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# Beyond the Bioorthogonal Inverse-Electron-Demand Diels–Alder Reactions of Tetrazines: 2-Pyrone-Functionalized Fluorogenic Probes

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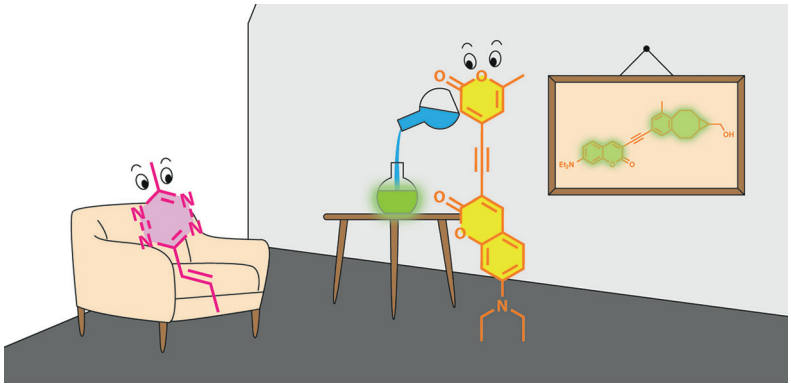

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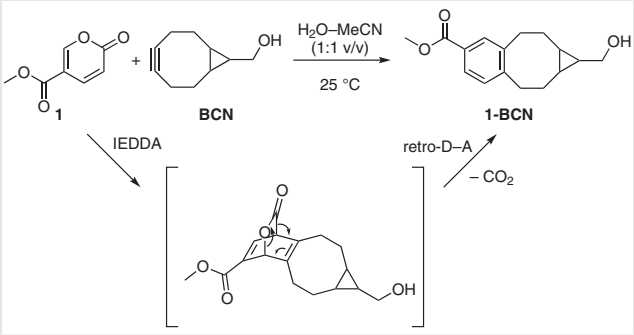
**Abstract** The applicability of pyrones as a bioorthogonal platform was explored in inverse-electron-demand Diels–Alder (IEDDA) reactions with a strained cyclooctyne. Studies showed that the pyrones are indeed suitable for IEDDA reactions under physiological conditions. Furthermore, the stable pyrone moiety could be utilized to construct easily accessible fluorogenic probes. Mutual orthogonality of the IEDDA reaction of 2-pyrones with SPAAC reactions of azides was also explored.

**Key words** IEDDA, bioorthogonal, 2-pyrone, fluorogenic probes, protein labeling

With its range of emerging reactions, bioorthogonal chemistry has revolutionized the field of chemical biology in the last two decades.[1] Manipulation of biomolecules through these rapid, high yielding, biocompatible, and highly selective reactions has greatly facilitated our ultimate goal of understanding biological processes. Among the existing bioorthogonal reactions, the inverse-electron-demand Diels–Alder (IEDDA) reactions of tetrazines and strained cyclooctenes or cyclooctynes deserve special attention for its remarkable reaction kinetics and full biocompatibility.[2] Moreover, the tetrazine moiety is able to quench the fluorescence of suitable fluorescent frames. This two-in-one feature of the tetrazine moiety was harnessed in the development of bioorthogonally applicable fluorogenic probes.[3] While the field of bioorthogonal chemistry and imaging probes have benefited greatly from tetrazines, the need for mutually orthogonal bioorthogonal reactions, for example in multi-color labeling schemes, called for the development of alternative bioorthogonal reactions.[4] This includes development of novel dienes for IEDDA schemes with substantially different reactivities toward strained alkenes/alkynes.[5a,b] Such needs were addressed recently by the development of triazines that can also react with strained dienophiles in IEDDA reactions.[5c,d] Sydnones can also react with strained alkynes in thermal [3 + 2] cycloadditions and, similarly, they can render fluorescent cores fluorogenic.[5e] These are very important additions to the bioorthogonal toolbox, offering more options to develop mutually orthogonal bioorthogonal chemistries.[5f,g] IEDDA reactions of 2-pyrones have been known for quite a while[6] and even used to access bioorthogonal functions, such as in the synthesis of a reactive cyclooctyne;[7] yet, to our knowledge, the use of 2-pyrones as bioorthogonally applicable dienes remained unexplored.

Our research group has been heavily involved[8] in the design and synthesis of bioorthogonally applicable fluorogenic probes for protein labeling purposes and, in this context, the 2-pyrone moiety seems a particularly useful platform. We reasoned that 2-pyrones form a benzene ring with suitable alkynes *via* an IEDDA–retroDA reaction sequence (Scheme 1), which, upon careful design, would directly result in scaffolds with extended conjugation, giving rise to dramatic changes in spectroscopic properties. In line with the above discussed need for surrogate bioorthogonal functions, and to establish the viability of our hypothesis regarding the fluorogenic probe design potential of 2-py-

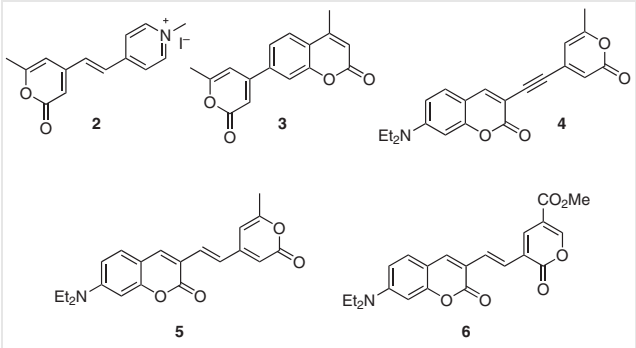
rones, we set forth a study aiming at exploring the applicability of the 2-pyrone scaffold from these two aspects. Literature examples describe related reactions of 2-pyrones with electron-deficient alkynes that required long reaction times at higher temperatures;[9] however, no studies under physiological conditions are reported. Therefore, we first set up a pilot experiment using commercially available methyl coumalate (**1**) with strained cyclooctyne BCN (Scheme 1) in aqueous solution at room temperature. We chose BCN as suitable dienophile because it produces an aromatic system (i.e., a substituted benzene ring) upon IEDDA reaction. Although more reactive *trans*-cyclooctenes could also be considered as complementary bioorthogonal platforms, their reaction would yield a cyclohexadiene. Since our goal is to fabricate fluorogenic systems taking advantage of the possible 2-in-1 feature of the pyrone scaffold, we foresaw that generation of a fully aromatic system would profoundly affect the spectroscopic characteristics of heterocycles.




**Scheme 1** Reaction of methyl coumalate (**1**) with BCN

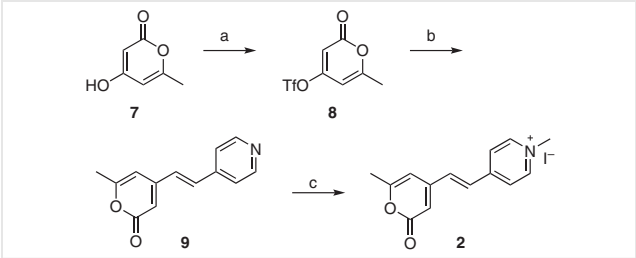
To our delight, the overnight reaction of **1** and BCN resulted in the desired **1-BCN** product in 90% yield following chromatography. Inspired by this finding, we moved onto our ultimate goal; namely, to explore the feasibility of using the pyrone moiety in the fabrication of bioorthogonally applicable fluorogenic probes. To this end, we designed and synthesized a set of probes **2–6** furnished with a 2-pyrone moiety. The probes were designed in a way that in the product of the reaction with BCN the benzene ring becomes directly attached to a conjugated system, giving rise to π-extended structures (Scheme 2).

