

Article

7-Aminopyrazolo[1,5-*d*][1,2,4]triazin-4(5*H*)-ones: Synthesis and Growth-Regulating Activity in *Chlorella vulgaris*

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Abstract

A series of 7-aminopyrazolo[1,5-*d*][1,2,4]triazin-4(5*H*)-ones was synthesized via a cascade condensation of methyl aroylpyruvates with 1,3-diaminoguanidine hydrochloride. The scope and limitations of this approach were investigated. Methyl mesitylpyruvate bearing a sterically hindered mesityl substituent diverted the reaction pathway, affording a 1,2,4-triazine derivative. Diethyl 2,4,6-trioxoheptanedioate resulted in an unexpected pyrazolo[1,5-*d*][1,2,4]triazepine scaffold. All synthesized compounds were evaluated for growth-regulating activity using the green microalga *Chlorella vulgaris* as a model organism. 7-Amino-2-(4-methoxyphenyl)pyrazolo[1,5-*d*][1,2,4]triazin-4(5*H*)-one has shown the best results in the initial microplate screening, showing increased cell density at 10 µmol/L. However, subsequent validation in 50 mL flask cultures revealed no significant effect on biomass accumulation, photosynthetic pigment content, carbohydrate levels, or neutral lipid production compared to the negative control. Only a modest increase in protein content was observed at the concentration of 100 µmol/L.

Keywords: acylpyruvate; *Chlorella*; cytokinin; diaminoguanidine; heterocycle; microalgae; pyrazolo[1,5-*d*][1,2,4]triazine

1. Introduction

The search for new plant growth regulators remains a central challenge in agrochemistry [1]. Among natural plant growth regulators, cytokinins—a class of purine-derived phytohormones—are paramount in governing crucial physiological processes such as cell division, shoot growth and signal exchange between different parts and organs of plants [2]. The structural core of cytokinins is the adenine (6-aminopurine) scaffold (Figure 1) [2,3].

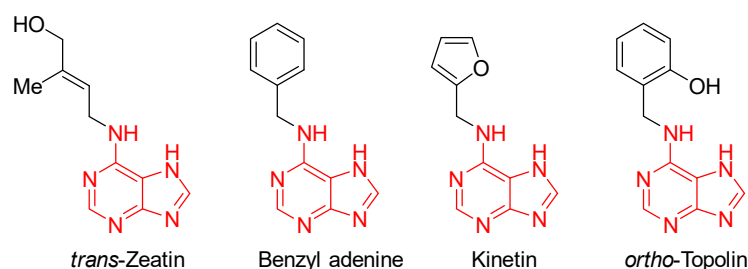


Figure 1. Structures of selected cytokinins (adenine scaffold is shown in red).



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In recent decades, the strategy of bioisosteric replacement has proven highly fruitful in agrochemical discovery [4–6]. Within this context, 7-aminopyrazolo[1,5-*d*][1,2,4]triazines are prominent adenine mimetics (Figure 2). Thus, compounds based on this structure can be expected to show cytokinin-like activity.

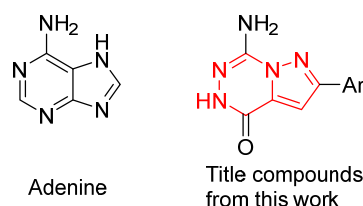
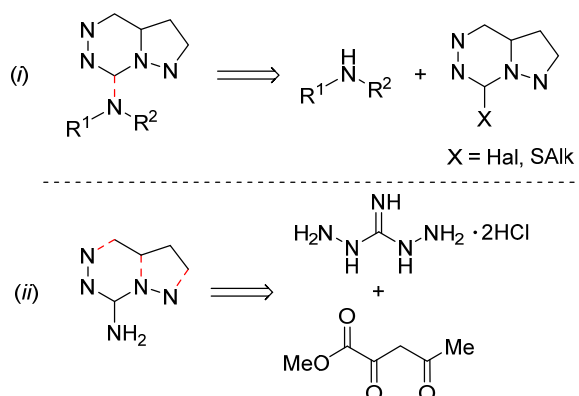


Figure 2. Structures of adenine and the title compounds (pyrazolo[1,5-*d*][1,2,4]triazine core) are shown in red.

Current synthetic strategies for 7-aminopyrazolo[1,5-*d*][1,2,4]triazines can be divided into two principal approaches (for the mini-review on the approaches to the pyrazolo[1,5-*d*][1,2,4]triazine core, see publication [7]). The first one involves nucleophilic substitution reactions, utilizing either a halogen [8–13] or an alkylthio group [14–16] as a leaving group (Equation (i), Scheme 1). The second approach is based on a condensation reaction of acetylpyruvate with 1,3-diaminoguanidine dihydrochloride [17] (Equation (ii), Scheme 1).



Scheme 1. Approaches to 7-aminopyrazolo[1,5-*d*][1,2,4]triazines (formed bonds are shown in red).

