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# SUPPORTING INFORMATION

## A Click-and-Release Approach to CO Prodrugs

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## Materials and Methods

All reagents and solvents were reagent grade. Column chromatography was carried out on flash silica gel (Sorbent 230–400 mesh) and P-2 Gel (Bio-Gel, particle size range 45-90 μM). TLC analysis was conducted on silica gel plates (Sorbent Silica G UV254). NMR spectra were recorded at 400 MHz for $^{1}H$ and 100 MHz for $^{13}C$ on an Avance Bruker instrument. Chemical shifts (δ values) and coupling constants (*J* values) are given in ppm and hertz, respectively, using solvents ($^{1}H$ NMR, $^{13}C$ NMR) as the internal reference. Compounds **1** and **13** were commercially available. Compounds **2,**[1] **9,**[2] **11,**[3] and **14**[3] were synthesized according to literature procedures.

### Inverse electron demand Diels-Alder reaction between 2,3,4,5-tetraphenylcyclopenta-2,4-dienone (TPCPD, 1) and *exo*-bicyclo[6.1.0]non-4-yn-9-ylmethanol (*exo*-BCN, 2)

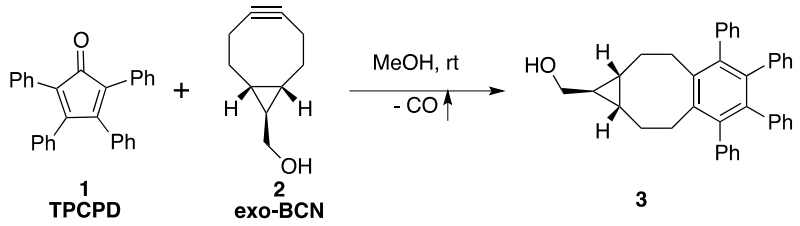



***Fig. S1.*** Inverse electron demand Diels-Alder reaction between TPCPD (**1**) and exo-BCN (**2**)

To a solution of TPCPD (**1,** 0.5 mmol) in $CH_2Cl_2$ (0.5 mL), *exo*-BCN (**2,** 0.5 mmol) in $CH_2Cl_2$ (0.5 mL) was added. Reaction mixture was stirred at room temperature for 5 min. The progress of the reaction was monitored by TLC (hexane/ethyl acetate 1:1, $R_{fproduct}$ = 0.4). Upon completion, the reaction mixture was directly loaded on the flash column for chromatography (eluted by hexane/ethyl acetate 10:1) to give white solid product (237 mg, yield: 94%). $^{1}H$ NMR ($CDCl_3$): δ 7.17 – 7.03 (m, 10H), 6.82 – 6.70 (m, 10H) 3.45 (d, *J* = 4.0 Hz, 2H), 2.84 – 2.77 (m, 2H), 2.70 – 2.66 (br, 2H), 2.23-2.22 (br, 2H), 1.26 (br, 2H), 0.89 – 0.87 (br, 2H), 0.79 – 0.77 (br, 1H). $^{13}C$ NMR ($CDCl_3$): δ 141.7, 140.9, 140.7, 140.3, 138.6, 131.4, 131.0, 130.5, 130.4, 127.2, 127.1,126.4, 126.2, 125.8, 124.9, 66.6, 30.7, 29.9, 22.6, 21.5. MS calcd. for $C_{38}H_{34}O$ $[M+H]^+$ 506.2610, found 506.2614.

