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Click JAHAs: Conformationally Restricted Ferrocene-Based Histone Deacetylase Inhibitors†

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General synthetic procedures and biology details, unless described herein, have previously been reported.[1,2,3,4]

The syntheses of **1a, 3a, 4a** were analogous to that of **1b**, **3b** and **4b** except that ethyl-4-bromobutyrate was used as starting material.

4-Azido-butyric acid ethyl ester, **1a** (76% yield) ^{1}H NMR (δ, 400.0 MHz, $CDCl_3$); 1.20-1.31 (3H, m, C*H*$_3$), 1.91 (2H, *app* q, *J* 8.0 Hz, C*H*$_2$), 2.39 (2H, dt, *J* 8.0, 2.6 Hz, C*H*$_2$), 3.36 (2H, dt, *J* 8.0, 2.6 Hz, C*H*$_2$), 4.08-4.19 (2H, m, C*H*$_2$); ^{13}C NMR (δ, 75 MHz, $CDCl_3$); 14.0, 24.1, 31.0, 50.5, 60.4, 172.5.

Ferrocenyl 1,4-triazole-butyric acid ethyl ester **3a** (43% yield); dark orange solid. M.p. 100-102°C; ^{1}H NMR (δ, 300.0 MHz, DMSO); 1.17 (3H, t, *J* 9.0 Hz, C*H*$_3$), 2.00-2.10 (4H, m, 2xC*H*$_2$), 2.31 (2H, t, *J* 9.0 Hz, C*H*$_2$), 4.03 (5H, s, C$_5$*H*$_5$), 4.30 (2H, t, *J* 1.50 Hz, Fc), 4.38 (2H, t, *J* 9.0 Hz, C*H*$_2$), 4.70 (2H, t, *J* 1.50 Hz, Fc), 8.18 (1H, s, C*H*); ^{13}C NMR (δ, 75 MHz, $CDCl_3$); 13.9, 25.2, 30.5, 48.8, 60.5, 66.3, 68.4, 69.3, 75.1, 118.8, 146.5, 172.1; HRMS (m/z, HNESP) $[M+H]^+$ for $C_{18}H_{22}N_3O_2Fe$= Calc. 366.1103 observed. 366.1104; elemental analysis for $C_{18}H_{21}N_3O_2Fe$ Calc. C, 58.9%, H, 5.7%, N, 11.4%. Observed C, 58.8%, H, 5.8%, N, 11.4%.

4-(4-(Ferrocenyl-*1H*-1,2,3-triazol-1-yl)-N-hydroxybutanamide, **4a** (40% yield); dark orange solid. M.p. 120-122°C; ^{1}H NMR (δ, 300.0 MHz, DMSO-d_6); 1.96-2.10 (4H, m, 2xC*H*$_2$), 4.04 (5H, s, C$_5$*H*$_5$), 4.30 (2H, brs, Fc), 4.35 (2H, t, *J* 6.00 Hz, C*H*$_2$), 5.70 (2H, brs, Fc), 8.18 (1H, s, C*H*), 8.73 (1H, s, O*H*), 10.42 (1H, s, N*H*); ^{13}C NMR (δ, 75 MHz, DMSO-d_6); 25.8, 28.9, 48.8, 66.3, 68.1, 69.2, 76.1, 120.5, 145.2, 168.1; HRMS (m/z, HNESP) $[M+H]^+$ for $C_{16}H_{19}N_4O_2Fe$= Calc. 353.0899 observed. 353.0895; elemental analysis for $C_{16}H_{18}N_4O_2Fe$ 0.11 CH_2Cl_2; Calc. C, 53.1%, H, 5.0%, N, 15.4 % observed C, 53.1%, H, 5.1%, N, 15.0%.

