Introduction of the Functional Amino Group at the *meso* Position of Cy3 and Cy5 Dyes: Synthesis, Stability, Spectra and Photolysis of 4-Amino-1-diazo-2-butanone Derivatives**

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Searching for photoconvertible or photoactivatable dyes, we considered trimethine (Cy3) and pentamethine (Cy5) fluorophores with a 4-amino-1-diazo-2-butanone fragment (H_2N-$(CH_2)_2COCH{=}N_2$) attached to the *meso* (γ) position of the polymethine chain via the amino group. The Cy3 derivative was prepared. All Cy5 derivatives with the basic amino group at this position decomposed with breaking the polymethine chain. However, amino-squaraine based Cy5 dyes were found to be stable and accessible. Photolysis of Cy3 and squaraine-based Cy5 dyes with a 4-amino-1-diazo-2-butanone fragment was accompanied with Wolff rearrangement and led to pyrrolidones (in methanol and aprotic solvents) formed via intramolecular cyclization of the intermediate ketenes. In aqueous acetonitrile, ketenes reacted with water and gave carboxylic acids. Both products were non-fluorescent, though the model Cy5 dyes (without the squaraine fragment) with acylated amino and *N*-methylamino groups were found to be fluorescent.

Introduction

The ability to induce transitions between non-fluorescent (dark) and fluorescent (bright) molecular states forms the basis of the modern superresolution microscopy.[1] For image acquisition, the fluorescent markers (as single emitters or ensembles) are temporary brought into a bright state for registration, while all adjacent ones are kept in a dark state. The transitions into the bright state may occur spontaneously (blinking), or can be induced photochemically; for example, by irreversible photoactivation or reversible photoswitching between the dark and bright states of the molecule.[1]

A prominent class of blinking dyes is represented by carbocyanines; in particular, pentamethinecyanines, like Cy5, sulfo-Cy5, or Alexa 647.[2] When Cy5 dyes mixed with thiols and oxygen scavengers are irradiated with strong pulses of red light, they form the non-fluorescent products.[2d] Under these conditions, thiols add to the double carbon-carbon bond of the pentamethine chain. The products of addition are unstable and, as single molecules, return to initial fluorescent species upon elimination of thiols.

Further progress in development of the fluorescent markers for superresolution microscopy and molecular tracking is based on the introduction of photoactivatable functional groups into the dye core.[3a] As candidates for photoconvertible dyes, we considered cyanines with 4-amino-1-diazo-2-butanone[3b] fragment ($H_2N(CH_2)_2COCH{=}N_2$) attached to polymethine chain via the amino group. Upon irradiation with UV light, diazoketones eliminate nitrogen, and the intermediate acylcarbenes undergo the Wolff rearrangement and, in the presence of nucleophiles, form carboxylic acid derivatives.[3c] These transformations can be represented as follows [Eq. (1)]:

$$\begin{aligned} &RCOCH{=}N_2 \rightarrow RCOCH :{\rightarrow} \\ &RCH{=}C{=}O\ (+H_2O) \rightarrow RCH_2COOH \end{aligned} \quad (1)$$

If the diazoketone group is separated from the amino group by two methylene residues, the intermediate ketene is expected to spontaneously cyclize to 5-membered pyrrolidone (Scheme 1). This kind of intramolecular amidation (unrelated to Wolff rearrangement) was reported for a Cy7 dye.[4] It was accompanied by a red shift (+174 nm) and significant increase in emission at 807 nm. The symmetrical amino-modified Cy7 dyes were obtained from the corresponding halogenated derivatives (Cl, Br) by nucleophilic substitution with amines.[5] However, the emission of Cy7 dyes in the IR region does not quite fit the common detection window of the current nanoscopy techniques (650–750 nm).[1]

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[**] *Cy3 = trimethinecyanine; Cy5 = pentamethinecyanine.*

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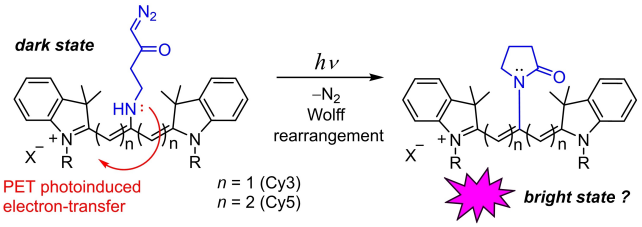


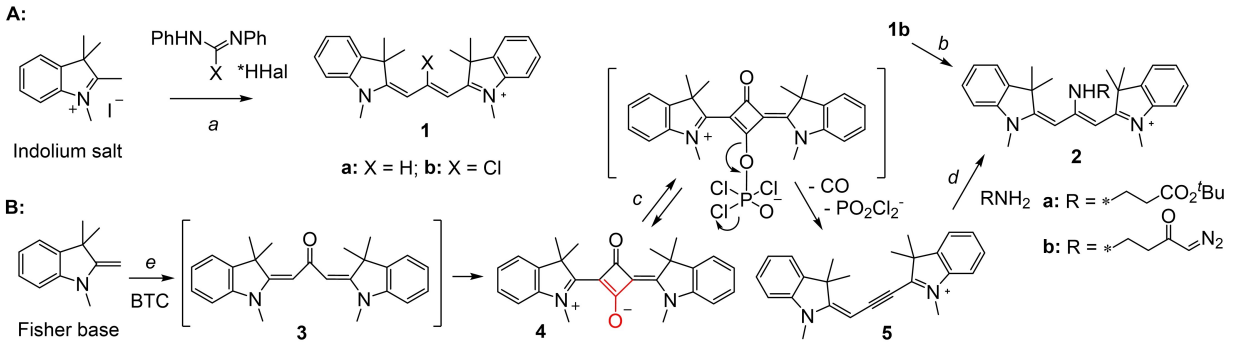
Scheme 1. The main concept for fluorescence uncaging of the present study, applied to Cy3 and Cy5 dyes.

