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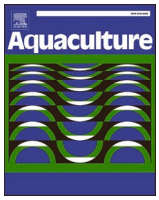

Short communication

Evaluation of photodegradable chitin synthetase inhibitors for the treatment of salmon lice (*Lepeophtheirus salmonis*)

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ABSTRACT

Some photolabile ethanolamine analogues of the chitin synthetase inhibitors diflubenzuron, teflubenzuron, and lufenuron were tested for activity as anti-lice compounds towards salmon lice (*Lepeophtheirus salmonis*). Two teflubenzuron analogues (**2** and **3**) exhibited interesting biological activity whereas their corresponding photodecomposition products were inactive. One of the analogues (**3**) decomposes completely when irradiated at pH 8, a relevant pH for seawater. In comparison, diflubenzuron showed a 66% photodecomposition under identical conditions. Thus, ethanolamine **3** is an interesting lead compound in the search for a powerful, environmentally friendly chemical to use in salmon-lice treatment.

1. Introduction

Salmon lice (*Lepeophtheirus salmonis*) infestation is the largest problem affecting the salmon farming industry worldwide (Bravo et al., 2015). In Norway alone more than 500 million USD are used to combat salmon lice annually (Sandlund et al., 2018). The problem is not limited to the fish-farming industry; salmon lice originating from farmed fish is also a threat to wild salmon and trout (Krkošek et al., 2007; Johansen et al., 2011; Torrissen et al., 2013; Taranger et al., 2015). The combat of salmon lice on salmon in sea cages in fjords is done by both non-medical and medical methods. Currently, hydrogen peroxide is the chemical used most frequently (Torrissen et al., 2013), but other compounds such as avermectins, pyrethroids, organophosphates, and chitin synthetase inhibitors (CSIs) (Fig. 1) are also applied. The fish are exposed to the chemicals either via the feed, which is the case for the CSIs, or through baths (Burridge et al., 2010). Regrettably, salmon lice have developed resistance towards most of the chemicals used, including hydrogen peroxide (Aaen et al., 2015; Fjørtoft et al., 2017; Fjørtoft et al., 2019; Fjørtoft et al., 2021), and this has led to application of higher doses and longer treatment times to offset the reduced efficacy. This unfortunate development is expected to cause problems because several of the commonly used medicines are relatively stable in the marine environment and may unintentionally harm non-targeted crustaceans. Laboratory studies have shown that the CSIs affect other crustaceans such as shrimp and crabs by reducing their ability to develop new cuticula after molting (Burridge et al., 2010; Soltani et al., 2009; Macken et al., 2015; Sun et al., 2015; Bechmann et al., 2018). This observation is not surprising because there is a high amino acid-sequence similarity among the chitinases found in salmon lice and other crustaceans (Eichner et al., 2015).

Non-chemical approaches may therefore seem more attractive, but treatment with hot water, fresh water, and UV light as well as the use of cleaning fish, wrasse, are facing other challenges. Hot water is stressful and painful for the salmon (Nilsson et al., 2019; Gismervik et al., 2019), UV light results in skin damage and discomfort (Barrett et al., 2020), and the use of wrasse is hampered by limited supply and high mortality (Geitung et al., 2020). In addition, most of the non-medical treatments involve large-scale handling of the fish with reduced fish welfare as a result. A method that is easy to implement in the cage without compromising fish welfare is delousing by laser, but the efficiency of this method compared to others is under debate (Bui et al., 2020).

