

## **Hypoxia-activated pro-drugs of the KDAC inhibitor Vorinostat (SAHA)**

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This paper is dedicated to Professor Steve Davies.

## **Supplementary Information**

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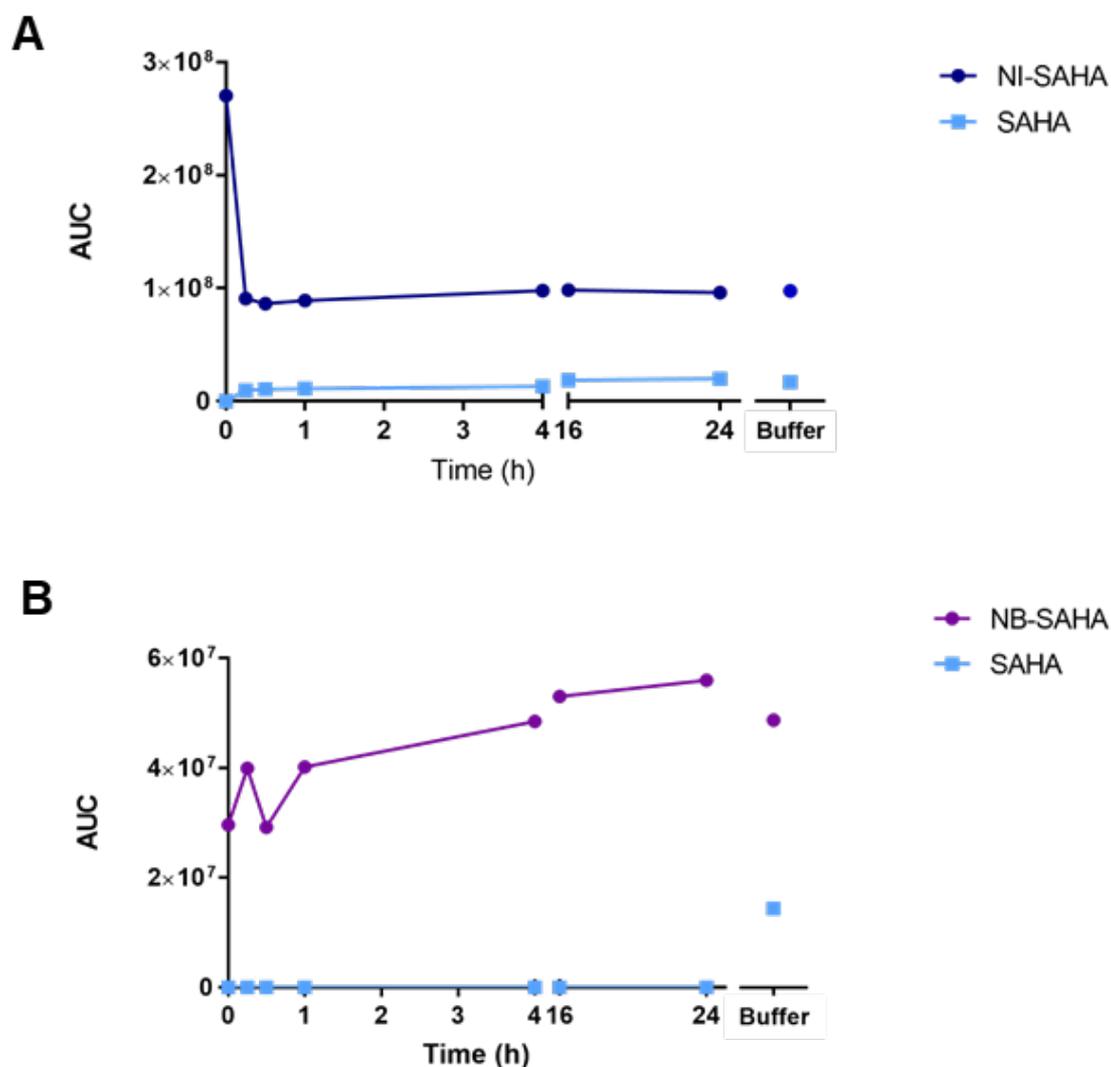
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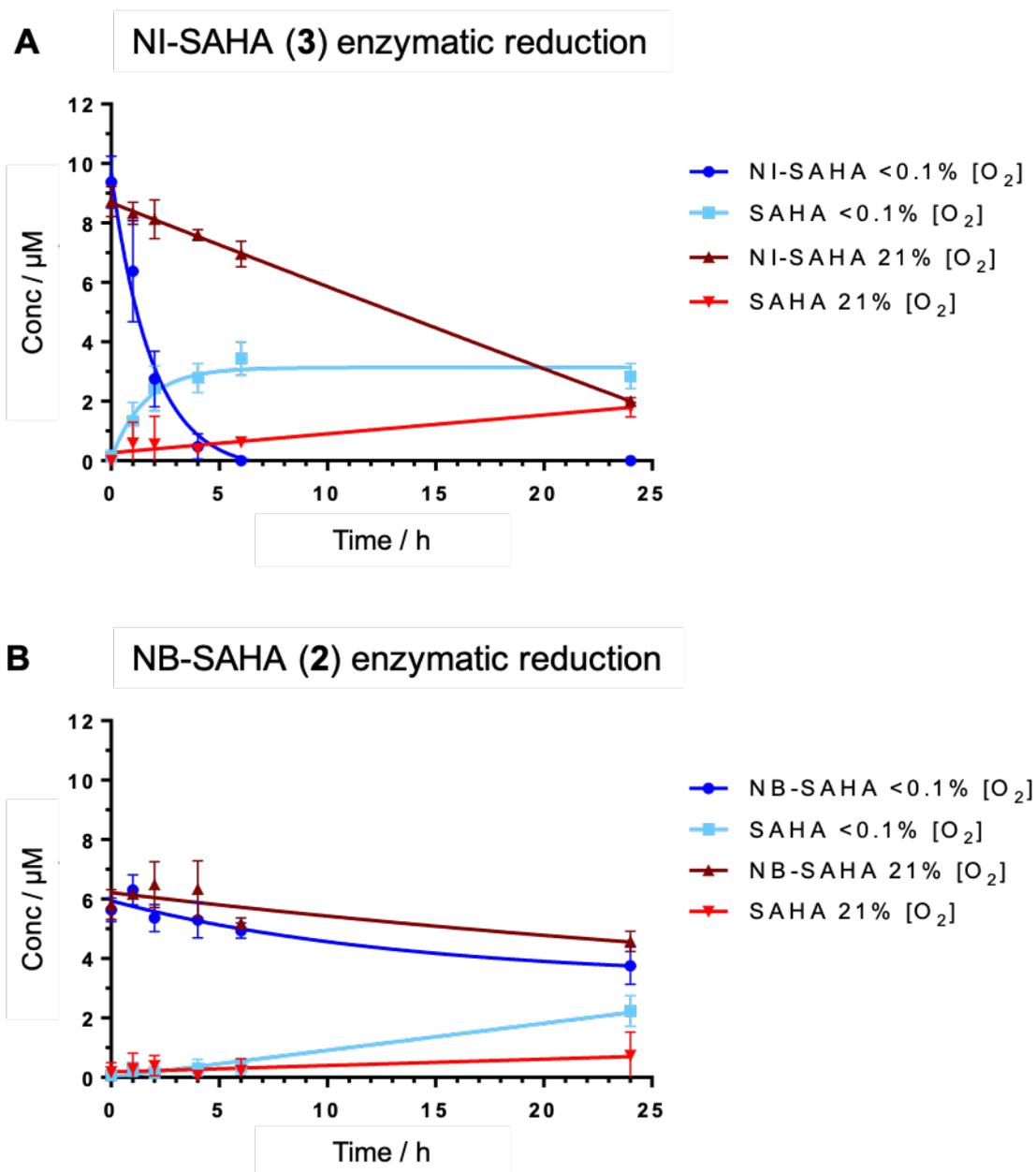
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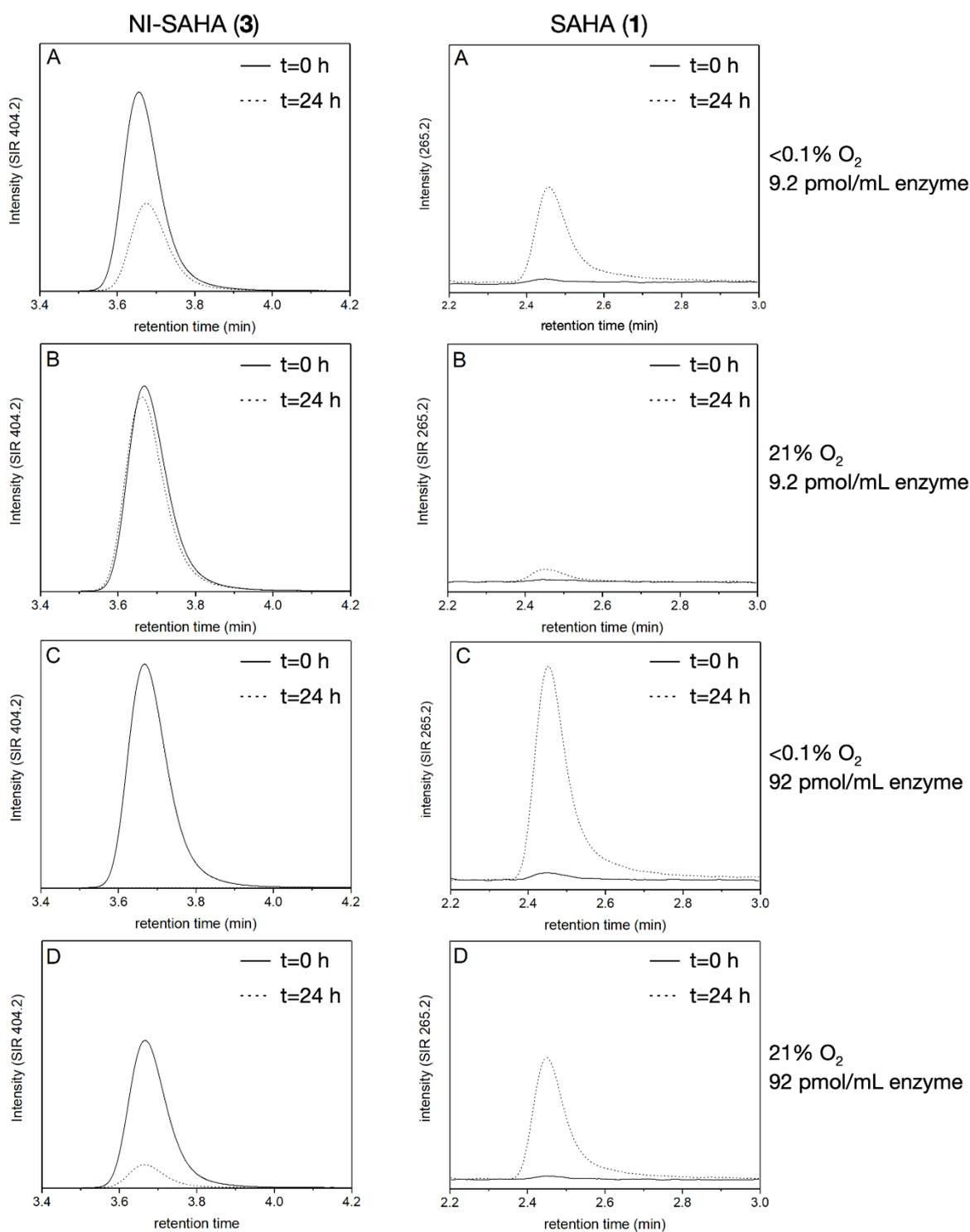
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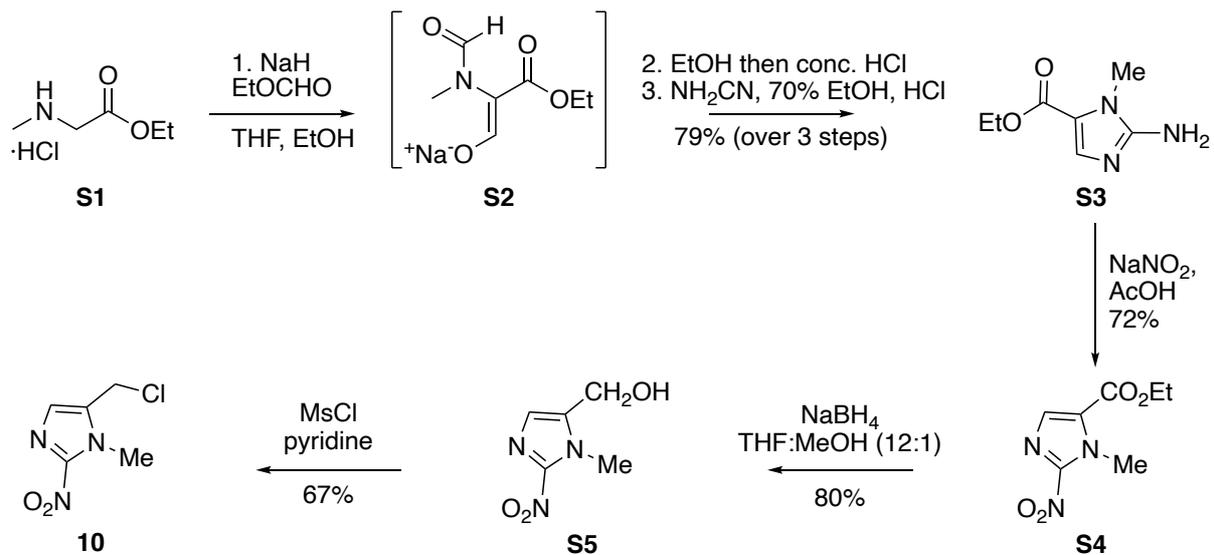
**Figure S1.** Reduction of NI-SAHA (**3**) and NB-SAHA (**2**) using zinc and ammonium chloride in DMF. Aliquots were taken at the timepoints shown, and were analysed using LCMS. An aliquot at t=16 h was combined with 0.1 M phosphate buffer and incubated at 37 °C for 24 h. **A.** NI-SAHA (**3**) ( $m/z$  404.2  $[M+H]^+$ , retention time 3.7 min) was reduced and fragmented to SAHA (**1**) ( $m/z$  265.2  $[M+H]^+$ , retention time 3.8 min) within 15 min. Upon addition of buffer, no additional fragmentation was observed **B.** No SAHA (**1**) ( $m/z$  265.2  $[M+H]^+$ , retention time 3.8 min) was observed upon reduction of NB-SAHA (**2**) ( $m/z$  400.2  $[M+H]^+$ , retention time 5.5 min) in DMF. Upon addition of buffer and incubation for 24 h, fragmentation to SAHA (**1**) was observed.



