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Embryotoxicity studies of tri-*n*-butyltin(IV) complexes of 5-[(*E*)-2-(aryl)-1-diazenyl]-2-hydroxybenzoic acid and 2-[(*E*)-2-(3-formyl-4-hydroxyphenyl)-1-diazenyl] benzoic acid on sea urchin development

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The toxicity studies of free 5-[(*E*)-2-(aryl)-1-diazenyl]-2-hydroxybenzoic acid and 2-[(*E*)-2-(3-formyl-4-hydroxyphenyl)-1-diazenyl]benzoic acid and their tri-*n*-butyltin(IV) complexes were evaluated by using sea urchin early developmental stages as recommended model organisms for toxicity tests. The novel complexes, as the parent tri-*n*-butyltin(IV) chloride (TBTCl), caused mitosis block and induced high embryonic mortality in sea urchin. **Copyright © 2005 John Wiley & Sons, Ltd.**

KEYWORDS: tri-*n*-butyltin; 5-[(*E*)-2-(aryl)-1-diazenyl]-2-hydroxybenzoic acid; 2-[(*E*)-2-(3-formyl-4-hydroxy phenyl)-1-diazenyl]benzoic acid; sea urchins; toxicity

INTRODUCTION

Organotin(IV) derivatives are used as active components of antifouling paints to prevent the settling of algae and benthic invertebrate organisms on surfaces immersed in fresh and marine water. These organotin compounds interfere with the reproductive cycle of the marine organism owing to their stability in the aquatic ecosystem. Apart from numerous literature reports on the toxicity of organotin(IV) compounds,[1–16] they have been detected also in aqueous ecosystems[17,18] and in marine plant and animal tissues.[19–21]

Sea urchin embryos are widely used systems for investigating the development of molecular mechanisms, mainly because a large number of eggs can be obtained easily, fertilized externally and both eggs and embryos are readily manipulated.[22] Because of their sensitivity, sea urchin developing embryos and larvae are also interesting models for ecotoxicological studies and are recommended for short-term toxicity testing.[23,24] The embryotoxicity of *n*-butyltin derivatives is concentration-dependent and increases in proportion with the number of butyl groups. As reported by Marin *et al.*,[25] the toxicity level of TBT (tri-*n*-butyltin(IV)$^+$) for *Paracentrotus lividus* embryos is at least 100 times higher than that of DBT (di-*n*-butyltin(IV)$^+$), and DBT is about 50 times more toxic than MBT (mono-*n*-butyltin(IV)$^+$). These differing effects may be related to the different membrane-crossing ability of *n*-butyltin derivatives, which increases with lipophilicity and therefore with the number of butyl groups. As a continuation of previous work on the toxicity studies on the second instar of *Aedes aegypti* and *Anopheles stephensi* mosquito larvae,[26,27] the present report details a study of the toxicity studies of free 5-[(*E*)-2-(aryl)-1-diazenyl]-2-hydroxybenzoic acid (L^1HH') and 2-[(*E*)-2-(3-formyl-4-hydroxyphenyl)-1-diazenyl]benzoic acid ($L^{2-5}HH'$) and their tri-*n*-butyltin(IV) complexes *n*-Bu_3SnL^1H (**1**), *n*-Bu_3SnL^2H (**2**), *n*-Bu_3SnL^3H (**3**), *n*-$Bu_3Sn\{L^4H\}OH_2 \cdot$ bipy (**4**) and *n*-Bu_3SnL^5H (**5**) using sea urchin early developmental

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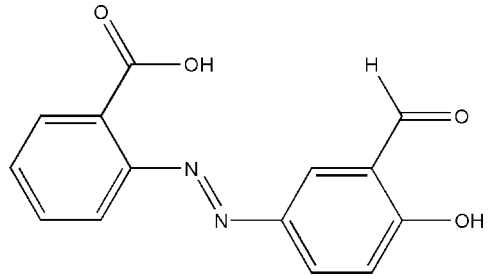


Figure 1. Structure of L^1HH' where H and H′ represent the hydroxyl and carboxyl protons, respectively.

