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A simple and efficient flow preparation of pyocyanin a virulence factor of *Pseudomonas aeruginosa*

Frederik B. Mortzfeld,[a,b] Jörg Pietruszka[b] and Ian R. Baxendale*[a]

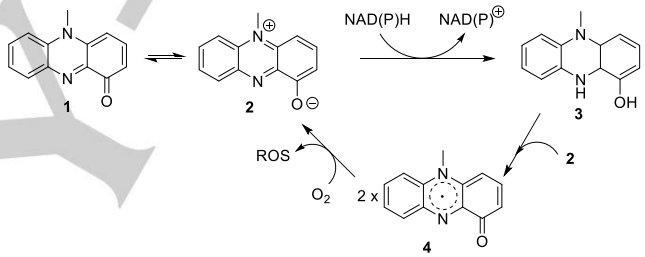
Abstract: The synthesis of the naturally occurring toxin pyocyanin has been realized in a short 4 step sequence. The key photochemical reaction and isolation of the final product has been facilitated by the use of flow chemistry techniques and immobilised reagents. Using these procedures gram quantities of pyocyanin were easily prepared in high yield and purity.

Introduction

Nature provides endless examples of organisms displaying amensalism behavior through antibiosis especially when vying for a common resource that is in limited supply, e.g. food or space. Scientists have therefore taken great inspiration from such interspecies chemical warfare to formulate new medicines or formulate new lines of defense against human and animal pathogens. Probably the most widely recognized example is the common bread mold *Penicillium* which secretes the antibiotic penicillin, a molecule which has become one of the front line treatments in combating bacterial infections.

Recently, the gram-negative bacterium *Pseudomonas aeruginosa* has received much publicity as an increasingly prevalent multidrug resistant pathogen associated with several life threating conditions and an upsurge in hospital-acquired infection.[1] Despite it's widely reported negative impact the organism may hold the key to a new generation of chemoprevention and chemotherapy treatments. *Pseudomonas aeruginosa* produces a wide range of virulence factors several of which have been identified as potent biochemical agents beneficial to the bacterium in dominating microbial competitive environments.[2] One molecule of particular interest is the growth pigment pyocyanin (Scheme 1; **1**,**2**),[3] a low molecular weight, redox active structure destructive to other bacteria and fungi through the formation of reactive oxygen species (ROS) (Scheme 1).[4,5] Its reactivity and pervasive toxic nature are related to its zwitterionic character allowing it to easily cross cell membranes and cause rapid tissue damage to the host.

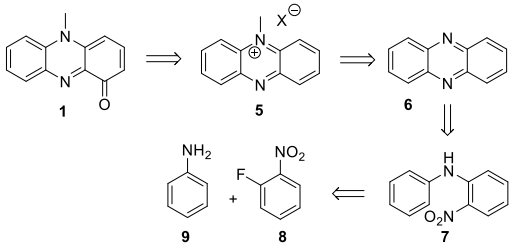
We became especially interested in pyocyanin (**1**) in relation to its potential use in the fast growing and potentially highly lucrative area of aquaculture as a biocontrol agent. The aquaculture market especially in Asia is seen as an essential emerging source of protein and carbohydrates but its intensification is being hindered by increasing outbreaks of viral, bacterial, fungal, parasitic and other emerging pathogen infections. Pyocyanin (**1**) has already shown some value in initial testing[6] but has been hampered from larger scale evaluation due to its limited supply and its relatively high cost (84 GBP for 5 mg).[7] Although the biosynthesis of pyocyanin has improved significantly in recent years, its production at scale (gram) is still very much limited, with isolation of the zwitterionic compound from the aqueous media still being a major bottleneck.[8] Furthermore, pyocyanin (**1**) is just one of a selection of toxins produced by during biological expression and therefore challenges exist regarding cross-contamination of extracts. Unfortunately, chemical synthesis approaches have also failed to supply the molecule in sufficient quantities despite the community's efforts over many years.[3f,9] We therefore decided to investigate the synthesis and where of value apply our in-house knowledge of flow chemistry[10] to expedite the synthesis.



Scheme 1. Proposed redox cycle of pyocyanin (**1**+**2**). Simplified mechanism adapted from Jacob *et al*.[5]

Results and Discussion

Our synthetic plan was to target a four step sequence to pyocyanin (**1**) (Scheme 2). We note that phenazine (**6**) is commercially available in multi-gram quantities at a reasonable price but its supply can be problematic.[11]



Scheme 2. Planned retrosynthesis to pyocyanin (**1**).

Consequently, the first stage was the synthesis of 2-nitro-*N*-phenylaniline (**7**) through aromatic substitution of 2-fluoronitrobenzene (**8**) with aniline (**9**). Utilizing a microwave heating procedure as previously reported by Kommi *et al.* a 90% isolated yield of the desired product could be obtained at 120 °C in 1.5 h.[12] Furthermore, we developed a simple work-up which

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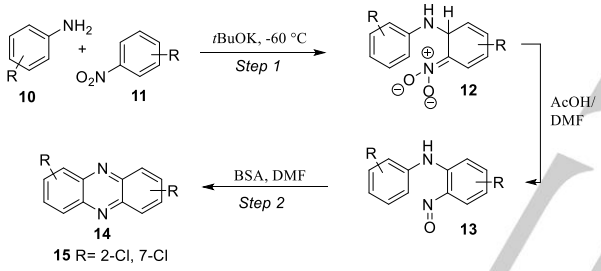
Supporting information.((Please delete this text if not appropriate))

consisted of only filtration of the solid and recrystallization from petroleum ether to give analytically pure material.

Next, Creencia *et al.*[13] had described a solvent free microwave assisted Cardogan reaction utilizing triphenylphosphine and isolating the phenazine **6** in good yield (75%). However, because of the type of domestic microwave used no information about the temperature of the reaction was presented. In attempts to replicate this result, we noted that the conversion of the starting material increased abruptly above 200 °C and that full conversion could be obtained at 250 °C after only 10 minutes of heating as confirmed by TLC and ^{1}H-NMR spectroscopy. However, after many attempts varying heating time and reagent stoichiometry a maximum threshold of only 38% of the desired product **6** was achieved.

We therefore decided to explore another alternative strategy involving a two-step procedure. Wrobel *et al.*[14] had reported the synthesis of various substituted phenazines via 2-nitroso-*N*-arylaniline intermediates (Scheme 3). In this sequence the substituted anilines **10** were reacted with nitrophenyls **11** at -60 °C in DMF under basic conditions. This led to the initial formation of σH-adducts **12**, which were subsequently quenched with acetic acid to form the intermediate nitroso compounds **13** (*step 1*). Subsequent ring closure could then be performed by treatment with BSA [*N,O-bis*(trimethylsilyl)acetamide] to give high yields of the corresponding phenazines **14** (*step 2*).

