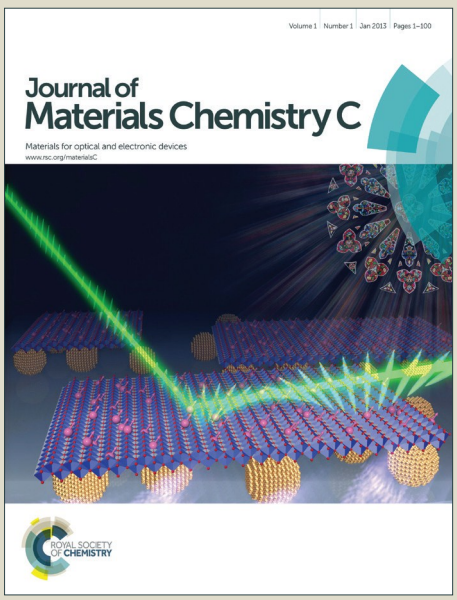
Journal of Materials Chemistry C

Accepted Manuscript



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Journal Name

ARTICLE

The modulation of fluorescent properties of diketopyrrolopyrroles via various electron-rich substituents

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Anna Purc,[a] Marzena Banasiewicz,[b] Eliza Glodkowska-Mrowka[c] and Daniel. T. Gryko*[a]

Four diketopyrrolopyrroles have been synthesized starting from heterocyclic aromatic nitriles. It was found that the negative influence of electron-donating groups on the reactivity of nitriles can be overcome by the presence of electron-deficient pyridine ring. The absorption and emission of diketopyrrolopyrroles and their *N*-substituted derivatives was evaluated in the range of solvents revealing that the exact position of electron-donating substituents significantly modulate their fluorescence response. The presence of dialkylamino moiety at position 3 of aryl substituents led to occurrence of very fast nonradiative deactivation processes. Formation of both anion (located on the core) and cation (located on pyridine ring) changes the relative energy of excited states leading to strong red fluorescence. On the other hand the presence of pyrrole moiety at position 4 of aryl substituents resulted in record high fluorescence quantum yield (0.88). The combination of two dialkylamino-pyridine moieties and oligoethylene glycol substituent made it possible to obtain compound possessing reasonable water-solubility, which was applied in fluorescence microscopy for selective staining of mitochondria in living cells.

Introduction

Chemistry of diketopyrrolopyrroles (DPPs) has gained a momentum since the advent of molecular electronics.[1] Indeed, the number of publications and patents on this scaffold increased 5-fold in years 2003 and 2014.[2] The revival of interest in last decade stems from the combination of their straightforward synthesis from aromatic nitriles and dialkyl succinates, and superb optical properties.[3] Still, in spite of this significant development, aromatic nitriles which have been utilized in DPPs' synthesis are surprisingly non-diversified.[4-6] Among heterocyclic nitriles, thiophene-2-carbonitryle is dominating the field[7] and derivatives of other scaffolds are only scarcely employed.[8] It is also well-known, that the presence of electron-donating groups, especially in para position to cyano group is problematic and leads to very low yields of DPPs.[1a,2] Numerous studies have shown that the replacement of benzene with aromatic five-membered rings at positions 2 and 6 for *N*,*N*-dialkylated DPPs decreases the dihedral angle from ca. 30 to ~7 degrees. This leads to significant changes in the optical properties including bathochromic shift of absorption,[9] better packing and also larger two-photon absorption cross-sections.[10] Yet the influence of the presence of six-membered heterocyclic rings has not been similarly explored. Surprisingly, despite widespread applications of DPPs no study exists that analyzes optical properties of these dyes possessing strongly electron-donating group at position 3 of aryl substituents. We decided to undertake a new study aimed at simultaneously investigating some new structural possibilities among diketopyrrolopyrroles and at the same time to obtain compounds possessing fluorescence highly sensitive to the environment.