6-Bromo-hexanoic acid ferrocenylphenylamide, **5a** (42%). In an oven dried microwave tube (35 cm^3) equipped with a stirrer bar, 4-amino-phenylferrocene (0.28 g, 1.00 mmol) was dissolved in anhydrous THF (1 cm^3) followed by 6-bromohexanoyl chloride (0.18 cm^3, 1.10 mmol) and reagent grade triethylamine (0.5 cm^3, 4.00 mmol). The reaction mixture was placed into the microwave cavity (CEM Explorer) and was ramped to 150°C, 150 W and held for 30 min with microwave irradiation. The cooled reaction mixture was extracted using ethyl acetate (10 cm^3) and washed with sat. brine (3 x 10 cm^3). The organic layer was dried with magnesium sulphate then filtered and concentrated. The crude mixture was purified using over silica using 3:7 ethyl acetate/methylene chloride to give the product (0.19 g, 42%) as a dark brown solid after recrystallisation from 1:20 ethyl acetate/hexane. M.p. 150-152°C; ^{1}H NMR (δ, 400.0 MHz, $CDCl_3$); 1.71-1.88 (4H, m, 2xCH_2), 2.38 (2H, t, *J* 8.0 Hz, CH_2), 3.56 (2H, t, *J* 8.0 Hz, CH_2), 4.03 (5H, s, C_5H_5), 4.29 (2H, brs, Fc), 4.61 (2H, brs, Fc), 7.15 (1H, s, N*H*), 7.43 (4H, m, 4xArC*H*); ^{13}C NMR (δ, 100.5 MHz, $CDCl_3$); 24.7, 26.3, 32.2, 37.3, 44.7, 66.1, 68.8, 69.6, 84.9, 119.9, 126.3, 126.9, 128.1, 171.1.

6-Azido-hexanoic acid ferrocenylphenylamide, **5b** was made as for **1b**. (71% yield) and used in the next step without any further purification. ^{1}H NMR (δ, 400.0 MHz, $CDCl_3$); 1.68-1.80 (2H, m, CH_2), 2.34 (2H, td, *J* 4.0, 1.7 Hz, CH_2), 3.22 (2H, t, *J* 8.0 Hz, CH_2), 3.50 (2H, t, *J* 8.0 Hz, CH_2), 4.01 (5H, s, C_5H_5), 4.28 (2H, brs, Fc), 4.59 (2H, brs, Fc), 7.15 (1H, s, N*H*), 7.43 (4H, brs, 4xArC*H*); ^{13}C NMR (δ, 100.5 MHz, $CDCl_3$); 25.2, 26.5, 32.4, 37.3, 45.0, 51.3, 66.4, 69.0, 69.7, 85.2, 120.2, 126.5, 135.2, 136.2, 171.6.

1-(6-(Ferrocenylphenyl-4-ylamino)-6-oxohexyl)-*1H*-1,2,3-triazole-4-carboxylate, **6a** (53% yield), made as for **3a**. M.p. 156-158°C; ^{1}H NMR (δ, 400.0 MHz, $CDCl_3$); 1.75-1.82 (4H, m, 2xCH_2), 1.97-2.05 (2H, m, CH_2), 2.36 (2H, t, *J* 8.0 Hz, CH_2), 3.95 (3H, s, OCH_3), 4.03 (5H, s, C_5H_5), 4.30 (2H, t, *J* 4.0 Hz, Fc), 4.47 (2H, t, *J* 8.0 Hz, CH_2), 4.61 (2H, t, *J* 4.0 Hz, Fc), 7.12 (1H,s, C*H*), 7.43 (4H, m, 4xArC*H*), 8.10 (1H, s, N*H*); ^{13}C NMR (δ, 100.5 MHz, $CDCl_3$); 24.7, 25.9, 29.9, 37.0, 50.5, 52.3, 66.3, 68.9, 69.7, 85.1, 120.0, 126.5, 127.6, 135.2, 136.1, 139.9, 161.3, 171.0; HRMS (m/z, HNESP) $[M+H]^+$ for $C_{26}H_{29}N_4O_3Fe$= Calc. 499.1630 observed. 499.1633; elemental Anal. Calc. For $C_{26}H_{28}N_4O_3Fe$.007 moles DCM; C, 61.8%, H, 5.6%, N, 11.1% observed C, 61.9%, H, 5.6%, N, 10.8%.