The nucleophilic substitution approaches (Equation (i), Scheme 1) require prefunctionalized heterocyclic cores, the synthesis of which often involves multiple steps and harsh conditions, limiting the overall atom economy and sustainability of the process. The cascade condensation reported in [17] (Equation (ii), Scheme 1) is highly attractive from the perspective of step-economy, and assembles the target heterocycle from simple acyclic precursors. Yet, despite its potential, this cascade condensation remains virtually unexplored. To the best of our knowledge, the scope of this reaction has been limited to a single example with methyl acetylpyruvate [17], and the potential of using various acylpyruvates in this cascade has not been systematically investigated. This represents a significant missed opportunity for the rapid and diverse synthesis of 7-aminopyrazolo[1,5-*d*][1,2,4]triazines.

The development of a versatile and efficient synthetic protocol based on this cascade condensation [17] (Equation (ii), Scheme 1) would provide a valuable library of novel compounds for biological screening. Given the structural similarity of the target 7-aminopyrazolo[1,5-*d*][1,2,4]triazine scaffold to adenine, such a library holds considerable promise for the discovery of new compounds with cytokinin-like activity. The green microalga *Chlorella vulgaris* serves as an excellent model organism for evaluating such activity, as its growth parameters are sensitive to phytohormones and their analogues, allowing for a rapid and informative assessment of biological effects [18–22].

The present study reports the development of a synthetic method to 7-amino-2-arylpyrazolo[1,5-*d*][1,2,4]triazin-4(5*H*)-ones via the cascade condensation of various aroylpyruvates with 1,3-diaminoguanidine hydrochloride. The synthesized compounds were evaluated for the growth-regulating activity on the microalga *C. vulgaris*, focusing on key physiological parameters such as biomass accumulation, chlorophyll and carotenoid content, and lipid production, to identify potential new leads with cytokinin-like properties.

2. Materials and Methods

2.1. Synthetic Methods and Analytic Data of Compounds

2.1.1. General Information

^1H and ^{13}C 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 $\text{DMSO-}d_6$, using solvent residual signals (in ^{13}C NMR, 39.51 ppm; in ^1H NMR, 2.50 ppm) as internal standards. 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 unit cell parameters and the X-ray diffraction intensities were measured on an Xcalibur Ruby diffractometer (Agilent Technologies, Wroclaw, Poland). The empirical absorption correction was introduced by multi-scan method using SCALE3 ABSPACK algorithm [23]. Using the Olex2 [24], the structures were solved with the SHELXT [25] program and refined by the full-matrix least-squares method in the anisotropic approximation for all non-hydrogen atoms with the SHELXL program [26]. Hydrogen atoms bound to carbon were positioned geometrically and refined using a riding model. Hydrogen atoms of NH , NH_2 , and OH groups were refined independently with isotropic displacement parameters.

Methyl aroylpyruvates **1a–i** were prepared by the Claisen condensation of diethyl oxalate with acetophenones in the presence of MeONa [27–30]. Diethyl 2,4,6-trioxoheptanedioate (**1j**) was prepared by the Claisen condensation of two equivalents of diethyl oxalate with acetone in the presence of EtONa [30,31]. All other solvents and reagents were purchased from commercial vendors and were used as received.

2.1.2. Synthesis of Compounds **2a–h**

General procedure to compounds 2a–h. A suspension of the corresponding methyl aroylpyruvate **1a–h** (0.48 mmol) and 1,3-diaminoguanidine hydrochloride (0.48 mmol, 60 mg) in EtOH (5 mL) was stirred at 90 °C for 24 h. Then, the mixture was cooled to ambient temperature. The resulting precipitate was filtered off and recrystallized from EtOH (3–10 mL) to afford the corresponding compound **2a–h**.

7-Amino-2-phenylpyrazolo[1,5-*d*][1,2,4]triazin-4(5*H*)-one (2a). Yield: 30% (33 mg); pale yellow solid; mp 320 °C (decomp., EtOH). ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ = 11.48 (s, 1H, NH), 8.09–8.06 (m, 2H^{Ar}), 7.62 (s, 1H, C³H), 7.52–7.48 (m, 2H^{Ar}), 7.46–7.41 (m, 1H^{Ar}), 6.66 (s, 2H, NH₂) ppm. ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$): δ = 153.3 (C⁴), 153.0 (C²), 139.5 (C⁷), 135.6 (C^{3a}), 131.2 (C^{Ar}), 129.3 (C^{Ar}), 129.0 (2C^{Ar}), 126.3 (2C^{Ar}), 103.0 (C³) ppm. IR (mineral oil): 3454, 3268, 3152 (N–H), 1656 (C=O) cm^{-1} . Anal. Calcd (%) for $\text{C}_{11}\text{H}_9\text{N}_5\text{O}$: C 58.14; H 3.99; N 30.82. Found: C 58.35; H 3.82; N 30.59. Crystal structure of compound **2a** was deposited at the Cambridge Crystallographic Data Centre with the deposition number CCDC 2554056.