Results and discussion

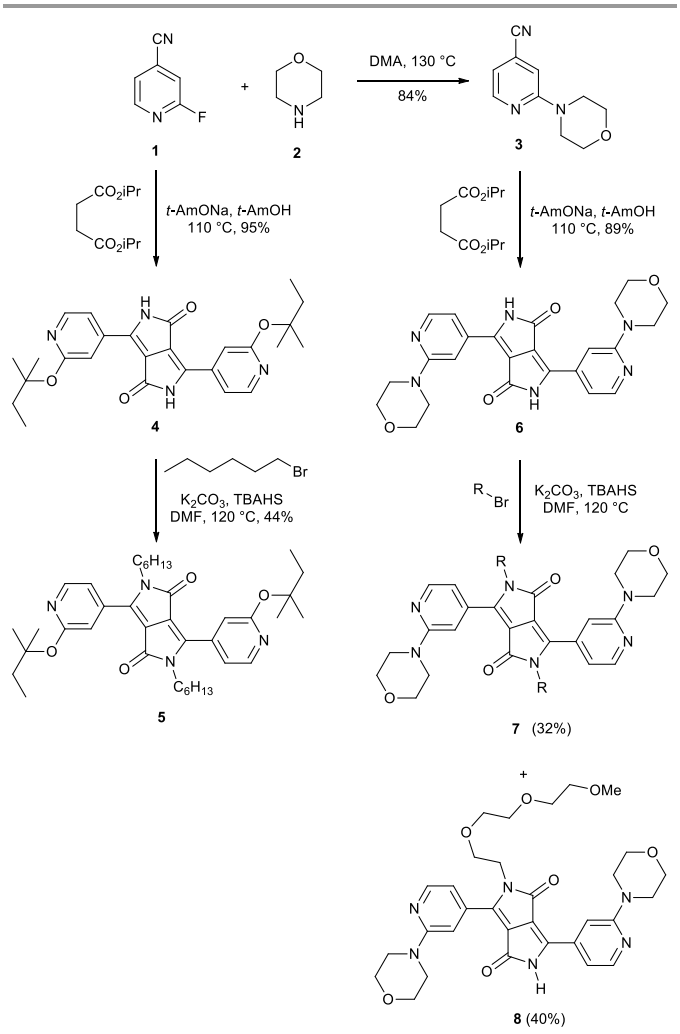
Design and synthesis

We designed a few nitriles which possess either electron-donating group and/or activating pyridine moiety. We would like to investigate the influence of both type and location of electron-rich substituent/ring on both reactivity of aromatic nitrile as well as on fluorescent properties of resulting DPPs and *N*-alkylated DPPs. The key element of design was to use, as electron-excessive moieties, both basic and non-basic functionalities. The reactivity of 2-fluoro-4-cyanopyridine (**1**) in nucleophilic aromatic substitution gave us the strategy to introduce tertiary amino group. Reaction of nitrile **1** with morpholine smoothly afforded nitrile **3**, which was

[a] Institute of Organic Chemistry, Polish Academy of Sciences, Kasprzaka 44/52, 01-224 Warsaw, Poland. E-mail: dtgryko@icho.edu.pl.
[b] Institute of Physics, Polish Academy of Sciences, Al. Lotników 32/46, 02-668 Warsaw (Poland).
[c] Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age
Medical University of Warsaw
Marszałkowska 24, 00-576 Warsaw (Poland).

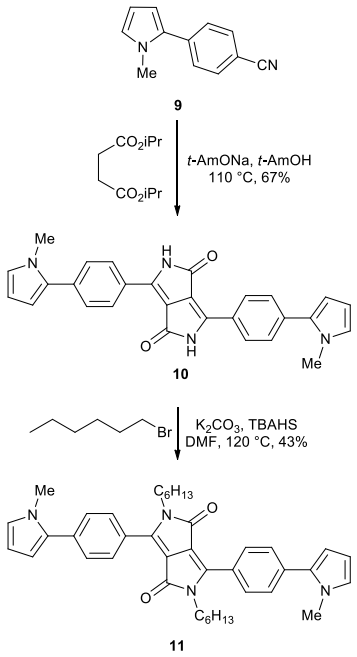
Electronic Supplementary Information (ESI) available: [absorption and emission spectra, copies of ^{1}H NMR and ^{13}C NMR spectra for compounds **4**-**8**, **10**, **11**, **13** and **14** as well as optical data]. See DOI: 10.1039/x0xx00000x

transformed into DPP **6** (Scheme 1). This result is in strong contrast to attempted synthesis of DPP from 3-*N*-morpholino-benzonitrile which failed to give any product.[2] Pigment **6** was alkylated with both lypophilic and hydrophilic alkylating agents (the latter one to achieve suitable water solubility). Interestingly, alkylation with *n*-hexyl bromide led to *N*,*N*'-bisalkylated compound **7** while analogous reaction with 12-bromo-2,4,7,10-tetraoxadodecane led to mono-alkylated product **8** (32% and 40% yields respectively). In parallel, the fact that fluorine at position 2 is highly activated towards nucleophilic aromatic substitution, inclined us to study the concomitant transformation of nitrile **1** under classical DPP-forming reaction conditions. It was discovered that in the presence of sodium *tert*-amylate, both reactions undergo simultaneously and diketopyrrolopyrrole **4** is the only isolable product (Scheme 1). The latter one was alkylated to give DPP **5**.

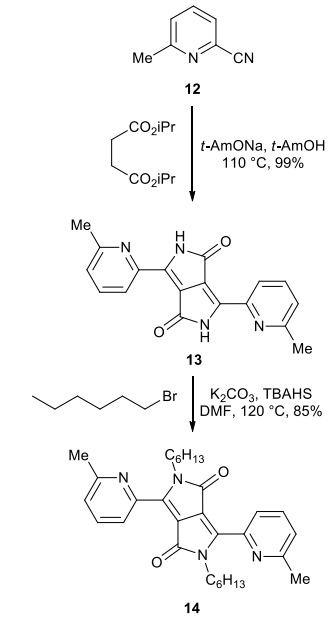


Scheme 1

The electron-donating effect of pyrrole, as substituent, located at *para*-position to reacting group is almost as strong as dialkylamino group.[11] Along these lines we obtained nitrile **9**[12] and we subjected it to reaction with diisopropyl succinate (Scheme 2). In comparison to analogous synthesis from 4-dimethylaminobenzonitrile (DPP yield = 4%),[1a] the expected dye **10** has been formed in surprisingly high yield (67%) and was smoothly alkylated with *n*-hexyl bromide to afford derivative **11**.



Scheme 2



Scheme 3

Finally, 6-methyl-2-cyanopyridine (**12**) seemed for us to be a perfect substrate to study the possibility to extend the core of DPPs via subsequent Knoevenagel reaction. The synthesis of corresponding DPP **13** occurred in almost quantitative yield (Scheme 3). Unfortunately all attempts to perform condensation with aromatic aldehydes using **13** or its *N*,*N*'-

dialkylated derivative **14** failed regardless the reaction conditions.

