cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 1.25 and 1.66 (2 s, 6 H, 2 CH_3C), 2.67 (m, CH_2), 2.75 (s, 3 H, CH_3N), 3.62–4.17 (m, CHO and CHN), 4.45 (s, 1 H, exchangeable), 5.38 (d, 1 H, exchangeable), 5.76 (d, 1 H, H-1′), 5.9 (d, 1 H, exchangeable), 7.15 (s, 1 H, H-3), 7.72 (s, 1 H, H-11), 7.72 (s, 1 H, CHO); ^{13}C NMR (Me_2SO-d_6) δ 191.1 (C-5), 179.2 (C-12), 163.8 (HCO), 159.0 (C-6), 154.6 (C-4), 146.5 (C-10a), 146.3 (C-1), 136.0 (C-2), 131.3 (C-11a), 131.0 (C-6a), 124.1 (C-3), 119.8 (C-11), 116.3 (C-12a), 114.8 (C-4a), 111.7 (C-5a), 95.8 (C-1′), 75.5 (C-5′), 70.4 (C-2′), 67.7 (C-4′), 66.1 (C-9), 61.4 (C-3′), 43.5 (C-10), 33.7 (C-8), 29.4 (C-9 CH_3), 24.4 (CH_3N), 23.7 (C-5′ CH_3), 20.4 (C-7); mass spectrum, m/e 525 (M, calcd 525), 507 (M − H_2O). Anal. Calcd for $C_{27}H_{27}NO_{10}$: C, 61.71; H, 4.79; N, 2.06. Found: C, 57.99; H, 5.01; N, 2.46; $CHCl_3$, 4.85.

Methylamine from 25. This was done as previously using 600 mg of **25** and 20 mL of 2 N NaOH. ^{1}H NMR on the volatile base hydrochloride gave a chemical shift of δ 2.65, identifying the product as CH_3NH_2·HCl.

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Agents for the Treatment of Brain Injury. 1. (Aryloxy)alkanoic Acids

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Blunt and ischemic injuries of the brain have been shown to result in swelling that is predominantly limited to a single cell type, the astrocyte, within the complex cellular mosiac of cerebral gray matter. Evaluation of various diuretic (aryloxy)acetic acids in vitro using incubating cat brain slices and primary astrocyte cultures identified compounds with marked ability to inhibit brain tissue swelling. Some of the compounds significantly reduced the mortality and morbidity following acceleration/deceleration brain injury in anesthesized cats. A variety of (indanyloxy)alkanoic acids were synthesized which were analogous to the dually active (indanyloxy)acetic acids. Some of the 4-(indanyloxy)butanoic acids were found to be devoid of diuretic activity but to possess equal or greater activity than the dually active compounds in the in vitro and in vivo brain assays. Selected examples from both the (indanyloxy)acetic and 4-(indanyloxy)butanoic acid series showed marked chiral effects, with one enantiomer generally exhibiting a much greater activity than the other. A clinical study of severely head-injured patients treated with ethacrynic acid demonstrated a significantly improved outcome when compared to controls. These data suggest a clinical advantage for the nondiuretic (aryloxy)alkanoic acids which possess in vitro and in vivo activities in the cat brain assays that are comparable or superior to dually active compounds.

Accidental death in the United States is the leading cause of mortality for individuals under the age of 45 and the fourth leading cause of death in all age groups following heart disease, cancer, and stroke.[1,2] Fatal vehicular-related brain injury is the single most common source of accidental mortality;[1,2] approximately 52 000 Americans die in automobile accidents yearly.[3] Moreover, morbidity following brain injury is the chief cause of persisting disability of the survivors of accidental injury in developed countries.[1,2] It was estimated in 1974 that survivors of brain injury accounted for the loss of 9.6 million days of normal activities and required 6.6 million days of hospitalization; moreover, the lifetime cost of medical care for a patient with serious brain injury may be very high.[1]

Diverse types of brain insult, including ischemic stroke, cardiac arrect, Reye's Syndrome, and hydrocephalus, exhibit pathological and even life-threatening increases in tissue-water content of the skull-encased brain which are similar to those resulting from accidental head injury. Of these cerebral edema-producing insults, ischemic cerebrovascular stroke is the third leading cause of death in the United States, being responsible for 200 000 deaths and double that number of hospitalized patients annually.[4,5] Those people who have survived one or more strokes number approximately 1.8 million.[4,5]

There is an incomplete understanding of the molecular mechanisms associated with both the cause and resolution of edema of cerebral white matter related to altered cerebrocapillary permeability. Specifically effective therapeutic agents do not exist. Therefore, a large number of nonspecific compounds have been evaluated. The principal aim in their use is the control of brain bulk and/or intracranial pressure. These agents include various osmotic diuretics,[6-12] steroids,[13-15] barbiturates,[16-20] carbonic an-

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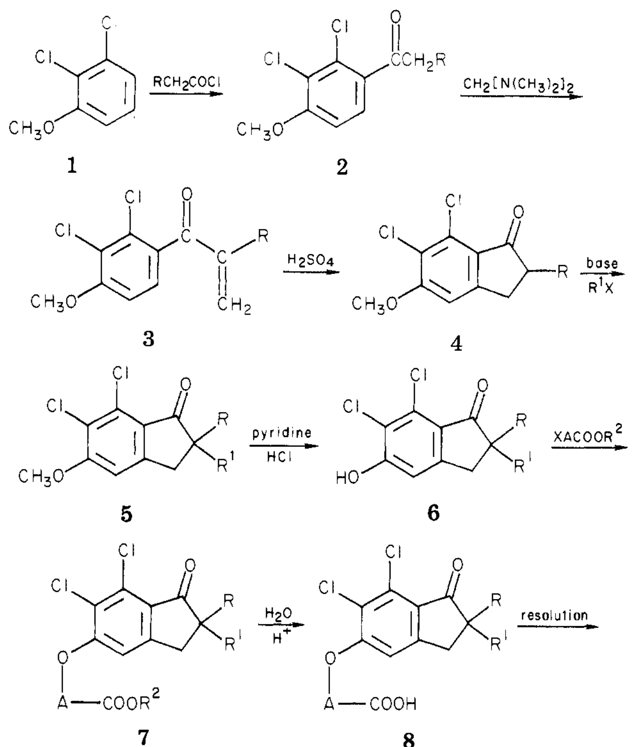
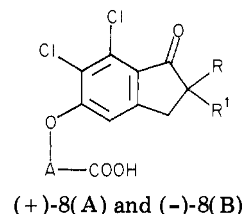
hydrase inhibitors,[21-23] and loop diuretics, including furosemide[6,8,24] and ethacrynic acid.[25-27a] Current evidence would suggest that steroids are of limited therapeutic effectiveness.[14] The usefulness of osmotic and renal diuretics, as well as barbiturates, is frequently limited by the medical complications associated with high-volume diuresis and induced barbiturate coma.

Rationale for New Therapy. There is evidence that pathologically altered fluid with solute compartmentation in the injured brain may be correlated with subsequent morbidity and mortality.[27b] Most studies of pathological fluid compartmentation in the injured brain have focused on cerebral white matter, which constitutes about one-half of the brain volume, as the site of clinically relevant cerebral edema.[27c] However, pathologically altered fluid compartmentation within gray matter, which also comprises about one-half of the brain volume, also appears to be a site of major clinical importance. Following blunt injury and stroke, swelling of a single cell type, the astrocyte, within the complex cellular mosiac of cerebral gray matter has been described.[28-32] Cellular extensions of astrocytes are physical satellites of neuronal perikarya which cover virtually the entire surface area of nutrient cerebrocortical capillaries. Astrocytes, which normally constitute an estimated 20–25% of the volume of cerebral cortex,[33a] are, therefore, strategically located to alter the concentrations of critical capillary-borne solutes and gases to and from metabolically dependent neurons. Furthermore, modest cerebrocortical tissue edema predominantly limited to swollen astrocytes does alter capillary-tissue microgeometry to the possible detriment of capillary-tissue solute transfer.[33b]

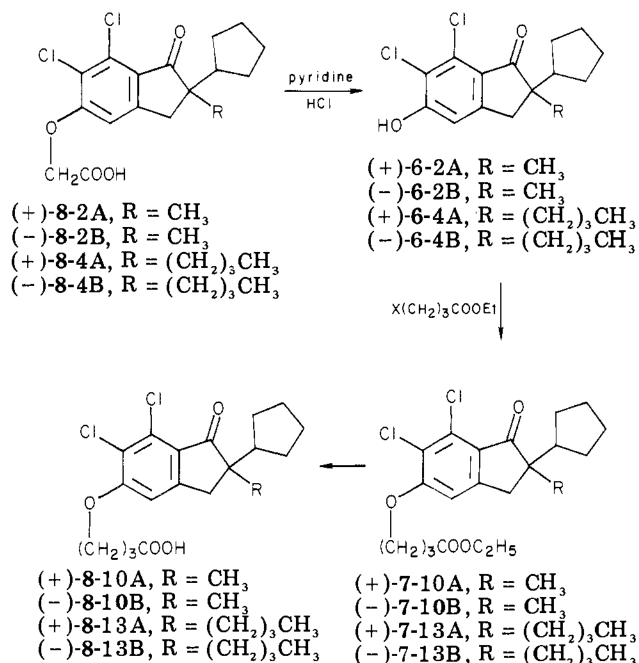
Scheme I. General Synthetic Methods





(+)-8(A) and (−)-8(B)

Scheme II. Synthesis of 8-10A, 8-10B, 8-13A, and 8-13B



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Our in vitro and in vivo studies have shown that K^+ and/or putative neurotransmitter-dependent, HCO_3^--stimulated net coupled Cl^--cation transport into astrocytes associated with an osmotic equivalent of water establish astrocytic swelling. Moreover, compounds related to the (aryloxy)alkanoic acid diuretic ethacrynic acid block Cl^- with cation and water uptake in various test systems, including the in vitro cerebrocortical slice preparation,[34,35a,b] cultured astrocytes,[34,49] and intact superfused cerebral cortex in vivo.[36] Preliminary studies[27,37] have shown that the administration of these compounds after experimental and clinical head injury resulted in decreased mortality and morbidity as compared to controls.

