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A prodrug system for hydroxylamines based on esterase catalysis

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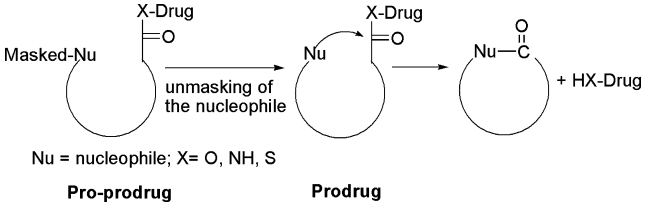
Abstract—The synthesis and reactivity of hydroxy hydroxamates as models for a prodrug form of hydroxylamine are described. γ-Hydroxy hydroxamates were found to enable hydroxylamine release via lactonisation. Hydroxamates were found to undergo esterase catalysed hydrolysis.

Prodrugs have little or no pharmaceutical activity but undergo transformation in the body to a therapeutically active product.[1] Use of the prodrug form of pharmaceuticals can enable to be overcome pharmacokinetic and pharmacodynamic barriers.[2] However, a single modification is not always sufficient to achieve the desired alteration in the physicochemical or biological properties of the drug. One solution is to prepare a prodrug of a prodrug (or pro-prodrug) by chemical modification of a prodrug.[3] Several pro-prodrug systems for amines, alcohols and peptides have been developed by taking advantage of facile intramolecular cyclisation reactions.[4–6] In this strategy, a latent nucleophile is unmasked by biological or chemical processes that in turn can initiate a cyclisation reaction to release the parent drug (Scheme 1).



Scheme 1. A prodrug strategy that takes advantage of intramolecular cyclisation.

Keywords: Prodrug; Intramolecular cyclisation; Lactonisation; Esterase; Hydroxylamine; Hydroxamates.
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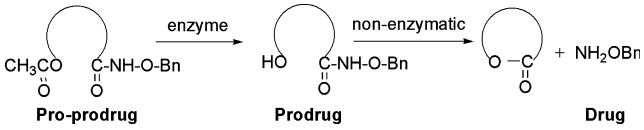
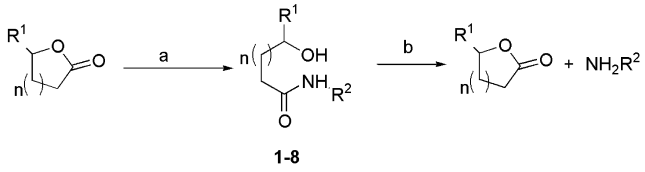


Scheme 2. Schematic representation of hydroxyl *N*-hydroxylamides as prodrugs.

To date there have been no reports of this type of prodrug system based on the release of hydroxylamine containing pharmaceuticals. We have been interested in developing hydroxamic acids and hydroxylamines as inhibitors of enzymes involved in antibiotic resistance[7] and the hypoxic response.[8] Certain hydroxylamines are known to possess important biomedical properties,[9–15] for example, derivatives of *O*-benzyl-*N*-hydroxylamine have shown the ability to inhibit the growth of the malaria parasite *Plasmodium falciparum* in vitro (IC_{50} < 10 μM)[9] and *o*-(2-chloro-6-fluorobenzyl)hydroxylamine was found to have potent inhibitory effects on the replication of human immunodeficiency virus (HIV-1) in cell cultures.[10]

Here we report studies on a prodrug system for hydroxylamines based on cyclisation of an alcohol onto a hydroxamate to give a lactone. It was envisioned that a pro-prodrug ester form would be metabolised to the prodrug via esterase mediated hydrolysis (Scheme 2).

Compounds **1**–**7** were synthesized via a one-step procedure[16] for the conversion of esters to hydroxamates using organoaluminium-promoted amidation. From the reports of the direct conversion of esters to Weinreb amides ($N(OCH_3)CH_3$) using aluminium activated hydroxyl-



Compound[18]	R^1	R^2	n	order of reactivity for hydroxylamine release
1	H	OBn	1	2nd
2	Me	OBn	1	1st
3	Et	OBn	1	3rd
4	Ph	OBn	1	n*
5	H	OBn	0	n*
6	H	OBn	2	4th
7	H	OBn	3	n*
8	H	Bn	1	n*

* n = no hydroxylamine release observed after 48 hours in CD_2Cl_2.

