

Article

Angular 6/6/5/6-Annelated Pyrrolidine-2,3-Diones: Growth-Regulating Activity in *Chlorella vulgaris*

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Abstract

Chlorella vulgaris, a unicellular microalga with broad industrial applications, is a valuable source of bioactive compounds, including proteins, pigments, and lipids. However, optimizing its growth and metabolite production remains a challenge. This study investigates the potential of angular 6/6/5/6-anneled pyrrolidine-2,3-diones—structurally complex small molecules resembling alkaloids and 13(14 → 8)*abeo*-steroids—as novel growth stimulants for *C. vulgaris*. A series of these compounds (20 structurally diverse derivatives, including 7 previously unreported ones) were synthesized and screened for their ability to enhance microalgal growth. Primary screening identified one compound as a promising candidate, significantly increasing algae cell concentration in microplate cultures. Subsequent validation in flask-scale experiments revealed that this candidate induced a 19% increase in protein content at 1 μmol/L, suggesting potential for protein enrichment in algal biomass. Stability studies of the candidate compound revealed its significant hydrolytic degradation in aqueous media. These findings highlight the potential of angular 6/6/5/6-anneled pyrrolidine-2,3-diones as modulators of microalgal metabolism, offering a new avenue for enhancing *C. vulgaris* biomass quality, particularly for protein-rich applications in the food and feed industries.

Keywords: alkaloid-like; *Chlorella*; cycloaddition; nitrogen heterocycle; oxygen heterocycle; protein enrichment; 1*H*-pyrrole-2,3-dione; pyrrolidine-2,3-dione; spiro-heterocycle; 13(14 → 8)*abeo*-steroids



Academic Editor: Igor Alabugin

Received: 5 May 2025

Revised: 18 June 2025

Accepted: 19 June 2025

Published: 21 June 2025

Citation: Novokshonova, A.D.; Khramtsov, P.V.; Khramtsova, E.E. Angular 6/6/5/6-Anneled Pyrrolidine-2,3-Diones: Growth-Regulating Activity in *Chlorella vulgaris*. *Chemistry* **2025**, *7*, 102. <https://doi.org/10.3390/chemistry7040102>

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1. Introduction

Chlorella vulgaris, a unicellular microalga, has garnered significant attention due to its extensive industrial applications, driven by its rapid growth rate, adaptability to diverse environmental conditions, and capacity to synthesize a wide array of valuable metabolites. This microalga is a rich source of bioactive compounds, including pigments (chlorophylls, carotenoids), proteins, carbohydrates, dietary fibers, vitamins, and lipids [1]. Its primary commercial applications span human and animal nutrition—such as dietary supplements—as well as cosmetics, while its secondary uses include biofertilizers and pharmaceuticals [2,3]. Despite the growing global market for *Chlorella*-based products, its utilization in emerging fields such as biofuels, bioremediation, and biodegradable polymer production remains relatively underdeveloped. A major bottleneck lies in the need for innovative strategies to enhance growth rates and increase the accumulation of target metabolites.

To overcome these limitations, various approaches have been explored to stimulate microalgal growth and optimize metabolite production. These strategies include the isolation of novel natural strains, genetic engineering, the optimization of cultivation parameters, and the application of growth stimulants such as phytohormones (for example, auxins [4–6], cytokinins [5,6], gibberellins [5,6], strigolactones [6], brassinosteroids [7], etc.) (Figure 1) [8–10]. Recently, another promising avenue has emerged: the identification of synthetic small molecules capable of modulating microalgal metabolism [11]. This approach draws inspiration from drug discovery pipelines and involves the high-throughput screening (HTS) of compound libraries to uncover molecules with stimulatory effects [12]. A particularly effective strategy is to investigate compounds with broad biological activity that are structural analogs of molecules known to exhibit growth-promoting properties [13]. In this context, our attention was directed towards angular 6/6/5/6-annulated pyrrolidine-2,3-diones as potential candidates for stimulating the growth of *C. vulgaris*.

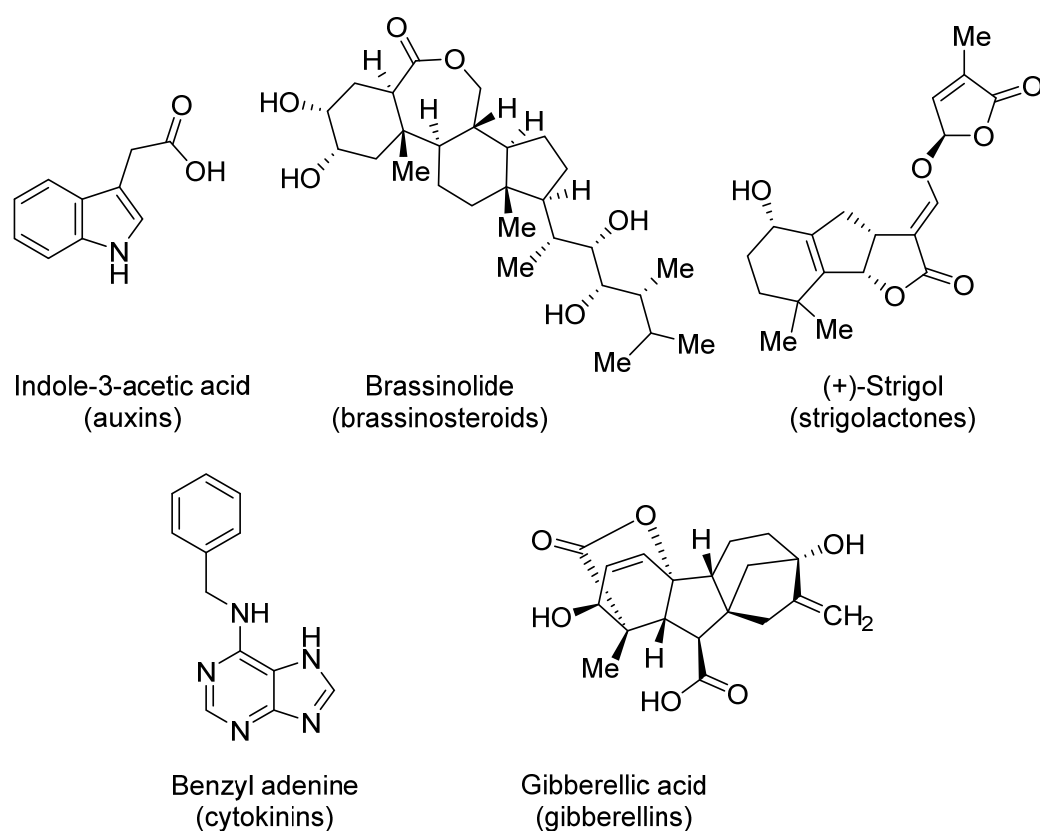


Figure 1. Selected examples of phytohormones.

Angular 6/6/5/6-annulated pyrrolidine-2,3-diones **1** (Figure 2) are an emerging class of biologically active compounds found to exhibit antinociceptive [14] and antiviral [15] activities. Molecules with such polycyclic annulated structures can be classified as structurally complex small molecules (in terms of 3D shape), which are highly sought after in medicinal chemistry, as their structural complexity can enhance biological activity by optimizing molecular interactions with biological targets [16,17]. Furthermore, annulated pyrrolidine-2,3-diones **1** bear a 6/6/5/6-spiroframework resembling either alkaloids (alkaloid-like [15,18,19]) or 13(14 → 8)*abeo*-steroids (13(14 → 8)*abeo*-steroid-like [20–22]), rendering them highly promising candidates for the exploration of various biological activities.

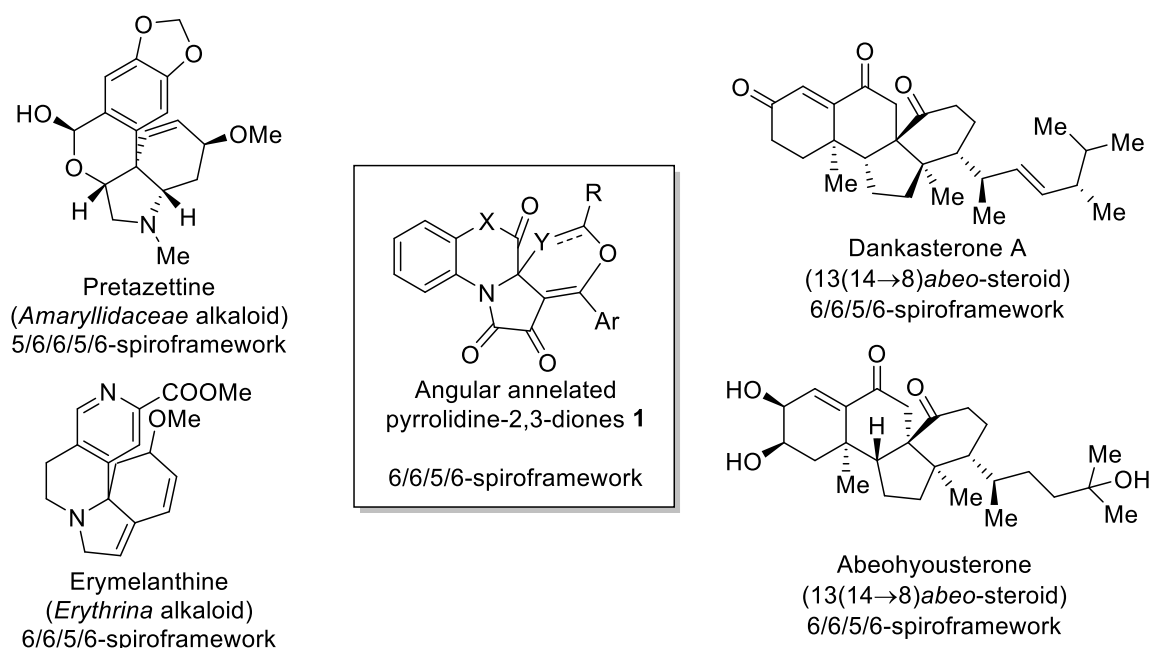


Figure 2. Angular 6/6/5/6-annelated pyrrolidine-2,3-diones **1** and their structural analogs (alkaloids and *abeo*-steroids).

