Supplementary Information for

Versatile naphthalimide tetrazines for fluorogenic bioorthogonal labelling

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Supplementary schemes

Scheme S1. Synthesis of naphthalimide tetrazine precursors.
Scheme S2. Naphthalimide tetrazines and corresponding pyridazines after reaction with BCN. Reaction conditions: 5 equiv. BCN, DMSO, rt, 10 min.
Scheme S3. Synthesis of LysoNpTz.

Scheme S4. Synthesis of MitoNpTz.
Scheme S5. Representative synthesis of BCN-tagged insulin monomers.
### Supplementary tables

Table S1. Maximum excitation and emission wavelengths observed for each of the compounds in the four solvents used in this study. "-" indicates the dye was quenched in that solvent.

| Compound  | CH₂Cl₂ | MeCN | EtOH | HEPES (20 mM, pH = 7.4) | QY (HEPES) |
|-----------|--------|------|------|-------------------------|-------------|
|           | λₑₓ (nm) | λₑₘ (nm) | λₑₓ (nm) | λₑₘ (nm) | λₑₓ (nm) | λₑₘ (nm) | λₑₓ (nm) | λₑₘ (nm) |
| Np₃mTz   | 421    | 520   | 432  | 530         | 445        | 528       | -       | -      | <0.01 |
| Np₃mPz   | 431    | 532   | 438  | 537         | 449        | 539       | 458     | 545    | 0.037 |
| Np₃pTz   | 418    | 511   | 432  | 535         | 451        | 533       | -       | -      | <0.01 |
| Np₆mPz   | 420    | 521   | 438  | 541         | 449        | 545       | 454     | 552    | 0.038 |
| Np₆mTz   | 443    | 525   | 453  | 526         | 460        | 530       | -       | -      | <0.01 |
| Np₆pTz   | 442    | 513   | 450  | 532         | 461        | 540       | 463     | 556    | 0.032 |
| Np₆pPz   | 438    | 529   | 453  | 529         | 463        | 533       | -       | -      | <0.01 |
| MitoNpTz | 443    | 510   | 449  | 529         | 460        | 537       | 467     | 559    | 0.022 |
| MitoNpPz | 440    | 530   | 438  | 534         | 450        | 543       | 473     | 563    | 0.085 |
| LysNpTz  | 439    | 506   | 439  | 533         | 457        | 528       | 459     | 543    | <0.01 |
| LysNpPz  | 445    | 526   | 444  | 528         | 458        | 532       | 462     | 563    | 0.025 |

Table S2. Exact masses of the naphthalimide tetrazines and corresponding pyridazine products.

|       | N₃mTz | N₃mPz |
|-------|-------|-------|
| Mol. Formula | C₂₉H₃₀N₆O₂ | C₃₉H₄₄N₄O₃ |
| [M]^+     | 494.2340 | 616.3413 |
| [M+H]^+   | 495.2508 | 617.3492 |
| [M+Na]^+  | 517.2328 | 639.3311 |

Table S3. Theoretical vertical excitation energies (eV) determined with TD-DFT and with the additional CC2 corrections and TD-DFT oscillator strengths for the two lowest lying excited states of the tetrazine derivatives. Note that the first excited state is localized on the tetrazine, whereas the second is localized on the naphthalimide. The experimental excitation energies in eV are also given. The most stable conformer was considered.

|       | S₀-S₁ | S₁-S₂ | Eₑₓc (exp) |
|-------|-------|-------|------------|
|       | DE (TD) | f(TD) | DE (CC2) | DE (TD) | f(TD) | DE (CC2) |       |
| Np₃mTz | 2.16 | 0.00 | 2.30 | 3.22 | 0.35 | 3.01 | 2.87 |
| Np₃pTz | 2.13 | 0.00 | 2.28 | 3.22 | 0.33 | 3.01 | 2.87 |
| Np₆mTz | 2.16 | 0.00 | 2.20 | 3.21 | 0.37 | 3.02 | 2.74 |
| Np₆pTz | 2.17 | 0.00 | 2.30 | 3.19 | 0.35 | 3.01 | 2.74 |

Table S4. Theoretical vertical emission energies (eV) determined on the corresponding excited-state minima. See caption of Table S2 for details.

|       | S₀-S₀ | S₂-S₀ | Eₑₘₙ (exp) |
|-------|-------|-------|------------|
|       | DE (TD) | f(TD) | DE (CC2) | DE (TD) | f(TD) | DE (CC2) |       |
| Np₃mTz | 1.82 | 0.00 | 1.97 | 2.53 | 0.43 | 2.31 | 2.34 |
| Np₃pTz | 1.82 | 0.01 | 1.96 | 2.53 | 0.42 | 2.31 | 2.33 |
| Np₆mTz | 1.82 | 0.01 | 1.96 | 2.57 | 0.45 | 2.35 | 2.34 |
| Np₆pTz | 1.82 | 0.01 | 1.97 | 2.57 | 0.45 | n.d. | 2.36 |
**Supplementary figures**

**Figure S1.** Absorption spectra of a) Np3mTz (orange) and Np3mPz (black), b) Np3pTz (orange) and Np3pPz (black), c) Np6mTz (orange) and Np6mPz (black), and d) Np6pTz (orange) and Np6pPz (black) in absolute ethanol. Insets present zoom of tetrazine shoulder peak between 500-600 nm. All absorption spectra were collected at a concentration of 10 μM.
Figure S2. LCMS traces of Np3mTz before (left) and after (right) reaction with BCN.
Figure S3. LCMS traces of Np3pTz before (left) and after (right) reaction with BCN.
Figure S4. LCMS traces of Np6mTz before (left) and after (right) reaction with BCN.
Figure S5. LCMS traces of Np6pTz before (left) and after (right) reaction with BCN.
Figure S6. ESI-HRMS of (top) reaction mixture of \textbf{Np3mTz} after reaction with BCN (5 equiv.) in DMSO for 10 min at room temperature and (bottom) calculated mass of pyridazine product [M+Na]$^+$. 
**Figure S7.** ESI-HRMS of (top) reaction mixture of Np3pTz after reaction with BCN (5 equiv.) in DMSO for 10 min at room temperature and (bottom) calculated mass of pyridazine product [M+H]⁺.
Figure S8. APCI-HRMS of (top) reaction mixture of Np6mTz after reaction with BCN (5 equiv.) in DMSO for 10 min at room temperature and (bottom) calculated mass of pyridazine product [M+H]^+.
Figure S9. APCI-HRMS of (top) reaction mixture of Np6pTz after reaction with BCN (5 equiv.) in DMSO for 10 min at room temperature and (bottom) calculated mass of pyridazine product [M+H]+.
Figure S10. Derivation of second-order rate constants for the reaction of 1 µM a) Np3mTz, b) Np3pTz, c) Np6mTz or d) Np6pTz with 100-400 equivalents of BCN in 1:1 MeCN:H₂O. The slope value (shown in tables) of the curve of observed pseudo first-order rate constants is the second-order rate constant in M⁻¹s⁻¹.

Figure S11. Excitation and emission spectra of a) Np3mPz, b) Np3pPz, c) Np6mPz and d) Np6pPz in CH₂Cl₂, MeCN, EtOH or HEPES buffer (20 mM, pH = 7.4).
Fold turn-on ratios of each of the tetrazine naphthalimide dyes after reaction with BCN in CH$_2$Cl$_2$, MeCN, EtOH and HEPES buffer (20 mM, pH = 7.4). Fold turn-on values were calculated as the ratio between the integrated spectra of the pyridazine dye and corresponding tetrazine dye. The data is presented as the mean ± SD of three independent solutions.

![Figure S12](image)

Figure S12. Fold turn-on ratios of each of the tetrazine naphthalimide dyes after reaction with BCN in CH$_2$Cl$_2$, MeCN, EtOH and HEPES buffer (20 mM, pH = 7.4). Fold turn-on values were calculated as the ratio between the integrated spectra of the pyridazine dye and corresponding tetrazine dye. The data is presented as the mean ± SD of three independent solutions.

Relative free energies of various conformers of Np3mTz and Np6mTz.

![Figure S13](image)

Figure S13. Relative free energies of various conformers of Np3mTz and Np6mTz.
Figure S14. Cell viability of A549 cells after 3 h exposure to the relevant probe or control (1% DMSO). All probes were dosed at a concentration of 25 μM, with the exception of BCN, which was dosed at 250 μM.