Optical properties

The photophysical properties were examined for compounds **4**-**8**, **10**, **11**, **13** and **14** and compared to those determined for other diketopyrrolopyrroles,[13] and structurally similar *N*,*N*-dialkylated diketopyrrolopyrroles[2] (Table 1, Figs. 1-5). All studied compounds can be divided into two families: diketopyrrolopyrroles, and *N*-alkyl- or *N*,*N*'-dialkyl-diketopyrrolopyrroles. Both absorption and emission spectra of DPPs lacking *N*-substituents were measured only in DMF and DMSO for solubility reason. Results gathered in Table 1 clearly show that the introduction of *N*-alkyl substituents always leads to hypsochromic shift of absorption. This is related to increase in dihedral angle between DPP core and aryl groups, hence lack of planarity of the chromophores. The same reasons are responsible for increase in Stokes shift while moving from **4** to **5** or from **10** to **11**. The most intriguing observation was the presence of additional, low-energy absorption and emission bands in case of dyes **4** and **6**. The presence of such bands in undoubtedly pure compounds can only be explained by the formation of corresponding anions in slightly basic DMF (unless DMF is freshly purified it always contains traces of Me_2NH). Such behaviour was already observed for DPPs in the presence of OH^- and even F^-.[15] Analogous bands are absent on spectra of compounds **10** and **13** (Table 1, ESI). This difference has an origin in the acidity of the diketopyrrolopyrroles, which is dependent of the aryl substituents. The special character of 2-(morpholino)pyridin-4-yl substituent (and to lesser extent 2-(*tert*-amyloxy)pyridin-4-yl substituent) makes compounds **4** and **6** more acidic.

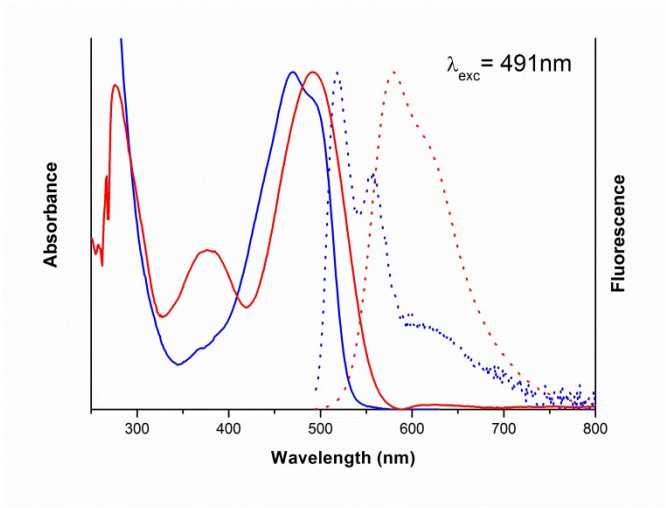


Figure 1. Normalized absorption (solid lines) and fluorescence (dotted lines, λ_{exc}= 491 nm) spectra of compound **8** (blue) and **11** (orange) in acetonitrile.

On the other hand the introduction of strongly electron-donating pyrrole moiety further increased push-pull character of parent dyes leading to a uniformly large bathochromic shift of absorption (~30-70 nm) and an even larger shift in fluorescence (60-70 nm) (compound **11**, Table 1, Fig. 1). While absorption of **11** is not comparably bathochromically shifted versus that of 2,5-dibutyl-1,4-diketo-3,6-di(4-piperidinophenyl)pyrrolo[3,4-*c*]pyrrole, the λ_{em} is on the same order (596 nm vs. 599 nm).

For compound **5** the absorption maxima shift hypsochromically while moving from non-polar solvent to polar solvents reaching 466 nm in MeOH (Fig. 2, Table 1). At the same time fluorescence is also slightly hypsochromically shifted. Given centrosymmetric nature of dyes, this is most probably related to difference in refractive index between solvents. Similar hypsochromic shift of absorption has been observed for remaining studied diketopyrrolopyrroles **5**, **7** and **14** (Table 1).

Table 1. Photophysical properties (in $CHCl_3$) determined for compounds **4**-**8**, **10**, **11**, **13** and **14** and their analog

Comp.	Solvent	λ_{abs}, nm	λ_{em}, nm	*Stokes shift* (cm^{-1})	(Φ_{fl})
4	DMF	511, 625	532, 650	770	-
	DMSO	514, 620	534	730	0.87[b]
5	toluene	482	549	2500	0.55[c]
	DCM	473	543	2700	0.49[c]
	acetonitrile	470	543	2700	0.52[c]
	1-butanol	472	542	2700	0.55[c]
	DMF	475	541	2600	0.51[a]
	methanol	466	544	3100	0.54[c]
6	DMF	514, 620	645	600	-
	DMSO	516, 617	575, 644	680	0.003[b]
7	toluene	480	561, 603	3000	0.025[c]
	DCM	473	549, 659	2900	<0.001[a]
	acetonitrile	466	519	2200	0.002[c]
	1-butanol	472	537	2900	<0.001[c]
	DMF	470	550	3100	-
	methanol	466	-	-	0[c]
8	toluene	475	524, 640	2000	0.014[b,c]
	DCM	471	519, 697	2000	<0.001[a]
	acetonitrile	470	518	2000	0.010[b]
	1-butanol	475	527	2100	0.001[b]
	DMF	476, 610	519, 664	1700	<0.001[a]
	methanol	472	528	2200	0.003[b]
10	DMF	546	575	900	0.88[b]
11	toluene	508	580	2400	0.93[b]
	DCM	498	556	2100	0.92[b]
	acetonitrile	491	580	3100	0.84[b]
	1-butanol	503	592	3000	0.51[b]
	DMF	504	594	3000	-
13	DMF	513	530	600	-
	DMSO	516	533	600	0.95[b]
14	toluene	533	558	800	0.88[b]
	DCM	524	557	1100	0.93[b]
	acetonitrile	517	548	1100	0.86[b]
	1-butanol	519	548	1000	0.84[b]
	DMF	524	556	1100	-
	DMSO	523	555	1100	0.90[b]
	methanol	507	546	1400	0.93[b]
PipDPP[d]	DMSO	536	599	2000	0.41

[a] Acridine orange as a reference. [b] Rhodamine 6G as a reference. [c] Coumarine 153 as a reference. [d] 2,5-Dibutyl-1,4-diketo-3,6-di(4-piperidinophenyl)pyrrolo[3,4-*c*]pyrrole, Ref. 14.

Strikingly different situation has been noticed for dye **8** possessing two strongly basic 2-(morpholine)pyridin-4-yl substituents.