7-Amino-2-(4-methylphenyl)pyrazolo[1,5-*d*][1,2,4]triazin-4(5*H*)-one (2b). Yield: 35% (41 mg); pale yellow solid; mp 318 °C (decomp., EtOH). ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ = 11.46 (s, 1H, NH), 7.96–7.94 (m, 2H^{Ar}), 7.56 (s, 1H, C³H), 7.31–7.29 (m, 2H^{Ar}), 6.63 (s, 2H, NH₂),

2.36 (s, 3H, Me) ppm. ^{13}C NMR (100 MHz, DMSO- d_6): δ = 153.4 (C^4), 152.9 (C^2), 139.4 (C^7), 138.8 (C^{Ar}), 135.5 (C^{3a}), 129.3 (2C^{Ar}), 128.3 (C^{Ar}), 126.2 (2C^{Ar}), 102.6 (C^3), 20.8 (Me) ppm. IR (mineral oil): 3445, 3262, 3168 (N–H), 1651 (C=O) cm^{-1} . Anal. Calcd (%) for $\text{C}_{12}\text{H}_{11}\text{N}_5\text{O}$: C 59.74; H 3.60; N 29.03. Found: C 59.51; H 3.62; N 28.88.

7-Amino-2-(4-methoxyphenyl)pyrazolo[1,5-*d*][1,2,4]triazin-4(5H)-one (2c). Yield: 39% (48 mg); pale yellow solid; mp 264 °C (decomp., EtOH). ^1H NMR (400 MHz, DMSO- d_6): δ = 11.45 (s, 1H, NH), 8.02–7.99 (m, 2H $^{\text{Ar}}$), 7.53 (s, 1H, C^3H), 7.07–7.04 (m, 2H $^{\text{Ar}}$), 6.62 (s, 2H, NH_2), 3.82 (s, 3H, Me) ppm. ^{13}C NMR (100 MHz, DMSO- d_6): δ = 160.1 (C^{Ar}), 153.3 (C^4), 152.9 (C^2), 139.4 (C^7), 135.4 (C^{3a}), 127.7 (2C^{Ar}), 123.6 (C^{Ar}), 114.2 (2C^{Ar}), 102.3 (C^3), 55.2 (Me) ppm. IR (mineral oil): 3443, 3265, 3168 (N–H), 1633 (C=O) cm^{-1} . Anal. Calcd (%) for $\text{C}_{12}\text{H}_{11}\text{N}_5\text{O}_2$: C 56.03; H 4.31; N 27.22. Found: C 56.27; H 4.12; N 26.98.

7-Amino-2-(4-chlorophenyl)pyrazolo[1,5-*d*][1,2,4]triazin-4(5H)-one (2d). Yield: 35% (44 mg); pale yellow solid; mp 331 °C (decomp., EtOH). ^1H NMR (400 MHz, DMSO- d_6): δ = 11.49 (s, 1H, NH), 8.11–8.09 (m, 2H $^{\text{Ar}}$), 7.65 (s, 1H, C^3H), 7.58–7.56 (m, 2H $^{\text{Ar}}$), 6.68 (s, 2H, NH_2) ppm. ^{13}C NMR (100 MHz, DMSO- d_6): δ = 152.9 (C^4), 152.2 (C^2), 139.4 (C^7), 135.7 (C^{3a}), 133.9 (C^{Ar}), 130.1 (C^{Ar}), 129.0 (2C^{Ar}), 128.1 (2C^{Ar}), 103.2 (C^3) ppm. IR (mineral oil): 3427, 3225, 3178 (N–H), 1662 (C=O) cm^{-1} . Anal. Calcd (%) for $\text{C}_{11}\text{H}_8\text{ClN}_5\text{O}$: C 50.49; H 3.08; N 26.76. Found: C 50.73; H 3.14; N 26.91.

7-Amino-2-(3-methoxyphenyl)pyrazolo[1,5-*d*][1,2,4]triazin-4(5H)-one (2e). Yield: 31% (38 mg); pale yellow solid; mp 268 °C (decomp., EtOH). ^1H NMR (400 MHz, DMSO- d_6): δ = 11.47 (s, 1H, NH), 7.68–7.64 (m, 3H, C^3H + 2H $^{\text{Ar}}$), 7.43–7.39 (m, 1H $^{\text{Ar}}$), 7.03–7.00 (m, 1H $^{\text{Ar}}$), 6.67 (s, 2H, NH_2), 3.85 (s, 3H, Me) ppm. ^{13}C NMR (100 MHz, DMSO- d_6): δ = 159.7 (C^{Ar}), 153.2 (C^4), 153.0 (C^2), 139.5 (C^7), 135.5 (2C , C^{3a} + C^{Ar}), 130.0 (C^{Ar}), 118.7 (C^{Ar}), 115.1 (C^{Ar}), 111.6 (C^{Ar}), 103.3 (C^3), 55.3 (Me) ppm. IR (mineral oil): 3436, 3380, 3176 (N–H), 1645 (C=O) cm^{-1} . Anal. Calcd (%) for $\text{C}_{12}\text{H}_{11}\text{N}_5\text{O}_2$: C 56.03; H 4.31; N 27.22. Found: C 56.36; H 3.92; N 27.28.

