Supporting Information

Fast Copper-Free Click DNA Ligation by the Ring-Strain Promoted Alkyne-Azide Cycloaddition Reaction

Montserrat Shelbourne*, Xiong Chen*, Tom Brown* and Afaf H. El-Sagheer*a,b*

*aSchool of Chemistry, University of Southampton, Highfield, Southampton. SO17 1BJ, UK.

*bChemistry Branch, Dept. of Science and Mathematics, Faculty of Petroleum and Mining Engineering, Suez Canal University, Suez 43721, Egypt.

* TB and AHE-S are joint main authors.
**General Information:** All reagents were purchased from Aldrich, Fluka, Avocado, Acros Organics or Baseclick GmbH (www.baseclick.eu) and used without purification with the exception of the following solvents, which were purified by distillation: THF (over sodium wire), DCM, DIPEA, Et$_3$N and pyridine (over calcium hydride). Chemical transformations were carried out under an atmosphere of argon using oven-dried glassware. Thin layer chromatography (TLC) was performed using Merck Kieselgel 60 F24 silica gel plates (0.22 mm thickness, aluminium backed) and the compounds were visualised by irradiation at 254 nm or by staining with ceric sulfate or ninhydrin solution. Column chromatography was carried out under argon pressure using Fisher Scientific DAVISIL 60Å (35-70 micron) silica.

$^1$H NMR spectra were measured at 300 MHz on a Bruker AC300 spectrometer or at 400 MHz on a Bruker DPX400 spectrometer. The $^{13}$C NMR spectra were measured at 75 MHz and 100 MHz respectively on the same spectrometers. The $^{31}$P NMR spectrum was recorded on a Bruker AC300 spectrometer at 121 MHz. Chemical shifts are given in ppm relative to tetramethylsilane, and $J$ values are quoted in Hz. All spectra were internally referenced to the appropriate residual undeuterated solvent signal, assignment of the compounds was aided by COSY ($^1$H-$^1$H) and HMQC/HMBC ($^1$H-$^{13}$C) experiments.

All the low-resolution mass spectra were recorded using electrospray ionisation on a Fisons VG platform instrument or a Waters ZMD quadrupole mass spectrometer in HPLC grade acetonitrile. High-resolution mass spectra were recorded in HPLC grade acetonitrile using electrospray ionisation on a Bruker APEX III FT-ICR mass spectrometer. Electrospray Mass spectrometry of oligonucleotides was recorded in water using a Bruker micrOTOF™ II focus ESI-TOF MS instrument in ES$^+$ mode.

![Chemical Structure](image)

**SI 1.** Synthesis of the DIBO monomer 3: (i) 3-amino-1-propanol, DCM, Et$_3$N, 88% yield (ii) 2-cyanoethyl-$N,N$-diisopropyl chlorophosphoramidite, DCM, DIPEA, 73% yield.
3-\{[N-(3-hydroxypropyl)carbamoyl]oxy\}-7,8-didehydro-1,2:5,6-dibenzocyclooctyne (2b)

To a solution of active ester 2 (0.17 g, 0.44 mmol) in anhydrous DCM (5 mL) was added anhydrous Et$_3$N (0.4 mL) followed by 3-amino-1-propanol (0.04 mL, 0.53 mmol) under an argon atmosphere at room temperature. The reaction mixture was stirred for 30 min at room temperature, diluted with DCM (10 mL) and the resulting mixture was extracted with saturated brine (2 x 10 mL). The combined extracts were dried over Na$_2$SO$_4$, and the solvent was removed \textit{in vacuo}. The crude product was purified by column chromatography (50-65\% EtOAc/Hexane) to give compound 2b as a white solid (0.13 g, 0.39 mmol, 88\%). $R_f$ 0.29 (65\% EtOAc/Hexane).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.42 (d, 1H, $J = 7.6$ Hz, CH-Ar), 7.37 - 7.11 (m, 7H, CH-Ar), 5.44 (m, 1H, CHO=C=O), 3.77 - 3.52 (m, 2H, CH$_2$OH), 3.29 (m, 2H, NHCH$_2$), 3.09 (dd, 1H, $J = 14.9, 1.8$ Hz, CH$_2$), 2.85 (dd, 1H, $J = 15.2, 3.5$ Hz, CH$_2$), 2.26 (br. s., 1H, OH), 1.65 (m, 2H, NHCH$_2$CH$_2$CH$_2$OH).

