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NIR Mega-Stokes Fluorophores for Bioorthogonal Labeling and Energy Transfer Systems–An Efficient Quencher for Daunomycin

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This work is dedicated to Prof. Otto S. Wolfbeis on the occasion of this 65th birthday

Abstract: A set of new azide- and alkyne-bearing lepidinium-based fluorophores were synthesized for bioorthogonal labeling schemes. These fluorescent dyes all show large Stokes-shifts with emission maxima in the near-infrared (NIR) region of the electromagnetic spectrum. The applicability of these dyes in the construction of energy-transfer systems was tested using one of these new fluorescent tags and daunomycin (Dau), an anticancer drug with fluorescent features. These daunomycin conjugates are the very first examples of fluorescently modulated constructs of this anticancer agent. The dually labeled architectures proved that the applied fluorescent dye can be utilized as an efficient quencher for daunomycin. Enzymatic cleavage of a dually labeled enzyme substrate resulted in full recovery of the fluorescence of daunomycin. Such fluorescently modulated Dau conjugates can provide useful information for the mechanism of action of Dau-regulated cell death processes.

Keywords: bioorthogonal · daunomycin · fluorescent probes · FRET · quenching

Introduction

Manipulation of biological matter by means of chemical transformations is of great importance today in many aspects of chemical biology. Since the introduction of bioorthogonality with all its criteria laid down by Bertozzi et al.,[1] researchers can choose from a set of chemical reactions. Today, we are able to modify biomolecules of interest using these special reactions proceeding quasi-quantitatively even under physiological conditions (*bio*). The utilized functionalities offer highly selective manipulations as these groups react solely with one another without any interference (*orthogonality*) with other functional groups (e.g., amines, thiols, and hydroxy groups) that are abundant in biological systems.[1–3] Of the bioorthogonally applicable chemical reactions the most frequently used one is still the 1,3-dipolar cycloaddition between azides and alkynes, mostly just referred to as click reaction. Depending on the system to be modified, the researcher can choose from copper-catalyzed and copper-free ring strain-promoted versions of this reaction.[4–8] A combination of both has also been reported in the literature for multiple labeling of biomolecules.[9,10] Nowadays, bioorthogonal chemistry schemes to probe biomolecules follow a two-step procedure. First, the target is modified with a chemical reporter module by using the metabolic machinery of cells with bioorthogonally modified metabolites (e.g., azido-sugars), chemical modifications of unique motifs (e.g., cysteine) or gene technology (e.g., tRNAs preloaded with modified amino acids).[1,2] The second step involves bioorthogonal modification with an appropriately modified probe (e.g., fluorescent-labeled, radiolabeled, etc.).[11–13]

In vitro and in vivo fluorescence imaging of biological structures is becoming increasingly important due to its high sensitivity, excellent temporal and spatial resolution, and its potential for multichannel imaging. Fluorescence labeling techniques often face problems, raised by background- or autofluorescence of unreacted dyes or naturally occurring fluorophores, respectively. Self-quenching or UV excitation can also cause problems when tagging biological samples. In many cases the use of exogenously delivered synthetic probes is the method of choice.[14,15] Fluorophores excitable in the red and emitting in the near infrared (NIR) region are particularly suitable for biological (in vitro and in vivo) applications as there is little or no interference from biological luminescence (autofluorescence). At the same time they enable deep tissue imaging owing to the larger penetration

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distance in this portion of the spectrum. Therefore, there is an increasing demand for labels emitting in the NIR region.[16] Another important feature of fluorophores is that they are, for practical purposes, best to be excited at wavelengths compatible with commercial laser sources (e.g., Ar, He-Ne, and diode lasers) widely used in fluorescence instrumentations such as cell sorters and imagers. Separation of the excitation and emission bands of the applied fluorescent units is also crucial in many applications. In our recent works we have demonstrated the applicability of so-called mega-Stokes red and far-red emitting probes in cell labeling experiments.[17,18] Fluorophores exhibiting large Stokes shifts make these dyes ideal candidates for Förster-type resonance energy transfer (FRET) applications for having essentially completely separated excitation and emission bands. Direct excitation of acceptor emission by the light used to excite the donor is an often encountered problem in FRET assays. This problem results in a decrease of the dynamic range of assays in which most of the acceptor is unbound. Complete separation of the excitation and emission bands therefore makes mega-Stokes dyes ideal candidates for FRET applications.[14,19]

The combination of NIR emission and mega-Stokes properties would result in fluorescent labels ideal for biological applications. Building on the frameworks of the first generation of our dyes, we aimed at tuning the emission maxima towards the NIR region and increasing the Stokes shifts of our previously reported dyes.[15,18] In this paper we report on the synthesis of a set of mega-Stokes NIR emitting labels that are suitable for incorporation by means of azide–alkyne click chemistry (Figure 1). We have demonstrated the usefulness of these tagging molecules in click chemistry-based energy transfer studies where the fluorescence of a cancer therapeutic agent (i.e., daunomycin) was modulated with one of our new labels.

Abstract in Hungarian: Munkánk során bioortogonális jelzési technikákban alkalmazható azid, illetve alkin funkciós csoporttal rendelkező lepidínium-alapú fluoreszcens jelzővegyületeket állítottunk elő. Jelzővegyületeink nagy Stokes-eltolódással, illetve távoli vörös, közeli infravörös tartományba eső gerjesztési és emissziós maximumokkal rendelkeznek. Fluoreszcens vegyületeink alkalmazhatóságát energiatranszfer rendszerek előállításával vizsgáltuk. Ezen kísérletek során egy széleskörben alkalmazott, fluoreszcens kemoterápiás szer, a daunomicin konjugátumait állítottuk elő. E daunomicin származékok az első példák olyan konjugátumokra, amelyekben a kemoterápiás vegyület fluoreszcenciáját egy másik jelzővegyülettel modulálták. Vizsgálataink során megállapítottuk, hogy a daunomicin mellett alkalmazott jelzővegyület rendkívül hatékonyan oltja ki a rákellenes szer fluoreszcenciáját, valamint azt is megfigyelhettük, hogy megfelelő festékkel és daunomicinnel is módosított enzimszubsztrátumok enzimatikus hidrolízise során a daunomicin fluorszcencia-intenzitása teljesen visszanyerhető. Ilyen fluoreszcensen modulált daunomicin konjugátumok a jövőben igen alkalmasak a rákellenes szer által kiváltott sejthalált eredményező folyamatok tanulmányozására, valamint a daunomicin hatásmechanizmusának felderítésével még hatékonyabb kemoterápiás szerek kifejlesztésére nyílik lehetőség.

