A Versatile Route to Red-Emitting Carbopyronine Dyes for Optical Microscopy and Nanoscopy


Keywords: Fluorescence / Chromophores / Carbocycles / Fluorescent probes / Fluorescence spectroscopy

Biological microscopy favors photostable fluorescent markers with large fluorescence quantum yields, low dark triplet state population, and good biocompatibility and absorption and emission maxima in the near-infrared, where cellular autofluorescence is minimized. In the present study, carbopyronines absorbing around 640 nm and emitting at around 660 nm, with a low intersystem crossing rate ($k_{isc} \approx 0.5 \times 10^6 \text{s}^{-1}$) and excellent properties for cellular imaging were synthesized. A general synthetic route to carbopyronines with functional groups variable in the final steps of the synthesis or in the resulting fluorescent dye is presented. Possessing two 2-methoxyethyl groups, the parent dye is soluble in water and most organic solvents. Demethylation of the dye or its precursors is straightforward, clean, and furnishes compounds with one or two 2-hydroxyethyl groups, which can be used for further transformations. Modifications in the linker-containing carboxy group are also possible. A multistep synthesis of the dye starting from a simple precursor and utilizing a single temporary protective group is described. The presented approach may be further applied to the design of caged carbopyronines.

Introduction

Fluorescent dyes that absorb in the far-red or near-infrared (IR) optical region are indispensable for a variety of microscopic techniques in biology, physics, and chemistry. Irradiation at wavelengths above 600 nm is much less invasive and minimizes the undesired background signal originating from cellular autofluorescence. Far-field optical nanoscopy,[1] in its particular applications[2] such as STED (stimulated emission depletion) nanoscopy,[2a,2d,2h,2p] PALM (photoactivation localization microscopy),[2a,2s] STORM (stochastic reconstruction microscopy),[2a,2v] or GSDIM (ground state depletion with individual molecular return)[3] pose very strict and sometimes contradictory requirements on the utilized fluorescent markers. Among them, the most important are: large quantum yield of fluorescence (greater than 0.5), low population of the dark triplet state, high photostability, good solubility in water, and a reactive group with a linker for conjugation to biological molecules such as proteins or nucleic acids. Finally, to make the conjugation possible, the markers (usually dye active esters) need to be stable enough in aqueous solutions. However, in practice, all of these requirements can seldom be fulfilled by a single substance. Therefore, it is advantageous to supply a wide variety of fluorescent dyes, allowing the selection of the marker with the most suitable characteristics. Attempts to design and improve photostable red-emitting dyes are being undertaken in a number of research groups. Recent publications on this topic describe water-soluble terylenediimides,[4a,4b] soluble quaterpylenediimides[4c] and bisanthenes,[4d] new hydrophilic BODIPY derivatives,[4e–4g] squaraine dyes,[4h] and dicyanomethylene dihydrofuranes.[4i] However, some important pieces of data on the photophysical properties of their bioconjugates and microscopic applications of these compound classes are still lacking.

Despite many attempts to design novel and improved red-emitting dyes on the basis of different chemical classes, the number of compounds that perform satisfactorily in fluorescence-based microscopy is still limited. Here we report on the synthesis of a structural scaffold that realizes a whole range of novel red-emitting carbopyronine dyes with improved solubility in water and variable chemical groups. For the first time, an improved and detailed synthesis of a carbopyronin scaffold is described that allows modifications on the final product, i.e., a photostable dye with large fluorescence quantum yield and the required absorption and emission bands in the red.

Results and Discussion

Choosing Carbopyronines

Very recently we described novel red-emitting rhodamine dyes of various polarities.[4j] Due to large fluorescence quantum yields, we decided to explore the potential of a complementary structural scaffold that would offer significantly improved solubility in water and a broader range of dye properties.

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quantum yields, high photostabilities and variable hydrophilicities, they compete well in microscopy with the commercially available near IR fluorophores such as ATTO 633, ATTO 647N, Alexa 633, and Alexa 647. The presented rhodamine dyes have maxima of absorption and emission at 630–640 nm and 660 nm, respectively. This allows the use of convenient excitation sources such as He–Ne, diode or krypton ion lasers. However, a limit has been reached for further red-shifting the absorption and emission maxima of the practically useful rhodamines.[5]

In our search for novel IR fluorophores, we decided to switch to a class of dyes other than rhodamines. Carbopyronine dyes are chemically very similar to the rhodamines (see Scheme 1 for structures). Compared to rhodamines, the oxygen atom at position 10 of the xanthene fragment is replaced by the geminal dimethyl group \([\text{C(\text{CH}_3)_2}]\), introducing a large bathochromic shift in the absorption and emission bands of approximately 50 nm[6] and probably changing the rate of triplet state formation. Due to the bathochromic shift provided by the carbopyronine core, there is no need for four fluorine atoms in the \(\sigma\)-substituted benzoic acid residue, as in the previously synthesized rhodamine dyes.[4]

In fact, the presence of fluorine atoms is a drawback, because the fluorinated aromatic ring drastically decreases the solubility of a dye in water and increases its lipophilicity. As a consequence, a very hydrophilic group (or groups; such as two sulfonic acid residues) was necessary to compensate these factors.[9] Besides that, four fluorine atoms increase the molecular mass, which is not desirable for a fluorescent label. The carbopyronines, we anticipated, would not require such a polar group (SO₃H) to be water-soluble.

Synthetic Pathway to Carbopyronines and Possibilities for Dye Design

The synthesis of carbopyronines is a rather challenging task. In all available publications, the syntheses of these dyes are described either incompletely or for very simple derivatives only.[6a–6c] As can be seen in the patents, many important details, particularly in regard to the preparation of key intermediates, are not disclosed at all.[6d,6e,7] Despite the large number of compounds claimed as examples, it is not clear whether it is easy or not to vary the residues in carbopyronine-containing scaffolds so that their properties, particularly polarity and/or water solubility, would be controlled.

Double bonds in bridges and open chains are more prone to photooxidation (as occurs in cyanine dyes) than the unsaturated cyclic \(\pi\)-systems of the main chromophores in rhodamines and carbopyronines. The exclusion of such double bonds from the scaffold was thus expected to improve the photostability. In addition, double bonds are known to reduce the fluorescence quantum yield of some carbopyronines.[7] Other important requirements for potential candidates are the presence of a hydrophilic group (or groups), the presence of a sterically unhindered reactive site for conjugation, and a moderate molecular mass (the absence of excessive side chains or extra aromatic rings).

A literature survey, complimented by our own experience in the synthesis of rhodamine derivatives,[2n–2r] led us to a particular structural scaffold of pyronine fluorescent dyes (Scheme 1). The scaffold combines several features that, together, meet all the requirements of an optimized fluorophore in the context of micro- and nanoscopic methods. Scaffold 1, accompanied by its retrosynthetic analysis (Scheme 1), illustrates our approach, which was intended to be uniform for carbopyronines and capable of providing derivatives with variable functional groups and variable properties. Apart from the absence of double bonds, some other structural features providing high fluorescence quantum yields and chemical stability were introduced.[8]
First, we decided to install methylene bridges that would “rigidize” the fluorophore molecule on both sides, thus increasing the fluorescence quantum yield (Rhodamine 101, whose $\phi_R$ is 1.0, and a significant number of other commercial dyes have these bridges). The chemical stability is also increased, when the planarity of the $\pi$-system improves the delocalization of the positive charge. Moreover, the two aromatic CH groups are blocked by the alkyl groups and are thus no longer prone to oxidation. Despite the merits of the julolidine fragment (which is present in Rhodamine 101 and carbopyronine fluorescent dye ATTO 647N), where the nitrogen atom is incorporated into the junction of three cycles, we decided not to use this structural feature because it would have been impossible to attach two (various) groups to the nitrogen atoms. In the synthetic approach that we suggest here, the modifications at these heteroatoms were expected to play the key role (see the modifications of scaffold $\textbf{I}$ outlined in Scheme 1).

The initial transformations in Scheme 1 are based on the approach first described by Frantzkeskos for the simplest carbopyronine dyes – $N,N'$-dimethyl derivatives (without methylene bridges). As explained above, our approach was flexible in regard to the variability of substituents. $R^4$ at the nitrogen atoms in the final steps of the synthesis or even in the final compound, i.e., the fluorescent dyes $1\text{a} - \text{c}$. It is noteworthy that this option has not been explored before. From the details that are available in the patent literature, it can be concluded that the substituents at the nitrogen atoms in carbopyronine dyes are attached in early steps of the synthesis, and thus cannot be easily modified. In our approach, the whole pathway starts with one simple precursor (compound $\textbf{A}$), which is transformed into two building blocks ($\textbf{B}$ and $\textbf{C}$) that are, in turn, utilized for the subsequent condensation step. The building blocks $\textbf{B}$ and $\textbf{C}$ should bear temporary protective groups (not necessarily the same) that can withstand the drastic condensation conditions. These groups ($R^1$ in Scheme 1) have to be changed in later steps to the required functional groups ($R^2$) that allow further chemical transformations. We purposely planned scaffold $\textbf{I}$ to be symmetrical because we expected to obtain maximum effect with two residues $R^4$. The solubility in water may be controlled by the hydrophilicity of these groups. If only one of the two groups is modified, an additional coupling site can be provided. This might be important, especially when the carboxy group in the $\alpha$-substituted phenyl ring remains free for other kinds of modifications (e.g., for caging, which involves the preparation of the colorless and photosensitive spiroamides for optical nanoscopy and novel caged dyes).

