LETTER

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A Synthetic Derivative of Plant Allylpolyalkoxybenzenes Induces Selective Loss of Motile Cilia in Sea Urchin Embryos

Marina N. Semenova[†,*], Dmitry V. Tsyganov[‡], Alexandr P. Yakubov[‡], Alexandr S. Kiselyov[‡], and Victor V. Semenov[‡,§,*]

[†]Institute of Developmental Biology, Russian Academy of Sciences, Moscow, Russia, [‡]Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia, and [§]Chemical Block Ltd., Limassol, Cyprus

ABSTRACT Polyalkoxybenzenes are plant components displaying a wide range of biological activities. In these studies, we synthesized apiol and dillapiol isoxazoline analogues of combretastatins and evaluated their effect on sea urchin embryos. We have shown that *p*-methoxyphenyl isoxazoline caused sea urchin embryo immobilization due to the selective excision of motile cilia, whereas long immotile sensory cilia of apical tuft remained intact. This effect was completely reversed by washing the embryos. The compound did not alter cell division, blastulae hatching, and larval morphogenesis. In our hands, the molecule would serve as a convenient tool for *in vivo* studying morphogenetic processes in the sea urchin embryo. We anticipate that both the assay and the described derivative could be used for studies in ciliary function in embryogenesis.

We have recently shown that the sea urchin embryo is a simple organism model that provides for the rapid "one-pot" assessment of antiproliferative, antimitotic, and tubulin destabilizing effects of small molecules on a living organism (*1*). The assay includes (i) the fertilized egg test for antimitotic activity displayed by cleavage alteration/arrest, and (ii) behavioral and morphological monitoring of a free-swimming embryos after their hatching. Tubulin destabilizing molecules combretastatin A-4 and A-2 (Scheme 1) isolated from the South African willow *Combretum caffrum* are potent antimitotic agents (*2*–*4*). In our hands, combretastatin A-4 phosphate demonstrated a profound phenotypic effect on the sea urchin embryo. It correlated well with the reported antitumor activity of this agent against human tumor cell lines (*1, 5*). Molecules of the combretastatin family generally share three common structural features: a trimethoxy "A" ring, a "B" ring containing substitutents at C3′ and C4′, and a *cis*-ethene bridge between the two rings which provides necessary structural rigidity (*3, 4*). A number of biologically active combretastatin analogues featuring modified "A" and "B" rings and bridge isosteres have been synthesized (*6*).

In addition to combretastatins, other plant polyalkoxybenzenes display a broad spectrum of biological activities. For example, apiol (Scheme 1) was reported to be a calcium antagonist. It also shows diuretic, abortive, sedative, antioxidant, and insecticidal activity. Dillapiol (Scheme 1) has reported activities as chemosensitizer and anticancer drug synergyst. It exhibits antibacterial, sedative, and pesticidal activity (*7, 8*). We reasoned that due to their polyoxygenated nature, both apiol and dillapiol were good starting points for the synthesis of molecules with the potential for antiproliferative activity (*3, 4*). On the basis of this premise, we have synthesized and tested structural analogues of combretastatin A-2 and *Z*,*E*-diene compound (Scheme 1). In order to maintain proper orientation of the biaryl motif that is important for physiological activity (*6*), we have introduced isoxazoline moiety into our final products (**1**, **2**, Schemes 1 and 2).

Biological Evaluation. In our hands, parent molecules apiol and dillapiol influenced neither cleavage nor blastula formation *in vivo*. Instead, both compounds inhibited spiculae growth at the early pluteus stage, when applied at 100 μM concentration immediately after hatching (Table 1). *trans*-Isoapiol (2–8 μM), but not the respective cis-isomer, displayed a specific antiproliferative activity (M. N. Semenova, personal communication). Encouraged by these data, we further studied the effect of 15 isoxazo-

*Corresponding authors, ms@chemical-block.com and vs@chemical-block.com.