1-(6-(Ferrocenylphenyl-4-ylamino)-6-oxohexyl)-*N*-hydroxy-*1H*-1,2,3-triazole-4-carboxamide, **7a** (58% yield), made as for **4a**. M.p. 158-160°C; ^{1}H NMR (δ, 400.0 MHz, DMSO-d_6); 1.30-1.36 (2H, m, CH_2), 1.68 (2H, *pent*, *J* 8.0 Hz, CH_2), 1.94 (2H, *pent*, *J* 8.0 Hz, CH_2), 2.35 (2H, t, *J* 8.0 Hz, CH_2), 4.08 (5H, s, C_5H_5), 4.37 (2H, brs, Fc), 4.47 (2H, t, *J* 8.0 Hz, CH_2), 4.78 (2H, brs, Fc), 7.50 (2H, d, *J* 8.0 Hz, 2xArC*H*), 7.53 (2H, d, *J* 8.0 Hz,

2xArC*H*), 8.61 (1H, s, C*H*), 9.09 (1H, s, O*H*), 9.89 (1H, s, N*H*), 11.30 (1H, s, N*H*); ^{13}C NMR (δ, 100.5 MHz, DMSO-d_6); 24.4, 25.4, 29.3, 36.1, 49.4, 65.8, 68.5, 69.2, 84.9, 119.0, 125.9, 126.1, 133.3, 137.2, 141.2, 157.7, 170.8; HRMS (m/z, HNESP) $[M+H]^+$ for $C_{25}H_{28}N_5O_3Fe$= Calc. 500.1583 observed. 500.1580; elemental Anal. Calc. For $C_{25}H_{27}N_5O_3Fe$ C, 59.9%, H, 5.4%, observed C, 59.5%, H, 5.4%. HPLC (anal.); 99:1 hexane/MeOH, 1 mL/min Rt= 17 min 9 secs purity 95%.

6-Azido-1-ferrocenylhexan-1-one, **8b** (91% yield) was an orange oil and made as for **1a** and used without further purification. ^{1}HNMR (δ, 400.0 MHz, $CDCl_3$); 1.45 (2H, m, CH_2), 1.64-1.74 (4H, m, 2xCH_2), 2.70 (2H, t, *J* 7.0 Hz, CH_2), 3.30 (2H, t, *J* 7.0 Hz, CH_2), 4.15 (5H, s, C_5H_5), 4.50 (2H, t, *J* 4.0 Hz, Fc), 4.77 (2H, t, *J* 4.0 Hz, Fc); ^{13}C NMR(δ, 400 MHz, $CDCl_3$); 23.8, 26.5, 28.7, 39.3, 51.2, 69.2, 69.7, 72.1, 80.0, 204.0.

Methyl-1-(6-oxo-6-ferrocenylhexyl)-*1H*-1,2,3-triazole-4-carboxylate, **9b**, made as for **3a**. Yellow solid (80% yield). M.p. 73-75°C; ^{1}H NMR (δ, 300.0 MHz, CD_3OD); 1.40 (2H, m, CH_2), 1.71 (2H, m, CH_2), 1.98 (2H, m, CH_2), 2.76 (2H, t, *J* 7.2 Hz, CH_2), 3.89 (3H, s, OCH_3), 4.19 (5H, s, C_5H_5), 4.56 (2H, brs, CH_2), 4.79 (2H, brs, Fc), 4.85 (2H, brs, CH_2), 8.67 (1H, s, C*H*); ^{13}C NMR (δ, 75.0 MHz, CD_3OD); 24.8, 27.1, 30.9, 40.1, 51.5, 52.0, 70.5, 70.9, 73.8, 79.9, 129.6, 140.4, 162.4, 207.3. HRMS (m/z, HNESP) $[M+H]^+$ for $C_{20}H_{24}N_3O_3Fe$= Calc. 410.1279 observed 410.1275.

