

# Synthesis of new tetronamides displaying inhibitory activity against bloom-forming cyanobacteria

Running title: Synthesis of new tetronamides with cyanobactericidal activity

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ps.5580

## ABSTRACT

**BACKGROUND:** The increasing frequency and intensity of cyanobacterial blooms pose a serious threat to aquatic ecosystems. These blooms produce potent toxins that can contaminate drinking water and endanger the life of wild and domestic animals as well as humans. Consequently, the development of effective methods for their control is a matter of high priority. We have previously shown that some  $\gamma$ -benzylidenebutenolides, related to the rubrolide family of natural products, are capable of inhibiting the photosynthetic electron transport chain (Hill reaction), a target of commercial herbicides. Here we report the synthesis and biological properties of a new class of rubrolide-inspired molecules featuring a tetronamide motif.

**RESULTS:** A total of 47 *N*-aryl tetronamides, including 38 aldol adducts, were prepared bearing phenyl, biphenyl, naphthyl, aliphatic and heteroaromatic groups. Some of the aldol adducts were dehydrated to the corresponding  $\gamma$ -benzylidenetetronamides, although satisfactory yields were obtained in only three cases (52-97%). None of the synthesized compounds were capable of blocking the Hill reaction. This notwithstanding, several aldol adducts equipped with a biphenyl substituent displayed excellent inhibitory activity against *Synechococcus elongatus* and other cyanobacterial strains ( $IC_{50} = 1-5 \mu M$ ). Further, these tetronamides were found to be essentially inactive against eukariotic microorganisms.

**CONCLUSION:** Several newly synthesized biphenyl-containing tetronamides were shown to display potent and selective inhibitory activity against cyanobacteria. These compounds appear to exert their biological effects without interfering with the Hill

reaction. As such, they represent novel leads in the search of environmentally benign agents for controlling cyanobacterial blooms.

**Keywords:** Cyanobactericidal activity, Hill reaction, *Synechococcus elongatus*, Tetronamides, Butenolides, Vinylogous aldol reaction.

## 1 INTRODUCTION

The tetronamide, or 4-amino-2(5*H*)-furanone, motif (**1**, Fig. 1) is a key structural feature of an increasing number of bioactive molecules, nearly all of them being of synthetic origin. In fact, the only tetronamide known to occur in nature is basidalin (**2**), an antitumor antibiotic isolated from terrestrial fungi.<sup>1,2</sup> Synthetic tetronamides are frequently used as building blocks for the construction of complex molecules,<sup>3-5</sup> as well as in chemical,<sup>6-9</sup> medicinal<sup>10,11</sup> and agrochemical research.<sup>8,12,13</sup> Many such compounds possess significant antibacterial (e.g. **3**),<sup>14</sup> herbicidal (**4**) and insecticidal activities.<sup>8,12,13</sup> The latter include Bayer's recently commercialized systemic insecticide flupyradifurone (Sivanto<sup>®</sup>, **5**).<sup>12,13</sup>

Pest control is a key factor toward the quantitative and qualitative improvement of agricultural yields, taking into account environmental safety.<sup>15</sup> Weeds cause significant crop losses worldwide. Currently, the most common method for controlling weeds involves the use of synthetic herbicides.<sup>16,17</sup> However, continuous application of herbicides has resulted in the selection of resistant weed biotypes and environmental

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pollution.<sup>18-21</sup> As a result, there is a need for new herbicidal agents, especially those that exhibit novel mechanisms of action and/or favorable environmental and toxicological properties. A well-established target of herbicides is the chloroplast electron transport chain. Inhibition of this process leads to energy starvation and formation of reactive oxygen species, causing plant death. New compounds can be screened for such activity *in vitro* by measuring the light-driven reduction of ferricyanide in isolated chloroplasts, known as the Hill reaction.<sup>22,23</sup>

Hill inhibitors are expected to interfere with the growth of all photosynthetic organisms, including cyanobacteria. Broadly distributed worldwide, cyanobacteria (a.k.a. blue-green algae) are the only prokaryotes able to perform oxygenic photosynthesis.<sup>24</sup> Under favorable conditions, cyanobacteria are capable of blooming and forming a dense scum on water surface, causing the establishment of anoxic conditions which alter the ecological structure and contribute to the ecosystem's decline.<sup>25</sup> Moreover, harmful cyanobacterial toxins are released<sup>26</sup> that may endanger animal and human health.<sup>27</sup> Because the frequency, intensity and duration of cyanobacterial blooms are increasing, possibly due to pollution and climate change,<sup>28</sup> the development of environmentally benign methods for their control is a matter of high priority.

The rubrolides are a family of marine antibiotics characterized by a  $\beta$ -aryl- $\gamma$ -benzylidenebutenolide framework.<sup>29-31</sup> Some of their analogues (cf. **6-7**, Figure 2) were found to inhibit the Hill reaction with comparable efficiency to that of commercial herbicides.<sup>32-33</sup> Although we expected these compounds to inhibit the growth of

cyanobacteria, e.g. *Synechococcus elongates*, they were found to be essentially devoid of such activity ( $\leq 20\%$  inhibition at  $50 \mu\text{M}$ ).<sup>34</sup> Considering the diverse biological properties of tetronamides, we sought to synthesize a new class of rubrolide analogues, in which the  $\beta$ -arylbutenolide is replaced by an *N*-aryl tetronamide.