Figure S15. Representative images of A549 cells dosed with A) 10 μM Np3mTz for 20 min, B) 10 μM Np3mTz for 90 min, C) 1 μM Np3mTz for 20 min with wash, D) 1 μM Np3mTz for 20 min without wash. λ_ex = 488 nm, λ_em = 510-610 nm. Scale bars represent 20 μm. White arrows indicate punctate regions of fluorescence observed in these images.
Figure S16. Representative images of A549 cells dosed with 1 µM naphthalimide tetrazines or 1 µM naphthalimide pyridazines for 20 min as indicated. 3-Position naphthalimides were excited at $\lambda_{ex} = 405$ nm and 6-position naphthalimides excited at $\lambda_{ex} = 488$ nm. $\lambda_{em} = 510\text{-}610$ nm. Scale bars represent 20 µm.

Figure S17. Images of A549 cells dosed with 2.5 µM Np6mTz for 30 min, followed by Fluorobrite media (rows 1 and 2) or 50 µM BCN in Fluorobrite media (rows 3 and 4) and imaged at the times indicated. Rows 1 and 3 are fluorescence images and rows 2 and 4 are overlays with brightfield images. $\lambda_{ex} = 488$ nm, $\lambda_{em} = 510\text{-}610$ nm. Scale bars represent 20 µm.
Figure S18. Low resolution mass spectrometry of targeted naphthalimide tetrazines. A) LysoNpTz only, B) LysoNpPz formed in situ from reaction of LysoNpTz and BCN, C) MitoNpTz only and D) MitoNpPz formed in situ from reaction of MitoNpTz and BCN. Indicated m/z ratios are for the most abundant predicted peak.
Figure S19. Absorbance spectra of A) LysoNpTz (10 µM) and LysoNpPz (10 µM); and B) MitoNpTz (10 µM) and MitoNpPz (10 µM) in EtOH.

Figure S20. Fluorescence spectra of A) LysoNpTz and LysoNpPz and B) MitoNpTz and MitoNpPz in EtOH.

Figure S21. Normalised fluorescence spectra of A) LysoNpPz and B) MitoNpPz in CH₂Cl₂, MeCN, EtOH and HEPES buffer (20 mM, pH 7.4).
Figure S22. Cell viability of A549 cells after exposure to 1% DMSO, 25 µM LysoNpTz or 25 µM LysoNpTz for 3 h. Data presented as the mean ± SD of triplicate values from one experiment.

Figure S23. Representative images of A549 cells dosed with LysoNpTz (2.5 µM), LysoNpTz (2.5 µM) and BCN (2.5 µM), MitoNpTz (10 µM) or MitoNpTz (10 µM) and BCN (10 µM) for 30 min as indicated. λ_{ex} = 488 nm, λ_{em} = 510-610 nm. Scale bars represent 20 µm.
Figure S24. Representative images of A549 cells dosed with LysoNpTz (2.5 µM) and BCN (2.5 µM) and A) LysoTracker Deep Red (LTDR) (50 nM) or B) MitoTracker Deep Red (MTDR) (100 nM). Representative images of A549 cells dosed with MitoNpTz (10 µM) and BCN (10 µM) and C) MitoTracker Deep Red (100 nM) or D) LysoTracker Deep Red (100 nM). Green channel: $\lambda_{ex} = 488$ nm, $\lambda_{em} = 510-610$ nm. Red channel: $\lambda_{ex} = 640$ nm, $\lambda_{em} = 650-750$ nm. Scale bars represent 20 µm.
Figure S25. A) Full MALDI-TOF-MS spectrum of insulin-BCN after spin-column purification. B) Zoom of 5500-6500 m/z region and assignment of major peaks to insulin and insulin-BCN.

Figure S26. Zoom of 5500-7000 m/z region from MALDI-TOF-MS analysis of insulin-TAMRA mixture and assignment of major peaks to insulin and insulin-TAMRA species.
**Figure S27.** Representative TIRF images of insulin-BCN fibrils at the indicated times. Insulin-BCN fibrils were stained with **Np6mTz** (2 µM). Excitation provided by 473 nm laser. Scale bars represent 5 µm.

**Figure S28.** Representative TIRF images of insulin-TAMRA fibrils at the indicated times. Excitation provided by 561 nm laser. Scale bars represent 5 µm.
**Figure S29.** Representative TEM images of insulin, insulin-BCN and insulin-TAMRA fibrils. Scale bars represent 500 nm.
Experimental details

General synthesis
All reactions were performed under a nitrogen atmosphere unless otherwise indicated. All reagents were obtained from Sigma-Aldrich, Merck or Combi-Blocks. Tetrahydrofuran (THF) and dichloromethane (CH$_2$Cl$_2$) were obtained from a Pure-Solv 400 Solvent Purification System. Anhydrous 1,4-dioxane was obtained from Sigma Aldrich. Peptide-grade N,N-dimethylformamide (DMF) was obtained from LabScan. All other solvents were laboratory grade and used without further purification. Reactions were monitored by silica gel thin-layer chromatography plates (Merck, TLC Silica gel 60 F$_{254}$). All column chromatography was performed on silica gel 60 (Merck, 0.040-0.063 mm) or using a Biotage Isolera One. Compounds 2b, 3, 2, 3-bromo-5-nitro-1,8-naphthalimide (4), 6, 4 and 13 were prepared according to literature methods.

General characterisation
All NMR spectra were obtained at 300 K on Bruker AVANCE III 400 or Bruker AVANCE III 500 spectrometers equipped with a 5 mm BBFO probe with z-gradients. Deuterated solvents (CDCl$_3$, DMSO-d$_6$, MeOD) were obtained from Cambridge Isotope Laboratories. All chemical shifts are reported in ppm and all coupling constants are reported in Hz. $^1$H NMR spectra are calibrated to trace isotopic impurities of the solvent used ($\delta = 7.26$ ppm for CDCl$_3$, $\delta = 2.50$ ppm for DMSO-d$_6$, $\delta = 3.31$ ppm for MeOD). $^1$H NMR data are reported as: chemical shift, multiplicity, coupling constant(s) ($J$) and relative integral. The multiplicities are reported as one or more of the following: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad. $^{13}$C$\{^1$H$\}$ NMR spectra are calibrated to trace isotopic impurities of the solvent used ($\delta = 77.16$ ppm for CDCl$_3$, $\delta = 39.52$ ppm for DMSO-d$_6$, $\delta = 49.00$ ppm for MeOD). $^{13}$C$\{^1$H$\}$ NMR data is reported as chemical shift.

Low resolution ESI and APCI mass spectrometry was performed on a Bruker AmaZon SL ion trap mass spectrometer. For low resolution ESI, samples were injected via flow injection at 0.3 mL/min in methanol or acetonitrile into an Apollo II source with nitrogen drying gas at 180 °C. For low resolution APCI, samples were placed in a melting point tube and inserted into the Bruker Apollo II APCI source with an atmospheric solid analysis probe attachment added with vaporisation temperature 400 °C and corona current 4 µA. High resolution ESI and APCI mass spectrometry was performed on a Bruker solarix 2XR Fourier Transform Ion Cyclotron Resonance Mass Spectrometer. For high resolution ESI, samples were injected using the supplied syringe pump at 180 µL/h with nebuliser flow 1 L/min and drying gas 4L/min at 180 °C. For high resolution APCI, samples were placed in a melting point tube and inserted into the Bruker Apollo II APCI source with an atmospheric solid analysis probe attachment added with vaporisation temperature 400 °C and corona current 4 µA.

Photophysical studies
The solvents used in photophysical studies were dichloromethane (HPLC grade, Sigma Aldrich), acetonitrile (Spectroscopy Grade, AJAX), absolute ethanol (200 proof, HPLC/spectrophotometric grade, Sigma Aldrich) or HEPES (20 mM, pH 7.4). All compounds were prepared as stock solutions in DMSO (Spectroscopy grade, Sigma Aldrich) and the concentrated stock solution was diluted to the required concentration in the appropriate solvent. The DMSO concentration in all experiments was <0.5% v/v.

Absorption spectra were obtained for each compound in absolute ethanol on a Varian Cary 400 UV-Vis spectrophotometer using 10 mm pathlength quartz cuvettes. Fluorescence spectra were obtained for each compound in CH$_2$Cl$_2$, MeCN, absolute EtOH or HEPES buffer on a Varian Cary Eclipse fluorometer using quartz cuvettes.
Extinction coefficients were determined from the absorption spectra of three independent solutions (5, 7.5 and 10 µM) of each compound. The absorption value at $\lambda_{\text{max}}$ above 400 nm was obtained for each concentration and a linear regression of these values was performed with GraphPad Prism 8.