7-Amino-2-(4-ethoxyphenyl)pyrazolo[1,5-*d*][1,2,4]triazin-4(5H)-one (2f). Yield: 35% (46 mg); pale yellow solid; mp 258 °C (decomp., EtOH). ^1H NMR (400 MHz, DMSO- d_6): δ = 11.44 (s, 1H, NH), 7.99–7.97 (m, 2H $^{\text{Ar}}$), 7.52 (s, 1H, C^3H), 7.04–7.02 (m, 2H $^{\text{Ar}}$), 6.61 (s, 2H, NH_2), 4.09 (q, J 7.0 Hz, 2H, CH_2), 1.35 (t, J 7.0 Hz, 3H, Me) ppm. ^{13}C NMR (100 MHz, DMSO- d_6): δ = 159.5 (C^{Ar}), 153.3 (C^4), 153.0 (C^2), 139.5 (C^7), 135.5 (C^{3a}), 127.8 (2C^{Ar}), 123.5 (C^{Ar}), 114.7 (2C^{Ar}), 102.4 (C^3), 63.5 (CH_2), 14.6 (Me) ppm. IR (mineral oil): 3432, 3282, 3157 (N–H), 1630 (C=O) cm^{-1} . Anal. Calcd (%) for $\text{C}_{13}\text{H}_{13}\text{N}_5\text{O}_2$: C 57.56; H 4.83; N 25.82. Found: C 57.73; H 4.80; N 25.98.

7-Amino-2-(thiophen-2-yl)pyrazolo[1,5-*d*][1,2,4]triazin-4(5H)-one (2g). Yield: 18% (20 mg); pale yellow solid; mp 315 °C (decomp., EtOH). ^1H NMR (400 MHz, DMSO- d_6): δ = 11.50 (s, 1H, NH), 7.78–7.77 (m, 1H $^{\text{Ar}}$), 7.66–7.65 (m, 1H $^{\text{Ar}}$), 7.52 (s, 1H, C^3H), 7.20–7.17 (m, 1H $^{\text{Ar}}$), 6.57 (s, 2H, NH_2) ppm. ^{13}C NMR (100 MHz, DMSO- d_6): δ = 152.7 (C^4), 149.0 (C^2), 139.2 (C^7), 135.6 (C^{3a}), 133.7 (C^{Ar}), 127.9 (C^{Ar}), 127.5 (C^{Ar}), 127.4 (C^{Ar}), 102.7 (C^3) ppm. IR (mineral oil): 3455, 3265, 3155 (N–H), 1645 (C=O) cm^{-1} . Anal. Calcd (%) for $\text{C}_9\text{H}_7\text{N}_5\text{OS}$: C 46.34; H 3.03; N 30.03. Found: C 46.00; H 3.11; N 29.98.

7-Amino-2-(4-cyanophenyl)pyrazolo[1,5-*d*][1,2,4]triazin-4(5H)-one (2h). Yield: 33% (40 mg); pale yellow solid; mp 320 °C (decomp., EtOH). ^1H NMR (400 MHz, DMSO- d_6): δ = 11.54 (s, 1H, NH), 8.29–8.26 (m, 2H $^{\text{Ar}}$), 7.99–7.97 (m, 2H $^{\text{Ar}}$), 7.79 (s, 1H, C^3H), 6.74 (s, 2H, NH_2) ppm. ^{13}C NMR (100 MHz, DMSO- d_6): δ = 152.8 (C^4), 151.4 (C^2), 139.2 (C^7), 135.8 (C^{3a}), 135.5 (C^{Ar}), 132.8 (2C^{Ar}), 126.9 (2C^{Ar}), 118.5 (CN), 111.5 (C^{Ar}), 103.9 (C^3) ppm. IR (mineral oil): 3439, 3245, 3120 (N–H), 2241 ($\text{C}\equiv\text{N}$), 1675 (C=O) cm^{-1} . Anal. Calcd (%) for $\text{C}_{12}\text{H}_8\text{N}_6\text{O}$: C 57.14; H 3.20; N 33.32. Found: C 56.89; H 3.10; N 32.98.