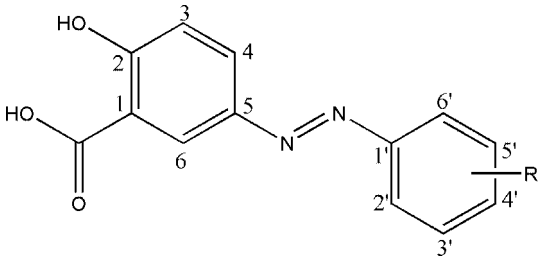


Figure 2. Structure of $L^2HH'(R = 3'\text{-}CH_3)$, $L^3HH'(R = 4'\text{-Cl})$, $L^4HH'(R = 4'\text{-Br})$ and $L^5HH'(R = 4'\text{-}NO_2)$, where H and H′ represent the hydroxyl and carboxyl protons, respectively.

stages. The preparation of L^1HH' (Fig. 1) and $L^{2-5}HH'$ (Fig. 2) and their tri-*n*-butyltin complexes (**1**–**5**) has been described in an earlier report,[26,28] along with the spectroscopic (IR, ^{1}H-, ^{13}C- and ^{119}Sn-NMR and ^{119}Sn Mössbauer) data and some representative X-ray crystal structures.

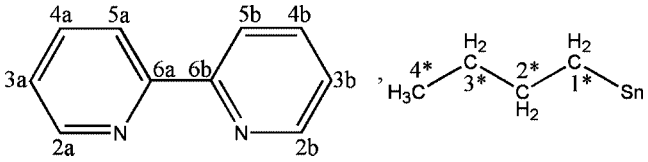
Moreover, the aim of this work is to extend analyses of effects of new organotin compounds towards two species of sea urchin, *Paracentrotus lividus* and *Sphaerechinus granularis*, in order to compare variation in the impact incidence of contaminant exposure among different species.

EXPERIMENTAL

Materials

Both L^1HH'[29] and $L^{2-5}HH'$[28] and their tri-*n*-butyltin complexes, i.e. *n*-Bu_3SnL^1H (**1**),[26] *n*-Bu_3SnL^2H (**2**),[28] *n*-Bu_3SnL^3H (**3**)[28] and *n*-Bu_3SnL^5H (**5**),[28] were prepared by reported methods. Complex *n*-$Bu_3Sn\{L^4H\}OH_2 \cdot$ bipy (**4**) was prepared by reacting L^4HH', $(n\text{-}Bu_3Sn)_2O$ and bipyridine in 2 : 1 : 1 molar proportions in anhydrous toluene under reflux conditions for 5 h. The water generated during the reaction was removed by a Dean-Stark apparatus. The reaction mixture was filtered and concentrated to give a jelly-like mass that was dried under vacuum. The orange-coloured mass was dissolved in hexane and kept in a refrigerator for 30 min to deposit an orange microcrystalline product. The product was isolated by filtration and dried under vacuum. Yield: 71%, m.p.: 100–102 °C. Anal. found: C, 53.55; H, 5.60, N, 7.20%. Calc. for $C_{35}H_{44}N_4O_4BrSn$: C, 53.60; H, 5.66; N, 7.15%. IR (cm^{-1}): 1628 $\nu(OCO)_{asym}$. ^{1}H-NMR ($CDCl_3$), δ_H: ligand skeleton: 7.05 [dd, 1H, H-3], 7.62 [d, 2H, H-2′ & H-6′], 7.77 [d, 2H, H-3′ & H-5′], 8.03 [dd, 1H, H-4], 8.49 [d, 1H, H-6], 12.06 [brs, 1H, OH]; bipy skeleton: 7.31 [td, 2H, H-3a and H-3b], 7.82 [td, 2H, H-4a and H-4b], 8.40 [dd, 2H, H-5a and H-5b], 8.68 [dd, 2H, H-2a and H-2b]; Sn-^{n}Bu skeleton: 0.95 [t, 9H, H-4*], 1.39 [m, 12H, H-2* and H-3*], 1.73 [m, 6H, H-1*] ppm. ^{13}C-NMR ($CDCl_3$), δ_C: ligand skeleton: 114.8 [C-1], 117.9 [C-3], 123.6 [C-2′ and C-6′], 124.6 [C-6], 127.7 [C-4], 128.5 [C-4′], 132.2 [C-3′ and C-5′], 145.2 [C-5], 151.6 [C-1′], 164.6 [C-2], 174.1 [CO_2]; bipy skeleton: 121.1 [C-3a and C-3b], 124.1 [C-5a and C-5b], 136.7 [C-4a and C-4b], 149.1 [C-2a and C-2b], 156.3 [C-6a and C-6b]; Sn-^{n}Bu skeleton: 13.5 [C-4* (−)], 17.1 [C-1* (349)], 26.9 [C-3* (63)], 27.7 [C-2* (−)], ppm. ^{119}Sn NMR ($CDCl_3$), δ_{Sn}: 138.7 ppm. Note: The composition of this compound matches that of the $Ph_3Sn\{O_2CC_6H_3\text{-}p\text{-}OH[N = N(C_6H_4\text{-}2\text{-}CH_3)]\}OH_2 \cdot C_{10}H_8N_2$ analogue.[30] The ^{119}Sn NMR data indicate a tetrahedral geometry in solution, in line with its triorganotin complex.[28,30] Several attempts to obtain a single crystal failed and hence conclusive information about the structure could not be obtained in the solid state.

