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Synthesis of *N,N*-Dialkylamino-*nor*-Dihydroxanthene-Hemicyanine Fused Near-Infrared Fluorophores and Their First Water-Soluble and/or Bioconjugatable Analogues

Michelle Jui Hsien Ong,[a] Sylvain Debieu,[b] Mathieu Moreau,[b] Anthony Romieu*[b],[c] and Jean-Alexandre Richard*[a]

Abstract: The effective synthesis of extended conjugated *N,N*-dialkylamino-*nor*-dihydroxanthene-based fluorophores is described from diversely functionalized salicylic aldehydes. The access to these original fluorescent derivatives proceeded in two steps through a one-pot construction of the unusual *nor*-dihydroxanthene (*nor*-DHX) scaffold followed by a diversification step providing a wide variety of *nor*-DHX-hemicyanine fused dyes emitting in the range 730–790 nm. The versatility of our approach has enabled a further extension to the late-stage introduction of negatively/positively charged polar groups onto their terminal nitrogen heterocyclic subunit giving access to the first water-soluble and/or bioconjugatable members of this emerging class of NIR fluorophores. Our water-solubilizing method is easily-implementable and the *nor*-DHX-hemicyanine skeleton keeps satisfying fluorescence quantum yields (5–20%) under physiological conditions. Finally, the bioconjugation ability of fluorescent derivatives bearing a free carboxylic acid was demonstrated through the covalent labeling of a model protein namely bovine serum albumin.

Introduction

Compared to the vast number of options offered for the choice of organic-based fluorophores emitting in the UV and visible region of the electromagnetic spectrum, near-infrared (NIR) families of dyes are in much shorter supply.[1] The need for new classes of NIR fluorophores is however apparent considering the benefits they provide in biological systems because of the gain of sensitivity (i.e., directly linked to low background fluorescence in the therapeutic window 700–900 nm), safety profile compared to dyes absorbing at more energetic wavelengths, and deeper tissue penetration.[2] The families of NIR fluorophores emitting above 700 nm[3] have so far been dominated by the cyanine[4],[5] and (aza-)BODIPY[6],[7] dyes and despite intense research interest for alternative scaffolds, new classes of high performance NIR fluorescent organic dyes are slow to emerge.[8] In this context, we became interested in the dihydroxanthene (DHX) fluorescent scaffold first reported by Czerney and Grummt in 1996.[9] This fluorescent core is a modified rhodamine skeleton where the C1'–C2' double bond is reduced and the π-conjugated system extended at the position C4', resulting in a fluorescence emission above 700 nm. Since the rediscovery of the DHX skeleton by Lin and co-workers in 2012,[10] particularly through a fortuitous synthesis during the reaction between a *meso*-chloro substituted heptamethine cyanine dye and

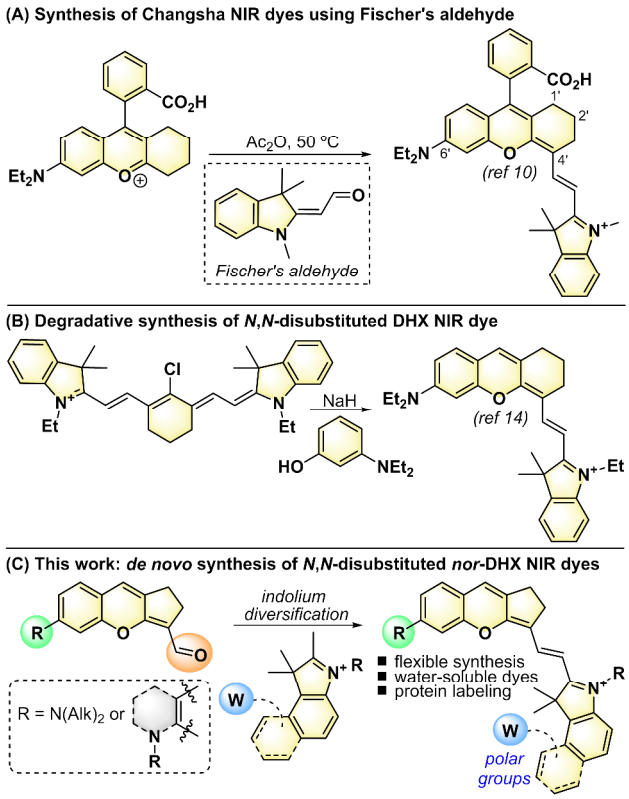

Figure 1. (A) and (B) Previous approaches to access dihydroxanthene-hemicyanine fused NIR fluorophores; (C) Alternative *de novo* synthesis allowing the easy access to structural analogues.

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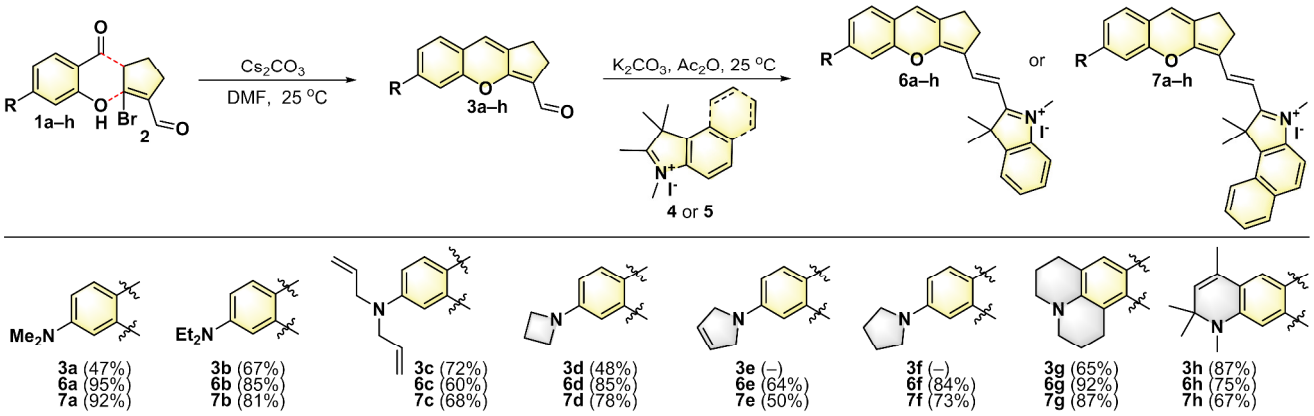

Figure 2. Synthesis of *N,N*-dialkylamino-*nor*-dihydroxanthene-hemicyanine fused NIR fluorophores.