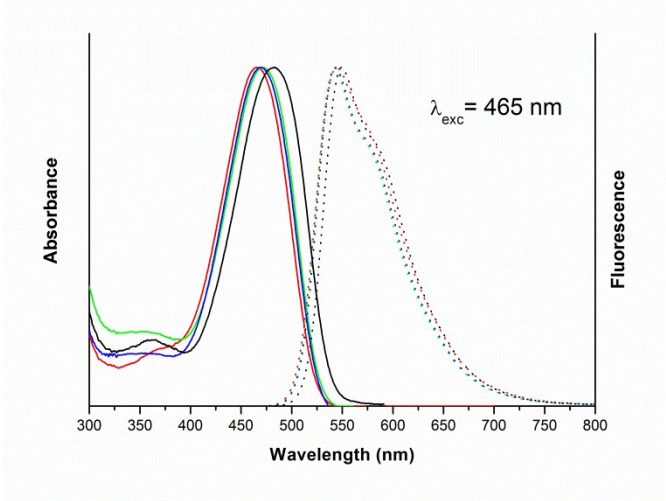


Figure 2. Normalized absorption (solid lines) and fluorescence (dotted lines, λ_{exc}= 465 nm) spectra of compound **5** in toluene (black line), acetonitrile (blue line), methanol (red line) and 1-buthanol (green line).

Its λ_{max} does not change differ significantly in toluene, MeCN, MeOH and 1-butanol. Interesting behavior has been observed for this compound in DMF. The absorption spectrum is consisted of 'normal band' and additional structured band with λ_{max} at 610 nm. In order to gain in-depth insight in this phenomenon we carried out titration experiments with $BnMe_3N^+OH^-$ (Fig. 3 and ESI). It turned out that both absorption and fluorescence spectra undergo marked changes due to the formation of anion **[8–H]**$^-$ (Scheme 4). In the presence of 35 equivs. of strong base compound **8** forms anion **[8–H]**$^-$ with λ_{max} which is almost identical with that of the band in DMF. Consequently, we have proved that the presence of small amount of dimethylamine in DMF is responsible for the formation of DPP-anion.

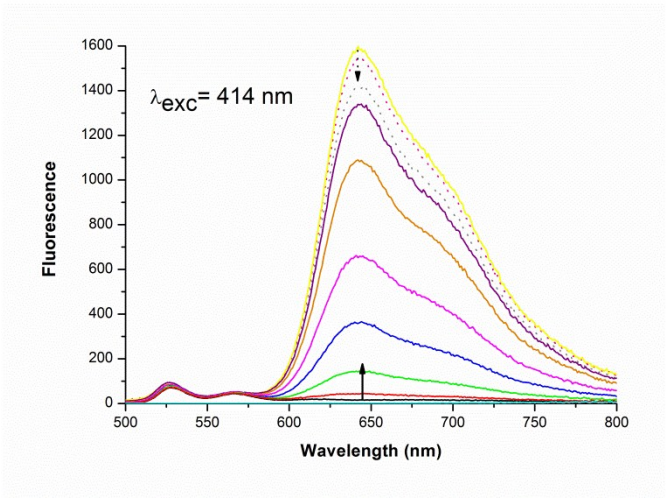
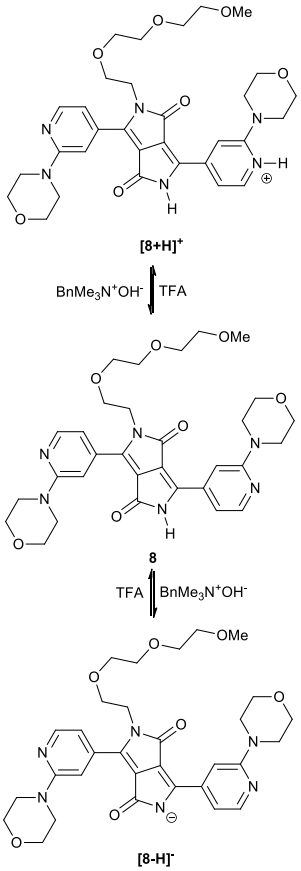


Figure 3. The change of fluorescence (λ_{exc}= 414nm) of compound **8** in DCM in presence of benzyltrimethylammonium hydroxide (BTMA). Solid lines show the fluorescence spectral titration from 0 (solid black line) to 35 equivalents of BTMA (solid yellow line), dotted lines show farther titration up to 50 equivalents of BTMA (dotted gray line).



Scheme 4. Protonation and deprotonation of diketopyrrolopyrrolo **8**.

The intensity of both absorption and fluorescence have increased and fluorescence quantum yield reached 0.35 in the presence of 35 equivs. of base.The excitation spectrum in DMF (Fig. 4) constitutes the final proof that low-energy band corresponds to the emission of anion. Excitation at 275 nm induces both weak fluorescence at 526 nm and strong one at 641 nm. On the other hand excitation at >540 nm results exclusively in fluorescence of DPP-anion. Similar effect can be observed in acidic conditions. After the addition of 0.5 eq. of trifluoroacetic acid (TFA) one can observe quick decrease in intensity of the band located at 530 nm combined with quick appearance of the new band at 601 nm (Fig. 5). Further acidification of the solution of dye **8** with TFA results in significant increase in fluorescence quantum yield to 0.20. The concomitant changes are noticeable in absorption spectrum. Band at 471 slowly disappears and the band located at double maximum at 517 and 539 nm increases (Fig. S1). Both these changes are undoubtedly corresponding to formation of cation **[8+H]**$^+$ *via* protonation of strongly basic 2-(morpholine)pyridin-4-yl substituents (Scheme 4). This is different behavior than described for corresponding 2,5-dibenzyl-1,4-diketo-3,6-di(4-morpholine-phenyl)pyrrolo[3,4-*c*]pyrrole which in the presence of acids fluorescence is decreasing and upon addition of more than 1250 equivalents the new bands which is hypsochromically shifted.[7k]