Moving the fish farms on shore, caging the fish deeper in the ocean offshore, using snorkel sea cages, or barrier nets, and making the salmon more resistant against lice by genetic modification are approaches currently under evaluation (Yue and Shen, 2022). The former alternatives result in higher cost, and the latter will require a change of opinion concerning gene-modified food among the consumers. It is therefore likely that medical treatment will remain an important alternative for

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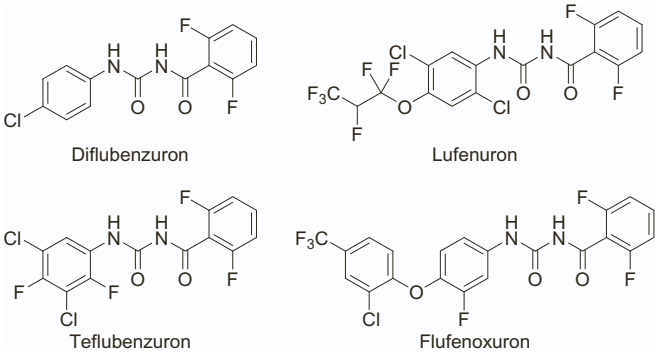


Fig. 1. Some chitin synthetase inhibitors used for salmon lice treatment.

controlling salmon lice in the future. Thus, there is an increasing need for new active chemicals that are at least as efficient as existing ones, but more environmentally friendly to improve the sustainability of the salmon farming industry.

Salmon lice have not yet developed resistance against the CSIs, a group of medicines that interferes with the formation of new exoskeleton and results in death after molting (Grant, 2002). With no resistance among the salmon-lice stock, the CSIs have remained attractive treatment agents despite their persistence in the marine environment. Studies conducted by Samuelsen (2016) have shown that teflubenzuron and diflubenzuron adsorbed to organic particles in marine sediments do not decompose significantly over a 24-week period. Another study stipulated a half-life of about 170 days for teflubenzuron in sediments collected under a fishpen (Samuelsen et al., 2015). Modification of the structures of CSIs making them more degradable in the marine environment is envisaged to be a viable solution both to combat salmon lice and protect non-targeted crustaceans and the environment. Herein we report our biological evaluation of a series of CSI analogues (diflubenzuron, teflubenzuron, and lufenuron analogues) where the urea moiety in the commercially available CSIs have been replaced with an ethanolamine unit (red in the structures depicted in Fig. 2) that facilitates photodecomposition.

2. Materials and methods

2.1. Preparation of compounds *1–6*

Compounds **1**–**6** were prepared as reported in the literature by Eikemo et al. (2021, 2022). In short, the relevant aniline and oxirane were treated with lithium perchlorate in diethyl ether (5 M) at 40–60 °C for 18–24 h. Purification of the crude material with column chromatography gave the desired ethanolamines **1**–**6**, which exhibited λ_{max} in the 240–260 nm region and therefore will absorb radiation in the UV part of the sunlight spectrum.

2.2. Procedure for photodecomposition

Irradiation of ethanolamines **2**–**4** in acetonitrile/water (7/3, ~ 0.7 mM) with a low-pressure mercury-vapor lamp under an atmosphere of air according to our reported method for 24 h securing full decomposition (Eikemo et al., 2022), the corresponding mixtures of decomposition products (**2d**-**4d**) were formed. Photolyses were carried out at pH 8 and 13. The resulting reaction mixtures **2d**-**4d** were extraction with ethyl acetate, dried, filtered, and concentrated prior to preparation of samples for biological evaluation. The decomposition rate for the compounds was evaluated based on ^{1}H NMR analysis of the crude product obtained after the work-up described above.

2.3. Breading of nauplii

All lice used in the presented study belongs to an established lice strain (LsGulen) as described by Hamre et al. (2009). Breeding and maintenance of the lice is described in detail by Hamre et al. (2009) and egg strings and planktonic life stages are kept in a flow-through system. The seawater used is obtained from 105 m depth in Byfjorden, outside Nordnes in Bergen, Norway and has a salinity of 34 parts per thousand (ppt) and a temperature of 10 °C and is filtered and irradiated with UV light prior to use. Hatched, viable nauplius I larva were collected and used for the assays described below.