Absolute quantum yields were measured in absolute EtOH or HEPES buffer (20 mM, pH 7.4) on a PTI QuantaMaster 400 fluorometer with an integrating sphere setup in 3.5 mL quartz cuvettes. Reported values are the average of three measurements of three independent optically dilute (A < 0.1, ~2-5 µM) solutions. The values were corrected for self-absorption as described previously, using estimates of the self-absorption parameter ($a$) from the area overlapping between the excitation and emission spectra.

**LCMS reaction analysis**

For the naphthalimide tetrazines, 2 µL of a 10 mM stock was diluted to approximately 300 µL in 1:1 MeCN:H$_2$O. For the corresponding pyridazines, 2 µL of a naphthalimide tetrazine stock (10 mM) was mixed with 2 µL of a BCN stock (50 mM) and incubated for 10 min at room temperature. This mixture was diluted to approximately 300 µL. Samples were syringe filtered before analysis.

Liquid-chromatography mass spectrometry (LCMS) was conducted on a Shimadzu LC-MS 2020 instrument consisting of a LC-M20A pump and a SPD-20A UV-VIS detector coupled to a Shimadzu 2020 mass spectrometer. Separations were performed on a Waters Sunfire C18 column (5 µm, 2.1x150 mm), operating at a flow rate of 0.2 mL min$^{-1}$ using a mobile phase of 0.1% formic acid in Milli-Q H$_2$O and 0.1% formic acid in MeCN (HPLC grade, Sigma Aldrich). All LCMS traces are taken over a solvent gradient of 0% MeCN to 100% MeCN run for 30 min.

**Kinetics**

Second order rate constants were derived using the fluorescence output of the reaction under pseudo-first order conditions. All fluorescence values were determined in black 96-well plates (655209, Greiner Bio-One) on a Perkin Elmer EnSpire Plate Reader. The naphthalimide tetrazines were diluted to 2 µM in 1:1 MeCN:H$_2$O. Solutions of BCN were prepared to concentrations of 200, 400, 600 and 800 µM in 1:1 MeCN:H$_2$O. For each compound, 100 µL of the tetrazine solution was added to five wells of the plate. 100 µL of 1:1 MeCN:H$_2$O was added to the control, and 100 µL of each of the BCN stock solutions were added to different wells, giving solutions with 0, 100, 200, 300 and 400 equivalents of BCN. The solutions were excited at 450 nm and fluorescence intensity at 540 nm was recorded every 10 s for 20 min. The resultant curves were fitted to ‘one-phase association’ models in GraphPad Prism 8 to determine the experimental first order rate constant, $k_{\text{obs}}$. A linear plot of BCN concentration against $k_{\text{obs}}$ was used to determine the second order rate constants. The data are presented as the mean ± standard error of a single experiment, with the error values of the rate constants extracted from the linear regression analysis.

**Fold turn-on**

Fold turn-on values for each of the naphthalimide tetrazines were measured in black 96-well plates (655209, Greiner Bio-One) on a Perkin Elmer EnSpire Plate Reader. Stock solutions of the naphthalimide tetrazines and the corresponding pyridazines were diluted into CH$_2$Cl$_2$, MeCN, absolute EtOH or HEPES buffer to give 5 µM solutions of each compound and the corresponding reacted form in triplicate. 200 µL of each solution was added to each well. Excitation was provided at the maximal excitation wavelength for each compound in each solvent, and emission was collected at 1 nm intervals from 500-700 nm. Blank solutions, containing an equal amount of DMSO and the relevant solvent were also collected. Each set of intensity values were added together to give an integrated intensity, and the blank solvent sums were subtracted from these. The integrated intensities
of the unreacted tetrazines were averaged, and each of the integrated intensities of the reacted forms were divided by this average to give the fold turn-on value. The values are presented as the mean ± SD of three independent experiments.

First-principle calculations
To model the excited state spectra of the dyes, we have applied a protocol used extensively in the Nantes’ group. The total and transition energies are determined with second-order Coupled-Cluster calculations (CC2), whereas the geometries, vibrations, and environmental effects are computed at the Time-Dependent Density Functional Theory (TD-DFT) level. No structure simplification was made. All CC2 calculations were achieved with Turbomole applying the resolution-of-identity approach and selecting the aug-cc-pVDZ atomic basis set. All (TD-)DFT calculations have been performed using the Gaussian16.A03 program. For these Gaussian calculations, we used tightened self-consistent field (10⁻¹⁰ a.u.) and geometry optimization (10⁻⁵ a.u.) convergence thresholds, and a large DFT integration grid (so-called ultrafine grid, a pruned 99,590 grid). These (TD-)DFT calculations relied on the M06-2X hybrid functional. Following the basis set combination approach proposed previously, we used the 6-311G(d,p) atomic basis set for determining the geometrical and vibrational parameters whereas the transition energies have been computed with 6-311++G(d,p). The nature of the ground-state stationary points was confirmed by analytical Hessian calculations that returned 0 (minima) imaginary vibrational modes. Several conformers were typically considered. Environmental effects on the transition energies have been accounted for using the polarizable continuum model (PCM). We have chosen acetonitrile as solvent in the modeling, because this solvent is aprotic (protic solvents are not ideally modelled with PCM) and gives results similar to the one obtained with MeCN and EtOH experimentally, e.g., see Figure 2 of the main text. For the geometrical and vibrational calculations, we did use the standard LR (linear-response) PCM model in its equilibrium limit, which is the default in Gaussian16. For both absorption and emission we used a more refined LR+cLR model in its non-equilibrium limit, so as to capture both linear-response and state-specific solvent effects. Excited-states have been represented using density difference plots, in which the excited-state density was determined at the TD-DFT level. In these plots blue and red regions respectively indicate decrease and increase of electron density upon photon absorption (a contour threshold of 8×10⁻⁴ au is used in all representations). The EET couplings were determined using Gaussian 16 with the same level of theory as for the TD-DFT calculations, and applying Curutchet and Mennucci’s approach.

Relative energies of the most stable conformers
For the various fluorophores, we have performed conformational search focussing on the relative orientations of the various moieties (and not on the flexibility of the butyl chains). We show in Figure S13 the relative free energies (as obtained with PCM-DFT) and the structures of the most stable conformers of the two meta structures. Conformational search for the para compounds revealed less possibilities, as expected. Note that the relative orientations of the tetrazine and naphthalimide are significantly different when going from one structure to another, but that the obtained energies are very similar clearly indicating the existence of several conformers in solution at room temperature.

Comparison between theoretical and experimental excitation energies.
In the Tables S3 and S4 we provide a comparison between the vertical excitation energies of the four considered dyes (computed on the ground-state geometry of the most stable conformer) and the experimental excitation energies. We underline that such comparison does not consider vibronic couplings, hence one expects the theoretical values to be (slightly) larger than their experimental counterparts. This is obviously the case for the S₀-S₂ excitation centred on the naphthalimide core, consistent with the measurement, whereas the S₀-S₁ excitation is much lower than the experimental value and remains unseen experimentally due to its dark (f=0) nature.

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**Cell culture**

A549 cells were maintained at 37 °C in 5% carbon dioxide. Cells were cultured in Advanced Dulbecco’s Modified Eagle’s Medium (ADMEM, Thermo Fisher Scientific) supplemented with 2% foetal calf serum (FCS, Thermo Fisher Scientific) and 2.5 mM L-glutamine (Sigma-Aldrich). Fluorobrite DMEM (FDMEM, Thermo Fisher Scientific) was supplemented with 2% FCS and 2.5 mM L-glutamine, unless otherwise stated.

Cells for imaging and toxicity experiments had a passage number lower than 20. Probe stock solutions were made in DMSO and were freshly dissolved and used within a week. For treatments, the DMSO stock was diluted into ADMEM or FDMEM at the appropriate concentrations and then added to cells.