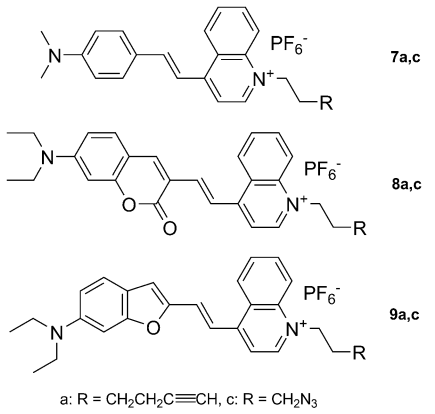
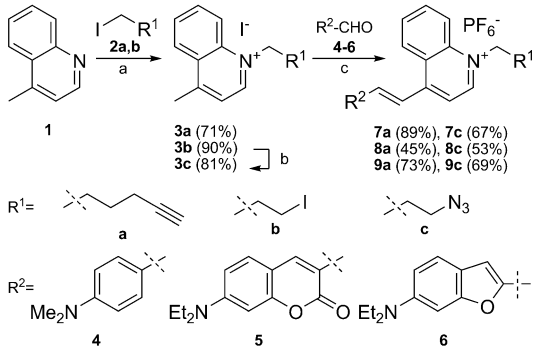


Figure 1. Novel mega-Stokes fluorophores for azide–alkyne dipolar cycloaddition-based labeling.

Results and Discussion

The excitation and fluorescent spectra as well as the Stokes-shifts of mega-Stokes dyes are highly dependent on the size of the conjugated system. One way to tune the photophysical properties of fluorophores is to change the length of the polymethine chain. Alternatively, few attempts were also made by increasing the size of the aromatic system.[20,21] We were curious whether extension of the aromatic scaffold of our previous dyes would result in red-shifted excitation and emission properties, while keeping these bands well separated (mega-Stokes properties).

We have devised a synthetic routine that uses lepidine as starting scaffold. The syntheses (Scheme 1) started with the formation of the lepidinium moieties **3** by N-alkylation of



Scheme 1. Synthetic route for clickable fluorophores. Reaction conditions: a) 6-iodo-1-hexyne (**2a**) or 1,3-diiodopropane (**2b**), MeCN, reflux; b) NaN_3, MeCN, reflux; c) **4–6** carbaldehyde, piperidine, EtOH, reflux; then NH_4PF_6, H_2O.

lepidine (**1**), either by reaction with 6-iodo-1-hexyne (**2a**) or with 1,3-diiodopropane (**2b**), to obtain the corresponding salts. In both cases the products **3a,b** were obtained in good yields. For the synthesis of **3c**, compound **3b** was further subjected to halogen–azide exchange, which was effected by treatment with NaN_3. Precursors **3a,c** were then condensed with the corresponding benz-, coumarin-, or benzofuran aldehydes (**4**–**6**). For better biocompatibility, the iodine counteranion was replaced by hexafluorophosphate by treatment with NH_4PF_6. These synthetic routes afforded dye molecules **7–9** in acceptable to good yields in high purity.

The dyes were then tested for their photophysical properties. To our delight both excitation and emission spectra showed considerable red-shifts compared to their predecessor picolinium congeners.[18] Furthermore, a remarkable increase in the Stokes-shifts were observed as well (to around 150 nm, Table 1). The excitation spectra show that **7a,c** and

Table 1. Photophysical properties of fluorophore frameworks **7**–**9**.[a]

	7	**8**	**9**
λ_{max} (abs) [nm][b]	553	549	602
ε[b,d] [10^4 M^{-1} cm^{-1}]	4.0	2.7	3.5
λ_{max} (exc) [nm][b]	560	579	655
λ_{max} (exc) [nm][c]	545	554	643
λ_{max} (em) [nm][b]	706	732	805
λ_{max} (em) [nm][c]	695	712	798
Stokes-shift [nm][b]	146	153	150
Stokes-shift [nm][c]	150	158	155
Φ_f[b,g] [%]	0.77	9.0	0.07
Φ_f[e,g] [%]	0.33	3.00	0.055
Φ_f[f,g] [%]	0.13	0.8	0.04
τ_f [ps][e,h]	50	200	90

[a] Data acquired using the **a** series of compounds **7**–**9**. [b] In DMSO. [c] In MeOH. [d] Measured at λ_{max} (abs). [e] In DMSO/H_2O (1:1). [f] In DMSO/H_2O (1:9). [g] Relative to cresyl violet in MeOH (Φ_f = 0.54)[30]. [h] Measured at 750 ± 20 nm.

8a,c can be excited by the highly efficient and relatively cheap 532 nm green diode-pumped solid-state laser (DPSSL), whilst compound **9a,c** was found to be fully compatible with the widely used red diode laser (625 nm) or the highly bright 640 nm red DPSSL (Figure S1, Supporting Information). We note that all photophysical characteristics of the fluorophores were found to be essentially identical for the **a** and **c** series. The fluorescence quantum yields are relatively low for all dyes, with the 7-aminocoumarin derivatives (**8a**,**c**) exhibiting the best performance. The small fluorescence quantum yields are in line with the short lifetime of these compounds, probably induced by an effective internal conversion process. In all cases the hydrogen bond formation with water further decreases the fluorescence yields, as it is shown in Table 1.

In a parallel ongoing project we were interested in the possibility to modulate the fluorescence of daunomycin (**Dau**), a biologically active anticancer agent.[22,23] Due to its high toxicity, **Dau** should be conjugated to targeting moieties (e.g., specific peptides) in order to minimize its undesired toxic effect on healthy cells.[26–28] The toxic effect is believed to be in connection with the release of the drug from the conjugates inside the tumorous cells, though this hypothesis needs further experimental support.[24–29]

A thorough understanding of **Dau**-mediated processes (e.g., its release from conjugates) would greatly facilitate the design of more efficient daunomycin-based drugs. To gain a better insight into the mechanism of action of this anticancer agent, we aimed at identifying constructs that probe the possible drug (**Dau**) release using fluorescence modulation-based techniques. We envisioned that energy transfer (FRET)-based constructs will greatly facilitate these action mechanism-related studies. To the best of our knowledge, no fluorescently modulated **Dau** constructs have been reported. The difficulty of finding a good match for **Dau** in such modulated systems lies in the fluorescent properties of daunomycin, as its rather wide excitation and emission spectra do not allow the use of conventional fluorophores. This can be circumvented by appropriately chosen mega-Stokes fluorophores, which could be excited within the 550–650 nm range and possess no significant emission around 600 nm.

Our new lepidinium dyes were analyzed from these aspects and fluorophore **7** was chosen for its good spectral overlap with daunomycin, high absorption coefficient, and virtually zero emission below 600 nm (Figure 2, note that

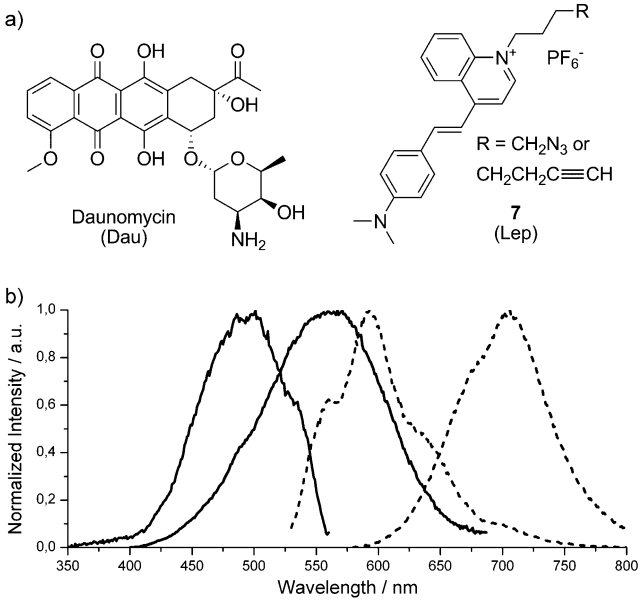
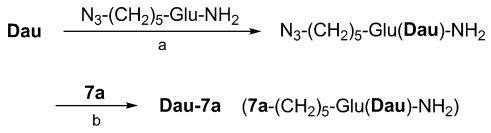


Figure 2. a) Structures of daunomycin and **7**. b) Fluorescent excitation (solid lines) and emission (dotted lines) spectra of daunomycin (grey) and **7** (black) in DMSO/water (1:1 v/v) solutions.

the Figure shows normalized spectra as the fluorescence intensity of **7** is orders of magnitude lower than that of **Dau** at the same concentration).