resorcinol,[11] an increasing number of publications reporting their use in a wide range of biosensing/bioimaging applications have demonstrated the growing interest for this family of fluorophores,[12] especially those bearing an aniline/phenol moiety in C6' and acting as an effective fluorescence switch (i.e., fluorogenic center).[13] However, the two synthetic routes reported so far to form the DHX core rely either on the use of Fischer's aldehyde precursors with limited diversification potential (Figure 1A)[10] or on the degradation of structurally advanced, valuable heptacarbocyanine dyes (Figure 1B).[11],[14] These approaches haven't given much leeway to access analogues featuring improved photophysical properties or provide site-specifically functionalized dyes. To streamline the access to the DHX-hemicyanine fused dyes and study more in depth their fluorescence properties, we developed a methodology allowing the one-pot access to DHX skeletons featuring an aldehyde function at the position C4' and/or a bromine atom as C6' substituent, both of them used as handle(s) for late-stage diversification.[15] Using that approach, we also synthesized the first *nor*-dihydroxanthene (*nor*-DHX) scaffold, a more compact skeleton than its original sibling and for which we have already reported *N,N*-dialkylamino-dihydroxanthene-pyrylium conjugated fluorophores emitting above 800 nm.[16] However, a systematic diversification of the electron-pulling and electron-donating parts of the *nor*-DHX-based dyes has not been explored yet. In order to fill this gap, we considered the synthesis of a small library of such NIR fluorophores through substituent variations for the *N,N*-dialkylamino donor and by changing the nature of indolium acceptor moiety. This library enabled us to conduct a structure-fluorescence relationship (SFR) study providing valuable information related to the fine-tuning of photophysical properties of these unconventional DHX-type fluorophores. In this article, we report the results of this ambitious study as well as a further extension of the claimed synthetic methodology to the preparation of the first water-soluble DHX-hemicyanine fused

Table 1. Photophysical properties of *nor*-DHX-hemicyanine fused dyes **6a–h** and **7a–h** at 25 °C. For structures, see Figure 2.

		Abs λ_{max} [nm][a]			Em λ_{max} [nm][c]			ε [M^{-1} cm^{-1}]			Stokes' shift [cm^{-1}]			Φ_F [%][d]		
entry	dye	PBS[b]	EtOH	$CHCl_3$	PBS[b]	EtOH	$CHCl_3$	PBS[b]	EtOH	$CHCl_3$	PBS[b]	EtOH	$CHCl_3$	PBS[b]	EtOH	$CHCl_3$
1	**6a**	719	712	722	731	739	737	89 865	90 530	101 580	228	513	282	12	19	30
2	**6b**	726	720	731	736	744	745	76 530	84 020	100 430	187	448	257	12	20	28
3	**6c**	715	710	720	728	736	735	81 480	107 430	125 050	250	498	283	11	19	32
4	**6d**	721	712	727	735	744	745	82 430	75 310	86 165	264	604	332	13	20	31
5	**6e**	717	713	723	728	738	738	130 770	109 480	127 070	211	475	281	16	18	30
6	**6f**	725	720	731	734	748	745	116 460	98 875	118 200	169	520	257	16	20	28
7	**6g**	747	740	750	758	768	764	94 530	115 840	137 540	194	493	244	8	11	19
8	**6h**	750	742	757	764	773	774	73 050	93 080	110 380	244	540	290	9	12	17
9	**7a**	737	730	741	748	760	756	52 810	110 590	120 360	200	541	268	5	8	14
10	**7b**	742	737	748	749	764	764	66 970	90 205	109 080	126	480	280	5	8	14
11	**7c**	735	729	739	746	753	755	72 150	108 210	123 635	201	437	287	6	6	14
12	**7d**	739	731	744	752	765	764	62 895	70 150	81 885	128	608	352	6	7	15
13	**7e**	735	731	742	742	758	757	36 485	69 365	82 930	128	487	267	4	9	16
14	**7f**	742	738	749	749	763	765	56 680	92 960	112 900	126	444	279	6	5	11
15	**7g**	765	757	768	778	783	781	79 420	103 160	128 780	218	439	217	5	7	15
16	**7h**	766	759	774	779	791	789	65 920	102 400	130 170	218	533	246	4	8	13

[a] Assigned to S0–S1 transition but vibronic and/or S0–S2 transitions are also observed (see Figure 3 for examples of Abs spectra). [b] PBS buffer containing 5% BSA. [c] Excitation at 650 nm. [d] Determined at 25 °C by using ICG (Φ_F = 10.6% in DMSO, λ_{ex} = 650 nm) as standard (see the Supporting Information).

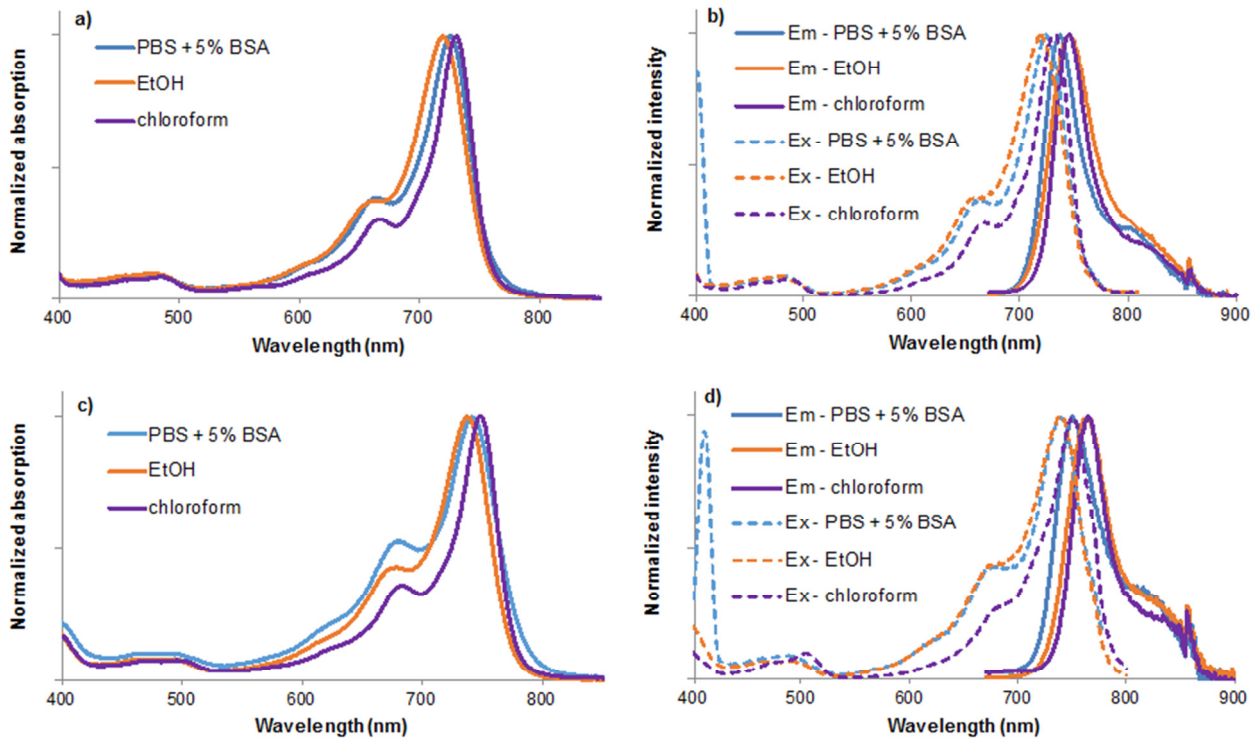

Figure 3. Normalized absorption, fluorescence emission (excitation at 650 nm) and excitation (emission at 800, 820 or 830 nm) spectra of *N,N*-diethylamino-*nor*-DHX NIR fluorophores **6b** (a and b) and **7b** (c and d) at 25 °C. *Please note*: *absorption and fluorescence emission spectra were recorded with solutions whose concentrations are in the range 10^{-5}–10^{-6} M and 10^{-6}–10^{-7} M respectively. All emission spectra (also those of standard ICG) are corrected until 850 nm, which explains the artefact observed at this wavelength.*

dyes (Figure 1C). Furthermore, the availability of a free carboxylic acid moiety within the terminal indolic subunit of some of these compounds has enabled us to use them in fluorescent covalent labeling of proteins.

