Supplemental Information

A Photoreactive Small Molecule Probe for 2-Oxoglutarate Oxygenases

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Table S1. Peptide sequences for 2-OG oxygenases identified by mass spectrometry[a].

DR025

ID	Protein	Peptide	Score[b]
Q9GZT9	**PHD2**	K.FTDGQLVSQKSDSSKDIR.G	17
		K.FTDGQLVSQKSDSSKDIR.G	26
P41229	**JARID1C**	R.GRALERRR.R	23
		R.QVEQARWLDEVKR.T	24
Q9Y2K7	**FBXL11**	R.IANVTLIDLR.G	31
O60568	**LH3**	R.GGDVARTVGGGQKVR.W	26
		R.GGDVARTVGGGQKVR.W	30
Q6N021	**TET2**	R.RNSPYSQTMK.S	10
		R.KYGTLTNRRCALNEER.T	21
Q9UPP1	**PHF8**	R.ILKIHRNGKLLL.	7
		R.QADCKMKLGDFVK.Y	21
Q15652	**JMJD1C**	R.SRANQNVNAVHSHYTR.A	25
		R.SRANQNVNAVHSHYTR.A	28
		R.SRANQNVNAVHSHYTR.A	21
		R.SRANQNVNAVHSHYTR.A	19
		R.SRANQNVNAVHSHYTR.A	41
Q9UGL1	**JARID1B**	K.LHFTPRIQRLNELEAQTR.V	29
O94953	**JMJD2B**	R.HPVDISAIPEQR.W	12
		K.NRAASFQAERKFNAAAAR.T	13
		K.NRAASFQAERKFNAAAAR.T	17
		K.NRAASFQAERKFNAAAAR.T	15

[a]See also Table 2.

[b]The scores were determined using Mascot version 2.3 (Matrix Science) and defined as the absolute probability that the observed match is a random event. The score is reported as $-10*LOG_{10}(P)$, where P is the absolute probability

Table S2.Proteins identified by mass spectrometry after probe capture and UV exposure.
The protein identified are listed with the following information (excel spreadsheet): column A: protein hit number; column B: protein accession number for SwissProt; column C: protein description; column D: protein molecular weight; column E: number of peptide matches; column F: protein sequence coverage in %; column G: number of MS/MS queries; column H: observed peptide masses [m/z]: column I: calculated peptide masses; column J: peptide charge states; column K: theoretical peptide masses; column L: delta masses; column M: number of missed cleavages; column N: peptide scores; column O: peptide sequence: column P: observed variable modifications.
.

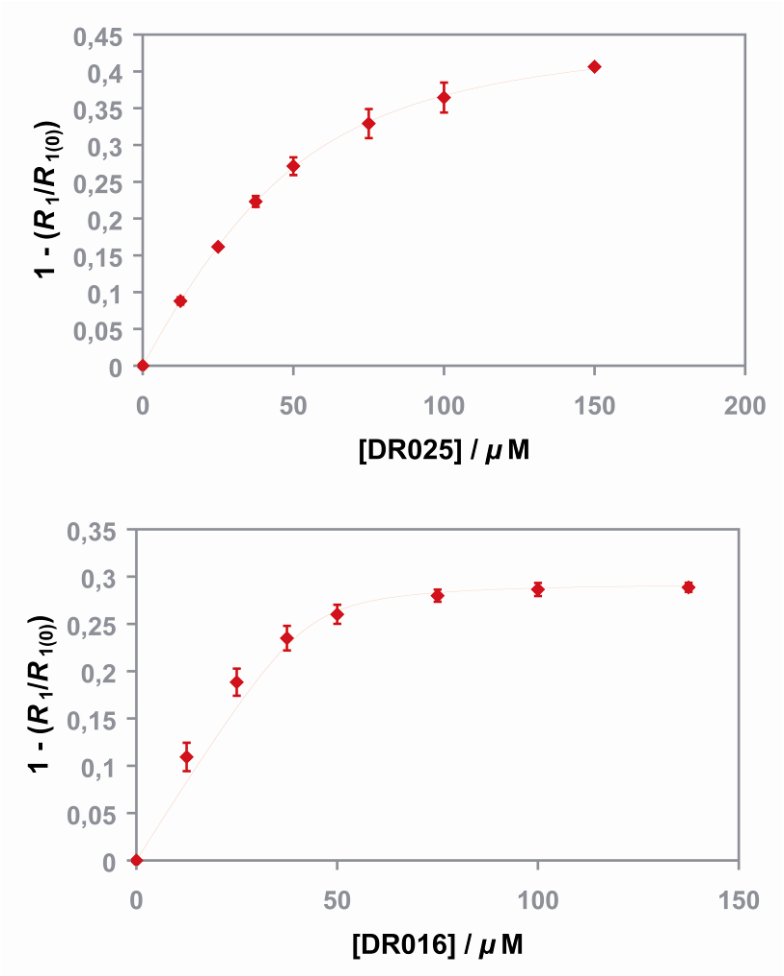


Figure S1A (related to Table 1B). Curves for NMR-based K_D Values Determination with PHD2

Experimental conditions were as reported in Experimental Procedures and in Leung et al., 2010.

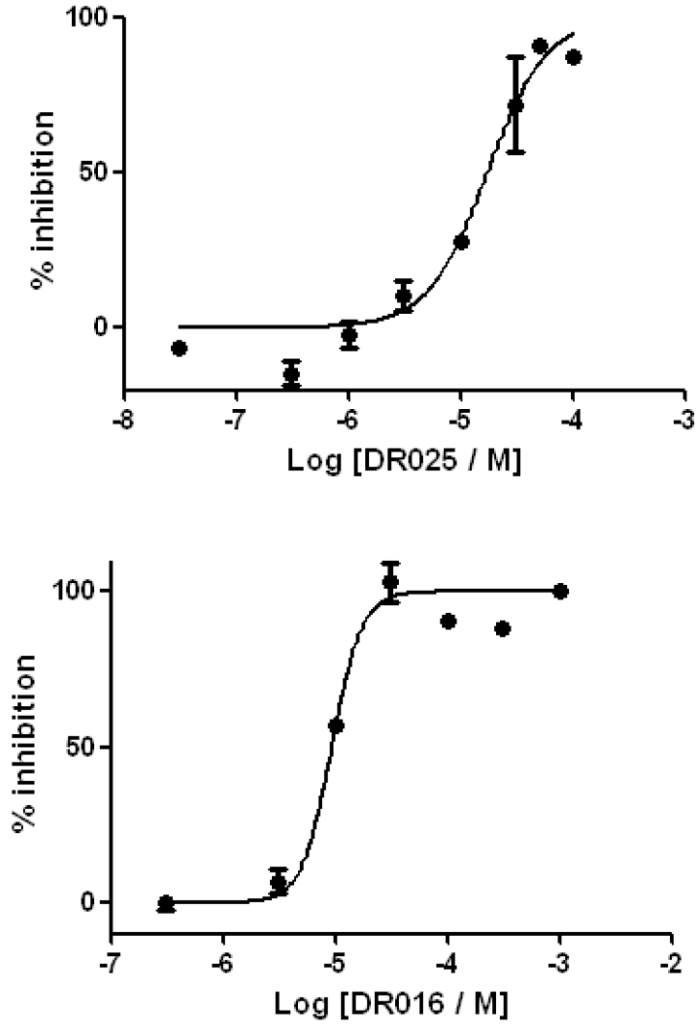


Figure S1B (related to Table 1B). Curves for MALDI-based IC_{50} Values Determination with PHD2

Experimental conditions were as reported in Experimental Procedures and in Flashman et al., 2010.

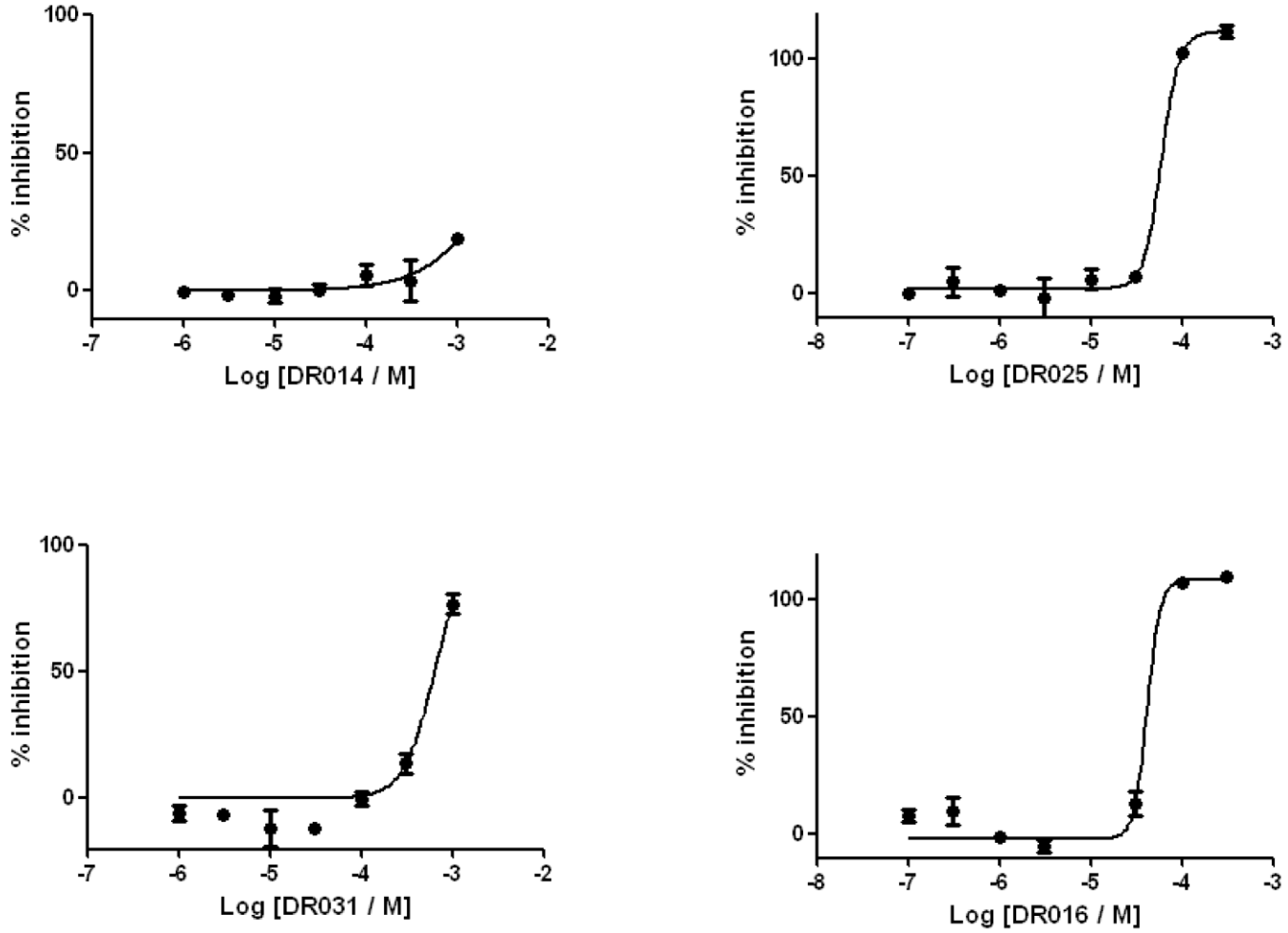


Figure S1C (related to Table 1B). Curves for FDH Coupled IC_{50} Values Determination with JMJD2E

Experimental conditions were as reported in Experimental Procedures and in Rose et al., 2008.

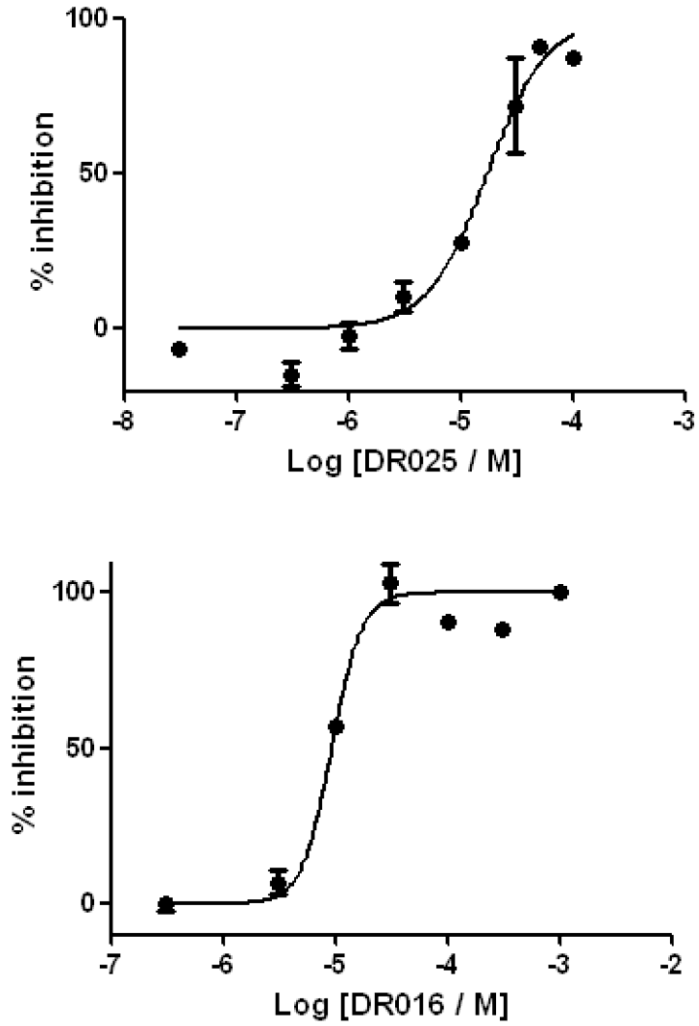


Figure S1D (related to Table 1B). Curves for FDH Coupled IC_{50} Values Determination with FBXL11 (KDM2A)

Experimental conditions were as reported in Experimental Procedures, and in Couture et al., 2007 and Rose et al., 2008.

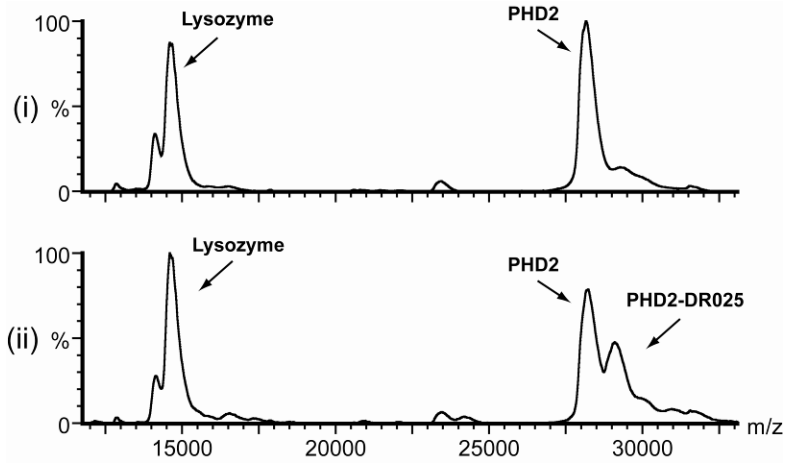


Figure S2A (related to Figure 1). Selective Photo Labelling of PHD2 by DR025 in a Simplified Protein Mixture Analysed by MALDI

Selective capture of $PHD2_{181-426}$ was investigated in the presence of other different proteins. $PHD2_{181-426}$ (5 μM), lysozyme (5 μM), lactic dehydrogenase (5 μM) and DR025 (25 μM) were incubated (45 min, r.t.) in Tris buffer in the presence of Mn(II) (5 μM). After irradiation on ice (20 min, 365 nm), the resulting solutions were analyzed by MALDI-MS. (i) and (ii) show results before and after UV irradiation, respectively.