To validate our hypothesis, a model system was prepared by using a glutamic acid derivative as a linker between the two fluorophore units. First, a N_3-$(CH_2)_5$-CO-Glu-NH_2 derivative was prepared by solid-phase peptide synthesis. In

the next step the daunomycin moiety was attached to the free carboxylic acid function in the side chain via its amino group by solution-phase synthesis, and finally the chosen **7a** fluorophore was conjugated via copper-catalyzed azide–alkyne cycloaddition (CuAAC) to obtain the model **Dau-7a** conjugate (Scheme 2).



Scheme 2. Synthetic route for the **Dau-7a** conjugate. Reaction conditions: a) HOBt, DIC, DMF, rt; b) $CuSO_4$, TBTA, Na-ascorbate, DMSO/water (1:1 v/v), rt.

Analyses of the fluorescence spectra showed that the emission intensity of **Dau** was largely affected by the presence of dye **7** and approximately 90% quenching of the original signal was observed at 592 nm (Figure 3). The lack of a simultaneous increase in fluorescence intensity at around 706 nm (the emission maximum of the acceptor dye **7a**) is most probably due to the much lower quantum yield of our new dye. These results suggest that **7a** is an efficient quencher for **Dau**, which offers the possibility to construct quenched **Dau** systems.

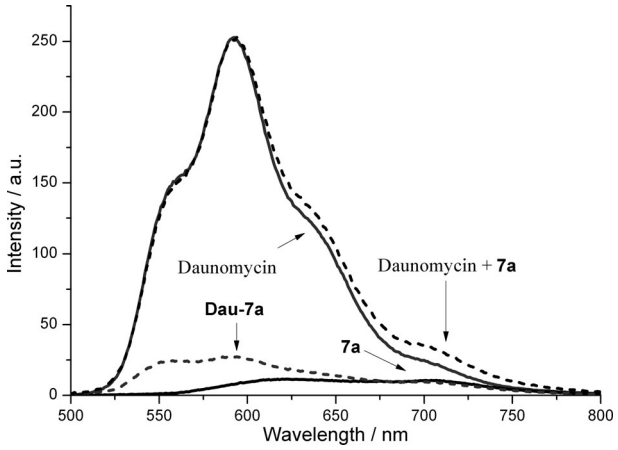
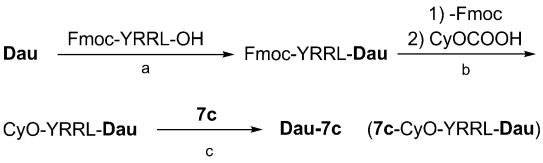


Figure 3. Emission spectra of daunomycin (solid grey line), **7a** (solid black line), daunomycin+**7a** (dashed black line), and **Dau-7a** conjugate (dashed grey line) at a concentration of 10^{-6} M in DMSO/water (1:1 v/v) under excitation at 480 nm.

Next, we were curious whether it is possible to recover the fluorescence signal of **Dau**; therefore, we have devised a tetrapeptide-based conjugate. The YRRL tetrapeptide motif was chosen because it is a substrate of cathepsin B, an enzyme capable of hydrolyzing between the Arg-Arg residues.[29] The tetrapeptide sequence was built using solid-phase Fmoc chemistry. Following cleavage from the resin, daunomycin was attached to the C-terminus in solution. Removal of the Fmoc protecting group from the N-terminus was followed by acylation with a cyclooctyne derivative, CyOCOOH.[31] At last, **7c** was incorporated by means of strain-promoted azide–alkyne cycloaddition to obtain the **Dau-7c** conjugate (Scheme 3). This latter reaction was monitored by following the changes in the fluorescence signal at 592 nm (Figure 4a). As expected, the fluorescent intensity of **Dau** dropped to almost 10% of its original value.



Scheme 3. Synthetic route for the **Dau-7c** conjugate. Reaction conditions: a) HOBt, DIC, DMF, rt; b) 1) 20% piperidine in DMF; 2) HOBt, DIC, DIEA, DMF, rt; c) DMF, rt.

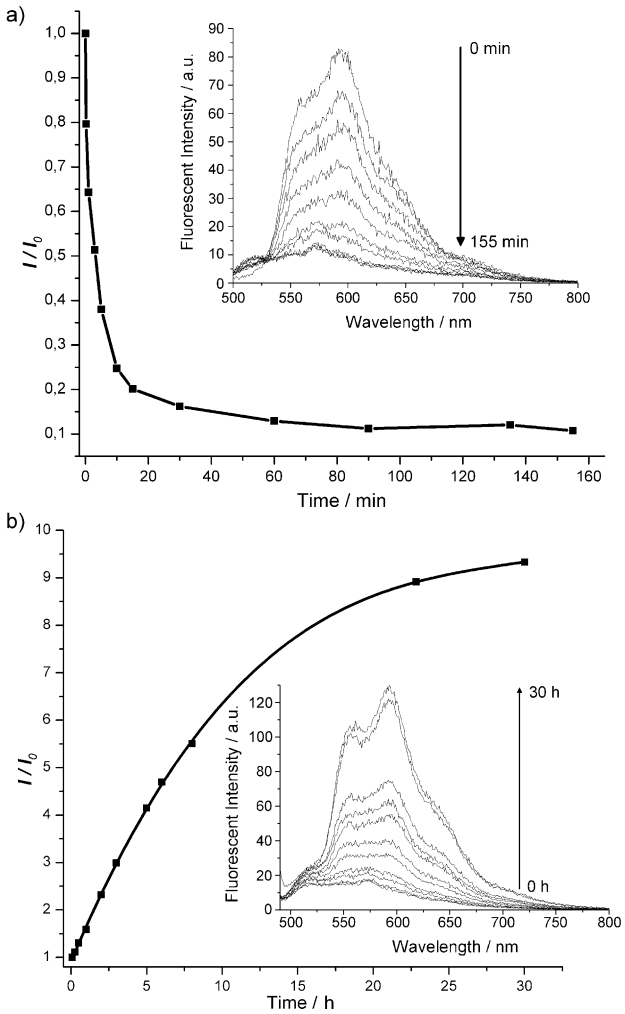


Figure 4. a) Changes in fluorescent intensity at 592 nm of the reaction mixture containing CyO-YRRL-**Dau** and **7c** (excitation at 480 nm). b) Changes in fluorescent intensity at 592 nm upon treatment of the **Dau-7c** conjugate with cathepsin B (excitation at 480 nm). The insets show the corresponding fluorescent spectra.

