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CYTOTOXIC ACTIVITY OF INTERMEDIATES AND SIDE PRODUCTS OF ECHINOCHROME SYNTHESIS

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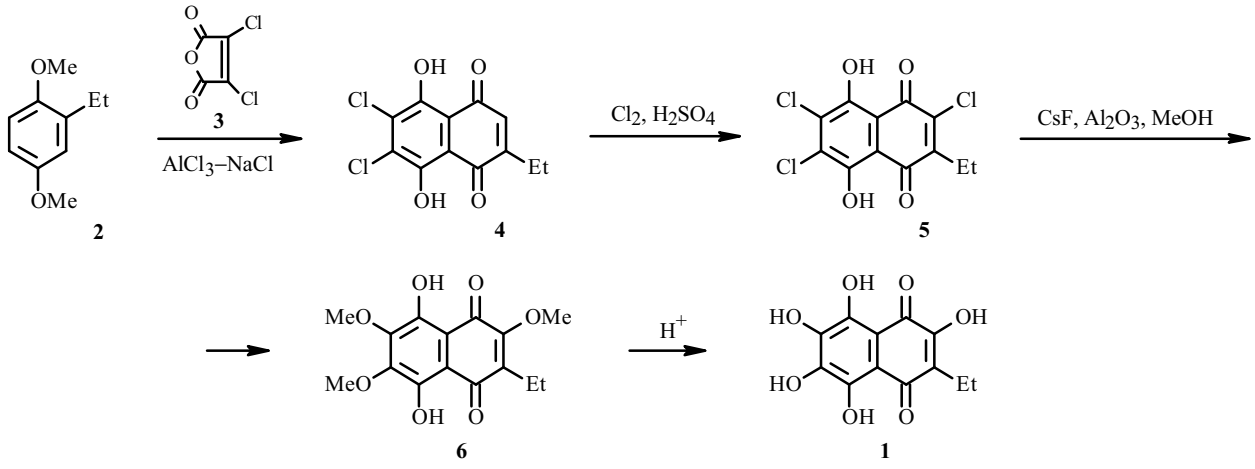
The cytotoxic activity of the principal intermediates and side products of echinochrome synthesis toward gametes of the sea urchin Strogylocentrotus intermedius *was studied. Di- and trichloro naphthazarin derivatives with two vicinal Cl atoms were the most active.*

Key words: naphthazarins, cytotoxic activity.

Echinochrome (2,5,6,7,8-pentahydroxy-3-ethyl-1,4-naphthoquinone) (**1**), the quinoid pigment of the sea urchin *Scaphechinus mirabilis* [1], is the biologically active substance (BAS) of the original antioxidant preparation "Gistokhrom" that is used to treat acute myocardial infarct, ischemic cardiac disease, and ophthalmologic diseases[2].

One of the most promising synthetic routes to **1** is depicted in Scheme 1. According to it, ethylhydroquinone dimethyl ether (**2**) is cycloacylated by dichloromaleic anhydride (**3**) to form dichloroethylnaphthazarin (**4**), which is further chlorinated. A halide atom is replaced by methoxy in the resulting trichloro derivative (**5**) (yield of the last step 68%) [3]. The final step is demethylation of the trimethylether (**6**) of **1** by appropriate reagents [4].



Scheme 1

Our goal was to isolate and establish the chemical structures of the principal side products that are formed during preparation of both echinochrome trimethylether (**6**) and **1** itself and to determine the cytotoxic activity of the isolated compounds and intermediates.

Side products were isolated by repeated chromatography of the reaction mixture using TLC and HPLC for monitoring. According to Scheme 1 for preparing **6**, there was a high probability of observing in the reaction mixture products of incomplete substitution of Cl by methoxy, i.e., di- and monochloro derivatives of **1**. As hypothesized, we isolated and identified

