Supporting Information

Water-soluble, stable, and azide-reactive strained dialkynes for biocompatible double strain-promoted click chemistry

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1. General Information

1.1 Solvents and reagents

Tetrahydrofuran was dried over sodium wire and distilled from a mixture of calcium hydride and lithium aluminium hydride with triphenylmethane as indicator. Diethyl ether was distilled from a mixture of calcium hydride and lithium aluminium hydride. Dichloromethane, methanol, hexane, acetonitrile and toluene were distilled from calcium hydride. Petroleum ether refers to the fraction of petroleum ether boiling in the range 40-60 °C. All other reagents and solvents were used as supplied, without prior purification.

1.2 Chromatography

Flash column chromatography was carried out using using Kieselgel 60 silica (230-400 mesh) with distilled solvents under a positive pressure of nitrogen. TLC was carried out on glass Merck Kieselgel 60 F254 plates, visualised by ultraviolet irradiation (254 and 365 nm)

1.3 NMR Spectroscopy

NMR spectra were recorded on a Bruker Ultrashield 500 (\(^1\)H: 500 MHz and \(^{13}\)C: 126 MHz) spectrometers. Chemical shifts are quoted in ppm and are referenced to the residual non-deuterated solvent peak, and are reported based on appearance rather than interpretation. \(^1\)H spectra are reported as follows: \(\delta_H\) (spectrometer frequency, solvent): ppm (no. of protons, multiplicity, J-coupling constant(s), assignment). \(^{13}\)C spectra are reported as follows \(\delta_C\) (spectrometer frequency, solvent): ppm (assignment). Spectral assignment was aided by the results of DEPT, COSY, HMBC and HSQC experiments where appropriate.
1.4  **IR Spectroscopy**

IR spectra were recorded neat on a Perkin Elmer Spectrum One FT-IR spectrophotometer fitted with an attenuated total reflectance (ATR) sampling accessory. Absorption maxima are reported in wavenumbers (cm$^{-1}$).

1.5  **High Resolution Mass Spectrometry**

Accurate masses were recorded on a Waters LCT Premier Time of Flight mass spectrometer or Micromass Quadrupole-Time of Flight mass spectrometer. Reported mass values are within the error limits of ±5 ppm.

1.6  **Liquid Chromatography-Mass Spectrometry**

LCMS chromatographs were obtained on an Agilent 1200 series LC using a Supelcosil ABZ+PLUS column (33 mm x 4.6 mm, 3 μm), together with an ESCi Multi-Mode Ionisation Waters ZQ spectrometer using MassLynx 4.1 software. Chromatographs were monitored by absorbance using diode array detection at a wavelength range of 190-600 nm.

1.7  **UV Spectroscopy**

All UV measurement were recorded using a Varian Cary 300 UV–visible spectrophotometer.

1.8  **High-performance Liquid Chromatography**

Analytical HPLC chromatographs were obtained on an Agilent 1260 Infinity, eluting with a gradient of 5-40% MeCN (with 0.05% TFA) in water (with 0.1% TFA) over 15 minutes. Semi-preparative HPLC was run on an Agilent 1260 Infinity, eluting with a gradient of 5-65% MeCN (with 0.05% TFA) in water (with 0.1% TFA) over 20 minutes. Retention times are reported to the nearest 0.01 min.
1.9 Melting points

All melting points were measured on a Büchi B545 melting point apparatus and are uncorrected. Solvents are reported in parentheses where solids were purified by recrystallization.

1.10 Naming and numbering of compounds

Where given, systematic compound names are those generated by ChemBioDraw Ultra 13.0 following IUPAC conventions. The numbering of atoms for spectral assignment purposes is arbitrary and not necessarily consistent with the IUPAC name.
2. Experimental procedures and data

2.1 Synthesis of trimethylammonium substituted Sondheimer dialkyne

2.2 Determination of octanol-water partition coefficient (log P) for amine substituted Sondheimer dialkyne 20 and 23

n-Octanol-water partition coefficients for substituted Sondheimer dialkyne 20 and 23 were determined via shake-flask method.

2.2.1 log P determination of dimethylamine substituted Sondheimer dialkyne 20

Analytical grade n-octanol was pre-saturated with distilled water. Similarly, distilled water (adjusted to pH 7.4 with phosphate buffer) was pre-saturated with analytical grade n-octanol. Both phases were left for separation for 24 hours at rt. A stock solution of dimethylamine substituted Sondheimer dialkyne 20 (0.3 mg/mL) was prepared in pre-saturated n-octanol. 2.0 mL of this solution was taken and shaken with 2.0 mL of pre-saturated distilled water and left for separation for 24 hours. Similarly, another 1.8 mL sample of the stock solution of 20 in n-octanol was shaken with 1.8 mL of pre-saturated distilled water and left for separation for 24 hours. The two layers were separated and the resulting amount of diyne 20 in n-octanol layer was measured for both the samples using UV spectroscopy.

Absorbance of stock solution of dialkyne 20 in pre-saturated n-octanol at 336 nm = 4.46
Run 1

Absorbance of 2.0 mL n-octanol sample of dialkyne 20 at 336 nm after shaking with 2.0 mL pre-saturated distilled water = 4.20

Fraction of dialkyne 20 in n-octanol phase after partitioning = 4.20/4.46 = 0.9417

Fraction of dialkyne 20 in water phase after partitioning = 1 - 0.9417 = 0.0583

n-Octanol-water partition coefficient; P = 16.15

log P = 1.21

Run 2

Absorbance of 1.8 mL n-octanol sample of dialkyne 20 at 336 nm after shaking with 1.8 mL pre-saturated distilled water = 4.06

Fraction of dialkyne 20 in n-octanol phase after partitioning = 4.06/4.46 = 0.9103

Fraction of dialkyne 20 in water phase after partitioning = 1 - 0.9103 = 0.0897

n-Octanol-water partition coefficient (P) = 10.15

log P = 1.00

Mean log P = 1.10

Error = ±0.10
Solubility comparison of dialkynes 20 and 23 in water:

Solubility limit of 20 in water- 0.02 mg/mL.

Solubility of 23 in water- up to 0.5 mg/mL with no precipitation observed.
2.2.2 log P determination of trimethylammonium substituted Sondheimer dialkyne 23

Analytical grade n-octanol was pre-saturated with distilled water. Similarly, distilled water (adjusted to pH 7.4 with phosphate buffer) was pre-saturated with analytical grade n-octanol. Both phases were left for separation for 24 hours at rt. A stock solution of trimethylammonium substituted Sondheimer dialkyne 23 (0.3 mg/mL) was prepared in pre-saturated distilled water. 2.0 mL of this solution was taken and shaken with 2.0 mL of pre-saturated n-octanol and left for separation for 24 hours. Similarly, another 1.8 mL sample of the stock solution of 23 in water was shaken with 1.8 mL of pre-saturated n-octanol and left for separation for 24 hours. The two layers were separated and the resulting amount of diyne 23 in water layer was measured for both the samples using UV spectroscopy.

Absorbance of stock solution of dialkyne 23 in pre-saturated water at 288 nm = 3.73

Run 1

Absorbance of 2.0 mL water sample of dialkyne 23 at 288 nm after shaking with 2.0 mL pre-saturated n-octanol = 3.62

Fraction of dialkyne 23 in water phase after partitioning = 3.62/3.73 = 0.9705

Fraction of dialkyne 23 in n-octanol phase after partitioning = 1 - 0.9705 = 0.0295

n-Octanol-water partition coefficient; P = 0.030

log P = -1.52

Run 2

Absorbance of 1.8 mL water sample of dialkyne 23 at 288 nm after shaking with 1.8 mL pre-saturated n-octanol = 3.61
Fraction of dialkyne 23 in water phase after partitioning = 3.61/3.73 = 0.9678

Fraction of dialkyne 23 in n-octanol phase after partitioning = 1 - 0.9678 = 0.0322

n-Octanol-water partition coefficient; P = 0.033

log P = -1.48

Mean log P = -1.50

Error = ±0.02
2.3 Stability of trimethylammonium substituted Sondheimer dialkyne 22 and 23 in aqueous buffer

Solutions of dialkynes 22 and 23 (1 mM concentration for each) were prepared in 20% MeOH/PBS (pH 7.4). The decomposition of 22 and 23 was monitored by UV spectroscopy by following the decrease in absorbance of 22 and 23 with time at 385 and 372 nm respectively.

**Figure S2.3.1.** Absorbance of dialkynes 22 (left), 23 (right) with time in aqueous buffer measured after leaving the sample (1 mM solution in 20% MeOH/PBS) for 7 days at room temperature.
Methyl 3-nitro-2-((phenylsulfonyl)methyl)benzoate

To a suspension of methyl 2-methyl-3-nitrobenzoate (1.0 g, 5.1 mmol) in CCl$_4$ (51 mL) at 80 °C under N$_2$ were added NBS (0.96 g, 5.4 mmol) and BPO (0.12 mg, 0.51 mmol), and then the resulting mixture was heated at 100 °C. After stirring at this temperature for 6 h, the reaction mixture was cooled to rt. The reaction mixture was diluted with saturated aqueous NH$_4$Cl (50 mL) and extracted with CH$_2$Cl$_2$ (3 × 50 mL). The organic layers were combined, dried (MgSO$_4$), filtered and concentrated in vacuo. To the resulting crude product (1.4 g, 5.0 mmol) were added benzenesulfinic acid sodium salt dihydrate (0.98 g, 6.0 mmol) and DMF (8.5 mL). After the resulting mixture had been stirred at 80 °C overnight, it was cooled to rt. The reaction mixture was diluted with water (25 mL) and extracted with EtOAc (3 × 25 mL). The organic layers were combined, dried (MgSO$_4$), filtered and concentrated in vacuo. Purification via flash column chromatography on silica gel (EtOAc/hexane = 2:1) gave 4 (0.89 g, 57%) as a yellow solid.

mp: 102-104 °C
ν$_{max}$ (neat): 1720 (C=O), 1533 (NO$_2$), 1447, 1302 (S=O), 1268, 1149 (S=O), 1083.

δ$_H$ (500 MHz, CDCl$_3$): 8.11 (1H, d, J 7.86, H1), 8.00 (1H, d, J 7.93, H3), 7.68-7.56 (4H, m, Ar H), 7.52-7.44 (2H, m), 5.70 (2H, br. s, H6), 3.86 (3H, d, J 1.83, H11), 3.05 (0.6 H, s).

δ$_C$ (126 MHz, CDCl$_3$): 166.2 (C12), 151.7 (C4), 138.0 (Ar C), 134.7 (C1), 134.2 (Ar CH), 134.1 (Ar C), 133.7 (Ar CH), 129.6 (C2), 129.3 (Ar CH), 128.3 (C3), 127.3 (Ar CH), 123.1 (Ar C), 53.1 (C11), 52.2 (C6), 44.5.

HRMS (ES+): found 358.0363; C$_{15}$H$_{13}$NO$_6$Na$_3$S [M+Na]$^+$ requires 358.0361.
(3-Nitro-2-((phenylsulfonyl)methyl)phenyl)methanol

To a solution of 4 (0.49 g, 1.5 mmol) in CH$_2$Cl$_2$ (4.5 mL), was added DIBAL-H (1.0 M in hexane, 3.4 mL, 3.4 mmol) at -78 °C. After the mixture had been stirred at this temperature for 2 h, saturated aqueous NH$_4$Cl (10 mL) was poured into the mixture followed by 1M HCl (10 mL). The product was then extracted with CH$_2$Cl$_2$ (3 × 20 mL). The organic layers were combined, dried (MgSO$_4$), filtered and concentrated in vacuo to afford the pure product 6 as a white solid (0.38 g, 84%).

mp: 88-89 °C

$\nu_{\text{max}}$ (neat): 3525 (O-H), 1736 (C=O), 1532 (NO$_2$), 1355, 1308 (S=O), 1148 (S=O), 1085.

