Optimized Aqueous Kinugasa Reactions for Bioorthogonal Chemistry Applications

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Materials and Synthetic Methods

All reagents and solvents were purchased from Sigma-Aldrich, unless otherwise stated, and used without further purification. Deuterated solvents were purchased from Cambridge Isotope laboratories. Thin layer chromatography was performed on SiliCycle Siliplate® silica gel plates (60 Å F254, layer thickness 200µm). Flash chromatography was performed using silica gel (60 Å, particle size 40–63 µm). Fluorescence microscopy was performed with a Nikon Ni-U ratiometric fluorescence microscope equipped with a LED excitation light source and Ultra-sensitive Andor iXon Ultra 897 cooled EMCCD camera. Images were acquired using a Nikon 60x oil dip objective lens and, if indicated, a 2x relay lens. Fluorescence images were obtained under strictly identical conditions of gain and exposure time, on focused beads, typically 2-5 s, and brightfield images were obtained using a 20-50 ms exposure. Images were acquired using Nikon NIS Elements software and processed with ImageJ. All 1H and 13C NMR spectra were obtained on a Bruker Avance 300 or 400 spectrometer using a frequency of 300 MHz or 400 MHz for 1H and 100 MHz for 13C and processed using INMR 4.2.0 software. The following abbreviations were used to designate chemical shift multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet or unresolved, br = broad signal and J = coupling constants in Hz.

Synthetic Methods

Synthesis of Propiolamides

\[
\text{N-Benzylprop-2-ynamide.} \quad 0.3 \text{ g of propionic acid was added to 5mL DMF in a 25mL round bottom flask equipped with a stir bar. 1.71 g HATU, 0.49 mL benzylamine were added successively to reaction mixture. Lastly, 0.82 mL DIPEA was added and the reaction was let stir for 1 hour at room temperature. Following reaction completion by TLC, the reaction was concentrated in vacuo. Reaction contents were dissolved in 20 mL of ethyl acetate and washed with 10 mL 70% brine 3 times. 60% yield of pure product was recovered following column chromatography using 1:1 EtOAc: hexanes. } \quad \text{^1H NMR (300 MHz; CDCl}_3\text{): } \delta = 7.37-7.30 (m, 5H), \text{ 6.10 (s, 1H), 4.50 (d, J = 5.9 Hz, 2H), 2.80 (s, 1H). Spectral data was consistent with previously reported data.} \]

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\text{N-(2-methoxyethyl)prop-2-ynamide.} \quad \text{Under an Argon atmosphere, propionic acid (0.3 g, 4.28 mmol) was dissolved in 10 mL of dry DMF, followed by the addition of HATU (1.71g, 4.50 mmol). 2-Methoxyethylamine (391 µL, 4.50 mmol) was slowly added over 15 minutes, followed by the addition of DIPEA (819 µL). The reaction was allowed to stir at room temperature for 6 hours, after which 10 mL of EtOAc was added. The mixture was extracted three times with 10 mL of a 50% brine solution. The organic layer was recovered, dried over Na}_2\text{SO}_4\text{, filtered and concentrated. Pure product (0.24 g) was obtained as a light yellow solid in 44% yield following flash column chromatography using 3:97 MeOH:DCM. } \quad \text{^1H NMR (400 MHz; CDCl}_3\text{): } \delta = 6.26 (s, 1H), \text{ 3.52-3.46 (m, 4H), 3.37 (s, 3H), 2.79 (s, 1H); } \quad \text{^13C NMR (CDCl}_3\text{, 100MHz) } \delta = 152.1, \text{ 73.2, 70.5, 58.8, 39.5. HRMS: for C}_9\text{H}_9\text{NO}_2\text{ (M+H): calculated: 128.0633; found: 128.0693.} \]
Synthesis N-Phenyl-Nitrone Derivatives

General Nitrone Synthesis A

300 mg (2.8 mmol) of phenylhydroxylamine and 3 mmol of para-substituted benzaldehyde were added to a dry 25 mL round-bottom flask. 5 mL of dry ethanol was added to the flask and the mixture was stirred for 2 hours at 35 °C. Pure products were obtained following filtration of the precipitated crude product and recrystallization from warm ethanol and hexanes.

