Exporting Metal-Carbene Chemistry to Live Mammalian Cells: Copper-Catalyzed Intracellular Synthesis of Quinoxalines Enabled by N–H Carbene Insertions

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1. General information

Synthetic procedures for the preparation of precursors were performed under an atmosphere of dry nitrogen using vacuum-line and standard Schlenk techniques, unless otherwise indicated. Dry solvents were directly purchased from Sigma Aldrich and used without further purification. Water was deionized and purified on a Millipore Milli-Q® Integral system. Phosphate buffered saline (PBS 10X, pH 7.2) was prepared following standard procedures and diluted ten times with water before use. Dulbecco’s Modified Eagle’s Medium (Gibco™ DMEM) was purchased from ThermoFisher Scientific. Fetal Bovine Serum (FBS) was purchased from Sigma Aldrich. HeLa cell lysates were obtained from 2 days cultured HeLa cells: after two washings with PBS, cells were scraped from the well, sonicated and diluted with PBS to reach the indicated concentration.

Chemicals were purchased from Sigma Aldrich, Alfa Aesar, Fluka, Iris Biotech and Carbosynth and used without further purification. BSA was purchased from Sigma Aldrich as lyophilized powder with purity ≥98% (SKU-A3059). 6-Carboxytetramethylrhodamine succinimidyl ester (6-TAMRA-NHS) was purchased from Carbosynth.

Amino acids were purchased as protected Fmoc amino acids with the standard side chain protecting scheme: Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Asp(Ot-Bu)-OH and Fmoc-Lys(Alloc)-OH.

Compounds 2a-c,1,2 and BTTA3 are known compounds and were synthesized according to the literature. Their 1H and 13C NMR data were in complete agreement with the reported values.

All catalytic reactions were carried out without particular precautions to extrude moisture or oxygen and open to air unless otherwise stated. Reaction mixtures were stirred using Teflon-coated magnetic stir bars. The abbreviation “r.t.” refers to reactions carried out approximately at 23 °C. Temperature was maintained using Thermowatch-controlled heating blocks. Thin-layer chromatography (TLC) was performed on silica gel plates (Merck 60 silica gel F254) and components were visualized by observation under UV light. Flash column chromatography was carried out on silica gel (Merck Geduran® Si 60, 40 – 63 μm silica gel, normal phase).

Concentration refers to the removal of volatile solvents via distillation using a rotary evaporator Büchi R-210 equipped with a thermostated bath B-491, a vacuum regulator V-850, followed by residual solvent removal under high vacuum. Dryings were performed with anhydrous Na2SO4.

1H NMR (300 MHz) and 13C NMR (75 MHz) spectra were recorded at room temperature on a Varian Mercury 300 MHz spectrometer. 1H NMR (500 MHz) and 13C NMR (126 MHz) spectra were
recorded at room temperature on a Bruker DRX-500 spectrometer. Data are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, bs = broad singlet), coupling constants in Hertz (Hz). The chemical shifts for protons (δ) are reported in parts per million downfield from tetramethylsilane and are referenced to residual protium in the NMR solvent (CDCl₃ δ = 7.26). Chemical shifts for carbon are reported in parts per million downfield from tetramethylsilane and are referenced to the carbon resonances of the solvent (CDCl₃ δ = 77.16). NMR spectra were analyzed using MestreNova© NMR data processing software (www.mestrelab.com).

High resolution mass spectra (HRMS) were acquired using electrospray (ESI) and were recorded at either the CACTUS facility of the University of Santiago de Compostela or the CACTUS facility of the University of Vigo.

UV Measurements were performed using a Jasco V-670 spectrometer.

Fluorescence measurements were performed using a Horiba FluoroMax®-3 spectrophotometer thermostated cell compartment at 20 ± 0.5 °C using 1 cm quartz cells. The measurements were made with the following settings: increment 1.0 nm, averaging time 0.2 s, excitation slit width 5.0 nm, emission slit width 5.0 nm, PMT voltage 620 V.

Electrospray Ionization Mass Spectrometry (ESI/MS) was performed with an Agilent Technologies 6120 Quadrupole using direct injection of a solution of the compound into the MS.

Analytical HPLC was performed on an Agilent 1260 Infinity II coupled to an Agilent Technologies 6120 Quadrupole LC-MS using a flow rate of 0.35 mL/min at room temperature. The initial conditions for the solvent system were H₂O/MeCN (95:5) followed by a gradual change over 12 min to H₂O/MeCN (5:95). The chromatogram was recorded via UV absorption at λ = 270 nm.

Electrospray Ionization Mass Spectrometry (ESI/MS) was performed using an Agilent 1260 Infinity II coupled to an Agilent Technologies 6120 Quadrupole LC-MS by analyzing the peaks obtained after analytical HPLC (method described above).

Quantification experiments by HPLC-MS were performed at the CACTUS facility of the University of Vigo. ICP analysis were performed at the CACTUS facility of the University of Lugo.
2. Synthesis of substrates (2a-c)

Synthesis of ethyl 2-diazo-3-oxo-3-phenylpropanoate (2a)

Procedure adapted from Jiang et al.\textsuperscript{[1]}

![Chemical structure](image)

To a solution of \(p\)-acetamidobenzenesulfonyl azide (\(p\)-ABSA, 2.4 g, 10.0 mmol, 1.2 eq.) in dry acetonitrile (17.0 mL), at 0 °C under nitrogen, was added ethyl benzoylacetate (1.8 mL, 8.3 mmol, 1.0 eq.). Then, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 1.5 mL, 10.0 mmol, 1.2 eq.) was added dropwise and the resulting red solution was stirred at 0 °C for 3 h and slowly brought to room temperature. Upon completion as indicated by TLC, the reaction was quenched with water and extracted with ethyl acetate. The combined organic phases were dried over anhydrous Na\(_2\)SO\(_4\), filtered and concentrated under reduced pressure. The residue was purified by silica flash column chromatography using hexane / EtOAc (9:1) as eluent to afford product 2a as a yellow oil. \(^1\)H NMR in concordance with the data reported in the literature.\textsuperscript{[1]}

\(R_f = 0.17\) (Hexane / EtOAc 9:1).

\textbf{Yield} = 99% (1.8 g, 8.2 mmol).

\(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.64 – 7.61 (m, 2H), 7.53 (t, \(^3\)J = 7.4 Hz, 1H), 7.46 - 7.39 (m, 2H), 4.25 (q, \(^3\)J = 7.1 Hz, 2H), 1.26 (t, \(^3\)J = 7.1 Hz, 3H).

Synthesis of ethyl 2-diazo-3-oxobutanoate (2b)

Procedure adapted from Jiang et al.\textsuperscript{[1]}

![Chemical structure](image)

To a solution of \(p\)-acetamidobenzenesulfonyl azide (\(p\)-ABSA, 865.0 mg, 3.6 mmol, 1.2 eq.) in dry acetonitrile (6.0 mL), at 0 °C under nitrogen, was added ethyl benzoylacetate (476.0 µL, 3.0 mmol, 1.0 eq.). Then, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 538.0 µL, 3.6 mmol, 1.2 eq.)
was added dropwise and the resulting orange solution was stirred at 0 °C for 5 h and slowly brought to room temperature. Upon completion as indicated by TLC, the reaction was quenched with water and extracted with ethyl acetate. The combined organic phases were dried over anhydrous Na$_2$SO$_4$, filtered and concentrated under reduced pressure. The residue was purified by silica flash column chromatography using hexane / EtOAc (9:1) as eluent to afford product 2b as a yellow oil. $^1$H NMR in concordance with the data reported in the literature.$^{[1]}$

R$_f$ = 0.32 (Hexane / EtOAc 8:2).

Yield = 78% (365.1 mg, 2.3 mmol).

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 4.30 (q, $^3$J = 7.1 Hz, 2H), 2.48 (s, 3H), 1.33 (t, $^3$J = 7.1 Hz, 3H).

