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Highly Fluorescent and Water-Soluble Diketopyrrolopyrrole Dyes for Bioconjugation**

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Abstract: *The preparation of highly water-soluble and strongly fluorescent diketopyrrolopyrrole (DPP) dyes using an unusual taurine-like sulfonated linker has been achieved. Exchanging a phenyl for a thienyl substituent shifts the emission wavelength to near $\lambda = 600$ nm. The free carboxylic acid group present in these new derivatives was readily activated and the dyes were subsequently covalently linked to a model protein (bovine serum albumin; BSA). The bioconjugates were characterized by electronic absorption, fluorescence spectroscopy and MALDI-TOF mass spectrometry, thus enabling precise determination of the labeling density (ratio DPP/BSA about 3 to 8). Outstanding values of fluorescence quantum yield (30 % to 59 %) for these bioconjugates are obtained. The photostability of these DPP dyes is considerably greater than that of fluorescein under the same irradiation conditions. Remarkably low detection limits between 80 and 300 molecules/μm^2 were found for the BSA bioconjugates by fluorescence imaging with a epifluorescence microscope.*

Of common dyes, diketopyrrolopyrroles (DPPs) based on a bis(lactam) backbone are interesting in regard to their synthetic availability, ease of modification, outstanding robustness, and attractive spectroscopic properties, thus making them excellent building blocks for a myriad of applications.[1] DPPs have been widely used as pigments in various paints, plastics, and inks, and exhibit exceptional semiconducting properties in field-effect transistors and photovoltaic solar cells.[2] Given limitations in the availability of the starting materials, very few synthetic methodologies for DPP dyes which are significantly soluble in organic solvents have been developed.[3] Thus, while various DPP-based fluorescent probes have been recently prepared for the detection of diverse analytes (anions, metal ions, reactive oxygen species, thiols, protons, various gases) in organic media, water-solubility remains a prerequisite for their use in relevant biosensing and bioimaging applications.[1b] Few attempts to solubilize DPP frameworks in aqueous solutions have been made, although some published strategies are based on the site-specific introduction of short polyethylene glycol chains through N-alkylation of both lactam moieties.[4,5] Further conjugation of such a non-ionic DPP dye to a cell-penetrating peptide [HIV-I Tat (44-61) fragment][4b] or a porphyrin[4d] has been achieved, thus providing a two-photon fluorescent tag suitable for live-cell microscopy and photodynamic therapy. Also, water-soluble conjugated and multicationic fluorene-DPP polymers have been used for the detection of DNA by fluorescence techniques.[6,7] The synthesis of anionic polyelectrolyte conjugates has also been investigated but the formation of aggregates limits their use as fluorescent bioprobes.[8] With the same objective of introducing ionized polar groups known to be more effective than pseudo-PEG linkers in enhancing water solubility of highly hydrophobic architectures,[9] we have functionalized DPP dyes with *N*,*N*-dimethylaminopropyne arms which, after alkylation, afforded weakly fluorescent water-soluble dyes useful for cascade energy-transfer processes[10] or lasing properties.[11] Unfortunately, such a promising protocol does not allow the preparation of water-soluble DPP-based fluorescent dyes for bioconjugation. Herein, we disclose a straightforward approach based on a postsynthetic sulfona-

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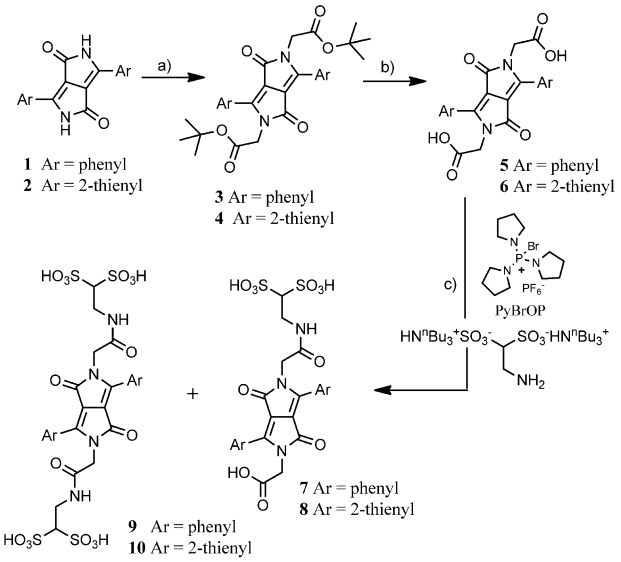
tion procedure involving a taurine-like disulfonated linker by providing highly fluorescent DPP dyes which are soluble in water (50–100 mg mL^{-1}) and have a free carboxylic acid function available for conjugation to amine-containing biopolymers. Bovine serum albumin (BSA) was chosen as model protein. To the best of our knowledge, this is the first time that water-soluble DPP derivatives have been conjugated to a protein. Even more impressive are the outstanding photophysical properties (particularly, quantum yields) of the grafted disulfonated DPP dyes compared to those of conventional green-yellow-emitting organic fluorophores.

