

Fluorescence Properties of a Novel Isoquinoline Derivative Tested in an Invertebrate Chordate, *Ciona intestinalis*

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3-Hydroxyisoquinolines (ISOs) and their tautomeric isoquinolin-3-ones are heterocycles with attractive biological properties. Here we reported the revisited synthesis of a highly functionalized ISO that showed blue fluorescence and the characterization of its biological properties in an invertebrate animal model, the ascidian *Ciona intestinalis*. Larvae exposed to ISO at

concentrations higher than 1 μM showed an intense fluorescence localized in the cell nuclei of all tissues. Moreover, exposure to ISO interfered with larval ability to swim; this neuromuscular effect was reversible. Overall, these results suggested that ISOs can have promising applications as novel fluorescent dyes of the cell nuclei.

Introduction

3-Hydroxyisoquinolines (ISOs) and their tautomeric isoquinolin-3-ones are rather unexplored heterocycles, even if they exhibit attractive biological activities. They have proven to strongly inhibit human 11 β -hydroxydehydrogenase 1 (11 β -HSD1), a privileged target for diabetes and/or metabolic syndrome.^[1] ISO derivatives are also potent renal vasodilators^[2] and inhibitors against *Plasmodium falciparum* cysteine protease falcipain-2.^[3] Moreover, isoquinoline alkaloids have been isolated in members of the *papaveraceae* family and they showed various biological properties including acetylcholinesterase inhibitory effects, anti-proliferative activities, antiviral activities and antiplasmodial activities.^[4] Recently, we have published a very fast and efficient synthesis of a series of highly functionalized ISOs, heterocyclic structures with excellent properties as blue-fluorescence emitters and remarkably high fluorescence quantum yields. Our approach consists in a two-step procedure where a four component Ugi reaction is followed by a palladium-catalysed reductive Heck cyclization.^[5] Actually, ISOs have showed remarkable deep-blue fluorescence under UV excitation, displaying absorption maxima in the range from 358 and 383 nm, and emission maxima in the range from 395 to 446 nm. The

fluorescence efficiency was strongly affected by the substitution of the isoquinoline core as the quantum yields determined with anthracene as a relative standard ($\Phi_f=0.36$) fall in the range of 0.20 to 0.90 (Scheme 1). The promising photophysical properties of ISOs prompted us to investigate their potential use as fluorescent sensors in different biological applications.

Herein, we describe the revisited synthesis of ISO-1, chosen as model compound, and explore its biological properties in invertebrate animal model, the ascidian *Ciona intestinalis*.

Ascidians are marine organisms closely related to vertebrates.^[6,7] They have been successfully used to assess the effects of several chemicals on animal development and behaviour.^[8,9] Adults are sessile filter-feeding animals that develop through a free-swimming larva. Larvae display the typical chordate body plan consisting of a trunk that houses the anterior part of the dorsal central nervous system with the sensory organs, and a muscular tail used for locomotion.^[10] *Ciona intestinalis*, one of the most studied ascidian species, has been established as a reliable model animal for toxicological tests.^[11–13] By in vitro fertilization, it is possible to obtain hundreds of synchronous embryos that complete their development in almost 18 hours at 18 °C^[14] thus allowing extremely quick and powerful toxicological and behavioural tests. Moreover, *Ciona intestinalis* larvae have a stereotyped behaviour making them a suitable model to test the effects of chemicals on their neuromuscular system. Soon after hatching, larvae show a positive phototropism and swim by lateral bending of their tail towards the surface. After few hours, they turn into negative phototropic and start to move toward the substrate searching for an appropriate site to which they adhere by means of three adhesive papillae.^[15,16] In addition to their numerous practical advantages, ascidians phylogenetic position makes these organisms particularly suitable for testing chemicals. Information about chemical effects in both invertebrates and vertebrates can indeed be extrapolated by results obtained in these animals.

