In Vivo Activation of Duocarmycin-Antibody Conjugates by Near-Infrared Light
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**General Materials and Methods.** Unless stated otherwise, reactions were conducted in oven-dried glassware under an atmosphere of nitrogen or argon using anhydrous solvents (passed through activated alumina columns). All commercially obtained reagents were used as received. Cyanine 1 and S7 were synthesized according to known procedures.1,2 IR-783, IR-806, and N-[3-(anilinomethylene)-2-chloro-1-cyclohexen-1-yl)methylene]aniline monohydrochloride S4 were purchased from Sigma-Aldrich (St. Louis, MO). Duocarmycin DM was purchased from Levena Biopharma (San Diego, CA). Azido-PEG4-acid 15 was purchased from Aurum Pharmatech, Inc. (Howell, NJ). Flash column chromatography was performed using reversed phase (100 Å, 20-40 micron particle size, RediSep® Rf Gold® Reversed-phase C18 or C18Aq) on a CombiFlash® Rf 200i (Teledyne Isco, Inc., Lincoln, NE). High-resolution LC/MS analyses were conducted on a Thermo-Fisher LTQ-Orbitrap-XL hybrid mass spectrometer system with an Ion MAX API electrospray ion source in negative ion mode. Analytical LC/MS was performed using a Shimadzu LCMS-2020 Single Quadrupole utilizing a Kinetex 2.6 μm C18 100 Å (2.1 x 50 mm) column obtained from Phenomenex, Inc (Torrance, CA). Runs employed a gradient of 0→90% MeCN/0.1% aqueous formic acid over 4.5 min at a flow rate of 0.2 mL/min. 1H NMR and 13C NMR spectra were recorded on Bruker spectrometers (at 400 or 500 MHz or at 100 or 125 MHz) and are reported relative to deuterated solvent signals. Data for 1H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity, coupling constant (Hz), and integration. Data for 13C NMR spectra are reported in terms of chemical shift. IR spectra were recorded on a Jasco FT/IR-4100 spectrometer and are reported in terms of frequency of absorption (cm⁻¹). Absorbance curves were obtained on a Shimadzu UV-2550 spectrophotometer operated by UVProbe 2.32 software. Fluorescence traces were recorded on a PTI QuantaMaster steady-state spectrofluorimeter operated by FelixGX 4.2.2 software, with 5 nm excitation and emission slit widths, and a 0.1 s integration rate. Data analysis and curve fitting were performed using MS Excel 2011 and GraphPad Prism 7. Light intensity measurements were performed with a Thorlabs PM200 optical power and energy meter fitted with an S120VC standard Si photodiode power sensor (200 – 1100

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S3
nm, 50 nW – 50 mW). See JOC Standard Abbreviations and Acronyms for abbreviations (http://pubs.acs.org/userimages/ContentEditor/1218717864819/joceah_abbreviations.pdf).

**Computational Chemistry**

Quantum chemistry calculations were performed using a local version of the GAMESS package and molecular structures were illustrated using MacMolPlt. Geometries of the C4'-dimethylamine and diethylamine-substituted heptamethine cyanines were optimized using the second-order Møller-Plesset perturbation theory (MP2) method, where water solvent effects were treated using the Polarizable Continuum Model (PCM) with a high density of tesserae (NTSALL = 960 in $PCM). The double-zeta 6-31G(d) basis sets were used throughout. The MP2/6-31G(d)-PCM geometries are shown in Figure S1 and Cartesian coordinates are provided below.

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Vertical excitation energies between the two lowest singlet states (S0 \rightarrow S1) for each system were computed using the Occupation Restricted Multiple Active Space (ORMAS)\textsuperscript{15} level of theory with second-order perturbation theory corrections (PT2)\textsuperscript{16} and water solvent modeling via the PCM method. The ORMAS-PT2-PCM computations used (12 electron, 11 orbital) active spaces which consisted of the \(\pi\)-chain orbitals/electrons between (and including) the end nitrogen atoms. State-averaged reference configuration interaction spaces in a multi-configurational singles-doubles style were used via the CIDET/ORMAS specifications:

\textit{C4'-dimethylamine heptamethine cyanine}

\begin{verbatim}
$det  ncore=127  nels=12  nact=11  nstate=3  wstate(1)=1,1 $end
$ormas  nspace=3  mstart(1)=128,133,135  mine(1)=8,0,0  maxe(1)=10,4,2 $end
\end{verbatim}

\textit{C4'-diethylamine heptamethine cyanine}

\begin{verbatim}
$det  ncore=135  nels=12  nact=11  nstate=3  wstate(1)=1,1 $end
$ormas  nspace=3  mstart(1)=136,141,143  mine(1)=8,0,0  maxe(1)=10,4,2 $end
\end{verbatim}

The ORMAS-PT2-PCM absolute and relative energies of the two lowest singlet states for each system are as follows:

\textit{C4'-dimethylamine heptamethine cyanine}

\begin{align*}
E(S0) &= -1480.0335919433 \\
E(S1) &= -1479.9684141067 \\
E(S1) - E(S0) &= 1.774 \text{ eV} = 699 \text{ nm}
\end{align*}

\textit{C4'-diethylamine heptamethine cyanine}

\begin{align*}
E(S0) &= -1558.3715833009 \\
E(S1) &= -1558.3091785992 \\
E(S1) - E(S0) &= 1.698 \text{ eV} = 730 \text{ nm}
\end{align*}

The lower vertical excitation energy for the Et,Et system can be rationalized by the decreased amount of C4'-N lone pair interaction with the cyanine \(\pi\) chain (in the ground state), thus perturbing the cyanine \(\pi\) system less than the Me,Me analogue. This effect is apparent structurally in three ways: (1) increased C4'-N bond length in the diethyl analogue (1.373

\textsuperscript{15} Ivanic, J. Direct configuration interaction and multiconfigurational self-consistent-field method for multiple active spaces with variable occupations. I. Method. \textit{J. Chem. Phys.} \textbf{2003}, \textit{119}, 9364; Ivanic, J. Direct configuration interaction and multiconfigurational self-consistent-field method for multiple active spaces with variable occupations. II. Application to oxoMn(salen) and N\textsubscript{2}O\textsubscript{4}. \textit{J. Chem. Phys.} \textbf{2003}, \textit{119}, 9377.

\textsuperscript{16} Roskop, L.; Gordon, M. S. Adsorption and diffusion of gallium atoms on the Si(100)-2 x 1 reconstructed surface: a multiconfiguration self-consistent field study utilizing molecular surface clusters. \textit{J. Chem. Phys. C} \textbf{2011}, \textit{135}, 044101.
angstroms vs. 1.364 angstroms), (2) an increased dihedral angle around the central C-C-N-C bond (42° vs 35°), and (3) decreased helical twist of the cyanine polyene (57° vs 62°) (Figure S1).

Figure S1. Structures of dimethyl- and diethylamine-substituted heptamethine cyanines.\(^{17}\)

\(^{17}\) Images were rendered in Spartan ’14 (Wavefunction Inc., Irvine, CA)
(2): To a solution of IR-783 (S1, 20 mg, 0.026 mmol) in DMF (0.5 mL) was added N,N'-diethylethylene-diamine (19 µL, 0.13 mmol). The green solution was heated to 85 °C in a sealed vial for 10 minutes, after which time LC/MS analysis of the dark blue reaction showed complete consumption of S1. The reaction was cooled to room temperature and precipitated into Et₂O (40 mL) with a 0.5 mL DMF vial wash. The slurry was centrifuged, the supernatant discarded, and the crude intermediate diamine placed under vacuum (< 1 Torr) for 1 h. This solid was redissolved in THF/H₂O (1:1, 6 mL) and NaHCO₃ (45 mg) was added to the solution. To this solution of the intermediate diamine was added a solution of 4-methyl umbelliferone chloroformate¹ (19 mg, 0.078 mmol) in THF (1.5 mL) with vigorous stirring. After removal of the THF in vacuo the crude aqueous mixture was purified by reversed-phase chromatography (C₁₈, 0→40% MeCN/water). The solvent was removed in vacuo to afford 2 (15 mg, 56% yield) as a blue solid. ¹H NMR (CD₂OD, 500 MHz, compound exists as a mixture of rotamers, major rotamer denoted by *, minor rotamer denoted by §) δ 7.87 – 7.70 (m, 3H*, 3H§), 7.48 – 7.32 (m, 4H*, 4H§), 7.29 – 7.04 (m, 6H*, 6H§), 6.30 (s, 1H§), 6.27 (s, 1H*), 6.07 (d, J = 13.6 Hz, 2H*, 2H§), 4.16 – 4.02 (m, 4H*, 4H§), 3.98 – 3.85 (m, 2H*, 2H§), 3.84 – 3.72 (m, 2H*, 2H§), 3.72 – 3.62 (m, 2H*, 2H§), 3.53 – 3.41 (m, 2H*, 2H§), 2.94 – 2.80 (m, 4H*, 4H§), 2.64 – 2.51 (m, 4H*, 4H§), 2.47 (s, 3H§), 2.44 (s, 3H*), 2.02 – 1.80 (m, 10H*, 10H§), 1.68 (s, 6H§), 1.67 (s, 6H*), 1.41 – 1.33 (m, 3H*, 3H§), 1.25 (t, J = 7.1 Hz, 3H*), 1.20 (t, J = 7.1 Hz, 3H§), IR (thin film) 2934, 2827, 1725, 1506, 1347, 1252 cm⁻¹; HRMS (ESI) calculated for C₅₅H₆₇N₄O₁₀S₂ (M–H)⁻ 1007.4304, observed 1007.4327.