**alamarBlue cell viability assay**

A549 cells were seeded in 96 well plates in ADMEM (100 µL) at a density of 25000 cells/well and allowed to adhere for 16 h. Solutions of Probes were diluted to 50 µM in ADMEM, containing 1% DMSO, whereas BCN was diluted to 500 µM in ADMEM, containing 1% DMSO. 100 µL of either ADMEM (for controls), ADMEM containing 1% DMSO, probe solution or BCN solution were added to wells in triplicate. The cells were incubated for 3 h, before the addition of alamarBlue reagent. After a further 3 h incubation, the fluorescence of each well was measured (λex = 570 nm, λem = 590 nm) in a Perkin EnSpire Plate Reader. Values were normalised to controls containing ADMEM only and the data is presented as the average percentage viability of at least triplicate values from a single experiment.

**Confocal microscopy**

For all imaging experiments, 1 × 10^5 cells were seeded into 35 mm glass bottom dishes (MatTek) and allowed to adhere overnight. Images were obtained at 37 °C in a 5% CO2 atmosphere on an Olympus FluoView FV3000 Confocal Laser Scanning Microscope, using an Olympus 60× water objective (UPLSAPO60XW) and 405, 488 and 640 nm lasers, unless otherwise stated. Images were processed using FIJI software.

For the static images, the media was removed from the cells and replaced with 1 µM or 10 µM solutions of the probe in ADMEM (1.0 mL). Cells were incubated for 20 min. When complete, the media was removed, cells washed with PBS (3×0.5 mL) and resuspended in FDMEM (1.0 mL).

For images where BCN was dosed after the addition of probe, the cells were incubated with the relevant dose of probe in ADMEM (1.0 mL) for 30 min. The media was removed, and cells were washed with PBS (3×0.5 mL). 50 µM BCN solution in FDMEM (1.0 mL) was added and the cells were incubated for a further 60 min before imaging.

For the time course experiments, the cells were dosed with either **Np6mTz** (2.5 µM) or BCN (50 µM) for 30 mins. The cells were then quickly washed with PBS (3×0.5 mL) and the cells were suspended in BCN (50 µM) or **Np6mTz** (2.5 µM) solutions in FDMEM (1.0 mL) containing 10% FCS and 2.5 mM L-glutamine. For the control, the cells were suspended in FDMEM (1.0 mL). The cells were imaged every 2 min with the first image obtained 2 minutes post-washing. These images were collected with an Olympus 40× dry objective (UPLSAPO40X2) and 488 nm laser.

For the lysosome colocalization experiments, 5 µM **LysoNpTz** (0.5 mL in ADMEM) and 5 µM BCN (0.5 mL in ADMEM) were added individually to cells which were allowed to incubate at 37 °C for 30 min. 1 µL of either LysoTracker Deep Red (50 µM in DMSO) or MitoTracker Deep Red (100 µM in DMSO) was added to the media in the last 10 min of incubation. The media was aspirated, the cells washed with PBS (3×0.5 mL) and resuspended in FDMEM (1.0 mL).
For the mitochondria colocalization experiments, 20 µM MitoNpTz (0.5 mL in ADMEM) and 20 µM BCN (0.5 mL in ADMEM) were added individually to cells which were allowed to incubate at 37 °C for 30 min. 1 µL of either LysoTracker Deep Red (100 µM in DMSO) or MitoTracker Deep Red (100 µM in DMSO) was added to the media in the last 10 min of incubation. The media was aspirated, the cells washed with PBS (3×0.5 mL) and resuspended in FDMEM (1.0 mL).

For the ‘green channel’ excitation was provided by a 488 nm laser and emission collected between 510-610 nm. For the ‘red channel’ excitation was provided by a 640 nm and emission collected between 650-750 nm. Colocalization analysis was performed using the inbuilt Coloc2 plugin in FIJI.

**Preparation of tagged insulin**

Tagging insulin was performed as per a previously established protocol. Recombinant human insulin was prepared as a 15 mg/mL solution in 0.1 M bicarbonate buffer (pH 9.5). For insulin-BCN, 5/6-carboxy-tetramethylrhodamine N-succinimidyl ester (Sigma-Aldrich) was prepared as a 5 mg/mL solution in DMSO. 0.5 mL of insulin solution was mixed with 40 µL of the rhodamine solution in a sealed vial at room temperature with stirring. The reaction was stirred at rt for 3 h.

MALDI analysis

Tagged insulin solutions were desalted using Merck C18 Zip Tips according to the manufacturer's protocol. Low resolution mass spectra of insulin fibrils were acquired in reflectron mode on a Bruker Autoflex Speed MALDI-TOF using a matrix of saturated trans-3,5-dimethoxy- 4-hydroxy cinnamic acid in 1:1 MeCN:H2O containing 0.1% TFA (TA50). Equal volumes of the insulin-BCN sample in TA50 and the matrix were mixed and spotted to a ground steel MALDI plate and the solvent was allowed to evaporate. Data was acquired with Protein 1 calibrants (Bruker). Raw data was exported and graphed using GraphPad Prism 8.

**Insulin fibril growth conditions**

Human recombinant insulin was dissolved in 20 mM glycine buffer (pH 2.0) to a concentration of 1 mg/mL. To form amyloid fibrils, the sample was incubated in a thermomixer at 60 °C with shaking at 700 rpm for 8 h.

**Thioflavin T kinetics assay**

To measure the kinetics of amyloid assembly, monomeric Insulin samples unlabelled, insulin-BCN and insulin-TAMRA samples purified using the Zeba 7K MWCO columns (2 mL, Thermo Fisher) were prepared in triplicate in 96-well plates in aqueous 20 mM glycine buffer (pH 2.0). Thioflavin T was added to the buffer to a final concentration of 40 µM. Samples were incubated in a POLARstar Omega microplate reader (BMG Labtech), with excitation at 440 nm and the fluorescence emission recorded at 480 nm with double orbital shaking at 700 rpm for ~10 h.
**TIRF imaging of insulin fibrils**

TIRF imaging was performed on the Oxford Nanoimager equipped with a 100 × 1.4 NA oil immersion objective (Olympus), and a Hamamatsu Flash 4 V3 sCMOS camera. Insulin-TAMRA or insulin-BCN fibrils treated with Np6mTz (2 μM) were placed on a glass slide and sealed with Marienfeld Precision cover glasses (thickness No. 1.5H, tol. ±5 μm). Samples were irradiated with 473 nm laser in TIRF imaging mode. The images obtained were analysed on FIJI ImageJ. The fibril areas were determined by thresholding the images in FIJI ImageJ to select fluorescent regions (excluding holes) following which the measure function was used to calculate the mean area of fluorescent pixels within the image. Data is presented as the mean ± SD of 25 images from one experiment.

**TEM imaging of insulin fibrils**

Unlabelled and labelled insulin fibrils were prepared in 20 mM glycine buffer (pH 2.0) to a concentration of 1 mg/mL and incubated in a thermomixer at 60 °C with shaking at 700 rpm for 500 min. Carbon/formvar coated copper grids (ProSciTech Pty Ltd.) were floated on 15 µL of protein sample for 1 min. Excess sample was wicked away and grids washed with MilliQ® water and stained with 2% aqueous uranyl acetate solution for 1 min. Samples were imaged on a FEI Tecnai T12 electron microscope operating at 120 kV. Digital images were captured using a Veleta CCD camera and the RADIUS 2.0 imaging software (EMSIS GmbH).

**Detailed synthetic procedures**

3-Bromo-4-butylamino-1,8-naphthalimide (1a)

\[
\text{\begin{tikzpicture}
\node at (0,0) {\includegraphics[width=0.5\textwidth]{naphthalimide.png}};
\end{tikzpicture}}
\]

3 (500 mg, 1.5 mmol) was dissolved in DMF (7 mL) and cooled to 0 °C. A solution of freshly-recrystallised N-bromosuccinimide (0.37 g, 2.1 mmol) in DMF (10 mL) was added dropwise over 1 h. The reaction was warmed to rt and stirred for 3 h. The reaction mixture was diluted with 5% LiCl solution (100 mL) and extracted with EtOAc (50 mL). The organic extracts were washed with 5% LiCl solution (5×50 mL), dried over MgSO₄ and concentrated in vacuo. The residue was purified with column chromatography (SiO₂, 6% EtOAc in hexanes). 1a was obtained as a bright yellow powder (0.49 g, 79%).

\[^{1}H\text{ NMR (400 MHz, CDCl}_{3}]: \delta 8.62 (s, 1H), 8.57 (d, J = 7.3 Hz, 1H), 8.45 (d, J = 8.6 Hz, 1H), 7.64 (t, J = 7.6 Hz, 1H), 4.15 (t, J = 7.6 Hz, 2H), 3.67 (t, J = 6.9 Hz, 2H), 1.78 – 1.64 (m, 4H), 1.53 – 1.37 (m, 4H), 0.99 – 0.94 (m, 6H).\]

\[^{13}C\text{ NMR (101 MHz, CDCl}_{3}): 164.3, 163.2, 149.8, 135.6, 131.5, 130.7, 129.8, 125.3, 123.5, 123.4, 114.4, 110.0, 50.9, 40.3, 33.7, 30.6, 20.5, 20.1, 14.0, 13.9.\]

LRMS (APCI) m/z: [M+H]^+ Calcd for C_{20}H_{23}BrN_{2}O_{2}^+ 403 and 405; Found 403 and 405.