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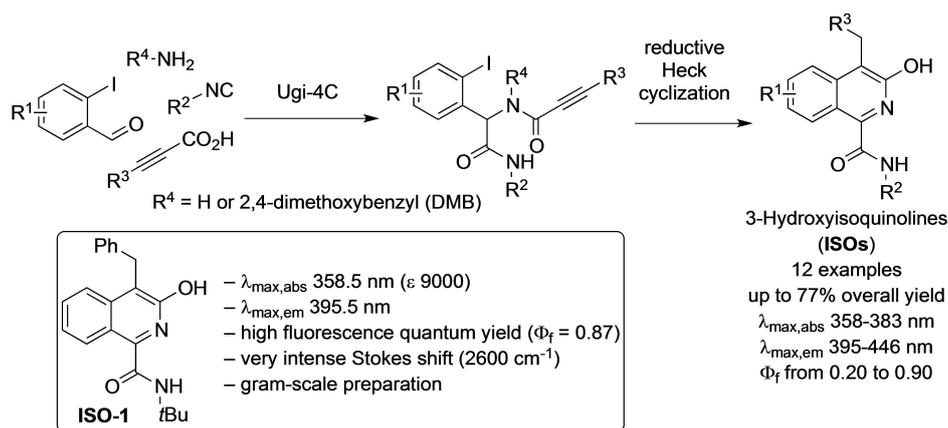
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Scheme 1. Synthetic strategy for the preparation of a library of substituted 3-hydroxyisoquinolines and structure and photophysical properties of compound ISO-1.

Results and Discussion

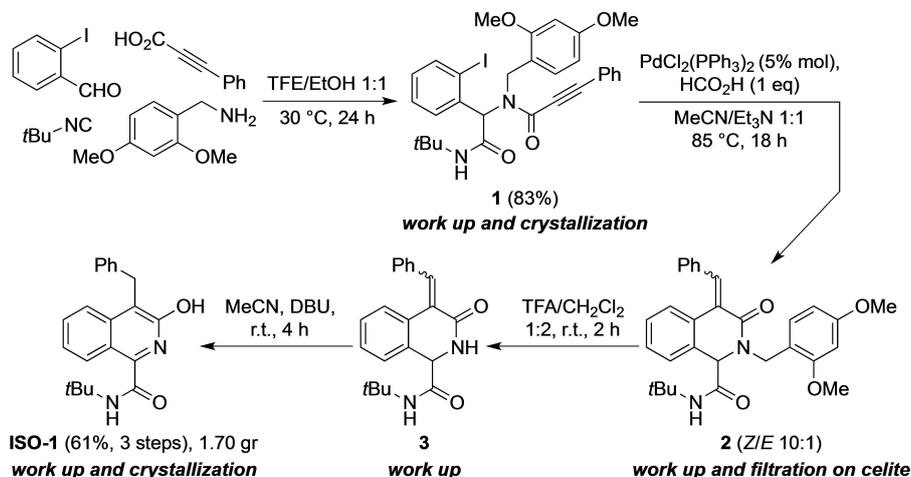
Gram-scale synthesis of ISO-1

Previous approach to synthesize the highly functionalized 3-hydroxyisoquinolines have only resulted in milligram quantities, whereas gram quantities would be required for *in vivo* studies. With this aim, a gram-scale synthesis of ISO-1 has been realized. The synthetic strategy has been depicted in Scheme 2. The Ugi reaction has been carried out in trifluoroethanol (TFE)/ethanol as solvent, employing 2,4-dimethoxybenzylamine as an ammonia surrogate, obtaining **1** in high yield and purity, after a simple work up to remove the excess of acid and a final crystallization. For the subsequent intramolecular reductive Heck reaction, a stoichiometric amount of formic acid is found to be crucial in order to promote the complete reductive elimination of palladium affording intermediate **2** as a mixture of *Z/E* diastereoisomers. After filtration on celite to remove palladium-related impurities, the mixture was treated with

trifluoroacetic acid (TFA) to give **3**, then with 1,8-diazabicyclo [5.4.0]undec-7-ene (DBU) in MeCN to promote the aromatization. Apart from four very fast work-up and two crystallizations, the sequence was performed without isolating any intermediate, and especially without any chromatographic purification (Scheme 2).

ISO-1 effects on development

Control larvae as well as those developed in Artificial Sea Water-HEPES (ASWH) plus 0.02% DMSO displayed the typical larval morphology. They were characterized by an elongated trunk with two pigmented sensory organs, clearly visible in the sensory vesicle, and a straight long tail (Figure 1A). ISO-1 concentration significantly affected *Ciona intestinalis* development in terms of number of normal developed individuals (ANOVA: $F=351.72$; $P < 0.001$), number of mildly affected individuals (ANOVA: $F=57.64$; $P < 0.001$), and number of



Scheme 2. Gram-scale synthesis of ISO-1.