**Synthesis of TPCPD-M (7)**

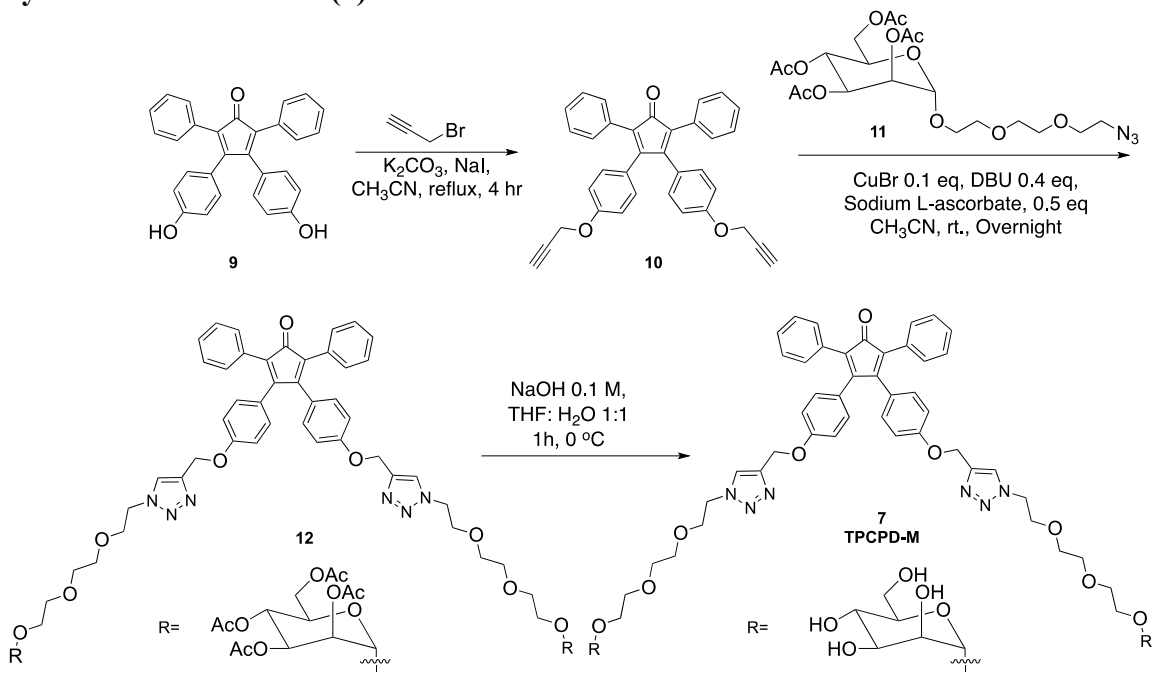



***Fig. S2.*** Preparation of TPCPD-M (**7**)

**2,5-Diphenyl-3,4-bis(4-(prop-2-yn-1-yloxy)phenyl)cyclopenta-2,4-dienone (10)**

To a 10 mL reaction tube equipped with a stir bar, 3,4-bis(4-hydroxyphenyl)-2,5-diphenylcyclopenta-2,4-dienone (**9,** 50 mg, 0.12 mmol) in $CH_3CN$ (2 mL), propargyl bromide (179 mg (80 wt.% in toluene), 1.2 mmol), $K_2CO_3$ (50 mg, 0.36 mmol), and NaI (1.8 mg, 0.012 mmol) were added. The vessel was sealed and the mixture was stirred in an oil bath at 80 ºC for 2 hours. The progress of the reaction was monitored by TLC (hexane/ethyl acetate 8:1, $R_{fproduct}$ = 0.5). Upon completion, the seal was removed and the reaction solution was cooled to room temperature. The reaction mixture was filtered. The filtrate was collected and dried under vacuum to give a crude product. The crude product was directly loaded on the flash column for chromatography (eluted by hexane/ethyl Acetate 10:1) to give dark brown solid product (50 mg, yield: 84%). $^1$H NMR ($CDCl_3$): δ 7.27 (br, 10H), 6.89 (d, *J* = 8.0 Hz, 4H), 6.81 (d, *J* = 8.0 Hz, 4H) 4.68 (m, 4H), 2.56 (S, 2H), $^{13}$C NMR ($CDCl_3$): δ 200.1, 157.8, 153.7, 131.1, 131.0, 130.1, 128.0, 127.3, 126.1, 124.9, 114.3, 78.1, 15.7, 55.8. MS calcd. For $C_{35}H_{24}O_3$ $[M+H]^+$ 493.1804, found 493.1807.

**(2R,3R,5R,6S)-2-(Acetoxymethyl)-6-(2-(2-(2-(4-((4-(3-oxo-2,4-diphenyl-5-(4-((1-(2-(2-(2-(((2R,3S,5S,6S)-3,4,5-triacetoxy-6-(acetoxymethyl)tetrahydro-2H-pyran-2-yl)oxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)cyclopenta-1,4-dien-1-yl)phenoxy)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (12)**

To a solution of **10** (50 mg, 0.1 mmol) in 1 mL $CH_3CN$, compound **11** (113 mg, 0.22

mmol) was added, followed by the addition of CuI (0.1 eq.), DBU (0.4 eq.) and sodium ascorbate (0.5 eq.). Then the solution was stirred at room temperature overnight. The progress of the reaction was monitored by TLC (hexane/ethyl acetate 2:1, $R_{fproduct}$ = 0.4) Upon completion, the reaction mixture was directly loaded on the flash column for chromatography (eluted by hexane/ethyl acetate 4:1) to give dark brown solid product. (98 mg, yield: 65%) $^1$H NMR ($CDCl_3$): δ 7.82 (s, 2H, NH), 7.22 (br, 10H), 6.85 (d, *J* = 8.0 Hz, 4H) 6.80 (d, *J* = 8.0 Hz, 4H), 5.34 – 5.24 (m, 8H), 5.14 (s, 4H), 4.85 (s, 2H), 4.58 – 4.55 (m, 4H), 4.27 – 4.23 (m, 2H), 4.10 – 4.03 (m, 4H), 3.91 – 3.88 (m, 4H), 3.80 – 3.77 (m, 2H), 3.647 – 3.60 (m, 4H), 2.12 (s, 6H), 2.07 (s, 6H), 2.01 (s, 6H), 1.96 (s, 6H). $^{13}$C NMR ($CDCl_3$): δ 199.7, 170.6, 160.0, 169.9, 169.6, 158.6, 153.8, 143.3, 131.1, 131.0, 130.0, 127.9, 127.2, 125.7, 124.7, 124.03, 114.1, 97.6, 77.3, 77.0, 76.7, 70.6, 70.5, 69.9, 69.5, 69.4, 69.0, 68.40, 67.3, 66.0, 62.3, 61.8, 50.3, 20.8, 20.7, 20.6. MS calcd. For $C_{75}H_{86}N_6O_{27}$ $[M+H]^+$ 1503.5619, found 1503.5627.

