

with water and the aqueous phase extracted with chloroform. The combined chloroform extracts were dried ( $\text{Na}_2\text{SO}_4$ ) and evaporated and the residue was crystallized from ethyl acetate to give 2-chloro-4-(4-formylpiperazin-1-yl)pyrimidine (24.0 g, 32%), mp 125–126 °C. Anal. ( $\text{C}_9\text{H}_{11}\text{ClN}_4\text{O}$ ) C, H, N.

(b) A solution of sodium phenoxide (2.50 g, 22 mmol) in 1,2-dimethoxyethane (160 mL) was treated with a sample (5.0 g, 22 mmol) of the product from (a) and then heated under reflux for 24 h. The solvent was evaporated, the residue partitioned between chloroform (50 mL) and water (30 mL), and the aqueous phase extracted with chloroform. The combined chloroform extracts were dried ( $\text{Na}_2\text{SO}_4$ ) and evaporated, and the residue was triturated with ether followed by crystallization from ethyl acetate to give 2-phenoxy-4-(4-formylpiperazin-1-yl)pyrimidine 0.25-hydrate (2.93 g, 47%), mp 149–151 °C. Anal. ( $\text{C}_{15}\text{H}_{16}\text{N}_4\text{O}_2 \cdot 0.25\text{H}_2\text{O}$ ) C, H, N.

(c) A sample (2.6 g, 9.2 mmol) of the above product in methanol (27 mL) and 2 N hydrochloric acid (6.9 mL) was left at room temperature for 24 h and then heated on a steam bath for 0.5 h. The mixture was evaporated and the residue crystallized from propan-2-ol to give 2-phenoxy-4-piperazin-1-ylpyrimidine (1.5 g, 64%) characterized spectroscopically (12).

**3-Isopropoxy-6-piperazin-1-ylpyridazine (13).** 3-Chloro-6-piperazin-1-ylpyridazine (4.0 g, 20 mmol) and sodium isopropoxide [from sodium (0.7 g, 30 mmol) and propan-2-ol (70 mL)] were heated in a sealed bomb at 130–140 °C for 10 h. The mixture was then evaporated, the residue taken up in dichloromethane (300 mL), and the solution washed with water ( $2 \times 50$  mL). The organic layer was dried ( $\text{Na}_2\text{SO}_4$ ) and evaporated to give 3-isopropoxy-6-piperazin-1-ylpyridazine (3.3 g, 74%). A sample of the product was converted to the dimaleate salt hemihydrate, which was recrystallized from ethanol, mp 144–145 °C. Anal. ( $\text{C}_{11}\text{H}_{18}\text{N}_4\text{O} \cdot 2\text{C}_4\text{H}_4\text{O}_4 \cdot 0.5\text{H}_2\text{O}$ ).

2-Chloro-4-propoxy-pyrimidine, bp 101–103 °C (14 mm), was prepared in a similar manner from 2,4-dichloropyrimidine and sodium propoxide at 40 °C and characterized spectroscopically.

**Biology.** Experimental details for evaluation of  $\alpha$ -adrenoceptor binding and antihypertensive activities have been detailed pre-

viously.<sup>1</sup> Okamoto SHR were used for the evaluation of 15, 16, 28, 29 and New Zealand AS genetically hypertensive rats for the remaining compounds in Table II.

**Acknowledgment.** We gratefully thank Drs. V. A. Alabaster and P. M. Greengrass for biological results, D. J. Sreenan for  $\text{pK}_a$  data, and Dr. M. S. Tute for theoretical calculations and modeling studies. In addition, K. A. Bevan, V. A. Horne, and M. J. Palmer provided valuable technical support.

**Registry No.** 4·2HCl (het = pyrimidin-2-yl,  $\text{R}^1 = \text{R}^2 = \text{H}$ ), 94021-22-4; 4·2HCl (het = pyridazin-3-yl,  $\text{R}^1 = \text{R}^2 = \text{H}$ ), 90434-90-5; 4·2HCl (het = pyrazin-2-yl,  $\text{R}^1 = \text{R}^2 = \text{H}$ ), 109467-19-8; 4·2HCl (het = pyrimidin-3-yl,  $\text{R}^1 = 6\text{-O-}i\text{-C}_3\text{H}_7$ ,  $\text{R}^2 = \text{H}$ ), 109467-20-1; 5, 23680-84-4; 6 (het = pyrimidin-2-yl,  $\text{R}^1 = 4\text{-OCH}_3$ ,  $\text{R}^2 = \text{H}$ ), 22536-63-6; 6 (het = pyrimidin-2-yl,  $\text{R}^1 = 4\text{-OC}_3\text{H}_7$ ,  $\text{R}^2 = \text{H}$ ), 83774-10-1; 6 (het = pyrimidin-2-yl,  $\text{R}^1 = 4\text{-N(CH}_3)_2$ ,  $\text{R}^2 = \text{H}$ ), 31058-81-8; 6 (het = pyrimidin-2-yl,  $\text{R}^1 = 4\text{-CH}_3$ ,  $\text{R}^2 = \text{H}$ ), 13036-57-2; 6 (het = pyrimidin-2-yl,  $\text{R}^1 = 4\text{-C}_6\text{H}_5$ ,  $\text{R}^2 = \text{H}$ ), 13036-50-5; 6 (het = pyrimidin-4-yl,  $\text{R}^1 = 6\text{-OC}_3\text{H}_7$ ,  $\text{R}^2 = \text{H}$ ), 83774-14-5; 6 (het = pyrimidin-4-yl,  $\text{R}^1 = 6\text{-O-}i\text{-C}_3\text{H}_7$ ,  $\text{R}^2 = \text{H}$ ), 83774-13-4; 6 (het = pyrimidin-4-yl,  $\text{R}^1 = \text{R}^2 = 2,6\text{-(OCH}_3)_2$ ), 6320-15-6; 6 (het = pyrimidin-4-yl,  $\text{R}^1 = 6\text{-N(CH}_3)_2$ ,  $\text{R}^2 = \text{H}$ ), 31058-83-0; 6 (het = *s*-triazin-2-yl,  $\text{R}^1 = \text{R}^2 = 4,6\text{-(OCH}_3)_2$ ), 3140-73-6; 6 (het = *s*-triazin-2-yl,  $\text{R}^1 = \text{R}^2 = 4,6\text{-(OC}_6\text{H}_5)_2$ ), 2972-65-8; 6 (het = *s*-triazin-2-yl,  $\text{R}^1 = \text{R}^2 = 4,6\text{-(NH}_2)_2$ ), 3397-62-4; 7, 60547-97-9; 8, 7755-92-2; 9, 3934-20-1; 10, 83774-24-7; 11, 83774-15-6; 11 (4-formyl), 109467-27-8; 12, 56392-83-7; 13, 83774-20-3; 13·2C<sub>4</sub>H<sub>4</sub>O<sub>4</sub>, 83774-27-0; 14, 109467-21-2; 14·2HCl, 83773-87-9; 15, 83774-07-6; 16, 109467-22-3; 16·HCl, 83773-96-0; 17, 109467-23-4; 17·HCl, 83773-99-3; 18, 83773-65-3; 19, 109467-26-7; 19·2HCl, 83773-71-1; 20, 109467-24-5; 20·HCl, 83773-68-6; 21, 83773-69-7; 22, 83773-64-2; 23, 83773-82-4; 24, 83773-81-3; 25, 83773-86-8; 26, 83773-72-2; 13·2C<sub>4</sub>H<sub>4</sub>O<sub>4</sub>, 83774-27-0; 27, 83773-80-2; 28, 83774-03-2; 29, 83773-93-7; 30, 109467-25-6; 30·HCl, 83773-76-6; 31, 83773-73-3; 32, 83773-75-5; 4-amino-6,7-dimethoxy-2-[4-(2-chloropyrimidin-4-yl)piperazin-1-yl]quinazoline, 83774-02-1.

## Effects of [(N-Alkyl-1,3-dioxo-1*H*,3*H*-isoindolin-5-yl)oxy]alkanoic Acids, [(N-Alkyl-1-oxo-1*H*,3*H*-isoindolin-5-yl)oxy]butanoic Acids, and Related Derivatives on Chloride Influx in Primary Astroglial Cultures

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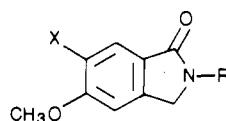
It has been shown that agents that inhibit chloride influx and therefore lower intracellular chloride levels in a major cell type in cerebral gray matter, the astrocyte, inhibit astrocytic swelling in vitro and in vivo. In our laboratories, 4-[(N-alkyl-1,3-dioxo-1*H*,3*H*-isoindolin-5-yl)oxy]alkanoic acids and related derivatives have been synthesized and tested for ability to lower intracellular astrocytic chloride levels in an established in vitro cultured rat astrocyte model. In general, derivatives with nitrogen substituents such as relatively small alkyl groups are active at 0.1 mM and/or 0.5 mM levels whereas larger substituents such as cyclopentyl and cyclohexyl are less active. Halogen substitution on the aromatic ring did not enhance activity. Derivatives with acid side chains of four carbons demonstrated superior activity to those of two carbons.

It has been shown that cerebral swelling is due largely to swelling of the glial cell, the astrocyte, in the cerebral gray matter.<sup>1</sup> Astrocytic swelling is a consequence of increased chloride influx followed by passive influx of an osmotic equivalent of water. Loop diuretics such as ethacrynic acid and furosemide markedly inhibit the influx of chloride in both cultured rat astrocytes and those associated with intact cerebral tissue.<sup>2,3</sup> In addition to the

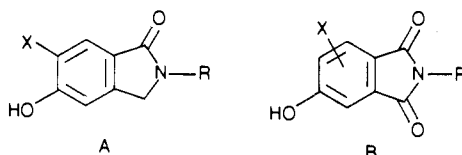
increased intracranial pressure that results from such swelling, astrocytic swelling alters capillary-tissue interaction and is detrimental to capillary-tissue solute transfer since astrocytes are involved in the structure of the blood-brain barrier.<sup>4</sup>

(1) Bourke, R. S.; Kimelberg, H. K. *Neurol. Trauma*; Raven: New York, 1979; p 95.