Therefore, as primary synthetic goals, we have chosen amino-modified Cy3 and Cy5 dyes (Scheme 1) with estimated emission maxima at 550 nm and 660 nm, respectively (upon amidation). The substituent at the *meso* position of the chromophore may reduce photobleaching and increase the fluorescence quantum yield.[6] Thus, the introduction of (ω-aminoalkyl)diazoketone moiety and amino group alone to the *meso* position of the chromophore was interesting and challenging. The same, as for amino substituted Cy7 dyes,[4] we expected the initial compounds in Scheme 1 to be non-fluorescent due to photoinduced electron transfer (PET).[2] The electron pair of the amide/lactam should have a lower reduction potential (as it is partially conjugated with the carbonyl), and thus the quenching is expected to disappear upon irradiation and subsequent amidation.

Results and Discussion

Trimethinecyanines (Cy3)

The most straightforward route to Cy3 dyes with an amino group at the *meso* position (Scheme 1, $n = 1$; compounds **2a**,**b** in Scheme 2) is based on the nucleophilic substitution of the halogen atom. This reaction is widely used for Cy7 dyes.[5] It proceeds most probably as addition – elimination sequence. We decided to apply this method to Cy3 dyes. The synthesis of unsubstituted Cy3 dye **1a** includes the condensation of a formamidinium salt with 1,2,3,3-tetraalkyl-3*H*-indolium salts (A, Scheme 2).[7] It was found that a chlorine atom cannot be introduced to the *meso* position of Cy3 chromophore via route A. The reaction of *N,N'*-diphenylchloroformamidinium hydrochloride[8] with indolium iodide did not lead to linear cyanine **1b** (Scheme 2), but gave several side products including trinuclear cyanines instead (See Supporting Information, Scheme SI-1 for details). The substitution of halogen X in the formamidinium salt is possible, if external nucleophiles (amines or thiols) are used. However, the products in the reactions with indolium salts – guanidines or isothioureas – also gave trinuclear cyanines as main components of complex reaction mixtures (Scheme SI-1, Figure SI-1). Since not only halogen atoms, but also other leaving groups at the *meso* position of Cy3 dyes are prone to substitution, we had to find another way. Coenen studied the reactions of methylene bases (like Fisher base in Scheme 2) with isocyanates, phosgene, and other electrophiles.[9] The use of isocyanates R–N=C=O, might help to introduce an amino group to the *meso* position of Cy3 in two steps (Scheme SI-2), but the small number of functionally substituted isocyanates limits the scope of this reaction. However, phosgene and triphosgene[10] condense with active methylene bases. One equivalent of phosgene reacts with 2 equivalents of Fisher base to give an unsaturated ketone **3** (B, Scheme 2).[11,12] It was mentioned that ketone **3** and $POCl_3$ gave compound **1b** – a cyanine with chlorine atom at the *meso* position, that undergoes the reaction with primary or secondary amines to give *meso* substituted amino cyanines **2** (Scheme 2).[11] On the other hand, acetylenic dye **5** was suggested as an intermediate in the transformation of ketone **3** to amine **2**.[12] The formation of acetylenic analogues of cyanine dyes either from ketones or chlorides depends on the structure of the dye.[13] Surprisingly, in our hands, the reaction of triphosgene with Fisher base gave squaraine **4** as a final product (even if the excess of base was used; see Scheme SI-3 for details). Ketone **3** was registered in the reaction mixture only as an intermediate (NMR, LCMS). Remarkably, compound **4** reacts with $POCl_3$ and gives the required acetylenic carbocyanine **5**. The plausible mechanism includes phosphorylation followed by the loss of CO and elimination of good leaving groups (Scheme 2). We intro-



Scheme 2. A: Common short route to Cy3 scaffold; **B**: Alternative Cy3 synthesis. The desired compound **1b** was not detected. Compound **3** was reported in Ref. [12]. Reagents and conditions: a) Ac_2O/AcOH 1:1, 120 °C; b) $CHCl_3$, RNH_2 (Ref. [11]); c) BTC=$Cl_3COCOOCCl_3$ (triphosgene), Et_3N, THF, 0 °C – r.t.; d) $POCl_3$, Et_3N, 1,4-dioxane, 80 °C; e) K_2CO_3, THF, r.t. Counterions are omitted (undefined).

duced compound **5** in the reaction with β-alanine *t*-butyl ester and obtained aminoester **2a**. It showed a significant hypsochromic shift compared to unsubstituted Cy3 and a very weak fluorescence (Table 1), which confirmed the initial assumption. The target compound **2b** was obtained by direct addition of freshly prepared 4-amino-1-diazobutan-2-one[3b] (the new synthesis is given in Supporting Information) to compound **5** (Scheme 2).

Table 1. Photophysical properties of **Cy3** derivatives in MeCN.