The obvious objective of our present investigation was to design compounds with specific activity in blocking Cl^- transport related cerebral edema which were free of diuretic activity. This report summarizes the results of our chemical and current biological studies in vitro and in vivo, as well as the initial clinical studies. Our previous experience with the general class of (aryloxy)alkanoic acid diuretics,[38] some of which have been shown to block renal Cl^- transport,[39a,39b] suggested that we focus our attention initially on this class of compounds.

Chemistry. The (indanyloxy)alkanoic acids that were studied are shown in Table I. Compounds **8-1** to **8-6**, **8-2A** and **8-2B**,[40] **8-7**,[41] and **8-10**[42] have been described previously. The general synthetic routes which were used to prepare these compounds are shown in Schemes I and II. The general synthetic procedures for the new compounds are described under Experimental Section, and the detailed data are summarized in Table VI. The difficulties encountered in the attempts to resolve **8-10** to **8-10A** and **8-10B** were obviated by resolving **8-2** and then cleaving the chiral homologues, **8-2A** and **8-2B**, to the corresponding phenols (**6-2A** and **6-2B**), which were converted directly to their respective chiral oxybutyric aids, **8-10A** and **8-10B**. Similarly, **8-13** was resolved to **8-13A** and **8-13B** by resolving **8-4**, cleaving the chiral homologues **8-4A** and **8-4B** to the analogous phenols (**6-4A** and **6-4B**), and then converting these to their respective chiral oxybutyric acids, **8-13A** and **8-13B**.

Biology. Four biological assay procedures for testing the synthetic compounds are presented, together with a controlled clinical study of ethacrynic acid. The specific criteria for drug efficacy varied with each assay system in order to provide a broad view of response to a specific compound at the following levels of biological integration: rodent astroglial cell (astrocyte), cat cerebral cortical tissue in vitro and in vivo, and the experimentally head-injured cat.

In Vitro Cat Cerebrocortical Tissue Slice Assay. This test provided the principal in vitro evaluation for each compound and consisted of a determination of the concentration vs. response curve. The addition of HCO_3^- to isotonic, K^+-rich saline–glucose incubation media has been shown to specifically stimulate the mediated transport of Cl^- coupled with Na^+ and an osmotic equivalent of water in incubating slices of mammalian cerebral cortex.[34,35a,43] Experiments in vitro[43] and in vivo[36,44,45] demonstrated that the tissue locus of swelling is an expanded astroglial compartment. As shown in groups C and D of Table II, the addition of 10 mM HCO_3^- as $NaHCO_3$ or as the organic bicarbonate (TEAB) to incubation media stimulated significant ($p < 0.01$) and comparable increases in cerebrocortical tissue swelling and ion levels when compared with values for these parameters in respective controls (groups A and B). Furthermore, the reproducibility of values for tissue water and ion levels was demonstrated when these parameters in identical control experiments were determined and compared after a hiatus of more than 6 months (i.e., group A vs. group B of Table II). The addition of 10 mM HCO_3^- per se stimulated significant ($p < 0.01$) cerebrocortical tissue fluid uptake of approximately 114 μL/g with approximately 15 μmol of Cl^-/g and approximately 17 μmol of Na^+/g; there was no significant change in tissue levels of K^+ (Table II, group B vs. D). Assuming the HCO_3^--stimulated tissue increases in NaCl to be solute associated with the HCO_3^--stimulated fluid expansion, the calculated osmotic load would be an isosmotic 281 mOsm/L.

The tissue assay system already described discriminated clearly between the enantiomers of MK473 (compound **8-2**, referred to earlier as DCPI[43]), as measured by their abilities to block HCO_3^--stimulated, ion-rich tissue swelling. Thus, a 10^{-5} M concentration of the (+) enantiomer (compound **8-2A**, Table I) totally ($p < 0.01$) inhibited HCO_3^--stimulated tissue uptake of water and ions when compared with uninhibited controls (groups E, H vs. C, D of Table II). Total inhibition of HCO_3^--stimulated fluid and ion alterations were observed in the presence of either an inorganic or organic source of HCO_3^-. A lesser concentration (10^{-7} M) of the (+) enantiomer (**8-2A**) provided partial inhibition of HCO_3^--stimulated, ion-rich swelling (group F of Table II). In contrast to the fully inhibitory effects of a concentration of 10^{-5} M of the (+) enantiomer (**8-2A**) on these parameters, a concentration of 10^{-5} M of the (−) enantiomer (**8-2B**) exhibited little, if any ($p < 0.01$), inhibitory effects (groups G, I vs. E, H of Table II). It is interesting to note that the values for HCO_3^--stimulated tissue swelling following the use of 10^{-5} M (+) enantiomer (**8-2A**) were significantly ($p < 0.01$) less than even comparable values in HCO_3^--free controls (groups E, H vs. A, B of Table II). Even though no HCO_3^-/CO_2 was added to controls, the pCO_2 in incubation media predominantly derived from tissue respiration and determined at the end of the incubation period was pCO_2 = 3 to 4 torr. Thus, a small CO_2/HCO_3^- stimulus to swelling existed in the controls. HCO_3^--stimulated tissue swelling occurs at low concentrations of HCO_3^-/CO_2 and is proportional[35a] to added HCO_3^-.

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Table I

no.	R	R[1]	A	enantiomer	rel salidiuretic act.[a] Rat	Dog	Chimp	Man	% distribution in octanol vs. 7.4 buffer[b]	pK_a[c] in 30% alcohol	in vitro assay[d] I_{50}, M
8-1[e]	c-C_5H_9	H	CH_2	±	1	2	1	2	83	3.9	10^{-5}
8-2[e,f]	c-C_5H_9	CH_3	CH_2	±	2	2	2	2	90	3.9	2×10^{-7}
8-2A[e,g]	c-C_5H_9	CH_3	CH_2	+	3	2	3		90	3.9	10^{-7}
8-2B[e,g]	c-C_5H_9	CH_3	CH_2	−	2	1	2		90	3.9	$>>10^{-5}$ [h]
8-3[e]	c-C_5H_9	C_2H_5	CH_2	±	2	2	2		97	3.9	10^{-9}
8-4	c-C_5H_9	$(CH_2)_3CH_3$	CH_2	±	1				99	3.9	3×10^{-9}
8-4A[g]	c-C_5H_9	$(CH_2)_3CH_3$	CH_2	+	1				99	3.9	3×10^{-10}
8-4B[g]	c-C_5H_9	$(CH_2)_3CH_3$	CH_2	−	1				99	3.9	5×10^{-5}
8-5[e]	CH_2-c-C_5H_9	H	CH_2	±	1	±	1		97	3.8	10^{-8}
8-6[e]	CH_3	$CH_2C_6H_5$	CH_2	±	±	±	2		91	3.8	10^{-9}
8-7[i]	C_6H_5	CH_3	CH_2	±	4	3	4	4	84	3.7	10^{-6}
8-8	c-C_5H_9	CH_3	$C(CH_3)_2$	±					98.6	3.7	$>10^{-4}$
8-9	c-C_5H_9	CH_3	c-$C(CH_2)_3$	±					98.6	3.8	10^{-6} [j]
8-10[k]	c-C_5H_9	CH_3	$(CH_2)_3$	±	±	±	±		99	5.7	2×10^{-8}
8-10A	c-C_5H_9	CH_3	$(CH_2)_3$	+	0[l]	0			99	5.7	10^{-8}
8-10B	c-C_5H_9	CH_3	$(CH_2)_3$	−	±	1			99	5.7	2.5×10^{-7}
8-11	c-C_5H_9	C_2H_5	$(CH_2)_3$	±	±				98.7	3.8	$>10^{-8}$
8-12	c-C_5H_9	$(CH_2)_2CH_3$	$(CH_2)_3$	±	±				99.6	4.1	8×10^{-9} [j]
8-13	c-C_5H_9	$(CH_2)_3CH_3$	$(CH_2)_3$	±	0				99.8	4.4	4×10^{-9}
8-13A	c-C_5H_9	$(CH_2)_3CH_3$	$(CH_2)_3$	+	0				99.9	4.4	10^{-6}
8-13B	c-C_5H_9	$(CH_2)_3CH_3$	$(CH_2)_3$	−	0				99.9	4.4	2×10^{-9}
8-14	C_6H_5	CH_3	$(CH_2)_3$	±		±			99	6.4	2.5×10^{-7}
8-15	CH_3	$CH_2C_6H_5$	$(CH_2)_3$	±					99.5	6.3	$>10^{-4}$
8-16	c-C_5H_9	CH_3	$CH_2CH{=}CHCH_2$	±					98	5.1	$>4 \times 10^{-5}$
8-17	c-C_5H_9	CH_3	CH_2-C_6H_4-*p*	±					100	6.2	$>10^{-5}$
9 (ethacrynic acid)					±	4	4	4	61	3.5	10^{-6}
10 (dihydroethacrynic acid)[m]					0	1	1		96	3.5	5×10^{-7}
11 (acetazolamide)					1	1	1	1	22	7.2	2×10^{-3}
12 (furosemide)					4	4	4	4	28	4.5	10^{-5}

[a] The relative salidiuretic efficacies are scored in a fashion similar to those reported previously:[38] 0 = inactive; ± = marginal; 1 = weak; 2 = moderate; 3 = marked; 4 = very marked. The rat and dog data were obtained by L. S. Watson and E. H. Blaine. The chipanzee and initial human data by G. M. Fanelli, Jr. Most of these data have been reported elsewhere. [b] These data were obtained by E. L. Cresson. [c] These data were obtained by Y. C. Lee. [d] These data were obtained as described under Experimental Section,[34,35a,43] and the I_{50} values were obtained with $p = 0.005$. [e] This compound is described in ref 40. [f] MK 473, referred to as "DCPI" in ref 36 and 43. [g] J. M. Hirschfield and J. M. Springer have determined by X-ray analysis that 8-2A and 8-4A are *R*(+) and 8-2B and 8-4B are *S*(−), while 8-13A is *R*(+) and 8-13B is *S*(−). [h] No inhibition occurred at this concentration. [i] Indacrinone; see ref 41. [j] At no concentration was swelling inhibited 100%. [k] This compound is described in ref 42 and has been referred to as "DCPIB" (36). [l] No significant salidiuretic activity was observed upon the intravenous adminstration of 20 mg/kg of the Na salt to cats (data by Dr. E. H. Blaine and his colleagues). [m] [2,3-Dichloro-4-(2-methylbutyryl)phenoxy]acetic acid.