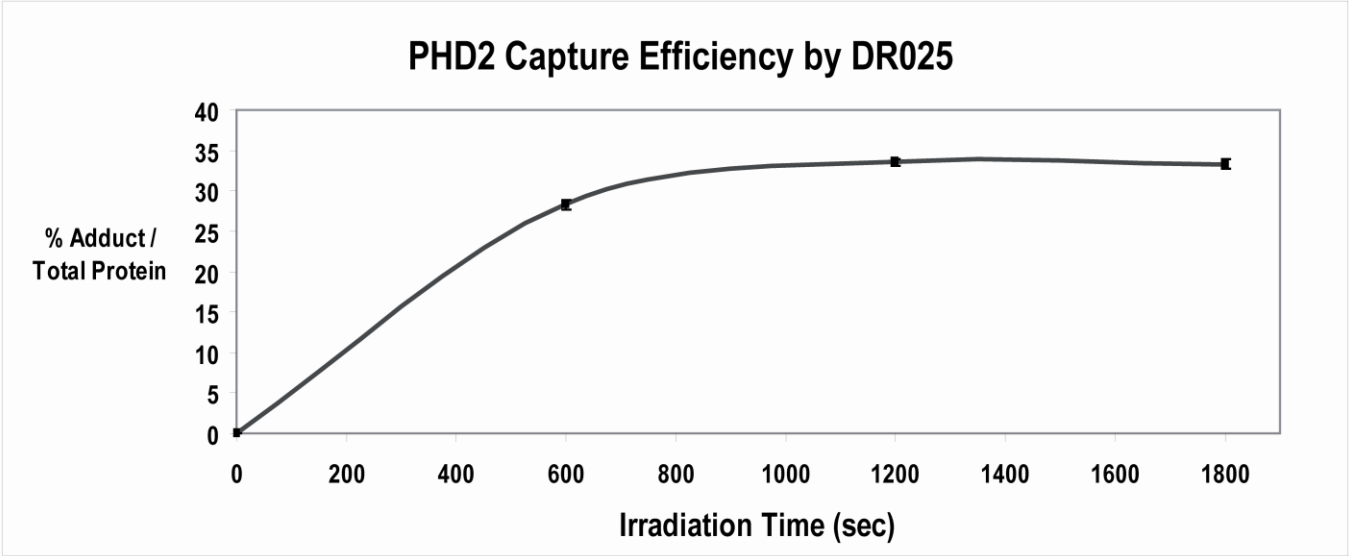


Figure S2B (related to Figure 1). Efficiency of PHD2 Capture by DR025

$PHD2_{181-426}$ (5 μM) and DR025 (25 μM) were incubated (45 min, r.t.) in Tris buffer in the presence of Mn(II) (5 μM) with analysis by MALDI. The yield of cross-linking was evaluated from the ratio between the area of the MALDI peak of the covalent adduct and the total area of the two peaks corresponding to the uncaptured and captured protein. All experiments were carried out in triplicate.

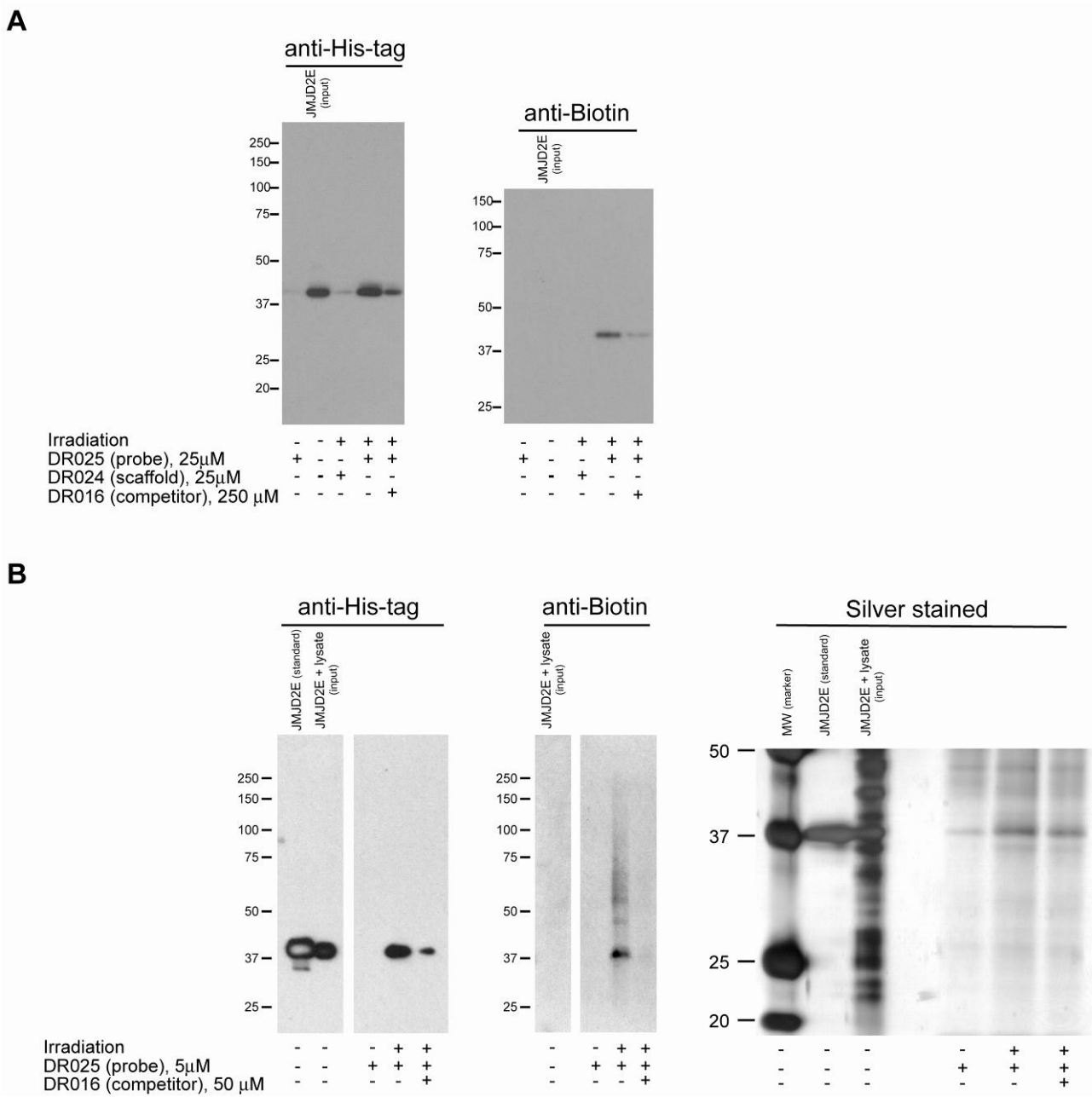

A
anti-His-tag
JMJD2E (input)
250
150
100
75
50
37
25
20
anti-Biotin
JMJD2E (input)
150
100
75
50
37
25
Irradiation
DR025 (probe), 25μM
DR024 (scaffold), 25μM
DR016 (competitor), 250 μM
B
anti-His-tag
JMJD2E (standard)
JMJD2E + lysate (input)
anti-Biotin
JMJD2E + lysate (input)
Silver stained
MW (marker)
JMJD2E (standard)
JMJD2E + lysate (input)
50
37
25
20
Irradiation
DR025 (probe), 5μM
DR016 (competitor), 50 μM

Figure S3 (related to Figure 3). Photo-Affinity Labelling and Enrichment of Purified His-tag JMJD2E Analysed by Western Blotting and Silver Staining

(A) Western blot detection of the capture of purified His-tag JMJD2E by DR025. His-tag JMJD2E$_{1-337}$ (5 μM, 8.4 μg) and DR025 (25 μM) were incubated (45 min, r.t.) in HEPES buffer (50mM, pH = 7.5, 500mM NaCl) in the presence of $NiCl_2$ (15 μM). After UV irradiation on ice (20 min, 365 nm) the resulting solutions were incubated for 30 min (r.t.) with avidin-coated agarose beads. After washing with buffer (10mM HEPES, pH 7.2, 1M NaCl, 1% Triton X-100, 2mM EDTA, 4mM dithiotreitol) the released proteins were analyzed by SDS-PAGE and Western blots using anti-His-tag (left panel) and anti-biotin (right panel) antibodies. The input purified His-tag JMJD2E was ~ 3% of the total protein corresponding to the other lanes.

(B) Western blot and silver staining detection and of the capture of purified His-tag JMJD2E by DR025 in the presence of HEK293T cell lysates. The anti-His-tag (left panel), the anti-biotin (central panel) and the silver stained SDS-PAGE gel (right panel) results are relative to an experiment carried out under the same conditions as in (A) but in the presence of HEK293T cell lysates (~ 63 μg total protein) and a reduced concentration of DR025 (5 μM). The affinity purification was carried out with streptavidin-coated magnetic The input lysate supplemented with JMJD2E was ~ 4% of the total protein corresponding to the other lanes; the purified His-tag JMJD2E as standard was ~ 2% of the protein corresponding to the other lanes.

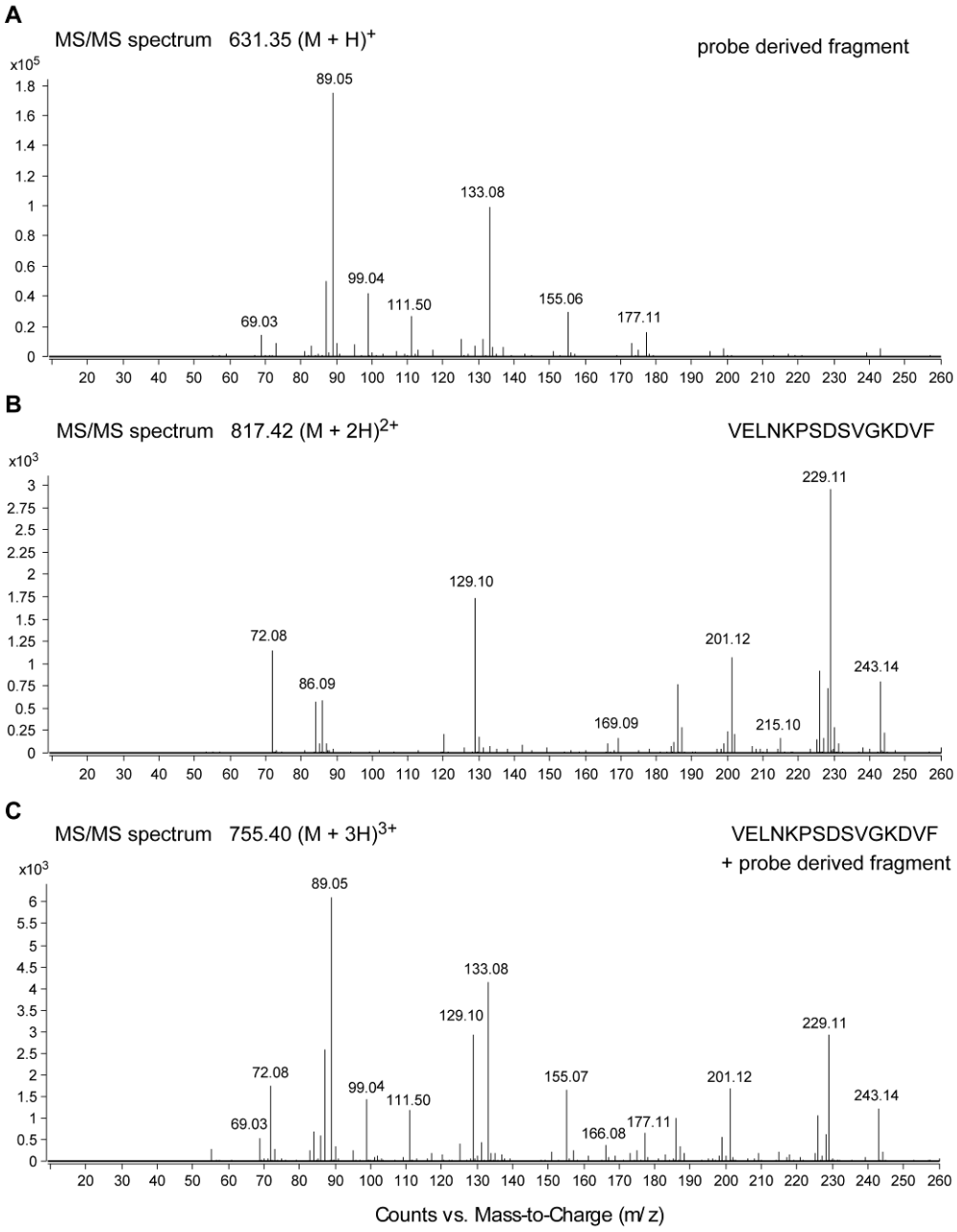

A
MS/MS spectrum 631.35 (M + H)+
probe derived fragment
x10^5
89.05
133.08
99.04
111.50
155.06
69.03
177.11
B
MS/MS spectrum 817.42 (M + 2H)2+
VELNKPSDSVGKDVF
x10^3
229.11
129.10
72.08
201.12
243.14
86.09
169.09
215.10
C
MS/MS spectrum 755.40 (M + 3H)3+
VELNKPSDSVGKDVF
+ probe derived fragment
x10^3
89.05
133.08
129.10
229.11
72.08
155.07
201.12
99.04
111.50
243.14
69.03
166.08
177.11
Counts vs. Mass-to-Charge (m/z)

Figure S4 (related to Figure 4). MS/MS Spectra in the Low Molecular Range of the Precursor Ion Masses Associated to the Cross-Linked PHD2

(A) MS/MS spectrum of the single charged precursor ion at m/z = 631.35 Da (MW = 630.35 Da).

(B) MS/MS spectrum of the doubly charged precursor ion at m/z = 817.42 Da (MW = 1632.84 Da).

(C) MS/MS spectrum of the triply charged precursor ion at m/z = 755.40 Da (MW = 2263.20 Da). The spectrum shows peaks which are specific for the precursor at 631.35 Da in addition to peaks which are specific for the precursor at 817.42 Da.