**Figure S2.** The enzymatic (92 pmol/mL bactosomal human NADPH-CYP reductase [CYP004, Cypex]) reduction of NI-SAHA (3) and NB-SAHA (2) at 21% oxygen (red lines) and  $<0.1\%$  oxygen (blue lines). **A.** NI-SAHA (3) was unstable at 21% oxygen and release of SAHA was detected (red lines). At  $<0.1\%$  oxygen it underwent rapid bioreduction and showed significant release of SAHA (1). **B.** NB-SAHA (2) was unstable at both 21% oxygen and  $<0.1\%$ , showing release of SAHA (1) under both of these conditions.



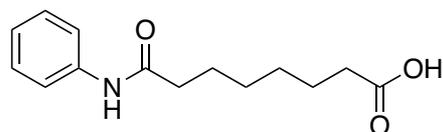
**Figure S3.** The enzymatic (bactosomal human NADPH-CYP reductase [CYP004, Cypex])-mediated reduction of NI-SAHA (**3**) at 21% oxygen and <0.1% oxygen analysed by LCMS. Chromatograms show the reduction in NI-SAHA ( $m/z$  404.2 [M+H]<sup>+</sup>, retention time 3.7 min) and increase in SAHA ( $m/z$  265.15, [M+H]<sup>+</sup>, retention time 2.5 min) over time. **A.** 9.2 pmol/mL enzyme, <0.1% oxygen. **B.** 9.2 pmol/mL enzyme, 21% oxygen. **C.** 92 pmol/mL enzyme <0.1% oxygen. **D.** 92 pmol/mL enzyme, 21% oxygen.



**Scheme S1.** The synthesis of 5-(chloromethyl)-1-methyl-2-nitro-1H-imidazole (**10**).

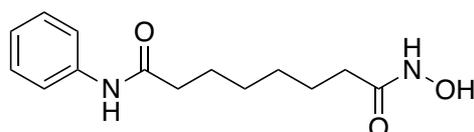
## EXPERIMENTAL SECTION

### Suberanilic acid (**6**)<sup>1</sup>



A suspension of suberic acid (**4**, 3.08 g, 17.7 mmol, 1.0 eq) in acetic anhydride (6 mL) was heated under reflux for 1 h then cooled to rt and concentrated *in vacuo*. The crude material was crystallised from boiling acetonitrile to yield crude suberic anhydride (**5**, 2.47 g, 88%) which was used without further purification. Aniline (2.80 mL, 31.8 mmol, 2.0 eq) was added to a solution of crude suberic anhydride (**5**, 2.47 g, 15.6 mmol, 1.0 eq) in tetrahydrofuran (15 mL) and stirred at rt for 4 h. Water (30 mL) was added to the reaction and the precipitate was removed by filtration. The remaining solution was acidified with aqueous hydrochloric acid (1 M, 50 mL) and extracted with ethyl acetate (2 × 100 mL). The combined organic components were washed with water (100 mL), and brine (100 mL), dried (Na<sub>2</sub>SO<sub>4</sub>), filtered, and concentrated *in vacuo*. The resulting residue was then crystallised from boiling water to yield suberanilic acid (**6**, 1.20 g, 27%) as a colourless solid. *R<sub>f</sub>* 0.50 (ethyl acetate); mp 122–123 °C (from PhMe) [lit.,<sup>1</sup> 122–123 °C]; <sup>1</sup>H NMR (400 MHz, DMSO-D<sub>6</sub>) δ<sub>H</sub> 11.98 (1H, s), 9.84 (1H, s), 7.63–7.47 (2H, m), 7.35–7.22 (2H, m), 7.06–6.94 (1H, m), 2.28 (2H, t, *J* 7.4), 2.19 (2H, t, *J* 7.4) 1.65–1.43 (4H, m) 1.38–1.20 (4H, m); LRMS *m/z* (ESI<sup>+</sup>) 272 (100%, [M+Na]<sup>+</sup>). The spectroscopic data are consistent with the literature.<sup>1</sup>

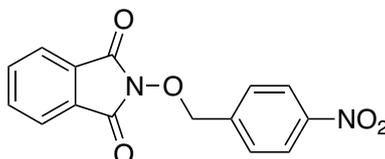
### *N*-Hydroxy-*N'*-phenyloctanediamide (SAHA, **1**)<sup>2</sup>



Triethylamine (0.16 mL, 1.2 mmol, 1.4 eq) was added to a solution of suberanilic acid (**6**, 0.20 g, 0.80 mmol, 1.0 eq) in tetrahydrofuran (4 mL) and cooled to 0 °C. Ethyl chloroformate (0.099 mL, 1.0 mmol, 1.3 eq) was then added to the solution before warming to rt and continuing stirring for 15 minutes. In a separate flask, hydroxylamine hydrochloride (0.11 g, 1.6 mmol, 2.0 eq) and potassium hydroxide (0.090 g, 1.6 mmol, 2.0 eq) were combined in methanol (3 mL) and stirred at rt for 20 mins. Both solutions were then filtered and combined at 0 °C in a third flask with rapid stirring. The solution was allowed to return to rt and stirred for 30 mins then concentrated *in vacuo*. The residue was crystallised from acetonitrile to yield compound **1** (0.14 g, 66%) as a colourless solid. *R<sub>f</sub>* 0.23 (ethyl acetate); mp 143–145 °C (from MeCN) [lit.,<sup>2</sup> 159–161 °C from MeCN]; <sup>1</sup>H NMR (400 MHz, DMSO-D<sub>6</sub>) δ<sub>H</sub> 10.36 (1H, s), 9.92

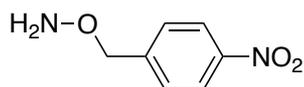
(1H, s), 8.67 (1H, s), 7.59 (2H, d,  $J$  7.6), 7.27 (2H, dd,  $J$  7.6, 7.4), 7.00 (1H, t,  $J$  7.4), 2.29 (2H, t,  $J$  7.4), 1.93 (2H, t,  $J$  7.4), 1.62–1.41 (4H, m), 1.34–1.22 (4H, m); LRMS  $m/z$  (ESI<sup>+</sup>) 287 (100%, [M+Na]<sup>+</sup>), 265 (71%, [M+H]<sup>+</sup>). The spectroscopic data are consistent with the literature.<sup>2</sup>