Dye	λ_{abs} [nm]	λ_{em} [nm]	ε,[a] [$M^{-1}\,cm^{-1}$]	Φ_{fl}[b]
1a	545	560	90 000	0.10
2a	458	–	65 000	0
2b	458	–	65 000	0
4	473	483	66 000	0.01[c]; 0.04[d]
5	516	–	84 000	0
6	535[c]	–	–	0[c]
7	458[e]	–	–	0[e]

[a] Molar extinction coefficient; [b] Absolute values of the fluorescence quantum yields; [c] In MeOH; [d] In dioxane; [e] In MeCN-buffer mixture (4:1 v/v).

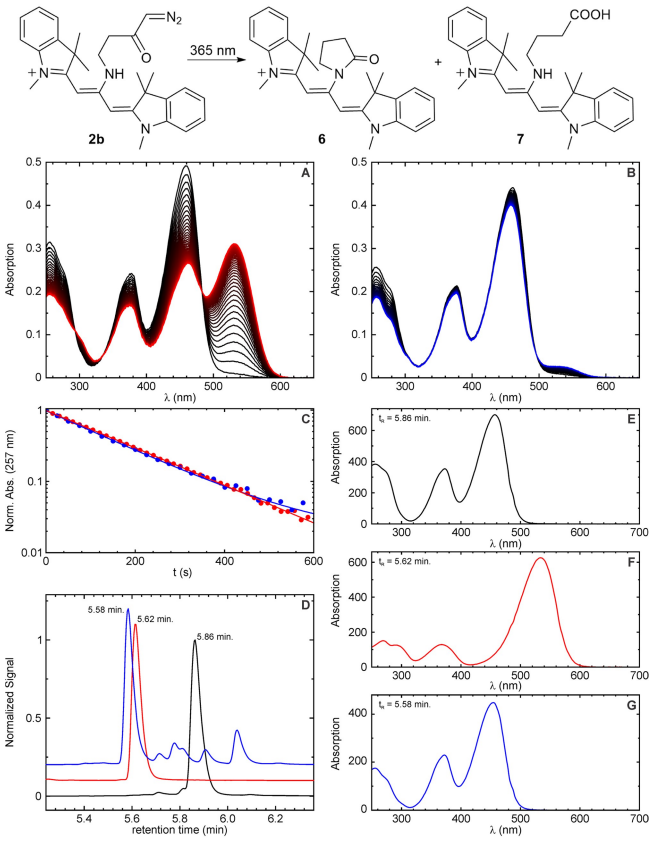


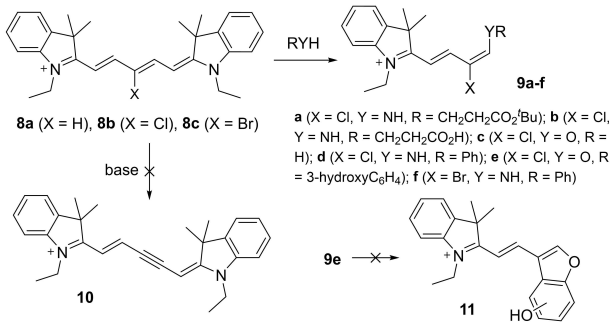
Figure 1. Photolysis of compound **2b** in MeOH (A) and in an MeCN – aq. buffer (4:1) mixture (B). The initial spectra are plotted in black; the data for products **6** and **7** are in red and blue, respectively. (C) Normalized transients (abs. at 257 nm) upon consumption of **2b**. (D) HPLC traces of **2b** (black line), **6** (photolysis in MeOH; red line) and **7** (photolysis in MeCN – aq. buffer (4:1); blue line). (E–G): Absorption spectra of the main peaks with indicated retention times corresponding to each HPLC trace in (D).

In general, the addition of amines to the electron-poor conjugated system **5** proceeds under mild conditions and gives symmetric *meso* amino substituted Cy3 analogues with functional groups. Photolysis of compound **2b** was performed in methanol and in an acetonitrile – aq. buffer (4:1) (Figure 1). The reaction rates in both solvents were nearly the same. In methanol, a clean reaction to a more polar compound with $\lambda_{abs}=535$ nm and $m/z=440$ was observed. As expected, diazoketone dye **2b** underwent Wolff rearrangement under irradiation with UV light and formed pyrrolidone **6**. In acetonitrile – aq. buffer (4:1), along with minor amounts of pyrrolidone **6**, an even more polar compound with $m/z=458$ and an absorption spectrum similar to the starting diazoketone dye **2b** ($\lambda_{abs}=458$ nm), was observed as the main photolysis product. We assign its structure to the amino acid **7**, formed from the intermediate ketene, which added water. The bathochromic shift in the transformation **2b** – **6** was large (77 nm); however, both products (**6** and **7**) were non-fluorescent.

