SUPPORTING INFORMATION

Click reaction between 1,2,4,5-tetrazine and cyclooctynes with tunable reaction rates

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Materials and Methods

All reagents and solvents were reagent grade or were purified by standard methods before use. Column chromatography was carried out on flash silica gel (Sorbent 230–400 mesh). TLC analysis was conducted on silica gel plates (Sorbent Silica G UV254). NMR spectra were recorded at $^1\text{H}$ (400 MHz) and $^{13}\text{C}$ (100 MHz) on a Bruker instrument. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and hertz, respectively, using solvents ($^1\text{H}$ NMR, $^{13}\text{C}$ NMR) as the internal standard. DIFO, methyl 1-fluorocyclooct-2-ynecarboxylate, compound 1, compound 2, compound 4, and compound 9 were synthesized according to literature procedures.

Preparation of Cyclooctyne 1

![Figure S1. Reagents and conditions for the synthesis of cyclooctyne 1](image)

Cyclooctyne (1): $^1\text{H}$ NMR (CDCl$_3$) δ 2.14 (s, 4H), 1.83 (s, 4H), 1.60 (s, 4H). $^{13}\text{C}$ NMR (CDCl$_3$) δ 94.6, 34.6, 29.8, 21.0.

Preparation of (1R,8S,9S)-Bicyclo[6.1.0]non-4-yn-9-ylmethanol (endo-4)

![Figure S2. Reagents and conditions for the synthesis of (1R,8S,9S)-bicyclo[6.1.0]non-4-yn-9-ylmethanol (endo-4)](image)

(1R,8S,9S)-Bicyclo[6.1.0]non-4-yn-9-ylmethanol (endo-4): $^1\text{H}$ NMR (CDCl$_3$): δ 3.66 (d, J = 8.0 Hz, 2H), 2.28-2.14 (m, 6H), 1.55-1.54 (m, 2H), 1.31-1.25 (m, 1H), 0.89-0.85 (m, 2H). $^{13}\text{C}$ NMR (CDCl$_3$) δ 98.9, 59.7, 29.0, 21.5, 21.3, 20.0.

Preparation of 3,6-diphenyl-1,2,4,5-tetrazine 2.
3,6-Diphenyl-1,2,4,5-tetrazine (2): $^1$H NMR (CDCl$_3$): $\delta$ 8.65 (t, $J = 6.0$ Hz, 4H), 7.65-7.59 (m, 6H). $^{13}$C NMR (CDCl$_3$) $\delta$ 164.0, 132.7, 131.8, 129.3, 128.0.

Preparation of 3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine 9.

3,6-Di(pyridin-2-yl)-1,2,4,5-tetrazine (9): $^1$H NMR (CDCl$_3$): $\delta$ 8.99 (d, $J = 4.8$ Hz, 2H), 8.76 (d, $J = 8$ Hz, 6H), 8.04-7.99 (m, 2H). $^{13}$C NMR (CDCl$_3$) $\delta$ 163.8, 151.0, 150.0, 137.4, 126.5, 124.5.

Preparation of N-isopropyl-4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin3-yl) benzamide 10.
Figure S5. Reagents and conditions for the synthesis of N-isopropyl-4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzamide 10.

4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic acid (19): To a solution of 2-pyrimidinecarbonitrile (935 mg, 8.9 mmol) in 30 mL ethanol, 4-cyanobenzoic acid (1.96 g, 13.3 mmol) was added, followed by addition of hydrazine monohydrate (5.5 mL). The solution was heated under reflux for 20 h with stirring. After cooling to r.t., the solid was collected by filtration and washed with acetone (2 × 50 mL). The dihydrotetrazine without carboxyl group (16) went into acetone. The remaining solid was added acetic acid (10 mL) followed by an aqueous solution of NaNO₂ (2.76 g, 40 mmol) at 10 °C. The purple colored tetrazine was collected and washed with water (3 × 10 mL). The solid was added into nearly boiled DMF and kept at this temperature for another 5 min. The DMF solution was filtered while it was hot. The filtrate, which contained mono-carboxyl substituted product, was collected and dried under vacuum to give a purple product (300 mg, 12%). The benzoic acid tetrazine was used for next step without further purification.