Interestingly, several phytohormones—such as strigolactones (6/5/5-annelated core) and brassinosteroids (6/7/6/5-annelated core)—also possess angular annelated frameworks (Figure 1). Given the structural parallels between these phytohormones and pyrrolidine-2,3-diones **1**, coupled with the latter's demonstrated bioactivity, we proposed that these compounds warranted investigation for potential plant growth-stimulating effects.

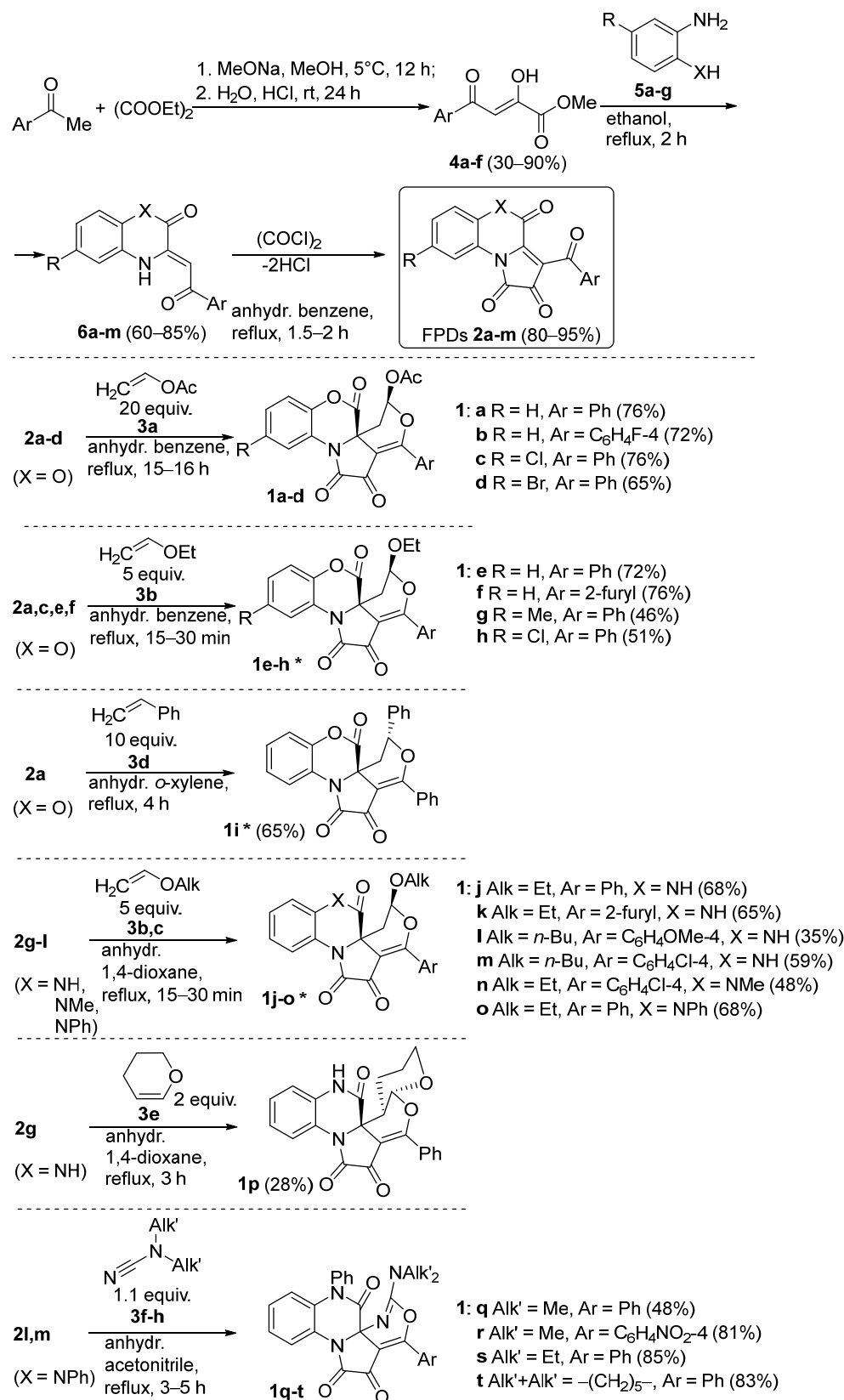
In this study, we investigate the growth regulation activity of a series of angular 6/6/5/6-annelated pyrrolidine-2,3-diones **1** in *C. vulgaris*.

2. Results and Discussion

2.1. Synthesis of 6/6/5/6-Annelated Pyrrolidine-2,3-Diones **1**

[*e*]-Fused 1*H*-pyrrole-2,3-diones (FPDs) are well-known precursors for the rapid construction of diverse heterocyclic scaffolds, including angular polycyclic ones [23–26]. Therefore, as a key step to prepare a series of diversely substituted angular 6/6/5/6-annelated pyrrolidine-2,3-diones **1a–t**, we used a [4+2]-cycloaddition reaction of FPDs **2a–m** with electron-rich dienophiles **3a–h** (vinyl acetate **3a** [27], alkoxyethylenes **3b,c** [20,28,29], styrene **3d** [30], 3,4-dihydro-2*H*-pyran **3e** [31]) and *N,N*-dialkylcyanamides **3f–h** [32] (Scheme 1, Supplementary Materials, S37).

A series of angular 6/6/5/6-annelated pyrrolidine-2,3-diones **1a–t** were obtained in several steps via earlier-reported synthetic protocols (Scheme 1, Supplementary Materials, S37) [20,27–32]. In brief, initially, methyl esters of aroylpyruvic acids **4a–f** were obtained via the Claisen condensation of the corresponding acetophenones and diethyl oxalate in the presence of sodium methoxide [33]. Then, the reaction of compounds **4a–f** with anilines **5a–g** afforded heterocyclic enaminones **6a–m** [29,34–39]. After that, the acylation of compounds **6a–m** by oxalyl chloride resulted in FPDs **2a–m** [29,34,35,37–40]. And finally, the hetero-Diels-Alder reaction of FPDs **2a–m** and dienophiles **3a–h** afforded the desired compounds **1a–t** [20,27–32]. Compounds **1e,f,i–m,o** were isolated as mixtures of two diastereomers. It should be mentioned that compounds **1b,d,f,g,k,n,o** were not reported earlier.



* For compounds **1e,f,i-m,o**, a mixture of two diastereomers was isolated and examined in the biological assays.

Scheme 1. Synthesis of angular 6/6/5/6-annulated pyrrolidine-2,3-diones **1a–t** (isolated yields are given).

2.2. Biology

The identification of bioactive compounds capable of stimulating the growth of *C. vulgaris* was carried out through a two-stage screening approach. In the initial stage, microalgae were cultivated in 96-well plates for five days in the presence of various concentrations (0.1–10 µmol/L) of the tested compounds. A culture medium without additives served as the negative control, while 2 g/L of glucose was used as the positive control. Optical density at 750 nm (OD750), a parameter reflecting both cell concentration and, to some extent, cell size and composition, was employed to evaluate growth. Compounds that induced OD750 values exceeding the mean OD750 of the negative control plus three standard deviations at two or more concentrations were shortlisted for further investigation.

The primary screening results (Table 1) identified four compounds (**1n,q,s,t**) that enhanced OD750 beyond the threshold, with only compound **1s** meeting the selection criteria for further analysis.

Table 1. The difference in algae cell concentrations between the cultures containing the compounds under study and the control cultures.

Entry	Difference ¹ in Algae Cell Concentration Between Cultures Containing Compounds Under Study and Control Cultures		
	Concentration of Compounds in Culture Medium		
	0.1 µmol/L	1 µmol/L	10 µmol/L
1a	9.65	−3.09	2.70
1b	0.77	1.93	3.47
1c	5.02	6.18	1.93
1d	5.79	0.00	4.63
1e	9.65	0.77	−1.93
1f	−2.32	−1.93	9.65
1g	6.83	−1.84	−1.45
1h	−0.66	−4.20	−0.66
1i	−4.20	−1.05	1.71
1j	3.47	6.56	4.25
1k	−0.77	−1.93	8.11
1l	−8.05	−4.94	2.86
1m	4.42	−4.94	−6.49
1n	2.50	4.47	13.53²
1o	9.20	6.83	5.26
1p	2.08	−1.82	1.30
1q	16.23	−0.31	6.13
1r	−0.27	2.95	12.60
1s	16.62	23.06	29.49
1t	13.03	−0.18	2.99
Glucose (2 g/L, 1.1×10^{-2} mol/L)		128–193	

¹ Expressed as a percentage of the negative control. ² Bold indicates conditions that result in cell concentrations exceeding the established threshold (mean of negative control plus three standard deviations).