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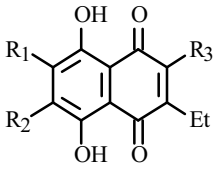
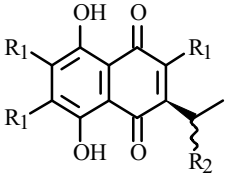
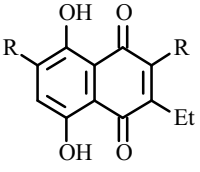
2,6-dimethoxy-7-chloro-3-ethylnaphthazarin (**7**) [5], 2,7-dimethoxy-6-chloro-3-ethylnaphthazarin (**8**) [5], 7-hydroxy-2,6-dichloro-3-ethylnaphthazarin (**9**), 6-hydroxy-2,7-dichloro-3-ethylnaphthazarin (**10**), 2,6,7-trimethoxy-3-(1′-methoxyethyl)naphthazarin (**11**), and 2,7-dimethoxy-6-ethylnaphthazarin (**12**) [6]. The last compound is the dimethylether of ethylmompain (2,7-dihydroxy-3-ethylnaphthazarin). Ethylmompain (**13**) itself, which was first isolated from spines of the sea urchin *Echinus diadema* [7], was observed among the impurities of natural BAS echinochrome [8].



7 - 10, 14, 17 - 19



11, 15, 16



12, 13

7: R_1 = Cl, R_2 = R_3 = OMe; **8:** R_1 = R_3 = OMe, R_2 = Cl; **9:** R_1 = OH, R_2 = R_3 = Cl; **10:** R_1 = R_3 = Cl, R_2 = OH
11: R_1 = R_2 = OMe; **12:** R = OMe; **13:** R = OH; **14:** R_1 = R_2 = Cl, R_3 = OH; **15:** R_1 = R_2 = Cl
16: R_1 = OMe; R_2 = OH; **17:** R_1 = R_2 = OMe, R_3 = OH; **18:** R_1 = R_3 = OH, R_2 = OMe; **19:** R_1 = OMe, R_2 = R_3 = OH

Naphthazarins **9**-**11** are new compounds, the structures of which were established using IR and NMR spectroscopy and mass spectrometry. However, the mutual locations of the Cl atom and the hydroxyl on C-6 and C-7 could not be definitively assigned for the two isomeric hydroxychloronaphthazarins **9** and **10**. The proposed assignments in the PMR and ^{13}C NMR spectra of the compounds are ambiguous. The third possible hydroxychloronaphthazarin isomer 2-hydroxy-6,7-dichloro-3-ethylaphthazarin (**14**) was synthesized previously [9] and is a key intermediate in the synthesis of echinochrome by the literature method [10] and was not observed among the studied side products.

2,6,7-Trimethoxy-3-(1′-methoxyethyl)naphthazarin (**11**) was apparently formed via substitution of the Cl atoms by methoxyls in 2,6,7-trichloro-3-(1′-chloroethyl)naphthazarin (**15**). According to the scheme [3], it can be assumed that **15** was formed as a side product via chlorination of 2,6,7-trichloro-3-ethylnaphthazarin (**5**). Compound **11** is the first synthesized methylether of lomazarin [2,6,7-trimethoxy-3-(1′-hydroxyethyl)naphthazarin, **16**], which was isolated earlier from roots of *Lomandra hastilis* (Liliaceae) [11].

Side products from hydrolysis of echinochrome trimethylether (**6**) [4], as expected, were dimethoxy and monomethoxy echinochrome derivatives 2-hydroxy-6,7-dimethoxy-3-ethylnaphthazarin (**17**), 2,7-dihydroxy-6-methoxy-3-ethylnaphthazarin (**18**), and 2,6-dihydroxy-7-methoxy-3-ethylnaphthazarin (**19**). Monomethylethers **18** and **19** are natural compounds that were isolated earlier from *Diadema antillarum* [12].

Gametes of sea urchin, which are widely used for biological testing because of the timing of their isolation [13], were used to study the cytotoxic activity of the synthetic naphthazarins. Natural echinochrome is known not to be cytotoxic toward *Strogyloctytrotus intermedius* sea urchin nymphs [14]. We investigated the cytotoxic activity of the isolated side products **7**-**12** and **17**-**19** and samples of intermediates in the synthesis of echinochrome **4**-**6** [3, 4] and **14** [4, 10]. Table 1 gives the results.