(2) Cragoe, E. J.; Gould, N. P.; Waltersdorf, O. W.; Ziegler, C.; Bourke, R. S.; Nelson, L. R.; Kimelberg, H. K.; Waldman, J. B.; Popp, A. J.; Sedransk, N. *J. Med. Chem.* 1982, 25, 567.  
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Table I. *N*-Alkyl-5-methoxy-1*H*,3*H*-isoindolin-1-ones

no.	R	X	starting material	mp, °C	yield, %	formula
25	CH <sub>3</sub>	H	10	105–107	62	C <sub>10</sub> H <sub>11</sub> NO <sub>2</sub>
26	C <sub>2</sub> H <sub>5</sub>	H	10	85–86	41	C <sub>11</sub> H <sub>13</sub> NO <sub>2</sub>
27	<i>n</i> -C <sub>3</sub> H <sub>7</sub>	H	10	91–93	32	C <sub>12</sub> H <sub>15</sub> NO <sub>2</sub>
28	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	H	10	oil	53	C <sub>13</sub> H <sub>17</sub> NO <sub>2</sub>
29	<i>c</i> -C <sub>5</sub> H <sub>9</sub>	Cl	7	104–107	74	C <sub>14</sub> H <sub>16</sub> ClNO <sub>2</sub>

Table II. *N*-Alkyl-5-hydroxy-1*H*,3*H*-isoindolin-1-ones and *N*-Alkyl-5-hydroxy-1*H*,3*H*-isoindoline-1,3-diones

no.	type	R	X	starting material	mp, °C	yield, %	formula
30	A	CH <sub>3</sub>	H	25	244–246	100	C <sub>9</sub> H <sub>9</sub> NO <sub>2</sub>
31	A	C <sub>2</sub> H <sub>5</sub>	H	26	224–225	23	C <sub>10</sub> H <sub>11</sub> NO <sub>2</sub>
32	A	<i>n</i> -C <sub>3</sub> H <sub>7</sub>	H	27	205–207	87	C <sub>11</sub> H <sub>13</sub> NO <sub>2</sub>
33	A	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	H	28	134–137	72	C <sub>12</sub> H <sub>15</sub> NO <sub>2</sub>
34	A	<i>c</i> -C <sub>5</sub> H <sub>9</sub>	Cl	29	274–275	65	C <sub>13</sub> H <sub>14</sub> ClNO <sub>2</sub>
35	B	<i>c</i> -C <sub>5</sub> H <sub>9</sub>	H	4HPA <sup>a</sup>	255–257	84	C <sub>13</sub> H <sub>13</sub> NO <sub>3</sub>
36	B	<i>c</i> -C <sub>6</sub> H <sub>11</sub>	H	4HPA	277–280	82	C <sub>14</sub> H <sub>15</sub> NO <sub>3</sub>
37	B	<i>c</i> -C <sub>5</sub> H <sub>9</sub>	6-Cl	11	gum	48	C <sub>13</sub> H <sub>12</sub> ClNO <sub>3</sub>
38	B	<i>c</i> -C <sub>5</sub> H <sub>9</sub>	4-Cl	11 <sup>b</sup>	116–118	13	C <sub>13</sub> H <sub>12</sub> ClNO <sub>3</sub>
39	B	<i>c</i> -C <sub>5</sub> H <sub>9</sub>	4,6-Cl <sub>2</sub>	12	213–215	77	C <sub>13</sub> H <sub>11</sub> Cl <sub>2</sub> NO <sub>3</sub>
40	B	<i>c</i> -C <sub>5</sub> H <sub>9</sub>	6,7-Cl <sub>2</sub>	2	274–275	70	C <sub>13</sub> H <sub>11</sub> Cl <sub>2</sub> NO <sub>3</sub>
41	B	CH <sub>3</sub>	H	4HPA	245–246	93	C <sub>9</sub> H <sub>7</sub> NO <sub>3</sub>
42	B	C <sub>2</sub> H <sub>5</sub>	H	4HPA	195–197	83	C <sub>10</sub> H <sub>9</sub> NO <sub>3</sub>
43	B	<i>n</i> -C <sub>3</sub> H <sub>7</sub>	H	4HPA	gum	72	C <sub>11</sub> H <sub>11</sub> NO <sub>3</sub>
44	B	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	H	4HPA	gum	95	C <sub>12</sub> H <sub>13</sub> NO <sub>3</sub>
45	B	<i>n</i> -C <sub>5</sub> H <sub>11</sub>	H	4HPA	86–88	62	C <sub>13</sub> H <sub>15</sub> NO <sub>3</sub>
46	B	<i>n</i> -C <sub>6</sub> H <sub>13</sub>	H	4HPA	104–106	72	C <sub>14</sub> H <sub>17</sub> NO <sub>3</sub>
47	B	H	H	4HPA	288–290	86	C <sub>8</sub> H <sub>5</sub> NO <sub>3</sub>
48	B	CH <sub>3</sub>	6,7-Cl <sub>2</sub>	2	241–243	64	C <sub>9</sub> H <sub>5</sub> Cl <sub>2</sub> NO <sub>3</sub>
49	B	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	6,7-Cl <sub>2</sub>	2	217–219	8	C <sub>12</sub> H <sub>11</sub> Cl <sub>2</sub> NO <sub>3</sub>
50	B	<i>n</i> -C <sub>3</sub> H <sub>7</sub>	6,7-Cl <sub>2</sub>	2	240–241	48	C <sub>11</sub> H <sub>9</sub> Cl <sub>2</sub> NO <sub>3</sub>
51	B	C <sub>2</sub> H <sub>5</sub>	6,7-Cl <sub>2</sub>	2	266–269	44	C <sub>10</sub> H <sub>7</sub> Cl <sub>2</sub> NO <sub>3</sub>
52	B	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	6-Cl	11	162–164	17	C <sub>12</sub> H <sub>12</sub> ClNO <sub>3</sub>
53	B	<i>n</i> -C <sub>3</sub> H <sub>6</sub> OH	H	4HPA	133–136	51	C <sub>11</sub> H <sub>11</sub> NO <sub>4</sub>
54	B	<i>n</i> -C <sub>4</sub> H <sub>8</sub> OH	H	4HPA	150–152	41	C <sub>12</sub> H <sub>13</sub> NO <sub>4</sub>

<sup>a</sup> 4-Hydroxyphthalic acid. <sup>b</sup> The starting material was the undesired 3-chloro isomer mentioned under preparation of 11.

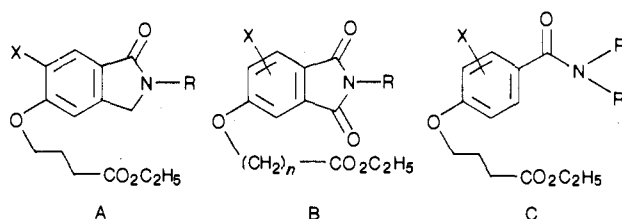
Kimelberg et al. have proposed a positive correlation between agents that inhibit astrocytic chloride influx by the cation coupled cotransport mechanism and their ability to inhibit cerebral swelling in vitro and in vivo.<sup>2,3</sup> Subsequent work<sup>5</sup> indicates that, in both erythrocytes and astrocytes, there exists both a high and low affinity site anion carrier, which may be responsible for selective inhibition of chloride influx in these cells, and that specific agents that inhibit this mechanism but are inactive with regard to the cation coupled system have the ability to selectively inhibit astrocytic swelling with little or no salidiuretic activity. Here, we report the effects of [(*N*-alkyl-1,3-dioxo-1*H*,3*H*-isoindolin-5-yl)oxy]alkanoic acids, 4-[(*N*-alkyl-1-oxo-1*H*,3*H*-isoindolin-5-yl)oxy]butanoic acids, and 4-[4-[(alkylamino)carbonyl]phenoxy]butanoic acids on chloride influx and steady-state levels in cultured rat astrocytes. These compounds are heterocyclic analogues of the indanones of Cragoe and Kimelberg<sup>2</sup> that have been shown to inhibit chloride flux into cultured

astrocytes and to inhibit astrocytic swelling in vitro and are distantly related structurally to the loop diuretic ethacrynic acid.

### Chemistry

Preparation of the *N*-alkyl-5-hydroxy-1*H*,3*H*-isoindoline-1,3-dione intermediates 35–54 (Table II, Scheme I) was accomplished by heating the appropriately substituted phthalic anhydride with the primary alkylamine in toluene with azeotropic removal of water to afford the product generally in excellent yield. In cases where the methoxy- rather than hydroxyphthalic anhydride was employed, the product was O-demethylated by heating with pyridine hydrochloride. The phenolic hydroxyl was then alkylated with either ethyl bromoacetate or ethyl 4-bromobutyrate in the presence of K<sub>2</sub>CO<sub>3</sub> in DMF by the procedure of Cragoe.<sup>2</sup> This step required much shorter reaction times in the case of the more reactive ethyl bromoacetate. Again, yields for both reagents were generally excellent (60–82, Table III, Scheme I). Hydrolysis of the ester to afford the product acid (92–114, Table IV, Scheme I) was readily accomplished by heating with 10% HCl-HOAc. In the case of the precursor anhydride (2)

