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Kondrat'eva Ligation: Diels–Alder-Based Irreversible Reaction for Bioconjugation

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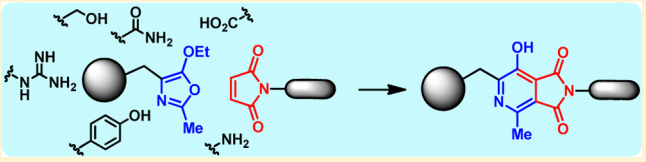
Supporting Information

ABSTRACT: Diversification of existing chemoselective ligations is required to efficiently access complex and well-defined biomolecular assemblies with unique and valuable properties. The development and bioconjugation applications of a novel Diels–Alder-based irreversible site-specific ligation are reported. The strategy is based on a Kondrat'eva cycloaddition between bioinert and readily functionalizable 5-alkoxyoxazoles and maleimides that readily react together under mild and easily tunable reaction conditions to afford a fully stable pyridine scaffold. The potential of this novel bioconjugation is demonstrated through the preparation of fluorescent conjugates of biomolecules and a novel Förster resonance energy transfer (FRET)-based probe suitable for the in vivo detection and imaging of urokinase-like plasminogen activator (uPA), which is a key protease involved in cancer invasion and metastasis.



INTRODUCTION

Chemoselective transformations have been used in a significant number of applications within the past decade in diverse research fields, including materials science,[1] biotechnology,[2] chemical biology,[3] and drug discovery.[4] In particular, site-selective modification of nucleic acids, glycans, peptides, or proteins with various reporter groups has become an intensive area of research to provide access to multicomponent biomolecular systems suitable for addressing numerous biological issues.[5] In this respect, the nucleophilic additions of hydrazines/alkoxyamines to carbonyl derivatives to form their corresponding hydrazone/oxime derivatives were among the first chemoselective chemical strategies reported.[6] However, to circumvent hydrolytic instability of the resulting imine linkage, Pictet–Spengler-like ligations have recently been reported to irreversibly trap the hydrazone/oxime intermediate.[7] Alternatively, transformations based on more sophisticated bimolecular processes, such as the Staudinger-modified reaction,[8] "sulfo-click" ligation,[9] native chemical ligation,[10] and several 1,3-dipolar cycloadditions, including Cu(I)-catalyzed, strain-promoted azide–alkyne cycloaddition,[11] nitrile imines and alkenes,[12] alkyne and nitrone,[13] or alkyne and alkene-nitrile oxide,[14] have been the subject of intensive research. Some of these processes are currently being used to design biosensors, molecular bioprobes, and drug vectors suitable for biomedical applications, including diagnostic assays, multimodality molecular imaging, and drug delivery.[15] In contrast, Diels–Alder-based peptide modification methods have had little investigation despite their attractive features, which is most likely due to two major drawbacks: (1) the formation of an uncontrolled mixture of endo/exo adducts or regioisomers during the cycloaddition process[16] and (2) the inherent reversible nature of Diels–Alder reactions that may not be suitable for effective bioconjugation.[17,18] The inverse electron-demand hetero-Diels–Alder (HDA) reactions occurring between tetrazines and strained alkenes that release dinitrogen,[19] or between *o*-quinone methides and thio vinyl ethers, constitute rare examples of irreversible [4+2] cycloaddition in bioconjugation.[20] Despite these remarkable recent advances, the development of new ligation tools, especially those involving peptides, is still urgently needed to overcome current limitations associated with the reaction conditions involved (i.e., the use of toxic metal catalysts, oxidizing agents,

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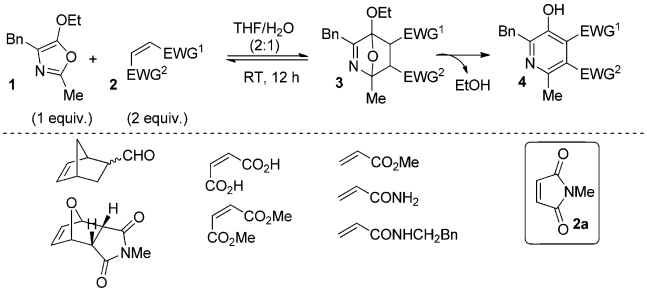
or photochemical activation),[21] poor commercial availability, and the complicated synthesis of bioconjugation partners and (bio)chemical reporters functionalized with unusual bioconjugatable handles.[22] Furthermore, diversification of chemoselective ligation reactions is also highly motivated by growing interest in their combined use while avoiding cross-reactivity between reagent pairs. In this respect, sophisticated heteromultifunctional cross-linking reagents have recently been designed and have already found several applications in chemical biology, therapeutics, and diagnosis.[23] Moreover, due to the need for rapid polyfunctionalization of biomolecules/biopolymers, efforts are currently being made to develop multiple ligation strategies for their sequential or parallel labeling.[23,24]

In this article, we investigate whether the HDA reaction of 5-alkoxyoxazoles with alkenes is amenable to the growing field of site-selective biomolecule ligation, thus offering prompt access to a novel pair of ligation partners that are particularly capable of functionalizing peptides. Furthermore, as part of an increasing demand for the development of highly valuable heteromultifunctional cross-linking reagents, the chemical orthogonality of 5-alkoxyoxazoles toward reported ligation partners is investigated, thus opening up new opportunities for performing two or more chemical ligations in parallel. As illustrative examples of the utility of this novel bioconjugation reaction, we have prepared fluorescent conjugates of multiple biomolecules, including α-tocopherol (one of the four tocopherols found in vitamin E) and bovine serum albumin (BSA) protein. A more challenging application devoted to the preparation of protease-activatable far-red fluorogenic probes, based on the Förster resonance energy transfer (FRET) phenomenon, that are suitable for biomolecular imaging applications has also been investigated.

RESULTS AND DISCUSSION

The Kondrat'eva HDA reaction was developed almost 60 years ago,[25] involves the azadiene alkoxyoxazole **1** and an electron-deficient olefinic dienophile **2**, and affords an oxabicyclic intermediate **3** that subsequently aromatizes into 3-hydroxypyridine **4** with concomitant loss of an alcohol molecule (Scheme 1). Interestingly, 5-alkoxyoxazole derivatives are

Scheme 1. Kondrat'eva HDA Reaction between 5-Alkoxyoxazole 1 and a Set of Activated Dienophiles (EWG = Electron-Withdrawing Group)



conveniently prepared from readily available α-amido esters through the Gabriel–Robinson synthesis[26] or from a variety of other recently reported methods.[27] Although the two-step HDA cycloaddition/aromatization reaction fulfills the important criterion of irreversibility required for the effective ligation of two (bio)molecular partners, the reaction conditions reported to date (i.e., temperature,[28] pressure,[29] and solvent[30]) definitely prevent its application for bioconjugation purposes.

To explore the utility of the Kondrat'eva reaction in bioconjugation, a model cycloaddition reaction was performed between the readily accessible 4-benzyl-5-ethoxy-2-methyloxazole **1** and dienophiles with various electrophilic character under mild aqueous conditions that are fully compatible with the stability of the peptides (THF/H_2O at room temperature, Scheme 1). First, strained dienophiles such as (oxa)-norbornenes[19] led to unsatisfying results because the starting oxazole was recovered unchanged. Next, the reaction of **1** with symmetrically disubstituted olefins, such as maleic acid and dimethyl maleate, or monosubstituted olefins did not afford any product. Finally, *N*-methylmaleimide reacted completely with **1** to afford oxabicyclic intermediate **3** as the sole product.[31] Indeed, no trace of the desired 3-hydroxypyridine **4** was observed. Satisfyingly, the Kondrat'eva reaction carried out in THF/aq 1% TFA (2:1) at room temperature (condition A) enables aromatization to provide bicyclic 3-hydroxypyridine **4a** in 67% isolated yield from **1** (Table 1, entry 1). Furthermore, a study of the reaction's pH dependence showed that the aromatization step occurred under acidic conditions up to pH 5.0 (sodium acetate buffer, condition B)[32] at 37 °C, which provided **4a** from **1** in 82% nonisolated yield. Next, the bioorthogonality of the reaction between the 5-alkoxyoxazole scaffold and *N*-methylmaleimide was studied by performing an HDA reaction in the presence of amino acids, among which the majority have reactive side chains, to confirm that the Kondrat'eva ligation reaction is suitable in the context of peptide/protein bioconjugation. The course of the reaction between **1** and **2a** in sodium acetate buffer (pH 5.0) was unaffected when the following amino acids were added to the mixture: Tyr, Trp, His, Pro, Ser, or Asn (see Supporting Information (SI)). However, the reaction carried out in the presence of Cys provided **4a** in a useful 50% yield, which is presumably due to the competitive Michael addition of the free thiol of this amino acid to *N*-methylmaleimide (entry 2).[5] Next, the reactivity of **1** with diversely functionalized maleimide derivatives was investigated at pH ∼1 and 5.0. Gratifyingly, the Kondrat'eva reaction conditions were fully compatible with popular bioorthogonal chemical reporters such as ketone (entry 6), alkyne (entry 7), or azide (Scheme 3). Indeed, **1** provided keto and acetylenic pyridine **4e** and **4f** in 78 and 75% yield, respectively. Then, the stability of **1** and 3-hydropyridine **4a** was examined. Although no noticeable degradation of **1** was observed upon its storage in sodium acetate buffer (pH 5.0) at 37 °C for 15 h (see SI), we preferred to store the α-amido ester precursors of 5-alkoxyoxazoles because the latter is susceptible to decomposition after long-term storage.[33] Although maleimide–thiol Michael adducts and maleimide–dienophile cycloadducts are generally prone to rapid hydrolysis in aqueous media, even at physiological pH,[18e,34] and to retro/exchange reactions in reducing environments,[35] it is worth noting that the azaphthalimide scaffold of **4a** exhibits good stability even upon standing at pH 2.0 and 10.0 for 48 h (see SI). Consequently, this ligation strategy results both in an appreciable improvement in the stability of bioconjugates assembled from maleimide derivatives and in a nonreversible ligation reaction utilizing this popular thiol reactive function, which is often present in commercially available bioconjugatable fluorophores.[36]

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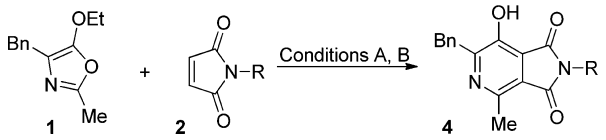
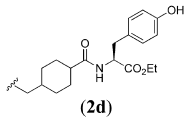
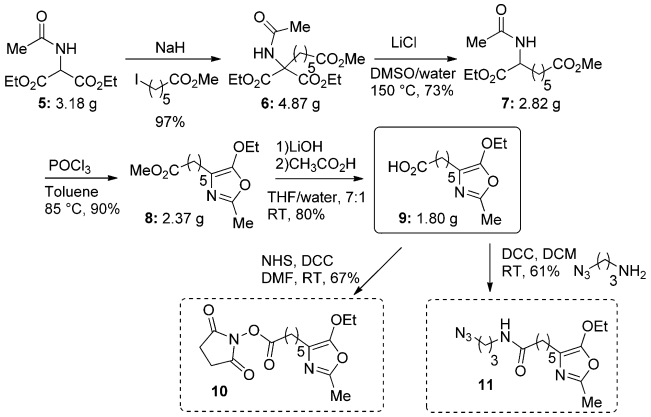
Table 1. Optimization and Orthogonality of the Kondrat'eva Ligation



entry	R (maleimide)	pyridine	yield[a] (pH ~1)	yield[b] (pH 5.0)
1	Me (**2a**)	**4a**	67	82
2	Me (**2a**)	**4a**	-	50[c]
3	Ph (**2b**)	**4b**	72	66
4	$(CH_2)_5CO_2H$ (**2c**)	**4c**	41	66
5	 (**2d**)	**4d**	46	59
6	$(CH_2)_5CONHCH_2COCH_3$ (**2e**)	**4e**	52	78
7	$(CH_2)_5CONHCH_2CCH$ (**2f**)	**4f**	53	75

[a]Condition A (pH ~1): 5-alkoxyoxazole **1a** (0.46 mmol), maleimide **2** (0.60 mmol, 1.3 equiv), THF/aq 1% TFA (2:1), rt, for 5 h. Isolated yield. [b]Condition B (pH 5.0): 5-alkoxyoxazole **1a** (0.07 mmol), maleimide **2** (0.09 mmol, 1.3 equiv), DMSO/NaOAc buffer pH 5.0 (1.0 M) (1:5), 37 °C, for 5 h. Yield determined by ^{1}H NMR spectroscopy of the crude reaction mixture relative to internal standard 1-phenylcyclohexene. [c]5-Alkoxyoxazole (0.46 mmol), L-cysteine (0.46 mmol, 1 equiv), *N*-methylmaleimide (0.60 mmol, 1.3 equiv).