The $^nJ(^{13}C\text{-}^{119/117}Sn)$ mean values (Hz) are given in parentheses. For assignments of NMR signals, refer to Fig. 2 for the numbering schemes of the ligand skeleton, whereas for bipy and Sn-^{n}Bu skeletons, see diagram below.



Preparation of stock solutions of the starting acids and organotin(IV) complexes

Filtered Sea Water (FSW, salinity 37%) was obtained by filtering sea water, collected from the Gulf of Palermo, through a millipore (0.22 μ). A 0.1 mM tri-*n*-butyltin(IV)chloride (TBTCl) solution was prepared by dissolving TBTCl (Merck) in FSW containing 0.5% dimethylsulfoxide (DMSO). Then 10^{-5} and 10^{-7} M solutions were obtained by dilution and their total tin content was checked using a Perkin Elmer 3100 atomic absorption spectrometer equipped with a Perkin Elmer 100 flow injection analysis system for atomic absorption spectrometry, according to a standard procedure.[31] The solvent DMSO (Merck) was used owing to the low solubility of TBTCl in FSW.

Biological tests

Paracentrotus lividus and Sphaerechinus granularis

Adult specimens of *Paracentrotus lividus (P. lividus)* and *Sphaerechinus granularis (S. granularis)* (Echinoidea, Echinodermata) were collected from the Gulf of Palermo. Male and female gametes were obtained by introcoelomic injection

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of 0.5 M potassium chloride. Eggs transferred onto Syracuse dishes were reared in FSW, while the sperm were diluted before insemination to a final suspension of approximately 0.1% (v/v). Soon after fertilization, the eggs, at the two- to four-cell stage, were incubated for 48 h in different solutions at 10^{-5} and 10^{-7} M concentrations of free $L^{1-5}HH'$ and their tri-*n*-butyltin(IV) complexes **1**–**5**, in simple FSW or in FSW containing DMSO (0.5%). Several other cells were incubated for comparison purposes in 10^{-5} and 10^{-7} M TBTCl solutions containing DMSO (0.5%). All the experiments were performed at 22 °C and the pH of the solutions obtained was controlled and maintained at the normal pH of seawater (7.76–7.78) by alternating 10 h of light and 14 h of dark during the day. The observations were made with a Leitz Diaplan microscope and photographs were taken using Kodak Tmax film.

All the experiments were repeated three times ($n = 100$ embryos for each replicate) and the values are means ± SD.

RESULTS

In order to evaluate possible cytotoxicity of the tri-*n*-butyltin(IV) complexes, the development of *P. lividus* and *S. granularis* embryos after treatment with complexes **1**–**5** was investigated at different concentrations. The embryos in FSW, used as controls or in FSW containing 0.5% DMSO developed regularly. In fact, once fertilized, the sea urchin eggs started to cleave at a very high frequency. The first cleavage occurred within about 60 min, giving rise to the two-cell stage. Cell divisions occurred about every 30 min after this stage, which is called the cleavage stage. After 24 h the embryos of *P. lividus* and *S. granularis* were at blastula (Fig. 3) and gastrula (Fig. 4) stages, and after 48 h they were at prism stage (10%) and at pluteus larva stage (90%) (Fig. 5).

The 10^{-5} M TBTCl solution immediately blocked the mitosis and the embryos presented a chaotic blastomere position; in 10^{-7} M TBTCl solution the embryos arrested at the anomalous 4–8–16-cell stage.

