Supplementary Information

Application of bioorthogonal hetero-Diels–Alder cycloaddition of 5-arylidene derivatives of 1,3-dimethylbarbituric acid and vinyl thioether for imaging inside living cells

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**Experimental Section**

**Chemistry**

Unless specified, all reagents and starting materials were purchased from commercial sources and used as received without purification. All reactions were monitored by thin-layer chromatography on 0.25 mm silica plates (F-254) visualized with UV light. Melting points were determined on a Boetius hot stage apparatus. IR spectra were measured using a Nicolet IR 200 FT-IR Thermo Scientific (with diamond stand) as film or powder. $^1$H and $^{13}$C NMR spectra were recorded in CDCl$_3$, DMSO-$d_6$, D$_2$O or CD$_3$CN on Bruker Avance II 600 MHz ($^1$H: 600 MHz, $^{13}$C: 151 MHz) or 300 MHz ($^1$H: 300.18 MHz, $^{13}$C: 75.48 MHz). Microanalyses were performed with Euro EA 3000 Elemental Analyzer or compounds purity was assessed by performing an HRMS (ESI-QTOF) spectra.

5-[(4-Hydroxyphenyl)methylidene]-1,3-dimethyl-1,3-diazinane-2,4,6-trione (2)

Compound 2 was prepared according to the procedure described in the literature$^{23}$: To a stirred solution of 1,3-dimethylbarbituric acid 1 (0.465 g, 3 mmol) in water (8 mL) was added 4-hydroxybenzaldehyde (0.366 g, 3 mmol) rapidly and all at once. After 15 min of stirring at room
temperature, the solid product 2 was isolated by simple filtration and dried. Compound 2 needed no further purification and was obtained with 95% yield as a pale yellow solid.

**4-[(1,3-Dimethyl-2,4,6-trioxo-1,3-diazinan-5-ylidene)methyl]benzoic acid (3)**

![Chemical Structure of 3](image3.png)

Compound 3 was obtained according to the procedure described in the literature\textsuperscript{22}: Solid 1,3-dimethylbarbituric acid (0.465 g, 3 mmol), 4-carboxybenzaldehyde (0.448 g, 3 mmol) and amidosulfonic acid (0.298 g) were ground for 10 minutes with a mortal and pastel. It was then placed in a desiccator for 2 hours. The ground mixture was then dissolved in DMSO and poured into 50 mL distilled water. The formed precipitate was filtered, washed with boiling water and MeOH and dried in vacuum. Compound 3 needed no further purification and was obtained with 89% yield as a light yellow solid.

**5-[(4-Aminophenyl)methylidene]-1,3-dimethyl-1,3-diazinane-2,4,6-trione (4)**

![Chemical Structure of 4](image4.png)

5-[(4-Aminophenyl)methylidene]-1,3-dimethyl-1,3-diazinane-2,4,6-trione (4)
*p*-Aminobenzaldehyde polymer (1.815 g, 15 mmol) and 100 mL of water were placed in a 150 mL round-bottomed flask on a magnetic stirrer. Then, to carry out the polymer hydrolysis, the mixture was acidified by adding approx. 5 mL conc. HCl. The suspension was stirred vigorously for 10 minutes until the polymer dissolved and hydrolyzed. 1,3-Dimethylbarbituric acid 1 (2.31 g, 15 mmol) was added to the solution with vigorously stirring. The mixture was then neutralized with saturated sodium bicarbonate solution, added gradually over about 15 min until the foaming mixture ceased. At the same time, the pH of the solution was checked to obtain pH = 7. The resulting dark red precipitate was filtered off and recrystallized from ethanol. Compound 4: red solid, mp: > 360 °C (2.75 g, 67%); IR (powder) \( \nu \) 3424, 3342, 3234, 1632, 1608, 1498, 1449, 1412, 1304, 1336, 1182, 1157, 1077 cm\(^{-1}\); \(^1\)H NMR (600 MHz, DMSO-\(d_6\)) \( \delta \) 8.33 (d, \( J = 9.0 \) Hz, 2H), 8.18 (s, 1H), 7.03 (br, 2H), 6.64 (d, \( J = 9.0 \) Hz, 2H), 3.22 (s, 6H); \(^{13}\)C NMR (151 MHz, DMSO-\(d_6\)) \( \delta \) 163.8, 161.7, 157.2, 156.5, 151.7, 140.5, 120.6, 113.6, 109.0, 29.0, 28.3; HRMS (ESI-QTOF) m/z \([M+Na]^+\) calcd for C\(_{13}\)H\(_{13}\)N\(_3\)NaO\(_3\) 282.0855, found 282.0849.

5-{[4-(Hydroxymethyl)phenyl]methylidene}-1,3-dimethyl-1,3-diazinane-2,4,6-trione (5)

To a stirred solution of 1,3-dimethylbarbituric acid 1 (0.465 g, 3 mmol) in water (10 mL) was added aldehyde 11 (0.405 g, 3 mmol) all at once. After 2 h of stirring at room temperature, the solid product 5 was isolated by simple filtration and dried. Compound 5 needed no further
purification: white solid, mp: 148-150 °C (0.694g, 85%); IR (powder) ν 3352, 2963, 2922, 1727, 1662, 1577, 1553, 1468, 1430, 1380, 1362, 1149, 1082 cm⁻¹; \(^1^H\) NMR (600 MHz, DMSO-d₆) δ 8.35 (s, 1H), 8.08 (d, \(J\) 7.5 Hz, 2H), 7.43 (d, \(J\) 7.5 Hz, 2H), 4.59 (s, 3H), 3.24 (s, 3H), 3.20 (s, 3H); \(^1^C\) NMR (151 MHz, DMSO-d₆) δ 162.8, 160.9, 156.1, 151.6, 148.3, 133.9, 131.5, 126.2, 118.5, 63.0, 29.1, 28.5; Anal. Calcd for C₁₄H₁₄N₂O₄: C, 61.31; H, 5.14; N, 10.21. Found: C, 61.34; H, 5.19; N, 10.26.

\(\{4-[(1,3\text{-Dimethyl-2,4,6-trioxo-1,3-diazinan-5-ylidene)methyl]phenoxy}acetaldehyde (6)\)

To a solution of acetal 15 (1.61 g, 4.3 mmol) in 15 mL of DCM, trifluoroacetic acid (9.92 g, 6.7 mL, 86 mmol) was added dropwise. The reaction mixture was stirred for 24 h at room temperature (monitored by TLC, hexane/ethyl acetate = 2:1). Then 50 mL of water was added and mixture was vigorously stirred. Precipitated yellow solid was filtered off and crystallized from ethanol. Compound 6: yellow solid, mp: 159-161 °C, (1.18 g, 91%); IR (powder) ν 3452, 3108, 3063, 2966, 1651, 1568, 1542, 1442, 1365, 1271, 1200, 1115, 1085, 1041 cm⁻¹; \(^1^H\) NMR (600 MHz, DMSO-d₆) δ 9.69 (s, 1H), 8.31 (s, 1H), 8.30 (d, \(J\)=8.3 Hz, 2H), 7.07 (d, \(J\)=7.0 Hz, 2H), 5.05 (s, 2H), 3.23 (s, 3H), 3.21 (s, 3H); \(^1^C\) NMR (151
MHz, DMSO-d$_6$) δ 198.5, 163.2, 162.1, 161.2, 156.2, 156.0, 151.6, 137.9, 137.5, 126.2, 125.6, 116.3, 115.8, 114.9, 88.3, 73.1, 72.4, 29.1, 28.5; **HRMS** (ESI-QTOF) m/z [M+Na]$^+$ calcd for C$_{15}$H$_{14}$N$_2$NaO$_5$ 325.0800, found 325.0797.