On the way to Scaffold $\textbf{I}$, one last obstacle remained. Protective groups and residues ($R^1$ and $R^4$) that were suitable for the whole synthetic sequence starting from compound $\textbf{A}$ (Scheme 1) needed to be found. Not only do these groups need to withstand the drastic conditions of some reactions, but also need to be smoothly removed under conditions that keep the other parts of the molecule unchanged. The latter requirement is especially important at the very end of the sequence. As an important building block in the dye design, we utilized an $N$-methyl-$\beta$-alanine bridge, which was used in our previous studies. Esterification or amidation of the carboxylic group in the $\alpha$-substituted phenyl ring provides an additional redshift in absorption and emission spectra (ca. 5–10 nm; compared to a compound with the free carboxylate). Also importantly, the $\beta$-alanine residue has a sterically unhindered carboxyl group that is suitable for further conjugation reactions.

Description of the Synthesis

The first crucial step of the synthesis is the condensation of building blocks $\textbf{B}$ and $\textbf{C}$, both of which are prepared from the same precursor $\textbf{A}$. According to the patent literature, the condensation is best assisted by $\text{BCl}_3$ and followed by cyclization at elevated temperatures in mineral acid media. For most amino-protective groups these conditions are prohibitively severe. The requirement of smooth and clean deprotection, on the other hand, leaves only the benzyl group (Bn) as a viable candidate. The whole sequence outlined in Scheme 1 is unfeasible without the temporary protective groups ($R^1$) and their removal in a later step. The compatibility of the protective groups (PGs) and reaction conditions is critical due to the wide variety of reagents used. There are clearly no groups that could resist all the reagents (see Schemes 1, 2 and 3) and, importantly, be smoothly removed in the end. As regards benzyl as PG, we could not retain this group after the condensation step because the CH$_2$ groups would have been oxidized. Moreover, the debenzylation on Pd/C in the presence of hydrogen or its donors is very likely to also reduce the carbopyronine core, as long as the third aromatic ring is attached. Due to these precautions, the benzyl group was used only for temporary protection ($R^1$ in Scheme 1) and was removed after the condensation step (Scheme 2).

As will be shown below, the removal of the temporary protective group (Bn) from dyes of this class went smoothly (see Scheme 2), which already meant that some success in carbopyronine dye design had been achieved. As long as this step is performed (Scheme 1), any residues ($R^4$) that are compatible with the reagents used in further steps can be attached. This, in turn, will increase diversity within Scaffold $\textbf{I}$.

Both building blocks $\textbf{B}$ and $\textbf{C}$ originate from compound $\textbf{A}$. We considered 1,2,3,4-tetrahydroquinoline derivatives as candidates for the starting compound $\textbf{A}$ with methylene bridges of a reasonable length. As regards building block $\textbf{B}$, there is one difficulty that had to be addressed. Whereas para-electrophilic substitution in $\textbf{A}$ presents no difficulty, the meta-type substitution (directed by the protonated secondary aromatic amino group) does not proceed very easily and cleanly (see ref. and Exp. Section for details).

Thereafter, the best candidate for $R^4$, the second and the final (if ever possible) PG in our synthesis, had to be selective. First, the dye (target compound) would be more practically important if $R^4$ alone was polar (and/or hydrophilic).
According to a review,[14] some “soft” demethylating agents might be compatible with carboxymethyl and amido groups. Particularly, the selective demethylation of a methyl ether that also had a carboxymethyl (CO₂Me) function was described.[15] Once the reaction proceeds, the resulting 2-hydroxyethyl group could be subjected to various transformations. The CO₂Me function, at the same time, would remain unchanged and could be hydrolyzed for conjugation purposes in the very final stage. The methoxy group (CH₃O) itself is far more polar than an alkyl group, and is known to increase the solubility and reduce the crystallinity of organic compounds. We had every reason to expect this effect to be demonstrated in case of the 2-methoxyethyl substituent, thus increasing the polarity and hydrophilic properties of the final fluorescent dyes.

Thus, we developed a scheme for the preparation of carboxyronine dyes with variable substituents from one nitrogen-containing starting compound (A, Scheme 1). After the scheme was put into practice, we found that, despite the wide range of reagents utilized, the protective groups needed to be switched only once or twice throughout the whole synthesis. Schemes 2 and 3 depict the actual preparation of a red-emitting fluorophore (compound 1c, Scheme 3) that proved to be excellent for bioconjugation and STED imaging, as expected. Except for the very first steps (depicted in Scheme 1), the yields are average values from 2–3 experiments (for details, see Exp. Section and Supporting Information).

Our synthesis started with 1,2,3,4-tetrahydroquinoline (4), as building block A (Scheme 2). To convert this into building block B, a halogen (Br or I) was introduced into position 7 in the aromatic ring. The stepwise transformation of the aromatic bromide into the isopropenyl residue could be achieved by lithiation followed by reaction with acetone and dehydration of the tertiary carbinol so formed.[16] Initially, we started with nitration, as the only known preparative method for the selective meta-substitution in compound 4 (an aromatic amine).[18] The transformation of a nitro group into a halogen is a classical method that involves a Sandmeier reaction on the corresponding amine. In the case of substrate 4, the required sequence was longer: the NH group first had to be temporary protected with an acetyl (Ac) or other electron-withdrawing group.

The synthesis of iodide 5-H,I from the corresponding nitro compound 5a was carried out analogously to 6-iodo-2,3-dihydro-1H-indole, the preparation of which is lengthy, yet generally high-yielding.[17] The synthesis of the bromo-substituted analogue via a Sandmeier reaction has not been reported. The best yields of N-benzyl derivatives (5-Bn, I, 6, and 7-Bn) were achieved by direct benzylolation; the reductive benzylolation with benzaldehyde gave lower yields, especially for 6. The conditions used for the dehydroxylation of carbonyl 8 were much improved compared to the single literature report that describes its simplest analogue – 3-(isopropenyl)-N,N'-dimethylaniline.[6a] Particularly, using this approach there was no need for vacuum distillation, and the reaction time was much shorter when a high-boiling solvent was used (see Exp. Section). Also importantly, we

Secondly, a very important option would be the ability to remove or to modify this group (or a part of it) so that the resulting molecule becomes even more polar and hydrophilic. As a suitable candidate for R₄, we chose the 2-methoxyethyl group (-CH₂CH₂OCH₃). This group can resist oxidants under mild conditions, and can also be smoothly demethylated.

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explored a shorter path to carbinol 8 via direct bromination of 4 to bromide 7-H. The bromination of indoline and some similar substrates in the presence of Ag\textsubscript{2}SO\textsubscript{4} in sulfuric acid is widely used as a carboxyl synthon. The deprotection is normally performed to 1a (80 % one-pot). (h) POCl\textsubscript{3}, ClCH\textsubscript{2}CH\textsubscript{2}Cl, 70 °C, 3 h; (i) MeNH(CH\textsubscript{2})\textsubscript{3}CO\textsubscript{2}Me, Et\textsubscript{3}N, CH\textsubscript{2}Cl\textsubscript{2}, –10 °C; (j) HATU [2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate] Et\textsubscript{3}N, MeCN, 0 °C.

Scheme 3. The actual synthesis of the carbopyronine dye 1c: (a) K\textsubscript{2}MnO\textsubscript{4}, Me\textsubscript{2}CO, 0 °C; (b) 15, THF, –78 to 0 °C; MeOH, AcOH, NaOH; (c) aq. KOH, r.t. 1 h; (d) 20 % aq. HCl, 80 °C, 16 h; (e) Me\textsubscript{3}SiCl, K\textsubscript{2}CO\textsubscript{3}, r.t.; (f) aq. K\textsubscript{2}CO\textsubscript{3}, r.t.; (g) 2 % aq. H\textsubscript{2}SO\textsubscript{4}/AcOH, 80 °C, 8 h; (h) POCl\textsubscript{3}, CICH\textsubscript{2}CH\textsubscript{2}Cl, 70 °C, 3 h; (i) Me\textsubscript{3}NH(CH\textsubscript{2})\textsubscript{3}CO\textsubscript{2}Me, Et\textsubscript{3}N, CH\textsubscript{2}Cl\textsubscript{2}, –10 °C; (j) HATU [2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate] Et\textsubscript{3}N, MeCN, 0 °C.