Furanolate chemistry provides an effective means of constructing  $\gamma$ -alkylidenebutenolides. However, to the best of our knowledge, there is only a single study exploiting tetronamide-derived furanolates for the synthesis of (*Z*)- $\gamma$ -alkylidene tetronamides.<sup>35</sup> In particular, Dechoux and co-workers described a decarboxylative Knoevenagel-type aldol condensation of *N*-substituted  $\gamma$ -acyltetronamides with aldehydes to afford (*Z*)- $\gamma$ -alkylidene tetronamides in moderate to good yields (Scheme 1A).<sup>35</sup> Our group recently reported the vinylogous aldol reaction (VAR) of unactivated tetronamides with aldehydes, giving adducts **9** in high yields and usually excellent *syn*-diastereoselectivity (Scheme 1B).<sup>36</sup> The ready availability of these adducts prompted us to explore their dehydration for acquiring the desired  $\gamma$ -alkylidenetetronamides (**10**).

Herein we describe the synthesis of a range of tetronamide aldol adducts and their conversion to  $\gamma$ -benzylidenetetronamides. We further describe the ability of synthesized compounds to block the Hill reaction *in vitro* and the growth of cyanobacteria *in vivo*.

## 2 EXPERIMENTAL METHODS

### 2.1 General experimental procedures

To perform all reactions, analytical grade solvents were used without further purifications, unless otherwise stated. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a

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Varian Mercury 300 instrument (300 MHz and 75 MHz, respectively) or on a Bruker NMR spectrometer (400 MHz and 100 MHz, respectively). The samples were dissolved in deuterated chloroform, acetone or dimethyl sulfoxide (DMSO), and tetramethylsilane (TMS) was used as internal standard ( $\delta = 0$ ). The experiments were performed at controlled probe temperature of 25 °C. Chemical shifts of  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra are reported in ppm. All coupling constants ( $J$  values) are expressed in Hertz (Hz). Multiplicities are reported as follows: singlet (s), doublet (d), doublet of doublets (dd), triplet (t), multiplet (m) and broad (br). Infrared spectra were recorded on a Varian 660-IR, equipped with GladiATR scanning from 4000 to 500  $\text{cm}^{-1}$ . Melting points are uncorrected and were obtained from MQAPF-301 melting point apparatus. High resolution mass spectra were recorded on a Bruker MicroTof (resolution = 10 000 FWHM) under electrospray ionization (ESI). The reactions were monitored by analytical thin layer chromatography analysis performed on aluminum packed pre-coated silica gel plates. All compounds were purified by column chromatography using silica gel (230–400 mesh) as a solid stationary phase.

## 2.2 Synthetic procedures

### 2.2.1 General synthesis procedure for the preparation of compound **8a–8h** and **8j**.

To a 100 mL round bottomed flask was added 3,4-dichlorofuran-2(5H)-one (**11a**) or 3,4-dibromofuran-2(5H)-one (**11b**) (1.0 mmol) dissolved in 5 mL MeOH, followed by  $\text{NaHCO}_3$  (0.6 mmol) and the required *p*-substituted aniline (1.1 mmol). The reaction mixture was stirred at room temperature for 12 h. After the consumption of the starting

butenolide (**11a** or **11b**), the reaction mixture was quenched by addition of aqueous HCl solution (1 M, 10 mL). The methanol was then removed under reduced pressure in a rotary evaporator and the aqueous mixture was extracted with ethyl acetate (3 × 10 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtrated and the solvent removed under reduced pressure. The crude residue was purified by silica-gel column chromatography, eluted with a mixture of ethyl acetate/hexane (1:4 to 1:1 v/v) to afford the required compounds **8a-8h** and **8j**. For structures, yields and physical data see Supporting Information material (pages S5–S8).

### 2.2.2 Procedure for the preparation of the dehalogenated compound **8i**.<sup>37</sup>

To a 25 mL two-necked round-bottom flask charged with compound **8a** (1.0 g, 3.73 mmol) were added Pd(CH<sub>3</sub>CN)<sub>2</sub>Cl<sub>2</sub> (24.13 mg, 2.5 mol %), Pd(OAc)<sub>2</sub> (21.00 mg, 2.5 mol %), PPh<sub>3</sub> (48.87 mg, 5 mol%), DIPEA (3.25 mL, 18.7 mmol), formic acid (0.71 mL, 18.7 mmol) and acetonitrile (10 mL). The reaction mixture was then degassed for 10 min under a flow of argon and then stirred at 100 °C for 12 h before it was filtered through a celite pad. The filtrate was extracted with ethyl acetate (3 × 30 mL) and the combined organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure in a rotary evaporator. The crude product was purified by silica-gel column chromatography eluted with hexane/ethyl acetate (3:2 v/v) to afford compound **8i** as a pale yellow solid in 66% yield (465.8 mg, 2.46 mmol). For physical data see Supporting Information material (pg. S8).

### 2.2.3 General synthetic procedure for preparation of aldol adducts **9**.

To a 25 mL one neck round bottomed flask were added the tetronamides **8** (1.0 mmol), 6 mL of a mixture of CH<sub>3</sub>OH and H<sub>2</sub>O (2:1, v/v), followed by NaOH (1.2 mmol). After stirring the reaction mixture for 5 min at room temperature, the aromatic aldehydes (1.0 mmol) were added slowly. The reaction mixture was stirred at room temperature until TLC analysis revealed total consumption of starting material. The reaction was then quenched by addition of an aqueous solution of HCl (1 M, 10 mL). The volatiles were removed under reduced pressure and the aqueous mixture was extracted with ethyl acetate (3 × 15 mL). The combined organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtrated and the solvent removed under reduced pressure in a rotary evaporator. The crude residue was purified by silica-gel column chromatography eluting with ethyl acetate/hexane (1:4 to 1:1 v/v) to afford racemic tetronamides **9**. For physical data of *syn* and *anti*-isomers see Supporting Information material (S15–S25).

