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

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Biological microscopy favors photostable fluorescent markers with large fluorescence quantum yields, low dark triplet state population, 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 ($k_{isc} \approx 0.5 \times 10^6\ s^{-1}$) 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 (photoactivtion localization microscopy),[2i,2s] STORM (stochastic reconstruction microscopy)[2j,2u,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 device and improve photostable red-emitting dyes are being undertaken in a number of research groups. Recent publications on this topic describe water-soluble terrylenediimides,[4a,4b] soluble quaterrylenediimides[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

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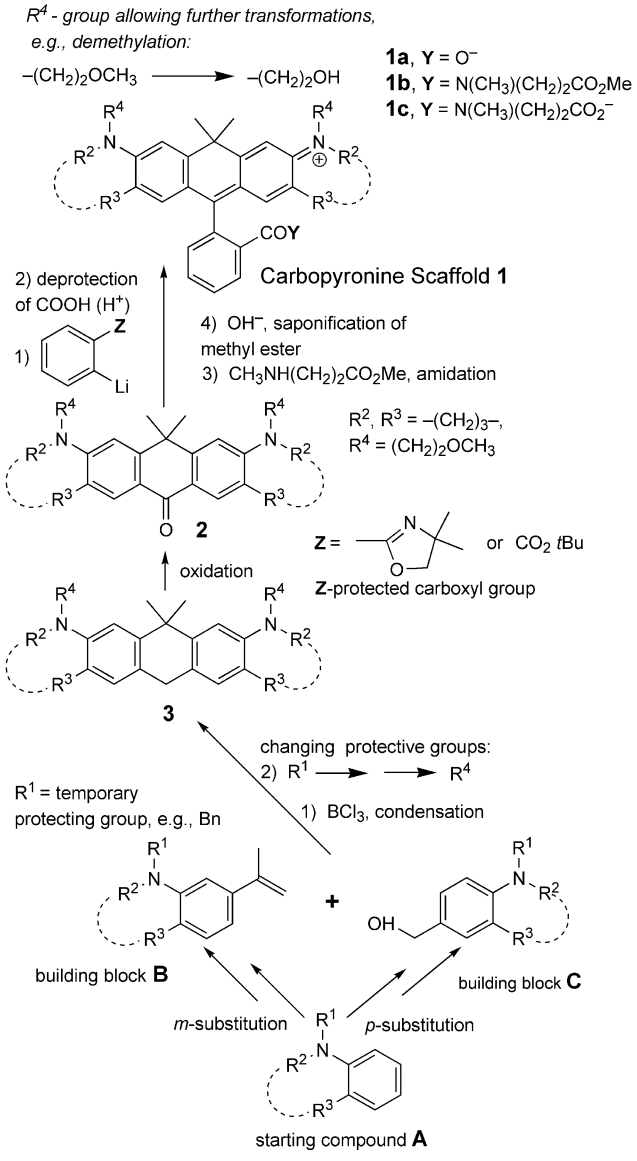
quantum yields, high photostabilities and variable hydrophilicities, they compete well in microscopy with the commercially available near IR fluorophores such as ATTO 633, ATTO 647N, Alexa 633, and Alexa 647. The presented rhodamine dyes have maxima of absorption and emission at 630–640 nm and 660 nm, 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 [$C(CH_3)_2$], 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 *o*-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.[4] 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 (SO_3H) to be water-soluble.



Scheme 1. General retrosynthetic scheme for a carbopyronine scaffold (**1**) with variable substituents.

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 π-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 $\Phi_{fl.}$ 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 π-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 **1** outlined in Scheme 1).

The initial transformations in Scheme 1 are based on the approach first described by Frantzeskos for the simplest carbopyronine dyes – *N*,*N*′-dimethyl derivatives (without methylene bridges).[6b] 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 **1a**–**c**. It is noteworthy that this option has not been explored before. From the details that are available in the patent literature,[6d–6e,7] 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 **A**), which is transformed into two building blocks (**B** and **C**) that are, in turn, utilized for the subsequent condensation step. The building blocks **B** and **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^4) that allow further chemical transformations. We purposely planned scaffold **1** 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 *o*-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[2m–2r] and novel caged dyes[9]).

On the way to Scaffold **1**, one last obstacle remained. Protective groups and residues (R^1 and R^4) that were suitable for the whole synthetic sequence starting from compound **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-β-alanine bridge, which was used in our previous studies.[2p,4] Esterification or amidation of the carboxylic group in the *o*-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 β-alanine residue has a sterically unhindered carboxyl group that is suitable for further conjugation reactions.

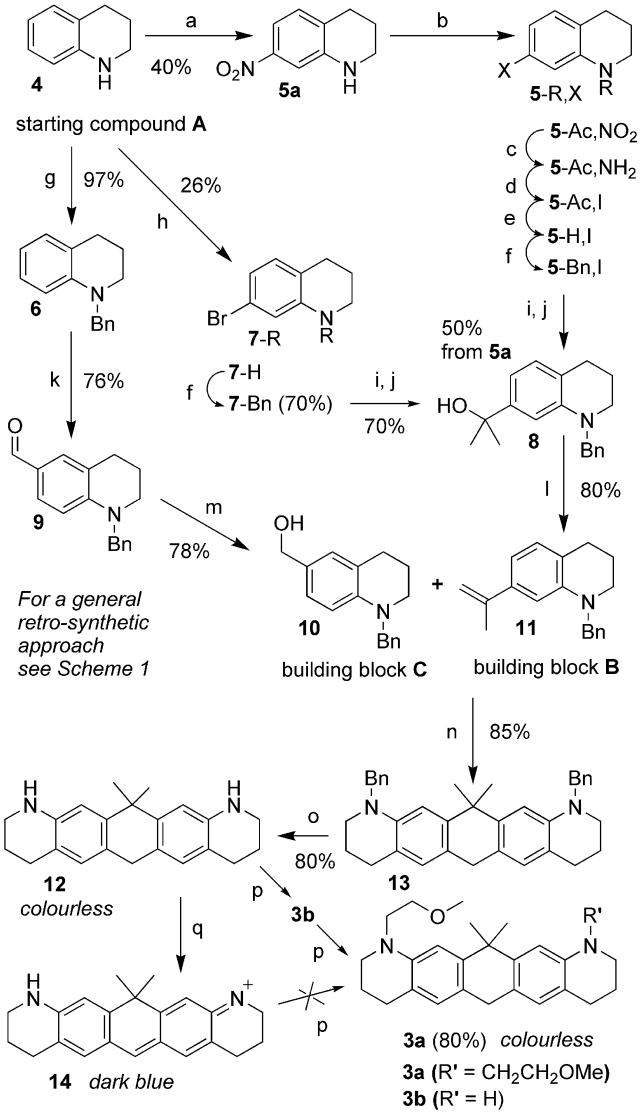
Description of the Synthesis

The first crucial step of the synthesis is the condensation of building blocks **B** and **C**, both of which are prepared from the same precursor **A**. According to the patent literature,[6d–6e,7] the condensation is best assisted by 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[10] (e.g., HCOOH or $HCOONH_4$)[11] 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 **1**.

Both building blocks **B** and **C** originate from compound **A**. We considered 1,2,3,4-tetrahydroquinoline derivatives as candidates for the starting compound **A** with methylene bridges of a reasonable length. As regards building block **B**, there is one difficulty that had to be addressed. Whereas *para*-electrophilic substitution in **A** presents no difficulty,[12] the *meta*-type substitution (directed by the protonated secondary aromatic amino group) does not proceed very easily and cleanly (see ref.[13] 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).



Scheme 2. Synthesis of the key precursor **3a** for carbopyronine dyes. Reagents and conditions (isolated yields are represented by average values obtained from 2–3 experiments): (a) 98% HNO_3/96% H_2SO_4, 10 °C, 2 h; (b) Ac_2O, pyridine, 90 °C, 1 h; (c) H_2, Pd/C (10% Pd), r.t., 16 h; (d) KNO_2/glacial HOAc, KI, 0 °C, 24 h; (e) 5% NaOH in aq. MeOH, reflux, 30 h; (f) BnCl, KI, K_2CO_3, DMF, r.t., 2 h; (g) BnCl, KOAc, HOAc, DMF, r.t., 20 h; (h) Br_2, Ag_2SO_4 in H_2SO_4; (i) *n*BuLi (2.5 M in hexanes), –78 °C,1 h; (j) Me_2CO, –78 °C, 0.5 h; (k) $POCl_3$/DMF, $ClCH_2CH_2Cl$, 0 °C; reflux, 1 h; (l) $KHSO_4$, PhCl, 130–140 °C, 15 min; (m) $NaBH_4$, EtOH, r.t., 1 h; (n) BCl_3 in CH_2Cl_2, r.t., 16 h, then P_2O_5 and H_3PO_4, 110 °C, 2 h; (o) Pd/C (10% Pd), MeOH/Et_2O, 40–50 °C, 0.5 h; (p) $Br(CH_2)_2OMe$, K_2CO_3, DMF, 100 °C, 2.5 h; (q) air oxygen.