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**Table III.** Ethyl [(*N*-Alkyl-1,3-dioxo-1*H*,3*H*-isoindolin-5-yl)oxy]alkanoates, Ethyl 4-[(*N*-Alkyl-1-oxo-1*H*,3*H*-isoindolin-5-yl)oxy]butanoates, and Ethyl 4-[4-[(Alkylamino)carbonyl]phenoxy]butanoates

no.	type	R	R'	X	n	starting material	mp, °C	yield, %	formula
55	A	CH <sub>3</sub>		H		30	oil	59	C <sub>15</sub> H <sub>19</sub> NO <sub>4</sub>
56	A	C <sub>2</sub> H <sub>5</sub>		H		31	gum	89	C <sub>16</sub> H <sub>21</sub> NO <sub>4</sub>
57	A	<i>n</i> -C <sub>3</sub> H <sub>7</sub>		H		32	oil	78	C <sub>17</sub> H <sub>23</sub> NO
58	A	<i>n</i> -C <sub>4</sub> H <sub>9</sub>		H		33	gum	93	C <sub>18</sub> H <sub>25</sub> NO
59	A	<i>c</i> -C <sub>5</sub> H <sub>9</sub>		Cl		34	gum	53	C <sub>19</sub> H <sub>24</sub> ClNO
60	B	<i>c</i> -C <sub>5</sub> H <sub>9</sub>		H	3	35	gum	68	C <sub>19</sub> H <sub>23</sub> NO <sub>5</sub>
61	B	<i>c</i> -C <sub>5</sub> H <sub>9</sub>		H	1	35	77-79	72	C <sub>17</sub> H <sub>19</sub> NO <sub>5</sub>
62	B	<i>c</i> -C <sub>6</sub> H <sub>11</sub>		H	3	36	97-99	68	C <sub>20</sub> H <sub>25</sub> NO <sub>5</sub>
63	B	<i>c</i> -C <sub>6</sub> H <sub>11</sub>		H	1	36	80-83	82	C <sub>18</sub> H <sub>21</sub> NO <sub>5</sub>
64	B	<i>c</i> -C <sub>5</sub> H <sub>9</sub>		6-Cl	3	37	107-108	47	C <sub>19</sub> H <sub>22</sub> ClNO <sub>5</sub>
65	B	<i>c</i> -C <sub>5</sub> H <sub>9</sub>		4-Cl	3	38	gum	20	C <sub>19</sub> H <sub>22</sub> ClNO <sub>5</sub>
66	B	<i>c</i> -C <sub>5</sub> H <sub>9</sub>		4,6-Cl <sub>2</sub>	3	39	oil	81	C <sub>19</sub> H <sub>21</sub> Cl <sub>2</sub> NO <sub>5</sub>
67	B	<i>c</i> -C <sub>5</sub> H <sub>9</sub>		6,7-Cl <sub>2</sub>	3	40	110-112	66	C <sub>19</sub> H <sub>21</sub> Cl <sub>2</sub> NO <sub>5</sub>
68	B	CH <sub>3</sub>		H	3	41	75-77	75	C <sub>15</sub> H <sub>17</sub> NO <sub>5</sub>
69	B	C <sub>2</sub> H <sub>5</sub>		H	3	42	gum	67	C <sub>16</sub> H <sub>19</sub> NO <sub>5</sub>
70	B	<i>n</i> -C <sub>3</sub> H <sub>7</sub>		H	3	43	gum	68	C <sub>17</sub> H <sub>21</sub> NO <sub>5</sub>
71	B	<i>n</i> -C <sub>4</sub> H <sub>9</sub>		H	3	44	gum	80	C <sub>18</sub> H <sub>23</sub> NO <sub>5</sub>
72	B	<i>n</i> -C <sub>5</sub> H <sub>11</sub>		H	3	45	oil	77	C <sub>19</sub> H <sub>25</sub> NO <sub>5</sub>
73	B	<i>n</i> -C <sub>6</sub> H <sub>13</sub>		H	3	46	oil	100	C <sub>20</sub> H <sub>27</sub> NO <sub>5</sub>
74	B	H		H	3	47	133-135	11	C <sub>14</sub> H <sub>15</sub> NO <sub>5</sub>
75	B	CH <sub>3</sub>		6,7-Cl <sub>2</sub>	3	48	oil	46	C <sub>15</sub> H <sub>15</sub> Cl <sub>2</sub> NO <sub>5</sub>
76	B	<i>n</i> -C <sub>4</sub> H <sub>9</sub>		6,7-Cl <sub>2</sub>	3	49	98-100	99	C <sub>18</sub> H <sub>21</sub> Cl <sub>2</sub> NO <sub>5</sub>
77	B	<i>n</i> -C <sub>3</sub> H <sub>7</sub>		6,7-Cl <sub>2</sub>	3	50	107-110	57	C <sub>17</sub> H <sub>19</sub> Cl <sub>2</sub> NO <sub>5</sub>
78	B	C <sub>2</sub> H <sub>5</sub>		6,7-Cl <sub>2</sub>	3	51	gum	29	C <sub>16</sub> H <sub>17</sub> Cl <sub>2</sub> NO <sub>5</sub>
79	B	<i>n</i> -C <sub>4</sub> H <sub>9</sub>		6-Cl	3	52	oil	100	C <sub>18</sub> H <sub>22</sub> ClNO <sub>5</sub>
80	B	<i>n</i> -C <sub>3</sub> H <sub>6</sub> OH		H	3	53	45-48	58	C <sub>17</sub> H <sub>21</sub> NO <sub>6</sub>
81	B	<i>n</i> -C <sub>4</sub> H <sub>8</sub> OH		H	3	54	oil	97	C <sub>18</sub> H <sub>23</sub> NO <sub>6</sub>
82	B	<i>n</i> -C <sub>3</sub> H <sub>7</sub>		H	1	43	68-70	96	C <sub>15</sub> H <sub>17</sub> NO <sub>5</sub>
83	C	<i>c</i> -C <sub>5</sub> H <sub>9</sub>	H	2,3-Cl <sub>2</sub>		17	gum	96	C <sub>18</sub> H <sub>23</sub> Cl <sub>2</sub> NO <sub>4</sub>
84	C	<i>c</i> -C <sub>5</sub> H <sub>9</sub>	H	3-Cl		22	gum	100	C <sub>18</sub> H <sub>24</sub> ClNO <sub>4</sub>
85	C	<i>c</i> -C <sub>5</sub> H <sub>9</sub>	H	3-Br		23	gum	79	C <sub>18</sub> H <sub>24</sub> BrNO <sub>4</sub>
86	C	<i>c</i> -C <sub>5</sub> H <sub>9</sub>	CH <sub>3</sub>	2,3-Cl <sub>2</sub>		21	gum	100	C <sub>19</sub> H <sub>25</sub> Cl <sub>2</sub> NO <sub>4</sub>

which could not be obtained by chlorination of commercially available 4-hydroxyphthalic acid, the corresponding phthalic acid was obtained by treatment of 2-methyl-5-methoxy-6,7-dichloroindan-1-one (1) with KMnO<sub>4</sub>, and the product diacid was sublimed to afford the anhydride.

The *N*-alkyl-5-methoxy-1*H*,3*H*-isoindolin-1-ones 25-29 (Table I, Scheme II) were prepared by the procedure of Danishefsky.<sup>6</sup> The appropriate methyl 2-(bromomethyl)benzoate was reacted with the primary alkylamine in either methanol or benzene to afford the ring-closed product with the benzylamino intermediate as the major side product. Yields were more satisfactory when benzene rather than methanol was used as the solvent. Subsequent O-demethylation was accomplished with BBr<sub>3</sub> (30-34, Table II, Scheme II) in methylene chloride followed by O-alkylation with ethyl 4-bromobutyrate (55-59, Table III, Scheme II) and ester hydrolysis with 10% HCl-HOAc to afford the products 87-91 (Table IV, Scheme II). The appropriate methyl 2-(bromomethyl)benzoates 7 and 10 were prepared by Friedel-Crafts acylation of *m*-methyl-anisole using acetic anhydride-AlCl<sub>3</sub>,<sup>7</sup> treatment with bromoform and subsequent hydrolysis affording the benzoic acid (5, 8), esterification in methanol-H<sub>2</sub>SO<sub>4</sub> (6, 9),

and finally benzylic bromination of the methyl ester using *N*-bromosuccinimide by the procedure of Grethe.<sup>8</sup>

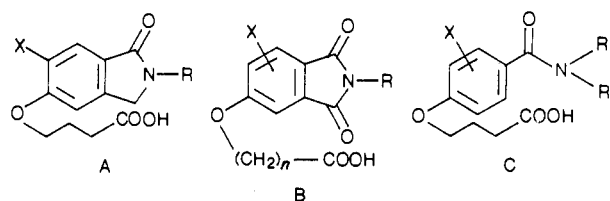
The 4-[4-[(alkylamino)carbonyl]phenoxy]butanoic acids 115-118 represented in Table IV (Scheme III) were prepared as above by alkylation of the corresponding phenolic precursors 21-24 with ethyl 4-bromobutyrate in the presence of K<sub>2</sub>CO<sub>3</sub> to afford the esters 83-86 (Table III, Scheme III), which were subsequently hydrolyzed by heating with 10% HCl-HOAc. The phenolic precursor 24 was prepared by iron-catalyzed bromination of 2,3-dichloroanisole to afford 13 and reaction of the corresponding Grignard reagent with CO<sub>2</sub> to afford the benzoic acid intermediate 14 followed by reaction of the acid chloride with cyclopentylamine and O-demethylation with BBr<sub>3</sub> (24). Compound 17 was *N*-methylated by using NaH-CH<sub>3</sub>I to afford the tertiary amide 20 followed by O-demethylation (BBr<sub>3</sub>) to afford 21. The 3-chloro and 3-bromo phenolic precursors 22 and 23 were prepared by treatment of 4-anisic acid with chlorine or bromine in acetic acid (15, 16) followed by conversion as above to the benzamides 18 and 19 and O-demethylation with BBr<sub>3</sub>.

In instances where syntheses of the corresponding propionic acids were attempted by using ethyl 3-bromopropionate in the presence of K<sub>2</sub>CO<sub>3</sub>, the products were

(6) Danishefsky, S.; Bryson, T. A.; Puthenpurayil, J. *J. Org. Chem.* 1975, 40, 797.

(7) Noller, C. R.; Adams, R. *J. Am. Chem. Soc.* 1924, 46, 1889.

(8) Grethe, G.; Lee, H. L.; Uskovic, M.; Brosse, A. *J. Org. Chem.* 1968, 33, 499.