### ***N*-Phthalimido-*O*-(4'-nitrobenzyl)-hydroxylamine (**8**)<sup>3</sup>**



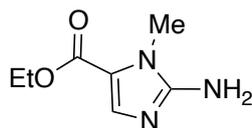
*N,N*-Diisopropylethylamine (5.78 mL, 33.1 mmol, 1.8 eq) was added to a stirred solution of *N*-hydroxyphthalimide (**7**, 3.00 g, 18.4 mmol, 1.0 eq) in *N,N*-dimethylformamide (18 mL). 4-Nitrobenzyl chloride (4.12 g, 23.9 mmol, 1.3 eq) was added and then the solution was heated to 70 °C for 2 h. The reaction was cooled to rt, diluted with ethyl acetate (200 mL) and washed with aqueous 0.5 M lithium chloride (4 × 200 mL) then dried (MgSO<sub>4</sub>), filtered, and concentrated *in vacuo*. Crystallised from hot ethanol yielded compound **8** (4.61 g, 84%) as a colourless solid.  $R_f$  0.12 (20% ethyl acetate:petroleum ether); mp 191–193 °C (from EtOH) [lit.,<sup>3</sup> 191–193 °C]; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta_H$  8.30–8.21 (2H, m), 7.87–7.80 (2H, m), 7.79–7.76 (2H, m), 7.76–7.72 (2H, m), 5.31 (2H, s); LRMS  $m/z$  (ESI<sup>+</sup>) 321 (100%, [M+Na]<sup>+</sup>). The spectroscopic data are consistent with the literature.<sup>3</sup>

### ***O*-(4-Nitrobenzyl)-hydroxylamine (**9**)<sup>3</sup>**



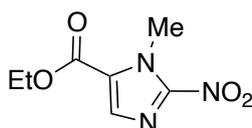
Concentrated hydrochloric acid (10 mL) was added to a suspension of *N*-phthalimido-*O*-(4'-nitrobenzyl)-hydroxylamine (**8**, 1.00 g, 3.35 mmol) in ethanol (5 mL) and heated to 78 °C for 18 h. The reaction was cooled to rt, diluted with water (50 mL) and extracted with chloroform (50 mL). The aqueous fraction was neutralised with saturated aqueous sodium carbonate solution and extracted with dichloromethane (2 × 50 mL), the combined organic fractions were washed with brine (50 mL), dried (MgSO<sub>4</sub>), filtered, and concentrated *in vacuo* to yield compound **9** (506 mg, 90%) as a yellow solid.  $R_f$  0.44 (100% ethyl acetate); mp 47–50 °C (from dichloromethane/hexane) [lit.,<sup>4</sup> 56 °C from light petroleum], [lit.,<sup>5</sup> 38–40 °C]; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta_H$  8.53–8.02 (2H, m), 7.59–7.40 (2H, m), 5.53 (2H, br s), 4.77 (2H, s); LRMS  $m/z$  (ESI<sup>+</sup>) 393 (60%), 209 (100%), 152 ([M-NH<sub>2</sub>]<sup>+</sup>, 42%). The spectroscopic data are consistent with the literature.<sup>3</sup>

### Ethyl (2-amino-1-methyl-imidazol-5-yl)carboxylate (**S3**)<sup>6,7,8</sup>



Ethyl *N*-methylglycine hydrochloride (**S1**, 6.00 g, 39.0 mmol, 1.0 eq) was dried by lyophilisation then suspended in a combination of dry tetrahydrofuran (37 mL), dry absolute ethanol (4.2 mL), and ethyl formate (22 mL) and cooled to 0 °C under a stream of argon. Sodium hydride (3.74 g, 156 mmol, 4.0 eq) was added in small portions to the cooled suspension and, once gas evolution had ceased, the reaction mixture was warmed to rt and stirred for 18 h. The reaction was quenched by the addition of wet diethyl ether (200 mL) and the mixture filtered. The collected solids were washed with diethyl ether (2 × 100 mL) then dried under vacuum. The solids were then suspended in ethanol (130 mL), and concentrated hydrochloric acid (26 mL) was slowly added to the suspension. The suspension was stirred at rt for 2 h then filtered to remove salt. The resulting solution was concentrated *in vacuo* then dissolved in ethanol (210 mL) and water (90 mL) and the pH adjusted to a value of 3 with aqueous sodium hydroxide (~40 mL, 6 M NaOH). Cyanamide (3.27 g, 77.9 mmol, 2.0 eq) was added to the solution and the reaction was heated to 100 °C for 2 h then cooled to rt and concentrated *in vacuo*. The residue was dissolved in ethyl acetate (200 mL) and saturated aqueous potassium carbonate solution (100 mL) then extracted with ethyl acetate (3 × 100 mL). The combined organic fractions were washed with brine (300 mL), dried (Na<sub>2</sub>SO<sub>4</sub>), filtered, and concentrated *in vacuo* to yield compound **S3** (5.18 g, 79%) as a pale-yellow solid which slowly decomposes at rt. Further purification or re-purification could be achieved by trituration with minimal chloroform but was usually unnecessary. *R<sub>f</sub>* 0.27 (5% ethanol:chloroform); mp 153–155 °C (from methanol) [lit.,<sup>7</sup> 130–133 °C (from water)]; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ<sub>H</sub> 7.44 (1H, s), 4.39 (2H, br s), 4.26 (2H, q, *J* 7.1), 3.67 (3H, s), 1.33 (3H, t, *J* 7.1); LRMS *m/z* (ESI<sup>+</sup>) 170 ([*M*+*H*]<sup>+</sup>, 100%), 142 (30%). The spectroscopic data are consistent with the literature.<sup>7</sup>

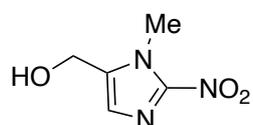
### Ethyl (1-methyl-2-nitro-imidazol-5-yl)carboxylate (**S4**)<sup>6,7,8</sup>



A solution of ethyl (2-amino-1-methyl-imidazol-5-yl)carboxylate (**S3**, 1.73 g, 10.2 mmol, 1.0 eq) in glacial acetic acid (18 mL) was added, dropwise at 0 °C, to a solution of sodium nitrite (7.06 g, 102 mmol, 10 eq) in water (9 mL). The solution was stirred at 0 °C for 1 h then warmed to rt over 3 h. The solution was extracted with dichloromethane (3 × 50 mL).

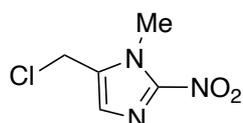
Combined organic fractions were washed with a saturated aqueous solution of sodium sulfite (100 mL), and brine (100 mL), then dried (MgSO<sub>4</sub>), filtered, and concentrated *in vacuo*. The residue was dissolved in dichloromethane (100 mL) and filtered through a short pad of silica to yield compound **S4** (1.46 g, 72%) as an off-white solid. *R*<sub>f</sub> 0.27 (100% dichloromethane); mp 51–53 °C (from dichloromethane) [lit.,<sup>7</sup> 56–58 °C (from dichloromethane)]; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ<sub>H</sub> 7.71 (1H, s), 4.37 (2H, q, *J* 7.2), 4.32 (3H, s), 1.38 (3H, t, *J* 7.2); LRMS *m/z* (ESI<sup>+</sup>) 200 ([M+H]<sup>+</sup>, 100%), 172 (65%). The spectroscopic data are consistent with literature.<sup>7</sup>