**Synthesis of 2-diazo-acetophenone (2c)**

Procedure adapted from Zhang et al.$^{[2]}$

![Chemical structure](image)

To a solution of benzoylacetone (649.0 mg, 4.0 mmol, 1.0 eq.) and $p$-ABSA (961.0 mg, 4.0 mmol, 1.0 eq.) in ethanol (4.0 mL) was added MeNH$_2$ (40% aqueous solution, 0.42 mL, 4.8 mmol, 1.2 eq.). Upon addition of methylamine, an exothermic reaction was observed; the reaction mixture turned orange, then pale-yellow and the mixture turned into a thick paste. To ensure an adequate stirring, 4.0 mL of ethanol were added and the mixture was stirred at room temperature for 1 h. Upon completion of the reaction, as indicated by TLC, the mixture was concentrated under reduced pressure and the residue was purified by silica flash column chromatography using hexane / EtOAc (9:1) as eluent to afford 2c as a yellow solid. $^1$H NMR in concordance with the data reported in the literature.$^{[2]}$

R$_f$ = 0.24 (Hexane / EtOAc 9:1).

Yield = 85% (495.0 mg, 3.4 mmol).

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.79 – 7.74 (m, 2H), 7.59 – 7.51 (m, 1H), 7.48 – 7.42 (m, 2H), 5.91 (s, 1H).
3. Metal screening and optimization of the reaction conditions

Representative general procedure for the synthesis of benzo[g]quinoxaline 3a via N-H carbene insertion / annulation in water using different metal catalysts

To a 2.0 mL vial containing a stir bar and diazocarbonyl 2a (21.8 mg, 0.1 mmol, 1.0 eq.), Milli-Q water (1.0 mL) was added, followed by 2,3-diaminonaphthalene 1 (15.8 mg, 0.1 mmol, 1.0 eq.) and the corresponding metal catalyst (10 mol%). The Thermowatch-controlled heating block was fixed at 40 °C, and the heterogeneous reaction mixture was stirred for 24 h, open to air. Then, the mixture was transferred to a separating funnel and extracted with ethyl acetate (3 x 3 mL). The combined organic phases were dried over anhydrous Na₂SO₄, filtered, concentrated under reduced pressure, and the crude was analyzed by ¹H NMR. The yield was determined by ¹H NMR using 1,3,5-trimethoxybenzene as internal standard.
Table S1. Yields of 3a via N-H carbene insertion / annulation in water in the presence of different metal catalysts.[a]

| Entry | Metal catalyst             | Yield (%)[^b] |
|-------|----------------------------|--------------|
| 1     | FeCl₃                      | 0            |
| 2     | Fe(acac)₃                  | 0            |
| 3     | FeTPPCl                    | 0            |
| 4     | [Rh(COD)Cl]₂               | 24           |
| 5     | [Cp*IrCl₂]₂               | 20           |
| 6     | [Ir(COD)(OMe)]₂           | 33           |
| 7     | JohnPhosAu(MeCN)SbF₆       | 18           |
| 8     | CuCl₂                      | 62           |
| 9     | Cu(SO₄)·5H₂O              | 67           |
| 10    | [(MeCN)₄Cu]OTf            | 64           |
| 11    | [(MeCN)₄Cu]PF₆            | 62           |
| 12    | Cu-BTTAA[^c]              | 59           |

[a] 1 (0.1 mmol), 2a (0.1 mmol), Milli-Q water (1.0 mL), metal catalyst (10 mol%), 40 °C, 24 h.
[b] Yields determined by ¹H NMR using 1,3,5-trimethoxybenzene as internal standard.
[c] Cu-BTTAA was freshly prepared as follows: a solution of CuSO₄·5H₂O (0.5 mL, 20 mM in H₂O) was added to a solution of BTTAA (0.5 mL, 40 mM in H₂O) and the mixture was stirred for 20 min at room temperature; this solution was added to a vial containing diazo 2a, followed by addition of diamine 1. BTTAA = 3-4-[(bis[(1-1,1-dimethylene)-1H-1,2,3-triazol-4-yl]methy]amino)methyl]-1H-1,2,3-triazole-acetic acid.

Control reaction in the absence of diamine 1

Following the previous procedure, a control reaction was carried out in the absence of diamine 1: To a 2.0 mL vial containing a stir bar and diazocarbonyl 2a (21.8 mg, 0.1 mmol, 1.0 eq.), Milli-Q water (1.0 mL) was added, followed by Cu(OAc)₂·H₂O (10 mol%). The Thermowatch-controlled heating block was fixed at 40 °C and the reaction mixture was stirred for 24 h, open to air. Then, the mixture was transferred to a separating funnel and extracted with ethyl acetate (3 x 3 mL). The combined organic phases were dried over anhydrous Na₂SO₄, filtered, concentrated under reduced pressure, and the crude was analyzed by ¹H NMR. HPLC-MS analysis showed a mixture of O-H insertion product 4 and dimerization product 4'.

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Figure S1. HPLC-MS analysis of the reaction of 2a with Cu(OAc)$_2$·H$_2$O (10 mol%) at 40 °C for 24 h.
Representative general procedure for the optimization of the Cu(OAc)$_2$ catalyzed N-H carbene insertion / annulation of 1 and 2a in water

To a 20 mL tube containing diazocarbonyl 2a (43.6 mg, 0.2 mmol, 1.0 eq.) was added a solution of Cu(OAc)$_2$·H$_2$O in Milli-Q water (2.0 mL), followed by 2,3-diaminonaphthalene 1 (31.6 mg, 0.2 mmol, 1.0 eq.). The Radleys Carousel 12 Plus Reaction was fixed at 40 °C and the heterogeneous reaction mixture was stirred for 24 h, open to air. Then, 3.0 mL of water were added, and the mixture was extracted with ethyl acetate (3 x 10 mL). The combined organic phases were dried over anhydrous Na$_2$SO$_4$, filtered, concentrated under reduced pressure, and the crude was analyzed by $^1$H NMR using 1,3,5-trimethoxybenzene as internal standard.

Table S2. Optimization of the reaction conditions for the Cu(OAc)$_2$ catalyzed N-H carbene insertion of 1 and 2a in water.[a]

| Entry | Catalyst loading (mol%) | Concentration (mM) | Ratio 1:2a | Yield (%)$^{[b]}$ |
|-------|--------------------------|--------------------|------------|------------------|
| 1     | 10                       | 100                | 1:1        | 70               |
| 2     | .[c]                     | -                  | -          | 51               |
| 3     | 5$^{[d]}$                | -                  | -          | 66               |
| 4     | .[e]                     | -                  | 1:1.5      | 59               |
| 5     | .[f]                     | -                  | -          | 62               |
| 6     | 5$^{[g]}$                | -                  | -          | 72               |
| 7     | 5                        | -                  | 1.5:1      | 75               |
| 8     | 5$^{[g]}$                | -                  | 1:1.5      | 80               |
| 9     | 5                        | 50                 | -          | 61               |
| 10    | 5                        | 25                 | -          | 52               |
| 11    | -                        | 10                 | -          | 74$^{[a]}$       |

[a] Only deviations from the standard conditions (shown in entry 1, 0.2 mmol scale) are indicated in the table. [b] Yield of 3a determined by $^1$H NMR using 1,3,5-trimethoxybenzene as internal standard. [c] DMSO (10% v/v) was used as cosolvent. [d] MeCN (20% v/v) was used as cosolvent. [e] The reaction was carried out in the absence of solvent. [f] The reaction was performed using deoxygenated Milli-Q water, under N$_2$ atmosphere; the extraction was carried out using a solution of ethylenediaminetetraacetic acid tetrasodium salt (EDTA tetrasodium salt, 10.0 mg) in water (3.0 mL) to ensure the absence of copper catalyst during the work-up of the reaction. [g] 0.02 mmol scale.
Table S3. Reaction kinetics of the Cu(OAc)$_2$ catalyzed N-H carbene insertion of 1 and 2a in water.$^{[a]}$

| Entry | Time | Yield (%)$^{[b]}$ |
|-------|------|-----------------|
| 1     | 30 min | 8               |
| 2     | 1 h    | 32              |
| 3     | 2 h    | 44              |
| 4     | 3 h    | 55              |
| 5     | 4 h    | 61              |
| 6     | 6 h    | 62              |
| 7     | 8 h    | 62              |
| 8     | 24 h   | 70              |

$^{[a]}$ Cu(OAc)$_2$·H$_2$O (10 mol%), 100 mM substrates in H$_2$O, 40 °C. $^{[b]}$ Yield determined by $^1$H NMR using 1,3,5-trimethoxybenzene as internal standard.