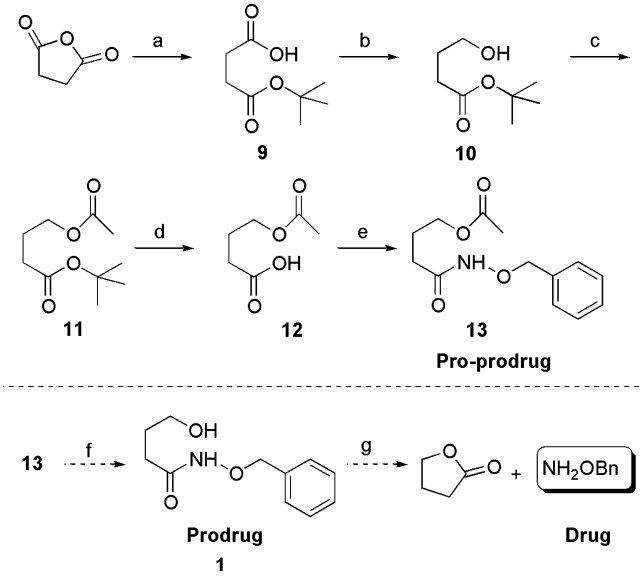
Scheme 3. Synthesis and reactivity of compounds **1**–**8**. Reagents and conditions: (a) $NH_2OBn \cdot HCl$ for **1**–**7** or $NH_2Bn \cdot HCl$ for **8**, $AlMe_3$ (2 M in toluene), dry THF, 0 °C, 1–4 h, 50–99%; (b) spontaneous lactonisation in CD_2Cl_2.

amine, a similar reagent[17] derived from *O*-benzyl-*N*-hydroxylamine permits the simple preparation of the hydroxamates. Thus, *O*-benzylhydroxylamine hydrochloride was treated with trimethylaluminium in THF to provide $(CH_3)_2AlNHOBn \cdot HCl$. This reagent was then treated in situ with the appropriate lactone at 0 °C to afford the corresponding hydroxy hydroxamate (**1**–**7**),[18] which were purified by chromatography (Scheme 3).

The rates of lactonisation of **1**–**7** were monitored by ^1H NMR in CD_2Cl_2. The results revealed that the rate of liberation of hydroxylamine followed the order **2** > **1** > **3** > **6** ≫ **4**/**5**/**7**. In the case of **1**–**3**, release of the hydroxylamine was observed within 30–60 min, but in the case of **6** lactonisation was slow with significant amount of lactone (>5%) only being observed after a week. Thus, it was concluded that under the conditions of the assay, the preferred length of the alkyl chain between the carbonyl and the nucleophile (OH) is 3 (*n* = 1, **1**–**4**) consistent with reaction via a 5-exo-trig cyclisation. With *n* = 2 lactonisation occurs (**6**), but at a slower rate. Longer (*n* = 3, **7**) or shorter (*n* = 0, **5**) chains do not permit the efficient liberation of the hydroxylamine. Unlike the hydroxamate, amide analogue, **8**, did not undergo lactonisation despite having a suitably positioned hydroxyl group. Substitution on the carbon adjacent to the alcohol with small alkyl groups, such as methyl or ethyl (**2** and **3**) had little effect on the rate of hydroxylamine release (over 30 min). However, the use of a phenyl substituent (**4**) halted lactonisation.

As anticipated,[5,6] the rate of reaction was significantly accelerated by acid catalysis, with even trace amounts of acetic acid (e.g., from the ethyl acetate used in purification) being enough to promote lactonisation, indicating that liberation of hydroxylamine in the acidic pH environment of the stomach could be possible.

