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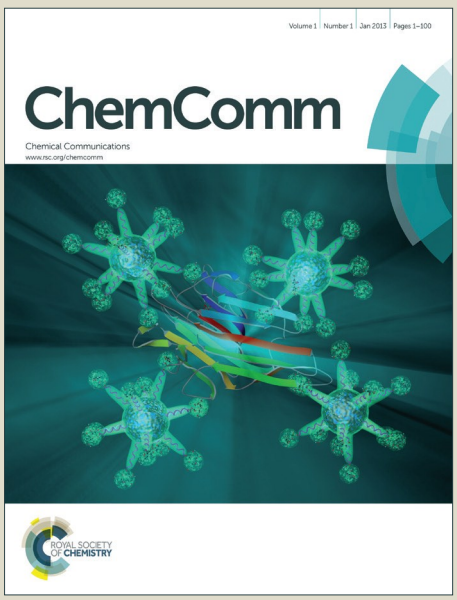

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Betti reaction enables efficient synthesis of 8-hydroxyquinoline inhibitors of 2-oxoglutarate oxygenases

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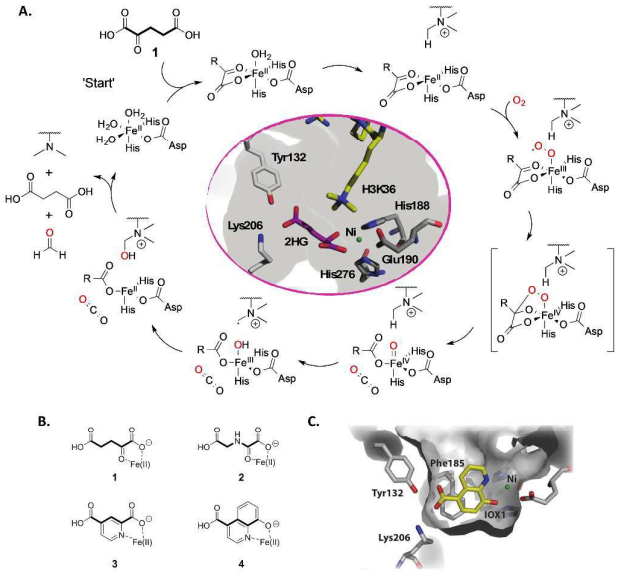
There is interest in developing potent, selective, and cell-permeable inhibitors of human ferrous iron and 2-oxoglutarate (2OG) oxygenases for use in functional and target validation studies. The 3-component Betti reaction enables efficient one-step C-7 functionalisation of modified 8-hydroxyquinolines (8HQs) to produce cell-active inhibitors of KDM4 histone demethylases and other 2OG oxygenases; the work exemplifies how a template-based metallo-enzyme inhibitor approach can be used to give biologically active compounds.

The application of modern molecular biology techniques has led to the identification of a plethora of potential pharmaceutical targets. Applying efficient methods to validate these targets is an objective of academic and commercial research at the chemistry-biology-medicine interface, in many cases requiring both genetic and chemical approaches.[1] We are interested in defining readily applicable methods for the synthesis of small molecules for use as functional probes for metallo-enzymes, in particular the 60-70 human ferrous iron and 2-oxoglutarate **1** (2OG)- dependent oxygenases. 2OG oxygenases play roles in all stages of protein biosynthesis in humans, including translation, splicing, and transcription.[2] The reversible methylation of histones is of central importance in eukaryotic transcriptional regulation. The JmjC 2OG oxygenases are the largest family of histone lysyl demethylases (KDMs) with >15 human members grouped into 6 subfamilies (KDM2-7), some of which are anticancer targets.[3]

Defining the functions of the JmjC domains is challenging, in part because of the presence of multiple other domains in the JmjC-containing proteins, and in part because of redundancy. There is interest in developing inhibitors not only for specific 2OG oxygenases, but for specific enzyme sets, in order to up- or down-regulate expression of specific sets of genes.[4]

