Stochastic protein alkylation by antimalarial peroxides

Supplementary Material

[4 figures and 3 tables; 8 pages plus 3 additional Excel files]

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Chemical syntheses

As shown in Scheme 1, ozonide alkyne click chemistry probes OZ726 and OZ727 were prepared in one or two steps from keto ozonide 3. Amino ozonide 4 and OZ727 were isolated as their mesylate salts; structures depicted are the free base forms. Griesbaum coozonolysis (Griesbaum et al., 1997) of dione 1 and oxime ether 2 afforded 3 in 65% yield. Reductive amination reactions of 3 afforded 4 and OZ726 in yields of 75 and 76%, respectively. Acylation of 4 afforded OZ727 in 59% yield. The non-peroxide control carbaOZ727 was prepared in a three-step sequence starting with monoketalization of 1 with diol 5 to form keto dioxolane 6 in 86% yield (Scheme 2). This was followed by reductive amination and acylation to afford carbaOZ277 in a combined yield of 21%.

Scheme 1

\[ \text{Scheme 1a} \]

\[ \text{Reagents and conditions: (a) O}_3, 3:1 \text{ cyclohexane:} \text{CH}_2\text{Cl}_2, 0 \, ^\circ\text{C}, 5 \text{ min; (b) propargylamine, sodium triacetoxyborohydride, AcOH, 1:1 CH}_2\text{Cl}_2:\text{CICH}_2\text{Cl}_2\text{C}_2, \text{rt, 14 h, then MsOH, EtOAc, rt, 0.5 h; (c) NaCNBH}_3, \text{NH}_4\text{OAc, AcOH, MeOH, rt, 14 h, then MsOH, EtOAc, rt, 0.5 h; (d) 4-pentynoic acid, HOBr, EDCI, NEt}_3, \text{DMF, rt, 24 h.}} \]

Scheme 2

\[ \text{Scheme 2a} \]

\[ \text{Reagents and conditions: (a) } p\text{-TSA, CH}_2\text{Cl}_2, \text{rt, 20 h; (b) NaCNBH}_3, \text{NH}_4\text{OAc, AcOH, MeOH, rt, 13 h; (c) 4-pentynoic acid, HOBr, EDCI, DMF, rt, 21 h.} \]
General. Melting points are uncorrected. 1D $^1$H and $^{13}$C NMR spectra were recorded on a 500 MHz spectrometer using CDCl$_3$ or DMSO-$d_6$ as solvents. All chemical shifts are reported in parts per million (ppm) and are relative to internal (CH$_3$)$_4$Si (0 ppm) for $^1$H and CDCl$_3$ (77.2 ppm) or DMSO-$d_6$ (39.5 ppm) for $^{13}$C NMR. Silica gel (sg) particle size 32–63 μm was used for all flash column chromatography. Reported reaction temperatures are those of the oil bath.

cis-6-Oxoadamantane-2-spiro-3'-1',2',4'-trioxaspiro[4.5]decane (3). A solution of adamantane-2,6-dione (1) (3.20 g, 19.5 mmol) and O-methyl cyclohexanone oxime (2) (1.27 g, 10.0 mmol) in cyclohexane (120 mL) and CH$_2$Cl$_2$ (40 mL) was treated with ozone according to the method of Dong et al (Dong et al., 2005). After solvent removal, the crude product was purified by sg flash chromatography to afford 3 (1.32 g, 65%) as colorless solid. mp 70–71 °C; $^1$H NMR (CDCl$_3$) $\delta$ 1.35–1.51 (m, 2H), 1.56–1.71 (m, 4H), 1.71–1.82 (m, 4H), 1.92 (d, J = 13 Hz, 2H), 1.98 (d, J = 13 Hz, 2H), 2.12 (s, 2H), 2.25 (d, J = 13 Hz, 2H), 2.36 (d, J = 13 Hz, 2H), 2.44–2.52 (m, 2H); $^{13}$C NMR (CDCl$_3$) $\delta$ 23.82, 24.88, 34.65, 35.67, 35.71, 35.94, 44.73, 45.16, 108.93, 109.79, 216.04. Anal. Calcd for C$_{16}$H$_{22}$O$_4$: C, 69.04; H, 7.97. Found: C, 69.02; H, 7.75.