### 2.2.4 General procedure for synthesis of tetronamides **9p** and **9u**.

A 25 mL round bottom flask was charged with tetronamide **9o** (100 mg, 0.24 mmol) or **9t** (100 mg, 0.22 mmol) diluted with 5 mL of anhydrous dichloromethane. The resultant solution was stirred, cooled in an ice bath at 0 °C before dropwise addition of BBr<sub>3</sub> in anhydrous dichloromethane (1.0 mL, 32% v/v, 0.44 mmol). The resulting mixture was allowed to warm to room temperature and stirred for an additional 4 h under an argon atmosphere. The reaction was quenched by addition of saturated aqueous solution of NH<sub>4</sub>Cl (5 mL), followed by removal of the volatiles under reduced



pressure. The aqueous phase was extracted with ethyl acetate (3 x 10 mL) and the organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated under reduced pressure. The crude product was purified by recrystallization with ethyl ether/hexane (1:1 v/v) to afford tetronamide **9p** or **9u**. For yields and physical data see supporting information (S20 and S24).

### 2.2.5 General procedure for synthesis of 4-amino-3-halo-5-benzylidene-furan-2-(5H)-ones (**10a-10c**).

To a 25 mL two neck round bottomed flask were added 200 mg of aldol products **9a-9c** (1.0 equiv.), and dissolved with dry acetonitrile (6 mL), then *p*-toluenesulfonyl chloride (2.0 equiv.) was added and leaving stirring under argon atmosphere for 5 min, thereafter, DBU (2.0 equiv.) was added dropwise and the reaction mixture was stirred for 12 h at 60 °C. When the reaction was completed, it was quenched by addition of an aqueous solution of HCl (1 M). The acetonitrile was removed under reduced pressure in a rotary evaporator and the aqueous mixture was extracted with ethyl acetate (3 × 15 mL). The combined organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtrated and the solvent removed under reduced pressure. The crude residue was purified by column chromatography using neutral alumina as stationary phase and eluted with ethyl acetate/hexane (1:9 v/v) to afford product **10**. For yields and physical data see Supporting Information material (S38–S40).

## 2.3 Biological assays

### 2.3.1 Measurement of the rate of photosynthetic electron transport.

Photosynthetically active thylakoids were isolated from market spinach (*Spinacia oleracea* L.) leaves. Briefly, 20 g of plant material were resuspended in 100 mL of ice-cold 20 mM Tricine/NaOH buffer (pH 8.0) containing 10 mM NaCl, 5 mM MgCl<sub>2</sub> and 0.4 M sucrose, and homogenized for 30 s in a blender at maximal speed. The homogenate was filtered through surgical gauze, and the filtrate was centrifuged at 4 °C for 1 min at 500 g; the supernatant was further centrifuged for 10 min at 1500 g. Pelleted chloroplasts were resuspended in sucrose-lacking buffer, immediately diluted 1:1 with sucrose-containing buffer, and kept on ice in the dark until used. Following dilution with 80% (v/v) acetone, chlorophyll content was calculated using the Arnon's formula. The rate of photosynthetic electron transport was measured following the light-driven reduction of ferricyanide. Aliquots of membrane preparations corresponding to 15 µg chlorophyll were incubated at 24 °C in 1 mL cuvettes containing 20 mM Tricine/NaOH buffer (pH 8.0), 10 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.2 M sucrose and 1 mM K<sub>3</sub>[Fe(CN)<sub>6</sub>]. The assay was started by light exposure (800 µmol m<sup>-2</sup> s<sup>-1</sup>), and the rate of ferricyanide reduction was measured at 1 min intervals for 20 min at 420 nm against an exact blank. Activity was calculated over the linear portion of the curve from a molar extinction coefficient of 1000 M<sup>-1</sup> cm<sup>-1</sup>.

Compounds were dissolved in DMSO and then diluted with water, as required. Their effect on the photosynthetic electron transport chain was measured by adding increasing concentrations (0.8, 1.6, 3.1, 6.3, 12.5, 25, 50, 100 and 200 µM) to the above reaction mixture, and comparing the results with untreated parallel controls. Each dose was

assayed in triplicate, and results were expressed as percentage of untreated controls (averaged from at least 12 replications). To evaluate the concentrations causing 50% inhibition (IC<sub>50</sub>) and their confidence intervals, data were fitted to the four-parameter logistic (variable slope sigmoidal) equation:  $Y=100/(1+10^{((\text{LogIC}_{50}-X)*\text{HillSlope}))}$ , where X is the logarithm of the concentration and the Y values of the curve will go from 100 down to 0, as described,<sup>38</sup> using Prism 6 for Windows, version 6.03 (GraphPad Software).

### 2.3.2 Measurement of Cyanobacterial Growth

The model strain *Synechococcus elongatus* PCC 6301 and the other cyanobacterial strains used, as listed in Table 5, were grown in Bg11 mineral medium as previously described.<sup>39</sup> To measure chlorophyll content, culture aliquots (0.5–1.0 mL) were withdrawn. Cells were sedimented by centrifugation for 3 min at 14000 x g, and pellets were solubilized with 1.0 mL of methanol for 30 min in the dark, with occasional mixing. Samples were centrifuged again, and chlorophyll content in the supernatant was estimated spectrophotometrically at 663 nm. Late log-grown cells were sedimented by centrifugation 5 min at 4,000 x g, and used to inoculate 96-well plates, 0.2 mL per well, to an initial density of about 1.0 mg L<sup>-1</sup> chlorophyll. Aliquots (2 μL) of suitable dilutions of a given compound in DMSO were added so as to obtain the desired final concentrations (0.2, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25, 50, 100 and 200 μM). A complete randomized design with four replications (eight for untreated controls) was adopted. Cell growth in each well was followed for one week by daily determination of

absorbance using a Ledetect 96 plate reader (Labexim, Lengau, Austria) equipped with a LED plugin at 660 nm. Following logarithmic transformation of data, growth constants were calculated, and expressed as percent of the mean value for controls treated with the same volume of DMSO. IC<sub>50</sub> and their confidence limits were estimated as described above.