**Table IV.** [(N-Alkyl-1,3-dioxo-1H,3H-isoindolin-5-yl)oxy]alkanoic Acids, 4-[(N-Alkyl-1-oxo-1H,3H-isoindolin-5-yl)oxy]butanoic Acids, and 4-[4-(Alkylamino)carbonyl]phenoxy]butanoic Acids

no.	type	R	R'	X	n	starting material	mp, °C	yield, %	formula	anal.
87	A	CH <sub>3</sub>		H		55	168-170	22	C <sub>13</sub> H <sub>15</sub> NO <sub>4</sub>	C, H, N
88	A	C <sub>2</sub> H <sub>5</sub>		H		56	88-91	53	C <sub>14</sub> H <sub>17</sub> NO <sub>4</sub>	C, H, N
89	A	n-C <sub>3</sub> H <sub>7</sub>		H		57	105-107	34	C <sub>15</sub> H <sub>19</sub> NO <sub>4</sub>	C, H, N
90	A	n-C <sub>4</sub> H <sub>9</sub>		H		58	112-114	56	C <sub>16</sub> H <sub>21</sub> NO <sub>4</sub>	C, H, N
91	A	c-C <sub>5</sub> H <sub>9</sub>		Cl		59	130-132	43	C <sub>17</sub> H <sub>20</sub> ClNO <sub>4</sub>	C, H, N
92	B	c-C <sub>5</sub> H <sub>9</sub>		H	3	60	156-158	51	C <sub>17</sub> H <sub>19</sub> NO <sub>5</sub>	C, H, N
93	B	c-C <sub>5</sub> H <sub>9</sub>		H	1	61	155-157	55	C <sub>15</sub> H <sub>15</sub> NO <sub>5</sub>	C, H, N
94	B	c-C <sub>6</sub> H <sub>11</sub>		H	3	62	184-186	68	C <sub>18</sub> H <sub>21</sub> NO <sub>5</sub>	C, H, N
95	B	c-C <sub>6</sub> H <sub>11</sub>		H	1	63	197-199	44	C <sub>16</sub> H <sub>17</sub> NO <sub>5</sub>	C, H, N
96	B	c-C <sub>5</sub> H <sub>9</sub>		6-Cl	3	64	183-187	63	C <sub>17</sub> H <sub>18</sub> ClNO <sub>5</sub>	C, H, N <sup>a</sup>
97	B	c-C <sub>5</sub> H <sub>9</sub>		4-Cl	3	65	158-160	73	C <sub>17</sub> H <sub>18</sub> ClNO <sub>5</sub>	C, H, N <sup>b</sup>
98	B	c-C <sub>5</sub> H <sub>9</sub>		4,6-Cl <sub>2</sub>	3	66	125-127	55	C <sub>17</sub> H <sub>17</sub> Cl <sub>2</sub> NO <sub>5</sub>	C, H, N
99	B	c-C <sub>5</sub> H <sub>9</sub>		6,7-Cl <sub>2</sub>	3	67	173-175	60	C <sub>17</sub> H <sub>17</sub> Cl <sub>2</sub> NO <sub>5</sub>	C, H, N
100	B	CH <sub>3</sub>		H	3	68	188-190	70	C <sub>13</sub> H <sub>13</sub> NO <sub>5</sub>	C, H, N
101	B	C <sub>2</sub> H <sub>5</sub>		H	3	69	148-150	66	C <sub>14</sub> H <sub>15</sub> NO <sub>5</sub>	C, H, N
102	B	n-C <sub>3</sub> H <sub>7</sub>		H	3	70	112-114	57	C <sub>15</sub> H <sub>17</sub> NO <sub>5</sub>	C, H, N
103	B	n-C <sub>4</sub> H <sub>9</sub>		H	3	71	110-112	51	C <sub>16</sub> H <sub>19</sub> NO <sub>5</sub>	C, H, N
104	B	n-C <sub>5</sub> H <sub>11</sub>		H	3	72	115-117	34	C <sub>17</sub> H <sub>21</sub> NO <sub>5</sub>	C, H, N
105	B	n-C <sub>6</sub> H <sub>13</sub>		H	3	73	105-106	82	C <sub>18</sub> H <sub>23</sub> NO <sub>5</sub>	C, H, N
106	B	H		H	3	74	204-206	69	C <sub>12</sub> H <sub>11</sub> NO <sub>5</sub>	C, H, N
107	B	CH <sub>3</sub>		6,7-Cl <sub>2</sub>	3	75	204-206	27	C <sub>13</sub> H <sub>11</sub> Cl <sub>2</sub> NO <sub>5</sub>	C, H, N
108	B	n-C <sub>4</sub> H <sub>9</sub>		6,7-Cl <sub>2</sub>	3	76	138-140	26	C <sub>16</sub> H <sub>17</sub> Cl <sub>2</sub> NO <sub>5</sub>	C, H, N
109	B	n-C <sub>3</sub> H <sub>7</sub>		6,7-Cl <sub>2</sub>	3	77	167-168	58	C <sub>15</sub> H <sub>15</sub> Cl <sub>2</sub> NO <sub>5</sub>	C, H, N
110	B	C <sub>2</sub> H <sub>5</sub>		6,7-Cl <sub>2</sub>	3	78	158-160	100	C <sub>14</sub> H <sub>13</sub> Cl <sub>2</sub> NO <sub>5</sub>	C, H, N
111	B	n-C <sub>4</sub> H <sub>9</sub>		6-Cl	3	79	117-119	39	C <sub>16</sub> H <sub>18</sub> ClNO <sub>5</sub>	C, H, N
112	B	n-C <sub>3</sub> H <sub>6</sub> OH		H	3	80	60-67	15	C <sub>15</sub> H <sub>17</sub> NO <sub>6</sub>	C, H, N
113	B	n-C <sub>4</sub> H <sub>8</sub> OH		H	3	81	90-95	17	C <sub>16</sub> H <sub>19</sub> NO <sub>6</sub>	C, H, N
114	B	n-C <sub>3</sub> H <sub>7</sub>		H	1	82	133-134	61	C <sub>13</sub> H <sub>13</sub> NO <sub>5</sub>	C, H, N
115	C	c-C <sub>5</sub> H <sub>9</sub>	H	2,3-Cl <sub>2</sub>		83	164-166	54	C <sub>16</sub> H <sub>19</sub> Cl <sub>2</sub> NO <sub>4</sub>	C, H, N
116	C	c-C <sub>5</sub> H <sub>9</sub>	H	3-Cl		84	154-160	68	C <sub>16</sub> H <sub>20</sub> ClNO <sub>4</sub>	C, H, N
117	C	c-C <sub>5</sub> H <sub>9</sub>	H	3-Br		85	153-155	68	C <sub>16</sub> H <sub>20</sub> BrNO <sub>4</sub>	C, H, N
118	C	c-C <sub>5</sub> H <sub>9</sub>	CH <sub>3</sub>	2,3-Cl <sub>2</sub>		86	oil	36	C <sub>17</sub> H <sub>21</sub> Cl <sub>2</sub> NO <sub>4</sub>	C, H, N

<sup>a</sup> Calcd: C, 58.07; H, 5.12; N, 3.98. Found: C, 57.33; H, 5.23; N, 3.92. <sup>b</sup> Calcd: C, 58.07; H, 5.12; N, 3.98. Found: C, 57.29; H, 5.34; N, 3.80.

not obtained due to acrylate formation.

## Results and Discussion

A recent report by the National Research Council<sup>9</sup> stresses the need for an increase in research activity directed at prevention and treatment of injuries in the United States. Among such injuries are those that involve traumatic impact to the skull-encased brain. Morbidity and mortality associated with such injuries are largely a consequence of the resultant astrocytic swelling in the cerebral gray matter. Astrocytes are glial, nonsynaptic cells of ectodermal origin that possess only one type of process and, along with oligodendrocytes, compose the macroglia. The processes form end feet with neurocapillaries and may have gap junctions between them.<sup>10</sup> In addition to the proposal that astrocytes function in neuronal support, scar formation,<sup>11</sup> phagocytosis,<sup>12</sup> and isolation of receptive surfaces,<sup>13</sup> there is strong evidence that the astrocytic sheath that surrounds the cerebral endothelium (the

blood-brain barrier) plays a vital role via the end feet in the neuronal control of the functional and structural characteristics of the neuronal capillaries.<sup>4</sup> Therefore, astrocytic swelling has a pronounced effect upon solute and gas exchange between capillaries and neurons. Kimelberg et al.<sup>1</sup> have demonstrated that astrocytic swelling in cerebral gray matter is due to chloride influx followed by an osmotic equivalent of water in vivo and in vitro, and this swelling is promoted by the presence of bicarbonate. Chloride may enter the cell by either an anion carrier or a cation coupled cotransport mechanism.<sup>3,4,14</sup> Loop diuretics such as furosemide and ethacrynic acid inhibit both mechanisms and decrease intracellular chloride levels and, therefore, swelling in vitro and in vivo. A series of indanone<sup>2</sup> and fluorene<sup>15</sup> analogues have been shown effective also in inhibiting chloride influx in vitro in primary rat astrocyte cultures and astrocytic swelling in vitro and in vivo in cat cerebral tissue. It should be noted that there exists a species-related difference in sensitivity to swelling inhibition between rat astrocyte tissue and that of other species such as the cat and guinea pig.<sup>15</sup> Significantly higher dose levels of antismelling agents are required to inhibit in vitro cerebrocortical tissue swelling in rat

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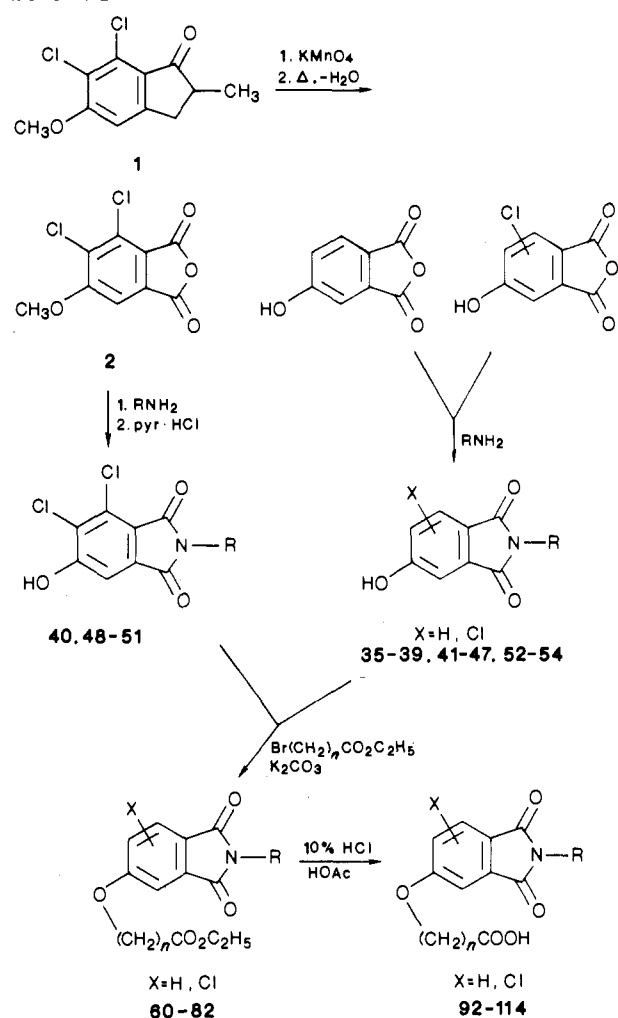
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## Scheme I



tissue than in the cat or guinea pig, but to date, attempts to establish primary cat or guinea pig astrocyte cultures in order to examine drug effects upon ion flux have been unsuccessful.<sup>15</sup>