The pro-prodrug **13** was then synthesized (Scheme 4). Direct acylation of alcohol **1** was difficult, due to facile lactonisation. Hence ester **13** was synthesized by heating succinic anhydride at reflux in *tert*-butyl alcohol to give **9,** which was then reduced by borane to afford alcohol



Scheme 4. Reagents and conditions: (a) *N*-hydroxysuccinimide, *N*,*N*′-dimethylaminopyridine, *tert*-butyl alcohol, toluene, reflux, 24 h: (b) BH_3:DMS, THF, rt, 24 h; (c) Ac_2O, *N*,*N*′-dimethylaminopyridine, pyridine, rt, 3 h; (d) cat. TsOH, wet toluene, reflux, 2 h; (e) $NH_2OBn \cdot HCl$, Et_3N, EDCI, HOBt, $CHCl_3$, rt, 7 h; (f) esterase from porcine liver; (g) spontaneous hydroxylamine release.

10.[19] The protection of the hydroxyl group as the acetyl derivative **11**[20] was followed by deprotection of the *tert*-butyl ester to give carboxylic acid **12**,[21] which underwent condensation with hydroxylamine to give the desired hydroxamate **13**.[22]

The ability of an esterase enzyme to enable hydroxylamine release was examined by reaction of **13** with porcine liver enzyme (Fig. 1). Surprisingly, ^1H NMR analyses in D_2O, revealed that, rather than ester hydrolysis, the hydroxamate bond was preferentially cleaved by the enzyme to liberate acid **14** (Fig. 1, ii). The esterase catalysed hydrolysis of the acetyl group of **13** to give **1** occurred at a slower rate (Fig. 1, iii). Both **14** and **1** were converted to **15** (Figs. 1, iii and iv). It was not possible to determine if the production of **15** from **1** arose from spontaneous lactonisation and/or esterase activity. Since **1** cyclises to γ-butyrolactone under the incubation conditions, the observation of **15** implies that the esterase possess lactonase activity. The proposal was supported by ^1H NMR analyses of the incubation of γ-butyrolactone with the esterase, which clearly demonstrated catalytic hydrolysis of the lactone to give **15**.

The esterase was also incubated with prodrug **2** (Fig. 2). This analogue of **1** was chosen because it not only posseses a similar lactonisation reactivity to **1** (in CD_2Cl_2) but also allows for easier monitoring of the reaction by following the ^1H NMR signal for the methyl group. Three different methyl doublets were observed in the ^1H NMR spectrum, corresponding to starting material **2**, lactone **16** and γ-hydroxy acid **17**.

This experiment demonstrated that lactone formation can occur in D_2O at pH 7. In the absence of enzyme, the rate of lactone formation from **2** at pH 7 is very