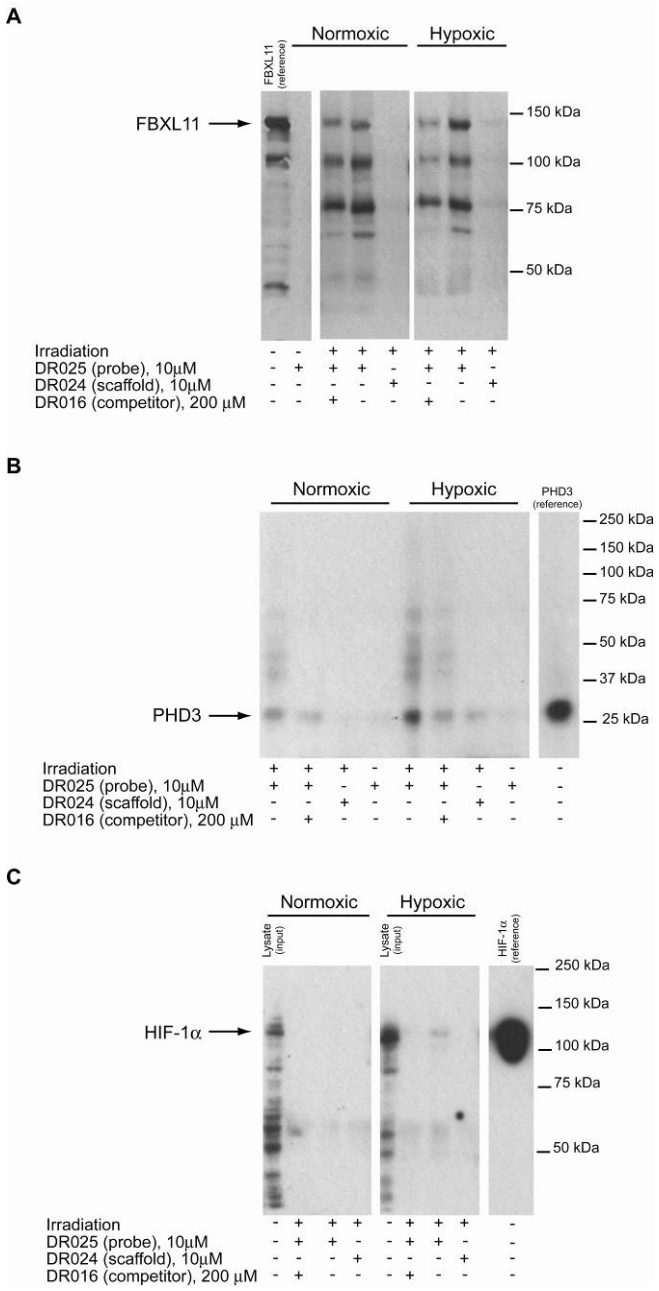

A
Normoxic
Hypoxic
FBXL11 (reference)
FBXL11
150 kDa
100 kDa
75 kDa
50 kDa
Irradiation
DR025 (probe), 10μM
DR024 (scaffold), 10μM
DR016 (competitor), 200 μM
B
Normoxic
Hypoxic
PHD3 (reference)
PHD3
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
Irradiation
DR025 (probe), 10μM
DR024 (scaffold), 10μM
DR016 (competitor), 200 μM
C
Normoxic
Hypoxic
Lysate (input)
Lysate (input)
HIF-1α (reference)
HIF-1α
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
Irradiation
DR025 (probe), 10μM
DR024 (scaffold), 10μM
DR016 (competitor), 200 μM

Figure S5 (related to Figure 5). Entire Immunoblot Images of Photo-Affinity Tagging Experiments in Lysates from HEK293T Cells Grown under Normoxic and Hypoxic Conditions

(A) Evidence for cross-linking of endogenous FBXL11 (KDM2A) in a nuclear protein extract of HEK293T cells. DR025 (10 μM) was incubated (45 min, r.t.) with nuclear extracts (~ 200 μg total protein) of HEK293T cells grown under normoxic (left) and hypoxic (right) conditions. After UV treatment (20 min, 365 nm) and affinity purification by the means of streptavidin-coated magnetic beads, the proteins released were analyzed by SDS-PAGE and subsequent anti-FBXL11 (polyclonal antibody from rabbit) immunoblotting (Blackledge et al., 2010). The protein reference was a nuclear extract of HEK293T cells over-expressing FBXL11 (KDM2A).

(B) Cross-linking of PHD3 at an endogenous level in whole HEK293T cell lysates. DR025 (10 μM) was incubated (45 min, r.t.) with a whole lysate (~ 200 μg total protein) of HEK293T cells grown under normoxic (left) and hypoxic (right) conditions. After UV treatment (20 min, 365 nm) and affinity purification by the means of streptavidin-coated magnetic beads, the proteins released were analyzed by SDS-PAGE and subsequent anti-PHD3 (monoclonal antibody from mouse) antibody blotting. The protein reference was a cell lysate (RCC4) over-expressing PHD3 (Appelhoff et al., 2004).

(C) Identification of endogenous HIF-1α in whole HEK293T cell lysates. DR025 (10 μM) was incubated (45 min, r.t.) with whole lysates (~ 200 μg total protein) of HEK293T cells grown under normoxic (left) and hypoxic (right) conditions. After UV treatment (20 min, 365 nm) and affinity purification by the means of streptavidin-coated magnetic beads, the proteins released were analyzed by SDS-PAGE and subsequent anti-HIF-1α (monoclonal antibody from mouse) immunoblotting. The input HEK293T lysate was ~ 3% of the total protein corresponding to the other lanes; the protein reference was a cell lysate (RCC4) over-expressing HIF-1α (Appelhoff et al., 2004).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Materials

All standard chemical reagents and solvents were obtained from commercial suppliers (Sigma-Aldrich, etc) and were used without further purification. Recombinant enzymes lysozyme and lactic dehydrogenase were purchased from Sigma (Saint Louis, Missouri, USA).The histone H3 fragment peptide (H3K9me3) was prepared as reported (Rose et al., 2008). The other histone peptide H3(30-41)K36me2 with a *C*-terminal amide was synthesised using a CS-Bio automated solid phase peptide synthesizer as described (Ng et al., 2007) and purified using reverse phase HPLC (C18 reverse phase column).

General Description of the Synthesis of the Probes

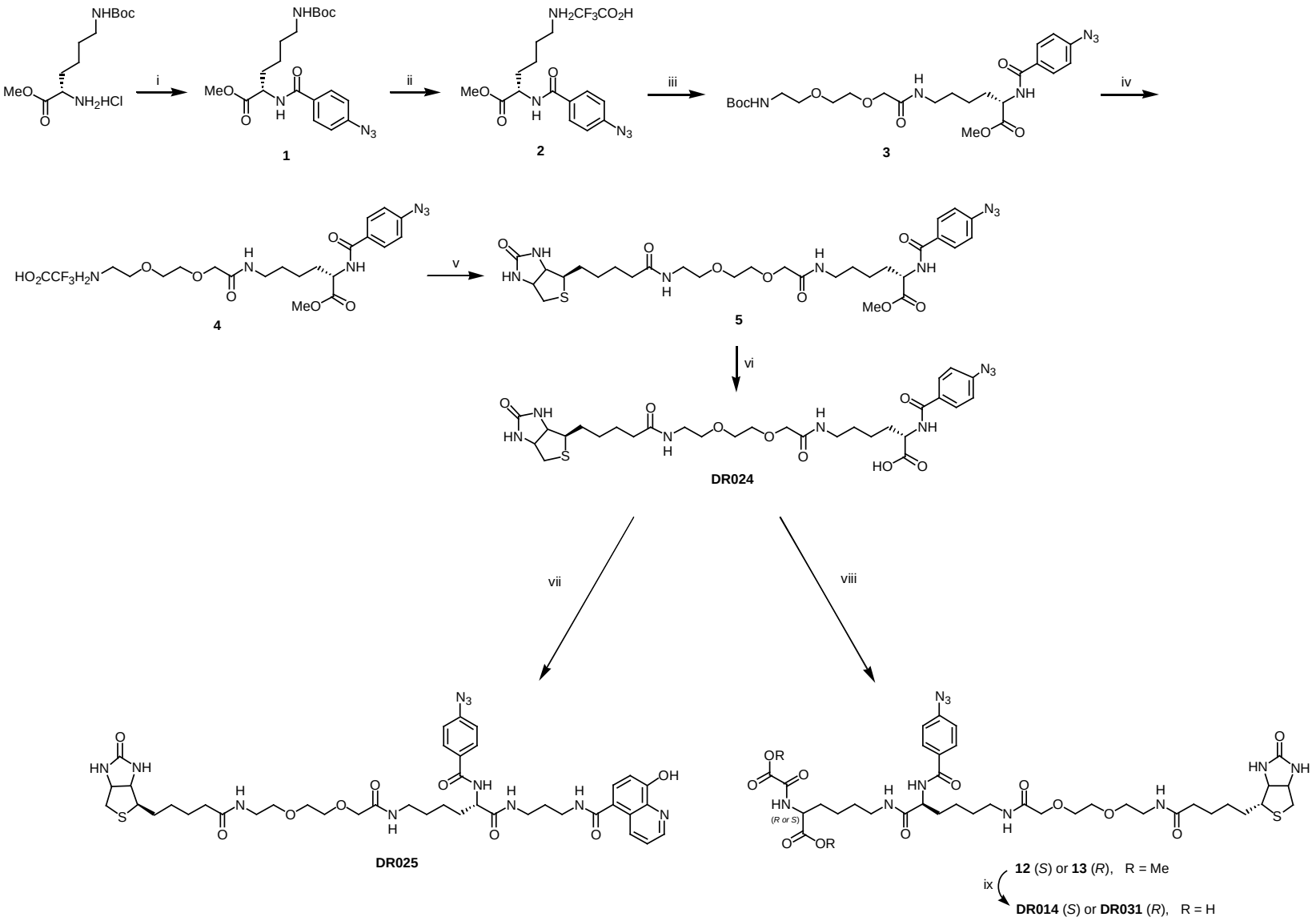
The routes for the synthesis of the probes are shown in Schemes S1-S3, below. The probes were prepared by coupling the common acidic scaffold **DR024** with the appropriate amines as their trifluoroacetate salts. **DR024** was synthesized starting from the commercially available hydrochloride salt of *N*-Boc protected (*L*)-lysine methyl ester. This latter was first coupled by standard methods (EDCI, HOBT) with commercially available 4-azidobenzoic acid to produce **1**, which was then deprotected with CF_3CO_2H to give the intermediate **2**. **2** was reacted with the commercially available [2-(2-tert-butoxycarbonylamino-ethoxy)-ethoxy]-acetic acid in the presence of EDCI, HOBT and Et_3N to give 2-(4-azidobenzoylamino)-6-{2-[2-(2-tert-butoxycarbonylaminoethoxy)-ethoxy]-acetylamino}-hexanoic acid methyl ester (**3**), which then was converted into the trifluoroacetate salt **4**. **4** was coupled with the commercially available (*D*)-biotin using PyBOP and Et_3N in dry DMF to give methyl ester **5**, which underwent hydrolysis under basic conditions to give **DR024**. The final compound **DR025** (Clemo and Howe, 1955) was synthesized by the coupling the trifluoroacetate salt of the *N*-(3-aminopropyl)-8-hydroxyquinoline-5-carboxyamide (**7**) with **DR024**. Derivative **7** was prepared starting from

commercially available 3-amino-4-hydroxybenzoic acid. This was subjected to a reaction with glycerol in the presence of iodine and an excess of concentrated sulphuric acid at 180 ºC (Skraup conditions) to give 8-hydroxy-5-carboxyquinoline (**6**). Reaction of **6** with commercially available *N*-Boc-1,3-diaminopropane under standard coupling conditions (EDCI, HOBT, Et_3N) provided intermediate **DR016** that was deprotected to give **7** using CF_3CO_2H (Scheme S2).

The two probe compounds differing in the configuration at the stereogenic centre of the *N*-oxalyllysine moiety (**DR014** and **DR031**) were prepared by reaction of **DR024** with the enantiomeric intermediates **10** and **11** followed by alkaline hydrolysis of the corresponding esters **12** and **13** (Scheme S1).

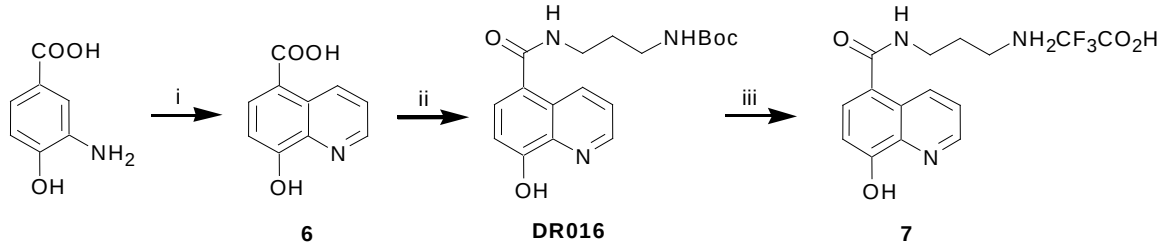
The two intermediates **10** (*L*) and **11** (*D*) were synthesized by acylation of the corresponding *(L*) and *(D*) *N*-Boc lysine methyl esters with the methyl chlorooxoacetate followed by subsequent deprotection (CF_3CO_2H) (Scheme S3). The (*L*) *N*-Boc-lysine methyl ester is commercially available as its hydrochloride salt. The free amine of the (*D*) enantiomer was prepared as reported (Manesis and Goodman, 1987).

Scheme S1.



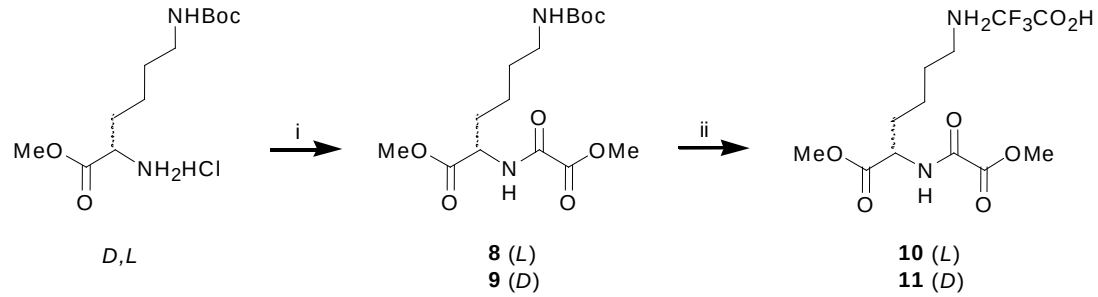
i) 4-Azidobenzoic acid, EDCI, HOBT, dry CH_2Cl_2, Et_3N, rt, 89%; ii) CF_3CO_2H, dry CH_2Cl_2, rt, 93%; iii) [2-(2-tertbutoxycarbonylaminoethoxy)ethoxy]acetic acid, EDCI, HOBT, CH_2Cl_2, rt, 81%; iv) CF_3CO_2H, dry CH_2Cl_2, rt, 98%; v) biotin, PyBOP, Et_3N, dry DMF, rt, 75%; vi) NaOH, MeOH, rt, 96%; vii) **7**, PyBOP, Et_3N, dry DMF, rt, 58%; viii) **10** or **11**, PyBOP, Et_3N, dry DMF, rt, 79%; ix) LiOH, THF/MeOH/H_2O, rt, 93%.

Scheme S2.



i) Glycerol, I_2, H_2SO_4 conc., 180 °C, 32%; ii) (3-aminopropyl)-carbamic acid *tert*butyl ester, EDCI, HOBT, dry DMF, Et_3N, rt, 82%; iii) CF_3CO_2H, dry CH_2Cl_2, rt, 90%.

Scheme S3.

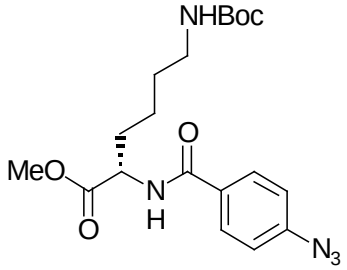


i) Methyl chlorooxoacetate, dry CH_2Cl_2, Et_3N, rt, 89%; ii) CF_3CO_2H, dry CH_2Cl_2, rt, 90%.