¹ With N-alkyl carbamates 2-8, S10-11, and 16-17 significant peak broadening is observed due to rotamers at 25 °C. High temperature NMR (70 °C in CD₂OD or (CD₃)₂SO) can partially resolve the carbamate rotamers for ¹H NMR. However, these elevated temperatures did not provide a fully resolved ¹³C NMR spectrum. Furthermore, the prolonged heating during ¹³C NMR experiments resulted in partial decomposition of the compounds.
(3): To a solution of IR-806 (S2, 20 mg, 0.027 mmol) in DMF (0.5 mL) was added N,N'-dimethylethylene-diamine (12 µL, 0.11 mmol). The green solution was heated to 70 °C in a sealed vial for 5 minutes, after which time LC/MS analysis of the dark blue reaction showed complete consumption of S2. The reaction was cooled to room temperature and precipitated into Et₂O (40 mL) with a 0.5 mL DMF vial wash. The slurry was centrifuged, the supernatant discarded, and the crude intermediate diamine placed under vacuum (< 1 Torr) for 1 h. This solid was redissolved in THF/H₂O (1:1, 6 mL) and NaHCO₃ (45 mg) was added to the solution. To this solution of the intermediate diamine was added a solution of 4-methyl umbelliferone chloroformate (19 mg, 0.081 mmol) in THF (1.5 mL) with vigorous stirring. After removal of the THF in vacuo the crude aqueous mixture was purified by reversed-phase chromatography (C₁₈, 0→40% MeCN/water). The solvent was removed in vacuo to afford 3 (16 mg, 59% yield) as a blue solid. ¹H NMR (CD₃OD, 500 MHz) δ 7.88 – 7.70 (m, 2H), 7.62 (d, J = 8.5 Hz, 1H), 7.36 – 7.25 (m, 4H), 7.17 – 6.97 (m, 6H), 6.09 (s, 1H), 5.77 (d, J = 13.0 Hz, 2H), 4.31 – 4.23 (m, 2H), 4.07 – 3.96 (m, 4H), 3.81 – 3.73 (m, 2H), 3.64 (s, 3H), 3.00 (s, 3H), 2.90 – 2.86 (m, 6H), 2.26 (s, 3H), 1.99 – 1.86 (m, 10H), 1.49 (s, 12H); IR (thin film) 2930, 2823, 1721, 1512, 1380, 1268 cm⁻¹; HRMS (ESI) calculated for C₅₃H₆₁N₄O₁₆S₄ (M–H) – 965.3824, observed 965.3815.

(S6): A mixture of 4-(sulfobutyl)-2-methylbenzothiazole S3² (300 mg, 1.05 mmol), N-[(3-(anilinomethylene)-2-chloro-1-cyclohexen-1-yl)methylene]aniline monohydrochloride S4 (343
mg, 0.96 mmol), and anhydrous sodium acetate (86 mg, 1.05 mmol) was heated to reflux for 3 h in ethanol (50 mL). The mixture was then allowed to cool to room temperature and was quenched with water (10 mL). The solvent was reduced to a volume of 20 mL under vacuum. The resulting mixture was cooled at 5 °C and the precipitate was filtered off. The filtrate was washed with 
H₂O/ethanol (50 mL, 1:2 v/v) and dried in vacuo. The resulting dark purple solid (407 mg) was carried forward without further purification. A portion of this crude intermediate (100 mg) was mixed with 2,3,3-trimethyl-1-(4-sulfobutyl)-3H-indolinium S₅¹⁹ (172 mg, 0.58 mmol) and anhydrous sodium acetate (48 mg, 0.58 mmol) and was heated to reflux for 4 h in ethanol (30 mL). The mixture was then allowed to cool to room temperature, diluted with water (10 mL), and the solvent was removed under vacuum to reduce the volume to 5 mL. The crude mixture was purified directly by reversed phase chromatography (C₁₈, 20→60% MeCN/water). The solvent was removed in vacuo to afford compound S₆ (54 mg, 39% yield) as a dark green solid. 

¹H NMR (500 MHz, methanol-d₄) δ 8.27 (d, J = 14.1 Hz, 1H), 8.12 (d, J = 13.5 Hz, 1H), 7.98 (d, J = 8.4 Hz, 1H), 7.91 (d, J = 8.5 Hz, 1H), 7.66 (d, J = 7.6 Hz, 1H), 7.53 (t, J = 7.6 Hz, 1H), 7.38 (d, J = 7.0 Hz, 1H), 7.33 – 7.28 (m, 1H), 7.13 (d, J = 7.9 Hz, 1H), 7.11 (d, J = 7.5 Hz, 1H), 6.79 (d, J = 14.1 Hz, 1H), 5.99 (d, J = 13.5 Hz, 1H), 4.63 (t, J = 7.7 Hz, 2H), 4.07 – 3.97 (m, 2H), 2.96 – 2.84 (m, 4H), 2.72 (t, J = 6.1 Hz, 2H), 2.70 – 2.64 (m, 2H), 2.15 – 2.05 (m, 2H), 2.02 – 1.88 (m, 8H), 1.68 (s, 6H); ¹³C NMR (125 MHz, methanol-d₄) δ 169.6, 169.2, 148.4, 146.0, 144.4, 143.0, 141.5, 140.4, 130.1, 129.5, 128.1, 128.0, 126.7, 124.4, 124.3, 123.1, 115.9, 110.6, 105.7, 98.6, 52.0, 51.4, 44.2, 28.6, 28.0, 28.0, 27.2, 27.0, 23.7, 23.3, 22.2.; IR (thin film) 2965, 2927, 2866, 1542, 1504, 1389, 1223 cm⁻¹; HRMS (ESI) calculated for C₃₅H₄₀ClN₂O₆S₃ (M–H)⁻ 715.1732, observed 715.1731.

