formulation of co-trimoxazole tablets[14] (BP 1973), it is predicted that no side effects would occur since only 4.75 μg of formaldehyde is contained in this formulation. The other formalized microcapsules may have a lower amount of remaining formaldehyde, although no such data are available. The method of removing the remaining formaldehyde from the formalized microcapsules also is important for microencapsulation if formaldehyde is applied as a hardening agent.

Effect of Spray-Drying Procedure on Microcapsule Surface Topography and Crystalline Drug Form—On the surface of the spray-dried microcapsules prepared from the unformalized coacervates (Fig. 3C), a smooth surface with some fissures of the coating was observed. Moreover, a few microcapsules possessed a characteristically folded and invaginated surface. This phenomenon may be deduced from a diagram (20–22) of a spray-dried slurry droplet.

X-ray diffraction patterns of the ground microcapsules were obtained to investigate the crystalline forms of sulfamethoxazole in the microcapsule prepared by conventional and spray-drying techniques. The intensities of the diffraction peaks of the spray-dried sulfamethoxazole were weaker than those of the original material. A rapid evaporation of solvent from spray droplets might change the crystalline form to the disordered form (Fig. 4). On the other hand, the intensities of the peaks found in the microcapsules dried by the conventional method coincided with those of the original sulfamethoxazole. This result may be explained in that, although the coacervation temperature was 50°, the crystalline forms could not be disordered due to the mild drying process employed. In addition, the IR spectrum of all of the microcapsules coincided with that of the original sulfamethoxazole, indicating that complexes between sulfamethoxazole and the polymers (gelatin or acacia) were not formed through spray drying and the complex coacervation processes.

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[14] One tablet of co-trimoxazole contains 400 mg of sulfamethoxazole and 80 mg of trimethoprim.

Effects of Dansylated Acetylcholine Analogs on *Schistosoma mansoni*

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Received April 18, 1979, from the **Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, TX 77550,* and the ‡*Division of Biomedical Sciences, Brown University, Providence, RI 02912.* Accepted for publication December 3, 1979.

Abstract □ A series of dansylated fluorescent analogs was synthesized and tested for cholinergic and anticholinergic activity in *Schistosoma mansoni*. The compounds were compared with a previously reported analog, 5-(dimethylamino)-*N*-(2-dimethylaminoethyl)naphthalenesulfonamide hydrochloride (I). All of the compounds produced some fluorescent labeling of a structure in the head region of the worms. Major differences were not seen among the compounds. The compounds also were tested as antagonists of carbachol-induced paralysis in an activity monitor. Significant carbachol antagonism was seen for all compounds. Effects on the serotonin response also were recorded. Only one compound, a dibutylaminopropylamine derivative, produced striking antagonism of serotonin-induced motor activity.

Keyphrases □ Cholinergics—antagonists, dansylated acetylcholine analogs, *in vitro* scanning fluorescence method, neuroactive drugs in schistosomes □ Carbachol—effect on schistosomes, effect of anticholinergic agents, scanning fluorescence method, motility studies □ Serotonin—effect on schistosomes, anticholinergic agents, scanning fluorescence method □ Structure–activity relationships—dansylated acetylcholine analogs, effect on motility and neuroactivity in schistosomes, scanning fluorescence method, motility studies

Various cholinomimetic and anticholinergic drugs affect the nervous system of schistosomes. However, some drugs, such as muscarine, nicotine, and tubocurarine, which are highly active at mammalian cholinergic synapses, are conspicuously inactive in schistosomes (1), although acetylcholine and carbachol are effective immediately. This finding suggests that sensitivity to cholinergic drugs in schistosomes is different from that in nicotinic or muscarinic mammalian synapses.