Table II. Effect of Added HCO_3^- as $NaHCO_3$ or as Triethylammonium Bicarbonate (TEAB) with Enantiomers of 8-2 on Swelling and Ion Content of Cerebrocortical Slices in Vitro [a]

group	conditions	N	tissue swelling,[e,f] μL/g	% HCO_3^--stimulated swelling	tissue content,[e,f] μmol/g Cl^-	Na^+	K^+
A	no additions	21	170 (±4)	0	90.2 (±1.1)	100.4 (±1.3)	94.4 (±1.2)
B [b]	no additions	46	179 (±4)	0	89.9 (±0.8)	101.6 (±1.1)	93.5 (±1.4)
C [c]	+$NaHCO_3$	24	268 (±5)	100	98.2 (±1.1)	116.3 (±1.7)	97.1 (±1.3)
D [d]	+TEAB	48	293 (±5)	100	104.8 (±0.9)	118.8 (±1.1)	91.4 (±1.3)
E	+$NaHCO_3$ + 10^{-5} M (+)-8-2A	8	157 (±9)*	0	89.0 (±0.9)*	101.8 (±1.1)*	92.4 (±1.8)
F	+$NaHCO_3$ + 10^{-7} M (+)-8-2A	10	232 (±10)*	68 [h]	94.4 (±1.2)*	113.7 (±3.0)*	98.4 (±2.2)
G	+$NaHCO_3$ + 10^{-5} M (−)-8-2B	5	266 (±15)**	100	104.6 (±2.5)**	115.2 (±2.6)	96.4 (±2.1)
H	+TEAB + 10^{-5} M (+)-8-2A	5	156 (±9) [g]	0	84.1 (±2.1) [g]	100.9 (±3.0) [g]	95.9 (±2.1)
I	+TEAB + 10^{-5} M (−)-8-2B	5	279 (±18)	100	98.3 (±3.6)	115.5 (±3.5)	93.6 (±1.9)

[a] Slices (~150 mg; 0.5-mm thick) of cat cerebral cortex were incubated aerobically for 1 h at 37 °C in Hepes-buffered saline–glucose media containing 27 mM K^+ and varied concentrations of the enantiomers of 8-2 (compounds 8-2A or 8-2B, Table I) indicated. Additions of HCO_3^- as $NaHCO_3$ or triethylammonium bicarbonate (TEAB) to a final concentration of 10 mM were made 20 min after the start of incubation. Values for tissue swelling and ion contents (as microliters or micromoles per gram initial fresh weight) are expressed as means ± SE. Percent bicarbonate-stimulated swelling was calculated as follows: (μL swelling determined − μL swelling of control, group A or B)/(μL swelling HCO_3^- stim. exptl, group C or D, − μL swelling of controls, group A or B) × 100. Significance was calculated by the Student's t test. [b] Values in group B do not differ significantly from corresponding values in group A. [c] Values in experimental group C differ significantly ($p < 0.01$) from corresponding control values in group A. [d] Values in experimental group D differ significantly ($p < 0.01$) from corresponding control values in group B. [e] (*) Values in this group differ significantly ($p < 0.01$) from corresponding values in groups C and A. [f] (**) Values in group G differ significantly ($p < 0.01$) from corresponding values in group E. [g] Values in group H differ significantly ($p < 0.01$) from corresponding values in groups I and D. [h] Utilizing all pooled controls, $n = 67$; 10^{-7} M (+)-MK 473 yielded approximately 60% HCO_3^--stimulated swelling.

The concentration–response curve for a specific (indanyloxy)alkanoic acid displaying inhibition of K^+-dependent, HCO_3^--stimulated ion-rich cerebrocortical tissue swelling was frequently parabolic, as shown earlier for ethacrynic acid.[35a] Data obtained with **8-10** (Figure 1) is typical of those obtained with many compounds in this series. Pharmacological concentrations of drug in excess of those that produce maximal inhibition seemingly caused some increase in tissue swelling. This may, in part, result from high concentration-related effects of these agents on tissue systems other than the astroglial cell Cl^- pore.[35a] Multiple effects of drugs may occur for other classes of compounds as well. Previously, we reported that the carbonic anhydrase inhibitor acetazolamide blocked K^+-dependent, HCO_3^--stimulated cerebrocortical swelling in vivo[44] and in vitro[35a] but only at concentrations far in excess of those which were adequate for inhibition of tissue carbonic anhydrase. Subsequently, it was demonstrated that pharmacological concentrations of sulfonamide diuretics, including acetazolamide, block the chloride pore in the mammalian red cell[46] and, most likely, in the astroglial cell as well. Thus, drug concentration-related epiphenomena, if not excluded by a systematic approach, may obscure the valid effective inhibitory range. Mindful of these pitfalls, detailed concentration–response curves, similar to that reported in Figure 1, were obtained for each compound that was tested. The data were expressed as percent HCO_3^--stimulated swelling (legend, Table II) vs. drug concentration, from which the concentration of drug providing 50% inhibition of HCO_3^--stimulated swelling (I_{50} in molarity) was interpolated and expressed in Table I.

In Vitro Primary Rat Astrocyte Culture Assay. The primary ionic mechanism promoting astrocytic swelling seems to be the mediated net Cl^- transport into cerebrocortical astrocytes.[47] Primary astrocyte cultures have a SITS-sensitive anion-exchange system[48-50] and show accumulation of chloride ion.[49,50] The data in Figure 2

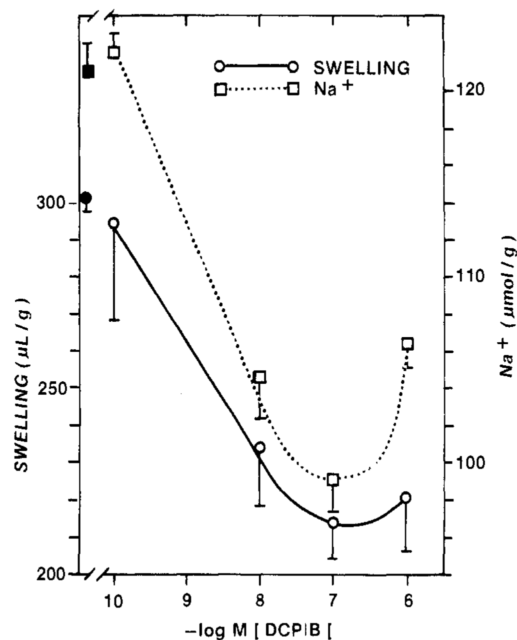

Figure 1. Swelling and tissue content of slices of cat cerebral cortex vs. compound **8-10** concentration. Swelling and tissue Na^+ content, expressed as microliters or micromoles per gram initial fresh tissue weight, of slices of cat cerebral cortex as a function of compound **8-10** (DCPIB) concentration (−log M). Slices (0.5-mm thick; ~150 mg fresh weight) were incubated for 1 h at 37 °C in K^+-rich, Hepes-buffered saline–glucose media containing various concentrations of [(6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1*H*-inden-5-yl)oxy]butanoic acid (Table I, compound **8-10**). HCO_3^- was added 20 min after the start of the incubation to give a final concentration of 10 mM. The open symbols represent the mean (±SE) values for three or more determinations at a single concentration. Closed symbols represent values from controls not containing **8-10** where $n = 18$.

(46) Cousin, J. L.; Motais, R. *J. Physiol.* (*London*) **1976**, *256*, 61.
(47) Bourke, R. S.; Daze, M. A.; Kimelberg, H. K. In ref 33a; p 337.

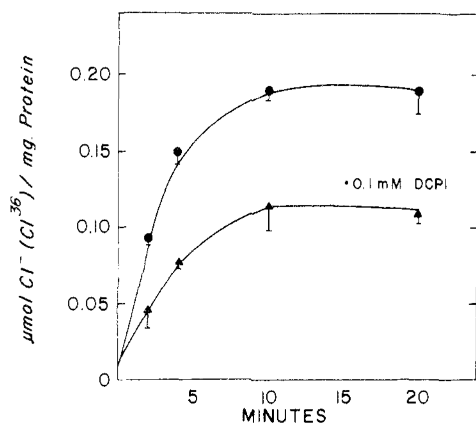

Figure 2. Inhibition of Cl$^-$ uptake in primary rat astrocyte cultures by compound **8-2A**. Three-week-old cultures containing 0.87 ± 0.27 mg of protein per dish (mean ±SD, n = 30) were used. Uptake of Cl$^-$ (micromoles of Cl$^-$ per milligram of protein) was calculated based on the specific activity of ^{36}Cl$^-$ in the medium and the ^{36}Cl$^-$ content of the cells. The radioactivity found in cells washed immediately after adding ^{36}Cl$^-$ was 0.011 ± 0.002 μmol of Cl$^-$/mg of protein. This presumably represents extracellular Cl$^-$ due to adherent medium remaining after washing and is equivalent to 0.1 μL/mg of protein. (●) Control; (▲) **8-2A** was added to a final concentration of 0.1 mM, 15 min before adding the radioisotopes. Values are means ± SD; n = 3.