N-Isopropyl-4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzamide (10): To a suspension of 4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic acid
(19, 60 mg, 0.214 mmol) in 5 mL DCM, thionyl chloride (187 µL, 257 mmol) was added. This mixture was refluxed for 20 h with stirring, at which point TLC indicated completion of the reaction. The solution was cooled to r.t. and dried under vacuum. The residue was suspended in dry 3 mL DCM, then propan-2-amine (55 µL, 0.64 mmol) was added and the resulting mixture was stirred at r.t. for 4 h. DCM was removed in vacuum and the reaction mixture was purified by silica gel column chromatography (DCM: MeOH, 20:1) to give a purple solid product (40 mg, 59 %).

$^1$H NMR (CDCl$_3$): $\delta$ 9.14 (d, $J = 2.0$ Hz, 2H, 4-Pyr-H, 6-Pyr-H), 8.78 (d, $J = 4.0$ Hz, 2H, 2’-Ph-H, 6’-H), 8.00 (d, $J = 4.0$ Hz, 2H, 3’-Ph-H, 5’-Ph-H), 7.60 (br, 1H, 5-Pyr-H), 6.17 (d, $J = 4.0$ Hz, 1H, -NH), 4.34-4.32 (m, 1H, -CH-NH-), 1.32 (s, 3H, -CH$_3$), 1.31 (s, 3H, -CH$_3$); $^{13}$C NMR (CDCl$_3$) $\delta$ 165.7, 164.0, 163.1, 159.4, 158.4, 139.2, 133.7, 128.9, 127.8, 122.6, 42.2, 22.8. MS calcd. For C$_{16}$H$_{15}$N$_7$O [M+H]$^+$ 322.1, found 322.3

**General procedure for strain-promoted inverse electron demand Diels-Alder reactions with 1,2,4,5-tetrazine.**

To a solution of tetrazine (2, 9, 10) in CH$_2$Cl$_2$ (1 mL), alkyne (1, 4) in CH$_2$Cl$_2$ (1 mL) was added. Reactions were stirred at room temperature for 5 to 30 min. The progress of the reaction was monitored by TLC. Upon completion, the reaction mixture was directly loaded on the flash column chromatography for purification.

**Characterization Data for cyclization products**

**Compound 3**

![Structure](image)

Purified by eluting with DCM:MeOH (10:1, $R_f = 0.65$) (white solid, 91%). $^1$H NMR (CDCl$_3$) $\delta$ 7.55-7.47 (m, 10H, Ph-H), 2.83-2.82 (m, 4H, -CH$_2$-C=C-), 1.60 (br, 4H, -CH$_2$-), 1.43 (br, 4H, -CH$_2$-). $^{13}$C NMR (CDCl$_3$) $\delta$ 161.1, 139.0, 138.2, 129.1, 128.3, 128.2, 30.3, 27.1, 25.9. MS calcd. For C$_{22}$H$_{22}$N$_2$ [M+H]$^+$ 315.2, found 315.3

**Compound 5**

![Structure](image)

Purified by eluting DCM:MeOH (10:1, $R_f = 0.55$) (white solid 88%). $^1$H NMR (CDCl$_3$) $\delta$ 7.48-7.41 (m, 10H, Ph-H), 3.63 (d, $J = 3.6$ Hz, 2H, -CH$_2$-OH), 2.93-2.85 (m, 2H, -CH$_2$-C=C-), 2.75 (br, 2H, -CH$_2$-C=C-), 2.50 (br, 1H, -CH-CH$_2$OH), 2.18-2.15 (br, 2H, -C-CH-C-), 1.43-1.42 (br, 2H, -CH$_2$-), 1.13 (br, 2H, -CH$_2$-). $^{13}$C NMR (CDCl$_3$) $\delta$ 160.8, 140.5, 137.9, 129.2, 128.4, 128.2, 58.8, 28.2, 24.3, 22.9, 20.4. MS calcd. For C$_{26}$H$_{23}$NO$_3$ [M+H]$^+$ 357.2, found 357.3.
**Compound 21**