Detailed Synthetic Methods and Analytical Data

^{1}H-NMR spectra were recorded on a Bruker AV400 (400 MHz) and a Bruker AV500 (500 MHz) spectrometer and referenced to residual solvent peaks. Chemical shifts are quoted in parts per million (ppm). Assignments were made on the basis of chemical shifts, coupling constants (*J*), ^{13}C, DEPT, COSY, HMQC data and comparison with spectra of related compounds. Resonances are described as s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublets of doublets), dt (doublet of triplets), td (triplet of doublets), t (triplet), q (quartet), quin (quintet), m (multiplet), broad and br. s. (broad singlet). Multiplets are either reported as a range or the centre of the multiplet is given. Coupling constants are given in Hz and are reported to the nearest 0.5 Hz. ^{13}C-NMR spectra were recorded on a Bruker AV400 spectrometer and a Bruker AV500 spectrometer (fitted with an inverse cryoprobe for ^{13}C observation) and referenced to $CDCl_3$, CD_2Cl_2 or DMSO-*d*6. Infra-red (IR) spectra were recorded on a Bruker Tensor 27 FT-IR spectrophotometer as KBr disks or as thin films. Selected absorption maxima (ν_{max}) are given in wavenumbers (cm^{-1}). High resolution mass spectra (HR-MS) were recorded using a Bruker MicroTOF. Microanalysis experiments were performed by Mr. Stephen Boyer (London Metropolitan University). Melting points were recorded using a Gallenkamp Hot Stage apparatus. All moisture or oxygen sensitive reactions were carried out under a nitrogen atmosphere. Oven-dried glassware was used throughout. Anhydrous solvents were obtained from solvent stills in the Chemistry Research Laboratory and were activated by passing over a short column of activated alumina. Cooling was performed in ice-water baths (0 °C). Reagents were from Acros, Aldrich, Fluka, Lancaster or TCI fine chemical suppliers. All other chemicals were used as received. Thin layer chromatography (TLC) was performed on Merck DC-Kieselgel 60 F254 0.2 mm pre-coated plates with fluorescence indicator. Visualization of spots was achieved using UV light (254 nm) and by developing in an acidic solution of ceric ammonium nitrate (CAN) and ammonium heptamolybdate (Seebach's Magic Stain), followed by heating.

2-(4-Azidobenzoylamino)-6-tertbutoxycarbonylamino-hexanoic acid methyl ester (1)



To a mixture of the commercially available (*L*)-H-Lys(Boc)-OMe hydrochloride (593,6 mg, 2.0 mmol, 1 eq.) and 4-azidobenzoic acid (358.8 mg, 2.2 mmol, 1.1 eq.) in dry CH_2Cl_2 (16 mL) were added in sequence HOBT, (324.3 mg, 2.4 mmol, 1.2 eq.), EDCI (460.17 mg, 2.4 mmol, 1.2 eq.) and Et_3N (0.97 mL, 7.0 mmol, 3.5 eq.). After stirring overnight at r.t. under inert atmosphere (N_2) the reaction mixture was diluted with additional CH_2Cl_2 (15 mL) and washed in sequence with $KHSO_4$ 1N (2 x 30 mL), $NaHCO_3$ saturated solution (3 x 30 mL), and brine (2 x 20 mL).The organic phase was then dried over Na_2SO_4 and removed *in vacuo* to give a residue that was purified by automated column chromatography [Biotage SNAP 50 g silica gel cartridge eluting with a gradient 10-80% AcOEt in *n*-hexane] to afford **1** (721.7 mg, 89 %) as a colourless oil.

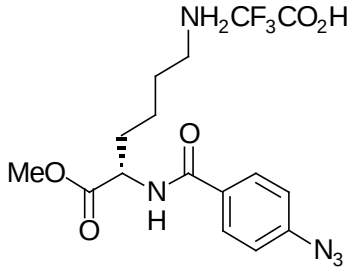
^{1}H-NMR (500 MHz, $CDCl_3$) δ_H/ppm: 1.49 (m, 13H, OC(C*H*$_3$)$_3$ and C*H*$_2$C*H*$_2$CH$_2$NHBoc), 1.81 (m, 1H, C*H*$_2$CH$_2$CH$_2$CH$_2$NHBoc), 1.96 (m, 1H, C*H*$_2$CH$_2$CH$_2$CH$_2$NHBoc), 3.12 (m, 2H, C*H*$_2$NHBoc), 3.81 (s, 3H, COOC*H*$_3$), 4.62 (br s, 1H, N*H*Boc), 4.78 (m, 1H, C*H*COOMe), 6.79 (d, 1H, CON*H*CHCOOMe, *J*=4.0 Hz), 7.07 (br d, 2H, aromatics, *J*=9.0 Hz), 7.83 (br d, 2H, aromatics, *J*=9 Hz).

^{13}C-NMR (126 MHz, $CDCl_3$) δ_C/ppm: 22.3, 28.3, 29.6, 32.0, 39.9, 52.5, 79.0, 118.9, 128.8, 130.2, 140.9, 156.1, 166.2, 172.9.

IR ν_{max}/cm^{-1} (thin film): 3336, 2951, 2125, 1645, 1604, 1499, 1285, 1173, 849, 766, 737.

HR-MS (ESI$^+$) $C_{19}H_{27}N_5O_5Na^+$ ([M+Na]$^+$) requires: 428.1909; found: 428.1917.

2-(4-Azidobenzoylamino)-6-aminohexanoic acid methyl ester trifluoroacetate salt (2)



CF_3CO_2H was added (5.27 mL, 71.0 mmol, 18 eq.) to a solution of the 2-(4-azidobenzoylamino)-6-tertbutoxycarbonylamino-hexanoic acid methyl ester **1** (1.6 g, 3.9 mmol, 1 eq.) in dry CH_2Cl_2 (21 mL). The resulting mixture was stirred at r.t. for 5 h. CF_3CO_2H was removed by washings with CH_2Cl_2 and evaporation under reduced pressure to give the desired amine salt as a sticky oil (1.52 g, 93 %).

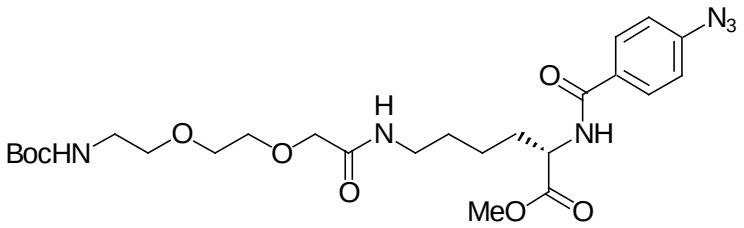
^{1}H-NMR (500 MHz, $CDCl_3$) δ_H/ppm: 1.50 (m, 2H, C*H*$_2$CH$_2$CH$_2$NH$_3^+$), 1.77 (m, 3H, CH$_2$C*H*$_2$CH$_2$NH$_3^+$ and C*H*$_2$CH$_2$CH$_2$CH$_2$NH$_3^+$), 1.93 (m, 1H, C*H*$_2$CH$_2$CH$_2$CH$_2$NH$_3^+$), 3.02 (m, 2H, CH$_2$C*H*$_2$NH$_3^+$), 3.78 (s, 3H, COOC*H*$_3$), 4.72 (td, 1H, NHC*H*COOMe, *J*=8.0 and 5.0 Hz), 7.06 (br d, 2H, aromatics, *J*=8.5 Hz), 7.12 (d, 1H, N*H*CHCOOMe, *J*=5.0 Hz), 7.72 (br s, 3H, N*H*$_3^+$), 7.77 (br d, 2H, aromatics, *J*=8.5 Hz).

^{13}C-NMR (126 MHz, $CDCl_3$) δ_C/ppm: 22.0, 26.4, 31.9, 39.7, 52.3, 52.8, 118.8, 128.9, 129.2, 144.3, 167.4, 172.7.

IR ν_{max}/cm^{-1} (thin film): 3301, 2947, 2126, 1640, 1603, 1474, 1284, 1178, 851, 767, 736.

HR-MS (ESI$^+$) (relative to free amine) $C_{14}H_{20}N_5O_3$ ([M+H]$^+$) requires: 306.1567; found: 306.1578.

2-(4-Azidobenzoylamino)-6-{2-[2-(2-tertbutoxycarbonylamino-ethoxy)-ethoxy]-acetylamino}-hexanoic acid methyl ester (3)



To a mixture of the trifluoroacetate salt of the 2-(4-azidobenzoylamino)-6-aminohexanoic acid methyl ester **2** (992,6 mg, 2.367 mmol, 1 eq.) and the commercially available [2-(2-*tert*butoxycarbonylamino)ethoxy]ethoxyacetic acid (dicyclohexylammonium) salt (947 mg, 2.13 mmol, 0.9 eq.) in dry CH_2Cl_2 (30 mL) were added in sequence, HOBT (319.8 mg, 2.367 mmol, 1 eq.), EDCI (453.8 mg, 2.367 mmol, 1 eq.) and Et_3N (0.824 mL, 5.917 mmol, 2.5 eq.). After stirring overnight at r.t. under an inert atmosphere (N_2), the reaction mixture was diluted with 20mL of CH_2Cl_2 and washed in sequence with $KHSO_4$ 1N (2 x 40 mL), $NaHCO_3$ saturated solution (3 x 40 mL), and brine (2 x 30 mL).The organic phase was then dried over Na_2SO_4, then removed *in vacuo* to give a residue that was purified by automated column chromatography [Biotage SNAP 100 g silica gel cartridge eluting with a gradient of 40-100% AcOEt:MeOH (9:1) in petroleum spirit 40-60 ºC] to afford **3** (950 mg, 81 %) as a colourless oil.

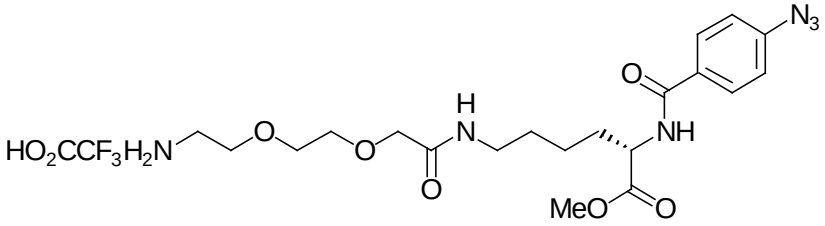
^{1}H-NMR (500 MHz, $CDCl_3$) δ_H/ppm: 1.48 (s, 9H, OC(C*H*$_3$)$_3$), 1.58 (m, 4H, C*H*$_2$C*H*$_2$CH$_2$NHCOCH$_2$), 1.89 (m, 1H, C*H*$_2$CH$_2$CH$_2$CH$_2$NHCOCH$_2$), 1.97 (m, 1H, C*H*$_2$CH$_2$CH$_2$CH$_2$NHCOCH$_2$), 3.20-3.43 (m, 4H, C*H*$_2$NHCOCH$_2$ and C*H*$_2$NHBoc), 3.55 (m, 2H, OC*H*$_2$CH$_2$NHBoc), 3.62 (m, 4H, OCH$_2$CH$_2$O), 3.79 (s, 3H, COOC*H*$_3$), 3.90 (d, 1H, COC*H*$_2$O, *J*=16 Hz), 3.97 (d, 1H, COC*H*$_2$O, *J*=16 Hz), 4.75 (td, 1H, NHC*H*COOMe, *J*=7.5 and 5.0 Hz), 4.98 (br s, 1H, N*H*Boc), 6.89 (br d, 1H, N*H*CHCOOMe), 6.96 (m, 1H, N*H*COCH$_2$), 7.08 (br d, 2H, aromatics, *J*=8.5 Hz), 7.90 (br d, 2H, aromatics, *J*=8.5 Hz).

^{13}C-NMR (126 MHz, $CDCl_3$) δ_C/ppm: 22.4, 28.4, 29.2, 31.6, 37.9, 40.2, 52.4, 52.9, 69.8, 70.3, 70.9, 79.4, 118.9, 129.0, 130.2, 143.5, 155.9, 166.1, 170.1, 173.0.

IR ν_{max}/cm^{-1} (thin film): 3332, 2932, 2125, 1654, 1539, 1500, 1284, 1174, 850, 767.

HR-MS (ESI^+) $C_{25}H_{38}N_6O_8Na^+$ ($[M+Na]^+$) requires: 573.2649; found: 573.2657.

6-{2-[2-(2-Aminoethoxy)-ethoxy]-acetylamino}-2-(4-azidobenzoylamino)-hexanoic acid methyl ester trifluoroacetate salt (4)



CF_3CO_2H was added (2.136 mL, 28.768 mmol, 18 eq.) to a solution of the 2-(4-azidobenzoylamino)-6-{2-[2-(2-tertbutoxycarbonylamino-ethoxy)-ethoxy]-acetylamino}-hexanoic acid methyl ester **3** (880 mg, 1.598 mmol, 1 eq.) in dry CH_2Cl_2 (8.5 mL) and the resulting mixture was stirred at r.t. for 6 h. Removal of the CF_3CO_2H in excess by several washings with CH_2Cl_2 and evaporation under reduced pressure to give the desired amine salt as a sticky colourless oil (884 mg, 98 %).

^{1}H-NMR (500 MHz, $CDCl_3$) δ_H/ppm: 1.45 (m, 2H, C*H*$_2$CH$_2$CH$_2$NHCOCH$_2$), 1.61 (m, 2H, CH$_2$C*H*$_2$CH$_2$NHCOCH$_2$), 1.78 (m, 1H, C*H*$_2$CH$_2$CH$_2$CH$_2$NHCOCH$_2$), 1.96 (m, 1H, C*H*$_2$CH$_2$CH$_2$CH$_2$NHCOCH$_2$), 3.20-3.35 (m, 4H, C*H*$_2$NHCOCH$_2$ and C*H*$_2$$NH_3^+$), 3.65 (m, 4H, OC*H*$_2$C*H*$_2$O), 3.78 (m, 5H, COOC*H*$_3$ and OC*H*$_2$CH$_2$$NH_3^+$), 4.00 (d, 1H, COC*H*$_2$O, *J*=15.5 Hz), 4.06 (d, 1H, COC*H*$_2$O, *J*=15.5 Hz), 4.72 (td, 1H,

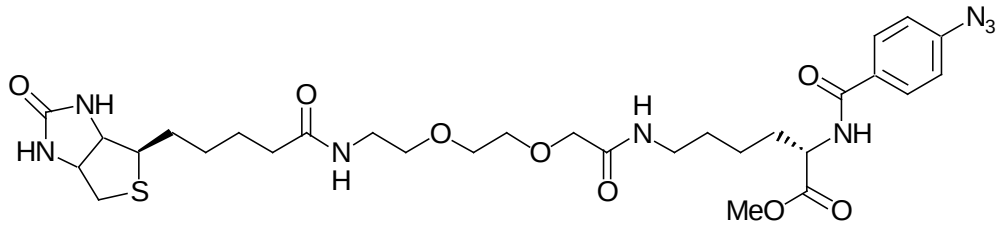
NHC*H*COOMe, *J*=7.5 and 5.0 Hz), 7.05 (d, 1H, N*H*CHCOOMe, *J*=8.0 Hz), 7.09 (br d, 2H, aromatics, *J*=10.0 Hz), 7.27 (m, 1H, N*H*$COCH_2$), 7.81 (br d, 2H, aromatics, *J*=10.0 Hz), 8.00 (br s, 3H, $OCH_2CH_2NH_3^+$).