With the exception of **6**, all tested naphthazarins, depending on the concentration, inhibited partially or completely embryo development. In contrast with known cytostatics, which block division of ova and turnover of sea urchin embryos immediately after adding them [15], the inhibiting action of the tested naphthazarins on developing embryos was observed both immediately in the early stages and gradually during the whole incubation period. Lysis of blastomers was observed after adding high doses of the compounds; anomalous development of embryos, lower doses. Nevertheless, all compounds could be divided into two groups. These were **7**-**12** and **17**-**19** that were weakly cytotoxic and **4**, **5**, and **14** with two vicinal Cl atoms that were 1-2 orders of magnitude more active than the others. In this instance, the mutual locations of the Cl atoms in the naphthazarins had a decisive effect on their biological properties. Therefore, the presence of even traces of **4**, **5**, and **14** in the final product, i.e., echinochrome, can affect its quality.

Thus, structures of the principal side products formed during synthesis of echinochrome were established. Compounds with the highest cytotoxic activity were identified.

TABLE 1. Cytotoxic Activity of **4-12**, **14**, and **17-19**

Compound	MW	MED, μg/mL*		LD_{50}, μg/mL**
		1 blastomer	1-8 blastomers	
4	287.1	2.40±0.02 (L)	0.60±0.01 (1-4 bl)	0.30±0.01
5	321.54	3.3±0.3 (L)	0.38±0.02 (1-2, L)	0.048±0.002
6	308.29	>1925	>1925	>1925
7	312.71	80.0±7.0 (L)	58.5±5.5 (1-4 bl, an)	50.0±3.0
8	312.71	110.0±7.0 (L)	82.5±3.5 (1-4 bl, an)	25.0±6.0
9	303.10	42.0±1.5	24.0±1.0 (1-4 bl, an)	10.0±0.3
10	303.10	115.0±9.5 (L)	60±2.8 (1-8 bl, an)	38.0±0.2
11	338.310	163.0±5.0 (L)	95.5±4.5 (1-2 bl, an)	27.0±1.5
12	278.26	400±100 (L)	150±25 (1-2 bl, an)	58.0±7.5
14	303.10	3.0±0.13 (L)	1.55±0.06 (1-4 bl, an)	0.23±0.11
17	294.26	<500	450±50 (1-8 bl, an)	250±50
18	280.23	130.0±8.0	80.0±6.5 (1-2 bl, an)	60.0±2.5
19	280.23	195.0±5.0 (L)	160.0±5.0 (1-2 bl, an, L)	97.5±0.5

*Minimal effective dose stopping division and killing fertilized ova in 1 and 1-8 blastomers.

**Concentration lethal to 50% of embryos within a day. >1925 means that the compound at a concentration of 1925 μg/mL did not exhibit cytotoxic activity. The activity of the compounds at concentrations >1925 μg/mL was not investigated. bl, blastomers; L, lysis of blastomers; an, anomalous development of blastomers. Data from 5-6 experiments are given. Confidence range $p \leq 0.05$.

EXPERIMENTAL

Melting points were determined on a Boetius heating stage and are uncorrected. PMR and ^{13}C NMR spectra in $CDCl_3$ (internal standard Me_4Si) were recorded on a Bruker DRX-500 spectrometer (500.13 MHz and 125 MHz). Mass spectra (EI) were obtained in an LKB-9000S instrument with direct sample introduction at ionizing-electron energy 70 eV. The purity of the compounds was monitored by TLC on Merck 60F-254 plates using hexane:acetone (3:1) and HPLC. Pure compounds were isolated by preparative TLC on plates (20 × 20 cm) with a fixed layer of silica gel (H^+-form, 5-40 μm) [16] or column chromatography over a column of silica gel (L 40/100 μm, H^+-form). Elemental analyses were performed on a Flash EA1112 C,H,N-analyzer and agree with those calculated for all compounds.

HPLC was carried out on a LaChrom (Merck Hitachi) liquid chromatograph equipped with an L-7100 pump, L-7400 UV detector, L-7300 thermostat, D-7500 integrator, and Agilent Technologies Zorbax Eclipse XDB-C18 column (3.5 μm, 75 cm × 4.6 mm) with a Hypersil ODS guard column (4.0 cm × 4.0 mm). The column was thermostatted at 30°C. Impurities were separated by a mixture of solvents A (water + glacial acetic acid, 1%) and B (CH_3CN + glacial acetic acid, 1%) using the program isocratic, 90% A, 10% B, 0-5 min; gradient 90% A, 10% B to 10% A, 90% B, 5-35 min. The analysis time was 35 min.