There are 6 human KDM4 enzymes, KDM4A-F (KDM4E/F being pseudogenes); their preferred substrates are histone 3 lysine 9 (H3K9) and histone 3 lysine 36 (H3K36) (for KDM4A-C only) N^{ε}-tri- and dimethylated lysines (H3K9me3 being demethylated most efficiently).[5] Although some 2OG oxygenase-selective inhibitors are reported,[4] including for JmjC KDMs, no such

Fig.1 JmjC histone demethylase catalysis and inhibition. (A) Outline mechanism for JmjC KDMs. The centre insert shows a view from a KDM4A structure complexed with a



H3K36me3 fragment substrate and 2-(*R*)-hydroxyglutarate (a 2OG analogue) (PDB ID: 2YBP). (B) 2OG competitor inhibitors *N*-oxalylglycine (NOG) **2**, 2,4-pyridinedicarboxylic acid (2,4-PDCA) **3**, and 8-hydroxyquinoline-4-carboxylic acid (4C8HQ) **4**. (C) View from a structure of KDM4A (PDB ID: 3NJY) complexed with **IOX1**. Key interactions include hydrogen bonding between the **IOX1** carboxylate and Tyr132/Lys206, π-stacking with Phe185, and bidentate metal chelation (Ni substitutes for Fe).

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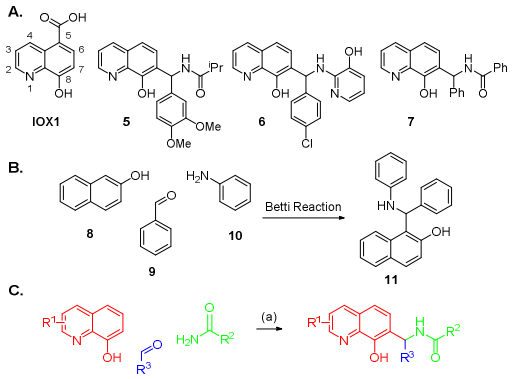


Fig. 2 The Betti reaction and 2OG oxygenase inhibitors. (A) Broad-spectrum inhibitor **IOX1**, cell-active modulators of the hypoxic response **5** and **6**, and original compound **7**. (B) Example of the classical Betti reaction leading to the aminoalkylation of 2-naphthol **8** with benzaldehyde **9** and aniline **10**. (C) General scheme for the synthesis of 7-substituted 8-hydroxyquinolines using a modified Betti reaction. (a): Neat, >130 °C, 3 h.

compounds are reported for the KDM4 subfamily, and there is little work on developing methods for generating inhibitors for different sets of 2OG oxygenases. We report that a modified Betti reaction is useful for efficient modification of 8-hydroxyquinoline templates for the generation of cell-active KDM4 and other 2OG oxygenase inhibitors.

Structural analyses reveal that 2OG oxygenase substrate binding sites vary considerably, but their Fe(II) and 2OG binding pockets are more conserved;[6] during catalysis, 2OG **1** binding is followed by substrate, then O_2 (Fig. 1A). 2OG **1** chelates to the metal in a bidentate manner *via* its ketone and C-1 carboxylate; the 2OG binding site is thus attractive for targeting template inhibitors (Fig. 1B), with the substrate binding site offering a region into which selectivity-enabling side chains could extend.[7] We recently reported that the 2OG analogue 8-hydroxyquinoline-5-carboxylic acid, **IOX1**, is a broad-spectrum 2OG oxygenase inhibitor active against enzymes including the JmjC KDMs (Fig. 1C, 2A, ST1).[8, 9] Some 7-substituted 8-hydroxyquinoline (8HQ) derivatives (e.g. **5** and **6**, Fig. 2A) inhibit a 2OG oxygenase, the prolyl hydroxylase domain-containing protein (PHD2, which is involved in transcription factor modification).[10] **IOX1** binds to the metal of 2OG oxygenases *via* its pyridinyl nitrogen and phenolic oxygen, and its C-5 carboxylate binds similarly to that of 2OG (Fig. 1C).[9] We therefore considered 8HQ as a potential template for efficient modification to obtain 2OG oxygenase inhibitors.