The target hydrophilic DPP dyes **7** and **8** were conveniently prepared through the short synthetic route depicted in Scheme 1. The key acids **5** and **6** were prepared by



Scheme 1. Synthesis of water-soluble DPP dyes. Reagents and conditions: a) *tert*-butyl bromoacetate, K_2CO_3, 120 °C, 90–120 min, 51 % for **3** and 64 % for **4**; b) TFA, CH_2Cl_2, 4 °C to RT, 2–4 h, 69 % for **5** and 95 % for **6**; c) 2-aminoethane-1,1-disulfonic acid (tributylammonium salt), PyBrOP, DIEA, NMP, 4 °C to RT, 3–4 h, followed by RP-HPLC purification and desalting over Dowex H^+, 35 % for **7**, 43 % for **8** and 14 % for **9**. DIEA = diisopropylethylamine, NMP = *N*-methylpyrrolidone, TFA = trifluoroacetic acid.

These compounds were easily separated by reversed-phase HPLC chromatography and desalting over Dowex H^+ resin (to recover carboxylic/sulfonic acid forms), and unambiguously characterized by NMR and ESI-HRMS analyses (see the Supporting Information). As intended when designing these novel polar DPP dyes, a very high solubility in water or related aqueous buffers (50–100 mg mL^{-1}) was obtained for both the disulfonic and tetrasulfonic acid derivatives.

The electronic absorption spectra of the compounds in phosphate-buffered saline (PBS, pH 7.3, simulated physiological conditions) are dominated by a broad and intense band centered at $\lambda = 460$ and 530 nm for the phenyl and thienyl DPP dyes, respectively (Figure 1). When excited in

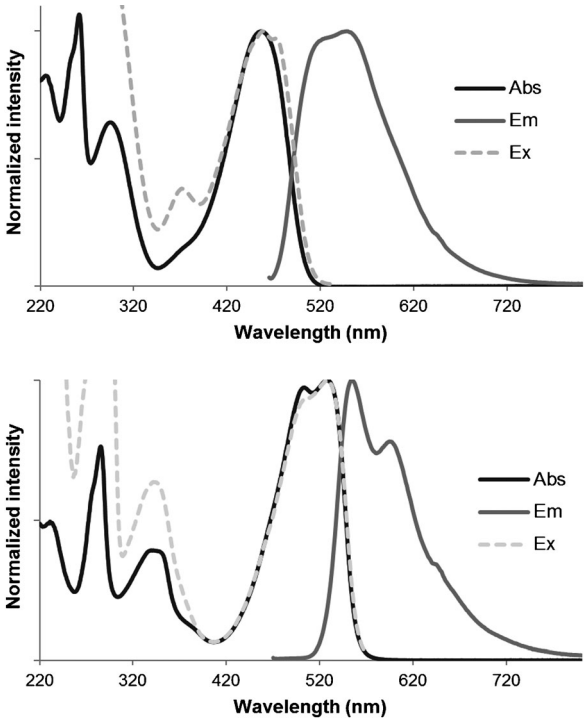


Figure 1. Absorption, corrected emission ($\lambda_{exc} = 450$ nm), and excitation ($\lambda_{em} = 550$ nm for **7**, $\lambda_{em} = 590$ nm for **8**) spectra of the DPP-phenyl di-SO_3H **7** (top) and DPP-thienyl di-SO_3H **8** (bottom) in PBS (100 mM phosphate + 150 mM NaCl, pH 7.3) at 25 °C.

