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STRUCTURE–ACTIVITY RELATIONSHIP FOR BROMOINDOLE CARBALDEHYDES: EFFECTS ON THE SEA URCHIN EMBRYO CELL CYCLE

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Abstract—Natural derivatives of indole-3-carbaldehyde were isolated from the tropical marine ascidian *Stomoza murrayi*. A series of 13 derivatives, three natural and 10 synthetic (brominated and N-methylated), were examined for their effects on cell division of sea urchin eggs. These derivatives were shown to inhibit the first mitotic cycle in a concentration-dependent manner. By comparing the IC50 values with the structure of the various molecules, we were able to determine that bromination increased the cytotoxicity of the compound with a maximum occurring when bromine was added to carbon number 2, while addition of N-methylation was shown to markedly reduce the cytotoxicity of these same compounds brominated at carbon 2 only. Biological activity of this family of compounds has been characterized, via detailed study of addition of the most active derivative, 2,5,6-tribromoindole-3-carbaldehyde, on macromolecule synthesis and cytoskeleton reorganization during the first mitotic cycle of fertilized sea urchin eggs. Fluorescence localization of chromatin and microtubules revealed that 2,5,6-tribromoindole-3-carbaldehyde allowed pronuclei migration and fusion but prevented the condensation of chromatin, nuclear envelope breakdown, and bipolar mitotic spindle assembly, inducing an arrest of sea urchin embryogenesis at the beginning of mitosis. It is postulated here that this phenotype is likely to be due to a strong inhibition of DNA replication and protein synthesis.

Keywords—Bromoindole-3-carbaldehydes Cytotoxicity Sea urchin eggs *Stomoza murrayi* Structure–activity relationship

INTRODUCTION

The marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural features not found previously in terrestrial natural products [1]. Some marine natural products have undergone biological evaluation and yielded compounds with potential use as therapeutic agents or molecular probes for the study and understanding of complex cellular and biochemical processes at the molecular level [1–4].

It is well known that many marine invertebrates, especially sessile animals, possess a chemical defense mechanism against larval settlement or overgrowth by other species. These marine organisms have developed chemical defenses by producing and exuding secondary metabolites against fouling organisms. They also can display potential ecological roles because of their antimicrobial and/or feeding deterrent activities.

Marine life is also the largest source of naturally occurring organohalogen compounds [5]. Red algae [6], sponges [7], and tunicates [8] are able to metabolize such compounds. The ascidian *Stomozoa murrayi*, which we observed for the first time in the Caribbean Sea [9], yielded small amounts of 6-bromoindole-3-carbaldehyde, compound (4) (Fig. 1). This compound, together with the de-bromo analogue, has been previously described in many organisms such as bacteria [10], sponges [11], coral [12], and tunicates [13].

In the course of synthesizing this compound, we also obtained three other known mono- and dibrominated compounds, (2), (3), and (5), and a new tribrominated derivative (6) (Fig. 1). This compound (6) is closely related to 2,5,6-tribromo-1-methylgramine, a potent inhibitor of larval settlement isolated from the bryozoan *Zoobotryon pellucidum.* So, because of the biological interest in brominated compounds of the *N*-methylindole series [14,15] and their antifouling activity, we synthetized the corresponding bromo-*N*-methylindolecarbaldehyde derivatives (7) to (13).

We investigated the effects of this series of 13 bromoindolecarbaldehydes on sea urchin eggs (*Paracentrotus lividus*), which may easily be obtained in the state of synchronous division. This model is rapid and of low cost and provides information on disruption of cell proliferation. Moreover, the sea urchin egg, being a marine model, allows investigation on the disturbance of the reproduction of marine organisms by marine toxins [16].

MATERIALS AND METHODS

Chemical instrumentation

High-pressure liquid chromatography (HPLC) was performed using Jasco 880-PU pumps (Jasco, Tokyo, Japan), 7125 Rheodyne® injectors (Rheodyne L.P., Rohnert Park, CA, USA), and a Waters 996 photodiode array detector (Waters, Milford, MA, USA) for purification and comparative retention times determination. Nuclear magnetic resonance (NMR) spectra were recorded in dimethyl sulfoxide (DMSO)-d_6 on a Jeol EX 400 spectrometer (Joel, Toyko, Japan). All spectra were obtained with a NM-40TH5 dual ^{1}H, ^{13}C probe operating at 400 MHz for proton and 100.53 MHz for carbon-13 at 293 K.

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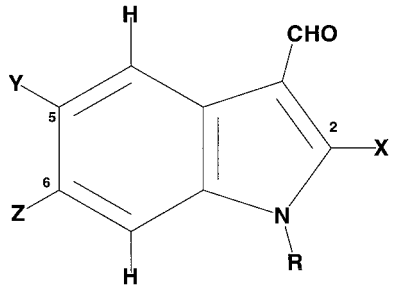

Number	R	X	Y	Z	Compounds
1	H	H	H	H	Indole-3-carbaldehyde
2	H	Br	H	H	2-bromoindole-3-carbaldehyde
3	H	H	Br	H	5-bromoindole-3-carbaldehyde
4	H	H	H	Br	6-bromoindole-3-carbaldehyde
5	H	H	Br	Br	5,6-dibromoindole-3-carbaldehyde
6	H	Br	Br	Br	2,5,6-tribromoindole-3-carbaldehyde
7	CH_3	H	H	H	N-methylindole-3-carbaldehyde
8	CH_3	Br	H	H	N-methyl-2-bromoindole-3-carbaldehyde
9	CH_3	H	Br	H	N-methyl-5-bromoindole-3-carbaldehyde
10	CH_3	H	H	Br	N-methyl-6-bromoindole-3-carbaldehyde
11	CH_3	Br	H	Br	N-methyl-2,6-dibromoindole-3-carbaldehyde
12	CH_3	H	Br	Br	N-methyl-5,6-dibromoindole-3-carbaldehyde
13	CH_3	Br	Br	Br	N-methyl-2,5,6-tribromoindole-3-carbaldehyde

Fig. 1. Chemical structure of compounds (1) to (13).