Dimethylaminonaphthalene - 5 - sulfonamidoethyltri-

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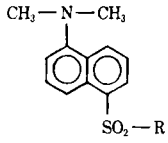


Table I—Analogs of I

Compound	R	Formula	Melting Point	Yield, %	Analysis, % Calc.	Found
II	$NH(CH_2)_2N(C_2H_5)_2$	$C_{18}H_{27}N_3SO_2 \cdot 2HCl \cdot H_2O$	140–142°	70	C 49.44 H 7.47 N 9.58	49.47 7.30 9.38
III	$NH(CH_2)_3N(CH_3)_2$	$C_{17}H_{25}N_3SO_2Cl \cdot 2HCl \cdot H_2O$	170°	70	C 47.88 H 6.86 N 9.85	48.14 7.25 9.75
IV	$NH(CH_2)_3N(C_2H_5)_2$	$C_{19}H_{29}N_3SO_2 \cdot HCl$	150°	73	C 57.08 H 7.51 N 10.51	57.03 7.46 10.17
V		$C_{17}H_{23}N_3SO_2 \cdot 0.75HCl \cdot 0.25H_2O$	200°	52	C 55.90 H 6.69 N 11.50	56.23 6.99 10.79
VI	$NH(CH_2)_3N(C_4H_9)_2$	$C_{23}H_{37}N_3SO_2 \cdot HCl \cdot 1.75H_2O$	124°	30	C 59.00 H 8.42 N —	59.03 8.47 —

methylammonium perchlorate, a dansylated choline derivative, acts as a fluorescent probe of acetylcholine receptors (2, 3). Related compounds having selective agonist or antagonist properties also have been studied (4), as has fluorescein-conjugated snake toxin (5). An analog of this compound, 5-(dimethylamino)-*N*-(2-dimethylaminoethyl)naphthalenesulfonamide hydrochloride (I), is a more effective labeling agent (6–8). For the present study, a series of dansylated fluorescent acetylcholine analogs was synthesized having systematic variations in the choline-like portions of the molecule.

A scanning fluorescence method and a technique for monitoring the movements of adult schistosomes *in vitro* (8, 9) are two approaches for assessing the effects of neuroactive drugs. With these methods, it was hypothesized (10) that hycanthone has selective anticholinergic action in schistosomes. This finding suggests that the cholinergic pharmacology of schistosomes may be important in chemotherapy, and the cholinomimetic or anticholinergic actions of structurally related compounds therefore are being studied. These structure–activity relationships are intended to suggest molecular variations that may lead to selective toxicity in schistosomes. This information will be used as a guide in the synthesis of other compounds.

EXPERIMENTAL[1]

Compound I and its analogs were synthesized by a method related to that reported previously (5, 7). 5-(Dimethylamino)-1-naphthalenesulfonyl (dansyl) chloride was refluxed with the appropriate *N,N*-dialkylethylenediamines, *N,N*-dialkylpropylenediamines, or 1-methylpiperazine in 50 ml of acetone for 1 hr. The acetone was evaporated to dryness at reduced pressure, and the resulting residue was washed with 100 ml of ether. The resulting hydrochloride was neutralized with 20 ml of 3% Na_2CO_3. Then it was repeatedly extracted with ether and dried over magnesium sulfate.

The solid was removed by filtration, and the filtrate was cooled in an ice bath and bubbled with excess hydrogen chloride gas. On cooling, it solidified slowly to yield analytically pure material. The product was highly hydroscopic and tended to form an oil on exposure to air. It was homogeneous by paper electrophoresis and had fluorescence excitation and emission spectra similar to those reported for dimethylaminonaphthalene-5-sulfonamidoethyltrimethylammonium perchlorate. The analyses and physical properties are given in Tables I and II.

Table II—Physical Properties of I Analogs

Compound	λ_{max}, nm (ϵ, 10^{-3}) pH 1	pH 11	Ethanol	R_f[a]
II	287 (12.6) 321 (3.8)	243 (18.4) 320 (6.0)	255 (19.8) 340 (4.0)	0.81
III	287 (11.6) 321 (2.6)	244 (15.8) 325 (4.4)	252 (13.0) 340 (3.8)	0.45
IV	289 (9.0) 322 (2.0)	244 (16.7) 323 (5.2)	254 (15.8) 340 (4.8)	0.31
V	292 (6.8) 321 (2.2)	249 (15.0) 330 (12.8)	254 (16.0) 345 (4.4)	0.79
VI	287 (7.3) 321 (1.3)	Low solubility	250 (10.7) 340 (3.2)	0.95

[a] TLC was run on silica gel and developed with ethanol.