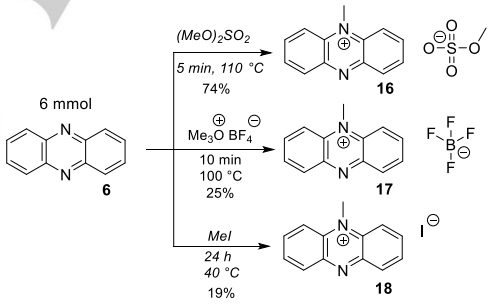


Scheme 3. Alternative general route to phenazines **14**.

To enable a direct comparison and standardization of our working practice against the literature we elected to initially reproduce the synthesis of 2,7-dichlorophenazine (**15**) which had been achieved in 57% overall yield (64% *step 1*,[13a] 89% *step 2*[13b]). After optimization and making some modifications to the general procedure we were pleased to be able to run the two stage process (*step 1 and 2*) to yield 85% of the desired 2,7-dichlorophenazine (**15**). We found that by adding the aniline (**10**, R = 4-Cl) dropwise to a well stirred DMF solution containing excess *tert*-BuOK (3 equiv.) at -60 °C followed by addition of a DMF solution of the nitroarene (**11**, R = 4-Cl) a very clean reaction occurred. After 30 minutes the reaction was quenched with sat. ammonium chloride and extracted with EtOAc enabling isolation of the intermediate product in 86%. Although the cyclisation could be effected under various conditions; basic (K_2CO_3, r.t., 24 h, 83 %), acidic (AcOH, reflux, 1.5 h, 90 %) we found that treatment of the nitroso compound with BSA at 70 °C gave complete and clean conversion within 1.5 h (>99%). Indeed, the product produced under this set of conditions started to crystalline out from solution during the treatment. However, to our great disappointment we discovered that translating the conditions to the synthesis of the parent phenazine **6** gave very poor results. It immediately became apparent that the initial vicarious nucleophilic substitution proceeded in only low conversion (<30%), we attributed this to the higher electron density of the nitrobenzene ring which upon further inspection of the literature accords with the generally observed pattern of reactivity.[13a,13b,14] Also despite running extensive optimizations we were unable to improve upon this result and so this initially promising approach was abandoned.

Finally, we decided to evaluate a palladium catalyzed homocoupling procedure involving double amination of 2-bromoaniline followed by *in situ* oxidation. This had been shown to be an effective strategy by Winkler[15] and others[16] to furnish phenazine (**6**) in 95%. Their original conditions involved heating the substrate in toluene (120 °C) with 5 mol% $Pd(OAc)_2$ and a bulky phosphine ligand such as BINAP, SPhos or XPhos. A heterogeneous base, namely Cs_2CO_3 was also added. We immediately substituted the base for DBU and used the SPhos ligand. This gave a fully homogenous mixture and also allowed an excellent yield of isolated phenazine (**6**) in 93% after 24 h.

Having ready access to phenazine (**6**) we turned our attention to its conversion into the corresponding methylated ammonium salt **5**. This could be easily accomplished by adding 1 equivalent of dimethyl sulfate to a hot solution of phenazine in 1,2-dichlorobenzene (DCB) at 110 °C with stirring for 5 minutes (Scheme 4). After cooling the solution was placed in a fridge for 3 days which yielded solid **16** that could be isolated pure in 74% after washing with diethyl ether. For comparison, we also tested the use of Meerwein's salt (trimethyloxonium tetrafluoroborate) and MeI but these prove less efficient giving only 25 and 19% isolated yields respectively.



Scheme 4. Methylation to the corresponding ammonium salts **16**-**18.**

As expected, we encountered several issues regarding consistency of material (purity) and yield when scaling the reaction up in batch due to control over the rapid addition (dimethyl sulfate) and the rate-short reaction time (5 min). This was deemed to be due to ineffective mixing and problems maintaining the necessary exacting reaction temperature (110 °C). We solved this problem by adopting a very simple flow set-up. A 1.1 M stock solution of phenazine (**6**) was prepared in DCB (incubated at 50 °C to maintain solubility) this was delivered (flow rate 5.0 mL/min) to a mixing T-piece to combine with a DCB solution of dimethyl sulfate (5 M; flow rate 1.1 mL/min). The united flow stream was directed into a 52 mL PTFE reactor which was maintained at 110 °C (residence time 8.5 min). The exiting solution was collected directly as a batch into a conical flask. However, during the processing it was noted some solid was produced which aggregated and started to clump on the walls of the tubular reactor. Over prolonged usage this resulted in reactor clogging. One simple option would be to

decrease the working concentration but this has a dual impact on the throughput and also decreases the efficiency of subsequent isolation through crystallization. We therefore elected to explore options regarding increasing the turbulent mixing within the reactor. Although various options are available including (in-line) agitators, sonication and other acoustic vibrators to stop particle accumulation we elected to use a pulsed flow approach.[17] For our small reactor, fluidic flow oscillation was achieved by means of a modified HRP series mini diaphragm pump set to operate in a horizontal orientation with the input and output connected in-line to the main flow stream (see the SI for more details). The pump pulsation was controlled using its stroke speed setting (1-720 per minute) with the induced oscillatory flow preventing clogging and enabling prolonged operation of the reactor.

The reaction was thus scaled to 0.55 M (2 h processing including start and shut down) and enabled isolation of 145.3 g of the phenazine methosulfate salt **16** representing an improved 86% yield. Of particular interest was that an additional 12% (standardized) material could be extracted from the DCB by aqueous extraction. This indicated that it may be conceivable to consider linking an aqueous extraction and the direct processing of the extract in a photochemical reactor perhaps as a telescoped process (see photoreaction discussion below). Provisional testing indicated this is feasible but it was not fully developed as an approach in this work.

For the key transformation of the methylated salt **16** to the final product **1** we elected to employ a Vapourtec UV150 photoflow reactor (Figure 1).[18] We quickly established that the optimized conditions were using the low pressure mercury lamp 100 W at 68% power and fitted with the blue wavelength filter selector (λ=380 nm transmitted), and a 10 mL reaction coil. Pressure was regulated in the system by the addition of a 100 psi in-line back pressure regulator (BPR) and a maximum reaction temperature of 50 °C maintained using the in-built gas regulated temperature control. The reaction (**16**→**1**) involves an interesting sequence of colour changes as shown in Figure 1. The aqueous solution of phenazine methosulfate **16** (canary yellow) rapidly changes to purple/red under irradiation in the photoreactor (380 nm) then when basified (Na_2CO_3) the colour of the solution changes to the characteristic blue of the final product **1**.

