A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

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SYNTHETIC BIOLOGY & BIO-NANOTECHNOLOGY

Accepted Article

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To be cited as: *ChemBioChem* 10.1002/cbic.201900013

Link to VoR: http://dx.doi.org/10.1002/cbic.201900013

A Journal of ChemPubSoc Europe

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10.1002/cbic.201900013

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FULL PAPER

Optimized synthesis of the bacterial *magic spot* (p)ppGpp chemosensor PyDPA

Gabriele Conti,[a],† Marco Minneci[a],† and prof. Sara Sattin[a],*

Abstract: (p)ppGpp is a nucleotide signalling molecule with a marked effect on bacterial physiology during stress. Its accumulation slows down cell metabolism and replication, supposedly leading to the formation of the antibiotic tolerant persister phenotype. A specifically tailored fluorescent chemosensor, PyDPA, allows to detect (p)ppGpp in solution with high selectivity compared to other nucleotides. Here we present an optimized synthetic approach that improves the overall yield from 9% to 67% over seven steps. The simplicity and the robustness of this approach will allow the groups investigating the many facets of (p)ppGpp easy access to this probe.

Introduction

Molecular-recognition and sensing of nucleotides has been an active research field over the past decades[1] due to their biological significance.

Beside the well-known DNA, RNA and co-factor (NADH and FAD) nucleotides, several nucleotide-related molecules, such as c-di-GMP, c-AMP, Ap4A, and (p)ppGpp, are being actively investigated for their role within second messenger signalling systems. These molecules are produced by bacteria, along with quorum sensing (QS) autoinducers, in response to environmental stimuli and, in turn, lead to a variety of phenotypic changes that allow bacteria to survive or even proliferate. In the post-antibiotic era,[2] it is of the outmost importance to acquire a detailed understanding of how bacteria respond to stress in order to set out effective strategies not only to prevent the insurgence of antibiotic resistance, but also to eradicate recurrent and chronic infections.

Dissecting each signalling pathway requires selective detection of each of such signalling molecules in a milieu overpopulated with many other, structurally similar, compounds.

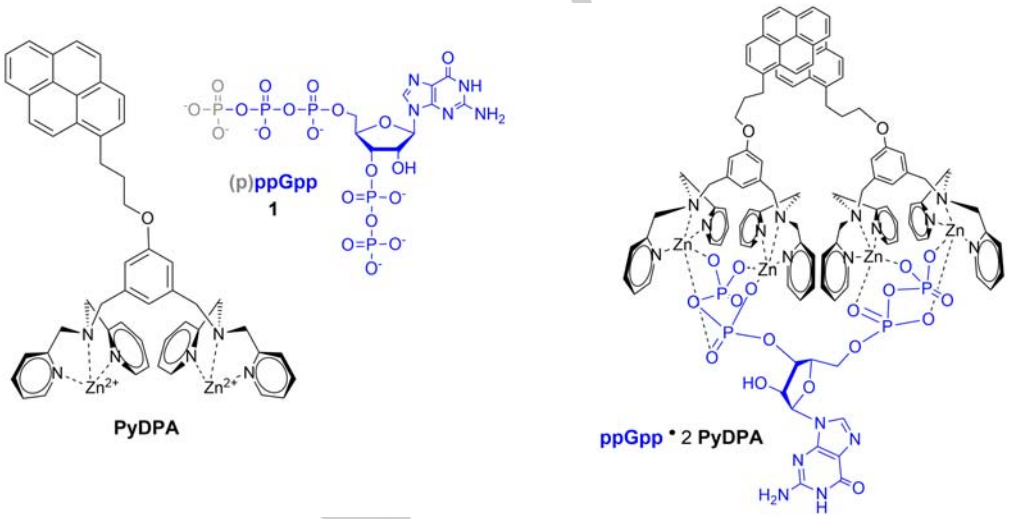

Figure 1. left) Structure of the nucleotide signalling molecule **1** (p)ppGpp and of its specific chemosensor PyDPA. Right) Structure of the 2:1 PyDPA : ppGpp complex.