To validate and explore the effects of compound **1s** in greater detail, secondary experiments were conducted in 50 mL flasks to ensure sufficient biomass for biochemical analyses (Table 2). The concentration range of compound **1s** was extended to 0.1–100 µmol/L, with experimental controls identical to those in the primary screening. Cell concentrations were quantified via direct cell counting, while metabolite content was assessed using standard biochemical methods.

Table 2. The effect of compound **1s** on the growth and accumulation of metabolites in *C. vulgaris* cells.

Concentration of 1s	Concentration of Cells, 10 ⁶ Cell/mL	Chlorophyll a and b, µg/10 ⁷ Cells	Carotenoids, µg/10 ⁷ Cells	Carbohydrates, µg/10 ⁶ Cells	Protein, µg/10 ⁶ Cells
100 µmol/L	17.85 ± 0.76 ³	3.558 ± 0.449	0.142 ± 0.013	3.29 ± 0.331	0.513 ± 0.041
10 µmol/L	19.13 ± 0.98	3.520 ± 0.171	0.170 ± 0.032	3.52 ± 0.293	0.469 ± 0.056
1 µmol/L	17.48 ± 0.23	3.552 ± 0.015	0.171 ± 0.023	3.97 ± 0.390	0.551 ± 0.065 ⁴
0.1 µmol/L	17.88 ± 0.74	3.476 ± 0.095	0.170 ± 0.026	3.27 ± 0.367	0.488 ± 0.044
Negative control ¹	18.68 ± 0.78	3.469 ± 0.084	0.185 ± 0.024	3.65 ± 0.239	0.462 ± 0.043
Positive control ²	69.83	3.689 ± 0.042	0.111 ± 0.003	5.74 ± 0.312	0.370 ± 0.015

¹ Culture medium with 1% of DMSO. ² Culture medium with 1% of DMSO and 2 g/L of glucose.

³ Mean ± standard deviation. ⁴ Bold indicates difference from negative control identified by nested ANOVA (*p* value less than 0.05).

Interestingly, despite its promising performance in microplate screening, compound **1s** did not significantly impact the cell concentration in the flask cultures compared to the negative control (Table 2). This discrepancy may be attributed to differences in mixing conditions and oxygen availability between the microplate and flask setups. Nevertheless, biochemical analysis revealed a statistically significant 19% increase in protein content at 1 µmol/L of compound **1s**.

A comparative review of the literature data on phytohormones demonstrated variability in their reported effects on protein accumulation in *C. vulgaris*. For instance, only salicylic acid at 5–10 mg/L induced a moderate 20–60% increase in protein content in some studies [5,41], whereas 2,4-epibrassinolide, benzyladenine, ethylene, gibberellin, indoleacetic acid, and naphthaleneacetic acid had no or little effect. At the same time, others reported markedly higher enhancements promoted by 2,4-epibrassinolide and indoleacetic acid [42].

The observed effect of compound **1s** on protein accumulation suggests its potential as an alternative protein-enhancing agent for *C. vulgaris*. Given the high nutritional quality of *C. vulgaris* proteins, which include all essential amino acids [43,44], this finding holds promise for its application in the food and feed industries where protein enrichment is a key objective.

2.3. Hydrolysis Study of Compound **1s**

Chemical instability in aquatic environments—driven by hydrolysis, oxidation, and other transformation reactions—can critically influence the stability and efficacy of algal growth regulators, including both natural phytohormones and synthetic analogs. Given this challenge, we evaluated the environmental stability of compound **1s**, which has demonstrated superior activity in biological assays, under simulated aquatic conditions (Table 3). As a result, we found that compound **1s** underwent a decomposition under all tested conditions, yielding a mixture of unidentified transformation products (HPLC-UV chromatogram plots are available in Supplementary Materials). While demonstrating moderate stability in DMSO with traces of water, the decomposition of compound **1s** accelerated in aqueous mixtures. Notably, intense illumination triggered the complete degradation of compound **1s** in all tested media.

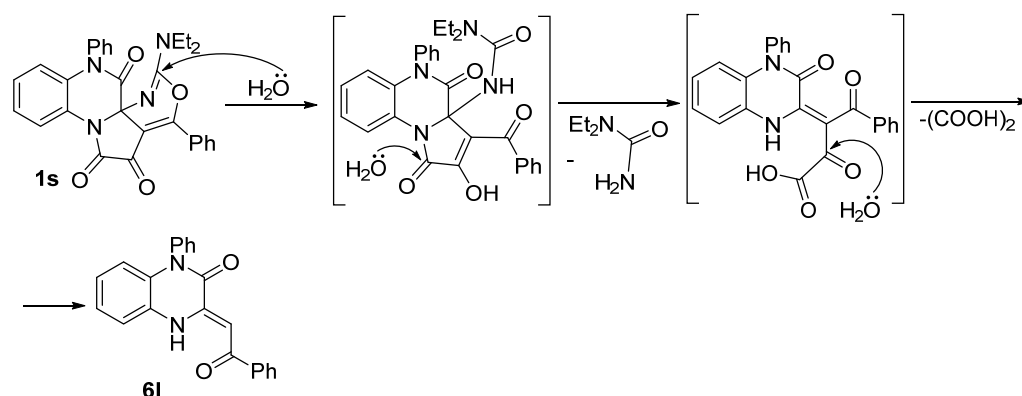
Although compound **1s** decomposed under all tested conditions to yield complex mixtures of transformation products, we successfully identified one of these products. An analysis of the reaction mixtures obtained under ambient conditions (Entries 1–3, Table 3) revealed the presence of heterocyclic enaminone **6l** (Scheme 2, HPLC-UV chromatogram plots are available in Supplementary Materials). However, this compound was absent in the mixtures exposed to the culture chamber conditions (Entries 4–6, Table 3),

suggesting that enaminone **6l** could undergo photodegradation under a more intense illumination regime.

Table 3. Conversion of compound **1s** under aquatic conditions.

Entry	Conditions ¹	Conversion ² of 1s , %	Content ² of 6l , %
1	DMSO with traces of H ₂ O ³	1	traces
2	DMSO/H ₂ O, ³ 8:1 v/v	49	~5
3	DMSO/BG-11, ³ 8:1 v/v	50	~5
4	DMSO with traces of H ₂ O, illumination ⁴	100	0
5	DMSO/H ₂ O, 8:1 v/v, illumination ⁴	100	0
6	DMSO/BG-11, 8:1 v/v, illumination ⁴	100	0

¹ A solution of 1 mg of **1s** in 1 mL of the corresponding aquatic mixture was stored in a capped clear chromatography glass vial under the conditions for five days. ² Determined by HPLC-UV (Supplementary Materials). ³ Room illumination, temperature, and humidity. ⁴ Illumination, temperature, and humidity as in culture incubation chamber.



Scheme 2. Possible hydrolysis pathway for the formation of compound **6l** from compound **1s** under ambient conditions.

3. Materials and Methods

3.1. Synthetic Methods and Analytic Data of Compounds

3.1.1. General Information

¹H and ¹³C NMR spectra (Supplementary Materials) were acquired on a Bruker Avance III 400 HD spectrometer (Bruker BioSpin AG, Faellanden, Switzerland) (at 400 and 100 MHz, respectively) in CDCl₃ or DMSO-*d*₆, using TMS (in ¹H NMR, 0.00 ppm) or solvent residual signals (in ¹³C NMR, 77.00 ppm for CDCl₃, 39.52 ppm for DMSO-*d*₆; in ¹H NMR, 7.26 ppm for CDCl₃, 2.50 ppm for DMSO-*d*₆) as internal standards. ¹⁹F NMR spectra (Supplementary Materials) were acquired on a Bruker Avance III 400 HD spectrometer (Bruker BioSpin AG, Faellanden, Switzerland) (at 376 MHz) in CDCl₃, using C₆F₆ (in ¹⁹F NMR, −162.90 ppm) as an internal standard. IR spectra were recorded on a Perkin-Elmer Spectrum Two spectrometer (PerkinElmer Inc., Waltham, MA, USA) from mulls in mineral oil. Melting points were measured on a Mettler Toledo MP70 apparatus (Mettler-Toledo (MTADA), Schwerzenbach, Switzerland). Elemental analyses were carried out on a Vario MICRO Cube analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany). The study of the hydrolysis of compound **1s** was carried out using HPLC-UV on a Hitachi Chromaster (Hitachi High-Tech, Tokyo, Japan) [NUCLEODUR C18 Gravity column (particle size 3 μm; eluent acetonitrile–water, flow rate 1.5 mL/min); Hitachi Chromaster 5430 diode array detector (λ 210–750 nm)] (Supplementary Materials). Thin-layer chromatography (TLC) was performed on ALUGRAM Xtra SIL G/UV254 silica gel

60 plates (Macherey-Nagel, Düren, Germany) using EtOAc/toluene, 1:5 *v/v*, EtOAc, and toluene as eluents; spots were visualized with iodine vapor and/or UV light (254, 365 nm) in the light of a TLC viewing cabinet Petrolaser TLC-254/365 Thin Layer Chromatography Dark Room (Petrolaser, St. Petersburg, Russia).

