Synthetic analogues of cyanobacterial alkaloid cylindrospermopsin and their toxicological activity

Cartmell, Christopher ; Evans, Daniel; Elwood, Jessica ML; Fituri, Hisham; Murphy, Patrick; Caspari, Thomas; Poniedziaekc, Barbara ; Rzymskic, Piotr

Toxicology in Vitro

DOI:
10.1016/j.tiv.2017.07.007

Published: 01/10/2017

Peer reviewed version

Synthetic analogues of cyanobacterial alkaloid cylindrospermopsin and their toxicological activity

Christopher Cartmell, Daniel M. Evans, Jessica M. L. Elwood, Hisham S. Fituri, Patrick J. Murphy*
chs027@bangor.ac.uk

School of Chemistry, Bangor University, Bangor, Gwynedd, LL57 2UW, UK

Thomas Caspari
t.caspari@bangor.ac.uk

School of Medical Sciences, Bangor University, Bangor, Gwynedd, LL57 2UW, UK

Barbara Poniedziałek, Piotr Rzymski
Department of Environmental Medicine, Poznan University of Medical Sciences, Poznań, Poland

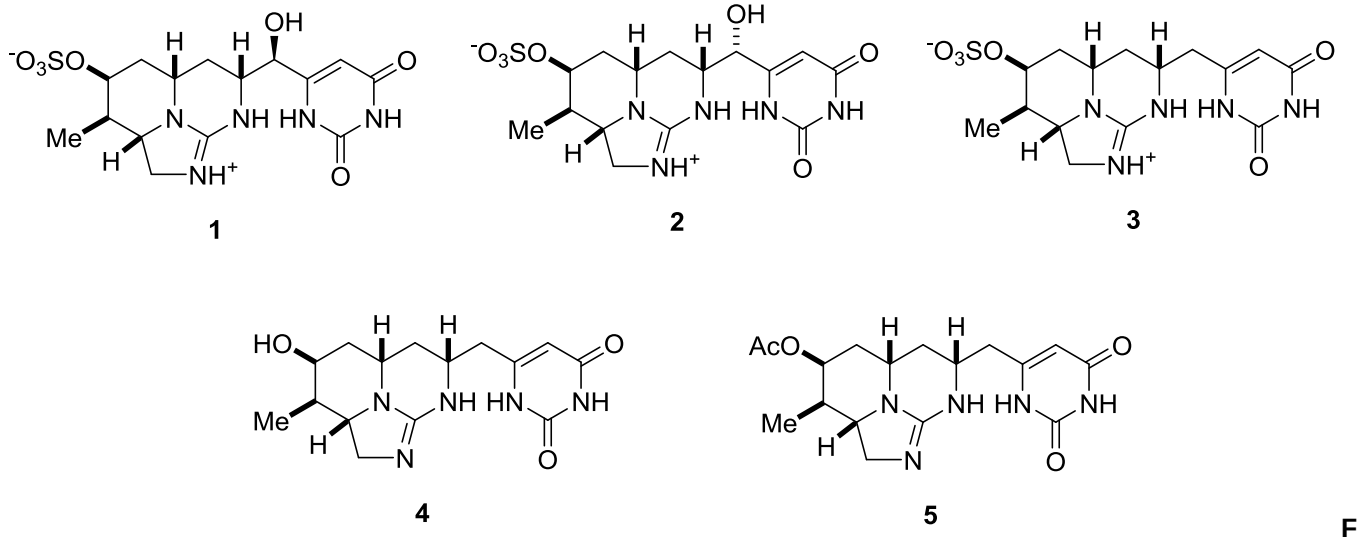
bpon@ump.edu.pl

Abstract: Cylindrospermopsin (CYN) is a naturally occurring alkaloid produced by a variety of cyanobacteria and known to induce oxidative stress-mediated toxicity in eukaryotic cells. Despite extensive research on the mechanism of CYN toxicity, an understanding of the structural features responsible for this toxicity and the mechanism by which it can enter the cell are still not clear. It was established that the presence of both the uracil and guanidine groups is essential in biological activity of CYN while not much is known in this regard on the role of tether that separates them and the attached hydroxyl group. Therefore, in the present study we have prepared three synthetic analogues possessing uracil and guanidine groups separated by a variable length tether (4-6 carbons) and containing a hydroxyl function in a position orientation to CYN, together with a tetracyclic analogue of CYN lacking the hydroxyl group at C-7. The toxicity of these compounds was then compared with CYN and guanidinoacetate (GAA; the primary substrate in CYN biosynthesis) in an *in vitro* model using human neutrophils isolated from healthy subjects. The lowest activity measured by means of reactive oxygen species generation, lipid peroxidation and cell death was observed for GAA and the tetracyclic analogue. The greatest toxicity was found in an analogue with a 6-carbon tether, but all three analogues and CYN caused rapid onset of redox imbalance. These results add to the general understanding of CYN toxicity and preliminary suggest that the -OH group at C-7 may be significant for the cellular transport of CYN and/or be involved in its toxic activity inside the cell, a hypothesis which requires further testing.

Keywords Cylindrospermopsin; guanidine synthesis, neutrophils; oxidative stress; reactive oxygen species (ROS), superoxide dismutase (SOD), catalase (CAT). glutathione peroxidase (GPx).

Introduction

Cylindrospermopsin (CYN) **1**, is a naturally occurring cyanotoxin originating from the cyanobacteria *Cylindrospermopsis raciborskii*.[1] This was the first member of a small group of related alkaloids to be isolated, along with *7-epi*-cylindrospermopsin **2**, *7*-deoxy-cylindrospermopsin **3**, 7-*deoxy-desulfo*-cylindrospermopsin **4** and *7-deoxy-desulfo*-12-acetylcylindrospermopsin **5** from this and other cyanobacteria species.[2-4] In 1979 a bloom of *C. raciborskii* occurred and contaminated a local water supply and was thought to be responsible for an outbreak of hepatoenteritis in 1979.[5] The outbreak, coined the "Palm Island mystery disease" was responsible for the hospitalization of 148 people, the vast majority children.[5] The highly toxic potencies of CYN in different types of mammalian cells were later extensively demonstrated.[6] Over ten freshwater species are currently known to be able to produce **1** including *Aphanizomenon ovalisporum, Raphidiopsis curvata* and terrestrial strain of *Hormoscilla pringsheimii* (Figure 1). [7,8,9,10] The reasons behind its production are yet to be fully elucidated though the toxin is known to be actively released from intact cells, to up-regulate alkaline phosphatase in sympatric phytoplankton and to contribute to allelopathic interactions.[11]



F

igure 1: The cylindrospermopsin alkaloids

Previous studies have attempted to link the activity of CYN **1** to specific functional groups within the molecule, most notably a study by Sukenik who proposed that the uracil group was partially responsible for its potent toxic activity, reasoning that there might be competitive or inhibitory binding to a catalytic site.[12] Since this study, there have been only a small number of

reports on the synthesis of analogues of the cylindrospermopsin family, most probably due to their structural complexity. However, Runnegar *et al.*[13] conducted a structure activity relationship investigation (SAR) utilising a series of simplified synthetic analogues of the natural products. Within this work the unsulfonated analogues **±6** and **7** were studied along with tricyclic analogue **±8**. The simplified uracil analogue **±9** was also investigated, as was the bicyclic sulfonate analogue **±10**, which lacks the uracil group (Figure 2).[13]

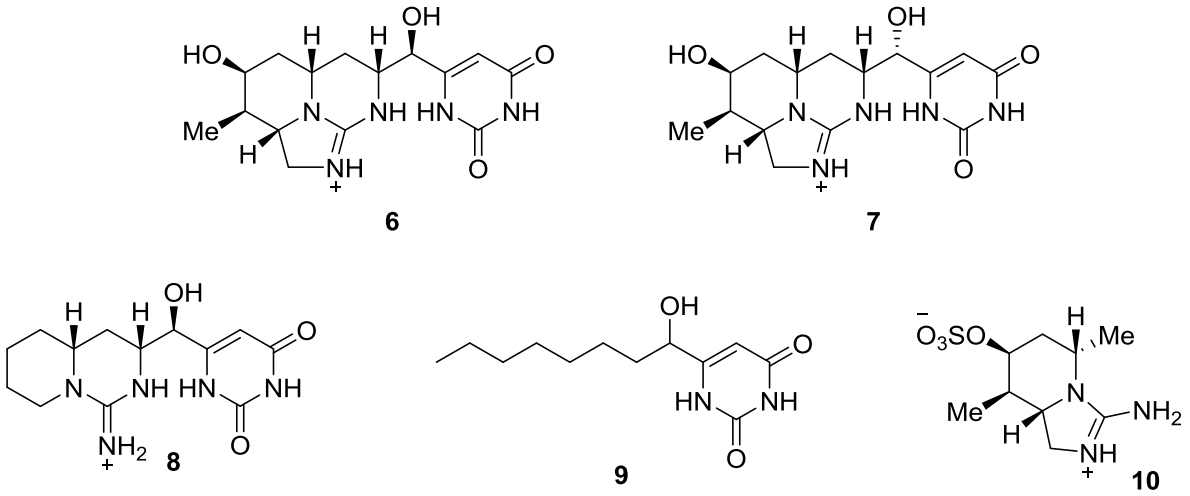


Figure 2: Cylindrospermopsin analogues

Initially, the toxicity of synthetic racemic CYN **±1** was compared to that of the stereoisomer 7-*epi*-cylindrospermopsin **2**. The results obtained provided evidence to state that the exact stereochemistry of the hydroxyl group located at *C*-7 had little effect on the biological activity or transport of the toxin. Furthermore, a comparison of the activity of **±1** and **2** alongside the corresponding diols **±6** and **7** indicated that **±**1 and **±6** both inhibit protein synthesis with almost identical IC_{50} values of 0.20 and 0.21 μM, whilst 7-*epi*-cylindrospermopsin **2** and diol ±**6** each depleted cell antioxidant glutathione by similar amounts. These results indicate that the sulfate group present within majority of the CYN alkaloids plays no appreciable role in either their biological activity or cellular uptake. Of the other compounds studied, the tricyclic analogue **±8** demonstrated an inhibitory effect on protein synthesis but only at concentrations between 500-1000 times greater than those of **1**. Similarly analogues **±9** which lacks the guanidine functionality, and **±10** which lacks the uracil ring displayed no biological activity at concentrations between 800 and 2000 μM.[13] From these results it was concluded, that the presence of both the uracil and guanidine groups are essential and that the guanidine must be contained in a lipophilic environment to enable any biological activity. Shaw also investigated the role of the hydroxyl group located at C-7. This was done by analysing the effect of 7-deoxy-cylindrospermopsin **3** in four different mammalian cell lines, the results demonstrated that **3** displays the same levels of toxicity as CYN **1**. The levels of protein synthesis inhibition were

also similar, with IC_{50} values of 340 and 220 nM for **3** and **1** respectively.[14] Williams *et al.* also determined that both toxins inhibit protein synthesis within one order of magnitude of each other and that both have a similar inhibitory effect on cell glutathione.[15] However, *in vivo* comparison of both compounds revealed that **3** did not produce any toxic effects at levels exceeding the lethal concentrations established for **1**.[3] Recently it was suggested that exposure to CYN at environmentally relevant concentrations, has the potential to lead to a reduced ability to fight pathogens and therefore increasing susceptibility to potential infections.[16,17] Further work in this area has sought to elucidate the sequence of events underlying CYN induced oxidative stress in human cells. The toxin has been shown to significantly increase levels of intracellular reactive oxygen species (ROS) and significantly suppresses the activation of superoxide dismutase (SOD) and catalase (CAT) whilst up-regulating glutathione peroxidase (GPx).[18]

It is apparent that it is still unclear as to role of the uracil group in these alkaloids, particularly as to its role in transportation into the cell or if it also plays a key role in the *in vitro* activity. It is however apparent the sulfate group does not play a significant role in either the biological activity or the transport of the CYN alkaloids, but that the hydroxyl group does play a vital role, but the hydroxyl-stereochemistry does not seem to be a key factor in this activity.[13,14] With these observations in mind, we were motivated to prepare a series of analogues of **1** which contain the key guanidine, uracil and hydroxyl groups found in **1** separated by a tether. Our plan was to simplify the metabolites by removing the tricyclic ring system and obtaining the structures **11a-c** which are separated by a variable length tether (n = 1-3) (Figure 3). We eventually compared the bioactive properties of **11a-c** with CYN **1**, a tetracyclic analogue **22** and guanidinoacetate (GAA; a primary substrate of CYN in cyanobacterial biosynthesis pathway[19]). We used an *in vitro* human cell model to elucidate the potential role of tether length in compound toxicity.