In order to study the recovery of the fluorescence signal, we subjected this dually labeled substrate to cathepsin B. A buffered solution of the conjugate was treated with the enzyme and the process was followed by monitoring the changes in the fluorescent intensity at 592 nm (Figure 4b). To our delight, full recovery of **Dau** fluorescence was observed after 24 hours, which corresponds to an about 10-fold increase of the original signal. In a control assay, the same conditions were applied without the enzyme, and no significant changes in fluorescence were observed (Figure S2, Supporting Information).

To gain a better insight into the nature of the fluorescent quenching process, we have measured some of the key photophysical parameters of the conjugates (Table 2). Daunomycin itself has a moderate fluorescence yield with a lifetime in the nanosecond range, which decreases slightly with increasing water content. The conjugates show a weak dual emission, with two exponential decay characters. At 590 nm detection, the fluorescence is dominated by a long-lived emission (1.31 ns) coming from the unperturbed singlet excited daunomycin, while the shorter component (0.19 ns) must be connected with the quenching process. At the detection wavelength of 750 nm, the emission decays are governed more by a short component coming from the excited dye. The less intense longer component (around 1.3 ns) most probably belongs to the tail of the emission of the isolated singlet excited daunomycin, as mentioned above. Note that in the conjugates almost two thirds of the light is absorbed directly by the dye ($\varepsilon_{480\,nm}=16000\,mol^{-1}\,dm^3\,cm^{-1}$), while the molar absorbance of **Dau** at the maximum (482 nm) is 8800 $mol^{-1}\,dm^3\,cm^{-1}$.

Table 2. Some photophysical properties of fluorophores **Dau** and its conjugates.

	Dau	**Dau-7a**	**Dau-7c**
Φ_f[a,d] [%]	8.50	0.60	0.70
Φ_f[b,d] [%]	7.00	0.47[e] and 0.1[f]	0.58[e] and 0.40[f]
Φ_f[c,d] [%]	4.50	0.14	0.18
τ_f [ns][b]	1.325	0.19 and 1.30[g]	0.19 and 1.31[g]
		0.067 and 1.28[h]	0.064 and 1.26[h]

[a] In DMSO. [b] In DMSO/H_2O (1:1). [c] In DMSO/H_2O (1:9). [d] Relative to cresyl violet in MeOH ($\Phi_f=0.54$, using n^2 correction)[30]. [e] Excited at 480 nm. [f] Excited at 560 nm. [g] Detected at 590±10 nm. [h] Detected at 750±20 nm.

Quenching of the daunomycin emission is partly caused by the Förster energy transfer process (the emission of **Dau** overlaps with the absorption spectrum of the dye, while the distance between the two parts of the conjugate is expected to be small in several conformers). However, the possibility of an intramolecular electron transfer cannot be ruled out. In a simple experiment we have checked whether the dialkylaniline-derived part of the molecule could quench the excited daunomycin subunit (Figure S4, Supporting Information). In this experiment, the regularly increasing concentration of *N,N*-dimethylaniline (DMAN) causes a decrease in the lifetime (as well as fluorescence intensity) from 1.425 ns ($\Phi_f=0.085$ in DMSO) to 0.886 ns ($\Phi_f=0.039$ at 0.095 $mol\,dm^{-3}$ DMAN in DMSO). The derived second-order rate parameter is $(4.47\pm0.10)\times10^9\,mol^{-1}\,dm^3\,s^{-1}$, which agrees well with the $4.4\times10^9\,mol^{-1}\,dm^3\,s^{-1}$ value belonging to the Stokes–Einstein limit of a diffusion-controlled bimolecular reaction rate in DMSO (viscosity of 2.133 cP). Consequently, dyes having an *N,N*-dialkylaniline subunit in a proper conformation can easily undergo an excited-state electron transfer followed by a ground-state electron back-transfer process (on the whole an internal conversion process).

Conclusions

In the present study we report the syntheses and photophysical characterization of three bioorthogonally applicable fluorescent NIR frameworks. These fluorophores possess large Stokes-shifts and optimal spectral bands for excitation by cheap, commercially available laser sources. Complete separation of excitation and emission bands justifies the use of these dyes in energy-transfer applications. Consequently, dually labeled model systems were designed and prepared using a fluorescent anticancer drug, daunomycin, and one of our new dyes (**7**). These represent the first examples of fluorescently modulated **Dau**-constructs. Our studies have shown that the fluorescence of **Dau** is efficiently quenched by the chosen dye, resulting in a 90% drop of fluorescent intensity. Probably, Förster-type energy-transfer processes can be accounted for the observed quenching phenomena; however, excited-state electron-transfer processes cannot be ruled out.

It was also demonstrated that the fluorescence signal of **Dau** can be fully reinstated upon hydrolysis of the dually labeled tetrapeptide-based enzyme substrate. These results point out that the fluorescence of **Dau** may be efficiently modulated by bioorthogonally applicable NIR fluorophores under in vivo conditions. To the best of our knowledge, this is the first energy transfer-based study of daunomycin conjugates. The fact that the fluorescent signal can be completely restored is very encouraging for the construction of appropriately designed **Dau** systems. We strongly believe that such modulated daunomycin conjugates will provide us with useful information regarding the release mechanism of daunomycin within target cells and its subsequent effects on **Dau**-induced cell death processes. Better understanding of **Dau**-mediated apoptotic processes can greatly facilitate the design of more efficient therapeutics. **Dau**-related studies on the mechanism of action are currently underway in our laboratory and results will be reported in due time.

In the current study, the bioorthogonal potential of the dyes was not realized as the azide–alkyne cycloaddition reaction was used for the in vitro assembly of the conjugates. To fully take advantage of the bioorthogonal nature of the dyes, we have already initiated experiments where cell-penetrating peptides are labeled in situ inside the cells. Preliminary results of these studies imply that the in-situ assembly

of such constructs results in much more efficient uptake of peptides as their penetrating property is not spoiled by the presence of a dye.

Experimental Section

General

Unless otherwise indicated, all starting materials were obtained from commercial suppliers (Sigma–Aldrich, Alfa Aesar, Merck) and used without further purification. Analytical thin-layer chromatography (TLC) was performed on Kieselgel 60 F_{254} precoated glass TLC plates with 0.25 mm silica. Column chromatography was carried out with flash silica gel (0.040–0.063 mm) from Merck. The NMR spectra were recorded on a Bruker DRX-250 or Varian VNMRS 600 MHz spectrometer. Chemical shifts (d) are given in parts per million (ppm) using solvent signals as the reference. Coupling constants (J) are reported in Hertz (Hz). Splitting patterns are designated as s (singlet), d (doublet), t (triplet), q (quadruplet), quint (quintuplet), m (multiplet), dd (doublet of a doublet), and td (triplet of doublets). The spectrophotometric measurements were carried out on a Varian Eclipse spectrofluorimeter. Mass spectrometry of the peptide derivatives were carried out on a Bruker Daltonics Esquire 3000+ ion trap mass spectrometer, while high-resolution mass spectrometry (HRMS) measurements were carried out on an Agilent 6230 MS-TOF system. All melting points were measured on a Büchi 501 apparatus and are uncorrected. IR spectra were obtained on a Bruker IFS55 spectrometer equipped with a single-reflection diamond ATR unit.