N-Hydroxy-1-(6-oxo-6-ferrocenylhexyl)-*1H*-1,2,3-triazole-4-carboxamide, **10b**, made as for **4a**. Yellow solid (53% yield). ^{1}HNMR (δ, 400.0 MHz, DMSO-d6); 1.33 (2H, m, CH_2), 1.56 (2H, m, CH_2), 1.89 (2H, m, CH_2), 3.30 (2H, t, *J* 7.4 Hz, CH_2CO), 4.13 (5H, s, C_5H_5), 4.29 (2H, t, *J* 4.0 Hz, Fc), 4.40 (2H, t, *J* 7.0 Hz, CH_2), 4.49 (2H, t, *J* 4.0 Hz, Fc), 8.53 (1H, s, O*H*), 9.01 (1H, s, C*H*), 11.23 (1H, s, N*H*); ^{13}C NMR (δ, 500.0 MHz, CD_3OD); 27.5, 28.1, 30.6, 30.9, 67.6, 70.2, 70.5, 82.4, 127.1, 142.1, 160.3, 160.7, 210.1. HRMS (m/z, HNESP) $[M+H]^+$ for $C_{19}H_{23}N_4O_3Fe$= Calc. 411.1279, observed= 411.1276. Anal. Calc: for $C_{19}H_{22}FeO_3N_4$0.1 CH_2Cl_2 C, 54.8%; H, 5.3%. Found C, 54.5%; H, 5.7%.

(6-Azidohexyl)ferrocene, **8a**. (91% yield) orange oil used without further purification. ^{1}H NMR (δ, 400.0 MHz, $CDCl_3$); 1.35 (2H, m, CH_2), 1.58 (2H, m, CH_2), 1.60 (2H, m, CH_2), 2.16 (2H, m, CH_2), 2.32 (2H, t, *J* 7.1 Hz, CH_2), 3.26 (2H, t, *J* 7.1 Hz, CH_2), 4.02 (2H, t, *J* 4.0 Hz, Fc), 4.04 (2H, t, *J* 4.0 Hz, Fc), 4.08 (5H, s, C_5H_5); ^{13}C NMR(δ, 100.5 MHz, $CDCl_3$); 26.4, 28.6, 28.8, 29.2, 30.8, 51.2, 66.9, 67.8, 68.3, 88.9.

1-(6-Ferrocenylhexyl)-methyl-*1H*-1,2,3-triazole-4-carboxylate **9a.** Yellowish solid (86% yield). ^{1}HNMR (δ, 400.0 MHz, $CDCl_3$); 1.20 (2H, m, CH_2), 1.33 (2H, m, CH_2), 1.47 (2H, m, CH_2), 1.92 (2H, m, CH_2), 2.30 (2H, t, *J* 7.3 Hz, CH_2), 3.13 (2H, t, *J* 7.3 Hz, CH_2), 3.86 (3H,

s, OCH_3), 3.95 (2H, t, *J* 4.0 Hz, Fc), 4.02 (2H, t, *J* 4.0 Hz, Fc), 4.07 (5H, s, C_5H_5), 8.05 (1H, s, C*H*). δC ^{13}C NMR (δ, 75.0 MHz, CD_3OD); 23.8, 29.1, 31.0, 43.1, 51.4, 55.3, 70.5, 71.7, 73.8, 79.9, 96.7, 128.6, 140.4, 161.4. HRMS (m/z, HNESP) $[M+H]^+$ for $C_{20}H_{26}N_3O_2Fe$= Calc. 397.1327 observed. 396.1323.