Compounds 12 and 13 are readily oxidized by air oxygen, which is typical for carbopyronines, as established by Frantzskos.[6b] In our synthesis, the preparation and isolation of the colored form 14 was unnecessary, but its formation was witnessed by the deep-blue color that rapidly appears upon exposure to air, and was also confirmed by mass spectroscopic analysis. For compound 14, further alkylation at both nitrogen atoms is impossible once their nucleophilicity is lost (due to the presence of the delocalized positive charge). Therefore, in our pathway, the alkylation has to precede the oxidation (Scheme 1 and Scheme 2), and had to be performed in an inert atmosphere. Before the reaction was complete, we could observe the formation of the partially alkylated (unsymmetrical) product 3b, the structure of which was confirmed by MS and NMR spectroscopic analysis. As regards the oxidation of compound 3a to ketone 2, which is another important step of the synthesis, a modest yield was reported for a far less sophisticated carbopyronine-containing substrate by using permanganate as oxidant.[6b] Expecting even poorer yields with compound 3a, we first tried some different oxidants—selenium dioxide was unnecessary, but its formation was witnessed by the deep-blue color that rapidly appears upon exposure to air, and was also confirmed by mass spectroscopic analysis. For compound 14, further alkylation at both nitrogen atoms is impossible once their nucleophilicity is lost (due to the presence of the delocalized positive charge). Therefore, in our pathway, the alkylation has to precede the oxidation (Scheme 1 and Scheme 2), and had to be performed in an inert atmosphere. Before the reaction was complete, we could observe the formation of the partially alkylated (unsymmetrical) product 3b, the structure of which was confirmed by MS and NMR spectroscopic analysis. As regards the oxidation of compound 3a to ketone 2, which is another important step of the synthesis, a modest yield was reported for a far less sophisticated carbopyronine-containing substrate by using permanganate as oxidant.[6b] Expecting even poorer yields with compound 3a, we first tried some different oxidants—selenium dioxide was unnecessary, but its forma...
been cleaved (see Scheme 3). There are two details about this reaction that are worth mentioning: First, during the preparation of reagent 15 (which is used in large excess), the use of excess tBuLi should be avoided because the latter readily reacts with ketone 2 to form a side-product. Secondly, as our subsequent experiments showed, compound 16a is sensitive to bases (note the base-assisted cyclization to 17, see Scheme 3 and Exp. Section for details). Therefore, to avoid complications, the reaction mixture was neutralized (or acidified) before being diluted with water. The best procedure we found was to pour the reaction mixture into a cool methanolic solution of acetic acid.

Under basic conditions, instead of the expected normal hydrolysis of amino ester 16a, a fast rearrangement to compound 17, a spiroamide, was observed, which represents the “closed” (colorless) form of the corresponding primary amide. Such cyclization of primary amides is typical for rhodamines and, probably, also for carbopyronines. Its driving force is the formation of stable non-ionic spiro compounds, such as lactones 18a and 18b (see Scheme 3). Being an amide, compound 17 is stable towards alkaline hydrolysis; at elevated temperatures it decomposes to a mixture of colorless products. In contrast, heating in glacial acetic acid in the presence of H2SO4, or with POCl3 in 1,2-dichloroethane, causes a “recyclization” to the corresponding oxazoline derivative 19.

Initial experiments on the acid hydrolysis of 16a with aqueous hydrochloric acid were surprising because the reaction proceeded through compound 18 as a major intermediate. It required a long time (more than 10 h) to complete, as indicated by HPLC monitoring. The picture got even more complicated when dilute (2–5%) hydrochloric acid was used: again, spiroamide 17 was formed, the hydrolysis of which required much longer and the reaction did not proceed cleanly. Oxazoline derivative 19, together with demethylated compounds 18a and 18b, was also detected in the reaction mixture. In concentrated HCl the hydrolysis proceeds faster, but the results of the experiments proved difficult to reproduce and, more importantly, the 2-methoxymethyl groups were found to have undergone complete demethylation to form compound 18b (R1 = R2 = H). Fortunately, we found that when the HCl concentration is moderate (20–22%) and the reaction temperature is lower (80 °C), compound 1a is obtained with good yields in an acceptable timescale (15–18 h). We also established that, in acidic media, an equilibrium exists between 16a and 19 (see Scheme 3 and Exp. Section). A useful feature of this method is that partial demethylation does occur, and the mono methylated product 18a (with R’ = H) is always formed in considerable amounts (1:4–1:2), depending on the reaction time. In fact, all three possible reaction products, 1a, 18a, and 18b are normally detected in the reaction mixture (see Scheme 3 and Exp. Section for details) and can be separated by column chromatography. In nonpolar solvents, the three compounds exist predominantly in their lactone forms, as confirmed by NMR analysis and witnessed by their very pale colors (almost colorless). The partially demethylated product 18a is a valuable intermediate in which the hydroxyl and the carboxyl groups can be modified (or protected) in a stepwise fashion.

Apart from acid hydrolysis of oxazoline derivatives, an alternative method for demasking carboxyl functions for acid-sensitive substrates is known: this approach makes use of methylation with methyl iodide to a quaternary salt, which is subjected to alkaline hydrolysis. We were unsure about applying this protocol to compound 16a for the following reasons: First, in compound 16a, the oxazoline ring is already cleaved, which means that the substrate differs from that described by Meyers et al. Secondly, and more importantly, compound 16a could be isolated and stored only as its salt, which means that a base is required to liberate the amino group for the alkylation. As mentioned above, we have established that bases, particularly K2CO3, promote a fast rearrangement to spiroamide 17 (as shown in Scheme 3). In order to inhibit this undesired process before the alkaline hydrolysis, we applied acetic anhydride and ethyl isocyanate as reagents to protect the amino group in 16a. Unfortunately, neither of these approaches were successful: in both cases, complex reaction mixtures were obtained in which compound 17 was detected as the major component. However, we found that full methylation of amine 16a can be an option. A large excess of MeI in N,N-dimethylformamide (DMF) cleanly methy whole the amine to the quaternary salt 16b (see Scheme 3), which can be saponified with dilute alkali in a one-pot fashion with very high yield in approximately two hours at 0 °C. The separation of 1a from partially demethylated product 18b – a side product of acid hydrolysis – is avoided because the demethylation does not occur under basic conditions. However, due to its longer reaction time, the acid hydrolysis proved to be the only feasible way to prepare an asymmetrical derivative such as 18a (an example of scaffold 1 bearing two different residues R2 at the nitrogen atoms). The reaction can be monitored analytically and stopped at the appropriate time, which, as mentioned above, might be advantageous for further dye design.

In the next step of the synthesis, an N-methyl-β-alanine bridge was attached to the carboxyl group of compound 1a by amidation (see Schemes 1 and 2). This involved the formation of an acid chloride, followed by a one-pot reaction with an amine. Taking some risk of demethylation, we used POCl3 as in our previous study.[4] Despite the high reaction temperature (70 °C) and the relatively long reaction time (2–3 h), the one-pot procedure was clean and high yielding (see Scheme 3 and Exp. Section). The saponification of ester 1b to free acid 1c also proceeded smoothly to provide the required carbopyronine-containing dye. To avoid hydrolysis of the amide bond and formation of the colorless spiro lactones (analogues of compound 1a), the alkaline hydrolysis was performed in a dilute solution under mild conditions. Dye 1a was obtained as a dark-blue, watersoluble (>5%) solid with a high extinction coefficient and intense red fluorescence in solutions (see Table 1 for the spectral properties). The compound is also readily soluble in most organic solvents (except alkanes). Furthermore, its hydroxysuccinimide ester (1-NHS) – the amino reactive de-
rivative that can be used for conjugation – formed smoothly and proved to be stable enough to be isolated by standard column chromatography using a water-containing mobile phase.

Table 1. Spectroscopic properties of the fluorescent dyes (for structures see Schemes 2–5 and Figure 1) and the conjugate of dye 1c with sheep anti-mouse antibodies.[a]

<table>
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<th>λ_{max}(abs) [nm]</th>
<th>λ_{max}(fl) [nm]</th>
<th>ε × 10^{-5} [L mol^{-1} cm^{-1}]</th>
<th>Φ_{fl} [%][b]</th>
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<th>MeOH</th>
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<td>1.5</td>
<td>65</td>
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</table>

**Scheme 4. Demethylation of bis-N-methoxymethylated carbopyronines 1b and 1c. Reagents and conditions: (a) BBr_{3}, CH_{2}Cl_{2}, 0 °C; (b) Me_{3}SiCl, CH_{2}Cl_{2}; (c) BBr_{3}, NaI, 15-crown-5, CH_{2}Cl_{2}, –40 to 0 °C; (d) 0.05 M aq. NaOH, THF, 10 °C.**

Apart from demethylation, we also used dye 1c to attempt a peptide-type modification in the linker that contains the carboxyl group (N-methyl-β-alanine bridge). The reagent of choice was l-cysteic acid, which is an easily available bifunctional building block that bears a sulfonic acid residue (see Scheme 5).[23b] Even though dye 1c is well-soluble in water (>5%), the presence of a sulfonic acid group might be crucial for applications requiring very hydrophilic compounds (e.g., for cell microinjections). The high polarity provides compounds with good affinity to glass (which is important, for example, in non-linear optical lithography) and negative affinity to lipophilic fragments in biomolecules. Furthermore, in the long run, it would be very interesting to establish whether or not the SO_{3}H group, as a part of a linker, has an influence on the fluorescence quantum yield, the imaging performance in STED, or the chemical and photostability of the dye. In addition, one could expect that an extra amino acid fragment may elongate the linking bridge and thus reduce undesired interference between the dye core and the labeled site. L-Cysteic acid methyl ester hydrochloride was prepared by a known procedure.[23b] Starting from compound 1c, the modified methyl ester 21a, the free amido acid 21b, and its active 2,3,5,6-tetrafluorophenyl ester 21-TFP were obtained. These kinds of active esters may be used for labeling purposes as alternatives to NHS esters.[24] Active ester 21-TFP proved to be very unstable due to the close proximity of the SO_{3}H group to the free carboxy group in the dye molecule. As a result, this compound did not have a purity exceeding 70% even immediately after the chromatographic fractions were pooled at low temperatures; in the course of freeze-drying, the concentration of this active ester dropped even further. Unlike compound 1c, we failed to obtain the corresponding NHS-ester in this case. The lower stability of 21-TFP resulted in a lower degree of labeling (by a factor of two) compared to that achieved with active ester 1-NHS.
Scheme 5. Peptide-type modification of carbopyronine dye 1c with l-cysteic acid. Reagents and conditions: (a) 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), Et₃N, MeCN, l-cysteic acid methyl ester, 0 °C; (b) 0.05 m aq. NaOH, THF, 10 °C; (c) HATU, Et₃N, 2,3,5,6-tetrafluorophenol, MeCN, 0 °C; (d) HBTU, Et₃N, l-β-alanine methyl ester, MeCN, 0 °C; (e) HATU, Et₃N, MeCN, 0 °C; * HPLC purity of the product immediately after isolation.