4-[{(1,3-Dimethyl-2,4,6-trioxo-1,3-diazinan-5-ylidene)methyl]phenoxy}acetic acid (7)

To a stirred solution of $N,N$-dimethylbarbituric acid 1 (1.56 g, 10 mmol) in 20 mL of water aldehyde 16 (1.80 g, 10 mmol) was added. The mixture was allowed to stir at 60 °C for 24 h, then the precipitated light-yellow solid was filtered off and washed with water, pale yellow solid (3.02 g, 95%); mp 212-216 °C; IR (powder) ν 3431, 1729, 1650, 1574, 1550, 1507, 1474, 1433, 1360, 1319, 1228, 1184, 1085 cm$^{-1}$; $^1$H NMR (600 MHz, DMSO-d$_6$) δ 8.32 (s, 1H), 8.29 (d, $J$ = 9.0 Hz, 2H), 7.04 (d, $J$ = 9.0 Hz, 2H), 4.84 (s, 2H), 3.23 (s, 3H), 3.21 (s, 3H); $^{13}$C NMR (151 MHz, DMSO-d$_6$) δ 170.1, 163.0, 162.3, 161.2, 156.0, 151.6, 137.5, 126.1, 116.2, 114.8, 65.0, 29.1, 28.5; **HRMS** (ESI-QTOF) m/z [M+Na]$^+$ calcd for C$_{15}$H$_{14}$N$_2$NaO$_6$ 341.0749, found 341.0742.
2-(4-((1,3-Dimethyl-2,4,6-trioxotetrahydropyrimidin-5(6H)-ylidene)methyl)phenoxy)ethylaminium 2,2,2-trifluoroacetate (8)

To a mixture of 6 mL of trifluoroacetic acid and 14 mL of DCM was added Boc-protected compound 25 (0.40 g, 0.99 mmol). The reaction mixture was stirred for 2 h at room temperature (monitored by TLC). Then it was brought to dryness by high vacuum. The crude product was redissolved in 1 mL of anhydrous methanol and 10 mL of anhydrous diethyl ether were added to precipitate the desired product 8. Compound 8 was collected through filtration and dried under high vacuum as pale yellow solid, mp: 189-190 °C (335.0 mg, 87%); IR (powder) ν 3067, 2968, 1677, 1578, 1561, 1509, 1439, 1256, 1206, 1180, 1131 cm⁻¹; \( ^1\text{H NMR} \) (300 MHz, DMSO-d₆) δ 8.34 (d, \( J = 9.0 \) Hz, 2H), 8.33 (s, 1H), 8.08 (br, 3H), 7.11 (d, \( J = 9.1 \) Hz, 2H), 4.30 (t, \( J = 5.0 \) Hz, 2H), 3.28 (m, 2H), 3.24 (s, 3H), 3.21 (s, 3H); \( ^{13}\text{C NMR} \) (151 MHz, DMSO-d₆) δ 162.6, 161.8, 160.8, 158.2 (q, \( J_{CF} = 31.3 \) Hz), 155.5, 151.1, 137.1, 125.8, 115.9, 114.4, 64.8, 38.2, 28.7, 28.0; HRMS (ESI-QTOF) m/z [M-CF₃COO]⁺ calcd for C₁₅H₁₈N₃O₄ 304.1297, found 304.1303.
2-(4-((1,3-Dimethyl-2,4,6-trioxotetrahydropyrimidin-5(6H)-ylidene)methyl)phenoxy)butylaminium 2,2,2-trifluoroacetate (9)

To a mixture of 6 mL of trifluoroacetic acid and 14 mL of DCM was added Boc-protected compound 26 (0.43 g, 0.99 mmol). The reaction mixture was stirred for 2 h at room temperature (monitored by TLC). Then it was brought to dryness by high vacuum. The crude product was redissolved in 1 mL of anhydrous methanol and 10 mL of anhydrous diethyl ether were added to precipitate the desired product 9. Compound 9 was collected through filtration and dried under high vacuum as pale yellow solid, mp: 173-175 °C (374.6 mg, 85%); IR (powder) ν 2955, 1658, 1573, 1541, 1509, 1432, 1260, 1184, 1134 cm⁻¹; ¹H NMR (300 MHz, DMSO-d₆) δ 8.32 (d, J = 9.0 Hz, 2H), 8.30 (s, 1H), 7.82 (br, 3H), 7.04 (d, J = 9.0 Hz, 2H), 4.12 (t, J = 5.0 Hz, 2H), 3.24 (s, 3H), 3.19 (s, 3H), 2.86 (m, 2H), 1.79 (m, 2H), 1.69 (m, 2H); ¹³C NMR (151 MHz, DMSO-d₆) δ 163.2, 163.1, 161.3, 156.2, 151.6, 137.9, 125.7, 115.9, 114.8, 67.9, 39.0, 29.1, 28.5, 25.9, 24.3; HRMS (ESI-QTOF) m/z [M-CF₃COO]⁺ calcd for C₁₂H₂₂N₃O₄ 332.1610, found 332.1616.

4-(hydroxymethyl)benzaldehyde (11)
Alcohol 11 was synthesized by reduction of terephthalaldehyde using sodium borohydride\textsuperscript{31}.

To a suspension of terephthalaldehyde 10 (2.00 g, 14.92 mmol, 1 eq) in anhydrous ethanol (50 mL) in a 250 mL Erlenmeyer flask cooled to 0°C in an ice bath, powdered sodium borohydride (0.200 g, 5.22 mmol; 0.33 eq) was added in one portion, and the reaction continued to stir at 0°C. The reaction was allowed to stir for one hour, and was followed by TLC with 1:1 ethyl acetate and hexanes as the eluent. After 1 h, the reaction was warmed to room temperature and water (50 mL) and next ethyl acetate was added to this mixture, and it was vigorously shaken. The aqueous and organic layers were separated and the aqueous layer was extracted 3 \(\times\) 20 mL with ethyl acetate. The combined organic layers were dried with MgSO\(_4\) and evaporated under reduced pressure. Crude mixture was purified by flash chromatography (0 to 30\% ethyl acetate/hexanes) to afford 91\% of the 11 product as a white solid.

\textbf{4-(2,2-diethoxyethoxy)benzaldehyde (14)}

![4-(2,2-diethoxyethoxy)benzaldehyde (14)]

Compound 14 was synthesized according to a procedure described in the publication\textsuperscript{26}.

To a stirred suspension of 98\% sodium hydride (2.5 g, 100 mmol) in DMF (100 mL) was added a solution of 4-hydroxybenzaldehyde 12 (10 g, 82 mmol) in DMF (100 mL) slowly dropwise at room temperature and stirred for 30 min and bromoacetalddehyde diethylacetal 13 (19.7 g, 100 mmol) was added to the reaction mixture, and the mixture was heated at 60 °C for 48 h. The reaction mixture was cooled to room temperature, quenched with water (200 mL), and extracted with
ethyl acetate (3×300 mL). The combined organic layers were washed with brine, dried over MgSO₄, and concentrated. The crude compound was purified by chromatography using EtOAc/pet. ether (1:2) as eluent to yield the compound 14 (12.65 g, 58%) as a brown colored liquid.

5-{{[4-(2,2-Diethoxyethoxy)phenyl]methylidene}-1,3-dimethyl-1,3-diazinane-2,4,6-trione (15)

To a solution of 1,3-dimethylbarbituric acid 1 (1.0 g, 6.4 mmol) in water (25 mL) was added aldehyde 14 (1.77 g, 7.45 mmol). The reaction was carried out for 3 h at room temperature with vigorously stirring. The product 15 was collected through filtration and dried under high vacuum. Compound 15 was used in subsequent reaction without purification. 15: yellow solid, mp: 130-132 °C (1.61 g, 67%); IR (powder) ν 3105, 3068, 2964, 1545, 1445, 1365, 1274, 1205, 1114, 1081, 1040 cm⁻¹; ¹H NMR (600 MHz, CDCl₃) δ 8.44 (s, 1H), 8.24 (d, J = 9.0 Hz, 2H), 6.93 (d, J = 9.0 Hz, 2H), 4.79 (t, J = 5.0 Hz, 1H), 4.03 (d, J = 5.0 Hz, 2H), 3.71 (dq, J = 9.3, 7.0 Hz, 2H), 3.58 (dq, J = 9.3, 7.0 Hz, 2H), 3.34 (s, 3H), 3.32 (s, 3H), 1.18 (t, J = 7.0 Hz, 6H); ¹³C NMR (151 MHz, CDCl₃) δ 163.3, 163.1, 161.0, 158.9, 151.4, 137.9, 125.8, 114.6, 114.5, 100.2, 68.7, 62.9, 29.1, 28.4, 15.3; Anal. Calcd for C₁₉H₂₄N₂O₆: C, 60.63; H, 6.43; N, 7.44. Found: C, 60.69; H, 6.52; N, 7.51.
(4-formylphenoxy)acetic acid (16)