Synthesis of Fluorescent Dyes

*1-(Hex-5-ynyl)-4-methylquinolinium iodide (**3a**)*

Lepidine (**1**, 270 μL, 2.0 mmol) and 6-iodo-1-hexyne[32] (**2a**, 0.830 g, 4.0 mmol) were heated at reflux in acetonitrile (6.0 mL) for 21 h. After cooling to room temperature, the solvent was removed in vacuo, and the crude product was suspended in ethyl acetate, filtered, and recrystallized from ethanol to give 0.509 g (71%) of the desired product as a pale green solid. M.p. 150–153°C; IR (neat): $\tilde{\nu}$=1367, 1404, 1586, 1603, 2920, 3199 cm^{-1}; ^1H NMR ([D_6]DMSO, 250 MHz): δ=1.56 (quint, 2H, J=7.4 Hz), 2.03 (quint, 2H, J=7.5 Hz), 2.24 (td, 2H, J=1.7 Hz, 6.6 Hz,), 2.80 (s, 1H), 3.01 (s, 3H), 5.04 (t, 2H, J=7.3 Hz), 8.06 (m, 2H), 8.27 (t, 1H, J=7.9 Hz), 8.55 (d, 1H, J=8.4 Hz), 8.61 (d, 1H, J=8.9 Hz), 9.42 ppm (d, 1H, J=6.0 Hz); ^{13}C NMR ([D_6]DMSO, 62.5 MHz): δ=17.2, 19.7, 24.6, 28.5, 56.4, 71.7, 83.8, 119.2, 122.6, 127.1, 128.9, 129.5, 135.0, 136.7, 148.3, 158.6 ppm; HRMS (ESI) $[M]^+$ calcd. for $C_{16}H_{18}N^+$: 224.1434; found: 224.1435.

*1-(3-Iodopropyl)-4-methylquinolinium iodide (**3b**)*

Lepidine (**1**, 1200 μL, 9.0 mmol) and 1,3-diiodopropane[32] (**2b**, 8.09 g, 27.0 mmol) were heated at reflux in acetonitrile (30 mL) for 20 h. After cooling to room temperature, the solvent was removed in vacuo, and the crude product was suspended in ethyl acetate, filtered, and recrystallized from ethanol to give 3.545 g (90%) of the desired product as a yellowish green solid. M.p. 161–165°C (decomp); IR (neat): $\tilde{\nu}$=1369, 1402, 1530, 1602, 3002 cm^{-1}; ^1H NMR ($CDCl_3$, 250 MHz): δ=2.69 (m, 2H), 3.03 (s, 3H), 3.47 (t, 2H, J=6.2 Hz), 5.47 (t, 2H, J=7.5 Hz), 8.01 (m, 2H), 8.24 (t, 1H, J=8.1 Hz), 8.38 (d, 1H, J=8.7 Hz), 8.57 (d, 1H, J=8.7 Hz), 10.29 ppm (d, 1H, J=5.7 Hz); ^{13}C NMR ([D_6]DMSO, 62.5 MHz): δ=1.5, 19.6, 32.9, 57.3, 119.0, 122.6, 127.1, 128.9, 129.5, 135.1, 136.7, 148.6, 158.7 ppm; HRMS (ESI) $[M]^+$ calcd. for $C_{13}H_{15}IN^+$: 312.0244; found: 312.0241.

*1-(3-Azidopropyl)-4-methylquinolinium iodide (**3c**)*

1-(3-Iodopropyl)-4-methylquinolinium iodide (**4b**, 0.440 g, 1.0 mmol) and NaN_3 (0.195 g, 3.0 mmol) were dissolved in acetonitrile (20 mL), and the resulting solution was heated at reflux overnight. After cooling to room temperature, the solution was filtered to remove salts and the filtrate was brought to dryness. To the remainder solid was added dichloromethane, and the solution was filtered through a plug of silica. Evaporation of the solvent gave 0.228 g (81%) product as a dark green crystalline solid. M.p. 118–119°C (decomp); IR (neat): $\tilde{\nu}$=1404, 1530, 1604, 1739, 2113, 3023 cm^{-1}; ^1H NMR ($CDCl_3$, 250 MHz): δ=2.42 (quint, 2H, J=7.0 Hz), 3.03 (s, 3H), 3.77 (t, 2H, J=6.0 Hz), 5.48 (t, 2H, J=7.9 Hz), 7.99 (m, 2H), 8.23 (t, 1H, J=8.5 Hz), 8.37 (d, 1H, J=8.5 Hz), 8.49 (d, 1H, J=8.7 Hz), 10.33 ppm (d, 1H, J=6.0); ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ=21.2, 29.8, 48.7, 55.4, 119.6, 123.7, 127.4, 129.9, 130.6, 136.3, 137.5, 149.1, 159.0 ppm; HRMS (ESI) $[M]^+$ calcd. for $C_{13}H_{15}N_4^+$: 227.1291; found: 227.1290.

*(E)-4-(4-(Dimethylamino)styryl)-1-(hex-5-ynyl)quinolinium hexafluorophosphate (**7a**)*

1-(Hex-5-ynyl)-4-methylquinolinium iodide (**3a**, 0.181 g, 0.5 mmol) and 4-(dimethylamino)-benzaldehyde (**4**, 0.150 g, 1.0 mmol) were stirred in EtOH (25 mL) at reflux temperature in the presence of a catalytic amount of piperidine (five drops) for 6 h. The solvent was evaporated and the product was purified on silica (MeCN, then 1% NH_4PF_6 in MeCN). To the combined fractions was added an excessive amount of NH_4PF_6, followed by removal of the solvent in vacuo. The remainder of the solid was suspended in water, filtered, washed with several portions of water, and dried in vacuo to afford 0.229 g (89%) product as a blackish powder with blue glittering. M.p. 215–218°C; IR (neat): $\tilde{\nu}$=1315, 1523, 1735, 2106, 2922, 3189 cm^{-1}; ^1H NMR ([D_6]DMSO, 600 MHz): δ=1.57 (quint, 2H, J=7.4 Hz), 2.01 (quint, 2H, J=7.6 Hz), 2.25 (td, 2H, J=2.6 Hz, 7.1 Hz,), 2.80 (t, 1H, J=2.6 Hz), 3.07 (s, 6H), 4.89 (t, 2H, J=7.5 Hz), 6.82 (d, 2H, J=8.9 Hz), 7.87 (d, 2H, J=8.9 Hz), 7.95 (t, 1H, J=7.8 Hz), 8.00 (d, 1H, J=15.6 Hz), 8.18 (d, 1H, J=15.6 Hz), 8.19 (t, 1H, J=7.8 Hz), 8.34 (d, 1H, J=6.8 Hz), 8.44 (d, 1H, J=8.9 Hz), 9.02 (d, 1H, J=8.4 Hz), 9.13 ppm (d, 1H, J=6.7 Hz); ^{13}C NMR ([D_6]DMSO, 150 MHz): δ=17.2, 24.7, 28.3, 39.6, 55.3, 71.6, 83.8, 111.8, 113.0, 114.0, 118.7, 123.0, 126.1, 126.6, 128.3, 131.3, 134.6, 137.7, 144.9, 145.9, 152.3, 153.3 ppm; HRMS (ESI): $[M]^+$ calcd. for $C_{25}H_{27}N_2^+$: 355.2169; found: 355.2169.