$\delta$H (500 MHz, CDCl$_3$): 7.88 (1H, dd, $J$ 8.13, 1.34, Ar H), 7.84-7.78 (3H, m, Ar H), 7.74-7.59 (4H, m, Ar H), 5.22 (2H, br. s, H6), 4.86 (2H, s, H12), 3.07 (0.7 H, s), 2.83 (1H, br. s, H11)

$\delta$C (126 MHz, CDCl$_3$): 151.1 (C4), 143.9 (C13), 138.0 (Ar C), 134.7 (Ar CH), 134.5 (Ar CH), 130.0 (Ar CH), 129.6 (Ar CH), 128.3 (Ar CH), 125.2 (Ar CH), 120.4 (Ar C), 63.0 (C12), 53.4 (C6), 44.5.

HRMS (ES+): found 330.0415; C$_{14}$H$_{13}$NO$_5$Na$^{32}$S [M+Na]$^+$ requires 330.0412.
3-Nitro-2-((phenylsulfonyl)methyl)benzaldehyde

To a solution of 6 (0.78 g, 2.5 mmol) in CH$_2$Cl$_2$ (7.6 mL) was added DMP (1.2 g, 2.8 mmol) at rt. After the reaction mixture had been stirred for 3 h, the reaction mixture was diluted with CH$_2$Cl$_2$ (25 mL) and poured into saturated Na$_2$S$_2$O$_3$ solution (35 mL). Mixture was stirred to dissolve the solid and layers were separated. The CH$_2$Cl$_2$ layer was then extracted with saturated aqueous Na$_2$S$_2$O$_3$ (2 × 35 mL) followed by saturated aqueous NaHCO$_3$ (2 × 35 mL). CH$_2$Cl$_2$ was then removed in vacuo to yield pure product 8 (0.67 g, 87%) as a white solid.

mp: 200-202 °C
ν$_{\text{max}}$ (neat): 1730 (C=O), 1534 (NO$_2$), 1447, 1354, 1304 (S=O), 1150 (S=O), 1084.

δ$_H$ (400 MHz, CDCl$_3$): 10.17 (1H, s, H11), 8.20-8.11 (2H, m, Ar H), 7.82-7.66 (4H, m, Ar H), 7.59-7.52 (2H, m, Ar H), 5.65 (2H, s, H6).

δ$_C$ (126 MHz, CDCl$_3$): 189.9 (C11), 151.7 (C4), 138.0 (Ar C), 137.0 (Ar CH), 136.8 (Ar C), 134.4 (Ar CH), 130.2 (Ar CH), 129.7 (Ar CH), 129.4 (Ar CH), 128.4 (Ar CH), 127.4 (Ar C), 123.2 (Ar C), 50.9 (C6).

HRMS (ES+): found 304.0280; C$_{14}$H$_{12}$NO$_5^{32}$S [M+H]$^+$ requires 304.0280.
(3-Amino-2-((phenylsulfonyl)methyl)phenyl)methanol

![Chemical structure](image)

Zn powder (0.39 g, 0.59 mmol) and NH₄Cl (79 mg, 1.5 mmol) were added to a solution of nitro-compound 8 (18 mg, 0.06 mmol) in a 5:1 mixture of acetone:water (1.2 mL) at rt. After 2 h the reaction mixture was filtered through a pad of Celite® and concentrated in vacuo. The residue was extracted with diethyl ether (5 mL), washed with brine (5 mL), dried (MgSO₄), filtered and concentrated in vacuo. Purification via flash column chromatography on silica gel (EtOAc/hexane = 1:1) gave 30 (14 mg, 86%) as a white solid.

mp: 139-141 °C
ν<sub>max</sub> (neat): 3525 (O-H), 3356 (NH₂), 1470, 1307 (S=O), 1143 (S=O), 1083.

δ<sub>H</sub> (500 MHz, CDCl₃): 7.99 (2H, dd, J 8.44, 1.22, Ar H), 7.77-7.56 (3H, m, Ar H), 7.20 (1H, t, J 7.76, Ar H), 6.89-6.79 (2H, m, Ar H), 4.66 (2H, s, H6), 4.48 (2H, s, H12).
δ<sub>C</sub> (126 MHz, CDCl₃): 147.9 (Ar C), 142.0 (Ar C), 139.1 (Ar C), 134.2 (Ar CH), 130.2 (Ar CH), 129.5 (Ar CH), 128.2 (Ar CH), 121.1 (Ar CH), 118.2 (Ar CH), 112.6 (Ar C), 64.0 (C12), 55.9 (C6).

HRMS (ES+): found 278.0845; C₁₄H₁₆NO₃S [M+H]<sup>+</sup> requires 278.0851.
Methyl 3-amino-2-((phenylsulfonyl)methyl)benzoate

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\includegraphics[width=0.3\textwidth]{molecule.png}
\end{center}}
\]

10

Zn powder (0.21 g, 3.3 mmol) and NH\(_4\)Cl (0.26 g, 4.9 mmol) were added to a solution of nitro-compound 4 (0.10 g, 0.30 mmol) in a 5:1 mixture of acetone:water (6.6 mL) at rt. After 2 h the reaction mixture was filtered through a pad of Celite\textsuperscript{®} and concentrated \textit{in vacuo}. The residue was extracted with diethyl ether (20 mL), washed with brine (20 mL), dried over MgSO\(_4\). The solvent was evaporated \textit{in vacuo} to give pure product 10 as a liquid (69 mg, 75\%).

mp: 103°C

\(\nu_{\text{max}}\) (neat): 3427 (NH\(_2\)), 3372 (NH\(_2\)), 1711 (C=O), 1666, 1302 (S=O), 1146 (S=O), 1081.

\([\delta_H\ (500\ MHz,\ CDCl_3):\ 7.82-7.75\ (2H,\ m,\ Ar\ H),\ 7.62\ (1H,\ app\ tt,\ J\ 7.47,\ 1.22,\ Ar\ H),\ 7.54-7.44\ (2H,\ m,\ Ar\ H),\ 7.29\ (1H,\ d,\ J\ 8.60,\ Ar\ H),\ 7.19\ (1H,\ t,\ J\ 7.86,\ Ar\ H),\ 6.98\ (1H,\ d,\ J\ 7.96,\ Ar\ H),\ 5.24\ (2H,\ br.\ s,\ H6),\ 4.51\ (2H,\ br.\ s,\ H14),\ 3.63\ (3H,\ s,\ H11),\ 2.44\ (1H,\ br.\ s,\ H11).\]

\([\delta_C\ (126\ MHz,\ CDCl_3):\ 167.6\ (C12),\ 148.1\ (Ar\ C),\ 138.3\ (Ar\ C),\ 133.6\ (Ar\ CH),\ 131.5\ (Ar\ C),\ 129.2\ (Ar\ CH),\ 128.8\ (Ar\ CH),\ 128.7\ (Ar\ CH),\ 122.1\ (Ar\ CH),\ 121.8\ (Ar\ CH),\ 114.9\ (Ar\ C),\ 54.3\ (C6),\ 52.0\ (C11).\]

HRMS (ES\(^+\)): found 306.0791; C\(_{13}\)H\(_{16}\)NO\(_4\)S \([\text{M+H}]^+\) requires 306.0795.
Methyl 3-(methylamino)-2-((phenylsulfonyl)methyl)benzoate

A solution of **10** (30 mg, 0.10 mmol) in methanol (0.50 mL) was heated to 50 °C and then 37% aqueous formaldehyde solution (0.016 mL, 0.60 mmol) was added. The mixture was stirred for 5 minutes at rt and then sodium cyanoborohydride (7.4 mg, 0.12 mmol) was added. 1M HCl was added to adjust the pH (6-8) of the reaction mixture. After the reaction mixture had been stirred for overnight, the reaction mixture was poured into a water-ice mixture (~5.0 mL) and extracted with CH$_2$Cl$_2$ (3 × 6.0 mL). The remaining aqueous layer was neutralized with saturated NaHCO$_3$ solution and extracted with CH$_2$Cl$_2$ (3 × 6.0 mL). The organic layers were combined, dried (MgSO$_4$), filtered and concentrated in vacuo. Purification via flash column chromatography on silica gel (EtOAc/hexane = 1:3) gave minor product **31** (5.0 mg, 16%) as a colourless liquid.

$\nu_{\text{max}}$ (neat): 2989 (C-H), 1714 (C=O), 1591, 1447, 1320 (S=O), 1150 (S=O), 1082.

$\delta_H$ (500 MHz, CDCl$_3$): 7.77 (2H, dd, $J$ 8.39, 1.22, H8), 7.62 (1H, t, $J$ 7.47, H10), 7.48 (2H, t, $J$ 7.86, H9), 7.30 (1H, t, $J$ 7.95, H1), 7.23 (1H, dd, $J$ 7.75, 1.21, H2), 6.96 (1H, d, $J$ 8.16, H3), 5.22 (2H, br. s, H6), 3.62 (3H, s, H11), 2.90 (3H, s, H15).

$\delta_C$ (126 MHz, CDCl$_3$): 167.9 (C12), 150.0 (Ar C), 138.3 (Ar C), 133.6 (Ar CH), 131.4 (Ar C), 129.5 (Ar CH), 128.8 (Ar CH), 128.7 (Ar CH), 120.2 (Ar CH), 115.8 (Ar CH), 114.6 (Ar C), 54.6 (C6), 52.0 (C11), 31.1 (C15).
Methyl 3-(dimethylamino)-2-((phenylsulfonyl)methyl)benzoate

To a solution of 10 (103 mg, 0.34 mmol) in glacial acetic acid (2 mL) under Ar (g) were added paraformaldehyde (102 mg, 3.4 mmol) and sodium cyanoborohydride (107 mg, 1.7 mmol). After the reaction mixture had been stirred for overnight, the reaction mixture was poured into a water-ice mixture (~5 mL) and extracted with CH₂Cl₂ (2 × 5 mL). The remaining aqueous layer was neutralized with saturated NaHCO₃ solution and extracted with CH₂Cl₂ (2 × 5 mL). The organic layers were combined, dried (MgSO₄), filtered and concentrated in vacuo. Purification via flash column chromatography on silica gel (EtOAc/hexane = 1:3) gave 12 (0.11 g, 93%) as a colourless liquid.

ν_max (neat): 2944, 1714 (C=O), 1446, 1306, 1263 (S=O), 1115, 1033, 1003 (S=O), 689.

δ_H (400 MHz, CDCl₃): 7.67 (1H, d, J 8.1, H1), 7.57 (2H, d, J 7.6, H8), 7.52 (1H, t, J 7.5, H10), 7.41-7.32 (3H, m, Ar H), 7.16 (1H, d, J 8.1, H3), 5.37 (2H, s, H6), 3.94 (3H, s, H11), 2.32 (6H, s, H14).

δ_C (101 MHz, CDCl₃): 168.5 (C12), 155.2 (Ar C), 139.2 (Ar C), 133.9 (Ar C), 133.1 (Ar CH), 129.3 (Ar CH), 128.5 (Ar CH), 128.4 (Ar CH), 126.4 (Ar CH), 124.7, 124.3 (Ar CH), 52.7 (C6), 52.5 (C11), 45.2 (C14).