show the time course of inhibition of the unidirectional influx of ^{36}Cl$^-$ by compound **8-2** (in Table I) when the cells are in a steady state. Compound **8-2**, at a final and fully inhibitory concentration of 0.1 mM, inhibited both the initial rate of influx and significantly ($p < 0.01$) reduced the steady-state content of chloride by more than 50%, i.e., from 0.18 to 0.10 μmol of Cl$^-$/mg of protein after preequilibration. This suggests that compound **8-2** not only inhibits a Cl$^-$ ↔ Cl$^-$ exchange system, as does the anion-exchange inhibitor SITS,[48] but also inhibits a net uptake system. This determination with selected compounds confirmed a cellular locus and mechanism of drug action without the interpretative problems attending flux and incorporation studies in the heterogeneous cerebrocortical tissue cellular mosaic.[51]

In Vivo Inhibiton of Cerebrocortical Astroglial Swelling. In order to determine whether the inhibition of cerebrocortical tissue slice swelling observed in vitro (Table II) could be achieved in vivo, the following experiment was carried out and reported elsewhere.[36] The exposed and intact cerebral cortices of cats in vivo were superfused under normothermic conditions with isotonic artificial CSF containing added 0.125 mM adenosine, which is a stimulus for cerebrocortical tissue slice swelling in vitro.[34] This was found to result in significant ($p < 0.01$) chloride-rich cerebrocortical swelling (75 μL/g) that was limited to swollen astrocytes as shown by electron microscopy. The addition of compound **8-10** at a concentration of 10^{-6} M to the superfusate, together with systemic pretreatment (2.5 mg/kg iv) totally blocked astroglial swelling and the associated increases in tissue ion contents. This confirmed both the validity of the in vitro tissue slice swelling assay recorded in Table II and the idea that tissue swelling in vivo was apparently limited to the astrocytic cell type.

(48) Kimelberg, H. K.; Biddlecome, S.; Bourke, R. S. *Brain Res.* **1979**, *173*, 111.
(49) Kimelberg, H. K.; Bowman, C.; Biddlecome, S.; Bourke, R. S. *Brain Res.* **1979**, *177*, 533.
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(51) Bourke, R. S. *Exp. Brain Res.* **1969**, *8*, 219.

Table III. Cat Head Injury Assay

no.	compd	dose, mg/kg iv	mortality: no. dead/total	mortality: % [b]
	control	0	38/75	51
11	acetazolamide	1	8/16	50 [c]
14	methylprednisolone sodium succinate	30 [a]	9/18	50 [c]
9	ethacrynic acid	1	14/39	36 [c]
8-7	indacrinone	1	8/22	36 [c]
8-2	MK 473 (DCPI)	1	11/42	26 [d]
8-10	DCPIB	5	4/16	
8-10A	(+)-DCPIB	1	5/16	25 [e]

[a] 30 mg/kg given q.i.d. [b] Significance determined by chi-square testing. [c] p = not significant. [d] $p < 0.05$. [e] The first study was conducted with a 5 mg/kg iv dose of the racemate (**8-10**) and the second study with a 1 mg/kg iv dose of the (+) enantiomer (**8-10A**). The mortality for the combined study was 9/32 (28%); $p < 0.05$.

In Vivo Cat Head Injury Assay. Experimental biomechanical studies have demonstrated that concussive brain injury results from acceleration/deceleration injury to the skull-encased brain. We have demonstrated[37] that the experimental addition of a period of controlled posttraumatic hypoxemia following acceleration/deceleration brain injury in the cat results in prolonged coma with delayed death similar to that observed in clinical experience.[52] Thus, an experimental paradigm of cat head injury has been developed in which administered drugs can be assayed in vivo for effectiveness in altering mortality and neurological morbidity.[37]

As seen in Table III, the postinjury intravenous administration of either compound **8-2** or **8-10** significantly ($p < 0.05$) reduced mortality. Acetazolamide or methylprednisolone sodium succinate (**8-14**) had no discernible effect on mortality. Both ethacrynic acid (**9**) and indacrinone (MK196, **8-7**) reduced head injury-related mortality but in a less than statistically significant fashion. To a large extent these findings reflect the intrinsic biological activity of these compounds, as determined by the in vitro cerebrocortical tissue slice assay (Table I). However, these findings may reflect both differing rates of achievement and ultimate concentrations of biologically available drug at brain tissue sites. In support of this arguement is the clear evidence that ethacrynic acid significantly ($p < 0.025$) improved both the rate and ultimate level of neurological recovery in treated cats surviving the head-injury insult (Figure 3).

Clinical Study. A controlled clinical study of ethacrynic acid as Edecrin was conducted based on our preliminary results.[27] All severely head-injured patients treated at the Albany Medical Center Hospital from July 1976 to January 1980 who met the restrictive criteria were included in the study. The criteria for inclusion in the study were as follows: patients were selected who were between the ages of 15 and 50 years and had suffered a severe, isolated, closed-head injury which rendered each one unresponsive to verbal commands and bilaterally decerebrate upon initial neurological assessment at our hospital within 24 h of injury. It was required that each patient have retained brainstem function as evidenced by at least one reactive pupil, intact corneal reflexes, and the presence of either oculocephalic or oculovestibular reflexes.

(52) Sinha, R. P.; Ducker, T. B.; Perot, P. L. *J. Am. Med. Assoc.* **1973**, *224*, 1258.

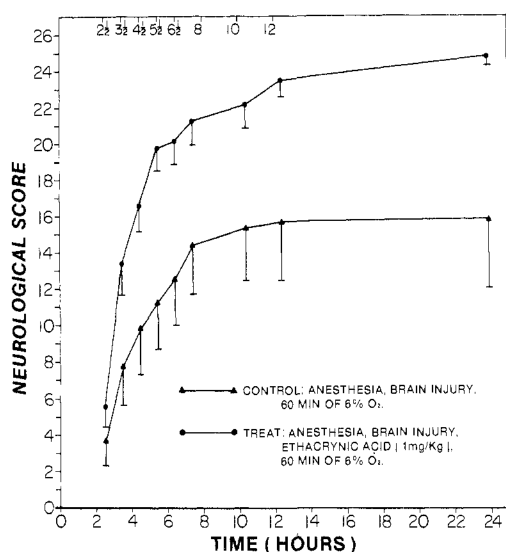

Figure 3. Clinical neurological score vs. time. Clinical neurological score (units) is presented as a function of time (hours) following combined translational head injury followed by controlled hypoxemia. Conditioned adult cats were anesthetized with a brief acting agent and subjected to a 1-min rapid acceleration/deceleration translational head injury followed 30 min later by a 60-min controlled period of hypoxemia (a pO_2 24 ± 1 torr), induced by ventilation (60 min) with a gas mixture of 6% O_2, balance N_2, followed by ventilation by room air. Survivors were scored from 2.5-h posttrauma with hypoxemia to the end of the experiment at 24 h at the times indicated. The clinical exam comprised 26 points accured by sum of the scores for each of the following: arousal, reaction to pain, walking position, pupil reflex, cornea reflex, ear reflex, and respiratory pattern. Each plotted point represents mean values plus or minus standard errors for 12 or more determinations in control animals (▲) or animals treated with ethacrynic acid (●), 1 mg/kg iv, given slowly 10 min after the head injury and before the start of induced hypoxemia. The mean values for neurological score that were determined for treated animals differ significantly ($p < 0.025$) from comparable control values that were determined at 3.5 h and for each subsequent time plotted.

The criteria for selection of patients for the study were chosen in an attempt to limit the effect of recognized variables known to influence the outcome of severe head trauma.[53-59] Moreover, it was expected that limiting the patients selected to those with two primary diagnostic findings, namely, diffuse brain injury (determined by a computerized axial tomograph of the brain) and decerebrate posturing with an initial clinical Glasgow Coma Score of 4, would lead to an expected mortality rate of 50% in controls treated with vigorous standard care.[53,60,61] Patients were excluded when thoracic, abdominal, or major orthopedic injuries accompanied the head injury. Pediatric patients and patients with surgically treatable intracranial hematomas were excluded because these two groups exhibit an expected mortality different from those included in the study.[53,62] These restrictive criteria limited to 18 the number of patients included in the study out of a possible 615 head-injured patients hospitalized at the Albany Medical Center Hospital for 4 or more days during the 3.5-year study period.

Standard care was identical for all patients. The goals of pulmonary management were to maintain arterial pO_2 >90 torr and arterial pCO_2 between 25 and 30 torr by ventilatory support via endotracheal intubation. All patients received an intravenously administered steroid (methylprednisolone sodium succinate as Solu-Medrol), 125-mg loading dose followed by 40 mg every 6 h for 5 days, and anticonvulsant medication (diphenylhydantoin sodium as Dilantin), 300 mg daily. Blood gases were determined for each patient at least every 6 h. Hemoglobin/hematocrit, serum electrolytes, and serum and urine osmolality determinations were made every 4 h. Urine output via an indwelling transurethral catheter was measured hourly. All patients had hourly clinical neurological assessments by the nursing staff with frequent verification by the neurosurgical staff using the quantified Albany Head Injury Watch Sheet (AHIWS).[63] This clinical scoring instrument permits the determination of the clinical rate (units per day) of recovery, as well as the initial clinical neurological status. The latter parameter was also rated by the Glasgow Coma Score,[64] an internationally accepted index of initial clinical neurological status following brain injury. Patients were alternately assigned to control or ethacrynic acid treatment groups. Patients assigned to the ethacrynic acid treatment group received the standard care already described plus an initial loading dose of 50 mg of ethacrynic acid, followed by a sustaining dose of 25 mg of ethacrynic acid every 12 h via the intravenous route for 7 days. Fluid balance was rigidly controlled so that patients in the control group received a volume of 2.5% dextrose in 0.5 N saline computed to constitute 67% of the daily requirement. Those patients in the ethacrynic acid treatment group received replacement fluid particularly rich in NaCl and KCl, since these ions were lost by saluresis. Thus, these patients were given a volume of normal saline equal to the previously hourly urine output plus an additional 10 mL of saline with supplemental K^+. Adjustments were made in the type and volume of parenteral intravenous solution administered in order to maintain serum electrolytes and osmolality within the normal range. The intent was to determine if ethacrynic acid beneficially influenced the clinical outcome from severe head injury independent of diuresis-related alterations in systemic solute and water balance.