¹⁹ Cao, H. S.; Xiong, Y. J.; Wang, T.; Chen, B. W.; Squier, T. C.; Mayer, M. U. A red Cy3-based biarsenical fluorescent probe targeted to a complementary binding peptide. J. Am. Chem. Soc. 2007, 129, 8672.
(4): Cyanine S6 (54 mg, 0.073 mmol) was dissolved in NMP (3 mL). To this solution was added \( N\text{-Boc-}N,N'\text{-dimethylethlenediamine} \) (137 mg, 0.728 mmol) and the reaction was heated at 80 °C for 4 h. The mixture was cooled to room temperature and diluted with water (3 mL). The solution was purified directly by reversed phase chromatography (C\(_{18}\), 20→50% MeCN/water). The solvent was removed \textit{in vacuo} to afford the intermediate cyanine Boc-diamine compound as a dark blue solid. To this intermediate was added trifluoroacetic acid (200 µL, 2.62 mmol) under an argon atmosphere. After stirring at room temperature for 30 min, the solvent was removed \textit{in vacuo}. The resulting solid was dissolved in aqueous NaHCO\(_3\) (286 mg in 3 mL of H\(_2\)O). To this aqueous solution was added a solution of 4-methyl umbelliferone chloroformate (17 mg, 0.073 mmol) in THF (2 mL) with vigorous stirring. After stirring for 2 h at ambient temperature, the volatiles were removed \textit{in vacuo} and the crude mixture was directly purified by reverse phase column chromatography (C\(_{18}\), 25→45% MeCN/water). The solvent was removed \textit{in vacuo} to afford 4 (4.4 mg, 6% yield) as a blue solid. \(^1\)H NMR (500 MHz, methanol-\(d_4\)) \( \delta \) 7.69 (d, \( J = 13.4 \) Hz, 1H), 7.58 – 7.18 (m, 8H), 7.12 – 6.99 (m, 3H), 6.97 – 6.91 (m, 1H), 6.22 (d, \( J = 13.6 \) Hz, 1H), 5.93 – 5.77 (m, 2H), 4.39 – 4.26 (m, 2H), 4.09 – 3.74 (m, 6H), 3.53 (s, 3H), 2.98 (s, 3H), 2.95 – 2.83 (m, 4H), 2.68 – 2.59 (m, 2H), 2.59 – 2.46 (m, 2H), 2.08 (s, 3H), 2.03 – 1.88 (m, 10H), 1.62 (s, 6H); IR (thin film) 2928, 2823, 1722, 1505, 1378, 1213, 1127 cm\(^{-1}\); HRMS (ESI) calculated for C\(_{50}H_{57}N_4O_{10}S_3\) (M–H)\(^–\) 969.3231, observed 969.3232.
(5): Cyanine S7 (56 mg, 0.077 mmol)\(^2\) was dissolved in DMSO (10 mL). To this solution was added N-Boc-N,N'-dimethylethylenediamine (187 mg, 0.99 mmol) and the reaction was heated at 80 °C for 1 h. The mixture was cooled to room temperature and diluted with water (3 mL). The solution was purified directly by reversed phase chromatography (C\(_{18}\), 20→50% MeCN/water). The solvent was removed \textit{in vacuo} to afford the intermediate cyanine Boc-diamine compound as a dark blue solid. To this intermediate (15 mg, 0.018 mmol) was added trifluoroacetic acid (200 µL, 2.62 mmol) under an argon atmosphere. After stirring at room temperature for 20 min, the reaction was cooled to −10 °C. To the resulting mixture were added THF (3 mL) and aqueous NaHCO\(_3\) solution (286 mg in 3 mL of H\(_2\)O) in succession. To this mixture was added a solution of 4-methyl umbelliferone chloroformate (13 mg, 0.054 mmol) in THF (2 mL) with vigorous stirring. After stirring for 2 h at ambient temperature, the volatiles were removed \textit{in vacuo} and the crude mixture was directly purified by reverse phase column chromatography (C\(_{18}\), 25→45% MeCN/water). The solvent was removed \textit{in vacuo} to afford 5 (17 mg, 23% yield) as a blue solid. \(^1\)H NMR (400 MHz, methanol-\(d_4\)) \(\delta 7.51 – 7.29\) (m, 9H), 7.26 – 7.12 (m, 2H), 7.02 (dd, \(J = 8.7, 2.3\) Hz, 1H), 6.91 (d, \(J = 2.3\) Hz, 1H), 6.18 – 5.99 (m, 2H), 5.86 (d, \(J = 1.5\) Hz, 1H), 4.30 – 4.15 (m, 4H), 4.03 – 3.95 (m, 2H), 3.90 – 3.71 (m, 2H), 3.47 (s, 3H), 2.98 (s, 3H), 2.95 – 2.86 (m, 4H), 2.62 – 2.46 (m, 4H), 2.07 (s, 3H), 2.03 – 1.84 (m, 10H). IR (thin film) 2930, 2820, 1720, 1503, 1386, 1214, 1123 cm\(^{-1}\); HRMS (ESI) calculated for C\(_{47}\)H\(_{51}\)N\(_4\)O\(_{10}\)S\(_4\) (M–H)\(^-\) 959.2494, observed 959.2488.
(6): To a solution of cyanine S8 (20 mg, 0.021 mmol) in DMSO (1.0 mL) and DMF (0.5 mL) was added N,N'-dimethylethylene-diamine (11 µL, 0.11 mmol). The green solution was heated to 70 °C in a sealed vial for 5 min, after which time LC/MS analysis of the dark blue reaction showed complete consumption of S8. The reaction was cooled to room temperature and precipitated into Et<sub>2</sub>O (40 mL) with a 0.5 mL DMF vial wash. The slurry was centrifuged, the supernatant discarded, and the crude intermediate diamine placed under vacuum (< 1 Torr) for 1 h. This solid was redissolved in MeCN/H<sub>2</sub>O (1:1, 6 mL) and NaHCO<sub>3</sub> (45 mg) was added to the solution. To this solution of the intermediate diamine was added a solution of 4-methyl umbelliferone chloroformate (15 mg, 0.063 mmol) in THF (1.0 mL) with vigorous stirring. After removal of the THF in vacuo the crude aqueous mixture was purified by reversed-phase chromatography (C<sub>18</sub>Aq, 0→25% MeCN/water). The solvent was removed in vacuo to afford 6 (13 mg, 51 % yield) as a blue solid. <sup>1</sup>H NMR (CD<sub>3</sub>OD, 500 MHz) δ 7.91 – 7.51 (m, 7H), 7.27 – 6.94 (m, 4H), 6.18 (s, 1H), 6.08 – 5.93 (m, 2H), 4.32 – 4.13 (m, 2H), 4.10 – 3.99 (m, 4H), 3.90 – 3.55 (m, 5H), 3.06 (s, 3H), 2.95 – 2.84 (m, 4H), 2.63 – 2.53 (m, 4H), 2.35 (s, 3H), 2.06 – 1.80 (m, 10H), 1.57 (s, 12H); IR (thin film) 2929, 2829, 1725, 1511, 1382, 1285 cm<sup>-1</sup>; HRMS (ESI) calculated for C<sub>53</sub>H<sub>61</sub>N<sub>4</sub>O<sub>16</sub>S<sub>4</sub> (M–3H)<sup>3-</sup> 379.0983, observed 379.0987.

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<sup>20</sup> Hilderbrand, S. A.; Kelly, K. A.; Weissleder, R.; Tung, C. H. Monofunctional near-infrared fluorochromes for imaging applications. *Bioconjug. Chem.* **2005**, *16*, 1275–1281.
(9): To a microwave vial containing indolenine S9\textsuperscript{21} (75 mg, 0.24 mmol) was added 1,4-
butanesultone (1.5 mL). The headspace was flushed with argon, and the light brown suspension
was heated in a microwave reactor at 175 °C for 7 h. Diethyl ether (10 mL) was charged to vial,
and the thick slurry was centrifuged to afford a brown pellet. The crude solid was dissolved in
water and purified by reversed-phase chromatography (C\textsubscript{18} Aq, 0→20% MeCN/water). The
solvent was removed \textit{in vacuo} to afford 9 (85 mg, 79 % yield) as a tan solid. \textsuperscript{1}H NMR (500 MHz,
DMSO-\textit{d}_6) \(\delta\) 8.41 (d, \(J = 1.8\) Hz, 1H), 8.36 (d, \(J = 8.9\) Hz, 1H), 8.33 (d, \(J = 8.8\) Hz, 1H), 8.20 (d, 
\(J = 8.9\) Hz, 1H), 7.94 (dd, \(J = 8.7, 1.7\) Hz, 1H), 4.59 (t, \(J = 8.0\) Hz, 2H), 2.94 (s, 3H), 2.55 (t, \(J = 
7.3\) Hz, 2H), 2.02 (p, \(J = 7.6\) Hz, 2H), 1.80 (p, \(J = 7.6\) Hz, 2H), 1.75 (s, 6H); \textsuperscript{13}C NMR (125 MHz,
DMSO-\textit{d}_6) \(\delta\) 196.5, 146.5, 139.0, 136.8, 132.5, 131.3, 127.0, 126.3, 125.7, 123.2, 113.8, 55.5, 50.2, 47.5, 26.2, 22.0, 21.6, 13.7; IR (thin film) 2946, 2834, 1717, 1466, 1199, 1098, 1025 cm\textsuperscript{-1};
HRMS (ESI) calculated for C\textsubscript{19}H\textsubscript{22}NO\textsubscript{6}S\textsubscript{2} (M–H)\textsuperscript{–} 424.0883, observed 424.0873.

(11): To a microwave vial was added indolenine 9 (130 mg, 0.29 mmol) and chloride 10\textsuperscript{22} (48 mg,
0.12 mmol). MeOH (5 mL), triethylamine (400 \(\mu\)L, 2.91 mmol), and acetic anhydride (550 \(\mu\)L,
5.82 mmol) were then added in succession. The yellow solution was heated to 110 °C for 20 min,
during which time the reaction transitioned to a deep green color. The reaction was cooled and
diluted into saturated aqueous NaHCO\textsubscript{3} (15 mL), yielding a tan precipitate. The supernatant was
decanted and the crude solid was dissolved in water (10 mL) and purified by reversed-phase
chromatography (C\textsubscript{18} Aq, 0→30% MeCN/water). The product-containing fractions were
lyophilized to afford 11 (95 mg, 74 % yield) as green solid. \textsuperscript{1}H NMR (500 MHz, DMSO-\textit{d}_6) \(\delta\) 8.39

\cite{Mujumdar1996}
\cite{Yang2013}

\textsuperscript{21} Mujumdar, S. R.; Mujumdar, R. B.; Grant, C. M.; Waggoner, A. S. Cyanine-labeling reagents:
Sulfobenzindocyanine succinimidyl esters. Bioconjug. Chem. 1996, 7, 356-362.
\textsuperscript{22} Yang, Z.; Lee, J. H.; Jeon, H. M.; Han, J. H.; Park, N.; He, Y.; Lee, H.; Hong, K. S.; Kang, C.; Kim, J.
S. Folate-based near-infrared fluorescent theranostic gemcitabine delivery. J. Am. Chem. Soc. 2013, 135,
11657-11626.
(d, J = 14.1 Hz, 2H), 8.32 – 8.23 (m, 4H), 8.16 (d, J = 8.8 Hz, 2H), 7.88 – 7.78 (m, 4H), 6.45 (d, J = 14.2 Hz, 2H), 4.54 – 4.24 (m, 6H), 4.12 (m, 1H), 3.50 (t, J = 2.3 Hz, 1H), 3.11 – 2.99 (m, 2H), 2.86 (dd, J = 15.8, 6.9 Hz, 2H), 2.54 (t, J = 7.3 Hz, 4H), 1.96 (s, 6H), 1.95 (s, 6H), 1.93 – 1.85 (m, 4H), 1.86 – 1.77 (m, 4H); ¹³C NMR (125 MHz, DMSO-d₆) δ 173.6, 146.6, 144.4, 142.8, 140.3, 133.7, 131.2, 130.7, 127.2, 126.0, 125.8, 123.2, 122.1, 112.3, 101.4, 81.0, 77.2, 70.3, 55.5, 50.7, 44.0, 31.0, 27.1, 27.0, 26.4, 22.5; IR (thin film) 3256, 2976, 2929, 2863, 2112, 1542, 1390, 1221, 1162, 1072 cm⁻¹; HRMS (ESI) calculated for C₄₉H₅₀ClN₂O₁₃S₄ (M–3H)⁻ 345.7289, observed 345.7283.