Scheme 6. Attempted alternative synthesis of carbopyronine dye 24 (N,N'-dibenzyl analogue of compound 19 in Scheme 2, b) via benzhydrol derivative 23. Reagents and conditions: (a) THF, –78 °C; (b) BCl₃ in CH₂Cl₂, r.t.; (c) P₂O₅ and H₃PO₄, 110–150 °C; (d) Bu₄NIO₄.

Along with the general synthetic approach depicted in Scheme 1, we also tried an alternative pathway that utilized a decorated benzhydrol derivative (structure 23, Scheme 6) as a building block in the cyclization step. As shown in Scheme 6, compound 23 already has an extra benzene ring with a masked carboxylate function (in contrast to the primary carbinol 10 in Scheme 2). In this approach, we attempted to build a carbopyronine core with three aromatic rings (24) in an early step to bypass the oxidation with KMnO₄ (which was considered to be potentially problematic because of the moderate or poor yields reported for...
Red-Emitting Carbopyronine Dyes

Simpler analogs. Also, importantly, benzhydrol 23 itself can be prepared from aldehyde 9, which is an early precursor of our synthesis (Scheme 2), by conventional methods (see the Supporting Information for details). Despite all attempts, the BCl3-assisted cyclization did not proceed. Longer exposure (several days) at ambient temperature resulted in only very slight conversion. Several products were formed, however, none of them produced the required blue color upon oxidation by periodate. Use of polyphosphoric acid (in a mixture with H3PO4) and P2O5 as condensation agents, was also unhelpful. These results are in accordance with previous reports however, as reported in one patent cyclization of this type in the presence of P2O5 was possible although details were not available.

Spectroscopic Properties of the Carbopyronine Dyes

Table 1 lists the maxima of the absorption and emission bands, the maximum absorption coefficients ($\epsilon$), and the fluorescence quantum yields ($\Phi_{fl}$) of the known red-emitting dyes KK114 [see ref. for compound and ref. for applications] and ATTO 647N (a widely used commercial dye provided by Atto-Tec GmbH, Siegen, Germany; for structures, see Figure 1). The positions of the absorption (636–641 nm) and emission (657–664 nm) maxima, the absorption coefficients ($\epsilon = 0.7–0.8 \times 10^5 \text{ m}^{-1} \text{ cm}^{-1}$) and fluorescence quantum yields ($\Phi_{fl} = 0.5–0.6$) of the newly synthesized carbopyronine dyes are very similar, regardless of the presence or absence of the sulfonic acid residues and groups attached to the benzoic acid site (see Scheme 2). This is in contrast to the previously obtained rhodamine dyes, the sulfonation of which had been observed to markedly (up to more than 20%) increase the fluorescence quantum yield. One can assume that the two sulfonic groups in the allylic positions of the rhodamines have a significant effect on the fluorescence. Furthermore, in the new carbopyronines, changing the COOCH3 group to COOH, or OCH3 to OH does not seem to have any noticeable influence on $\Phi_{fl}$. The values of $\epsilon$ are slightly below those of the reference dyes KK114 and ATTO 647N ($\epsilon = 1–1.5 \times 10^5 \text{ m}^{-1} \text{ cm}^{-1}$) and the values of $\Phi_{fl}$ are in the same range as that of ATTO 647N but below those of KK114 ($\Phi_{fl} = 0.8$). The fluorescence quantum yields are large enough to record fluorescence correlation spectroscopy (FCS) data of, e.g., compounds 1c, 21b, and 22, allowing the determination of the rates $k_{isc}$ and $k_T$ of the intersystem-crossing to and from the dark triplet state. We recorded the FCS data and determined the rates in the same way as described in our previous paper. These values, which were obtained from aqueous solutions, are listed in Table 2. The triplet decay time of $k_T = 0.3 \times 10^6 \text{ s}^{-1}$ proved to be the same for all the dyes and correlates well with the values of $k_T$ determined for other organic dyes in water. In contrast, the intersystem-crossing rates differ by a factor of three, i.e., $k_{isc} = 0.5 \times 10^6 \text{ s}^{-1}$ for compound 1c and ATTO 647N, and $k_{isc} = 1–2 \times 10^6 \text{ s}^{-1}$ for compounds 21b, 22, and KK114, respectively. Low intersystem-crossing rates are favorable for fluorescence microscopy because they minimize dark state population and thus maximize signal yield and minimize photobleaching yield.

The increased intersystem-crossing rate of compounds 21b, 22, and KK114, compared to compound 1c, are very likely due to the presence of the sulfonic acid groups (see Table 2). Interestingly, the presence or absence of these groups seems to be more important than the nature of the fluorophore core (rhodamine or carbopyronine).

Table 2. Rates of intersystem crossing ($k_{isc}$) and triplet state depopulation ($k_T$) as determined by FCS for the new carbopyronine and rhodamine dyes with and without sulfonic acid groups.

<table>
<thead>
<tr>
<th></th>
<th>$k_{isc}$ ($\times 10^6$)</th>
<th>$k_T$ ($\times 10^6$)</th>
<th>HO$_3$S group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1c</td>
<td>0.6</td>
<td>0.3</td>
<td>–</td>
</tr>
<tr>
<td>21b</td>
<td>1.1</td>
<td>0.3</td>
<td>+</td>
</tr>
<tr>
<td>22</td>
<td>1</td>
<td>0.3</td>
<td>+</td>
</tr>
<tr>
<td>KK 114</td>
<td>2</td>
<td>0.3</td>
<td>+</td>
</tr>
<tr>
<td>ATTO 647N</td>
<td>0.5</td>
<td>0.3</td>
<td>–</td>
</tr>
</tbody>
</table>

Use of the New Carbopyronine Dyes in Fluorescence Microscopy

To evaluate the performance of the described dyes in microscopic and nanoscopic applications, three dyes (1c, 21b, and 22) were coupled to antibodies and applied in immuno-fluorescence labeling studies. In the conjugated state, the
fluorescence quantum yield for dye 1c was found to be 26%. The decrease in $\Phi_{fl.}$ after bio-conjugation is well-known and was expected. ATTO 647N and the spectrally similar rhodamine dye KK114 (compound 6 in ref.[4] and Figure 1), were used as references.

After immunofluorescence labeling of the tubulin cytoskeleton in PtK2 cells (a well-known model structure), the fixed cells were imaged using conventional confocal microscopy and stimulated emission depletion (STED) nanoscopy (Figure 2).

![Figure 2](image)

Figure 2. Conventional confocal microscopy (left panels) and STED nanoscopy images (right panels) of microtubule in fixed PtK2 cells immunolabeled with compound 1c (a), compound 22 (b), ATTO 647N (c), and KK114 (d).

We found that all the new dyes tested here were suitable for immunofluorescence labeling using convenient standard protocols, and the conventional as well as high-resolution STED images were of sufficient brightness and signal-to-noise ratio. For example, excellent high resolution images were obtained for dyes 1c and 22 (see Figure 2). However, unexpectedly, dye 21b gave the unstable NHS ester and provided images with considerably lower brightness.

To evaluate and quantify the photostability of the newly developed dyes, bleaching curves under confocal (without STED, Figure 3, a) and STED conditions (Figure 3, b) were recorded. For this purpose, we compared the total fluorescence signal of the same area in the immunolabeled cell samples over the course of several scans. Note the unexpected behavior of ATTO 647N in the absence of the STED beam (Figure 3, a) and at high excitation powers (see also Figure S2 in the Supporting Information). This effect may be explained in terms of breaking the nonfluorescent aggregates of the dye molecules, which start to emit after dissociation. Without STED light, under confocal conditions, the new dyes are more photostable than rhodamine KK114, but bleach faster than ATTO 647N. However, under STED conditions, the bleaching rates of the new dyes are enhanced as compared to the exceptionally photostable fluorescent dyes ATTO 647N and KK114. The increased photoreactivity under STED conditions may result from the higher absorption of the STED light by the dye in its first excited singlet or triplet states.[27] Nevertheless, the resolution and brightness of the STED images are excellent for all new dyes, except 21b (see Figure 2 above and Figure S1 in the Supporting Information).