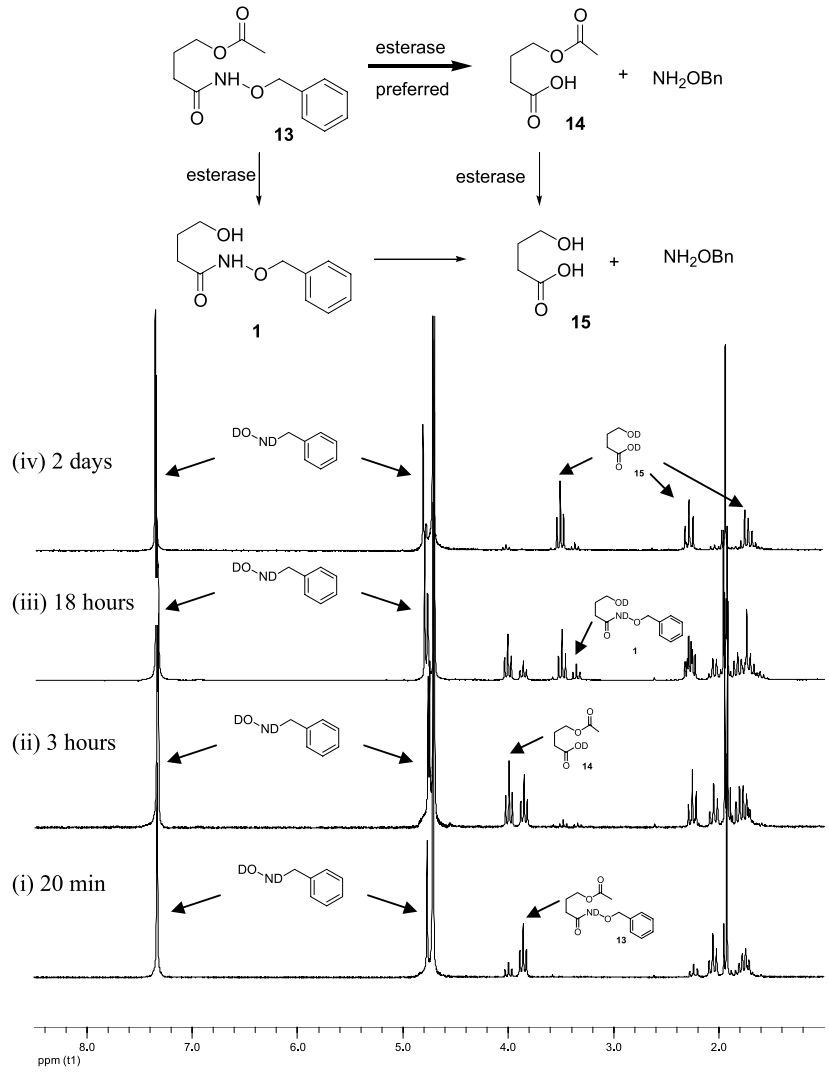


Figure 1. ^{1}H NMR (D_2O) spectra for the treatment of the pro-prodrug **13** with esterase.

slow; as in CD_2Cl_2 it is acid catalysed proceeding significantly faster at pH 4 in D_2O (data not shown). Thus, some of the lactone formation at pH 7 in D_2O may be enzyme catalysed.

Although not the focus of the current investigation, this observation suggests that the reactivity of γ-lactones with esterases to form acyl–enzyme complexes may be reversible, as has been observed for certain γ-lactams with proteases.[23,24] In contrast β-lactam inhibitors react effectively irreversible with proteases (and mechanistically related enzymes). This difference has been suggested as an origin of the 'special' nature of β-lactam inhibitors; the current data suggests a similar rationale that may apply to β- versus γ-lactone inhibitors of esterases.

Amide bonds are not efficiently hydrolysed by the esterase was confirmed; ^{1}H NMR analyses of the incubation of amide analogue **18**[25] with esterase in D_2O at pH 7, led only to the observation of acetyl group hydrolysis to give **8**. There was no evidence of hydrolysis of the amide bond even after all the ester group had been hydrolysed (Scheme 5). Therefore, we conclude that porcine liver esterase catalyses the hydrolysis of hydroxamates in preference to amides and, at least in some cases, to that of an acetyl group.

The hydroxamate analogue **19**[26] that does not contain the nucleophilic alcohol, was synthesized by the same procedure[16] used for preparation of hydroxy hydroxamates **1**–**7**. Incubation of **19** with esterase in D_2O revealed hydrolysis, demonstrating that hydroxamates could also be used as a prodrug form of hydroxylamines (Scheme 6).

An *N*-hydroxylated peptide bond has been reported to be cleaved faster by α-chymotrypsin than the analogue peptide.[27] In this work it was proposed that an intramolecular H-bond enabled the increased hydrolysis rate for the *N*-hydroxypeptide, although the inductive effect of the oxygen was also considered. In our case, the results indicate H-bonding is not important (Scheme 6) hence the enhanced hydrolysis rate for the hydroxamates is