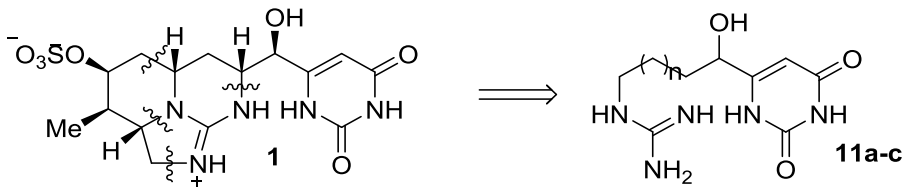
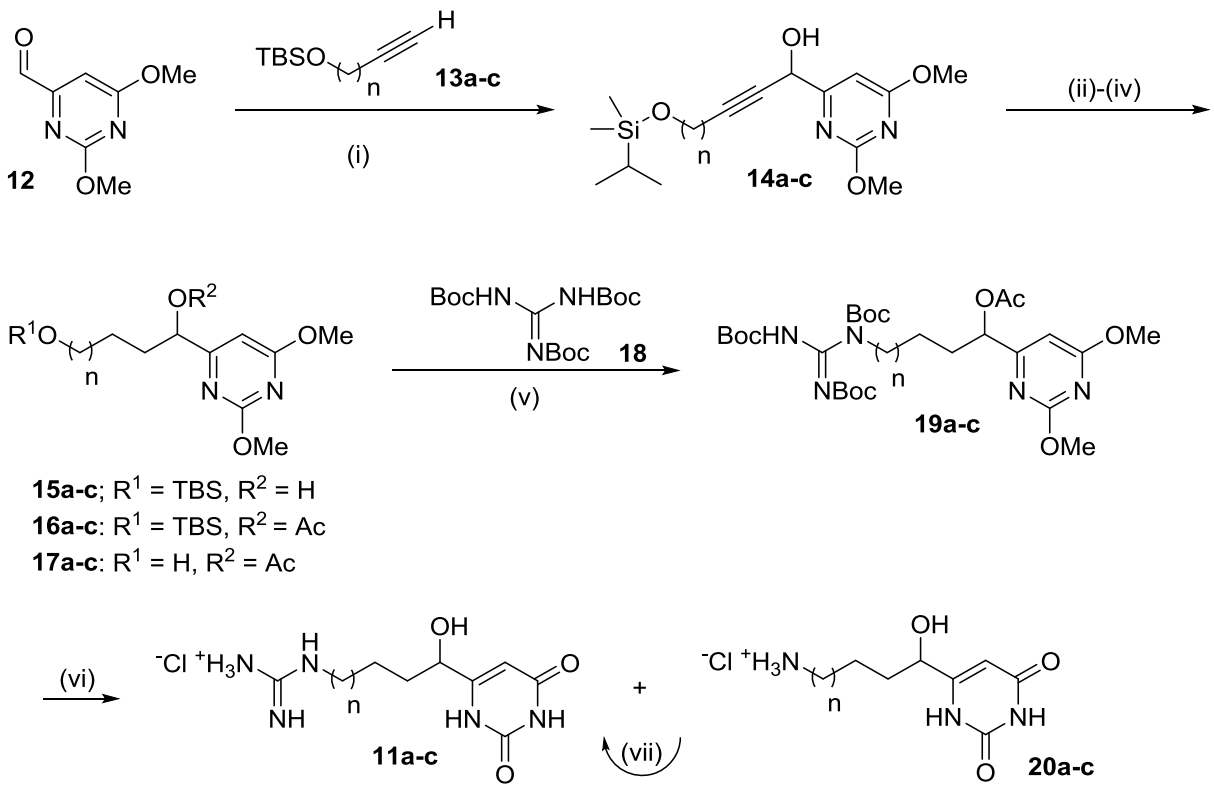


Figure 3: Proposal of the structure of analogues **11**.

Results and discussion

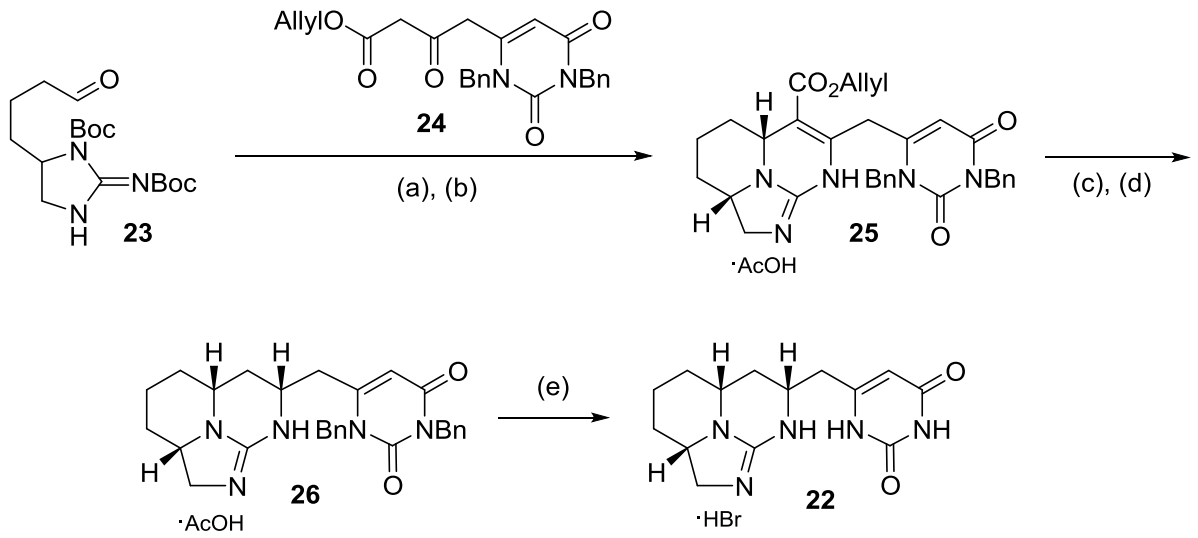
Synthesis

The synthesis of the analogues **11a-c** began with the known aldehyde **12**[20] which was treated with the corresponding acetylinic Grignard reagents **13a-c** (n = 1-3) in THF to give the corresponding alcohols **14a-c** in 55-75 % yield. Hydrogenation of these gave the alcohols **15a-c** (79-100 % yield), which were acetylated using acetic anhydride in pyridine to give acetates **16a-c** (68-88 % yields) and then desilylated with TBAF to give the alcohols **17a-c** (71-80 % yield). Alcohols **17a-c** were reacted with N,N',N''-tri-Boc-guanidine **18** in the presence of DIAD and triphenylphosphine to give guanidines **19a-c**. These were deprotected by refluxing in 0.5 M HCl to give the desired guanidinium salts **11a-c**. Unfortunately, on deprotection, the guanidine groups underwent partial hydrolysis to the amines **20a-c** which were inseparable from guanidines **11a-c**. However, treatment of this mixture with 1H-pyrazole-1-carboxamidine hydrochloride **21** and DIPEA, followed by purification on Amberlite© CG-50 gave the desired guanidines **11a-c** in 12-54% yields over 3 steps (Scheme 1).



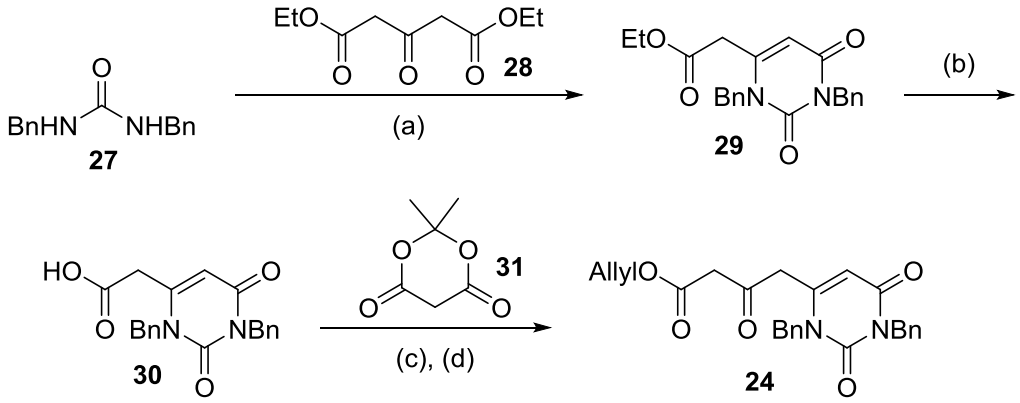
Scheme 1: Synthesis of the analogues 11a-c. (i) **13a-c**, EtMgBr in THF (1M), -78 °C, 2 h, 0 °C – rt, 16 h. then NH_4Cl aq. n = 1, 75 %; n = 2, 71 % n = 3, 55 %. (ii) Pd/C (10%), EA, H_2, 2 h. n = 1, 79 %, n = 2, 100 %, n = 3, 86 %. (iii) Ac_2O, pyridine, DMAP, CH_2Cl_2, rt, 1 h. n = 1, 68 %, n = 2, 74 %, n = 3, 88 %. (iv) TBAF (1M), THF, 0 °C - rt, 2 h, n = 1, 80 %; n = 2, 74 %, n = 3, 71 %. (v) DIAD, PPh_3, N,N',N"-tri-Boc-guanidine **18**, THF, rt 16 h. (vi) HCl (0.5 M), 100 °C, 16 h. (vii) 1*H*-Pyrazole-1-carboxamidine hydrochloride **21**, DIPEA, DMF, 24 h, then Amberlite© CG-50l; 11**a**; n = 1, 54 %; 11**b**; n = 2, 12 %; 11**c**; n = 3, 23 % (over three steps).

Concomitant to the preparation of the tethered analogues described above we prepared a tetracyclic analogue **22** of *7-deoxy*-cylindrospermopsin **3**. The synthesis of **22** began with our previously prepared aldehyde **23** which was subjected to a tethered Biginelli condensation with the β-keto ester **24** using our previously reported conditions.[21,22] Under these conditions it was possible to isolate the tetracyclic guanidine **25** in 19% yield, the relative stereochemistry of which was determined by 2D NOESY NMR. Deallylation/decarboxylation of **25** was achieved by treatment with $Pd(PPh_3)_4$, followed by reduction with $NaBH_3CN$ to give guanidine **26** in 75% yield. Again 2D NOESY NMR was used to determine the orientation of the newly introduced methine proton. Deprotection of the *N*-benzyl groups was achieved by treatment with BBr_3 which furnished the *7-deoxy*-cylindrospermopsin analogue **22** in 93% yield (Scheme 2).