N-alkylation of the dyes **1**[12] and **2**,[13] respectively, with an excess of *tert*-butyl bromoacetate under basic conditions and in hot NMP to achieve complete dissolution of the starting DPP pigments, followed by TFA-mediated removal of the *tert*-butyl esters. The use of the bench-stable phosphonium salt bromo-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBrOP) in the presence of DIEA enabled generation of the more reactive acyl bromide derivatives, which readily reacted with 2-aminoethane-1,1-sulfonic acid to provide the desired unsymmetrical disulfonated DPP dyes **7** and **8**. In the case of **6**, the reactivity of its carboxylic acid group is sufficiently low to limit formation of the symmetrical tetrasulfonated DPP dye **10**, which was only detected at trace levels. Conversely, bis-amidification of **5** was significant and **9** was obtained in a mixture with **7** (ratio **7**/**9** = 2.4:1).

this low-energy absorption band an intense fluorescence centered at $\lambda = 548$ and 553 nm was observed with corresponding quantum yields of 57 % and 49 % for the carboxylic acids **5** and **6**, respectively, and of 76 % and 56 % for the disulfonated derivatives **7** and **8**, respectively (Table 1). Note that for the phenyl derivatives the emission band is not structured and the Stokes shifts are larger than in the case of the thienyl analogues, where a vibronic sequence of 1350 cm^{-1} is clearly evident (Figure 1).[10,14] Particularly remarkable is the fact that the shape of the absorption and emission bands are similar for the series and that the quantum yields remain high in all cases even compared with the nonpolar dyes **3** and **4** (see the Supporting Information and Table 1). The emission lifetime (4.5 to 6.5 ns) was comparable to those recorded for related fluorescent organic-soluble dyes including Bodipys.[15]

Table 1: Summary of optical data of the DPP dyes **3**–**10**.

Cmpd	Solvent	λ_{abs} [nm][a]	ε [$M^{-1}cm^{-1}$]	λ_{em} [nm]	Φ_F [%]	τ [ns]
3	THF	456	20000	517	88[b]	6.2
	DMSO	460	18400	518	92[b]	6.4
4	THF	500; 538	24400; 28000	548; 594	77[b]	6.4
	DMSO	504; 541	26400; 29200	553; 599	74[b]	6.4
5	PBS	455	22700	515	57[b]	6.5
6	PBS	503; 530	29200; 30700	553; 596	49[b]	4.4
7	PBS	456	14700	548[c]	76[d]	6.5
	H_2O + 2.5% SDS[e]	456	14700	548[c]	94[d]	6.8
8	PBS	503; 527	17500; 18100	554; 594	56[d]	4.8
	H_2O + 2.5% SDS[e]	503; 527	17500; 18100	554; 594	80[d]	5.0
9	PBS	451	13900	541[c]	90[d]	6.6
	H_2O + 2.5% SDS[e]	451	13900	541[c]	97[d]	6.9
10	PBS	501; 523	–[f]	554; 594	61[d]	4.8
	H_2O + 2.5% SDS[e]	501; 523	–[f]	554; 594	61[d]	4.9

[a] UV absorption maxima have been omitted. [b] Determined by using cresyl violet (Φ_F = 50% in ethanol) as a standard (Ex. at λ = 546 nm).[19] [c] Broad emission curves with two maxima more or less well defined according to concentration and aqueous buffer used, close to λ = 520 and 550 nm for **7** and λ = 515 and 540 nm for **9**. [d] Determined by using fluorescein (Φ_F = 90% in 0.1N NaOH) as a standard (Ex. at λ = 450 nm).[19] [e] Measurements with this additive were performed in water because of its poor solubility in PBS. [f] Not obtained in a sufficient amount for a measurement of the molar absorption coefficient.

In line with this observation is the fact that in aqueous sodium dodecylsulfate solution (SDS, 2.5% w/v, 87 mM) the absorption and emission profiles do not change (see the Supporting Information), but the fluorescence quantum yields dramatically increase (up to 40%), reaching 97% for **9** (Table 1).