(12): To a 1-dram vial was added chloride 11 (15 mg, 0.014 mmol) and DMF (0.8 mL). N,N′-dimethylethylene diamine (5.0 µL, 0.047 mmol) was added and the reaction was heated to 75 °C for 10 min, during which time the reaction color transitioned from green to dark blue. The reaction was cooled and diluted with H₂O (8 mL), and the solution was directly purified by reversed-phase chromatography (C₁₈ gold, 0→30% MeCN/water). The product-containing fractions were lyophilized to afford 12 (12 mg, 76% yield) as blue solid. ¹H NMR (400 MHz, methanol-d₄) δ 8.43 (d, J = 1.8 Hz, 2H), 8.28 (d, J = 8.9 Hz, 2H), 8.09 (d, J = 8.8 Hz, 2H), 8.00 (dd, J = 8.9, 1.6 Hz, 2H), 7.89 (d, J = 13.6 Hz, 2H), 7.66 (d, J = 8.9 Hz, 2H), 6.20 (d, J = 13.7 Hz, 2H), 4.34 (d, J = 2.4 Hz, 2H), 4.32 – 4.26 (m, 4H), 4.24 – 4.16 (m, 1H), 4.02 – 3.92 (m, 2H), 3.49 (s, 3H), 3.42 – 3.33 (m, 4H), 2.99 – 2.83 (m, 5H), 2.84 – 2.67 (m, 5H), 2.15 – 2.03 (m, 4H), 2.00 (s, 6H), 1.99 (s, 6H), 1.98 – 1.90 (m, 4H); ¹³C NMR (125 MHz, methanol-d₄) δ 173.3, 144.5, 142.8, 141.9, 134.1, 132.6, 131.7, 129.9, 128.5, 127.1, 125.8, 123.5, 122.6, 112.9, 98.3, 81.2, 76.1, 73.4, 56.9, 55.7, 51.9, 51.4, 49.1 (HSQC), 45.6, 44.6, 34.9, 31.7, 29.0, 29.0, 27.3, 23.6; IR (thin film) 3308, 2936, 2830, 2109, 1545, 1351, 1227, 1101 cm⁻¹; HRMS (ESI) calculated for C₅₃H₆₁N₆O₁₃S₄ (M–3H)⁻ 363.1045, observed 363.1050.
(13): To a 1-dram vial was added chloride 11 (22 mg, 0.020 mmol) and DMF (1.5 mL). N,N'-diethylethylene diamine (14 µL, 0.10 mmol) and diisopropylethylamine (7.0 µL, 0.040 mmol) were added and the reaction was heated to 110 °C for 20 min, during which time the reaction color transitioned from green to dark blue. The reaction was cooled and diluted with H₂O (8 mL), and the solution was directly purified by reversed-phase chromatography (C₁₈ gold column, 0→30% MeCN/water). The product-containing fractions were lyophilized to afford 13 (14 mg, 60% yield) as bluish-green solid. ¹H NMR (500 MHz, methanol-d₄) δ 8.42 (d, J = 1.8 Hz, 2H), 8.29 (d, J = 9.0 Hz, 2H), 8.07 (d, J = 8.8 Hz, 2H), 8.00 (dd, J = 8.9, 1.9 Hz, 2H), 7.94 (d, J = 13.7 Hz, 2H), 7.65 (d, J = 8.9 Hz, 2H), 6.17 (d, J = 13.8 Hz, 2H), 4.33 (d, J = 2.3 Hz, 2H), 4.26 (t, J = 7.2 Hz, 4H), 4.21 – 4.15 (m, 1H), 3.89 (t, J = 7.4 Hz, 2H), 3.84 (dd, J = 7.4, 6.9 Hz, 2H), 3.09 (t, J = 7.3 Hz, 2H), 2.96 – 2.87 (m, 7H), 2.82 (m, 2H), 2.74 (dd, J = 15.3, 6.1 Hz, 2H), 2.12 – 1.90 (m, 20H), 1.44 (t, J = 6.9 Hz, 3H), 1.18 (t, J = 7.2 Hz, 3H); ¹³C NMR (125 MHz, Methanol-d₄) δ 173.4, 144.7, 142.8, 142.0, 134.1, 132.7, 131.8, 130.0, 128.7, 128.5, 125.9, 123.5, 123.4, 112.9, 98.4, 81.2, 76.0, 73.3, 56.8, 52.2, 51.9, 51.5, 48.5 (HSQC), 44.8, 44.5, 40.4, 31.6, 29.1, 29.0, 27.3, 23.6, 15.4, 14.2.; IR (thin film) 3270, 2935, 2822, 2110, 1502, 1352, 1178, 1029 cm⁻¹; HRMS (ESI) calculated for C₅₅H₆₅N₄O₁₃S₄ (M–3H)⁻ 372.4472, observed 372.4460.

(7): To a solution of cyanine 12 (6.0 mg, 0.0052 mmol) in 1:1 H₂O/MeCN (2 mL) was added NaHCO₃ (15 mg). To this mixture was added a solution of 4-methyl umbelliferone chloroformate (3.8 mg, 0.016 mmol) in THF (1.0 mL) with vigorous stirring. After removal of the THF in vacuo the crude aqueous mixture was purified by reversed-phase chromatography (C₁₈Aq, 0→25%
MeCN/water). The solvent was removed in vacuo to afford 7 (3.9 mg, 55 % yield) as a blue solid. 

$^1$H NMR (500 MHz, methanol-$d_4$) $\delta$ 8.47 – 8.30 (m, 2H), 8.23 – 8.10 (m, 2H), 8.09 – 7.97 (m, 2H), 7.97 – 7.86 (m, 3H), 7.87 – 7.49 (m, 4H), 7.30 – 7.06 (m, 2H), 6.37 – 5.95 (m, 3H), 4.52 – 4.04 (m, 9H), 3.99 – 3.57 (m, 5H), 3.10 (s, 3H), 3.01 – 2.85 (m, 7H), 2.77 – 2.67 (m, 2H), 2.53 – 2.18 (m, 3H), 2.10 – 1.77 (m, 20H); IR (thin film) 3279, 2935, 2829, 2111, 1711, 1502, 1350, 1171, 1098 cm$^{-1}$; HRMS (ESI) calculated for $C_{64}H_{67}N_4O_{17}S_4$ (M–3H)$^-$ 430.4456, observed 430.4451.

(8): To a solution of cyanine 13 (6.0 mg, 0.0050 mmol) in 1:1 H$_2$O/MeCN (2 mL) was added NaHCO$_3$ (15 mg). To this mixture was added a solution of 4-methyl umbelliferone chloroformate (3.6 mg, 0.015 mmol) in THF (1.0 mL) with vigorous stirring. After removal of the THF in vacuo the crude aqueous mixture was purified by reversed-phase chromatography (C$_{18}$Aq, 0$\rightarrow$25% MeCN/water). The solvent was removed in vacuo to afford 8 (3.5 mg, 50 % yield) as a blue solid. 

$^1$H NMR (500 MHz, methanol-$d_4$) $\delta$ 8.41 (s, 2H), 8.23 (d, $J = 9.0$ Hz, 2H), 8.07 (t, $J = 8.6$ Hz, 2H), 8.02 – 7.89 (m, 4H), 7.86 – 7.70 (m, 1H), 7.68 – 7.58 (m, 2H), 7.26 – 7.10 (m, 2H), 6.34 – 6.19 (m, 1H), 6.15 (d, $J = 13.7$ Hz, 2H), 4.43 – 4.12 (m, 6H), 4.07 – 3.69 (m, 5H), 3.58 – 3.42 (m, 2H), 3.01 – 2.84 (m, 7H), 2.79 – 2.69 (m, 2H), 2.52 – 2.35 (m, 3H), 2.14 – 1.89 (m, 20H), 1.47 (t, $J = 6.9$ Hz, 3H), 1.32 – 1.19 (m, 3H); IR (thin film) 2940, 2830, 2112, 1725, 1668, 1501, 1349, 1099 cm$^{-1}$; HRMS (ESI) calculated for $C_{66}H_{71}N_4O_{17}S_4$ (M–3H)$^-$ 439.7894, observed 439.7886.

S16
(S10): To a 1-dram vial was added duocarmycin DM (1.0 mg, 0.0017 mmol) and MeCN (0.15 mL). Diisopropylethylamine (0.9 µL, 0.005 mmol) and a solution of 4-nitrophenylchloroformate (0.70 mg, 0.0035 mmol) in MeCN (0.1 mL) were added in succession. The clear, light yellow solution was stirred for 40 min at room temperature, after which time HPLC indicated 80% conversion to mixed carbonate 14. In a separate vessel diamine 12 (2.5 mg, 0.0022 mmol) was dissolved in DMF (0.4 mL) under argon, to which diisopropylethylamine (1.2 µL, 0.0070 mmol) was added. This DMF solution was combined with mixed carbonate 14, and the dark blue mixture was heated to 40 °C for 60 min. The reaction was cooled and diluted with H2O (7 mL), and the solution was directly purified by reversed-phase chromatography (C18 gold column, 0→40% MeCN/water). The product-containing fractions were lyophilized to afford S10 (2.2 mg, 77% yield) as blue solid. LCAP (liquid chromatography area percent) at 254 nm: 99%; HRMS (ESI) calculated for C80H85ClN7O17S4 (M–3H)– 526.1530, observed 526.1521.