Characterisation data matches that reported in the literature.²
3-Bromo-5-butilamino-N-butil-1,8-naphthalimide (1b)

4 (0.20 g, 0.62 mmol) and Et₃N (0.18 mL, 1.3 mmol) were suspended in absolute EtOH (10 mL). n-
-n-Butylamine (0.13 mL, 1.3 mmol) was added dropwise and the reacted heated to reflux for 16 h. The reaction was diluted with 1 M HCl (50 mL) and extracted with CH₂Cl₂ (3×30 mL). The organic extracts were washed with 1 M HCl (2×100 mL) and brine (100 mL), dried over Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (SiO₂, 0-7.5% EtOAc in hexanes) to afford 1b (0.13 g, 53%) as an orange powder.

¹H NMR (400 MHz, CDCl₃): δ 8.56 (d, J = 1.8 Hz, 1H), 8.40 (d, J = 8.4 Hz, 1H), 8.16 (d, J = 1.8 Hz, 1H), 6.69 (d, J = 8.4 Hz, 1H), 4.13 (t, J = 7.5 Hz, 2H), 3.38 (t, J = 7.2 Hz, 2H), 1.83 – 1.76 (m, 2H), 1.71 – 1.64 (m, 2H), 1.58 – 1.49 (m, 2H), 1.46 – 1.37 (m, 2H), 1.02 (t, J = 7.4 Hz, 3H), 0.95 (t, J = 7.4 Hz, 3H).

¹³C NMR (101 MHz, CDCl₃): δ 163.8, 163.6, 148.6, 134.7, 133.8, 128.3, 124.9, 121.7, 118.7, 110.4, 105.2, 43.7, 40.3, 31.1, 30.4, 20.5, 20.5, 14.0, 14.0.

LRMS (APCI) m/z: [M+H]⁺ Calcd for C₂₀H₂₃BrN₂O₂ 403 and 405; Found 403 and 405.

Characterisation data matches that reported in the literature.

3-(3-Bromophenyl)-6-methyl-1,2,4,5-tetrazine (5)

3-Bromobenzonitrile (1.0 g, 5.5 mmol) was suspended in absolute EtOH (0.75 mL) and MeCN (2.3 mL, 44 mmol) and cooled to 0 °C. 3-mercaptopropionic acid (0.48 mL, 5.5 mmol) and hydrazine hydrate (4.3 mL, 88 mmol) were sequentially added dropwise. The reaction was stirred for 30 min before being warmed to 40 °C for 16 h. The reaction was slowly poured into a solution of NaNO₂ (5.7 g, 15 equiv.) in water, open to air. 1 M HCl was then added dropwise until the mixture was pH 3, with no effervescence and was vivid pink/red colour (SAFETY: During the addition of acid, small amounts of toxic NOₓ gases are produced. Perform this step in a well-ventilated fume hood). The mixture was extracted with CH₂Cl₂ (3 × 50 mL) and the organic extracts were washed with brine (150 mL), dried over Na₂SO₄ and concentrated to dryness in vacuo. Purification by column chromatography (SiO₂, 0-35% CH₂Cl₂ in hexanes) afforded 5 (0.78 g, 56%) as pink crystals.

¹H NMR (400 MHz, CDCl₃): δ 8.75 (s, 1H), 8.53 (d, J = 7.8 Hz, 1H), 7.75 (d, J = 7.8 Hz, 1H), 7.47 (t, J = 7.9 Hz, 1H), 3.12 (s, 3H).

¹³C NMR (101 MHz, CDCl₃): δ 167.8, 163.3, 135.6, 133.9, 131.0, 130.9, 126.5, 123.6, 21.4.

LRMS (APCI) m/z: [M+H]⁺ Calcd for C₉H₈BrN₄ 251 and 253; Found 251 and 253.
3-Methyl-6-(3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)-1,2,4,5-tetrazine (2a)

\[
\text{N} \quad \text{N} \quad \text{N} \\
\text{N} \quad \text{B} \quad \text{O} \\
\text{N} \quad \text{N} \\
\text{N}
\]

5 (0.25 g, 1.0 mmol), B\textsubscript{2}pin\textsubscript{2} (0.30 g, 1.2 mmol), KOAc (0.15 g, 1.5 mmol) and PdCl\textsubscript{2}(dppf) (36 mg, 5 mol%) were put under N\textsubscript{2} atmosphere. Degassed (3 × freeze-pump-thaw cycles) THF (7 mL) was added, and the reaction heated to 65 °C for 16 h. The reaction mixture was diluted with water (20 mL) and extracted with CH\textsubscript{2}Cl\textsubscript{2} (3 × 15 mL). The organic extracts were washed with brine (50 mL), dried over Na\textsubscript{2}SO\textsubscript{4} and concentrated in vacuo. Purification by column chromatography (SiO\textsubscript{2}, 2-10% EtOAc in hexanes) afforded 2a as a pink solid (0.22 g, 74%).

\(^1\)H NMR (500 MHz, CDCl\textsubscript{3}): δ 9.03 (s, 1H), 8.68 – 8.64 (m, 1H), 8.05 (d, J = 7.2 Hz, 1H), 7.59 (t, J = 7.6 Hz, 1H), 3.10 (s, 3H), 1.38 (s, 12H).

\(^{13}\)C NMR (101 MHz, CDCl\textsubscript{3}): δ 167.4, 164.4, 138.9, 134.5, 131.4, 130.7, 128.8, 84.3, 25.1, 21.3.

HRMS (ESI) m/z: [M+Na]\textsuperscript{+} Calcd for C\textsubscript{15}H\textsubscript{19}BNaN\textsubscript{4}O\textsubscript{2} 321.1493; Found 321.1495.

**General method for Suzuki coupling**

The relevant brominated naphthalimide (1 equiv.), boronate ester (1.5-2 equiv.), K\textsubscript{2}CO\textsubscript{3} (2 equiv.) and Pd(PPh\textsubscript{3})\textsubscript{4} (5-10 mol% catalyst loading) were added to a flask under a N\textsubscript{2} atmosphere. The flask was evacuated and refilled with N\textsubscript{2} thrice. A degassed (nitrogen sparging) solvent mixture was added (4:1 THF:H\textsubscript{2}O; final reaction concentration approximately 0.01-0.02 M relative to brominated naphthalimide), and the mixture was heated at the indicated temperature for the indicated time. Upon completion of the reaction (as determined by TLC analysis), the reaction mixture was diluted with water (30 mL) and extracted thrice with CH\textsubscript{2}Cl\textsubscript{2} (3 × 30 mL). The organic extracts were washed with brine (100 mL), dried over Na\textsubscript{2}SO\textsubscript{4} and concentrated in vacuo. The crude residue was purified by column chromatography to afford the desired products.

**Np3mTz**

\[
\text{N} \quad \text{N} \quad \text{N} \\
\text{O} \quad \text{N} \\
\text{N} \quad \text{N} \\
\text{N}
\]

1a (30 mg, 0.074 mmol), 2a (44 mg, 0.15 mmol), K\textsubscript{2}CO\textsubscript{3} (21 mg, 0.15 mmol) and Pd(PPh\textsubscript{3})\textsubscript{4} (4 mg, 5 mol%) in 4:1 THF:H\textsubscript{2}O (7 mL) were subjected to the General Method for Suzuki Coupling at 65 °C for 5 h. Purification by column chromatography (SiO\textsubscript{2}, 0-4% EtOAc in CH\textsubscript{2}Cl\textsubscript{2}) afforded Np3mTz as a red powder (22 mg, 60%).
$^1$H NMR (500 MHz, CDCl$_3$): δ 8.72 (s, 1H), 8.66 – 8.65 (m, 1H), 8.61 (d, $J$ = 7.2 Hz, 1H), 8.45 (s, 1H), 8.40 (d, $J$ = 8.4 Hz, 1H), 7.76 – 7.72 (m, 2H), 7.69 (t, $J$ = 7.7 Hz, 1H), 4.69 (br s, 1H), 4.18 (t, $J$ = 7.5 Hz, 2H), 3.28 (t, $J$ = 7.0 Hz, 2H), 3.12 (s, 3H), 1.72 (quint, $J$ = 7.6 Hz, 2H), 1.51 – 1.42 (m, 4H), 1.24 – 1.17 (m, 2H), 0.97 (t, $J$ = 7.4 Hz, 3H), 0.78 (t, $J$ = 7.4 Hz, 3H).