Compound 16 was synthesized according to a procedure described in literature\textsuperscript{26}. To a stirred solution of \textit{p}-hydroxybenzaldehyde 12 (1.22 g, 10 mmol) and bromoacetic acid (1.67 g, 12 mmol) in water (20 mL) was dropwise added 20 mL water solution of sodium hydroxide (0.96 g, 24 mmol) within 30 min at room temperature. Stirring of the reaction mixture continued for 1.5 h under reflux and then cooled to room temperature. The solution was acidified with concentrated hydrochloric acid to pH 1–2, and the resulting solid was filtered and recrystallized from 96% ethanol to give \textit{p}-formylphenoxyacetyl acid as a white solid (1.40 g, 78%).

\[ \textit{p}-\text{formylphenoxyacetyl acid} \]

tert-Butyl (2-hydroxyethyl)carbamate (19) and tert-butyl (4-hydroxybutyl)carbamate (20)

Compounds 19 and 20 were synthesized according to the procedure from the publication\textsuperscript{24}. To 50 mL of anhydrous DCM was added di-\textit{t}-butyl carbonate (3.57 g, 16.4 mmol). Ethanolamine 17 (1.00 g, 16.4 mmol) was added dropwise to the solution at 0 °C over 10 min. The reaction was
allowed to warm to room temperature and stirred for 2 h. The solvent was removed under reduced pressure and the crude product was purified via column chromatography with hexane and ethyl acetate (3:2, v/v) to yield the product 19 as colorless oil (98%).

tert-Butyl (2-bromoethyl)carbamate (21) and tert-Butyl (4-bromobutyl)carbamate (22)

Alcohols 19 and 20 have been converted to bromides 21 and 22 via mesylate by the modified procedure described in literature\textsuperscript{25}: A flask was charged with \textit{tert}-butyl (2-hydroxyethyl)carbamate 19 (0.81 g, 5.0 mmol), methanesulfonyl chloride (0.47 mL, 6.0 mmol), and DCM (15 mL). To this stirring solution was added NEt\textsubscript{3} (0.9 mL, 6.5 mmol). Stirring was continued for 45 min, and then LiBr (4.35 g, 50 mmol) and acetone (15 mL) were added. The reaction mixture was stirred for an additional 24 h, and then the solvents were removed by rotary evaporation. The contents were partitioned between Et\textsubscript{2}O (30 mL) and H\textsubscript{2}O (20 mL), and the Et\textsubscript{2}O layer was washed with brine, dried (MgSO\textsubscript{4}), filtered, evaporated to dryness, and placed under vacuum to yield 1.09 g (4.95 mmol, 99%) of 21 as a white solid.
Compounds 23 and 24 were synthesized according to a modified procedure from the publication26:

To a stirred suspension of 98% sodium hydride (0.25 g, 10 mmol) in DMF (10 mL) was added a solution of 4-hydroxybenzaldehyde (1 g, 8.2 mmol) in DMF (10 mL) slowly dropwise at room temperature and stirred for 30 min and bromide 21 (2.24 g, 10 mmol) was added to the reaction mixture, and the mixture was heated at 60 °C for 48 h. The reaction mixture was cooled to room temperature, quenched with water (20 mL), and extracted with ethyl acetate (3·30 mL). The combined organic layers were washed with brine, dried over MgSO₄, and concentrated. The crude compound was purified by chromatography using EtOAc/pet. ether = 1:2 as eluent to yield the compound 23 (1.565 g, 59%) as a white solid.

tert-Butyl[{4-[(2,4,6-trioxo-1,3-dimethyl-1,3-diazinan-5-ylidene)methyl]phenoxy}ethyl]carbamate (25)
To a solution of 1,3-dimethylbarbituric acid 1 (0.47 g, 3.00 mmol) in water (15 mL) was added aldehyde 23 (0.80 g, 3.00 mmol). The reaction was carried out for 24 h at 60 °C with vigorously stirring. After this time, the reaction mixture was cooled down to room temperature. The product 25 was collected through filtration and dried under high vacuum. Compound 25 was used in subsequent reactions without purification.

25: yellow solid, mp: 129-130 °C (798.8 mg, 67%); IR (powder) 3372, 3014, 2982, 2930, 2870, 1725, 1689, 1665, 1567, 1533, 1458, 1437, 1281, 1177, 1153, 1086, 1055 cm\(^{-1}\); \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 8.32 (d, \(J = 8.9\) Hz, 2H), 8.31 (s, 1H), 7.06 (d, \(J = 9.0\) Hz, 2H), 7.03 (s, 1H), 4.10 (t, \(J = 5.7\) Hz, 2H), 3.35 (m, 2H), 3.23 (s, 3H), 3.21 (s, 3H), 1.39 (s, 9H); \(^{13}\)C NMR (151 MHz, DMSO-\(d_6\)) \(\delta\) 162.7, 162.6, 160.8, 155.7, 151.1, 137.4, 125.2, 115.4, 114.3, 77.9, 66.9, 30.7, 28.6, 28.2, 28.0; HRMS (ESI-QTOF) m/z \([M+Na]^+\) calcd for C\(_{20}\)H\(_{25}\)N\(_3\)NaO\(_6\) 426.1641, found 426.1635.

**tert-Butyl(4-[2,4,6-trioxo-1,3-dimethyl-1,3-diazinan-5-ylidene)methyl]phenoxy]butyl)carbamate (26)**

![Chemical Structure of 26](image)

To a solution of 1,3-dimethylbarbituric acid (0.47 g, 3.00 mmol) in water (15 mL) was added aldehyde 24 (0.88 g, 3.00 mmol). The reaction was carried out for 24 h at 60 °C with vigorously stirring. After this time, the reaction mixture was cooled down to room temperature. The product 26
was collected through filtration and dried under high vacuum. Compound 26 was used in subsequent reactions without purification. 26: yellow solid, mp: 120-123 °C (866.7 mg, 67%); IR (powder) 3366, 2932, 1726, 1686, 1659, 1535, 1472, 1434, 1261, 1167, 1150, 1083, 1038 cm⁻¹; ¹H NMR (300 MHz, DMSO-d₆) δ 8.43 (s, 1H), 8.24 (d, J = 8.9 Hz, 2H), 6.88 (d, J = 9.0 Hz, 2H), 4.57 (br, 1H), 4.01 (t, J = 5.7 Hz, 2H), 3.34 (s, 3H), 3.32 (s, 3H), 3.13 (m, 2H), 1.78 (m, 2H), 1.61 (m, 2H), 1.38 (s, 9H); ¹³C NMR (151 MHz, DMSO-d₆) δ 163.8, 163.2, 161.0, 159.0, 156.0, 151.5, 138.0, 125.5, 114.4, 79.3, 67.9, 40.2, 29.1, 28.45, 28.4, 26.8, 26.3; HRMS (ESI-QTOF) m/z [M+Na]+ calcd for C₂₂H₂₉N₃NaO₆ 454.1954, found 454.1950.

{4-[(1,3-Dimethyl-2,4,6-trioxo-1,3-diazinan-5-ylidene)methyl]phenoxy}acetyl chloride (27)

![Formula Image]

To a suspension of acetic acid derivative 7 (3.18 g, 10 mmol) in dry DCM (30 mL) oxalyl chloride (2.5 g, 1.7 mL, 20 mmol) and three drops DMF were added and stirred at room temperature overnight. The yellow suspension was filtered to obtain yellow solid product 27 which was dried under high vacuum (3.04 g, 90%); mp 170-175 °C; IR (powder) ν 3128, 3006, 2963, 2933, 1800, 1724, 1652, 1601, 1571, 1541, 1424, 1365, 1284, 1244, 1181, 1085 cm⁻¹; ¹H NMR (600 MHz, DMSO-d₆) δ 8.32 (s, 1H), 8.30 (d, J = 9.0 Hz, 2H), 7.05 (d, J = 9.0 Hz, 2H), 4.84 (s,
N-Fluoresceinylated-[4-](1,3-dimethyl-2,4,6-trioxo-1,3-diazinan-5-ylidene)methyl]phenoxy]acetamide (28)