We decided to focus our attention in particular on guanosine tetra-(ppGpp) or penta-phosphate (pppGpp), collectively known as (p)ppGpp, a signalling *alarmone* produced in response to stress conditions[3] (*e.g.* heat shock, nutrient starvation, etc.). Discovered in 1969 by Cashel and Gallant,[4] they were initially nicknamed *magic spot I* and *II* but, even after their structure was elucidated (**1**, Fig. 1), the nickname lingered due to the complexity of the pleiotropic effects these molecules have on bacterial physiology.[5] Indeed, (p)ppGpp impacts transcription, translation, and DNA replication,[3] and generates virulence factors by interfering with QS networks.[6] Accumulation of (p)ppGpp is also at the upstream of the *stringent response*, a signalling cascade implicated with the formation of a dormant bacterial phenotype called *persisters*.[7] This phenotype, transiently tolerant to antibiotic treatment, is not only largely responsible for the

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10.1002/cbic.201900013

difficulties encountered in eradicating recurrent and chronic infections but also favours to the insurgence of resistant strains.

In the course of our research project on small molecules able to control the onset of the persistent phenotype by interfering with the stringent response pathway, selective detection of (p)ppGpp has become a critical factor. Detection of (p)ppGpp in solution historically relied on radiolabelled compounds (either with ^{3}H or ^{32}P)[8] or HPLC methods.[9] Only in recent years, a fluorescent chemosensor (**PyDPA**, Fig. 1) – a compound bearing a binding moiety connected and communicating with a fluorophore[10] – has been specifically designed to bind selectively (p)ppGpp over other nucleotides, such as ATP, GTP, UTP, TTP, cAMP and cGMP[11]

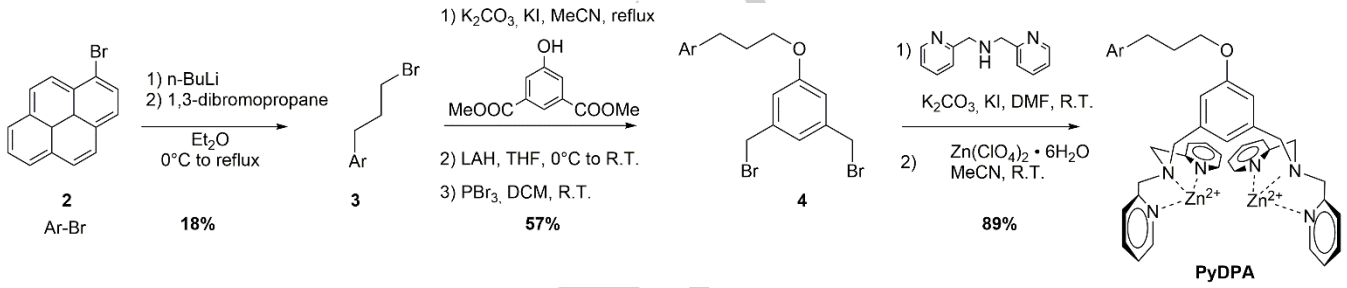
From a structural point of view, PyDPA comprises two Zn^{2+}-dipicolylamine (Zn^{2+}-DPA) units, well known for their ability to bind pyrophosphate groups in water,[12] bridged to a pyrene moiety through an alkyloxy-phthalate. One molecule of (p)ppGpp with its two terminal pyrophosphate groups is able to chelate two molecules of PyDPA, forcing the proximity of the corresponding pyrene units that is exploited for its distinctive excimer emission (Em = 470 nm).[13]

Quantification of (p)ppGpp in solution is therefore possible with the appropriate calibration curve up to the low micromolar range.

The synthesis of PyDPA, as originally reported in 2008 by Rhee and co-workers,[11] was achieved with a modest yield of about 9% over 6 steps, starting from 1-bromopyrene (Scheme 1) while a marginal improvement of the overall yield was reported a few years later by the same group[14] starting from 1-pyrenecarboxaldheyde (19% over 8 steps). Here we report an optimized synthetic sequence that overcomes the critical steps of the original and the modified synthetic approaches, increasing the overall yield from 9-19% to 67% over 7 steps.