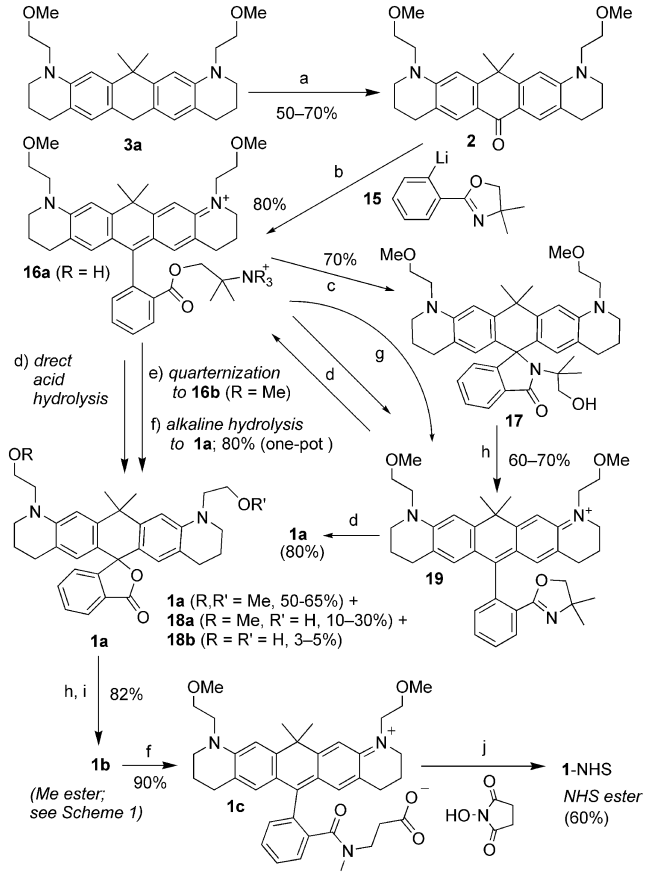
Secondly, a very important option would be the ability to remove or to modify this group (or a part of it) so that the resulting molecule becomes even more polar and hydrophilic. As a suitable candidate for R^4, we chose the 2-methoxyethyl group (-$CH_2CH_2OCH_3$). This group can resist oxidants under mild conditions, and can also be smoothly demethylated.

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 (CO_2Me) function was described.[15] Once the reaction proceeds, the resulting 2-hydroxyethyl group could be subjected to various transformations. The CO_2Me function, at the same time, would remain unchanged and could be hydrolyzed for conjugation purposes in the very final stage. The methoxy group (CH_3O) 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 carbopyronine 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 dehydratation 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).[13b] 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 by 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-1*H*-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 benzylation; the reductive benzylation with benzaldehyde gave lower yields, especially for **6**. The conditions used for the dehydratation of carbinol **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 is 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



Scheme 3. The actual synthesis of the carbopyronine dye **1c**: (a) $KMnO_4$, Me_2CO, 0 °C; (b) **15**, THF, –78 to 0 °C; MeOH, AcOH; (c) aq. NaOH, r.t. 1 h; (d) 20% aq. HCl, 80 °C, 16 h; (e) MeI/DMF, K_2CO_3, r.t.; (f) aq. KOH, r.t.; (g) 2% aq. H_2SO_4/AcOH, 80 °C, 8 h; (h) $POCl_3$, $ClCH_2CH_2Cl$, 70 °C, 3 h; (i) $MeNH(CH_2)_2CO_2Me$, Et_3N, CH_2Cl_2, –10 °C; (j) HATU [2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] Et_3N, MeCN, 0 °C.

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_2SO_4 in sulfuric acid was studied by Miyake and Kikugawa.[13a]

According to these authors, the separation of the isomeric bromides by means of gas chromatography was problematic (in fact, the separation was easier for the corresponding dihydroindoles in the next step of their synthesis). However, when we applied the bromination procedure to 1,2,3,4-tetrahydroquinoline (**4**), it was possible to isolate the pure 7-bromo derivative **7**-H by means of standard column chromatography. Even though the isolated yield of the required regioisomer was modest (although only slightly less than that achieved in HF/SbF_5 superacidic medium, see later),[13c] the bromination protocol is straightforward and allows five steps to be omitted (see Scheme 2). The condensation of compounds **10** and **11**, and the subsequent cyclization, were executed in one pot with excellent yields, so the purification of carbopyronine **13** was not required. The Pd-assisted debenzylation to compound **12** proceeded far easier and cleaner than expected, considering the relatively long timescales and the yields reported in the literature for some simple substrates.[11a,11b]

Compounds **12** and **13** are readily oxidized by air oxygen, which is typical for carbopyronines, as established by Frantzeskos.[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 and chromic anhydride in 1,4-dioxane and acetic acid, respectively. Unfortunately, only trace amounts of **2** were detected, even though CrO_3 worked well for some similar substrates,[18] and SeO_2 is known to be a "softer" oxidant. In our experiments with $KMnO_4$, when the protocol mentioned above was reproduced,[6b] the yield was poor (15%). However, the yield was increased to 70% by lowering the reaction temperature and controlling the rate of $KMnO_4$ addition. It is noteworthy that, in our experiments, neither acetic acid nor base (NaOH or Na_2CO_3) were helpful for this oxidation reaction. In the next step of the synthesis, the third aromatic ring, which bears a protected (preconstructed) carboxyl function, is attached. To this end, the corresponding aryllithium reagent (reagent **15**, Scheme 3) was preprepared, then reacted with the ketone and, finally, the carboxyl function was liberated. As seen in a number of examples,[19] 4,4-dimethyl-2-oxazoline is widely used as a carboxyl synthon. The deprotection is normally performed by heating in aqueous HCl. Drexhage and co-workers used 2-(2-bromophenyl)-4,4-dimethyloxazoline (reagent **15**, Scheme 3; for its preparation see ref.[19d]) to synthesize some carbopyronine dyes; these syntheses always involved acid-assisted demasking procedures.[6b,7]

It was not clear that the same approach would be applicable to our substrates (**16a** or **19**) because they contain 2-methoxyethyl groups (R^4), which are sensitive to mineral acids. In fact, most alkyl ethers undergo dealkylation in strong acid media.[14] On the other hand, *tert*-butyl esters are known to be cleaved by acids under milder conditions.[20] However, attempts to lithiate *tert*-butyl 2-bromobenzoate (Scheme 1) and use it as a substitute for the Li-containing reagent **15** failed. Arylation of ketone substrate **2** with reagent **15**, followed by aqueous workup, led to amino ester **16a**, the oxazoline ring of which has already

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 *t*BuLi 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[21] 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 H_2SO_4, or with $POCl_3$ 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-methoxyethyl groups were found to have undergone complete demethylation to form compound **18b** ($R^1 = R^2 = H$). 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;[22] 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.[22] 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 K_2CO_3, 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 methylates the amine to the quarternary 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 R^4 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 $POCl_3$ 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[2p] 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, water-soluble (>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-

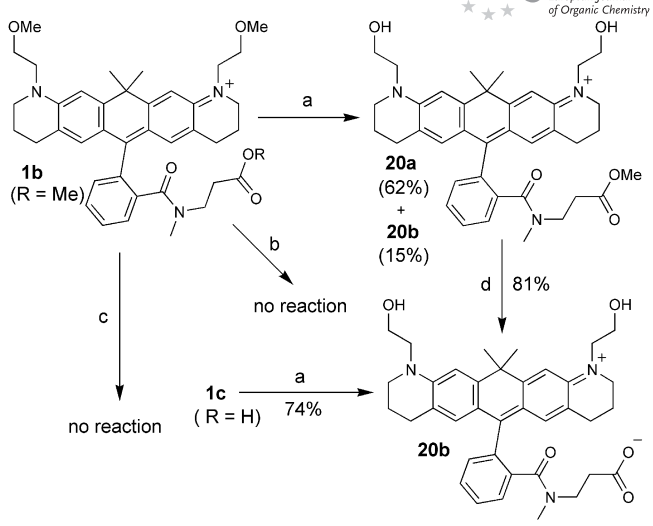
rivative that can be used for conjugation – formed smoothly and proved to be stable enough to be isolated by standard column chromatography using a water-containing mobile phase.

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]

	λ_{max} [nm] (abs.)	λ_{max} [nm] (fl.)	$\varepsilon \times 10^{-5}$ [$M^{-1}cm^{-1}$]	Φ_{fl} [%][b] H_2O	MeOH
16a	636	657	1.14	62	73
1b	639	661	0.89	58	70
1c	640	662	0.89	53	68
20a	640	661	0.70	53	68
20b	641	663	0.80	53	65
21a	640	663	0.70	56	70
21b	640	664	0.68	56	69
22	640	664	0.67	54	68
KK 114	637	660	0.94	80	–
ATTO 647N	644	669	1.5	65	–
Conjugate antibody					
1c	642	665	–	26[c]	–

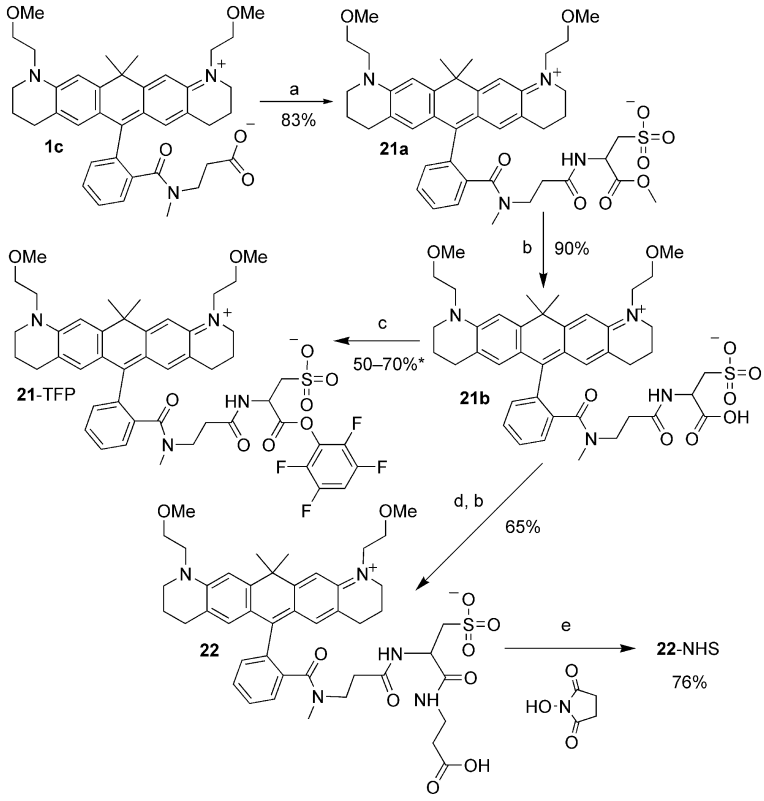
[a] λ_{max}(abs.), λ_{max}(fl.), ε, and Φ_{fl}. are absorption maxima, emission maxima, extinction coefficient, and fluorescence quantum yield, respectively, in H_2O. [b] ATTO 633 was used as a reference. [c] In aqueous PBS buffer with degree of labelling (DOL) 4.4, see Exp. Section for details.