HPLC chromatogram of S10

SAFETY NOTE: While no unexpected safety hazards were encountered, Duocarmycin DM is a potent cytotoxic small molecule. Great care should be taken when handling the compound during synthetic procedures, and waste streams should be properly segregated and labeled.
(S11): To a 1-dram vial was added duocarmycin DM (4.9 mg, 0.0085 mmol) and MeCN (0.2 mL). Diisopropylethylamine (3.7 µL, 0.021 mmol) and a solution of 4-nitrophosphorylchloroformate (2.5 mg, 0.013 mmol) in MeCN (0.25 mL) were added in succession. The clear, light yellow solution was stirred for 40 min at room temperature, after which time HPLC indicated 80 % conversion to mixed carbonate 14. In a separate vessel diamine 13 (6.7 mg, 0.0056 mmol) was dissolved in DMF (1.0 mL) under argon, to which diisopropylethylamine (8.0 µL, 0.046 mmol) was added. This DMF solution was combined with mixed carbonate 14, and the dark blue mixture was heated to 65 °C for 60 min. The reaction was cooled and diluted with H2O (7 mL), and the solution was directly purified by reversed-phase chromatography (C18 gold column, 0→35 % MeCN/water). The product-containing fractions were lyophilized to afford S11 (5.5 mg, 59 % yield) as a bluish-green solid. LCAP at 250 nm: 95%; HRMS (ESI) calculated for C82H89ClN7O17S4 (M–3H)3− 535.4968, observed 535.4965.

HPLC chromatogram of S11
(16): To a 1-dram vial containing $S_{10}$ (2.1 mg, 0.013 mmol) and 15 (0.5 mg, 0.002 mmol) was added a water/t-butanol mixture (1.0 mL, 3:1 v/v). The deep blue solution was sparged with argon for 1 minute with stirring. Cupric sulfate (0.03 mg, 0.0002 mmol) and sodium ascorbate (0.02 mg, 0.001 mmol) were added from 5 mg/mL aqueous stock solutions. The reaction was stirred for 30 min at 22 °C, at which time LC/MS indicated consumption of $S_{10}$. The reaction was diluted with water (4 mL) and the solution was directly purified by reversed-phase preparative HPLC (5→90 % MeCN/0.1% aqueous (NH$_4$)HCO$_3$). The product-containing fractions were lyophilized to afford 16 (1.5 mg, 60 % yield) as a blue solid. HRMS (ESI) calculated for C$_{91}$H$_{106}$ClN$_{10}$O$_{23}$S$_4$ (M–3H)$^-$ 623.1996, observed 623.2007.

(17): To a 1-dram vial containing $S_{11}$ (5.5 mg, 0.0033 mmol) and 15 (1.3 mg, 0.0043 mmol) was added a water/t-butanol mixture (1.2 mL, 1:1 v/v). The deep blue solution was sparged with argon for 1 minute with stirring. Cupric sulfate (0.08 mg, 0.0005 mmol) and sodium ascorbate (0.45 mg,
0.0023 mmol) were added from 5 mg/mL aqueous stock solutions. The reaction was stirred for 30 min at 22 °C, at which time LC/MS indicated consumption of S11. The reaction was diluted with water (4 mL) and the solution was directly purified by reversed-phase preparative HPLC (5→90 % MeCN/0.1% aqueous (NH₄)HCO₃). The product-containing fractions were lyophilized to afford 17 (3.5 mg, 55 % yield) as a blue solid. HRMS (ESI) calculated for C₉₃H₁₁₀ClN₁₀O₂₃S₄ (M–3H)⁻ 632.5433, observed 632.5416.

(18): To a 1 dram vial was added 16 (1.0 mg, 0.00046 mmol) and N,N,N',N'-tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (0.2 mg, 0.0006 mmol). DMF (0.5 mL) was charged to the vial, followed by N,N-diisopropylethylamine (0.1 µL, 0.0006 mmol). The deep blue solution was stirred for 30 min at 22 °C, at which time LC/MS indicated consumption of 16. The reaction was precipitated into ethyl acetate (1.0 mL). The fine suspension was centrifuged, the supernatant decanted, and the pellet resuspended in diethyl ether (0.5 mL). The procedure was repeated twice with diethyl ether, and the pellet was placed under vacuum (< 0.1 Torr) for 1 h to afford 18 (0.9 mg, 85 % yield) as a dark blue solid. HRMS (ESI) calculated for C₉₅H₁₀₉ClN₁₁O₂₅S₄ (M–3H)⁻ 655.5384, observed 655.5390.
To a 1 dram vial was added 17 (2.0 mg, 0.0010 mmol) and N,N,N',N'-tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (0.6 mg, 0.002 mmol). DMF (0.5 mL) was charged to the vial, followed by N,N-diisopropylethylamine (0.3 µL, 0.002 mmol). The deep blue solution was stirred for 30 min at 22 °C, at which time LC/MS indicated consumption of 17. The reaction was precipitated into diethyl ether (1.0 mL). The fine suspension was centrifuged, the supernatant decanted, and the pellet resuspended in ethyl acetate (0.5 mL). The procedure was then repeated twice with diethyl ether, and the pellet was placed under vacuum (< 0.1 Torr) for 1 h to afford 19 (1.7 mg, 83 % yield) as a dark blue solid. HRMS (ESI) calculated for C_{97}H_{113}ClN_{11}O_{25}S_{4} (M–3H)^{3–} 664.8821, observed 664.8806.
Absorbance and normalized emission curves of 2 µM 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), 6 (F), 7 (G), 8 (H), 16 (I) and 17 (J) in pH 7.4 PBS.

Procedure for Determining Photooxidation Efficiency

Stock solutions of cyanine-umbelliferone conjugates 1-8 were diluted into pH 7.4 PBS (50 mM) to afford 1 µM solutions. Samples in 96-well plates (Corning black plate, clear bottom, polystyrene, #3603) were irradiated using a 690 ± 20 nm LED (L690-66-60, Marubeni America Co.) at a light intensity of 20 mW/cm² as measured using a power meter. Single point absorption measurements at the $\lambda_{\text{max}}$ were recorded at regular intervals (1-10 min intervals for 12-150 min depending on photobleaching rate) until ablation of the absorbance signal. Experiments were run in quadruplicate and plotted with error bars derived from the standard deviation (<5% in all cases). Half-lives were obtained by fitting the curves with one phase decay parameters ($R^2 > 0.99$ in all cases). To account for differences in the absorption spectra overlap of 1-8 with the 690 ± 20 nm LED emission profile a correction factor can be applied to the $k_{\text{rel}}$ values (Table S1). The correction factor for each compound was obtained by integrating the absorbance curve from 670 nm to 710 nm. The $k_{\text{rel}}$ values were multiplied by this correction factor relative to compound 1 to obtain the corrected values. Ideally, these values would be presented as photobleaching quantum yields,
however there are no useful water-soluble chemical actinometry standards in these wavelengths.24,25

One phase decay curves obtained from 690 nm irradiation of compounds 1–8 (A–H). Half-lives are expressed in minutes and k, the calculated rate constant, is expressed in min⁻¹.

| Compound | Uncorrected k rel | Corrected k rel |
|----------|-------------------|-----------------|
| 1        | 1.0               | 1.0             |
| 2        | 2.8               | 2.4             |
| 3        | 3.5               | 3.1             |
| 4        | 3.7               | 4.5             |
| 5        | 6.8               | 8.2             |
| 6        | 0.43              | 0.66            |
| 7        | 0.60              | 0.83            |
| 8        | 0.77              | 0.82            |

Table S1. Absorbance corrected photooxidation half-lives

Procedure for Determining Uncaging Kinetics

Stock solutions of cyanine-umbelliferone conjugates 1-8 were diluted into pH 7.4 PBS (50 mM) to afford 1 µM solutions. Samples were dispensed into 96-well plates (Corning black plate, clear bottom, polystyrene, #3603) and sealed with ThermalSeal RT film (Sigma, Z707465). The plate was immersed in an ice bath and irradiated using a 690 ± 20 nm LED at a light intensity of 20 mW/cm² (the light dose required for exhaustive bleaching ranged from 15–180 J/cm² and was

24 Kuhn, H. J.; Braslavsky, S. E.; Schmidt, R. Chemical Actinometry. Pure Appl. Chem, 1989, 61, 187.
25 Klan, P. & Wirz, J. Photochemistry of Organic Compounds: From Concepts to Practice. Ch. 3 (Wiley) 2009.
determined from the photooxidation efficiency experiment). After irradiation, the plate was equilibrated to 37 °C within the plate reader and held at that temperature for the duration of the experiment. 4-Methylumbelliferone (Umb) fluorescence was read at 30 min intervals (ex 365 nm, em 460 nm). Experiments were run in quadruplicate and plotted as % Umb released, with error bars derived from the standard deviation (<5% in all cases). A calibration curve was generated from solutions of Umb in pH 7.4 PBS and the fluorescence values were obtained using identical parameters (e.g. gain, well volume, temperature, etc) of this specific procedure. Percent yield of release was obtained from the input of the experimental fluorescence values into the equation derived from the standard calibration curve, with 1 µM defined as 100% yield. Half-lives were obtained by fitting the curves with one phase decay parameters (R² >0.99 in all cases).

Umb uncaging yield curves following 690 nm irradiation and incubation at 37 °C of compounds 1–8 (A–H). Half-lives are expressed in h and k, the calculated rate constant, is expressed in h s⁻¹. Light dose values used to achieve complete photobleaching in J/cm² are listed.

Standard calibration curve of Umb in pH 7.4 PBS.