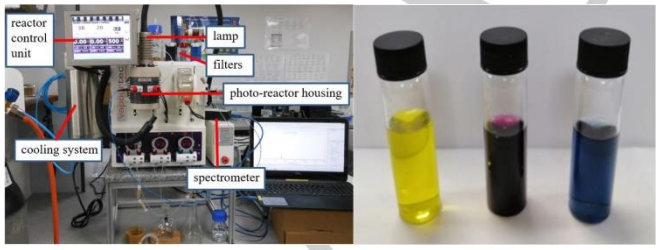
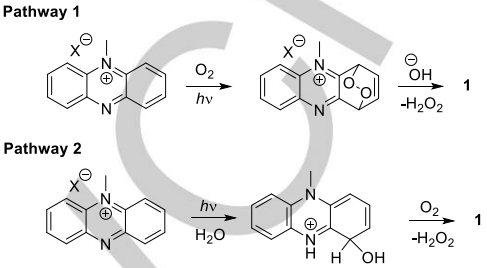


Figure 1. Flow reactor (Left). Flow photochemical colour change in the processing of phenazine salt **16** (right). Starting material (SM), Irradiated SM, Product development upon basification of the red/purple sample.

Intrigued by such interesting intrinsic colour changes and also acknowledging that the photochemical mechanism of the process has not been previously elucidated we considered our finding in the context of the wider literature.

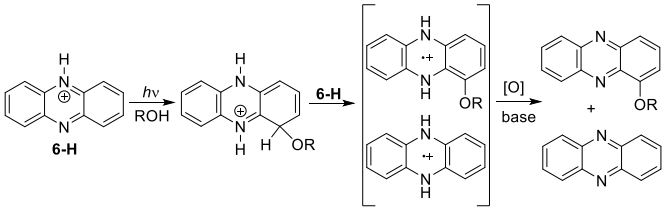
We highlighted two speculative mechanisms for the photochemical formation of pyocyanin (**1**) from salt **16**. The first involves a formal photocatalyzed [4+2] addition of oxygen across the phenyl ring followed by ring opening (*pathway 1*) and the alternative requires photoinduced water addition followed by stepwise oxidation using molecular oxygen (*pathway* 2, Scheme 5). Both processes would produce hydrogen peroxide which was experimentally confirmed by testing with peroxide dip sticks.



Scheme 5. Alternative synthesis proposals for formation of pyocyanin (**1**).

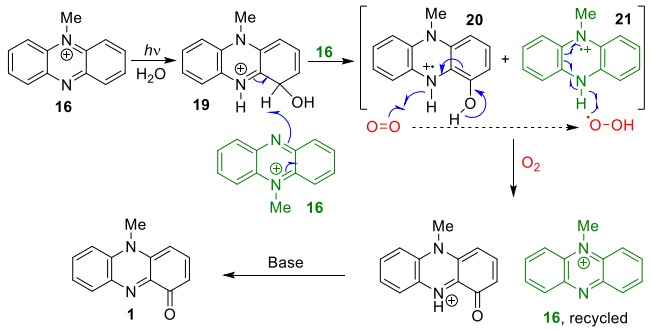
In relation to the first proposal (*pathway 1*). There is significant literature precedent that anthracene and naphthalene systems undergo facile photooxidation in the presence of oxygen to furnish intermediate bridged 1,4-endoperoxides. However, these typically then fragment to yield 1,4-dioxygenated products under a range of conditions.[19] Informatively we were unable to locate any related example of either 1,4-benzodiazines, isoquinolines or phenazines undergoing 1,4-endoperoxide formation, although a limited selection (3 examples) of quinolines have been reported.[20] This data alongside the observation that irradiation of the starting material **16** in the presence or absence of oxygen gives the same product upon basification in air (although in lower yield (39-48%) if air is exclude during irradiation) is indicative that *pathway 1* may not be the dominant process.

The second hypothesised process is based upon mechanistic studies previously undertaken on the photochemical reduction and addition of protic solvents to phenazine (**6**) albeit under acidic conditions.[21] As a pertinent illustration from the previous work,[21c] in the presence of H_3PO_4 or TsOH and under N_2 an irradiated (100 W Hg lamp) sample of phenazine yielded 1-hydroxyphenazine (48% conversion) or the corresponding ether (25-35% conversion) if MeOH or EtOH was present. Following irradiation, work-up involved air oxidation and basification enabling isolated of the product along with the residual starting material. Of note, the reactions did not occur in the absence of acid. The authors proposed a general mechanistic sequence as shown below (Scheme 6).



Scheme 6. Wake *et al.* proposed reaction sequence for photoaddition to phenazine (**6**).[21c]

Based upon this work and the wider literature we suggest, that in our work the phenazine methosulfate (**16**) absorbs light (380 nm) to reach an excited state, which reacts with water to furnish the intermediate **19** (Scheme 7). This species then reacts with a second molecule of **16** to produce a pair of cation radicals **20** and **21**. In the presence of O_2 these species are oxidized with concomitant formation of hydrogen peroxide and the regeneration of an equivalent of *N*-methylphenazonium salt **16** (which enters the cycle again) and one equivalent of the protonated product, pyocyanin (**1**).



Scheme 7. Mechanism for the photosynthesis of pyocyanin (**1**).

To add credence to this proposal we performed the irradiation of salt **16** in ^{18}O labelled water, quenching with a large excess of aqueous $NaHCO_3$ (non-labelled). This gave the desired product with full incorporation of the ^{18}O label validating that the oxygen in the product is derived from the water solvent during the irradiation step. In addition we determined that if the reaction was performed in a rigorously deoxygenated aqueous solution, and only allowed to contact oxygen after quenching a green coloured solution was obtained (Figure 2). Analysis of the green liquid indicated an equal composition of compounds **6** (yellow in solution) and **1** (blue coloured in solution see Figure 1). This result is consistent with the existence of the radical ion pair **20**+**21** (Scheme 7) which in the absence (limitation) of oxygen are persistent for the duration of the reaction. Furthermore, under basic reaction conditions (i.e. upon quenching) it was shown that compound **16** readily decomposed to yield **6**. In the absence of additional kinetic studies these data support the proposed mechanistic pathway as outlined in Scheme 7.

