Table V—Power Analysis Table[a]

Parameter	N for 20% Difference		Minimum Detectable Difference, %	
	Group I	Group II	Group I	Group II
1 hr	25	70	44.0	77.3
2 hr	18	50	37.0	60.9
3 hr	10	40	26.8	54.4
4 hr	8	22	23.7	40.8
6 hr	13	20	29.9	38.3
8 hr	22	28	36.3	46.7
10 hr	6	28	19.7	46.3
24 hr	16	24	34.2	42.8
32 hr	45	35	51.9	52.5
Peak level	11	17	27.0	35.5
Time of peak	>150	80	104.7	80.4
$AUC_{0-32\ hr}$	5	14	16.5	32.6
$AUC_{0-\infty}$	6	13	18.3	31.2

[a] Calculated for $\alpha = 0.05$ and $\beta = 0.2$.

products to each subject indicated mean half-lives ranging from 8.9 (*SD* 1.8) to 10.8 (*SD* 1.2) hr for Group I subjects and from 9.7 (*SD* 2.5) to 14.1 (*SD* 4.1) hr for Group II subjects. These data are in agreement with a previous study in which the half-life of meprobamate ranged from 6.4 to 16.6 hr with a mean of 11.3 hr (11). No trends were apparent in half-lives determined for each subject over the 6-week study period, indicating that the weekly administration of the drug had no progressive influence on its metabolism in a given subject.

Statistically significant differences ($p < 0.05$) were also found among study weeks in Group I for the 4-, 10-, and 24-hr sampling times as well as for the *AUC* values. The cause of these differences was not known, but they did not appear to result from any cumulative effect of repeated drug administration. For example, the $(AUC)_{0-32\ hr}$ calculated by week for Group I ranged from 92 to 117 (μg/ml) (hr), with the ranking of the weeks, from lowest *AUC* to highest, being Week 2, 6, 4, 5, 1, and 3.

Data Variability and Power Analysis—To maintain the study design within manageable limits, two groups of six subjects were employed. With the exception of the *AUC* and the drug level at 10 hr for Group I, more than six subjects would have been required to permit a difference of 20% or less to be statistically significant ($p < 0.05$) (Table V). A 20% difference in parameters was selected as the maximum difference allowable and still have the products be considered bioequivalent. For most parameters evaluated, the difference between the reference product and the other products was less than 15%.

There was considerable intersubject variability in the absorption and/or disposition of meprobamate, as may be seen from the coefficients of variation given in Tables II–IV. There was no clear trend to this variability among individual products. In Group I, the reference product (Product 1) was less variable than the other five products tested at several sampling times. However, in Group II, the variability of the data for Product 1 was similar to that of the other five products.

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New Drug Metabolism Inhibitor of Marine Origin

PUSHKAR N. KAUL ˣ and SHRINIVAS K. KULKARNI

Received November 4, 1977, from the *Marine Pharmacology Laboratories, College of Pharmacy, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190.* Accepted for publication January 13, 1978.

Abstract □ Dactylyne, an acetylenic dibromochloro ether, was isolated from the sea hare *Aplysia dactylomela* and characterized pharmacologically. It had no direct effect on the cardiovascular, respiratory, and central nervous systems of mice, rats, and guinea pigs. However, a 25-mg/kg ip dose of dactylyne prolonged pentobarbital sleep time in mice by more than 10 hr. This potentiation was subsequently determined to be due to the inhibition of pentobarbital metabolism by dactylyne since the elimination half-life of pentobarbital in the dactylyne-treated mice increased several folds. Dactylyne was nontoxic up to 200 mg/kg iv. The unique structure, high potency, and relatively nontoxic nature of dactylyne make it an interesting pharmacological substance of marine origin.

Keyphrases □ Dactylyne—isolated from *Aplysia dactylomela* sea hare, pharmacological activity in mice, rats, and guinea pigs, effect on drug metabolism in mice □ *Aplysia dactylomela*—sea hare, dactylyne isolated, pharmacological activity in mice, rats, and guinea pigs, effect on drug metabolism in mice □ Metabolism, drug—effect of dactylyne isolated from *Aplysia dactylomela* sea hare

Exploration of the sea as a source of potential drugs is relatively recent. Over the past several years, cardioactive substances (1, 2), anticancer agents (3), and toxins (4) have been isolated from marine invertebrates. Moreover, the extracts of some marine organisms possess potent central nervous system (CNS) depressant activity in laboratory animals (2).