The high solubility in aqueous media, the interesting optical properties, and the availability of a free carboxylic acid group on their core structure, are positive features for the use of **7** and **8** in fluorescent bio-labeling applications.[16] To demonstrate their ability both to readily react with proteins under mild reaction conditions and to give highly fluorescent bioconjugates, the labeling of BSA as a model protein was explored. BSA contains 59 lysine residues, and 30–35 of them are accessible for conjugation.[17] Since the ε-amino groups of lysine residues of BSA are known to easily form amides with a wide range of activated esters in aqueous media, the conversion of **7** and **8** into their corresponding NHS esters by treatment with *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate TSTU (1.1 equiv) and DIEA (3 equiv) in DMSO was considered. BSA was labeled through overnight incubation with either a 15- or 30-fold molar excess of such NHS esters in phosphate buffer (pH 7.0) at 4 °C. The DPP-conjugated proteins BSA-**5** and BSA-**8** were washed with an ultra-centrifugal filter device (30 kDa cut-off) to remove the excess free, unbound DPP dyes. Since the method of choice for analyzing protein conjugates is by mass spectrometry, BSA-**5** and BSA-**8** were subjected to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analyses and compared to the parent BSA (see Figure S36 in the Supporting Information for illustrative examples with conjugates BSA-**6** and BSA-**8**). In all mass spectra, three main peaks assigned to $[M+2H]^{2+}$ (charge state +2), $[M+H]^+$ (molecular ion), and $[2M+H]^+$ (noncovalently bonded dimer) were observed. The mass difference between molecular ions of BSA and its fluorescent conjugates provided direct access to labeling densities, defined as the (average) number of dye molecules attached to a protein (F/P). Values are compiled in Table 2 and compared with those determined through the common spectrophotometric method.[18] Good F/P values within the range 2.9–7.9 according to the structure and excess of DPP dye used were obtained, thus substantiating the covalent fluorescent labeling. Gratifyingly, the lack of free, unbound fluorophore in the fluorescent protein samples BSA-**6** and BSA-**8** was confirmed by gel electrophoresis and subsequent imaging of the gels by fluorescence scanning at λ = 365 nm [see Figure S37 (left) in the Supporting Information]. Coomassie staining of the gel has clearly shown that the labeling protocol did not alter the structural integrity of the protein because the same migration pattern observed for unlabeled BSA was also obtained for BSA-**6** and BSA-**8** [see Figure S37 (right)].

The spectroscopic features (absorption/emission maxima and quantum yields) of these labeled proteins are summarized in Table 2 and illustrative

Table 2: Spectroscopic properties and labeling densities of fluorescent BSA conjugates.

Conjugated BSA-DPP dye[a]	Labeling conditions	λ_{abs} [nm][a]	λ_{em} [nm]	F/P [MS][b]	F/P [UV/vis][c]	Φ_F [%][d]
BSA-**5**	15 equiv of **5**, pH 7.0	460	522	6.8	4.4	37
BSA-**5**	30 equiv of **5**, pH 7.0	460	522	7.9	7.5	35
BSA-**6**	15 equiv of **6**, pH 7.0	534	595	4.3	3.3	30
BSA-**6**	30 equiv of **6**, pH 7.0	534	595	5.2	3.6	30
BSA-**7**	15 equiv of **7**, pH 7.0	455	517	2.9	3.9	62
BSA-**7**	30 equiv of **7**, pH 7.0	455	517	3.9	6.3	59
BSA-**8**	15 equiv of **8**, pH 7.0	529	554; 595	3.6	3.3	44
BSA-**8**	30 equiv of **8**, pH 7.0	529	554; 595	4.8	4.1	44

[a] UV absorption maxima have been omitted. [b] Determined by MALDI-TOF mass spectrometry. [c] Determined by absorption spectroscopy (for details see Supporting Information). Variances in the results obtained using the two methods are primarily explained as follows: for the photometric method, molar absorptivities determined for the free DPP dyes were used for the quantification but their covalent binding to protein has changed the value of this spectral parameter. [d] Determined by using fluorescein (Φ_F = 90% in 0.1 N NaOH) as a standard (Ex. at 450 nm).[19]