Results and Discussion

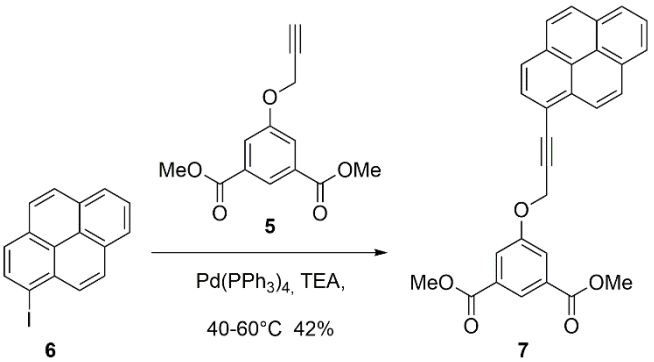
The synthesis of Rhee *et al* is outlined in Scheme 1. Halogen-lithium exchange on 1-bromopyrene (*n*BuLi), followed by treatment with 1,3-dibromopropane yields the monoarylation product **3**, which is used to alkylate 5-hydroxyisophthalic acid dimethyl ester. Ester reduction ($LiAlH_4$) and reaction of the resulting diol with PBr_3 affords dibromide **4**. Alkylation of dipicolylamine with **4** affords the structure of the ligand, which is finally transformed in the Zn complex by treatment with with $ZnClO_4$. All the steps are described to proceed in good to excellent yields, except for the first one which proceeds with a modest 18% yield, undermining the whole synthetic sequence.



Scheme 1. Reported synthesis of PyDPA according to *ref*[11]

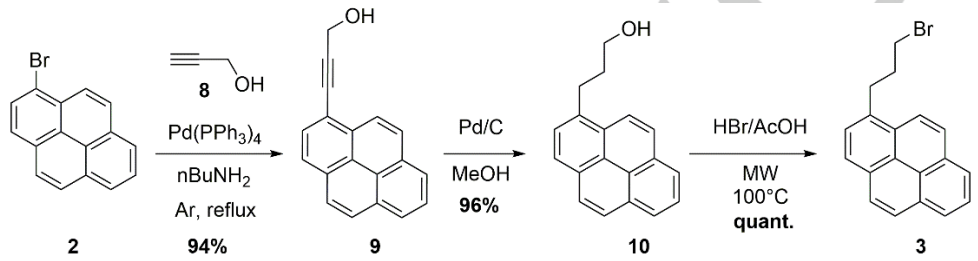
In order to improve on these results, we envisaged to exploit the reactivity of the C-Br bond of 1-bromopyrene **2** to perform a Pd-catalysed coupling reaction with dimethyl-5-propargyloxy-isophthalate **5** (Scheme 2), under Sonogashira conditions. Different Pd sources ($Pd(PPh_3)_2Cl_2$ or $Pd(PPh_3)_4$), in the presence or the absence of copper salts (CuI), different solvents (*e.g.* tertiary amines, THF, DMF) and a range of reaction temperatures (from 40 to 90°C) were explored without success. Indeed, the high activation temperatures required to activate the C-Br bond were not compatible with the thermal instability of the alkyne **5** and either no conversion of the starting material or alkyne decomposition was observed.

Using the more reactive 1-iodopyrene **6**,[15], allowed to obtain the desired product **7** under mild conditions, although in modest yield (42%), but it also introduced an additional step to the synthetic sequence, which reduced the overall yield to 35% (Scheme 2).