Scheme 2: Preparation of analogue 22. *reagents and conditions*: (a) AcOH, 24 h; (b) Morpholine acetate, **24,** Na_2SO_4, CF_3CH_2OH, 100 °C, 12 d, 19%; (c) $Pd(PPh_3)_4$, pyrollidine, THF-MeOH (1:1), 4 h; (d) $NaBH_3CN$, AcOH-MeOH (1:1), 0 °C to rt, 16 h, 75% (2 steps); (e) i) BBr_3, Xylenes, 130 °C, 16h; ii) MeOH, rt, 16 h, 93%.

The β-keto ester **24** required for the synthesis of **22** was prepared by condensation of *N*,*N*'-dibenzylurea **27**[23] with diethyl 1,3-acetonedicarboxylate **28** in the presence of *p*-TsOH, to give the benzyl protected pyrimidine ester **29** in 67 % yield. Saponification of **29** by treatment with ethanolic NaOH, gave the corresponding carboxylic acid **30** in 78 % yield. The desired β-keto ester functionality was incorporated using CDI mediated coupling[24] of **30** with Meldrum's acid **31** followed by heating the intermediate adduct in neat allyl alcohol to give **24** in 75% yield.



Scheme 2: preparation of analogue 24. (a) *p*-TsOH, **28**, 4 Å MS, benzene, 4 d, 67%; (b) NaOH, EtOH, 0 °C to rt, 5 d, 78%; (c), CDI, **31**, THF, 50 °C, 16 h; (d) Allyl alcohol, 110 °C, 16 h, 75%.

Toxicity studies

The present study compared the toxicity of the synthetic CYN analogues (**11a**, **11b**, **11c** and **22**), **1** and GAA in human-derived neutrophils *in vitro*. Neutrophils are the most abundant and chemo-sensitive type of leukocytes with a circulating half-life of only 6-8 h,[25] previously shown to be a suitable *in vitro* model to evaluate the toxicity of cyanobacterial metabolites.[26-28] The employed model is rapid in contrast to those employing cell lines that usually require culturing and a longer assay time to allow for cell responses. Moreover, in contrast to numerous toxicological *in vitro* studies involving human cell lines, our experimental model used cells isolated from healthy donors, thereby taking into account individual variability and susceptibility and for this reason the observed effects may be more relevant in human risk assessment. This study is amongst those that highlight that toxicology *in vitro* can be used as a convenient tool to investigate the biological activity of synthetic toxin analogues, prepared in order to elucidate which structures may play a pivotal in the toxicity of the parent compound.

The first assay monitored the intracellular levels of ROS and was employed to directly evaluate the potential of tested compounds in altering the cellular redox balance. The assay is based on a fluorogenic cell permeant dye which is oxidized to fluorescent compound (dichlorofluorescein) in the presence of different ROS. The vast majority of cyanobacterial metabolites and other toxic compounds (e.g. tBHP used in our study as positive control) are known to trigger ROS generation above physiological levels.[29-31] Increased ROS can lead to oxidative stress which overwhelms the ability of biological system to readily detoxify the reactive intermediates resulting in damage to cellular structures and the genetic information. [32]

As shown, all the compounds tested, at concentration of 2.0 µg mL^{-1}, had the ability to temporarily increase the intracellular ROS levels to different extents (Figure 4A), which is in contrast to tBHP which caused a gradual increase in ROS over the exposure time. All the CYN

analogues tested showed a peak-pattern effect on the redox balance with the greatest levels of ROS noted after 15 minutes after the exposure was initiated. The most significant increase was observed in the presence of **11a** which amounted to 121.6% of the control level. A slightly lower effect was found for **1** (120.5% of control level) and **11c** (119.1% of control level). Importantly, **11a** was the only compound that caused a statistically significant increase in ROS level after just 5 min of exposure, indicating the highest bioavailability of all tested compounds. After 1 h of exposure the ROS level remained significantly increased after exposure to **1**, **11a** and **11c**. The lowest effect on neutrophils redox balance was observed for **22** and GAA (Figure 4A).

The kinetics of the increase in ROS levels may be determined by the toxic properties of the compound but potentially also by how rapidly the toxic compound is being taken up and metabolized by cells. The potential detrimental outcomes of elevated generation of ROS levels include DNA damage, protein modifications, lipid peroxidation, and necrotic or apoptotic cell death.[32] In the present study the two latter effects were also assessed to determine whether potential change in ROS levels produced adverse effects or whether neutrophils had the ability to cope with them through adaptive responses.

Peroxidation of lipids was measured by means of the malondialdehyde (MDA) content. MDA levels were significantly increased following the 1 h exposure to 2 μg mL^{-1} of **1**, **11a**, **11b** and **11c**. The **11c** compound caused its elevation by as much as 33.3% when compared to the negative control, at level similar to positive control (Figure 4B). Peroxidation of lipids is a chain reaction which is initiated by the hydrogen abstraction or addition of an oxygen radical, resulting in the oxidative damage of polyunsaturated fatty acids. Increased lipid oxidation can decrease the membrane fluidity and the final products of peroxidation (predominantly MDA and 4-hydroxy-2-nonenal) can induce genotoxicity, and cell death.[33,34] Consistent with their ability to increase ROS and MDA levels, **1**, **11a**, **11b** and **11c** also reduced cell viability accordingly (Figure 4C). In summary, the ability of compounds **11a**, **11b** and **11c** to elevate ROS levels correlated closely with an increase in lipid peroxidation and cell death. If one considers the short-term exposure model applied in this study, the magnitude of observed effects highlights the potentially fast bioavailability of the tested compounds and onset of effects on cellular level. It should however be stressed that the employed concentration is much above the maximal observed for **1** in aquatic environment (usually found in the range of 1–10 μg L^{-1}, maximal reported concentration exceeded 500 μg L^{-1}) [35] and concentrations lower by order(s) of magnitude may not produce such adverse effects. Nevertheless, in the present study, a concentration of 2.0 μg mL^{-1} was used intentionally to maximize the potential differences in action of particular compounds.

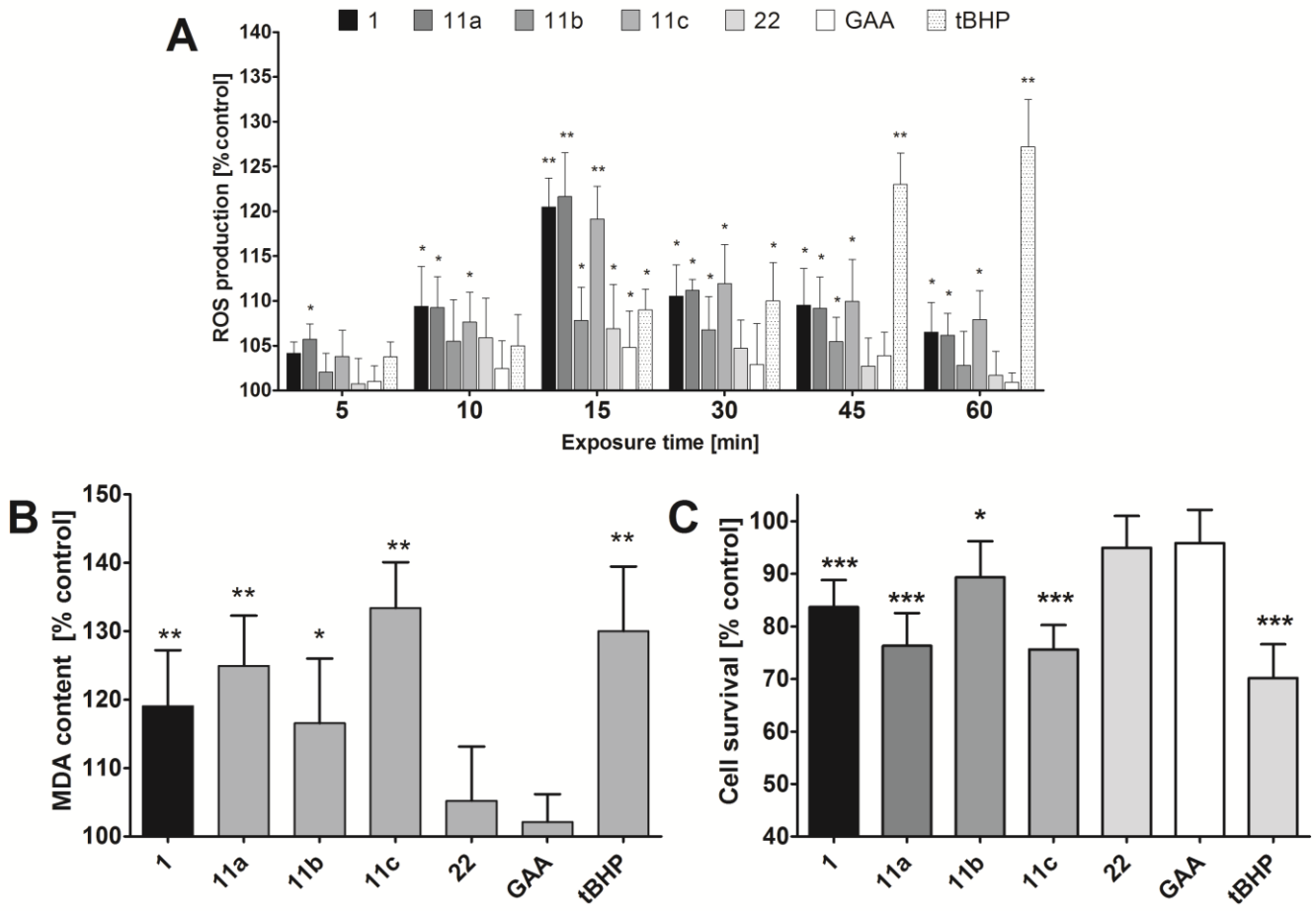


Figure 4. Generation of intracellular ROS (A), lipid peroxidation (B) and cell survival (C) after 1 h exposure to different CYN analogues (1, 11a-11c and 22), GAA and 10 µM tBHP (positive control) expressed as percentage of control. Bars represent mean ± SD from three independent experiments corresponding to three different donors (n = 3). Asterisks represent statistically significant difference to the control (* - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$; Wilcoxon signed-rank test).