Mice infested with *Schistosoma mansoni* were obtained from a mouse–snail cycle[2]. The adult, paired schistosomes were removed 45–60 days postinoculation. The infected mice were administered heparin and ether before being sacrificed by cervical dislocation. Using gentle-hook dissection, the adult, paired schistosomes were removed from the portal and mesenteric veins. The worms were maintained at 37° and pH 7.4 in cell culture medium[3] with added antibiotics and buffering (11). About 1 ml of culture medium was used per worm pair.

For scanning fluorescence measurements, the worm pairs from a mouse were divided into test and control groups and incubated at 37° for 60 min in a culture medium containing the appropriate compound. Each test group was paired with a control group of worms incubated in I. After incubation, the worms were rinsed with saline and placed under a cover slip on a microscope slide for evaluation of fluorescence as described previously (8).

The mounted schistosome was placed on the microscope[4] stage and aligned in the field of view. A small synchronous motor attached to the stage controlled the movement of the image of the worm across the light-sensitive spot of the photometer. As the microscope stage moved, the fluorescence (as a function of distance across the specimen) was recorded from the photometer output. The photometric information was entered directly into a computer for interpretation. The computer au-

[1] Melting points were determined on a Gallenkamp apparatus and are uncorrected. UV spectra were determined on a Perkin-Elmer model 402 spectrophotometer. Elemental analyses were performed by Baron Consulting Co., Orange, Conn. TLC was run on silica gel, and the plates were developed with ethanol.

[2] Obtained from Dr. S. File and Dr. J. Smith, University of Texas Medical Branch, Pathology Department, Galveston, Tex.

[3] Fischer, Grand Island Biological Co.

[4] Olympus model BMB with vertical fluorescence illumination. Emitted fluorescent light, returning through the microscope objective, was directed to the camera port at the top of the microscope. On the camera port, a Farrand MSA microscope spectrum analyzer was mounted. An Aminco potted 1P21 photomultiplier was adapted to the Farrand housing. The photomultiplier was connected to an Aminco J-22A photometer. The photometer output was directly connected to a Hewlett-Packard 9825A computer.

Table III—Specific Fluorescence and Partition Coefficients of the Compounds

Compound	Specific Fluorescence, % transmittance	Partition Coefficient (P)
I	26.0	0.0941
II	31.0	0.2374
III	40.0	0.0190
IV	46.5	0.0345
V	14.0	0.6108
VI	46.5	0.0676

tomatically recorded 250 fluorescence values for each scan, located the fluorescent peak, and determined the height and area of the scanned fluorescent region.

Schistosomes were routinely scanned twice: once across the esophageal region and once across the region of maximal brightness posterior to the esophagus (Fig. 1). Only male schistosomes were scanned due to the small size of the females. The data obtained included the peak height or maximum recorded fluorescence intensity at any point in the scanned region and the peak area or total fluorescence over the scanned region. Both parameters were used to evaluate the esophageal and central regions of the head.

The specific fluorescence of the compounds was compared by two methods. The fluorescence of a 10^{-6} M solution of each compound in pH 7.4 phosphate buffer was measured using a spectrophotofluorometer. The maximum excitation and emission wavelengths for each compound were used to determine the fluorescence. The compounds also were compared using the microfluorometer. A 2-mm thick plastic ring[5] was placed on a microscope slide, filled with drug solution, and covered with a cover slip; this procedure gave a reproducible depth of solution for observation. The specific fluorescence of each compound at 10^{-3} M in phosphate buffer (pH 7.4) was recorded and evaluated at an emission wavelength of 530 nm.