The followup study of bioassay-guided isolation and

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Table I—General Pharmacological Activities of Dactylyne

Treatment	Behavior (Mice)	Ptosis (Mice/Rats)	Hypnosis[a] (Mice)	Spontaneous Motor Activity[b] (Mice)	Motor Activity[c] (Mice)	Temperature (Rats)	Cardiovascular[d]		Intravenous Lethality
							Heart	Blood Pressure	
Saline	Normal, $n = 6$	Absent, $n = 5$	1 ± 0.16 hr, $n = 10$	82 ± 22.5, $n = 6$	303 ± 45, $n = 10$	35.9 ± 0.3°, $n = 5$	None	None	—
Dactylyne (25 mg/kg)	Normal, $n = 6$	Absent, $n = 15$	>10 hr, $n = 20$	88 ± 22.7, $n = 6$ ($p > 0.05$)	274 ± 18, $n = 8$ ($p > 0.05$)	36.1 ± 0.2°, $n = 5$	None	None	>200 mg/kg[e]
Chlorpromazine (3 mg/kg)	Sedated, $n = 6$	—	3 ± 0.66 hr, $n = 10$	40 ± 5.2, $n = 6$	141 ± 38, $n = 6$	33.75 ± 0.3°, $n = 5$	—	—	—

[a] Pentobarbital, 60 mg/kg, was used. [b] In movements per hour per group of three mice. [c] Locomotor activity as mean score per mouse per 10 min. [d] Cardiac activity of dactylyne (10 and 100 μg and 1 mg) was tested on the isolated perfusing guinea pig heart. The effect of dactylyne (up to 1 mg/kg) on mean blood pressure was determined in anesthetized rats and dogs. [e] Doses above 50 mg/kg induced clonic convulsions of short duration on intravenous dactylyne administration. All animals recovered.

purification of extracts from one marine animal, *Aplysia dactylomela*, known commonly as the sea hare, led to the isolation of dactylyne ($C_{15}H_{19}Br_2ClO$) (1), an acetylenic dibromochloro cyclic ether (5). This paper describes the pharmacological characterization and the mechanism of action of this marine-derived novel substance.

EXPERIMENTAL

Although dactylyne was isolated and structurally characterized earlier (6), the compound used in these studies was obtained by an improved method employing chromatography[1] as described elsewhere (5).

Pharmacological Procedures—Male albino Sprague–Dawley rats, 150–200 g, and Swiss–Webster mice, 25–30 g, were maintained on a 12-hr light and dark cycle and given food and water *ad libitum*. Random principle was used in the selection of animals from among acceptable groups as well as in the drug treatments. The Student *t* test was used to test for significance. Each experimental observation was reconfirmed by repeat experiments.

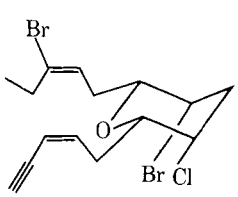
Drug solutions were prepared fresh in saline or distilled water. Dactylyne was dissolved in a few drops of dimethyl sulfoxide and diluted to volume with distilled water. The injection volumes of 1 (mouse) and 0.5 (rat) ml/100 g were kept constant. All drugs were administered intraperitoneally unless indicated otherwise.

Gross Behavior—The gross behavioral scoring method of Irwin (7) was used. Groups of six mice were injected with 25- or 50-mg/kg doses of dactylyne. The changes in the gross behavior produced by dactylyne were compared with those produced by chlorpromazine (3 mg/kg), which served as a standard.

Spontaneous Motor Activity—This test was performed in groups of three mice placed in 600-ml beakers, thereby restricting their locomotor activity (4). The spontaneous head movements and grooming were recorded every minute for 60 min. In this test, the control group invariably showed an activity score of 80–120/hr whereas the chlorpromazine (3 mg/kg) and amphetamine (2 mg/kg) groups showed activities of 40–60 and 140–160/hr, respectively. The activity scores due to dactylyne (25 mg/kg) were compared with these activities.