The general toxicity decreased in the following order: **11c** > **11a** > **1** > **11b** > **22** > GAA. Since all of the CYN analogues tested contained guanidine and uracil, either group could be involved in generating the observed *in vivo* effects. The uracil group was demonstrated to be a pharmacophore of CYN,[12] likely facilitating direct binding to nucleic acids and its further effects such as DNA strand breaks and other genotoxic effects[36], all events usually accompanied by an imbalance in cellular ROS levels.[37] It is however likely that other structures of CYN are also involved in its bioactivity. It remains so far unclear how CYN moves across the cellular membrane and which structures are crucial in this process[6]. It was however, demonstrated that the sulfate group has no importance for this import process[13]. The role of the hydroxyl group in this matter is unknown. Interestingly, the naturally occurring *7*-deoxy-cylindrospermopsin **3**, that lacks the hydroxyl group, did not produce any toxic effects in an *in vivo* rodent model at levels

previously shown to exceed the lethal concentrations established for **1**[3]. Consistent with the conclusion that the hydroxyl group is important for cytotoxicity all active compounds in this study, **11a-11c** and **1**, contained this group. The **22** analogue, which is most closely related to **1** in terms of its general structure and molecular weight, but lacks the hydroxyl group, did also not cause toxic outcomes. These findings support the view that the hydroxyl group may play an important role in the bioactivity of CYN and highlight the need for further studies focusing in detail on the mechanism of cellular uptake, intracellular localization and intracellular concentration of tested compounds. It should also be noted that exposure to **11a-11c** analogues and **1** led to a more rapid onset of ROS production, indirectly indicating that their transport across the plasma membrane might be more efficient. Other compounds, such as cholesterol, are known to enter cellular membrane by inserting their hydroxyl group into hydrophilic phospholipid head group[38]. Therefore one hypothesis is that the -OH group at C-7 may be involved in the cellular transport of CYN possibly resembling the uptake of cholesterol. Moreover, it was established that CYN can be, at least partially, transported across the cellular membrane via passive diffusion owing to the low molecular weight and high hydrophilicity. The presence of –OH group in **1** and **11a-11c** supports the hydrophilicity of those analogues due to the polarity of this functional group and likely increases the facilitated diffusion over this presented by **22** and **GAA** that lack it. As proposed, hydroxylation of C-7 is the last step of cyanobacterial biosynthesis of CYN and is catalyzed by a product encoded by *cyrI* gene of *cyr* cluster.[19] This might also support the significance of –OH group in CYN bioactivity. Further studies are however necessary to elucidate the exact sequence of events in which hydroxyl group is involved in triggering the toxic potencies of CYN in eukaryotic cells. The present study did not find a linear association between toxicity and the length of tether. Since the greatest toxicity was found for the analogue **11c**, which from the point of view of the proximity of the uracil, hydroxyl and guanidine groups, might be best able to adopt a similar conformation to that found in the naturally occurring compound **1**.

Conclusions

This study describes the synthesis of four CYN analogues differing in length of tether between guanidine and uracil groups, and the presence or absence of a hydroxyl group. The comparison of bioactivity of the synthetic analogues revealed that only those having a hydroxyl group at C-7 produced significant *in vitro* toxicity in human cells. The largest toxic effect was found in compound **11c**, which has a 6-carbon tether separating the guanidine and uracil groups. This tether length might allow the molecule to adopt a similar conformation to that of CYN as might a 4-carbon tether as **11a** had similar activity. The tether length of 5-carbons gave

an appreciably lower activity. Overall the present study adds to the general understanding that CYN damages human tissue by increasing oxidative stress levels which in turn damages membrane lipids and can trigger cell death. The results also highlight the potentially important role of the hydroxyl group at C-7 in cellular uptake and toxicity. Further studies on detailed mechanism of cellular uptake, intracellular localization and intracellular concentration are required to fully elucidate these points.

Acknowledgements

Thanks are given to the EPSRC for a fellowship (DE, EP/J01821X/1), to the BEACON (ERDF) program for support (PJM, DE), the Libyan Authority for Research, Science & Technology (HSF) and the Foundation for Polish Science (PR "Start" Program: 091.2016). We would also like to thank the EPSRC National Mass Spectrometry Facility at Swansea.

Experimental

All glassware used was washed with acetone and dried with N_2 or in a vacuum oven. Reagents and starting materials were from commercial suppliers and used as supplied. Diethyl ether and tetrahydrofuran were distilled from benzophenone and sodium wire, whilst dichloromethane was dried over CaH and freshly distilled. Dry DMF and methanol were purchased from Aldrich. Petroleum ether (PE) refers to the fraction distilled between boiling range of 40-60 ºC. Column chromatography and TLC chromatography was carried out using silica and silica plates purchased from Fluorochem Ltd (particle size 35-70 μ) using HPLC grade acetic acid (AA) chloroform (CFM), dichloromethane (DCM), diethyl ether (DE), ethyl acetate (EA), hexane (HX) and methanol (ME). Compounds were visualized using UV, iodine or stained using polyphosphomolybdic acid (PMA) in EtOH or vanillin in EtOH/H_2SO4, with heating. IR spectra were recorded on a Perkin-Elmer 1600 series FTIR instrument as KBr discs, thin films, Nujol mulls, or chloroform solution on NaCl plates with absorption frequencies reported in wavenumbers (cm^{-1}). Routine NMR samples were performed on a Bruker AC250, AC400 or on a Bruker Avance-500 spectrometer. Chemical shifts for spectra are all reported in δ values (ppm) relative to the residual solvent peak in each case. Electron Ionisation (EI) and Chemical Ionisation (CI) mass spectra were recorded on an Agilent Tech. 6890N spectrometer or an XCalibur MAT900 XLT spectrometer and ESI were recorded on a LTQ Orbitrap XL at the EPSRC Mass Spectrometry Service (Swansea).

General method for the Grignard reaction.

A solution of EtMgBr in THF (1M, 1.9 eqv.) was added to a stirred and cooled (0 ˚C) solution of the alkyne **13** (2 eqv.) in dry THF (2.5 mL per mmol of **13**) and the resulting mixture was stirred for 1 h. This solution was then added via cannula to a cooled (-78 ˚C) solution of aldehyde **12** (1.0 eqv) in dry THF (4 mL per mmol of **12**) and the mixture stirred for 2 h. After warming to rt overnight NH_4Cl solution (aqueous, saturated, 20 mL) was added and the mixture extracted with EA (3 × 50 mL) and the combined organic extracts washed with brine (25 mL) and water (25 mL), dried ($MgSO_{4|}$) and evaporated under reduced pressure. The crude residue was purified by flash chromatography on silica gel (EA/PE 5-30%) to give the acetylinic alcohols **14a-c** as unstable oils.

4-((*tert*-Butyldimethylsilyl)oxy)-1-(2,6-dimethoxypyrimidin-4-yl)but-2-yn-1-ol **14a**: 75 %; R_f 0.32 (25% EA/PE); δ_H 0.09 (6H, s, 2 × CH_3), 0.87 (9H, s, 3 × CH_3), 1.95 (1H, br s, OH), 3.97 (3H, s, CH_3), 3.99 (3H, s, CH_3), 4.35 (2H, d *J* 1.1 Hz, CH_2), 5.31 (1H, t, *J* 1.1 Hz, CH), 6.54 (1H, s, CH); δ_C -5.1, 18.4, 25.9, 51.8, 54.3, 55.1, 63.3, 82.8, 85.1, 98.2, 165.2, 168.7, 172.5; ν_{max} 3392, 2955, 2930, 2885, 2858, 1597, 1572, 1484, 1463, 1382, 1358, 1255, 1203, 1129, 1059, 815, 779; **m/z (CI)** 339.2 (100%, $[M+H]^+$), 577.2 (90%, $[2M+H]^+$); **HRMS (CI)** $C_{16}H_{27}N_2O_4Si$ ($[M+H]^+$) requires 339.1735, found 339.1735.

5-((*tert*-Butyldimethylsilyl)oxy)-1-(2,6-dimethoxypyrimidin-4-yl)pent-2-yn-1-ol **14b**: 71 %; R_f 0.32 (25% EA/PE); δ_H 0.04 (6H, s, 2 × CH_3), 0.87 (9H, s, 3 × CH_3), 2.45 (2H, dt, *J* 2.0, 7.1 Hz, CH_2) 3.72 (2H, t, *J* 7.1 Hz, CH_2), 3.90 (1H, br s, OH) 3.99 (3H, s, CH_3), 4.01 (1H, s, CH), 5.27 (1H, br t, *J* 2.0 Hz, CH), 6.56 (1H, s, CH); δ_C -5.2, 18.4, 23.3, 26.0, 54.3, 55.2, 61.7, 63.3, 79.2, 84.5, 98.2, 165.1, 169.1, 172.6; ν_{max} 3384, 2953, 2929, 2883, 2857, 1596, 1572, 1482, 1462, 1381, 1356, 1253, 1203, 1102, 1056, 836, 777; **m/z (CI)** 339.2 (100%, $[M+H]^+$), 577.2 (90%, $[2M+H]^+$) **HRMS (CI)** $C_{17}H_{28}N_2O_4Si$ ($[M+H]^+$) requires 353.1890, found 353.1891.

6-((*tert*-Butyldimethylsilyl)oxy)-1-(2,6-dimethoxypyrimidin-4-yl)hex-2-yn-1-ol **14c**: 55 %; R_f 0.34 (25% EA/PE); δ_H 0.00 (6H, s, 2 × CH_3), 0.84 (9H, s, 3 × CH_3), 1.69 (2H, tt, *J* 6.0, 7.1 Hz, CH_2), 2.29 (2H, dt, *J* 2.0 Hz, CH_2), 3.63 (2H, t, *J* 6.0 Hz, CH_2), 3.90 (1H Br s OH), 3.95 (3H, s, CH_3), 3.98 (3H, s CH_3), 5.25 (1H, br t, *J* 2.0 Hz, CH), 6.54 (1H, s, CH); δ_C -5.3, 15.3, 26.0, 31.5, 54.2, 55.0, 61.6, 63.5, 78.3, 87.0, 98.0, 165.1, 169.5, 172.5; ν_{max} 3434, 2955, 2930, 2897, 2858, 2216, 1590, 1570, 1483, 1463, 1386, 1360, 1253, 1203, 1100, 1056, 836, 758; **m/z (CI)** not determined (unstable to oxidation).

General method for hydrogenation.

Palladium on activated carbon (1:1 by mass with **14**) was added to a solution of alkyne **14** (1.0 eqv.) in EA (4 mL per mmol) under an argon blanket. The flask was purged with hydrogen gas (balloon) and the mixture stirred vigorously under a hydrogen atmosphere for 2 h.

The mixture was then filtered through a Celite© pad which was washed with further portions of EA and the filtrate concentrated by evaporation under reduced pressure. The crude product was purified by column chromatography on silica gel (10-40% EA/PE) to give the alkanes **15a-c** as colourless oils.

4-((tert-Butyldimethylsilyl)oxy)-1-(2,6-dimethoxypyrimidin-4-yl)butan-1-ol **15a**: 79 %; R_f 0.25 (20% EA/PE); δ_H 0.05 (6H, s, 2 × CH_3), 0.89 (9H, s, 3 × CH_3), 1.60-1.78 (3H, m, CH, CH_2), 1.93-2.03 (1H, m, CH), 3.65 (2H, t, *J* 6.5 Hz, CH_2), 3.95 (3H, s, Me), 3.97 (3H, s, Me), 4.13 (1H, br s, OH), 4.56 (1H, m, CH), 6.44 (1H, s, CH); δ_C 5.3, 18.4, 26.0, 28.6, 34.7, 54.0, 54.8, 63.4, 72.7, 97.5, 165.1, 172.3, 174.2; ν_{max} 3402, 2955, 2930, 2885, 2857, 1598, 1570, 1481, 1462, 1382, 1355, 1255, 1203, 1153, 1097, 1006, 836, 776; **m/z (CI)** 343.2 (100%, $[M+H]^+$), 365.2 (15%, $[M+Na]^+$), 707.4 (30%, $[2M+Na]^+$); **HRMS (CI)** $C_{16}H_{31}N_2O_4Si$ ($[M+H]^+$) requires 343.2048, found 343.2049.