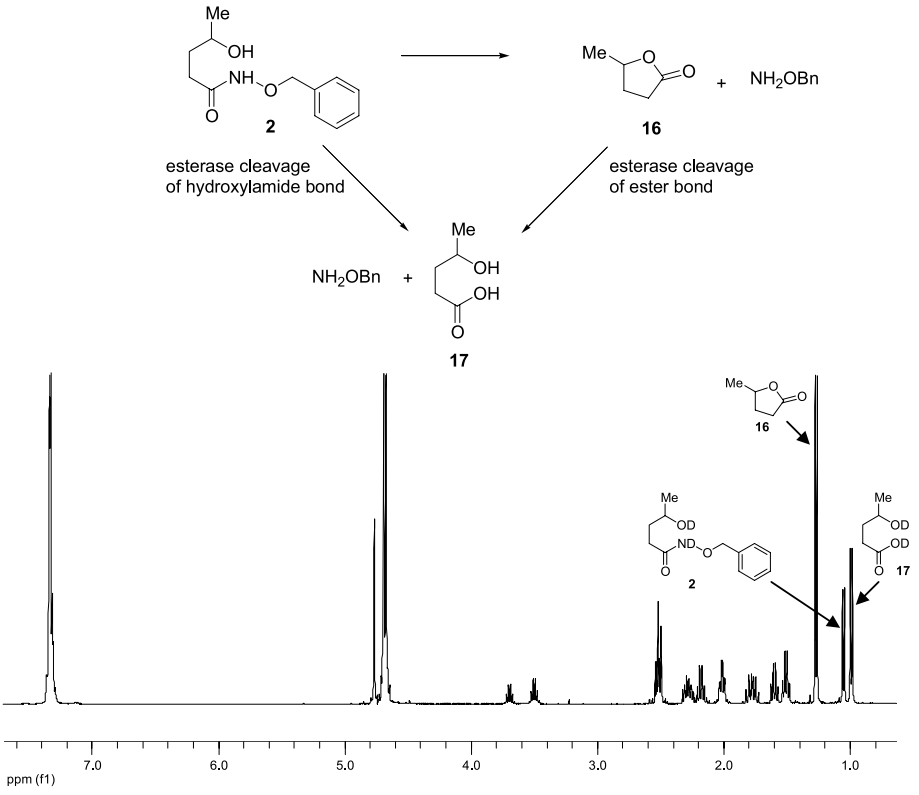
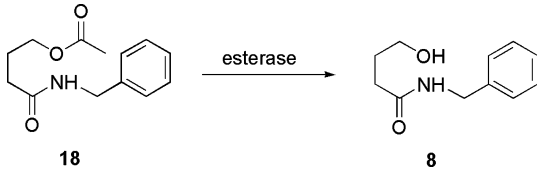
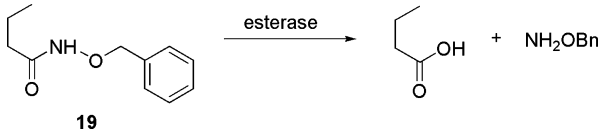


Figure 2. Result of the ^{1}H NMR (D_2O) experiment of **2** in the presence of esterase.



Scheme 5. Esterase activity on acyl protected γ-hydroxylamide **18**.



Scheme 6. Result of esterase activity on *N*-hydroxylamide **19**.

likely related to the inductive effect of the oxygen bonded to nitrogen.

In conclusion, a series of hydroxy hydroxamates (**1**–**7**) were synthesized as potential prodrug forms of hydroxylamine. γ-Hydroxy hydroxamates (**1**–**3**) were found to enable release of hydroxylamine via lactonisation. The pro-prodrug **13** of prodrug **1** was synthesized and the enzyme catalysed release of hydroxylamine was observed. Although the predominant enzyme activity was not ester hydrolysis, the release of the hydroxylamine drug occurred in an enzyme catalysed manner. The hydroxamate hydrolysis activity of esterases may thus enable the use of simple hydroxamate derivatives as prodrug forms of hydroxylamine, as well as enable the release of acetyl protecting groups from hydroxylamine under mild conditions.