In 10^{-7} M concentration, the *P. lividus* and *S. granularis* embryos incubated for 48 h in the free acid containing solutions developed like the controls. In fact, they gave rise to normal plutei and embryos at prism stage. The solutions of L^1HH' and L^2HH' at 10^{-5} M concentration blocked the development of the embryos of the two species at the swimming blastula and gastrula stage after 48 h of treatment. In contrast, the embryos incubated in L^3HH' solution for 24 h arrested at the blastula stage; after 48 h they recovered and developed up to normal pluteus stage. The 10^{-5} M L^5HH' and L^4HH' solutions inhibited the development in the blastula–gastrula–prism stage after 48 h of incubation. In conclusion, the free acids at 10^{-5} M inhibited or blocked the formation of the germinal layers in sea urchin, i.e. ectoderm, mesoderm and endoderm.

Tri-*n*-butyltin(IV) complexes **1**–**5** at 10^{-5} M concentration blocked embryo development of the two species at the two- to four-cell stages after 48 h of exposure (Fig. 6). The blastomeres were arranged in a chaotic pattern, with a non-uniform distribution of hyaline and dark areas. At 10^{-7} M concentration the **1**–**5** complex solutions inhibited cleavage of the eggs in the 8–16–32-blastomere strongly anomalous stages (Fig. 7). In conclusion, complexes **1**–**5** exerted a toxic activity on *P. lividus* and *S. granularis* embryos at the first development stages and the sensitivity of the embryos was the same.

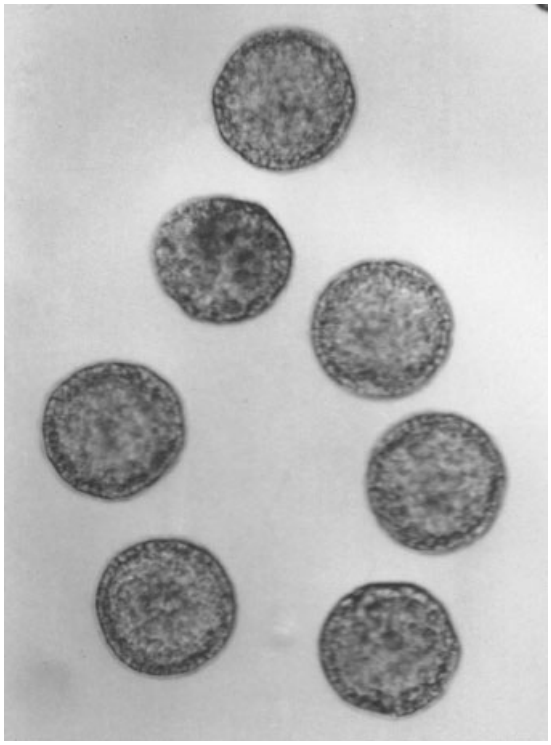

Figure 3. Sea urchin control embryos and treated embryos with tri-*n*-butyltin complexes (**1**–**5**). Anomalous embryos exposed to tri-*n*-butyltin complexes **1**–**5** solutions did not show any significant difference under optical microscopy (magnification × 144): *S. granularis* control blastula stage 24 h after fertilization.

Results obtained after embryo development analyses are reported in Table 1 as percentages and compared with the controls.

DISCUSSION

The embryo toxicity of the five tri-*n*-butyltin(IV) complexes (**1**–**5**) was investigated. Biological activity tests of the complexes demonstrated the following:

(i) Embryos exposed to the tri-*n*-butyltin(IV) complexes (**1**–**5**) at 10^{-5} and 10^{-7} M solutions presented blocks and strong developmental anomalies.