As stated above, we anticipated that the electronic differences between the pyrone and the benzene ring would dramatically change the photophysical properties of the probes, resulting in highly fluorogenic probes. For synthetic considerations, 4-hydroxy-6-methyl-pyrone (**7**) was chosen as a readily available starting material, which offers a conjugation site *via* its hydroxyl group. We believed that cross-coupling reactions of pseudohalogenated pyrones would allow a versatile approach for the synthesis of a wide variety of 2-pyrone derivatized scaffolds.[10] Therefore, **7** was converted into its triflate derivative **8**,[10a] through treatment



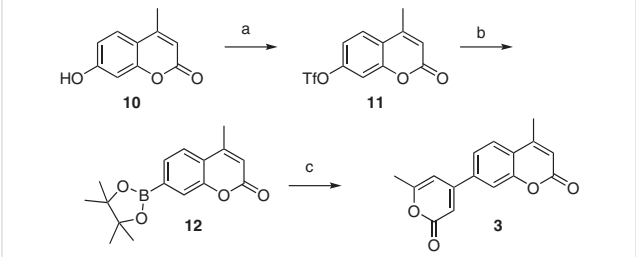

**Scheme 2** Target bioorthogonal probes **2–6**

with $PhNTf_2$. After optimizing the reaction conditions, **8** could be prepared in good yields on the multi-gram scale and served as a common building block for further probes. First, **8** was coupled to 4-vinylpyridine in a Heck reaction to access **9**, which was N-alkylated with methyl iodide to yield probe **2** (Scheme 3).




**Scheme 3** Synthesis of probe **2**. *Reagents and conditions*: (a) $PhNTf_2$, $NEt_3$, DCM, 40 °C, 1 h, 88%; (b) 4-vinylpyridine, $Pd_2(dba)_3$, QPhos, $Cy_2NMe$, DMF, 100 °C, $N_2$, 1 h, MW, 50%; (c) MeI, MeCN, 60 °C, 1 h, 20%.

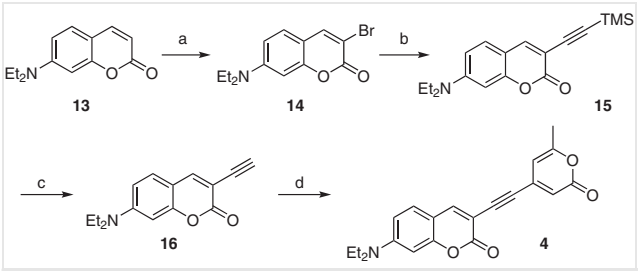
Next, 4-methyl-7-hydroxycoumarin (**10**) was converted into its pinacol boronic ester counterpart **12**[11] in two steps. This was further reacted with **8** in a Suzuki cross-coupling reaction to result in target probe **3** (Scheme 4).




**Scheme 4** Synthesis of probe **3**. *Reagents and conditions*: (a) $PhNTf_2$, $NEt_3$, DCM, 40 °C, 1 h, 85%; (b) $B_2pin_2$, KOAc, $Pd(dppf)Cl_2$, dioxane, $N_2$, 100 °C, 2 h, 90%; (c) **8**, $Pd(dppf)Cl_2$, KOAc, 1,4-dioxane, $N_2$, 100 °C, 2 h, 81%.

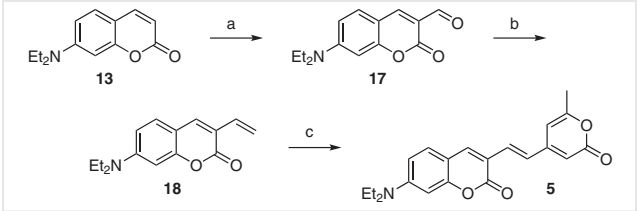
To further extend the conjugated system, 7-diethylaminocoumarin (**13**) was prepared according to reported procedures.[12] Site-selective bromination was effected with

*N*-bromosuccinimide (NBS) to yield 3-bromo derivative **14**, which was subjected to a Sonogashira cross-coupling with TMS-acetylene to deliver **15**. Removal of the TMS group gave **16**,[13] which was immediately used in a second Sonogashira coupling reaction with pyrone triflate **8**, to give acetylene-linked probe **4** in excellent yield (Scheme 5).



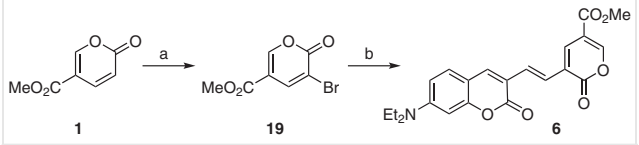

**Scheme 5** Synthesis of probe **4**. *Reagents and conditions*: (a) NBS, $NH_4OAc$, MeCN, 25 °C, 2 h, dark, 30%; (b) TMS-acetylene, $Pd(PPh_3)_2Cl_2$, CuI, EDIPA, DMF, $N_2$, 45 °C, 1 h, 91%; (c) 1 M TBAF in THF, 25 °C, 2 h, 26%; (d) **8**, $Pd(PPh_3)_2Cl_2$, CuI, EDIPA, MeCN, $N_2$, 45 °C, 1 h, quant.

To access vinylene extended probe **5**, intermediate **18** was synthesized through a Vilsmeier–Haack formylation,[12] Wittig reaction sequence, *via* **17**. Finally, the pyrone ring was installed in a Heck reaction to yield **5** (Scheme 6).




**Scheme 6** Synthesis of probe **5**. *Reagents and conditions*: (a) $POCl_3$, DMF, $N_2$, 0 °C/30 min, 60 °C/16 h, 54%; (b) *i*. $Ph_3P^+Me\ Br^-$, *n*-BuLi/hexane, THF, $N_2$, 0 °C, 20 min; *ii*. **17**, THF, 25 °C, 18 h, 47%; (c) **8**, $Pd_2(dba)_3$, QPhos, $Cy_2NMe$, DMF, 100 °C, $N_2$, 1 h, MW, 32%.

To study the effects of different connectivity of the pyrone ring and to be able to install an electron-withdrawing group, which facilitates IEDDA reaction, we also prepared bromo-pyrone **19**.[14] Vinyl coumarin **18** was then allowed to react with **19** to yield probe **6** in good yield (Scheme 7).




**Scheme 7** Synthesis of probe **6**. *Reagents and conditions*: (a) PBPB, AcOH, 100 °C, 12 h, 41%; (b) **18**, $Pd_2(dba)_3$, QPhos, $Cy_2NMe$, DMF, 100 °C, $N_2$, 1 h, MW, 85%.