To a stirred suspension of 5-aminofluorescein (0.1 g, 0.29 mmol) in glacial acetic acid (10 mL), chloride 27 (0.097 g, 0.29 mmol) was added at room temperature and mixture was stirred for 30 min. Next, anhydrous sodium acetate (0.037 g, 0.45 mmol) was added and mixture was vigorously stirred at room temperature overnight. Afterwards to the reaction mixture water (20 mL) was added and suspension was stirred for 1 h. Finally, product 28 was filtered off and brought to dryness by high vacuum. Compound 28: Dark red solid, mp: > 360 °C (156 mg, 84%); IR (powder) ν 3312, 1673, 1597, 1504, 1457, 1384, 1313, 1258, 1176, 1111, 1085 cm\(^{-1}\); \(^1\)H NMR (600 MHz, DMSO-\(d_6\)) δ 10.62 (br, 1H), 10.00 (s, 2H), 8.31-8.34 (m, 3H), 7.89-7.93 (m, 2H), 7.13-7.25 (m, 3H), 6.52-6.67 (m, 6H), 4.93 (br, 2H), 3.23, 3.21 (s, 6H); \(^{13}\)C NMR (151 MHz, DMSO-\(d_6\)) δ 170.0, 163.0, 162.3, 161.2, 156.0, 151.6, 137.5, 126.1, 116.2, 114.8, 66.1, 29.1, 28.5; HRMS (ESI-QTOF) m/z [M+Na]\(^+\) calcd for C\(_{15}\)H\(_{13}\)ClN\(_2\)NaO\(_5\) 359.0411, found 359.0419.
DMSO-d$_6$ δ 191.9, 169.0, 167.1, 163.1, 160.0, 159.7, 152.4, 147.8, 140.4, 137.5, 132.3, 130.7, 129.6, 129.5, 127.4, 127.3, 125.0, 115.7, 115.0, 114.6, 113.1, 112.8, 110.1, 102.7, 83.6, 67.5, 29.1, 28.5; HRMS (ESI-QTOF) m/z [M+H]$^+$ calcd for C$_{35}$H$_{26}$N$_3$O$_{10}$ 648.1618, found 648.1615.

1-(3',6'-Dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-5-yl)-3-(2-(4-((1,3-dimethyl-2,4,6-trioxotetrahydropyrimidin-5(6H)-ylidene)methyl)phenoxy)ethyl)thiourea (30)

![Chemical structure of 30]

To a solution of fluorescein 5-isothiocyanate isomer (0.11 g, 0.28 mmol) in anhydrous DMF (1.5 mL) was added salt 8 (0.10 g, 0.24 mmol) in anhydrous DMF (1 mL). N,N-Diisopropylethylamine (0.13 mL, 0.72 mmol) was added dropwise subsequently. The reaction was stirred in the dark at room temperature for 24 h. The solvent was removed in vacuo and the crude product was purified by silica gel column chromatography (CHCl$_3$/MeOH=4:1) to give thiourea 30 as orange oil, (85.0 mg, 51%); IR (film) ν 3241, 2926, 1756, 1736, 1660, 1604, 1506, 1452, 1385, 1320, 1256, 1180, 1110 cm$^{-1}$; $^1$H NMR (300 MHz, DMSO-d$_6$) δ 10.21 – 9.82 (m, 3H), 8.46 – 8.16 (m, 3H), 7.89 (d, $J$ = 8.6 Hz, 1H), 7.73 (d, $J$ = 8.6 Hz, 1H), 7.30 – 7.12 (m, 2H), 6.86 (m, 1H), 6.73 – 6.50 (m, 5H), 4.63 – 4.23 (m, 2H), 4.18 – 3.80 (m, 2H), 3.40-3.30 (br, 2H), 3.27 – 3.09 (m,
$^{13}$C NMR (151 MHz, DMSO-$d_6$) δ 191.4, 180.8, 168.5, 163.3, 159.5, 155.7, 151.9, 147.4, 141.2, 137.4, 131.9, 129.9, 129.1, 126.6, 124.2, 115.6, 115.1, 114.4, 112.6, 109.7, 102.3, 83.0, 79.2, 66.2, 43.1, 28.7, 28.3, 28.1, 26.3; HRMS (ESI-QTOF) m/z [M+Na]$^+$ calcd for C$_{36}$H$_{28}$N$_4$NaO$_9$S 715.1475, found 715.1471.

2-Vinylsulfany lethanol (32)

![Structure of 32]

Compound 32 was prepared according to the procedure described in the literature$^{31}$: To a stirred solution of sodium ethanoate in ethanol made by dissolving sodium (0.730 g, 31.8 mmol) in ethanol (15 mL), 2-mercaptopoethanol 31 (1.985 g, 25.4 mmol) was added at room temperature. At 0 ºC the vinyl bromide solution (40 mL, 1M in THF, 40 mmol) was added and the reaction was transferred to an autoclave pipe, where was is heated to 105 ºC for 4 hours. After removal of solvent in vacuo, 15 mL water was added and the solution was extracted with 4 x 15 mL diethyl ether. The organic layer was dried with anhydrous magnesium sulfate and the solvent was removed in vacuo. This gave 32 (2.222 g, 84%), a brown oil with good purity as seen by $^1$H NMR. A colorless oil was obtained by bulb-to-bulb distillation, aprox. bp. 110-125 ºC at 20-25 mbar. The structure of the compound 32 was confirmed by $^1$H NMR spectrum (see ESI page S79); $^1$H NMR (600 MHz, CDCl$_3$) δ 6.32 (dd, $J = 16.8, J = 10.0$ Hz, 1H), 5.25 (d, $J = 9.8$ Hz, 1H), 5.23 (d, $J = 16.8$, 1H), 3.80 (q, $J = 5.8$ Hz, 2H), 2.92 (t, $J = 5.85$ Hz, 2H), 2.05 (br, s, 1H).
Succinimidy 2-vinylsulfanyethyl carbonate (33)

Compound 33 was prepared according to the procedure described in the literature\textsuperscript{31}: To a stirred solution of 32 (1.100 g, 10.56 mmol) in dry acetonitrile (40 mL), \textit{N,N}'-disuccinimidylcarbonate (3.953 g, 15.43 mmol) was added at room temperature. To the reaction mixture, which was a cloud suspension, triethylamine (3.206 g, 31.7 mmol) was added, and after a couple of minutes the reaction mixture turns clear. Analysis by TLC (eluent: diethyl ether) shows that the reaction is complete after 30 min. The solvent was removed in vacuo. The residue was dissolved in 50 mL DCM and washed with 3 \times 25 mL saturated NaHCO\textsubscript{3}. The organic phase was washed with 30 mL brine and dried with anhydrous MgSO\textsubscript{4}, and the solvent was removed in vacuo. The crude product was purified with flash column chromatography to obtain 33 (1.87 g, 7.6 mmol, 72\%). The structure of the compound 33 was confirmed by \textsuperscript{1}H NMR spectrum (see ESI page S80): \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}): \(\delta\) 6.29 (dd, \(J = 10.0\) Hz, \(J = 16.8\) Hz, 1H), 5.29 (d, \(J = 10.0\) Hz, 1H), 5.25 (d, \(J = 16.8\) Hz, 1H), 4.45 (t, \(J = 7.2\) Hz, 2H), 3.03 (t, \(J = 7.4\) Hz, 2H), 2.84 (s, 4H).
2’-Carboxybenzoyltaxol (35)

Compound 35 was prepared according to the procedure described in the literature\textsuperscript{32}: A solution of taxol 34 (100 mg, 0.117 mmol) and pyridine (950 \(\mu\)L, 11.7 mmol, 100 eq.) in DCM (5 ml) at room temperature under argon was treated, at 10 min intervals, with benzyl chloroformate (170 \(\mu\)L aliquots, 1.17 mmol, 10 eq.). When thin layer chromatography (TLC) (EtOAc/hexane 1:1) showed no remaining starting material, the reaction was quenched with aqueous \(\text{NH}_4\text{Cl}\) (1 mL). After dilution with ethyl acetate (10 mL), the reaction mixture was washed with ammonium chloride (2 x 5 mL), copper sulfate (4 x 10 mL), and sodium chloride (5 mL). The combined organic layer was dried (\(\text{MgSO}_4\)), concentrated, and purified by flash chromatography (EtOAc/hexane 1:1) to give 2’carboxybenzoyltaxol 35 as a white powder (80 mg, 70%). The structure of the compound 35 was confirmed by \(^1\text{H NMR}\) spectrum (see ESI page S81): \(^1\text{H NMR}\) (300 MHz, CDCl\(_3\)) \(\delta\) 1.14 (s, 3 H, 16-H) 1.25 (s, 3 H, 17-H) 1.69 (s, 3 H, 19-H), 1.76 (s, 1 H, l-OH), 1.93 (s, 3 H, 18-H), 1.90-1.94 (m, 1 H, 6\(\beta\)-H) 2.2-2.3, 2.4-2.5 (m, 2 H, 14-H), 2.24 (s, 3 H, 10-OAc-H), 2.46 (s, 3 H, 4-OAc-H), 2.51 (s, 1 H, 7-OH), 2.60 (m, 1 H, 6a-H), 3.82 (d, \(J = 7.0\) Hz, 1 H, 3-H) 4.20 (d, \(J = 8.5\) Hz, 1 H, 20-H), 4.32 (d, \(J = 8.5\), 1 H, 20-H), 4.41 (m, 1 H, 7-H), 4.98 (d, \(J = 8.0\) Hz, 1 H, 5-H) 5.18 (dd, \(J = 9.0\) Hz, 10 Hz, 2 H, Bn), 5.45 (d, \(J = 2.5\) Hz, 1 H, 2’-H), 5.69 (d, \(J = 7.0\) Hz, 1 H, 5a-H).
Hz, 1 H, 2-H), 5.98 (dd, J= 2.5 Hz, 9.5 Hz, 1 H, 3'-H), 6.30 (br, 2 H, 10-H, 13-H), 6.92 (d, J= 9.5 Hz, 1 H, NH), 7.30-7.42 (m, 12 H, Ar-H), 7.44-7.54 (m, 3 H, Ar-H), 7.61 (t, J = 7.0 Hz, 1 H, Ar-H), 7.73 (d, J = 8.5 Hz, 2 H, Bz-H), 8.14 (d, J = 8.5 Hz, 2 H, Bz-H).