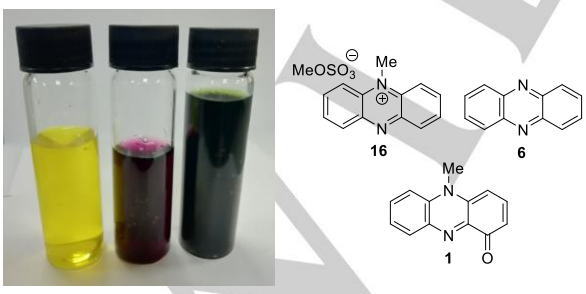
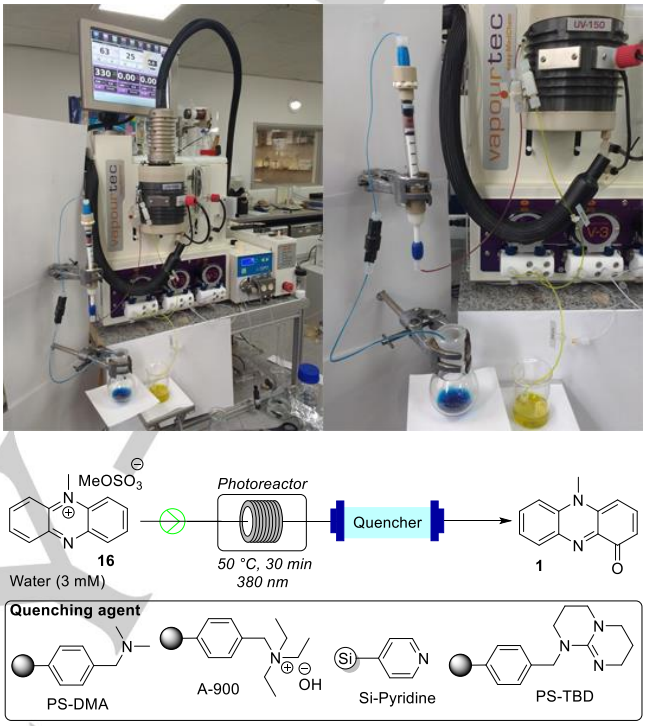


Figure 2. Photochemical colour change in the processing of phenazine salt **16**. *(left to right)* Starting material (SM), Irradiated mixture **20**+**21**, Product development upon basification in the presence of oxygen (**6**+**1**).

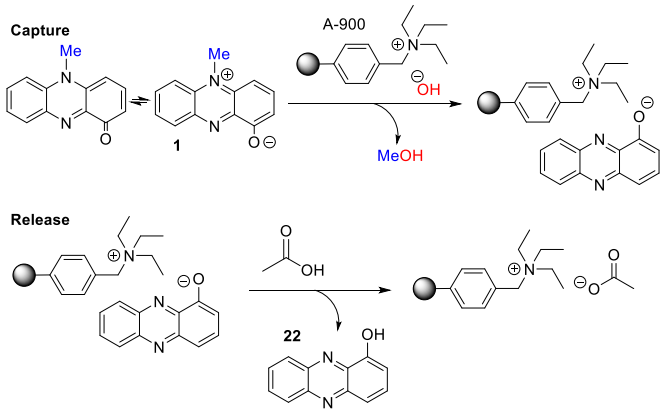
As noted previously the zwitterionic nature of pyocyanin **1** means exhaustive extraction typically with chloroform is used to isolate it from the aqueous reaction media (biological or chemical). However, this is an intensive, wasteful and very inefficient process which also results in low recoveries (47-55%). Therefore instead of quenching the reaction with an aqueous soluble base such as sodium carbonate followed by repetitive extraction and purification by column chromatography, we sought a new simplified work-up facilitated by polymer-supported reagents.[22] Various basic resins were assessed as direct in-line quenchers (Scheme 8).



Scheme 8. Flow photochemical processing of phenazine salt **16**. Lower image: Reactor schematic with quenching bases. Upper image: Photo of reactor showing quenching with PS-DMA; yellow - compound **16**; Red solution **20**+**21** and Blue product solution of **1**.

Although all species worked to some degree the most promising candidate for a future integrated flow process was the dimethylamino polystyrene (PS-DMA) resin. The silica immobilised pyridine reagent failed to fully quench the mixture, being a weak base, whereas the stronger PS-TDB was a successful quencher but was also responsible for partially sequestering of the product (see below discussion). The Ambersep® 900 hydroxide form (A-900) behaved similarly enabling fast quenching but also leading to complete sequestering of the product. It was shown that this occurred through a secondary reaction of the initially formed pyocyanin (**1**) to generate 1-hydroxyphenazine (**22**) with the production of methanol (Scheme 9). Indeed, the rapid and stoichiometric production of methanol was shown using ^1H-NMR in various water suppression experiments. Furthermore, treatment of the captured material with an acid such as acetic acid (or trifluoroacetic acid) allowed release of pure compound **22**. We identified that a previous literature synthesis of compound **19** had allowed its isolation in 40% from the phenazine salt **16**.[23] Using the capture and release methodology we could readily isolate the compound in a pure form in 96% yield. It should also

be noted at no stage did we identify any of the possible *O*-methylated derivative of compound **19** in any analysis.



Scheme 9. The reaction and capture of pyocyanin (**1**) derivative with A-900 resin and its subsequent release by treatment with acetic acid**.**

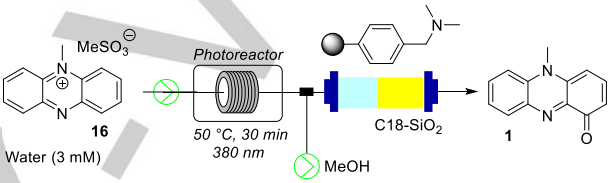
Based upon these results we therefore deduced that the PS-DMA resin worked well (>99% conversion to **1** determined by ^{1}H-NMR using water suppression sampling of the product stream, with no capture) due to its sufficient basicity and poor nucleophilicity. Although a clean product stream was being generated this still left the issue of the isolation of compound **1** from the dilute aqueous media.

To purify and concentrate the product an alternative capture and release protocol was initially pursued. A strongly-acidic sulfonic acid resin (Amberlyst® 15) was chosen which proved very effective at sequestering the product and allowing washing with different solvents. The product could then be released by treatment with a base such as triethylamine (yielding **1**:47% and **22**:18%). However, again, compound **1** was found to be unstable under the basic release conditions especially upon concentration (variety of solvents). Attempts were made to use a more volatile base; methylamine in THF to release the product. Gratifyingly the purity of the isolated product **1** was improved and could be further increased by reducing the time the product remained under the basic conditions by bubbling nitrogen through the released product solution, thus removing gaseous $MeNH_2$ to yield pyocyanin (**1**) in 65% accompanied with 10% 1-hydroxyphenazine (**22**).