$^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 156.7 (C=O), 152.2 (C-Ar), 151.2 (C-Ar), 130.2 (CH-Ar), 128.4 (CH-Ar), 128.2 (CH-Ar), 127.4 (CH-Ar), 127.4 (CH-Ar), 126.6 (CH-Ar), 126.3 (CH-Ar), 124.1 (C-Ar), 123.9 (CH-Ar), 121.6 (C-Ar), 113.3 (C-Ar), 110.2 (C-Ar), 77.1 (CHO=C=O), 59.9 (CH$_2$OH), 46.5 (CH$_2$), 38.1 (HNCH$_2$), 32.9 (HNCH$_2$CH$_2$CH$_2$OH).

LRMS [ES$^+$, MeCN]: $m/z$ (%): 322.2 ([M+H]$^+$, 64\%), 344.2 ([M+Na]$^+$, 50\%).

HRMS [ES$^+$]: C$_{20}$H$_{19}$NNaO$_3$ requires 344.1263 found 344.1252.
3-(N-{3-O-[(2-cyanoethyl-N,N-diisopropyl)phosphoramidyl]propyl}carbamoyloxy)-7,8-didehydro-1,2:5,6-dibenzocyclooctyne (3)

Alcohol 2b (0.9 g, 2.8 mmol) was co-evaporated with anhydrous DCM (3 x 3 mL) before being dissolved in anhydrous DCM (20 mL) followed by the addition of anhydrous DIPEA (1 mL, 5.6 mmol). 2-Cyanoethyl-N,N-diisopropylchlorophosphoramidite (0.75 mL, 3.36 mmol) was added dropwise, and the reaction mixture was left to stir at room temperature for 40 min, then transferred under argon into a separating funnel that contained degassed DCM (30.0 mL). The mixture was washed with degassed saturated aqueous KCl (30.0 mL), and the organic layer was separated, dried over Na₂SO₄, filtered and the solvent removed in vacuo. The phosphoramidite product was purified by column chromatography under argon pressure (40% EtOAc/Hexane, 0.5% pyridine) to give the title compound 3 (1.07 g, 2.04 mmol, 73%). Rᵣ 0.26 (30% EtOAc/Hexane).

³¹P NMR (121 MHz, CD₃CN) δ 148.5

¹H NMR (400 MHz, CD₃CN) δ 7.58 (d, 1H, J = 7.6 Hz, CH-Ar), 7.46 - 7.29 (m, 7H, CH-Ar), 6.05 - 5.90 (m, 1H, NH), 5.36 (br. s., 1H, CHOC=O), 3.86 - 3.69 (m, 2H, POCH₂), 3.76 - 3.63 (m, 2H, CΗ₂O), 3.68 – 3.54 (m, 2H, CH(CH₃)₂), 3.31 - 3.09 (m, 3H, NHCH₂ & CH₂), 2.83 (dd, 1H, J = 14.9, 3.8 Hz, CH₂), 2.63 (t, 2H, J = 6.1 Hz, CH₂-CN), 1.86 - 1.70 (m, 2H, NHCH₂CH₂CH₂O), 1.26 - 1.09 (m, 12H, (CH(CH₃)₂).

LRMS [ES⁺, MeCN] m/z (%): 544.3 ([M+Na]⁺, 100%).

HRMS [ES⁺]: C₂₃H₃₆N₃NaO₄P requires 544.2341 found 544.2335.
SI 2. Synthesis of the NSCO active ester 1: (i) EDC, N-hydroxysuccinimide, DIPEA, DCM, 51 % yield.

2,5-Dioxopyrrolidin-1-yl 4-(cyclooct-2-ynylmethyl)benzoate (1)

\[
\text{N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl) (0.39 g, 2.06 mmol) was added with stirring to a suspension of carboxylic acid 4 (0.2 g, 0.82 mmol), } N,N'\text{-diisopropylethylamine (DIPEA) (0.51 mL, 2.96 mmol) and N-hydroxysucinimide (0.24 g, 2.06 mmol) in DCM (7.0 mL) over molecular sieves. The reaction was left to stir at room temperature for 4 hr. The suspension was then diluted with DCM (40 mL) and washed with dilute HCl (20 mL, 1M) then distilled water (3x30 mL). The aqueous layer was back-extracted by DCM (1 x 20 mL). The combined extracts were dried over Na}_2\text{SO}_4, \text{and the solvent was removed in vacuo. The crude product was purified by column chromatography (Pet Ether: Toluene:EtOAc 3:1:0.5) to give compound 1 as a white solid (0.14 g, 50.6%). R}_f 0.26 (40\% EtOAc/Petroleum Ether).}
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\[\text{\textsuperscript{1}H NMR (400 MHz, CDCl}_3)\delta 8.06 (d, 2H, } J = 8.0 \text{ Hz, CH-Ar), 7.37 (d, 2H, } J = 8.5 \text{ Hz, CH-Ar), 2.90 (br s, 4H, 2xCH}_2\text{), 2.82-2.67 (m, 2H, CH}_2\text{), 2.81-2.72 (m, 1H, CH), 2.24-1.72 (m, 7H, CH}_2\text{), 1.67-1.57 (m, 1H, CH}_2\text{), 1.48-1.38 (m, 2H, CH}_2\text{).}\]