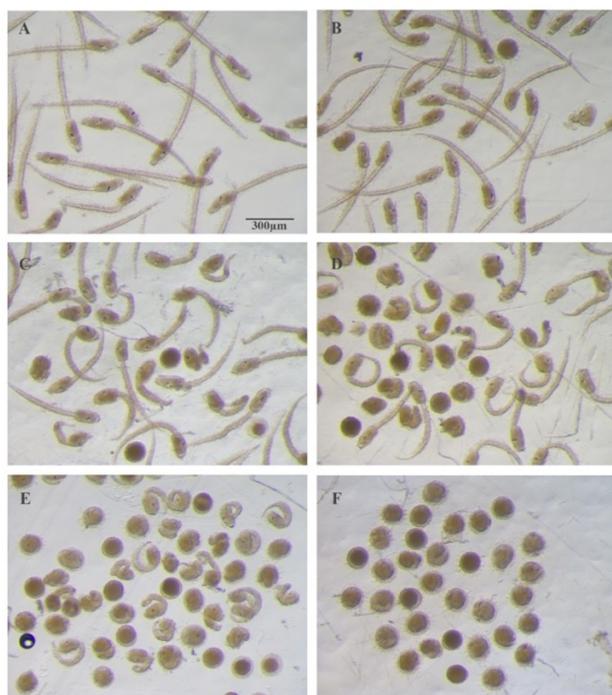


Figure 1. Phenotypes of larvae developed from embryos exposed to different concentrations of ISO-1. A) Control larvae exposed to DMSO. B) Larvae exposed to 1 μM ISO-1 showing a normal phenotype. C) Larvae exposed to 5 μM ISO-1. D) Larvae exposed to 10 μM ISO-1. E) Larvae exposed to 20 μM ISO-1. F) Larvae exposed to 25 μM ISO-1.

severely damaged individuals (ANOVA: $F = 641.56$; $P < 0.001$). Larvae exposed to the lowest tested concentration of ISO-1 (1 μM ; Figure 1B) were similar to the control ones and the incidence of adverse effects (either mildly or severely affected larvae) was not significantly different from controls (Tukey's post hoc test: $P > 0.05$). Exposure to 5 μM ISO-1 caused a statistically significant increase of mild malformations (Tukey's post hoc test: $P < 0.001$; Figure 2): almost 50% of larvae showed short and bent tail, round trunk with a small sensory vesicle with close pigmented cells (Figure 1C).

Exposure to higher concentrations caused a significant increase of severe malformations (Tukey's post hoc test: $P < 0.001$) characterized by a curled tail, round trunk, small sensory vesicle with no pigmented organs (Figure 1D).

These kinds of malformations have been reported after exposure to different chemicals and are considered to be due to toxic effects caused by a pleiotropic action of the molecule.^[8] Twenty per cent of larvae treated with 10 μM ISO-1 showed severe malformations. This percentage gradually increased with ISO-1 doses reaching 100% in larvae exposed to 25 μM ISO-1 (Figure 2). Embryos exposed to 50 μM ISO-1 died at early developmental stages (data not showed).

Performing Probit analysis, we determined ISO-1 toxicological features during *Ciona intestinalis* embryogenesis (Figure 3). The concentration of ISO-1 that caused adverse effects in 50% of exposed sample (median effective concentration, EC_{50}) was

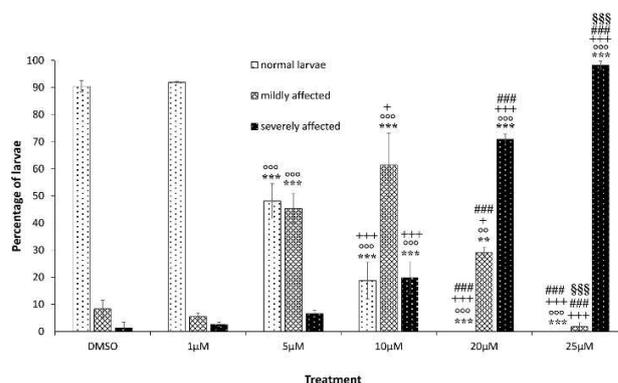


Figure 2. Graph showing the incidence of different phenotypes (normal, mildly, severely affected) in larvae developed from embryos treated with different concentrations of ISO-1. Mean values of three replicates and standard errors are indicated. Legend of symbols: * = differences from control, ° = different from 1 μM ; + = different from 5 μM ; # = different from 10 μM ; § = different from 20 μM . The repetition of each symbol indicates the level of significance according to ANOVA significance codes: $p < 0.001$ ***, $p < 0.01$ **, $p < 0.05$ *.