Results and Discussion

Synthesis of *N,N*-dialkylamino-*nor*-DHX-hemicyanine fused NIR fluorophores

The access to a wide variety of fluorescent organic dyes could be ensured at the outset by choosing an appropriate range of salicylic aldehydes displaying diversity on the amino group donor. We therefore sourced or prepared salicylic aldehyde precursors featuring either an acyclic *N,N*-dialkylamino (**1a**–**c**), a cyclic *N,N*-dialkylamino (**1d**–**f**) or a fused *N,N*-dialkylamino (**1g**–**h**) moiety, the latter presenting the additional advantage to potentially lead to fluorophores not accessible using our previously reported late-stage amination strategy.[15b] The construction of the *nor*-DHX scaffold could be secured using our optimized conditions (i.e., Cs_2CO_3, DMF) where *N,N*-substituted salicylic aldehydes condensed one-pot with 2-bromocyclopent-1-ene-1-carbaldehyde **2** to form the formyl derivatives **3a**–**h** in yields ranging from 47% to 87%. With eight *nor*-DHX aldehyde precursors **3a**–**h** in hand, we then performed a diversification step to vary the electron-acceptor moiety of the targeted NIR fluorophores. In accordance with the recent results reported by Yuan and co-workers,[17] the work conducted in our group identified the 1,3,3-trimethyl-2-methyleneindoline unit (also known as Fischer's base) and related derivatives as the ideal structural moieties to maximize both absorption and emission of the π-extended *nor*-DHX fluorophores in the NIR range. We therefore chose two of such indolinium salts in order to produce sixteen new NIR fluorophores upon condensation with **3a**–**h**, whose fluorescence properties would give us insight regarding the SFR of this family of dyes. We chose 1,2,3,3-tetramethyl-3*H*-indolium **4** and 1,1,2,3,-tetramethyl-1*H*-benz[*e*]indolium **5** (both as iodide salt) as condensing partners because of their simplicity and the availability of related precursors functionalized at various positions of the indolinium or benzoindolinium skeleton. The Knoevenagel condensation proceeded in good to excellent yields (50–95%) under mild conditions (i.e., K_2CO_3, Ac_2O). The structures of these sixteen novel DHX-type fluorophores were unambiguously confirmed by ESI-HRMS and NMR spectroscopic analyses (see the Supporting Information).

Photophysical properties of *N,N*-dialkylamino-*nor*-DHX-hemicyanine fused NIR fluorophores

The photophysical properties of these novel DHX-based fluorophores were evaluated in different media including phosphate-buffered saline (PBS) with 5% (w/v) bovine serum albumin (BSA) as simulated body fluid, EtOH, and $CHCl_3$. These spectroscopic data are gathered in Table 1 and selected examples of electronic absorption, excitation and emission

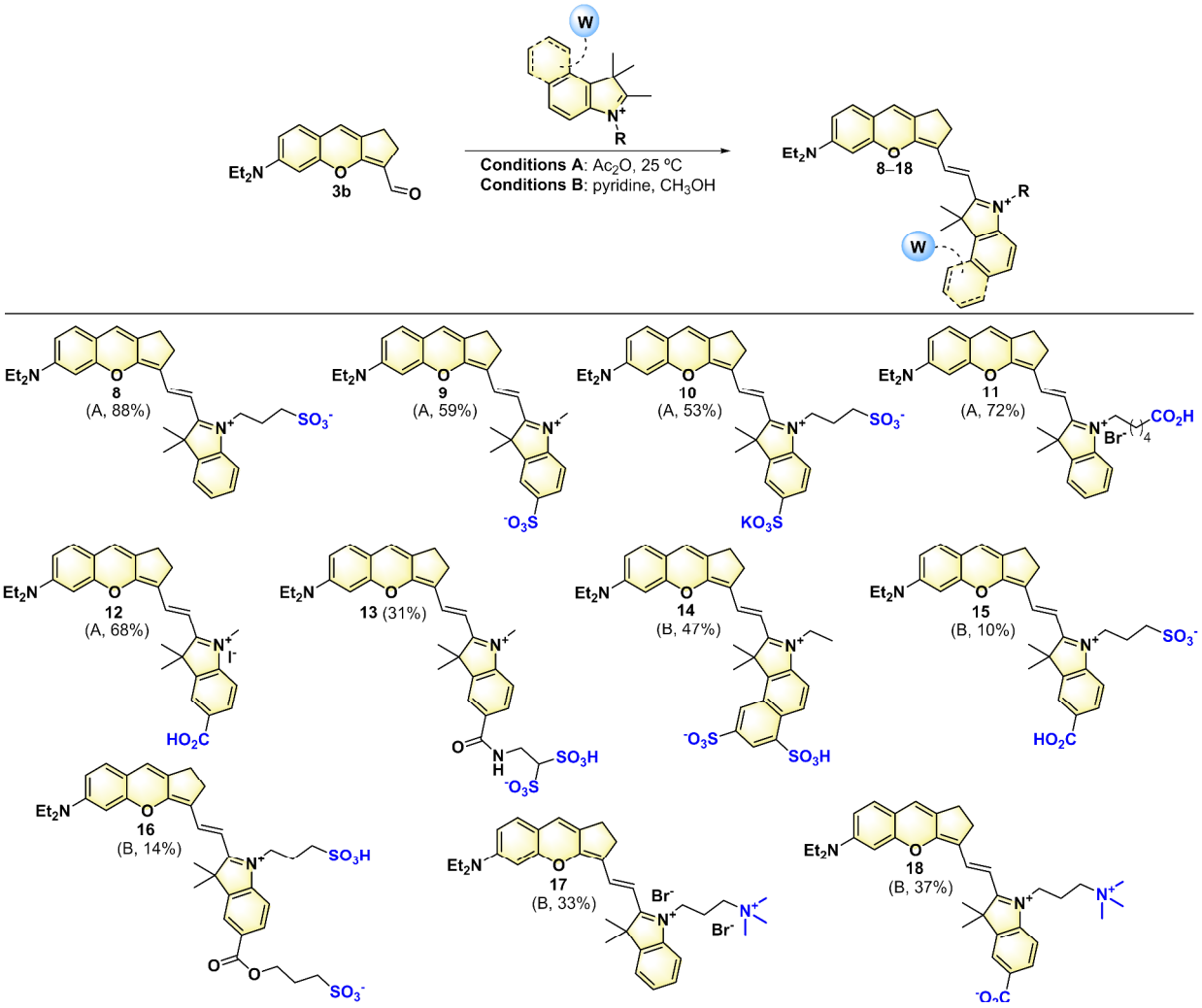

Figure 4. Synthesis of water-soluble *N,N*-diethyllamino-*nor*-dihydroxanthene NIR fluorophores (compounds **13**, **15**, **16** and **18** were isolated as TFA salts; the number of TFA molecules was determined by elemental analysis).