With the desired molecules in hand, we tested their reactions with BCN and monitored the changes in their fluorescence spectra. Results showed that all compounds reacted with BCN; however, the change in the fluorescence properties varied considerably (Table 1 and see the Supporting Information for spectra). Compound **2** and its reaction product with BCN (**2-BCN**) both showed large Stokes shifts; however, the intensity of the fluorescence increased by only about threefold upon reaction. Probes **3** and **3-BCN** showed similar absorption and emission properties, with somewhat larger (13-fold) increase in fluorescence. These latter two probes, however, required UV excitation, which is not ideal for biological applications. Probe **4**, on the other hand, showed significantly redshifted excitation and emission maxima, both of which were slightly blueshifted upon reaction with BCN. Notably, however, this reaction was accompanied by a huge increase in the intensity of fluorescence (over 100-fold). Reaction of probe **5** with BCN resulted in decreased fluorescence intensity and the disappearance of the originally large Stokes shift. Changing the substitution pattern of the 2-pyrone frame and introducing an electron-withdrawing group onto it (**6**) resulted in a hypsochromic shift compared to **5**, and only a slight increase in fluorescence was observed upon reaction. These results indicate that the position and the nature of the linkage between the 2-pyrone and the fluorescent frame should be carefully considered during the design process.

**Table 1** Excitation and Emission Maxima[a] of the New Fluorescent Dyes and the Change in Fluorescence Intensity upon Reaction of BCN

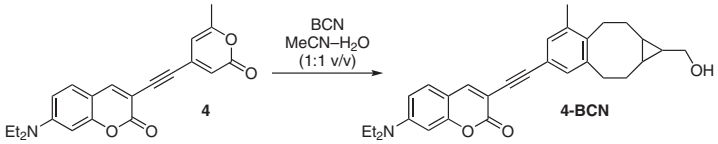
| Compound | $\lambda_{max}$(ex) (nm) | $\lambda_{max}$(em) (nm) | Change in fluorescence |
|---|---|---|---|
| **2** | 360 | 491 | slight increase (ca. 3.3×) |
| **2-BCN** | 365 | 484 | |
| **3** | 335 | 429 | increase (ca. 12–13×) |
| **3-BCN** | 334 | 424 | |
| **4** | 465 | 536 | huge increase (>100×) |
| **4-BCN** | 426 | 487 | |
| **5** | 469 | 593 | decrease |
| **5-BCN** | 538 | 565 | |
| **6** | 407 | 490 | slight increase (ca. 2.5×) |
| **6-BCN** | 422 | 528 | |

[a] Measurement conditions: 50% MeCN in $H_2O$, 25 °C.

Based on these findings, we chose **4** for further experiments. First, we quantified its reaction speed with BCN and found the second-order rate constant to be $k_2 = 0.095\ M^{-1}s^{-1}$, which is in the same order of magnitude as the well-established strain-promoted cycloaddition reactions of azides and strained alkynes (e.g., DIFO and BCN have a $k_2$ of 0.076 and 0.14, respectively).[15] We also measured the molar absorption coefficients, fluorescence quantum yields, and the fluorescence enhancement (Table 2).

To test whether the pyrone-derivatized bioorthogonally applicable fluorogenic probe **4** is suitable for protein labeling, we first functionalized a human serum protein, Transferrin (TF, 76 kDa) with BCN using BCN-NHS. Following removal of unreacted reagents, probe **4** was added to the

**Table 2** Spectral Properties[a] ($\varepsilon$ and $\varphi$ values) of **4** and **4-BCN** and the Calculated Fluorescence Enhancement upon Reaction with BCN




| **4** | | **4-BCN** | |
|---|---|---|---|
| $\varepsilon_{465nm}$ ($M^{-1}cm^{-1}$) | $\varphi$[b] | $\varepsilon_{426nm}$ ($M^{-1}cm^{-1}$) | $\varphi$[b] |
| 52730 | 0.009 | 33150 | 1.0 |
| Fluorescence enhancement ($\varphi_{4\text{-BCN}}/\varphi_4$): | | | 111× |

[a] Measurement conditions: 50% MeCN in $H_2O$, 25 °C.
[b] Using coumarin-153 in EtOH ($\varphi$ = 0.544) as reference standard.[16]

samples at different concentrations (i.e., 125, 250 or 500 μM). Following 24 hour incubation, the reaction mixtures were worked up, then the samples were subjected to an SDS-polyacrylamide gel, and in-gel fluorescence detection (Figure 1). Fluorescent Transferrin bands occurred in the lanes only where Transferrin was co-incubated both with NHS-BCN and **4**. As expected, fluorescence intensity was proportional to the concentration of the probe added. These results indicated that the fluorogenic probe **4** indeed reacted specifically with transferrin-conjugated BCN, in a concentration-dependent manner. As control experiments we mixed probe **4** with Transferrin that was not treated with BCN previously. Only a very low fluorescent signal was observed, indicating that fluorogenic **4** in its quenched (i.e., 2-pyrone) form does not contribute to background fluorescence even when it is adhered non-specifically to the protein by adsorption. We have also studied the labeling scheme of further proteins including β-lactoglobulin B, α1-acid glycoprotein, myoglobin and a trypsin inhibitor, each of which showed similar results to transferrin labeling (see the Supporting Information). To explore the labeling efficacy, we have also labeled a BCN-tagged 17-mer oligonucleotide and a BCN-tagged cyclic peptide, phalloidin. The reactions were followed either by capillary electrophoresis or by LC-MS. The labeling reactions were accomplished either in 50% DMSO-water (oligonucleotide) or in methanol (phalloidin). Mass spectrometry proved the formation of the expected products; however, the conversion rates were very different, i.e., 33% vs. 100% for the aqueous solution and methanol, respectively, which is attributed to the limited solubility of compound **4** (see the Supporting Information).

The stability of compound **4** was assessed in the presence of excess amounts of GSH (1–10 mM). LC-MS analysis of the samples indicated no substantial change of the pyrone in the presence of up to 5 mM GSH after 24 hours. Higher amounts of GSH (10 mM), however, led to ca. 20% decomposition of **4** after 24 hours and MS indicated the

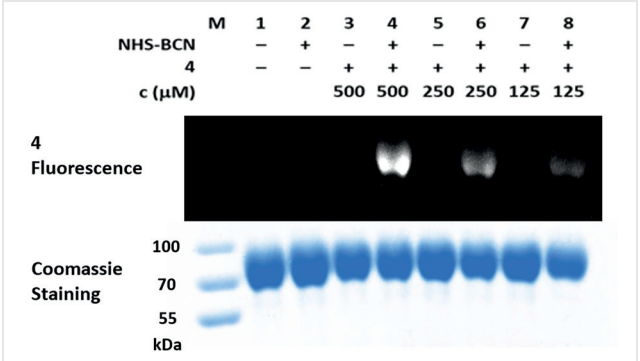



**Figure 1** In-gel SDS PAGE representation of the ability of the fluorogenic coumarin-pyrone derivative **4** to label the serum protein Transferrin (TF) modified with NHS-BCN. Top image is the fluorescent channel detected using 460–490 nm excitation and emission detection with a 532/28 nm band pass filter. Coomassie staining to indicate equal protein loading is shown in the lower image. Molecular weights corresponding to the visible bands of the marker are indicated.

presence of a **4**-GSH adduct. We also checked fluorescence spectra of these samples and, to our delight, found no change in the emission spectrum, indicating that the product is not fluorescent.