The ^1H and ^{13}C NMR chemical shifts are referenced to solvent peaks: δ_H 2.49 ppm (residual DMSO-d_5H), δ_C 39.5 ppm (DMSO-d_6). Electron impact mass spectra were performed at 70 eV on a ATI Unicam Automass spectrometer (Thermo Quest, San Jose, CA, USA).

Preparation of brominated compounds

Most of the brominated derivatives were prepared by treating the starting commercial compounds (Sigma-Aldrich Chimie S.a.r.l., France) with various amounts of bromine in CCl_4 at 70°C during 24 h. After cooling and alkaline hydrolysis (1 M NaOH), the organic material was extracted with ethyl acetate (three times), and the combined extracts were dried (Na_2SO_4). Evaporation of the solvent in vacuo led to the crude mixture that was chromatographied on a silica gel column (pentane/ethyle acetate). The fractions containing bromoindoles were then purified by HPLC (UB 250 × 10, RP-8, 5 μ, Shandon), using H_2O-acetonitrile-trifluoroacetic acid (1:1: 0.01) as eluent.

Monobrominated derivatives in the carbon-2 position were prepared from commercially available 2-oxindole compounds (Sigma-Aldrich) according to the reaction conditions of Vilsmeier-Haack [17]. N-alkylation of (2) using ICH_3 (1.5 equivalent) in a mixture of water and $CHCl_3$, potassium hydroxide, and t-BuN^+Br^- as phase transfer catalyst led to (8).

Compounds (1) to (13) were diluted in DMSO. Dimethyl sulfoxide was included in the control in all experiments; its concentration never exceeded 0.02% of the total volume of experimental medium.

Biological material

The Mediterranean sea urchin *Paracentrotus lividus*, collected in the bay of Villefranche-sur-Mer, France, provided the eggs and sperm cells. After dissection of the gonads, eggs were placed in filtered (0.22 μm) fresh seawater and dejellied by four successive passages through a 90-μm mesh nylon filter. Sperm was collected dry and kept at 4°C. Shortly before use, it was diluted 1:50 in seawater, and 20 μl of this suspension were added per milliliter of egg suspension [18]. Experiments were carried out at room temperature with a 4% (v/v) egg suspension containing 20,000 eggs/ml in seawater stirred with a three-blade propeller.

Determination of sea urchin egg division rate

The extent of fertilization was judged by the proportion of elevated fertilization envelopes as seen under a light microscope. The rate of division was determined as described by Biyiti et al. [18]. Toxins were added 30 s postfertilization to an egg suspension adjusted to 20,000 eggs/ml. When the control eggs were undergoing the first cleavage and then 5, 10, 15, 25, and 35 min later, the number of cleaving embryos (treated or controls) was determined under a light microscope, and the results were expressed as a percentage of cleaving treated eggs relative to controls. The half inhibiting concentration (IC50) values (expressed in μM) were recorded for treated eggs at the time at which 90 to 95% of control eggs reached the two-cell stage.

The period during which the embryos are sensitive to the presence of 2,5,6-tribromoindole-3-carbaldehyde (6) was determined by adding, 30 s to 60 min postfertilization, to aliquots of sea urchin embryos (2 μM, a concentration earlier shown to cause complete inhibition of cleavage) and counting the percentage of cleavage inhibition during the first cell cycle.

Cumulative incorporation of ^{35}S-methionine into proteins and ^3H-thymidine into DNA

The rate of DNA or protein synthesis was measured according to the procedure previously described by Pesando et al. [19]. After 1 h incubation of a 5% (v/v) suspension of unfertilized eggs with ^3H-thymidine or ^{35}S-methionine, the eggs were washed, then fertilized in the presence or absence of 2 μM (6). Samples (500 μl) were taken when the controls completed their first and second divisions and treated for radioactivity counting and protein content measurement [20].

Cytochemistry

For cytolocalization of chromatin, microtubules, and filamentous actin, aliquots of sea urchin egg cultures were taken at different times after fertilization, decanted, and rinsed in artificial seawater deprived of Ca^{2+} (484 mM NaCl, 10 mM KCl, 27 mM $MgCl_2$, 29 mM $MgSO_4$, 2.4 mM $NaHCO_3$, pH 8.2). The eggs were fixed and permeabilized, for 30 min at room temperature, by dilution in a fixation medium composed of 2% formaldehyde, 20 mM Pipes (piperazine *N*,*N*′-bis[2-ethane-sulfonic acid]; piperazine 1,4-diethane sulfonic acid), 5 mM EGTA, 0.5 mM $MgSO_4$, and 0.1% Triton X-100 (TX 100, Rohm and Haas, Philadelphia, PA, USA), pH 6.5 [21]. The decanted eggs were then transferred to TBS (Tris Base Saline = 10 mM Tris-Cl, 140 mM NaCl, 2.7 mM KCl, pH 7.4) containing 0.1% TX100 and were stored at 4°C or used immediately as described in the following. A sample of 25 μl of egg suspension in TBS was layered on each polylysine-coated coverslip and rinsed in TBS 0.1% TX 100. Each spec-