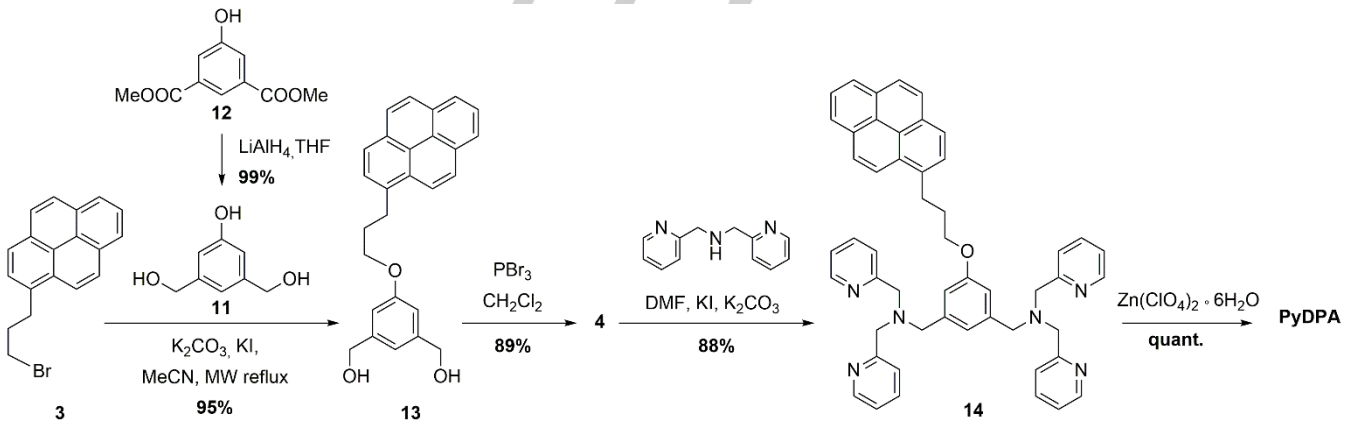
Scheme 2. Pd-mediated coupling of 1-Iodopyrene **6** with isophthalate **5**.

Finally, a sequential approach was adopted to introduce the alkynyl chain first and form the ether linkage at a later stage. Although classical Sonogashira conditions are described as effective for the reaction of propargylic alcohol **8** with 1-bromopyrene **2**[16], in our hands only copper-free conditions, using $Pd(PPh_3)_4$ as catalyst in *n*-$BuNH_2$ as solvent at reflux gave the desired product **9** in excellent yield (94%, Scheme 3).[17] It is worth noting that the same reaction conditions applied to phthalate **5** only produced alkyne decomposition and a mixture of unidentified by-products. Catalytic reduction of the triple bond of compound **9** has been described using PtO_2 (15 mol%) in THF[18] but we found that the much cheaper Pd(C) in methanol worked just as well with a lower catalyst loading (5 mol%) and shorter reaction times (20 minutes) with an overall dramatic decrease of the reaction cost. Finally, reaction of alcohol **10** with HBr/AcOH under MW irradiation as described in[19] with modifications, proceeded smoothly affording bromide **3** in quantitative yield (Scheme 3). Alternative conditions using PBr_3 for the functional group transformation provided lower yields and complicated the reaction work-up. Thus, compared to the reported procedure, we obtained the same intermediate **3** with 92% overall yield, as opposed to 18%. Two additional steps are required, but only one chromatographic purification is involved over the three steps.



Scheme 3. Optimized approach for the preparation of 1-(3-bromopropyl)pyrene **3.**

At this stage, to increase the convergence of the synthetic path and to skip the carboxymethylesters reduction step that was described to proceed in 68% yield (Scheme 1), triol **11** was used for the synthesis of ether **13**, exploiting the lower pKa of the phenol moiety (Scheme 4). The triol could be obtained in almost quantitative yield by reduction of the commercially available dimethyl-5-hydroxy-isophthalate **12** with $LiAlH_4$[19]. Alkylation of **11** with **3** was best performed using excess K_2CO_3 and 1 mol equiv of KI in refluxing acetonitrile. Under these conditions, ether **13** was isolated in 95% yield, after 17 h.



Scheme 4. Preparation of compound **13** via alkylation of phenol **11** with compound **3** and final steps of the synthesis of PyDPA.