N-Phenylhydroxylamine. A mixture of nitrobenzene (10.8 mL, 0.105 mol), NH4Cl (6.5 g, 0.12 mol) and degassed H2O (200 mL) under argon at r.t. was stirred vigorously while zinc dust (15.4 g, 0.21 mol) was added portion wise over 20 minutes. After addition was complete, the reaction mixture was stirred for an additional 20 minutes and was filtered while still warm. The resultant filter cake was washed with hot distilled water (50 mL) and the combined filtrate was saturated with NaCl, and extracted with 3x100 mL of EtOAc. The organic layers were combined, dried over Na2SO4 and concentrated under reduced pressure. The crude N-phenyl hydroxylamine was recrystallized from petroleum ether/EtOAc (8.2 g, 72%), dried thoroughly and stored under an atmosphere of argon at -20°C. 1H NMR and MS data are in agreement with that reported previously. 21HNMR (300 MHz; CDCl3): δ 7.32–7.26 (m, 2H), 7.02–6.97 (m, 3H), 6.77 (dq, J = 2.5, 0.8 Hz, 1H).

General Nitrone Synthesis B

16 mmol of para-substituted nitrobenzene and 1.7 g (16 mmol) of benzaldehyde were added to a dry 100 mL round-bottom flask. 29 mL of EtOH and 29 mL of H2O were added, followed by 0.9 g (18 mmol) of NH4Cl. The reaction mixture was cooled to 0°C, and 1.9 g of Zn power (29 mmol) was added over the course of 20 minutes. The reaction was allowed to warm to room temperature and stirred for 16 hours. The reaction was then filtered through Celite, and washed 3 times with 60 mL DCM. The organic layers were combined, dried over Na2SO4, filtered, and concentrated. Pure product was obtained following recrystallization from warm EtOAc and Hexanes.

C,N-diphenyl Nitrone. The nitrone was synthesized according to General Nitrone Synthesis B. The product was obtained in 50% yield. 1HNMR (300 MHz; CDCl3): δ 8.42–8.39 (m, 2H), 7.93 (s, 1H), 7.80–7.77 (m, 2H), 7.50–7.48 (m, 6H). Spectral data was consistent with previously reported data.
C-(4-nitrophenyl)-N-phenylnitrone. The nitrone was synthesized according to General Nitrone Synthesis A. The product was obtained in 75% yield. $^1$H NMR (300 MHz; CDCl$_3$): $\delta$ 8.58-8.53 (m, 2H), 8.34-8.30 (m, 2H), 8.07 (s, 1H), 7.80-7.77 (m, 2H), 7.53 (dd, $J$=4.2, 2.5 Hz, 3H). Spectral data was consistent with previously reported data.$^4$

C-(4-cyanophenyl)-n-phenylnitrone. The nitrone was synthesized according to General Nitrone Synthesis A. The product was obtained in 52% yield. $^1$H NMR (400 MHz; CDCl$_3$): $\delta$ 8.48 (d, $J$=8.4 Hz, 2H), 8.00 (s, 1H), 7.78-7.74 (m, 4H), 7.52 (t, $J$=3.3 Hz, 3H). Spectral data was consistent with previously reported data.$^4$

C-(4-methoxycarbonylphenyl)-N-phenylnitrone. The nitrone was synthesized according to General Nitrone Synthesis A. The product was obtained in 63% yield. $^1$H NMR (300 MHz; CDCl$_3$): $\delta$ 8.45 (d, $J$=8.5 Hz, 2H), 8.14 (d, $J$=8.7 Hz, 2H), 8.00 (s, 1H), 7.78 (dd, $J$=6.9, 2.9 Hz, 2H), 7.52-7.49 (m, 3H), 3.95 (s, 3H). Spectral data was consistent with previously reported data.$^5$

C-(4-fluorophenyl)-N-phenylnitrone. The nitrone was synthesized according to General Nitrone Synthesis A. The product was obtained in 54% yield. $^1$H NMR (300 MHz; CDCl$_3$): $\delta$ 8.48-8.43 (m, 2H), 7.91 (s, 1H), 7.79-7.76 (m, 2H), 7.52-7.48 (m, 3H), 7.20-7.15 (m, 2H). Spectral data was consistent with previously reported data.$^4$