^{13}C-NMR (126 MHz, $CDCl_3$) δ_C/ppm: 22.6, 28.4, 38.2, 38.6, 39.9, 52.4, 52.7, 66.5, 69.9, 70.3, 70.7, 118.8, 128.9, 129.5, 144.2, 167.1, 171.3, 173.0.

IR ν_{max}/cm^{-1} (thin film): 3299, 2927, 2126, 1737, 1645, 1604, 1500, 1285, 1202, 849, 799, 767.

HR-MS (ESI^-) $C_{22}H_{30}F_3N_6O_8$ ($[M-H]^-$) requires: 563.2076; found: 563.2059.

2-(4-Azidobenzoylamino)-6-[2-(2-{2-[5-(2-oxohexahydrothieno[3,4-*d*]imidazol-6-yl)-pentanoylamino]-ethoxy}-ethoxy)-acetylamino]-hexanoic acid methyl ester (5)



To a solution of biotin (468 mg, 1.92 mmol, 1.2 eq.) in dry DMF (10 mL) were added in sequence, PyBOP (1.197 g, 2.3 mmol, 1.44 eq.) and Et_3N (0.80 mL, 5.75 mmol, 3.6 eq.). After 30 min of stirring at r.t., the activated biotin was added to a solution in dry DMF of the salt **4** (902.0 mg, 1.598 mmol, 1 eq.) in presence of Et_3N (0.445 mL, 3.196 mmol, 2.0 eq.) and the resulting mixture was stirred at r.t. overnight. After completion, the solvent was evaporated under reduced pressure and the residue was dissolved in 40 mL of CH_2Cl_2. The organic phase was then washed sequentially with saturated solutions of $NaHCO_3$ (3 x 40 mL) and NaCl (2 x 40 mL), dried over Na_2SO_4, and finally removed *in vacuo* to give a residue that was purified by automated column chromatography [Biotage SNAP 100 g silica gel cartridge eluting with a gradient 50-100% AcOEt:MeOH (9:1) in

petroleum spirit 40-60 ºC for 10 column volumes then 10 more column volumes of AcOEt:MeOH (9:1)] to afford **5** (811 mg, 75 %) as a colourless oil.

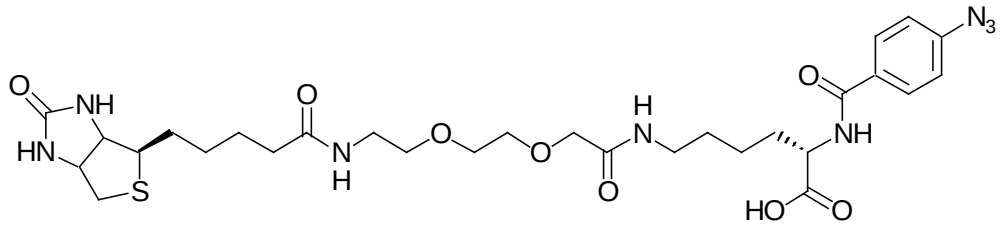
^{1}H-NMR (500 MHz, $CDCl_3$) δ_H/ppm: 1.35-1.75 (m, 10H, C*H*$_2$C*H*$_2$CH$_2$NHCOCH$_2$O and C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$CONH), 1.91 (m, 2H, C*H*$_2$CH$_2$CH$_2$CH$_2$NHCOCH$_2$O), 2.23 (t, 2H, CH$_2$C*H*$_2$CONH), 2.73 (d, 1H, SC*H*$_2$, *J*=13.0 Hz), 2.91 (dd, 1H, *J*=13.0 and 5 Hz), 3.15 (m, 1H, C*H*S), 3.34 (m, 2H, C*H*$_2$NHCOCH$_2$O), 3.4-3.7 (m, 8H, OC*H*$_2$C*H*$_2$OC*H*$_2$C*H*$_2$NHCO), 3.78 (s, 3H, COOCH$_3$), 3.99 (br s, 2H, OC*H*$_2$CO), 4.33 (dd, 1H, NHC*H*(CH)CHS, *J*=7.5 and 5.0 Hz), 4.52 (dd, 1H, NHC*H*(CH)CH$_2$S *J*=7.5 and 5.0 Hz), 4.70 (td, 1H, NHC*H*COOMe, *J*=8 and 5.0 Hz), 5.36 (br s, 1H, N*H*), 6.03 (br s, 1H, N*H*), 6.57 (br s, 1H, N*H*), 7.07 (m, 3H, N*H* and aromatics), 7.65 (d, 1H, N*H*CHCOOMe, *J*=7.5 Hz), 7.94 (br d, 2H, aromatics, *J*=10.0 Hz).

^{13}C-NMR (126 MHz, $CDCl_3$) δ_C/ppm: 22.7, 25.5, 27.7, 28.9, 31.3, 35.4, 38.2, 39.2, 40.3, 45.8, 52.4, 52.8, 55.3, 60.6, 62.0, 69.7, 70.2, 70.6, 70.8, 118.9, 129.2, 130.1, 143.5, 163.6, 166.5, 170.3, 173.2, 173.8.

IR ν_{max}/cm^{-1} (thin film): 3391, 2869, 2126, 1640, 1544, 1497, 1282, 1101, 855, 769.

HR-MS (ESI$^+$) $C_{30}H_{44}N_8O_8SNa^+$ ([M+Na]$^+$) requires: 699.2901; found: 699.2913.

2-(4-Azidobenzoylamino)-6-[2-(2-{2-[5-(2-oxohexahydrothieno[3,4-*d*]imidazol-6-yl)-pentanoylamino]-ethoxy}-ethoxy)-acetylamino]-hexanoic acid (DR024)



To a solution of 2-(4-azidobenzoylamino)-6-[2-(2-{2-[5-(2-oxohexahydrothieno[3,4-*d*]imidazol-6-yl)-pentanoylamino]-ethoxy}-ethoxy)-acetylamino]-hexanoic acid methyl ester **5** (883 mg, 1.3 mmol, 1 eq.) in MeOH (7.5 mL) was added a solution of NaOH (78.3 mg, 1.975 mmol, 1.5 eq.) in H_2O (2 mL) and the resulting mixture was stirred at r.t. for 3 h. The solution was poured into water (20 mL) and extracted with diethyl ether (2 x 15 mL). HCl (1 N) was then added to the aqueous layer until the pH was 4 and the acidic phase was extracted with $CHCl_3$-isopropanol (4:1) (4 x 25mL). After washings with a saturated solution of NaCl (1 x 20 mL), the organic phase was dried over Na_2SO_4, and finally removed *in vacuo* to give the title compound **DR024** (827 mg, 96%) as a TLC pure white solid.

^{1}H-NMR (400 MHz, DMSO-d_6) δ_H/ppm: 1.25-1.60 (m, 10H, C*H*$_2$C*H*$_2$CH$_2$NHCOCH$_2$O and C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$CONH), 1.77 (m, 2H, C*H*$_2$CH$_2$CH$_2$CH$_2$NHCOCH$_2$O), 2.06 (t, 2H, CH$_2$C*H*$_2$CONH, *J*=7.5 Hz), 2.57 (d, 1H, SC*H*$_2$, *J*=12.5 Hz), 2.81 (dd, 1H, *J*=12.5 and 5 Hz), 3.09 (m, 3H, C*H*S and C*H*$_2$NHCOCH$_2$O), 3.18 (m, 2H, OCH$_2$C*H*$_2$NHCO), 3.53 (m, 6H, OC*H*$_2$C*H*$_2$OC*H*$_2$CH$_2$NHCO), 3.84 (s, 2H, OC*H*$_2$CO), 4.12 (dd, 1H, NHC*H*(CH)CHS, *J*=8.0 and 4.5 Hz), 4.31 (m, 2H, NHC*H*(CH)CH$_2$S and NHC*H*COOH), 6.43 (br s, 2H, N*H*CON*H*), 7.22 (br d, 2H, aromatics, *J*=8.5 Hz), 7.72 (t, 1H, N*H*COCH$_2$, *J*=6.0 Hz), 7.85 (t, 1H, N*H*COCH$_2$, *J*=5.5 Hz), 7.95 (br d, 2H, aromatics, *J*=8.5 Hz), 8.60 (d, 1H, N*H*CHCOOH, *J*=7.0 Hz).

^{13}C-NMR (101 MHz, DMSO-d_6) δ_C/ppm: 21.7, 25.5, 26.7, 29.9, 31.2, 35.4, 37.2, 38.3, 41.3, 45.8, 48.5, 48.9, 52.8, 58.3, 69.7, 70.2, 70.6, 73.8, 120.9, 132.2, 133.4, 145.5, 163.6, 168.5, 171.3, 173.8, 177.8.

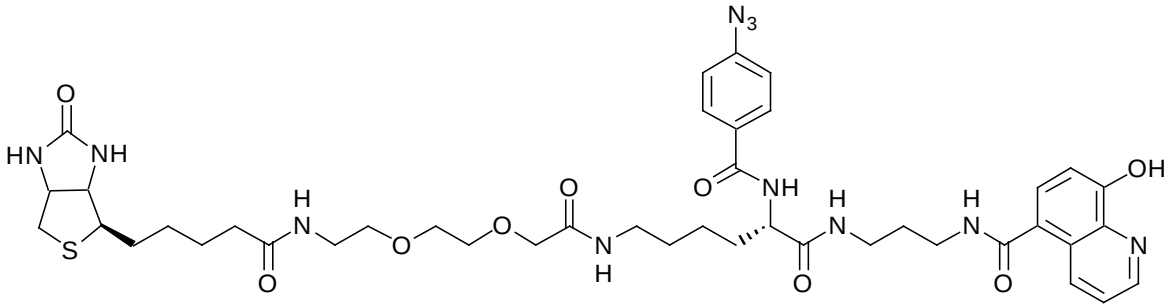
m.p.: 76-79 ºC.

FT-IR ν_{max}/cm^{-1} (KBr disk): 3406, 2934, 2866, 2127, 1644, 1547, 1499, 1460, 1286, 1104, 850, 766.

HR-MS (ESI$^-$) $C_{29}H_{41}N_8O_8S^-$ ([M-H]$^-$) requires: 661.2767; found: 661.2753.

Microanalysis $C_{29}H_{42}N_8O_8S$ requires: C, 52.55%; H, 6.39%; N, 15.72%; S, 3.60%; found: C, 52.71%; H, 6.46%; N, 17.41%; S, 3.56%.

8-Hydroxyquinoline-5-carboxylic acid (3-{2-(4-azido-benzoylamino)-6-[2-(2-{2-[5-(2-oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]-ethoxy}-ethoxy)-acetylamino]-hexanoylamino}-propyl)-amide (DR025)



To a solution of the acid **DR024** (150 mg, 0.226 mmol, 1 eq.) in dry DMF (1 mL) were added in sequence, PyBOP (141 mg, 0.271 mmol, 1.2 eq.) and Et_3N (0.11 mL, 0.792 mmol, 3.5 eq.). After 30 min of stirring at r.t., the activated acid was added to a solution in dry DMF of the salt **7** (105.7 mg, 0.294 mmol, 1.3 eq.) in the presence of Et_3N (0.11 mL, 0.792 mmol, 3.5 eq.). The resulting mixture was stirred at r.t. overnight. After completion, the solvent was evaporated under reduced pressure and the residue was dissolved in 20 mL of a mixture $CHCl_3$-isopropanol (4:1). The organic phase was then washed sequentially with saturated solutions of $NaHCO_3$ (3 x 10 mL) and NaCl (2 x 10 mL), dried over Na_2SO_4, and finally removed *in vacuo* to give a residue that was purified by automated column chromatography [Biotage SNAP 25 g silica gel cartridge eluting with a gradient 50-100% CH_2Cl_2:MeOH (5:1) in hexane for 10 column volumes then 10 more column volumes of CH_2Cl_2:MeOH (4:1)] to afford **DR025** (117 mg, 58 %) as a faint yellow powder after drying under high vacuum.

^{1}H-NMR (500 MHz, CD_3OD) δ_H/ppm: 1.35-1.95 (m, 14H, C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$NHCOCH$_2$O, C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$CONH and NHCH$_2$C*H*$_2$CH$_2$NH), 2.21 (t, 2H, CH$_2$C*H*$_2$CONH, *J*=7.5 Hz), 2.69 (d, 1H, SC*H*$_2$, *J*=12.5 Hz), 2.90 (dd, 1H, *J*=12.5 and 5 Hz), 3.12-3.64 (m, 15H, C*H*S, C*H*$_2$NHCOCH$_2$O,

OCH_2CH_2OCH_2CH_2NHCO and NHCH_2CH$_2$CH_2NH), 3.97 (s, 2H, OCH_2CO), 4.27 (dd, 1H, NHCH(CH)CHS, J=8.0 and 4.5 Hz), 4.48 (m, 2H, NHCH(CH)CH$_2$S and NHCHCONH), 7.10 (d, 1H, quinoline ring, J=8.0 Hz), 7.16 (br d, 2H, benzene ring, J=8.5 Hz), 7.57 (dd, 1H, quinoline ring, J=8.5 and 4.0 Hz), 7.81 (d, 1H, quinoline ring, J=8.0 Hz), 7.94 (br d, 2H, benzene ring, J=8.5 Hz), 8.78 (dd, 1H, quinoline ring, J=8.5 and 1.5 Hz), 8.85 (dd, 1H, quinoline ring, J=4.0 and 1.5 Hz).

^{13}C-NMR (126 MHz, CD$_3$OD) δ_C/ppm: 24.3, 26.8, 27.2, 29.5, 29.8, 30.1, 30.3, 32.6, 36.7, 37.7, 38.0, 39.6, 40.3, 41.0, 47.9, 55.8, 57.0, 61.6, 63.4, 70.6, 71.3, 110.6, 119.9, 123.6, 125.2, 128.2, 129.2, 130.6, 131.7, 135.7, 136.2, 139.8, 145.1, 149.6, 166.0, 169.4, 171.3, 172.7, 174.8, 176.2.

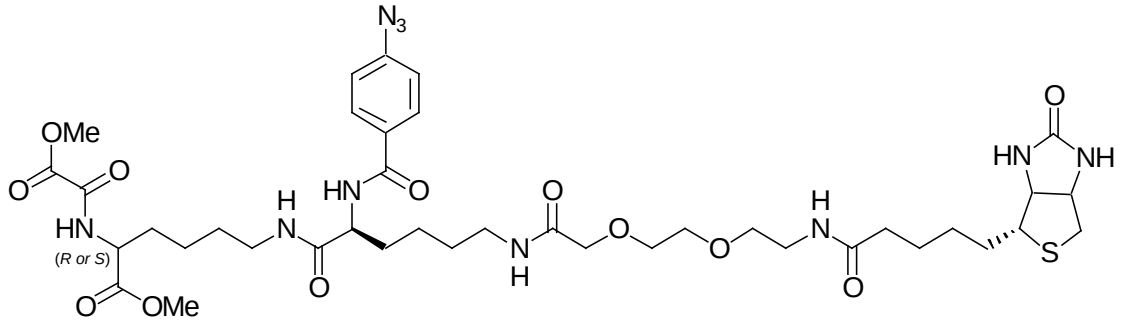
m.p.: 118-120 ºC.