Table 2. Photophysical properties of water-soluble *nor*-DHX-hemicyanine fused dyes **8**–**18** at 25 °C. For structures, see Figure 4.

		Abs λ_{max} [nm][a]		Em λ_{max} [nm]		ε [M^{-1} cm^{-1}]		Stokes' shift [cm^{-1}]		Φ_F [%][b]	
entry	dye	PBS	PBS + 5% BSA	PBS	PBS + 5% BSA	PBS	PBS + 5% BSA	PBS	PBS + 5% BSA	PBS	PBS + 5% BSA
1	**8**	719, 787[c]	731	743	738	—[d]	81 835	449	130	9	18
2	**9**	648, 704	729	745	744	—[d]	—	2009, 782	277	6	13
3	**10**	663, 716	734	750	751	—[d]	85 600	1750, 633	308	7	11
4	**11**	652, 713	669, 732	742	740	—[d]	45 130, 168 540	1860, 548	1434, 148	7	16
5	**12**	648, 709	730	747	742	—[d]	97 520	2045, 717	222	6	10
6	**13** (1.5 TFA)	666, 717	722	752	748	—[d]	150 520	1717, 649	481	5	10
7	**14**	677, 728	740	760	755	—[d]	106 400	1613, 578	268	3	13
8	**15** (1.5 TFA)	664, 719	736	747	752	58 930, —[d]	122 270	1673, 521	289	5	11
9	**16** (1.25 TFA)	660, 707	738	746	761	79 540, —[d]	147 300	1747, 739	410	3	8
10	**17**	705	724	745	740	85 700	77 350	762	299	7	12
11	**18** (3.5 TFA)	717	724	751	744	128 730	131 200	631	371	5	11

[a] Assigned to S0–S1 transition but vibronic and/or S0–S2 transitions are also observed (see Figure 5 and the Supporting Information for the corresponding Abs spectra). [b] Determined at 25 °C by using ICG (Φ_F = 10.6% in DMSO, λ_{ex} = 650 nm) as standard (see the Supporting Information). [c] Only one absorption maxima at 715 nm was observed at a lower 10^{-6}–10^{-7} M concentration. [d] A non-linear relationship between absorption and concentration was obtained due to the formation of H-type dimer aggregates.

spectra of **6b** and **7b** are available in Figure 3. As expected, fluorophores **6a**–**h** and **7a**–**h** display absorption and fluorescence emission peaks in the NIR region at 710–774 nm and 728–791 nm depending on the substitution pattern of the amino group and the medium. However, no significant solvatochromism effect was observed for this class of DHX-hemicyanine hybrids. The reduction of the dihydro ring size has no real positive or negative impact on the spectral features of these DHX-based fluorophores because values close to those of cyclohexenyl-based congeners previously studied have been obtained. Comparing the properties of the **6** and **7** series differing only by the additional aromatic ring on the hemicyanine part of the dye showed a 13–21 nm red-shift between **6a**–**h** and their π–extended analogues **7a**–**h**. The relative fluorescence quantum yields under physiological conditions were better for the indolinium analogues **6a**–**h** (8–16%) than for benzoindolinium **7a**–**h** (4–6%), and sufficient for considering the further use of these NIR emitters in biosensing/bioimaging applications. Furthermore, it is known that the presence of BSA or related proteins in aq. media enhances the emission of organic-based fluorophores owing to a combination of rigidification, a reduction in the polarity of the dye's microenvironment (binding in the hydrophobic BSA pocket), and deaggregation.[18] Interestingly, we noted that the quantum yields of the *nor*-DHX indolinium analogues **6a**–**h** were notably superior compared to the values (4–11%) already reported for a similar subset of *N,N*-dialkylamino-dihydroxanthene-based fluorophores.[15b] This may be explained by the less flexibility of the 5-ring system that limits molecular motions involved in the non-radiative decay pathways. The nature of the *N,N*-dialkylamino electron-donating group appeared to be also important to influence the brightness of the *nor*-DHX fluorophores. For instance, it is worth highlighting the superiority of dyes **6e** and **6f** featuring either a 3,4-dihydro pyrrolidinyl or pyrrolidinyl over the other *N,N*-dialkylamino donating moieties, including the azetidinyl ring which had previously been reported as being the optimum electron-donating group for brighter rhodamine-based fluorophores namely Janelia Fluor™dyes (Table 1).[19]

Synthesis of water-soluble *N,N*-dialkylamino-*nor*-DHX-hemicyanine fused NIR fluorophores

Capitalizing on the promising fluorescence properties obtained for the *nor*-DHX- and DHX-based fluorophores in PBS + 5% BSA, we were then interested in exploring the synthesis of the first hydrophilic variations of these unusual fluorescent scaffolds to make easier their applications in biological media. To the best of our knowledge, no water-soluble DHX analogue has been reported to date and we hypothesized that the hemicyanine part could be functionalized through the site-specific introduction of polar group(s) onto the phenyl ring and/or through the *N*-quaternarization of (benzo)indole unit with a hydrophilic

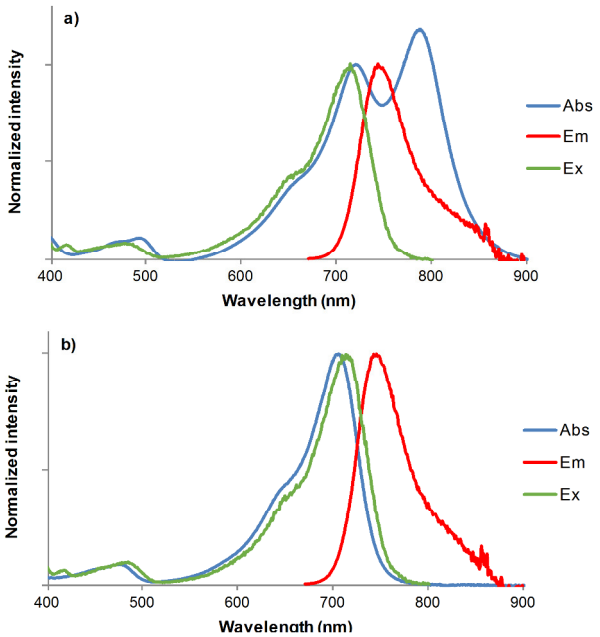

Figure 5. Normalized absorption, fluorescence emission (excitation at 650 nm) and excitation (emission at 820 nm) spectra of water-soluble *N,N*-diethylamino-*nor*-DHX NIR fluorophores **8** (a) and **17** (b) in PBS at 25 °C. *Please note*: *absorption and fluorescence emission spectra were recorded with solutions whose concentrations are in the range 10^{-5}–10^{-6} M and 10^{-6}–10^{-7} M respectively. All emission spectra (also those of standard ICG) are corrected until 850 nm, which explains the artefact observed at this wavelength. If the absorption spectrum of **8** in PBS is recorded in the range of 10^{-6}–10^{-7} M, the red-shift band at 787 nm disappears and a single maximum at 715 nm is observed.*

