of three to six mice/dose level. Deaths within a 21-day period were recorded and LD_{50} values were estimated using the method of Litchfield and Wilcoxon.[7]

Antitumor activities of the compounds against the Walker 256 carcinosarcoma were determined as follows: Actively growing tumor tissue (200 mg) obtained from a tumor-bearing rat killed by cervical fracture was implanted into the right flank of anesthetized male Holtzman rats by means of trochar and cannula. On the day following implantation of the tumor, the drugs were administered intraperitoneally in cottonseed oil. Each drug was administered at three different dose levels with each dose level being administered to three rats. Ten days after implantation the animals were killed and the tumors were excised and weighed. Tumor growth in drug-treated animals was compared with tumor growth in control animals which received the vehicle only.

TABLE V

ANTITUMOR AND TOXICITY DATA OF N,N-BIS(2-HALOETHYL)AMINOPHENOLS AND THEIR ESTERS

$ROC_6H_4N(CH_2CH_2X)_2$

No.	R	X	Mouse toxicity LD_{50}, mg/kg	Rat toxicity LD_{50}, mg/kg	Rat antitumor act. ED_{50}, mg/kg	LD_{50}/ED_{90}
A	H	Cl	17.5	20	8.5	2.3
B	H	Br	3.5	3.0	3.0	1.0
1	C_6H_5CO	Br	2.0	1.5	1.5	1.0
2	2-MeC_6H_4CO	Br	6.0	5.2	4.8	1.1
3	2,6-$(Me)_2C_6H_3CO$	Br	130	120	70.0	1.7
4	2-EtC_6H_4CO	Br	3.5	10.4	5.0	2.1
5	2-*i*-PrC_6H_4CO	Br	6.5	10.0	19.0	<1.0
6	3-MeC_6H_4CO	Br	2.7	3.0	2.9	<1.0
7	4-MeC_6H_4CO	Br	1.4	2.1	5.0	<1.0
8	3-ClC_6H_4CO	Br	4.2	3.5	5.0	<1.0
9	4-ClC_6H_4CO	Br	4.0	3.0	>10.0	<1.0
10	3-$MeOC_6H_4CO$	Br	3.8	2.5		
11	4-$MeOC_6H_4CO$	Br	2.8	2.8	3.4	<1.0
12	3-$CF_3C_6H_4CO$	Br	3.9	3.8	5.0	<1.0
13	4-$CF_3C_6H_4CO$	Br	5.0			
14	3-$O_2NC_6H_4CO$	Br	4.0	4.0	>10.0	<1.0
15	4-$O_2NC_6H_4CO$	Br	3.0	2.0	6.4	<1.0
16	4-$C_6H_5C_6H_4CO$	Br	16.0			
20	C_6H_5CO	Cl	18.5	29.0	4.6	6.3
21	2-MeC_6H_4CO	Cl	36.0	68.0	10.0	6.8
22	2,6$Me_2C_6H_3CO$	Cl	500	290	180	1.6
23	4-MeC_6H_4CO	Cl	15.5	22.0	5.9	3.7
24	3-BrC_6H_4CO	Cl	>30.0	~60.0	25.0	~2.4
25	4-BrC_6H_4CO	Cl	19.0	27.0	6.0	4.5
26	3-ClC_6H_4CO	Cl	21.0	42.0	10.5	4.0
27	3-FC_6H_4CO	Cl	10.5	15.0	4.7	3.2
28	4-FC_6H_4CO	Cl	11.5	22.0	4.5	5.8
29	4-$MeOC_6H_4CO$	Cl	21.5	28.5	4.8	5.9
30	3-$O_2NC_6H_4CO$	Cl	15.5	15.0	6.0	2.5

and homogenized in sucrose (0.25 *M*) to give a 30% homogenate. Esterase activity was determined colorimetrically using the Folin–Ciocalteau reagent[6] to determine the amount of 4-(N,N-diethylamino)phenol released on hydrolysis of the benzoate esters of 4-(N,N-diethylamino)phenol which were used as analogs of the corresponding alkylating agents. The incubation mixture contained the substrate (10^{-3} *M* final concentration), DMSO (150 μl), Tris buffer (2 ml, 0.1 *M*, pH 8.5), and homogenate (0.5 ml). The homogenate had previously been incubated with KCN (150 μl, 1.0 m*M*) for 5 min to prevent oxidation of the subsequently liberated aminophenol. After incubation (5 min) at 32°, an aliquot of the mixture (2 ml) was added to trichloroacetic acid