Synthesis of ethyl 3-phenylbenzo[g]quinoxaline-2-carboxylate 3a following the optimized conditions for the copper-catalyzed N-H carbene insertion

To a 20 mL tube containing diazocarbonyl 2a (43.6 mg, 0.2 mmol, 1.0 eq.) was added a solution of Cu(OAc)$_2$·H$_2$O (2.0 mg, 0.01 mmol, 5 mol%) in Milli-Q water (2.0 mL), followed by 2,3-diaminonaphthalene 1 (31.6 mg, 0.2 mmol, 1.0 eq.). The Radleys Carousel 12 Plus Reaction was fixed at 40 °C and the heterogeneous reaction mixture was stirred for 24 h, open to air. Then, 3.0 mL of water were added, and the mixture was extracted with ethyl acetate (3 x 10 mL). The combined organic phases were dried over anhydrous Na$_2$SO$_4$, filtered and concentrated under reduced pressure. The residue was purified by silica flash column chromatography using hexane / EtOAc (9:1) as eluent to afford product 3a as a yellow solid. $^1$H NMR in concordance with the data reported in the literature.$^{[4]}$

R$_f$ = 0.51 (Hexane / EtOAc 7:3).

Yield = 66% (43.4 mg, 0.13 mmol).
\( ^1H \text{ NMR} \) (300 MHz, CDCl\(_3\)): \( \delta \) 8.81 (s, 1H), 8.76 (s, 1H), 8.14 (dd, \( J = 5.4, 2.8 \) Hz, 2H), 7.81 (dd, \( J = 6.7, 3.0 \) Hz, 2H), 7.66 – 7.57 (m, 2H), 7.57 – 7.50 (m, 3H), 4.37 (q, \( ^3J = 7.1 \) Hz, 2H), 1.21 (t, \( ^3J = 7.1 \) Hz, 3H).

\( ^{13}C \text{ NMR} \) (75 MHz, CDCl\(_3\)) \( \delta \) 166.7 (C=O), 152.5 (C \text{ ipso}), 146.8 (C \text{ ipso}), 138.5 (C \text{ ipso}), 138.1 (C \text{ ipso}), 136.5 (C \text{ ipso}), 135.1 (C \text{ ipso}), 134.3 (C \text{ ipso}), 129.9 (CH), 128.9 (CH), 128.8 (2xCH), 128.7 (CH), 128.7 (3xCH), 128.0 (CH), 127.8 (CH), 127.3 (CH), 62.6 (CH\(_2\)), 13.9 (CH\(_3\)).

HRMS-ESI Calculated for C\(_{21}\)H\(_{16}\)N\(_2\)O\(_2\) [M+H]\(^+\): 329.1285; found: 329.1235.

**Synthesis of ethyl 3-methylbenzo[g]quinoxaline-2-carboxylate 3b**

Following the standard procedure described above, starting from diazocarbonyl \( 2b \) (31.2 mg, 0.2 mmol, 1.0 eq.) and using in this case 10 mol\% of Cu(OAc)\(_2\):H\(_2\)O (4.0 mg, 0.02 mmol), product \( 3b \) was obtained after purification by silica flash column chromatography (hexane / EtOAc (9:1)) as a yellow solid. \( ^1H \text{ NMR} \) in concordance with the data reported in the literature.\([4]\)

\( R_f = 0.32 \) (Hexane / EtOAc 7:3).

Yield = 57% (30.2 mg, 0.11 mmol).

\( ^1H \text{ NMR} \) (500 MHz, CDCl\(_3\)): \( \delta \) 88.74 (s, 1H), 8.56 (s, 1H), 8.07 (dd, \( J = 7.6, 2.1 \) Hz, 2H), 7.61 – 7.51 (m, 2H), 4.57 (q, \( ^3J = 7.2 \) Hz, 2H), 2.96 (s, 3H), 1.51 (t, \( ^3J = 7.2 \) Hz, 3H).

\( ^{13}C \text{ NMR} \) (126 MHz, CDCl\(_3\)) \( \delta \) 165.7 (C=O), 152.9 (C \text{ ipso}), 145.5 (C \text{ ipso}), 138.5 (C \text{ ipso}), 136.6 (C \text{ ipso}), 135.1 (C \text{ ipso}), 133.8 (C \text{ ipso}), 129.0 (CH), 128.9 (CH), 128.6 (CH), 127.7 (CH), 126.9 (CH), 126.7 (CH), 62.6 (CH\(_2\)), 24.2 (CH\(_3\)), 14.4 (CH\(_3\)).

HRMS-ESI Calculated for C\(_{16}\)H\(_{14}\)N\(_2\)O\(_2\) [M+H]\(^+\): 267.1128; found: 267.1128.
4. Copper catalyzed N-H carbene insertion under biologically relevant conditions

Representative general procedure for the copper catalyzed N-H carbene insertion in different biological media (exemplified using PBS as solvent)

\[
\text{(1, 1.0 eq.)} + \text{(2a, 1.0 eq.)} \rightarrow \text{(3a)}
\]

To a 20 mL tube containing diazocarbonyl \text{2a} (43.6 mg, 0.2 mmol, 1.0 eq.) in PBS (1.9 mL) was added 2,3-diaminonaphthalene \text{1} (31.6 mg, 0.2 mmol, 1.0 eq.), followed by a solution of Cu(OAc)\(_2\)-H\(_2\)O (4.0 mg, 0.02 mmol, 10 mol%) in water (0.1 mL). The Radleys Carousel 12 Plus Reaction was fixed at 40 °C and the reaction mixture was stirred for 24 h, open to air. Then, 3.0 mL of water were added, and the mixture was extracted with ethyl acetate (3 × 10 mL). The combined organic phases were dried over anhydrous Na\(_2\)SO\(_4\), filtered, concentrated under reduced pressure, and the crude was analyzed by \(^1\)H NMR using 1,3,5-trimethoxy benzene as internal standard.

The reaction was further studied in HeLa cell lysates at different concentrations and catalyst loading.

**Table S4.** Cu(OAc)\(_2\) catalyzed N-H carbene insertion of \text{1} and \text{2a} in HeLa cell lysates at different concentrations / catalyst loading.[a]

| Entry | Catalyst loading (mol%) | Concentration (mM) | Yield (%)[^b] |
|-------|-------------------------|--------------------|--------------|
| 1     | 10                      | 100                | 81           |
| 2     | 5                       | 100                | 73           |
| 3     | 10                      | 50                 | 80           |
| 4     | 10                      | 25                 | 63           |

[a] HeLa cell lysates: 4.0 mg/mL in PBS, Cu(OAc)\(_2\)-H\(_2\)O (5 or 10 mol%), for 100 mM, 40 °C, 24 h. [^b] Yield determined by \(^1\)H NMR using 1,3,5-trimethoxy benzene as internal standard.
N-H Carbene insertion of 1 and 2a in water in presence of biologically relevant additives (exemplified using cytosine as additive)

To a 20 mL tube containing diazocarbonyl 2a (43.6 mg, 0.2 mmol, 1.0 eq.) in Milli-Q water (2.0 mL) was added cytosine (1.0 eq. or 0.1 eq.) followed by 2,3-diaminonaphthalene 1 (31.6 mg, 0.2 mmol, 1.0 eq.). To this mixture, Cu(OAc)$_2$·H$_2$O (4.0 mg, 0.02 mmol, 10 mol%) was added, the Radleys Carousel 12 Plus Reaction was fixed at 40 °C and the reaction mixture was stirred for 24 h, open to air. Then, the work-up of the reaction was performed as described above, and the crude was analyzed by $^1$H NMR using 1,3,5-trimethoxybenzene as internal standard.