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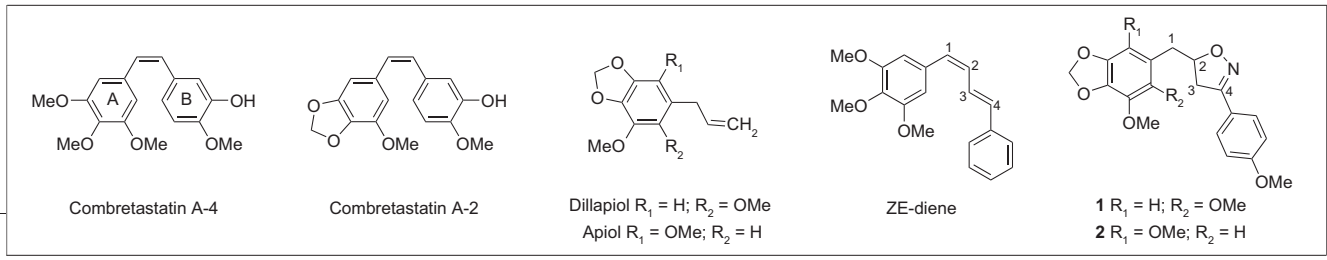
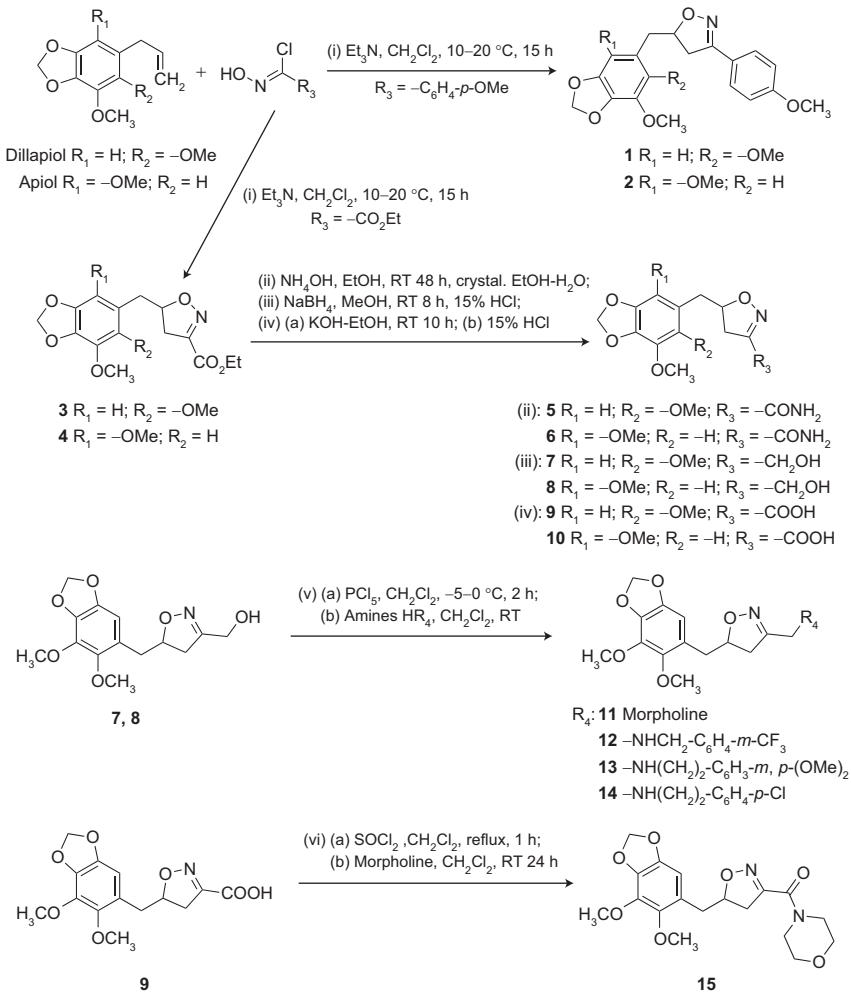


Scheme 1.

line derivatives of apiol and dillapiol (Scheme 2) on specific developmental stages of the sea urchin *Paracentrotus lividus* embryos. These included fertilized egg, hatched blastula, prism, and early pluteus (Figure 1, panels a, e, h, and j).