Compounds **4-8**, **12**, **14**, and **17-19** were identified by direct comparison with authentic samples and comparison of their physical chemical properties with the literature values.

7-Hydroxy-2,6-dichloro-3-ethylnaphthazarin (9). Retention time 20.20 min, mp 278-280°C (dec.) (hexane:acetone). IR spectrum ($CHCl_3$, ν, cm^{-1}): 3398 (OH), 1607 (C=O), 1457, 1426. PMR spectrum (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 1.22 (3H, t, J = 7.6, CH_3), 2.97 (2H, q, J = 7.6, CH_2), 12.09 (1H, s, α-OH), 13.06 (1H, s, α-OH). ^{13}C NMR spectrum (125 MHz, $CDCl_3$): 181.17, 178.9, 157.7, 155.7, 153.5, 146.9, 132.2, 119.3, 107.97, 107.76, 21.5, 11.9. Mass spectrum (EI, 70 eV, *m*/*z*, I_{rel}, %): 306/304/302 (100) $[M]^+$.

6-Hydroxy-2,7-dichloro-3-ethylnaphthazarin (10). Retention time 19.30 min, mp 170-174°C (hexane:acetone). PMR spectrum (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 1.22 (3H, t, J = 7.6, CH_3), 2.98 (2H, q, J = 7.6, CH_2), 12.14 (1H, s, α-OH), 13.09 (1H, s, α-OH). ^{13}C NMR spectrum (125 MHz, $CDCl_3$): 181.4, 179.1, 157.6, 155.6, 153.6, 147.9, 132.6, 132.1, 108.0, 107.8, 21.6, 12.1. Mass spectrum (EI, 70 eV, *m*/*z*, I_{rel}, %): 306/304/302 (100) $[M]^+$.

2,6,7-Trimethoxy-3-(1′-methoxyethyl)naphthaarin (11). Retention time 21.94 min, mp 53-63°C (hexane:acetone). PMR spectrum (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 1.60 (3H, d, J = 6.9, CH_3), 4.97 (1H, q, J = 6.9, CH), 3.31 (3H, s, OCH_3), 4.07 (3H, s, OCH_3), 4.09 (3H, s, OCH_3), 4.14 (3H, s, OCH_3), 12.91 (1H, s, α-OH), 13.28 (1H, s, α-OH). ^{13}C NMR spectrum (125 MHz, $CDCl_3$): 181.0, 179.7, 162.6, 156.8, 156.7, 148.7, 147.2, 133.0, 110.1, 106.4, 71.2, 61.644, 61.638, 61.605, 57.1, 19.4. Mass spectrum (EI, 70 eV, *m*/*z*, I_{rel}, %): 338 (100) $[M]^+$, 323 (52), 306 (52), 292 (93), 263 (25).

Determination of Cytotoxic Activity. Cytotoxic activity of synthetic naphthazarins was determined using fertilized ova of *S. intermedius* sea urchin collected in Peter the Great Bay (Sea of Japan). Male and female gametes and further fertilization were performed as before [17]. Compounds in various concentrations were added to an incubated mixture 3-5 min after fertilization of ova in the zygote stage. The incubation mixture contained an ethanolic solution (0.01 mL) of the studied compound or ethanol (0.01 mL, control) and a suspension (0.99 mL, 2500/mL of seawater) of fertilized ova. Fertilized ova were incubated with the tested compounds in open containers with filtered seawater for 24 h until late gastrula under optimal (20°C) temperature conditions. The activities of the compounds were followed using a microscope. The condition of fertilized ova and embryos was determined visually by comparison with the control (embryos in seawater and embryos in seawater with 1-2% alcohol). Cytotoxic activity of the naphthazarins was estimated from their ability to induce blockage, blastomer lysis, anomalies, or reduced division of ova and to delay embryo development 3, 12, and 24 h after fertilization [18].

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