cis-Dispiro[adamantane-2,3'-[1,2,4]trioxolane-5',1''-cyclohexan]-6-amine mesylate (4). To a solution of 3 (0.15 g, 0.54 mmol) in MeOH (10 mL) were added ammonium acetate (0.5 g, 6.49 mmol) and acetic acid (1.0 mL). The resulting mixture was stirred at rt for 2 h before sodium cyanoborohydride (0.10 g, 1.60 mmol) was added. The reaction mixture was stirred overnight and then quenched with 1 M NaOH (2 mL). After removal of solvents in vacuo, the residue was dissolved in EtOAc (20 mL), and washed with water (10 mL) and brine (10 mL). The organic layer was dried over MgSO$_4$, filtered and concentrated. The residue was dissolved in EtOAc (5 mL) and then a solution of methanesulfonic acid (0.05 g) in ethyl acetate (20 mL) was added. The precipitate was collected by filtration to afford 4 (0.15 g, 75%) as a colorless solid. mp 70–71 °C; $^1$H NMR (CDCl$_3$) $\delta$ 1.30–2.08 (m, 22H), 2.50 (s, 3H), 3.33 (s, 1H), 7.41 (brs, 3H); $^{13}$C NMR (CDCl$_3$) $\delta$ 23.82, 24.88, 34.65, 35.67, 35.71, 35.94, 44.73, 45.16, 108.93, 109.79, 216.04. Anal. Calcd for C$_{16}$H$_{22}$O$_4$: C, 69.04; H, 7.97. Found: C, 69.02; H, 7.75.

cis-N-(prop-2-yn-1-yl)dispiro[adamantane-2,3'-[1,2,4]trioxolane-5',1''-cyclohexan]-6-amine mesylate (OZ726). To a solution of 3 (0.22 g, 0.79 mmol) in CH$_2$Cl$_2$ (5 mL) and ClCH$_2$CH$_2$Cl (5 mL) were added propargylamine (0.20 g, 3.64 mmol) and acetic acid (1.0 mL). The resulting mixture was stirred at rt for 2 h before sodium triacetoxyborohydride (0.22 g, 1.03 mmol) was added. The reaction mixture was stirred overnight and then quenched with 1 M NaOH (2 mL). The organic layer
was separated and washed with water (10 mL), brine (10 mL), dried over MgSO$_4$, and concentrated. The residue was dissolved in EtOAc (5 mL) and then a solution of methanesulfonic acid (0.06 g) in ethyl acetate (20 mL) was added. The precipitate was collected by filtration to afford **OZ726** (0.25 g, 76%) as a colorless solid. mp 167–168°C. $^1$H NMR (CDCl$_3$) $\delta$ 1.32–1.50 (m, 2H), 1.54–2.30 (m, 20H), 2.51 (s, 1H), 2.78 (s, 3H), 3.54 (s, 1H), 3.96–4.12 (m, 2H), 9.05 (brs, 2H); $^{13}$C NMR (CDCl$_3$) $\delta$ 23.78, 24.88, 27.19, 27.37, 27.47, 27.74, 33.89, 34.02, 34.47, 34.63, 34.99, 35.13, 39.34, 59.63, 73.65, 109.56. Anal. Calcd for C$_{20}$H$_{31}$NO$_6$S: C, 58.09; H, 7.56; N, 3.39. Found: C, 58.07; H, 7.52; N, 3.30.

cis-N-dispiro[adamantane-2,3'-[1,2,4]trioxolane-5',1''-cyclohexan]-6-yl)pent-4-ynamide