The final steps of the synthesis were reproduced as previously described with comparable yields (Scheme 4). Diol **13** was therefore treated with PBr_3 to afford dibromide **4** (89%), which gave **14** upon reaction with excess bis(2-picolyl)amine (88%). Treatment of **14** with $Zn(ClO_4)_2 \cdot 6\,H_2O$ allowed to obtain chemosensor **PyDPA**, which was used for (p)ppGpp detection without further purification.

Spectral data

The spectral properties of **PyDPA**, including absorption spectra and fluorescence spectra in the presence of ppGpp were consistent with the data reported in the literature for the PyDPA chemosensor (Fig. 2).

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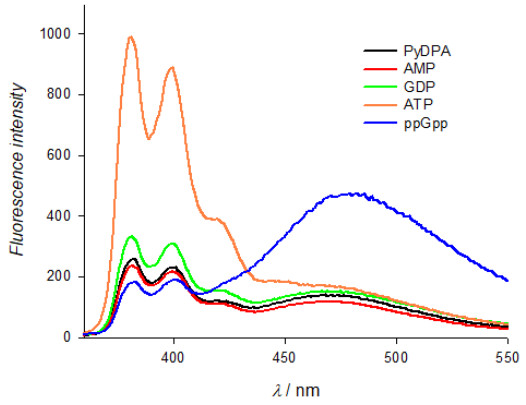

Figure 2. Fluorescence emission spectra of PyDPA (20 μM in 1 mM HEPES pH 7.5) alone and upon addition of each nucleotide and ppGpp (7 μM). Ex = 344 nm.

Indeed, while the presence of ppGpp led to the distinctive excimer emission band at 470nm, the presence of other nucleotides, such as AMP, GDP or ATP only showed the monomer emission bands.

Conclusions

In conclusion, we have been able to streamline the synthesis of the PyDPA chemosensor, overcoming the critical steps of the original synthetic approach and increasing the overall yield from 9% to 67% from the same starting material, 1-bromopyrene. We believe this approach will be easily reproducible and useful for the many groups that are investigating worldwide the biological activity of the still puzzling nucleotide signalling molecule (p)ppGpp.

Experimental Section

Chemicals were purchased by commercial sources and used without further purification, unless otherwise indicated. When anhydrous conditions were required, the reactions were performed under Nitrogen or Argon atmosphere. Anhydrous solvents were purchased from Merck. Reactions were monitored by analytical thin-layer chromatography (TLC) performed on Silica Gel 60 F254 plates (Merck) with UV detection (254 nm) and/or staining with ammonium molybdate acid solution, potassium permanganate alkaline solution. Silica gel 60 (40-63 µm) (Merck) was used for flash column chromatography. NMR experiments were recorded on a Bruker AVANCE-400 MHz instrument at 298 K. Chemical shifts (δ) are reported in ppm. The ^{1}H and ^{13}C NMR resonances of compounds were assigned with the assistance of COSY and HSQC experiments. Multiplicity are assigned as s (singlet), d (doublet), t (triplet), q (quartet), quint (quintet), m (multiplet). Mass spectra were recorded on Apex II ICR FTMS (ESI ionization-HRMS), Waters Micromass Q-TOF (ESI ionization-HRMS) or ThermoFischer LCQ apparatus (ESI ionization). Compound **11** was prepared as described in literature[19]