**2,5-Diphenyl-3-(4-((1-(2-(2-(2-(((2R,3S,5R,6S)-3,4,5-trihydroxy-6 (hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)ethoxy)ethoxy)ethyl)-1H-1,2,3 triazol-4-yl)methoxy)phenyl)-4-(4-((1-(2-(2-(2-(((2S,3R,5S,6R)-3,4,5-trihydroxy-6 (hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)ethoxy)ethoxy)ethyl)-1H-1,2,3 triazol-4-yl)methoxy)phenyl)cyclopenta-2,4-dienone (7, TPCPD-M)**

To a solution of **12** (50 mg, 0.033 mmol) in 0.5 mL THF cooled to 0 °C, NaOH aqueous solution (0.2 M, 0.5 mL) was added dropwise. Then this mixture was stirred for 1 h at 0 °C. The progress of the reaction was monitored by TLC (hexane/ethyl acetate 2:1, starting material **12,** $R_f$ = 0.2). Upon completion, $H^+$ resin was added to adjust the pH to 7. The reaction mixture was filtered. The filtrate was collected and dried under vacuum to give a crude product. The crude product was directly loaded on P2 column for chromatography (eluted by $H_2O$) to give dark brown solid product. (34 mg, yield: 90% after lyophilization). $^1$H NMR ($CD_3OD$): δ 8.12 (s, 2H), 7.24 – 7.18 (m, 8H), 6.87 (br, 10H), 5.14 (s, 4H), 4.78 (m, 3H), 4.61 – 4.59 (m, 5H), 3.91 – 3.89 (m, 5H), 3.82 – 3.77 (m, 8H), 3.72 – 3.68 (m, 6H), 3.67 – 3.35 (m, 23H). $^{13}$C NMR ($CD_3OD$) δ 197.4, 160.2, 155.8, 144.5, 132.6, 132.3, 131.3, 129.0, 128.4, 127.1, 126.3, 126.2, 115.5, 101.7, 74.6, 72.6, 72.1, 71.6, 71.5, 71.4, 70.4, 68.6, 67.7, 62.9, 62.4, 51.5, 49.6, 49.4, 49.2, 49.0, 48.8, 48.6, 48.4. MS calcd. For $C_{59}H_{70}N_6O_{19}$ $[M-H]^+$ 1165.4617, found 1165.4538.

**Synthesis of BCN-M (8)**

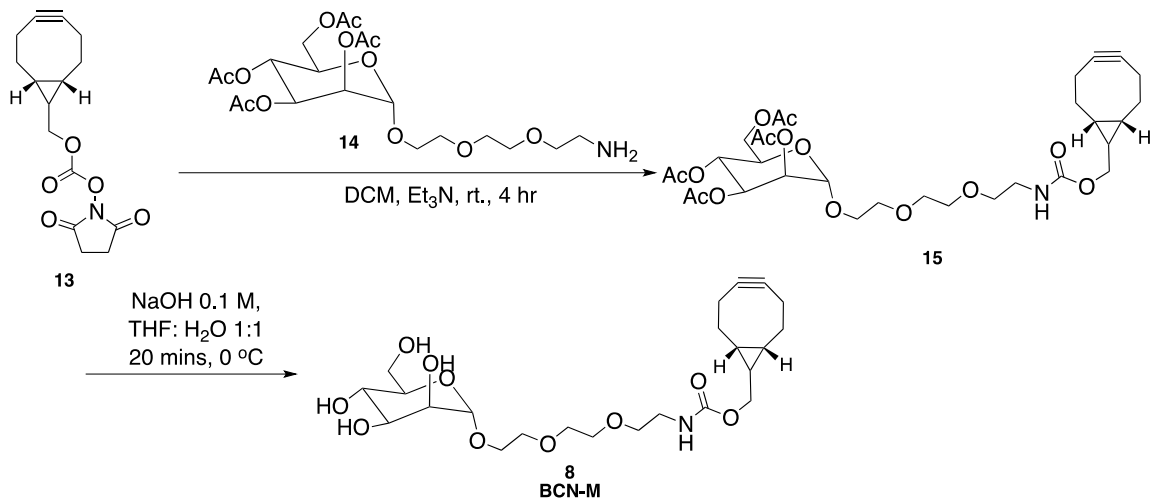



***Fig. S3***. Preparation of BCN-M (**8**)

**(2R,3R,4S,5R,6S)-2-(Acetoxymethyl)-6-((1-(bicyclo[6.1.0]non-4-yn-9-yl)-3-oxo-2,7,10-trioxa-4-azadodecan-12-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (15)**

To a solution of *endo*-bicyclo[6.1.0]non-4-yn-9-ylmethyl (2,5-dioxopyrrolidin-1-yl) carbonate (**13**, 50 mg, 0.17 mmol) in 2 mL DCM, compound **14** (123 mg, 0.25 mmol) was added 1 mL DCM, followed by the addition of $Et_3N$ (52 mg, 0.52 mmol). This mixture was stirred at rt for 4 h. The progress of the reaction was monitored by TLC (hexane/ethyl acetate 1:1, $R_{f\text{product}}$ = 0.2). Upon completion, the reaction mixture was directly loaded on a flash column for chromatography (hexane/ethyl acetate 2:1) to give colorless oil product. (111 mg, yield: 68 %). 1H NMR ($CDCl_3$): δ 5.37 – 5.15 (m, 8H), 4.84 (d, *J* = 15.7 Hz, 2H), 4.27 (dd, *J* = 12.2, 4.8 Hz, 2H), 4.17 – 4.00 (m, 4H), 3.80 (dd, *J* = 12.2 Hz, 7.4 Hz, 1H), 3.75 – 3.56 (m, 8H), 3.54 (t, *J* = 5.0 Hz, 2H), 3.36 (d, *J* = 4.7 Hz, 2H), 2.29 – 2.16 (m, 4H), 2.13 (d, *J* = 7.3 Hz, 3H), 2.08 (s, 3H), 2.02 (s, 3H), 1.97 (s, 3H), 1.56 (d, *J* = 10.3 Hz, 2H), 1.33 (dd, *J* = 17.7, 7.7 Hz, 1H), 0.90 (dd, *J* = 22.0, 12.4 Hz, 2H). MS calcd. For $C_{31}H_{45}NO_{14}$ $[M+H]^+$ 656.2918, found 656.2922.