Results of Clinical Study. There were 10 patients (4 females, 6 males; average age, 18.3 (±2.2) years; age range 16–23 years) in the ethacrynic acid treatment group and 8 patients (4 females, 4 males; average age 26.9 (±11) years; age range 16–48 years) in the control group. As seen in Table IV, there were no significant differences between the treated and control patients in regard to clinical neurological and physiological parameters measured during the critical initial 1–2 weeks after head-injury intensive care, save for significantly increased ($p < 0.05$) daily urinary output in patients treated with ethacrynic acid. Despite differences in urinary output, there were no significant differences in indexes of systemic fluid balance

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(61) Bowers, S. A.; Marshall, L. F. *Neurosurgery* **1980**, *6*, 237.
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(63) Yen, J. K.; Bourke, R. S.; Nelson, L. R.; Popp, A. J. *J. Neurol. Neurosurg. Psychiatry* **1978**, *41*, 1125.
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Table IV. Clinical Study with Ethacrynic Acid: Comparison of General Physiological and Clinical Parameters during the Initial 1 to 2 Postinjury Weeks, Respectively

parameter[a]	treated patients	control patients	signif[b]
duration of decerebration, days	6.2 ± 4.5	6.5 ± 2.1	NS
duration of coma,[c] days	11.7 ± 5.4	14.8 ± 3.9	NS
pO_2,[d] torr	114.4 ± 22.3	113.7 ± 20.6	NS
pCO_2, torr	30.7 ± 2.6	29.9 ± 4.6	NS
pH, units	7.471 ± 0.020	7.466 ± 0.030	NS
osmolality, mOsm/L	304.8 ± 11.4	295.8 ± 17.5	NS
serum Na^+, mequiv/L	144.6 ± 2.0	142.6 ± 4.5	NS
serum K^+, mequiv/L	4.1 ± 0.2	3.9 ± 0.3	NS
BUN, mg %	22.1 ± 3.4[e]	19.1 ± 5.8	NS
HCt,[f] %	36.9 ± 3.8	35.8 ± 2.8	NS
urine output, L/day	4.1 ± 1.8	2.5 ± 0.9	$p = 0.05$

[a] Mean values (± SD) for seven or more determinations are presented. [b] Test of significance was the Student's *t* test. [c] Coma is defined as the inability to follow verbal commands. [d] Mean values for arterial pO_2 determined on arrival in the emergency room for treated and controls were 74.4 and 63.4 torr, respectively; a nonsignificant difference. [e] Excluding one surviving patient with a BUN of 54 mg %, which subsequently returned to normal. [f] Hematocrit.

Table V. Clinical Study of Ethacrynic Acid: Comparison of Initial and Subsequent Diagnostic and Outcome Factors

group	parameters	treated patients	control patients	signif
A[a]	initial AHIWS score (group mean)	5.4	5.1	NS[b]
B	initial Glasgow coma score (group mean)	4.0	4.0	
C	initial oculovestibular/oculocephalic reflexes			
	no. of patients intact	6	6	NS[c]
	no. of patients impaired	4	2	
D	initial computerized axial tomograph of brain			
	no. with diffuse brain swelling	6	5	NS[c]
	no. with brain contusion	4	3	
E	no. of survivors	9	4	$p < 0.09$[c,d]
	no. of deaths	1	4	
F[e]	no. of good results	9	2	$p < 0.01$[c]
	no. of poor results	1	6	
G[e]	no. of functional survivors	9	2	$p < 0.05$[c]
	no. of nonfunctional survivors	0	2	
H[a]	rate of neurological recovery (AHIWS, units/day)	0.41 (± 0.33)	0 (± 0.23)	$p < 0.05$[f]
		survivors	deaths	
I	av age, years	22.8 (± 9.5)	20.4 (± 4.6)	NS[f]
J	av interval from time of accident to arrival in emergency room, h	1.6 (± 1.1)	1.9 (± 1.2)	NS[f]
K	initial oculovestibular/oculocephalic reflexes			
	intact	8	4	NS[c]
	impaired	5	1	
L	initial computerized axial tomograph of brain			
	diffuse brain swelling	10	1	
	contusion	3	4	$p < 0.05$[c]

[a] Albany Head Injury Watch Sheet (AHIWS).[63] [b] Mann-Whitney *U* test. [c] Fisher exact test or chi-square. [d] Chi-square was calculated to be 3.545 vs. 3.841 where $p = 0.05$. [e] Based on Glasgow Outcome Scale,[65] where GR = good recovery; MD = moderate disability; SD = severe disability; PVS = persistent vegetative state; D = death. Thus, good result = GR + MD; nonfunctional survival = SD + PVS. [f] Student's *t* test.

between treated and control groups when serum Na^+, K^+, BUN and HCt are compared. Furthermore, there were no significant differences between the values for serum osmolality in treated and control groups (Table IV). Thus, the effect of ethacrynic acid treatment can not be simply ascribed to diuretic related hyperosmolality. Induced serum hyperosmolality has been shown to alter intracranial fluid and pressure relationships.[10]

Not only were treated and control groups comparable during the first 2 weeks following injury in terms of physiological and clinical neurological parameters already described but they were also comparable in terms of initial neurological and diagnostic criteria. The data in Table V demonstrate that there were no significant differences between treated and control groups in terms of initial general neurological status (groups A and B), specific clinical brain-stem function (group C), and findings on computerized axial tomography of the brain (group D). However, the outcome of the treated group was significantly ($p < 0.05$ to 0.01) better than the control group in terms "good" vs. "poor" results as defined by the Glasgow Outcome Scale[65] (group F), functional survival (group G), and the rate of clinical neurological recovery as defined by the Albany Head Injury Watch Sheet (group H). There was a 10% mortality rate (1 of 10) in the treated group vs. a 50% mortality rate (4 of 8) in the control group as already predicted. Survival was not significantly related to age (group I) or interval from injury to start of resuscitation (group J). The differences in mortality rates only marginally approached statistical significance (group E). Survival vs. mortality in either group was not significantly correlated with clinical brain-stem function (group K) but was significantly ($p < 0.05$) correlated with the appearance of brain contusion determined by initial computerized axial tomography of the brain (group L) as has been reported.[66,67]

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Discussion

Structure–Activity Relationships (SAR). The in vitro data shown in Table I obtained using the cat cerebrocortical tissue slice assay reveals some interesting SAR trends in regard to the ability of the compounds to inhibit swelling. Among the three "standard" diuretics and "dihydroethacrynic acid" shown at the end of the table, the relative activities are as follows: dihydroethacrynic acid (**10**) > ethacrynic acid (**9**) > furosemide (**12**) ≫ acetazolamide (**11**). Among the (indanyloxy)acetic acids, where A = CH_2 and R is cyclopentyl, the order of the activity-enhancing effect of R^1 is as follows: $(CH_2)_3CH_3$ (**8-4**) > C_2H_5 (**8-3**) > CH_3 (**8-2**) > H (**8-1**). The marked chiral effect of the 2-position carbon atom is seen by a study of the enantomers of **8-2** and **8-4**. The (+) enantiomer, **8-2A**, is 10 times as active as the racemate, **8-2**, and the (–) enantiomer, **8-2B**, is over two orders of magnitude less active than **8-2A**. Likewise, the (+) enantiomer, **8-4A**, is twice as active as the racemate, **8-4**, and the (+) enantiomer, **8-4A**, is about five orders of magnitude more active than the (–) enantiomer, **8-4B**.

The isomer of **8-2**, where R = cyclopentylmethyl and R^1 = H (**8-5**), is somewhat more active than **8-2**. When either R or R^1 is methyl and the other group is varied, the order of activity-enhancing effects is as follows: benzyl (**8-6**) > cyclopentyl (**8-2**) > phenyl (**8-7**).

Although the influence of the A moiety is dependent upon the nature of R and R^1, the following order of activity-enhancing effect is seen when R = cyclopentyl and R^1 = CH_3: $(CH_2)_3$ (**8-10**) > CH_2 (**8-2**) > $C(CH_2)_3$ (**8-9**) > $C(CH_3)_2$ (**8-8**) = $CH_2CH{=}CHCH_2$ (**8-16**) = $CH_2{-}C_6H_4$ (**8-17**). Thus, lengthening A from methylene (**8-2**) to trimethylene (**8-10**) produced a 10-fold increase in activity, but all other changes were detrimental to activity. Again, the chiral effect of the 2-position carbon atom is seen, since the (+) enantiomer (**8-10A**) is twice as active as the racemate **8-10**, while the (–) enantiomer (**8-10B**) is only one-fortieth as active as the racemate.

Since **8-10** was an order of magnitude more active than **8-2** and **8-3** was more active than **8-2**, a series of compounds was prepared in which A = $(CH_2)_3$ and R = cyclopentyl, while R^1 was varied. The general order of activating influence of R^1 was found to be butyl (**8-13**) > propyl (**8-12**) > methyl (**8-10**) > ethyl (**8-11**). This order is slightly deceptive, since 100% inhibition cannot be achieved with the propyl compound (**8-12**), i.e., only 60% inhibition being the maximum achievable inhibition.

As usual, it can be seen that one enantiomer of the 2-butyl compound, **8-13B**, is twice as active as the racemate, **8-13**, and **8-13B** is 2000-fold more active than the other enantiomer, **8-13A**. However, it is interesting to note that the active enantiomer of the 2-methyl compound (**8-10**) is the (+) form (**8-10A**), but with the homologous 2-butyl compound (**8-13**), it is the (–) form (**8-13B**). It was tempting to speculate as to the absolute configuration of **8-10A** and **8-13B**. Fortunately, J. M. Hirschfield and J. P. Springer of our Rathway Laboratories have determined the absolute configuration of these compounds from the X-ray analysis of **6-2A** (the precursor of **8-10A**) and **6-13B** using Hamilton R-Factor ratios. This study revealed **8-10A** to be *R*(+) and **8-13B** to be *S*(–); thus, the active enantiomers of these homologues have opposite absolute configurations.