1-(6-(Ferrocenyl)hexyl)-*N*-hydroxy-*1H*-1,2,3-triazole-4-carboxamide **10a** (55% yield). M.p. 130-132°C; ^{1}HNMR (δ, 400.0 MHz, DMSO-d6); 1.11 (2H, m, CH_2), 1.22 (2H, m, CH_2), 1.43 (2H, m, CH_2), 1.82 (2H, m, CH_2), 2.25 (2H, t, *J* 7.4 Hz, CH_2), 4.01 (2H, t, *J* 4.0 Hz, Fc), 4.05 (2H, t, *J* 4.0 Hz, Fc), 4.07 (5H, s, C_5H_5), 4.38 (2H, t, *J* 7.8 Hz, CH_2), 8.53 (1H, s, C*H*), 9.01 (1H, s, O*H*), 11.21 (1H, s, N*H*); ^{13}C NMR (δ, 125.0 MHz, CD_3OD); 27.3, 29.8, 30.1, 31.2, 32.0 49.4, 68.1, 69.1, 69.4, 127.1, 210.0; HRMS (m/z, HNESP) $[M+H]^+$ for $[C_{19}H_{25}N_4O_2Fe]$= Calc. 397.1325 observed. 397.1323. Anal. Calc: for $C_{19}H_{24}FeO_2N_4$.0.115 moles hexane C, 58.2%; H, 6.4%, N, 13.8%. Found C, 58.2%; H, 6.6%, N, 13.6%.

HDAC Activity: HDAC1-9 activity was determined in vitro with an optimized homogenous assay performed in a 384-well plate. Recombinant, full-length HDAC protein (BPS Biosciences) was incubated with fluorophore conjugated substrate, MAZ1600 and MAZ1675, at K_m = [Substrate]. (MAZ1600; 11 μM for HDAC1, 18 μM for HDAC2, 9 μM for HDAC3, 4 μM for HDAC6; MAZ1675; 21 μM for HDAC4, 66 μM for HDAC5, 60 μM for HDAC7, 263 μM for HDAC8, 55 μM for HDAC9). Reactions were performed in assay buffer (50 mM HEPES, 100 mM KCl, 0.001% Tween-20, 0.05% BSA and pH 7.4. Additional 200 μM TCEP was added for HDAC6) and followed by fluorogenic release of 7-amino-4-methylcoumarin from substrate upon deacetylase and trypsin enzymatic activity. Flourescence measurements were obtained every five minutes using a multilabel plate reader and plate-stacker (Envision; Perkin-Elmer). Each plate was analyzed by plate repeat, and the first derivative within the linear range was imported into analytical software (Spotfire DecisionSite). Replicate experimental data from incubations with inhibitor were normalized to DMSO controls ([DMSO] < 0.5%). IC_{50} is determined by logistic regression with unconstrained maximum and minimum values.

Flow Cytometry. K562 cells were grown in 6-well tissue culture plates (BD Falcon) at a starting concentration of $5.0x10^5$ cells/mL. Cells were treated with SAHA (10μM), **11** (10 μM), **4b** (10 μM) or DMSO (0.1%) for 18 hours. After incubation, cells were spun at 1000RPM for 5 minutes, fixed in 3.8% formaldehyde for 20 minutes at 4°C, and washed (PBS + 0.1% Triton-X 100). Cells were incubated for 1+ hour at 4°C in blocking solution (PBS + 2% BSA + 0.1% Triton X 100), washed three times, and spun down at 1000RPM for 10 minutes. Cells were then incubated for 1+ hour 4°C in primary antibody for acetylated-

tubulin (Sigma T7451) and acetylated-lysine (Cell Signaling #9441L) at a 1:1000 dilution in blocking solution, washed three times, and spun down at 1000RPM for 10 minutes. Cells were then incubated for 90 minutes at room temperature in secondary antibody (Invitrogen A-21202, A-21244) at a 1:1000 dilution in blocking solution. After incubation, cells were washed 3 times, transferred to PBS, and analyzed on a BD FACS Canto II. Histograms were generated using FlowJo flow cytometry analysis software (Tree Star, Inc.).