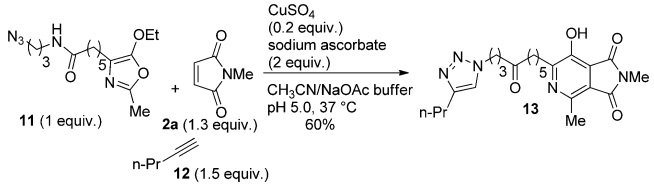
Scheme 2. Gram-Scale Synthesis of Key Acid-Terminated 5-Ethoxyoxazole 9 and Heterobifunctional Reagents 10 and 11



Having established suitable reaction conditions for performing Kondrat'eva ligation in the context of peptidyl biopolymers, we next investigated a gram-scale synthesis of the readily functionalizable 5-alkoxyoxazole **9** (Scheme 2). A 5-alkoxyoxazole separated by a five methylene spacer unit from the carboxylic acid group was selected to limit both electron-withdrawing and steric-hindrance effects that may be detrimental to the HDA process. First, alkylation of commercially available diethyl acetamidomalonate **5** in the presence of methyl 6-iodohexanoate afforded **6** in a near quantitative yield. Next, amido diester **7**, which was obtained in 73% yield under Krapcho's decarboxylation conditions, subsequently gave 5-alkoxyoxazole **8** in 90% yield upon cyclodehydration. Finally, carboxylic acid-terminated 5-alkoxyoxazole **9** was obtained by alkaline hydrolysis of ester in 80% yield (overall yield is 51% for the four steps). It is worth noting that the carboxylic acid moiety of **9** can be readily converted into amine-reactive *N*-hydroxysuccinimidyl (NHS) ester using standard conditions to provide heterobifunctional reagent **10** in 67% yield. A second valuable heterobifunctional reagent **11** bearing both azido- and alkoxyoxazole moieties was also prepared from **9** in 61% yield.[5]

With reagent **11** in hand, its dual derivatization through a "one-pot" parallel Kondrat'eva HDA and copper-catalyzed azide–alkyne 1,3-dipolar cycloaddition (CuAAC) was demonstrated (Scheme 3).[24] Indeed, reagent **11** mixed with *N*-methylmaleimide **2a** and pent-1-yne in the presence of $CuSO_4$ (0.2 equiv) and sodium ascorbate (2 equiv) afforded compound **13** in 60% isolated yield.

Scheme 3. Dual Derivatization of 11 via HDA and CuAAC Reactions Involving *N*-Methylmaleimide 2a and Terminal Alkyne 12



During further optimization, we observed that reactions between maleimides and 5-alkoxyoxazoles proceeded effectively under mild conditions in either organic or aqueous media (see SI). To confirm the robustness and versatility of the Kondrat'eva cycloaddition-based ligation strategy, labeling of biologically relevant lipo- and hydrophilic biomolecules such as

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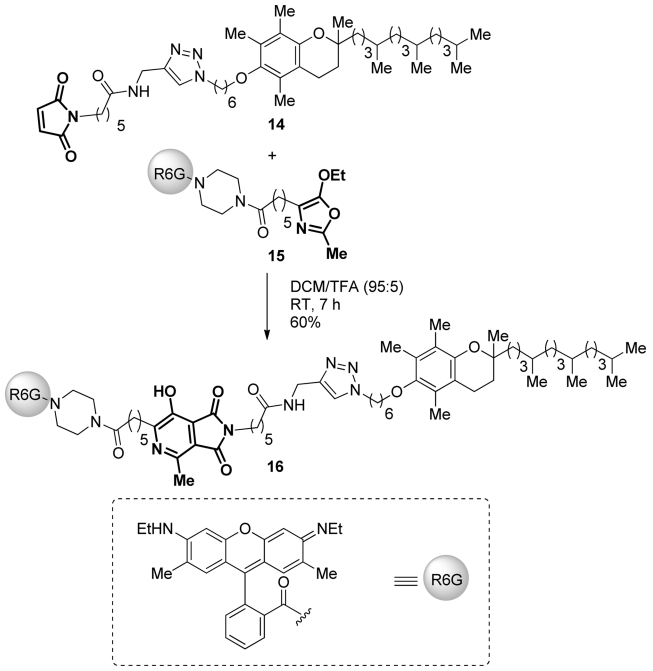
α-tocopherol[37] and BSA protein with a green-emitting fluorophore (i.e., rhodamine 6G (R6G)) was investigated. Interestingly, different fluorescent or fluorogenic α-tocopherol analogues have been designed and prepared either to track the intracellular location and transfer of vitamin E or to study in cellulo lipid peroxidation by taking advantage of the antioxidant properties of α-tocopherol.[38] In this context, it is worthwhile to study the fluorescent labeling of this biologically relevant target through the Kondrat'eva cycloaddition reaction. Thus, maleimide-derivatized tocopherol **14** was readily prepared in two steps from α-tocopherol (see Experimental Section (ES)) and reacted with "in-house synthesized" alkoxyoxazole-based R6G derivative **15** in DCM/TFA (95:5) to give fluorescent compound **16** in 60% yield (Scheme 4).

Scheme 4. Fluorescent Labeling of α-Tocopherol with R6G Dye and through the Kondrat'eva Cycloaddition Reaction



We then extended the scope of this labeling approach with alkoxyoxazole-based R6G dye **15** to a model protein, namely BSA, which is known to be both very stable and water-soluble (Scheme 5). BSA contains 59 lysine residues, 30−35 of which are accessible for conjugation. The reactive ε-amino group of lysine residues in this biopolymer was reacted with the heterobifunctional cross-linker sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC, 100 equiv (i.e., ∼3 equiv relative to the lysine residues)) to introduce maleimide moieties. Importantly, 34 of the 35 cysteine residues in BSA are inert under these conditions because they are involved in disulfide linkages.

The resulting maleimide-activated BSA was washed using an ultracentrifugal filter device to remove excess unconsumed cross-linker reagent and subsequently treated with **15** (37.5 or 125 equiv relative to BSA (i.e., 1.1−1.2 or 3.6−4.2 equiv relative to theoretically conjugated maleimides)) in sodium acetate buffer (pH 5.0) at 37 °C for 12 h.

The resulting R6G-conjugated proteins **17a** and **17b** were then washed with an ultracentrifugal filter device with a 10 kDa cutoff to remove excess alkoxyoxazole-based R6G derivative **15**.

Scheme 5. Fluorescent Labeling of BSA Protein with R6G Dye Using a Two-Step Sequential Procedure Involving Aminolysis of Sulfo-SMCC and the Kondrat'eva Cycloaddition Reaction

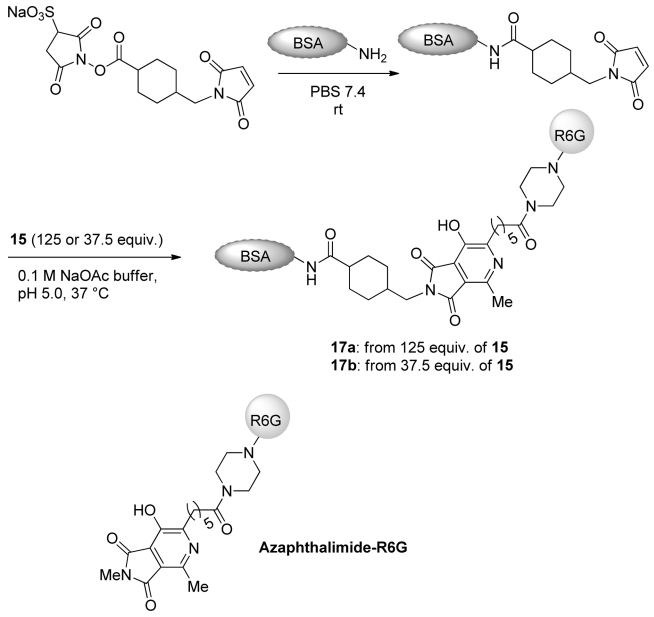


Table 2 displays the absorption and emission wavelengths and quantum yields of fluorescent proteins **17a** and **17b** both in phosphate buffered saline (PBS, pH 7.4) and in deionized water with sodium dodecyl sulfate (SDS, 2.5% w/v), which is a well-known deaggregating and denaturing agent, along with the fluorophore to protein molar ratio (*F*/*P*) estimated from the relative intensities of protein and dye absorption (see Figure 1 for the corresponding spectra of the fluorescent bioconjugate in aq 2.5% SDS and PBS).[39]

Table 2. Spectroscopic Data of R6G-Containing Compounds

compound	*F*/*P*	solvent[a]	λ_{abs} (nm)	λ_{em} (nm)	Stokes shift (nm)	Φ_F (%)[ab]
17a	9.6	PBS	508/534	557	23	2
17a	9.6	aq 2.5% SDS[c]	539	560	21	9
17b	5.7	aq 2.5% SDS[c]	539	559	20	7
R6G		PBS	526	556	30	83
R6G		aq 2.5% SDS[c]	534	561	27	87
15		PBS	533	557	24	52
15		aq 2.5% SDS[c]	539	559	20	85
azapht.-R6G		PBS	533	558	25	33
azapht.-R6G		aq 2.5% SDS[c]	539	559	20	53

[a]All measurements were taken at 25 °C. [b]R6G was used as the standard (94% in EtOH)[40] with excitation at 500 nm. [c]Measurements were taken in water with this additive due to its poor solubility in PBS.

The presence of absorption bands in the 460−560 nm region, which correspond to the rhodamine scaffold, confirmed the labeling of the BSA protein. Contrary to that of free R6G dye, the absorption spectrum of fluorescent-protein conjugate **17a** in PBS revealed two distinct peaks at 508 and 534 nm (Figure 1). The breadth of the absorption bands and the blue-

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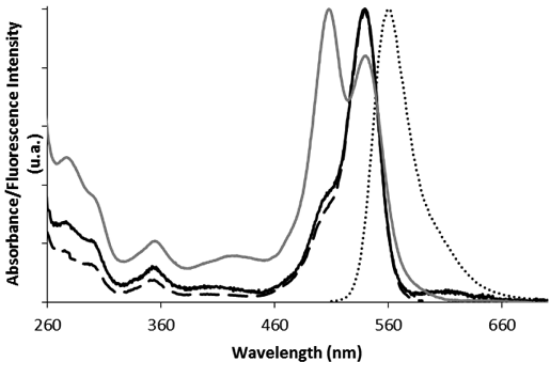

Figure 1. Normalized absorption (—), emission (···) and excitation (- - -) spectra of R6G-labeled BSA **17a** in deionized water containing SDS (2.5% w/v) at 25 °C, and normalized absorption (—) of **17a** in PBS (pH 7.4) at 25 °C.

shift of one of them to 16 nm (compared to the R6G absorption maximum) are in keeping with the formation of nonemissive aggregates (i.e., *H*-type homodimers).[41] The poor value of the measured quantum yield (2%) in spite of the convenient *F*/*P* ratio (9.6) 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,[42] and (3) azaphthalamide-scaffold electronic effects (quantum yield was decreased ∼35% when alkoxyoxazole-R6G **15** was converted to azaphthalimide-R6G), which could promote fluorescence quenching through photoinduced electron transfer (PeT). Interestingly, the addition of SDS (2.5% w/v in water) led to both the disappearance of the blue-shifted dimer absorption band and an increase in the quantum yield to 7−9%. Obviously, protein unfolding in conjunction with dye-SDS interactions is needed to partially break up already-formed dye aggregates bound to the protein.