To explore the possibility of further modifications of dye **1a**, we attempted a demethylation, which was potentially the most troublesome step of the whole sequence. In the course of the demethylation, it was important to keep the carboxyl methyl ester group intact because successfully achieving this would demonstrate the possibility of further stepwise modifications on the dye, the carboxyl group of which is protected up to a certain synthetic step. To this end we used methyl ester **1b** as a test substrate. First, we attempted to reproduce the classical protocols that utilize trimethylsilyl iodide (TMS-I)[14] or BBr_3 in the presence of NaI and 15-crown-5 (see Scheme 4).[15] None of these approaches were successful, which was unexpected. Instead, we found that simple treatment with a solution of BBr_3 in dichloromethane furnished the desired demethylated compound **20a** in good yield. The corresponding acid **20b** was also formed as a by-product in moderate yields. Another possible by-product, the 2-bromoethyl derivative (where OH is replaced with Br, as takes place in many other substrates),[14] was not detected at all, which demonstrates the high selectivity of the demethylation. Accordingly, under the same reaction conditions, compound **1c** (the free acid) was cleanly demethylated to **20b**. This may be significant for preparative chemistry because, as our literature survey showed, no reports on the demethylation of substrates containing a tertiary nitrogen connected to a methoxyalkyl group have been published. Thus, the opportunity to smoothly vary substituents in the final dye (depicted by Scaffold **1**) has been demonstrated.



Scheme 4. Demethylation of bis-*N*-methoxyethylated carbopyronines **1b** and **1c**. Reagents and conditions: (a) BBr_3, CH_2Cl_2, 0 °C; (b) Me_3SiI, CH_2Cl_2; (c) BBr_3, NaI, 15-crown-5, CH_2Cl_2, –40 to 0 °C; (d) 0.05 M aq. NaOH, THF, 10 °C.

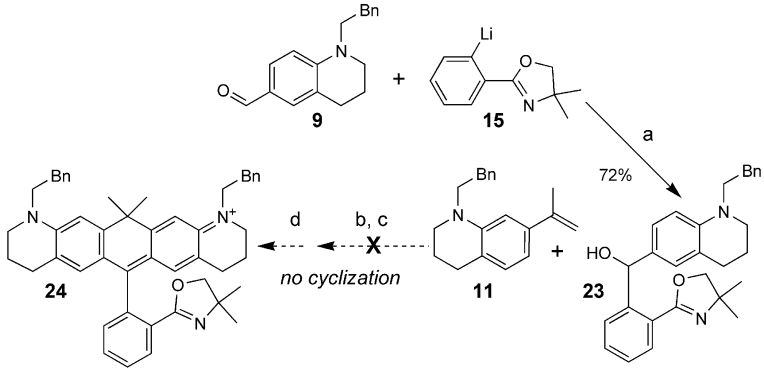
Apart from demethylation, we also used dye **1c** to attempt a peptide-type modification in the linker that contains the carboxyl group (*N*-methyl-β-alanine bridge). The reagent of choice was L-cysteic acid, which is an easily available bifunctional building block that bears a sulfonic acid residue (see Scheme 5).[23a] 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 nanolithography) and negative affinity to lipohilic fragments in biomolecules. Furthermore, in the long run, it would be very interesting to establish whether or not the SO_3H 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_3H 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-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), Et_3N, MeCN, L-cysteic acid methyl ester, 0 °C; (b) 0.05 M aq. NaOH, THF, 10 °C; (c) HATU, Et_3N, 2,3,5,6-tetrafluorophenol, MeCN, 0 °C; (d) HBTU, Et_3N, L-β-alanine methyl ester, MeCN, 0 °C; (e) HATU, Et_3N, MeCN, 0 °C; * HPLC purity of the product immediately after isolation.

(which is far easier to manipulate). In addition, the conjugates obtained with **21**-TFP were insufficiently bright under the conditions of STED imaging. To improve the performance of the modified dye **21b**, we extended the linking group by an additional β-alanine fragment, which only slightly increased the overall molecular weight. Dye **22**, which was spectrally identical to **21b** (see below), and its active ester **22**-NHS were prepared by conventional methods. As expected, ester **22**-NHS demonstrated very good hydrolytic stability, which is helpful for the standard conjugation protocol.

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 $KMnO_4$ (which was considered to be potentially problematic because of the moderate or poor yields reported for

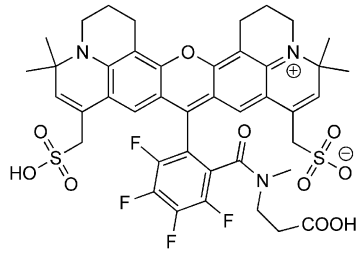


Scheme 6. Attempted alternative synthesis of carbopyronine dye **24** (*N*,*N*′-dibenzyl analogue of compound **19** in Scheme 2, b) via benzhydrol derivative **23**. Reagents and conditions: (a) THF, –78 °C; (b) BCl_3 in CH_2Cl_2, r.t.; (c) P_2O_5 and H_3PO_4, 110–150 °C; (d) Bu_4NIO_4.

simpler analogs).[6b] 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 BCl_3-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 polyphosporic acid (in a mixture with H_3PO_4) and P_2O_5 as condensation agents, was also unhelpful. These results are in accordance with previous reports,[6b] however, as reported in one patent,[7] cyclization of this type in the presence of P_2O_5 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 (ε), and the fluorescence quantum yields of the free acids **1c**, **20b**, **21b**, and **22**, methyl esters (**1b**, **20a**, and **21a**), and amino ester **16a**. These data highlight the influence of the polar (hydrophilic) groups. For comparison, the table also details the maximum absorption coefficients (ε) and the fluorescence quantum yields (Φ_{fl}) of the known red-emitting dyes KK114 [see ref.[4j] for compound **6** and ref.[25] 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 ($\varepsilon \approx 0.7–0.8 \times 10^5$ $M^{-1}cm^{-1}$) and fluorescence quantum yields ($\Phi_{fl} \approx 0.5–0.6$) 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.[4] 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 $COOCH_3$ group to COOH, or OCH_3 to OH does not seem to have any noticeable influence on Φ_{fl}. The values of ε are slightly below those of the reference dyes KK114 and ATTO 647N ($\varepsilon \approx 1–1.5 \times 10^5$ $M^{-1}cm^{-1}$) and the values of Φ_{fl} are in the same range as that of ATTO 647N but below those of KK114 ($\Phi_{fl} \approx 0.8$).[4] 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.[26a] We recorded the FCS data and determined the rates in the same way as described in our previous paper.[4j] These values, which were obtained from aqueous solutions, are listed in Table 2. The triplet decay time of $k_T = 0.3 \times 10^6$ 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.[26a] In contrast, the intersystem-crossing rates differ by a factor of three, i.e., $k_{isc} \approx 0.5 \times 10^6$ s^{-1} for compound **1c** and ATTO 647N, and $k_{isc} \approx 1–2 \times 10^6$ 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.[26a] 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).



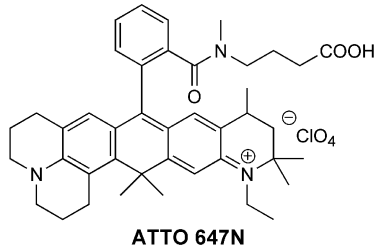


Figure 1. Structures of rhodamine KK114 and the carbopyronine dye ATTO 647N.

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

	k_{isc} ($\times 10^{-6}$)	k_T ($\times 10^{-6}$)	HO_3S group
1c	0.6	0.3	–
21b	1.1	0.3	+
22	1	0.3	+
KK 114	2	0.3	+
ATTO 647N	0.5	0.3	–

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 immunofluorescence labeling studies. In the conjugated state, the

fluorescence quantum yield for dye **1c** was found to be 26%. The decrease in Φ_{fl}. after bio-conjugation is well-known and was expected.[2p] 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).

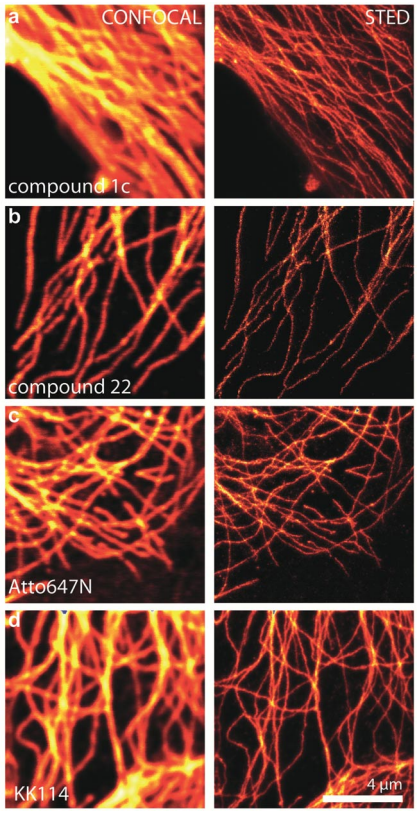


Figure 2. Conventional confocal microscopy (left panels) and STED nanoscopy images (right panels) of microtubule in fixed PtK2 cells immunolabeled with compound **1c** (a), compound **22** (b), ATTO 647N (c), and KK114 (d).

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).