C-(4-hydroxyphenyl)-N-phenylnitrone. The nitrone was synthesized according to General Nitrone Synthesis A. The product was obtained in a 47% yield. $^1$H NMR (300 MHz; CD$_3$OD): $\delta$ 8.40-8.35 (m, 2H), 8.27 (s, 1H), 7.84-7.81 (m, 2H), 7.57-7.51 (m, 3H), 6.95-6.90 (m, 2H). Spectral data was consistent with previously reported data.$^5$
**C-(4-methoxyphenyl)-N-phenylnitrone.** The nitrone was synthesized according to General Nitrone Synthesis A. The product was obtained in 77% yield. $^1$H NMR (300 MHz; CDCl$_3$): $\delta$ 8.43-8.39 (m, 2H), 7.86 (s, 1H), 7.80-7.76 (m, 2H), 7.51-7.44 (m, 3H), 7.02-6.99 (m, 2H), 3.89 (s, 3H). Spectral data was consistent with previously reported data.$^3$

**N-(4-chlorophenyl)-a-phenylnitrone.** The nitrone was synthesized according to General Nitrone Synthesis B. The product was obtained in 53% yield. $^1$H NMR (300 MHz; CDCl$_3$): $\delta$ 8.40-8.37 (m, 2H), 7.90 (s, 1H), 7.77-7.73 (m, 2H), 7.49 (q, $J=3.3$ Hz, 4H), 7.45 (t, $J=2.5$ Hz, 1H). Spectral data was consistent with previously reported data.$^3$

**N-(4-methoxycarbonylphenyl)-a-phenylnitrone.** The nitrone was synthesized according to General Nitrone Synthesis B. The product was obtained in 83% yield. $^1$H NMR (300 MHz; CDCl$_3$): $\delta$ 8.43-8.39 (m, 2H), 8.19-8.15 (m, 2H), 7.98 (s, 1H), 7.89-7.85 (m, 2H), 7.52-7.49 (m, 3H), 3.96 (s, 3H). Spectral data was consistent with previously reported data.$^5$
Synthesis of Biotin-CMPO

The Biotin-CMPO was synthesized according to previously reported procedures. Biotin-PEG (91 mg, 0.20 mmol, 1 eq), CMPO (35 mg, 0.24 mmol, 1.2 eq) and HATU (76 mg, 0.20 mmol, 1 eq) were dissolved in DMF (175 μL). DIPEA (52 μL, 0.30 mmol, 1.75 eq) was added all at once and the mixture was stirred for 45 minutes. Reaction progress was confirmed by LC-MS. The reaction was concentrated under reduced pressure and stored at -20 °C overnight. The crude was purified using preparatory HPLC with MeCN/H₂O/Formic acid (0.1%) as eluent, running gradient of 10 to 60% acetonitrile over 15 minutes. The product eluted at 7.5-8 minutes and its presence was confirmed by MS; the fractions were pooled and concentrated under reduced pressure. Some starting material was also isolated. The product was obtained as colourless oil (20.1 mg, 0.035 mmol, 17.5 % yield). MS (ESI+) calcd (C₁₀H₁₅N₆O₅S): 572.30 [M+H]⁺, found 572.1; ¹H NMR (400 MHz, MeOD-d₄) δ 7.24 (s, 1H), 4.52 (dd, 1H, J=4.9, 7.7 Hz), 4.33 (dd, 1H, J=4.4, 7.8 Hz), 3.64 (m, 9H), 3.55 (dt, 4H, J=4.7, 6.0, 6.1 Hz), 3.36 (m, 2H), 3.25 (m, 3H), 2.95 (dd, 1H, J=4.9, 12.7 Hz), 2.71 (dd, 3H, J=10.6, 14.1 Hz), 2.22 (t, 3H, J=7.4, 7.4 Hz), 1.79 (ddd, 5H, J=4.0, 6.4, 12.8 Hz), 1.69 (s, 3H), 1.62 (m, 3H), 1.47 (dd, 2H, J=7.5, 15.2 9 Hz); ¹³C NMR (100 MHz, MeOD-d₄) δ 174.6, 170.8, 141.1, 79.1, 70.1, 69.9, 69.8, 68.6, 68.5, 62.0, 60.2, 55.6, 39.7, 37.0, 36.4, 35.5, 31.0, 29.0, 28.8, 28.4, 28.1, 25.5, 25.0, 21.5. Spectral data was consistent with previously reported data.