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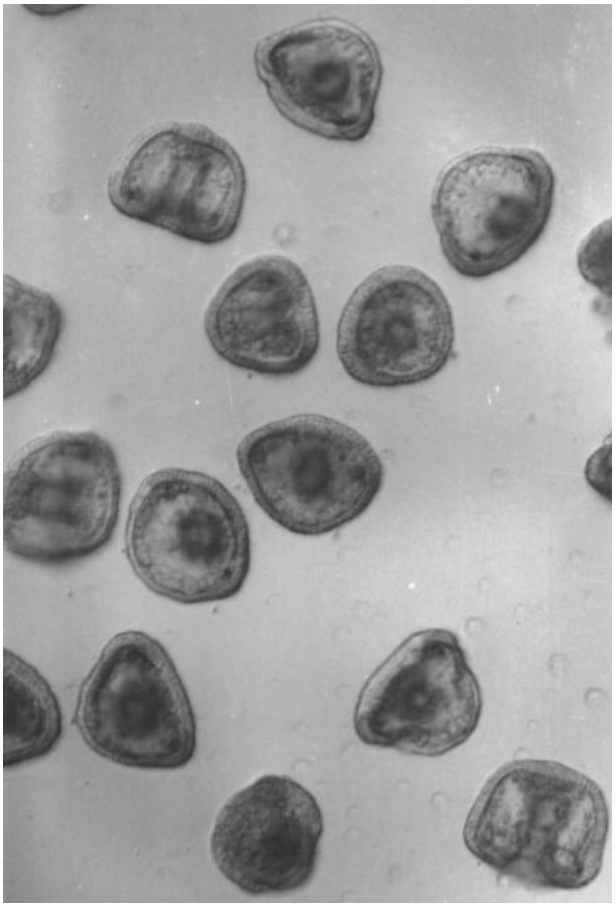

Figure 4. Sea urchin control embryos and treated embryos with tri-*n*-butyltin complexes (**1**–**5**). Anomalous embryos exposed to tri-*n*-butyltin complexes **1**–**5** solutions did not show any significant difference under optical microscopy (magnification × 144): *P. lividus* control gastrula stage 24 h after fertilization.

Figure 5. Sea urchin control embryos and treated embryos with tri-*n*-butyltin complexes (**1**–**5**). Anomalous embryos exposed to tri-*n*-butyltin complexes **1**–**5** solutions did not show any significant difference under optical microscopy (magnification × 144): *P. lividus* swimming pluteus larvae 48 h after fertilization.

(ii) Embryos treated with free $L^{1-5}HH'$ at 10^{-5} M concentration stopped developing at the blastula stage. At 10^{-7} M they developed regularly as the control.
(iii) Embryos treated with TBTCl (positive control) did not develop any more.
(iv) The sensitivity of *S. granularis* embryos was like that of *P. lividus*.

The mechanism of action of the tested compounds is still unknown, but literature data on the highly toxic tri-*n*-butyltin(IV)$^+$ indicate that TBT caused mitosis block and cellular death. As suggested in a previous report,[32] the present data imply that the cytotoxic alterations involving embryos at early stages of development are also related to chromosomal disorders, mainly resulting in structural alterations of the chromosomes and/or inhibition of mitotic spindle, the latter presumably through modification of the chemical structure of the tubulin. The results in this study for *P. lividus* embryos are comparable with those observed for these species in similar studies, where toxic effects of organotin compounds were examined towards embryos.[32,33] Blockage of the mitosis and significant chromosomal alterations or anomalies on *P. lividus* embryos treated with triorganotin(IV) *L*-homocysteate derivatives at 10^{-5} and 10^{-7} M concentrations were identified by Pellerito *et al*.[33] In addition, blockage of egg cleavage by TBTCl solutions ranging from 10^{-4} up to 10^{-9} M inhibited first and second cleavage in a dose-dependent manner, accompanied by an inhibition of protein and DNA synthesis.[34] Similar toxic effects have been observed in TBTCl-exposed ascidian embryos: consistent ultrastructural damage involving the mitochondria and the membranes was observed in ascidian eggs after treatment with 10^{-5} and 10^{-7} M TBTCl solutions by Mansueto *et al*.[35] and Villa *et al*.[36]

Even if the 10^{-5} M solution could be higher than those of the environmental antifouling coating found in the aqueous environment, many researchers have shown that the toxic effect on many processes of different species are dose and time dependent:[24,25,32–36] the permanence of gametes, embryos and larvae in polluted environments varies in different species and consequently their integrity could be compromised in relation to the incubation time. In addition, it is known that the sediments accumulate organotin compounds and these can be remobilized and resuspended.