***endo*-Bicyclo[6.1.0]non-4-yn-9-ylmethyl (2-(2-(2-(((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)ethoxy)ethoxy)ethyl)carbamate (8, BCN-M)**

To a solution of **15** (50 mg, 0.076 mmol) in 0.5 mL THF cooled to 0 °C, NaOH aqueous solution (0.2 M, 0.5 mL) was added dropwise. Then this mixture was stirred for 20 min at 0 °C. The progress of the reaction was monitored by TLC (hexane / ethyl acetate 1:1, $R_{f\text{product}}$ = 0.4). Upon completion, $H^+$ resin was added to adjust the pH to 7. The reaction mixture was filtered, and the filtrate was collected and dried under vacuum to give a crude product. The crude product was directly loaded on a P2 column for chromatography (eluted by $H_2O$) to give dark brown solid product. (25 mg, yield: 70% after lyophilization). $^1$H NMR ($D_2O$): δ 4.23 (d, *J* = 7.8 Hz, 2H), 4.04 – 3.96 (m, 1H), 3.95 – 3.82 (m, 2H), 3.82 – 3.60 (m, 8H), 3.37 (d, *J* = 6.5 Hz, 2H), 2.28 (dd, *J* = 24.4 Hz,

12.4 Hz, 4H), 1.63 (d, $J$ = 10.6 Hz, 2H), 1.43 (d, $J$ = 8.7 Hz, 1H), 1.10 – 0.93 (m, 2H). MS calcd. For $C_{23}H_{37}NO_{10}$ $[M-H]^+$ 486.2339, found 486.2342.

**Kinetic studies of the cycloaddition reaction**

UV/Vis kinetic measurements: Separate solutions of TPCPD (**1**) and *exo*-BCN (**2**) (>95-98% by 1H-NMR) were prepared in HPLC-grade solvent (methanol, acetonitrile, 1,2-dichloroethane, or dioxane) at room temperature. The reaction between **1** (200 μM) and **2** (5 mM) leads to significant changes in the UV-vis spectrum of TPCPD (**1**). For kinetic studies, the solutions containing TPCPD (**1**, 50 μM, 400 μL) and *exo*-BCN (**2**, 900 μM, 400 μL) were added into quartz cuvettes, thoroughly mixed and sealed with a PTFE cap. All kinetic runs were in triplicates. Curve fitting was operated using the Prism5 software.

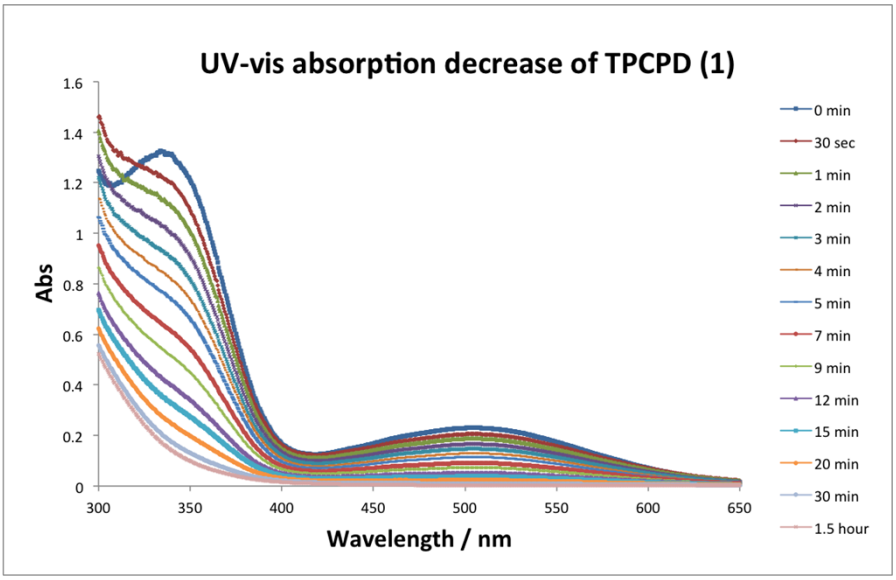



***Fig. S4***. Reaction between TPCPD and BCN. UV-vis absorption decreases of TPCPD (**1**). TPCPD: 200 μM, *exo*-BCN: 5 mM in dried MeOH.

**Kinetic study based on solvent effect**

***SI-Table 1.*** Second order rate constants of TPCPD with BCN in different solvents.

| **Solvent** | $H_2O$** **(7 + 8)** 37 °C | MeOH* **1 + 2** | | $CH_3CN$* **1 + 2** | DCE* **1 + 2** | Dioxane* **1 + 2** |
|---|---|---|---|---|---|---|
| | | r.t. | 37 °C | r.t. | r.t. | r.t. |
| **Half life** | 38 min | 55 min | 23 min | 99 min | 130 min | 282 min |
| $k_2$ ($M^{-1}s^{-1}$) | 0.61± 0.003 | 0.50± 0.01 | 1.1± 0.002 | 0.26± 0.004 | 0.17± 0.003 | 0.09± 0.001 |

* all half-life were determined at 450 μM of *exo*-BCN (**2**).
** all half-life were determined at 500 μM of BCN-M (**8**).