Next, the efficiency of the Kondrat'eva cycloaddition reaction for grafting various reporter groups onto biologically relevant molecules was illustrated and supported by the preparation of a FRET-based urokinase-like plasminogen activator (uPA)-sensitive probe **18**. Indeed, uPA is a serine protease that plays a crucial role in malignancies, and its overexpression has been linked to poor clinical prognosis, particularly in breast cancer.[43] Thus, noninvasive imaging of uPA overexpression would have significant potential to improve novel cancer therapies. In this context, we have recently reported that heptapeptide H-Ser-Gly-Arg-Ser-Ala-Asn-Ala-OH (SGRSANA, a highly potent uPA substrate with a cleavage site between the Arg and Ser residues)[44] could easily be labeled with a FRET pair that consists of a far-red emitting cyanine dye (i.e., sulfoindocyanine dye Cy5.0) and an azo-based dark quencher (Black Hole Quencher-3, BHQ-3) to provide effective, synthetic access to uPA-activatable fluorescence imaging probes (Figure 2).[45,46]

These promising results prompted us to validate in vitro the efficiency of the FRET-based uPA-sensitive probe **18**, its preparation involving for the first time, as the last step, the Kondrat'eva ligation reaction (Scheme 6).

The heptapeptide SGRSANA was prepared according to standard solid-phase peptide synthesis protocols (see ES) with an incorporated Dde-protected lysine residue at the C-terminus for the installation of a maleimide-terminated linker. Fluorescent labeling with Cy5.0 dye was achieved on the N-terminal α-amino group of the peptide under standard conditions involving the in situ generation of 1-hydroxybenzotriazole (HOBt) esters of this fluorophore (Scheme 6a).[47] The 5-alkoxyoxazole-based BHQ-3 derivative **22** was readily prepared through an amidification reaction between the carboxylic acid-terminated 5-alkoxyoxazole **9** and the amino derivative of BHQ-3 **21** (Scheme 6b). Then, the Kondrat'eva ligation between **20** and **22** was performed in a mixture of THF/H_2O/TFA (Scheme 6c), and its progress was monitored by RP-HPLC (Figure 3). Complete consumption of the starting 5-alkoxyoxazole **22** and clean formation of **18** were observed within 6 h. The resulting uPA fluorogenic substrate **18** was isolated in pure form by semipreparative RP-HPLC (63% overall yield, 98% purity). Its structure was unambiguously confirmed by HRMS (ESI-TOF) analysis. The same protocol was used to prepare negative control probe **18-D** that bears non-natural D-amino acids (see ES).

The results from the fluorogenic cleavage assay with commercial uPA (from human urine) are summarized in Figure 4. Almost complete quenching (calculated efficiency = 99%) was found for **18** until it was cleaved by uPA, which caused a 66-fold increase in fluorescence at λ_{em} = 665 nm over time (plateau reached within 20 min). As expected, no significant fluorescence signal changes were observed in the absence of protease.

Encouraged by these in vitro results, we next studied the ability of **18** to probe uPA activity inside living cells. The human prostate cancer cell line PC3 was incubated with **18** (20 μM) for 2 h. Satisfyingly, we clearly observed red fluorescence staining inside tumor cells, which indicates that the probe was efficiently cleaved by overexpressed uPA to release the Cy5.0-labeled residue and split the FRET pair. Moreover, no red fluorescence was observed in the control experiment conducted with the **18-D** probe bearing D-amino acids, which confirms the specificity of the protease-induced hydrolysis process.

■ CONCLUSION

In this article, we have optimized and reported bioconjugation applications for a rare example of irreversible Diels−Alder-based ligation, which meets the important criterion of linkage stability that is required for applications in biological media. Indeed, this process involves an HDA/aromatization sequence

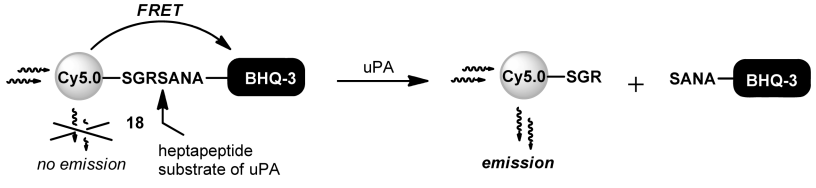

Figure 2. Principle of the FRET-based uPA-sensitive probe.

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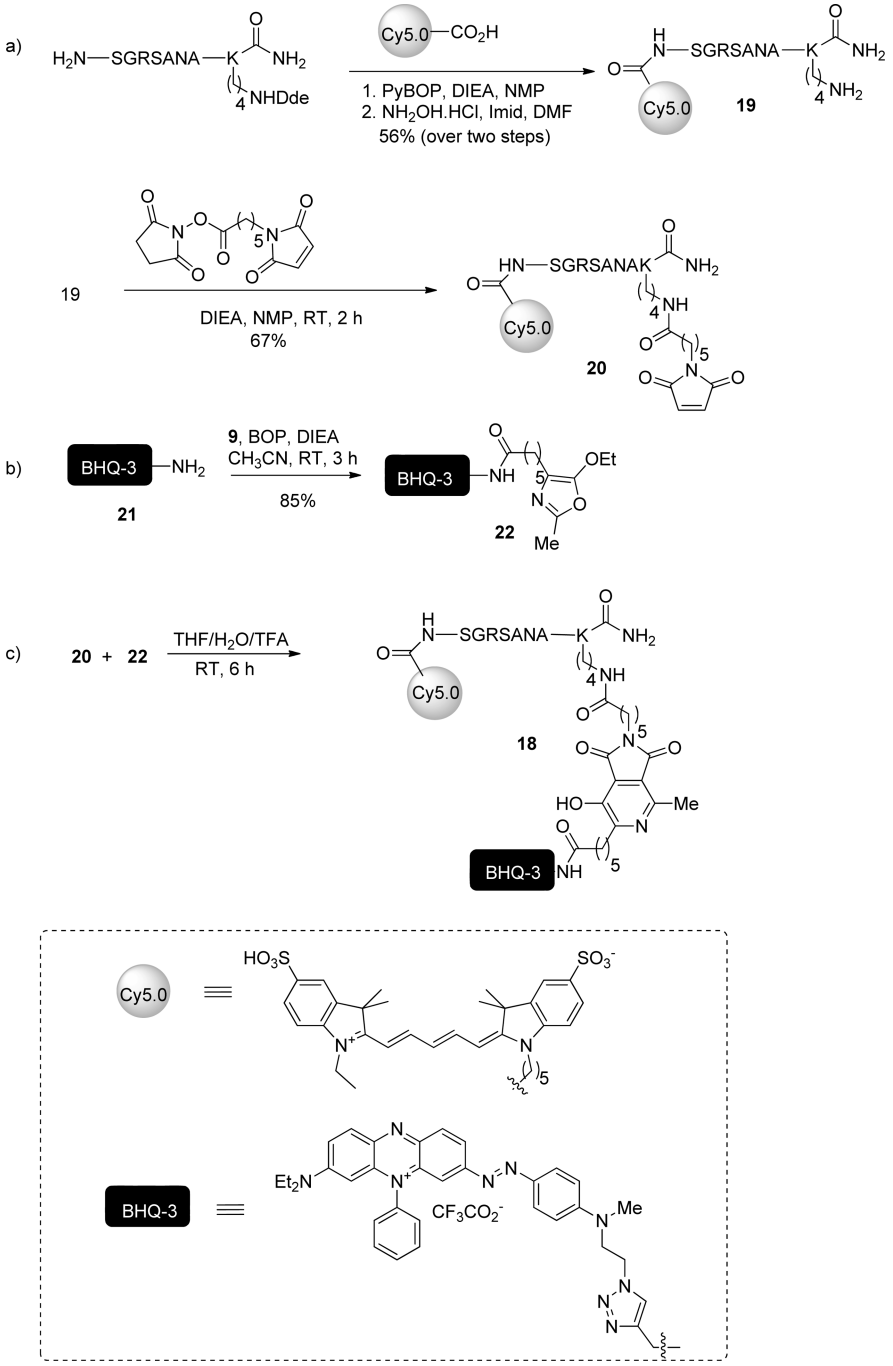
Scheme 6. Synthesis of uPA-Activatable Far-Red Fluorescent Probe 18 with the Kondrat'eva Cycloaddition Reaction as the Key Step



between 5-alkoxyoxazole and maleimide derivatives, which advantageously give rise to a particularly hydrolytically stable azaphthalimide scaffold. This reaction, which performed indifferently in aqueous or organic medium, proved to be of practical use in preparing both small molecules and complex bioconjugates under mild conditions, and it fulfills most of the requirements to belong in the "click chemistry" repertoire.[48] Importantly, this article also highlights for the first time that 5-alkoxyoxazoles, readily prepared by scalable synthesis, are potent, bioinert reactive partners in bioconjugation reactions.

EXPERIMENTAL SECTION

General Information. All solvents were dried following standard procedures: CH_2Cl_2, CH_3CN, and Et_3N were distilled over CaH_2, DMF and MeOH were dried by storage over a 3 Å molecular sieve, and THF was distilled over Na°/benzophenone. All reactions involving air- and moisture-sensitive reagents were performed under argon gas using the syringe−septum cap technique. Column chromatography purifications were performed on silica gel (40−63 μm) and carried out on Merck DC Kieselgel 60 F-254 aluminum sheets. Thin-layer chromatography (TLC) analyses were carried out on Merck DC Kieselgel 60 F-254 aluminum sheets. Spots were visualized through illumination with a UV lamp (λ = 254 nm) and/or staining with $KMnO_4$. *N*,*N*-Diisopropylethylamine (DIEA) and *N*-methyl-2-pyrrolidone (NMP, peptide synthesis grade) were purchased

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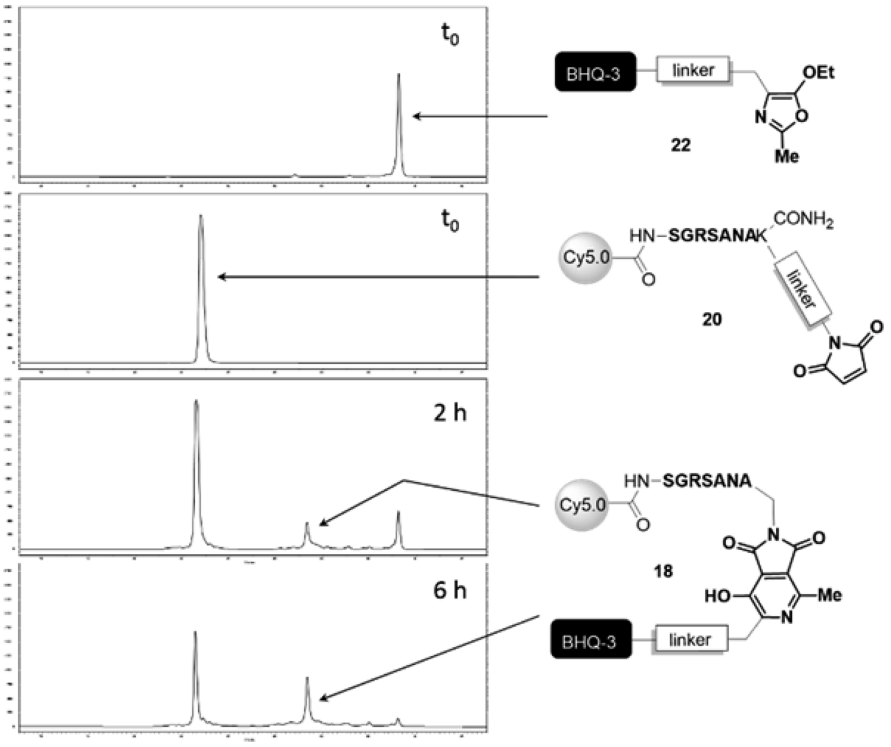

Figure 3. Monitoring of the Kondrat'eva ligation applied to the preparation of the FRET-based uPA-sensitive probe **18**. RP-HPLC using visible detection at 645 nm at t_0 and after 2 and 6 h.

from Iris Biotech GmbH. PBS (10 mM phosphate, 15 mM NaCl, pH 7.5), aq 0.1 and 1.0 M sodium acetate buffer, and aq mobile phases for HPLC were prepared using water purified with a Milli-Q system (purified to 18.2 MΩ cm). Protein samples were concentrated using a Millipore Spin-X UF ultracentrifugal filter device with a 10 kDa cutoff and a volume of 500 μL.