Table 1. Experimental data of bromination

Reactants		Products	Retention time (min)	Yield (%)
Starting compounds[a]				
(1)	1.5 eq Br_2 10% sol[a] in CCl_4	(3)	7.1	13
(1)	1.5 eq Br_2 10% sol[a] in CCl_4	(4)	7.3	9
(1)	1.5 eq Br_2 10% sol[a] in CCl_4	(5)	11.6	3
(1)	10 eq Br_2 10% sol[a] in CCl_4	(6)	13.8	25
2-Oxindole	$POBr_3$/DMF[b] in $CHCl_3$	(2)	6.3	74
(7)	1.5 eq Br_2 10% sol[a] in CCl_4	(9)	9.9	17
(7)	1.5 eq Br_2 10% sol[a] in CCl_4	(10)	10.4	12
(7)	1.5 eq Br_2 10% sol[a] in CCl_4	(11)	15.1	6
(7)	1.5 eq Br_2 10% sol[a] in CCl_4	(12)	15.5	<5
(7)	10 eq Br_2 10% sol[a] in CCl_4	(13)	25.0	33
(2)	ICH_3/KOH, t-BuN^+Br^- in H_2O: $CHCl_3$	(8)	8.8	61

[a] Compound numbers refer to those of figure 1.
[b] DMF = *N*,*N*-dimethylformamide.

imen was then incubated with 10 μl of primary antibody (described in the following) for 30 min at room temperature, rinsed in TBS 0.1% TX100, and incubated with 10 μl of the appropriate secondary antibody for 30 min at room temperature [21]. After rinsing, the specimens were stained for chromatin by 5 μg/ml Hoechst 33258 [22] in the same buffer for 2 min and mounted in 90% glycerol containing 20 mM Tris-HCl, pH 8, and 0.1% *p*-phenylenediamine as an antibleach.

Antibodies were diluted in TBS, 0.1% TX-100, 1% normal donkey serum (Jackson). YL1/2, a rat monoclonal antitubulin [23] (Sera-Lab, Salisbury, UK), was diluted at 1:1,000, and donkey anti-rat–purified immunoglobulins (Jackson) labeled with fluorescein or Texas Red (Jackson) were diluted at 1:250.

The observations were performed on a Zeiss Axiophot (Zeiss, Petersburg, VA, USA) using a 40× or 63× Plan-Neofluar objective. Pictures were taken on Kodak T MAX 100 Film and processed with the Kodak T MAX developer (Eastman Kodak, Rochester, NY, USA).

RESULTS

Chemistry

The brominated indoles (Fig. 1) were prepared from compound (1) for the N-H series and from (7) for the N-Me series by standard bromination and purified by C18 RP HPLC. The reactant used and the HPLC retention time of each final product are given in Table 1. By addition of 1.5 equivalents of bromine in the N-H series, we obtained the 5-bromoindole-3-carbaldehyde (3) as the major compound together with the 6-bromo (4) and 5,6-dibromoindole-3-carbaldehyde (5). In the N-Me series, the 5-bromo (9), 6-bromo (10), 2,6-dibromo (11), and 5,6-dibromo derivatives (12) were isolated from the reactional mixture also with 1.5 equivalents of bromine. The 2,5,6-tribromo derivatives (6) and (13) were obtained by addition of a large excess of bromine (10 equivalents). In order to optimize the formation of the dibromo derivatives and especially to obtain the 2,6-dibromoindole-3-carbaldehyde not detected in any of the two reactions with 1.5 and 10 equivalents of bromine, the bromation was attempted with 2.5 equivalents of bromine. The 5,6-dibromo (5) and the 2,5,6-tribromo (6) derivatives were formed (Table 1), but not the desired 2,6-dibromoindole-3-carbaldehyde, even in trace amount. The 2-bromoindole-3-carbaldehyde (2) was obtained from 2-oxindole by Vilsmeier reaction [17] and the 2-bromo N-Me derivative (8) by N-alkylation of (2) with ICH_3. These two 2-monobrominated derivatives were not detected in the standard brominations of indole-3-carbaldehyde.

Nine synthetic derivatives were obtained. Each compound was identified by NMR ^1H and ^{13}C spectroscopy and analysis of isotopic clusters on EI mass spectra. 1H and 13C NMR data are reported in Tables 2 and 3.

The natural compound 6-bromoindole-3-carbaldehyde (4) was isolated from different marine invertebrates, such as sponges [24], ascidian, and corals, and the 2,5,6-tribromo-*N*-methylindole-3-carbaldehyde (13) from a bryozoan [15]. The 5- and 6-monobromoindole-3-carbaldehyde were already specifically synthetized: 5-bromoindole-3-carbaldehyde (3) by formylation of 5-bromoindole [25] and 6-bromoindole-3-carbaldehyde (4) by mild bromination [10]. The 2-bromoindole-3-carbaldehyde (2) was used as a building block in the synthesis of 2-substituted indoles [26]. The 5,6-dibromoindole-3-carbaldehyde (5) was described as a by-product of the bromination of (1) [27]. Bromo-*N*-methylindolecarbaldehyde

Table 2. ^1H-Nuclear magnetic resonance data of compounds (1) to (13)[a]

