Flow Reactions

A Simple and Efficient Flow Preparation of Pyocyanin a Virulence Factor of Pseudomonas aeruginosa

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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 have 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-threatening conditions and an upsurge in hospital-acquired infection.[1] Despite its 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.\textsuperscript{[14]} We, therefore, decided to investigate the synthesis and where of value apply our in-house knowledge of flow chemistry\textsuperscript{[10]} to expedite the synthesis.

**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.\textsuperscript{[11]}

Consequently, the first stage was the synthesis of 2-nitro-\textsuperscript{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.\textsuperscript{[12]} Furthermore, we developed a simple workup which consisted of only filtration of the solid and recrystallization from petroleum ether to give analytically pure material.

Next, Creencia et al.\textsuperscript{[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 \textsuperscript{1}H-NMR spectroscopy. However, after many attempts varying the 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.\textsuperscript{[5b,14]} had reported the synthesis of various substituted phenazines via 2-nitroso-\textsuperscript{N}-arylaniline intermediates (Scheme 3). In this sequence, the substituted anilines 10 were treated with nitrophenyls 11 at –60 °C in DMF under basic conditions. This led to the initial formation of o-H-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).

To enable a direct comparison and standardization of our work 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,\textsuperscript{[13]} 89 % step 2\textsuperscript{[14]}). 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, \(R = 4-\text{Cl}\)) dropwise to a well stirred DMF solution containing excess \(t\text{BuOK}\) (3 equiv), at –60 °C followed by addition of a DMF solution of the nitroarene (11, \(R = 4-\text{Cl}\)) 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\textsubscript{2}CO\textsubscript{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.\textsuperscript{[13,14]} 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\textsuperscript{[15]} and others\textsuperscript{[16]} to furnish phenazine (6) in 95 %. Their original conditions involved heating the substrate in toluene (120 °C) with 5 mol-% Pd(OAc)\textsubscript{2} and a bulky phosphine ligand such as Binap, SPhos or XPhos. A heterogeneous base, namely Cs\textsubscript{2}CO\textsubscript{3} was also added. We immediately substituted the base for DBU and used the SPhos ligand. This gave a fully homogeneous 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 Mel but these prove less efficient giving only 25 and 19 % isolated yields respectively.

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 existing 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 (\(\lambda=380\) nm transmitted), and a 10 mL reaction coil. The 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.

Scheme 4. Methylation to the corresponding ammonium salts 16–18.
The aqueous solution of phenazine methosulfate 16 (canary yellow) rapidly changes to purple/red under irradiation in the photoreactor (380 nm) then when basified (Na₂CO₃) the color of the solution changes to the characteristic blue of the final product 1.

Intrigued by such interesting intrinsic color 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.

Pathway 1

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.²¹ 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.²² 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 excluded during irradiation) is indicative that pathway 1 may not be the dominant process.

The second hypothesised process (pathway 2; Scheme 5) 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 in the presence of H₂PO₄ or TsOH and under N₂ 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).

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 moleculer of 16 to produce a pair of cation radicals 20 and 21. In the presence of O₂, 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).

To add credence to this proposal we performed the irradiation of salt 16 in ¹⁸O labelled water, quenching with a large excess of aqueous NaHCO₃ (non-labelled). This gave the desired product with the full incorporation of the ¹⁸O 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 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.

Scheme 6. Wake et al. proposed reaction sequence for photoaddition to phenazine (6).²²¹
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).

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 $^1$H-NMR in various water suppression experiments. Furthermore, treatment of the captured material with an acid such as acetic acid (or trifluoroacetic acid) allowed the 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 derivatives of compound 19 in any analysis.

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 \( \text{1} \) 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 \( \text{1}:47 \% \) and \( \text{22}:18 \% \)). However, again, compound \( \text{1} \) 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 \( \text{1} \) 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 \( \text{1}:\text{22} \) and the duration of the capture period. The more time product \( \text{1} \) spent on the resin in the presence of water (or a protic solvent – MeOH or EtOH) the higher the proportional recovery of compound \( \text{22} \). Indeed, times in excess of 6 h led to only compound \( \text{22} \) being isolated upon release. We, therefore, propose that protonation of pyocyanin (1) acts the species to attack at the methyl by the solvent generating \( \text{22} \).

We, therefore, concluded that although a Brønsted based catch and release was feasible it was far from ideal. Instead, considering the structure of compound \( \text{1} \) 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 \( \text{16} \) was not. After the photochemical reaction and capture the elution of the product \( \text{1} \) could be readily achieved by simply washing with MeOH or other organic solvents (delivered from the 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.

Although phenazine methosulfate (16) has a high solubility of \( >200 \text{ mg/mL} \) a maximum concentration for the photoreaction was established at ca. \( 3 \text{ mg/mL} \) (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
Scheme 11. Final reactor configuration for continuous mode running. Interchangeable columns 1 and 2 filled with PS-DMA, columns 3 and 4 containing C18-SiO2. Sample valves allowed independent quenching and capture as well as washing and conditioning the columns for reuse (see SI for additional details).

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 principal 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-SiO2 capture, indicating when to exchange to a new sequestering column (Scheme 11). In addition, a fraction collector was added to simplify the collection of the product and enable a more automated process.

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-SiO2 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-SiO2 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 automatic capabilities the reactor ran repeatedly overnight without human intervention.