4-Amino-6-(2-mesityl-2-oxoethyl)-5-oxo-4,5-dihydro-1,2,4-triazin-3(2H)-iminium chloride (3). A suspension of methyl mesitoylpyruvate (**1i**) (0.48 mmol, 119 mg) and 1,3-diaminoguanidine

hydrochloride (0.48 mmol, 60 mg) in EtOH (5 mL) was stirred at 90 °C for 24 h. Then, the mixture was cooled to ambient temperature. The resulting precipitate was filtered off and recrystallized from EtOH (5 mL) to afford compound **3**. Yield: 41% (64 mg); pale yellow solid; mp 228 °C (decomp., EtOH). ¹H NMR (400 MHz, DMSO-*d*₆): δ = 9.08 (s, 2H, NH₂), 6.91 (s, 2H^{Ar}), 5.80 (br.s, 3H, NH + NH₂), 4.14 (s, 2H, CH₂), 2.25–2.23 (m, 9H, 3Me) ppm. ¹³C NMR (100 MHz, DMSO-*d*₆): δ = 204.4 (C=O), 153.1 (C⁵), 152.4 (C³), 143.9 (C⁶), 138.4 (C^{Ar}), 138.0 (C^{Ar}), 132.5 (2C^{Ar}), 128.2 (2C^{Ar}), 45.9 (CH₂), 20.5 (Me), 18.5 (2C, 2Me) ppm. IR (mineral oil): 3539, 3324, 3162 (N–H), 1703, 1665 (C=O) cm^{−1}. Anal. Calcd (%) for C₁₄H₁₈ClN₅O₂: C 51.93; H 5.60; N 21.63. Found: C 52.22; H 5.47; N 21.67. Crystal structure of compound **3** was deposited at the Cambridge Crystallographic Data Centre with the deposition number CCDC 2554058.

*2,5-Bis(ethoxycarbonyl)-3a-hydroxy-3,3a,4,7-tetrahydro-8H-pyrazolo[1,5-*d*][1,2,4]triazepin-8-iminium chloride (4)*. A suspension of diethyl 2,4,6-trioxoheptanedioate **1j** (0.48 mmol, 124 mg) and 1,3-diaminoguanidine hydrochloride (0.48 mmol, 60 mg) in EtOH (5 mL) was stirred at 90 °C for 24 h. Then, the mixture was cooled to ambient temperature. The resulting precipitate was filtered off and recrystallized from EtOH (3 mL) to afford compound **4**. Yield: 18% (30 mg); pale yellow solid; mp 181 °C (decomp., EtOH). ¹H NMR (400 MHz, DMSO-*d*₆): δ = 12.61 (br.s, 1H, NH), 8.95 (s, 2H, NH₂), 8.14 (s, 1H, OH), 4.38–4.31 (m, 2H, CH₂Me), 4.24 (q, *J* 6.8 Hz, 2H, CH₂Me), 3.74 (d, *J* 16.6 Hz, 1H, C³H₂), 3.67 (d, *J* 19.6 Hz, 1H, C⁴H₂), 3.43 (d, *J* 19.6 Hz, 1H, C⁴H₂), 3.09 (d, *J* 16.6 Hz, 1H, C³H₂), 1.30 (t, *J* 7.0 Hz, 3H, Me), 1.27 (t, *J* 7.0 Hz, 3H, Me) ppm. ¹³C NMR (100 MHz, DMSO-*d*₆): δ = 164.0 (CO), 160.1 (CO), 153.6 (C⁸), 151.4 (C²), 145.3 (C⁵), 92.2 (C^{3a}), 62.6 (CH₂Me), 62.3 (CH₂Me), 48.4 (C³), 40.2 (C⁴), 14.5 (2C, 2Me) ppm. IR (mineral oil): 3361, 3237, 3122 (N–H), 1750, 1722, 1678 (C=O) cm^{−1}. Anal. Calcd (%) for C₁₂H₁₈ClN₅O₅: C 41.45; H 5.22; N 20.14. Found: C 41.63; H 5.38; N 20.18. Crystal structure of compound **4** was deposited at the Cambridge Crystallographic Data Centre with the deposition number CCDC 2554057.

2.2. Biology

Screening of test compounds was conducted in 96-well microplates, followed by validation of the most promising candidates in 50 mL Erlenmeyer flasks. *C. vulgaris* strain IMBR-19 (The A.O. Kovalevsky Institute of Biology of the Southern Seas, RAS, Sevastopol, Russia) served as the test object. Experimental procedures were based on our previously established protocols [30,32].