Prior to expanding our chemistry effort, we evaluated the effect of compounds **1** and **2** on the sea urchin embryo (Table 1). Instead of the anticipated antiproliferative and/or antitubulin activity, dillapiol derivative **1** caused embryo immobilization at free-swimming stages (Figure 1, panels e–k) but did not influence larval morphogenesis. Similarly, apiol derivative **2** induced embryo immobilization accompanied by little or no cleavage/larval abnormalities. Since the effect of dillapiol analogue **1** on sea urchin embryos appeared to be more selective, we studied it in more detail. Specifically, **1** did not affect cell division and blastula formation (Figure 1, panels b–d) when added to fertilized eggs at 1–4 μM concentrations. Both treated and intact blastulae hatched at the same time followed by a proper developmental pattern. At 2–4 μM concentration of **1,** hatched embryos remained immobile on the bottom of the culture dish for the next 30 h up to the midpluteus stage (Figure 1, panel k). We observed only a moderate developmental delay at the four-arm midpluteus stage (Figure 1, panel k) presumably due to the insufficient air supply. A similar effect has been noted for the intact embryo cultures growing at a high embryo concentration. At 1 μM concentration, the molecule **1** caused embryo immobilization up to the prism stage (about 12 h after hatching; Figure 1, panel h), when applied to fertilized eggs. Subsequently, embryo swimming was gradually restored resulting in midplutei indistinguishable from the intact larvae. The same concentration-dependent responses have been detected when compound **1** was added to an embryo suspension just after hatching (midblastula stage; Figure 1, panel e) at 1–5 μM concentrations. Intriguingly, this effect was completely reversible. Washing the immobilized blastulae treated by test molecule **1** (2 μM) with filtered seawater recovered embryo locomotion within 1 h. Further at the early prism stage, the swimming pattern was completely restored. Specifically, embryos resumed moving near the bottom within 1 h. During the next several hours their swimming reached the intact pattern.

The immobilizing effect of compound **1** was not immediate. After addition of **1**(2 μM), blastulae (Figure 1, panel e) featured a normal swimming pattern for 12 min, whereas prisms (Figure 1, panel h) and early plutei (Figure 1, panel j) swam normally during 8 and 5 min, respectively. Then embryo swimming gradually slowed down, and embryos settled to the bottom of the dish. Within 20 min of treatment for blastulae and prisms or 13 min for plutei, they be-



Scheme 2. Preparation of isoxazoline derivatives of dillapiol and apiol. Yields of 5–15 were 50–90%. Reagents and conditions of reaction i are reported in ref *9*.

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TABLE 1. The effect of apiol, dillapiol, and their isoxazoline derivatives on sea urchin embryo development

	Threshold concentrations, μM[a]		
Compound	**Cleavage alteration**	**Embryo abnormalities[b]**	**Embryo immobilization**
Dillapiol	>100	100	>100
Apiol	>100	100	>100
1	>4	>5	1
2	4	2	
3	5	5	>5
4	5	5	>5
5	>5	>5	>5
6	>5	>5	>5
7	>5	>5	>5
8	>5	>5	>5
9	>5	>5	>5
10	>5	>5	>5
11	>5	>5	>5
12	>5	>5	>5
13	>5	>5	>5
14	>5	5	>5
15	>5	>5	>5

[a]Repeated tests showed no differences in threshold concentration values. [b]Embryo/larval abnormalities were monitored after treatment of hatched blastulae (Figure 1, panel e) up to midpluteus.

came immotile. This observation suggests that the sea urchin embryos are more sensitive to the compound at later developmental stages.

Interestingly, in a more concentrated embryo culture (1500 instead of 600–850 embryos/mL) complete embryo immobilization occurred at double the concentration of compound **1**, namely at 4 μM. The same concentration applied to a culture of 3000 embryos/mL caused considerable slowing down of embryo swimming.

We further attempted to rationalize these observations. Sea urchin embryo motility is mediated by coordinated ciliary beating. Cilia are evolutionary conserved cell surface organelles adapted to perform both dynamic and sensory perception. They consist of a microtubule-based axoneme covered by a plasma membrane. Cilia anchorage in a cytoplasm is mediated by an elaborated basal apparatus (*10, 11*). Swimming blastulae and gastrulae are uniformly covered by cilia, each ectodermal cell bearing one cilium. Cells of the animal plate, a presumptive larval sensory structure, form long immotile cilia of apical tuft, which are two to three times longer than motile cilia of the remaining ectoderm (*12, 13*). At the late gastrula, a specific motor ciliary band consisted of three to four rows of columnar epithelial cells is formed. We speculated that the observed embryo immobilization was a consequence of (i) suppressing of ciliary beating or (ii) cilia loss. On the basis of a phase-contrast microscopy examination, we concluded that compound **1** induced embryo immobilization due to the selective loss of short motile cilia, while long cilia of apical tuft remained intact (Figure 1, panels l and m). Previously it was reported that neither electron microscopy nor electrophoresis revealed any difference in morphology and protein content between cilia types at blastula stage (*14*). However, in animalized embryos characterized by an enlarged apical region with long cilia, a protein marker specific for surrounding aboral ectoderm is not expressed (*15*). Moreover, cells of the animal plate represent a distinct ectodermal regulatory subregion highly resistant to expression factors or signaling molecules (*16*). Cells of the late gastrula apical plate retain long cilia, and some of the cells further differentiate to serotonergic neurons of the apical organ (*17, 18*). Later at the four- to eight-arm pluteus stage, the cluster of neuronal cells is characterized by coiled immotile sensory cilia of specific structure and orientation (*17, 19*). Thus, the failure of compound **1** to remove the apical tuft cilia could arise from the peculiar features of the apical cells.