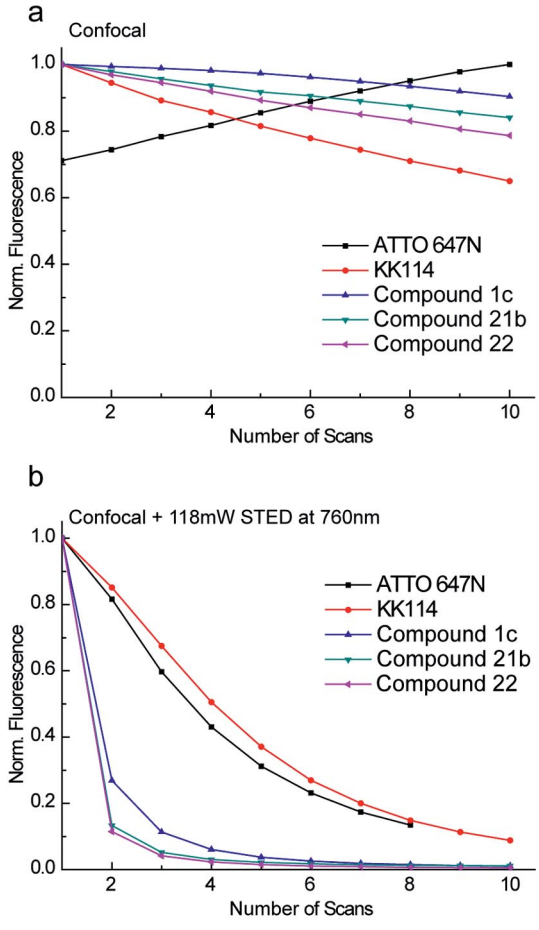


Figure 3. Photostabilities of the dyes under conventional confocal (a) and STED conditions (b). Relative change in the total fluorescence signal of the same area of the microtubule-immunolabeled cell samples in the course of the progressive scanning. Average excitation power 1 µW at 640 nm and average 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 carboxyl group in the benzene ring. The hydroxyl group can be protected, and then the carboxyl 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 spiroamides,[2m–2o,2r,21] carboryronine amido derivatives must exist predominantly in the "closed" colorless form (e.g., spiroamide **17**). Some of the spiroamides 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 spiroamides to be a valuable addition to the multicolor toolbox of photochromic spiroamides. On the other hand, the novel caging groups keep the initial cationic dyes 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-counters under argon, using a standard manifold with vacuum and argon lines. Routine NMR spectra were recorded with a Varian MERCURY-300 spectrometer operating at 300.5 (^{1}H) and 75.5 (^{13}C and APT) MHz. ^{1}H and ^{13}C 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 $CHCl_3$ (δ = 7.26 ppm) in $CDCl_3$, CHD_2OD (δ = 3.31 ppm) in CD_3OD, HOD (δ = 4.75 ppm) in D_2O, [D_5]acetone (δ = 2.04 ppm) in [D_6]acetone or [D_5]DMSO (δ = 2.50 ppm) in [D_6]DMSO. Multiplicities in the ^{13}C NMR spectra were determined by Attached Proton Test (ATP) measurements. Low-resolution mass spectra (electrospray ionization, ESI) were obtained with LCQ and ESI-TOF mass spectrometers [MICROTOF (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: Eurospher-100 C18, 5 μm, 250×4 mm, 1.1 mL/min; solvent A: H_2O (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 (F_{254}). Column chromatography: MERCK silica gel, grade 60, 0.04–0.063 mm. While using MeCN/H_2O 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 coverslips overnight and fixed with absol. methanol (–20 °C). After washing in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7) and blocking with 5% (w/v) bovine serum albumin in PBS, the cells were incubated with mouse monoclonal antibodies against α-Tubulin (Sigma–Aldrich, St. Louis, MO, USA). The primary antibodies were detected with secondary antibodies (sheep anti-mouse; Jackson ImmunoResearch, West Grove, PA, USA) custom-labeled with ATTO 647N (AttoTec, Siegen, Germany), KK114, **1c**, **21b**, and **22**. After immunolabeling, the samples were mounted in MOWIOL containing 0.1% (w/v) 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma–Aldrich). The STED setup used for the high-resolution measurements was described before.[28] The pulsed excitation was implemented 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-iodo-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 K_2CO_3 (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 H_2O (30 mL), extracted with CH_2Cl_2 (3×30 mL), and the combined organic solu-

tions were dried (Na_2SO_4) and the solvents evaporated. The residue was dissolved in Et_2O (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. $NaHCO_3$ (15 mL) and CH_2Cl_2 (20 mL), the aqueous layer was extracted with CH_2Cl_2 (2 × 15 mL) and the combined organic solutions were dried (Na_2SO_4), decolorized 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 CH_2Cl_2 (which reacts with BuLi in the next step). Benzylated amine (**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). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.98 (quint, 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, CH_2Ph), 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. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 21.9 (CH_2), 27.9 (CH_2), 49.4 (CH_2), 54.8 (CH_2Ph), 92.2 (CI), 119.1, 121.8, 124.6, 126.6 (2×), 127.0, 128.7 (2×), 130.4, 138.0, 146.8 ppm. MS (ESI+): m/z = 350 $[M + H]^+$. HRMS: calcd. for $C_{16}H_{16}IN$ [M + H] 350.0400; found 350.0397.

2-(*N*-Benzyl-1,2,3,4-tetrahydroquinolin-7-yl)propan-2-ol (8): In a typical experiment, commercial *n*BuLi (2.5 M, 4.0 mL, 10 mmol) was injected into a cooled 50 mL Schlenk Flask (external dry ice bath, –78 °C) that had been flushed with argon and charged with anhydrous THF (6 mL). A solution of **5**-Bn,I (1.04 g, 2.9 mmol) in anhydrous THF (12 mL) was introduced over a period of 6 min with stirring. After 1 h stirring at this temperature, acetone (3 mL) was injected in one portion and, after 30 min, the mixture was poured into ice-cold 20% aq. NH_4Cl (50 mL). The aqueous layer was separated and extracted with EtOAc (3 × 20 mL), the combined organic extracts dried (Na_2SO_4), and the solvents evaporated. Carbinol **8** was isolated as a colorless oil in 75% yield (0.61 g) by means of flash chromatography (30 g of SiO_2; hexane/EtOAc, 8:1→1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.26 (s, 6 H, CH_3), 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, CH_2Ph), 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. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 22.7 (CH_2), 28.1 (CH_2), 31.9 (CH_3), 50.2 (CH_2), 55.7 (CH_2Ph), 72.8 (C-OH), 107.7, 112.3, 121.1, 127.1 (2×), 128.5, 128.9 (2×), 129.1, 139.3, 145.6, 148.6 ppm. MS (ESI+): m/z (%) = 304 (100) $[M + Na]^+$. HRMS: calcd for $C_{19}H_{23}NO$ [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 indoline bromination[13a] was applied to 1,2,3,4-tetrahydroquinoline (**4**). The substrate **4** (6.03 g, 45 mmol) was added with vigorous stirring to a cooled solution of Ag_2SO_4 (7.67 g, 25 mmol) in concd. H_2SO_4 (70 mL) at 0 °C (ice bath) in a flask equipped with a reflux condenser, and Br_2 (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 Br_2 (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 CH_2Cl_2 (4 × 80 mL) and the combined extracts were washed with brine, dried (Na_2SO_4), and the solvents evaporated. The title compound was isolated as a colorless solid by column chromatography (260 g of SiO_2; hexane/CH_2Cl_2, 4:1). 5-Bromo-1,2,3,4-tetrahydroquinoline and the starting material **4**, both of which are more polar then 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/$CHCl_3$, 3:1; R_f = 0.15). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.91 (m, 2 H, CH_2), 2.64 (m, 2 H, CH_2), 3.23 (m, 2 H, CH_2), 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. ^{13}C NMR (75.5 MHz, CD_3CN): δ = 21.7 (CH_2), 26.6 (CH_2), 41.6 (CH_2), 116.3, 119.4, 120.1, 130.7, 145.9 ppm.

***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/CH_2Cl_2, 3:1), and the product was isolated as a pale-brown oil in 70% yield (4.01 g) after column chromatography (200 g of SiO_2; hexane/CH_2Cl_2, 4:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.96 (quint, J = 6 Hz, 2 H, CH_2), 2.64 (t, J = 6 Hz, 2 H, CH_2), 3.23 (t, J = 6 Hz, 2 H, CH_2), 4.39 (s, 2 H, CH_2Ar), 6.57 (m, 2 H), 6.78 (d, J = 8 Hz, 1 H), 7.16–7.40 (m, 5 H, Ph) ppm. MS (ESI+): m/z = 302 $[M + H]^+$. HRMS: calcd. for $C_{16}H_{16}BrN$ [M + H] 302.0466; found 302.0463.

The lithiation of **7**-Bn (see Scheme 2, a) was performed as for **5**-Bn,I (the iodo-substituted analog, see above) to afford carbinol **8**, which was identical to the previously obtained samples (TLC, ^{1}H NMR), in 88% yield.

***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 $KHSO_4$ (136 mg, 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 SiO_2 (12 g), which was eluted with hexane/CH_2Cl_2 (6:1) to afford 218 mg (83%) of a "TLC-pure" alkene **11** as a colorless photosensitive oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.06 (m, 2 H), 2.12 (s, 6 H, CH_3), 2.85 (t, J = 6 Hz, 2 H), 3.42 (t, J = 6 Hz, 2 H), 4.60 (s, 2 H, CH_2Ph), 5.00, 5.22 (2 × s, 2 H, C=CH_2), 6.76 (s, 1 H), 6.80 (dd, J = 8 Hz, 1 H), 7.02 (d, J = 8 Hz, 1 H), 7.32–7.40 (m, 5 H, Ph) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 21.9 (CH_3), 22.3 (CH_2), 27.9 (CH_2), 49.9 (CH_2), 55.3 (CH_2N), 108.4 (CH), 111.2 (CH_2), 113.4 (CH), 121.8 (C), 126.6 (2 × CH), 126.7 (CH), 128.5 (2 × CH), 128.7 (CH), 139.0 (C), 140.3 (C), 143.9 (C), 145.3 (C) ppm. MS (ESI+): m/z (%) = 264 (100) $[M + H]^+$. HRMS: calcd. for $C_{19}H_{21}N$ [M + H] 264.1747; found 264.1749.