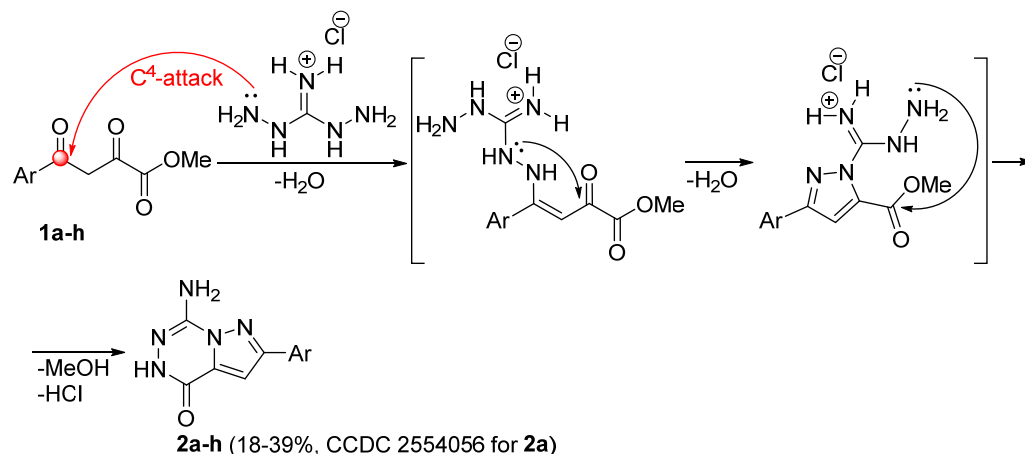
3. Results and Discussion

3.1. Chemistry

To achieve the target 7-aminopyrazolo[1,5-*d*][1,2,4]triazin-4(5*H*)-ones **2**, we examined the reaction of substituted methyl aroylpyruvates **1a–i** with 1,3-diaminoguanidine hydrochloride. As a result, we found that the reaction pathway and the structure of the final product were dependent on the aryl substituent in aroylpyruvate **1**.

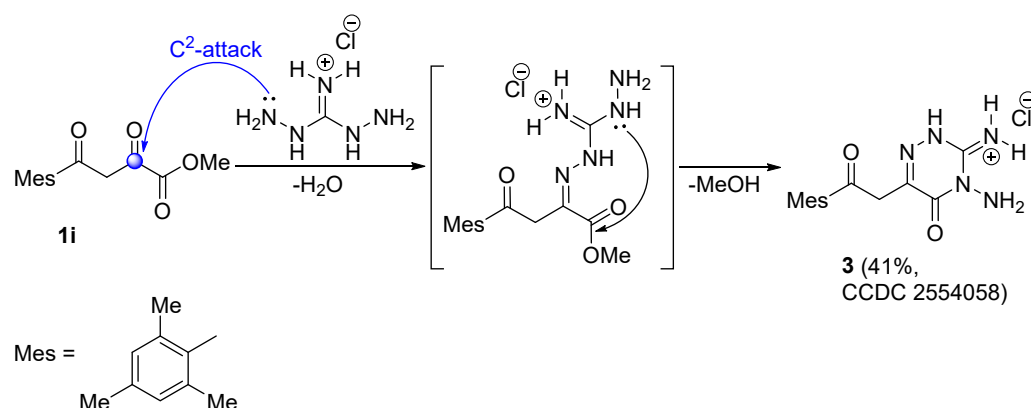
For the majority of methyl aroylpyruvates **1a–h**, the reaction proceeded as anticipated, yielding the desired 7-aminopyrazolo[1,5-*d*][1,2,4]triazin-4(5*H*)-ones **2a–h** (Scheme 2). A notable exception to this general reactivity was observed with methyl mesitoylpyruvate **1i**, which contains a sterically hindered mesityl substituent. Under the same reaction conditions, mesitoylpyruvate **1i** afforded 1,2,4-triazine **3** (Scheme 3). We suppose that this divergence in reactivity could be caused by an alteration of the direction of the initial nucleophilic attack of 1,3-diaminoguanidine hydrochloride on aroylpyruvates **1a–i**. Specifically, in the reaction with aroylpyruvates **1a–h**, the initial nucleophilic attack of the terminal NH₂ group of 1,3-diaminoguanidine hydrochloride [33] is directed at the C⁴=O carbonyl group

(Scheme 2), which initiates the cascade leading to pyrazolotriazines **2a–h**. In the case of mesitoylpyruvate **1i**, the C⁴=O carbonyl group is sterically shielded for the nucleophile by two *ortho*-methyl substituents, which shifts the initial nucleophilic attack of the terminal NH₂ group of 1,3-diaminoguanidine hydrochloride to the C²=O carbonyl group (Scheme 3), which initiates the cyclization to triazine **3**.



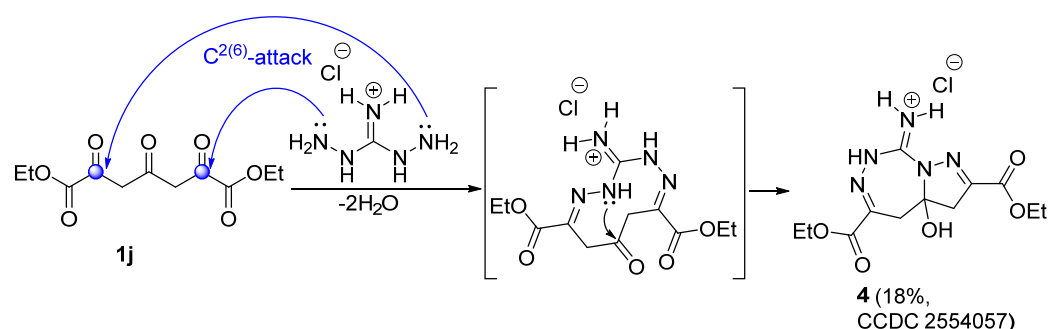
1, 2: Ar = Ph (**a**), C₆H₄Me-4 (**b**), C₆H₄OMe-4 (**c**), C₆H₄Cl-4 (**d**), C₆H₄OMe-3 (**e**), C₆H₄OEt-4 (**f**), thiophen-2-yl (**g**), C₆H₄CN-4 (**h**).