With most compounds, the concentration vs. tissue swelling inhibition curves were parabolic, with high concentrations apparently inducing some concentration-related swelling action. The most interesting compounds not only had high intrinsic inhibitory activity but also a wide range of concentrations where maximal inhibition occurred.

It will be noted that the structural features which decreased the acidity (i.e., increased pK_a) and increased the lipophilicity (as measured by octanol/H_2O distribution) generally contributed to an increase in intrinsic activity.

Most importantly, it was found that some structural changes which either produced increases in or at least maintained a high order of intrinsic brain tissue swelling inhibitory activity resulted in decreases in diuretic activity. Thus, the structural change in homologizing **8-2** to **8-10** not only improved intrinsic activity but virtually eliminated the unwanted salidiuretic activity. Similarly, when **8-4** was homologized to **8-13**, intrinsic inhibition of brain swelling was maintained, while diuretic activity was lost. In fact, none of the 4-(indanyloxy)butanoic acids possessed appreciable salidiuretic activity. Interestingly, the (+) enantiomer (**8-10A**) of **8-10**, which was the most active in inhibiting astroglial swelling, was not diuretic in rats, dogs, and cats, while the less intrinsically active (–) enantiomer (**8-10B**) had marginal salidiuretic activity in rats and weak salidiuretic activity in dogs.

Representatives of the most interesting (aryloxy)alkanoic acids were evaluated in the cat head-injury assay; the results are summarized in Table III. The assay was exquisitely designed so that only half (51%) of the untreated (control) animals survived. From the table it can be seen that at the doses used, acetazolamide and the steroid methylprednisolone sodium succinate had no effect on mortality. On the other hand, ethacrynic acid and indacrinone (**8-7**) produced rather sizable (15%), although not statistically significant, decreases in mortality as compared to controls. Compound **8-2** reduced the mortality to 25%, which is not only significant but is approximately one-half of the mortality rate of the controls. These data confirm and extend our preliminary results.[27]

Each of the three compounds described above that were active in the cat head-shake assay were potent diuretics, producing marked salidiuresis at the doses used. As mentioned earlier, the objective was to develop an effective agent which was not diuretic. In a study using compound **8–10** (referred to as DCPIB in preliminary studies[36]) or its (+) enantiomer (**8-10A**), neither of which were significantly salidiuretic, a marked effect in reducing mortality was observed. The reduction in mortality was 23% below that of the controls, which is not only significant but as good as any of the compounds that were studied. Thus, as measured by this assay, a compound has been discovered which, at effective doses, is not diuretic but is more active than ethacrynic acid in reducing the mortality of experimental traumatic brain injury.

It is important to note that the surviving animals in the control group failed to achieve complete restitution of neurological integrity during the 24-h observation period, whereas each of the animals receiving an effective drug (**8-2**, **8-7**, **8-10**, and **8-10A**) did. In fact, even with those animals receiving ethacrynic acid (**9**) or indacrinone (**8-7**), whose effects were sizable but not statistically significant, complete restitution of neurological integrity was observed.

Within the members of the (aryloxy)alkanoic acid series that were studied there appears to be a good correlation between the in vitro and in vivo data.

Clinical Studies. A controlled clinical study was conducted using ethacrynic acid as a model in order to determine if a correlation exists between the therapeutic results observed in the cat head-injury model and the

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traumatic heat injury seen in the clinic.

Table V provides a comparison between the mortality rate and clinical neurological status of the control and the treated groups. The survival rate in the treated group was 90% as compared to 50% in the controls. Moreover, half of the controls that survived remained in a persistant vegetative state, whereas none of the treated survivors was so afflicted.

It should be noted that all deaths in the control series occurred in patients who exhibited progressive neurological deterioration and not sudden death from systemic factors. All the surviving patients in the treated group made a functional survival, defined as recovery to a level of self-care, as compared with the control group in which a similar state was attained in only half of the survivors. Therefore, ethacrynic acid therapy significantly improved the clinical outcome of seriously head-injured patients.

Ethacrynic acid is a diuretic agent; thus, the urine output in the treated groups was approximately double that of the controls. Nevertheless, serum electrolytes and osmolality data do not suggest a major difference in serum composition between control and treated groups. Thus, the neurological recovery appears to be due to a direct effect of the drug and not a secondary effect via diuretic-related systemic changes in osmolality. However, the diuresis and salidiuresis caused by the drug produces an added stress on a very ill patient and requires monitoring and replacing the diuretically induced water and electrolyte loss. This study, although limited in scope, does suggest a correlation between the drug related effects observed in the cat assay and the clinical situation. It also indicates that a potent, nondiuretic agent would have decided advantages.

We do not recommend the general use of ethacrynic acid or other loop diuretics in the treatment of the seriously head-injured patients. Rapid renal fluid and ion losses, followed by alterations in cardiovascular function, could seriously jeopardize neurological recovery in a patient with an insulted brain. Furthermore, the use of loop diuretics in the multiple injured head trauma victim, who is already in a state of unstable hemodynamic function, is not to be encouraged. In addition, many diuretics, i.e., furosemide and acetazolamide (Table I), do not possess high intrinsic activity in brain tissue. The development of nondiuretic agents which are active in blocking pathological shifts of brain fluids and solutes is recommended and appears to be achievable as demonstrated by our studies. The usefulness of the various biological assays in confirming both mechanism and site of action of ion-related fluid expansion of cerebral cortex, specifically the astrocytic compartment, is emphasized. It has been demonstrated that compounds, e.g., the (indanyloxy)butanoic acids described in this study, which possess specific brain-related biological activity but are devoid of diuretic activity can be designed and synthesized. Since good correlation between in vitro, in vivo, and clinical results was observed with ethacrynic acid, it is reasonable to expect these nondiuretic analogues to be clinically effective in head-injured patients.

Experimental Section

Biology. Cat Cerebrocortical Tissue Slice Assay. Adult cats of 2–3 kg body weight were employed in tissue slice studies. Prior to sacrifice by cervical crush and brain sampling,[68] the animals were anesthetized with ketamine hydrochloride (Ketaset), 10 mg/kg im. Eight (three control, five experimental) pial surface cerebrocortical tissue slices (0.5-mm thick; approximately 150 mg initial fresh weight) were cut successively with a calibrated Stadie-Riggs fresh tissue microtome without moistening and weighed successively on a torsion balance. During the slice preparation all operations except weighing were confined to a humid chamber. Each slice was rapidly placed in an individual Warburg flask containing 2 mL of incubation medium at room temperature. The basic composition of the incubation media, in millimoles per liter, was as follows: glucose, 10; $CaCl_2$, 1.3; $MgSO_4$, 1.2; KH_2SO_4, 1.2; Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, titrated with NaOH to pH 7.4), 20. Except when adding HCO_3^-, the osmolarity of the media was maintained isosmotic (approximetly 285 mOsm/L) by reciprocal changes of Na^+ or K^+ to achieve a concentration of K^+ of 27 mM. The basic medium was bubbled for 30 min with 100% O_2 before use. When added, $NaHCO_3$ or triethylammonium bicarbonate (TEAB) was initially present in the sidearm of each flask at an initial concentration of 50 mM in 0.5 mL of complete medium. Nonbicarbonate control slices were incubated at 37 °C in 2.5 mL of basic medium for 60 min. Bicarbonate control slices were simiarly incubated for an initial 20 min at 37 °C in 2.0 mL of basic medium to which was added from the sidearm an additional 0.5 mL of incubation medium containing 50 mM HCO_3^-, which, after mixing, resulted in a HCO_3^- concentration of 10 mM and a total volume of 2.5 mL. The incubation continued for an additional 40 min. The various compounds tested (Table I) were dissolved by forming the sodium salts by treatment with a molar equivalent of $NaHCO_3$ and diluting to the appropriate concentrations. Just prior to incubation, all flasks containing HCO_3^- were gassed for 5 min with 2.5% CO_2/97.5% O_2 instead of 100% O_2.

Following the 60-min incubation period, tissue slices were separated from incubation medium by filtration, reweighed, and homogenized in 1 N $HClO_4$ (10% w/v) for electrolyte analysis. The tissue content of ion is expressed in micromoles per gram initial preswelling fresh weight. Control slice swelling is expressed as microliters per gram initial preswelling fresh weight. The effectiveness of an inhibitor at a given concentration was measured by the amount of HCO_3^--stimulated swelling that occurred in its presence, computed as a percent of the maximum possible (legend, Table II). Tissue and media Na^+ and K^+ were determined by emission flame photometry with Li^+ internal standard; Cl^- was determined by amperometric titration. Tissue viability during incubation was monitored by manometry. A full discussion of methods is to be found elsewhere.[69]

In Vitro Cultured Rat Astrocyte Assay. Primary astrocyte cultures were started from the dissociated cerebral hemispheres of 1–3 day old rats and grown on 50-mm diameter petri dishes as previously described.[70,71] We have shown by immunocytochemical staining for the astroglial-specific marker glial fibrillary acidic protein that the proportion of cells in these cultures which are astrocytes is around 80%.[71] Uptake of $^{36}Cl^-$ was measured as previously described.[48] Essentially, the growth medium was replaced with 2 mL of Hepes-buffered medium at pH 7.4 containing 10 mM $NaHCO_3$ of the following composition (mM): NaCl, 122; KCl, 4.5; $NaHCO_3$, 10; Hepes (titrated to pH 7.4 with NaOH), 20; $CaCl_2$, 1.3; KH_2PO_4, 1.2; $MgSO_4$, 0.4; glucose, 10; sucrose, 60. It was then equilibrated for 30 min at 37 °C in a 2.5% CO_2/97.5% air atmosphere, and 3 μCi of $^{36}Cl^-$ was then added to each dish. After varying periods of time, the dishes were rapidly washed seven times within 20 s with 3 mL of ice-cold sucrose solution using a repipet. The cells were then scraped into water and dispersed by sonication, and portions were used for determining $^{36}Cl^-$ radioactivity and total protein content of the cultures.