Carbopyronines 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 CH_2Cl_2 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 BCl_3 (1 M in CH_2Cl_2, 2.70 mL, 2.70 mmol) at 0 °C, and the reaction mixture was stirred at r.t. overnight. Polyphosphoric acid (VWR 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 CH_2Cl_2 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 H_2O (120 mL) and CH_2Cl_2 (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 CH_2Cl_2 (3 × 50 mL) and the combined organic extracts were washed with brine (30 mL) and dried with anhydrous K_2CO_3. The solvent was completely removed in vacuo to afford 1.10 g of residue, which consisted of compound **13** and a very small amount of its oxidized (colored) form **14** (TLC; EtOAc/hexane, 1:1). In initial experiments, compound **13** was isolated in 85% yield by flash chromatography (hexane/EtOAc/CH_2Cl_2, 2:1:1) as a pale-yellow amorphous material that rapidly oxidized in air to a dark-blue dye with intense red florescence; the mass spectrum of the oxidation product was in agreement with structure **14**: MS (ESI+): *m*/*z* (%) = 497 (100) $[M]^+$. HRMS: calcd. for $C_{36}H_{37}N_2$ $[M]^+$ 498.2951; found 497.2943. ^{1}H NMR (for compound **13**, *N*-benzyl derivative, 300 MHz, CD_3CN): δ = 1.12 (s, 6 H, 2 × CH_3), 1.82 (m, 4 H, 2 × CH_2), 2.70 (t, *J* = 6 Hz, 4 H, 2 × CH_2), 3.36 (t, *J* = 6 Hz, 4 H, 2 × CH_2), 3.63 (s, 2 H, $ArCH_2Ar$), 4.42 (s, 4 H, CH_2Ph), 6.52 (s, 2 H), 6.76 (s, 2 H), 7.20–7.40 (m, 5 H, Ph) ppm. ^{13}C NMR (75.5 MHz, CD_3CN): δ = 23.4 (CH_2), 28.2 (CH_2), 29.6 (CH_3), 33.6 (CH_2), 39.5 (C_q), 51.3 (CH_2), 56.2 (CH_2), 108.9 (CH), 121.2 (C), 124.2 (C), 127.5 (CH), 127.6 (2 × CH), 127.8 (CH), 129.0 (CH), 129.3 (CH), 129.4 (2 × C), 140.9 (C), 144.0 (C), 144.6 (C) ppm. MS (ESI+): *m*/*z* (%) = 497 (100) [M^+ for the oxidized form **14**]. See above for the HRMS of compound **14**.

The removal of the benzyl protective group was carried out as follows: In an argon atmosphere, upon slight heating and with stirring, the crude compound **13** was dissolved in a mixture of MeOH (100 mL) and Et_2O (40 mL) in a flask equipped with a gas inlet, a reflux condenser, and a bubble counter. Upon cooling to r.t., Pd/C (10% Pd, oxidized form, VWR International; 1.50 g) and ammonium formate (NH_4OOCH; 2.0 g, 32 mmol) were added, the flask was flushed with Ar, and the reaction mixture was stirred for 50 min at 50 °C and at r.t. for 1 h. The mixture was diluted with CH_2Cl_2 (100 mL), filtered through Celite, and the solvents were evaporated. (***CAUTION:*** the Pd/C catalyst may cause ignition!). In the course of the evaporation *n*-heptane (2 × 20 mL) was added to remove MeOH. The residue (air-sensitive!) was subjected to column chromatography (40 g of SiO_2; hexane/EtOAc/CH_2Cl_2, 10:5:1) to furnish 615 mg (82%) of **12** as a pale-green crystalline powder (m.p. 157–158 °C). ^{1}H NMR (300 MHz, CD_3CN): δ = 1.41 (s, 6 H, 2 × CH_3), 1.82 (quint, *J* = 6 Hz, 4 H, 2 × CH_2), 2.62 (t, *J* = 6 Hz, 4 H, 2 × CH_2), 3.20 (t, *J* = 6 Hz, 4 H, 2 × CH_2), 3.64 (s, 2 H, $ArCH_2Ar$), 4.22 (br. s, 2 H, NH), 6.60 (s, 2 H), 6.74 (s, 2 H) ppm. ^{13}C NMR (75.5 MHz, CD_3CN): δ = 23.3 (CH_2), 27.2 (CH_2), 29.2 (CH_3), 31.7 (CH_2), 34.3 (C), 42.6 (CH_2), 110.9 (CH), 119.6 (C), 125.4 (C), 129.2 (CH), 144.4 (C), 144.5 (C) ppm. MS (ESI+): *m*/*z* (%) = 319 (100) $[M + H]^+$, 317 (90) [M^+ for the oxidized form]$^+$. HRMS: calcd. for $C_{22}H_{26}N_2$ [M + H] 319.2169; found 319.2168.

***N*-(2-Methoxyethyl) Carbopyronine Derivatives 3a and 3b:** Compound **12** (0.61 g, 1.92 mmol) in DMF (7 mL) was placed in a screw-cap test tube containing 2-methoxyethyl bromide (3.90 g, 28 mmol) and finely powdered K_2CO_3 (0.99 g, 7.2 mmol). The test tube was flushed with argon, sealed, and the mixture was magnetically stirred at 100 °C (CAUTION: slight internal pressure!) for 2.5 h, until the alkylation was complete [TLC monitoring (EtOAc/hexane, 1:2)]. In the course of the alkylation the partially alkylated product **3b** (R′ = H, Scheme 3, a) was detected: in the preliminary experiments this compound was isolated by chromatography (hexane/EtOAc, 7:1) and identified in ^{1}H NMR and MS spectra. ^{1}H NMR **3b** (300 MHz, CD_3CN): δ = 1.43 (s, 6 H, 2 × CH_3), 1.82 (quint, *J* = 6 Hz, 4 H, 2 × CH_2), 2.63 (t, *J* = 6 Hz, 4 H, 2 × CH_2), 3.20 (t, *J* = 6 Hz, 2 H, CH_2), 3.28 (t, *J* = 6 Hz, 2 H, CH_2), 3.30 (s, 3 H, OCH_3), 3.45 (t, *J* = 6 Hz, 2 H, CH_2N), 3.56 (t, *J* = 6 Hz, 2 H, CH_2O), 3.75 (s, 2 H, $ArCH_2Ar$), 4.22 (br. s, 1 H, NH), 6.60 (s, 2 H), 6.68–7.00 (m, 1 H) ppm. MS (ESI+): *m*/*z* (%) = 399 [M + Na]$^+$. HRMS: calcd for $C_{25}H_{32}N_2O$ [M + H] 377.2587; found 377.2582.

The reaction mixture was diluted with H_2O (10 mL), extracted with CH_2Cl_2 (3 × 20 mL), and the combined organic extract was washed with H_2O (2 × 10 mL), dried (Na_2SO_4) and evaporated to furnish **3a** (820 mg, 98%) as an amorphous grey solid, which was air-sensitive, slightly soluble in hexane, and well-soluble in chlorinated solvents. ^{1}H NMR (300 MHz, CD_3CN): δ = 1.43 (s, 6 H, 2 × CH_3), 1.82 (quint, *J* = 6 Hz, 4 H, 2 × CH_2), 2.63 (t, *J* = 6 Hz, 4 H, 2 × CH_2), 3.28 (t, *J* = 6 Hz, 4 H, 2 × CH_2), 3.31 (s, 6 H, 2 × OCH_3), 3.45 (t, *J* = 6 Hz, 4 H, 2 × CH_2N), 3.56 (t, *J* = 6 Hz, 4 H, 2 × CH_2O), 3.75 (s, 2 H, $ArCH_2Ar$), 6.76–6.78 (br. s, 4 H) ppm. ^{13}C NMR (75.5 MHz, CD_3CN): δ = 23.3 (CH_2), 28.2 (CH_2), 29.7 (CH_3), 33.6 (CH_2), 50.9 (C), 52.0 (CH_2), 59.0 (OCH_3), 70.5 (CH_2), 108.2, 121.0, 124.0, 129.0 (2 ×), 144.2, 144.5 ppm. HRMS: calcd. for $C_{28}H_{38}N_2O_2$ [M + Na] 457.2825; found 457.2829.

Ketone 2: 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 $KMnO_4$ (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 CH_2Cl_2 (800 mL), and filtered through a paper filter. The brown precipitate was washed with additional CH_2Cl_2 (2 × 40 mL) and the solvents were evaporated. Ketone **2** was isolated by chromatography on SiO_2 (90 g; hexane/EtOAc/CH_2Cl_2, 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/$CHCl_3$, 1:6). ^{1}H NMR (300 MHz, CD_3CN): δ = 1.61 (s, 6 H, 2 × CH_3), 1.82 (quint, *J* = 6 Hz, 4 H, 2 × CH_2), 2.76 (t, *J* = 6 Hz, 4 H, 2 × CH_2), 3.33 (s, 6 H, 2 × OCH_3), 3.42 (t, *J* = 6 Hz, 4 H, 2 × CH_2), 3.60 (m, 8 H, 2 × CH_2N, 2 × CH_2O), 6.78 (s, 2 H), 7.72 (s, 2 H) ppm. ^{13}C NMR (75.5 MHz, CD_3CN): δ = 22.6 (CH_2), 28.3 (CH_2), 33.6 (CH_2), 38.3 (CH_2), 51.0 (C), 51.6 (CH_2), 59.1 (OCH_3), 70.6 (CH_2), 107.8, 119.8, 122.0, 127.8 (2 × C), 149.6, 151.5, 180.5 (C=O) ppm. HRMS: calcd. for $C_{28}H_{26}N_2O_3$ [M + H] 449.2799; found 449.2792.