**(1-Methyl-2-nitro-imidazol-5-yl)methanol (S5)**<sup>6,7,8</sup>



A solution of sodium borohydride (0.160 g, 4.22 mmol, 3.0 eq) in dry ethanol (5.3 mL) was added dropwise with vigorous stirring to a solution of ethyl 1-methyl-2-nitroimidazole-5-carboxylate (**S4**, 0.280 g, 1.41 mmol, 1.0 eq) in dry tetrahydrofuran (7.0 mL) at 0 °C. The reaction was stirred at 0 °C for 3 h then added slowly with stirring to a mixture of diethyl ether (100 mL) and wet methanol (100 mL) at 0 °C. The resulting solution was stirred at 0 °C for 30 min then gradually acidified to pH 5 with aqueous hydrochloric acid (2 M). The solvent was removed *in vacuo* to give a mostly aqueous solution. The residue was extracted with ethyl acetate (5 × 50 mL), the combined organic components were dried (Na<sub>2</sub>SO<sub>4</sub>), filtered, and concentrated *in vacuo* to yield compound **S5** (0.176 g, 80%) as a pale-yellow solid. *R*<sub>f</sub> 0.37 (5% ethanol:chloroform); mp 126–130 °C (from chloroform) [lit.,<sup>7</sup> 141–143 °C (from ethyl acetate)]; <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD) δ<sub>H</sub> 7.10 (1H, s), 4.66 (2H, s), 4.04 (3H, s); LRMS *m/z* (ESI<sup>+</sup>) 180 ([M+Na]<sup>+</sup>, 45%), 170 (80%), 158 ([M+H]<sup>+</sup>, 100%), 113 (31%). The spectroscopic data are consistent with the literature.<sup>7</sup>

**(1-Methyl-2-nitro-imidazol-5-yl)methyl chloride (10)**<sup>6,7,8</sup>



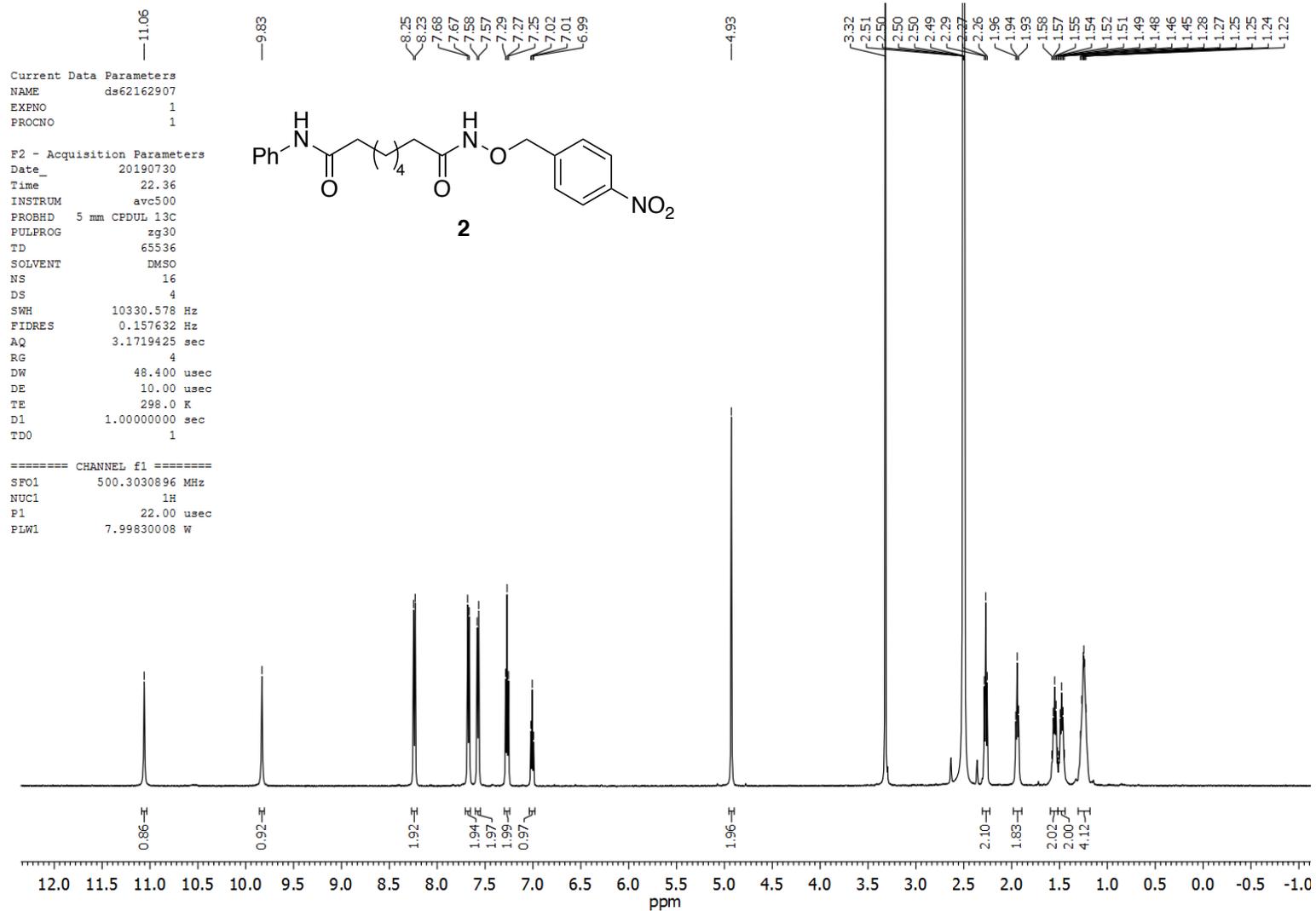
Methanesulfonyl chloride (74 μL, 0.96 mmol, 1.5 eq) was added dropwise to a stirred solution of (1-methyl-2-nitro-imidazol-5-yl)methanol (**S5**, 0.10 g, 0.64 mmol, 1.0 eq) in pyridine

(1.3 mL) and stirred at rt for 3 h then concentrated *in vacuo*. Purification by filtration through a short pad of silica (elution with 50% ethyl acetate: petroleum ether) yielded compound **10** (75 mg, 67%) as a colourless solid.  $R_f$  0.43 (50% ethyl acetate:petroleum ether). mp 68–77 °C (from dichloromethane) [lit.,<sup>7</sup> 87–90 °C (from ethyl acetate)];  $^1\text{H NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta_{\text{H}}$  7.19 (1H, s), 4.62 (2H, s), 4.07 (3H, s); LRMS  $m/z$  (ESI<sup>+</sup>) 176 ([M+H]<sup>+</sup>, 100%), 140 (43%). The spectroscopic data are consistent with the literature.<sup>7</sup>

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### N-(4'-Nitrobenzyloxy)-N'-phenyloctanediamide (2)