5-((tert-butyldimethylsilyl)oxy)-1-(2,6-dimethoxypyrimidin-4-yl)pentan-1-ol **15b**: 100 %; R_f 0.27 (20% EA/PE); δ_H 0.00 (6H, s, 2 × CH_3), 0.85 (9H, s, 3 × CH_3), 1.39-1.58 (4H, m, 2 x CH_2), 1.61-1.70 (1H, m, CH), 1.74-1.83 (1H, m, CH), 3.37 (1H, br s, OH), 3.52 (2H, t, *J* 6.3 Hz, CH_2), 3.94 (3H, s, Me), 3.97 (3H, s, Me), 4.53 (1H, dd, *J* 4.6, 7.6 Hz, CH_2) 6.32 (1H, s, CH); δ_C -5.2, 18.4, 21.6, 26.0, 32.6, 37.4, 54.1, 54.9, 63.1, 72.5, 97.5, 165.0, 172.2, 173.5; ν_{max} 3422, 3018, 2953, 2931, 2898, 2858, 1598, 1570, 1482, 1462, 1382, 1357, 1255, 1215, 1100, 1056, 1006, 837, 758; **m/z (CI)** 357.2 (100%, $[M+H]^+$), 735.4 (20%, $[2M+Na]^+$); **HRMS (CI)** $C_{17}H_{33}N_2O_4Si$ ($[M+H]^+$) requires 357.2204, found 357.2202.

6-((*tert*-Butyldimethylsilyl)oxy)-1-(2,6-dimethoxypyrimidin-4-yl)hexan-1-ol **15c**: 86 %; R_f 0.34 (25% EA/PE); δ_H 0.00 (6H, s, 2 × CH_3), 0.86 (9H, s, 3 × CH_3), 1.31-1.43 (4H, m, 2 x CH_2), 1.46-1.53 (2H, m, CH_2), 1.60-1.69 (1H, m, CH), 1.73-1.81 (1H, m, CH), 3.47 (1H, br s, OH), 3.57 (2H, t, CH_2 *J* 6.3 Hz), 3.95 (3H, s, Me), 3.98 (3H, s, Me), 4.52 (1H, dd, *J* 4.6, 7.6 Hz, CH), 6.32 (1H, s, CH); δ_C -5.2, 18.5 (C), 25.0, 25.8, 32.8, 37.7, 54.1, 54.9, 63.2, 72.5, 97.4, 165.0, 172.2, 173.5; ν_{max} 3418, 2951, 2932, 2858, 1598, 1570, 1480, 1461, 1381, 1356, 1253, 1203, 1099, 1056, 1006, 837, 758; **m/z (CI)** 371.2 (100%, $[M+H]^+$); **HRMS (CI)** $C_{18}H_{35}N_2O_4Si$ ($[M+H]^+$) requires 371.2361, found 371.2360.

General method for acetylation.

Acetic anhydride (3.7 eqv.) was added drop wise to a cooled (0˚C) and stirred mixture of the alcohol **15** (1.0 eqv.), pyridine (3.9 eqv.) and DMAP (0.1 eqv.) in dry DCM (10 mL per mmol of **15**). The mixture was warmed to rt and stirred for 1 h, whereupon HCl (aqueous. 1 M, 10 eqv.) was added and the mixture stirred for 30 min. The mixture was then extracted with DCM (3 x 25 mL) and the combined extracts washed with HCl (aqueous, 1M, 3 × 10 mL) and water

(20 mL). After drying ($MgSO_4$) and evaporated under reduced pressure, column chromatography on silica gel (2.5-17.5% DE in PE) gave the acetates **16a-c** as oils.

1-(2,6-Dimethoxypyrimidin-4-yl)-4-((tert-butydimethylsilyl)oxy)butyl acetate **16a**: 68 %; R_f 0.28 (20% DE/PE); δ_H 0.02 (6H, s, 2 × CH_3), 0.87 (9H, s, 3 × CH_2), 1.51-1.62 (2H, m, CH_2), 1.84-1.94 (1H, m, CH), 1.96-2.04 (1H, m, CH), 2.13 (3H, s, CH_3), 3.60 (2H, t, *J* 6.3 Hz, CH_2), 3.94 (3H, s, Me), 3.96 (3H, s, Me), 5.61 (1H, dd, *J* 4.8, 7.8 Hz, CH), 6.30 (1H, s, CH); δ_C -5.2, 18.4, 21.1, 26.0, 28.4, 30.6, 54.0, 54.9, 62.7, 75.2, 97.7, 165.4, 170.3, 170.6, 172.3; ν_{max} 2956, 2930, 2898, 2858, 2253, 1744, 1598, 1572, 1482, 1463, 1388, 1358, 1242, 1206, 1162, 1104, 1059, 964, 910, 836; **m/z (CI)** 385.2 (100%, $[M+H]^+$), 791.4 (15%, $[2M+H]^+$), **HRMS (CI)** $C_{18}H_{33}N_2O_5Si$ ($[M+H]^+$) requires 385.2153, found 385.2146.

1-(2,6-Dimethoxypyrimidin-4-yl)-4-((tert-butydimethylsilyl)oxy)butyl acetate **16b**: 74 %; R_f 0.64 (20% EA/PE); δ_H 0.01 (6H, s, 2 × CH_3), 0.86 (9H, s, 3 × CH_2), 1.34-1.42 (2H, m, CH_2), 1.46-1.57 (2H, m, CH_2), 1.80-1.98 (2H, m, CH_2), 2.13 (3H, s, CH_3), 3.57 (2H, t, *J* 6.3 Hz, CH_2), 3.94 (3H, s, Me), 3.96 (3H, s, Me), 5.58 (1H, dd, *J* 4.8, 7.8 Hz, CH), 6.30 (1H, s, CH); δ_C -5.2, 0.1, 18.5, 21.2, 21.6, 26.1, 32.6, 33.9, 54.0, 54.9, 62.9, 75.5, 97.7, 165.4, 170.4, 170.7, 172.3; ν_{max} 2956, 2929, 2857, 1747, 1597, 1573, 1461, 1386, 1357, 1233, 1205, 1101, 837; **m/z (CI)** 399.2 (100%, $[M+H]^+$), 819.4 (10%, $[2M+H]^+$); **HRMS (CI)** $C_{19}H_{35}N_2O_4Si$ ($[M+H]^+$) requires 399.2310, found 399.2299.

1-(2,6-Dimethoxypyrimidin-4-yl)-4-((tert-butydimethylsilyl)oxy)butyl acetate **16c**: 88 %; R_f 0.44 (20% EA/PE); δ_H -0.02 (6H, s, 2 × CH_3), 0.87 (9H, s, 3 × CH_2), 1.30-1.40 (4H, m, 2 x CH_2), 1.45-1.52 (2H, m, CH_2), 1.79-1.97 (2H, m, CH_2), 2.13 (3H, s, CH_3), 3.57 (2H, t, *J* 6.3 Hz, CH_2), 3.95 (3H, s, Me), 3.97 (3H, s, Me), 5.58 (1H, dd, *J* 4.8, 7.8 Hz, CH), 6.30 (1H, s, CH); δ_C -5.2, 18.5, 21.2, 25.0, 25.7, 32.7, 34.2, 54.0, 54.9, 63.2, 75.4, 97.6, 165.4, 170.4, 170.7, 172.3; ν_{max} 2952, 2932, 2858, 1747, 1597, 1574, 1481, 1462, 1386, 1357, 1232, 1205, 1101, 1045, 835; **m/z (CI)** 413.2 (100%, $[M+H]^+$); **HRMS (CI)** $C_{20}H_{37}N_2O_5Si$ ($[M+H]^+$) requires 413.2466, found 413.2458.

General method for silyl deprotection.

A solution of TBAF (1M, 1.5 eqv.) in THF was added to a cooled (0˚C) solution of the silane **16** (1.0 eqv.) in THF (10 mL per mmol) and the resulting mixture stirred for 2 h. The reaction mixture was evaporated onto silica gel (ca 1-2 g per mmol of **16**) under reduced pressure, then purified by flash chromatography on silica gel, (gradient elution; 30-40% EA/PE) to give **17a-c** as colorless oils.

1-(2,6-Dimethoxypyrimidin-4-yl)-4-hydroxybutyl acetate **17a**: 80 %; R_f 0.26 (40% EA in PE); δ_H 1.64-1.89 (4H, m, 2 × CH_2), 2.00 (3H, s, CH_3), 3.59 (1H, br s, OH), 3.94 (3H, s, CH_3),

3.97 (3H, s, CH_3), 4.04-4.11 (2H, m, CH_2), 4.55 (1H, dd, *J* 2.6, 6.3 Hz, CH), 6.33 (1H, s, CH); **δ_C** 21.0, 24.8, 34.0, 54.1, 54.9, 64.3, 72.0, 97.4, 165.1, 171.3, 172.3, 173.1; ***ν_{max}*** 3440, 3428, 2955, 2927, 1738, 1596, 1569, 1482, 1381, 1351; **m/z (CI)** 271.1 (100%, $[M+H]^+$), 293.1 (40%, $[M+Na]^+$), 563.2 (20%, $[2M+Na]^+$); **HRMS (CI)** $C_{12}H_{19}N_2O_5$ ($[M+H]^+$) requires 271.1288, found 271.1287.

1-(2,6-Dimethoxypyrimidin-4-yl)-4-hydroxybutyl acetate **17b**: 74. %; ***R_f*** 0.26 (40% EA in PE); **δ_H** 1.35-1.43 (2H, m, CH_2), 1.49-1.62 (2H, m, CH_2), 1.79-1.97 (2H, m, CH_2) 2.11 (3H, s, CH_3), 2.12 (1H, br s, OH), 3.57 (2H, t, *J* 6.5 Hz, CH_2), 3.92 (3H, s, CH_3), 3.94 (3H, s, CH_3), 5.56 (1H, dd, *J* 4.8, 7.5 Hz, CH), 6.28 (1H, s, CH); **δ_C** 21.1, 21.4, 32.3, 33.8, 54.0, 54.9, 62.5, 75.2, 97.5, 165.3, 170.4, 170.5, 172.2; ***ν_{max}*** 3408, 2948, 2870, 1744, 1597, 1571, 1483, 1386, 1359; **m/z (CI)** 285.1 (100%, $[M+H]^+$), **HRMS (CI)**; $C_{13}H_{20}N_2O_5$ ($[M+H]^+$) requires 285.1443, found 285.1445.

1-(2,6-Dimethoxypyrimidin-4-yl)-6-hydroxyhexyl acetate **17c**: 71 %; ***R_f*** 0.26 (40% EA in PE); **δ_H** 1.27-1.39 (4H, m, 2 x CH_2), 1.49-1.56 (2H, m, CH_2), 1.78-1.95 (3H, m, CH_2, OH), 2.12 (3H, s, CH_3), 3.58 (2H, t, *J* 6.6 Hz, CH_2), 3.93 (3H, s, CH_3), 3.95 (3H, s, CH_3), 5.56 (1H, dd, *J* 4.9, 7.8 Hz, CH), 6.28 (1H, s, CH); **δ_C** 21.1, 21.9, 25.5, 32.5, 34.0, 54.0, 54.9, 62.7, 75.3, 97.5, 165.3, 170.4, 170.6, 172.3; ***ν_{max}*** 3414, 2934, 2862, 1746, 1598, 1573, 1483, 1386, 1358; **m/z (CI)** 299.2 (100%, $[M+H]^+$), **HRMS (CI)** $C_{14}H_{23}N_2O_5$ ($[M+H]^+$) requires 299.1601, found 299.1599.

General method for the guanylation, deprotection, reguanylation and purification.