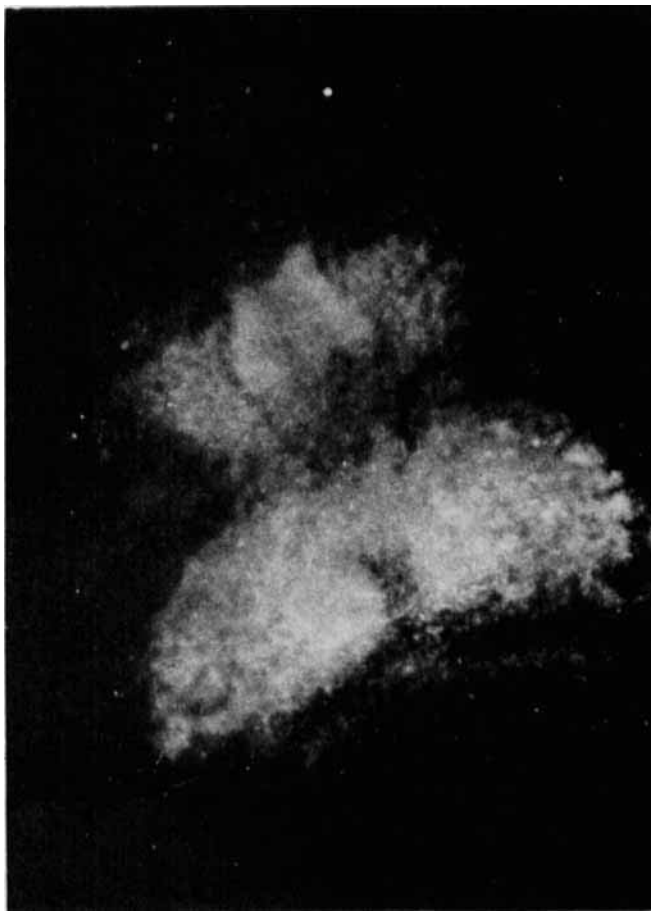


Figure 1—*Fluorescence micrograph of the head region of a male* S. mansoni *labeled with II. The worm was incubated for 1 hr in cell culture medium with* 1×10^{-5} M *II.*

[5] Delrin.

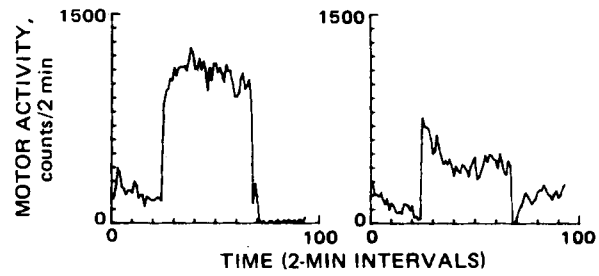


Figure 2—*Effects of IV on carbachol-induced paralysis. Serotonin (1* $\times 10^{-3}$ M*) was added after 18 intervals to produce stimulation. Carbachol (1* $\times 10^{-4}$ M*) was added later, producing paralysis in the control (left). Compound IV (1* $\times 10^{-4}$ M*) was present throughout the experiment (right).*

The motor response of paired *S. mansoni* to the compounds was measured in a specially constructed activity cage described previously (9, 11–13). The apparatus contained four chambers mounted in a temperature-regulated block. Two worm pairs were placed in each glass-bottom chamber, which was located directly above an array of fiberoptics connected to photocells. Movement of the worms in the chamber obscured a light beam, producing a change in the light intensity measured by the photocells. The resultant electronic changes registered by the photocells were translated into numerical counts that were proportional to the total amount of movement. The counts for each chamber were accumulated for 2 min, and the data were automatically transferred to a computer. The data were collected and plotted automatically by the computer.

Four experiments were run simultaneously in the presence and absence of the test compounds. The test compounds, serotonin and carbachol, in culture medium solution were perfused through the 37° chambers, and the response data were accumulated. The activity of the worm was plotted as a function of time.

The partition coefficient was determined for each compound. Equal amounts of pH 7.4 phosphate buffer and ether were shaken for 30 min and allowed to equilibrate. The equilibrated phosphate buffer was used to make a 10^{-4} M solution of each compound. The absorbance of these solutions at 350 nm was recorded. A combination of 4.5 ml of the drug solution and 0.5 ml of the buffer-saturated ether was vortexed for 3 min. The absorbance of the aqueous phase of the mixture was measured after mixing.