## Scheme II

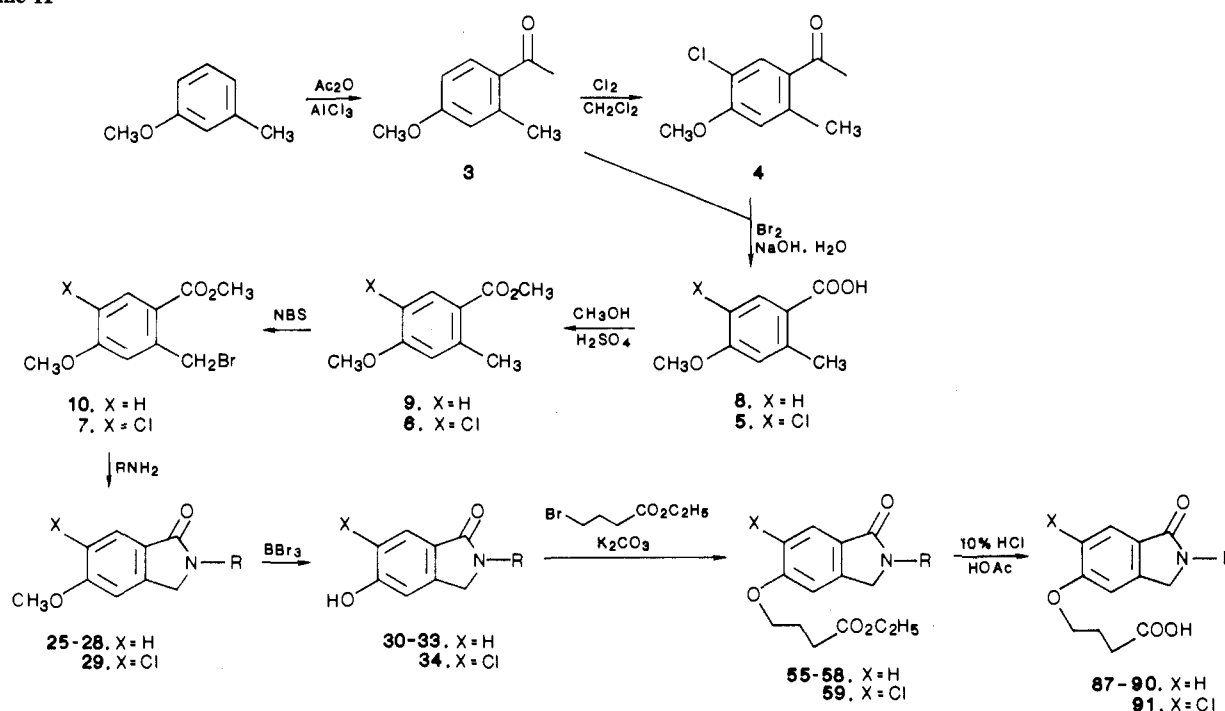


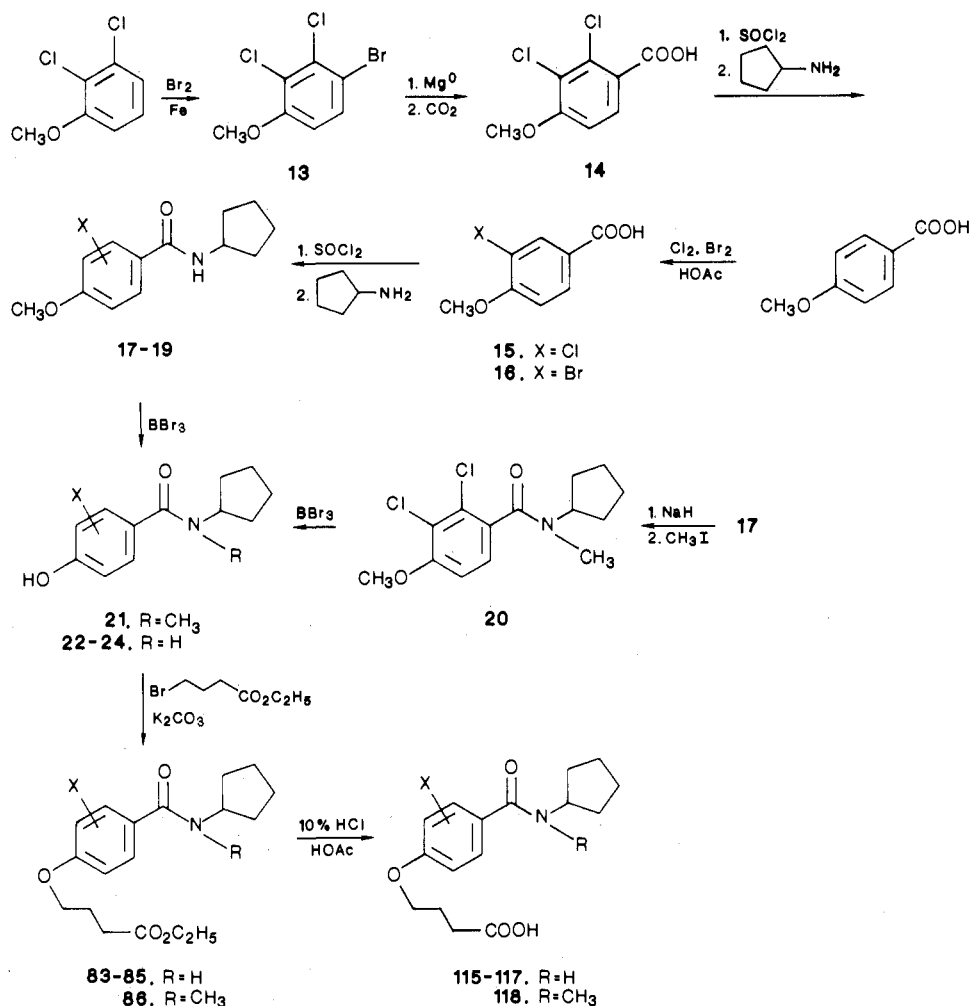
Table V. Effects of Test Compounds on Astrocyte Intracellular Chloride Concentration

compd	% of control intracellular chloride concn $\pm$ SD at dose given ( $n = 4$ )		
	0.1 mM	0.5 mM	1.0 mM
control <sup>f</sup>	100 $\pm$ 5 <sup>b</sup>		
furosemide	79 $\pm$ 6 <sup>c</sup>		21 $\pm$ 8 <sup>a</sup>
ethacrynic acid	78 $\pm$ 2 <sup>b</sup>		
87	84 $\pm$ 1		
88 <sup>g</sup>	67 $\pm$ 8 <sup>b</sup>	67 $\pm$ 10 <sup>b</sup>	57 $\pm$ 5 <sup>a</sup>
89	98 $\pm$ 11	76 $\pm$ 5 <sup>a</sup>	
90	97 $\pm$ 11	76 $\pm$ 13 <sup>d</sup>	
91	115 $\pm$ 2		
92	101 $\pm$ 5		
93	91 $\pm$ 1	92 $\pm$ 9	
94	105 $\pm$ 11		
95	94 $\pm$ 2	87 $\pm$ 5 <sup>e</sup>	
96	111 $\pm$ 8		
97	107 $\pm$ 11		
98	94 $\pm$ 3		
99	97 $\pm$ 3		
100	90 $\pm$ 2 <sup>c</sup>	118 $\pm$ 14	
101	104 $\pm$ 11	76 $\pm$ 8 <sup>b</sup>	
102	67 $\pm$ 11 <sup>c</sup>	80 $\pm$ 7 <sup>c</sup>	
103	88 $\pm$ 7	69 $\pm$ 6 <sup>b</sup>	
104	85 $\pm$ 2	77 $\pm$ 9 <sup>c</sup>	
105	109 $\pm$ 6	118 $\pm$ 12	
106	93 $\pm$ 2	88 $\pm$ 3 <sup>b</sup>	
107	91 $\pm$ 10		
108	86 $\pm$ 14	74 $\pm$ 9 <sup>c</sup>	
109	74 $\pm$ 7 <sup>e</sup>	89 $\pm$ 8	
110	103 $\pm$ 8	83 $\pm$ 13	
111	95 $\pm$ 6	81 $\pm$ 7 <sup>b</sup>	
112	75 $\pm$ 3 <sup>a</sup>	83 $\pm$ 8 <sup>c</sup>	
113	106 $\pm$ 7	101 $\pm$ 7	
114	103 $\pm$ 8	110 $\pm$ 6	
115	93 $\pm$ 3	78 $\pm$ 0 <sup>c</sup>	
116	83 $\pm$ 4		
117	74 $\pm$ 5		
118	79 $\pm$ 11		

<sup>a</sup> $p < 0.001$ . <sup>b</sup> $p < 0.005$ . <sup>c</sup> $p < 0.01$ . <sup>d</sup> $p < 0.025$ . <sup>e</sup> $p < 0.05$ . <sup>f</sup>0.005 M NaOH. <sup>g</sup>109%  $\pm$  9 (0.01 mM dose) and 93%  $\pm$  9 (0.05 mM dose). <sup>h</sup>0.221  $\pm$  0.0086  $\mu\text{mol}$  of  $\text{Cl}^-$ /mg of protein.