Guanylation: DIAD (1.5 eqv.) was added in a drop wise manner to a cooled (0 ˚C) and stirred solution of the alcohol **17** (1.0 eqv.), PPh_3 (1.53 eqv.) and *N,N′,N″*-tri-Boc-guanidine **18** (2 eqv.) in dry THF (8 mL per mmol of **17**). After stirring to rt overnight, water (3-4 eqv.) was added and the mixture stirred for 30 min after which the solution was evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (5-20% EA/PE) to give **19a-c** as gums, which were contaminated with quantities of DIAD byproducts and were used in the next step without further purification. *Deprotection:* Hydrochloric acid (6 mL per 0.1 mmol, 0.5 M) was added to **19a-c** (1.0 eqv.) and the mixture heated (100°C) at reflux for 16h. After cooling and evaporation under reduced pressure the residue was dried in a desiccator (P_2O_5) overnight, dissolved in ME (ca 2 mL) and DE (3-5 ml) was added to the cloud point whereupon an oily precipitate on cooling (-20˚C) overnight. This was found to be a mixture of **11a-c** and **20a-c**. *Reguanylation and ion exchange purification:* The crude mixture from the previous step was dissolved in DMF (2-3 mL per 0.1 mmol) and 1*H*-pyrazole-1-carboxamidine hydrochloride (0.5-0.7 eqv) was added together with DIPEA (1.5-2.0 eqv) and the mixture

stirred at rt for 24 hrs. DE (5-10 ml) was added which resulted in the formation of an oily precipitate formed, whereupon the supernatant liquid was decanted. The precipitate was dissolved in ME (1-2 ml) and DE (3-5 ml) was added to the cloud point, and an oily precipitate formed on cooling (-20˚C) overnight. After decanting the supernatant liquid, the precipitate was dissolved in water (5 mL) then passed several times through a short column (3 g) of activated Amberlite© CG-50 (the resin was pre-washed with MeOH, then HCl (2 M), then water to *p*H = 7). The column was then eluted with water (20 mL), ammonia (1 M (30 mL) and 2M (30 mL)), evaporation of the 2M ammonia washings gave on evaporation the crude guanidines **11a-c** (the water fraction was found to contain residual product, which could be purified by repeating the Amberlite© CG-50 purification steps). The crude guanidine obtained was dissolved in ME (1-2 ml) containing a drop of HCl (12 M) and DE (3-5 ml) was added to the cloud point. After cooling (-20˚C) overnight, the supernatant liquid was decanted to give **11a-c** as gums after drying.

1-(2,6-dimethoxypyrimidin-4-yl)-4-(1,2,3-tris(tertbutoxycarbonyl)guanidino)butyl acetate **19a**: R_f 0.26 (20% EA/PE); δ_H 1.49 (18H, s, 6 × CH_3), 1.50 (9H, s, 3 × CH_3), 1.71-1.91 (2H, m, CH_2), 2.04 (3H, s, CH_3), 2.17-2.38 (2H, m, CH_2), 3.94 (3H, s, CH_3), 3.96 (3H, s, CH_3), 4.11 (2H, t, *J* 6.4 Hz, CH_2), 5.40-5.68 (1H, m, CH), 6.50 (1H, s, CH), 10.73 (1H, s, NH); δ_C (partial data) 21.1, 25.7, 27.9, 27.9, 28.1, 28.2, 54.0, 54.9, 61.1, 72.3, 99.1, 149.7, 152.5, 155.0, 170.8, 172.2, 172.3 (5 x C not observed); **m/z (CI)** 612.3 (100%, $[M+H]^+$), 1245.6 (90%, $[2M+Na]^+$), 312 (96%, $[M-2Boc+H]^+$); **HRMS (CI)** $C_{28}H_{46}N_5O_{10}$ ($[M+H]^+$) requires 612.3239, found 612.3225.

1-(2,6-Dimethoxypyrimidin-4-yl)-5-(1,2,3-tris(*tert*-butoxycarbonyl)guanidino)pentyl acetate **19b**: R_f 0.26 (20% EA/PE); δ_H 1.46 (9H, s, 3 × CH_3), 1.49 (18H, s, 6 × CH_3), 1.35-1.42 (2H, m, CH_2), 1.59-1.73 (2H, m, CH_2), 1.79-2.02 (2H, m, CH_2) 2.14 (3H, s, CH_3), 3.75 (2H, t, *J* 7.4 Hz, CH_2), 3.95 (3H, s, CH_3), 3.97 (3H, s, CH_3), 5.58 (1H, dd, *J* 4.6, 7.7 Hz, CH), 6.30 (1H, s, CH), 10.61 (1H, s, NH); δ_C (partial data) 21.2, 22.5, 28.1, 28.1, 28.2, 28.6, 33.8, 47.7, 54.0, 54.9, 75.4, 97.6, 149.6, 152.8, 153.6, 170.4, 170.6, 172.3 (5 x C not observed); **m/z (CI)** 626.3 (7%, $[M+H]^+$), 648.3 (100%, $[M+Na]^+$); **HRMS (CI)** $C_{29}H_{47}N_5O_{10}Na$ ($[M+Na]^+$) requires 648.3215, found 648.3221.

1-(2,6-Dimethoxypyrimidin-4-yl)-4-(1,2,3-tris(tertbutoxycarbonyl)guanidino)hexyl acetate **19c**: R_f 0.26 (20% EA/PE); δ_H 1.26-1.36 (4H, m, 2 x CH_2), 1.45 (9H, s, 3 × CH_3), 1.47 (18H, s, 6 × CH_3), 1.55-1.66 (2H, m, CH_2), 1.77-1.92 (2H, m, CH_2), 2.13 (3H, s, CH_3), 3.72 (2H, t, *J* 7.5 Hz, CH_2), 3.95 (3H, s, CH_3), 3.96 (3H, s, CH_3), 5.55 (1H, dd, *J* 4.8, 7.7 Hz, CH), 6.27 (1H, s, CH), 10.59 (1H, s, NH); δ_C (partial data) 21.1, 24.9, 26.6, 28.1, 28.1, 28.2, 28.7, 34.1, 47.9, 54.0, 54.9, 75.4, 97.6, 149.1, 152.7, 153.6, 170.4, 170.6, 172.3 (5 x C not observed).

Analogues 11a-c

Amino((4-(2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)-4-hydroxybutyl)amino)methaniminium chloride **11a**: 54 % (over three steps); δ_H (CD_3OD) 1.59-1.74 (2H, m, CH_2), 1.76-1.86 (1H, m, CH_2), 2.00-2.08 (1H, m, CH), 3.63 (2H, t, *J* 6.0 Hz, CH_3), 4.33 (1H, dd, *J* 3.8, 9.0 Hz, CH), 5.56 (1H, s, CH); δ_C (CD_3OD) 29.4, 31.8, 54.0, 61.8, 98.0, 152.2, 157.1, 158.7, 166.8; **m/z (CI)** 242.1 (100%, $[M]^+$); **HRMS (CI)** $C_9H_{16}N_5O_3$ ($[M]^+$) requires 242.1248, found 242.1250.

Amino((5-(2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)-5-hydrooxypentyl)amino)methaniminium chloride **11b**: 12 % (over three steps); δ_H (CD_3OD) 1.42-1.80 (6H, m, 3 x CH_2), 3.19 (2H, t, *J* 7.0 Hz, CH_2), 4.38 (1H, dd, *J* 4.5, 7.6 Hz, CH), 5.63 (1H, s, CH); δ_C (CD_3OD) 23.3, 29.6, 36.5, 42.3, 70.2, 97.3, 153.4, 158.6, 161.6, 167.4; **m/z (CI)** 256.1 (100 %, $[M]^+$); **HRMS (CI)** $C_{10}H_{18}N_5O_3$ ($[M]^+$) requires 256.1404, found 256.1411.

Amino((5-(2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)-6-hydrooxypentyl)amino)methaniminium chloride **11c**: 23 % (over three steps); δ_H (CD_3OD) 1.37-1.76 (8H, m, 4 x CH_2), 3.17 (2H, t, *J* 7.0 Hz, CH_2), 4.36 (1H, dd, *J* 4.8, 7.2 Hz, CH), 5.62 (1H, s, CH); δ_C (CD_3OD) 25.8, 27.4, 29.7, 36.9, 42.4, 70.4, 97.3, 153.4, 158.6, 161.8, 167.4; **m/z (CI)** 270.2 (100 %, $[M]^+$); **HRMS (CI)** $C_{10}H_{18}N_5O_3$ ($[M]^+$) requires 270.1561, found 270.1557.

Allyl (5aR,8aS)-4-((1,3-dibenzyl-2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)methyl)-3,5a,6,7,8,8a-hexahydro-1H-2,2a1,3-triazaacenaphthylene-5-carboxylate acetate 25.

A solution of aldehyde **23**[16,17] (255.1 mg, 0.719 mmol) in AA (3 mL) was stirred at rt for 24 h. After evaporation under reduced pressure, residual AA was removed by co-evaporation with CFM (3 x 15 mL) and the residue dried under high vacuum for 4 h. The resultant salts where dissolved in trifluoroethanol (1.5 mL) and morpholine acetate (264.7 mg, 1.798 mmol), **24** (1.55 g, 3.595 mmol) and anhydrous sodium sulphate (1.0 g) were added and the resultant mixture stirred at 70°C in a sealed Carius tube for 12 d. After cooling to rt and evaporation, purification was achieved by flash column chromatography on silica gel using ME/CFM (stepwise gradient of 1% increments 0:100 to 8:92) containing 1% AA. Fractions eluting in 5:95 ME/CFM gave **25** as a tan oil (82.5 mg, 0.135 mmol, 19%). R_f 0.53 (10% ME/CFM); δ_H 1.36-1.41 (2H, m, 2 x CH), 1.52-1.64 (3H, m, 3 x CH), 1.78-1.81 (2H, m, 2 x CH), 1.96 (3H, s, CH_3), 3.19 (1H, dd, *J* = 9.6, 1.3 Hz, CH_2), 3.54-3.58 (1H, m, CH), 3.79 (1H, d, *J* = 16.6 Hz, CH_2), 3.83-3.88 (1H, m, CH), 3.98 (1H, d, *J* = 16.6 Hz, CH_2), 4.40 (1H, dd, *J* = 11.2, 2.4 Hz, CH), 4.46-4.48 (2H, m, CH_2), 5.09-5.22 (6H, m, 3 x CH_2), 5.82 (1H, ddt, *J* = 16.1, 10.8, 5.6 Hz, CH), 7.17-7.37 (10H, m, 10 x CH); δ_C (partial data) 21.8, 22.5, 30.2, 31.8, 39.1, 45.5, 47.3, 48.9 (HSQC), 54.2, 58.5, 65.5, 101.0, 103.0, 118.2, 127.4, 128.5, 128.6, 129.3, 129.4, 129.8, 133.9, 137.6, 138.3, 154.0, 156.5, 159.8, 164.6, 166.8 (1 x C not observed); ν_{max} 3064, 3010, 2957, 2928, 2856,

1700, 1659, 1617, 1562, 1496, 1449, 1395, 1361, 1314, 1248; **m/z (ESI)** 1103 (16%, $[2M+H]^+$), 574 (14%, $[M+Na]^+$), 552 (100%, $[M+H]^+$); **m/z (ESI negative)** 121 (100), 75 (88), 59 (85%, $[M-H]^-$); **HRMS (ESI)** $C_{32}H_{34}N_5O_4$ $[M+H]^+$ requires 552.2605, found 552.2602.