Instruments and Methods. The synthesis of peptide H-Ser-Gly-Arg-Ser-Ala-Asn-Ala-Lys(Dde)-NH_2 was carried out on an Applied Biosystems 433A synthesizer using standard Fmoc/tBu chemistry.[49] IR spectra were recorded with a universal ATR sampling accessory. ^{1}H and ^{13}C NMR spectra (C13APT or C13CPD experiments) were recorded on either a 200 or 300 MHz spectrometer. Chemical shifts are expressed in parts per million (ppm) from the residual nondeuterated solvant signal: $CDCl_3$ (δ_H = 7.26, δ_C = 77.16), DMSO-d_6 (δ_H = 2.50, δ_C = 39.52), MeOH-d_4 (δ_H = 3.31, δ_C = 49.00), CD_3CN (δ_H = 1.94, δ_C = 1.32 and 118.26). Multiplicities are described as s (singlet), d (doublet), dd, ddd, and so forth (doublet of doublets, doublet of doublets of doublets, etc.), t (triplet), q (quadruplet), quin (quintuplet), sex (sextuplet), sep (septuplet), dt (doublet of triplets), td (triplet of doublets), m (multiplet), and b (broad). Coupling constants (J values) are reported in hertz. High-resolution mass spectra (HRMS) were obtained using an orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer equipped with an electrospray source in positive and negative modes (ESI±). Low-resolution mass spectra (LRMS) were obtained with an ion trap mass spectrometer equipped with an ESI source. Analytical HPLC was performed on an instrument equipped with a PDA detector. Semipreparative HPLC was performed on a chromatography system equipped with a UV−visible detector. UV−visible spectra were obtained using either a rectangular quartz cell (open top, 10 × 10 mm, 3.5 mL) or a quartz micro cell (light path 10 mm, 500 μL). Fluorescence spectroscopic studies (emission/excitation spectra) were performed with either a semimicro quartz fluorescence cell (base thickness 10 × 4 mm, 1400 μL) or an ultramicro quartz fluorescence cell (light path 3 × 3 mm, 45 μL) for uPA assays. Emission spectra were recorded under the same conditions after excitation at the corresponding wavelength (excitation and emission filters: auto, excitation, and emission slit = 5 nm). Fluorescence quantum yields of R6G in denaturing medium (H_2O, 2.5% SDS) and R6G-BSA conjugates (in PBS or H_2O plus 2.5% SDS) were measured at 25 °C by a relative method using R6G (Φ_F = 94% in EtOH)[40] as a standard. The following equation was used to determine the relative fluorescence quantum yield:

$$\Phi_F(x) = (A_s/A_x)(F_x/F_s)(n_x/n_s)^2\Phi_F(s)$$

where A is the absorbance (in the range of 0.01−0.1 AU), F is the area under the emission curve, n is the refractive index of the solvents (at 25 °C) used in measurements, and the subscripts s and x represent standard and unknown, respectively. The following refractive index values were used: 1.333 for water, 1.337 for PBS, and 1.337 for water plus 2.5% SDS.

High-Performance Liquid Chromatography Separations. *Several Chromatographic Systems Were Used for the Analytical Experiments and Purification Steps.* System A: analytical RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 2.1 × 100 mm) was performed with CH_3CN and 0.1% aq trifluoroacetic acid (0.1% aq TFA, pH 2.2) functioning as eluents. No CH_3CN was included for the first 2.5 min and then was followed by a linear gradient from 0 to 80% CH_3CN over 35 min at a flow rate of 0.25 mL/min. Triple UV−vis detection was achieved at 230, 254, and 666 nm with the "Max Plot" (i.e., chromatogram at absorbance maximum for each compound) mode (220−700 nm). System B: semipreparative RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 21.2 × 250 mm) was performed with CH_3CN and 0.1% aq TFA functioning as eluents. No CH_3CN was included for the first 10 min and was then followed by a linear gradient from 0 to 100% CH_3CN over 100 min at a flow rate of 15.0 mL/min. UV detection was achieved at 250 nm. System C: semipreparative RP-HPLC (Varian Kromasil C_{18} column, 10 μm, 21.2 × 250 mm) was performed with CH_3CN and 0.1% aq TFA functioning as eluents. No CH_3CN was included for the first 5 min and was then followed by a linear gradient from 0 to 100% CH_3CN over 100 min at a flow rate of 20.0 mL/min. Dual visible detection was achieved at 600 and 645 nm. System D: semipreparative RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 10.0 × 250 mm) was performed with CH_3CN and 0.1% aq TFA functioning as eluents. No CH_3CN was included for the first 5 min and was then followed by a

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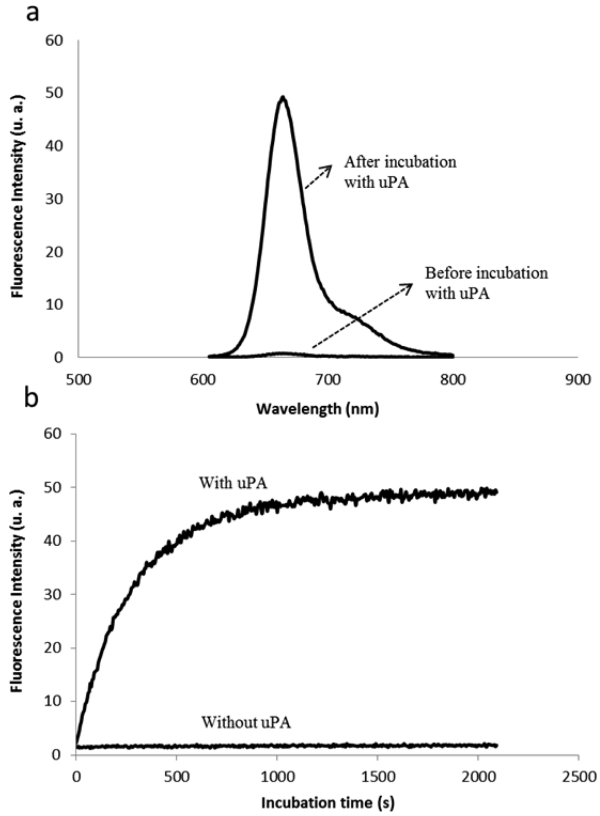

Figure 4. (a) Fluorescence emission spectrum ("Scan" mode, λ_{ex} = 595 nm) of probe **18** before and after incubation with uPA. (b) Fluorescence emission time course ("Kinetics" mode, λ_{ex} = 595 nm, λ_{em} = 665 nm) of probe **18** with 0.6 units of uPA from human urine and without, incubated for 25 min in PBS at 37.5 °C (probe concentration = 1.0 μM).

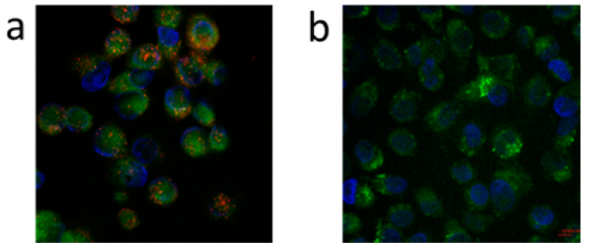

Figure 5. Confocal microscopy of treated human prostate cancer cell line PC3 stained with LysoTracker (green) and DAPI (blue) for labeling lysosomes and cell nuclei, respectively. (a) PC3 cells with L-amino acid-containing uPA-sensitive FRET probe **18**. (b) PC3 cells with D-amino acid-containing uPA-sensitive FRET probe **18-D**.

linear gradient from 0 to 100% CH_3CN over 100 min at a flow rate of 4.0 mL/min. Dual visible detection was achieved at 600 and 700 nm.

Maleimides **2a** and **2b**, sulfo-SMCC, and diethyl acetamidomalonate **5** are commercially available and were used without further purification. 4-Benzyl-5-ethoxy-2-methyloxazole **1**,[30] maleimides **2c**[50] and **2f**,[51] 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate,[52] and NHS ester of maleimide **2c**[53] were prepared according to procedures described in the literature. uPA from human urine (500 units/mg of protein, lyophilized form) was provided by Sigma-Aldrich (ref U0633).

Diethyl Maleate ***6***. To a solution of diethyl acetamidomalonate **5** (3.18 g, 14.6 mmol, 1 equiv) in dry DMF (20 mL) at 0 °C was added NaH (60% dispersion in mineral oil, 643 mg, 16.1 mmol, 1.1 equiv). Then, the reaction mixture was allowed to warm to rt and stirred for 1 h. The solution was then cooled to 0 °C, and a solution of methyl 6-iodohexanoate[54] (4.87 g, 19.0 mmol, 1.3 equiv) in dry DMF (2 mL) was added dropwise. The reaction mixture was then stirred at rt for 6 h. Next, the reaction mixture was diluted with deionized water (80 mL) and extracted with EtOAc (3 × 80 mL), and the combined organic layers were washed with brine (50 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. Finally, the crude product was purified by chromatography over silica gel (EtOAc/cyclohexane 1:1, v/v) to give desired product **6** as a white solid (14.1 mmol, 4.87 g, 97% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.75 (s, 1H), 4.24 (q, J = 7.2 Hz, 4H), 3.66 (s, 3H), 2.34−2.24 (m, 4H), 2.03 (s, 3H), 1.63−1.57 (m, 2H), 1.37−1.23 (m, 2H), 1.25 (t, J = 7.1 Hz, 6H), 1.14−1.09 (m, 2H). This ^{1}H NMR analysis was in accordance with literature data.[55]

α-Amido Ester ***7***. To a solution of compound **6** (4.87 g, 14.1 mmol, 1 equiv) in DMSO/H_2O (27 mL, 25:2, v/v) was added LiCl (897 mg, 21.2 mmol, 1.5 equiv). The resulting reaction mixture was then stirred at 150 °C for 16 h. Thereafter, the solution was cooled to rt and diluted with deionized water (60 mL), and the aqueous layer was extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (40 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The crude residue was purified by chromatography over silica gel (EtOAc/cyclohexane 1:1, v/v) to give desired product **7** as a yellow oil (10.3 mmol, 2.82 g, 73% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.98 (d, J = 7.8 Hz, 1H), 4.58 (td, J = 7.2, 5.4 Hz, 1H), 4.20 (q, J = 7.2 Hz, 2H), 3.66 (s, 3H), 2.30 (t, J = 7.5 Hz, 2H), 2.02 (s, 3H), 1.70−1.52 (m, 2H), 1.40−1.23 (m, 4H), 1.28 (t, J = 7.2 Hz, 3H). This ^{1}H NMR analysis was in accordance with literature data.[56]

5-Ethoxyoxazole ***8***. To a solution of compound **7** (2.82 g, 10.3 mmol, 1 equiv) in dry toluene (25 mL) under an inert atmosphere was added $POCl_3$ (1.29 mL, 13.4 mmol, 1.3 equiv). The resulting reaction mixture was then stirred at 80 °C, and consumption of the starting material was monitored by TLC analysis. The reaction was then quenched by adding a solution of saturated aq $NaHCO_3$ (50 mL), and the resulting mixture was extracted with EtOAc (2 × 80 mL). The combined organic layers were washed with brine (50 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. Finally, the crude product was purified by chromatography over silica gel (100% EtOAc) to give desired product **8** as a colorless oil (9.3 mmol, 2.38 g, 90% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.09 (q, J = 7.1 Hz, 2H), 3.66 (s, 3H), 2.36−2.28 (m, 4H), 2.31 (s, 3H), 1.70−1.54 (m, 4H), 1.37−1.32 (m, 2H), 1.34 (t, J = 7.1 Hz, 3H). This ^{1}H NMR analysis was in accordance with literature data.[27g]

6-(5-Ethoxy-2-methyloxazol-4-yl)hexanoic Acid ***9***. To a solution of compound **8** (2.38 g, 9.3 mmol) dissolved in a mixture of THF/water (99 mL, 7:1, v/v) under an inert atmosphere was added LiOH (46 mg, 18.6 mmol, 2 equiv). The resulting reaction mixture was heated at 70 °C for 3 h. Consumption of the starting material was monitored by TLC analysis. Thereafter, the mixture was allowed to cool at rt, and glacial AcOH was added (2.13 mL, 37.2 mmol, 4 equiv). The reaction mixture was further stirred for 1 h under an inert atmosphere. The reaction was quenched with a solution of saturated aq $NaHCO_3$ (50 mL), and the resulting mixture was extracted with EtOAc (2 × 100 mL). The combined organic layers were washed with brine (10 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. Finally, the resulting crude product was purified by chromatography on silica gel (100% EtOAc) and coevaporated several times with toluene to remove excess AcOH and give desired product **9** as a pale yellow oil (1.80 g, 80% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.10 (q, J = 7.1 Hz, 2H), 2.35 (m, 4H), 2.33 (s, 3H), 2.35−2.24 (m, 4H), 2.28 (s, 3H), 1.73−1.55 (m, 4H), 1.44−1.25 (m, 2H), 1.35 (t, J = 7.2 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 178.0, 153.4, 152.9, 116.3, 70.4, 34.1, 28.6, 28.1, 24.5, 23.9, 14.9, 13.9. IR (neat) ν_{max}: 2925, 2858, 2563, 1723, 166, 1582, 1381, 1266, 1199, 1085, 1018 cm^{-1}. HRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{12}H_{20}NO_4$, 242.1392; found, 242.1395.