2.4. General procedure for testing with the live-dead assay

The nauplius I stage of the salmon lice was used for a live-dead assay to evaluate the ability of the benzoyl urea analogues as anti-lice chemicals. Around 5–30 individuals were exposed for 1 h at 10 °C at concentrations 1.0 and 0.1 ppt according to the details given below. The lice were subsequently transferred to incubators with a constant flow of seawater at 9 °C. After 7 days the number of surviving nauplii or copepodids was counted as well as a subjective evaluation of their condition. This time point is used since all normal developing lice larvae will be at the copepodid stage and an increase in non-molted nauplis II indicate that the molting process is influenced/interrupted as would be expected from the compounds tested in the present study. If the compounds have no effect on the nauplii, they will develop normally into copepodids through ecdysis, i.e. shedding their chitin-rich exoskeleton, and an exuvium can be observed. An active compound, such as diflubenzuron, would prevent formation of the exoskeleton when nauplii develop, resulting in observation of dead nauplii and no copepodids.

2.5. Preliminary screening of biological activity of compounds *1–6*

Compounds **1**–**6** dissolved in DMSO to a concentration of 39–40 ppt

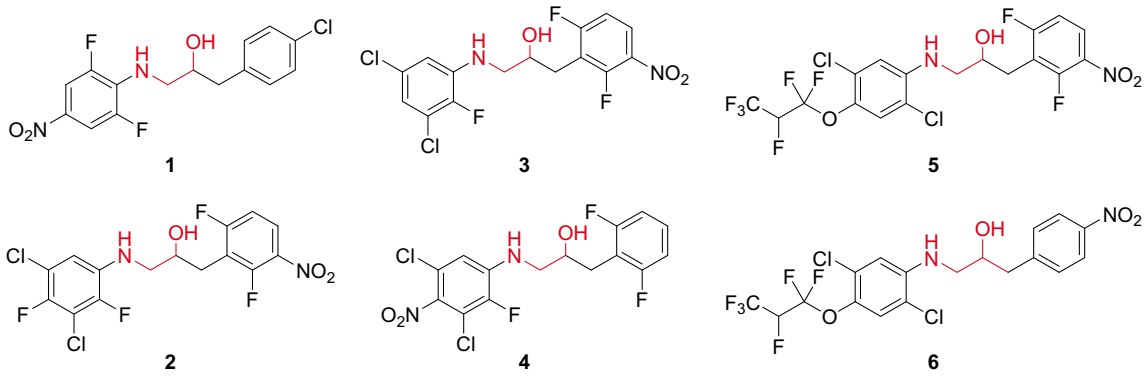
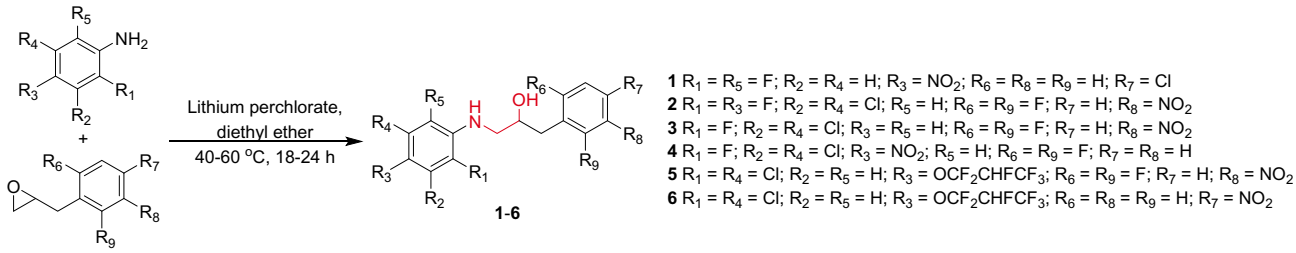


Fig. 2. All compounds (**1**–**6**) investigated contain an ethanolamine moiety (highlighted in red), which facilitates their photodegradation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



Scheme 1. Schematic overview of the synthesis of compounds **1**–**6**.

Table 1
Results from the biological testing of compounds **2**–**4**, diflubenzuron (positive control), and DMSO.