Procedure for Determining Background Hydrolysis
Stock solutions of cyanine-umbelliferone conjugates 1-8 were diluted into pH 7.4 PBS (50 mM) to afford 10 µM solutions. Samples were dispensed into 96-well plates (Corning black plate, clear bottom, polystyrene, #3603) and sealed with ThermalSeal RT film (Sigma, Z707465). After a 3 h dark incubation at 37 °C, Umb fluorescence was read at 2.5 h intervals (ex 365 nm, em 460 nm). Experiments were run in quadruplicate and plotted as % Umb released, with error bars derived from the standard deviation (<5% in all cases). A calibration curve was generated from solutions of Umb in pH 7.4 PBS and the fluorescence values were obtained using identical parameters (e.g. gain, well volume, temperature, etc) of this specific procedure. Percent yield of release was obtained from the input of the experimental fluorescence values into an equation derived from a standard calibration curve of Umb, with 10 µM defined as 100% yield. The time to 10% yield of released Umb (t_{10%}) was obtained from the equation derived by linear regression (R^2 >0.97 in all cases).

Umb background hydrolysis release curves, expressed as % Umb released, during 37 °C incubation of compounds 1–8 (A–H)

![Graphs of umb background hydrolysis release curves](image)

Standard calibration curve of Umb in pH 7.4 PBS and linear regression equation
Procedure for LC/MS Relative Ion Analysis of Cyclopentyl Umb (3) Photolysis

A 20 mM stock of 3 in DMSO was diluted into water to yield a 10 µM solution. Phenylalanine (40 µM) was used as an internal standard. The photolysis was run for 30 min at 22 °C with 10 mW/cm² 690 ± 20 nm light in a 12-well plate. After 30 min, the reaction was transferred to a vial and heated to 37 °C. Aliquots were taken at 0 (prior to irradiation), 30, 60, and 120 min and immediately analyzed by a direct loop injection method with a Shimadzu LCMS-2020 Single Quadrupole instrument (normal resolution). The relative ion counts in Figure S2 were calculated by integrating the extracted ion chromatogram (EIC) of the m/z of 3, S12, S13, and Umb and dividing by the ion count of the phenylalanine internal standard.

![Chemical structures and ion counts](image)

**Figure S2.** Relative Ion Counts of 3, S12, S13, and Umb.
Figure S3. One phase decay curves obtained from photobleaching of 8 using either 690 nm (A) or 740 nm (B) irradiation. Experiments were performed according to the “Procedure for Determining Photooxidation Efficiency” section on page S22. Half-lives are expressed in min and k, the calculated rate constant, is expressed in min$^{-1}$.

Determination of Molar Absorption Coefficients and Absolute Fluorescence Quantum Yields

Molar absorption coefficients ($\epsilon$) were determined in PBS (pH 7.4) or 1:1 (v/v) MeOH/PBS (pH 7.4) using Beer’s law, from plots of absorbance vs. concentration. Measurements were performed in 10 mm path length quartz cuvettes (Hellma 111-QS), maintained at 25 °C, with absorbance at the highest concentration $\leq$ 0.20.

Absolute quantum yields ($\Phi_F$) were measured using a Quantaurus-QY spectrometer (Hamamatsu, model C11374).$^{26}$ This instrument is equipped with an integrating sphere to determine photons absorbed and emitted by a sample. Measurements were carried out at a concentration of 500 nM in either methanol or PBS (50 mM, pH 7.4) and self-absorption corrections were performed using the instrument software.

$^{26}$ Suzuki, K.; Kobayashi, A.; Kaneko, S.; Takehira, K.; Yoshihara, T.; Ishida, H.; Shiina, Y.; Oishic, S.; Tobita, S. Reevaluation of absolute luminescence quantum yields of standard solutions using a spectrometer with an integrating sphere and a back-thinned CCD detector. Phys Chem. Chem. Phys. 2009, 11, 9850.
Procedure for Panitumumab Conjugation\textsuperscript{27} and Purification

All steps were performed under reduced lighting. To 100 µL of 1 M PBS (pH 8.5) in a 1.5 mL microcentrifuge tube was added 200 µL of panitumumab (from a 20 mg/mL commercial stock acquired from Amgen). In a separate tube, 16 µL of an 8 mM DMSO solution of 18 or 19 was quickly premixed with 100 µL of 1 M PBS (pH 8.5), and then immediately transferred to the panitumumab solution. The resulting mixture was gently pipetted and inverted and incubated at room temperature for 1 h. The conjugation solution was eluted through a pH 7.4 PBS equilibrated Zeba spin DS column (7K MWCO, Thermo Fisher Scientific). The eluent was transferred to a Slide-A-Lyzer dialysis cassette (10K MWCO, 0.5 mL, Thermo Fisher Scientific) and immersed in pH 7.4 PBS (400 mL) for 5 h at room temperature. The cassette was transferred to fresh PBS buffer, and the dialysis was performed at 4 °C for 16 h. After dialysis, the conjugate solution was purified by preparative SEC using an Agilent 1260 Infinity HPLC equipped with a fraction collector and employing a TSKgel BioAssist DS column (Tosoh Biosciences, P/N 21464). Runs employed an isocratic gradient of pH 7.4 PBS at a flow rate of 2.5 mL/min. The majority of the first eluting peak was collected and fractionated as shown from the tailing portion of the peak, which contains conjugates possessing a DOL >4 and residual free dye. Runs employed an isocratic gradient of pH 7.4 PBS at a flow rate of 0.8 mL/min. HPLC analyses of the conjugates revealed no detectable amounts of free duocarmycin DM.

\textsuperscript{27} Bhattacharyya, S.; Patel, N.; Wei, L.; Riffle, L. A.; Kalen, J. D.; Hill, G. C.; Jacobs, P. M.; Zinn, K. R.; Rosenthal, E. Synthesis and biological evaluation of panitumumab-IRDye800 conjugate as a fluorescence imaging probe for EGFR-expressing cancers. \textit{Med. Chem. Comm.} \textbf{2014}, \textit{5}, 1337-1346.
HPLC chromatogram (280 nm and 710 nm channels) of preparative SEC purification of CyEt-Pan-Duo with fractionation pattern.

**Procedure for Cy-Pan Conjugate Spectral Analysis**

An aliquot of the purified conjugate was diluted tenfold into 1:1 (v/v) MeOH/PBS (50 mM, pH 7.4) and analyzed spectrophotometrically in a plastic UV cuvette (BrandTech Scientific, catalog number 759200). The absorption values at 280 nm ($A_{280}$) and the cyanine $\lambda_{\text{max}}$ (705 nm for 16, 745 nm for 17) were obtained, and the dye and antibody concentrations were determined from Beer’s law ($C = A/\varepsilon l$). The values obtained were $\varepsilon_{\text{dye}} = 49,000$ M$^{-1}$ cm$^{-1}$ for 16, $\varepsilon_{\text{dye}} = 70,000$ M$^{-1}$ cm$^{-1}$ for 17, and $\varepsilon_{\text{antibody}} = 200,000$ M$^{-1}$ cm$^{-1}$, corresponding to the molar absorption coefficients of the dyes and panitumumab in 1:1 (v/v) MeOH/PBS, respectively. A correction factor (CF) of 40% and 28% for 16 and 17, respectively, was applied to account for the absorption contribution of the dye at 280 nm relative to the $\lambda_{\text{max}}$ (see equation 1 below, adapted from SI reference 25). The degree of labeling (DOL), the average number of dye molecules per antibody, was determined from the quotient of dye concentration to antibody concentration. CyMe-Pan-Duo possessed a DOL of 4.1 and a protein concentration of 8.5 $\mu$M and CyEt-Pan-Duo a DOL of 4.2 and protein concentration of 7.1 $\mu$M. The antibody conjugate solutions were filtered through a 0.22 $\mu$m sterile filter (Acrodisc) and stored at 4 °C.
Equation 1: Calculations used for dye absorption correction at 280 nm and DOL calculations

\[
\text{DOL} = \frac{(A_{\lambda_{\text{max}}}/\varepsilon_{\text{dye}})}{(A_{280} - CF \times A_{\lambda_{\text{max}}})/\varepsilon_{\text{protein}}}
\]

\[\text{[protein] (mg/mL)} = \frac{(A_{280} - CF \times A_{\lambda_{\text{max}}})}{\varepsilon_{\text{protein}}} \times \text{MW}_{\text{protein}} \times \text{dilution factor}\]

Absorption spectra of free dye and conjugates. A) 16 and 17 B) CyMe-Pan-Duo and CyEt-Pan-Duo.

SDS-PAGE
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to assess the purity of the antibody conjugates. NuPAGE 4-12% Bis-Tris gels (ThermoFisher Scientific)
were loaded with 5 µg of each indicated antibody conjugate and Pan (in a 1:4 (v/v) solution of NuPAGE LDS sample buffer and 50 mM pH 7.4 PBS) and run under non-reducing conditions in 1X MES SDS buffer at 200 V for 35 min. BenchMark Pre-Stained Protein Standard (ThermoFisher Scientific) was used for molecular weight comparison. Fluorescence images were obtained using an ImageQuant LAS 4000 (GE Healthcare) with red epi light (630 nm) excitation and a 670 nm longpass emission filter. Exposure time was 300 s. Gels were stained with SimplyBlue SafeStain (ThermoFisher Scientific) for 1 h and imaged with white transillumination (1 s exposure time).

SDS-PAGE analysis of DOL 4 CyEt-Pan-Duo (1), CyMe-Pan-Duo (2), Pan (3), and molecular weight standards (4).