Modelling of 8HQ inhibitors based on KDM4A structures indicates that modifications at C-7 of 8HQ will extend towards the substrate binding site (Fig. 4C). We considered methods for C-7 derivatisation and investigated the 3-component Betti reaction, which classically comprises C-α functionalisation of phenols *via* a Mannich-type process (Fig. 2B).[11] Preliminary studies employed aromatic amides and aldehydes under high-temperature and solvent-free conditions to give e.g. **7** (Fig. 2C).[12, 13] Heating 8HQ **15**, benzamide, and benzaldehyde **9** (180 °C, 3h) afforded a crude mixture; treatment with toluene precipitated the desired racemic product **7** on cooling, with unreacted starting materials remaining in solution (Fig. 2A). *In vitro* activity assay results with **7** as an inhibitor of KDM4C/E (see below) encouraged further structure-activity relationship (SAR) studies employing commercial and synthetic aldehydes (prepared by nitrile reduction using diisobutylaluminium hydride (supplementary information **S1**)), or amides using sodium perborate tetrahydrate with microwave irradiation (**S2**). Modified 8HQs were prepared from the requisite 2-aminophenols and acroleins *via* Skraup condensation (**S3**-**S15**); 4- and 5-substituted 8HQs and biphenyl-containing aldehydes were prepared *via* palladium-mediated cross-coupling (**S16**-**S26**). When conducting the Betti-type reaction at 130 °C, below which little product formation was observed, a wide variety of non-nucleophilic aromatic (**S28**-**S73**), heterocyclic (**S74**-**S115**), electron-rich (**S116**-**S132**), and –poor aldehydes (**S133**-**S138**), with primary amides, gave the desired 7-substituted 8HQs.

Aliphatic amides, but not aliphatic aldehydes, yielded the desired products (**S139**-**S149**). Amines, instead of amides, can be used employing the standard Betti conditions (Fig. 3A, **S150**- **S153**). Reactions in the presence of nucleophiles, e.g. free alcohols or amines, did not yield the desired products; nucleophilic substituents were introduced after Betti reaction, e.g. amines by amide coupling involving the free acid **23** to give **S154**-**S158** (Fig. 3D), and the nitro group was reduced to the free amine using sodium dithionite (**S159**). The conditions tolerate 'bifunctional' component urea **18** in a double Betti reaction to give **19** as a mixture of stereoisomers (Fig. 3B) and a 2-component process with both amide and aldehyde functionalities in the same molecule **21** to give **22** (Fig. 3C).

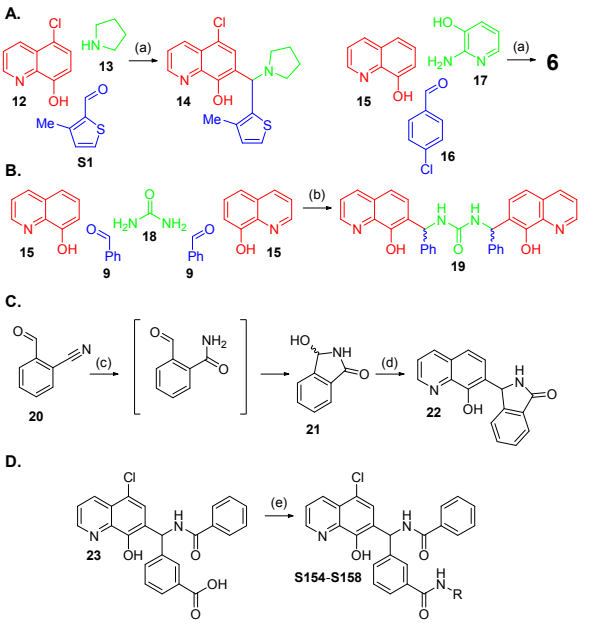
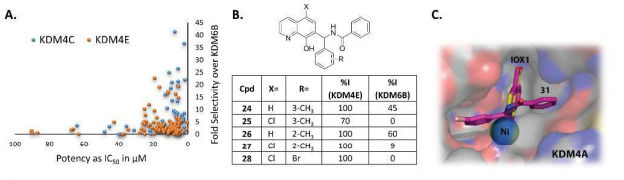