Compound	Conc. ppt	Parallel 1		Parallel 2		Parallel 3		Comment
		Naup.	Cop.	Naup.	Cop.	Naup.	Cop.	
2[a]	0.1	7	0	14	0	14	1	Active at this conc.
	0.01	12	1	13	1	7	2	Active at this conc.
	0.001	2	5	1	10	0	15	Not active at this conc.
3[b]	0.1	8	0	13	0	9	3	Active at this conc.
	0.01	11	2	4	2	4	1	Active at this conc.
	0.001	0	7	1	17	0	10	Not active at this conc.
4[c]	0.1	0	1[d]	6	16	3	8	Slightly active at this conc.
	0.01	3	23	1	10	1	12	Not active at this conc.
	0.001	0	13	1	18	1	15	Not active at this conc.
Diflubenz-	0.01	15	3	16	7	NT	NT	Active at this conc.
uron	0.001	16	2	12	3	NT	NT	Active at this conc.
	0.0001	14	2	6	3	NT	NT	Active at this conc.
2d	0.1	1	8	3	16	6	10	Slightly active at this conc.
	0.01	1	13	2	14	1	11	Not active at this conc.
	0.001	0	10	3	5	1	6	Not active at this conc.
3d	0.1	0	28	0	3	0	2	Not active at this conc.
	0.01	1	8	2	10	0	8	Not active at this conc.
	0.001	1	10	1	6	2	13	Not active at this conc.
4d	0.1	13	0	16	0	15	0	Active at this conc.
	0.01	3	20	1	10	0	9	Not active at this conc.
	0.001	1	14	0	11	1	15	Not active at this conc.
DMSO	0.1	1	10	0	6	2	10	Not active at this conc.

[a]1-((3,5-Dichloro-2,4-difluorophenyl)amino)-3-(2,6-difluoro-3-nitrophenyl)propan-2-ol; [b]1-((3,5-dichloro-2-fluorophenyl)amino)-3-(2,6-difluoro-3-nitrophenyl) propan-2-ol; [c]1-(3,5-Dichloro-2-fluoro-4-nitrophenyl)-amino-3-(2,6-difluorophenyl)propan-2-ol; [d]leaking test well; NT = not tested.

were diluted with seawater to a concentration of 1.0 ppt and 0.1 ppt and tested according to the general procedure for live dead assay.

2.6. Activity testing of compounds 2–4 and 2d-4d

Compounds **2**–**4** and photodecomposition mixtures **2d**-**4d** dissolved in DMSO to a concentration of 39–40 ppt were diluted with seawater to a concentration of 0.1, 0.01, and 0.001 ppt. Diflubenzuron dissolved in DMSO to a concentration of 40 ppt was diluted with seawater to a concentration of 0.01, 0.001, and 0.0001 ppt. Photodecomposed products were dissolved in DMSO to a concentration of 39–40 ppt and diluted with seawater to a concentration of 0.1, 0.01, and 0.001 ppt. All solutions were tested according to the general procedure for live dead assay.

3. Results

Compounds **1**–**6** were prepared by treating the relevant aniline and oxirane with lithium perchlorate at 40–60 °C according to our reported procedure (Eikemo et al., 2021; Eikemo et al., 2022) (Scheme 1). The resulting products (Scheme 1, Fig. 2), where compound **1** is a diflubenzuron analogue, compounds **2**–**4** are teflubenzuron analogues, and compounds **5** and **6** are lufenuron analogues, were first subjected to a preliminary screening. All compounds were tested for biological activity using a live-dead assay of nauplius I larvae at concentrations 1.0 and 0.1 ppt. Ethanolamines **2**–**4** showed activity at both concentrations while **1**, **5**, and **6** exhibited little or no activity (results not shown). The latter group of compounds was therefore discarded whereas ethanolamines **2**–**4** were subjected to further testing.

In order to assess the biological activity more precisely, more dilute samples of compounds **2**–**4** and the corresponding crude photodecomposition mixtures (**2d**-**4d**) were tested. In the biological evaluation diflubenzuron was used as a positive control and DMSO was used to detect potential solvent effects. As the results compiled in Table 1 show, compounds **2** and **3** exhibited activity at 0.1 and 0.01 ppt, but not at 0.001 ppt whereas compound **4** showed some activity at 0.1 ppt, but none at 0.01 and 0.001 ppt. Diflubenzuron, on the other hand, showed activity at concentrations all the way down to 0.0001 ppt, whereas DMSO did not exhibit any activity even at 0.1 ppt ruling out any solvent effect in the biological tests.