Cell Culture
MDA-MB-468 (EGFR overexpression) and MCF-7 (normal EGFR expression) human breast cancer cell lines were obtained from the NCI DTP, DCTD Tumor Repository. MDA-MB-468 was cultured in RPMI supplemented with 2 mM L-glutamine, 11 mM D-glucose, 24 mM sodium bicarbonate, 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B. MCF-7 was cultured in DMEM supplemented with
4 mM L-glutamine, 25 mM D-glucose, 44 mM sodium bicarbonate, 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B. Both cell lines were grown at 37 °C in an atmosphere of 20% O₂ and 5% CO₂. Stock cultures were maintained in continuously exponential growth by weekly passage of the appropriate number of cells following trypsinization with 0.25% Trypsin-EDTA (0.9 mM) in PBS.

**Fluorescence Microscopy**

MDA-MB-468 or MCF-7 cells (2 x 10⁴ cells/well) were plated on a #1.5 cover glass-bottomed 96-well plate (Cellvis) and allowed to adhere overnight. Cells were incubated with 1 µM of the indicated antibody conjugates for 1 h on ice, the media replaced, and imaged. Alternatively, cells were incubated with conjugate for 1 h on ice, the media replaced, incubated for 6 and 24 h at 37 °C, the media replaced, and imaged. Fluorescence microscopy was performed using an Evos FL Auto Cell Imaging System (ThermoFisher Scientific) at 100X magnification using a plan-fluorite oil immersion objective. Near-IR fluorescence was obtained with a Cy7 LED light cube (λ<sub>ex</sub> 710 ± 20 nm, λ<sub>em</sub> 775 ± 23 nm). Image processing was conducted with ImageJ.
Figure S4A. Fluorescence microscopy images of live MDA-MB-468 (EGFR+) cells treated with DOL 4 CyMe-Pan-Duo and CyEt-Pan-Duo for 1 h on ice or 6 and 24 h at 37 °C.
Figure S4B. Fluorescence microscopy images of live MCF-7 (EGFR−) cells treated with DOL 4 CyMe-Pan-Duo and CyEt-Pan-Duo for 1 h on ice or 6 and 24 h at 37 °C.

Cell Photolysis and Cytotoxicity

MDA-MB-468 or MCF-7 cells were seeded into 96-well plates (5 x 10⁴ cells/well) and allowed to adhere overnight. Initial seeding densities were such to ensure cells remained in exponential growth for the duration of the assay. For the continuous dose assay, media was replaced with that containing the indicated antibody conjugates, duocarmycin DM (Duo), or DMSO. Cells were placed in a 37 °C humidified incubator with an atmosphere of 1 or 20% O₂ (5% CO₂, balance N₂) and either exposed to the indicated dosage of irradiation from a 690 or 780 nm LED (15 mW/cm²) or kept dark. Irradiation times may be calculated according to the following formula:
Irradiation time (s) = \frac{\text{Light Dose (J/cm}^2\text{)}}{\text{Light Power (W/cm}^2\text{)}}

For the pre-incubation assay, media was replaced with that containing test compounds, incubated for 24 h at 37 °C in the dark, media replaced with fresh, inhibitor free media, and irradiated as above. Following a 72 h incubation period at 37 °C, 20 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) from a 5 mg/mL stock in PBS was added to each well and incubated for 4 h at 37 °C. Media was removed, 100 µL of DMSO added to each well to solubilize MTT formazan, and absorbance at 550 nm was recorded using a microplate reader. Drug effects were expressed as % cell viability relative to the DMSO (no inhibitor) control. Half maximal inhibitory concentrations (IC\textsubscript{50}s) were obtained from sigmoidal curve fits of % viability vs. concentration data using GraphPad Prism 6. All experiments were conducted in quadruplicate, with error bars representing the standard deviation.
Figure S5. Dose response curves for MDA-MB-468 (EGFR+) cells (A,C) and MCF-7 (EGFR-) cells (B,D) irradiated with 20 J/cm² (15 mW/cm², 22 min) of 690 nm (A,B) or 780 nm (C,D) light. Error bars represent the standard deviation resulting from quadruplicate measurements.
**Table S2. In vitro efficacy at 1% O₂.**

| Compound     | Cell Line & Irradiation | IC₅₀ (nM)ᵃ | Fold Δᶜ |
|--------------|--------------------------|------------|---------|
|              | MDA-MB-468               | 0.048 ± 0.0014 | 1.0     |
|              |                          | 13 ± 2.0   | 270     |
|              |                          | 55 ± 1.7   | 1200    |
|              |                          | 84 ± 2.2   | 1700    |
| CyMe-Pan-Duo |                          | 0.034 ± 0.00080 | 1.0     |
|              | MDA-MB-468               | 18 ± 1.0   | 520     |
|              |                          | 58 ± 1.5   | 1700    |
|              |                          | > 100      | > 2900  |
| CyEt-Pan-Duo |                          | ±hv        |         |
|              | MDA-MB-468               | 0.013 ± 0.00040 |         |
|              |                          | 0.023 ± 0.0021 |         |
| Duo          | MDA-MB-468               | >100       |         |
|              |                          |            |         |
| Pan          | MDA-MB-468               | >100       |         |
|              |                          |            |         |
|              | MCF-7                    |            |         |

²²0 J/cm² (15 mW/cm², 22 min); ³Average IC₅₀ value ± standard deviation (n = 4); ⁴Ratio value / (MDA-MB-468 +hv)

**Animal Tumor Models**

All *in vivo* procedures were conducted in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), US National Research Council, and approved by the National Cancer Institute/NIH Animal Care and Use Committee. Six- to 8-week-old female homozygote athymic nude mice were purchased from Charles River Laboratories International, Inc. (NCI-Frederick). During treatment, mice were anesthetized with isoflurane. MDA-MB-468 cells (6 x 10⁶) were injected subcutaneously in the right dorsum of each mouse. Experiments were performed at 9-10 days after cell injection. Tumors reaching approximately 5-7 mm were selected for the study.
**Biodistribution**

Serial ventral and dorsal fluorescence images of MDA-MB-468 tumor-bearing mice were obtained before and 0, 1, 3, 6, 9, 24, 48, 72, 96, 120, 144, and 168 h after i.v. injection of 100 µg of CyEt-Pan-Duo (DOL 1, 2, or 4) via the tail vein. Images were collected with a Pearl Imager (LI-COR Biosciences) using a 800 nm fluorescence channel. Regions of interest (ROIs) were placed on the fluorescent images with a white light reference to measure fluorescence intensities of the tumor, the liver, and the left dorsum (i.e. background tissue on the opposite side of the target). Pearl Cam software (LI-COR Biosciences) was used to calculate the average fluorescence intensity within each ROI. Target-to-background ratio (TBR) was calculated using the following formula ($n = 5$):

$$\text{TBR} = \frac{\text{mean target intensity} - \text{mean background intensity}}{\text{mean non-target intensity} - \text{mean background intensity}}$$

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**Figure S6A.** Dorsal fluorescence images of MDA-MB-468 tumor-bearing mice treated with 100 µg of CyEt-Pan-Duo by i.v. injection at the indicated DOL. Tu = tumor. $n = 5$ mice per group.
**Figure S6B.** Ventral fluorescence images of mice in Figure S6A treated with 100 µg of CyEt-Pan-Duo by i.v. injection at the indicated DOL. Li = liver, Bl = bladder. n = 5 mice per group.
Figure S6C. Quantification of images in Figure Figures S6A and S6B. Fluorescence intensity at the tumor (A), TBR (B), fluorescence intensity at the liver (C), and LBR (D) are plotted as a function of time post-conjugate injection. Error bars represent the standard error of the mean (n = 5).

In Vivo Near-IR Irradiation
CyEt-Pan-Duo (DOL 4, 100 µg) was administered i.v. via the tail vein to MDA-MB-468 tumor-bearing mice (n = 5 mice per condition). Four days post-injection, the tumor was exposed to 0, 8, 16, 24, 40, and 80 J/cm² doses of 690 ± 10 nm (0.8 W/cm²) using a laser system (BWF5-690-8-600-0.37; B&W Tek, Inc.). A second cohort was maintained without irradiation. Immediately after exposing tumors to the indicated dose, serial dorsal fluorescence images of irradiated and corresponding unirradiated mice were obtained with a Pearl Imager using a 800 nm fluorescence
channel. Regions of interest (ROIs) were placed on the tumor and average fluorescence intensity determined using Pearl Cam software, with the intensity of the 0 J/cm² dose point set to 100%.

**Figure S7.** (A) Fluorescence images before and after external irradiation at the tumor site using the near-IR (800 nm) signal of CyEt-Pan-Duo (DOL 4). (B) Quantification of fluorescence images. *** $p \leq 0.001$, **** $p \leq 0.0001$. Error bars represent the standard error of the mean ($n = 5$).

**In Vivo Efficacy**

MDA-MB-468-*luc* tumor-bearing mice (right dorsum tumor xenograft) were randomized into 5 groups of at least 9 animals per group. On day 10 post-cell injection, the following treatments were conducted: vehicle treated (A), i.v. injection (tail vein) of 100 µg CyEt-Pan-Duo (B), and i.v. injection of 10 µg (C), 30 µg (D), and 100 µg (E) CyEt-Pan-Duo followed by irradiation. All irradiations consisted of 80 J/cm² (0.8 W/cm²) of 690 nm light applied to the tumor region on day 14 post-cell injection. Fluorescence images, bioluminescence images, and tumor volume measurements were obtained immediately prior to and following irradiation, and at 1, 2, 3, 4, 5, 6, and 7 d post-irradiation. White light images and 800 nm fluorescence images were obtained with
a Pearl Imager. Bioluminescence images were obtained with a Photon Imager (Biospace Lab) using intraperitoneally injected D-luciferin (15 mg/mL, 200 µL, Gold Biotechnology). For analyzing the bioluminescence signal, an ROI of consistent size was placed over the entire tumor. The bioluminescence signal immediately prior to irradiation was set to 100%.