*(E)-1-(3-Azidopropyl)-4-(4-(dimethylamino)styryl)quinolinium hexafluorophosphate (**7c**)*

To a solution of 1-(3-azidopropyl)-4-methylquinolinium iodide (**3c**, 0.060 g, 0.17 mmol) in warm EtOH (10 mL) was added 4-(dimethylamino)-benzaldehyde (**4**, 0.076 g, 0.50 mmol) and a catalytic amount of piperidine (1 drop), and the resulting solution was stirred at reflux temperature for 3 h. The solvent was evaporated under reduced pressure and the crude product was purified by silica gel column chromatography (CH_2Cl_2, then CH_2Cl_2/MeOH 9:1 v/v). The exchange of the counteranion was performed by addition of an excess amount of NH_4PF_6 to the aqueous solution of the purified product. The resulting precipitate was filtered, washed with water and Et_2O, and dried in vacuo to yield 0.057 g (67%) of an almost black, dark purple powder. M.p. 177–178°C (decomp); IR (neat): $\tilde{\nu}$=1374, 1528, 1739, 2096, 2921 cm^{-1}; ^1H NMR ([D_6]DMSO, 600 MHz): δ=2.20 (bs, 2H), 3.07 (s, 6H), 3.56 (bs, 2H), 4.92 (bs, 2H), 6.83 (d, 2H, J=7.6 Hz), 7.88 (d, 2H, J=6.8 Hz), 7.96 (t, 1H, J=6.8 Hz), 8.02 (d, 1H, J=15.9 Hz), 8.20 (m, 2H), 8.35 (d, 1H, J=4.6 Hz), 8.42 (d, 1H, J=8.4 Hz), 9.03 (d, 1H, J=7.6 Hz), 9.11 ppm (d, 1H, J=5.3 Hz); ^{13}C NMR ([D_6]DMSO, 62.5 MHz): δ=28.2, 39.6, 47.8, 53.4, 111.8, 113.0, 114.0, 118.6, 123.0, 126.0, 126.6, 128.3, 131.4, 134.7, 137.8, 145.1, 146.2, 152.3, 153.5 ppm; HRMS (ESI): $[M]^+$ calcd. for $C_{22}H_{24}N_5^+$: 358.2026; found: 358.2026.

*(E)-4-(2-(7-(Diethylamino)-2-oxo-2H-chromen-3-yl)vinyl)-1-(hex-5-ynyl)quinolinium hexafluorophosphate (**8a**)*

1-(Hex-5-ynyl)-4-methylquinolinium iodide (**3a**, 0.100 g, 0.28 mmol) and 7-(diethylamino)-coumarin-3-carbaldehyde (**5**, 0.084 g, 0.34 mmol) were dissolved in EtOH (15 mL) by heating and, after the addition of a catalytic amount of piperidine (three drops), the mixture was stirred at reflux

temperature for 12 h. After evaporation of the solvent, the crude product was purified on silica (MeCN, then 1% NH_4PF_6 in MeCN). To the combined fractions was added an excess amount of NH_4PF_6, followed by removal of the solvent in vacuo. The remainder of the solid was suspended in water, filtered, washed with several portions of water, and dried in vacuo to give 0.077 g (45%) of a blackish blue glittering powder. M.p. 232–234°C; IR (neat): $\tilde{\nu}$=1352, 1508, 1555, 1713, 2925, 3286 cm^{-1}; ^1H NMR ([D$_6$]DMSO, 600 MHz): δ=1.17 (t, 6H, J=7.1 Hz), 1.59 (quint, 2H, J=7.4 Hz), 2.04 (quint, 2H, J=7.7 Hz), 2.26 (td, 2H, J=2.6 Hz, 7.1 Hz), 2.81 (t, 1H, J=2.6 Hz), 3.52 (q, 4H, J=7.1 Hz), 4.96 (t, 2H, J=7.4 Hz), 6.65 (d, 1H, J=2.0 Hz), 6.84 (dd, 1H, J=2.3 Hz, 9.1 Hz), 7.57 (d, 1H, J=9.0 Hz), 8.04 (d, 1H, J=15.6 Hz), 8.06 (t, 1H, J=7.7 Hz), 8.25 (t, 1H, J=7.9 Hz), 8.40 (d, 1H, J=6.5 Hz), 8.47 (d, 1H, J=15.6 Hz), 8.52 (s, 1H), 8.53 (d, 1H, J=9.1 Hz), 8.75 (d, 1H, J=8.7 Hz), 9.28 ppm (d, 1H, J=6.6 Hz); ^{13}C NMR ([D$_6$]DMSO, 150 MHz): δ=12.3, 17.2, 24.7, 28.4, 44.4, 55.9, 71.7, 83.8, 96.3, 108.5, 110.2, 113.9, 115.6, 118.5, 119.1, 125.9, 126.4, 129.2, 130.9, 134.9, 137.8, 138.5, 145.2, 147.0, 152.2, 152.7, 156.5, 160.0 ppm; HRMS (ESI) $[M]^+$ calcd. for $C_{30}H_{31}N_2O_2^+$: 451.2380; found: 451.2378.

*(E)-1-(3-azidopropyl)-4-(2-(7-(diethylamino)-2-oxo-2H-chromen-3-yl)vinyl)quinolinium hexafluorophosphate (**8c**)*

To a warm solution of 1-(3-azidopropyl)-4-methylquinolinium iodide (**3c**) (0.060 g, 0.17 mmol) and 7-(diethylamino)-coumarin-3-carbaldehyde (**5**) (0.084 g, 0.34 mmol) in EtOH (20 mL) was added a catalytic amount of piperidine (2 drops) and the solution was stirred at reflux temperature for 4 h. After evaporation of the reaction solvent and separation using silica gel column chromatography (DCM, then DCM/MeOH : 9/1 v/v%) the exchange of the counter anion was performed by addition of excessive amount of NH_4PF_6 to the aqueous solution of the purified product. The resulting precipitate was filtered, washed with water and Et_2O and dried in vacuo to give 0.054 g (53%) dark purple powder. M.p. 196–199°C (decomp); IR (neat): $\tilde{\nu}$=1511, 1559, 1616, 1703, 2099, 2850, 2917 cm^{-1}; ^1H NMR ([D$_6$]DMSO, 600 MHz): δ=1.23 (s, 6H), 2.23 (m, 2H), 3.52 (s, 4H), 3.57 (s, 2H), 4.99 (s, 2H), 6.65 (s, 1H), 6.85 (s, 1H), 7.57 (m, 1H), 8.06 (s, 2H), 8.25 (s, 1H), 8.40–8.52 (m, 4H), 8.76 (s, 1H), 9.27 ppm (s, 1H). ^{13}C NMR ([D$_6$]DMSO, 150 MHz): δ=12.3, 28.9, 44.3, 47.7, 54.0, 96.3, 108.5, 110.2, 112.7, 113.8, 115.5, 118.5, 119.0, 126.4, 129.2, 130.9, 135.0, 136.6, 138.6, 145.2, 147.3, 149.4, 152.8, 159.9, 162.0 ppm; HRMS (ESI) $[M]^+$ calcd. for $C_{27}H_{28}N_5O_2^+$: 454.2238; found: 454.2239.