NHS Ester ***10***. To a solution of carboxylic acid **9** (1.80 g, 7.5 mmol) dissolved in dry DMF (30 mL) under an inert atmosphere were sequentially added *N*-hydroxysuccinimide (946 mg, 8.2 mmol, 1.1 equiv) and dicyclohexylcarbodiimide (DCC, 2.0 g, 9.8 mmol, 1.3 equiv). The resulting reaction mixture was stirred at rt for 14 h. The

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reaction was quenched by adding deionized water (100 mL), and the resulting mixture was extracted with EtOAc (2 × 150 mL). The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. Finally, the resulting crude product was purified by chromatography on silica gel (EtOAc/cyclohexane 1:1, v/v) to give desired product **10** as a colorless oil (1.7 g, 67% yield). R_f (EtOAc/cyclohexane 1:1, v:v) = 0.2. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.10 (q, J = 7.1 Hz, 2H), 2.83 (s, 4H), 2.60 (t, J = 7.5 Hz, 2H), 2.36 (t, J = 7.5 Hz, 2H), 2.31 (s, 3H), 2.60 (quin, J = 7.6 Hz, 2H), 1.65−1.54 (m, 2H), 1.47−1.40 (m, 2H), 1.35 (t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.1, 168.4, 153.3, 152.1, 116.2, 70.2, 30.6, 28.1, 27.8, 25.4, 24.1, 24.0, 14.8, 14.0. IR (neat) ν_{max}: 2932, 2866, 1823, 1787, 1735, 1668, 1585, 1375, 1268, 1202, 1065, 1021 cm^{-1}. HRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{16}H_{23}N_2O_6$, 339.1556; found, 339.1562.

N-(3-Azidopropyl)-6-(5-ethoxy-2-methyloxazol-4-yl)hexanamide ***11***. To a solution of carboxylic acid **9** (100 mg, 0.4 mmol) in dry CH_2Cl_2 (10 mL) under an inert atmosphere were sequentially added DCC (101 mg, 0.5 mmol, 1.2 equiv) and 3-azidopropylamine (45 mg, 0.45 mmol, 1.1 equiv). The resulting reaction mixture was then stirred at rt for 12 h. Thereafter, the formed dicyclohexyl urea (DCU) precipitate was removed by filtration. The solid was washed with CH_2Cl_2 (3 mL), and filtrates were concentrated under reduced pressure. Finally, the resulting crude product was purified by chromatography on silica gel (EtOAc/petroleum ether 1:1, then 100% EtOAc, then EtOAc/NH_4OH, aq 28−30%, 95:5) to give **11** as a colorless oil (82 mg, 61% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.60 (s, 1H), 4.10 (q, J = 7.1 Hz, 2H), 3.39−3.30 (m, 2H), 2.34 (t, J = 7.5 Hz, 2H), 2.31 (s, 3H), 2.17 (t, J = 7.5 Hz, 2H), 1.83−1.74 (m, 1H), 1.79 (t, J = 6.6 Hz, 2H), 1.66−1.55 (m, 5H), 1.40−1.32 (m, 2H), 1.34 (t, J = 7.2 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.4, 153.4, 152.3, 116.6, 70.4, 49.3, 36.9, 36.5, 28.8, 28.7, 28.2, 25.4, 24.3, 15.0, 14.2. IR (neat) ν_{max}: 3287, 3078, 2933, 2854, 2094, 1645, 1544, 1440, 1377, 1267, 1090, 1021 cm^{-1}. HRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{15}H_{26}N_5O_3$, 324.2036; found, 324.2026.

Compound ***15***. To a solution of NHS ester **10** (51 mg, 0.15 mmol, 1.6 equiv) dissolved in a mixture of THF/deionized water (2.5 mL, 1.5:1 v/v) were added $NaHCO_3$ (10 mg, 0.11 mmol, 1.2 equiv) and rhodamine 6G-piperazine[57] (50 mg, 0.09 mmol, 1 equiv). The resulting reaction mixture was stirred for 12 h at rt. Thereafter, the solution was concentrated under reduced pressure. Finally, the crude product was purified by chromatography over silica gel (CH_2Cl_2/MeOH 98:2 to 93:7) to give desired product **15** as a red solid (0.04 mmol, 35 mg, 46% yield). Mp: 186−188 °C. IR (neat) ν_{max}: 3241, 2924, 2854, 1604, 1527, 1498, 1427, 1365, 1303, 1241, 1182, 1005 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.81−7.78 (m, 2H), 7.73−7.70 (m, 1H), 7.52−7.49 (m, 1H), 7.05 (s, 2H), 6.94 (s, 2H), 4.12 (q, J = 6.9 Hz, 2H), 3.55 (q, J = 7.2 Hz, 4H), 3.43−3.31 (m, 8H), 2.36−2.30 (m, 2H), 2.30 (s, 3H), 2.19 (s, 6H), 1.63−1.54 (m, 4H), 1.38 (t, J = 7.2 Hz, 6H), 1.32 (t, J = 6.9 Hz, 3H), 1.40−1.29 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.1, 169.6, 158.8, 157.7, 156.1, 155.1, 154.5, 136.4, 132.5, 131.6, 131.3, 131.2, 130.7, 128.9, 127.3, 126.7, 126.6, 118.6, 117.2, 114.8, 111.7, 94.9, 71.6, 39.4, 33.7, 29.7, 29.2, 26.0, 24.9, 17.5, 15.3, 14.0, 13.8. HRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{42}H_{52}N_5O_5$, 706.3968; found, 706.3973. UV−vis (PBS): λ_{max} = 533 nm, fluorescence (PBS) λ_{em} = 557 nm (Φ_F = 52%).

Compound ***22***. The amino derivative of BHQ-3 **21**[58] (20 mg, 28.5 μmol, 1 equiv), carboxylic acid **9** (6.9 mg, 28.5 μmol, 1 equiv), and BOP reagent (12.6 mg, 28.5 μmol, 1 equiv) were dissolved in freshly distilled CH_3CN (200 μL). The mixture was stirred under an argon atmosphere at 0 °C. Then, DIEA (15 μL, 85.5 μmol, 3 equiv) was added slowly, and the resulting mixture was stirred under an argon atmosphere at rt for 2 h. The reaction was checked for completion by RP-HPLC (system A). After the reaction was complete, the mixture was diluted with 50 mL of CH_2Cl_2 and sequentially washed twice with aq 10% citric acid (2 × 50 mL) and saturated aq $NaHCO_3$ (2 × 50 mL). The organic phase was then dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. The resulting blue solid was dissolved in a minimum volume of CH_2Cl_2 (about 200 μL), and 2 mL of Et_2O was added. The resulting precipitate was isolated by centrifugation and dried under vacuum to afford compound **22** as a dark-blue amorphous powder with a satisfying degree of purity (22 mg, 84% yield). IR (neat) ν_{max}: 3273, 2926, 1669, 1630, 1593, 1514, 1352, 1333, 1177, 1136, 1096, 795, 699 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.05 (bs, 3H), 1.32 (m, 8H), 1.56 (m, 4H), 2.14 (t, J = 7.3 Hz, 2H), 2.28 (bs, 5H), 2.85 (s, 3H), 3.36 (bs, 2H), 3.70 (bs, 2H), 3.87 (bs, 2H), 4.07 (q, J = 7.1 Hz, 2H), 4.42 (d, J = 4.9 Hz, 2H), 4.53 (bs, 2H), 5.73 (s, 1H), 6.31 (bs, 1H), 6.52 (d, J = 8.4 Hz, 2H), 7.17 (bs, 1H), 7.47 (m, 2H), 7.60 (m, 4H), 7.8−8.0 (m, 5H), 8.08 (d, J = 8.7 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.4, 156.3, 155.5, 153.6, 152.6, 152.4, 145.5, 144.1, 143.7, 139.1, 137.5, 135.4, 135.0, 133.2, 132.7, 132.2, 132.1, 127.4, 126.8, 123.7, 123.4, 120.2, 116.8, 112.2, 111.7, 92.3, 70.6, 52.5, 47.5, 47.3, 38.9, 36.3, 35.1, 29.0, 28.4, 25.4, 24.5, 15.4, 14.4, 13.8, 11.5. HRMS/ESI (positive mode) m/z: $[M]^{+\bullet}$ calcd for $C_{46}H_{54}N_{11}O_3$, 808.4406; found, 808.4443. UV−vis (CH_3CN/H_2O 1:1): λ_{max} = 642 nm. HPLC (system A): t_R = 23.6 min (94.2% purity).

Maleimide ***2d***. To a solution of 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylic acid (450 mg, 1.90 mmol, 1 equiv) in dry THF (15 mL) under an inert atmosphere at 0 °C were added, dropwise and successively, *N*-methylmorpholine (NMM, 0.23 mL, 2.1 mmol, 1.1 equiv) and isobutyl chloroformate (0.25 mL, 1.9 mmol, 1 equiv). The resulting reaction mixture was then stirred at 0 °C for 1 h. Then, the HCl salt of H-L-Tyr-OEt (560 mg, 2.28 mmol, 1.2 equiv) and NMM (0.25 mL, 2.28 mmol, 1.2 equiv) were added. The resulting mixture was stirred at 0 °C for 1 h and then at rt for 12 h. The reaction mixture was quenched by adding a solution of saturated aq $NaHCO_3$ (20 mL) and extracted with EtOAc (2 × 25 mL). The combined organic layers were washed with brine (10 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. Finally, the crude product was purified by chromatography over silica gel (EtOAc/petroleum ether 1:1, v/v) to give **2d** as a white solid (1.37 mmol, 586 mg, 72% yield). Mp: 70−72 °C. IR (neat) ν_{max}: 3319, 2931, 1702, 1643, 1514, 1441, 1407, 1210, 1109, 1024 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.94 (d, J = 8.7 Hz, 2H), 6.73 (d, J = 8.4 Hz, 2H), 6.69 (s, 2H), 5.86 (d, J = 7.5 Hz, 1H), 4.80 (dt, J = 7.8, 5.7 Hz, 1H), 4.18 (q, J = 7.1 Hz, 2H), 3.36 (d, J = 6.6 Hz, 2H), 3.12−2.96 (m, 2H), 2.02 (dt, J = 12.3, 3.6 Hz, 1H), 1.91−1.81 (m, 2H), 1.75−1.70 (m, 3H), 1.45−1.31 (m, 2H), 1.26 (t, J = 7.1 Hz, 3H), 1.04−0.90 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 175.8, 171.8, 171.1, 155.7, 133.9, 130.2, 126.7, 115.4, 61.5, 53.1, 44.7, 43.5, 37.0, 36.2, 29.7, 29.6, 28.6, 28.4, 14.0. HRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{23}H_{29}N_2O_6$, 429.2026; found, 429.2031.