In our laboratory, a series of [(*N*-alkyl-1,3-dioxo-1*H*,3*H*-isoindolin-5-yl)oxy]alkanoic acids, 4-[(*N*-alkyl-1-

## Scheme III



oxo-1*H*,3*H*-isoindolin-5-yl)oxy]butanoic acids, and 4-[4-(alkylamino)carbonyl]phenoxy]butanoic acids have been prepared (Tables IV and V) and tested for ability to lower intracellular steady-state chloride levels in primary rat astrocyte cultures in the presence of bicarbonate-containing media. As shown in Table V, the loop diuretics furosemide and ethacrynic acid at a 0.1 mM dose similarly reduce intracellular chloride levels to 79% and 78% of control values, respectively, while furosemide at 1.0 mM reduces levels to 21% of control values. The compounds reported here were all tested at a 0.1 mM dose, which is the routine screening dose used by Kimelberg,<sup>2,15</sup> and certain derivatives were screened also at additional dose levels (0.01, 0.05, 0.5, and 1.0 mM). Structure-activity studies include variation of the N-substituents, aromatic substitution with halogen(s), and variation of the length of the carboxylic acid side chain. The isoindolin-1-ones (87-91) may be considered as carbonyl-reduced analogues of the isoindoline-1,3-diones (92-114) and the benzamides (115-118) as acyclic analogues of the isoindolin-1-ones. In the isoindoline-1,3-dione series, activity appeared to reside solely in derivatives with small alkyl N-substituents. In the homologous series CH<sub>3</sub>-*n*-C<sub>6</sub>H<sub>13</sub>, the *N*-*n*-C<sub>3</sub>H<sub>7</sub> derivative 102 was most active at 0.1 mM, reducing chloride levels to 67% of control at 0.1 mM and 80% of control at 0.5 mM. This decrease in activity at the higher dose may be due to concurrent inhibition of the sodium pump as proposed by others.<sup>15</sup> The C<sub>2</sub>H<sub>5</sub> (101), *n*-C<sub>4</sub>H<sub>9</sub> (103), and *n*-C<sub>5</sub>H<sub>11</sub> (104) derivatives were each less active than 102 at 0.1 mM, but activity increased at the 0.5 mM dose level. The largest acyclic substituent, *n*-C<sub>6</sub>H<sub>13</sub> (105), showed no

activity at either dose level. The methyl-substituted analogue 100 as well as the *N*-unsubstituted analogue 106 were less active than 102 at 0.1 mM. The *N*-cyclopentyl (92) and *N*-cyclohexyl (94) analogues with a four-carbon acid side chain were inactive at 0.1 mM. Therefore, the optimal N-substituent of those tested in the isoindoline-1,3-dione series appears to be a three-carbon chain (102) or three carbons and one oxygen (112).

Testing of alkyl-substituted derivatives 87-91 at 0.1 mM in the isoindolin-1-one series reveals that, of the homologues CH<sub>3</sub> through *n*-C<sub>4</sub>H<sub>9</sub>, optimal activity resides with the C<sub>2</sub>H<sub>5</sub> (88) compound, which reduced chloride levels to 67% of control values with statistical significance of  $p < 0.005$ . Upon examination of the response at other doses, 88 was inactive (109%) at 0.01 mM, slightly active (93%) at 0.05 mM, and similarly active (67%) at 0.5 mM to the 0.1 mM dose and showed greatest activity at the 1.0 mM dose level, reducing chloride levels to 57% of control values. The *N*-CH<sub>3</sub> analogue 87 was weakly active at 0.1 mM (84%) while the *n*-C<sub>3</sub>H<sub>7</sub> and *n*-C<sub>4</sub>H<sub>9</sub> analogues 89 and 90 were inactive at 0.1 mM but increased in activity at 0.5 mM, both reducing chloride levels to 76% of control values. The *N*-cyclopentyl derivative 91 showed no activity at 0.1 mM. Therefore, when one carbonyl of the isoindoline-1,3-diones is reduced to produce the isoindolin-1-ones, the optimal N-substituent changes from *n*-C<sub>3</sub>H<sub>7</sub> to C<sub>2</sub>H<sub>5</sub>.

Unlike the indanone<sup>2</sup> and fluorene carboxylic acids<sup>15</sup> reported by Cragoe et al., which required ortho dichlorination of the aromatic ring for activity, none of the chlorinated isoindoline-1,3-diones (96-99, 107-111) or isoindolin-1-ones (91) showed any increase in activity as

compared to the unchlorinated analogues discussed above at 0.1 mM. The *n*-C<sub>3</sub>H<sub>7</sub> dichlorinated analogue 109 again maintained optimal activity among the dichlorinated analogues, reducing chloride levels to 74% of control values at the 0.1 mM level. Of the halogenated benzamide analogues tested (115–118), the 3-bromo analogue 117 was the most active, reducing chloride levels to 74% of control values. The *N*-cyclopentyl 2,3-dichlorinated analogue 115 was inactive at 0.1 mM but moderately active (78%) at 0.5 mM. The corresponding *N*-cyclopentyl-*N*-methyl 2,3-dichlorinated derivative 118 was more active (79%) at 0.1 mM. It appears, therefore, that the benzamide series benefits by disubstitution on the nitrogen. The analogous position of the indanones<sup>2</sup> also benefited by substitution with both cyclopentyl and methyl groups.

Upon examination of the effect produced by variation of the four-carbon acid side chain to a two-carbon chain in the isoindoline-1,3-dione series, the *N*-cyclopentyl (93) and *N*-cyclohexyl (95) analogues showed relatively little change in activity as compared to 92 and 94. When the most active analogue (102) having the *N*-*n*-C<sub>3</sub>H<sub>7</sub> substituent and a four-carbon acid side chain was compared to the analogous two-carbon chain (114), activity was completely abolished.

In summary, structure-activity studies reveal that in the isoindoline-1,3-dione and isoindolin-1-one series, *N*-substitution is optimized by a three-carbon and two-carbon chain, respectively. Halogenation of the aromatic ring in the above series produces no increase in activity. Monobromination in the benzamide series produces slightly greater activity than monochlorination. Substitution of a two-carbon acid side chain for the four-carbon acid side chain in the isoindoline-1,3-dione series decreases activity. Compounds 88 and 102 are significantly more active at 0.1 mM than furosemide and ethacrynic acid. It remains to be seen as to whether these compounds actually do possess antishwelling activity *in vitro* and *in vivo*, and studies are planned that include testing for inhibition of astrocytic swelling *in vivo* in the cat head injury model as well as *in vitro* in the cat cerebrocortical tissue slice assay.

## Experimental Section

All chemicals were used as received from the manufacturers. Thin-layer chromatography was performed by using silica gel 60 coated plates, and column chromatography was performed using silica gel 60 (70–230 mesh). All melting points were obtained on a Mel-Temp apparatus and are uncorrected. <sup>1</sup>H NMR spectra were obtained on a JEOL FX60 60-MHz spectrometer and were consistent with assigned structures. Elemental analyses were obtained from M-H-W Laboratories, Phoenix, AZ and are correct within ±0.4% of theoretical values unless otherwise noted. Protein analyses were performed by using a Bausch & Lomb 1001 UV-vis spectrometer. Radioisotopes were quantitated by using a Packard Tricarb 4000 liquid scintillation spectrometer. Pregnant Sprague-Dawley rats were purchased from Charles River Co., Wilmington, MA.

**2-Methyl-6,7-dichloro-5-methoxy-1-indanone (1).** 2-Methyl-6,7-dichloro-5-methoxy-1-indanone (1) was prepared according to the procedure of Waltersdorf<sup>16</sup> in 66% yield: mp 127–128 °C (lit. mp 128–129 °C).

**3,4-Dichloro-5-methoxyphthalic Anhydride (2).** Compound 1 (3.0 g, 0.012 mol) was added to a solution of 11.7 g (0.074 mol) of KMnO<sub>4</sub> in 50 mL of water. The suspension was stirred at reflux for 5 h, after which time 5.0 g of Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> was added and the solution adjusted to pH 1.0 with concentrated HCl. The resulting precipitate was collected, dissolved in 50 mL of hot methanol, and filtered. The filtrate was evaporated *in vacuo* and the solid residue sublimed at 190 °C (0.6 mmHg) to afford 300 mg (10%)

of colorless solid: mp 215–218 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, TMS) δ 7.81 (s, 1 H, Ar *H*) and 4.14 (s, 3 H, OCH<sub>3</sub>).

**2-Methyl-4-methoxyacetophenone (3).** Compound 3 was prepared according to the procedure of Noller<sup>7</sup> in 42% yield: bp 107 °C (1.2 mmHg) (lit. bp 116 °C (3 mmHg)).

**2-Methyl-4-methoxy-5-chloroacetophenone (4).** Compound 4 was prepared by the method of Pearson.<sup>17</sup> A solution of 4.5 g (0.065 mol) of chlorine gas in 75 mL of CH<sub>2</sub>Cl<sub>2</sub> was prepared at –76 °C. This solution was added in one portion to a solution of 10.7 g (0.065 mol) of 3 in 25 mL of CH<sub>2</sub>Cl<sub>2</sub> at –76 °C, and the reaction mixture was stirred until the color disappeared (15 min). The volatiles were removed *in vacuo*, and the residue was recrystallized from hexane-ether to afford 5.1 g (40%) of product: mp 88–90 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, TMS) δ 7.78 (s, 1 H, Ar *H*), 6.73 (s, 1 H, Ar *H*), 3.92 (s, 3 H, OCH<sub>3</sub>), 2.60 (s, 3 H, COCH<sub>3</sub>), and 2.54 (s, 3 H, Ar CH<sub>3</sub>).

**4-Methoxy-5-chloro-2-methylbenzoic Acid (5).** Bromoform was prepared by the addition over 5 min of 5.1 mL of bromine to a solution of 15.6 g (0.39 mol) of NaOH in 70 mL of water at 5 °C. This solution was added dropwise to 5.0 g (0.025 mol) of 4 while the temperature was maintained below 30 °C. The reaction mixture was then stirred at 40 °C for 0.5 h, followed by addition of 15.0 g of NaHSO<sub>3</sub> and stirring at room temperature overnight. Concentrated HCl was added to pH 1.0, followed by reduction of the volume to 250 mL *in vacuo*. The resulting white precipitate was collected and recrystallized from benzene to afford 3.6 g (72%) of product: mp 220–222 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, TMS) δ 7.87 (s, 1 H, Ar *H*), 7.09 (s, 1 H, Ar *H*), 3.92 (s, 3 H, OCH<sub>3</sub>), and 2.58 (s, 3 H, Ar CH<sub>3</sub>).

**Methyl 4-Methoxy-5-chloro-2-methylbenzoate (6).** To a solution of 3.5 g (0.0175 mol) of 5 in 100 mL of dry methanol was added 10 drops of concentrated H<sub>2</sub>SO<sub>4</sub>. After the mixture was stirred at reflux for 24 h, the methanol was removed *in vacuo*, the colorless, solid residue was dissolved in ether, washed with 0.1 M Na<sub>2</sub>CO<sub>3</sub>, and dried (Na<sub>2</sub>SO<sub>4</sub>), and the ether was evaporated *in vacuo* to afford 3.2 g (85%) of product: mp 88–90 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, TMS) δ 8.00 (s, 1 H, Ar *H*), 6.77 (s, 1 H, Ar *H*), 3.96 (s, 3 H, CO<sub>2</sub>CH<sub>3</sub>), and 3.90 (s, 3 H, OCH<sub>3</sub>).