### 2.3.3 Measurement of Bacterial and Yeast Growth

*Escherichia coli* strain BL21(DE3)pLysS was grown at 37 °C in the dark in standard Davis and Mingioli medium supplemented with microelements as in Bg11 medium. *Bacillus subtilis* strain ATCC 7003 was grown at 30 °C in the same medium supplemented with 500 mg L<sup>-1</sup> yeast extract. Baker yeast Ura3<sup>-</sup> strain S23344C was grown at 30 °C in standard Yeast Carbon Base (with 50 mg L<sup>-1</sup> uracil but without amino acids, and with 1.0 g L<sup>-1</sup> ammonium sulfate as the nitrogen source). The effect of selected compounds on bacterial and yeast growth was evaluated as described for cyanobacteria, except that the inoculum was made to an initial density of about 0.1 Abs (600 nm), and growth was followed for up to 10 h by determining at 60 min intervals the increase in absorbance using the plate reader equipped with a LED plugin at 600 nm.

## 3 RESULTS AND DISCUSSION

### 3.1 Chemical synthesis

The synthesis of new tetronamides (**9a-9v**) was carried out as previously reported.<sup>36,40</sup> Congujate addition of substituted anilines to commercially available  $\alpha,\beta$ -

dichlorobutenolide (**11a**) or  $\alpha,\beta$ -dibromorobutenolide (**11b**), followed by *in situ*  $\beta$ -elimination, afforded intermediates **8a-8h** and **8j** in high yields.<sup>41</sup> Submission of **8a** to our reductive hydrodehalogenation procedure<sup>37</sup> provided compound **8i** in 66% yield (Scheme 2).

Tetronamides **8a-8i** were then submitted to our VAR method<sup>36</sup> using nine substituted benzaldehydes, eleven biphenylcarbaldehydes, and two naphthalenecarbaldehydes. The resulting aldol adducts **9a-9v** were obtained in yields of 54-99%. In keeping with previous observations, the *syn* adduct was the major isomer in most cases.<sup>36,40</sup> Their structures and yields are shown in Table 1. Compounds **9a**, *anti-9a*, **9c**, **9d**, **9e**, *anti-9e*, **9g**, **9h**, **9i**, **9j**, **9o**, **9k** and *anti-9o* were previously reported and fully characterized.<sup>36</sup>

In order to generate tetronamide analogues of rubrolides, we explored the dehydration of aldol adducts **9** to the corresponding  $\gamma$ -benzylidenetetronamides **10**. For this purpose, compound **9a** was initially treated with pyridine (Py) as base and MsCl as a pro-leaving group, following a procedure recently reported for the synthesis of the natural antitumor-antibiotic basidalin.<sup>2</sup> Under these conditions the starting material was totally consumed, but the desired product could not be isolated (Table 2, entry 1). Increasing the amount of MsCl, Py, temperature and reaction time and using DIPEA as base did not remedy matters; only decomposition of the starting aldol adduct was observed (Table 2, entries 2-4).

When TsCl was used instead of MsCl and DCM as solvent, the required product **10a** was isolated in 8% yield along with recovered starting material (67%, entry 5). Changing the base to DIPEA and using THF or acetonitrile as solvent resulted in

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somewhat better yields (16-48%; entries 6-9). Even though a higher amount of TsCl and the use of more polar solvents were clearly beneficial, conversion to **10a** was still incomplete. In contrast, the use of a stronger base (DBU) and acetonitrile as solvent improved the yield to 57% (entry 10). Gratifyingly, optimization of reaction time and temperature (entries 11-13) led to an excellent yield of **10a** (87%, entry 12). Furthermore, in all instances dehydration afforded solely the *Z*-configured isomer (**10a**).<sup>42,43</sup>

Several other aldol adducts were subjected to dehydration under our best conditions (cf. entry 12). Unfortunately, only substrates bearing simple aromatic substituents worked well (52-97% for **10a-c**; Table 1). All other substrates tried, including those having a naphthyl or biphenyl group, resulted in unstable products that degrade under the reaction conditions or during the purification process (*e.g.* **10q**). We also tried to convert **8a** directly into **10a** by using several one-pot aldol condensation procedures (*e.g.* TBSOTf/DIPEA/DBU/CH<sub>2</sub>Cl<sub>2</sub>, piperidine/CH<sub>3</sub>OH) that were successful in the synthesis of rubrolides and related natural products.<sup>42-48</sup> However, none of them proved successful in isolating **10a** from the resulting complex product mixtures.

### 3.2 Biological assays

Despite the difficulties encountered in obtaining  $\gamma$ -alkyldenetetronamides **10**, the availability of three such compounds and numerous aldol adducts **9**, allowed a preliminary investigation of their biological effects. In addition, some simple tetronamides **8** were also tested. Although the diversity of substitution on the *N*-

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aromatic ring was small (H, CH<sub>3</sub>, OCH<sub>3</sub>, Br), a preliminary analysis of the structure-activity relationship was feasible, as well as the study of the influence of the halogen (Cl and Br) at the  $\alpha$  position of the butenolide core. In particular, the capability of all these compounds to inhibit either the photosynthetic electron transport chain in isolated spinach chloroplasts and/or the photoautotrophic growth of the cyanobacterium *Synechococcus elongatus* PCC 6301 was evaluated.

The results pertaining to synthesized aldol adducts (**9a-k**) and  $\gamma$ -alkylidenetetronamides (**10a-c**) are summarized in Table 3. Additional results focusing on biphenyl-substituted aldol adducts (**9l-v**) are presented in Table 4. For the starting tetronamides **8a-i**, the results are summarized in Table S1 (Supplementary material). It is important to note that most aldol adducts **9** were tested as single *syn* isomers, except for compounds with a nitro substituent **9c**, **9d**, **9i** and the bi-halogenated **9j**, for which separation of the *syn/anti* diastereoisomers was not possible. In only a few instances the *anti*-isomers were purified and tested (entries 2, 9, 11 and 14, Table 3). Thus, unless indicated otherwise, aldol adducts refer to pure *syn*-isomers.