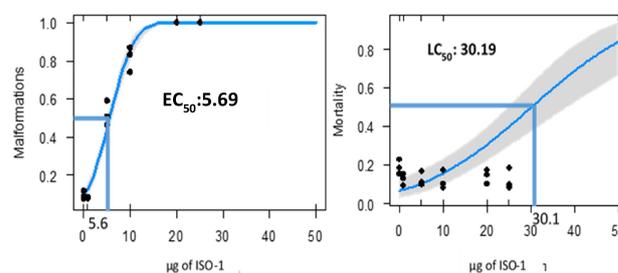


Figure 3. EC_{50} and LC_{50} predicted values calculated with Probit analysis.

5.69 μM . The median lethal concentration (LC_{50}) predicted by the analysis was 30.19 μM .

Fluorescence properties of ISO-1

Hatched larvae developed in ASWH were exposed to different concentrations of ISO-1 for 1 hour. After this time, a fluorescence signal could be observed using a microscope equipped with a UV lamp and a DAPI filter (band pass 352 to 477 nm). The intensity of the signal appeared proportional to the tested concentrations and was detectable only in larvae exposed to concentrations higher than 1 μM . At high magnification, fluorescent signal was clearly localized in the nuclei of the cells (Figure 4).

ISO-1 effects on larvae behaviour

Exposure to ISO-1 interfered with larval ability to swim. After 1 hour, 90% of larvae exposed to 1 μM ISO-1 could swim, a percentage comparable to that of larvae exposed to DMSO alone. On the contrary, only 40% of larvae exposed to 5 μM

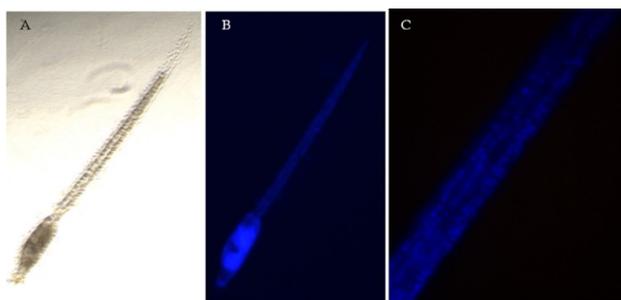


Figure 4. A living larva exposed to 1 μM ISO for 15 minutes. A) Larva observed at the light microscope. B) The same larva of A observed with a UV light and a DAPI filter. The fluorescence signal is concentrated in the trunk. C) Higher magnification of the tail of B shows the signal in the nuclei.

ISO-1 showed a normal swimming behaviour (Figure 5). Concentrations of ISO-1 higher than 5 μM blocked tail movements

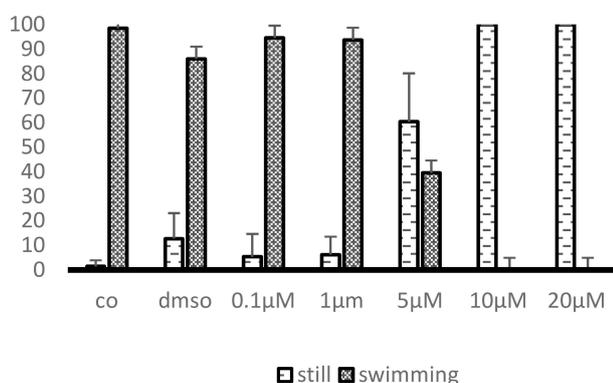


Figure 5. Graph showing the percentage of swimming and resting larvae after an exposure of 15 minutes to different concentrations of ISO-1.

Table 1. Effects of ISO-1 exposure on the number of *Ciona intestinalis* larvae that were not able to swim.