![Figure 3](image)

Figure 3. Photostabilities of the dyes under conventional confocal (a) and STED conditions (b). Relative change in the total fluorescence signal of the same area of the microtubule-immunolabeled cell samples in the course of the progressive scanning. Average excitation power 1 µW at 640 nm and average STED power 118 mW at 760 nm (in the focal spot of the objective lens).
Conclusion and Outlook

The new red-emitting carbopyronine dyes can be used as fluorescent labels in various microscopic and nanoscopic applications. Although less photostable under STED conditions than the spectrally similar dyes KK114 and ATTO 647N, they are excellent for STED nanoscopy and possess certain valuable features that fluorophores KK114 and ATTO 647N do not have. The flexible synthetic approach presented here affords intermediate 18a with a free hydroxyl group in the side chain and a carbonyl group in the benzene ring. The hydroxyl group can be protected, and then the carbonyl group may be transformed into new derivatives with remarkable properties. For example, photosensitive spiroamides[2m,2o,2r,21] or novel caged (masked) carbopyronines[9b] may be obtained. Deprotection of the hydroxyl group followed by its transformation into an amino or thiol reactive site will provide the derivatives required for conjugation with biomolecules. Such options are impossible for KK114 and ATTO 647N in which additional anchoring sites cannot be incorporated unless the synthetic schemes are changed completely.

Analogously to rhodamine spiropiones,[2m,2o,2r,21] carbopyronine amido derivatives must exist predominantly in the “closed” colorless form (e.g., spiropione 17). Some of the spiropiones can be photoactivated and transformed into the colored and fluorescent (zwitter)ionic state. Thermal relaxation reaction of this “open” fluorescent form is very likely to restore the initial nonfluorescent compound so that the whole ring-opening and ring-closing sequence may be repeated several times. Therefore, we expect carbopyronine spiropiones to be a valuable addition to the multicolor toolbox of photochromic spiropiones. On the other hand, the novel caging groups keep the initial cationic compounds nonfluorescent and provide an irreversible transformation into the colored and highly fluorescent derivatives by irradiation with violet or blue (visible) light.[9b]

The real potential of the photochromic and caged red-emitting dyes may be revealed in applications where tracking of dynamic processes is required or when these compounds are used together with the spectrally similar conventional fluorophores (e.g., KK114 or ATTO 647N). Thus, the present work opens the way to novel photochromic and/or caged carbopyronines, the utilization of which will help to push the frontiers of optical microscopy and nanoscopy.

Experimental Section

**General:** UV/Vis absorption spectra were recorded with a Varian Cary 4000 UV/Vis spectrophotometer, and fluorescence spectra with a Varian Cary Eclipse fluorescence spectrophotometer. Reactions were carried out with magnetic stirring in Schlenk flasks equipped with septa or reflux condensers with bubble-covers under argon, using a standard manifold with vacuum and argon lines. Routine NMR spectra were recorded with a Varian VECURY-300 spectrometer operating at 300.5 (1H) and 75.5 (13C) MHz. 1H and 13C NMR spectra were also recorded with Varian INOVA 600 (600 MHz) and Varian INOVA 500 (125.7 MHz) instruments, respectively. All NMR spectra are referenced to tetramethylsilane as an internal standard (δ = 0 ppm) using signals of the residual protons of CHCl3 (δ = 7.26 ppm) in CDCl3, CH2OD (δ = 3.31 ppm) in CD2OD, HOD (δ = 4.75 ppm) in D2O, [D6]acetone (δ = 2.04 ppm) in [D6]acetone or [D6]DMSO (δ = 2.50 ppm) in [D6]DMSO. Multiplicities in the 13C NMR spectra were determined by Attached Proton Test (APT) measurements. Low-resolution mass spectra (electrospray ionization, ESI) were obtained with LCQ and ESI-TOF mass spectrometers [MICRO-TOF (focus), Bruker]. A MICROTOF spectrometer equipped with an ESI ion source (Apollo) and direct injector with LC autosampler (Agilent RR 1200) was used to obtain high-resolution mass spectra (ESI-HRMS). ESI-HRMS were also obtained with an APEX IV spectrometer (Bruker). HPLC system (Knauer): Smartline pump 1000 (2 ×), UV detector 2500, column thermostat 4000 (25 °C), mixing chamber, injection valve with 20 μL loop for the analytical column; 6-port-3-channel switching valve; analytical column: EuroSensor-100 C18, 5 μm, 250 × 4 mm, 1.1 mL/min; solvent A: H2O (HPLC grade) + 0.1% v/v trifluoroacetic acid (TFA); solvent B: MeCN + 0.1% v/v TFA; detection at 636 nm (if not stated otherwise). The reactions were monitored by TLC on MERCK ready-to-use plates with silica gel 60 (F254). Column chromatography: MERCK silica gel, grade 60, 0.04-0.063 mm. While using MeCN/H2O mixtures as mobile phase, compressed air (CAUTION: pressure!) was applied due to the high column resistance. All compounds (including intermediates) were stored in a refrigerator at about +5 °C, unless otherwise stated.

**Fluorescence Microscopy, Sample Preparation, Materials and Methods:** For immunolabeling, cultured PtK2 cells originating from the marsupial (kidney epithelia), *Potorous tridactylus*, and cells of the human osteosarcoma cell line U2OS, were grown on cover slides overnight and fixed with absol. methanol (~20 °C). After washing in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, pH 7) and blocking with 5 % (w/v) bovine serum albumin (Invitrogen Inc., Karlsruhe, Germany), the samples were mounted in MOWIOL containing 0.1 % (w/v) 1,4-diazabicyclo[2.2.2]octane (DABCO). The STED setup used for the high-resolution measurements was described before.[28] The pulsed excitation was performed by a 640 nm laser diode (Picoquant GmbH, Germany), which was triggered by the photo diode signal of the STED laser (MIRA 900, Coherent, USA) running at 760 nm. The donut-shaped intensity distribution required for the STED beam was achieved by adding a phase plate with helical phase retardation (RPC photonics, Rochester, NY, USA) to the STED beam. Scanning was realized by a two-axis beam scanner (Yanus IV, Till Photonics, Germany). Detection of the fluorescence photons was performed with four avalanche photodetectors (APD, Perkin-Elmer), coupled to a multi-mode fiber splitter acting as the confocal pinhole (0.7 times Airy discs), due to the high photon flux.

**N-Benzyl-7-ido-1,2,3,4-tetrahydroquinoline (5-Bn,I):** 7-Iodo-1,2,3,4-tetrahydroquinoline (5-H, I; for the preparation see the Supporting Information) was benzylated with benzyl chloride (BnCl), and the reaction product (5-Bn,I) was isolated as a hydrochloride. In a typical experiment, 5-H, I (hydrochloride; 1.66 g, 5.6 mol) was stirred overnight with finely powdered K2CO3 (1.70 g, 12 mmol), KI (1.66 g, 10 mmol), and BnCl (1.26 g, 10 mmol) in DMF (10 mL). The reaction mixture was diluted with H2O (30 mL), extracted with CH2Cl2 (3 × 30 mL), and the combined organic solu-
tions were dried in Et₂O (50 mL), and the solution was decolorized with silica gel (0.5 g) upon stirring (r.t., 15 min). The mixture was filtered and commercial 5 m HCl solution in 2-propanol (3 mL, ACROS Organics) was added. After 30 min, the fine precipitate was filtered, washed with hexane, and dried in air to furnish 5-H,I (1.35 g, 69%, M = 385) as a salt. To obtain the free amine (5-H,I), the product was shaken with sat. aq. NaHCO₃ (15 mL) and CH₂Cl₂ (20 mL), the aqeous layer was extracted with CH₂Cl₂ (2×15 mL) and the combined organic solutions were dried (Na₂SO₄), decolored with silica gel (0.5 g) upon stirring (r.t., 15 min), and filtered. The solvents were evaporated in vacuo at temperatures not exceeding 35 °C. In the course of the evaporation, hexane (2×10 mL) was added to the residue to completely remove the CH₂Cl₂ (which reacts with BuLi in the next step). Benzyliamined (5-Bn,I; 1.25 g, 64% from 5-H,I) was obtained as beige crystals with m.p. 61–62 °C; (CAUTION: the product is photosensitive). 1H NMR (300 MHz, CDCl₃): δ = 1.98 (qunt, J = 6 Hz, 2 H), 2.78 (t, J = 6 Hz, 2 H), 3.26 (t, J = 6 Hz, 2 H), 4.22 (s, 2 H, CH₂Ph), 6.64 (d, J = 8 Hz, 1 H), 6.82 (s, 1 H), 6.85 (dd, J = 8 Hz, 1 H), 7.20–7.40 (m, 5 H, Ph) ppm. 13C NMR (75.5 MHz, CDCl₃): δ = 21.9 (CH₂), 27.9 (CH₃), 49.4 (CH₂), 54.8 (CH₂Ph), 92.2 (C), 119.1, 121.8, 124.6, 126.6 (2×), 128.3, 128.7, 130.4, 138.0, 146.8 ppm. MS (ESI+): ml/z = 350 [M + H]+. HRMS: calcd. for C₁₉H₂₁N [M + H] 282.1854; found 282.1852.

**N-Benzyl-7-bromo-1,2,3,4-tetrahydroquinoline (7-Bn):** Compound 7-H (17 mmol, 3.70 g) was benzylated with benzyl chloride as described for the iodo-substituted analogue 5-H,I (see above). The reaction was monitored by TLC (hexane/CH₂Cl₂, 3:1), and the product was isolated as a pale-brown oil in 70% yield (4.01 g) after column chromatography (200 g of SiO₂; hexane/CH₂Cl₂, 3:1), and the product was isolated as a pale-brown oil in 70% yield (4.01 g) after column chromatography (200 g of SiO₂; hexane/CH₂Cl₂, 3:1).