\[\text{\textsuperscript{13}C NMR (100 MHz, CDCl}_3)\delta 169.3 (NC=O), 161.8 (OC=OC-Ar), 148.1 (Ar-CC=O), 130.6 (C-Ar), 129.5 (C-Ar), 122.9 (CH}_2\text{C-Ar), 95.7 (C=C), 95.3 (C=C), 41.7 (CH}_2\text{), 40.4 (CH}_2\text{), 36.4 (CH), 34.7 (CH}_2\text{), 29.9 (CH}_2\text{), 28.44 (CH}_2\text{), 25.7 (CH}_2\text{), 20.8 (CH}_2\text{).}\]

LRMS [ES\textsuperscript{+}, MeCN] \textit{m/z} (%): 403.3 ([M+MeCN+Na]\textsuperscript{+}, 100 %).

HRMS [ES\textsuperscript{+}]: C\textsubscript{20}H\textsubscript{21}N\textsubscript{1}NaO\textsubscript{4} requires 362.1363 found 362.1357.
Oligonucleotide synthesis and azide labelling:
Standard DNA phosphoramidites, solid supports, and additional reagents were purchased from Link Technologies Ltd or Applied Biosystems (UK) Ltd. For 5’-amino group addition TFA-protected aminohexyl phosphoramidite was used and for 3’-amino group addition TFA-protected aminolink C7-solid support was employed (both from Link Technologies). Oligonucleotides were synthesized on an Applied Biosystems 394 automated DNA/RNA synthesizer using a standard 0.2 or 1.0 μmol phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping, and iodine oxidation. Stepwise coupling efficiencies and overall yields were determined by the automated trityl cation conductivity monitoring facility on the DNA synthesizer and in all cases were >98.0 %. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. The coupling times were 35 seconds for normal A, G, C, and T monomers and the coupling time for the alkyne phosphoramidite monomer 3 and 5’-TFA-aminohexyl phosphoramidite was extended to 6 min. Cleavage of oligonucleotides from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia solution for 5 hr at 55 °C. For the modified ODN-4 containing DIBO phosphoramidite monomer 3, N(4)-acetyl dC, N(2)-dmf dG and N(6)-benzoyl dA amidites were used (fast deprotecting monomers), and deprotection was achieved by exposure to concentrated aqueous ammonia solution for 1 hr at 55 °C. The oligonucleotides were subsequently heated in ammonia for prolonged periods (> 5 hr) to determine their stability. The oligonucleotides were gel-filtered using disposable NAP-10 columns (GE Healthcare) according to the manufacturer’s instructions then freeze-dried before labelling. For azide-labelling, 6-azidohexanoic acid NHS ester1 (1 mg) in DMSO (80 µL) was added to the freeze-dried 3’-amino-modified oligonucleotide (1.0 μmol) in 80 µL of 0.5 M Na2CO3/NaHCO3 buffer at pH 8.75 (4 hr, room temperature) to give ODN-3. The fully-labelled oligonucleotides were gel-filtered using disposable NAP-10 sephadex columns (GE Healthcare) and purified by reversed-phase HPLC.

Labelling of oligonucleotide with alkyne NSCO (1)
The NSCO active ester 1 (2 mg) in DMF (160 µL) was added post-synthetically to the freeze dried amino-modified oligonucleotide (1.0 μmol) in 0.5 M Na2CO3/NaHCO3 buffer (160 µL) at pH 8.75 (4 hr, room temperature), The fully-labelled oligonucleotide (ODN-2) was desalted by using NAP-10 sephadex columns and purified by reversed-phase HPLC.
Labelling of oligonucleotide with alkyne DIBO (2)
The DIBO active ester 2 (1 mg) in DMF (80 µL) was added post-synthetically to the freeze dried amino-modified oligonucleotide (1.0 µmol) in 0.5 M Na₂CO₃/NaHCO₃ buffer (80 µL) at pH 8.75 (4 hr, 55 °C). The fully-labelled oligonucleotide (ODN-1) was desalted using a NAP-10 sephadex column and purified by reversed-phase HPLC.