In Vivo Cat Cerebrocortical Superfusion Assay. For details not already described, see ref 36.

In Vivo Cat Experimental Head Injury Assay. Healthy, conditioned adult cats of 2.5–4 kg of body weight were anesthetized with intravenous methohexital (13.25 mg/kg) followed by a supplemental dose of 2.75 mg/kg, if required, 25 min later. Transoral tracheal intubation was carried out, a femoral artery and vein were cannulated to permit blood sampling, fluid and inhibitor administration, and arterial blood pressure monitoring.

(68) Tower, D. B. *J. Neurochem.* **1958**, *3*, 185.

(69) Bourke, R. S.; Tower, D. B. *J. Neurochem.* **1966**, *13*, 1071.

(70) Kimelberg, H. K.; Narumi, S.; Bourke, R. S. *Brain Res.* **1978**, *153*, 55.

(71) Stieg, P. E.; Kimelberg, H. K.; Mazurkiewicz, J. E.; Banker, G. A. *Brain Res.* **1980**, *199*, 493.

A transurethral bladder catheter was placed to facilitate urine collection for determination of urine volume and electrolyte composition. Intracranial subarachnoid pressure was monitored. Electroencephalographic (EEG) activity, electrocardiographic (ECG) activity, and intracranial pressure were monitored and periodically sampled by microcomputer for off-line data analysis and fast Fourier transform processing of EEG data. Head injury related cerebral death was defined as disappearance of EEG activity in the presence of sustained cardiovascular function with maintenance of arterial blood pressure following head injury. Aliquots of blood and urine were periodically taken for determination of blood gases, osmolarity, hematocrit, and electrolytes.

Machines have been developed[37] which deliver a translational or translational plus rotational insult to the skull-encased brain. Characteristically, these engines deliver 1250–1450 positive-negative oscillations per minute for 60–67 s with an average peak magnitude of 75–80 G. Strobe and accelerometer calibration is maintained. Forty minutes following machine-induced brain injury, a 60-min period of controlled hypoxemia is achieved by ventilation of the chemically paralyzed (gallemin triethiodide, 6.5 mg/kg) animal with 6% O_2/94% N_2. This results in a sustained arterial pO_2 of 23 ±1 torr. Drugs tested (Table III) in this paradigm were administered intravenously 20 min after head injury and prior to the period of hypoxemia. All surviving animals were scored using a quantifiable neurological examination[37] by a competent evaluator who was blind to the treatment or control status of the animal. The 26-point range of the neurological examination scored the following parameters: arousal, reaction to pain, walking position, pupillary reflex, corneal reflex, ear reflex, and respiratory pattern at specific intervals following head injury to the conclusion of the experiment at 24 h. All animals underwent neurological and systemic autopsy. Full details are found elsewhere.[37]

Clinical Study. The methodology details already have been presented, since they are critical for an understanding of the biological results of the study.

Chemistry. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Where analyses are indicated only by symbols of the elements, analytical results obtained are within ±0.4% of the theoretical values. The synthetic routes are disclosed in Schemes I and II. Detailed experimental procedures are given only for selected compounds, which will serve to illustrate the general synthetic methods employed. The remaining data are summarized in Table VI.

I. 2,3-Dihydro-5-methoxy-1*H*-inden-1-ones (5). The two compounds of this type which had not been previously described in the literature were prepared as follows.

6,7-Dichloro-2-cyclopentyl-2,3-dihydro-5-methoxy-2-propyl-1*H*-inden-1-one (5-12). 6,7-Dichloro-2-cyclopentyl-2,3-dihydro-5-methoxy-1*H*-inden-1-one (8.98 g, 0.03 mol)[40] was dissolved in a mixture of dry DMF (55 mL) and $C_6H_5CH_3$ (55 mL), and NaH (50% in mineral oil, 1.54 g, 0.032 mol) was added under dry nitrogen. The mixture was stirred at ambient temperature until the evolution of H_2 ceased and then cooled and treated with PrI (5.95 g, 0.035 mol). After stirring at 25 °C for 18 h, the mixture was treated with Et_2O (100 mL) and filtered. The filtrate was washed with 5% aqueous $Na_2S_2O_3$, then with H_2O, dried over anhydrous $MgSO_4$, and finally concentrated in vacuo to a yellow oil (10.6 g). The product was slurried with petroleum ether and filtered to give 5-12: yield 5.12 g (50%); mp 118–119 °C after recrystallization from acetonitrile. Anal. ($C_{18}H_{22}Cl_2O_2$) C, H.

2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-5-methoxy-1*H*-inden-1-one (5-13) was prepared by a procedure analogous to that used for 5-12, except that the PrI was replaced by an equimolar amount of BuI. The yield of 5-13 was 53%; after recrystallization from acetonitrile, the melting point was 122–123 °C. Anal. ($C_{19}H_{24}Cl_2O_2$) C, H.

II. 2,3-Dihydro-5-hydroxy-1*H*-inden-1-ones (6). The two compounds not previously described in the literature were prepared as follows.

6,7-Dichloro-2-cyclopentyl-2,3-dihydro-5-hydroxy-2-propyl-1*H*-inden-1-one (6-12). Pyridine hydrochloride (50 g) was melted by heating at approximately 195 °C, and 5-12 (5 g, 0.0147 mol) was added with stirring in an atmosphere of N_2. After heating at 195 °C for 1.5 h, the mixture was added to crushed ice (400 g), and the resulting aqueous suspension was extracted with Et_2O. After the extract was washed with H_2O and dried over $MgSO_4$, the Et_2O was removed from the organic extract by evaporation in vacuo, and 6-12 (4.52 g, 94%) was recrystallized from acetonitrile, mp 148.5–150 °C. Anal. ($C_{17}H_{20}Cl_2O_2$) C, H.

2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-5-hydroxy-1*H*-inden-1-one (6-13) was prepared by a procedure analogous to that used for 6-12, except that the 5-12 was replaced by an equimolar amount of 5-13: yield 4.69 g (92%); mp 159.5–160 °C after recrystallization from acetonitrile. Anal. $C_{18}H_{22}Cl_2O$) C, H.

III. Alkyl [(2,3-Dihydro-1-oxo-1*H*-inden-5-yl)oxy]alkanoates (7). Ethyl 2-[(2-Cyclopentyl-6,7-dichloro-2,3-dihydro-2-methyl-1-oxo-1*H*-inden-5-yl)oxy]-2,2-dimethylacetate (7-8). To a solution of 6,7-dichloro-2-cyclopentyl-2,3-dihydro-5-hydroxy-2-methyl-1*H*-inden-1-one (6.0 g, 0.02 mol)[40] and K_2CO_3 (8.3 g, 0.06 mol) in DMF (10 mL) was added ethyl 2-bromoisobutyrate (4.3 g, 0.022 mol), and the mixture was heated at 50–55 °C for 16 h. The reaction mixture was poured into water to give 7-8, which was recrystallized to constant melting point.

Specific information on this compound and the eight compounds prepared by an analogous procedure are recorded in Table VI.

IV. 2-[(2,3-Dihydro-1-oxo-1*H*-inden-5-yl)oxy]alkanoic Acids (8). 2-[(2-Cyclopentyl-6,7-dichloro-2,3-dihydro-2-methyl-1-oxo-1*H*-inden-5-yl)oxy]-2,2-dimethylacetic Acid (8-8). To a solution of AcOH (30 mL) and 6 N HCl (10 mL) was added 7-8 (3.7 g, 0.009 mol), and the mixture was refluxed for 3 h. The mixture was poured into H_2O (200 mL), and the resulting solid was recrystallized to constant melting point.

Specific information on this compound and the eight compounds prepared by an analogous procedure are recorded in Table VI.

V. Resolution of 4-[(2,3-Dihydro-1-oxo-1*H*-inden-5-yl)-oxy]butanoic Acids (8). (+)-6,7-Dichloro-2-cyclopentyl-2,3-dihydro-5-hydroxy-2-methyl-1*H*-inden-1-one (6-2A). Pyridine hydrochloride (80 g) was melted by heating at 190 °C, and 8-2A (10 g, 0.28 mol)[40] was added with stirring. After heating at 190 °C for 45 min, the mixture was poured into ice (800 g), and the 6-2A was removed by filtration: yield 7.9 g (78%); mp 211–215 °C. Anal. ($C_{15}H_{16}Cl_2O_2$) C, H.

(−)-6,7-Dichloro-2-cyclopentyl-2,3-dihydro-5-hydroxy-2-methyl-1*H*-inden-1-one (6-2B) was prepared as described for 6-2A, except that 8-2B[40] was used in place of 8-2A: yield 86%; mp 211–215 °C. Anal. ($C_{15}H_{16}Cl_2O_2$) C, H.

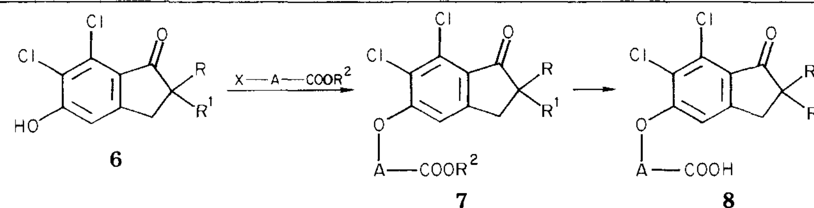
(+)-4-[(6,7-Dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1*H*-inden-5-yl)oxy]butanoic Acid–0.25-Water (8-10A). To a solution of 6-2A (4.8 g, 0.0145 mol) in DMF (50 mL) was added K_2CO_3 (4.22 g, 0.0305 mol) and ethyl 4-bromobutanoate (5.9 g, 0.0302 mol). The mixture was stirred and heated at 60 °C for 4.5 h, then cooled, and poured into ice–H_2O (500 mL). The mixture was extracted with Et_2O and dried over $MgSO_4$, and the Et_2O was removed by evaporation in vacuo. The residue, 7-10A, was dissolved in AcOH (50 mL), and 5% aqueous HCl (20 mL) was added. The mixture was heated and stirred on a steam bath for 2 h and cooled to give 8-10A: yield 3.4 g (60%); mp 75–77 °C; $[\alpha]^{25}_D$ +30° (c 1, Me_2CO). Anal. ($C_{19}H_{22}Cl_2O_4 \cdot 0.25H_2O$) C, H.