The partition coefficient was calculated from:

$$p = \frac{V_2(x - y)}{V_1 y} \qquad \text{(Eq. 1)}$$

where V_1 is the volume of the aqueous phase of the mixture, V_2 is the volume of ether, x is the absorbance of the compound in the buffer before partitioning, and y is the absorbance of the aqueous phase of the buffer

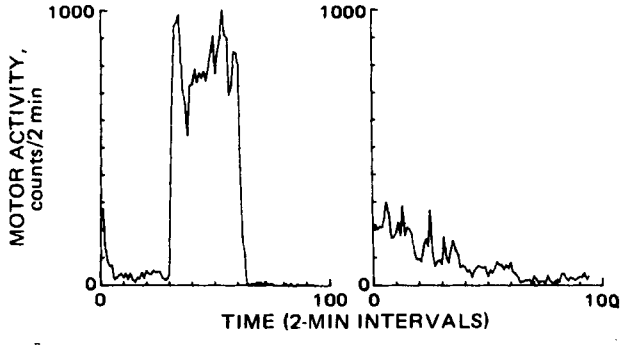


Figure 3—*Effects of VI on serotonin-induced motility. Serotonin (1* $\times 10^{-3}$ M*) was added to the basal medium at 30 intervals, and carbachol (3* $\times 10^{-4}$ M*) was added at 60 intervals. Key: left, normal response; and right, VI present throughout the experiment.*

Table IV—Relative Labeling Intensity of Schistosomes by Dansylated Choline Analogs

Compound	Region 1[a]								Region 2[a]							
	Peak Area				Peak Height				Peak Area				Peak Height			
	N_1[b]	N_2[c]	$\bar{F}$[d]	SE[e]	N_1	N_2	$\bar{F}$	SE	N_1	N_2	$\bar{F}$	SE	N_1	N_2	$\bar{F}$	SE
II	22	4	1.027	0.130	29	5	1.072	0.098	22	4	0.984	0.136	24	4	1.162	0.154
III	24	4	0.737	0.057	23	4	0.710	0.078	24	4	0.672	0.074	23	4	0.698	0.125
IV	51	9	0.772	0.051	54	9	0.730	0.038	55	7	0.727	0.059	54	5	0.901	0.098
V	27	4	1.004	0.114	27	4	0.981	0.110	23	3	1.096	0.118	21	3	0.925	0.035
VI	13	2	1.883	0.424	13	2	1.803	0.114	11	2	1.719	0.321	13	2	1.428	0.046

[a] Regions 1 and 2 represent the anterior and posterior fluorescently labeled areas in the head of the male schistosome, respectively. [b] N_1 is the total number of test worms in N_2 experiments; an equal number of control (I) worms were measured concurrently. [c] N_2 is the number of experiments, each involving about six test worms and controls. [d] $\bar{F}$ is the mean of the results for N_2 experiments. Values are normalized so that I = 1.000. [e] SE is the standard error of the mean $\bar{F}$.

Table V—Significance of Drug Effects on Carbachol Motility Response[a] for Several Drug to Carbachol Concentration Ratios

Compound	0.1 m*M*/0.1 m*M*			0.1 m*M*/0.3 m*M*			0.3 m*M*/0.3 m*M*		
	Percent Change			Percent Change			Percent Change		
	Drug, $\bar{x} \pm SD$	Control, $\bar{x} \pm SD$	p	Drug, $\bar{x} \pm SD$	Control, $\bar{x} \pm SD$	p	Drug, $\bar{x} \pm SD$	Control, $\bar{x} \pm SD$	p
I	75.6 ± 5.2	69.6 ± 22.63	NS	95.7 ± 3.69	82.13 ± 14.13	NS	76.80 ± 19.49	94.43 ± 4.40	<0.01
II	76.42 ± 13.93	78.52 ± 19.05	NS	81.90 ± 14.36	87.13 ± 13.57	NS	76.10 ± 12.01	96.13 ± 1.88	<0.01
III	51.52 ± 20.6	76.00 ± 19.60	<0.01	92.33 ± 10.51	94.25 ± 6.18	NS	91.33 ± 3.46	92.43 ± 4.38	NS
IV	52.58 ± 18.45	78.63 ± 10.77	<0.001	92.78 ± 6.73	97.32 ± 2.55	NS	46.45 ± 18.10	94.45 ± 1.99	<0.001
V	73.73 ± 13.69	87.63 ± 7.68	<0.05	93.87 ± 5.54	94.37 ± 3.11	NS	84.53 ± 11.08	95.48 ± 6.35	NS
VI	54.58 ± 15.55	87.32 ± 8.83	<0.001	57.73 ± 18.33	82.95 ± 19.13	<0.05	79.17 ± 8.39	89.30 ± 9.77	NS[b]

[a] Numbers are the mean and standard deviation of four or more experiments. Each experimental value is the percent change in average motor activity from 46 min before to 46 min after the addition of carbachol. [b] This value could not be determined since the activity of the worms had already been diminished such that a carbachol effect could not be measured.

after partitioning. The absorbance was assumed to be directly proportional to the compound concentration.