HRMS (ES+): found 334.1106; C_{17}H_{20}NO_{4}S [M+H]^+ requires 334.1108.
(3-(Dimethylamino)-2-((phenylsulfonyl)methyl)phenyl)methanol

To a solution of 12 (1.89 g, 5.68 mmol) in CH₂Cl₂ (18.0 mL), was added DIBAL-H (1.0 M in hexane, 12.50 mL, 12.50 mmol) at -78 °C. After the mixture had been stirred at this temperature for 2 h, saturated aqueous NH₄Cl (60 mL) was poured into the mixture followed by 1M HCl (60 mL). The product was then extracted with CH₂Cl₂ (3 × 120 mL). The organic layers were combined, dried (MgSO₄), filtered and concentrated *in vacuo* to afford the pure product 14 as a pale-yellow oil (1.66 g, 96%).

δₜ ᵃ (400 MHz, CDCl₃): 7.67-7.53 (3H, m, Ar H), 7.49-7.40 (2H, m, Ar H), 7.38-7.32 (2H, m, Ar H), 7.00-6.94 (1H, m, Ar H), 4.94 (2H, s, H6), 4.83 (2H, d, J 6.68, H12), 3.25 (1H, t, J 6.70, H11), 2.18 (6H, s, H14).

δᵣ (126 MHz, CDCl₃): 150.0 (Ar C), 141.7 (Ar C), 139.2 (Ar C), 134.1 (Ar CH), 130.4 (Ar CH), 129.4 (Ar CH), 128.2 (Ar CH), 119.6 (Ar CH), 112.3 (Ar CH), 112.2 (Ar C), 64.2 (C12), 56.0 (C6), 31.1 (C14).
3-(Dimethylamino)-2-((phenylsulfonyl)methyl)benzaldehyde

To a solution of 14 (1.66 g, 5.45 mmol) in CH₂Cl₂ (20 mL) was added DMP (2.54 g, 6.0 mmol) at rt. After the reaction mixture had been stirred for 3 h, the reaction mixture was poured into saturated Na₂S₂O₃ solution (20 mL). Mixture was stirred to dissolve the solid and layers were separated. The CH₂Cl₂ layer was then extracted with saturated aqueous Na₂S₂O₃ (2 × 20 mL) followed by saturated aqueous NaHCO₃ (2 × 20 mL). CH₂Cl₂ was then removed in vacuo and purification via flash column chromatography on silica gel (EtOAc/hexane = 1:4) gave pure product 16 (1.35 g, 82%) as a white solid.

ν max (neat): 2938, 1692 (C=O), 1583, 1447, 1307 (S=O), 1157 (S=O), 1083.

δH (400 MHz, CDCl₃): 10.17 (1H, s, H11), 7.68 (1H, dd, J 7.7, 1.4, H1), 7.63-7.59 (2H, m, Ar H), 7.59-7.52 (1H, m, Ar H), 7.48 (1H, t, J 7.8, H2), 7.42 (2H, t, J 7.8, H9), 7.31 (1H, dd, J 7.9, 1.4, H3), 5.28 (2H, s, H6), 2.36 (6H, s, H13).

δC (126 MHz, CDCl₃): 193.4 (C11), 150.5 (Ar C), 138.3 (Ar C), 134.9 (Ar C), 133.8 (Ar CH), 130.1 (Ar CH), 128.8 (Ar CH), 128.7 (Ar CH), 125.7 (Ar CH), 117.6 (Ar CH), 112.9 (Ar C), 53.8 (C6), 31.0 (C13).

HRMS (ES+): found 304.0997; C₁₆H₁₈NO₃S [M+H]⁺ requires 304.1002.
(5E,11E)-N⁴,N⁴,N⁷,N⁷-tetramethyl-6,12-bis(phenylsulfonyl) dibenzo[ae][8]annulene-1,7-diamine

A mixture of 16 (1.30 g, 4.29 mmol) and ClP(O)(OEt)₂ (0.644 mL, 4.72 mmol) in THF (86 mL) was cooled to −78 °C, and then LiHMDS (1.0 M in THF, 9.44 mL, 9.44 mmol) was added. After stirring at −78 °C for 30 min, the reaction mixture was warmed to rt and stirred for a further 1.5 h. Saturated aqueous NH₄Cl (10 mL) was poured into the mixture. The reaction mixture was diluted with water (10 mL) and extracted with EtOAc (3 × 20 mL). The organic layers were combined, dried (MgSO₄), filtered and concentrated in vacuo. Purification via flash column chromatography on silica gel (EtOAc/hexane = 1:2) gave 19 as a pale yellow solid (318 mg, 26%).

mp: 197-199°C
νₘₐₓ (neat): 1574, 1296 (S=O), 1140 (S=O), 727.

δH (400 MHz, CDCl₃): 7.55 (2H, tt, J 6.0, 2.6, Ar H), 7.46-7.38 (10H, m, Ar H), 7.18 (2H, t, J 7.9, H2), 6.76 (2H, d, J 7.9, H1), 6.61 (2H, d, J 7.9, H3), 2.42 (12H, s, H13).

δC (101 MHz, CDCl₃): 154.1 (Ar C), 147.3 (Ar C), 140.9 (Ar CH), 140.6 (Ar C), 138.8 (Ar C), 133.0 (Ar CH), 129.9 (Ar CH), 128.7 (Ar CH), 128.2 (Ar CH), 120.9 (Ar C), 119.0 (Ar CH), 118.1 (Ar CH), 42.9 (C13).

HRMS (ES+): found 571.1715; C₃₂H₃₁N₂O₄S₂ [M+H]⁺ requires 571.1720.
A solution of 19 (100 mg, 0.17 mmol) in THF (3.5 mL) was cooled to −78 °C, and then LDA (1.0 M in THF/hexane, 0.85 mL, 0.85 mmol). The reaction mixture was stirred at this temperature for 2 h, and saturated aqueous NH₄Cl (1 mL) was poured into the mixture. The reaction mixture was cooled to rt, diluted with water (4 mL) and extracted with CH₂Cl₂ (3 × 5 mL). The organic layers were combined, dried (MgSO₄), filtered and concentrated in vacuo. Purification via flash column chromatography on silica gel (CH₂Cl₂/hexane = 1:1) gave 21 as an orange solid (27 mg, 54%).

mp: 186-190°C (dec.)
νmax (neat): 3049, 2960, 2804, 2137 (C≡C), 1433, 1346, 1130, 1002, 785.

δH (400 MHz, CDCl₃): 6.79 (2H, dd, J 8.6, 7.4, H2), 6.47 (2H, d, J 8.6, Ar H), 6.30 (2H, d, J 7.4, Ar H), 2.92 (12H, s, H9).

δC (101 MHz, CDCl₃): 150.28 (C4), 134.56 (Ar C), 129.63 (C2), 119.3 (Ar C), 118.9 (Ar CH), 117.6 (Ar CH), 110.9 (C≡C), 108.7 (C≡C), 42.6 (C9).

HRMS (ES+): found 287.1540; C₂₀H₁₉N₂ [M+H]+ requires 287.1543.
7-(dimethylamino)-N¹,N¹,N¹-trimethyl-5,6,11,12-tetrahydrodibeno[a,e]cyclooctene-1-aminium trifluoroacetate

A solution of 21 (18.0 mg, 0.06 mmol) in CH₂Cl₂ (1.0 mL) was taken in a dry round bottom flask. Methyl trifluoromethanesulfonate (0.012 mL, 0.11 mmol) was added to the reaction mixture dropwise at rt and the mixture was stirred for 1 h. CH₂Cl₂ was then removed \textit{in vacuo} and preparative HPLC purification (30-60\% MeCN) gave pure product 24 (10.0 mg, 37\%) as an orange solid.

mp: 115-116 °C (dec.)

ν_max (neat): 3034, 2851, 2801, 2150 (C≡C), 1690, 1681, 1469, 1193, 1109, 782.

δ_H (500 MHz, CD₃CN): 7.32 (1H, dd, J 8.9, 0.9, H11), 7.18 (1H, dd, J 8.9, 7.6, H10), 6.98-6.92 (2H, m, Ar H), 6.67 (1H, dd, J 8.9, 1.0, H1), 6.39 (1H, dd, J 7.2, 1.0, H3), 3.58 (9H, s, H18), 2.98 (6H, s, H17).

δ_C (126 MHz, CD₃CN): 151.1 (C12), 143.7 (C4), 138.0 (Ar C), 132.4 (Ar C), 132.0 (C10), 131.8 (Ar CH), 129.2 (Ar CH), 125.8 (Ar C), 121.1 (C11), 120.4 (C1), 120.0 (C3), 119.8 (Ar C), 114.7 (C≡C), 112.9 (C≡C), 108.1 (C≡C), 101.8 (C≡C), 57.1 (C18), 42.3 (C17).

HRMS (ES+): found 301.1698; C_{21}H_{21}N₂ [M]⁺ requires 301.1699.
A solution of 21 (18.0 mg, 0.06 mmol) in CH₂Cl₂ (1.1 mL) was taken in a dry round bottom flask. Methyl trifluoromethanesulfonate (0.013 mL, 0.12 mmol) was added to the reaction mixture dropwise at rt and the mixture was stirred for 2 h. CH₂Cl₂ was then removed in vacuo. The resulting crude product was dissolved in MeCN (1.6 mL) and methyl trifluoromethanesulfonate (0.027 mL, 0.25 mmol) was added dropwise. After having stirred the reaction mixture for 5 h MeCN was removed in vacuo and preparative HPLC purification (30-60% MeCN) gave pure product 25 (20 mg, 55%) as a light brown solid.

mp: 168-170 °C (dec.)

νₘₐₓ (neat): 3049, 2159 (C≡C), 1771, 1731, 1185, 1135, 787.

δₕ (500 MHz, CD₃CN): 7.51 (2H, dd, J 8.9, 0.9, H3), 7.32 (2H, dd, J 8.9, 7.6, H2), 7.14 (2H, dd, J 7.6, 0.9, H1), 3.60 (18H, s, H9).

δₖ (126 MHz, CD₃CN): 144.3 (C4), 135.4 (C8), 133.1 (C2), 130.8 (C1), 126.0 (C5), 123.8 (C3), 116.8 (C7), 104.0 (C6), 57.4 (C9).

HRMS (ES+): found 158.0960; C₂₂H₂₄N₂ [M]²⁺ requires 158.0964.
**Methyl 5-nitro-2-((phenylsulfonyl)methyl)benzoate**

![Chemical Structure](image)

To a suspension of methyl 2-methyl-5-nitrobenzoate (5.00 g, 25.61 mmol) in CCl$_4$ (128.0 mL) at 80 °C under N$_2$ were added NBS (4.8 g, 26.9 mmol) and BPO (620 mg, 2.56 mmol), and then the resulting mixture was heated at 100 °C. After stirring at this temperature for 6 h, the reaction mixture was cooled to rt. The reaction mixture was diluted with saturated aqueous NH$_4$Cl (120 mL) and extracted with CH$_2$Cl$_2$ (3 × 120 mL). The organic layers were combined, dried (MgSO$_4$), filtered and concentrated *in vacuo*. To the resulting crude product (7.74 g, 28.20 mmol) were added benzenesulfinic acid sodium salt dihydrate (5.56 g, 33.86 mmol) and DMF (42.3 mL). After the resulting mixture had been stirred at 80 °C overnight, it was cooled to rt. The reaction mixture was diluted with water (50 mL) and extracted with EtOAc (3 × 50 mL). The organic layers were combined, dried (MgSO$_4$), filtered and concentrated *in vacuo*. Purification via flash column chromatography on silica gel (EtOAc/hexane = 1:1) gave 5 (6.35 g, 74%) as a yellow solid.