	Chemical shifts (ppm)					
Proton	(1)[b]	(2)[b]	(3)[b]	(4)[b]	(5)[b]	(6)[b]
NH	12.14	13.05	12.28	12.22	12.42	13.39
C2H	8.29		8.34	8.27	8.29	
C4H	8.08	8.07	8.21	7.98	8.21	8.34
C5H	7.25	7.26		7.35		
C6H	7.20	7.22	7.39			
C7H	7.49	7.42	7.48	7.70	7.81	7.79
CHO	9.92	9.89	9.91	9.96	9.77	9.85
	(8)[b]	(9)[b]	(10)[b]	(11)[b]	(12)[c]	(13)[b]
NCH_3	3.86	3.90	3.85	3.85	3.83	3.83
C2H		8.33	8.30		7.63	
C4H	8.11	8.22	8.01	8.03	8.57	8.34
C5H	7.33		7.40	7.43		
C6H	7.27	7.47				
C7H	7.65	7.58	7.88	8.00	7.64	8.20
CHO	9.92	9.87	9.88	9.90	9.92	9.86

[a] Compound numbers refer to those of figure 1.
[b] Recorded at 400 MHz in DMSO-d_6.
[c] Recorded at 400 MHz in $CDCl_3$.

Table 3. Comparison between nuclear magnetic resonance chemical shifts and atomic charges for the amine hydrogen and the aldehyde carbon

	Amine hydrogen		Aldehyde carbon	
Compound[a]	δ NH (ppm)[b]	Atomic charge	δ C (ppm)[c]	Atomic charge
(1)	12.14	+0.356	184.90	+0.239
(2)	13.05	+0.328	184.42	+0.233
(3)	12.28	+0.355	185.11	+0.238
(4)	12.22	+0.353	185.07	+0.239
(5)	12.42	+0.353	184.84	+0.238
(6)	13.39	+0.325	184.57	+0.232

[a] Compound numbers refer to those of figure 1.
[b] δ NH (ppm) = chemical shift in ppm for the amine proton.
[c] δ C (ppm) = chemical shift in ppm for the aldehyde carbon.

derivatives (9) to (12) were mentioned in synthetic works [28] but only characterized by elemental analyses. The 2,5,6-tribromo-N-H derivative (6) is a new compound.

Compounds (1) to (13) were analyzed after energy minimization with MOPAC (Fujitsu, Toyko, Japan) using AM1 (Austin Model 1) as approximation method. For each compound, the two stable conformers (*s-trans* and *s-cis*) were minimized and the minimum energy values (data not shown) used to compare the stability of conformers of the same model. In the N-H series, the *s-cis* conformation of compounds (1), (3), (4), and (5) was approx. 1 kcal/mole more stable than the *s-trans* conformation. With compounds (2) and (6), the *s-trans* conformation was the most stable conformation with a difference of about 1 kcal/mole. Similar results were obtained in the N-Me series. On the minimized models, charge distribution was calculated and analyzed for each molecule, focusing on the carbaldehyde and amine functions. Charges on the amine nitrogen and the aldehyde carbon of compounds (1) to (6) are shown in Table 3. The substitution of a bromine atom at the C-2 position showed the greatest increase—+0.014 from (1) to (6)—in positive charge on the amine proton while at the same time the greatest reduction—−0.007 from (1) to (6)—of the positive charge on the aldehyde carbon.

The lipophilic character of compounds (1) to (13) was estimated by the retention time on reverse-phase HPLC (Table 1). As expected, the lipophilicity in the N-H or N-Me series increased with the number of bromine, (6) > (5) > (4) > (3) > (2) and (13) > (12) > (11) > (10) > (9) > (8). The 2-bromoindole in the NH (2), as well as in the N-Me series (8), significantly was the least lipophilic monobromo derivative.

Table 4. IC50 of indole-3-carbaldehydes calculated from the dose–response curves[a]

Products	IC50 μM
1	40 ± 4.8
2	0.6 ± 0.09
3	24 ± 3.1
4	17 ± 1.44
5	10 ± 1.2
6	0.31 ± 0.028
7	>100
8	67 ± 8.9
9	12.5 ± 1.31
10	12 ± 0.94
11	40 ± 5.1
12	24 ± 2.17
13	56 ± 3.6

[a] Values are the means ± standard error from three experiments.

Activity of compounds (1) to (13) measured on sea urchin egg cleavage

To study cleavage inhibition, we ensured that at least 95% of the eggs could be properly fertilized in all batches before adding the compounds. The percentage of divided cells was calculated for each compound concentration and at different times after the beginning of cell cleavage in the control batch.

All compounds except (7) inhibited cell cleavage. Figure 2 shows the inhibition of cleavage in the presence of increasing concentrations of the most toxic compound (6). Dose–response curves were obtained by considering the percentage of cleavage 85 min after the fertilization at the time when 90 to 95% of control eggs had reached the two-cell stage. According to the model given by Stastistica® 5.1 (1995, StatSoft, Tulsa, OK, USA) that can be used to estimate organism's responsiveness to a drug and the IC50 value, the dose–response curve follows a sigmoid function. An example of such a curve is given in Figure 2B in the case of compound (6). The IC50 values, the mean of three experiments, are indicated in Table 4. The IC50 ranged from 0.3 to more than 100 μM, showing a great variability between the different compounds. When comparing the toxicity indexes expressed as log 1/IC50, of the 13 indole carbaldehydes tested, (2) and (6) were the most active, and (7) was the least active, which suggests the importance of the position and number of brominated groups and the influence of nitrogen methylation (Fig. 3).