Scheme 2. Reaction of methyl arylpyruvates **1a–h** with 1,3-diaminoguanidine hydrochloride. Reaction conditions: ethanol, 90 °C, 24 h.



Scheme 3. Reaction of methyl mesitoylpyruvate **1i** with 1,3-diaminoguanidine hydrochloride. Reaction conditions: ethanol, 90 °C, 24 h.

Furthermore, to explore the limits of this methodology, we extended the study to include a *bis*-pyruvate entity—diethyl 2,4,6-trioxoheptanedioate **1j**. Under the same reaction conditions as for compounds **1a–i**, the reaction of *bis*-pyruvate **1j** with 1,3-diaminoguanidine hydrochloride followed a different pathway, leading to the formation of an unexpected bicyclic system, pyrazolo[1,5-*d*][1,2,4]triazepine **4** (Scheme 4). We suppose that in this case, as in the case of mesitoylpyruvate **1i**, the initial nucleophilic attack of the terminal NH₂ group of 1,3-diaminoguanidine hydrochloride is directed at the C²=O carbonyl group (Scheme 4), which initiates the cascade leading to pyrazolo[1,5-*d*][1,2,4]triazepine **4**. Possibly, such reactivity of *bis*-pyruvate **1j** could be attributed to electronic effects influencing the C⁴=O carbonyl group, making it less attractive for the attack of the nucleophile.



Scheme 4. Reaction of diethyl 2,4,6-trioxoheptanedioate **1j** with 1,3-diaminoguanidine hydrochloride. Reaction conditions: ethanol, 90 °C, 24 h.

In summary, the cascade condensation of aroylpyruvates **1** with 1,3-diaminoguanidine hydrochloride provides a straightforward, one-pot access to a library of 7-aminopyrazolo[1,5-*d*][1,2,4]triazin-4(5*H*)-ones **2**. However, the reaction is not universally applicable to all substrates. The structure of the final product is critically dependent on the substituents in the aryl ring of the starting aroylpyruvate **1**.

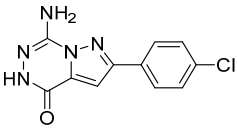
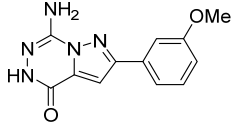
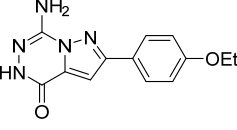
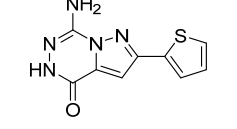
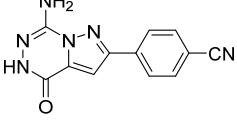
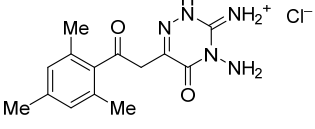
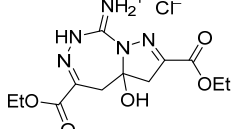
3.2. Biology

The biotechnological potential of ten synthesized heterocyclic derivatives was assessed by analyzing their effects on *C. vulgaris* cultures (Table 1) according to the protocol we developed earlier [30,32]. In brief, compound stock solutions were prepared in DMSO and added to cultures at final concentrations ranging from 0.1 to 100 µmol/L. Cultures were grown in 96-well plates, with 1% DMSO serving as a negative control and 2 g/L glucose as a positive control. After five days of culture growth, *C. vulgaris* cell concentrations were determined by measuring optical density at 750 nm (OD750). Compounds were considered active if they increased OD750 relative to the negative control (defined as an OD750 greater than the mean of the negative control plus three standard deviations) at at least one concentration tested.

Table 1. The difference in algae cell concentration between cultures containing the compounds **2a–h,3,4** and the negative control cultures (1% of DMSO).

Compound	Structure	Difference ¹ in Algae Cell Concentration Between Cultures Containing Test Compounds and Control Cultures			
		Concentration of Compounds in Culture Medium			
		100 µmol/L	10 µmol/L	1 µmol/L	0.1 µmol/L
2a		20 ²	−23	−9	−23
2b		10	−19	−28	−31
2c		35	−8	−3	−11

Table 1. Cont.