**Physical Properties**

- **mp:** 134-135 °C
- **$\nu$**$_{\text{max}}$ (neat): 2952, 1722 (C=O), 1527 (NO$_2$), 1346, 1306 (NO$_2$), 1254 (S=O), 1135 (S=O), 1067, 732.
- **$\delta$**$_{H}$ (400 MHz, CDCl$_3$): 8.74 (1H, d, $J$ 2.5, H1), 8.32 (1H, dd, $J$ 8.5, 2.5, H3), 7.71-7.61 (3H, m, Ar H), 7.56 (1H, d, $J$ 8.5, H4), 7.53-7.47 (2H, m, Ar H), 5.15 (2H, s, H6), 3.84 (3H, s, H11).
- **$\delta$**$_{C}$ (126 MHz, CDCl$_3$): 165.2 (C2), 147.8 (Ar C), 138.1 (Ar C), 136.1 (Ar C), 134.7 (Ar CH), 134.1 (Ar CH), 132.1 (Ar C), 129.2 (Ar CH), 128.6 (Ar CH), 126.3 (Ar CH), 125.9 (Ar CH), 58.7 (C6), 52.9 (C11).
- **HRMS (ES+):** found 336.0546; C$_{15}$H$_{14}$NO$_6^{32}$S [M+H]$^+$ requires 336.0542.
Methyl 5-amino-2-((phenylsulfonyl)methyl)benzoate

Zn powder (1.95 g, 29.82 mmol) and NH₄Cl (2.39 g, 44.73 mmol) were added to a solution of nitro-compound 5 (1.0 g, 2.98 mmol) in a 5:1 mixture of acetone:water (60 mL) at rt. After 2 h the reaction mixture was filtered through a pad of Celite® and concentrated in vacuo. The residue was extracted with diethyl ether (200 mL), washed with brine (200 mL), dried over MgSO₄. The solvent was evaporated in vacuo to give pure product 11 as a liquid (0.64 g, 70%).

mp: 168-169 °C

ν<sub>max</sub> (neat): 3100-2800 (N-H), 3076, 2957 1720 (C=O), 1286 (S=O), 1137 (S=O), 1066, 691.

δ<sub>H</sub> (400 MHz, CDCl₃): 7.65-7.61 (2H, m, Ar H), 7.60-7.54 (1H, m, Ar H), 7.46-7.39 (2H, m, Ar H), 7.15 (1H, d, J 2.6, H1), 7.07 (1H, d, J 8.2, H4), 6.74 (1H, dd, J 8.2, 2.6, H3), 4.90 (2H, s, H6), 3.84 (2H, br. s, NH₂), 3.69 (3H, s, H11).

δ<sub>C</sub> (101 MHz, CDCl₃): 167.3 (C12), 147.1 (C2), 138.6 (Ar C), 134.7 (C4), 133.5 (Ar CH), 131.7 (Ar C), 128.8 (Ar CH), 128.8 (Ar CH), 118.2 (C3), 118.1 (Ar C), 117.1 (C1), 59.1 (C6), 52.2 (C11).

HRMS (ES+): found 306.0790; C₁₃H₁₆NO₄³²S [M+H]<sup>+</sup> requires 306.0800.
To a solution of 5 (0.28 g, 0.84 mmol) in CH₂Cl₂ (2.6 mL), was added DIBAL-H (1.0 M in hexane, 1.95 mL, 1.95 mmol) at -78 °C. After the mixture had been stirred at this temperature for 2 h, saturated aqueous NH₄Cl (10 mL) was poured into the mixture followed by 1M HCl (10 mL). The product was then extracted with EtOAc (3 × 20 mL). The organic layers were combined, dried (MgSO₄), filtered and concentrated in vacuo. Purification via flash column chromatography on silica gel (EtOAc/hexane = 1:1) gave 7 as a white solid (0.235 g, 91%).

mp: 146-147 °C
ν_max (neat): 3487 (O-H), 3099, 2870, 1590, 1518, 1446, 1347, 1304 (S=O).

δH (400 MHz, CD₃OD): 8.34 (1H, d, J 2.6, Ar H), 8.03 (1H, dd, J 8.5, 2.6, Ar H), 7.79-7.71 (3H, m, Ar H), 7.60 (2H, t, J 7.8, Ar H), 7.31 (1H, J 8.5, Ar H), 4.76 (2H, s, H6), 4.67 (2H, s, H12).

δC (101 MHz, CD₃OD): 150.5, 146.5, 140.6, 136.4 (Ar CH), 135.7 (Ar CH), 135.3, 131.4 (Ar CH), 130.5 (Ar CH), 124.2 (Ar CH), 123.5 (Ar CH), 62.8 (C12), 60.0 (C6).

HRMS (ES+): found 328.0240; C₁₄H₁₁NO₅S²⁺[M+Na]⁺ requires 328.0250.
5-nitro-2-((phenylsulfonyl)methyl)benzaldehyde

To a solution of 7 (0.16 g, 0.52 mmol) in CH$_2$Cl$_2$ (8 mL) was added DMP (0.27 g, 0.63 mmol) at rt. After the reaction mixture had been stirred for 3 h, the reaction mixture was poured into saturated Na$_2$S$_2$O$_3$ solution (8 mL). Mixture was stirred to dissolve the solid and layers were separated. The CH$_2$Cl$_2$ layer was then extracted with saturated aqueous Na$_2$S$_2$O$_3$ (2 × 8 mL) followed by saturated aqueous NaHCO$_3$ (2 × 8 mL). CH$_2$Cl$_2$ was then removed in vacuo and purification via flash column chromatography on silica gel (PE/EtOAc = 1:1) gave pure product 9 (0.14 g, 87%) as a white solid.

mp: 187-188 °C

$\nu_{\text{max}}$ (neat): 1702 (C=O), 1611, 1589, 1522, 1446, 1400, 1350, 1303 (S=O).

$\delta_{H}$ (400 MHz, CDCl$_3$): 9.95 (1H, s, H12), 8.62 (1H, d, J 2.42, Ar H), 8.42 (1H, d, J 2.43, Ar H), 8.40 (1H, d, J 2.43, Ar H), 7.79-7.61 (4H, m, Ar H), 7.57-7.47 (2H, m, Ar H), 5.08 (2H, s, H6)

$\delta_{C}$ (101 MHz, CDCl$_3$): 189.5 (C12), 148.4 (Ar C), 137.9 (Ar C), 135.6 (Ar C), 135.3 (Ar C), 135.2 (Ar CH), 134.4 (Ar CH), 129.3 (Ar CH), 128.5 (Ar CH), 128.2 (Ar CH), 127.5 (Ar CH), 57.4 (C6).

HRMS (ES+): found 330.0401; C$_{14}$H$_{13}$NO$_5$S $[\text{M+Na}]^+$ requires 330.0407.
Methyl 5-(dimethylamino)-2-((phenylsulfonyl)methyl)benzoate

To a solution of 11 (6.1 g, 19.94 mmol) in glacial acetic acid (119.4 mL) under Ar (g) were added paraformaldehyde (6.0 g, 199.4 mmol) and sodium cyanoborohydride (6.3 g, 99.7 mmol). After the reaction mixture had been stirred for overnight, the reaction mixture was poured into a water-ice mixture (~240 mL) and extracted with CH$_2$Cl$_2$ (2 × 240 mL). The remaining aqueous layer was neutralized with saturated NaHCO$_3$ solution and extracted with CH$_2$Cl$_2$ (2 × 240 mL). The organic layers were combined, dried (MgSO$_4$), filtered and concentrated in vacuo. Purification via flash column chromatography on silica gel (EtOAc/hexane = 1:1) gave 13 (5.48 g, 82%) as a colourless liquid.

mp: 150-152 °C

ν$_{\text{max}}$ (neat): 2950 (C-H), 1714 (C=O), 1606, 1515, 1446, 1305 (S=O), 1258 (S=O), 1083.

δ$_H$ (500 MHz, CDCl$_3$): 7.66-7.60 (2H, m, Ar H), 7.57 (1H, t, J 7.00, Ar H), 7.47-7.40 (2H, m, Ar H), 7.16 (1H, s, H1), 7.12 (1H, d, J 8.6, Ar H), 6.76 (1H, dd, J 8.55, 2.85, Ar H), 4.90 (2H, s, H6), 3.70 (3H, s, H11), 2.98 (6H, s, H14).

δ$_C$ (126 MHz, CDCl$_3$): 167.8 (C12), 150.2 (Ar C), 138.7 (Ar C), 134.3 (Ar CH), 133.3 (Ar CH), 131.3 (Ar C), 129.6 (Ar C), 128.7 (Ar CH), 128.7 (Ar CH), 115.2 (Ar CH), 114.2 (Ar CH), 59.1 (C6), 52.0 (C11), 40.2 (C14).

HRMS (ES+): found 334.1105; C$_{17}$H$_{20}$NO$_4^{32}$S [M+H]$^+$ requires 334.1113.
(5-(Dimethylamino)-2-((phenylsulfonyl)methyl)phenyl)methanol

To a solution of 13 (0.34 g, 1.02 mmol) in CH₂Cl₂ (3.1 mL), was added DIBAL-H (1.0 M in hexane, 2.24 mL, 2.24 mmol) at -78 °C. After the mixture had been stirred at this temperature for 2 h, saturated aqueous NH₄Cl (10 mL) was poured into the mixture followed by 1M HCl (10 mL). The product was then extracted with CH₂Cl₂ (3 × 20 mL). The organic layers were combined, dried (MgSO₄), filtered and concentrated in vacuo to afford the pure product 15 as an off-white solid (0.29 g, 94%).

mp: 140-141 °C
ν max (neat): 3452 (O-H), 2898 (C-H), 1610, 1515, 1360, 1298 (S=O), 1205 (C-N), 1147.

δH (500 MHz, CDCl₃): 7.80-7.73 (2H, m, Ar H), 7.64 (1H, t, J 7.48, Ar H), 7.56-7.48 (2H, m, Ar H), 6.79-6.73 (2H, m, Ar H), 6.50 (1H, dd, J 8.58, 2.72, Ar H), 4.57 (2H, s, H12), 4.42 (2H, s, H6), 2.97 (6H, s, H14).

δC (126 MHz, CDCl₃): 151.0 (Ar C), 141.7 (Ar C), 138.4 (Ar C), 133.8 (Ar CH), 133.5 (Ar CH), 129.1 (Ar CH), 128.6 (Ar CH), 113.8 (Ar CH), 112.3 (Ar C), 111.7 (Ar CH), 63.8 (C12), 59.6 (C6), 40.2 (C14).

HRMS (ES+): found 306.1149; C₁₆H₂₀NO₃³²S [M+H]⁺ requires 306.1164.
5-(Dimethylamino)-2-((phenylsulfonyl)methyl)benzaldehyde

To a solution of 15 (0.29 g, 0.95 mmol) in CH$_2$Cl$_2$ (2.9 mL) was added DMP (0.44 g, 1.05 mmol) at rt. After the reaction mixture had been stirred for 3 h, the reaction mixture was diluted with CH$_2$Cl$_2$ (4 mL) and poured into saturated Na$_2$S$_2$O$_3$ solution (5 mL). Mixture was stirred to dissolve the solid and layers were separated. The CH$_2$Cl$_2$ layer was then extracted with saturated aqueous Na$_2$S$_2$O$_3$ (2 × 7 mL) followed by saturated aqueous NaHCO$_3$ (2 × 7 mL). CH$_2$Cl$_2$ was then removed in vacuo and purification via flash column chromatography on silica gel (EtOAc/hexane = 1:2) gave pure product 17 (0.21 g, 73%) as a white solid.

mp: 122-125 °C

$\nu_{\text{max}}$ (neat): 2922 (C-H), 1692 (C=O), 1606, 1517, 1305 (S=O), 1206 (C-N), 1148, 1083.