alkylating agent. For this purpose, we set out to take advantage of the divergency of our synthetic route to introduce one or two polar groups negatively- and/or positively-charged at physiological pH, to the skeleton of the fluorophore. The three hydrophilic moieties (i.e, carboxylate, sulfonate and trimethylalkylammonium) selected to be added to the *nor*-DHX-hemicyanine hybrid scaffold during the diversification step were therefore positioned at various specific positions of the indolium partner. Because of its convenient availability on gram scale, we performed the water-solubilization step on *nor*-DHX aldehyde precursor **3b** (see Figure 4 as an illustration of the scope of our investigations). Our previsouly established conditions for the Knoevenagel condensation (i.e., K_2CO_3, Ac_2O) proved to be disappointing since only traces of mono-sulfonated fluorophore **8** could be identified in the reaction mixture. To our delight, we realized that the presence of base was detrimental to the formation of the dye and that by simply removing it (i.e., Ac_2O, conditions A), the Knoevenagel-type condensation between **3b** and a set of indolinium salts bearing either a single or two sulfonates or a carboxylic acid functionality worked well. The resulting hydrophilic derivatives were isolated by flash-column chromatography on silica gel in good yields (53–88%). It is worth noting the additional benefit of the carboxylic acid function in **11**

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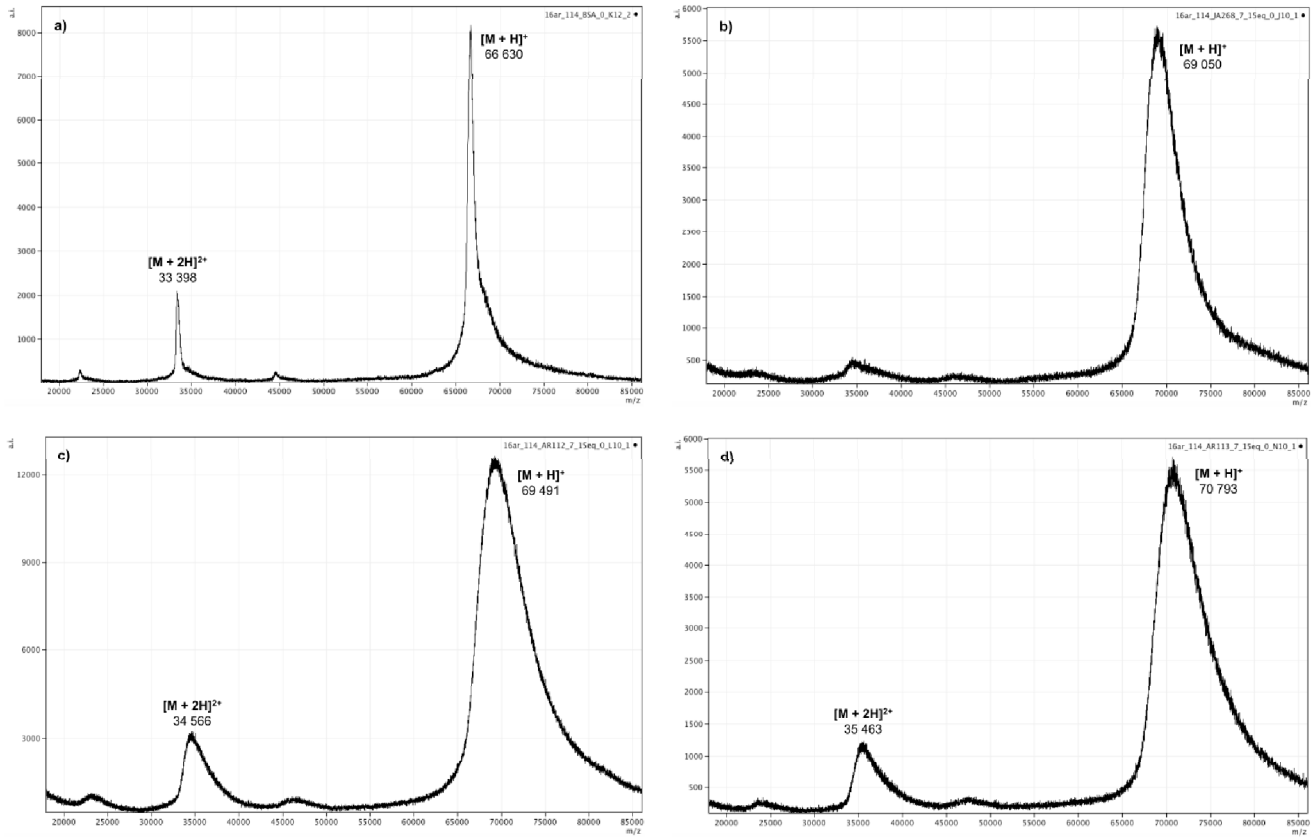

Figure 6. MALDI-TOF mass spectra of BSA (a) and fluorescent conjugates **BSA-12** (b, labeling conditions: 15 eq. of NHS ester of *nor*-DHX-hemicyanine fused dye **12** in phosphate buffer at pH 7.0), **BSA-15** (c, labeling conditions: 15 eq. of NHS ester of *nor*-DHX-hemicyanine fused dye **15** in phosphate buffer at pH 7.0) and **BSA-18** (d, labeling conditions: 15 eq. of NHS ester of *nor*-DHX-hemicyanine fused dye **18** in phosphate buffer at pH 7.0) recorded in the positive mode (matrix: sinapinic acid).

and **12** which can also be used as a reactive handle for further functionalization through amidification reactions. As an illustrative example, we performed an amide coupling between **12** and 2-aminoethane-1,1-disulfonic acid (DIEA salt)[20] and mediated by uronium reagent HATU,[21] that allowed the post-synthetic sulfonation of this DHX-hemicyanine fused dye **12** under very mild conditions. After purification by semi-preparative RP-HPLC with aq. 0.1% TFA and CH_3CN as eluents, compound **13** was obtained in pure form and in a satisfying 31% isolated yield. Further extension of the Knoevenagel-type reaction with more polar indolinium condensing partner failed due to solubility issues even at higher reaction temperatures. Inspired by a literature precedent[22], we found that the use of CH_3OH as solvent was not only able to solubilize all the starting materials but also successfully led to the desired water-soluble DHX-based fluorophores **14**–**18** in the presence of a trace amount of a weak base (i.e., pyridine, CH_3OH, conditions B). Isolation in a pure form of **15**, **16** and **18** was achieved by semi-preparative RP-HPLC with aq. 0.1% TFA and CH_3CN as eluents (isolated yields: 10%, 14% and 37% respectively) whereas compounds **14** and **17** were purified by flash-column chromatography on silica gel. It is important to underline that the preparation of the indolium precursor involved in the synthesis of **15** was not trivial because the *N*-quaternarization of 2,3,3-trimethyl-3*H*-indole-5-carboxylic acid with an excess of 1,3-propanesultone (acting as both reagent and solvent) at 120–145 °C also led to complete esterification of the carboxylic acid (see the Supporting Information for the preparation of such starting indolinium salt). A further acidic hydrolysis (aq. 6 N HCl) followed by a purification by semi-preparative RP-HPLC with aq. triethylammonium bicarbonate buffer (TEAB, 50 mM, pH 7.5) and CH_3CN as eluents were required to readily obtain this starting material. Trimethylalkylammonium groups have recently emerged as valuable water-solubilizing moieties to dramatically improve the solubility of a wide range of organic-based fluorophores under physiological conditions.[23] That approach, mainly applied to heptamethine cyanine dyes, has led to better performances *in vivo* in the context of NIR fluorescence molecular imaging[5b, 5e, 5f, 5i, 24] and prompted us to introduce the positively-charged trimethylpropylammonium group onto the *nor*-DHX-hemicyanine hybrid scaffold. Fluorophores **17** and **18** bearing a positive net charge $q = +2$ and $q = +1$ at physiological pH were therefore synthesized, the latter one featuring an additional carboxylic acid group for further functionalization (*vide infra*).