Amino Ester 16a: In an argon-flushed Schlenk flask (50 mL) fitted with a septum, 2-(2-bromophenyl)-4,4-dimethyl-2-oxazoline[19d] (635 mg, 2.5 mmol) in anhydrous THF (12 mL) was lithiated with *t*BuLi (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, 0.500 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 SiO_2 (30 g; MeCN/H_2O, 10:1) to separate the yellow and blue impurities, then eluted with a MeCN/H_2O (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 CH_2Cl_2 (80 mL) and sat. $NaHCO_3$ (20 mL), and the organic layer was separated, dried

(Na_2SO_4), and evaporated to afford **16a** (380 mg, 91%, bis-trifluoroacetate) as a dark-blue amorphous solid. HPLC: t_R = 11.2 min, A/B 70:30–0:100 in 25 min. ^{1}H NMR (300 MHz, CD_3CN): δ = 1.32 (s, 6 H, 2 × CH_3), 1.76 (s, 3 H, CH_3), 1.80 (s, 3 H, CH_3), 1.88 (m, 4 H, 2 × CH_2), 2.50 (t, J = 6 Hz, 4 H, 2 × CH_2), 3.40 (s, 6 H, 2 × OCH_3), 3.64 (t, J = 6 Hz, 4 H, 2 × CH_2), 3.78 (t, J = 6 Hz, 4 H, 2 × CH_2N), 3.92 (t, J = 6 Hz, 4 H, 2 × CH_2O), 4.20 (s, 2 H, CH_2O), 6.70 (s, 2 H), 7.28 (s, 2 H), 7.40 (d, J = 9 Hz, 1 H), 7.76–7.90 (m, 2 H), 8.40 (br. s, 1 H, NH_3^+), 8.40 (d, J = 9 Hz, 1 H) ppm. ^{13}C NMR (75.5 MHz, CD_3CN): δ = 21.6 (CH_2), 26.3 (CH_3), 27.6 (CH_2), 28.2 (CH_2), 33.7 (CH_3), 33.9 (CH_3), 41.9 (C), 50.9 (CH_2), 52.0 (CH_2), 52.6 (CH_2), 59.2 (OCH_3), 70.7 (CH_2), 111.5, 121.2, 124.5 (CF_3), 127.8, 130.1, 131.2, 131.8, 133.3, 134.5, 138.4, 154.3, 156.1, 164.5 (C=O), 165.8 (CF_3C=O) ppm. MS (ESI+): m/z (%) = 624 (100) $[M]^+$. HRMS: calcd. for $C_{39}H_{50}N_3O_4$ $[M]^+$ 624.3796; found 624.3799.

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 H_2O (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 H_2O, then CH_2Cl_2 (40 mL) and EtOAc (60 mL) were added, and the mixture was neutralized with solid $NaHCO_3$ (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 CH_2Cl_2 (5 × 40 mL), until the extract became colorless. The combined organic layer was dried (Na_2SO_4), evaporated in vacuo, and the residue was dissolved in a mixture of MeCN (20 mL) and CH_2Cl_2 (15 mL) and separated on SiO_2 (80 g; MeCN/H_2O, 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 70:30–0:100 in 25 min); t_R = 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.

Reaction time [h]	Product yields (isolated) [%]		
	1a	**18a**	**18b**
14	65	13	–
18	62	27	3
22	52	26	5

1a: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.70 (s, 3 H, CH_3), 1.78 (s, 3 H, CH_3), 1.80 (m, 4 H, 2 × CH_2), 2.48 (m, 4 H, 2 × CH_2), 3.30 (m, 4 H, 2 × CH_2), 3.38 (s, 6 H, 2 × OCH_3), 3.46 (m, 4 H, 2 × CH_2N), 3.60 (m, 4 H, CH_2O), 6.20 (s, 2 H), 6.76 (s, 2 H), 7.06 (d, J = 9 Hz, 1 H), 7.52 (m, 2 H), 7.98 (d, J = 9 Hz, 1 H) ppm. ^{13}C NMR (75.5 MHz, CD_3CN): δ = 22.0 (CH_2), 27.6 (CH_2), 33.3 (CH_3), 35.1 (CH_3), 37.7 (CH_2), 50.3 (CH_2), 51.3 (CH_2), 59.1 (OCH_3), 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 (%) = 553 (100) $[M + H]^+$. HRMS: calcd. for $C_{35}H_{40}N_2O_4$ $[M + H]$ 553.3061; found 553.3059.

18a: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.62 (s, 3 H, CH_3), 1.68 (s, 3 H, CH_3), 1.80 (m, 4 H, 2 × CH_2), 2.50 (m, 4 H, 2 × CH_2), 3.08 (m, 4 H, 2 × CH_2), 3.38 (s, 6 H, 2 × OCH_3), 3.46 (m, 4 H, 2 × CH_2N), 3.60 (m, 2 H, CH_2OMe), 3.82 (t, J = 6 Hz, 2 H, CH_2OH), 6.22 (d, J = 9 Hz, 2 H), 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. ^{13}C NMR (75.5 MHz, CD_3CN): δ = 22.0 (CH_2), 27.6 (CH_2), 33.3 (CH_3), 35.1 (CH_3), 37.9 (CH_2), 50.3 (CH_2), 50.3 (CH_2), 51.3 (CH_2), 59.1 (OCH_3), 69.9 (C), 107.4, 108.1, 118.3, 121.6, 121.9, 124.1, 125.0, 127.2, 128.4, 128.5, 128.6, 134.3, 145.0, 145.3, 145.9, 146.5, 154.9, 171.0 (C=O) ppm. MS (ESI+): m/z (%) = 539 (100) $[M + H]^+$. HRMS: calcd. for $C_{34}H_{38}N_2O_4$ $[M + H]$ 539.2904; found 539.2903.

18b: ^{1}H NMR (300 MHz, CD_3CN): δ = 1.60 (s, 3 H, CH_3), 1.78 (s, 3 H, CH_3), 1.80 (m, 4 H, 2 × CH_2), 2.04 (br. s, 1 H, OH), 2.50 (m, 4 H, 2 × CH_2), 3.30 (m, 4 H, 2 × CH_2), 3.40–3.60 (m, 4 H, 2 × CH_2N), 3.70–3.90 (m, 4 H, 2 × CH_2O), 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) $[M]^+$. HRMS: calcd. for $C_{33}H_{37}N_2O_4$ $[M]^+$ 525.2748; found 525.2743.

Carbopyronine 1a (Alkaline Hydrolysis of 16b): Amino ester **16a** (20.0 mg, 23 μmol) was quaternized with MeI (0.10 mL, 1.50 mmol) in the presence of K_2CO_3 (28 mg, 0.20 mmol) with vigorous stirring at r.t. for 2–4 h. The solvent was evaporated to dryness at 0.2–0.4 mbar, and a solution of aq. NaOH (1 M, 0.30 mL, 0.30 mmol) in a mixture of H_2O (1.5 mL) and THF (2 mL) was added to the residue (containing the quaternary salt **16b**; not isolated, one spot on TLC). The resulting homogeneous solution was left overnight at 4 °C, then partly neutralized with HOAc (20 μL, 0.36 mmol), evaporated in vacuo, and shaken with CH_2Cl_2 (10 mL) and H_2O (6 mL). The aqueous layer was separated, extracted with an equal volume of CH_2Cl_2, and the organic extracts were washed with aq. HCl (0.5 M, 2 mL), sat. aq. $NaHCO_3$ (2 mL), dried (Na_2SO_4) and evaporated to furnish 10.3 mg (81%) of a compound that was identical to the previously obtained samples of **1a** (HPLC, TLC, and ^{1}H NMR analysis; see above).

Carbopyronine 1b (One-Pot Amidation of 1a): A 100-mL Schlenk flask fitted with a septum was flushed with argon and charged with **1a** (100 mg, 0.180 mmol) in 1,2-dichloroethane (18 mL) and $POCl_3$ (0.60 mL, 6 mmol). The dark-blue solution was stirred for 2.5 h at 70–72 °C (the pressure was relieved through a cannula in the first 15 min of heating). The solvent was then removed in vacuo, and the residue was kept for an additional 1 h at 0.4–0.6 mbar and dissolved in CH_2Cl_2 (15 mL) under argon. The flask was then cooled to –10 °C (external dry-ice bath), and a solution containing methyl 3-(methylamino)propionate hydrochloride[2p] (92 mg, 0.60 mmol) and Et_3N (0.13 mL, 0.9 mmol) in anhydrous MeCN (HPLC grade; 3 mL) was added in one portion with stirring. The solution was warmed to r.t., stirred for an additional 15 min, and washed with H_2O (10 mL) containing HOAc (0.60 mL, 10 mmol). The aqueous layer was extracted with CH_2Cl_2 (15 mL) and the combined organic layer was dried (Na_2SO_4) and the solvents evaporated. The residue was purified by chromatography over SiO_2 (35 g; MeCN/H_2O, 5:1 → 2:1). The main fraction was evaporated in vacuo at temperatures below 37 °C to a volume of 10 mL, stepwise filtered through Rotilabo® syringe filters (0.80 and 0.22 μm), and freeze-dried to furnish the pure (HPLC, TLC) title compound (101 mg, 82%, hydrochloride) as a bulky dark-blue solid. TLC: R_f = 0.30 (MeCN/H_2O, 5:1); HPLC: t_R = 18.2 min (A/B 70:30–0:100 in 25 min). ^{1}H NMR (300 MHz, $CDCl_3$; signals of the major invertomer are marked with an asterisk *): δ = 1.61 (s, 3 H, CH_3), 1.78 (s, 3 H, CH_3), 1.88 (m, 4 H, 2 × CH_2), 2.21 (t, J = 6 Hz, 2 H, CH_2 β-Ala),