FT-IR ν_{max}/cm^{-1} (KBr disk): 3419, 2928, 2126, 1645, 1541, 1502, 1459, 1286, 1089, 797.

HR-MS (ESI$^+$) $C_{42}H_{55}N_{11}O_9SNa^+$ ([M+Na]$^+$) requires: 912.3803; found: 912.3809.

Microanalysis $C_{42}H_{55}N_{11}O_9S$ requires: C, 56.68%; H, 6.23%; N, 17.31%; S, 3.60%; found: C, 56.79%; H, 6.27%; N, 17.27%; S, 3.58%.

General procedure for the preparation of 6-{2-(4-Azidobenzoylamino)-6-[2-(2-{2-[5-(2-oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]-ethoxy}-ethoxy)-acetylamino]-hexanoylamino}-2-(methoxyoxalyl-amino)-hexanoic acid methyl esters (12,13)



12 *(S or L)*
13 *(R or D)*

Example: 6-{2-(4-Azidobenzoylamino)-6-[2-(2-{2-[5-(2-oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]ethoxy}-ethoxy)-acetylamino]-hexanoylamino}-2-(methoxyoxalyl-amino)-hexanoic acid methyl ester (13) (*D*-Lysine derivative)

To a solution of the acid **DR024** (175 mg, 0.264 mmol, 1 eq.) in dry DMF (2 mL) were added in sequence, PyBOP (165 mg, 0.316 mmol, 1.2 eq.) and Et_3N (0.12 mL, 0.871 mmol, 3.3 eq.). After 30 min of stirring at r.t., the activated acid was added to a solution in dry DMF of the salt **11** (105.7 mg, 0.294 mmol, 1.3 eq.) in the presence of Et_3N (0.11 mL, 0.792 mmol, 3.0 eq.). The resulting mixture was stirred at r.t. overnight. The solvent was then evaporated under reduced pressure and the residue was dissolved in 20 mL of CH_2Cl_2. The organic phase was then washed sequentially with saturated solutions of $NaHCO_3$ (3 x 10 mL) and NaCl (2 x 10 mL), dried over Na_2SO_4, and finally removed *in vacuo* to give a residue that was purified by automated column chromatography [Biotage SNAP 25 g silica gel cartridge eluting with a gradient 50-100% CH_2Cl_2:MeOH (9:1) in

hexane for 9 column volumes then 15 more column volumes of CH_2Cl_2:MeOH (9:1)] to afford **13** (185 mg, 79 %) as a colourless oil after drying under high vacuum.

^{1}H-NMR (500 MHz, $CDCl_3$) δ_H/ppm: 1.30-1.95 (m, 18H, C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$NHCOCH$_2$O, C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$CONH and NHCH$_2$C*H*$_2$C*H*$_2$C*H*$_2$CHCO$_2$Me), 2.23 (m, 2H, CH$_2$C*H*$_2$CONH), 2.80 (m, 1H, SC*H*$_2$), 2.93 (m, 1H, SC*H*$_2$), 3.14-3.48 (m, 5H, C*H*S, C*H*$_2$NHCOCH$_2$O and NHC*H*$_2$CH$_2$CH$_2$CH$_2$CHCO$_2$Me), 3.5-3.7 (m, 8H, OC*H*$_2$C*H*$_2$OC*H*$_2$C*H*$_2$NHCO), 3.76 (s, 3H, CO_2CH_3), 3.91 (s, 3H, $COCO_2CH_3$), 4.00 (s, 2H, OC*H*$_2$CO), 4.37 (dd, 1H, NHC*H*(CH)CHS, *J*=7.5 and 5.0 Hz), 4.57 (m, 2H, NHC*H*(CH)CH$_2$S and NHCH$_2$CH$_2$CH$_2$CH$_2$C*H*CO$_2$Me), 4.68 (m, 1H, C*H*CH$_2$CH$_2$CH$_2$CH$_2$NHCOCH$_2$O), 6.41 (br s, 1H, N*H*), 6.55 (br s, 1H, N*H*), 6.85 (br s, 1H, N*H*),7.07 (m, 3H, N*H* and aromatics), 7.74 (br s, 1H, N*H*), 7.89 (m, 3H, aromatics and N*H*), 8.01 (br d, 1H, N*H*, *J*=8.5 Hz).

^{13}C-NMR (126 MHz, $CDCl_3$) δ_C/ppm: 22.1, 22.4, 22.9, 25.1, 25.6, 27.8, 28.1, 28.6, 28.9, 31.1, 31.8, 35.6, 38.3, 39.3, 40.4, 41.0, 52.6, 53.5, 55.6, 60.7, 62.5, 69.7, 70.2, 70.6, 70.9, 118.9, 129.3, 130.0, 143.6, 156.6, 160.5, 163.8, 166.4, 170.1, 171.6, 172.1, 173.7.

FT-IR ν_{max}/cm^{-1} (KBr disk): 3299, 2929, 2125, 1742, 1650, 1538, 1285, 1118.

HR-MS (ESI$^+$) $C_{39}H_{58}N_{10}O_{12}SNa^+$ ([M+Na]$^+$) requires: 913.3854; found: 913.3848.

6-{2-(4-Azidobenzoylamino)-6-[2-(2-{2-[5-(2-oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]ethoxy}-ethoxy)-acetylamino]-hexanoylamino}-2-(methoxyoxalyl-amino)-hexanoic acid methyl ester (12)(*L*-Lysine derivative)

12 (190 mg, 81%) was prepared from **DR024** (175 mg, 0.264 mmol, 1.0 eq.) and **10** (105.7 mg, 0.294 mmol, 1.3 eq.) in the same manner used to prepare **13**.

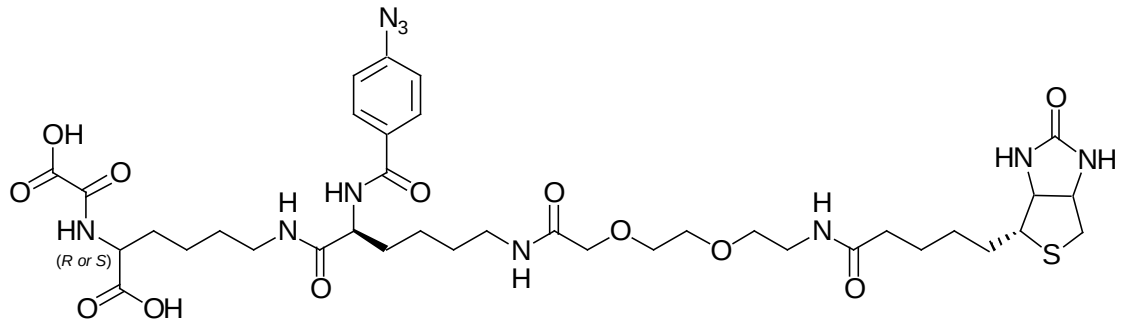
^{1}H-NMR (500 MHz, $CDCl_3$) δ_H/ppm: 1.25-1.90 (m, 18H, C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$NHCOCH$_2$O, C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$CONH and NHCH$_2$C*H*$_2$C*H*$_2$C*H*$_2$CHCO$_2$Me), 2.20 (m, 2H, CH$_2$C*H*$_2$CONH), 2.82 (m, 1H, SC*H*$_2$), 2.91 (m, 1H, SC*H*$_2$), 3.10-3.45 (m, 5H, C*H*S, C*H*$_2$NHCOCH$_2$O and NHC*H*$_2$CH$_2$CH$_2$CH$_2$CHCO$_2$Me), 3.5-3.7 (m, 8H, OC*H*$_2$C*H*$_2$OC*H*$_2$C*H*$_2$NHCO), 3.76 (s, 3H, CO_2CH_3), 3.90 (s, 3H, $COCO_2CH_3$), 3.99 (s, 2H, OC*H*$_2$CO), 4.37 (m, 1H, NHC*H*(CH)CHS, *J*=7.5 and 5.5 Hz), 4.52-4.72 (m, 3H, NHC*H*(CH)CH$_2$S, NHCH$_2$CH$_2$CH$_2$CH$_2$C*H*CO$_2$Me and C*H*CH$_2$CH$_2$CH$_2$CH$_2$NHCOCH$_2$O), 6.40 (br s, 1H, N*H*), 6.54 (br s, 1H, N*H*), 6.86 (br s, 1H, N*H*), 7.06-7.24 (m, 3H, N*H* and aromatics), 7.79 (br s, 1H, N*H*), 7.92 (m, 3H, aromatics and N*H*), 8.23 (br d, 1H, N*H*, *J*=7.0 Hz).

^{13}C-NMR (126 MHz, $CDCl_3$) δ_C/ppm: 22.2, 22.5, 22.8, 25.3, 25.8, 27.7, 28.1, 28.6, 28.9, 31.1, 31.8, 35.1, 38.4, 39.2, 40.1, 41.0, 52.6, 53.5, 55.6, 60.7, 62.5, 69.7, 70.2, 70.6, 70.9, 118.9, 129.2, 130.0, 143.6, 156.6, 160.5, 163.8, 166.8, 170.3, 171.7, 172.0, 173.7.

FT-IR ν_{max}/cm^{-1} (KBr disk): 3300, 2933, 2125, 1649, 1543, 1499, 1285.

HR-MS (ESI^+) $C_{39}H_{58}N_{10}O_{12}SNa^+$ ($[M+Na]^+$) requires: 913.3854; found: 913.3857.

General procedure for the preparation of 6-{2-(4-Azido-benzoylamino)-6-[2-(2-{2-[5-(2-oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-ethoxy}-ethoxy)-acetylamino]-hexanoylamino}-2-(oxalyl-amino)-hexanoic acids (DR014, DR031)



DR014 *(S or L)*
DR031*(R or D)*

Example: 6-{2-(4-Azido-benzoylamino)-6-[2-(2-{2-[5-(2-oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-ethoxy}-ethoxy)-acetylamino]-hexanoylamino}-2-(oxalyl-amino)-hexanoic acid (DR014) (*L*-Lysine derivative)

To a solution of 6-{2-(4-azidobenzoylamino)-6-[2-(2-{2-[5-(2-oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]ethoxy}-ethoxy)-acetylamino]-hexanoylamino}-2-(methoxyoxalyl-amino)-hexanoic acid methyl ester **12** (218 mg, 0.24 mmol, 1 eq.) in a mixture THF/MeOH/H_2O (1.85/0.9/1.35 mL) was added a solution of LiOH (61.6 mg, 1.468 mmol, 3.0 eq.) in H_2O (1.5 mL). The resulting mixture was stirred at r.t. for 5 h. The volume of the solution was then reduced under vacuum, acidified with HCl (1 N) and extracted with a mixture $CHCl_3$-isopropanol (4:1) (10 x 5 mL). After rapid washing with a saturated solution of NaCl (1 x 10 mL), the organic phase was dried over Na_2SO_4, and finally removed *in vacuo* to give **DR014** (196 mg, 93%) as a TLC white solid.

^{1}H-NMR (500 MHz, CD_3OD) δ_H/ppm: 1.20-2.00 (m, 18H, CH_2CH_2CH_2CH_2NHCOCH_2O, CH_2CH_2CH_2CH_2CONH and NHCH_2CH_2CH_2CH_2CHCO_2Me), 2.23 (m, 2H, CH_2CH_2CONH), 2.72 (m, 1H, SCH_2), 2.94 (dd, 1H, SCH_2, *J*=13.0 and 5.0 Hz), 3.15-3.40 (m, 7H, C*H*S, CH_2NHCOCH_2O, NHCH_2CH_2CH_2CH_2CHCO_2Me and OCH_2CH_2OCH_2CH_2NHCO), 3.53-3.67 (m, 6H, OCH_2CH_2OCH_2CH_2NHCO), 3.98 (s,

2H, OC*H*$_2$CO), 4.32 (m, 1H, NHC*H*(CH)CHS), 4.41-4.54 (m, 3H, NHC*H*(CH)CH$_2$S, NHCH$_2$CH$_2$CH$_2$CH$_2$C*H*CO$_2$Me and C*H*CH$_2$CH$_2$CH$_2$CH$_2$NHCOCH$_2$O), 7.18 (br d, 2H, aromatics, *J*=8.5 Hz), 7.95 (m, 2H, aromatics, *J*=8.5 Hz).

^{13}C-NMR (126 MHz, CD$_3$OD) δ_C/ppm: 22.2, 23.8, 24.8, 25.3, 26.8, 27.7, 28.1, 28.6, 29.9, 31.1, 31.8, 35.1, 38.4, 39.2, 40.1, 41.0, 55.5, 62.7, 64.6, 69.7, 70.2, 71.6, 72.9, 119.9, 130.0, 131.8, 145.1, 156.6, 160.5, 163.8, 166.1, 169.1, 171.7, 172.7, 176.2.

m.p.: 85.5-87 ºC.

FT-IR ν_{max}/cm^{-1} (KBr disk): 3395, 2934, 2126, 1685, 1646, 1541, 1498, 1457, 1285, 1112, 850, 764, 598.

HR-MS (ESI$^+$) C$_{37}$H$_{53}$N$_{10}$O$_{12}$S ([M-H]$^-$) requires: 861.3564; found: 861.3571.

Microanalysis C$_{37}$H$_{54}$N$_{10}$O$_{12}$S requires: C, 51.50%; H, 6.31%; N, 16.23%; S, 3.72%; found: C, 51.59%; H, 6.34%; N, 16.17%; S, 3.68%.

6-{2-(4-Azido-benzoylamino)-6-[2-(2-{2-[5-(2-oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-ethoxy}-ethoxy)-acetylamino]-hexanoylamino}-2-(oxalyl-amino)-hexanoic acid (DR031) (*D*-Lysine derivative)

DR031 (85 mg, 89%) was prepared from **13** (99 mg, 0.11 mmol, 1 eq.) in the same manner used to prepare **DR014**.

^{1}H-NMR (500 MHz, CD$_3$OD) δ_H/ppm: 1.25-1.95 (m, 18H, C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$NHCOCH$_2$O, C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$CONH and NHCH$_2$C*H*$_2$C*H*$_2$C*H*$_2$CHCO$_2$Me), 2.22 (t, 2H, CH$_2$C*H*$_2$CONH, *J*=7.5 Hz), 2.70 (d, 1H, SC*H*$_2$, *J*=12.5 Hz), 2.90 (dd, 1H, SC*H*$_2$, *J*=12.5 and 5.0 Hz), 3.15-3.45 (m, 7H, C*H*S, C*H*$_2$NHCOCH$_2$O, NHC*H*$_2$CH$_2$CH$_2$CH$_2$CHCO$_2$Me and OCH$_2$CH$_2$OCH$_2$C*H*$_2$NHCO), 3.50-3.70 (m, 6H, OC*H*$_2$C*H*$_2$OC*H*$_2$CH$_2$NHCO), 3.97 (s, 2H, OC*H*$_2$CO), 4.30 (dd, 1H, NHC*H*(CH)CHS, *J*=7.5 and 4.5 Hz), 4.40-4.51 (m, 3H, NHC*H*(CH)CH$_2$S, NHCH$_2$CH$_2$CH$_2$CH$_2$C*H*CO$_2$Me and C*H*CH$_2$CH$_2$CH$_2$CH$_2$NHCOCH$_2$O), 7.17 (br d, 2H, aromatics, *J*=8.0 Hz), 7.94 (m, 2H, aromatics, *J*=8.0 Hz).