\[ \text{2'-Carboxybenzoyl-7-}(N\text{-carboxybenzoyl-}\beta\text{-alanyl)-taxol (36)} \]

Compound 36 was prepared according to the procedure described in the literature\textsuperscript{32}: A solution of 2'-carboxybenzoyltaxol 35 (25 mg, 0.025 mmol) and 4-\((N,N\text{-dimethylamino})\text{pyridine (3 mg, 0.025 mmol, 1 eq.) in DCM (2 mL) at room temperature under argon was treated with N-carboxybenzoyl-β-alanine (24 mg, 0.11 mmol, 4.4 eq.) and dicyclohexylcarbodiimide (22 mg, 0.11 mmol, 4.4 eq.). After 18 h, the mixture was diluted with DCM (5 mL) and filtered to remove the urea. The crude product was evaporated to dryness in vacuo and purified by flash chromatography (40-45 % EtOAc in hexane) to give 2'-carboxybenzoyl-7-(N-carboxybenzoyl-β-alanyl)-taxol as a white powder (28 mg, 95%).

The structure of the compound 36 was confirmed by \(^1\)H NMR spectrum (see ESI page S82): \(^1\)H NMR (300 MHz, CDCl\textsubscript{3}) \(\delta\) 1.15 (s, 3 H, 16-H) 1.21 (s, 3 H, 17-H), 1.81 (s, 3 H, 19-H), 1.99 (s, 3 H, 18-H) 2.06 (s, 3 H, 10-OAc-H), 2.21 (m, 1 H, 6β-H), 2.4-2.6 (m, 5 H, 14-H, 6α-H, alanyl
CH₂), 2.46 (s, 3 H, 4-OAc-H), 3.4-3.5 (m, 2 H, alanyl CH₂), 3.96 (d, J = 7.0 Hz, 1 H, 3-H), 4.20 (d, J = 8.5 Hz, 1 H, 20-H), 4.33 (d, J = 8.5 Hz, 1 H, 20-H), 4.95 (d, J = 8.5 Hz, 1 H, 5-H) 5.18 (dd, J = 9 Hz, J = 10 Hz, 2 H, Bn), 5.45 (d, J = 2.5 Hz, 1 H, 2'-H), 5.63 (dd, J = 5.0 Hz, J = 7.0 Hz, 1 H, 7-H), 5.70 (d, J = 7.0 Hz, 1 H, 2-H), 5.98 (dd, J = 2.5 Hz, J = 9.0 Hz, 1 H, 3'-H), 6.22-6.30 (m, 2 H, 10-H, 13-H), 6.98 (d, J = 9.0 Hz, 1 H, NH), 7.30-7.42 (m, 17 H, Ar-H), 7.43-7.52 (m, 3 H, Ar-H), 7.61 (t, J = 7.0 Hz, 1 H, Ar-H), 7.73 (d, J = 8.5 Hz, 2 H, Bz-H), 8.14 (d, J = 8.5 Hz, 2 H, Bz-H).

**7-β-alanyltaxol (37)**

Compound 37 was prepared according to the procedure described in the literature: A solution of compound 36 (25 mg, 0.021 mmol) in methanol (1 mL) at room temperature under argon was treated with 10 % palladium on carbon (1 mg, 4 wt %) and placed under hydrogen. After 2 h, the mixture was filtered to remove the catalyst and evaporated to dryness *in vacuo* to give 7-β-alanyltaxol 37 as a white powder (19 mg, 98%). The structure of the compound 37 was confirmed by ^1^H NMR spectrum (see ESI page S83): ^1^H NMR (300 MHz, CD₃OD) δ 1.09 (s, 3 H, 32
16-H), 1.16 (s, 3 H, 17-H), 1.78 (s, 3 H, 19-H), 1.78-1.89 (m, 1 H, 6β-H), 1.87 (s, 3 H, 18-H), 1.91-2.00 (m, 1 H, 14-H), 2.18 (s, 3 H, 10-OAc-H), 2.19-2.24 (m, 1 H, 14-H), 2.38 (s, 3 H, 4-OAc-H), 2.50-2.59 (m, 1 H, 6α-H), 2.65-2.67 (m, 2 H, alanyl CH₂), 3.12-3.22 (m, 2 H, alanyl CH₂), 3.90 (d, J = 7.0 Hz, 1 H, 3-H) 4.19 (dd, J = 7.0 Hz, J = 14.0 Hz, 2 H, 20-H), 4.73 (d, J = 6.0 Hz, 1 H, 2'-H), 5.00 (d, J = 8.5 Hz, 1 H, 5-H), 5.63-5.65 (m, 3 H, 2-H, 3'-H, 7-H), 6.14 (br t, J = 6.0 Hz, 1 H, 13-H), 6.19 (s, 1 H, 10-H), 7.28-7.67 (m, 11 H, Ar-H, NH), 7.85 (dd, J = 1.5 Hz, J = 7.5 Hz, 2 H, Bz-H), 8.14 (dd, J = 1.5 Hz, J = 7.5 Hz, 2 H, Bz-H).

Vinyl thioether taxol (38)

Compound 38 was prepared according to the procedure described in the literature⁹: To a solution of 7-β-alanyltaxol 37 (30 mg, 0.032 mmol) in anhydrous acetonitrile (2 mL) was added compound 33 (12 mg, 0.048 mmol) in anhydrous acetonitrile (1 mL) at room temperature. After 24 h, the mixture was evaporated to dryness in vacuo and purified by flash chromatography (silica gel, 40% EtOAc in petrol ether) to afford the desired product 38 as a white powder (20 mg, 59%). The structure of the compound 38 was confirmed by ¹H NMR spectrum (see ESI page S84):
\(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 1.16 (s, 3H), 1.21 (s, 3H), 1.81 (s, 3H), 1.83 (s, 3H), 1.78-1.86 (m, 2H), 2.18 (s, 3H), 2.33 (d, \(J = 9.2\) Hz, 2H), 2.38 (s, 3H), 2.52-2.58 (m, 3H), 2.92 (t, \(J = 7.2\) Hz, 2H), 3.44 (m, 2H), 3.65 (d, \(J = 5.2\) Hz, 1H), 3.92 (d, \(J = 6.4\) Hz, 1H), 4.17-4.23 (m, 3H), 4.31 (d, \(J = 8.4\) Hz, 1H), 4.80 (m, 1H), 4.92 (d, \(J = 8.8\) Hz, 1H), 5.21 (m, 2H), 5.58 (m, 1H), 5.67 (d, \(J = 6.4\) Hz, 2H), 5.79 (dd, \(J = 8.8\) Hz, 2.0 Hz, 1H), 6.18 (t, \(J = 8.4\) Hz, 1H), 6.24 (s, 1H), 6.33 (dd, \(J = 16.8\) Hz, 9.6 Hz, 1H), 7.07 (d, \(J = 8.8\) Hz, 1H), 7.33-7.64 (m, 11H), 7.76 (d, \(J = 7.6\) Hz, 2H), 8.11 (d, \(J = 8.0\) Hz, 2H).