2.56 (t, J = 6 Hz, 2 H, CH_2 β-Ala), 2.62 (m, 4 H, 2 × CH_2), 2.82 (s, 3 H, NCH_3), 3.38 (s, 6 H, 2 × OCH_3), 3.58 (s, 3 H, CO_2CH_3), 3.60 (m, 4 H, 2 × CH_2N), 3.72 (m, 4 H, CH_2O), 3.42/3.82* (m, Σ = 4 H, 2 × CH_2), 6.70 (s, 2 H), 7.18 (s, 2 H), 7.24 (m, 1 H), 7.43 (m, 1 H), 7.58 (m, 2 H) ppm. ^{1}H NMR (300 MHz, CD_3CN; signals of the major invertomer are marked with an asterisk *): δ = 1.58/1.60* (s, Σ = 3 H, CH_3), 1.74*/1.80 (s, Σ = 3 H, CH_3), 1.85 (m, 4 H, 2 × CH_2), 2.04 (t, J = 6 Hz, 2 H, CH_2 β-Ala), 2.50–2.60 (overlapped: m, 2 H, CH_2 β-Ala, m, 4 H, 2 × CH_2), 2.84*/3.06 (s, Σ = 3 H, NCH_3), 3.34 (s, 6 H, 2 × OCH_3), 3.52 (s, 3 H, CO_2CH_3), 3.56 (m, 4 H, 2 × CH_2N), 3.72 (m, 4 H, CH_2O), 3.38/3.80* (m, Σ = 4 H, 2 × CH_2Ar), 6.76 (s, 2 H), 7.18 (s, 2 H), 7.36 (m, 1 H), 7.45 (m, 1 H), 7.62 (m, 2 H) ppm. ^{13}C NMR (75.5 MHz, CD_3CN): δ = 21.7 (CH_2), 27.6 (CH_2), 30.3 (CH_2), 31.8 (CH_3), 32.3 (CH_2), 35.5/38.3 (CH_3), 42.0 (CH_2), 43.9 (CH_2), 50.9 (CH_2), 52.8 (CO_2CH_3), 59.3 (OCH_3), 70.8 (C), 70.9 (C), 111.7, 121.7, 124.2, 127.7, 129.7, 131.2, 124.9, 125.5, 131.8, 132.5, 135.6, 154.5, 156.3, 162.0, 169.2 (C=O), 172.8 (C=O) ppm. MS (ESI+): m/z (%) = 652 (100) $[M]^+$. HRMS: calcd. for $C_{40}H_{50}N_3O_5$ $[M]^+$ 652.3745; found 652.3742.

Dye 1c (Free Acid): The amidation of **1a** (0.180 mmol) was carried out as described above. The crude amido ester **1b** in CH_2Cl_2 (30–40 mL) was washed with sat. $NaHCO_3$ (10 mL), dried (Na_2SO_4) and the solvents evaporated. The residue was dissolved in aq. NaOH (1 M, 0.70 mL, 0.70 mmol) in a mixture of H_2O (10 mL) and THF (15 mL). The saponification came to completion in 3 h at 5–10 °C [TLC: R_f = 0.15 (**1a**; MeCN/H_2O, 5:1)]. The solution was acidified with HOAc (0.6 mL, 1 mmol), evaporated to dryness in vacuo at temperatures below 37 °C, and the residue was separated on SiO_2 (50 g; MeCN/H_2O, 5:1 → 2:1). The main fraction was evaporated, filtered, and freeze-dried as described for **1b** (see above) to afford the title compound (88 mg, 76%) as a dark-blue solid. HPLC: t_R = 15.5 min (A/B 70:30–0:100 in 25 min). ^{1}H NMR (300 MHz, $CDCl_3$, signals of the major invertomer are marked with an asterisk *): δ = 1.51*/1.60 (s, Σ = 3 H, CH_3), 1.66/1.72 (s, 3 H, CH_3), 1.88 (m, 4 H, 2 × CH_2), 1.98*/2.22 (m, Σ = 2 H, CH_2 β-Ala), 2.44 (m, 4 H, 2 × CH_2), 2.62–2.78 (m, 2 H, CH_2 β-Ala), 2.67*/2.98 (s, Σ = 3 H, NCH_3), 3.36 (s, 6 H, 2 × OCH_3), 3.60 (m, 4 H, 2 × CH_2N), 3.72 (m, 4 H, CH_2O), 3.28/3.96* (m, Σ = 4 H, 2 × $ArCH_2$), 6.72 (s, 1 H), 6.80 (s, 1 H), 7.04 (d, J = 8 Hz, 2 H), 7.18–7.24 (m, 1 H), 7.42–7.60 (br. m, 3 H) ppm. ^{13}C NMR (75.5 MHz, CD_3CN): δ = 21.7 (CH_2), 27.6 (CH_2), 30.3 (CH_3), 31.8/32.3* (CH_2), 36.8/38.0 (CH_3), 41.9 (CH_2), 43.0 (CH_2), 52.10 (C), 52.7 (CH_2), 59.3 (OCH_3), 70.8 (C), 111.5, 121.5, 121.6, 124.1, 124.2, 127.7, 127.9, 129.5, 129.6, 129.8, 131.2, 134.9, 135.5, 136.0, 154.4, 156.1, 156.2, 168.6/169.3* (C=O), 175.0/175.8* (C=O) ppm. MS (ESI+): m/z (%) = 638 (100) $[M + H]^+$. HRMS: calcd. for $C_{39}H_{47}N_3O_5$ [M + H] 638.3588; found 638.3604.

Dye 1-NHS (The Active Ester): In a typical experiment, dye **1c** (5 mg, 8 μmol) in anhydrous MeCN (HPLC grade; 3–4 mL) was treated with *N*-hydroxysuccinimide (14 mg, 0.120 mmol) in the presence of HATU (12 mg, 30 μmol) and Et_3N (20 μL, 0.140 mmol) in a screw-cap vial at room temp. A conversion of ca. 90% was detected in 2 h, as shown by HPLC (t_R = 17.7 min; A/B 70:30–0:100 in 25 min) and by TLC (R_f = 0.50; silica plates; MeCN/H_2O, 5:1). The reaction solution was loaded straight into a short column with SiO_2 (5 g). The main fraction (MeCN/H_2O, 10:1) was collected and quickly evaporated at r.t. in vacuo to a volume of ca. 10 mL, filtered through a Rotilabo® syringe filter (0.22 μm), and freeze-dried to afford 6–8 mg of a blue solid containing ca. 10–20% of the starting acid (t_R = 15.5 min) and variable amounts of *N*-hydroxysuccinimide. MS (ESI+): m/z (%) = 735 $[M]^+$. HRMS: calcd. for $C_{43}H_{51}N_4O_7$ $[M]^+$ 735.3752; found 735.3748. The material was stored under argon at –20 °C for use in labeling studies.

Dyes 20a and 20b (Demethylation Products): To an ice-cold solution of **1b** (20 mg, 29 μmol) in CH_2Cl_2 (10 mL), a commercial BBr_3 solution (1 M in CH_2Cl_2, 0.17 mL, 0.17 mmol) was added and the mixture was stirred for 2 h at 0 °C with TLC monitoring (MeCN/H_2O, 7:1). The yellow solution was thoroughly washed with sat. aq. $NaHCO_3$ (6 mL), the aqueous layer was extracted with CH_2Cl_2 (3 × 10 mL), the combined organic layer dried (Na_2SO_4), and the solvents evaporated. The residue was purified by chromatography over SiO_2 (12 g; MeCN/H_2O, 6:1 → 2:1). 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, acid **1c** was demethylated to **20b** (see below for the spectroscopic data) with 74% isolated yield.

The alkaline hydrolysis of **20a** was carried out as for **1b** (see above): To a solution of **20a** (11 mg, 17 μmol) in a mixture of THF (3 mL) and H_2O (2 mL), aq. NaOH (0.1 M, 0.6 mL, 0.06 mmol) was added and the mixture was kept for 3 h at 5–10 °C, acidified with HOAc (0.06 mL, 0.10 mmol), evaporated and separated over SiO_2 (8 g; MeCN/H_2O, 5:1 → 2:1). Solvent removal (as described above for **1b**) afforded acid **20b** (8.4 mg, 81%). HPLC: t_R (**20a**) = 11.5 min, t_R (**20b**) = 9.7 min (A/B: 70:30–0:100 in 25 min). ^{1}H NMR (300 MHz, CD_3CN; **20a**: signals of the major invertomer are marked with an asterisk *): δ = 1.58/1.61* (s, Σ = 3 H, CH_3), 1.66*/1.80 (s, Σ = 3 H, CH_3), 1.82 (m, 4 H, 2 × CH_2), 2.02 (t, J = 6 Hz, 2 H, CH_2 β-Ala), 2.50–2.60 (overlapped: m, 2 H, CH_2 β-Ala, m, 4 H, 2 × CH_2), 2.82 (s, 3 H, NCH_3), 3.30–3.45 (br. m, 4 H, 2 × CH_2N), 3.50 (s, 3 H, CO_2CH_3), 3.52–3.80 (br. m, 4 H, CH_2O), 3.75 (m, 4 H, 2 × CH_2), 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) $[M]^+$. HRMS: calcd. for $C_{38}H_{46}N_3O_5$ $[M]^+$ 624.3432; found 624.3430. **20b**: ^{1}H NMR (300 MHz, CD_3CN): δ = 1.58/1.61* (s, Σ = 3 H, CH_3), 1.66*/1.80 (s, Σ = 3 H, CH_3), 1.82 (m, 4 H, 2 × CH_2), 2.02 (t, J = 6 Hz, 2 H, CH_2 β-Ala), 2.44 (t, J = 6 Hz, 2 H, CH_2 β-Ala), 2.58 (m, 4 H, 2 × CH_2), 2.90 (s, 3 H, NCH_3), 3.33–3.60 (br. m, 4 H, 2 × CH_2N), 3.52–3.80 (br. m, 4 H, 2 × CH_2O), 3.75 (m, 4 H, 2 × CH_2), 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 (%) = 632 (100) $[M + Na - H]^+$, 610 (30) $[M]^+$. HRMS: calcd. for $C_{37}H_{44}N_3O_5$ $[M]^+$ 610.3275; found 610.3271.