### 3.2.1 Inhibition of the photosynthetic electron transport chain in isolated spinach chloroplasts

As seen in Table S1, the starting tetronamides **8a-8i** were not effective photosynthesis inhibitors (assay 1). The three  $\gamma$ -alkylidenetetronamides tested (**10a-10c**) were also inactive (Table 3, entries 3, 5 and 7), while the corresponding aldol adducts showed mild activity (entries 1, 2, 4 and 6). These findings were not entirely unexpected.

In the case of rubrolides a negative correlation has been found between reduction potential ( $E_{pc}$ ) and photosynthesis-inhibitory effectiveness,<sup>33</sup> and the introduction of an electron-donating nitrogen on the butenolide ring must decrease  $E_{pc}$ . For a rubrolide analogue having an  $IC_{50} > 100 \mu M$  (Fig. 2, **6**,  $R^1 = H$ ,  $R^2 = m-OH$ ),  $E_{pc}$  is in fact -1.33 V, whereas for the most active ( $IC_{50} = 1.1 \mu M$ ) rubrolide with a nitro group on the benzylidene ring (Fig. 2, **6**,  $R^1 = H$ ,  $R^2 = p-NO_2$ )  $E_{pc}$  is -0.88 V.<sup>33</sup> However, in the case of teronamides, the presence of a nitro group (**10c**) was not sufficient to afford an active compound. Among the tetronamide aldol adducts derived from benzaldehydes, compound **9i**, which bears a *para*-nitro group in  $Ar^2$  and an  $\alpha$ -chlorine on the tetronamide nucleus, was the most active ( $IC_{50} = 23.7 \mu M$  for *syn/anti* 97:3 mixture). In addition, two naphthalene derivatives (**9e** and **9f**) were found to be about as active, exhibiting  $IC_{50}$  values of 28.8 and 23.8  $\mu M$  for the *syn* isomers, respectively. In both cases, the *anti* isomers were slightly less active (entries 9 and 14). In this initial series of compounds, one biphenyl derivative (**9q**) was also tested (*syn* or *anti* isomers, entries 10-11). The former isomer was nearly half as potent as the *syn* naphthyl derivatives **9e** and **9f** (entry 10 vs entries 9 and 14).

Although none of these compounds were as active as the previously reported rubrolide analogues,<sup>15, 32-34</sup> the results suggest that the presence of a naphthalene group improves the ability of tetronamide aldol adducts to inhibit the Hill reaction.

### 3.2.2 Inhibition of Cyanobacterial Growth



Substances that interfere with the photosynthetic apparatus have the potential of inhibiting the growth of photoautotrophic organisms, such as cyanobacteria. Thus, we evaluated the effects of increasing concentrations of each compound to the growth medium of the model cyanobacterial strain *Synechococcus elongatus* PCC 6301. As indicated in Table S1, the intermediates **8a-8i** were poor inhibitors of cyanobacterial growth (assay 2). In fact, only compounds **8d** and **8h**, both bearing an electron-withdrawing bromine atom at the *para* position, showed IC<sub>50</sub> values lower than 50 μM. As previously found for rubrolide analogues,<sup>34</sup> all three γ-alkylidenetetronamides **10a-10c** were inactive (Table 3; entries 3, 5, and 7; assay 2).

In contrast, some teronamide aldol adducts **9a-9k** were capable of significantly reducing cyanobacterial growth, but in most cases the IC<sub>50</sub> values were higher than 10 μM (Table 3). A notable exception is the biphenyl-containing aldol adduct **9q**, which displays superior cyanobacterial activity (2.8 μM, entry 10) than the best rubrolide analogues uncovered so far.<sup>34</sup> Consistent with the inhibition of the Hill reaction, the *syn*-isomer was more potent than its *anti* counterpart (entries 10 and 11).

In order to assess the importance of the biphenyl substituent, a series of 10 new analogues of **9q** were prepared and evaluated. In this new compound set, the *para* substituent of the aniline moiety and the α-halogen of the tetronamide were modified (Table 4).

Since the lead compound **9q** had some inhibitory effect on Hill reaction, these new compounds were also tested as photosynthesis inhibitors (Table 4, assay 1). Although none of the compounds were particularly effective in blocking the Hill reaction, five of

them strongly inhibited the growth of *S. elongates* ( $IC_{50} < 20 \mu M$ ), with **9m** being twice as potent as **9q** ( $IC_{50} = 1.3 \mu M$ , Table 4, entry 2). Although the number of compounds bearing a biphenyl group was limited, these results allowed a preliminary structure-activity relationship to be established. The simple *N*-phenyl analogue **9n** was ineffective, as were those with a *p*-OMe (**9o**) or a *p*-OH (**9p**) group. In contrast, the presence of bromine at the *para* position on the aniline moiety (**9m**) was beneficial, whereas its replacement with a methyl group (**9l**) resulted in lower activity. Replacing the  $\alpha$ -chlorine with bromine improved activity in most cases (cf. **9q**, **9s**, **9t** and **9u** vs **9l**, **9n**, **9o** and **9p**). Only in the case of **9m** and its congener **9r** the opposite was true. The importance of an  $\alpha$ -halogen in the tetronamide was confirmed by its replacement with a hydrogen atom, showing a seven to eleven-fold decrease in activity (**9l** and **9q** vs **9v**). It was also found that replacement of the methoxy group by hydroxyl substantially reduces activity (**9t** vs **9u**, Table 4).

Heteroaromatic and aliphatic aldol derivatives (**9w-9ab**) were evaluated but no significant activity was found with respect to the inhibition of both the Hill reaction and cyanobacterial growth (Table S2 in supporting information). These results, although negative, confirm the requirement of the biphenyl moiety for growth inhibition.