Synthesis of N-hydroxysuccinimide ester alkyne

1-[(1-oxo-2-propynyl)oxy]-2,5-pyrrolidinedione. Synthesis was accomplished following a modified procedure. Pro-picolic acid (500 mg, 7.14 mmol) and N-hydroxysuccinimide (822 mg, 7.14 mmol) were suspended in EtOAc (39 mL) and cooled to 0°C. A solution of N,N-Dicyclohexylcarbodiimide (1.47 g, 7.14 mmol) in EtOAc (13 mL) was added dropwise over the course of 1 hour. The mixture was then stirred at 0°C for 6 hours. The urea byproduct was removed by filtration and the filtrate was concentrated under reduced pressure to approximately 10 mL and then washed twice with brine (5 mL). The organic phase was dried with Na₂SO₄, then concentrated to approximately 1-2 mL. The concentrated organic phase was cooled to -5 °C; 1-2 mL of heptanes was then added and the mixture was
further cooled to -10 °C and the resulting solids were stirred for 2 hours. The solid precipitate was filtered, rinsed with cold heptanes and dried under vacuum to yield the product as a white solid (895 mg, 75% yield). \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta 2.87 (s, 4H), 3.31 (s, 1H)\). Spectral data was consistent with previously reported data.\(^7\)

**Procedure for isolation of \(\beta\)-lactam products for characterization**

The reaction was conducted in 6 mL of argon degassed H\(_2\)O. 4 mL of acetonitrile was added to help solubilize organic reagents. L-Proline (36 mg, 0.63 mmol), CuSO\(_4\)+5H\(_2\)O (39 mg, 0.16 mmol), sodium ascorbate (249 mg, 1.26 mmol) and pyridine (51 \(\mu\)L, 0.63 mmol) were then added successively. Following the addition of N-benzylprop-2-ynamide (50 mg, 0.31 mmol), C,N-diphenylazetidine (62 mg, 0.31 mmol) was added and the mixture was allowed to stir for 1 hour at 25 °C. The reaction was then extracted 3x20 mL of EtOAc. The organic fractions were then dried over Na\(_2\)SO\(_4\) and concentrated under reduced pressure. The dried residue was then purified by flash column chromatography using 30% EtOAc in hexanes. The product was recovered as a white solid for analytical and characterization purposes.

\(N\)-benzyl-2-oxo-1,4-diphenylazetidine-3-carboxamide, (3S, 4R) -rel-

\(^1\)H NMR (400 MHz; CDCl\(_3\)): \(\delta 7.38 (d, J=13.6 Hz, 4H), 7.29 (d, J=14.0 Hz, 6H), 7.24 (s, 4H), 7.06 (s, 1H), 6.54 (t, J=0.3 Hz, 1H), 5.41 (d, J=2.6 Hz, 1H), 4.56 (dd, J=14.8, 6.1 Hz, 1H), 4.41 (dd, J=14.8, 5.5 Hz, 1H), 3.86 (d, J=2.6 Hz, 1H); \(^{13}\)C NMR (100 MHz; CDCl\(_3\)): \(\delta 163.5, 163.1, 137.6, 136.9, 133.9, 129.5, 129.25, 129.21, 129.01, 128.89, 128.82, 128.6, 127.86, 127.81, 127.5, 126.9, 126.2, 124.6, 77.3, 59.4, 43.2, 29.9; HRMS (ESI-TOF): for C\(_{23}\)H\(_{20}\)N\(_2\)O\(_2\) (M+Na\(^+\)): calculated: 379.1525; found: 379.1422.