**Methyl 2-(Bromomethyl)-4-methoxy-5-chlorobenzoate (7).** The procedure of Grethe<sup>8</sup> was used. *N*-Bromosuccinimide (2.57 g, 0.014 mol) was suspended in a solution of 3.1 g (0.014 mol) of 6 in 50 mL of CCl<sub>4</sub>. Benzoyl peroxide (30 mg) was added, and the reaction mixture was stirred at reflux for 2.5 h. The reaction mixture was cooled and filtered, and the filtrate was washed with 25 mL of 0.1 N NaOH and 25 mL of water. The volatiles were removed *in vacuo* to afford a yellow solid residue, which was recrystallized from hexane-ether to afford 1.7 g (40%) of product: mp 93–95 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, TMS) δ 8.07 (s, 1 H, Ar *H*), 7.01 (s, 1 H, Ar *H*), 4.98 (s, 2 H, CH<sub>2</sub>Br), 4.00 (s, 3 H, CO<sub>2</sub>CH<sub>3</sub>), and 3.94 (s, 3 H, OCH<sub>3</sub>).

**4-Methoxy-2-methylbenzoic Acid (8).** Compound 8 was prepared according to the procedure of Grethe<sup>8</sup> in 64% yield: mp 176–178 °C (lit. mp 176 °C).

**Methyl 4-Methoxy-2-methylbenzoate (9).** Compound 9 was prepared from 8 by the procedure of Grethe<sup>8</sup> in 73% yield: bp 87 °C (1.2 mmHg) (lit. bp 143 °C (16 mmHg)).

**Methyl 2-(Bromomethyl)-4-methoxybenzoate (10).** The procedure of Grethe<sup>8</sup> was used to afford 10 from 9 in 73% yield: mp 64–66 °C (lit. mp 66–67 °C).

**4-Chloro-5-hydroxyphthalic Acid (11).** 4-Hydroxyphthalic acid (3.5 g, 0.019 mol) was dissolved with slight warming in 25 mL of glacial acetic acid. A solution of 1.4 g (0.02 mol) of chlorine in 30 mL of acetic acid was added dropwise, and the reaction mixture was stirred at room temperature overnight. The volatiles were removed *in vacuo*, and the residue was dissolved in methanol-CHCl<sub>3</sub>. Upon standing, the undesired 3-chloro isomer precipitated and was filtered off. The filtrate was evaporated *in vacuo* to afford 1.4 g (32%) of slightly impure, hygroscopic product as a colorless solid: <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, TMS) δ 7.73 (s, 1 H, Ar *H*) and 7.13 (s, 1 H, Ar *H*).

**3,5-Dichloro-4-hydroxyphthalic Acid (12).** 4-Hydroxyphthalic acid (1.0 g, 0.0054 mol) was dissolved in 15 mL of glacial

(16) Waltersdorf, O. W.; DeSolmes, S. J.; Schultz, E. M.; Cragoe, E. J. *J. Med. Chem.* 1977, 20, 1400.

(17) Pearson, D. E.; Wysong, R. D.; Breder, C. V. *J. Org. Chem.* 1967, 32, 2358.

acetic acid and 5 mL of water. To this solution was added dropwise a solution of 0.945 g (0.0135 mol) of chlorine in 30 mL of acetic acid. After the mixture was stirred at room temperature overnight, the volatiles were removed in vacuo to afford a yellow residue, which was recrystallized from methanol-CHCl<sub>3</sub> to afford 1.0 g (74%) of product: mp 186–188 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, TMS) δ 7.81 (s, 1 H, Ar H).

**2,3-Dichloro-4-bromoanisole (13).** Iron filings (0.3 g) were added to a melt of 30.0 g (0.169 mol) of 2,3-dichloroanisole at 90 °C. Bromine (8.7 mL, 0.169 mol) was added dropwise with stirring, and the reaction mixture was then stirred at 90 °C for 4 h. The reaction mixture was then cooled to 50 °C, and 150 mL of ether was carefully added. The resulting solution was washed with water, 10% NaOH, saturated Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, and again water. The organic phase was dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated in vacuo to afford 28.4 g (66%) of product: mp 74–76 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, TMS) δ 7.84 (d, 1 H, Ar H<sub>5</sub>), 7.25 (d, 1 H, Ar H<sub>6</sub>), and 3.92 (s, 3 H, OCH<sub>3</sub>).

**2,3-Dichloro-4-methoxybenzoic Acid (14).** Magnesium turnings (1.7 g, 0.07 mol) were placed in a flame-dried three-neck 1.0-L flask equipped with nitrogen inlet and reflux condenser. Compound 13 (18.2 g, 0.071 mol) in 100 mL of dry THF was added dropwise. Iodine was added to initiate the reaction, and the reaction mixture was stirred at reflux until no magnesium remained. The warm solution was poured over excess crushed dry ice and was allowed to evaporate. The resulting brown gum was dissolved in 200 mL of 0.1 M Na<sub>2</sub>CO<sub>3</sub> and was washed with ether. The aqueous solution was acidified with concentrated HCl and the product extracted into ether. The ether solution was dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated in vacuo to afford a solid, which was recrystallized from methanol-benzene to afford 9.1 g (58%) of product: mp 221–224 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, TMS) δ 7.84 (d, 1 H, Ar H<sub>6</sub>), 7.75 (d, 1 H, Ar H<sub>5</sub>), and 3.92 (s, 3 H, OCH<sub>3</sub>).

**3-Chloro-4-anisic Acid (15).** Compound 15 was prepared by chlorination of 4-anisic acid (20.0 g, 0.131 mol) in a manner similar to that of preparation of 11 in 58% yield: mp 211–214 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, TMS) δ 7.98 (m, 2 H, Ar H<sub>2,6</sub>), 7.02 (d, 1 H, Ar H<sub>5</sub>), and 4.00 (s, 3 H, OCH<sub>3</sub>).

**3-Bromo-4-anisic Acid (16).** Compound 16 was prepared in a manner similar to that of preparation of 15 by using 20.0 g (0.131 mol) of 4-anisic acid and bromine instead of chlorine in 40% yield: mp 201–206 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, TMS) δ 8.05 (m, 2 H, Ar H<sub>2,6</sub>), 7.00 (d, 1 H, Ar H<sub>5</sub>), and 4.00 (s, 3 H, OCH<sub>3</sub>).

***N*-Cyclopentyl-2,3-dichloro-4-methoxybenzamide (17).** Compound 14 (2.0 g, 0.0091 mol) was dissolved in 25 mL of ethyl acetate, and to this solution were added 0.73 mL (0.01 mol) of SOCl<sub>2</sub> and 1 drop of DMF. The reaction mixture was stirred for 10 h at reflux, after which time the volatiles were removed in vacuo. A solution of the residue in 10 mL of ethyl acetate was added dropwise to a solution of 2.0 mL (0.015 mol) of triethylamine and 1.34 mL (0.01 mol) of cyclopentylamine, and the reaction mixture was allowed to stand overnight at room temperature. The reaction mixture was then dissolved in 15 mL of ethyl acetate and washed with 0.1 M Na<sub>2</sub>CO<sub>3</sub>. The organic phase was dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated in vacuo to afford 2.0 g (75%) of the amide: mp 155–157 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, TMS) δ 7.50 (d, 1 H, Ar H<sub>6</sub>), 6.84 (d, 1 H, Ar H<sub>5</sub>), 3.88 (s, 3 H, OCH<sub>3</sub>), and 1.70 (m, 9 H, cyclopentyl H).

***N*-Cyclopentyl-3-chloro-4-methoxybenzamide (18).** Compound 15 (14.1 g, 0.076 mol) was dissolved in 100 mL of SOCl<sub>2</sub> and the reaction mixture stirred at reflux for 2 h. The SOCl<sub>2</sub> was removed in vacuo and azeotroped twice with toluene. The residue was dissolved in 70 mL of benzene, and 7.1 g (0.084 mol) of cyclopentylamine in 30 mL of triethylamine was added dropwise. After being stirred at room temperature for 1.0 h, the reaction mixture was extracted with water, 10% NaOH, and 0.1 N HCl. The organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated in vacuo to afford a solid, which was recrystallized from ether to afford 2.9 g (15%) of product: mp 168–172 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, TMS) δ 7.75 (m, 2 H, Ar H<sub>2,6</sub>), 6.90 (d, 1 H, Ar H<sub>5</sub>), 3.94 (s, 3 H, OCH<sub>3</sub>), and 2.31–1.35 (m, 9 H, cyclopentyl H).

***N*-Cyclopentyl-3-bromo-4-methoxybenzamide (19).** Compound 19 was prepared in a manner similar to that of preparation of 18 in 16% yield: mp 154–158 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, TMS) δ 7.75 (m, 2 H, Ar H<sub>2,6</sub>), 6.90 (d, 1 H, Ar H<sub>5</sub>), 3.94 (s, 3 H, OCH<sub>3</sub>), and 2.31–1.35 (m, 9 H, cyclopentyl H).

***N*-Methyl-*N*-cyclopentyl-2,3-dichloro-4-methoxybenzamide (20).** Sodium hydride as a 50% mineral oil dispersion (0.60 g, 0.012 mol) and 25 mL of benzene were placed in a flame-dried three-neck flask equipped with nitrogen inlet and reflux condenser. Compound 17 (2.2 g, 0.008 mol) was added in one portion, and the resulting suspension was stirred at reflux for 16 h. The suspension was cooled to 10 °C, and 1.0 mL (0.016 mol) of CH<sub>3</sub>I was added. The solution was then stirred at reflux for 5 h and filtered. The filtrate was evaporated in vacuo to afford 2.0 g (95%) of an oil: <sup>1</sup>H NMR (CDCl<sub>3</sub>, TMS) δ 6.84–7.42 (m, 2 H, Ar H), 3.94 (s, 3 H, OCH<sub>3</sub>), 2.87 (d, 3 H, NCH<sub>3</sub>), and 1.22–1.90 (m, 9 H, cyclopentyl H).