With this in mind, the effects of the two most effective biphenyl derivatives were investigated on a set of ten other cyanobacterial strains belonging to different taxonomic subsections (Table 5). Both compounds were able to reduce cell growth rate when added to the culture medium at micromolar concentrations, and significant inhibition was found for the strains *Microcystis* and *Anabaena* (entries 1 and 9), which are often

responsible for cyanobacterial blooming.<sup>49</sup> In addition, **9m** and **9q** demonstrated high efficacy against *Synechococcus* sp. (Entries 2-4). Interestingly, the effectiveness varied from a quantitative point of view, with IC<sub>50</sub> values differing up to two orders of magnitude. Even within the same species a significantly different effect was found, with two *S. elongatus* strains showing five to eight-fold difference in sensitivity. Moreover, if the two derivatives were initially found to have a similar effect on *S. elongatus*, in the case of other species, such as *Tolypothrix* sp., they showed strikingly different effectiveness (Entry 11). Several causes might explain these results, ranging from a different susceptibility of the molecular target(s) to various rates of uptake or enzymatic detoxification of the inhibitor. Whatever the reason, the occurrence of heterogeneous effects is highly desirable for a lead compound, as it facilitates the development of selective inhibitors.

### 3.2.3 Effects on Eubacteria and Yeasts

To explore the possibility that inhibition of cyanobacterial growth might be due to general cytotoxicity, we also screened these compounds at various concentrations against Gram negative and Gram positive eubacteria, namely *Escherichia coli* and *Bacillus subtilis*, and the eukariotic microorganism, *Saccharomyces cerevisiae*. The results are shown in Fig. 3. In the case of *E. coli*, growth inhibition occurred only at substantially higher concentrations, resulting in IC<sub>50</sub> values above 200 μM. *B. subtilis* showed a slightly higher sensitivity, yet only compounds **9o** and **9q** had IC<sub>50</sub> values lower than 100 μM (72 and 93 μM, respectively). Remarkably, compound **9q** had been

found ineffective against *S. elongatus* (Table 4). For yeast, a similar pattern to that of *E. coli* was evident, except for compounds **9l** and **9r** ( $IC_{50} = 15$  and  $11 \mu\text{M}$ , respectively) that were about as potent against cyanobacteria (Table 4). However, the two most active compounds against *S. elongatus* (**9m** and **9q**) were essentially ineffective against *S. cerevisiae*. Taken together, these findings suggest that biphenyl tetronamides are not generally antimicrobial, but exert specific activity against oxygenic photosynthetic bacteria.

#### 4 CONCLUSIONS

Several new tetronamides were synthesized and shown to display potent and selective inhibitory activity against bloom-forming cyanobacteria ( $IC_{50} = 1\text{-}5 \mu\text{M}$ ). Interestingly, these compounds do not significantly inhibit the chloroplastic electron transport chain (Hill reaction), thereby suggesting that they may exert their cyanobacterial effects by a different mechanism. Alternatively, they may be transformed inside the cyanobacterial cell into active metabolites capable of blocking the Hill reaction. The combination of ready accessibility, high potency and intriguing mechanism of action makes these tetromamides attractive leads in the search of environmentally benign agents for controlling cyanobacterial blooms.

#### ACKNOWLEDGEMENTS

We are grateful to the following Brazilian agencies for financial support to this study: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq);

Coordenação de aperfeiçoamento de Pessoal de Nível Superior (CAPES); Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG). We also thank NSERC (Canada) for a Discovery grant to JB and the University of Ferrara (FAR 2018) for financial support.

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## SUPPORTING INFORMATION

The supporting information is available in the online version of this article.

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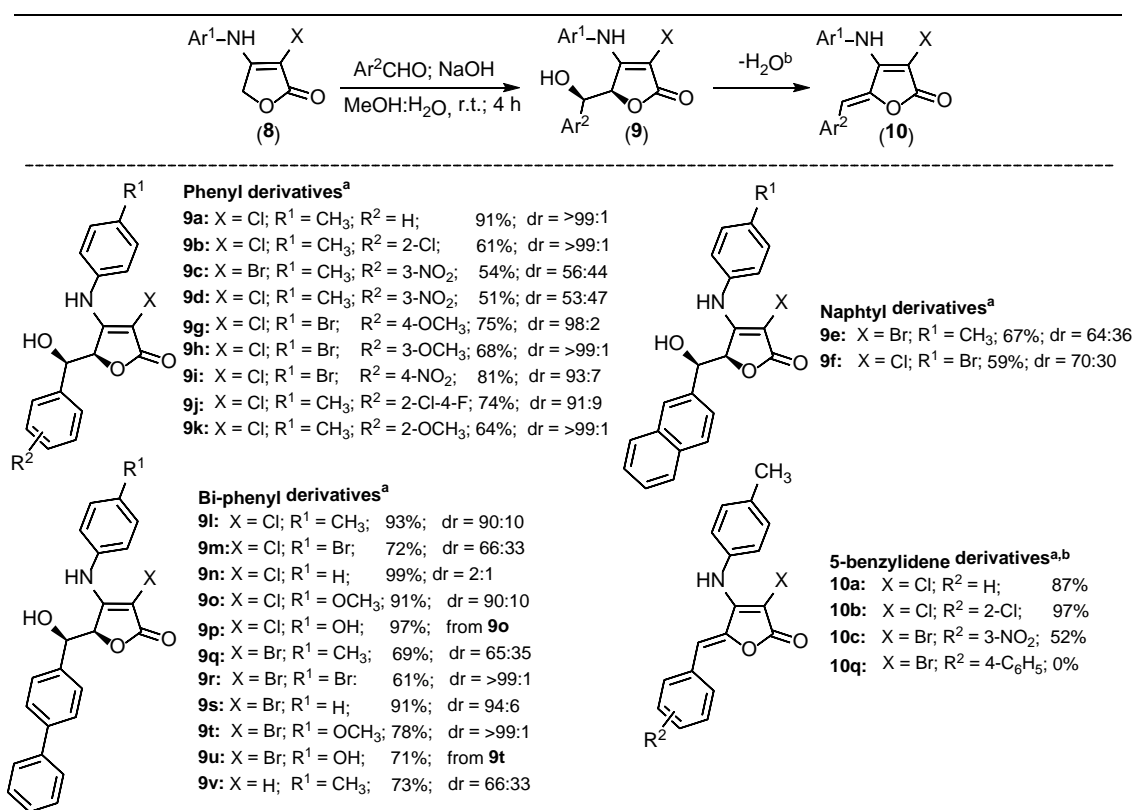
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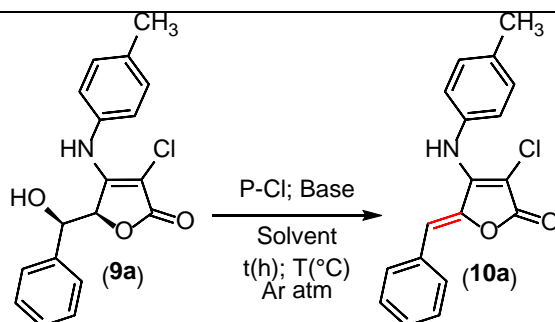
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**Table 1.** Structure, yield and diastereoselectivity ratio (dr) of the aldol adducts (**9**) obtained from tetronamides (**8**) and some  $\gamma$ -benzylidene tetronamides (**10**).