$\delta_H$ (500 MHz, CDCl$_3$): 9.76 (1H, s, H11), 7.72-7.64 (2H, m, Ar H), 7.58 (1H, t, $J$ 7.47, Ar H), 7.49-7.40 (2H, m, Ar H), 7.23 (1H, d, $J$ 8.53, Ar H), 6.98 (1H, d, $J$ 2.87, H1), 6.83 (1H, dd, $J$ 8.53, 2.87, Ar H), 4.87 (2H, s, H6), 3.03 (6H, s, H13).

$\delta_C$ (126 MHz, CDCl$_3$): 192.5 (C11), 150.7 (Ar C), 138.5 (Ar C), 135.2 (Ar C), 134.7 (Ar CH), 133.5 (Ar CH), 128.75 (Ar CH), 128.71 (Ar CH), 117.0 (C1), 116.2 (Ar CH), 114.9 (Ar C), 57.4 (C6), 40.1 (C13).

HRMS (ES+): found 304.1014; C$_{16}$H$_{18}$NO$_3^+$ [M+H]$^+$ requires 304.1007.
(5E,11E)-N²,N²,N⁸,N⁸-tetramethyl-5,11-bis(phenylsulfonyl)dibenzo[a,e][8]annulene-2,8-diamine

A mixture of 17 (3.54 g, 11.67 mmol) and ClP(O)(OEt)₂ (2.02 mL, 14.0 mmol) in THF (233 mL) was cooled to −78 °C, and then LiHMDS (1.0 M in THF, 23.34 mL, 23.34 mmol) was added. After stirring at −78 °C for 30 min, the reaction mixture was warmed to rt and stirred for a further 1.5 h. Saturated aqueous NH₄Cl (25 mL) was poured into the mixture. The reaction mixture was diluted with water (210 mL) and extracted with EtOAc (3 × 230 mL). The organic layers were combined, dried (MgSO₄), filtered and concentrated in vacuo. Purification via flash column chromatography on silica gel (EtOAc/hexane = 2:3) gave 18 as a pale yellow solid solid (0.86 g, 13%).

mp: 156-158 °C

ν max (neat): 2923 (C-H), 1595, 1303 (S=O), 1147 (S=O), 1206 (C-N), 1084.

δH (500 MHz, CDCl₃): 7.60 (2H, t, J 7.39, Ar H), 7.48-7.42 (4H, m, Ar H), 7.42-7.37 (6H, m, Ar H), 6.57 (2H, dd, J 8.86, 2.70, Ar H), 6.21 (2H, d, J 2.62, H1), 2.90 (12H, s, H13).

δc (126 MHz, CDCl₃): 150.2 (C2), 144.4 (Ar C), 139.7 (Ar C), 139.1 (Ar CH), 136.9 (Ar C), 133.4 (Ar CH), 131.7 (Ar CH), 128.7 (Ar CH), 127.9 (Ar CH), 115.5 (Ar C), 111.7 (Ar CH), 109.4 (Ar CH), 40.0 (C13).

HRMS (ES+): found 571.1711; C₃₂H₃₁N₂O₄S² [M+H]⁺ requires 571.1720.
A solution of 18 (269 mg, 0.47 mmol) in THF (9.4 mL) was cooled to −78 °C, and then LDA (1.0 M in THF/hexane, 2.36 mL, 2.36 mmol). The reaction mixture was stirred at this temperature for 2 h, and saturated aqueous NH₄Cl (3 mL) was poured into the mixture. The reaction mixture was cooled to rt, diluted with water (10 mL) and extracted with CH₂Cl₂ (3 × 12 mL). The organic layers were combined, dried (MgSO₄), filtered and concentrated in vacuo. Purification via flash column chromatography on silica gel (CH₂Cl₂/hexane = 1:1) gave 20 as an orange solid (67 mg, 50%).

mp: 160 °C (dec.)

ν_{max} (neat): 2917 (C-H), 2132 (C≡C), 1588, 1215 (C-N), 1051, 805.

δ_H (400 MHz, CDCl₃): 6.64 (2H, d, J 8.52, Ar H), 6.21 (2H, d, J 2.63, H1), 6.14 (2H, dd, J 8.45, 2.59, Ar H), 2.92 (12H, s, H9).

δ_C (126 MHz, CDCl₃): 150.4 (C2), 134.9 (Ar C), 127.5 (Ar CH), 117.7 (Ar C), 111.6 (C1), 109.3 (Ar CH), 107.2 (C≡C), 40.1 (C9).

HRMS (ES+): found 286.1460; C₂₀H₁₈N₂[M+H]⁺ requires 286.1470.
8-(dimethylamino)-N2,N2,N2-trimethyl-5,6,11,12-tetrahydrodibenzo[a,e]cyclooctene-2-aminium trifluoroacetate

A solution of 20 (25 mg, 0.09 mmol) in CH2Cl2 (1.3 mL) was taken in a dry round bottom flask. Methyl trifluoromethanesulfonate (0.019 mL, 0.17 mmol) was added to the reaction mixture dropwise at rt and the mixture was stirred for 2 h. CH2Cl2 was then removed in vacuo and preparative HPLC purification (30-60% MeCN) gave pure product 22 (11 mg, 30%) as an orange solid.

mp: 160 °C (dec.)

νmax (neat): 2916 (C-H), 2134 (C≡C), 1664, 1170 (C-N), 1125 (C-N), 802.

δH (500 MHz, CD3CN): 7.28 (1H, dd, J 8.40, 3.05, Ar H), 7.12 (1H, d, J 2.90, Ar H), 6.93 (1H, d, J 8.65, Ar H), 6.73 (1H, d, J 8.64, Ar H), 6.38 (1H, d, J 2.70, ArH), 6.30 (1H, dd, J 8.70, 2.75, Ar H), 3.42 (9H, s, H18), 2.92 (6H, s, H17).

δC (126 MHz, CD3CN): 152.1 (Ar C), 147.9 (Ar C), 137.3 (Ar C), 135.1 (Ar C), 133.4 (Ar C), 129.4 (Ar CH), 128.3 (Ar CH), 120.6 (Ar CH), 118.9 (Ar CH), 116.6 (Ar C), 115.1 (C≡C), 113.9 (Ar CH), 112.9 (C≡C), 111.6 (Ar CH), 107.3 (C≡C), 105.8 (C≡C), 57.5 (C18), 40.1 (C17).

HRMS (ES+): found 301.1705; C21H21N2 [M]+ requires 301.1705.
\(N^2,N^2,N^8,N^8\)-hexamethyl-5,6,11,12-tetrahydrodibenz[a,e]cyclooctene-2,8-diaminium trifluoroacetate

A solution of 20 (21 mg, 0.07 mmol) in \(\text{CH}_2\text{Cl}_2\) (1.1 mL) was taken in a dry round bottom flask. Methyl trifluoromethanesulfonate (0.016 mL, 0.15 mmol) was added to the reaction mixture dropwise at rt and the mixture was stirred for 2 h. \(\text{CH}_2\text{Cl}_2\) was then removed \textit{in vacuo}. The resulting crude product (32 mg, 0.11 mmol) was dissolved in MeCN (1.6 mL) and methyl trifluoromethanesulfonate (0.023 mL, 0.21 mmol) was added dropwise. After having stirred the reaction mixture for 3 h MeCN was removed \textit{in vacuo} and preparative HPLC purification (30-60% MeCN) gave pure product 23 (20 mg, 49%) as a light brown solid.

mp: 120 °C (dec.)

\(\nu_{\text{max}}\) (neat): 2970 (C-H), 1670, 1172 (C-N), 1120 (C-N), 799.

\(\delta_H\) (500 MHz, CD\textsubscript{3}CN): 7.49 (2H, dd, \(J\) 8.75, 2.80, H3), 7.34 (2H, d, \(J\) 2.76, H1), 7.08 (2H, d, \(J\) 8.67, H4), 3.46 (18H, s, H9).

\(\delta_C\) (126 MHz, CD\textsubscript{3}CN): 148.3 (C2), 135.1 (Ar C), 135.0 (Ar C), 129.3 (Ar CH), 122.9 (Ar CH), 121.0 (Ar CH), 110.1 (C≡C), 110.0 (C≡C), 57.6 (C9).

HRMS (ES\textsuperscript{+}): found 158.0968; \(\text{C}_{22}\text{H}_{24}\text{N}_2\) [M]\textsuperscript{2+} requires 158.0964.
Fmoc-Orn(N₃)-OH

Fmoc-Orn-OH·HCl (4.0 g, 10.2 mmol) was dissolved in a biphasic mixture of H₂O (50 mL), MeOH (100 mL) and CH₂Cl₂ (85 mL). CuSO₄·5H₂O (16 mg, 0.06 mmol) and imidazole-1-sulfonyl azide hydrochloride (8.6 g, 32.0 mmol) were added, and the mixture was adjusted to pH 9 with aqueous K₂CO₃ solution. After stirring vigorously for 18 h, the organic solvents were removed in vacuo. The remaining aqueous phase was washed with diethyl ether (2 × 20 mL), acidified to pH 2 with concentrated HCl and extracted with diethyl ether (3 × 30 mL). The organic extracts were dried over MgSO₄ and concentrated in vacuo. The oily residue was dissolved in CH₂Cl₂, and the solvent was removed under a stream of nitrogen to give 64 (2.05 g, 53%) as a white solid.

mp: 133-135 °C

δH (500 MHz, CDCl₃): 7.79 (2H, d, J 7.48, H14), 7.61 (2H, d, J 6.5, H11), 7.43 (2H, app t, J 7.1, H13), 7.38-7.30 (2H, app tt, J 7.44, H12), 5.35 (1H, d, J 8.09, H6), 4.70-4.34 (3H, m, H8, H5), 4.24 (1H, t, J 6.71, H9), 3.44-3.09 (2H, m, H1), 2.16-1.19 (4H, m, H2, H3).

δC (126 MHz, CDCl₃): 176.0 (C10), 156.1 (C5), 143.6 (Ar C), 141.3 (Ar C), 127.8 (Ar CH), 127.1 (Ar CH), 125.0 (Ar CH), 120.0 (Ar CH), 67.1 (C8), 53.3 (C9), 50.8 (C1), 47.1 (C5), 29.6 (C3), 24.8 (C2).

Characterisation data is in accordance with that previously reported.¹
2.4 Peptide synthesis procedure

Peptide synthesis was carried out on solid-phase using an Fmoc-protecting group strategy on a CEM Liberty Automated Microwave Peptide Synthesiser. Merck Rink Amide MBHA resin LL (0.29-0.39 mmol/g) was used. Peptide couplings were conducted with Fmoc-protected amino acids (5 equiv) in DMF, HATU or HBTU (5 equiv) in DMF as the coupling reagent, and \(N,N\)-diisopropylethylamine (10 equiv) in NMP as the base. Double coupling was used for arginine for 15 min each without microwave irradiation. Single coupling was used for all other amino acids, with 25 W power at 75 °C over 15 min. Fmoc deprotection was carried out using 20% piperidine in DMF, with 45 W power at 75 °C over 3 min.