General method for the templated and non-templated ligation reactions in the absence of Cu¹ (SPAAC)
A solution of template (ODN-5) and azide (ODN-3) oligonucleotides (0.2 nmol of each) in 0.2 M NaCl (100 µL) was annealed for 30 min at room temperature, after which the alkyne oligonucleotide (0.2 nmol) was added. The reaction mixture was left at room temperature for a specified period of time before adding 50 µL formamide and loading directly onto a 20% polyacrylamide/7 M urea gel. It was electrophoresed at a constant power of 20W for 3 hr in 0.09 M Tris-borate-EDTA buffer. The gel-purified product was analysed and characterised by mass spectrometry. For the non-templated reactions the same conditions were used without the addition of the template oligonucleotide.

Ligation reactions in the presence of Cu¹
A solution of template ODN-5 and azide ODN-3 (0.2 nmol of each) in 0.2 M NaCl (50 µL) was annealed for 30 min at room temperature. In the meantime tris-hydroxypropyl triazole ligand² (28 nmol in 42 µL 0.2 M NaCl), sodium ascorbate (40 nmol in 4 µL 0.2 M NaCl) and CuSO₄.5H₂O (4 nmol in 4.0 µL 0.2 M NaCl), were added under argon to the alkyne oligonucleotide (0.2 nmol) which was added immediately to the solution containing the template and azide oligonucleotides. The reaction mixture was kept under argon at room temperature for the desired time before analysing the reaction by adding 50 µL formamide and loading directly onto a 20% polyacrylamide electrophoresis gel.

In another experiment, Cu¹ solution was added to the azide oligonucleotide in the presence of the template oligonucleotide then the alkyne oligonucleotide was added. This gave similar results to adding the Cu¹ solution to the alkyne oligonucleotide then adding this mixture to the azide and template oligonucleotides.
Ligation reactions in the presence of Cu^I and EDTA
Template ODN-5 and azide ODN-3 (0.2 nmol of each) in 0.2 M NaCl (50 μL) were annealed for 30 min at room temperature. In the meantime tris-hydroxypropyl triazole ligand (28 nmol in 42 μL 0.2 M NaCl), sodium ascorbate (40 nmol in 4 μL 0.2 M NaCl) and CuSO_4·5H_2O (4 nmol in 4.0 μL 0.2 M NaCl), were added under argon to the alkyne oligonucleotide (0.2 nmol) and left at room temperature for 10 min before adding EDTA (400 nmol in 4 μL 0.2 M NaCl). The mixture was left for 10 min at room temperature then added to the solution containing the template and azide oligonucleotides. The reaction mixture was kept under argon at room temperature for 30 min before adding 50 μL formamide and loading directly onto a 20% polyacrylamide gel for electrophoresis.

Binding of cylcooctyne to Cu^I
Tris-hydroxypropyl triazole ligand (28 nmol in 57 μL 0.2 M NaCl), sodium ascorbate (40 nmol in 2 μL 0.2 M NaCl) and CuSO_4·5H_2O (4 nmol in 1 μL 0.2 M NaCl), were added to the alkyne oligonucleotides (0.2 nmol in 440 μL 0.2 M NaCl) under argon and the reaction was left at room temperature for 20 min before the reagents were removed using a NAP-10 sephadex gel-filtration column. The aqueous solution was then freeze dried overnight before re-dissolving the solid in 100 μL 0.2 M NaCl which was then added to template ODN-5 and azide ODN-3 (0.2 nmol of each). The reaction mixture was then left at room temperature for 30 min before adding 50 μL formamide and loading directly onto a 20% polyacrylamide gel for electrophoretic analysis of the reactions. For the non-templated reactions the same conditions were used without addition of the template oligonucleotide.