Another observation was made regarding a general correlation of the ratio of recovered **1**:**22** and the duration of the capture period. The more time product **1** spent on the resin in the presence of water (or a protic solvent - MeOH or EtOH) the higher the proportional recovery of compound **22**. Indeed, times in excess of 6 h led to only compound **22** being isolated upon release. We therefore propose that protonation of pyocyanin (**1**) actives the species to attack at the methyl by the solvent generating **22**.

We therefore concluded that although a Brønsted based catch and release was feasible it was far from ideal. Instead, considering the structure of compound **1** we conceived of the possibility of using a C18 functionalized silica packed bed as a capture media to remove the product from the aqueous solution.

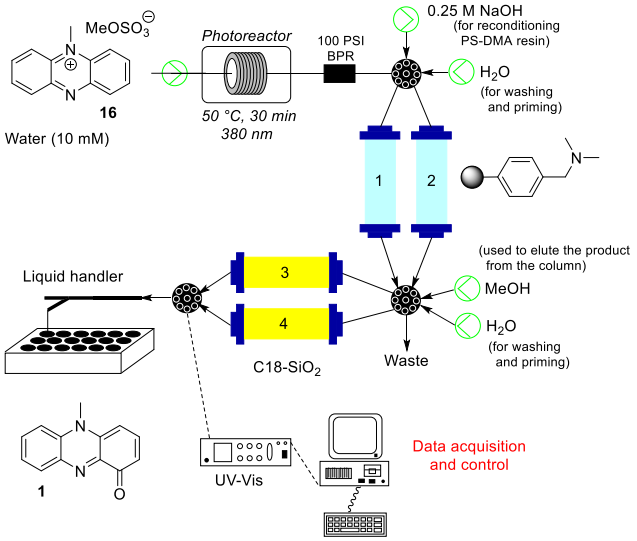
Immediately we found that pyocyanin (**1**) was fully retained by the reverse phase silica but advantageously unreacted phenazine salt **16** was not. After the photochemical reaction and capture the elution of the product **1** could be readily achieved by simply washing with MeOH or other organic solvents (delivered from secondary pump). Of additional benefit was that the C18-SiO_2 could be reused. This created a simple, user friendly purification which could be incorporated at the end of the existing synthesis sequence allowing isolation of pyocyanin (**1**) in a yield of 97% (Scheme 10). This then encouraged us to investigate increasing the throughput.



Scheme 10. Reactor schematic for the synthesis of pyocyanin (**1**) with PS-DMA quenching and C-18 capture column.

Although phenazine methosulfate (**16**) has a high solubility of >200 mg/mL a maximum concentration for the photoreaction was established at ~3 mg/mL (10 mM). The main issue was the very low solubility of the pyocyanin (**1**) in water; at higher concentrations we encountered problems with precipitation. At 10 mM concentration flow rates of up to 0.5 mL/min gave full conversion and excellent isolated yields of pyocyanin (**1**, 95-97%) equating to a minimum residence time of 20 minutes.

With modification to the design of the reactor we sought to demonstrate we could run the process in a continuous mode. This required the addition of a set of automated switching valves facilitating programmed column changes and extra conditioning pumps for washing and priming the column reactors. However, the principle change was the integration of a UV detector which was used to monitor breakthrough (λ=311 nm: See SI for full details) of the pyocyanin (**1**) when preforming the C18-SiO_2 capture, indicating when to exchange to a new sequestering column (Scheme 11). In addition, a fraction collector was added to simplify collection of the product and enable a more automated process.



Scheme 11. Final reactor configuration for continuous mode running. Interchangeable columns 1 and 2 filled with PS-DMA, columns 3 and 4 containing C18-SiO_2. Sample valves allowed independent quenching and capture as well as washing and conditioning the columns for reuse (see SI for additional details).

Pleasingly, the new reactor set-up worked well enabling the photochemical reaction to be run uninterrupted and the capture and release to be fully automated. Of particular note was that the final system was configured so that whilst one set of columns acted to quench (i.e. PS-DMA *column 1*) and subsequently sequester (i.e. C18-SiO_2 *column 3*) the product the second reserve set (i.e. *columns 2 and 4*) could be washed and conditioned for direct in-line replacement. In this way it was possible to repeatedly interchange between the two sets of columns to create an unhindered processing sequence. Of particular value was the installation of the in-line UV detector positioned at the exit of the C18-SiO_2 capture column. This was set to initiate the column exchange process when breakthrough (threshold setting) of the pyocyanin (**1**) product was detected. As an illustration of the systems automatous capabilities the reactor ran repeatedly overnight without human intervention. In one run of 18 h a total of 1080 mL of reactant was processed which following evaporation of the solvent yielded a massed aggregate collection of 2.88 g (87%) of pyocyanin (**1**). This means gram quantities of pyocyanin (**1**) can now be conveniently generated using an on-demand synthesis approach.

Conclusions

This work described in this manuscript demonstrates the synergistic value of employing photochemistry, immobilized reagents and flow reactors for the synthesis and isolation of natural products. We have shown how both product synthesis and isolation can be integrated to allow access to pyocyanin (**1**) at gram scale in short time frames. Based upon the greater understanding of the mechanism and processing we now hope to further exploit this chemistry into a wider range of pyocyanin related derivatives.