Table S5. Cu(OAc)$_2$ catalyzed N-H carbene insertion of 1 and 2a in the presence of 1.0 or 0.1 eq. of biologically relevant additives.^[a]

| Entry | Additive             | Yield 3a (%)^[b] |
|-------|----------------------|------------------|
|       |                      | 1.0 eq. | 0.1 eq. |
| 1     | Glycine              | 0       | 65     |
| 2     | L-Valine             | 0       | 68     |
| 3     | D-Glutamic acid      | 0       | 68     |
| 4     | Nα-Boc-L-lysine      | 56      | .^[c]  |
| 5     | L-Histidine          | 0       | 70     |
| 6     | L-Tyrosine           | 61      | .^[c]  |
| 7     | N-Acetyl-L-cysteine  | 0       | 60     |
| 8     | Boc-L-methionine     | 19      | 57     |
| 9     | Boc-glycine          | 61      | .^[c]  |
| 10    | D-Glucose            | 62      | .^[c]  |
| 11    | Cytosine             | 51      | .^[c]  |
| 12    | Adenine              | 0       | 0      |
| 13    | Guanine              | 50      | .^[c]  |
| 14    | Thymine              | 60      | .^[c]  |
| 15    | Sodium ascorbate     | 55      | .^[c]  |
| 16    | L-Glutathione reduced| 0       | 0 (66^[d]) |

[a] Reaction conditions: 1 eq. 1 and 2a, 1.0 or 0.1 eq. additive, Cu(OAc)$_2$·H$_2$O (10 mol%), 100 mM substrates in H$_2$O, 40 °C, 24 h. [b] Yield determined by $^1$H NMR using 1,3,5-trimethoxybenzene as internal standard. [c] The reaction was not carried out using 0.1 eq. of the additives. [d] The reaction was performed using PBS as the reaction media.
5. Spectroscopic studies of substrates 1, 2a and product 3a

For the UV-Vis spectroscopic studies, solutions of substrates 1, 2a and product 3a (5 mM in DMSO) were freshly prepared and diluted with 1.0 mL H₂O into a quartz Hellma® fluorescence cuvette with a pathlength 10 x 4 mm, chamber volume 1.5 mL (final concentration: 40 μM). The samples were analyzed in a Jasco V-630 UV-Vis spectrophotometer at 25 °C. UV-Vis analysis were performed in the interval of 260-600 nm. For the fluorescence measurements, solutions of 1, 2a and 3a (5 mM in DMSO) were freshly prepared and diluted with water into quartz Hellma® fluorescence cuvette with a path length 10 x 4 mm, chamber volume 1.5 mL (final concentration: 20 μM). The samples were analyzed in a Horiba FluoroMax-3 fluorescence spectrophotometer at 25 °C. The solutions of substrates were excited at 385 nm and the emission spectrum was recorded in the interval 425-720 nm.

![UV-Vis absorption spectra of 1 (blue), 2a (red) and 3a (black) (40 μM in H₂O).](image_url)

**Figure S2.** UV-Vis absorption spectra of 1 (blue), 2a (red) and 3a (black) (40 μM in H₂O).
Figure S3. Fluorescence spectra of 1 (blue), 2a (red) and 3a (black) (20 µM in H₂O, λ_{exc} = 385 nm).
6. Synthesis of Tyrphostin AG1385 (3c)

2-Phenylbenzo[g]quinoxaline (3c, Tyrphostin AG1385)

To an open vial containing 2,3-diaminonaphthalene 1 (31.6 mg, 0.2 mmol, 1.0 eq.), was added a solution of Cu(OAc)$_2$·H$_2$O (8.0 mg, 0.04 mmol, 20 mol%) in Milli-Q water (1.6 mL). The Thermowatch-controlled heating block was fixed at 40 °C, and the mixture was stirred at this temperature, open to air, with slow addition (over 4 h) of a solution of diazocarbonyl 2c (29.2 mg, 0.2 mmol, 1.0 eq.) in acetonitrile (0.4 mL). After 24 h, 3.0 mL of water were added and the mixture was extracted with ethyl acetate (3 x 10 mL). The combined organic fractions were dried and concentrated under reduced pressure. The residue was purified by silica flash column chromatography using a gradient from hexane to hexane / EtOAc (9:1) as eluent to afford Tyrphostin AG1385 (3c) as a dark-yellow solid. $^1$H NMR in concordance with the data reported in the literature.$^[5]$  

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 9.40 (s, 1H), 8.73 (s, 1H), 8.69 (s, 1H), 8.27 (dd, $J$ = 8.0, 1.7 Hz, 2H), 8.12 (dd, $J$ = 6.3, 3.5 Hz, 2H), 7.64 – 7.55 (m, 5H).

$^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 151.9 (C ipso), 144.6 (CH), 138.9 (C ipso), 138.2 (C ipso), 136.9 (C ipso), 134.4 (C ipso), 133.8 (C ipso), 130.6 (CH), 129.3 (2xCH), 128.7 (CH), 128.6 (CH), 128.1 (CH), 127.8 (2xCH), 127.7 (CH), 127.1 (CH), 126.9 (CH).

HRMS-ESI Calculated for C$_{18}$H$_{12}$N$_2$ [M+H]$^+$: 257.1073; found: 257.1071.
7. Synthesis of peptide ligand (5)

Abbreviations

BTTAA = 3-4-{{Bis[[1-(1,1-dimethylethyl)-1H-1,2,3-triazol-4-yl]methyl]amino}-methyl}-1H-1,2,3-triazole-acetic acid.
DIEA = Diisopropylethylamine.
HOBT = 1-Hydroxybenzotriazole.
HBTU = N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate.
NaDEDTC = Sodium diethyldithiocarbamate.
6-TAMRA-NHS = 6-Carboxytetramethylrhodamine N-succinimidyl ester.
TFA = Trifluoroacetic acid.
TIS = Triisopropylsilane.

General peptide synthesis procedures

Peptide ligand 5 (BTTAA-K(TMR)-R-G-D-CONH₂) was synthesized on a 0.1 mmol scale using the standard Fmoc-based amino acid protection strategy on H-Rink amide ChemMatrix® resin (0.49 mmol/g) using a mixture of HOBT/HBTU (1:1) as activating agent, diisopropylethylamine as base, and N,N-dimethylformamide (DMF) as solvent. The removal of the temporary Fmoc protecting group was performed by treating the resin with 20% piperidine in DMF for 20 min.

Alloc deprotection step was performed by treating the resin-bound peptide for 20 min with a mixture of Pd(PPh₃)₄ (11.6 mg, 0.01 mmol, 10 mol%) and phenylsilane (248.0 µL, 2.0 mmol, 20.0 eq.) in dichloromethane (6.0 mL). The resin was then washed with dichloromethane three times, and the process was repeated. Finally, the resin was washed twice with a solution of sodium diethyldithiocarbamate in DMF.

Cleavage/deprotection step was performed by treating the resin-bound peptide for 2 h with the following cleavage cocktail: 900.0 µL trifluoroacetic acid (TFA), 50.0 µL dichloromethane, 25.0 µL H₂O and 25.0 µL triisopropylsilane (1 mL of cocktail / 40 mg resin). The resin was filtered, and the TFA filtrate was concentrated with a nitrogen current to an approximate volume of 1.0 mL, which was added to ice-cold diethyl ether (10.0 mL). After 10 min, the precipitate was centrifuged and washed again with 15.0 mL of ice-cold ether. The solid residue was dried under argon, dissolved in acetonitrile/water 1:1 and purified by semi-preparative reverse-phase HPLC,
3.5 mL/min, gradient 5 to 75% B over 20 min (A: H₂O 0.1% TFA, B: MeCN 0.1% TFA) on a semipreparative Agilent Eclipse–C18 (250 × 10 mm) reverse-phase column.