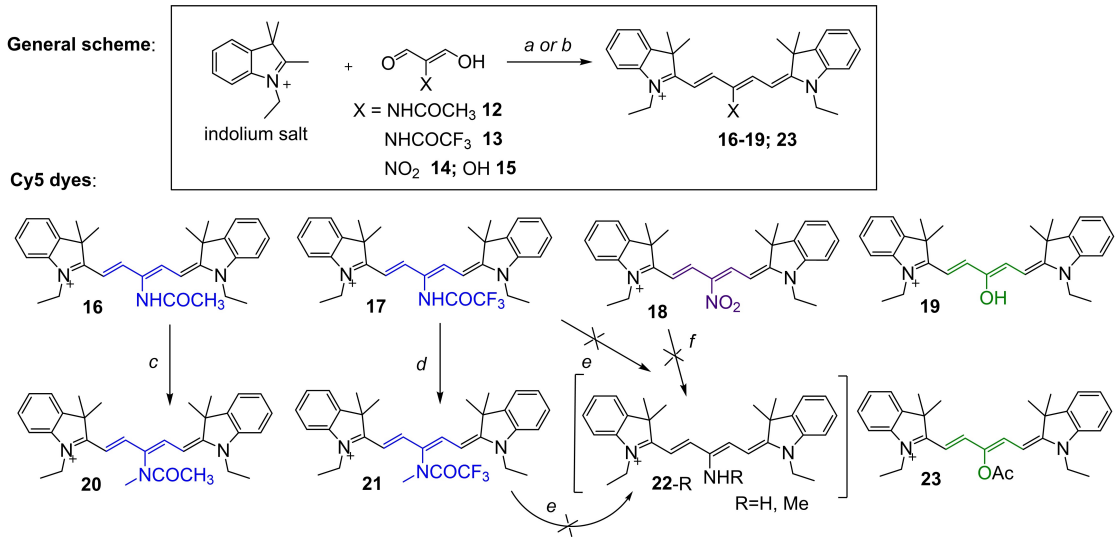
Pentamethinecyanines (Cy5)

Unlike amino substituted Cy3 and Cy7 dyes, Cy5 dyes with a basic amino group at the *meso* position of the pentamethine chain are unknown.[14] On the other hand, the symmetric Cy5 dyes with halogen atoms at the *meso* position are well known,[15,16] and, like Cy7 analogues,[5] they might serve as direct precursors for amino modified Cy5 dyes. We prepared compounds **8b,c** (Scheme 3) according to reported methods,[15] but all attempts to substitute the halogen atom for amino or hydroxyl group failed.

Further investigation revealed that no substitution occurred, the halogen atom remained attached, while the polymethine chain became shorter, and one indolenine fragment was replaced with a nucleophile (compounds **9a–f** in Scheme 3). This kind of reactivity of Cy5 core has been unknown so far. The products **9a–f** are termed hemicyanines. In related transformations, the aromatic nucleophiles such as resorcinol, 3-mercaptophenol, and 3-nitrophenol were found to attack and cut the long methine chain of Cy7.[17] The donor substituent (in resorcinol) caused the intramolecular



Scheme 3. Cy5-X decomposition by nucleophiles; counterions are omitted (undefined).



Scheme 4. Synthesis of Cy5 derivatives via substituted malondialdehydes. Compounds **22**-R were not detected. Reagents and conditions: a) Ac_2O, NaOAc, 120 °C; b) BuOH/ toluene 1 : 1, pyridine, 100 °C; c) MeI, NaH, DMF, 0 °C – r.t. d) MeI, Cs_2CO_3, DMF, 100 °C; e) 1 M aq. NaOH, MeCN, r.t.; f) N_2H_4*H_2O, $FeCl_3$*$6H_2O$, active carbon, MeOH, 60 °C or H_2, Pd/C, MeOH, 60 °C. Counterions are omitted (undefined).

cyclisation leading to cyclic hemicyanines. However, such kind of transformation did not occur for Cy5: in the reaction of **8b** with resorcinol, we observed only the product of destruction **9e** which did not undergo the intramolecular cyclisation to **11**. As an alternative, introduction of *meso* brominated cyanine **8c** to metal-catalysed Ullmann and Buchwald-Hartwig reactions[18] did not lead to the desired aminocyanines.

We also tried to obtain acetylenic type of Cy5 (as we did for Cy3; see compound **5** in Scheme 2). The dehydrohalogenation methods reported for vinyl halides[19] incorporated into a conjugated system were applied to compounds **8b** and **8c** in Scheme 3. The use of strong bases such as DBN did not result in the required acetylenic compound **10**. Both strong bases and nucleophiles led to the destruction of the pentamethine chain.

Other methods of introducing a functional group to the *meso* position of the polymethine chain in Cy5 include 2-substituted malondialdehydes OHCCHRCHO (R=NO_2, CN, aryl, etc.) or their synthetic equivalents.[20]

Free aminomalondialdehydes OHCCHRCHO (R=NHR′) are unstable, but the corresponding *bis*-dimethylacetals, and *N*-(acylamino)malondialdehydes were obtained through Vilsmeier-Haack formylation of glycine.[21] In our case, trifluoroacetylation looked promising, because it would allow further functionalisation of the amino group via *N*-alkylation,[22] and the trifluoromethyl residue as a protective group is easily removable by alkaline hydrolysis.[23] For example, *N*-trifluoroacetylamino group would permit alkylation of the nitrogen atom with *O*-THP-protected 3- iodopropanol. Further transformations would allow introducing diazoketone moiety into the molecule.[24,25]

We prepared both *N*- acetyl- and *N*-trifluoroacetyl aminomalondialdehydes **12** and **13** according to the method reported by Arnold et al.[21] and condensed them with indolium salt to afford dyes **16** and **17** (Scheme 4). The possibility of further modification via *N*-alkylation was proved by methylation with methyl iodide to afford compounds **20** and **21** (Scheme 4).

Amide **20** was of particular interest, because it structurally relates to *N*-pyrrolidone (Scheme 1), and it was important to study its photophysical properties. Compared to the non-fluorescent *meso* pyrrolidone substituted Cy3 (**6**, QY 0, Table 1), and Cy7 (QY 0.075 in MeCN),[4] the fluorescence quantum yield of **20** was found to be high enough for Cy5 dyes: 0.22 in MeCN (Table 2). However, the cleavage of the trifluoroacetyl protecting group in compounds **17** and **21** (Scheme 4) did not produce *meso* aminopentamethines **22** (R=H, CH_3). Under basic conditions, the hydrolysis resulted in decomposition of the polymethine chain and disappearance of the typical blue colour to give hemicyanines (Figure 2). Several compounds were detected by LCMS, but none of them was of particular interest as a fluorescent dye.