Photophysical properties of water-soluble *N,N*-dialkylamino-*nor*-DHX-hemicyanine fused NIR fluorophores

The introduction of polar groups onto the skeleton of the *nor*-DHX-based fluorophores gave us the opportunity to assess their influence on the solubility and aggregation behavior in physiological conditions. As a general trend, *nor*-DHX derivatives bearing a single hydrophilic group are soluble in water and related aq. buffers in the concentration range 1–100 μM, and the upper limit is 1–2 mM for the bis-sulfonated derivatives **10**, **13**, **14** and **16**, and carboxylic acids **15** and **18**. The photophysical properties were determined in pure PBS and in PBS containing 5% (w/v) BSA and gathered in Table 2 (see Figure 5 and the Supporting Information for the electronic absorption, excitation and emission spectra). In the concentration range 5–15 μM, we found that a partial aggregation occurred in PBS with fluorophores **8**–**16** featuring carboxylate and/or sulfonate group(s) (i.e., negatively-charged at physiological pH), which prevents an accurate determination of their molar extinction coefficients at absorption maxima. However, these compounds remained fluorescent and satisfactory fluorescence quantum yields (3–9%) were obtained. Such aggregation phenomenon in PBS could be completely avoided through the introduction of the positively-charged trimethylpropylammonium moiety. Indeed, for compounds **17** and **18**, a good matching between the absorption and excitation spectra was observed and a linear relationship between absorbance and concentration was obtained (see Figure 5b and the Supporting Information). Compared to these measurements, those achieved in PBS/BSA revealed a notable red-shift for the absorption spectra, resulting in lower Stokes' shifts (7–26 nm, 130–480 cm^{-1} vs. 24–41 nm, 449–782 cm^{-1}). Using the optical properties of **6b** as benchmark (i.e., Abs/Em λ_{max} = 726/736 nm, ε = 76 530 M^{-1} cm^{-1} and Φ_F = 12% in PBS + 5% BSA) to compare with those of more hydrophilic *nor*-DHX-hemicyanine fused dyes **8**–**18** in PBS/BSA, we observed that the absorption and emission maxima were in the range 722–740 nm and 738–761 nm respectively, directly dependent on the presence of ionized substituent(s) on *N*-alkyl arm and/or phenyl ring of indolinium unit. Gratifyingly, a 50% increase of fluorescence quantum yield was obtained with fluorophore **8**, showing the beneficial effect of such functionalization to obtain bright and biocompatible DHX-hemicaynine fused dyes.

Water-soluble *N,N*-dialkylamino-*nor*-DHX-hemicyanine fused dyes as fluorescent covalent labeling reagents of biomolecules

The marked solubility in aqueous media, the valuable optical properties, and the availability of a free carboxylic acid group on their core structure, are positive features for the use of DHX derivatives **12**, **15** and **18** in fluorescent bio-labeling applications.[25] To demonstrate their ability both to readily react with proteins under mild conditions and to give fluorescent bioconjugates, the labeling of BSA as a model protein was explored. BSA contains 59 lysine residues, and more than thirty

Table 3. Photophysical properties and labeling densities of fluorescent BSA conjugates.

dye	Abs λ_{max} [nm][a], [b]	Em λ_{max} [nm]	F/P [MS][c]	F/P [UV/vis][d]	Φ_F [%][e]
12, 15 eq., pH 7.0	665, 720	746	5.4	3.1	1.5
12, 30 eq., pH 7.0	658, 716	748	5,2	6.0	1.5
12, 15 eq., pH 7.7	666, 720	748	5.0	4.7	1.5
12, 30 eq., pH 7.7	658, 718	749	4.9	4.2	1.7
15, 15 eq., pH 7.0	682 (sh)[f], 731	747	5.1	1.4	2.0
15, 30 eq., pH 7.0	680 (sh)[f], 729	746	5.0	1.3	1.5
15, 15 eq., pH 7.7	676, 729	751	5.2	2.5	1.0
15, 30 eq., pH 7.7	675, 727	746	6.5	2.4	1.0
18, 15 eq., pH 7.0	673, 723	753	7.8	2.7	1.0
18, 30 eq., pH 7.0	682 (sh)[f], 718	751	13.8	3.9	2.0
18, 15 eq., pH 7.7	679, 724	751	10.1	3.7	1.0
18, 30 eq., pH 7.7	680, 720	751	14.4	3.5	1.5

[a] UV absorption maxima have been omitted. [b] Shorter absorption maximum assigned to H-type dimer aggregates. [c] Determined by MALDI-TOF mass spectrometry. [d] Determined by absorption spectroscopy (for details see the Supporting Information). [e] Determined by using ICG (Φ_F = 10.6% in DMSO, λ_{ex} = 650 nm) as a standard (see the Supporting Information). [f] sh = shoulder

are accessible for conjugation.[26] Since the ε-amino groups of lysine residues of BSA are known to form amides through reaction with a wide range of activated esters in aqueous buffers the conversion of **12**, **15** and **18** into their corresponding NHS esters using uronium-based coupling agent TSTU[27] (1.1 eq.) and DIEA (2–3 eq.) in DMSO was considered. BSA was labeled after overnight incubation with either a 15- or 30-fold molar excess of the NHS ester in phosphate buffer (pH 7.0 or pH 7.7) at 4 °C. The *nor*-DHX-conjugated proteins **BSA-12**, **BSA-15** and **BSA-18** were washed with an ultra-centrifugal filter device (30 kDa cut-off) to remove most of the excess of free unbound *nor*-DHX-hemicyanine fused dye. Since the method of choice for analyzing protein conjugates is mass spectrometry, all labeled protein samples were subjected to matrix-assisted laser desorption time-of-flight (MALDI-TOF) analyses and their spectra were compared with that of the parent BSA (Figure 6 and Figures S1–S3 in the Supporting Information). In all of the mass spectra, a main peak assigned to $[M+H]^+$ (molecular ion) was observed. The mass difference between molecular ions of BSA and its fluorescent conjugates allowed to estimate labeling densities, defined as the (average) number of fluorophore molecules attached to a protein (F/P). The corresponding values are gathered in Table 3. Moderate to high F/P values (4.9–14.4) were obtained but a poor correlation was observed with those determined by the UV-vis spectrophotometric method (see the Supporting Information), mainly for fluorescent conjugates **BSA-15** and **BSA-18**. Even if there are no scientific certainties to interpret these unexpected results, we assumed the hetereogeneity of labeled protein samples containing residual amounts of free *nor*-DHX-hemicyanine fused dye (monomeric and/or aggregated forms) not removed by ultracentrifugation and adsorbed on the protein. The latter hypothesis was supported by gel electrophoresis and subsequent imaging of the gel by fluorescence scanning with IVIS Lumina III *in vivo* imaging system (Ex/Em filters 660/790 nm, bandwidth 20/40 nm, Figure S4 in the Supporting Information) showing the presence of both targeted protein-dye conjugates and free dye. Despite the