(−)-4-[(6,7-Dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1*H*-inden-5-yl)oxy]butanoic Acid–0.25-Water (8-10B) was prepared as described for 8-10A, expect that 6-2B was substituted for 6-2A: yield 59%; mp 75–77 °C; $[\alpha]^{25}_D$ −29.6° (c 1, Me_2CO). Anal. ($C_{19}H_{22}Cl_2O_4 \cdot 0.25H_2O$) C, H.

(+)-4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]acetic Acid (8-4A). A mixture of 8-4 (26.1 g, 0.065 mol) and cinchonine (19.2 g, 0.065 mol) was dissolved in hot DMF (400 mL) and cooled. The salt that separated was recrystallized 10 times from DMF to give 10.7 g of salt, which was then partitioned between H_2O and Et_2O and acidified with aqueous HCl. The Et_2O extract was washed with dilute aqueous HCl and then H_2O and dried over $MgSO_4$. Evaporation of the solvent gave 5.7 g of 8-4A: mp 173–174 °C; $[\alpha]^{24}_D$ +19.1° (c 5, EtOH). Anal. ($C_{20}H_{24}Cl_2O_4$) C, H.

(−)-4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]acetic Acid (8-4B). The initial DMF filtrate from the resolution of 8-4A was evaporated to dryness,

Table VI



no.	R	R¹	R²	A	X	source of 6	% yield	recrystn solvent	mp or bp (mm), °C	emp formula	anal.
7-4	c-C_5H_9	$(CH_2)_3CH_3$	C_2H_5	CH_2	Br	6-4	96	*d*	*d*	*d*	*d*
7-8	c-C_5H_9	CH_3	C_2H_5	$C(CH_3)_2$	Br	ref 35	51	$Et_2O-C_6H_{12}$	89-91	$C_{21}H_{26}Cl_2O_4$	C, H
7-9	c-C_5H_9	CH_3	C_2H_5	c-$C(CH_2)_3$	Br	ref 35	35		255-258 (3.5)	$C_{22}H_{26}Cl_2O_4$	C, H
7-11	c-C_5H_9	C_2H_5	C_2H_5	$(CH_2)_3$	Br	ref 35	89[d]	*d*	*d*	$C_{22}H_{28}Cl_2O_4$	*d*
7-12	c-C_5H_9	$(CH_2)_2CH_3$	C_2H_5	$(CH_2)_3$	Br	6-11	96[d]	*d*	*d*	$C_{23}H_{30}Cl_2O_4$	*d*
7-13	c-C_5H_9	$(CH_2)_3CH_3$	C_2H_5	$(CH_2)_3$	Br	6-12	67[d]	*d*	*d*	$C_{24}H_{32}Cl_2O_4$	*d*
7-14[b]	C_6H_5	CH_3	C_2H_5	$(CH_2)_3$	Br	ref 36	90[d]	*d*	*d*	$C_{22}H_{22}Cl_2O_4$	*d*
7-15[c]	CH_3	$CH_2C_6H_5$	C_2H_5	$(CH_2)_3$	Br	ref 35	88[d]	*d*	*d*	$C_{23}H_{24}Cl_2O_4$	*d*
7-16	c-C_5H_9	CH_3	CH_3	$CH_2CH=CHCH_2$	Br	ref 35	43	EtOH	89-91	$C_{20}H_{22}Cl_2O_4$	C, H
7-17	c-C_5H_9	CH_3	C_2H_5	CH_2-C_6H_4-*p*	Cl	ref 35	63	EtOH	123-126	$C_{25}H_{26}Cl_2O_4$	C, H, Cl
8-4	c-C_5H_9	$(CH_2)_3CH_3$	C_2H_5	CH_2	Br		96	$MeOH-H_2O$	175-177	$C_{20}H_{24}Cl_2O_4$	C, H
8-8	c-C_5H_9	CH_3	C_2H_5	$C(CH_3)_2$			30	$Et_2O-C_6H_{12}$	165-168	$C_{19}H_{22}Cl_2O_4$	C, H
8-9	c-C_5H_9	CH_3	C_2H_5	c-$C(CH_2)_3$			30	$Et_2O-C_6H_{12}$	223-225	$C_{20}H_{22}Cl_2O_4$	C, H
8-11	c-C_5H_9	C_2H_5	C_2H_5	$(CH_2)_3$			82	BuCl	189-193	$C_{20}H_{24}Cl_2O_4$	C, H
8-12	c-C_5H_9	$(CH_2)_2CH_3$	C_2H_5	$(CH_2)_3$			87	BuCl	140-141	$C_{21}H_{26}Cl_2O_4$	C, H
8-13	c-C_5H_9	$(CH_2)_3CH_3$	C_2H_5	$(CH_2)_3$			87	BuCl	156.5-159	$C_{22}H_{28}Cl_2O_4$	C, H
8-14[b]	C_6H_5	CH_3	C_2H_5	$(CH_2)_3$			82	BuCl	129-131	$C_{20}H_{18}Cl_2O_4$	C, H
8-15[c]	CH_3	$CH_2C_6H_5$	C_2H_5	$(CH_2)_3$			43	BuCl	148-150	$C_{21}H_{20}Cl_2O_4$	C, H
8-16	c-C_5H_9	CH_3	C_2H_5	$CH_2CH=CHCH_2$			58	$THF-Et_2O-C_6H_{12}$	209-211	$C_{19}H_{20}Cl_2O_4$	C, H
8-17	c-C_5H_9	CH_3	C_2H_5	CH_2-C_6H_4-*p*			60	Me_2CO-H_2O	235-238	$C_{23}H_{22}Cl_2O_4$	C, H

[a] Analyses data were provided by K. B. Streeter and Y. C. Lee. [b] Prepared by S. J. deSolms. [c] Prepared by W. J. Holtz. [d] This compound was isolated but not purified or analyzed before use in the hydrolysis step.

partitioned between Et_2O and H_2O, and acidified with aqueous HCl. The Et_2O extract was washed with dilute HCl and then H_2O and finally dried over $MgSO_4$. Evaporation of the Et_2O extract gave 8-4 enriched with **8-4B** (16.2 g, 0.040 mol), which was dissolved in boiling EtOH (110 mL) and treated with cinchonidine (11.9 g, 0.04 mol) dissolved in boiling EtOH (110 mL). The solid that separated was recrystallized 10 times from DMF, then partitioned between H_2O and Et_2O, and acidified with dilute HCl. The Et_2O extract was washed with dilute HCl and then with H_2O and finally dried over $MgSO_4$. Evaporation of the Et_2O extract gave **8-4B**: mp 172–173.5 °C; $[\alpha]^{24}{}_D$ −18.6° (*c* 5, EtOH). Anal. ($C_{20}H_{24}Cl_2O_4$) C, H.

(+)-2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-5-hydroxy-1*H*-inden-1-one (6-4A). Using the procedure for the preparation of **6-2A** but substituting an equimolar amount of **8-4A** for the **8-2A**, we obtained **6-4A**, which was used in the next step without purification.

(−)-2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-5-hydroxy-1*H*-inden-1-one (6-4B). Employing the procedure described for the preparation of **6-2A** but substituting an equimolar amount of **8-4B** for **8-2A**, we obtained **6-4B**, which was used in the next step without purification.

(+)-4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]butanoic Acid (8-13A). By following the procedure described for the preparation of **8-10A** but substituting an equimolar amount of **6-4A** for the **6-2A**, we obtained **8-13A**: mp 139–139.5 °C; $[\alpha]^{25}{}_D$ +18.4° (*c* 5, EtOH). Anal. ($C_{22}H_{28}Cl_2O_4$) C, H.

(−)-4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]butanoic Acid (8-13B). By following the procedure described for the preparation of **8-10A** but substituting an equimolar amount of **6-4B** for **6-2A**, we obtained **8-13B**: mp 139–139.5 °C; $[\alpha]^{25}{}_D$ −17.7° (*c* 5, EtOH). Anal. ($C_{22}H_{28}Cl_2O_4$) C, H.

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Structural Modifications of Anguidin and Antitumor Activities of Its Analogues

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Approximately 60 derivatives of anguidin were prepared for evaluation of antitumor activities. Positions 3, 4, 8–10, and 15 were modified, and the resultant derivatives were screened against P-388 leukemia. It was found that introduction of the C3-keto and C3,C8-diketo groups markedly improved the antileukemic activity, whereas epoxidation of the C9–C10 double bond or oxidation of the C15 position diminished its activity. Selected derivatives were further tested in the L1210, B16, Lewis lung, Colon 36, and Colon 38 tumor lines. Among these compounds, 4β,15-diacetoxyscirpene-3,8-dione (**54**) and 4β-(chloroacetoxy)-15-acetoxyscirpene-3,8-dione (**55**) were found to be most active in various tumors. Inhibitory action of several analogues on protein synthesis was also examined using H-HeLa cells.

Anguidin (4β,15-diacetoxyscirpen-3α-ol, **1**) is a fungal metabolite produced by *Fusarium equiseti*.[1] It belongs to the family of trichothecenes, many of which have been shown to have cytotoxic and antitumor activities.[2] Anguidin shows marked activities against P-388 and L1210 leukemias;[3] however, it is only marginally active against B16 melanoma and Lewis lung tumor. Phase I and Phase II clinical studies have been carried out with limited success.[4] Trichothecenes are inhibitors of eukaryotic protein synthesis;[5] more specifically, anguidin has been reported to inhibit the initiation of protein synthesis at low concentrations (e.g., 5 μg/mL) in HeLa cells, and at high concentrations (100 μg/mL), it behaves as an inhibitor of polypeptide chain elongation.[6]

Although extensive work on the modification of anguidin was carried out at the time of its discovery,[7,8] little has been

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