In one run of 18 h a total of 1080 mL of the 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 CaCO3 (10 mL) and extracted with EtOAc (2 x 35 mL). The combined organic extracts were washed with H2O (50 mL), dried with Na2SO4 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.14) 72–74°C). 1H NMR (400 MHz, CDCl3)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, dd, J = 8.6, 8.0, 1.6 Hz, 3-H), 7.42 (1 H, dd, J = 8.0, 1.6 Hz, 4-H), 4.73. 13C NMR (100 MHz, CDCl3)δ [ppm] = 139.46 (C-1, C-4), 138.75 (C-2, C-3), 134.78 (C-5, C-6), 119.92, 53.26. (very broad signals due to solvent effect) 

**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 washed in MeOH and the product was captured onto Amberlite 15 sulfonic acid resin (2.5 g; ca. 7.5 mmol), washed with methanol (2 × 15 mL), and then dried in vacuo (2.27 g, 74 %). The product was obtained as a green-brown powder (71 mg, 0.25 mmol, 25 %). LC-MS: Rt = 3.19 min, 267.0104, found 267.0092 (Δ = 2.8 ppm). IR: ν (cm−1) = 3358, 3054, 1592, 1570, 1494, 1254, 1227, 1176, 754, 738, 517, 495.

**Flow Procedure for Phenazine Methosulfate (16):** A stock solution of phenazine (6) (180 g, 1 mmol, 1 equiv.) was dissolved in 1,2-dichlorobenzene (500 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 filtered, washed with diethyl ether (2 × 5 mL) and then dried in vacuo (2.27 g, 74 %).

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**Two-Step Batch Procedure of Dichlorophenazine (15):** Adapted from the procedure of Wrobel et al.23,24 Synthesis of 5-chloro-N-(4-chlorophenyl)-2-nitrosoaniline: A cooled solution of HClO4 (337 mg, 3 mmol, 3 equiv.) in DMF (3 mL) was charged into a round-bottomed flask fitted with a magnetic stirrer bar. A solution of 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 chloronitrobenzene (157 mg, 1 equiv.) in DMF (1 mL) was added. The solvent was removed under reduced pressure and the crude product purified by flash chromatography (hexane/EtOAc, 9:1) and then quenched with H2O (50 mL). The product was isolated as yellow crystal (475 mg, 1.91 mmol, 95 %), m.p: 258.9–260.5 °C. 1H NMR (600 MHz, CDCl3)δ [ppm] = 8.26 (2 H, d, J = 1.9 Hz, 3/14-H), 8.20 (2 H, d, 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); 13C NMR (151 MHz, CDCl3)δ [ppm] = 46.19, 65.33, 137.02, 133.05, 130.36, 130.21 (C-10, C-12), 126.80 (C-1, C-4); LC-MS: Rt = 3.22 min, m/z 215.2 [M + H]+; HR-ES+MS calculated for C3H5NO2 215.0827, found 215.0821 (Δ = 2.8 ppm). IR: ν (cm−1) = 3358, 3054, 1592, 1570, 1494, 1254, 1227, 1176, 754, 738, 517, 495.

**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 trimethylxonium 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: Rt = 3.22 min, m/z 195.7 [M + H]+; HR-MS calculated for C13H11N2O2F4 195.0930, found 195.0929 (Δ = 1.9 ppm). IR: ν (cm−1) = 3377, 2981, 1585, 1488, 1336, 1105, 1089, 796, 557, 457.

**2,7-Dichlorophenazine (15):** 5-Chloro-N-(4-chlorophenyl)-2-nitrosoaniline (532 mg, 2 mmol, 1 equiv.) was dissolved in DMF (10 mL) and BSTFA (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 H2O (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. 1H NMR (600 MHz, CDCl3)δ [ppm] = 8.26 (2 H, d, J = 1.9 Hz, 3/14-H), 8.20 (2 H, d, 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); 13C NMR (151 MHz, CDCl3)δ [ppm] = 43.22 (C-5, C-8), 138.86 (C-8), 135.80 (C-3), 133.36 (C-6), 129.86 (C-10, C-12), 126.80 (C-1, C-4), 125.79 (C-11), 124.53 (C-9, C-13), 117.63, 112.67 (C-14); LC-MS: Rt = 3.22 min, m/z 215.2 [M + H]+; HR-ES+MS calculated for C3H5NO2 215.0827, found 215.0821 (Δ = 2.8 ppm). IR: ν (cm−1) = 3358, 3054, 1592, 1570, 1494, 1254, 1227, 1176, 754, 738, 517, 495.
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, 99 %). 1H NMR (600 MHz, D2O): δ [ppm] = 8.80 (8 H, 6-H/14-H, 2H), 8.73 (8 H, 3-H/11-H, 2H), 8.61 (8 H, 1H-13-H, 2H), 8.40 (2 H, 2-H/12-H), 7.53 (8 H, 15-H, 3H). 13C NMR (151 MHz, D2O): 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: Rf = 3.11 min, m/z 195 [M + H]+; HR-MS calculated for C13H11N2O = 211.0876, found 211.0871 ([M + H]+ 3.3 ppm).

Acidification of 5-Methylphenazin-5-ium Iodide (18) with 18O2 was carried out in a 1:1 solution of MeOH:AcOH under reduced pressure. The product was isolated as a yelow-brown powder (35 mg, 90 %). m.p. 157.8 – 159.6 °C; ethanol (Lit.[10] 132–133 °C). 1H NMR (400 MHz, [D6]DMSO): 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, 2-H), 8.00 (1 H, 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). 13C NMR (176 MHz, [D6]DMSO): 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), 130.13 (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: Rf = 1.86 min, m/z 197 [M + H]+; HR-MS calculated for C13H13N2O2 = 213.0713, found 213.0713 (Δ = 0.0 ppm); IR: δ (cm–1) = 3563, 3035, 1516, 1518, 1470, 1429, 1391, 1150, 759, 735, 415.

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Keywords: Flow chemistry · Heterocycles · Photochemistry · Pyocyanin · Natural products

References


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