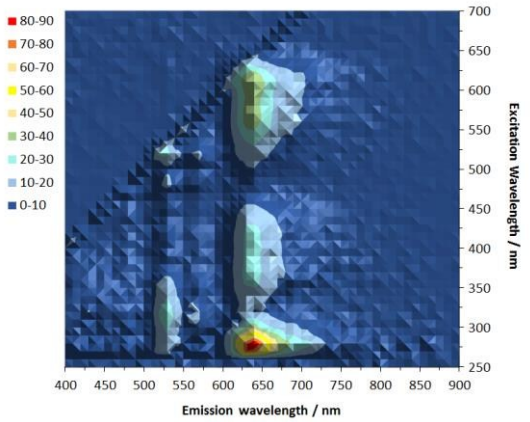


Figure 4. Excitation–emission–intensity maps of dye **8**. The photoluminescence intensity is reported in false color scale.

In the case of molecules which undergo internal rotation, the solvent viscosity has often an effect on fluorescence characteristics. In this context it is worth to emphasize that in case of dyes **5**, **7**, **8** and **14** prototypical viscous solvent *n*-butanol, has negligible influence on both absorption and emission (Table 1).

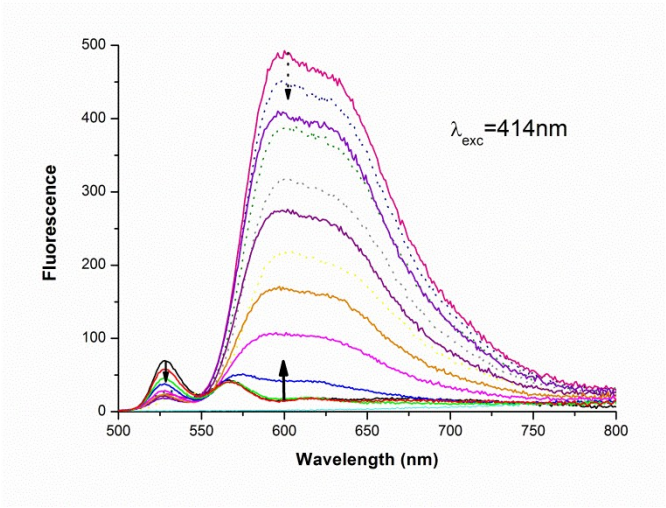


Figure 5. The change of fluorescence (λ_{exc}= 414nm) of compound **8** in DCM in presence of trifluoroacetic acid (TFA). Solid lines show the fluorescence spectral titration from 0 (solid black line) to 250 equivalents of TFA (solid pink line), dotted lines show farther titration up to 2000 equivalents of TFA (dotted yellow line).

Fluorescence quantum yields determined for dyes **6**-**8** were low or very low (Φ_{fl} = below 0.001-0.09) compared to classical DPPs.[2]

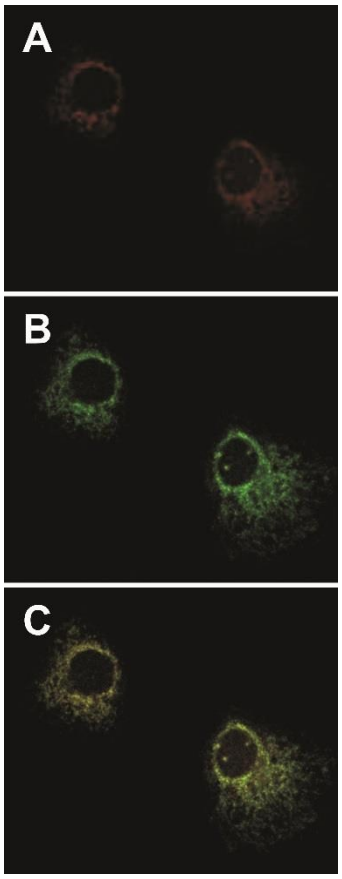


Figure 6. Confocal microscopy image of living human cervical cancer cells HeLa showing mitochondrial staining after 1 h incubation with 1 μM of dye **8** (A), and 250 nM MitoTracker Green (B). Panel C shows a merged image indicating co-localization of compound **8** with mitochondria.

Comparison of fluorescence of various DPPs within the Table 1, has clearly proven that neither the presence of dialkylamino group in position 4 of benzene ring nor the presence of pyridine ring decreases the fluorescence quantum yield of these dyes. The observed effect is clearly associated with the presence of dialkylamino group in position 3, which most probably causes efficient S_1-ICT→S_0 internal conversion (due to vibronic interactions between close lying excited states) similar to observed in the case of 6-aminocoumarin.[16]

To assess the utility of compound **8** in bioimaging, we stained living human cervical cancer cells HeLa with that compound and visualized by confocal microscopy. Co-localization experiments with MitoTracker Green (for mitochondria) have shown that compound **8**, at micro- and sub-micromolar concentrations, penetrate the cell membrane of living cells and localizes selectively in mitochondria (Figure 6). Given all optical properties of dye **8**, its structure, its weak fluorescence in the cell (visible on Fig. 6) as well as slightly basic environment in mitochondria (pH=7.8),[17] the only plausible explanation behind this phenomenon is formation of small amount of anion inside of mitochondria. In such case there is no real selectivity of staining but rather the dye is distributed evenly in the cell, while emission is only visible in organelle which has slightly basic pH.

Experimental

Synthetic procedures.

General.

All chemicals were used as received unless otherwise noted. All reported ^{1}H NMR and ^{13}C NMR spectra were recorded on 500 MHz spectrometer. Chemical shifts (δ ppm) were determined with TMS as the internal reference; J values are given in Hz. Chromatography was performed on silica (Kieselgel 60, 200-400 mesh).