(4-{7-[2-Hydroxyethyl)sulfanyl]-1,3-dimethyl-2,4-dioxo-1,3,4,5,6,7-hexahydro-2H-pyrano[2,3-d]pyrimidin-5-yl}phenoxy)ethanaminium 2,2,2-trifluoroacetate (39)

To a solution of heterodiene 8 (0.035 g, 0.084 mmol) in water (2 mL) was added thioether 32 (0.0875 g, 0.84 mmol). The reaction was stirred at 37 °C for 24 h (monitored by TLC, CHCl\(_3\)/MeOH = 2:1). The solvent was removed \textit{in vacuo} and the crude product was purified by silica gel column chromatography (DCM/MeOH = 2:1) to give 39 as pale yellow oil (23.2 mg, 53%); IR (neat) \(\nu\) 3390, 2952, 1682, 1634, 1509, 1248,
1205, 1140, 1023 cm$^{-1}$; $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 8.13 (s, 4H), 7.16 (d, $J = 8.7$ Hz, 2H), 6.89 (d, $J = 8.6$ Hz, 2H), 5.39 (dd, $J = 10.3$, 2.4 Hz, 1H), 4.91 (t, $J = 5.5$ Hz, 1H), 4.23 – 4.13 (m, 2H), 4.00 – 3.91 (m, 2H), 3.67 – 3.47 (m, 4H), 3.31 (s, 3H), 3.11 (s, 3H), 2.87 – 2.80 (m, 2H);

$^{13}$C NMR (151 MHz, DMSO-$d_6$) $\delta$ 161.4, 157.9 (d, $J_{C-F} = 30.8$ Hz), 156.5, 156.0, 150.6, 136.8, 128.7, 118.3, 116.3, 114.5, 87.6, 82.0, 64.4, 61.0, 38.4, 33.5, 33.0, 28.6, 27.5; HRMS (ESI-QTOF) m/z [M-CF$_3$COO]$^+$ calcd for C$_{19}$H$_{26}$N$_3$O$_5$S 408.1593, found 408.1589.

5-[(3,4-diacetoxyphenyl)methylidene]-1,3-dimethyl-1,3-diazinane-2,4,6-trione (44)

To a stirred solution of 1,3-dimethylbarbituric acid 1 (0.465 g, 3 mmol) in water (10 mL) was added 3,4-diacetoxybenzaldehyde 43 (0.666 g, 3 mmol). After 24h of stiring at room temperature, the solid product 44 was isolated by filtration and dried. Compound 44 needed no further purification and was obtained with 95% yield. Compound 44: white solid, mp 149-150 °C; IR (powder) ν 3012, 2968, 2930, 1773, 1735, 1670, 1654, 1566, 1505, 1377, 1201, 1150, 1085 cm$^{-1}$; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.48 (s, 1H), 8.23 (s, 1H), 7.95 (d, $J = 9.0$ Hz, 1H), 7.32 (d, $J = 9.0$ Hz, 1H), 3.43 (s, 3H), 3.39 (s, 3H), 2.33 (s, 3H), 2.32 (s, 3H); $^{13}$C NMR (151 MHz, CDCl$_3$) $\delta$ 168.6, 168.1, 162.9, 160.9, 157.4, 151.7, 146.4,
142.3, 133.8, 131.5, 129.4, 123.8, 118.5, 29.8, 29.1, 21.3, 21.2; **HRMS** (ESI-QTOF) m/z [M+Na]+ calcd for C_{17}H_{16}N_{2}O_{7}Na 383.0855, found 383.0855.

5-[(4-acetoxy-3-hydroxyphenyl)methylidene]-1,3-dimethyl-1,3-diazinane-2,4,6-trione (49)

Heterodiene 49 was obtained from compound 48. Heterodiene 48 was synthesized by a Knoevenagel condensation of a commercially available aldehyde 42 and 1,3-dimethylbarbituric acid in water according to procedure described in literature.\(^{34}\) The acetylation of compound 48 by Schoetten-Baumann method yielded only the monoacetyl derivative 49. Compound 49 needed no further purification and was obtained with 90% yield. Compound 49: white solid, mp 200-201 °C; **IR** (powder) ν 3235, 3089, 2954, 2930, 1724, 1664, 1543, 1506, 1421, 1299, 1226, 1156, 1081 cm\(^{-1}\); **¹H NMR** (300 MHz, CDCl\(_3\)) δ 11.10 (br, 1H), 8.38 (d, \(J = 3.0\) Hz, 1H), 8.25 (s, 1H), 8.00 (dd, \(J = 9.0, 3.0\) Hz, 1H), 7.01 (d, \(J = 9.0\) Hz, 1H), 3.21 (s, 6H), 2.30 (s, 3H); **¹³C NMR** (151 MHz, CDCl\(_3\)) δ 163.9, 161.9, 157.9, 153.6, 152.2, 145.9, 137.4, 132.5, 125.4, 122.3, 116.5, 114.5, 29.7, 29.1, 21.6; **HRMS** (ESI-QTOF) m/z [M+Na]+ calcd for C\(_{15}\)H\(_{14}\)N\(_2\)O\(_6\)Na 341.0749, found 341.0750.
Stability studies of heterodienes and cycloaddition products

Stability studies of 5-arylidenebarbituric acids and cycloaddition products in different pH values

To analyze the stability of 5-arylidenebarbituric acids or cycloaddition products in different pH values, appropriate compounds (0.4 mmol) were added in to 20 mL of pH=4.0 or pH=7.4 buffer solution. The prepared mixtures were heated at 37 °C for 24 h. Extractions with dichloromethane (3x10 mL) were performed, the organic phases were dried over anhydrous magnesium sulfate and then evaporated. $^1$H NMR spectra of the reaction mixture were recorded and compared to the spectra of pure heterodienes.

Stability studies of 5-arylidenebarbituric acids and cycloaddition products in the presence of cysteine or lysine

Aqueous solutions of heterodienes and amino acids (20 mL, 20 mM) were prepared. To 0.4 mmol of the heterodiene in 20 mL of water, respectively 48 mg (0.4 mmol) cysteine or 58 mg (0.4 mmol) lysine was added. The prepared solutions were heated at 37 °C for 24 h. Extractions with dichloromethane (3x10 mL) were performed, the organic phases were dried over anhydrous magnesium sulfate and then evaporated. $^1$H NMR spectra of the reaction mixture were recorded and compared to the spectra of pure heterodienes.
Selected research results

Fig. S1 Stability study of 5-phenylmethylene-1,3-dimethyl-1,3-diazinane-2,4,6-trione in pH=4.0 buffer solution, 37 °C, 24h.
Fig. S2 Stability study of 5-[(4-methoxyphenyl)methylidene]-1,3-dimethyl-1,3-diazinane-2,4,6-trione -2,4,6-trione in pH=7.4 buffer solution, 37 °C, 24h.
Fig. S3 Stability study of 5-phenylmethylidene-1,3-dimethyl-1,3-diazinane-2,4,6-trione in H₂O in the presence of lysine, 37 °C, 24h.
Fig. S4 Stability study of 5-phenylmethylidene-1,3-dimethyl-1,3-diazinane-2,4,6-trione in H₂O in the presence of cysteine, 37 °C, 24h.
Fig. S5 Stability study of mixture of cis and trans diastereoisomers of 7-ethoxy-1,5,6,7-tetrahydro-1,3-dimethyl-5-phenyl-2H-pyrano[2,3-d]pyrimidine-2,4(3H)-dione in H₂O in the presence of lysine, 37 °C, 24h.
Stability studies of the cycloaddition product 39 in different pH values

To analyze the stability of cycloaddition product 39 in different pH values, 39 (0.0035 mmol) was dissolved in 1 mL of pH=4.0 and pH=7.4 buffer solution at 37 °C. The resulting solutions were monitored by LC-MS after 24 h.

**Fig. S6** Stability studies of the cycloaddition product 39 in pH=4.0 buffer solution.
Fig. S7 Stability studies of the cycloaddition product 39 in pH=7.4 buffer solution.

Fig. S8. HRMS (ESI-QTOF) of cycloadduct 39, m/z [M-CF₃COO]+ calcld for C₁⁹H₂₆N₃O₅S 408.1593, found 408.1589.
It was found that 39 is stable at pH 4.0−7.4 (Fig. S8 and Fig. S9; mass peaks characteristic for the product 39 are marked with red boxes, bold red box – main peak).