Treatment ^[a]	Estimate	SE	t	P
1 μM ISO-1	8.99E-15	3.89E+00	0	1
5 μM ISO-1	2.27E+01	3.89E+00	5.825	< 0.001
10 μM ISO-1	4.43E+01	3.89E+00	11.393	< 0.001
20 μM ISO-1	3.90E+01	3.89E+00	10.022	< 0.001
Control	-1.33E+0	3.89E+00	-0.343	0.737
DMSO	2.00E+00	3.89E+00	0.514	0.615

[a] The table reports the control-treatment contrasts; significant effects are in bold.

Table 2. Effects of ISO-1 on swimming behaviour and fluorescent signal during the exposure and after rinsing.

Treatment	During exposure		After exposure	
	Swimming	Fluorescence	Swimming	Fluorescence
1 μM ISO-1	+	+	+	-
5 μM ISO-1	-	+	+	+
10 μM ISO-1	-	+	-	+
20 μM ISO-1	-	+	-	+
DMSO	+	-	+	-

resulting in an increasing number of larvae that appeared resting on the bottom of Petri dishes (Figure 5).

The number of resting larvae was significantly higher at concentrations of 5 μM , 10 μM and 20 μM than at control conditions (Table 1).

These effects were partially reversible. In fact, after two short rinses in ASWH, all larvae exposed to 5 μM ISO-1 were able to swim again (Table 2) even if they maintained a faint fluorescence visible mainly in the trunk, where the cells are smaller and densely packed (Figure 6).

Cell viability assay

The experiment of cells viability revealed a viability rate of 96.05% in control treatment, 89.36% in treatment with 0.01 μM ISO-1, 89.36% in treatment with 0.1 μM ISO-1 and 83.33% in treatment with 1 μM ISO-1. Fisher's exact test showed that ISO-1 treatments determined always a significant difference in the rate of viable and dead cells by respect to control treatment ($P \geq 0.01$).

In the present work, we explored the biological properties of 3-Hydroxyisoquinoline derivative ISO-1 in the animal model *Ciona intestinalis*, documenting its potentialities in different aspects of scientific research.

Using an established ascidian-based approach, we identified ISO-1 ability to stain specifically cellular nuclei. This peculiar feature, coupled with our revisited synthesis, which increases the amount of ISO-1 to gram scale, makes ISO-1 an effective vital fluorescent dye with numerous possible applications in *in vivo* tests. Based on our embryotoxicity analysis, ISO-1 affected ascidian development at concentrations higher than 5 μM , with a predicted median lethal concentration of 30.19 μM . These concentrations are far from the ISO-1 dose necessary to induce fluorescence in multicellular living organisms such as ascidians larvae. In fact, the lowest tested concentration that elicited a fluorescent signal was 1 μM . The signal was strong enough for being detected with a simple UV

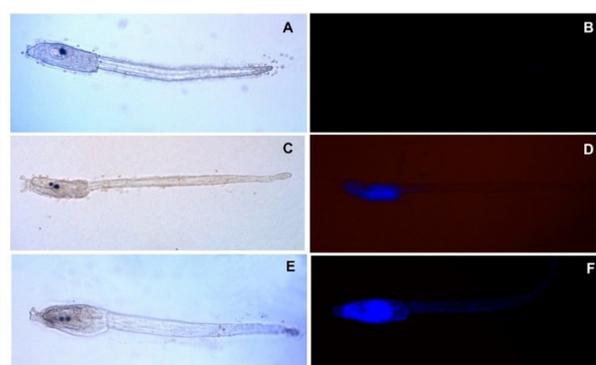


Figure 6. Larvae rinsed in ASWH after exposure to ISO-1 observed at light (A, C, E) and fluorescent microscope (B, D, F). A, B) Larvae exposed to 1 μM ISO-1, the fluorescent signal disappeared completely. C, D) A faint signal was still present in larvae exposed to 5 μM ISO-1. E, F) The fluorescent signal persisted in larvae exposed to 10 μM ISO-1.

lamp and a DAPI filter. Indeed, even low concentrations of ISO-1 can affect the viability of cultured HEK-293 cells, which are more sensitive to the action of drugs than multicellular organisms. Fluorescence localized mainly in cell nuclei and larvae exposed to this concentration appeared normal and healthy, as well as able to swim. These promising results raised the possibility to use ISO-1 as a vital dye to mark nuclei in a reversible way as we demonstrated that ISO-1 labelling could be eliminated by rapid wash in sea water. All these features strongly suggested a high affinity of ISO-1 for the nuclei. Future investigations will be focused on clarifying ISO-1 binding affinity for nucleic acids and its interactions and/or possible damages to DNA after prolonged exposure.