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Discussion

As anticipated the 2-methyl- and 2,6-dimethylbenzoates of 4-[N,N-bis(2-chloroethyl)amino]phenol (**21** and **22**, Table V) and 4-[N,N-bis(2-bromoethyl)amino]-phenol (**2** and **3**, Table V) were less toxic than the unsubstituted compounds (**1** and **20**, Table V), particularly dramatic decreases being observed with the 2,6-dimethyl analogs. That these decreases in toxicity may be due to increased resistance to hydrolysis is suggested by the data of Table IV which reveal that the 2,6-dimethylbenzoate of 4-(N,N-diethylamino)phenol[8] is hydrolyzed only very slowly by the crude liver esterase preparation. These results are paralleled by the antitumor activities (Table V) which show that the 2,6-dimethylbenzoates (**3** and **22**) are active only at markedly higher dose levels. These findings are thus consistent with the previously expressed hypothesis[2] that hydrolysis of esters of 4-[N,N-bis(2-haloethyl)amino]phenol is a prerequisite for significant antitumor activity.

Examination of the enzyme and animal data for the series of *meta*- and *para*-substituted benzoates of 4-[N,N-bis(2-halogenoethyl)amino]phenol shows that, as anticipated,[9] hydrolysis is facilitated by electron-withdrawing substituents and decreased by electron-releasing substituents. However, these substituents did not exert a large effect upon the sensitivity of the compounds to enzymatic hydrolysis (Table IV). In view of this finding, large differences in the biological activities of these compounds would not be expected. In fact, the toxicities and antitumor activities of the esters of 4-[N,N-bis(2-bromoethyl)amino]phenol (**1**, **6**–**16**, Table V) are not significantly different from those of the parent phenol (B, Table V). Similarly, there is little difference in the biological activities of the members of the chloro series (**19**, **22**–**29**, Table V; **23** appears to be an exception), although because of their lower reactivity these compounds are significantly less toxic than their bromo analogs.

Finally, it will be observed that the more favorable therapeutic indices (as measured by LD_{50}/ED_{90}) are found with the esters of 4-[N,N-bis(2-chloroethyl)-amino]phenol. This is, in part, probably due to the fact that the derived phenol, A (Table V), has a more favorable activity than the bromo analog (B).

(8) Esters of 4-(N,N-diethylamino)phenol were used as appropriate model compounds in the enzyme studies to avoid the complication of using the alkylating agents in this system.[2]

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2-Chloroadenosine 5′-Phosphate and 2-Chloroadenosine 5′-Diphosphate, Pharmacologically Active Nucleotide Analogs

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2-Chloroadenosine 5′-phosphate and diammonium 2-chloroadenosine 5′-diphosphate have been synthesized, and isolated analytically pure. Their effects on the *in vitro* aggregation of sheep platelets in platelet-rich plasma and on rat arterial blood pressure were compared with those of adenosine, 2-chloroadenosine, adenosine 5′-phosphate (AMP), and adenosine 5′-diphosphate (ADP). 2-Chloroadenosine 5′-phosphate (2-chloro-AMP) inhibited the ADP-mediated aggregation of sheep platelets and was initially equipotent with AMP and was longer acting; 2-chloro-AMP was a more potent and longer acting vasodepressor than AMP. 2-Chloroadenosine 5′-diphosphate (2-chloro-ADP) was of similar potency to ADP as a vasodepressor and was longer acting; 2-chloro-ADP reversibly aggregated sheep platelets and was nine times as potent as ADP.

Adenosine, adenosine 5′-phosphate (AMP), and adenosine 5′-diphosphate (ADP) are physiologically active in a number of *in vivo* and *in vitro* preparations. For example, adenosine on intravenous administration in the cat[1] caused a transitory drop in blood pressure, and in the anesthetized open-chested dog adenosine, AMP, and ADP have been found to have brief coronary vasodilator effects.[2] ADP in concentrations as low as 10^{-6} *M* causes mammalian platelets in plasma to aggregate reversibly,[3] a phenomenon which is believed to play a key role in hemostasis.[4] Adenosine and AMP have been shown to inhibit the ADP-mediated aggregation of platelets,[3] although their inhibitory effects are of short duration. The analog 2-chloroadenosine has more potent effects than adenosine, both on the vascular[1,5] and platelet-aggregation systems,[6] and its action in both systems is of greater duration than that of adenosine. The ready deamination of adenosine to inosine by adenosine deaminase is thought to explain the transient nature of the adenosine effect, since inosine is without activity either as a vasodilator[1] or as an inhibitor of platelet aggregation.[3] 2-Chloroadenosine is not deaminated by adenosine deaminase,[1,7] and this resistance to deamination may explain the greater duration of action of 2-chloroadenosine; it does not, however, ex-

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