# N-(4'-Nitrobenzyloxy)-N'-phenyloctanediamide (2)

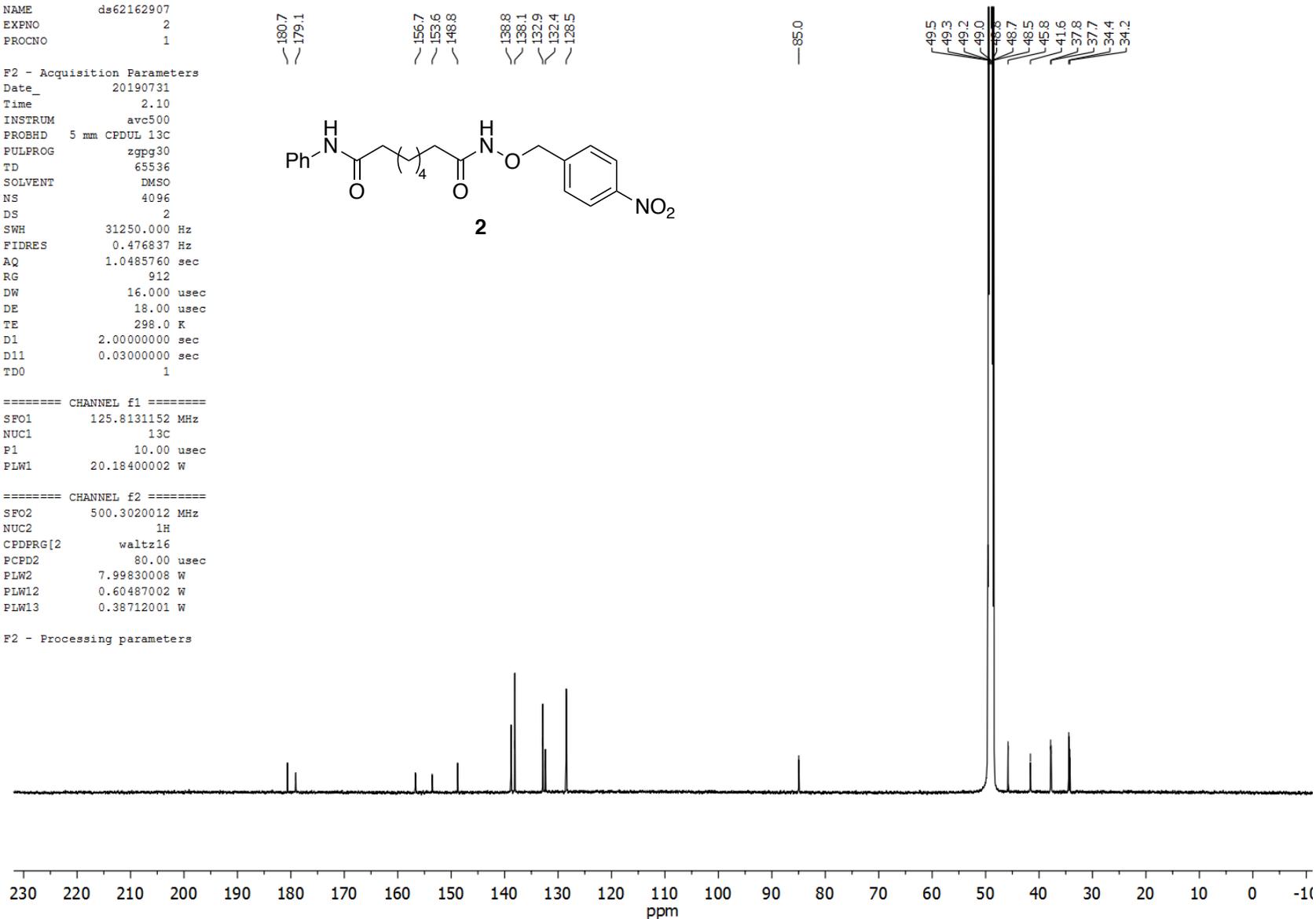
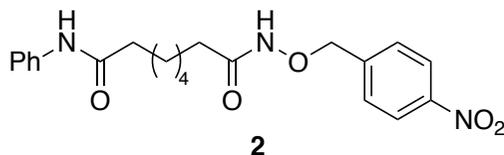
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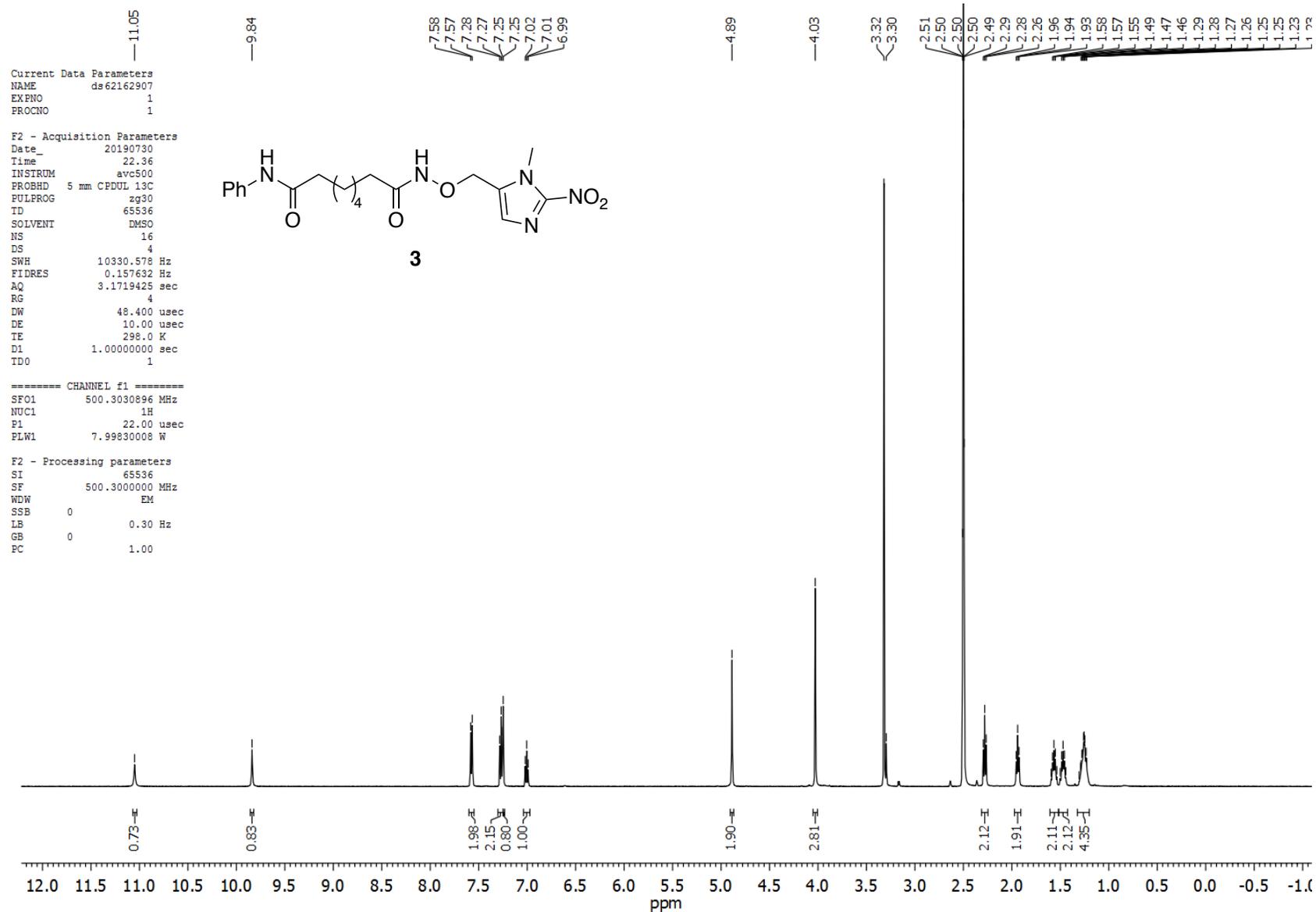
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**N-((1'-Methyl-2'-nitroimidazol-5'-yl)methoxy)-N'-phenyloctanediamide (3)**