**Studies of cycloaddition reaction of 8 and 32 in the presence of cysteine 40**

Heterodiene 8 (0.1 mM) and dienophile 32 (1 mM) were treated with cysteine (0.1 mM) in H₂O at 37 °C, and the resulting solution was monitored via LC-MS after 24 h.
Fig. S9 Studies of cycloaddition reaction of 8 and 32 in the presence of cysteine 40.

Fig. S10 Studies of cycloaddition reaction of 8 and 32 in the presence of cysteine 40.
The reaction of heterodiene 8 and dienophile 32 in the presence of cysteine 40 was investigated (Fig. S9 and Fig. S12). The HDA cycloadduct 39 (mass peaks characteristic for this compound are marked with red boxes) and also unreacted cysteine 40 (mass peak characteristic for this compound is marked with blue box) were observed in H$_2$O at 37 °C after 24 h.

**Experiment concerning thiol Michael addition heterodiene 8 and cysteine 40**

A mixture of heterodiene 8 (0.01 mmol) and cysteine 40 (0.011 mmol) in 3 mL of H$_2$O was stirred at 37 °C, and the resulting solution was monitored via LC-MS after 24 h.
Fig. S11 Experiment concerning thiol Michael addition heterodiene 8 and cysteine 40.

Fig. S12 Experiment concerning thiol Michael addition heterodiene 8 and cysteine 40.
A reaction of heterodiene 8 and cysteine 40 was carried out to check the reactivity between these two compounds (Fig. S11 and Fig. S12). The intense mass peaks characteristic for both substrates 8 and 40 were observed after 24 h (peak for cysteine 40 in blue box, peak for heterodiene 8 in green box, Fig. S11). There was also a very small peak for thiol Michael addition product 41 (marked with orange box, Fig. S12).

**Cycloaddition reactions rate study**

The cycloaddition reaction of 8 and 32 rate study (indirect method)

$^1$H NMR was used to calculate the reaction rate constant of the hetero-Diels–Alder cycloaddition of heterodiene 8 and ether 32. Heterodiene 8 (0.17 mmol) and ether 32 (1.7 mmol) were first dissolved in H$_2$O (50 mL), and the reaction mixture was stirred at 37 °C. A sample of 8 mL solution of the reaction mixture was collected every 20~30 min, evaporated under high vacuum and was analyzed by $^1$H NMR in DMSO-$d_6$. Three samples were used to calculate the reaction rate constant after 80 min. Percent conversion was monitored by both disappearance of starting material and appearance of product as determined by integration at multiple chemical shifts. Pseudo-first order rate constant ($k_{obs}$) for the reaction was determined by plotting the ln [1/(1-x)] versus time and analysis by linear regression, x represents the percent conversion of compound 8. The reaction was repeated in triplicate. The three lines had an average of $k_{obs} = 5.4333 \times 10^{-4}$ s$^{-1}$. Second order rate constant was estimated by $k_{obs}/[32]$. As a result, the second order rate constant $k_2$ for the cycloaddition of 8 and 32 is $(1.6 \pm 0.1) \times 10^{-2}$ M$^{-1}$ s$^{-1}$ in H$_2$O.
Fig. S13 The cycloaddition reaction of 8 and 32 rate study (indirect method).
The cycloaddition reaction of 8 and 32 rate study (direct method)

$^1$H NMR was used to calculate the reaction rate constant of the hetero-Diels–Alder cycloaddition of heterodiene 8 and ether 32. The reaction was carried out directly in the cuvette. Heterodiene 8 (0.0095 mmol) was first dissolved in D$_2$O (0.6 mL) and then added to solution of 32 (0.0095 mmol) in D$_2$O (0.2 mL). The temperature in the spectrometer was 27 °C. Spectra were collected every 7 min. 3 spectra were chosen and used to calculate the reaction rate constant. Percent conversion was monitored by both disappearance of starting material (heterodyne 8) and appearance of product 39 as determined by integration at multiple chemical shifts. The reaction was repeated in triplicate. The key signals used to determine the disappearance of starting material and appearance of product are shown in the following spectra:

- time: 49 min, x = 61% (% of substrate remaining in the sample)

- time: 98 min, x = 49,5%

![Spectrum](image-url)
time: 154 min, x = 38.9%

Second order rate constant $k_2$ for the reaction 8 and 32 was determined by plotting the $1/c_D - 1/c_{D,0}$ versus time and analysis by linear regression. $c_{D,0}$ represents the initial concentration of diene and $c_D$ represents the concentration of the diene at the appropriate measuring point. $c_D$ was calculated based on $c_{D,0}$ and $x$ (\% of substrate remaining in the sample). A slope factor (a) is equal to the second order rate constant $k_2$, in accordance with second order rate kinetics. The three lines had an average of slope factor $a = 1.4 \times 10^{-2}$. As a result, the second order rate constant for the cycloaddition of 8 and 32 is $(1.4 \pm 0.1) \times 10^{-2} \text{ M}^{-1} \text{s}^{-1}$ in $\text{D}_2\text{O}$. 
The cycloaddition reaction of 9 and 32 rate study

$^1$H NMR was used to calculate the reaction rate constant of the hetero-Diels-Alder cycloaddition of hetrodiene 9 and ether 32. Hetrodiene 9 (0.225 mmol) and ether 32 (2.25 mmol) were first dissolved in H$_2$O (50 mL), and the reaction mixture was stirred at 37 °C. A sample of 8 mL solution of the reaction mixture was collected every 20–30 min, evaporated under high vacuum and was analyzed by $^1$H NMR in DMSO-$d_6$. 

Fig. S14 The cycloaddition reaction of 8 and 32 rate study (direct method).
Three samples were used to calculate the reaction rate constant after 80 min. Percent conversion was monitored by both disappearance of starting material and appearance of product as determined by integration at multiple chemical shifts. Pseudo-first order rate constant ($k_{\text{obs}}$) for the reaction was determined by plotting the $\ln \left[1/(1-x)\right]$ versus time and analysis by linear regression, $x$ represents the percent conversion of compound 17. The reaction was repeated in triplicate. The three lines had an average of $k_{\text{obs}} = 4.0667 \times 10^{-4} \text{ s}^{-1}$. Second order rate constant was estimated by $k_{\text{obs}}/[32]$. As a result, the second order rate constant for the cycloaddition of 9 and 32 is $(0.9 \pm 0.1) \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$ in H$_2$O.

**Fig. S15** The cycloaddition reaction of 9 and 32 rate study.
Rate study of cycloaddition reaction of 44 and 32 and reaction of 46 and 32

An attempt was made to determine the rate constant $k_2$ of reaction of compounds 44 and 32. First, the same measurement conditions were applied as for the studies of the kinetics of the reaction of compounds 8 and 32 or 9 and 32. However, measurement of the first $^1$H NMR spectrum of the reaction mixture indicated that the reaction was complete because no signals for the heterodiene 44 were visible in the spectrum (Fig. S16). The same result was obtained for the reaction of the monoacetyl derivative 46 and ether 32. Only cycloadduct 47 signals were visible in the measured spectrum (Fig. S17).
Fig. S16 ¹H NMR spectrum of the first sample taken from the reaction mixture of heterodiene 44 and vinyl thioether 32.
Fig. S17 $^1$H NMR spectrum of the first sample taken from the reaction mixture of heterodiene 46 and vinyl thioether 32.
The cycloaddition reaction of 44 and 32 rate study by the direct method in the NMR cuvette, the molar ratio of the reactants 1:1, D$_2$O/CD$_3$CN (3:1)

$^1$H NMR was used to calculate the reaction rate constant $k_2$ of the hetero-Diels–Alder cycloaddition of 44 and 32. The reaction was carried out directly in the NMR cuvette. 44 (4.5 mg, 0.0125 mmol) was first dissolved in CD$_3$CN (0.2 mL) and then added to solution of 32 (1.3 mg, 0.0125 mmol) in D$_2$O (0.6 mL). The temperature in the spectrometer was 27 °C. Spectra were collected every 27 s. 5 spectra were chosen and used to calculate the reaction rate constant $k_2$. Percent conversion was monitored by both disappearance of starting material 44 and appearance of products (two diastereoisomers of 45) as determined by integration at multiple chemical shifts. The reaction was repeated in triplicate. The key signals used to determine the disappearance of starting material and appearance of products are shown in the following spectra:

time: 81 s, $x = 43\%$ (% of substrate 44 remaining in the sample)

![NMR spectrum showing signals for 44 and two diastereoisomers of 45](image-url)
time: 189 s, x = 21%

Second order rate constant $k_2$ for the reaction 44 and 32 was determined by plotting the $1/c_D - 1/c_{D,0}$ versus time and analysis by linear regression. $C_{D,0}$ represents the initial concentration of heterodiene 44 and $c_D$ represents the concentration of the heterodiene 44 at the appropriate measuring point. $c_D$ was calculated based on $c_{D,0}$ and $x$ (% of substrate remaining in the sample). A slope factor (a) is equal to the second order rate constant
(\(k_2\)), in accordance with second order rate kinetics. The three lines had an average of slope factor \(a = 1.4\). As a result, the second order rate constant \(k_2\) for the cycloaddition of 44 and 32 is \(1.4 \pm 0.1 \text{ M}^{-1} \text{s}^{-1}\) in D\(_2\)O/CD\(_3\)CN (3:1).