Maleimide ***2e***. Step 1: To a solution of **2c** (528 mg, 2.50 mmol, 1 equiv) in dry THF (25 mL) under an inert atmosphere at 0 °C were added, dropwise and successively, NMM (0.27 mL, 2.5 mmol, 1 equiv) and isobutyl chloroformate (0.33 mL, 2.5 mmol, 1 equiv). The mixture was then stirred at 0 °C for 1 h. Then, 1-aminopropan-2-ol (0.20 mL, 2.5 mmol, 1 equiv) was added, and the resulting reaction mixture was stirred at 0 °C for 1 h and then at rt for 12 h. The reaction was quenched by adding a solution of saturated aq $NaHCO_3$ (20 mL) and extracted with EtOAc (2 × 25 mL). The combined organic layers were washed with brine (10 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. Finally, the crude product was purified by chromatography over silica gel (CH_2Cl_2/MeOH 99:1, then 98:2) to give the desired product as a white solid (1.37 mmol, 367 mg, 55% yield). Mp: 84−86 °C. IR (neat) ν_{max}: 3336, 3106, 2931, 2853, 1695, 1643, 1559, 1406, 1325, 1136, 1058 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.68 (s, 2H), 6.03 (s, 1H), 3.94−3.84 (m, 1H), 3.50 (t, J = 7.1 Hz, 2H), 3.46−3.39 (m, 1H), 3.12−3.03 (m, 1H), 2.88 (s, 1H), 2.19 (t, J = 7.1 Hz, 2H), 1.71−1.54 (m, 4H), 1.35−1.25 (m, 2H), 1.17 (d, J = 6.3 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.9, 170.8, 134.0, 66.9, 46.9, 37.4, 36.1, 28.1, 26.1, 25.0, 20.7. HRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{13}H_{21}N_2O_4$, 269.1501; found, 269.1491.

Step 2: To a solution of the secondary alcohol prepared above (200 mg, 0.75 mmol, 1 equiv) in dry CH_2Cl_2 (15 mL) were added (diacetoxyiodo)benzene (267 mg, 0.83 mmol, 1.1 equiv) and (2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl (TEMPO, 23 mg, 0.15 mmol, 0.2 equiv). The resulting reaction mixture was then stirred at rt for 4 h and

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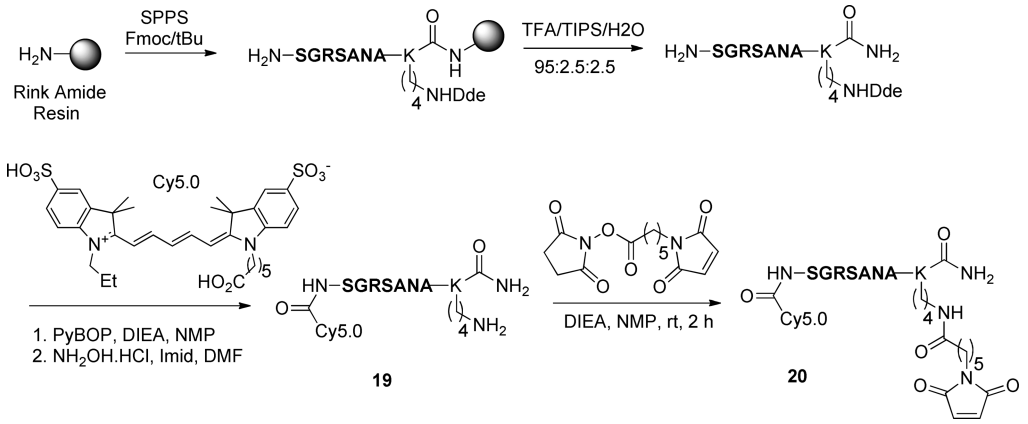
Scheme 7. Preparation of Maleimide Derivative 20



monitored by TLC to check for complete consumption of the starting material. Thereafter, the reaction mixture was quenched by adding saturated aq $NaHCO_3$ (10 mL), extracted with CH_2Cl_2 (2 × 20 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. Finally, the crude product was purified by chromatography over silica gel (CH_2Cl_2/MeOH 99:1, v/v) to give ketone-containing maleimide **2e** as a white solid (145 mg, 0.54 mmol, 73% yield). Mp: 98−100 °C. IR (neat) ν_{max}: 3303, 2943, 1730, 1700, 1643, 1549, 1413, 1371, 1371, 1180, 1120 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 6.68 (s, 2H), 6.15 (s, 1H), 4.16 (d, J = 4.5 Hz, 2H), 3.51 (t, J = 7.2 Hz, 2H), 2.23 (t, J = 7.5 Hz, 2H), 2.21 (s, 3H), 1.72−1.55 (m, 4H), 1.37−1.26 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 203.1, 172.7, 170.7, 133.9, 49.6, 37.4, 35.7, 28.0, 27.1, 26.1, 24.8. HRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{13}H_{19}N_2O_4$, 267.1345; found, 267.1334.

Maleimide ***14***. Step 1: To a solution of α-tocopherol (420 mg, 0.98 mmol, 1 equiv) in dry DMF (5 mL) under an inert atmosphere were successively added K_2CO_3 (677 mg, 4.90 mmol, 5 equiv) and 6-azidohexyl 4-methylbenzenesulfonate (874 mg, 2.94 mmol, 3 equiv). The resulting reaction mixture was heated at 50 °C for 24 h. The solution was diluted with EtOAc (40 mL), washed successively with deionized water (30 mL) and brine (20 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. Finally, the crude product was purified by chromatography over silica gel (100% cyclohexane) to give a colorless oil of an unresolved mixture of the desired product α-tocopherol-azide and the starting α-tocopherol (461 mg, 1:2 molar ratio). Step 2: to a solution of the previous mixture in CH_2Cl_2/EtOH/deionized H_2O (3 mL, 1:1:1, v/v) under an inert atmosphere at rt were successively added **2f** (197 mg, 0.80 mmol, 2.4 equiv relative to α-tocopherol-azide) and sodium ascorbate (57 mg, 0.29 mmol, 0.9 equiv relative to α-tocopherol-azide). Argon gas was bubbled through the solution for 5 min, and finally, $CuSO_4{\cdot}5H_2O$ (24 mg, 0.15 mmol, 0.45 equiv relative to α-tocopherol-azide) was added. Then, the reaction mixture was stirred at rt with light protection for 12 h. The reaction was quenched by adding deionized water (5 mL), and the aq layer was extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layers were washed with brine (10 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The crude product was purified by chromatography over silica gel (CH_2Cl_2/EtOAc 7:3, then 1:1, v/v) to afford product **14** as a white solid (0.28 mmol, 219 mg, 29% overall yield over the two steps). Mp: 78−80 °C. IR (neat) ν_{max}: 3312, 2927, 2861, 1700, 1645, 1552, 1461, 1417, 1375, 1254, 1210, 1132, 1090, 1053 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 7.52 (s, 1H), 6.67 (s, 2H), 6.17 (b, 1H), 4.49 (d, J = 5.7 Hz, 2H), 4.35 (t, J = 7.2 Hz, 2H), 3.61 (t, J = 6.2 Hz, 2H), 3.50 (t, J = 7.2 Hz, 2H), 2.56 (t, J = 6.5 Hz, 2H), 2.17 (t, J = 7.5 Hz, 2H), 2.14 (s, 3H), 2.10 (s, 3H), 2.07 (s, 3H), 1.95 (quin, J = 7.2 Hz, 2H), 1.86−1.71 (m, 4H), 1.68−1.01 (m, 34H), 0.87−0.82 (m, 12H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.9, 170.7, 148.1, 147.6, 144.9, 133.9, 127.6, 125.6, 122.7, 122.3, 117.4, 74.6, 72.5, 50.2, 40.0, 39.3, 37.5, 37.4, 37.4, 37.3, 37.2, 36.0, 34.7, 32.7, 32.6, 31.2, 30.2, 30.0, 28.2, 27.9, 26.4, 26.2, 25.7, 24.9, 24.7, 24.3, 23.8, 22.7, 22.6, 20.9, 20.6, 19.7, 19.6, 12.7, 11.8, 11.7. HRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{48}H_{78}N_5O_5$, 804.6003; found, 804.6003.

Preparation of Fluorescently Labeled Maleimide-Peptide ***20***. Step 1: solid-phase synthesis (SPS) of the uPA peptidyl substrate. SPS of peptide H-Ser-Gly-Arg-Ser-Ala-Asn-Ala-Lys(Dde)-NH_2 with L-amino acids was performed using standard Fmoc/tBu chemistry and Rink Amide MBHA resin (Novabiochem, 100−200 mesh, 0.50 or 0.59 mmol/g loading) on a scale of 0.25 mmol. Coupling reactions were performed with commercial Fmoc-protected L-amino acids from Iris Biotech or Novabiochem (4 equiv) and *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU, 4 equiv), HOBt (4 equiv), and DIEA (12 equiv) in peptide-grade DMF.

Step 2: cleavage of the uPA peptidyl substrate from resin. A precooled TFA solution (~6 mL for 350 mg of resin) containing TIPS (2.5% v/v) and deionized water (2.5% v/v) was added to the resin. After being stirred at rt for 3 h, the resin was filtered and washed with TFA. The filtrate was evaporated to dryness, and cold Et_2O was added to the oily residue. The resulting white precipitate was isolated by centrifugation, lyophilized, and purified by semipreparative RP-HPLC (system B, 3 injections). The product-containing fractions were lyophilized to give a TFA salt of the uPA peptidyl substrate as a white amorphous powder (127 mg, 53% yield). HRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{40}H_{69}N_{14}O_{13}$, 953.5190; found, 953.5192. HPLC (system A): t_R = 19.3 min (96.3% purity).

Step 3: N-terminal peptide labeling with sulfoindocyanine dye Cy5.0 and Dde removal. The TFA salt of the peptide (5 mg, 5.25 μmol, 1 equiv), Cy5.0 dye[59] (3.8 mg, 5.75 μmol, 1.1 equiv), and PyBOP reagent (3.0 mg, 5.75 μmol, 1.1 equiv) were dissolved in NMP (70 μL). DIEA (2.0 M solution in NMP, 4.5 μL, 26 μmol, 5 equiv) was added, and the resulting reaction mixture was stirred at rt for 2 h. The reaction was checked for completion by RP-HPLC (system C). After the reaction was completed, Et_2O was added, and the resulting precipitate was centrifugated and then dissolved in DMF (500 μL). Hydroxylamine hydrochloride (1.7 mg, 26 μmol, 5 equiv) and imidazole (1.8 mg, 26 μmol, 5 equiv) were added, and the reaction mixture was stirred at rt for another 2 h. The reaction was checked for completion by RP-HPLC (system A). After the reaction was completed, the mixture was diluted with aq 0.1% TFA and purified by semipreparative RP-HPLC (system C). The product-containing fractions were lyophilized to give 4.2 mg (56% overall yield for the two steps) of the Cy5.0-labeled peptide **19** as a blue amorphous powder. HRMS/ESI (negative mode) m/z: $[M - H]^-$ calcd for $C_{63}H_{93}N_{16}O_{18}S_2$, 1425.6295; found, 1425.6284. UV−vis (PBS): λ_{max} = 649 nm, fluorescence (PBS) λ_{em} = 665 nm (Φ_F = 28%). HPLC (system A): t_R = 20.9 min (98.8% purity).

Step 4: functionalization of the Cy5.0-labeled peptides with a maleimide moiety. Cy5.0-labeled peptide **19** (3 mg, 2.1 μmol, 1 equiv) and NHS ester of maleimide **2c** (0.8 mg, 2.5 μmol, 1.2 equiv) were dissolved in NMP (50 μL). DIEA (2.0 M solution in NMP, 8.4 μL, 16.8 μmol, 8 equiv) was then added, and the resulting reaction mixture was stirred at rt for 2 h. The reaction was checked for completion by

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RP-HPLC (system C). After the reaction was completed, the mixture was diluted with aq 0.1% TFA and purified by semipreparative RP-HPLC (system C). The product-containing fractions were lyophilized to give 2.3 mg (67% yield) of maleimide-functionalized Cy5.0-labeled peptide **20** as a blue amorphous powder. HRMS/ESI (negative mode) m/z: $[M - H]^-$ calcd for $C_{73}H_{104}N_{17}O_{21}S_2$, 1618.7034; found, 1618.7024. UV−vis (PBS): λ_{max} = 649 nm, fluorescence (PBS) λ_{em} = 665 nm (Φ_F = 24%). HPLC (system A): t_R = 21.9 min (99.1% purity).