***N*-((1'-Methyl-2'-nitroimidazol-5'-yl)methoxy)-*N'*-phenyloctanediamide (3)**

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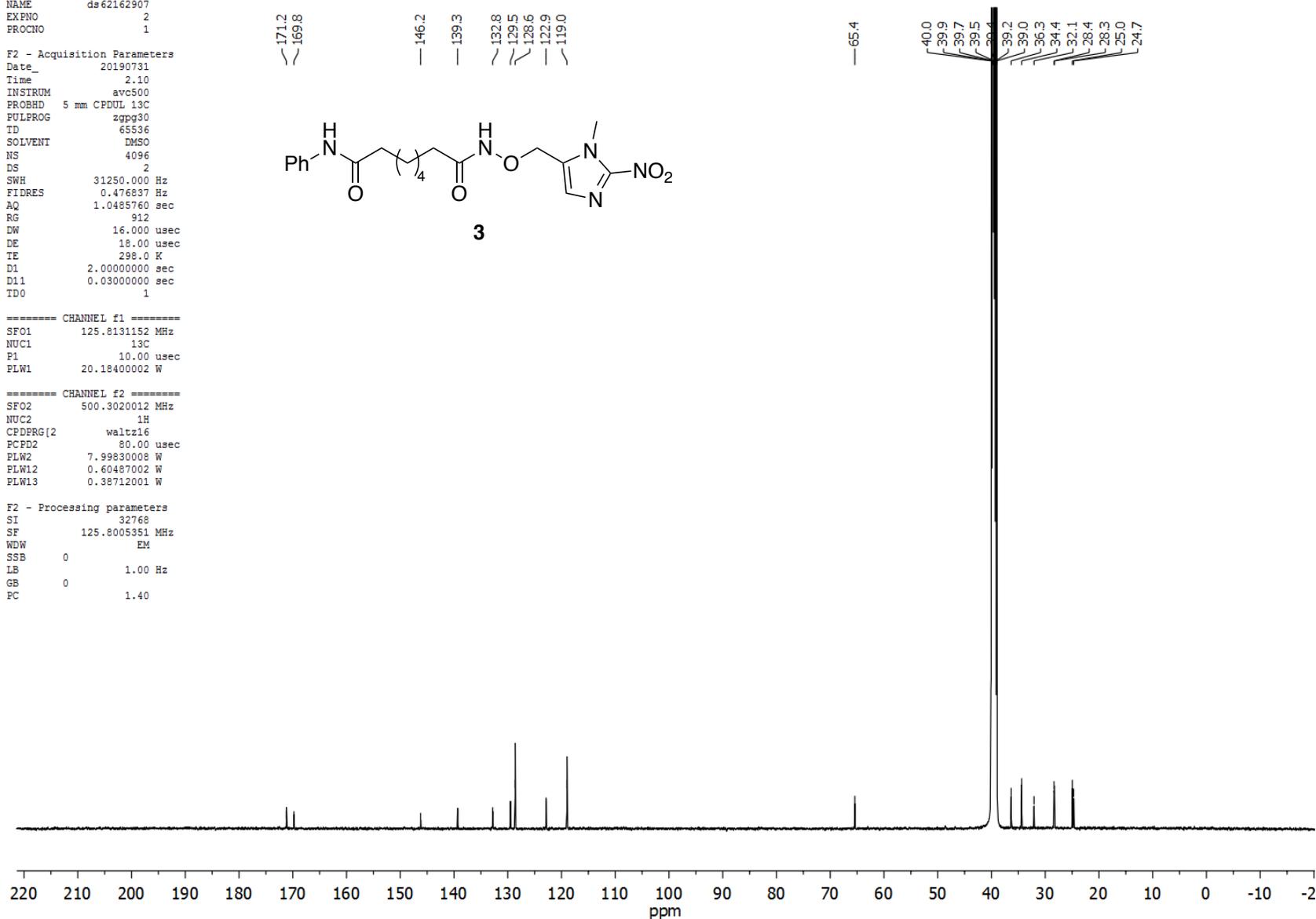
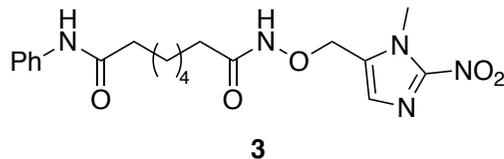
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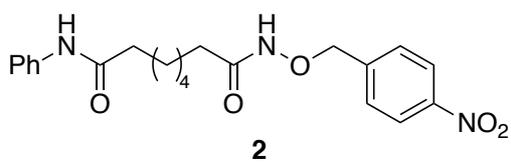
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 34.4  
 32.1  
 28.4  
 28.3  
 25.0  
 24.7



## N-(4'-Nitrobenzyloxy)-N'-phenyloctanediamide (2)

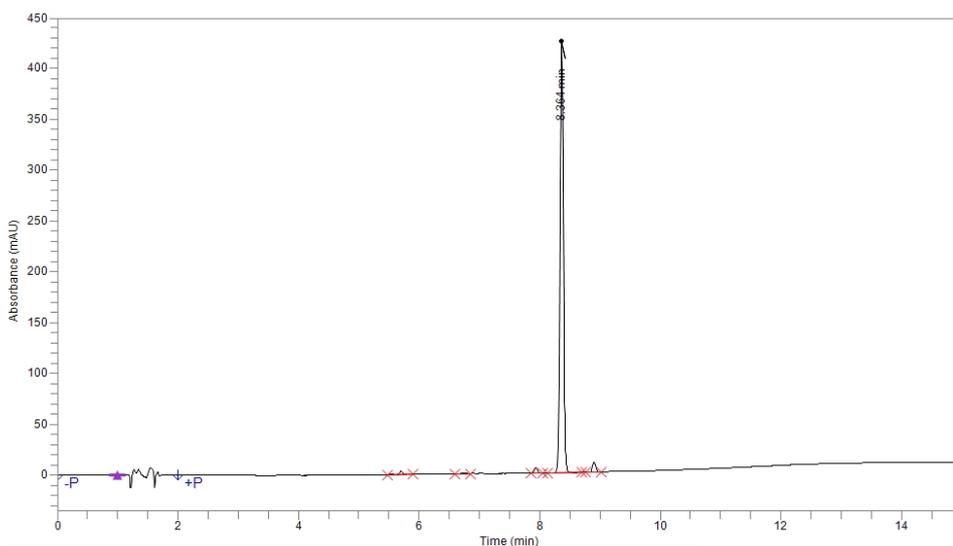


EC1-029\_254\_15uL

7/27/2019

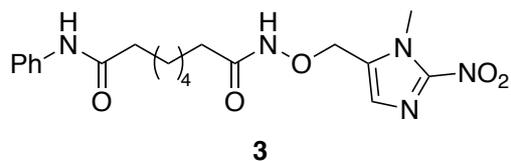
Acquisition Method	Purity short run @254 nm
Acquisition Date/Time	7/27/2019 5:56 pm
Injection Volume	15
Sample Name	EC1-029_254_15uL
Sample Description	
Batch Description	

EC1-029\_254\_15uL : Injection 1



Time	Height	Area	Area %
5.698	3,452.3	13,617.4	0.83
6.721	787.0	4,870.4	0.30
7.933	5,539.0	19,970.2	1.22
8.364	425,055.4	1,567,394.6	95.42
8.903	10,068.5	36,771.3	2.24
<b>Total</b>		1,642,623.9	100.00