Fig. 3 Modified Betti reaction enables efficient inhibitor synthesis. (A) Synthesis of **14** and **6** employing standard Betti reaction conditions. (a): EtOH, RT, 72 h. (B) Double Betti reaction using bifunctional urea **18**. (b): Neat, 180 °C, 3 h. (C) Two-component Betti reaction with aldehyde and amide functionalities in one substrate **21**. (c): $NaBO_3{\cdot}4H_2O$, EtOH, MW irradiation, 100 °C, 10 min. (d): 8HQ, neat, 180 °C, 3 h. (D) Derivatisation of carboxylate **23** *via* amide coupling. (e): EDCI, HOBt, DMF, CH_2Cl_2, 50 °C, 18 h.



Cpd	X=	R=	%I (KDM4E)	%I (KDM6B)
24	H	3-CH_3	100	45
25	Cl	3-CH_3	70	0
26	H	2-CH_3	100	60
27	Cl	2-CH_3	100	9
28	Cl	Br	100	0

Cpd	X=	R^1=	R^2=	IC_{50} / μM (KDM4C)	IC_{50} / μM (KDM4E)	IC_{50} / μM (KDM6B)	Cpd	X=	R^1=	R^2=	IC_{50} / μM (KDM4C)	IC_{50} / μM (KDM4E)	IC_{50} / μM (KDM6B)
29	Cl	Ph	Ph	6	18	120	35	Cl	Bn	S	40	NI	NI
30	Cl	Ph	Ph	39	NI	238	36	Cl	O NH	S	7	ND	253
31	Cl	Ph	OPh	5	NI	424	37	Cl	O NH	S	7	82	ND
32	NO_2	Ph	OPh	6	11	18	38	Cl	Ph	S	7	14	52
33	Br	Ph	OPh	5	NI	8	39	Cl	Ph	S	9	90	124
34	Br	Bn	OPh	182	NI	NI	40	Cl	Ph	S	40	NI	NI
CCT1	Cl	Ph	S	5	5	106	41	F	Ph	Br	ND	8	NI

Fig. 4 Inhibition of JmjC KDMs by Betti products. (A) Potency for KDM4C/E (IC_{50}/μM) *vs*. selectivity against KDM6B shown as the ratio of IC_{50} for KDM6B and the respective IC_{50} for KDM4C/E. (B) Selection of 7-substituted 8HQs illustrating structure-activity and -selectivity relationships of early-stage compounds. (C) Docking of (*S*)-**31** (pink) overlayed with **IOX1** (yellow) co-crystallised with KDM4A (PDB ID: 3NJY). (D) Effect of various 8HQ 7-substituents on potency/selectivity *vs*. KDM4C/E and KDM6B. NI: no inhibition. ND: not determined.

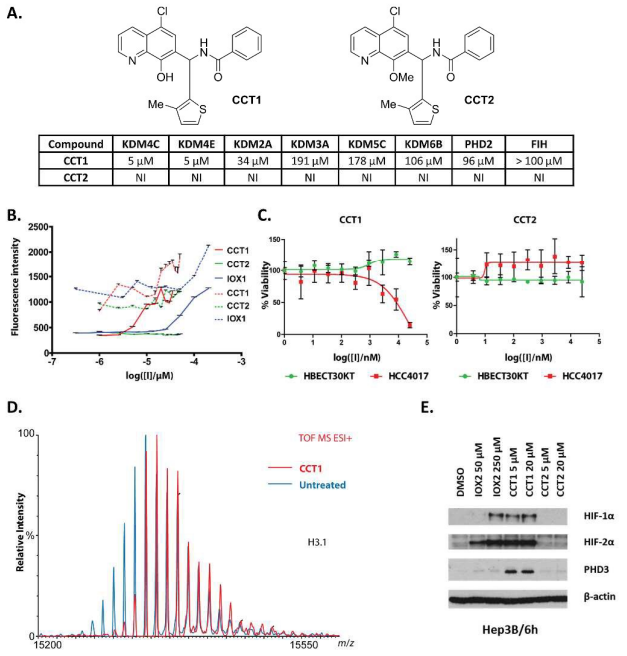
To validate the Betti approach for generation of inhibitors, we screened with KDM4C/E, which act on methylated H3K9 (KDM4C also acts on H3K36), and counter-screened against KDM6B, which acts on methylated H3K27. While some 7-substituted 8HQs inhibited both KDM4 and KDM6B, plotting potency *vs*. selectivity reveals a trend towards KDM4C/E-selective compounds (Fig. 4A). SAR analysis suggested selectivity for KDM4C/E over KDM6B may emerge from a combination of halogen substitution at the 8HQ C-5 position and modification at the *meta*-position of the aldehyde-derived component; e.g. comparing **24** and **25**, or **26** and **27** (Fig. 4B). Note that bulky *meta*-substitution on the aldehyde Betti component is tolerated for KDM4, but not KDM6B inhibition, consistent with the relatively large 2OG substrate binding pocket for the KDM4s, compared to KDM6B (Fig. 4C/D). In contrast, alteration of the amide component did not lead to significant changes in activity (e.g. **35**, **36**, **37**) (7-substituted 8HQs derived from a combination of both relatively large aldehydes and large amides were inactive, e.g. **34**) (Fig. 4D).