Compound	Structure	Difference ¹ in Algae Cell Concentration Between Cultures Containing Test Compounds and Control Cultures			
		Concentration of Compounds in Culture Medium			
		100 $\mu\text{mol/L}$	10 $\mu\text{mol/L}$	1 $\mu\text{mol/L}$	0.1 $\mu\text{mol/L}$
2d		17	1	−7	−16
2e		5	−15	−14	−28
2f		29	13	11	9
2g		−75	−66	−15	−21
2h		−32	−26	−27	−34
3		−8	−3	−20	−8
4		−35	−23	−25	−15

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

Next, to investigate its biological activity in more detail, compound **2c** was further studied for its effects on cell concentration, as well as the content of pigments, proteins, polysaccharides, and neutral lipids (Table 2). For these experiments, the selected compound **2c** was added to *C. vulgaris* cultures at concentrations ranging from 0.1 to 100 $\mu\text{mol/L}$. Cultures were grown in 50 mL flasks, and after five days of cultivation, samples were analyzed using standard biochemical methods with minor modifications.

Interestingly, despite its promising performance in microplate-based screenings, the tested compound did not exhibit a statistically significant effect on cell concentration in flask cultures when compared to the negative control (Table 2). This discrepancy between the microplate and flask culture systems may be explained by differences in mixing dynamics and oxygen availability, which could influence the presence or concentration of specific signaling or regulatory molecules [34] that serve as targets for the tested compound. Additionally, no effects were observed on content of key metabolites, except for slight (13%) growth of protein concentration at 100 $\mu\text{mol/L}$.

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

Concentration of 2c	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	Neutral Lipids, F ₅₈₀ /10 ⁶ Cells
100 µmol/L	22.56 ± 4.11 ³	2.475 ± 0.247	0.194 ± 0.035	1.74 ± 0.120	0.488 ± 0.050 ⁴	468.3 ± 86.3
10 µmol/L	22.88 ± 0.36	2.515 ± 0.077	0.194 ± 0.012	1.82 ± 0.219	0.433 ± 0.065	272.6 ± 30.0
1 µmol/L	23.28 ± 0.71	2.229 ± 0.104	0.185 ± 0.019	1.70 ± 0.203	0.389 ± 0.050	431.0 ± 60.9
0.1 µmol/L	21.22 ± 1.81	2.303 ± 0.216	0.174 ± 0.010	1.84 ± 0.161	0.421 ± 0.093	270.2 ± 25.5
Negative control ¹	21.53 ± 0.49	2.485 ± 0.117	0.187 ± 0.017	1.99 ± 0.122	0.431 ± 0.054	415.8 ± 75.4
Positive control ²	68.00	3.253 ± 0.002	0.095 ± 0.017	4.62 ± 0.040	0.627 ± 0.006	550.8 ± 23.4

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

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

4. Conclusions

In this study, we investigated a one-pot cascade condensation of methyl aroylpyruvates with 1,3-diaminoguanidine hydrochloride, providing access to a library of 7-aminopyrazolo[1,5-*d*][1,2,4]triazin-4(5*H*)-ones **2a–h**. The method is operationally simple, uses ethanol as a solvent, and assembles the adenine-mimetic heterocyclic core from readily available acyclic precursors in 18–39% yield.

However, the reaction outcome is critically dependent on the nature of the substituent in the aroylpyruvate. While most substrates followed the expected pathway (initial nucleophilic attack at the C⁴=O carbonyl group), two notable deviations were observed. First, methyl mesitylpyruvate **1i** bearing a sterically hindered mesityl group shifted the initial attack to the C²=O carbonyl group, leading to a 1,2,4-triazine derivative **3** instead of the expected pyrazolotriazine. Second, the bis-pyruvate **1j** (diethyl 2,4,6-trioxoheptanedioate) underwent an alternative cyclization to form an unexpected pyrazolo[1,5-*d*][1,2,4]triazepine scaffold **4**. These findings delineate both the scope and the limitations of the methodology, providing valuable guidance for future synthetic applications.

All synthesized compounds were evaluated for cytokinin-like growth-regulating activity using the green microalga *C. vulgaris* as a model system. Compound **2c** has shown the best results in the initial microplate screening, showing increased cell density at 10 µmol/L. Nevertheless, when subjected to more detailed flask-based validation, compound **2c** did not produce statistically significant effects on biomass accumulation, chlorophyll and carotenoid content, carbohydrate levels, or neutral lipid production compared to the negative control. The only observed effect was a modest (13%) increase in protein content at the highest tested concentration (100 µmol/L).

5. Patents

The method for preparing products **2** has been patented [35].

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/chemistry8070090/s1>, The following are available online, Figure S1: Molecular structure of compound **2a** showing 30% probability amplitude displacement ellipsoids (CCDC 2554056); Figure S2: Molecular structure of compound **3** showing 30% probability amplitude displacement ellipsoids (CCDC 2554058); Figure S3: Molecular structure of compound **4** showing 30% probability amplitude displacement ellipsoids (CCDC 2554057); Table S1: Crystal data and structure refinement for compounds **2a**, **3**, and **4**.

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