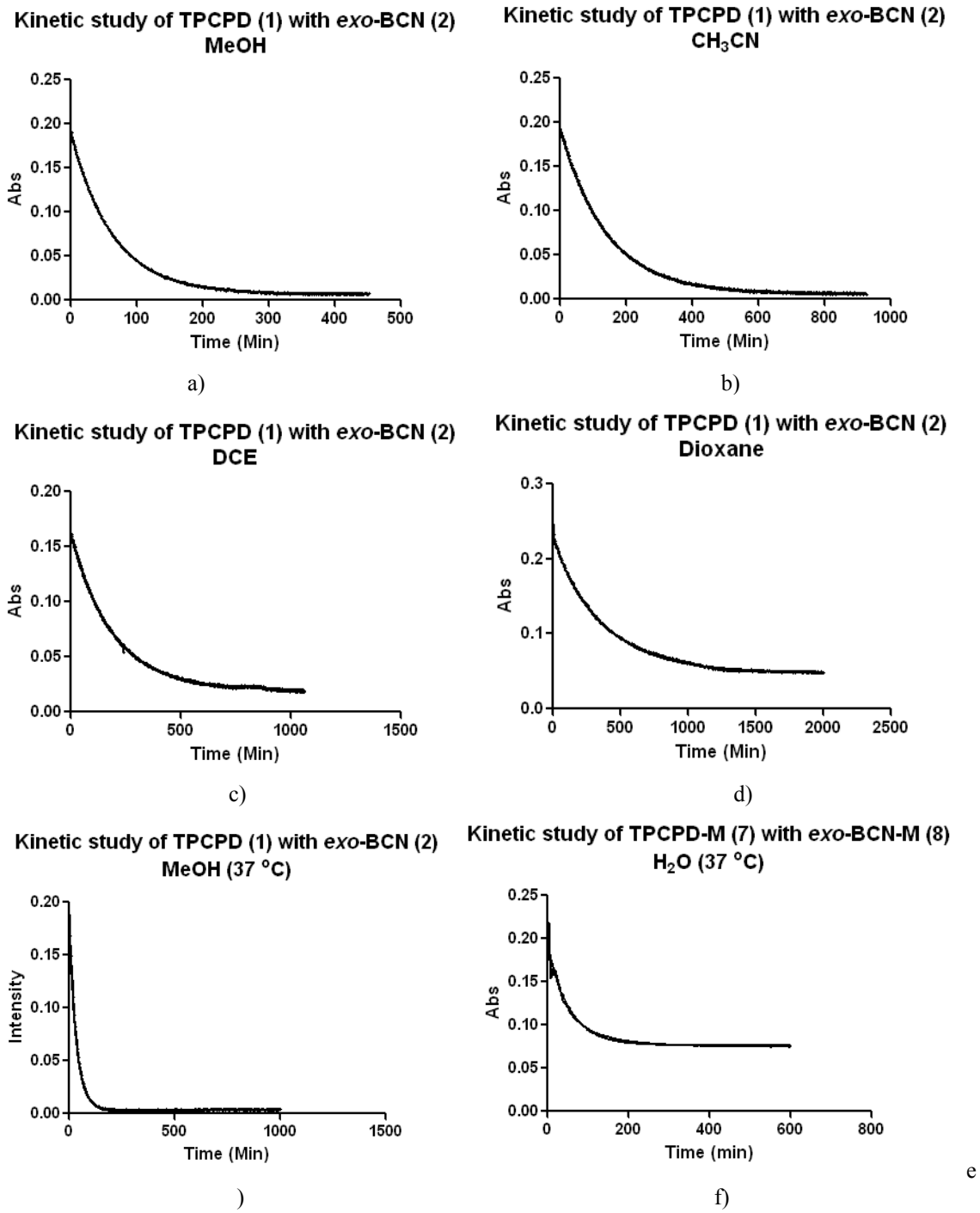



***Fig. S5.*** Pseudo-first kinetic studies of the cycloadition between TPCPD (25 μM) and *exo*-BCN (450 μM).
a) Methanol ($t_{1/2}$ = 55 min ± 4 min)
b) Acetonitrile ($t_{1/2}$ = 99 min ± 3 min)
c) 1,2-Dichloroethane ($t_{1/2}$ = 129 min ± 2 min)
d) Dioxane ($t_{1/2}$ = 282 ± 7 min)
e) Methanol (37 °C, $t_{1/2}$ = 23 min ± 1 min)
f) $H_2O$ (37 °C, $t_{1/2}$ = 38 min ± 1 min, TPCPD-M (25 μM) and *exo*-BCN-M (500 μM))

**Monitoring the release CO by using myoglobin[4]**

**Preparation of fresh stock solutions of deoxy-myoglobin (Mb).** A stock solution of myoglobin (lyophilised horse heart) (Sigma) (20 μM final concentration) was prepared fresh by dissolving the protein in phosphate buffered saline (PBS) (0.01 M, pH = 7.4) (Sigma). Sodium dithionite (0.1% final concentration, w/v) was added to convert the myoglobin stock to deoxy-Mb.