\(N\)-benzyl-2-oxo-1,4-diphenylazetidine-3-carboxamide, (3R, 4R) -rel-

\(^1\)H NMR (400 MHz; CDCl\(_3\)): \(\delta 7.33 (s, 5H), 7.28 (d, J=7.1 Hz, 4H), 7.25 (s, 3H), 7.08 (d, J=6.9 Hz, 1H), 7.03 (d, J=9.5 Hz, 2H), 6.77 (s, 1H), 5.37 (d, J=6.3 Hz, 1H), 4.52 (d, J=6.2 Hz, 1H), 4.39 (dd, J=14.9, 6.5 Hz, 1H), 4.14 (dd, J=14.8, 5.1 Hz, 1H); \(^{13}\)C NMR (101 MHz; CDCl\(_3\)): \(\delta 163.5, 163.1, 137.6, 136.9, 133.9, 129.5, 129.25, 129.21, 129.01, 128.89, 128.82, 128.6, 127.86, 127.81, 127.5, 126.9, 126.2, 124.6, 77.3, 59.4, 43.2, 29.9; HRMS (ESI-TOF): for C\(_{23}\)H\(_{20}\)N\(_2\)O\(_2\) (M+Na\(^+\)): calculated: 379.1525; found: 379.1422.
In vitro Micelle-Assisted Kinugasa/CuANCR reactions

General Procedure

Reactions were conducted in 20 mL of argon degassed H₂O containing 10 mM sodium dodecyl sulfate (58 mg, 0.2 mmol). Sodium ascorbate (40 mg, 0.2 mmol), pyridine (8 μL, 0.1mmol), L-proline (6 mg, 0.05 mmol) and CuSO₄ (6 mg, 0.025 mmol) were then added successively. Following the addition of alkyne 1-8 (0.05 mmol), C,N- diphenylnitrone (10 mg, 0.05 mmol) was added and the reaction was stirred for 30 minutes at 25 °C. 3 mL of brine was then added to the mixture, followed by an extraction with 3x20 mL of EtOAc. The organic fractions were then dried over Na₂SO₄ and concentrated under reduced pressure. An internal standard, 1,4-Dimethoxybenzene, was accurately weighed (approximately 0.1 mmol) and added to the dried reaction. NMR yields were obtained by comparing relevant new cis/trans product peaks (β-lactam doublet peaks, 4.5-5.5 ppm range, Hα from the representative product spectra) to the internal standard peak (6.83 ppm, s, 4H). Nitrone conversion was determined by comparing the remaining nitrone peak (8.41 ppm, m, 2H) to the initial amount used (0.05 mmol). The diastereomeric ratio was determined by comparing the calculated NMR yields of both the β-lactam products, while assuming that the minor product was cis.

Table S1. Diastereomeric ratios for screen of alkynes used in micelle-assisted Kinugasa reactions

<table>
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<th>Entry</th>
<th>Alkyne</th>
<th>Yield</th>
<th>trans: cis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ph</td>
<td>22b</td>
<td>55:45</td>
</tr>
<tr>
<td>2</td>
<td>CF₃</td>
<td>16b</td>
<td>70:30</td>
</tr>
<tr>
<td>3</td>
<td>Et</td>
<td>20b</td>
<td>55:45</td>
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<tr>
<td>4</td>
<td>CN</td>
<td>32b</td>
<td>77:23</td>
</tr>
<tr>
<td>5</td>
<td>OMe</td>
<td>60</td>
<td>80:20</td>
</tr>
<tr>
<td>6</td>
<td>NHPh</td>
<td>64</td>
<td>68:32</td>
</tr>
<tr>
<td>7a/b</td>
<td>OEt</td>
<td>65/21c</td>
<td>74:26/55:45</td>
</tr>
</tbody>
</table>

a) Isolated yields extracted from micellar emulsions. b) Entries 1, 2, 3 and 4 were conducted in 3.5 mM CTAB (26 mg, 0.07 mmol) instead of SDS and in the absence of L-proline. c) Entry 7b was conducted in the absence of surfactant.

Table S2. Screen of nitrones used in micelle-assisted Kinugasa Reaction
<table>
<thead>
<tr>
<th>Entry</th>
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<tr>
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</table>
Micelle Assisted Kinugasa/CuANCR Reaction on Alkyne Beads