*(E)-4-(2-(6-(Diethylamino)benzofuran-2-yl)vinyl)-1-(hex-5-ynyl)quinolinium hexafluorophosphate (**9a**)*

1-(Hex-5-ynyl)-4-methylquinolinium iodide (**3a**, 0.100 g, 0.28 mmol) and 6-(diethylamino)benzofuran-2-carbaldehyde hydrochloride (**6**, 0.128 g, 0.50 mmol) were dissolved in warm EtOH (15 mL) and then piperidine (55 μL, 0.56 mmol) was added. The reaction mixture was stirred for 4 h at reflux temperature. Subsequently, the solvent was evaporated and the crude product was purified on silica (MeCN, then 1% NH_4PF_6 in MeCN). To the combined fractions was added an excess amount of NH_4PF_6 and afterwards the solvent was removed in vacuo. The remainder of the solid was suspended in water, filtered, washed with several portions of water, and dried in vacuo to yield 0.118 g (73%) product as a reddish black powder. M.p. 166–170°C; IR (neat): $\tilde{\nu}$=1352, 1396, 1551, 1630, 2972, 3288 cm^{-1}; ^1H NMR ([D$_6$]DMSO, 600 MHz): δ=1.16 (t, 6H, J=7.0 Hz), 1.58 (quint, 2H, J=7.4 Hz), 2.03 (quint, 2H, J=7.6 Hz), 2.26 (td, 2H, J=2.6 Hz, 7.1 Hz,), 2.83 (t, 1H, J=2.6 Hz), 3.48 (q, 4H, J=7.0 Hz), 4.93 (t, 2H, J=7.4 Hz), 6.81 (brs, 2H), 7.38 (s, 1H), 7.54 (d, 1H, J=6.2 Hz), 7.91 (d, 1H, J=15.3 Hz), 8.00 (t, 1H, J=7.7 Hz), 8.16 (d, 1H, J=15.3 Hz), 8.23 (t, 1H, J=7.9 Hz), 8.40 (d, 1H, J=6.5 Hz), 8.50 (d, 1H, J=8.8 Hz), 8.85 (d, 1H, J=8.4 Hz), 9.23 ppm (d, 1H, J=6.0 Hz); ^{13}C NMR ([D$_6$]DMSO, 150 MHz): δ=12.3, 17.2, 24.7, 28.4, 40.0, 44.3, 55.7, 71.7, 83.8, 91.9, 110.6, 115.0, 115.2, 115.4, 119.0, 123.1, 126.1, 126.2, 128.9, 129.6, 134.8, 137.8, 146.5, 148.5, 150.9, 151.6, 158.7 ppm; HRMS (ESI) $[M]^+$ calcd. for $C_{29}H_{31}N_2O^+$: 423.2431; found: 423.2432.

*(E)-1-(3-azidopropyl)-4-(2-(6-(diethylamino)benzofuran-2-yl)vinyl)quinolinium hexafluorophosphate (**9c**)*

1-(3-azidopropyl)-4-methylquinolinium iodide (**3c**, 0.060 g, 0.17 mmol) and 6-(diethylamino)benzofuran-2-carbaldehyde hydrochloride (**6**, 0.127 g, 0.50 mmol) were stirred at reflux temperature in ethanol (15 mL) in the presence of an excess amount of piperidine (55 μL, 0.56 mmol) for 5 h. The solvent was removed in vacuo, and the product was purified on silica (CH_2Cl_2, then CH_2Cl_2/MeOH 9:1). The product was dissolved in water and an excess amount of NH_4PF_6 was added to the solution. The resulting precipitate was filtered, washed with water and Et_2O, and dried in vacuo to give 0.067 g (69%) product as a blackish dark red powder. M.p. 162–166°C (decomp); IR (neat): $\tilde{\nu}$=1396, 1511, 1582, 1737, 2097, 2920 cm^{-1}; ^1H NMR ([D$_6$]DMSO, 250 MHz): δ=1.16 (bs, 6H), 2.22 (s, 2H), 3.46 (s, 4H), 3.57 (s, 2H), 4.95 (s, 2H), 6.79 (bs, 2H), 7.35 (s, 1H), 7.51 (s, 1H), 7.87 (d, 1H, J=15.8 Hz), 7.98 (s, 1H), 8.14 (d, 1H, J=15.8 Hz), 8.21 (s, 1H), 8.37 (s, 1H), 8.45 (d, 1H, J=6.8 Hz), 8.81 (d, 1H, J=6.0 Hz), 9.20 ppm (s, 1H); ^{13}C NMR ([D$_6$]DMSO, 62.5 MHz): δ=12.3, 28.3, 44.3, 47.8, 53.8, 91.9, 110.6, 114.9, 115.5, 117.5, 118.7, 123.0, 124.3, 126.0, 126.1, 128.9, 129.6, 134.8, 137.8, 146.7, 148.5, 150.9, 151.7, 158.7 ppm. HRMS (ESI) $[M]^+$ calcd. for $C_{26}H_{28}N_5O^+$: 426.2288; found: 426.2289.

Solid-phase Peptide Syntheses

General

All natural amino acid derivatives, chlorotrityl chloride resin, and Rink amide MBHA resin were purchased from Reanal (Budapest, Hungary) and IRIS Biotech GmbH (Marktredwitz, Germany). *N,N′*-diisopropylcarbodiimide (DIC), triisopropylsilane (TIS), *N,N*-diisopropylethylamine (DIEA), and phenol were purchased from Fluka (Buchs, Schwitzerland). 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) and 1-hydroxybenzotriazole (HOBt) were purchased from IRIS Biotech. Solvents were obtained from the commercial suppliers mentioned earlier (Sigma–Aldrich, Alfa Aesar, Merck) and were used as received.

Syntheses

The glutamic acid derivative N_3-$(CH_2)_5$-CO-Glu-NH_2 was built up on a Rink amide MBHA resin (0.7 mmol g^{-1} capacity), and the tetrapeptide Fmoc-Tyr-Arg-Arg-Leu-OH (Fmoc-YRRL-OH) was synthesized on a 2-chlorotrityl chloride resin (0.6 mmol g^{-1} capacity) by using standard Fmoc chemistry. The synthetic protocol was as follows:

a) Washing of the resin (3×1 min) with DMF; b) removal of the Fmoc group with 2% DBU and 2% piperidine in DMF (4 times for 2+2+5+10 min); c) Washing (8×1 min) with DMF; d) coupling of the Fmoc-protected amino acid derivative using HOBt and DIC (3 equiv each; for 60 min); e) Washing (3×1 min) with DMF; f) Washing (2×0.5 min) with CH_2Cl_2; and g) Examination of the efficiency of the coupling cycle by using the ninhydrin assay.