FPDs **2a–m** were obtained according to reported procedures [29,34,35,37–40]. Enaminones **6a–m** were obtained according to the reported procedures [29,34–39]. Methyl aroylpyruvates **4a–f** were obtained according to the reported procedures [33]. Dienophiles **3a–e** were distilled before their use. The benzene, toluene, *o*-xylene, and 1,4-dioxane for the procedures with compounds **2a–m** were distilled over Na before use. The acetonitrile for the procedures with FPDs **2a–m** was dehydrated over molecular sieves 4Å before its use. All procedures with FPDs **2a–m** were performed in oven-dried glassware. All other solvents and reagents were purchased from commercial vendors and were used as received.

3.1.2. Analytic Data of Compounds **1a–t**

Compounds **1a,c** were reported earlier [27]. Compounds **1b,d** were not reported.

General procedure for compounds 1a–d. A suspension of the corresponding FPD **2a–d** (3.1 mmol) and vinyl acetate **3a** (5.7 mL, 62 mmol) in 10 mL of anhydrous benzene was refluxed for 15–16 h (until the violet color typical of starting compound **2** disappeared). Then, the mixture was cooled and evaporated to dryness. The resulting residue was ground with toluene, and the precipitate was filtered off and recrystallized from toluene (5–15 mL) to afford the corresponding compound **1a–d**.

(5*R**,6*aR**)-1,2,7-Trioxo-3-phenyl-1,2,5,6-tetrahydro-7*H*-benzo[*b*]pyrano[4',3':2,3]pyrrolo[1,2-*d*][1,4]oxazin-5-yl acetate (**1a**) [27]. Yield: 76% (0.95 g); yellow solid; mp 256–258 °C (decomp., toluene) (Lit.mp 257–258 °C (decomp., toluene) [27]). ¹H NMR (400 MHz, CDCl₃): δ = 8.01 (m, 2H), 7.91 (m, 1H), 7.65 (m, 1H), 7.52 (m, 2H), 7.34 (m, 3H), 6.80 (d.d, 1H, ³*J*_{eq.eq.} = 1.0 Hz, ³*J*_{ax.eq.} = 4.4 Hz), 2.71 (d.d, 1H, ²*J* = 14.1 Hz, ³*J*_{eq.eq.} = 0.9 Hz), 2.18 (d.d, 1H, ²*J* = 14.1 Hz, ³*J*_{ax.eq.} = 4.5 Hz), 2.14 (s, 3H) ppm.

(5*R**,6*aR**)-3-(4-Fluorophenyl)-1,2,7-trioxo-1,2,5,6-tetrahydro-7*H*-benzo[*b*]pyrano[4',3':2,3]pyrrolo[1,2-*d*][1,4]oxazin-5-yl acetate (**1b**). Yield: 72% (0.94 g); yellow solid; mp 278–279 °C (decomp., toluene). ¹H NMR (400 MHz, CDCl₃): δ = 8.07 (m, 2H), 7.90 (m, 1H), 7.39 (m, 1H), 7.31 (m, 2H), 7.20 (m, 2H), 6.79 (d.d, 1H, ³*J*_{eq.eq.} = 0.7 Hz, ³*J*_{ax.eq.} = 4.4 Hz), 2.71 (d.d, 1H, ²*J* = 14.1 Hz, ³*J*_{eq.eq.} = 0.9 Hz), 2.18 (d.d, 1H, ²*J* = 14.1 Hz, ³*J*_{ax.eq.} = 4.5 Hz), 2.14 (s, 3H) ppm. ¹³C NMR (100 MHz, DMSO-*d*₆): δ = 176.1, 168.0, 164.8 (d, ¹*J*_{CF} = 252.0 Hz), 163.9, 160.8, 158.7, 143.0, 133.2 (d, 2C, ³*J*_{CF} = 9.2 Hz), 127.4, 126.5 (d, ⁴*J*_{CF} = 2.3 Hz), 124.8, 122.3, 121.2, 116.7, 115.1 (d, 2C, ²*J*_{CF} = 24.7 Hz), 102.8, 90.9, 54.1, 33.6, 20.3 ppm. ¹⁹F NMR (376 MHz, CDCl₃): δ = −103.55 ppm. IR (mineral oil): 1796, 1770, 1735, 1717 cm^{−1}. Anal. Calcd (%) for C₂₂H₁₄FNO₇: C 62.42; H 3.33; N 3.31. Found: C 62.69; H 3.38; N 3.51.

(5*R**,6*aR**)-11-Chloro-1,2,7-trioxo-3-phenyl-1,2,5,6-tetrahydro-7*H*-benzo[*b*]pyrano[4',3':2,3]pyrrolo[1,2-*d*][1,4]oxazin-5-yl acetate (**1c**) [27]. Yield: 76% (1.03 g); yellow solid; mp 278–280 °C (decomp., toluene) (Lit.mp 278–280 °C (decomp., toluene) [27]). ¹H NMR (400 MHz, CDCl₃): δ = 8.00 (m, 2H), 7.94 (m, 1H), 7.65 (m, 1H), 7.52 (m, 2H), 7.36 (m, 1H), 7.23 (m, 1H), 6.81 (d.d, 1H, ³*J*_{eq.eq.} = 0.9 Hz, ³*J*_{ax.eq.} = 4.3 Hz), 2.70 (d.d, 1H, ²*J* = 14.1 Hz, ³*J*_{eq.eq.} = 0.9 Hz), 2.18 (d.d, 1H, ²*J* = 13.9 Hz, ³*J*_{ax.eq.} = 4.4 Hz), 2.13 (s, 3H) ppm.

(5*R**,6*aR**)-11-Bromo-1,2,7-trioxo-3-phenyl-1,2,5,6-tetrahydro-7*H*-benzo[*b*]pyrano[4',3':2,3]pyrrolo[1,2-*d*][1,4]oxazin-5-yl acetate (**1d**). Yield: 65% (0.98 g); yellow solid; mp 263–265 °C (decomp., toluene). ¹H NMR (400 MHz, CDCl₃): δ = 8.09 (m, 1H), 8.00 (m, 2H), 7.66 (m, 1H), 7.52 (m, 3H), 7.17 (m, 1H), 6.81 (d.d, 1H, ³*J*_{eq.eq.} = 1.0 Hz, ³*J*_{ax.eq.} = 4.4 Hz), 2.70 (d.d, 1H, ²*J* = 13.9 Hz, ³*J*_{eq.eq.} = 1.0 Hz), 2.17 (d.d, 1H, ²*J* = 14.2 Hz, ³*J*_{ax.eq.} = 4.4 Hz), 2.13 (s, 3H) ppm. ¹³C NMR (100 MHz, DMSO-*d*₆): δ = 175.6, 168.0, 165.4, 160.2, 158.8, 142.3, 133.2, 130.4, 130.0, 129.9, 127.8, 124.5, 122.6, 118.8, 115.8, 102.6, 90.8, 53.9, 33.6, 20.3 ppm. IR (mineral oil):

1796, 1758, 1731, 1714 cm^{-1} . Anal. Calcd (%) for $\text{C}_{22}\text{H}_{14}\text{BrNO}_7$: C 54.57; H 2.91; N 2.89. Found: C 54.87; H 2.58; N 2.55.

Compounds **1e,h** were reported earlier [20]. Compounds **1f,g** were not reported.

General procedure for compounds 1e–h. A suspension of the corresponding FPD **2a,c,e,f** (3.1 mmol) and ethoxyethylene **3b** (1.5 mL, 15.5 mmol) in 10 mL of anhydrous benzene was refluxed for 15–30 min (until the violet color typical of starting compound **2** disappeared). Then, the mixture was cooled to room temperature. The formed precipitate was filtered off and recrystallized from toluene (5–20 mL) to afford the corresponding compound **1e–h**.

(5*S**,6*aR**)- and (5*R**,6*aR**)-5-Ethoxy-3-phenyl-5,6-dihydro-7*H*-benzo[*b*]pyrano[4',3':2,3]pyrrolo[1,2-*d*][1,4]oxazine-1,2,7-triones (**1e**) [20]. Obtained as a mixture of diastereomers, (5*S**,6*aR**):(5*R**,6*aR**) ~ 5:2 (by NMR). Yield: 72% (0.87 g); yellow solid; mp 246–249 °C (decomp., toluene) (Lit.mp 248–250 °C (decomp., toluene) [20]). ^1H NMR (400 MHz, $\text{DMSO-}d_6$) of (5*S**,6*aR**)-diastereomer (major): δ = 7.92 (m, 2H), 7.81 (m, 1H), 7.68 (m, 1H), 7.58 (m, 2H), 7.40 (m, 2H), 7.36 (m, 1H), 5.79 (m, 1H, X-part of ABX system), 3.86–3.67 (m, 2H), 2.39 (m, 2H, AB-part of ABX system), 1.11 (t, 3H, 3J = 7.0 Hz) ppm. ^1H NMR (400 MHz, $\text{DMSO-}d_6$) of (5*R**,6*aR**)-diastereomer (minor): δ = 7.86 (m, 2H), 7.77 (m, 1H), 7.68 (m, 1H), 7.58 (m, 2H), 7.40 (m, 1H), 7.36 (m, 2H), 5.60 (d.d, 1H, $^3J_{ax.ax.}$ = 10.2 Hz, $^3J_{ax.eq.}$ = 4.1 Hz), 4.09–4.01 (m, 1H), 3.84–3.77 (m, 1H), 2.61 (d.d, 1H, 2J = 13.0 Hz, $^3J_{ax.eq.}$ = 4.2 Hz), 2.18 (d.d, 1H, 2J = 13.0 Hz, $^3J_{ax.ax.}$ = 10.4 Hz), 1.21 (t, 3H, 3J = 7.2 Hz) ppm.