5 µL of alkyne-tagged beads (corresponding to 3-5 µM of reactive alkyne groups, Click Chemistry tools) were washed in PBS prior to use. The reaction was carried out in PBS and consisted of 100 µM CuSO₄, 2 mM freshly solubilized sodium ascorbate, 200 µM L-proline, 50 µM biotin-CMPO (or vehicle DMSO) to which the indicated amount of surfactant or water solvent was added. The reaction was started by addition of washed beads and was carried out with gentle shaking at 37 °C for the indicated amount of time. Beads were washed 1x with PBS containing 0.05% Tween20, then 3x with PBS prior addition of 5 µg/mL FITC-streptavidin in PBS. The binding of streptavidin-FITC was carried out at room temperature for 30 minutes in the dark. The beads were washed three more times with PBS and resuspended in PBS containing 5% glycerol. 8 µL of this solution was applied to a microscopy slide which were imaged using the Nikon Ni-U ratiometric fluorescence microscope equipped with a LED excitation light source and Ultra-sensitive Andor iXon Ultra 897 cooled EMCCD camera. Images were acquired using a Nikon 60x oil dip objective lens and, if indicated, a 2x relay lens. Fluorescence images were obtained under strictly identical conditions of gain and exposure time, on focused beads, typically 2-5 s, and brightfield images were obtained using a 20-50 ms exposure. Images were acquired using Nikon NIS Elements software and processed with ImageJ.

Figure S1. Quantification of Figure 2 CuANCR magnetic beads. Images were set to the same fluorescence intensity levels then converted to 8bit. Integrated density from the same area was then measured for 5 beads per image, which is from different fields of view for each sample
Figure S2. Fluorescence microscopy of micelle-assisted CuANCNR with surfactants. Alkyne-tagged beads were labelled with 50 µM biotin-CMPO in presence of 1.5 mM of indicated surfactant (at or above CMC for all detergents) from Table S3. Labelling was carried out for 30 minutes at 37°C after which beads were washed and incubated with 5 µg/mL FITC-streptavidin for another 30 minutes at room temperature in the dark. Images were acquired as indicated in the labelling protocol using a 60x objective with oil dip lens and background fluorescence (sample without biotin-CMPO) was subtracted using software ImageJ. Scale bar indicates 5 µm.
Table S3. Lipids used in screening of Kinugasa reaction on alkyne-tagged beads. Structures are shown as well as approximate critical micellar concentrations (CMC) in water for each lipid.

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>CMC in water(^a)</th>
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<tbody>
<tr>
<td>FCH16</td>
<td><img src="image" alt="FCH16 structure" /></td>
<td>~ 0.013 mM</td>
</tr>
<tr>
<td>FCH14</td>
<td><img src="image" alt="FCH14 structure" /></td>
<td>~ 0.12 mM</td>
</tr>
<tr>
<td>FCH12</td>
<td><img src="image" alt="FCH12 structure" /></td>
<td>~ 1.5 mM</td>
</tr>
<tr>
<td>α-DDM</td>
<td><img src="image" alt="α-DDM structure" /></td>
<td>~ 0.152 mM</td>
</tr>
<tr>
<td>β-TDM</td>
<td><img src="image" alt="β-TDM structure" /></td>
<td>~ 0.01 mM</td>
</tr>
<tr>
<td>DMNG</td>
<td><img src="image" alt="DMNG structure" /></td>
<td>~ 0.36 mM</td>
</tr>
</tbody>
</table>

Figure S3. Time course of micelle-assisted CuANCR reaction in presence of β-TDM surfactant. Alkyne-tagged beads were labelled with 50 μM biotin-CMPO in presence of 150 μM β-TDM, 100 μM CuSO₄, 2 mM sodium ascorbate and 200 μM L-proline for 0-60 min, as indicated, after which beads were washed with PBS and then incubated with 5 μg/mL FITC-streptavidin in the dark. Beads were again washed with 3x PBS and imaged using Nikon Ni-U ratiometric microscope equipped with a 60x objective lens and a 2x relay lens. Average background fluorescence obtained in absence of biotin-CMPO was subtracted from all images. Scale bar indicates 5 μm.
Figure S4. Micelle-dependence of aqueous CuANCR labelling reaction. Labelling of alkyne beads with biotin-CMPO was performed as indicated in the experimental section, in a range of concentrations of β-TDM, both below and above the CMC of β-TDM of 10 µM (See Table S3). Fluorescence images were acquired using the Nikon ratiometric microscope Ni-U equipped with an oil-dip 60x objective lens. Background fluorescence was determined as average fluorescence of beads in absence of biotin-CMPO, and was subtracted from all images. Scale bar indicates 5 µm.
Figure S5. Micelle-dependence of aqueous CuANCR labelling reaction. Labelling of alkyne-tagged beads with 50 μM biotin-CMPO in 100 μM CuSO₄, 2 mM sodium ascorbate and 200 μM L-proline was performed in a range of concentrations of β-TDM, both below (blue area) and above (light red area) the CMC of β-TDM of 10 μM (See Table S3). Each data point is an average above background fluorescence determined from at least five different beads.
Figure S6. Kinetics of aqueous Kinugasa on alkyne-tagged beads. Alkyne-tagged beads with β-TDM (150 µM, red curve) and without lipid (0 µM, blue curve). Alkyne beads (3-5 µM alkyne groups) were incubated with 50 µM biotin-CMPO, 100 µM CuSO₄, 2 mM sodium ascorbate and 200 µM L-proline in PBS for the indicated amount of time at 37°C, and then stained with 5 µg/mL FITC-streptavidin. Beads were washed in PBS before fluorescence imaging. Above background fluorescence of five beads from two independent view fields (10 beads total) was determined using ImageJ software, normalized and plotted against time of labelling (minutes). Data points were fitted to first order kinetics equation in Prism 4. See also Figure S3 for fluorescence imaging data.
**E5-TAT peptide modification procedure**