Dye 21a (Peptide-Type Coupling with L-Cysteic Acid): In a typical experiment, compound **1c** (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 hydrochloride[23b] (1.55 mg, 7.0 μmol) in the presence of HBTU (2.5 mg, 6.6 μmol) and Et_3N (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/H_2O (10:1) followed by evaporation, filtration (Rotilabo® 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 H_2O, MeOH, MeCN, and $CHCl_3$. TLC: R_f = 0.30 (MeCN/H_2O, 10:1); HPLC: t_R = 12.3 min (A/B: 70:30–0:100 in 25 min). ^{1}H NMR (300 MHz, CD_3CN): δ = 1.56/1.62* (s, Σ = 3 H, CH_3), 1.72*/1.82 (s, Σ = 3 H, CH_3), 1.90 (m, 4 H, 2 × CH_2), 2.20–2.30 (m, 2 H, CH_2 β-Ala), 2.54–2.60 (overlapped: m, 2 H, CH_2 β-Ala, m, 4 H, 2 × CH_2), 2.82*/3.60 (s, Σ = 3 H, NCH_3), 3.36 (s, 6 H, 2 × OCH_3), 3.58 (m, 4 H, 2 × CH_2N), 3.60 (s, 3 H, CO_2CH_3), 3.68 (t, J = 6 Hz, 2 H,

CH_2SO_3), 3.50–3.80 (m, 4 H, CH_2O), 3.75/4.01 (m, 4 H, 2 × CH_2Ar), 4.42 (br. s, 1 H, NH), 5.32 (m, 1 H, CH Cys), 6.78 (s, 2 H), 7.20 (d, J = 6 Hz, 2 H), 7.36 (m, 1 H), 7.62 (m, 2 H), 7.75 (d, J = 6 Hz, 1 H) ppm. ^{13}C NMR (75.5 MHz, CD_3CN): δ = 21.7 (CH_2), 27.6 (CH_2), 31.8 (CH_3), 32.3 (CH_2), 35.5/38.1 (CH_3), 42.0 (CH_2), 44.1 (CH_2), 51.0 (CO_2Me), 52.0 (CH_2), 52.5 (CH_2), 52.7 (CH), 59.2 (OMe), 70.8 (C), 70.9 (C), 111.5, 111.6, 121.7, 124.0, 127.8, 129.5, 131.0, 135.1, 135.2, 135.4, 137.3, 154.3, 156.0, 161.7*/168.8 (C=O), 170.8/171.8* (C=O) ppm. MS (ESI+): *m/z* (%) = 825 (100) $[M + Na]^+$. HRMS: calcd. for $C_{43}H_{54}N_4O_9S$ [M + Na] 825.3504; found 825.3505.

Dye 21b (Free Acid): To a solution of **21a** (74 mg, 0.92 mmol) in a mixture of THF (15 mL) and H_2O (10 mL), aq. NaOH (1 M, 0.30 mL, 0.30 mmol) was added, and the mixture was kept for 2 h at 0 °C, acidified with HOAc (0.10 mL, 1.80 mmol), evaporated and purified by chromatography over SiO_2 (36 g; MeCN/H_2O, 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 H_2O and MeOH, sparingly soluble in MeCN, and insoluble in $CHCl_3$. TLC: R_f = 0.12 (MeCN/H_2O, 5:1); HPLC: t_R = 10.4 min (A/B: 70:30–0:100 in 25 min). ^{1}H NMR (300 MHz, [D$_4$]MeOH; signals of the major invertomer are marked with an asterisk *): δ = 1.59/1.62* (s, Σ = 3 H, CH_3), 1.78/1.92* (s, Σ = 3 H, CH_3), 1.82 (m, 4 H, 2 × CH_2), 2.08–2.12 (m, 2 H, CH_2 β-Ala), 2.36–2.40 (m, 2 H, CH_2 β-Ala), 2.60 (m, 4 H, 2 × CH_2), 2.66/2.92* (s, Σ = 3 H, NCH_3), 3.32 (m, 4 H, 2 × CH_2N), 3.38 (s, 6 H, 2 × OCH_3), 3.68 (t, J = 6 Hz, 2 H, CH_2SO_3), 3.72 (m, 4 H, 2CH_2), 3.96 (m, 4 H, CH_2O), 4.45 (br. s, 1 H, NH), 4.82 (overlapped: m, 1 H, CH Cys + HOD), 6.78 (d, J = 7 Hz, 2 H), 7.24 (d, J = 7 Hz, 2 H), 7.40 (br. m, 1 H), 7.64–7.66 (m, 3 H) ppm. ^{13}C NMR (75.5 MHz, CD_3CN): δ = 21.7 (CH_2), 27.6 (CH_2), 31.8 (CH_3), 32.2 (CH_2), 35.3/38.4* (CH_3), 41.6 (CH_2), 44.0 (CH_2), 52.1 (CH_2), 52.6 (CH_2), 53.0 (CH), 59.2 (OCH_3), 70.9 (C), 71.0 (C), 111.4, 111.6, 121.9, 124.0, 128.1, 129.6, 131.1, 135.4, 135.0, 135.4, 137.3, 154.3, 156.2, 162.1*/169.0 (C=O), 171.0/171.9* (C=O) ppm. MS (ESI+): *m/z* (%) = 833 $[M + 2Na – H]^+$; MS (ESI–): *m/z* (%) = 787 $[M – H]^–$. HRMS: calcd. for $C_{42}H_{52}N_4O_9S$ [M + H] 789.3528; found 789.3526.

Active Ester 21-TFP (2,3,5,6-Tetrafluorophenyl Ester of 21b): Dye **21b** (3.6 mg, 4.6 μmol) in anhydrous MeCN (HPLC grade; 1 mL) was treated with 2,3,5,6-tetrafluorophenol (15 mg, 100 μmol) in CH_2Cl_2 (1.5 mL) in the presence of HATU (17 mg, 45 μmol), and Et_3N (10 vol.-%. in MeCN, 40 μL, 28 μmol) under an argon atmosphere at 4–5 °C. The dye dissolved completely in a few minutes upon stirring, and the solution was kept for 1 h at this temperature and loaded straight into a column with SiO_2 (1.8 g). Elution with cold (10 °C) MeCN/H_2O (10:1) followed by filtration (Rotilabo® syringe filters, 0.22 μm) and freeze-drying, afforded 5 mg of a solid blue material containing 50–70% of active ester **22**. HPLC: t_R = 17 min (A/B: 70:30–0:100 in 25 min). The material (which was very unstable in aqueous solutions) was stored under argon at –20 °C for use in labeling studies.

Dye 22 (Linker Extension via Peptide-Type Coupling): Dye **21b** (16 mg, 20 μmol) in MeCN (3 mL) was stirred overnight at r.t. with β-alanine methyl ester hydrochloride (6.4 mg, 46 μmol), HBTU (23 mg, 60 μmol) and Et_3N (15 μL, 103 μmol). The reaction solution was diluted with MeCN (3 mL) and loaded directly into a column of SiO_2 (10 g). Upon elution with MeCN/H_2O (10:1), fractions containing compounds far less polar than the starting material were collected, filtered and the solvents evaporated. The dark-blue amorphous solid (Methyl ester of **22**) was saponified with aq. NaOH (1 M, 60 μL, 60 μmol) in a mixture of H_2O (3 mL) and THF (2 mL) at 4–5 °C overnight. The solution was neutralized with HOAc (60 μmol), evaporated to dryness in vacuo, and separated on SiO_2 (7 g; MeCN/H_2O, 5:1 → 3:1). Filtration (Rotilabo® syringe filters, 0.22 μm), centrifugation, and freeze-drying, afforded the free acid **22** (11 mg, 65%) as a dark-blue, fine crystalline powder that was well-soluble in H_2O and MeOH, very slightly soluble in MeCN, and insoluble in $CHCl_3$. TLC: R_f = 0.10 (MeCN/H_2O, 5:1); HPLC: t_R = 9.2 min (A/B: 70:30–0:100 in 25 min). MS (ESI+): *m/z* = 882 $[M + Na]^+$. HRMS (negative mode): calcd. for $C_{45}H_{57}N_5O_{10}S$ [M – H] 858.3753; found 858.3783. ^{1}H NMR (300 MHz, [D$_4$]MeOH; signals of the major invertomer are marked with an asterisk *): δ = 1.58/1.63* (s, Σ = 3 H, CH_3), 1.76*/1.80 (s, Σ = 3 H, CH_3), 1.80 (m, 4 H, 2 × CH_2), 2.20–2.30 (m, 4 H, 2 × CH_2, β-Ala), 2.36–2.40 (m, 4 H, 2 × CH_2, β-Ala), 2.60 (m, 4 H, 2 × CH_2), 2.65/2.91* (s, Σ = 3 H, NCH_3), 3.32 (m, 4 H, 2 × CH_2N), 3.38 (s, 6 H, 2 × OCH_3), 3.70 (t, J = 6 Hz, 2 H, CH_2SO_3), 3.75 (m, 4 H, 2 × CH_2), 3.96 (m, 4 H, CH_2O), 4.40–4.60 (br. s, 2 H, 2 × NH), 4.82 (overlapped: m, 1 H, CH Cys + HDO), 6.80 (d, J = 7 Hz, 2 H), 7.25 (d, J = 7 Hz, 2 H), 7.40 (br. m, 1 H), 7.65–7.70 (m, 3 H) ppm. ^{13}C NMR (75.5 MHz, CD_3CN): δ = 22.2 (CH_2), 24.0 (CH_2), 28.2 (CH_2), 32.0 (CH_3), 34.2 (CH_2), 36.1/36.8* (CH_3), 42.4 (CH_2), 45.1 (CH_2), 49.4 (CH_2), 49.6 (CH_2), 52.5 (CH_2), 52.6 (CH_2), 53.0 (CH), 59.5 (OCH_3), 71.3 (C), 112.0, 121.8, 121.9, 124.5, 128.5, 130.1, 130.1, 131.5, 135.6, 135.7, 135.8, 137.2, 154.9, 156.7, 161.5*/170.5 (C=O), 172.0*/173.0 (C=O), 179.9*/180.1 (C=O) ppm.

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 Et_3N (7 μL, 49 μmol) in a screw-cap vial at room temp. The maximum conversion (82%) was reached in 2 h, as shown by HPLC (t_R = 10.9 min; A/B: 70:30–0:100 in 25 min) and TLC (R_f = 0.20; MeCN/H_2O, 5:1) analyses. The reaction solution was loaded directly into a short column with SiO_2 (4 g; MeCN/H_2O, 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 (t_R = 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 $C_{49}H_{60}N_6O_{12}S$ [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 carbopyronine dye **1c**.

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