Acknowledgments

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16. General procedure for the synthesis of compounds **1**–**8** and **19**: To a stirring suspension of *O*-benzyl-*N*-hydroxylamine hydrochloride (3 equiv) in dry THF at 0 °C, a solution of trimethylaluminium (2 M, 3 equiv) in toluene was added; stirred was continued at 0 °C for 30 min under nitrogen. The reaction was later stirred at room temperature for 20 min and cooled to −15 °C when a solution of the lactone (1 equiv) in dry THF was added dropwise. The suspension was warmed to 0 °C and stirred for 1.5 h. The reaction was quenched with HCl (0.5 M) and extracted with ethyl acetate. The organic extracts were dried over Na_2SO_4 and evaporated. The crude product was purified by flash column chromatography on silica gel (ethyl acetate–hexane/4:1) to afford the desired compound.
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18. Spectroscopic data for compounds **1**–**8:** *N*-(benzyloxy)-4-hydroxybutanamide **1**. Colourless oil (58%), ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.78 (dt, J = 6, 6.5 Hz, 2H), 2.16–2.17 (m, 2H), 3.13 (br s, 1H), 3.58 (t, J = 6 Hz, 2H), 4.86 (s, 2H), 7.36–7.37 (m, 5H), 9.30 (br s, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 27.8, 30.1, 61.6, 78.1, 128.5 (2×), 128.7, 129.2 (2×), 135.3, 171.3; High Resolution Electrospray Ionisation MS (HRESMS) (*m*/*z*): calcd for $C_{11}H_{14}NO_3$ 208.0974 $[M-N]^-$; found 208.0968. *N*-(benzyloxy)-4-hydroxypentanamide **2**. White solid (74%); mp: 53–55 °C; ^{1}H NMR (500 MHz, CD_2Cl_2): δ 1.17 (d, J = 6 Hz, 3H), 1.63–1.67 (m, 1H), 1.77–1.78 (m, 1H), 2.19–2.20 (m, 2H), 2.65 (br s, 1H), 3.76–3.80 (m, 1H); 4.89 (s, 2H), 7.40–7.43 (m, 5H), 8.66 (br s, 1H); ^{13}C NMR (128.5 MHz, CD_2Cl_2): δ 23.4, 29.8, 34.0, 67.2, 78.0, 128.5 (2×), 128.6, 129.2 (2×), 135.6, 171.2; HRESMS (*m*/*z*): calcd for $C_{12}H_{16}NO_3$ 222.1130 $[M-N]^-$; found 222.1134. *N*-(benzyloxy)-4-hydroxyhexanamide **3**. White solid (80%); mp: 57.5–59 °C; ^{1}H NMR (500 MHz, CD_2Cl_2): δ 0.94 (t, J = 7.5 Hz, 3H), 1.44–1.48 (m, 2H), 1.60–1.63 (m, 1H), 1.81–1.84 (m, 1H), 2.20–2.21 (m, 2H), 2.74 (br s, 1H), 3.49 (br s, 1H); 4.89 (s, 2H), 7.38–7.42 (m, 5H), 9.00 (br s, 1H); ^{13}C NMR (128.5 MHz, CD_2Cl_2): δ 9.8, 29.8, 30.4, 31.8, 72.5, 78.0, 128.5 (2×), 128.6, 129.2 (2×), 135.6, 171.4; HRESMS (*m*/*z*): calcd for $C_{13}H_{18}NO_3$ 236.1287 $[M-N]^-$; found 236.1282. *N*-(benzyloxy)-4-hydroxy-4-phenylbutanamide **4**. White solid (83%); mp: 43–45 °C; ^{1}H NMR (400 MHz, CD_2Cl_2): δ 1.97–2.03 (m, 2H), 2.18 (br s, 2H), 3.44 (br s, 1H); 4.68–4.71 (m, 1H), 4.87 (s, 2H), 7.28–7.40 (m, 10H), 8.91 (br s, 1H); ^{13}C NMR (100.6 MHz, CD_2Cl_2): δ 30.0, 34.6, 75.6, 78.4, 126.1 (2×), 127.7 (2×), 128.8 (2×), 128.9 (2×), 129.0, 129.6, 135.9, 144.9, 171.6; HRESMS (*m*/*z*): calcd for $C_{17}H_{18}NO_3$ 284.1283 $[M-N]^-$; found 284.1283. *N*-(benzyloxy)-3-hydroxypropanamide **5**. White solid (87%), mp: 69–71 °C; ^{1}H NMR (400 MHz, CD_2Cl_2): δ 2.30–2.31 (m, 2H), 2.62 (br s, 1H), 3.83 (t, J = 5.5 Hz, 2H); 4.91 (s, 2H), 7.42–7.43 (m, 5H), 8.65 (br s, 1H); ^{13}C NMR (100.6 MHz, CD_2Cl_2): δ 36.0, 58.8, 78.5, 128.9 (2×), 129.0, 129.6 (2×), 135.9, 176.4; HRESMS (*m*/*z*): calcd for $C_{10}H_{12}NO_3$ 194.0817 $[M-N]^-$; found 194.0822. *N*-(benzyloxy)-5-hydroxypentanamide **6**. Colourless oil (50%); ^{1}H NMR (400 MHz, CD_2Cl_2): δ 1.49–1.57 (m, 2H), 1.64–1.67 (m, 2H), 2.09 (t, J = 6.5 Hz, 2H), 2.78 (br s, 1H); 3.57 (t, J = 6 Hz, 2H), 4.88 (s, 2H), 7.40–7.41 (m, 5H), 9.23 (s, 1H); ^{13}C NMR (100.4 MHz, CD_2Cl_2): δ 22.0, 32.1, 32.9, 62.1, 78.4, 128.7 (2×), 128.9, 