RESULTS

Specific Fluorescence—The relative fluorescences observed using the fluorescence microscope were in excellent agreement with those recorded using the spectrophotometer. However, due to the dependence of these measurements on variables such as microscope focus, the spectrophotofluorometric measurements were used for further calculations. The specific fluorescence measurements are shown in Table III.

Schistosome Fluorescence—Both test and control (I) schistosomes were from the same mouse and were measured in the same experiment. The fluorescence of the esophageal region and the region of maximal brightness posterior to the esophagus of the schistosome was measured with each fluorescent compound. The maximum fluorescence intensity (scanning peak height) and integrated total fluorescence (scanning peak area) were calculated for both regions. All fluorescence values then were corrected by the specific fluorescence of the tested compound. Each test value was compared to the corresponding mean value from a group of scanned I-labeled worms from the same host to determine a fluorescence intensity ratio. Table IV summarizes the ratios of the fluorescence intensity of the test compounds to that of I. All compounds fluorescently labeled specific regions in the schistosome in a manner similar to I (7).

The fluorescent labeling of worms by each compound relative to labeling by I was calculated according to:

$$\bar{F} = \frac{1}{N_2}\sum_{1}^{N_2}\left(\frac{1}{N_0}\sum_{1}^{N_0}\frac{F_{\text{compound}}}{\bar{F}_I}\right) \qquad \text{(Eq. 2)}$$

where N_0 is the number of test worms (*i.e.*, exposed to the compound being tested) in an experiment, F_{compound} is the fluorescence of a test worm adjusted for specific fluorescence of the compound, $\bar{F}_I$ is the mean fluorescence of all I-treated (control) worms in the same experiment adjusted for the specific fluorescence of I (usually equal in number to N_0), and N_2 is the number of experiments conducted with the same test compound.

Table IV gives the values for N_1 where $N_1 = \Sigma_1^{N_2} N_0$. The standard error of $\bar{F}$ for $n = N_2$ also is shown in Table IV.

Motility—The acetylcholine-like drug carbachol was used to evaluate the anticholinergic effect of the compounds on the schistosomes in the activity monitor (Table V). Carbachol has a paralytic effect on schistosomal movements (1). Worm pairs were exposed to a 1 m*M* solution of serotonin to stimulate the worm activity maximally (10). Carbachol at 10^{-4} or 3×10^{-4} *M* then was added, and the activity response of the schistosomes was recorded with and without the test compound.

The movement of the worms was monitored in 2-min intervals. The values for these intervals were averaged for each period (at least 36 min) of drug exposure. The percent decrease in movement due to carbachol administration was calculated from these means. This experiment was conducted in the presence and absence of the test drug on different worms from the same host. The effect of carbachol on motility with and without each test drug is shown in Table V. The difference between the carbachol effect with and without the test drug was tested for significance by the t test with n as the number of repetitions of the experiments. The p value derived from the t test also is shown in Table V.

All compounds were effective in blocking the carbachol action. Since VI had a strong paralytic action of its own, its effects on the carbachol response could not be assessed reliably at higher concentrations.

The effects of the drugs on the serotonin response could be assessed by the same technique. However, since the percent increase with serotonin was more variable (from 60 to 2000%, depending critically on the value of the motility rate before serotonin) than the decrease with carbachol (reliably about 90%), quantitation of drug effects on the serotonin response was difficult. However, it was clear that VI strongly blocked the response to serotonin. None of the other compounds had this effect.

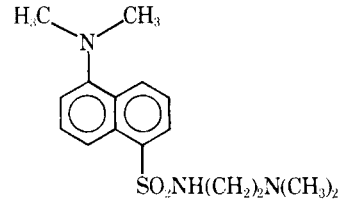
Typical experiments are shown in Figs. 2 and 3. The reduction of the carbachol response by IV and the inhibition of the serotonin response by VI can be seen clearly.