^{13}C-NMR (126 MHz, CD_3OD) δ_C/ppm: 22.9, 23.2, 24.9, 25.5, 26.4, 27.9, 28.5, 28.9, 30.4, 31.6, 32.5, 35.7, 38.9, 39.9, 40.7, 41.8, 55.0, 62.9, 64.1, 70.3, 70.9, 71.5, 72.9, 120.2, 130.7, 131.5, 145.6, 156.2, 160.7, 163.9, 166.7, 168.1, 171.4, 172.8, 176.0.

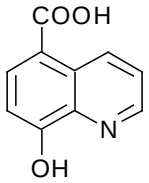
m.p.: 83.5-85 ºC.

FT-IR ν_{max}/cm^{-1} (KBr disk): 3385, 2933, 2361, 2126, 1647, 1542, 1499, 1458, 1285, 1114, 851, 668.

HR-MS (ESI$^+$) $C_{37}H_{53}N_{10}O_{12}S$ ([M-H]$^-$) requires: 861.3564; found: 861.3573.

Microanalysis $C_{37}H_{54}N_{10}O_{12}S$ requires: C, 51.50%; H, 6.31%; N, 16.23%; S, 3.72%; found: C, 51.57%; H, 6.33%; N, 16.18%; S, 3.70%.

8-Hydroxyquinoline-5-carboxylic acid (6)



A solution of the commercial 3-amino-4-hydroxybenzoic acid (2.0 g, 13.05 mmol, 1 eq.), glycerol (1.8 g, 19.58 mmol, 1.5 eq.) and iodine (58.7 mg, 0.23 mmol, 0.017 eq.) in concentrated sulphuric acid (2.5 mL) was heated at 180 ºC for 3 h. The reaction was allowed to cool at r.t., diluted with water (70 mL), made basic to pH 8-9 with an ammonia solution and stirred with activated charcoal (870 mg). The mixture was then filtered through Celite and the filtrate acidified to pH 4-5 with acetic acid. The resulting precipitate was obtained by filtration and dried in a desiccator to yield the desired product **6** as a brown powder (790 mg, 32%) that was used in the next step without further purification.

^{1}H-NMR (400 MHz, DMSO-d_6) δ_H/ppm: 7.13 (d, 1H, quinoline ring, J=8.0 Hz), 7.69 (dd, 1H, quinoline ring, J=8.5 and 4 Hz), 8.25 (d, 1H, quinoline ring, J=8.0 Hz), 8.90 (dd, 1H, quinoline ring, J=4 and 1.5 Hz), 9.46 (dd, 1H, quinoline ring, J=8.5 and 1.5 Hz).

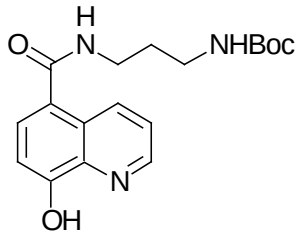
^{13}C-NMR (101 MHz, DMSO-d_6) δ_C/ppm: 111.0, 117.2, 124.1, 128.9, 134.4, 135.3, 139.1, 149.0, 158.7, 168.5.

m.p.: 278-280 ºC (decomposition)(Literature value: 272-273 °C, Clemo and Howe, 1955).

FT-IR ν_{max}/cm^{-1} (KBr disk): 3210, 2568, 1684, 1577, 1511, 1267, 1148, 823, 776, 600.

HR-MS (ESI$^-$) $C_{10}H_6NO_3$ ([M-H]$^-$) requires: 188.0347; found: 188.0329.

{3-[(8-Hydroxyquinoline-5-carbonyl)-amino]propyl}-carbamic acid *tert*butyl ester (DR016)



To a mixture of the commercially available *N*-Boc-1,3-propanediamine (0.42 mL, 2.44 mmol, 1.1 eq.) and **6** (420 mg, 2.22 mmol, 1 eq.) in dry DMF (10 mL) were added in sequence HOBT (330 mg, 2.44 mmol, 1.1 eq.), EDCI (468 mg, 2.44 mmol, 1.1 eq.) and Et_3N (1.08 mL, 7.8 mmol, 3.5 eq.). After stirring overnight at r.t. under inert atmosphere (N_2), the solvent was evaporated under reduced pressure and the residue dissolved in AcOEt (40 mL). The organic phase was then washed in sequence with $NaHCO_3$ saturated solution (3 x 20 mL), and brine (2 x 10 mL), dried over Na_2SO_4 and removed *in vacuo* to give a residue that was purified by automated column chromatography [Biotage SNAP 50 g silica gel cartridge eluting with a gradient 50-100% CH_2Cl_2:MeOH (9:1) in *n*-hexane] to afford **DR016** (628.7 mg, 82 %) as a white solid.

^{1}H-NMR (500 MHz, DMSO-d_6) δ_H/ppm: 1.38 (s, 9H, C(CH_3)$_3$, 1.66 (m, 2H, $CH_2CH_2CH_2$), 3.02 (m, 2H, CH_2NHBoc), 3.28 (m, 2H, CONHCH_2), 6.85 (br t, 1H, CH_2N*H*Boc), 7.08 (d, 1H, quinoline ring, *J*=8.0 Hz), 7.62 (dd, 1H, quinoline ring, *J*=9.0, 4.0 Hz), 7.67 (d, 1H, quinoline ring, *J*=8.0 Hz), 8.42 (br t, 1H, CON*H*CH_2), 8.84 (d, 1H, quinoline ring, *J*=9.0 Hz), 8.90 (d, 1H, quinoline ring, *J*=4.0 Hz), 10.27 (br s, 1H, O*H*).

^{13}C-NMR (126 MHz, DMSO-d_6) δ_C/ppm: 28.3, 29.6, 36.8, 37.7, 77.5, 109.8, 122.4, 124.0, 126.6, 127.8, 134.4, 138.2, 148.4, 155.1, 155.6, 167.5.

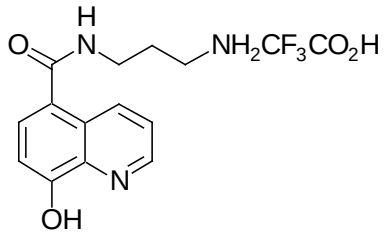
m.p.: 162-164 ºC.

FT-IR ν_{max}/cm^{-1} (KBr disk): 3360, 3316, 2980, 2934, 1687, 1633, 1534, 1367, 1277, 1171, 789, 714.

HR-MS (ESI$^-$) $C_{18}H_{22}N_3O_4$ ([M-H]$^-$) requires: 344.1610; found: 344.1601.

Microanalysis $C_{18}H_{23}N_3O_4$ requires: C, 62.59%; H, 6.71%; N, 12.17%; found: C, 62.68%; H, 6.75%; N, 12.01%.

***N*-(3-Aminopropyl)-8-hydroxyquinoline-5-carboxamide trifluoroacetate salt (7)**



CF_3CO_2H was added (0.49 mL, 6.55 mmol, 18 eq.) to a solution of **DR016** (126 mg, 0.36 mmol, 1 eq.) in dry CH_2Cl_2 (2.5 mL). The resulting mixture was stirred at r.t. for 4 h. Removal of the CF_3CO_2H in excess by several washings with CH_2Cl_2 and evaporation under reduced pressure gave the desired amine salt as a sticky brown oil (117,7 mg, 90 %).

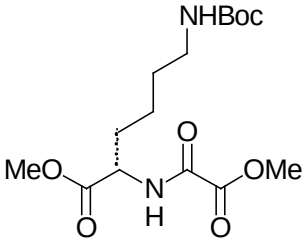
^{1}H-NMR (500 MHz, CD_3OD) δ_H/ppm: 2.03 (tt, 2H, $CH_2CH_2CH_2$, *J*=7.5, 7.0 Hz), 3.09 (t, 2H, $CONHCH_2$, *J*=7.5 Hz), 3.58 (t, 2H, $CH_2CH_2NH_3^+$, *J*=7.0 Hz), 7.36 (d, 1H, quinoline ring, *J*=8.0 Hz), 7.96 (m, 2H, quinoline ring), 9.07 (dd, 1H, quinoline ring, *J*=5.0, 1.5 Hz), 9.45 (dd, 1H, quinoline ring, *J*=8.5, 1.0 Hz).

^{13}C-NMR (126 MHz, CD_3OD) δ_C/ppm: 28.9, 30.8, 37.5, 38.4, 112.4, 123.9, 124.7, 128.7, 130.6, 136.5, 139.7, 147.9, 155.2, 171.1.

FT-IR ν_{max}/cm^{-1} (thin film): 3292, 2943, 1687, 1638, 1536, 1271, 1175, 791, 715.

HR-MS (ESI^+) (relative to free amine) $C_{13}H_{16}N_3O_2$ ($[M+H]^+$) requires: 246.0939; found: 246.0928.

General procedure for the preparation of 6-tert-butoxycarbonylamino-2-(methoxyoxalyl-amino)-hexanoic acid methyl esters (8,9)



8 (*L*)
9 (*D*)

Example: (*D*)-6-tert-butoxycarbonylamino-2-(methoxyoxalyl-amino)-hexanoic acid methyl esters (9)

(*D*)-H-Lys(Boc)-OMe (572 mg, 2.2 mmol, 1 eq.), prepared as reported (Manesis and Goodman, 1987), and Et_3N (0.46 mL, 3.3 mmol, 1.5 eq.) were dissolved in dry CH_2Cl_2 (14 mL). The resulting solution was added dropwise, while cooling on ice, a solution in dry CH_2Cl_2 (3 mL) of commercially available methyl chlorooxoacetate (0.22 mL, 2.4 mmol, 1.1 eq.). After stirring overnight at r.t. under inert atmosphere (N_2) the reaction mixture was diluted with additional CH_2Cl_2 (20 mL) and washed in sequence with $KHSO_4$ 1N (2 x 35 mL), $NaHCO_3$ saturated solution (3 x

35 mL), and brine (2 x 25 mL).The organic phase was then dried over Na_2SO_4 and removed *in vacuo* to give a residue that was purified by automated column chromatography [Biotage SNAP 50 g silica gel cartridge eluting with a gradient 10-100% AcOEt in *n*-hexane] to afford **9** (675.1 mg, 89 %) as a colourless oil.

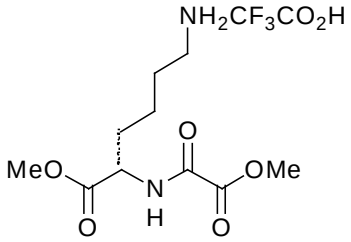
^{1}H-NMR (500 MHz, $CDCl_3$) δ_H/ppm: 1.26-1.54 (m, 13H, OC(CH_3)$_3$ and $CH_2CH_2CH_2NHBoc$), 1.78 (m, 1H, $CH_2CH_2CH_2CH_2NHBoc$), 1.95 (m, 1H, $CH_2CH_2CH_2CH_2NHBoc$), 3.10 (m, 2H, CH_2NHBoc), 3.78 (s, 3H, CO_2CH_3), 3.93 (s, 3H, $COCO_2CH_3$), 4.51 (br s, 1H, N*H*Boc), 4.63 (m, 1H, CONHC*H*COOMe), 7.56 (d, 1H, CON*H*CHCOOMe, *J*=8.0 Hz).

^{13}C-NMR (126 MHz, $CDCl_3$) δ_C/ppm: 22.4, 28.4, 29.6, 31.9, 40.0, 52.5, 52.7, 53.7, 79.2, 155.9, 160.3, 160.5, 171.5.

IR ν_{max}/cm^{-1} (thin film): 3358, 2956, 1743, 1697, 1524, 1438, 1282, 1173, 987, 783.

HR-MS (ESI^+) $C_{15}H_{25}N_2O_7$ ($[M-H]^-$) requires: 345.1661; found: 345.1656.

General procedure for the preparation of 2-(methoxyoxalyl-amino)-hexanoic acid methyl ester trifluoroacetate salts (10,11)



10 (*L*)
11 (*D*)

Example: (*L*)-2-(methoxyoxalyl-amino)-hexanoic acid methyl ester trifluoroacetate salt (10)

CF_3CO_2H was added (1.28 mL, 17.3 mmol, 18 eq.) to a solution of the (*L*)-6-tert-butoxycarbonylamino-2-(methoxyoxalyl-amino)-hexanoic acid methyl esters **8** (334 mg, 0.96 mmol, 1 eq.) in dry CH_2Cl_2 (5.5 mL) and the resulting mixture was stirred at r.t. for 3 h. Removal of the CF_3CO_2H in excess by several washings with CH_2Cl_2 and evaporation under reduced pressure gave the desired amine salt as a sticky oil (313 mg, 90 %).

^{1}H-NMR (500 MHz, $CDCl_3$) δ_H/ppm: 1.46 (m, 2H, C*H*$_2$CH$_2$CH$_2$NH$_3^+$), 1.74 (m, 3H, CH$_2$C*H*$_2$CH$_2$NH$_3^+$ and C*H*$_2$CH$_2$CH$_2$CH$_2$NH$_3^+$), 1.95 (m, 1H, C*H*$_2$CH$_2$CH$_2$CH$_2$NH$_3^+$), 3.01 (m, 2H, CH$_2$C*H*$_2$NH$_3^+$), 3.77 (s, 3H, CO$_2$C*H*$_3$), 3.90 (s, 3H, COCO$_2$C*H*$_3$), 4.59 (m, 1H, NHC*H*COOMe), 7.78 (m, 4H, N*H*CHCOOMe and N*H*$_3^+$).

^{13}C-NMR (126 MHz, $CDCl_3$) δ_C/ppm: 21.9, 26.4, 31.6, 39.5, 50.8, 52.2, 53.7, 156.6, 159.9, 171.4.

IR ν_{max}/cm^{-1} (thin film): 3301, 2934, 1697, 1524, 1436, 1281, 1175, 984, 785.

HR-MS (ESI$^+$) (relative to free amine) $C_{10}H_{19}N_2O_5$ ([M+H]$^+$) requires: 247.1295; found: 247.1299.

Purified Enzymes Used in this Study

Recombinant human PHD2$_{181–426}$ was produced and purified by cation exchange and size exclusion chromatography, as described (McNeill et al., 2005). Apo-PHD2 was obtained by incubation with EDTA after cation exchange chromatography. Protein purity (> 95%) was assessed by SDS-PAGE.

Recombinant His-tag JMJD2E$_{1-337}$ was produced and purified by nickel affinity chromatography and size exclusion chromatography, essentially as described (Rose et al., 2008). The enzyme was shown to be > 95% pure by SDS-PAGE analysis.

FBXL11 (KDM2A) containing the JmjC-catalytic domain (1-517aa) was cloned into pNIC-BSA4 using ligation independent cloning (Graslund et al., 2008) and transformed into BL21-Gold(DE3) *E. coli* cells (Stratagene). FBXL11 (KDM2A) expression was conducted as reported

(Han et al., 2007) with modifications. Cells were grown in terrific broth supplemented with 50µg/mL kanamycin and induced with 0.5 mM IPTG at mid log-phase. Cells were grown for further 16 hours at 25 °C and harvested. Cells were resuspended in 50 mM HEPES (pH 7.5), 500 mM NaCl, 20 mM imidazole, 5% glycerol and lysed by sonication. After centrifuging to remove cell debris (14,000 rpm, 10 minutes, 4 °C), His-tagged (FBXL11) KDM2A was purified using Ni-affinity gel (Sigma, USA). The purified fractions were treated with 1 mM EDTA and the TEV protease overnight. His-tag cleaved FBXL11 (KDM2A) was further purified by gel filtration chromatography in 50 mM HEPES (pH 7.5), 500 mM NaCl, 5% glycerol (Superdex 200, Pharmacia) and the most catalytically active fractions of KDM2A were pooled. Protein purity was >90% as determined by SDS-PAGE.