Table 2. Photophysical properties of **Cy5** derivatives in MeCN.

Dye	Substituent at the *meso* position	σ_p[a]	λ_{abs} [nm]	λ_{em} [nm]	ε[b] [$M^{-1}cm^{-1}$]	Φ_{fl}[c]
8a	H	0	639	664	191 000	0.17
8b	Cl	0.23	640	659	193 700	0.07
8c	Br	0.23	636	658	179 000	0.06
16	NHCOMe	0	637	660	180 000	0.15
17	$NHCOCF_3$	0.12	631	651	175 000	0.15
18	NO_2	0.78	594	641	160 000	0.02
19	OH	−0.37	667	692	190 000	0.14
20	NMeCOMe	0.26	631	651	175 000	0.22
21	$NMeCOCF_3$	0.39	625	645	175 000	0.22
23	OCOMe	0.31	642	663	176 000	0.11
24	*o*-$CO_2HC_6H_4$	–	640	664	168 000	0.11

[a] Data from Ref. [26]; [b] Molar extinction coefficient; [c] Absolute values of the fluorescence quantum yields.

Table 3. Photophysical properties of squarylium derivatives in MeCN.

Dye	λ_{abs}, [nm]	λ_{em} [nm]	ε[a] [$M^{-1}\,cm^{-1}$]	Φ_{fl}[b]
30	631	644	210 000	0.08
32 a	643	659	150 000	0.01
32 b	643	659	150 000	0.01
33	620	654[c]	≈100 000[d]	0.007[c]
34	643	651[e]	≈139 000[d]	0.008[e]

[a] Molar extinction coefficient; [b] Absolute values of the fluorescence quantum yields; [c] In MeOH; [d] Assuming full conversion; [e] In MeCN-buffer mixture (4:1 v/v).

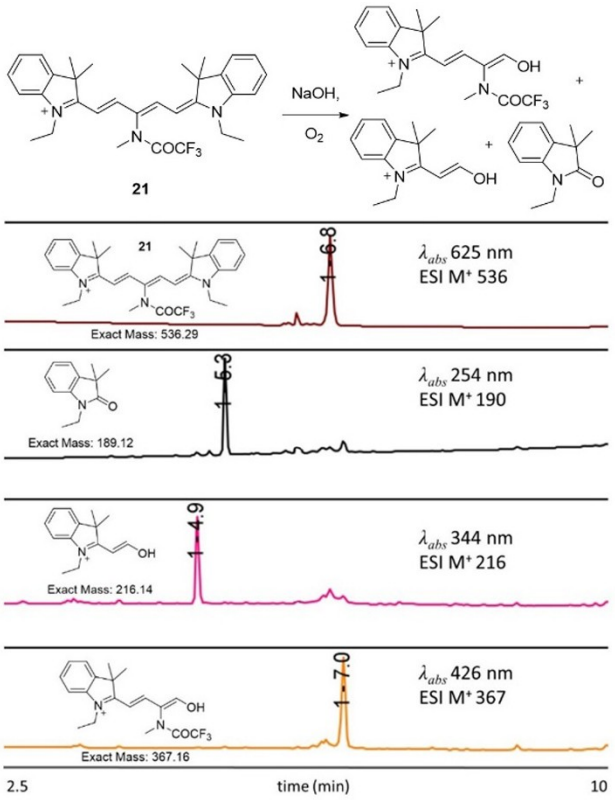


Figure 2. Reaction scheme and LCMS traces of the alkaline hydrolysis of Cy5 derivative **21** in the presence of air oxygen; detection at different wavelengths.

We also checked whether an amino group might be introduced via the reduction of a nitro group. For this purposes, the nitro compound **18** (Scheme 4) was prepared[20g] and subjected to catalytic reduction with hydrazine[27] or hydrogen.[28] The desired amine **22**-H with Cy5 fluorophore was not detected. Among the decomposition products, compounds with Cy3 fluorophore were found. Interestingly, the related "downgrade" of fluorophores was observed in the row Cy7–Cy5–Cy3 by photolysis, but in our case this transformation occurred without applied light.[29,30]

Our attempts to introduce a basic amino group to the *meso* position of Cy5 failed due to instability of the desired compounds. We introduced another donor group (OH) to Cy5 fluorophore (**19**, Scheme 4). To the best of our knowledge, it has been the first strong donor ($\sigma_p = -0.37$)[26] attached to the *meso* position of Cy5. Compound **19** with fluorescence quantum yield of 0.14 (Scheme 4, Table 2) was obtained through the condensation of commercially available hydroxymalondialdehyde **15** and indolium salt (Scheme 2) in butanol – toluene solution. If this reaction was carried in acetic anhydride – acetic acid (typical solvent mixture for cyanine synthesis), the hydroxyl group was *in situ* acylated, and the ester **23** formed (Scheme 4).