1,3-Dibenzyl-6-(((4R,5aR,8aS)-3,4,5,5a,6,7,8,8a-octahydro-1H-2,2a1,3-triazaacenaphthylen-4-yl)methyl)pyrimidine-2,4(1H,3H)-dione acetate **26**.

Tetrakis(triphenylphosphine)palladium(0) (8.1 mg, 0.007 mmol) and pyrrolidine (0.027 mL, 0.337 mmol) were added to a stirred solution of **25** (206.0 mg, 0.337 mmol) in a 1:1 mixture of anhydrous ME/THF (6 mL). The reaction was stirred at rt and progress monitored by TLC. After 4 h the solvent was removed under reduced pressure and the residue dissolved in ME (3 mL) and AA (3 mL) and cooled (0 °C), whereupon sodium cyanoborohydride (105.8 mg, 1.685 mmol) was added and the mixture stirred to rt over 16 hours. After evaporation, the reaction material was purified by flash column chromatography on silica gel using ME/CFM (0:100 to 15:85) containing 1% AA. Fractions eluting in 10:90 ME/CFM gave **26** (133.8 mg, 0.253 mmol, 75%) as a pale yellow oil. R_f 0.18 (10% ME/CFM); δ_H 0.95-1.10 (2H, m, 2 x CH), 1.17-1.26 (1H, m, CH_2), 1.32-1.42 (1H, m, CH_2), 1.77-1.81 (1H, m, CH_2), 1.85-1.89 (1H, m, CH_2), 1.94 (3H, s, CH_3), 1.97-2.01 (1H, m, CH_2), 2.03-2.07 (1H, m, CH_2), 2.45 (1H, dd, J = 9.9, 2.7 Hz, CH_2), 2.98-3.03 (1H, m, CH), 3.12-3.20 (2H, m, 2 x CH), 3.40-3.45 (1H, m, CH), 3.52-3.60 (1H, m, CH), 3.73-3.78 (1H, m, CH_2) 4.94-5.37 (4H, m, 2 x CH_2), 5.64 (1H, s, CH), 7.16-7.49 (10H, m, 10 x CH); δ_C 22.0, 23.3, 29.5, 30.9, 34.2, 36.9, 44.7, 47.7, 47.9, 48.5, 49.6, 56.7, 102.7, 126.4, 127.6, 127.8, 128.3, 129.0, 129.0, 136.0, 136.6, 150.7, 152.6, 155.6, 161.8, 178.0; ν_{max} 3155, 3065, 3034, 2947, 2931, 2862, 1704, 1668, 1620, 1586, 1557, 1497, 1451, 1398, 1340 and 1262; **m/z (ESI)** 470 (100%, $[M+H]^+$); **m/z (ESI negative)** 157 (18), 141 (20), 121 (100), 75 (86), 73 (12), 59 (83%, $[M-H]^-$); **HRMS (ESI)** $C_{28}H_{32}N_5O_2$ $[M+H]^+$ requires 470.2551, found 470.2546.

6-(((4R,5aR,8aS)-3,4,5,5a,6,7,8,8a-Octahydro-1H-2,2a1,3-triazaacenaphthylen-4-yl)methyl)pyrimidine-2,4(1H,3H)-dione hydrobromide **22**.

Boron tribromide (106 µL, 1.120 mmol) was added to a stirred solution of **26** (118.4 mg, 0.224 mmol) dissolved in xylenes (12 mL) and the mixture heated at reflux for 16 h. On cooling to rt ME (5 mL) was slowly added and the mixture stirred for a further 16 h. After evaporation, the crude product was purified by flash column chromatography on silica gel (ME:CFM (0:100 to 30:70 containing 1% AA). Fractions eluting in 12:88 ME/CFM gave **22** (77.9 mg, 0.210 mmol, 94%) as a waxy solid. R_f 0.11 (20% ME/CFM); δ_H 1.21-1.29 (1H, m, CH_2), 1.33-1.41 (1H, m, CH_2), 1.47-1.56 (2H, m, 2 x CH), 1.93-1.99 (2H, m, 2 x CH), 2.06-2.09 (1H, m, CH_2), 2.29 (1H, dt, J = 13.5, 3.6 Hz, CH_2), 2.69 (2H, d, J = 6.8 Hz, CH_2), 3.18-3.25 (1H, m, CH_2), 3.40 (1H, tt, J

= 10.9, 3.6 Hz, CH), 3.80-3.86 (3H, m, 3 x CH), 5.59 (1H, s, CH); δ_C 23.1, 30.4, 31.7, 35.1, 38.3, 49.3, 49.6, 51.2, 58.3, 101.8, 153.3, 153.4, 156.4, 166.8; ν_{max} 3322, 3159, 3054, 2963, 2927, 2859, 1713, 1659, 1607, 1445, 1342, 1331; **m/z (ESI)** 579 (4%, $[2M+H]^+$), 394 (5), 372 (10), 312 (9%, $[M+Na]^+$), 290 (100%, $[M+H]^+$); **m/z (ESI negative)** 125 (53), 85 (27), 81 (95), 79 (100%, $[M-H]^-$), 62 (20), 32 (21); **HRMS (ESI)** $C_{14}H_{20}N_5O_2$ $[M+H]^+$ requires 290.1612, found 290.1607.

1,3-Dibenzylurea **27**.[18]

A vigorously stirred suspension of finely powdered carbohydrazide (9.0 g, 100.0 mmol) in DMF (200 mL) and benzylamine (109 mL, 1000 mmol) was cooled to 0 °C, to which I_2 (100.0 g, 0.40 mol) was added in portions over 1 h until a brown colour persisted. Excess Iodine was removed by the addition of $Na_2S_2O_3$ (Sat, ca. 2-3 mL) and the desired compound was precipitated from the resulting straw yellow solution by the addition of water (800 mL). After filtration and drying under vacuum, **27** (15.3 g, 64 mmol, 64%) was obtained as a white solid which was used without further purification. Recrystallisation from THF/toluene gave An analytically pure of sample. R_f 0.20 (50% EA/PE); **Mp** 165-168 °C (lit.[39] 168-169 °C); δ_H 4.23 (2H, s, CH_2), 4.24 (2H, s, CH_2), 6.43 (2H, t, *J* = 5.8 Hz, 2 x NH), 7.20-7.33 (10H, m, 10 x CH); δ_C 43.0, 43.0, 126.5, 127.0, 128.2, 140.9, 158.1; ν_{max} 3337, 3086, 3062, 3030, 2919, 2874, 1627, 1613, 1589, 1574, 1493, 1472, 1453, 1421; **m/z (ESI)** 503.2 (25%, $[2M+Na]^+$), 481.3 (100%, $[2M+H]^+$), 263.1 (8%, $[M+Na]^+$), 241.1 (95%, $[M+H]^+$); **HRMS (ESI)** $C_{15}H_{17}N_2O$ $[M+H]^+$ requires 241.1335, found 141.1333.

Ethyl 2-(1,3-dibenzyl-2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)acetate **29**.

Urea **27** (11.5 g, 47.7 mmol), diethyl 1,3-acetonedicarboxylate **28** (17.3 mL, 95.4 mmol) and *p*-TsOH (907 mg, 4.8 mmol) were dissolved in benzene (250 mL) and the mixture heated to reflux under a soxhlet extractor containing powdered 4 Å molecular sieves (ca 20 g). The molecular sieves were replaced every 2 days and after 4 days ^{1}H NMR analysis indicated complete consumption of the starting material. The reaction mixture was cooled to rt, diluted with DCM (800 mL) and washed with $NaHCO_3$ (sat. 3 x 150 mL) and brine (100 mL). After drying and evaporation the resulting viscous oil was stirred with PE (300 mL). After 1 hour the solvent was decanted and the resultant precipitate triturated with DE (4 x 100 mL) to give ester **29** as a pale yellow solid (12.1 g, 32.0 mmol, 67%). R_f 0.41 (50% EA/PE); **Mp** 119-122 °C; δ_H 1.26 (3H, t, *J* 7.2 Hz, CH_3), 3.4 (2H, s, CH_2), 4.14 (2H, q, *J* 7.2 Hz, CH_2), 5.13 (2H, s, CH_2), 5.17 (2H, s, CH_2), 5.73 (1H, s, CH), 7.11-7.50 (10H, m, 10 x CH); δ_C 14.0, 38.7, 44.8, 48.1, 62.2, 104.3, 126.1, 127.6, 127.9, 128.4, 129.0, 129.1, 135.8, 136.7, 147.7, 152.5, 161.8, 167.4; ν_{max} 3090, 3061, 3023, 3008, 2979, 2935, 1738, 1702, 1693, 1654, 1662, 1617, 1584, 1495, 1451,

1423, 1396, 1367, 1333; **m/z (ESI)** 779.3 (6%, $[2M+Na]^+$), 757.3 (100%, $[2M+H]^+$), 401.1 (5%, $[M+Na]^+$), 379.2 (95%, $[M+H]^+$); **HRMS (ESI)** $C_{22}H_{23}N_2O_4$ $[M+H]^+$ requires 379.1652, found 379.1650.

2-(1,3-Dibenzyl-2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)acetic acid **30**.

Sodium hydroxide (2.25 g, 56.1 mmol) was added to a cooled (0 °C), stirred suspension of ester **29** (10.62 g, 28.1 mmol) in ethanol (500 mL) and the mixture stirred to rt over 5 d. The mixture was quenched with H_2O (600 mL) and the pH adjusted to pH 1-2 by the slow addition of 1 M HCl, then extracted with DCM (3 x 100 mL) and the combined organic extracts dried over magnesium sulfate. After evaporation the resulting pale brown solid was triturated with DE (3 x 70 mL) to give acid **30** as an off white solid (7.70 g, 22.0 mmol, 78%). R_f 0.26 (20% ME/DCM); **Mp** 138-141 °C; δ_H 3.57 (2H, s, CH_2), 5.02 (2H, s, CH_2), 5.04 (2H, s, CH_2), 5.88 (1H, s, CH), 7.15-7.36 (10H, m, 10 x CH), 13.07 (1H, s, COOH); δ_C 38.0, 43.8, 47.5, 103.0, 125.9, 127.2, 127.4, 127.6, 128.4, 128.7, 136.4, 137.0, 149.6, 152.0, 161.3, 169.4; ν_{max} 3442, 3096, 3063, 3034, 2921, 2793, 2695, 2576, 1722, 1705, 1693, 1642, 1598, 1496, 1472, 1454, 1423, 1399, 1367, 1351, 1315; **m/z (ESI)** 701.3 (100%, $[2M+H]^+$), 373.1 (15%, $[M+Na]^+$), 351.1 (100%, $[M+H]^+$); **HRMS (ESI)** $C_{20}H_{19}N_2O_4$ $[M+H]^+$ requires 351.1399, found 351.1340.

Allyl 4-(1,3-dibenzyl-2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)-3-oxobutanoate **24**.