We also explored the mutual orthogonality of the 2-pyrone moiety in the presence of other bioorthogonal functions. We assumed that, similar to tetrazines, 2-pyrones are also inert towards sterically demanding dibenzocyclooctynes, such as DBCO.[5f] Preliminary studies confirmed that **4** indeed does not react with DBCO. We then combined excess amounts of DBCO and BCN and added 2-pyrone, bearing probe **4**, to this solution. Next, all the remaining BCN was consumed by adding a tetrazine bearing fluorogenic probe, PheCou,[17a] in excess. Finally, we added an azide-bearing probe, CBRD.[17b] The reaction mixture was then analyzed by LC-MS. To our delight, only the three expected products could be detected; namely, **4**-BCN, PheCou-BCN, and CBRD-DBCO (see the Supporting Information).

In conclusion, we have explored the potential of the 2-pyrone moiety in bioorthogonal reaction schemes in combination with strained alkyne, BCN. The inverse-electron-demand Diels–Alder reaction follows a moderate kinetics with second-order rate constant around 0.1 $M^{-1}$ $s^{-1}$, which is similar to, for example, SPAAC reactions of azides with BCN. We also prepared several 2-pyrone appended probes, one of which showed remarkable fluorescence increase upon IEDDA reaction with BCN. We also demonstrated the applicability of our new 2-pyrone probe in protein labeling schemes *in vitro* with various BCN-modified proteins. Sequential addition of **4**, a tetrazine- and an azide-appending probe, to a mixture of BCN and DBCO revealed that the SPAAC reaction of azides and the IEDDA of 2-pyrones can be conducted orthogonally upon careful selection of reaction partners.

Since tetrazines are very hard to connect directly to fluorescent frames, as only a few examples are reported,[3b,18] we believe that the finding that the 2-pyrone moiety allows several cross-coupling reactions (e.g., Heck, Suzuki, Sonogashira) giving rise to various 2-pyrone-appending profluorescent frames, further highlights the significance of this study. The pyrone moiety is stable, easy to handle, and, similar to the tetrazine function, can also participate in IEDDA reactions, which upon careful design, can result in fluorogenic probes. Upon reacting with alkynes, the formed aromatic moiety allows direct extension of conjugated systems. Furthermore, by adding the pyrone moiety to the bioorthogonal reaction tool-box, we envision that it can be applied to develop mutually orthogonal bioorthogonal reactions taking advantage of its substantially different reaction kinetics.

All starting materials were obtained from commercial suppliers (Sigma–Aldrich, Fluka, Merck, Alfa Aesar, Reanal, Molar Chemicals, Fluorochem) and used without further purification. (1*R*,8*S*,9*S*)-Bicyclo[6.1.0]non-4-yn-9-ylmethanol (BCN) and the corresponding succynimidyl carbonate (BCN-NHS) was obtained from Sigma. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F254 precoated aluminum TLC plates from Merck. Flash column chromatography was performed with a Teledyne Isco CombiFlash® Rf⁺ automated flash chromatographer with silica gel (25–40 μm) from Zeochem. Microwave experiments were carried out with an AntonPaar (Graz, Austria) Monowave 300 microwave reactor (maximum power 850 W). NMR spectra were recorded with a Varian Inova 500 MHz spectrometer. Chemical shifts (δ) are given in parts per million (ppm) using solvent signals or TMS as the reference. Coupling constants (*J*) are reported in hertz (Hz). Analytical RP-HPLC-UV/Vis-MS measurements were conducted with a Shimadzu LCMS-2020 instrument applying a Gemini C18 column (100 × 2.00 mm I.D.) in which the stationary phase was 5 μm silica with a pore size of 110 Å. The chromatograms were detected with UV/Vis diode array (190–800 nm) and ESI-MS detectors. Linear gradient elution (0 min 0% B; 2.0 min 100% B; 3.5 min 100% B; 4.5 min 0% B; 5.0 min 0% B) with eluents A (2% HCOOH, 5% MeCN, and 93% $H_2O$) and B (2% HCOOH, 80% MeCN, and 18% $H_2O$) was used at a flow rate of 1.0 mL $min^{-1}$ at 30 °C. The samples were dissolved in MeCN–$H_2O$ mixture. Spectroscopic measurements were performed with a Jasco FP 8300 spectrofluorometer. Quartz cuvettes with path length of 1 cm were used. The exact masses were determined with an Agilent 6230 time-of-flight mass spectrometer.

**Methyl 1-(Hydroxymethyl)-1a,2,3,8,9,9a-hexahydro-1*H*-benzo[*a*]-cyclopropa[*e*][8]annulene-5-carboxylate (1-BCN)**

In a round-bottom flask, methyl coumalate (**1**; 5.0 mg, 0.033 mmol, 1 equiv) was dissolved in 50% MeCN–$H_2O$ (1 mL) and BCN (15 mg, 0.10 mmol, 3 equiv) was added. The reaction mixture was stirred at 25 °C for 18 hours, then concentrated *in vacuo* on a rotary evaporator. The crude product was purified by flash column chromatography (0→60%, EtOAc–hexane) to give the desired product.

Yield: 7.6 mg (90%); white solid.

$^1$H NMR ($CDCl_3$, 500 MHz): δ = 7.80–7.75 (m, 2 H), 7.16 (d, *J* = 8.4 Hz, 1 H), 3.89 (s, 3 H), 3.70 (dd, *J* = 7.8, 1.3 Hz, 2 H), 3.06–2.95 (m, 2 H), 2.81 (m, 2 H), 2.31–2.20 (m, 2 H), 1.49 (m, 2 H), 1.13–1.02 (m, 1 H), 0.91 (m, 2 H).

$^{13}$C NMR ($CDCl_3$, 126 MHz): δ = 167.5, 147.7, 142.2, 131.3, 130.4, 128.0, 127.5, 59.9, 52.0, 33.6, 33.4, 24.54, 24.53, 22.1, 19.4.

LC-MS (ESI): *m*/*z* = 261 ($C_{16}H_{21}O_3$) $[M + H]^+$.

**6-Methyl-2-oxo-2*H*-pyran-4-yl Trifluoromethanesulfonate (8)**[10a]

4-Hydroxy-6-methyl-pyrone (**7**; 631 mg, 1.20 mmol, 1 equiv) and *N*-phenyl-bis(trifluoromethanesulfonimide) (1.97 g, 1.32 mmol, 1.1 equiv) were dissolved in DCM (40 mL, stabilized with amylene), then triethylamine (1.00 mL, 1.80 mmol, 1.5 equiv) was added, and the reaction mixture was stirred at 40 °C for 1 h. After cooling to r.t., it was diluted with EtOAc (200 mL), washed with sat. $NaHCO_3$ (3 × 100 mL) and dried over $MgSO_4$. After filtration and evaporation, the crude product was purified by flash column chromatography (hexane–EtOAc, 10:1 v/v) to give the desired product.

Yield: 1.14 g (88%); colorless oil.

Repeating the same procedure with **7** (3.00 g) under similar conditions gave **8** (5.29 g, 86%) as a pale-yellow oil, which solidified in the freezer.

It is important to note, that **8** slowly decomposed in the fridge, but was stable at –20 °C.

$R_f$ 0.76 (hexane–EtOAc, 1:1 v/v).