When irradiated with light, polymethine dyes are capable of reacting with singlet oxygen, and the intermediate cyclic peroxides decompose with breaking the polymethine chain. This transformation reminds ozonolysis. Cy7 dyes with a basic amino group at the *meso* position showed lower photostability than the acylated analogues.[31] The hypothesis was that an iminium intermediate facilitates the incorporation of the singlet oxygen species into the polymethine chain.[31] Acylation on the heteroatom (N, O) reduces the electron density on the free amino or hydroxyl group and minimizes the photoinduced addition reactions to the chromophore. Thus, *N*-, or *O*-acylation increases photo- and chemical stability, as we observed for dyes **16**, **17**, **20**, **21**, **23**. They are stable as solids and solutions in MeCN for weeks and can be kept at r.t. without exclusion of air oxygen. Compound **19** is unstable; it decomposed faster than other Cy5 derivatives mentioned here.

The photophysical properties of Cy5 derivatives are given in Table 2. The introduction of acylamino group ($\sigma_p = 0.0$)[26] to the *meso* position of the pentamethine chain (compounds **16**, **17**, **20**, **21**, Scheme 4) produces a small blue shift of the absorption and emission maxima compared to unsubstituted dye **8 a** (Scheme 3). For the nitro group ($\sigma_p = 0.78$[26] compound **18**, Scheme 4), this effect is much stronger (−45 nm). The only group with donor properties (OH, $\sigma_p = -0.37$)[26] induces a red shift in both absorption and emission maxima (compound **19**, Scheme 4). In our previous publication, we reported the linearized correlations between the positions of absorption and/or fluorescence maxima and Hammett σ_p constants.[22b] In the present case, we plotted the values of the Hammett σ_p constants and the positions of absorption and emission maxima (in eV) for Cy5 derivatives (data from this study) and Cy7 analogues (data from Ref. [32]) in Figure 3. If we consider the influence of the substituents at the *meso* position on the spectra of cyanine dyes, we can conclude that Cy7 and Cy3 dyes demonstrate similar trends, while the Cy5 fluorophore demonstrates the opposite trend. The explanation of this phenomena can be found in the localization of HOMO and LUMO.[33] According to DFT-calculations, the highest occupied molecular orbital (HOMO) is localized on the entire pentamethine chromophore including the *meso* carbon atom (and an adjacent substituent). The lowest unoccupied molecular orbital (LUMO) is localized on the pentamethine chain, excluding the *meso* carbon atom. Thus, the electron donor groups at the *meso* position of the Cy5 chromophore increase the energy of the HOMO, reduce the energy gap between HOMO and LUMO, and produce bathochromic shift of the absorption and emission maxima. The localizations of HOMO and LUMO at

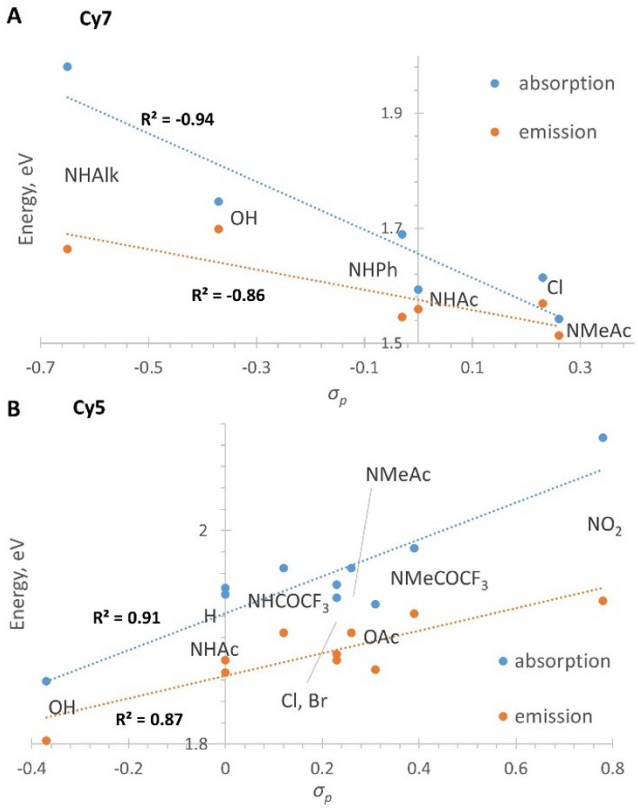


Figure 3. Correlation of σ_p values with the positions of absorption and emission maxima (in eV) of Cy7 (**A**, data from Ref. [31]) and Cy5 (**B**, this work).