Locomotor Activity—Motor activity was studied with the help of an actophotometer (8). Groups of six to eight mice each were injected with dactylyne (25 mg/kg), saline (control), and chlorpromazine (3 mg/kg) 30 min prior to measuring their activity. Each mouse was individually placed in the actophotometer, and the activity was recorded for 10 min. Mean scores per mouse in different treatment groups were compared.

In the drug combination studies aimed at understanding the mechanism of action of dactylyne, groups of six to eight mice each received 5-mg/kg doses of either amphetamine or quipazine 30 min after dactylyne. Each mouse was tested in the actophotometer as described.



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Palpebral Response (Ptosis Induction)—The intensity of ptosis was calculated according to the method of Rubin *et al.* (9); the relative degree of ptosis is given scores ranging from 0 to 4. Groups of three to five mice each were given dactylyne (10, 25, or 50 mg/kg ip), saline (control), and reserpine (1 mg/kg ip). The activity was evaluated by summing the scores of each group and expressing these values as percent of reserpine (standard) activity.

Body Temperature Determination—The variation of rectal temperature was recorded by a telethermometer. Three to five rats were used per treatment. The temperatures were recorded every 30 min for 2 hr to note changes. Dactylyne was tested at 25- and 50-mg/kg dose levels.

Anticonvulsant Activity—Groups of five mice each were treated with 25-, 50-, and 100-mg/kg ip doses of dactylyne. Thirty minutes later, pentylenetetrazol (100 mg/kg) was administered. The onset, duration, severity, and death due to convulsions were recorded over 24 hr (10). A group of mice treated with phenobarbital (25 mg/kg) was used as a standard for comparison.

Pentobarbital Hypnosis—The prolongation of pentobarbital-induced hypnosis was carried out in mice (10). Groups of eight to 10 animals each, pretreated with either dactylyne or chlorpromazine (standard), received pentobarbital 30 min later. The onset and duration of sleep due to pentobarbital were noted. Duration of sleep in each animal was recorded as the time elapsing from the loss of the righting reflex to its recovery.

The pentobarbital-induced sleep time experiments were carried out in two sets. In the first set, a fixed dose of dactylyne (25 mg/kg) was followd 30 min later by varying doses of pentobarbital (20, 40, or 60 mg/kg).

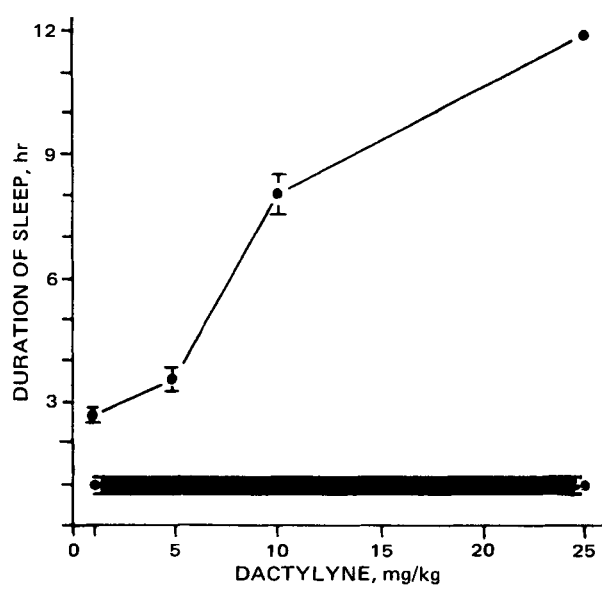

Figure 1—*Effect of increasing doses of dactylyne on pentobarbital-(60 mg/kg) induced hypnosis. Each point is the mean ± SD of eight to 10 animals. The effect was significant ($p < 0.001$) at all dose levels of dactylyne. Key:* ■, *pentobarbital alone; and* —, *pentobarbital plus dactylyne.*

[1] Sephadex LH_{20} columns.