\(N\)-terminal capping was carried out manually by treating the resin-bound peptide with acetic anhydride (10 equiv) and \(N,N\)-diisopropylethylamine (10 equiv) in dichloromethane for 45 min. Cleavage was carried out with a cocktail of 95% trifluoroacetic acid, 2.5% water and 2.5% triisopropylsilane for 2 h. The cleavage solution was then evaporated under a stream of nitrogen and triturated with diethyl ether prior to purification by preparative HPLC.

Peptide yields were estimated based on absorbance in the HPLC chromatograph at 220 nm. Concentration of peptides in stock solutions were determined by amino acid analysis at the Peptide Nucleic Acid Chemistry Facility at the Department of Biochemistry, University of Cambridge.

2.5 General strain-promoted double-click peptide stapling procedure

A solution of diazido PDI-E peptide (1 eq) and dialkyne (1.1 eq) in 1:1 \(t\)-BuOH/H\(_2\)O (1 mL/mg peptide) was stirred at rt for 16 h (1:1 MeCN/H\(_2\)O employed for trimethylammonium substituted diynes). Reaction mixture was lyophilised and purified by HPLC to give the stapled peptide.
2.6 Peptide LCMS and HPLC data

Data on PDI-E was previously reported.\textsuperscript{2}

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Mass</th>
<th>m/z found</th>
<th>m/z calcd</th>
</tr>
</thead>
<tbody>
<tr>
<td>26 PDI-E</td>
<td>1577.8</td>
<td>790.3</td>
<td>790.4 [M+2H]\textsuperscript{2⁺}</td>
</tr>
<tr>
<td>27</td>
<td>1864.2</td>
<td>933.5</td>
<td>933.1 [M+2H]\textsuperscript{2⁺}</td>
</tr>
<tr>
<td>28</td>
<td>1879.2</td>
<td>940.6</td>
<td>940.6 [M+2H]\textsuperscript{2⁺}</td>
</tr>
<tr>
<td>29</td>
<td>1894.2</td>
<td>947.8</td>
<td>948.1 [M+2H]\textsuperscript{2⁺}</td>
</tr>
</tbody>
</table>
Analytical HPLC Spectra (5-95% MeCN over 15 mins)

LCMS Spectra
Analytical HPLC Spectra (40-70% MeCN over 20 mins)

LCMS Spectra
Analytical HPLC Spectra (40-70% MeCN over 20 mins)

LCMS Spectra

Peak ID  Compound  Time  Mass Found  BPM

1  MS ES+  3.3e+007
Analytical HPLC Spectra (40-70% MeCN over 20 mins)

LCMS Spectra

Peak ID | Compound | Time | Mass Found | BPM
---|---|---|---|---
3 | | 1.74 | 947.8 | 948

1: MS ESI+ 1.7e+008
2.7 **X-ray crystallography**

2.7.1 **X-ray crystallography of meta-trimethylammonium functionalized peptide 28 in complex with MDM2**

2.7.1.1 Expression and purification of MDM2<sub>17-108</sub>-E69AK70A

The protein was expressed and purified following the methods described previously. The plasmid pGEX6P1- MDM2<sub>17-108</sub>-E69AK70A, kindly provided by Dr J. Reeks (Newcastle University, UK), was transformed into BL21 (DE3) cells for expression. The cells were cultured in two litres of Hyper-Broth medium (Molecular Dimension) with 100 µg/ml ampicillin at 37 °C until the OD<sub>600</sub> reached 0.4. The cell culture was then incubated at 18 °C for one hour before induction with 0.2 mM IPTG. Protein was expressed at 18 °C for 16 hours before the cells were centrifuged at 4000 g for 10 minutes at 4 °C. The cell pellet was resuspended in 50 mL lysis buffer (20 mM Tris pH 8, 200 mM NaCl, 5 mM DTT, 10% sucrose, cOmplete mini protease inhibitors cocktail (Roche Diagnostics)) and then lysed by EmusiFlex. The soluble fraction of the lysate was collected after centrifugation at 15000 g for 30 minutes at 4°C and then filtered through Minisart 0.45 µm syringe filter (Sartorius Stedium).

The GST-tagged MDM2<sub>17-108</sub>-E69AK70A was purified by affinity chromatography using PureCube Glutathione Agarose beads (Cube Biotech). 2 mL beads were equilibrated with 10 mL of the lysis buffer and then mixed with the lysate. After incubation at 18 °C for one hour, the beads were washed with 40 mL of the lysis buffer and then eluted by the lysis buffer with 10 mM reduced glutathione. The eluted fractions were analysed by SDS-PAGE and the ones containing GST-tagged MDM2<sub>17-108</sub>-E69AK70A were combined and concentrated to 4 mL using Amicon Ultra-4 centrifugal filter with 3 kDa cut off at 18 °C. The GST tag was cleaved by incubation with PreScission 3C protease at the molar ratio of 30:1 at 4 °C overnight. 0.9 mg of the stapled peptide 51 was added to the cleaved GST-tagged MDM2<sub>17-108</sub>-E69AK70A and incubated for one hour on ice. The mixture was then purified by size exclusion chromatography using Superdex 75 10/300 column (GE Healthcare) in the buffer of 20 mM HEPES pH 7.4, 200 mM NaCl, 5 mM DTT. The peak fractions were analysed by SDS-PAGE and the ones containing MDM2<sub>17-108</sub>-E69AK70A were combined and concentrated to 12 mg/ml using Amico Ultra-4 centrifugal filter with 3 kDa cut off.

2.7.1.2 Crystallisation and structure determination of MDM2-28 peptide complex

Crystallisation of the MDM2<sub>17-108</sub>-E69AK70A-28 stapled peptide complex was performed in 96-well MRC 2-drop crystallisation plates using JCSG+ Crystal Screen (Molecular Dimensions). Mosquito Crystal (TTPLabtech Ltd) system was used to set up two crystallisation drops for each condition. One drop contains 200 nL of protein plus 200 nL of reservoir and the other drop contains 200 nL of protein plus 100 nL of reservoir. Crystals appeared after eight days in the conditions of 1.1 M sodium malonate dibasic, 0.1 M HEPES pH 7.0, 0.5% v/v Jeffamine ED-2003. Three crystals were harvested and flash-cooled in liquid nitrogen. Diffraction data was collected at Diamond synchrotron I04 beamline and processed by autoPROC<sup>3</sup> to the resolution of 2.0 Å.

The structure of MDM2<sub>17-108</sub>-E69AK70A-28 stapled peptide was solved by molecular replacement in Phaser<sup>4</sup>. The coordinates of MDM2 in the structure of MDM2<sub>17-108</sub>-E69AK70A-E1 stapled peptide (PDB code 5AFG) were used as a search model. The structure solution from Phaser was refined without the stapled peptide by several rounds of manual
building using Coot\textsuperscript{5} and refinement using REFMAC5\textsuperscript{6}. The refined data shows clear electron density to build the stapled peptide in both the 2Fo-Fc map and Fo-Fc map (Figure S1.2.1a and b). The stapled peptide was therefore manually built using Coot and refined using PHENIX\textsuperscript{7} (Figure S2.7.1.2.1c). The geometrical restraint file for the stapled linker was generated using Grade Web Server (Global Phasing Ltd.). The statistics of data processing and refinement are shown in Table S2.7.1.2.

**Figure S2.7.1.2.** Electron density maps of the bound 28 peptide. The structure of MDM2 is shown as surface and coloured in grey. The structure of stapled peptide is shown in sticks. a. Difference density map (Fo-Fc) contoured at 2σ before modeling the peptide. b. Electron density map (2Fo-Fc) contoured at 0.5σ before modeling the peptide. c. Final electron density map (2Fo-Fc) contoured at 1σ after peptide modeling and structure refinement.

**Table S2.7.1.2.** Crystallographic statistics of peptide-MDM2 complex (PDB code: 6H22)

<table>
<thead>
<tr>
<th>Data Collection</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>Wavelength (Å)</td>
<td>0.9795</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>48.06 - 2.006 (2.078 - 2.006)</td>
</tr>
<tr>
<td>Space group</td>
<td>P 2₁ 2₁ 2₁</td>
</tr>
<tr>
<td>Unit cell (Å)</td>
<td>a=64.416 b=72.186 c=45.124 α=β=γ=90°</td>
</tr>
<tr>
<td>Molecules per asymmetric unit</td>
<td>2</td>
</tr>
<tr>
<td>Total reflections</td>
<td>77879 (6731)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>14427 (1253)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>5.4 (5.4)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.54 (86.83)</td>
</tr>
<tr>
<td>Mean I/σ(I)</td>
<td>9.46 (2.20)</td>
</tr>
<tr>
<td>R-merge</td>
<td>0.1103 (0.7797)</td>
</tr>
<tr>
<td>CC₁/₂</td>
<td>0.998 (0.898)</td>
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</table>

<table>
<thead>
<tr>
<th>Refinement</th>
<th>Value</th>
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<tbody>
<tr>
<td>Resolution range (Å)</td>
<td>48.06 - 2.006 (2.078 - 2.006)</td>
</tr>
<tr>
<td>R-work</td>
<td>0.2107 (0.3239)</td>
</tr>
<tr>
<td>R-free</td>
<td>0.2384 (0.3977)</td>
</tr>
<tr>
<td>Number of non-hydrogen atoms</td>
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<tr>
<td>Macromolecules</td>
<td>1700</td>
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<td>Ligands</td>
<td>74</td>
</tr>
<tr>
<td>Water</td>
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<tr>
<td>Protein residues</td>
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</table>
### Root-mean-square deviation

<p>| | |</p>
<table>
<thead>
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<tbody>
<tr>
<td>Bonds</td>
<td>0.005</td>
</tr>
<tr>
<td>Angles</td>
<td>0.88</td>
</tr>
<tr>
<td>Average B-factor</td>
<td>30.1</td>
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<tr>
<td>Macromolecules</td>
<td>29.6</td>
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<tr>
<td>Ligands</td>
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<tr>
<td>Solvent</td>
<td>35</td>
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<tr>
<td>Ramachandran favored (%)</td>
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</tr>
<tr>
<td>Ramachandran outliers (%)</td>
<td>0</td>
</tr>
<tr>
<td>Clashscore</td>
<td>1.99</td>
</tr>
</tbody>
</table>

#### 2.7.1.3 Analysis of the MDM2-28 peptide structure

The analysis of the crystallographic data of MDM2-28 peptide complex shows that one asymmetric unit contains two macromolecules that have the same structures apart from the small variation at the N-termini (r.m.s.d of 0.108) (Figure 2.7.1.3.1a and b). As the complex is eluted from size exclusion chromatography as monomers (data not shown), the possibility that the complex forms a dimer is emitted. The fact that two complexes present in one asymmetric unit is due to the structural heterogeneity.

![Figure S2.7.1.3.1](image.png)

**Figure S2.7.1.3.1.** Crystal structure of MDM2-28 peptide in one asymmetric unit. Structures of MDM2 are shown as cartoon and coloured in light orange and grey. Structures of peptides are shown as cartoon and coloured in orange and magenta with the staple shown as sticks. a. Asymmetric unit of the crystal structure of MDM2-28 peptide complex. b. Superimposition of two MDM2-28 peptide complex structures in one asymmetric unit. The root-mean-square deviation (r.m.s.d) is 0.108.