IR (neat): 3104, 1743, 1646, 1575, 1429, 1318, 1206, 1134, 1109, 961, 803 $cm^{-1}$.

$^1$H NMR ($CDCl_3$, 500 MHz): δ = 6.10 (s, 1 H), 6.05 (s, 1 H), 2.31 (s, 3 H).

$^{13}$C NMR ($CDCl_3$, 126 MHz): δ = 165.7, 161.7, 161.1, 120.6, 102.5, 99.7, 20.4.

HRMS (ESI): *m*/*z* $[M + H]^+$ calcd for $C_7H_6F_3O_5S$: 258.9888; found: 258.9893.

**(*E*)-6-Methyl-4-(2-(pyridin-4-yl)vinyl)pyrone (9)**

In a microwave pressure tube with a magnetic stir bar, 4-vinylpyridine (122 mg, 1.16 mmol, 3 equiv) and **8** (100 mg, 0.39 mmol, 1 equiv) were dissolved in abs. DMF (4 mL) under $N_2$. *N*,*N*-Dicyclohexylmethylamine (410 μL, 1.94 mmol, 5 equiv), QPhos (14 mg, 0.02 mmol, 0.05 equiv) and $Pd_2(dba)_3$ (17 mg, 0.02 mmol, 0.05 equiv) were added and the reaction mixture was heated in a microwave reactor at 100 °C for 1 hour. The solvent was evaporated *in vacuo* and the crude product was purified by flash chromatography twice (first with DCM–MeOH, 20:1, then hexane–EtOAc, 1:1 v/v eluent) to give the product.

Yield: 42 mg (50%); sticky white solid; $R_f$ 0.31 (DCM–MeOH, 20:1 v/v).

IR (neat): 2925, 2852, 2781, 1737, 1594, 1448, 1261, 1199, 1049, 890 $cm^{-1}$.

$^1H$ NMR ($CD_3CN$, 500 MHz): δ = 8.59 (d, *J* = 6.1 Hz, 2 H), 7.50 (d, *J* = 6.1 Hz, 2 H), 7.31 (d, *J* = 16.4 Hz, 1 H), 7.17 (d, *J* = 16.4 Hz, 1 H), 6.47 (s, 1 H), 6.16 (s, 1 H), 2.25 (s, 3 H).

$^{13}C$ NMR ($CD_3CN$, 126 MHz): δ = 163.13, 153.34, 152.25, 151.05, 144.28, 134.76, 130.08, 122.51, 111.42, 101.25, 20.12.

HRMS (ESI): *m*/*z* $[M + H]^+$ calcd for $C_{13}H_{12}NO_2$: 214.0868; found: 214.0865.

### (*E*)-1-Methyl-4-(2-(6-methyl-2-oxo-2*H*-pyran-4-yl)vinyl)pyridin-1-ium Iodide (2)

Compound **9** (52 mg, 0.24 mmol, 1 equiv) was dissolved in anhydrous acetonitrile (4 mL) in a pressure tube, then methyl iodide (150 µL, 2.40 mmol, 10 equiv) was added and the vial was sealed. The reaction mixture was stirred at 60 °C for 1 h, then cooled to r.t. and the solvent was evaporated. The crude product was suspended in EtOAc, filtered, and washed with EtOAc to give the desired product, which required no further purification.

Yield: 18 mg (20%); yellow powder.

IR (neat): 3041, 3012, 1734, 1705, 1639, 1517, 1312, 1228, 984, 846 $cm^{-1}$.

$^1H$ NMR ($D_2O/CD_3CN$, 500 MHz): δ = 9.05 (s, 2 H), 8.49 (s, 2 H), 7.88 (s, 2 H), 7.03 (s, 1 H), 6.77 (s, 1 H), 4.67 (s, 3 H), 2.68 (s, 3 H).

$^{13}C$ NMR ($D_2O/CD_3CN$, 126 MHz): δ = 165.1, 162.6, 150.9, 150.6, 144.5, 134.6, 130.4, 124.6, 111.1, 100.9, 47.0, 18.5.

HRMS (ESI): *m*/*z* $[M]^+$ calcd for $C_{14}H_{14}NO_2$: 228.1025; found: 228.1016.

### 4-Methyl-coumarin-7-yl Trifluoromethanesulfonate (11) and 4-Methyl-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)coumarin (12)

Prepared according to reported procedures[11] with slight modifications.

7-Hydroxy-4-methyl-coumarin (**10**; 176 mg, 1.0 mmol, 1 equiv) and *N*-phenyl-bis(trifluoromethanesulfonimide) (393 mg, 1.1 mmol, 1.1 equiv) were dissolved in DCM (20 mL, stabilized with amylene), then triethylamine (0.21 mL, 1.5 mmol, 1.5 equiv) was added and the reaction mixture was stirred at 40 °C for 1 h. After cooling to r.t., it was diluted with EtOAc (200 mL), washed with sat. $NaHCO_3$ (3 × 100 mL) and dried over $MgSO_4$. After filtration and evaporation of the solvent, the crude product was purified by flash column chromatography (0→50%, EtOAc–hexane) to give **11**.

Yield: 262 mg (85%); white crystalline solid; $R_f$ 0.63 (hexane–EtOAc, 1:1 v/v).

$^1H$ NMR ($CDCl_3$, 500 MHz): δ = (d, *J* = 8.8 Hz, 1 H), 7.29 (d, *J* = 2.4 Hz, 1 H), 7.24 (dd, *J* = 8.8, 2.4 Hz, 1 H), 6.36 (d, *J* = 1.2 Hz, 1 H), 2.46 (d, *J* = 1.2 Hz, 3 H).

LC-MS (ESI): *m*/*z* = 309 ($C_{11}H_8F_3O_5S$) $[M + H]^+$.

Then compound **11** (202 mg, 0.66 mmol, 1 equiv), bis(pinacolato)diboron (200 mg, 0.79 mmol, 1.2 equiv) and anhydrous KOAc (159 mg, 1.62 mmol, 3.6 equiv) were dissolved in abs. 1,4-dioxane (6 mL) under $N_2$ atmosphere. $Pd(dppf)Cl_2$ (30 mg, 0.04 mmol, 0.06 equiv) was added and the reaction mixture was stirred at 100 °C for 2 hours. After cooling to r.t., water (100 mL) was added and the mixture was extracted with EtOAc (3 × 75 mL). The combined organic phases was washed with brine (100 mL) and dried over $MgSO_4$. The crude product was purified by flash column chromatography (0→5%, DCM–MeOH) to give the product as a mixture of the pinacolatoboron and the free boronic acid derivatives, which was used in the next step.

Yield: 133 mg (90%); off-white solid.

IR (neat): 2979, 1730, 1622, 1508, 1338, 1280, 1125, 850 $cm^{-1}$.

LC-MS (ESI): *m*/*z* = 287 ($C_{16}H_{20}BO_4$) $[M + H]^+$ for **12** and *m*/*z* = 203 ($C_{10}H_8BO_4$) $[M–H]^-$ for the free boronic acid derivative.