The synthetic peptide was analyzed by analytical HPLC-MS using the standard conditions described in the section 1. General Information.

**Synthesis of peptide ligand BTTAA-K(TMR)-R-G-D-CNH₂ (5)**

After introducing the N-terminal Fmoc-Lys(Alloc)-OH to the RGD resin-bound peptide, Fmoc was cleaved by treatment with 20% piperidine in DMF. Then, BTTAA was coupled using 2.0 eq. (86.0 mg, 0.2 mmol), 2.0 eq. of HBTU and 2.0 eq. of HOBT (0.2 M solution in DMF) and 3.0 eq. of DIEA (0.2 M in DMF) for 60 min. Finally, alloc deprotection was carried out, followed by coupling of 6-carboxytetramethylrhodamine N-succinimidyl ester, using 1.0 eq. of 6-TAMRA-NHS (52.8 mg, 0.1 mmol), and 3.0 eq. of DIEA (0.2 M in DMF) for 60 min.
HPLC-MS characterization of peptide ligand BTTAA-K(TMR)-R-G-D-CONH₂

**Figure S4.** Left) HPLC chromatogram of the purified peptide. Gradient 5 to 95% B over 12 min. Right) Mass spectrum of the purified peptide.

ESI/MS (m/z): Calculated for C₆₂H₮₃N₂₁O₁₁ [M+H]⁺: 1298.67; found: 1298.70 [M+H]⁺; 650.00 [M+2H]²⁺; 433.80 [M+3H]³⁺.

**ESI-MS detection of 5-Cu**

To a vial containing 200 µL of a 1 mM solution of 5 in MeCN/H₂O (1:1), 20 µL of a solution of Cu(SO₄)·5H₂O (20 mM in H₂O) was added. Ten minutes later, 20 µL of the solution was injected in an Agilent Technologies 6120 Quadrupole MS, and the ESI-MS spectrum was recorded. The resulting ESI-MS spectrum shows two peaks that correspond to 5-Cu: 680.45 [M+2H]²⁺; 454.40 [M+3H]³⁺.

**Figure S5.** ESI-MS spectrum of 5-Cu. Turquoise: 5-Cu ([M+2H]²⁺, [M+3H]³⁺); Blue: 5 ([M+2H]²⁺).
8. Characterization of new compounds (3a-c)

NMR Spectra of ethyl 3-phenylbenzo[g]quinoxaline-2-carboxylate (3a)

$^1$H (CDCl$_3$, 300 MHz)
$^{13}$C (CDCl$_3$, 75 MHz)

DEPT-135 (CDCl$_3$, 75 MHz)
NMR Spectra of ethyl 3-methylbenzo[g]quinoxaline-2-carboxylate (3b)

$^1$H (CDCl$_3$, 500 MHz)
$^{13}$C (CDCl$_3$, 126 MHz)

DEPT-135 (CDCl$_3$, 126 MHz)
NMR Spectra of 2-phenylbenzo[g]quinoxaline (3c)

$^1H$ (CDCl$_3$, 300 MHz)
$^{13}\text{C} (\text{CDCl}_3, 75 \text{ MHz})$

DEPT-135 (CDCl$_3$, 75 MHz)
9. General information for the biological experiments

**General executions and substances:** All steps were performed on a sterile clean bench *Telstar AV-100* at room temperature. Solutions stored in a fridge were warmed beforehand in a water bath (37 °C). Unless otherwise specified, all incubations were performed in DMEM.

**Cell Culture:** All cell lines were cultured in DMEM (Dulbecco’s modified Eagle’s medium) containing 5 mM glutamine, penicillin (100 units/mL) and streptomycin (100 units/mL) (all from *Invitrogen*). Proliferating cultures were maintained in a 5% CO₂ humidified incubator at 37 °C.

For all the experiments, cells were seeded in the corresponding well at the indicated concentration two days before treatment.

**Protein quantification:** For protein concentration measurements the Bio-Rad DC Protein Assay Kit was used (Bio-Rad 500-0114).

**Fluorescence microscopy:** All images were obtained with an *Andor Zyla* mounted on a *Nikon TiE*. Confocal images were acquired in an *Andor Dragonfly* High Speed Confocal Platform. Images were further processed with *Image J* or *NIS* software (*Nikon*).

**Microscopy settings:** The filter sets for the observation of the fluorescence of the products were as follows:

- **Widefield:** LED λ excitation: 385 nm. Filter cube DAPI-1160B-000 (*Semrock*): BP 387/11-25 nm, LP 447/60-25 nm and DM 409 nm. Confocal: Laser excitation: 405 nm. LP 450/50 and DM 418 nm.
- **Widefield:** LED λ excitation: 385 nm. Filter cube: BP 375/28x nm, LP 515lp nm and DM 415 nm. Confocal: Laser excitation: 405 nm. LP 525/50 and DM 501 nm.
- **Widefield:** LED λ excitation: 470 nm. Filter cube FITC-3540C-000 (*Semrock*): BP 482/35 nm, LP 536/40 nm and DM 506 nm. Confocal: Laser excitation: 488 nm. LP 525/50 and DM 501 nm.
- **TMRE** (tetramethylrhodamine, ethyl ester) LED λ excitation: 550 nm. Filter cube TRITC-B-000 (*Semrock*): BP 543/22-25 nm, LP 593/40-25 nm and DM 562 nm. Confocal: Laser excitation: 561 nm. LP 620/60 and DM 567 nm.
10. Viability assays

The toxicity of copper salt Cu(OAc)$_2$•H$_2$O was tested using MTT and Propidium iodide assays in HeLa, MCF7 and HEK293 cell lines. The toxicity of copper complex 5-Cu was tested using the MTT assay in HeLa, MCF7 and HEK293 cell lines. The toxicity of substrates 1, 2a and 2c, and products 3a and 3c was tested using the MTT assays in HeLa cell line.

For the studies using HeLa cell line, 10000 cells per well were seeded in 96 well plates 2 days before performing the assay. For the studies using MCF7 and HEK293 cell lines, 15000 cells per well were seeded in 96 well plates 2 days before performing the assay.

**Propidium iodide assay:** Cells were washed once with Krebs-Ringer-HEPES (KRH) buffer. To each well, 100 µL of propidium iodide solution in KRH buffer (50 µM) were then added. After 20 min of incubation at 37 °C, initial fluorescence from each well was measured in a microtiter plate reading spectrophotometer (Tecan Infinite 200 PRO, $\lambda_{\text{exc}} = 560$ nm, $\lambda_{\text{em}} = 610$ nm). Then, different concentrations of Cu(OAc)$_2$ (25 or 50 µM) were added. Subsequently, fluorescence was measured after 1.5, 3.5, 6 and 8 h. Between measurements, microtiter plate was incubated at 37 °C. At the end of the experiment, 100 µL of digitonin in KRH (1 mM) was added to each well to permeabilize all cells and label all nuclei with propidium iodide. Fluorescence was measured again to obtain a value corresponding to 100% cell death.

Krebs-Ringer-HEPES buffer: 136 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl$_2$, 12.5 µM MgCl$_2$, 10 mM HEPES buffer pH = 7.9, 10 mM glucose. Final pH = 7.5.