Cytotoxicity of compound (6)

The percentage of sea urchin eggs undergoing first cleavage decreased with increasing concentrations of (6) with a total inhibition observed for concentrations above 1.3 μM (Fig. 2A). The IC50 calculated from the dose response shown in Figure 2B was 0.31 ± 0.028. In order to determine whether the inhibition of the first cell cycle was induced immediately after fertilization or during a critical period of sensitivity to the drug later in the cell cycle, 2 μM of (6) were added at different times from fertilization to the premitotic stage. The extent of division was then determined over a period corresponding to the first cell cycle in absence of (6). Figure 2A shows that control eggs started to divide around 60 min and reached the two-cell stage around 80 min postfertilization. Treated eggs did not divide if 2 μM (6) were added within the first 30 min following fertilization and were arrested in a way similar to that observed when fertilization was performed in the presence of the drug. However, the extent of inhibition was progressively reduced in samples in which (6) was added between 30 and 60 min (Fig. 2C). Our results suggest that (6) had no effect after 40 min of development, which corresponds to chromo-

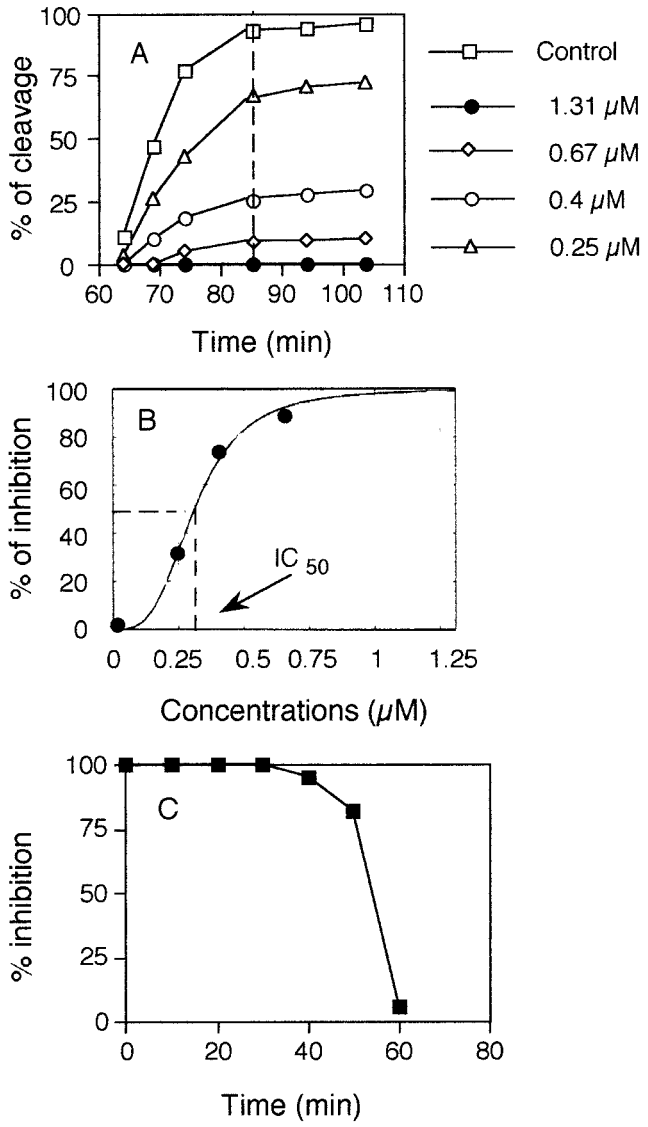

Fig. 2. Effect of increasing concentrations of 2,5,6-tribromoindole-3-carbaldehyde (6) on cleavage of the sea urchin eggs (*Paracentrotus lividus*). (**A**) Eggs were fertilized at time 0, and (6) was added 30 s after sperm addition. (**B**) Dose–response curve and C50 were obtained by plotting the percentage of cleavage in (**A**) at 85 min after fertilization. (**C**) Rate of inhibition of egg cleavage as a function of time at which 2 μM (6) are added after fertilization. Data shown are averages of three similar experiments.

some migration and cytokinesis, a microfilament-dependent event. Because eggs treated at 30 min postfertilization were fully arrested, (6) must inhibit an event taking place between 30 and 40 min after fertilization (before metaphase in control eggs). This led us to investigate which activities were altered by (6) and thus prevented normal progression through mitosis.

Effect of 2,5,6-tribromo-indole-carbaldehyde (6) during the first cell cycle

In order to know more precisely at which particular stage embryos were arrested by (6) and which effects were induced by the compound, control and embryos treated by 2 μM of (6) were fixed at various times postfertilization, permeabilized, and stained for microtubules and chromatin (Fig. 4).

At 30 min, both control (Fig. 4a and a′) and treated (Fig. 4d and d′) eggs showed a large spermaster of microtubules

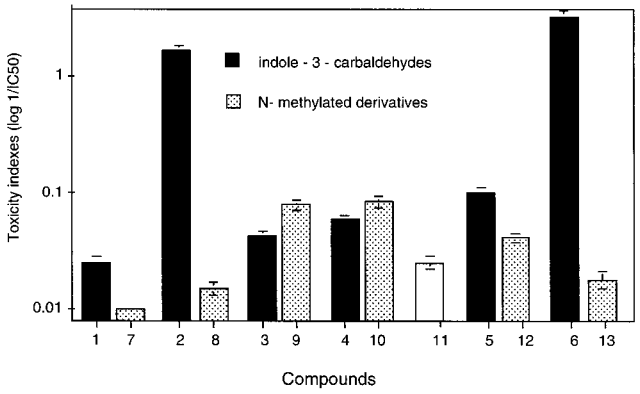

Fig. 3. Toxicity indexes (log1/IC50) obtained from IC50 expressed in micromoles of indole-3-carbaldehydes and their methylated derivatives. Values are the means ± standard error of three independent experiments.

radiating throughout the eggs, allowing pronuclei migration and fusion in the center of the eggs.