### 4-Methyl-7-(6-methyl-2-oxo-2*H*-pyran-4-yl)coumarin (3)

Compound **12** (128 mg, 0.45 mmol, 1 equiv), **8** (115 mg, 0.45 mmol, 1 equiv), and anhydrous KOAc (232 mg, 2.36 mmol, 3.6 equiv) were dissolved in abs. 1,4-dioxane (6 mL). $Pd(dppf)Cl_2$ (19.8 mg, 0.027 mmol, 0.06 equiv) was added and the reaction mixture was stirred at 100 °C for 2 hours. After cooling to r.t., water (100 mL) was added and the mixture was extracted with EtOAc (3 × 75 mL). The combined organic phases was washed with brine (100 mL) and dried over $MgSO_4$. The crude product was purified by flash column chromatography (0→5%, DCM–MeOH) to give the desired product.

Yield: 98 mg (81%); pale-yellow solid; $R_f$ 0.26 (hexane–EtOAc, 1:1 v/v).

IR (neat): 3051, 2921, 1707, 1634, 1323, 1026, 871 $cm^{-1}$.

$^1H$ NMR ($CDCl_3$, 500 MHz): δ = 7.70 (d, *J* = 8.0 Hz, 1 H), 7.52–7.48 (m, 2 H), 6.40 (s, 1 H), 6.37 (d, *J* = 1.0 Hz, 1 H), 6.28 (s, 1 H), 2.48 (d, *J* = 1.1 Hz, 3 H), 2.35 (s, 3 H).

$^{13}C$ NMR ($CDCl_3$, 126 MHz): δ = 163.7, 162.1, 153.6, 151.6, 150.6, 125.6, 122.5, 121.6, 116.5, 115.3, 110.7, 109.5, 103.0, 101.5, 20.4, 18.7.

HRMS (ESI): *m*/*z* $[M + H]^+$ calcd for $C_{16}H_{13}O_4$: 269.0814; found: 269.0807.

### 3-Bromo-7-(diethylamino)-coumarin (14), 7-(Diethylamino)-3-((trimethylsilyl)ethynyl)-coumarin (15) and 7-(Diethylamino)-3-ethynyl-coumarin (16)

Prepared according to reported procedures[13] with slight modifications.

In a round-bottom flask 7-(diethylamino)-coumarin (**13**; 2.60 g, 12.0 mmol, 1 equiv) and $NH_4OAc$ (92 mg, 1.2 mmol, 0.1 equiv) were dissolved in acetonitrile (250 mL) and *N*-bromosuccinimide (2.60 g, 14.3 mmol, 1.2 equiv) was added while stirring. The reaction mixture was stirred at r.t. for 2 hours in the dark (covered with aluminum foil), then concentrated onto silica. The crude product was partially purified by column chromatography (hexane–EtOAc, 4:1 v/v), then recrystallized from acetonitrile and washed with cold $Et_2O$ to give **14**.

Yield: 1.03 g (30%); light-brown solid; $R_f$ 0.76 (hexane–EtOAc, 1:1 v/v); $R_f$ 0.50 (hexane–EtOAc, 3:1 v/v).

LC-MS (ESI): *m*/*z* = 296 and 298 ($C_{13}H_{15}BrNO_2$) $[M + H]^+$.

Compound **14** (500 mg, 1.7 mmol, 1 equiv), CuI (74 mg, 0.39 mmol, 0.2 equiv), and $Pd(PPh_3)_2Cl_2$ (119 mg, 0.17 mmol, 0.1 equiv) was mixed in a round-bottom flask under $N_2$ atmosphere. Then anhydrous DMF (15 mL), EDIPA (1.2 mL, 6.8 mmol, 4 equiv) and trimethylsilylacetylene (0.70 mL, 5.1 mmol, 3 equiv) were added. The reaction mixture was stirred at 45 °C for 1 h, then water (150 mL) was added and the mixture was extracted with DCM (3 × 100 mL). The combined organic phases was washed with sat. EDTA (100 mL), brine (100 mL) and dried over $MgSO_4$. The crude product was purified by flash column chromatography (hexane–EtOAc, 4:1 v/v) to give **15**.

Yield: 482 mg (91%); brown solid; $R_f$ 0.81 (hexane–EtOAc, 1:1 v/v); $R_f$ 0.53 (hexane–EtOAc, 3:1 v/v).

LC-MS (ESI): *m*/*z* = 314 ($C_{18}H_{24}NO_2Si$) $[M + H]^+$.

To TMS-protected coumarin **15** (197 mg, 0.63 mmol, 1 equiv) was added TBAF (1 M in THF, 1.26 mL, 1.26 mmol, 2 equiv) and the mixture was stirred at r.t. for 2 hours. The reaction mixture was concentrated onto Celite and purified by flash column chromatography (0→2% MeOH–DCM) to give the product **16**. It is important to note that **16** quickly decomposed and was used immediately in the next step.

Yield: 40 mg (26%); yellow-brownish oil; $R_f$ 0.20 (DCM).

LC-MS (ESI): $m/z$ = 242 ($C_{15}H_{16}NO_2$) $[M + H]^+$.

### 7-(Diethylamino)-3-((6-methyl-2-oxo-2*H*-pyran-4-yl)ethynyl)coumarin (4)

In a round-bottom flask, **8** (36 mg, 0.138 mmol, 1 equiv), $Pd(PPh_3)_2Cl_2$ (5.0 mg, 0.007 mmol, 0.05 equiv), and CuI (3.0 mg, 0.014 mmol, 0.1 equiv) were mixed and flushed with $N_2$. Then coumarin **16** (40 mg, 0.16 mmol, 1.2 equiv) dissolved in anhydrous acetonitrile (5 mL) was added, followed by EDIPA (96 µL, 0.552 mmol, 4 equiv). The reaction mixture was stirred at 45 °C for 1 h, then the solvent was evaporated and the crude product was purified by flash column chromatography (0→3% DCM–MeOH) to give the desired product.

Yield: 48 mg (quant.); off-white solid; $R_f$ 0.35 (hexane–EtOAc, 1:1 v/v).

IR (neat): 2969, 2931, 2192, 1708, 1578, 1513, 1414, 1280, 1134, 816 $cm^{-1}$.

$^1H$ NMR ($CDCl_3$, 500 MHz): δ = 7.83 (s, 1 H), 7.27 (d, $J$ = 8.9 Hz, 1 H), 6.62 (dd, $J$ = 8.9, 2.5 Hz, 1 H), 6.49 (d, $J$ = 2.5 Hz, 1 H), 6.27 (s, 1 H), 6.08 (t, $J$ = 1.0 Hz, 1 H), 3.45 (q, $J$ = 7.1 Hz, 4 H), 2.24 (d, $J$ = 1.0 Hz, 3 H), 1.24 (t, $J$ = 7.1 Hz, 6 H).

$^{13}C$ NMR ($CDCl_3$, 126 MHz): δ = 162.4, 161.9, 157.1, 152.2, 147.9, 138.9, 129.8, 113.9, 109.8, 108.4, 105.5, 97.4, 94.8, 89.5, 45.2, 20.0, 12.6.

HRMS (ESI): $m/z$ $[M + H]^+$ calcd for $C_{21}H_{20}NO_4$: 350.1392; found: 350.1386.