Physiologically, the ciliary loss occurs *via* deciliation/excision or disassembly/resorption (*20*). Deciliation involves severance of axoneme microtubules at the cilia base. It usually occurs in response to stress factors. In the sea urchin embryos, cilia excision by a short (2 min) treatment of hypertonic seawater (*21*) is commonly used for elucidating ciliary structure and regeneration. Another deciliating agent, chloral hydrate, is known to disassemble microtubules and induce cleavage alterations in the sea urchin embryo (*22, 23*). In contrast to compound **1** that affected only short motile cilia, the former two agents produce total deciliation (Figure 1, panel n). A continuous observation of embryos treated by molecule **1** revealed a gradual cilia shedding from the surface of ectodermal cells. Detached cilia moved away by the neighboring cilia beating, resulting in a number of excised cilia scattered in the vicinity of immobilized embryos (Figure 1, panel o). Further determination of the cellular target(s) for compound **1** is necessary in order to understand its spe-

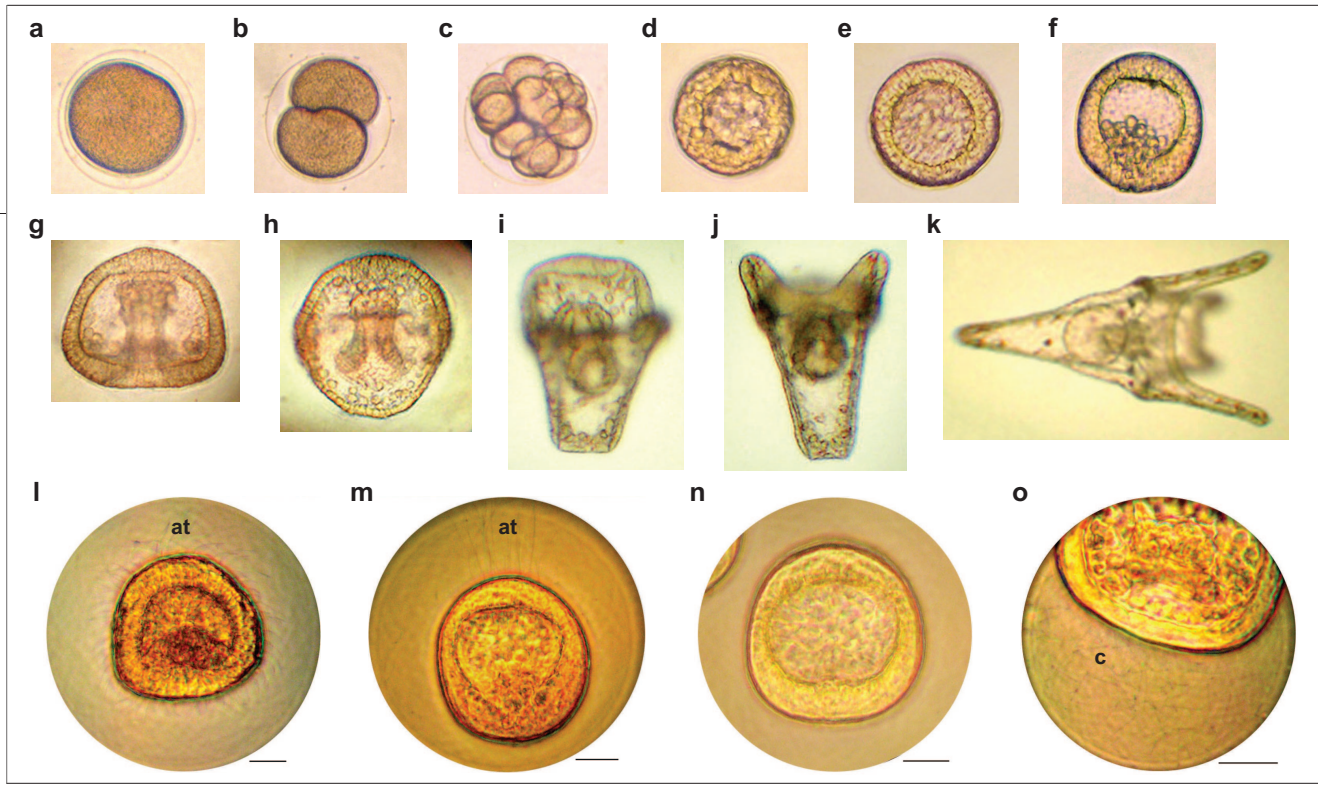

Figure 1. Normal development of the sea urchin embryo (*Paracentrotus lividus* at 20 °C) and motile cilia loss caused by compound 1. Time after fertilization is shown in parentheses. a) Fertilized egg. b) 2-cell stage (1 h 20 min). c) 16-cell stage (3 h). d) Early blastula (6 h). e) Hatched midblastula (10 h). f) Late mesenchyme blastula (13 h). g) Late gastrula (17 h). h) Prism (22 h). i) Early pluteus (26 h). j) Early pluteus (30 h). k) Four-arm midpluteus (40 h). Beginning of the active feeding. l) Control embryo at free-swimming late-blastula stage fixed by a drop of 5% glutaraldehyde/filtered seawater. Note numerous short cilia covered the embryo surface. m) Living immotile embryo exposed to 2 μM 1 for 30 min. n) Deciliated blastula treated with hypertonic seawater. o) Detached short cilia in the vicinity of the embryo exposed to 4 μM 1 for 20 min. Key: at, apical tuft; c, cilia. For panels a–f the average embryo diameter is 115–120 μm. For panels g–k the maximum embryo sizes are ~125, ~140, ~160, ~250, and ~ 450 μm, respectively. Bars in panels l–o represent 30 μm.