<sup>a</sup> For the sake of clarity only the *syn* (major) isomer is shown along with the *syn:anti* ratio.

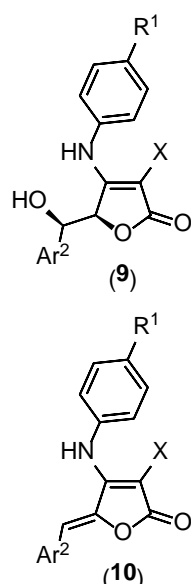

















<sup>b</sup> More details about **9** to **10** transformation see Table 2

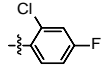
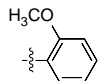
**Table 2.** Optimization of dehydration of aldol adduct **9a**.

Entry	<b>P</b> (equiv) <sup>a</sup>	Base (equiv) <sup>b</sup>	solvent <sup>d</sup>	temp. (°C)	time(h)	% recovery	% yield
1	Ms (1) <sup>a</sup>	Py (1) <sup>c</sup>	DCM	r.t.	4	0	N.R. <sup>e</sup>
2	Ms (2)	Py (2)	DCM	r.t.	8	0	N.R.
3	Ms (2)	Py (2)	DCM	60	8	0	N.R.
4	Ms (2)	DIPEA (2)	THF	60	8	0	N.R.
5	Ts (1) <sup>b</sup>	Py (1)	DCM	r.t.	4	67	8
6	Ts (1)	DIPEA (1)	THF	r.t.	8	57	16
7	Ts (1)	DIPEA (1)	MeCN	r.t.	2	40	26
8	Ts (1)	DIPEA (1)	MeCN	r.t.	6	55	34
9	Ts (2)	DIPEA (2)	MeCN	r.t.	8	30	48
10	Ts (2)	DBU (2)	MeCN	r.t.	6	0	57
11	Ts (2)	DBU (2)	MeCN	60	8	0	62
12	Ts (2)	DBU (2)	MeCN	60	12	0	87
13	Ts (2)	DBU (2)	MeCN	60	16	0	74
14	Ts (2)	DBU (2)	MeCN	110	12	0	N.R.

P = Pro-leaving group, <sup>a</sup> MsCl = Mesyl chloride; <sup>b</sup> TsCl = Tosyl chloride; <sup>c</sup> Py = Pyridine; <sup>d</sup> All the solvents were drying; <sup>e</sup> N.R. = No product formed.

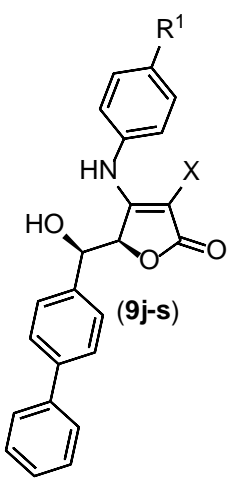
**Table 3.** Concentrations of tetronamides (**9a-9k**) and  $\gamma$ -alkylidene derivatives (**10a-10c**) able to inhibit by 50% ( $IC_{50}$ ) the photosynthetic electron transport chain in isolated spinach chloroplasts (assay 1) and the photoautotrophic growth of the model cyanobacterium *S. elongatus* (assay 2). Data include the compounds derived from benzaldehydes (**9a-9d** and **9g-9k**), 2-naphtalenecarbaldehydes (**9e-9f**) and the only available  $\gamma$ -alkylidene derivatives (**10a-10c**).