Fmoc-Arg(Pbf)-OH, Fmoc-Glu(O*t*Bu)-OH, and Fmoc-Tyr(*t*Bu)-OH was used as side chain-protected amino acid derivatives. The peptides were cleaved from the resin either with a phenol/TIS/thioanizole/water/TFA (0.25:0.25:0.25:0.25:9.0 v/v, 3 h) cleavage mixture (Fmoc-YRRL-OH) or TFA/water (9.5:0.5 v/v, 3 h) mixture in case of the modified Glu derivative. The modified glutamic acid derivative was obtained by evaporation of the cleavage mixture and was used without further purification. In case of the tetrapeptide, the crude product was precipitated by adding dry diethyl ether to the cleavage mixture and was purified by RP-HPLC. Daunomycin (**Dau**) was attached to the C-terminus of the tetrapeptide and to the side chain of the modified glutamic acid in DMF solution, using five equivalents of daunomycin and coupling agents (HOBt and DIC). The reactions were carried out at room temperature for 3 h, and then the reaction mixtures were directly purified by RP-HPLC. Deprotection of the N-terminus of the Fmoc-YRRL-**Dau** conjugate was carried out by using 20% piperidine in DMF (5 min). Subsequently, the solvent was evaporated, and the conjugate was purified by RP-HPLC. The cyclo-

octyne derivative CyOCOOH[31] was then attached to the N-terminus of the YRRL-**Dau** conjugate in DMF solution by applying CyOCOOH (3 equiv) and coupling agents (HOBt and DIC) in the presence of DIEA (3 equiv with regard to the peptide) for the coupling. The reactions were carried out at rt for 3 h, and the reaction mixtures were then directly purified by RP-HPLC.

*Preparation of the Dually Labeled (**Dau-7**) Systems*

*Lep-$(CH_2)_5$-Glu(Dau)-NH_2 Conjugate (**Dau-7a**)*

To a solution of 31.5 mL DMSO/water (1:1 v/v) containing 0.5 M $CuSO_4$, TBTA (TBTA = Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine), and Na-ascorbate was added N_3-$(CH_2)_5$-CO-Glu(**Dau**)-NH_2 (5.0 mg, 6.3 μmol), **7a** (**Lep**, 4.7 mg, 9.5 μmol), and the solution was stirred overnight at room temperature. The crude product was purified by RP-HPLC to give 4.2 mg (52%) lyophilized product as a purple powder.

*Lep-CyO-YRRL-Dau Conjugate (**Dau-7c**)*

CyO-YRRL-**Dau** (2.0 mg, 1.5 μmol) and **7c** (**Lep**) (2.3 mg, 4.5 μmol) were dissolved in 0.5 mL DMF and stirred for 72 h at room temperature. After evaporation of the solvent, the crude product was purified by RP-HPLC to yield 1.0 mg (37%) lyophilized product as a purple powder.

Purification and Analysis by RP-HPLC

Analytical RP-HPLC was performed on Knauer systems (Knauer, Bad Homburg, Germany) using a Phenomenex Jupiter C_4 column (250× 4.6 mm I.D.) with 5 μm silica (300 Å pore size) as a stationary phase. A linear gradient (0 min, 0% B; 5 min, 0% B; 50 min, 90% B) with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile/water (80:20, v/v)) was used for elution at a flow rate of 1 mL min^{-1} at ambient temperature. Peaks were detected at $\lambda = 220$ nm. The samples were dissolved in a mixture of eluents A and B (1:1, v/v). The crude products of the glutamic acid derivates and **Dau-7a** were purified on a semi-preparative Phenomenex Jupiter C_4 column (250×21.2 mm I.D.) with 10 μm silica (300 Å pore size), while the crude products of the YRRL derivatives and **Dau-7c** were purified on a semi-preparative Phenomenex Luna C_{18} column (250×21.2 mm I.D.) with 10 μm silica (100 Å pore size). The flow rate was 9 mL min^{-1}. The same eluents with a linear gradient of 0–90% B in 60 min were used in all cases.

Mass Spectrometry (MS)

Mass spectrometric analyses were carried out on a Bruker Daltonics Esquire 3000+ (Bremen, Germany) ion trap mass spectrometer. Spectra were acquired in the 50–3000 m/z range. Samples were dissolved in acetonitrile/water (1:1 v/v) containing 0.1% acetic acid. The exact mass of the final products was measured on an Agilent 6230 MS-TOF system. Spectra were acquired in the 100–1200 m/z range from acetonitrile solutions.

Photophysical and Fluorescence Studies

The excitation and emission spectra were measured using 10^{-6} M solutions in all cases. For the pure lepidinium fluorophores, methanol and DMSO/water (1:1 v/v) were used as solvents. The absorbance maxima and ε values were determined using dilution series (10^{-6}–3×10^{-5} M DMSO/water solutions). The reaction of CyO-YRRL-**Dau** with **7c** was followed by measuring the fluorescence intensity at 592 nm. The reaction was carried out using 10^{-6} M peptide and 1.5×10^{-6} M lepidinium dye solution (DMSO/water, 0.5:9.5 v/v) at room temperature. The cleavage of the cathepsin B substrate **Dau-7c** system and the recovery of daunomycin signal were studied under in vitro conditions by applying 5×10^{-6} M cathepsin B enzyme to 5×10^{-5} M **Dau-7c** in buffered medium (pH 5, 0.1 M NaOAc, 0.01 M DTT, and 0.002 M EDTA) at 37 °C. The fluorescence intensity was measured by taking 50 μL aliquots of the reaction mixture, which were brought to 2.5 mL using the same buffer (final concentration 10^{-6} M).

Quantum Yield and Fluorescence Lifetime Determinations

The absorption spectra were recorded on a thermoregulated Unicam UV500 spectrophotometer, while the corrected fluorescence spectra were obtained on a Shimadzu RF-5000PC spectrofluorometer. Unless otherwise indicated, the excitation wavelength was around 480 nm or 560 nm. Room-temperature fluorescence quantum yields were determined relative to that of cresyl violet in methanol ($\Phi_f = 0.54$, using n^2 correction).[30] In all measurements (except some absorption ones) freeze-pump-thaw degassed samples were used in $1\times1\times4$ cm Suprasil quartz cells. The fluorescence decay times were measured by using an FLS 920 time-resolved spectrofluorimeter (Edinburgh Instruments), with an EPL-470 nm semiconductor laser for excitation and a Hamamatsu R3809U-50 microchannel plate-photomultiplier tube (MCP-PMT) for detection.[33] For lifetime determination, samples were excited at 473 nm and signals were detected at 590 ± 10 for **Dau** and its conjugates, and at 750 ± 10 for **7a** or **7c** and their conjugates.

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