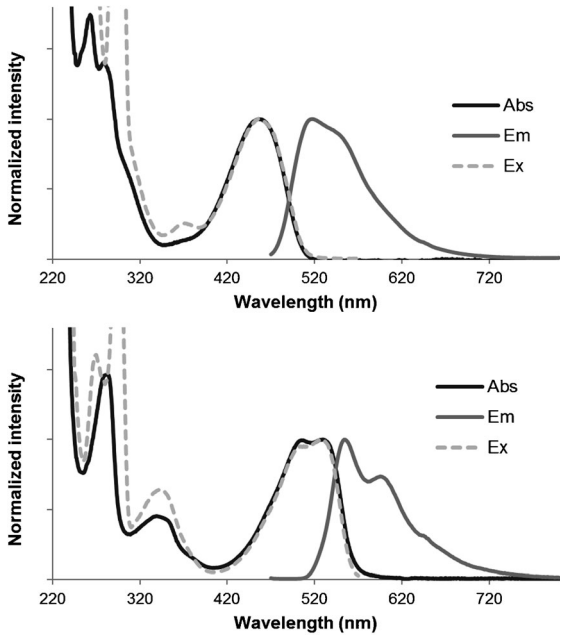


Figure 2. Absorption, corrected emission (λ_{exc} 450 nm), and excitation ($\lambda_{em} = 550$ nm for BSA-**7**, $\lambda_{em} = 590$ nm for BSA-**8**) spectra of BSA-**7** (top) and BSA-**8** (bottom), in PBS at 25 °C (labeling conditions: 15 equiv, pH 7.0).

examples of spectra (for BSA-**7** and BSA-**8**, obtained by reaction of BSA with 15 equiv of the corresponding DPP dyes) are shown in Figure 2. Interestingly, the spectra are remarkably similar to those obtained for the free, unbound DPP dyes in aqueous solution (in particular, a good matching between the absorption and excitation spectra was observed), thus allowing the conclusion that 1) the formation of dimers or small aggregates consisting of only few BSA-bound dye molecules does not occur, 2) the protein environment does not perturb the dyes to a large extent, and 3) the grafted DPP molecules are probably located at the periphery of the BSA surface. These conclusions were further supported by the values of fluorescence quantum yields which remain very high, and are only about 20% lower than those of the free DPP dyes as determined under the same conditions. To the best of our knowledge, very few organic fluorophores that exhibit such valuable spectral behavior upon covalent attachment to proteins have been reported in the literature.[20]

The suitability of the novel DPP dyes for fluorescent labeling of bioprobes has been assessed by preliminary fluorescence imaging experiments (Figure 3). Spots containing known amounts of BSA conjugates were imaged with a epifluorescence microscope employing fluorescein isothiocyanate (FITC)- and rhodamine-B-specific excitation/emission filter combinations for DPP-phenyl and DPP-thienyl conjugates, respectively. The BSA conjugates of DPP-thienyl derivatives showed remarkably low detection limits, ranging from 7.0×10^{-17} to 2.6×10^{-16} moles/spot (evaluated at a signal-to-background ratio = 3). These conjugate amounts corresponded to a detection limit between 80 and 300 molecules μm^{-2}. For comparison, we also measured the detection limit of a commercially available FITC-labeled IgG antibody (labeling ratio F/P = 2.3), thus obtaining a value (1.1×10^{-16} moles/spot) in line with those measured for the DPP-thienyl BSA conjugates. Despite their high fluorescence quantum yields, the fluorescence emissions obtained for the DPP-phenyl conjugates were weaker and, correspondingly, their detection limits were one order of magnitude higher. Such behavior could be reasonably ascribed to the non-optimal matching between the FITC excitation/emission filter combination employed for the measurement ($\lambda_{exc} = 470$–490 nm, $\lambda_{em} = 515$–550 nm) and the absorption spectra of the