General structures	Entry	Compound	X	R <sup>1</sup>	Ar <sup>2</sup>	IC <sub>50</sub> ( $\mu$ M) Assay 1	IC <sub>50</sub> ( $\mu$ M) Assay 2
	1	<b>9a</b>	Cl	CH <sub>3</sub>		72.8 $\pm$ 11.0	198 $\pm$ 79
	2	<i>anti-9a</i>	Cl	CH <sub>3</sub>		165 $\pm$ 29	86.7 $\pm$ 26.3
	3	<b>10a</b>	Cl	CH <sub>3</sub>		>200	>200
	4	<b>9b</b>	Cl	CH <sub>3</sub>		>200	>200
	5	<b>10b</b>	Cl	CH <sub>3</sub>		>200	180 $\pm$ 3
	6	<b>9c<sup>a</sup></b>	Br	CH <sub>3</sub>		152 $\pm$ 40	89.6 $\pm$ 10.8
	7	<b>10c</b>	Br	CH <sub>3</sub>		>200	>200
	8	<b>9e</b>	Br	CH <sub>3</sub>		28.8 $\pm$ 5.7	50.7 $\pm$ 5.2
	9	<i>anti-9e</i>	Br	CH <sub>3</sub>		48.0 $\pm$ 28.2	25.0 $\pm$ 2.3
	10	<b>9q</b>	Br	CH <sub>3</sub>		58.4 $\pm$ 53.7	2.8 $\pm$ 0.3
	11	<i>anti-9q</i>	Br	CH <sub>3</sub>		166 $\pm$ 500	10.9 $\pm$ 3.3
	12	<b>9d<sup>a</sup></b>	Cl	CH <sub>3</sub>		86.8 $\pm$ 35.1	>200
	13	<b>9f</b>	Cl	Br		23.8 $\pm$ 7.4	16.1 $\pm$ 1.8
	14	<i>anti-9f</i>	Cl	Br		60.8 $\pm$ 18.5	15.6 $\pm$ 1.1
	15	<b>9g</b>	Cl	Br		106 $\pm$ 78	>200
	16	<b>9h</b>	Cl	Br		104 $\pm$ 50	>200
	17	<b>9i<sup>a</sup></b>	Cl	Br		23.7 $\pm$ 4.9	32.0 $\pm$ 2.4

18	<b>9j<sup>a</sup></b>	Cl	CH <sub>3</sub>		71.0 ± 35.8	>200
19	<b>9k</b>	Cl	CH <sub>3</sub>		172 ± 36	>200

<sup>a</sup> Compounds tested as mixture of diastereomers due to difficulties in separation process.

**Table 4.** Concentrations of biphenyl-containing tetronamides (**9l-9p**; **9r-9v**) able to inhibit by 50% (IC<sub>50</sub>) the photosynthetic electron transport chain in isolated spinach chloroplasts (assay 1) and the photoautotrophic growth of the cyanobacterium *S. elongatus* (assay 2).

General structure	Entry	Compound	X	R <sup>1</sup>	IC <sub>50</sub> (μM)	
					Assay 1	Assay 2
	1	<b>9l</b>	Cl	CH <sub>3</sub>	>200	4.4 ± 0.1
	2	<b>9m</b>	Cl	Br	186 ± 308	1.3 ± 0.1
	3	<b>9n</b>	Cl	H	>200	>200
	4	<b>9o</b>	Cl	OCH <sub>3</sub>	>200	>200
	5	<b>9p</b>	Cl	OH	>200	>200
	6	<b>9r</b>	Br	Br	77.0 ± 15.0	5.0 ± 0.6
	7	<b>9s</b>	Br	H	>200	20.0 ± 3.0
	8	<b>9t</b>	Br	OCH <sub>3</sub>	>200	5.1 ± 0.5
	9	<b>9u</b>	Br	OH	>200	116 ± 16
	10	<b>9v<sup>a</sup></b>	H	CH <sub>3</sub>	>200	31.0 ± 4.0

<sup>a</sup> Tested as a mixture of diastereoisomers.

**Table 5.** Concentrations of aldol biphenyl-containing tetronamides **9m** and **9q** able to inhibit by 50% (IC<sub>50</sub>) the photoautotrophic growth of a group of eleven cyanobacterial strains.

Entry	Strain (subsection)		<b>9m</b> IC <sub>50</sub> (μM)	<b>9q</b> IC <sub>50</sub> (μM)
1	<i>Microcystis aeruginosa</i> PCC 7941	(I)	25.4 ± 4.2	75.1 ± 7.7
2	<i>Synechococcus elongatus</i> PCC 6301	(I)	1.3 ± 0.1	2.8 ± 0.3
3	<i>Synechococcus</i> sp. PCC 6715	(I)	2.1 ± 0.4	1.9 ± 0.2
4	<i>Synechococcus elongatus</i> UTEX 2431	(I)	10.3 ± 1.5	16.3 ± 3.4
5	<i>Synechocystis</i> sp. PCC 6803	(I)	23.9 ± 3.6	41.2 ± 5.7
6	<i>Chroococidiopsis thermalis</i> PCC 7203	(II)	>200	86.5 ± 15.7
7	<i>Cylindrospermum licheniforme</i> ATCC 29412	(III)	7.7 ± 1.7	4.3 ± 0.5
8	<i>Lyngbya</i> sp. PCC 7419	(III)	57.7 ± 13.2	21.5 ± 3.2
9	<i>Anabaena</i> sp. PCC 7120	(IV)	15.9 ± 1.2	9.9 ± 0.8
10	<i>Nostoc</i> sp. PCC 6719	(IV)	30.6 ± 5.4	9.4 ± 3.4
11	<i>Tolypothrix</i> sp. PCC 7601	(IV)	101.9 ± 26.3	10.1 ± 2.6

## FIGURE LEGENDS

**Figure 1.** Parent tetronamide (**1**) and some biologically active natural (**2**) and synthetic derivatives (**3-5**).

**Figure 2.** Rubrolide analogues.

**Figure 3.** Effect of increasing levels of compounds **9l-v** on the growth of different microorganisms, as indicated. Each compound was added to the growth medium at concentrations in the range of 0.2-200  $\mu\text{M}$ . The resulting growth rate was determined, and expressed as percent of that for untreated controls. Data are mean  $\pm$  SE over 4 replications.

**Scheme 1.** Synthetic routes to  $\gamma$ -alkylidenetetronamides.

**Scheme 2.** Synthesis of  $\gamma$ -substituted tetronamides.