General procedure for synthesis of DPP

Tert-amyl alcohol (8 ml), sodium (250 mg, 11 mmol) and a catalytic amount of $FeCl_3$ were placed in a three necked flask. The mixture was refluxed under an argon atmosphere until sodium has completely reacted. Then the reaction mixture was cooled to 90 °C and nitrile (5.0 mmol) was added. The mixture was then heated to 110 °C and diisopropyl succinate (0.37 ml, 2.2 mmol) was added dropwise. After 16h of reaction at 110 °C, the mixture was cooled to 40 °C and 20 ml of mixture water/methanol/acetic acid (1:1:1) was added. The resulting suspension was refluxed for a few minutes and cooled to room temperature. The precipitate of the obtained pigment was then filtered, washed several times with hot water and methanol and dried under vacuum.

1,4-Diketo-3,6-di(2-(*tert*-amyloxy)-4-pirydyl)pyrrolo[3,4-*c*]pyrrole (4). Prepared from 4-cyano-2-fluoropyridine (0.61 g, 5.0 mmol). Yield: 0.95 g (95%). Red powder. Molar absorption coefficient in DMF: 22 000 $M^{-1}cm^{-1}$. Mp: Decomposition > 260 °C δ_H (500 MHz, DMSO-d6) 11.44 (2H, s, N*H*), 8.33 (2H, d, *J* = 5.2 Hz, Ar-*H*), 7.91 (d, 2H, *J* = 5.2 Hz, Ar-*H*), 7.72 (2H, s, Ar-*H*), 1.97 (4H, q, *J* = 7.4, CH_2), 1.54 (12H, s, CH_3), 0.90 (6H, t, *J* = 7.4, CH_3). δ_C (126 MHz, DMSO-d6) 164.3, 162.1, 147.7, 136.7, 113.6, 113.2, 110.2, 82.1, 33.0, 25.8, 8.2. HRMS (ESI) calcd for $C_{26}H_{30}N_4O_4$ $(M+Na)^+$: 485.2165, found: 485.2162, Elemental analysis calcd (%) for $C_{26}H_{30}N_4O_4$: C 67.51, H 6.54, N 12.11; found: C 67.36, H 6.72, N 12.14.

1,4-Diketo-3,6-di(2-morpholino-4-pirydyl)pyrrolo[3,4-*c*]pyrrole (6). Prepared from 2-(4-morpholinyl)-4-cyanopyridine (0.95 g, 5 mmol). Yield: 1.00 g (88%). Red powder. Molar absorption coefficient in DMF: 18 000 $M^{-1}cm^{-1}$. Mp: Decomposition > 360 °C, δ_H (500 MHz, $CDCl_3$: TFA 4:1) 11.66 (2H, s, CON*H*), 8.47 (2H, s, Ar-*H*), 8.13 (s, 2H, Ar-*H*), 7.42 (2H, d, *J* = 4,5, Ar-*H*), 4.14 (4H, s, CH_2), 3.92 (2H, s, CH_2). δ_C (126 MHz, $CDCl_3$: TFA 4:1) 163.4, 152.3, 143.7, 139.7, 137.8, 115.8, 112.5, 109.3, 65.3, 45.8. HRMS (EI) calcd for $C_{24}H_{25}N_6O_4$ $(M+H)^+$: 461.1937, found: 461.1930, Elemental analysis calcd (%) for $C_{24}H_{24}N_6O_4$: C 62.60, H 5.25, N 18.25; found: C 62.70, H 5.32, N 18,24.

1,4-Diketo-3,6-di(4-(1-methyl-1H-pyrrol-2-yl)phenyl)pyrrolo[3,4-c]pyrrole (10). Prepared from 4-(1-methyl-1H-pyrrol-2-yl)benzonitrile (0.91 g, 5 mmol). Yield: 0.65 g (67%). Dark red powder. Molar absorption coefficient: 35 000 $M^{-1}cm^{-1}$. Mp: Decomposition > 330 °C. The NMR spectra of the product could not be obtained due to the low solubility. HRMS (EI) calcd for $C_{28}H_{22}N_4O_2$ (M+): 446.1743, found: 446.1751, Elemental analysis calcd (%) for $C_{28}H_{22}N_4O_2$: C 75.32, H 4.97, N 12.55; found: C 75.14, H 5.12, N 12.41.

1,4-Diketo-3,6-di(6-methyl-pyridin-2-yl)pyrrolo[3,4-*c*]pyrrole (13). Prepared from 2-cyano-6-methylpyridine (0.65 g, 5 mmol). Yield: 0.69g (99%). Red powder. Molar absorption coefficient: 29 000 $M^{-1}cm^{-1}$. Mp: Decomposition > 370 °C. The NMR spectra of the product could not be obtained due to the low solubility. HRMS (EI) calcd for $C_{18}H_{14}N_4O_2$ (M+): 318.1117, found: 318.1125, Elemental analysis calcd (%) for $C_{18}H_{14}N_4O_2$: C 67.91, H 4.43, N 17.60; found: C 67.66, H 4.44, N 17.48.

General method of alkylation of DPP (5, 7, 8, 11, 14) synthesis

A mixture of DPP (1 mmol), tetrabutylammoniumbisulfate (TBAHS, 17 mg, 0.05 mmol), K_2CO_3 (2.07 g, 15 mmol) and DMF (25 ml) were heated to 120 °C under an argon atmosphere. Then *n*-bromohexane (1.4 ml, 10 mmol) was added dropwise via a syringe. The reaction mixture was stirred overnight, then cooled and diluted with water and CH_2Cl_2. The aqueous layer was extracted with CH_2Cl_2, combined organic layers were washed with water and brine and dried over $MgSO_4$. Solvents were evaporated, the product was separated by the column chromatography in given eluting system.