(OZ727). A mixture of 4-pentynoic acid (0.10 g, 1.0 mmol), HOBt (0.14 g, 1.0 mmol), EDCI (0.20 g, 1.0 mmol) in DMF (5 mL) was stirred at rt for 2 h. Then 4 (0.20 g, 0.69 mmol) and NEt$_3$ (1 mL) were added and the resulting mixture was stirred at rt for 24 h. Under ice cooling, the reaction was quenched with water (20 mL). The precipitate was collected by filtration, washed with cold water, and dried in vacuo at 40 °C to afford **OZ727** (0.15 g, 59%) as colorless solid. mp 151–152°C. $^1$H NMR (CDCl$_3$) $\delta$ 1.32–1.48 (m, 2H), 1.54–1.88 (m, 14H), 1.90–1.96 (m, 3H), 1.96–2.05 (m, 3H), 2.08–2.14 (m, 1H), 2.43 (t, $J$ = 7.0 Hz, 2H), 2.54 (td, $J_1 = 7.0$ Hz, $J_2 = 2.5$ Hz, 2H), 4.00 (d, $J$ = 8.0 Hz, 1H), 6.01 (d, 1H); $^{13}$C NMR (CDCl$_3$) $\delta$ 15.12, 23.80, 23.86, 24.92, 29.17, 30.20, 30.59, 33.94, 34.05, 34.70, 34.73, 35.29, 35.46, 35.64, 52.48, 69.63, 83.19, 109.29, 110.41, 170.21. Anal. Calcd for C$_{21}$H$_{29}$NO$_4$: C, 70.17; H, 8.13; N, 3.90. Found: C, 70.39; H, 8.15; N, 4.07. HRMS (ESI-TOF) m/z: [M]$^+$ Calcd for C$_{21}$H$_{29}$NO$_4$ 359.2097; Found 359.2105.

N-dispiro[adamantane-2,2'-[1,3]dioxolane-4',1''-cyclohexan]-6-yl)pent-4-ynamide

(carbaOZ727). **Step 1.** A mixture of 1-(hydroxymethyl)cyclohexan-1-ol (5) (0.31 g, 2.38 mmol), adamantane-2,6-dione (1) (0.78 g, 4.76 mmol) and p-toluenesulfonic acid monohydrate (0.09 mg, 0.47 mmol) in CH$_2$Cl$_2$ (30 mL) was stirred at rt for 20 h. The reaction mixture was then washed with saturated NaHCO$_3$ (10 mL), water (10 mL) and brine (10 mL), dried over MgSO$_4$, filtered and concentrated. The residue was purified by sg chromatography eluting with CH$_2$Cl$_2$ to afford 6-oxoadamantane-2-spiro-5'-1',3'-dioxaspiro[4.5]decane (6) (0.57 g, 86%) as white solid. mp 63–64 °C; $^1$H NMR (CDCl$_3$) $\delta$ 1.46–1.36 (m, 4H), 1.77–1.65 (m, 5H), 1.91–1.86 (m, 5H), 2.32 (d, $J = 10$ Hz, 2H), 2.40 (d, $J = 15$ Hz, 2H), 2.46 (d, $J = 15$ Hz, 2H), 3.80 (s, 2H); $^{13}$C NMR (CDCl$_3$) $\delta$ 23.64, 25.38, 35.82, 35.94, 36.87, 37.59, 45.13, 45.47, 73.79, 80.97, 109.16, 217.27. **Step 2.** A mixture of 6 (0.800 g, 2.89 mmol), NH$_4$OAc (2.68 g, 34.68 mmol) and HOAc (0.10 mL) in methanol (30 mL) was stirred at rt for 30 min before the addition of NaCNBH$_3$ (455 mg, 11.56 mmol. The resulting mixture was
stirred for 12 h before quenching with 1 M NaOH (10 mL). After solvent removal in vacuo, the residue was extracted with EtOAc (50 mL) and washed with 1 M NaOH (10 mL) and brine (10 mL). The organic layer was dried over MgSO$_4$, filtered and concentrated to afford 6-aminoadamantane-2-spiro-5'-1',3'-dioxaspiro[4.5]decane (7) as a white solid (0.796 g, 96%). $^1$H NMR (CDCl$_3$) $\delta$ 2.16–1.39 (m, 22H), 2.97 (s, 1H), 3.75 (s, 2H). **Step 3.** To a mixture of 4-pentyloic acid (0.19 g, 2.0 mmol) and HOBt (0.27 g, 2.0 mmol) in DMF (7 mL) was added EDCI (0.52 g, 2.7 mmol) and the resulting mixture was stirred at rt for 1 h. Then 7 (0.50 g, 1.8 mmol) in DMF (3 mL) was added to the reaction mixture dropwise and the mixture was stirred at rt for 20 h. The reaction mixture was diluted with ethyl acetate (40 mL) and washed with brine (3 x 20 mL), dried over MgSO$_4$, filtered and concentrated. The residue was purified by sg chromatography (10:1 hexane:ethyl acetate) to afford carbaOZ727 (0.14 g, 22%) as a colorless oil. $^1$H NMR (CDCl$_3$) $\delta$ 1.98–1.37 (m, 20H), 2.09–2.05 (m, 2H), 2.15 (dd, $J_1$ = 3 Hz, $J_2$ = 13.0 Hz, 2H), 2.46 (t, $J$ = 7.0 Hz, 2H), 2.58 (td, $J_1$ = 1.5 Hz, $J_2$ = 7.0 Hz, 2H), 3.74 (s, 2H), 4.01 (s, 1H), 6.06 (d, $J$ = 7 Hz, 1H); $^{13}$C NMR (CDCl$_3$) $\delta$ 15.14, 23.71, 23.73, 25.42, 29.22, 29.27, 30.48, 30.81, 34.01, 34.04, 35.70, 36.97, 37.00, 37.06, 37.38, 52.83, 69.57, 73.46, 80.55, 83.24, 110.68, 170.13. HRMS (ESI-TOF) m/z: [M]$^+$ Calcd for C$_{22}$H$_{31}$NO$_3$ 357.2304; Found 357.2319.