$^{13}$C NMR (126 MHz, CDCl$_3$): δ 167.7, 164.7, 164.2, 164.0, 149.7, 140.5, 135.3, 133.7, 132.8, 131.4, 130.2, 129.9, 129.5, 127.4, 125.4, 125.3, 123.7, 123.4, 113.5, 50.2, 40.2, 33.2, 30.4, 21.4, 20.5, 20.0, 14.0, 13.8.

HRMS (ESI) m/z: [M+Na]$^+$ Calcd for C$_{29}$H$_{29}$N$_6$NaO$_2$ 517.2322; Found 517.2323.

Np$_3$pTz

1a (30 mg, 0.074 mmol), 2b (44 mg, 0.15 mmol), K$_2$CO$_3$ (21 mg, 0.15 mmol) and Pd(PPh$_3$)$_4$ (4 mg, 5 mol%) in 4:1 THF:H$_2$O (7 mL) were subjected to the General Method for Suzuki Coupling at 65 °C for 4 h. Purification by column chromatography (SiO$_2$, 0-4% EtoAc in CH$_2$Cl$_2$) afforded Np$_3$pTz as a red powder (17 mg, 48%).

$^1$H NMR (500 MHz, CDCl$_3$): δ 8.74 – 8.71 (m, 2H), 8.61 (dd, $J$ = 7.3, 0.8 Hz, 1H), 8.44 (s, 1H), 8.39 (dd, $J$ = 8.4, 0.8 Hz, 1H), 7.73 – 7.67 (m, 3H), 4.75 (br s, 1H), 4.16 (t, $J$ = 7.5 Hz, 2H), 3.26 (t, $J$ = 7.0 Hz, 2H), 3.13 (s, 3H), 1.75 – 1.69 (m, 2H), 1.52 – 1.41 (m, 4H), 1.27 – 1.19 (m, 2H), 0.96 (t, $J$ = 7.5 Hz, 3H), 0.80 (t, $J$ = 7.3 Hz, 3H).

$^{13}$C NMR (126 MHz, CDCl$_3$): δ 167.5, 164.7, 164.1, 164.0, 149.6, 143.9, 135.2, 131.4, 131.4, 130.4, 129.9, 129.3, 128.7, 125.4, 125.1, 123.6, 123.4, 113.5, 50.1, 40.2, 33.3, 30.4, 21.4, 20.5, 20.0, 14.0, 13.8.

HRMS (ESI) m/z: [M+Na]$^+$ Calcd for C$_{29}$H$_{29}$N$_6$NaO$_2$ 517.2322; Found 517.2323.

Np$_6$mTz

1b (35 mg, 0.087 mmol), 2a (44 mg, 0.15 mmol), K$_2$CO$_3$ (24 mg, 0.17 mmol) and Pd(PPh$_3$)$_4$ (5 mg, 5 mol%) in 4:1 THF:H$_2$O (8 mL) were subjected to the General Method for Suzuki Coupling at 60 °C for 3 h. Purification by column chromatography (SiO$_2$, 0-6% EtoAc in CH$_2$Cl$_2$) afforded Np$_6$mTz as a red powder (18 mg, 43%).
$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 8.84 (s, 1H), 8.80 (d, $J$ = 1.2 Hz, 1H), 8.55 (d, $J$ = 7.8 Hz, 1H), 8.41 (d, $J$ = 8.5 Hz, 1H), 8.31 (d, $J$ = 1.2 Hz, 1H), 7.89 (d, $J$ = 7.8 Hz, 1H), 7.66 (t, $J$ = 7.8 Hz, 1H), 6.73 (d, $J$ = 8.5 Hz, 1H), 5.64 (br s, 1H), 4.18 (t, $J$ = 7.6 Hz, 2H), 3.46 (t, $J$ = 7.3 Hz, 2H), 3.12 (s, 3H), 1.89 – 1.81 (m, 2H), 1.76 – 1.69 (m, 2H), 1.59 – 1.41 (m, 4H), 1.03 (t, $J$ = 7.4 Hz, 3H), 0.98 (t, $J$ = 7.4 Hz, 3H).

$^{13}$C NMR (126 MHz, CDCl$_3$): 167.7, 164.7, 164.1, 163.9, 149.9, 141.1, 136.8, 134.6, 132.6, 131.6, 130.3, 130.1, 129.2, 127.4, 126.5, 124.2, 123.9, 120.8, 110.1, 104.9, 43.8, 40.2, 31.2, 30.5, 21.4, 20.6, 20.6, 14.0, 14.0.

HRMS (ESI) m/z: [M+Na]$^+$ Calcd for C$_{29}$H$_{29}$N$_6$NaO$_2$ 517.2322; Found 517.2323.

**Np6pTz**

![Np6pTz](image)

$1b$ (40 mg, 0.099 mmol), $2b$ (50 mg, 0.17 mmol), K$_2$CO$_3$ (27 mg, 0.20 mmol) and Pd(PPh$_3$)$_4$ (6 mg, 5 mol%) in 4:1 THF:H$_2$O (7 mL) were subjected to the General Method for Suzuki Coupling at 60 °C for 16 h. Purification by column chromatography (SiO$_2$, 0-4% EtOAc in CH$_2$Cl$_2$) afforded Np6pTz as a red powder (25 mg, 69%).

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 8.78 (s, 1H), 8.61 (d, $J$ = 7.6 Hz, 2H), 8.40 (d, $J$ = 8.2 Hz, 1H), 8.27 (s, 1H), 7.84 (d, $J$ = 7.6 Hz, 2H), 6.74 (d, $J$ = 7.8 Hz, 1H), 4.16 (t, $J$ = 7.4 Hz, 2H), 3.46 (t, $J$ = 7.2 Hz, 2H), 3.10 (s, 3H), 1.90 – 1.81 (m, 2H), 1.75 – 1.67 (m, 2H), 1.61 – 1.52 (m, 2H), 1.49 – 1.39 (m, 2H), 1.04 (t, $J$ = 7.2 Hz, 3H), 0.97 (t, $J$ = 7.2 Hz, 3H).

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 167.4, 164.7, 164.1, 163.8, 149.9, 143.8, 136.5, 134.7, 131.3, 130.2, 129.3, 128.6, 128.1, 124.2, 123.9, 120.8, 110.3, 105.0, 43.8, 40.2, 31.2, 30.5, 21.3, 20.6, 14.0.

HRMS (APPI) m/z: [M]$^+$ Calcd for C$_{29}$H$_{29}$N$_6$O$_2$ 494.2425; Found 494.2424.

**Compound 7**

![Compound 7](image)

$4$ (225 mg, 0.70 mmol) was dissolved in anhydrous 1,4-dioxane (10 mL) under a nitrogen atmosphere. 3-Morpholinopropylamine (113 μL, 0.77 mmol) was added dropwise and the resultant solution was heated to 100 °C and stirred for 3 h (TLC analysis showed complete conversion of starting material).
The reaction mixture was then diluted with EtOAc (50 mL), washed with H₂O (50 mL), brine (50 mL), dried (Na₂SO₄), filtered, and concentrated in vacuo. Purification via flash chromatography (SiO₂, 5 - 80% EtOAc in hexanes) gave 7 as an orange/yellow solid (255 mg, 81%).

^1^H NMR (500 MHz, CDCl₃): δ 9.09 (s, 1H), 8.79 (s, 1H), 8.68 (d, J = 8.0 Hz, 1H), 8.48 (d, J = 8.0 Hz, 1H), 4.29 – 4.26 (m, 2H), 3.59 – 3.57 (m, 4H), 2.51 (t, J = 6.8 Hz, 2H), 2.43 (br s, 4H), 1.97 – 1.91 (m, 2H).