Synthesis of 3-Pyren-1-yl-prop-2-yn-1-ol (9): 1-Bromopyrene (**3**) (100 mg, 0.35 mmol) was dissolved with $Pd(PPh_3)_4$ (13 mg, 0.0105 mmol) in nBuNH$_2$ (12 mL) degassed with Ar. Propargyl alcohol (**8**) (102 µL, 1.78 mmol) was added to the mixture and the reaction was left stirring at reflux. After 3h the reaction was complete (TLC: 2:1 Hex:EtOAc). The solvent was evaporated at reduced pressure and the crude was purified by automated flash chromatography (Hex: EtOAc gradient from 92:8 to 40:60). Product **9** was obtained as a slightly yellow solid (85.1 mg, 95%). Spectral data matched those reported in literature.[17] ^{1}H-NMR (400MHz, $CDCl_3$): δ(ppm)= 8.55 (d, 1H, H_{3Ar}, ^{3}J=9.2Hz), 8.25-8.19 (m, 2H, H_{6Ar}, H_{8Ar}), 8.19-8.15 (m, 2H, H_{5Ar}, H_{9Ar}), 8.14-8.01 (m, 4H, H_{2Ar}, H_{4Ar}, H_{7Ar}, H_{10Ar}), 4.52 (d, 2H, CH_2OH, ^{3}J=6.5Hz), 1.82 (t, 1H. OH). ^{13}C-NMR (100MHz, $CDCl_3$): δ (ppm)= 132.2 (C_1), 131.5 (C_{5a}), 131.3 (C_{8a}), 131.1 (C_{3a}), 129.9 (C_{10a}), 128.5 (C_2), 128.4 (C_9), 127.3 (C_5), 126.4 (C_4), 126.2 (C_7), 126.9 ($C_{5a'}$), 126.7 ($C_{3a'}$), 125.7 (C_6), 125.5 (C_8), 124.5 (C_{10}), 124.4 (C_3), 92.9 ($\underline{C}$≡CH_2OH), 52.2 ($\underline{C}H_2OH$), 84.9 ($\underline{C}CH_2OH$). MS (ESI) *m/z*: calculated for $[C_{19}H_{12}ONa]^+$=279.08, found=279.28

Synthesis of 3-(pyren-1-yl)propan-1-ol (10): Compound **9** (184 mg, 0.72 mmol) was dissolved in freshly distilled MeOH (24 mL). Pd/C 10% (38 mg, 0.04 mmol) was added and the reaction mixture was stirred under hydrogen atmosphere (1 atm) for 20 minutes (TLC: 6:4 Hex:AcOEt). The catalyst was removed by filtration over celite and the solvent was evaporated at reduced pressure. Pure product **10** was obtained in 96% yield (179mg). Spectral data matched those reported in literature [20] ^{1}H-NMR (400MHz, $CDCl_3$): δ(ppm)= 8.32 (d, 1H, H_{3Ar}, ^{3}J=9.4Hz), 8.19-8.15 (dd, 2H, H_{6Ar}, H_{8Ar}, ^{3}J=7.5Hz), 8.14-8.09 (dd, 2H, H_{2Ar}, H_{9Ar}, ^{3}J=7.9Hz), 8.06-8.01 (m, 2H, H_{4Ar}, H_{5Ar}), 7.99 (t, 1H, H_{7Ar}), 7.90 (d, 1H, H_{10Ar}), 3.80 (t, 2H, $C\underline{H}_2OH$, ^{3}J=6.1Hz), 3.50-3.43 (m, 2H, $ArC\underline{H}_2$), 2.18-2.10 (m, 2H, $C\underline{H}_2CH_2OH$). ^{13}C-NMR (100MHz, $CDCl_3$): δ (ppm)= 136.3 (C_1), 131.6 (C_{5a}), 131.0 (C_{8a}), 131.1 (C_{3a}), 128.8 (C_{10a}), 127.7 (C_2), 127.6 (C_9), 127.5 (C_5), 127.4 (C_4), 126.2 (C_7), 125.4 ($C_{5a'}$), 125.3 ($C_{3a'}$), 125.1 (C_6), 125.0 (C_8), 124.9 (C_{10}), 123.4 (C_3), 62.6 ($\underline{C}H_2OH$), 34.7 ($Ar\underline{C}H_2$), 29.8 ($\underline{C}H_2$-CH_2OH). MS (ESI) *m/z*: calculated for $[C_{19}H_{16}ONa]^+$= 283.10; found: 282.96.