129.5 (2×), 135.3, 176.1; HRESMS (*m*/*z*): calcd for $C_{12}H_{16}NO_3$ 222.1130 $[M-N]^-$; found 222.1135. *N*-(benzyloxy)-6-hydroxyhexanamide **7**. Colourless oil (72%); ^{1}H NMR (400 MHz, CD_2Cl_2): δ 1.35–1.42 (m, 2H), 1.52–1.57 (m, 2H), 1.62–1.66 (m, 2H), 1.72 (br s, 1H), 2.07 (m, 2H), 3.61 (t, J = 6.5 Hz, 2H), 4.89 (s, 2H), 7.39–7.42 (m, 5H), 8.39 (br s, 1H); ^{13}C NMR (128.5 MHz, CD_2Cl_2): δ 24.9, 25.2, 32.3, 33.0, 62.3, 78.0, 128.5 (2×), 128.6, 129.2 (2×), 135.6, 170.7; HRESMS (*m*/*z*): calcd for $C_{13}H_{18}NO_3$ 236.1287 $[M-H]^-$; found 236.1286. *N*-benzyl-4-hydroxybutanamide **8**. White solid, (99%); mp: 58.5–60 °C; ^{1}H NMR (400 MHz, CD_2Cl_2): δ 1.86 (dt, J = 6, 7 Hz, 2H), 2.39 (t, J = 7 Hz, 2H), 2.86 (br s, 1H), 3.67 (t, J = 6 Hz, 2H), 4.43 (d, J = 6 Hz, 2H), 6.10 (br s, 1H), 7.30–7.31 (m, 3H), 7.35–7.38 (m, 2H); ^{13}C NMR (100.6 MHz, CD_2Cl_2): δ 28.5, 34.3, 43.9, 62.7, 127.7, 128.0 (2×), 129.0 (2×), 139.0, 173.6; HRESMS (*m*/*z*): calcd for $C_{11}H_{14}NO_2$ 192.1025 $[M-N]^-$; found 192.1025.
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20. Synthesis and characterization of *tert*-butyl 4-(acetyloxyl)butanoate **11**: To a solution of **10** (350 mg, 2.18 mmol) and *N*,*N*′-dimethylaminopyridine (26.7 mg, 0.218 mmol) in pyridine (2 mL), was added acetic anhydride (0.617 mL, 6.54 mmol) at room temperature. The reaction mixture was stirred for 3 h, then poured into ice-water (5 mL) and extracted with ether (3 × 5 mL). The organic phase was washed with HCl aq (1 M, 15 mL), water (15 mL), satd $NaHCO_3$ (15 mL), water (15 mL), and brine (10 mL), dried over $MgSO_4$ and evaporated to afford **11** as a slightly brown oil (340 mg, 77%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.44 (s, 9H), 1.91 (dt, J = 6.5 Hz, 7, 2H), 1.95 (s, 3H), 2.29 (t, J = 7 Hz, 2H), 4.09 (t, J = 6.5 Hz, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 20.9, 24.1, 28.1 (3×), 32.0, 63.5, 80.4, 171.0, 172.1; HRCIMS (*m*/*z*): calcd for $C_{10}H_{22}NO_4$ 220.1549 $[M+NH_4]^+$; found 220.1544.
21. Synthesis and characterization of 4-(acetyloxy)butanoic acid **12**: To a solution (10 mL) of *tert*-butyl 4-hydroxybutanoate **11** (250 mg, 1.23 mmol) in toluene were added *p*-TsOH (23.5 mg, 0.123 mmol) and one drop of water. The mixture was heated at reflux for 2 h. After cooling, ethyl acetate (10 mL) was added and the organic layer washed with water (2 mL), dried ($MgSO_4$) and evaporated to afford a colourless oil (179.8 mg, 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.99 (dt, J = 6.5, 7 Hz, 2H), 2.06 (s, 3H), 2.47 (t, J = 7 Hz, 2H), 4.13 (t, J = 6.5 Hz, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 20.8, 23.7, 30.4, 63.2, 171.0, 178.2; HRCIMS (*m*/*z*): calcd for $C_6H_{14}NO_4$ 164.0923 $[M+NH_4]^+$; found 164.0919.
22. Synthesis and characterization of 5-[(benzyloxy)amino]-4-oxopentyl acetate **13**: To a solution of 4-(acetyloxy)butanoic acid **12** in $CHCl_3$ (10 mL), HOBt (110.9 mg, 0.821 mmol), EDCI (157.4 mg, 0.821 mmol), TEA (480 mL, 3.421 mmol), and NH_2OBn were added and stirred at room temperature for 7 h. The reaction mixture was washed with HCl aq (1 M, 2 × 5 mL), satd $NaHCO_3$, (2 × 5 mL), water (2 × 5 mL), dried ($MgSO_4$) and evaporated to afford a colourless oil (114.5 mg, 67%). ^{1}H NMR (400 MHz, D_2O): δ 1.83 (dt, J = 6.5, 7 Hz, 2H), 2.02 (s, 3H), 2.14 (t, J = 7 Hz, 2H), 4.13 (t, J = 6.5 Hz, 2H); 4.86 (s, 2H), 7.42 (s, 5H); ^{13}C NMR (100.6 MHz, D_2O): δ 20.6, 24.0, 29.3, 64.4, 78.4, 129.0 (2×), 129.5, 130.1 (2×), 135.0, 172.3, 174.8; HRESMS (*m*/*z*): calcd for $C_{13}H_{16}NO_4$ 250.1079 $[M-N]^-$, found 250.1084.

