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Supporting Information

For

Dissociative Reactions of Benzonorbornadienes with Tetrazines: Scope of Leaving Groups and Mechanistic Insights

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Figure S1. Determination of second-order rate constants k_2 of the reactions of the BNBD derivatives 2c, 2d, 3a-3b, 5b, 6a-6b and 7b and PEG-Tz determined from plots of pseudo-first order k_{obs} versus concentrations. The results are expressed as the mean \pm standard deviation (n = 3).

¹H NMR Release Studies

Stock solutions of benzonorbornadiene derivatives **2c**, **3a/b**, **4a/b**, **5b**, **6a/6b**, **7b** and **8a** (40 mM) and DPTz (24 mM) in DMSO- d_6 were prepared. Aliquots of probe stock solution (75 µL), DPTz solution (375 µL) and D₂O (50 µL) were pre-warmed to 37 °C and combined to give final concentrations of 6 mM for the probe and 18 mM for DPTz. 18-crown-6-ether or furan was added as internal standard for peak integration. The samples were incubated at 37 °C and ¹H NMR spectra recorded at the indicated time points (5 min and 6 h) at 37 °C.



□; **DPPz**: ■; I.S: Internal standard (18-crown-6).



Figure S3. The reaction of **3a/b** with DPTz in 90% DMSO- d_6/D_2O . Legend: **3a/b**: \blacktriangle ; Released phenoxy acetic acid: \bigcirc ; DPTz: \Box ; DPPz: \blacksquare ; I.S: Internal standard.



Figure S4. The reaction of 4a/b with DPTz in 90% DMSO- d_6/D_2O . Legend: 4a/b: \blacktriangle ; Released PEG4-acid: \bigcirc ; DPTz: \Box ; DPPz: \blacksquare ; \bigcirc : Impurity in 4b; I.S: Internal standard (Furan).



Figure S5. The reaction of **5b** with DPTz in 90% DMSO- d_6/D_2O . Legend: **5b**: \blacktriangle ; Released ibuprofen: \bigcirc ; **DPTz**: \Box ; **DPPz**: \blacksquare ; I.S: Internal standard.



Figure S6. The reaction of **6a/b** with DPTz in 90% DMSO- d_6/D_2O . Legend: **6a/b**: \blacktriangle ; Released benzyl alcohol: \bigcirc ; DPTz: \Box ; DPPz: \blacksquare ; I.S: Internal standard.



Figure S7. The reaction of **7b** with DPTz in 90% DMSO- d_6/D_2O . Legend: **7b**: \blacktriangle ; Released menthyl alcohol: \bigcirc ; DPTz: \Box ; DPPz: \blacksquare ; Sol: Methanol; I.S: Internal standard (18-crown-6).



Figure S8. The reaction of **8a** with DPTz in 90% DMSO- d_6/D_2O . Legend: **8a**: \blacktriangle ; Released benzyl phosphate: \bigcirc ; DPTz: \Box ; DPPz: \blacksquare ; I.S: Internal standard.

Release studies of 4a and PEG-Tz in D_2O

Stock solutions of **4a** (12 mM) and PEG-Tz (12 mM) in D₂O were prepared. Aliquots of the **4a** stock solution (125 μ L) and PEG-Tz stock solution (375 μ L) were combined to give final concentrations of 3 mM for the **4a** and 9 mM for PEG-Tz. N-methylpyrrole was added as internal standard for peak integration. The samples were incubated at 37 °C and ¹H NMR spectra recorded at 2 h. Notice: since the solvent effect will dramatically accelerate kinetics with vigorous formation of bubbles (N₂ and CO₂), impacting the NMR acquisition at first 1 h, to quantify the release rate of the PEG-4 acid, same aliquot of **4a** stock solution was token and diluted with D₂O (375 μ L) to make the dummy sample as reference.



Figure S9. Bioorthogonal release reaction of **4a** with **PEG-Tz** in 100% D₂O. Legend: **PEG-Tz**: \Box ; Released PEG4 acid: \bullet ; **PEG-Pz**: \blacksquare .

Temperature dependence of reaction mechanism

Stock solutions of benzonorbornadiene derivatives **2a/b** (40 mM) and DPTz (24 mM) in DMSO- d_6 were prepared. Aliquots of probe stock solution (75 µL), DPTz solution (375 µL) and D₂O (50 µL) were prewarmed to corresponding temperature and combined to give final concentrations of 6 mM for the probe and 18 mM for DPTz. 18-crown-6-ether was added as internal standard for peak integration. The samples were incubated at 22, 37 and 50 °C and ¹H NMR spectra recorded at 2 h at 22, 37 and 50 °C.



Figure S10. Temperature dependence of the reaction of 2a with DPTz in 90% DMSO- d_6/D_2O . Legend: 2a: \bigstar ; p-nitroaniline: \bigcirc ; DPTz: \Box ; tentative 1,4-tautomer byproduct: \bigstar ; DPPz: \blacksquare ; I.S: Internal standard.