cific molecular mechanism of action. At this stage, it seems reasonable to conclude that the immobilizing effect of **1** is not a result of direct antitubulin activity, as the agent did not influence cell division (cleavage). Literature data also suggest that the tubulin-destabilizing agents are unable to disrupt ciliary microtubules (*24, 25*). Moreover, they cause sea urchin embryo spinning over several hours (*1*).

For a preliminary estimation of a structure–activity relationship in the isoxazoline series, we further analyzed analogues **3**–**15** in our assay system (Table 1). Molecules **5**–**13** and **15** did not result in a developmental alteration at the concentration up to 5 μM. At 5 μM, the respective ethoxy derivatives furnished developmental delay without visible embryo abnormalities (**3**) or embryo malformations observed after 6 h of exposure (**4**), both independent of the application stage. Both molecules appeared to affect embryos *via* different mechanisms. Compound **3** featured a developmental delay without visible abnormalities. For the molecule **4** malformations appeared within 6 h postapplication. Compound **14** inhibited spiculae growth in plutei at 5 μM when applied immediately after hatching. None of the molecules altered embryo swimming behavior. These data suggest that the *p*-methoxyphenyl group directly connected to the isoxazoline ring in our series is essential for the selective loss of motile cilia.

In conclusion, screening of a library based on natural products apiol and dillapiol in the sea urchin embryo model yielded molecule **1** that selectively affected motile cilia of the organism. To the best of our knowledge, this mode of action has never been described in the literature. Notably, compound **1** did not produce any adverse short- or long-term cellular effects. In addition, cell division was not affected. We believe this agent is a convenient tool for studying morphogenetic processes *in vivo*, specifically when embryo immobilization is required. Since cilia are highly conserved across various species (*26–28*), molecule **1** may yield clues to elucidating stage-specific role(s) of ciliary motility in embryogenesis and developmental disorders of various organisms. Moreover, agents featuring cilia-specific effects could be of interest in treating respiratory, reproductive, and renal abnormalities in humans (*29, 30*). Finally, our results showed that the sea urchin embryo is a simple and reproducible model for the phenotypic evaluation of small molecule activities.