### 7-(Diethylamino)coumarin-3-carbaldehyde (17)

Synthesized as described in the literature.[12]

$POCl_3$ (4.7 mL, 50.4 mmol, 3.1 equiv) under $N_2$ atmosphere in a round-bottom flask was cooled to 0 °C in an ice-water bath and anhydrous DMF (4.8 mL, 61.8 mmol, 3.8 equiv) was added dropwise and the mixture was stirred for 30 minutes. Then 7-(diethylamino)coumarin (**13**; 3.53 g, 16.2 mmol, 1 equiv) was dissolved in anhydrous DMF (20 mL) and slowly added. The reaction mixture was stirred at 60 °C for 16 hours, then poured onto ice and the pH was adjusted to 6 with 20% NaOH solution. The precipitate was filtered, washed with cold EtOH, then recrystallized from abs. EtOH to give the product.

Yield: 2.16 g (54%); orange powder; $R_f$ 0.52 (hexane–EtOAc, 1:1 v/v); $R_f$ 0.18 (hexane–EtOAc, 3:1 v/v).

$^1H$ NMR ($CDCl_3$, 500 MHz): δ = 10.14 (s, 1 H), 8.26 (s, 1 H), 7.41 (d, $J$ = 9.0 Hz, 1 H), 6.64 (dd, $J$ = 9.0, 2.5 Hz, 1 H), 6.49 (d, $J$ = 2.5 Hz, 1 H), 3.48 (q, $J$ = 7.2 Hz, 4 H), 1.26 (t, $J$ = 7.2 Hz, 6 H).

LC-MS (ESI): $m/z$ = 246 ($C_{14}H_{16}NO_3$) $[M + H]^+$.

### 7-(Diethylamino)-3-vinyl-coumarin (18)

Methyltriphenylphosphonium bromide (464 mg, 1.30 mmol, 1.3 equiv) was dissolved in anhydrous THF (4 mL) under $N_2$, cooled to 0 °C and *n*-BuLi (2.5 M in hexane, 0.65 mL, 1.30 mmol, 1.3 equiv) was added dropwise, then stirred for 20 minutes. Compound **17** (245 mg, 1.00 mmol, 1 equiv) was dissolved in anhydrous THF (3 mL) and added dropwise to the above reaction mixture at 0 °C, then stirred at 25 °C for 18 hours. The reaction was quenched with sat. $NH_4Cl$ (50 mL) and extracted with EtOAc (3 × 50 mL) and dried with brine and $MgSO_4$ to give the product. It should be noted that **18** quickly decomposes on dry silica and in the fridge over time.

Yield: 115 mg (47%); off-white sticky solid; $R_f$ 0.57 (hexane–EtOAc, 3:1 v/v).

$^1H$ NMR ($CDCl_3$, 500 MHz): δ = 7.53 (s, 1 H), 7.22 (d, $J$ = 8.8 Hz, 1 H), 6.64 (dd, $J$ = 17.6, 11.3 Hz, 1 H), 6.54 (dd, $J$ = 8.8, 2.5 Hz, 1 H), 6.44 (d, $J$ = 2.5 Hz, 1 H), 5.99 (dd, $J$ = 17.6, 1.3 Hz, 1 H), 5.26 (dd, $J$ = 11.3, 1.3 Hz, 1 H), 3.37 (q, $J$ = 7.1 Hz, 4 H), 1.17 (t, $J$ = 7.1 Hz, 6 H).

$^{13}C$ NMR ($CDCl_3$, 126 MHz): δ = 161.3, 155.8, 150.6, 138.6, 131.2, 128.9, 117.9, 115.7, 109.1, 108.8, 97.1, 44.8, 12.5.

HRMS (ESI): $m/z$ $[M + H]^+$ calcd for $C_{15}H_{18}NO_2$: 244.1338; found: 244.1336.

### (*E*)-7-(Diethylamino)-3-(2-(6-methyl-2-oxo-2*H*-pyran-4-yl)vinyl)coumarin (5)

Compound **18** (75 mg, 0.30 mmol, 2 equiv) and **8** (39 mg, 0.15 mmol, 1 equiv) were dissolved in abs. DMF (2 mL) under $N_2$. *N*,*N*-Dicyclohexylmethylamine (130 µL, 0.60 mmol, 4 equiv), QPhos (11 mg, 0.015 mmol, 0.1 equiv) and $Pd_2(dba)_3$ (14 mg, 0.015 mmol, 0.1 equiv) were added and the reaction mixture was heated in a microwave reactor at 100 °C for 1 hour. The solvent was evaporated *in vacuo* and the crude product was purified by flash chromatography (0→100%, EtOAc–hexane) to give the desired product.

Yield: 17 mg (32%); orange solid; $R_f$ 0.38 (hexane–EtOAc, 1:1 v/v).

IR (neat): 3114, 2923, 1705, 1651, 1626, 1528, 1444, 1363, 1250, 1141, 841 $cm^{-1}$.

$^1H$ NMR ($CDCl_3$, 500 MHz): δ = 7.75 (s, 1 H), 7.31 (d, $J$ = 8.9 Hz, 1 H), 7.17 (s, 2 H), 6.61 (dd, $J$ = 8.9, 2.6 Hz, 1 H), 6.50 (d, $J$ = 2.6 Hz, 1 H), 6.26 (s, 1 H), 6.08 (s, 1 H), 3.45 (q, $J$ = 7.1 Hz, 4 H), 2.27 (s, 3 H), 1.24 (t, $J$ = 7.1 Hz, 6 H).

$^{13}C$ NMR ($CDCl_3$, 126 MHz): δ = 163.9, 161.3, 156.5, 152.3, 151.7, 142.8, 141.2, 136.4, 131.3, 129.8, 125.3, 115.7, 109.7, 109.1, 100.9, 97.3, 45.2, 20.2, 12.6.

HRMS (ESI): $m/z$ $[M + H]^+$ calcd for $C_{21}H_{22}NO_4$: 352.1549; found: 352.1549.

### Methyl 3-Bromo-2-oxo-2*H*-pyran-5-carboxylate (19)

Pyridinium bromide perbromide (PBPB; 2.16 g, 6.76 mmol, 1.3 equiv) was dissolved in glacial acetic acid (60 mL) and heated to 100 °C. Meanwhile methyl coumalate (800 mg, 5.20 mmol, 1 equiv) was dissolved in glacial acetic acid (40 mL) and added dropwise to the hot solution. The reaction mixture was stirred at 100 °C for further 12 hours, then cooled to r.t. and most of the acetic acid was removed on a rotary evaporator. Water (200 mL) was added, the mixture was extracted with EtOAc (3 × 100 mL), and the combined organic phases were washed with brine and dried over $MgSO_4$. The crude product was purified by flash chromatography (0→30%, EtOAc–hexane) to give the product.

Yield: 484 mg (41%); off-white solid; $R_f$ 0.68 (hexane–EtOAc, 1:1 v/v).

IR (neat): 3075, 2961, 1744, 1707, 1436, 1283, 1103, 955, 857 $cm^{-1}$.

$^1H$ NMR ($CDCl_3$, 500 MHz): δ = 8.26 (d, $J$ = 2.2 Hz, 1 H), 8.13 (d, $J$ = 2.2 Hz, 1 H), 3.87 (s, 3 H).

$^{13}C$ NMR ($CDCl_3$, 126 MHz): δ = 162.5, 156.8, 156.6, 142.7, 112.9, 111.7, 52.9.