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25. Synthesis and characterization of 4-(benzylamino)-4-oxobutyl acetate **18**: To a solution of 8 (75 mg, 0.388 mmol) and *N*,*N*′-dimethylaminopyridine (4.7 mg, 0.003 mmol) in pyridine (0.5 mL), acetic anhydride (40.3 mL, 0.427 mmol) was added at room temperature; the reaction mixture was stirred for 2 h, then poured into ice-water (2 mL) and extracted with ether (3 × 5mL). The organic phase was washed with HCl aq (1 M, 2 × 5 mL), water (2 × 5 mL), satd $NaHCO_3$ (2 × 5 mL), water (2 × 5 mL) and brine (5 mL), then dried ($MgSO_4$) and evaporated to afford **18** as a colourless oil (58.5 mg, 64%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.97–2.03 (m, 2H), 2.03 (s, 3H), 2.29 (t, J = 7.5 Hz, 2H), 4.11 (t, J = 6.5 Hz, 2H), 4.43 (d, J = 5.5 Hz, 2H), 6.96 (br s, 1H), 7.27–7.34 (m, 3H), 7.34 (m, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$): d 20.9, 24.7, 32.9, 43.6, 63.6, 127.6, 127.8 (2×), 128.7 (2×), 138.2, 171.2, 171.8; HRESMS (*m*/*z*): calcd for $C_{13}H_{17}NO_3Cl$ 270.0897 $[M+Cl]^-$; found 270.0894.
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