Synthesis of 1-(3-bromopropyl)pyrene (3): 33% HBr in AcOH (0.8 mL) was added to alcohol **10** (179 mg, 0.68 mmol) in a microwave vial. The reaction was stirred under MW irradiation at 100°C for 45' (TLC: 7:3 Hex:AcOEt). The reaction mixture was diluted with 15 mL of EtOAc and the organic phase was washed with 50% $NaHCO_3$ solution (3x15mL), water (1x15mL) and then dried over anhydrous Na_2SO_4. The solvent was evaporated at reduced pressure to yield pure product **3** as a brown viscous oil (218 mg, quant.). Spectral data matched those reported in literature.[20] ^{1}H-NMR (400MHz, $CDCl_3$): δ(ppm)= 8.29 (d, 1H, H_{3Ar}, ^{3}J=9.2Hz), 8.20-8.15 (dd, 2H, H_{6Ar}, H_{8Ar}, ^{3}J=7.6Hz), 8.15-8.10 (dd, 2H, H_{2Ar}, H_{9Ar}, ^{3}J=7.7Hz), 8.03 (s, 2H, H_{4Ar}, H_{5Ar}), 7.99 (t, 1H, H_{7Ar}), 7.90 (d, 1H, H_{10Ar}), 3.56-347 (m, 4H, $C\underline{H}_2OH$, $ArC\underline{H}_2$), 2.41 (qui, 2H, $C\underline{H}_2CH_2OH$, ^{3}J=6.9Hz). ^{13}C-NMR (100MHz, $CDCl_3$): δ (ppm)= 134.9 (C_1), 131.6 (C_{5a}), 131.3 (C_{3a}), 131.0 (C_{8a}), 128.8 (C_{10a}), 127.7 (C_2), 127.6 (C_9), 127.0 (C_5), 126.0 (C_4), 125.2 (C_7), 125.1 (C_6), 125.0 (C_8, $C_{5a'}$), 124.9 (C_{10}), 123.4 (C_3), 123.3 ($C_{3a'}$), 34.7 ($H_2\underline{C}$-CH_2Br), 33.6 ($\underline{C}H_2Br$), 31.8 ($Ar\underline{C}H_2$). MS (ESI) *m/z*: calculated for $[C_{19}H_{16}OBr]^+$= 323.04; found: 323.03.

Synthesis of (5-(3-(pyren-1-yl)propoxy)-1,3-phenylene)dimethanol (13): Compounds **3** (100 mg, 0.31 mmol), **11** (57.2 mg, 0.37 mmol), oven-dried K_2CO_3 (128 mg, 0.93 mmol) and KI (56 mg, 0.34 mmol) were dissolved in 2 mL of dry CH_3CN (0.15 M). The reaction mixture was stirred at reflux under microwave irradiation for 17h (TLC: 95:5 CH_2Cl_2:MeOH). The solvent was evaporated at reduced pressure and the resulting brown solid was dissolved in 15 mL of EtOAc. The solution was washed with water (3 x5 mL) and brine (1 x 15 mL). The organic phase was dried over anhydrous $MgSO_4$ and the solvent was evaporated at reduced pressure. The crude was purified by automated flash chromatography (95:5