1H NMR (300 MHz, CDCl₃): δ = 1.96 (qunt, J = 6 Hz, 2 H), 2.64 (t, J = 6 Hz, 2 H, CH₂), 3.23 (t, J = 6 Hz, 2 H, CH₂), 4.39 (s, 2 H, CH₂Ar), 6.57 (m, 2 H), 6.78 (d, J = 8 Hz, 1 H), 7.16–7.40 (m, 5 H, Ph) ppm. MS (ESI+): ml/z = 302 [M + H]+. HRMS: calcd. for C₁₉H₁₆BrN [M + H] 302.0466; found 302.0463.

**N-Benzyl-7-isopropenyl-1,2,3,4-tetrahydroquinoline (11):** In a typical experiment, carbinol 8 (281 mg, 1.00 mmol) in chlorobenzene (1 mL) was placed in a screw-cap test tube containing KHSO⁴ (136 mg, 1.1 mmol) and a magnetic stirring bar. The test tube was flushed with argon, sealed, and vigorously stirred for 15 min in a preheated oil bath at 140 °C (CAUTION: slight internal pressure!). The reaction mixture was cooled, diluted with hexane (2 mL), and transferred by means of a Pasteur pipette (decanting from the inorganic precipitate) straight into a column charged with SiO₂ (200 g; hexane/CH₂Cl₂, 4:1). The title compound was isolated as a pale-brown oil in 70% yield (4.01 g) after being eluted with hexane/CH₂Cl₂ (6:1) to afford 218 mg (83%) of a “TLC-pure” alkene 11 as a colorless photosensitive oil. 1H NMR (300 MHz, CDCl₃): δ = 0.26 (s, 6 H, CH₃), 1.82 (br, s, 1 H, OH), 2.04 (quint, J = 6 Hz, 2 H, 2.83 (t, J = 6 Hz, 2 H), 3.42 (t, J = 6 Hz, 2 H), 4.58 (s, 2 H, CH₂Ph), 6.76–6.80 (m, 2 H), 6.82 (s, 1 H), 7.02 (d, J = 8 Hz, 1 H), 7.32–7.40 (m, 5 H, Ph) ppm. 13C NMR (75.5 MHz, CDCl₃): δ = 22.7 (CH₂), 28.1 (CH₃), 31.9 (CH₂), 50.2 (CH₂Ph), 55.7 (CH₂Ph), 72.8 (C-OH), 107.7, 112.3, 121.1, 121.7 (2×), 128.5, 128.9 (2×), 129.1, 139.3, 145.6, 148.6 ppm. MS (ESI+): ml/z (%): 304 (100) [M + Na]+. HRMS: calcd. for C₁₉H₂₃NO [M + H] 282.1852, found 282.1854.

**7-Bromo-1,2,3,4-tetrahydroquinoline (7-H):** (Direct bromination of 1,2,3,4-tetrahydroquinoline). The protocol for iodine bromination⁵ was applied to 1,2,3,4-tetrahydroquinoline (4). The substrate 8 (2.5 g, 45 mmol) was added with vigorous stirring to a cooled solution of Ag₂SO₄ (7.67 g, 25 mmol) in conc. H₂SO₄ (70 mL) at 0 °C (ice bath) in a flask equipped with a reflux condenser, and Br₂ (8.5 g, 53 mmol) was added within 10 min at 0 °C. The mixture was then stirred until most of bromine had reacted (2.5 h). An extra portion of Br₂ (1.0 g, 6.3 mmol) was added and stirring was continued for an additional 4 h. The reaction mixture was poured onto crushed ice (300 g) and the solution was filtered. The filtrate was poured onto crushed ice (500 g) and KOH (150 g), and the filtration was repeated. The precipitate and the solution were extracted with CH₂Cl₂ (4×80 mL) and the combined extracts were washed with brine, dried (Na₂SO₄), and the solvents evaporated. The title compound was isolated as a colorless solid by column chromatography (260 g of SiO₂; hexane/CH₂Cl₂, 4:1). 5-Bromo-1,2,3,4-tetrahydroquinoline and the starting material 4, both of which are more polar than the title compound, were also collected in subsequent fractions. The yield of compound 7-H (colorless solid, m.p. 68–69 °C) was 2.52 g (26%). The expected position of bromine atom was confirmed by NMR analysis, and the purity of compound 7-H was assessed by TLC (hexane/CHCl₃, 3:1; Rₚ = 0.15). 1H NMR (300 MHz, CDCl₃): δ = 1.91 (m, 2 H, CH₂), 2.64 (m, 2 H, CH₃), 3.23 (m, 2 H, CH₂), 3.90 (br. s, 1 H, NH), 6.68 (d, J = 9 Hz, 1 H), 6.73 (d, J = 9 Hz, 1 H), 6.78 (d, J = 9 Hz, 1 H) ppm. 13C NMR (75.5 MHz, CDCl₃): δ = 21.7 (CH₂), 26.6 (CH₂), 41.6 (CH₃), 116.3, 119.4, 120.1, 130.7, 145.9 ppm.

**Carboxylic acids 12, 13, and 14:** The condensation was carried out as follows: A 100 mL-Schlenk flask fitted with a septum was flushed with argon and charged with a CH₂Cl₂ solution (40 mL) of compounds 11 (618 mg, 2.35 mmol) and 10 (600 mg, 2.37 mmol; prepared by routine methods from 1,2,3,4-tetrahydroquinoline (4), as described in the Supporting Information), then with BCl₃ (1 M in CH₂Cl₂, 2.70 mL, 2.70 mmol) at 0 °C, and the reaction mixture was stirred at r.t. overnight. Polyphosphoric acid (WVR International, 20 g) was mixed with 85% aq. phosphoric acid and heated to 80–100 °C with manual stirring; the melt was allowed to cool to r.t. and poured into the reaction flask. Through a thick cannula as an outlet, the CH₂Cl₂ was slowly evaporated in an argon purge with stirring and slight heating. The temperature was raised to 110 °C and the mixture was maintained at this temperature for 2 h.
with stirring under a slow argon flow (HCl gas evolved and the viscous material completely dissolved within 15 min). The reaction mixture was allowed to cool and then poured onto ice-cold H2O (120 mL) and CH2Cl2 (100 mL). The viscous material remaining in the flask was thoroughly washed out with MeOH (2 × 10 mL), the combined mixture was well stirred, and the organic phase was separated. The aqueous layer was extracted with CH2Cl2 (3 × 50 mL) and the combined organic extracts were washed with brine (30 mL) and dried with anhydrous K2CO3. The solvent was completely removed in vacuum to afford 1.10 g of residue, which consisted of compound 14 and a very small amount of its oxidized (colored) form (TLC; EtOAc/hexane, 1:1). In initial experiments, compound 14 was isolated in 85% yield by flash chromatography (hexane/EtOAc/CH2Cl2, 2:1:1) as a pale-yellow amorphous material that rapidly oxidized in air to a dark-blue dye with intense red fluorescence; the mass spectrum of the oxidation product was in agreement with structure 14: MS (ESI+): m/z (%) = 497 (100) [M+]. HRMS: calcld for C28H32N2O3 [M+H]+ 498.2951; found 497.2943.

1H NMR (for compound 13, N-benzyl derivative, 300 MHz, CDCl3): δ = 1.12 (s, 6 H, 2 × CH3), 1.82 (m, 4 H, 2 × CH2), 2.70 (t, J = 6 Hz, 4 H, 2 × CH2), 3.36 (t, J = 6 Hz, 4 H, 2 × CH2), 3.63 (s, 6 H, ArCH3), 4.42 (s, 4 H, CH2Ph), 6.52 (s, 2 H), 6.76 (s, 2 H), 7.20–7.40 (m, 5 H, Ph) ppm. 13C NMR (75.5 MHz, CDCl3): δ = 23.4 (CH2), 28.2 (CH2), 39.5 (Cq), 51.0 (C), 51.6 (CH2), 59.1 (OCH3), 70.5 (CH2), 127.8, 131.0, 144.2 ppm.

13C NMR (75.5 MHz, CDCl3): δ = 23.4 (CH2), 28.2 (CH2), 29.7 (CH2), 33.6 (CH3), 50.9 (C), 52.0 (CH2), 59.0 (OCH3), 70.5 (CH2), 108.2, 121.0, 124.0, 129.0 (2 × C), 144.2, 144.5 ppm. HRMS: calcld for C28H32N2O3 [M + Na]+ 457.2825; found 457.2829.