***N*-((1'-Methyl-2'-nitroimidazol-5'-yl)methoxy)-*N'*-phenyloctanediamide (3)**

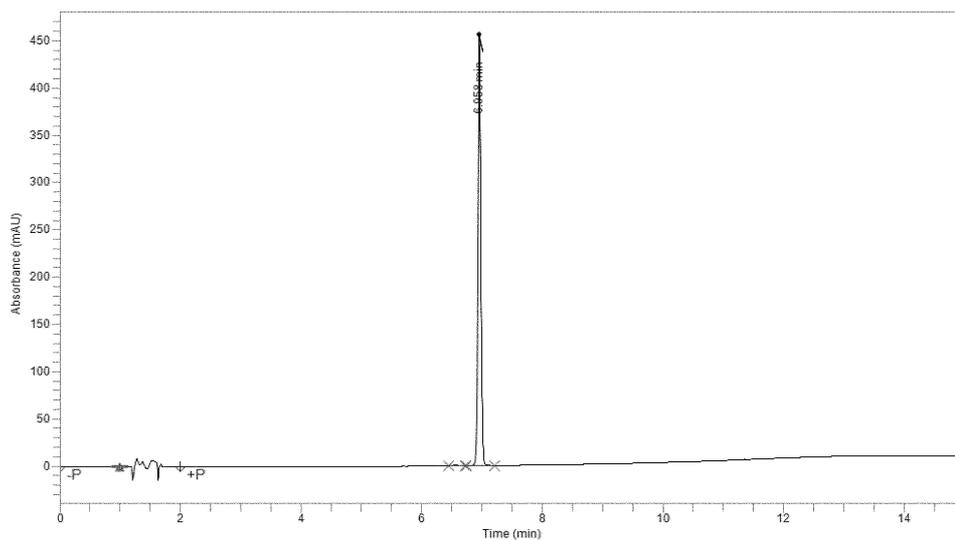


DSCH\_05\_007\_254nm

8/19/2019

Acquisition Method Purity short run @254 nm  
 Acquisition Date/Time 8/19/2019 4:19 pm  
 Injection Volume 20  
 Sample Name DSCH\_05\_007\_254nm  
 Sample Description  
 Batch Description

DSCH\_05\_007\_254nm : Injection 1



Time	Height	Area	Area %
6.553	1,471.1	5,205.5	0.33
6.958	457,037.7	1,563,067.5	99.67
<b>Total</b>		1,568,273.0	100.00

**KDAC profiling report** (Performed by Reaction Biology Corp. Malvern, PA, USA)

Both compounds were tested in a singlet 5-dose (IC50) mode with 3-fold serial dilution starting at 10  $\mu$ M against HDACs 1-9 and 11. HDAC reference compound Trichostatin A (TSA) and TMP269 were tested in a 10-dose IC50 with 3 fold serial dilution starting at 10  $\mu$ M.

**NB-SAHA (2)**

Conc (M)	KDAC									
	KDAC 1	KDAC 2	KDAC 3	KDAC 4	KDAC 5	KDAC 6	KDAC 7	KDAC 8	KDAC 9	KDAC 11
<b>1.00E-05</b>	1844929	1208900	5628838	14095215	4912195	1998918	2449056	2734875	2439608	5307412
<b>3.33E-06</b>	1716780	1207478	5696041	14993210	5075856	2202914	2440806	2834340	2777949	5251099
<b>1.11E-06</b>	1837430	1207080	5699150	15158434	5249288	2197819	2412317	2558824	2371997	5118535
<b>3.70E-07</b>	1786771	1253842	5760616	15141906	5795967	2268130	2426753	2610762	2278761	5358045
<b>1.23E-07</b>	1749987	1221894	5600139	13854056	5836247	2157911	2520117	2699626	2488317	5164092
<b>DMSO</b>	1646708	1165518	5590958	14906007	6297886	2058071	2136746	2369313	2583811	5067025

**NI-SAHA (3)**

Conc (M)	KDAC									
	KDAC 1	KDAC 2	KDAC 3	KDAC 4	KDAC 5	KDAC 6	KDAC 7	KDAC 8	KDAC 9	KDAC 11
<b>1.00E-05</b>	2154511	1333078	5268378	14180858	5399441	2069630	2294534	2974190	2499451	4230977
<b>3.33E-06</b>	1892772	1257706	5720616	13824798	5439285	1981128	2169972	2741198	2670169	4444601
<b>1.11E-06</b>	1805305	1141701	5658357	13810000	5601370	2165047	2260160	2731086	2706796	4621614
<b>3.70E-07</b>	1888789	1231926	5545370	14753919	5548980	2112836	2021625	2856756	2329144	4606461
<b>1.23E-07</b>	1769011	1222138	5689608	14118797	5598196	2195083	2321836	2542797	2620626	4665322
<b>DMSO</b>	1646708	1165518	5590958	14906007	6297886	2058071	2136746	2369313	2583811	5067025

## Trichostatin A

Conc. (M)	KDAC					
	KDAC 1	KDAC 2	KDAC 3	KDAC 6	KDAC 8	KDAC 11
1.00E-05	36147	14879	58249	5642	136407	1379600
3.33E-06	25888	31990	138690	-3139	693091	2871578
1.11E-06	85398	113928	393247	6940	1215421	3669751
3.70E-07	143777	201950	800340	15889	1519356	4277810
1.23E-07	298774	358923	1562109	31150	1730243	4697974
4.12E-08	638537	645369	2629743	142472	2058130	4958158
1.37E-08	943976	856093	3744811	360776	2094719	4618999
4.57E-09	1230468	1031861	4461765	778821	2040952	4858265
1.52E-09	1478828	1048333	4821484	1429612	2047267	4834810
5.08E-10	1548446	1148251	5127618	1825931	2087926	5052546
DMSO	1634113	1130500	5778964	2034237	1950604	4858778

## TMP269

Conc. (M)	KDAC			
	KDAC 4	KDAC 5	KDAC 7	KDAC 9
1.00E-05	442243	79379	-16122	-2984
3.33E-06	1156645	243009	36096	17551
1.11E-06	2590091	659144	127909	86063
3.70E-07	4850469	1516517	397012	84520
1.23E-07	8350205	2847962	676418	276019
4.12E-08	11088173	4465207	1215122	752701
1.37E-08	12747059	5019806	1916762	1332371
4.57E-09	13279732	5192229	1970818	1800665
1.52E-09	12715312	5394591	2101515	2524598
5.08E-10	13638495	5435239	2011773	2353222
DMSO	14112746	5188645	2440949	2554105

**Turbidimetric solubility assay** (performed by Cyprotex Discovery Ltd., Macclesfield, UK)

The test compound is diluted in buffer to give a range of concentrations (typically 1, 3, 10, 30 and 100  $\mu\text{M}$ , final DMSO concentration 1 %) and incubated at 37 °C for 2 h. Absorbance is measured at a wavelength of 620 nm and the solubility is estimated from the concentration of test compound that produces an increase in absorbance above the vehicle control (i.e., 1 % DMSO in buffer). Nicardipine and pyrene are included as control compounds. The solubility of nicardipine is pH dependent whereas the solubility of pyrene is pH independent.

Compound	Estimated Precipitation Range ( $\mu\text{M}$ )		
	Lower bound	Upper bound	Calculated Mid-range
NB-SAHA	10	30	20
NI-SAHA	100	>100	>100
Nicardipine	10	30	20
Pyrene	3	10	6.5