Table II—Effect of Dactylyne on Pentylenetetrazol- (100 mg/kg) Induced Convulsions in Mice

Treatment (*n*)	Dose, mg/kg ip	Convulsions		Death	
		Onset, sec	Severity	*n*	Minutes
Saline (10)	—	48 ± 6.8	+++	10/10	2
Dactylyne (5)	50	49 ± 3.0	+++	5/5	1.5
Dactylyne (5)	100	48 ± 9.3	++	5/5	2.5
Phenobarbital (10)	25	127 ± 57.3	+	1/10	Recovered

In the second set, the dose of dactylyne (1, 5, 10, or 25 mg/kg) was varied and a fixed dose of pentobarbital (60 mg/kg) was injected 30 min later.

Determination of Blood Pentobarbital Levels—Pentobarbital was determined by the modified fluorometric method of Hollister *et al.* (11). To a 0.25–0.5-ml blood sample in a 13-ml screw-capped centrifuge tube was added an equal volume of distilled water. After the pH was adjusted to 5.4 ± 0.2 with 0.25 ml of phosphate buffer, pH 5.3 (27.0 g of monobasic sodium phosphate monohydrate and 1 g of dibasic sodium phosphate/liter), 5 ml of hexane containing 1.5% of isoamyl alcohol was added. The tube was capped and shaken for 10 min at 150 cpm. It was centrifuged for 10 min at 2200 rpm, and a 4-ml aliquot of organic layer then was transferred to another tube containing 3 ml of 0.5 *N* NaOH and shaken for 10 min at 150 cpm. After centrifugation, the fluorescence in the aqueous layer was determined spectrophotofluorometrically at an excitation λ_{max} of 262 nm and an emission λ_{max} of 420 nm.

A standard curve was derived by adding known concentrations (1–5 μg/ml) of pentobarbital sodium to fresh blood samples and processing as described. A blood blank and at least two known concentrations of pentobarbital sodium were carried through the procedure each time. All samples were assayed in replicate sets of two or three.

Disappearance of pentobarbital from the blood was studied in the saline- and dactylyne- (10 mg/kg) treated groups of mice, each receiving 60 mg of pentobarbital sodium/kg ip 30 min after treatment. The animals were sacrificed, and carotid blood samples were collected in heparinized tubes separately from each animal at 5 min and 1, 3, 6, and 24 hr after pentobarbital injection. These collection times were chosen after preliminary experiments so as to reflect the blood levels at the time of sleep onset (3–5 min), at the time of the recovery of the righting reflex in the saline-treated controls (1 hr), and at later times (3, 6, and 24 hr), particularly in the dactylyne-treated mice which slept for much longer periods.

Toxicity Studies—Since the dactylyne supply was limited, only pilot toxicity studies were possible. Groups of three mice were given intravenous doses of 25, 50, 100, 120, 160, and 200 mg of dactylyne/kg, and the gross behavior as well as survival was observed for 48 hr.

Another group of 10 mice was injected with 120 mg of dactylyne/kg ip once a day for 15 days. This dose was selected on the basis that it was larger than the one used in the toxicity study of a well-known enzyme inhibitor proadifen hydrochloride[2] (II) (12). A group of 10 mice, serving as the control, was injected daily with 1 ml of saline/100 g ip. Twenty-four hours after the last dose, all mice were sacrificed. The heart, liver, kidneys, lungs, brain, intestines, stomach, adrenals, and skin were removed and fixed in formalin (10%). Macroscopic and microscopic examinations of the organs were carried out for observation of any possible lesions.

Cardiovascular Activity—With modified Langendorff preparation (4) of the isolated guinea pig heart, varying concentrations (10 and 100 μg and 1 mg) of dactylyne were injected into the perfusion fluid. The effects on the inotropy, chronotropy, and coronary flow were recorded on an amplifier–recorder system. The effect of dactylyne in doses up to 1 mg/kg on mean blood pressure was determined in anesthetized rats and dogs. The animals were anesthetized with urethan (1 g/kg ip) or pentobarbital sodium (45 mg/kg iv). The mean arterial pressure was measured by inserting a cannula into the carotid artery and connecting it to a pressure transducer. One femoral vein was cannulated for drug administration. Electrodes were pinned in the appropriate limbs to provide lead II of the ECG record. Both blood pressure and ECG were monitored continuously.