**Red-Emitting Carbopyronine Dyes**

**Amino Ester 16a:** To a solution of the crude compound 3a (820 mg, 1.89 mmol) from the previous step in acetone (20 mL), maintained at -12 to -15 °C (external ice/salt bath), finely powdered KMnO4 (633 mg, 4.00 mmol) was added with vigorous stirring in small portions (8 × 80 mg, 15 min between portions) over a period of 2 h. The solution was stirred for an additional 15 min, diluted with CH2Cl2 (800 mL) and filtered through a paper filter. The brown precipitate was washed with additional CH2Cl2 (2 × 40 mL) and the solvents were evaporated. Ketone 2 was isolated by chromatography on SiO2 (90 g; hexane/EtOAc/CH2Cl2, 1:1:5) to remove the less and more polar impurities. The main fraction afforded 2 (530 mg, 62% from compound 12) as a bright-yellow solid (m.p. 186–188 °C), which was photosensitive and gave intense green fluorescence in solution. The reaction was monitored by TLC (EtOAc/CHCl3, 1:6). 1H NMR (300 MHz, CDCl3): δ = 1.61 (s, 6 H, 2 × CH3), 1.82 (quint, J = 6 Hz, 4 H, 2 × CH2), 2.62 (t, J = 6 Hz, 4 H, 2 × CH2), 3.20 (t, J = 6 Hz, 4 H, 2 × CH2), 3.64 (s, 6 H, ArCH3), 4.22 (br. s, 2 H, NH), 6.60 (s, 2 H), 6.74 (s, 2 H) ppm. 13C NMR (75.5 MHz, CDCl3): δ = 23.3 (CH2), 28.7 (CH2), 29.7 (CH2), 33.6 (CH3), 50.9 (C), 52.0 (CH2), 59.0 (OCH3), 70.5 (CH2), 108.2, 121.0, 124.0, 129.0 (2 × C), 144.2, 144.5 ppm. HRMS: calcld for C34H33N2O3 [M + Na]+ 471.2721; found 449.2792.

**Amino Ester 16b:** In an argon-flushed Schlenk flask (50 mL) fitted with a septum, 2-(2-bromophenyl)-4,4-dimethyl-2-oxazoline (635 mg, 2.5 mmol) in anhydrous THF (12 mL) was lithiated with tBuLi (1.5 M in pentane, 1.75 mL, 2.63 mol) at -78 °C, and the mixture was stirred for 2 h at this temperature to form the reagent 15. A solution of ketone 2 (224 mg, 0.50 mmol) in anhydrous THF (6 mL) was added and stirring was continued for an additional 6 h at -78 °C, then overnight at 0 °C (ice bath). The reaction mixture was poured into a stirred ice-cold solution of glacial HOAc (1 mL) in MeOH (15 mL), and the resulting dark-blue solution was evaporated in vacuo. The residue was separated on SiO2 (30 g; MeCN/H2O, 10:1) to separate the yellow and blue impurities, then eluted with a MeCN/H2O (3:1) mixture containing 0.2% (vol.) TFA. The dark-blue fluorescent eluate was collected and the solvents were evaporated at a temperature below 38 °C in vacuo to a volume of 10 mL. The residue was shaken with CH2Cl2 (80 mL) and sat. NaHCO3 (20 mL), and the organic layer was separated, dried
Carbopyronine Derivatives 1a, 18a, and 18b (Acidic Hydrolysis of 16a): In a typical experiment, the hydrolysis was carried out as follows: Compound 16a (380 mg, 0.45 mmol) was dissolved in a mixture of concd. HCl (20 mL, 0.24 mol) and H2O (10 mL) and heated with stirring in a flask fitted with a reflux condenser for 18 h at 80 °C. The yellow solution was diluted with an equal volume of H2O, then CH2Cl2 (40 mL) and EtOAc (60 mL) were added, and the mixture was neutralized with solid NaHCO3 (22 g, 0.26 mol) under vigorous stirring in a 600-mL beaker. The liquid was decanted from the small amount of solid and the aqueous layer was separated and extracted with CH2Cl2 (5 × 40 mL), until the extract became colorless. The combined organic layer was dried (Na2SO4), evaporated in vacuo, and the residue was dissolved in a mixture of MeCN (20 mL) and CH2Cl2 (15 mL) and separated on SiO2 (80 g; MeCN/H2O, 20:1→5:1). The homogeneous fractions were pooled, stepwise filtered through Rotilabo® syringe filters (0.80 and 0.22 μm), and the solvents were evaporated in vacuo at a temperature not exceeding 38 °C. The following HPLC-pure compounds were isolated (in order of elution): 1a (155 mg, 62%), 18b (66 mg, 27%), and 18c (7 mg, 3%), see Table 3. All the compounds were pale-blue solids with decomposition temperatures of 176–178 °C; they were almost colorless in non-polar solvents, dark-blue in MeOH and MeCN, and turned blue on silica gel, particularly on TLC plates. The reaction was monitored by HPLC (A/B 70:30–10:00 in 25 min); tR = 15.8, 13.2, and 10.4 min, respectively. The yields values were in a good agreement [±(10–15)%] with the HPLC areas.

Table 3. Influence of reaction time on yields.

<table>
<thead>
<tr>
<th>Reaction time [h]</th>
<th>Product yields (isolated) [%]</th>
</tr>
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<tbody>
<tr>
<td>1a</td>
<td>18a</td>
</tr>
<tr>
<td>14</td>
<td>65</td>
</tr>
<tr>
<td>18</td>
<td>62</td>
</tr>
<tr>
<td>22</td>
<td>52</td>
</tr>
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</table>

1a: 1H NMR (300 MHz, CDCl3): δ = 1.70 (s, 3 H, CH3), 1.78 (s, 3 H, CH3), 1.80 (m, 4 H, 2 × CH2), 2.48 (m, 4 H, 2 × CH2), 3.30 (m, 4 H, 2 × CHN), 3.38 (s, 6 H, 2 × OCH3), 3.46 (m, 4 H, 2 × CH2N), 3.60 (m, 4 H, CH2O), 6.22 (d, J = 9 Hz, 2 H), 6.76 (s, 2 H), 7.06 (d, J = 9 Hz, 1 H), 7.54 (m, 2 H), 7.98 (d, J = 9 Hz, 1 H). 13C NMR (75.5 MHz, CDCl3): δ = 22.0 (CH2), 27.6 (CH3), 33.3 (CH2), 35.1 (CH1), 37.7 (CH2), 50.3 (CH2), 51.3 (CH3), 59.1 (OCH3), 69.9 (C), 107.4, 118.4, 121.5, 123.9, 124.7, 127.1, 128.1, 128.5, 134.3, 144.7, 145.6, 155.6, 171.0 (C=O) ppm. MS (ESI+): m/z (%) = 535 (100) [M + H]+. HRMS: calcd. for C35H40N2O4 [M + H]+ 553.2748; found 553.2743.

18a: 1H NMR (300 MHz, CDCl3): δ = 1.62 (s, 3 H, CH3), 1.68 (s, 3 H, CH3), 1.80 (m, 4 H, 2 × CH2), 2.50 (m, 4 H, 2 × CH2), 3.08 (m, 4 H, 2 × CH3), 3.38 (s, 6 H, 2 × OCH3), 3.46 (m, 4 H, 2 × CH2N), 3.60 (m, 2 H, CH2O), 6.22 (d, J = 6 Hz, 2 H, CH2OH), 6.76 (s, 2 H, 6.82 (s, 2 H), 7.06 (d, J = 9 Hz, 1 H), 7.54 (m, 2 H), 7.98 (d, J = 9 Hz, 1 H) ppm. 13C NMR (75.5 MHz, CDCl3): δ = 22.0 (CH2), 27.6 (CH3), 33.3 (CH2), 35.1 (CH1), 37.7 (CH2), 50.3 (CH2), 51.3 (CH3), 59.1 (OCH3), 69.9 (C), 107.4, 118.1, 121.2, 121.5, 125.0, 127.2, 128.4, 128.5, 134.3, 145.0, 145.3, 145.9, 151.0 (C=O) ppm. MS (ESI+): m/z (%) = 539 (100) [M + H]+. HRMS: calcd. for C39H50N3O4 [M + H]+ 624.3799; found 624.3796.

18b: 1H NMR (300 MHz, CDCl3): δ = 1.60 (s, 3 H, CH3), 1.78 (s, 3 H, CH3), 1.80 (m, 4 H, 2 × CH2), 2.04 (br. s, 1 H, OH), 2.50 (m, 4 H, 2 × CH2), 3.30 (m, 4 H, 2 × CH2), 3.40–3.60 (m, 4 H, 2 × CH2N), 3.70–3.90 (m, 4 H, 2 × CH2O), 6.60 (m, 2 H), 7.06 (m, 2 H), 7.54 (m, 1 H), 7.60–7.80 (m, 3 H) ppm. MS (ESI+): m/z (%) = 525 (100) [M + H]+. HRMS: calcd. for C31H38N2O3 [M + H]+ 525.2748; found 525.2743.
Dyes 20a and 20b (Demethylation Products): To an ice-cold solution of 1b (20 mg, 29 μmol) in CH2Cl2 (10 mL), a commercial BB3 solution (1 mL in CH2Cl2, 0.17 mL, 0.17 mmol) was added and the mixture was stirred for 2 h at 0 °C with TLC monitoring (MeCN/H2O, 7:1). The yellow solution was thoroughly washed with sat. aq. NaHCO3 (6 mL), the aqueous layer was extracted with CH2Cl2 (3 × 10 mL), the combined organic layer dried (Na2SO4), and the solvents evaporated. The residue was purified by chromatography over SiO2 (12 g, MeCN/H2O, 61 → 21). The main fraction was evaporated, filtered and freeze-dried, as described for 1b (see above), to afford ester 20a (11 mg, 60%) as an amorphous solid. The corresponding acid 20b was also isolated as a by-product in 15% yield. Under the same conditions and with the same workup, acyl 1c was demethylated to 20b (see below for the spectroscopic data) with 74% isolated yield.