(5*S**,6*aR**)- and (5*R**,6*aR**)-5-Ethoxy-3-(furan-2-yl)-5,6-dihydro-7*H*-benzo[*b*]pyrano[4',3':2,3]pyrrolo[1,2-*d*][1,4]oxazine-1,2,7-triones (**1f**). Obtained as a mixture of diastereomers, (5*S**,6*aR**):(5*R**,6*aR**) ~ 5:2 (by NMR). Yield: 76% (0.90 g); yellow solid; mp 199–201 °C (decomp., toluene). ^1H NMR (400 MHz, $\text{DMSO-}d_6$) of (5*S**,6*aR**)-diastereomer (major): δ = 8.26 (m, 1H), 8.13 (m, 1H), 7.81 (m, 1H), 7.37 (m, 3H), 6.89 (m, 1H), 5.77 (m, 1H, X-part of ABX system), 3.92–3.71 (m, 2H), 2.38 (m, 2H, AB-part of ABX system), 1.14 (t, 3H, 3J = 7.1 Hz) ppm. ^1H NMR (400 MHz, $\text{DMSO-}d_6$) of (5*R**,6*aR**)-diastereomer (minor): δ = 8.26 (m, 1H), 8.13 (m, 1H), 7.78 (m, 1H), 7.36 (m, 3H), 6.88 (m, 1H), 5.61 (d.d, 1H, $^3J_{ax.ax.}$ = 10.0 Hz, $^3J_{ax.eq.}$ = 4.2 Hz), 4.08–4.00 (m, 1H), 3.84–3.77 (m, 1H), 2.61 (d.d, 1H, 2J = 13.2 Hz, $^3J_{ax.eq.}$ = 4.4 Hz), 2.15 (d.d, 1H, 2J = 13.0 Hz, $^3J_{ax.ax.}$ = 10.0 Hz), 1.21 (t, 3H, 3J = 7.1 Hz) ppm. ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$) of (5*S**,6*aR**)-diastereomer (major): δ = 174.7, 160.7, 159.1, 153.8, 147.9, 144.9, 143.1, 127.4, 124.7, 122.1, 121.7, 121.2, 116.6, 112.9, 101.5, 100.1, 64.8, 54.2, 34.8, 14.6 ppm. ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$) of (5*R**,6*aR**)-diastereomer (minor): δ = 174.9, 162.7, 159.9, 154.4, 147.8, 144.9, 142.8, 127.2, 124.8, 122.7, 121.5, 121.1, 116.7, 112.8, 101.7, 101.5, 65.5, 57.0, 34.0, 14.9 ppm. IR (mineral oil): 1789, 1771, 1723, 1699 cm^{-1} . Anal. Calcd (%) for $\text{C}_{20}\text{H}_{15}\text{NO}_7$: C 62.99; H 3.96; N 3.67. Found: C 63.26; H 3.87; N 3.95.

(5*S**,6*aR**)-5-Ethoxy-11-methyl-3-phenyl-5,6-dihydro-7*H*-benzo[*b*]pyrano[4',3':2,3]pyrrolo[1,2-*d*]oxazine-1,2,7-trione (**1g**). Yield: 46% (0.58 g); yellow solid; mp 191–193 °C (decomp., toluene). ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ = 7.93 (m, 2H), 7.68 (m, 1H), 7.62 (m, 1H), 7.57 (m, 2H), 7.28 (m, 1H), 7.21 (m, 1H), 5.78 (m, 1H, X-part of ABX system), 3.87–3.68 (m, 2H), 2.38 (m, 5H, AB-part of ABX system + Me), 1.11 (t, 3H, 3J = 7.1 Hz) ppm. ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$): δ = 175.8, 166.4, 160.8, 159.0, 141.0, 134.1, 132.9, 130.7, 130.2, 127.9, 127.8, 122.1, 120.9, 116.3, 102.9, 100.2, 64.6, 54.4, 35.0, 20.4, 14.5 ppm. IR (mineral oil): 1789, 1724, 1709 cm^{-1} . Anal. Calcd (%) for $\text{C}_{23}\text{H}_{19}\text{NO}_6$: C 68.14; H 4.72; N 3.46. Found: C 68.01; H 4.83; N 3.53.

(5*S**,6*aR**)-11-Chloro-5-ethoxy-3-phenyl-5,6-dihydro-7*H*-benzo[*b*]pyrano[4',3':2,3]pyrrolo[1,2-*d*]oxazine-1,2,7-trione (**1h**) [20]. Yield: 51% (0.67 g); yellow solid; mp 243–244 °C (decomp., toluene) (Lit.mp 243–245 °C (decomp., toluene) [20]). ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ = 7.91 (m, 2H), 7.79 (m, 1H), 7.69 (m, 1H), 7.58 (m, 2H), 7.46 (m, 2H), 5.80 (m, 1H, X-

part of ABX system), 3.86–3.67 (m, 2H), 2.42 (m, 2H, AB-part of ABX system), 1.10 (t, 3H, $^3J = 7.0$ Hz) ppm.

Compound **1i** was reported earlier [30].

Procedure for compound 1i. A suspension of FPD **2a** (3.1 mmol, 1.0 g) and styrene **3c** (3.6 mL, 31.0 mmol) in 15 mL of anhydrous *o*-xylene was refluxed for 4 h (until the reaction mixture became dark yellow). Then, the mixture was cooled to room temperature. The formed precipitate was filtered off and recrystallized from toluene (10 mL) to afford compound **1i**.

(5*R**,6*aR**)- and (5*S**,6*aR**)-3,5-Diphenyl-5,6-dihydro-7*H*-benzo[*b*]pyrano[4',3':2,3]pyrrolo[1,2-*d*][1,4]oxazine-1,2,7-triones (**1i**) [30]. Obtained as a mixture of diastereomers, (5*R**,6*aR**):(5*S**,6*aR**) ~ 5:1 (by NMR). Yield: 65% (0.85 g); yellow solid; mp 275–277 °C (decomp., toluene) (Lit.mp 278–279 °C (decomp., toluene) [30]). ^1H NMR (400 MHz, DMSO- d_6) of (5*R**,6*aR**)-diastereomer (major): $\delta = 7.75$ (m, 3H), 7.61 (m, 5H), 7.36 (m, 6H), 5.53 (d.d, 1H, $^3J_{ax.ax.} = 12.7$ Hz, $^3J_{ax.eq.} = 4.1$ Hz), 2.60 (d.d, 1H, $^2J = 12.9$ Hz, $^3J_{ax.eq.} = 3.9$ Hz), 2.47 (m, 1H) ppm. ^1H NMR (400 MHz, DMSO- d_6) of (5*S**,6*aR**)-diastereomer (minor): $\delta = 7.99$ (m, 2H), 7.75 (m, 1H), 7.61 (m, 5H), 7.36 (m, 6H), 5.92 (m, 1H, X-part of ABX system), 2.79 (m, 2H, AB-part of ABX system) ppm.

Compounds **1j,l,m** were reported earlier [28]. Compounds **1k,n,o** were not reported.

General procedure for compounds 1j–o. A suspension of the corresponding FPD **2g–l** (3.1 mmol) and alkoxyethylenes **3b,d** (15.5 mmol) in 10 mL of anhydrous 1,4-dioxane was refluxed for 15–30 min (until the violet color typical of starting compound **2** disappeared). Then, the mixture was cooled to room temperature. The formed precipitate was filtered off and recrystallized from 1,4-dioxane (5–10 mL) to afford the corresponding compound **1j–o**.

(5*S**,6*aR**)- and (5*R**,6*aR**)-5-Ethoxy-3-phenyl-5,6-dihydropyrano[4',3':2,3]pyrrolo[1,2-*a*]quinoxaline-1,2,7(8*H*)-triones (**1j**) [28]. Obtained as a mixture of diastereomers, (5*S**,6*aR**):(5*R**,6*aR**) ~ 5:1 (by NMR). Yield: 68% (0.82 g); yellow solid; mp 243–244 °C (decomp., 1,4-dioxane) (Lit.mp 243–244 °C (decomp., 1,4-dioxane) [28]). ^1H NMR (400 MHz, DMSO- d_6) of (5*S**,6*aR**)-diastereomer (major): $\delta = 10.88$ (s, 1H), 7.94 (m, 2H), 7.72 (m, 1H), 7.67 (m, 1H), 7.56 (m, 2H), 7.30 (m, 1H), 7.14 (m, 2H), 5.73 (m, 1H, X-part of ABX system), 3.88–3.67 (m, 2H), 2.30 (m, 2H, AB-part of ABX system), 1.12 (t, 3H, $^3J = 7.1$ Hz) ppm. ^1H NMR (400 MHz, DMSO- d_6) of (5*R**,6*aR**)-diastereomer (minor): $\delta = 11.04$ (s, 1H), 7.87 (m, 2H), 7.72 (m, 1H), 7.67 (m, 1H), 7.56 (m, 2H), 7.30 (m, 1H), 7.14 (m, 2H), 5.60 (d.d, 1H, $^3J_{ax.ax.} = 10.3$ Hz, $^3J_{ax.eq.} = 3.9$ Hz), 4.08–4.01 (m, 1H), 3.84–3.77 (m, 1H), 2.42 (d.d, 1H, $^2J = 12.7$ Hz, $^3J_{ax.eq.} = 4.2$ Hz), 2.05 (d.d, 1H, $^2J = 12.7$ Hz, $^3J_{ax.ax.} = 10.3$ Hz), 1.22 (t, 3H, $^3J = 7.1$ Hz) ppm.