**Fig. S18** The cycloaddition reaction of 44 and 32 rate study by the direct method in the NMR cuvette, the molar ratio of the reactants 1:1, D\(_2\)O/CD\(_3\)CN (3:1).
The cycloaddition of 46 and 32 rate study by the direct method in the NMR cuvette, the molar ratio of the reactants 1:1, D$_2$O/CD$_3$CN (3:5)

$^1$H NMR was used to calculate the reaction rate constant $k_2$ of the hetero-Diels–Alder cycloaddition of 46 and 32. The reaction was carried out directly in the NMR cuvette. 46 (2.25 mg, 0.00745 mmol) was first dissolved in CD$_3$CN (0.5 mL) and then added to solution of 32 (3 mg, 0.02885 mmol) in D$_2$O (0.3 mL). The temperature in the spectrometer was 27 °C. Spectra were collected every 27 s. 4 spectra were chosen and used to calculate the reaction rate constant $k_2$. Percent conversion was monitored by both disappearance of starting material 46 and appearance of products (two diastereoisomers of 47) as determined by integration at multiple chemical shifts. The reaction was repeated in triplicate. The key signals used to determine the disappearance of starting material and appearance of products are shown in the following spectra:

time: 317 s, x = 46% (% of substrate remaining in the sample)

![46 signal](image1)

![two diastereoisomers 47 signals](image2)

time: 441 s, x = 37%

![46 signal](image3)

![two diastereoisomers 47 signals](image4)
time: 546 s, x = 31.5%

Second order rate constant $k_2$ for the reaction 46 and 32 was determined by plotting the $1/c_D - 1/c_{D,0}$ versus time and analysis by linear regression. $C_{D,0}$ represents the initial concentration of diene 46 and $c_D$ represents the concentration of the diene 46 at the appropriate measuring point. $c_D$ was calculated based on $c_{D,0}$ and $x$ (% of substrate remaining in the sample). A slope factor (a) is equal to the second order rate constant ($k_2$), in accordance with second order rate kinetics. The three lines had an average of slope factor $a = 4.3 \times 10^{-1}$. As a result, the second order rate constant $k_2$ for the cycloaddition of 46 and 32 is $0.43 \pm 0.1 \, \text{M}^{-1} \, \text{s}^{-1}$ in D$_2$O/CD$_3$CN (3:5).
Fig. S19 The cycloaddition reaction of 46 and 32 rate study by the direct method in the NMR cuvette, the molar ratio of the reactants 1:1, D$_2$O/CD$_3$CN (3:5).

Bioorthogonality of the proposed cycloadditions

Cell culture

Human osteosarcoma cell line U-2 OS (ECACC, cat. no 92022711, lot no. 10K035) and human cervix epitheloid carcinoma cell line HeLa (ECACC, cat. no 93021013) were cultured in Mc Coy’s medium (BioWest) supplemented with 10% Foetal Bovine Serum (FBS, BioWest). The cells were grown at 37°C in a humified atmosphere containing 5% CO$_2$.  

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Cell viability MTT assay and dose-response curve fitting

HeLa and U-2 OS cells were seeded on 96-well transparent plates (Falcon) at the density of 5000 per well. The next day, the cells treated with the increasing concentrations of the indicated compounds, prepared as 1000x stock solutions in DMSO. Also, DMSO was used as a control. The cells were treated for 48 hours and then Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma Aldrich) was added for 30 minutes at a final concentration of 0.5 mg/ml. The medium was carefully removed and MTT crystals were dissolved in isopropanol supplemented with 40 mM HCl. The absorbance was measured with the Spark microplate reader (Tecan Group Ltd.) at 570 nm with the reference wavelength 650 nm for background subtraction. For data analysis, a dose-response fitting was performed using the Origin 2020 software.

Fluorescence labeling studies

U-2 OS or HeLa cells were plated on Cell Culture Slides (Biologix, cat. 07-2104) at a density of 40 000 cells/cm² in the culture medium. The next day, the cells were treated with DMSO or dienophile-taxol 38 (20 μM for U-2 OS and 0.5 μM for HeLa) at 37 °C for 2 h and then washed three times with PBS. The cells were then treated with FITC (100 μM) or heterodiene-fluorescein 30 (33.3 μM for U-2 OS and 20 μM for HeLa) for additional 10 hours at the culture conditions, washed three times with PBS and submerged with fresh PBS containing Hoechst 33342 for the microscopy analysis performed with the use of Leica DM IL Led fluorescence microscope, equipped with Leica DFC3000 G digital camera. The images were captured using Leica Application Suite X 3.3.3.16958 software and analysed with ImageJ 1.52i software.37
Fluorescence labeling studies. U-2 OS cells were first treated with either the dienophile-taxol 38 (20 µM) or DMSO for 2 hours, washed, treated with either FITC (33.3 µM) or its heterodiene derivative 30 (33.3 µM) for additional 10 hours, washed again, stained with Hoechst 33342, and visualized with fluorescence microscopy.

Fig. S20 Fluorescence labeling studies. U-2 OS cells were first treated with either the dienophile-taxol 38 (20 µM) or DMSO for 2 hours, washed, treated with either FITC (33.3 µM) or its heterodiene derivative 30 (33.3 µM) for additional 10 hours, washed again, stained with Hoechst 33342, and visualized with fluorescence microscopy.
$^1$H NMR (600 MHz, DMSO-$d_6$)
$^1$H NMR (600 MHz, DMSO-$d_6$)
$^{13}$C NMR (151 MHz, DMSO-$d_6$)
$^1$H NMR (600 MHz, DMSO-$d_6$)
$^{13}$C NMR (151 MHz, DMSO-d$_6$)
$^{13}$C NMR (151 MHz, DMSO-$d_6$)

![Chemical structure diagram]

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$^{1}$H NMR (300 MHz, DMSO-$d_6$)
$^{13}$C NMR (151 MHz, DMSO-d$_6$)
$^1$H NMR (600 MHz, DMSO-$d_6$)

![NMR Spectrum](image)

**Chemical Structure**

\[ \text{CF}_3\text{COO}^+\text{H}_3\text{N}^+ \text{O} \]

**compound 9**
$^{13}$C NMR (151 MHz, DMSO-$d_6$)
$^1$H NMR (600 MHz, CDCl$_3$)
$^{13}$C NMR (151 MHz, CDCl$_3$)
$^1$H NMR (300 MHz, DMSO-d$_6$)
$^{13}$C NMR (151 MHz, DMSO-$d_6$)
$^3$H NMR (600 MHz, CDCl$_3$)
$^{13}$C NMR (151 MHz, CDCl$_3$)
$^1$H NMR (600 MHz, DMSO-$d_6$)
\(^{13}\)C NMR (151 MHz, DMSO-\(d_6\))

\[ \text{Diagram} \]

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$^1$H NMR (600 MHz, DMSO-d6)
$^{13}$C NMR (151 MHz, DMSO-$d_6$)
$^1$H NMR (300 MHz, DMSO-d$_6$)
$^{13}$C NMR (151 MHz, DMSO-$d_6$)
$^1$H NMR (300 MHz, CDCl$_3$)
$^1$H NMR (300 MHz, CDCl$_3$)
$^1$H NMR (300 MHz, CDCl$_3$)
$^{1}H$ NMR (300 MHz, CDCl$_3$)
$^{1}H$ NMR (300 MHz, CD$_3$OD)
$^1$H NMR (300 MHz, CDCl$_3$)
$^1$H NMR (300 MHz, DMSO-d$_6$)
$^{13}$C NMR (151 MHz, DMSO-$d_6$)
$^1$H NMR (300 MHz, CDCl$_3$)
$^1$H NMR (300 MHz, DMSO-d$_6$)
$^{13}$C NMR (151 MHz, DMSO-$d_6$)