RESULTS AND DISCUSSION

When freeze dried and redissolved in saline, the aqueous alcoholic crude extract of *A. dactylomela* consistently exhibited a general CNS depressant profile; *i.e.*, it prolonged the pentobarbital sleep time, depressed the body temperature, and decreased the spontaneous and locomotor activities, although only moderately. However, when fractionation of the extract was carried out under the guidance of pentobarbital hypnosis bioassay in mice, the relatively purer fractions became devoid of any significant CNS depressant activity except the potentiation of barbiturate-induced hypnosis. It is possible that the crude extract contained some substance(s) with the CNS depressant activity; but as the purification progressed, the elimination of the substance(s) from the fractions led to a gradually increasing potentiation of pentobarbital hypnosis and, thereby, an improvement in the purity of that component as an index of isolation. The CNS depressant components in the original crude extract were not isolated.

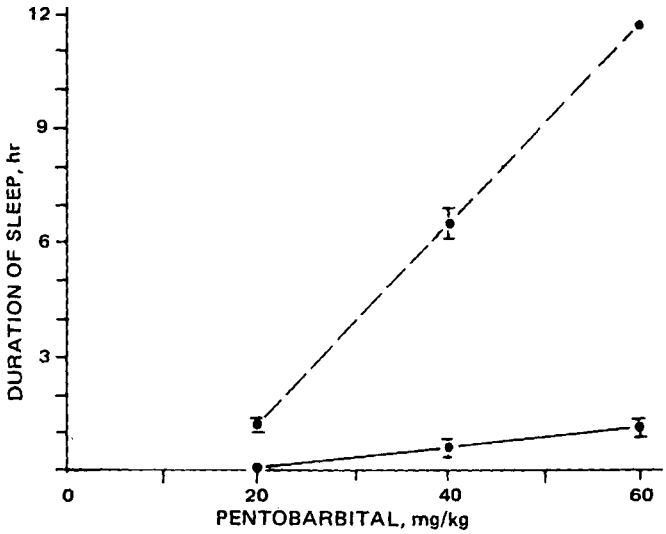

Figure 2—*Effect of a fixed dose (25 mg/kg) of dactylyne on various doses of pentobarbital. Each point is the mean ± SD of six to eight animals. The effect at the nonhypnotic dose of 20 mg of pentobarbital/kg was significant (*$p < 0.001$*). Key: —, pentobarbital alone; and – –, pentobarbital plus dactylyne.*

Dactylyne did not exhibit any significant pharmacological activity of its own in the test systems studied. In doses of 25–50 mg/kg in mice and rats, no major gross behavioral effects were visible. Furthermore, dactylyne did not significantly affect the spontaneous or locomotor activity ($p > 0.05$). Likewise, there was little effect on body temperature of mice (±0.2°) (Table I).

Dactylyne did not significantly modify the stimulant effects of amphetamine ($p > 0.05$) or quipazine ($p > 0.05$). It also failed to protect mice from pentylenetetrazol-induced convulsions (Table II). Neither the onset time nor the severity of convulsions was affected. All mice pretreated with dactylyne died, as did the controls. As expected, the phenobarbital-given mice showed both the delayed onset of convulsion and protection against death.

Dactylyne had no effect on the inotropy, chronotropy, and coronary flow in the isolated perfusing guinea pig heart. Nor did it show any change in the blood pressure or ECG, these effects being observed for 15 min following injections.

The only pronounced activity of dactylyne was a marked prolongation of pentobarbital sleep time in mice. The duration of action of pentobarbital increased with increasing doses of dactylyne (Fig. 1). A significant ($p < 0.001$) prolongation of barbiturate hypnosis was seen even at 1 mg of dactylyne/kg. A dose of 25 mg/kg induced sleep with what was otherwise a nonhypnotic dose of pentobarbital sodium (20 mg/kg) (Fig. 2).