(5*S**,6*aR**)- and (5*R**,6*aR**)-5-Ethoxy-3-(furan-2-yl)-5,6-dihydropyrano[4',3':2,3]pyrrolo[1,2-*a*]quinoxaline-1,2,7(8*H*)-triones (**1k**). Obtained as a mixture of diastereomers, (5*S**,6*aR**):(5*R**,6*aR**) ~ 5:1 (by NMR). Yield: 65% (0.77 g); yellow solid; mp 225–227 °C (decomp., 1,4-dioxane). ^1H NMR (400 MHz, DMSO- d_6) of (5*S**,6*aR**)-diastereomer (major): $\delta = 10.87$ (s, 1H), 8.24 (m, 1H), 8.12 (m, 1H), 7.71 (m, 1H), 7.30 (m, 1H), 7.17 (m, 1H), 7.10 (m, 1H), 6.86 (m, 1H), 5.71 (m, 1H, X-part of ABX system), 3.93–3.68 (m, 2H), 2.29 (m, 2H, AB-part of ABX system), 1.15 (t, 3H, $^3J = 7.1$ Hz) ppm. ^1H NMR (400 MHz, DMSO- d_6) of (5*R**,6*aR**)-diastereomer (minor): $\delta = 11.03$ (s, 1H), 8.27 (m, 1H), 8.12 (m, 1H), 7.71 (m, 1H), 7.30 (m, 1H), 7.17 (m, 1H), 7.10 (m, 1H), 6.86 (m, 1H), 5.61 (d.d, 1H, $^3J_{ax.ax.} = 10.3$ Hz, $^3J_{ax.eq.} = 4.4$ Hz), 4.08–4.00 (m, 1H), 3.84–3.77 (m, 1H), 2.42 (d.d, 1H, $^2J = 12.7$ Hz, $^3J_{ax.eq.} = 3.9$ Hz), 2.03 (d.d, 1H, $^2J = 12.7$ Hz, $^3J_{ax.ax.} = 10.3$ Hz), 1.21 (t, 3H, $^3J = 7.1$ Hz) ppm. ^{13}C NMR (100 MHz, DMSO- d_6) of (5*S**,6*aR**)-diastereomer (major): $\delta = 175.0, 163.8, 159.5, 154.1, 147.7, 145.3, 130.4, 127.1, 122.5, 122.3, 121.5, 121.4, 115.8, 112.8, 102.3, 100.9, 64.8, 55.4, 36.0, 14.7$ ppm. ^{13}C NMR (100 MHz, DMSO- d_6) of (5*R**,6*aR**)-diastereomer (minor): $\delta = 174.9, 165.4, 160.1, 154.7, 150.7, 145.0, 130.0, 127.1, 122.8, 122.7, 121.5, 121.4, 115.9, 112.7, 102.0, 101.6, 65.4, 58.0$,

34.7, 14.9 ppm. IR (mineral oil): 1715, 1696 cm^{-1} . Anal. Calcd (%) for $\text{C}_{20}\text{H}_{16}\text{N}_2\text{O}_6$: C 63.16; H 4.24; N 7.37. Found: C 62.86; H 4.36; N 7.41.

(5*S**,6*aR**)- and (5*R**,6*aR**)-5-Butoxy-3-(4-methoxyphenyl)-5,6-dihydropyrano[4',3':2,3]pyrrolo[1,2-*a*]quinoxaline-1,2,7(8*H*)-triones (**1l**) [28]. Obtained as a mixture of diastereomers, (5*S**,6*aR**):(5*R**,6*aR**) ~ 1:2 (by NMR). Yield: 35% (0.47 g); yellow solid; mp 256–258 °C (decomp., 1,4-dioxane) (Lit.mp 250–251 °C (decomp., 1,4-dioxane) [28]). ^1H NMR (400 MHz, $\text{DMSO}-d_6$) of (5*S**,6*aR**)-diastereomer (minor): δ = 10.91 (s, 1H), 7.97 (m, 2H), 7.71 (m, 1H), 7.30 (m, 1H), 7.17 (m, 1H), 7.12 (m, 3H), 5.67 (m, 1H, X-part of ABX system), 3.89 (s, 3H), 3.81 (m, 1H), 3.65 (m, 1H), 2.28 (m, 2H, AB-part of ABX system), 1.47 (m, 2H), 1.31 (m, 2H), 0.84 (t, 3H, $^3J = 7.2$ Hz) ppm. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) of (5*R**,6*aR**)-diastereomer (major): δ = 11.08 (s, 1H), 7.89 (m, 2H), 7.71 (m, 1H), 7.30 (m, 1H), 7.17 (m, 1H), 7.12 (m, 3H), 5.56 (d.d, 1H, $^3J_{ax.ax.} = 10.2$ Hz, $^3J_{ax.eq.} = 4.3$ Hz), 4.01 (m, 1H), 3.89 (s, 3H), 3.73 (m, 1H), 2.42 (d.d, 1H, $^2J = 12.7$ Hz, $^3J_{ax.eq.} = 4.1$ Hz), 2.02 (d.d, 1H, $^2J = 12.5$ Hz, $^3J_{ax.ax.} = 10.6$ Hz), 1.56 (m, 2H), 1.37 (m, 2H), 0.89 (t, 3H, $^3J = 7.2$ Hz) ppm.

(5*S**,6*aR**)- and (5*R**,6*aR**)-5-Butoxy-3-(4-chlorophenyl)-5,6-dihydropyrano[4',3':2,3]pyrrolo[1,2-*a*]quinoxaline-1,2,7(8*H*)-triones (**1m**) [28]. Obtained as a mixture of diastereomers, (5*S**,6*aR**):(5*R**,6*aR**) ~ 10:1 (by NMR). Yield: 59% (0.83 g); yellow solid; mp 243–245 °C (decomp., 1,4-dioxane) (Lit.mp 241–242 °C (decomp., 1,4-dioxane) [28]). ^1H NMR (400 MHz, $\text{DMSO}-d_6$) of (5*S**,6*aR**)-diastereomer (major): δ = 10.93 (s, 1H), 7.94 (m, 2H), 7.71 (m, 1H), 7.65 (m, 2H), 7.30 (m, 1H), 7.14 (m, 2H), 5.71 (m, 1H, X-part of ABX system), 3.79 (m, 1H), 3.64 (m, 1H), 2.28 (m, 2H, AB-part of ABX system), 1.47 (m, 2H), 1.30 (m, 2H), 0.83 (t, 3H, $^3J = 7.4$ Hz) ppm. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) of (5*R**,6*aR**)-diastereomer (minor): δ = 11.09 (s, 1H), 7.85 (m, 2H), 7.71 (m, 1H), 7.65 (m, 2H), 7.30 (m, 1H), 7.14 (m, 2H), 5.60 (d.d, 1H, $^3J_{ax.ax.} = 10.2$ Hz, $^3J_{ax.eq.} = 3.9$ Hz), 3.99 (m, 1H), 3.72 (m, 1H), 2.42 (d.d, 1H, $^2J = 12.5$ Hz, $^3J_{ax.eq.} = 3.9$ Hz), 2.07 (d.d, 1H, $^2J = 12.7$ Hz, $^3J_{ax.ax.} = 10.4$ Hz), 1.55 (m, 2H), 1.37 (m, 2H), 0.88 (t, 3H, $^3J = 7.2$ Hz) ppm.

(5*S**,6*aR**)-3-(4-Chlorophenyl)-5-ethoxy-8-methyl-5,6-dihydropyrano[4',3':2,3]pyrrolo[1,2-*a*]quinoxaline-1,2,7(8*H*)-trione (**1n**). Yield: 48% (0.65 g); yellow solid; mp 235–236 °C (decomp., 1,4-dioxane). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ = 7.95 (m, 2H), 7.76 (m, 1H), 7.65 (m, 2H), 7.42 (m, 2H), 7.25 (m, 1H), 5.72 (m, 1H, X-part of ABX system), 3.81 (m, 1H), 3.66 (m, 1H), 3.35 (s, 3H), 2.25 (m, 2H, AB-part of ABX system), 1.10 (t, 3H, $^3J = 6.8$ Hz) ppm. ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ = 176.2, 165.3, 163.2, 158.8, 137.6, 132.4, 132.0, 129.8, 128.0, 127.3, 122.9, 122.5, 122.1, 116.0, 104.6, 101.1, 64.3, 55.4, 35.9, 30.1, 14.5 ppm. IR (mineral oil): 1720, 1698, 1683 cm^{-1} . Anal. Calcd (%) for $\text{C}_{23}\text{H}_{19}\text{ClN}_2\text{O}_5$: C 62.95; H 4.36; N 6.38. Found: C 63.18; H 4.26; N 6.55.