Experimental Section

2-Nitro-*N*-phenylaniline (7): Aniline (**8**) (931 mg, 10 mmol) and *o*-fluoronitrobenzene (**9**) (1.41 g, 10 mmol) in water (20 mL) were charged to a 20 mL Biotage microwave vial fitted with a stirrer bar, which was sealed with a fitted Teflon cap. The vial was heated at 120 °C for 2.5 h with stirring. The reaction mixture was cooled to r.t. and treated with saturated aqueous $CaCO_3$ (10 mL) and extracted with EtOAc (2 × 35 mL). The combined organic extracts were washed with H_2O (50 mL), dried over Na_2SO_4 and concentrated under rotary vacuum evaporation. The crude product was purified by flash chromatography (hexane–EtOAc, 90:5) to obtain a red crystalline material (1.94 g, 90%). m.p.: 73.5-74.1 °C from EtOAc (Lit.[24]: 72-74 ° C). ^{1}H NMR (400 MHz, $CDCl_3$):δ [ppm] = 9.50 (1 H, s, 7-H), 8.21 (1 H, dd, *J* = 8.6, 1.6 Hz, 1-H), 7.43 (1 H, ddd, *J* = 8.6, 8.0, 1.6 Hz, 3-H), 7.42 (1 H, dd, *J* = 8.0, 1.6 Hz, 4-H), 7.37 (1 H, ddd, *J* = 8.6, 6.9, 1.6 Hz, 2-H), 7.28 (2 H, ddd, *J* = 8.4, 6.9, 1.6 Hz, 10/12-H), 7.24 (2 H, dd, *J* = 8.4, 1.4 Hz, 9/13-H), 6.78 (1 H, tt, *J* = 8.4, 1.4 Hz, 11-H); ^{13}C NMR (151 MHz, $CDCl_3$): δ [ppm] = 143.22 (C-5), 138.86 (C-8), 135.80 (C-3), 133.36 (C-6), 129.86 (C-10, C-12), 126.80 (C-1), 125.79 (C-11), 124.53 (C-9, C-13), 117.63 (C-2), 116.18 (C-4); LC-MS: Rt = 3.22 min, m/z 215.2 [M+H$^+$]; HR-ES+MS calculated for $C_{12}H_{11}N_2O_2$ 215.0827, found 215.0821 (Δ = 2.8 ppm). IR: ṽ (cm^{-1}) = 3358, 3046, 1592, 1570, 1494, 1254, 1227, 1176, 754, 738, 517, 495.

One step batch procedure from 2-nitro-*N*-phenylaniline (7) to phenazine (1). A mixture of 2-nitro-*N*-phenylaniline (**7**) (215 mg, 1 mmol) and triphenylphosphine (0.787 g, 3 mmol) was charged into a 5 mL microwave vial one with toluene (2 mL). The vial was sealed and heated with stirring at 250 °C for 10 min. The mixture was dissolved in MeOH and the product was captured onto Amberlyst 15 sulfonic acid resin (2.5 g; ~7.5 mmol), washed with methanol (2 × 15 mL) and released with triethylamine (1.5 mL in 20 mL of MeOH). The volatiles were removed by rotary vacuum evaporation and the crude product purified by flash chromatography (hexane-EtOAc, 90:10). The product crystallised in long yellow needles. Product can be recrystallized from petrol ether. The Product crystallised in long yellow needles (68 mg, 38%). m.p.: 175 °C from EtOAc (Lit.[16]: 172-175 °C). ^{1}H NMR (700 MHz, DMSO-d_6): δ [ppm] = 8.28 – 8.21 (4 H, m, 3/6/11/14-H), 7.98 – 7.93 (4 H, m, 1/2/11/14-H); ^{13}C NMR (176 MHz, DMSO-d_6): δ [ppm] =142.82 (C-4, C-5, C-8, C-9), 130.95 (C-1, C-2, C-11, C-14), 129.27 (C-3, C-6, C-11, C-12); LC-MS: Rt = 1.93 min, m/z 181 [M+H$^+$]; HR-MS calculated for $C_{12}H_9N_2$ 181.0771, found 181.0766 (Δ = 2.8 ppm); IR: ṽ (cm-1) = 3059, 1627, 1512, 1470, 1430, 1359, 1145, 1108, 956, 902, 818, 738, 591.

Two step batch procedure of dichlorophenazine (15). Adapted from the procedure of Wróbel *et al.*[14] Synthesis of 5-chloro-*N*-(4-chlorophenyl)-2-nitrosoaniline: A cooled solution of *tert*-BuOK (337 mg, 3 mmol, 3 equiv.) in DMF (3 mL) was charged into a round bottom flask fitted with a magnetic stirrer bar. A solution of *p*-chloroaniline (127 mg, 1 mmol, 1 equiv.) in DMF (1 mL) was added dropwise at –60 °C and stirred for 5 min. Then a solution of *p*-chloronitrobenzene (157 mg, 1 equiv.) in DMF (1 mL) was added rapidly under fast stirring. The dark purple solution was stirred for 30 min, and poured into sat. NH_4Cl solution (20 mL) and directly extracted with EtOAc (2 × 15 mL). The combined EtOAc fractions were washed with brine (20 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure and the crude product purified by SiO_2 column chromatography (97:3 Hex:EtOAc) to yield the title compound as a dark red-brown powder (216 mg, 81%); after recrystallization in petrol ether brown needles. m.p.: 118-119 °C. ^{1}H NMR (700 MHz, $CDCl_3$): δ [ppm] = 11.81 (br. s, 1 H, 7-H), 8.64 (bs, 1 H, 1-H), 7.43-7.39 (m, 2 H, 10/12-H), 7.23 – 7.17 (m, 2 H, 9/13-H), 7.05 (1 H, d, *J* = 1.9 Hz, 4-H), 6.99 (1 H, d, *J* = 8.7 Hz, 2-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ [ppm] = 155.16 (C-3), 145.01 (C-5), 135.21 (C-8), 132.44 (C-11), 130.36, 130.21 (C-10, C-12), 126.33 (C-9, C-13), 119.26 (C-2), 114.25 (C-4), C-1 and C-6 signal not observed; LC-MS: R_t = 3.33 min, *m/z* 267.4 [M+H$^+$]; HR-MS calculated for $C_{12}H_9N_2OCl_2$ 267.0104, found 267.0092 (Δ = 4.5 ppm); IR: ṽ (cm^{-1}) = 2981, 1585, 1558, 1488, 1336, 1150, 1089, 796, 557, 453.

2,7-Dichlorophenazine (15): 5-Chloro-*N*-(4-chlorophenyl)-2-nitrosoaniline (532 mg, 2 mmol, 1 equiv.) was dissolved in DMF (10 mL) and BSA (2.48 mL, 10 mmol) was added. The reaction mixture was stirred at 70 °C for 1.5 h (the reaction was monitored by TLC (Hex:EtOAc 9:1)) and then quenched with H_2O (50 mL). The light yellow precipitate was filtered off, washed with water (2 × 10 mL) and a small amount of ice cold MeOH (5 mL), and dried *in vacuo*. The product was isolated as yellow crystal (475 mg, 1.91 mmol, 95%), m.p: 258.9-260.5 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 8.26 (2 H, d, *J* = 1.9 Hz, 3/14-H), 8.20 (2 H, dd, *J* = 9.3, 2.3 Hz, 1/12-H), 7.80 (2 H, ddd, *J* = 9.3, 2.2, 1.1 Hz, 6/11-H); ^{13}C NMR (151 MHz, $CDCl_3$, long run of 12h): δ [ppm] = 143.16 (C-5, C-8), 142.10 (C-2, C-15), 136.95 (C-4, C-9), 132.65 (C-6, C-11), 130.77 (C-1, C-12), 128.00 (C-3, C-4); LC-MS: R_t = 3.19 min, *m/z* 249 [M+H$^+$]; HR-MS calculated for $C_{12}H_7N_2Cl_2$ 249.0000, found 248.9986 (Δ = 5.6 ppm); IR: $\tilde{\nu}$ (cm^{-1}) = 3059, 1626, 1512, 1470, 1430, 1359, 1145, 1108, 818, 737, 591, 391; X-Ray data: CCDC 1907540; Formula: $C_{12}H_6Cl_2N_2$, Unit Cell Parameters: a 3.7799(2) b 5.9426(4) c 11.0787(7) P-1.