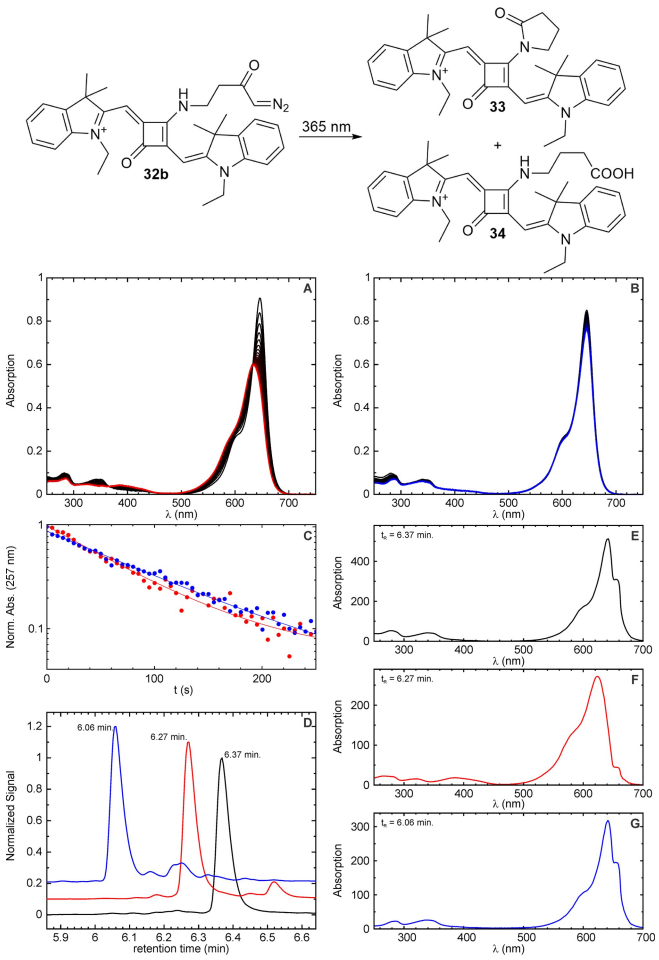


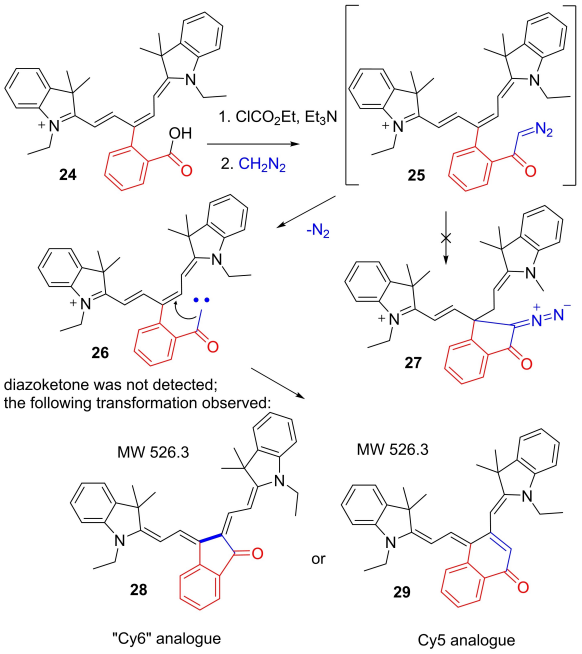
Figure 4. Photolysis of compound **32b** in MeOH (A) and in an MeCN – aq. buffer (4:1) mixture (B). The initial spectra are plotted in black. The data for products **33** and **34** are in red and blue, respectively. (C) Normalized transients (absorption at 257 nm) by consumption of **32b**. (D) HPLC traces of compound **32b** (black line) and the reaction mixtures after photolysis in MeOH (red line, compound **33**) and in the MeCN – aq. buffer (4:1) mixture (blue line, compound **34**). (E–G) Absorption spectra of **32b** (black line) and the main peaks (products) in red and blue HPLC traces in (D) (with indicated retention times).

the *meso* amino substituted Cy7 and Cy3 dyes are opposite to Cy5. In these dyes, the electron donors increase the energy of the LUMO and produce hypsochromic shift of the absorption and emission bands. Indeed, the blue shifts in absorption and emission maxima are observed for compounds **2a** and **2b**, compared to unsubstituted dye **1b** (Scheme 2, Table 1). Thus, our experimental data confirm the trends based on DFT calculations.[33] The correlation coefficients are positive for Cy5 dyes (ca. 0.9) and negative for Cy7 derivatives (ca. −0.9; Figure 3).

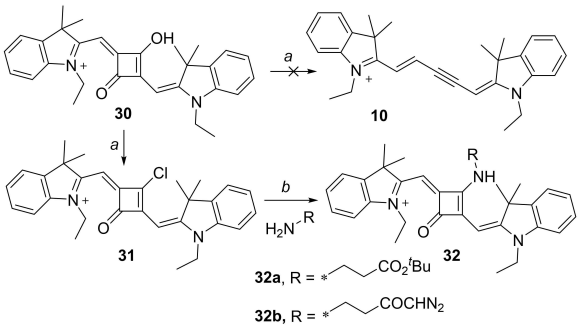
Interlude: *meso*-(2-carboxyphenyl)-Cy5 dye

We briefly checked another option which might lead to photoactivatable Cy5 dyes having 2-diazo-3-spiro-1-indanone unit (compound **27** in Scheme 5). This group incorporated into rhodamine and carborhodamine dyes made them colorless (pale yellow) and non-fluorescent, due to spontaneous cyclization to the spiro-form with broken conjugation.[34,35] The existence of spirolactam capped Cy7 dyes[36] with similar molecular architecture confirmed the possibility to mask the cyanine chromophore by intramolecular addition of a nucleophilic group (–**CH**=N_2, –CO**NH**R) to polymethine chain. Photolysis of 2-diazo-3-spiro-1-indanones (in protic solvents) obtained from rhodamines and carborhodamines was accompanied by Wolff rearrangement[3b] and led to fluorescent analogues of the initial dyes with an additional CH_2 group between the phenyl ring and the carboxylic acid function (COOH, COOR or COONHR).[34,35]

The starting *meso*-(2-carboxyphenyl)-Cy5 dye **24** was obtained from the corresponding brominated derivative **8c** and 2-carboxyphenyl boronic acid via the Suzuki coupling[37] (Scheme SI-13). The diazoketone **25** was generated in two steps (Scheme 5). It turned out to be unstable and decomposed in the reaction mixture giving only a non-fluorescent product, to which we can assigned either structure **28**, or **29**. The former (**28**) corresponds to carbene insertion into the carbon-carbon bond, and the latter (**29**) – to carbene insertion into carbon-hydrogen bond. The similar process – formation of a dark by-product via carbene insertion into a carbon-carbon bond – was observed in the photolysis of 2-



Scheme 5. Generation of instable diazoketone **25** leads to formation of non-fluorescent products. Counterions are omitted (undefined).