In comparison with the structure of MDM2-E1 peptide complex (PDB code 5AFG), both peptides form hydrophobic interactions with MDM2 through residue F3, W7, and L10 (Figure 2.7.1.3.2a). However, the staple compound of 28 peptide shifts away from the MDM2 protein surface (Figure 2.7.1.3.2b). The trimethylammonium group moves even further compared to the dimethylamine group, indicating that the hydrophobic interface on MDM2 seem to expel the staple compound due to its charged groups.
Figure S2.7.1.3.2. Comparison of structures of MDM2-28 peptide complex (PDB code 6H22) and MDM2-E1 peptide complex (PDB code 5AFG). The structure of MDM2 is shown as surface and coloured in grey. The peptides are shown as cartoon and the staple shown as sticks. E1 peptide is coloured in cyan and 28 peptide is in orange. a. 28 peptide associates with MDM2 through hydrophobic interactions mediated by residue F3, W7, and L10, which is conserved in E1 peptide-MDM2 interactions. b. The staple of 51 peptide shifts away from the MDM2 surface formed by F55, Q59, and M62 compared to the staple of E1 peptide.
2.8 Competitive fluorescence polarization assay

Competitive fluorescence polarization (FP) assays were performed as previously described\(^2,8\) in 384-well microplates (Corning) on a CLARIOstar microplate reader (BMG labtech) using excitation filter 540–20 nm, dichroic mirror LP 566 nm, and emission filter 590–20 nm. All peptides were dissolved in DMSO as stock solutions. The stock concentration of the TAMRA-labeled tracer (TAMRA-RFMDYWEGL-NH\(_2\)) was determined based on the 5-TAMRA absorbance at 556 nm (extinction coefficient \(\varepsilon = 89,000 \text{ M}^{-1} \text{ cm}^{-1}\)) measured on a NanoDrop 2000 (Thermo Scientific) and concentrations of all peptides were determined by amino acid analysis (Department of Biochemistry, University of Cambridge). \(K_d\) of the TAMRA-labelled tracer was obtained from previously reported experiments\(^2\). For the competitive fluorescence polarization assay, TAMRA-labelled tracer (50 nM) was incubated with MDM2 (95 nM) in PBS buffer containing 0.05% (v/v) Tween 20 at 25 °C for 1 hr. The unlabeled peptides or positive controls (nutlin) were diluted 2-fold serially in PBS buffer containing 0.05% (v/v) Tween 20 for a 16-point titration curve (20 μL per well). To each well containing the unlabeled compound was added the TAMRA-labelled tracer/MDM2 solution (20 μL) and the mixture was incubated for 1 hr at 25 °C before the measurement was taken. Titrations were performed in triplicate. Data were fitted in GraphPad Prism 5.0 using the equations as described previously.\(^9\) Data for 27 (inseparable mixture of two conformational isomers) was fitted using a competitive two-site binding model from GraphPad Prism 7.0, and the \(K_d\) from the tighter binding is reported below.

<table>
<thead>
<tr>
<th>Functionalized Stapled Peptide</th>
<th>(K_d) / nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>35.3 ± 0.1</td>
</tr>
<tr>
<td>28</td>
<td>7.0 ± 3.9</td>
</tr>
<tr>
<td>29</td>
<td>17.0 ± 6.2</td>
</tr>
</tbody>
</table>

Binding data for unstapled diazido peptide 26 (PDI-E) and its corresponding unsubstituted stapled form 30 (PDI-E1) with Sondheimer dialkyne\(^8\)

<table>
<thead>
<tr>
<th></th>
<th>(K_d) / nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>6.5 ± 0.6</td>
</tr>
<tr>
<td>30</td>
<td>7.5 ± 0.7</td>
</tr>
</tbody>
</table>
2.9 Cellular p53 reporter assay

The basic assay was carried out as previously described in the literature\textsuperscript{10, 11} and is briefly described as follows. T22 cells stably transfected with a p53-responsive β-galactosidase reporter,\textsuperscript{10} kindly provided by Prof. Sir David Lane, were grown in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum and penicillin/streptomycin. Cells were seeded for 24 h at 8000 cells per 100 μL well in a 96-well black-walled clear flat-bottom polystyrene plate (Greiner #655090), then treated with peptide in triplicate for 18 h at 37 °C in DMEM with 10% serum and a final DMSO concentration of 1%. β-galactosidase activity was quantified using a FluoReporter LacZ/Galactosidase Quantitation kit (Invitrogen). Fluorescence measurements were read on a Tecan Infinite 200 Pro plate reader.

The \textit{in situ} stapling version of the assay was identical to the basic assay conditions in all aspects, except that the cells were treated with pure unstapled PDI-E peptide (50 μM) and substituted Sondheimer dialkynes (0.5 mM). Control cells were treated with only substituted Sondheimer dialkynes (0.5 mM), only unstapled PDI-E peptide (50 μM), or only 1% DMSO.
2.9.1 p53 activation data for pre-stapled purified peptides

![Graph showing p53 activation data for pre-stapled purified peptides](image)

**Figure S2.9.1** p53 activation in the cellular reporter assay with pre-stapled purified peptides 27-29. Data is reported as fold activation over 1% DMSO.
2.10 Kinetic analysis

The rate measurement of substituted Sondheimer dialkynes 20-25 in a double strain-promoted click reaction was performed by monitoring the absorbance of dialkynes in the presence of an excess amount of benzyl azide.\textsuperscript{12, 13} It has been demonstrated previously with Sondheimer diyne 1 that the first cycloaddition leading to the formation of monoyne intermediate is the rate-determining step of the reaction.\textsuperscript{12} All UV measurement were recorded using a Varian Cary 300 UV–visible spectrophotometer.

2.10.1 Kinetic analysis of 1\textsuperscript{12}

To 1.5 mL 1.5 mM solution of substituted dialkyne 1 in MeOH taken in a quartz cuvette was added 1.5 mL of MeOH solution of benzyl azide in 3 different concentrations (20, 100, and 200 mM; final concentration at 10, 50, and 100 mM, respectively). The consumption of dialkyne 1 was monitored by UV spectroscopy at a wavelength of 370 nm that is characteristic for the absorbance of dialkyne 1 but almost no significant absorption is observed for benzyl azide and products. The experiments were repeated in duplicate for each concentration of azide. The observed absorbance data at 370 nm were plotted versus time and fitted to a first order exponential decay curve. The pseudo-first order rate constants ($k_0$) were determined by least-squares fitting of the data to the following exponential equation ($y = A\times\exp(-k_0\times x) + y_0$) using Origin where $A$, $y_0$ are constants. The pseudo-first order rate constants determined were plotted versus concentration of azide and fitted to a straight line by linear regression method using Microsoft Office Excel 16. The slope of the straight line, (0.048 ± 7.5E-5) M$^{-1}$ s$^{-1}$, indicates the second order rate constant ($k$) for the first cycloaddition in a double strain-promoted click reaction of dialkyne 1 with benzyl azide, which is the rate-determining step of the reaction.
- Time-dependent absorbance of dialkyne 1 (initial concentration of 1.5 mM) at 370 nm of wavelength monitored in duplicate during the reaction with 10 mM benzyl azide

Run 1

![Graph](image1.png)

Run 2

![Graph](image2.png)

- Time-dependent absorbance of dialkyne 1 (initial concentration of 1.5 mM) at 370 nm of wavelength monitored in duplicate during the reaction with 50 mM benzyl azide

Run 1

![Graph](image3.png)

Run 2

![Graph](image4.png)

- Time-dependent absorbance of dialkyne 1 (initial concentration of 1.5 mM) at 370 nm of wavelength monitored in duplicate during the reaction with 100 mM benzyl azide

Run 1

![Graph](image5.png)

Run 2

![Graph](image6.png)
• Pseudo-first order rate constants ($k_0$) determined for dialkyne 1 by fitting of the absorbance versus time data to the following exponential equation ($y = A \exp(-k_0 x) + y_0$) using Origin

<table>
<thead>
<tr>
<th>Benzyl azide</th>
<th>Run</th>
<th>Pseudo-first order rate constant $k_0$ (min$^{-1}$)</th>
<th>Standard error</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM</td>
<td>1</td>
<td>0.028</td>
<td>4.28E-6</td>
<td>0.99998</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.028</td>
<td>4.23E-6</td>
<td>0.99998</td>
</tr>
<tr>
<td>50 mM</td>
<td>1</td>
<td>0.137</td>
<td>1.18E-5</td>
<td>0.99998</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.140</td>
<td>2.66E-5</td>
<td>0.9999</td>
</tr>
<tr>
<td>100 mM</td>
<td>1</td>
<td>0.287</td>
<td>3.81E-5</td>
<td>0.99998</td>
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<tr>
<td></td>
<td>2</td>
<td>0.288</td>
<td>6.03E-5</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

• Plot of pseudo-first order rate constants ($k_0$) versus the concentration of benzyl azide for determination of second order rate constant ($k$).

<table>
<thead>
<tr>
<th>Run</th>
<th>Second order rate constant ($M^{-1} min^{-1}$)</th>
<th>Second order rate constant ($M^{-1} s^{-1}$)</th>
<th>Mean second order rate constant $k$ ($M^{-1} s^{-1}$)</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.883</td>
<td>0.04805</td>
<td>0.048125</td>
<td>7.5E-5</td>
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<tr>
<td>2</td>
<td>2.892</td>
<td>0.0482</td>
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</tr>
</tbody>
</table>
2.10.2 Kinetic analysis of 20

To 1.5 mL 0.4 mM solution of substituted dialkyne 20 in MeOH taken in a quartz cuvette was added 1.5 mL of MeOH solution of benzyl azide in 3 different concentrations (20, 100, and 200 mM; final concentration at 10, 50, and 100 mM, respectively). The consumption of dialkyne 20 was monitored by UV spectroscopy at a wavelength of 372 nm that is characteristic for the absorbance of dialkyne 20 but almost no significant absorption is observed for benzyl azide and products. The experiments were repeated in duplicate for each concentration of azide. The observed absorbance data at 372 nm were plotted versus time and fitted to a first order exponential decay curve. The pseudo-first order rate constants \( k_0 \) were determined by least-squares fitting of the data to the following exponential equation \( y = A \exp(-k_0 x) + y_0 \) using Origin where \( A, y_0 \) are constants. The pseudo-first order rate constants determined were plotted versus concentration of azide and fitted to a straight line by linear regression method using Microsoft Office Excel 16. The slope of the straight line, \( (0.039 \pm 3.50 \times 10^{-4}) \text{ M}^{-1} \text{ s}^{-1} \), indicates the second order rate constant \( k \) for the first cycloaddition in a double strain-promoted click reaction of dialkyne 20 with benzyl azide, which is the rate-determining step of the reaction.
• Time-dependent absorbance of dialkyne 20 (initial concentration of 0.4 mM) at 372 nm of wavelength monitored in duplicate during the reaction with 10 mM benzyl azide

Run 1

Run 2

• Time-dependent absorbance of dialkyne 20 (initial concentration of 0.4 mM) at 372 nm of wavelength monitored in duplicate during the reaction with 50 mM benzyl azide

Run 1

Run 2

• Time-dependent absorbance of dialkyne 20 (initial concentration of 0.4 mM) at 372 nm of wavelength monitored in duplicate during the reaction with 100 mM benzyl azide

Run 1

Run 2
- Pseudo-first order rate constants \((k_0)\) determined for dialkyne 20 by fitting of the absorbance versus time data to the following exponential equation \(y = A \times \exp(-k_0 \times x) + y_0\) using Origin.