2,5-Dihexyl-1,4-diketo-3,6-di(2-(*tert*-amyloxy)-4-pirydyl)pyrrolo[3,4-*c*]pyrrole (5) Prepared from **4** (0.33 g, 1 mmol). Purified by the column chromatography (methylene chloride → methylene chloride:methanol 98:2) and crystallization from cyclohexane : methylene chloride. Yield: 0.25 g (44%). Orange crystals. Molar absorption coefficient in DMF: 15 000 $M^{-1}cm^{-1}$; in DCM: 15 000 $M^{-1}cm^{-1}$. Mp: 144-145 °C. δ_H (500 MHz, $CDCl_3$) 8.29 (2H, d, *J* = 5.2, Ar-*H*), 7.18 (2H, dd, J_1 = 5.2, J_2 = 1.4, Ar-*H*), 6.95 (s, 2H, Ar-*H*), 3.73-3.79 (4H, m, CONCH_2), 1.97 (4H, q, *J* = 7.5, CH_2), 1.59-1.53 (18H, m, CH_2+H_2O) 1.26-1.20 (12H, m, CH_3), 0.94 (6H, t, *J* = 7.5, CH_3), 0.83 (6H, t, *J* = 6.8, CH3), δ_C (126 MHz, $CDCl_3$) 164.6, 162.0, 147.6, 137.4, 114.6, 111.8, 110.7, 82.9, 41.9, 33.8, 31.1, 29.4, 26.2, 26.0, 22.4, 13.9, 8.4. HRMS (ESI) calcd for $C_{38}H_{54}N_4O_4Na$ $(M+Na)^+$: 653.4043, found: 653.4047, Elemental analysis calcd (%) for $C_{38}H_{54}N_4O_4$: C 72.35, H 8.63, N 8.88; found: C 72.53, H 8.51, N 8.67.

2,5-Dihexyl-1,4-diketo-3,6-di(2-morpholino-4-pirydyl)pyrrolo[3,4-*c*]pyrrole (7) Prepared from **6** (0.46 g, 1 mmol). Purified by the column chromatography (methylene chloride → methylene chloride: THF 5:2) and crystallization from cyclohexane: methylene chloride. Yield: 0.20 g (32%). Orange crystals. Molar absorption coefficient in DMF: 16 000 $M^{-1}cm^{-1}$; in DCM: 16 000 $M^{-1}cm^{-1}$. Mp: 178-179 °C, δ_H (500 MHz, $CDCl_3$) 8.34 (2H, d, *J* = 5.3, Ar-*H*), 7.25 (2H, s, Ar-*H*), 6.82 (2H, dd, J_1 = 5.1, J_2 = 0.9),

3.88-3.85 (8H, m, OCH_2CH$_2$N), 3.77-3.73 (4H, m, CONCH_2), 3.65-3.61 (8H, m, OCH$_2$CH_2N), 1.60-1.53 (4H, m, CH_2), 1.26-1.20 (12H, m, CH_2), 0.84 (6H, t, *J* = 6.8, CH_3), δ_C (126 MHz, $CDCl_3$) 162.2, 159.9, 148.6, 148.5, 147.5, 136.6, 110.8, 110.5, 106.7, 66.7, 45.4, 42.1, 31.2, 29.5, 26.3, 22.5, 13.9, HRMS (EI) calcd for $C_{36}H_{49}N_6O_4$ $(M+H)^+$: 629.3815, found: 629.3814, Elemental analysis calcd (%) for $C_{36}H_{48}N_6O_4$: C 68.76, H 7.69, N 13.37; found: C 68.63, H 7.80, N 13.24.

2-(2-(2-(2-(methoxy)ethoxy)ethoxy)ethyl)-1,4-diketo-3,6-di(2-morpholino-4-pirydyl)pyrrolo[3,4-*c*]pyrrole (8) Prepared from **6** (0.46 g, 1 mmol). Purified by the column chromatography (methylene chloride → methylene chloride: methanol 95:5) and crystallization from methylene chloride:methanol. Yield: 0.24 g (40%). Red powder. Molar absorption coefficient in DMF: 19 000 $M^{-1}cm^{-1}$; in DCM: 21 000 $M^{-1}cm^{-1}$. Mp: 167-168 °C, δ_H (500 MHz, $CDCl_3$) 9.99 (1H, s, CON*H*), 8.36 (1H, d, *J*=4.9, Ar-H), 8.31 (1H, d, *J* = 4.7, Ar-*H*), 7.93 (1H, s, Ar-*H*), 7.31 (1H, s, Ar-*H*), 7.15 (1H, d, *J* =4.6, Ar-*H*), 7.08 (1H, d, J = 4.5, Ar-*H*), 3.99 (2H, s, CONCH_2), 3.80 (8H, br. s, OCH_2CH$_2$N), 3.63-3.54 (16H, m, NCH_2CH$_2$O + OCH_2CH_2O), 3.49-3.45 (2H, m, CONCH$_2$CH_2O), 3.31 (3H, s, OCH_3), δ_C (126 MHz, $CDCl_3$) 163.1, 162.4, 160.4, 160.0, 149.1, 148.8, 148.6, 144.5, 136.1, 135.1, 112.0, 111.8, 111.3, 108.8, 106.9, 105.6, 77.5, 77.4, 71.8, 70.7, 70.6, 70.4, 68.8, 66.7, 66.6, 45.5, 45.3, HRMS (ESI) calcd for $C_{31}H_{39}N_6O_7$ $(M+H)^+$: 607.2880, found: 607.2883, Elemental analysis calcd (%) for $C_{31}H_{39}N_6O_7$: C 61.37, H 6.31, N 13.85; found: C 60.72, H 6.29, N 13.35.