Partition Coefficients—Table III shows the partition coefficients for each drug solution. Compound V demonstrated the highest lipid solubility. The lipid solubility was not related in any obvious way to the structure of the drugs.

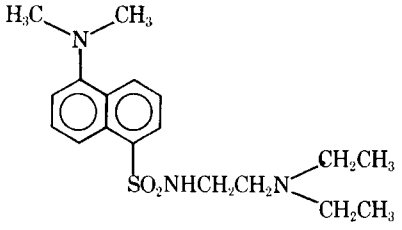
DISCUSSION

Previous studies showed that a dansylated choline analog (I) fluorescently labels schistosomes in regions containing nerve endings and behaves as an antagonist at acetylcholine receptors. In view of the evidence that some antischistosomal drugs (*e.g.*, hycanthone and metrifonate) may act by affecting the cholinergic nervous system, this study was conducted to determine the structural requirements for anticholinergic activity in schistosomes. This problem was approached by the synthesis of structurally related analogs of I, with testing of the compounds in two independent schistosomal experimental systems.

The fluorescent labeling experiments were conducted on the premise that I and its analogs label the physiological acetylcholine binding sites. This hypothesis is supported by prior observations that dansylated compounds not resembling acetylcholine, such as dansyl glycine, do not label specific structures in schistosomes and that anticholinergic drugs



I



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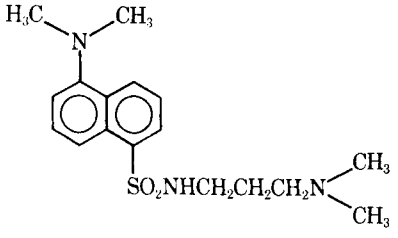

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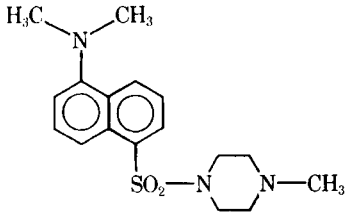
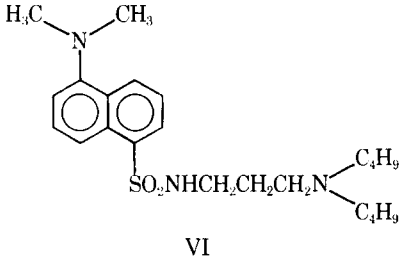


V



VI

such as atropine and mecamylamine block labeling of schistosomes by I. The results (Table IV) indicate that there were some differences among the compounds. Analogs with two-carbon chains (I and V, in which the chain is part of a piperazine ring) gave more effective labeling; the three-carbon analogs (III and IV) were less effective as labels.

Addition of larger groups to the terminal nitrogen produced some increase in labeling effectiveness. Compound VI shows that large *N*-substitutions result in increased labeling, even with a three-carbon aliphatic chain. The variations do not correlate in an obvious way with lipid solubility, as shown in Table III. It is suggested that the labeling site preferentially binds molecules with an aliphatic two-carbon side chain and may have a hydrophobic region near an anionic site; more extensive structure–activity studies are needed to characterize this site.

The motility (activity cage) studies provide an independent approach to measuring the neuroactivity of drugs by allowing observation of the antagonism of acetylcholine or serotonin responses. The ability of I to block carbachol-induced paralysis is reported in Table V. All compounds produced a significant block of the carbachol response under at least one set of conditions, indicating that the compounds do have anticholinergic activity. Compound IV had the most clearcut effect. Because IV was one of the less effective fluorescent labels, some of the sites involved in the control of body movements probably are not labeling sites. Only VI effectively blocked the serotonin response. This drug had strong paralytic effects of its own, which made it impossible to determine its ability to block carbachol paralysis.

The structural series reported here is too small to provide a full description of activity requirements in the schistosome system, particularly since several compounds had very similar activity. However, the results with VI suggest that the addition of bulkier groups on the amine nitrogen may be a useful modification. In future studies, similar compounds, with or without the fluorescent dansyl group, will be tested *in vivo* for toxicity to schistosomes within the host animal.

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