The product mixtures obtained by photolysis of compounds **2**–**4**, denoted **2d**-**4d**, showed interesting differences in biological activity. Whereas sample **3d** was inactive at all tested concentrations (Table 1), **2d** and **4d** displayed some activity at 0.1 ppt, but none at the two lowest concentrations. Photodecomposition of ethanolamines **2** and **3** and diflubenzurone at pH 8 for 24 h resulted in full decomposition of compound **3**, while ethanolamine **2** and diflubenzurone showed 19% and 66% decomposition, respectively.

4. Discussion

The initial screening of compounds **1**–**6** at 1.0 ppt and 0.1 ppt showed that diflubenzeron analogues **2**–**4** prevented the nauplii to develop into live copepodids, and this warranted further examination. The remaining three compounds, viz. **1**, **5**, and **6** did not show activity

that was interesting for further evaluation. Compounds **2**–**4** and the corresponding photodecomposition mixtures, viz. **2d**- **4d**, derived from photolysis at pH 13 to secure that the mother compounds were fully decomposed were therefore subjected to testing at 0.1, 0.01, and 0.001 ppt. Diflubenzuron, a CSI that is in use to combat salmon lice in fish farms (Parsons et al., 2021), was included as a positive control (0.01, 0.001, and 0.0001 ppt), and since DMSO was used as a co-solvent in the test solutions, it was included at a concentration of 0.1 ppt to evaluate its impact on the testing.

As expected, diflubenzuron stopped the development of nauplius larvae to copepodids even at the lowest concentration (0.0001 ppt) whereas DMSO did not impact the natural development of the nauplius larva at all. As for compounds **2** and **3**, the development of nauplius larvae was prevented both at 0.1 and 0.01 ppt, whereas ethanolamine **4** had only a slight effect at the highest concentration (Table 1). Of the photodecomposition product mixtures from compounds **2**–**4** (Eikemo et al., 2022), only the sample from ethanolamine **3** (**3d**) was biological inactive. Since **3** is active and the corresponding photodecomposition products are inactive, this compound appears to be a very interesting lead compound provided it photodegrades efficiently at the pH prevailing in seawater.

Our previous work has shown that compounds **2**–**4** fully decomposed at pH 13 (Eikemo et al., 2022). However, the pH in sea water ranges from 7.8 to 8.3 (Marion et al., 2011) so interesting compounds for further investigation must undergo photodecomposition under these conditions. Photodecomposition studies showed that compound **3** was fully decomposed at pH 8 after 24 h, while ethanolamine **2** and diflubenzuron under the same conditions showed only 19% and 66% decomposition, respectively. This makes ethanolamine **3** the most interesting lead for further optimization studies. Such studies must include testing of potent compounds against later life stages of lice, thorough toxicity screening, and photodecomposition studies under natural conditions among other activities.

5. Conclusions

In conclusion, we have developed two compounds, viz. **2** and **3**, that have interesting biological activity. The decomposition products derived from the two compounds have no activity towards salmon lice. Ethanolamine **3** fully decomposes over the course of 24 h at pH 8, which is favorable compared to diflubenzuron that only undergo 66% photodecomposition under the same conditions. Ethanolamin **3** is an excellent lead compound in the process of developing potent salmon lice treatments with reduced environmental consequences.

Credit authorship contribution statement

Magne O. Sydnes: Conceptualization, Methodology, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. Vebjørn Eikemo: Investigation, Methodology, Analysis, Writing – review. Per Gunnar Espedal: Testing methodology, Biological testing, Analysis, Writing – review. Leiv K. Sydnes: Conceptualization, Writing – review, Writing - review & editing. Frank Nilsen: Testing methodology, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests of personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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