^1^C NMR (126 MHz, CDCl₃): δ 162.4, 162.2, 148.3, 135.7, 131.6, 129.9, 127.8, 127.5, 125.42, 125.40, 125.0, 124.5, 67.1, 56.5, 53.7, 39.7, 24.5.

HRMS (ESI): calculated for C₁₉H₁₉N₃O₅Br [M+H]^⁺ m/z 448.0503, observed 428.0504.

Compound 8

7 (100 mg, 0.22 mmol) was dissolved in EtOH (10 mL) under a nitrogen atmosphere. n-Butylamine (87 μL, 0.88 mmol) was added dropwise and the resultant mixture heated to 80 °C and stirred for 16 h. The reaction mixture was then diluted with H₂O (50 mL), extracted with EtOAc (3 × 30 mL), the combined organics washed with brine (50 mL), dried (Na₂SO₄), filtered, and concentrated in vacuo. Purification via flash chromatography (SiO₂, 5 - 80% EtOAc in hexanes) gave 8 as an orange solid (93 mg, 89%).

^1^H NMR (400 MHz, CDCl₃): δ 8.59 (d, J = 1.6 Hz, 1H), 8.41 (d, J = 8.5 Hz, 1H), 8.18 (d, J = 1.6 Hz, 1H), 6.71 (d, J = 8.5 Hz, 1H), 5.19 (t, J = 1.4 Hz, 1H), 4.22 – 4.19 (m, 2H), 3.61 – 3.59 (m, 4H), 3.39 (dd, J = 12.4, 7.0 Hz, 2H), 2.48 (t, J = 7.1 Hz, 2H), 2.43 (4H, br s, 4H), 1.94 – 1.87 (2H, m, 2H), 1.84 – 1.77 (2H, m, 2H), 1.58 – 1.49 (m, 2H), 1.03 (t, J = 7.3 Hz, 3H).

^1^C NMR (100 MHz, CDCl₃): δ 163.7, 163.6, 148.5, 134.6, 133.7, 128.29, 128.25, 124.8, 121.7, 118.6, 110.3, 105.1, 67.0, 56.5, 53.6, 43.5, 38.8, 31.0, 24.7, 20.4, 13.8.

HRMS (ESI) m/z: [M+H]^⁺ Calcd for C₂₃H₂₉N₃O₅Br 474.1387; Found 474.1389.
LysoNpTz

8 (47 mg, 0.10 mmol), 2a (36 mg, 0.12 mmol), and Cs₂CO₃ (98 mg, 0.30 mmol) were dissolved in thoroughly degassed (3 × freeze-pump-thaw cycles) THF:H₂O (9:1, 3 mL) under a nitrogen atmosphere. Pd(PPh₃)₄ (11.5 mg, 10 mol%) was added and the resultant mixture heated to 70 °C and stirred for 2 h (TLC analysis 5% MeOH in CH₂Cl₂ showed complete consumption of starting material). The reaction mixture was then filtered through a celite plug, eluted with EtOAc (30 mL), washed with H₂O (20 mL), brine (20 mL), dried (Na₂SO₄), filtered, and concentrated in vacuo.

Purification via flash chromatography (SiO₂, 10-100% EtOAc in hexanes) gave LysoNpTz as a red solid (36 mg, 64%).

1H NMR (400 MHz, CDCl₃) δ 8.85 (t, J = 1.6 Hz, 1H), 8.80 (d, J = 1.6 Hz, 1H), 8.59 – 8.54 (m, 1H), 8.41 (d, J = 8.4 Hz, 1H), 8.32 (d, J = 1.6 Hz, 1H), 7.94 – 7.87 (m, 1H), 7.67 (t, J = 7.8 Hz, 1H), 6.73 (d, J = 8.5 Hz, 1H), 5.67 (t, J = 5.1 Hz, 1H), 4.26 – 4.22 (m, 2H), 3.64 – 3.61 (m, 4H), 3.48 – 3.43 (m, 2H), 3.12 (s, 3H), 2.51 (t, J = 7.1 Hz, 2H), 2.44 (br., s, 4H), 1.98 – 1.91 (m, 2H), 1.88 – 1.81 (m, 3H), 1.59 – 1.50 (m, 2H), 1.02 (t, J = 7.4 Hz, 3H).

13C NMR (100 MHz, CDCl₃): δ 167.6, 164.6, 164.0, 163.8, 149.8, 140.9, 136.7, 134.5, 132.6, 131.5, 130.1, 130.0, 129.1, 127.3, 126.5, 124.1, 123.8, 120.7, 109.9, 104.7, 67.0, 56.6, 53.6, 43.7, 38.7, 31.0, 24.8, 21.2, 20.4, 13.9.

HRMS (ESI) m/z: [M+H]⁺ Calcd for C₃₂H₃₆N₇O₅ 566.2880; Found 566.2873.

Compound 9

4 (0.50 g, 1.6 mmol) was dissolved in anhydrous 1,4-dioxane (20 mL). N-Boc-ethylenediamine (0.30 mL, 1.9 mmol) and Et₃N (0.24 mL, 1.7 mmol) were added and the reaction heated to 100 °C for 4 h. The reaction mixture was diluted with water (50 mL, pH 4, acidified with 1 M HCl) and extracted with EtOAc (50 mL). The organic extracts were washed with water (50 mL, pH 4) and brine (2 × 50 mL), dried over Na₂SO₄ and concentrated in vacuo. Purification by column chromatography (SiO₂, 0-7% EtOAc in CH₂Cl₂), afforded 9 (0.38 g, 52%) as a beige powder.

1H NMR (500 MHz, CDCl₃): δ 9.07 (s, 1H), 8.79 (s, 1H), 8.69 (d, J = 7.8 Hz, 1H), 8.46 (d, J = 7.8 Hz, 1H), 4.83 (s, 1H), 4.35 (t, J = 5.2 Hz, 2H), 3.58 – 3.47 (m, 2H), 1.22 (s, 9H).
\(^{13}\)C NMR (126 MHz, CDCl\(_3\)): 162.7, 162.5, 156.3, 148.3, 135.8, 131.6, 130.1, 127.9, 127.3, 125.4, 125.3, 124.9, 124.4, 79.5, 40.9, 39.2, 28.3.

HRMS (ESI) m/z: \([\text{M}+\text{Na}]^+\) Calcd for C\(_{19}\)H\(_{18}\)BrN\(_3\)O\(_6\)Na 486.0271; Found 486.0270.

**Compound 10**

9 (0.18 g, 0.38 mmol) was suspended in MeCN (17 mL). \(n\)-Butylamine (0.12 mL, 1.13 mmol) was added dropwise, and the reaction heated to 70 °C for 16 h. The solvent was removed \textit{in vacuo} and the residue purified by column chromatography (SiO\(_2\), 2-15% EtOAc in CH\(_2\)Cl\(_2\)). This gave 10 (0.15 g, 80%) as an orange-yellow powder.

\(^1\)H NMR (500 MHz, DMSO-d\(_6\)): \(\delta\) 8.97 (s, 1H), 8.37 – 8.34 (m, 1H), 8.22 (t, \(J = 8.5\) Hz, 1H), 7.75 (t, \(J = 4.9\) Hz, 1H), 6.82 (t, \(J = 6.0\) Hz, 1H), 6.77 (d, \(J = 8.7\) Hz, 1H), 4.07 (t, \(J = 6.0\) Hz, 2H), 3.34 (q, \(J = 6.8\) Hz, 2H), 3.20 (q, \(J = 5.9\) Hz, 2H), 1.72 – 1.66 (m, 2H), 1.47 – 1.42 (m, 2H), 1.24 (s, 9H), 0.95 (t, \(J = 7.4\) Hz, 3H).

\(^{13}\)C NMR (126 MHz, DMSO-d\(_6\)): 162.8, 162.7, 155.6, 149.6, 134.4, 132.3, 130.4, 128.2, 124.1, 121.6, 117.0, 107.7, 104.5, 77.4, 42.7, 37.9, 29.8, 28.1, 19.8, 13.7. 1 \(\times\) C not observed in 1D spectrum but found under solvent at 39.5 ppm by HSQC analysis.

HRMS (ESI) m/z: \([\text{M}+\text{Na}]^+\) Calcd for C\(_{23}\)H\(_{28}\)BrN\(_4\)O\(_4\)Na 512.1155; Found 512.1154.