**Two-Step Biotin Labeling of E5-TAT peptide**

E5-TAT peptide (GLFEAIAEFGWEGLGWGGKRRQRRR) (GenScript) samples were diluted (20 μM) in Phosphate-Buffered Saline (PBS) containing varying concentrations of Fos-Choline 12 (FC12) (0.01% to 0.2%, CMC=0.047%). The peptide samples were then treated with 1-{[(1-oxo-2-propynyl)oxy]-2,5-pyrrolidinedione (N-hydroxysuccinimide ester alkyne) (300 μM, DMSO stock) and allowed to stand for 1 hour at room temperature. Sodium ascobate (300 μM), L-proline (40 μM), CuSO$_4$·5H$_2$O (20 μM) and Biotin-CMPO (300 μM, DMSO stock) were then sequentially added to the samples, which were allowed to sit for an additional hour at room temperature. The samples were then prepared for SDS-PAGE and Western blotting analysis.

**Immunoblotting**

Labeled E5-TAT peptide (GenScript) samples were loaded and analyzed using SDS-PAGE and western blotting. Samples were run using 12% stain-free polyacrylamide gel electrophoresis (TGX Stain-Free Fastcast Acrylamide kit, Bio-Rad). The proteins were then transferred to a PVDF membrane using the Trans-blot Turbo RTA Transfer Kit (Bio-Rad). Membrane was blocked using Tris-buffered saline with 0.05% Tween-20 (TBS-T) containing 3% W/V Bovine serum albumin (Sigma-Aldrich). Peptides were probed using anti-biotin antibody (1:1000, Invitrogen, MA5-11251) overnight at 4 °C. Blot was washed in TBS-T and probed for one hour at room temperature with HRP-conjugated goat anti-mouse secondary antibody (1:20000, Jackson Immunoresearch Laboratories, Westgrove, PA). Bands were visualized using Clarity ECL western blotting substrate (Bio-Rad) according to the manufacturer’s protocols. Integrated signal was calculated relative to negative control (no Biotin-CMPO) taking into account peptide loading using Image Lab software (Bio-Rad). Figure S7 shows a repeat experiment with the unmodified E5-TAT negative control.

**Peptide Modification Analysis**

2 mg of E5-TAT peptide dissolved in 1x phosphate-buffered saline (pH 7.5) was treated with 15 equivalents of N-hydroxysuccinimide ester alkyne and left for one hour at room temperature. The peptide sample was then subjected to FPLC purification. The size exclusion chromatography profile was obtained for the alkyne modified E5-TAT using a Superdex 75 size exclusion column (FPLC ÄKTA pure, GE) at a concentration of 1 mg/ml in 1x phosphate-buffered saline (pH 7.5). Absorbance was recorded at 280 nm, with flow rate maintained at 0.8 mL/min (See Figure S8). 10 μg of purified modified and unmodified peptide were then subjected to desalting using C18 spin columns (ThermoFisher Scientific) according to manufacturer’s protocol. Samples were then subjected to mass spectrometry analysis. Pro- teome Discoverer 2.1 (ThermoFisher Scientific) was used to evaluate the modification of peptide with the N-hydroxy-succinimide ester alkyne. Search engine: SEQUEST-HT implemented in Proteome Discover was applied for all MS raw files. Search parameters were set to allow for dynamic modification of the N-hydroxysuccinimide ester alkyne (51.995 Da). The peptide-spectrum matches (PSMs) was used to evaluate the alkyne modification of peptides and only peptides for Sequest results of XCorr ≥ 2.5 were retained. The obtained PSMs showed modification of lysine residues K26 and K27 (both mono and di-substitution), modifications not found for the unmodified peptide sample.
Figure S7. Biotin conjugation of E5-TAT membrane peptide using CuANCR. Alkyne functionalization of E5-TAT (20 μM) was carried out using N-hydroxysuccinimide ester alkyne (300 μM). Biotin labelling was achieved by treating modified peptide with CuSO₄·H₂O (20 μM), sodium ascorbate (300 μM), L-proline (40 μM) and biotin-CMPO (300 μM).