***N*-Methyl-*N*-cyclopentyl-2,3-dichloro-4-hydroxybenzamide (21).** In a flame-dried three-neck flask under a N<sub>2</sub> atmosphere was placed 2.0 g (0.0066 mol) of 20 dissolved in 25 mL of CH<sub>2</sub>Cl<sub>2</sub>. The solution was cooled to -70 °C (dry ice-acetone), and 8.0 mL of 1.0 M BBr<sub>3</sub> in CH<sub>2</sub>Cl<sub>2</sub> was added via syringe. The solution was allowed to warm to room temperature and was stirred for 16 h, after which time 10 mL of H<sub>2</sub>O was carefully added. The solvents were removed under reduced pressure, and the brownish-white residue was dissolved in 50 mL of 1.0 N NaOH and washed with 2 × 50 mL of CH<sub>2</sub>Cl<sub>2</sub>. The aqueous layer was acidified with 1.0 N HCl to pH 1.0. The volume of H<sub>2</sub>O was reduced to 10 mL, the solution was cooled, and the precipitate was collected. Recrystallization from MeOH-H<sub>2</sub>O yielded 1.2 g (63%) of product: mp 203–205 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, TMS) δ 6.92–6.70 (m, 2 H, Ar H), 2.87 (d, 3 H, NCH<sub>3</sub>), and 1.50–1.90 (m, 9 H, cyclopentyl H).

***N*-Cyclopentyl-3-chloro-4-hydroxybenzamide (22).** Compound 22 was prepared from 18 in a similar manner to that of preparation of 21 in 44% yield: mp 173–174 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, TMS) δ 7.70 (m, 2 H, Ar H<sub>2,6</sub>), 7.01 (d, 1 H, Ar H<sub>5</sub>), and 2.30–1.32 (m, 9 H, cyclopentyl H).

***N*-Cyclopentyl-3-bromo-4-hydroxybenzamide (23).** Compound 23 was prepared from 19 in a similar manner to that of preparation of 21 in 77% yield: mp 157–160 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, TMS) δ 7.65 (m, 2 H, Ar H<sub>2,6</sub>), 7.05 (d, 1 H, Ar H<sub>5</sub>), and 2.30–1.33 (m, 9 H, cyclopentyl H).

***N*-Cyclopentyl-2,3-dichloro-4-hydroxybenzamide (24).** Compound 24 was prepared from 17 in a similar manner to that of preparation of 21 in 82% yield: mp 192–194 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, TMS) δ 7.24 (d, 1 H, Ar H<sub>6</sub>), 6.90 (d, 1 H, Ar H<sub>5</sub>), and 2.30–1.25 (m, 9 H, cyclopentyl H).

**General Procedure for Preparation of *N*-Alkyl-5-methoxy-1*H*,3*H*-isoindolin-1-ones 25–29.** The title compounds (Table I) were prepared by the procedure of Danishefsky.<sup>6</sup> To a solution of the appropriate bromo ester (7 or 10, 0.018 mol) in 20 mL of methanol or benzene were added 0.018 mol of the alkylamine and 0.018 mol of triethylamine. The solution was then stirred at reflux for 3–5 h with monitoring by thin-layer chromatography. The volatiles were then removed in vacuo, and the residue was washed with 1.0 N HCl. Recrystallization from methanol-water or column chromatography (CH<sub>2</sub>Cl<sub>2</sub>-ether, 8:2) afforded the product in purity suitable for the next synthetic step.

**General Procedure for Preparation of *N*-Alkyl-5-hydroxy-1*H*,3*H*-isoindolin-1-ones 30–34.** The title compounds (Table II) were prepared by demethylation as follows. Each of the corresponding methoxy compounds (0.01 mol, Table I) was dissolved in 25 mL of CH<sub>2</sub>Cl<sub>2</sub> and placed in a flame-dried flask equipped with nitrogen inlet and rubber septum. The solution was cooled to -76 °C (dry ice-acetone), and 20 mL of 1.0 M BBr<sub>3</sub> was introduced via syringe. The reaction mixture was then stirred for 3 h at room temperature, after which time 10 mL of water was added carefully, followed by stirring for 5 min. The volatiles were removed in vacuo, and the residue was dissolved in 20 mL of 1.0 N NaOH and extracted with CH<sub>2</sub>Cl<sub>2</sub>. The aqueous layer was then acidified with concentrated HCl, and the precipitate was collected and dried to afford the phenolic product in suitable purity for the next synthetic step.

**General Procedure for Preparation of *N*-Alkyl-5-hydroxy-1*H*,3*H*-isoindoline-1,3-diones 35–54.** The title compounds (Table II) were prepared as follows. The appropriate phthalic anhydride was prepared by sublimation (200 °C (0.6 mmHg)) of the corresponding hydroxy- or methoxyphthalic acid. The anhydride (0.0036 mol) and appropriate alkylamine or ammonia<sup>18</sup> (0.029 mol) in 20 mL of dry toluene were stirred at reflux



overnight, with removal of water via a Dean-Stark trap. The volatiles were removed in vacuo, and the crude product was either carried on or recrystallized from ethanol. In cases where a methoxy-substituted phthalic anhydride (2) was used, the product was demethylated to the phenolic compound by heating to 190 °C in a beaker with excess pyridine hydrochloride for 30 min. The hot solution was then poured into ice water and the precipitate collected, dried, and recrystallized from methanol to afford the product in >70% yield.

**General Procedure for Preparation of Ethyl [(N-Alkyl-1,3-dioxo-1H,3H-isoindolin-5-yl)oxy]alkanoates 60–82, Ethyl 4-[(N-Alkyl-1-oxo-1H,3H-isoindolin-5-yl)oxy]butanoates 55–59, and Ethyl 4-[4-[(Alkylamino)carbonyl]phenoxy]butanoates 83–86.** The title compounds (Table III) were prepared by alkylation of the corresponding phenolic derivatives (Table II) as follows. The phenolic precursor (0.66 mmol) was dissolved in 5.0 mL of dry DMF, and 2.0 mmol of K<sub>2</sub>CO<sub>3</sub> was added. The resulting suspension was stirred for 5 min, and 0.75 mmol of ethyl 4-bromobutyrate or ethyl bromoacetate was added. The reaction mixture was then stirred at 50 °C for 24–48 h (shorter times required for ethyl bromoacetate). The DMF was removed in vacuo, and the residue was partitioned between CH<sub>2</sub>Cl<sub>2</sub> and water. The organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated in vacuo to afford the product usually in >90% purity. When necessary, the product was purified by column chromatography (CH<sub>2</sub>Cl<sub>2</sub>-ether, 7:3). Yields were generally >60%.

**General Procedure for Preparation of [(N-Alkyl-1,3-dioxo-1H,3H-isoindolin-5-yl)oxy]alkanoic Acids 92–114, 4-[(N-Alkyl-1-oxo-1H,3H-isoindolin-5-yl)oxy]butanoic Acids 87–91, and 4-[4-[(Alkylamino)carbonyl]phenoxy]butanoic Acids 115–118.** The title compounds (Table IV) were prepared by acid hydrolysis of the corresponding esters (Table III). The ethyl ester (1.3 mmol) was dissolved and stirred at reflux in 6.0 mL of acetic acid and 2.0 mL of 10% HCl for 2–4 h. Upon cooling, the resulting precipitate was collected. When necessary, water was added to aid in precipitation. The product was then either recrystallized from ethanol–benzene or column chromatographed (CH<sub>2</sub>Cl<sub>2</sub>–methanol, 9:1) to afford the pure product as a nearly colorless solid.

**In Vitro Rat Astrocyte Chloride Flux Assay.** Primary rat astrocyte tissue cultures were prepared according to the procedure of McCarthy and DeVellis.<sup>19</sup> This procedure was modified in that oligodendrocytes were not separated from astrocytes in keeping with cultures prepared by Kimelberg.<sup>14</sup> Briefly, 1–4-day-old Sprague–Dawley rat pups were decapitated with surgical scissors, and the brain was removed. A small tissue sample was taken from each cerebral hemisphere, and the samples were collectively trypsinized (0.1%) for 30 min at 37 °C followed by dispersion into single cells by pipetting. The suspension in Eagle's Basic Medium (BME) was centrifuged at 700 rpm for 10 min to obtain the glial pellet, which was resuspended in fresh BME supplemented with 10% fetal calf serum, glucose, glutamine, and antibiotics and filtered through a 35- $\mu$ m screen. The filtrate was added in 3.0-mL portions to 60-mm sterile culture dishes, and cultures were grown in an atmosphere of 95% air–5% CO<sub>2</sub> at 37 °C until a confluent monolayer of astrocytes was obtained. The medium was changed every 2 days until the cells were utilized (3–5 weeks). Staining techniques indicate that these cultures contain >90% astrocytes.

For assay of test compounds,<sup>14</sup> the BME was poured off the dishes and replaced by a chloride–HCO<sub>3</sub><sup>-</sup>-containing Hepes buffered medium and the cells were equilibrated for 60 min at 37 °C in a 95% air–5% CO<sub>2</sub> atmosphere. Test compounds were then added as their sodium salts in 0.005 M sodium hydroxide (100  $\mu$ L, 0.1 mM dose or 200  $\mu$ L, 0.5 mM dose) to each dish ( $n = 4$ ), 0.005 M sodium hydroxide was added to the control group,

and the cells were incubated at 37 °C for 15 min. Following the second incubation, 1.5  $\mu$ Ci of <sup>36</sup>Cl<sup>-</sup> as HCl (New England Nuclear) was added to each dish followed by incubation at 37 °C for 20 min in order to reach steady-state concentration.<sup>14,2</sup> A time 0 control group was included in which the medium was poured off immediately after isotope addition. Isotope quantitated in this group represents noninternalized radioactivity or that present in adherent medium and is subtracted from all other test groups. All of the dishes were then washed (7  $\times$  3.0 mL) within 25 s with ice-cold 0.32 M sucrose solution, and the cells from each dish were dissolved in 2.8 mL of 0.5 N NaOH and added to test tubes. A 2.0-mL aliquot from each tube was taken for liquid scintillation counting after quenching, and 0.2-mL aliquots were taken in duplicate for Lowry<sup>20</sup> protein determinations. Intracellular chloride concentrations were determined as micromoles of Cl<sup>-</sup>/milligram of protein and expressed as percent of control values  $\pm$  standard deviation. The  $p$  values were determined by Student's  $t$  test.<sup>21</sup>

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