(5*S**,6*aR**)- and (5*R**,6*aR**)-5-Ethoxy-3,8-diphenyl-5,6-dihydropyrano[4',3':2,3]pyrrolo[1,2-*a*]quinoxaline-1,2,7(8*H*)-triones (**1o**). Obtained as a mixture of diastereomers, (5*S**,6*aR**):(5*R**,6*aR**) ~ 5:2 (by NMR). Yield: 68% (0.98 g); yellow solid; mp 236–238 °C (decomp., 1,4-dioxane). ^1H NMR (400 MHz, $\text{DMSO}-d_6$) of (5*S**,6*aR**)-diastereomer (major): δ = 7.93 (m, 2H), 7.84 (m, 1H), 7.67 (m, 1H), 7.56 (m, 3H), 7.49 (m, 2H), 7.30 (m, 2H), 7.23 (m, 2H), 6.51 (m, 1H), 5.82 (d, 1H, $^3J_{ax.eq.} = 4.4$ Hz), 3.85 (m, 1H), 3.73 (m, 1H), 2.53 (d, 1H, $^2J = 13.2$ Hz), 2.37 (d.d, 1H, $^2J = 13.7$ Hz, $^3J_{ax.eq.} = 4.9$ Hz), 1.15 (t, 3H, $^3J = 7.1$ Hz) ppm. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) of (5*R**,6*aR**)-diastereomer (minor): δ = 7.87 (m, 2H), 7.80 (m, 1H), 7.67 (m, 1H), 7.56 (m, 5H), 7.49 (m, 1H), 7.30 (m, 1H), 7.23 (m, 2H), 6.47 (m, 1H), 5.63 (d.d, 1H, $^3J_{ax.ax.} = 10.3$ Hz, $^3J_{ax.eq.} = 3.9$ Hz), 4.08 (m, 1H), 3.82 (m, 1H), 2.73 (d.d, 1H, $^2J = 13.0$ Hz, $^3J_{ax.eq.} = 4.2$ Hz), 2.14 (d.d, 1H, $^2J = 12.7$ Hz, $^3J_{ax.ax.} = 10.3$ Hz), 1.25 (t, 3H, $^3J = 6.8$ Hz) ppm. ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) of (5*S**,6*aR**)-diastereomer (major): δ = 176.2, 166.6, 163.1, 159.0, 137.7, 133.1, 132.9, 131.0, 130.2, 130.0, 129.6, 128.8, 128.4, 127.8, 126.8, 123.2, 122.4, 122.2, 116.7, 103.8, 101.1, 64.6, 55.9, 35.7, 14.6 ppm. ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) of (5*R**,6*aR**)-diastereomer (minor): δ = 170.1, 167.2, 164.7, 159.8, 137.0, 132.9, 132.7, 130.9,

129.6, 128.7, 128.6, 128.1, 127.8, 126.7, 123.2, 122.8, 122.2, 122.2, 116.7, 103.2, 102.0, 65.4, 58.5, 34.7, 15.0 ppm. IR (mineral oil): 1715 cm⁻¹. Anal. Calcd (%) for C₂₈H₂₂N₂O₅: C 72.09; H 4.75; N 6.01. Found: C 71.93; H 4.89; N 6.15.

Compound **1p** was reported earlier [31].

Procedure for compound 1p. A suspension of FPD **2g** (3.1 mmol, 1.0 g) and 3,4-dihydro-2H-pyran **3e** (5.7 mL, 6.2 mmol) in 10 mL of anhydrous 1,4-dioxane was refluxed for 3 h (until the violet color typical of starting compound **2** disappeared). Then, the mixture was cooled to room temperature. The formed precipitate was filtered off to afford compound **1i**. Compound **1i** was used in further studies without additional purification.

(9aR*,13aR*,13bR*)-8-Phenyl-9a,12,13,13a-tetrahydro-11H-pyrano[3'',2'':5',6']pyrano[4',3':2,3]pyrrolo[1,2-a]quinoxaline-6,7,14(15H)-trione (**1p**) [31]. Yield: 28% (0.35 g); yellow solid; mp 270–271 °C (decomp., 1,4-dioxane) (Lit.mp 278–280 °C (decomp., 1,4-dioxane) [31]). ¹H NMR (400 MHz, DMSO-*d*₆): δ = 11.15 (s, 1H), 7.91 (m, 2H), 7.69 (m, 2H), 7.57 (m, 2H), 7.33 (m, 1H), 7.19 (m, 1H), 7.13 (m, 1H), 5.77 (d, 1H, ³J_{ax.eq.} = 3.9 Hz), 3.79 (m, 2H), 2.26 (d.t, 1H, ³J = 12.5 Hz, ³J_{ax.eq.} = 4.3 Hz), 1.60 (m, 2H), 1.42 (m, 1H), 1.11 (m, 1H) ppm.

Compounds **1q–t** were reported earlier [32].

General procedure for compounds 1q–t. A suspension of the corresponding FPD **2l,m** (3.1 mmol) and the corresponding cyanamide **3f–h** (3.2 mmol) in 15 mL of anhydrous acetonitrile was refluxed for 3–5 h (until the violet color typical of starting compound **2** disappeared). Then, the reaction mixture was cooled to room temperature, and the resulting precipitate was filtered off to afford the corresponding compound **1q–t**. Compounds **1q–t** were used in further studies without additional purification.

5-(Dimethylamino)-3,8-diphenyl-[1,3]oxazino[4',5':2,3]pyrrolo[1,2-a]quinoxaline-1,2,7(8H)-trione (**1q**) [32]. Yield: 78% (1.14 g); yellow solid; mp 220–221 °C (decomp., acetonitrile) (Lit.mp 220–221 °C (decomp., acetonitrile) [32]). ¹H NMR (400 MHz, DMSO-*d*₆): δ = 8.08 (m, 2H), 7.81 (m, 1H), 7.74 (m, 1H), 7.65 (m, 2H), 7.59 (m, 2H), 7.51 (m, 1H), 7.27 (m, 2H), 7.20 (m, 2H), 6.41 (m, 1H), 3.01 (s, 6H) ppm.

5-(Dimethylamino)-3-(4-nitrophenyl)-8-phenyl-[1,3]oxazino[4',5':2,3]pyrrolo[1,2-a]quinoxaline-1,2,7(8H)-trione (**1r**) [32]. Yield: 81% (1.26 g); yellow solid; mp 210–211 °C (decomp., acetonitrile) (Lit.mp 210–211 °C (decomp., acetonitrile) [32]). ¹H NMR (400 MHz, DMSO-*d*₆): δ = 8.46 (m, 2H), 8.29 (m, 2H), 7.81 (m, 1H), 7.60 (m, 2H), 7.51 (m, 1H), 7.27 (m, 2H), 7.22 (m, 2H), 6.43 (m, 1H), 3.02 (s, 6H) ppm.

5-(Diethylamino)-3,8-diphenyl-[1,3]oxazino[4',5':2,3]pyrrolo[1,2-a]quinoxaline-1,2,7(8H)-trione (**1s**) [32]. Yield: 85% (1.30 g); yellow solid; mp 200–204 °C (decomp., acetonitrile) (Lit.mp 200–204 °C (decomp., acetonitrile) [32]). ¹H NMR (400 MHz, DMSO-*d*₆): δ = 8.07 (m, 2H), 7.82 (m, 1H), 7.74 (m, 1H), 7.66 (m, 2H), 7.59 (m, 2H), 7.51 (m, 1H), 7.23 (m, 4H), 6.43 (m, 1H), 3.56–3.47 (m, 2H), 3.36–3.26 (m, 2H), 1.15 (t, 6H, ³J = 7.1 Hz,) ppm.

3,8-Diphenyl-5-(piperidin-1-yl)-[1,3]oxazino[4',5':2,3]pyrrolo[1,2-a]quinoxaline-1,2,7(8H)-trione (**1t**) [32]. Yield: 83% (1.30 g); yellow solid; mp 221–224 °C (decomp., acetonitrile) (Lit.mp 221–224 °C (decomp., acetonitrile) [32]). ¹H NMR (400 MHz, DMSO-*d*₆): δ = 8.04 (m, 2H), 7.82 (m, 1H), 7.74 (m, 1H), 7.65 (m, 2H), 7.59 (m, 2H), 7.51 (m, 1H), 7.22 (m, 4H), 6.41 (m, 1H), 3.49 (m, 4H), 1.57 (m, 6H) ppm.

3.2. Biology

3.2.1. Screening of Substances in 96-Well Plates

Chlorella vulgaris (strain IMBR-19, obtained from the A.O. Kovalevsky Institute of Biology of the Southern Seas, RAS, Sevastopol, Russia) was cultivated in BG-11 medium [45] under aseptic conditions. The only exception to sterility was the use of DMSO solutions of the tested compounds, which were not sterilized after dilution. The test compounds were initially dissolved in 99% DMSO. For poorly soluble compounds, the solutions were either

incubated on a rotator for 16 h or subjected to ultrasonic treatment using an ultrasound homogenizer with a 3 mm probe (VCX-130, Sonics and Materials, Newtown, CT, USA).