2,5-Dihexyl-1,4-diketo-3,6-di(4-(1-methyl-1H-pyrrol-2-yl)phenyl)pyrrolo[3,4-*c*]pyrrole (11) Prepared from **10** (0.45 g, 1 mmol). Purified by the column chromatography (methylene chloride: hexanes 3:2) and crystallization from methylene chloride:cyclohexane. Yield: 0.26 g (43%). Purple crystals. Molar absorption coefficient in DMF: 32 000 $M^{-1}cm^{-1}$; in DCM: 35 000 $M^{-1}cm^{-1}$. Mp: 136-137 °C, δ_H (500 MHz, $CDCl_3$) 7.89 (4H, d, J = 8.3, Ph-H), 7.57 (4H, d, J = 8.3, Ph-H), 6.77 (2H, s, Py-H), 6.38-6.35 (2H, m, Py-H), 6.23 (2H, t, J = 3.0, Py-H), 3.83-3.78 (4H, m, $CONH_2$), 3.75 (6H, s, NCH_3), 1.70-1.62 (4H, m, CH_2), 1.31-1.21 (12H, m, CH_2), 8.32 (6H, t, J = 6.7, CH_3)). δ_C (126 MHz, $CDCl_3$) 162.9, 147.9, 136.0, 133.6, 128.9, 128.2, 126.1, 125.1, 110,1, 109.8, 108.3, 42.2, 35.5, 31.2, 29.5, 26.4, 22.5, 14.0. HRMS (ESI) calcd for $C_{40}H_{42}N_4O_2$ (M+): 615.3699, found: 615.3697, Elemental analysis calcd (%) for $C_{40}H_{42}N_4O_2$: C 78.14, H 7.54, N 9.11; found: C 78.14, H 7.48, N 8.95.

2,5-Dihexyl-1,4-diketo-3,6-di(6-methyl-pyridin-2-yl)pyrrolo[3,4-*c*]pyrrole (14) Prepared from **13** (0.32 g, 1 mmol). Purified by the column chromatography (methylene chloride → methylene chloride : methanol 98:2) and crystallization from cyclohexane : methylene chloride. Yield: 0.41 g (85%). Orange crystals. Molar absorption coefficient in DMF: 14 000 $M^{-1}cm^{-1}$; in DCM: 21 000 $M^{-1}cm^{-1}$.Mp: Decomposition > 130 °C. δ_H (500 MHz, $CDCl_3$) 8.82 (2H, d, *J* = 7.9, Ar-*H*), 7.78 (2H, t, *J* = 7.8, Ar-*H*), 7.21 (2H, d, *J* = 7.6, Ar-*H*), 4.34-4.31 (4H, m, CONCH_2), 2.60 (6H, s, CH_3), 1.71-1.65 (4H, m, CH_2) 1.37-1.25 (12H, m, CH_2), 0.86 (6H, t, J = 7.1, CH_3), δ_C (126 MHz, $CDCl_3$) 162.5, 157.9, 147.0, 145.6, 137.1, 124,5, 124.4, 110.9, 42,8, 31.6, 29.9, 26.6, 24.4, 22.6, 14.0. HRMS (EI) calcd for $C_{30}H_{38}N_4O_2$ (M+): 486.2995, found: 486.3003, Elemental analysis calcd (%) for $C_{30}H_{38}N_4O_2$: C 74.04, H 7.87, N 11.51; found: C 74.14, H 7.84, N 11.49.

Optical studies. Absorption and fluorescence spectra were measured at room temperature using a PerkinElmer UV/VIS Spectrometer Lambda 25 and Hitachi F-7000 fluorescence spectrometer, respectively. Fluorescence quantum yields were measured by using acridine orange, rhodamine 6G or coumarine 153 in ethanol as a standard.

Cells and microscopy.

For microscopic and cytotoxic experiments, human cervical cancer cells HeLa were cultured in DMEM supplemented with 10% (v/v) FBS (both from Life Technologies) and 1% antibiotic/antimycotic solution (Sigma Aldrich), and maintained in humidified 5% CO_2 atmosphere at 37 °C. The cells were seeded onto 35 mm Petri dishes ($50x10^4$ cells/dish), allowed to attached overnight and further incubated at 37 °C in the dark for 1 hour in the presence or absence of **8** [1 μM in DMSO] and/or MitoTracker Green (Life Technologies) [250 nM in DMSO]. Next, the cells were washed twice with PBS, and culture medium was replaced with dye-free FluoroBrite DMEM supplemented with 10% (v/v) FBS (both from Invitrogen) and 1% antibiotic/ antimycotic solution (Sigma Aldrich) for further analysis. The images were collected using confocal microscope (Zeiss LSM 7 MP with upright Axio Examiner.Z1) and a mode-locked Ti: sapphire laser, tunable from 690 to 1050 nm (Coherent Chameleon) equipped with W Plan-Apochromat 20x/1.0 objective. The excitation wavelength was 500 nm and emission signal was separated and collected on NDD Bi-GaAsP detectors (emission wavelength range of 600-700 nm for the first channel (for compound **8**) and 500-550 nm for the second channel (for MitoTracker Green).

Conclusions

The presence of tertiary amino functionality in *meta*-position to CN group allows to synthesize diketopyrrolopyrroles as long as reacting nitrile is a derivative of pyridine. The placement of electron-donating groups at position 3 of aryl substituents dramatically alters DPPs' photophysics. DPP possessing two (2-morpholine)pyridin-4-yl substituents have negligible emission, which increases after protonation and deprotonation. Deprotonation is probably responsible for selective visualization of mitochondria *via* fluorescence microscopy with this non-fluorescent compound. Acidity of diketopyrrolopyrroles possessing electron-donating groups at position 3 of aryl substituents is higher than typically for this family of dyes so that anions are formed even in not freshly distilled DMF.

Acknowledgements

The work was financially supported by Polish National Science Centre (grant MAESTRO-2012/06/A/ST5/00216).

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Graphical abstract:

Electron-donating functionalities allow to modulate emission properties of diketopyrrolopyrroles.