In conclusion, the tri-*n*-butyltin complexes of the present investigation (**1**–**5**) induced high embryonic mortality in *P. lividus* and *S. granularis* and were as toxic as the

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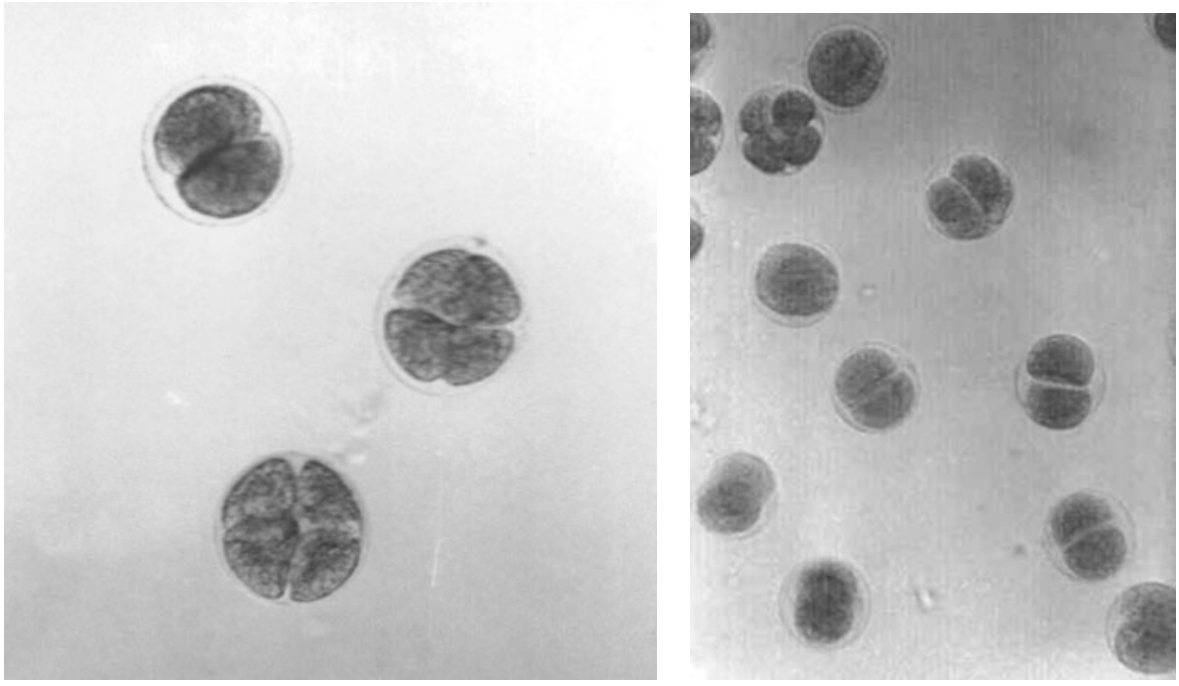
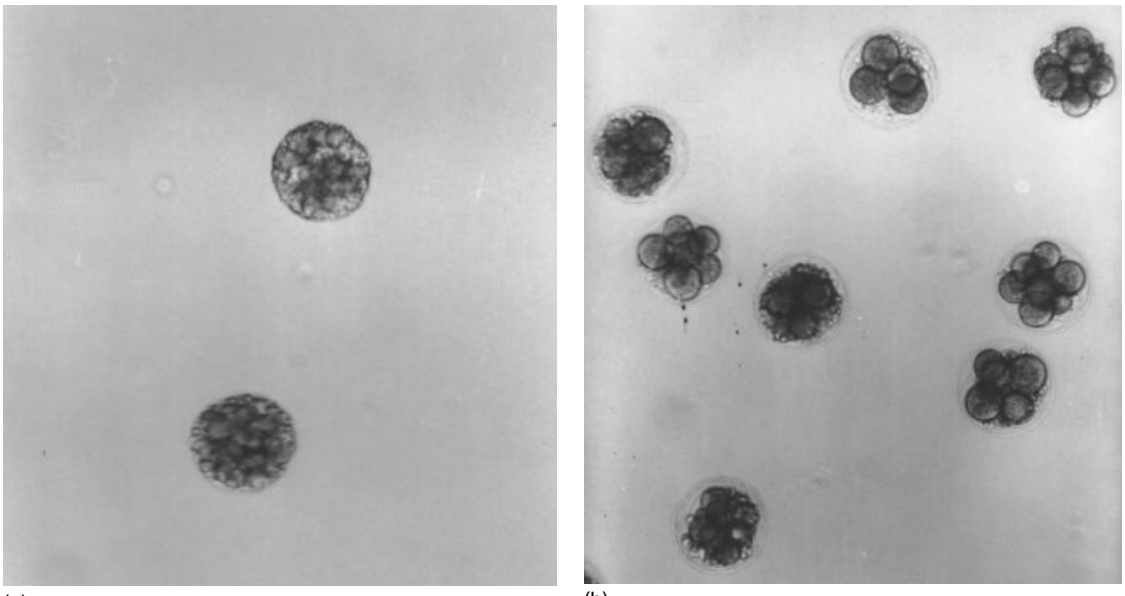