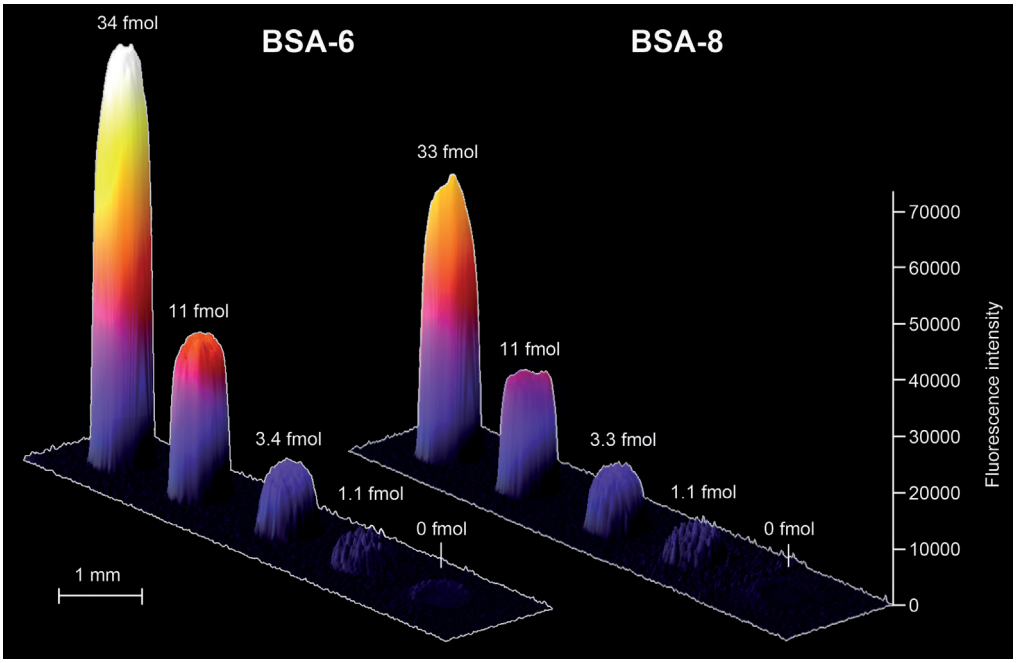


Figure 3. Pseudocolored three-dimensional plots of fluorescence images of spots containing different amounts of the BSA-**6** (labeling conditions: 30 equiv, pH 7.0) and BSA-**8** (labeling conditions: 30 equiv, pH 7.0) conjugates. The spots (diameter ca. 800 μm) were obtained with a manual microarray device that employs an array of pins to transfer small volumes of solution (ca. 3 nL) on conventional microscope glass slides. A solvent spot (10% v/v glycerol in water) was also measured to evaluate the background fluorescence signal. Images were acquired using an epifluorescence microscope and rhodamine-B-specific excitation and emission filters ($\lambda_{exc} = 510$–550 nm and $\lambda_{em} > 590$ nm, respectively). The plots represent the actual fluorescence intensity of each image pixel (expressed in arbitrary units) mapped to a colour according to its value.

conjugates. Thus, the detection limits could be improved by using excitation and emission wavelengths more suitable for the phenyl DPP dyes.

Finally, photostability of the dyes was next tested with respect to fluorescein in PBS buffer (pH 7.2) using a cut-off filter at $\lambda = 399$ nm and an irradiation bench with a 250 W focused halogen lamp. The disulfonated DPP-thienyl dye **8** is highly stable with a maximum loss of 4.4% in fluorescence intensity, whereas photobleaching of fluorescein reached 96% under the same irradiation conditions (see Figure S53 in the Supporting Information). Furthermore, no photoproduct in the case of **8** was detected (see Figure S51 in the Supporting Information), unlike fluorescein (Figure S52 in the Supporting Information).[21]

In short, the key design feature of the present fluorescent reporters is the facile and site-specific introduction of a disulfonated linker and a carboxylic acid function within the same molecular scaffold, both to dramatically solubilize DPP dyes in aqueous solutions and to use them, after conventional activation as NHS esters, in covalent labeling of biomolecules/biopolymers. Here, we largely solve the deficiencies and problems frequently encountered with fluorescent organic dyes used as biolabels, in particular the severe quenching of their fluorescence, perturbation of their spectroscopic features upon conjugation to proteins, and their poor photochemical stability. Fluorescence imaging microscopy shows that these novel biocompatible dyes are comparable (and possibly superior using optimized excitation/emission filter combinations) to conventional fluorophores and offer many opportunities for multichromatic patterning of biomaterials.

Experimental Section

Details of the synthesis of DPP dyes **1**–**10**, analytical data, and photophysical characterizations of all compounds/bioconjugates studied in this work are available in the Supporting Information section.

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