**Figure S8A.** Fluorescence images (800 nm) pre- and post-irradiation with 80 J/cm$^2$ of 690 nm light. The post-hv timepoint was recorded immediately following irradiation. Mice were treated as follows: vehicle treated control (A), 100 µg CyEt-Pan-Duo (B), and 10 µg (C), 30 µg (D), and 100 µg (E) CyEt-Pan-Duo + hv ($n = 9$ mice per group).

**Figure S8B.** Bioluminescence images at pre- and post-irradiation with 80 J/cm$^2$ of 690 nm light. Mice were treated as follows: untreated control (A), 100 µg CyEt-Pan-Duo (B), and 10 µg (C), 30 µg (D), and 100 µg (E) CyEt-Pan-Duo + hv ($n = 9$ mice per group).
Figure S9. Average body weight of mice treated in Figure 4 and S8 as a function of time post-irradiation. Mice were treated as follows: vehicle-treated control (black), 100 µg CyEt-Pan-Duo (red), and 10 µg (green), 30 µg (purple), and 100 µg (orange) CyEt-Pan-Duo + hv. Error bars represent the standard error of the mean (n = 9 mice per group).
\(^1\)H and \(^{13}\)C NMR Spectra
**Cartesian Coordinates**

*C4'-dimethylamine heptamethine cyanine: E(MP2-PCM) = -1479.98085*

| ATOM | CHARGE | X          | Y          | Z           |
|------|--------|------------|------------|-------------|
| C    | 6.0    | 0.0896965894 | 0.0662845212 | -0.2781299338 |
| C    | 6.0    | -1.0676205807 | -0.387571451 | -0.9970884686 |
| C    | 6.0    | 1.2505338601  | 0.4573599771 | -1.0251319984 |
| C    | 6.0    | -2.3325545267 | -0.2563589907 | -0.4426516727 |
| C    | 6.0    | 2.5072621829  | 0.2971622702 | -0.4540279745 |
| H    | 1.0    | -2.376357619  | 0.2075807858 | 0.5384571669 |
| H    | 1.0    | 2.5400908835  | -0.1625140368 | 0.5295551214 |
| C    | 6.0    | -3.5510280083 | -0.6205635007 | -1.0535500188 |
| C    | 6.0    | 3.7215336479  | 0.6252652074 | -1.0867455636 |
| H    | 1.0    | -3.5073184224 | -1.1517108313 | -1.995577865 |
| H    | 1.0    | 3.6688790811  | 1.1540176872 | -2.0339904532 |
| C    | 6.0    | -4.8104109697 | -0.3540252096 | -0.5407061255 |
| C    | 6.0    | 4.9865380107  | 0.3309245365 | -0.5966250660 |
| N    | 7.0    | -5.9497702544 | -0.7635840889 | -1.1756269750 |
| N    | 7.0    | 6.1183074423  | 0.6921221437 | -1.2649146332 |
| C    | 6.0    | -5.9558799884 | -1.5207262154 | -2.4174835738 |
| C    | 6.0    | 6.1147210409  | 1.4114597915 | -2.5301621112 |
| C    | 1.0    | -6.9782560870 | -1.8122761787 | -2.6509478870 |
| C    | 1.0    | 7.1420187061  | 1.6075381953 | -2.830697931 |
| C    | 1.0    | -5.3492418405 | -2.4231354205 | -2.3034044160 |
| C    | 1.0    | 5.6217646701  | 0.8136931973 | -3.3026199454 |
| H    | 1.0    | -5.5567748323 | -0.9159709368 | -3.2377286701 |
| H    | 1.0    | 5.5889801630  | 2.3633988617 | -2.4160928809 |
| C    | 6.0    | -7.1039095296 | -0.3293759681 | -0.5012164606 |
| C    | 6.0    | 7.2778896532  | 0.2593936162 | -0.5950375451 |
| C    | 6.0    | -8.4460435019 | -0.5177031318 | -0.8338447374 |
| C    | 6.0    | 8.6169521892  | 0.4173540468 | -0.9538127898 |
| H    | 1.0    | -8.7584210790 | -1.0555745674 | -1.7246514634 |
| H    | 1.0    | 8.9242520125  | 0.9301427717 | -1.8609239243 |
| C    | 6.0    | -9.4013667394 | 0.0268599652 | 0.0344656546 |
| C    | 6.0    | 9.5763906048  | -0.1205108785 | -0.0861877323 |
| H    | 1.0    | -10.4565575485 | -0.1003670557 | -0.1961345131 |
| H    | 1.0    | 10.6298447962 | -0.0160467744 | -0.3351621825 |
| C    | 6.0    | -9.0245353114 | 0.7310413372 | 1.1856872292 |
| C    | 6.0    | 9.2056466127  | -0.7878477332 | 1.0889432363 |
| H    | 1.0    | -9.7893776310 | 1.1424900135 | 1.8397367581 |
| H    | 1.0    | 9.9738940890  | -1.194676385 | 1.7418690963 |
| C    | 6.0    | -7.6691050853 | 0.9083595214 | 1.4997579519 |
| C    | 6.0    | 7.8530301577  | -0.9342148224 | 1.4289191776 |
| H    | 1.0    | -7.3785859266 | 1.4571393652 | 2.3942820555 |
| H    | 1.0    | 7.5679572271  | -1.4537139834 | 2.3423683026 |
| C    | 6.0    | -6.7119375577 | 0.3747739602 | 0.6430904412 |
| C    | 6.0    | 6.8914980172  | -0.4062133914 | 0.5734505890 |
| C    | 6.0    | -5.2037437654 | 0.4029380802 | 0.7264424156 |
| C    | 6.0    | 5.3850091003  | -0.4053796753 | 0.6797403544 |
| C    | 6.0    | -4.7474612581 | -0.3354239670 | 1.9960654322 |
| C    | 6.0    | 4.8561799393  | -1.8481771572 | 0.6973460505 |
| C    | 6.0    | -4.7022914452 | 1.8551287038 | 0.7072919543 |
| C    | 6.0    | 4.9591347067  | 0.3678466529 | 1.9389816603 |
C4'-diethylamine heptamethine cyanine: $E_{\text{MP2-PCM}} = -1558.31457$

COORDINATES OF ALL ATOMS ARE (ANGS)

| ATOM | CHARGE | X     | Y     | Z     |
|------|--------|-------|-------|-------|
| C    | 6.0    | 0.0908988442 | 0.0631388213 | -0.3785253721 |
| C    | 6.0    | -1.0723450828 | -0.3716528364 | -1.0941453913 |
| C    | 6.0    | 1.2577805700 | 0.4414073891 | -1.1147085309 |
| C    | 6.0    | -2.3250255193 | -0.2558427418 | -0.5064556395 |
| C    | 6.0    | 2.5034016285 | 0.3023138626 | -0.5103599476 |
| H    | 1.0    | -2.3367035584 | 0.1797508779 | 0.4887566233 |
| H    | 1.0    | 2.5075041079 | -0.1248954271 | 0.4882978470 |
| C    | 6.0    | -3.5590387197 | -0.6013686507 | -1.091224333 |
| C    | 6.0    | 3.7306140470 | 0.6125959947 | -1.119487034 |
| H    | 1.0    | -3.5460942931 | -1.102727381 | -2.0545362597 |
| H    | 1.0    | 3.7055132488 | 1.1048504946 | -2.0876113257 |
| C    | 6.0    | -4.8009835172 | -0.3440783581 | -0.5302737789 |
| C    | 6.0    | 4.9808819897 | 0.3325652715 | -0.5804540328 |
| N    | 7.0    | -5.9620031343 | -0.7191347803 | -1.1441297358 |
| N    | 7.0    | 6.1324101545 | 0.6686130916 | -1.2233407498 |
| C    | 6.0    | -6.0126568607 | -1.4249176191 | -2.4153295619 |
| C    | 6.0    | 6.1718206744 | 1.3481156380 | -2.5106143988 |
| H    | 1.0    | -7.0471937854 | -1.6759100771 | -2.6415874537 |
| H    | 1.0    | 7.2085698217 | 1.5390498958 | -2.7800037021 |
| H    | 1.0    | -5.4306383599 | -2.3482200112 | -2.3502999744 |
| Element | Angle | x     | y     | z     |
|---------|-------|-------|-------|-------|
| C       | 6.0   | -0.3153982498 | 1.9100339832 | 2.6358988858 |
| H       | 1.0   | 0.1476299457  | 2.7927551734 | 3.0877796736  |
| H       | 1.0   | -0.6560331158 | 1.2621990658 | 3.4472136390  |
| H       | 1.0   | -1.1836156780 | 2.2349017258 | 2.0548483361  |
| C       | 6.0   | 0.5418911399  | -1.6232299879 | 2.7232621816  |
| H       | 1.0   | 0.8986947990  | -0.9322869806 | 3.4908802505  |
| H       | 1.0   | 1.3986900113  | -1.9847972482 | 2.1469032689  |
| H       | 1.0   | 0.0837952152  | -2.4771400510 | 3.2319614507  |