**MTT assay:** Cells were incubated with different concentrations of Cu(OAc)$_2$ (12.5 – 100 µM), 5-Cu (10 – 20 µM), substrates or products (25 – 100 µM) in DMEM. After 12, 18 or 24 h of incubation, HEPES containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to a final concentration of 0.5 mg/mL. Cells were then incubated for 4 h to allow the formation of formazan precipitates by metabolically active cells. A detergent solution of 10% SDS and 0.01 M HCl was then added and the plate was incubated overnight at 37 °C to allow the solubilization of the precipitates. The quantity of formazan in each well (directly proportional to the number of viable cells) was measured by recording changes in absorbance at 570 nm in a microtiter plate reading spectrophotometer (Tecan Infinite 200 PRO).
a Pi assay

Cu(OAc)$_2$ in different cell lines

Cell viability (%)

| Duration | HeLa | MCF7 | HEK293 |
|----------|------|------|--------|
| 1.5 h    | 100  | 100  | 100    |
| 3.5 h    | 100  | 100  | 100    |
| 6 h      | 100  | 100  | 100    |
| 8 h      | 100  | 100  | 100    |

b MTT assay

Cu(OAc)$_2$ in different cell lines

Cell viability (%)

| Duration | HeLa 12 h | HeLa 24 h | MCF7 24 h | HEK293 24 h |
|----------|-----------|-----------|-----------|-------------|
| 12.5 µM  | 100       | 100       | 100       | 100         |
| 25 µM    | 100       | 100       | 100       | 100         |
| 37.5 µM  | 100       | 100       | 100       | 100         |
| 50 µM    | 100       | 100       | 100       | 100         |
| 100 µM   | 100       | 100       | 100       | 100         |
Figure S6. Viability Assays. a, Propidium iodide assay. Bars representation of the cell viability with increasing incubation times using different concentrations of Cu(OAc)$_2$ (25 or 50 µM) in HeLa, MCF7 and HEK293 cell lines. After 8 h in the case of HeLa and 6 h in the case of MCF7 and HEK293 cell lines, digitonin was added and the amount of viable cells was analyzed by fluorescence. b-d, MTT assays. Bars representation of the viability of HeLa, MCF7 or HEK293 cells incubated in cell culture medium containing the indicated amounts of: b, Cu(OAc)$_2$ (12.5 – 100 µM) for 12 or 24 h; c, 5-Cu (10 – 20 µM) for 12 or 18 h; d, substrates 1 or 2a or product 3a (25 – 100 µM) for 18 h. The viability is expressed as the fold change of the absorbance value with respect to untreated cells (value 1.0). The error bars represent the standard deviation of three different samples.
11. Carbene transfer reactions catalyzed by Cu(OAc)$_2$ in mammalian cells

Cu-catalyzed synthesis of 3a in mammalian cells

HeLa, MCF7 and HEK293 cells were seeded on glass coverslips two days before treatment. Then, they were incubated with Cu(OAc)$_2$ (50 µM) for 50 min. Cells were washed twice with DMEM and incubated with substrates 1 and 2a (100 µM) for the indicated reaction time.* Prior to observation by fluorescence microscopy, the samples were washed twice with fresh DMEM. The coverslips were observed *in vivo* in a fluorescence microscope equipped with adequate filters. Digital pictures of the different samples were taken under identical conditions of gain and exposure.

*A freshly prepared stock solution of substrates 1 and 2a (10 mM in DMSO) was used.

Figure S7. Synthesis of 3a inside live HeLa cells. Fluorescence micrographies of HeLa cells (brightfield) after incubation with: 1 and 2a (A-C); Cu(OAc)$_2$, washing and treatment with 1 and 2a for the indicated time (D-F). Reaction conditions: Cells were pretreated with 50 µM of Cu(OAc)$_2$ (stock solution in H$_2$O) for 50 min, washed twice with DMEM and incubated with 100 µM of substrates 1 and 2a (stock solutions in DMEM) for the indicated time. Scale bar: 20 µm. $\lambda_{exc} = 385$ nm, $\lambda_{em} > 520$ nm.
Figure S8. Fluorescence micrographies of control experiments in HeLa cells, brightfield (A-C) and green channel (D-F) after incubation with: 1 and 2a (A,D); Cu(OAc)$_2$, washing and treatment with either 1 (B,E) or 2a (C,F) for 1.5 h. Reaction conditions: Cells were pretreated with 50 µM of Cu(OAc)$_2$ (stock solution in H$_2$O) for 50 min, washed twice with DMEM and incubated with 100 µM of substrates 1 or 2a (stock solutions in DMEM) for the indicated time. Scale bar: 20 µm. $\lambda_{\text{exc}}$ = 385 nm, $\lambda_{\text{em}}$ > 520 nm.

Cu-catalyzed synthesis of 3c in mammalian cells

HeLa, MCF7 or HEK293 cells were seeded on glass coverslips two days before treatment. Then, they were incubated with Cu(OAc)$_2$ (25-37.5-50 µM) for 50 min. Cells were then washed twice with DMEM and incubated with substrates 1 and 2c (50-75-100 µM) for the indicated reaction time. Then TMRE (100 nM) was added, and cells were incubated for 10 min.* Prior to observation by fluorescence microscopy, the samples were washed twice with fresh DMEM. The coverslips were observed in vivo in a fluorescence microscope equipped with adequate filters. Digital pictures of the different samples were taken under identical conditions of gain and exposure.

* A freshly prepared stock solution of substrates 1 and 2c (10 mM in DMSO) was used.
Figure S9. Mitochondrial depolarization in HeLa cells. Fluorescence micrographies (brightfield) after incubation with: 1 (A); 2c (B); 1 and 2c (C); 3c (D) (100 µM, stock solutions in DMEM) for 3.5 h followed by TMRE (100 nM) for 10 min. Scale bar: 20 µm (100 µm in panel D). $\lambda_{\text{exc}} = 385$ nm, $\lambda_{\text{em}} > 520$ nm.

Figure S10. Mitochondrial depolarization in HEK293 cells. Fluorescence micrographies (brightfield) using TMRE as marker, after incubation with: 1 and 2c (A); 3c (B); Cu(OAc)$_2$, washing and treatment with 1 and 2c (C) for 3.5 h. Reaction conditions: Cells were mixed with Cu(OAc)$_2$ (50 µM) for 50 min, washed twice with DMEM and treated with substrates 1 and 2c (100 µM) for 3.5 h followed by TMRE (100 nM) for 10 min. Scale bar: 100 µm. $\lambda_{\text{exc}} = 550$ nm, $\lambda_{\text{em}} = 570-590$ nm.

For the mitochondrial fragmentation, after incubation for the specified time, cells were treated either with TMRE (Figure S11) or MitoTracker Green (Figure S12) (100 nM) for 10 min. The coverslips were observed under confocal microscopy.
Figure S11. Mitochondrial fragmentation. Fluorescence micrographies of HeLa cells (brightfield) using TMRE as marker, after incubation with: 1 and 2c (A) or Cu(OAc)$_2$, washing and treatment with 1 and 2c (C) for 1.5 h; (B) and (D) are zoom in of panels (A) and (C), respectively. Reaction conditions: Cells were mixed with Cu(OAc)$_2$ (50 µM) for 50 min, washed twice with DMEM, treated with substrates 1 and 2c (100 µM) for 1.5 h, and incubated with TMRE (100 nM) for 10 min. Scale bar: 20 µm. $\lambda_{exc} = 550$ nm, $\lambda_{em} = 570-590$ nm.

Figure S12. Mitochondrial fragmentation. Fluorescence micrographies of HeLa cells (brightfield) using MitoTracker Green as marker, after incubation with: 1 and 2c (A); 3c (B) or Cu(OAc)$_2$, washing and treatment with 1 and 2c (C) for 3 h; (D-F) Mitochondrial staining from (A-C) displayed as a skeleton network using ImageJ skeleton filter. Reaction conditions: Cells were mixed with Cu(OAc)$_2$ (50 µM) for 50 min, washed twice with DMEM, treated with substrates 1 and 2c (100 µM) for 3 h, and incubated with MitoTracker Green (100 nM) for 10 min. Scale bar: 20 µm (15 µm in panel C). $\lambda_{exc} = 470$ nm, $\lambda_{em} = 490-580$ nm.
12. Cell selective studies using 5-Cu

HeLa cells were seeded on glass coverslips two days before treatment. Then, they were incubated with 5-Cu (15 µM) for 1.5 h. Cells were then washed twice with DMEM and incubated with substrates 1, and 2a (100 µM) or 2c (50-75-100 µM) for the indicated reaction time. Then TMRE (100 nM) was added and cells were incubated for 10 min.* Prior to observation by fluorescence microscopy, the samples were washed twice with fresh DMEM. The coverslips were observed in vivo in a fluorescence microscope equipped with adequate filters. Digital pictures of the different samples were taken under identical conditions of gain and exposure.