Xenopus Methods.[2,3,4]

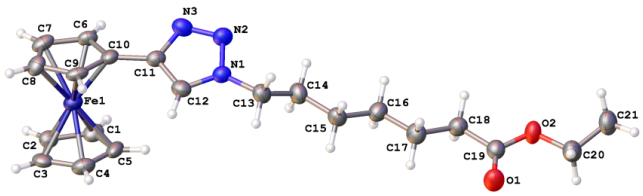
Embryos were prepared and cultured according to Guille and compounds added in DMSO solutions at the 2-cell stage. Embryos were harvested when they reached stage 14 and each pool was homogenised in EB (120ul). Yolk was extracted with Freon and 12ul of extract proteins separated by 12% SDS-PAGE, transferred to nitrocellulose membrane (Hybond ECL, GE) and the alpha tubulin and acetylated alpha tubulin detected using ab24610 and ab15246 (Abcam) respectively.

Table S1. Crystal data and structure refinement details.

Identification code	**2010src0948a**	
Empirical formula	$C_{21}H_{27}FeN_3O_2$	
Formula weight	409.31	
Temperature	120(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	***C*2/*c***	
Unit cell dimensions	***a* = 51.309(6) Å**	**α = 90°**
	***b* = 7.2426(10) Å**	**β = 94.098(6)°**
	***c* = 10.7246(12) Å**	**γ = 90°**
Volume	3975.2(8) Å^3	
Z	8	
Density (calculated)	1.368 Mg / m^3	
Absorption coefficient	0.779 mm^{-1}	
F(000)	1728	
Crystal	Plate; yellow	
Crystal size	0.37 × 0.16 × 0.04 mm^3	
θ range for data collection	3.06 – 25.00°	
Index ranges	$-60 \le h \le 60$, $-8 \le k \le 8$, $-12 \le l \le 12$	
Reflections collected	16878	

Independent reflections	3341 [R_{int} = 0.1113]
Completeness to θ = 25.00°	95.1 %
Absorption correction	Semi–empirical from equivalents
Max. and min. transmission	0.9695 and 0.7614
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	3341 / 0 / 245
Goodness-of-fit on F^2	1.046
Final R indices [$F^2 > 2\sigma(F^2)$]	$R1$ = 0.0816, $wR2$ = 0.1966
R indices (all data)	$R1$ = 0.1332, $wR2$ = 0.2289
Largest diff. peak and hole	1.112 and –0.454 e Å^{-3}

Diffractometer: *Nonius KappaCCD* area detector (ϕ scans and ω scans to fill *asymmetric unit* sphere). **Cell determination:** DirAx (Duisenberg, A.J.M.(1992). J. Appl. Cryst. 25, 92-96.) **Data collection:** Collect (Collect: Data collection software, R. Hooft, Nonius B.V., 1998). **Data reduction and cell refinement**: *Denzo* (Z. Otwinowski & W. Minor, *Methods in Enzymology* (1997) Vol. **276**: *Macromolecular Crystallography*, part A, pp. 307–326; C. W. Carter, Jr. & R. M. Sweet, Eds., Academic Press). **Absorption correction**: *SADABS* (Sheldrick, G. M. (2007). SADABS. Version 2007/2. Bruker AXS Inc., Madison, Wisconsin, USA.). **Structure solution**: *SHELXS97* (Sheldrick, G.M. (2008). Acta Cryst. A64, 112-122.). **Structure refinement**: *SHELXL97* (G Sheldrick, G.M. (2008). Acta Cryst. A64, 112-122.). **Graphics:** *CAMERON* (Watkin, D. M., Pearce, L. & Prout, C. K. (1993). Chemical Crystallography Lab, University of Oxford).



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