(a) (b)

Figure 6. Sea urchin control embryos and treated embryos with tri-*n*-butyltin complexes (**1**–**5**). Anomalous embryos exposed to tri-*n*-butyltin complexes **1**–**5** solutions did not show any significant difference under optical microscopy (magnification × 144): *P. lividus* (a) and *S. granularis* (b) anomalous embryos after incubation in 10^{-5} M complex **2** for 48 h. The blastomeres are different sizes and are blocked at the two to four-cell stage.



(a) (b)

Figure 7. Sea urchin control embryos and treated embryos with tri-*n*-butyltin complexes (**1**–**5**). Anomalous embryos exposed to tri-*n*-butyltin complexes **1**–**5** solutions did not show any significant difference under optical microscopy (magnification × 144): *P. lividus* (a) and *S. granularis* (b) anomalous embryos after incubation in 10^{-7} M complex **2** for 48 h and arrested at anomalous embryos.

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Table 1. Results of sea urchin embryo development incubated in 10^{-5} and 10^{-7} M $L^{1-5}HH'$ solutions and their tri-*n*-butyltin(IV) complexes **1**–**5**

Free acid complex	Conc. (M)	Par 2	Sph 2	Par 4	Sph 4	Par 8	Sph 8	Par 16	Sph 16	Par 32	Sph 32	Par B	Sph B	Par G	Sph G	Par P	Sph P	Par L	Sph L
L^1HH'	10^{-5}											60 ± 5	65 ± 5	40 ± 5	35 ± 5				
	10^{-7}															10 ± 2	15 ± 3	90 ± 2	85 ± 3
L^2HH'	10^{-5}											90 ± 6	90 ± 5	10 ± 6	10 ± 3				
	10^{-7}															10 ± 5	15 ± 7	90 ± 5	85 ± 7
L^3HH'	10^{-5}													20 ± 3	15 ± 5	10 ± 2	10 ± 2	70 ± 6	75 ± 7
	10^{-7}															10 ± 3	10 ± 2	90 ± 3	90 ± 2
L^4HH'	10^{-5}											90 ± 2	80 ± 2	5 ± 2	10 ± 3	5 ± 4	10 ± 3		
	10^{-7}															10 ± 2	10 ± 2	90 ± 2	90 ± 2
L^5HH'	10^{-5}											35 ± 3;	40 ± 2	10 ± 2	5 ± 1	55 ± 5	55 ± 2		
	10^{-7}															10 ± 3	10 ± 2	90 ± 3	90 ± 2
1	10^{-5}	80 ± 6	75 ± 2	20 ± 6	25 ± 2														
	10^{-7}					25 ± 3	30 ± 2	15 ± 1	15 ± 1	60 ± 4	55 ± 1								
2	10^{-5}	85 ± 5	80 ± 5	15 ± 5	20 ± 5														
	10^{-7}					10 ± 2	10 ± 3	25 ± 3	30 ± 2	65 ± 4	60 ± 3								
3	10^{-5}	70 ± 5	75 ± 7	30 ± 5	25 ± 7														
	10^{-7}					25 ± 5	20 ± 1	15 ± 2	15 ± 2	60 ± 6	65 ± 1								
4	10^{-5}	80 ± 4	75 ± 5	20 ± 4	25 ± 5														
	10^{-7}					20 ± 3	20 ± 3	25 ± 1	20 ± 1	55 ± 3	20 ± 3								
5	10^{-5}	90 ± 2	90 ± 4	10 ± 2	10 ± 4														
	10^{-7}					20 ± 3	15 ± 3	10 ± 2	15 ± 2	70 ± 3	70 ± 3								

The controls (embryos in FSW) give rise to 90% swimming pluteus larva. Results are given as the mean percentage of developed or arrested embryos (±SD) in three different experiments from the two- to four-cell stage for 48 h of exposure; Par = *Paracentrotus. lividus*; Sph = *Sphaerechinus granularis*; cleavage stage: 2, 4, 8, 16 and 32 (blastomeres), B: blastula stage, G: gastrula stage, P: prism stage, L: pluteus larva.

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triorganotin(IV) parent compound, independently of the presence of the ligands.

Acknowledgements

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