Internalization studies

![Fluorescence micrographies of HEK293 cells (brightfield). Reaction conditions: cells were incubated with 15 µM of 5-Cu for 1.5 h. Scale bar: 20 µm.](image)

Figure S13. Fluorescence micrographies of HEK293 cells (brightfield). Reaction conditions: cells were incubated with 15 µM of 5-Cu for 1.5 h. Scale bar: 20 µm.
Synthesis of 3a promoted by 5-Cu in mammalian cells

![Figure S14](image1.png)

**Figure S14.** Synthesis of 3a inside live HeLa cells promoted by 5-Cu. Fluorescence micrographies of HeLa cells (brightfield) after incubation with: 1 and 2a (A); 5-Cu, washing and treatment with either 1 (B,D) or 1 and 2a (C,E) for 3.5 h. Scale bar: 20 µm. Reaction conditions: cells were mixed with 5-Cu (15 µM) for 1.5 h, washed twice with DMEM and treated with either 1 or 1 and 2a (100 µM) for 3.5 h followed by TMRE (100 nM) for 10 min. λ_{exc} = 385 nm, λ_{em} > 520 nm for panels (A-C); λ_{exc} = 550 nm, λ_{em} = 570-590 nm for panels (D,E).

Synthesis of 3c promoted by 5-Cu in mammalian cells

![Figure S15](image2.png)

**Figure S15.** Selective mitochondrial depolarization. Fluorescence micrographies of HEK293 cells (brightfield) after incubation with: 1 and 2c (A); 5-Cu, washing and treatment with 1 and 2c (B); 3c (C) for 3.5 h. Reaction conditions: cells were mixed with 5-Cu (15 µM) for 1.5 h, washed twice with DMEM and treated with substrates 1 and 2c (100 µM) for 3.5 h followed by TMRE (100 nM) for 10 min. Scale bar: 100 µm. λ_{exc} = 550 nm, λ_{em} = 570-590 nm.
13. ICP Analysis

Cells were seeded in 6 well plates two days before treatment.

For the ICP measurements, a total of $3 \times 10^6$ HeLa cells growing in 6 well plates were treated with 50 µM of Cu(OAc)$_2$ in DMEM for 50 minutes. Prior to digestion, the samples were washed with fresh DMEM and then twice with PBS. The obtained fractions were digested in duplicate in HNO$_3$/H$_2$O$_2$ by microwave heating and analyzed.

ICP values: Cu(OAc)$_2$ (50 µM, 50 min) = $15.088 \pm 0.047 \, \mu g \, 10^6 \, \text{cells}^{-1}$. 
14. Quantification studies using LC/MS

Quantification of the copper catalyzed N-H carbene insertion of 1 and 2a to afford benzoquinoxaline 3a in HeLa cells

For the quantification of the copper-catalyzed N-H carbene insertion of 1 and 2a in HeLa cells, a total of $12 \times 10^6$ HeLa cells growing in 6 plates of 100 mm were used.

The experiments were performed in plates of 100 mm as follows: $10^6$ cells per well were seeded in 100 mm plated two days before treatment. For each measurement, six plates were used. Cells were incubated with catalyst Cu(OAc)$_2$ (50 μM) for 50 minutes followed by two washing steps with DMEM. Then, cells were incubated with substrates 1 (100 μM) and 2a (100 μM) for 3 h or 5 h. Afterwards, the reaction media was collected for analysis in a 50 mL Falcon. Prior to extraction, cells were washed with 3 mL of DMEM, followed by two washing steps with 3 mL of PBS and the washings were also collected separately in 50 mL Falcons. Then the cell monolayer was treated with 1 mL of MeOH. After 5 min and pipetting up this solution was transferred to a 15 mL Falcon. Finally, we obtained 6 mL of methanolic extracts from the six plates employed. All the samples were lyophilized for 3 days and dissolved in MeCN until reaching a theoretical concentration of 250 μM.

![Figure S16. Schematic representation of the protocol for the quantification of the copper catalyzed reaction in mammalian cells.](image)

For the quantification of the product, the obtained samples (250 μM in MeCN) were centrifuged at 13500 rpm for 15 minutes and the supernatant was collected. In the case of the methanolic extract, it was diluted 1:4 using MeCN/H$_2$O 6:4. However, in the case of the samples of the reaction media, first, second and third washings, no dilution was required.
Each sample was injected in a Bruker Elute coupled with *timsTOF* using a column *Zarbax Eclipse XDB-C18* 2.1 x 100 mm 1.8 μm and a flow rate of 0.4 mL/min at room temperature. For the solvent system, initial conditions 80% B (A: H₂O 0.1% TFA, B: MeCN 0.1% TFA) were used for 1 min and followed by a gradual change over 7 min to 100% B, followed by a gradual change over 12 secs to 80% B and maintained for 2 min.

It’s important to mention that in all the cases we have detected significant amounts of substrate 3a.

**Results obtained after 3 h of reaction in HeLa cells**

A 250 μM standard solution in MeCN of product 3a was prepared for the calibration curve. The following dilutions were prepared using a mixture of MeCN/H₂O 6:4. For the calibration curve, we represented the intensity obtained in the MS spectra vs the concentration using an internal standard.

![Figure S17. Calibration curve of product 3a. Circle: point for calibration; diamond: injected samples.](image)

As an example, table S6 shows the values of the product 3a detected in the methanolic extracts, the reaction media and three washing steps after 3 h of reaction in HeLa cells.

**Table S6.**

| Sample             | Value (pmol) | Concentration (µM) |
|--------------------|--------------|--------------------|
| Methanolic extraction | 15.199       | 3.040              |
| Methanolic extraction | 14.695       | 2.939              |
| Methanolic extraction | 14.902       | 2.980              |
| Methanolic extraction | 14.977       | 2.820              |
| Methanolic extraction | 15.495       | 3.141              |
| Methanolic extraction | 15.704       | 3.099              |
| **Average**       | **15.015 ± 0.582** | **3.003 ± 0.116**  |
|                | Value     | Standard Deviation |
|----------------|-----------|--------------------|
| **Reaction media** | 6.436     | 0.644              |
| **Reaction media** | 6.201     | 0.620              |
| **Reaction media** | 6.140     | 0.614              |
| **Reaction media** | 6.227     | 0.623              |
| **Reaction media** | 6.135     | 0.614              |
| **Reaction media** | 6.017     | 0.602              |
| **Average**      | 6.193 ± 0.140 | 0.619 ± 0.014     |
| **First washing** | 3.437     | 0.172              |
| **First washing** | 3.116     | 0.156              |
| **First washing** | 3.055     | 0.153              |
| **First washing** | 3.036     | 0.152              |
| **First washing** | 3.415     | 0.171              |
| **First washing** | 3.333     | 0.167              |
| **Average**      | 3.232 ± 0.184 | 0.162 ± 0.009     |
| **Second washing** | 6.168     | 0.308              |
| **Second washing** | 5.831     | 0.292              |
| **Second washing** | 6.029     | 0.301              |
| **Second washing** | 6.141     | 0.307              |
| **Second washing** | 5.892     | 0.295              |
| **Second washing** | 6.067     | 0.303              |
| **Average**      | 6.021 ± 0.135 | 0.301 ± 0.007     |
| **Third washing** | 7.134     | 0.357              |
| **Third washing** | 7.285     | 0.364              |
| **Third washing** | 7.012     | 0.351              |
| **Third washing** | 7.182     | 0.359              |
| **Third washing** | 7.130     | 0.357              |
| **Third washing** | 7.275     | 0.364              |
| **Average**      | 7.170 ± 0.102 | 0.358 ± 0.005     |
| **Total Value**  |           | 4.443 ± 0.151     |
We considered the total amount of product detected in all the fractions analyzed, and we used the value $4.443 \pm 0.151 \ \mu M$ as total concentration of product generated after 3 h of reaction.