Figure S1. Optimization of assay parameters as determined by Western blot. A) Different incubation times of *P. falciparum* cultures with 1000 ng/ml ozonide alkyne, exposure 10 s; B) Different concentrations of ozonide alkyne at 4 h drug incubation, exposure 30 s. Parasite cultures were lysed with RIPA-lysis buffer (see Material and Methods for details).
Figure S2. Washing of the neutravidin-coupled agarose beads after reaction with biotinylated peptides as determined by silver stain. *P. falciparum* cultures were incubated with 100 ng/ml AA2 or 0.1% DMSO for 4 h. Protein extracts were prepared with 1.2% SDS in PBS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Wash</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Beads only</td>
</tr>
<tr>
<td>2</td>
<td>No treatment</td>
</tr>
<tr>
<td>3</td>
<td>Artemisinin alkyne 2</td>
</tr>
<tr>
<td>4</td>
<td>DMSO</td>
</tr>
<tr>
<td>5</td>
<td>DMSO, beads preincubated with BSA</td>
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<tr>
<td>6</td>
<td>DMSO, beads preincubated and incubated with BSA</td>
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<tr>
<td>7</td>
<td>DMSO, beads preincubated with milk powder</td>
</tr>
<tr>
<td>8</td>
<td>DMSO, beads preincubated and incubated with milk powder</td>
</tr>
<tr>
<td>9</td>
<td>DMSO</td>
</tr>
</tbody>
</table>

5x wash (too harsh):
- Denaturation solution
- PBS
- 1% SDS
- PBS
- H₂O

7x wash (final protocol):
- 1% SDS
- 1% SDS
- 1% SDS
- Na-citrate 25 mM
- 6 M Urea
- PBS
- H₂O

*Guanidine thiocyanate 4 M
Na-citrate 25 mM
Sarcosyl 0.5%*
Figure S3. Washing of the neutravidin-coupled agarose beads after reaction with biotinylated peptides as determined by Western blot. *P. falciparum* cultures were treated with 100 ng/ml AA2 or 0.1% DMSO for 4 h and extracts were prepared with 1.2% SDS in PBS. Exposure time 1 min.
**Figure S4.** Chemical proteomics with artemisinin alkyne (AA2) on synchronized early ring stages of two *P. falciparum* strains, the K13 propeller mutant Cam3.1\(^{R539T}\) (red) and the engineered revertant Cam3.1\(^{rev}\) (blue). The numbers in the Venn diagram are proteins enriched at least 4-fold in the AA2-treated samples over the DMSO-treated control. The single protein present in all three replicates was a putative nucleoside triphosphate hydrolase (the ortholog of PF3D7_1352700) in *P. falciparum* Cam3.1\(^{R539T}\) and a putative zinc finger protein (the ortholog of PF3D7_1450400) in *P. falciparum* Cam3.1\(^{rev}\). The NTP hydrolase was also present in two replicates of *P. falciparum* Cam3.1\(^{rev}\). See Table S3 for all data and protein descriptions.