Pyrimidine **30** (1.50 g, 4.28 mmol), Meldrum's acid **31** (0.68 g, 4.71 mmol) and CDI (1.04 g, 6.42 mmol) were dissolved in THF (20 mL) and the mixture heated at 50 °C for 16 h. Upon cooling to rt the solvent was removed under reduced pressure and the residue diluted with DCM/H_2O (1:1, 160 mL) and the pH of the aqueous layer adjusted to pH 2 by the slow addition of 1 M HCl. The organic layer was then separated and washed with H_2O (50 mL), dried over magnesium sulfate and the solvent removed under vacuum. The resulting yellow solid was dissolved in allyl alcohol (20 mL) and the mixture heated at 110 °C for 16 h. After cooing the mixture was evaporated under reduced pressure and the resulting material purified by flash column chromatography on silica gel using EA/PE (15:85 to 30:70). Fractions eluting in 25:75 EA/PE gave **24** as a viscous yellow oil (1.38 g, 3.19 mmol, 75%). R_f 0.08 (30% EA/PE); δ_H 3.77 (2H, s, CH_2), 3.98 (2H, s, CH_2), 4.58 (2H, dt, *J* 5.5, 1.4 Hz, CH_2), 4.69 (2H, s, CH_2), 5.02 (2H, s, CH_2), 5.19-5.33 (2H, m, CH_2), 5.81 (1H, s, CH), 5.83-5.92 (1H, m, CH), 7.14-7.36 (10H, m, 2 x Ph); δ_C 43.8, 46.1, 47.6, 48.2, 65.2, 103.2, 118.3, 126.0, 127.3, 127.4, 127.5, 128.4, 128.7, 132.1, 136.6, 137.1, 149.3, 151.9, 161.2, 166.6, 198.4; ν_{max} 3094, 3067, 3023, 3002, 2968, 2939, 1746, 1727, 1705, 1702, 1663, 1628, 1586, 1497, 1453,1396, 1347 and 1328; **m/z (ESI)**

865.3 (50%, $[2M+H]^+$), 455.2 (6%, $[M+Na]^+$), 433.2 (100%, $[M+H]^+$); **HRMS (ESI)** $C_{25}H_{25}N_2O_5$ $[M+H]^+$ requires 433.1758, found 433.1756.

Toxicological studies: The toxicity of CYN variants was evaluated using human neutrophils in an *in vitro* model. Heparinized blood samples (6.0 mL) were collected in lithium heparin tubes from 3 healthy (screened by physical examination, medical history and initial blood tests), non-smoking and normal weighted (BMI 18.5–24.9) male donors (aged 21–25 years old) at the Regional Centre of Blood and Blood Treatment in Poznan, Poland, according to accepted safeguard standards and legal requirements. Human neutrophils were isolated immediately after collection using a one-step density-gradient centrifugation on Gradisol G of a specific gravity of 1.115 g mL^{-1} (Polfa, Poland) at 400 g at room temperature for 30 min. The residual erythrocytes were removed from the cell population by hypotonic lysis. The purity of the neutrophils (>90%) was verified by counting under a light microscope after May-Grunwald-Giemsa staining. Neutrophils were exposed for 1 h to 2.0 µg/mL of **11a-c**, **22**, **1** or GAA and their toxicity was compared by monitoring the intracellular generation of reactive oxygen species (ROS), peroxidation of lipids and cell survival. Each experimental assay was performed on three independent neutrophil isolates with three technical replicates. The negative control was constituted of unexposed cells. The positive control was constituted of cells exposed to 10 µM tert-butyl hydroperoxide (tBHP), a well established inducer of oxidative stress and specifically, lipid peroxidation and oxidative stress-induced death in eukaryotic cells.

Intracellular reactive oxygen species assay: Human neutrophils were loaded for 30 min at 37 °C in darkness with 20 µM of 2',7' –dichlorofluorescin diacetate (DCFDA; Abcam, UK), a fluorogenic dye that measures hydroxyl, peroxyl and other reactive oxygen species (ROS) activity within the cell. Cells were then washed, dissolved in PBS, seeded in a black clear bottom 96-well plate at a density of 20 x 10^4 neutrophils per well (90 µL aliquots/well) and exposed to tested compounds for 1h at 37°C for 1 h. Fluorescence of DCFDA was measured kinetically after 5, 15, 30, 45 and 60 min of incubation using a Synergy HTX multi-mode plate reader (BioTek, USA) at an excitation of 495 nm and emission of 528 nm. The background signal, measured in exposed neutrophils not loaded with DCFDA, was withdrawn from the corresponding samples. The final results were presented as a percentage of the negative control.

Lipid peroxidation assay: Lipid peroxidation was analyzed using a Lipid Peroxidation Colorimetric/Fluorometric Assay Kit (BioVision, UK) by means of malondialdehyde (MDA)

content. Human neutrophils were seeded in a 96-well plate at a density of 20 x 10^4 neutrophils per well (90 µL aliquots/well) and exposed to each tested compound for 1 h at 37 °C. The control was constituted of cells incubated with 10 µL of PBS. After the experiments, cells were harvested from each well and homogenized on ice in 300 µL of provided lysis buffer and centrifuged to remove insoluble material. The resulting 200 µL of supernatants were transferred to a microcentrifuge tube and supplemented with 600 µL of thiobarbituric acid (TBA) to generate an MDA–TBA adduct. To accelerate the reaction, samples were incubated at 95 °C for 60 min and the final product was measured colorimetrically at 532 nm. The calculated values were compared to a calibration curve prepared using MDA standard (BioVision, UK). The coefficient of variation (r^2) for the calibration curve was 0.99. The final results were presented as a percentage of the negative control.

Cell viability assay: Cell viability was determined using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) metabolic activity assay (BioVision, UK). Human neutrophils were seeded in a 96-well plate at a density of 20 x 10^4 neutrophils per well (90 µL aliquots/well) and exposed to each tested compound for 1 h at 37 °C. Afterwards, cells were washed, seeded again, and 10 µL of MTT was added to each well for 3 h. Neutrophils were then treated with 10% sodium dodecyl sulfate in 0.01 M HCl and incubated for another 6 h in the darkness to dissolve formazan crystals. The optical density (OD) of the final product (the formazan crystals) was measured at 570 nm using a Synergy HTX microplate reader (BioTek, USA). The final results were presented as a percentage of the negative control.

References

1. I. Ohtani, R. E. Moore and M. T. C. Runnegar, *J. Am. Chem. Soc*., 1992, **114**, 7941.
2. R. Banker, B. Teltsch, A. Sukenik and S. Carmeli, *J. Nat. Prod*., 2000, **63**, 387.
3. R. L. Norris, G. K. Eaglesham, G. Pierens, G. R. Shaw, M. J. Smith, R. K. Chiswell, A. A. Seawright and M. R. Moore, *Environ. Toxicol*., 1999, **14**, 163.
4. K. M. Wimmer, W. K. Strangman, J. L.C. Wright, *Harmful Algae*., 2014, **37**, 203.
5. S. Byth, *Med. J. Aust*., 1980, **2**, 40.
6. B. Poniedziałek, P. Rzymski, M. Kokociński, *Environ. Toxicol. Pharmacol*., 2012, **34**, 651.
7. R. Banker, S. Carmeli, O. Hadas, B. Teltsch, R. Porat, A. Sukenik, *J. Phycol*., 1997, **33**, 613.
8. R. Li, W. W. Carmichael, S. Brittain, G. K. Eaglesham, G. R. Shaw, Y. Liu and M. W. Watanabe, *J. Phycol*., 2001, **37**, 1121.
9. P. Rzymski, B. Poniedziałek, B*. J Phycol*., 2015, **51**, 1037.
10. M. Bohunicka, J. Mareš, P. Hrouzek, P. Urajová, M. Lukeš, J. Šmarda, J. Komárek, L. Gaysina , O. Strunecky, O. *J. Phycol*. 2015, **51**, 1040.
11. P. Rzymski, B. Poniedziałek, M. Kokociński, T. Jurczak, D. Lipski, K. Wiktorowicz K. *Harmful Algae* 2014, **35**, 1.

12. R. Banker, S. Carmeli, M. Werman, B. Teltsch, R. Porat and A. Sukenik, J. Toxicol. Environ. Health, Part A, 2001, 62, 281.
13. M. T. Runnegar, C. Xie, B. B. Snider, G. A. Wallace, S. M. Weinreb, J. Kuhlenkamp, *Toxicol. Sci*., 2002, **67**, 81.
14. C. Neumann, P. Bain and G. Shaw, *J. Toxicol. Environ. Health, Part A*., 2007, **70**, 1679.
15. R. E. Looper, M. T. C. Runnegar, R. M. Williams, *Angew. Chem. Int. Ed*., 2005, **44**, 3879.
16. B. Poniedziałek, P. Rzymski, K. Wiktorowicz, *Toxicol. In Vitro.,* 2014, **28**, 968.
17. B. Poniedziałek, P. Rzymski, J. Karczewski, *Toxicon.,* 2014, **87**, 113.
18. B. Poniedziałek, P. Rzymski, J. Karczewski, *Toxicol. In Vitro.,* 2015, **29**, 926.
19. T.K. Mihali, R. Kellmann, J. Muenchhoff, K.D. Barrow B.A. Neilan, *Appl. Environ. Microbiol*., 2008, **74**, 716.
20. B. B. Snider, C. Xie, *Tetrahedron Lett*., 2000, **41**, 1849.
21. D. M. Evans, P. J. Murphy, *Chem. Commun*., 2011, **47**, 3225.
22. D. M. Evans, P. N. Horton, M. B. Hursthouse, P. J. Murphy, *RSC Adv*., 2014, 4, 20744.
23. Y. Wolman, P. M. Gallop *J. Org. Chem*., 1962, **27**, 1902.
24. V. Z. Shimkin, A. K. Shirinian, D. V. Mailian, D. V. V.Lonshakov, M. M. Gorokhov, M. Krayushkin, *Russ. Chem. Bull*., 2011, **60**, 139.
25. C. Nathan, *Nat. Rev. Immunol.,* 2006, **3**, 173.
26. B. Poniedziałek, P. Rzymski, M. Kokociński, J. Karczewski, *Chemosphere*., **120**, 608.
27. P. Rzymski, P. Niedzielski, N. Kaczmarek, T. Jurczak, P. Klimaszyk, *Harmful Algae*, 2015, **46**, 34.
28. P. Rzymski, B. Poniedziałek, J. Mankiewicz-Boczek, E.J. Faassen, T. Jurczak, I. Gągała-Borowska, A. Ballot, M. Lürling, M. Kokociński, *Algal Res*., 2017, **24**, 72.
29. D. Lobner, P. M. Piana, A. K. Salous, R. W. Peoples, *Neurobiol. Dis*., 2007, **25**, 360.
30. A. Rymuszka, A. Sieroslawska, *Endocrinol. Lett*. 2011, **32**, Suppl. 1, 84.
31. B. Poniedziałek, P. Rzymski, J. Karczewski, *Toxicol. In Vitro*., 2015, **29**, 926.
32. S. Halliwell, J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*., Oxford University Press, New York, 4th Ed., 2007.
33. P. C. Burcham, *Mutagenesis*, 1998, **13**, 287.
34. A. Ayala, M. F. Muñoz, S. Argüelles, *Oxid. Med. Cell. Longev*., 2014, 360438.
35. P. Rzymski, B. Poniedziałek, *Water Res*., 2014, **66**, 320.
36. X. Shen, P.K. Lam, G.R. Shaw, W. Wickramasinghe W, *Toxicon* 2002, **40**, 1499.
37. B. Žegura, G. Gajski, A. Štraser, V, Garaj-Vrhovac V, *Toxicon*, 2011, **58**, 471.
38. G.M. Cooper, 2002, Sunderland, Sinauer Associates.
39. L. Hong-Xi, C. Mei-Ling, W. He-Ming, Y. Xiao-Juan, R. Zhi-Gang, and L. Jian-Ping, *Organometallics*., 2011, 30, 208.