Selected compounds displaying reasonable activity against isolated KDM4s were tested in an immunofluorescence (IF) assay using HeLa cells with transiently overexpressed KDM4A (SF1). Some compounds (**27**, **28**, **S85**, **S120** (SF1)) showed apparent inhibition as evidenced by increased H3K9me3 levels, with EC_{50}s in the μM range, i.e. the compounds are more potent than **IOX1**.[9] Racemic **CCT1** was selected for further investigation as the resolved enantiomers displayed little difference in KDM4 inhibition (Fig. 5A, SF2A/B); with HeLa cells the **CCT1** EC_{50} value was 9 μM, ~10-fold more potent than **IOX1** (EC_{50} = 86 μM), at a concentration range where catalytically inactive KDM4A showed no significant changes in H3K9me3 levels. The IF cell assays show a dose-dependent increase in H3K9me3 fluorescence and a corresponding decrease in cell numbers (SF3A/B/C/D) suggesting a narrow window between cellular effect and toxicity. The **CCT1** analogue **CCT2**, in which the phenolic oxygen is methylated, was not cytotoxic, displayed no inhibition of isolated KDM4, and did not affect H3K9me3 levels in cells (Fig. 5A, SF3A/B/C/D). KDM4s are overexpressed in both breast and lung cancer cells:[14] **CCT1**, but not **CCT2**, increased H3K9me3 levels in an MCF7 breast cancer cell line (EC_{50} = 12 μM) (SF3B). The effect of **CCT1** and **CCT2** on proliferation in some patient-matched cell lines, one derived from cancerous, the other from normal, lung tissue of the same patient was tested.[15] **CCT1** exerted a marked effect on the viability of the lung cancer cells (EC_{50} = 6 μM), whereas the non-cancer cells were relatively unaffected; **CCT2** had little effect (Fig. 5C).

Intact protein mass spectrometry indicated that histones H3.1 and H3.2 from HEK293T cells treated with **CCT1** have an overall positive shift in mass, indicative of increased levels of post-translational modification compared to untreated cells (Fig. 5D); **CCT1** manifested no effect on modifications to H2A, H2B, and H4 by this technique (SF4). Since histone methylation principally occurs on H3 proteins, the results support the proposal that **CCT1** is a JmjC KDM inhibitor in cells. **CCT1** (Fig. 5A) and some analogues (Fig. 4B/D) demonstrated selectivity for KDM4 against other isolated recombinant KDM subfamilies.