(a) Western blot analysis of biotin labelled E5-TAT samples shown under varying percentages (%W/V) of detergent (FC12) ranging from (0.01-0.2%, CMC=0.05%). (b) TGX Stain-free protein loading control.

Figure S8. Size exclusion chromatography profile for modified E5-TAT run in 1x PBS, pH 7.5 on a Super-dex 75 column (GE).
Table S4. Results from SEQUEST-HT implementation in Proteome Discovery for unmodified E5-TAT samples. XCorr values ≥ 2.5 are shown.

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<th>Mascot Score</th>
<th>Rank</th>
<th>Area</th>
<th>Raw RT (s)</th>
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Table S5. Results from SEQUEST-HT implementation in Proteome Discovery for modified E5-TAT samples. XCorr values ≥ 2.5 are shown.

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<th>Protein</th>
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NMR Spectra
Synthesized Activated Alkynes

N-(2-methoxyethyl)prop-2-ynamide
$^1$H, 400 MHz, CDCl$_3$

N-(2-methoxyethyl)prop-2-ynamide
$^{13}$C, 300 MHz, CDCl$_3$
$^1$H NMR of Synthesized Nitrones
Characterization β-Lactams for Table 1
NMR Data for Table 1. Screen of Alkyne Reactivity

Entry 1
$^1$H, 300 MHz, CDCl$_3$, std= 2.76 mg

Entry 2
$^1$H, 300 MHz, CDCl$_3$, std= 2.02 mg
Entry 5
$^1$H, 300 MHz, CDCl$_3$, std= 2.71 mg

Entry 6
$^1$H, 300 MHz, CDCl$_3$, std= 3.42 mg
NMR Data for Table S2. Screen of Nitrone Reactivity

Table S2 yields were calculated based on $^1$H NMR Spectra recorded with a frequency of 300MHz. NMR yields are reported using 1,4-Dimethoxybenzene as internal standard.

Table S2. Reaction 1A.
Std= 2.34 mg

![NMR Spectrum](image-url)
Table S2. Reaction 1B.
Std= 2.32 mg

Table S2. Reaction 1C.
Std= 2.50 mg
Table S2. Reaction 2A.
Std= 1.76 mg

Table S2. Reaction 2B.
Std= 2.90 mg
Table S2. Reaction 2C.
Std= 2.47 mg

Table S2. Reaction 3A
Std= 1.82 mg
Table S2. Reaction 3B.
Std = 1.81 mg

Table S2. Reaction 3C.
Std = 1.52 mg
Table S2. Reaction 4A.
Std = 1.90 mg

Table S2. Reaction 4B.
Std = 1.92 mg
Table S2. Reaction 4C.
Std = 2.78 mg

Table S2. Reaction 5A.
Std = 2.14 mg
Table S2. Reaction 5B.
Std = 2.23 mg

Table S2. Reaction 5C.
Std = 1.84 mg
Table S2. Reaction 6A.
Std = 2.61 mg

Table S2. Reaction 6B.
Std = 1.41 mg
Table S2. Reaction 6C.
Std= 2.96 mg

Table S2. Reaction 7A.
Std= 1.47 mg
Table S2. Reaction 7B.
Std = 3.34 mg

Table S2. Reaction 7C.
Std = 2.42 mg
Table S2. Reaction 8A.
Std= 3.02 mg

Table S2. Reaction 8B.
Std= 3.23 mg
Table S2. Reaction 8C.
Std= 1.69 mg
References