The Same Protocols Were Used for the Preparation of Peptide ***19-D*** *(Bearing D-Amino Acids).* Step 1: SPS of peptide H-Ser-Gly-Arg-Ser-Ala-Asn-Ala-Lys(Dde)-NH_2 with D-amino acids was also performed using standard Fmoc/tBu chemistry and Rink Amide MBHA resin (Novabiochem, 100−200 mesh, 0.50 or 0.59 mmol/g loading) on a scale of 0.1 mmol. Coupling reactions were performed with commercial Fmoc-protected D-amino acids from Iris Biotech (10 equiv) and HBTU (10 equiv), HOBt (10 equiv), and DIEA (30 equiv) in peptide-grade DMF.

Step 2: A precooled TFA solution (∼3 mL for 120 mg of resin) containing TIPS (2.5% v/v) and deionized water (2.5% v/v) was added to the resin. After being stirred at rt for 3 h, the resin was filtered and washed with TFA. The filtrate was evaporated to dryness, and cold Et_2O was added to the oily residue. The resulting white precipitate was isolated by centrifugation, lyophilized, and purified by semipreparative RP-HPLC (system B, 1 injection). The product-containing fractions were lyophilized to give the TFA salt of the uPA peptidyl substrate as a white amorphous powder (41 mg, 43% yield). LRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{40}H_{69}N_{14}O_{13}$, 953.52; found, 953.53. HPLC (system A): t_R = 19.1 min (99.1% purity).

Step 3: **19-D** was prepared from the TFA salt of the uPA peptidyl substrate (5 mg, 5.75 μmol, 1 equiv) according to the procedure described above for the synthesis of compound **19**; 3.5 mg (47% overall yield for the two steps) of Cy5.0-labeled peptide **19-D** as a blue amorphous powder was obtained. LRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{63}H_{95}N_{16}O_{18}S_2$, 1427.64; found, 1428.20. UV−vis (PBS): λ_{max} = 649 nm, fluorescence (PBS) λ_{em} = 664 nm (Φ_F = 26%). HPLC (system A): t_R = 21.2 min (96.1% purity).

Step 4: **20-D** was prepared from Cy5.0-labeled peptide **19-D** (3 mg, 2.1 μmol, 1 equiv) according to the procedure described above for the synthesis of compound **20**; 2.6 mg (76% yield) of maleimide-functionalized Cy5.0-labeled peptide **20-D** as a blue amorphous powder was obtained. LRMS/ESI (negative mode) m/z: $[M - H]^-$ calcd for $C_{73}H_{104}N_{17}O_{21}S_2$, 1618.70; found, 1618.53. UV−vis (PBS): λ_{max} = 648 nm, fluorescence (PBS) λ_{em} = 665 nm (Φ_F = 23%). HPLC (system A): t_R = 22.7 min (98.9% purity).

Compound ***3****.* To a solution of **1** (17 mg, 0.08 mmol, 1 equiv) in $CDCl_3$ (0.54 mL) was added *N*-methylmaleimide (22 mg, 0.19 mmol, 2.5 equiv). The reaction was run at rt for 10 h in an NMR tube due to the poor stability of the corresponding bicyclic adduct. This intermediate is not stable on silica gel but can be aromatized within 15 min after the addition of TFA and can also undergo aromatization when heated at 40 °C while being concentrated under reduced pressure. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.32−7.21 (m, 5H), 3.94−3.84 (m, 1H), 3.74−3.64 (m, 3H), 3.51 (d, J = 7.8 Hz, 1H), 3.37 (d, J = 7.8 Hz, 1H), 2.61 (s, 3H), 1.95 (s, 3H), 1.32 (t, J = 7.2 Hz, 3H).

Kondrat'eva Reaction Conditions. *Condition A (pH ∼1) for the Preparation of 3-Hydroxypyridines* ***4a−f****.* To a solution of **1** (0.46 mmol) dissolved in a mixture of THF/water/TFA (3.88 mL, 170:100:1, v/v) was added corresponding maleimide **2** (0.60 mmol, 1.3 equiv). The resulting reaction mixture was stirred at rt for 5 h. Thereafter, the reaction mixture was quenched by adding a solution of saturated aq $NaHCO_3$ (5 mL), and the resulting aqueous mixture was extracted with EtOAc (2 × 30 mL). The combined organic layers were washed with brine (5 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. Finally, the crude product was purified according to reported procedures.

Hydroxypyridine ***4a****.* This compound was prepared according to the procedure described above from **1** (100 mg, 0.46 mmol) and *N*-methylmaleimide **2a** (67 mg, 0.60 mmol, 1.3 equiv) in a mixture of THF/water/TFA. The crude product was purified by chromatography on silica gel (100% CH_2Cl_2, then EtOAc/CH_2Cl_2 1:1, v/v) and triturated with Et_2O (2 × 1 mL) to give desired product **4a** as a pale yellow solid (0.29 mmol, 82 mg, 67% yield). Mp: 162−164 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.41−7.17 (m, 5H), 4.26 (s, 2H), 3.12 (s, 3H), 2.76 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.1, 168.0, 157.2, 147.5, 145.3, 137.7, 129.2, 128.5, 126.7, 120.7, 120.2, 39.0, 23.9, 20.2. IR (neat) ν_{max}: 2927, 1758, 1701, 1615, 1490, 1427, 1376, 1242, 1125, 1039, 1005 cm^{-1}. HRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{16}H_{15}N_2O_3$, 283.1083; found, 283.1080.

Hydroxypyridine ***4b****.* This compound was prepared according to the procedure described above from **1** (100 mg, 0.46 mmol) and *N*-phenylmaleimide **2b** (104 mg, 0.60 mmol, 1.3 equiv) in a mixture of THF/water/TFA. The crude product was purified by chromatography on silica gel (100% CH_2Cl_2) to give the desired product as a yellow solid (0.33 mmol, 115 mg, 72% yield). Mp: 64−66 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.53−7.48 (m, 2H), 7.44−7.36 (m, 5H), 7.31−7.21 (m, 3H), 4.31 (s, 2H), 2.82 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 168.0, 166.7, 157.5, 148.1, 145.7, 137.6, 131.0, 129.2, 129.2, 128.5, 128.4, 126.6, 126.4, 120.2, 119.5, 38.9, 20.3. IR (neat) ν_{max}: 2930, 1768, 1706, 1602, 1496, 1386, 1110 cm^{-1}. HRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{21}H_{17}N_2O_3$, 345.1239; found, 345.1242.

3-Hydroxypyridine ***4c****.* This compound was prepared according to the procedure described above from **1** (100 mg, 0.46 mmol) and 6-(*N*-maleimide)caproic acid **2c** (127 mg, 0.60 mmol, 1.3 equiv) in a mixture of THF/water/TFA. The crude product was purified by precipitation at −20 °C (first dissolved by heating, 3 mL, EtOAc/Et_2O 5:1) to give desired product **4c** as a white solid (0.19 mmol, 74 mg, 41% yield). Mp: 137−139 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.31−7.12 (m, 5H), 4.25 (s, 2H), 3.62 (t, J = 7.2 Hz, 2H), 2.71 (s, 3H), 2.28 (t, J = 7.4 Hz, 2H), 2.18 (s, 3H), 1.71−1.59 (m, 4H), 1.41−1.31 (m, 2H). ^{13}C NMR (75 MHz, MeOH-d_4): δ 177.4, 169.2, 168.4, 159.7, 146.9, 146.6, 139.5, 130.0, 129.3, 127.3, 122.7, 122.4, 39.4, 38.5, 34.6, 29.0, 27.3, 25.5, 19.6. IR (neat) ν_{max}: 3402, 1691, 1438, 1407, 1268, 1216, 1123, 1042 cm^{-1}. HRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{21}H_{23}N_2O_5$, 383.1607; found, 383.1605.

3-Hydroxypyridine ***4d****.* This compound was prepared according to the procedure described above from **1** (100 mg, 0.46 mmol) and maleimide **2d** (257 mg, 0.60 mmol, 1.3 equiv) in a mixture of THF/water/TFA. The crude product was purified by precipitation at −20 °C (first dissolved by heating, 3 mL, EtOAc/Et_2O 5:1) to give the desired product as a white solid (0.21 mmol, 128 mg, 46% yield). Mp: 241−243 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.35 (d, J = 6.9 Hz, 2H), 7.28−7.15 (m, 3H), 6.89 (d, J = 8.7 Hz, 2H), 6.70 (d, J = 8.4 Hz, 2H), 5.96 (d, J = 7.5 Hz, 1H), 4.75 (dt, J = 7.8, 5.7 Hz, 1H), 4.25 (s, 2H), 4.15 (q, J = 7.2 Hz, 2H), 3.45−3.41 (m, 2H), 3.07−2.93 (m, 2H), 2.74 (s, 3H), 2.05−1.95 (m, 2H), 1.88−1.65 (m, 5H), 1.41−1.28 (m, 2H), 1.24 (t, J = 7.2 Hz, 3H), 1.05−0.92 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$ plus one drop of [D_4]MeOH): δ 175.5, 172.0, 168.9, 168.2, 157.9, 155.8, 147.2, 145.3, 137.7, 130.3, 129.1, 128.5, 126.8, 126.6, 120.4, 120.1, 115.3, 61.6, 53.0, 44.8, 43.8, 38.9, 37.0, 36.3, 29.8, 29.8, 28.7, 28.4, 19.9, 14.1. IR (neat) ν_{max}: 3314, 2934, 1706, 1640, 1551, 1515, 1401, 1218, 1159, 1059 cm^{-1}. HRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{34}H_{38}N_3O_7$, 600.2710; found, 600.2703.

3-Hydroxypyridine ***4e****.* This compound was prepared according to the procedure described above from **1** (100 mg, 0.46 mmol) and 6-(*N*-maleimide) (*N*-2-oxopropyl)hexanamide **2e** (241 mg, 0.60 mmol, 1.3 equiv) in a mixture of THF/water/TFA. The crude product was purified by precipitation at −20 °C (first dissolved by heating, 3 mL, EtOAc/Et_2O 5:1) to give the desired product as a white solid (0.24 mmol, 105 mg, 52% yield). Mp: 149−151 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.36 (d, J = 6.9 Hz, 2H), 7.30−7.17 (m, 3H), 6.15 (s, 1H), 4.26 (s, 2H), 4.14 (d, J = 4.5 Hz, 2H), 3.61 (t, J = 7.2 Hz, 2H), 2.75 (s, 3H), 2.23 (t, J = 7.4 Hz, 2H), 2.18 (s, 3H), 1.73−1.60 (m, 4H), 1.40−1.28 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 203.0, 172.7, 169.1, 168.0, 157.1, 147.5, 145.3, 137.8, 129.2, 128.5, 126.6, 120.6, 120.1, 49.9, 39.0, 37.8, 36.1, 28.2, 27.4, 26.4, 25.0, 20.2. IR (neat) ν_{max}: 3310, 1697, 1645, 1549, 1406, 1370, 1207, 1172, 1124, 1050 cm^{-1}. HRMS/

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ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{24}H_{28}N_3O_5$, 438.2029; found, 438.2030.

3-Hydroxypyridine ***4f***. This compound was prepared according to the procedure described above from **1** (100 mg, 0.46 mmol) and 6-(*N*-maleimide)-1-(prop-2-yn-1-yl)hexanamide **2f** (149 mg, 0.60 mmol, 1.3 equiv) in a mixture of THF/water/TFA. The crude product was purified by precipitation at −20 °C (first dissolved by heating, 3 mL, EtOAc/Et_2O 5:1) to give the desired product as a white solid (0.25 mmol, 105 mg, 54% yield). Mp: 186−188 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.38−7.35 (m, 3H), 7.30−7.20 (m, 2H), 5.70 (s, 1H), 4.26 (s, 2H), 4.04 (dd, J = 5.4, 2.6 Hz, 2H), 3.62 (t, J = 7.2 Hz, 2H), 2.76 (s, 3H), 2.21−2.16 (m, 3H), 1.72−1.60 (m, 4H), 1.40−1.31 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$ plus one drop of MeOH-d_4): δ 172.8, 168.8, 168.1, 157.8, 147.2, 145.3, 137.8, 129.2, 128.5, 126.6, 120.5, 120.2, 79.6, 71.4, 38.9, 37.7, 35.9, 29.0, 28.0, 26.2, 24.9, 20.0. IR (neat) ν_{max}: 3287, 3241, 2944, 1762, 1712, 1628, 1543, 1439, 1404, 1370, 1218, 1051 cm^{-1}. HRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{24}H_{26}N_3O_4$, 420.1923; found, 420.1907.