The alkaline hydrolysis of 20a was carried out as described above for 1b (see above): To a solution of 20a (11 mg, 17 μmol) in a mixture of THF (3 mL) and H2O (2 mL), aq. NaOH (0.1 mL, 0.6 mL, 0.06 mmol) was added and the mixture was kept for 3 h at 5–10 °C, acidified with H2OAc (0.06 mL, 0.10 mmol), evaporated and separated over SiO2 (8 g; MeCN/H2O, 5:1 → 21). Solvent removal (as described above for 1b) afforded acid 20b (8.4 mg, 81%). HPLC: τR (20a) = 11.5 min, τR (20b) = 9.7 min (A/B; 70:30–0:100 in 25 min).1H NMR (300 MHz, CDCl3; 20b: signals of the major invertermer are marked with an asterisk *): δ = 1.58/1.61* (s, Σ = 3 H, CH3), 1.66/1.80 (s, Σ = 3 H, CH3), 1.82 (m, 4 H, 2 × CH2), 2.02 (t, J = 6 Hz, 2 H, CH2-β-Ala), 2.50–2.60 (overlapped: m, 2 H, CH2-β-Ala, m, 4 H, 2 × CH2), 2.82 (s, 3 H, NCH3), 3.30–3.45 (br. m, 4 H, 2 × CH2N), 3.50 (s, 3 H, CH3O), 3.52–3.80 (br. m, 4 H, MeOH), 3.75 (m, 4 H, 2 × CH2), 4.80–5.01 (br. m, 2 H, 2 × OH), 6.68 (s, 2 H), 7.20 (s, 2 H), 7.33 (m, 1 H), 7.48 (m, 2 H), 7.61 (m, 1 H) ppm. MS (ESI+): m/z (%) = 624 (100) [M+H]+. HRMS: calcld. for C18H22N2O5 [M+H]+ 624.3432; found 624.3430.20b: H NMR (300 MHz, CDCl3; 20b: signals of the major invertermer are marked with an asterisk *): δ = 1.58/1.61* (s, Σ = 3 H, CH3), 1.66/1.80 (s, Σ = 3 H, CH3), 1.82 (m, 4 H, 2 × CH2), 2.02 (t, J = 6 Hz, 2 H, CH2-β-Ala), 2.44 (t, J = 6 Hz, 2 H, CH2-β-Ala), 2.58 (m, 4 H, 2 × CH2N), 2.90 (s, 3 H, NCH3), 3.33–3.60 (br. m, 4 H, 2 × CH2N), 3.52–3.80 (br. m, 4 H, MeOH), 3.75 (m, 4 H, 2 × CH2), 4.80–5.01 (br. m, 2 H, 2 × OH), 6.68 (s, 2 H), 7.20 (s, 2 H), 7.33 (m, 1 H), 7.48 (m, 2 H), 7.61 (m, 1 H) ppm. MS (ESI+): m/z (%) = 630 (100) [M + Na–H]+. HRMS: calcld. for C17H21N2O5 [M+Na]+ 610.3275; found 610.3271.

Dye 21a (Peptide-Type Coupling with t-Cysteine Acid): In a typical experiment, compound 1e (4.0 mg, 6.3 μmol) in a screw-cap vial was sonicated for 3 min in anhydrous MeCN (HPLC grade; 0.5 mL) and reacted with methyl cysteate hydrochloride2b (1.55 mg, 7.0 μmol) in the presence of HBTU (2.5 mg, 6.6 μmol) and Et3N (6.0 μL, 42 μmol) with stirring at 0 °C for 1 h. The reaction solution was diluted with MeCN (3 mL) and charged straight onto a column of silica gel (5 g). Elution with MeCN/H2O (10:1) followed by evaporation, filtration (Rothlab® syringe filters, 0.22 μm) and freeze-drying afforded 21a (4.2 mg, 83%) as a dark-blue amorphous solid, which was well-soluble in H2O, MeOH, MeCN, and CHCl3. TLC: τR = 0.30 (MeCN/H2O, 10:1); HPLC: τR = 12.3 min (A/B; 70:30–0:100 in 25 min).1H NMR (300 MHz, CDCl3; 21a): δ = 1.56/1.82* (s, Σ = 3 H, CH3), 1.72/1.82 (s, Σ = 3 H, CH3), 1.90 (m, 4 H, 2 × CH2), 2.20–2.30 (m, 2 H, CH2-β-Ala), 2.54–2.60 (overlapped: m, 2 H, CH2-β-Ala, m, 4 H, 2 × CH2), 2.82/3.60 (s, Σ = 3 H, NCH3), 3.36 (s, 6 H, 2 × CH2O), 3.58 (m, 4 H, 2 × CH2N), 3.60 (s, 3 H, CO2CH3), 3.68 (t, J = 6 Hz, 2 H, -Ala), 4.00 (d, J = 6 Hz, 2 H, -N-Chloro) ppm.
Dye 21b (Free Acid): To a solution of 21a (74 mg, 0.92 mmol) in a mixture of THF (15 mL) and H₂O (10 mL),aq. NaOH (1 mL, 0.30 mmol) was added, and the mixture was kept for 2 h at 0 °C, acidified with HOAc (10.1 mmol, 1.80 mmol), evaporated and purified by chromatography over SiO₂ (36 g; MeCN/H₂O, 5:1→3:1). Solvent removal (as described for 1b, with filtration and centrifugation) afforded acid 21b (66 mg, 91%) as a dark-blue fine crystalline powder that was well-soluble in H₂O and MeOH, sparingly soluble in MeCN, and insoluble in CHCl₃. TLC: Rₗ = 0.12 (MeCN/H₂O, 5:1); HPLC: tᵣ = 10.4 min (A/B: 70:30→0:100 in 25 min). ¹³C NMR (300 MHz, [D₆]MeOH): signals of the major isomer are marked with an asterisk *: δ = 1.59/1.62* (s, Σ = 3 H, CH₃), 1.78/1.92* (s, Σ = 3 H, CH₂), 1.82 (m, 4 H, 2 CH₂), 2.08–2.12 (m, 2 H, CH₂ β-Ala), 2.36–2.40 (m, 2 H, CH₂ β-Ala), 2.60 (m, 4 H, 2 × CH₂), 2.66/2.92* (s, Σ = 3 H, NCH₃), 3.32 (m, 2 H, 2 × CH₂), 3.38 (s, 6 H, 2 × OCH₃), 3.68 (t, J = 6–7 Hz, 2 H, CH₂SO₃), 3.72 (m, 4 H, 2 CH₂), 3.96 (m, 4 H, CH₂O), 4.45 (br. s, 1 H, NH), 4.82 (overlapped: m, 1 H, CH₂S + HO), 6.78 (d, J = 7 Hz, 2 H, 7.24 (d, J = 7 Hz, 2 H), 7.40 (m, 1 H), 7.64–6.76 (m, 3 H)) ppm. ¹²³NMR (75.5 MHz, MeCN-CN): δ = 21.7 (CH₃), 25.8 (CH₂), 28.6 (CH₂), 31.8 (CH₃), 32.2 (CH₂), 35.3/38.4* (CH₃), 41.6 (CH₃), 44.0 (CH₂), 52.1 (CH₂), 52.6 (CH₃), 53.0 (CH₂), 59.2 (OCH₃), 70.9 (C), 71.0 (C), 111.4, 111.6, 121.9, 124.0, 128.1, 131.1, 133.5, 130.5, 135.0, 137.3, 145.3, 156.2, 162.1/169.0 (C=O), 171.0/171.9* (C=O) ppm. MS (ESI+): m/z (%) = 833 [M + 2Na⁺]−; MS (ESI−): m/z (%) = 787 [M − Na⁺]. HRMS: calcd. for C₁₇H₁₈N₂O₄S [M + Na⁺] 979.3897; found 979.3896.

Active Ester 22-NHS: Dye 22 (5 mg, 6 µmol) in anhydrous MeCN (4 mL, HPLC grade) was treated with N-hydroxysuccinimide (7 mg, 35 µmol) in the presence of HATU (17 mg, 45 µmol) and Et₃N (7 µmol) in a screw-cap vial at room temp. The maximum conversion (82%) was reached in 2 h, as shown by HPLC (Rₗ = 10.9 min; A/B: 70:30–0:100 in 25 min) and TLC (Rₗ = 0.20; MeCN/H₂O, 5:1) analyses. The reaction solution was loaded directly into a short column with SiO₂ (4 g; MeCN/H₂O, 10:1) and the first colored fraction was collected, quickly evaporated in vacuo at room temp. to the volume of about 10 mL, filtered through a Rotilabo® syringe filter (0.22 µm), and freeze-dried to afford 6 mg of a blue solid material containing few percent of the starting acid (tᵣ = 9.2 min) and N-hydroxysuccinimide (HPLC analysis with detection at 636 nm showed a 93% area for the peak of 22-NHS). MS (ESI+): m/z (%) = 979 [M + Na⁺]. HRMS: calcd. for C₄₉H₆₀N₆O₁₂S [M + Na⁺] 979.3882; found 979.3897. The material was stored under argon at −20 °C for use in labeling studies.

Supporting Information (see also the footnote on the first page of this article): Syntheses and properties of some early precursors and side-products, additional STED images with dyes 22 and KK114, additional bleaching curves for dyes ATTO 647N, KK114, 1c, 21b, and 22, full absorption and emission (UV/Vis) spectra of fluorescent carbonylone dye 1c.

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