Another fascinating property of ISO-1 is related to its effects on larval behaviour. We found that ISO-1, even at low concentrations such as 5 μM , could reversibly impair larvae swimming abilities. ISO-1 immobilizing effects were transient, lasting as long as the larvae were maintained in the ISO-1 solution. Ascidian larvae movements are determined by lateral contractions of their tail muscles. Muscles contractions are under the control of the central nervous system,^[19] and particularly, are driven by five pairs of GABAergic motor neurons that project axons to the muscle fibres in the tail.^[10,20] Even if the mechanism by which ISO-1 interfered with larvae swimming behaviour remains unclear, we can hypothesize that ISO-1 has neuroactive properties similarly to those displayed by other isoquinoline derivatives.^[21] For example, papaverine is a well-known isoquinoline derivative that has antispasmodic effects on smooth muscles. It exerts its activity by inhibiting oxygen uptake of mitochondria oxidizing glutamate under phosphorylative conditions.^[22] Drotaverine, is an isoquinoline derivative structurally related to papaverine. It inhibits phosphodiesterases hydrolysing cAMP, thereby increasing cAMP concentration, decreasing Ca^{2+} cellular uptake and changing its distribution among the cells. It is used as an antispasmodic drug, with no anticholinergic effects.

Conclusion

Even though the molecular mechanisms by which ISO-1 could exert its biological properties are still to be clearly determined, this novel molecule have showed attractive and promising features worth to be further analysed. The results obtained in ascidian model underlined the potentialities of this chemical, paving the way for future investigations on ISO-1 biological interactions, and its applications as fluorescent biosensor.

Experimental Section

Chemicals and animals

Isochinoline (ISO-1) was synthesized according to our procedure.^[5] Gram-scale synthesis of Ugi product: a solution of 2-iodobenzaldehyde (2.85 g, 12.28 mmol) in TFE/EtOH (1:1, 26 mL) was treated at r.t. with 2,4-dimethoxybenzylamine (1.94 mL, 12.89 mmol). After 30 min the solution was treated at r.t. with phenylpropionic acid

(1.88 g, 12.89 mmol) and *tert*-butylisocyanide (1.46 mL, 12.89 mmol) and the mixture was stirred at 30 °C. After 24 h, the solvent was removed by evaporation. The crude was treated with saturated aqueous NaHCO_3 (60 mL) and extracted three times with CH_2Cl_2 (150 + 50 + 50 mL). The combined organic phases were dried (Na_2SO_4), filtered and concentrated. The residue was purified by crystallization from Et_2O to give the Ugi product (6.19 g, 83%) as a white solid. The analytical data of this compound are in agreement with those already reported by us. Gram-scale synthesis of 3-hydroxyisoquinoline: a solution of Ugi product (5.19 g, 8.50 mmol) in MeCN/ Et_3N (1:1, 80 mL) under argon atmosphere, was treated with $\text{PdCl}_2(\text{PPh}_3)_2$ (316 mg, 0.42 mmol) and formic acid (3.20 mL, 8.50 mmol) at 85 °C for 18 h. After this time, the solvent was evaporated and the crude was diluted with saturated aqueous NH_4Cl (80 mL) and extracted three times with CH_2Cl_2 (150 + 150 + 50 mL). The combined organic phases were filtered through a celite cake, washing thoroughly with CH_2Cl_2 , dried (Na_2SO_4), filtered and concentrated. The residue was diluted with CH_2Cl_2 (40 mL) and treated with TFA (20 mL) at room temperature for 2 h. Then, TFA was evaporated and the crude was diluted with saturated aqueous NaHCO_3 (100 mL), extracted three times with CH_2Cl_2 (150 + 80 + 80 mL), dried (Na_2SO_4), filtered and concentrated. The residue was directly dissolved in dry MeCN (30 mL) and DBU (2.50 mL, 17.00 mmol) was added. The reaction was stirred at r.t. for 4 h, then diluted with CH_2Cl_2 and washed with saturated aqueous NH_4Cl . The combined organic phases were filtered through a celite cake, washing thoroughly with CH_2Cl_2 , dried (Na_2SO_4), filtered and concentrated. The residue was purified by crystallization from $\text{CH}_2\text{Cl}_2/\text{Et}_2\text{O}$ to give the ISO-1 (1.72 g, 61%) as a yellow solid. The analytical data of this compound are in agreement with those already reported by us.^[5] HPLC (see SI) showed a purity of 99.6%.