At 45 min, control eggs showed the typical metaphase bipolar mitotic spindle resulting in chromosome alignment (Fig. 4b and b′), while treated eggs (Fig. 4e and e′) showed no development compared with the eggs fixed at 30 min.

At 75 min, control eggs cleaved (Fig. 4c and c′), whereas treated eggs (Fig. 4f and f′) did not show any sign of embryonic development beyond pronuclei fusion (compare Fig. 4f and f′ with Fig. 4d and d′). In particular, they showed no condensation of chromatin, no nuclear envelope breakdown, and no mitotic spindle formation, that is, no entry into mitosis.

2,5,6-Tribromo-indole-carbaldehyde (6) inhibits protein and DNA synthesis

To examine the possibility that the cell cycle arrested by (6) may result from the inhibition of protein synthesis or DNA replication, we measured the incorporation of labeled methionine into proteins and labeled thymidine into DNA. Figure 5A shows that protein synthesis was drastically inhibited by (6) during the first cell cycle, whereas its rate is elevated in the control at the time when the second cell cycle occurs in the control batch. In Figure 5B, we observed that the nucleic acid synthesis was suppressed by (6) during the first two-cell cycle.

DISCUSSION

Structure–activity relationships for brominated-indole carbaldehydes and their methylated derivatives

As expected, the toxicity of indole carbaldehyde derivatives varied with structural differences, such as addition of 1-, 2-, or 3-brominated groups or N-methylation. Bromination of indole-3-carbaldehyde increases the cytotoxicity, which is maximal when compounds are brominated in position 2. Generally, capping of the NH group reduced activity 2- to 10-fold except for the 5- and 6-monobromo derivatives; the greatest variations were observed for N-methylation of (2) and (6), which are brominated in position 2, which is vicinal to the NH group.

Among the NH derivatives, (2) and (6) exhibited the highest cytotoxicity with IC50 of 0.6 and 0.3 μM, more than 15-fold more active than (3), (4), and (5). Cytotoxicity increased weakly with the addition of one (from [1] to [3] or [4]) or two bromines (from [1] to [5]) and strongly with the addition of a third one (from [5] to [6]). But the fact that the 2-bromoin-