![Image of Compound 21]

Purified by eluting DCM:MeOH (10:1, \( R_f = 0.54 \)) (white solid 90%). \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 8.66 (d, \( J = 2.2 \) Hz, 2H, 6-Py-H), 7.84-7.79 (m, 4H, 3-Py-H, 4-Py-H), 7.33-7.30 (m, 2H, 5-Py-H), 3.01-2.98 (m, 4H, -CH\(_2\)-C=C-), 1.72 (br, 4H, -CH\(_2\)-), 1.38 (br, 4H, -CH\(_2\)-). \(^1\)C NMR (CDCl\(_3\)) \( \delta \) 158.7, 156.9, 148.4, 140.9, 136.6, 124.8, 123.1, 30.3, 26.4, 25.9. MS calcd. For C\(_{20}\)H\(_{20}\)N\(_4\) [M+H]\(^+\) 317.2, found 317.2

**Compound 22**

![Image of Compound 22]

Purified by eluting DCM:MeOH (10:1, \( R_f = 0.35 \)) (white solid 89%). \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 8.72 (d, \( J = 2.0 \) Hz, 2H, 6-Py-H), 7.95-7.85 (m, 4H, 3-Py-H, 4-Py-H), 7.39-7.36 (m, 2H, 5-Py-H), 3.72 (d, \( J = 3.6 \) Hz, 2H, -CH\(_2\)-C-OH), 3.06-3.05 (m, 4H, -CH\(_2\)-C=C-), 2.39-2.36 (br, 2H, -CH\(_2\)-), 1.62 (br, 2H, -CH\(_2\)-), 1.10 (br, 3H, -CH\(_3\), -CH\(_2\)-). \(^1\)C NMR (CDCl\(_3\)) \( \delta \) 159.1, 157.0, 148.7, 142.2, 136.8, 125.0, 123.3, 59.5, 27.8, 24.4, 22.7, 19.7. MS calcd. For C\(_{22}\)H\(_{22}\)N\(_4\)O [M+H]\(^+\) 359.2, found 359.3.

**Compound 23**

![Image of Compound 23]

Purified by eluting DCM:MeOH (10:1, \( R_f = 0.32 \)) (white solid 82%). \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 8.94 (d, \( J = 2.4 \) Hz, 2H, 4-Pyr-H, 6-Pyr-H), 7.86 (d, \( J = 4.2 \) Hz, 2H, 6’-Ph-H), 7.53 (d, \( J = 4.0 \) Hz, 2H, 3’-Ph-H, 5’-Ph-H), 7.42 (t, \( J = 5.0 \) Hz, 1H, 5-Pyr-H), 6.25 (d, \( J = 3.8 \) Hz, 1H, -NH), 4.35-4.26 (m, 1H, -NH-CH-), 2.99-2.89 (m, 2H, -CH\(_2\)-C=O), 2.32-2.31 (br, 1H, -CH\(_2\)-), 2.19-2.17 (br, 1H, -CH\(_3\)-), 1.84 (br, 1H, -CH\(_3\)-), 1.59-1.56 (br, 2H, -CH\(_2\)-), 1.29 (s, 3H, -CH\(_3\)), 1.27 (s, 3H, -CH\(_3\)), 1.15 (br, 2H, -CH\(_2\)-). \(^1\)C NMR (CDCl\(_3\)) \( \delta \) 166.4, 164.8, 161.0, 158.0, 157.2, 141.2, 141.0, 140.5, 135.2, 129.4, 127.0, 120.4, 59.2, 42.0, 28.0, 24.1, 22.8, 21.0, 19.7, MS calcd. For C\(_{26}\)H\(_{29}\)N\(_5\)O\(_2\) [M+H]\(^+\) 444.2, found 444.3.