<table>
<thead>
<tr>
<th>Benzyl azide</th>
<th>Run</th>
<th>Pseudo-first order rate constant (k_0) (min(^{-1}))</th>
<th>Standard error</th>
<th>(R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM</td>
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<td>0.057</td>
<td>4.59E-4</td>
<td>0.97066</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.029</td>
<td>4.40E-5</td>
<td>0.99931</td>
</tr>
<tr>
<td>50 mM</td>
<td>1</td>
<td>0.148</td>
<td>8.51E-6</td>
<td>0.99998</td>
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<tr>
<td></td>
<td>2</td>
<td>0.127</td>
<td>6.09E-5</td>
<td>0.99977</td>
</tr>
<tr>
<td>100 mM</td>
<td>1</td>
<td>0.264</td>
<td>3.77E-4</td>
<td>0.99939</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.241</td>
<td>3.84E-4</td>
<td>0.99899</td>
</tr>
</tbody>
</table>

- Plot of pseudo-first order rate constants \((k_0)\) versus the concentration of benzyl azide for determination of second order rate constant \((k)\).

Run 1

![Graph of pseudo-first order rate constant vs. benzyl azide concentration](image1)

Run 2

![Graph of pseudo-first order rate constant vs. benzyl azide concentration](image2)

<table>
<thead>
<tr>
<th>Run</th>
<th>Second order rate constant ((M^{-1} \text{ min}^{-1}))</th>
<th>Second order rate constant ((M^{-1} \text{ s}^{-1}))</th>
<th>Mean second order rate constant (k) ((M^{-1} \text{ s}^{-1}))</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.300</td>
<td>0.038333</td>
<td>0.038683</td>
<td>3.50E-4</td>
</tr>
<tr>
<td>2</td>
<td>2.342</td>
<td>0.039033</td>
<td>0.038683</td>
<td>3.50E-4</td>
</tr>
</tbody>
</table>
2.10.3 Kinetic analysis of 22

To 1.5 mL 1.5 mM solution of substituted dialkyne 22 in MeOH taken in a quartz cuvette was added 1.5 mL of MeOH solution of benzyl azide in 3 different concentrations (20, 100, and 200 mM; final concentration at 10, 50, and 100 mM, respectively). The consumption of dialkyne 22 was monitored by UV spectroscopy at a wavelength of 385 nm that is characteristic for the absorbance of dialkyne 22 but almost no significant absorption is observed for benzyl azide and products. The experiments were repeated in duplicate for each concentration of azide. The observed absorbance data at 385 nm were plotted versus time and fitted to a first order exponential decay curve. The pseudo-first order rate constants ($k_0$) were determined by least-squares fitting of the data to the following exponential equation ($y = A \exp(-k_0 x) + y_0$) using Origin where $A, y_0$ are constants. The pseudo-first order rate constants determined were plotted versus concentration of azide and fitted to a straight line by linear regression method using Microsoft Office Excel 16. The slope of the straight line, $(0.097 \pm 1.50E-4) \text{ M}^{-1} \text{ s}^{-1}$, indicates the second order rate constant ($k$) for the first cycloaddition in a double strain-promoted click reaction of dialkyne 22 with benzyl azide, which is the rate-determining step of the reaction.
• Time-dependent absorbance of dialkyne 22 (initial concentration of 1.5 mM) at 385 nm of wavelength monitored in duplicate during the reaction with 10 mM benzyl azide

Run 1

Run 2

• Time-dependent absorbance of dialkyne 22 (initial concentration of 1.5 mM) at 385 nm of wavelength monitored in duplicate during the reaction with 50 mM benzyl azide

Run 1

Run 2

• Time-dependent absorbance of dialkyne 22 (initial concentration of 1.5 mM) at 385 nm of wavelength monitored in duplicate during the reaction with 100 mM benzyl azide

Run 1

Run 2
- Pseudo-first order rate constants ($k_0$) determined for dialkylene 22 by fitting of the absorbance versus time data to the following exponential equation ($y = A\exp(-k_0x) + y_0$) using Origin

<table>
<thead>
<tr>
<th>Benzyl azide</th>
<th>Run</th>
<th>Pseudo-first order rate constant $k_0$ (min$^{-1}$)</th>
<th>Standard error</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM</td>
<td>1</td>
<td>0.052</td>
<td>2.14E-5</td>
<td>0.99989</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.053</td>
<td>2.26E-5</td>
<td>0.99988</td>
</tr>
<tr>
<td>50 mM</td>
<td>1</td>
<td>0.280</td>
<td>3.25E-4</td>
<td>0.99899</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.259</td>
<td>1.94E-4</td>
<td>0.99938</td>
</tr>
<tr>
<td>100 mM</td>
<td>1</td>
<td>0.577</td>
<td>4.15E-4</td>
<td>0.99943</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.578</td>
<td>3.58E-4</td>
<td>0.99956</td>
</tr>
</tbody>
</table>

- Plot of pseudo-first order rate constants ($k_0$) versus the concentration of benzyl azide for determination of second order rate constant ($k$).

<table>
<thead>
<tr>
<th>Run</th>
<th>Second order rate constant ($\text{M}^{-1} \text{min}^{-1}$)</th>
<th>Second order rate constant ($\text{M}^{-1} \text{s}^{-1}$)</th>
<th>Mean second order rate constant $k$ ($\text{M}^{-1} \text{s}^{-1}$)</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.838</td>
<td>0.0973</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.856</td>
<td>0.0976</td>
<td>0.09745</td>
<td>1.50E-4</td>
</tr>
</tbody>
</table>
2.10.4 Kinetic analysis of 23

To 1.5 mL 1.5 mM solution of substituted dialkyne 23 in MeOH taken in a quartz cuvette was added 1.5 mL of MeOH solution of benzyl azide in 3 different concentrations (20, 100, and 200 mM; final concentration at 10, 50, and 100 mM, respectively). The consumption of dialkyne 23 was monitored by UV spectroscopy at a wavelength of 372 nm that is characteristic for the absorbance of dialkyne 23 but almost no significant absorption is observed for benzyl azide and products. The experiments were repeated in duplicate for each concentration of azide.

The observed absorbance data at 372 nm were plotted versus time and fitted to a first order exponential decay curve. The pseudo-first order rate constants \( k_0 \) were determined by least-squares fitting of the data to the following exponential equation \( y = A \cdot \exp(-k_0 \cdot x) + y_0 \) using Origin where \( A, y_0 \) are constants. The pseudo-first order rate constants determined were plotted versus concentration of azide and fitted to a straight line by linear regression method using Microsoft Office Excel 16. The slope of the straight line, \( (0.097 \pm 1.50E-4) \, \text{M}^{-1} \, \text{s}^{-1} \), indicates the second order rate constant \( k \) for the first cycloaddition in a double strain-promoted click reaction of dialkyne 23 with benzyl azide, which is the rate-determining step of the reaction.
• Time-dependent absorbance of dialkyne 23 (initial concentration of 1.5 mM) at 372 nm of wavelength monitored in duplicate during the reaction with 10 mM benzyl azide

Run 1

Run 2

• Time-dependent absorbance of dialkyne 23 (initial concentration of 1.5 mM) at 372 nm of wavelength monitored in duplicate during the reaction with 50 mM benzyl azide

Run 1

Run 2

• Time-dependent absorbance of dialkyne 23 (initial concentration of 1.5 mM) at 372 nm of wavelength monitored in duplicate during the reaction with 100 mM benzyl azide

Run 1

Run 2
- Pseudo-first order rate constants \((k_0)\) determined for dialkyne 23 by fitting of the absorbance vs time data to the following exponential equation \((y = A*\exp(-k_0*x) + y_0)\) using Origin.

<table>
<thead>
<tr>
<th>Benzyl azide</th>
<th>Run</th>
<th>Pseudo-first order rate constant (k_0) (min(^{-1}))</th>
<th>Standard error</th>
<th>(R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM</td>
<td>1</td>
<td>0.045</td>
<td>1.40E-5</td>
<td>0.99995</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.046</td>
<td>2.56E-5</td>
<td>0.99984</td>
</tr>
<tr>
<td>50 mM</td>
<td>1</td>
<td>0.455</td>
<td>2.12E-4</td>
<td>0.99979</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.468</td>
<td>2.24E-4</td>
<td>0.99977</td>
</tr>
<tr>
<td>100 mM</td>
<td>1</td>
<td>0.968</td>
<td>6.00E-4</td>
<td>0.9998</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.998</td>
<td>7.33E-4</td>
<td>0.99972</td>
</tr>
</tbody>
</table>

- Plot of pseudo-first order rate constants \((k_0)\) versus the concentration of benzyl azide for determination of second order rate constant \((k)\).

Run 1

\[
y = 10.256x - 0.0576 \\
R^2 = 1
\]

Run 2

\[
y = 10.579x - 0.0602 \\
R^2 = 1
\]

<table>
<thead>
<tr>
<th>Run</th>
<th>Second order rate constant ((M^{-1} \text{ min}^{-1}))</th>
<th>Second order rate constant ((M^{-1} \text{ s}^{-1}))</th>
<th>Mean second order rate constant (k) ((M^{-1} \text{ s}^{-1}))</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.256</td>
<td>0.170933</td>
<td>0.173625</td>
<td>2.69E-4</td>
</tr>
<tr>
<td>2</td>
<td>10.579</td>
<td>0.176317</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.10.5 Kinetic analysis of 21

To 1.5 mL 1.3 mM solution of substituted dialkyne 21 in MeOH taken in a quartz cuvette was added 1.5 mL of MeOH solution of 200 mM benzyl azide (final concentration at 100 mM). The consumption of dialkyne 21 was monitored by UV spectroscopy at a wavelength of 372 nm that is characteristic for the absorbance of dialkyne 21 but almost no significant absorption is observed for benzyl azide and products. The observed absorbance data at 372 nm were plotted versus time.

- Time-dependent absorbance of dialkyne 21 (initial concentration of 1.3 mM) at 372 nm of wavelength monitored during the reaction with 100 mM benzyl azide
2.10.6 Kinetic analysis of 24

To 1.5 mL 1.5 mM solution of substituted dialkyne 24 in MeOH taken in a quartz cuvette was added 1.5 mL of MeOH solution of 200 mM benzyl azide (final concentration at 100 mM). The consumption of dialkyne 24 was monitored by UV spectroscopy at a wavelength of 372 nm that is characteristic for the absorbance of dialkyne 24 but almost no significant absorption is observed for benzyl azide and products. The observed absorbance data at 372 nm were plotted versus time.

- Time-dependent absorbance of dialkyne 24 (initial concentration of 1.5 mM) at 372 nm of wavelength monitored during the reaction with 100 mM benzyl azide
2.10.7 Kinetic analysis of 25

To 1.5 mL 1.5 mM solution of substituted dialkyne 25 in MeOH taken in a quartz cuvette was added 1.5 mL of MeOH solution of 200 mM benzyl azide (final concentration at 100 mM). The consumption of dialkyne 25 was monitored by UV spectroscopy at a wavelength of 372 nm that is characteristic for the absorbance of dialkyne 25 but almost no significant absorption is observed for benzyl azide and products. The observed absorbance data at 372 nm were plotted versus time.

- Time-dependent absorbance of dialkyne 25 (initial concentration of 1.5 mM) at 372 nm of wavelength monitored during the reaction with 100 mM benzyl azide
Figure S2.10. A direct comparison of the rate of SPAAC reaction observed for *ortho*-substituted dialkynes 21, 24, 25 against their *meta*-substituted counterparts 20, 22, 23 (rate plots with 100 mM benzyl azide in MeOH at rt).