CH_2Cl_2:MeOH) affording pure product **13** in 95% yield (116.3 mg). Spectral data matched those previously reported.[11] ^{1}H-NMR (400MHz, $CDCl_3$): δ(ppm)= 8.32 (d, 1H, H_{3Ar}, ^3J=9.6Hz), 8.17 (d, 2H, H_{6Ar}, H_{8Ar}, ^3J=7.6Hz), 8.13-8.07 (dd, 2H, H_{2Ar}, H_{9Ar}, ^3J=7.7Hz), 8.03 (s, 2H, H_{4Ar}, H_{5Ar}), 7.99 (t, 1H, H_{7Ar}), 7.90 (d, 1H, H_{10Ar}), 6.95 (s, 1H, H_p-Ph), 6.87 (s, 2H, H_o-Ph), 4.67 (s, 4H, CH_2OH), 4.07 (t, 2H, CH_2OPh, ^3J=6.1Hz), 3.56 (t, 2H, $ArCH_2$, ^3J=7.4Hz), 2.35 (quint, 2H, $ArCH_2CH_2$). ^{13}C-NMR (100MHz, $CDCl_3$): δ (ppm)= 159.7 (Cq-OCH_2), 142.9 (2x Cq), 135.9 (C_1), 131.6 (C_{5a}), 131.0 (C_{8a}), 130.1 (C_{3a}), 128.9 (C_{10a}), 127.6 (C_2), 127.5 (C_9), 127.4 (C_5), 126.8 (C_4), 126.0 (C_7), 125.3 ($C_{5a'}$), 125.1 ($C_{3a'}$), 125.0 (C_6, C_8), 124.9 (C_{10}), 123.5 (C_3), 117.6 (CH_p-Ph), 112.4 (2xCH_o-Ph), 67.1 (CH_2OPh), 65.3 (2xCH_2OH), 31.3 ($ArCH_2CH_2$), 29.9 ($ArCH_2$). MS (ESI) *m/z*: calculated for $[C_{27}H_{24}O_3Na]^+$= 419.16; found: 419.64.

Chemosensor PyDPA. ^{1}H-NMR (400MHz, CD_3CN): δ(ppm)= 8.76-8.72 (d, 4H, CH-*o*-Py, ^3J=5.3Hz), 8.37 (d, 1H, H_{3Ar}, ^3J=9.2Hz), 8.21-8.09 (m, 3H, H_{6Ar}, H_{8Ar}, H_{9Ar}), 8.07-7.90 (m, 9H, H_{2Ar}, H_{4Ar}, H_{5Ar}, H_{7Ar}, H_{10Ar}, CH-*p*-Py), 7.67 (t, 4H, CH-*m*-Py, ^3J=6.3Hz), 7.33 (d, 4H, CH-*m'*-Py, ^3J=7.8Hz), 6.73 (s, 2H, H_o-Ph), 6.71 (s, 1H, H_p-Ph), 4.16 (d, 4H, $PyCH_2N$, J_{gem}=16Hz), 4.02 (t, 2H, $ArCH_2CH_2CH_2O$, ^3J=5.5Hz), 3.82 (s, 4H, NCH_2Ph,), 3.64 (d, 4H, $PyCH_2N$), 3.61 (t, 2H, $ArCH_2CH_2CH_2O$, ^3J=7.3Hz), 2.36 (quint, 2H, $ArCH_2CH_2CH_2O$, ^3J=6.9Hz). ^{13}C-NMR (100MHz, CD_3CN): δ(ppm)= 160.4 (Cq-OCH_2), 155.4 (Cq Py), 149.1 (C_o Py), 143.0 (C_p Py), 137.3 (2x Cq Ph), 134.3 (C_1), 132.3 (C_{5a}), 131.8 (C_{8a}), 130.9 (C_{3a}), 129.8 (C_{10a}), 129.0 (C_2), 128.5 (C_9), 128.2 (C_5), 127.7 (C_4), 127.4 (2xCH_o-Ph), 127.2 (C_7), 126.4 (CH-*m*-Py), 126.1 (C_{10}), 126.0 (CH-*m'*-Py), 126.0 (C_6), 125.8 (C_8), 125.7 ($C_{5a'}$), 125.5 ($C_{3a'}$), 124.7 (C_3), 119.0 (CH_p-Ph), 68.0 ($ArCH_2CH_2CH_2OPh$), 56.9 ($PhCH_2N$), 55.8 ($PyCH_2N$), 31.9 ($ArCH_2CH_2CH_2OPh$), 29.8 ($ArCH_2CH_2CH_2OPh$). *m/z*: calculated for $[C_{51}H_{46}N_6OZn_2^{4+} + 3ClO_4^-]^+$ = $[M^{4+}+3ClO_4^-]^+$: 1187.07; found: 1187.28.

Acknowledgements

This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement No 758108)". We thank Alessio Maria Caramiello for technical assistance.

Keywords: (p)ppGpp • persisters • alarmone • fluorescent chemosensor • PyDPA

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