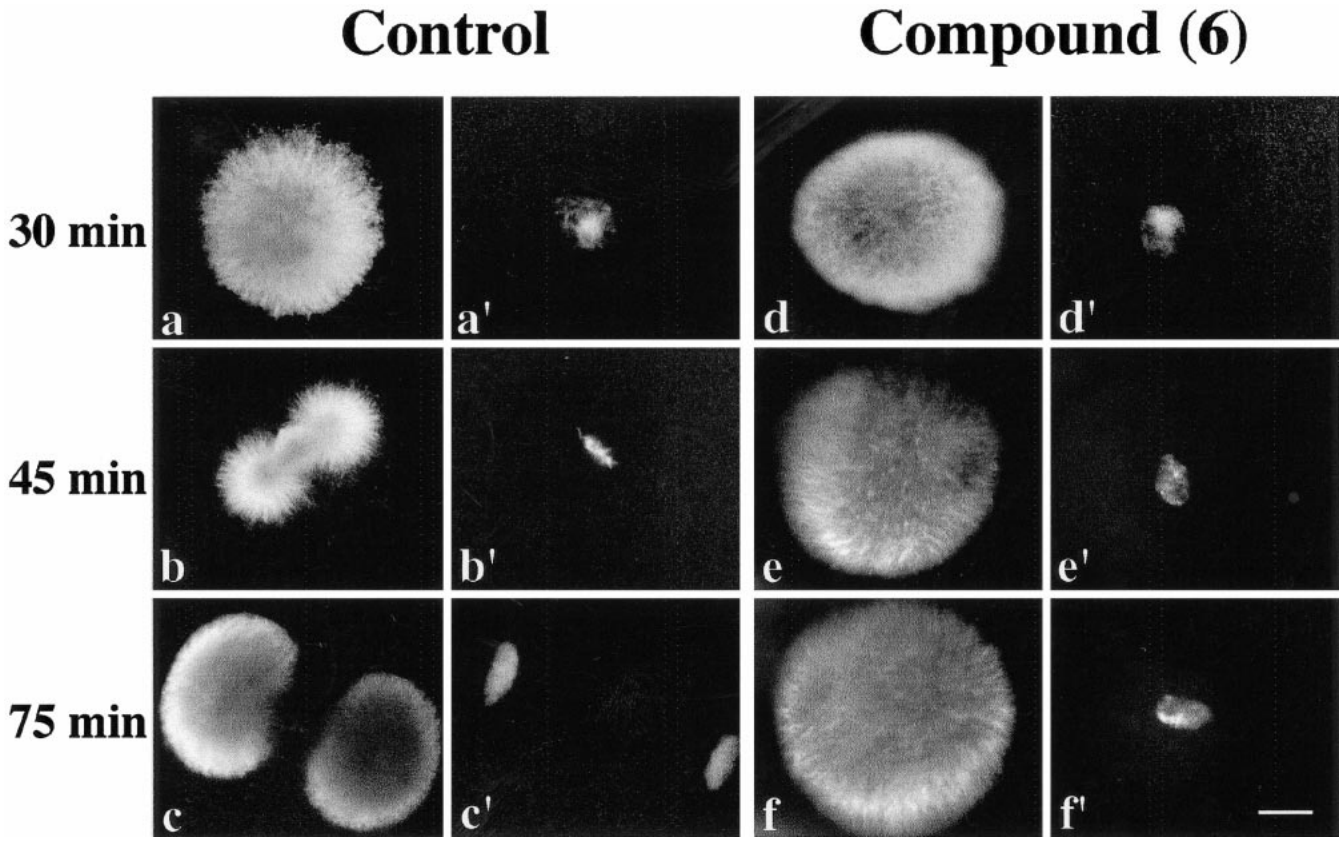

Fig. 4. Effect of 2 μM 2,5,6-tribromoindole-3-carbaldehyde (6) on the first cell cycle of sea urchin eggs. Control (**a**, **a′**, **b**, **b′**, **c**, **c′**) or treated (**d**, **d′**, **e**, **e′**, **f**, **f′**) eggs were fixed at different times—at 30 min after fertilization (at fusion of pronuclei; **a**, **a′**, **d**, **d′**), at 45 min after fertilization (at metaphase; **b**, **b′**, **e**, **e′**), and at 75 min after fertilization (at first cleavage; **c**, **c′**, **f**, **f′**)—and processed as described in Materials and Methods for immunofluorescence. Antitubulin antibodies allowed the visualization of microtubules (**a**, **b**, **c**, **d**, **e**, **f**), while the Hoechst 33258 dye allowed the visualization of nuclei (**a′**, **b′**, **c′**, **d′**, **e′**, **f′**). Scale bar = 20 μm.

dole-3-carbaldehyde (2) is nearly as cytotoxic as the 2,5,6-tribromoindole-3-carbaldehyde (6) indicated that more than the number, the position of the halogen atoms on the indole group is important. The positive contribution of the bromines to the overall lipophilicity of the molecule is not the direct cause of the cytotoxicity. A bromine in position 2 greatly enhances the biological activity as indicated by the large increase in toxicity between the 5,6-dibromo and the 2,5,6-tribromo analogue and especially between the 5- or 6-bromo and the 2-bromo analogue. N-methylation leads to a drastic loss of toxicity in compounds with a bromine in position 2. Note that the toxicity of the 2,6-dibromo-*N*-methylindole-3-carbaldehyde (11) and that of the other 2-brominated *N*-methyl derivatives (8) and (13) are similar. The results of this study suggest that the bromine adjacent to the unprotected amine appears to be required for optimal biological activity.

Within this small series, the introduction of a bromine atom at C-2 appears to be required for optimal biological activity. As molecular models show the bromine atom embedded between the unprotected amine and the aldehyde function, a hydrophobic interaction of the halogen with a lipophilic site seems unlikely. However, the effect of this bulky atom on the displacement of the conformational equilibria toward the *s-trans* orientation of the aldehyde, as indicated by energy minimization, may contribute to the activity of compounds (2) and (6). In indole-3-carbaldehydes, the aldehyde function, conjugated and coplanar with the indole ring, adopt two stable conformers, *s-trans* and *s-cis*. With compounds (2) and (6), where the *s-trans* conformation was the most stable conformation, the oxygen atom of the aldehyde points preferably in the direction of the H4 hydrogen atom.

On the other hand, the electrophilic aromatic substitution on position 2 might allow some modifications on the electrophilicity of the aldehyde and on the acidity of the N-H bond. Concerning the aldehyde function of compounds (1) to (6), the electrophilicity of the carbonyl group is reduced, relatively to nonaromatic aldehydes, by the conjugation with the electron-rich ring of the indole. This can be attributed to resonance interaction between the electron-realizing indole ring and the carbonyl group. The resonance interaction is most pronounced in the case of 3-acylindoles [29]. Furthermore, as indicated by the charge distribution on molecular models, the bromo substitution on position 2 weakly decreases the partial positive charge on the carbonyl carbon. This is confirmed by the analysis of the chemical shifts of the carbonyl carbons in the 13C NMR spectra. The mean value of this carbon, in compounds (1) to (6), is about 185 ppm, a typical value for an unsaturated carbonyl system. With the introduction of a bromine atom at C-2 in compounds (2) and (6), the carbonyl resonance is weakly upfield shifted to 184.42 and 184.57 ppm (the two lowest values), respectively. The electron-withdrawing ability of the bromo-substituent in position 2 seems to have no (if not an opposite) effect on the electrophilicity of the aldehyde function. The reactivity of the aldehyde cannot explain the significant increase of activity of compounds (2) and (6).