For the screening experiments, 96-well culture plates were prepared by combining BG-11 medium, a starter culture of *C. vulgaris*, and DMSO-diluted test compounds to a final volume of 300 μL per well. The initial cell density was set at 5×10^4 cells/well, with a final DMSO concentration of 1% (v/v) across all wells. The test compounds were evaluated at final concentrations of 0.1, 1, and 10 $\mu\text{mol/L}$. Negative control wells received an equivalent volume of pure DMSO without test compounds, while positive control wells contained BG-11 medium supplemented with 2 g/L glucose in addition to DMSO. Each test condition was performed in duplicate (two wells per concentration). To minimize edge effects, sterile distilled water was added to all edge and corner wells of the plates. The plates were sealed with gas-permeable films to maintain sterility and allow gas exchange.

The cultures were incubated for five days in a humidified chamber at 28 °C with orbital shaking at 150 rpm. Illumination was provided in a 12 h light/12 h dark cycle at an intensity of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ using an array of evenly distributed white LEDs positioned below the culture plates for bottom illumination. The lighting system included a cooling mechanism to prevent heat transfer to the culture plates.

At the conclusion of the incubation period, the contents of each well were thoroughly mixed using a multichannel pipette to ensure uniform cell suspension. Cell growth was assessed by measuring the OD750 using a microplate spectrophotometer [46].

3.2.2. Evaluation of Compound 1s in 50 mL Flasks

The experiments were conducted in 50 mL Erlenmeyer flasks containing BG-11 medium, a starter culture of *C. vulgaris*, and compound **1s** dissolved in DMSO. The final volume in each flask was adjusted to 30 mL, with the initial cell density standardized at 1×10^7 cells per flask. The DMSO concentration in all flasks, including the controls, was maintained at 1% (v/v). The test compound was evaluated at final concentrations of 0.1, 1, 10, and 100 $\mu\text{mol/L}$. Negative control flasks received an equivalent volume of DMSO without any test compound, while positive control flasks were supplemented with 2 g/L glucose in addition to DMSO. All treatments, including the controls, were prepared in triplicate (three flasks per condition). To ensure gas exchange while minimizing contamination, the flasks were sealed with gas-permeable cellulose caps. The cultures were incubated for five days under conditions identical to those described in prior studies.

Cell Count. At the end of the incubation period, the contents of each flask were thoroughly mixed to ensure homogeneity. A 10 mL aliquot of the culture was transferred into a 15 mL centrifuge tube and subjected to two washing steps with distilled water (10 mL per wash) by centrifugation at $450 \times g$ for 15 min. The washed cell pellet was resuspended in 5 mL of distilled water (the resulting cell pellet was concentrated two-fold and vortexed for 1 min to ensure uniformity). Cell counts were determined using a hemocytometer.

Pigment Analysis. To determine the concentration of cellular pigments, 2 mL of washed cells obtained from the cell count experiment were subjected to centrifugation at $7000 \times g$ for 10 min. Following centrifugation, 1.8 mL of the supernatant was removed, and an equal volume (1.8 mL) of 99% methanol was added to achieve a final methanol concentration of 90%. The samples were incubated at 60 °C for 30 min in a solid-state thermostat, followed by cooling to room temperature. After cooling, the samples were centrifuged at $10,000 \times g$ for 10 min to remove cellular debris. The absorbance of the supernatant containing the extracted pigments was measured spectrophotometrically at 665 nm, 652 nm, and 470 nm. Chlorophyll and carotenoid concentrations were calculated using established equations [47,48]. The pigment content was normalized to the cell count and expressed as μg per 1×10^7 cells.

Preparation of *C. vulgaris* biomass for protein and carbohydrate analysis. Cells were washed in deionized water and concentrated two-fold. Cell suspensions washed from BG-11 medium were transferred to 2 mL screw-cap tubes. Only the pellet (cell biomass) was left in the tubes. Then, 90% methanol (*v/v*) was added to the pellet for pigment extraction. The samples were incubated at 60 °C for 30 min in a solid-state thermostat and then cooled to room temperature. After cooling, the samples were centrifuged at $10,000\times g$ for 10 min to remove the supernatant. The pellet was treated with 500 μL of 2 mol/L hydrochloric acid and boiled at 100 °C for 2 h.

Protein Analysis. A modified Bradford colorimetric assay was employed to quantify total protein content [49]. Acid hydrolysates (160 μL) were loaded into deep-well 96-well plates, followed by the sequential addition of 2 mol/L NaOH (160 μL) and deionized water (480 μL) with thorough mixing. A bovine serum albumin (BSA) standard curve (8–32 $\mu\text{g}/\text{mL}$ in 8 $\mu\text{g}/\text{mL}$ increments) and blank (deionized water) were prepared in parallel, each receiving 160 μL of 2 mol/L NaCl and being brought to an 800 μL final volume with deionized water. Using a multichannel pipette, 160 μL aliquots from the deep-well plate were transferred to a flat-bottom 96-well plate, combined with 40 μL of Bradford reagent (Bio-Rad Protein Assay Dye Concentrate), and incubated (30 °C, 850 rpm, 5 min). Absorbance at 595 nm was measured using a Synergy H1 microplate reader (BioTek Instruments). Protein concentrations were determined by referencing a calibration curve and normalized to cellular content as μg per 1×10^6 cells.

Carbohydrate Analysis. A modified colorimetric anthrone assay was employed to quantify the total carbohydrate content [50]. Anthrone (150 mg) was dissolved in 3.75 mL of 95% ethanol under magnetic stirring (80 °C, 5 min) in a 100 mL beaker. Sulfuric acid (75%, 71.25 mL) was added dropwise to the mixture with continuous stirring, followed by heating for an additional 5 min. The final reagent volume was 75 mL. The reagent was stored at 4 °C for ≤ 24 h prior to use. Acid hydrolysates (80 μL for compound **1s**/DMSO-treated cells; 10 μL for glucose-treated cells) were diluted to 200 μL with distilled water in 2.0 mL screw-cap micro-centrifuge tubes. A calibration curve was prepared using glucose standards (0, 50, 100, 150, and 200 $\mu\text{g}/\text{mL}$ in distilled water). To each tube, 1.0 mL of freshly prepared anthrone reagent (prepared as described above) was added, followed by gently mixing all tubes for 20 s. The reaction mixtures were heated at (100 ± 1) °C for 15 min in a water bath and then cooled in an ice-water bath for 5 min. The absorbance was measured at 625 nm using a spectrophotometer. All technical replicates were performed in triplicate. Carbohydrate concentrations were determined by referencing a calibration curve and normalized to cellular content as μg per 1×10^6 cells.

3.2.3. Data Analysis

For the screening experiments, two wells were used for each concentration of the tested compounds, while six wells were allocated for both the negative and positive controls. Compounds were selected for further analysis if their mean OD750 exceeded the mean OD750 of the negative control by more than three standard deviations.

In the detailed investigation of compound **1s** bioactivity, the biological replicates consisted of three independently cultured flasks. For the positive controls, the biological replicates were derived from a single culture flask per condition. Protein quantification was performed using triplicate technical replicates ($n = 3$) from each biological replicate (flask), resulting in nine data points per condition. For pigment and carbohydrate analyses, duplicate technical replicates ($n = 2$) were obtained from each biological replicate (flask), yielding six data points per condition. Statistical comparisons between groups were conducted using nested one-way ANOVA.

4. Conclusions

The present study explored the potential of angular 6/6/5/6-annulated pyrrolidine-2,3-diones as growth-stimulating agents for *C. vulgaris*. We identified compound **1s** as a promising candidate capable of modulating microalgal metabolism. While our initial microplate assays suggested a stimulatory effect on biomass accumulation, flask-scale experiments revealed that compound **1s** did not significantly enhance cell concentration but instead induced a 19% increase in protein content at 1 $\mu\text{mol/L}$. While demonstrating promising bioactivity, compound **1s** exhibited substantial hydrolytic instability in aqueous media. This finding aligns with the growing demand for sustainable protein sources, as *C. vulgaris* is already recognized for its high-quality protein profile. Overall, this work contributes to the development of novel small-molecule strategies for enhancing microalgal biomass composition, with potential applications in nutraceuticals, aquaculture feed, and sustainable protein production.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/chemistry7040102/s1>. The following are available online: copies of NMR spectra for compounds **1a–t**; copies of HPLC-UV chromatogram plots for hydrolysis study of compound **1s**; and clarified substituents in compounds **1–6**.

Author Contributions: Conceptualization, E.E.K. and P.V.K.; investigation, E.E.K. (chemistry), A.D.N. and P.V.K. (biology); writing—original draft preparation, E.E.K., P.V.K. and A.D.N.; writing—review and editing, E.E.K., P.V.K. and A.D.N.; supervision, E.E.K. and P.V.K.; project administration, E.E.K. and P.V.K. All authors have read and agreed to the published version of the manuscript.

Funding: This study was performed in the framework of State Assignment No. 124020500028-4.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The presented data are available in this article.

Acknowledgments: The authors thank Ekaterina A. Lystsova (Perm State University, Perm, Russia) for their help with the HPLC and NMR experiments.

Conflicts of Interest: The authors declare no conflicts of interest.

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