LC-MS (ESI): $m/z$ = 233 and 235 ($C_7H_6BrO_4$) $[M + H]^+$.

HRMS (ESI): did not ionize.

**Methyl (*E*)-3-(2-(7-(Diethylamino)-coumarin-3-yl)vinyl)-2-oxo-2*H*-pyran-5-carboxylate (6)**

Compound **18** (84 mg, 0.34 mmol, 2 equiv) and **19** (40 mg, 0.17 mmol, 1 equiv) were dissolved in abs. DMF (4 mL) under $N_2$. *N*,*N*-Dicyclohexylmethylamine (146 µL, 0.68 mmol, 4 equiv), QPhos (12 mg, 0.017 mmol, 0.1 equiv) and $Pd_2(dba)_3$ (16 mg, 0.017 mmol, 0.1 equiv) were added and the reaction mixture was heated in a microwave reactor at 100 °C for 1 hour. The solvent was evaporated *in vacuo* and the crude product was purified by flash column chromatography (0→20% EtOAc–hexane) to give the desired product.

Yield: 58 mg (85%); reddish brown solid; $R_f$ 0.55 (hexane–EtOAc, 1:1 v/v).

IR (neat): 2975, 2931, 1714, 1603, 1589, 1509, 1412, 1134, 998, 733 $cm^{-1}$.

$^1H$ NMR ($CDCl_3$, 500 MHz): δ = 8.20 (d, $J$ = 2.2 Hz, 1 H), 7.87 (d, $J$ = 2.2 Hz, 1 H), 7.71 (s, 1 H), 7.62 (d, $J$ = 16.1 Hz, 1 H), 7.49 (d, $J$ = 16.1 Hz, 1 H), 7.29 (d, $J$ = 8.8 Hz, 1 H), 6.60 (dd, $J$ = 8.8, 2.3 Hz, 1 H), 6.50 (d, $J$ = 2.3 Hz, 1 H), 3.89 (s, 3 H), 3.43 (q, $J$ = 7.1 Hz, 4 H), 1.22 (t, $J$ = 7.1 Hz, 6 H).

$^{13}C$ NMR ($CDCl_3$, 126 MHz): δ = 163.7, 161.1, 159.8, 156.0, 154.9, 151.1, 141.1, 135.7, 129.7, 129.4, 124.3, 123.3, 116.9, 113.0, 109.5, 97.2, 52.6, 45.1, 12.6.

HRMS (ESI): $m/z$ $[M + H]^+$ calcd for $C_{22}H_{22}NO_6$: 396.1447; found: 396.1441.

**7-(Diethylamino)-3-((1-(hydroxymethyl)-7-methyl-1a,2,3,8,9,9a-hexahydro-1*H*-benzo[*a*]cyclopropa[*e*][8]annulen-5-yl)ethynyl)-coumarin (4-BCN)**

In a round-bottom flask **4** (20 mg, 0.057 mmol, 1 equiv) was dissolved in acetonitrile (5 mL) and BCN (17 mg, 0.114 mmol, 2 equiv) was added. The reaction mixture was stirred at 40 °C for 6 hours, then 2.5 days at 25 °C. After concentration onto silica, the crude product was purified by flash column chromatography (0→60%, EtOAc–hexane) to give the desired product.

Yield: 23 mg (88%); bright-yellow, sticky solid; $R_f$ 0.21 (hexane–EtOAc, 1:1 v/v).

IR (neat): 3414, 2970, 2924, 1716, 1614, 1516, 1254, 1131, 1014, 727$cm^{-1}$.

$^1H$ NMR ($CDCl_3$, 500 MHz): δ = 7.78 (s, 1 H), 7.29 (d, $J$ = 8.9 Hz, 1 H), 7.26 (s, 1 H), 7.22 (s, 1 H), 6.61 (dd, $J$ = 8.9, 2.1 Hz, 1 H), 6.53 (d, $J$ = 2.1 Hz, 1 H), 3.76 (m, 2 H), 3.46 (q, $J$ = 7.1 Hz, 4 H), 3.02 (m, 1 H), 2.96 (m, 1 H), 2.81 (m, 2 H), 2.34 (s, 3 H), 2.32–2.21 (m, 2 H), 1.71 (s, 1 H), 1.64 (m, 2 H), 1.60–1.44 (m, 2 H), 1.25 (t, $J$ = 7.1 Hz, 6 H), 1.11 (m, 2 H).

$^{13}C$ NMR ($CDCl_3$, 126 MHz): δ = 161.1, 156.3, 151.1, 145.0, 136.1, 131.7, 131.2, 128.9, 125.2, 119.9, 110.1, 109.3, 108.7, 105.4, 97.6, 94.1, 83.7, 60.0, 45.1, 34.0, 32.4, 26.6, 23.6, 20.0, 12.6.

HRMS (ESI): $m/z$ $[M + H]^+$ calcd for $C_{30}H_{34}NO_3$: 456.2539; found: 456.2531.

**Activation and Conjugation of Transferrin for SDS-PAGE and In-Gel Fluorescence Study**

Human Transferrin (TF, 70 µM) and BCN-NHS (350 µM; from 20 mM stock solution in DMSO) were mixed and incubated for 60 min at r.t. in 110 mM $Na_2CO_3$/$NaHCO_3$ buffer (pH 9.0). The excess reagents were removed by using a SpinPrep column (Sigma, St Louis, MO, USA) filled with Sephadex G-25 'Fine' desalting gel (Pharmacia Fine Chemicals, Sweden). This procedure resulted in a buffer exchange also to PBS (pH 7.4). In the next step, **4** (500, 250 or 125 µM) was added and co-incubated for 24 hours. The excess reagents were removed again by using a SpinPrep column filled with Sephadex G-25 'Fine' desalting gel. The labeled proteins were subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

**SDS Polyacrylamide Gel Electrophoresis and In-Gel Fluorescence Detection**

The samples were diluted with a sample buffer (250 mM Tris-HCl (pH 6.8), 10% SDS, 40% glycerol, 0.02% bromophenol blue, 400 mM DTT) in a 3:1 ratio. The size of the polyacrylamide gels was 8.5 cm × 7.5 cm × 0.1 cm. They were the combination of 4% concentration and 8% separation PAGE gels (acrylamide/bisacrylamide ratio was 29:1; and concentration gel buffer was 125 mM Tris-HCl + 0.1% SDS (pH 6.8) and the separation gel buffer was 375 mM Tris-HCl + 0.1% SDS (pH 8.8)). PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific) was applied as the molecular weight standard. Separations were carried out in a Mini-Protean Tetra Cell (Bio-Rad, Hercules, CA, USA) using 25 mM Tris /192 mM glycine + 0.1% SDS (pH 8.3) as the running buffer and 150 V voltage for 70 min at 25 °C.

The gels were documented by using a Biorad Bio-Rad ChemiDoc™ Imager. **4/4-BCN** fluorescence was detected in the Pro-Q Emerald 488 channel using blue led epi illumination (460–490 nm) and emission detection with a 532/28 nm band pass filter. Afterwards, the gels were stained for proteins with Coomassie Brilliant-Blue and documented using the colorimetric setting with white epi illumination.

## Conflict of Interest

The authors declare no conflict of interest.

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## Supporting Information

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