presence of unbound fluorophore molecules in the labeled protein samples, the spectroscopic features (absorption/emission maxima and quantum yields) of the BSA conjugates were determined (see Table 3 and Figure S5 in the Supporting Information for selected examples of electronic absorption, excitation and emission spectra). The absorption spectrum of fluorescent protein conjugates in PBS revealed two distinct peaks at 658–682 nm and 716–731 nm. The dual absorption, the breadth of the absorption bands and the blue-shift of one of them to 35–50 nm (compared to the absorption maximum of **12**, **15** or **18**) support the formation of non-emissive aggregates (i.e., H-type homodimers).[28] The low values of the measured quantum yields (1–2% vs. 5–6% for the corresponding free *nor*-DHX-hemicyanine fused dyes) may be attributed to a combination of several effects, including (1) H-dimer formation, (2) autoquenching induced by interactions between fluorophores and aromatic rings on the side chains of amino acids such as tryptophan and phenylalanine.[29] These results are mixed but the use of DHX-based fluorophores as fluorescent labeling reagents of amine-containing biomolecules was demonstrate dual absorptiond for the first time.

Conclusions

In summary, we developed a new family of organic NIR fluorophores based on the unusual *nor*-dihydroxanthene scaffold. We designed a synthetic route allowing the expedient formation of the *nor*-DHX scaffold which was suitably functionalized for a subsequent diversification step allowing the formation of wide range of DHX-hemicyanine fused dyes. We applied our strategy to the rapid synthesis of *nor*-DHX-based fluorophores featuring either a *N*,*N*-dialkylamino, a cyclic amino or a fused (bi)cyclic amino group as electron-donating moiety and identified 5-membered-ring heterocyclic amines as being optimum substituent to maximize the fluorescence emission (fluorescence quantum yields 15–20%). A further and easily-implementable extension of this methodology has led to the first water-soluble *nor*-DHX-based fluorophores through the introduction of hydrophilic groups performed on the hemicyanine part of the molecule. Despite the "anisotropic" character of this water-solubilizing methodology known to favor the formation of non-fluorescent micellar aggregates,[30] we managed to identify the positively-charged trimethylpropylammonium group as an effective moiety to readily water-solubilize the DHX-hemicyanine fused dyes as well as to avoid their aggregation at physiological pH. However, to improve the brightness of these water-soluble DHX-based fluorophores, further work is in progress in our laboratories to devise a complementary functionalization approach of the DHX core through the introduction of polar subtituent(s) on the donor part of the fluorophore skeleton. Finally, we demonstrated the bioconjugation ability of *nor*-DHX-hemicyanine fused dyes bearing a free carboxylic acid by performing the fluorescent labeling of the BSA protein. Thus, this contribution paves the way for a further use of DHX-based NIR fluorophores in the rational design of either molecular imaging agents (mainly, antibody-NIR dye conjugates)[31] or water-soluble fluorogenic probes (also known as fluorescent chemodosimeters) for various analytes.[32]

Experimental Section

See the Supporting Information for the details about sections "General", "Instruments and methods", "HPLC separations" and all experimental data associated with synthesized compounds and fluorescent BSA conjugates.

Procedure for the synthesis of *nor*-DHX aldehydes 3a–h

To a solution of salicylic aldehydes **1a–d**, **1g** or **1h** in dry DMF at 25 °C were added Cs_2CO_3 (3 eq.) and crude 2-bromocyclopent-1-ene-1-carbaldehyde **2** (2 eq.) in solution in dry DMF. The resulting reaction mixture was stirred for 48 h at 25 °C to reveal an intense yellow spot (TLC hexane/EtOAc, 8:2, v/v). The insoluble material was then filtered on a pad of silica gel and the filtrate was concentrated under vacuum. The resulting residue was dissolved in CH_2Cl_2 and washed with deionized water. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo. Purification by flash-column chromatography on silica gel (CH_2Cl_2/EtOAc, 9:1, v/v) provided the desired aldehydes **3a–d**, **3g** or **3h** as deep orange solids.

Salicylic aldehydes **3e** and **3f** were prepared as following: to a solution of aldehyde **3c** (37 mg, 0.13 mmol) in CH_2Cl_2 (4 mL) were added 1st generation Grubbs' catalyst (5.2 mg, 0.0063 mmol, 0.05 eq.) and *p*-benzoquinone (1.4 mg, 0.013 mmol, 0.1 eq.). The reaction mixture was stirred at 25 °C for 1 h before it was filtered over a short pad of silica gel. The filtrate was concentrated under vacuum and provided **3e** which was used directly in the next step, either for the formation of dyes **6e** and **7e** (*vide infra*) or hydrogenated in toluene for 1 h in the presence of Adam's catalyst ($PtO_2 \cdot H_2O$). After removing the catalyst on a short pad of silica gel, the filtrate was concentrated and cleanly afforded aldehyde **3f** which was used without further purification in the formation of *nor*-DHX dyes **6f** and **7f**.

General procedure for the synthesis of *nor*-DHX-hemicyanine fused dyes 6a–h and 7a–h

To *nor*-DHX aldehydes **3a–h** in anhydrous Ac_2O (0.025–0.05 M) were added 1,2,3,3-tetramethyl-3*H*-indolium iodide **4** or 1,1,2,3,-tetramethyl-1*H*-benz[*e*]indolium iodide **5** (1.2 eq.) along with K_2CO_3 (2 eq.) and the mixture was stirred at 25 °C for 16 h to reveal an intense green spot (TLC CH_2Cl_2/CH_3OH, 9:1, v/v). The reaction mixture was concentrated and the resulting residue dissolved in CH_2Cl_2 and washed with deionized water. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo. Purification by flash-column chromatography on silica gel (step gradient of CH_3OH in CH_2Cl_2 from 0% to 3%) afforded NIR *nor*-DHX-based fluorophores **6a–h** and **7a–h**.

General procedure for the synthesis of water-soluble *nor*-DHX-hemicyanine fused dyes 8–12

To *nor*-DHX aldehyde **3b** in anhydrous Ac_2O (0.025–0.1 M) were added the corresponding indolinium salt (1.2 eq.) and the mixture was stirred at 25 °C for 16 h to reveal an intense green spot (TLC CH_2Cl_2/CH_3OH, 9:1, v/v). The reaction mixture was concentrated and directly loaded on silica gel and purified (step

gradient of CH_3OH in CH_2Cl_2 from 0% to 10–15%) to afford *nor*-DHX-based fluorophores **8**–**12**.