METHODS

Synthesis of Isoxazolines 1–4. Typical Procedure for Reaction of Apiol and Dillaiol with Carbethoxyhydroxymoyl Chloride and 4-Methoxybenzylhydroxymoyl Chloride. A solution of triethylamine (17.1 mmol) in 20 mL of methylene dichloride was added to a solution containing unsaturated compound (apiol or dillapiol; 18.0 mmol) and carbethoxyhydroxymoyl chloride or 4-methoxybenzylhydroxymoyl chloride (16.3 mmol) in 60 mL of methylene dichloride. The reaction mixture was stirred at +10 °C for 3 h, then at RT for 10 h, washed with water (2 × 20 mL), and evaporated under vacuum. Recrystallization of the oil from hexane/ethyl acetate (1:1, 15 mL) yielded a white solid powder: **1**, mp 98–100 °C, yield 67%; **2**, mp 102–103 °C, yield 78%; **3**, mp 79–81 °C, yield 62%; **4**, mp 74–76 °C, yield 59%.

Synthesis of Isoxazolines 5–15. The compounds **5**–**15** were prepared by the standard procedures. Reaction conditions are listed in Scheme 2: **5**, mp 124–126 °C (ethanol), yield 80%; **6**, mp 126–128 °C (ethanol), yield 71%; **7**, mp 48–50 °C (hexane/ethyl acetate 1:2), yield 74%; **8**, mp 79–80 °C (hexane/ethyl acetate 1:2), yield 66%; **9**, mp 116–118 °C (hexane/ethyl acetate 1:1), yield 85%; **10**, mp 86–88 °C (hexane/ethyl acetate 1:1), yield 74%; **11**, oil (LC), yield 47%; **12**, hydrochloride, mp 159–161 °C (ethanol), yield 35%; **13**, hydrochloride, mp 83–85 °C (ethanol), yield 43%; **14**, hydrochloride, mp 156–158 °C (ethanol), yield 51%; **15**, mp 95–96 °C (hexane/ethyl acetate 1:1), yield 54%.

The structures were approved by ^{1}H NMR and mass spectra using NMR spectrometer Bruker DRX500 (500.13 MHz) and mass spectrometer Kratos MS-30 (electron impact of 70 eV). The spectra are presented in Supporting Information.

Sea Urchin Embryo Assay. Adult sea urchins *Paracentrotus lividus* were collected from the Mediterranean Sea off the Cyprus coast and kept in an aerated seawater tank. Gametes were obtained by intracoelomic injection of 0.5 M KCl. Eggs were washed with filtered seawater and fertilized by adding drops of a diluted sperm. Embryos were cultured at RT (19–21 °C) under gentle agitation with a motor-driven plastic paddle (60 rpm) in filtered seawater. For compound treatment, 5 mL aliquots of embryo suspension were transferred to six-well plates and incubated at a concentration of 600–850 embryos/mL. The ability of a molecule to cause developmental abnormalities was assessed by exposing fertilized eggs (10–15 min after fertilization) and free-swimming blastulae just after hatching (10 h after fertilization) to double decreasing concentrations of a compound. The development was monitored until the beginning of active feeding (midpluteus stage). Deciliation by a short (2 min) treatment of hypertonic seawater was performed as described previously (*21*). The embryos were observed with light microscope Biolam LOMO. For cilia observation, LOMO LUMAM I2 microscope with a phase-contrast device MFA-2 was used. Electronic images were obtained using a digital camera (Olympus Camedia C-760 Ultra Zoom with microscope adaptor Optica M).

Apiol and dillapiol, isolated from CO_2 extracts of the seeds of parsley *Petroselinum sp*. and dill *Anethum graveolens*, respectively (*31*), were dissolved in 95% EtOH to prepare a 20 mM solution. Mother stocks (10 mM in DMSO) of the test articles **1**–**15** were diluted with 95% EtOH to a 1 mM concentration. The resulting solutions were added to the embryo suspension in filtered seawater to obtain the required final concentrations. In our hands, addition of EtOH to the dilution scheme dramatically enhanced compound solubility as evidenced by the microscopy studies. Maximal tolerated concentrations of DMSO and EtOH for the *in vivo* assay were 0.05% (v/v) and 1% (v/v), respectively. Higher concentrations of the organic solvents led to nonspecific alteration and delay of the sea urchin embryo development.

For quantitative estimation of compound activity, we used the threshold concentrations that resulted in phenotypic and/or behavioral abnormalities. At these concentrations all tested molecules caused 100% developmental changes, whereas they failed to produce any effect at 2-fold lower concentrations. In our experience, a conventional IC_{50} determination was impractical, as we have never observed a quantifiable partition between embryos developing normally versus aberrant ones using a 2-fold decreasing concentration range.

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Supporting Information Available: This material is available free of charge *via* the Internet.

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