**Monitoring of the CO released from the cycloaddition reaction.** Separate solutions of TPCPD (**1**) and *exo*-BCN (**2**) (>95-98% by 1H-NMR) were prepared in HPLC-grade DMSO at room temperature. The solutions containing TPCPD (**1**, 1mM (stock solution), 200 μL) and *exo*-BCN (**2**, 100 mM (stock solution), 20 μL) were added into a deoxy-myoglobin solution (1.8 mL, conc. = 24 μM) in a quartz cuvette. After thorough mixing, 400 μL light mineral oil was added to the cuvette, which was then sealed with a PTFE cap.

Significant spectroscopic changes were observed during reaction indicating CO release (Fig. S5). Specifically, the maximal absorption peak of deoxy-Mb at 560 nm is converted to two maximal absorption peaks of Mb-CO at 540 and 578 nm. The final concentration was 100 μM for TPCPD and 1 mM for BCN.

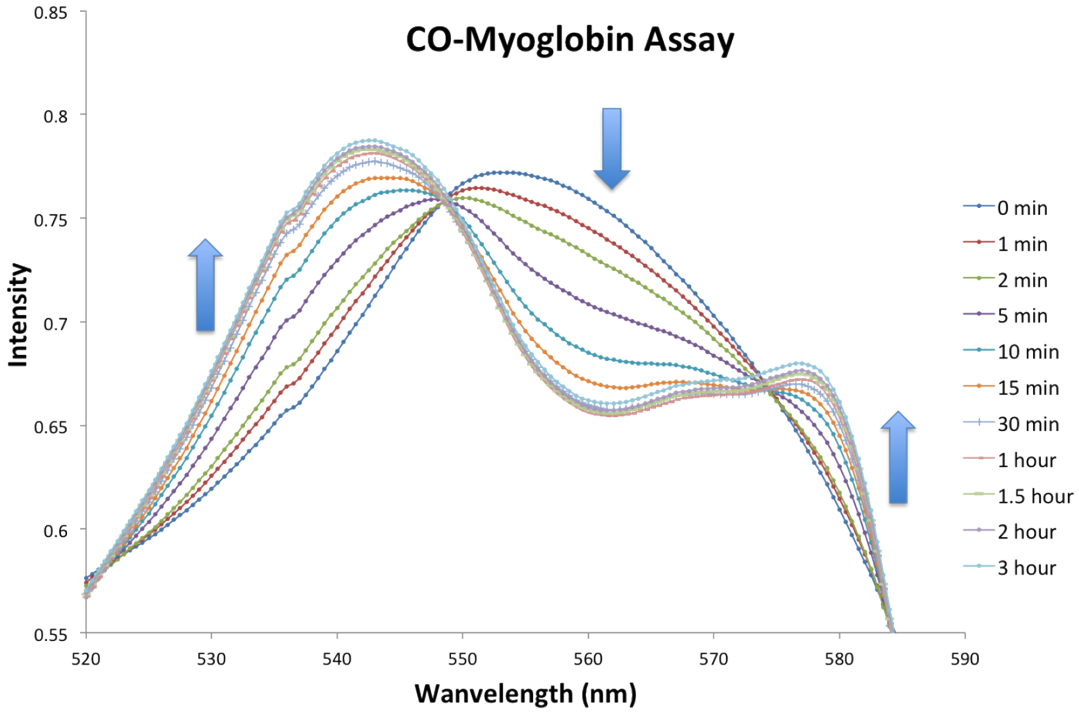
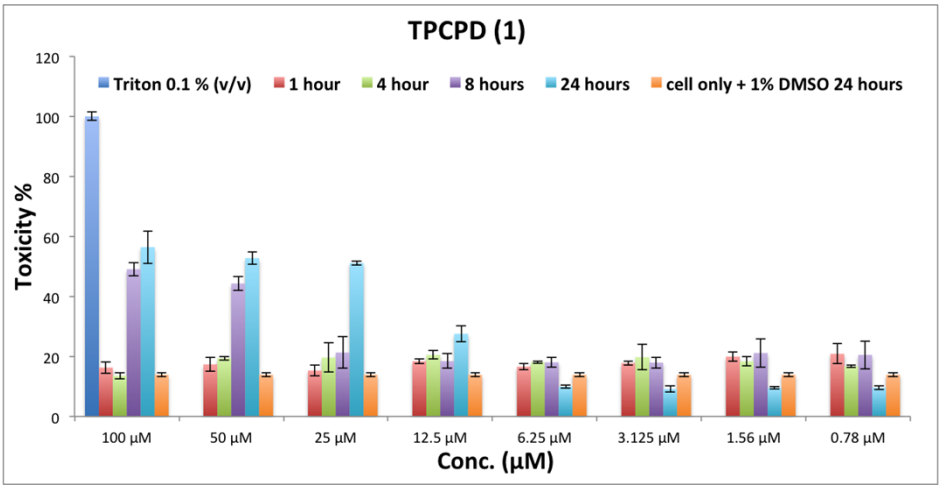



***Fig.S6***. UV–Vis spectra demonstrating the conversion of deoxy-myoglobin (Mb) to carbon monoxide myoglobin (MbCO) caused by the binding of CO released from the click reaction. Conditions: 20 μM Mb 2 mL (concentration determined by UV540 nm, the absorption coefficient for MbCO at 540 nm ($\varepsilon_{max}$ = 15400 $M^{-1}cm^{-1}$)—literature reported standard[5]) 0.1 % $Na_2S_2O_4$, 200 μL TPCPD stock (1 mM DMSO) final conc. 100 μM, 20 μL *exo*-BCN stock (100 mM DMSO) final conc. 1 mM, Solvent: 10% DMSO PBS buffer (1X PBS buffer, pH = 7.4)