Scheme 6. Synthesis of amino-squaraine based Cy5 dyes. Reagents and conditions: a) $POCl_3$, Et_3N, 1,4-dioxane, 80 °C; b) K_2CO_3, THF, r.t. Counterions are omitted (undefined).

diazo-3-spiro-1-indanones obtained from rhodamines and carborhodamines, but these diazoketones were quite stable.[34,35]

Cy5 Squaraines

Squarylium dyes combine good photostability, high quantum yields, and some stabilized squaraines are used as fluorescent probes and labels for biomedical applications.[38] The stabilization (often in the supramolecular guest-host complex) is required, as the "naked" squarylium dyes are hydrolytically unstable and readily react with nucleophiles. However, the facile synthesis makes them attractive platform for the design of the far-red emitting fluorescent probes.[39] The incorporation of the squaraine residue in compound 4 in Scheme 2 was only temporarily and allowed obtaining the amino substituted cyanine dye **2**. We decided to incorporate the squarylium moiety into Cy5 fluorophore, because this approach helped us to achieve the synthetic goal for the Cy3 chromophore (Scheme 2). Squaraine **30** (Scheme 6) was obtained according to the reported method.[38] In the reaction with $POCl_3$ compound **30** did not lose CO and gave no acetylenic derivative **10** (Scheme 6), but rather formed chloride **31**, which readily reacted with β-alanine *t*-butyl ester and afforded amino-squaraine **32a**. In comparison with the parent squarylium cyanine **30**, amino-squaraine **32a** displays a small bathochromic shift of the absorption band and shows very weak fluorescence (Table 3), as required for the dark state. A further transformation was based on the introduction of the diazoketone moiety and resulted in dye **32b**. This modification did not increase the quantum yield. Diazoketone **32b** was subjected to photolysis at 365 nm in methanol and in aqueous acetonitrile (Figure 4). The products are similar to the products obtained from compound **2b** (Figure 1). In methanol, the main product with $t_R = 6.27$ min, $\lambda_{abs} = 620$ nm and $m/z = 520$ is pyrrolidone **33**. In acetonitrile – aqueous buffer (4:1) solution, the main product with $t_R = 6.06$ min, $\lambda_{abs} = 643$ nm and $m/z = 538$ is amino acid **34**. Unfortunately, the photolysis products **33** and **34** are poorly fluorescent (Figure SI-7, Table 3), with emission quantum yields as low, as that of compound **32b**.

Conclusions

Unlike Cy3 dyes, all Cy5 derivatives with the basic amino group at the *meso* position of the fluorophore bridge were found to be unstable and decomposed with breaking the polymethine chain. The presence of an amino group at this position quenches the fluorescence of cyanines and, as an additional functionality, provides high degree of synthetic freedom. To demonstrate this, we prepared 4-amino-1-diazo-2-butanone ($H_2N(CH_2)_2COCH{=}N_2$) and attached it via an amino group to Cy3 and squaraine based Cy5 dyes. Photolysis of these compounds was accompanied with Wolff rearrangement and led to pyrrolidones (in methanol and aprotic solvents) formed via intramolecular cyclization of the intermediate ketenes. In aqueous acetonitrile, ketenes reacted with water and gave carboxylic acids. Though the products of these reactions were found to be non-fluorescent, synthesis and incorporation of the fragment $H_2N(CH_2)_2COCH{=}N_2$ into other chromophores, except Cy3 and squaraine based Cy5 dyes, is interesting. In our cases, the initial dyes were non-fluorescent; probably, due to photo-induced electron transfer (PET) from the basic amino group.[2] For other dyes, like BODIPYs or rhodamines, these aspects remain unexplored. Importantly, we established that the intramolecular acylation of the amino group takes place if the photolysis is carried out in methanol or aprotic solvents. Thus, the use of photoconvertible dyes (dye-$HN(CH_2)_2COCH{=}N_2$) in aqueous solutions may be limited, but the applications in material science (in organic solvents, polymer matrices, etc.) are more promising. The use as protein labels looks less attractive, if photoactivation of the emission is desired, but for binding

with free amino groups in proteins (lysine residues), diazoketone photolysis represents an interesting option.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: cyanines · cyclization reactions · diazo compounds · photolysis · squaraines

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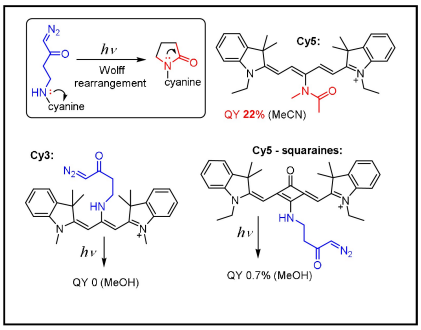
RESEARCH ARTICLES

Make and break: Trimethinecyanine (Cy3) and squaraine-based pentamethinecyanine (Cy5) dyes with the (functionally substituted) amino group at the *meso* position have been prepared. Photolysis of these dyes with a 4-amino-1-diazo-2-butanone fragment was studied in methanol and aprotic acetonitrile.



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