Figure S11. Temperature dependence of the reaction of **2b** with DPTz in 90% DMSO- d_6/D_2O . Legend: **2b**: **\bigstar**; p-nitroaniline: **\bigcirc**; **DPTz**: \Box ; **DPPz**: **\blacksquare**; I.S: Internal standard.

Formation of Byproduct

Stock solutions of benzonorbornadiene derivatives 2a (40 mM) and DPTz (24 mM) in DMSO- d_6 were prepared. Aliquots of probe stock solution (75 µL), DPTz solution (375 µL) and D₂O (50 µL) were combined to give final concentrations of 6 mM for the probe and 18 mM for DPTz. 18-crown-6-ether was added as internal standard for peak integration. The samples were incubated at 22 °C and ¹H NMR spectra recorded at the indicated time points at 22 °C.



Figure S12. Full spectrum range of the analysis of the bioorthogonal release reaction of **2a** with **DPTz** in 90% DMSO- d_6/D_2O (Corresponds to Figure 5a in main text). Legend: **2a**: \triangle ; **DPTz**: \Box ; **1,4-tautomer**:•; **DPPz**:•.

Computational Methodology

The calculated $\Delta\Delta G$ values where obtained by the super-molecule approach using the D.01 version of the Gaussian 09 package¹. Formation of I3 from I2 and formation of I3 from 1,4-tautomer (hypothesized to happen) were build using Gaussview and a full geometric optimization was performed using the m06-2x^{2,3} functional with a 6-31G(d,p) basis set. I4, I3, 1,4-tautomer, I2 and DPPz were calculated. No negative frequencies were observed ensuring a proper optimization of the structure and the sum of electronic and thermal free energies was calculated using the same level of theory and basis set. For each elimination reaction proposed, the general scheme to obtain the $\Delta\Delta G$ was:

 $\Delta\Delta G = \Delta G_{start-molecule} - (\Delta G_{productA} + \Delta G_{productB})$



Figure S13. Proposed mechanism of formation of I3 and I4 from tetrazine-induced cargo release.

Table S1. The calculated ΔG values of each structure and $\Delta \Delta G$ values of react	tions.
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Compound	ΔG (Hartrees)
I2 (X = O)	-1859.840556
I2 ($X = NAc$)	-1992.534671
I3 (X = O)	-1101.825977
I3 ($X = NAc$)	-1234.526816
I4 (X = O)	-421.903449
I4 ($X = NAc$)	-554.609656
1,4-tautomer $(X = O)$	-1859.844438
1,4-tautomer ($X = NAc$)	-1992.538413
DPPz	-758.022099
Carbamic	-679.886543

When X= O:

Step 1: $\Delta\Delta G = -4.7188$ (kcal/mol) Step 2: $\Delta\Delta G = 2.2828$ (kcal/mol)

Step 3: $\Delta\Delta G = 22.5806$ (kcal/mol)

When X= NAc:

Step 1: $\Delta\Delta G$ = -8.93811 (kcal/mol) Step 2: $\Delta\Delta G$ = 6.59005 (kcal/mol) Step 3: $\Delta\Delta G$ = 19.2121(kcal/mol)



Table S2. The simulated structures from Gaussview

Formation of Intermediates and Adducts

Stock solutions of benzonorbornadiene derivatives **2b** (40 mM) and DPTz (24 mM) in DMSO- d_6 were prepared. Aliquots of probe stock solution (75 µL), DPTz solution (375 µL) and D₂O (50 µL) were combined to give final concentrations of 6 mM for the probe and 18 mM for DPTz. 18-crown-6-ether was added as internal standard for peak integration. The samples were incubated at 37 °C and HSQC spectra recorded from 2 h to 6 h.



Figure S14. Full HSQC spectrum of the bioorthogonal release reaction of 2b with DPTz in 90%DMSO d_6/D_2O (equivalent of Figure 6 in main text).



Figure S15. HPLC trace of the reaction of 2b with DPTz in DMSO/H₂O system and LC/MS analysis of two tetrazine adducts. Adduct 1: MW = 379.86; Adduct 2: MW = 338.84;



Figure S16. HPLC trace of the reaction of 2a with DPTz in DMSO/H₂O system and LC/MS analysis of plausible tautomer structures. 14: MW = 546.8; 15: MW = 564.8;

 $<^{1.570}_{1.554}$ -240 -220 200 180 -160 -140 120 -100 -80 -60 40 -20 -0 1.13⊣ 1.00-≢ 3.06≖ 2.08⊣ 1.105 98-08-0.81 -20 5.0 4.5 f1 (ppm) 1.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 400000 125.379 125.262 125.174 120.316 119.776 152.179 150.149 147.648 144.846 143.634 143.180 142.223 -82.066 -94.453 -69.689 -16.055-380000 -360000 -340000 -320000 -300000 -280000 -260000 -240000 -220000 200000 180000 -160000 -140000 120000 -100000 -80000 60000 40000 -20000 -0 -20000 80 70 f1 (ppm) 160 150 140 130 120 110 100 90 60 50 40 30 20 10 0 -10

¹*H* and ¹³*C* NMR spectra of synthesized compounds 2c





3b



4a



4b











-1.949

-400

-350

-300

-250

-200

-150





8a





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