**Kinetics measurements of 2, 9 and 10 with 1 or 4.**

UV/Vis kinetic measurements: Separate solutions of pure tetrazines 2, 9, 10 and pure alkynes 1, 4 (>95-98% by 1H-NMR) were prepared in HPLC-grade MeOH at 297 K. The stability of 2, 9, and 10 in MeOH was examined by monitoring its absorption maximum at 295 nm. Solutions containing 2, 9, 10 (25 uM) and 10-18 fold excess of an alkyne (1 or 4) were pipetted into quartz cuvettes for UV measurements and thoroughly mixed. All kinetic runs were triple triplicates. Curve fitting was operated in Prism3 software.

A) Tetrazine 2 with alkyne 1
**Figure S6.** Ph-tetrazine 25 μM, alkyne 250 μM, 300 μM, 350 μM, 400 μM, 450 μM.

B) Tetrazine 9 with alkyne 1

\[
k_1 = (3.15 \pm 0.3) \times 10^{-5} \text{ s}^{-1}
\]

\[
k_2 = k_1 / 450 \text{ μM} = (7.0 \pm 0.7) \times 10^{-2} \text{ M}^{-1}\text{s}^{-1}
\]

**Figure S7.** Py-tetrazine 25 μM, alkyne 250 μM, 300 μM, 350 μM, 400 μM, 450 μM,

Second order rate constant \(k_2 = 2.0 \pm 0.3 \text{ M}^{-1}\text{s}^{-1}\)

C) Tetrazine 2 with alkyne 4
**Figure S8.** Ph-tetrazine 25 μM, alkyne 250 μM, 300 μM, 400 μM, 450 μM, Second order rate constant $k_2 = 3.3 \pm 0.4 \text{ M}^{-1}\text{s}^{-1}$

D) Tetrazine 9 with alkyne 4

**Figure S9.** Py-tetrazine 25 μM, alkyne 250 μM, 300 μM, 400 μM, 450 μM, Second order rate constant $k_2 = 44.8 \pm 4.9 \text{ M}^{-1}\text{s}^{-1}$

E) Tetrazine 10 with alkyne 4
IsoP-tetrazine click reaction
Second order rate constant

Figure 10 IsoP-tetrazine 25 uM, alkyne 250 uM, 300 uM, 350 uM, 400 uM, 450 uM,
Second order rate constant $k_2 = 40.9 \pm 13.8 \text{ M}^{-1}\text{s}^{-1}$

Computational detail
All calculations were performed using the Gaussian 03 program. Initial geometry optimizations
were carried out at the AM1 semiempirical level. DFT calculations were carried out with use of
the B3LYP with the standard 6-31G** basis set. The stationary points were characterized by
frequency calculations to verify that TSs had one and only one imaginary frequency.

Unique imaginary frequencies

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<th>TS2</th>
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<td>$-113.1305 \text{ cm}^{-1}$</td>
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<tr>
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<tr>
<td>$-265.2117 \text{ cm}^{-1}$</td>
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References

$^1$H and $^{13}$C Spectra
Ph-tetrazine-1H

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EXPNO 3
PROCNO 1

F2 - Acquisition Parameters
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Time_ 18.46
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PULPROG zg30
TD 65536
SOLVENT CDC13
NS 16
DS 2
SWH 8223.685 Hz
FIDRES 0.125483 Hz
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RG 203
DW 60.800 usec
DE 6.50 usec
TE 298.1 K
D1 1.0000000 sec

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P1 13.50 usec
PLW1 16.0000000 W
SFO1 400.1424710 MHz

F2 - Processing parameters
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WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
Ph-tetrazine-C13

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**Current Data Parameters**

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**EXPNO**  4
**PROCNO**  1

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**CHANNEL f2**

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**F2 - Processing parameters**

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Py-tetrazine-C13
DZ-III-80-Ph-tetrazine-alkyne with 3 ring-1H
DZ-III-79-isop-tetrazine-alkyne with 3 ring-1H