NMR PHD2 Binding Assay

Dissociation Constants (K_D) of the tested compounds with apo-PHD2$_{181-426}$ were determined in water relaxation experiments essentially as described (Leung et al., 2010). NMR experiments were conducted at 500 MHz using a Bruker Avance II spectrometer equipped with a standard 5 mm z-gradient TXI probe at 298 K. 3 mm diameter Bruker MATCH tubes (Hilgenberg, Germany) containing 50 µM PHD2 and 50 µM Mn(II). Solutions were buffered using Tris-D11 (pH 7.5) dissolved in 12.5% H_2O and 87.5% D_2O. Saturation recovery experiments were performed with 1 scan with a relaxation delay of at least 5 times T_1 between transients. Pulse tip-angle calibration using the single-pulse nutation method was undertaken for each sample (Wu et al., 2005). K_D curves were fitted using OriginPro 8.0 (OriginLab, Massachusetts, USA) using a reported equation (Fielding, 2003).

MALDI-based Hydroxylation Inhibition Assays

PHD2 inhibition, in terms of reduced CODD peptide substrate hydroxylation, was measured by matrix-assisted laser desorption/ionization MS (MALDI-TOF MS). Standard assay mixtures contained: 100 μM CODD substrate (DLDLEMLAPYIPMDDDFQL), 300 μM of 2-OG, different concentrations of the putative inhibitor, 4 mM of ascorbic acid, 1 mM of DTT, 50 μM of $FeSO_4 \cdot 7H_2O$ (dissolved first to 250 mM with 20 mM HCl, then 2.5 mM with Milli-Q water) and enzyme (1 μM). Assay mixtures were made up to 100 μL with 50 mM Tris pH 7.5. Reagents were pipetted into the base of a 5 mL tube as three separate spots: enzyme, substrate, and all the other reagents prepared as a master mixture. Assays were incubated at 37 °C for 10 minutes in a shaking incubator then quenched by 1:1 addition of 0.1% formic acid and stored at -20 °C. Samples were finally analysed by MALDI-TOF MS. For comparison, positive controls were without inhibitor, and negative controls without enzyme. Assays were carried out at one time in triplicate. MALDI MS was performed on a MALDI-TOF microMX mass spectrometer (Waters Micromass) in negative ion reflectron mode with the following parameters: Laser energy 155, Pulse 2000, Detector 2700 and Suppression 700. Calibration was carried out using 1 μl PepMix4 (Trypsinogen, Enolase, BSA) in 9 μl CHCA (recrystallized α-cyano-4-hydroxy-cinnamic acid) matrix (both Laser BioLabs). Sample were prepared by mixing 1 μL of solution with CHCA MALDI matrix (1 μL) and spotting all together onto a 96 well sample plate and allowing to air dry before measurements. Data were analyzed using MassLynx version 4.1. Relative intensities of the hydroxylation states observed in the mass spectra were then used to calculate the residual percentage of hydroxylation in the presence of each inhibitor concentration and then the corresponding percentage of inhibition in comparison with the experiment without any inhibitor (Rose et al., 2008).

Formaldehyde Dehydrogenase (FDH) Coupled JMJD2E Inhibition Assays Formaldehyde release by demethylation of the N^{ε}- Lys9 trimethylased histone fragment peptide substrate (H3K9me3) was monitored by its oxidation to give formate as catalyzed by FDH, which occurs concomitantly with the reduction of nicotinamide adenine dinucleotide (NAD^+). The production of NADH was monitored by fluorescence

spectroscopy. The assay was carried out with modifications (Rose et al., 2008). FDH from *Pseudomonas Putida* (50 nM), NAD^+ (500 μM), *L*-ascorbic acid (100 μM), ferrous ammonium sulphate (FAS, 10 μM), JMJD2E (1 μM), ARK(me3)STGGK-NH_2 histone H3 fragment peptide substrate (100 μM), 2-OG (20 μM) and the inhibitors (different concentrations) were incubated in 25 μL reactions at 25 ºC for 15 min while fluorescence was recorded (355 nm excitation, 460 nm emission) at 30 s intervals. Inhibitors were dissolved in DMSO at various concentrations, and added to the assay mixture such that the final DMSO concentration was < 1 %. All other reagents were used as solutions in HEPES buffer (50 mM, pH 7.5) containing 0.01% of Tween-20, with the exception of Fe(II) solutions, which were made using $(NH_4)_2Fe(SO_4)_2$ dissolved in 20 mM HCl to make 400 mM stock solutions, which were then diluted to the proper concentration with MilliQ water. All data were processed with GraphPad Prism 5.0.

Formaldehyde Dehydrogenase (FDH) Coupled FBXL11 (KDM2A) Inhibition Assays

Detailed kinetic characterisation of FBXL11 protein will be published elsewhere. Inhibition of FBXL11 (KDM2A) activity was measured using FDH as described (Couture et al., 2007; Rose et al., 2008). The assay reaction contained FBXL11/KDM2A (0.5 μM), 2OG (10 μM), H3(30-41)K36me2 (50 μM), 10 μM $(NH_4)_2SO_4.FeSO_4.6H_2O$, 100 μM ascorbate, 0.025U FDH from *Pseudomonas putida* (Sigma), 0.5 mM NAD^+ (Sigma) in 50mM HEPES (pH 7.5), 0.01% Tween-20 in a 25 μl reaction. Test compounds were pre-incubated with enzymes for 15 minutes. The remaining substrates and co-factors were added to start the reaction and incubated at rt for 20 minutes while fluorescence was recorded (355 nm excitation, 460 nm emission) at 50s intervals. The final DMSO concentration in the reaction was 1%. Data were processed using GraphPad Prism 5.0.

Cell Cultures

Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, CA, USA) supplemented with 10% (v/v) fetal calf serum (FCS, Sigma), penicillin G (100 UmL^{-1}) and streptomycin (100 μgmL^{-1}) at 37 ºC and 5% CO_2. Hypoxic conditions were achieved by culturing cells in an $InVivo_2$ 500 Hypoxia Workstation (Ruskinn Technology, UK) with a mixture of 1% O_2 and 5% CO_2 for 24 hours. HEK293T cells were transfected with the plasmid pcDNA3 (Epstein et al., 2001) by using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions for the over-expression of human full length PHD2. After harvesting, cell pellets were washed twice with PBS and frozen with liquid nitrogen and stored at -80 ºC until use.

Preparation of Lysates and Nuclear Protein Extracts

To obtain HEK293T cell lysates, cells were lysed for 30 minutes on ice in 0.125% NP40 PBS (pH 7.0) supplemented with an EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). After centrifugation (1000g, 5 min, 4 ºC), the supernatant was collected and further centrifuged (5000g, 5 min, 4 ºC). After transfer of supernatant material, when appropriate, a slurry of avidin-coated agarose beads (Pierce, Thermo Scientific, Rockford, USA) was added to the lysate to remove the biotin-containing proteins (*e.g.* carboxylases), the resulting mixture was then rotated at 4 ºC for 30 minutes, centrifuged at 5000g (5 minutes, 4 ºC) and finally decanted.

HEK293T cells were harvested, washed with PBS and resuspended in 10 volumes of lysis buffer [10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and an EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany)]. The cells were then incubated on ice for 10 minutes and centrifuged (1500g, 5 min, 4°C). The supernatant was discarded and the cells were resuspended in 3 volumes of lysis buffer and nuclei were released by 40 strokes with a potter-elvehjem (glass-teflon) dounce homogenizer. The homogenate was centrifuged (1500g, 5

min), the supernatant was discarded and the pellet containing the nuclei was washed 3 times with lysis buffer (each time spun at 1500 g except after the last wash when the nuclei were spun at 5000g). The nuclear pellet was resuspended 1:1 in the nuclear lysis buffer [5 mM Hepes pH 7.9, 1.5 mM $MgCl_2$ and 26% glycerol and an EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany)]. Saturated brine was then added dropwise up to a final NaCl concentration of 400 mM under gentle agitation, incubated on ice for 1 hour and centrifuged (21,000 g, 20 min). The supernatant containing the nuclear proteins was finally frozen at -80 °C for further processing.

The total protein concentration was always determined according to the BCA™ Protein Assay Kit (Pierce, Thermo Scientific, Rockford, USA).

MALDI TOF Analysis of Photo-Affinity Tagging

Photo-affinity labelling experiments with $PHD2_{181-426}$ were analysed by MS, using a MALDI-TOF microMX mass spectrometer (Waters Micromass) in positive ion linear mode with the following parameters: Laser energy 170, Pulse 2050, Detector 2700 and Suppression 4500. Calibration was carried out using 1 µl ProteinMix (Cythochrome C, Myoglobin, Trypsinogen) in 9 µl SA (recrystallized sinapinic acid) matrix (both Laser BioLabs). Samples were prepared by mixing 1 µL of the irradiated mixture, or of a water suspension of the beads, with SA MALDI matrix (1 µL) followed by spotting a MALDI 96 well sample plate and allowing to air dry before measurements. Data were analyzed using MassLynx v4.1. The yield of cross-linking was estimated from the areas of the MALDI peak of PHD2-DR025 adduct and for the uncaptured and captured proteins. For yield determinations, the photo cross-linking experiments were carried out in triplicate.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For analysis of photo-affinity tagging experiments by SDS-polyacrylamide gel electrophoresis followed by silver staining, or by Western blotting the beads with captured proteins were resuspended in an appropriate volume (depending on the number of beads) of reducing sample buffer (Laemmli, 1970) and heated (95 °C, 10 min). Subsequently, the beads were separated from the released proteins using a magnet and loaded onto the gel. Alternatively the supernatant was carefully loaded directly onto the gel. Gels were stained using the Pierce® Silver Stain for Mass Spectrometry (Thermo Scientific, Rockford, USA) according to the manufacturer's instructions.

Western Blot Analysis of Photo-Affinity Tagging

Western blots were carried out according to standard procedures. After separation by SDS-PAGE, the proteins were transferred to PVDF membranes (Immobilon-P™ Transfer Membrane, Millipore, Billerica, MA, USA). The membranes were blocked for 1 h at r.t. with a solution of 5% (wt/vol) skimmed milk powder in PBS buffer supplemented with 0.1% [vol/vol] Tween 20 [PBS-T]. Incubation with the primary antibody was performed for 1 h at r.t. or overnight at 4 ºC, followed by three wash steps in PBS-T and incubation with the secondary antibody for 1 h at r.t.. Antibodies were diluted in 5% skimmed milk powder in PBS-T as follows: polyclonal anti-PHD2 from rabbit (Abcam plc, Cambridge, UK) 1:1000, secondary anti-rabbit antibody conjugated to horseradish peroxidise (DakoCytomation, Glastrup, Denmark) 1:10000; monoclonal anti-PHD2 (clone 76A, prepared as reported in Appelhoff et al., 2004) from mouse 1:50, secondary anti-mouse antibody conjugated to horseradish peroxidise (DakoCytomation, Glastrup, Denmark) 1:10000; monoclonal anti-PHD3 (clone 188E, prepared as reported in Appelhoff et al., 2004) from mouse 1:10, secondary anti-mouse antibody conjugated to horseradish peroxidise (DakoCytomation, Glastrup, Denmark) 1:10000; monoclonal anti-HIF-1α (clone 54, BD Transduction Laboratories™, BD Biosciences) from mouse 1:1000, secondary anti-mouse antibody conjugated to horseradish

peroxidise (DakoCytomation, Glastrup, Denmark) 1:10000; monoclonal anti-polyHystidine antibody from mouse (clone HIS-1, Sigma, Saint Louis, Missouri, USA) 1:3000, secondary anti-mouse antibody conjugated to horseradish peroxidise (DakoCytomation, Glastrup, Denmark) 1:10000; polyclonal anti-KDM2A from rabbit (Blackledge et al., 2010) 1:1000, secondary anti-rabbit antibody conjugated to horseradish peroxidise (DakoCytomation, Glastrup, Denmark) 1:5000. After three washes in PBS-T and one in PBS, membranes were usually treated with ECL Plus Western Blotting Detection System (Amersham™, GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. For detection of endogenous levels of human PDH2, PHD3, HIF-1α and FBXL11 (KDM2A) membranes were treated with Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, USA) according to the manufacturer's instructions. Kodak X-Omat LS films (Sigma-Aldrich, St. Louis, USA) were used to detect the chemiluminescence. In case of the detection of biotinylated proteins, Amersham ECL Streptavidin Horseradish Peroxidase Conjugate (GE Healthcare, Piscataway, USA) was used instead of a primary antibody at a dilution of 1:500 in 5% skimmed milk powder in PBS-T and blots were developed directly after washing with PBS-T (x3) and PBS (x1).

Protein Digestion

Following the photo-affinity tagging experiments, two protocols of protein digestion were applied: (i) in-solution digestion (or 'on bead-digestion') was carried out for 2-OG oxygenase profiling in whole HEK293T cell lysates and the identification of the $PHD2_{181-426}$ peptide sequence covalently cross-linked by DR025; (ii) in-gel digestion was used for the identification of captured $PHD2_{181-426}$ (Figure 3B, right panel) and JMJD2E (see Supplemental Figure S3B, right panel) in the photo-affinity labelling tests in the presence of HEK293T cell lysates as background. The in-solution and the in-gel digestion methods were carried out essentially as reported (Cockman et al., 2009 for the in-solution, and Batycka et al., 2006 for the in-gel digestion).

Nano LC-MS/MS

LC-MS/MS analyses for the profiling of the 2-OG oxygenases was conducted using a Waters nano-Acquity UPLCsystem coupled to a Q-TOF Premier^{TM} tandem mass spectrometer (Waters) essentially as reported (Cockman et al., 2009). The LC-MS/MS experiments for the identification after in-gel digestion of PHD2 and JMJD2E photo labelled in the presence of HEK293T cell lysates as background, were also carried out on an Ultimate HPLC (LC-Packings, Dionex, Amsterdam, The Netherlands) system directly coupled to a 3D high-capacity ion trap (HCTplus, Bruker Daltonics, Bremen, Germany) essentially as reported (Batycka et al., 2006).

The identification of the $PHD2_{181-426}$ peptide sequence covalently cross-linked by DR025 was performed on an Agilent 6520 Q-TOF mass analyser after separation on a 43mm x 75µm Zorbax 300SB-C18 5µm column (Agilent). For chromatographic separation a gradient of 5.5% to 40% acetonitrile in 0.1% formic acid over 7.5 min was applied. MS data were acquired with a spectrum frequency of 5 Hz for MS spectra and 2 Hz for MS/MS spectra.

Peptide Identification via Database Search

Proteins were identified by automated database searching against the human UniProtKB/Swiss-Prot using Mascot (Matrix Science) database essentially as reported (Cockman et al., 2009). Identification of proteins was based on the detection of at least two peptides.

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