Discrimination between fully matched and single base pair mismatched templates
A solution of the template oligonucleotide (fully matched ODN-5 or mismatched ODN-11) and alkyne oligonucleotide ODN-1 (0.2 nmol of each) in 0.2 M NaCl (100 μL) was annealed for 5 min at 45 °C, after which the azide oligonucleotide ODN-3 (0.2 nmol) was added. The reaction mixture was left at 45 °C for 5 min before adding 50 μL formamide and loading directly onto a 20% polyacrylamide/7 M urea gel which was electrophoresed at a constant power of 20W for 3 hr in 0.09 M Tris-borate-EDTA buffer. Identical results were obtained from the SPAAC reaction if the azide oligonucleotide was annealed to the templates and the alkyne oligonucleotide was added subsequently.
SI 3. SPAAC reaction of DIBO and NSCO alkyne oligonucleotides (ODN-1) and (ODN-2), respectively, in the presence of Cu$^+$ and EDTA. Lane 1: control starting oligonucleotide (ODN-3), lanes 2 and 3: templated SPAAC reactions, lanes 4 and 5: Cu$^+$ templated reactions, lanes 6 and 7: Cu$^+$ templated reaction in the presence of EDTA. All reactions were performed at 2 µM oligonucleotide concentration for 30 min at room temperature, in 0.2 M NaCl buffer, before loading onto a 20% polyacrylamide gel.

SI 4. Comparison between SPAAC templated and non-templated reactions for the DIBO alkyne added as an active ester (ODN-1) and as a phosphoramidite (ODN-4). Lane 1: control starting oligonucleotide (ODN-3), Lanes 2-4: templated reactions using (ODN-1); 0 min, 5 min, 30 min, lanes 5-7: Non-templated reactions using (ODN-1); 0 min, 5 min, 30 min, lanes 8-10: template reactions using (ODN-4); 0 min, 5 min, 30 min, lanes 11-13: non-templated reactions using (ODN-4); 0 min, 5 min, 30 min. All reactions performed at 2 µM oligonucleotide in 0.2 M NaCl at room temperature, before loading on a 20% polyacrylamide gel.
Labelling of ODN-4 with 6-carboxyfluorescein amidopropylazide

6-Carboxyfluorescein amidopropylazide (56.8 nmol, 10 eq.) (Baseclick GmbH, Munich) was added post-synthetically to the alkyne-modified oligonucleotide (ODN-4, 5.68 nmol) in 40 µL of DMSO: 20 µL 0.2 M NaCl buffer and set aside for 1 hr at 37 °C. The fully labelled oligonucleotide (ODN-9) was then desalted by using a NAP-10 sephadex column and analysed by capillary electrophoresis and mass spectrometry.

SI 5. Capillary electrophoresis (CE) analysis; A) Starting ODN-4, B) Products: two isomers ODN-9, C) mixed injection between the product ODN-9 and starting ODN-4. The x-axis is time [min], y-axis is UV absorbance at 254 nm. The CE was performed by injecting 0.4 OD/100 mL onto a ssDNA 100-R Gel, Tris-borate-7 M Urea (Kit No 477480) on a Beckman Coulter P/ACE™ MDQ Capillary Electrophoresis system using 32 Karat software. Inject-voltage 10.0 kv and separation-voltage 9.0 kv (45.0 min duration).
**Ultraviolet Melting studies**

UV melting experiments were performed on a Varian Cary 4000 Scan UV-Visible spectrophotometer at 1 μM oligonucleotide concentration in 10 mM sodium phosphate with 200 mM NaCl buffer at pH 7.0. The melting temperature ($T_m$) was calculated at 260 nm using Cary WinUV Thermal application software. The samples were initially denatured by heating to 84 °C at 10 °C/min then cooled to 20 °C at 1 °C/min$^{-1}$ and heated to 80 °C at 1 °C/min$^{-1}$. Three successive melting curves were measured and averaged.

$T_m$ values:

ODN-5 + ODN-10 (native control): 66.1 °C

ODN-5 + ODN-2 (NSCO) 59.2 °C

ODN-5 + ODN-1 (DIBO active ester) 58.5 °C

ODN-5 + ODN-4 (DIBO phosphoramidite) 58.5 °C

SI 6. UV melting analysis; A) Melting Curves and B) Derivatives.
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<th>Code</th>
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F = fluorescein, \(z\) = amino C7 labelled with 6-azidohexanoic acid, \(^{1}\)K = aminohexyl labelled with NSCO (1), \(^{DIBO}\)\(^{1}\)K = aminohexyl labelled with DIBO (2), \(^{DIBO}\)\(^{1}\)K\(_{1}\) = dibenzocyclooctynyl derived from phosphoramidite (3). \(X\), \(Y\) and \(Z\) = ligated triazole products derived from ODN-3 with ODN-1, ODN-2 and ODN-4 respectively. Fz\(^{DIBO}\) = fluoresceinamidopropyl azide + DIBO.

References