Batch procedure for phenazine methosulfate (16): The phenazine (**6**) (1.80 g, 10 mmol, 1 equiv.) was dissolved in 1,2-dichlorobenzene (10 mL, 1 M) and heated up to 140 °C for 10 min to induce removal of residual water. The yellow solution was cooled to 110 °C and the dimethyl sulfate (1.26 g, 1 equiv.) was added to the vigorously stirred solution. The mixture was stirred for 5 min and then cooled quickly with the aid of an ice bath. Note we observed that a green colouration was indicative of decomposition. The solution was capped and cooled in the fridge for 3 days. The precipitated phenazine methosulfate (**16**) was filtrated, washed with diethyl ether (2 x 5 mL), and then dried *in vacuo* (2.27 g, 74%).

Flow procedure for phenazine methosulfate (16): A stock solution of phenazine (**6**) (99.0 g, 0.55 mol) in 1,2-dichlorobenzene (500 mL, 1.1 M) was prepared and incubated at 50 °C to maintain solubility. A second stock solution of dimethyl sulfate (78.75 g, 0.625 mol) in 1,2-dichlorobenzene (125 mL, 5 M) was also prepared. The phenazine solution (flow rate 5.0 mL/min) and dimethyl sulfate (flow rate 1.1 mL/min) were mixed at a T-piece and progressed into a 52 mL PTFE reactor which was maintained at 110 °C (residence time 8.5 min). The reactor output was collected into a conical flask which was maintained in an ice bath and upon finishing the reaction the flask was transferred to a fridge for 3 days. The precipitated phenazine methosulfate (**16**) was filtrated, washed with diethyl ether (2 x 50 mL), and then dried *in vacuo* (145.3 g, 86%). ^{1}H NMR (700 MHz, DMSO-d_6)): δ [ppm] = 8.93 (d, $^3J_{6,1/14,13}$= 9.2 Hz, 2 H, 6/14-H), 8.93 (bs, 2 H, 3/11-H), 7.80 (bs, 2 H, 2/12-H), 8.39 – 8.29 (m, 2H, 1/13-H), 5.04 (s, 3H, 14-H), 3.38 (s, 3H, SO_4CH_3). ^{13}C NMR (176 MHz, DMSO-d_6): δ [ppm] = 145.33, 139.89, 133.51, 132.94, 132.69 – 131.09 (m), 119.94, 53.26. (very broad signals due to solvent exchange); LC-MS: R_t = 0.29 min, *m/z* 195.7 [M+H$^+$]; HR-MS calculated for $C_{13}H_{11}N_2$ 195.0919, found 195.0922 (Δ = -1.5 ppm); IR: $\tilde{\nu}$ (cm^{-1}) = 3092, 1546, 1472, 1437, 1391, 1256, 1215, 1178, 998, 764, 729, 575, 431; X-Ray structure data: CCDC 1907541; Formula: $C_{13}H_{11}N_2^+$,$CH_3O_4S^-$, Unit Cell Parameters: a 10.8453(6) b 12.2006(7) c 12.4630(7) P-1.

5-methylphenazin-5-ium tetrafluoroborate (**17**): Phenazine (**6**) (180 mg, 1 mmol, 1 equiv.) was dissolved in 1,2-dichlorobenzene (1 mL, 1 M) and heated up to 140 °C. After cooling down to 100 °C trimethyloxonium tetrafluoroborate (175 mg, 1.2 mmol) was added. After 5 min stirring the reaction was cooled in an ice bath and the resulting precipitate was filtered off, washed with hexane (5 mL) and dried *in vacuo*. The product was obtained as a green-brown powder (71 mg, 0.25 mmol, 25%). LC-MS: R_t = 3.19 min, *m/z* 195 [M+H$^+$]; HR-MS calculated for $C_{13}H_{11}N_2$ 195.0930, found 195.0922 (Δ = 4.1 ppm). IR: $\tilde{\nu}$ (cm^{-1}) = 3416, 1619, 1601, 1429, 1253, 1167, 1000, 760, 727, 574, 431.

5-methylphenazin-5-ium iodide (**18**): Phenazine (**6**) (180 mg, 1 mmol, 1 equiv.) was dissolved in 1.2-dichlorobenzene (2 mL) and heated up to 40 °C. Methyl iodide (170 mg, 0.08 mL, 1.2 mmol) was added and the reaction stirred for 24 h at 40 °C. The cooled mixture was filtered to remove the precipitate, which was washed with hexane (5 mL) and dried *in vacuo*. The product was obtained as a red-brown powder (61 mg, 0.19 mmol, 19%). ^{1}H NMR (600 MHz, D_2O): δ [ppm] = 8.80 (bs, 6-H/14-H, 2H), 8.73 (bs, 3-H/11-H, 2H), 8.61 (bs, 1-H/13-H, 2H), 8.40 (bs, 2-H/12-H, 2H), 5.13 (bs, 15-H, 3H); ^{13}C NMR (151 MHz, D_2O): δ [ppm] = 144.80 (C-4, C-8), 139.85 (C-1/C-13), 133.25 (C-5/C-9), 132.58 (C-2/C-12), 131.43 (C-3/C-11), 118.26 (C-6/C-14), 38.66 (C-15); LC-MS: R_t = 3.19 min, *m/z* 195 [M+H$^+$]; HR-MS calculated for $C_{13}H_{11}N_2$ 195.0912, found 195.0912 (Δ = 0.0 ppm). IR: $\tilde{\nu}$ (cm^{-1}) = 3077, 1601, 1469, 1437, 1365, 1351, 1094, 833, 752, 590, 390.