For the N-H bond, it is interesting to underline the down-

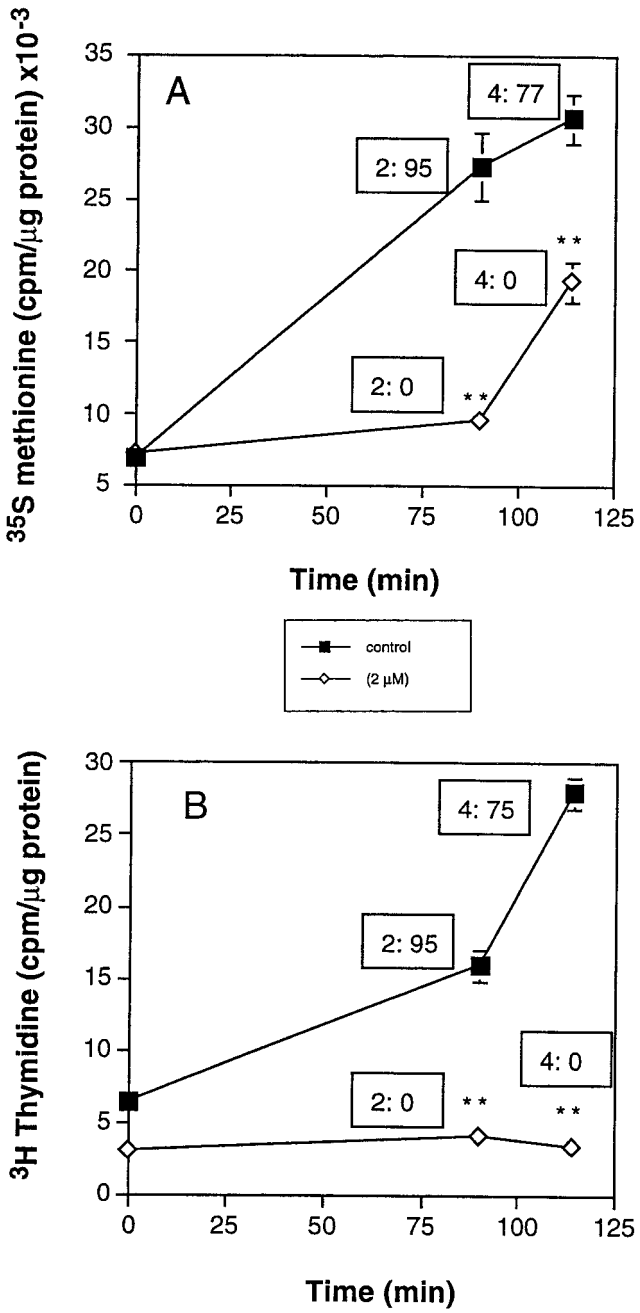

Fig. 5. Effect of 2,5,6-tribromoindole-3-carbaldehyde (6) on the cumulative incorporation of ^{35}S-methionine into proteins (**A**) and ^{3}H-thymidine into DNA (**B**) synthesis during the first and second division cycles. Eggs were fertilized at time 0; numbers in squares indicate the stage of cleavage (2,4 cells) and the percentage of cleaved eggs in this stage. ** Significantly different from untreated control ($p < 0.01$, Dunnet's t test, $n = 3$).

field chemical shift (13.0 and 13.4 ppm) of the NH proton in the 1H NMR spectra of compounds (2) and (6) in comparison with the 5- and 6-monobromo and 5,6-dibromo analogues (12.3 ppm). This lower electronic density on the NH proton is also observed in the analysis of charge distribution on molecular models, with an increase in the partial positive charge of the amine hydrogen of compounds (2) and (6). This is indicative of an increase of the acidity of the N-H bond and so of a better ability of this proton to proceed in hydrogen bonding.

Tribromo-indole carbaldehyde (6) blocks the sea urchin egg cell cycle

Given the fact that (6) had no effect on fertilization but blocked the first division, we determined the period of the cell cycle during which eggs were sensitive to (6) for the arrest of egg division. Two micromoles of (6) blocked the first cell cycle if added up to 30 min postinsemination. Our results strongly suggest that, during the first cell cycle, early events directly linked to fertilization and late events directly involved in cytokinesis [30] were not affected by (6). This means that the microfilament network, present in the egg cortex, is probably not sensitive to (6).

On the other hand, events taking place soon before mitosis were sensitive to the action of (6). The cytological and immunofluorescence localization revealed that sea urchin embryos treated any time during the first 30 min of development by (6) did not progress beyond the fusion of pronuclei. Treated eggs showed no sign of entry into mitosis, which is consistent with our biochemical results discussed in the following.

Tribromo-indole carbaldehyde (6) inhibited protein and DNA synthesis

We demonstrated that embryos preloaded with ^{35}S-methionine or ^{3}H-thymidine and treated with (6) added 2 min after fertilization showed a greatly reduced protein and DNA synthesis at the normal time of the first mitosis in control eggs.

As described by Wagenaar [31], protein synthesis is required for chromosome condensation and nuclear envelope breakdown in the cell cycle of the sea urchin embryos. Wagenaar showed that emetine, an inhibitor of protein synthesis, arrested cells in prophase with cells presenting enlarged nuclei containing partially or fully condensed chromosomes. Moreover, Dubé [32], who determined precisely the phase of the cell cycle that was most affected by protein synthesis inhibitors, showed that the timing of pronuclei fusion is not affected by emetine; rather, this event was concomitant with the first round of DNA synthesis.

In agreement with Nishioka et al. [33] and Ettouati and Jacobs [34], we have shown that the first DNA replication period (S1) during the first cell cycle of sea urchin eggs occurs at the time of pronuclei fusion, whereas the second round of DNA synthesis (S2) occurs during telophase and cytokinesis of the first division cycle [35]. Indeed, (6)-treated eggs were arrested at the beginning of fusion of pronuclei before chromosome condensation and nuclear envelope breakdown. In other words, they did not enter into mitosis, showing a phenotype comparable to that induced by aphidicolin, a well-known DNA replication inhibitor [33,35–37].

Taken together, our results suggest that (6) induced the arrest of the cell cycle at the entry in mitosis because of a defect in DNA replication. The observed phenotype is very similar to the one we previously observed in the presence of butyrolactone 1 [35], a known inhibitor of maturating promoting factor activation. Therefore, our results with (6), which are comparable with those obtained by Patel et al. [38] with aphidicolin, confirm the existence of an S-phase checkpoint in the early sea urchin embryo that prevents mitosis entry in the presence of unreplicated DNA. Indeed, if DNA synthesis has not been properly completed, no subsequent histone kinase (H1K)/maturating promoting factor activation occurs preventing the G2/mitosis transition during cellular cycle [39].

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