**Cytotoxicity test**[6]

The WST-1 assay was performed with the following exposure time: 1, 4, 8 and 24 h. RAW 264.7 cells were seeded in 96-well plates and incubated overnight at 37 ºC. Different concentrations of each compound have been assessed in the range of 0.78 to 100 µM for **1** and **3** and 7.8 to 1000 µM for **2**. Triton 0.1% was used as a positive control of cytotoxicity and the subsequent value was established as 100% of cytotoxicity. WST-1 proliferation reagent was added to well after specific exposure time and incubated for 4 h at 37 ºC. The wavelength for measuring absorbance of the formazan product was 440 nm.



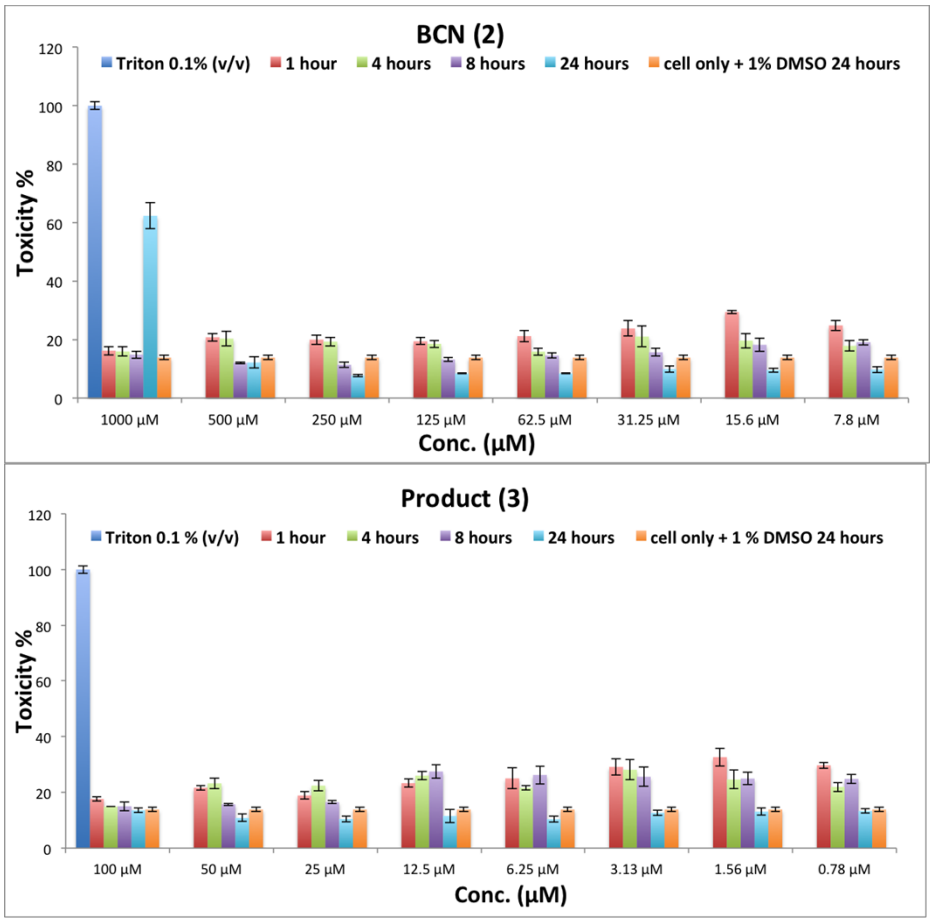



***Fig. S7.*** Cytotoxicity test of TPCPD (**1**), exo-BCN (**2**), and product (**3**)

RAW 264.7 cell line was seeded in 96-well plates for overnight at 37 °C. Compounds **1**, **2**, **3**, **7**, **8** and CORM-3 at same concentration (1 mM, **1**, **2**, and **3** dissolved in DMSO, **7**, **8**, and CORM-3 dissolved in DI-$H_2O$) were added to wells and incubated 24 h. Then, WST-1 proliferation reagent was added to well and incubated for 4 h at 37 °C. The wavelength for measuring absorbance of the formazan product was 440 nm.

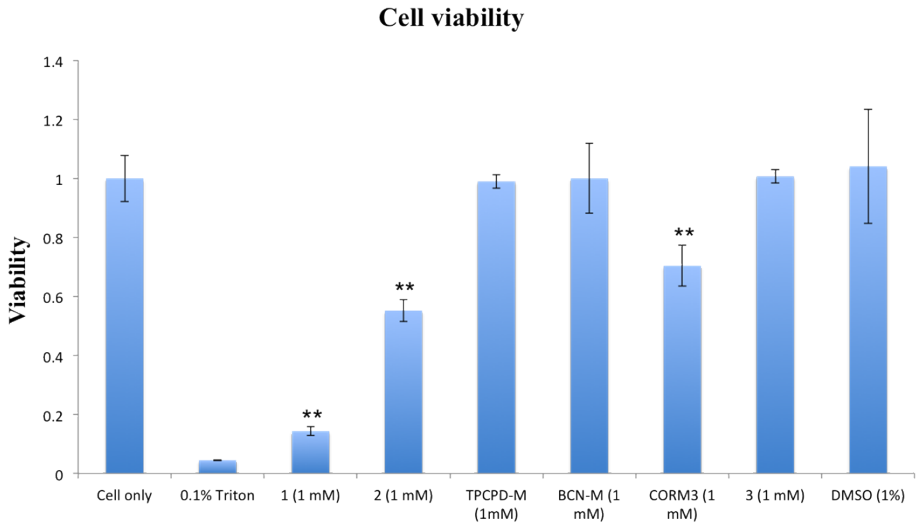



***Fig. S8.*** Cell viability test of TPCPD, TPCPD-M (**1** and **7**), BCN, BCN-M (**2** and **8**), product and CORM3. * Indicates $P < 0.01$ vs control (cell only).