Potentiation of drug activity by another agent is usually a result of receptor sensitization, decreased urinary excretion of the drug, or reduced metabolic inactivation (13, 14). The fact that blood pentobarbital levels in dactylyne-pretreated mice at the time of awakening (16.8 ± 3.99 μg/ml) were nearly the same as those in the control mice (15.9 ± 3.37 μg/ml) rules out the possibility of receptor sensitization. Biliary and urinary excretions together of pentobarbital are limited to 20–30% in most species studied (14). Therefore, if dactylyne should affect kidneys in some manner, reducing the urinary excretion even to the maximum possible extent, one would expect only a marginal increase in the blood pentobarbital levels at any particular time. However, the actual levels in the dactylyne-pre-

[2] SKF 525-A.

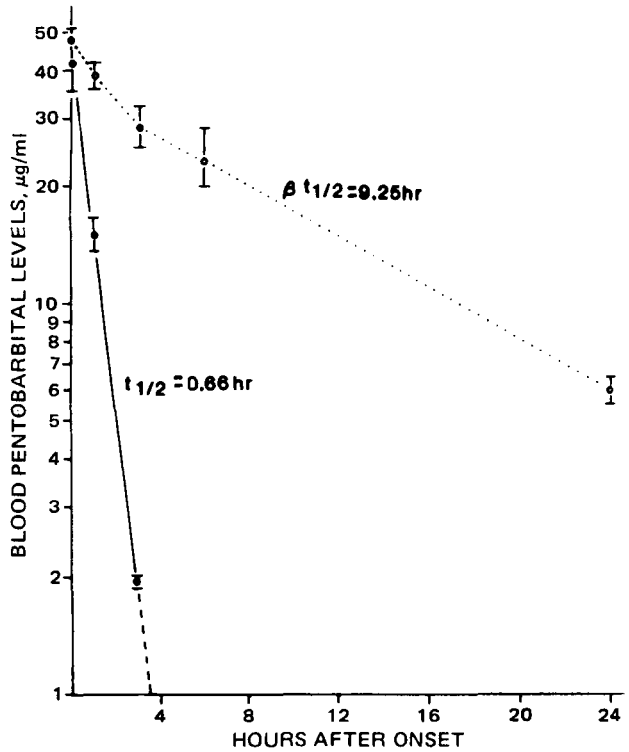

Figure 3—*Disappearance of pentobarbital from the blood of control (saline) and dactylyne- (10 mg/kg) pretreated mice. Each point is the mean ± SD of eight to 10 animals. Key: —, control; and . . . , dactylyne.*

treated mice, for example, at 1 hr following pentobarbital injection were 37.7 ± 3.70 μg/ml as against 15.9 ± 3.3 μg/ml in the saline-pretreated mice, *i.e.*, an increase of more than 100%.

It thus seems reasonable to assume that dactylyne, or perhaps a metabolite, actually inhibits the metabolic elimination of pentobarbital. Figure 3 shows the disappearance of pentobarbital in mice pretreated with dactylyne 30 min prior to pentobarbital administration. Dactylyne increased both the half-life (9.25 *versus* 0.66 hr) and the duration of action of pentobarbital several fold (from 1 to 8 hr).

The inhibition of pentobarbital metabolism by dactylyne apparently is quite analogous to a similar effect of II, except that it is more active on a milligrams per kilogram dose basis. For example, 10 mg of dactylyne/kg prolonged the pentobarbital sleep time from 1 to 8 hr whereas 15 mg of II/kg prolonged the sleep time from only 1 to 6 hr; the molecular weight of dactylyne is only 5% more than that of II. Moreover, dactylyne appeared to be less toxic than II, being nonlethal up to a dose of 200 mg/kg iv; the intravenous LD_{50} of II is 60 mg/kg (12). In the pilot toxicity studies, no gross morphological or histological lesions were found in the body and/or organs of animals treated with 120 mg of dactylyne/kg daily for 15 days.

In conclusion, a substance of marine origin with an ability to inhibit pentobarbital metabolism has been described. Data from further experimentation indicated that dactylyne also potentiated the action of other hypnotics, *e.g.*, phenobarbital and chloral hydrate. The unique structure, high potency, and relatively nontoxic nature of dactylyne make it an interesting pharmacological agent with perhaps a feasible clinical applicability.

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