The results with isolated enzymes indicate that **CCT1** is selective for the JmjC KDMs over, at least some, other human 2OG oxygenases (Fig. 5A); in particular **CCT1** was inactive against isolated recombinant hypoxia inducible transcription factor (HIF) prolyl hydroxylase PHD2 (MS-based assay: IC_{50} >100 μM; antibody-based AlphaScreen® assay: IC_{50} = 96 μM (Fig. 5A, SF5A)) under standard conditions, and non-denaturing mass spectrometry of PHD2 did not indicate binding of **CCT1** (SF5B/C); similarly, **CCT1** was inactive against isolated recombinant factor inhibiting HIF (FIH) (MS-based assay: IC_{50} >100 μM). HIF-α levels are regulated by PHD catalysis.[16] Further studies revealed that the cell effects of **CCT1** may not be solely due to 'direct' JmjC KDM inhibition. Treatment of HeLa cells with **CCT1**, but not **CCT2**, caused stabilisation of the hypoxia-inducible transcription factor HIF-1α (SF6A); treatment of Hep3b cells with **CCT1**, but not **CCT2**, caused stabilisation of HIF-1α, HIF-2α, and led to induction of PHD3 (Fig. 5E). The extent of HIF upregulation by **CCT1** is comparable to that of the PHD2-selective inhibitor **IOX2** (Fig. 5E).[17] In renal cell carcinoma cells (RCC4), where HIF-1α is stabilised due to absence of functional Von-Hippel-Lindau (VHL) complex, all 3 identified sites of HIF-1α hydroxylation (two due to PHD catalysis and one due to catalysis by factor inhibiting HIF (FIH)) were reduced by **CCT1**, but not **CCT2** (SF6C). The results suggest that PHD2, and to a lesser extent FIH, activities are inhibited by **CCT1** in cells. Addition of Fe(II) to the media reversed the effects of **CCT1** on HIF induction, as well as apparent PHD and FIH inhibition (SF6B/C).

These results suggest that the effects of **CCT1** on the hypoxic response pathway are, at least in part, related to Fe(II) availability in a cellular context.



Compound	KDM4C	KDM4E	KDM2A	KDM3A	KDM5C	KDM6B	PHD2	FIH
CCT1	5 µM	5 µM	34 µM	191 µM	178 µM	106 µM	96 µM	> 100 µM
CCT2	NI	NI	NI	NI	NI	NI	NI	NI

Fig. 5 Biochemical effects of Betti products on 2OG oxygenases. (A) Activity of **CCT1** and **CCT2** on isolated 2OG oxygenases. Values are given as IC_{50}. NI: no inhibition. (B) Effects of **CCT1**, **CCT2**, and **IOX1** on HeLa H3K9me3 levels with transiently overexpressed KDM4A (solid lines), or the corresponding catalytically inactive form (dotted lines) as determined by antibody-based immunofluorescence assays in biological triplicates. Data points represent the mean for triplicate assays with standard error as error bars. (C) Cell viability dose response curves for patient-matched non-tumorigenic human bronchial epithelial cells (HBECT30KT) and human lung carcinoma cells (HCC4017) treated with **CCT1** and **CCT2** over four days. Error bars represent standard error across eight replicates. (D) Mass spectrometric analysis of histones extracted from HEK293T cells after 24h treatment with **CCT1** (30 µM). (E) Immunoblot showing upregulation of HIF-1α, HIF-2α, and induction of HIF target protein PHD3 by **CCT1** in Hep3B cells after 6h treatment.

Overall, the results validate a one-pot template modification-based approach for the identification of selective 2OG oxygenase inhibitors, as exemplified by use of the Betti reaction to modify 8HQs in order to generate selective KDM4 inhibitors. An advantage of the modified Betti procedure is its operational simplicity. Application of the procedure led to compound **CCT1**, which has an EC_{50} ~10-fold more potent than the parent template **IOX1** in cells, as judged by histone H3 modification and cell growth assays, including with patient-matched lung cancer cell lines, with **CCT1** showing selective inhibition of cell proliferation of the cancer *vs*. the normal cells. Importantly, the results reveal how efficient synthetic access to compounds for testing in cells can raise new biological questions. Thus, the lack of inhibition of isolated PHD2 and FIH by **CCT1** *in vitro* does not correlate with the strong stabilisation of HIF and inhibition of HIF hydroxylation observed on treatment of cells by **CCT1**, raising the possibility of (an) unidentified iron-availability mechanism(s) for regulating HIF levels and activity. Given the importance of HIF in the hypoxic response and regulation of proteins such as erythropoietin, such mechanisms are of biomedicinal interest and subject of ongoing investigations.

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