**Compound 11**

10 (70 mg, 0.14 mmol), 2a (64 mg, 0.21 mmol), Cs\(_2\)CO\(_3\) (93 mg, 0.27 mmol) and Pd(PPh\(_3\))\(_4\) (19 mg, 10 mol%) were dissolved in thoroughly degassed (3 × freeze-pump-thaw cycles) THF:H\(_2\)O (9:1, 7 mL) and the mixture heated to 65 °C for 4 h. The reaction mixture was diluted with water (15 mL) and extracted thrice with CH\(_2\)Cl\(_2\) (3 × 15 mL). The organic extracts were washed with brine (50 mL), dried over Na\(_2\)SO\(_4\) and concentrated \textit{in vacuo}. Purification by column chromatography (SiO\(_2\), 3-20% EtOAc in CH\(_2\)Cl\(_2\)) afforded 11 (49 mg, 59%) as a red powder.
1H NMR (500 MHz, CDCl3): δ 8.89 (s, 1H), 8.85 (s, 1H), 8.62 (d, J = 7.7 Hz, 1H), 8.44 (d, J = 8.4 Hz, 1H), 8.33 (s, 1H), 7.95 (d, J = 7.7 Hz, 1H), 7.72 (t, J = 7.7 Hz, 1H), 6.75 (d, J = 8.4 Hz, 1H), 5.65 – 5.59 (m, 1H), 5.20 (br s, 1H), 4.38 (br s, 2H), 3.55 (br s, 2H), 3.47 (q, J = 6.7 Hz, 2H), 3.13 (s, 3H), 1.85 (quint, J = 7.3 Hz, 2H), 1.59 – 1.51 (m, 2H), 1.33 (s, 9H), 1.03 (t, J = 7.3 Hz, 3H).

13C NMR (126 MHz, CDCl3): δ 167.7, 165.1, 164.5, 164.0, 156.3, 150.1, 141.0, 141.0, 136.9, 135.0, 132.7, 131.7, 130.6, 130.3, 129.4, 127.5, 126.6, 124.4, 123.7, 120.8, 109.8, 104.9, 79.2, 43.8, 40.4, 39.8, 31.2, 28.5, 21.4, 20.6, 14.0.

HRMS (ESI) m/z: [M+H]+ Calcd for C32H36N7O5 582.2823; Found 582.2820.

Compound 12

11 (17 mg, 0.030 mmol) was dissolved in CH2Cl2 (2.5 mL). Trifluoroacetic acid (0.75 mL) was added and the reaction stirred for 30 min at rt. The solvent was removed under a stream of N2. This afforded 12 in quantitative yield as a red residue that was used without further purification.

1H NMR (500 MHz, MeOD): δ 8.80 (t, J = 1.7 Hz, 1H), 8.74 (d, J = 1.7 Hz, 1H), 8.66 (d, J = 1.7 Hz, 1H), 8.47 (dt, J = 7.8, 1.0 Hz, 1H), 8.23 (d, J = 8.7 Hz, 1H), 8.04-8.00 (m, 1H), 7.71 (t, J = 7.8 Hz, 1H), 6.71 (d, J = 8.7 Hz, 1H), 4.43 (t, J = 5.9 Hz, 2H), 3.44 (t, J = 7.5 Hz, 2H), 3.34 (t, J = 5.7 Hz, 2H), 3.08 (s, 3H), 1.82 – 1.75 (m, 2H), 1.56 – 1.48 (m, 2H), 1.03 (t, J = 7.5 Hz, 3H).

13C NMR (126 MHz, MeOD): δ 169.0, 166.6, 165.7, 165.1, 153.2, 141.5, 137.1, 136.2, 134.2, 132.0, 131.1, 130.7, 128.2, 127.4, 127.0, 123.6, 122.2, 108.4, 105.5, 44.4, 40.3, 38.8, 31.6, 21.5, 21.2, 14.3.

I × C not observed.

HRMS (ESI) m/z: [M+H]+ Calcd for C27H28N7O2 482.2299; Found 482.2294.
4-Carboxybutyltriphenylphosphonium bromide (11.6 mg, 29.8 µmol), HATU (21.3 mg, 56.0 µmol) and DIPEA (25 µL, 140 µmol) were dissolved in DMF (1.0 mL). A solution of 12 (17 mg, 30 µmol) in DMF (1.7 mL) was added dropwise and the reaction was protected from light and stirred at rt for 3 h. The mixture was diluted with 5% LiCl solution (20 mL) and extracted with CHCl$_2$ (2 × 25 mL). The organic extracts were washed with 5% LiCl solution (2 × 50 mL) and brine (50 mL), dried over Na$_2$SO$_4$ and concentrated in vacuo. The compound was purified by column chromatography (SiO$_2$, 0-5% MeOH in CH$_2$Cl$_2$). The pure fractions were triturated thrice into Et$_2$O with the solid isolated by centrifugation each time. This afforded **MitoNpTz** (10 mg, 44%) as a red solid.

$^1$H NMR (500 MHz, CDCl$_3$): δ 8.49 (s, 1H), 8.46 (s, 1H), 8.33 (d, $J$ = 7.7 Hz, 1H), 8.23 (s, 1H), 8.07 (d, $J$ = 8.5 Hz, 1H), 7.84 (d, $J$ = 7.6 Hz, 1H), 7.75 – 7.69 (m, 3H), 7.64 – 7.55 (m, 13H), 6.78 (t, $J$ = 4.9 Hz, 2H), 6.51 (d, $J$ = 8.7 Hz, 1H), 6.06 (t, $J$ = 4.9 Hz, 2H), 4.22 (t, $J$ = 5.6 Hz, 2H), 3.58 (q, $J$ = 5.2 Hz, 2H), 3.37 (q, $J$ = 6.5 Hz, 2H) 3.19 – 3.13 (m, 2H), 3.06 (s, 3H), 2.26 (t, $J$ = 6.7 Hz, 2H), 1.83 (quint, $J$ = 6.7 Hz, 2H), 1.76 (quint, $J$ = 7.3 Hz, 2H), 1.73 – 1.63 (m, 2H), 1.50 – 1.44 (m, 2H), 0.97 (t, $J$ = 7.3 Hz, 3H).

$^{13}$C NMR (126 MHz, CDCl$_3$): δ 172.9, 167.5, 165.0, 164.1, 163.7, 150.6, 139.9, 135.9, 135.8, 135.3 (d, $J_{C-P} = 2.7$ Hz), 134.6, 133.5 (d, $J_{C-P} = 9.9$ Hz), 132.1, 131.6, 130.6 (d, $J_{C-P} = 12.5$ Hz), 130.3, 129.6, 129.0, 127.2, 125.7, 124.7, 122.7, 120.6, 118.0 (d, $J_{C-P} = 86.1$ Hz), 108.3, 104.6, 43.7, 39.5, 39.0, 34.7, 30.8, 25.9 (d, $J_{C-P} = 16.7$ Hz), 21.8 (d, $J_{C-P} = 56.0$ Hz), 21.6, 21.3, 20.5, 14.0.

HRMS (ESI) m/z: [M]$^+$ Calcd for C$_{50}$H$_{49}$N$_7$O$_3$P 826.3629; Found 826.3617.
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NMR spectra of novel compounds
Compound 5 – $^1$H NMR

![1H NMR spectrum](image)

Compound 5 – $^{13}$C NMR

![13C NMR spectrum](image)
Compound 2a – $^1$H NMR

Compound 2a – $^{13}$C NMR
Np3mTz – $^1$H NMR

Np3mTz – $^{13}$C NMR
Np3pTz – $^1$H NMR

Np3pTz – $^{13}$C NMR
Np6mTz – $^1$H NMR

Np6mTz – $^{13}$C NMR
Compound 7 – \( ^1H \) NMR

Compound 7 – \( ^{13}C \) NMR
Compound 8 – $^1$H NMR

Compound 8 – $^{13}$C NMR
LysoNpTz – $^1$H NMR

LysoNpTz – $^{13}$C NMR
Compound 9 – \(^1\text{H} \text{NMR}\)

\[
\text{HN} \quad \text{O}
\]

Compound 9 – \(^{13}\text{C} \text{NMR}\)

\[
\text{O} \quad \text{Br}
\]
Compound 10 – $^1$H NMR

Compound 10 – $^{13}$C NMR
Compound 11 – $^1$H NMR

Compound 11 – $^{13}$C NMR
Compound 12 – $^1$H NMR

Compound 12 – $^{13}$C NMR
MitoNpTz – $^1$H NMR

MitoNpTz – $^{13}$C NMR