Stock solution of 100 mM ISO-1 was made in dimethyl sulfoxide (DMSO) and then diluted in filtered artificial sea water with 1 M HEPES pH 8.0 (ASWH) to reach the final test concentrations.

Ciona intestinalis adults were collected from natural populations in Roscoff (France) by the fishing service of the Station Biologique de Roscoff, and reared in aquaria equipped with mechanical, chemical and biological filters. Animals were maintained at 16 ± 1 °C and in constant light conditions to avoid gamete release and stimulate their production.^[17] Eggs and sperm were obtained by dissecting the gonoducts and cross-fertilization was performed. All experiments were carried out at 18 ± 1 °C.

Effects of ISO-1 exposure on *Ciona intestinalis* development

To test the effect of ISO-1 on the development of *Ciona intestinalis*, embryos at two-cell stage (~ 1 hr post fertilization (hpf)^[14]) were exposed to 1, 5, 10, 20, 25 and 50 μM ISO-1 in ASWH. Fresh solutions were prepared every time. Control embryos were maintained in ASWH plus 0.02% DMSO. For each treatment, ~ 100 embryos were transferred into glass Petri dishes ($\varnothing 9$ cm) filled with in 50 ml of experimental solution. Each set of treatments were replicated three time with different batches. Embryos were reared at 18 °C in a thermostatic chamber until they reached the swimming larva stage (~ 18 hpf), and then fixed in 4% paraformaldehyde in phosphate buffer saline (PBS) for 1 h for the following analyses.

The morphological effects of ISO-1 exposure were evaluated under a dissection microscope. Then, fixed larvae were rinsed in PBS 3 times for 10 minutes, mounted on glass slides and carefully observed at a transmission microscope for detailed analysis.

Control and treated larvae were scored according to their morphology and classified in three groups: normal phenotype; mildly affected larvae showing malformations in the trunk and in

the pigmented sensory organs; severely affected larvae displaying short trunk and a curled and/or short tail.

ISO-1 effects on larval behaviour

To test the effects of ISO-1 on larval behaviour, 30 swimming larvae, 1 hour after hatching, were exposed to different concentrations of ISO-1 for 15 min. Then, larvae were observed under an inverted microscope equipped with a UV lamp, a DAPI filter and a DMC5400 camera (Leica). We recorded the larvae for 5 min and scored the number of fluorescent larvae as well as the number of swimming ones. Then, the larvae were rinsed twice in ASWH for 5 min and observed again under the microscope. The number of swimming, resting and fluorescent larvae was annotated.

Cell viability assay

To test the effects on cell viability of ISO-1, 80% confluent HEK-293 cells were exposed to 1, 0.1 and 0.01 μM ISO-1 for 15 min. Suspended cells were centrifuged at 9 g and resuspended in 1 ml PBS. 10 μl of this suspension were mixed with 10 μl of trypan blue stain and allowed to stay at room temperature for 3 min. Viable (unstained) and nonviable cells were counted in a hemocytometer and the percentage of viable cells (viability) was calculated. A Fisher exact test was applied to assess the significance of the observed differences.

Statistical analysis

First, we assessed the effect of ISO-1 on *Ciona intestinalis* larvae development. We performed analyses of variance (ANOVA) followed by HSD Tukey's post hoc test considering the effects of the different ISO-1 concentrations on both mildly and severely affected larvae. Prior to analyses, we verified the homogeneity and normality of the variances. Subsequently, we performed a Probit analysis following the simple least squares regression method to calculate the median lethal concentration (LC_{50}) and the median effective concentration (EC_{50}) of ISO-1 on *Ciona intestinalis* embryos. To assess the effect of ISO-1 on larvae behaviour, we built a linear model (LM) using as dependent variable the number of resting larvae and the ISO-1 concentration as fixed factor. We then used control-treatment contrasts to compare the different exposure conditions against the controls. All the analyses were performed in the R 3.6.3 environment using the packages *vegan*, *visreg* and *lme4*.^[18]

Conflict of Interest

The authors declare no conflict of interest.

Keywords: ascidian · bioassay · fluorescent molecule · nuclear dye · swimming behaviour

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