Synthesis of *nor*-DHX-hemicyanine fused dye (13)

Carboxylic acid-functionalized *nor*-DHX-hemicyanine NIR dye **12** (60 mg, 0.1 mmol, 1 eq.) was dissolved in dry DMF (2 mL). DIEA (34 µL, 0.2 mmol, 2 eq.) and HATU (43 mg, 0.11 mmol, 1.1 eq.) were sequentially added. The resulting reaction mixture was stirred at 25 °C for 15 min. The resulting crude HOAt activated ester was added dropwise to a pre-cooled solution of 2-aminoethane-1,1-disulfonic acid (DIEA salt) in dry DMF (0.19 M in DMF, 1 mmol, 10 eq.) and DIEA (85 µL, 0.5 mmol, 5 eq.) and the resulting mixture was stirred at 25 °C for 1 h. The reaction was checked for completion by TLC (CH_2Cl_2/CH_3OH, 9:1, v/v), quenched by adding glacial AcOH (50 µL) and finally evaporated under reduced pressure. The resulting residue was dissolved in a (1:1, v/v) mixture of aq. 0.1% TFA and CH_3CN (ca. 5 mL) and purified by semi-preparative RP-HPLC (for more detail see the Supporting Information). The product-containing fractions were lyophilized to give the TFA salt (1.5 TFA) of compound **13**, as a green amorphous powder (26 mg, yield 31%).

General procedure for the synthesis of *nor*-DHX-hemicyanine fused dyes 14–18

To *nor*-DHX aldehyde **3b** in HPLC-grade CH_3OH (0.025–0.05 M) were added the corresponding indolinium or benzoindolinium salt (1.2 eq.) and dry pyridine (one or two drops). The resulting reaction mixture was stirred under reflux (except for **18**, stirring at 25 °C) for 2 h and at 25 °C for 16 h (please note: the color gradually changed to green). The reaction was checked for completion by TLC (CH_2Cl_2/CH_3OH, 8:2, v/v), evaporated under reduced pressure and purified by flash-column chromatography on silica gel or by semi-preparative RP-HPLC.

Isolation of dye 14: The crude product was purified by flash-column chromatography over silica gel (dry loading, step gradient of CH_3OH in CH_2Cl_2 from 0% to 20%). Bis-sulfonated *nor*-DHX-hemicyanine NIR dye **14** was obtained as a green solid (42 mg, yield 47%).

Isolation of dye 15: The crude product was dissolved in a (1 : 1) mixture of aq. 0.1% TFA and CH_3CN (ca. 5 mL) and purified by semi-preparative RP-HPLC (for more detail see the Supporting Information). The product-containing fractions were lyophilized to give the TFA salt (1.5 TFA) of compound **15**, as a green amorphous powder (22 mg, yield 10%). Please note: the low isolated yield was explained by partial degradation of *nor*-DHX-hemicyanine fused dye in the crude reaction mixture due to too prolonged heating at reflux.

Isolation of dye 16: The crude product was purified by flash-column chromatography over silica gel (dry loading, bed size 20 × 170 mm, step gradient of CH_3OH in CH_2Cl_2 from 0% to 30%). Carboxylic acid 3-sulfonatopropyl ester-functionalized mono-sulfonated *nor*-DHX-hemicyanine NIR dye **16** was obtained as a green solid which was submitted to a further purification by semi-preparative RP-HPLC (for more detail see the Supporting Information). The product-containing fractions were lyophilized to give the TFA salt (1.25 TFA) of compound **16**, as a green amorphous powder (17 mg, yield 14%).

Isolation of dye 17: The crude product was purified by flash-column chromatography over silica gel (dry loading, step gradient of CH_3OH in CH_2Cl_2 from 0% to 50%). *N*-(Trimethylammonio)propyl *nor*-DHX-hemicyanine NIR dye **17** was obtained as a green solid (44 mg, yield 33%).

Isolation of dye 18: The crude product was dissolved in a (1 : 1) mixture of aq. 0.1% TFA and CH_3CN (ca. 5 mL) and purified by semi-preparative RP-HPLC (for more detail see the Supporting Information). The product-containing fractions were lyophilized to give the TFA salt (3.5 TFA) of compound **18**, as a green amorphous powder (60 mg, yield 37%).

Preparation of fluorescent BSA conjugates

(a) Synthesis of NHS esters: carboxylic acid-functionalized *nor*-DHX-hemicyanine NIR dye **12**, **15** or **18** (1.2–2.0 µmole, 1 eq., weighed in a 0.5 mL "eppendorf"-type microtube) was dissolved in dry DMSO (final concentration 25 mM). 1.1 eq. of TSTU (7.6–9.0 µL of a 180 mM solution in DMSO) and 2 eq. (or 3 eq. for **18**) of DIEA (1.47–2.0 µL of a 2.0 M solution in NMP) were sequentially added and the resulting mixture was periodically vortexed for 1 h. The reaction was checked for completion by ESI-MS. The resulting NHS esters were used in the next BSA labeling step without purification.

(b) Fluorescent labeling of BSA: The solution of NHS ester (see above, 15- or 30-fold excess according to protein) was added to a solution of BSA (500 µL, 1.8 mg/mL, 13.5 nmol) in phosphate buffer (pH 7.05 or 7.70). The resulting mixture was protected from light and periodically vortexed. The reaction was left at 4 °C overnight and further 2 h at 20 °C. Thereafter, the mixture was diluted with phosphate buffer (1 mL), centrifugated to remove insoluble materials (excess of NHS ester and/or starting dye). Thereafter, the solution was transferred to an ultra centrifugal filter device (Amicon Ultra 2 mL, Ultracel cut-off 30 kDa from Merck Millipore, ref. UFC203024) and centrifugated at 4000 rpm for 15 min. For each fluorescent BSA conjugate, 50–100 µL of solution was recovered. Confirmation of conjugation to the protein was achieved by MALDI-TOF mass spectrometry.

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Keywords: *nor*-dihydroxanthene • fluorescence • NIR dyes • protein labeling • water solubility

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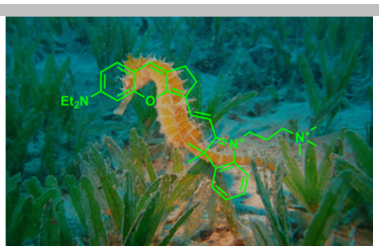
Layout 1:

FULL PAPER

Like a fish to water! A concise and efficient synthetic route toward *nor*-dihydroxanthene-hemicyanine hybrids has been developed. A facile further extension to water-soluble derivatives has led to a novel class of NIR fluorescent dyes (see figure) with valuable spectral features for applications in biosensing and bioimaging.



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Synthesis of *N,N*-Dialkylamino-*nor*-Dihydroxanthene-Hemicyanine Fused Near-Infrared Fluorophores and Their First Water-Soluble and/or Bioconjugatable Analogues