Taking into account the number of cells used in these experiments ($12 \times 10^6$ cells), we obtained a $0.370 \pm 0.013 \ \mu M \ 10^6 \ \text{cells}^{-1}$ of product.

The average of total concentration of intracellular generated product in three different experiments is: $0.405 \pm 0.045 \ \mu M \ 10^6 \ \text{cells}^{-1}$.

ICP value: $0.083 \pm 0.016 \ \mu M$ of Cu $10^6 \ \text{cells}^{-1}$.

Estimated TON = mol product / mol Cu = $0.405 \pm 0.045 / 0.083 \pm 0.016 = 4.860 \pm 0.304$.

Results obtained after 5 h of reaction in HeLa cells using 50 μM of Cu(OAc)$_2$

A 250 μM standard solution in MeCN of product 3a was prepared for the calibration curve. The following dilutions were prepared using a mixture of MeCN/H$_2$O 6:4. For the calibration curve, we represented the intensity obtained in the MS spectra vs the concentration using an internal standard.

As an example, table S7 shows the values of the product 3a detected in the methanolic extracts, the reaction media and three washing steps after 5 h of reaction in HeLa cells.

Table S7.

| Sample                  | Value (pmol) | Concentration (µM) |
|-------------------------|--------------|--------------------|
| Methanolic extraction   | 31.467       | 6.293              |
| Methanolic extraction   | 30.207       | 6.041              |
| Methanolic extraction   | 29.485       | 5.897              |
| Methanolic extraction   | 29.587       | 5.917              |
| Methanolic extraction   | 29.149       | 5.830              |
| Methanolic extraction   | 29.572       | 5.914              |
| Average                 | $29.911 \pm 0.836$ | $5.982 \pm 0.167$ |
| Reaction media          | 11.778       | 1.178              |
| Reaction media          | 11.227       | 1.123              |
| Reaction media          | 11.070       | 1.107              |
| Reaction media          | 11.255       | 1.126              |
|                          | 1.107 | 1.155 |
|--------------------------|-------|-------|
| Reaction media           | 11.073| 1.107 |
| Reaction media           | 11.547| 1.155 |

|                          | 1.133 ± 0.028 |
|--------------------------|---------------|
| **Average**              |               |
| First washing            | 8.278         | 0.414 |
| First washing            | 8.420         | 0.421 |
| First washing            | 8.064         | 0.403 |
| First washing            | 7.905         | 0.395 |
| First washing            | 7.971         | 0.399 |
| First washing            | 7.811         | 0.391 |

|                          | 0.404 ± 0.012 |
|--------------------------|---------------|
| **Average**              |               |
| Second washing           | 9.993         | 0.500 |
| Second washing           | 9.503         | 0.475 |
| Second washing           | 9.565         | 0.478 |
| Second washing           | 9.718         | 0.486 |
| Second washing           | 9.690         | 0.485 |
| Second washing           | 9.433         | 0.472 |

|                          | 0.483 ± 0.010 |
|--------------------------|---------------|
| **Average**              |               |
| Third washing            | 7.583         | 0.379 |
| Third washing            | 6.933         | 0.347 |
| Third washing            | 7.167         | 0.358 |
| Third washing            | 7.264         | 0.363 |
| Third washing            | 7.117         | 0.356 |
| Third washing            | 7.027         | 0.351 |

|                          | 0.359 ± 0.011 |
|--------------------------|---------------|
| **Average**              |               |
| **Total Value**          | 8.361 ± 0.228 |

We considered the total amount of product detected in all the fractions analyzed, and we used the value 8.361 ± 0.228 μM as total concentration of product generated after 5 h of reaction.

Taking into account the number of cells used in these experiments (12 x 10⁶ cells), we obtained a 0.697 ± 0.019 μM 10⁶ cells⁻¹ of product.
The average of total concentration of intracellular generated product in two different experiments is: $0.597 \pm 0.141 \mu M \cdot 10^6 \text{ cells}^{-1}$.

ICP value: $0.083 \pm 0.016 \mu M$ of Cu $10^6 \text{ cells}^{-1}$.

Estimated TON = mol product / mol Cu = $0.597 \pm 0.141 / 0.083 \pm 0.016 = 7.193 \pm 0.429$.

Quantification of the copper catalyzed N-H carbene insertion of 1 and 2c to afford Tyrphostin AG1385 (3c) in HeLa cells.

Results obtained after 4.5 h of reaction using 50 μM of Cu(OAc)$_2$

A 250 μM standard solution in MeCN of product 3c was prepared for the calibration curve. The following dilutions were prepared using a mixture of MeCN/H$_2$O 6:4. For the calibration curve, we represented the intensity obtained in the MS spectra vs the concentration using an internal standard.

![Figure S18. Calibration curve of product 3c. Circle: point for calibration; diamond: injected samples.](image)

As an example, table S8 shows the values of the product 3c detected in the methanolic extracts, the reaction media and three washing steps after 4.5 h of reaction in HeLa cells.
Table S8.

| Sample                        | Value (pmol) | Concentration (µM) |
|-------------------------------|--------------|-------------------|
| Methanolic extraction        | 17.631       | 3.526             |
| Methanolic extraction        | 18.256       | 3.651             |
| Methanolic extraction        | 18.309       | 3.662             |
| Methanolic extraction        | 18.113       | 3.623             |
| Methanolic extraction        | 18.383       | 3.677             |
| Methanolic extraction        | 17.338       | 3.468             |
| Average                      | 18.005 ± 0.423 | 3.601 ± 0.085    |
| Reaction media               | 9.018        | 0.902             |
| Reaction media               | 8.835        | 0.884             |
| Reaction media               | 9.132        | 0.913             |
| Reaction media               | 8.778        | 0.878             |
| Reaction media               | 8.525        | 0.853             |
| Reaction media               | 8.595        | 0.860             |
| Average                      | 3.800 ± 0.163 | 0.881 ± 0.023    |
| First washing                | 10.688       | 0.534             |
| First washing                | 10.857       | 0.543             |
| First washing                | 10.886       | 0.544             |
| First washing                | 11.444       | 0.572             |
| First washing                | 11.297       | 0.565             |
| First washing                | 11.136       | 0.557             |
| Average                      | 11.051 ± 0.289 | 0.553 ± 0.014    |
| Second washing               | 2.658        | 0.133             |
| Second washing               | 2.775        | 0.139             |
| Second washing               | 2.527        | 0.126             |
| Second washing               | 2.711        | 0.136             |
| Second washing               | 2.625        | 0.131             |
| Second washing               | 2.724        | 0.136             |
| Average                      | 2.670 ± 0.087 | 0.134 ± 0.004    |
We considered the total amount of product detected in all the fractions analyzed, and we used the value $5.310 \pm 0.129 \, \mu M$ as total concentration of product generated after 4.5 h of reaction.

Taking into account the number of cells used in these experiments ($12 \times 10^6$ cells), we obtained a $0.443 \pm 0.011 \, \mu M \, 10^6 \text{cells}^{-1}$ of product.

The average of total concentration of product generated in three different experiments is: $0.352 \pm 0.080 \, \mu M \, 10^6 \text{cells}^{-1}$.

ICP value: $0.083 \pm 0.016 \, \mu M$ of Cu $10^6 \text{cells}^{-1}$.

Estimated TON = mol product / mol Cu = $0.352 \pm 0.080 / 0.083 \pm 0.016 = 4.241 \pm 0.420$.

**Figure S19.** Quantification of turnover numbers for the generation of 3c in HeLa cells. The error bars represent the standard deviation of three different samples.
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