## In vitro anti-inflammatory effects of on macrophage Raw264.7 [6]

RAW264.7 cells were seeded in gas permeable 96-well plates and incubated overnight at 37 °C. Cells were stimulated with LPS (10 ng/mL) for 1 h. TPCPD-M (**7**) and BCN-M (**8**) (1 mM) were added to a separate standard 96-well plates and attached to the bottom of the gas permeable plate. The gas permeable membrane will only allowed the CO but not compounds permeate and contacted with cell. Gas permeable membrane prevented the potential inflammation effect of compounds themselves; it also prevented compounds themselves lead to any inhibition of the LPS-induced accumulation of TNF-α. . As controls, **7** and **8** were examined at the same concentration. TNF-α secretion in the medium was measured with an eBioscience kit (mouse TNF-α ELISA kit, eBioscience, San Diego, CA, USA).

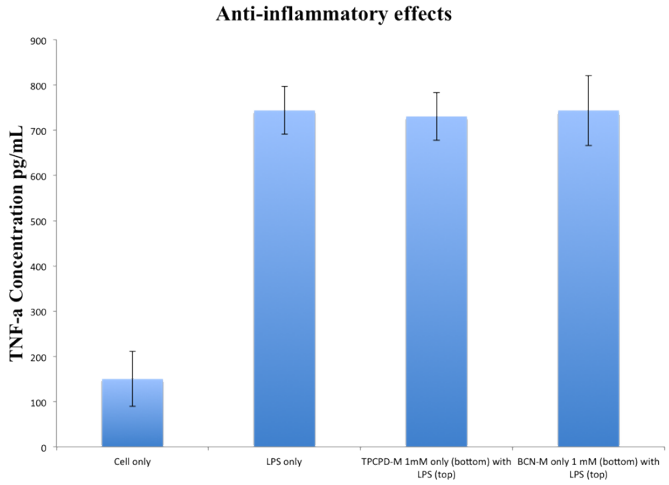



***Fig. S9.*** Anti-inflammatory effects of TPCPD-M only and BCN-M only after treatment with LPS 1h.

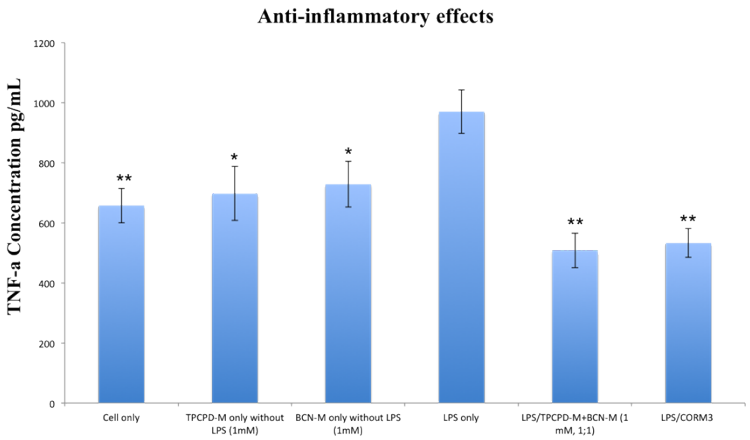



***Fig. S10.*** Anti-inflammatory effects of CO released from the $DAR_{inv}$ click reaction.
* Indicates $P < 0.05$ vs LPS only; ** indicates $P<0.01$ vs LPS only.

## References:


[1] J. Dommerholt, S. Schmidt, R. Temming, L. J. Hendriks, F. P. Rutjes, J. van Hest, D. J. Lefeber, P. Friedl, F. L. van Delft, *Angew. Chem.Int. Ed. Engl.* **2010**, *49*, 9422-9425.

[2] K. J. Thomas, M. Velusamy, J. T. Lin, C. Chuen, Y.-T. Tao, *J. Mater. Chem.* **2005**, *15*, 4453-4459.

[3] M. Yin, K. Ding, R. A. Gropeanu, J. Shen, R. d. Berger, T. Weil, K. Müllen, *Biomacromolecules* **2008**, *9*, 3231-3238.

[4] G. T. Noble, S. L. Flitsch, K. P. Liem, S. J. Webb, *Org. Biomol. Chem.* **2009**, *7*, 5245-5254.

[5] R. Motterlini, J. E. Clark, R. Foresti, P. Sarathchandra, B. E. Mann, C. J. Green, *Circ. Res.* **2002**, *90*, e17-e24.

[6] H. Laroui, A. L. Theiss, Y. Yan, G. Dalmasso, H. T. Nguyen, S. V. Sitaraman, D. Merlin, *Biomaterials* **2011**, *32*, 1218-1228.