Condition B (pH 5.0) for the Preparation of 3-Hydroxypyridines ***4a−f***. To a solution of **1** (0.07 mmol) in a mixture of DMSO/NaOAc buffer (1.895 mL, 1.0 M, pH 5.0, 21:79, v/v) was added the corresponding maleimide (0.09 mmol, 1.3 equiv). The resulting reaction mixture was left at 37 °C for 5 h in a heating chamber Thereafter, the reaction was quenched by adding saturated aq $NaHCO_3$ (2 mL) and extracted with CH_2Cl_2 (10 mL) and EtOAc (2 × 10 mL). The combined organic phases were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Finally, 1-phenyl-1-cyclohexene (10.92 mg, 0.07 mmol) was added to the sample as in internal standard peak for ^{1}H NMR analyses.

Tocopherol Conjugate ***16***. To a solution of oxazole-R6G **15** (35 mg, 0.043 mmol) in CH_2Cl_2 (0.38 mL) under an inert atmosphere at rt were sequentially added tocopherol-maleimide **14** (68 mg, 0.086 mmol, 2 equiv) and TFA (25 μL). This resulting reaction mixture was stirred at rt for 7 h. Thereafter, the reaction mixture was concentrated under reduced pressure. The crude product was purified by chromatography over silica gel (CH_2Cl_2/MeOH 100:0, then 98:2) to afford the product as a dark pink solid (0.027 mmol, 42 mg, 63% yield). Mp: 150−152 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.71−7.53 (m, 5H), 7.37−7.32 (m, 1H), 6.91−6.85 (m, 3H), 6.64 (s, 2H), 4.49 (s, 2H), 4.35−4.29 (m, 2H), 3.60 (t, J = 6.2 Hz, 4H), 3.51−3.23 (m, 15H), 2.96−2.88 (m, 2H), 2.71 (s, 3H), 2.55 (t, J = 7.1 Hz, 2H), 2.30−2.16 (m, 12H), 2.13 (s, 3H), 2.09 (s, 3H), 2.06 (s, 3H), 1.96−1.90 (m, 2H), 1.80−1.03 (m, 55H), 0.87−0.82 (m, 13H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 167.6, 157.2, 156.2, 148.2, 147.7, 134.9, 131.5, 130.4, 130.3, 130.2, 130.1, 130.0, 129.2, 127.8, 125.8, 122.8, 117.5, 113.8, 94.0, 74.8, 72.7, 50.5, 50.5, 40.2, 39.4, 38.8, 37.5, 37.3, 32.8, 32.7, 31.3, 30.2, 30.2, 29.7, 28.0, 26.6, 25.8, 24.8, 24.5, 23.9, 22.8, 22.7, 21.1, 20.7, 19.8, 19.7, 17.6, 13.7, 12.8, 11.9, 11.8. IR (neat) ν_{max}: 3296, 2926, 1697, 1641, 1605, 1528, 1499, 1440, 1303, 1239, 1180, 1128, 1082, 1009 cm^{-1}. LRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{88}H_{123}N_{10}O_9$, 1463.94; found, 1464.67.

Azaphthalimide-R6G. To a solution of **15** (30 mg, 0.04 mmol, 1 equiv) in a solution of DMSO/CH_2Cl_2/TFA (1.0 mL, 100:100:1, v/v) was added *N*-methylmaleimide **2a** (8 mg, 0.07 mmol, 2 equiv). The resulting reaction mixture was stirred at rt for 7 h. Thereafter, the crude mixture was then concentrated under reduced pressure and purified by chromatography over silica gel (CH_2Cl_2/MeOH 99:1 to 90:10, v/v). A trace amount of residual DMSO was removed by coevaporating the product with EtOAc (3 mL) to give product azaphthalimide-R6G as a dark pink solid (0.20 mmol, 17 mg, 54% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.79−7.77 (m, 2H), 7.72−7.69 (m, 1H), 7.53−7.49 (m, 1H), 7.02 (s, 1H), 6.85 (s, 1H), 3.50 (q, J = 6.9 Hz, 4H), 3.44−3.29 (m, 8H), 3.03 (s, 3H), 2.89−2.82 (m, 2H), 2.60 (s, 3H), 2.33 (t, J = 7.4 Hz, 2H), 1.69−1.52 (m, 4H), 1.39−1.26 (m, 2H), 1.35 (t, J = 7.2 Hz, 2H). HRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{45}H_{51}N_6O_6$, 771.3870; found, 771.3893. UV−vis (PBS): λ_{max} = 533 nm, fluorescence (PBS) λ_{em} = 558 nm (Φ_F = 33%).

R6G-BSA Conjugates ***17a/17b***. Step 1: To a solution of BSA protein (2.0 mg, 30 nmol, 1 equiv) in phosphate buffer (0.1 M, pH 7.4, 200 μL) was added sulfo-SMCC (1.31 mg, 3000 nmol, 100 equiv) predissolved in DMSO (6 μL). The reaction mixture was left at rt for 5 h. Thereafter, the reaction mixture was filtered directly on an ultracentrifugal filter device with a 10 kDa cutoff to remove excess, unconsumed cross-linker reagent; the phosphate buffer was exchanged with NaOAc buffer (pH 5.0, 0.1 M) through four washes of 500 μL. Step 2: To a solution of BSA maleimide (∼1 mg, 15 nmol) in NaOAc buffer (0.1 M, pH 5.0, 1 mL) was added oxazole-R6G **15** (1.33 mg, 1875 nmol, 125 equiv or 0.40 mg, 563 nmol, 37.5 equiv) predissolved in DMSO (10 μL). The resulting reaction mixture was then left at 37 °C for 12 h in a heating chamber. The reaction mixture was then filtered directly on an ultracentrifugal filter device (10 kDa cutoff, 500 μL) to remove excess oxazole-R6G **15**. The BSA was washed first with a mixture of PBS buffer/DMSO 9:1 (3 × 500 μL) and then with PBS buffer (4 × 500 μL). *F*/*P* molar ratios were estimated from the relative intensities of the labeled protein and azaphthalimide-R6G dye absorption at 539 nm (see SI).

3-Hyroxypyridine ***13*** *(through a Dual Conjugation via HDA and CuAAC Reactions)*. To a solution of *N*-(3-azidopropyl)-6-(5-ethoxy-2-methyloxazol-4-yl)hexanamide **11** (22.3 mg, 0.069 mmol) in a mixture of CH_3CN/NaOAc buffer (1.0 M, pH 5.0, 396:1500 μL) were sequentially added *N*-methylmaleimide **2a** (9.97 mg, 0.090 mmol, 1.3 equiv), $CuSO_4{\cdot}5H_2O$ (3.50 mg, 0.014 mmol, 0.2 equiv), sodium ascorbate (27.34 mg, 0.138 mmol, 2 equiv), and pent-1-yne **12** (10.3 μL, 0.104 mmol, 1.5 equiv). The resulting reaction mixture was left at 37 °C for 12 h in a heating chamber. Thereafter, the solution was diluted in EtOAc (30 mL) and washed with aq 10% EDTA (5 mL). The organic phases were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Finally, the crude product was purified by chromatography on silica gel (100% CH_2Cl_2, then CH_2Cl_2/MeOH 98:2, v/v) to give product **13** as a pale yellow solid (0.042 mmol, 19 mg, 60% yield). Mp: 128−130 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.35 (s, 1H), 5.83 (s, 1H), 4.38 (t, J = 6.0 Hz, 2H), 3.24 (q, J = 6.1 Hz, 2H), 3.14 (s, 3H), 2.92 (m, 2H), 2.74 (s, 3H), 2.69 (t, J = 7.8 Hz, 2H), 2.16 (t, J = 7.5 Hz, 2H), 2.08 (t, J = 6.3 Hz, 2H), 1.80−1.63 (m, 6H), 1.48−1.37 (m, 2H), 0.97 (t, J = 7.2 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.6, 169.1, 168.1, 159.3, 147.1, 145.3, 120.2, 119.7, 47.8, 36.5, 36.5, 32.4, 30.1, 29.0, 27.8, 27.7, 25.4, 23.9, 22.7, 20.2, 13.9 cm^{-1}. IR (neat) ν_{max}: 3315, 2941, 2871, 1702, 1638, 1517, 1424, 1379, 1245, 1149, 1120, 1024 cm^{-1}. HRMS/ESI (positive mode) m/z: $[M + H]^+$ calcd for $C_{23}H_{33}N_6O_4$, 457.2563; found, 457.2550.

General Procedure for the Synthesis of FRET Probes 18 and 18-D. FRET probe **18** was prepared according to the procedure described above using 5-alkoxyoxazole-based BHQ-3 **22** (0.62 mg, 0.76 μmol, 1 equiv) and fluorescently labeled maleimide-based peptide **20** (2.6 mg, 1.52 μmol, 2 equiv) in a mixture of THF/water/TFA (30 μL) with stirring for 6 h. The reaction was checked for completion by RP-HPLC (system A). Thereafter, the mixture was diluted with aq 0.1% TFA and purified by semipreparative RP-HPLC (system D). The product-containing fractions were lyophilized to give the probe **18** as a dark-blue amorphous powder with L-amino acids (1.2 mg, 63% yield). HRMS/ESI (negative mode) m/z: $[M - 2H]^{2-}$ calcd for $C_{117}H_{150}N_{28}O_{23}S_2/2$, 1189.5435; found, 1189.5426. HPLC (system A): t_R = 27.4 min (98.0% purity).

Probe ***18-D*** *with D-Amino Acids*. **18-D** (1.1 mg, 58% yield). LRMS/ESI (positive mode) m/z: $[M + 2H]^{2+}$ calcd for $C_{117}H_{154}N_{28}O_{23}S_2/2$, 1191.56; found, 1192.00. HPLC (system A): t_R = 27.3 min (99.6% purity).

General Procedure for in Vitro Peptide Cleavage by uPA. A 1.0 μM solution of the fluorogenic peptide was prepared in 45 μL of PBS and transferred into an ultramicro quartz fluorescence cell. Five microliters (0.6 units) of uPA solution (25 μg in 100 μL of buffer: 500 mM Tris-HCl, 1.0 M NaCl, 1% PEG 6000, and 2.0 M mannitol) was added, and the resulting mixture was incubated at 37.5 °C. After excitation at 595 nm, fluorescence emission at 665 nm was monitored over time with measurements recorded every 5 s. Emission spectra of the probe were recorded before and after cleavage to determine the quenching efficiency (QE). QE was calculated based on the equation 100 × [1 − (fluorescence emission intensity of the probe)/

(fluorescence emission intensity of the probe after complete digestion with uPA)].

Intracellular Fate of the Probes in Cells by Confocal Microscopy. PC-3 cells of prostate cancer origin were seeded at a concentration of 6×10^4 cells per well and allowed to attach over 2 days. Cells were co-incubated with 120 nM Lysotracker Green and 20 μM probes for 2 h. Then, the cells were washed with HBSS, and HBSS with 1% BSA, and were subsequently fixed using 4% paraformaldehyde solution. After fixation, cells were rinsed three times with HBSS and once with water. Lastly, they were mounted using Vectashield mounting medium containing DAPI. Thereafter, the cells were imaged by confocal fluorescence microscopy using a Zeiss Axiovert 710 microscope (Carl Zeiss, Jena, Germany).

■ ASSOCIATED CONTENT

Supporting Information

Copies of ^{1}H and ^{13}C NMR spectra for all compounds; absorption, excitation, and emission spectra for all rhodamine- and cyanine-labeled derivatives; HPLC chromatograms for compounds **18**, **18-D**, **19**, **19-D**, **20**, **20-D**, and **22**; and mass spectra for compounds **18**, **18-D**, **19**, **19-D**, **20**, and **20-D**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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