Pyocyanin, 5-Methylphenazin-1-(5*H*)-one (**1**): Indicative small scale reaction. Phenazine methosulfate (**16**) (60 mg, 0.196 mmol) was dissolved in H_2O (30 mL, c = 2 mg/mL) and injected in the Vapourtec UV150 at a flow rate of 0.5 mL/min (Lamp 100 W (68%), 10 mL reaction coil, filter blue (λ=380 nm transmitted), fitted with a 100 psi BPR, coil temperature of 50 °C). The intermediate purple solution was quenched by flowing through a glass Omnifit® column with adjustable end pieces (Length: 100 mm; Bore: 6.6 mm) packed with PS-DMA (3 g) and the solution was concentrated on a C_{18}-SiO_2 (4 g) contained in a glass Omnifit® column with adjustable end pieces (length: 100 mm; bore: 6.6 mm). The blue product was eluted with methanol (0.5 mL/min flow rate) until no more material could be visually seen in the output solution. The solvent was removed under reduced pressure to yield the product as a dark blue powder (40 mg, 97%). m.p. decomposition: 133.2 °C; methanol (Lit. [25]: 132-133° C). ^{1}H NMR (700 MHz, DMSO-d_6): δ [ppm] = 8.12 (1 H, dd, *J* = 8.1, 1.5 Hz, 3-H), 7.95 (1 H, dd, *J* = 8.7, 1.2 Hz, 6-H), 7.88 (1 H, ddd, *J* = 8.7, 7.0, 1.5, 1-H), 7.66 (1 H, dd, *J* = 9.2, 7.8 Hz, 13-H), 7.55 (1 H, ddd, *J* = 8.1, 7.0, 1.2 Hz, 2-H), 6.26 (1 H, dd, *J* = 9.2, 1.0 Hz, 12-H), 6.12 (1 H, d, *J* = 7.8, 3.9 Hz, 14-H), 3.90 (3 H, s, 15-H); ^{13}C NMR (176 MHz, DMSO-d_6): δ [ppm] = 178.70 (C-11), 146.75 (C-8), 142.94 (C-13), 135.21 (-9), 135.13 (C-4), 134.78 (C-1), 133.12 (C-5), 132.30 (C-3), 124.44 (C-2), 115.66 (C-12), 114.97 (C-6), 91.04 (C-14), 34.58 (C-15); LC-MS: R_t = 1.02 min, *m/z* 211.2 [M+H$^+$]; HR-MS calculated for $C_{13}H_{11}N_2O$ 211.0876, found 211.0871 (Δ = 2.4 ppm); IR: $\tilde{\nu}$ (cm^{-1}) = 3467, 1619, 1603, 1444, 1257, 1169, 1115, 763, 727, 590, 513, 403.

5-Methylphenazin-1-(5*H*)-one (**1**) (^{18}O labelled): Water-^{18}O 97 atom% ^{18}O. ^{1}H and ^{13}C NMR data was consistent with the previously prepared material purchased from Aldrich Product code 329878 for PET application. LC-MS: R_t = 1.01 min, *m/z* 213.3 [M+H$^+$]; HR-MS calculated for $C_{13}H_{11}N_2{}^{18}O$ 213.0914, found 213.0921 (Δ = 3.3 ppm).

1-Hydroxyphenazine (**22**): Phenazine methosulfate (**16**) (60 mg, 0.196 mmol) was dissolved in H_2O (30 mL, c = 2 mg/mL) and injected in the Vapourtec UV150 at a flow rate of 0.5 mL/min (Lamp 100 W (68%), 10 mL reaction coil, filter blue (λ=380 nm transmitted), fitted with a 100 psi BPR, coil temperature of 50 °C). The intermediate was quenched in-line by passage of the solution through a column filled with Ambersep 900 hydroxide resin (1.5 g, in a Omnifit® Column with adjustable end pieces (length: 150 mm; bore: 10.0 mm, bed volume 9.5 mL). The purple coloured intermediate solution changed to blue with the colour remaining on the resin. The blue resin was washed with MeOH (1.5 mL/min – 10 min) and the product was released with a 1:1 solution of MeOH:AcOH (0.5 mL/min – 30 min). The solvent and the excess AcOH was removed under reduced pressure. The product was isolated as a yellow-brown powder (35 mg, 90%). m.p.: 157.8 -159.6 °C; ethanol (Lit. [26]: 132-133° C). ^{1}H NMR (400 MHz, DMSO-d_6): δ [ppm] = 10.64 (1 H, bs, 16-H), 8.29 (1 H, dd, *J* = 7.8, 2.1 Hz, 6-H), 8.23 (1 H, dd, *J* = 7.7, 2.1 Hz, 3-H), 7.99-7.92 (2 H, m, 1/2-H), 8.00 (1 H, t, *J* = 7.8, 13-H), 7.69 (1 H, d, *J* = 8.7 Hz, 14-H), 7.20 (1 H, d, *J* = 7.4 Hz, 12-H); ^{13}C NMR (176 MHz, DMSO-d_6): δ [ppm] = 153.57 (C-11), 143.77 (C-9), 142.86 (C-4) 141.15 (C-5), 135.75 (C-8), 131.93 (C-13), 131.03 (C-1 or C-2), 130.42 (C-1 or C-2), 129.38 (C-6), 129.11 (C-3), 118.97 (C-14), 110.41 (C-12); LC-MS: R_t = 1.86 min, *m/z* 197 [M+H$^+$]; HR-MS calculated for $C_{12}H_9N_2O$ 197.0713, found 197.0715 (Δ = -1.0 ppm); IR: $\tilde{\nu}$ (cm^{-1}) = 3053, 1561, 1518, 1470, 1429, 1391, 1150, 759, 735, 415.

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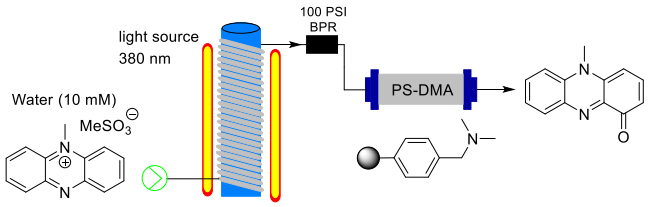

The photochemical generation and isolation of the pyocyanin has been performed in an efficient, scalable and high yielding synthesis through the use of an integrated flow chemistry approach in a semi-automated reactor.

Flow Heterocyclic synthesis

*Frederik B. Mortzfeld, Jörg Pietruszka and Ian R. Baxendale**

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A simple and efficient flow preparation of pyocyanin a virulence factor of Pseudomonas aeruginosa